# Assessment of Endometrial Receptivity in Assisted Reproductive Cycles
## The Value of Biophysical Markers

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Thesis submitted for the degree of Doctor of Philosophy in
**Medicine**

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# Avaliação da Receptividade Endometrial em ciclos de Procriação Medicamente Assistida
## O Valor dos Marcadores Biofísicos

**Renato Alessandre Silva Martins**

Tese para obtenção do Grau de Doutor em
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(3o ciclo de estudos)

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To my Grandparents, who have always inspired me and taught me how to guide by intuition, wisdom, and the value of books.

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To all the people that helped me in this journey.

# Publications

**The investigation done for this Thesis lead to the publication of the following papers in indexed and peer-reviewed journals:**

<u>Silva Martins R</u>, Helio Oliani A, Vaz Oliani D, Martinez de Oliveira J. Subendometrial resistance and pulsatility index assessment of endometrial receptivity in assisted reproductive technology cycles. Reprod Biol Endocrinol. 2019 Aug 2;17(1):62. doi: 10.1186/s12958-019-0507-6.
PMID: 31375113. (Appendix 1)

<u>Silva Martins R</u>, Helio Oliani A, Vaz Oliani D, Martinez de Oliveira J. Continuous endometrial volumetric analysis for endometrial receptivity assessment on assisted reproductive technology cycles. BMC Pregnancy Childbirth. 2020 Nov 3;20(1):663. doi: 10.1186/s12884-020-03372-2.
PMID: 33143675. (Appendix 2)

<u>Silva Martins R</u>, Helio Oliani A, Vaz Oliani D, Martinez de Oliveira J. The predictive value of serial serum estradiol and serial endometrial volume on endometrial receptivity on assisted reproductive technology cycles. BMC Pregnancy Childbirth. 2021 Mar 5;21(1):184. doi: 10.1186/s12884-021-03672-1.
PMID: 33673820. (Appendix 3)

# Presentations

**The investigation done for this Thesis lead to the following poster presentations made at scientific events:**

Silva Martins R, Helio Oliani A, Martinez de Oliveira J, Vaz Oliani D. Serial endometrial volume assessment as a predictor of endometrial receptivity in assisted reproductive technology cycles. Hum Reprod. Abstract Book of 35th Annual Meeting European Society of Human reproduction and Endocrinology. Vol34, Supl.1 2019. Presented at 35th Annual Meeting of European Society of Human Reproduction and Endocrinology – Vienna, Austria – 23 to 26 June 2019. (Appendix 4)

Silva Martins R, Helio Oliani A, Vaz Oliani D, Martinez de Oliveira J. Endometrial Volume and Adjusted Endometrial Volume assessment of Endometrial Receptivity in Assisted Reproductive Technology Cycles. Presented at 27th World Congress on Controversies in Obstetrics, Gynecology and Infertility – Paris, France – 21 to 23 November 2019. (Appendix 5)

**The investigation done for this Thesis lead to the following oral presentation made at scientific events:**

Silva Martins R, Helio Oliani A, Vaz Oliani D, Martinez de Oliveira J. Biophysical Ultrasonographic Markers Assessment of Endometrial Receptivity in Assisted Reproductive Technology Cycles. Presented at 27th World Congress on Controversies in Obstetrics, Gynecology and Infertility – Paris, France – 21 to 23 November 2019. (Appendix 6)

**The investigation done for this Thesis lead to the following Conference made at scientific events:**

Silva Martins R. (invited Speaker) Subendometrial Resistance and Pulsatility Index Assessment of Endometrial Receptivity in Assisted Reproductive Technology Cycles. Presented at Gynecology 2020 – European Gynecology and Obstetrics Congress – Paris, France – 17 and 18 February 2020. (Appendix 7)

# Prizes

## During this investigation received the following prize:

**<u>"Clinical Research Prize SPMR 2020"</u>** for the best clinical investigation with the manuscript entitled "Characterization of the infertile couple and first IVF's results in public fertility centers of Portuguese Northern and Central regions - a five-year cohort study", as part of a multicentric collaborative group, signed by the Portuguese Society of Reproductive Medicine – Sociedade Portuguesa de Medicina da Reprodução (SPMR), 2020 (Appendix 8).

# Abstract

**Objective:** Human implantation is a complex process requiring synchrony between a healthy embryo and a functionally competent or receptive endometrium. We evaluated the potential role of serial biophysical and biochemical parameters to predict endometrial receptivity in Assisted Reproductive Technology (ART) cycles.

**Design:** Endometrial receptivity assessment protocol to predict clinical pregnancy with serial sequential biochemical (serum estradiol) and biophysical (endometrial volume and adjusted endometrial volume, uterine artery fluxometry and fluxometry of subendometrial flow) parameters was designed. All parameters were compared between negative and positive outcome for clinical pregnancy. Serial serum estradiol and 2D and 3D-transvaginal ultrasound and Doppler fluxometry were performed in a prospective cohort study of 169 subjects.

**Results:** No statistical difference was noted between case and controls, in terms of demographics and ART procedures and scores. Endometrial volume and adjusted endometrial volume were significantly higher in the positive group as soon as day 6 of ovarian controlled stimulation. Serum estradiol was significantly higher in the positive group from day 8 after ovarian controlled stimulation. Uterine artery resistance and pulsatility index showed statistical difference between the two groups. Also, statistical significance was obtained between the two groups in terms of subendometrial vascularization.

**Conclusions:** Serial endometrial volumetry assessment and serial endometrial doppler fluxometry provides a predictive clinical tool enhancing elective embryo transfers in fresh ART cycle. Continuous serum estradiol and 3D endometrial volume and adjusted endometrial volumes may reflect endometrial changes during ART procedures.

# Keywords

Endometrial receptivity;Assisted reproductive technology;Embryo implantation Endometrial volume;Adjusted endometrial volume;Serum estradiol;Subendometrial blood flow;Uterine artery fluxometry

# Resumo

**Objetivo:** O processo de implantação é complexo e exige sincronia entre um embrião saudável e um endométrio recetivo e competente. A possibilidade da avaliação seriada de parâmetros biofísicos e bioquímicos no contexto da avaliação da recetividade endometrial em ciclos de procriação medicamente assistida (PMA) é proposta neste trabalho.

**Metodologia do Estudo:** Elaborado protocolo seriado sequêncial de parâmetros bioquímicos (estradiol sérico), e de parâmetros biofísicos (volume endometrial, volume endometrial ajustado, fluxometria doppler da artéria uterina e fluxometria doppler do território subendometrial) de avaliação da recetividade endometrial. Comparação das varáveis em estudo, em termos de gravidez clínica. Estudo prospetivo cohort de 169 casais, com avaliação seriada dos níveis séricos de estradiol, ecografia 2D e 3D e fluxometria doppler.

**Resultados:** Ausência de diferença estatisticamente significativa entre os grupos em termos de dados demográficos e parâmetros do tratamento realizado em PMA. Diferenças com significado estatístico entre os grupos em termos de volume endometrial e volume endometrial ajustado após o 6º dia de estímulo controlado do ovário. Valores estatisticamente mais elevados de estradiol sérico no grupo com gravidez clínica após o 8º dia de estímulo. Diferenças com significado estatístico entre os dois grupos em termos de índice de resistência e pulsatilidade da artéria uterina, bem como em termos de vascularização subendometrial.

**Conclusões:** Avaliações seriadas da volumetria endometrial e da fluxometria uterina e subendometrial podem fornecer dados com relevância clínica na decisão de transferência embrionária no ciclo de PMA. Avaliação seriada da volumetria endometrial e o doseamento seriado do estradiol sérico podem refletir alterações endometriais no decurso do ciclo de estimulação ovárica.

# Palavras-chave

Recetividade endometrial;Procriação medicamente assistida;Implantação embrionária;Volume endometrial;Volume endometrial ajustado;Estradiol sérico;Fluxo subendometrial;Fluxometria da artéria uterina

# Resumo Alargado

## Avaliação da receptividade endometrial em ciclos de procriação medicamente assistida - o valor dos marcadores biofísicos

### Introdução

A infertilidade é definda pela Organização Mundial de Saúde como a incapacidade de conceber por parte de um casal após 12 meses de tentativas. Estima-se que a sua prevalência possa atingir até 48,5 milhões de casais.

O processo de implantação é complexo e exige sincronia entre um embrião saudável e um endométrio recetivo e competente, na tentativa de potenciar o sucesso das técnicas de reprodução humana. O conceito de selectividade endometrial, no qual o endométrio funciona como biossensor da qualidade embrionária é igualmente relevante.

Múltiplos marcadores foram definidos com limitações importantes à sua aplicabilidade prática, nomeadamente marcadores bioquímicos, genéticos e outros marcadores.

No que diz respeito aos marcadores biofísicos a possibilidade do estudo de forma não invasiva e com resultados que reflectem a metodologia quotidiana da prática diária das unidades de reprodução é uma vantagem. Vários estudos foram realizados ao longo das últimas décadas, com potenciais marcadores em estudo; no entanto a maioria destes reflecte resultados contraditórios, pela utilização de avaliações pré-definidas em análises únicas. A ausência de uma sequência de eventos e ou de avaliações seridas torna difícil a interpretação dos resultados desses mesmos estudos.

Dos vários marcadores analisados, o mais referido é a espessura endometrial que alia não apenas a metodologia de análise única em momento pré-definido no tempo, à utilização de valores médios na grande maioria dos casos com sobreposição de valores, o que torna difícil a interpretação dos dados obtidos de forma clara e conclusiva.

Outro marcador atualmente em voga, que alia a maior nitidez e contraste das imagens obtidas através das sondas de alta definição é a volumetria endometrial. Análises computadorizadas das áreas de interesse tornam mais precisa a avaliação em comparação com os estudos realizados em 2 dimensões apenas.

Outro conceito igualmente inovador, decorrente dos avanços tecnológicos alia a possibilidade de avaliação do grau de compactação endometrial na sequência transformativa de um endométrio inicialmente secretor, proliferativo, e estratificado em endométrio compactado, e homogéneo. No entanto os estudos nesta área são heterógenos, e ainda sem resultados conclusivos com aplicação prática.

Ainda na área dos marcadores biofísicos um conceito interessante acerca da angiogénese prefencial, com recurso à observação das alterações dos índices fluxométricos nos territórios da artéria uterina e do território subendometrial, verificando-se uma vasculzarização preferencial nos territórios em que ocorre o fenómeno de implantação.

Deste modo, pelo seu caracter não invasivo, os marcadores biofísicos são assim de fácil aplicabilidade à prática quotidiana das unidades de reprodução, de elevada reprodutibilidade e baixo custo associados aos avanços tecnológicos com a disponibilidade de sondas de alta definição, fazendo resurgir o potencial da utilização destes marcadores no contexto actual.

Este trabalho tem assim como objetivos:

1. Avaliar o potencial da utilização seriada e sequencial dos marcadores biofísicos enquanto marcadores da receptividade endometrial em ciclos de reprodução medicamente assistida.
2. Avaliar os efeitos dos níveis séricos do estradiol no contexto de avaliação da receptividade endometrial nos ciclos de reprodução medicamente assistida.
3. Avaliar o conceito de angiogénese preferencial com o estudo dos parâmetros fluxométricos do território da artéria uterina e do território subendometrial, enquanto potenciais marcadores diagnósticos do conceito de receptividade endometrial em contexto de ciclos de tratamento de reprodução medicamente assistida.

**Material e Métodos**

Para a realização deste estudo foram recrutadas inicialmente 189 participantes, e após a aplicação dos critérios de inclusão e exclusão foram avaliadas ao longo do tempo 169 indivíduos. O principal outcome deste trabalho foi estabelecido pelo critério de gravidez clínica, com obtenção de 46 casos positivos versus 123 casos negativos.

A todas as participantes foi aplicado o mesmo critério em termos de avaliação e protocolo de estudo, com avaliações sequenciais e seriadas ao longo do processo de tratamento de reprodução humana.

Todas as participantes foram avaliadas prévio ao início do estímulo controlado do ovário, e ao sexto, oitavo e décimo dia após início do estímulo controlado do ovário. Foram ainda avaliadas no dia da administração com hormona gondocoriónica humana para desencadeamento da ovulação e no dia da transferência embrionária.

Todas as participantes foram submetidas a um protocolo de estimulação controlada do ovário em protocolo curto com antagonista da GnRH, e apenas incluídas as participantes elegíveis para transferência embrionária ao terceiro dia de desenvolvimento embrionário com 2 embriões de boa qualidade.

Elaborado protocolo seriado sequêncial de parâmetros bioquímicos (estradiol sérico), e de parâmetros biofísicos (volume endometrial, volume endometrial ajustado, fluxometria doppler da artéria uterina e fluxometria doppler do território subendometrial) de avaliação da recetividade endometrial.

**Resultados**

Importa salientar que decorrente dos resultados deste trabalho é possível verificar que em termos de dados demográficos e dados relativos ao tratamento de reprodução medicamente assistida os dois grupos não apresentam diferenças com significado estatístico; nomeadamente no que diz respeito a idade do elemento feminino(34.94±4.03 vs 34.28±3.35, valores em anos), reserva ovárica (2.45±2.45 vs 2.62±2.46 em Unidades Internacionais), dose de gonadotrofinas necessárias para produzir estímulo (2500.81±812.19 vs 2508.15±757.91 em Unidades Internacionais), quantidade de ovócitos recuperados (8.25±5.14 vs 10.50±5.20) e número de ovócitos maduros (6.57±4.22 vs 7.06±4.77), bem como quantidade de embriões clivados (3.18±2.40 vs 3.84±2.65) e blastocistos gerados para vitrificação futura (0.65±1.51 vs 0.86±1.71); em comparação do grupo com desfecho negativo versus grupo com desfecho positivo respectivamente para os valores apresentados.

1. Relativamente aos marcadores biofísicos em contexto de análise sequencial ao longo do tempo com avaliação prévia ao ciclo de tratamentos e posteriormente no decurso dos mesmos importa salientar que dos marcadores analisados verificou-se: ausência de diferença com significado estatístico entre os dois

grupos em análise para a morfologia endometrial; porém para a espessura endometrial verificou-se diferença com relevância estatística entre os dois grupos em fases mais avançadas do estímulo (4.32±0.72 vs 4.22±0.51 para p value 0.387; 6.32±0.96 vs 6.28±0.75 para p value 0.827; 7.47±0.80 vs 7.96±0.79 para p value 0.01; 8.01±1.04 vs 8.61±0.98 para p value 0.01; 8.53±1.32 vs 9.59±1.14 para p value 0.001 e 9.06±1.30 vs 10.15±1.35 para p value 0.001) com valores médios e respectivo desvio padrão expressos em milímetros, mais elevados de espessura endometrial no grupo associado ao desfecho positivo, respectivamente. Na análise da volumetria endometrial, a reportar valores médios e desvio padrão em mililitros, no momento basal prévio ao ciclo de tratamento comparáveis entre os dois grupos (2.52±0.71 vs 2.77±0.63 para p value de 0.54), e após início do estímulo diferenças com significado estatístico entre os mesmos (3.08±0.66 vs 3.33±0.57 para p value de 0.024; 3.90±0.94 vs 4.40±0.71 para p value 0.002; 4.12±1.01 vs 4.91±0.82 para p value de 0.001; 4.52±1.00 vs 5.33±0.76 para p value 0.001 e 4.84±1.01 vs 5.59±0.77 para p value 0.001) com maiores valores médios e desvio padrão em mililitros, de volumetria endometrial associados ao desfecho positivo, respectivamente.

