

Early Diagnosis and Effective Management of Diminished Ovarian Reserve (DOR)

Alexandra Fernandes de Carvalho

Tese para obtenção do Grau de Doutor em

Biomedicina

(3º ciclo de estudos)

Orientador: Prof. Doutora Ana Teresa Moreira Almeida Santos

Co-orientador: Prof. Doutora Mariana Costa Brandão de Moura Ramos

Co-orientador: Prof. Doutora Ana Cristina Monteiro Ramalhinho Tavares Patrício

Júri:

Prof. Doutor Joaquim Mateus Paulo Serra (Presidente do Júri)

Prof. Doutora Sílvia Cristina da Cruz Marques Socorro

Prof. Doutora Luiza Augusta Tereza Gil Breitenfeld Granadeiro

Prof. Doutora Patrícia Carla Coelho Rodrigues

Prof. Doutora Juliana Baptista Pedro

Prof. Doutora Maria João da Silva Fernandes Leal Carvalho

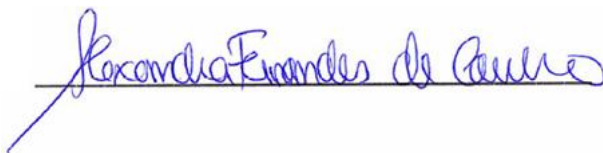
19/12/2024

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Universidade da Beira Interior, Covilhã 19 /07 /2024

A handwritten signature in blue ink, reading "Alexandra Fernandes de Carvalho", is written over a horizontal line.

Dedicatória

Enquanto houver memória, há vida e será eterna quem nela vive.

À Teresa.

Agradecimentos

Começo esta seção dos agradecimentos assumindo que esta tese é muito mais do que minha. Trata-se de um trabalho conjunto, fruto da vontade concertada de muitas pessoas que merecem ser referidas.

Os primeiros elementos, basais, a referir neste projeto são as minhas orientadora e coorientadoras. Tenho a agradecer à Professora Doutora Ana Teresa Almeida-Santos por ser uma inspiração no meu percurso académico, profissional e pessoal. A imagem e referência de sempre. À Doutora Mariana Moura-Ramos, por ter aceitado o desafio de me orientar neste projeto e a quem devo um apoio incondicional. Quem muito admiro e com quem foi um enorme gosto trabalhar neste projeto. À Doutora Ana Cristina Ramalhinho, pela disponibilidade sem fim e um carinho inigualável. Teve a palavra certa em cada fase.

Agradeço à Roche, na pessoa do Dr. Carlos Catalão, por acreditar neste projeto e garantir os recursos necessários para o implementarmos.

No departamento de ginecologia, obstetrícia, neonatologia e reprodução da ULS Coimbra, gradeço à senhora diretora do serviço de ginecologia Dra. Fernanda Águas, e à Dra. Maria João Carvalho que me levou à pessoa certa, no momento certo! À Enfermeira Ana Paula Gaudêncio e a todas as enfermeiras da consulta externa da Maternidade Bissaya Barreto (MBB). A esta casa (MBB) gradeço não só a disponibilidade na colaboração para a realização do projeto, como a simpatia com que me acolheram.

No âmbito do recrutamento agradeço à Mariana Trindade que abriu a porta ao apoio das USFs Coimbra Norte, Topázio, Fernão de Magalhães, São Martinho, e Condeixa -um apoio imprescindível a este estudo!

Fico muito grata a todas as participantes voluntárias do estudo que aceitaram este desafio! Sem elas não haveria resultados.

Ao Dr. Francisco Caramelo que deu um apoio imprescindível na análise estatística, com uma enorme generosidade e profissionalismo.

Do serviço de patologia clínica, agradeço ao senhor diretor, Dr. Fernando Rodrigues. Às técnicas superior de diagnóstico e terapêutica - análises clínicas, Sandra Silva, Sabina

Vítor e Sónia Silva. As que estiveram permanentemente disponíveis e abraçaram este projeto como delas.

Ao serviço de anatomia patológica, que já me apoia deste o projeto de tese de mestrado, tenho tudo a agradecer. À senhora diretora Dra. Maria Augusta Cipriano pela empatia e sensibilidade para a investigação. À Dra. Fátima Pereira da Silva pela dedicação preciosa a este projeto. Ao Dr. Rui Almeida pela disponibilidade, simpatia e orientação do trabalho investigação.

Às minhas amigas Néné e Silvana, que me abriram a porta da biologia molecular. Que me transmitiram, da melhor forma possível, toda a sua experiência. Pela sua enorme generosidade e contributos fundamentais para esta tese.

À minha querida Bárbara Gomes, à Vanessa Vieira, à Francisca Vilaça, à Ana Filipa Sousa e à Ana Duarte por dedicarem tanto tempo a este projeto – quando o tempo é o bem mais valioso que dispomos.

À minha equipa de sempre. Aos que são tão parte de mim que já nem dou conta. Ao senhor diretor do serviço de medicina da reprodução, Dr. Paulo Cortesão que cultiva, no serviço que dirige, a comunhão da ciência com a clínica. Às minhas incansáveis enfermeiras, Ana Luísa, Glória, Catarina, Carmo e Cláudia. As que embarcaram em todos os desafios e aceitaram todos os pedidos. Ao corpo clínico e não clínico da medicina da reprodução que me acompanharam sempre. Ao grupo de investigação de biologia da reprodução e células estaminais que permitiu um encontro muito feliz com influências fundamentais neste trabalho. Agradeço em particular ao núcleo mais próximo. À Ana Sofia Pais, que é inspiração pelo perfeccionismo em tudo o que faz, e que me guiou neste percurso. À Sandra Reis pelo exemplo de resiliência, e entreaajuda. À Maria, pela companhia em horas de trabalho, pela referência de rigor científico. Em último, mas às que estão mais próximas de mim todos os dias, às minhas embriologistas. À Rachel, Rita e Ana Paula. Um obrigada especial à Ana Paula, a quem devo a entrega desta tese. Devo-lhe por ter acreditado que era possível, como ninguém, por todo o apoio e compreensão.

À Filipa, que conheci no âmbito profissional e que se tornou uma amiga. A pessoa que admiro em todas as dimensões, pelo profissionalismo e dedicação que coloca em tudo o que faz. Pela retitude e exigência que impõe a si própria. Por ser a alma mais pura que conheço, e por conseguir ver através de mim. Conseguiu nos momentos mais difíceis tornar tudo mais suave. Partilhámos os projetos mais bonitos e acredito que assim será sempre. Obrigada!

Agradeço aos amigos e à família que permitiram, ao integrar o meu projeto de vida, que chegasse aqui. Em especial ao João, que não só esteve sempre ao meu lado e conheceu a face mais difícil da elaboração deste projeto, como contribuiu com a sua *expertise* na construção das ferramentas tecnológicas deste trabalho. Aos meus pais, pelo suporte inesgotável, inesgotável, inesgotável. À Inês e ao André por me mostrarem que temos forças inimagináveis. À Inês que foi a primeira a dizer-me “Vais conseguir”.

Como veem esta tese não é minha (e só por isso consegui dar-lhe vida!). Foi um projeto de quatro anos do trabalho de muitas pessoas. Quatro anos em que aprendi que a vida continuamente nos impõe desafios incalculáveis e que, em tudo, há oportunidades de crescimento. Este projeto integrou, até agora, a fase mais desafiante da minha vida e, por isso, terá sempre um valor especial.

Obrigada a todos!

Funding

The funding for this project has been generously provided by Roche – Sistemas de Diagnósticos, Sociedade Unipessoal, Lda, with a specific focus on supporting the early diagnosis of diminished ovarian reserve. Roche contribution included the assessment of ovarian reserve, anti-Müllerian hormone testing.

Resumo

A reserva ovárica diminuída, *diminished ovarian reserve*, (DOR) refere-se à redução do número de folículos disponíveis, levando a uma diminuição precoce do potencial reprodutivo (Cohen et al., 2015). Esta condição afeta cerca de 10% das mulheres em idade jovem, não se sabendo a sua etiologia em 50% desses casos (Ferreri et al., 2020; Greene et al., 2014; Nikolaou & Templeton, 2003).

A tendência global de adiamento da maternidade afeta particularmente as mulheres com DOR, muitas vezes atrasando o diagnóstico e aumentando a probabilidade de necessitarem de tratamentos de fertilidade (Barbakadze et al., 2015; Geyter et al., 2020). Além disso, a falta de conhecimento em saúde reprodutiva (Halleran et al., 2022; Iino et al., 2022; Pedro et al., 2018; Ren et al., 2023) resulta, em muitos jovens em idade reprodutiva, numa infertilidade involuntária (Beaujouan, 2020). Intervenções no conhecimento sobre saúde reprodutiva e fertilidade, bem como o suporte na definição do plano reprodutivo podem ajudar a mitigar problemas de infertilidade. No entanto, medidas desta natureza não têm sido consistentemente implementadas (Bakkensen & Goldman, 2022; Harper et al., 2021). O aconselhamento reprodutivo personalizado tem sido sugerido para mitigar o risco de infertilidade no futuro (Azhar et al., 2015), dado que a quantificação da hormona anti-Mülleriana (AMH) e a determinação da contagem de folículos antrais (CFA) são considerados os métodos padrão para avaliar a reserva ovárica (Tremellen & Savulescu, 2014), permitindo oferecer às mulheres informação ajustada ao seu caso específico e assim apoiar a sua tomada de decisão. No entanto, até ao momento, nenhum estudo baseado neste tipo de abordagem foi feito junto da população portuguesa.

Investigar o perfil genético numa população com DOR tem sido proposto para esclarecer a etiologia desta condição, potencialmente melhorando o diagnóstico e permitindo uma terapia mais direcionada (Nesbit et al., 2020). Atualmente, a informação que existe sobre a prevalência de fatores genéticos na DOR é limitada, uma vez que a maioria dos estudos genéticos se têm focado na insuficiência ovárica prematura, *premature ovarian insufficiency*, (POI) (Huhtaniemi et al., 2018). Polimorfismos de nucleotídeo único, *single nucleotide polymorphisms*, (SNPs) em genes que codificam recetores hormonais, como o recetor de hormona folículo-estimulante, *follicle-stimulating hormone receptor*, (*FSHR*) ou o recetor de estrogénios 1, *estrogen receptor 1*, (*ESR1*), foram já relacionados com recrutamento, desenvolvimento e maturação de folículos (Greene et al., 2014). No

entanto, são necessárias mais evidências para estabelecer o seu papel em situações de reserva ovárica alterada.

Novas abordagens têm surgido para promover o rejuvenescimento do tecido ovárico e estimular a foliculogênese. O plasma rico em plaquetas (PRP) evidenciou potencial para estimular a ativação folicular e a angiogênese do tecido ovárico através da libertação de fatores de crescimento (Alam et al., 2009; Atkinson et al., 2021; Sills & Wood, 2019). Embora a literatura demonstre um interesse crescente no uso de PRP para melhorar os resultados em contexto de estimulação ovárica controlada, *controlled ovarian stimulation*, (COS) em mulheres com DOR (Alam et al., 2009; Cakiroglu et al., 2020; Callejo et al., 2013; Hsu et al., 2020; Melo et al., 2020; Pantos et al., 2019; Sfakianoudis et al., 2018, 2019, 2020; Sills et al., 2018; Sills & Wood, 2019), a sua eficácia continua sob investigação (Reig et al., 2021).

A estratégia mais eficaz para mitigar os efeitos da DOR ainda está por ser determinada. O nosso objetivo foi avaliar diferentes abordagens para oferecer melhores cuidados de saúde a mulheres com esta condição, considerando estratégias antes e após o seu diagnóstico.

Este projeto foi estruturado em três tarefas com os respetivos objetivos:

Tarefa 1: intervenção no conhecimento: informação genérica e informação personalizada sobre reserva ovárica e reserva ovárica diminuída (DOR)

Desenvolver e testar o impacto de duas intervenções no conhecimento sobre reserva ovárica e DOR: 1) oferta de informação genérica através de um panfleto, e 2) oferta de informação personalizada e aconselhamento reprodutivo com base na avaliação da reserva ovárica.

Tarefa 2: estudo genético de populações inférteis com reserva ovárica alterada

Rever o papel dos polimorfismos dos recetores hormonais na função ovárica.

Verificar a ocorrência de alterações genéticas relativas aos polimorfismos *FSHR* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 em casos de reserva ovárica alterada. Especificamente, investigar a associação de variantes genéticas polimórficas em recetores hormonais com marcadores de reserva ovárica (HAM e CFA) em diferentes grupos, mulheres com DOR e mulheres com síndrome do ovário poliquístico (SOP) submetidas a tratamentos de fertilidade.

Estabelecer um protocolo de estudo que inclua sequenciação de nova geração, *next generation sequencing*, (NGS) para verificar a ocorrência de variantes genéticas na

população com DOR, que inclui rever os genes que melhor se adequam ao estudo desta população e definir o painel de genes a analisar posteriormente.

Tarefa 3: estudo in vitro da ativação folicular, através da suplementação do meio de cultura de tecido ovárico humano com plasma-rico em plaquetas (PRP)

Avaliar a eficácia da suplementação com PRP na ativação da via fosfatidilinositol-3-quinase (PI3K)-proteína quinase B (Akt), *Phosphatidylinositol 3-Kinase / Protein Kinase B*, e na melhoria da vascularização do estroma, utilizando um sistema de cultura *in vitro*, utilizando tecido ovárico humano.

Os resultados estão descritos pela ordem das três tarefas mencionadas.

Realizou-se um estudo observacional longitudinal para avaliar a eficácias das intervenções no conhecimento. O nosso estudo envolveu 105 mulheres sem filhos com uma idade média de $27,33 \pm 3,67$ anos, com a maioria das participantes (79%) a integrar o estudo por autoproposta. O conhecimento das participantes sobre reserva ovárica, DOR e fertilidade; as suas intenções e atitudes em parentalidade foram avaliados em três momentos, através de questionário. Especificamente, foi feita uma avaliação antes de qualquer intervenção (t0), depois do panfleto contendo informação genérica (t1) e depois da oferta de informação personalizada (t2).

As participantes constituem um grupo altamente instruído, com 55,2% tendo o grau de mestre, e o seu conhecimento inicial sobre fertilidade era próximo de 64%. As participantes demonstraram melhorias significativas no seu conhecimento, avaliado pela percentagem de respostas corretas sobre a definição de reserva ovárica, os fatores que a afetam e a forma como pode ser avaliada, definição de DOR e a sua incidência, após ambas as intervenções no conhecimento ($p < .001$ para t0 vs t1 e $p = .008$ para t1 vs t2).

A maioria das participantes estava numa relação (84,5%), embora para 31,4% delas os planos de casamento não sejam uma prioridade. Uma proporção significativa das participantes considerou ter filhos muito importante (40%) ou extremamente importante (37,1%), com 58,1% estando certas de que queriam filhos no futuro. As participantes estavam conscientes de que a idade ideal para ter o primeiro filho é antes dos 30 anos, com a idade ideal reportada sendo próxima de 29 anos, nos três momentos ($p = .129$). No entanto, em média, as participantes demonstraram planejar ter o primeiro filho aos 31 anos. Ou seja, as participantes expressaram, consistentemente, uma idade ideal significativamente menor para ter o primeiro filho em comparação com a idade que realmente planeiam ter filhos ($p < .001$), o que indica alguma perceção das limitações para atingir os seus planos reprodutivos. O número de filhos desejado foi significativamente maior do que o número que sentiam ser possível ter, considerando fatores como situação

económica, idade e antecedentes pessoais. Esta tendência persistiu de t_0 ($p=.004$) a t_1 ($p<.001$), e em t_2 ($p<.001$).

A maioria das participantes reconheceram que a infertilidade poderia limitar os seus planos de parentalidade, sendo o obstáculo mais considerado em t_0 (51,4%) e t_1 (54,3%) e o segundo mais considerado em t_3 (43,8%). Se confrontadas com um diagnóstico de DOR, a decisão de desistir da parentalidade manteve-se baixa e consistente ao longo do período de intervenção ($p=.618$). Apesar das nossas intervenções, as participantes não alteraram a intenção de antecipar a idade em que pretendem tornar-se mães em caso de infertilidade devido por DOR ($p=.532$). Em situações de infertilidade, as participantes mostraram preferência por realizar tratamentos de procriação medicamente assistida (PMA) em vez de adoção ($p<.001$) e em vez doação de ovócitos ($p<.001$).

Além de avaliar o impacto geral das intervenções no conhecimento, também investigámos como é que a informação fornecida sobre a avaliação da reserva ovárica afetou o conhecimento, intenções e atitudes em parentalidade. Para isso, formámos subgrupos para análise: mulheres que receberam informação tranquilizadora, com níveis de HAM e CFA acima do percentil 25 para a sua idade (NOR), comparativamente a mulheres com níveis de HAM ou CFA abaixo do percentil 25 para a sua idade (DOR1); ou mulheres com ambos os níveis de HAM e CFA abaixo do percentil 25 (DOR2); e mulheres com discrepâncias entre os níveis de HAM e CFA, onde um desses parâmetros estava abaixo do percentil 25 (INT).

Ao longo do estudo, apenas a preferência pela criopreservação de ovócitos mudou significativamente, com uma diminuição estatisticamente significativa da intenção de criopreservação de ovócitos ($p=.002$). Especificamente, entre a avaliação inicial (t_0) e o fornecimento do folheto informativo (t_1), o valor médio diminuiu ($p=.046$). Esta tendência continuou entre a fase pós-informação genérica (t_1) e a fase após a informação personalizada e o aconselhamento reprodutivo (t_2) ($p=.024$). Analisando a criopreservação de ovócitos entre subgrupos formados de acordo com o estado da reserva ovárica, revelaram-se diferenças significativas ao explorar a interação tempo e grupo. Mulheres com valores de HAM e CFA acima do percentil 25 (NOR), mostraram maior preferência pela criopreservação de ovócitos em comparação com mulheres com ambos os níveis de HAM e CFA abaixo do percentil 25 (DOR2) ($p=.043$). No subgrupo DOR1, em que HAM ou CFA estão abaixo do esperado para a idade, a possibilidade de criopreservação de ovócitos foi menos considerada após receberem a informação personalizada sobre a sua reserva ($p<.001$), talvez devido a uma visão realista de que esta não é a melhor opção para elas.

O conhecimento não melhorou após fornecer informações específicas sobre o estado da reserva ovárica, com a interação tempo-grupo a não evidenciar mudanças significativas para a comparação DOR1 e NOR ($p=.247$), bem como para DOR2, INT e NOR ($p=.574$). As intenções de ter filhos mantiveram-se estáveis ao considerar a interação tempo-grupo, de acordo com os resultados da avaliação da reserva ovárica. Por exemplo, o número de filhos que as participantes gostariam de ter ($p=.707$ e $p=.116$), o número de filhos que as participantes consideraram possível ter ($p=.473$ e $p=.349$), a idade ideal para ter filhos ($p=.152$ e $p=.155$) e a idade em que planeiam ter o primeiro filho ($p=.648$ e $p=.373$), que não apresentaram mudanças significativas, considerando a informação personalizada sobre a reserva ovárica.

Importa notar que a informação dada no aconselhamento reprodutivo foi bem direcionada e recebida pelas participantes. No subgrupo NOR, onde a informação fornecida foi mais tranquilizadora, observou-se uma diminuição na percentagem de participantes que consideravam a infertilidade um obstáculo, de 59.3% para 55.9% ($p=.004$). Para o grupo INT, fornecer informação aumentou o número de participantes que consideravam a infertilidade um obstáculo de t_0 para t_1 ($p=.039$), e essa percepção foi mantida ao longo do tempo de t_1 (60.8%) para t_2 (54.9%), $p=.375$. Além disso, a percepção de que tudo está bem foi predominantemente escolhida pelas participantes NOR ($p<.001$). Por outro lado, as participantes que receberam informações sobre ter uma reserva ovárica abaixo do esperado para a sua idade, tanto DOR1 como DOR2, frequentemente avaliaram a sua situação como necessitando de mais tempo para alcançar a gravidez ou necessitando de ação a curto ou médio prazo ($p<.001$). Além disso, uma minoria de participantes DOR1 e nenhum de DOR2 acreditaram ser seguro adiar a maternidade ($p=.015$ para DOR1 e NOR, e $p=.040$ para DOR2, INT e NOR).

Não houve aumento de ansiedade resultante das intervenções. Os níveis de ansiedade não mostraram diferenças significativas após as intervenções ($p=.714$ para STAI-S e $p=.104$ para STAI-T). A interação tempo-grupo, considerando os subgrupos formados de acordo com a informação fornecida sobre a reserva ovárica, também não mostrou diferenças significativas para STAI-S ($p=.272$ para DOR1 e NOR, e $p=.558$ para DOR2, INT e NOR), nem para STAI-T ($p=.344$ para DOR1 e NOR, e $p=.744$ para DOR2, INT e NOR).

A maioria das participantes (58.1%) classificou a avaliação da reserva ovárica como extremamente importante, e uma percentagem ainda maior (75.8%) relatou tê-la realizado no momento adequado das suas vidas.

As tarefas subsequentes (2 e 3) focaram-se em ações estratégicas para potencialmente gerir a DOR após o seu diagnóstico.

O estudo de variantes genéticas, particularmente nos recetores de gonadotrofinas, tem sido sugerido para compreender situações em que há alterações da reserva ovárica, bem como quando se pretende otimizar a resposta à COS. Os SNPs em recetores como *FSHR*, recetor da hormona luteinizante, *luteinizing hormone receptor*, (*LHR*), e *ESR* estão descritos como determinantes do desenvolvimento folicular, mas a sua associação à função ovárica precisa de ser aprofundada.

A partir da revisão da literatura concluiu-se que os polimorfismos *FSHR* rs6166, *ESR1* rs2234693 e *ESR1* rs9340799 estão entre os mais estudados em relação à função ovárica. Contrariamente, a associação dos polimorfismos do *LHR* à função ovárica está pouco descrita na literatura.

Especificamente, a associação do *FSHR* rs6166 à resposta à COS está extensivamente estudada, com o alelo Ser680 frequentemente associado a piores resultados em comparação com o alelo Ans680. Apesar da associação do *FSHR* rs6166 com marcadores de reserva ovárica estar menos documentada na literatura, o seu potencial papel na regulação da via PI3K-Akt, e por isso na ativação folicular, continua a ser um tema de investigação e debate.

A revisão da literatura permitiu-nos perceber que os polimorfismos do *ESR* na função ovárica estão pouco documentados, apesar de existirem evidências do envolvimento do *ESR1* rs2234693 e *ESR1* rs9340799 no desenvolvimento folicular, e na resposta à COS.

O impacto dos polimorfismos *FSHR* rs6166, *ESR1* rs2234693 e *ESR1* rs9340799 em populações inférteis com reserva ovárica alterada (DOR e SOP) foi avaliado no nosso estudo, num estudo observacional retrospectivo.

Não encontramos diferenças estatisticamente significativas na distribuição das variantes genéticas *FSHR* rs6166 ($p=.807$), *ESR1* rs2234693 ($p=.633$) e *ESR1* rs9340799 ($p=.672$) entre DOR ($N=88$) e o grupo de controlo ($N=219$). Da mesma forma, entre mulheres com menos de 35 anos, não houve diferenças significativas na distribuição das variantes *FSHR* rs6166 ($p=.301$), *ESR1* rs2234693 ($p=.582$) e *ESR1* rs9340799 ($p=.593$) entre DOR ($N=27$) e o grupo de controlo ($N=112$).

Além da DOR, a SOP é também um distúrbio ovárico que compromete a fertilidade feminina. Acredita-se que a DOR e a SOP partilhem disfunções comuns em fatores-chave que afetam o desenvolvimento folicular. Relativamente à população com SOP, estudos anteriores mostraram dados discordantes em relação à associação entre os

polimorfismos do *FSHR* e *ESR1* e esta condição. O nosso estudo não encontrou uma associação significativa na distribuição de genótipos do *FSHR* rs6166 ($p=.522$) e *ESR1* rs2234693 ($p=.697$) entre mulheres inférteis com SOP ($N=88$) e o grupo controlo ($N=80$). No entanto, o genótipo SS do polimorfismo *FSHR* rs6166 foi associado a níveis mais elevados de FSH, doseado ao terceiro dia do ciclo menstrual ($p=.011$). Não foram encontradas outras diferenças relativamente aos genótipos *FSHR* rs6166 e *ESR1* rs2234693.

Por fim, para aprofundar o estudo da população com DOR, revimos a literatura existente por forma a identificar os genes *Forkhead Box L2 (FOXL2)*, *Forkhead Box O3 (FOXO3)*, *Factor in the Germline Alpha (FIGLA)*, *Phosphatase and Tensin Homolog (PTEN)*, *Insulin-like Growth Factor 1 (IGF1)* e *Insulin-like Growth Factor 2 (IGF2)*, *Growth Differentiation Factor 9 (GDF9)*, *Bone Morphogenetic Protein 15 (BMP15)* e *AKT Serine/Threonine Kinase 1 (AKT1)* para integrar um painel de genes para análise por NGS. Esta abordagem evita o viés de uma população infértil e é aplicável às amostras de sangue colhidas para a quantificação da HAM, das participantes da Tarefa 1.

Em suma, o estudo de variantes genéticas quer em populações inférteis, quer, futuramente, na população não infértil com reserva ovárica alterada tem como objetivo encontrar alvos para uma terapia direcionada, como a farmacogenética, e melhorar a resposta à estimulação ovárica dos folículos sensíveis às gonadotrofinas, bem como definir um diagnóstico mais preciso destes casos. Complementarmente, novas terapias têm sido sugeridas para ativar folículos primordiais, visando populações com DOR, em que a COS, por si só, não é suficiente. Para aprofundar esta hipótese, seguiu-se a Tarefa 3, na qual otimizámos um protocolo para obter PRP, investigando o seu impacto numa via envolvida na ativação folicular.

O PRP emergiu como uma terapia promissora na medicina regenerativa e que se estendeu para muitas áreas, incluindo a medicina da reprodução. No entanto, a falta de padronização nos protocolos de preparação de PRP apresenta um desafio para obter resultados reprodutíveis e precisos. O nosso objetivo inicial foi otimizar o protocolo de preparação de PRP investigando o impacto de dois anticoagulantes diferentes, o citrato de sódio (CS) e o ácido etilenodiamino tetra-acético, *ethylenediamine tetraacetic acid*, (EDTA), e dois meios de ressuspensão, plasma e cloreto de sódio (NaCl). As taxas de recuperação de plaquetas foram calculadas e comparadas entre os grupos, além da atividade plaquetária e fator de crescimento endotelial vascular, *vascular endothelial growth factor*, (VEGF) libertado no plasma após a ativação das plaquetas. A taxa de recuperação de plaquetas foi maior com EDTA em comparação com CS (51.04% vs 29.85%, $p=.005$). A atividade plaquetária também foi maior, com maior expressão de

dois anticorpos plaquetários, P-38 Selectina de superfície plaquetária (CD62p) e PAC-1 no grupo EDTA. A concentração de VEGF foi maior com CS em comparação com EDTA (628.73 vs 265.44 pg/mL, $p=.013$). As taxas de recuperação de plaquetas e os níveis de VEGF foram maiores no PRP ressuspensão em plasma quando comparados com NaCl (61.60% vs 48.61%, $p=.011$ e 41 363.32 vs 159.83 pg/mL, $p=.005$, respectivamente).

Após estabelecer um protocolo, partindo de 15 mL de sangue, centrifugação a 300g por 10 minutos seguido de 700g por 15 minutos, procedemos com um estudo experimental *in vitro* para avaliar o impacto do PRP em fragmentos de tecido ovárico humano. Testamos dois tempos de cultura, 4 e 48 horas ($t=4h$ e $t=48h$) em relação ao tecido avaliado imediatamente após a descongelação ($t=0h$). Adicionalmente analisou-se o tecido com e sem PRP ($t=4h$ Vs $t=4h+PRP$ e $t=48h$ Vs $t=48h+PRP$).

Ao longo do estudo, a necrose do tecido aumentou significativamente de $t=0h$ para $t=48h$ ($p<.001$), começando nas 4 horas ($t=0h$ vs $t=4h$, $p=.002$), tendo estabilizado a partir das 4 horas iniciais ($t=4h$ vs $t=48h$, $p=.71$). A degeneração folicular aumentou significativamente de $t=0h$ para $t=48h$ ($p<.001$) e de $t=4h$ para $t=48h$ ($p<.001$), sem mudança significativa entre $t=0h$ e $t=4h$ ($p=.262$). O edema intersticial aumentou significativamente ao longo do tempo, de $t=4h$ para $t=48h$ ($p<.001$), sem diferença entre $t=0h$ e $t=4h$ ($p=.070$).

A percentagem de folículos positivos para Ki-67 aumentou significativamente de $t=0h$ para $t=48h$ ($p=.002$), enquanto nenhuma variação foi observada na percentagem de estroma positivo para Ki-67 ($p=.716$). A marcação para a caspase 3 manteve-se inalterada, ao nível dos folículos e do estroma, não mostrando diferenças significativas entre os três tempos de cultura ($p=.851$ e $p=.663$, respectivamente). A densidade vascular, número de vasos por mm², e a área vascular, percentagem de estroma positivo para o fator VIII (FVIII), também não apresentaram mudanças significativas ao longo do tempo ($p=.937$ e $p=.284$, respectivamente).

A suplementação de PRP no meio de cultura impactou significativamente no estroma ovárico, mostrando mudanças significativas em resposta ao tratamento com PRP. A necrose do tecido apresentou uma diminuição às 4 horas ($p=.002$), enquanto a degeneração folicular e o edema intersticial diminuíram com a suplementação de PRP, no período de 48 horas (respectivamente, $p=.002$ e $p<.001$).

A marcação com Ki-67 aumentou nas células estromais com a suplementação de PRP ($p<.001$) tanto às 4 horas ($p<.001$) quanto às 48 horas ($p=.007$), quando comparado com o grupo sem PRP. Além disso, foi notado um aumento significativo na área vascular com

a suplementação de PRP ($p=.014$), especificamente no período de 48 horas de cultura ($p=.024$).

O PRP não teve impacto na percentagem de folículos e células estromais positivas para caspase 3 ($p=.898$ e $p=.986$, respetivamente), nem na densidade vascular ($p=.272$).

A expressão de p-Akt mostrou um aumento de intensidade ao longo do tempo ($p<.001$), com esse efeito amplificados pelo PRP tanto às 4 horas ($p=.003$) quanto às 48 horas ($p=.034$).

Em conclusão, este estudo visou abordar aspetos não solucionados dos casos de DOR, desenvolvendo estratégias desde o aumento do conhecimento sobre o seu impacto na fertilidade até à investigação de potenciais terapias pós-diagnóstico. Investir em informação sobre este tema provou ser eficaz em aumentar o conhecimento, o que nos leva a defender a continuação de esforços em educação para a fertilidade. A informação personalizadas não mostrou efeitos secundários, particularmente aumento da ansiedade pós-intervenção. No entanto, permanece a questão sobre o impacto desta intervenção a longo prazo nos planos reprodutivos. Este tópico poderia ser esclarecido num estudo de seguimento futuro.

A NGS é promissora e alinha-se com nossos objetivos de investigar alvos para diagnóstico focado e terapia personalizada em casos de DOR, bem como elucidar a etiologia da condição.

Por fim, embora alguns aspetos ainda necessitem de uma investigação mais aprofundada para sustentar os resultados do estudo *in vitro* da cultura de tecido ovárico com PRP, obtivemos informações valiosas sobre seu efeito na via de ativação folicular PI3K-Akt.

Palavras-chave

Reserva ovárica diminuída ; intervenções no conhecimento ; avaliação da reserva ovárica ; polimorfismos de nucleotídeo único ; ativação folicular *in vitro* ; plasma rico em plaquetas ; fosfatidilinositol-3-quinase (PI3K)-proteína quinase b, (Akt)

Abstract

Diminished ovarian reserve (DOR) refers to a reduction in number of available follicles, leading to decreased reproductive potential at an early age (Cohen et al., 2015). This condition affects about 10% of women, with over 50% of these cases being idiopathic (Ferreri et al., 2020; Greene et al., 2014; Nikolaou & Templeton, 2003).

A global trend of delayed childbearing particularly impacts women with DOR, often delaying their diagnosis, and making them more likely to require fertility treatments (Barbakadze et al., 2015; Geyter et al., 2020). Furthermore, a significant lack of fertility knowledge (Halleran et al., 2022; Iino et al., 2022; Pedro et al., 2018; Ren et al., 2023), frequently leads to unintended childlessness (Beaujouan, 2020). Interventions to provide information and support fertility management can help mitigate infertility problems; however, they have not been consistently implemented (Bakkensen & Goldman, 2022; Harper et al., 2021). Tailored counselling in reproductive health has also been recommended to help reduce the risk of infertility (Azhar et al., 2015). Once anti-Müllerian hormone (AMH) measurement and antral follicle count (AFC) determination are considered the gold standard methods for assessing ovarian reserve (Tremellen & Savulescu, 2014), they can be used to provide tailored information to women, and support decision-making in reproductive plans. However, at the moment, the Portuguese population lacks comprehensive studies in this particular field.

Investigating the genomic profile of a DOR cohort has been suggested to elucidate on the aetiology of this condition, potentially enhancing diagnostics and enabling a targeted therapy (Nesbit et al., 2020). Currently, limited information exists regarding the prevalence of genetic factors contributing to DOR, as most genetic studies have focused on premature ovarian insufficiency (POI) (Huhtaniemi et al., 2018). Single nucleotide polymorphisms (SNPs) in genes encoding hormonal receptors, such as follicle-stimulating hormone receptor (*FSHR*) or estrogen receptor 1 (*ESR1*), have been associated with follicle recruitment, development, and maturation (Greene et al., 2014). However, further evidence is required to establish their role in situations of abnormal ovarian reserve.

Novel approaches have emerged for rejuvenating ovarian tissue and activating folliculogenesis. Platelet-rich plasma (PRP) has shown potential to stimulate follicle activation and angiogenesis by the presence of growth factors (Alam et al., 2009; Atkinson et al., 2021; Sills & Wood, 2019). While the literature demonstrates a growing

interest in the use of PRP to improve outcomes for women with DOR undergoing assisted reproduction (Alam et al., 2009; Cakiroglu et al., 2020; Callejo et al., 2013; Hsu et al., 2020; Melo et al., 2020; Pantos et al., 2019; Sfakianoudis et al., 2018, 2019, 2020; Sills et al., 2018; Sills & Wood, 2019), its efficacy remains under investigation (Reig et al., 2021).

Therefore, the most effective strategy for mitigating the effects of DOR has yet to be determined. Our objective was to evaluate different approaches to manage women with DOR, regarding strategies to be implemented before and after the diagnosis. Accordingly, this project was structured into three tasks with their respective objectives:

Task 1: intervention in knowledge through general and tailored information on ovarian reserve and diminished ovarian reserve (DOR)

Develop and test the impact of two interventions on knowledge about ovarian reserve DOR: 1) providing general leaflet information, and 2) offering tailored information and reproductive counselling based on the assessment of ovarian reserve.

Task 2: genetic analysis of infertile populations with abnormal ovarian reserve
Review the role of hormonal receptor polymorphisms in ovarian function.

Identify genetic variants concerning *FSHR* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 polymorphisms in cases of altered ovarian reserve (DOR and PCOS), among infertile populations. Specifically, investigate the association of polymorphic genetic variants in hormonal receptor genes with ovarian reserve markers in different groups, women with DOR and polycystic ovarian syndrome (PCOS), undergoing fertility treatments.

Establish an NGS study protocol to identify genetic variants in the population with DOR, which includes reviewing the genes most relevant to this population and defining the gene panel to be analysed subsequently.

Task 3: in vitro study of follicular activation, through the incubation of human ovarian tissue with platelet-rich plasma (PRP)

Evaluate the efficacy of PRP supplementation in activating the phosphatidylinositol-3-kinase (PI3K)-protein kinase B (Akt) Pathway and enhancing stromal vascularization using an *in vitro* culture system with human ovarian tissue.

The results are in the order of the three described tasks.

An observational longitudinal study was conducted. Our study involved 105 childless women with an average age of 27.33 ± 3.67 years, with the majority (79%) participating by self-proposal. The participants knowledge, reproductive intentions, and attitudes

were assessed at three-time-points by questionnaire. Specifically, assessment before any intervention (t0), and after the general information leaflet (t1) and after the tailored information (t2). This group was highly educated, with 55.2% holding a master's degree. Their baseline knowledge about ovarian reserve, assessed by the percentage of correct answers regarding its definition, the factors that affect it, how it decreases, the markers used to evaluate it, and specific situations of DOR and its incidence, was close to 64%. Participants demonstrated significant improvements in their knowledge, after both interventions ($p < .001$ for t0 Vs t1 and $p = .008$ for t1 Vs t2).

Most participants were in a relationship (72.38%), even though for 31.4% of them marriage plans are not something that they prioritize. A significant portion of the participants considered having children very important (40%) or extremely important (37.1%), with 58.1% being certain they wanted children in the future. Participants were aware that the ideal age to have their first child is before 30 years, with the ideal age reported as close to 29 years old, at the 3 time-points ($p = .129$). However, on average, they planned to have their first child at 31 years, consistently expressing a significantly lower mean ideal age for first childbearing compared to the age they actually plan to have children ($p < .001$). This discrepancy indicates some awareness of the limitations to their reproductive plans. Accordingly, the desired number of children was significantly higher than the number they felt confident they could have, considering factors such as economic situation, age, and personal background. This trend persisted from t0 ($p = .004$) to t1 ($p < .001$), and at t2 ($p < .001$).

Participants acknowledged that infertility could limit their parenthood plans, with it being the most considered obstacle at t0 (51.4%) and t1 (54.3%) and the second most considered at t2 (43.8%). If confronted with a diagnosis of DOR, the decision to give up on parenthood remained low and consistent throughout the intervention period ($p = .618$). Moreover, participants did not adjust their intended childbearing age in response to potential infertility caused by DOR ($p = .532$). When presented with a hypothetical possibility of infertility, participants showed a preference for ART over adoption ($p < .001$) and egg donation ($p < .001$).

In addition to evaluating the overall impact of the interventions on knowledge, we also aimed to investigate how the information provided about ovarian reserve assessment affected knowledge, reproductive intentions, and attitudes. To achieve this, we formed subgroups for analysis: women who received reassuring information, with AMH and AFC levels above the 25th percentile for their age (NOR); in comparison to women with either AMH or AFC levels below the 25th percentile for their age (DOR1); women with both AMH and AFC levels below the 25th percentile (DOR2); and women with discrepancies

between AMH and AFC levels, where one of these measures was below the 25th percentile (INT).

Throughout the study, the only significant change observed was in the preference for oocyte cryopreservation, with a significant decrease in considering social freezing as an option ($p=.002$). Specifically, between the initial assessment (t_0) and the provision of general informational leaflets (t_1), the mean value decreased ($p=.046$). This trend continued between the post-general information stage (t_1) and the phase following the provision of tailored information and reproductive counselling (t_2) ($p=.024$). Analysing oocyte freezing across subgroups formed according to ovarian reserve status revealed significant differences when exploring the interplay between time and group ($p=.016$), particularly noticeable between t_1 and t_2 ($p=.002$). Within the DOR1 subgroup, participants considered the option of oocyte cryopreservation less after receiving information ($p<.001$). This attitude may reflect a more realistic understanding that it is not the most suitable option for them.

The knowledge did not improve after providing tailored information about ovarian reserve status, with the interaction between time and group not showing significant changes for the comparisons of DOR1 with NOR subgroups ($p=.247$), as well as between DOR2, INT, and NOR subgroups ($p=.574$). Childbearing intentions remained stable when considering the interaction between time and group, regarding the groups formed according to the ovarian reserve testing results. For instance, the number of children participants would like to have ($p=.707$ and $p=.116$), the number of children participants consider possible to have ($p=.473$ and $p=.349$), the ideal childbearing age ($p=.152$ and $p=.155$), and the age at which they plan to have their first child ($p=.648$ and $p=.373$) did not show significant changes.

Importantly, the message was well-targeted and well-received by participants. In the NOR subgroup, where the information provided was more reassuring, we observed a decrease in the percentage of participants who considered infertility as an obstacle, from 59.3% to 55.9% ($p=.004$). For INT subgroup, providing information increased the number of participants considering infertility an obstacle, from t_0 (47.06%) to t_1 (60.78%), $p=.039$, and this perception was maintained over time: t_1 (60.78%) to t_2 (54.9%), $p=.375$.

The perception that everything is fine was predominantly chosen by NOR participants ($p<.001$). On the other hand, participants who received information indicating that their ovarian reserve was below the expected percentile for their age—both in the DOR1 and DOR2 subgroups—frequently assessed their situation as requiring additional time to

achieve pregnancy or as necessitating to take action in short- to medium-term ($p < .001$). Additionally, a minority of DOR1 participants and none of DOR2 believed it was safe to postpone motherhood ($p = .015$ for DOR1 and NOR, and $p = .040$ for DOR2, INT and NOR).

The interventions did not lead to an increase in anxiety. Anxiety levels showed no significant differences after the key interventions ($p = .714$ for STAI-S and $p = .104$ for STAI-T). The interaction between time and groups formed according to the information received also showed no significant differences for STAI-S ($p = .272$ for DOR1 and NOR, and $p = .558$ for DOR2, INT, and NOR), nor for STAI-T ($p = .344$ for DOR1 and NOR, and $p = .744$ for DOR2, INT, and NOR).

The majority of participants (58.1%) rated the evaluation of ovarian reserve as extremely important, and an even larger majority (75.8%) reported having undergone it at the appropriate time in their lives, as noted at the final assessment point of this study.

The subsequent tasks (2 and 3) focused on strategic actions to potentially manage DOR after its diagnosis had been established.

The study of genetic variants, particularly in gonadotropin receptors, has been suggested to understand situations where there are alterations in ovarian reserve, as well as to optimize the response to controlled ovarian stimulation. Single nucleotide polymorphisms (SNPs) in receptors such as *FSHR*, luteinizing hormone receptor (*LHR*), and *ESR* are described as determinants of follicular development, but their association with ovarian function needs further exploration.

From the literature review, it was concluded that the *FSHR* rs6166, *ESR1* rs2234693, and *ESR1* rs9340799 polymorphisms are among the most studied in relation to ovarian function. In contrast, polymorphic variation of the *LHR* is poorly associated with reproductive outcomes.

The association of *FSHR* rs6166 with the response to controlled ovarian stimulation (COS) is extensively studied, with the Ser680 allele often associated with poorer outcomes compared to the Asn680 allele. Although the association of *FSHR* rs6166 with ovarian reserve markers is less documented in the literature, its potential role in regulating the PI3K-Akt pathway, and affecting follicular activation, remains a topic of investigation and debate.

The literature review allowed us to understand that *ESR* polymorphisms in ovarian function are poorly documented, although there is evidence of the involvement of *ESR1* rs2234693 and *ESR1* rs9340799 in follicular development and response to COS.

The impact of *FSHR* rs6166, *ESR1* rs2234693, and *ESR1* rs9340799 polymorphisms in infertile populations with altered ovarian reserve was evaluated in our study, a retrospective observational study.

We found no statistically significant differences in the distribution of *FSHR* rs6166 ($p=.807$), *ESR1* rs2234693 ($p=.633$), and *ESR1* rs9340799 ($p=0.672$) genetic variants between DOR ($N=88$) and the control group ($N=219$). Similarly, among women under 35 years old, there were no significant differences in the distribution of *FSHR* rs6166 ($p=.301$), *ESR1* rs2234693 ($p=.582$), and *ESR1* rs9340799 ($p=.593$) variants, between DOR ($N=27$) and the control group ($N=112$).

In addition to DOR, PCOS is also an ovarian disorder that impairs female fertility by disrupting normal follicular development. Accordingly, DOR and PCOS are hypothesized to share common dysregulations in key factors affecting this process. In the PCOS population, previous studies showed conflicting data regarding the relationship between *FSHR* and *ESR1* polymorphisms and this condition. Our study found no significant association in the genotype distribution of *FSHR* rs6166 ($p=.522$) and *ESR1* rs2234693 ($p=.697$) between PCOS patients ($N=88$) and controls ($N=80$). However, the SS genotype of the *FSHR* rs6166 polymorphism was associated with higher FSH levels on the third day of the menstrual cycle ($p=.011$). No other differences in AMH, or AFC were found among different *FSHR* rs6166 and *ESR1* rs2234693 genotypes.

To deepen the study of the population with DOR, we reviewed the existing literature and have identified Forkhead Box L2 (*FOXL2*), Forkhead Box O3 (*FOXO3*), Factor in the Germline Alpha (*FIGLA*), Phosphatase and Tensin Homolog (*PTEN*), Insulin-like Growth Factor 1 (*IGF1*) and Insulin-like Growth Factor 2 (*IGF2*), Growth Differentiation Factor 9 (*GDF9*), Bone Morphogenetic Protein 15 (*BMP15*) and AKT Serine/Threonine Kinase 1 (*AKT1*) as potential targets, establishing a gene panel for analysis using NGS. This approach avoids the bias of an infertile population and is applicable to blood samples collected during AMH measurement, from participants of Task 1.

In summary, the study of genetic variants in both infertile populations and, in the future, in a non-infertile population with altered ovarian reserve aims to identify targets for tailored therapies, such as pharmacogenetics, and improve the response to ovarian stimulation of follicles sensitive to gonadotropins, as well as to provide a more accurate diagnosis of these cases. Additionally, new therapies have been suggested to activate primordial follicles, targeting populations with DOR where COS alone is insufficient. To explore this hypothesis, Task 3 involved optimizing a protocol to obtain PRP and investigating its impact on a pathway involved in follicular activation.

PRP has emerged as a promising therapy in regenerative medicine, and its applications have been extended to other areas, including reproductive medicine. However, the lack of standardization in PRP preparation protocols presents a challenge in achieving reproducible and accurate results. We aimed to optimize the PRP preparation protocol by investigating the impact of two different anticoagulants, sodium citrate (SD) and ethylenediamine tetraacetic acid (EDTA), and resuspension media, plasma versus sodium chloride (NaCl). Platelet recovery rate (PRR) was calculated and compared between groups, in addition to platelet activity and vascular endothelial growth factor (VEGF) released into plasma after PRP activation. The PRR was higher with EDTA in comparison to SC (51.04%vs29.85%, $p=.005$). Platelet activity was also higher, with a higher expression of two platelet antibodies, platelet surface P-38 Selectin (CD62p) and PAC-1 in the EDTA group. The concentration of VEGF was higher with SC in comparison to EDTA (628.73vs265.44 pg/mL, $p=.013$). PRR and VEGF levels were higher in PRP resuspended in plasma when compared to NaCl (61.60%vs48.61%, $p=.011$ and 41363.32vs159.83 pg/mL, $p=.005$, respectively).

After establishing a protocol starting with 15 mL of blood, centrifuged at 300g for 10 minutes followed by 700g for 15 minutes, we proceeded with an experimental *in vitro* study to evaluate the impact of PRP on human ovarian tissue fragments.

Two culture times were tested, 4 and 48 hours ($t=4h$ and $t=48h$) compared to the tissue evaluated immediately after thawing ($t=0h$). Additionally, the tissue was analysed with and without PRP ($t=4h$ Vs $t=4h+PRP$ and $t=48h$ Vs $t=48h+PRP$).

Throughout the study, tissue necrosis increased significantly from $t=0h$ to $t=48h$ ($p<.001$), starting at the 4-hour mark ($t=0h$ vs $t=4h$, $p<.002$), but plateaued beyond the initial 4 hours ($t=4h$ vs $t=48h$, $p=.71$). Follicular degeneration increased significantly from $t=0h$ to $t=48h$ ($p<.001$) and from $t=4h$ to $t=48h$ ($p<.001$), with no significant change between $t=0h$ and $t=4h$ ($p=.262$). Interstitial edema increased significantly over time, from 4 to 48 hours ($p<.001$), with no difference between $t=0h$ and $t=4h$ ($p=.070$).

Ki-67 positive follicles increased significantly from $t=0h$ to $t=48h$ ($p=.002$), while no variation was observed in Ki-67 positive stromal cells ($p=.716$). Caspase 3 positive follicles and stromal cells showed no significant differences across the three time-points ($p=.851$ and $p=.663$, respectively). The vascular density, number of blood vessels per square millimeter (mm^2), and the vascular area, the percentage of stromal cells positive for Factor VIII (FVIII) also did not show significant changes over time ($p = .937$ and $p = .284$, respectively).

PRP supplementation in the culture media significantly impacted the stromal characteristics of ovarian tissue. Tissue necrosis, show a decrease at 4-hour mark ($p=.002$), while follicular degeneration and interstitial edema decreased with supplementation with PRP, at 48- hour mark (respectively, $p=.002$ and $p<.001$).

Ki-67 staining increased in stromal cells with PRP supplementation ($p<.001$) at both 4 hours ($p<.001$) and 48 hours ($p=.007$), when compared with the group without PRP. Also, a significant increase in vascular area was noted with PRP supplementation ($p=.014$), specifically at the 48-hour culture period ($p=.024$).

PRP had no impact on the percentage of caspase 3 positive follicles and stromal cells ($p=.898$ and $p=.986$, respectively), nor on vascular density ($p=.272$).

p-Akt expression showed a significant reduction in weak staining and an increase in intense staining over time (both $p<.001$), with these effects amplified by PRP at both 4 hours ($p=.003$) and 48 hours ($p=.034$).

In conclusion, this study aimed to address unanswered aspects of DOR, developing strategies from enhancing knowledge of its impact on fertility, to exploring potential post-diagnosis therapies. Investing in information on this topic has proven effective in increasing knowledge, and we advocate for continuing these efforts. Tailored information did not result in any side effects, particularly in terms of post-intervention anxiety. However, the question of its overall value remains to be clarified. This could be addressed in a future follow-up study to better understand its long-term impact on reproductive plans.

NGS analysis aligns with our goals to investigate targets for focused diagnosis and personalized therapy in DOR cases, as well as elucidating the condition's aetiology.

Lastly, while some details may need further exploration to support the *in vitro* study of ovarian tissue culture with PRP, we have gained valuable insights into its effect as a stimulant of the PI3K-Akt follicular activation pathway.

Keywords

Diminished ovarian reserve (DOR) ; Interventions in knowledge ; ovarian reserve assessment ; single nucleotide polymorphisms (SNPs) ; follicular *in vitro* activation ; Platelet-rich plasma (PRP) ; phosphatidylinositol-3-kinase (PI3K)-protein kinase B(Akt) pathway (9)

Table of Contents

Dedicatória.....	v
Agradecimentos	vii
Funding	xi
Resumo.....	xiii
Abstract	xxiii
Table of Contents.....	xxxiii
List of Figures	xxxvii
List of Tables	xli
List of Abbreviations	xlvi
List of Scientific Publications.....	li
Thesis Overview.....	liii
1 Introduction to fundamental literature on ovarian reserve, with a focus on the study of diminished ovarian reserve (DOR).....	1
1.1 An overview of the sociodemographic context in female reproductive health and fertility	2
1.1.1 Definition of infertility.....	2
1.1.2 Global trends in declining fertility rates.....	2
1.1.3 Maternal age progression over time	3
1.1.4 Impact of increasing maternal age.....	3
1.1.5 Reproductive intentions and outcomes	6
1.1.6 Understanding fertility awareness.....	7
1.1.7 Reproductive health care.....	9
1.1.8 Initiatives in fertility education.....	12
1.1.9 Considerations in ovarian reserve screening	16
1. Introduction to fundamental literature on ovarian reserve, with a focus on the study of diminished ovarian reserve (DOR).....	19
1.2 Exploring ovarian reserve through testing, genetic markers, and follicular activation regulatory signalling pathways	20
1.2.1 Ovarian reserve	20

1.2.2	Revision of indicators assessing female reproductive function	24
1.2.3	Diminished Ovarian Reserve (DOR)	33
2	Strategies to increase the understanding of diminished ovarian reserve (DOR)	59
2.1	Overview of the project on fertility awareness, genetic testing, and interventions in the context of diminished ovarian reserve	60
2.	Strategies to increase the understanding of diminished ovarian reserve (DOR)	63
2.2	Task 1: intervention in knowledge through general and tailored information on ovarian reserve and diminished ovarian reserve (DOR)	64
2.2.1	Overview of existing literature: understanding and enhancing fertility knowledge	64
2.2.2	Objectives	75
2.2.3	Methods	75
2.2.4	Results	87
2.2.5	Discussion	146
2.	Strategies to increase the understanding of diminished ovarian reserve (DOR)	155
2.3	Task 2: genetic study of infertile populations with abnormal ovarian reserve	156
2.3.1	Introductory note, context, and objectives	156
2.3.2	Current insights into genetic exploration in cases of altered ovarian reserve.....	157
2.3.3	Methods	161
2.3.4	Results	172
2.3.5	Discussion	186
2.	Strategies to increase the understanding of diminished ovarian reserve (DOR)	189
2.4	Task 3: <i>in vitro</i> study of follicular activation through the incubation of human ovarian tissue with platelet-rich plasma (PRP).....	190
2.4.1	Objectives	190
2.4.2	Introduction to follicular activation, platelet-rich plasma (PRP) protocol, ovarian tissue culture and assessment techniques	190
2.4.3	Methods	198
2.4.4	Results	218
2.4.5	Discussion	263
3	Conclusions and future perspectives.....	269
3.1	What is already known on this topic?	269
3.2	What are the main conclusions drawn from this study?	270

3.3	What are the limitations?	272
3.3.1	Task 1: intervention in knowledge through general and tailored information on ovarian reserve and diminished ovarian reserve (DOR).....	272
3.3.2	Task 2: genetic study of infertile populations with abnormal ovarian reserve	273
3.3.3	Task 3: <i>in vitro</i> study of follicular activation through the incubation of human ovarian tissue with platelet-rich plasma (PRP)	274
3.4	What this study adds? How might this study affect research, practice or policy?	275
3.5	Anticipating tomorrow: What lies ahead?	276
3.6	Final remarks.....	277
4	Supplementary data	279
4.1	The study leaflet	279
4.2	Images illustrating the tool for determining AMH and AFC percentiles	280
4.3	Guide for reproductive counselling	281
4.4	The study video.....	287
4.5	Questionnaires.....	288
5	Bibliography	307

List of Figures

Figure 1 Age-related impairments and its impact on oocyte and embryo competence	4
Figure 2 First birth rate for all women aged 48+, in European countries	5
Figure 3 Oocyte retrieval for IVF an ICSI at ages 40+, in European countries	6
Figure 4 Dynamics of follicle numbers across a woman's lifespan, from gestation to menopause	21
Figure 5 Natural follicle depletion with aging	22
Figure 6 Schematic representation illustrating the principal pathways of communication via chemical messengers within the female reproductive system	23
Figure 7 Illustration of FSH actions in promoting follicular growth	25
Figure 8 Diagram illustrating the two-cell two-gonadotropin hypothesis	27
Figure 9 Regulation of ovarian reserve from initial recruitment to ovulation (involvement of Hippo and AKT & mTOR pathways)	46
Figure 10 Stimulation of the Akt pathway and disruption of Hippo signalling effect on follicular development	49
Figure 11 Image illustrating the role of growth factors derived from PRP in mediating follicular development	52
Figure 12 Schematic representation of the project, including the future perspectives	61
Figure 13 Inviting card from the project “aTuaFertilidade”	76
Figure 14 Diagram of the study design	77
Figure 15 “aTuaFertilidade” study logo	77
Figure 16 “aTuaFertilidade” leaflet	78
Figure 17 ROC curve of AMH for the prediction of DOR among infertile women, considering an AFC cut off value of 5	80
Figure 18 Participants flow chart	88
Figure 19 The distribution of contraceptive methods among participants	90
Figure 20 Graphical representation of the changes in the percentage of correct answers across the three time-points	99
Figure 21 Graphical representation of how preferences for oocyte cryopreservation vary at three time-points	105
Figure 22 Graph of opinions on fertility preservation for social reasons being available for all women, over study time	107
Figure 23 Diagram of the study design, extending from the initial phase to incorporate additional analysis	111
Figure 24 A graphical representation of the changes in knowledge about ovarian reserve across three time-points, considering the interaction time-group	122

Figure 25 Preferences for adoption (I), and oocyte cryopreservation (II) across three time-points, considering the interaction time-group	126
Figure 26 Participants acceptability to undergo an innovative treatment (PRP) if they knew in advance that they would have DOR	142
Figure 27 Diagram regarding the genetic polymorphism research	159
Figure 28 Flow diagram from initial search to final analysed articles included in the review about polymorphisms impact on ovarian reserve.....	163
Figure 29 Study design to evaluate the impact of <i>FSHR</i> rs6166, <i>ESR1</i> rs2234693 and <i>ESR1</i> rs9340799 polymorphisms on DOR.....	165
Figure 30 <i>FSHR</i> rs6166 genotypes	168
Figure 31 <i>ESR1</i> rs2234693 and <i>ESR1</i> rs9340799 genotypes.....	169
Figure 32 Schematic representation of the protocol to obtain PRP	200
Figure 33 Processing of human ovarian tissue.....	203
Figure 34 Representative scheme of the experimental design (ovarian tissue culture with PRP).....	205
Figure 35 Positive controls for immunohistochemistry	211
Figure 36 Schematic representation of digitalization process for glasses of H&E and IHC	212
Figure 37 PRR using different anticoagulants	219
Figure 38 PRR using different resuspension solutions	220
Figure 39 Representative image of ovarian tissue (H&E staining)	223
Figure 40 Follicular density over culture conditions	226
Figure 41 Representative images of stromal appearance over culture time, with and without PRP (H&E Staining)	230
Figure 42 Comparison of the values of tissue necrosis between the between various time-points and PRP supplementation conditions	233
Figure 43 Comparison of the values of follicular degeneration, between various time-points and PRP supplementation conditions	235
Figure 44 Comparison of the values of interstitial edema between various time-points and PRP supplementation conditions.....	237
Figure 45 Comparison of the values of total score the between various time-points and PRP supplementation conditions.....	239
Figure 46 Representative image of Ki-67 immunostaining.....	240
Figure 47 Comparison of the percentage values of Ki-67 expression in follicles between the various time-points and PRP supplementation conditions	244
Figure 48 Comparison of the values of Ki-67 expression in stromal cells over various time-points and PRP supplementation conditions	246
Figure 49 Representative image of Caspase 3 immunostaining	247
Figure 50 Comparison of the percentage values of caspase 3 expression in follicles (A) and stromal cells (B) over different culture times	249
Figure 51 Representative image of FVIII immunostaining.....	250

Figure 52 Comparison of the values of vascular density in various time-points and PRP supplementation conditions	252
Figure 53 Comparison of the mean values of vascular area between the between various time-points and PRP supplementation conditions.....	255
Figure 54 Representative image of p-Akt immunostaining.....	256
Figure 55 Comparison of the mean values of p-Akt week staining between various time-points and PRP supplementation conditions	260
Figure 56 Comparison of the on the mean percentage of follicle with p-Akt intense staining among the various time-points and PRP supplementation conditions ...	262

List of Tables

Table 1 Genetic contributions to DOR and POI	35
Table 2 PRP interventions to improve ovarian function: an overview of studies	53
Table 3 Summary of effectiveness of fertility education for increasing knowledge through various studies	66
Table 4 Summary of effectiveness of fertility education for changing intentions through various studies	68
Table 5 Summary of studies analysing the impact of fertility education and reproductive counselling on anxiety	70
Table 6 Impact of hormonal contraception on ovarian reserve markers	73
Table 7 Participants descriptives: distribution and characteristics of collected data for age, AMH, AFC, and origin.....	89
Table 8 Participants descriptives: contraceptive use distribution and percentages	90
Table 9 Participants descriptives: distribution of educational level and employment status among the study participants	91
Table 10 Participants descriptives: relationship status and current relationship ...	91
Table 11 Participants descriptives: habits and lifestyle	92
Table 12 Participants descriptives: menstrual cycle details	93
Table 13 Correlation analysis of key variables related to ovarian reserve.....	94
Table 14 Descriptives of perceived importance and certainty of future childbearing plans.....	95
Table 15 Participants levels of planning across various life domains	96
Table 16 Participants preferences and plans for childbearing: ideal and realistic expectations.....	97
Table 17 Assessment on knowledge about ovarian reserve and reproductive health, and knowledge average age at first childbirth in Portugal	98
Table 18 Percentage of global knowledge on ovarian reserve and DOR.....	99
Table 19 Association between percentage of global knowledge and participants' age and level of education.....	100
Table 20 Participants' responses regarding their intentions and plans for having children at three different time-points	101
Table 21 Comparison of ideal vs. planned childbearing age and number of children, at three time-points.....	101
Table 22 Distribution of responses regarding obstacles influencing future parenthood decisions at three different time-points	102
Table 23 Perception of infertility (DOR) as an obstacle to have children at the intended age over three different time-points.....	102

Table 24	Participants' preferred attitudes towards reproductive plans in case of DOR	103
Table 25	Comparison of the mean differences between seeking fertility treatments and two other reproductive options (oocyte donation and adoption) at three different time-points	104
Table 26	Data on participants' willingness to undergo fertility treatments to "freeze" their oocytes if they knew in advance about having DOR	106
Table 27	Participants' opinions regarding whether fertility preservation for social reasons should be available to all women	106
Table 28	Data on participants' feelings regarding the possibility of using donated gametes	108
Table 29	Analysis of participants' motivation to consider voluntary donation of gametes (oocytes) to help women with DOR	108
Table 30	Participants' opinions regarding whether all women should have access to an easy and inexpensive way to evaluate ovarian reserve	109
Table 31	Detailed analysis of various subgroups categorized by ovarian reserve status	112
Table 32	Results on BMI and age at first menstruation for ovarian reserve subgroups	113
Table 33	The data on the significance of parenthood and participants' confidence in their decision to have children ovarian reserve subgroups	114
Table 34	The data on prioritized life goals are detailed across ovarian reserve subgroups	115
Table 35	Participants' ideal number of children, perceived capacity to have children, ideal age for childbearing, and planned age for starting a family across ovarian reserve subgroups	116
Table 36	Comparison of AMH, and AFC values across ovarian reserve subgroups (DOR1 and NOR)	116
Table 37	Comparison of AMH, and AFC values across ovarian reserve subgroups (DOR2, INT and NOR)	117
Table 38	Comparison of the age at which their mother's entered menopause across ovarian reserve subgroups	117
Table 39	Comparison of use of hormonal contraception across ovarian reserve subgroups	118
Table 40	Correlation between the use of hormonal contraception and AMH and AFC values, across different ovarian reserve subgroups	119
Table 41	Correlation analysis between BMI and AMH and AFC among the ovarian reserve subgroups	120
Table 42	Changes in the percentage of global knowledge, considering the interaction time-group	121

Table 43 Participants' responses regarding their intentions and plans for having children at three different time-points, considering the considering the interaction time-group	123
Table 44 Distribution of responses regarding obstacles influencing future parenthood decisions, considering the interaction time-group	124
Table 45 Responses of participants regarding adopting a child and cryopreserve oocytes, if they knew in advance that they would have DOR, considering the interaction time-group	125
Table 46 Participants responses regarding their options if they knew in advance that they would have DOR, considering the interaction time-group	128
Table 47 Data on participants' feelings regarding the possibility of using donated gametes, considering the interaction time-group	129
Table 48 Participants' opinions regarding whether fertility preservation for social reasons should be available to all women, considering the interaction time-group	130
Table 49 Participants' opinions regarding whether all women should have access to an easy and inexpensive way to evaluate ovarian reserve, considering the interaction time-group	131
Table 50 Participants' willingness to consider voluntary donation of gametes (oocytes) to help women with DOR, considering the interaction time-group	131
Table 51 Interpretation of the information received by participants regarding their ovarian reserve status	133
Table 52 Participants' experience regarding the evaluation of their ovarian reserve and undergoing reproductive counselling	135
Table 53 Data on the factors motivating individuals to evaluate their ovarian reserve	136
Table 54 Participants' views on whether they should have received information about ovarian reserve earlier	137
Table 55 Participants opinions on the importance of being informed about and evaluating ovarian reserve, considering the interaction of between time and groups	138
Table 56 Perspectives on the importance of reproductive counselling consultations	139
Table 57 STAI scores with various parameters such as mean and SD for different subgroups	140
Table 58 Participants' acceptability to undergo an innovative treatment (PRP) if they knew that they would have DOR	141
Table 59 Participants' acceptability to undergo an innovative treatment (PRP) if they knew that they would have DOR, considering the interaction between time and groups	142

Table 60 Assessing the informative content: participants age and knowledge levels	143
Table 61 Assessing the informative content: summary of the website evaluation results	144
Table 62 Assessing the informative content: summary of the video evaluation results	145
Table 63 Description of investigated SNPs of <i>FSHR</i> and <i>ESR1</i>	166
Table 64 Restriction endonuclease enzymes utilized for RFLP analysis	167
Table 65 Summary of selected genes for NGS analysis: implications in reproductive health, and their association with DOR and POI	171
Table 66 Summary of the findings related to <i>FSHR</i> polymorphisms, the number of studies examining each polymorphism, the main findings observed, and references	176
Table 67 Summary of the findings related to <i>LHR</i> polymorphisms, the number of studies examining each polymorphism, the main findings observed, and references	177
Table 68 Summary of the findings related to <i>ESR</i> polymorphisms, the number of studies examining each polymorphism, the main findings observed, and references	180
Table 69 Genotype distribution of <i>FSHR</i> rs6166, <i>ESR1</i> rs2234693 and <i>ESR1</i> rs9340799 polymorphisms among DOR and control groups	181
Table 70 Genotype distribution of <i>FSHR</i> rs6166, <i>ESR1</i> rs2234693 and <i>ESR1</i> rs9340799 polymorphisms among DOR and control groups, normalized per age < 35 years old	183
Table 71 Genotype distribution of <i>FSHR</i> rs6166 and <i>ESR1</i> rs2234693 polymorphisms among PCOS case and control groups	184
Table 72 Results of analysis of the association between <i>FSHR</i> rs6166 and <i>ESR1</i> rs2234693 polymorphisms and clinical and biochemical data in PCOS patients	185
Table 73 Clinical characteristics of patients whose ovarian tissue was used for the experiment	202
Table 74 Overview of the study culture conditions	204
Table 75 H&E protocol used at pathological anatomy of ULS Coimbra	206
Table 76 Summary of the immunohistochemistry protocol for Ki-67 and caspase 3 from the department of pathology of ULS Coimbra	208
Table 77 Summary of the immunohistochemistry protocol for Factor VIII from the department of pathology of ULS Coimbra	209
Table 78 Summary of the immunohistochemistry protocol for p-Akt from the department of pathology of ULS Coimbra	210
Table 79 Follicular classification according to its developmental stage	214
Table 80 Stromal classification	216

Table 81 Comparison of PRP obtained after blood sample collection with different anticoagulants, SC versus EDTA	219
Table 82 Comparison of PRP resuspension solution with plasma versus NaCl.....	220
Table 83 Observed power for various parameters under the effects of time, PRP supplementation, and their interaction (time*PRP), regarding two-way ANOVA analysis.....	221
Table 84 Follicular density, both total and categorized by developmental stage ..	222
Table 85 Effect of time on follicular parameters in ovarian tissue, presenting mean values over time	224
Table 86 Effect of PRP supplementation on follicular density	225
Table 87 Effect of the interaction between time and PRP supplementation on follicular density	226
Table 88 Mean values for tissue necrosis, follicular degeneration, interstitial edema, and total score	227
Table 89 Mean values for tissue necrosis, follicular degeneration, interstitial edema, and the total score across different time-points.....	227
Table 90 Mean values for tissue necrosis, follicular degeneration, and interstitial edema, and the total score between groups supplemented with PRP and those that were not supplemented with PRP.....	228
Table 91 Effect of the interaction between time and PRP supplementation on tissue necrosis, follicular degeneration, interstitial edema, and the total score	229
Table 92 Results of post-hoc Tukey tests conducted to analyse the differences in tissue necrosis between various time-points and PRP supplementation conditions	232
Table 93 Results of post-hoc Tukey tests examining differences in follicular degeneration across various time-points and PRP supplementation conditions .	234
Table 94 Post-hoc Tukey test results examining differences in interstitial edema across various time-points and PRP supplementation conditions	236
Table 95 Results of post-hoc Tukey tests investigating differences in the total score of tissue damage across various time-points and PRP supplementation conditions	238
Table 96 percentage of Ki-67 positive follicles and Ki-67 positive stromal cells ...	240
Table 97 Mean percentage of Ki-67 positive follicles and Ki-67 positive stromal cells at different time-points.....	241
Table 98 Mean percentage of Ki-67 positive follicles and Ki-67 positive stromal cells in the groups supplemented with PRP and in those that were nor supplemented with PRP	241
Table 99 Effect of the interaction between time and PRP supplementation for the percentage of positive follicles and positive stromal cells for Ki-67.....	242

Table 100 Results of post-hoc Tukey tests examining differences in the percentage of Ki-67 positive follicles among various time-points and PRP supplementation conditions.....	243
Table 101 Post-hoc Tukey tests results evaluating differences in the percentage of Ki-67 positive stromal cells across various time-points and PRP supplementation conditions.....	245
Table 102 Mean values for the percentage of positive follicles and positive stromal cells for caspase 3	247
Table 103 Mean values for the percentage of caspase 3 positive follicles and positive stromal cells at different time-points.....	248
Table 104 Comparison of the mean percentage of caspase 3 positive follicles and positive stromal cells between the groups supplemented with PRP and those not supplemented with PRP.....	248
Table 105 Effect of the interaction between time and PRP supplementation for the percentage of caspase 3 positive follicles and positive stromal cells.....	249
Table 106 Mean values for vascular density and vascular area.....	250
Table 107 Effect of time on vascular density and vascular area	251
Table 108 Effect of PRP on vascular density and vascular area	251
Table 109 Effect of the interaction between time and PRP supplementation on vascular density and vascular area	252
Table 110 Results of post-hoc Tukey tests assessing differences in the vascular area among various time-points and PRP supplementation conditions	254
Table 111 Mean values for the percentage of follicles with weak and intense p-Akt staining.....	256
Table 112 Mean values for the percentage of follicles with weak and intense p-Akt staining at different time-points	257
Table 113 Mean values for the percentage of follicles with weak and intense p-Akt staining in groups supplemented with PRP and those that were not supplemented with PRP	257
Table 114 Effect of the interaction between time and PRP supplementation for the percentage of follicles with p-Akt weak and intense staining.....	258
Table 115 Results of post-hoc Tukey tests analysing differences in the percentage of follicles with weak p-Akt staining across various time-points and PRP supplementation conditions	259
Table 116 Results of post-hoc Tukey tests analysing differences in the percentage of follicles with intense p-Akt staining across various time and PRP supplementation	261

List of Abbreviations

2D	Two-dimension
3D	Three-dimensional
ACD	Citrate dextrose
ADP	Adenosine diphosphate
AFC	Antral follicle count
AKT1	Akt serine/threonine kinase 1
AMH	Anti-Müllerian hormone
AMHR2	Anti-Müllerian hormone receptor 2
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
AUC	Area under de curve
bFGF	basic fibroblast growth factor
BIRC	Baculoviral inhibitors of apoptosis repeat-containing
BMI	Body mass index
BMP15	Bone morphogenic protein 15
BMPR1B	Bone morphogenetic protein receptor type 1b
BNC1	Basonuclin 1
bp	Base pairs
BRCA1/2	Breast cancer gene 1/2
cAMP	Cyclic adenosine monophosphate
CCN	Cysteine-rich angiogenic protein 61, connective tissue growth factor, and nephroblastoma
CD42b	Glycoprotein 1b
CD62P	Platelet surface p-selectin
CDKN1B/P27	Cyclin dependent kinase inhibitor 1b/p27
CFKS-J	Cardiff fertility knowledge scale
CITED2	Cbp/p300 Interacting transactivator with Glu/Asp rich carboxy-terminal domain 2
COL4A6	Collagen type IV alpha 6 chain
COS	Controlled ovarian stimulation
CREB	Camp-responsive element binding-protein
CSS	Cascading style sheets
CTGF	Connective tissue growth factor
CV	Coefficient of variation
CYP19A1	Cytochrome p450 family 19 subfamily a member 1
DACH2	Dachshund family transcription factor 2
DGORN	Department of gynaecology obstetrics, reproduction, and neonatology
DIAPH2	Diaphanous related formin 2
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DOR	Diminished ovarian reserve

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EIF4ENIF1	Eukaryotic translation initiation factor 4e nuclear import factor 1
ELISA	Enzyme-linked immunosorbent assay
ESHRE	European society of human reproduction and embryology
ESR	Estrogen receptors
FABMs	Fertility awareness-based methods
FAC	Fertility assessment and counselling
FANCM	Fanconi anaemia complementation group m
FertiSTAT	Fertility status awareness tool
FET	Frozen embryo transfer
FGF	Fibroblast growth factor
FIGLA	Folliculogenesis-specific basic helix-loop-helix
FMR1	Fragile x mental retardation 1
FOXL2	Forkhead box l2
FOXO1	Phosphorylation and regulation of Forkhead box O1
FOXO3	Forkhead box O3
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
FVIII	Factor VIII
FXPOI	Fragile X-associated premature ovarian insufficiency
GDF9	Growth differentiation factor 9
GnRH	Gonadotropin-releasing hormone
GPR3	G protein-coupled receptor 3
GPs	general practitioners
GREM1	Gremlin 1
Gas	G alpha subunit
H&E	Haematoxylin and eosin
hCG	Human chorionic gonadotropin
HIF1 α	Hypoxia-inducible factor 1 α
hMG	Human menopausal gonadotropin
HRP	Horseradish peroxidase
HSA	Human serum albumin
HTML	Hypertext markup language
IFFS	The international federation of fertility societies
IGF1/2	Insulin-like growth factor 1/2
IHC	Immunohistochemistry
IUI	Intra-uterine insemination
IL-8	Interleukin-8
INHA	Inhibin a
IQR	Interquartile range
IRHEC	The international reproductive health education collaboration
IVF	In vitro fertilization
KGF	keratinocyte growth factor
LATS1/LATS2	Large tumor suppressor kinase 1/2
LBR	Live birth rate

LH	Luteinizing hormone
LHCGR	Luteinizing/choriogonadotropin receptor
LHR	Luteinizing hormone receptors
LHX9	Lim homeobox 9
LIF	Leukemia inhibitory factor
MEM	Eagle's minimum essential medium
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
MST1/2	Mammalian ste-20 like kinase 1/2
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NaCl	Sodium chloride
NANOS3	Nanos homolog 3
NGS	Next generation sequencing
NOBOX	Newborn ovary homeobox
NOR	Normal ovarian reserve
NOTCH2	Neurogenic locus notch homolog protein 2
NR5A1	Nuclear receptor subfamily 5 group a member 1
OHSS	Ovarian hyperstimulation syndrome
p-Akt	Phosphorylated-Akt
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PDAF	Platelet-derived angiogenic factor
PDGF	Platelet-derived growth factor
PGCs	Primordial germ cells
PhD	Doctor of philosophy
PI3K-Akt	Phosphatidylinositol 3-kinase - protein kinase B
PKA	Protein kinase A
POF1B	Premature ovarian failure 1B
POI	Premature ovarian insufficiency
POSEIDON	Patient-oriented strategies group encompassing individualized oocyte number
PRP	Platelet-rich plasma
PRR	Platelet recovery rate
PTEN	Phosphatase and tensin homolog
RCT	Randomized controlled trial
RFLP	Restriction fragment length polymorphism
r-hFSH	Recombinant human FSH
ROC	Receiver operating characteristic
RPM	Rotations per minute
SALL4	Sal-like protein 4
SC	Sodium citrate
SD	Standard deviation
SDG	Sustainable development goal
SF1	Splicing factor 1
SNPs	Single nucleotide polymorphisms
STAI; STAI-S; STAI-T	State-trait anxiety inventory; state; trait
STIs	Sexually transmitted infections

TAZ	Pdz-binding motif
TEAD	Transcriptional factors
TGFβ	Transforming growth factor β
TSC1/2	Tuberous sclerosis complex 1/2
UK	United Kingdom
ULS Coimbra	Unidade local de saúde de Coimbra
USA	United States of America
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor A
vWF	Von Willebrand factor (factor VIII-related antigen)
WHO	World health organization
WT1	Wilms tumour 1
YAP	Yes-associated protein

List of Scientific Publications

Papers related to this doctoral thesis

Vieira, I. H., Carvalho, A. F., Almeida Reis, S., Carreira, A. L., Dias, C., Fernandes, S., Ferreira, A. F., Rodrigues, D., Sousa, A. P., Ramalho-Santos, J., Ramalhinho, A. C., Moura Ramos, M., Paiva, I., Cortesão, P., & Almeida-Santos, A. T. (2023). Association Between Follicle-Stimulating Hormone Receptor (FSHR) rs6166 and Estrogen Receptor 1 (ESR1) rs2234693 Polymorphisms and Polycystic Ovary Syndrome Risk, Phenotype, and Reproductive Outcomes in an Infertile Portuguese Population. *Cureus*, 15(3). <https://doi.org/10.7759/cureus.35690>

Carvalho, A., Ferreira, A. F., Soares, M., Santos, S., Tomé, P., Machado-Simões, J., Pais, A. S., Sousa, A. P., Paiva, A., & Almeida-Santos, T. (2024). Optimization of Platelet-Rich Plasma Preparation for Regenerative Medicine: Comparison of Different Anticoagulants and Resuspension Media. *Bioengineering*, 11(3), 209. <https://doi.org/10.3390/bioengineering11030209>

Papers unrelated to this doctoral thesis

Almeida-Reis, S., Carvalho, A., Dias, C., Brito, R., Silva, R., Almeida-Santos, T., Ramalho-Santos, J., & Sousa, A. P. (2024). Mitochondrial Dysfunction in Advanced Maternal Aged Cumulus Cells: A Possible Link to ATP Synthase Impairment? *Biomolecules*, 14(3), 281. <https://doi.org/10.3390/biom14030281>

Ferreira, A. F., Machado-Simões, J., Moniz, I., Soares, M., Carvalho, A., Diniz, P., Ramalho-Santos, J., Sousa, A. P., Lopes-da-Costa, L., & Almeida-Santos, T. (2024). Chemical reversion of age-related oocyte dysfunction fails to enhance embryo development in a bovine model of postovulatory aging. *Journal of Assisted Reproduction and Genetics*. <https://doi.org/10.1007/s10815-024-03151-4>

Ferro, B., Sousa, F., Carvalho, A., Ferreira, A. F., Sousa, L., Sousa, A. P., Parada, B., Cortesão, P., & Santos, T. A. (2023). Altered Sperm Analysis, Where Are We Now? The Reality of the Reproductive Medicine Unit of a Tertiary University Center. *International Journal of Infertility & Fetal Medicine*, 14(2), 85–93. <https://doi.org/10.5005/jp-journals-10016-1315>

Thesis Overview

This thesis is organized into three sections:

1. Introduction, *Introduction to fundamental literature on ovarian reserve, with a focus on the study of diminished ovarian reserve (DOR)*: Provides a comprehensive review of the fundamental literature on ovarian reserve, with a focus on DOR, representing the current state of the art.
2. Methods, *Strategies to increase the understanding of diminished ovarian reserve (DOR)*: outlines the applied methodologies used to advance understanding of DOR.
3. Conclusions, *Conclusions and future perspectives*: Discusses the findings in terms of strengths, weaknesses, clinical and research applicability, and future directions.

Section 1 is divided in two parts.

Part 1 gives the sociodemographic context in reproductive health and fertility. It focuses into various factors influencing fertility, including global trends in declining fertility rates, the impact of increasing maternal age, and the rising use of fertility treatments. Advanced maternal age is introduced as a trigger for global efforts in reproductive health care, considering its role in demographics and its influence on fertility education and reproductive counselling. Additionally, it further explores reproductive intentions and outcomes, shedding light on delayed childbearing motivations and implications, and the gap between reproductive intentions and family size. Fertility awareness is a key theme, studying its levels, interventions, and reasons for prioritization. The role of reproductive health care and initiatives in fertility education is also discussed, with a global perspective, an overview of the international reproductive health education collaboration (IRHEC), and insights into the Portuguese scenario. The section concludes by examining the impact of fertility awareness and ovarian reserve testing, considering its effectiveness in increasing knowledge, altering intentions, and potential side-effects. The final section specifically focuses on the significance of ovarian reserve screening.

Part 2 presents a literature review, focusing on key concepts of the ovarian reserve. It explores ovarian reserve and the regulators of female reproductive function, analysing potential biomarkers for ovarian reserve assessment. A critical examination of DOR follows, emphasizing the challenges faced by women with DOR when attempting to

conceive. DOR is thoroughly addressed, encompassing its definition, origin, genetic markers, and the role of various genes in this condition. Strategies for managing women with DOR are explored, including pharmacogenetics, adapted protocols of controlled ovarian stimulation (COS), acupuncture, and follicular activation. The section concludes with a detailed section on platelet-rich plasma (PRP), including its application in cases of DOR.

The transition from the introductory phase, which revisited the literature and laid the groundwork for the project, to the project tasks is given by the next section. Section 2 establishes the research questions and objectives, providing the methodological strategies adopted to deepen the knowledge on DOR. Specifically, this section is divided into 3 parts, covering to the 3 Tasks of these project, including methodologies employed, data analyses, and discussion of the key findings.

Section 3 synthesizes the findings from the research project, offering conclusions based on the data. It also outlines the weaknesses and limitations, as well as the potential opportunities for future research, suggesting areas for further exploration and development in the topic of DOR.

1 Introduction to fundamental literature on ovarian reserve, with a focus on the study of diminished ovarian reserve (DOR)

1.1 An overview of the sociodemographic context in female reproductive health and fertility

A comprehensive exploration of the present status of sociodemographic factors influencing female reproductive health and fertility awareness.

1.2 Exploring ovarian reserve through testing, genetic markers, and follicular activation regulatory signalling pathways

Exploring fundamental concepts within diminished ovarian reserve.

1.1 An overview of the sociodemographic context in female reproductive health and fertility

1.1.1 Definition of infertility

According to the world health organization (WHO), infertility is characterized as a medical condition affecting the male or female reproductive system, marked by the inability to conceive after regular unprotected sexual intercourse for 12 months or more (WHO, 2023). Globally, nearly 1 in 6 adults, accounting for approximately 17.5% of the adult population, suffer from infertility. Addressing infertility plays a critical role in advancing both sustainable development goal (SDG) 3, which aims to ensure healthy lives and promote well-being across all age groups, and SDG 5, dedicated to achieving gender equality and empowering women and girls (WHO, 2023).

Infertility stands out as one of the most prevalent chronic diseases in the age group 25-44 compared to other serious chronic diseases (WHO, 2018), underscoring the urgency for action. WHO emphasizes the importance of recognizing infertility as a preventable disease in health policies (WHO, 2018).

1.1.2 Global trends in declining fertility rates

We are currently facing the reality of declining fertility rates in numerous countries, coupled with a rising trend in maternal age and an increased reliance on fertility treatments (WHO, 2018). This raises pertinent questions about the implications of diminishing family planning, the dynamics of fertility, and the surge in infertility, all of which have significant sociodemographic consequences (Cheng et al., 2022).

In demographic terms, it is important to look at the total fertility rate, defined by the international glossary on infertility and fertility care as the “average number of live births per woman” (Zegers-Hochschild et al., 2017).

Currently, approximately half of the countries worldwide have fertility rates that are below the replacement level. Without accounting for migration, numerous nations are expected to experience a population decline of over 50% from 2017 to 2100 (Fauser, 2019; Vollset et al., 2020). According to the United Nations data total fertility rate has consistently decreasing across various countries and, in western Europe, it stands at approximately 2, a number notably proximate to the critical threshold for renewing a generation. As well as western Europe we have the same trend in eastern Europe, Latin America, Asia and Africa (United Nations, 2022). Portugal registered a total fertility rate of 1.43 in 2022 (PORDATA, 2022).

The International Federation of Fertility Societies (IFFS) has recently released a document addressing the global decline in population, which synthesizes current research and emphasizes the urgency of implementing various interventions. Highlighted among these interventions is

education on family building, acknowledging that tailored approaches are essential given the diversity of contexts and needs (Fauser et al., 2024).

Additionally, we are witnessing a significant rise in childbearing among individuals aged 40 and above across Europe, the United States (US), and Japan. This trend is particularly pronounced in South European countries (Philipov 2017).

1.1.3 Maternal age progression over time

The age at which women give birth to their first child has seen a notable increase in recent decades, especially in western Europe countries (Eurostat, 2023). This trend is evident, reflecting a substantial rise in first birth rates among women aged 30 and above, exceeding 40% in Europe (OCDE, 2019). In Portugal, the average age of women at first childbirth was 30.6 years old in 2023 (PORDATA, 2023).

1.1.4 Impact of increasing maternal age

1.1.4.1 Decreasing chance of getting pregnant

The likelihood of spontaneous pregnancy begins to decline between the ages of 30 and 35 (Menken et al., 1986), a trend that is similarly noted when employing treatments involving assisted reproductive technology (ART) (Gliozheni et al., 2023; Piette et al., 1990).

For a healthy 20 years old woman, the probability of becoming pregnant per menstrual cycle is approximately 34%. By the age of 30, this probability is halved to around 17% per cycle. As a woman reaches 37 years, the likelihood further diminishes to 8% per cycle. By the age of 45, the chance of achieving a live birth drops significantly, with only about 0.5% probability per menstrual cycle (Choi et al., 2023; Petersen, 2016).

Even though there is no consensus about the definition of advanced maternal age, varies from 35 to 45 years old, there is no doubt about age impact in the capacity to conceive (Ferraretti et al., 2011). This occurs due to a decrease in the quantity and quality of oocytes (Coxworth & Hawkes, 2010).

The oocyte competence is strongly affected by age due to an increased incidence of aneuploidies and a decreased mitochondrial activity (Eichenlaub-Ritter et al., 2011). Therefore, oocyte ageing is associated with a defective energy production and altered metabolism, as well as abnormal meiosis and poor embryo development, as illustrated in Figure 1 (Capalbo et al., 2017; Cimadomo et al., 2018; Franasiak et al., 2014). Moreover, age-related abnormal vascularization, oxidative stress and free radical imbalance contribute to a decreasing of reproductive success in natural conception (Hansen et al., 2011; La Marca & Sunkara, 2014; Pellestor et al., 2006; Sauer, 2015).

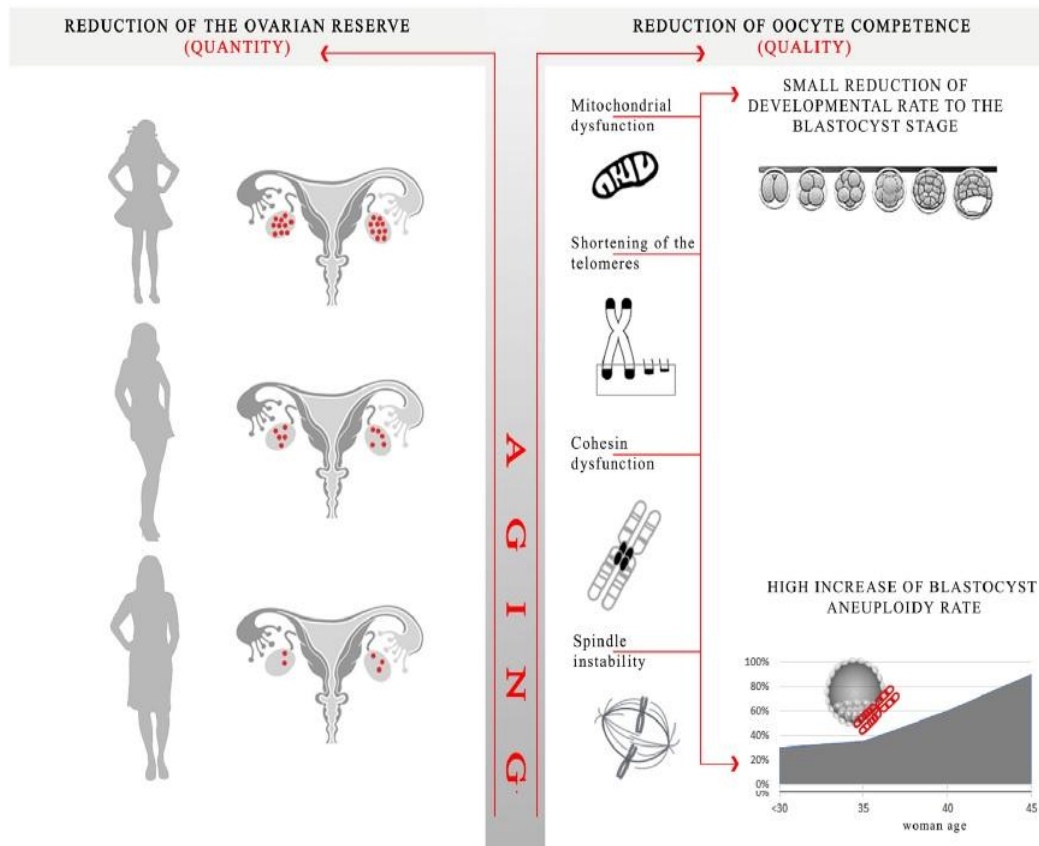


Figure 1 Age-related impairments and its impact on oocyte and embryo competence

The image illustrates age-related impairments and their impact on oocyte and embryo competence. It is divided into two main sections: "Reduction of the Ovarian Reserve (Quantity)" and "Reduction of Oocyte Competence (Quality)," both of which contribute to the effects of aging on reproductive health.

Reduction of the Ovarian Reserve (Quantity): indicates a reduction in the ovarian reserve with increasing age.

Reduction of Oocyte Competence (Quality): details 1) Mitochondrial Dysfunction (mitochondrial function decline with age); Shortening of Telomeres (a telomere at the end of a chromosome getting shorter, which is a marker of cellular aging); Cohesin Dysfunction (defective cohesin protein complex, which is crucial for chromosome segregation); Spindle Instability (disorganized spindle apparatus, crucial for proper chromosome alignment and segregation during cell division); Developmental Rate Reduction (reduction in the developmental rate to the blastocyst stage); High Increase in Blastocyst Aneuploidy Rate (increase in blastocyst aneuploidy rate as a woman ages, particularly from below 30 to above 45 years).

Adapted from Cimadomo et al., 2018.

1.1.4.2 Rising usage of fertility treatments

As a consequence of increasing childbearing age, there is an increased demand for ART treatments (Barbakadze et al., 2015; Geyter et al., 2020).

According to the human fertility database, when considering European countries, there is an observable increase in the number of births occurring beyond the biological limits (Figure 2), with this trend primarily linked to ART (Philipov, 2017).

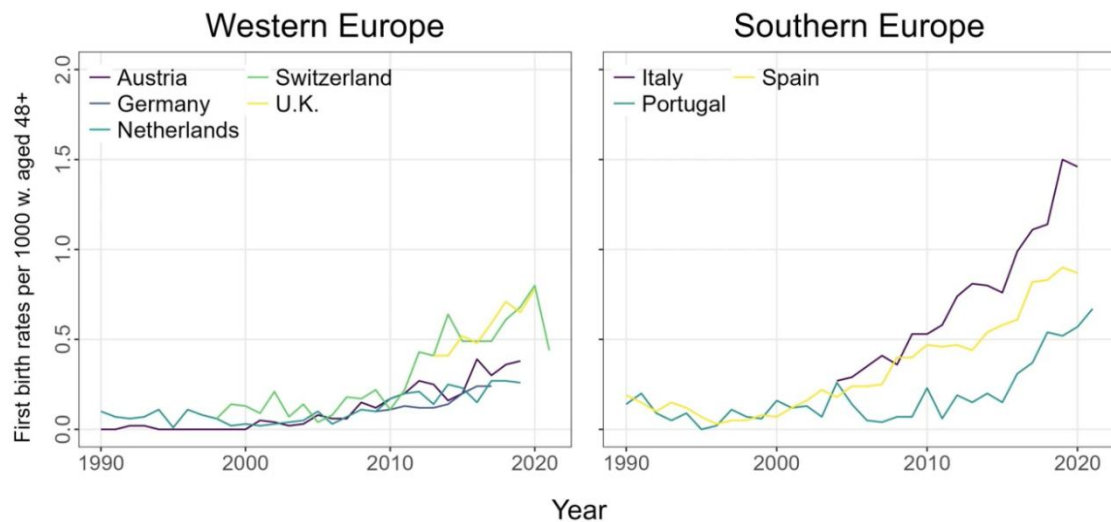


Figure 2 First birth rate for all women aged 48+, in European countries

The image contains two graphs comparing first birth rates per 1,000 women aged 48+ in Western Europe and Southern Europe from 1990 to 2020, indicating a trend of increasing first birth rates at that age.

Western Europe: shows an increasing trend in first birth rates among women aged 48+ starting from the early 2000s, with more pronounced rises in some countries like the U.K. and Switzerland.

Southern Europe: Also shows increasing trends, with notable rises in Italy and Spain starting from around 2000 and continuing to grow steadily. Data from human fertility database, <https://www.humanfertility.org/>

U.K.: United Kingdom

The annual count of ART cycles has been increasing, surpassing 2.5 million cycles conducted globally in 2019 (Fauser, 2019). Despite the increasing usage of fertility treatments worldwide, the likelihood of success is still modest, with only about 25–30% of attempts resulting in a live birth (De Mouzon et al., 2020). Nonetheless, many individuals undergoing ART tend to have unrealistically high expectations for success (Dvroe et al., 2022).

Importantly, the use of ART is growing, with an increasing prevalence among women aged 40 and above over time, as indicated in the European society of human reproduction and embryology (ESHRE) (Gliozheni et al., 2023) and as illustrated in Figure 3.

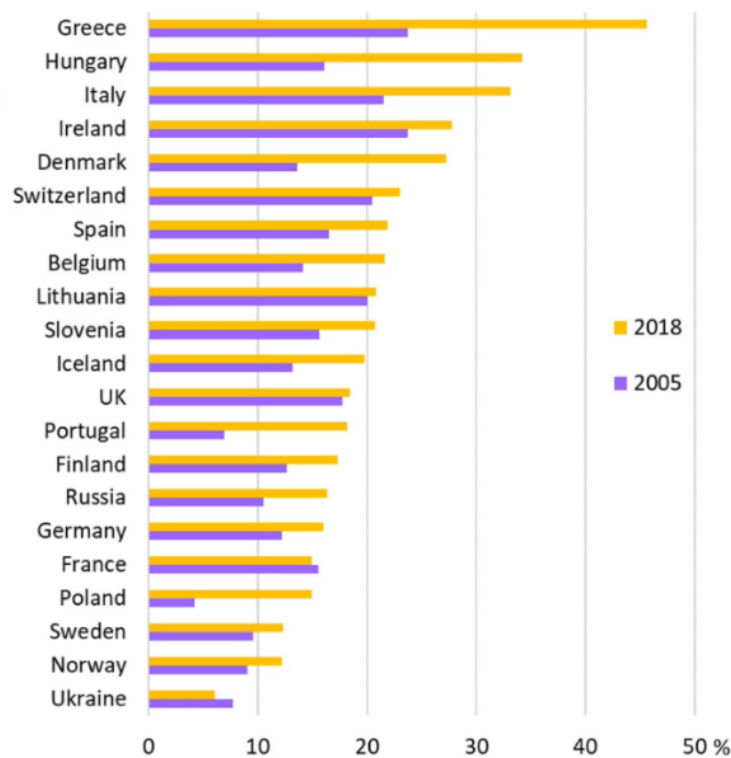


Figure 3 Oocyte retrieval for IVF and ICSI at ages 40+, in European countries

The image is a horizontal bar chart comparing the percentage of IVF and ICSI treatments at age 40+ across various European countries for the years 2005 and 2018. Each country is represented by two bars: a purple bar for 2005 and an orange bar for 2018.

The y-axis shows the percentage difference over the 13-year period. Over the 13-year period, all countries show an increase from 2005 to 2018. The largest increases are observed in Greece, Hungary, and Italy. The smallest increases are seen in France, Poland, Sweden, Norway, and Ukraine. Data from ESHRE.

IVF: *in vitro* fertilization; ICSI: *intracytoplasmic injection*; UK: United Kingdom.

1.1.5 Reproductive intentions and outcomes

1.1.5.1 Delayed childbearing implications: the gap between reproductive intentions and family size

Delayed childbearing often leads individuals and couples to have fewer children than expected, or none at all, with many expressing regret for that (Mills et al., 2011).

On an individual level, women who try to conceive later in life are less likely to have children by the conclusion of their reproductive years. The evidence supporting this is the robust association between the timing of childbirth in a country and the percentage of women who are childless and facing infertility. The later individuals had their children, the greater is the likelihood of experiencing infertility and remaining childless (Beaujouan, 2020).

A growing proportion of people may be reaching biological reproductive limits, and this number will probably continue to grow as fertility is delayed further. In consequence to that, 1-8% of people tried to have a first child but did not manage, and this was larger in countries where parenthood was postponed most (Philipov, 2017).

The number of unintentionally childless women has increased over time, and this phenomenon is closely linked to the increasing age of mothers at the time of childbirth. Consequently, more women express a desire to have a child between the ages of 35 and 39, but do not have one by the time they reach 45 (Beaujouan, 2022).

1.1.5.2 Motivations for delayed childbearing

Not all women who delay childbirth necessarily desire to have children later in life. Numerous factors come into play, encompassing extended periods spent on education (Bhrolcháin & Beaujouan, 2012), and economic uncertainty and youth unemployment (Kreyenfeld, 2015). Additionally, competing activities (Testa & Stephany, 2017), longer life expectancy (Beaujouan & Toulemon, 2021), changes in partnerships (Klesment & Bavel, 2017), and shifts in values and attitudes towards maternity (Lesthaeghe, 2010), all contribute to the trend of delayed childbearing. In a recent study on the impact of the COVID-19 pandemic, English women identified several factors influencing their decision about the age of childbearing. The most frequently chosen factor was 'developing my career,' followed by the desire for stability and balance in life, and the importance of finding the right time and gaining life experiences (Harper & Botero-Meneses, 2022).

Accordingly, Temmeesen et al. reviewed the existing information about timing of motherhood, among childless women. Women in their reproductive years are aware of the significant decision they face about when or if to have children, emphasizing the importance of choosing the 'right time.' While some women may hesitate to start a family for diverse reasons, there is an underlying fear of regretting the decision to delay childbearing (Temmesen et al., 2023).

1.1.6 Understanding fertility awareness

1.1.6.1 Definition of fertility awareness

Fertility awareness is defined by the international glossary on infertility and fertility care as “the understanding of reproduction, fecundity, fecundability, and related individual risk factors (e.g. advanced age, sexual health factors such as sexually transmitted infections, and life style factors such as smoking, obesity) and non-individual risk factors (e.g. environmental and work place factors); including the awareness of societal and cultural factors affecting options to meet reproductive family planning, as well as family building needs” (Zegers-Hochschild et al., 2017).

Enhancing public awareness of modifiable factors affecting fertility among both women and men of reproductive age, has the potential to reduce the incidence of infertility and the reliance on infertility treatments (Barker et al., 2018; Fleming et al., 2018; Stephenson et al., 2018).

1.1.6.2 Fertility awareness levels

Fertility awareness is low in different countries and in different populations (Bretherick et al., 2010; Hammarberg et al., 2013, 2017; Lampic et al., 2006; Pedro et al., 2018), Portugal included (Almeida-Santos et al., 2017). Additionally, there is a tendency for individuals to overestimate the likelihood of achieving parenthood through *in vitro* fertilization (IVF), leading to unrealistic expectations (Fauser et al., 2019).

The literature is vast evaluating the fertility awareness levels, reporting a low to moderate fertility awareness, particularly low to moderate knowledge regarding infertility definition and causes; moderate/high knowledge regarding lifestyle risk factors and consequences of delaying childbearing to later ages; low knowledge about age-related fertility decline (Pedro et al., 2018). In a recent study involving women attempting to conceive spontaneously (N=1542), it was observed that 21.9% of these women believe that, in general, women can easily get pregnant beyond the age of 45 (Iino et al., 2022).

Importantly, fertility awareness was reported to be dependent on gender (women have greater fertility awareness than men); age (older people have higher fertility awareness); education (higher education have higher fertility awareness); and reproductive status (people who had undergone ART/faced infertility had higher fertility awareness; people who had planned their pregnancy shown higher fertility awareness; parents and childless people have similar levels of fertility awareness) (Pedro et al., 2018).

Health care professionals knowledge does not surpass the tendency observed in the general population (Grace et al., 2023; Kim et al., 2022). There is a significant gap in knowledge among health care professionals, as highlighted by Garcia et al., with 77.3% of physicians and 72.9% of nurses incorrectly reporting the age limit for an easily spontaneous pregnancy as over 39 years (García et al., 2017). Similar findings were reported among pharmacists (Ceulemans et al., 2020), and regarding ART knowledge (Péloquin et al., 2021; Smith et al., 2022).

1.1.6.3 Interventions to raise fertility awareness

Fertility awareness is advocated as a global priority, involving educators, policymakers, researchers, and health professionals to enhance the effectiveness of messages and to implement optimal strategies, integrated into various life stages (Hall et al., 2023).

The third goal of the sustainable development 2030 agenda of “Good health and well-being” refer ensure universal access to sexual and reproductive health-care services, including for family planning, information and education, and the integration of reproductive health into national strategies and programmes (target 3.7). Accordingly, the WHO advocates for the inclusion of fertility awareness in national sexuality education programs, highlighting these as policy and programmatic interventions that governments can implement (UNESCO et al., 2021).

Fertility awareness interventions have shown to increase fertility knowledge (Boivin et al., 2018; Conceição et al., 2017; Daniluk & Koert, 2015; Fooladi et al., 2018; Hammarberg & Stocker, 2021; Maeda et al., 2016, 2018; Pedro et al., 2020; Skogsdal et al., 2019; Stern et al., 2013; Wojcieszek & Thompson, 2013). However, lasting impact of these interventions remains uncertain for individuals without children, presumed to be fertile (García et al., 2018; Pedro et al., 2020). More comprehensive follow-ups, particularly exploring the role of fertility awareness in reproductive decision-making, are needed (García et al., 2018).

1.1.7 Reproductive health care

1.1.7.1 Definition of reproductive health care

According to the international glossary on infertility and fertility care, fertility care is defined by the “interventions that include fertility awareness support and fertility management with an intention to assist individuals and couples to realize their desires associated with reproduction and/or to build a family” (Zegers-Hochschild et al., 2017).

1.1.7.2 Reproductive health care programs

Globally the majority of topics in reproductive health care include preventing unplanned pregnancies and sexually transmitted infections (Desrosiers et al., 2020). Although family planning services are accessible through the national health system, the emphasis lies predominantly on preventing unplanned pregnancies. Conversations about contraception and preconception care typically commence only when women actively seek out family planning consultations with the intention of conceiving. This emphasis is due to the significantly worse outcomes for both a mother and a baby when a pregnancy is unplanned (Kallner & Danielsson, 2016; Otu et al., 2021), aligning with guidelines from *direção geral de saúde* advocating for assistance in preconception care (Direcção Geral da Saúde, 2015).

Consequently, the term "family planning" is narrowly linked with pregnancy prevention and contraception, sometimes carrying negative connotations regarding family initiation (Grace, Shawe, Barrett, et al., 2022). This highlights a lack of comprehensive healthcare initiatives aimed at supporting women in their considerations about the timing of motherhood (Temmesen et al., 2023). The diversity in individual perspectives towards parenthood, as described by Grace et al., (Grace, Shawe, Johnson, et al., 2022) emphasizes the absence of a one-size-fits-all model for reproductive health care. Mertes et al. capture this entire topic with a reflection on the importance of language when communicating in reproductive health (Mertes et al., 2023).

1.1.7.2.1 Reproductive life plan

The concept of a reproductive life plan, as defined by Liu et al. serves as a "framework for promoting reproductive health across the lifespan" (Liu et al., 2016). Interventions utilizing a reproductive life plan approach have demonstrated effectiveness in influencing perceptions,

acceptability, knowledge change, and behaviour change (Hipp et al., 2019). Acceptability was demonstrated in a randomized controlled trial (RCT) of reproductive life plan-based counselling involving 201 individuals aged 18-50, with 76% reporting a positive experience (Bodin et al., 2018). Additionally, in a population-based study of over 700 people of reproductive age, 74% would not mind if their general practitioner asked about pregnancy intentions (Hammarberg et al., 2020).

The effectiveness of reproductive life plan was evident in improving fertility knowledge for both women (Stern et al., 2013) and men (Bodin et al., 2018). Behaviour change was observed, with prior knowledge about female age-related fertility decline being linked to having the first child more than 2 years earlier (Maeda et al., 2016).

1.1.7.2.2 One Key Question®

One Key Question® is a successful intervention in reproductive healthcare provision. This approach involves pregnancy intention screening and has been implemented in primary care in many states of the United States (Hunter et al., 2022).

The aim of One Key Question® was promoting reproductive healthcare for all patients (Bellanca & Hunter, 2013). Developed by the Oregon Foundation for Reproductive Health, One Key Question®, serves as a clinical tool in primary care settings, facilitating proactive screening and dialogue between patients and clinicians regarding their reproductive goals. Through the simple inquiry, "Would you like to become pregnant in the next year?" patients' responses guide the provision of appropriate counselling: contraceptive, preconception, or both, based on whether they answer "yes", "no", "either way" or "unsure" (Allen et al., 2017; Bellanca & Hunter, 2013).

Stulberg et al. noted a rise in patient-reported rates of contraceptive counselling, increasing from 52% pre- One Key Question® to 76% post- One Key Question® ($p=0.04$) (Stulberg et al., 2019). In contrast, Song et al. observed no significant alteration in patient-reported reproductive counselling, encompassing both contraceptive and preconception counselling (69% pre- One Key Question® to 76% post- One Key Question®, $p=0.58$) (Song et al., 2021). Thorman et al. reported an initial documentation rate for contraceptive counselling of 39.7% (Thorman et al., 2022), which was lower than the rates reported in these earlier studies, despite operating within a similar primary care clinic setting.

In summary, One Key Question®'s primary advantage lies in its simplicity. Compared to established tools like the "Desires to Avoid Pregnancy scale", One Key Question® reliably identifies patients' definitive wishes, serving as a useful starting point for discussions on reproductive plans (Stulberg et al., 2019). Alternative approaches have been suggested, and researchers are still searching for an instrument that primary care providers could universally implement across various settings to support reproductive health decision-making (Callegari et al., 2017).

1.1.7.2.3 Shared decision-making tools in fertility awareness

The use of shared decision-making tools enhances patients' understanding of available treatment options and empowers them to play a more active role in their healthcare (Elwyn et al., 2013). These tools have been applied across various healthcare domains, proving to enhance the overall patient experience, family planning included (Webb et al., 2020).

Patients frequently follow fertility awareness-based methods (FABMs) of family planning, but clinicians often have limited familiarity or engagement with these methods (Choi et al., 2010; Duane et al., 2022; Fehring, 1995; Stanford et al., 1999). A study led by Duanne et al. was designed to assess the impact of education about FABMs and training on the use of a shared decision-making tool among clinicians. The study aimed to evaluate how education and shared decision-making tool training influenced knowledge scores, even among experienced clinicians. The findings confirmed an increase in knowledge among clinicians, supporting the efficacy of incorporating shared decision-making tools in healthcare practices (Duane et al., 2022).

Following the initial phase, the same research group investigated the influence of the shared decision-making tool on patients utilizing FABMs for family planning (Duane et al., 2023). The authors concluded that employing the shared decision-making tool through FABMs counselling resulted in higher patient adherence to their selected method and increased satisfaction among those who change the method (Duane et al., 2023).

1.1.7.2.4 Fertility assessment and counselling (FAC) clinic

Establishing fertility awareness and reproductive counselling, such as the Danish fertility assessment and counselling (FAC) clinic, serves as a reference for providing reproductive health care. This clinic enables childless individuals to evaluate their fertility in alignment with age expectations, offering educational approaches and enhancing perception and debate by sharing biological facts. The FAC clinic was established in 2011 and has offered personalized fertility assessments and guidance to more than 3000 people (Hvidman et al., 2015; Koert et al., 2022; Petersen et al., 2017).

Considering female population, the clinic provides a 30-minute consultation, during which women undergo a comprehensive review of their risk factors for female subfertility. These risk factors are categorized into three zones (green, yellow/orange, and red) and are scored on the risk evaluation, inspired by the fertility status awareness tool (FertiSTAT). Women attending the FAC clinic do not receive a specific prognosis, but they are provided with an estimate of their reproductive potential and general information regarding fertility at specific female ages. Anti-Müllerian hormone (AMH) values are decreased, women are warned that they are expected to have a poorer chance of achieving the two children they desire within the next 5 years compared to those with higher levels of AMH (Hvidman et al., 2015).

A follow-up evaluation of FAC clinic highlights that women acknowledged the importance of not waiting 'too long' to have children. Moreover, participants believed that the information received at the FAC clinic assisted in their decision-making (Koert et al., 2020).

1.1.8 Initiatives in fertility education

1.1.8.1.1 Global perspective

According to the WHO definition of health education, fertility education encompasses communication strategies and materials used to inform and guide decisions to enhance fertility literacy and/or awareness (Nutbeam & Muscat, 2021; WHO, 2022). The dissemination of information on fertility includes doctor-patient communication during family planning consultations, brochures at primary healthcare centres, and school lectures. Additionally, fertility education resources, such as websites, fertility patient apps, videos, and social media accounts, are designed to effectively deliver evidence-based information on fertility (Conard, 2019). Recognizing the critical importance of fertility education, the WHO has recently underscored its necessity (WHO, 2022).

Cassandra's Prophecy, authored by "Jane Everywoman," serves as an exploration into the dissemination of fertility information. Detailing a personal story of a woman dealing with infertility. The report emphasizes certain aspects of this experience, including the lack of information and a lack of urgency regarding childbearing age, mixed messages from health professionals, the expectation of IVF as a curative solution, and the disappointment of failure (Everywoman, 2013). Building upon this narrative, more research has revealed a concerning lack of fertility awareness among individuals without children (Bunting et al., 2013; Daniluk & Koert, 2013; Harper et al., 2017). Recognizing the role of information provision, it becomes clear that empowering prospective parents with knowledge is indispensable for making informed decisions about their reproductive futures (Delbaere et al., 2020; Schmidt et al., 2012).

Initiatives focusing on fertility education have been implemented. Australia pioneered a government-funded program, featuring an interactive website (www.yourfertility.org.au) with over 5 million annual visits and educational modules for healthcare professionals (Hammarberg et al., 2017). In the United Kingdom (UK), researchers from Cardiff university and University College London along with physicians have been actively contributing to produce informative content for fertility education and investigating effects of fertility information (Harrison et al., 2023; Maslowski et al., 2022, 2023). Denmark, in 2016, released a national report on infertility prevention and established the world's inaugural FAC Clinic in 2011 (Hvidman et al., 2015; Koert et al., 2022; Petersen et al., 2017). In the UK, the fertility education initiative was founded in 2016 (www.fertilityed.uk) and in 2019, the department for education incorporated fertility education into the UK curriculum. Various other countries, such as Greece, the Czech Republic (www.mojereprodukcnizdravi.cz), and Sweden (<http://www.reproduktivlivsplan.se/>), have also launched fertility education websites. In Estonia, the digital textbook viljakusest.ee stands as an innovative and comprehensive resource on human fertility, comprising three interactive modules.

Each section includes a link to a learning platform for users to assess their understanding. This initiative received the "Yearly Contribution Award for Human Health Education" in 2022, recognizing its significant impact on health education.

Meanwhile, in Belgium, fertility experts collaborated with VIVES University College to create an online learning platform supporting teachers in addressing fertility-related topics. The platform, My fertility | Reproductive Health Education (allesovervruchtbaarheid.be), includes sections for teachers, individuals seeking accurate fertility information, and healthcare providers.

The Portuguese scenario will be explored in a later section.

1.1.8.1.2 The international reproductive health education collaboration (IRHEC)

The international reproductive health education collaboration (IRHEC) is a global initiative dedicated to increase the awareness in reproductive health. At the core of IRHEC's mission is the commitment to raising awareness of reproductive health through a life course approach. The aim is to improve reproductive health and facilitate informed decision-making in family planning. This goal is pursued through the development, evaluation, and dissemination of comprehensive educational resources designed for adolescents, individuals in their reproductive years, primary healthcare providers, educators, and policymakers (Harper et al., 2021).

1.1.8.1.3 Overview of the Portuguese scenario

In Portugal, substantial efforts have been undertaken to enhance fertility education and awareness. A study (Almeida-Santos et al., 2017) focused on evaluating three main aspects: 1) parenthood intentions (desired and planned number of children); 2) knowledge about fertility and ART (understanding of fertility, chances of spontaneous conception, and ART); 3) sources of information on fertility and infertility risk. The study involved 2,404 participants (808 men and 1,596 women) aged 18–45, without children, representing a national sample. Noteworthy findings include 95.5% of participants expressing an intention to have children in the future, with the ideal age for the first child perceived as 29, although the majority anticipated having their first child at 33. The study revealed limited knowledge about fertility and ART, with 46% correct responses. Factors associated with higher correct answer included being female, age >35 years, university education, employment, and higher income. Despite clinicians being considered the most reliable source, less than 30% of participants consulted their doctors for information, while around 46% relied on medical websites.

In a separate study, Portuguese oncologists' knowledge concerning fertility preservation was evaluated. The need to assess oncologists' knowledge about fertility preservation arose from the observation that oncology patients were rarely referred for fertility preservation. In 2013 and 2015, a questionnaire was administered to 179 physicians from major hospitals nationwide (Melo et al., 2018). The study identified communication skills as a significant barrier related to a lack of

information. However, a follow-up study in 2018 by the same group of researchers suggested an apparent increase in clinicians knowledge about the reproductive health of female oncology patients and improved doctor-patient communication (Cabral, 2019).

Additionally, it was also developed national guidelines and information programs for health professionals to raise awareness about fertility's importance in patients' life projects and enhance patient care were developed. Initiatives included developing an informational program for patients and clinicians in collaboration with the Portuguese league against cancer in 2017 (Silva et al., 2017), national recommendations published in the Journal of the Portuguese Society of Oncology (Sousa et al., 2016), and launching the fertility preservation center website in 2013 (<http://www.centropreservacaofertilidade.pt>).

In the promotion of literacy in the general population, a nationwide campaign titled "Take Care of Your Fertility" was launched in 2017. This campaign significantly contributed to understanding factors affecting fertility. Aimed at providing clear information on reproductive health, particularly environmental factors and lifestyles impacting fertility, the campaign featured a website (www.cuidadatuafertilidade.pt) and widespread promotion through television, national newspaper, social media, and music festivals throughout 2017. The campaign effectively dispelled myths about fertility, reaching various media with 16 prime-time television ads, 90,000 weekly newspaper publications, 11,000 website visits, and extensive engagement on Facebook.

Additionally, an illustrated column was published in the Público newspaper as part of the "Health in Knowledge" project. The series covered fertility preservation, clinical advancements, and research in the field (<https://www.publico.pt/2021/01/20/ciencia/noticia/futuro-hoje-preservar-fertilidade-1946841e>) e (<https://www.publico.pt/2018/05/04/ciencia/fotogaleria/viagens-pela-infertilidade-385652>).

Two interventions on fertility knowledge, evaluating the impact of educational videos, were conducted for university students (Conceição et al., 2017) and for partnered women (Pedro et al., 2020). Both works had shown a positive impact of the interventions in fertility knowledge.

1.1.8.2 Impact of fertility education

1.1.8.2.1 Fertility education effectiveness in increasing knowledge

Previous studies, employing various approaches and designs, consistently demonstrate that fertility education improves knowledge in the short term and, partially, in the long term (Boivin et al., 2018; Conceição et al., 2017; Daniluk & Koert, 2015; Fooladi et al., 2018; García et al., 2016; Hammarberg & Stocker, 2021; Maeda et al., 2016, 2018; Pedro et al., 2018; Skogsdal et al., 2019; Stern et al., 2013; Wojcieszek & Thompson, 2013)

Maeda et al., 2016, and Maeda et al., 2018, are both RCTs utilizing an online brochure. The content included 1) basic facts on fertility: "What is infertility?" and "What can cause infertility?"; 2) age-related decline in fertility: "What is the ideal age for women to conceive", "Both men and

women are affected by aging", "What is aging of the ovum?", and 3) preconception health (Maeda et al., 2016, 2018). In these two relevant studies, participants aged 20 to 39 years old, hoping to have (more) children, were included, while pregnant women were excluded. Participants knowledge was evaluated three times. T1 comprised a survey followed by information exposure for the intervention group (the control group received information about folic acid intake). T2, conducted immediately after exposure to the brochure, involved another survey to assess the impact of providing information. T3 included a survey, but the follow-up rate was low (51%). Knowledge was measured using the Japanese version of the 13-item Cardiff fertility knowledge scale (CFKS-J) (Maeda et al., 2016).

Over a longer period of time, covering two years, the intervention group significantly increased their knowledge but lost it after two years, although there was some retention of the knowledge (Maeda et al., 2018).

1.1.8.2.2 Fertility education effectiveness altering reproductive intentions

Regarding the impact of fertility education on reproductive intentions, there are mixed results across the published data (Boivin et al., 2018; Fooladi et al., 2018; García et al., 2016; Maeda et al., 2016, 2020; Pedro et al., 2020; Skogsdal et al., 2019; Stern et al., 2013; Wojcieszek & Thompson, 2013).

The use of a chatbot increased the proportion of people prepared to get pregnant, and the intentions to optimize preconception behaviours. In this study the chatbot was more effective than a brochure (Maeda et al., 2020).

An RCT study from Maeda et al. (Maeda et al., 2018) and two protocol studies from different authors (Chan et al., 2022; Gharacheh et al., 2020) revealed that the proportion of new births and new fertility consultations did not change after providing fertility education. However, the timing of childbirth among those who had a partner at T1 was accelerated by the brochure (Maeda et al., 2018). The significant limitation of this study, as well as other reported on the same topic, is the attrition, which is particularly pronounced in young ages. In this study, the 2-year follow-up rate was 51% (Maeda et al., 2018). Other studies reported the similar results. For instance, a face-to-face study over 2 months had a follow-up rate of 88% (Stern et al., 2013); 2), an online study revealed 55% in 6 months (Daniluk & Koert, 2015); and another one 23% in 1 year (Pedro et al., 2020).

A before and after prospective study, evaluating the effect of information provided by a brochure, has shown alterations in the desired age of pregnancy (Wojcieszek & Thompson, 2013). Stern et al. also reported a significant change in the desired age of pregnancy and reproductive behaviours (Stern et al., 2013).

1.1.8.2.3 Fertility education side-effects

Providing information about reproductive health and fertility can lead to changes in behaviour, influencing the timing of births and prompting medical help-seeking among partnered individuals (Maeda et al., 2018). However, there is another aspect to consider: the potential for inducing anxiety, particularly in women (Maeda et al., 2016). The level of concern also increases, especially in groups of young people who are already facing growing pressure about achieving their family goals or potential fertility issues, such as emerging adults, aged 18-24 years old (Boivin et al., 2019).

Considerations have been given to the potential side effects of fertility education, including anxiety, misplaced feelings, and hostility related to perceived infringement on reproductive autonomy (Delbaere et al., 2020). In Maeda et al., 2016, state anxiety levels were similar between groups but significantly higher in the intervention group after exposure to the information. Subjective anxiety levels were also significantly higher in the intervention group, particularly among women in their late twenties (Maeda et al., 2016).

The risk of causing an unnecessary reaction is highlighted by the data on egg storage. There are alarming numbers of people freezing their eggs, with a massive increase of +240% from 2013 to 2018 in the UK (Chronopoulou et al., 2021). People are concerned and take proactive measures in case of unforeseen circumstances. Proof of that is that, between 3.2% to 15% of people return to use their cryopreserved eggs (Weizman et al., 2021) indicating that the threat is real and can lead to unnecessary actions.

Importantly, while an unquestionable association exists between anxiety and fertility information, it is also recognized that tailored messages do not induce anxiety in individuals (Koert et al., 2020; Maeda et al., 2020). Specifically, reproductive life plan counselling has demonstrated a positive impact on the participants (Fooladi et al., 2018; Skogsdal et al., 2019; Stern et al., 2013).

In addition to personalised and carefully tailored information, language use is also crucial to reduce anxiety and guilt (Mertes et al., 2023). In line with this, Martins et al. provide valuable insights for developing fertility education tools, summarizing as key considerations: 1) understanding the target population; 2) incorporating theories of behavioural change; 3) involving user perspectives and co-creation; and 4) utilizing guidelines to enhance engagement (Martins et al., 2024).

1.1.9 Considerations in ovarian reserve screening

Suggesting ovarian reserve testing was highlighted as an option to assist women in making informed decisions about their reproductive life (Tremellen & Savulescu, 2014), especially to manage the impact of situations in which ovarian reserve is compromised (Grisendi et al., 2019; Ligon et al., 2019; Nikolaou & Templeton, 2003; Sinha et al., 2022).

The widely utilized biomarker for assessing ovarian reserve is AMH (Çelik et al., 2023). AMH is a good predictor of the number of retrievable eggs (Baird et al., 2005; Broer et al., 2014; Tal & Seifer, 2017), but is not a reliable predictor of the likelihood of pregnancy in non-infertile populations (Harris et al., 2023; Lin et al., 2021; Steiner et al., 2011, 2017; Zarek et al., 2015). Moreover, ovarian reserve testing is subject to multifactorial influences (Landersoe et al., 2019).

Despite of these limitations, AMH testing is becoming more and more common to evaluate fertility (Peipert et al., 2023). The recent availability of direct-to-consumer AMH testing in various countries has broadened public access to the test (Kyweluk, 2020; Peipert et al., 2023; Stanczyk et al., 2022). It also enables individuals to undergo testing without the requirement for interaction or counselling from a healthcare practitioner (Copp et al., 2023; Johnson et al., 2023). For those with normal or high results, it may falsely reassure them about the possibility of delaying pregnancy. In contrast, individuals with low results may experience unwarranted anxiety, possibly leading to earlier attempts at conception or consideration of egg freezing (O'Brien et al., 2018).

In Portugal, a cost-saving solution to expedite the fertility journey has been studied (Xavier et al., 2023). The study aimed to assess the financial impact of implementing nationwide screening for diminished ovarian reserve (DOR) in Portugal's national health system using AMH levels. The researchers established the clinical pathway based on existing literature, and a panel of four clinicians validated assumptions and inputs. The results suggested that screening for ovarian reserve could potentially save €9.4 million for the national health system, primarily due to a 24% reduction in the use of medically assisted reproduction. Additionally, early referrals for IVF and increased utilization of first-line techniques by women were observed, leading to a 12% decrease in treatment failure. The conclusion drawn from the model suggests that AMH screening may result in more informed decisions, a shorter fertility journey, more efficient treatment utilization, and significant cost savings for the national health system (Xavier et al., 2023).

Concerns regarding ovarian reserve screening are also highlighted. Firstly, existing research has failed to definitively establish any immediate implications on conception potential within the next six months following a poor ovarian screening result (Gnoth et al., 2003). Moreover, such screenings have the potential to introduce unwarranted anxiety in women, possibly leading to premature decisions to restrict educational or career pursuits (Goold & Savulescu, 2009). Financial considerations also weigh heavily against ovarian reserve screening, with costs diverting resources away from other pressing health priorities. Additionally, there is concern that ovarian reserve screening may inadvertently contribute to a rise in women seeking oocyte vitrification and ART (Dondorp et al., 2012), escalating both individual and community costs.

1. Introduction to fundamental literature on ovarian reserve, with a focus on the study of diminished ovarian reserve (DOR)

1.1 An overview of the sociodemographic context in female reproductive health and fertility

A comprehensive exploration of the present status of sociodemographic factors influencing female reproductive health and fertility awareness.

1.2 Exploring ovarian reserve through testing, genetic markers, and follicular activation regulatory signalling pathways

Exploring fundamental concepts within diminished ovarian reserve.

1.2 Exploring ovarian reserve through testing, genetic markers, and follicular activation regulatory signalling pathways

1.2.1 Ovarian reserve

1.2.1.1 Definition of ovarian reserve

Ovarian reserve refers to the number of follicles remaining in the ovary, which reflects their ability to grow and develop into competent oocytes; thus, it reflects the fertility potential of women (Gong et al., 2023). Ovarian reserve is susceptible to various influences, including age, environmental factors, the initial pool of primordial follicles, diseases, drugs, and unidentified elements (Zhu et al., 2023).

The number of ovarian germ cells follows a dynamic process during women's life, as illustrated in Figure 4 (Johnson, 2007). Every woman is born with about 2 million of primordial follicles, formed during fetal life, and that number defines each woman ovarian reserve. This number naturally declines with increasing age (300 000-500 000 primordial follicles at puberty and 25000 at the age of 35) (Faddy et al., 1992). From menarche on, the primordial follicles, an oocyte with a flattened single layer of granulosa cells, are activated and develop into primary, secondary and antral follicles. Then, antral follicles reach the preovulatory stage to origin a Graafian follicle that releases a mature oocyte for fertilization (Ford et al., 2020; McGee & Hsueh, 2000).

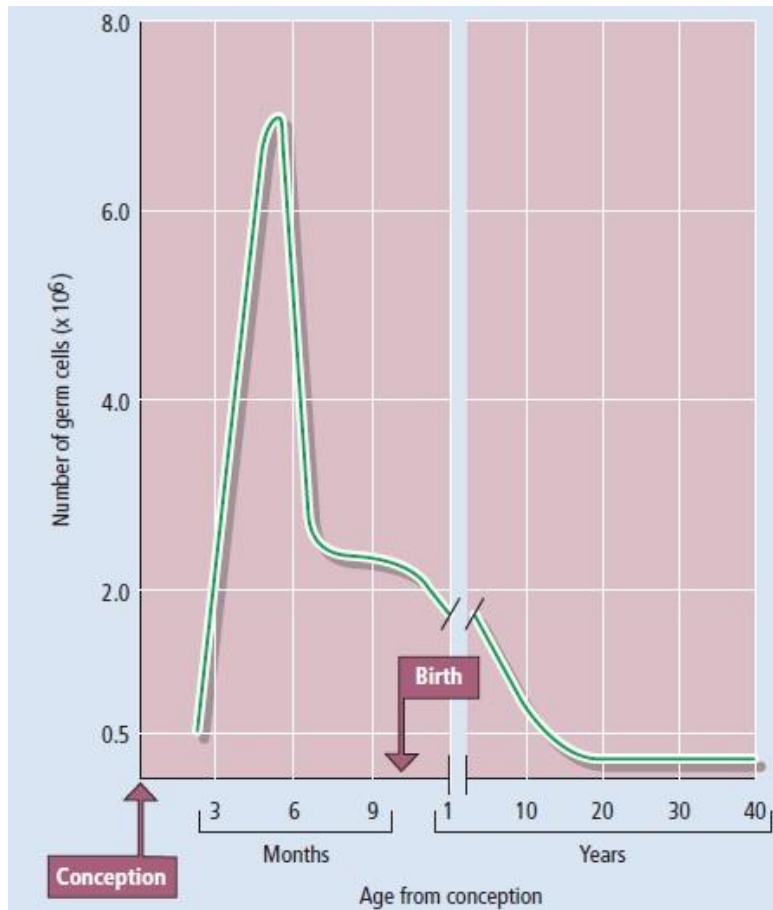


Figure 4 Dynamics of follicle numbers across a woman's lifespan, from gestation to menopause

Number of ovarian germ cells gradually increase during gestation, peak at around 20 weeks, and then progressively decline postnatally. After an initial period of migration, mitotic proliferation commences and at 16-20 week there are $6-8 \times 10^6$ oogonia. Then the first meiotic prophase can be detected and the first diplotene-stage chromosomes at around the end of the first trimester. By puberty, the follicle count stabilizes and remains relatively constant until the 30s, where a gradual decline begins. This decline accelerates in the late 30s, culminating in menopause when the follicle count falls below a critical threshold. By birth, there are $1-2 \times 10^6$ of oocytes at the dictyate stage. Atresia of oocytes in the meiotic prophase is a natural process throughout fetal and neonatal life Adapted from Johnson, 2007.

The decline in ovarian reserve naturally occurs with increasing age (Figure 5) in wherein the vast majority of early antral follicles (~99%) undergo programmed cell death (atresia) before ovulation (Hsueh et al., 1994; Kaipia & Hsueh, 1997). Consequently, ovarian reserve relies on a delicate balance between the initial pool of primordial follicles and the natural rate of atretic loss and/or a possible depletion due to an insult to the ovary (Faddy et al., 1992; La Marca et al., 2011).

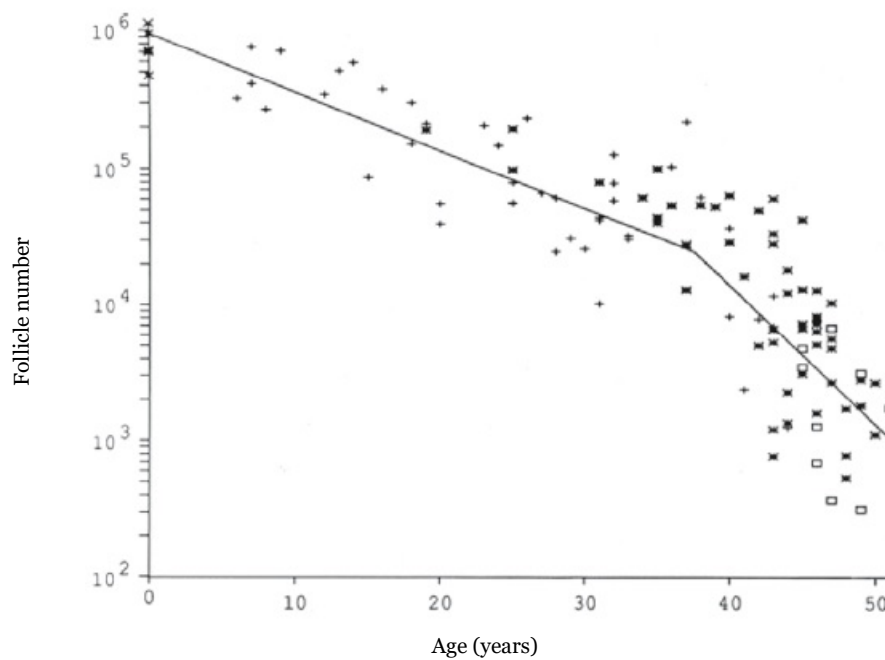


Figure 5 Natural follicle depletion with aging

Follicle depletion begins to accelerate in the 30s, further declining in the late 30s, coinciding with a notable rise in miscarriage rates. Menopause marks the point at which the primordial follicle pool diminishes below a critical threshold (typically 750-1000 remaining follicles). Adapted from Faddy et al., 1992.

The ovarian reserve determines the response to controlled ovarian stimulation (COS), which is a pharmacological treatment with the intention of inducing the development of ovarian follicles. In ART, COS is used to obtain multiple oocytes at follicular aspiration (Zegers-Hochschild et al. 2017). A poor ovarian response, obtaining less than 4 oocytes following ovarian stimulation, correlates with low ovarian reserve test. In opposition, an exaggerated ovarian response, obtaining more than 15-20 oocytes, is likely to cause ovarian hyperstimulation syndrome (OHSS), and is associated with high values in ovarian reserve test (ESHRE, 2019).

According to the international glossary on infertility and fertility care, “ovarian reserve can be assessed by any of several means. They include: female age; number of antral follicles on ultrasound; AMH levels; follicle stimulating hormone (FSH) and estradiol levels; clomiphene citrate challenge test; response to gonadotropin stimulation, and oocyte and/or embryo assessment during an ART procedure, based on number, morphology or genetic assessment of the oocytes and/or embryos” (Zegers-Hochschild et al. 2017).

Figure 6 presents a schematic representation of the principal chemical messengers within the female reproductive system. The hormones introduced in this schematic representation are further detailed in the following sections. (Draper et al., 2018)

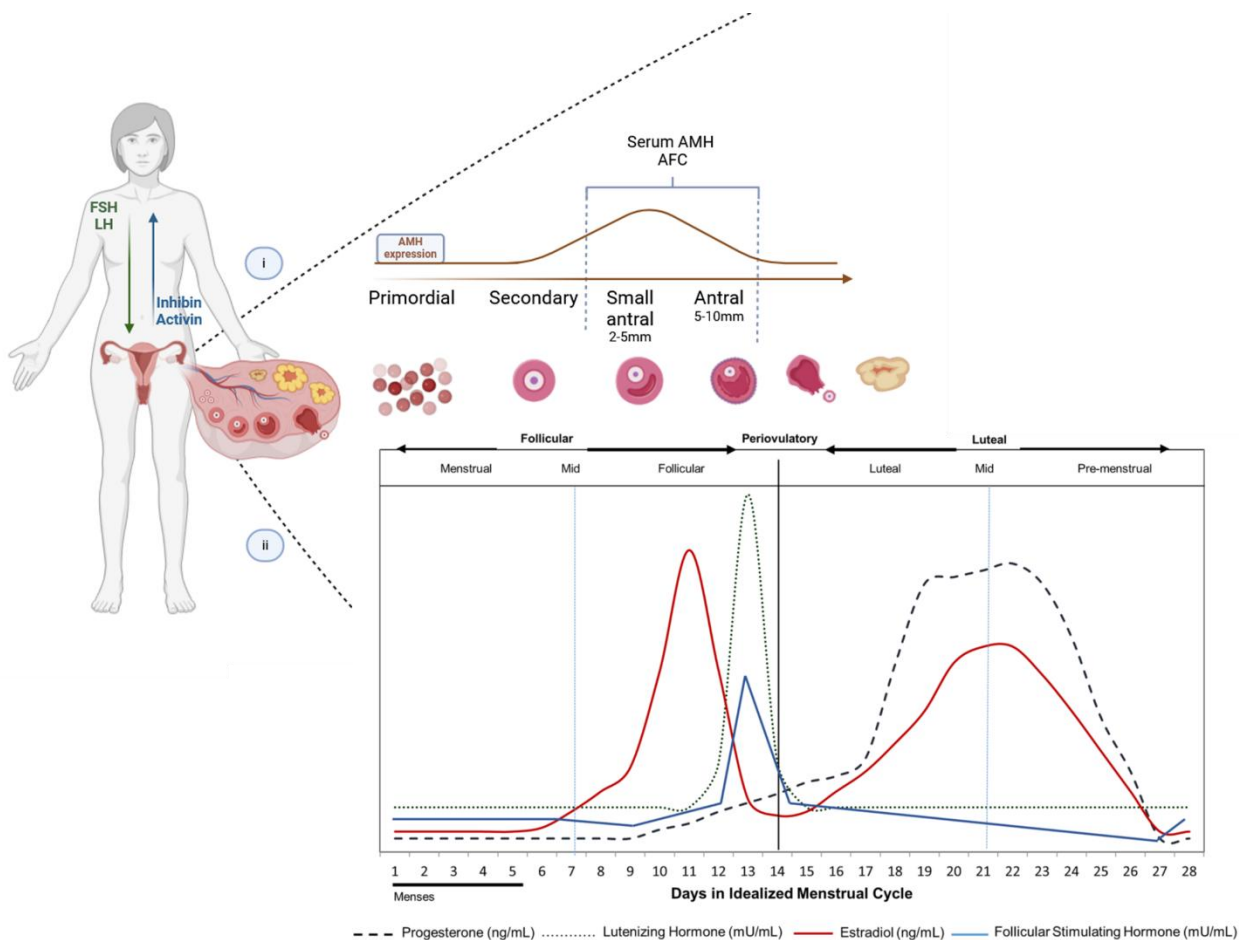


Figure 6 Schematic representation illustrating the principal pathways of communication via chemical messengers within the female reproductive system

i: The Hypothalamus stimulates the pituitary gland to secrete gonadotropins, which in turn regulate ovarian activity. Additionally, the ovaries provide feedback to both the pituitary gland and the hypothalamus, thereby regulating reproductive function. Key hormones involved include FSH, LH, and AMH. AMH serum levels have a significant contribution from the early antral and antral follicle pool. In preovulatory follicles, AMH is only expressed in cumulus cells surrounding the oocyte.

Activin and Inhibin modulate the hypothalamo-pituitary-gonadal axis. The hypothalamus secretes GnRH, which prompts the anterior pituitary to release FSH, thereby influencing ovarian activity. Activin enhances FSH secretion, whereas inhibin acts to suppress FSH release. The illustrations were created using BioRender.

ii: Diagram illustrating the various stages of follicular development alongside corresponding blood hormone levels—Progesterone, LH, estradiol, and FSH —throughout the menstrual cycle and their roles in determining follicular development.

During the follicular phase, FSH and LH stimulate inhibin production, which gradually rises as ovulation approaches. Under the influence of FSH and LH, the dominant antral follicle produces increasing levels of estrogen, ultimately leading to an estrogen surge. Subsequently, the dominant follicle enters the preovulatory phase characterized by elevated levels of progesterone from mural granulosa cells and the emergence of prolactin and progesterone receptors. The graph was adapted from Draper et al., 2018.

FSH: follicle stimulating hormone; LH: luteinizing hormone; AMH: anti-Müllerian hormone

The next sections discuss the clinical utility of the regulators of the female reproductive system. All the molecules considered are crucial to define the correct regulation of the hypothalamic-pituitary-ovarian function. However, a more detailed description will be provided for the most promising ones, regarding their applicability in assessing ovarian reserve.

1.2.2 Revision of indicators assessing female reproductive function

1.2.2.1 Follicle stimulating hormone (FSH)

1.2.2.1.1 Follicle stimulating hormone receptor (FSHR)

The FSH structure is composed by the subunits alpha (FSH α) and beta (FSH β). FSH β provides specificity for the *FSH* receptor (*FSHR*) and is encoded by a gene located on the chromosome 11p.21 (Watkins et al., 1987). Post-translational modifications of FSH, namely differences in glycosylation, alter the secretion rate, molecule half-life, transduction signal and so, may affect the ovarian response in ART (Mastrangeli et al., 2017; Miguel et al., 2017).

FSHR is a 76 kDa G-protein coupled receptor, consisting of 695 amino acids, which is primarily located on granulosa cells of antral follicles. The *FSHR* gene is located at chromosome 2.p21 and includes 10 exons and 9 introns. Exons 1 to 9 encode for the extracellular domain while the exon 10 encodes for the transmembrane domain and for the intracellular C-terminal tail (Simoni et al., 1997).

FSH binding to its receptor enrolls the activation of multiple signalling pathways that start with the activation of G alpha subunit (Gas) protein followed by the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Consequently, the cAMP-responsive element binding-protein (CREB) (transcription factor) modules the expression of genes encoding for enzymes involved in steroidogenesis (Gloaguen et al., 2011). Particularly, *FSHR*, after FSH binding, activates adenylyl cyclase, protein kinase A (PKA), and phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) pathways (Lee et al., 2021).

1.2.2.1.2 Role of follicle stimulating hormone (FSH) in follicular development

FSH promotes the follicular recruitment, growth, and selection of one follicle (Figure 7). This hormone is particularly important at the final phase of follicular growth, with its effect on FSH receptors on cumulus and granulosa cells of antral follicles (Kallio et al., 2013). For this reason, FSH defines the gonadotropin-dependent phase of follicular growth that does not occur below the FSH threshold level (Karakaya et al., 2014)

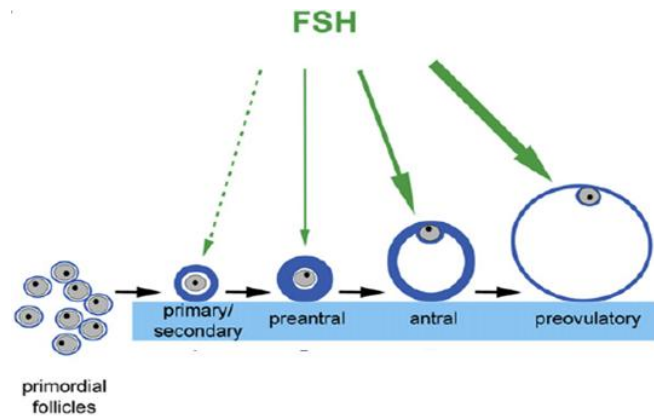


Figure 7 Illustration of FSH actions in promoting follicular growth

FSH stimulates mitogenesis of granulosa cells, fosters production of follicular fluid, facilitates expansion of the follicle, and induces the secretion of growth factors crucial for follicular development. FSH plays a pivotal role in the maturation of late preantral and early antral follicles, characterized by the presence of FSH receptors on their granulosa cells. Moreover, as follicles progress, they exhibit an increase in AMH levels, with higher concentrations secreted by antral follicles. Adapted from Kallio et al., 2013.

FSH: follicle stimulating hormone; AMH: anti-Müllerian hormone

At the time of recruitment, the early antral follicles exhibit the highest sensitivity for FSH, and it then declines in the late antral stage. The follicle with the highest sensitivity will benefit from this increasing in FSH levels and gain dominance (Raju et al., 2013). The final stages of follicle development are regulated by activins and estradiol. The dominant follicle has an increased aromatase activity and starts expressing luteinizing hormone receptors (*LHR*) (Gervásio et al., 2014).

Activins and inhibins are important regulators of gonadotropin synthesis at late stages of follicular development (Makanji et al., 2011). Activins modulate the follicle development while inhibins A and B negatively regulate follicle growth. Inhibin B is produced by granulosa cells of small and large antral follicles and, at this stage, estradiol and inhibin B inhibit pituitary secretion of FSH (Knight & Glister, 2003).

1.2.2.1.3 Follicle stimulating hormone (FSH) levels and its predictive capacity

FSH is a well-studied biomarker, available on multiples automated platforms and is considered fast, cost-effective and reproducible (Sharara et al., 1998). Considered an indirect marker of ovarian reserve, FSH was initially proposed to predict the ovarian response to COS (Muasher et al., 1988; Scott et al., 1989; Toner et al., 1991).

FSH is dependent on inter and intra-menstrual variations (Kwee et al., 2004; Scott et al., 1990). In the early follicular phase, FSH levels exceeding 10-20 (mIU)/(mL) are indicative of a diminished number of follicles (Broekmans et al., 2009; Roberts et al., 2005), and associated with

poor ovarian response (Esposito et al., 2002; Gurgan et al., 1997). Importantly, a single abnormal FSH value in women <40 years old may not have a predictive value (Roberts et al., 2005). Moreover, FSH measurement per se is associated with a moderate clinical sensitivity and specificity in predicting ovarian response to COS (Chuang et al., 2003; Toner & Seifer, 2013), that justifies combining FSH with estradiol measurement to enhance sensitivity and reduce the incidence of false-negative test results (Licciardi et al., 1995).

1.2.2.2 Estradiol

1.2.2.2.1 Estradiol and estrogen receptors (ESR)

Estradiol is an endogenous estrogen derived from cholesterol that increases during the folliculogenesis to promote granulosa cells growth. This hormone is not only produced by granulosa cells but also by adipose, liver, adrenal, breast, and neural tissues (Jamil et al., 2016; Mais et al., 1987; Ryan, 1982). Its secretion by early antral follicles is under the influence of FSH (Dewailly et al., 2014).

Estrogen receptors (ESR) have two nuclear subtypes ER α , encoded by *ESR1* gene located on chromosome 6q25.1 and typically expressed in theca cells, and ER β , encoded by *ESR2* gene located on chromosome 14q25-24 and predominantly expressed in granulosa cells (Altmäe et al., 2007). *ESR* regulate the expression of a variety of genes involved in follicular growth and proliferation (De Mattos et al., 2014). Likewise, estrogen play an important role on follicular growth and maturation as well as regulates follicle apoptosis (Rabet et al., 2012).

1.2.2.2.2 Estradiol levels and its predictive capacity

Estradiol testing is available on multiple automated platforms and it is fast, cost-effective and reproducible (Roudebush et al., 2008).

The circulating levels estradiol had been successfully used to predict COS, particularly cases of OHSS (Lee et al., 2008). The rationale behind employing estradiol for COS monitoring lies in the observation that follicles exhibiting a mean diameter of 20-22 (mm) typically yield an estradiol concentration of approximately 200 (pg/mL) (Esposito et al., 2002). Importantly, estradiol alone cannot predict ovarian response (Evers et al., 1998; Licciardi et al., 1995; Smotrich et al., 1995).

Estradiol has never been used alone as a biomarker for ovarian reserve evaluation (Ryan, 1982) and its blood concentrations rise only when ovarian reserve is severely compromised (Licciardi et al., 1995). Estradiol could also be altered in cases of polycystic ovary syndrome (PCOS), hypogonadotropic or hypergonadotropic hypogonadism (La Marca et al., 2009).

1.2.2.3 Luteinizing Hormone (LH).

1.2.2.4 Luteinizing hormone (LH) and luteinizing hormone receptor (LHR)

Luteinizing hormone (LH) is a pituitary hormone that acts in theca cells promoting its development. Theca cells, under LH stimulation, synthesize androgens from cholesterol and release it during folliculogenesis. LH has also a crucial role in steroidogenesis in the preovulatory follicle, through *LHR* on theca cells and FSH (possibly plus LH) receptors on granulosa cells (Raju et al., 2013).

The steroidogenic process depends on androstenedione produced in theca cells, which subsequently diffuses into granulosa cells to be converted (by aromatase) in estradiol, as illustrated in Figure 8 (Elder et al., 2010). Furthermore, the surge levels of LH shift follicular steroidogenesis from predominately estrogen to progesterone production. This actively promotes the resumption of meiosis, by stimulating the oocyte to produce a signal that induces germinal vesicle breakdown (Raju et al., 2013).

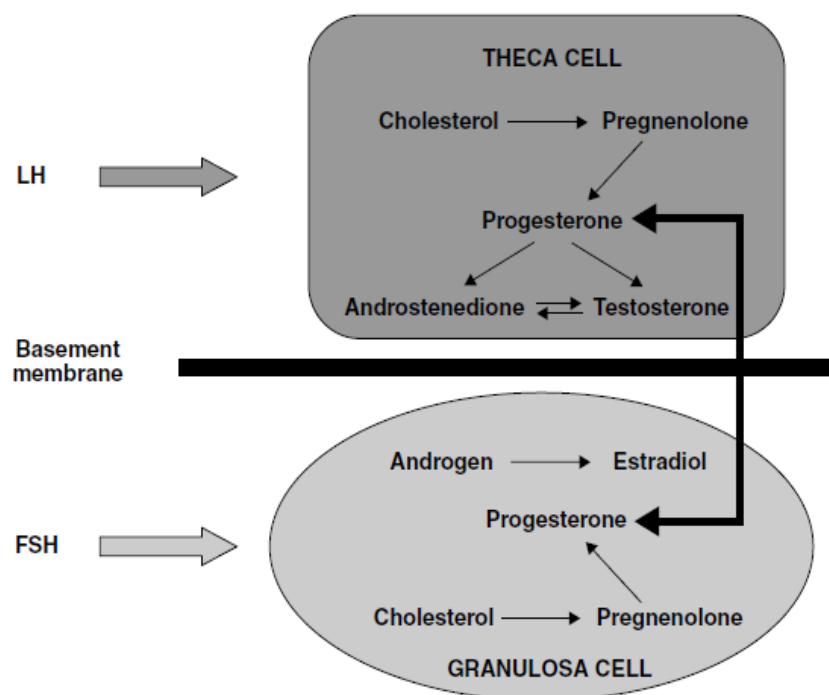


Figure 8 Diagram illustrating the two-cell two-gonadotropin hypothesis

This hypothesis proposes the essential roles of both FSH and LH in estrogen synthesis, highlighting the cooperative interaction between theca and granulosa cells. From Elder & Dale, 2011.

FSH: follicle stimulating hormone; LH: luteinizing hormone.

Antral follicles, under the influence of LH, rapidly synthesize estradiol, essential for oocyte maturation and endometrium readiness for implantation (Smith et al., 2014). Therefore, LHR activation in the dominant follicle is important in oocyte maturation, ovulation, ovarian steroidogenesis, luteinization and progesterone synthesis (Ascoli et al., 2008).

After the ovulation, the follicle collapses and origins the corpus luteum or the postovulatory follicle. In this phase there is an luteinization of the theca cells to produce large amounts of progesterone in response to LH stimulus (Taylor et al., 2020).

1.2.2.5 Luteinizing hormone (LH) levels and its predictive capacity

It is known that basal LH levels face a natural increment during the female reproductive lifespan, getting particularly high at menopause (Baird et al., 2005). Beyond that, high concentrations of LH could also indicate PCOS (Chun, 2014; Gervásio et al., 2014).

LH has low predictive value as a marker of ART outcomes mainly due to its significant intra- and inter-cycle fluctuations (Weghofer et al., 2007).

An additional factor to take into consideration is the combination of LH with FSH. The FSH:LH ratio is usually tested on day 3 of the cycle and, when elevated, can be an early signal of DOR (Roudebush et al., 2008).

1.2.2.6 Androgens

Natural androgens, such as 5 α -dihydrotestosterone, testosterone, androstenedione, are important to regulate the secretion of gonadotrophins. Androgens, under the regulation of LH, are converted in estrogens (aromatization) promoting growth of granulosa cells, progesterone production and conversion to estradiol (Gervásio et al., 2014).

1.2.2.7 Progesterone

Progesterone is a hormone involved in preparing the uterus for implantation and stimulating the growth of mammary glands. Serum progesterone levels, usually measured around day 21st of the menstrual cycle or approximately one week before the expected onset of menses, can be employed to assess ovulatory function (Taylor et al., 2020).

Progesterone concentration greater than 3 (ng)/mL provides presumptive evidence of recent ovulation (Taylor et al., 2020). Importantly, progesterone levels fluctuate throughout upon the ovulatory cycle, and its concentration depends on the pulsatile secretion from the corpus luteum (Filicori et al., 1984; Wathen et al., 1984).

1.2.2.8 Anti-Müllerian Hormone (AMH)

1.2.2.8.1 Significance of anti-Müllerian hormone (AMH)

AMH is a glycoprotein secreted by cumulus and mural granulosa cells, spanning from primary to antral follicles. However, a substantial 60% of the AMH present in the bloodstream originates from follicles within the 5–8 (mm) size range, encompassing pre-antral and small antral follicles (Dewailly et al., 2014; Harold Moreno-Ortiz et al., 2018). While all growing follicles release AMH, the serum AMH level more accurately reflects the secretion from larger follicles in direct contact with the vascular bed. Consequently, AMH is considered to be reflective of the cumulative activity of growing follicles (La Marca et al., 2010; La Marca & Volpe, 2006; Roudebush et al., 2008; Tal & Seifer, 2017; Visser et al., 2006).

AMH reflects the ovarian reserve upon the natural female reproductive lifetime - levels begin to get high in adolescence with a peak in the early 20s and declines with age toward menopause (La Marca & Volpe, 2006; Overbeek et al., 2012; Visser et al., 2006). This means that AMH strongly correlates with ovarian follicle pool and, because of that, has an inverse correlation with age (Hansen et al., 2011; Kelsey et al., 2012; Okunola et al., 2017).

With advancing age, the follicle pool declines and it means that granulosa cells secrete less AMH and inhibin B. Lower inhibin B levels lead to an earlier increase of FSH levels during the follicular phase (Steiner et al., 2017).

1.2.2.8.2 Anti-Müllerian hormone (AMH) levels

Fluctuations in AMH measurement upon the menstrual cycle have been reported, but they have not any clinical meaning (Disseldorp et al., 2010; Melado et al., 2018; Overbeek et al., 2012; Sowers et al., 2010). This is a reasonable and frequently cited advantage of AMH over other biomarkers (Fanchini et al., 2005; Feyereisen et al., 2006; La Marca et al., 2007; Roudebush et al., 2008; Tsepelidis et al., 2007).

The AMH cut-off values reported in the literature exhibit considerable variability, indicating a significant assay variability and lack of standardization (La Marca & Sunkara, 2014).

Elevated AMH levels (AMH > 5 ng/mL) have been noted in women diagnosed with PCOS (Dewailly et al., 2011; Pigny et al., 2006). Conversely, diminished AMH levels are documented in women experiencing DOR and premature ovarian insufficiency (POI) (AMH < 0.5 ng/mL) (Toner & Seifer, 2013).

Hormonal contraception and gonadotropin-releasing hormone (GnRH) agonists are known to temporarily reduce AMH levels (Bentzen et al., 2012; Johnson et al., 2014; Kallio et al., 2013; Landersoe et al., 2019), with values typically returning to baseline within 2-4 months after discontinuation (Landersoe et al., 2020). Additionally, the impact of alcohol consumption

(Dolleman et al., 2013), and smoking (Freour et al., 2008; La Marca et al., 2013; Waylen et al., 2010) on AMH levels has also been documented.

1.2.2.8.3 Anti-Müllerian hormone (AMH) predictive capacity

1.2.2.8.3.1 Anti-Müllerian hormone (AMH) predicting spontaneous conception

Korsholm et al. analysed the relationship between AMH and time-to-pregnancy in a cohort of healthy women. This prospective study, covering a two-year follow-up period, revealed a decrease of time-to-pregnancy as AMH increases ($p=0.01$) (Korsholm et al., 2018).

Steiner et al. evaluated the association between age, AMH and FSH, and a positive pregnancy test (urine test), in a group of women monitored for 6 months. The findings of this study unveiled that age, AMH and FSH are three independent predictors of the possibility of getting pregnant. Notably, age > 35 years old, FSH > 10IU/L and AMH < 0.7 ng/mL were identified as predictive factors of a low capacity to get pregnant (Steiner et al., 2011).

Subsequently, the same research group published the final data of this study. More women were included, and the primary outcome shifted to assessing the probability of conception in 6 to 12 months. These subsequent results contradicted the first ones, revealing that AMH and FSH were not significantly correlated with the capacity of getting pregnant (Steiner et al., 2017).

While AMH has shown limited capacity in predicting the spontaneous pregnancies, Schumacher et al. have established a significant association between AMH levels and the risk of miscarriage. Their study, following patients through to live births, showed that, irrespective of age, individuals with lower AMH levels (< 0.4 ng/mL) exhibited an elevated risk of experiencing a miscarriage (Schumacher et al., 2018).

1.2.2.8.3.2 Anti-Müllerian hormone (AMH) predicting menopause

Since AMH reflects the decline in ovarian follicles over the years, it has been also considered to diagnose menopause (Broer et al., 2011; Depmann et al., 2016).

Disseldorp et al. established that, for women with similar ages, the lower AMH values the earlier age at menopause (Disseldorp et al., 2008). Broer et al. also proved, in a longitudinal study where women were prospectively followed up for 11 years, that women who experienced menopause were the ones with lower values of AMH at the beginning of the study (Broer et al., 2011).

1.2.2.8.4 Anti-Müllerian Hormone (AMH) predicting outcomes in assisted reproductive technology (ART) procedures

The blood levels of AMH are considered the most sensitive biomarker to evaluate ovarian reserve (Moreno-Ortiz et al., 2018), being indicated as the preferred test for assessing ovarian reserve in

51% of infertility centers (Çelik et al., 2023). ESHRE recognises that AMH alone is a reliable predictor of ovarian response (ESHRE, 2019).

In the last years, there has been significant progress in enhancing the sensitivity of AMH in predicting ovarian response, covering both poor and hyper responses (La Marca & Sunkara, 2014). Consequently, a strong and significant correlation between AMH and the number of retrieved oocytes after COS was reported across different populations (Almog et al., 2011; Barbakadze et al., 2015; de Castro et al., 2012; Lee et al., 2015).

The AMH testing has been used to decide the pharmaceutical protocol of COS in IVF (Nelson et al., 2009; Yates et al., 2011), reducing the risks of OHSS and increasing the number of normal and safe ovarian response (La Marca & Sunkara, 2014).

AMH <0.5 ng/mL and/or AMH between $0.7 - 1.3$ ng/mL was associated with a poor ovarian response in IVF (Al-azemi et al., 2011; Gnoth et al., 2008; La Marca et al., 2010; La Marca & Sunkara, 2014; Nelson et al., 2007). A threshold defining poor ovarian response was established at 0.93 ng/ml, demonstrating 90% specificity and 74.1% sensitivity through (Baker et al. 2018).

AMH ≥ 1 ng/mL but ≤ 3.5 ng/mL, if age appropriate, was associated with normal ovarian response (Almog et al., 2011; Barad et al., 2011; Fong et al., 2012; La Marca et al., 2010, 2012; Lee et al., 2012; Seifer et al., 2011).

AMH levels ≥ 3.5 ng/mL have been predictive of a hyper response and an increased risk of OHSS (La Marca et al., 2010). Furthermore, higher AMH levels have been associated with an increased probability of obtaining supernumerary blastocysts and achieving a higher cumulative live birth rate (Arce et al., 2013; Gat et al., 2017; Hamdine et al., 2015; La Marca et al., 2017).

1.2.2.9 Antral follicle count (AFC)

Ultrasonographic measures of ovarian reserve include antral follicle count (AFC) and ovarian volume. Ovarian volume, is calculated using ellipsoid formula (for each ovary), correlates with follicle count and oocyte retrieval, but with inconsistent variability between cycles (Penzias et al., 2020). Ovarian size was linked to the results of ART, with smaller ovaries being related to unfavourable outcomes (Craig et al., 2003). As women age, ovarian volume decreases, making it a potential marker for ovarian reserve. However, this measure is rarely used for clinical prediction because of significant variability between and within cycles, as well as its generally low sensitivity (Jayaprakasan et al., 2008).

AFC is obtained from the ultrasound counting of all the identifiable antral follicles of $2-10$ mm, in largest mean diameter on 2-dimension (2D) (Broekmans et al., 2010; Disseldorp et al., 2010).

AFC is undeniably correlated with age (La Marca et al., 2011; Scheffer et al., 1999), as it represents the size of the remaining number of follicles (Scheffer et al., 1999).

1.2.2.9.1 Antral follicle count (AFC) levels and its predictive capacity

AFC measurement is not much invasive, it is easy to perform and provides an immediate result (La Marca et al., 2011; Melo et al., 2009). AFC assessment involves some degree of inter-observer/ultrasound technology variability (Broer et al., 2009; Hansen et al., 2003; Iliodromiti et al., 2015), that is attenuated if performed by the same observer or by adhering to established protocols (Scheffer et al., 2002).

To avoid the influence of dominant follicles or corpus luteum from the previous cycle, AFC could be performed during the early follicular phase, or it can be measured at any point in the menstrual cycle (Szmelskyj et al., 2015).

AFC exhibits a good predictive power for poor ovarian response and a high response in IVF (Andersen et al., 2011; Arce et al., 2013; Broer et al., 2013; Hsu et al., 2011; Mutlu et al., 2013; Nelson et al., 2015; Tsakos et al., 2014). Low AFC (3-6 follicles) revealed a high specificity to predict poor ovarian response (3/4 retrieved oocytes) (Frattarelli et al., 2003; Hendriks et al., 2005; La Marca et al., 2010). High AFC (15-20 follicles) demonstrated a strong predicting value for OHSS (Leonhardt et al., 2014).

Finally, AFC is strongly correlated with AMH (Feyereisen et al., 2006; Fleming et al., 2015), particularly in patients with low ovarian reserve (Baker et al., 2018; Tadros et al., 2016). In IVF, the use of AMH and AFC is almost ubiquitous to enhance the fertility assessment (Keane et al., 2017; Spresão et al., 2016; Zheng et al., 2017) and deciding the FSH starting doses for COS (Lan et al., 2013).

In line with that, both AMH and AFC were recognised as reliable indicators, to predict response to ovarian stimulation (ESHRE, 2019).

1.2.2.10 Final considerations on the markers used to assess reproductive function

In conclusion, this subsection revisited the literature on markers used to assess female ovarian/reproductive function (FSH, estradiol, LH, progesterone, thyroid hormone, androgens, AMH, ovarian volume and AFC). The aim was to identify the preferred markers for assessing ovarian reserve. Among these, AMH and AFC are the most promising for evaluating ovarian reserve (not oocyte quality) (Cedars, 2022). These markers demonstrate predictive value and are widely utilized to predict the response to COS (Penzias et al., 2020). Simultaneously, AMH and AFC still be a focus of continuous research into its predictive value — for instance, in relation of AMH to live birth rates (Peigné et al., 2023), and blastocyst quality (Arnanz et al., 2023) - as well as the confirming of the roles of AMH and AFC in predicting the response to COS (Hochberg et al., 2024).

1.2.3 Diminished Ovarian Reserve (DOR)

1.2.3.1 Definition of diminished ovarian reserve (DOR)

There is no uniformly accepted definition of DOR, therefore the term DOR may refer to three related but distinctly different outcomes, oocyte quality, oocyte quantity, and reproductive potential (Gong et al., 2023; Xu et al., 2021).

Globally, DOR affects about 10% of women of reproductive age (Kesharwani et al., 2022), that have regular menses and exhibit a significantly lower count of available follicles or oocytes compared to those in the normal group of a similar age (Nikolaou & Templeton, 2003; Zhu et al., 2023). Women with DOR have been associated with decreased values of AMH and AFC, AMH < 1ng/mL (Laqqan & Yassin, 2023; Pastore et al., 2018), and AFC < 5 (Laqqan & Yassin, 2023).

Women with DOR may experience ovarian atrophy within 1 to 6 years, and their disease progress to POI simultaneously (Pastore et al., 2018). POI is more severe condition that is defined by amenorrhea for at least 6 months and a day 3 FSH level in the menopausal range (>40 IU/L), for women under 40 years old (ESHRE, 2015). Despite DOR and POI may share clinical features, women with DOR may never meet the criteria of POI (Ferrarini et al., 2013; Goswami & Conway, 2005; Nelson, 2009; Nikolaou & Templeton, 2003; Persani et al., 2010; Shamilova et al., 2013).

DOR stands as a prominent contributor to female fertility challenges (Bunnewell et al., 2020), representing about 30% of women in IVF cycles (Laqqan & Yassin, 2023; Pastore et al., 2018). Given the contemporary tendency to delay pregnancy, DOR has emerged as a prevalent issue in present reproductive medicine (Attali & Yogev, 2021). Due to its unclear mechanism and clinical characteristics, including the unclear aetiology and pathogenesis of DOR, physicians face challenges in providing precise and targeted treatment. Effective treatment methods for DOR are still needed to improve female reproductive health (Xu et al., 2021; Zhang et al., 2023).

DOR can be associated with factors such as autoimmunity, iatrogenic influences, or idiopathic (of unknown origin) reasons, but the exact cause of DOR remains unclear in 50% of the cases (Çelik et al., 2023; Nelson, 2009). It is hypothesized that the impairment of ovarian reserve is due to the abnormal balance between the initial follicle pool and its rate of depletion, resulting in a decrease of AFC (AFC < 5) and AMH (AMH < 0.5–1 ng/mL) and/or an increase of FSH (FSH > 10 IU/L on cycle day 2 to 4) (Ferraretti et al., 2011; Gleicher et al., 2011; Sills et al., 2009; Wang et al., 2010). This dysregulation may result from various factors, including (1) an initially low number of primordial follicles, (2) an accelerated follicular atresia, (3) an insufficient maturation/recruitment of primordial follicles or (4) a disabled communication between oocyte and granulosa cells (Goswami & Conway, 2005; Nelson, 2009). The most plausible explanation for DOR is a premature depletion of the primordial follicles but the origin of this depletion is not yet elucidated (Melo et al., 2009; Persani et al., 2010).

1.2.3.2 Genetic factors involved in ovarian function, diminished ovarian reserve (DOR), and premature ovarian insufficiency (POI)

Genetic alterations, loss-of-function mutations, and whole chromosomal abnormalities play crucial roles in modulating the activation rate of primordial follicles (Huhtaniemi et al., 2018). Meiotic genes, such as those involved in the assembly of the meiotic cohesion and synaptonemal complex, are significant contributors to POI, leading to multi-chromosome aneuploidy and follicle atresia (Jiao et al., 2021; Yatsenko & Rajkovic, 2019). Additionally, defects in deoxyribonucleic acid (DNA) damage repair mechanisms can activate cell apoptosis, depleting germ cells and causing a dramatic loss of ovarian reserve (Yatsenko & Rajkovic, 2019).

Single nucleotide polymorphisms (SNPs) are variations in the genetic code that can be used as genetic signatures. Targeted SNP assays hold promise in predicting premature DOR for young women, allowing for early identification and intervention (McCallie et al., 2021). SNPs in genes such as *AMH*, AMH receptor 2 (*AMHR2*), Growth Differentiation Factor 9 (*GDF9*), Bone Morphogenic Protein 15 (*BMP15*), *FSHR*, LH/Choriogonadotropin Receptor (*LHCGR*), and *ESR1* have been associated with an increased risk of DOR (McCallie et al., 2021). While DOR is more common than POI, genetic studies have identified candidate genes associated with both conditions, including Fragile X Mental Retardation 1 (*FMR1*), *FSHR*, *GDF9*, Nuclear Receptor Subfamily 5 Group A Member 1 (*NR5A1*), and Breast Cancer Gene 1/2 (*BRCA1/2*) mutations (Tang & Yu, 2020).

Several well-known genes are implicated in POI, including Bone Morphogenetic Protein Receptor Type 1B (*BMPR1B*), Forkhead Box L2 (*FOXO2*), Cytochrome P450 Family 19 Subfamily A Member 1 (*CYP19A1*), Sal-Like Protein 4 (*SALL4*), Wilms Tumor 1 (*WT1*), LIM Homeobox 9 (*LHX9*), Nanos Homolog 3 (*NANOS3*), Fanconi Anemia Complementation Group M (*FANCM*), Eukaryotic Translation Initiation Factor 4E Nuclear Import Factor 1 (*EIF4ENIF1*), Splicing Factor 1 (*SF1*), Basonuclin 1 (*BNC1*), Neurogenic Locus Notch Homolog Protein 2 (*NOTCH2*), G Protein-Coupled Receptor 3 (*GPR3*), Premature Ovarian Failure 1B (*POF1B*), Diaphanous Related Formin 2 (*DIAPH2*) Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 2 (*CITED2*) Cyclin Dependent Kinase Inhibitor 1B/P27 (*CDKN1B/P27*), Dachshund Family Transcription Factor 2 (*DACH2*), Collagen Type IV Alpha 6 Chain (*COL4A6*), *FMR1*, and others, each contributing to different aspects of ovarian function and development (França & Mendonca, 2020; Huhtaniemi et al., 2018; Venturella et al., 2019).

Furthermore, mutations in the *BRCA1/2* genes are associated with DOR, affecting homologous recombination and DNA repair mechanisms (La Marca & Mastellari, 2021; Yatsenko & Rajkovic, 2019). Fragile X-associated premature ovarian insufficiency (FXPOI) is linked to mutations in the *FMR1* gene with cytosine (C), guanine (G), and guanine (G) (CGG) repeats, influencing ovarian reserve (Nesbit et al., 2020).

Variations in other genes, such as AKT Serine/Threonine Kinase 1 (*AKT1*), Forkhead Box O3 (*FOXO3*), Folliculogenesis-Specific Basic Helix-Loop-Helix (*FIGLA*), Phosphatase and Tensin Homolog (*PTEN*), Matrix Metalloproteinases (MMPs), Insulin-Like Growth Factor 1/2 (*IGF1/2*), *GDF9*, and *BMP15*, contribute to follicular activation and ovarian development, with potential implications in DOR and POI (Ford et al., 2020; Greene et al., 2014; Huhtaniemi et al., 2018; Priya et al., 2023; Venturella et al., 2019).

While specific gene variants, such as *AMH*, *AMHR2*, *LHCGR*, *BMP15*, *GDF9*, *FSHR*, and new born ovary homeobox (*NOBOX*), have been linked to an increased risk of DOR (Moiseeva et al., 2021; Nesbit et al., 2020), the fundamental genetic mechanisms behind this association remain largely unknown.

The prevalence of a genetic origin for DOR has been supported by genome-wide studies for POI patients. Next generation sequencing (NGS) technology can be used to identify genetic variants within specific patient populations and has already begun to emerge for POI cases (Persani et al., 2010). Even though the study of DOR is mostly based on the model of POI, it is important to acknowledge that POI and DOR are not directly comparable (Pellicer et al., 2023).

The impact of mutations and polymorphic variant genes involved in folliculogenesis in DOR is still uncertain (Pellicer et al., 2023). To help track the information related to the genetic contribution to DOR and POI, we have constructed the table below (Table 1). This table provides a concise overview of the key genes involved in DOR and POI, their functions, and their impacts on ovarian health, along with the associated references. Some of the genes will be detailed in the following section.

Table 1 Genetic contributions to DOR and POI

This table provides a detailed overview of specific genes associated with DOR/POI. Each entry in the table includes the gene name, its function, its impact on DOR/POI, and references or descriptions supporting the association.

Gene	Function	Impact on DOR/POI	References
<i>AMH</i> (Anti-Müllerian Hormone)	Regulates folliculogenesis and ovarian reserve	Decreased expression in DOR; associated with age at menopause	McCallie et al., 2021; Cheng et al., 2019; Olsen et al., 2021; Skiadas et al., 2012
<i>AMHR2</i> (AMH Receptor 2)	Mediates AMH effects	Variants associated with DOR and natural menopause	McCallie et al., 2021; Cheng et al., 2019
<i>GDF9</i> (Growth Differentiation Factor 9)	Essential for folliculogenesis, granulosa cell proliferation, and follicle maturation	SNPs associated with DOR; rare variants in POI	McCallie et al., 2021; Persani et al., 2011; Wang et al., 2010; Yan et al., 2001

Gene	Function	Impact on DOR/POI	References
<i>BMP15</i> (Bone Morphogenetic Protein 15)	Influences follicle maturation and granulosa cell apoptosis prevention	Mutations associated with primary and secondary amenorrhea; linked to POI	McCallie et al., 2021; Chang et al., 2002; Dube et al., 1998; Shimasaki et al., 2004
<i>FSHR</i> (Follicle Stimulating Hormone Receptor)	Influences follicular recruitment, development, and maturation	Variants associated with ovarian response; Ser/Ser homozygous variant linked to higher FSH levels and reduced response	McCallie et al., 2021; Aittomäki et al., 1995; Casarini et al., 2015; Mayorga et al., 2000
<i>LHCGR</i> (Luteinizing Hormone/Choriogonadotropin Receptor)	Regulates ovulation and fertilization	Increased expression in DOR; inactivating variants associated with POI, oligomenorrhea, and lower fertilization rate	McCallie et al., 2021; Skiadas et al., 2012; Latronico et al., 1996
<i>ESR1</i> (Estrogen Receptor 1)	Regulates menstrual cycle and follicle senescence	Polymorphisms associated with POI and low fecundity; results inconsistent	McCallie et al., 2021; Altmäe et al., 2007a; Livshyts et al., 2013; M'Rabet et al., 2012
<i>FMR1</i> (Fragile X Mental Retardation 1)	Associated with FXPOI; CGG repeats influence ovarian reserve	Mutations linked to premature ovarian insufficiency	Tang & Yu, 2020; Nesbit et al., 2020
<i>BRCA1/2</i> (Breast Cancer Genes 1 and 2)	Involved in DNA repair and homologous recombination	Mutations associated with DOR	Tang & Yu, 2020; La Marca & Mastellari, 2021; Yatsenko & Rajkovic, 2019
<i>NR5A1</i> (Nuclear Receptor Subfamily 5 Group A Member 1)	Regulates folliculogenesis and ovarian function	Mutations identified in women with POI	Tang & Yu, 2020; Lourenço et al., 2009
<i>FOXL2</i> (Forkhead Box L2)	Important for granulosa cell differentiation and follicle pool maintenance	Mutations observed in non-syndromic POI	Huhtaniemi et al., 2018; Harris et al., 2002; Schmidt et al., 2004
<i>IGF1/2</i> (Insulin-Like Growth Factors 1 and 2)	Involved in granulosa cell proliferation and theca cell steroidogenesis	Downregulated in granulosa cells of DOR patients	Greenseid et al., 2011; Ford et al., 2020
<i>NOBOX</i> (Newborn Ovary Homeobox)	Encodes oocyte regulatory transcription factors important for early folliculogenesis	Mutations described in POI patients	Qin et al., 2007, 2009; Zhao et al., 2005
<i>FIGLA</i> (Factor In Germline Alpha)	Encodes oocyte regulatory transcription factors important for early folliculogenesis	Rare contribution to POI	Qin et al., 2007; Zhao et al., 2005
<i>INHA</i> (Inhibin A)	Regulates FSH levels; produced by granulosa cells	Polymorphisms debated in POI; some studies show association with ovarian insufficiency	Shelling et al., 2000; Dixit et al., 2006; Marozzi et al., 2002

Gene	Function	Impact on DOR/POI	References
CYP19A1 (Cytochrome P450 Family 19 Subfamily A Member 1)	Involved in estrogen biosynthesis	Variants associated with POI	França & Mendonca, 2020; Venturella et al., 2019
BMPRI1B (Bone Morphogenetic Protein Receptor Type 1B)	Part of the TGF- β superfamily; involved in folliculogenesis	Implicated in POI	França & Mendonca, 2020; Venturella et al., 2019
SALL4 (Sal-Like Protein 4)	Involved in early ovarian development	Mutations associated with POI	França & Mendonca, 2020; Venturella et al., 2019
WT1 (Wilms Tumor 1)	Involved in urogenital development	Variants associated with POI	França & Mendonca, 2020; Venturella et al., 2019
LHX9 (LIM Homeobox 9)	Transcription factor important for early ovarian development	Implicated in POI	França & Mendonca, 2020; Venturella et al., 2019
NANOS3 (Nanos Homolog 3)	Involved in germ cell development	Mutations linked to POI	França & Mendonca, 2020; Venturella et al., 2019
FANCM (Fanconi Anemia Complementation Group M)	Involved in DNA repair mechanisms	Variants associated with POI	França & Mendonca, 2020; Venturella et al., 2019
EIF4ENIF1 (Eukaryotic Translation Initiation Factor 4E Nuclear Import Factor 1)	Involved in translation initiation and protein synthesis	Implicated in POI	França & Mendonca, 2020; Venturella et al., 2019
SF1 (Splicing Factor 1)	Involved in RNA splicing	Associated with POI	França & Mendonca, 2020; Venturella et al., 2019
BNC1 (Basonuclin 1)	Involved in keratinocyte proliferation and differentiation	Implicated in POI	França & Mendonca, 2020; Venturella et al., 2019
NOTCH2 (Neurogenic Locus Notch Homolog Protein 2)	Involved in cell differentiation processes	Associated with POI	França & Mendonca, 2020; Venturella et al., 2019
GPR3 (G Protein-Coupled Receptor 3)	Involved in maintaining meiotic arrest in oocytes	Implicated in POI	França & Mendonca, 2020; Venturella et al., 2019
POF1B (Premature Ovarian Failure 1B)	Associated with ovarian function	Mutations linked to POI	França & Mendonca, 2020; Venturella et al., 2019
DIAPH2 (Diaphanous Related Formin 2)	Involved in actin cytoskeleton remodelling	Associated with POI	França & Mendonca, 2020; Venturella et al., 2019
CITED2 (Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 2)	Involved in transcriptional regulation	Implicated in POI	França & Mendonca, 2020; Venturella et al., 2019
CDKN1B/P27 (Cyclin Dependent Kinase Inhibitor 1B/P27)	Involved in cell cycle regulation	Associated with POI	França & Mendonca, 2020; Venturella et al., 2019
DACH2 (Dachshund Family Transcription Factor 2)	Involved in developmental processes	Implicated in POI	França & Mendonca, 2020; Venturella et al., 2019
COL4A6 (Collagen Type IV Alpha 6 Chain)	Involved in extracellular matrix composition	Associated with POI	França & Mendonca, 2020; Venturella et al., 2019

Gene	Function	Impact on DOR/POI	References
<i>PTEN</i> (Phosphatase and Tensin Homolog)	Involved in cell growth and proliferation	Variations contribute to follicular activation and ovarian development; implicated in DOR and POI	França & Mendonca, 2020; Venturella et al., 2019
<i>AKT1</i> (AKT Serine/Threonine Kinase 1)	Involved in cell survival and metabolism	Variations implicated in follicular activation and ovarian development	França & Mendonca, 2020; Venturella et al., 2019
<i>FOXO3</i> (Forkhead Box O3)	Involved in cell cycle regulation and apoptosis	Variations contribute to follicular activation and ovarian development; implicated in DOR and POI	França & Mendonca, 2020; Venturella et al., 2019
<i>MMPs</i> (Matrix Metalloproteinases)	Involved in extracellular matrix degradation	Variations contribute to follicular activation and ovarian development; implicated in DOR and POI	França & Mendonca, 2020; Venturella et al., 2019
<i>FIGLA</i> (Folliculogenesis-Specific Basic Helix-Loop-Helix)	Involved in oocyte regulatory functions	Variations contribute to follicular activation and ovarian development; implicated in DOR and POI	Qin et al., 2007; Zhao et al., 2005

DOR: diminished ovarian reserve; POI: premature ovarian insufficiency; FSH: follicle stimulating hormone; SNPs: single nucleotide polymorphisms.

1.2.3.3 Specific gene insights

1.2.3.3.1 Anti-Müllerian Hormone (AMH) and Anti-Müllerian Hormone receptor (AMHR)

AMH belongs to the transforming growth factor β (TGF β) superfamily as well as GDFs, BMPs, inhibins or activins. Since the paracrine action of these factors is required for correct folliculogenesis, their encoding genes were proposed to be investigated in cases of DOR (Shimasaki et al., 2004).

A decreased expression of *AMH* has been documented in granulosa cells of patients with DOR (Skiadas et al., 2012). Furthermore, variants of the *AMH/AMHR* have been associated with the age at natural menopause (Cheng et al., 2019). Nevertheless, *AMH* involvement in the pathophysiology of DOR remains to be elucidated (Olsen et al., 2021).

1.2.3.3.2 Growth Differentiation Factor 9 (GDF9)

GDF9 gene is essential in folliculogenesis, being expressed in oocyte and also acting on granulosa cells regulating the primordial follicle development, granulosa cells proliferation and follicle maturation (Persani et al., 2011; Wang et al., 2010, 2013; Yan et al., 2001). *GDF9* is also involved in steroidogenesis and regulation of FSH sensitivity (Chand et al., 2006).

SNPs of *GDF9* have been associated with DOR (Altmäe et al., 2007; Wang et al., 2013).

In cases of POI, variants of *GDF9* were considered a rare contributor for this condition (Chand et al., 2006; Wang et al., 2010), with a prevalence of 1-4% among POI women (Kovanci et al., 2007; Laissue et al., 2006; Zhao et al., 2007).

1.2.3.3.3 Gremlin 1 (*GREM1*)

Gremlin 1 (*GREM1*) is a downstream effector of *GDF9* and is differentially expressed in DOR patients. *GREM1* is downregulated in granulosa cells of DOR patients (Jindal et al., 2012) and that is associated with lower quality embryos (McKenzie et al., 2004).

1.2.3.3.4 Bone Morphogenetic Protein 15 (*BMP15*)

BMP15 encodes oocyte-derived growth and differentiation factors essential in follicular development (Chang et al., 2002; Dube et al., 1998; McNatty et al., 2004; Shimasaki et al., 2004). Specifically, *BMP15* influences the follicle maturation, regulating gonadotropin independent phase and the FSH sensitivity of granulosa cells; prevents apoptosis in granulosa cells; and determines oocyte competence and ovulation (Hashimoto et al., 2005; Hussein et al., 2005, 2006; Shimasaki et al., 2004). *BMP15* gene mutations were already associated with primary and secondary amenorrhea, in 1.5 to 12% of women with POI (Di Pasquale et al., 2004; Dixit et al., 2006; Rossetti et al., 2009; Tiotiu et al., 2010).

1.2.3.3.5 Inhibin A (*INHA*)

Inhibin A (*INHA*) coordinates important mechanism of ovarian function (Rah et al., 2014) and folliculogenesis (Shelling et al., 2000). Specifically, *INHA*, produced by granulosa cells, regulates the FSH levels (Robertson et al., 1997). A decrease in inhibin levels triggers a rise in FSH, leading to uncontrolled follicular recruitment and the depletion of ovarian reserve (Shelling et al., 2000).

The association of *INHA* with POI remains a subject of debate. Some studies report an *INHA* polymorphism (*INHA* 769G>A, A257T) in cases of ovarian insufficiency (Chand et al., 2010; Dixit et al., 2006; Marozzi et al., 2002; Shelling et al., 2000). However, other authors report no significant association of the *INHA* polymorphism with oocyte depletion (Corre et al., 2009; Persani et al., 2011; Rah et al., 2014), possibly due to variations in its representation among different ethnicities.

1.2.3.3.6 Innsulin-like growth factors 1 and 2 (*IGF1*, *IGF2*).

IGF1 *IGF2* and their receptors (*IGF1R*, *IGF2R*) are involved in granulosa cells proliferation and theca cells steroidogenesis. Additionally, *IGF1*, *IGF2* and *IGF1R*, *IGF2R* were differentially expressed (downregulated) in granulosa cells from women with DOR (Greenseid et al., 2011).

1.2.3.3.7 Nuclear Receptor Subfamily Five, Group A Member 1 (*NR5A1*), New Born Ovary Homeobox (*NOBOX*), Factor In Germline Alpha (*FIGLA*) And The Forkhead Box L2 (*FOXL2*)

NR5A1 is a transcriptional regulator of genes involved in hypothalamic-pituitary-steroidogenic axis that regulates folliculogenesis and ovarian function (Persani et al., 2010). *NR5A1* mutations have been identified in women with POI (Lourengo et al., 2009).

NOBOX and *FIGLA* encode oocyte regulatory transcription factors important for the early folliculogenesis (Qin et al., 2007, 2009; Rajkovic et al., 2004; Zhao et al., 2008).

NOBOX mutations were described in POI patients (Qin et al., 2007), but the literature suggests a rare contribution of these transcriptional factors for POI (Qin et al., 2009; Zhao et al., 2005)

FOXL2 gene is important for granulosa cells differentiation, being involved in the maintenance of the follicle pool (Schmidt et al., 2004). *FOXL2* mutations were observed in about 5% of non-syndromic POI (Harris et al., 2002).

1.2.3.3.8 Hormonal receptors genes: follicle stimulating hormone receptor (*FSHR*), estrogen receptor (*ESR*); and luteinizing hormone receptor (*LHR*)

Alterations in genes encoding for hormonal receptors influence the recruitment, development and maturation of follicles, motivating their study in patients with abnormal ovarian function, including those diagnosed with PCOS and DOR/POI (Hoogendoorn et al., 2003).

The first *FSHR* mutation identified was due to the substitution of Alanine to Valine at position 189 (p.Ala189Val), at the receptor glycosylation site. This mutation was described in women with ovarian dysgenesis and primary amenorrhea (Aittomäki et al., 1995).

More than two thousand SNPs within coding and non-coding regions of the *FSHR* gene were described (Casarini et al., 2015). Particularly, two variants of *FSHR* have intensively been studied, both in exon 10. One at position 680, *FSHR* rs6166 (c.2039 A>G, p.Asn680Ser), in which Asparagine is replaced by Serine in the intracellular domain of the *FSHR* protein, and the other at position 307, *FSHR* rs6165 (c.919G>A, p.Thr307Ala), causing the replacement of Threonine by Alanine. The former (*FSHR* rs6166) introduces a potential phosphorylation site and the latter (*FSHR* rs6165) results in a change from a polar to a non-polar hydrophobic amino acid, thereby removing a potential O-linked glycosylation site (Simoni & Casarini, 2014).

FSHR rs6166 is the most studied in relation to ovarian stimulation. *FSHR* rs6166 Ser/Ser homozygous genotype was associated with higher FSH serum levels and dosage of FSH to get a similar ovarian response (Mayorga et al., 2000) suggesting that Ser/Ser homozygous variant is less sensitive to FSH (Simoni & Casarini, 2014). These results were verified in *in vitro* studies by a lower cAMP increase in the cells from *FSHR* rs6166 Ser/Ser homozygous carriers, compared to

Asn/Asn homozygous. Consequently, the gene expression and the progesterone production were altered in the Ser/Ser genotype (Casarini et al., 2014).

Accordingly, women homozygous for the serine (*FSHR* rs6166) have been associated with a reduced response, higher concentrations of basal levels of FSH and higher requirement of gonadotrophins, when compared with women with the two other variants (Mayorga et al., 2000). Even so the referred studies suggest that Ser/Ser homozygous variant indicates a less sensitiveness to FSH, the results are inconsistent (Daelemans et al., 2004; Greb et al., 2005; Klinkert et al., 2006; Mohiyiddeen et al., 2012, 2013; Trevisan et al., 2014).

The *FSRH* rs1394205 (c.-29G > A) is another common polymorphic variant of the gene, which could affect reproductive function. Individuals with the AA genotype were associated with higher levels of AMH and AFC compared to those with the AG genotype. On the contrary, individuals with the AG and GG genotypes showed lower levels of AMH and AFC in comparison to the AA genotype (Guo et al., 2021). Another study suggested that A variant could be related to high serum FSH and low estradiol levels (Achrekar et al., 2009; Grigorova et al., 2014) but there is not a consensus about its influence on ovarian response to FSH (Desai et al., 2011; Trevisan et al., 2014; Wunsch et al., 2005).

ESR1 polymorphisms have been also studied because of their role in regulation of the menstrual cycle, low fecundity and PCOS (Altmäe et al., 2007; Livshyts et al., 2013; M'Rabet et al., 2012; Yoon et al., 2010). The two most common SNPs among *ESR1* are *ESR1* rs2234693 (PvuII, -397 TC, NM_000125.3:c.453-397 T > C) and *ESR1* rs9340799 (XbaI, -351 AG, NM_000125.3:c.453-351 A > G), respectively, PvuII and XbaI, named accordingly to their detecting restriction enzyme. These SNPs are located in the first intron of *ESR1* and have been associated with ovarian follicles senescence and POI (Altmäe et al., 2011; Bretherick et al., 2008; Cordts et al., 2012; M'Rabet et al., 2012). This association was not consensual (Corbo et al., 2007; De Mattos et al., 2014; Silva et al., 2010).

The combined model of *FSHR* with *ESR1* variants is expected to predicted 10–15% of poor responders to recombinant FSH in IVF treatments (de Castro et al., 2004).

Finally, the expression of *LHCGR* has been described to be increased in DOR patients (Skiadas et al., 2012). Moreover, *LHCGR* inactivating variants were associated with POI, leading to a compromised ovulation, oligomenorrhea (Latronico et al., 1996, 1998) and a lower fertilization rate (Maman et al., 2012).

1.2.3.4 Therapeutic approaches for women with diminished ovarian reserve (DOR)

1.2.3.4.1 Adapted protocols of controlled ovarian stimulation (COS)

The reduced follicular pool of DOR patients negatively impacts IVF outcomes by a pathological mechanisms that is poorly understood (Abhari et al., 2022). DOR patients respond poorly to COS

resulting in retrieval of fewer oocytes, producing poorer quality embryos, and reduced implantation rates and pregnancy rates (Patrizio et al., 2015; Steiner et al., 2017).

In order to standardize the concept of “poor responders”, ESHRE defined the “Bologna criteria” (Ferraretti et al., 2011). To be included in this group, patients must meet ≥ 2 of the following: 1 – Previous episode of low ovarian response to COS protocol using a standard dose of drug (collection of ≤ 3 oocytes after ovarian stimulation cycle); 2 – DOR, with an AFC < 5 -7 follicles or an AMH value < 0.5 -1.1 ng/mL; 3 – Woman > 40 years old or with other risk factors for low ovarian response, such as previous ovarian surgery, genetic changes, chemotherapy, radiotherapy and autoimmune diseases (Selman & Rinaldi, 2016; Ubaldi et al., 2014). All studies on patients who met the Bologna criteria reported very low pregnancy rates, regardless of the woman's age and the ovarian stimulation protocol (Fusi et al., 2020).

The patient-oriented strategies group encompassing individualized oocyte number (POSEIDON) suggested stratifying poor responders based on quantitative and qualitative parameters, namely: age and expected rate of aneuploidy; ovarian biomarkers (AFC and AMH); and ovarian response (information obtained from an previous ovarian stimulation cycle) (Humaidan et al., 2016). By dividing these into 4 subgroups, the POSEIDON group allowed the distinction between women who, despite having a suboptimal ovarian response to COS, have an adequate ovarian reserve (Groups 1 and 2: AFC ≥ 5 and AMH ≥ 1.2 ng/mL) and women who have a low ovarian reserve (Groups 3 and 4: AFC < 5 and AMH < 1.2 ng/mL). This is a distinction that the Bologna criteria did not take into consideration (Grynberg & Labrosse, 2019). Accordingly, DOR patients could be considered POSEIDON group 3, women < 35 years old, AFC < 5 , and AMH < 1.2 ng/mL (Esteves et al., 2019).

It is also argued that the quality of oocytes in women with DOR, specifically those aged < 35 years old, remains unaffected despite the reduction in quantity. This suggests that younger women with DOR have a significantly higher chance of achieving pregnancy with their own eggs if they anticipate conception (Pastore et al., 2018). In another words young women diagnosed with DOR do not consistently exhibit a poor response. A study by Jin et al. compared a DOR group (AMH < 1.1 ng/mL) with a non-DOR group (AMH ≥ 1.1 ng/mL), involving 443 patients in the DOR group and 7,736 patients in the non-DOR group. The incidence of live birth and clinical pregnancy after fresh cycle transfer did not significantly differ between the groups. Nevertheless, the cumulative live birth rate was notably lower in the DOR group (Jin et al., 2023).

In the context of ovarian stimulation during an IVF treatment, LH supplementation at the mid-follicular phase may enhance pregnancy rates was suggested for DOR patients (Yenigul et al., 2022). A total of 393 women were recruited for a study, with 274 receiving LH supplementation (recombinant FSH + highly purified -human menopausal gonadotropin (hMG) and 119 undergoing treatments with recombinant FSH only. Results revealed that women with DOR who used LH supplementation experienced inferior days of stimulation and a lower total gonadotropin dose compared to those receiving recombinant FSH only. LH supplementation was associated

with an increased number of fertilized eggs, as well as a higher count of good quality embryos and embryos that were successfully transferred (Yenigul et al., 2022).

The use of growth hormone in IVF treatments has been also proposed, as it regulates signalling pathways associated with primordial follicular activation, steroidogenesis, and oocyte maturation. The effects of growth hormone on the regulation of primordial follicles operate through the PI3K-Akt pathway (Chang et al., 2022; Pan & Huang, 2022). Supplementing growth hormone in patients with poor ovarian response has been studied as a noteworthy therapy aimed at increasing the number of retrieved oocytes, enhancing the availability of embryos for transfer, and elevating the clinical pregnancy rate (Han et al., 2023). Moreover, co-treatment with growth hormone during ovarian stimulation has been observed to alter follicular fluid metabolite profiles and increase the number of retrieved oocytes, particularly in patients with DOR (He et al., 2023).

1.2.3.4.2 Pharmacogenetics

Pharmacogenetic approaches, detail how patients genotype influences the response to exogenous stimulation and take advantage of that to define the ovarian stimulation protocol (Altmäe et al., 2011). This rational is based on the fact that clinical characteristics of women (age, AFC, AMH and FSH levels) could not be enough to predict the ovarian response to exogenous gonadotropins and to design the most suitable protocol of ovarian stimulation (Alviggi et al., 2013; Conforti et al., 2017), particularly in patients with DOR/POI (Fauser et al., 2010).

Panels of genetic tests to define the optimal stimulation protocol were already proposed (Sun et al., 2008).

1.2.3.4.3 Acupuncture

Acupuncture due to its proven inference with BMP15 and Akt in mice was also suggested in cases of POI (Li et al., 2022; Xu et al., 2021). In a study by Zhang et al. electro-acupuncture in mice was shown to enhance primordial follicle counts, elevate E2 and AMH levels, and reduce FSH and LH levels, by targeting the PI3K-Akt signalling pathway (Zhang et al., 2019). Other authors have also suggested that acupuncture can modulate the PI3K-Akt pathway (Chen et al., 2021; Wang et al., 2019).

1.2.3.4.4 Follicular activation

New approaches have been proposed to revert the follicular loss and induce postnatal oogenesis, potentially increasing the number of available oocytes (Zou et al., 2009) (Virant-Klun et al., 2009). This possibility is based on the prevalence of germline/oogonial stem cells in the ovary, indicating the dynamic nature of the follicle pool (Bukovsky et al., 2005).

Looking at the oocyte development, it starts during the early embryonic stage with the migration of primordial germ cells (PGCs) to the gonadal ridge. PGCs will then proliferate to originate the oogonia and, at the end, the primary follicle (Hummitzsch et al., 2015). The differentiation of

ovarian cells compromises the synergy between the ovary, responsible for the tissue remodelling and support (Hsueh et al., 2015), and the oocyte, determinant for the differentiation of granulosa cells (Guigon & Cohen-Tannoudji, 2011).

Interestingly, it has been suggested that there is a reminiscent source of germ cells in the outer cortex region of the ovary, possibly PGCs, that persist after the postnatal period (Bhartiya et al., 2013; Byskov et al., 2011; Usongo & Farookhi, 2012; White et al., 2012). These cells are quiescent until the surge of stimulus (Drukała et al., 2012; Wojakowski et al., 2010) to repair the ovarian epithelium, for example as it happens after the ovulation (Szotek et al., 2008). Similarly, there are mesenchymal stem cells (MSCs) present in tunica albuginea that can undergo ovarian epithelium renewal (Bukovsky et al., 2005). Therefore, it has been hypothesised that the ovarian tissue maintains a regenerative capacity comparable to the recovery processes observed in many other tissues (Hensley & Floyd, 2002).

Although the exact mechanisms that control the follicular activation still unknown (Li et al., 2021; Patton et al., 2021), important intracellular signalling pathways have already been described as targets for therapy for DOR patients (Adhikari & Liu, 2009). The transition from dormant to growing state in primordial follicles is under control of serine/threonine kinases through two signalling pathways: 1) Mammalian target of rapamycin (mTOR) and 2) PI3K-Akt (Hsueh et al., 2015).

Kit ligand, neurotrophins, IGF, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), BMP4, BMP7, leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF) and keratinocyte growth factor (KGF), have been also shown to be important for primordial follicle activation from the dormant state (Hsueh et al., 2015).

1.2.3.4.4.1 Signalling pathways controlling follicular activation

1.2.3.4.4.2 Protein kinase B (Akt) or phosphoinositide-3-kinase (PI3K)-Akt and mammalian target of rapamycin (mTOR)

Akt or PI3K-Akt pathway plays an important role in metabolic control, immunity, cancer, and follicular survival, growth, and proliferation (Tanaka et al., 2012). Specifically, this signalling pathway is responsible for the primordial follicle's activation and growth to primary and secondary stages (Hsueh et al., 2015; Kawamura et al., 2013).

PI3K-Akt is negatively regulated by PTEN (Wulschleger et al., 2006). Therefore, the incubation of ovarian tissue with a PTEN inhibitor effectively initiates the primordial follicle growth (Kawamura et al., 2013; Lerer-Serfaty et al., 2013; Li et al., 2010; McLaughlin et al., 2014; Novella-Maestre et al., 2015). Pregnancies were reported after ovarian tissue exposure to PTEN inhibitors, in patients with POI (Kawamura et al., 2013; Suzuki et al., 2015).

The investigation of PTEN and/or tuberous sclerosis complex 1/2 (TSC1/2) deletion in mice has revealed that such deletions lead to the overexpression of PI3K-Akt and/or mTOR, that results in a significant activation of primordial follicles and subfertility (Adhikari et al., 2010; Adhikari & Liu, 2009; Reddy et al., 2008, 2009; Tanaka et al., 2012). Removal of ovarian tissue from its *in vivo* environment can disrupt the normal regulation of this pathway (Grosbois et al., 2023; Hovatta et al., 1999; Telfer et al., 2008).

mTOR is an effector of the PI3K-Akt pathway and has been demonstrated to regulate cellular growth and proliferation across various cell types (Wullschleger et al., 2006). The activity of mTOR complex 1 (mTORC1) is negatively modulated by the TSC1 and TSC2 (Kwiatkowski & Manning, 2005). Inactivating mutations in *TSC1* and *TSC2* genes have been associated with benign tumors in various organs, including the brain, heart, kidney, lung, and skin (Kwiatkowski & Manning, 2005).

The PI3K-Akt and mTOR pathways are intricately interconnected. In granulosa cells, FSH activates mTOR through the PI3K signalling pathway. This activation, in turn, leads to the phosphorylation and regulation of Forkhead box O1 (FOXO1) and RAS (a small GTP-binding protein), orchestrating the differentiation of granulosa cells (Wayne et al., 2007). This regulatory cascade also up-regulates hypoxia-inducible factor 1 α (HIF1 α) and VEGF and LHR expression in granulosa cells, crucial for follicular maturation (Alam et al., 2004, 2009).

Moreover, the LH surge stimulates both PI3K-Akt and RAS signalling pathways, contributing to the coordination of ovulation (Fan et al., 2008). Notably, independently of PI3K-Akt signalling, mTOR is activated in luteal cells by LH (Hou et al., 2010).

The PI3K-Akt pathway was also proved to be required for 17 β estradiol - induced vascular endothelial growth factor A (VEGF-A) expression in rat uterus to increase vascular permeability in preparation for implantation (Kazi & Koos, 2007).

1.2.3.4.4.2.1 Hippo-Pathway

Generally speaking, the Hippo-pathway controls the cell fate to proliferate, remain quiescent or undergo cell death, based on a serine/threonine kinase cascade (Halder & Johnson, 2011). Hippo-pathway is involved in 1) organ size control, which is conserved in all metazoan (Halder & Johnson, 2011; Hergovich, 2012; Pan, 2007); 2) regulation and maintenance of various stem cell populations (Meng et al., 2016) and 3) tumor suppression (Jonhson & Halder, 2014).

The Hippo signalling pathway comprises a series of negative growth regulators. Activation of the mammalian Ste-20 like kinase (MST1/2) results in the phosphorylation and activation of the large tumor suppressor kinase (LATS1/ LATS2). Subsequently, LATS1/2 phosphorylates and inactivates the transcriptional co-activators, Yes-associated protein (YAP) and PDZ-binding motif (TAZ), which are sequestered in the cytoplasm. When the Hippo-pathway is disrupted, unphosphorylated YAP and TAZ move into the nucleus where they activate genes involved in survival and proliferation (Hong & Guan, 2012; Oka et al., 2008). The referred genes (YAP, TAZ,

MST1/2, and LATS1/2) were already identified in murine and human ovaries, being expressed in follicles at different stages (Kawamura et al., 2013)

The Hippo pathway acts as a restrictive force on follicular development, limiting it from progressing beyond the secondary to the preantral stage, as represented in Figure 9.

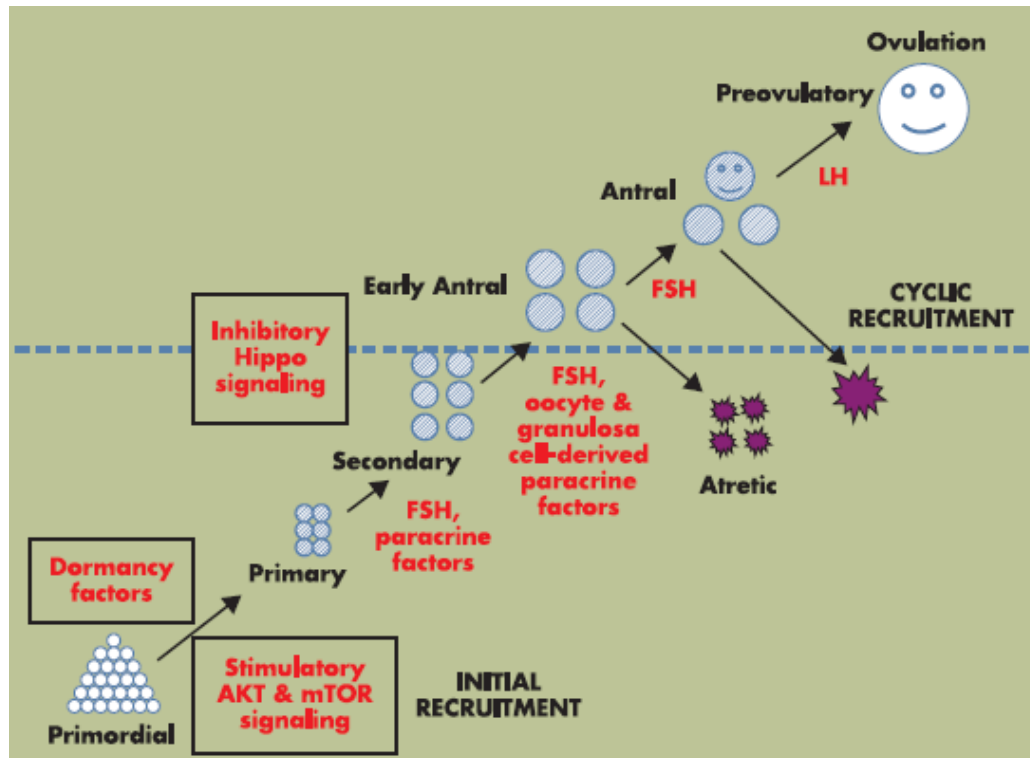


Figure 9 Regulation of ovarian reserve from initial recruitment to ovulation (involvement of Hippo and AKT & mTOR pathways)

The transition from primordial to primary follicular state relies on a delicate balance between dormancy factors and stimulatory signalling of AKT & mTOR pathways. Subsequent development through primary and secondary stages to early antral follicles is influenced by hormonal regulation, particularly FSH, which induces granulosa cell proliferation. While most early antral follicles naturally undergo atresia, a subset is selected by FSH and LH (cyclic recruitment) to become capable of releasing a mature oocyte. The fate of preantral and antral follicles is further controlled by the Hippo pathway. From Hsueh et al., 2015.

FSH: follicle stimulating hormone; LH: luteinizing hormone; AKT: protein kinase B; mTOR: Mammalian Target of Rapamycin

Given its negative regulatory impact on follicular growth (Hsueh et al., 2015; Pan, 2007), disrupting the Hippo pathway becomes imperative to trigger the secretion of growth factors capable of stimulating follicular development (Hsueh et al., 2015; Kawamura et al., 2013). This disruption is evident in medical interventions such as ovarian drilling to induce follicular growth in patients with PCOS (Farquhar et al., 2012). Additionally, the fragmentation of ovarian tissue into small pieces during cryopreservation has proven to facilitate follicular growth upon grafting for fertility preservation (Donnez et al., 2011). Supporting this, the hypothesis that there is

follicular growth (from pre-antral to late secondary, antral, and preovulatory stages) associated with ovarian injury was validated in mouse and rat models (Kawamura et al., 2013).

The inhibition of YAP in granulosa cells was associated with the suppression of proliferation and stimulation of differentiation. Additionally, the phosphorylation and degradation of YAP, in response to ovulatory signals, allow the final differentiation of cumulus cells (Ji et al., 2017; Lv et al., 2019; Sun & Diaz, 2019).

TAZ and YAP exert their transcriptional activity by binding to transcriptional factors (TEAD) (Mahoney et al., 2005; Vassilev et al., 2001). Consequently, the TEAD family plays a pivotal role in regulating the activity of both TAZ and YAP (Zhao et al., 2008). When YAP combines with TEAD, it triggers the expression of various cysteine-rich angiogenic protein 61, connective tissue growth factor, and nephroblastoma (CCN) growth factors overexpressed and baculoviral inhibitors of apoptosis repeat-containing (BIRC) apoptosis inhibitors. CCN and BIRC factors, in turn, play crucial roles in stimulating cell growth, promoting survival, and facilitating proliferation (Pan, 2007). Particularly, CCN proteins have been implicated in the development of preantral follicles (Kawamura et al., 2013). Notably, studies in human have demonstrated an increase in CCN growth factors in ovarian cortices after the cutting and thawing of human cortical strips into smaller cubes (Kawamura et al., 2013).

The fragmentation of ovarian tissue, involving Hippo pathway disruption triggers the translocation of non-phosphorylated YAP into the nucleus, activating CCN growth factors and BIRC apoptosis inhibitor genes (Pan, 2007). Furthermore, it has been demonstrated that the ovarian fragmentation induce an increase in the actin polymerization, resulting in an increase of nuclear YAP and CCN growth factors and BIRC apoptosis inhibitors (Kawamura et al., 2013).

Actin polymerization is intricately linked with Hippo signalling disruption, a phenomenon conserved in human HeLa cells. The deletion of genes involved in Hippo pathway has been found to lead to the formation of extra F-actin, (Sansores-Garcia et al., 2011). The transition of globular actin to the filamentous form mediates cell adhesion, shape maintenance, and locomotion (Kawamura et al., 2013; Wada et al., 2011).

The physical and mechanical microenvironment is also documented as a regulator of the Hippo pathway, influencing factors such as cell adhesion, shape, density, and polarity (Halder et al., 2012). Simultaneously, follicle activation is intricately tied to the physical environment of the ovary. This is evident in the distinction between the cortical and medullar regions, where differences in rigidity and follicle density impact the activation of dormant follicles. Specifically, the cortical region, being more rigid than the medullar region, exhibits a higher concentration of primordial follicles that are in a dormant state (Woodruff & Shea, 2011).

As follicular growth advances, a dynamic shift unfolds from the cortical to the medullar region. This transition is intimately associated with the perturbation of the Hippo pathway within the follicles located in the cortical region. Consequently, this disruption triggers an upregulation of YAP activity and the secretion of CCN growth factors, ultimately culminating in cell proliferation (Hsueh et al., 2015).