2. Na análise dos efeitos séricos dos níveis de estradiol verificou-se neste estudo, ausência de diferença com significado estatístico expresso pelos valores médios e desvio padrão em Unidades Internacionais, entre os dois grupos prévio ao estímulo (10.47±2.89 vs 10.39±2.87 para p value 0.727), e em fases iniciais do estímulo controlado do ovário (305.10±130.49 vs 381.65±122.46 para p value de 0.055). No entanto em fase mais avançada do estímulo é possível verificar valores médios e desvio padrão em Unidades Internacionais, superiores dos níveis de estradiol sérico com significado estatístico no grupo associado ao desfecho positivo (840.68±230.49 vs 896.32±222.46 para p value de 0.034; 1652.56±607.90 vs 1848.65±599.42 para p value 0.01 e 1746.76±654.34 vs 2214.65±612.34 para p value de 0.01) respectivamente. Na distribuição de valores em gráfico de *scatter plot* é possível igualmente verificar a maioria dos resultados com desfecho positivo para valores de volumetria endometrial superiores a 5 mililitros e para valores de estradiol sérico inferiores a 3000 Unidades Internacionais. Utilizando um modelo de regressão logística linear pela aplicação das *Receiver Operating Curves*, verificamos para estes parâmetros em análise valores de área abaixo da curva de 0.7 e 0.72 respectivamente, indicativos de moderada acuidade diagnóstica de cada um destes marcadores enquanto possível marcador da previsibilidade de um resultado positivo.

3. Na análise dos parâmetros fluxométricos verificamos neste estudo que para os índices em análise quer no território da artéria uterina quer no território subendometrial valores médios e respectivo desvio padrão, de índice de pulsatilidade e resistência inferiores com significância estatística associados ao desfecho positivo de forma consistente. Relativamente ao território da artéria uterina verificou-se valores médios e respectivo desvio padrão, comparáveis entre os grupos prévio ao início dos tratamentos (0.97±0.16 vs 0.91±0.12 e 1.46±0.51 vs 1.33±0.34 com p value de 0.1 e 0.06 respectivamente para os índices de resistência e pulsatilidade). No entanto após início do estímulo verificaram-se diferenças com significado estatístico entre os 2 grupos (1.01±0.15 vs 0.94±0.11 e 1.64±0.45 vs 1.47±0.40 com p value 0.04 e 0.023; 1.09±0.14 vs 0.97±0.12 e 1.74±0.47 vs 1.44±0.44 com p value 0.001 e 0.001; 1.19±0.16 vs 1.07±0.16 e 1.87±0.43 vs 1.51±0.37 com p value de 0.001 e 0.001; 0.93±0.10 vs 0.88±0.09 e 1.48±0.38 vs 1.18±0.27 com p value de 0.001 e 0.001; 1.12±0.12 vs 1.02±0.09 e 1.91±0.54 vs 1.49±0.42 com p value de 0.001 e 0.001 respectivamente para os índices de resistência e pulsatilidade). Os resultados são sobreponíveis aos verificados no território subendometrial com ausência de diferença entre os grupos antes do início dos tratamentos (0.77±0.17 vs 0.71±0.17 e 1.16±0.25 vs 1.01±0.26 com p value de 0.82 e 0.2 respectivamente para os índices de resistência e pulsatilidade); porém com valores estatisticamente inferiores reportados ao grupo com desfecho positivo após início dos tratamentos (0.84±0.17 vs 0.73±0.14 e 1.14±0.20 vs 0.98±0.22 com p value de 0.001 e 0.001; 0.95±0.16 vs 0.79±018 e 1.24±0.20 vs 1.03±0.25 com p value de 0.001 e 0.001; 1.04±0.72 vs 0.88±0.19 e 1.32±0.23 vs 1.12±0.31 com p value de 0.001 e 0.001; 0.78±0.16 vs 0.65±0.12 e 0.95±0.14 vs 0.83±0.14 com p value de 0.001 e 0.001; e 0.99±0.15 vs 0.87±0.12 e 1.19±0.17 vs 1.07±0.20 com p value de 0.001 e 0.001 respectivamente para o indíce de resistência e pulstilidade do território subendometrial).

## Conclusão

A recetividade endometrial desempenha um papel fulcral no sucesso dos tratamentos de reprodução medicamente assistida, e verificou-se um incremento nas áreas da cultura embrionária, desenvolvimento embrionário e na transferência de embriões porém o mecanismo subjacente à interacção embrião-endométrio permanece envolto em controvérsia.

A janela de implantação é o perído mais crítico da reprodução humana, auto-limitado e de muito difícil caraterização, logo o período mais sucesptivel de falha.

Diversas abordagens para caraterização da receptividade endometrial foram pospostos no entanto todos os marcadores apresentam falhas significativas ou limitações importantes que impedem a sua real aplicação no contexto diário dos tratamentos de reprodução humana.

A compreensão do endométrio enquanto tecido biologicamente activo e progressivamente transformativo, num processo contínuo e sequencial, constitui uma abordagem alternativa na tentativa de reconhecimento, identificação e diagnóstico preciso do padrão mais favorável para ocorrência da implantação.

A avaliação ecográfica morfocinética do endométrio, baseada na avaliação seriada ecográfica permite a identificação de padrões evolutivos e sequenciais do processo de transformação endometrial.

O nosso estudo foi capaz de identificar alguns marcadores ecográficos do padrão morfocinético endometrial, nomeadamente associados à volumetria endometrial e ao conceito de angiogénese preferencial com recurso aos parâmetros fluxométricos da artéria uterina e do território subendometrial.

Assim, o recurso a uma metodologia sequencial, não invasiva e adaptada ao normal funcionamento das unidades de reprodução humana vão permitir uma decisão mais informada e correcta ao clínico acerca da transferência embrionária nesse mesmo ciclo de tratamento, ou transferência em deferido assim que as condições endometriais sejam mais favoráveis.

Pretende-se com este trabalho o desenvolvimento e aplicabilidade global desta metodologia integrada em score multifactorial com inclusão de outros potenciais marcadores na implementação de um algoritmo de decisão clinica da previsibilidade real da recetividade endometrial.

# Palavras-chave

Recetividade endometrial;Procriação medicamente assistida;Implantação embrionária;Volume endometrial;Volume endometrial ajustado;Estradiol sérico;Fluxo subendometrial;Fluxometria da artéria uterina

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# Abbreviations/Acronym List

| | |
|---|---|
| Adj.EV | Adjusted Endometrial Volume |
| ART | Assisted Reproductive Technologies |
| ASEBIR | Asociación para el Estudio de la Biología de la Reproducción |
| AUC | Area Under Curve |
| BLC 6 | B-cell Lymphoma 6 Protein |
| CDC | Center for Disease Control and Prevention |
| CHCB | Centro Hospitalar Cova da Beira |
| ER | Endometrial Receptivity |
| ERA | Endometrial Receptivity Array |
| EV | Endometrial Volume |
| GCP | Good Clinical Practices |
| GE | General Electrics |
| GR | Growth Rate |
| hCG | human Chorionic Gonadotropin |
| hCG-LH | human Chorionic Gonadotropin-Luteinizing Hormone |
| HOXA-10 | gene for Homebox Protein HOX A-10 |
| IBM | International Business Machines Corporation |
| ICH | International Conference Harmonisation |
| IP-10 | Interferon gamma-induced protein 10 |
| LIF | Leukemia inhibitory Factor |
| ML | Multi-layered endometrium |
| MII | Metaphase II |
| NM | Non-Multi-layered endometrium |
| NS | No statistical analysis |
| OHSS | Ovarian Hyperstimulation Syndrome |
| PEG | Polyethylene Glycol |
| PI | Pulsatility Index |
| RI | Resistance Index |
| ROC | Receiver Operating Curve |
| SE/Ut | SubEndometrial – Uterine Artery ratio |
| SD | Standard Deviation |
| SPMR | Sociedade Portuguesa de Medicina da Reprodução |
| SPSS | Statistical Package for the Social Sciences |
| TM | Trademark |
| TNF | Tumor Necrosis Factor |
| TNF-α | Tumor Necrosis Factor – alpha or Cachectin |
| VEGF-A | Vascular Endothelial Growth Factor A |
| VOCAL | Virtual Organ Computer-aided Analysis |
| WHO | World Health Organization |
| WOI | Window of Implantation |
| 2D | Two Dimensional |
| 3D | Three Dimensional |

# Chapter 1.
# INTRODUCTION

*"As armas e os Barões assinalados*
*Que da Ocidental praia Lusitana*
*Por mares nunca de antes navegados*
*Passaram ainda além da Taprobana,*
*Em perigos e guerras esforçados*
*Mais do que prometia a força humana,*
*E entre gente remota edificaram*
*Novo Reino, que tanto sublimaram."*

in Luís Vaz de Camões – Os Lusíadas Canto I

# 1. Introduction

The World Health Organization (WHO) has defined infertility as a disease of the reproductive system, as an inability for a couple to conceive after one year of regular unprotected intercourse (1,2). This matter has been portrayed as a global public health issue affecting both developed and undeveloped countries. Infertility is the source of individual, social and economic distress (3,4). This concept of infertility does not apply to all scenarios, and a broader non heteronormative definition will soon be required as the growing numbers of single people, same-sex couples and women that wish to socially delay pregnancy, seek in Assisted Reproductive Technologies (ART) treatments a pathway to parenthood.

A published study from the WHO in 2012, revealed a burden of women from 190 countries affected by infertility like previous estimates from 1990 to 2010. Around 48,5 million couples will have some sort of reproductive problem, and around 20 million will never be able to conceive (5).

In Portugal, the Afrodite study was a pioneer in the characterization of infertility. It has estimated its prevalence around 9-10%, but with no regional differences. Thus, it estimates that between 266 000 to 292 000 couples will be affected by this pathology, but only 43 to 48% will seek medical aid (6). In 2006 a published study revealed that the majority ART facilities in the Public National Health system were in principal metropolitan urban centres, and most of them by the coastline. The high demand on ART treatment access, the distance between other parts of the country to any existing facility, and the economic burden, led to the creation of the first (and up to these days) the only public or private ART unit in the inland of Portugal.  Still the debate continues, and further ART public facilities are expected to be created, in the foreseeable near future as part of Government's policies.

One of the most challenging tasks is to properly advise couples with infertility, not only due to the delay of parenthood, with notorious decline in the female reproductive ability, but also because of the increasingly higher number of cases of male associated infertility.

Decline of female fertility with age is a known factor not only for biological issues associated with the decrease in oocyte pool, but also due to the reduction of oocyte quality related with increased maternal age (7). On the other hand, a study published in 1991 showed a significant decrease in seminal parameters over a period of 50 years (from 1938 to 1990). This led to the revision by WHO of the seminal parameters with the reduction of its values (8-9).

Reproductive Medicine plays an important role in solving these issues, as it offers a way for some of these couples to conceive. The Center for Disease Control and Prevention (CDC) has claimed infertility as a "public health priority" and is promoting initiatives to identify, manage and treat this issue with relevance (10). As a result of this increasing demand, also an increase in ART treatments is growing each day, posing new challenges to satisfy the needs.

Assisted Reproductive Technologies are a complex series of procedures used to help fertility or prevent genetic problems and assist with the conception of a child. In general terms it consists of ovarian stimulation, oocyte retrieval, in vitro fertilization, embryo culture and embryo transfer. Human fertilization consists in the union of a sperm nucleus of paternal origin with an egg nucleus of maternal origin to form the embryo. (11) This is in fact the fusion of the hereditary material of two different sex cells or gametes each of which carries half the number of chromosomes typical of the species. During ART procedures, in vitro procedures replace the normal setting of human conception. In vitro derives from the Latin and translates to "within the glass" and refers to all procedures that occur outside a living organism, in the laboratory setting.

Early reproductive failure is one of the most known pitfalls in ART. (12) Only around 30-40% of ART treatments will reach pregnancy, and a healthy baby. To establish a successful pregnancy, proper implantation of the embryo on a receptive endometrium is required. (13)

Endometrial receptivity (ER) has been studied for almost 80 years, and many people have been made aware of implantation, and embryo-endometrium cross talk. Still, meaningful diagnostic tests, or treatments for sub-optimal endometrial receptivity are still clinically unavailable so far. (14)

## 1.1 Endometrial Receptivity

Human implantation is a complex process requiring synchrony between a healthy embryo and a functionally competent or receptive endometrium.

Since the introduction of assisted conception, many techniques have been developed to further improve ovarian stimulation, oocyte retrieval, and embryo culture. However there has always been a lack in understanding the endometrial characteristics compatible with a successful pregnancy. To prepare for pregnancy, the endometrial lining in the uterus thickens and becomes receptive to implantation of a fertilized egg. This happens in response to hormone secretion, with estrogen and progesterone being the primary hormones that are released to ensure the endometrial lining is receptive to pregnancy. (15)

Generally, we address the matter of endometrial receptivity in a wider term. This concept involves not only endometrial receptivity but also endometrial selectivity. (16)

Endometrial selectivity and receptivity are complimentary concepts that allows the endometrium to work as a biosensor of embryo quality. Selectivity relates to the endometrium's natural inherited property to detect and reject embryos of poor quality, whilst receptivity per se, is the actual function that enables the endometrium to become capable of implantation. For ease of reference, we will only use the general term of endometrial receptivity throughout this document (17).

Endometrial receptivity is still a controversial topic and the mechanism which leads the normal endometrium to become receptive to a healthy embryo is unclear. Xavier, et al in 2005 evaluated subendometrial and intraendometrial blood flow in women with endometriosis, and the excessive endometrial angiogenesis could be an explanation as to why endometriotic women had endometrium with higher potential to implant outside the normal uterus (18).

Endometrial differentiation, embryo development and especially embryo-endometrium interaction leading to implantation requires synchronous and continuous dialogue between these two important components.

Diagnosis of endometrial receptivity (ER) has posed a challenge and so far, most available tests have been subjective and lack accuracy and a predictive value.

Successful outcome in assisted reproductive technology cycles depends on the intricate interplay between embryo quality and endometrial receptivity. Endometrium is a dynamic tissue that grows, differentiates, and suffers regression throughout the menstrual cycle in response to hormonal regulation to prepare the uterus for embryo implantation (19).

Endometrium is a highly dynamic tissue undergoing physiological changes in response to ovarian steroid hormones. It has been proven that the supraphysiological hormonal levels as in the ART cycle has a harmful effect on endometrial receptivity. (20) Endometrial characteristics compatible with a successful pregnancy have proven to be difficult to properly assess.

Adequate endometrial development seems to be important for implantation given that previous studies have shown an association between abnormal glandular or vascular development and defective placentation disorders.

## 1.2 Biophysical Markers

Ultrasound can assess changes in the endometrium during stimulated cycles. It also has minimal interobserver and intra-observer variability. Monitoring of both the endometrial and ovarian response to ovarian stimulation on ART cycles with transvaginal ultrasound has become an important predictor of the success of ART.

Published studies have conflicting results on this subject, but the common feature in all, is the lack of continuity on endometrial assessment. The use of high-resolution transvaginal probes makes it possible to follow endometrium changes throughout the cycle (21).

From a clinical point of view some objective parameters must be obtained to ascertain the likelihood of an ongoing pregnancy in ART cycles, preferably in a non-invasive and cost-efficient way. Hou et al. have also confirmed the possibility of non-invasive prediction of success in ART cycles, with serial assessments of the echogenicity pattern transformation, after human Chorionic Gonadotropin (hCG) (22).