1.2.3.4.4.3 Regulatory mechanisms of follicular activation

The interest in activating dormant follicles within the ovarian cortex was first described by Li and colleagues, when successfully reactivated follicles (Li et al., 2010). This achievement was realized through an *in vitro* activation protocol involving PTEN inhibitors and PI3K stimulators (Akt stimulation). It marked a significant development, supported by the discovery that a considerable number of women with POI retained dormant follicles in the ovarian cortex (Li et al., 2010).

Importantly, the regulatory mechanisms of primordial follicle activation comprise a) follicular microenvironment, namely autocrine and paracrine growth factors involved in the aforementioned signalling pathways (Levy et al., 1997; Liu et al., 2014; Zeng et al., 2017); b) communication between follicular cells and oocytes, a synergetic effect of hormones and growth factors produced by granulosa cells that impact on development of oocytes and *vice-versa* (Das et al., 2008; Donaubauer & Hunzicker-Dunn, 2016; Zeng et al., 2017), and c) physical and mechanical stimulus in ovarian tissue, such as cell adhesion or gradient of density (Woodruff & Shea, 2011; Yin et al., 2016), which regulate signalling pathways including PI3K–Akt pathway and the Hippo-pathway (Cheng et al., 2015; Hu et al., 2019). Alterations in the extracellular matrix (ECM) resulting from ovarian culture were also correlated with the activation of primordial follicles (Grosbois et al., 2023).

In vitro activation of ovarian follicles has been induced 1) pharmacologically, via PTEN inhibitors and PI3K activators to activate dormant follicles, in murine and human (Kawamura et al., 2013; Li et al., 2010); 2) mechanically, inducing Hippo-pathway inhibition by ovarian tissue fragmentation to promote growth of preantral follicles in mice and human (Kawamura et al., 2013), or 3) combining both strategies (Adhikari et al., 2012; Dolmans et al., 2019; Kawamura et al., 2013; Li et al., 2010; Suzuki et al., 2015). The combined method was proposed by Kawamura and Suzuki to promote development of antral follicles in women with DOR (Kawamura et al., 2013; Suzuki et al., 2015).

The question persists as to whether patients with DOR would benefit from a combined approach involving the stimulation of Akt and the disruption of the Hippo pathway. This potential combination holds promise for advantages in both primordial follicle activation and the subsequent growth of secondary follicles, as illustrated in Figure 10 (Broekmans et al., 2009; Ferreri et al., 2020; Hsueh & Kawamura, 2020; Kawamura et al., 2020). Importantly, the Akt pathway, with its impact on granulosa cells, emerges as a significant contributor to the growth of secondary follicles (Hsueh et al., 2015).

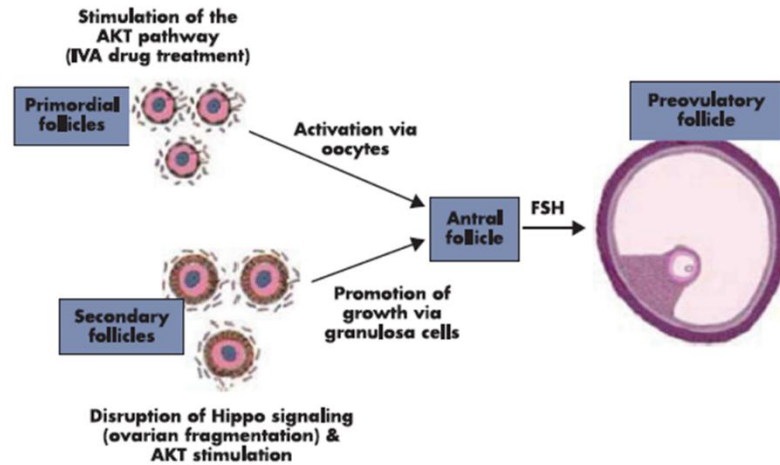


Figure 10 Stimulation of the Akt pathway and disruption of Hippo signalling effect on follicular development

Activation of the Akt pathway promotes the awakening of dormant primordial follicles, whereas disruption of Hippo signalling facilitates the growth of secondary follicles. From Hsueh et al., 2015

Akt: protein kinase B

Considering the usage of Akt stimulating drugs, several studies have investigated its impact. In a study by Kawamura et al. and Suzuki et al., which included POI patients under 40 years old (N=37), 9 out of 37 individuals developed follicles. Among them, 3 out of 37 achieved a biochemical pregnancy, and 2 out of 37 had a live birth (Kawamura et al., 2013; Suzuki et al., 2015). Another study by Zhai et al. focusing on POI (N=14) reported that 6 out of 14 patients developed follicles, with 4 out of 14 having embryos frozen and 1 out of 14 achieving a live birth (Zhai et al., 2016).

Considering drug-free activation approaches, various studies have explored their effectiveness in individuals with specific reproductive conditions. Fabregues et al. reported follicular development in 7 out of 14 patients, with 4 pregnancies and 4 live births (Fabregues et al., 2018).

In a study conducted by Lunding et al., focusing on poor responders (N=20), 7 out of 20 had a significant increase of AMH levels. Among them, 12 out of 20 achieved a clinical pregnancy without ART. Furthermore, 16 out of 20 undergone an IVF cycle, with 7 out of 20 having a live birth after IVF, resulting in an 18.4% cumulative live birth rate (LBR) per IVF cycle. Additionally, 1 out of 20 had a live birth after intrauterine insemination (IIU) , and 3 out of 20 had a live birth after natural conception (Lunding et al., 2019).

In the study conducted by Kawamura et al, which included poor responders (N=11), 9 out of 11 individuals exhibited increased AMH levels (baseline prior to the procedure). Among them, 3 out of 11 achieved a clinical pregnancy after IVF, and 1 out of 11 resulted in a natural conception (Kawamura et al., 2020).

Ferreri et al. conducted a study on individuals with POI (N=14), where 4 out of 14 resulted in a clinical pregnancy after IVF (Ferreri et al., 2020).

In the research by Diaz-Garcia et al. involving poor ovarian responders (N=34), reported a significant increase in AFC. The clinical pregnancy rate after IVF was 2 out of 15 with intervention versus 3 out of 16 in controls. Similarly, the LBR after IVF was 1 out of 15 with intervention versus 3 out of 16 in controls (Díaz-García et al., 2022).

Importantly, the focus of follicular activation research has predominantly centred on women with POI. However, there is a pressing need for clinical insights into DOR populations. Consequently, clinicians often apply similar principles of follicular activation developed for women with POI to those with DOR. It is important to note that due to the distinct etiologies of POI and DOR, direct comparisons may not be correct (Pellicer et al., 2023). The benefit the follicle activation is currently affirmed to be controversial, as it may lead to a rapid depletion of the follicle pool and have detrimental effects on the oocyte competence (Lerer-Serfaty et al., 2013; McLaughlin et al., 2014).

1.2.3.4.4.4 Intra-ovarian infusion with platelet-rich plasma (PRP)

Platelet-rich plasma (PRP) administration is a promising medical therapy proposed to delay follicular atresia and oocyte degeneration, and re-establish the ovarian function (Bos-Mikich et al., 2018). The theory underlying this treatment is the natural capacity of platelets to induce cell proliferation, revascularization and tissue repair (Qian et al., 2017; Sánchez-González et al., 2012). The use of platelets has been demonstrating promising results, triggering tissue repair, cell proliferation (Crowley et al., 1994) and angiogenesis (Stellos et al., 2010) due to the various growth factors contained in the platelet α granules (Alves & Grimalt, 2018).

The remarkable potential of platelets arises from their rich content of cytokines and growth factors, including platelet-derived growth factor (PDGF), which activate neutrophils, fibroblasts, and macrophages, serving as both chemoattractants and stimulators of cell proliferation, particularly in mesenchymal cell lineages. Transforming growth factors (TGF β 1, TGF β 2, TGF β 3) support cellular differentiation and replication, stimulate collagen synthesis, and enhance fibroblast activity, crucial for tissue repair (Alves & Grimalt, 2018).

VEGF and platelet-derived angiogenic factor (PDAF), fibroblast growth factor (FGF), crucial in angiogenesis, facilitating the formation of new blood vessels and stimulating tissue regeneration (Amable et al., 2013; Jo et al., 2013). IGF-1 stimulates cellular growth and proliferation, contributing to overall tissue regeneration processes. Connective tissue growth factor (CTGF), and interleukin-8 (IL-8), which collectively contribute to the cellular responses and tissue repair mechanisms (Amable et al., 2013; Jo et al., 2013). Fibrin is another important constituent derived from platelets (Raja & Naidu, 2008).

In response to a stimuli, platelets α -granules release its content in growth factors by a process of degranulation (Wang et al., 2015). Importantly, the active secretion of these growth factors by platelets begins within 10 minutes after activation, with more than 95% of the growth factors secreted within 1 hour (Marx, 2004). These factors additionally promote the migration and differentiation of MSCs (Boer et al., 2006; Ponte et al., 2007). MSCs (multipotent stem cells)

further play a role in the regenerative process by virtue of their ability to differentiate into various tissues such as bone, cartilage, adipose, or dermis (Caplan, 1991; Kim & Cho, 2013).

Laminin, thromboxane A₂, epinephrine, adenosine diphosphate (ADP), or contact with a negatively charged surface are additional activators of platelets (Davis et al., 2014).

The therapeutic potential of PRP was firstly described in the 1970s to treat thrombocytopenia (Alves & Grimalt, 2018). Since 1990's, it has been extensively applied in regenerative medicine particularly in the recovery from athletic injuries (Dhillon et al., 2012; Miller et al., 2017).

Easily to obtain, PRP could be prepared using a commercial kit or simply isolating the blood constituents according to its density, in a two-step centrifugation process (Pavlovic et al., 2016). After the first centrifugation, it is possible to distinguish the erythrocytes sediment, at the bottom of the tube, and, above the erythrocytes, a thin, white-tinted buffy coat. That buffy coat and plasma are collected and proceed for a second centrifugation to increase a platelet concentration (Alves & Grimalt, 2018; Karimi & Rockwell, 2019; Pavlovic et al., 2016).

According to the therapeutic purpose, it is possible to selectively isolate specific constituents (Anitua et al., 2011). Through diverse preparations, it is possible to obtain samples enriched in platelets; leukocytes and platelets; platelets and fibrin; or leukocytes, platelets and fibrin. Dohan Ehrenfest et al. have established a classification for the different types of platelet preparations (Ehrenfest et al., 2014).

PRP application in reproductive medicine was initial reported for endometrial preparation (Chang et al., 2015). The intrauterine infusion of PRP has been demonstrating good results in cases of refractory endometrium (Barad et al., 2014; Chang et al., 2015; Molina et al., 2017) and repeated implantation failure (Nazari et al., 2016). In this case, PRP is intended to effectively prepare endometrium in terms of growth and receptivity, improving the pregnancy rate (Nazari et al., 2016).

Complementary, PRP revealed a good potential in ovarian rejuvenation, suggested by murine (White et al., 2012) and rat experiments (Ozcan et al., 2020), as well as in inducing the primordial to primary transition in rat model (Nilsson et al., 2006). It was also reported the effect of PRP in patients with POI (Hsu et al., 2020). Van Kasteren and Schoemaker demonstrated, through ovarian biopsies in women with POI, that approximately 40% retained some follicles (30%) or a substantial number of follicles (9%) in the ovarian cortex (Van Kasteren & Schoemaker, 1999).

The idea that dormant stem cells may exist in human ovarian tissue provides an opportunity to introduce natural signalling molecules (cytokines) released from activated platelets directly into the ovarian tissue (Sills & Tan, 2023). Therefore, these activins and growth factors, such as GDF-9, BMPs, play crucial roles in oocyte activation and development (Figure 11). Specifically, the combination of GDF-9, BMPs, and activins 1) facilitates the development from the primary follicle to the early antral stage; 2) are involved in antral follicle growth and the selection of the dominant follicle; and 3) contribute to the expression of *FSHR*. Levels of GDF-9 and BMP15 in follicular

fluid are significantly correlated with oocyte nuclear maturation and embryo quality (Vo et al., 2021).

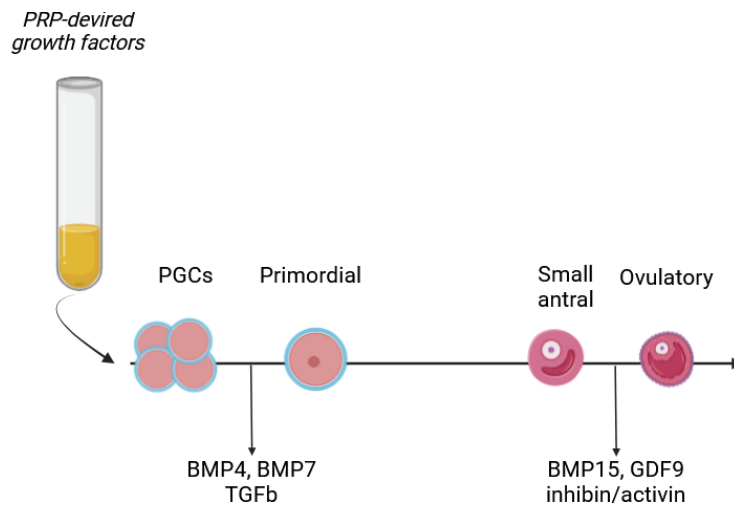


Figure 11 Image illustrating the role of growth factors derived from PRP in mediating follicular development

BMP4, BMP7, TGF- β , BMP15, and GDF9 are recognized for their ability to regulate the transcription of genes involved in differentiation, chemotaxis, and proliferation, as well as oocyte and follicular growth and maturation. The illustrations were created using BioRender.

PRP: platelet-rich plasma; BMP4 - Bone Morphogenetic Protein 4, BMP7 - Bone Morphogenetic Protein 7, TGF- β - Transforming Growth Factor beta, BMP15 - Bone Morphogenetic Protein 15, GDF9 - Growth Differentiation Factor 9

Moreover, the release of cytokines and proangiogenic factors by PRP has proven have a promising impact on ovarian reserve, resulting in increased circulating levels of AMH and decreased FSH levels (Li et al., 2010). Specifically, VEGF and PDGF, exhibit proangiogenic properties, contributing to increased vascularization during follicular maturation up to ovulation (Pauli et al., 2005). Studies involving induced ischemia in rat ovaries and subsequent PRP infusion demonstrated a significant rise in peritoneal VEGF, emphasizing PRP's proangiogenic effects (Alam et al., 2009; Bakacak et al., 2016). Beyond angiogenesis, PRP also influences mitogenesis, chemotaxis, and extracellular matrix formation in the ovary (Sills & Wood, 2019).

In brief, PRP usage for ovarian improvement highlights its benefits as a biological product (Vo et al., 2021). In order to be classified as a biological product, the serum used for ovarian improvement should contain around 1 million platelets (Marx, 2001).

Preclinical trials have been successfully conducted and have proven that intra-ovarian injection of PRP is both effective and safe in enhancing ovarian reserve markers such as FSH, AMH, and AFC in patients with DOR (Melo et al., 2020). The findings derived from the available data regarding the utilization of PRP to enhance ovarian function are outlined in the Table 2.

Table 2 PRP interventions to improve ovarian function: an overview of studies

This table provides a summary of various studies that investigate the use of PRP interventions aimed to improve ovarian function. The columns include details on the study authors and year of publication, the population involved, the sample size, the PRP protocol followed, the volume of blood collected, the volume of PRP administered, and the main results observed.

Study (reference)	Population	Sample size	PRP protocol	Blood Volume (mL)	PRP Volume (mL)	Main Results
Callejo et al., 2013	Autografted frozen ovarian tissue, 20 years old when the ovarian tissue was cryopreserved; 30 years old at the time of the procedure.	frozen-thawed small cubes of ovarian tissue (1–2mm ³)	280×g for 15 minutes and 680×g for 20 minutes	60 (citratate dextrose)	5	PRP enhances angiogenesis
Pantos et al., 2016	Peri-menopausal women, aged 45.13±4.42	8	KIT	Not specified	Not specified	PRP injection for 1-3 months resulted in embryos obtained and cryopreserved after a natural cycle in IVF.
Hosseini et al., 2017	Primordial follicles in 3D culture	frozen-thawed small cubes of ovarian tissue (1-1.1 mm)	200×g for 20 min and 3000×g for 15 min	5 (citrate solution)	0.5 Activated with thrombin	Measurable growth observed in primordial follicles after 10 days in a 3D culture enriched with PRP.
Sills et al., 2018	DOR patients, >35 years, prior IVF failure, amenorrhea	4	KIT	8	1 Activated with calcium gluconate	Increased serum AMH observed, and at least one blastocyst suitable for cryopreservation after PRP treatment.
Sfakianoudis et al., 2018	Poor responders, 1 aged 40 years old; 2 aged 37 years old	3	KIT	70	10	Increased AMH levels, natural conception, and successful live birth observed in three poor responder patients.
Sfakianoudis et al., 2019	with POI, 40 years old diagnosed at the age of 35	1	KIT	60	8	PRP injections resulted in increased a biochemical pregnancy after an IVF cycle, that resulted in a spontaneous abortion during the fifth week of pregnancy
Stojkovska et al., 2019	Poor responders undergoing IVF/ICSI, aged 35-42	40 (20 received PRP)	KIT	Not specified	3-5 Activated with calcium gluconate	No observed differences in live birth rates between PRP-treated and control groups.
Farimani et al., 2019	Poor responders, 34 to 40 years old	19	Not specified	Not specified	4	15.8% pregnancy rate, including 3 clinical pregnancies, 2 of which natural conceptions, observed post-PRP treatment.
Farimani et al., 2021	Poor responders (POSEIDON groups)	96	Not specified	Not specified	2	No significant changes in hormone concentrations, notable increase in retrieved and mature oocytes in all POSEIDON groups. The overall pregnancy rate was 14.6%, with the most benefit observed in group 1 (young patients), with 71.4% pregnancy rate.

Study (reference)	Population	Sample size	PRP protocol	Blood Volume (mL)	PRP Volume (mL)	Main Results
Pantos et al., 2019	2 women with POF, aged 40 and 27 years; 1 menopausal woman aged 46 years; prior IVF failure	3	KIT	60	8	Decrease in FSH levels, increase in AMH levels. Spontaneous pregnancies in POF and menopausal cases.
Melo et al., 2020	Low ovarian reserve, aged 39–44	83 (46 PRP treatment; 37 no intervention)	270×g10 min	18 (sodium citrate)	0.4 Activated with calcium chloride	Significant improvement in AMH and AFC, higher pregnancy rates compared to the non-intervention group.
Petryk & Petryk, 2020	Low ovarian reserve, 31 to 45 years old; ≥2 IVF attempts	38	800×g for 3 min and 1400×g 15 min	17 (ACD-A)	1.5	PRP influenced pregnancy rate, six healthy babies born, and ten pregnancies observed (four from natural conception)
Hsu et al., 2020	POI, 37 years old	1	3,000 rpm for 8 min and 4,000 rpm for 5 min	40	6 Activated with calcium gluconate	Successful pregnancy and live birth of twins observed.
Cakiroglu et al., 2020	POI, IVF, aged 24-40	311	KIT	20	2-4	Improved AMH and AFC after intraovarian injection of PRP.
Pacu et al., 2021	Poor responders, 31 to 44 years old	20	KIT	60-80	2-4	Differences in ovarian reserve markers.
Cakiroglu et al., 2022	Poor responders, 30-45 years old	510	KIT	20	2-4	Significant increase in AMH and AFC, 20.5% pregnancy rate, and 12.9% live birth rate observed post-PRP infusion.
Navali et al., 2022	Poor responders, 30-42 years old	35	KIT	20	4-8	No significant differences in AMH levels, increase in retrieved oocytes and developed embryos.
Tremellen & Pacella-Ince, 2022	Severe DOR, prior IVF failures, all 45 years old	20	KIT	50	3	No significant improvement in oocyte numbers, increased number of embryos compared to pre-PRP IVF cycle.
Barad et al., 2022	Low ovarian reserve, 28-54 years old	80	KIT	10 (citrate)	2.5-3	No clinically significant effects observed in ovarian function following non-activated PRP treatment.

Study (reference)	Population	Sample size	PRP protocol	Blood Volume (mL)	PRP Volume (mL)	Main Results
Sabouni et al., 2022	DOR, 35 years old	1	1500 RPM for 15 minutes and 2000 RPM for 10 minutes	20	1	PRP injection increased AMH levels.
Merhi et al., 2022	Infertile, ≥1 prior failed IVF, Aged 38 to 45	12	1,500×g for 5 minutes	32	8	Improved biomarkers of ovarian reserve observed.
Parvanov et al., 2022	Poor responders, <46 years old	66	500×g for 5 min 700×g for 7 min	22 (ACD-A)	1	Significant increase in AMH, AFC, MII, and D5 embryos observed post-PRP treatment.
Tülek & Kahraman, 2022	POI and poor responders, <40 years old	71 (21 were diagnosed with POI)	KIT	20	4	Significant increase in retrieved and fertilized oocytes, quantity of cleavage-stage embryos increased.
Hosseinisadat et al., 2023	Poor ovarian reserve, mean age 33.91±6.58 years old	21	800 RPM for 15 min and 2000 RPM for 5 min	20 (sodium citrate)	0.5	Increased AMH levels observed after PRP treatment.
Davari Tanha et al., 2023	Poor responders, ≥ 40 years old	20	1200 rpm for 10 minutes 3300 rpm for 6 minutes	80	Not specified	Trend towards improvement in ovarian reserve markers, including AMH, estradiol, and oocyte quality. 15% chemical pregnancy.
Najafian et al., 2023	POSEIDON groups 3 and 4, 35-43 years old	50	KIT	80	8	Significant increase in high-quality embryos. 20 % pregnancy rate, and 14% spontaneous pregnancy rate.
Safarova et al., 2023	DOR, 20-50 years old	60	5 minutes at 2100 revolutions per minute (857×g)	8,5 (citrate-phosphate-dextrose)	1.5 Activated with calcium gluconate	Statistically significant increases in endometrial thickness, basal follicle number, estradiol, oocyte count, and MII oocyte count observed.
Tehraninejad et al., 2023	Women with poor ovarian response according to the Bologna criteria	45 (23 in the intervention group, 22 in the control group)	KIT	35 ccs of blood	4 cc	Significant improvement in AMH levels in the PRP group after two cycles No difference in the cumulative number and quality of embryos between the two groups At one-year follow-up, overall pregnancy rates were the same in both groups
Herlihy et al., 2023	Women under 38 years old with poor ovarian response undergoing IVF, meeting specific inclusion criteria (two or more prior cycles with fewer than three oocytes retrieved; without single gene disorders, prior ovarian surgery, endometriomas, BMI >35 kg/m ² , or severe male factor infertility)	83 patients (41 in the PRP group, 42 in the control group)	KIT	16 mL of blood	1mL	No significant difference in posttreatment AFC and AMH levels No significant difference in the number of MII oocytes retrieved per cycle between the PRP and control No significant difference in the number of blastocysts No significant difference in the rate of sustained implantation

AMH: Anti-Müllerian hormone; AFC: antral follicle count; BMI: body mass index; DOR: diminished ovarian reserve; FSH: follicle stimulating hormone; ICSI: intracytoplasmic injection; IVF: *in vitro* fertilization; POI: premature ovarian insufficiency; POSEIDON: patient-oriented strategies encompassing individualized oocyte number; PRP: platelet-rich plasmas; MII: metaphase II; RPM: rotations per minute; 3D: three-dimensional

In essence, studies on patients with altered ovarian reserve indicate a restoration of ovarian function within the initial 2–3 months following a PRP intervention (Cakiroglu et al., 2022; Hosseinisadat et al., 2023; Melo et al., 2020; Sabouni et al., 2022; Sills et al., 2018; Tülek & Kahraman, 2022). The studies suggest that PRP injections contribute to increased AMH levels (Cakiroglu et al., 2020, 2022; Melo et al., 2020; Merhi et al., 2022; Pacu et al., 2021; Pantos et al., 2019; Parvanov et al., 2022; Sabouni et al., 2022; Sfakianoudis et al., 2018; Sills et al., 2018). Importantly, the trend toward improved ovarian reserve markers is observed even in patients with specific criteria, such as advanced age and a history of cycle cancellation, IVF failure, and poor ovarian response (Cakiroglu et al., 2020, 2022; Melo et al., 2020; Pacu et al., 2021; Pantos et al., 2019; Parvanov et al., 2022; Seckin et al., 2022; Sfakianoudis et al., 2018; Sills et al., 2018; Tanha et al., 2023). In different terms, hormonal improvement was not observed among all patients (Barad et al., 2022; Farimani et al., 2021; Navali et al., 2022; Tanha et al., 2023).

PRP injections have demonstrated an impact on the yield of usable embryos in various studies (Najafian et al., 2023; Navali et al., 2022; Parvanov et al., 2022; Sills et al., 2018; Tremellen & Pacella-Ince, 2022; Tülek & Kahraman, 2022). Additionally, PRP stimulus appears to prevent follicle atresia in poor-responder patients (Melo et al., 2020).

Various studies have reported positive outcomes in terms of pregnancy rate after PRP treatment (Cakiroglu et al., 2022; Farimani et al., 2019, 2021; Najafian et al., 2023; Petryk & Petryk, 2020; Sfakianoudis et al., 2019; Tanha et al., 2023). For instance, Cakiroglu et al. demonstrated a significant pregnancy rate of 20.5% in poor responders following PRP infusion (Cakiroglu et al., 2022). Similarly, studies by Parvanov et al. and Najafian et al. revealed increased pregnancy rates, with significant improvements in AMH, AFC, and embryo numbers among poor responders (Najafian et al., 2023; Parvanov et al., 2022). Significantly, it is noteworthy that in addition to pregnancies resulting from IVF, spontaneous pregnancies were successfully achieved, as reported by Pantos et al. (Pantos et al., 2019). In particular, one study documented a substantial 14% pregnancy rate through natural conception (Najafian et al., 2023).

LBR, a crucial outcome for evaluating the effectiveness of PRP, was assessed in various studies (Cakiroglu et al., 2022; Farimani et al., 2019; Hsu et al., 2020; Petryk & Petryk, 2020). Notably, Farimani et al. reported the birth of 3 babies among 19 treated patients (Farimani et al., 2019) while Petryk & Petryk documented the birth of 6 babies out of 38 treated patients (Petryk & Petryk, 2020). The study led by Cakiroglu et al. revealed a LBR of 12.9% (Cakiroglu et al., 2022).

The increasing interest in the field has led to the publication of numerous reviews and meta-analyses on ovarian PRP and its outcomes (Dastjerdi et al., 2024; Éliás et al., 2024; Herlihy & Seli, 2022; Maged et al., 2024; Wang et al., 2024; Wu et al., 2024). Specifically, the efficacy of intra-ovarian injection of autologous PRP in improving ovarian reserve markers and ART outcomes in women with poor ovarian response was corroborated by Dastjerdi et al. (Dastjerdi et al., 2024). Maged et al. evidenced the significant improvements in AFC, AMH, and in the number of retrieved oocytes, and cleavage-stage embryos in women with low ovarian reserve (Maged et al., 2024). Similarly, Elias et al. demonstrated that PRP treatment significantly increased AMH

levels, decreased FSH, and improved oocyte and embryo counts, indicating a positive impact on fertility parameters and reproductive outcomes in DOR patients (Éliás et al., 2024). Wu et al. confirmed the promising effects of PRP on patients that poorly respond to IVF, highlighting its potential to improve live birth and clinical pregnancy rates, number of MII oocytes, and embryo count (Wu et al., 2024). Lastly, Wang et al. reviewed the therapeutic roles of PRP in various female reproductive disorders, emphasizing its potential in promoting endometrial hypertrophy and follicle development, thereby offering new therapeutic options for female infertility (Wang et al., 2024).

Recently, two studies on the impact of PRP treatment on ovarian reserve were published: a non-randomized trial by Tehraninejad et al. and a RCT by Herlihy et al. (Herlihy & Seli, 2022; Tehraninejad et al., 2023). Tehraninejad et al. found that PRP treatment improved ovarian reserve markers (Tehraninejad et al., 2023). In contrast, Herlihy et al. demonstrated that PRP treatment had no significant effect on ovarian reserve markers, embryo quality, or implantation rates in patients with poor ovarian reserve (Herlihy et al., 2024). Importantly, the study from Herlihy et al. is particularly robust due to its RCT design, which is considered the gold standard for clinical research. It stands out in a field lacking other RCTs to clarify the benefits of PRP in women with DOR (Kheil et al., 2022; Sharara et al., 2021; Tremellen & Pacella-Ince, 2022).

PRP was also investigated as a treatment for frozen-thawed ovarian tissue, in different studies and objectives, namely: 1) to enhance transplantation outcomes, and 2) to facilitate the acquisition of follicles *in vitro* (Callejo et al., 2013; Hosseini et al., 2017).

The varied PRP protocols and diverse population characteristics across studies lead to differing conclusions. This variability makes it challenging to draw definitive conclusions for future studies (Pellicer et al., 2023).

2 Strategies to increase the understanding of diminished ovarian reserve (DOR)

2.1 Overview of the project on fertility awareness, genetic testing, and interventions in the context of diminished ovarian reserve

Project research question, objectives, and tasks.

2.2 Task 1: intervention in knowledge through general and tailored information on ovarian reserve and diminished ovarian reserve (DOR)

Development and testing the impact of two interventions on knowledge about ovarian reserve DOR: 1) providing general leaflet information, and 2) offering tailored information and reproductive counselling based on the assessment of ovarian reserve.

2.3 Task 2: genetic analysis of infertile populations with abnormal ovarian reserve

Review of the role of hormonal receptor polymorphisms in ovarian function.

Identification of genetic variants concerning FSHR rs6166, ESR1 rs2234693 and ESR1 rs9340799 polymorphisms in cases of altered ovarian reserve (DOR and PCOS), among infertile populations.

Establishment of an NGS study protocol to identify genetic variants in the population with DOR, which includes reviewing the genes most relevant to this population and defining the gene panel to be analysed subsequently.

2.4 Task 3: *in vitro* study of follicular activation through the incubation of human ovarian tissue with platelet-rich plasma (PRP)

*Evaluation of the efficacy of PRP supplementation in activating the PI3K-Akt pathway and enhancing stromal vascularization, using an *in vitro* culture system, with human ovarian tissue.*

2.1 Overview of the project on fertility awareness, genetic testing, and interventions in the context of diminished ovarian reserve

The global decline in fertility rates and delayed parenthood underscore the urgent need for action within the non-infertile populations. The lack of fertility awareness is one of the most significant contributors to this scenario, often resulting in involuntary childlessness. Interventions to provide information and support fertility management have been pointed out to help mitigating infertility problems. However, they have not been consistently implemented.

DOR accounts for 10% of women in their 30s, with unknown causes in 50% of these cases. The asymptomatic nature of DOR often delays diagnosis, commonly resulting in the need for fertility treatments to conceive. Currently, the ART fail to offer a solution for DOR-related infertility. Strategies to address DOR-related infertility, such as pharmacogenetic approaches, including genetic-directed diagnosis and therapies, and follicular activation have emerged. However, their widespread integration into clinical practice requires further research to ensure efficacy and safety across DOR patients.

Therefore, an essential question surged: What steps can be taken to enhance healthcare support for patients with DOR? Consequently, additional questions emerge: Does knowledge intervention (generic or tailored) alter reproductive knowledge, intentions, and attitudes? Considering the current reality, where approximately 30% of infertile women are already diagnosed with DOR, which options can be offered to them? Are there genetic variants, specifically associated with *FSHR*, *ESR1*, and *ESR2* polymorphisms, more prevalent in populations with altered ovarian reserve, and can these variants be utilized to optimize responses to COS? Or can these genetic variants be used to diagnose situations of DOR? Is PRP effective in activating a pathway involved in follicular activation (PI3K-Akt), in human ovarian tissue?

Addressing these questions three primary objectives were defined:

1) to develop and test the effectiveness of two interventions in knowledge about ovarian reserve and DOR, a) providing general leaflet information, and b) offering tailored information and reproductive counselling based on the assessment of ovarian reserve.

2) to review the role of hormonal receptor polymorphisms in ovarian function. Additionally, we aimed to look for genetic variants concerning *FSHR* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 polymorphisms in cases of altered ovarian reserve, among infertile populations.

Our last goal was to establish a NGS study protocol to verify the occurrence of genetic variants in the population with DOR, which included reviewing the genes most relevant to this population and defining the gene panel to be analysed subsequently.

3) to evaluate the efficacy of PRP supplementation in activating the PI3K-Akt pathway and enhancing stromal vascularization, using an *in vitro* culture system, with human ovarian tissue.

To reach the objectives, this project was structured into three tasks:

- 1) Intervention in knowledge through general and tailored information on ovarian reserve and DOR.
- 2) Genetic study of infertile populations with abnormal ovarian reserve.
- 3) *In vitro* study of follicular activation, through the incubation of human ovarian tissue with PRP.

A schematic representation of the project, from the initial research question to the multifocal approach applied to address women with DOR, spanning from the undiagnosed period to the established condition, is illustrated in Figure 12. This figure also highlights ongoing tasks that require further continuation.

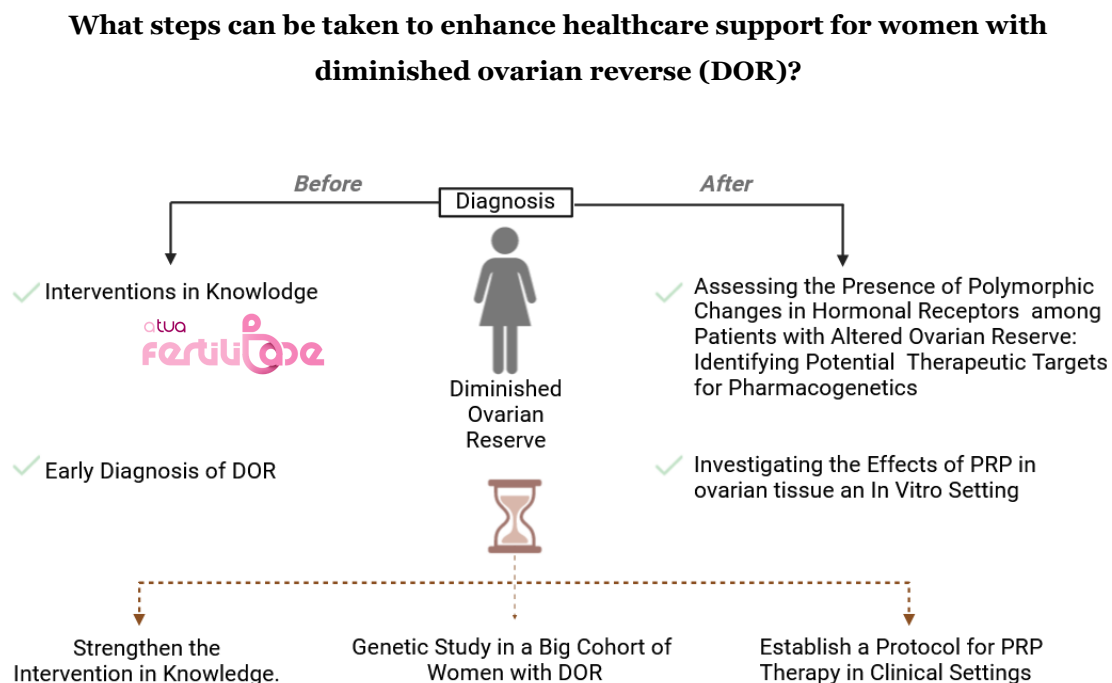


Figure 12 Schematic representation of the project, including the future perspectives

This image provides an overview of the project. It is divided into two main sections: actions to take "Before" and "After" the diagnosis of DOR, to improve healthcare support for women with this condition. Additional actions and future perspective are also represented. Image created in BioRender.

DOR: diminished ovarian reserve; PRP: platelet-rich plasma

Before detailing the three tasks, and to facilitate understanding of the adopted organization, we provide a brief overview of the thesis structure:

The thesis is divided into three main sections: 1) introduction (*Introduction to fundamental literature on ovarian reserve, with a focus on the study of diminished ovarian reserve*); 2)

materials and methods (*strategies to increase the knowledge on diminished ovarian reserve (DOR)*), and 3) conclusion (*conclusions and future perspectives*).

Section 2, *strategies to increase the understanding of diminished ovarian reserve (DOR)*, presents a brief theoretical contextualization, results, and discussion for Tasks 1, 2, and 3. This contextualization, present in each task, was more concise compared to section's 1 review, aiming to introduce essential concepts for understanding the implemented techniques and to facilitate the discussion of results. The obtained results are presented and followed by the discussion, which briefly emphasizes key conclusions and limitations.

Section 3, *conclusions and future perspectives*, presents the final conclusions of the project, including the main outcomes of all three tasks, their limitations, and implications for clinical and research settings.

2. Strategies to increase the understanding of diminished ovarian reserve (DOR)

2.1 Overview of the project on fertility awareness, genetic testing, and interventions in the context of diminished ovarian reserve

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2.3 Task 2: genetic analysis of infertile populations with abnormal ovarian reserve

Review of the role of hormonal receptor polymorphisms in ovarian function.

Identification of genetic variants concerning FSHR rs6166, ESR1 rs2234693 and ESR1 rs9340799 polymorphisms in cases of altered ovarian reserve (DOR and PCOS), among infertile populations.

Establishment of an NGS study protocol to identify genetic variants in the population with DOR, which includes reviewing the genes most relevant to this population and defining the gene panel to be analysed subsequently.

2.4 Task 3: *in vitro* study of follicular activation through the incubation of human ovarian tissue with platelet-rich plasma (PRP)

*Evaluation of the efficacy of PRP supplementation in activating the PI3K-Akt pathway and enhancing stromal vascularization, using an *in vitro* culture system, with human ovarian tissue.*

2.2 Task 1: intervention in knowledge through general and tailored information on ovarian reserve and diminished ovarian reserve (DOR)

2.2.1 Overview of existing literature: understanding and enhancing fertility knowledge

2.2.1.1 Target population of the interventions

There is substantial evidence demonstrating a lack of fertility knowledge among the general population (Halleran et al., 2022; Iino et al., 2022; Pedro et al., 2018; Ren et al., 2023), with people consistently overestimating their ability to conceive (Bunting et al., 2013; Daniluk et al., 2012; Harper et al., 2017; Iino et al., 2022).

While schools and family planning include programmes about contraception and how to avoid sexual transmitted infections (STIs), fertility education remains not considered (Bakkensen & Goldman, 2022; Harper et al., 2021). It is recognized that there is a need to produce content and develop initiatives to expand fertility education (Martins et al., 2024).

A crucial factor to consider, when addressing fertility-related topics, is the age of the target population, once it determines the receptiveness to information (Stokes et al., 2023). It is advocated that strategies should preferably be implemented before the onset of age-related fertility decline (Chan et al., 2022; Delbaere et al., 2020), but taking into consideration the intended age to have children (Stokes et al., 2023). The ideal age to have children is increasing worldwide, with mean ages nearing 30 years old in most European countries (Harper & Botero-Meneses, 2022).

Recent studies in the field have shown that the most significant participation in interventions in knowledge about fertility occurs in age ranges around 30 years old (Chan et al., 2022; Herzberger et al., 2022; Stokes et al., 2023). Chan et al. have defined a protocol for a study to address female fertility awareness, and the target population were women aged 25–34 years at the point of recruitment (Chan et al., 2022). Stokes et al. conducted a study to assess the knowledge and attitudes regarding fertility in both men and women, comprising a total of 480 participants. Among the respondents, 70.7% were women, and the age group most prominently represented was 25 years old (44.8%), followed by the age range of 30–34 years (24.1%) (Stokes et al., 2023). In the study conducted by Herzberger et al the largest age group was 30–34.9 years old, comprising 39.7% of the participants (Herzberger et al., 2022).

Younger cohorts have been associated with benefiting more from general guidance, whereas older groups tend to benefit more from focused information (Stokes et al., 2023). Interventions that include counselling and personalized educational materials, such as brochures, websites, or videos, have shown the potential to enhance fertility knowledge in the short term, more than

generic interventions (Chan et al., 2022). The internet has consistently been the most popular source for accessing (in)fertility-related information (Grace et al., 2023; Melo et al., 2018). The dissemination of content on digital platforms and applications with information about fertility is also an attractive solution as they allow for very active user interaction (Conard, 2019). The presence of digital tools in fertility literacy has increased (Geampana, 2024), but it should be noted that many of them are not properly validated (Jandoo, 2020).

A noted limitation, when considering interventions among younger populations, is the attrition rate, a concern raised by several authors (Daniluk & Koert, 2015; Maeda et al., 2016, 2018; Pedro et al., 2020; Stern et al., 2013). Self-selection bias is another potential challenge when evaluating fertility knowledge, particularly when including individuals from the field, those with high levels of education, or those naturally interested in the topic (Stokes et al., 2023). A review of the literature concluded that fertility awareness levels are generally higher among women and medical students (Ren et al., 2023).

2.2.1.2 Impact of interventions in knowledge about fertility

As already described in detail in sections 1.1.7 and 1.1.8, from section one, addressing fertility challenges can help to avert or mitigate infertility problems by integrating information, education, and communication into counselling initiatives (Khadivzadeh et al., 2021).

Published information tailored specifically for DOR is scarce within the literature on fertility education (Duane et al., 2022). Herzberger et al. examined the impact of educational interventions on ovarian reserve knowledge. Their evaluation was conducted both before and after exposure to online educational resources about ovarian reserve and fertility preservation treatments. Following exposure, participants demonstrated a significant increase in knowledge concerning female age-related fertility decline (51.6% vs. 79.6%, $p < .001$). Furthermore, there was an increase in the willingness to pursue fertility preservation treatments (38.6% vs. 42.9%, $p < 0.001$), coupled with an increased tendency to recommend such treatments to friends (55% vs. 65.2%, $p < .001$) (Herzberger et al., 2022).

In general, fertility education has proven successful in improving knowledge within the domain, as summarized in Table 3 (Boivin et al., 2018; Conceição et al., 2017; Daniluk & Koert, 2015; Fooladi et al., 2018; Fujishima et al., 2024; García et al., 2016; Hammarberg & Stocker, 2021; Maeda et al., 2016, 2018; Pedro et al., 2020; Skogsdal et al., 2019; Stern et al., 2013; Wojcieszek & Thompson, 2013). Table 3 provides a comprehensive summary of various studies on the effectiveness of fertility education in increasing knowledge. It includes RCTs and pre-post studies involving diverse participants such as adolescents, emerging adults, university students, partnered women, health professionals, and reproductive-aged men and women. Interventions ranged from online brochures and learning modules, to video-based education and preconception check-ups. The studies consistently showed an increase in fertility knowledge in the short term, with some demonstrating knowledge gains over periods from two months to two years.

Maeda et al. and Pedro et al. reported partial long-term retention of knowledge (Maeda et al., 2016, 2018; Pedro et al., 2020), while others found improvements predominantly in the short term (Boivin et al., 2018; Fooladi et al., 2018; García et al., 2016; Hammarberg & Stocker, 2021; Skogsdal et al., 2019; Stern et al., 2013).

Table 3 Summary of effectiveness of fertility education for increasing knowledge through various studies

This table summarizes various studies that assess the effectiveness of fertility education programs in enhancing knowledge. It includes information on the authors, the study details, participants involved, interventions implemented, and the observed effects in both the short-term and long-term.

AUTHORS	STUDY	PARTICIPANTS	INTERVENTIONS	EFFECTS	
				SHORT-TERM	LONG-TERM
Maeda et al., 2016	RCT	Men and women 20-39 years old	Online brochure	Increased	Partially (2 year)
Maeda et al., 2018	RCT				
Hammarberg & Stocker, 2021	Pre-post	Health professionals (midwives)	Online learning modules	Increased	
Boivin et al., 2018	RCT	Adolescents	Online pages	No changes	
		Emerging adults		Increased	
Pedro et al., 2020	RCT	Partnered women	video	Increased	Partially (1 year)
Conceição et al., 2017	RCT	University students		Increased	
Daniluk et al., 2015	Pre-post	Childless people	Web articles	Increased	Partially (6 months)
Wojcieszek & Thompson, 2013	Two groups Pre-post	University students	Brochure	Increased	
Fujishima et al., 2024	Before and after	Women	Preconception check-up and counselling at work	Increased	
Garcia et al., 2016	RCT	women attending a fertility centre for oocyte donation	Brochure		No changes
			Brochure and oral information		Increased
Fooladi et al., 2018	RCT	Reproductive aged women	Reproductive life plan counselling	Increased (2 months)	
Skogsdal et al., 2019	RCT	Women 20-40 years old		Increased (2 months)	
Stern & Tyde, 2013	RCT	Students		Increased (2 months)	

RCT: randomized controlled trial

About the impact of fertility education in intentions, considering desired age of pregnancy and change behaviours, there are inconsistent results, as shown in Table 4. Some authors reported changes (Fujishima et al., 2024; Maeda et al., 2020; Stern et al., 2013; Wojcieszek & Thompson, 2013) while others don't (Boivin et al., 2018; Fooladi et al., 2018; García et al., 2016; Maeda et al., 2016; Pedro et al., 2020; Skogsdal et al., 2019). The table 4 details various studies on the effectiveness of fertility education in altering intentions regarding reproductive plans. These studies, encompassing RCTs and pre-post designs, involve diverse participant groups such as men and women, adolescents, emerging adults, university students, and reproductive-aged women. Interventions include chatbots, brochures, online pages, videos, and preconception check-ups. The findings reveal a varied impact on participants' desired age of pregnancy and behaviour changes. For instance, Maeda et al. showed an increase in behavioural changes through a chatbot intervention (Maeda et al., 2020), while Pedro et al. and García et al. reported no significant changes in either desired age of pregnancy or behaviour following video-based education and tailored educational interventions, respectively (García et al., 2016; Pedro et al., 2020). Wojcieszek & Thompson, and Stern et al. noted a decrease in the desired age of pregnancy among university students, with Stern et al. also observing increased behaviour changes (Stern et al., 2013; Wojcieszek & Thompson, 2013). However, Boivin et al. and Fooladi et al. found no changes in either outcome among adolescents, emerging adults, and reproductive-aged women (Boivin et al., 2018; Fooladi et al., 2018). These mixed results highlight the complexities and varied effectiveness of fertility education programs in influencing reproductive intentions and behaviours.

Table 4 Summary of effectiveness of fertility education for changing intentions through various studies

This table provides a summary of various studies that evaluate the impact of fertility education programs on changing individuals' intentions regarding fertility-related decisions. It includes information on the authors, study details, participants involved, interventions implemented, and the observed effects, particularly focusing on the desired age of pregnancy and changes in behaviour.

AUTHORS	STUDY	PARTICIPANTS	INTERVENTIONS	EFFECTS	
				DESIRED AGE OF PREGNANCY	CHANGE BEHAVIOURS
Maeda et al., 2020	RCT	Men and women	Chatbot, brochure		Increased
Maeda et al., 2016	RCT	Men and women 20-39 years old	Online brochure	No change	
Boivin et al., 2018	RCT	Adolescents	Online pages	No change	
		Emerging adults		No change	
Pedro et al., 2020	RCT	Partnered women	video	No change	No change
Wojcieszek & Thompson, 2013	Two groups (pre-post)	University students	Brochure	Decreased	
Garcia et al., 2016	RCT	Women attending a fertility centre for oocyte donation	Brochure	No change	
			Brochure and oral information	No change	
Fooladi et al., 2018	RCT	Reproductive aged women	Reproductive life plan counselling	No change	
Skogsdal et al., 2019	RCT	Women 20-40 years old		No change	
Stern & Tyde, 2013	RCT	Students		Decreased	Increased
Fujishima et al., 2024	Before and after	Women, 19-39 years old	Preconception check-up and counselling at work	Anticipated the desired plan to try to conceive	

RCT: randomized controlled trial

While fertility education shows promising results in increasing knowledge, and potentially modifying behaviours, challenges such as psychological side effects must be considered. Anxiety due to fertility education and/or reproductive counselling is described (Boivin et al., 2018; Maeda et al., 2016). However, considering the existing studies there are more positive than negative effects (Fooladi et al., 2018; Fujishima et al., 2024; Koert et al., 2020; Maeda et al., 2020; Skogsdal et al., 2019; Stern et al., 2013). Table 5 reports the summary of studies analysing the

impact of fertility education and reproductive counselling on anxiety. These studies, ranging from RCTs to cohort studies, include diverse interventions such as online brochures, chatbots, preconception check-ups, and reproductive life plan counselling. Maeda et al. and Boivin et al. reported increased anxiety and perceived infertility threats among participants receiving fertility education via online brochures and websites (Maeda et al., 2016, 2018). Conversely, another study conducted by Maeda et al. found that using a chatbot and brochure significantly decreased anxiety among men and women (Maeda et al., 2020). Fujishima et al. noted high satisfaction levels (85%) among women receiving preconception check-ups and counselling at workplaces (Fujishima et al., 2024). Similarly, Koert et al. highlighted that women perceived the benefits of fertility assessment and counselling to outweigh the risks (Koert et al., 2020). Fooladi et al. and Stern et al. reported overwhelmingly positive reactions (97% and 90% positive, respectively) to reproductive life plan based information (Fooladi et al., 2018; Stern et al., 2013). Skogsdal et al. observed that 59% of women had positive experiences with contraceptive counselling, while 38% were neutral and only 3% had negative experiences (Skogsdal et al., 2019). These findings suggest that while some anxiety can result from fertility education, most interventions are perceived positively, providing benefits that outweigh the risks.

Table 5 Summary of studies analysing the impact of fertility education and reproductive counselling on anxiety

This table presents a summary of studies that investigate the effects of fertility education and reproductive counselling on anxiety levels. It includes details on the authors, study titles, participant demographics, interventions administered, and the observed impacts on anxiety.

AUTHORS	STUDY	PARTICIPANTS	INTERVENTIONS	EFFECT
Maeda et al., 2016	RCT	Men and women 20-39 years old	Online brochure	Increased anxiety
Boivin et al., 2018	RCT	Adolescents Emerging adults	Online pages	Increased infertility threats (=perceived susceptibility to infertility) Increased infertility threats (=perceived susceptibility to infertility)
Maeda et al., 2020	RCT	Men and women	Chatbot, brochure	Decreased anxiety
Fujishima et al., 2024	Before and after	Women	Preconception check-up and counselling at work	85% reported satisfaction with the check-ups.
Koert et al., 2020	cohort	women	Fertility assessment and counselling	“Benefits outweighed the risks of receiving personalized fertility education”
Fooladi et al., 2018	RCT	Reproductive aged women	Reproductive life plan counselling	97% positive
Skogsdal et al., 2019	RCT	Women 20-40 years old		59% positive 38% neither 3% negative
Stern & Tyde, 2013	RCT	Students		90% positive

RCT: randomized controlled trial

2.2.1.3 Ovarian reserve testing: an opportunity to provide tailored information

The FAC clinic, recognized for its expertise in reproductive counselling, has been providing women’s fertility assessment based on ovarian reserve evaluation (Hvidman et al., 2015; Koert et al., 2022; Petersen et al., 2017). The results of Koert et al., summarizing the 6-year experience of the FAC clinic, underscore the significance of personalized interventions with women of reproductive age regarding optimal timing for conceiving. The authors claim that providing realistic, tailored information can prevent potential emotional distress and regret regarding reproductive plans (Koert et al., 2022). The evidence that 1 in 10 women are expected to experience reduced fertility by the age of 30 helps to justify the opportunity to conduct ovarian

reserve evaluation as a potential early diagnostic for fertility risk factors (Nikolaou & Templeton, 2003; Tal & Seifer, 2017).

As described in section 1.2.2.8 and 1.2.2.9, 1st section, AMH and AFC are currently the simplest, most sensitive, and specific measures of ovarian reserve, as well as good predictors of oocyte yield (Broer et al., 2011; Penzias et al., 2020; Sun et al., 2022). AMH measurement is considered the ideal primary tool for ovarian reserve evaluation because it is a non-invasive marker with high precision results, reduced costs and can be taken at any time of the cycle - has very little variation intracycle (La Marca et al., 2010) or between menstrual cycles (Hehenkamp et al., 2006). Moreover, AMH assay is reproducible and reliable (Dewailly et al., 2014).

Besides the uncertainty about the predictive capacity of AMH in natural conceiving (Loh & Maheshwari, 2011; Nelson et al., 2012), Tremellen et al. discussed the opportunity to consider AMH as part of an ovarian reserve screening test (Tremellen & Savulescu, 2014). In line with that, we have seen a widespread advertisement of AMH testing to evaluate ovarian reserve (Çelik et al., 2023; Copp et al., 2023, 2024), despite its lack of capacity to predict oocyte quality (Broer et al., 2014; Loh & Maheshwari, 2011).

While the association between AMH and AFC levels is strong and positive (Fanchini et al., 2005; Panchal & Nagori, 2012), occasional discrepancies are observed (Schipper et al., 2012; Zhang et al., 2019). These variations may arise, in part, due to technical challenges, but other physiological factors could also impact the anticipated relationship (Dewailly et al., 2014).

Variations in serum AMH levels have been documented in response to various circumstances, such as ethnicity (Schuh-Huerta et al., 2012; Seifer et al., 2009), body mass index (BMI) (Freeman et al., 2007; Steiner et al., 2010) or genetic variants (Schuh-Huerta et al., 2012). Although menstrual cycle fluctuations seem random and minor, allowing for AMH measurement independent of cycle phase, pharmacological interventions like hormonal contraception can impact AMH values (Bentzen et al., 2012; Berg et al., 2010; Broer et al., 2014; Kallio et al., 2013; Landersoe et al., 2019, 2020). Hormonal contraception has also been noted to influence the count of antral follicles (Bentzen et al., 2012; Deb et al., 2012). Besides that, it is argued that stopping contraception could be an obstacle to most of women and lead to the exclusion of almost one-third of the women to get reproductive counselling (Petersen et al., 2015).

In Western countries, 50–89% of women use hormonal contraception at some point in their lifetime (Petersen et al., 2015) and in Portugal contraceptive pill remains the preferred choice. A study conducted in 2016 indicates that the contraceptive pill remains the preferred choice among Portuguese women, with approximately 58% using it. The survey involved 4,003 women across seven geographical regions of Portugal (Águas & Bombas, 2016). In 2022 another research revealed that the most widely used contraceptive method among Portuguese women was the oral hormonal contraceptive (57.5%). The study included 127 women from three different primary health units in the central region of Portugal (Cunha et al., 2022).

With the significant number of women using hormonal contraception, researchers have suggested studying its effects on ovarian reserve markers (Siegel et al., 2023). In several studies, it has been demonstrated that hormonal contraception can influence AMH levels (Table 6), making the interpretation of this marker challenging. For example, numerous studies have reported a decrease in AMH levels ranging from 19% to 31% among hormonal contraception users (Arbo et al., 2007; Bentzen et al., 2012; Bernardi et al., 2021; Dólleman et al., 2013; Hariton et al., 2021; Kallio et al., 2013; Kristensen et al., 2012; Landersoe, Larsen, et al., 2020; Landersoe, Petersen, et al., 2020; Nelson et al., 2023; Petersen et al., 2015; Siegel et al., 2023; Van Rooij et al., 2005), along with reductions in AFC and ovarian volume (Kristensen et al., 2012; Landersoe, Larsen, et al., 2020; Landersoe, Petersen, et al., 2020; Petersen et al., 2015). However, there is no consensus on this matter, as some studies present contradictory findings, showing non-significant differences in serum AMH levels between hormonal contraception users and non-users (Deb et al., 2012; Van Den Berg et al., 2010).

The impact of hormonal contraception on ovarian reserve markers is considered temporary, as AMH levels tend to normalize after discontinuation of hormonal contraception. Additionally, the duration of hormonal contraception use should not be linked to its impact (Barnhart & Schreiber, 2009; Landersoe et al., 2020).

Table 6 Impact of hormonal contraception on ovarian reserve markers

This table summarizes the impact of different types of hormonal contraception on markers of ovarian reserve, such as AMH and AFC. It includes data from various studies, detailing changes observed in these markers among different populations of contraceptive users. The table may also provide additional notes or observations related to each study's findings, helping to elucidate the effects of hormonal contraception on ovarian health.

Study (Authors, Year)	Population	Sample Size	Hormonal Contraception Type	AMH Change	AFC Change	Notes
Petersen et al., 2015	19–46 years old	N=909 278 users 631 non-users	OC (80.95%) IUD (4.31%) POP & implants (7.91%) Vaginal ring (6.83%)	Decreased: 19%	Decreased: 18%	Cross-sectional More AMH < 5 pmol/l in young OC users
Bentzen et al., 2012	21–41 years old	N=732 228 OC users 504 non-users	COC (95.20%) Vaginal Ring (4.80%)	Decreased: 29.8%	Decreased: 30.4%	Cohort study Impact more pronounced over time
Dölleman et al., 2013	mean age 37.3 years old	N=2320	OC (39.13%)	Decreased: 11 percentiles	-	cross-sectional Lower age-specific AMH percentiles
Kallio et al., 2013	20–33 years	N=42	Oral pill (30.96%) Transdermal (35.71%) Vaginal Ring (33.33%)	Decreased: ~ 50% in all groups	-	Randomized study Different administration methods
Arbo et al., 2007	normovulatory infertile women age > 35	N=20	OC pills	Decreased: 15.44%	No impact on AFC;	Prospective clinical trial
Bernardi et al., 2021	African American women 23–34 years old	N=1643	COCs (39%), LNG-IUD (25%) DMPA (22%)	Decreased: 25.2%	-	Cross-sectional Reversible suppressive effect on AMH
Kristensen et al., 2012	mean age 20.1 years old	N= 256 180 users 76 non-users	OC	Decreased: 55%	-	Cross-sectional Lower AMH in hormonal contraceptive users
Landersoe et al., 2020	19–46 years old	N= 1548 983 non-users 565 users	COC pill (71.50%) LNG-IUS (15.93%) POP (9.73%) vaginal ring (2.84%)	Decreased: 17.1–35.6% 31.1% COC 35.6% POP 17.1% LNG-IUS no differences vaginal ring	Decreased: 31.3% COC 29.7% POP no differences LNG-IUS or vaginal ring	Cohort study Variability among different contraceptive types
Siegel et al., 2023	Women with unexplained infertility 18–45 years old	N=123 107 users 16 non-users	COC; IUD; vaginal ring Long term users: 98 short term users: 9	Trend towards lower AMH	-	Cohort study Duration impacts AMH levels
Nelson et al., 2023	21–45 years old	N=42,684 15,170 users 27,514 non-users	COC (45.15%) POP (3.06%) hormonal (32.08%) IUD (8.36%) implant (5.50%) vaginal ring (5.85%)	Decreased: COC 17% No effect for the hormonal IUD	-	Cross-sectional Impact varies with contraceptive type

Study (Authors, Year)	Population	Sample Size	Hormonal Contraception Type	AMH Change	AFC Change	Notes
Hariton et al., 2021	20–46 years old	N= 27,125 8598 users 18,527 non-users	Copper IUD (10.15%) Hormonal IUD (32.37%) POP (2.64%) Ring (6.18%) Implant (5.14%) COC (43.52%)	Decreased: 7% IUD 15% POP 22% vaginal ring 23% implants 24% COC No differences copper IUD	-	Cohort study Different effects based on contraceptive type
Deb et al., 2012	18 and 35 years	N= 70 34 users 36 non-users	COC with HFI	No differences in AMH	-	Case-Control No significant impact on AMH
Van de Berg et al., 2019	18–14 years old	N=25	HC	No differences in AMH	-	Longitudinal prospective No significant impact on AMH

AMH - Anti-Müllerian hormone; AFC - Antral Follicle Count; OC - Oral Contraceptives, COCs: Combined Oral Contraceptives; HC: hormonal contraception; POP: Progestin-Only Pills; LNG-IUD: Levonorgestrel-Releasing Intrauterine Device; DMPA: Depot Medroxyprogesterone Acetate; COCP with HFI Combined Oral Contraceptive Pills with Hormone-Free Interval; IUD: Intrauterine Device. The impact on AMH and AFC is reported as a percentage change or as noted in the respective studies. Different studies may use different methodologies and populations.

Lastly, there are nor many studies meticulously describing the distribution of AMH and AFC per age for non-infertile populations (Anckaert et al., 2016; La Marca et al., 2010, 2011, 2012; Seifer et al., 2011; Yoo et al., 2011), nor are there exact cut-offs to define DOR in these women. Olsen et al. described the classification of ovarian reserve referring to normal ovarian reserve when higher than the 25th percentile (Olsen et al., 2021). In this field, it is crucial to acknowledge the significant variability in AFC cut-off values, primarily due to a tendency to overestimate them. This is often because similarly sized atretic follicles that could also be counted (Fleming et al., 2015; Jayaprakasan et al., 2010).

Based on the summarized information highlighting weaknesses and gaps in the field, opportunities to assist individuals with their reproductive plans, particularly women with DOR, have emerged. Therefore, the next sections will outline the objectives, methods utilized, and the results and discussion arising from this task.

2.2.2 Objectives

This task goal is to develop and test the effectiveness of two interventions in knowledge about ovarian reserve and DOR, 1) providing general leaflet information, and 2) offering tailored information and reproductive counselling based on the assessment of ovarian reserve.

2.2.3 Methods

2.2.3.1 Participants

Recruitment of participants took place in Coimbra.

Participants were included in the study if they were nulliparous women between 18 and 35 years old who a) undergo family planning consultation; b) potentially seek for fertility assessment and/or for knowing about their reproductive potential. Participants were excluded if they had known history of infertility.

Participants were not asked to interrupt their contraceptive method for the study. This decision facilitated the recruitment process, and enhanced the translational relevance of the findings, reflecting conditions that could be implemented in future consultations or services beyond the study setting. The information about hormonal contraception usage was considered while communicating the ovarian reserve testing results and offered the opportunity for a confirmatory testing after 3 months suspension.

The power analysis was conducted a priori, to compute the required sample size given the statistical test ANOVA of repeated measures, within-between interaction, an effect size f of .15, an α error probability of .05, a power ($1-\beta$ error probability) of .80, 2 groups, 3 measurements, a correlation among repeated measures of .5, and a no sphericity correction ϵ of 1. With these parameters, and using the G*Power 3.1.9.4, the calculated total sample size was 74 participants.

2.2.3.2 Recruitment

Collaborative partnerships were established for recruitment purposes: the department of gynecology obstetrics, reproduction, and neonatology (DGORN), from Unidade Local de Saúde de Coimbra (ULS Coimbra), and family health units, Unidades de Saúde Familiar, from Coimbra Norte, Topázio, Fernão de Magalhães, and São Martinho.

Our recruitment process involved spontaneous calls via email (info@atuaafertilidade.pt) and face-to-face recruitment, in collaboration with the aforementioned services. The recruitment process involved utilizing the study's contact card, as demonstrated in Figure 13.



Figure 13 Inviting card from the project “aTuaFertilidade”

The recruitment process started in March 2023 and ended in June 2024. We have successfully enrolled 105 participants, and, for each participant, we have:

- 1) Conducted initial and two follow-up questionnaires to evaluate the impact of interventions in knowledge;
- 2) Conducted AMH quantification, using Roche kits (Elecsys® AMH);
- 3) Performed AFC and the provide tailored information based on the ovarian reserve assessment;
- 4) Collected peripheral blood for DNA extraction and further genetic analysis.

2.2.3.3 Study design

The timeline of the study followed what is illustrated in Figure 14. Women who met the inclusion criteria gave their written informed consent, completed the initial questionnaire, and underwent blood sample collection for AMH measurement. In the same day (t₀), participants received a leaflet with general information about ovarian reserve. Two weeks later (t₁), participants were given a 2nd questionnaire to evaluate the impact of the provided information. Within a 2-to-4-week interval, participants were contacted to undergo an ultrasound for AFC and to receive the results of the ovarian reserve testing, along with reproductive counselling. Two weeks after (at t₂), participants were requested to fill the last questionnaire, to evaluate the impact of the tailored information.

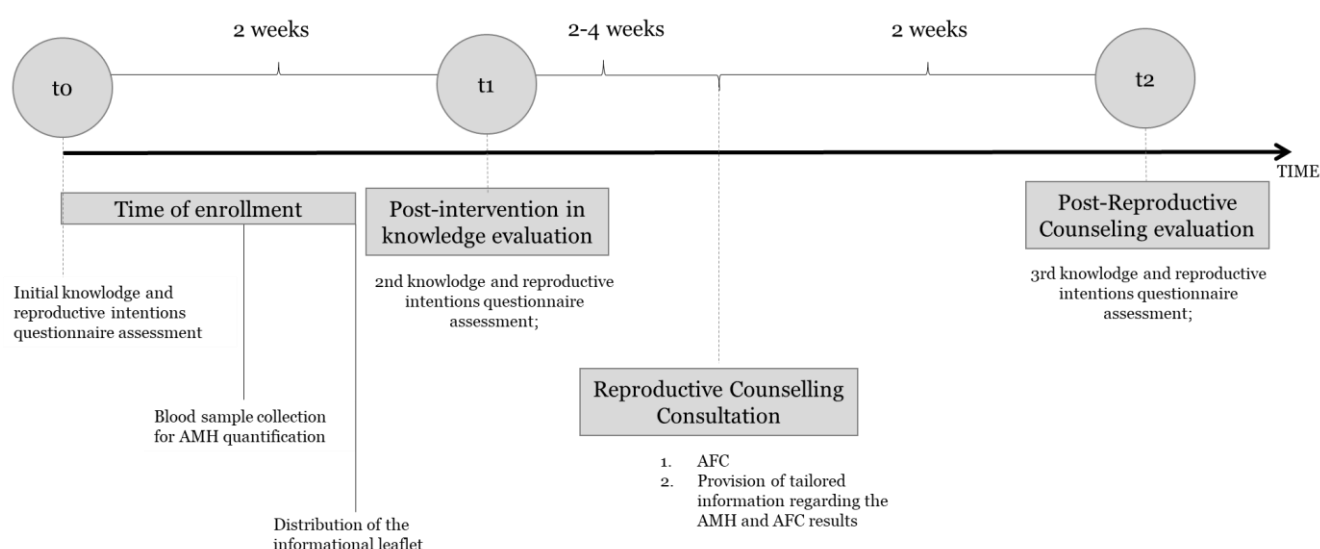


Figure 14 Diagram of the study design

t0, t1, and t2 represent three distinct time-points, spaced two weeks apart, during which participants completed knowledge and reproductive intentions questionnaires. At the study's outset, patients underwent blood sample collection for quantification of AMH. On the day of the reproductive counselling consultation, AFC assessments were conducted, and the results were subsequently provided to participants.

AMH: anti-mullerian hormone; AFC: antral follicle count

2.2.3.3.1 Measurement tools and procedures

2.2.3.3.1.1 Project name, logo, and collaborative actions

"aTuaFertilidade" is an original name, and both the logo (Figure 15) and content design have been created specifically for this purpose.



Figure 15 "aTuaFertilidade" study logo

This project involved developing informative content disseminated across various platforms, including the creation of a leaflet, a website, and a video addressing the topic. Detailed descriptions of all the content developed in this project, as well as the evaluation instruments employed, are provided in the following sections.

2.2.3.3.1.2 The leaflet – first intervention in knowledge

The leaflet (Figure 16) included general information about ovarian reserve, the number of available oocytes throughout a woman's lifespan, the impact of age on fertility, the implications of DOR, and possibilities for ovarian reserve testing.

The primary objective of the leaflet was to act as an educational intervention aimed at increasing knowledge about ovarian reserve and DOR. The impact of the leaflet was assessed through a questionnaire administered before and two weeks after providing the information. The secondary objective was to compare the impact of this generic intervention on knowledge with the impact of a tailored intervention, obtained through AMH and AFC testing, on knowledge assessed through questionnaire responses, as well as on childbearing intentions and reproductive plans.

The leaflet is available as supplementary material (section 4.1).



Figure 16 “aTuaFertilidade” leaflet

Leaflet used as intervention in knowledge with general information about ovarian reserve and DOR, factors that affect it, how fertility declines, and how to evaluate ovarian reserve.

2.2.3.3.1.3 Ovarian reserve assessment and reproductive counselling – second intervention in knowledge

A sponsorship protocol was signed with Roche, for the project duration from March 2023 to December 2024, to support the AMH quantification.

2.2.3.3.1.3.1 The tool to interpret anti-Müllerian hormone (AMH) and antral follicle count (AFC) values

AMH and AFC were interpreted in percentiles, by specific age categories. Drawing from the two most comprehensive papers on AMH (La Marca et al., 2012) and AFC (La Marca et al., 2011), which outlined age-specific expected values in percentiles in non-infertile populations, we have created a calculator specifically tailored for clinicians. Through the integration of Hypertext Markup Language (HTML), Cascading Style Sheets (CSS), and JavaScript, we have created a completely new tool specifically designed for this purpose. By inputting the participant's age, AMH, and AFC values, this tool provides the corresponding percentile (5th, 25th, 50th, 75th, and 95th) based on the available literature. To address whether patients ovarian reserve testing was lower-than-expected, we used the cutoff 25th percentile (<25th).

To further enhance the usability of this information, we have implemented a colour code system for the results. Results falling below the 25th percentile are denoted in red, those falling between the 25th and 75th percentiles are shown in yellow, and results surpassing the 75th percentile are indicated in green. This color-coded approach aids in quickly assessing and interpreting the results, ensuring efficient communication with patients and between medical professionals. Images illustrating the tool for determining AMH and AFC percentiles are available in the supplementary data (Section 4.2)

2.2.3.3.1.3.2 Receiver operating characteristic (ROC) curve of anti-Müllerian hormone (AMH) and antral follicle count (AFC) to predict diminished ovarian reserve (DOR) (within the infertile population)

Receiver operating characteristic (ROC) curve analysis was used to predict the specificity, sensitivity, and accuracy of the AMH with the AFC as a diagnostic test for identifying DOR patients. This evaluation was conducted at the beginning of the study as an initial approach to the subject.

Within the scope of the infertile population, our analysis focused on an IVF cohort of women treated at the reproductive medicine unit of CHUC from 2015 to 2019. This analysis enabled us to suggest an AMH cut-off value of <.85 ng/mL, demonstrating a sensitivity of 84% and specificity of 72%, and an area under de curve (AUC) of .857, for an AFC of <5, as a predictive indicator for DOR. The ROC curve (Figure 17) was constructed using data from 485 patients aged between 22

to 44 years old. AMH levels ranged from .01 to 3.97, and AFC ranged from 0 to 34. The exclusion criteria for this analysis involved individuals with PCOS and those who had undergone chemotherapy or radiotherapy. Both AMH and AFC measurements were determined within a one-year timeframe, with an average time difference of 6 months between these analyses. Patients undergoing fertility preservation were excluded from the analysis.

These results were useful for the study of the infertile population carried out in Task 2.

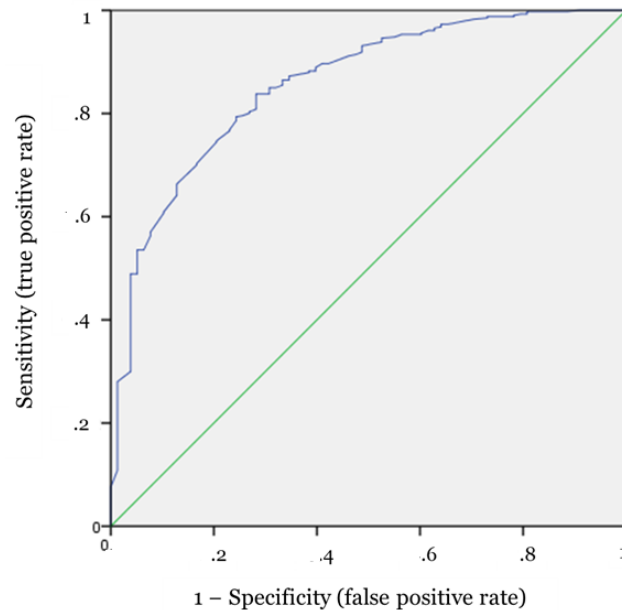


Figure 17 ROC curve of AMH for the prediction of DOR among infertile women, considering an AFC cut off value of 5

The ROC curve represents the trade-off between sensitivity (the true positive rate) and 1 – specificity (the false positive rate) for the AMH test in distinguishing between DOR and normal ovarian reserve (binary classification model) in infertile women, considering an AFC cut-off value of 5.

It plots the true positive rate against the false positive rate at various threshold settings. The AUC the ROC curve summarizes the performance of the model across all the thresholds.

AUC: area under the curve; ROC: receiver operating characteristic curve; AMH: anti-Müllerian hormone; AFC: antral follicle count; DOR: diminished ovarian reserve

2.2.3.3.1.3.3 Anti-Müllerian hormone (AMH) and antral follicle count (AFC) testing

For AMH determination, blood samples (2.5 mL) were collected in tubes with separating gel on an independent cycle day. The collected blood was centrifuged at 2000 rotations per minute (RPM) for 10 minutes and the serum was stored in polypropylene tubes at –20 °C, as recommended by the manufacturer, until analysis. AMH levels were measured at the clinical

pathology laboratory of ULS Coimbra, using Roche kits (Elecsys® AMH, Roche Diagnostics, Basel, Switzerland). The fully automated Elecsys® AMH assay from Roche detects AMH in a range of .01 to 23 ng/mL (.071 to 164.2 pmol/L), with a detection limit of .010 ng/mL (.071 pmol/L). The assay's imprecision is less than 5%. The limits of blank, limit of detection, and limit of quantification are .049 pmol/L (.007 ng/mL), .07 pmol/L (.010 ng/mL), and .21 pmol/L (.030 ng/mL), respectively. Repeatability is within 1 - 2.6% coefficient of variation (CV) (.33 - 91.1 pmol/L or .046 - 20.8 ng/mL), while intermediate precision ranges from 2.5 to 3.9% CV (.33 - 91.1 pmol/L or .046 - 20.8 ng/mL).

AFC was performed by transvaginal ultrasound. An experienced clinician, consistent across all patients, counted the follicles ranging from 2 to 10 mm in both ovaries, on the day of the reproductive counselling consultation, aligning with established literature (Broekmans et al., 2010; Disseldorp et al., 2010).

The choice to perform AFC at any point in the cycle was supported by the demonstrated accuracy of measurements reported in literature (section 1, section 1.2.2.9). Moreover, the practicality of scheduling ultrasounds without specific cycle day restrictions contributed to the efficiency and convenience of the study.

2.2.3.3.1.3.4 Reproductive counselling

The communication of the ovarian reserve testing results involved a standardized guide developed by a multidisciplinary team, which included gynaecological and obstetric physicians, along with a psychologist, all specialized in reproductive medicine and experienced in fertility counselling for fertility preservation.

All the results were analysed and communicated by a specialist in gynaecology and obstetrics. The counselling information included both general information, applicable to all participants and tailored information, tailored according to AMH and AFC values.

The general information included referring that ovarian reserve is the quantity of oocytes available throughout women's reproductive years. This reserve is finite and limited, and gradually decreases with increasing age. AMH and AFC values are extensively used and exhibit good discriminatory capacity in predicting ovarian response in ART. However, they may not necessarily provide accurate predictions for spontaneous pregnancy. This limitation arises due to all the other variables involved in pregnancy that are not evaluated by ovarian reserve testing, including tubal factors, uterine conditions, male factors, and even idiopathic factors. The natural decline in ovarian reserve with age was highlighted, theoretically starting to decrease from age 30 and more significantly at age 35.

The tailored information included whether participants were above or lower than the expected range (25th percentile). The baseline questionnaire included asking about the use of contraceptive methods. This information was confirmed in the day of the consultation. Additionally, it was

acknowledged that hormonal contraception can potentially affect and undervalue the AMH and AFC measurements.

If AMH and AFC values were lower than the 25th percentile participants were informed that, according to AMH and AFC values, the ovarian reserve was lower than expected. They were warned that these values, alone, do not necessarily mean the inability to get pregnant, as many other factors contribute to fertility. Two approaches were discussed: 1) "Wait and see" if the participant was not in a condition or do not intend to create a family at that time; 2) anticipate the childbearing age, if trying to get pregnant is an option. It was given the information that 80% of women with DOR get pregnant within the first 6 months when attempting to conceive before the age of 35.

If AMH and AFC values were in 25th percentile or higher, participants were informed that the ovarian reserve was in a normal range. They were also advised that these values, alone, do not mean they will be able to get pregnant, as many other factors contribute to fertility. However, at that time, they seem to be in a good position to get pregnant.

In cases where there were discrepancies between AMH and AFC results, participants were informed about this inconsistency. Both scenarios were explained: one where the AMH/AFC value was lower than the 25th percentile and another where it was in the 25th percentile or higher. If participants expressed interest in further exploration, they were offered the option to repeat the analysis.

Given the widespread discussion surrounding oocyte cryopreservation and its relevance within the age ranges of the participants, this possibility was also mentioned. We highlighted that oocyte cryopreservation is a viable option for preserving fertility. However, for non-medical reasons, this procedure is not covered by our national health system. Participants were informed that oocyte freezing involves ovarian stimulation and, in the future, ART to get pregnant. It was emphasized that being well-informed is crucial and that, in reproductive counselling, the primary focus should still be the anticipation of pregnancy.

Towards the conclusion of the counselling sessions, participants were informed about the subsequent steps in the study. They were made aware that they would be required to complete a final questionnaire. Additionally, they were assured that they would have access to the results of the ovarian reserve testing.

The information used to provide reproductive counselling is available in the section 4.3

2.2.3.3.1.4 The website

The website, *www.atuafertilidade.pt* was exclusively designed and conceptualized for this study. All the information within it was carefully reviewed from the literature to provide reliable information, encouraging reproductive health literacy. Created using WordPress technology, the website serves as a resource offering information on reproductive health and fertility, tailored to childbearing-age women in various life stages.

Structured around four key questions, the website's main sections are:

1. What should I know about fertility?
2. How to evaluate my fertility?
3. I want to get pregnant, what should I know?
4. How to take care of my fertility?

Within these main sections, detailed information is provided, covering essential topics such as:

1. From the oocyte to pregnancy
2. Menstrual cycle and the fertile period
3. Ovarian reserve
4. Factors that affect fertility
5. Information about family planning and guidelines based on the time women are planning to have children (next year, in the future, already trying for a year)
6. General care practices are outlined to maintain a healthy reproductive life.

2.2.3.3.1.5 The video

A video was created with the same information as the leaflet, intending to be used in oral communications.

The link to the video is available in the supplementary data (Section 4.4).

2.2.3.3.1.6 Data collection instruments

2.2.3.3.1.6.1 Questionnaires

Recognizing that the existing tools for evaluating knowledge do not align with our specific goal of assessing knowledge about ovarian reserve and DOR, we developed an assessment protocol for three time-points, in Portuguese. These instruments are adapted from prior questionnaires used within our research group in this field (Oliveira et al., 2016), based on the existing measures (Bunting & Boivin, 2010; Peterson, 2017).

The questionnaires include essential demographic and clinical information, while also evaluate participants knowledge about ovarian reserve and DOR, in addition to their childbearing intentions and attitudes towards reproductive plans. Multiple question formats were included. Namely, the first questionnaire included,

1. Personal information and demographics: covers ethnicity, diagnosed diseases, contraceptive use, academic and professional status, and marital status (14 questions with 5 multiple choices).

2. Menstrual cycle details: explores age of menarche, mother's age at menopause, date of the last period, duration of the menstrual cycle, presence of pelvic pain during menstruation, and history of ovarian surgery (7 questions with 4 multiple choices and 1 Likert scale).
3. Childbearing intentions: investigates whether participants have tried to get pregnant, the importance of future plans (career, education, motherhood, living abroad, buying a house, getting married), importance of having children, desired number of children, ideal age to have the first child, planned age to have the first child, perceived obstacles to having children (infertility, stable relationship, academic objectives, career progression, financial instability, not being prepared), and confidence in achieving planned parenthood (11 questions with 6 multiple choices and 3 Likert scales).
4. Habits and lifestyle: explores habits such as smoking, physical exercise, weight, and height (5 questions with 2 multiple choices).
5. Knowledge about female reproductive potential: assesses knowledge about ovarian reserve concepts, perceptions of fertility decline with age, average age of mother first childbirth in Portugal; awareness of ovarian reserve testing and DOR incidence, familiarity with number of available oocytes during woman lifespan, factors that contribute to ovarian reserve decreasing, ovarian reserve evaluation (7 questions with 3 multiple choices, 1 Linkert scale, 2 scales of pre-defined options to choose the age at which fertility declines, and 1 true or false).
6. Attitudes towards ART, oocyte donation, oocyte freezing, and alternative parenthood perspectives: explores attitudes towards management the management of a DOR diagnosis; considerations about donating oocytes; sentiments about using donated oocytes; thoughts on freezing oocytes; opinions on social freezing, and openness to novel therapies (8 questions with 5 multiple choices and 2 Likert scales).
7. Final considerations: gathers insights on the importance of being informed and the significance of the fertility counselling (3 questions with 1 multiple choice and 2 Likert scales).

The 2nd questionnaire did not include the personal information and demographics nor the menstrual cycle details. The childbearing intentions, knowledge about ovarian reserve and DOR (specifically 4 out of the 7 items initially referred), attitudes towards ART, oocyte donation, oocyte freezing, and alternative parenthood perspectives, and final considerations were repeated.

The 3rd questionnaire included the same as the 2nd questionnaire. The final considerations section added six questions, including questions about whether the participant believes she should have received information about ovarian reserve earlier in life, the motivations behind evaluating ovarian reserve, perceptions about information received post-consultation, and interpretations of the provided information. These additional questions comprised 3 multiple-choice questions and 2 Likert scale questions.

Additionally, to the assessment protocol, we added the state-trait anxiety inventory (STAI) questionnaires. STAI is a commonly used measure of trait (STAI-T) and state (STAI-S) anxiety (Spielberger et al., 1983), Portuguese version by Silva & Spielberger (Silva & Spielberger, 2011). It can be used in clinical settings to diagnose anxiety, and it was also used as an indicator of anxiety related to the communication of fertility information (Boivin et al., 2018; Maeda et al., 2016, 2020).

The questionnaires used in this study are included in the supplementary data of the thesis (section 4.5).

2.2.3.3.1.6.2 Evaluation of the website and the video content: semi-structured interviews

Different participants from those who participated in the interventions in knowledge evaluated the website and the video content. Semi-structured interviews were conducted with women who were childless, had no history of infertility, and were aged between 18 and 35 years. Recruitment was carried out through the study's promotion, with participation on a self-proposal basis via email. This approach involved conducting semi-structured interview with predefined categories for answers as well as opportunities for open-ended responses. The duration of the interview was the same for all the participants. The duration of the interview, conducted via video call, was consistent for all participants. They first watched the video, then answered predefined questions. Subsequently, the website was shared, and participants were given 10 minutes to navigate it. The interview followed this structured format:

1. Introduction:
 - Welcomed participants and briefly explained the purpose of the evaluation
2. Initial questions about knowledge in the field:
 - Asked participants if they had heard about ovarian reserve
 - Inquired about their understanding of fertility decline with age
 - Questioned their awareness of the average maternal age at the birth of the first child in Portugal
 - Asked whether young women could experience DOR, and its prevalence
3. Video evaluation:
 - Participants viewed the video
 - Questions with predefined categories for answers were posed regarding the video:
 - Clarified if the content was clear
 - Rated the relevance of the information to their understanding of reproductive health
 - Assessed the video's duration
 - Determined if they gained new insights into female reproductive health, particularly ovarian reserve

- Considered if the content was suitable for individuals without prior knowledge of reproductive health
 - Stated whether they would recommend the video to a friend
 - Questions with open-ended answers,
 - Identified any confusing or unclear sections and provided reasons
 - Highlighted the most/least understandable aspects of the video
- 4. Website evaluation:
 - Participants navigated through the website for 15 minutes
 - Questions with predefined categories for answers,
 - Evaluated the ease of website navigation
 - Rated the relevance of the information to their current understanding of reproductive health
 - Assessed the understandability of website content
 - Determined if the information provided was relevant and informative
 - Examined if the website offered new insights or information
 - Considered if the content was suitable for individuals without prior knowledge of reproductive health
 - Stated whether they would recommend the website to a friend
 - Revealed if they learned anything new about female reproductive health, particularly ovarian reserve
 - Open-ended questions were asked about the website:
 - Identified the most/least understandable aspects of the website
 - Identify any confusing or unclear sections of the website and provided explanations
- 5. General feedback:
 - General questions were posed about the overall experience:
 - Addressed if the content met their concerns about female reproductive health
 - Solicited suggestions for material improvement
 - Invited any additional thoughts or suggestions
- 6. Questions about knowledge in the field:
 - At the conclusion, the initial questions were revisited to assess the impact of the information provided

2.2.3.3.1.7 Statistical analysis

Descriptive statistics were used to present the distribution of sociodemographic backgrounds, lifestyles, and reproductive histories, as well as data from the post-consultation assessment. Independent and paired samples t-tests, along with the Mann-Whitney U test and Wilcoxon signed-rank test, were employed to assess differences in numeric variables (e.g., age, AMH, AFC distribution) between the subgroups of interest in the study. Additionally, correlations between

categorical variables were analysed using Fisher's exact test and the chi-squared test. For numerical variables, Pearson's correlation coefficient was used.

The data from the interviews underwent descriptive analysis, with comparisons made using paired t-tests to assess knowledge differences (% of correct answers before and after the video and website exposure)

To evaluate post-intervention changes in fertility knowledge and childbearing desire, responses from three different questionnaires were analysed using repeated measures ANOVA and the Friedman test.

The specific tests used in each analysis are indicated in the tables accompanying the results for easier follow-up of the study.

The observed power was calculated and verified to be greater than .8 for the major study questions. An exception is acknowledged for a subgroup analysis, detailed further in the results section. In that case, a small subgroup was formed, and we believe the clinical value surpasses the potential loss of statistical power.

All analyses were conducted using IBM SPSS Statistics for Windows, Version 28.0 (IBM Corp, Armonk, NY, USA), with the level of significance set at 5%.

2.2.4 Results

2.2.4.1 Baseline characteristics of the study population

An initial cohort of 118 women, from 18 to 35 years old, without children and with no history of infertility, were included in the study (Figure 18). Volunteers were recruited 1) among patients attending family planning consultations (n=35), and 2) through spontaneous calls (n=83).

Thirteen participants withdrew from or dropped out of the study, resulting in a final participant count of 105. The attrition rate was calculated at 11% using the following equation:

$$\text{Attrition rate} = \frac{\text{participants who have dropped out or withdrawn from the study}}{\text{total number of participants at the beginning of the study}} \times 100$$

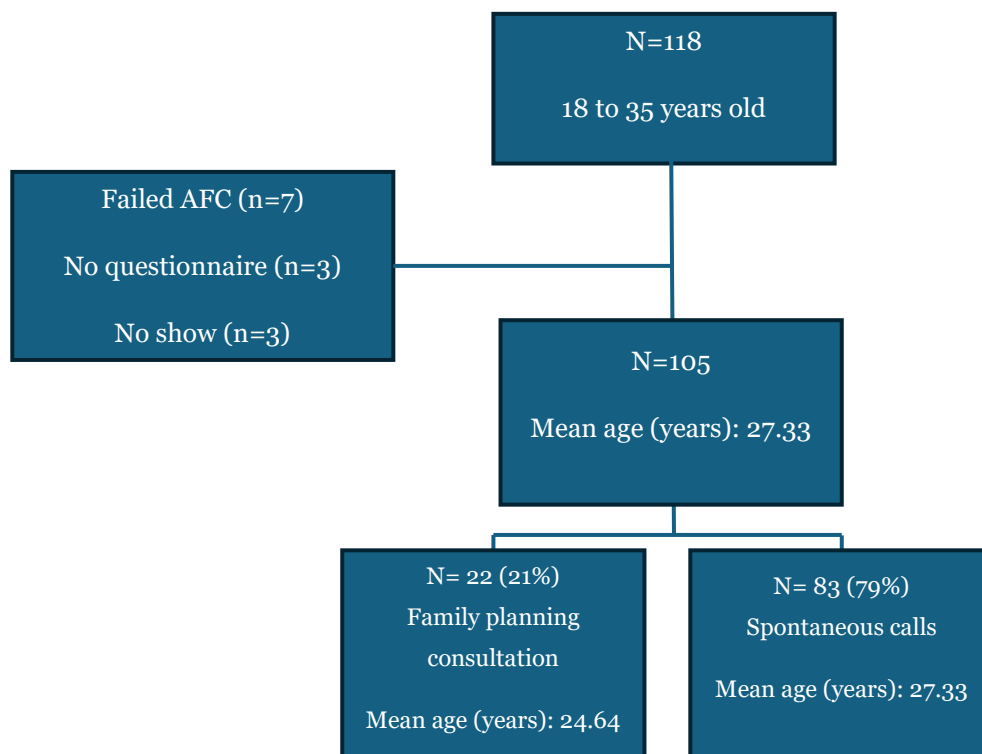


Figure 18 Participants flow chart

Initial number of participants recruited, including those who withdrew (and their reasons for withdrawal). Distribution of participants based on their source of recruitment (family planning consultation or spontaneous call) and their mean age.

The 105 participants responded to the assessment protocol to evaluate their knowledge and reproductive attitudes across three time-points – to (at study enrolment), t1 (after the first intervention in knowledge), and t2 (after the second intervention in knowledge). The data collected from the questionnaires were analysed to identify changes in their knowledge and perspectives over time. We begin by reporting general data about our participants at to, the initial time-point, which serves as the baseline, with no intervention.

At the time of blood sampling women were asked to fill in a questionnaire regarding sociodemographic characteristics, fertility knowledge, childbearing intentions, and attitudes through reproductive plans. All women gave their written informed consent before the blood sampling. The baseline characteristics of this study population are described in Tables 7 to 12. The information was divided to facilitate easier reading of the data. Statistical measures including N (%), minimum, maximum, mean, and standard deviation (SD) are provided for each variable analysed within the characteristics of the total participants.

2.2.4.1.1 Age, AMH and AFC, and ethnicity

The mean age of participants was 27.33 ± 3.67 years (Table 7). Within this population, which mostly comprised individuals who participated by self-proposal (79%), the mean AMH level was 3.08 ± 2.32 ng/mL, and the mean AFC was 19.86 ± 10.70 (Table 7). The values are reported as mean $\pm$ SD. The age of participants who enrolled by self-proposal was significantly higher than that of participants recruited during family planning consultations (27.33 years vs. 24.64 years, respectively; $p < .001$).

The distribution of participants' ethnicity (Table 7) revealed the following: there were no participants of Asian ethnicity (0%). African and Hispanic participants each constituted 5 individuals, representing 4.76% of the total population respectively. The majority of participants were Caucasian (white), accounting for 93 individuals, which is 88.57% of the total. Additionally, there were 2 participants (1.91%) identified as belonging to multiple ethnicities.

Table 7 Participants descriptives: distribution and characteristics of collected data for age, AMH, AFC, and origin

For each variable listed (age in years, AMH (ng/mL), AFC, origin), the table displays the distribution or characteristics of the data collected.

	Total (N=105) N (%)	Total Min	Total Max	Total Mean	Total SD
Age in years					
	105 (100%)	20	34	27.34	3.67
AMH (ng/mL)					
	105 (100%)	.12	11.56	3.08	2.32
AFC					
	105 (100%)	2	53	19.86	10.69
Origin					
1: Family planning	22 (20.95%)				
2: External	83 (79.05%)				
Ethnicity					
1: Asian	0				
2: African	5 (4.76%)				
3: Hispanic	5 (4.76%)				
4: Caucasian (White)	93 (88.57%)				
5: Multiple ethnicities	2 (1.91%)				

AMH: Anti-Müllerian hormone; AFC: antral follicle count; SD: standard deviation; Min: Minimum; Max: Maximum

2.2.4.1.2 Contraceptive use

Concerning contraceptive use, 27.62% of participants (29 individuals) indicated they were not using any contraceptive method. The majority, 57.14% (60 individuals), reported using the pill. The ring was used by 5.71% of participants (6 individuals), while 8.57% (9 individuals) were using an intrauterine device. Finally, .95% of participants (1 individual) indicated they were using an implant. The distribution of contraceptive methods among participants is visually represented in the accompanying pie chart (Table 8 and Figure 19).

Table 8 Participants descriptives: contraceptive use distribution and percentages

For each category of contraceptive use (none, pill, ring, intrauterine device, implant), the table presents the count (N) and percentage (%) of participants who reported using that specific method.

Contraceptive use	Total (N=105) N (%)
0: None	29 (27.62%)
1: Pill	60 (57.14%)
2: Ring	6 (5.71%)
3: Intrauterine device	9 (8.57%)
4: Implant	1 (.95%)

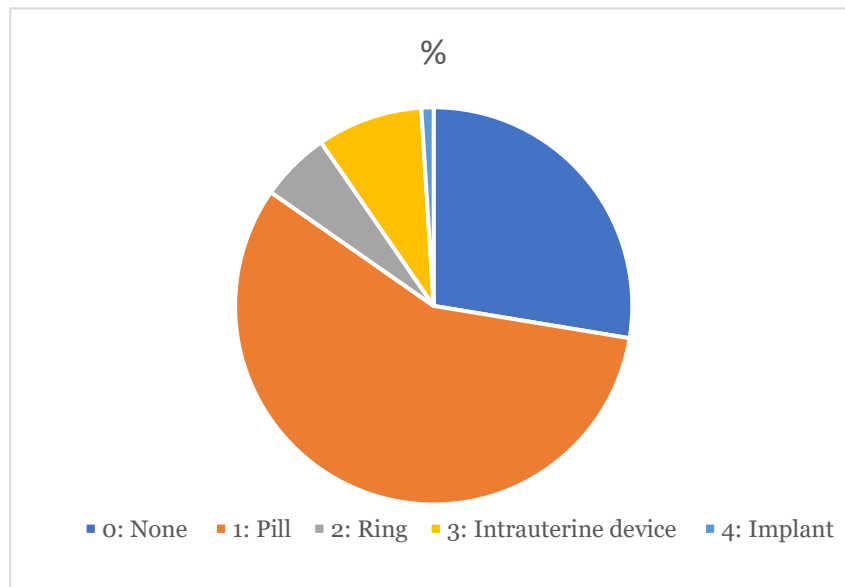


Figure 19 The distribution of contraceptive methods among participants

Results are expressed in percentage.

2.2.4.1.3 Education level and employment status

Academic and professional status varied among participants (Table 9). The educational background exhibited a substantial portion of participants held advanced degrees, with 28.57% possessing a bachelor's degree and 55.24% holding a master's degree. Additionally, 3.81% of participants had a PhD. In terms of employment status, the majority of participants, 58.1%, were actively employed. A smaller percentage, 5.71%, were unemployed, while 36.19% identified as students.

Table 9 Participants descriptives: distribution of educational level and employment status among the study participants

Count (N) and percentage (%) of participants who reported having attained that level of education; and who reported being in that specific employment category.

Education	Total (N=105) N (%)
1: Basic education (9th grade)	1 (.95%)
2: High school diploma (12th grade)	12 (11.43%)
3: Bachelor's degree	30 (28.57%)
4: Master's degree	58 (55.24%)
5: PhD	4 (3.81%)
Employment status	Total (N=105) N (%)
1: Employed	61 (58.1%)
2: Unemployed	6 (5.71%)
3: Student	38 (36.19%)

PhD: Doctor of Philosophy

2.2.4.1.4 Relationship status

Participants' relationship status (Table 10) indicated that a significant majority of participants, 72.38%, were currently in a relationship. Being married represented a minority, with 15.24% of participants either married or in a common-law relationship (Table 10).

Table 10 Participants descriptives: relationship status and current relationship

For each category (single, married/common-law, in a relationship), the table shows the count (N) and percentage (%), as well as the mean and SD of participants who reported being in that specific relationship status.

Relationship status	Total (N=105) N (%)
Single	89 (84.76%)
Married/common-law	16 (15.24%)
In a relationship	Total (N=105) N (%)
No	29 (27.62%)
Yes	76 (72.38%)

2.2.4.1.5 Habits and lifestyle

Regarding habits and lifestyle (Table 11), 15.24% of participants reported smoking habit. Regarding physical exercise, more than half of the population (61.9%) reported exercising 1-3 times per week. The mean BMI was 24.35 ± 4.46 .

Table 11 Participants descriptives: habits and lifestyle

For each category, the table shows the count (N) and percentage (%), as well as the Min, Max, mean and SD of participants smoking habits, physical exercise and BMI (kg/m²).

Smoker	Total (N=105) N (%)				
No	89 (84.76%)				
Yes	16 (15.24%)				
Physical exercise	Total (N=105) N (%)				
0: No	21 (20%)				
1: 1-3 times/week	65 (61.9%)				
2: 4-6 times/week	18 (17.14%)				
3: Competition	1 (.95%)				
BMI (kg/m ²)	Total (N=105) N (%)	Total Min	Total Max	Total Mean	Total SD
	105 (100%)	17.84	40.40	24.35	4.46

BMI: body mass index; SD: standard deviation; Min: minimum; Max: maximum

2.2.4.1.6 Menstrual cycle details

Participants provided various details regarding their menstrual cycles (Table 12). The mean age of menarche was 12.24 ± 1.34 years. Responses regarding the age at which participants' mothers reached menopause predominantly fell into the category of over 45 years old.

Regarding the presence of pelvic pain during menstruation, the majority of participants reported experiencing moderate discomfort, with 35.24% indicating a severity rating of 2 out of 5, and 28.57% reporting a rating of 3 out of 5.

A small subset of participants, specifically three individuals, reported a history of ovarian surgery. Two cases reported undergoing surgery within the first 6 months of life due to ovarian torsion resulting from cysts. Another case underwent surgery in adulthood due to a mucinous cystadenoma.

Within the cohort, one participant had actively tried to get pregnant. This participant reported a successful spontaneous conception, occurring after her participation in the study. The pregnancy was confirmed in February 2024, marking the 13th week of gestation.

Table 12 Participants descriptives: menstrual cycle details

Summary of the data related to age at menstruation, menopause, dysmenorrhea severity, ovarian surgery, and attempts to conceive among the study participants. Age at first menstruation is reported in min, max, mean values, and SD; the other variables are presented as categorical distributions

	Total (N=105) N (%)	Total Min	Total Max	Total Mean	Total SD
Age at first menstruation (years)					
	105 (100%)	8	16	12.24	1.34
Age at which the mother entered menopause (years)					
0: has not entered	16 (15.24%)				
1: <40 years	0				
2: 40-45 years	13 (12.38%)				
3: >45 years	76 (72.38%)				
Dysmenorrhea					
0: no pain	2 (1.9%)				
1	17 (16.19%)				
2	37 (35.24%)				
3	30 (28.57%)				
4	14 (13.33%)				
5: very intense painful periods	5 (4.76%)				
Had ovarian surgery					
No	102 (97.14%)				
Yes	3 (2.86%)				
Have tried to get pregnant					
No	104 (99.05%)				
Yes	1 (.95%)				

SD: standard deviation; Min: Minimum; Max: Maximum

2.2.4.1.7 Examining the relationship between determinant variables in ovarian reserve

A significant positive correlation ($r=.680$, $p<.001$) was identified between AMH and AFC (Table 13), showing the strong association between the AMH and AFC as measures of the ovarian reserve. Significant differences in AMH levels were observed when comparing users and non-users of hormonal contraception ($p<.001$, Table 13), with those using hormonal contraception exhibiting lower levels of AMH (2.58 ng/mL for users versus 4.39 ng/mL for non-users).

No significant correlations were found between smoking, BMI, age at first menstruation, age at mother entered in menopause, and levels of AMH and AFC (Table 13).

The association between a higher level of education and prior knowledge about ovarian reserve and about the possibility to evaluate ovarian reserve was not confirmed by the data analysis ($p=.800$, Table 13)

A final observation concerns the median planned age for having a child for those with a 12th grade education was 28.50 years, which was significantly lower when compared with participants

holding a master's degree (32 years) or a doctorate (PhD; 33 years; $p < .001$). However, higher education did not impact the number of children participants intended to have ($p = .125$).

Table 13 Correlation analysis of key variables related to ovarian reserve

p-values for the comparisons between the three time-points. Statistical significance ($p\text{-value} < .05$) is highlighted in blue.

		Pearson's r	p-value
AMH (ng/mL)	AFC	.680	$p < .001^c$
BMI	AMH (ng/mL)	.083	$p = .398^c$
	AFC	-.019	$p = .848^c$
Age at First Menstruation in years	AMH	-.022	$p = .824^c$
	AFC	.021	$p = .830^c$
Age at which the mother entered menopause	Age at First Menstruation in years		$p = .813^b$
	AMH (ng/mL)		$p = .184^b$
	AFC		$p = .190^b$
Hormonal contraception 0: No 1: Yes	AMH (ng/mL)		$p < .001^a$
	AFC		$p = .052^a$
Smoker 0: No 1: Yes	AMH (ng/mL)		$p = .314^a$
	AFC		$p = .409^a$
Education 1: Basic education (9th grade) 2: High school diploma (12th grade) 3: Bachelor's degree 4: Integrated master's degree 5: PhD	Already knew about ovarian reserve?		$p = .800^b$
	Aware about ovarian reserve testing?		$p = .343^b$
	Age at which intend to have the first child		$p < .001^a$
	Number of children do you consider possible to have		$p = .125^a$
In a relationship 0: No 1: Yes	Age at which intend to have the first child		$p = .617^a$
	Number of children do you consider possible to have		$p = .517^a$

AMH: Anti-Müllerian hormone; AFC: antral follicle count; BMI: body mass index; Doctor of Philosophy (PhD); a: t-test for independent samples or Mann-Whitney U test; b: Chi-square test or Fisher's exact; c: Pearson's correlation

2.2.4.1.8 Childbearing intentions

Childbearing intentions were assessed and are described in Tables 14 to 16. The results are divided to facilitate easier reading of the data. Statistical measures including N (%), minimum, maximum, mean, and SD are provided for each variable analysed within the assessment of childbearing intentions and future plans, at to, of the total participants.

Questions regarding the importance and certainty participants express about having children were only present in the first questionnaire – considered “to”, as it corresponds to the baseline evaluation, without any intervention. The same applied to a self-evaluation of how well-defined their plans are regarding career, education, motherhood, living abroad, buying or renting a house, and getting married.

About the importance of having children in the future (Table 14), evaluated in a Likert scale of 1 (not at all important) to 5 (extremely important), 40% of participants rated its importance at 4 out of 5, while 37.11% rated it at the highest level, 5 out of 5. Regarding the certainty of having children (Table 14), a multiple-choice question, a significant majority (58.1%) of participants expressed being sure about their desire to have children, contrasting with a minority (2.92%) who affirmed they do not wish to have children.

Table 14 Descriptives of perceived importance and certainty of future childbearing plans

Categories range from 1 (Not important at all) to 5 (Extremely important), indicating the extent to which participants have planned various aspects of their lives. The results are expressed in both numbers and percentages for each category.