Uterine receptivity is regulated by a number of factors including uterine perfusion, and better yet endometrial perfusion. Differences between infertile and fertile women uterine perfusion have been reported. It has been suggested that impaired uterine and endometrial perfusion may be the cause of failure. (23)

In ART cycles blood flow resistance in the uterine artery and in the endometrial territory has been reported to be a predictive indicator of implantation. However, using this method is still controversial in clinical applications, and many studies reported a small sample size or a single one-moment observation. (24)

Many studies have been conducted on the hemodynamic changes of utero-ovarian arteries during ART cycles. (25) For optimizing ART results, it is critical to decide the best timing for embryo transfer. With the introduction of high-resolution transvaginal probes, the non-invasive, accessible transvaginal sonography made it particularly suitable for serial follow up throughout the stimulated cycle.

## 1.2.1 Endometrial Thickness

The endometrium should be measured in the long axis or sagittal plane, ideally on transvaginal scanning, with the entirety of the endometrial lining through to the endocervical canal in view. The measurement is of the thickest echogenic area from one basal endometrial interface across the endometrial canal to the other basal surface.

The normal endometrium changes in appearance as well as in thickness throughout the menstrual cycle, and during stimulated cycles in ART treatments.

This has been the most studied parameter in ART treatments, for many decades. Most of the studies evaluate the mean value of endometrial thickness with the primary outcome, generally defined as clinical pregnancy.

The overlapping values of mean endometrial thickness, the use of different protocols, and the heterogenicity of study methodology make it difficult to extract useful clinical application. Still, values of endometrial thickness over 7 mm are considered a good predictive value for embryo implantation. Also, a limitation to most of the studies is the absence of continuous evaluations. Most studies are based in single scoop analysis of a predetermined time during ART procedures (26-28).

### 1.2.2 Endometrial Volume

Three-dimensional (3D) ultrasound represents a technical development which has led to an improvement in the quality and precision of ultrasound examination. The ability to determine volumes from ultrasonographic images has an important role in the diagnosis and treatment of many situations.

Another important function of 3D ultrasound is volume calculation using the Virtual Organ Computer-aided AnaLysis (VOCAL) even in irregularly shaped structures. This method has been demonstrated to be more accurate than 2D-volume estimations.

In a similar way, values between studies are conflicting and results difficult to extrapolate to the clinical setting. Some studies tried to establish cut-off points (29). Volume estimation of the endometrium can be made easily because of the good contrast between endometrial tissue and myometrium by 3D transvaginal ultrasound. A high degree of reproducibility and accuracy of volume estimation using 3D ultrasound has been demonstrated. The actual cut-off point is still unclear and many studies have been conducted in order to ascertain the optimal value. Many advocate values over 2.5 mL as a good predictive positive outcome, and values under 1.2 mL are a clear indication of implantation failure. (30)

Still more recent studies proposed a higher cut off value of 5 mL as a good indicator in the prediction of endometrial receptivity with good sensitivity and low specificity in a group application and with good predictive negative value and bad predictive positive value, in an individual setting. (31)

### 1.2.3 Endometrial Vascularity

Ultrasound examination of the endometrium allows a non-invasive evaluation of endometrial receptivity (32-34). Angiogenesis is essential in reproductive process. It plays a key role in the development of a mature ovulatory follicle, in the creation of corpus luteum, in the development of endometrium and in the implantation process (35-38). In this way, a good supply of blood to the endometrium is considered a good predictor of implantation and endometrial receptivity (39).

Power doppler in association with 3D ultrasound has been made available in many studies. The use of impedance on the downstream flow is used as a powerful tool, since vessel volume and chatter plot of vascularized fields was inaccurate and imprecise due to the anatomic dimension of vessels, and its tortuous path (40-44).

Raine-Fenning et al. (2004) showed that endometrial and subendometrial vascularity by 3D ultrasound increased during the proliferative phase (45). They further proposed that the degree of change in endometrial perfusion from the late follicular phase through to the early luteal phase was a more important determinant of endometrial receptivity.

Riad and Hak (2013) found lower pulsatility index (PI) and lower resistance index (RI) of the subendometrial blood flow on the day of rhCG injection in women who achieved a clinical pregnancy compared to those who did not. Similar findings were obtained by Engels et al. (2011) (46-47).

Still the absence of a continuous evaluation, and the heterogenicity and subjectivity associated with many of the studies were unable to sustain relevant data for the clinical setting.

### 1.2.4 Other Ultrasonographic Markers

In relation to endometrial thickness, some studies also tried to establish relation between endometrial receptivity and endometrial pattern, or degree of endometrial compaction (48).

Similarly, the heterogenicity of the studies and heterogenicity of definitions, and the subjectivity associated with observer-based studies made comparisons and results difficult to translate to the clinical setting.

## 1.3 Biochemical Markers

The physiological and biochemical steps which will allow endometrium to become receptive remains unclear, poorly understood and a matter for much debate and study.

The impact of serum estradiol levels in ART has been debated for over 25 years with conflicting results about the effect of supraphysiological levels of estradiol during controlled ovarian stimulation.

Some studies showed a negative impact, while others showed a positive impact in ART outcome. The majority showed no impact (49-51). High levels of estradiol and its influence on embryonic implantation, and embryonic development is still controversial and unclear.

Much has been published regarding the deleterious effects of supraphysiological levels of estradiol on ovarian stimulation for ART procedures. Also, questions have been raised concerning high levels of estradiol and the impairment caused by it on endometrial receptivity.

A metanalysis was conducted in 2019 and no good evidence was found to support or refute the value of estradiol levels on the day of rhCG administration, as a predictor of pregnancy in ART cycles (52). Conflicting results may relate to the difference between the way the trials were conducted, the difference of stimulation protocol, number of embryos transferred, and the definition of outcome in terms of pregnancy rates.

According to Paulson (2011), the supraphysiological elevation of serum estradiol may compromise endometrial receptivity. This elevation might play a definitive role in embryo implantation which is claimed to be dose dependent (53).

As a result of multiple follicle maturation, the rise of serum estradiol to supraphysiological levels alters endometrial receptivity by morphological and biochemical changes produced against this tissue (Simon et al.) (54).

Mirkin et al., 2005 reports that elevated serum estradiol levels have a negative impact on endometrial receptivity especially in fresh embryo transfer cycles (55). Still other clinical trials, contrast with these claims with conflicting results.

By Sharara & McClamrock 1999, no significant impact on implantation rates were reported due to the supraphysiological levels of estradiol (56).

Another important factor, and a matter for many studies and much controversy is the role of progesterone. Progesterone belongs to a group of steroid hormones called progestogens. It is mainly secreted by the corpus luteum in the ovary during the second half of the menstrual cycle.

It plays important roles in the menstrual cycle and in maintaining the early stages of pregnancy. The corpus luteum works as a transient gland, responsible for the secretion of adequate levels of progesterone to provide proper decidualization of the endometrium.

In ART cycles, the supraphysiological levels of estradiol are counterbalanced by increased levels of progesterone produced by the corpus luteum. However, mid-luteal progesterone measurements are complicated by the pulsatile nature of hormone secretion from the corpus luteum. Filicori et al. (57) showed that plasma progesterone concentrations exhibit large and rapid fluctuations during the mid-luteal phase of naturally cycling women.

In ART cycles, the pituitary is suppressed by the negative feedback from supra-physiological steroid levels. Nevertheless, serum progesterone levels are uncertain and unreliable. Many studies have shown conflicting results about the use of serum progesterone measured on trigger day as a way to predict ART positive outcome.

## 1.4 Genetic Screening of Endometrial Receptivity

Still the results of all these features remain uncertain and conflicting. Ultrasound can assess endometrium changes during a stimulated ART cycle, in a non-invasive manner. Monitoring both follicle development and endometrium during an ART cycle is a normal clinical procedure.

The window of implantation (WOI) is defined as a short period of time while the endometrium is receptive to the embryo (58). Diagnosis of endometrial receptivity (ER) has posed a challenge and so far, most available tests have been subjective and lack accuracy and a predictive value (59).

Endometrial Receptivity Array (ERA) is a genetic test performed prior to embryo transfer in selected patients. This test aims to identify the best time for embryo transfer, based on genetic testing to identify the window of implantation. During the WOI, the endometrium displays a receptive phenotype. Functional genomics of endometrial receptivity has been widely investigated (60-68).

Hashimoto et al. (2017) performed ERA testing on 50 women with recurrent implantation failure and reported non-receptive endometrium at a rate of 24%. Subsequently they performed programmed embryo transfer according to ERA evaluation and reported an overall rate of 50% clinical pregnancies (69). Similar results were reported by Tan et al. (2018), Diaz-Gimeno et al. (2017) and Mahajan (2015) (70-72).

The use of transcriptomic signature of the WOI by microarray technology is possible, however it demands an endometrial biopsy. This requires an invasive procedure, and it has an associated cost. Also, in women with irregular cycles it may not be cost-efficient (73-74).

## 1.5 Other Markers of Endometrial Receptivity

Several individual molecular markers have been investigated. Clinical pregnancies have been reported in association with the expression of BLC6, aromatase P450, α-inhibin and β-glycan, integrins, and L-selectin ligand (75-77). Also, the use of mean measurements of VEGF, matrix metalloproteinases and E-cadherin expression, alpha-2 PEG, human Chorionic Gonadotropin-Luteinizing Hormone (hCG-LH) receptor, Leukemia inhibitory Factor (LIF), macrophage colony-stimulating factor, HOXA-10 and vascular endothelial growth factor A (VEGF-A) (78-86), was investigated. No convincing evidence for clinical use emerged from these studies.

Also, the correlation between clinical pregnancy and markers from endometrial fluid aspirate was attempted. Some of the markers studied were: Activin A, Urocortin, human decidua associated protein and Interleukin 18 (87-90).

Levels of various cytokines, glycodelin, isoforms of leucine-rich alpha2-glycoprotein, LIF and tumor necrosis factor (TNF), interleukin-1β, tumor necrosis factor – alpha or Cachectin (TNF-α), interferon gamma-induced protein 10 (IP-10), were also subject to study but no clinical practical outcome was ever obtained (91-95).

All these markers are promising but still have not been able to provide an accurate diagnostic test, reliable for endometrial receptivity.

# Chapter 2.
# AIMS of the THESIS

*"Se também com tais obras nos engana*
*O desejo de um nome avantajado,*
*Mais razão há que queira eterna glória*
*Quem faz obras tão dinas de memória."*

in Luís Vaz de Camões – Os Lusíadas Canto II

# 2. Aims of the Thesis

Endometrial receptivity on ART cycles has posed a challenge and has been a target of many debates and controversy. Many methods and many parameters have been assessed to better understand the synchronous dialogue between and healthy embryo and a receptive endometrium.

The objectives of this study were:

1) To evaluate the capability of serial and continuous evaluation of biophysical markers as a non-invasive procedure to determine endometrial receptivity (Chapter 4);

2) To determine the changes and follow up the way both serum estradiol and ultrasound parameters influence this process (Chapter 5);

3) To evaluate the capability of serial measurement of subendometrial fluxometry Doppler flow as a non-invasive procedure to determine endometrial receptivity (Chapter 6).

# Chapter 3.

# MATERIALS AND METHODS

*"Agora tu, Calíope, me ensina*
*O que contou ao Rei o ilustre Gama;*
*Inspira imortal canto e voz divina*
*Neste peito mortal, que tanto te ama."*

in Luís Vaz de Camões – Os Lusíadas Canto III

# 3. Materials and Methods

This was a prospective cohort study of 169 women in ART cycles. All infertile couples submitted to ART treatment at the Reproductive Medicine Unit of the University Hospital Centre Cova da Beira, were included for a 4 Year period interval. [1]

Cancelled treatments prior to oocyte pickup; cycles with missing or erroneous data; and cycles with elective single embryo transfer were excluded. (Figure. 1.)

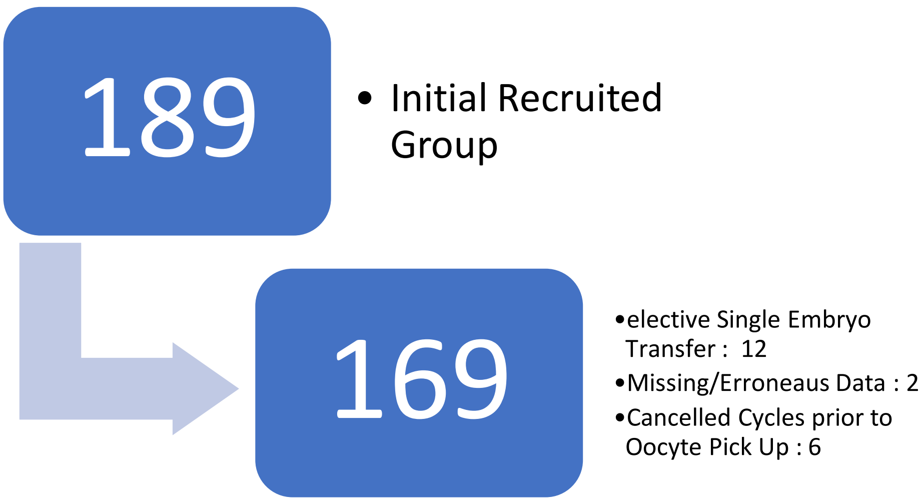


Figure 1. Study Population and Exclusion Criteria.[2]

The primary data source for this study was the local databases routinely used in the participating centre in ongoing treatments. The data output was anonymized in the extraction for statistical treatment purposes.

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[1] A corrective note has been sent to the Editor in Chief of the publication. Research period of 4 years.

[2] A corrective note has been sent to the Editor in Chief of the publication. It has been published 209 patients, but one should read 169 patients instead.

All data collected and written informed consent was obtained according to the Ethics Committee of our Institution. Subjects with double viable good grade embryo transfer on day 3 of embryo development were included. Only Grade A and B cleaved embryos were included in this selection according to the morphocinetic analysis using Asociación para el Estudio de la Biología de la Reproducción (ASEBIR) embryo assessment criteria.

All subjects have been in a short protocol regimen with antagonist for ovarian controlled stimulation using gonadotropins.

All subjects were submitted to recombinant human chorionic gonadotropin hormone (rhCG) for induction of ovulation 36 h prior to oocyte pick up.

Demographics and ART parameters were collected for all patients.

## 3.1 Continuous endometrial volumetric analysis for endometrial receptivity assessment on assisted reproductive technology cycles

Ultrasonographic protocol for serial ultrasound analysis (endometrial morphology, endometrial thickness, endometrial volume and uterine volume) was performed. Women were scanned by 2D and 3D ultrasound using a transvaginal 7.5 MHz transducer (General Electrics – GE Volusson E6, USA TM) by a single operator.

During ovarian controlled stimulation serial ultrasound exams were performed, using 2D and 3D transvaginal probe.

Ultrasonographic markers were obtained in all evaluations (Basal moment – day 2 or 3 of women menstrual cycle and prior to onset of ovarian controlled stimulation; at day 6, day 8 and day 10 after initiating ovarian controlled stimulation; at Trigger day with rhCG; and at embryo transfer day). (Figure 2.)


Figure 2. Study Protocol – timing of ultrasonographic evaluation

Endometrial morphology was based on the two-grade system by Sher et al. (96): non-multi-layered homogeneous hyperechogenic or iso-echogenic endometrium compared with the myometrium and multi-layered triple line pattern, 'halo pattern' with an outer peripheral layer of denser echogenicity and a central sonolucent area.

Endometrial thickness was obtained in millimetres (mm) on the long axis or sagittal plane, with the entirely of the endometrial lining through and endocervical canal in view.

The measurement was taken of the thickest echogenic area from one basal endometrial interface across the endometrial canal to the other basal surface.