	Total(N=105) N (%)
Importance of having children in future	
1: Not at all important	3 (2.86%)
2: Slightly important	7 (6.67%)
3: Moderately important	14 (13.33%)
4: Very important	42 (40%)
5: Extremely important	39 (37.14%)
Certainty of having children in the future	
I am certain that I want to have children	61 (58.1%)
I still have doubts. but most likely I will decide to have children	31 (29.52%)
At the moment. I am certain that I do not want to have children	3 (2.86%)
I still have doubts. but most likely I will decide not to have children	10 (9.52%)

The life planning across various domains (Table 15) was evaluated in a Likert scale 1 (not at all important) to 5 (extremely important). Participants demonstrated a high level of clarity in their career planning, with 48.57% indicating well-defined (4 out of 5) professional aspirations, and 21.9% expressing the highest level of certainty. Similarly, educational goals were highly defined, with 62.86% reporting clear academic plans, rated at the highest level. Parenthood intentions exhibited a diverse range of clarity levels, with 30.48% expressing a high certainty (4 out of 5)

about the timing preferences. In terms of housing, a significant portion (34.29%) reported moderate (3 out of 5) clarity in planning for home ownership or rental. Living abroad plans were moderately defined, with 27.62% grading with 3 out of 5 their intentions. However, marriage planning showed a higher level of uncertainty (1 out of 5), with 31.43% reporting lack of specific plans regarding the timing of marriage.

Table 15 Participants levels of planning across various life domains

Categories range from 1 (Not defined at all) to 5 (Completely defined), indicating the extent to which participants have planned various aspects of their lives. The results are expressed in both numbers and percentages for each category.

How defined are your plans?	Total (N=105) N (%)
Career (planned their professional life)	N (%)
1: Not defined at all	2 (1.9%)
2: Slightly defined	3 (2.86%)
3: Moderately defined	26 (24.76%)
4: Mostly defined	51 (48.57%)
5: Completely defined	23 (21.9%)
Education (planned their academic formation)	N (%)
1: Not defined at all	2 (1.9%)
2: Slightly defined	1 (0.95%)
3: Moderately defined	9 (8.57%)
4: Mostly defined	27 (25.71%)
5: Completely defined	66 (62.86%)
Parenthood (planned the moment they want to have children)	N (%)
1: Not defined at all	23 (21.9%)
2: Slightly defined	15 (14.29%)
3: Moderately defined	30 (28.57%)
4: Mostly defined	32 (30.48%)
5: Completely defined	5 (4.76%)
Buying/renting a house (planned the stage of life when they will have their own home)	N (%)
1: Not defined at all	11 (10.48%)
2: Slightly defined	8 (7.62%)
3: Moderately defined	36 (34.29%)
4: Mostly defined	29 (27.62%)
5: Completely defined	21 (20%)
Living abroad (planned when to go abroad for work or leisure)	N (%)
1: Not defined at all	22 (20.95%)
2: Slightly defined	14 (13.33%)
3: Moderately defined	29 (27.62%)
4: Mostly defined	24 (22.86%)
5: Completely defined	16 (15.24%)
Marriage (planned the age at which they intend to marry)	N (%)
1: Not defined at all	33 (31.43%)
2: Slightly defined	15 (14.29%)
3: Moderately defined	26 (24.76%)
4: Mostly defined	18 (17.14%)
5: Completely defined	13 (12.38%)

Moreover, on average, participants expressed a desire to have $2.23 \pm .72$ children, while they believed they could have $1.92 \pm .83$ children (Table 16). Regarding the age at which participants consider it ideal to have their first child, the mean was estimated at 28.95 ± 2.33 years old, while the mean age at which they plan to have their first child was determined to be 31.08 ± 2.76 years old (Table 16).

Table 16 Participants preferences and plans for childbearing: ideal and realistic expectations

Number of children participants would ideally like to have, and the number of children participants think they can have. Age at which participants consider ideal to have their first child, and age at which participants plan to have their first child.

Results are expressed in mean and SD.

	Total (N=105) (%)	Total Mean	Total SD
How many children would you ideally like to have?			
	105 (100%)	2.23	.72
How many children do you consider possible to have taking into account your economic situation, age, and personal background?			
	105 (100%)	1.92	.83
What age do you consider ideal for having the first child? (Age in years)			
	105 (100%)	28.95	2.33
At what age do you plan to have your first child? (Age in years)			
	105 (100%)	31.08	2.76

SD: standard deviation

2.2.4.1.9 Knowledge about reproductive health and ovarian reserve

Participant's baseline evaluation also included asking to grade their knowledge about female reproductive health and ovarian reserve (Table 17). This item was evaluated using a Likert scale, from 1: no knowledge to 5: high level of knowledge. Comparable percentages of 35.24% and 33.33% were related to grades of 2 out of 5 and 3 out of 5, respectively.

Also presented in the 1st questionnaire was a question regarding the average age at first childbirth in Portugal (Table 17). 39.05% of the participants provided the correct answer, with the mean age response standing at 30.82 years. This mean aligns closely with the actual average age of 30.8 years recorded in 2022, despite responses spanning from 25 to 39 years of age. The data is outlined in the table below (Table 17).

Table 17 Assessment on knowledge about ovarian reserve and reproductive health, and knowledge average age at first childbirth in Portugal

The data includes the count (N) and percentage (%) of participants who reported having each level of knowledge. Additionally, it provides the mean, maximum, minimum, and standard deviation (SD) for responses to the question, “What is the average age of the mother at the birth of her first child in Portugal?”. The mean of the correct answers for this question is also included.

	Total (N=105) (%)	Total Min	Total Max	Total Mean	Total SD
How do you classify your knowledge?					
1: No knowledge	8 (7.62%)				
2	37 (35.24%)				
3	35 (33.33%)				
4	21 (20%)				
5: High level of knowledge	4 (3.81%)				
What is the average age of the mother at the birth of her first child in Portugal? (years)					
Age in years	105 (100%)	25	39	30.82	2.64
What is the average age of the mother at the birth of her first child in Portugal? (years)					
Number (%) of correct answers	41 (39.05%)				

SD: standard deviation; Min: Minimum; Max: Maximum

2.2.4.2 Assessing the impact of the two interventions

The effects of both the leaflet (providing general information) and reproductive counselling (based on individuals' ovarian reserve assessment results) were evaluated. This assessment was conducted two weeks after patients completed the initial questionnaire and received the information, as well as two weeks after their medical appointment.

2.2.4.2.1 Knowledge about ovarian reserve and reproductive health

The knowledge evaluation included four different questions: two multiple choices questions about awareness of ovarian reserve testing and DOR incidence; a scale of pre-defined options to choose the age at which fertility declines; true or false category of 4 items regarding the number of available oocytes during woman lifespan, factors that contribute to ovarian reserve decreasing, and ovarian reserve evaluation. The percentage of global knowledge was calculated based on the total number of correct answers for the referred items.

The data corresponding to this analysis is summarized in the Table 18 and Figure 20. Participants demonstrated significant changes in their knowledge ($p < .001$), characterized by an increase in the percentage of correct answers, from $63.97\% \pm 17.77\%$ to $79.05\% \pm 21.81\%$ after the general informational leaflet intervention ($p < .001$). This positive trend continued at t2, with the mean

knowledge percentage increasing to 83.65%±16.82% after the communication of ovarian reserve assessment (p=.008).

Table 18 Percentage of global knowledge on ovarian reserve and DOR

Expressed in % of correct answers), at three different time-points (t0, t1, t2). p-values for the comparisons between the three time-points. Statistical significance (p-value<.05) is highlighted in blue.

% of global knowledge (expressed in % of correct answers) N=105								
t0		t1		t2				
Mean	SD	Mean	SD	Mean	SD	Time p-value d	t0 Vs t1 p-value d	t1 Vs t2 p-value d
63.97	17.77	79.05	21.81	83.65	16.82	p<.001	p<.001	p=.008

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

DOR: diminished ovarian reserve; SD: standard deviation; d: repeated measures ANOVA or Friedman Test

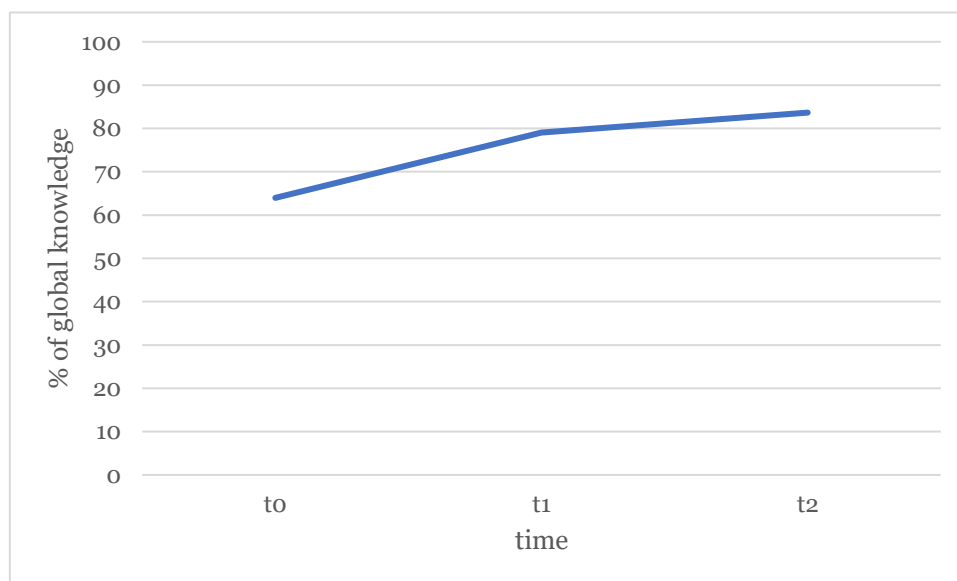


Figure 20 Graphical representation of the changes in the percentage of correct answers across the three time-points

The graph shows the mean percentage values of global knowledge at t0, t1, and t2.

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

The association between the percentage of global knowledge and the participants' age and level of education was examined (Table 19), yielding non-significant results both at t0 (p=.257), t1 (p=.826) and t2 (p=.574). We also analysed the global knowledge in the two groups of participants, who came from the family planning consultation, and the ones who came by self-proposal. No differences were found (p=.162).

Table 19 Association between percentage of global knowledge and participants' age and level of education

N=105			p-value <i>a</i>
% of global knowledge	Participant's origin 1: Family planning 2: External		p=.162
N=105			p-value <i>c</i>
% of global knowledge	Age (years)	Pearson's <i>r</i> -.072	p=.467
N=105			p-value <i>f</i>
% of global knowledge	Level of education 1: Basic education (9th grade) 2: High school diploma (12th grade) 3: Bachelor's degree 4: Integrated master's degree 5: Doctorate		p=.257 (to) p=.826 (t1) p=.574 (t2)

a: t-test for independent samples or Mann-Whitney U test; *c*: Pearson's correlation; *f*: Kruskal-Wallis test

2.2.4.2.2 Childbearing intentions

Participants did not exhibit changes in their childbearing intentions (Table 20), including the number of children they would ideally like to have (mean of $2.23 \pm .72$ at t0, $2.23 \pm .80$ at t1, and $2.19 \pm .73$ in t2, $p=.638$) and the number of children they consider possible to have (mean changed from $1.92 \pm .83$ at t0 to $1.84 \pm .79$ at t1, and to $1.78 \pm .78$ at t2, $p=.210$). It is noteworthy that the desired number of children reported by participants was significantly higher than the number they felt confident they could have, considering factors such as economic situation, age, and personal background (Table 20). This trend persisted from (p=.004) to t1 ($p<.001$), and at t2 ($p<.001$), as shown in the Table 21.

Similarly, no significant differences were observed after the two interventions in knowledge regarding the age participants consider ideal for having their first child (28.95 ± 2.33 at t0, 28.68 ± 2.51 at t1, and 28.72 ± 2.51 at t2, $p=.129$), and the age they intend to have their first child (31.09 ± 2.76 at t0, 31.30 ± 2.76 at t1, and 31.28 ± 2.80 , $p=.095$, Table 20). Nonetheless, participants consistently expressed a significantly lower mean ideal age for first childbearing compared to the age they actually plan to have their first child. This pattern persisted from t0 to both time-points, t1, and t2 ($p<.001$), as illustrated in the Table 21.

Table 20 Participants' responses regarding their intentions and plans for having children at three different time-points

Participants' responses at three different time-points (to, t1, t2) for two questions: the number of children they would ideally like to have versus the number they consider possible to have, and the age considered ideal for having the first child versus the age they plan to have the first child. The results are presented as mean and SD for the time-points to, t1, and t2. p-value for the comparison across the three time-points is reported.

		to		t1		t2		Time p-value d
About having children in future		Mean	SD	Mean	SD	Mean	SD	
How many children would you ideally like to have?	TOTAL (N=105)	2.23	.72	2.23	.79	2.19	.73	p=.638
How many children do you consider possible to have, taking into account your economic situation, age, and personal background?	TOTAL (N=105)	1.92	.83	1.84	.79	1.78	.78	p=.210
What age do you consider ideal for having the first child?	TOTAL (N=105)	28.95	2.33	28.68	2.51	28.72	2.51	p=.129
At what age do you plan to have your first child?	TOTAL (N=105)	31.08	2.76	31.30	2.76	31.28	2.80	p=.095

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

SD: standard deviation; d: repeated measures ANOVA or Friedman Test

Table 21 Comparison of ideal vs. planned childbearing age and number of children, at three time-points

Participants' responses at three different time-points (to, t1, t2) for two questions: the number of children they would ideally like to have versus the number they consider possible to have, and the age considered ideal for having the first child versus the age they plan to have the first child. The p-value from the analysis of time-related changes is also provided. Statistical significance (p-value<.05) is highlighted in blue.

		Paired Samples Test N=105		
		Mean	SD	p-value
to	Number of children they would ideally like to have			
	- Number of children they consider possible to have	.30	1.06	p=.004
t1	Number of children they would ideally like to have			
	- Number of children they consider possible to have	.39	1.03	p<.001
t2	Number of children they would ideally like to have			
	- Number of children they consider possible to have	.41	.89	p<.001
to	Age considered ideal for having the first child			
	- Age planned to have the first child	-2.13	2.59	p<.001
t1	Age considered ideal for having the first child			
	- Age planned to have the first child	-2.63	2.55	p<.001
t2	Age considered ideal for having the first child			
	- Age planned to have the first child	-2.55	2.58	p<.001

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

SD: standard deviation

Among the possible obstacles that might prevent participants from having children, such as the absence of a stable partner, continuing education, career advancement, financial instability, and not feeling ready to be a mother, infertility—particularly DOR—was frequently mentioned, as illustrated in Table 22. This item was assessed using a multiple-choice format.

Table 22 Distribution of responses regarding obstacles influencing future parenthood decisions at three different time-points

The table includes the number (N) and percentage (%) of "yes" responses for each factor, at three time-points (t0, t1, t2).

	N=105					
	t0		t1		t2	
	N (yes)	% (yes)	N (yes)	% (yes)	N (yes)	% (yes)
Infertility (DOR)	54	51.43	57	54.29	46	43.81
Absence of partner	25	23.81	25	23.81	28	26.67
Studies	11	10.48	12	11.43	15	14.29
Career	42	40	48	45.71	53	50.48
Financial	46	43.81	45	42.86	45	42.86
Not being prepared to be a mother	30	28.57	32	30.48	30	28.57

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

DOR: diminished ovarian reserve

Globally, at the first time-point of the assessment protocol (t0), 51.43% of participants considered infertility as a significant obstacle, and this perception remained relatively stable at t1, with 54.29% ($p=.648$). However, from t1 to t2, this percentage significantly decreased from 54.29% to 43.81% ($p=.007$, Table 23).

Table 23 Perception of infertility (DOR) as an obstacle to have children at the intended age over three different time-points

Count (N) and percentage (%) of participants considering this obstacle. p-value for results of the related-samples McNemar change Test, which assesses changes in perception between consecutive time-points (t0, t1, t2). Statistical significance ($p\text{-value}<.05$) is highlighted in blue.

	t0	t1	t2	Related-Samples McNemar Change Test	
	N(%)	N(%)	N(%)	t0 Vs t1 p-value	t1 Vs t2 p-value
Consider infertility (DOR) an obstacle to have children at the intended age you intend					
TOTAL (N=105)	54 (51.42%)	57 (54.29%)	46 (43.81%)	$p=.648$	$p=.007$

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

DOR: diminished ovarian reserve

2.2.4.2.3 Attitudes towards fertility treatments, oocyte freezing, oocyte donation, and alternative parenthood

Overall, our analysis revealed no statistically significant changes in attitudes towards ART ($p=.111$) or alternative paths to parenthood, such as considering egg donation ($p=.225$) or adopting a child ($p=.068$) when facing a potential DOR diagnosis. The participants preferences were assessed by Likert scale from 1: completely unlikely to 5: completely likely. When examining the acceptance levels of these options (Table 24), seeking fertility treatments has shown a higher acceptance, with respondents attributing values ranging from 3.75 ± 1.17 at t_0 , 3.93 ± 1.04 at t_1 , to $3.93\pm.99$ at t_2 , out of 5. In comparison, adoption falls within the ranges of 2.90 ± 1.14 at t_0 , 3.09 ± 1.12 at t_1 , and 2.98 ± 1.04 at t_2 ; and egg donation even lower values of 2.59 ± 1.14 at t_0 , 2.74 ± 1.14 at t_1 , and 2.63 ± 1.15 at t_2 .

Table 24 Participants' preferred attitudes towards reproductive plans in case of DOR

These options were evaluated on a scale of 1 to 5, where higher scores signify a stronger preference for the option. The mean and SD of responses are provided for three time-points: t_0 , t_1 , and t_2 . The p-value from the analysis of time-related changes is also provided. Statistical significance ($p\text{-value}<.05$) is highlighted in blue.

If you knew in advance that you would have DOR, what would be your options? Item evaluated on a scale of 1 to 5.		t ₀		t ₁		t ₂		Time p-value <i>d</i>	t ₀ Vs t ₁ p-value <i>d</i>	t ₁ Vs t ₂ p-value <i>d</i>
		Mean	SD	Mean	SD	Mean	SD			
When you want to have children, seek fertility treatments	TOTAL (N=105)	3.75	1.17	3.93	1.04	3.93	.99	$p=.111$		
Consider egg donation	TOTAL (N=105)	2.59	1.14	2.74	1.14	2.63	1.15	$p=.225$		
When you want to have children, try to adopt a child	TOTAL (N=105)	2.90	1.14	3.08	1.12	2.98	1.04	$p=.068$		
Give up on having children	TOTAL (N=105)	1.76	1.02	1.81	1.08	1.73	1.02	$p=.618$		
Anticipate the age of having a child	TOTAL (N=105)	3.58	1.31	3.68	1.25	3.65	1.22	$p=.532$		
Freeze your eggs to use in the future, when you want to have children	TOTAL (N=105)	4.09	1.03	3.93	1.11	3.76	1.17	$p=.002$	$p=.046$	$p=.024$

t_0 : baseline evaluation; t_1 : after receiving general information; t_2 : after receiving tailored information

DOR: diminished ovarian reserve; SD: standard deviation; *d*: repeated measures ANOVA

A preference for ART is evidenced, when comparing with adoption ($p<.001$) and egg donation ($p<.001$) within our sample population (Table 25).

Table 25 Comparison of the mean differences between seeking fertility treatments and two other reproductive options (oocyte donation and adoption) at three different time-points

Results expressed in mean values and SD. The p-values indicate the statistical significance of the mean differences. Statistical significance (p-value<.05) is highlighted in blue.

		Paired Samples Test N=105		
		Mean	SD	p-value
to	When you want to have children, seek fertility treatments - Consider egg donation	1.16	1.68	p<.001
t1	When you want to have children, seek fertility treatments - Consider egg donation	1.19	1.38	p<.001
t2	When you want to have children, seek fertility treatments - Consider egg donation	1.30	1.41	p<.001
to	When you want to have children, seek fertility treatments - When you want to have children, try to adopt a child	.85	1.55	p<.001
t1	When you want to have children, seek fertility treatments - When you want to have children, try to adopt a child	.848	1.518	p<.001
t2	When you want to have children, seek fertility treatments - When you want to have children, try to adopt a child	.95	1.41	p<.001

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

SD: standard deviation

Additionally, if confronted with a diagnosis of DOR, the decision to give up on parenthood (Table 24) remained low and consistent throughout the intervention period (p=.618) with participants consistently assigning low mean values ranging from 1.76±1.02 at to, 1.82±1.08 at t1, and 1.73±1.02 at t2. Despite the interventions, participants did not change their plans regarding the age at which they intend to become mothers, even when presented with a hypothetical scenario involving DOR (Table 24). Participants did not demonstrate a desire to have children earlier in life after receiving both general and tailored information about ovarian reserve (p=.532; mean values of 3.58±1.31 at to; 3.68±1.25 at t1; and 3.65±1.22 at t2;).

Throughout the study, the only significant change observed was in the preference for oocyte cryopreservation (Table 24), with a marked decrease in the consideration of on social freezing as an option (p=.002). Specifically, following the initial assessment (to) and the provision of general informational leaflets (t1), the mean value decreased from 4.10±1.03 to 3.93±1.11 (p=.046). This trend continued between the post-general information stage (t1) and the phase after tailored information and reproductive counselling (t2), where the mean decreased further from 3.93±1.11 to 3.76±1.17 (p=.024), as evidenced in Table 24, and in Figure 21.

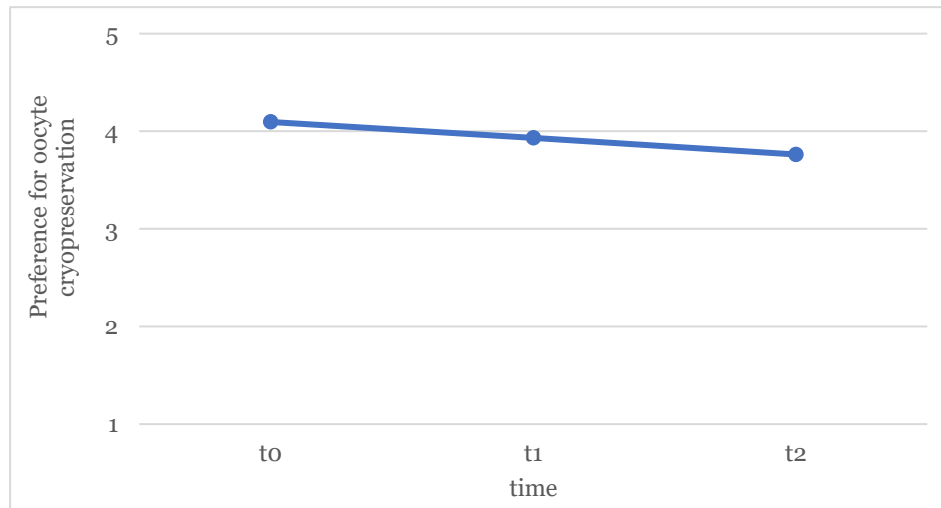


Figure 21 Graphical representation of how preferences for oocyte cryopreservation vary at three time-points

Preference for oocyte cryopreservation was evaluated on a scale of 1 to 5, where higher scores signify a stronger preference for the option. Mean values for t0, t1, and t2 are represented.

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

The attitude of participants regarding undergoing egg freezing if they knew in advance they would have DOR, was reassessed in another point of the questionnaire, using a multiple-choice format (Table 26). At the initial assessment (t0), 47 participants (44.76%) indicated that they would be very likely to undergo fertility treatments for egg freezing. Meanwhile, 20 participants (19.05%) expressed interest but cited lack of necessary economic resources as a barrier. 10 participants (9.52%) stated that they would not opt for egg freezing and instead preferred to reconsider the age at which they planned to become pregnant. In comparison, at t1, the percentages corresponded to those of t0, with minor fluctuations noted across the categories (40% expressing certainty, 22.86% desiring but facing financial constraints, and 8.57% opting to anticipate the childbearing age). At t2, similar results were found, with 45.71% expressing certainty in their preference, 21.9% desiring but facing financial limitations, and 11.43% expressing intention to become mothers earlier in life). No significant differences over time were noted ($p=.261$).

Table 26 Data on participants' willingness to undergo fertility treatments to "freeze" their oocytes if they knew in advance about having DOR

The table includes the number of respondents (N), their respective percentages (%), for each category, and p-value for comparison over time.

If you knew in advance that you would have DOR, would you consider undergoing fertility treatments to "freeze" eggs?	to		t1		t2		Time p-value d
	TOTAL (N=105) N	TOTAL (N=105) %	TOTAL (N=105) N	TOTAL (N=105) %	TOTAL (N=105) N	TOTAL (N=105) %	
Yes, very likely	47	44.76	42	40	48	45.71	p=.261
Yes, but I do not have the necessary economic resources	20	19.05	24	22.86	23	21.90	
No, although I am informed about the effect of a woman's age on her fertility	2	1.9	3	2.86	3	2.86	
Maybe, but I am not sufficiently informed on the subject	26	24.76	27	25.71	19	18.1	
No, I would prefer to reconsider the age at which I want to get pregnant	10	9.52	9	8.57	12	11.43	

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

DOR: diminished ovarian reserve; d: Friedman Test

Participants were asked about the accessibility of egg freezing for all women through a multiple-choice question with options: 1: yes for all, 2: no for nobody, 3: I'm not informed, and 4: yes, for specific cases (Table 27). These options were coded accordingly, so the mean values reflect their opinions: a mean close to 1 indicates agreement with universal accessibility, close to 2 indicates a preference for no accessibility, close to 3 indicates lack of information, and close to 4 indicates support for accessibility in specific cases. A significant influence of time in the opinion was observed ($p=.018$), particularly from to to t1 ($p=.031$), suggesting a decrease from a mean of 1.94 ± 1.22 to 1.68 ± 1.07 (Figure 22). This decrease was further investigated by t-test paired analysis which revealed that the change in opinion was not significant after providing additional information (to Vs t1 $p=.634$), nor after the reproductive counselling (t1 Vs t2, $p=.231$).

Table 27 Participants' opinions regarding whether fertility preservation for social reasons should be available to all women

The item was evaluated on a multiple-choice at three time-points: to, t1, and t2. Item evaluated on a multiple-choice question with options: 1: yes for all, 2: no for nobody, 3: I'm not informed, and 4: yes, for specific cases. The table includes the mean and standard deviation (SD) of responses for each time-point, along with p-values to assess changes over time. Statistical significance ($p\text{-value} < .05$) is highlighted in blue.

Do you think that fertility preservation for social reasons ('egg freezing') should be available to all women?	to		t1		t2		Time p-value d	to Vs t1 p-value d	t1 Vs t2 p-value d
	Mean	SD	Mean	SD	Mean	SD			
TOTAL (N=105)	1.94	1.21	1.68	1.07	1.62	1.09	p=.018	p=.031	p=.539

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

SD: standard deviation; d: ANOVA repeated measures and Friedman Test

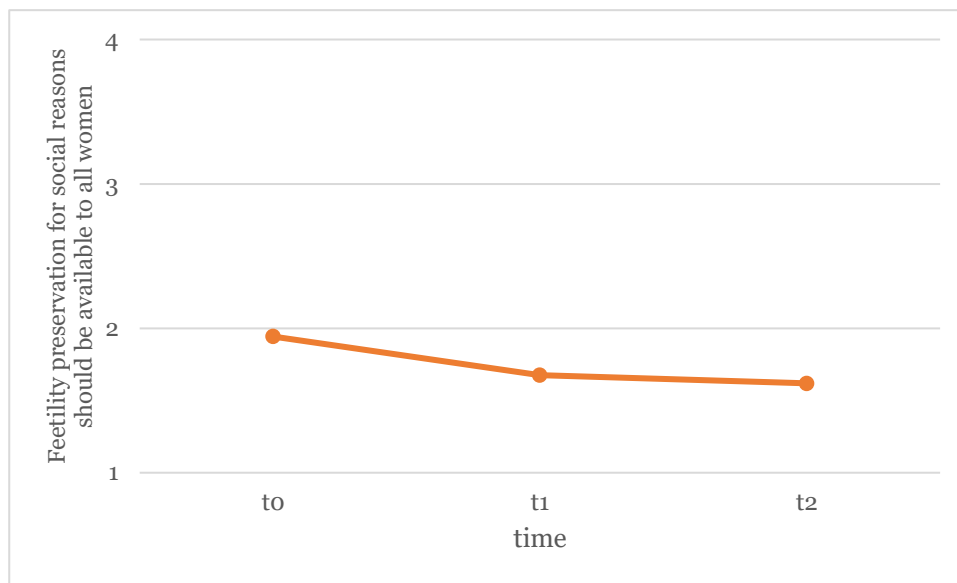


Figure 22 Graph of opinions on fertility preservation for social reasons being available for all women, over study time

The mean of responses for each time-point (t0, t1, and t2) is represented. Item evaluated on a multiple-choice question with options: 1: yes for all, 2: no for nobody, 3: I'm not informed, and 4: yes, for specific cases.

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

Alterations were not observed in attitudes following being exposed to hypothetical DOR diagnosis, particularly concerning feelings about using donated oocytes (Table 28). This assessment was conducted using a Likert scale, ranging from 1: not true at all, to 5: totally true. Specifically, participants' feelings about fulfilling their wish of becoming a mother remained relatively stable over time (t0: 3.48 ± 1.25 , t1: 3.60 ± 1.14 , t2: 3.49 ± 1.14 , $p = .301$). Similarly, attitudes towards caring for a child from birth showed consistency across assessments (t0: 3.82 ± 1.17 , t1: 3.90 ± 1.12 , t2: 3.88 ± 1.09 , $p = .490$). Additionally, sentiments about passing on family values demonstrated no significant variation (t0: 3.96 ± 1.15 , t1: 4 ± 1.03 , t2: 3.84 ± 1.18 , $p = .200$). Concerns regarding possible hereditary health problems also remained unchanged over time (t0: 3.27 ± 1.28 , t1: 3.30 ± 1.29 , t2: 3.22 ± 1.23 , $p = .607$). Within this topic, the only difference found was about feeling apprehensive about potentially feeling that the child is not biologically hers, from t0 to t1, with values of 2.72 and 2.94 respectively out of 5 ($p = .027$).

Table 28 Data on participants' feelings regarding the possibility of using donated gametes

The item was evaluated on a scale of 1 to 5 at three different time-points: t0, t1, and t2. The options were evaluated on a scale of 1 to 5, with higher scores indicating a stronger agreement with the option. For each time-point, the table includes the mean and SD of responses. The p-value from the analysis of time-related changes is provided. Statistical significance (p-value<.05) is highlighted in blue.

		t0		t1		t2		Time p-value d	t0 Vs t1 p-value d	t1 Vs t2 p-value d
How would you feel about the possibility of having to use donated gametes? Item evaluated on a scale of 1 to 5.		Mean	SD	Mean	SD	Mean	SD			
Happy to fulfill my dream of becoming a mother	TOTAL (N=105)	3.48	1.25	3.60	1.14	3.49	1.14	p=.301		
Happy to care for a child from birth	TOTAL (N=105)	3.82	1.17	3.90	1.12	3.88	1.09	p=.490		
Happy to be able to pass on my family values	TOTAL (N=105)	3.96	1.15	4.00	1.023	3.84	1.18	p=.200		
Apprehensive about potentially feeling that the child is not really mine	TOTAL (N=105)	2.72	1.36	2.94	1.34	2.85	1.26	p=.027	p=.015	p=.123
Concerned about possible hereditary health problems	TOTAL (N=105)	3.27	1.28	3.30	1.29	3.22	1.23	p=.607		

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

SD: standard deviation; d: ANOVA repeated measures and Friedman Test

In addition to analysing participants' views regarding the use of donated eggs, we also sought to understand if they altered their perspective about being a donor, recognizing its potential to assist women with DOR (Table 29). This item was evaluated via multiple-choice format. Participants revealed significant reluctance to act as donors, with the majority indicating uncertainty and expressing the need for more information to make a decision—48.57% at t0 and t1, and 42.86% at t2. No significant differences were found over time (p=.464).

Table 29 Analysis of participants' motivation to consider voluntary donation of gametes (oocytes) to help women with DOR

The responses were collected at three time-points: t0, t1, and t2. For each time-point, the table includes the total number of respondents (N), the percentage of respondents, and the mean and SD of responses. The p-value from the analysis of time-related changes is also provided.

Would you consider doing a voluntary donation of gametes ("eggs") if such an act helped women with DOR?	t0				t1				t2				Time p-value d
	TOTAL (N=105) N	TOTAL (N=105) %	Mean	SD	TOTAL (N=105) N	TOTAL (N=105) %	Mean	SD	TOTAL (N=105) N	TOTAL (N=105) %	Mean	SD	
No	19	18.1	1.30	.76	20	19.05	1.31	.79	24	22.86	1.25	.87	p=.464
Yes	35	33.33			33	31.43			36	34.29			
Need more information	51	48.57			51	48.57			45	42.86			

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

DOR: diminished ovarian reserve; SD: standard deviation; d: Friedman test

Opinions regarding the accessibility of an affordable option to evaluate ovarian reserve (Table 30), evaluated through a multiple-choice format, also showed stability over time ($p=.334$), with most of the participants supporting this possibility, 102 out of 105 that represents 97.14% agreed on this.

Table 30 Participants' opinions regarding whether all women should have access to an easy and inexpensive way to evaluate ovarian reserve

Responses were collected at three time-points: t0, t1, and t2. For each time-point, the table includes the total number of respondents (N), the percentage of respondents, and the mean and SD of responses. Additionally, the p-value from the analysis of time-related changes is provided.

Do you think all women should have access to an easy and inexpensive way to evaluate ovarian reserve?	t0				t1				t2				Time p-value ^d
	TOTAL (N=105) N	TOTAL (N=105) %	Mean	SD	TOTAL (N=105) N	TOTAL (N=105) %	Mean	SD	TOTAL (N=105) N	TOTAL (N=105) %	Mean	SD	
No	0	NA	1.03	.17	3	2.86	1	.24	0	NA	1.04	.19	p=.334
Yes	102	97.14			99	94.29			101	96.19			
Need more information	3	2.86			3	2.86			4	3.81			

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

SD: standard deviation; NA: not applicable; ^d: Friedman test

2.2.4.3 Evaluating knowledge, attitudes, and reproductive plans, according to the information received about the ovarian reserve testing

We firstly presented the general data from all participants, highlighting results in knowledge, childbearing intentions, and reproductive plans from t0 to t2, from the first to third time-points of the assessment protocol. From t0 to t1, participants were provided information about reproductive health, ovarian reserve and DOR, through a general informational leaflet – the first intervention in knowledge. At this stage, no specific information about individual ovarian reserve was available, allowing us to measure the impact of providing general information. After t1, participants received tailored information based on their ovarian reserve evaluation - the second intervention. Differences in responses from t1 to t2 reflect the impact of receiving tailored information, about the ovarian reserve status.

Beyond examining changes in knowledge and attitudes over time, we also aimed to explore how different types of information influenced participants knowledge, childbearing intentions, and reproductive plans. Specifically, we were interested in whether receiving reassuring information, about having a normal ovarian reserve (NOR), versus concerning information, having a DOR, affected the referred outcomes differently. To facilitate this analysis, we divided our participants into two subgroups based on their ovarian reserve assessment: the normal reserve subgroup (NOR), consisting of participants who were informed that their ovarian reserve was within a normal range, defined by having both AMH and AFC above the 25th percentile for their age (46 participants); and the DOR1, consisting of participants who were informed that their ovarian reserve was below the expected range, with at least one of either AMH or AFC below the 25th percentile for their age (59 participants).

While analysing the data, we identified two distinct subgroups within the DOR1 subgroup. The first subgroup, consisting of 8 participants, had both AMH and AFC values below the 25th percentile for their age, indicating “a true DOR” (DOR 2). The remaining 51 participants initially included in the DOR1 subgroup had discordant AMH and AFC values, with AMH being the marker below the 25th percentile in all cases. 84.3% of these participants were using hormonal contraception, which could explain the discrepancy in their values.

Therefore, analysis included the distinctions observed between two main datasets (as illustrated in Figure 23) One dataset included two subgroups: those who received information about a normal ovarian reserve (NOR) and those informed about a DOR (DOR 1). The other dataset involved the division into three subgroups: the normal reserve subgroup (NOR), the “true” DOR subgroup (DOR 2), and the subgroup with inconsistencies in ovarian reserve evaluation, which was considered an intermediate subgroup with values neither typical of DOR nor NOR (INT). Recognizing the potential loss of statistical power when analysing small subgroups, particularly the DOR2 subgroup (N=8), we proceeded with its analysis, advocating the clinical value of distinguishing this subgroup.

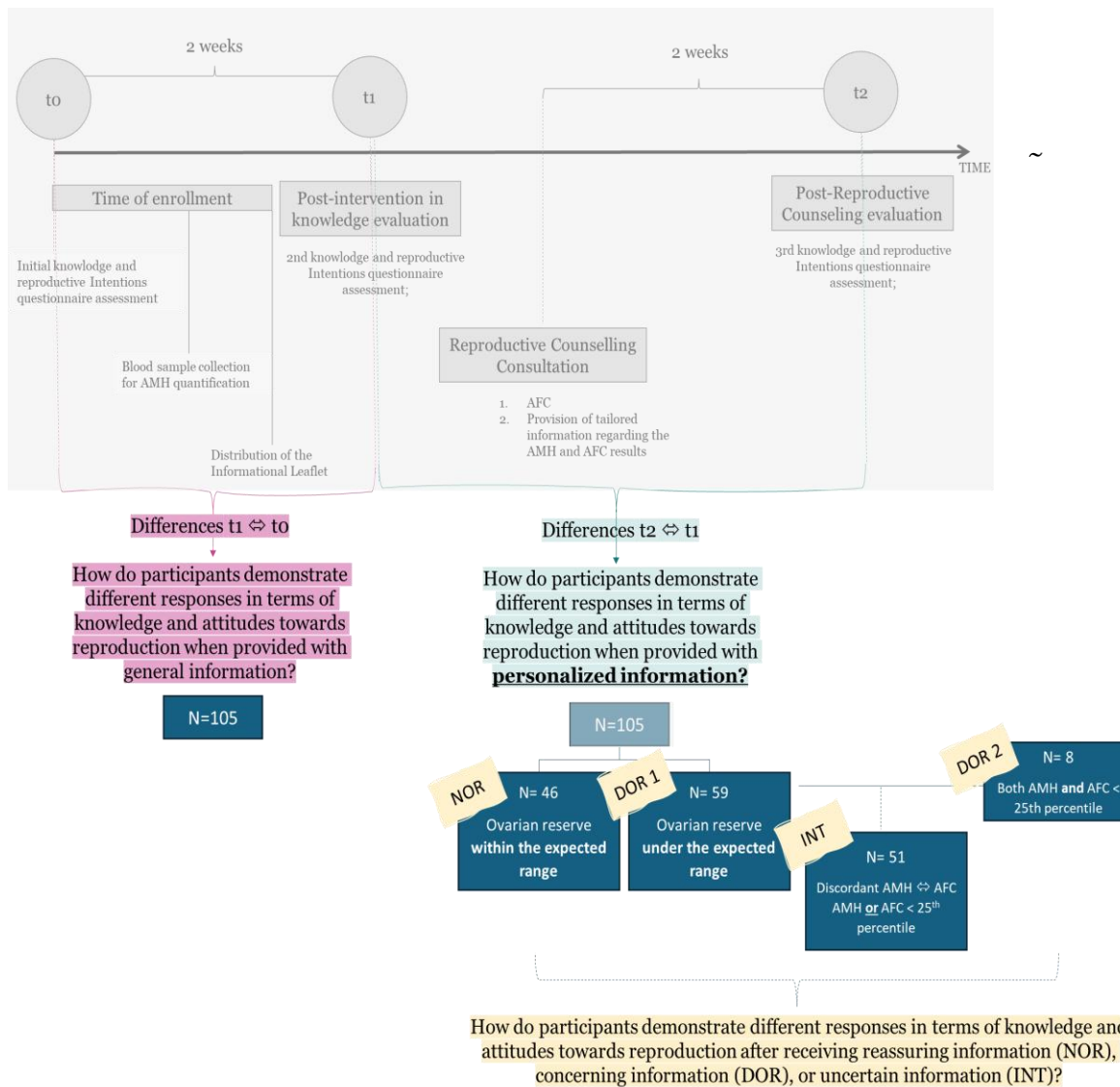


Figure 23 Diagram of the study design, extending from the initial phase to incorporate additional analysis

The second part of the study emphasizes the distinctions between participants' ovarian reserve status and the information they received. As part of this analysis, new subgroups were formed, including NOR, DOR1, DOR2, and INT.

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

AMH: Anti-Müllerian hormone; AFC: antral follicle count

2.2.4.3.1 Subgroups baseline comparison

Before proceeding further with this analysis, baseline characteristics of the subgroups compared.

The subgroups were found to be homogeneous as no differences were observed in the means across the NOR and DOR1 subgroups, as well as for NOR, DOR2 and INT considering age, ethnicity, education, employment status, relationship status, smoking, practice of physical exercise, dysmenorrhea, and ovarian surgery (Table 31).

Table 31 Detailed analysis of various subgroups categorized by ovarian reserve status

For each subgroup, the table includes the number and percentage of participants (N%), mean, and SD. Comparative p-values are presented for DOR1 vs NOR, DOR2 vs NOR, DOR2 vs INT, and NOR vs INT.

	DOR1 (N=59) N (%)	NOR (N=46) N (%)	DOR1 Vs NOR p-value <i>b</i>	DOR2 (N=8) N (%)	INT (N=51) N (%)	NOR (N=46) N (%)	DOR2 Vs NOR Vs INT p-value <i>b</i>
Ethnicity							
1: Asian	0	0	p=.547	0	0	0	p=.737
2: African	3 (5.08%)	2 (4.35%)		0	3 (5.88%)	2 (4.35%)	
3: Hispanic	2 (3.39%)	3 (6.52%)		0	2 (3.92%)	3 (6.52%)	
4: Caucasian (White)	52 (88.14%)	41 (89.13%)		8 (100%)	44 (86.27%)	41 (89.13%)	
5: Multiple ethnicities	2 (3.39%)	0		0	2 (3.92%)	0	
Education							
1: Basic education (9th grade)	1 (1.69%)	0	p=.358	0	1 (1.96%)	0	p=.065
2: High school diploma (12th grade)	7 (11.86%)	5 (10.87%)		1 (12.5%)	6 (11.76%)	5 (10.87%)	
3: Bachelor's degree	14 (23.73%)	16 (34.78%)		3 (37.5%)	11 (21.57%)	16 (34.78%)	
4: Integrated master's degree	36 (61.02%)	22 (47.83%)		4 (50%)	32 (62.75%)	22 (47.83%)	
5: Doctorate	6 (10.17%)	3 (6.52%)		0	1 (1.96%)	3 (6.52%)	
Employment status							
1: Employed	35 (59.32%)	26 (56.52%)	p=.780	5 (62.5%)	30 (58.82%)	26 (56.52%)	p=.870
2: Unemployed	4 (6.78%)	2 (4.35%)		1 (12.5%)	3 (5.88%)	2 (4.35%)	
3: Student	20 (33.9%)	18 (39.13%)		2 (25%)	18 (35.29%)	18 (39.13%)	
Relationship status							
Single	51 (86.44%)	38 (82.61%)	p=.588	7 (87.5%)	44 (86.27%)	38 (82.61%)	p=.860
Married/common-law	8 (13.56%)	8 (17.39%)		1 (12.5%)	7 (13.73%)	8 (17.39%)	
In a relationship							
No	51 (86.44%)	10 (21.74%)	p=.234	3 (37.5%)	16 (31.37%)	10 (21.74%)	p=0.462
Yes	8 (13.56%)	36 (78.26%)		5 (62.5%)	35 (68.63%)	36 (78.26%)	
Smoker							
No	50 (84.75%)	39 (84.78%)	p=.996	6 (75%)	44 (86.27%)	39 (84.78%)	p=.712
Yes	9 (15.25%)	7 (15.22%)		2 (25%)	7 (13.73%)	7 (15.22%)	
Physical exercise							
0: No	12 (20.34%)	9 (19.57%)	p=.781	1 (12.5%)	11 (21.57%)	9 (19.57%)	p=.701
1: 1-3 times/week	37 (62.71%)	28 (60.87%)		7 (87.5%)	30 (58.82%)	28 (60.87%)	
2: 4-6 times/week	9 (15.25%)	9 (19.57%)		0	9 (17.65%)	9 (19.57%)	
3: Competition	1 (1.69%)	0		0	1 (1.96%)	0	
Dysmenorrhea							
0: no pain	1 (1.69%)	1 (2.17%)	p=.359	0	1 (1.96%)	1 (2.17%)	p=.614
1	11 (18.64%)	6 (13.04%)		1 (12.5%)	10 (19.61%)	6 (13.04%)	
2	20 (33.9%)	17 (36.96%)		4 (50%)	16 (31.37%)	17 (36.96%)	
3	13 (22.03%)	17 (36.96%)		1 (12.5%)	12 (23.53%)	17 (36.96%)	
4	11 (18.64%)	3 (6.52%)		1 (12.5%)	10 (19.61%)	3 (6.52%)	
5:very intense painful periods	3 (5.08%)	2 (4.35%)		1 (12.5%)	2 (3.92%)	2 (4.35%)	
Had ovarian surgery							
No	57 (96.61%)	45 (97.83%)	p=.711	8 (100%)	49 (96.08%)	45 (97.83%)	p=.771
Yes	2 (3.39%)	1 (2.17%)		0	2 (3.92%)	1 (2.17%)	

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; *b*: Chi-square test and Fisher's exact

The results on BMI and age at first menstruation are presented separately in the Table 32 to facilitate easier reading of the data. No differences were observed in the means across the DOR1, and NOR subgroups ($p=.565$), as well as for DOR2 and NOR ($p=.568$), DOR2 and INT ($p=.595$) and NOR and INT ($p=.656$) considering age at first menstruation. There was no difference in the mean BMI across DOR1 and NOR ($p=.350$), as all as between DOR2 and NOR ($p=.228$) and NOR and INT ($p=.088$), but a significant difference was found when comparing DOR and INT subgroups ($p=.033$).

Table 32 Results on BMI and age at first menstruation for ovarian reserve subgroups

Results are expressed in mean and SD. p-values for comparisons between subgroups (DOR1, NOR, DOR2, INT) are reported. Statistical significance ($p\text{-value} < .05$) is highlighted in blue.

	DOR1 (N=59) Mean ±SD	DOR2 (N=8) Mean ±SD	INT (N=51) Mean ±SD	NOR (N=46) Mean ±SD	DOR1 Vs NOR p-value <i>a</i>	DOR2 Vs NOR p-value <i>a</i>	DOR2 Vs INT p-value <i>a</i>	NOR Vs INT p-value <i>a</i>
Age at First Menstruation in years	12.31 ± 1.10	12.50 ± 1.41	12.27 ± 1.06	12.15 ± 1.60	$p=.565$	$p=.568$	$p=.595$	$p=.656$
BMI (kg/m²)	23.99 ± 4.47	28.32 ± 7.56	23.31 ± 3.42	24.81 ± 4.45	$p=.350$	$p=.228$	$p=.033$	$p=.088$

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; BMI: body mass index; *a*: t-test for independent samples or Mann-Whitney U test

Regarding the childbearing intentions, no differences were found in the importance giving to have children in future, assessed using a Likert scale ranging from 1: not at all important to 5: extremely important, nor the certainty of having children, evaluated in a multiple-choice format (Table 33).

Table 33 The data on the significance of parenthood and participants' confidence in their decision to have children ovarian reserve subgroups

Table includes the representation of number and percentage of participants (N%), alongside mean and SD. p-values are provided to assess statistical differences between subgroups (DOR1, NOR, DOR2, INT).

	DOR1 (N=59) N (%)	NOR (N=46) N (%)	DOR1 Vs NOR p-value <i>b</i>	DOR2 (N=8) N (%)	INT (N=51) N (%)	NOR (N=46) N (%)	DOR2 Vs NOR Vs INT p-value <i>b</i>
Importance of having children in future							
1: Not at all important	1 (1.69%)	2 (4.35%)	p=.855	0	1 (1.96%)	2 (4.35%)	p=.822
2: Slightly important	5 (8.47%)	2 (4.35%)		1 (12.5%)	4 (7.84%)	2 (4.35%)	
3: Moderately important	8 (13.56%)	6 (13.04%)		2 (25%)	6 (11.76%)	6 (13.04%)	
4: Very important	23 (38.98%)	19 (41.3%)		4 (50%)	19 (37.25%)	19 (41.3%)	
5: Extremely important	22 (37.29%)	17 (36.96%)		1 (12.5%)	21 (41.18%)	17 (36.96%)	
Certainty of having children in the future							
I am certain that I want to have children	34 (57.63%)	27 (58.7%)	p=361	3 (37.5%)	31 (60.78%)	27 (58.7%)	p=.470
I still have doubts. but most likely I will decide to have children	16 (27.12%)	15 (32.61%)		4 (50%)	12 (23.53%)	15 (32.61%)	
At the moment. I am certain that I do not want to have children	1 (1.69%)	2 (4.35%)		0	1 (1.96%)	2 (4.35%)	
I still have doubts. but most likely I will decide not to have children	8 (13.56%)	2 (4.35%)		1 (12.5%)	7 (13.73%)	2 (4.35%)	

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; *b*: Chi-square test or Fisher's exact test

The prioritized plans in life were similar across the subgroups formed (Table 34).

Table 34 The data on prioritized life goals are detailed across ovarian reserve subgroups.

This item was assessed using a Likert scale ranging from 1: not defined at all to 5: completely defined. Table includes the representation of number and percentage of participants (N) in each category of the Likert scale, divided in each subgroup (DOR1, NOR, DOR2, INT). p-values are provided to assess statistical differences between the subgroups.

How defined are your plans?	DOR1 (N=59) N (%)	NOR (N=46) N (%)	DOR1 Vs NOR p-value <i>b</i>	DOR2 (N=8) N (%)	INT (N=51) N (%)	NOR (N=46) N (%)	DOR2 Vs NOR Vs INT p-value <i>b</i>
Career (planned their professional life)							
1: Not defined at all	2 (3.39%)	0	p=.669	1 (12.5%)	1 (1.96%)	0	p=.477
2: Slightly defined	2 (3.39%)	1 (2.17%)		0	2 (3.92%)	1 (2.17%)	
3: Moderately defined	16 (27.12%)	10 (21.74%)		3 (37.5%)	13 (25.49%)	10 (21.74%)	
4: Mostly defined	27 (45.76%)	24 (52.17%)		3 (37.5%)	24 (47.06%)	24 (52.17%)	
5: Completely defined	12 (20.34%)	11 (23.91%)		1 (12.5%)	11 (21.57%)	11 (23.91%)	
Education (planned their academic formation)							
1: Not defined at all	2 (3.39%)	0	p=.264	1 (12.5%)	1 (1.96%)	0	p=.151
2: Slightly defined	0	1 (2.17%)		0	0	1 (2.17%)	
3: Moderately defined	7 (11.86%)	2 (4.35%)		0	7 (13.73%)	2 (4.35%)	
4: Mostly defined	16 (27.12%)	11 (23.91%)		1 (12.5%)	15 (29.41%)	11 (23.91%)	
5: Completely defined	34 (57.63%)	32 (69.57%)		6 (75%)	28 (54.90%)	32 (69.57%)	
Parenthood (planned the moment they want to have children)							
1: Not defined at all	15 (25.42%)	8 (17.39%)	p=.109	4 (50%)	11 (21.57%)	8 (17.39%)	p=.188
2: Slightly defined	9 (15.25%)	6 (13.04%)		1 (12.5%)	8 (15.69%)	6 (13.04%)	
3: Moderately defined	18 (30.51%)	12 (26.09%)		1 (12.5%)	17(33.33%)	12 (26.09%)	
4: Mostly defined	17 (28.81%)	15 (32.61%)		2 (25%)	15 (29.41%)	15 (32.61%)	
5: Completely defined	0	5 (10.87%)		0	0	5 (10.87%)	
Buying/renting a house (planned the stage of life when they will have their own home)							
1: Not defined at all	8 (13.56%)	3 (6.52%)	p=.634	1 (12.5%)	7 (13.73%)	3 (6.52%)	p=.887
2: Slightly defined	4 (6.78%)	4 (8.7%)		0	4 (7.84%)	4 (8.7%)	
3: Moderately defined	22 (37.29%)	14 (30.43%)		4 (50%)	18 (35.29%)	14 (30.43%)	
4: Mostly defined	15 (25.42%)	14 (30.43%)		2 (25%)	13 (25.49%)	14 (30.43%)	
5: Completely defined	10 (16.95%)	11 (23.91%)		1 (12.5%)	9 (17.65%)	11 (23.91%)	
Living abroad (planned when to go abroad for work or leisure)							
1: Not defined at all	17 (28.81%)	5 (10.87%)	p=.161	2 (25%)	15 (29.41%)	5 (10.87%)	p=.542
2: Slightly defined	7 (11.86%)	7 (15.22%)		1 (12.5%)	6 (11.76%)	7 (15.22%)	
3: Moderately defined	17 (28.81%)	12 (26.09%)		2 (25%)	15 (29.41%)	12 (26.09%)	
4: Mostly defined	10 (16.95%)	14 (30.43%)		2 (25%)	8 (15.69%)	14 (30.43%)	
5: Completely defined	8 (13.56%)	8 (17.39%)		1 (12.5%)	7 (13.73%)	8 (17.39%)	
Marriage (planned the age at which they intend to marry)							
1: Not defined at all	23 (38.98%)	10 (21.74%)	P=.107	4 (50%)	19 (37.25%)	10 (21.74%)	P=.249
2: Slightly defined	6 (10.17%)	9 (19.57%)		1 (12.5%)	5 (9.8%)	9 (19.57%)	
3: Moderately defined	17 (28.81%)	9 (19.57%)		1 (12.5%)	16 (31.37%)	9 (19.57%)	
4: Mostly defined	8 (13.56%)	10 (21.74%)		2 (25%)	6 (11.76%)	10 (21.74%)	
5: Completely defined	5 (8.47%)	8 (17.39%)		0	5 (9.8%)	8 (17.39%)	

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; *b*: Chi-square test or Fisher's exact test

The number of children participants ideally would like to have, the number of children they consider they can have, as well as the ideal age and the planned childbearing age, did not show differences across the subgroups DOR1, NOR, DOR2, and INT (Table 35).

Table 35 Participants' ideal number of children, perceived capacity to have children, ideal age for childbearing, and planned age for starting a family across ovarian reserve subgroups

Results are expressed in mean and SD. p-values are included to assess statistical differences between subgroups (DOR1, NOR, DOR2, INT).

	DOR1 (N=59) Mean ±SD	NOR (N=59) Mean ±SD	DOR1 Vs NOR p-value <i>a</i>	DOR2 (N=8) Mean ± SD	INT (N=51) Mean ± SD	NOR (N=46) Mean ± SD	DOR2 Vs NOR p-value <i>a</i>	DOR2 Vs INT p-value <i>a</i>	NOR Vs INT p-value <i>a</i>
How many children would you ideally like to have?	2.37 ± .81	2.04 ± .56	p=.120	2.37 ± .91	2.37 ± .80	2.04 ± .56	p=.167	p=.994	p=.122
How many children do you consider possible to have, taking into account your economic situation, age, and personal background?	1.98 ± .92	1.84 ± .70	p=.402	1.50 ± .92	2.05 ± .90	1.85 ± .70	p=.221	p=.110	p=.205
What age (in years) do you consider ideal for having the first child?	28.93 ± 2.38	28.98 ± 2.28	p=.920	29.25 ± 2.05	28.88 ± 2.44	28.98 ± 2.28	p=.754	p=.688	p=.842
At what age (in years) do you plan to have your first child?	31.25 ± 2.77	30.87 ± 2.76	p=.482	31 ± 3.02	31.29 ± 2.77	30.87 ± 2.76	p=.904	p=.783	p=.452

NOR: normal ovarian reserve, AMH and AFC ≥ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; *a*: t-test for independent samples or Mann-Whitney U test

The subgroups DOR1, NOR, DOR2 and INT were formed according to AMH and AFC values. Consequently, differences in mean values of AMH and AFC were found, with significantly higher values in the NOR subgroup compared to DOR1, DOR2, and INT subgroups (p<.001 for both AMH and AFC, for both datasets, Table 36 and Table 37). Additionally, DOR2 and INT subgroups also differed in terms of AMH (p=.023, Table 37) and AFC (p<.001, Table 37), with higher mean values attributed to the NOR subgroup.

Table 36 Comparison of AMH, and AFC values across ovarian reserve subgroups (DOR1 and NOR)

The table reveals differences in age, AMH levels, and AFC between participants with DOR1 and NOR. The results are expressed in min, max, mean and SD. Statistical significance is indicated by the p-values, guiding the interpretation of these differences. Statistical significance (p-value<.05) is highlighted in blue.

	DOR1 (N=59) Min	DOR1 (N=59) Max	DOR1 (N=59) Mean ±SD	NOR (N=46) Min	NOR (N=46) Max	NOR (N=46) Mean ±SD	DOR1 Vs NOR p-value <i>a</i>
Age (years)	20	34	27.49 ± 3.84	20	33	27.15 ± 3.46	p=.356
AMH (ng/mL)	.12	2.81	1.53 ± .64	2.46	11.56	5.08 ± 2.16	p<.001
AFC	2	34	14.81 ± 7.21	9	53	26.33 ± 11	p<.001

NOR: normal ovarian reserve, AMH and AFC ≥ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; Min: Minimum; Max: Maximum SD: standard deviation; *a*: t-test for independent samples

Table 37 Comparison of AMH, and AFC values across ovarian reserve subgroups (DOR2, INT and NOR)

The table reveals differences in age, AMH levels, and AFC between participants with DOR2, INT and NOR. The results are expressed in min, max, mean and SD. Statistical significance is indicated by the p-values, guiding the interpretation of these differences. Statistical significance (p-value<.05) is highlighted in blue.

	DOR2 (N=8) Min	DOR2 (N=8) Max	DOR2 (N=8) Mean ±SD	INT (N=51) Min	INT (N=51) Max	INT (N=51) Mean ±SD	NOR (N=46) Min	NOR (N=46) Max	NOR (N=46) Mean ±SD	DOR2 Vs NOR p-value <i>a</i>	DOR2 Vs INT p-value <i>a</i>	NOR Vs INT p-value <i>a</i>
Age in years	21	31	26.63 ±3.07	20	34	27.63 ± 3.95	20	33	27.15 ± 3.46	p=.398	p=.156	p=.229
AMH (ng/mL)	.12	2	1.05 ± .76	.56	2.81	1.60 ± .60	2.46	11.56	5.08 ± 2.16	p<.001	p=.023	p<.001
AFC	2	7	5.63 ± 1.68	7	34	16.25 ± 6.66	9	53	26.33 ± 11	p<.001	p<.001	p<.001

NOR: normal ovarian reserve, AMH and AFC ≥ 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

AMH: Anti-Mullerian hormone; AFC: antral follicle count; Min: Minimum; Max: Maximum; SD: standard deviation; *a*: t-test for independent samples or Mann-Whitney U test

Further subtle differences were identified. For instance, the age at which the mother entered menopause differed among the subgroups (p=.012 when comparing DOR1 and NOR, and p=.011 regarding DOR2, INT, and NOR). However, the majority of participants, across all subgroups, still reported that their mothers entered menopause at over 45 years old, as reported in Table 38.

Table 38 Comparison of the age at which their mother's entered menopause across ovarian reserve subgroups

This item was evaluated by multiple-choice (0: has not entered, 1: <40 years old; 2: 40-45 years old; 3: > 45 years old). The results are expressed in both numbers and percentages for each category. p-values indicate the statistical significance level of the differences observed between the respective subgroups (DOR1, NOR, DOR2, INT) for the age at which the mother entered menopause. Statistical significance (p-value<.05) is highlighted in blue.

Age at which the mother entered menopause in years	DOR1 (N=59) N (%)	NOR (N=46) N (%)	DOR1 Vs NOR p-value <i>b</i>	DOR2 (N=8) N (%)	INT (N=51) N (%)	NOR (N=46) N (%)	DOR2 Vs NOR Vs INT p-value <i>b</i>
0: has not entered	4 (6.78%)	12 (26.09%)	p=.012	1 (12.5%)	3 (5.88%)	12 (26.09%)	p=.011
1: <40 years	0	0		0	0	0	
2: 40-45 years	10 (16.95%)	3 (6.52%)		3 (37.5%)	7 (13.73%)	3 (6.52%)	
3: >45 years	45 (76.27%)	31 (67.39%)		4 (50%)	41 (80.39%)	31 (67.39%)	

NOR: normal ovarian reserve, AMH and AFC ≥ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

AMH: Anti-Mullerian hormone; AFC: antral follicle count; *b*: Chi-square test or Fisher's exact test

Similarly, the distribution of types of contraception varied significantly (Table 39), with significant differences observed between DOR1 and NOR ($p < .001$), and among DOR2, INT and NOR ($p = .002$).

Table 39 Comparison of use of hormonal contraception across ovarian reserve subgroups

The results are expressed in both numbers and percentages for each category. p-values indicate the statistical significance level of the differences observed between the respective subgroups (DOR1, NOR, DOR2, INT) for the age at which the mother entered menopause. Statistical significance ($p\text{-value} < .05$) is highlighted in blue.

Hormonal contraception	DOR1 (N=59) N (%)	NOR (N=46) N (%)	DOR1 Vs NOR p-value <i>b</i>	DOR2 (N=8) N (%)	INT (N=51) N (%)	NOR (N=46) N (%)	DOR2 Vs NOR Vs INT p-value <i>b</i>
0: None	8 (13.56%)	21 (45.65%)	$p < .001$	0	8 (15.69%)	21 (45.65%)	$p = .002$
1: Pill	38 (64.41%)	22 (47.83%)		6 (75%)	32 (62.75%)	22 (47.83%)	
2: Ring	6 (10.17%)	0		0	6 (11.76%)	0	
3: Intrauterine device	6 (10.17%)	3 (6.52%)		2 (25%)	4 (7.84%)	3 (6.52%)	
4: Implant	1 (1.69%)	0		0	1 (1.96%)	0	

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile.

AMH: Anti-Mullerian hormone; AFC: antral follicle count; *b*: Chi-square test or Fisher's exact test

When analysing the relationship between the use of hormonal contraception and AMH values (Table 40), the significant difference previously observed in the entire sample ($N=105$), where non-users exhibited higher AMH levels ($p < .001$), was also found in the subgroups DOR1 ($p = .013$), INT ($p = .020$), and NOR ($p = .018$). In the DOR2 subgroup, all participants were users of hormonal contraception. AFC did not show differences in mean values between users and non-users of hormonal contraception across the entire sample ($p = .052$) and subgroups DOR1 ($p = .968$), INT ($p = .529$), and NOR ($p = .841$).

Table 40 Correlation between the use of hormonal contraception and AMH and AFC values, across different ovarian reserve subgroups

For each subgroup (NOR, INT, DOR2, DOR1), the table provides the Pearson's correlation coefficient (r) and the associated p-value for the correlation between the use of hormonal contraception and AMH and AFC, indicating the statistical significance level of the observed differences. Statistical significance (p-value<.05) is highlighted in blue.

		p-value <i>a</i>
TOTAL (N=105)		
Hormonal contraception o: No i: Yes	AMH (ng/mL)	p<.001
Hormonal contraception o: No i: Yes	AFC	p=.052
NOR (N=46)		
Hormonal contraception o: No i: Yes	AMH (ng/mL)	p=.018
Hormonal contraception o: No i: Yes	AFC	p=.841
INT (N=51)		
Hormonal contraception o: No i: Yes	AMH (ng/mL)	p=.020
Hormonal contraception o: No i: Yes	AFC	p=.529
DOR2 (N=8)		
Hormonal contraception o: No i: Yes	AMH (ng/mL)	NA
Hormonal contraception o: No i: Yes	AFC	NA
DOR1 (N=59)		
Hormonal contraception o: No i: Yes	AMH (ng/mL)	p=.013
Hormonal contraception o: No i: Yes	AFC	p=.968

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile

AMH: Anti-Mullerian hormone; AFC: antral follicle count; NA: not applicable; *a*: t-test for independent samples or Mann-Whitney U test

Additionally, a significant difference in BMI (Table 41) was specifically observed when comparing DOR2 and INT ($p=.033$), with a higher BMI in DOR2 (28.32 ± 7.56) compared to INT (23.31 ± 3.42). In line with this, a strong positive correlation was found, with a Pearson's value of .758 ($p=.029$). In practical terms, a positive correlation between AMH and BMI suggests that as BMI increases, AMH levels also tend to increase. In opposition, as BMI decreases, AMH levels tend to decrease.

Table 41 Correlation analysis between BMI and AMH and AFC among the ovarian reserve subgroups

For each subgroup (NOR, INT, DOR2, DOR1), the table provides the Pearson's correlation coefficient (r) and the associated p -value for the correlation between the BMI and AMH and AFC, indicating the statistical significance level of the observed differences. Statistical significance (p -value $<.05$) is highlighted in blue.

		Pearson's r	p -value c
TOTAL (N=105)			
BMI	AMH (ng/mL)	.083	$p=.398$
	AFC	-.019	$p=.848$
NOR (N=46)			
BMI	AMH (ng/mL)	-.003	$p=.995$
	AFC	.087	$p=.563$
INT (N=51)			
BMI	AMH (ng/mL)	.036	$p=.799$
	AFC	-.167	$p=.241$
DOR2 (N=8)			
BMI	AMH (ng/mL)	.758	$p=.029$
	AFC	.311	$p=.454$
DOR1 (N=59)			
BMI	AMH (ng/mL)	.092	$p=.491$
	AFC	.284	$p=.079$

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile

AMH: Anti-mullerian hormone; AFC: antral follicle count; BMI: body mass index; c : Pearson's test

2.2.4.3.2 Impact of information provided regarding ovarian reserve assessment

With the new formed subgroups, it was possible to analyse how knowledge, attitudes, and intentions regarding fertility and reproductive plans changed: a) over time, and b) in relation to the information received (categorized into subgroups: NOR, DOR1, DOR2, INT). Particularly, we referred to the changes over time and the alterations resulting from the interaction between time and group.

2.2.4.3.2.1 Knowledge about ovarian reserve and reproductive health

Regarding knowledge about female reproductive health and ovarian reserve, assessed through a global knowledge percentage of correct answers, no differences beyond what has already been described for the entire population were found (Table 42). In other words, statistical analyses revealed significant effects of time on knowledge levels ($p < .001$), but no effect of the interaction between time and group was observed, considering DOR1 and NOR subgroups ($p = .347$), as DOR2, INT and NOR ($p = .574$). This suggests that changes in knowledge over time were consistent across all subgroups, irrespective of the specific information received. This pattern is illustrated in Figure 24.

Table 42 Changes in the percentage of global knowledge, considering the interaction time-group

Mean and SD of the percentage of global knowledge (percentage of correct answers) across three time-points (to, t1, t2), considering ovarian reserve subgroups (NOR, DOR1, DOR2, INT). The results of the statistical analysis (p-values) to examine the within-subjects effects of time, the between-subjects effects of group, and the interaction effects between time and group are reported. Statistical significance ($p\text{-value} < .05$) is highlighted in blue.

% of global knowledge	to		t1		t2		Effect	Time p-value _d	to Vs t1 p-value _d	t1 Vs t2 p-value _d	Time*Group p-value _d
	Mean	SD	Mean	SD	Mean	SD					
TOTAL (N=105)	63.97	17.77	79.05	21.81	83.65	16.82	<i>Within Subjects</i>	$p < .001$	$p < .001$	$p = .008$	
NOR (N=46)	63.77	18.69	81.88	20.13	83.69	15.51	<i>Within Subjects</i>	$p < .001$	$p < .001$	$p = .013$	$p = .347$
DOR1 (N=59)	64.12	17.18	76.84	22.96	83.61	17.91	<i>Between Subjects</i>	$p = .579$			
NOR (N=46)	63.77	18.69	81.88	20.13	83.69	15.51	<i>Within Subjects</i>	$p < .001$	$p < .001$	$p = .005$	$p = .574$
DOR2 (N=8)	58.33	15.43	66.67	29.55	79.17	21.36	<i>Between Subjects</i>	$p = .310$			
INT (N=51)	65.03	17.40	78.43	21.68	84.31	17.45					

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile.

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; SD: standard deviation; d: ANOVA repeated measures and Friedman test

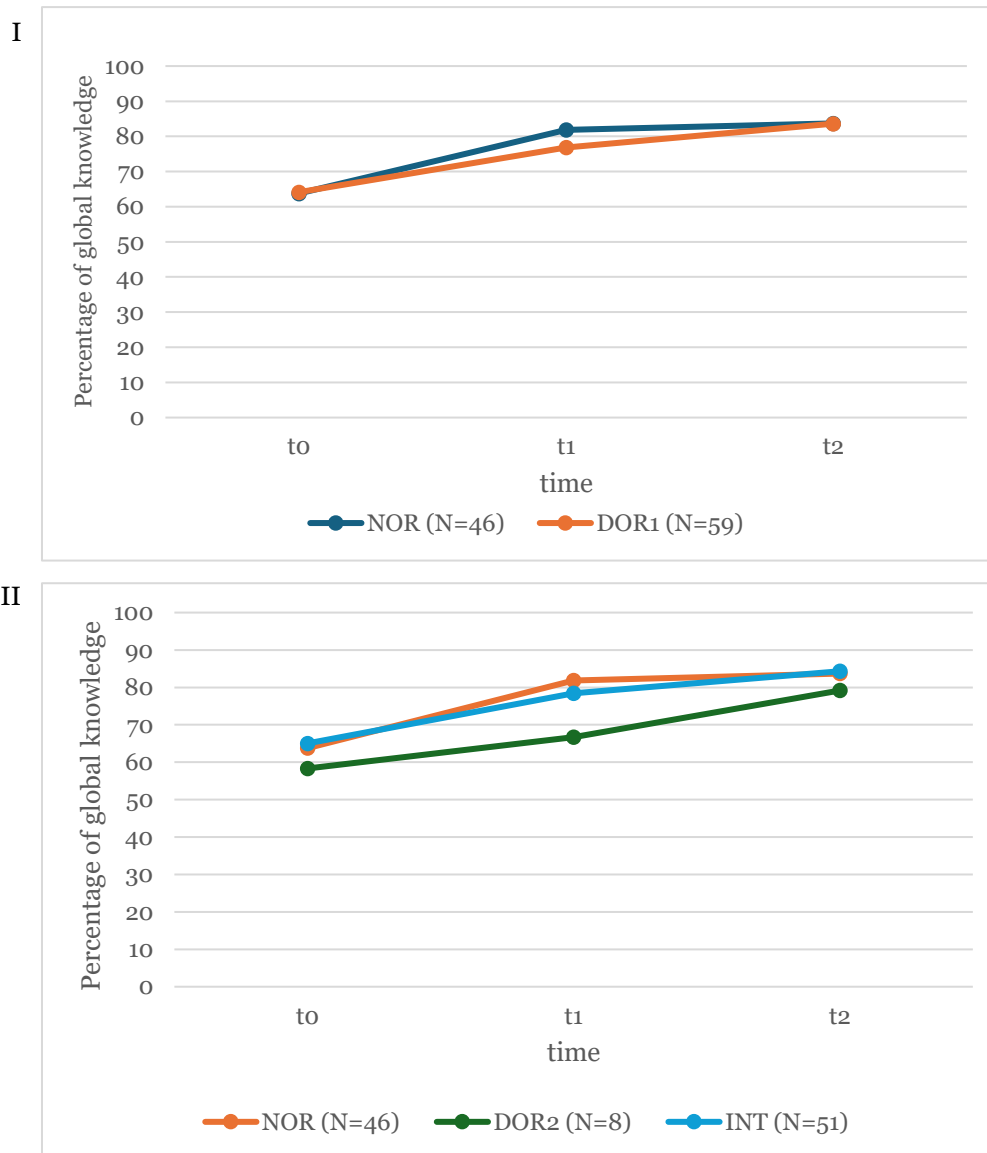


Figure 24 A graphical representation of the changes in knowledge about ovarian reserve across three time-points, considering the interaction time-group

The graphs represent the changes in the global percentage of correct answers across three time-points (t0, t1, t2), considering the subgroups NOR, DOR1 (I), and NOR, DOR2, and INT (II).

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile.

AMH: Anti-Müllerian hormone; AFC: antral follicle count

2.2.4.3.2.2 Childbearing intentions

Childbearing intentions remained stable when considering the interaction between time and group, regarding the subgroups formed according to the ovarian reserve testing results (Table 43). This was valid for DOR1 and NOR as well as for DOR2, INT, and NOR considering the number of children participants would like to have ($p=.707$ and $p=.116$), the number of children participants consider possible to have ($p=.473$ and $p=.349$), the childbearing age ($p=.152$ and $p=.155$) and the age they plan to have their first child ($p=.648$ and $p=.373$). The p-value is reported considering DOR1 and NOR, followed by DOR2, INT and NOR analysis.

Table 43 Participants' responses regarding their intentions and plans for having children at three different time-points, considering the interaction time-group

Participants' responses at three different time-points (to, t1, t2) for two questions: the number of children they would ideally like to have versus the number they consider possible to have, and the age considered ideal for having the first child versus the age they plan to have the first child. The results are presented as mean and SD for the time-points to, t1, and t2, considering the ovarian reserve statuses (NOR, DOR1, DOR2, INT). The results of the statistical analysis (p-values) to examine the within-subjects effects of time, the between-subjects effects of group, and the interaction effects between time and group are reported.

About having children in future		to		t1		t2		Effect	Time p-value	Time*Group p-value d
		Mean	SD	Mean	SD	Mean	SD			
How many children would you ideally like to have?	TOTAL (N=105)	2.23	.72	2.23	.80	2.19	.73	Within Subjects	p=.638	
	NOR (N=46)	2.04	.56	2.17	.71	2.15	.67	Within Subjects	p=.719	p=.707
	DOR1 (N=59)	2.37	.81	2.27	.87	2.22	.79	Between Subjects	p=.227	
	NOR (N=46)	2.04	.56	2.17	.71	2.15	.67	Within Subjects	p=.167	p=.116
	DOR2 (N=8)	2.34	.92	2.62	.92	2.25	.89	Between Subjects	p=.413	
	INT (N=51)	2.34	.80	2.21	.85	2.22	.78	Between Subjects	p=.413	
How many children do you consider possible to have, taking into account your economic situation, age, and personal background?	TOTAL (N=105)	1.92	.83	1.84	.79	1.78	.78	Within Subjects	p=.210	
	NOR (N=46)	1.85	.70	1.85	.63	1.80	.69	Within Subjects	p=.266	p=.473
	DOR1 (N=59)	1.98	.92	1.83	.89	1.76	.86	Between Subjects	p=.842	
	NOR (N=46)	1.85	.70	1.85	.63	1.80	.69	Within Subjects	p=.939	p=.349
	DOR2 (N=8)	1.50	.92	1.62	.92	1.75	.71	Between Subjects	p=.535	
	INT (N=51)	2.06	.90	1.86	.89	1.76	.89	Between Subjects	p=.535	
What age do you consider ideal for having the first child?	TOTAL (N=105)	28.95	2.33	28.68	2.51	28.72	2.51	Within Subjects	p=.129	
	NOR (N=46)	28.98	2.28	28.59	2.47	28.43	2.51	Within Subjects	p=.093	p=.152
	DOR1 (N=59)	28.93	2.38	28.74	2.56	28.95	2.51	Between Subjects	p=.646	
	NOR (N=46)	28.98	2.28	28.59	2.47	28.43	2.51	Within Subjects	p=.208	p=.155
	DOR2 (N=8)	29.25	2.05	28.87	2.42	29.87	1.89	Between Subjects	p=.752	
	INT (N=51)	28.88	2.44	28.72	2.61	28.80	2.58	Between Subjects	p=.752	
At what age do you plan to have your first child?	TOTAL (N=105)	31.08	2.76	31.30	2.76	31.28	2.80	Within Subjects	p=.095	
	NOR (N=46)	30.87	2.76	31.02	2.83	30.98	2.81	Within Subjects	p=.095	p=.648
	DOR1 (N=59)	31.25	2.78	31.52	2.71	31.51	2.80	Between Subjects	p=.378	
	NOR (N=46)	30.87	2.76	31.02	2.83	30.98	2.81	Within Subjects	p=.069	p=.373
	DOR2 (N=8)	31.31	3.02	31.62	2.67	31.87	3.09	Between Subjects	p=.678	
	INT (N=51)	31.29	2.77	31.51	2.74	31.45	2.78	Between Subjects	p=.678	

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile.

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; SD: standard deviation; d: ANOVA repeated measures and Friedman test

When examining infertility, particularly DOR, as a potential obstacle to having children at the desired time, a significant difference was observed in the perception of this obstacle across various subgroups, evaluated in a multiple-choice format (Table 44). Globally, we observed a significant decrease in the percentage of participants considering infertility an obstacle from t1 (54.29%) to t2 (43.81%) ($p=.007$). However, when examining specific subgroups such as DOR1 and DOR2, the percentages did not decrease after providing information from t1 to t2 ($p=.668$ and $p=1$, respectively). In the NOR subgroup, where the information provided was more reassuring, the pattern was different, and we observed a decrease in the percentage considering infertility an obstacle, from 59.32% to 55.93% ($p=.004$). Within DOR1 and DOR2, participants maintained their view of infertility as a potential obstacle from t1 to t2 ($p=.317$). For INT subgroup, providing information increased the number of participants considering infertility an obstacle, from to (47.06%) to t1 (60.78%), $p=.039$, and this perception was maintained without differences over time: t1 (60.78%) to t2 (54.9%), $p=.375$.

In both datasets, DOR1 and NOR, as well as DOR2, NOR, and INT, significant differences were observed in the distribution of participants considering infertility as an obstacle after receiving tailored information ($p=.006$ and $p=.017$, respectively).

Table 44 Distribution of responses regarding obstacles influencing future parenthood decisions, considering the interaction time-group

Responses at three different time-points (to, t1, t2) regarding whether participants consider DOR an obstacle to having children at the intended age. The data includes the number and percentage of participants N(%) for each subgroup (NOR, DOR1, DOR2, INT) and time-point (to, t1, t2). Additionally, the table provides the p-values from the Related-Samples McNemar Change Test and chi-square (χ^2) test for subgroup comparisons and changes over time. Statistical significance ($p\text{-value} < .05$) is highlighted in blue.

	to	t1	t2	χ^2						Related-Samples McNemar Change Test	
Consider infertility (DOR) an obstacle to have children at the intended age you intend	YES N(%)	YES N(%)	YES N(%)	p-value DOR1 Vs NOR to	p-value DOR1 Vs NOR t1	p-value DOR1 Vs NOR t2	p-value DOR2 Vs NOR Vs INT to	p-value DOR2 Vs NOR Vs INT t1	p-value DOR2 Vs NOR Vs INT t2	to Vs t1 p-value	t1 Vs t2 p-value
TOTAL (N=105)	54 (51.43%)	57 (54.29%)	46 (43.81%)							$p=.648$	$p=.007$
NOR (N=46)	24 (52.17%)	22 (47.83%)	13 (28.26%)	$p=.893$	$p=.324$	$p=.006$	$p=.336$	$p=.427$	$p=.017$	$p=.724$	$p=.004$
DOR1 (N=59)	30 (50.85%)	35 (59.32%)	33 (55.93%)							$p=.227$	$p=.668$
NOR (N=46)	24 (52.17%)	22 (47.83%)	13 (28.26%)							$p=.724$	$p=.004$
DOR2 (N=8)	6 (75%)	4 (50%)	5 (62.5%)							$p=.500$	$p=1$
INT (N=51)	24 (47.06%)	31 (60.78%)	28 (54.9%)							$p=.039$	$p=.375$

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile.

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups

AMH: Anti-Müllerian hormone; AFC: antral follicle count; DOR: diminished ovarian reserve

2.2.4.3.2.3 Attitudes towards fertility treatments, oocyte freezing, oocyte donation, and alternative parenthood

Regarding attitudes towards reproductive plans (Table 45), differences emerged regarding the options participants would follow in the case of a DOR diagnosis. The interaction between time and group was only significant for subgroups DOR1 and NOR, from t1 to t2. Specifically, regarding the consideration of adoption that differed when taking into account the interaction between time and groups ($p=.028$, Table 45 and Figure 25.I), with the differences evidenced from t1 to t2 ($p=.005$). For the NOR subgroup, the mean values remained with similar from 3.04 ± 1.11 at t1 to 3.17 ± 1.12 at t2, without statistical significance ($p=.155$) and for DOR1, the means decreased from t1: 3.12 ± 1.13 and t2: $2.83\pm.95$ ($p=.005$).

Table 45 Responses of participants regarding adopting a child and cryopreserve oocytes, if they knew in advance that they would have DOR, considering the interaction time-group

The options are evaluated on a scale of 1 to 5 (where higher scores signify a stronger preference for the option). This table explores participants' preferences may change over time (to, t1, t2) and vary across different ovarian reserve statuses (NOR, DOR1, DOR2, INT). The table includes the mean and SD. The results of the statistical analysis (p-values) to examine the within-subjects effects of time, the between-subjects effects of group, and the interaction effects between time and group are reported. Statistical significance ($p\text{-value}<.05$) is highlighted in blue.

If you knew in advance that you would have DOR, what would be your options? Item evaluated on a scale of 1 to 5		to		t1		t2		Effect	Time p-value d	to Vs t1 p-value d	t1 Vs t2 p-value d	Time*Gr oup p- value d	Time*Gr oup to Vs t1 p-value d	Time*Gr oup t1 Vs t2 p-value d
		Mean	SD	Mean	SD	Mean	SD							
When you want to have children, try to adopt a child	TOTAL (N=105)	2.90	1.14	3.08	1.12	2.98	1.04	Within Subjects	$p=.068$					
	NOR (N=46)	2.98	1.12	3.04	1.11	3.17	1.12	Within Subjects	$p=.097$			$p=.028$	$p=.175$	$p=.005$
	DOR1 (N=59)	2.85	1.16	3.12	1.13	2.83	.95	Between Subjects	$p=.501$					
	NOR (N=46)	2.98	1.12	3.04	1.11	3.17	1.12	Within Subjects	$p=.139$			$p=.120$		
	DOR2 (N=8)	3.37	.74	3.62	1.19	3.25	.71	Between Subjects	$p=.270$					
	INT (N=51)	2.76	1.19	3.04	1.11	2.76	.97							
Freeze your eggs to use in the future, when you want to have children	TOTAL (N=105)	4.09	1.03	3.93	1.11	3.76	1.17	Within Subjects	$p=.002$	$p=.046$	$p=.024$			
	NOR (N=46)	4	1.09	3.83	1.18	3.91	1.24	Within Subjects	$p=.004$	$p=.047$	$p=.050$	$p=.016$	$p=.896$	$p=.002$
	DOR1 (N=59)	4.17	.98	4.02	1.06	3.64	1.11	Between Subjects	$p=.876$					
	NOR (N=46)	4	1.09	3.83	1.18	3.91	1.24	Within Subjects	$p<.001$	$p=.100$	$p=.030$	$p=.071$		
	DOR2 (N=8)	4	1.19	3.75	1.16	3.37	1.19	Between Subjects	$p=.761$					
	INT (N=51)	4.20	.96	4.059	1.05	3.69	1.10							

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups

AMH: Anti-Müllerian hormone; AFC: antral follicle count; DOR: diminished ovarian reserve; SD: standard deviation; d: ANOVA repeated measures and Friedman test

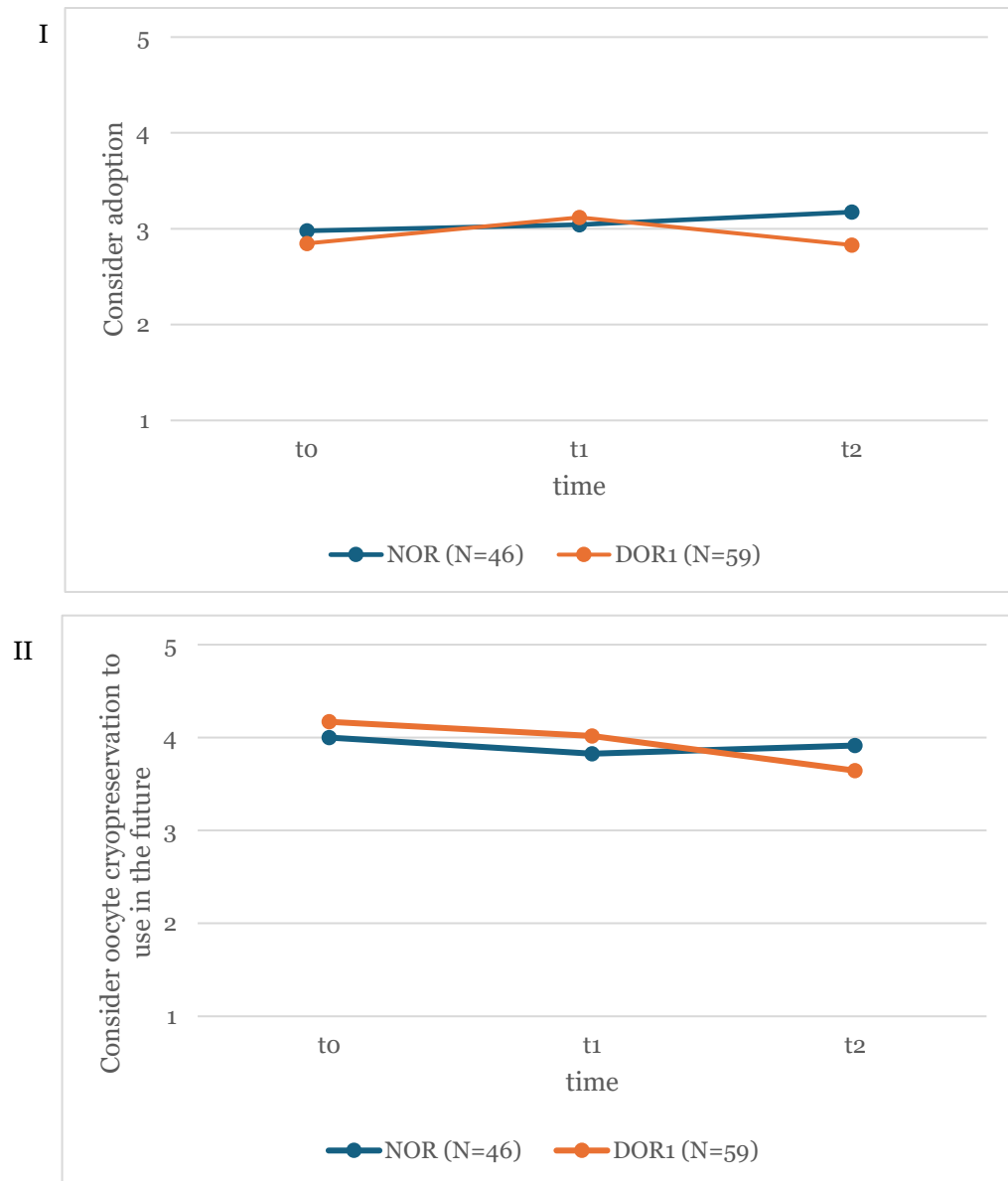


Figure 25 Preferences for adoption (I), and oocyte cryopreservation (II) across three time-points, considering the interaction time-group

The graph represents the mean values for the options are evaluated on a scale of 1 to 5 at three different time-points (to, t1, and t2), and regarding different ovarian reserve statuses (NOR, DOR1).

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile

AMH: Anti-Müllerian hormone; AFC: antral follicle count

Additionally, the preference for oocyte freezing changed when considering the interaction between time and group ($p=.016$, Table 45 and Figure 25.II), particularly between t1 and t2 ($p=.002$). Within the NOR subgroup, the mean score changed from 3.83 ± 1.18 at t1 to 3.91 ± 1.24 at t2; however, this change did not reach statistical significance based on paired t-test comparison ($p=.420$). For the DOR1 subgroup, the mean score decreased significantly from t1 (4.02 ± 1.06) to t2 (3.64 ± 1.11) ($p<.001$).

The remaining options related to reproductive plans, as well as considering undergoing COS to cryopreserve oocytes, accessibility to affordable methods for evaluating ovarian reserve, and the importance of being informed, alongside attitudes towards oocyte donation and feelings about using donated oocytes, remained stable over time, both independently and when accounting for the interaction between time and group, as observed across Table 46 to Table 50.

To facilitate easier reading of the data, the information was divided in participants responses regarding their options if they knew in advance that they would have DOR (Table 46); how they would feel about having to use donated oocytes (Table 47); their opinions about social freezing (Table 48); their views on ovarian reserve assessment for all women (Table 49); and their attitude on becoming an oocyte donor (Table 50). Statistical measures including N (%), minimum, maximum, mean, and SD are provided for each variable analysed within the characteristics of the total participants.

Participants exhibited comparable responses across all subgroups regarding their options in the context of a diagnosis of DOR, suggesting homogeneity in attitudes (Table 46).

Table 46 Participants responses regarding their options if they knew in advance that they would have DOR, considering the interaction time-group

The options were evaluated on a scale of 1 to 5, where higher scores signify a stronger preference for the option. The mean and SD of responses are provided for three time-points (to, t1, and t2), considering the ovarian reserve subgroups (NOR, DOR1, DOR2, INT) are reported. The results of the statistical analysis (p-values) to examine the within-subjects effects of time, the between-subjects effects of group, and the interaction effects between time and group are reported.

		to		t1		t2				
If you knew in advance that you would have DOR, what would be your options? Item evaluated on a scale of 1 to 5.		Mean	SD	Mean	SD	Mean	SD	Effect	Time p-value d	Time*Group p-value d
When you want to have children, seek fertility treatments	TOTAL (N=105)	3.75	1.17	3.93	1.04	3.93	.99	Within Subjects	p=.111	
	NOR (N=46)	3.85	1.09	3.85	1.07	3.98	1.02	Within Subjects	p=.145	p=.229
	DOR1 (N=59)	3.68	1.24	4.00	1.02	3.90	.98	Between Subjects	p=.857	
	NOR (N=46)	3.85	1.09	3.85	1.07	3.98	1.02	Within Subjects	p=.414	p=.448
	DOR2 (N=8)	3.38	1.06	3.50	0.93	3.38	1.06	Between Subjects	p=.334	
	INT (N=51)	3.73	1.27	4.08	1.02	3.98	.95			
Consider egg donation	TOTAL (N=105)	2.59	1.14	2.74	1.14	2.63	1.15	Within Subjects	p=.225	
	NOR (N=46)	2.67	1.19	2.80	1.24	2.67	1.21	Within Subjects	p=.239	p=.935
	DOR1 (N=59)	2.53	1.10	2.69	1.07	2.59	1.12	Between Subjects	p=.573	
	NOR (N=46)	2.67	1.19	2.80	1.24	2.67	1.21	Within Subjects	p=.514	p=.166
	DOR2 (N=8)	2.50	1.31	2.63	1.19	1.88	.83	Between Subjects	p=.614	
	INT (N=51)	2.53	1.08	2.71	1.06	2.71	1.12			
Give up on having children	TOTAL (N=105)	1.76	1.02	1.82	1.08	1.73	1.02	Within Subjects	p=.618	
	NOR (N=46)	1.78	1.03	1.87	1.19	1.78	1.01	Within Subjects	p=.619	p=.946
	DOR1 (N=59)	1.75	1.03	1.78	1.00	1.69	1.04	Between Subjects	p=.688	
	NOR (N=46)	1.78	1.03	1.87	1.19	1.78	1.01	Within Subjects	p=.715	p=.995
	DOR2 (N=8)	2.00	1.07	2.13	1.25	2.00	1.41	Between Subjects	p=.553	
	INT (N=51)	1.71	1.03	1.73	.96	1.65	.98			
Anticipate the age of having a child	TOTAL (N=105)	3.58	1.31	3.68	1.25	3.65	1.22	Within Subjects	p=.532	
	NOR (N=46)	3.59	1.24	3.70	1.19	3.59	1.24	Within Subjects	p=.540	p=.679
	DOR1 (N=59)	3.58	1.37	3.66	1.31	3.69	1.21	Between Subjects	p=.927	
	NOR (N=46)	3.59	1.24	3.70	1.19	3.59	1.24	Within Subjects	p=.955	p=.813
	DOR2 (N=8)	3.50	1.60	3.38	1.41	3.38	1.19	Between Subjects	p=.833	
	INT (N=51)	3.59	1.34	3.71	1.30	3.75	1.21			

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups

AMH: Anti-Müllerian hormone; AFC: antral follicle count; DOR: diminished ovarian reserve; SD: standard deviation; d: repeated measures ANOVA and Friedman test

Analysis indicated uniformity in participants feeling regarding the possibility of using donated oocytes, regardless of the tailored information received (Table 47).

Table 47 Data on participants' feelings regarding the possibility of using donated gametes, considering the interaction time-group

The options were evaluated on a scale of 1 to 5, with higher scores indicating a stronger agreement with the option. The mean and SD of responses are provided for three time-points (to, t1, and t2), considering the ovarian reserve subgroups (NOR, DOR1, DOR2, INT). The results of the statistical analysis (p-values) to examine the within-subjects effects of time, the between-subjects effects of group, and the interaction effects between time and group are reported. Statistical significance (p-value<.05) is highlighted in blue.

How would you feel about the possibility of having to use donated gametes? Item evaluated on a scale of 1 to 5.		Mean	SD	Mean	SD	Mean	SD	Effect	Time p-value	to Vs t1 p-value	t1 Vs t2 p-value	Time*Group p-value
Happy to fulfill my dream of becoming a mother	TOTAL (N=105)	3.48	1.25	3.60	1.14	3.50	1.14	Within Subjects	p=.301			
	NOR (N=46)	3.46	1.26	3.54	1.15	3.46	1.24	Within Subjects	p=.329			p=.932
	DOR1 (N=59)	3.49	1.25	3.64	1.14	3.53	1.06	Between Subjects	p=.745			
	NOR (N=46)	3.46	1.26	3.54	1.15	3.46	1.24	Within Subjects	p=.229			p=.079
	DOR2 (N=8)	3.75	.89	3.25	1.17	3	1.31	Between Subjects	p=.779			
	INT (N=51)	3.45	1.30	3.71	1.14	3.61	1					
Happy to care for a child from birth	TOTAL (N=105)	3.82	1.17	3.90	1.12	3.88	1.09	Within Subjects	p=.490			
	NOR (N=46)	3.76	1.21	3.89	1.20	3.78	1.23	Within Subjects	p=.468			p=.633
	DOR1 (N=59)	3.86	1.14	3.92	1.07	3.95	.97	Between Subjects	p=.633			
	NOR (N=46)	3.76	1.21	3.89	1.20	3.78	1.23	Within Subjects	p=.831			p=.600
	DOR2 (N=8)	4.12	.83	4.00	.93	3.88	1.13	Between Subjects	p=.862			
	INT (N=51)	3.82	1.18	3.90	1.10	3.96	.96					
Happy to be able to pass on my family values	TOTAL (N=105)	3.96	1.15	4.00	1.03	3.84	1.18	Within Subjects	p=.200			
	NOR (N=46)	3.87	1.20	4.04	1.09	3.74	1.36	Within Subjects	p=.468			p=.615
	DOR1 (N=59)	4.03	1.11	3.97	0.98	3.92	1.02	Between Subjects	p=.633			
	NOR (N=46)	3.87	1.20	4.04	1.09	3.74	1.36	Within Subjects	p=.831			p=.600
	DOR2 (N=8)	4.12	.99	3.88	.83	3.75	1.16	Between Subjects	p=.862			
	INT (N=51)	4.02	1.14	3.98	1.01	3.94	1.01					
Apprehensive about potentially feeling that the child is not really mine	TOTAL (N=105)	2.72	1.36	2.94	1.34	2.85	1.26	Within Subjects	p=.027	p=.015	p=.123	
	NOR (N=46)	2.57	1.42	2.76	1.34	2.78	1.32	Within Subjects	p=.029	p=.018	p=.182	p=.367
	DOR1 (N=59)	2.85	1.31	3.08	1.33	2.90	1.23	Between Subjects	p=.325			
	NOR (N=46)	2.57	1.42	2.76	1.34	2.78	1.32	Within Subjects	p=.220			p=.686
	DOR2 (N=8)	2.25	1.58	2.38	1.41	2.25	1.49	Between Subjects	p=.170			
	INT (N=51)	2.94	1.26	3.20	1.30	3	1.17					
Concerned about possible hereditary health problems	TOTAL (N=105)	3.27	1.28	3.30	1.29	3.22	1.23	Within Subjects	p=.607			
	NOR (N=46)	3.11	1.39	3.30	1.33	3.28	1.28	Within Subjects	p=.642			p=.080
	DOR1 (N=59)	3.39	1.19	3.31	1.28	3.17	1.21	Between Subjects	p=.806			
	NOR (N=46)	3.11	1.39	3.30	1.33	3.28	1.28	Within Subjects	p=.219			p=.152
	DOR2 (N=8)	3.25	.71	3.25	1.04	2.75	.89	Between Subjects	p=.841			
	INT (N=51)	3.41	1.25	3.31	1.32	3.24	1.24					

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups

AMH: Anti-Müllerian hormone; AFC: antral follicle count; SD: standard deviation; d: repeated measures ANOVA and Friedman test

Participants exhibited similar views on social freezing regardless of the tailored ovarian reserve information (Table 48).

Table 48 Participants' opinions regarding whether fertility preservation for social reasons should be available to all women, considering the interaction time-group

The item was evaluated on a multiple-choice with options: 0: no for nobody; 1: yes for all, 2: I'm not informed, and 3: yes, for specific cases. The table includes the mean and SD of responses for each time-point (t0, t1, t2) and subgroups (NOR, DOR1, DOR2, INT). The results of the statistical analysis (p-values) to examine the within-subjects effects of time, the between-subjects effects of group, and the interaction effects between time and group are reported. Statistical significance (p-value < .05) is highlighted in blue.

Do you think that fertility preservation for social reasons ('egg freezing') should be available to all women?	t0		t1		t2		Effect	Time p-value _d	t0 Vs t1 p-value _d	Time*Group p-value _d
	Mean	SD	Mean	SD	Mean	SD				
TOTAL (N=105)	1.94	1.21	1.68	1.07	1.61	1.09	<i>Within Subjects</i>	p=.018	p=.031	
NOR (N=46)	1.76	1.16	1.78	1.19	1.63	1.12	<i>Within Subjects</i>	p=.033	p=.048	p=.092
DOR1 (N=59)	2.08	1.25	1.59	.97	1.61	1.08	<i>Between Subjects</i>	p=.829		
NOR (N=46)	1.76	1.16	1.78	1.19	1.63	1.12	<i>Within Subjects</i>	p=.072		p=.223
DOR2 (N=8)	1.62	1.19	1	0	1.38	1.06	<i>Between Subjects</i>	p=.338		
INT (N=51)	2.16	1.25	1.69	1.01	1.65	1.09				

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC ≥ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups

AMH: Anti-Müllerian hormone; AFC: antral follicle count; SD: standard deviation; *d*: repeated measures ANOVA and Friedman test

Participants demonstrated similar opinions on assessing ovarian reserve for all women regardless of their tailored ovarian reserve information (Table 49).

Table 49 Participants' opinions regarding whether all women should have access to an easy and inexpensive way to evaluate ovarian reserve, considering the interaction time-group

The item was evaluated on a multiple-choice with options: 0: no for nobody; 1: yes for all, 2: I'm not informed, and 3: yes, for specific cases. The responses were evaluated at three different time-points: t0, t1, and t2, and across different ovarian reserve statuses (NOR, DOR1, DOR2, INT). The table includes the mean and SD of responses for each time-point and subgroup. The results of the statistical analysis (p-values) to examine the within-subjects effects of time, the between-subjects effects of group, and the interaction effects between time and group are reported.

Do you think all women should have access to an easy and inexpensive way to evaluate ovarian reserve?	t0		t1		t2		Effect	Time p-value <i>d</i>	Time*Group p-value <i>d</i>
	Mean	SD	Mean	SD	Mean	SD			
TOTAL (N=105)	1.03	.17	1	.24	1.04	.19	<i>Within Subjects</i>	p=.334	
NOR (N=46)	1	0	1	.21	1.02	.15	<i>Within Subjects</i>	p=.383	p=.092
DOR1 (N=59)	1.05	.22	1	.26	1.05	.22	<i>Between Subjects</i>	p=.279	
NOR (N=46)	1	0	1	.21	1.02	.15	<i>Within Subjects</i>	p=.734	p=.223
DOR2 (N=8)	1	0	1	0	1	0	<i>Between Subjects</i>	p=.397	
INT (N=51)	1.06	.24	1	.28	1.06	.24			

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile.

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups

AMH: Anti-Müllerian hormone; AFC: antral follicle count; SD: standard deviation; *d*: repeated measures ANOVA and Friedman test

The interaction between time and groups did not yield significant differences in participants attitudes toward becoming oocyte donors (Table 50).

Table 50 Participants' willingness to consider voluntary donation of gametes (oocytes) to help women with DOR, considering the interaction time-group

The item was evaluated on a multiple-choice with options: 0: no for nobody, 1: yes for all, 2: I'm not informed. The table includes mean and SD of responses for three time-points (t0, t1, and t2), considering the ovarian reserve subgroups (NOR, DOR1, DOR2, INT). The results of the statistical analysis (p-values) to examine the within-subjects effects of time, the between-subjects effects of group, and the interaction effects between time and group are reported.

Would you consider doing a voluntary donation of gametes (eggs) if such an act helped women with DOR?	t0		t1		t2		Effect	Time p-value <i>d</i>	Time*Group p-value <i>d</i>
	Mean	SD	Mean	SD	Mean	SD			
TOTAL (N=105)	1.30	.76	1.31	.79	1.25	.87	<i>Within Subjects</i>	p=.464	
NOR (N=46)	1.30	.78	1.22	.81	1.22	.96	<i>Within Subjects</i>	p=.496	p=.331
DOR1 (N=59)	1.30	.75	1.39	.77	1.27	.80	<i>Between Subjects</i>	p=.601	
NOR (N=46)	1.30	.78	1.22	.81	1.22	.96	<i>Within Subjects</i>	p=.809	p=.622
DOR2 (N=8)	1.50	.53	1.50	.53	1.50	.53	<i>Between Subjects</i>	p=.666	
INT (N=51)	1.27	.78	1.37	.80	1.24	.84			

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups

AMH: Anti-Müllerian hormone; AFC: antral follicle count; DOR: diminished ovarian reserve; SD: standard deviation; *d*: repeated measures ANOVA and Friedman test

2.2.4.3.3 Understanding information in reproductive counselling: perceived *versus* provided

In the 3rd time-point of the assessment protocol, after the communication of tailored results regarding ovarian reserve testing, participants were asked, in a multiple-choice format, to tell how they interpret the information provided. This data is summarized in Table 51. The most frequently chosen option was the perception that everything was fine regarding their fertility (selected 65 times). This was followed by the recognition of the need to take action in the short to medium term (chosen 39 times). The acknowledgment that it might take longer to achieve pregnancy was selected 25 times. The least considered interpretations of the information received were the assurance of being able to postpone motherhood (chosen 10 times) and indicating a need for additional information (selected 3 times).