Endometrial volume calculation by 3D ultrasound presented as voxels and geometric information of surfaces in a 3D dataset. The results obtained are then converted to millilitres.

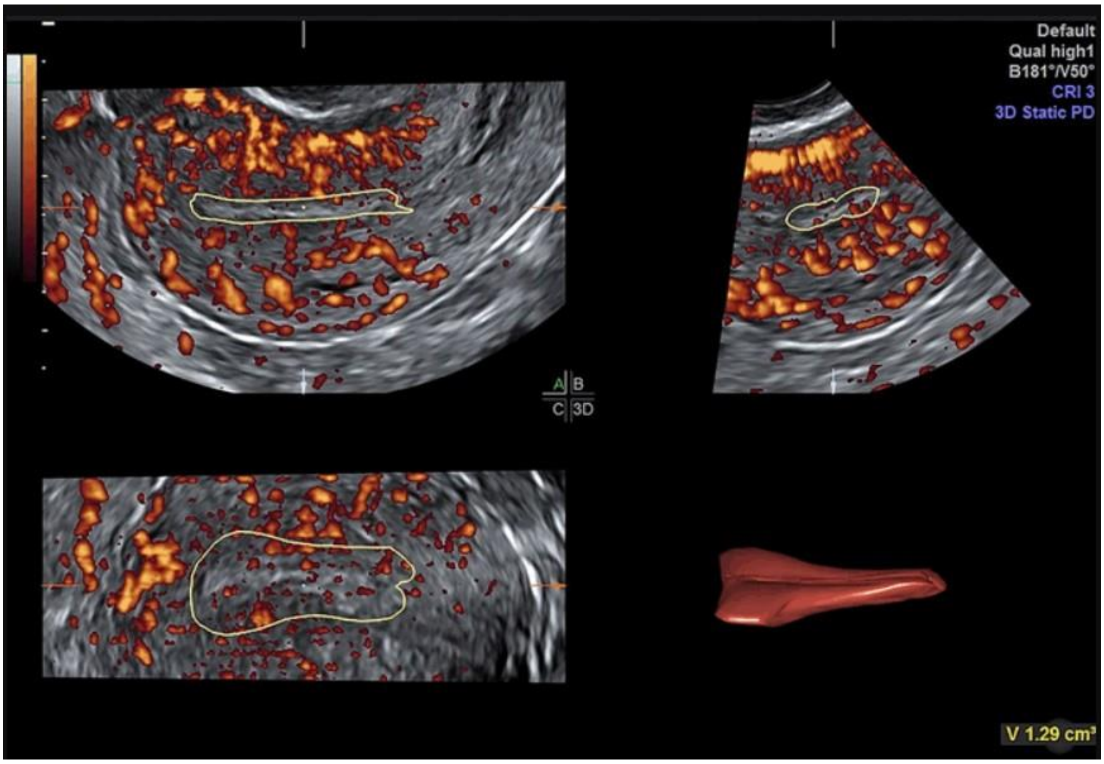



Figure 3. Example of VOCAL imaging to measure endometrial volume. Voluson E6 (General Electric Healthcare, USA) three-dimensional (3D) system with RIC 5-9 D transvaginal probe used. Image obtained at basal moment, previous to ovarian controlled stimulation.

The actual cut off point is unclear. Values over 2.5 mL have been considered as a good predictive positive outcome in some studies.

More recently, higher cut off value of 5 mL were proposed as good indicator in the prediction of endometrial receptivity. The authors performed a scatter plot distribution with majority of positive outcomes higher than 5 mL.

For purpose of this study, we used the higher value of over 5 mL.

Adjusted Endometrial volume (Adj.EV) was also obtained as a ratio between endometrial volume calculated on 3D analysis and uterine volume based on 3D volumetric acquisitions which then generated an estimated uterine volume (also in millilitres). Adjusted endometrial volume deflects the potential difference in uterine volume from each single individual.

## 3.2 The predictive value of serial serum estradiol and serial endometrial volume on endometrial receptivity on assisted reproductive technology cycles

Continuous serial serum estradiol levels were obtained, and ultrasound analysis were performed using the same protocol for all participating subjects, from basal moment (prior to ovarian controlled stimulation) and throughout ovarian controlled stimulation.

Serum levels of estradiol were measured by chemiluminescent enzyme immunoassays (IMMULITE; Diagnostic Products, Los Angeles, CA, USA). Inter-assay and intra-assay coefficients of variation were 9.8 and 9.4%

Sagittal midline 2D longitudinal cross section of the uterus was obtained and a volume box was superimposed on the scan image. The volume was captured through the automatic sweep of the transducer.

Endometrial volume calculation by 3D ultrasound presented as voxels and geometric information of surfaces in a 3D dataset. The results obtained are then converted to millilitres.

## 3.3 Subendometrial resistence and pulsatility index assessment of endometrial receptivity in assisted reproductive technology cycles

Serial ultrasound analysis (uterine artery fluxometry and subendometrial blood blow) was performed using the same protocol for all participating subjects.

Colour Doppler signals were measured in uterine artery and their ascending branches located in the outer third of the myometrium.

The impedance of blood flow through the uterine arteries may be expressed as the pulsatility index (PI), unitless and angle independent, and the resistance index (RI), unitless and angle dependent.

The PI was measured from the flow velocity waveforms as the systolic peak velocity minus end-diastolic velocity divided by the mean. It can be classified as low (0.00–1.99), medium (2.00–2.99) and high (over 3.0). The RI was calculated as the ratio of peak systolic flow velocity minus end-diastolic velocity divided by peak systolic velocity, ranging from 0.0 to 1.0.

Subendometrial blood flow represents vessels irrigating endometrium within 10 mm of the lateral endometrium border. Uterine and subendometrial resistance and pulsatility index was obtained with 2D sagittal uterine view with power coloured doppler in all evaluations (Basal moment – Day 2 or 3 of women menstrual cycle and prior to begin of ovarian controlled stimulation; at Day 6, Day 8 and Day 10 after the begin of ovarian controlled stimulation; at Trigger Day; and at Embryo Transfer).

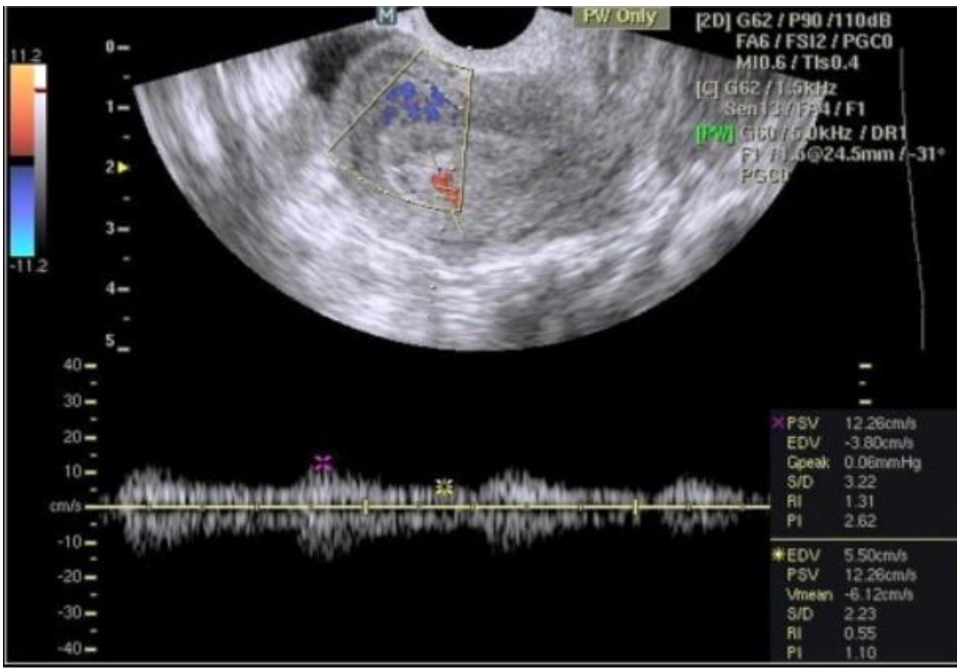



Figure 4. Doppler ultrasound velocimetry of the subendometrial blood vessels.

Blood flow evaluations were performed in the morning to avoid fluctuation due to circadian rhythm of uterine artery blood flow.
Further methodological details can be found on the published articles.

## 3.4 Study Protocol and Statistical Methodology

Positive outcome was considered for subjects with positive pregnancy test 12 days after successful embryo transfer and consequently ultrasonographic confirmation of pregnancy with the presence of gestational sac with at least one embryo with positive fetal heart activity.

Data was analysed in Excel 2019 (Microsoft Corp, Redmond, WA) and International Business Machines Corporation SPSS statistics v25 (IBM Corp. Armonk, NY).

Continuous variables were analysed with Levene's test (equality of variances) and visual assessment of the histogram (normality). Results were expressed as mean ± Standard Deviation (SD), frequency, and percentages. For statistical differences between the means of two groups, t-student for independent samples was applied. Categorical characteristics of patients were compared with $\chi^2$ test. Value of $p < 0.05$ was considered statistically significant.

Receiver operating characteristic (ROC) curve analysis and comparison of area under the curve (AUC) were performed to determine cut-off values of Estradiol, Endometrial Volume and Adjusted Endometrial Volume for the prediction of positive outcome.

This curve measures the accuracy of biomarkers to predict positive outcome in endometrial receptivity displayed by the relationship between sensitivity (true positive rate, y-axis) and 1-specificity (false positive rate, x-axis) across the possible threshold values considered.

Values of AUC equal to 1 indicate a perfect test, values of AUC higher than 0.9 demonstrate high accuracy and values between 0.7 and 0.9 reflect moderate accuracy of the test.

The study protocol was approved by the Ethics Committee of our Institution in accordance with the relevant guidelines and regulations. The authors do not report any conflict of interest.

The study was developed under the research practices described in International Conference Harmonisation (ICH) guidelines, Good Clinical Practices (GCP) and the Declaration of Helsinki.

# Chapter 4.

## CONTINUOUS ENDOMETRIAL VOLUMETRIC ANALYSIS FOR ENDOMETRIAL RECEPTIVITY ASSESSMENT ON ASSISTED REPRODUCTIVE TECHNOLOGY CYCLES

*"Nenhum cometimento alto e nefando*
*Por fogo, ferro, água, calma e frio,*
*Deixa intentado a humana geração.*
*Mísera sorte! Estranha condição!"*

in Luís Vaz de Camões – Os Lusíadas Canto IV

# 4. Continuous endometrial volumetric analysis for endometrial receptivity assessment on assisted reproductive technology cycles

Clear morphology and volume of the endometrium was obtained in all 169 cycles using 3D transvaginal ultrasound in continuous serial observations.

Women were divided into two groups depending on the value of hCG at Day 12 after embryo transfer and ultrasound confirmation of clinical pregnancy: 123 on the negative group (72.8%) and 46 on the positive group (27.2%). Authors report a total of 31 live births from 19 singleton pregnancies and 12 newborns from 6 twin pregnancies.

Demographics characteristics and ART parameters are shown in Table 1.

There was no statistical difference between the two set groups in terms of demographics and ART parameters.

Mean age of female partner or mean age of male partner, duration and type of infertility, total drug dose used for ovarian stimulation, overall median number of harvested oocytes per cycle defined as the total number of oocytes harvested during oocyte pick up procedure, rate of collected metaphase II (MII) oocytes, was similar between the two groups.

Also, the mean number of cleaved embryos at day 3 of embryo development, and mean number of blastocysts for cryopreservation showed no significant statistical difference between the two set groups.

Table 1. Demographics and ART parameters between two Groups
(Positive Group, $n$ = 46 and Negative Group, $n$ = 123).
Descriptive statistics between two Groups. Mean values with standard deviation (SD).

| | Negative Group *n* (%) (interval) | Positive Group *n* (%) (interval) | t-Test p Value |
|---|---|---|---|
| Total | 123 (72.8) | 46 (27.2) | |
| Female Age (in years) | 34.94±4.03 (19-39) | 34.28±3.35 (25-39) | 0.290 |
| Male Age (in years) | 36.14±4.76 (22-46) | 37.19±5.91 (29-62) | 0.832 |
| Time of Infertility (in months) | 54.46±33.82 (12-204) | 60.22±38.49 (14-192) | 0.375 |
| Type of Infertility: | | | |
| • Primary<br>• Secondary | 95(77.2)<br>28 (22.8) | 38 (82.6)<br>8 (17.4) | 0.297 |
| Antimullerian hormone (pg/mL) | 2.45±2.45 (0.09-16.65) | 2.62±2.46 (0.04-13.56) | 0.679 |
| Antral follicle count | 8.43±5.07 (2-40) | 8.63±3.74 (2-20) | 0.801 |
| Total dose of gonadotropins (in International Units) | 2500.81±812.19 (300-4500) | 2508.15±757.91 (450-4500) | 0.956 |
| Progesterone levels at Trigger day (ng/mL) | 0.88±0.44 (0.01-2.20) | 0.78±0.47 (0.01-2.10) | 0.188 |
| Number of collected Oocytes | 8.25±5.14 (2-22) | 10.50±5.20 (2-23) | 0.140 |
| Metaphase II Oocytes | 6.57±4.22 (2-17) | 7.06±4.77 (2-21) | 0.150 |
| Number of day 3 embryos | 3.18±2.40 (2-12) | 3.84±2.65 (2-12) | 0.120 |
| Number of blastocysts for vitrification | 0.65±1.51 (0-6) | 0.86±1.71 (0-9) | 0.200 |

Endometrial morphology showed no statistically significant difference between the two set groups.

Endometrial thickness on a single 2D sagittal profile showed no statistical difference at baseline and at Day 6 after ovarian controlled stimulation. Statistically significant difference was only met at a later phase of ovarian controlled stimulation – at day 8 and on following evaluations.

Uterine Volume was comparable between the two Groups with no statistical difference between the two. (Table 2).