The way participants perceived the information revealed a significant relationship with the results from ovarian reserve testing (Table 51). Specifically, significant differences were found for all interpretations, excepting for the need for more information ($p=.121$ for DOR1 and NOR and $p=.195$ for DOR2, INT and NOR). The perception that everything is fine was predominantly chosen by both NOR participants ($p<.001$). Participants who received information about having an ovarian reserve under the expected percentile for their age, both DOR1 and DOR2, often evaluate their situation as potentially requiring more time to achieve pregnancy or necessitating to do something in the short to medium term ($p<.001$). Additionally, a minority of DOR1 participants and none of DOR2 believe it is safe to postpone motherhood ($p=.015$ considering DOR1 and NOR and $p=.040$ for DOR2, INT and NOR).

Table 51 Interpretation of the information received by participants regarding their ovarian reserve status

Each row corresponds to a different interpretation, while columns represent the response distribution across various participant subgroups. The "Yes" column indicates the number and percentage of participants who endorsed with each interpretation. The "p-value DOR1 Vs NOR" and "p-value DOR2 Vs INT Vs NOR" columns display the statistical significance levels for comparisons between the DOR1 subgroup and the NOR subgroup, as well as between the combined DOR2 and INT subgroups and the NOR subgroup, respectively. Statistical significance (p-value < .05) is highlighted in blue.

		How do you interpret the information you received?		
		Yes N (%)	DOR1 Vs NOR p-value <i>b</i>	DOR2 Vs NOR Vs INT p-value <i>b</i>
It seems to me that everything is fine regarding my fertility	Total (N=105)	65 (61.9%)		
	DOR1 (N=59)	20 (33.9%)	p<.001	p<.001
	DOR2 (N=8)	0		
	INT (N=51)	20 (39.22%)		
	NOR (N=46)	45 (97.83%)		
I understand that it is safe to postpone motherhood	Total (N=105)	10 (9.52%)		
	DOR1 (N=59)	2 (3.39%)	p=.015	p=.040
	DOR2 (N=8)	0		
	INT (N=51)	2 (3.92%)		
	NOR (N=46)	8 (17.39%)		
I need to take action in the short/medium term	Total (N=105)	39 (37.14%)		
	DOR1 (N=59)	36 (61.02%)	p<.001	p<.001
	DOR2 (N=8)	7 (87.5%)		
	INT (N=51)	29 (56.86%)		
	NOR (N=46)	3 (6.52%)		
It may take me longer to get pregnant	Total (N=105)	25 (23.81%)		
	DOR1 (N=59)	23 (38.98%)	p<.001	p<.001
	DOR2 (N=8)	8 (100%)		
	INT (N=51)	15 (29.41%)		
	NOR (N=46)	2 (4.35%)		
It was limited; I need more information	Total (N=105)	3 (2.86%)		
	DOR1 (N=59)	3 (5.08%)	p=.121	p=.195
	DOR2 (N=8)	0		
	INT (N=51)	3 (5.88%)		
	NOR (N=46)	0		

NOR: normal ovarian reserve, AMH and AFC ≥ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile

AMH: Anti-Müllerian hormone; AFC: antral follicle count; *b*: Chi-square test or Fisher's exact test

2.2.4.3.4 Participants' experience and satisfaction with evaluation of ovarian reserve

Participants description of the experience of evaluating ovarian reserve was also analysed at the 3rd time-point of the assessment protocol, after the communication of the results regarding ovarian reserve testing (Table 52). This item referred to the question “How would you describe your experience after testing your ovarian reserve and having the reproductive counselling”. Participants were asked to rate their response on a Likert scale ranging from 1 ('Not true at all') to 5 ('Totally true').

Participants did not clearly specify that the evaluation of ovarian reserve was the leading factor behind their decisions regarding motherhood ($p=.358$ for DOR1 and NOR comparison and $p=.349$ for DOR2, INT, NOR). Therefore, receiving information about having a reserve under the expected values did not lead to significant changes in participants' attitudes or intentions. (Table 52).

Regarding learning about fertility, participants consistently rated the information with a high score of 5/5 across all subgroups (Table 52), indicating that they found it useful. There were no differences observed between the subgroups ($p=.224$ for DOR1 and DOR comparison and $p=.396$ for DOR2, INT, and NOR), suggesting that all participants considered the information valuable. Similarly, the presence of doubts, the desire for more concrete information, or the aim to discuss the options available was not associated with any specific subgroup (respectively, $p=.056$ for DOR1 and NOR comparison, and $p=.289$ for DOR2, INT, and NOR; $p=.269$ for DOR1 and NOR comparison and $p=.526$ for DOR2, INT, and NOR; and $p=.126$ for DOR1 and NOR comparison and $p=.059$ for DOR2, INT, and NOR).

Most participants across all subgroups indicated that they had no doubts, rating it as 1/5 (Table 52). This trend was consistent across all subgroups, indicating that the information was generally well-explained and met participants' expectations. Similarly, participants gave lower ratings (mostly 1 and 2 out of 5) regarding the need for more information or further discussion, suggesting that the information provided was sufficient (Table 52).

About maintaining the motherhood plans, differences were observed in the evaluations of this option across the subgroups ($p<.001$). DOR1, DOR2, and INT cohorts exhibited lower percentages of 5/5 ratings, indicating complete agreement, than NOR subgroup (Table 52), suggesting potential reconsideration of their plans.

Table 52 Participants' experience regarding the evaluation of their ovarian reserve and undergoing reproductive counselling

Responses evaluated on a scale ranging from 1 to 5. Each row represents a different aspect of the experience, such as learning about fertility, maintaining motherhood plans, finding motivation to make decisions about motherhood, seeking concrete information, experiencing doubts, and expressing the need for more information or discussion about available options. The columns display the distribution of responses across participant subgroups, including DOR1, DOR2, INT, and NOR subgroups. The "N (%)" columns indicate the number and percentage of participants who selected each response option within each subgroup. Additionally, the "p-value DOR1 Vs NOR" and "p-value DOR2 Vs NOR Vs INT" columns show the statistical significance levels for comparisons between the DOR1 subgroup and the NOR subgroup, as well as between the combined DOR2 and INT subgroups and the NOR subgroup, respectively. Statistical significance (p-value<.05) is highlighted in blue.

Item evaluated on a scale of 1 to 5.		How would you describe the experience of evaluating your ovarian reserve and undergoing reproductive counseling?					
		N (%)					
		Total (N=105)	DOR1 (N=59)	DOR2 (N=8)	INT (N=51)	NOR (N=46)	DOR1 Vs NOR p-value ^b
I learned more about my fertility	1 - Not True at All	0	0	0	0	0	p=.224
	2	2 (1.9%)	2 (3.39%)	0	2 (3.92%)	0	
	3	2 (1.9%)	2 (3.39%)	0	2 (3.92%)	0	
	4	20 (19.05%)	13 (22.03%)	1 (12.5%)	12 (23.53%)	7 (15.22%)	
	5 - Completely True	81 (77.14%)	42 (71.19%)	7 (87.5%)	35 (68.63%)	39 (84.78%)	
I kept my motherhood plans	1 - Not True at All	0	0	0	0	0	p<.001
	2	2 (1.9%)	2 (3.39%)	1 (12.5%)	1 (1.96%)	0	
	3	12 (11.43%)	11 (18.64%)	3 (37.5%)	8 (15.69%)	1 (2.17%)	
	4	36 (34.29%)	25 (42.37%)	3 (37.5%)	22 (43.14%)	11 (23.91%)	
	5 - Completely True	55 (52.38%)	21 (35.59%)	1 (12.5%)	20 (39.22%)	34 (73.91%)	
It was the motivation I needed to make a decision about motherhood	1 - Not True at All	24 (22.86%)	10 (16.95%)	0	10 (19.61%)	14 (30.43%)	p=.358
	2	40 (38.1%)	26 (44.07%)	3 (37.5%)	23 (45.1%)	14 (30.43%)	
	3	20 (19.05%)	11 (18.64%)	3 (37.5%)	8 (15.69%)	9 (19.57%)	
	4	12 (11.43%)	8 (13.56%)	2 (25%)	6 (11.76%)	4 (8.7%)	
	5 - Completely True	9 (8.57%)	4 (6.78%)	0	4 (7.84%)	5 (10.87%)	
I was looking for something more concrete	1 - Not True at All	56 (53.33%)	27 (45.76%)	5 (62.5%)	22 (43.14%)	29 (63.04%)	p=.269
	2	28 (26.67%)	16 (27.12%)	2 (25%)	14 (27.45%)	12 (26.09%)	
	3	15 (14.29%)	11 (18.64%)	1 (12.5%)	10 (19.61%)	4 (8.7%)	
	4	5 (4.76%)	4 (6.78%)	0	4 (7.84%)	1 (2.17%)	
	5 - Completely True	1 (.95%)	1 (1.69%)	0	1 (1.96%)	0	
I had doubts	1 - Not True at All	74 (70.48%)	38 (64.41%)	5 (62.5%)	33 (64.71%)	36 (78.26%)	p=.056
	2	17 (16.19%)	8 (13.56%)	1 (12.5%)	7 (13.73%)	9 (19.57%)	
	3	7 (6.67%)	6 (10.17%)	1 (12.5%)	5 (9.8%)	1 (2.17%)	
	4	6 (5.71%)	6 (10.17%)	1 (12.5%)	5 (9.8%)	0	
	5 - Completely True	1 (.95%)	1 (1.69%)	0	1 (1.96%)	0	
I need more information and/or to discuss the options available	1 - Not True at All	47 (44.76%)	20 (33.9%)	2 (25%)	18 (35.29%)	27 (58.7%)	p=.126
	2	32 (30.48%)	20 (33.9%)	2 (25%)	18 (35.29%)	12 (26.09%)	
	3	14 (13.34%)	10 (16.95%)	4 (50%)	6 (11.76%)	4 (8.7%)	
	4	7 (6.67%)	5 (8.47%)	0	5 (9.8%)	2 (4.35%)	
	5 - Completely True	5 (4.76%)	4 (6.78%)	0	4 (7.84%)	1 (2.17%)	

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile

AMH: Anti-Müllerian hormone; AFC: antral follicle count; ^b: Chi-square test or Fisher's exact test

2.2.4.3.5 Reasons for participating in the study

In the 3rd time-point of the assessment protocol, after the communication of tailored results regarding ovarian reserve testing, participants were asked to provide their motivations to evaluate ovarian reserve. The most frequently cited reasons were concern about their fertility, chosen 70 times, and search for information on the topic, selected 68 times (Table 53). The motivation to participate in the study remained consistent across the formed subgroups.

Table 53 Data on the factors motivating individuals to evaluate their ovarian reserve

Each row corresponds to a specific motivation, while columns indicate the number and percentage of participants who endorsed each motivation. The "Yes" column denotes the number and percentage of participants who endorsed each motivation. The p-value columns show the statistical significance of differences between subgroups, specifically between DOR1 and NOR subgroups, and between DOR2 and the combined NOR and INT subgroups.

		What motivated you to evaluate your ovarian reserve?		
		Yes N (%)	DOR1 Vs NOR p-value <i>b</i>	DOR2 Vs NOR Vs INT p-value <i>b</i>
Search for information on the topic	Total (N=105)	68 (64.76%)		
	DOR1 (N=59)	36 (61.02%)	p=.363	p=.159
	DOR2 (N=8)	7 (87.5%)		
	INT (N=51)	29 (56.86%)		
	NOR (N=46)	32 (69.57%)		
Confirmation of postponement of motherhood	Total (N=105)	9 (8.57%)		
	DOR1 (N=59)	6 (10.17%)	p=.508	p=.436
	DOR2 (N=8)	0		
	INT (N=51)	6 (11.76%)		
	NOR (N=46)	3 (6.52%)		
Concern about my fertility	Total (N=105)	70 (66.67%)		
	DOR1 (N=59)	38 (64.41%)	p=.661	p=.829
	DOR2 (N=8)	6 (75%)		
	INT (N=51)	32 (62.75%)		
	NOR (N=46)	31 (67.39%)		
Seeking help in making decisions about motherhood	Total (N=105)	22 (20.95%)		
	DOR1 (N=59)	13 (22.03%)	p=.758	p=.740
	DOR2 (N=8)	1 (12.5%)		
	INT (N=51)	12 (23.53%)		
	NOR (N=46)	9 (19.57%)		

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile

AMH: Anti-Müllerian hormone; AFC: antral follicle count; *b*: Chi-square test or Fisher's exact test

Additionally, participants were questioned about whether they believed they should have received information about ovarian reserve earlier in life (Table 54). 24.76% participants answered affirmatively, with the most common suggested age being 25 years old, selected by 38.46% of those respondents. It is noteworthy that the average age of participants was 27.3 $\pm$ 3.67 years old.

Table 54 Participants' views on whether they should have received information about ovarian reserve earlier

Each row represents a different age at which participants believe they should have received this information, and the columns indicate the number and percentage of participants who responded "YES" within each subgroup. The p-value columns indicate the statistical significance of differences between the DOR1 subgroup and the NOR subgroup (DOR1 Vs NOR), and between the DOR2 subgroup and the combined NOR and INT subgroups (DOR2 Vs NOR Vs INT).

	Do you think you should have received information about ovarian reserve earlier?						
	Total (N=105)	DOR1 (N=59)	DOR2 (N=8)	INT (N=51)	NOR (N=46)	DOR1 Vs NOR p-value <i>b</i>	DOR2 Vs NOR Vs INT p-value <i>b</i>
YES	26 (24.76%)	12 (20.34%)	3 (37.5%)	9 (17.65%)	14 (30.43%)	p=.463	p=.422
IF YES, AT WHAT AGE?							
18 years old	2 (7.69%)	0	0	0	2 (14.29%)	p=.452	p=.806
20 years old	5 (19.23%)	3 (25%)	1 (33.33%)	2 (22.22%)	2 (14.29%)		
21 years old	1 (3.85%)	0	0	0	1 (7.14%)		
25 years old	10 (38.46%)	5 (41.67%)	2 (66.67%)	4 (44.44%)	5 (35.71%)		
27 years old	2 (7.69%)	2 (16.67%)	0	1 (11.11%)	0		
28 years old	3 (11.54%)	1 (8.33%)	0	1 (11.11%)	2 (14.29%)		
30 years old	3 (11.54%)	1 (8.33%)	0	1 (11.11%)	2 (14.29%)		

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; *b*: Chi-square test or Fisher's exact test

2.2.4.3.6 Additional comments

The importance of being informed (in general) and receiving tailored information about ovarian reserve was evaluated by participants, in the three time-points from the assessment protocol. This evaluation utilized a Likert scale ranging from 1 ('Not important at all') to 5 ('Extremely important')."

Participants' views on the importance of being informed about this topic and the significance of ovarian reserve evaluation remained consistent over the study (Table 55), with non-significant changes observed (p=.516 for information and p =.624 for evaluation). The mean scores for information were 4.48 $\pm$.75 at the initial assessment, 4.44 $\pm$.71 at the t1, and 4.40 $\pm$.72 at the t2. Similarly, the mean scores for evaluation were 4.40 $\pm$.77 at the initial assessment, 4.34 $\pm$.78 at the t1, and 4.40 $\pm$.87 at t2.

Considering the various subgroups formed based on the results of ovarian reserve testing, there was no change in the importance participants gave on being informed about the topic and evaluating their ovarian reserve (Table 55). In another words, the interaction between time and groups showed a p-value of .264 for DOR1 and NOR, and p=.601 for DOR2, INT, and NOR regarding the importance of being informed. Similarly, it revealed a p-value of .143 for DOR1 and NOR, and p=.233 for DOR2, INT, and NOR concerning the evaluation of ovarian reserve.

Table 55 Participants opinions on the importance of being informed about and evaluating ovarian reserve, considering the interaction of between time and groups

The options were evaluated on a scale of 1 to 5, with higher scores indicating a stronger agreement with the option. The table includes mean and SD of responses for three time-points (t0, t1, and t2), considering the ovarian reserve subgroups (NOR, DOR1, DOR2, INT). The results of the statistical analysis (p-values) to examine the within-subjects effects of time, the between-subjects effects of group, and the interaction effects between time and group are reported.

How important is it to you Item evaluated on a scale of 1 to 5.		Mean	SD	Mean	SD	Mean	SD	Effect	Time p-value <i>d</i>	Time*Group p-value <i>d</i>
to be informed about ovarian reserve	TOTAL (N=105)	4.48	.75	4.44	.71	4.40	.72	Within Subjects	p=.516	
	NOR (N=46)	4.39	.77	4.39	.68	4.43	.62	Within Subjects	p=.638	p=.264
	DOR1 (N=59)	4.54	.73	4.47	.73	4.37	.79	Between Subjects	p=.633	
	NOR (N=46)	4.39	.77	4.39	.68	4.43	.62	Within Subjects	p=.635	p=.601
	DOR2 (N=8)	4.50	.76	4.50	.76	4.38	.74	Between Subjects	p=.893	
	INT (N=51)	4.55	.73	4.47	.73	4.37	.80			
to evaluate ovarian reserve	TOTAL (N=105)	4.40	.77	4.34	.78	4.40	.87	Within Subjects	p=.624	
	NOR (N=46)	4.33	.76	4.30	.84	4.48	.75	Within Subjects	p=.569	p=.143
	DOR1 (N=59)	4.46	.77	4.37	.74	4.34	.96	Between Subjects	p=.883	
	NOR (N=46)	4.33	.76	4.30	.84	4.48	.75	Within Subjects	p=.478	p=.233
	DOR2 (N=8)	4.50	.76	4.38	.74	4.63	.74	Between Subjects	p=.882	
	INT (N=51)	4.45	.78	4.37	.75	4.29	.99			

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; *d*: repeated measures ANOVA and Friedman test

At t2, after receiving tailored information, no significant differences were found according to the information received about the ovarian reserve status (Table 56). The comparison between DOR1 and NOR resulted in a p-value of .540, and the comparison among DOR2, INT, and NOR had a p-value of .727. The majority of participants (58.1%) classified reproductive counselling as "extremely important" (Table 56).

Table 56 Perspectives on the importance of reproductive counselling consultations

The responses are evaluated on a scale of 1 to 5, with 1 representing the lowest importance and 5 representing the highest. The percentages and p-values are reported across different subgroups.

	How important is it to you to have had a reproductive counselling consultation? N(%)						
Item evaluated on a scale of 1 to 5.	Total (N=105)	DOR1 (N=59)	DOR2 (N=8)	INT (N=51)	NOR (N=46)	DOR1 Vs NOR p-value <i>b</i>	DOR2 Vs NOR Vs INT p-value <i>b</i>
1: Not at all important	1 (.95%)	1 (1.69%)	0	1 (1.96%)	0	p= .540	p= .727
2: Slightly important	5 (4.76%)	3 (5.08%)	0	3 (5.88%)	2 (4.35%)		
3: Moderately important	6 (5.71%)	5 (8.47%)	1 (12.5%)	4 (7.84%)	1 (2.17%)		
4: Very important	32 (30.48%)	16 (27.12%)	1 (12.5%)	15 (29.41%)	16 (34.78%)		
5: Extremely important	61 (58.1%)	34 (57.63%)	6 (75%)	28 (54.90%)	27 (58.70%)		

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; *b*: Chi-square test or Fisher's exact test

2.2.4.3.7 Assessing the anxiety levels before and after the interventions

The STAI questionnaire, Portuguese version by Silva & Spielberger (Silva & Spielberger, 2011), was included in the three time-points from the assessment protocol to evaluate anxiety levels among participants both before and after the interventions in knowledge.

The results indicate that anxiety levels remained stable over time and were not significantly influenced by the information provided during the study, as illustrated in Table 57. Specifically, the anxiety levels revealed no significant differences after the key interventions ($p=.714$ for STAI-S and $p=.104$ for STAI-T). The interaction between time and groups formed according to the information received was also accessed. No significant differences were found for STAI-S ($p=.272$ considering DOR1 and NOR, and $p=.558$ DOR2, INT, and NOR), nor for STAI-T ($p=.344$ considering DOR1 and NOR, and $p=.744$ DOR2, INT, and NOR), nor for STAI-T

Table 57 STAI scores with various parameters such as mean and SD for different subgroups

The table includes mean and SD of STAI-S and STAI-T score for three time-points (t0, t1, and t2), considering the ovarian reserve subgroups (NOR, DOR1, DOR2, INT). The results of the statistical analysis (p-values) to examine the within-subjects effects of time, the between-subjects effects of group, and the interaction effects between time and group are reported.

		t0		t1		t2				
STAI SCORE		Mean	SD	Mean	SD	Mean	SD	Effect	DOR1 Vs NOR p-value b	DOR2 Vs NOR Vs INT p-value b
STAI-S	TOTAL (N=105)	47.74	4.26	47.7	3.38	47.53	3.16	Within Subjects	p=.714	
	NOR (N=46)	47.80	4.38	47.30	3.54	47.13	2.95	Within Subjects	p=.632	p=.272
	DOR1 (N=59)	47.69	4.20	48	3.24	47.85	3.30	Between Subjects	p=.493	
	NOR (N=46)	47.80	4.38	47.30	3.54	47.13	2.95	Within Subjects	p=.738	p=.558
	DOR2 (N=8)	48.75	2.49	48.75	2.96	48.37	3.29	Between Subjects	p=.604	
	INT (N=51)	47.5~3	4.41	47.88	3.30	47.76	3.33			
STAI-T	TOTAL (N=105)	47.86	3.55	47.01	3.52	47.57	3.17	Within Subjects	p=.104	
	NOR (N=46)	47.80	3.88	46.85	3.98	47.22	3.07	Within Subjects	p=.151	p=.344
	DOR1 (N=59)	47.69	3.30	47.25	3.14	47.85	3.24	Between Subjects	p=.633	
	NOR (N=46)	47.78	3.88	46.85	3.98	47.22	3.07	Within Subjects	p=.123	p=.744
	DOR2 (N=8)	48.25	3.92	48.12	4.29	48.87	4.61	Between Subjects	p=.744	
	INT (N=51)	47.86	3.23	47.12	2.95	47.69	3			

t0: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups

AMH: Anti-Müllerian hormone; AFC: antral follicle count; STAI: State-Trait Anxiety Inventory, S: state and T: trait; SD: standard deviation; d: ANOVA repeated measures and Friedman test

2.2.4.3.8 Assessing the acceptability of innovative treatments for diminished ovarian reserve (DOR)

In addition to assessing knowledge and attitudes in our study, we also investigated the impact of PRP on human ovarian tissue as part of the third task of our project. Therefore, we aimed to understand the acceptability of PRP therapy among general population. The acceptability was assessed by multiple-choice questions. The Table 58 shows the distribution of responses, that were coded in 0: No, 1: Yes, 2: I don't know/I'm not informed.

At t0, 55 participants, equivalent to 52.38% of the total, expressed a compliance to accept innovative therapies. This acceptability remained consistent across the subgroups, NOR and DOR1, across the three time-points analysis (Table 58). Differences emerged, at t2 regarding the comparison of NOR, DOR2, and INT (p=.033).

Table 58 Participants' acceptability to undergo an innovative treatment (PRP) if they knew that they would have DOR

The acceptability is measured over three time-points (to, t1, t2) and analysed across different subgroups. The items evaluated were assessed by multiple-choice questions. The table shows the distribution of responses (No, Yes, I don't know/I'm not informed) for each time-point (to, t1, t2) across different participant subgroups (NOR, DOR1, DOR2, INT). The percentages reflect the proportion of each subgroup's total number of participants. The p-values indicate whether there are statistically significant differences in responses between the specified subgroups at to,t1,and t2. Statistical significance (p-value<.05) is highlighted in blue.

If you knew in advance that you would have DOR. would you consider undergoing an innovative treatment (e.g., therapy with PRP)?	TOTAL (N=105) N (%)	NOR (N=46)		DOR1 (N=59)		DOR1 Vs NOR p-value <i>b</i>	NOR (N=46)		DOR2 (N=8)		INT (N=51)		DOR1 Vs NORVs INT p-value <i>b</i>	
		N	%	N	%		N	%	N	%	N	%		
to														
No	8 (7.62%)	5	10.87	3	5.08	p=.416	5	10.87	0	0	3	5.88	p= .661	
Yes	55 (52.38%)	25	54.35	30	50.85		25	54.35	5	62.5	25	49.02		
I don't know/I'm not informed	42 (40%)	16	34.78	26	44.07		16	34.78	3	37.5	23	45.1		
t1														
No	8 (7.62%)	4	8.7	4	6.78	p=.416	4	8.7	1	12.5	3	5.88	p= .701	
Yes	60 (57.14%)	29	63.04	31	52.54		29	63.04	4	50	27	52.94		
I don't know/I'm not informed	37 (35.24%)	13	28.26	24	40.68		13	28.26	3	37.5	21	41.18		
t2														
No	7 (6.67%)	4	8.7	3	5.08	p= .237	4	8.7	2	25	1	1.96	p=.033	
Yes	64 (60.95%)	31	67.39	33	55.93		31	67.39	5	62.5	28	54.9		
I don't know/I'm not informed	34 (32.38%)	11	23.91	23	38.98		11	23.91	1	12.5	22	43.14		

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq$ 25th percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC < 25th percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC < 25th percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC < 25th percentile.

AMH: Anti-Müllerian hormone; AFC: antral follicle count; *b*: Chi-square test or Fisher's exact test

We aimed to determine whether receiving less reassuring information about ovarian reserve would alter the acceptability of a specific treatment (Table 59). Once the item was assessed in a multiple-choice format, mean values close to 1 indicated acceptance, close to 2 indicated uncertainty, and close to 0 indicated non-acceptance (answers were coded as 0: No, 1: Yes, 2: I don't know/I'm not informed).

When examining the subgroups of NOR and DOR1, the previously described pattern persisted, with no significant differences across time (p=.977) or in the interaction of time and group (p=.887). However, when considering the subgroups NOR, DOR2, and INT the influence of time became evident (p=.025), particularly following the communication of ovarian reserve testing results (t2). This resulted in an increase in consideration of this treatment from t1 to t2 within the INT subgroup, and a decrease among DOR2 and NOR (p=.042), as illustrated in Figure 26. Importantly, this comparison involves subgroup analysis, which could imply a loss of statistical power. The effect of the interaction between time and group was close to significance (p=.080).

Table 59 Participants' acceptability to undergo an innovative treatment (PRP) if they knew that they would have DOR, considering the interaction between time and groups

The acceptability is measured over three time-points (to, t1, t2) and analysed across different subgroups. The items evaluated were assessed by multiple-choice questions, with the responses likely being quantified in a range It includes means, SD, effect sizes, and p-values to understand the significance and magnitude of changes over time, between subgroups, and the interaction effects between time and group. Statistical significance (p-value<.05) is highlighted in blue.

If you knew in advance that you would have DOR, would you consider undergoing an innovative treatment (e.g., therapy with blood cells)?											
Item evaluated by multiple choice (1 to 3)											
	to		t1		t2						
	Mean	SD	Mean	SD	Mean	SD	Effect	Time p-value d	to Vs t1 p-value d	t1 Vs t2 p-value d	Time*Group p-value d
TOTAL (N=105)	1.32	.61	1.28	.60	1.26	.57	Within Subjects	p=.383			
NOR (N=46)	1.24	.64	1.19	.58	1.15	.56	Within Subjects	p=.977			p=.887
DOR1 (N=59)	1.39	.59	1.34	.60	1.34	.57	Between Subjects	p=.114			
NOR (N=46)	1.24	.64	1.19	.58	1.15	.56	Within Subjects	p=.025	p=.356	p=.042	p=.080
DOR2 (N=8)	1.37	.52	1.25	.71	.88	.64	Between Subjects				
INT (N=51)	1.39	.60	1.35	.59	1.41	.54					

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR1: diminished ovarian reserve subgroup 1, AMH or AFC $< 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile.

The lighter grey shading distinguishes NOR and DOR1 from the darker grey, which is associated with the NOR, DOR2, and INT subgroups

AMH: Anti-Müllerian hormone; AFC: antral follicle count; DOR: diminished ovarian reserve; SD: standard deviation; d: ANOVA repeated measures and Friedman test

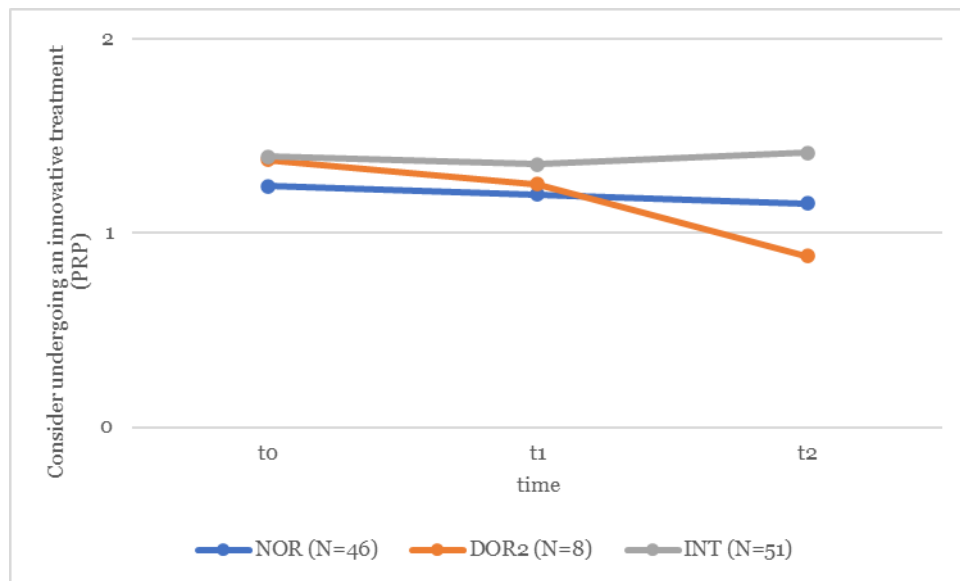


Figure 26 Participants acceptability to undergo an innovative treatment (PRP) if they knew in advance that they would have DOR

Representation of the acceptability of an innovative treatment, measured over three time points (to, t1, t2) and analysed across different subgroups (NOR, DOR2, INT). The acceptability was evaluated by multiple choice, 0: No, 1: Yes, 2: I don't know/I'm not informed

to: baseline evaluation; t1: after receiving general information; t2: after receiving tailored information

NOR: normal ovarian reserve, AMH and AFC $\geq 25^{\text{th}}$ percentile; DOR2: diminished ovarian reserve subgroup 2, AMH and AFC $< 25^{\text{th}}$ percentile; INT: intermediate subgroup, AMH and AFC discordant, AMH or AFC $< 25^{\text{th}}$ percentile

AMH: Anti-Müllerian hormone; AFC: antral follicle count

2.2.4.3.9 Assessing the informative content produced during the study

To assess the usability of both the website and the video, we conducted by semi-structured interviews involving 50 women without children and who had no history of infertility, aged between 18 and 35 years old. These women comprised a different group of participants from those who took part in the interventions in knowledge. Recruitment was done through the promotion of the study, and participants volunteered by emailing their interest. The semi-structured interviews were conducted through video calls.

The age distribution of participants is as follows: 18-24 years old (20%), 25-32 years old (60%), and 30-35 years old (20%). The reported mean age was 33.24 ± 3.35 years (Table 60).

In this evaluation, 78% of participants reported prior knowledge of ovarian reserve. Initial knowledge levels had a mean value of 28.34 ± 27.94 of percentage of correct regarding the age at which female fertility declines, the average age of women at first childbirth in Portugal, and DOR incidence. The percentage of correct answer significantly increased to 98.02 ± 7.91 , after exposure to both the video and the website content (Table 60, $p < .001$).

Table 60 Assessing the informative content: participants age and knowledge levels

Age (years)	Percentage of participants
18-24	20%
25-32	60%
33-35	20%
Mean age (years) $\pm$ SD	33.24 ± 3.35
Participant knowledge level	Percentage of participants (%)
Heard about ovarian reserve (Yes)	78%
Initial knowledge	Range: 0% to 100%
Mean % of correct answers $\pm$ SD	28.34 ± 27.94
Final knowledge	Range: 67% to 100%
Mean % of correct answers $\pm$ SD	98.02 ± 7.91

SD: standard deviation

2.2.4.3.9.1 The website

All 50 participants unanimously found the website's content to be clear and informative (Table 61). Additionally, participants evaluated the information relevance to their prior knowledge in reproductive health on a scale of 1 (not relevant at all) to 5 (extremely relevant). The majority of participants (64%) rated the content as highly relevant (4 out of 5) to their existing knowledge in the topic, with 10% rating it moderately relevant (3 out of 5) and 26% rating it extremely relevant (5 out of 5, Table 61).

Furthermore, every participant reported that they found it easy to navigate through the content and that they have learned something new about reproductive health and ovarian reserve. None of the participants found the information confusing (Table 61). Additionally, all participants recognized that the content was suitable for individuals without prior knowledge in the field. Lastly, every participant expressed they would recommend the content to friends or relatives (Table 61).

Table 61 Assessing the informative content: summary of the website evaluation results

Feedback category	Rating (1-5)	Percentage
Clarity (Yes)	NA	100%
Relevance to knowledge	3	10%
1: Not relevant at all	4	64%
2: Slightly relevant	5	26%
3: Moderately relevant		
4: Highly relevant		
5: Extremely relevant		
Ease of navigation (Yes)	NA	100%
Learnt something new about reproductive health (Yes)	NA	100%
Learnt something new about ovarian reserve (Yes)	NA	100%
Existence of unclear information (Yes)	NA	0%
Adequacy for people without prior knowledge about the subject (Yes)	NA	100%
Likelihood to recommend it (Yes)	NA	100%

NA: not applicable

2.2.4.3.9.2 The video

All 50 participants found the video content to be clear and informative (Table 62). Participants evaluated the video's information relevance to their prior knowledge in reproductive health on a scale of 1 (not relevant at all) to 5 (extremely relevant). The video evaluation revealed that the majority of participants (58%) rated the relevance of the content as 4 out of 5, indicating it was highly relevant. Additionally, 30% of participants rated it 5 out of 5, considering its extreme relevance, while 12% rated it 3 out of 5, indicating a moderate level of relevance (Table 62). Moreover, all participants unanimously found the video's duration appropriate (Table 62).

Furthermore, a significant majority (92%) of participants reported learning something new about reproductive health (Table 62).

None of the participants reported confusion, indicating clear and understandable content delivery (Table 62). Additionally, all participants affirmed that the video was suitable for individuals without prior knowledge in the field. Consequently, every participant expressed their willingness to recommend the video to others (Table 62).

Table 62 Assessing the informative content: summary of the video evaluation results

Feedback category	Rating (1-5)	Percentage
Clarity of content (Yes)	NA	100%
Relevance to knowledge	3	12%
1: Not relevant at all	4	58%
2: Slightly relevant	5	30%
3: Moderately relevant		
4: Highly relevant		
5: Extremely relevant		
Adequate duration of the video (Yes)	NA	100%
Learnt something new about reproductive health (Yes)	NA	92%
Learnt something new about ovarian reserve (Yes)	NA	100%
Existence of unclear information (Yes)	NA	0%
Adequacy for people without prior knowledge about the subject (Yes)	NA	100%
Likelihood to recommend it (Yes)	NA	100%

NA: not applicable

2.2.5 Discussion

Reproductive counselling is recommended to help manage expectations about reproductive plans. It is particularly important to advise young adults to avoid delaying childbearing, due to an increased risk of DOR (Nesbit et al., 2020). To address this issue, an intervention program was developed, its efficacy was tested, and the results will now be discussed.

When discussing the results of the impact of an intervention program, it is crucial to reflect on the studied population, as it can directly influence our findings. Firstly, there are no studies regarding interventions focused on DOR within the Portuguese population. In designing our study, we included women aged 18 to 35 as the priority target population. Our inclusion criteria partially align with a study by Chan et al. which chose an age range of 25–34 years at the time of recruitment, considering the primary rationale for this age range: it aligns with the ideal timeframe to encourage childbearing and addresses fertility-related topics before the onset of age-related fertility decline (Chan et al., 2022).

Analysing the study population's motivation, the importance of enrolling in the study, and the perception of the information provided are critical aspects. Additionally, determining the attrition rate and identifying the percentage of participants who participated in the study through spontaneous interest are valuable indicators. These factors help assess whether the intervention program aligns with the specific characteristics and needs of the target population (women aged 18 to 35) and how effectively the interventions address their concerns related to reproductive health and fertility.

At first glance, revisiting studies that emphasized a significant impact of fertility education on enhancing knowledge, it is possible to find shared similarities in the populations considered with ours. Specifically, the age range partially overlaps with studies such as the RCT by Maeda et al. which included both men and women aged 20-39 years (Maeda et al., 2016, 2018). Skogsdal et al. also included women aged 20-40 years (Skogsdal et al., 2019), and Fooladi et al. which included women with mean ages ranging from 27.6 to 31 years (Fooladi et al., 2018). Stokes et al. included both men and women aged 16 years and older (Stokes et al., 2023). It is worth mentioning that the FAC clinic, a pioneering example worldwide aiming to provide fertility assessment for both men and women, has no age limits. Among the first 916 women attending the FAC clinic, the mean female age was 33.4 years, with an age range of 19 to 46 (Hvidman et al., 2015). Additionally, like our study, childlessness was also considered a criterion in other studies (Daniluk & Koert, 2015; Pedro et al., 2020).

Our study included 105 participants aged between 20 and 34, with a mean age of 27.35 years. Of these, 79% joined the study through spontaneous interest, with a mean age of 28.06 years. In contrast, 21% were recruited during family planning consultations (where participation in the study was not their primary objective), and this group had a significantly lower mean age of 24.64 years. Interestingly, from the 13 individuals who withdrew from or dropped the study, 10 were recruited within the family planning consultation, having a mean age of 23.85 years old.

Self-selection bias, commonly observed in studies similar to ours, can occur when evaluating fertility knowledge, especially among individuals from related fields, those with high education levels, or those inherently interested in the topic (Stokes et al., 2023). To mitigate this, we analysed the relationship between prior global knowledge and the participants, according to their background, and no significant differences were found.

Comparing our results with the ones found in other studies, which aimed to assess knowledge and attitudes regarding fertility, differences were found in the representation of age groups. For instance, Stokes et al. reported that the most prominently represented age group was 25 years old (44.8%) (Stokes et al., 2023), whereas our study had a higher mean age of 27.34 years, with only 4.8% of participants being 25 years old. This percentage remained the same for those who enrolled spontaneously and was slightly lower (4.5%) for participants recruited through family planning consultations. While Stokes et al. and Skogsdal et al. reported a mean age of 25 years old for their population (Skogsdal et al., 2019), the mean age reported in the other studies was higher (Hvidman et al., 2015; Maeda et al., 2016, 2018). For instance, Fooladi et al., reported a mean age in the intervention group of 27.6 years old (Fooladi et al., 2018), which is very similar to ours.

Stokes et al. detailed that 24.1% of their participants were aged 30-34 years (Stokes et al., 2023), while our study showed a higher representation in this age group at 33.3%. Consequently, the authors of this study were able to conclude that the age groups >25 years old and 30-34 years old are optimal targets for fertility education (Stokes et al., 2023). In our study, we found that spontaneous participation was more common among women aged 20-29 years, with the highest percentages attributed to those aged 28-30 years (together accounting for 32.4%). This likely reflects the fact that individuals in this age group are more actively contemplating childbearing, aligning with the average childbearing age in Portugal, which is around 30 years old (PORDATA, 2023).

Importantly, the referred studies for the comparison analysis have some differences to ours, since they include both men and women (Maeda et al., 2016, 2018; Stokes et al., 2023). Additionally, they applied broader inclusion criteria: considering age, both with and without children (Fooladi et al., 2018; Fujishima et al., 2024; Maeda et al., 2016, 2018; Skogsdal et al., 2019). These factors could justify differences found between our study and theirs. Furthermore, our selection of this age interval was scrutinized by the participants when questioned whether they believed they should have received this information earlier in life, with only 24.76% responding positively. This sentiment remained consistent even among those who were informed that their ovarian reserve was under the expected values. Among participants who believed they should have received information earlier, the majority indicated that the ideal age is around 25 years old. This aligns with the findings of the Stokes et al, that advocate that ages around this range are the optimal targets for fertility education (Stokes et al., 2023).

Regarding the attrition rate, ours was 11%, which is similar to a face-to-face study conducted over 2 months, which had an attrition rate of 12% (Stern et al., 2013). It is considerably lower than the attrition rates found in other studies: a 2-year study with a 49% attrition rate (Maeda et al., 2018),

a 6-month online study with a 45% attrition (Daniluk & Koert, 2015); and a 1-year study with a 77% attrition rate (Pedro et al., 2020). Our study has shown a higher follow-up rate when compared to long term follow-up studies (Daniluk & Koert, 2015; Maeda et al., 2018; Pedro et al., 2020), and similar rates within the same term study (Stern et al., 2013).

Participants have higher education levels, with 55.24% holding a Master's degree, and the vast majority were either employed or studying (94.29%). Furthermore, a majority of participants (72.38%) were in a relationship. In terms of lifestyle factors, a large majority of our participants (84.76%) reported not smoking, and the average BMI was calculated at 24.35 kg/m^2 . These findings, as well as the maternal age at menopause and the age of menarche, are aligned with the ones observed in the FAC clinic (Hvidman et al., 2015; Petersen et al., 2017). A noteworthy observation regarding BMI was the positive correlation found between AMH levels and BMI. This suggests that individuals with higher body mass indexes might exhibit elevated levels of AMH, indicating a potentially better ovarian reserve—a phenomenon particularly pronounced in cases of PCOS (Abbara et al., 2019). However, it is important to highlight that this correlation was specifically noted within the population where both AMH and AFC levels fell below the 25th percentile for their age groups. One plausible explanation for this discrepancy could be the limited sample size within this group (N=8), which could have introduced biases into the analysis. Furthermore, no association was detected between smoking and ovarian reserve markers, supporting the findings of Oladipupo et al. (Oladipupo et al., 2022).

In terms of contraception usage, 57.14% of the participants were using the pill. This figure falls within the reference range of 50 to 89% reported for Western countries (Petersen et al., 2015), and closely resembles the percentages of 58% and 57.2% reported in two different studies on the Portuguese population (Águas & Bombas, 2016; Cunha et al., 2022). In fact, a pertinent factor requiring consideration in tailored counselling is the utilization of hormonal contraception, known to potentially influence AMH and AFC results interpretation. We chose not to ask participants to discontinue hormonal contraception, aligning with the established approach in this field. At the FAC clinic, 50–89% of women have used hormonal contraceptives at some point in their lives, and the clinic does not advise women using hormonal contraception to stop their method. However, individuals are informed that this may influence the results of the assessment (Hvidman et al., 2015) – a protocol consistent with our study methodology.

In this study, we observed that participants using hormonal contraception were more likely to exhibit discrepancies between AMH and AFC, with AMH levels being the ones under the 25th percentile. Users of hormonal contraception had AMH levels that were 41.23% lower than those of non-users, which closely aligns with the findings reported by Arbo et al. who reported a decrease ranging from 15 to 44% (Arbo et al., 2007). AFC values in users of hormonal contraception were found to be 19.58% lower compared to those in non-users, a result consistent with the 18% decrease reported by Petersen et al. (Petersen et al., 2015). Despite the slight underestimation of AMH and AFC values due to hormonal contraception, we firmly believe that the benefits far outweigh any drawbacks. Practical considerations such as encouraging

participants from suspending contraception, particularly impactful in a population generally not seeking immediate pregnancy, support the study's design.

The prevalent use of oral contraception among almost 60% of participants minimizes the potential influence of dominant follicles or corpus luteum from previous cycles on the counting process. Furthermore, the flexibility of scheduling ultrasounds without cycle day restrictions enhances the study's efficiency and convenience, ensuring robust results. Nevertheless, we acknowledge the importance of being prudent when interpreting AMH and AFC results in individuals using hormonal contraceptives. Therefore, as part of our counselling approach, we mentioned that AMH and AFC values in women using hormonal contraception may be up to 20% higher than the values measured. Moreover, we offer the option to repeat the measurements three months after discontinuing hormonal contraception. This strategy was employed in cases where there are discrepancies between AMH and AFC values, as well as when both AMH and AFC levels are found to be low. This methodology was based on the insights and expertise of researchers in the field (Hvidman et al., 2015; Petersen et al., 2015).

The study population was found to exhibit high motivation regarding the topic, with 77.14% rating the two highest scores on an importance scale for the desire to have children, as well as 58.1% positioned themselves as being sure to have children in future. Additionally, a significant percentage of participants (59.05%) expressed a high to moderate (4 and 3 out of 5) level of certainty regarding their plans about parenthood, with only 4.76% of them rating their plan as well-defined (5 out of 5). However, this 4.76% represents the lowest proportion compared to the responses for other categories, such as education (where 62.86% rated their plan as 5 out of 5), career goals (21.9%), purchasing a house (20%), living abroad (15.24%), and marriage (12.38%).

This conducts us to examine the motivations behind participating in this study. Studies by Hvidman et al. and Petersen et al. have highlighted the perspective of the FAC clinic, a view recently corroborated by a follow-up study. According to these studies, the most common motivation for accessing fertility information and seeking reproductive counselling was to estimate how long individuals could safely delay childbearing, with concerns about fertility also frequently cited (Hvidman et al., 2015, 2015; Koert et al., 2020; Sylvest et al., 2018). This partially aligns with what we observed in our study, where the most frequently chosen reason for participation was concern about fertility (selected 70 times), followed closely by the desire to seek information about the topic (selected 68 times), while confirmation of postponement of motherhood was chosen only 9 times.

In line with the desire for more information regarding reproductive health, participants exhibited an increase in baseline knowledge following the two interventions. Both the general informational leaflet and the tailored information and reproductive counselling proved effective in improving knowledge, as anticipated based on the success observed in other studies (Fooladi et al., 2018; Fujishima et al., 2024; García et al., 2016; Maeda et al., 2016, 2018; Pedro et al., 2020; Skogsdal et al., 2019).

Considering the higher education level of this study population, this factor could be considered a bias when evaluating global knowledge on the topic. However, we found no relationship between education level and awareness of ovarian reserve and ovarian reserve testing. Additionally, specific knowledge about reproductive health, as assessed in our questionnaire, was not related to educational level. Our results align with a recent review by Ren et al. which indicated that fertility awareness levels are generally low to moderate among university students, whereas women and medical students tend to exhibit higher levels of fertility awareness (Ren et al., 2023).

In this study it became evident that higher education is associated with delaying the intended age of motherhood of participants. However, despite this delay, there was no significant impact on the perceived number of children they could have. This observation aligns with a misconception about fertility, highlighted by Lino et al. In their study, involving women attempting to conceive spontaneously (N=1542), they found that there is a false perception that they can become mothers later in life. In the case of this study, 21.9% believed they could still have children at the age of 45 (Lino et al., 2022)

When referring to changes in childbearing intentions and reproductive attitudes and plans, the results differed from those found regarding the impact of these interventions on knowledge. Most of the reported data in about the effectiveness of interventions in knowledge to change the reproductive intentions show no significant impact (Boivin et al., 2018; Fooladi et al., 2018; García et al., 2016; Maeda et al., 2016; Pedro et al., 2020; Skogsdal et al., 2019), with a few exceptions that reported an anticipation of the age of pregnancy (Fujishima et al., 2024; Stern et al., 2013; Wojcieszek & Thompson, 2013), and behavioural changes (Maeda et al., 2016). Maeda et al. reported the use of a chatbot that has proved to be more effective than a brochure and was associated with increased intentions to optimize preconception behaviours (increase the proportion of people reporting to be prepared to get pregnant) (Maeda et al., 2020).

According to a protocol report, individualized interventions that include counselling and personalized educational materials, such as brochures, websites, or videos, have shown the potential to enhance fertility knowledge in the short term (Chan et al., 2022). The evidence suggests that tailored interventions tend to yield more favourable outcomes compared to generic interventions (Chan et al., 2022). Because of that, we dedicated our efforts to developing specific materials for this project, which included a leaflet, a website, and a video. The website and video were assessed through semi-structured interviews, a method commonly recommended in similar studies (Hamilton & Harper, 2024). This analysis primarily served as a proof of concept for the developed content, with a predominant focus on qualitative analysis. Findings from the website evaluation reveal a high level of satisfaction regarding its clarity, relevance, accessibility, and educational value. Similarly, feedback on the video indicates a generally positive reception and perceived utility, with all participants expressing their willingness to recommend it to others. In terms of quantitative assessment, knowledge levels were measured and found to have significantly increased following exposure to both the video and website content ($p < .001$). It is worth noting that this assessment offers a limited scope in evaluating the impact of the content, as it primarily

compromises a small subset of questions compared to the structured questionnaire used in the remaining analysis.

Furthermore, the calculator, which provided a colour code indicating the percentile for both AMH and AFC, proved to be highly beneficial for counselling, for clinicians, and for participants. We anticipate that this approach could be implemented on a larger scale to facilitate the conveyance and interpretation of medical results across different departments, reducing the likelihood of misinterpretation. In line with this is the given the increasing prevalence of AMH testing (Copp et al., 2023, 2024; Johnson et al., 2023). For instance, in Australia, approximately 15% of general practitioners (GPs) and 40% of gynaecologists and other specialists prescribe at least one AMH test per month. Surprisingly, patient requests were more common among GPs. It is worth noting that half of the clinicians surveyed expressed a lack of confidence in interpreting (51%) and explaining (48%) AMH results to patients. Besides that, 40% of clinicians had previously prescribed the test for reproductive planning purposes (Copp et al., 2024).

The information provided to our participants, even when less optimistic about their ovarian reserve, did not influence the number of children they believe they can have or their intention to anticipate the age at which they want to have their first child. What is intriguing is that, simultaneously, the notion of abandoning parenthood was not further contemplated after they received this information, and it was consistently unlikely to be considered. Even when not considering the anticipation of pregnancy, participants also do not consider the possibility of egg freezing as a strategy to manage the postponement of motherhood, despite the majority agreeing that making oocyte freezing accessible is important. Particularly, participants who received less reassuring information showed a decreased intention to consider undergoing COS for oocyte preservation. These findings are consistent with those reported by Kim et al., where a study involving medical staff found that nearly one-third (30.5%) expressed reluctance towards considering oocyte freezing (Kim et al., 2022). Also in this study, less than 20% of participants indicated their willingness to donate oocytes (Kim et al., 2022), something that is in line with our study results – a stable opinion over time, with participants mostly showing uncertainty about donation and expressing the need for more information.

In this section, our interventions requested participants to imagine receiving a diagnosis of DOR and to consider the strategies they might admit. Regarding adoption, there was a decrease in the intention to choose this option among participants who were informed that their ovarian reserve was actually below the expected level for their age. This result aligns with Yassini et al. who emphasized the low acceptance of adoption among those experiencing infertility (Yassini et al., 2012). When it came to the option of using donor oocytes, which is often considered the most impactful scenario in cases of DOR (Devenuto et al., 2020), participants showed a significant increase in discomfort about the idea that the child would not be biologically theirs, after the reproductive counselling. This may be attributed to their exposure to a new reality, which they may not have previously considered. This reaction is commonly described among couples facing the prospect of undergoing egg donation (Montagnini et al., 2023).

Although the tailored intervention did not significantly alter participants' childbearing intentions or reproductive plans, they accurately perceived the information provided about their ovarian reserve. Firstly, participants distinctly assessed infertility, particularly DOR, as a significant obstacle to parenthood based on the specific information about their ovarian reserve. For instance, the subgroup with a normal ovarian reserve decreased their consideration of infertility as an obstacle after receiving this information. However, both subgroups with ovarian reserve under expected values and the intermediate subgroup with inconsistent results haven't decreased their consideration of infertility as an obstacle, as they received less reassuring information. This suggests that receiving tailored information about their ovarian reserve, rather than general information, led participants to be less likely to consider infertility as a potential issue that could interfere with their reproductive plans. Indeed, the evidence suggests that tailored interventions tend to be more effective in increasing awareness than generic interventions (Chan et al., 2022).

Moreover, participants who received information indicating that their ovarian reserve was under the normal range tended to perceive it as less optimistic, acknowledging that it could take longer to achieve pregnancy. In contrast, the subgroup with ovarian reserve within the normal range for their age perceived that everything appeared to be normal. This correspondence gave us confidence that the message was well assimilated. Additionally, the interventions were well received by the participants, as they reported learning something about their fertility and did not feel that the information was lacking.

Indeed, the way participants perceive the results of the tailored intervention holds paramount importance in this study. Providing clear and concise explanations about AMH and AFC testing was a priority, as we aimed to ensure that women fully comprehend the significance of these tests in assessing ovarian reserve. Acknowledging the limitations of the AMH test, namely its inability to accurately predict current and future fertility due to its incapacity to assess egg quality and anticipate the decline in follicular count over time (Harris et al., 2023; Lin et al., 2021; Steiner et al., 2017), we informed our participants about these limitations. We emphasized that AMH is best suited for predicting the number of oocytes in COS, it does not provide insight into the complete fertility picture. Other factors also influence the ability to conceive.

Finally, and according to the approach established by the FAC clinic (Hvidman et al., 2015), we abstained from providing a specific prognosis regarding participants' likelihood of conceiving. Instead, participants were offered an assessment of their AMH and AFC values relative to age-specific percentiles, along with general information on fertility and reproductive health. Importantly, besides the limited capacity of AMH and AFC to predict natural fertility (Dewailly et al., 2014), ovarian assessment is not exclusively pertinent to infertile women. It plays a crucial role in supporting reproductive intentions, particularly considering the delayed childbearing age (Petersen et al., 2015).

Another significant aspect of this study was the assessment of anxiety, given its commonly reported side effect in interventions related to fertility knowledge (Delbaere et al., 2020). Our

study found no differences in anxiety levels, as evaluated by STAI-S and STAI-T. This contradicts findings from some previous research (Boivin et al., 2018; Maeda et al., 2016).

In summary, it is imperative to assess the potential of interventions like the one we developed. The literature on fertility awareness approaches lacks published data focusing on information customized for DOR (Duane et al., 2022). However, a recent study by Herzberger et al. evaluated the impact of online educational content on ovarian reserve and fertility preservation treatments. Knowledge about female age-related fertility decline significantly improved after exposure to educational content (51.6% vs. 79.6%, $p < .001$) (Herzberger et al., 2022). This highlights the urgent need for action in this area.

While we cannot categorize our interventions as extremely impactful in terms of immediate changes in childbearing intentions and reproductive plans, they did yield important outcomes. Participants found the study useful and comprehended the results of their ovarian reserve evaluation. An important message was delivered, particularly considering the advocacy by Sinha et al. who emphasize the necessity of screening for ovarian reserve in women with DOR to identify those with a reduced likelihood of achieving pregnancy (Sinha et al., 2022). Furthermore, Temmesen et al. highlight the importance to develop preventive health initiatives that can assist and guide women in their considerations about the timing of motherhood (Temmesen et al., 2023).

A path to understand the real impact of interventions and initiatives in fertility education must pass by a long-term follow-up, as advocated by other authors within this field (Koert et al., 2022). In another words, our study could be strengthened by a long-term follow-up. For instance, the 6-year follow-up from the FAC clinic (N=24) has shown that 88% of women have tried to become pregnant, with 43% starting their attempts in the first year. All participants became parents to at least one child (Koert et al., 2020). Additionally, Koert et al. underscored the importance of having thoughtful discussions with women of reproductive age about the timing of attempting to become pregnant. Their results highlight that it is crucial to collaboratively map out and plan future pregnancies, taking into consideration realistic expectations within specific age ranges (Koert et al., 2020). This proactive approach is advocated to prevent potential grief and regret by providing clear insights into what is realistically achievable (Koert et al., 2022).

While the young adults and adults benefit from more focused information, it was already advocated that the younger cohort requires general guidance (Stokes et al., 2023). Therefore, another job-to-be done in future is extending our efforts to include structured interventions within educational settings, specifically schools. To this end, we are actively engaged in a pilot study in partnership with the IRHEC group under the umbrella of the ESHRE. This collaboration aims to integrate fertility education into school curricula, in Portugal, thereby reaching young individuals at a crucial stage of their reproductive journey.

Regarding reproductive counselling, based on our project observations, we propose an improved model. This model includes information sessions customized for healthcare professionals and community workshops strategically integrated within family health units. Moreover, we advocate

for the distribution of informative materials like flyers and roll-ups during these consultations. Given the presence of participants from the family planning demographic among the withdrawal rate (13 out of the initial 118 recruited, with 10 recruited from family planning), it is evident that this specific group experiences higher disengagement from the study.

For women who voluntarily seek to assess their ovarian reserve, we suggest establishing a self-refer counselling consultation at the reproductive medicine unit, CHUC. This model would adopt a one-stop clinic approach where AMH and AFC assessments can be conducted on the same day.

Bridging to the third and final task of this project (section 2.4), we evaluated the responses of women confronted with information about DOR related to their acceptability to undergo PRP therapy. The feedback was positive, with the majority expressing openness to that possibility across all three questionnaires.

The next task (Task 2, section 2.3) marks the transition to explore strategies to address DOR after its diagnosis. Task 2 regards exploring genetic approaches to potentially identify the aetiology of this condition and reveal new therapeutic targets for cases of infertility caused by DOR.

2. Strategies to increase the understanding of diminished ovarian reserve (DOR)

2.1 Overview of the project on fertility awareness, genetic testing, and interventions in the context of diminished ovarian reserve

Project research question, objectives, and tasks.

2.2 Task 1: intervention in knowledge through general and tailored information on ovarian reserve and diminished ovarian reserve (DOR)

Development and testing the impact of two interventions on knowledge about ovarian reserve DOR: 1) providing general leaflet information, and 2) offering tailored information and reproductive counselling based on the assessment of ovarian reserve.

2.3 Task 2: genetic analysis of infertile populations with abnormal ovarian reserve

Review of the role of hormonal receptor polymorphisms in ovarian function.

Identification of genetic variants concerning FSHR rs6166, ESR1 rs2234693 and ESR1 rs9340799 polymorphisms in cases of altered ovarian reserve (DOR and PCOS), among infertile populations.

Establishment of an NGS study protocol to identify genetic variants in the population with DOR, which includes reviewing the genes most relevant to this population and defining the gene panel to be analysed subsequently.

2.4 Task 3: *in vitro* study of follicular activation through the incubation of human ovarian tissue with platelet-rich plasma (PRP)

*Evaluation of the efficacy of PRP supplementation in activating the PI3K-Akt pathway and enhancing stromal vascularization, using an *in vitro* culture system, with human ovarian tissue.*

2.3 Task 2: genetic study of infertile populations with abnormal ovarian reserve

2.3.1 Introductory note, context, and objectives

At present, the identification of women with DOR relies on markers such as AMH and AFC, chosen for their ability to predict the ovarian response to COS and guide treatment strategies in IVF. Accordingly, AMH and AFC have been selected in this project for ovarian reserve screening and counselling, as detailed in Task 1. Nevertheless, we acknowledge two critical considerations: 1) the search for specific markers dedicated to diagnosing this condition more accurately, and 2) the exploration of targets that can enhance treatment efficacy. A genetic approach to address these aspects could significantly contribute to advancing diagnostic precision and optimizing therapeutic interventions for women with DOR.

Therefore, in Task 2, we began by reviewing the literature to understand the role of hormonal receptor polymorphisms in ovarian function. After that, our objective was to investigate the influence of genetic variants on hormonal receptor genes within distinct groups, namely those with DOR and PCOS, who were undergoing fertility treatments. The genomic analysis of women already diagnosed with infertility aimed to explore the possibility to establish tailored therapies in IVF.

Simultaneously, we aimed to investigate the occurrence of genetic variants in the non-infertile population with DOR. With this goal in mind, we established an NGS study protocol to identify genetic variants in the DOR population. This involved reviewing the most relevant genes within this scope and defining the gene panel that will allow for genetic screening when the number of samples is adequate. We estimate that approximately 500 participants are needed to conduct the NGS analysis effectively.

2.3.2 Current insights into genetic exploration in cases of altered ovarian reserve

Female infertility contributes to about one-third of all infertility cases. Although female infertility is widespread globally, the diagnosis and treatment of conditions associated with ovarian dysfunction are still limited (Phillips et al., 2023). Among these conditions, DOR and PCOS are recognized ovarian disorders characterized by abnormal follicular development (Kawamura et al., 2013). Notably, DOR and PCOS are both conditions impacting female fertility by disrupting the normal growth of follicles (Kawamura et al., 2013), and sharing dysregulation of key factors affecting follicle development (Dumesic & Abbott, 2008).

The most plausible explanation for DOR is the premature depletion of primordial follicles, although the underlying cause of this depletion is not yet fully understood (Persani et al., 2010). Literature suggests a potential genetic origin for DOR (Moiseeva et al., 2021), and emerging genetic biomarkers have been recommended for further investigation (Çelik et al., 2023). Similarly to DOR, the pathophysiology of PCOS is still poorly understood; however, several potential genetic susceptibility factors have been identified, including mutations and polymorphisms on the genes involved in the gonadal axis, steroid metabolism, cardiovascular risk, and insulin resistance (Unsal et al., 2009).

PCOS is the most common endocrine disorder in women of reproductive age, with a prevalence that may be close to 18% (March et al., 2010). Women with PCOS display a significant presence of small antral follicles while lacking preovulatory follicles (Dewailly et al., 2014). Consequently, it has been suggested that alterations in follicular activation occur in women with PCOS, caused by an abnormal extracellular matrix and rigid cortex that controls the Hippo signalling pathway (Maas et al., 2018). The similarity in therapeutic strategies between DOR and PCOS, particularly in modulating the follicular activation, suggests a potential convergence in regulatory mechanisms, and possibly in their aetiology (Devos et al., 2022).

Genetic determination of ovarian reserve remains a pertinent topic, as highlighted by Moiseeva and colleagues in their literature review published in 2021 (Moiseeva et al., 2021). For instance, SNPs have been shown to play an important role in ovarian function (Moiseeva et al., 2021). Particularly, SNPs in genes encoding for hormonal receptors are capable to influence the recruitment, development and maturation of follicles, and, because of that, might be associated with alterations in ovarian reserve (Hoogendoorn et al., 2003).

FSHR stands out as the most extensively studied hormonal receptor, owing to its crucial involvement in ovarian function and follicular development (Casarini et al., 2015; Eisele et al., 2021; Livshyts et al., 2009). Additionally, the analysis of the *FSHR* proves essential considering situations of impaired ovarian responses, attributed to a reduced number of FSH receptors in granulosa cells or impaired signal transduction. The literature consistently suggests that *FSHR* genotyping is promising in a pharmacogenomic approach for tailoring therapy to infertile women

(Tafazoli et al., 2021). Among the numerous polymorphisms already described in the literature, the *FSHR* Thr307Ala (rs6165) and Asn680Ser (rs6166) are the most studied (Baldini et al., 2023).

A study conducted by Neves et al., within a normoresponder population, explored the correlation between *FSHR* sequence variants and reproductive outcomes post IVF, in 368 women. Their findings suggest that individuals with the *FSHR* rs6165 genotype GG exhibit higher clinical pregnancy rates and live birth rates. Additionally, this study indicates a positive impact on cumulative live birth rates for those with the *FSHR* rs6166 genotype AA (Neves et al., 2023).

FSHR rs6166 Ser/Ser homozygous genotype was associated with a reduced ovarian response, higher concentrations of basal levels of FSH and higher requirement of gonadotrophins, when compared with women with the two other variants (Mayorga et al., 2000). In another study, findings indicated a significant prevalence of the amino acid serine of *FSRH* rs6166 in the abnormal responder's group (N=31 cases and N=36 controls). Conversely, in the normal responders group, the majority of patients were observed to be homozygous for asparagine (Baldini et al., 2023). However, these results are inconsistent (Daelemans et al., 2004; Greb et al., 2005; Klinkert et al., 2006; Mohiyiddeen et al., 2012, 2013; Trevisan et al., 2014).

Variations in *FSHR* Ser680Asn (rs6166) have been shown to be linked with changes in the ovarian reserve (Čuš et al., 2019; Pabalan et al., 2014). Additionally, FSH and *FSHR* play a crucial role in the development of antral follicles in the mammalian ovary, particularly associated with the PI3K-Akt pathway (Johnson et al., 2022). An association between *FSHR* Asn680Ser (rs6165) polymorphism and PCOS risk has been studied (Unsal et al., 2009) with conflicting results (Valkenburg et al., 2011).

ESR1 has been associated with poor ovarian response (De Mattos et al., 2014); however, contrasting results have also been reported in the literature (Li et al., 2014). SNPs in *ESR1* genes have been studied regarding regulation of the menstrual cycle, low fecundity, PCOS and POI (Altmäe et al., 2007; Livshyts et al., 2013; M'Rabet et al., 2012; Yoon et al., 2010). The two most common SNPs among *ESR1* are *ESR1* rs2234693 (PvuII, -397 TC, NM_000125.3:c.453-397 T > C) and *ESR1* rs9340799 (XbaI, -351 AG, NM_000125.3:c.453-351 A > G), respectively, PvuII and XbaI, named accordingly to their detecting restriction enzyme. These polymorphism have been associated with follicular senescence (Altmäe et al., 2011) and POI (Bretherick et al., 2008; Cordts et al., 2012; M'Rabet et al., 2012), but this conclusions are inconsistent (Corbo et al., 2007; De Mattos et al., 2014) or do not find this association at all (Silva et al., 2010). There are also reports on the associating *ESR1* polymorphisms with ovulatory defects (Sundarrajan et al., 2001) and a possible role in PCOS susceptibility has been suggested (Liaqat et al., 2015).

Considering our research background, at the reproductive medicine unit (Figure 27), the investigation of *FSRH* rs6166, *ESR1* rs2234693, and *ESR1* rs9340799 polymorphisms started in 2008. Over the years, 3146 distinct DNA samples were collected. Among these samples, 2351 have been genotyped for *ESR1* rs2234693 and *ESR1* rs9340799. Additionally, 2308 samples have undergone genotyping for *FSRH* rs6166, considering only the results that yielded clear and conclusive results. In some cases, when feasible and available information allowed, the

association of these polymorphisms with ovarian response was investigated. A comprehensive examination was conducted on a total of 105 cases to evaluate the impact of *ESR1* rs2234693 and *ESR1* rs9340799 on ART outcomes (Castro, 2016). Furthermore, two distinct studies were undertaken, one comprising 498 patients (Santos, 2013) and the other 563 patients (Reis, 2017), to investigate the influence of *FSRH* rs6166 on ART outcomes.

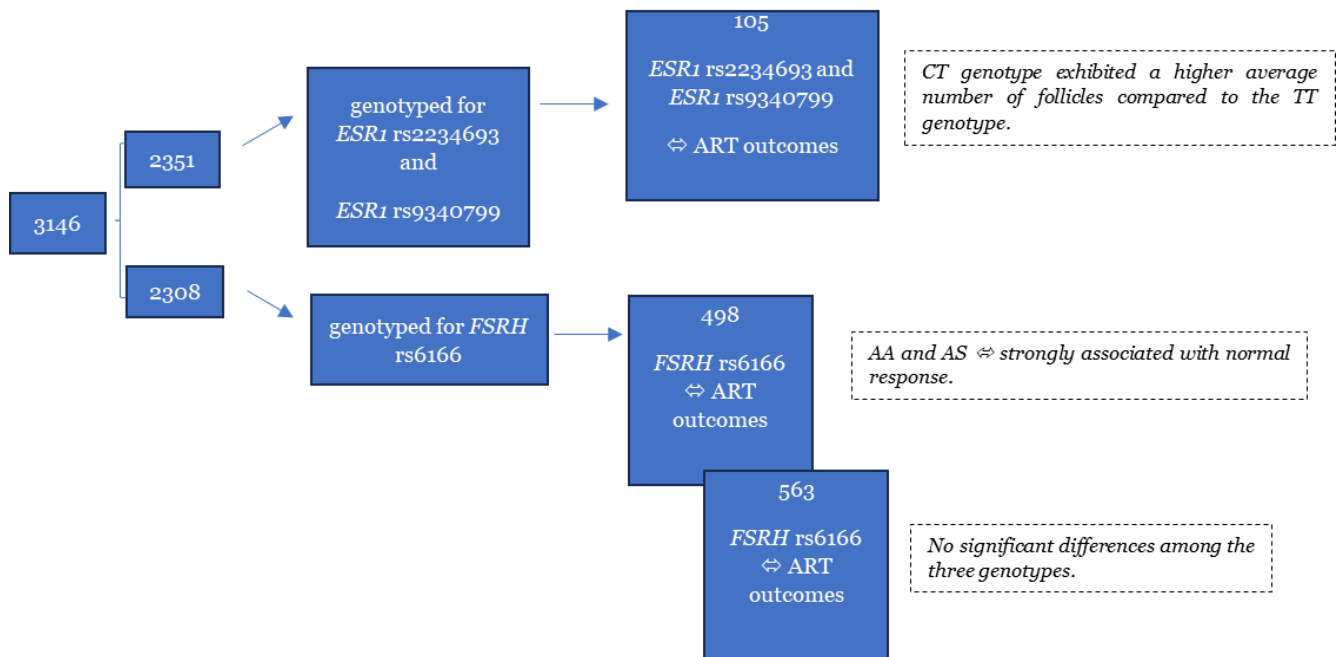


Figure 27 Diagram regarding the genetic polymorphism research

This diagram illustrates the genetic research background related to polymorphisms. Over the years, a total of 3146 distinct DNA samples were collected. Among these samples, 2351 underwent genotyping for *ESR1* rs2234693 and *ESR1* rs9340799. Additionally, 2308 samples were genotyped for *FSHR* rs6166. The association of these polymorphisms with ovarian response was investigated. A more detailed examination was carried out on a total of 105 cases to specifically assess the impact of *ESR1* rs2234693 and *ESR1* rs9340799 on ART outcomes (Castro, 2016). Furthermore, two separate studies were conducted, one involving 498 patients (Santos, 2013), and the other encompassing 563 patients (Reis, 2017), to explore the influence of *FSHR* rs6166 on ART outcomes.

FSHR: follicle stimulating hormone receptor; *ESR*: estrogen receptor; ART: assisted reproductive technology

A study about the role of *ESR1* rs2234693 and *ESR1* rs9340799 polymorphism in ART was conducted in 2016. This study included 105 cases of infertile patients. Two groups were formed: an experimental group of 93 infertile women and a control group of 12 women with infertility attributed to male factor causes. Clinical data was collected, and ovarian stimulation parameters were assessed. *ESR1* rs2234693 genotypes exhibited statistically significant variations in the mean total number of follicles on the day of the human chorionic gonadotropin (hCG) administration ($p=.031$). The CT genotype exhibited a higher average number of follicles compared to the TT genotype (CT vs TT $p=.010$) (Castro, 2016).

Another master's thesis, conducted in 2013, explored the relationship between the *FSHR* rs6166 polymorphism and treatment outcomes, regarding the associations between genotypes and ovarian response. The study involved 498 patients who underwent ART. Normal response was mostly represented by the Asn/Ser genotype (51.5%), while the Ser/Ser variant accounts for only 10.3% of the normal response ($p < .001$). The Ser/Ser variant was weakly associated with obtaining a high response in the ovarian stimulation process, with only 14% of patients with a high response carrying the Ser/Ser genotype (Santos 2013).

Ultimately, a retrospective study regarding *FSHR* rs6166 polymorphism, conducted in 2017, included 563 infertile women. This study analysed the number of preovulatory follicles, retrieved oocytes, and mature oocytes per treatment cycle, as well as the classification of ovarian response. All these parameters showed no significant differences among these three genotypes (AA, AS, and SS) (Reis 2017).

In fact, the literature highlights the relevance of studying *FSHR* and *ESR* polymorphisms within the domain of tailored treatments, particularly pharmacogenetics, given their ability to predicted 10–15% of poor responders in IVF (de Castro et al., 2004). Pharmacogenetics is the study of how the genetic profile of individuals affect their responses to medication. In ART it refers to the study and definition of a protocol of COS based on the genetic characteristics of the patient (Altmäe et al., 2011).

Women with DOR are included in poor responders group, which represent 9-24% of the total women submitted to COS, and are one of the greatest challenges in ART (Drakopoulos et al., 2017; Fusi et al., 2020). On the contrary, hyper response to COS is frequently observed in patients diagnosed with PCOS, presenting a considerable difficulty in ART. This arises from the complexities associated with managing the hormonal imbalances inherent in PCOS, which significantly impact follicular development and oocyte quality (Cordeiro et al., 2018).

Several genes have been investigated in the context of ovarian reserve assessment, response to ovarian stimulation, and the study of POI and DOR. Meireles et al. investigated the relationship between *LH*, *LHR*, *GDF9*, *FSHR*, *AMH*, *AMHR2*, and *BMP15* polymorphisms and reduced ovarian response during COS for IVF, involving 66 participants (52 in the normal ovarian response group and 14 in the poor ovarian response group). Their findings suggest that *GDF9* plays a crucial role in follicular development, and variations in its DNA sequence may have adverse effects on ovarian reserve (Meireles et al., 2021).

GDF9, *BMPs*, *TGF* and *AKT* are often studied in the context of DOR (Chu et al., 2018). Particularly, *GDF9* and *BMP15* expression was described to be altered in granulosa cells of DOR patients (Omrizadeh et al., 2022). Biallelic variants in MutS Homolog 4 (*MSH4*) were also identified as a potential contributor to DOR, regarding its association with compromised oocyte quality, along with an abnormal morphology observed in the first polar body (Wan et al., 2023). *KIT* ligand plays an indispensable role in coordinating granulosa cells and oocytes, initiating the activation of primordial follicles in adult ovaries (Chang et al., 2019).

This initial section aims to provide the rationale for the chosen study design in Task 2. The methods applied to achieve our objectives in elucidating the genetic determinants of abnormal ovarian reserve, including the conducted work, obtained results, and their critical discussion in light of existing literature, are described in the following sections.

2.3.3 Methods

2.3.3.1 Assessing the influence of hormonal receptor polymorphisms on ovarian function - designing a narrative review on the role of follicle stimulating hormone (FSH), luteinizing hormone (LH), and Estrogen (ES) receptor polymorphisms in ovarian function

As previously mentioned, our group has reported experience with studying the impact of SNPs on ART outcomes. Given the passage of time, we decided to revise the literature on hormonal receptors involved in ovarian function. This was a novel approach for our group, as we had not previously undertaken a comprehensive review of the literature on this topic.

A comprehensive narrative review of literature was undertaken to examine the roles played by *FSHR*, *LHR*, and *ESR* polymorphisms in ovarian function. To ensure the rigor of our review, we adopted a systematic approach for the identification and selection of the studies. Through a narrative review of the existing literature we aimed to contribute to the comprehension of how genetic factors influence ovarian function – ideally elucidating their role in situations of abnormal ovarian reserve.

2.3.3.1.1 Bibliographical research

For the literature search, three databases were selected: PubMed, MEDLINE and TripDataBase. The first search was conducted in May 2022, and all the resulting articles were selected and proved to be sufficient to conduct a review on the topic. The last search was conducted in November 2022, in which the most recent articles were added. No publication year limits were applied to the articles.

2.3.3.1.1.1 Search terms

For the search strategy, on the PubMed database it was used the following search formula: "(((Estrogen receptor*)) AND ((ovulation Induction or ovulation stimulation)) AND ((Polymorphism*))) OR ((Receptor* FSH OR Follicle Stimulating Hormone) AND (ovulation Induction or ovulation stimulation) AND (Polymorphism*)) OR (((Receptor* LH OR Luteinizing Hormone Receptor* OR Chorionic Gonadotropin Receptor)) AND ((ovulation Induction or ovulation stimulation)) AND ((Polymorphism*)))". For the MEDLINE database search, the Boolean operators AND (AND) and OR (OR) and the following MESH terms, in

different combinations, were used: "Polymorphism, Genetic", "Receptors, FSH", "Ovulation Induction", "Receptors, LH", "Receptors, Estrogen", and "Ovarian reserve". For TripDataBase, it was used the PICO strategy: Patients - women of reproductive age with evaluation of *FSHR*, *LHR*, *ESR*; Intervention - Ovarian Stimulation; Comparison - women of reproductive age with different genotypes; Outcome - response to ovarian stimulation.

2.3.3.1.1.2 Eligibility criteria

Studies that met the following criteria were included: 1) Studies examining polymorphisms of *FSHR*, *LHR*, and *ESR*; 2) Studies including women of childbearing age (aged between 18 and 40 years old); 3) Studies where the purpose of this review is evaluated, namely the ovarian function and the response to COS.

2.3.3.1.1.3 Exclusion criteria

Articles were excluded if: 1) Studies not focusing on the specified polymorphisms; 2) Studies conducted on species other than humans; 3) Literature reviews, systematic reviews, meta-analyses, commentaries, or guidelines.

The search led to 141 articles, after excluding duplicates. Articles were initially selected, according to the defined criteria, based on the title and abstract, by two independent researchers. After that, we selected 42 articles for full-text reading. In the end, 42 studies were included in this review: 26 on *FSHR*, 8 on *ESR*, 3 on *LHR* and 5 on *FSHR* and *ESR*. The process of paper selection is summarized in Figure 28. The key findings concerning *FSHR*, *LHR*, and *ESR* polymorphisms are succinctly presented in Table 66, 67, and 68, respectively, providing a comprehensive overview of the research outcomes.

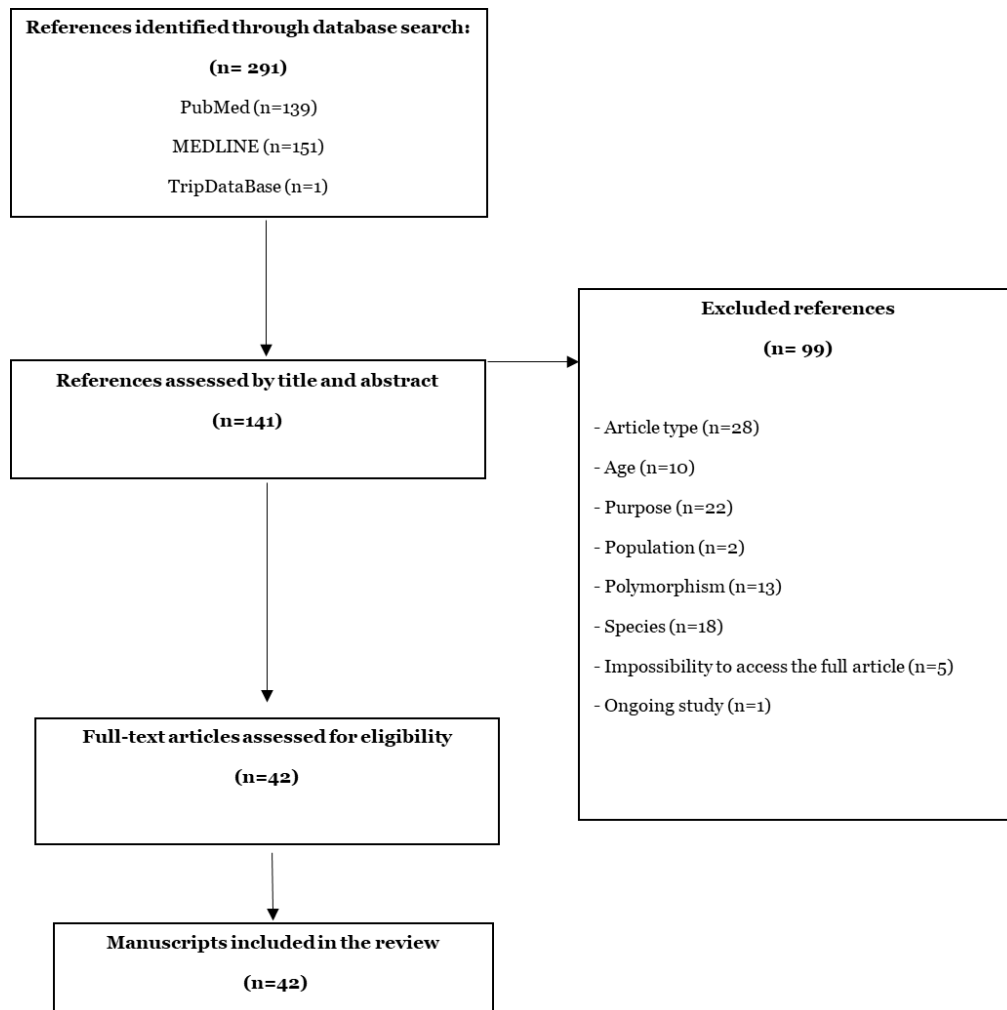


Figure 28 Flow diagram from initial search to final analysed articles included in the review about polymorphisms impact on ovarian reserve

2.3.3.1.2 Definition of study populations to evaluate the impact of *FSHR* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 polymorphisms on ovarian reserve

2.3.3.1.2.1 Regarding infertile patients with diminished ovarian reserve (DOR)

The objective of this analysis was to investigate the impact of *FSHR* rs6166, *ESR1* rs2234693, and *ESR1* rs9340799 polymorphisms on ovarian reserve. Accordingly, the aim was to identify a genetic variant that is more prevalent in the study group (women with DOR) and to understand how these specific genetic factors interact with AMH and AFC levels. To ensure meaningful associations, the cut-off values for DOR have been defined based on the preliminary analysis outlined in Section 2.2.3.3.1.3.2: AMH < .85 ng/mL and AFC < 5.

The study population included women aged 19 to 40 years, that underwent fertility treatments between 2012 and 2020. Within this cohort, the study group, characterized by DOR, was defined by an AMH level less than .85 ng/mL and an AFC below 5. In contrast, the control group was identified as having an AMH level equal to or greater than .85 ng/mL and an AFC of 5 or more.

To verify the occurrence of genetic variants in a DOR cohort, a total of 143 samples are required, assuming a power of 80% and a Type I error (α) of 5%. This sample size was calculated using G*Power 3.1.9.4, utilizing the Chi-Square analysis. Taking into account that the estimated prevalence of DOR is 30% among infertile women, a sample size of 233 or more is sufficient for the analysis.

The DNA samples had been collected within the reproductive medicine unit, at the time of the fertility treatment, with the explicit purpose of studying these specific polymorphisms. The first step was to blindly select DNA samples from the original database, ensuring that they had not been utilized in any previous published data or scientific production. We began our analysis with an initial pool of 500 subjects.

From these 500 selected samples, 387 cases were genotyped between June and December of 2022. From the 8 cases out of 500 revealed inconclusive genotypes, reducing the number of samples to 492. The samples remained anonymized during the analysis. Once the genotyping was completed, the principal investigator identified the samples to correlate them with AMH, AFC, and ethnicity data, collected from the internal database. This process was authorized by the ethical board of the hospital (CHUC-171-20).

From the 492 samples with conclusive genotype, complete information on AMH, AFC, and ethnicity was available for 472 cases (Figure 29). Regarding that information, two groups were defined, 1) the study group involved AMH levels less than .85 ng/mL and an AFC below 5; 2) the control group involved AMH levels $\geq .85$ ng/mL and an AFC ≥ 5 . By applying these criteria, which required the intersection of AMH and AFC data, the sample size was reduced to 307 participants, and this subset was used for the final analysis. A secondary goal included restricting the analysis to women under 35 years old, the final number of participants included in the study was 139.

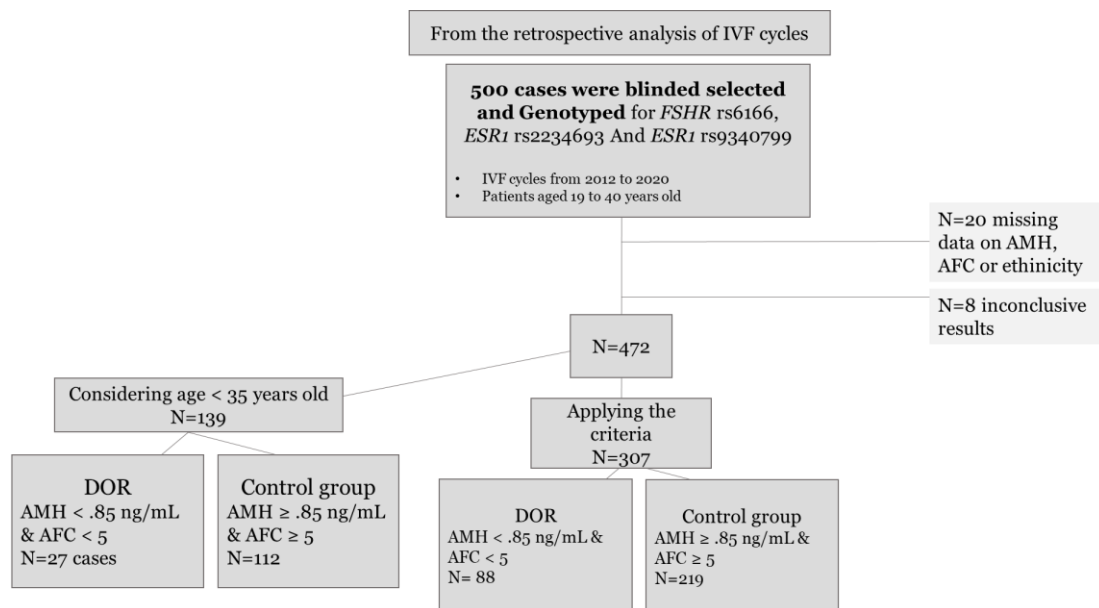


Figure 29 Study design to evaluate the impact of *FSHR* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 polymorphisms on DOR

Initial selection of 500 samples, with 8 revealing inconclusive results and 20 lacking AMH, AFC, or ethnicity data. Final analysis focused on 139 samples (comprising 88 cases in the DOR group and 219 in the control group). Sample size further reduced to 307 when limiting the study to women under 35 (27 cases in the DOR group and 112 in the Control group).

AMH: Anti-Müllerian Hormone; AFC: Antral Follicle Count; *FSHR*: Follicle-Stimulating Hormone Receptor; *ESR1*:

2.3.3.1.2.2 Regarding infertile patients with polycystic ovary syndrome (PCOS)

The study design for this part of the project is published as an open access original article with the DOI: 10.7759/cureus.35690.

The population investigated consisted of women who underwent ART between 2013 and 2019. The study group (n=88) comprised women diagnosed with PCOS. The control group (n=80) consisted of age- and BMI-matched women with regular menstrual cycles. Specifically, the control group included infertile women with tubal obstruction, regular menstrual cycles, no clinical or biochemical signs of hyperandrogenism, and no polycystic ovaries.

The primary objective of this study was to investigate the impact of *FSHR* rs6166, *ESR1* rs2234693, and *ESR1* rs9340799 polymorphisms on a population with PCOS. This was a novel investigation, as we had not previously explored the role of the specified SNPs in a PCOS population, despite such associations being reported in the literature.

2.3.3.1.2.3 Genotyping *FSHR* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 polymorphisms

The methodology for genotyping *FSHR* rs6166, *ESR1* rs2234693, and *ESR1* rs9340799 polymorphisms follows the procedures outlined in an open access original article with the DOI: 10.7759/cureus.35690.

All patients provided written informed consent, which was obtained at the time of blood collection. The blood samples (2.5 mL per participant) were collected on tube containing Ethylenediaminetetraacetic acid (EDTA).

Genomic DNA was isolated from peripheral blood collected in EDTA tubes on day-3 of the follicular phase and stored at 4°C until DNA extraction. Extraction of genomic DNA was performed using QIAamp® DNA Blood MiniKit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. QIAamp® DNA Blood Kits provide silica membrane-based DNA purification from biological fluids (including blood samples). We optimized buffers lyse samples, stabilized nucleic acids, and enhanced selective DNA adsorption to the membrane. Alcohol was added and sample lysates were loaded onto the spin column. Finally, wash buffers were added to remove impurities and purified DNA was then eluted in low-salt buffer. The quantity and purity of each eluted sample were assessed by NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

DNA amplification was carried out using the polymerase chain reaction (PCR) with specific primers to assess *FSHR* and *ESR1*, as described in Table 63.

Table 63 Description of investigated SNPs of *FSHR* and *ESR1*

Hormonal receptor gene	Primer sequence	Product size	PCR program
<i>FSHR</i>	Primer F 5' TTT GTG GTC ATC TGT GGC TGG 3' - Primer R 5' CAA AGG CAA GGA CTG AAT TAT CAT T 3'	520bp	94°C, 5 min, 1 cycle; 94°C, 1 min; 60°C 1 min and 72°C 1 min, 40 cycles; 72°C, 1 min, 1 cycle; 12°C, ∞
<i>ESR1</i>	Primer F 5' CGT CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC 3' - Primer R 5' TCT TTC TCT GCC ACC CTG GGG TCG ATT ATC TGA 3'	1373bp	95°C, 5 min, 1 cycle; 95°C, 30 sec; 63°C 1 min and 72°C 1 min, 44 cycles; 72°C, 10 min, 1 cycle; 12°C, ∞

FSHR: follicle-stimulating hormone receptor; *ESR*: estrogen receptor 1; PCR: polymerase chain reaction; SNPs: single nucleotide polymorphisms

Specifically, PCR was carried out in a .2-mL PCR tube with a 25µL reaction volume, containing .1µL of template genomic DNA, 1.25 U Taq polymerase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), .2µM deoxynucleotide triphosphates (dNTPs) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), .2µM primer reverse, and .2µM primer forward (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA); the reaction volume was adjusted with nuclease-free water. The amplification reaction for each primer set was conducted in a programmable c1000Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Nuclease-free water was used instead of genomic DNA as a blank to check for DNA contamination.

Restriction fragment length polymorphism (RFLP) was carried out on the purified PCR products using restriction endonuclease enzymes. To determine the genotype for each sample, PCR products were incubated and digested with the respective restriction enzyme; BseNI (Thermo Fisher Scientific, Waltham, MA, USA); PvuII (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and XbaI for *FSHR* rs6166, *ESR1* rs2234693, and *ESR1* rs9340799 respectively, according to the manufacturer's instructions (Table 64). The samples were then run on a 2% agarose gel containing ethidium bromide (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) at 80 V for 75 minutes and visualized by ultraviolet transilluminator (DNA MiniBIS Pro, DNR Bio Imaging System, Neve Yamin, Israel).

Table 64 Restriction endonuclease enzymes utilized for RFLP analysis

Hormonal receptor gene	Restriction endonucleases enzymes	Incubation conditions	Product size		
			Genotype		Band size
<i>FSHR</i>	<i>BseNI</i>	5 min at 65°C; 5 min at 80°C	AA AS SS	Homozigotes Heterozigotes Homozigotes	520 bp 520 + 413 bp 413 bp
<i>ESR1</i>	<i>PvuII</i>	2 hours at 37°C; 5 min at 80°C	CC CT TT	Homozigotes Heterozigotes Homozigotes	1374 bp, 1374+ 936 + 438 bp 936 + 438 bp
<i>ESR1</i>	<i>XbaI</i>	24 hours at 37°C; 5 min at 80°C	GG GA AA	Homozigotes Heterozigotes Homozigotes	1374 bp 1374 +981 +393 bp 981 + 393 bp

RFLP: restriction fragment length polymorphism; *FSRH*: follicle-stimulating hormone receptor; *ESR1*: estrogen receptor 1; bp: base pairs

FSRH rs6166 leads to a change from A to G. BseNI digestion produces an uncleaved 520 base pairs (bp) fragment that indicates homozygosity for asparagine (Asn/Asn). In contrast, two fragments of 413 and 520 bp indicate heterozygosity (Asn/Ser). The presence of one fragment of 413 bp reveals homozygosity for serine (Ser/Ser), as represented in Figure 30.

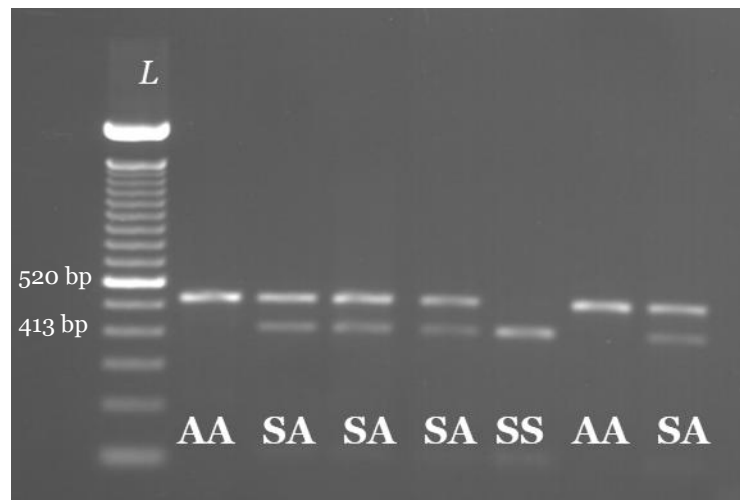


Figure 30 *FSHR* rs6166 genotypes

The AA genotype is depicted by a single band of 520 bp, the SA genotype is identified by two bands of 520 bp and 413 bp, and the SS genotype is represented by a single band of 413 bp.

FSHR: Follicle-Stimulating Hormone Receptor; L - molecular weight marker;
The sizes of the presented fragments were determined through searches in
bioinformatics databases, namely NCBI/BLAST
(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), Ensembl
(<http://www.ensembl.org/Multi/Info/Index>), NEBcutter
(<http://tools.neb.com/NEBcutter2/>), and REBsites
(<http://tools.neb.com/REBsites/index.php>)

ESR1 rs2234693 induces a T to C alteration, with genotypes denoted as TT and CC for the homozygous type and TC for the heterozygous type. The CC genotype is evidenced by a single band of 1374 bp, while the TT homozygous genotype exhibits two bands of 936 and 438 bp. In the heterozygous CT genotype, three bands of 1374, 936, and 438 bp.

ESR1 rs9340799 involves an A to G alteration, with genotypes identified as GG and AA for the homozygous type and GA for the heterozygous type. XbaI digestion yields a single band of 1374 bp in the homozygous GG genotype, while the AA homozygous genotype displays two bands of 393 and 981 bp. The heterozygous GA genotype is characterized by three bands of 393, 981, and 1374 bp.

The genotypes of *ESR1* rs2234693 and *ESR1* rs9340799 are represented in Figure 31.

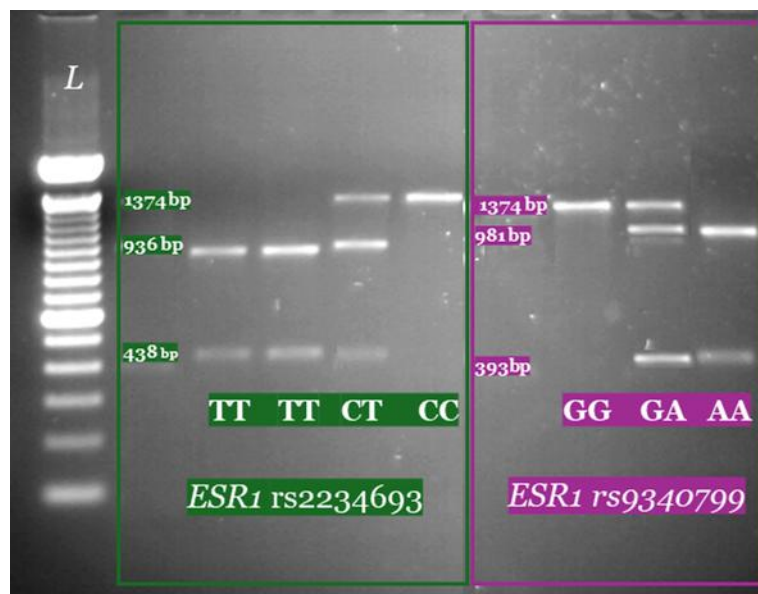


Figure 31 *ESR1* rs2234693 and *ESR1* rs9340799 genotypes

ESR1 rs2234693 genotypes (in green): The CC genotype, where enzymatic digestion does not occur, is depicted by a single band of 1374 bp, the CT genotype is recognized by three bands of 1374 bp, 936 bp, and 438 bp, and the TT genotype is illustrated by two bands of 936 bp and 438 bp.

ESR1 rs9340799 (in pink): The GG genotype, where enzymatic digestion does not occur, is indicated by a single band of 1374 bp, while the AA homozygous genotype exhibits bands of 981 bp and 393. The heterozygous GA genotype is distinguished by three bands of 1374 bp, 981 bp, and 393bp.

ESR1: estrogen receptor 1; L - molecular weight marker; The sizes of the presented fragments were determined through searches in bioinformatics databases, namely NCBI/BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), Ensembl (<http://www.ensembl.org/Multi/Info/Index>), NEBcutter (<http://tools.neb.com/NEBcutter2/>), and REBsites (<http://tools.neb.com/REBsites/index.php>).

2.3.3.1.3 Statistical analysis

Statistical analysis was performed IBM SPSS Statistics for Windows, (Version 28.0. IBM Corp, Armonk, NY, USA). Statistical significance was assumed for $p\text{-value} < .05$.

Hardy-Weinberg equilibrium was verified for the three polymorphisms, in different populations datasets.

The chi-square test was performed for categorical variables to test differences in the distribution of two independent variables. Fisher's exact test was used instead whenever expected frequencies below five occurred with enough frequency.

After verifying the required assumptions, linear regression was used whenever there was a need to confirm an independent effect of an independent variable on a continuous dependent variable.

2.3.3.1.4 Establishing the next-generation sequencing (NGS) and constructing the genes panel

In addition to studying infertile patients undergoing IVF, we aimed to investigate the cohort of non-infertile women recruited for ovarian reserve assessment. Consequently, we wanted to compare individuals identified with DOR (defined as both AMH and AFC values below the 25th percentile) against those with normal ovarian reserve (AMH and AFC equal to or above the 25th percentile). At the time of writing this manuscript, the project comprised a total of 105 participants, 8 with DOR (AMH and AFC < 25th percentile for their age), and 46 with normal ovarian reserve (AMH and AFC $\geq$ 25th for their age). To establish the NGS analysis, we have reviewed the literature and constructed a panel of genes to be analysed as the cohort of women with DOR expands. The collaboration protocol has been established with the pathology department of ULS Coimbra. We have identified a panel of 11 genes to include in our study, which are summarized in the table below (Table 65).

Table 65 Summary of selected genes for NGS analysis: implications in reproductive health, and their association with DOR and POI

Gene	Function/description	Associated conditions/pathways	References
AMH	- Indicator of ovarian follicle pool and POI risk - Regulator of follicular maturation and growth (Primary to antral follicles) - Downregulated in patients with DOR	POI, DOR	Venturella et al., 2019 Skiadas et al., 2012 Huhtaniemi, 2018
NOBOX	- Transcription factor involved in early folliculogenesis and follicular activation - Regulates ovarian genes like GDF9 and BMP15	Rare (5.6% and 6.2%) contribution for POI	Rajkovic et al., 2004 Qin et al., 2007, 2009 H. Zhao et al., 2008 Zhao et al., 2005 França et al., 2020 Jiao et al., 2020
FOXL2	- Transcription factor crucial for GCs differentiation, follicle development and activation (primordial follicles) - Directly or indirectly controls AMH expression	POI (rare contribution) Non-syndromic POI (5%)	Huhtaniemi, 2018 Schmidt et al., 2004 Ford, 2020 Venturella, 2019 Harris et al., 2002
FOXO3	- Regulates activation of primordial follicles (inhibiting factor) and oocyte quiescence (suppresses initiation of follicular maturation)	Non-syndromic POI	Venturella, 2019 Huhtaniemi, 2018
FIGLA	- Transcription factor involved in transition from primordial to primary follicles - Inhibits follicular activation - Effector of PI3K-Akt signalling pathway	POI	Huhtaniemi, 2018 Yatsenko, 2019 França et al., 2020
PTEN	- Encodes protein negatively regulating PI3K-Akt pathway - Maintains dormancy of primordial follicles - Deficiency leads to premature activation and depletion	Non-syndromic POI	Venturella et al. 2019
IGF1 & IGF2	- Induces follicular activation via androgens and PI3K-Akt - Promotes GCs proliferation and theca cells steroidogenesis - Downregulated in GCs of DOR patients	DOR	Ford, 2020 Greene, 2014

Gene	Function/description	Associated conditions/pathways	References
GDF9	- Oocyte-derived factor regulating primordial follicle development, GCs proliferation, and follicle maturation - Follicular growth from primary to secondary follicle stage - Regulator of steroidogenesis and FSH sensitivity	POI (1-4%), DOR (10%)	Greene, 2014 Venturella 2019 Huhtaniemi 2018 Wang et al., 2013 Wang et al., 2010 Persani et al., 2011 Yan et al., 2001 Yatsenko & Rajkovic, 2019 Chand et al., 2006 Laissue et al., 2006 Zhao et al., 2007 Kovanci et al., 2007
BMP15	- Influences follicle maturation, prevents GCs apoptosis, and enhances oocyte competence and ovulation - Regulator of steroidogenesis and FSH sensitivity in GCs	Non-syndromic POI and POI (1.5 to 12%)	Venturella et al., 2019 Di Pasquale et al., 2004 Dixit et al., 2006 Rossetti et al., 2009 Tiotiu et al., 2010. Yatsenko & Rajkovic, 2019 Huhtaniemi 2018 França, 2020
AKT1	- Protein kinase involved in various cellular processes including cell survival, proliferation, and metabolism - It is a key component of the PI3K-Akt pathway, involved in follicular activation	POI, DOR	Prya et al., 2023

TGF- β : Transforming Growth Factor Beta; POI: Primary Ovarian Insufficiency; DOR: Diminished Ovarian Reserve; GDF9: Growth Differentiation Factor 9; BMP15: Bone Morphogenetic Protein 15; GCs: Granulosa Cells; PI3K-Akt: Phosphatidylinositol 3-Kinase-Protein Kinase B.

These selected genes have been integrated into a draft from the HyperDesign platform by Roche (Roche Diagnostics, Basel, Switzerland) for further NGS analysis.

2.3.4 Results

2.3.4.1 Impact of *FSRH* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 polymorphisms on ovarian response: key findings from literature review

Among the studied polymorphisms, *FSHR* Asn680Ser emerges as the most extensively studied genetic predictor. While *ESR* and *LHR* variants are prevalent in the general population, their specific impact on ovarian function remains inadequately documented. Once the specificity and sensitivity of a single genetic marker would be too low to be employed as a predictive biomarker, it is crucial to provide further evidence about the role of *LHR* and *ESR* in ovarian function. The results are detailed below, categorized by each polymorphism.

2.3.4.1.1 *FSHR* polymorphisms

Several studies evaluated the role of *FSHR* SNPs on reproductive function (Casarini et al., 2011). The first *FSHR* mutation identified was the substitution of Alanine to Valine at position 189 (Ala189Val), at the receptor glycosylation site. This mutation was described in women with ovarian dysgenesis and primary amenorrhea (Aittomäki et al., 1995).