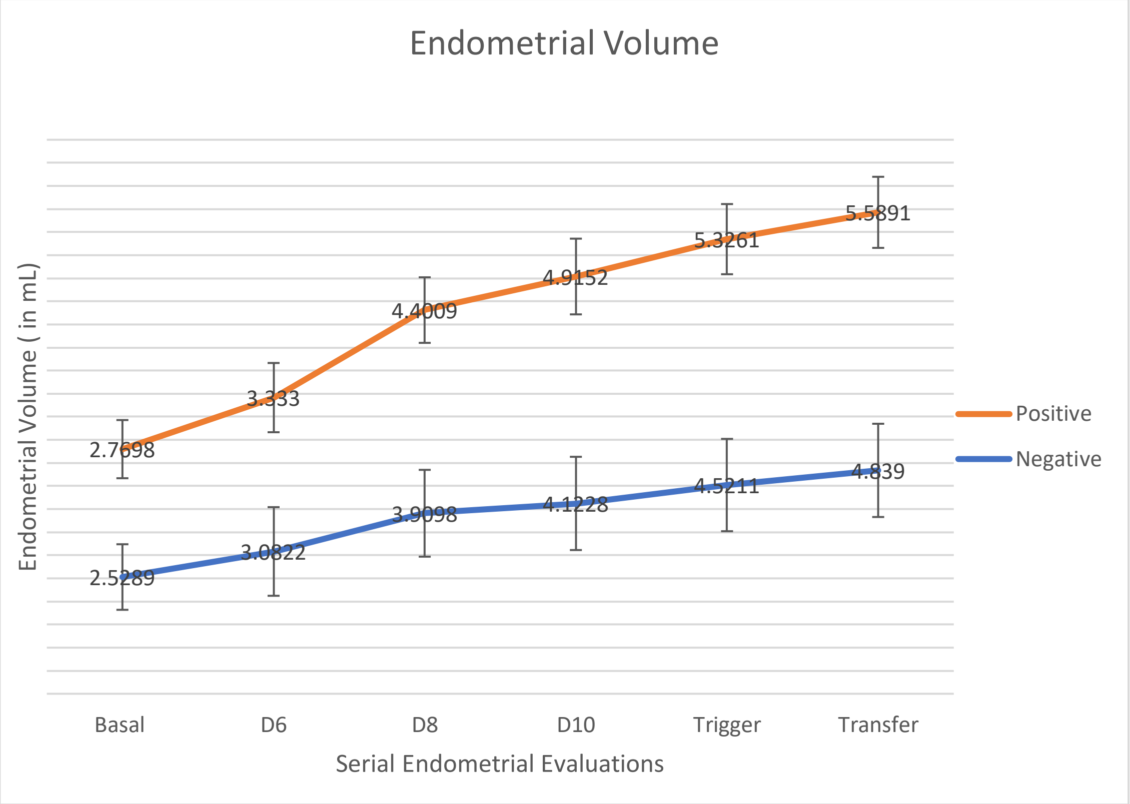
Table 2. Ultrasound parameters between two Groups - Endometrial morphology and Endometrial thickness at baseline, at day 6, 8 and 10 after controlled ovarian stimulation, at trigger day and at embryo transfer day. Ratios in percentages (%) and mean values with standard deviation (SD). NM – Non multi-layered endometrium; ML – Multi-layered endometrium; rhCG – recombinant human chorionic gonadotropin; NS – No statistical analysis performed.

| | | Negative Group *n* (%) (interval) | Positive Group *n* (%) (interval) | p Value |
|---|---|---|---|---|
| Total | | 123 (72.8) | 46 (27.2) | |
| Basal | Endometrial Morphology (ML/NM) | 0/123 (0/ 100) | 0/46 (0/ 100) | NS |
| | Endometrial Thickness (in mm) | 4.32±0,72 | 4.22±0,51 | 0.387 |
| Day 6 after Controlled Ovarian Stimulation | Endometrial Morphology (ML/NM) | 97/26 (78.9/ 21.1) | 43/3 (93.5/ 6.5) | 0.15 |
| | Endometrial Thickness (in mm) | 6.32±0.96 | 6.28±0.75 | 0.827 |
| Day 8 after Controlled Ovarian Stimulation | Endometrial Morphology (ML/NM) | 123/0 (100/ 0) | 46/0 (100 / 0) | NS |
| | Endometrial Thickness (in mm) | 7.47±0.80 | 7.96±0.79 | **0.01** |
| Day 10 after Controlled Ovarian Stimulation | Endometrial Morphology (ML/NM) | 123/0 (100/ 0) | 46/0 (100/ 0) | NS |
| | Endometrial Thickness (in mm) | 8.01±1.04 | 8.61±0.98 | **0.01** |
| Trigger Day with rhCG | Endometrial Morphology (ML/NM) | 123/0 (100/ 0) | 46/0 (100/ 0) | NS |
| | Endometrial Thickness (in mm) | 8.53±1.32 | 9.59±1.44 | **0.001** |
| Embryo Transfer Day | Endometrial Morphology (ML/NM) | 5/118 (4.1/ 95.9) | 2/44 (4.3/ 95.7) | 0.613 |
| | Endometrial Thickness (in mm) | 9.06±1.30 | 10.15±1.35 | **0.001** |

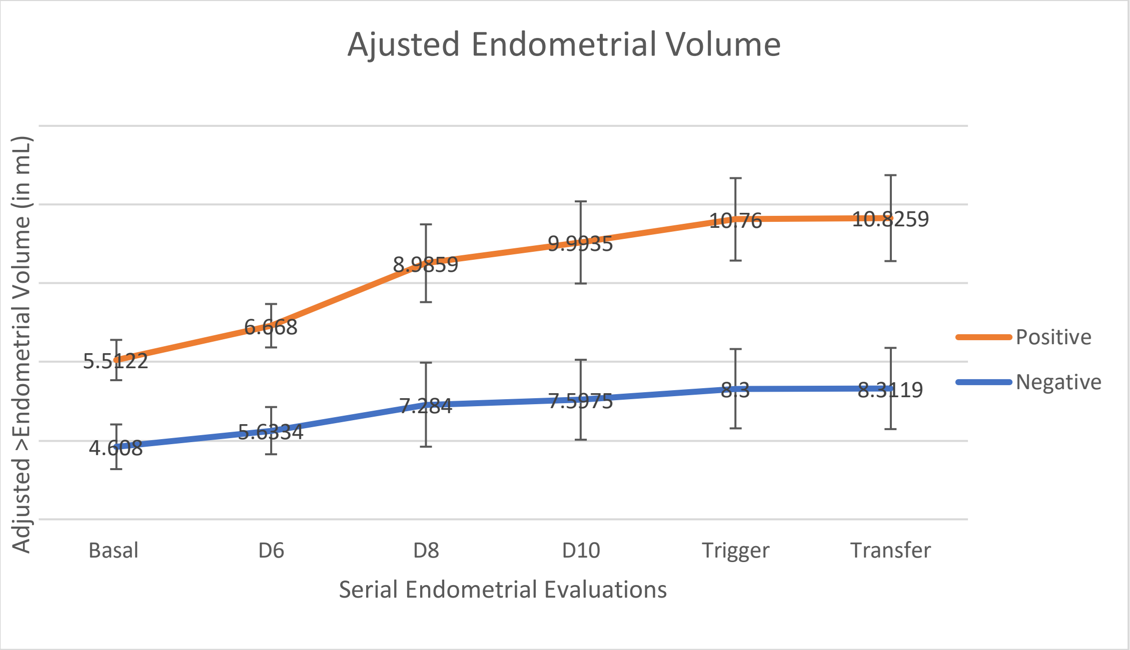
Table 3. Ultrasound parameters between two groups - Endometrial volume and adjusted endometrial volume at baseline, at day 6, 8 and 10 after controlled ovarian stimulation, at trigger day and at embryo transfer day.

Ratios in percentages (%) and mean values with standard deviation (SD). rhCG – recombinant human chorionic gonadotropin.

| | | Negative Group N=123 (72.8%) | Positive Group N= 46 (27.2%) | t-Test p Value |
|---|---|---|---|---|
| Basal | Endometrial Volume (in mm3) | 2.52±0.71 | 2.77±0.63 | 0.54 |
| | Adjusted Endometrial Volume | 4.60±1.42 | 5.51±1.28 | 0.21 |
| Day 6 after Controlled Ovarian Stimulation | Endometrial Volume (in mm3) | 3.08±0.66 | 3.33±0.57 | **0.024** |
| | Adjusted Endometrial Volume | 5.63±1.50 | 6.67±1.38 | **0.001** |
| Day 8 after Controlled Ovarian Stimulation | Endometrial Volume (in mm3) | 3.90±0.94 | 4.40±0.71 | **0.002** |
| | Adjusted Endometrial Volume | 7.28±2.67 | 8.98±2.47 | **0.001** |
| Day 10 after Controlled Ovarian Stimulation | Endometrial Volume (in mm3) | 4.12±1.01 | 4.91±0.82 | **0.001** |
| | Adjusted Endometrial Volume | 7.60±2.54 | 9.99±2.61 | **0.001** |
| Trigger Day with rhCG | Endometrial Volume (in mm3) | 4.52±1.00 | 5.33±0.76 | **0.001** |
| | Adjusted Endometrial Volume | 8.30±2.52 | 10.76±2.62 | **0.001** |
| Embryo Transfer Day | Endometrial Volume (in mm3) | 4.84±1.01 | 5.59±0.77 | **0.001** |
| | Adjusted Endometrial Volume | 8.32±2.58 | 10.83±2.73 | **0.001** |




Graph 1. Continuous endometrial volume analysis (Mean values with Standard Deviation)




Graph 2. Continuous adjusted endometrial volume analysis
(Mean values with Standard Deviation)

Endometrial Volume and Adjusted Endometrial Volume showed statistical difference from Day 6 after Ovarian controlled stimulation (Table 3, Graph 1and 2).

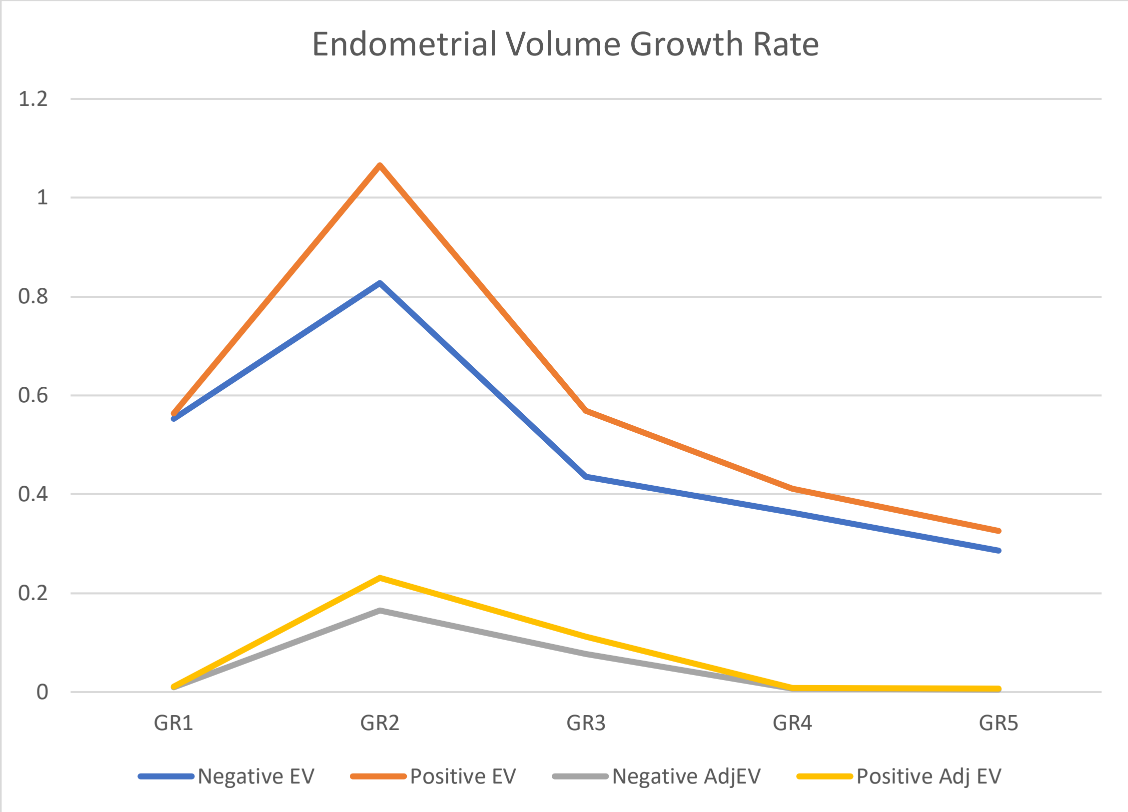
Consistently higher values were seen, for both ultrasonographic markers on the positive group.

In terms of endometrial volume, the positive group had statistically significant higher values in all observations except at the basal moment prior to ovarian controlled stimulation (2.77 ± 0.63 vs 2.52 ± 0.71 with p value of 0.54). Similar findings were noted on adjusted endometrial volume.

By comparing the difference between two consecutive measurements (endometrial growth rate) for endometrial and adjusted endometrial volumes, and the overall difference between the final value and the initial basal measurement we were able to note with statistical differences that values were higher on the positive group on initial phases of endometrial development and in the overall assessment (Table 4 and Graph 3).

Table 4. Endometrial and Adjusted Endometrial volume Growth rate (GR) (difference between two consecutive continuous evaluations, and Overall difference between final and first evaluation).
GR1 – Growth rate 1 (difference between Basal Moment and Day 6 after controlled ovarian stimulation) GR2 – Growth rate 2 (difference between Day 8 and Day 6 after controlled ovarian stimulation) GR3 – Growth rate 3 (difference between Day 10 and Day 8 after controlled ovarian stimulation) GR4 – Growth rate 4 (difference between Trigger with hCG day and Day 10 after controlled ovarian stimulation) GR5 – Growth rate 5 (difference between Transfer Day and Trigger with hCG day) Overall GR – Overall Growth day (difference between embryo transfer day and Basal moment) Negative EV – Negative Group of Endometrial Volume; Positive EV – Positive Group of Endometrial Volume; Negative AdjEV – Negative Group of Adjusted Endometrial Volume; Positive AdjEV – Positive Group of Adjusted Endometrial Volume.
Value of p <.05 considered statistically significant.

| **Endometrial Volume Growth Rate** | | | | | | |
|---|---|---|---|---|---|---|
| | GR1 | GR2 | GR3 | GR4 | GR5 | Overall GR |
| Negative EV | 0,5533 | 0,8275 | 0,4357 | 0,3626 | 0,2861 | 2,4654 |
| Positive EV | 0,5632 | 1,0657 | 0,5686 | 0,4108 | 0,326 | 2,9367 |
| p Value | 1,34 | **0,01** | 0,74 | 0,987 | 0,567 | **0,01** |
| **Adjusted Endometrial Volume Growth Rate** | | | | | | |
| | GR1 | GR2 | GR3 | GR4 | GR5 | Overall GR |
| Negative AdjEV | 0,0102 | 0,165 | 0,077 | 0,0063 | 0,005 | 0,0459 |
| Positive Adj EV | 0,0115 | 0,231 | 0,112 | 0,0083 | 0,007 | 0,061 |
| p Value | 1,43 | **0,01** | 0,53 | 0,873 | 0,678 | **0,01** |




Graph 3. Endometrial and Adjusted Endometrial Volume Growth Rate (difference between two consecutive measurements)

Negative EV – Negative Group of Endometrial Volume; Positive EV – Positive Group of Endometrial Volume; Negative AdjEV – Negative Group of Adjusted Endometrial Volume; Positive AdjEV – Positive Group of Adjusted Endometrial Volume.

In terms of endometrial volume assessment, a cut off ≥5 mL in the prediction of endometrial receptivity was used with good sensitivity (85%) and low specificity (69%) in a group application; in individual setting it had a good predictive negative value (90.1%) and low predictive positive value (81.1%), with a diagnostic accuracy of 75%.

The degree of agreement was assessed by calculation of Kappa (κ) statistics. κ is a statistical parameter of agreement that does not require any assumption of the correct diagnosis, expressed through a coefficient ranging between – 1.0 and + 1.0.

Perfect agreement corresponds to a coefficient of 1.0, a value of zero indicates agreement no better than that expected by chance, and negative values indicate agreement worse than that expected by chance.

There is no absolute way to interpret the values between 0 and 1. As a guideline Landis and Koch indicated that values of < 0.20 suggest poor agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 good agreement and > 0.81 excellent agreement.

In this study the intra-observer reliability was 0.96. In addition, because all measurements were performed by the same operator in this study there was no interobserver variability.

# Chapter 5.

## THE PREDICTIVE VALUE OF SERIAL SERUM ESTRADIOL AND SERIAL ENDOMETRIAL VOLUME ON ENDOMETRIAL RECEPTIVITY ON ASSISTED REPRODUCTIVE TECHNOLOGY CYCLES

*"Quão doce é o louvor e a justa glória*
*Dos próprios feitos, quando são soados!*
*Qualquer nobre trabalha que em memória*
*Vença ou iguale os grandes já passados."*

in Luís Vaz de Camões – Os Lusíadas Canto V

# 5. The predictive value of serial serum estradiol and serial endometrial volume on endometrial receptivity on assisted reproductive technology cycles

Subjects were divided into two groups depending on the value of hCG at Day 12 after embryo transfer and ultrasonographic confirmation of clinical pregnancy: 123 on the negative group (72.8%) and 46 on the positive group (27.2%).

Demographics characteristics and ART parameters of the 169 subjects are shown in Table 1 (Chapter 4), and no statistical difference between the two set groups in terms of demographics and ART parameters was met.

Serum estradiol levels on basal (prior to ovarian controlled stimulation) was similar between the two groups. Significantly higher levels were noted on the positive outcome group, but it only met statistical significance after Day 8 after ovarian controlled stimulation. (Table 5).

Table 5. Serum estradiol measurements between two Groups (in picograms per millilitre – pg/mL) (Positive Group, N = 46 and Negative Group, N = 123).
Mean values with standard deviation (SD).

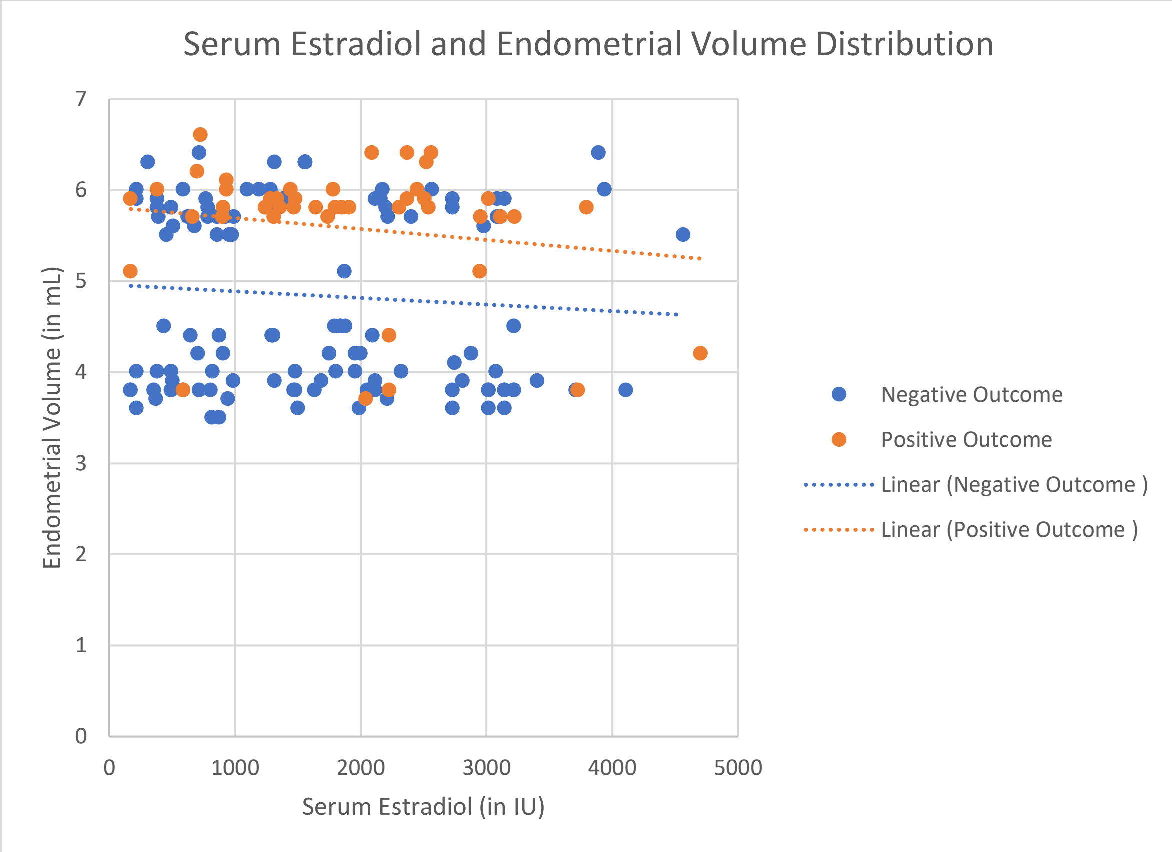
| | Serum Estradiol Levels (pg/mL) Negative Group N=123 (72.8%) | Serum Estradiol Levels (pg/mL) Positive Group N= 46 (27.2%) | t-Test p Value |
|---|---|---|---|
| Basal | 10.47±2.89 | 10.39±2.87 | 0.727 |
| Day 6 after Controlled Ovarian Stimulation | 305.10±130.49 | 381.65±122.46 | 0.055 |
| Day 8 after Controlled Ovarian Stimulation | 840.68±230.49 | 896.32±222.46 | **0.034** |
| Day 10 after Controlled Ovarian Stimulation | 1652.56±607.90 | 1848.65±599.42 | **0.01** |
| Trigger Day with rhCG | 1746.76±654.34 | 2214.65±612.34 | **0.01** |

Endometrial Volume and Adjusted Endometrial Volume showed statistical difference from Day 6 after Ovarian controlled stimulation (Table 6).

Table 6. Ultrasound parameters between two groups – endometrial thickness, volume and adjusted endometrial volume at baseline, at day 6, 8 and 10 after controlled ovarian stimulation, at trigger day and at embryo transfer day.
Ratios in percentages (%) and mean values with standard deviation (SD). rhCG – recombinant human chorionic gonadotropin.

| | | Negative Group N=123 (72.8%) | Positive Group N= 46 (27.2%) | t-Test p Value |
|---|---|---|---|---|
| Basal | Endometrial Thickness (mm) | 4.32±0.72 | 4.22±0.51 | 0.387 |
| | Endometrial Volume ($mm^3$) | 2.52±0.71 | 2.77±0.63 | 0.54 |
| | Adjusted Endometrial Volume | 4.60±1.42 | 5.51±1.28 | 0.21 |
| Day 6 after Controlled Ovarian Stimulation | Endometrial Thickness (mm) | 6.32±0.96 | 6.28±0.75 | 0.827 |
| | Endometrial Volume ($mm^3$) | 3.08±0.66 | 3.33±0.57 | **0.024** |
| | Adjusted Endometrial Volume | 5.63±1.50 | 6.67±1.38 | **0.001** |
| Day 8 after Controlled Ovarian Stimulation | Endometrial Thickness (mm) | 7.47±0.80 | 7.96±0.79 | **0.01** |
| | Endometrial Volume ($mm^3$) | 3.90±0.94 | 4.40±0.71 | **0.002** |
| | Adjusted Endometrial Volume | 7.28±2.67 | 8.98±2.47 | **0.001** |
| Day 10 after Controlled Ovarian Stimulation | Endometrial Thickness (mm) | 8.01±1.04 | 8.61±0.98 | **0.01** |
| | Endometrial Volume ($mm^3$) | 4.12±1.01 | 4.91±0.82 | **0.001** |
| | Adjusted Endometrial Volume | 7.60±2.54 | 9.99±2.61 | **0.001** |
| Trigger Day with rhCG | Endometrial Thickness (mm) | 8.53±1.32 | 9.59±1.44 | **0.001** |
| | Endometrial Volume ($mm^3$) | 4.52±1.00 | 5.33±0.76 | **0.001** |
| | Adjusted Endometrial Volume | 8.30±2.52 | 10.76±2.62 | **0.001** |
| Embryo Transfer Day | Endometrial Thickness (mm) | 9.06±1.30 | 10.15±1.35 | **0.001** |
| | Endometrial Volume ($mm^3$) | 4.84±1.01 | 5.59±0.77 | **0.001** |
| | Adjusted Endometrial Volume | 8.32±2.58 | 10.83±2.73 | **0.001** |

Consistently higher values were seen for both biophysical markers on the positive group. (Graph 4)

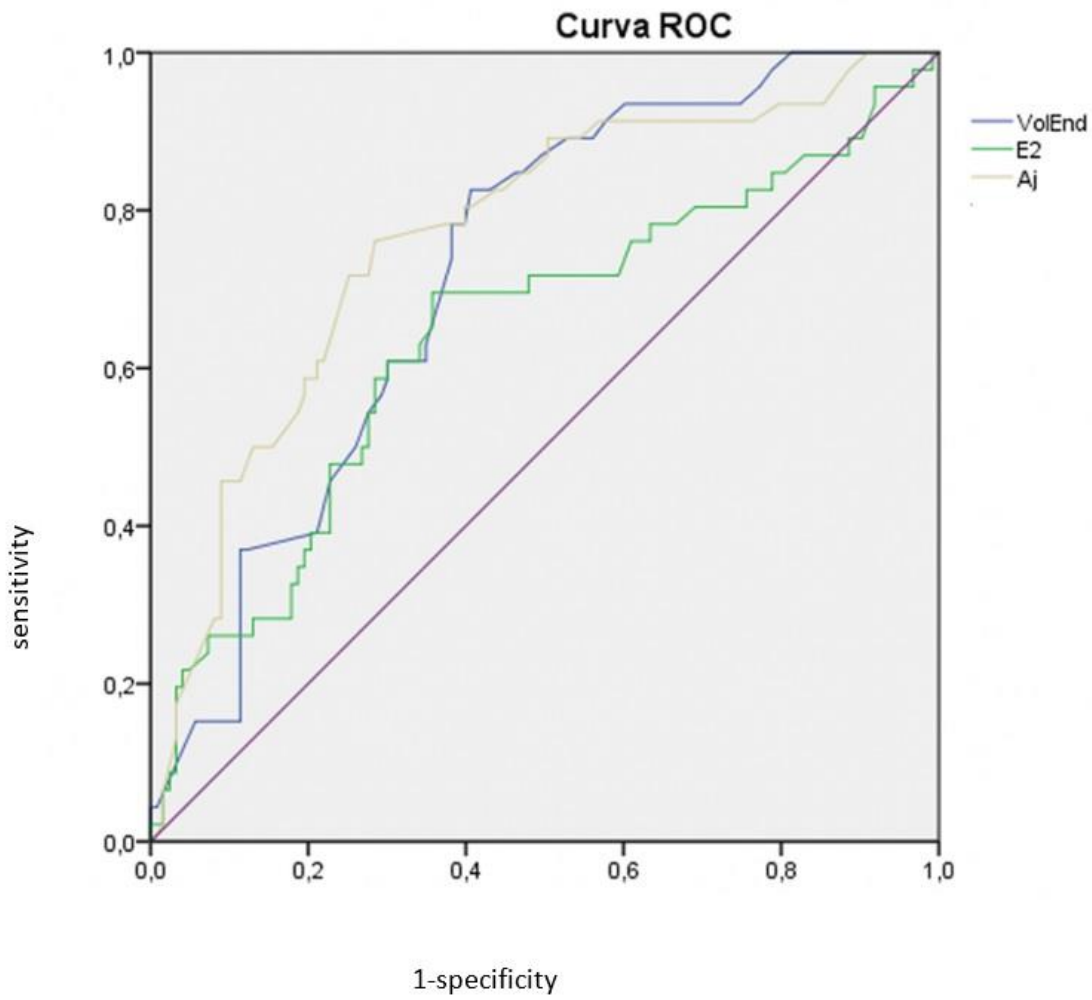



Graph 4. Scatter-plot chart for Serum Estradiol levels and endometrial volume distribution on trigger day with hCG are displayed. (negative outcome in blue, and positive outcome in orange)

Receiver operating characteristic (ROC) curve analysis and comparison of area under curves (AUC) were performed for Estradiol,

Endometrial Volume and Adjusted Endometrial Volume on prediction of positive outcome with sensitivity (true positive rate, y-axis) and 1- specificity (false positive rate, x-axis).

Values of 0,701; 0, 723 and 0,756 were obtained respectively for the examined parameters. (Graph 5)




Graph 5. The ROC area under curves for prediction of positive outcome.
Vol End – Endometrial Volume; E2 – Serum Estradiol; Aj – Adjusted Endometrial Volume.

In this study the intra-observer reliability was 0.96. This is the mean value for all ultrasonographic variables evaluated on this study. In addition, because all measurements were performed by the same operator in this study there was no interobserver variability.

# Chapter 6.

## SUBENDOMETRIAL RESISTANCE AND PULSATILITY INDEX ASSESSMENT OF ENDOMETRIAL RECEPTIVITY IN ASSISTED REPRODUCTIVE TECHNOLOGY CYCLES

*"Destarte se esclarece o entendimento,*
*Que experiências fazem repousado,*
*E fica vendo, como de alto assento,*
*O baxo trato humano embaraçado."*

in Luís Vaz de Camões – Os Lusíadas Canto VI

# 6. Subendometrial resistance and pulsatility index assessment of endometrial receptivity in assisted reproductive technology cycles

Uterine artery resistance and pulsatility index, as well as subendometrial blood flow resistance and pulsatility index was obtained in all 169 cycles using 2D power Doppler transvaginal ultrasound in continuous observations. Subjects were divided into two groups depending on the value of hCG at Day 12 after embryo transfer and ultrasonographic confirmation of clinical pregnancy: 123 on the negative group (72.8%) and 46 on the positive group (27.2%). Demographic characteristics and ART parameters are shown in Table 1 (Chapter 4) and no statistical difference between two set groups were reported.

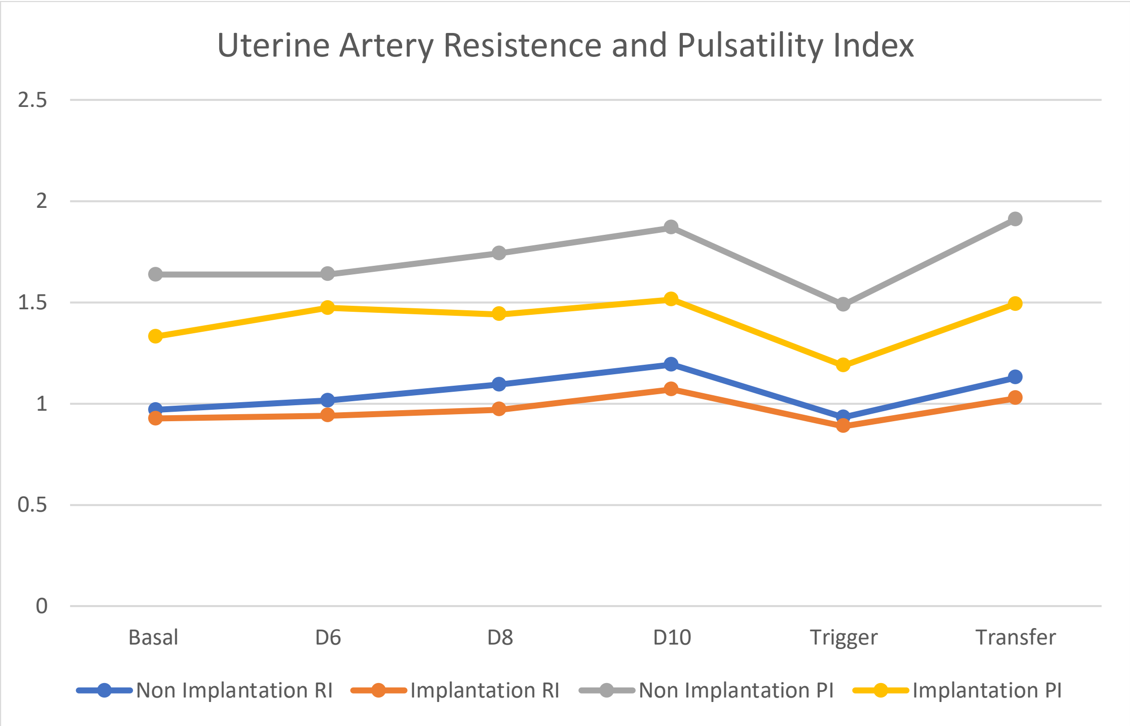
Table 7. Ultrasound parameters between two groups (Uterine artery resistance index and uterine artery pulsatility index) at baseline, at Day 6,8 and 10 after controlled ovarian stimulation, at trigger day and at embryo transfer day. Mean values with standard deviation (SD). rhCG – recombinant Human chorionic gonadotropin.

| | | Negative Group N=123 (72.8%) | Positive Group N= 46 (27.2%) | t Test p Value |
|---|---|---|---|---|
| Uterine Resistance Index (Ut RI) | Basal | 0.97±0.16 | 0.92±0.12 | 0.1 |
| | Day 6 | 1.01±0.15 | 0.94±0.11 | **0.04** |
| | Day 8 | 1.09±0.14 | 0.97±0.12 | **0.001** |
| | Day 10 | 1.19±0.16 | 1.07±0.16 | **0.001** |
| | Trigger Day with rhCG | 0.93±0.10 | 0.88±0.09 | **0.011** |
| | Embryo Transfer Day | 1.12±0.12 | 1.02±0.09 | **0.001** |
| Uterine Pulsatility Index (Ut PI) | Basal | 1.46±0.51 | 1.33±0.34 | 0.06 |
| | Day 6 | 1.64±0.45 | 1.47±0.40 | **0.023** |
| | Day 8 | 1.74±0.47 | 1.44±0.44 | **0.001** |
| | Day 10 | 1.87±0.43 | 1.51±0.37 | **0.001** |
| | Trigger Day with rhCG | 1.48±0.38 | 1.18±0.27 | **0.001** |
| | Embryo Transfer Day | 1.91±0.54 | 1.49±0.42 | **0.001** |

Uterine artery blood flow showed no statistical difference between two groups at baseline, for both resistance and pulsatility indexes.