Particularly, two variants of *FSHR* SNPs have intensively been studied, both in exon 10. One at position 680, Asn680Ser, where Asparagine is replaced by Serine in the intracellular domain of the *FSHR* protein, and the other at position 307, Ala307Thr, causing the replacement of Threonine by Alanine. *FSHR* Asn680Ser Ser/Ser homozygous genotype was associated with higher FSH serum levels and dosage of FSH to get a similar ovarian response (Mayorga et al., 2000) suggesting that Ser/Ser homozygous variant is less sensitive to FSH (Simoni & Casarini, 2014).

Provided that, women homozygous for the serine (Ser/Ser) have been associated with a reduced response, higher concentrations of basal levels of FSH and higher requirement of gonadotrophins, when compared with women with the two other variants (Mayorga et al., 2000). For this reason, it could be hypothesized that *FSHR* pharmacogenetic modulates the ovarian response (Casarini et al., 2016; Riccetti et al., 2017)

Considering *FSHR* G-29A SNP (rs1394205), it has been suggested that A variant could be related to high serum FSH and low estradiol levels (Achrekar et al., 2009; Grigorova et al., 2014) but there is not a consensus about its influence on ovarian response to FSH (Desai et al., 2011; Wunsch et al., 2005).

The *FSHR* polymorphisms reviewed in the literature that are related to response to COS were Asn680Ser (rs6166); Ala307Thr (rs6165); G-29A (rs1396205); Ala189Val (rs121909658); Ile160Thr (rs121909659), and Thr449Ile (rs28928870).

The Asn680Ser polymorphism was evaluated in 24 studies. Almost every study affirms the correlation between this polymorphism and the outcome of COS, with the prevailing trend indicating that the 680Ser allele is linked to an unfavourable outcome. This pattern was notably observed in the study conducted by Alviggi et al, where an analysis of 42 Italian women led them to this conclusion. They observed that this particular variant appears to correlate with a substantial reduction in ovarian response to recombinant human follicle-stimulating hormone (r-hFSH) in ART cycles. Consequently, this association is related with a significant increase in drug consumption (Alviggi et al., 2016). de Castro et al. studied a population of 102 Spanish women and also concluded that patients who carry the Ser/Ser genotype seem to have an increased risk of cycle cancellation during recombinant FSH treatment (de Castro et al., 2003). Lledó et al., added the information that, in subjects with Ser/Ser genotype, the use of a corifollitropin alfa-based ovarian stimulation protocol may be linked to a lower number of total oocytes and metaphase II oocytes retrieved (Lledó et al., 2022). Valkenburg et al. corroborated this finding, concluding that homozygous carriers of the 680Ser allele have a 90% higher probability of

clomiphene-resistant anovulation during ovulation induction with clomiphene citrate. Based on this, they could hypothesise that these patients are more effectively treated with exogenous gonadotropins (Valkenburg et al., 2015). Beyond this, Sudo *et al.*, in a study with 522 Japanese women, observed that the difference in ovarian response to hMG among these polymorphisms could be used not only for determination of its dose in an ovarian stimulating cycle, but also for prediction of OHSS (Sudo et al., 2002).

Behre et al. studied 93 women from Germany and compared different FSH doses based on their genotype. The authors observed that by elevating the daily dose from 150 to 225UI, they were able to overcome the lower estradiol response in women with the Ser/Ser FSH receptor variant. Accordingly, they concluded that, in women with normal ovarian function undergoing ART, it might be possible to define the optimal FSH starting dose based on the simple determination of the FSH receptor genotype (Behre et al., 2005).

In contrast, Binder et al., that also performed a study in a German population of 259 women, concluded that this variation may not be a decisive factor for poor or low response to fertility treatment (Binder et al., 2012). The same conclusion was achieved by Laven *et al.*, in a population of 148 Dutch patients, where *FSHR* genotypes are not related with altered ovarian sensitivity to exogenous FSH during COS in anovulatory patients (Laven et al., 2003).

Another important finding was reported by Nenonen et al. in a study that evaluated 586 Swedish women, where they observed that women with 680Asn allele are hyperresponsive to FSH and consequently are at increased risk for OHSS when undergoing IVF treatment. This leads to the conclusion that genetic testing could serve as an additional predictor for OHSS to identify susceptible women (Nenonen et al., 2019).

Therefore, it can be concluded that *FSHR* gene polymorphism at position 680 is associated with altered ovarian responses to COS (Lledo et al., 2013), namely that Ser/Ser variant influence negatively on COS outcome (Monge-Ochoa et al., 2021).

Regarding Ala307Thr polymorphism, it was studied by 14 authors. Kaviani *et al.* findings are consistent with most reported conclusions. They observed that this *FSHR* polymorphism showed a strong association with treatment response. Their findings indicated a significantly higher prevalence of the Ala/Ala genotype among poor responders compared to good responders in the context of IVF treatment. Additionally, women with poor responses exhibited a higher frequency of the 307Ala allele compared to good responders patients (Kaviani et al., 2018). Laven *et al.* found that Ala/Thr genotype was significantly more prevalent among anovulatory women, and that the Thr/Thr was significantly more prevalent in controls (30 healthy volunteers). Despite of that, they concluded that *FSHR* genotypes are not associated with altered ovarian sensitivity to exogenous FSH during ovulation induction in anovulatory patients (Laven et al., 2003). Song et al. observed that, in a population of 705 Chinese women, the frequency of 307Ala was significantly higher in low responders than in good responders. Also, it was noticed that the risk of a low response increased with the number of 307Ala alleles present (Song et al., 2019). The analysis performed by Monge-Ochoa et al. revealed that COS response was not independent of Ala307Thr

genotypes, since the Ala/Ala genotype was significantly increased in poor responder and underrepresented in the pregnancy group. This led them to the conclusion that Ala/Ala variant influence negatively on COS outcome (Monge-Ochoa et al., 2021). Motawi et al. found that, after analysing 216 Egyptian women, Ala/Ala variant was 2.5-fold higher in the poor responders group than in the good responders group (Motawi et al., 2017).

d'Alva et al. reported a finding related to OHSS association with this *FSHR* polymorphism. They studied a population of 29 Brazilian women who experienced moderate-to-severe OHSS during ovarian stimulation and came to the conclusion that the FSH receptor genotype did not play a significant role in the risk of iatrogenic OHSS, inferring that ovarian response to exogenous gonadotropin stimulation is still unpredictable (d'Alva et al., 2005).

Thus, it is corroborated the hypothesis that 307Ala allele is associated with worst COS outcomes (Song et al., 2019).

The G-29A polymorphism was analysed in 7 studies. It is speculated by some authors that the AA genotype at position -29 of the FSH receptor gene is probably responsible for poor ovarian response. A study by Achrekar et al. performed in 50 normogonadotrophic ovulatory females with male or tubal factor infertility, from Indian ethnicity, found that AA genotype is resistant to FSH treatment (Achrekar et al., 2009). These results indicate that the subjects with the AA genotype have a significantly lower rate of successful clinical pregnancy as compared with the GG and GA genotypes. The AA genotype appears to be associated with poor responder to gonadotrophin treatment based on endocrine and clinical parameters. Nevertheless, Čuš et al. studied 60 women undergoing ovarian stimulation from Slovenia and concluded that the GG genotype is strongly linked with poor response on ovarian stimulation (Čuš et al., 2019). Also, Zamaniara et al. observed that this polymorphism had no effects on poor responders, after comparing the genotypes with good responders (Zamaniara et al., 2018). Tohlob et al. observed that women carrying the variant A allele were more likely to have a clinical pregnancy and a live birth following COS. However, these relationships did not retain significance when the analysis was adjusted for the number of embryos transferred, concluding that the results of their study do not provide enough evidence to support the use of genotyping *FSHR* G-29A in the individualization of treatment for women undergoing IVF (Tohlob et al., 2016).

When analysed in combination, the Asn680Ser with the G-29A polymorphism, Desai et al. concluded that A/A-Asn/Asn genotype is associated with poor ovarian response and suggested that the 680Asn allele in combination with the 29A allele serves as a better marker to predict poor ovarian response (Desai et al., 2013). Furthermore, Livshyts et al. also analysed Asn680Ser in association with Ala307Thr, and presumed that the statistically significant prevalence of Ala307-Ser680 observed in the ovarian dysfunction and poor responders groups is an additional evidence that the polymorphisms in exon 10 are associated with diminished reserve (Livshyts et al., 2009).

Lastly, one study performed by Binder et al. analysed three less studied variants of *FSHR*, along with Asn680Ser: Ala189Val, Ile160Thr and Thr449Ile. They concluded that frequencies of these variants were not significantly different between the low responders and controls, hypothesizing

that these variants may not be a decisive factor for poor or low response to COS (Binder et al., 2012).

In brief, among the studied *FSHR* polymorphisms, Asn680Ser stands out, being the most studied one and, consequently, is where most authors agree. Ser680 allele is mostly found to be associated with a worse outcome in COS, while Asn680 is defined as more favourable to achieve good response. The other *FSHR* polymorphisms, namely Ala307Thr and G-29A were also found to be related to COS outcome, but are less studied, and it is more difficult to find consensus about the genotypes with better or worse response. Ala189Val, Ile160Thr and Thr449Ile were only reported in one study, where they hypothesized that these variants may not be a decisive factor for poor or low response to COS (Table 66).

Table 66 Summary of the findings related to *FSHR* polymorphisms, the number of studies examining each polymorphism, the main findings observed, and references

Polymorphism	Number of studies	Main findings	References
Asn680Ser (rs6166)	24	Correlation between Asn680Ser polymorphism and COS outcome observed in almost every study. Ser/Ser genotype associated with reduced ovarian response, higher basal FSH levels, and increased requirement of gonadotrophins.	Alviggi et al., 2016, de Castro et al., 2003, Lledó et al., 2022, Valkenburg et al., 2015, Sudo et al., 2002, Behre et al., 2005, Binder et al., 2012, Laven et al., 2003, Nenonen et al., 2019
Ala307Thr (rs6165)	14	Association between Ala307Thr polymorphism and COS outcome. Ala/Ala genotype more prevalent among poor responders. Thr/Thr genotype more prevalent in controls.	Laven et al. 2003, Kaviani et al., 2018, Song et al., 2019, Monge-Ochoa et al., 2021, Motawi et al., 2017
G-29A (rs1396205)	7	Speculation that AA genotype may lead to poor ovarian response. Contradictory findings regarding the association between GG genotype and poor response. No conclusive evidence to support genotyping <i>FSHR</i> G-29A in individualization of IVF treatment.	Achrekar et al., 2009, Čuš et al., 2019, Zamaniara et al., 2018, Tohlob et al., 2016, Desai et al., 2013, Livshyts et al., 2009
Combination of Asn680Ser and G-29A	1	A/A–Asn/Asn genotype associated with poor ovarian response. Suggested that 680Asn allele in combination with 29A allele may serve as a better marker to predict poor ovarian response.	Desai et al., 2013
Other less studied variants (Ala189Val, Ile160Thr, Thr449Ile)	1	Not significantly associated with poor or low response to COS.	Binder et al., 2012

COS: controlled ovarian stimulation; FSH: follicle-stimulating hormone

2.3.4.1.2 *LHR* polymorphisms

The expression of the *LHR* gene has been described to be increased in poor responders (Skiadas et al., 2012). *LHR* inactivating variants were associated with POI. Patients with this condition are characterized by high levels of LH (LH levels > FSH levels) leading to a compromised ovulation and oligomenorrhea (Latronico et al., 1996, 1998) and a lower fertilization rate (Maman et al., 2012). The evidence about the relationship between the *LHR* mutations and the reproductive outcomes is scarce (Table 67).

In this review, regarding *LHR* polymorphisms, two studies were found that analysed the N312S (rs2293275) polymorphism (Ghaderian et al., 2021). Despite different populations (Indians and Iranians), both studies conclude that women with A/A genotype or A-allele demonstrate a better outcome from COS, in particular a significant increase in total number of oocytes and metaphase II oocytes, compared to women that are homozygous and heterozygous for G allele. Alternatively, Ramaraju et al. found a significant increase in clinical pregnancy rate in women homozygous or heterozygous for G allele compared to women homozygous for A allele (Ramaraju et al., 2018).

Other polymorphisms, namely insLQ (rs4539842) and rs4073366, were studied by O'Brien et al. They concluded that insLQ was not associated with patient response to COS, nor was it a predictor for OHSS. Carriers of the rs4073366 C allele exhibited a 3-fold increased risk of developing OHSS. This means that rs4073366 can be considered a potential predictor of OHSS risk, while the functional consequences of this polymorphism on *LHR* function still have to be elucidated (O'Brien et al., 2013).

In short, due to the lack of evidence, it is more challenging to draw conclusions about *LHR* polymorphisms role in ovarian response to COS. The results from these studies suggest that it would be intriguing to perform further studies to explore the utility of these polymorphisms to predict the response to COS

Table 67 Summary of the findings related to *LHR* polymorphisms, the number of studies examining each polymorphism, the main findings observed, and references

Polymorphism	Number of studies	Main findings	References
N312S (rs2293275)	2	A/A genotype or A-allele associated with better COS outcomes in both studies. One study found G allele linked with higher clinical pregnancy rate.	Ramaraju et al., 2018, O'Brien et al., 2013
InsLQ (rs4539842)	1	No association found with COS response or OHSS prediction.	O'Brien et al., 2013
rs4073366	1	C allele associated with 3-fold increased risk of OHSS. No conclusion about its effect on COS outcome.	O'Brien et al., 2013

COS: controlled ovarian stimulation; OHSS: ovarian hyperstimulation syndrome

2.3.4.1.3 *ESR* polymorphisms

ESR polymorphisms have been studied because of their role in the regulation of the menstrual cycle, low fecundity and PCOS (Altmäe et al., 2007; Livshyts et al., 2013; M'Rabet et al., 2012; Yoon et al., 2010). Additionally, *ESR* variants are expected to predict 10 to 15% of poor responders to r-hFSH in IVF treatments (de Castro et al., 2004).

The two most common SNPs in *ESR* are rs2234693 and rs9340799, respectively, PvuII and XbaI, named accordingly to their detecting restriction enzyme. These SNPs are located in the first intron of *ESR* and have been associated with ovarian follicles senescence and POI (Bretherick et al., 2008; Cordts et al., 2012; M'Rabet et al., 2012) (Altmäe et al., 2011). Nevertheless, the results are still discrepant (Altmäe et al., 2011; Bretherick et al., 2008; Cordts et al., 2012; M'Rabet et al., 2012).

Other *ESR* polymorphisms were described in the literature, AluI (rs4986938), BstUI (rs12722) and RsaI (rs1256049).

There are eight studies analysing PvuII polymorphism (Altmäe et al., 2007; Anagnostou et al., 2012, 2013; Čuš et al., 2019; De Mattos et al., 2014; Georgiou et al., 1997; Lazaros et al., 2014; Sundarrajan et al., 1999). The majority of women studied are from Greece (four studies) but were also studied women from West Europe in two studies, from China in one study and from Brazil in one study. T allele was identified as having a negative outcome in COS in the most of them, while C allele was associated with a better outcome. However, Čuš et al. findings disagreed. They conclude that these SNPs did not show any association with COS outcome (Čuš et al., 2019). Nevertheless, this study has the smallest population sample, with only 60 women analysed, what may explain the lack of association found. Recent studies corroborate the premise that Sundarrajan et al. made in 1999, that we can conclude that this polymorphism down-regulates the *ESR* gene, interfering with the effective mediation of estrogen and its functions, on the follicle-oocyte unit (Sundarrajan et al., 1999).

XbaI polymorphism was analysed in six studies (Altmäe et al., 2007; Anagnostou et al., 2013; De Mattos et al., 2014; Lazaros et al., 2014; Zamaniara et al., 2018). Again, the most prevalent population is from Greece (two studies).

Again, the most prevalent population is from Greece (two studies). The most common conclusion is that A allele is related with worst COS outcome than G allele. Altmäe *et al.* described that GG genotype was associated with higher estradiol levels achieved during COS (Altmäe et al., 2007). In contrast, Zamaniar et al. studied a population of 202 Iranian women and described a higher prevalence of A allele in good responders to COS (Zamaniara et al., 2018). It can be hypothesized that women with XbaI AA genotype are possibly more resistant to FSH action. Consequently, they might require higher doses of gonadotropins during COS to achieve follicular sizes as high as those observed in XbaI GG (Lazaros et al., 2014).

Another *ESR* polymorphism studied was *AluI* that is described in six studies (de Castro et al., 2004; De Mattos et al., 2014; Kaviani et al., 2018; Lazaros et al., 2014; Motawi et al., 2017; Sundarrajan et al., 2001). The population sample is more diverse, with women from Greece, China, Iran, Spain, Egypt and Brazil. Despite this, all studies observed that AA genotype is more resistant to COS, having worst outcomes, when compared to AG and GG genotypes. The exception is De Mattos et al. study that studied 136 Brazilian women and concluded that GG genotype had longer COS protocols, used a higher dose of recombinant human FSH (r-hFSH) and was also more associated with OHSS (De Mattos et al., 2014).

Regarding *BstUI* polymorphism, it is reported only in two studies (Georgiou et al., 1997; Sundarrajan et al., 1999). Sundarrajan et al. led a study among 200 Chinese women undergoing IVF and did not find any case with this polymorphism present (Sundarrajan et al., 1999). Georgiou et al. found this polymorphism among 200 Greek women and concluded that it did not show an influence on the number of the follicles, oocytes and ratios of follicles to oocytes, when compared alone or in combination with *PvuII* (Georgiou et al., 1997).

Lastly, *RsaI* polymorphism is assessed in four studies (Altmäe et al., 2007; Anagnostou et al., 2012; De Mattos et al., 2014; Sundarrajan et al., 2001). Once again, the population sample is diverse, including women from Estonia, Greece, China and Brazil. Only De Mattos et al. found that GG genotype is associated with higher doses of r-hFSH in IVF cycles and concluded that more studies with larger samples are needed to confirm their results (De Mattos et al., 2014). The other studies describe that this polymorphism are not associated with the parameters describing ovarian stimulation outcomes (Altmäe et al., 2007; Anagnostou et al., 2012; Sundarrajan et al., 2001).

In summary, further studies are necessary to draw conclusions about the role of *ESR* polymorphisms in COS outcome and the advantage of using them for the prediction of ovarian response in ART protocols. For now, it only can be concluded that these polymorphisms, other than *BstUI*, have been described as having a role in COS by most authors (Table 68).

Table 68 Summary of the findings related to *ESR* polymorphisms, the number of studies examining each polymorphism, the main findings observed, and references

Polymorphism	Number of studies	Main findings	References
PvuII (rs2234693)	8	T allele associated with negative COS outcome in most studies, while C allele linked to better outcome. No association found in one study.	Čuš et al., 2019, O'Brien et al., 2013, De Mattos et al., 2014, Anagnostou et al., 2012, Anagnostou et al., 2013
XbaI (rs9340799)	6	A allele linked with worse COS outcome compared to G allele in most studies. GG genotype associated with higher estradiol levels during COS.	Zamanian et al., 2018, Altmäe et al., 2007, De Mattos et al., 2014, Lazaros et al., 2014, Anagnostou et al., 2013, Altmäe et al., 2007
AluI (rs4986938)	6	AA genotype associated with poorer COS outcomes in all studies. One study (61) found GG genotype related to longer COS protocols and higher OHSS risk.	Kaviani et al., 2018, Motawi et al., 2017, de Castro et al., 2004, De Mattos et al., 2014, Lazaros et al., 2014, Sundarrajan, 2001
BstUI (rs12722)	2	No association found between BstUI polymorphism and COS outcome.	Sundarrajan et al., 1999, Georgiou et al., 1997
RsaI (rs1256049)	4	Inconclusive findings regarding association with COS outcome. One study (61) found GG genotype linked to higher r-hFSH doses in IVF cycles.	de Mattos et al., 2014, Anagnostou et al., 2012, Altmäe et al., 2007, Sundarrajan, 2001

COS: controlled ovarian stimulation; OHSS: ovarian hyperstimulation syndrome; r-hFSH: recombinant human follicle-stimulating hormone; IVF: *in vitro* fertilization.

2.3.4.2 Impact of *FSHR* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 polymorphisms on ovarian reserve

2.3.4.2.1 Regarding infertile patients with diminished ovarian reserve (DOR)

We evaluated whether there is an association between the polymorphic variants of *FSHR* rs6166, *ESR1* rs2234693, and *ESR1* rs9340799 and DOR.

In the studied dataset of 472 cases, the distribution among ethnic groups is as follows: Caucasian - 454 cases (96.2%), Romani - 7 cases (1.5%), Mixed - 5 cases (1%), African - 6 cases (1.3%). The global mean age was 34.22±3.84 years old.

The sample was divided into two groups: the DOR group, characterized by AMH levels below .85 ng/mL and AFC less than 5, and the control group, with AMH levels equal to or greater than .85 ng/mL and AFC of 5 or more. This resulted in total a cohort of 307 women, aged 19 to 40, who underwent ART treatments between 2012 and 2020.

Without adjusting for age, 88 individuals with DOR were identified, while the control group comprised 219 women. The DOR group constituted 28.7% of the total participants, closely aligning with the 30% prevalence reported in the literature.

No statistically significant differences in mean age were observed between the DOR and control groups ($p=.188$). The overall mean age for the entire cohort ($N=307$) was 34.44 ± 3.95 years old. Specifically, the mean age of the DOR group ($N=88$) was 35.92 ± 3.50 years old, while the control group ($N=219$) had a mean age of 33.85 ± 3.97 years old. No statistically significant differences were observed in the distribution of ethnicity between the DOR and control group ($p=.495$).

There were no statistically significant differences in the distribution of *FSHR* rs6166 ($p=.807$), *ESR1* rs2234693 ($p=.633$), and *ESR1* rs9340799 ($p=.672$) genetic variants between DOR and the control group. Detailed genotype distribution results for *FSHR* rs6166, *ESR1* rs2234693, and *ESR1* rs9340799 are provided in the Table 69.

Table 69 Genotype distribution of *FSHR* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 polymorphisms among DOR and control groups

Genotypes of each polymorphism	Control group	DOR	p-value
<i>FSHR</i> rs6166			$p=.807$
AA	37.9% (83)	34.09% (30)	(Vs AS/SS) $p=.532$
AS	45.2% (99)	48.86% (43)	(Vs AA/SS) $p=.561$
SS	16.89% (37)	17.04% (15)	(Vs AA/AS) $p=.975$
<i>ESR1</i> rs2234693			$p=.633$
CC	13.7% (30)	11.36% (10)	(Vs CT/TT) $p=.583$
CT	49.77% (109)	55.68% (49)	(Vs CC/TT) $p=.349$
TT	36.53% (80)	32.95% (29)	(Vs CC/CT) $p=.554$
<i>ESR1</i> rs9340799			$p=.672$
AA	2.28% (5)	3.41% (3)	(Vs AG/GG) $p=.575$
AG	10.96% (24)	13.64% (12)	(Vs AA/GG) $p=.390$
GG	86.76% (190)	82.95% (73)	(Vs AA/AG) $p=.510$
Total	219	88	307

DOR: diminished ovarian reserve; *FSHR*: follicle-stimulating hormone receptor; *ESR1*: estrogen receptor 1

A secondary analysis was conducted on new subgroups refined by age (<35 years). Although this subgroup analysis has statistical power limitations, it provided valuable insights for this study, particularly for understanding the target population - women with a reduced number of oocytes at reproductive age.

The refined examination resulted in a subset of 139 participants who underwent ART procedures between 2012 and 2020. Within this subset, the two aforementioned groups were defined: DOR group (N=27), characterized by AMH<.85 ng/mL and AFC<5, representing 19% of the patients; and the control group (N=112), AMH ≥ .85 ng/mL and AFC≥5.

No statistically significant differences in mean age were observed between the DOR and control groups (p=.927). The overall mean age for the entire cohort (N=139) was 31.05±3.09 years old. Specifically, the mean age of the DOR group (N=27) was 31.81±3.03 years old, while the control group (N=112) had a mean age of 30.87±3.11 years old. No statistically significant differences were observed in the distribution of ethnicity (p=.890).

The genotype distribution of *FSHR* rs6166, *ESR1* rs2234693, and *ESR1* rs9340799 is presented in the corresponding Table 70. The statistical analysis did not reveal statistically significant differences in the distribution of *FSHR* rs6166 (p=.301), *ESR1* rs2234693 (p=.582), and *ESR1* rs9340799 (p=.593), between DOR and the control group.

Table 70 Genotype distribution of *FSHR* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 polymorphisms among DOR and control groups, normalized per age < 35

Genotypes of each polymorphism	Controls	DOR	p-value
<i>FSHR</i> rs6166			p=.301
AA	42% (47)	25.9% (7)	(Vs AS/SS) p=.125
AS	42% (47)	51.9% (14)	(Vs AA/SS) p=.353
SS	16.1% (18)	22.2% (6)	(Vs AA/AS) p=.448
<i>ESR1</i> rs2234693			p=.582
CC	10.7% (12)	7.4% (2)	(Vs CT/TT) p=.608
CT	44.6% (50)	55.6% (15)	(Vs CC/TT) p=.308
TT	44.6% (50)	37% (10)	(Vs CC/CT) p=.474
<i>ESR1</i> rs9340799			p=.593
AA	2.7% (3)	0	(Vs AG/GG) p= .390
AG	10.7% (12)	14.8% (4)	(Vs AA/GG) p= .847
GG	86.6% (97)	85.2% (23)	(Vs AA/AG) p= .549
Total	112	27	139

DOR: diminished ovarian reserve; *FSHR*: follicle-stimulating hormone receptor; *ESR1*: estrogen receptor 1

2.3.4.2.2 Regarding infertile patients with polycystic ovary syndrome (PCOS)

The following results are derived from published data available in an open access original article with the DOI: 10.7759/cureus.35690. Importantly, the results focused on the *FSHR* rs6166 and *ESR1* rs2234693 polymorphisms. *ESR1* rs9340799 analysis was not included as it did not reach Hardy-Weinberg equilibrium. The Hardy-Weinberg equilibrium assess whether the observed genotype frequencies deviate significantly from those expected under Hardy-Weinberg equilibrium. Deviations may suggest the presence of underlying factors affecting the population, that are the reason to do not consider the data for further assumptions.

The results of the genotype distribution of *FSHR* rs6166 genotype and *ESR1* rs2234693 are reported in Table 71. We did not identify statistically significant differences in the distribution between PCOS patients and controls.

Table 71 Genotype distribution of *FSHR* rs6166 and *ESR1* rs2234693 polymorphisms among PCOS case and control groups

Genotypes of each polymorphism	Controls	PCOS	p-value
<i>FSHR</i> rs6166			p=.522
AA	37.5% (30)	31.82% (28)	(Vs AS/SS) p=.516
AS	40% (32)	48.86% (43)	(Vs AA/SS) p=.279
SS	22.5% (18)	19.32% (17)	(Vs AA/AS) p=.705
<i>ESR1</i> rs2234693			p=.697
CC	18.75% (15)	23.86% (21)	(Vs CT/TT) p=.454
CT	48.75% (39)	45.45% (40)	(Vs CC/TT) p=.546
TT	32.5% (26)	30.68% (27)	(Vs CC/CT) p=.740
Total	80	88	168

PCOS: polycystic ovary syndrome; *FSHR*: follicle-stimulating hormone receptor; *ESR1*: estrogen receptor 1.

For PCOS patients, biochemical parameters to analyse the gonadal axes (FSH, LH, estradiol, and progesterone), total testosterone and AMH levels, and ultrasonographic data on AFC were compared between different *FSHR* rs6166 and *ESR1* rs2234693 genotypes. Patients with the SS variant of the *FSHR* polymorphism had higher FSH levels on the third day of the menstrual cycle (p=.011). We did not find other statistically significant differences in biochemical and ultrasonographic parameters in relation to the *FSHR* or the *ESR1* genotypes. The detailed results are presented in Table 72.

Table 72 Results of analysis of the association between *FSHR* rs6166 and *ESR1* rs2234693 polymorphisms and clinical and biochemical data in PCOS patients

Data expressed as mean $\pm$ SD

<i>FSHR</i> rs6166 polymorphism				
Parameter	PCOS			
	AA	AS	SS	p-value
FSH 23 rd day (mIU/mL)	5.91 $\pm$ 3.3	5.22 $\pm$ 1.8	6.42 $\pm$ 3.2	.173
FSH 3 rd day (mIU/mL)	6.2 $\pm$ 1.6	5.6 $\pm$ 1.61	9.2 $\pm$ 9.21	.011 ^{*(1)}
LH 23 rd day (mIU/mL)	12 $\pm$ 6.54	12.9 $\pm$ 10	14.41 $\pm$ 18.3	.843
LH 3 rd day (mIU/mL)	9.9 $\pm$ 7.8	7.6 $\pm$ 5.42	8.62 $\pm$ 6.41	.460
Estradiol 23 rd day (pg/mL)	96.61 $\pm$ 68.1	119.71 $\pm$ 96.3	103.3 $\pm$ 50.12	.390
Estradiol 3 rd day (pg/mL)	58.6 $\pm$ 44.3	49.82 $\pm$ 15.9	55.6 $\pm$ 24.61	.334
Progesterone (ng/mL)	4.1 $\pm$ 8.9	3.6 $\pm$ 5.43	3.74 $\pm$ 4.33	.710
Testosterone (ng/mL)	.6 $\pm$.31	.6 $\pm$.41	.8 $\pm$.81	.957
AMH (ng/mL)	8.3 $\pm$ 7.22	7.6 $\pm$ 5.41	5.2 $\pm$ 5.73	.459
AFC	16.9 $\pm$ 8.11	22.7 $\pm$ 14	14.42 $\pm$ 9.91	.357
<i>ESR1</i> rs2234693 polymorphism				
Parameter	PCOS			
	CC	CT	TT	p-value
FSH 23 rd day (mIU/mL)	5.71 $\pm$ 1.81	5.4 $\pm$ 2.8	5.81 $\pm$ 2	.524
FSH 3 rd day (mIU/mL)	5.82 $\pm$ 1.82	7.13 $\pm$ 6.4	6.2 $\pm$ 1.91	.762
LH 23 rd day (mIU/mL)	12.9 $\pm$ 5.81	14.21 $\pm$ 15.2	11.12 $\pm$ 6.6	.483
LH 3 rd day (mIU/mL)	8.62 $\pm$ 3.83	7.62 $\pm$ 5.54	9.21 $\pm$ 8.5	.391
Estradiol 23 rd day (pg/mL)	75.82 $\pm$ 23.3	126.4 $\pm$ 97	110 $\pm$ 76	.301
Estradiol 3 rd day (pg/mL)	46.41 $\pm$ 13.5	58.21 $\pm$ 38	52 $\pm$ 20	.600
Progesterone (ng/mL)	2.23 $\pm$ 3.3	4 $\pm$ 5.31	4.81 $\pm$ 9.5	.654
Testosterone (ng/mL)	.61 $\pm$.3	.61 $\pm$.4	.72 $\pm$.6	.630
AMH (ng/mL)	8.52 $\pm$ 3.9	7.22 $\pm$ 7.53	6.61 $\pm$ 5.21	.255
AFC	25.21 $\pm$ 12.71	17.91 $\pm$ 10.9	15.93 $\pm$ 10.9	.080

* $p < .05$ vs control group. (1) Post-hoc: statistically significant difference between SA and SS (p -value=.012).

PCOS: polycystic ovary syndrome; *FSHR*: follicle-stimulating hormone receptor; *ESR1*: estrogen receptor 1; BMI: body mass index; FSH: follicle-stimulating hormone; LH: luteinizing hormone; AMH: anti-müllerian hormone; AFC: antral follicle count; MII: metaphase II; MI: metaphase I; GV: germinal vesicle.

2.3.5 Discussion

The literature review confirmed our initial knowledge in selecting the polymorphisms for study, highlighting that the most extensively researched polymorphisms related to hormonal receptors are *FSHR* and *ESR1*. In contrast, evidence regarding the relationship between *LHR* mutations and reproductive outcomes is limited.

FSHR rs6166 is the most extensively studied genetic predictor, when referring to ovarian function, specifically ovarian response. Accordingly, the literature review allowed us to understand that *ESR1* rs2234693 and *ESR1* rs9340799 are the most studied polymorphisms within *ESR* (Altmäe et al., 2011). Specifically, the association of *FSHR* rs6166 with the response to COS is extensively studied, with the Ser680 allele often associated with poorer outcomes, compared to the Asn680 allele (Mayorga et al., 2000; Simoni & Casarini, 2014). The association of *FSHR* rs6166 with ovarian reserve markers is less documented in the literature, but its potential role in regulating the PI3K-Akt pathway, and in follicular activation, was pointed out (Johnson et al., 2022). Regarding the *ESR* polymorphisms, their impact on ovarian function is poorly documented. However, there is evidence of the involvement of *ESR1* rs2234693 and *ESR1* rs9340799 in follicular development and response to COS (Altmäe et al., 2007; De Mattos et al., 2014; Livshyts et al., 2013; M'Rabet et al., 2012; Yoon et al., 2010).

Considering that, a retrospective observational study was carried out to elucidate if *FSHR* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 were genetic determinants of ovarian reserve and therapeutic targets for patients in the context of IVF. We aimed to verify if there were any genetic variants of these polymorphisms that were more prevalent in the populations under study, DOR and PCOS, compared to controls.

The anticipated associations were not revealed, and we did not find statistically significant differences in the distribution of each of the genotypes of *FSHR* rs6166, *ESR1* rs2234693 and *ESR1* rs9340799 polymorphisms in our population. These results suggest that neither of the studied polymorphisms is associated with DOR and PCOS risk.

Differently, a recent study examining the association between *FSHR* and *LHR* polymorphisms and ovarian reserve yielded intriguing findings that contrast with our own. Specifically, the *FSHR* c.2039 G>A (p. Ser680Asn, rs6166) genotype, which was a focal point in our investigation, demonstrated significant associations with AMH levels and AFC. Specifically, individuals with the AA genotype exhibited higher levels of AMH and AFC, whereas those with AG and GG genotypes displayed lower levels, suggesting a potential role of this genetic variant as a predictor of ovarian reserve (Guo et al., 2021). Comparing this study to our own, both share similar methodologies, involving infertile women within a similar age range and a substantial sample size (N=210). The results obtained in this study suggest that *FSHR* gene variants, including the one we studied, could serve as reliable indicators of ovarian reserve (Guo et al., 2021).

While our study did not find significant differences in studying the impact of SNPs on DOR within the IVF population, the exploration of genetic variations within key genes such as *FSHR* continues

to offer valuable insights into its impact on COS, paving the way for more personalized approaches to infertility treatment (Tafazoli et al., 2021). *FSHR* and *LHR* have earned significant attention in investigations concerning altered ovarian reserve, owing to their demonstrated involvement in follicular recruitment and development, crucial for maintaining ovarian reserve balance. This increased interest arises from *FSHR* and *LHR* roles in regulating the PI3K-Akt pathway, which is associated with follicular activation (Lee et al., 2021). In fact, the early activation of primordial follicular pool and excessive recruitment of follicles are implicated in the development of DOR (Gong et al., 2023). However, the impact of genetic variations within these genes on ovarian reserve remains a subject of debate and ongoing research, particularly recognising that the initial stages of follicle development seems to occur independently of gonadotropins (Lee et al., 2021).

Referring to *ESR*, despite their influence on gonadotropin secretion and ovarian function, the initial stages of follicle development seem to be independent of gonadotropins (Lee et al., 2021). This underscores the complexity of the mechanisms governing ovarian reserve that can justified the lack of evidence linking the genetic variants in these genes and cases of altered ovarian reserve.

Considering the PCOS population, results from previous studies regarding the relationship between both *FSHR* and *ESR1* polymorphisms and this condition present conflicting data. In a group of adolescents in Turkey, Unsal et al. did not find a different distribution of several *FSHR* polymorphisms associated with PCOS (Unsal et al., 2009). Wu et al. also failed to find an association between the *FSHR* polymorphisms and PCOS in women in the north of China, but the authors did report an association with higher levels of FSH (Wu et al., 2014). An absence of association was also reported in Thai (Singhasena et al., 2014) and in Sri Lankan women (Branavan et al., 2018). More recently Wan et al. also reported an absence of association between several *FSHR* polymorphisms, including rs6166, and PCOS risk in Asian women (Wan et al., 2021). An association between *FSHR* rs6166, but not rs6165, was reported by Gu et al. in Korean women (Baek et al., 2010). Conversely, in an Italian cohort, reported by Dolfin et al. did find a relationship between the rs6165 variant and PCOS risk (Dolfin et al., 2011). Kim et al. found a significant association between rs6165 and rs6166 *FSHR* polymorphisms and PCOS in South Korea (Kim et al., 2017).

Regarding *ESR1*, the contribution of *ESR1* genetic variants to PCOS is also controversial. In a study from Korea, Kim et al. reported an association with the risk of PCOS (Kim et al., 2010). Jiao et al. also found such an association in the Chinese population (Jiao et al., 2018). Conversely, Silva et al. (Silva et al., 2015), Valkenburg et al. (Valkenburg et al., 2011), and Mir et al. (Mir et al., 2022) found no association with PCOS risk. A meta-analysis from Zhou et al reported no significant association between several *ESR1* polymorphisms and PCOS risk, even taking ethnicity into account (Zhou et al., 2021).

It has also been hypothesized that even if there is no association between *FSHR* polymorphisms or *ESR1* and PCOS risk, there may be an association with its phenotype and hormone levels (Silva et al., 2015; Valkenburg et al., 2011). As such, we aimed to understand whether the distinctive

hormonal pattern observed in these patients could be attributed to *FSHR* rs6166 or *ESR1* rs2234693 polymorphisms. Our findings partially support this theory. The SS genotype of the *FSHR* rs6166 polymorphism was associated with higher FSH levels on the third day of the menstrual cycle. We found no other differences.

Controversial findings in this field may be related to differences in study design and sample selection. Castillo et al. described that the contribution of each gene in multifactorial diseases, such as DOR and PCOS, is small, and very large samples may be necessary to detect a small effect. Furthermore, there is the possibility of incomplete penetrance and gene-gene interactions; therefore, studies of individual genes in DOR and PCOS populations may not reveal the general pattern of these conditions. Nevertheless, it is possible that, due to genetic heterogeneity, the same polymorphism has distinct contributions to DOR and PCOS risk and phenotype across different populations (Castillo-Higuera et al., 2021). This adds another level of complexity when defining the genetic risk factors based on complex diseases.

Our study has some limitations. The fact that the populations studied are composed exclusively of women referred for ART limits the generalization of our conclusions to all Portuguese DOR/PCOS patients. Additionally, controversial findings still exist when applying SNPs analysis in infertile groups, which may be related to the diverse populations and genotyping methods. To address this, we planned to conduct NGS in a non-infertile cohort of women as part of our future research efforts.

Finally, it is already established that DOR translates to poorer outcomes in IVF, with the possible approach being to adapt the protocol of COS (Devenutto et al., 2020). Nevertheless, the ovarian stimulation only acts on follicles sensible to gonadotrophins, and the primordial follicles are not considered (Fàbregues et al., 2021). Even POI women have dormant follicles in their ovaries, so thinking about strategies that take advantage of activating primordial follicles would benefit DOR patients (Devenutto et al., 2020).

In summary, the study of genetic variants in both infertile populations and, in the future, in a non-infertile population with altered ovarian reserve aims to identify targets for tailored therapies, such as pharmacogenetics, to improve the response to ovarian stimulation, and to provide a more precise diagnosis. Complementary to these exploring strategies, activating primordial follicles in populations with DOR, where COS alone is insufficient, could improve the effectiveness of ovarian stimulation. To explore this hypothesis, Task 3 focuses on optimizing a protocol to obtain PRP, to investigate its potential in activating one of the pathways involved in follicular activation.

2. Strategies to increase the understanding of diminished ovarian reserve (DOR)

2.1 Overview of the project on fertility awareness, genetic testing, and interventions in the context of diminished ovarian reserve

Project research question, objectives, and tasks.

2.2 Task 1: intervention in knowledge through general and tailored information on ovarian reserve and diminished ovarian reserve (DOR)

Development and testing the impact of two interventions on knowledge about ovarian reserve DOR: 1) providing general leaflet information, and 2) offering tailored information and reproductive counselling based on the assessment of ovarian reserve.

2.3 Task 2: genetic analysis of infertile populations with abnormal ovarian reserve

Review of the role of hormonal receptor polymorphisms in ovarian function.

Identification of genetic variants concerning FSHR rs6166, ESR1 rs2234693 and ESR1 rs9340799 polymorphisms in cases of altered ovarian reserve (DOR and PCOS), among infertile populations.

Establishment of an NGS study protocol to identify genetic variants in the population with DOR, which includes reviewing the genes most relevant to this population and defining the gene panel to be analysed subsequently.

2.4 Task 3: *in vitro* study of follicular activation through the incubation of human ovarian tissue with platelet-rich plasma (PRP)

*Evaluation of the efficacy of PRP supplementation in activating the PI3K-Akt pathway and enhancing stromal vascularization, using an *in vitro* culture system, with human ovarian tissue.*

2.4 Task 3: *in vitro* study of follicular activation through the incubation of human ovarian tissue with platelet-rich plasma (PRP)

2.4.1 Objectives

The preparation of the PRP could follow different methods, and there is no consensus on the best protocol to obtain PRP for clinical application. The steps to obtain PRP have to be accurately chosen as they directly affect the platelet concentration and viability, that consequently influence the concentration of growth factors in the target tissue and the efficacy of the PRP therapy (Davis et al., 2014; Dhurat & Sukesh, 2014).

Therefore, our first objective in this task was to explore the utilization of diverse anticoagulants and resuspension media in the preparation of PRP by conducting a comparative analysis of the platelet recovery rate (PRR), the percentage of active platelets, and the concentration of platelet derived VEGF.

After establishing the optimized protocol for PRP preparation, our aim was to utilize an *in vitro* approach to assess the effect of PRP in human ovarian tissue.

2.4.2 Introduction to follicular activation, platelet-rich plasma (PRP) protocol, ovarian tissue culture and assessment techniques

PRP has been extensively applied in regenerative medicine, particularly in the recovery from athletic injuries (Dhillon et al., 2012; Miller et al., 2017). The theory underlying this treatment is the natural capacity of platelets to induce cell proliferation, revascularization, and tissue repair due to the various growth factors contained in the platelet α granules (Dhillon et al., 2012).

Due to its effectiveness on tissue regeneration and repair, PRP has been progressively applied in other medical fields, such as to promote endometrial and follicle growth, although its specific mechanism remains unclear (Wang et al., 2024).

2.4.2.1 Ovarian rejuvenation and follicular activation

New approaches have been proposed to rejuvenate ovarian tissue and re-establish folliculogenesis, breaking with the dogma of a finite pool of follicles, and bringing a concept of activation of germ cells in adult ovaries (Pellicer et al., 2023). The scientific concept of 'ovarian rejuvenation' has been acknowledged in discussion, and there is now a widespread consensus confirming the existence of ovarian cells with stem-like characteristics (Martin et al., 2019). PRP therapy has been proposed as a positive stimulus for ovarian reserve (Atkinson et al., 2021).

The ovarian reserve is composed by primordial follicles, which include oocytes meiotically arrested in dictyate stage of Meiosis I and transcriptional quiescent (Fabbri et al., 2018). Follicular activation concerns the passage from dormancy to growing stage, considering this follicles capacity to respond to signalling pathways and/or mechanical stimuli to develop and complete folliculogenesis (Grosbois et al., 2020; Shah et al., 2018; Zhang & Liu, 2015).

PI3K-Akt-mTOR and Hippo pathway are the two known routes that regulate follicular development and ovarian reserve maintenance (Fàbregues et al., 2021), and their role in follicle activation is well reviewed (Devenutto et al., 2020; Fàbregues et al., 2021; Lee & Chang, 2019; Pellicer et al., 2023).

Drug-induced follicular activation was primarily described by Kawamura (Kawamura et al., 2013) and Suzuki (Suzuki et al., 2015) and then evolved to drug-free *in vitro* activation, that has shown promising results, with 30% of pregnancy rate (Kawamura et al., 2020). Besides the positive outcomes, *in vitro* activation remains experimental (Reig et al., 2021).

2.4.2.2 Who benefits from follicular activation?

These innovative options are particularly attractive for women with POI and DOR, especially considering that they are expected to have a poor ovarian response to COS and their primary alternative is oocyte donation (Devenutto et al., 2020). While the consensual used protocols for COS act on follicles sensible to gonadotrophins, the primordial follicles are neglected (Fàbregues et al., 2021). For instance, during the early stages of menopause, the ovary still containing around 1000 primordial or nongrowing dormant follicles (Hsu et al., 2021; Vollenhoven & Hunt, 2018). Accordingly, dormant primordial follicles are still present in 3 out of 4 women with POI (Lee & Chang, 2019), so thinking about strategies that take advantage of activating primordial follicles is attractive (Devenutto et al., 2020).

Follicular activation could also be useful to improve ovarian tissue cryopreservation and transplantation (De Roo et al., 2020). Ovarian tissue cryopreservation is a technique to preserve women's fertility, considering the primordial follicle pool preservation (Donnez et al., 2015), resulting in more than 130 live births worldwide (Donnez & Dolmans, 2017; Ladanyi et al., 2017). Despite these successes, ovarian transplantation still compromise limiting factors, as it promotes an unbalance activation of the dormant primordial follicles, and short graft lifespan (Meirow et al., 2015). The applicability of follicular activation before ovarian tissue transplantation remains a topic of debate. Considering that the burnout already occurs spontaneously after the transplantation, it could be advocated that there is no need to induced follicular activation before ovarian tissue transplantation (Masciangelo et al., 2020). Furthermore, it is warranted that it is a considerable challenge to develop culture systems that support ovarian cortex for autologous transplantation, preventing tissue damage, necrosis, and apoptosis (Griesinger & Fauser, 2020). Controlling follicular activation, which naturally occurs *in vitro* (Vos et al., 2014), is crucial to avoid abnormal development of oocytes and granulosa cells (Smitz & Cortvrindt, 2002). When

referring to Hippo pathway disruption, in a practical way, there is no advantage of transplant small fragments, rather than the regular size pieces (Meirow et al., 2015).

2.4.2.3 Platelet-rich plasma's (PRP's) applicability in stimulating follicular activation

Employing PRP is another option to enhance ovarian function, through the stimulation of dormant follicles (Sills & Wood, 2022), and by promoting angiogenesis (Battinelli et al., 2011). PRP, defined as the plasma fraction with a high platelet concentration – including at least 1 million of platelet per mL, releases various factors, such as TGF- β 1, GFD9, VEGF, IGF-1, PDGF and BMPs that play crucial roles in cell migration, vascular support, and overall ovarian function (Sills & Wood, 2022). These growth factors have the potential to ameliorate an hypoxic environment, reducing intraovarian reactive oxygen species and temporarily resolving tissue dysfunction associated with hypoperfusion in the senescent ovary (Kheil et al., 2022; Sills & Wood, 2021, 2022). Platelets serve as a concentrated source of growth factors and cytokines, highlighting the potential exploration of PRP on therapeutic applications for conditions affecting ovarian reserve (Sills & Tan, 2023). However, the diversity of protocols among the studies, characterized by different PRP protocols, complicates the assessment of the potential benefits of this therapy (Sharara et al., 2021).

2.4.2.4 Protocols to obtain platelet-rich plasma (PRP)

The protocols of PRP preparation were initially described in 1999 (Anitua, 1999) and now there is a vast number of different protocols employed. The preparation of the PRP could follow different methods as long as we vary the speed and timing of centrifugation (Amable et al., 2013; Dhurat & Sukesh, 2014).

Despite the diverse number of protocols utilized for the preparation of PRP, all involve blood collection, initial centrifugation to separate red blood cells, subsequent centrifugations to concentrate platelets, and, optionally, platelet activation. Usually, around 3 to 5 cc of PRP can be extracted from a 30cc blood draw, although this quantity can vary based on the individual's platelet count, the specific device used, and the method applied. The blood collection procedure entails incorporating an anticoagulant to inhibit platelet activation. PRP could be prepared reliably and cost-effectively by simply isolating the blood constituents according to their density using a commercial kit or following a two-step centrifugation protocol (Davis et al., 2014; Dhurat & Sukesh, 2014). The first centrifugation step is conducted at a constant acceleration to separate red blood cells from the remaining whole blood volume. After the first centrifugation, it is possible to distinguish three layers: a top layer of plasma (platelet-poor plasma), a middle thin layer of leukocytes and platelets (buffy coat) and a lower layer of erythrocytes. The top layer of plasma (with or without the buffy coat) is collected and proceeded for a second centrifugation to increase the platelet concentration.

The second centrifugation step aims to concentrate platelets. Subsequently, the upper portion of the volume, predominantly composed of platelet-poor plasma is removed. The pellets are then homogenized in the smallest final plasma volume. Typically, the PRP is diluted in the lower one-third to create the PRP (Davis et al., 2014; Dhurat & Suresh, 2014). The pellet is resuspended in a media, that could be plasma (Alves & Grimalt, 2018; Karimi & Rockwell, 2019; Pavlovic et al., 2016) or sodium chloride (NaCl) (Acebes-Huerta et al., 2020). Factors influencing PRP yield include blood draw, centrifugation parameters, and choice of anticoagulants (Dhurat & Suresh, 2014). Particularly for the preparation of PRP samples intended for intraovarian use, it is crucial to avoid subjecting platelets to stresses exceeding 1500 g, as platelet survival becomes compromised beyond 2200 g. Operating at these high speeds not only elevates the risk of tube shattering but also increases the likelihood of platelets exposure and other potential damages (Sills, 2022).

Selecting an appropriate anticoagulant is crucial to maintain the optimal functionality, integrity, and morphology of platelets. Regarding the choice of anticoagulant, most experts advise against using EDTA due to its potential to harm the platelet membrane. Instead, anticoagulants containing citrate and dextrose or sodium citrate (SC) are recommended for preserving platelet quality. EDTA, heparin, acid citrate dextrose (ACD) and SC have been described for blood collection prior to PRP preparation due to their capacity to avoid clot formation and their documented use for hematological studies, hemostasis, and coagulation tests (Do Amaral et al., 2016). The majority of commercial kits use SC and ACD (Dhurat & Suresh, 2014) to prepare PRP, but there is no agreement on the most effective anticoagulant for this purpose since studies have shown contradictory results regarding the PRP and growth factors (Amable et al., 2013; Araki et al., 2012; Dhurat & Suresh, 2014; Do Amaral et al., 2016; McShine et al., 1990; Ulasli et al., 2022; Zhang et al., 2019).

Flow cytometry analysis offers an evaluation of platelet function using various fluorochrome-antibody conjugates. This methodological approach demonstrates several advantages, including the requirement of a minimal volume of total blood, the ability to analyse platelets within their physiological environment, and the necessity for minimal sample manipulation (Vucetic et al., 2018). Moreover, it enables the determination of the activation and reactivity state of circulating platelets in response to agonists (Yaw et al., 2020). Testing can be conducted using whole blood, PRP, or washed platelets, providing flexibility and adaptability to different experimental conditions (Michelson et al., 2009). Specific components derived from platelets are employed for flow cytometry testing purposes. CD62P (platelet surface P-Selectin) is mobilized from the α -granules to the surface when platelet activation occurs, promoting platelet-platelet and platelet-fibrin binding (Michelson et al., 2009). CD42b (Glycoprotein Ib) is a component of the platelets GPIb-V-IX complex, which binds to von Willebrand factor, facilitating platelet adhesion after their activation. PAC1 targets activated GP-IIb/IIIa on activated platelets (Shattil et al., 1985).

Importantly, at the end of PRP isolation, after platelet activation, it is expected an intake of growth factors up to 10 times higher when compared to the baseline values (Herlihy & Seli, 2022; Zadehmodarres et al., 2017).

The concentration of PRP used in the ovarian perfusion procedures, as previously described in section 1.2.3.4.4.3, section 1, Table 2, varies among different studies (Herlihy & Seli, 2022; Kuang et al., 2022; Pellicer et al., 2023; Rinaudo & Albertini, 2021; Seckin et al., 2022; Sharara et al., 2021; Sills, 2022; Sills & Wood, 2021; Vo et al., 2021). Consistency and accuracy in results of appliance PRP across studies are dependent on the implementation of a standardized PRP preparation protocol (Sharara et al., 2021).

2.4.2.5 *In vitro* culture for human ovarian tissue

Since cortical ovarian tissue predominantly consists of primordial follicles, *in vitro* culture must incorporate parameters essential for the maintenance of these follicles (Grosbois et al., 2023).

Eagle's minimum essential medium (MEM) is a commonly used cell culture medium that provides a balanced and nutritive environment for the growth of various cell types (Smitz et al., 2010). In the context of ovarian tissue or follicle culture, the choice of MEM is often made, being described in studies of *in vitro* activation (Yang et al., 2020). In some cases, MEM is customized by adding specific factors, such as hormones or growth factors, to create a culture environment that closely mimics the *in vivo* conditions necessary for successful tissue or follicle development (Abir et al., 2011; Friedman et al., 2012; Kang et al., 2016).

The influence of specific factors on follicular activation has justified the supplementation of α -MEM. For instance, Human serum albumin (HSA) and FSH are considered fundamental to create an environment conducive to the proper maintenance of primordial follicles during the *in vitro* culture process (Smitz et al., 2010). Specifically, FSH addition to the media has demonstrated benefits preventing apoptotic atresia and has a mitogenic function (Wright et al., 1999). HSA has demonstrated similar effect in increasing the number of health follicles (Wright et al., 1999). The use of hMG has demonstrated a beneficial effect on angiogenesis, being associated with an increase of the surviving follicles as well as, revascularization in the ovary (Wang et al. 2002, 2012).

Insulin, transferrin, selenium (Grosbois et al., 2023; Yang et al., 2020) as well as, glutamine (Grosbois et al., 2023; Lunding et al., 2020; Schmidt et al., 2005), GDF9, ascorbic acid, and activin (Smitz et al., 2010) are used to favour follicle development. Activin A was described as regulator of granulosa cell growth and differentiation in *in vitro* culture (Telfer et al., 2008), while GDF9 was associated with *in vitro* maturation and cumulus cells expansion (Chatroudi et al., 2019). Ascorbic acid promotes follicular integrity, being described for some authors its usage in culture of ovarian tissue for follicular activation (Kawamura et al., 2013; Lunding et al., 2020; Suzuki et al., 2015).

In addition to the culture media, it is important to acknowledge that the continuous and dynamic process of folliculogenesis requires significant alterations in the ECM (Smith et al., 2002), particularly during the activation of primordial follicles (Grosbois et al., 2023). Grosbois et al. conducted a study examining changes in the ECM composition and architectural features across different regions of the ovarian cortex, in combination with follicle dynamics and localization

during culture. This study has shown that the *in vitro* culture could naturally induce mechanical alterations in ECM of ovarian cortex that might create a more favourable environment for primordial follicle activation (after 6 days of culture there was a decrease in primordial follicles number) (Grosbois et al., 2023).

In terms of the time needed to detect follicular activation, through Akt pathway products, there is a lack of consensus. Akt phosphorylation was reported to be significantly increased after 3 days of grafting (Masciangelo et al., 2019). Li et al. described a protocol involving 48 hours of incubation with PI3K stimulating drugs to activate follicles in human and mouse ovaries (Li et al., 2010). Dolmans et al. suggested 48h of culture but without significant differences in the proportion of primordial follicles after the xenografting (Dolmans et al., 2019). However, the same authors adverted to the 2-day culture with *in vitro* activation stimuli because of its potential deleterious effect on ovarian reserve. Massive follicle activation in this context could result in widespread atresia (Dolmans et al., 2019). In line with that, Devenuto et al. reported that 2-day culture system is associated with an increase in tissue damage, necrosis and apoptosis (Devenuto et al., 2020).

Even though numerous studies have underscored the beneficial effects of PRP on the proliferation and differentiation of diverse cell types (Liu et al., 2016), advocating its utility as a substitute for fetal bovine serum in cell cultures (Viau et al., 2019), the exploration of PRP's impact on ovarian tissue *in vitro* remains relatively limited. Hosseini et al. demonstrated notably higher growth and survival rates of follicles in both fresh and vitrified groups compared to others. Moreover, the inclusion of PRP in the culture medium notably enhanced the survival and growth of early preantral follicles *in vitro* (Hosseini et al., 2017).

Furthermore, a case report by Callejo et al. showcased successful autologous transplantation of frozen/thawed human ovarian tissue treated with PRP, resulting in a live birth (Callejo et al., 2013). Another experiment, where human preantral follicles were isolated from ovarian medullary tissue and cultured for 8 days, the survival rate of follicles in the human platelet lysate group surpassed those in groups treated with fetal bovine serum, HSA, and umbilical cord plasma (Adrados et al., 2023).

Ljubić et al. reported a 2-hour incubation of PRP with ovarian tissue followed by the transplantation of these fragments. Follicular growth was observed three months after the orthotopic transplantation, leading to the retrieval of a mature oocyte (Ljubić et al., 2017).

2.4.2.6 Human ovarian tissue analysis through histology and immunohistochemistry (IHC)

The critical aspect of the *in vitro* culture approaches lies in the evaluation of ovarian tissue integrity; particularly, histological and immunohistochemical analysis, that are crucial in assessing follicular classification, density, stromal evaluation, and ensuring the practical applicability of such interventions (Pais et al., 2021).

The assessment of human ovarian tissue was primarily reviewed by our research group in 2016, focusing on evaluating the tissue before and after slow cryopreservation. Specifically, our research group established a methodology for histological (follicular density, stromal integrity) and immunohistochemical (Ki-67, Bcl2, and P53) evaluation of human ovarian tissue, based on the existing literature (Carvalho, 2016). This methodology established in 2016 served as the basis for subsequent studies conducted at our center. Further research improved the evaluation of apoptosis by incorporating Caspase 3, vascular assessment using Factor VIII (FVIII) and stromal integrity was thoroughly assessed using a scoring system (Pais, 2022).

Histological criteria enable the assessment of ovarian tissue morphology through light microscopy, utilizing standard Hematoxylin and Eosin (H&E) staining techniques. Hematoxylin is a basic dye (cationic) with a positive charge, which is oxidized and converted into hematein that reacts with anionic groups such as nucleic acids, phosphate groups, glycosaminoglycan sulfates, and protein carboxyl groups in cells and tissues. Cellular structures such as nucleic acids. Hematoxylin stains these structures blue. Eosin, an acidic (anionic) dye, stains basic structures, such as connective tissue fibers, and matrices with a pink colour (Gamble, 2008). Gougeon established characteristics for classifying the developmental state of follicles within H&E (Gougeon, 1996).

Follicular density serves as a quantitative assessment of the distribution and abundance of follicles within the ovarian tissue, typically measured as the number of follicles per mm² (Fransolet et al., 2014). When assessing follicular density it is desirable to only consider follicles with a visible nucleus to account for the three-dimensional structure of follicles and avoid double counting (Candelaria & Denicol, 2024; Herraiz et al., 2020). The irregular follicular distribution is considered normal in the human ovarian cortex (McLaughlin et al., 2014).

Within H&E staining, follicles are classified as degenerated when they included cells with pyknotic nuclei, retracted ooplasm, and disorganized granulosa cells. The follicular degeneration count is calculated based on the relative quantity of degenerated follicles compared to the total number of follicles (Yildirim et al., 2015).

Morphologically, cellular death is characterized by cytoplasmatic vacuolization, chromatin condensation and by the presence of apoptotic bodies and eosinophiles. The atretic oocytes reveal a defeat on granulosa cells that is then followed by disintegration of the oocyte and its cytoplasm vacuolization (Devine et al., 2000; Hussein et al., 2003). Stromal cells, when damaged, show chromatin condensation, pyknotic nuclei and its cellular membranes lysis or even its matrix lose cytoplasm (Otalá et al., 2002; Talevi et al., 2013). The follicular atresia was described by Gougeon as irregular shape, pyknosis of granulosa cells, cytoplasmic contraction, the presence of vacuoles, and eosinophilia of the ooplasm (Gougeon, 1996).

The literature suggests a score for follicular generation, tissue necrosis and interstitial edema, on a scale of 0 to 3, where 0 corresponds to the absence of alterations, 1 indicates mild alterations or less than 1/3, 2 signifies moderate alterations or between 1/3 and 2/3, and 3 represents severe alterations or more than 2/3 (Yildirim et al., 2015). Follicular degeneration is detected

histologically by identifying a pyknotic nucleus, retraction of the ooplasm, disorganization of granulosa cells, and increased intercellular spaces (Yildirim et al., 2015). Tissue necrosis is described by a visually assessment of the area in necrosis (Asfour et al., 2015). Edema, which refers to the accumulation of intercellular fluid, is assessed based on its extent (Yildirim et al., 2015).

Immunohistochemistry is a well-established method for detecting antigens (proteins) using antibodies, accounting for their location in the tissue (Magaki et al., 2019). First described by Coons and Jones, comprises the labelling of target antigens due to the colour formation (Coons et al., 1941).

Ki-67 is a nuclear antigen associated with cell proliferation, present in the G1, S, G2, and M phases of the cell cycle – the phases of cellular activity – proving useful in assessing the fraction of cells that are in growth (Dath et al., 2010; Dolmans et al., 2007; Schluter et al., 1993). In ovarian follicles Cell proliferation is indicated by the percentage of follicles expressing Ki-67 in granulosa cells. Follicles with at least one granulosa cell marked for Ki-67 are considered to have initiated development (Candelaria & Denicol, 2024; Dath et al., 2010; Dolmans et al., 2015; Luyckx et al., 2013). Ki-67 expression was chosen to quantify the follicular activation when studying signalling pathways during *in vitro* human follicle activation (Grosbois & Demeestere, 2018; Lunding et al., 2020; Masciangelo et al., 2019).

The anti-caspase 3 labelling is a marker used to identify apoptotic cells in tissues. In apoptotic cells, it becomes activated (Elmore, 2007). A correlation between caspase 3 staining and follicular apoptosis was established either by marking the oocyte or when 50% of the granulosa cells tested positive (Kedem et al., 2011).

The FVIII is used as a marker for endothelial cells and was described to evaluate the stimulation of angiogenic process. This antigen is a glycoprotein produced exclusively by endothelial cells and megakaryocytes, making it suitable for vessel identification (Peyvandi et al., 2011). According to the literature, clusters of endothelial cells marked with FVIII, with or without a clear lumen, are considered individual vessels (Wu et al., 2010). The vascular area was found to be correlated with the percentage of the area marked with FVIII (Herraiz et al., 2020).

p-Akt immunostaining has been documented to evaluate *in vitro* activation (Masciangelo et al., 2019; Terren et al., 2021). De Roo et al. described a diffuse distribution of p-Akt throughout the entire oocyte cytoplasm in primordial, intermediate, and primary follicle, in human ovarian tissue, from day 0 to day 4. Limited expression was observed in granulosa cells and stromal cells at day 0. As the culture progressed from day 1 to day 6, the diffuse localization transformed into a cytoplasmic spot, independent of the follicle stage (De Roo et al., 2020).

Goto et al. proposed an evaluation of Akt and p-Akt regarding the staining intensity, based on the following criteria: negative, scored as 0; weak, 0–20% of cells stain, scored as +1; moderate, when 20–50% of cells stain, scored as +2; and strong, >50% of cells stain, scored as +3 (Goto et al., 2007). In this study, immunoreactivity of Akt was observed in oocytes, granulosa cells, and theca

cells in primordial follicles and follicles at each growth stage. Akt expression was also detected in luteal cells during the mid-luteal phase. Staining intensity was moderate in granulosa cells of primary follicles, whereas granulosa cells in antral and preovulatory follicles exhibited strong staining (Goto et al., 2007).

p-Akt was also assessed in comparison to PTEN. The staining intensity of p-Akt in granulosa cells was strong or moderate, accompanied by negligible or weak expression of PTEN. In contrast, weak staining for p-Akt occurred in granulosa cells that stained moderately for PTEN (Goto et al., 2007).

Considering a qualitative grade, p-Akt was also described by Barretta et al, on a scale of no staining, weak staining (on primordial follicles of the granulosa cell layer and oocyte cytoplasm), to intense staining of the granulosa cell layer and oocyte nucleus and cytoplasm (Barretta et al., 2023).

Based on this brief introduction to the topic, the next section will describe the methods applied to optimize the PRP preparation protocol and test its efficacy on human ovarian tissue, assessed in an *in vitro* culture system. The subsequent results are then presented and discussed in the context of the literature.

2.4.3 Methods

2.4.3.1 Optimization of platelet-rich plasma (PRP) protocol

The following results are derived from published data available in an Open Access Original Article with the DOI: <https://doi.org/10.3390/bioengineering11030209>.

This study was designed, conducted, and reported in accordance with the principles of good clinical practice guidance and with the 1964 Helsinki declaration and its later amendments. Ethical approval was granted by the Hospital Ethics Board (reference number CHUC-171-20), and written consent was obtained from all participants.

The research was carried out at the reproductive medicine unit of a tertiary hospital. Women planning to undergo frozen embryo transfer (FET) were informed about the study. As the FET requires measuring plasma progesterone levels on the day of transfer, the patients who gave the written informed consent, also donated blood for the study, in the same procedure.

This study employed a two-step approach to investigate the impact of different anticoagulants and PRP resuspension media on PRP preparation. In the first experiment, we evaluated the efficacy of SC (Sarstedt S-monovette®, Nümbrecht, Germany) versus EDTA (Sarstedt S-monovette®, Nümbrecht, Germany) as anticoagulants for blood collection providing a 28 mL venous blood sample. Half of this volume was collected in tubes containing SC, while the other half was collected in tubes containing EDTA. This dual collection method ensured consistency by using samples from the same patients.

According to the results obtained in the first experiment, we proceeded to the second experiment. Here, we compared plasma and NaCl as PRP resuspension media. Similarly to the first experiment, blood samples were obtained from 19 participants using the same collection technique. Samples collected in EDTA-containing tubes were utilized for this phase. Half of the volume from each participant was used to prepare PRP resuspended in plasma, while the other half was utilized for PRP resuspended in NaCl.

Throughout the study, meticulous attention was paid to maintaining controlled conditions. Peripheral blood samples were collected by two trained healthcare professionals using a 21 G butterfly catheter affixed to negative pressure-receiving tubes.

This standardized approach minimized potential variables during sample collection, ensuring the reliability and validity of the study findings. Additionally, this approach has been chosen to help prevent clotting and unintended platelet activation.

A small amount of the blood sample (1 mL) was separated to perform the initial evaluation of whole blood by flow cytometry and the remaining volume was transferred to two empty 15 mL centrifugal tubes (Sarstedt S-monovette®, Nümbrecht, Germany) and centrifuged at $300\times g$ for 10 min. The upper layer (plasma and buffy coat) was then transferred to a new empty sterile tube and centrifuged at $700\times g$ for 15 min, to pellet the platelets. The supernatant was discarded, and the pellet was resuspended in 1:4 volume with the lower portion of plasma in the first experiment. In the second experiment, we aimed to compare two different resuspension solutions and the pellet was resuspended in (1) plasma or (2) NaCl (.9%, B.Braun, Queluz de Baixo, Portugal). The entire procedure was performed at room temperature (21–24 °C).

A schematic representation of the PRP preparation is presented in Figure 32.

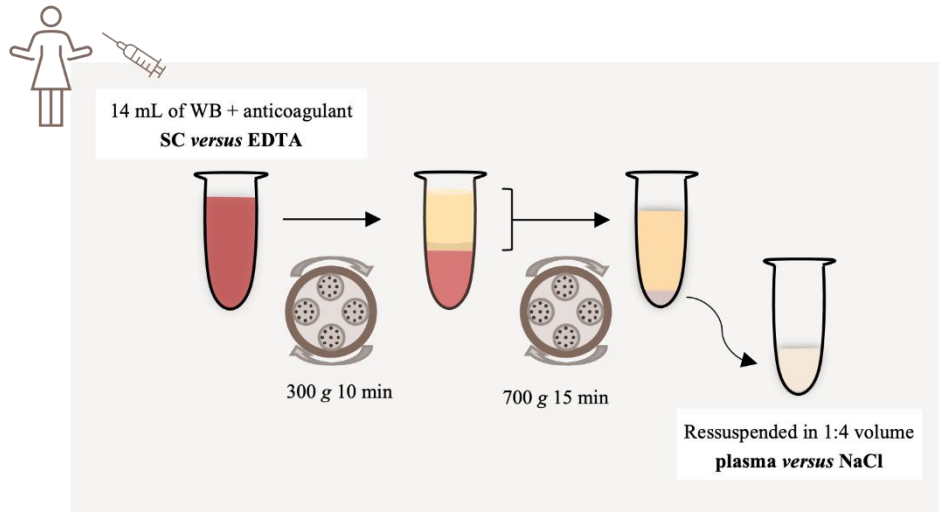


Figure 32 Schematic representation of the protocol to obtain PRP

Whole blood was collected into tubes with anticoagulant, SC versus EDTA. The samples (14 mL) were transferred to empty tubes and centrifuged at 300 g for 10 minutes. The upper layer (plasma and buffy coat) was then transferred to a new empty sterile tube and centrifuged at 700 g for 15 minutes, to pellet the platelets. The supernatant was discarded, and the pellet was resuspended in 1:4 volume of plasma versus NaCl.

PRP: platelet-rich plasma; EDTA ethylenediaminetetraacetic acid; SC: sodium citrate; NaCl: sodium chloride; WB: whole blood

A sample from the whole blood (1 mL) and the PRP samples were transported to the hospital's cytometry unit, avoiding agitation and maintaining a temperature range of 21–24°C. The total platelet cell count was determined in whole blood and in PRP samples to calculate the PRR. Platelet count was performed in a ABX Micro ES60 (Horiba, Japan) hemacytometer. The PRR was determined by calculating the ratio of the platelet concentration in the PRP sample to the platelet concentration in the whole blood sample, considering the respective volumes.

This calculation was performed using the formula:

$$\text{PRR (\%)} = \frac{\text{Platelets concentration in PRP} \times \text{volume of PRP}}{\text{Platelets concentration in whole blood} \times \text{volume blood}} \times 100$$

The adoption of PRR as a reporting metric enhances the clarity and interpretability of our findings within the context of PRP research. We chose this method to best capture the relative increase in platelet concentration within PRP compared to the baseline concentration in whole blood. Additionally, PRR considers the variability present in biological samples across individuals, as it relates the analysis to the initial platelet count.

To assess the platelet activity in PRP, we evaluated the presence of specific markers of platelet activation (CD62p and PAC1) before and after inducing platelet activation with thrombin.

For the platelet activity assay, samples were prepared by diluting the PRP samples in NaCl (.9%) at a ratio of 1:10. PRP samples were mixed with 5 µL of each monoclonal antibody, CD62Ppe (Becton, Dickinson – BD - bioscience, New Jersey, USA) and PAC1-FITC (Becton, Dickinson – BD - bioscience, New Jersey, USA) and analyzed using a flow cytometer (Becton, Dickinson – BD

- bioscience, New Jersey, USA). Identical PRP samples were incubated with 6.5 μ L of thrombin (50 μ M) (Sigma, Merck, Darmstadt, Germany). The incubation was carried out at room temperature, in a dark environment, for a duration of 15 min. Following this incubation period, samples were resuspended in 100 μ L of NaCl (.9%) and analyzed using a FACSCalibur flow cytometer (Becton, Dickinson – BD - bioscience, New Jersey, USA). Results were expressed as the percentage of CD62p positive or PAC-1 positive platelets after in vitro activation.

After activation, PRP samples were centrifuged at 1200 $\times$ g for 10 min and the supernatant was stored at –80 °C. The concentration of VEGF released into the plasma after PRP activation was measured using an enzyme-linked immunosorbent assay (ELISA). The ELISA kit used was the Human VEGF Immunoassay by R&D Systems® (RD systems Quantikine ELISA, Minneapolis, MN, USA). The sensitivity of the kit was determined by the minimum detectable dose of human VEGF, which is <9 pg/mL. The ELISA procedure followed the manufacturer's instructions without any modifications. Briefly, the VEGF concentrations of in the samples were determined by comparing the optical densities of the samples to the standard curve generated using the known concentrations of VEGF standards provided in the kit. To ensure the accuracy and reliability of the ELISA results, controls were used. The VEGF quantification assays were performed in duplicate for all the PRP preparation samples.

2.4.3.2 Ovarian tissue culture

A total of 85 frozen-thawed human ovarian cortex fragments, from 6 oncological patients, were used in this experiment. This sample size does not allow for the detection of small or medium effects; it only enables the detection of large effects. The calculation of sample size a priori, using G*Power for an F test with ANOVA for fixed effects and interactions and considering a desired power (1- β) of .80 with 5 groups, established sample size was 269. Despite this, we proceeded with the analysis due to the scarcity of human ovarian tissue and recognizing its significant value in translational research.

The human ovarian tissue was donated by patients who underwent fertility preservation and provided written informed consent for the use of surplus tissue (less than 10%). The ovarian cortical pieces were obtained from non-pregnant oncological patients under the age of 35. The characteristics of the patients who contributed human ovarian tissue for research are described in Table 73.

Table 73 Clinical characteristics of patients whose ovarian tissue was used for the experiment

Age and diagnosed disease corresponding to each code assigned for the anonymization of the samples.

Code	Age (years)	Diagnosis
A	30	Nasopharyngeal tumor
B	20	Osteosarcoma
C	22	Osteosarcoma
D	23	Bone tumor
E	33	Breast cancer
F	27	Intestinal tumor

Fragments of human ovarian tissue were originally cryopreserved in pieces with 10x5x1mm using slow freezing protocol, adapted from Rosendahl et al. (Rosendahl et al. 2011). Fragments of human ovarian tissue were cryopreserved in cryotubes (Nunc™, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), each containing 1.5 mL of freezing medium. The freezing medium comprised Dulbecco's Phosphate Buffered Saline (DPBS, Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) with .1M sucrose (Merck, Darmstadt, Germany), 1.5 M Ethylene glycol (Sigma-Aldrich/Merck, Darmstadt, Germany), and 10 mg/mL of HSA (Grifols, Barcelona, Spain).

For the slow freezing process, an automated system, the Planer Kryo 360-1.7 (Planer PLC, Sunbury-On-Thames, UK), equipped with the Freeze Control CL-8800 system (CryoLogic, Victoria, Australia) and controlled by CryoGenesis™ V5 software, was utilized. The applied protocol is detailed as follows:

	0°C for 15 minutes
i	2°C/minute until -9°C.
ii	manual seeding (repeatedly inverting each cryotube three times)
iii	.3°C/minute until -40°C
iv	10°C/minute until -140°C
v	liquid nitrogen container at -196°C.

Thawing was performed according to the established protocol for fertility preservation in the reproductive medicine unit, involving a sequential passage through solutions with decreasing concentrations of cryoprotectant. The first solution consists of ethylene glycol (Sigma-

Aldrich/Merck, Darmstadt, Germany) at a concentration of .75 M, sucrose at a concentration of .25 M, and HSA (Grifols, Barcelona, Spain) at a concentration of 10 mg/mL in DPBS (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The second solution is composed of sucrose at a concentration of .25 M and HSA (Grifols, Barcelona, Spain) at a concentration of 10 mg/mL in DPBS (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The last thawing solution corresponds to DPBS (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) with HSA (Grifols, Barcelona, Spain) at a concentration of 10 mg/mL.

The complete procedure for the cryopreservation and thawing of human ovarian tissue is illustrated in the Figure 33.

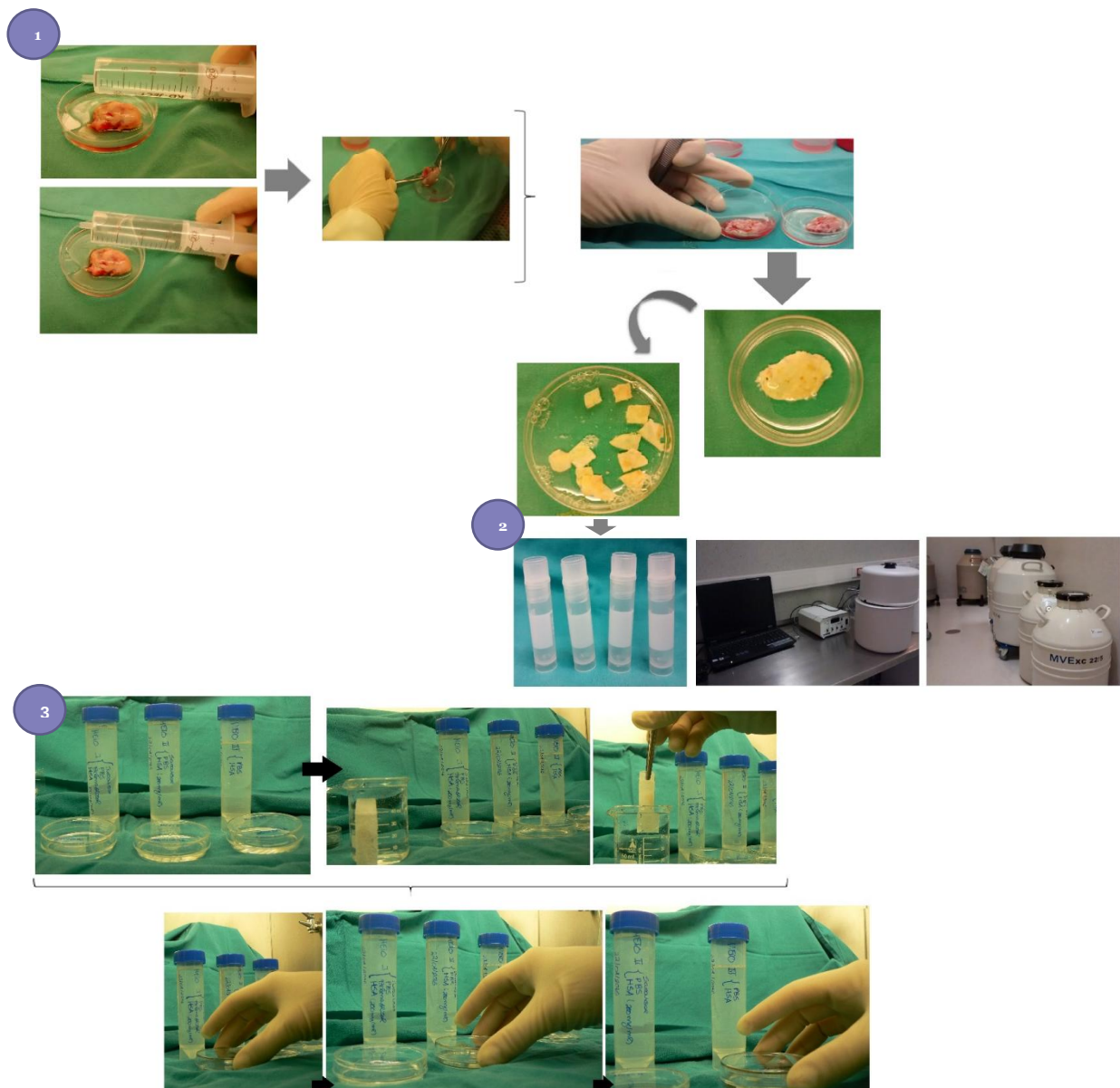


Figure 33 Processing of human ovarian tissue

(1) Cryopreservation - The medullary region is removed, and the cortex is divided into pieces measuring 10x5x1 mm. Fragments are then transferred to cryotubes and cryopreserved using a slow freezing protocol with 1.5M Ethylene glycol. (2) Cryopreservation in slow freezing protocol - (3) Thawing involves passing the fragments through three different media, involving a stepwise reduction in the concentration of Ethylene glycol and sucrose. Adapted from Carvalho, 2016.

Immediately after thawing (t=0h), a sample of ovarian cortex was used as a control and fixed in a 4% buffered formaldehyde solution (Sarstedt, Nümbrecht, Germany). For research purposes, the original fragment of human ovarian tissue was cut into pieces measuring 3x2x1 mm, a size compatible with our prior studies (Pais et al., 2021). This dimension aligns with the size range commonly used for human ovarian tissue cryopreservation in clinical settings (Donnez et al., 2022) and differs from the size specified for biopsy ovarian tissue to stimulate the Hippo-pathway, which is 1x1x1 mm (Lunding et al., 2018, 2020).

At least duplicate samples of the tissue were ensured for each experimental condition, as outlined in Table 74.

Table 74 Overview of the study culture conditions

"to" denotes immediately after thawing, "t4" represents 4 hours of culture, and "t48" represents 48 hours of culture, both with and without PRP. The ovarian tissue donors are coded from A to F, with their respective ages listed. The number of fragments used per woman and per condition is detailed in the table, highlighted in blue.

Age		Without PRP				PRP				Without PRP				PRP			
		to				t4				t48				t48			
A	30	1	2			3	4			5	6			7	8	9	
B	20	1	2			3	4	5	6	7	8	9	10	11	12	13	14
C	22	1	2			3	4	5				6	7	8		9	10
D	23	1	2			3	4	5				6	7	8		9	10
E	33	1	2			3	4					5	6			7	8
F	27	1	2			3	4					5	6			7	8

to: immediately after thawing; t4: 4 hours of culture; t48: 48 hours of culture; PRP: platelet-rich plasma

In addition to the 85 frozen-thawed human ovarian fragments used for this analysis, an additional fragment of ovarian tissue from each condition was stored at -80°C for further analysis. While the results of this analysis are not included in this manuscript, they were considered in the study design and are expected to be used in future research.

PRP was freshly prepared for this purpose, following the optimized protocol. We opted for non-autologous PRP due to the time-consuming nature of collecting, processing, and preparing autologous PRP.

Non-autologous PRP was obtained from blood collected from patients undergoing frozen embryo transfer, who were already required to provide blood for progesterone quantification at the time of transfer.

Conventional culture conditions were applied, using standard 4-well dishes (Nunc™, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The human ovarian tissue was cultured for 4 hours (t=4h) and 48 hours (t=48h) in 600 µL of medium containing Minimum Essential Medium α (α-MEM; 22561-021, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), supplemented with penicillin/streptomycin (Pen-Strep; 1:1000, 15140-122, Gibco, Thermo Fisher

Scientific Inc., Waltham, Massachusetts, USA), HSA 10% (Grifols, Barcelona, Spain), and human menopausal gonadotropin (hMG; .3 IU/mL, Menopur®, Ferring Pharmaceuticals, Saint-Prex, Switzerland). The culture was maintained in a controlled environment of 5% CO₂ and 37°C.

The medium was collected from each well at each time-point, and the tissue was fixed in a 4% buffered formaldehyde solution (Sarstedt, Nümbrecht, Germany). The entire medium was stored at -80°C. The study design is schematically described in Figure 34.

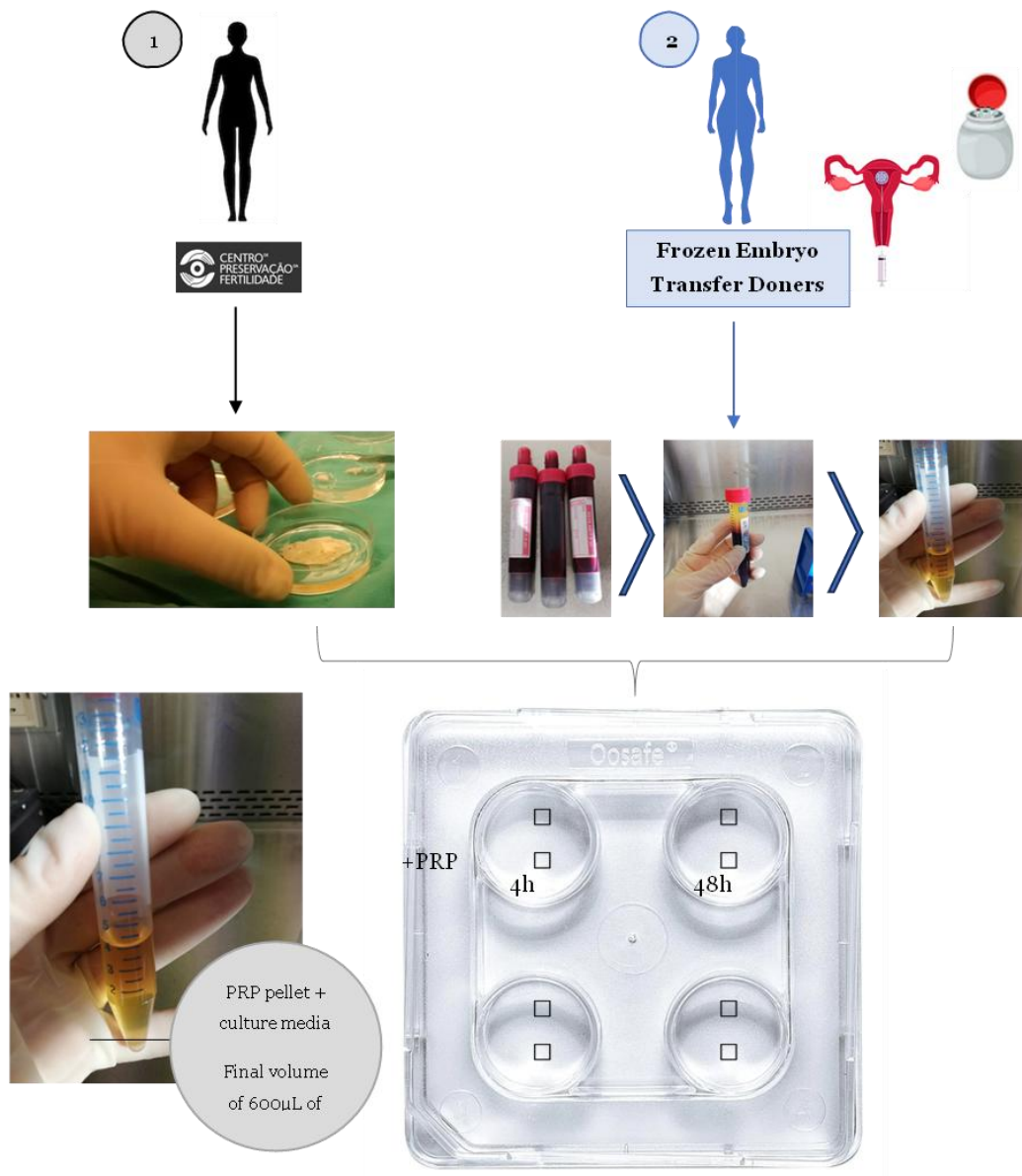


Figure 34 Representative scheme of the experimental design (ovarian tissue culture with PRP)

(1) patients who did fertility preservation and donate <10% of their ovarian tissue for research and (2) patients undergoing frozen embryo transfer who donated blood for the preparation of non-autologous PRP. PRP was prepared for the ovarian tissue culture on Day 0, simultaneously with ovarian tissue thawing. The culture conditions included both PRP-supplemented and non-supplemented conditions at 4h and 48h time-points. In cases where PRP supplementation occurred, it was diluted in the culture medium (final volume of 600uL). PRP: platelet-rich plasma.

The *in vitro* culture and assessment of ovarian tissue followed established protocols outlined in the literature, cited in the section 2.4.2.5 and 2.4.2.6, and aligns with the methodologies previously described by our research group (Azevedo et al. 2022; Carvalho 2016; Pais et al. 2021).

2.4.3.3 Evaluation of ovarian tissue: histology and immunohistochemistry (IHC)

To assess the ovarian tissue characteristics, we evaluated follicle density and stromal integrity using H&E staining. Additionally, we assessed, by, immunohistochemistry (IHC), 1) proliferation levels using Ki-67 staining; 2) apoptosis using caspase 3 staining; 3) angiogenesis using FVIII staining; and 4) PI3K-Akt activation through p-Akt staining.

All samples were processed at the department of pathology of ULS Coimbra. The ovarian tissue samples were embedded in paraffin, sectioned using a microtome into 5 µm thick slices, and mounted on glass slides before being stained.

2.4.3.3.1 Haematoxylin & eosin (H&E) staining

Histological analysis was performed using H&E staining, with a staining protocol as described in Table 75.

Table 75 H&E protocol used at pathological anatomy of ULS Coimbra

COMPOUND	NUMBER OF PASSAGES	TIME
Xylol (VWR, Fontenay-sous-Bois, France)	3	2min.
Absolute ethanol (Proclínica, Lisboa, Portugal)	2	2min.
Ethanol 95% (vol/vol)	1	2min.
Tap water	1	2min.
Haematoxylin Gill 1 (Leica, Richmond, VA, USA)	1	5min.
Tap water washing	1	5min.
Eosin 1% water solution (Bio-optica, Milan, Italy)	1	40sec.
Ethanol 95% (vol/vol)	1	30sec.
Absolute ethanol (Proclínica, Lisboa, Portugal)	2	30sec.
Xylol (VWR, Fontenay-sous-Bois, France)	3	30sec.

H&E: Haematoxylin and eosin

2.4.3.3.2 Immunohistochemistry (IHC)

The immunohistochemistry was conducted in accordance with the protocols established by the pathology unit. The labelling procedure was performed using the BenchMark ultra IHC/ISH automated staining system (Ventana Medical Systems, Roche Diagnostics, Basel, Switzerland) with the OptiView DAB IHC v5 detection kit (v1.00.0117, Ventana Medical Systems, Roche Diagnostics, Basel, Switzerland). This kit employs an indirect method for detecting primary antibodies. The tissue antigen specifically binds to a primary antibody in paraffin-embedded tissue sections. This primary antibody is recognized by secondary antibodies from the kit (HQ linker). Subsequently, the secondary antibodies are recognized by tertiary antibodies associated with an enzyme, in this case, horseradish peroxidase (HRP). This complex is visualized through the oxidation of substrates (hydrogen peroxide and 3,3'-diaminobenzidine, or DAB) by HRP, resulting in an insoluble, brown-coloured product, as described by the manufacturer.

The antigen retrieval process utilized a cell conditioner 1 (CC1, Ventana Medical Systems, Roche Diagnostics, Basel, Switzerland) buffer, followed by incubation with the primary antibody as per manufacturer instructions and the previously mentioned dilutions. Immunostaining detection was carried out using the OptiView DAB IHC Detection Kit (Ventana Medical Systems, Roche Diagnostics, Basel, Switzerland) according to the manufacturer's recommendations, involving the detection of the precipitated DAB chromogenic substrate.

For Ki-67, the immunohistochemical assay employed a rabbit monoclonal primary antibody against Ki-67 (diluted 1:300; clone 30-9, 5278384001, Roche, Roche Diagnostics, Basel, Switzerland).

In the case of caspase 3 analysis, a rabbit polyclonal antibody against caspase 3 (diluted 1:100; AHP2286, Bio-Rad Laboratories, Hercules, California, USA) was utilized. The IHC protocol for Ki-67 and caspase 3 is outlined in the provided Table 76.

Table 76 Summary of the immunohistochemistry protocol for Ki-67 and caspase 3 from the department of pathology of ULS Coimbra

Step number	Description
1	Deparaffinization: Heating to 72°C (4 minutes) with EZprep solution to emulsify paraffin.
2	Antigen retrieval: Induced by heat and incubation with CC1 buffer solution at 100°C (40 minutes).
3	Endogenous peroxidase inhibition: Pre-primary inhibitor - Inhibition with hydrogen peroxide (4 minutes).
4	Immunohistochemical labelling: Incubation with the primary antibody at 36°C: - Ki-67 – 40 minutes; caspase 3 – 44 minutes.
5	Antigen-antibody reaction detection (OptiView DAB IHC): Incubation with a cocktail of secondary antibodies, HQ Universal linker (8 minutes); Incubation with tertiary antibodies with the HRP Multimer peroxidase enzyme (8 minutes).
6	Chromogenic revelation: Application of a drop of hydrogen peroxide simultaneously with a drop of DAB and incubation (8 minutes); Addition of a drop of copper solution and incubation (4 minutes).
7	Nuclear contrast for nuclear architecture observation: Addition of a drop of haematoxylin and incubation (8 minutes); Incubation with a drop of post-contrast, bluing reagent (4 minutes).

FVIII staining utilized a rabbit polyclonal antibody against FVIII (760-2642, Cell Marque, Merck, Darmstadt, Germany). The staining procedure was conducted on the BenchMark ultra IHC/ISH automated staining system (Ventana Medical Systems, Roche Diagnostics, Basel, Switzerland) using the OptiView DAB IHC v5 detection kit (v1.00.0117, Ventana Medical Systems). The detailed protocol is presented in the accompanying Table 77.

Table 77 Summary of the immunohistochemistry protocol for Factor VIII from the department of pathology of ULS Coimbra

Step number	Description
1	Deparaffinization: Heating to 72°C for 4 minutes with the aid of EZprep solution to emulsify paraffin.
2	Antigen retrieval: Induced by heat and incubation with CC1 buffer solution for 24 minutes at 95°C.
3	Immunohistochemical labelling: Incubation with the primary antibody for 12 minutes at 36°C.
4	Endogenous peroxidase inhibition: Pre-primary inhibitor – Inhibition with hydrogen peroxide (4 minutes).
5	Antigen-antibody reaction detection (OptiView DAB IHC): Incubation with secondary antibodies, HQ Universal linker, for 8 minutes; Incubation with tertiary antibodies with the HRP Multimer peroxidase enzyme for 8 minutes.
6	Chromogenic revelation: Application of a drop of hydrogen peroxide simultaneously with a drop of DAB and incubation (8 minutes); Addition of a drop of copper solution and incubation (4 minutes).
7	Nuclear contrast for nuclear architecture observation: Addition of a drop of hematoxylin and incubation for 8 minutes; Incubation with a drop of post-contrast, bluing reagent, for 4 minutes.

For p-Akt immunohistochemistry, a rabbit monoclonal antibody (diluted 1:50; Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060, Cell Signaling, Danvers, Massachusetts, USA) was employed. The staining was performed on the BenchMark ultra IHC/ISH automated staining system (Ventana Medical Systems, Roche Diagnostics, Basel, Switzerland) using the OptiView DAB IHC v5 detection kit (v1.00.0117, Ventana Medical Systems, Roche Diagnostics, Basel, Switzerland). The protocol is summarized in Table 78.

Table 78 Summary of the immunohistochemistry protocol for p-Akt from the department of pathology of ULS Coimbra

Step number	Description
1	Deparaffinization: Heating to 72°C (4 minutes) with EZprep solution to emulsify paraffin.
2	Antigen retrieval for 25 minutes in a thermostated bath (Dako Agilent, Santa Clara, USA) at 95°C.
3	Endogenous peroxidase inhibition: Pre-primary inhibitor – Inhibition with hydrogen peroxide (4 minutes).
4	Immunohistochemical labelling: Incubation with the primary antibody at 36°C overnight.
5	Antigen-antibody reaction detection (OptiView DAB IHC): Incubation with a cocktail of secondary antibodies, HQ Universal linker (8 minutes); Incubation with tertiary antibodies with the HRP Multimer peroxidase enzyme (8 minutes).
6	Chromogenic revelation: Application of a drop of hydrogen peroxide simultaneously with a drop of DAB and incubation (8 minutes); Addition of a drop of copper solution and incubation (4 minutes).
7	Nuclear contrast for nuclear architecture observation: Addition of a drop of hematoxylin and incubation (8 minutes); Incubation with a drop of post-contrast, bluing reagent (4 minutes).

A negative control and a positive control were included in each staining cycle. The negative control involved applying only the secondary antibody, omitting the primary antibody. Different types of tissues samples were utilized as positive controls (Figure 35).

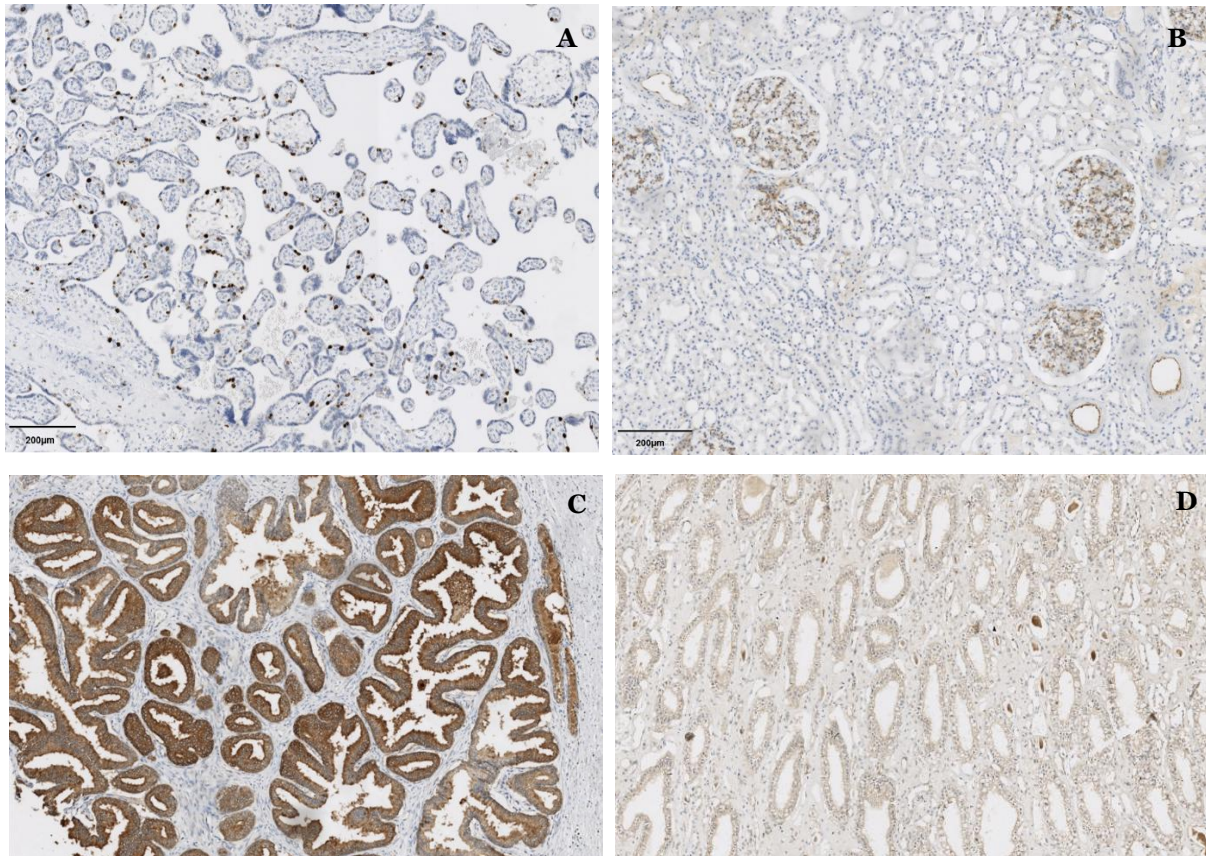


Figure 35 Positive controls for immunohistochemistry

For the Ki-67 (A), FVIII (B), caspase 3 (C), and p-Akt (D) antibodies, confirmation of labelling was performed on sections of the placenta, kidney, prostate, and kidney respectively. The images were captured at a 200× magnification, and the scale bar is 200µm.

2.4.3.3.3 Ovarian tissue photograph register

The slides were photographed to capture images of the stained tissue sections.

Glass slides were acquired using the VENTANA® DP 200 slide scanner (Roche Diagnostics, Basel, Switzerland; Figure 36), at the magnification of 20x, and photographed using the Ventana Image viewer software (Roche Diagnostics, Basel, Switzerland). The histological analysis and stained quantification were conducted, employing the QuPath, Open Software for Bioimage Analysis (QuPath-0.5.0) (Bankhead et al. 2017).

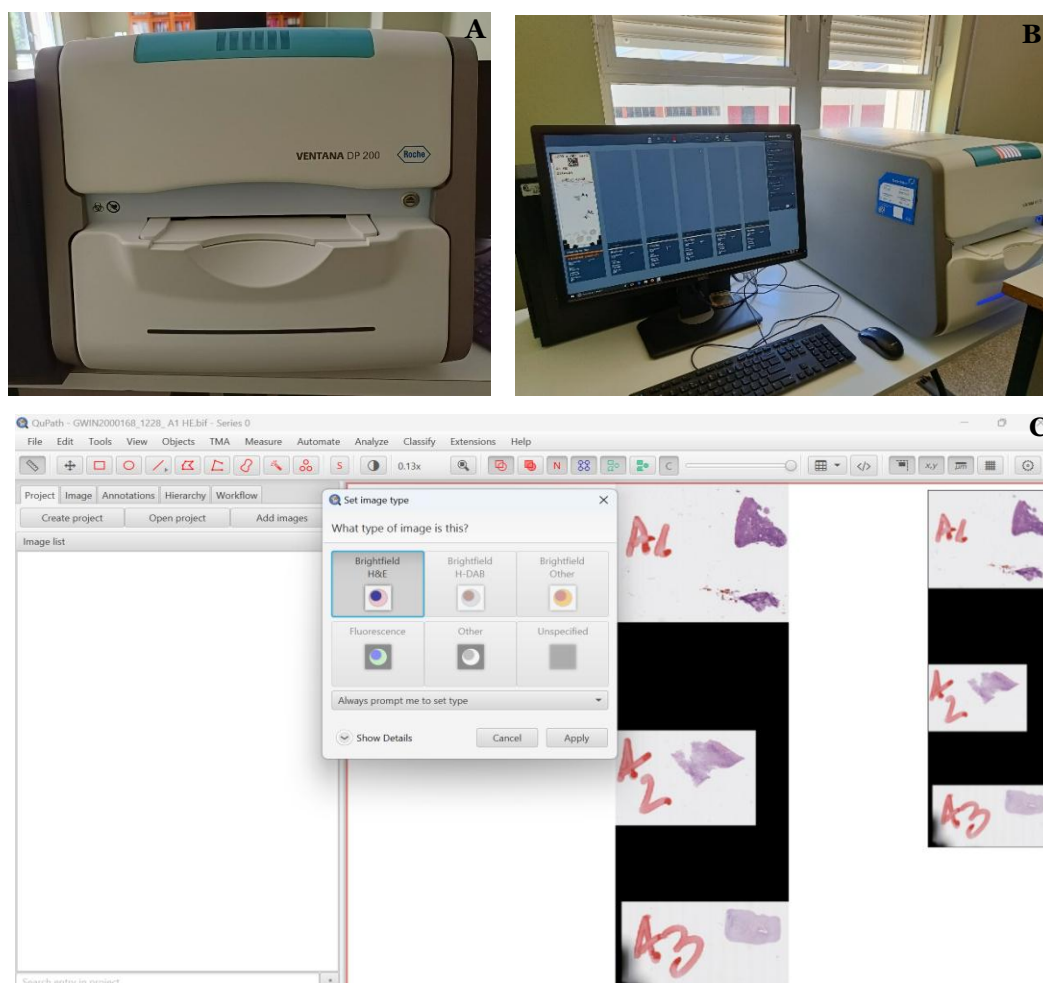


Figure 36 Schematic representation of digitalization process for glasses of H&E and IHC

(A) Digitizer for glasses of H&E and IHC; (B) Coupled program for digitalization; (C) QuPath Software used to analyse the digitalized images.

H&E: haematoxylin and eosin; IHC: Immunohistochemistry

2.4.3.3.4 Histological immunohistochemistry (IHC) analysis

In this study, histological and IHC analyses were performed in a blinded manner to minimize bias and ensure objectivity. Three evaluations were conducted at intervals of two months, spanning from December of 2023 to April of 2024. This approach enabled multiple independent evaluations over time, thereby ensuring consistency and reliability of the results. Additionally, the decision to space out the evaluations served to mitigate the risk of potential systematic errors or biases that could arise during a single evaluation session.

The mean of the three observations was used for further analysis.

The images were decoded only at the end of the analysis by the principal researcher.

2.4.3.3.4.1 Follicular density

The follicular density was calculated counting the number of follicles and expressing it as a density measurement per unit area, specifically in terms of the number of follicles per millimetre squared (number/mm²).

In this study, only follicles displaying normal morphology were quantified. Only follicles with a visible nucleus were considered.

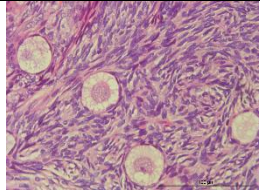
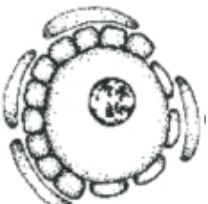
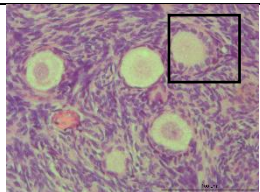
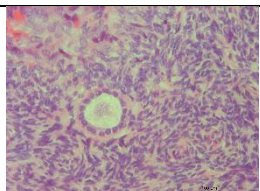
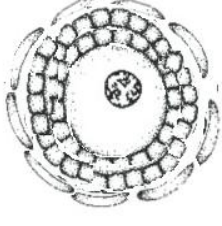
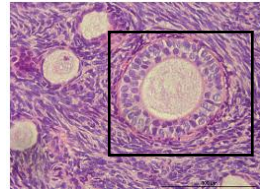
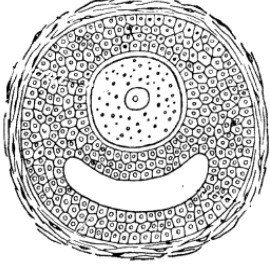
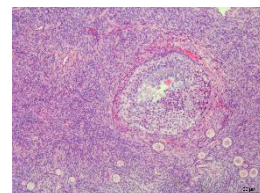
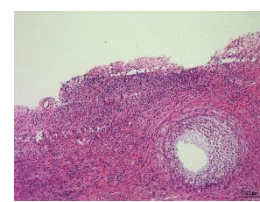
Follicular classification was carried out following the criteria outlined by Gougeon (Gougeon 1996), as summarized in Table 79.

The calculation of follicular density based on the developmental category was obtained counting the number of follicles of each category and expressing it as a density measurement per unit area, specifically in terms of the number of follicles per millimetre squared (number/mm²).

Follicular density and follicular classification were analysed with a 100x magnification.

Table 79 Follicular classification according to its developmental stage

This classification was originally established by Carvalho A. to evaluate human ovarian tissue before and after cryopreservation (Carvalho 2016), and has now been adapted for the present analysis. Scale bar 100µm.

FOLLICULAR CLASSIFICATION		
Primordial follicle: oocyte with a tiny single layer of flattened granulosa cells.		 (400×)
Intermediary follicle: oocyte with a single layer of flattened and cuboidal granulosa cells.		 (400×)
Primary follicle: oocyte with a layer of cuboidal granulosa cells.		 (400×)
Secondary follicle: oocyte with two layers of granulosa cells.		 (400×)
Pre-antral follicle: oocyte with more than two layers of granulosa cells, with vesicles but without an antrum.		 (100×)
Antral follicle: oocyte with more than two layers of granulosa cells and with an antral cavity.		 (100×)

2.4.3.3.4.2 Evaluation of follicles and ovarian tissue integrity

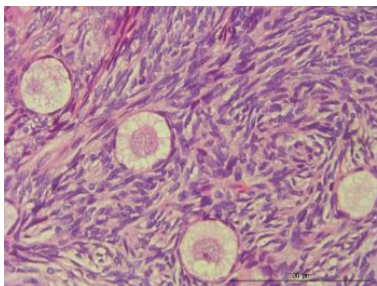
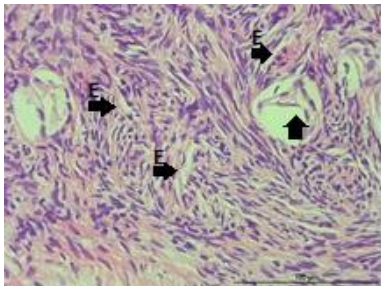
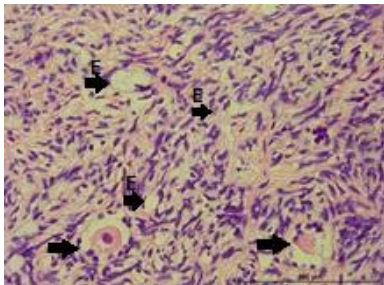
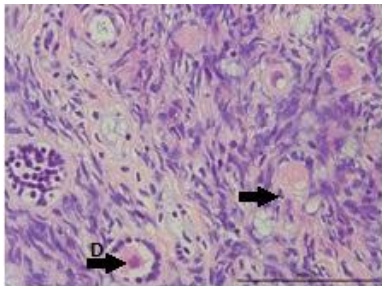
The follicular atresia evaluation involved examining various morphological aspects, such as irregular shape, the condensation of granulosa cells (pyknosis), cytoplasmic contraction, the presence of vacuoles (small cavities or spaces within cells), and eosinophilia of the ooplasm (increased staining with eosin dye, indicating certain cellular changes). The density of atretic follicles was calculated counting the number of atretic follicles and expressing it as a density measurement per unit area, specifically in terms of the number of follicles per millimetre squared (number/mm²).

The histopathological examination of tissue integrity assessed tissue necrosis, follicular degeneration, and interstitial edema, following established protocols outlined in section 2.4.2.6. The stromal classification, ranging from 0 (no alteration) to 3 (severe alterations), is summarized and illustrated in Table 80. All parameters were quantified for the entire section of ovarian tissue stained on the slide to enhance the representativeness of the analysis.

Follicles and stroma were analysed with a 400x magnification.

Table 8o Stromal classification

The magnification is 400x, and the scale bar is 100 µm. Grades range from 0 to 3, with 0 indicating absence, 1 representing mild, 2 indicating moderate, and 3 denoting severe alterations. This classification was originally applied by Carvalho A. to evaluate human ovarian tissue before and after cryopreservation (Carvalho 2016). It has been adapted regarding the study from Fabbri et al., 2003, for the present analysis.

GRADE	DESCRIPTION	ILLUSTRATION (400x, scale bar 100µm)
0	Without signals of interstitial edema or necrosis. Follicles with granulosa cells in a normal shape and oocytes without signals of a degenerative process.	
1	Stroma slightly altered and presence of edema in a limited area (E), characterized by increasing eosinophilic cytoplasm, and loss of microvilli in necrotic cells. These changes are indicative of mild alterations, covering less than 1/3 of the tissue. Follicles with granulosa cells separated from basal membrane (arrow).	
2	Stroma characterized by Interstitial edema (E). Eosinophilic cytoplasm, vacuoles, and loss of microvilli representing moderate alterations or between 1/3 and 2/3. Layer of granulosa cells totally disorganized and alteration upon the oocyte nucleus (arrow).	
3	Fibrotic aspect (deposition of collagen, hypereosinophilic cytoplasm, and total loss of microvilli). Severe alterations or more than 2/3. Follicles with an altered morphology, apparently degenerated (D).	

2.4.3.3.4.3 Immunohistochemistry (IHC) evaluation

IHC allowed a quantitative assessment of the positivity of the antibodies under investigation.

Regarding the stroma, the quantification of the area marked by each antibody was performed using the employing the QuPath, Open Software for Bioimage Analysis (QuPath-0.5.0). Five randomly selected zones from each sample were chosen using a rectangular grid application. The results were presented as the average of all sections.

Follicles and stroma were analysed with a 200x magnification.

2.4.3.3.4.3.1 Proliferation: Ki-67

Follicles were categorized as positive for Ki-67 if staining was observed in the oocyte and/or at least one granulosa cell.

Formula for calculating Ki-67 positive follicles:

$$\text{Ki-67 positive follicles (\%)} = \frac{\text{Number of Ki-67 positive follicles}}{\text{total number of follicles}} \times 100$$

2.4.3.3.4.3.2 Apoptosis: caspase 3

Follicles were considered positive for Caspase 3, signifying apoptosis, when the oocyte was marked with caspase 3 or when more than 50% of granulosa cells were also marked.

Formula for calculating caspase 3 positive follicles:

$$\text{Caspase 3 positive follicles (\%)} = \frac{\text{Number of caspase 3 positive follicles}}{\text{total number of follicles}} \times 100$$

2.4.3.3.4.3.3 Vascular density: Factor VIII (FVIII)

For the quantification of endothelial cell staining with FVIII, five regions of each fragment were randomly selected to calculate vascular density and area. Vascular density was calculated as the number of blood vessels per unit area (in mm²). Clusters of endothelial cells marked with FVIII, with or without a clear lumen, were considered individual vessels. Vascular area corresponds to the percentage of the area marked with FVIII quantified using the employing the QuPath, Open Software for Bioimage Analysis (QuPath-0.5.0).

2.4.3.3.4.3.4 Follicular activation: p-Akt

The p-Akt analysis included a qualitative assessment, evaluating staining intensity and distribution of p-Akt positive staining in oocytes and granulosa cells. Staining included three categories: no staining, weak staining at the granulosa cell layer and/or oocyte cytoplasm, and intense staining of the granulosa cell layer with visible staining of the oocyte nucleus and cytoplasm.

2.4.3.3.5 Statistical analysis

To compare the anticoagulants and resuspension media for optimizing the PRP preparation protocol, variables were analysed using paired Wilcoxon test, since samples for comparison of the anticoagulants (SC versus EDTA) and resuspension solutions (plasma versus NaCl) were obtained from the same patient.

Differences in follicular density, stromal integrity, and expression of Ki-67, caspase 3, FVIII, and p-Akt across different culture times (toh, t4h, and t48h) with PRP supplementation using two-way ANOVA followed by one-way ANOVA, and post-hoc Tukey tests.

Statistical analysis was performed with the support of IBM SPSS Statistics for Windows, (Version 28.0. IBM Corp, Armonk, NY, USA), with the level of significance fixed at 5%.

The graphs accompanying the results were created in GraphPad Prism 9 (San Diego, California, USA).

2.4.4 Results

2.4.4.1 Platelet-rich plasma (PRP) protocol

The following results are derived from published data available in an Open Access Original Article with the DOI: <https://doi.org/10.3390/bioengineering11030209>.

To compare different anticoagulants, blood samples were collected from 26 patients. One patient was excluded due to a previously unknown medical condition affecting platelets. Five patients were excluded due to insufficient blood volume from one of the anticoagulants. Therefore, anticoagulants were compared by analysing blood samples from 21 patients. To compare different resuspension media, blood samples were collected from 19 patients.

Values were expressed as median and interquartile range (Q1–Q3).

2.4.4.1.1.1 Platelet concentration

The mean platelet concentration in whole blood was 200 190 platelets/mm³ (range, 117 000 to 347 000) and 200 190 cells/mm³ (range, 86 000 to 406 000) when blood was collected in tubes containing SC and EDTA, respectively. The mean platelet concentration in PRP was 1,858 410 cells/mm³ (range, 102 000 to 4,260 000) and 3,458 222 cells/mm³ (range, 1,476 000 to 5,322 000) when blood was collected in tubes containing SC and EDTA, respectively. PRP led to a 9 to 17-fold concentration of platelets.

PRP obtained from blood samples collected from the same patient with different anticoagulants had significantly different PRR ($p=.005$), being higher when EDTA was used to collect the blood. The median recovery rate was 51.04% with EDTA versus 29.85% with SC (Table 81, Figure 37).

Table 81 Comparison of PRP obtained after blood sample collection with different anticoagulants, SC versus EDTA

	SC (n=21)	EDTA (n=21)	p-value
PRR (%)	29.85 (21.82-57.10)	51.04 (45.04-60.24)	.005
CD62p (%) platelets	16.20 (9.25-36.95)	39.60 (32.10-51.88)	.035
PAC-1 (%) platelets	55.50 (31.41-68.45)	79.20 (54.85-88.20)	.014
VEGF (pg/mL)	628.73 (291.52-1100.43)	265.44 (159.84-673.43)	.013

Values are expressed as median (Q1-Q3). VEGF: vascular endothelial growth factor; PRP: platelet-rich plasma; PRR: platelet recovery rate; SC: sodium citrate; EDTA: ethylenediaminetetraacetic acid

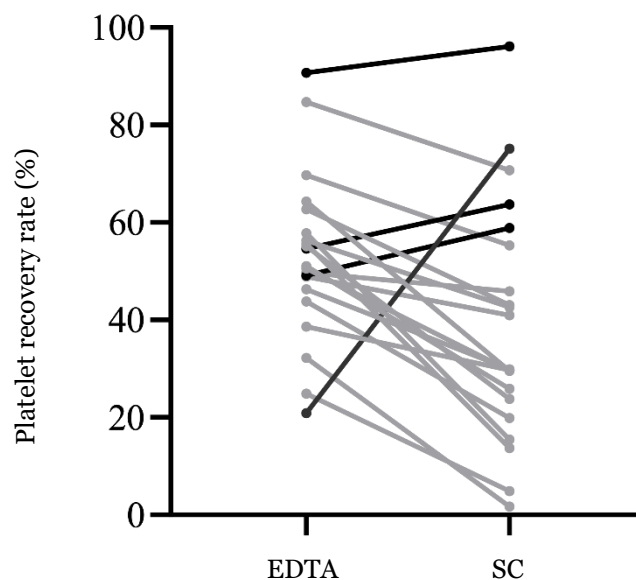


Figure 37 PRR using different anticoagulants

Each line represents a patient, connecting the values of PRR obtained with EDTA versus SC. Positive ranks (PRP with citrate higher than with EDTA) are depicted in black and negative ranks (PRP with citrate lower than with EDTA) are depicted in grey.

PRP: platelet-rich plasma; PRR: platelet recovery rate; SC: sodium citrate; EDTA: ethylenediaminetetraacetic acid

PRR were higher ($p=.011$) in PRP resuspended in plasma (61.60%) when compared to NaCl (48.61%) (Table 82, Figure 38).

Table 82 Comparison of PRP resuspension solution with plasma versus NaCl

	Plasma (n=19)	NaCl (n=19)	p-value
PRR (%)	61.60 (51.29-78.73)	48.61 (42.12-59.43)	.011
CD62p (%) platelets	24.60 (14.95-42.12)	36.30 (20.80-45.23)	.314
PAC-1 (%) platelets	65.85 (40.06-85.67)	76.22 (49.71-92.70)	.159
VEGF (pg/mL)	363.32 (129.56-926.59)	159.83 (116.18-435.82)	.005

Values are expressed as median (Q1-Q3). VEGF: vascular endothelial growth factor; PRP: platelet-rich plasma; NaCl: sodium chloride; PRR: platelet recovery rate

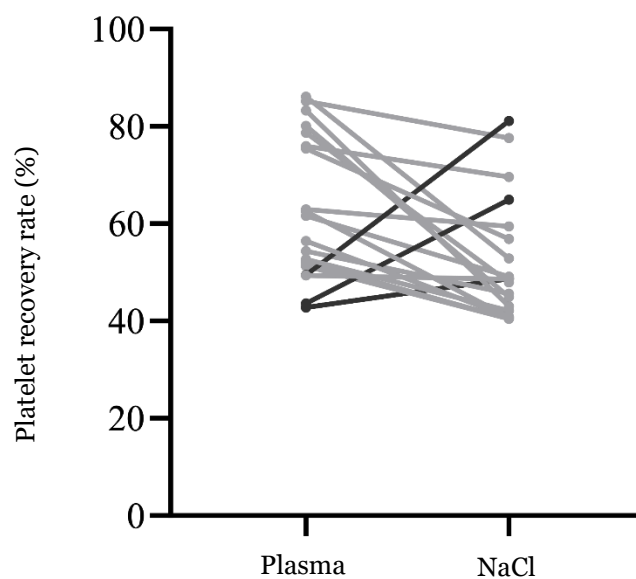


Figure 38 PRR using different resuspension solutions

Each line represents a patient, connecting the values of PRR obtained with plasma versus NaCl. Positive ranks (PRP with NaCl higher than with Plasma) are depicted in black and negative ranks (PRP with NaCl lower than with Plasma) are depicted in grey.

PRP: platelet-rich plasma; PRR: platelet recovery rate; NaCl: sodium chloride

2.4.4.1.1.2 Platelet activity assay of CD62p and PAC-1

The expression of platelet activity markers was significantly higher in PRP obtained when EDTA was used to collect the blood. Expression of CD62p and PAC-1 were 39.60 versus 16.20 ($p=.035$) and 79.20 and 55.50 ($p=.014$) in the EDTA versus SC groups, respectively (Table 81).

There was no difference in the expression of CD62p and PAC-1 when different resuspended solutions were compared (Table 82).

2.4.4.1.1.3 Determination of VEGF concentration

The concentration of VEGF released into plasma after PRP activation was significantly higher in samples collected with SC, with a median of 628.73 pg/mL, when compared to EDTA, with a median of 265.44 pg/mL ($p=.013$) (Table 81).

Resuspension of PRP in plasma had higher levels of VEGF, with a median of 363.32 pg/mL, when compared to NaCl, with a median of 159.83 pg/mL ($p=.005$; Table 82).

2.4.4.1.2 Ovarian tissue culture: results from histological analysis

For the analyses that follow, an initial statistical test was conducted using a two-way ANOVA to elucidate the effect of 1) culture time (4 hours and 48 hours in relation to 0 hours, immediately after thawing, and in relation to each other, 4 hours compared to 48 hours; 2) the effect of supplementation with PRP (comparing conditions with and without PRP); and 3) the effect of the interaction between time and supplementation with PRP. After confirming the effects of these factors on the dependent variables, a one-way ANOVA analysis was performed, which detailed where the differences identified by the previous analysis actually exist, with a Post-hoc test (Tukey test).

The observed power of the two-way ANOVA was estimated (Table 83), and the value was superior to .80 (80%), for time, and supplementation with PRP. The same was not always true for the interaction of these two parameters.

Table 83 Observed power for various parameters under the effects of time, PRP supplementation, and their interaction (time*PRP), regarding two-way ANOVA analysis

	Tissue necrosis	Follicular degenerati	Interstitial edema	Total score	Percentage of Ki-67 positive follicles	Percentage of Ki-67 positive stromal cells	Percentage of caspase 3 positive follicles	Percentage of caspase 3 positive stromal	Vascular density	Vascular area	p-Akt staining
Time	1	1	1	1	.974	.98	.972	.952	.959	.929	.997
PRP	.989	.932	.998	1	.954	1	.852	.902	.911	.924	.998
Time*PRP	.636	.508	.998	.958	.513	.582	.534	.534	.602	.540	.645

PRP: platelet-rich plasma; ANOVA: analysis of variance

For tissue necrosis, follicular degeneration, Ki-67 staining, vascular area, and p-Akt, the interaction of time and PRP supplementation did not reveal statistical significance in the two-way ANOVA test. However, subsequent one-way ANOVA and Tukey tests showed significant differences when comparing time and PRP supplementation conditions – as it will be shown further. This inconsistency could be justified by the low levels of observed power associated with the interaction of time with PRP supplementation (Table 83).

Therefore, for 1) tissue necrosis, 2) follicular degeneration, 3) Ki-67 staining, 4) vascular area, and 5) p-Akt staining, where the initial statistical analysis did not reveal significant differences resulting from the interaction of time with PRP supplementation and the observed power was accordingly lower than .80, the p-value from the post-hoc analysis is indicated with a hash mark (#). This suggests a potential relationship between time and PRP supplementation. These results would only be confirmed if the study was repeated in another cohort with similar results. Increasing the sample size might also be necessary to validate these findings. Given the absence of alternative statistical tests and the importance of this analysis for this specific task objective, we have chosen to maintain these results, referring to them with an adjusted uncertainty level, and marking them distinctly.

2.4.4.1.2.1 Follicular density

The follicular density, which represents the number of follicles per mm², exhibited an average value of 4.06 with a standard deviation of 2.03 (Table 84). In terms of primordial follicles (Table 84), the mean count was 3.13 follicles/mm², with a SD of 1.82. Primordial follicles were the predominant contributors to follicular density.

Moving on to intermediate follicles (Table 84), on average, there were .81 follicles/mm², with a SD of .56. Primary follicles exhibited a mean count of .13 follicles/mm², with a SD of .13. Similarly, secondary follicles also presented a mean count of .13 follicles/mm², with a SD of .13, indicating a similar density to primary follicles.

Pre-antral follicles (Table 84) showed a negligible mean count of .003 follicles/mm², with a SD of .01. Antral follicles showed no detectable density in the samples analysed.

Lastly, atretic follicles (Table 84) presented a mean count of .03 follicles/mm², with a SD of .03, indicating some presence but at a relatively low density compared to the other follicular stages.

Table 84 Follicular density, both total and categorized by developmental stage

Number of follicles per mm² in total and categorized into different developmental stages, including primordial, intermediate, primary, secondary, pre-antral, antral, and atretic follicles. The results are presented as mean and SD.

	Mean	SD
Follicular density (follicles/mm ²)	4.06	2.03
Primordial follicles (number /mm ²)	3.13	1.82
Intermediate follicles (number /mm ²)	.81	.56
Primary follicles (number /mm ²)	.13	.13
Secondary follicles (number /mm ²)	.13	.13
Pre-antral follicles (number /mm ²)	0	.015
Antral follicles (number /mm ²)	0	0
Atretic follicles (number /mm ²)	.03	.03

mm²: square millimeter; SD: standard deviation

Figure 39 represents a histological section of ovarian tissue post-thawing (t=oh), evidencing the follicular densities across cortical layers.

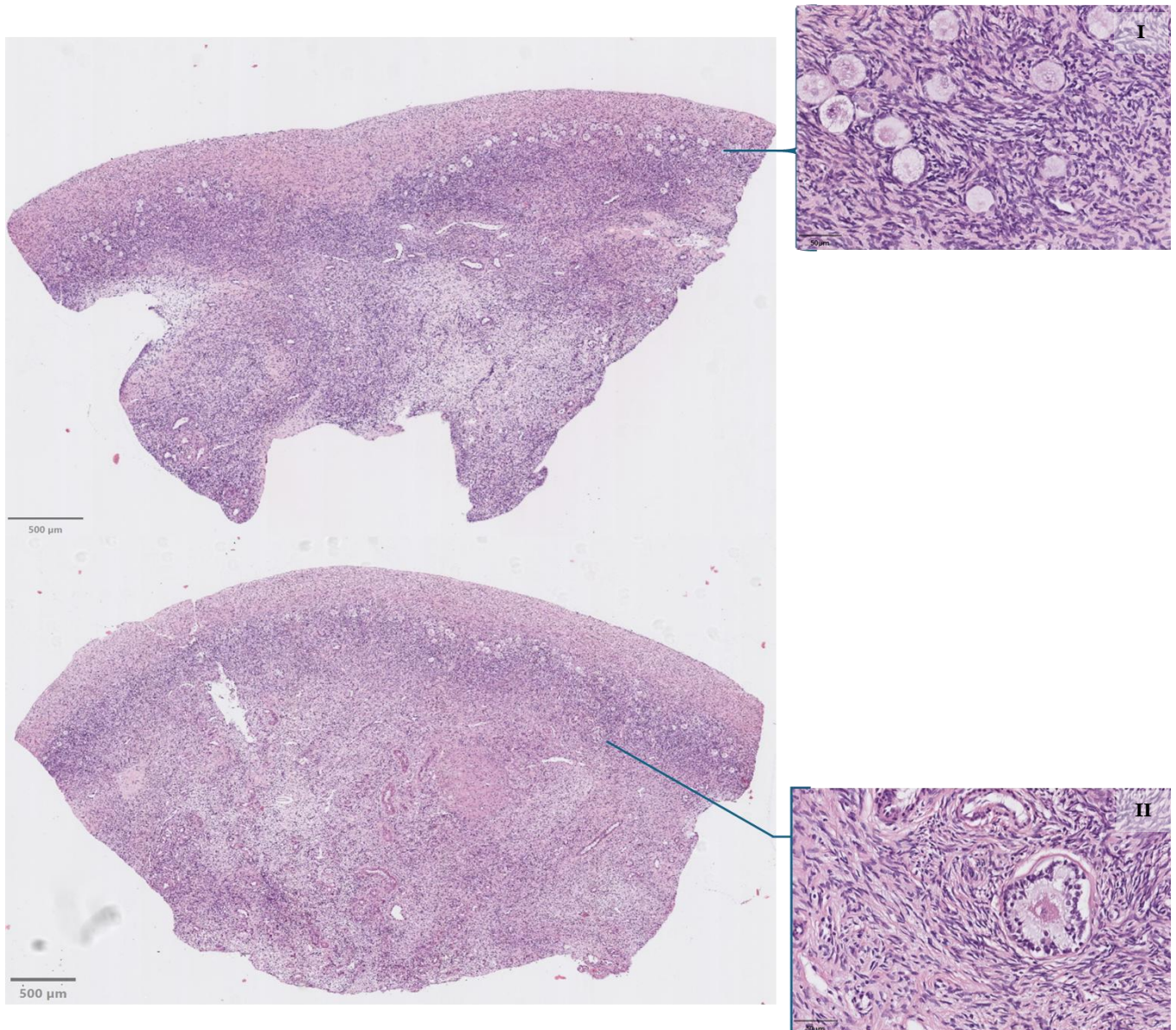


Figure 39 Representative image of ovarian tissue (H&E staining)

Histological section of ovarian tissue stained with H&E obtained immediately after thawing (toh), in a magnification of 10x with a bar scale of 500 μm.

The upper cortical region of the tissue exhibits a higher density of primordial follicles (I) compared to the inner layer of the cortex. As the observation moves towards the inner layer of the cortex, there is a transition to more developed follicles (II).

I and II were obtained with a magnification of 100x, with a scale bar of 50 μm. H&E: Haematoxylin and Eosin.

The follicular density at different time-points (t=0h, t=4h, and t=48h, Table 85), represented as mean±SD, was calculated at 4.03±1.01 follicles/mm² at t=0h. After 4 hours (t=4h), the mean follicular density was 3.96±.57 follicles/mm², and by 48 hours (t=48h), the mean follicular density was 4.17±.58 follicles/mm². No differences were found in follicular densities across the time (p=.0973).

When analysing the densities of specific follicular stages over time (Table 85), there was no significant difference in mean density from t=0h to t=48h across all stages (p=.859 for primordial follicles, p=.974 for intermediate follicles, p=.620 for primary follicles, p=.620 for secondary follicles, and p=.543 for pre-antral follicles).

No differences were found in the density of atretic follicles over time (p=.077, Table 85).

Table 85 Effect of time on follicular parameters in ovarian tissue, presenting mean values over time

Follicular density, expressed as follicles per mm², is measured alongside the distribution of different follicle types (primordial, intermediate, primary, secondary, pre-antral, antral, and atretic follicles) per mm². toh: immediately after thawing; 4 hours and 48 hours of culture (t4h and t48h).

The results are presented as mean and SD. Two-way ANOVA was conducted to determine statistical significance, with p-values provided for each comparison.

Effect of time	Mean toh	SD (toh)	Mean t4h	SD (t4h)	Mean t48h	SD (t48h)	Statistical analysis
Follicular density (follicles/mm ²)	4.03	2.48	3.96	1.99	4.17	2.01	p=.973
Primordial follicles (number /mm ²)	3.13	2.25	3.32	2.08	2.93	1.41	p=.859
Intermediate follicles (number /mm ²)	.74	.45	.81	.65	.85	.56	p=.974
Primary follicles (number /mm ²)	.14	.08127	.12	.14	.14	.14	p=.620
Secondary follicles (number /mm ²)	.14	.08	.12	.14	.14	.14	p=.620
Pre-antral follicles (number /mm ²)	0	0	.01	.02	0	0	p= .543
Atretic follicles (number /mm ²)	.02	.03	.03	.02	.05	.02	p=.077

ANOVA: analysis of variance; mm²: square millimeter; SD: standard deviation

The addition of PRP to the media (Table 86) did not result in significant changes in follicular density overall (p=.054), nor did it affect the density of primordial (p=.551), intermediate (p=.748), primary (p=.088), secondary (p=.088), or pre-antral (p=.876) follicles.

Table 86 Effect of PRP supplementation on follicular density

This table provides mean values and SD for the groups supplemented with and those not supplemented with PRP (for each time-point as well as the overall total) measured in follicles per mm² for various follicle types (primordial, intermediate, primary, secondary, pre-antral, antral, and atretic follicles). Statistical significance was assessed using two-way ANOVA, with corresponding p-values reported for each comparison.

	time	PRP	Mean	SD	effect of PRP
Follicular density (follicles/mm²)	toh		4.04	2.48	p=.954
	t4h	Without PRP	3.93	2.09	
		PRP	3.98	2.08	
	t48h	Without PRP	4.14	2.12	
		PRP	4.19	2.10	
	Total	Without PRP	4.04	2.10	
		PRP	4.09	2	
	time	PRP	Mean	SD	effect of PRP
Primordial follicles (number /mm²)	toh		3.13	2.25	p=.551
	t4h	Without PRP	3.64	2.70	
		PRP	3.00	1.42	
	t48h	Without PRP	3.09	1.58	
		PRP	2.78	1.35	
	Total	Without PRP	3.29	2.11	
		PRP	2.89	1.33	
	time	PRP	Mean	SD	effect of PRP
Intermediate follicles (number /mm²)	toh		.74	.45	p=.748
	t4h	Without PRP	.92	.65	
		PRP	.69	.68	
	t48h	Without PRP	.66	.39	
		PRP	1.04	.68	
	Total	Without PRP	.77	.49	
		PRP	.86	.67	
	time	PRP	Mean	SD	effect of PRP
Primary follicles (number /mm²)	toh		.14	.08	p=.088
	t4h	Without PRP	.07	.06	
		PRP	.18	.18	
	t48h	Without PRP	.10	.08	
		PRP	.17	.18	
	Total	Without PRP	.10	.08	
		PRP	.18	.17	
	time	PRP	Mean	SD	effect of PRP
Secondary follicles (number /mm²)	toh		.14	.08	p=.088
	t4h	Without PRP	.07	.06	
		PRP	.18	.18	
	t48h	Without PRP	.10	.08	
		PRP	.17	.18	
	Total	Without PRP	.10	.08	
		PRP	.18	.17	
	time	PRP	Mean	SD	effect of PRP
Pre-antral follicles (number /mm²)	toh		0	0	p=.274
	t4h	Without PRP	0	0	
		PRP	.01	.03	
	t48h	Without PRP	0	0	
		PRP	0	0	
	Total	Without PRP	0	0	
		PRP	.01	.02	
	time	PRP	Mean	SD	effect of PRP
Atretic follicles (number /mm²)	toh		.02	.03	p=.876
	t4h	Without PRP	.03	.02	
		PRP	.03	.03	
	t48h	Without PRP	.05	.03	
		PRP	.05	.02	
	Total	Without PRP	.03	.03	
		PRP	.04	.02	

ANOVA: analysis of variance; mm²: square millimeter; PRP: platelet-rich plasma; toh: immediately after thawing; 4 hours and 48 hours of culture (t4h and t48h)

The interaction between time and PRP supplementation in the culture media (Table 87) had no significant effect on follicular density ($p=.999$) or on the densities of primordial ($p=.836$), intermediate ($p=.216$), primary ($p=.678$), secondary ($p=.678$), and pre-antral ($p=.274$) follicles. Similarly, there was no significant change in the density of atretic follicles ($p=.534$, Table 87).

Table 87 Effect of the interaction between time and PRP supplementation on follicular density

The p-values associated with each follicle type indicate the level of significance for the interaction effect, determined through two-way ANOVA.

Time x PRP	Statistical analysis
Follicular density (follicles/mm ²)	$p=.999$
Primordial follicles (number /mm ²)	$p=.836$
Intermediate follicles (number /mm ²)	$p=.216$
Primary follicles (number /mm ²)	$p=.678$
Secondary follicles (number /mm ²)	$p=.678$
Pre-antral follicles (number /mm ²)	$p=.274$
Atretic follicles (number /mm ²)	$p=.534$

ANOVA: analysis of variance; mm²: square millimeter; PRP: platelet-rich plasma

The graph above (Figure 40) indicates the absence of significant differences in follicular densities across the various culture conditions.

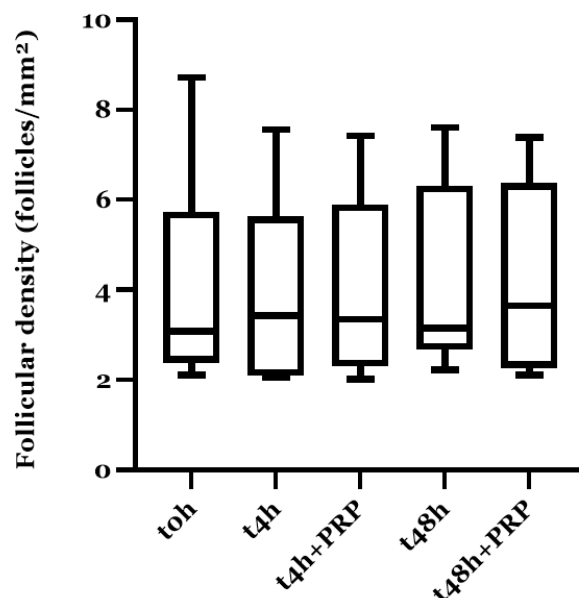


Figure 40 Follicular density over culture conditions

The graph illustrates the changes in follicular density (number of follicles per mm²) at three distinct time-points: immediately after thawing (toh), after 4 hours (t4h), and after 48 hours (t48h).

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

mm²: square millimeter; PRP: platelet-rich plasma

2.4.4.1.2.2 Evaluation of follicles and ovarian tissue integrity

The stromal characteristics of the ovarian tissue were analysed using a scoring system ranging from 0 to 3, with 0 indicating no changes and 3 indicating severe damage. Parameters such as tissue necrosis, follicular degeneration, and interstitial edema were evaluated based on this scale, yielding mean scores, and respective SD, of $.73 \pm .78$, $.77 \pm .82$, and $1.10 \pm .55$, respectively (Table 88).

Table 88 Mean values for tissue necrosis, follicular degeneration, interstitial edema, and total score

Each parameter was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations. The total score represents the cumulative assessment across tissue necrosis, follicular degeneration and interstitial edema.

The results are presented as mean and SD.

	Mean	SD
Tissue necrosis	.73	.78
Follicular degeneration	.77	.82
Interstitial edema	1.10	.55
Total score	2.60	1.89

ANOVA: analysis of variance; SD: standard deviation

When examining the impact of culture duration on tissue integrity (Table 89), substantial statistical differences were evident across all parameters ($p < .001$).

Table 89 Mean values for tissue necrosis, follicular degeneration, interstitial edema, and the total score across different time-points

toh, immediately after thawing; t4h; and t48h of culture. Each parameter was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations. The total score represents the cumulative assessment across tissue necrosis, follicular degeneration and interstitial edema.

The results are presented as mean and SD. The statistical analysis indicates the significance level (p-value) obtained from the two-way ANOVA for each parameter.

Effect of time	Mean toh	SD (toh)	Mean t4h	SD (t4h)	Mean t48h	SD (t48h)	Statistical analysis
Tissue necrosis	0	0	.50	.52	1.33	.78	$p < .001$
Follicular degeneration	.17	.41	.33	.65	1.50	.52	$p < .001$
Interstitial edema	.50	.55	1	0	1.50	.52	$p < .001$
Total score	.67	.52	1.83	.94	4.33	1.56	$p < .001$

ANOVA: analysis of variance; SD: standard deviation

PRP supplementation in the culture media exerted a significant impact on stromal characteristics in ovarian tissue (Table 90). Specifically, tissue necrosis ($p<.001$), follicular degeneration ($p=.001$), and interstitial edema ($p<.001$) all displayed changes in response to PRP supplementation.

Table 90 Mean values for tissue necrosis, follicular degeneration, and interstitial edema, and the total score between groups supplemented with PRP and those that were not supplemented with PRP

Each parameter was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations. The total score represents the cumulative assessment across tissue necrosis, follicular degeneration and interstitial edema.

	time	PRP	Mean	SD	effect of PRP
Tissue necrosis	toh		0	0	$p<.001$
	t4h	Without PRP	1	0	
		PRP	0	0	
	t48h	Without PRP	1.67	.52	
		PRP	1	.89	
	Total	Without PRP	.89	.76	
		PRP	.50	.80	
	time	PRP	Mean	SD	effect of PRP
Follicular degeneration	toh		.17	.41	$p=.001$
	t4h	Without PRP	.50	.84	
		PRP	.17	.41	
	t48h	Without PRP	2	0	
		PRP	1	0	
	Total	Without PRP	.89	.96	
		PRP	.58	.51	
	time	PRP	Mean	SD	effect of PRP
Interstitial edema	toh		.50	.55	$p<.001$
	t4h	Without PRP	1	0	
		PRP	1	0	
	t48h	Without PRP	2	0	
		PRP	1	0	
	Total	Without PRP	1.17	.71	
		PRP	1	0	
	time	PRP	Mean	SD	effect of PRP
Total score	toh		.67	.52	$p<.001$
	t4h	Without PRP	2.50	.84	
		PRP	1.17	.41	
	t48h	Without PRP	5.67	.52	
		PRP	3	.89	
	Total	Without PRP	2.94	2.21	
		PRP	2.08	1.16	

ANOVA: analysis of variance; PRP: platelet-rich plasma; SD: standard deviation; toh: immediately after thawing; 4 hours and 48 hours of culture (t4h and t48h)

In assessing the interaction between culture time and the addition of PRP (Table 91), the statistical analysis reveals varying impacts across different parameters. For interstitial edema and total score the interaction of PRP and culture time had a significant impact ($p < .001$ and $p = .021$, respectively). However, this was not the case for tissue necrosis ($p = .385$) and follicular degeneration ($p = .085$).

Table 91 Effect of the interaction between time and PRP supplementation on tissue necrosis, follicular degeneration, interstitial edema, and the total score

Each parameter was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations. The total score represents the cumulative assessment across tissue necrosis, follicular degeneration and interstitial edema.

The p-values indicate the significance of these interactions. Statistical significance was assessed using two-way ANOVA,

Time x PRP	Statistical analysis
Tissue necrosis	$p = .385$
Follicular degeneration	$p = .085$
Interstitial edema	$p < .001$
Total score	$p = .021$

ANOVA: analysis of variance; PRP: platelet-rich plasma

The representative images grouped in Figure 41 provide visual evidence of the impact of culture time, PRP supplementation, and their interaction on stromal quality, as evaluated through H&E staining.

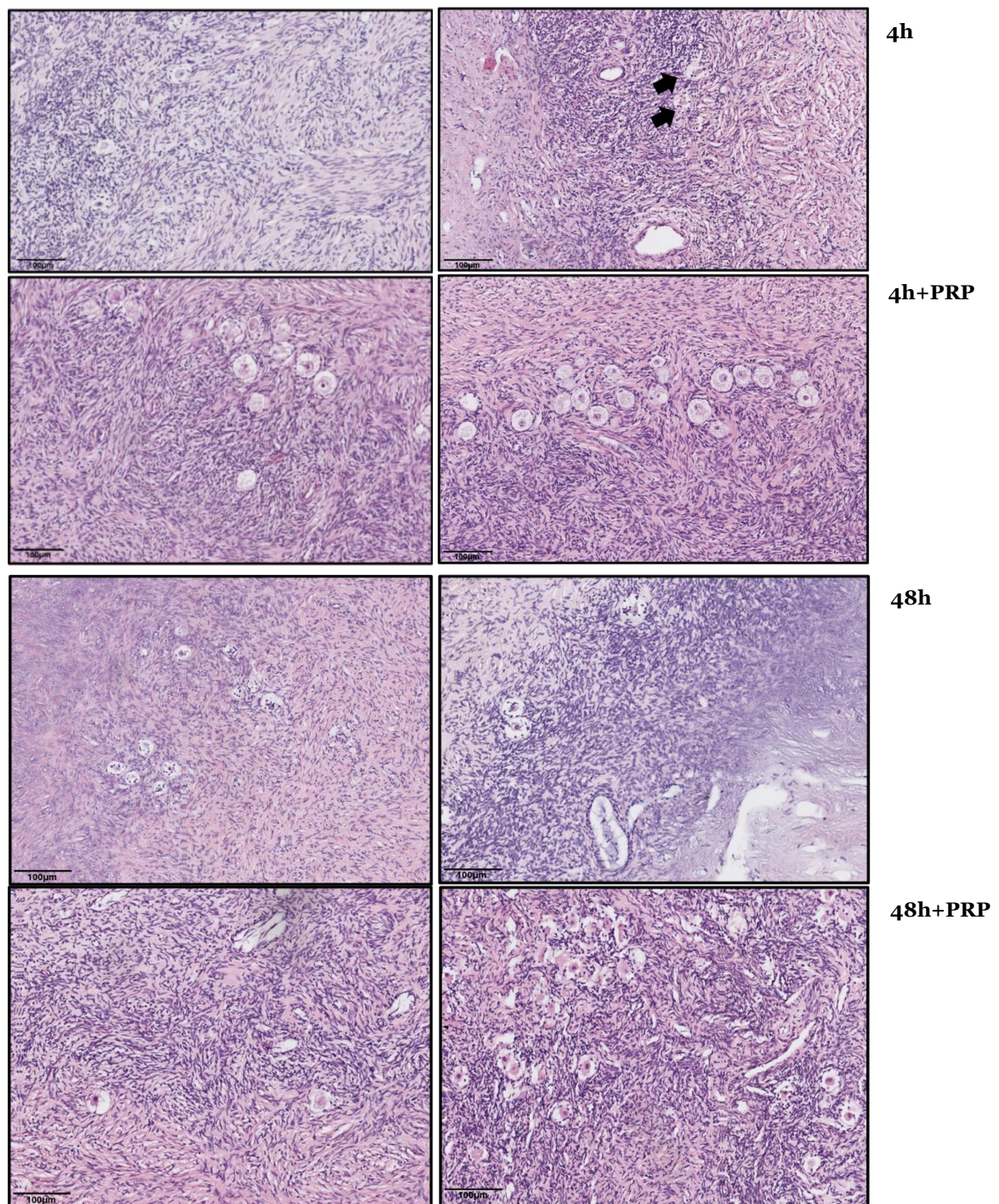


Figure 41 Representative images of stromal appearance over culture time with and without PRP (H&E Staining)

The tissue at 4 hours (t4h) begins to show some interstitial edema, and at the level of the follicles, a detachment of the basement membrane (indicated by arrows in the image). This detachment is visibly reversed with the addition of PRP (t=4h+PRP), resulting in the recovery of the follicles. Interstitial edema and tissue necrosis tend to increase at 48 hours (t48h), but these parameters improve again with the addition of PRP (t48h+PRP), as visually monitored in the representative images.

Images were obtained with a magnification of 100x, with a scale bar of 100 µm.

H&E: haematoxylin and eosin; PRP: platelet-rich plasma

The initial two-way ANOVA analysis evaluated the impact of time, PRP supplementation and interaction of both on tissue necrosis, follicular degeneration, interstitial edema, and total score. Subsequent one-way ANOVA and post-hoc analysis using with multiple comparisons Tukey test, detailed the differences between the culture conditions. The one-way ANOVA results were as follows: for tissue necrosis, the F-value was 19.27 with a significance level of $<.001$; for follicular degeneration, the F-value was 25.14 with a significance level of $<.001$; for interstitial edema, the F-value was 23.79 with a significance level of $<.001$; and for the total score, the F-value was 53.47 with a significance level of $<.001$.

The post-hoc analysis for tissue necrosis (Table 92), revealed that alteration significantly increased from $t=0h$ to $t=48h$ ($p<.001$), with this increase being evident as early as 4 hours of culture ($t=0h$ Vs $t=4h$, $p<.002$), indicating a gradual deterioration over time. No significant difference in tissue necrosis was observed between 4 hours and 48 hours of culture ($p=.71$), suggesting a plateau in tissue damage beyond the initial 4-hour period.

PRP supplementation (Table 92) has shown a tendency to effectively revert tissue necrosis to basal levels, within the first 4 hours of culture, as evidenced by the absence of significant differences between $t=0h$ and $t=4h+PRP$ ($p=1$). However, at 48 hours of culture, PRP supplementation did not yield a significant improvement in tissue necrosis, as indicated by the absence of significant differences between $t=48h$ and $t=48h+PRP$ ($p=.06$). When comparing the two time points with PRP, specifically at $t=4h+PRP$ versus $t=48h+PRP$, tissue necrosis was significantly higher after the longer culture period ($p=.002$).

Table 92 Results of post-hoc Tukey tests conducted to analyse the differences in tissue necrosis between various time-points and PRP supplementation conditions

Tissue necrosis was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations.

The table includes every possible comparison, distributed in columns I and J, as well as the mean difference (I-J) between different time-points (t=0h, t=4h and t=48h) or PRP conditions, along with the significance level (Sig.), indicating whether the observed differences are statistically significant. In addition to the p-value, the mean difference value (positive or negative) indicates the relationship (I and J pattern) between the two-time conditions being compared. If the mean difference of I - J is positive, it means I > J; if it is negative, it means I < J.

	(I)	(J)	Mean difference (I-J)	Relationship between the conditions	Sig.
Tissue necrosis	t=0h	t=4h	-1*	t0h < t4h	.002
		t=4h+PRP	0	t0h = t4h+PRP	1.000
		t=48h	-1.63*	t0h < t48h	<.001
		t=48h+PRP	-.98#	t0h < t48h+PRP	.002
	t=4h	t=0h	1*	t4h > t0h	.002
		t=4h+PRP	1*	t4h > t4h+PRP	.002
		t=48h	-.63	t4h = t48h	.071
		t=48h+PRP	.02	t4h = t48h+PRP	1.000
	t=4h+PRP	t=0h	0	t4h+PRP=t0h	1.000
		t=4h	-1*	t4h+PRP < t4h	.002
		t=48h	-1.63*	t4h+PRP < t48h	<.001
		t=48h+PRP	-.98*	t4h+PRP < t48h+PRP	.002
	t=48h	t=0h	1.63*	t48h > t0h	<.001
		t=4h	.63	t48h = t4h	.071
		t=4h+PRP	1.63#	t48h > t4h+PRP	<.001
		t=48h+PRP	.65	t48h = t48h+PRP	.060
	t=48h+PRP	t=0h	.98#	t48h+PRP > t0h	.002
		t=4h	-.02	t48h+PRP=t4h	1.000
		t=4h+PRP	.98*	t48h+PRP > t4h+PRP	.002
		t=48h	-.65	t48h+PRP=t48h	.060

Statistical significance is denoted by asterisks (*). The mean difference is significant at the .05 level. "I and J Pattern," indicates the relationship between the groups based on the mean difference. The comparisons were analysed by one-way ANOVA, and post-hoc Tukey test.

The hash mark (#) indicates cases where ANOVA and Tukey tests showed significant differences when comparing time and PRP supplementation conditions, despite the initial lack of significance in the two-way ANOVA.

The differences are highlighted in bold.

ANOVA: analysis of variance; PRP: platelet-rich plasma; t=0h (t0h): immediately after thawing; t=4h (t4h): 4 of *in vitro* culture; t=48h (t48h): 48 hours of *in vitro* culture

The following graph (Figure 42) summarizes the results previously described, illustrating the impact of culture conditions on tissue necrosis.

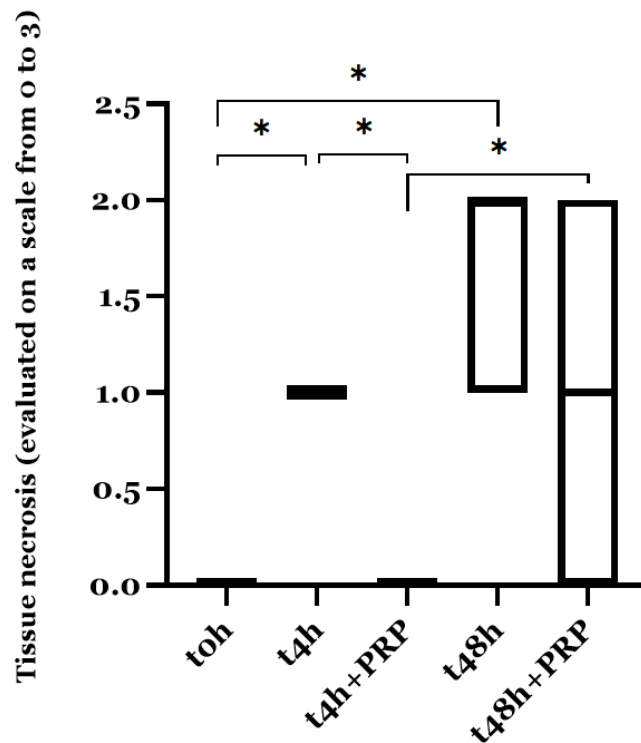


Figure 42 Comparison of the values of tissue necrosis between the between various time-points and PRP supplementation conditions

Tissue necrosis was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations. The conditions include the toh (the baseline condition, immediately after thawing,), and groups treated with or without PRP at different time-points: t4h, t48h, t4h+PRP, and t48h+PRP, respectively.

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

Statistical significance is denoted by asterisks (*) for $p < .005$, next to the legend representing significant differences.

PRP: platelet-rich plasma

Considering the follicular degeneration (Table 93), the post-hoc analysis elucidated the specific temporal and PRP-related differences in this factor. Follicular degeneration exhibited a substantial increase from $t=0h$ to $t=48h$ ($p < .001$), as well as from $t=4h$ to $t=48h$ ($p < .001$). However, no significant differences in follicular degeneration were detected between $t=0h$ and $t=4h$ ($p = .262$), underscoring a consistent level of degeneration during the initial hours of culture.

Regarding PRP supplementation (Table 93), a significant reduction in follicular degeneration was evident at the 48-hour mark ($t=48h$ vs $t=48h+PRP$, $p = .002$). On the contrary, no significant differences in follicular degeneration were observed between PRP-supplemented and non-PRP-supplemented groups at earlier time-points ($t=4h$ vs $t=4h+PRP$, $p = .471$).

PRP supplementation seems to effectively alter the follicular degeneration observed at 48 hours of culture, reducing it to levels similar to those observed at 4 hours ($p=.193$). Nonetheless, the extent of degeneration at 48 hours, when adding PRP, remained higher than at the 4-hour point ($p=.005$ for comparison of $t=48h+PRP$ with $t=4h+PRP$), and appears to be higher than that observed at $t=0h$ ($p=.002$).

Table 93 Results of post-hoc Tukey tests examining differences in follicular degeneration across various time-points and PRP supplementation conditions

Follicular degeneration was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations. The table includes every possible comparison, distributed in columns I and J, as well as the mean difference (I-J) between different time-points ($t=0h$, $t=4h$ and $t=48h$) or PRP conditions, along with the significance level (Sig.), indicating whether the observed differences are statistically significant. In addition to the p-value, the mean difference value (positive or negative) indicates the relationship (I and J pattern) between the two-time conditions being compared. If the mean difference of I - J is positive, it means $I > J$; if it is negative, it means $I < J$.

	(I)	(J)	Mean difference (I-J)	Relationship between the conditions	Sig.
Follicular degeneration	t=0h	t=4h	-.42	toh = t4h	.262
		t=4h+PRP	-.08	toh = t4h+PRP	.994
		t=48h	-1.78*	toh < t48h	<.001
		t=48h+PRP	-.89*	toh < t48h+PRP	.002
	t=4h	t=0h	.42	t4h = toh	.262
		t=4h+PRP	.34	t4h = t4h+PRP	.471
		t=48h	-1.35*	t4h < t48h	<.001
		t=48h+PRP	-.46	t4h = t48h+PRP	.193
	t=4h+PRP	t=0h	.08	t4h+PRP=toh	.994
		t=4h	-.34	t4h+PRP=t4h	.471
		t=48h	-1.69*	t4h+PRP < t48h	<.001
		t=48h+PRP	-.80*	t4h+PRP < t48h+PRP	.005
	t=48h	t=0h	1.78*	t48h > toh	<.001
		t=4h	1.35*	t48h > t4h	<.001
		t=4h+PRP	1.69*	t48h > t4h+PRP	<.001
		t=48h+PRP	.89*	t48h > t48h+PRP	.002
	t=48h+PRP	t=0h	.89*	t48h+PRP > toh	.002
		t=4h	.46	t48h+PRP=t4h	.193
		t=4h+PRP	.80*	t48h+PRP > t4h+PRP	.005
		t=48h	-.89*	t48h+PRP < t48h	.002

Statistical significance is denoted by asterisks (*). The mean difference is significant at the .05 level. "I and J Pattern," indicates the relationship between the groups based on the mean difference. The comparisons were analysed by one-way ANOVA, and post-hoc Tukey test.

The hash mark (#) indicates cases where ANOVA and Tukey tests showed significant differences when comparing time and PRP supplementation conditions, despite the initial lack of significance in the two-way ANOVA.

The differences are highlighted in bold.

ANOVA: analysis of variance; PRP: platelet-rich plasma; t=0h (toh): immediately after thawing; t=4h (t4h): 4 of *in vitro* culture; t=48h (t48h): 48 hours of *in vitro* culture

The following graph (Figure 43) summarize the results previously described, illustrating the impact of culture conditions on follicular degeneration.

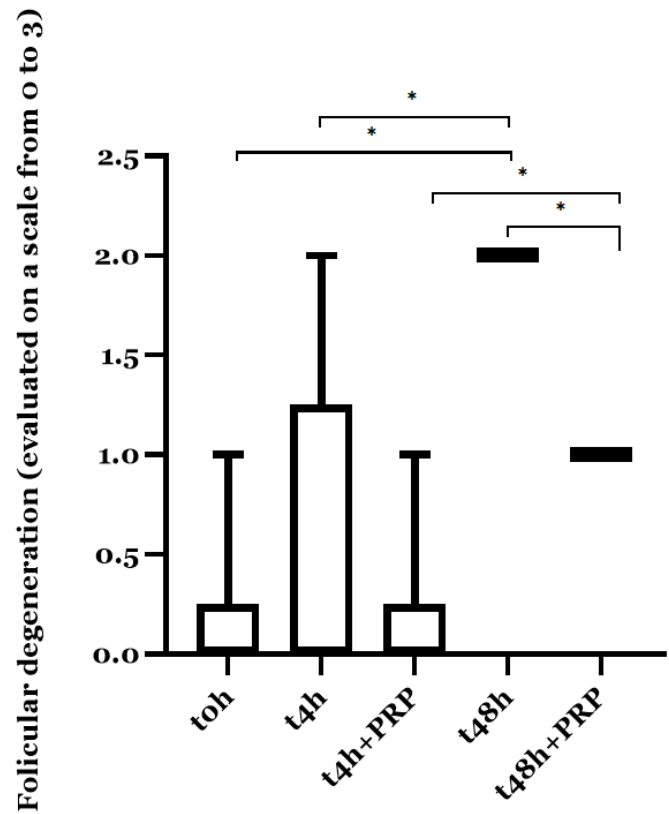


Figure 43 Comparison of the values of follicular degeneration, between various time-points and PRP supplementation conditions

Follicular degeneration was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations. The conditions include the (the baseline condition, immediately after thawing.), and groups treated with or without PRP at different time-points: t4h, t48h, t4h+PRP, and t48h+PRP, respectively.

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

Statistical significance is denoted by asterisks (*) for $p < .005$, next to the legend representing significant differences.

PRP: platelet-rich plasma

Regarding the analysis of interstitial edema significant effects of time, PRP supplementation, and their interaction were initially identified ($p < .001$). When detailing this analysis (Table 94), time emerged as a substantial influencer of interstitial edema, with a significant increase observed from t=0h to t=48h ($p < .001$) as well as from 4h of culture to 48h of culture ($p < .001$). At the 4-hour mark, interstitial edema was equivalent to t=0h ($p = .070$)

Following 48 hours of culture, PRP supplementation evidenced a significant reduction in interstitial edema compared to the group that was not supplemented with PRP (t=48h+PRP Vs t=48h, $p<.001$, Table 94), effectively restoring values analogous to those observed after 4 hours of culture (t=48h+PRP Vs t=4h, $p=.997$). However, despite this positive change, interstitial edema remained significantly higher than the baseline, at t=0h, ($p=.033$). The beneficial impact of PRP was not evident during the early stages of culture (t=4h+PRP Vs t=4h, $p=.693$).

Table 94 Post-hoc Tukey test results examining differences in interstitial edema across various time-points and PRP supplementation conditions

Follicular degeneration was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations. The table includes every possible comparison, distributed in columns I and J, as well as the mean difference (I-J) between different time-points (t=0h, t=4h and t=48h) or PRP conditions, along with the significance level (Sig.), indicating whether the observed differences are statistically significant. In addition to the p-value, the mean difference value (positive or negative) indicates the relationship (I and J pattern) between the two-time conditions being compared. If the mean difference of I - J is positive, it means I > J; if it is negative, it means I < J.

	(I)	(J)	Mean difference (I-J)	Relationship between the conditions	Sig.
Interstitial edema	t=0h	t=4h	-.44	toh = t4h	.070
		t=4h+PRP	-.24	toh = t4h+PRP	.588
		t=48h	-1.45*	toh < t48h	<.001
		t=48h+PRP	-.50*	toh < t48h+PRP	.033
	t=4h	t=0h	.44	t4h = toh	.070
		t=4h+PRP	.21	t4h = t4h+PRP	.693
		t=48h	-1*	t4h < t48h	<.001
		t=48h+PRP	-.056	t4h = t48h+PRP	.997
	t=4h+PRP	t=0h	.24	t4h+PRP=toh	.588
		t=4h	-.21	t4h+PRP=t4h	.693
		t=48h	-1.21*	t4h+PRP < t48h	<.001
		t=48h+PRP	-.26	t4h+PRP=t48h+PRP	.482
	t=48h	t=0h	1.45*	t48h > toh	<.001
		t=4h	1*	t48h > t4h	<.001
		t=4h+PRP	1.21*	t48h > t4h+PRP	<.001
		t=48h+PRP	.95*	t48h > t48h+PRP	<.001
	t=48h+PRP	t=0h	.50*	t48h+PRP > toh	.033
		t=4h	.056	t48h+PRP=t4h	.997
		t=4h+PRP	.26	t48h+PRP=t4h+PRP	.482
		t=48h	-.95*	t48h+PRP < t48h	<.001

Statistical significance is denoted by asterisks (*). The mean difference is significant at the .05 level. "I and J Pattern," indicates the relationship between the groups based on the mean difference. The comparisons were analysed by one-way ANOVA, and post-hoc Tukey test.

The hash mark (#) indicates cases where ANOVA and Tukey tests showed significant differences when comparing time and PRP supplementation conditions, despite the initial lack of significance in the two-way ANOVA.

The differences are highlighted in bold.

ANOVA: analysis of variance; PRP: platelet-rich plasma; t=0h (toh): immediately after thawing; t=4h (t4h): 4 of *in vitro* culture; t=48h (t48h): 48 hours of *in vitro* culture

The following graph (Figure 44) summarizes the results previously described, illustrating the impact of culture conditions on interstitial edema.

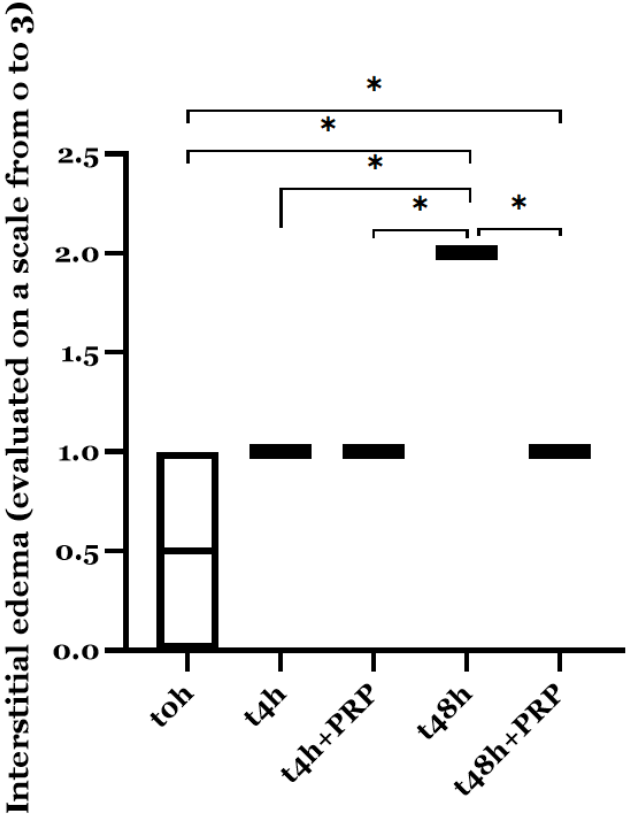


Figure 44 Comparison of the values of interstitial edema between various time-points and PRP supplementation conditions

Interstitial edema was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations. The conditions include the control group (toh), and groups treated with or without PRP at different time-points: toh: immediately after thawing, t4h, t48h, t4h+PRP, and t48h+PRP.

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

Statistical significance is denoted by asterisks (*) for $p < .005$, next to the legend representing significant differences.

PRP: platelet-rich plasma

Regarding the total score, both time and PRP supplementation ($p < .001$), as well as the interaction between these two factors ($p = .021$) influenced the results. Specifically (Table 95), total score increased over time, with a substantial difference observed between $t = 0h$ and $t = 4h$ ($p < .001$), as well as between $t = 0h$ and $t = 48h$ ($p < .001$).

PRP supplementation caused significant alterations in the total score (Table 95). At the 4-hour mark, PRP supplementation led to a significant reduction in the total score compared to the group that was not supplemented with PRP ($t = 4h + PRP$ Vs $t = 4h$, $p = .005$), effectively

restoring the total score to levels similar to those observed at baseline (t=0h Vs t=4h+PRP, p=.942). Furthermore, after 48 hours of culture, the addition of PRP resulted in a marked reduction in the total score (t=48h Vs t=48h+PRP, p<.001), aligning it with levels observed after 4 hours of culture (t=48h+PRP Vs t=4h, p=.603). However, despite this improvement, the total score remained significantly higher than that at t=0h (t=48h+PRP Vs t=0h, p<.001).

Table 95 Results of post-hoc Tukey tests investigating differences in the total score of tissue damage across various time-points and PRP supplementation conditions

The total score represents the cumulative assessment across tissue necrosis, follicular degeneration and interstitial edema (each parameter was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations). The table includes every possible comparison, distributed in columns I and J, as well as the mean difference (I-J) between different time-points (t=0h, t=4h and t=48h) or PRP conditions, along with the significance level (Sig.), indicating whether the observed differences are statistically significant. In addition to the p-value, the mean difference value (positive or negative) indicates the relationship (I and J pattern) between the two-time conditions being compared. If the mean difference of I - J is positive, it means I > J; if it is negative, it means I < J.

	(I)	(J)	Mean difference (I-J)	Relationship between the conditions	Sig.
Total score	t=0h	t=4h	-1.72*	toh < t4h	<.001
		t=4h+PRP	-.28	toh = t4h+PRP	.942
		t=48h	-4.79*	toh < t48h	<.001
		t=48h+PRP	-2.26*	toh < t48h+PRP	<.001
	t=4h	t=0h	1.72*	t4h > toh	<.001
		t=4h+PRP	1.44*	t4h > t4h+PRP	.005
		t=48h	-3.06*	t4h < t48h	<.001
		t=48h+PRP	-.54	t4h = t48h+PRP	.603
	t=4h+PRP	t=0h	.278	t4h+PRP=toh	.942
		t=4h	-1.44*	t4h+PRP < t4h	.005
		t=48h	-4.51*	t4h+PRP < t48h	<.001
		t=48h+PRP	-1.98*	t4h+PRP < t48h+PRP	<.001
	t=48h	t=0h	4.79*	t48h > toh	<.001
		t=4h	3.06*	t48h > t4h	<.001
		t=4h+PRP	4.51*	t48h > t4h+PRP	<.001
		t=48h+PRP	2.53*	t48h > t48h+PRP	<.001
	t=48h+PRP	t=0h	2.26*	t48h+PRP > toh	<.001
		t=4h	.54	t48h+PRP=t4h	.603
		t=4h+PRP	1.98*	t48h+PRP > t4h+PRP	<.001
		t=48h	-2.53*	t48h+PRP < t48h	<.001

Statistical significance is denoted by asterisks (*). The mean difference is significant at the .05 level. "I and J Pattern," indicates the relationship between the groups based on the mean difference. The comparisons were analysed by one-way ANOVA, and post-hoc Tukey test.

The hash mark (#) indicates cases where ANOVA and Tukey tests showed significant differences when comparing time and PRP supplementation conditions, despite the initial lack of significance in the two-way ANOVA.

The differences are highlighted in bold.

ANOVA: analysis of variance; PRP: platelet-rich plasma; t=0h (toh): immediately after thawing; t=4h (t4h): 4 of *in vitro* culture; t=48h (t48h): 48 hours of *in vitro* culture

The following graph (Figure 45) summarizes the results previously described, illustrating the impact of culture conditions on total score.

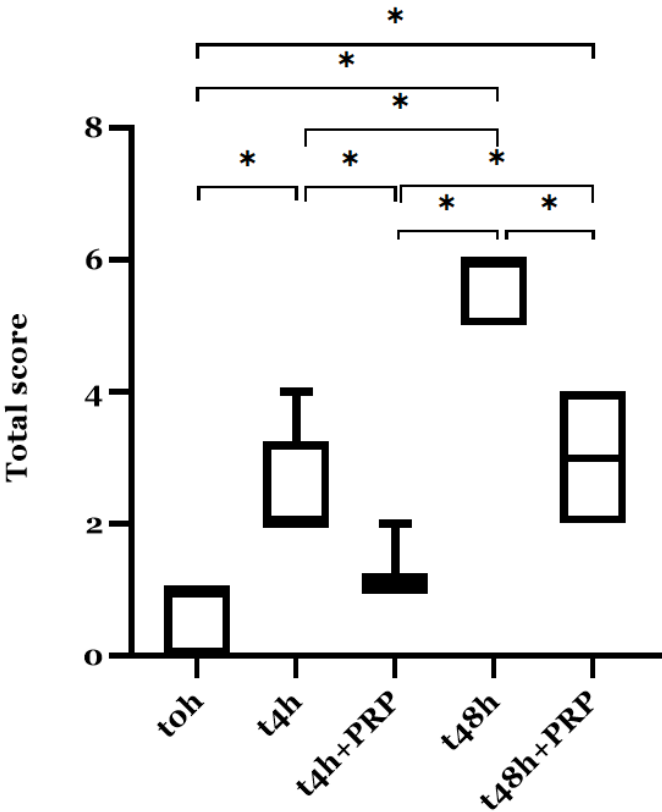


Figure 45 Comparison of the values of total score the between various time-points and PRP supplementation conditions

The total score represents the cumulative assessment across tissue necrosis, follicular degeneration and interstitial edema (each parameter was scored on a scale of 0 to 3, where 0 corresponds to the absence of alterations, and 3 represents severe alterations). The conditions include the toh (the baseline condition, immediately after thawing,) and groups treated with or without PRP at different time-points: t4h, t48h, t4h+PRP, and t48h+PRP, respectively.

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

Statistical significance is denoted by asterisks (*) for $p < .005$, next to the legend representing significant differences.

PRP: platelet-rich plasma

2.4.4.1.3 Ovarian tissue culture: results from immunohistochemistry (IHC) analysis

2.4.4.1.3.1 Proliferation: Ki-67

The mean percentage of Ki-67 positive follicles was 62.81%, with a SD of 10.20 (Table 96). In contrast, the mean percentage of positive stromal cells was .20%, accompanied by a SD of .09 (Table 96).

Table 96 percentage of Ki-67 positive follicles and Ki-67 positive stromal cells

The results are presented as mean and SD.

	Mean	SD
Percentage of Ki-67 positive follicles	62.81	10.20
Percentage of Ki-67 positive stromal cells	.20	.09

SD: standard deviation

The observed staining for Ki-67 in ovarian tissue follows the pattern represented in Figure 46. Primordial follicles have shown minimal staining, primarily nuclear, whereas larger follicles have positively stained granulosa cells.

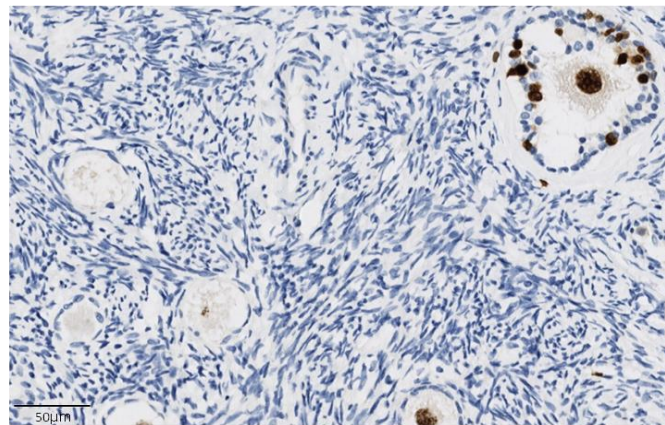


Figure 46 Representative image of Ki-67 immunostaining

Obtained with a magnification of 100x, with a scale bar of 50µm.

In examining the impact of culture time (Table 97), differences were observed in the percentage of positive follicles for Ki-67 ($p < .001$). Nevertheless, no significant variation was detected in the percentage of positive stromal cells across different culture durations ($p = .716$), indicating no substantial influence of culture time on this parameter (Table 97).

Table 97 Mean percentage of Ki-67 positive follicles and Ki-67 positive stromal cells at different time-points

toh: immediately after thawing; 4 hours and 48 hours of culture (t4h and t48h).

The results are presented as mean and SD. Statistical analysis using two-way ANOVA indicates the significance level of the differences observed.

Effect of time	Mean toh	SD (toh)	Mean t4h	SD (t4h)	Mean t48h	SD (t48h)	Statistical analysis
Percentage of Ki-67 positive follicles	51.22	5.77	61.12	8.05	70.28	7.55	p<.001
Percentage of Ki-67 positive stromal cells	.15	.031	.22	.11	.20	.09	p=.716

ANOVA: analysis of variance. SD: standard deviation

When considering the effect of PRP supplementation on the percentage of positive follicles (Table 98) no differences were found, when comparing with the groups that were not supplemented with PRP (p=.112). In contrast, the percentage of positive stromal cells (Table 98) exhibited a significant difference (p<.001) with PRP supplementation.

Table 98 Mean percentage of Ki-67 positive follicles and Ki-67 positive stromal cells in the groups supplemented with PRP and in those that were not supplemented with PRP

This table provides mean values and SD for the groups supplemented with PRP and those that were not supplemented with PRP (for each time-point as well as the overall total). Statistical significance was assessed using two-way ANOVA, with corresponding p-values reported for each comparison

	time	PRP	Mean	SD	effect of PRP
%of Ki-67 positive follicles	toh		51.22	5.77	p=.112
	t4h	Without PRP	57.53	7.05	
		PRP	64.71	7.88	
	t48h	Without PRP	68.97	7.60	
		PRP	71.60	7.97	
	Total	Without PRP	59.24	9.92	
		PRP	68.16	8.37	
	time	PRP	Mean	Std. Deviation	effect of PRP
%of Ki-67 positive stromal cells	toh		.15	.03	p<.001
	t4h	Without PRP	.14	.03	
		PRP	.30	.10	
	t48h	Without PRP	.14	.03	
		PRP	.27	.08	
	Total	Without PRP	.14	.03	
		PRP	.29	.09	

ANOVA: analysis of variance; PRP: platelet-rich plasma; SD: standard deviation; toh: immediately after thawing; 4 hours and 48 hours of culture (t4h and t48h)

Regarding the interaction between culture time and PRP supplementation (Table 99), no significant effects were observed for both the percentage of positive follicles ($p=.453$) and the percentage of positive stromal cells ($p=.590$), indicating that the combined influence of these factors did not substantially impact these parameters.

Table 99 Effect of the interaction between time and PRP supplementation for the percentage of positive follicles and positive stromal cells for Ki-67

The p-values indicate the level of significance for the observed differences. Statistical significance was assessed using two-way ANOVA

Time x PRP	Statistical analysis
Percentage of Ki-67 positive follicles	$p=.453$
Percentage of Ki-67 positive stromal cells	$p=.590$

ANOVA: analysis of variance; PRP: platelet-rich plasma

The subsequent one-way ANOVA analysis (F-value of 8.01, significance level $<.001$) and post-hoc Tukey testing revealed significant differences in the percentage of positive follicles between $t=0h$ and $t=48h$ ($p=.002$, Table 100). The 4-hour culture period did not induce significant changes in the percentage of follicles exhibiting Ki-67 staining ($t=0h$ Vs $t=4h$, $p=.570$).

Upon PRP supplementation (Table 100), differences seemed to emerge between the 4-hour time-point and baseline levels ($t=4h+PRP$ Vs $t=0h$, $p=.025$), demonstrating a possible efficacy of PRP in altering the initial state. However, PRP intervention did not yield any discernible impact on Ki-67 staining when comparing PRP-supplemented groups with those that were not supplemented with PRP, neither at the 4-hour mark ($p=.429$) nor at the 48-hour mark ($p=.968$).

Table 100 Results of post-hoc Tukey tests examining differences in the percentage of Ki-67 positive follicles among various time-points and PRP supplementation conditions

The table includes every possible comparison, distributed in columns I and J, as well as the mean difference (I-J) between different time-points (t=oh, t=4h and t=48h) or PRP conditions, along with the significance level (Sig.), indicating whether the observed differences are statistically significant. In addition to the p-value, the mean difference value (positive or negative) indicates the relationship (I and J pattern) between the two-time conditions being compared. If the mean difference of I - J is positive, it means I > J; if it is negative, it means I < J.

	(I)	(J)	Mean difference (I-J)	Relationship between the conditions	Sig.
Percentage of Ki-67 positive follicles	t=oh	t=4h	-6.33	toh = t4h	.570
		t=4h+PRP	-13.67[#]	toh < t4h+PRP	.025
		t=48h	-17.83[*]	toh < t48h	.002
		t=48h+PRP	-20.50[#]	toh < t48h+PRP	<.001
	t=4h	t=oh	6.33	t4h = toh	.570
		t=4h+PRP	-7.33	t4h = t4h+PRP	.429
		t=48h	-11.50	t4h = t48h	.078
		t=48h+PRP	-14.17[#]	t4h < t48h+PRP	.019
	t=4h+PRP	t=oh	13.67[#]	t4h+PRP > toh	.025
		t=4h	7.33	t4h+PRP=t4h	.429
		t=48h	-4.17	t4h+PRP=t48h	.858
		t=48h+PRP	-6.83	t4h+PRP=t48h+PRP	.498
	t=48h	t=oh	17.83[*]	t48h > toh	.002
		t=4h	11.50	t48h = t4h	.078
		t=4h+PRP	4.17	t48h = t4h+PRP	.858
		t=48h+PRP	-2.67	t48h = t48h+PRP	.968
	t=48h+PRP	t=oh	20.50[#]	t48h+PRP > toh	<.001
		t=4h	14.17[#]	t48h+PRP > t4h	.019
		t=4h+PRP	6.83	t48h+PRP=t4h+PRP	.498
		t=48h	2.67	t48h+PRP=t48h	.968

Statistical significance is denoted by asterisks (*). The mean difference is significant at the .05 level. "I and J Pattern," indicates the relationship between the groups based on the mean difference. The comparisons were analysed by one-way ANOVA, and post-hoc Tukey test.

The hash mark (#) indicates cases where ANOVA and Tukey tests showed significant differences when comparing time and PRP supplementation conditions, despite the initial lack of significance in the two-way ANOVA.

The differences are highlighted in bold.

ANOVA: analysis of variance; PRP: platelet-rich plasma; t=oh (toh): immediately after thawing; t=4h (t4h): 4 of *in vitro* culture; t=48h (t48h): 48 hours of *in vitro* culture

Figure 47 summarizes the results previously described, illustrating the impact of culture conditions on the percentage of Ki-67 positive follicles.

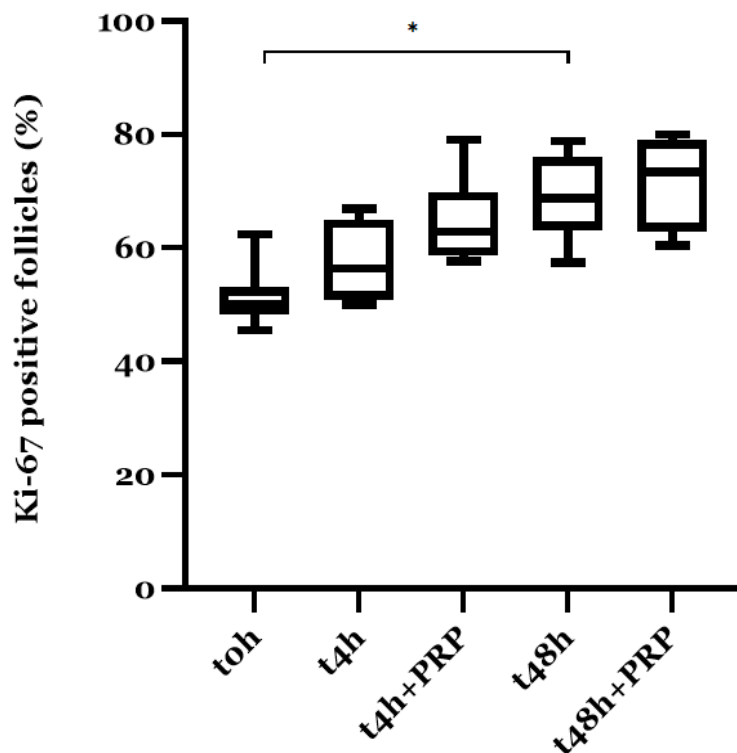


Figure 47 Comparison of the percentage values of Ki-67 expression in follicles between the various time-points and PRP supplementation conditions

The conditions include the toh (the baseline condition), and groups treated with or without PRP at different time-points: toh: immediately after thawing, t4h, t48h, t4h+PRP, and t48h+PRP.

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

Statistical significance is denoted by asterisks (*) for $p < .005$, next to the legend representing significant differences.

PRP: platelet-rich plasma

Regarding the percentage of stromal cells positive for Ki-67, a one-way ANOVA (F-value of 10.22, significance level $< .001$) and subsequent post-hoc Tukey test revealed distinct patterns compared to Ki-67 positive follicles. Significant differences were observed in comparisons involving PRP supplementation (Table 101). At the 4-hour mark of culture, a significantly higher percentage of Ki-67 staining was evident when stromal cells were incubated with PRP compared to the group that was not supplemented with PRP ($t=4h+PRP$ Vs $t=4h$, $p < .001$, Table 101). This effect was also observed after 48 hours of culture, noted by the significant difference between the PRP-supplemented group and the group not supplemented with PRP ($t=48h$ Vs $t=48h+PRP$, $p = .007$).

The addition of PRP to the 4-hour culture seems to determine a significant increase in Ki-67 staining of stromal cells compared to both the baseline (t=0h, p=.002) and the 48-hour mark (t=48h, p<.001).

The only significant variation in stromal cell positivity for Ki-67 was observed with the addition of PRP during the 4 and 48-hour culture period. Otherwise, the percentage of stromal cells marked remained unchanged across all conditions, from the initial (t=0h) to 4-hour and 48-hour time-points (t=0h Vs t=4h, p=.999; t=0h Vs t=48h, p=.993; t=4h Vs t=48h, p=1.000).

Table 101 Post-hoc Tukey tests results evaluating differences in the percentage of Ki-67 positive stromal cells across various time-points and PRP supplementation conditions

The table includes every possible comparison, distributed in columns I and J, as well as the mean difference (I-J) between different time-points (t=0h, t=4h, and t=48h) or PRP conditions, along with the significance level (Sig.), indicating whether the observed differences are statistically significant. In addition to the p-value, the mean difference value (positive or negative) indicates the relationship (I and J pattern) between the two-time conditions being compared. If the mean difference of I - J is positive, it means I > J; if it is negative, it means I < J.

	(I)	(J)	Mean Difference (I-J)	Relationship between the conditions	Sig.
Percentage of Ki-67 positive stromal cells	t=0h	t=4h	.01	toh = t4h	.999
		t=4h+PRP	-.15[#]	toh < t4h+PRP	.002
		t=48h	.015	toh = t48h	.993
		t=48h+PRP	-.12[#]	toh < t48h+PRP	.018
	t=4h	t=0h	-.01	t4h = toh	.999
		t=4h+PRP	-.16[*]	t4h < t4h+PRP	<.001
		t=48h	.00	t4h = t48h	1.000
		t=48h+PRP	-.13[#]	t4h < t48h+PRP	.010
	t=4h+PRP	t=0h	.15[#]	t4h+PRP > toh	.002
		t=4h	.16[*]	t4h+PRP > t4h	<.001
		t=48h	.17[#]	t4h+PRP > t48h	<.001
		t=48h+PRP	.03	t4h+PRP=t48h+PRP	.884
	t=48h	t=0h	-.01	t48h = toh	.993
		t=4h	-.00	t48h = t4h	1.000
		t=4h+PRP	-.17[#]	t48h < t4h+PRP	<.001
		t=48h+PRP	-.14[*]	t48h < t48h+PRP	.007
	t=48h+PRP	t=0h	.12[#]	t48h+PRP > toh	.018
		t=4h	.13[#]	t48h+PRP > t4h	.010
		t=4h+PRP	-.03	t48h+PRP=t4h+PRP	.884
		t=48h	.14[*]	t48h+PRP > t48h	.007

Statistical significance is denoted by asterisks (*). The mean difference is significant at the .05 level. "I and J Pattern," indicates the relationship between the groups based on the mean difference. The comparisons were analysed by one-way ANOVA, and post-hoc Tukey test.

The hash mark (#) indicates cases where ANOVA and Tukey tests showed significant differences when comparing time and PRP supplementation conditions, despite the initial lack of significance in the two-way ANOVA.

The differences are highlighted in bold.

ANOVA: analysis of variance; PRP: platelet-rich plasma; t=0h (toh): immediately after thawing; t=4h (t4h): 4 of *in vitro* culture; t=48h (t48h): 48 hours of *in vitro* culture

Figure 48 summarizes the results previously described, illustrating the impact of culture conditions on the percentage of stromal cells positive for Ki-67.

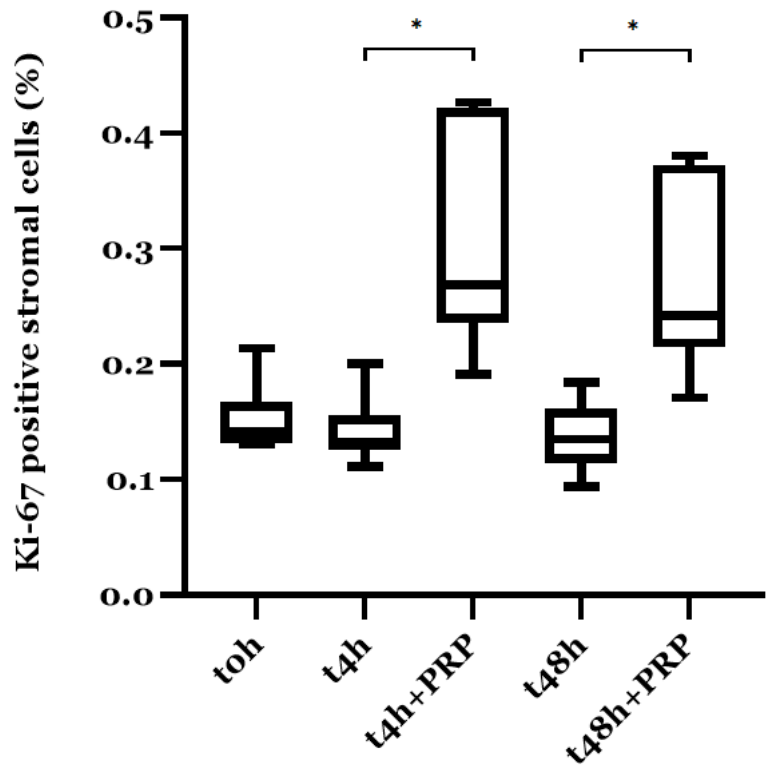


Figure 48 Comparison of the values of Ki-67 expression in stromal cells over various time-points and PRP supplementation conditions

The conditions include the toh (the baseline condition), and groups treated with or without PRP at different time-points: toh: immediately after thawing, t4h, t48h, t4h+PRP, and t48h+PRP.

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

Statistical significance is denoted by asterisks (*) for $p < .005$, next to the legend representing significant differences.

PRP: platelet-rich plasma

2.4.4.1.3.2 Apoptosis: caspase 3

The mean percentage of caspase 3 positive follicles was approximately 44.07%, accompanied by a SD of 10.41 (Table 102). The mean percentage of positive stromal cells was 1.77% with a SD of 1.57.

Table 102 Mean values for the percentage of positive follicles and positive stromal cells for caspase 3

The results are presented as mean and SD.

	Mean	SD
Percentage of caspase 3 positive follicles	44.07	10.41
Percentage of caspase 3 positive stromal cells	1.77	1.57

SD: standard deviation

The Figure 49 illustrates the pattern observed in caspase 3 immunostaining, indicating a reduced positive area in both stromal cells and follicles.

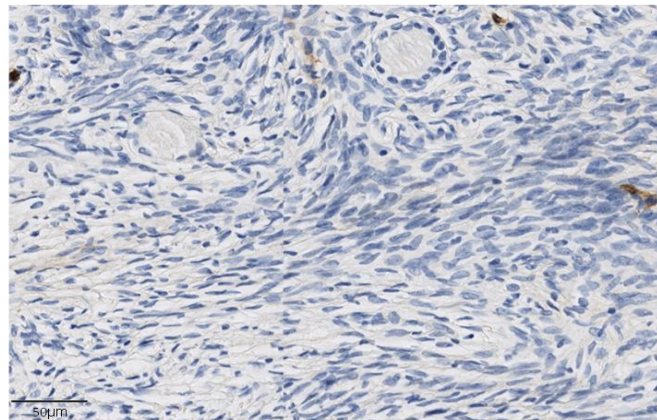


Figure 49 Representative image of Caspase 3 immunostaining

Obtained with a magnification of 100x, with a scale bar of 50µm

The effect of time on the percentage of caspase 3 positive follicles and stromal cells was investigated across different culture durations (Table 103). For the percentage of positive follicles, the mean values at t=0h, t=4h, and t=48h were found to be 43.10%, 43.17%, and 45.46% respectively, with corresponding SD of 12.29, 11.47, and 9.07. Statistical analysis revealed no significant differences across the three time-points ($p=.851$, Table 103). For the percentage of positive stromal cells, the mean values were 1.75%, 1.46%, and 2.08% respectively, with SD of 1.43, 1.08, and 2.05. No significant differences were observed ($p=.663$, Table 103).

Table 103 Mean values for the percentage of caspase 3 positive follicles and positive stromal cells at different time-points

toh, immediately after thawing; t4h; and t48h of culture. The results are presented as mean and SD. The statistical analysis indicates the significance level (p-value) obtained from the two-way ANOVA for each parameter.

Effect of Time	Mean toh	SD (toh)	Mean t4h	SD (t4h)	Mean t48h	SD (48h)	Statistical analysis
Percentage of caspase 3 positive follicles	43.10	12.29	43.17	11.47	45.46	9.07	p=.851
Percentage of caspase 3 positive stromal cells	1.75	1.43	1.46	1.08	2.08	2.05	p=.663

ANOVA: analysis of variance; SD: standard deviation

Regarding the effect of PRP on the percentage of caspase 3 positive follicles and stromal cells (Table 104), no significant differences for either parameter (p=.898 for follicles, p=.986 for stromal cells) were found.

Table 104 Comparison of the mean percentage of caspase 3 positive follicles and positive stromal cells between the groups supplemented with PRP and those not supplemented with PRP

This table provides mean values and SD for the groups supplemented with PRP and those that were not supplemented with PRP (for each time-point as well as the overall total). The statistical analysis presents the p-values, indicating the level of significance for the observed differences. Statistical significance was assessed using two-way ANOVA.

	time	PRP	Mean	SD	effect of PRP
% of caspase 3 positive follicles	toh		43.10	12.29	p=.898
	t4h	Without PRP	43.82	9.19	
		PRP	42.52	14.29	
	t48h	Without PRP	45.40	9.17	
		PRP	45.52	9.83	
	Total	Without PRP	44.11	9.75	
		PRP	44.02	11.80	
	time	PRP	Mean	Std. Deviation	effect of PRP
% of caspase 3 positive stromal cells	toh		1.76	1.43	p=.986
	t4h	Without PRP	1.47	.97	
		PRP	1.46	1.28	
	t48h	Without PRP	2.07	2.09	
		PRP	2.10	2.22	
	Total	Without PRP	1.76	1.49	
		PRP	1.78	1.76	

ANOVA: analysis of variance; PRP: platelet-rich plasma; SD: standard deviation; toh: immediately after thawing; 4 hours and 48 hours of culture (t4h and t48h)

The interaction between time and PRP was examined (Table 105). For both the percentage of positive follicles and stromal cells, no significant interaction effects were observed ($p=.877$ for follicles, $p=.971$ for stromal cells).

Table 105 Effect of the interaction between time and PRP supplementation for the percentage of caspase 3 positive follicles and positive stromal cells

The p-values indicate the level of significance for the observed differences. Statistical significance was assessed using two-way ANOVA.

Time x PRP	Statistical analysis
Percentage of caspase 3 positive follicles	$p=.877$
Percentage of caspase 3 positive stromal cells	$p=.971$

ANOVA: analysis of variance; PRP: platelet-rich plasma

The results previously described are transcribed in the Figure 50 A and B, demonstrating the lack of significant differences in caspase 3 expression across the culture conditions (in both follicles and stromal cells).

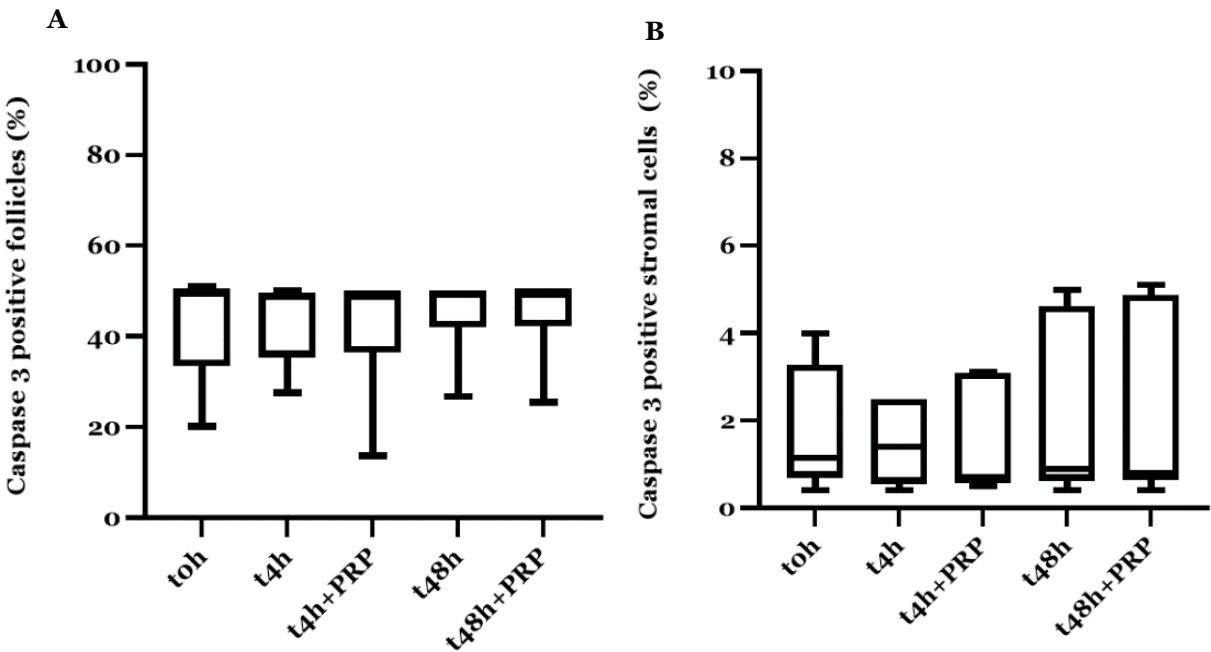


Figure 50 Comparison of the percentage values of caspase 3 expression in follicles (A) and stromal cells (B) over different culture times

The conditions include the toh (the baseline condition), and groups treated with or without PRP at different time-points: toh: immediately after thawing, t4h, t48h, t4h+PRP, and t48h+PRP.

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

Statistical significance is denoted by asterisks (*) for $p<.005$, next to the legend representing significant differences.

PRP: platelet-rich plasma

2.4.4.1.3.3 Vascular density and vascular area: Factor VIII (FVIII)

The baseline characteristics of vascular density and vascular area were assessed (Table 106). The mean values for vascular density were 17.37 blood vessels/mm² with a SD of 2.18, while for vascular area, the mean was 4.27% with a SD of 3.71.

Table 106 Mean values for vascular density and vascular area

Vascular density is defined by the number of blood vessels per unit area, in mm² and vascular area represents the percentage of the area marked with FVIII. The results are presented as mean and SD.

	Mean	SD
Vascular density (number of blood vessels/mm ²)	17.37	2.18
Vascular area (% of the area marked with FVIII)	4.27	3.71

FVIII: Factor VIII; mm²: square millimeter; SD: standard deviation

The immunostaining pattern for FVIII is illustrated in Figure 51.

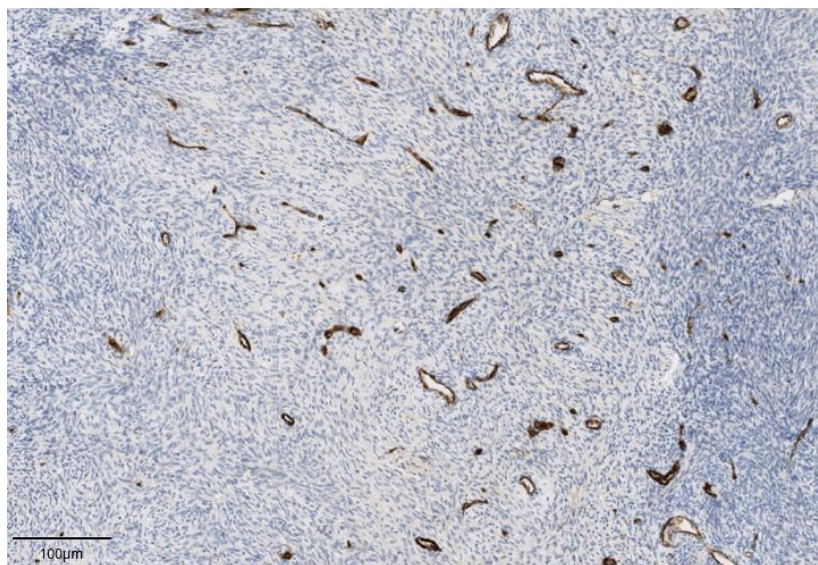


Figure 51 Representative image of FVIII immunostaining

Obtained with a magnification of 50x, with a scale bar of 100µm. FVIII: Factor VIII

When examining the effect of time on vascular characteristics (Table 107), no significant differences were observed across the various time-points for both vascular density ($p=.937$) and vascular area ($p=.284$).

Table 107 Effect of time on vascular density and vascular area

Vascular density is defined by the number of blood vessels per unit area, in mm² and vascular area represents the percentage of the area marked with FVIII. Mean values at three time-points (toh, immediately after thawing; t4h; and t48h) along with their corresponding SD are presented. Statistical analysis was conducted using two-way ANOVA, and p-values are reported for each variable.

Effect of time	Mean toh	SD (toh)	Mean t4h	SD (t4h)	Mean t48h	SD (t48h)	Statistical Analysis
Vascular density, (number of blood/mm ²)	16.94	2.30	17.64	1.61	17.32	2.71	p=.937
Vascular area (% of the area marked with FVIII)	1.90	.94	3.97	2.69	5.74	4.81	p=.284

ANOVA: analysis of variance; FVIII: Factor VIII; mm²: square millimeter; SD: standard deviation

Supplementation with PRP (Table 108) did not significantly alter vascular density (p =.272), although a significant increase was noted in vascular area with PRP supplementation (p =.014).

Table 108 Effect of PRP on vascular density and vascular area

This table provides mean values and SD for the groups supplemented with PRP and those that were not supplemented with PRP (for each time-point as well as the overall total) for vascular density (number of blood vessels per unit area, in mm²) and vascular area (percentage of the area marked with FVIII). Statistical analysis was conducted using two-way ANOVA, and p-values are reported for each variable.

	time	PRP	Mean	SD	effect of PRP
Vascular density (number of blood vessels/mm ²)	toh		16.94	2.30	p=.272
	t4h	Without PRP	17.76	1.86	
		PRP	17.52	1.50	
	t48h	Without PRP	16.19	3.32	
		PRP	18.44	1.43	
	Total	Without PRP	16.96	2.50	
		PRP	17.98	1.48	
	time	PRP	Mean	SD	effect of PRP
Vascular area (% of the area marked with FVIII)	toh		1.90	.94	p=.014
	t4h	Without PRP	3.49	2.51	
		PRP	4.46	3	
	t48h	Without PRP	2.88	2.13	
		PRP	8.59	5.17	
	Total	Without PRP	2.76	1.97	
		PRP	6.53	4.57	

ANOVA: analysis of variance; FVIII: Factor VIII; mm²: square millimeter; PRP: platelet-rich plasma; SD: standard deviation; toh: immediately after thawing; 4 hours and 48 hours of culture (t4h and t48h)

The interaction between time and PRP was analysed (Table 109). No significant impact of the interaction effect was found for vascular density ($p=.177$), and nor for vascular area ($p=.072$).

Table 109 Effect of the interaction between time and PRP supplementation on vascular density and vascular area

Vascular density is defined by the number of blood vessels per unit area, in mm^2 and vascular area represents the percentage of the area marked with FVIII.

Statistical analysis was conducted using two-way ANOVA, and p-values are reported for each variable.

Time x PRP	Statistical analysis
Vascular density, (number of blood vessels/ mm^2)	$p=.177$
Vascular area (% of the area marked with FVIII)	$p=.072$

FVIII: Factor VIII; mm^2 : square millimeter; PRP: platelet-rich plasma

The lack of a significant effect of time and PRP supplementation on vascular density is demonstrated in the graph (Figure 52).

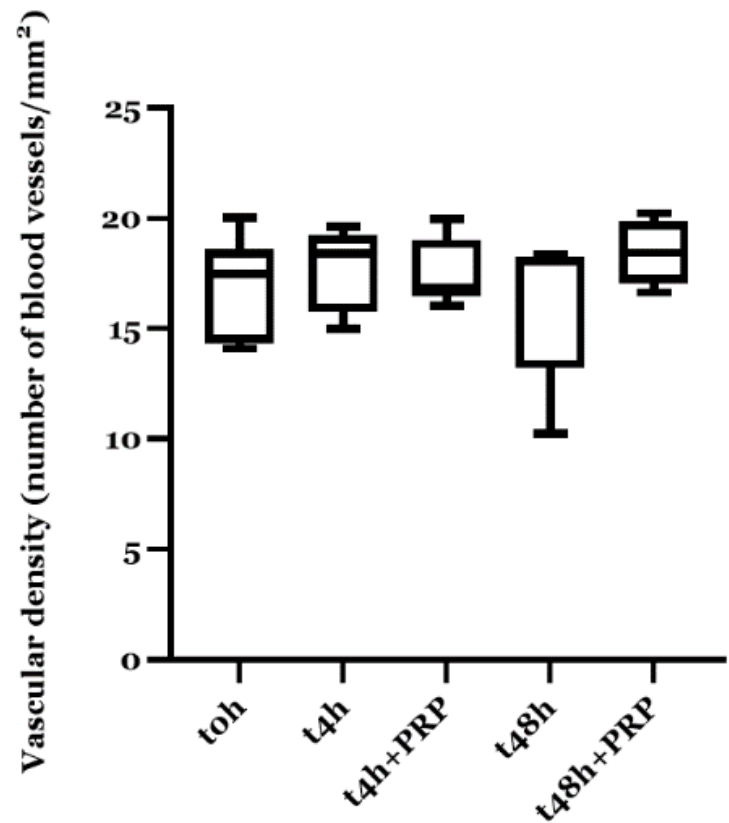


Figure 52 Comparison of the values of vascular density in various time-points and PRP supplementation conditions

The conditions include the toh (the baseline condition), and groups treated with or without PRP at different time-points: toh: immediately after thawing, t4h, t48h, t4h+PRP, and t48h+PRP.

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

Statistical significance is denoted by asterisks (*) for $p<.005$, next to the legend representing significant differences.

PRP: platelet-rich plasma

Regarding the vascular area, which showed changes due to the PRP supplementation, further analysis was conducted. After one-way ANOVA (F-value was 4.372 with a significance level of .008), the post-hoc analysis of the vascular area (% of the area marked with FVIII) was conducted across various time-points and PRP supplementation conditions (Table 110). No significant differences were observed between the baseline values (t=0h) and those at t=4h (p=.882) or t=48h (p=.980), confirming that time alone did not significantly impact the percentage of area marked with FVIII.

A significant difference emerged when PRP was introduced during the 48-hour culture period (Table 110). A significant increase in the percentage of area marked with FVIII was observed in the presence of PRP compared to the group that was not supplemented with PRP (t=48h+PRP Vs t=48h, p=.024). This value seems to exceed the baseline value (t=48h+PRP Vs t=0h, p=.006), suggesting a substantial effect of PRP supplementation on vascular area during the extended culture period.

Table 110 Results of post-hoc Tukey tests assessing differences in the vascular area among various time-points and PRP supplementation conditions

Vascular density is defined by the number of blood vessels per unit area, in mm² and vascular area represents the percentage of the area marked with FVIII.

The table includes every possible comparison, distributed in columns I and J, as well as the mean difference (I-J) between different time-points (t=0h, t=4h and t=48h) or PRP conditions, along with the significance level (Sig.), indicating whether the observed differences are statistically significant. In addition to the p-value, the mean difference value (positive or negative) indicates the relationship (I and J pattern) between the two-time conditions being compared. If the mean difference of I - J is positive, it means I > J; if it is negative, it means I < J.