Statistical difference between the two groups is shown after day 6 of ovarian controlled stimulation for both parameters in analysis (Table 7 and Graph 6).

We can see that both the resistance and pulsatility indexes increase its values slightly until trigger day with rhCG.




Graph 6. Serial Uterine Artery Resistance and Pulsatility Index Flow (Mean values). RI – Resistance Index; PI – Pulsatility Index.

The results are always lower for the positive group (trigger day with rhCG 0.93 ± 0.10 on the negative group versus 0.88 ± 0.09 for the positive group with p value of 0.011 in the resistance index and 1.48 ± 0.38 versus 1.18 ± 0.27 with p value of 0.001 for the pulsatility index).

After trigger day values tend to return to previously observed during controlled ovarian stimulation.

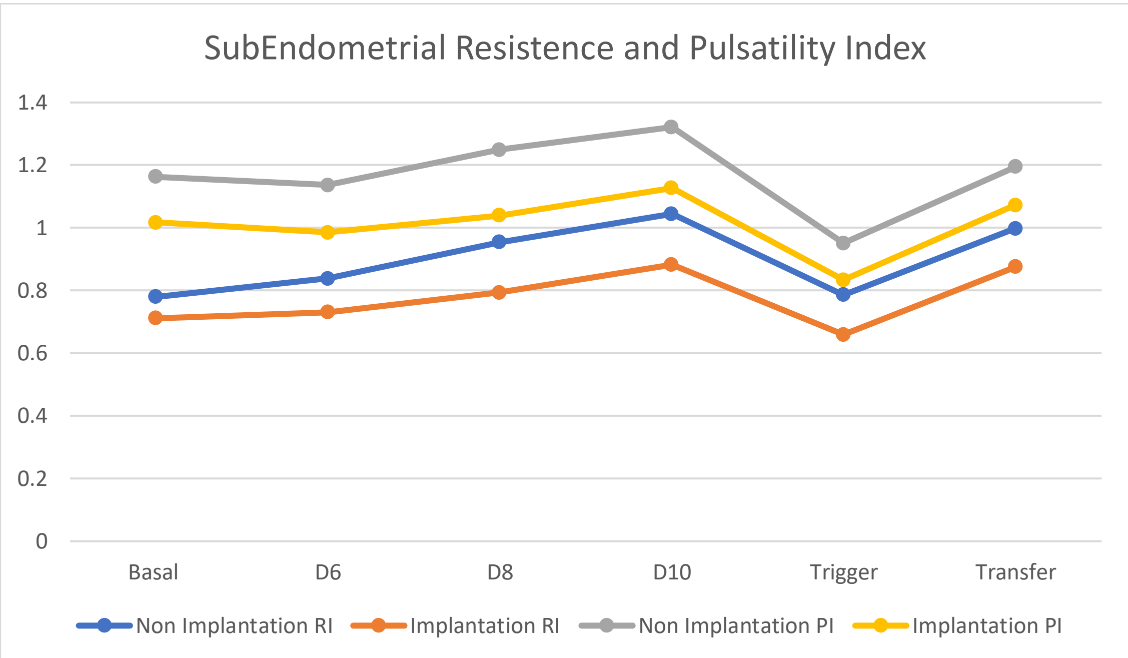
Subendometrial blood flow analysis (resistance and pulsatility indexes) showed no statistical difference between the two groups at baseline, with increasing values for both groups during controlled ovarian stimulation.

During that period at trigger day, and at embryo transfer day, there was statistical difference between the two groups with lower scores for the positive group (resistance index of 0.78 ± 0.16 versus 0.65 ± 0.12 with p value of 0.001 and pulsatility index of 0.95 ± 0.14 versus 0.83 ± 0.14 with p value of 0.001 for negative versus positive group at trigger day respectively) (Table 8 and Graph 7).

Table 8. Ultrasound parameters between two groups. (Subendometrial resistance index, subendometrial pulsatility index, subendometrial / uterine artery resistance index ratio and subendometrial / uterine artery pulsatility index ratio) at baseline, at day 6, 8 and 10 after controlled ovarian stimulation, at trigger day and at embryo transfer day. Mean values with standard deviation (SD). SE/Ut – Subendometrial / Uterine Arteries ratio; rhCG – recombinant Human chorionic gonadotropin

| | | Negative Group N=123 (72.8%) | Positive Group N= 46 (27.2%) | t Test p Value |
|---|---|---|---|---|
| Basal | Subendometrial Resistance Index | 0.77±0.17 | 0.71±0.17 | 0.82 |
| | Subendometrial Pulsatility Index | 1.16±0.25 | 1.01±0.26 | 0.1 |
| | SE/Ut RI ratio | 0.80±0.09 | 0.76±0.12 | 0.6 |
| | SE/Ut PI ratio | 0.73±0.12 | 0.77±0.11 | 0.117 |
| Day 6 after Controlled Ovarian Stimulation | Subendometrial Resistance Index | 0.84±0.17 | 0.73±0.14 | **0.001** |
| | Subendometrial Pulsatility Index | 1.14±0.20 | 0.98±0.22 | **0.001** |
| | SE/Ut RI ratio | 0.82±0.10 | 0.77±0.09 | **0.04** |
| | SE/Ut PI ratio | 0.72±0.14 | 0.68±0.12 | 0.132 |
| Day 8 after Controlled Ovarian Stimulation | Subendometrial Resistance Index | 0.95±0.16 | 0.79±0.18 | **0.001** |
| | Subendometrial Pulsatility Index | 1.24±0.20 | 1.03±0.25 | **0.001** |
| | SE/Ut RI ratio | 0.87±0.13 | 0.81±0.12 | **0.04** |
| | SE/Ut PI ratio | 0.75±0.15 | 0.73±0.10 | 0.615 |
| Day 10 after Controlled Ovarian Stimulation | Subendometrial Resistance Index | 1.04±0.718 | 0.88±0.19 | **0.001** |
| | Subendometrial Pulsatility Index | 1.32±0.23 | 1.12±0.31 | **0.001** |

| | | | | |
|---|---|---|---|---|
| | SE/Ut RI ratio | 0.88±0.11 | 0.82±0.08 | **0.04** |
| | SE/Ut PI ratio | 0.72±0.10 | 0.75±0.11 | 0.251 |
| Trigger Day with rhCG | Subendometrial Resistance Index | 0.78±0.16 | 0.65±0.12 | **0.001** |
| | Subendometrial Pulsatility Index | 0.95±0.14 | 0.83±0.14 | **0.001** |
| | SE/Ut RI ratio | 0.84±0.14 | 0.73±0.09 | **0.01** |
| | SE/Ut PI ratio | 0.72±0.12 | 0.68±0.11 | **0.01** |
| Embryo Transfer Day | Subendometrial Resistance Index | 0.99±0.15 | 0.87±0.12 | **0.001** |
| | Subendometrial Pulsatility Index | 1.19±0.17 | 1.07±0.20 | **0.001** |
| | SE/Ut RI ratio | 0.84±0.15 | 0.74±0.10 | **0.001** |
| | SE/Ut PI ratio | 0.66±0.12 | 0.70±0.08 | **0.018** |

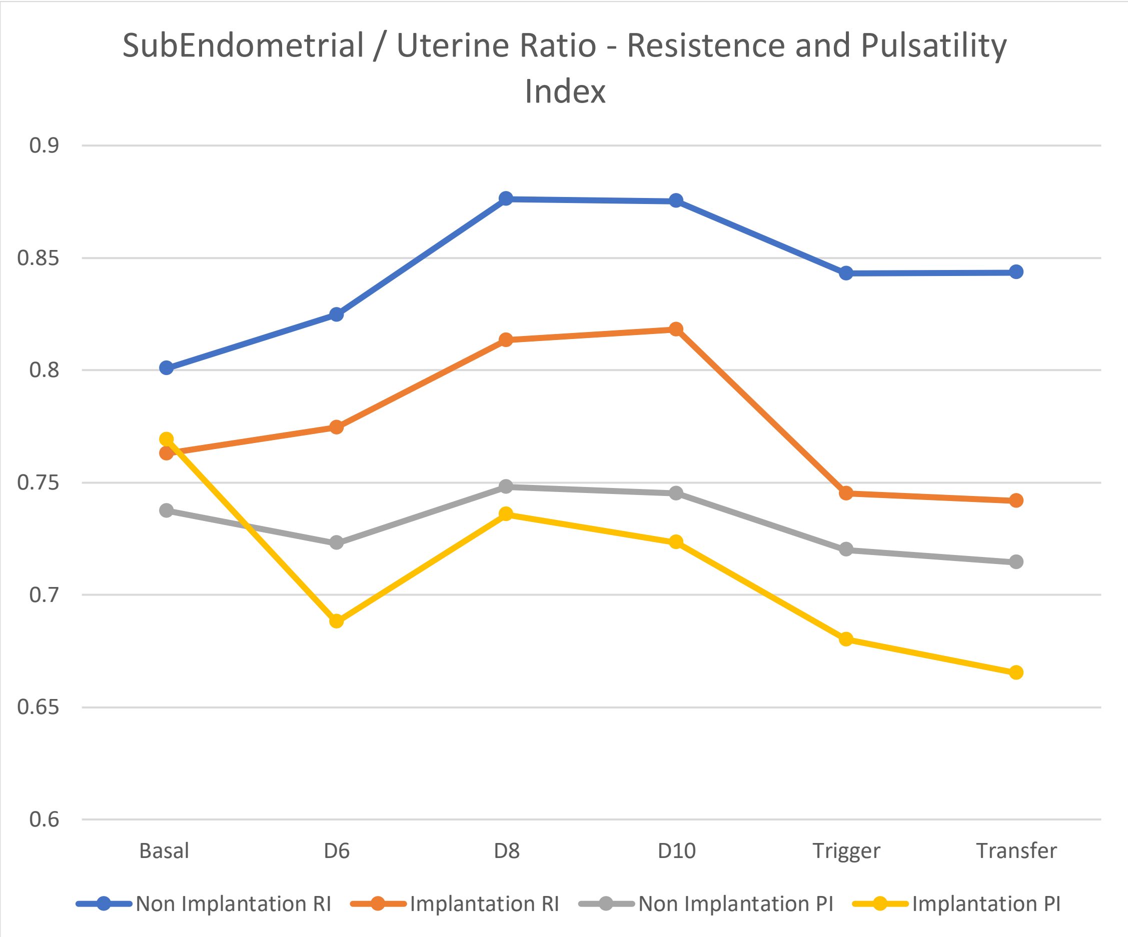



Graph 7. Serial Subendometrial Artery Resistance and Pulsatility Index Flow (Mean values). RI – Resistance Index; PI – Pulsatility Index

The ratio between subendometrial blood flow and uterine artery fluxometry showed no statistical difference between both groups at baseline for the Resistance index.

Statistical difference between the two groups was set after controlled ovarian stimulation, trigger day with rhCG and at embryo transfer for the resistance Index.

However, in terms of the Pulsatility Index, no statistical difference was met between the two groups except for the trigger day with rhCG and at embryo transfer day (0.72 ± 0.12 versus 0.68 ± 0.11 with p value of 0.01and 0.66 ± 0.12 versus 0.70 ± 0.08 with p value of 0.018, for the negative versus positive group respectively) (Graph 8).




Graph 8. Serial Subendometrial / Uterine Arteries Ratio – Resistance and Pulsatility Index Flow (Mean values) RI – Resistance Index; PI – Pulsatility Index

In this study the intra-observer reliability was 0.96. This is the mean value for all ultrasonographic variables evaluated on this study. In addition, because the same operator performed all measurements, in this study there was no inter-observer variability.

# Chapter 7.
# DISCUSSION

*"Aqueles sós direi que aventuraram*
*Por seu Deus, por seu Rei, a amada vida,*
*Onde, perdendo-a, em fama a dilataram,*
*Tão bem de suas obras merecida."*

in Luís Vaz de Camões – Os Lusíadas Canto VII

# 7. Discussion

The pregnancy potential of good quality embryos is still not high on ART cycles, even with the progress in the programs of ovarian stimulation, ART technique and embryo development and culture.

Implantation is still strongly reliable on the cross talk between a healthy good quality embryo and the receptive endometrium. Although several parameters have been used to assess the pregnancy rate in ART cycles, there is still some controversy about its efficacy, and about underlying mechanisms in endometrial receptivity (97-98).

The possibility to use non-invasive techniques to determine endometrial receptivity would allow better clinical judgment. From a clinical point of view, the decision to transfer a fresh embryo on the same ART cycle or to postpone it for a deferred transfer with better endometrial conditions is possible.

This non-invasive tool to predict endometrial receptivity will improve the clinical setting and allow a better understanding of endometrial receptivity. This may be the way to optimize and achieve greater results in ART cycles.

In our study we aimed to assess endometrial evolution to ascertain a plausible predictive non-invasive diagnostic tool for clinicians to better understand endometrium changes.

Vaginal 3D ultrasound is a non-invasive and an inexpensive tool at clinician's disposal (112). The process of endometrial transformation from a proliferative phase to a secretory phase under the hormonal influence of steroids, called endometrial decidualization, is a set goal for optimal implantation.

The cyclic change of the endometrium is regulated by ovarian hormones and their receptors. In addition, endometrial luteal phase development may alter in ART cycles due to supraphysiological hormone levels.

Contradictory findings are often reported in a single analysis of the endometrial pattern at the trigger day. Recent studies have proven that serial evaluations may provide better understanding rather than a single scoop at a pre-determined phase of the process. This relates to what one should expect from the transformation of living tissue and its natural developments onto the complex binding process of implantation. The main purpose of this study was to further evaluate potential ultrasonographic markers that might be used in the continuous changes that the endometrium goes through during an ART cycle.