		(I)	(J)	Mean Difference (I-J)	Relationship between the conditions	Sig.
Vascular area (% of the area marked with FVIII)	t=0h		t=4h	-1.67	t0h = t4h	.882
			t=4h+PRP	-2.67	t0h = t4h+PRP	.578
			t=48h	-1	t0h = t48h	.980
			t=48h+PRP	-6.83[#]	t0h < t48h+PRP	.006
	t=4h		t=0h	1.67	t4h = t0h	.882
			t=4h+PRP	-1	t4h = t4h+PRP	.980
			t=48h	.67	t4h = t48h	.996
			t=48h+PRP	-5.17	t4h = t48h+PRP	.056
	t=4h+PRP		t=0h	2.67	t4h+PRP=t0h	.578
			t=4h	1	t4h+PRP=t4h	.980
			t=48h	1.7	t4h+PRP=t48h	.882
			t=48h+PRP	-4.17	t4h+PRP=t48h+PRP	.169
	t=48h		t=0h	1	t48h = t0h	.980
			t=4h	-.67	t48h = t4h	.996
			t=4h+PRP	-1.67	t48h = t4h+PRP	.882
			t=48h+PRP	-5.83[*]	t48h < t48h+PRP	.024
	t=48h+PRP		t=0h	6.83[#]	t48h+PRP > t0h	.006
			t=4h	5.17	t48h+PRP=t4h	.056
			t=4h+PRP	4.17	t48h+PRP=t4h+PRP	.169
			t=48h	5.83[*]	t48h+PRP > t48h	.024

Statistical significance is denoted by asterisks (*). The mean difference is significant at the .05 level. "I and J Pattern," indicates the relationship between the groups based on the mean difference. The comparisons were analysed by one-way ANOVA, and post-hoc Tukey test.

The hash mark (#) indicates cases where ANOVA and Tukey tests showed significant differences when comparing time and PRP supplementation

conditions, despite the initial lack of significance in the two-way ANOVA.

The differences are highlighted in bold.

ANOVA: analysis of variance; PRP: platelet-rich plasma; t=0h (t0h): immediately after thawing; t=4h (t4h): 4 of *in vitro* culture; t=48h (t48h): 48 hours of *in vitro* culture

The results previously described are represented in Figure 53, illustrating comparisons across the culture conditions.

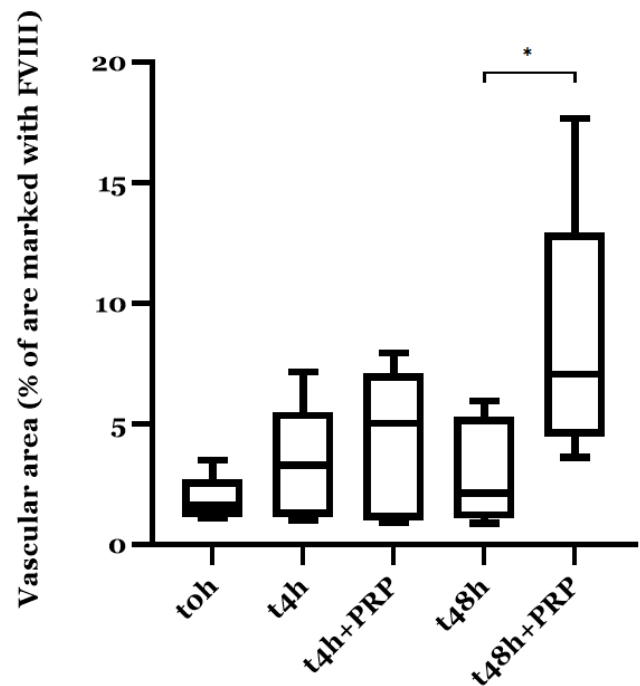


Figure 53 Comparison of the mean values of vascular area between the between various time-points and PRP supplementation conditions

Vascular area represents the percentage of the area marked with FVIII. The conditions include the toh (the baseline condition), and groups treated with or without PRP at different time-points: toh: immediately after thawing, t4h, t48h, t4h+PRP, and t48h+PRP.

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

Statistical significance is denoted by asterisks (*) for $p < .005$, next to the legend representing significant differences.

PRP: platelet-rich plasma; FVIII: Factor VIII

2.4.4.1.3.4 Follicular activation: p-Akt

The intensity of follicular p-Akt staining was analysed (Table 111). A mean percentage of 69.22% of follicles displayed weak staining, with a standard deviation of 29.11. In contrast, follicles with intense staining constituted a mean of 30.77%, with a similar SD of 28.38.

Table 111 Mean values for the percentage of follicles with weak and intense p-Akt staining

The results are presented as mean and SD.

	Mean	SD
Percentage of follicles with p-Akt weak staining	69.22	28.380
Percentage of follicles with p-Akt intense staining	30.77	28.38

p-AKT: phosphorylated AKT; SD: standard deviation

The pattern of immunostaining observed in ovarian tissue for p-Akt is represented in Figure 54. Particularly noteworthy is the side-by-side evidence of weak staining (i) and intense staining (ii).

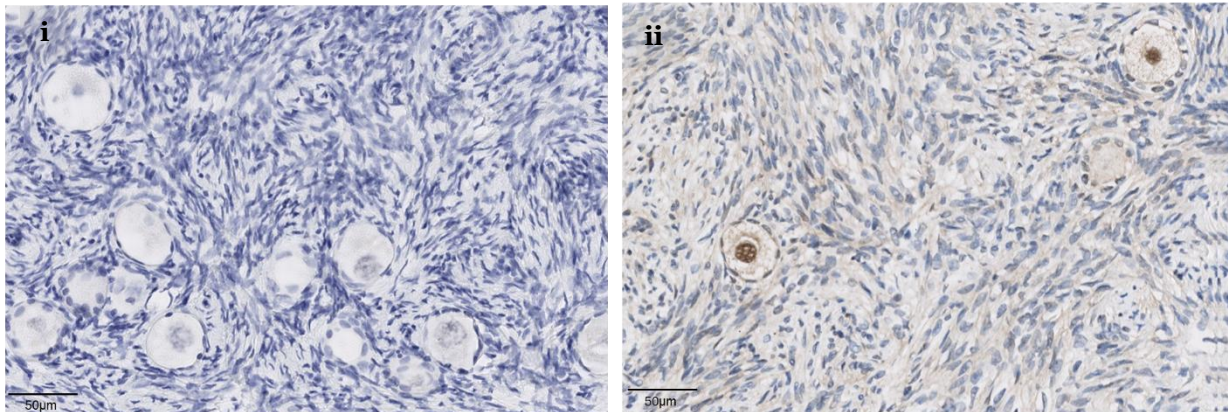


Figure 54 Representative image of p-Akt immunostaining

i) representative images showing negative staining for p-Akt in ovarian tissue; ii) representative images demonstrating positive staining for p-Akt in ovarian tissue. Images were obtained with a magnification of 100x, with a scale bar of 50µm. p-Akt: phosphorylated protein kinase B

Upon analysing the influence of culture duration (Table 112), significant differences emerged for both weak ($p < .001$) and intense ($p < .001$) staining.

Table 112 Mean values for the percentage of follicles with weak and intense p-Akt staining at different time-points

toh, immediately after thawing; t4h; and t48h of culture.

The results are presented as mean and SD. The statistical analysis indicates the significance level (p-value) obtained from the two-way ANOVA for each parameter.

Effect of time	Mean toh	SD (toh)	Mean t4h	SD (t4h)	Mean t48h	SD (t48h)	Statistical analysis
Percentage of follicles with p-Akt weak staining	100	0	75.23	22.11	47.83	24.06	p<.001
Percentage of follicles with p-Akt intense staining	0	0	24.76	22.12	52.17	24.06	p<.001

ANOVA: analysis of variance; p-Akt: phosphorylated protein kinase B; SD: standard deviation

PRP supplementation significantly affected p-Akt staining intensity (Table 113), leading to a decrease in the percentage of follicles with weak staining (p<.001) and a simultaneous increase in those with intense staining (p<.001).

Table 113 Mean values for the percentage of follicles with weak and intense p-Akt staining in groups supplemented with PRP and those that were not supplemented with PRP

This table provides mean values and SD for the groups supplemented with PRP and those that were not supplemented with PRP (for each time-point as well as the overall total). Statistical significance was assessed using two-way ANOVA, with corresponding p-values reported for each comparison.

	time	PRP	Mean	SD	effect of PRP
% of follicles with p-Akt weak staining	toh		100	0	p<.001
	t4h	Without PRP	93.18	.86	
		PRP	57.27	17.36	
	t48h	Without PRP	61.39	19.77	
		PRP	34.27	21.03	
	Total	Without PRP	84.86	20.37	
		PRP	45.77	21.96	
	time	PRP	Mean	Std. Deviation	effect of PRP
% of follicles with p-Akt intense staining	toh		0	0	p<.001
	t4h	Without PRP	6.81	.86	
		PRP	42.70	17.40	
	t48h	Without PRP	38.61	19.77	
		PRP	65.73	21.03	
	Total	Without PRP	15.14	20.37	
		PRP	54.21	21.98	

ANOVA: analysis of variance; p-Akt: phosphorylated protein kinase B; PRP: platelet-rich plasma; SD: standard deviation; toh: immediately after thawing; 4 hours and 48 hours of culture (t4h and t48h)

The interaction of time and PRP supplementation did not cause alteration in weak ($p=.481$) or intense ($p=.483$) p-Akt staining (Table 114).

Table 114 Effect of the interaction between time and PRP supplementation for the percentage of follicles with p-Akt weak and intense staining

The p-values indicate the level of significance for the observed differences. Statistical significance was assessed using two-way ANOVA.

Time x PRP	Statistical analysis
Percentage of follicles with p-Akt weak staining	$p=.481$
Percentage of follicles with p-Akt intense staining	$p=.483$

p-Akt: phosphorylated protein kinase B; PRP: platelet-rich plasma

A one-way ANOVA (F-value of 19.47 for weak staining and F-value of 19.44 for intense staining, both $p<0.001$) followed by a post-hoc Tukey test was employed (Table 115 and Table 116). The percentage of follicles positive for p-Akt exhibited a significant temporal trend, characterized by a reduction in weak staining ($t=0h$ Vs $t=48h$, $p=.001$) and an increase in intense staining ($t=0h$ Vs $t=48h$, $p=.001$). The 4-hour culture duration did not induce significant changes in p-Akt staining levels ($t=0h$ Vs $t=4h$, $p=.933$).

Examining the effect of PRP supplementation (Table 115 and Table 116), a significant difference was observed at the four-hour culture mark when comparing the PRP-supplemented condition with the group that was not supplemented with PRP ($t=4h$ Vs $t=4h+PRP$, $p=.003$). This comparison revealed an increase in intense staining accompanied by a reduction in weak staining. This pattern remained consistent when considering the 48-hour culture duration ($t=48h$ Vs $t=48h+PRP$, $p=.034$).

The supplementation with PRP appears to effectively increase the intensity of staining, reducing weak staining compared to baseline levels ($t=4h+PRP$ vs. $t=0h$, $p<.001$ and $t=48h+PRP$ vs. $t=0h$, $p<.001$). Furthermore, with PRP supplementation at the 48-hour mark, this pattern seems to persist, demonstrating an increase in intense staining and a reduction in weak staining compared to the 4-hour culture alone ($t=48h+PRP$ vs. $t=4h$, $p<.001$).

Table 115 Results of post-hoc Tukey tests analysing differences in the percentage of follicles with weak p-Akt staining across various time-points and PRP supplementation conditions

The table displays the mean difference (I-J) between different time-points (t=oh, t=4h and t=48h) or PRP conditions, along with the significance level (Sig.), indicating whether the observed differences are statistically significant. In addition to the p-value, the mean difference value (positive or negative) indicates the relationship (I and J pattern) between the two-time conditions being compared. If the mean difference of I - J is positive, it means I > J; if it is negative, it means I < J.

	(I)	(J)	Mean Difference (I-J)	Relationship between the conditions	Sig.
Percentage of follicles with weak p-Akt staining	t=oh	t=4h	6.82	t _{oh} = t _{4h}	.933
		t=4h+PRP	42.72[#]	t_{oh} > t_{4h}+PRP	<.001
		t=48h	38.61[*]	t_{oh} > t_{48h}	.001
		t=48h+PRP	65.73[#]	t_{oh} > t_{48h}+PRP	<.001
	t=4h	t=oh	-6.82	t _{4h} = t _{oh}	.933
		t=4h+PRP	35.91[*]	t_{4h} > t_{4h}+PRP	.003
		t=48h	31.80[*]	t_{4h} > t_{48h}	.010
		t=48h+PRP	58.91[#]	t_{4h} > t_{48h}+PRP	<.001
	t=4h+PRP	t=oh	-42.72[#]	t_{4h}+PRP < t_{oh}	<.001
		t=4h	-35.91[*]	t_{4h}+PRP < t_{4h}	.003
		t=48h	-4.11	t _{4h} +PRP=t _{48h}	.989
		t=48h+PRP	23	t _{4h} +PRP=t _{48h} +PRP	.092
	t=48h	t=oh	-38.61[*]	t_{48h} < t_{oh}	.001
		t=4h	-31.80[*]	t_{48h} < t_{4h}	.010
		t=4h+PRP	4.11	t _{48h} = t _{4h} +PRP	.989
		t=48h+PRP	27.11[*]	t_{48h} > t_{48h}+PRP	.034
	t=48h+PRP	t=oh	-65.73[#]	t_{48h}+PRP < t_{oh}	<.001
		t=4h	-58.91[#]	t_{48h}+PRP < t_{4h}	<.001
		t=4h+PRP	-23	t _{48h} +PRP=t _{4h} +PRP	.092
		t=48h	-27.11[*]	t_{48h}+PRP < t_{48h}	.034

Statistical significance is denoted by asterisks (*). The mean difference is significant at the .05 level. "I and J Pattern," indicates the relationship between the groups based on the mean difference. The comparisons were analysed by one-way ANOVA, and post-hoc Tukey test.

The hash mark (#) indicates cases where ANOVA and Tukey tests showed significant differences when comparing time and PRP treatment conditions, despite the initial lack of significance in the two-way ANOVA.

The differences are highlighted in bold.

ANOVA: analysis of variance; PRP: platelet-rich plasma; p-Akt: phosphorylated protein kinase B; t=oh (toh): immediately after thawing; t=4h (t4h): 4 of *in vitro* culture; t=48h (t48h): 48 hours of *in vitro* culture

Figure 55 summarizes the results previously described, illustrating the impact of culture conditions on the percentage of follicles with p-Akt weak staining.

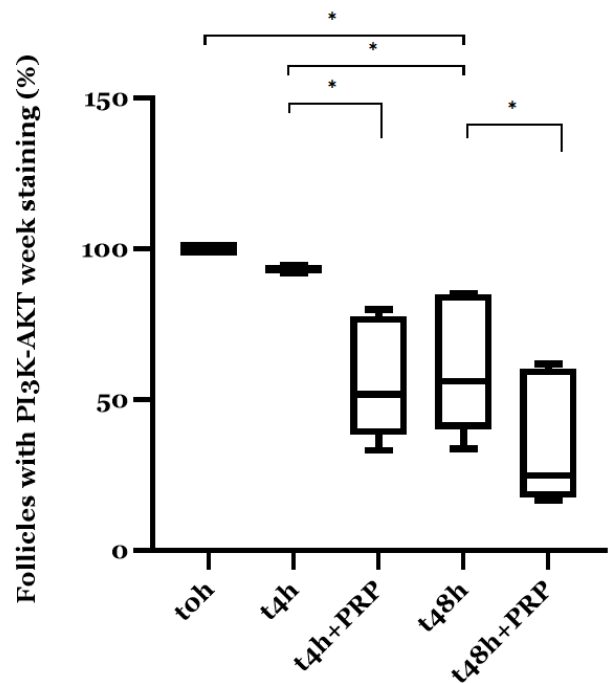


Figure 55 Comparison of the mean values of p-Akt weak staining between various time-points and PRP supplementation conditions

The conditions include the toh (the baseline condition), and groups treated with or without PRP at different time-points: toh: immediately after thawing, t4h, t48h, t4h+PRP, and t48h+PRP.

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

Statistical significance is denoted by asterisks (*) for $p < .005$, next to the legend representing significant differences.

PRP: platelet-rich plasma; p-Akt: phosphorylated protein kinase B

Table 116 Results of post-hoc Tukey tests analysing differences in the percentage of follicles with intense p-Akt staining across various time and PRP supplementation

The table includes every possible comparison, distributed in columns I and J, as well as the mean difference (I-J) between different time-points (t=oh, t=4h and t=48h) or PRP conditions, along with the significance level (Sig.), indicating whether the observed differences are statistically significant. In addition to the p-value, the mean difference value (positive or negative) indicates the relationship (I and J pattern) between the two-time conditions being compared. If the mean difference of I - J is positive, it means I > J; if it is negative, it means I < J.

	(I)	(J)	Mean Difference (I-J)	Relationship between the conditions	Sig.
Percentage of follicles with intense p-Akt staining	t=oh	t=4h	-6.81	toh = t4h	.933
		t=4h+PRP	-42.70[#]	toh < t4h+PRP	<.001
		t=48h	-38.61[*]	toh < t48h	.001
		t=48h+PRP	-65.73[#]	toh < t48h+PRP	<.001
	t=4h	t=oh	6.81	t4h = toh	.933
		t=4h+PRP	-35.88[*]	t4h < t4h+PRP	.003
		t=48h	-31.80[*]	t4h < t48h	.010
		t=48h+PRP	-58.91[#]	t4h < t48h+PRP	<.001
	t=4h+PRP	t=oh	42.70[#]	t4h+PRP > toh	<.001
		t=4h	35.88[*]	t4h+PRP > t4h	.003
		t=48h	4.085	t4h+PRP=t48h	.989
		t=48h+PRP	-23.03	t4h+PRP=t48h+PRP	.092
	t=48h	t=oh	38.61[*]	t48h > toh	.001
		t=4h	31.80[*]	t48h > t4h	.010
		t=4h+PRP	-4.08	t48h = t4h+PRP	.989
		t=48h+PRP	-27.11[*]	t48h < t48h+PRP	.034
	t=48h+PRP	t=oh	65.73[#]	t48h+PRP > toh	<.001
		t=4h	58.92[#]	t48h+PRP > t4h	<.001
		t=4h+PRP	23.03	t48h+PRP=t4h+PRP	.092
		t=48h	27.11[*]	t48h+PRP > t48h	.034

Statistical significance is denoted by asterisks (*). The mean difference is significant at the .05 level. "I and J Pattern," indicates the relationship between the groups based on the mean difference. The comparisons were analysed by one-way ANOVA, and post-hoc Tukey test.

The hash mark (#) indicates cases where ANOVA and Tukey tests showed significant differences when comparing time and PRP treatment conditions, despite the initial lack of significance in the two-way ANOVA.

The differences are highlighted in bold.

ANOVA: analysis of variance; PRP: platelet-rich plasma; p-Akt: phosphorylated protein kinase B; t=oh (toh): immediately after thawing; t=4h (t4h): 4 of *in vitro* culture; t=48h (t48h): 48 hours of *in vitro* culture

Figure 116 summarizes the results previously described, illustrating the impact of culture time on the percentage of follicles with p-Akt intense staining.

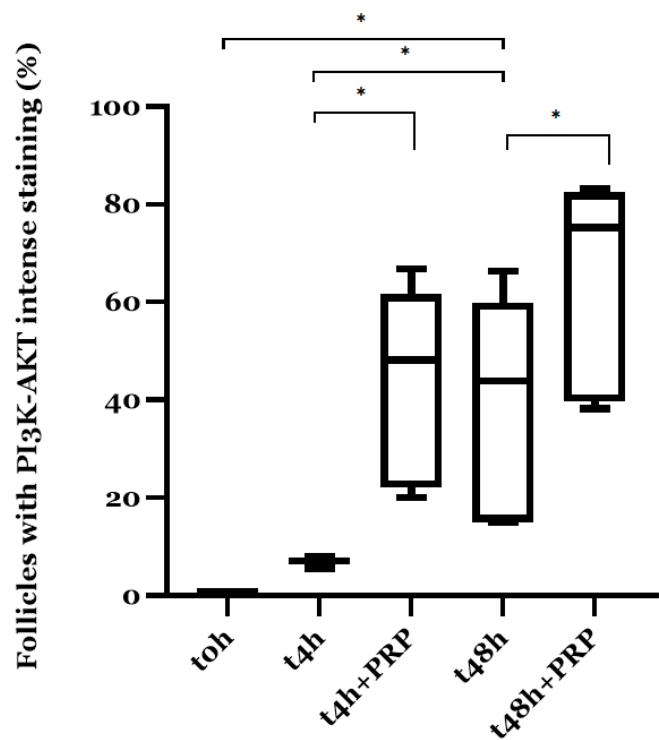


Figure 56 Comparison of the on the mean percentage of follicle with p-Akt intense staining among the various time-points and PRP supplementation conditions

The conditions include the toh (the baseline condition), and groups treated with or without PRP at different time-points: toh: immediately after thawing, t4h, t48h, t4h+PRP, and t48h+PRP.

The box and whiskers plot includes the minimum value, first quartile (Q1), median (Q2), third quartile (Q3), and maximum value. Additionally, the graph highlights potential outliers and shows the interquartile range (IQR), providing a comprehensive view of the data distribution over the specified time-points.

Statistical significance is denoted by asterisks (*) for $p < .005$, next to the legend representing significant differences.

PRP: platelet-rich plasma; p-Akt: phosphorylated protein kinase B

2.4.5 Discussion

The increasing use of PRP in various medical fields has stimulated interest in expanding its application to reproductive medicine, particularly to stimulate follicular activation in cases of DOR. Despite the growing adoption of this therapy, there is still a lack of sufficient evidence and research to confirm its efficacy and safety in clinical practice (Pellicer et al. 2023). Our first step was to optimize the protocol for obtaining PRP, taking into account our laboratory specific conditions.

When preparing PRP from whole blood, anticoagulants are used to prevent blood clotting and preserve the platelets in a nonactivated state, preventing the release of growth factors before exposure to target tissue. The choice of the anticoagulant can influence the PRR. EDTA and citrate are commonly used anticoagulants in PRP preparation both acting by chelation of calcium. ACD is often used to preserve blood for transfusion, since dextrose supports blood cell metabolism and viability (Arora & Agnihotri, 2017).

Our results revealed a lower PRR in relation to other published studies (Amable et al., 2013; Araki et al., 2012). Despite that, our protocol of PRP preparation yielded a 9- to 17-fold concentration of platelets, which is in accordance with the platelet concentrations reported by other authors (Amable et al., 2013; Araki et al., 2012; El-Sharkawy et al., 2007). A higher PRR and a higher expression of platelet activity markers was reported when EDTA was used, a finding also described by other researchers (Araki et al., 2012; Zhang et al., 2019). This can be attributed to the ability of EDTA to maintain platelets in a more natural state without altering their characteristics during the preparation process. Amaral et al. found a significantly lower PRR with ACD, the anticoagulant used in several commercial kits for PRP preparation (Do Amaral et al., 2016).

It is important to note that the optimal anticoagulant choice may vary depending on the specific goals of the PRP application and the intended use, with some authors reporting better results with citrate (Almhanawi et al., 2017).

According to the literature the concentration of VEGF in PRP preparations demonstrates a large variability between individuals and study protocols, ranging from 127 to 1300 pg/mL (El-Sharkawy et al., 2007; Eppley et al., 2004; Hariprasad et al., 2021; Ulasli et al., 2022). It would be expected that a PRP with a higher PRR would also result in a PRP with higher concentration of growth factors after platelet activation. Surprisingly, in our study, the median concentration of VEGF released into PRP obtained from blood collected with SC was higher than the PRP prepared from blood collected with EDTA (628.73 pg/mL versus 265.44 pg/mL). This suggests that EDTA may adversely affect platelet degranulation. In fact, the presence of anticoagulant when PRP is being prepared may affect the release and/or measurement of growth factors by platelets.

A higher concentration of several growth factors has been reported when using a half-dose of SC (Anitua et al., 2016; Ulasli et al., 2022). Ulasli and colleagues (Ulasli et al., 2022) compared the VEGF levels from PRP prepared with a standard dose of SC (140.7 pg/mL), half-dose of SC (240.2

pg/mL) and without the use of any anticoagulant (1303 pg/mL) – concluding that the use and dose of SC affect the growth factor composition. Like in our study, Ulasli et al. found no correlation between platelet concentration and growth factor concentration, since the protocol with a standard dose of SC had the highest PRR and the lowest VEGF concentration (Ulasli et al., 2022).

Furthermore, a higher concentration of a growth factor might not result in a better therapeutic effect since *in vitro* studies have shown that the dose–response curves of most growth factors are not linear (Arora & Agnihotri, 2017). The optimal dose of VEGF and other growth factors may vary depending on the target tissue and intended therapeutic effect, rather than relying on their absolute concentration values.

When considering the resuspension of platelets, both NaCl and plasma can be used, as they both prevent unintentional platelet activation (Arora & Agnihotri, 2017). We found a significantly higher level of VEGF when the platelet pellet was resuspended in plasma (363.32 pg/mL) in comparison to NaCl (159.83 pg/mL). In addition, we also found a higher PRR when plasma was used as the resuspension media, indicating that plasma might provide a more suitable environment for platelet stability and functionality, as suggested by Etulain et al. (Etulain et al., 2018).

Numerous methods are outlined in the literature to prepare PRP, emphasizing the importance of each laboratory defining a protocol. Many different factors, such as centrifuge type, temperature, duration, collecting technique, use of activators, etc., may affect the composition of PRP preparations, which may impact the review of PRP therapies. In addition, there is a large variability between individuals regarding blood cell counts and growth factors concentration. Therefore, we collected blood from a relatively high number of patients (n=21) and compared samples from the same patient supporting the interpretation of our results.

It is worth noting that VEGF is just one of the numerous growth factors and cytokines present in PRP. Further research is needed to evaluate the concentration of other growth factors, such as TGF- β 1, GDF9, IGF-1, PDGF and BMPs. Also, we have not determined the white blood cells count on PRP, which might affect VEGF levels since it is released by both platelets and leucocytes.

After establishing a protocol for obtaining activatable platelets, we proceeded to study PRP impact on ovarian tissue, including evaluating its capacity to stimulate PI3-Akt pathway.

Follicular activation is a controversial topic when referring to ovarian reserve, as it breaks with the dogma of a finite pool of follicles (Pellicer et al., 2023). Among the stimuli of follicular activation, modulation of PI3K-Akt pathway through PRP ovarian infusion is a promising option (Reig et al., 2021). However, its usage remains experimental (Sfakianoudis et al., 2020), reflecting a gap in understanding the regulatory process of follicular activation, assuring a safeguarding activation to avoid ovarian reserve burnout (Grosbois et al., 2020).

Recognising that an *in vitro* culture is a non-physiologic approach to study the impact of PRP in human ovarian tissue, we based our method in the existing literature. A 2D system was selected based on our research group's prior findings, which demonstrated that 3D culture of ovarian

tissue does not offer advantages over 2D culture, for cultures lasting up to 72 hours (Pais et al., 2021). Similarly, dynamic culture systems have not yet shown benefits in supporting the initial stages of folliculogenesis (Del Valle et al., 2022). Grosbois and Demeestere used 4-well dishes in their *in vitro* study with a similar aim to ours of examine the impact of the PI3K-Akt pathway on follicle activation, and had good results preserving follicular integrity throughout a 5-day culture period (Grosbois & Demeestere, 2018).

Most of the studies described in the literature primarily utilize pharmacological agents to induce follicular activation. To our knowledge, using PRP with this purpose, has only been reported in one study (Hosseini et al., 2017), lacking guiding references. Therefore, the incubation time for this study was chosen to ensure the maintenance of ovarian tissue while allowing PI3K-Akt pathway activation. Nevertheless, the exact trigger of primordial follicle activation, considering both stimuli and timing, remains unidentified (De Roo et al., 2020).

Based on the existing literature on similar *in vitro* studies of follicular activation (Devenutto et al., 2020; Devos et al., 2020; Dolmans et al., 2019; Grosbois & Demeestere, 2018; Hosseini et al., 2017; Kawamura et al., 2013; Masciangelo et al., 2019; McLaughlin et al., 2011; Pors et al., 2020), we opted for a minimum culture duration of 4 hours and a maximum of 48 hours when using PRP, acknowledging the potential detrimental effects of prolonged culture on ovarian tissue (Devenutto et al., 2020; Dolmans et al., 2019). Specifically, massive follicle activation in this context has been described as a trigger to widespread atresia (Dolmans et al., 2019).

The uncontrolled activation of quiescent follicles, in *in vitro* environment, is described to occur within 2 days in some species, but in humans the follicular development to secondary stage takes 6-8 days, and to reach antral stage more 4 to 8 days. Importantly, this is an unnatural process that results from the differences between the *in vivo* and *in vitro* conditions (Grosbois et al., 2020). Nevertheless, the impact of *in vitro* culture modifying the mechanisms of follicular activation, specifically accelerating follicular growth, was contradicted if up to 12 hours of culture (Pors et al., 2020). In our study, no significant differences were found in follicular densities over the 48 hours, nor in the density of atretic follicles. Our results revealed no significant differences over the 48 hours of culture in the indicator of apoptosis (caspase 3 staining). As well as that, Suzuki et al. have cultured ovarian tissue with Akt stimulators for 2 days, leading to beneficial effects, such as follicular growth and subsequent pregnancies following tissue transplantation (Suzuki et al., 2015).

While the number of atretic follicles and the expression of caspase 3 remained consistent across the 48 hours of culture, significant alterations were observed in ovarian tissue integrity, as indicated by an increase in the total score over the culture period. Detailing the impact of culture conditions in the ovarian tissue integrity, our group anterior studies demonstrated that the ovarian tissue could be cultured in 2D system without altering the follicular proliferation and apoptosis nor the follicular and cortex integrity, for no longer than 24h (Pais et al., 2021). Simultaneously, Devenutto et al. reported that 2-day culture system is associated with an increase in tissue damage, necrosis and apoptosis (Devenutto et al., 2020). Building on this, our current

study revealed that follicular degeneration and interstitial edema remained unchanged during the 4-hour culture period. However, both these factors, along with tissue necrosis, demonstrated a significant increase during the 48-hour period. These findings highlight the impact of culture duration on stromal characteristics, with the 48-hour period showing significant effects on the evaluated parameters. According to that, other studies evaluating *in vitro* activation, reported a higher sensibility of the ovarian tissue, being more degraded, than the follicles, over 5 and 6 days of culture (Grosbois & Demeestere, 2018; Kawamura et al., 2013).

Media supplementation, such as hMG, has been shown to offer benefits by reducing apoptosis in stromal cells. Gonadotropins play a physiological role in the ovary, and numerous studies in the field advocate for the use of FSH in the culture of human ovarian tissue to mitigate follicular atresia (Fabbri et al., 2009; Grosbois et al., 2023; Sanfilippo et al., 2013; Suzuki et al., 2015; Walker et al., 2019). We incorporated hMG into our regular media; however, the novel aspect of this study is that we found PRP supplementation in the culture media significantly impacted stromal characteristics in ovarian tissue, indicating a positive effect. In fact, there is limited research employing an *in vitro* approach to investigate the potential benefits of PRP for ovarian tissue. Hosseini et al. have reported that supplementing the medium with PRP has demonstrated improvements in the survival and growth of early follicles during a 10-day *in vitro* culture (Hosseini et al., 2017). Additionally, Callejo et al. documented successful live births following autologous transplantation of frozen/thawed human ovarian tissues supplemented with PRP (Callejo et al., 2013).

In our study, we found a tendency of PRP supplementation to restore tissue necrosis to basal levels within the first 4 hours of culture ($t_{4h}+PRP \sim t_{0h}$ and $t_{4h}+PRP < t_{4h}$). Regarding follicular degeneration and interstitial edema, the PRP beneficial effect was only evident at the 48-hour mark. In another words, while time alone has a significant impact on ovarian tissue characteristics, PRP supplementation demonstrates the potential to mitigate some of the adverse effects observed over time, particularly evident in reduction of interstitial edema and follicular degeneration after 48 hours of culture – but not completely restore them to baseline levels. Our results 1) corroborated the therapeutic potential of PRP in ovarian function, as described in literature (Sills & Wood, 2022); and 2) suggest a complex interplay between time and PRP supplementation, indicating the need for further investigation into their combined effects on tissue health and regeneration, as it is reinforced by De Roo (De Roo et al., 2020).

Additionally, the PRP supplementation has induced changes in the Ki-67 expression in stromal cells, suggesting an increase in its proliferative activity. At the 4-hour mark, a significantly higher percentage of Ki-67-positive stromal cells was observed in the PRP-supplemented condition compared to the group without PRP, and that persisted after 48 hours of culture. This pattern diverges from the findings of previous studies conducted by our research group, wherein the expression of Ki-67 in human ovarian tissue culture remained stable over 72 hours, using media solely supplemented with hMG (Pais et al., 2021).

PRP is described to exert a therapeutic effect on ovarian function (Kheil et al., 2022; Sills & Wood, 2021, 2022) through the activation of dormant follicles (Sills & Wood, 2022), and by promoting angiogenesis (Battinelli et al., 2011). Aligned with that, we assessed the alterations of vascular density, which remained stable for the 48-hour period of culture, and vascular area (% of the area marked with FVIII), that was different when added PRP. A substantial effect of PRP supplementation on vascular area was observed during the extended culture period (48h). Angiogenesis is a complex and dynamic process, influenced by multiple factors that contribute to the vascularization of tissues. Despite the being described that angiogenesis typically requires more than 48 hours to initiate (Yang et al., 2008), our results suggest that this time interval has been adequate to regulate FVIII gene expression, and modulate protein synthesis, that was quantified by IHC, if in presence of PRP supplementation. Time alone did not yield any observable changes, underscoring the significance of PRP supplementation in this regulatory process. In accordance to our results, Bentley & Chakravartula reported that short periods of time are sufficient to activate initial sprouting angiogenesis (Bentley & Chakravartula, 2017). Regarding vascular density, we observed that it remained stable throughout the culture period. Contrarily, there are studies reporting an increase in vascular densities by exposing human ovarian tissue to inducers of angiogenesis, such as bFGF and VEGF, for durations similar to ours. However, these studies include xenografting periods of 7 days (Kang et al., 2016), and 6 weeks (Tanaka et al., 2018; Wang et al., 2013). These conditions differ from ours in terms of the longer duration and interaction with other cell types, which might explain the lack of variation in vascular density observed in our study compared to what has been reported in other studies. Sanfilippo et al. reported results consistent to ours (Sanfilippo et al., 2013).

About p-Akt expression, an independent influence of culture duration and PRP supplementation on follicular staining intensity was evident. Over time, there was a significant change in p-Akt staining patterns, with a decrease in weak staining and a simultaneous increase in intense staining from the initial to the 48-hour culture period. De Roo, find the same pattern, of increasing intensity of p-Akt staining, but without enough to reach significant expression of the PI3K-Akt pathway, over the culture time (De Roo et al., 2020). Importantly, in our study, the 4-hour culture time was not enough to produce significant alterations in p-Akt staining levels, which was the same pattern observed in the percentage of Ki-67 positive follicles, with increased alterations being evident only after 48 hours of culture.

Regarding PRP supplementation, a significant difference was observed since the 4-hour culture mark, where PRP supplementation led to intensified staining alongside reduced weak staining compared to the groups without PRP. This effect was also sustained at the 48-hour mark. This increasing in p-Akt expression was not accompanied by the of Ki-67 follicular expression in the presence of PRP – while other studies have observed an increase in Ki-67 expression associated with *in vitro* follicular activation (Grosbois & Demeestere, 2018; Lunding et al., 2019; Masciangelo et al., 2019). The discrepancy between the results of these studies and ours could be attributed to factors such as longer culture periods (Grosbois & Demeestere, 2018; Lunding et al.,

2019), or the difference in stimuli applied, which was fragmentation of ovarian tissue (Lunding et al., 2019).

The primary limitation of this study lies in the fact that the investigation of follicular activation was carried out under unphysiological conditions, specifically through the *in vitro* culture of ovarian tissue. Another possible drawback is the absence of a fresh tissue group, which could have served as a comparative baseline for evaluating the observed effects in the cultured tissue, considering cryopreservation and thawing of ovarian tissue are enough to induce follicular activation (Terren et al., 2021).

Moreover, we acknowledge that our analysis of ovarian tissue from a limited number of participants (N=6) might not be robust enough for statistical techniques such as ANOVA. However, it is important to emphasize that ANOVA was the most appropriate statistical test for comparing the effects of time and PRP across all conditions. Despite the small sample size, the use of human samples offers significant clinical relevance to our findings, surpassing the applicability of animal models. Furthermore, our ratio of different patients (N=6) to the number of tissue fragments (N=85) is consistent with those employed in comparable studies (De Roo et al., 2020; Dolmans et al., 2019; Grosbois & Demeestere, 2018; Masciangelo et al., 2019, 2020), underscoring the validity of our approach.

A compelling direction for future analysis involves analysing the stored culture media from our experiments to assess the concentration of growth factors, including VEGF, IGF-1, PDGF, and TGF- β . Understanding the levels of these growth factors over the culture conditions could provide valuable perspectives into the temporal impact of PRP. As well as that, an upcoming strategy involves perform proteomic studies and/or to characterize the expression of genes related to follicular activation in ovarian tissue, after culture with different stimuli, using the samples stored for that purpose.

Given the insights from previous studies in the field, the natural progression of this research would include xenografting. Xenografting presents a distinctive opportunity to evaluate the efficacy of PRP under *in vivo* conditions, offering a comprehensive assessment of its impact on improving ovarian function. Moreover, xenografting facilitates the evaluation of the long-term effects of PRP on ovarian tissue and offers relevant information on its feasibility and effectiveness in a clinical setting.

3 Conclusions and future perspectives

The presented study aimed to contribute, in a multifocal approach, with potential strategies to manage to situations of DOR. DOR is a condition characterized by the premature reduction in the number of oocytes in the ovaries, affecting 10% of women of reproductive age, around 30 years old. Its impact on female fertility has taken particular interest for this group of researchers due to the following reasons: 1) there is a new sociodemographic pattern, with increasing maternal age; 2) it is predominantly an asymptomatic condition, which means that in most cases the diagnosis is delayed, often only discovered when women are trying to conceive; 3) 50% of cases are idiopathic, therefore lacking a targeted diagnosis and supplementation; 4) when ART is necessary, there are no strategies to mitigate this reduction in ovarian reserve; often the best option is the use of donor oocytes, which has a significant emotional and economic impact. Given this panorama, we defined and tested the effectiveness of strategies to establish practices a) prior to the diagnosis of this condition, and b) once it is already established (briefly addressing a diagnostic possibility as well), thus covering the possible approaches to improve healthcare in these situations.

The discussion of the results was presented in section 2, adjacent to each task. The concluding section is structured to synthesize the main outcomes of the project, while also identifying strengths and weaknesses, and outlining the future prospects.

3.1 What is already known on this topic?

Millions of women worldwide are infertile due to ovarian disorders, including DOR. This condition frequently leads to infertility and has a substantial impact on the quality of life of the affected couples, primarily because of their psychological implications and high financial costs. Recognizing infertility as a major public health priority, it is imperative to address the social, economic, and geostrategic impacts of declining fertility rates. While age remains a natural factor in fertility decline, societal trends such as delayed childbearing due to professional and personal considerations, economic crises, and the absence of supportive policies pose additional challenges. Additionally, there is an increasing reliance on ART and the use of donated oocytes, that underscore the complex and emotionally challenging nature of the contemporary reproductive experiences.

Within the Portuguese context, although contraception is effectively addressed in family planning consultations, both in primary care and gynaecology units within the national health system, the provision of information on reproductive health and fertility is not explicitly defined in the health plan. Given the lack of knowledge in this field, evidence indicates that there is significant potential for interventions. Decision deliberation opportunities, providing information that gives

individuals control of what they can do, helping them defining a plan, can effectively increase knowledge, and potentially empower them to make informed choices regarding family planning.

With the trend of postponing of pregnancy in modern society - in Portugal the average age at first childbirth has already surpasses 30 years old - DOR has become one of the most common challenges in current clinical reproductive medicine. Attributed to its unclear mechanism and complex clinical features (aetiology and pathogenesis of DOR are still vague), it is difficult for physicians to define a targeted supplementation. Given the impact of gonadotrophin receptors and *ESR* polymorphisms on COS, adopting a pharmacogenomic approach emerges as a promising strategy to significantly improve the success rates of fertility treatments.

The search of innovative treatments, such as intraovarian infusion of PRP, holds a potential for improving outcomes in assisted reproduction. Strategies to modulate follicle activation, especially for DOR patients in the context of IVF or fertility preservation, offer prospective benefits.

The question emerged: how can healthcare services actively contribute to this field? Whom should we prioritize as the target audience for our intervention, and where should our efforts be concentrated? In addressing these questions, we developed this research project, adopting a multifocal approach centred on a particularly vulnerable group: women with DOR.

3.2 What are the main conclusions drawn from this study?

Regarding interventions in knowledge, in addition to evaluating their impact, this study provided a description of women who would be receptive to this approach. The participants who accepted to be part of the study, and thus represent the population sensitive to the interventions, were predominantly women under 30 years old, mostly in a relationship, and with a strong desire for motherhood. The majority felt they received information about ovarian reserve at the right age. A minority, however, indicated that they would have preferred to receive this information earlier, specifically at the age of 25.

Most participants joined the study on their initiative, contrasting with those recruited through family planning services. Additionally, the participants recruited through family planning consultations were less committed to the project. This suggests that reinforcing the presence with generic information brochures or roll-ups would be sufficient within family planning environment. Besides that, we understood that efforts should be focused on enhancing the project's visibility through digital marketing, ensuring encouraging self-proposal.

It was consensual among this population that assessing and being informed about ovarian reserve is important. In fact, participants were relatively well-informed about the topic and aware that infertility could limit their parenthood plans. Their primary reason for participating in the study was concern about their fertility, instead of finding a justification to delay motherhood. However, they still planned to have children at an older age than the age they considered ideal.

Despite recognizing the importance of motherhood in their lives, participants did not intend to anticipate the age at which they planned to have children, at least in this short-term assessment. ART remains their backup plan in case of infertility, reflecting its popularity among the general population.

Regarding our primary outcome, we concluded that the interventions developed effectively enhanced knowledge in the field of fertility, ovarian reserve, and DOR. However, in terms of intentions, participants did not show an increased preference to anticipate their childbearing age or consider alternative reproductive plans. This lack of change was observed following both general and tailored information.

Particularly after providing tailored information about ovarian reserve being within or below expected levels for their age, there was no significant preference to alter reproductive plans. This absence of alteration might have been due to potential inadequacies in the communicated information. Nevertheless, the study demonstrated alignment between the information provided and the participants' understanding.

Interestingly, intention to undergo oocyte cryopreservation was the only behavioural intention that changed after the interventions. Despite the growing recommendation of this practice to address delayed motherhood, this study observed a decrease in the reliance on social freezing after both interventions, varying according to the information received. The group informed about having a lower-than-expected ovarian reserve showed a reduced consideration of this option, demonstrating an adjustment of their plans based on the information provided.

Side effects such as anxiety remained unaffected after the interventions. This, combined with the fact that women demonstrated clear comprehension of the information provided about their ovarian reserve status, suggests that the message was effectively transmitted.

Regarding the genetic study of populations with altered ovarian reserve, our research began with a literature review. Indeed, the review emphasized the role of polymorphic changes in ovarian function, highlighting the significance of studying *FSHR* rs6166, *ESR1* rs2234693, and *ESR2* rs9340799. However, we did not find any significant association between these polymorphisms and DOR, within IVF populations. Similarly, there was no meaningful association observed in populations with PCOS regarding these SNPs. Based on the literature suggesting the influence of these polymorphisms on the hormonal profile of women with PCOS, we investigated their association with 3rd-day FSH levels. We found a positive association between the SS genotype of the *FSHR* rs6166 polymorphism and higher levels of FSH on the 3rd day of the menstrual cycle.

Regarding the applicability of PRP in this field, we concluded that a two-step centrifugation protocol effectively isolates viable platelets from 15 mL of blood collected in EDTA tubes. The protocol involves centrifugation at 300g for 10 minutes followed by 700g for 15 minutes. Additionally, our *in vitro* assessment indicates that this method allowed the preparation of PRP capable of altering the expression (increasing the staining) of p-Akt in the follicles within the ovarian tissue.

Particularly, it has been confirmed that a 4-hour culture period is preferable to 48 hours in a 2D minimal culture system. When culturing ovarian tissue for 48 hours, supplementation with PRP helps reduce follicular degeneration and interstitial edema. However, PRP alone is insufficient to prevent tissue necrosis within this 48-hour culture period.

Ki-67 expression in follicles was unaltered at 4-hour mark, when compared with the tissue immediately after thawing. This might mean this culture time is the one that maintains the follicles closer to the state after thawing. In stromal cells, time alone did not impact Ki-67 expression. However, PRP demonstrated the ability to alter this pattern, increasing Ki-67 expression in stromal cells starting from the 4-hour culture mark.

To achieve an angiogenic response, as indicated by the percentage of area marked with Factor VIII, both a 48-hour period and additional PRP supplementation are necessary.

After a four-hour culture period, PRP supplementation resulted in intensified staining and reduced weak p-Akt staining compared to the group without PRP.

In conclusion, our results suggest that no additional benefits were observed from prolonging tissue culture for 48 hours when aiming to stimulate the PI3K-Akt pathway in ovarian tissue, *in vitro*.

3.3 What are the limitations?

3.3.1 Task 1: intervention in knowledge through general and tailored information on ovarian reserve and diminished ovarian reserve (DOR)

Interventions in knowledge lacked a control group, which may affect our conclusions. The non-randomized design introduces potential selection bias, challenging generalizability.

Furthermore, the results may have limited generalizability beyond the Portuguese population studied. The study's focus on a specific demographic could restrict its applicability to broader populations with different genetic, cultural, or environmental backgrounds.

Another potential limitation is the influence of bias, particularly performance bias, where participants behaviour and responses may have been influenced by the awareness of being studied (known as the Hawthorne effect). This awareness could have altered participants natural responses and behaviours, affecting the study outcomes. Additionally, there is the recruitment bias. Many women came through self-referral, meaning they already had an interest in the study, which could affect how they engage with the intervention (bias of the self-selected sample).

The sample size may have not been sufficiently large to detect small effects or differences between groups. Moreover, forming subgroups based on tailored information have further reduced the sample size within each subgroup, potentially impacting the study's statistical power.

Additionally, the timing of measurements in the study may have introduced temporal effects. Changes observed in knowledge, intentions, and plans might reflect short-term responses rather than reflecting long-term impacts or trends.

Reliance on self-reported data for capturing knowledge, intentions, and reproductive plans introduces potential for response bias and inaccuracies. Variations in participants' interpretations of questions or their willingness to report sensitive information could affect the accuracy and reliability of the data collected.

Lastly, the questionnaire was developed by the team based on previous existing validated questionnaires. The current version of the questionnaire that was used was not subject to psychometric analysis, namely validity and reliability study. Although this may be considered a limitation, it was mainly due to the fact that we were interested in assessing a set of specific questions that could not be tapped by a global construct. Conversely, when assessing anxiety, a validated and widely used measure was used.

3.3.2 Task 2: genetic study of infertile populations with abnormal ovarian reserve

About the evaluation of *FSHR* rs6166, *ESR1* rs2234693, and *ESR2* rs9340799 polymorphisms in infertile population, an observational study, with a retrospective design was conducted. Controversial data on the contribution of polymorphic genetic variants to DOR, already denoted in the literature, emphasizes the importance of large RCTs. Our study did not adopt this methodology, limiting the conclusiveness of our results.

Moreover, the population under the study of genetic variants consists of infertile women, selected to enhance outcomes following a DOR diagnosis and when confronting with infertility. However, there may be selection bias as the samples were randomly selected, but exclusively from an infertile group of women attending a reproductive medicine unit. This narrow selection could restrict the applicability of the findings to the broader population, limiting their generalizability. Additionally, the study's findings may only be relevant to the Portuguese population, as different populations might display varied genetic associations with ovarian reserve markers.

The presence of confounding variables, including unmeasured factors such as environmental influences, lifestyle choices, and additional genetic variations, could potentially influence ovarian reserve markers in ways that were not accounted in this study. Another limitation pertained to genotyping, particularly regarding SNPs analysis. The research focused solely on SNPs of *FSHR* rs6166, *ESR1* rs2234693, and *ESR2* rs9340799, potentially overlooking other pertinent genetic variations that could also contribute to altered ovarian reserve. In line with that, the contribution of a particular gene in multifactorial diseases, such as PCOS and DOR, is difficult to determine.

The study's statistical power, particularly concerning populations with SOP, may have been insufficient. For both, DOR and SOP, the sample size might not have been adequate to detect subtle effects or to generalize findings across diverse populations effectively.

3.3.3 Task 3: *in vitro* study of follicular activation through the incubation of human ovarian tissue with platelet-rich plasma (PRP)

Firstly, this study may not fully replicate the complex interactions and responses found in living organisms. *In vitro* conditions lack the dynamic biological environment present *in vivo*, thereby limiting their biological relevance and the ability to convert findings directly to clinical settings.

Another significant limitation is its focus on short-term analyses, limited to a period up to 48 hours. While this study can provide valuable insights into immediate effects, it may overlook long-term consequences and responses that are crucial for understanding the broader impacts of PRP. The long-term effects of PRP on ovarian tissue were not assessed in this study.

One notable limitation is the small sample size inherent in the study design. A larger sample size would have enhanced the statistical power and reliability of the findings.

We decided to use non-autologous PRP due to the difficulties in coordinating blood collection (patient availability) on the day of the *in vitro* culture (and ovarian tissue thawing). As a result, we do not perfectly replicate the real scenario where PRP is autologous. However, this decision helped to avoid delays in our experiments. Since we utilized an *in vitro* model, we found that the disadvantages associated with non-autologous PRP did not apply to our study. In an *in vitro* culture, concerns such as immunogenic reactions or transmissible infections were irrelevant.

The process of cryopreservation and subsequent thawing of tissue samples can independently alter their properties, introducing variability into experimental results. Additionally, variations in tissue fragments used in experiments, such as differences in age or quality, can further complicate comparisons and impact the reliability of findings. Establishing consistent baseline conditions is essential in *in vitro* studies. While immediate post-thawing analysis serves as a baseline, variations in initial tissue conditions among samples can influence subsequent measurements and interpretations. We did not include the analysis of human ovarian tissue before cryopreservation and thawing.

Biological markers selected for study, such as Ki-67, caspase 3, FVII, and p-Akt, may not comprehensively capture all relevant biological processes affected by the PRP. Additional confirmatory analysis to the IHC would strengthen the robustness of our conclusions.

The study did not include the evaluation of histological and immunohistochemical results by two separate investigators. This approach allows for better methodology and increases the technique reproducibility. Instead, in our study, samples were evaluated blindly and randomized three times by the same observer, with intervals of two months between assessments.

3.4 What this study adds? How might this study affect research, practice or policy?

This study was pioneering on the national scenario, as no previous studies were known in the Portuguese context regarding populations with DOR. Conducted with Portuguese participants, specifically in a demographic region, we aimed to align our objectives with existing studies in the literature, while addressing Portuguese characteristics. In other words, we responded to needs identified in the current European context—a shift in sociodemographic patterns requiring coordinated strategies for information and healthcare improvement to ensure a reproductive health response. Our objectives and study hypotheses were based on a review of existing literature and identified gaps, allowing our results to provide valid contributions, not only within the Portuguese context, despite the discussion being focused on this population context.

Regarding the interventions in knowledge, it was possible to show that women learnt about ovarian reserve and DOR and understood ovarian reserve assessment results. This aspect is crucial yet challenging to assess when designing a knowledge intervention to ensure its effectiveness. Initially, we based our criteria on existing literature. Upon completing the project and confirming its efficacy in increasing knowledge about ovarian reserve and understanding ovarian reserve assessment results, we were able to define a group of women who are sensitive to this information. This group can be useful for other studies aiming to improve knowledge about ovarian reserve and DOR.

Additionally, the reproductive counselling package that included informative content (a leaflet, a video, and a website), a consultation guide and a corresponding tool intended for use during consultation were developed and readily accessible to be replicated in other populations. The website and video were evaluated through interviews, and the feedback indicated a high level of satisfaction regarding their clarity, relevance, accessibility, and educational value. All participants expressed willingness to recommend them to others. It is important to note the limitations of this evaluation in assessing the overall impact of the content, as it primarily involved a small subset of inquiries compared to the principal tasks of the study.

Despite the informative and counselling content has been developed and applied for in a Portuguese population, the generic information regarding ovarian reserve and the reproductive counselling complements international efforts in this area. Moreover, the results demonstrated an understanding of the information provided and comprehension of the content included in the reproductive counselling, validating our approach. Overall, the interventions developed hold potential for replication in other countries and continued implementation within the Portuguese context.

The assessment of *FSHR* rs6166, *ESR1* rs2234693, and *ESR2* rs9340799 polymorphisms has provided new insights specific to the Portuguese population. To our knowledge, these genetic variants have not been previously studied among individuals in this demographic. The findings contribute to the ongoing discussion surrounding the association of these genetic variations with

DOR and PCOS, conditions characterized by altered ovarian reserve, within the Portuguese population.

The optimization of the protocol for PRP preparation proves useful in clinical and research contexts where isolating a sufficient and viable concentration of platelets is required, such as in reproductive medicine. Regarding its impact on *in vitro* culture of ovarian tissue with PRP, several conclusions can guide future research, particularly when employing an *in vitro* approach in a 2D minimal culture system.

3.5 Anticipating tomorrow: What lies ahead?

The integration of fertility education emerges as a potential solution, though its effectiveness depends on various factors. An integrated, community-based approach, involving education in schools, social media, national campaigns, and training for health professionals, is proposed. Accordingly, we have outlined objectives to further strengthen the intervention in knowledge, specifically:

- i. Enhancing educational resources: develop teacher's guide and a pilot study for secondary students (school year 2024/2025);
- ii. Supporting healthcare services: produce informative materials designed to primary health units and clinics with family planning consultation;
- iii. Engaging policy makers: present the project to policy makers to foster collaboration and gather support for the effective implementation of interventions.

For the participants from our study, doing a follow-up to see if they still remember what they learned could give us a better idea of how long the knowledge lasts. Furthermore, reassessing their fertility goals and the decisions made in practice is crucial to evaluate the true impact of the interventions, comparing initial expectations with actual outcomes (expectation vs. reality). Alongside this, evaluating decisional anxiety in contrast to the already assessed anticipatory anxiety (referring to immediate decisions) and anticipated anxiety (related to future unfulfilled plans) would offer a comprehensive understanding of the psychological effects of the interventions.

Lack of support of the contribution of polymorphisms to DOR motivated further investigation, emphasizing the importance of larger cohorts and use of other methodologies, such as NGS. The elucidation of the aetiology of DOR shows its potential in defining new biomarkers, potentially more directed and specific than AMH and AFC, and to define novel target therapies.

To enhance the assessment PI3K-Akt activation, we plan to measure the levels of growth factors present in the culture media, focusing on those commonly released by platelets. This will help us understand the concentration levels and duration of these factors in culture, with the intention of correlating them with observations from IHC. Additionally, we intend to analyse the releasates

from ovarian tissue, particularly factors and hormones involved in follicular development and angiogenesis, to further enrich our understanding. Furthermore, evaluating the proteomics and genomics of ovarian tissue samples from different culture conditions is a future perspective that we aim to explore.

About the clinical application of PRP, as research progresses there is optimism for its usage in the field of reproductive medicine. We understood from our questionnaire (Task 1) a significant acceptability of PRP towards innovative reproductive treatments among the participants. The validation of the capacity of our own prepared PRP to impact on the PI3K-Akt pathway, opened the door for its appliance in DOR patients. From a clinical point a view, after the *in vitro* tests we are currently developing a protocol for autologous PRP ovarian infusion. This protocol will be implemented for patients with DOR, at the Reproductive Medicine Unit of CHUC.

3.6 Final remarks

As initially mentioned, this study aimed to address aspects that remain unanswered in cases of DOR, ranging from raising knowledge about this fertility aspect to investigating potential therapies post-diagnosis of the condition, and its implications within the context of infertility.

We concluded that the interventions developed effectively increased knowledge on the subject, which validate our model for its continuation in reproductive healthcare for non-infertile populations without children, within reproductive age. The short-term analysis did not reveal significant changes in reproductive plans and intentions, supporting the need for a long-term follow-up study to ascertain if the internalized information translates into behavioural changes (follow-up at 3 to 5 years).

Regarding genetic variants, we did not identify a predominant determinant associated with altered ovarian reserve, at least among the genetic variants studied. This led us to reconsider the study of these polymorphisms in this population, redirecting our focus towards a different technological approach (NGS) even before the diagnosis of infertility, thereby reducing bias associated with variable confounders. Nevertheless, our findings clarified that in the Portuguese population, there is no association of these SNPs with ovarian reserve markers, particularly *FSHR* rs6166, *ESR1* rs2234693, and *ESR2* rs9340799 SNPs have no predictive value for DOR.

In addressing the infertile population, it was opportune to test the effect of PRP therapy *in vitro*, given its growing interest to treat women with DOR. We observed that PRP therapy had an effect on p-Akt expression, suggesting its implication in the PI3K-Akt pathway involved in follicular activation. The mechanism of follicular activation was briefly explored, providing positive indicators for the clinical applicability of this therapy.

4 Supplementary data

4.1 The study leaflet

a tua fertilidade

O que sabes sobre a tua Fertilidade?

O que é a reserva ovária?



A reserva ovária traduz o **número de óvulos disponíveis** ao longo da vida reprodutiva da mulher. A reserva ovária é **finita e limitada** e diminui naturalmente com o **aumento da idade**.

Ao nascimento, a reserva ovária é composta por **1 a 2 Milhões de óvulos**



O número de óvulos **vai diminuindo** ao longo da vida de mulher



Consequentemente, a fertilidade diminui com o aumento da idade, principalmente a partir dos **30 anos** e, de forma mais acentuada, aos **35 anos**

Sabias que podes avaliar a tua fertilidade?



Apesar de não ter sintomas associados, a **reserva ovária diminuída**, pode ser diagnosticada através de um **marcador sanguíneo** e **ecografia ovária**.



Contudo, **10% das mulheres** com idade próxima dos **30 anos** têm uma redução da fertilidade, são diagnosticadas com **reserva ovária diminuída**.

Está associada a uma maior dificuldade em engravidar, mesmo recorrendo a **tratamentos de fertilidade**.

Em **50%** dos casos, a **causa** da diminuição da reserva ovária é **desconhecida**.

Sabe mais sobre a tua reserva ovária!

Envia-nos um e-mail para: **info@atuafertilidade.pt**
Segue-nos em @atuafertilidade
Visita-nos em www.atuafertilidade.pt



Informar e aconselhar em fertilidade poderá ajudar a tomar decisões atempadas e a prevenir uma infertilidade futura!



4.2 Images illustrating the tool for determining AMH and AFC percentiles

Reserva Ovária

Idade (anos) 25

HAM (ng/ml) 1

● Percentil < 5% (1.26)

CFA 4

● Percentil < 5% (5)

Calcular

Reserva Ovária

Idade (anos) 25

HAM (ng/ml) 4

● Percentil > 25% (2.97), < 50% (4.753)

CFA 10

● Percentil > 25% (9), < 50% (13)

Calcular

Reserva Ovária

Idade (anos) 25

HAM (ng/ml) 5

● Percentil > 50% (4.753), < 75% (7.11)

CFA 15

● Percentil > 50% (13), < 75% (18)

Calcular

Reserva Ovária

Idade (anos) 25

HAM (ng/ml) 10

● Percentil > 75% (7.11), < 95% (11.65)

CFA 23

● Percentil > 75% (18), < 95% (26)

Calcular

Reserva Ovária

Idade (anos) 25

HAM (ng/ml) 5

● Percentil > 50% (4.753), < 75% (7.11)

CFA 23

● Percentil > 75% (18), < 95% (26)

Calcular

Reserva Ovária

Idade (anos) 25

HAM (ng/ml) 2

● Percentil > 5% (1.26), < 25% (2.97)

CFA 10

● Percentil > 25% (9), < 50% (13)

Calcular

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4.3 Guide for reproductive counselling

aTuaFertilidade

1. Verificação dos valores HAM e confirmação do percentil correspondente
 2. Ecografia CFA e confirmação do percentil correspondente
 3. Comunicação dos resultados
- a) **Ponto prévio:** esta consulta decorre no âmbito de um projeto de investigação. Como tal, trata-se de uma consulta direcionada e padronizada de acordo com os objetivos do estudo. Abordaremos alguns pontos que consideramos importantes para a compreensão dos resultados da avaliação da reserva ovária. Tentaremos também, dentro do possível responder às suas questões e ir ao encontro dos seus objetivos.
- b) **Comunicação dos valores de HAM/CFA:**
- O seu valor da HAM é ____ng/mL que corresponde, aproximadamente, ao percentil ____
 - A sua CFA é ____ que corresponde, que corresponde, aproximadamente, ao percentil ____
- c) **Interpretação dos resultados**

Percentil $\geq 25\%$

- ✓ De acordo com a literatura consultada considera-se **que tem uma reserva ovárica normal** para a sua idade. Ainda assim, há que considerar,
- ✓ A avaliação da reserva ovárica com recurso ao doseamento da HAM e CFA é sobretudo usada no contexto dos tratamentos de infertilidade. Como tal, os valores de HAM e CFA apresentam uma forte correlação com a resposta ao tratamento farmacológico (*valores de HAM e CFA mais altos $\Leftrightarrow$ maior número de ovócitos obtidos após estimulação ovárica*) **mas não necessariamente com a capacidade de engravidar**.

Isto é,

! existem múltiplos fatores que determinam a fertilidade feminina, a reserva ovárica é um dos parâmetros que se relaciona com o número de ovócitos disponíveis;

!! por si só, a avaliação da reserva ovárica, e os valores de HAM e CFA, não é/são suficiente/s para determinar a possibilidade de conseguir uma gravidez pois há outros fatores associados à infertilidade nomeadamente femininos (tubares, hormonais, uterinos) e masculinos. Pode não ser possível identificar nenhum fator, e considerar-se idiopático;
- ✓ Há que considerar que, sob o efeito de **contraceção oral**, os resultados da HAM e CFA poderão estar subvalorizados (segundo a literatura aproximadamente 20%). Caso pretenda, poderá reavaliar após suspensão de, pelo menos 3 meses, da toma da pílula.
- ✓ **Em suma**, para os valores de HAM e CFA que hoje apresenta, à partida, estará numa melhor situação para conseguir engravidar. **No entanto**, não conseguimos prever a sua situação futura, nem calcular como evoluirá a sua reserva ovárica ao longo do tempo. Sabemos que, naturalmente o número de óvulos que dispõe (reserva ovárica) diminui ao longo da vida até à menopausa. Começa a diminuir próximo dos 30 anos e, de forma mais acentuada, aos 35 anos.

Percentil < 25%

!! DOR se HAM < 0,85ng/mL e CFA < 5

- ✓ De acordo com a literatura consultada, considera-se que tem uma reserva ovária **abaixo do normal/diminuída para a sua idade**. Ainda assim, há que considerar,
- ✓ A avaliação da reserva ovária com recurso ao doseamento da HAM e CFA é sobretudo usada no contexto dos tratamentos de infertilidade. Como tal, os valores de HAM e CFA apresentam uma forte correlação com a resposta ao tratamento farmacológico (permitem prever nº ovócitos obtidos: valores de HAM e CFA mais altos ⇔ maior número de ovócitos obtidos após estimulação ovária) **mas não necessariamente com a capacidade de engravidar**.

Isto é,

! existem múltiplos fatores que determinam a fertilidade feminina, a reserva ovária é um dos parâmetros que se relaciona com o número de ovócitos disponíveis;

!! por si só, a avaliação da reserva ovária, e os valores de HAM e CFA, não é/são suficiente/s para determinar a possibilidade de conseguir uma gravidez pois há outros fatores associados à infertilidade nomeadamente femininos (tubares, hormonais, uterinos) e masculinos. Pode não ser possível identificar nenhum fator, e considerar-se idiopático;

!!! A HAM e CFA permitem identificar os casos em que há um decréscimo da reserva ovária (quer naturalmente pelo aumento da idade quer em mulheres jovens que têm reserva ovária diminuída) contudo, em populações não inférteis não há evidências da sua associação com a probabilidade de engravidar. Ou seja, **há mulheres que, com baixa reserva ovária, engravidam espontaneamente e sem dificuldade**.

- ✓ Para os valores de HAM e CFA que hoje apresenta, deve considerar que,
 - sob o efeito de **contraceção oral**, os resultados da HAM e CFA poderão estar subvalorizados (segundo a literatura aproximadamente 20%). Caso pretenda, poderá reavaliar após suspensão de, pelo menos 3 meses, da toma da pílula.
 - Esta foi uma avaliação isolada, não sabemos o padrão e tendência do decréscimo da sua reserva ovárica. Não conseguimos prever a sua situação futura, nem calcular como evoluirá a sua reserva ovárica ao longo do tempo. Sabemos que, naturalmente o número de óvulos que dispõe (reserva ovárica) diminui ao longo da vida até à menopausa. Começa a diminuir próximo dos 30 anos e, de forma mais acentuada, aos 35 anos.
 - A sua reserva ovárica está abaixo do que se considera normal para a sua idade o que poderá significar, à partida e eventualmente, uma maior dificuldade em engravidar.

- ✓ **Em suma**, face a estes dados e mediante os seus projetos de parentalidade, há diferentes opções,
 - 1) A primeira opção poderá ser uma abordagem **“wait and see”**, caso, neste momento, não pretenda tomar nenhuma atitude/constituir família;
 - 2) Como segunda opção poderá **antecipar o projeto de parentalidade**, se tentar engravidar for uma opção para si.

Há evidência que >88% das mulheres cujo diagnóstico de reserva ovárica diminuída foi feito antes dos 35 anos, engravidaram espontaneamente em 6 meses.

No entanto, e face a esta avaliação, ao fim de 6 meses a tentar engravidar sem sucesso, será aconselhável aprofundar o estudo da infertilidade.

Por último,

- ✓ Considerando que, naturalmente, o número de óvulos que dispõe (reserva ovárica) diminui ao longo da vida até à menopausa, se engravidar não estiver nos seus planos futuros, a criopreservação de ovócitos por motivos não médicos pode ser um assunto a discutir.

- A sua utilização, com a finalidade de obter uma gravidez, implica uma estimulação ovárica (hormonal) e, no futuro, a realização de técnicas de PMA;

- É muito importante estar bem informada antes de tomar a decisão criopreservar os seus ovócitos. O correto aconselhamento reprodutivo, baseado em dados concretos da reserva ovárica, bem como taxas de sucesso associadas à viabilidade ovocitária após criopreservação e descongelamento são indispensáveis e deve ser feito por um médico especialista em Medicina da Reprodução;

- A criopreservação de ovócitos é uma opção para preservar a fertilidade feminina. No entanto, atualmente, o Serviço Nacional de Saúde não oferece esta técnica por motivos não médicos.
No âmbito do aconselhamento reprodutivo, a atitude preferencial passa por antecipar a gravidez, face às evidências existentes.

***Mais perguntas fora deste âmbito

Opinião sobre criopreservação de ovócitos/taxas de sucesso das técnicas...

>>>>> A resposta a essas perguntas foge ao âmbito desta consulta (que é parte integrante de um projeto de investigação), não podendo, por isso, dar-lhe essa informação. Após esta consulta terá hipótese de preencher novo questionário que lhe permitirá assimilar e refletir sobre o que falámos. Se persistirem dúvidas sugiro-lhe que entre em contacto com a investigadora responsável pelo estudo para a tentarmos esclarecer, podendo ser agendada nova consulta.

4.4 The study video

<https://atuaafertilidade.pt/video/>

4.5 Questionnaires

Projeto de Investigação e Apoio aos Casos de Reserva Ovárica Diminuída

Avaliação do conhecimento sobre reserva ovárica diminuída e da sua importância na tomada de decisão sobre os seus projetos parentais futuros. Este estudo destina-se a mulheres que não tenham filhos, nem estejam a tentar engravidar com recurso a técnicas de Procriação Medicamente Assistida (PMA). A participação no estudo é voluntária e implica o preenchimento do presente questionário. O tempo previsto de preenchimento é de 15 minutos. Todos os dados recolhidos são confidenciais e serão analisados coletivamente, não havendo por isso lugar à identificação de nenhum dos participantes. É importante que responda da forma o mais honesta e sincera possível. Os dados serão apenas utilizados para fins de investigação e no âmbito do estudo.

Dados pessoais

1. Nome completo* _____
2. Data de nascimento* *Por favor, escreva a sua resposta utilizando apenas números* ____/____/____
3. E-mail* _____
4. Contacto telefónico* _____

5. Que etnia/raça melhor a descreve?* *Por favor, selecione apenas uma das seguintes opções:*

☐	Asiática
☐	Africana
☐	Hispânica
☐	Caucasiana (branca)
☐	Etnia múltipla
☐	Prefiro não responder

6. Tem alguma doença?* *Por favor, escreva todas as que se aplicam:* _____

7. Utiliza contraceptivo oral (pílula)?* *Por favor, selecione uma opção:*

☐	Sim
☐	Não

8. Se respondeu sim, desde há quanto tempo (em anos)?*

Por favor, escreva a sua resposta utilizando apenas números; se for há menos de 1 ano, coloque 0. _____

9. Habilitações literárias* *Por favor, selecione apenas uma das seguintes opções:*

☐	Ensino básico (9º ano escolaridade)
☐	12º ano de escolaridade
☐	Licenciatura
☐	Bacharelato
☐	Mestrado integrado
☐	Doutoramento

10. Profissão* *Por favor, escreva a sua resposta* _____

11. Situação profissional* *Por favor, escolha apenas uma das seguintes opções:*

☐	Empregada
☐	Desempregada
☐	Estudante
☐	Outro:

12. Estado civil* *Por favor selecione apenas uma das seguintes opções:*

☐	Solteira
☐	Casada/união de facto
☐	Divorciada
☐	Viúva

13. Se se encontra numa relação, desde há quanto tempo (em anos)?* *Por favor, escreva a sua resposta utilizando números; se for há menos de 1 ano, coloque 0.* _____

14. Se se encontra numa relação, qual a idade do seu companheiro(a)?* *Por favor, utilize apenas números.* _____

Ciclo menstrual

15. Com que idade menstruou pela primeira vez? * *Por favor, utilize apenas números.* _____

16. Idade a que a mãe "entrou" em menopausa * *Por favor selecione apenas uma das seguintes opções:*

☐	<40 anos
☐	40-45 anos
☐	>45 anos

17. Data da sua última menstruação * *Por favor indique no formato dia/mês/ano.* ____/____/____

18. Duração do ciclo menstrual * *Por favor selecione apenas uma das seguintes opções:*

☐	<25 dias
☐	25-35 dias
☐	> 35 dias

19. Dor abdominal e/ou pélvica intensa antes/durante a menstruação) numa escala de 1 (nada) a 5 (muito intensa)? * *Por favor selecione apenas uma das opções:*

	1	2	3	4	5	
Nada	☐	☐	☐	☐	☐	Muito intensa

20. Alguma vez foi operada ao ovário? * *Por favor selecione uma opção:*

☐	Sim
☐	Não

21. Se respondeu sim, porquê? *Por favor, escreva a sua resposta* _____

Intenções de parentalidade

22. Já tentou engravidar? * *Por favor selecione uma opção:*

☐	Sim
☐	Não

23. Em relação às seguintes áreas quão definidos estão os seus planos? *Por favor selecione a opção que mais se adequa às seguintes afirmações, de 1(Nada definidos) a 5 (Completamente definidos) **

Marcar apenas uma opção por linha.

	1 Nada definidos	2	3	4	5 Completamente definidos
Carreira (planeou a sua vida profissional)	☐	☐	☐	☐	☐
Educação (planeou a sua formação académica)	☐	☐	☐	☐	☐
Parentalidade (planeou o momento em que quer ter filhos)	☐	☐	☐	☐	☐
Viver fora do país (planeou quando ir para o estrangeiro em trabalho ou lazer)	☐	☐	☐	☐	☐
Comprar/arrendar casa (planeou a altura da vida em que terá a sua casa)	☐	☐	☐	☐	☐
Casar (planeou a idade com que pretende casar)	☐	☐	☐	☐	☐

24. Em relação a ter filhos no futuro * *Por favor selecione apenas uma das seguintes opções:*

☐	Tenho a certeza que quero ter filhos
☐	Ainda tenho dúvidas, mas muito provavelmente decidirei ter filhos
☐	Neste momento tenho a certeza que não quero ter filhos
☐	Ainda tenho dúvidas, mas muito provavelmente decidirei não ter filhos

25. Quão importante é, para si, ter filhos, numa escala de 1(nada) a 5(muitíssimo)? * *Por favor selecione apenas uma das opções:*

	1	2	3	4	5	
Nada importante	☐	☐	☐	☐	☐	Muitíssimo importante

26. Quantos filhos gostaria (idealmente) de ter? * *Por favor, selecione apenas uma das seguintes opções:*

☐	Nenhum
☐	1
☐	2
☐	3 ou mais
☐	não sei

2

*Obrigatório

27. Número de filhos que considera possível ter, atendendo à situação económica, idade, antecedentes pessoais, etc.*

Por favor, selecione apenas uma das seguintes opções:

☐	Nenhum
☐	1
☐	2
☐	3 ou mais
☐	não sei

28. Qual a idade que considera ideal para ter o primeiro filho?* Por favor, utilize apenas números. _____

29. Qual a idade em planeia ter o seu primeiro filho?* Por favor, utilize apenas números. _____

30. Considera que irá conseguir ter um filho no momento que planeia?* Por favor, selecione uma opção:

☐	Sim
☐	Não

31. Assinale o quão confiante se sente, numa escala de 1 (nada confiante) a 5 (totalmente confiante), em relação a conseguir ter filhos quando o pretender.* Por favor, selecione apenas uma opção:

	1	2	3	4	5	
Nada confiante						Totalmente confiante

32. Para si, quais são os principais obstáculos que a poderão impedir de ter filhos na idade que pretende?* Por favor, selecione todas as opções que se apliquem:

☐	Nenhum
☐	Infertilidade (Reserva Ovárica Diminuída)
☐	Ausência de parceiro estável
☐	Prosseguimento de estudos
☐	Progressão na carreira
☐	Instabilidade financeira
☐	Não estar preparada para ser mãe

Hábitos e estilo de vida

33. É fumadora?* Por favor, selecione uma opção:

☐	Sim
☐	Não

34. Se respondeu sim, quantos cigarros, em média, fuma por dia?* Por favor, utilize apenas números. _____

35. Pratica exercício físico?* Por favor, selecione apenas uma das seguintes opções:

☐	Não
☐	1-3 vezes/semana
☐	4-6 vezes/semana
☐	Competição

36. Qual é o seu peso habitual (em quilogramas)?* Por favor, utilize apenas números. _____

37. Qual é a sua altura (em centímetros)?* Por favor, utilize apenas números. _____

Conhecimento sobre Potencial Reprodutivo Feminino

38. Já ouviu falar sobre reserva ovárica ou seja, a quantidade de óvulos que cada mulher tem?* Por favor, selecione uma opção:

☐	Sim
☐	Não

39. Como classifica o seu conhecimento sobre reserva ovárica, numa escala de 1 (nenhum conhecimento) até 5 (elevado conhecimento)?* Por favor, selecione apenas uma opção:

	1	2	3	4	5	
Nenhum conhecimento						Elevado conhecimento

25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45

25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45

	Sim
	Não

	Não
	Não, apenas mulheres com condições genéticas graves
	Sim, <5%
	Sim, cerca de 10%
	Sim, cerca de 25%

	Verdadeira	Falsa
Uma mulher dispõe de aproximadamente 500 óvulos ao longo da sua vida		
A reserva ovárica só diminui com a idade		
A fertilidade feminina começa a diminuir aos 40 anos		
A reserva ovárica relaciona-se com a fertilidade feminina e pode ser avaliada através de uma colheita de sangue		

Marcar apenas uma opção por linha.

	1 Totalmente improvável	2	3	4	5 Totalmente provável
Desistir de ter filhos					
Antecipar a idade de ter um filho					
Congelar os meus óvulos para usar no futuro, quando quisesse ter filhos					
Quando quisesse ter filhos, procuraria tratamentos de fertilidade					
Quando quisesse ter filhos, tentaria adotar uma criança					
Consideraria recorrer a uma doação de óvulos					

	Sim
	Não
	Talvez, mas necessitaria de mais informação sobre o assunto

47. Como se sentiria face à possibilidade de ter que recorrer a gâmetas de dadora?*

Por favor, indique o quão verdadeiras são para si as seguintes afirmações, de 1 (Nada verdadeiro) a 5 (Totalmente verdadeiro)

Marcar apenas uma opção por linha.

	1 Nada verdadeiro	2	3	4	5 Totalmente verdadeiro
Feliz por concretizar o meu sonho de ser mãe					
Feliz por cuidar de uma criança desde o seu nascimento					
Feliz pelo facto de poder transmitir os meus valores familiares					
Receosa por poder vir a sentir que o filho não é realmente meu					
Apreensiva sobre eventuais problemas de saúde hereditários					

48. Se tivesse um diagnóstico de reserva ovárica diminuída, consideraria submeter-se a tratamentos de fertilidade para “congelar” óvulos, como forma de adiar a gravidez/preservar a sua fertilidade? Valor estimado para a congelação: 3000 euros *

Por favor, selecione uma das seguintes opções:

☐	Sim, muito provavelmente
☐	Sim, mas não possuo os recursos económicos necessários
☐	Não, embora esteja informada sobre o efeito da idade da mulher na sua fertilidade
☐	Talvez, mas não estou suficientemente informada sobre o assunto
☐	Não, preferia repensar a idade a que quero engravidar

49. Considera que a preservação da fertilidade por motivos sociais (“congelamento” de óvulos) deveria estar disponível para todas as mulheres?*

Por favor, selecione apenas uma das seguintes opções:

☐	Sim, para todas
☐	Não, para ninguém
☐	Não sei/não estou informada
☐	Sim, para casos específicos

50. Se respondeu “sim, para casos específicos”, por favor, diga quais _____

51. Se não conseguisse engravidar e tivesse reserva ovárica diminuída consideraria submeter-se a um tratamento inovador (pex.. terapia com células do sangue ou células estaminais)?* Por favor, selecione apenas uma das seguintes opções:

☐	Sim
☐	Não
☐	Não sei/não estou informada

52. Considera que todas as mulheres deveriam ter acesso a uma forma fácil e barata de avaliar a reserva ovárica?*

Por favor, selecione apenas uma das seguintes opções:

☐	Sim
☐	Não
☐	Não sei/não estou informada

Considerações Finais

53. Quão importante é, para si, estar informada sobre a reserva ovárica, numa escala de (nada importante) a 5 (muitíssimo importante)?* Por favor, selecione apenas uma opção:

	1	2	3	4	5
Nada importante					
Muitíssimo importante					

54. Quão importante é, para si, avaliar a sua reserva ovárica, numa escala de 1(nada importante) a 5 (muitíssimo importante)?* Por favor, selecione apenas uma opção:

	1	2	3	4	5
Nada importante					
Muitíssimo importante					

55. A sua participação é essencial para o desenvolvimento do estudo. A participação neste projeto depende da avaliação do seu conhecimento em diferentes momentos. Aceita a utilização dos seus dados pessoais para a contactar e, assim, participar na segunda fase do estudo?*

Por favor, selecione uma opção:

☐	Sim
☐	Não

56. Data ao preenchimento: __/__/__

QUESTIONÁRIO DE AUTO-AVALIAÇÃO
de Charles D. Spielberger
STAI Forma Y – 1, Versão Portuguesa de Danilo R. Silva

E _____ T _____

INSTRUÇÕES: Em baixo encontra uma série de frases que as pessoas costumam usar para se descreverem a si próprias. Leia cada uma delas e faça uma cruz (x) no número da direita que indique como se **sente agora**, isto é, neste preciso momento. Não há respostas certas nem erradas. Não leve muito tempo com cada frase, mas dê a resposta que melhor lhe parece descrever os seus sentimentos neste momento.

1 - Nada 2 – Um pouco 3 - Moderadamente 4 – Muito

	1	2	3	4
1. Sinto-me calmo				
2. Sinto-me seguro				
3. Sinto-me tenso				
4. Sinto-me esgotado				
5. Sinto-me à vontade				
6. Sinto-me perturbado				
7. Presentemente, ando preocupado com desgraças que podem vir a acontecer				
8. Sinto-me satisfeito				
9. Sinto-me assustado				
10. Estou descansado				
11. Sinto-me confiante				
12. Sinto-me nervoso				
13. Estou inquieto				
14. Sinto-me indeciso				
15. Estou descontraindo				
16. Sinto-me contente				
17. Estou preocupado				
18. Sinto-me confuso				
19. Sinto-me uma pessoa estável				
20. Sinto-me bem				

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STAI Forma Y – 2

INSTRUÇÕES: Em baixo encontra uma série de frases que as pessoas costumam usar para se descreverem a si próprias. Leia cada uma delas e faça uma cruz (x) no número da direita que indique como se **sente em geral**. Não há respostas certas nem erradas. Não leve muito tempo com cada frase, mas dê a resposta que lhe parece descrever como se sente **geralmente**.

1 – Quase nunca 2 – Algumas vezes 3 - Frequentemente 4 – Quase sempre

	1	2	3	4
21. Sinto-me bem				
22. Sinto-me nervoso e inquieto				
23. Sinto-me satisfeito comigo próprio				
24. Quem me dera ser tão feliz como os outros parecem sê-lo				
25. Sinto-me um falhado				
26. Sinto-me tranquilo				
27. Sou calmo, ponderado e senhor de mim mesmo				
28. Sinto que as dificuldades estão a acumular-se de tal forma que as não consigo resolver				
29. Preocupo-me demais com coisas que na realidade não têm importância				
30. Sou feliz				
31. Tenho pensamentos que me perturbam				
32. Não tenho muita confiança em mim				
33. Sinto-me seguro				
34. Tomo decisões com facilidade				
35. Muitas vezes sinto que não sou capaz				
36. Estou contente				
37. Às vezes, passam-me pela cabeça pensamentos sem importância que me aborrecem				
38. Tomo os desapontamentos tão a sério que não consigo afastá-los do pensamento				
39. Sou uma pessoa estável				
40. Fico tenso ou desorientado quando penso nas minhas preocupações e interesses mais recentes				

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Dados pessoais

- ### Intenções de parentalidade

- ### Conhecimento sobre Potencial Reprodutivo Feminino

9. Tem conhecimento sobre a existência de um teste para avaliar a reserva ovária? * *Por favor, selecione uma opção:*

☐	Sim
☐	Não

10. Mulheres jovens podem ter reserva ovária diminuída? * *Por favor, selecione apenas uma das seguintes opções:*

☐	Não
☐	Não, apenas mulheres com condições genéticas graves
☐	Sim, <5%
☐	Sim, cerca de 10%
☐	Sim, cerca de 25%

11. Em relação às afirmações que se seguem, deverá indicar se é verdadeira (V) ou falsa (F) *. *Por favor selecione apenas uma opção por linha.*

	Verdadeira	Falsa
Uma mulher dispõe de aproximadamente 500 óvulos ao longo da sua vida	☐	☐
A reserva ovária só diminui com a idade	☐	☐
A fertilidade feminina começa a diminuir aos 40 anos	☐	☐
A reserva ovária relaciona-se com a fertilidade feminina e pode ser avaliada através de uma colheita de sangue	☐	☐

Atitudes em relação a tratamentos de fertilidade, doação de gâmetas (óvulos), congelação de óvulos e a outras formas de parentalidade

12. Se soubesse antecipadamente que iria ter reserva ovária diminuída. *Por favor selecione a opção que mais se adequa às seguintes afirmações, de 1(Totalmente improvável) a 5 (Totalmente provável)**

Marcar apenas uma opção por linha.

	1 Totalmente improvável	2	3	4	5 Totalmente provável
Desistir de ter filhos	☐	☐	☐	☐	☐
Antecipar a idade de ter um filho	☐	☐	☐	☐	☐
Congelar os meus óvulos para usar no futuro, quando quisesse ter filhos	☐	☐	☐	☐	☐
Quando quisesse ter filhos, procuraria tratamentos de fertilidade	☐	☐	☐	☐	☐
Quando quisesse ter filhos, tentaria adotar uma criança	☐	☐	☐	☐	☐
Consideraria recorrer a uma doação de óvulos	☐	☐	☐	☐	☐

13. Consideraria fazer uma doação benévola de gâmetas (óvulos) se tal ato ajudasse mulheres com reserva ovária diminuída? *

Por favor, selecione uma opção:

☐	Sim
☐	Não
☐	Talvez, mas necessitaria de mais informação sobre o assunto

14. Como se sentiria face à possibilidade de ter que recorrer a gâmetas de dadora? *

Por favor, indique o quão verdadeiras são para si as seguintes afirmações, de 1 (Nada verdadeiro) a 5 (Totalmente verdadeiro)

Marcar apenas uma opção por linha.

	1 Nada verdadeiro	2	3	4	5 Totalmente verdadeiro
Feliz por concretizar o meu sonho de ser mãe	☐	☐	☐	☐	☐
Feliz por cuidar de uma criança desde o seu nascimento	☐	☐	☐	☐	☐
Feliz pelo facto de poder transmitir os meus valores familiares	☐	☐	☐	☐	☐
Receosa por poder vir a sentir que o filho não é realmente meu	☐	☐	☐	☐	☐
Aprensiva sobre eventuais problemas de saúde hereditários	☐	☐	☐	☐	☐

15. Se tivesse um diagnóstico de reserva ovárica diminuída, consideraria submeter-se a tratamentos de fertilidade para “congelar” óvulos, como forma de adiar a gravidez/preservar a sua fertilidade? Valor estimado para a congelação: 3000 euros * Por favor, selecione uma das seguintes opções:

☐	Sim, muito provavelmente
☐	Sim, mas não possuo os recursos económicos necessários
☐	Não, embora esteja informada sobre o efeito da idade da mulher na sua fertilidade
☐	Talvez, mas não estou suficientemente informada sobre o assunto
☐	Não, preferia repensar a idade a que quero engravidar

16. Considera que a preservação da fertilidade por motivos sociais (“congelação” de óvulos) deveria estar disponível para todas as mulheres? * Por favor, selecione apenas uma das seguintes opções:

☐	Sim, para todas
☐	Não, para ninguém
☐	Não sei/não estou informada
☐	Sim, para casos específicos

17. Se respondeu “sim, para casos específicos”, por favor, diga quais _____

18. Se não conseguisse engravidar e tivesse reserva ovárica diminuída consideraria submeter-se a um tratamento inovador (pex.. terapia com células do sangue ou células estaminais)? * Por favor, selecione apenas uma das seguintes opções:

☐	Sim
☐	Não
☐	Não sei/não estou informada

19. Considera que todas as mulheres deveriam ter acesso a uma forma fácil e barata de avaliar a reserva ovárica? * Por favor, selecione apenas uma das seguintes opções:

☐	Sim
☐	Não
☐	Não sei/não estou informada

Considerações Finais

20. Quão importante é, para si, estar informada sobre a reserva ovárica, numa escala de 1 (nada) a 5 (muitíssimo)? * Por favor, selecione apenas uma opção:

	1	2	3	4	5	
Nada importante						Muitíssimo importante

21. Quão importante é, para si, avaliar a sua reserva ovárica, numa escala de 1(nada) a 5 (muitíssimo)? * Por favor, selecione apenas uma opção:

	1	2	3	4	5	
Nada importante						Muitíssimo importante

22. A sua participação é essencial para o desenvolvimento do estudo. A participação neste projeto depende da avaliação do seu conhecimento em diferentes momentos. Aceita a utilização dos seus dados pessoais para a contactar e, assim, participar na próxima fase do estudo? * Por favor, selecione uma opção:

☐	Sim
☐	Não

23. Data ao preenchimento: ____/____/____

QUESTIONÁRIO DE AUTO-AVALIAÇÃO
de Charles D. Spielberger
STAI Forma Y – 1, Versão Portuguesa de Danilo R. Silva

E _____ T _____

INSTRUÇÕES: Em baixo encontra uma série de frases que as pessoas costumam usar para se descreverem a si próprias. Leia cada uma delas e faça uma cruz (x) no número da direita que indique como se **sente agora**, isto é, neste preciso momento. Não há respostas certas nem erradas. Não leve muito tempo com cada frase, mas dê a resposta que melhor lhe parece descrever os seus sentimentos neste momento.

1 - Nada 2 – Um pouco 3 - Moderadamente 4 – Muito

	1	2	3	4
1. Sinto-me calmo				
2. Sinto-me seguro				
3. Sinto-me tenso				
4. Sinto-me esgotado				
5. Sinto-me à vontade				
6. Sinto-me perturbado				
7. Presentemente, ando preocupado com desgraças que podem vir a acontecer				
8. Sinto-me satisfeito				
9. Sinto-me assustado				
10. Estou descansado				
11. Sinto-me confiante				
12. Sinto-me nervoso				
13. Estou inquieto				
14. Sinto-me indeciso				
15. Estou descontraindo				
16. Sinto-me contente				
17. Estou preocupado				
18. Sinto-me confuso				
19. Sinto-me uma pessoa estável				
20. Sinto-me bem				

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1 – Quase nunca 2 – Algumas vezes 3 - Frequentemente 4 – Quase sempre

	1	2	3	4
21. Sinto-me bem				
22. Sinto-me nervoso e inquieto				
23. Sinto-me satisfeito comigo próprio				
24. Quem me dera ser tão feliz como os outros parecem sê-lo				
25. Sinto-me um falhado				
26. Sinto-me tranquilo				
27. Sou calmo, ponderado e senhor de mim mesmo				
28. Sinto que as dificuldades estão a acumular-se de tal forma que as não consigo resolver				
29. Preocupo-me demais com coisas que na realidade não têm importância				
30. Sou feliz				
31. Tenho pensamentos que me perturbam				
32. Não tenho muita confiança em mim				
33. Sinto-me seguro				
34. Tomo decisões com facilidade				
35. Muitas vezes sinto que não sou capaz				
36. Estou contente				
37. Às vezes, passam-me pela cabeça pensamentos sem importância que me aborrecem				
38. Tomo os desapontamentos tão a sério que não consigo afastá-los do pensamento				
39. Sou uma pessoa estável				
40. Fico tenso ou desorientado quando penso nas minhas preocupações e interesses mais recentes				

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Projeto de Investigação e Apoio aos Casos de Reserva Ovárica Diminuída

parentais futuros. Este estudo destina-se a mulheres que não tenham filhos, nem estejam a tentar engravidar com recurso a técnicas de Procriação Medicamente Assistida (PMA). A participação no estudo é voluntária e implica o preenchimento do presente questionário. O tempo previsto de preenchimento é de 10 minutos. Todos os dados recolhidos são confidenciais e serão analisados coletivamente, não havendo por isso lugar à identificação de nenhum dos participantes. É importante que responda da forma o mais honesta e sincera possível. Os dados serão apenas utilizados para fins de investigação e no âmbito do estudo.

Dados pessoais

1. Nome completo* _____
2. Data de nascimento* *Por favor, escreva a sua resposta utilizando apenas números* ____/____/____

Intenções de parentalidade

1. Quantos filhos gostaria (idealmente) de ter? * Por favor, selecione apenas uma das seguintes opções:

	Nenhum
	1
	2
	3 ou mais
	não sei

2. Número de filhos que considera possível ter, atendendo à situação económica, idade, antecedentes pessoais, etc. * *Por favor, seleccione apenas uma das seguintes opções:*

	Nenhum
	1
	2
	3 ou mais
	não sei

3. Qual a idade que considera ideal para ter o primeiro filho? * *Por favor, utilize apenas números.*

4. Qual a idade em que planeia ter o seu primeiro filho? * *Por favor, utilize apenas números.*

	Sim
	Não

6. Assinale o quão confiante se sente, numa escala de 1 (nada confiante) a 5 (totalmente confiante), em relação a conseguir ter filhos quando o pretender. * Por favor, selecione apenas uma opção:

	1	2	3	4	5	
Nada confiante						Totalmente confiante

7. Para si, quais são os principais obstáculos que a poderão impedir de ter filhos na idade que pretende?*
- Por favor, selecione todas as opções que se apliquem:*

	Nenhum
	Infertilidade (Reserva Ovária Diminuída)
	Ausência de parceiro estável
	Prosseguimento de estudos
	Progressão na carreira
	Instabilidade financeira
	Não estar preparada para ser mãe

Conhecimento sobre Potencial Reprodutivo Feminino

8. Sabe a partir de que idade a fertilidade diminui? * Por favor, marque com um (X) a idade que considera correta:

25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45

9. Tem conhecimento sobre a existência de um teste para avaliar a reserva ovárica? * *Por favor, selecione uma opção:*

☐	Sim
☐	Não

10. Mulheres jovens podem ter reserva ovárica diminuída? * *Por favor, selecione apenas uma das seguintes opções:*

☐	Não
☐	Não, apenas mulheres com condições genéticas graves
☐	Sim, <5%
☐	Sim, cerca de 10%
☐	Sim, cerca de 25%

11. Em relação às afirmações que se seguem, deverá indicar se é verdadeira (V) ou falsa (F) *. *Por favor selecione apenas uma opção por linha.*

	Verdadeira	Falsa
Uma mulher dispõe de aproximadamente 500 óvulos ao longo da sua vida	☐	☐
A reserva ovárica só diminui com a idade	☐	☐
A fertilidade feminina começa a diminuir aos 40 anos	☐	☐
A reserva ovárica relaciona-se com a fertilidade feminina e pode ser avaliada através de uma colheita de sangue	☐	☐

12. Se soubesse antecipadamente que iria ter reserva ovárica diminuída. *Por favor selecione a opção que mais se adequa às seguintes afirmações, de 1 (Totalmente improvável) a 5 (Totalmente provável) **

Marcar apenas uma opção por linha.

	1 Totalmente improvável	2	3	4	5 Totalmente provável
Desistir de ter filhos	☐	☐	☐	☐	☐
Antecipar a idade de ter um filho	☐	☐	☐	☐	☐
Congelar os meus óvulos para usar no futuro, quando quisesse ter filhos	☐	☐	☐	☐	☐
Quando quisesse ter filhos, procuraria tratamentos de fertilidade	☐	☐	☐	☐	☐
Quando quisesse ter filhos, tentaria adotar uma criança	☐	☐	☐	☐	☐
Consideraria recorrer a uma doação de óvulos	☐	☐	☐	☐	☐

13. Consideraria fazer uma doação benévola de gâmetas (óvulos) se tal ato ajudasse mulheres com reserva ovárica diminuída? *

Por favor, selecione uma opção:

☐	Sim
☐	Não
☐	Talvez, mas necessitaria de mais informação sobre o assunto

14. Como se sentiria face à possibilidade de ter que recorrer a gâmetas de dadora? * *Por favor, indique o quão verdadeiras são para si as seguintes afirmações, de 1 (Nada verdadeiro) a 5 (Totalmente verdadeiro)*

Marcar apenas uma opção por linha.

	1 Nada verdadeiro	2	3	4	5 Totalmente verdadeiro
Feliz por concretizar o meu sonho de ser mãe	☐	☐	☐	☐	☐
Feliz por cuidar de uma criança desde o seu nascimento	☐	☐	☐	☐	☐
Feliz pelo facto de poder transmitir os meus valores familiares	☐	☐	☐	☐	☐
Receosa por poder vir a sentir que o filho não é realmente meu	☐	☐	☐	☐	☐
Apreensiva sobre eventuais problemas de saúde hereditários	☐	☐	☐	☐	☐

15. Se tivesse um diagnóstico de reserva ovárica diminuída, consideraria submeter-se a tratamentos de fertilidade para “congelar” óvulos, como forma de adiar a gravidez/preservar a sua fertilidade? *Valor estimado para a congelação: 3000 euros* * *Por favor, selecione uma das seguintes opções:*

☐	Sim, muito provavelmente
☐	Sim, mas não possuo os recursos económicos necessários
☐	Não, embora esteja informada sobre o efeito da idade da mulher na sua fertilidade
☐	Talvez, mas não estou suficientemente informada sobre o assunto
☐	Não, preferia repensar a idade a que quero engravidar

2

*Obrigatório

16. Considera que a preservação da fertilidade por motivos sociais (“congelamento” de óvulos) deveria estar disponível para todas as mulheres? * *Por favor, seleccione apenas uma das seguintes opções:*

☐	Sim, para todas
☐	Não, para ninguém
☐	Não sei/não estou informada
☐	Sim, para casos específicos

17. Se respondeu “sim, para casos específicos”, por favor, diga quais _____

18. Se não conseguisse engravidar e tivesse reserva ovárica diminuída consideraria submeter-se a um tratamento inovador (pex.. terapia com células do sangue ou células estaminais)? * *Por favor, seleccione apenas uma das seguintes opções:*

☐	Sim
☐	Não
☐	Não sei/não estou informada

19. Considera que todas as mulheres deveriam ter acesso a uma forma fácil e barata de avaliar a reserva ovárica? * *Por favor, seleccione apenas uma das seguintes opções:*

☐	Sim
☐	Não
☐	Não sei/não estou informada

Considerações Finais

20. Quão importante é, para si, estar informada sobre a reserva ovárica, numa escala de 1 (nada importante) a 5 (muitíssimo importante)? * *Por favor, seleccione apenas uma opção:*

	1	2	3	4	5	
Nada importante	☐	☐	☐	☐	☐	Muitíssimo importante

21. Considera que ter recebido informação e ter tido consulta de aconselhamento reprodutivo foi importante para si? * *Por favor, classifique de 1 (nada importante) a 5 (muito importante):*

	1	2	3	4	5	
Nada importante	☐	☐	☐	☐	☐	Muito importante

22. Considera que a deveria ter recebido informação sobre a reserva ovárica mais cedo? * *Por favor, seleccione apenas uma opção:*

☐	Sim
☐	Não

23. Se respondeu “sim”, por favor, diga, utilizando números, como que idade _____

24. O que a motivou a avaliar a reserva ovárica? * *Por favor, seleccione as opções mais adequadas para si:*

☐	Procura de informação sobre o tema
☐	Confirmação do adiamento da maternidade
☐	Preocupação com a minha fertilidade
☐	Procura de ajuda na tomada de decisão sobre a maternidade

25. O que a motivou a avaliar a reserva ovárica? * *Por favor, indique o quão verdadeiras são para si as seguintes afirmações, de 1 (Nada verdadeiro) a 5 (Totalmente verdadeiro)*

Marcar apenas uma opção por linha

	1 Nada verdadeiro	2	3	4	5 Totalmente verdadeiro
Procura de informação sobre o tema	☐	☐	☐	☐	☐
Confirmação do adiamento da maternidade	☐	☐	☐	☐	☐
Preocupação com a minha fertilidade	☐	☐	☐	☐	☐
Procura de ajuda na tomada de decisão sobre a maternidade	☐	☐	☐	☐	☐

26.Como descreve a experiência após a avaliação da fertilidade e aconselhamento reprodutivo? * *Por favor, indique o quão verdadeiras são para si as seguintes afirmações, de 1 (Nada verdadeiro) a 5 (Totalmente verdadeiro)*

	1 Nada verdadeiro	2	3	4	5 Totalmente verdadeiro
Fiquei a saber mais sobre a minha fertilidade					
Mantive os meus planos de maternidade					
Foi a motivação que precisava para tomar uma decisão sobre a maternidade					
Procurava algo mais concreto					
Fiquei com dúvidas					
Preciso de mais informação e/ou discutir as opções que disponho					

27.Como interpreta a informação que recebeu? * *Por favor, selecione as opções mais adequadas para si.*

☐	Parece-me estar tudo bem em relação à minha fertilidade
☐	Retive que é seguro adiar a maternidade
☐	Preciso de fazer algo a curto/médio prazo
☐	Poderei demorar mais tempo até conseguir engravidar
☐	Foi escassa, preciso de mais informação

28. Data ao preenchimento: ____/____/____

QUESTIONÁRIO DE AUTO-AVALIAÇÃO
de Charles D. Spielberger
STAI Forma Y – 1, Versão Portuguesa de Danilo R. Silva

E _____ T _____

INSTRUÇÕES: Em baixo encontra uma série de frases que as pessoas costumam usar para se descreverem a si próprias. Leia cada uma delas e faça uma cruz (x) no número da direita que indique como se **sente agora**, isto é, neste preciso momento. Não há respostas certas nem erradas. Não leve muito tempo com cada frase, mas dê a resposta que melhor lhe parece descrever os seus sentimentos neste momento.

1 - Nada 2 – Um pouco 3 - Moderadamente 4 – Muito

	1	2	3	4
1. Sinto-me calmo				
2. Sinto-me seguro				
3. Sinto-me tenso				
4. Sinto-me esgotado				
5. Sinto-me à vontade				
6. Sinto-me perturbado				
7. Presentemente, ando preocupado com desgraças que podem vir a acontecer				
8. Sinto-me satisfeito				
9. Sinto-me assustado				
10. Estou descansado				
11. Sinto-me confiante				
12. Sinto-me nervoso				
13. Estou inquieto				
14. Sinto-me indeciso				
15. Estou descontraindo				
16. Sinto-me contente				
17. Estou preocupado				
18. Sinto-me confuso				
19. Sinto-me uma pessoa estável				
20. Sinto-me bem				

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STAI Forma Y – 2

INSTRUÇÕES: Em baixo encontra uma série de frases que as pessoas costumam usar para se descreverem a si próprias. Leia cada uma delas e faça uma cruz (x) no número da direita que indique como se **sente em geral**. Não há respostas certas nem erradas. Não leve muito tempo com cada frase, mas dê a resposta que lhe parece descrever como se sente **geralmente**.

1 – Quase nunca 2 – Algumas vezes 3 - Frequentemente 4 – Quase sempre

	1	2	3	4
21. Sinto-me bem				
22. Sinto-me nervoso e inquieto				
23. Sinto-me satisfeito comigo próprio				
24. Quem me dera ser tão feliz como os outros parecem sê-lo				
25. Sinto-me um falhado				
26. Sinto-me tranquilo				
27. Sou calmo, ponderado e senhor de mim mesmo				
28. Sinto que as dificuldades estão a acumular-se de tal forma que as não consigo resolver				
29. Preocupo-me demais com coisas que na realidade não têm importância				
30. Sou feliz				
31. Tenho pensamentos que me perturbam				
32. Não tenho muita confiança em mim				
33. Sinto-me seguro				
34. Tomo decisões com facilidade				
35. Muitas vezes sinto que não sou capaz				
36. Estou contente				
37. Às vezes, passam-me pela cabeça pensamentos sem importância que me aborrecem				
38. Tomo os desapontamentos tão a sério que não consigo afastá-los do pensamento				
39. Sou uma pessoa estável				
40. Fico tenso ou desorientado quando penso nas minhas preocupações e interesses mais recentes				

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