In our study, endometrial morphology has not proven to be useful, and no significant difference was found between the two groups. Also 2-D endometrial thickness showed no difference at early stages of ovarian controlled stimulation, but significant difference could be seen after day 8 of stimulation. These findings are compatible with the ones provided by the literature.

The main reason for such a finding may be the fact that subjective tools produce conflicting results and therefore are not able to provide an accurate diagnostic tool for endometrial receptivity assessment.

Endometrial volume and adjusted endometrial volume have proven to be more effective with differences shown from the early stages of ovarian controlled stimulation. Both groups were similar at baseline, but as soon as controlled ovarian stimulation started, the differences between the positive and the negative groups were clearly discernible.

We have also been able to show differences between the two groups in terms of endometrial and adjusted endometrial volume in early stages of endometrial development under the influence of controlled ovarian stimulation.
Higher volumes were seen in the positive controls, but the changes were more evident in the early stages (especially between day 6 and day 8 of controlled ovarian stimulation).

This is also corroborated by the fact that the growth rate was statistically higher in the positive group, but the greater difference was found in the early stages of endometrial development (between day 6 and day 8). The supraphysiological environment produced by the controlled ovarian stimulation may have a leading role in this result.

Our study showed that serial continuous endometrial volume was significantly higher in the positive group, whereas studies from Kupesic et al., and Wu et al., (98-99) have conflicting results in the assessment of endometrial receptivity.

The difference can possibly be explained by the fact that serial continuous evaluations better reflect endometrial changes, rather than predetermined single scoop analysis. The use of a cut off ≥5 mL in endometrial volume in prediction of endometrial receptivity had a good sensitivity and low specificity and may be used as a good test to exclude success. The fact that we can have a tool that indicates a non-optimal endometrium aid in the decision-making process of postponing embryo transfer for a more suitable and receptive endometrium.

The possibility of real time non-invasive continuous assessment of the endometrium further assists clinicians in making better medical decisions. The current study demonstrated that it is possible to evaluate endometrial morphologic parameters, serial endometrial volume and adjusted serial endometrial volume in the coronal plane in accordance with published method by Mercer et al. (115).

All findings may prove to be a useful management tool for clinicians in order to establish a diagnostic tool for better decision making in selective embryo transfers.

Nevertheless, one must always be cautious that artefacts during 3D analysis may occur due to a 2D imaging process, patient motion during rendering of images and artefacts due to operator choice in the selection of which part of the volume to display (100).

Serial evaluations, of both biochemical and biophysical parameters, better manifest endometrium transformation and may be a basis for understating endometrial receptivity (101). Cyclic changes of the endometrium are regulated by ovarian hormones (102). Endometrial volume and adjusted endometrial volume have proven to be more effective with differences shown since the beginning of ovarian controlled stimulation..

In a similar way the rise of serum estradiol was significantly higher in the positive outcome group, even though the number of oocytes on pick up, mature oocytes and number of cleaved and blastocysts was similar between both groups.

This reflects the effects that serum estradiol has directly on the endometrium in later phases of its development prior to embryo implantation. Serum estradiol and endometrial volume may prove to be a useful management tool for clinicians in order to establish yet another diagnostic tool for better decision making in selective embryo transfer.

This new methodology process, and a new perspective of endometrial analysis is certainly the strongest factor of this study. Nevertheless, in our work, in line with previous publications, positive outcomes in term of clinical pregnancies were met for values of serum estradiol at trigger day bellow 3000 pg/mL (103).

This can be explained by the policy of tailor made controlled ovarian stimulation protocol, in mild stimulation to avoid potential Ovarian Hyperstimulation Syndrome (OHSS).

Vaginal 2D power Doppler ultrasound is a non-invasive and a relatively inexpensive tool at clinicians' disposal (104). Uterine artery fluxometry (resistance and pulsatility indexes) showed with statistical difference lower values in the positive group in comparison to the negative group. We could also monitor increasingly higher levels during controlled ovarian stimulation for both parameters.

These findings may relate to the hormonal status during ovarian controlled stimulation and the effects of higher serum estradiol. A significant decrease of all parameters for both groups was observed on the trigger day with rhCG, with the recovery of fluxometry parameters at embryo transfer day.

The decrease of both resistance and pulsatility flow may be associated with the rhCG effect on vascularization, due to its up-regulation effect on vascular endothelial growth factors.

Subendometrial blood flow displayed a similar pattern with comparable values at baseline and increasingly higher values during stimulation and a significant decrease after rhCG administration and recovery to previous values at embryo transfer.

In a similar pattern values were statistically similar at baseline, and significantly different between the two groups afterwards.

The ratio obtained between subendometrial flow and uterine artery fluxometry parameters showed, for all parameters (resistance and pulsatility) and for both groups that values in the subendometrial area were sustained and lower in comparison to the uterine artery flow (values always under 1).

This means that subendometrial territory has lower resistance to blood flow allowing further and privileged vascularization. We could also note that in terms of resistance index the values on the implantation group were lower with a statistical difference between the two sets.

In terms of pulsatility, values were also lower in comparison to uterine artery fluxometry, but no significant pattern was met. Since we are dealing with a reason between two values (pulsatility index from subendometrial flow and uterine artery), with increasingly higher values until trigger day with rhCG, followed by a significant drop and recovery afterwards at embryo transfer day, this might be an explanation of the observed pattern (105-106).

We can highlight as the major strength of this study the awareness of a controversial topic, with a different view on the question of non-invasive methods to assess endometrial receptivity.

This new methodology of continuous or better yet, consecutive serial evaluations ascertain the discriminatory value of biophysical and biochemical parameters. Also, the fact of prospective continuous assessment using the same protocol for all subjects can be pointed out as a strength of this study.

The limited number of subjects in our sample can be deemed as a limitation or weakness of this study.

Still, this new methodological approach can now be used in a larger setting to provide further information and knowledge to underscore the information that has already been seen.

This study provides a different view of the question of non-invasive methods to assess endometrial receptivity. We have successfully been able to obtain limited data concerning a preferential pathway on endometrial assessment. However larger studies should be carried out to further enlarge our findings.

# Chapter 8.
# CONCLUSIONS

*"Aqueles pais ilustres que já deram*
*Princípio à geração que deles pende,*
*Pela virtude muito antão fizeram*
*E por deixar a casa que descende."*

in Luís Vaz de Camões – Os Lusíadas Canto VIII

# 8. Conclusions

Endometrial receptivity plays an important role in the successful outcome in ART cycles. Much has improved over recent years in the area of embryo transfer, and embryo cultures. Yet the underlying mechanism that results in failure of implantation of a good quality embryo on a supposedly receptive endometrium is still unclear.

The implantation window is the most critical period of time in human reproduction. From a clinical point of view, practitioners need to have some objective measurements to determine the probability for a healthy pregnancy. Many techniques have been developed but results are still controversial, or in some cases proven to be too invasive and lacking reliability, especially in women with irregular menstrual cycles.

Accurately predicting implantation is one of the greatest challenges. Progress in embryo transfer and culture, still relies on the uncertainty of embryo implantation even in the presence of what appears to be a receptive endometrium. Some techniques have been developed but the results are still controversial, invasive and lacking reliability. Endometrium is a living, adaptative and transformative tissue. Understanding this ability to transform, and the continuity of the process may allow better knowledge of its ability to allow the implantation process of a good quality embryo.

The underlying mechanism that results in failure of implantation of a good quality embryo on a supposed receptive endometrium is still unclear. Endometrial receptivity is still up to this date a controversial subject and a hot topic for discussion and analysis. Most published studies present conflicting results and are still controversial. The adaptative changes and continuous evolution of the endometrium make it difficult to establish a normative pattern of development in a way that provides useful information regarding its receptiveness, either in real time or in a personalized individualized setting.

The continuous evolution of the endometrium makes it difficult to establish a pattern that might be useful in identifying a receptive endometrium. Ultrasound developments have been able to clarify and make available more information about the morphokinetics of this tissue and its changes throughout the cycle.

Ultrasonographic advances over the past decades provide a useful tool to evaluate the morphokinetics of this transformative tissue. Information of what makes an endometrium receptive may be the key to solving these issues, providing a diagnostic tool that will enhance ART cycles and elective embryo transfers. This will result in more effective transfers and better ART outcomes. Also, the ability to determine in real time endometrial receptivity will shorten the time to birth lapse, thus improving quality of life for infertile couples.

Better understanding of what makes an endometrium receptive may be the key to solve these issues, providing a diagnostic tool that will enhance ART cycles and elective embryo transfers more effective in producing better outcomes.

This study showed that endometrial 3D volume analysis as well as adjusted 3D endometrium volume may identify a receptive endometrium as soon as day 6 of ovarian controlled stimulation, and the results show a high accuracy in detecting the non-receptive endometrium (with a high Predictive Negative Value of 90.1%). In this way clinicians may be made aware of this possibility, and further enhance their procedures with better knowledge of whether or not to perform embryo transfer on that given cycle.

This study showed that endometrial volumetry may identify a receptive endometrium as soon as day 6 of ovarian controlled stimulation. Serum estradiol also showed some predictive value, with statistical significance. Nevertheless, the difference noted between groups did not affect the number of oocytes in oocyte retrieval, nor the number of mature oocytes. Also, the number of cleaved embryos and blastocysts was similar in both groups. This shows that serum estadiol has potential effects on latter phases of endometrium development.

This study showed that endometrial 2D power Doppler analysis may identify a receptive endometrium as soon as day 6 of ovarian stimulation. Uterine artery fluxometry and subendometrial blood flow as single evaluation parameters, or in combination as a ratio show a clear continuous mechanism that enables the endometrium to become receptive to a healthy embryo.

In this way clinicians may be made aware of this possibility and further enhance their procedures with better knowledge whether to perform embryo transfer on that given cycle. Many articles have been published regarding endometrial receptivity, but many questions remain unanswered.

## Future Perspectives

The intricate role the endometrium plays in the game of successful reproductive outcome is still a mystery and a source for much needed further investigation.

There is no doubt that, in order for pregnancy to occur, whether by natural means or by the means of ART therapies, both a healthy embryo and a selective and receptive endometrium are required, and that the communication between the two needs to be successful.

The knowledge and science behind omics technologies will perhaps provide more questions, and better understanding of the intricate communication between embryo and endometrium.

Regarding the clinical pragmatic aspect of it, the enhancement of ultrasonography allows a better and more comprehensive view of the morphology of the transforming endometrium.

In the absence of a fully comprehensive powerful tool to outweigh early pregnancy losses, perhaps a combination of known available techniques will provide better results. In addition, the ability to continuously evaluate the endometrium will provide a powerful diagnostic tool to enhance clinical guidance, or even develop a mathematical algorithm that might be translational and complementary to ultrasound machines used in daily practice.

To sum it all up, our group will further continue not only to evaluate the morphocinetic aspect of the endometrium but will develop a parallel protocol using omics technologies.

With this approach we strongly believe that better understanding and a powerful useful tool might be developed in future years.

Other future projects and further lines of investigation in this area will be developed with the aid of the engineering and informatics department of our University. Artificial neural networks show considerable promise in ultrasound imaging, with their use enabling improved speed and accuracy of diagnosis.

Developing an artificial intelligence (AI) system can identify automatically standard reference planes during a routine ultrasound scan. With the bank of images used to develop this study authors will be able to develop an automated system capable of identifying standard planes and the requisite anatomical landmarks, all of which can be displayed in real time on the screen.

Trainees and infrequent users may find it beneficial to scan with a system capable of highlighting structures and confirming that an acquisition is adequate to be considered as a standard plane.

Contrary to concerns that AI may replace healthcare workers, a more likely scenario will be that in the foreseeable future, sonograms will increasingly interact with AI to ensure that tests are performed adequately, and the correct diagnosis reached.

# Chapter 9.
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*"Impossibilidades não façais,*
*Que quem quis, sempre pôde; e numerados*
*Sereis entre os Heróis esclarecidos*
*E nesta «Ilha de Vénus» recebidos."*

in Luís Vaz de Camões – Os Lusíadas Canto IX

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# Chapter 10.
# APPENDIX

*"Ou fazendo que, mais que a de Medusa,*
*A vista vossa tema o monte Atlante,*
*Ou rompendo nos campos de Ampelusa*
*Os muros de Marrocos e Trudante,*
*A minha já estimada e leda Musa*
*Fico que em todo o mundo de vós cante,*
*De sorte que Alexandro em vós se veja,*
*Sem à dita de Aquiles ter enveja."*

in Luís Vaz de Camões – Os Lusíadas Canto X

# Appendix 1

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Reproductive Biology and Endocrinology

RESEARCH Open Access

## Subendometrial resistence and pulsatility index assessment of endometrial receptivity in assisted reproductive technology cycles

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**Abstract**

**Objective:** To evaluate Subendometrial and Uterine artery resistance and pulsatility index continuous analysis as a predictor of Endometrial receptivity in Assisted Reproductive Technology (ART) Cycles.

**Design:** Serial 2D transvaginal coloured power doppler ultrasound performed in women on ART cycle to evaluate a pattern that better predicts implantation rates. One hundred sixty-nine subjects on a prospective case control study were assessed. Uterine artery and Subendometrial resistance and pulsatility index was performed to all subjects at baseline (prior to ovarian controlled stimulation), at day 6, 8 and 10 of controlled ovarian stimulation, at trigger day and at embryo transfer day. Also the ratio of fluxometric parameters between Subendometrial blood flow and uterine artery was measured.

**Results:** No statistical difference was noted between two groups in terms of demographics and ART procedures and scores. Uterine artery resistance and pulsatility index showed statistical difference between the two groups (implantation versus non-implantation group). Also statistical significance was obtained between two groups in terms of Subendometrial vascularization. Ratio between Subendometrial and Uterine artery showed lower values of fluxometric parameters in all range for the Subendometrial territory.

**Conclusions:** Serial Subendometrial and Uterine artery fluxometry may be a useful tool for clinicians in predicting endometrial receptivity enhancing elective embryo transfers in the same ART cycle.

**Keywords:** Endometrial receptivity, Assisted reproductive technology, Subendometrial blood flow, Uterine artery fluxometry, Embryo implantation

### Introduction

Human implantation is a complex process requiring synchrony between a healthy embryo and a functionally competent or receptive endometrium [1]. Since the introduction of assisted conception, many techniques have been developed to further improve ovarian stimulation, oocyte retrieval, and embryo culture [2]. However there has always been a lack in understanding the endometrial characteristics compatible with a successful pregnancy. To prepare for pregnancy, the endometrial lining in the uterus thickens and becomes receptive to implantation of a fertilized egg. This happens in response to hormone secretion, with oestrogen and progesterone being the primary hormones that are released to ensure the endometrial lining is receptive to pregnancy [3]. Diagnosis of endometrial receptivity (ER) has posed a challenge and so far, most available tests have been subjective and lack accuracy and a predictive value. Microarray technology has allowed identification of the transcriptomic signature of the window of receptivity - window of implantation (WOI). This technology has led to the development of a molecular diagnostic tool, the ER array (ERA) for diagnosis of ER [4]. The ERA is a

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tissue test, which evaluates the receptivity of the endometrial lining to determine the window of implantation. It is performed based on the assumed WOI for a woman during a natural cycle or an HRT (hormone replacement therapy) cycle [5]. The test consists of an endometrial biopsy to determine the optimal timing for implantation in a round of Assisted Reproductive Technology (ART) cycle [6]. Nevertheless, ERA requires an invasive procedure, it has an associated cost, and in women with irregular cycles may not prove to be cost-efficient. Ultrasound is a non-invasive technique that can assess changes in the endometrium during stimulated cycles. The use of high-resolution transvaginal probes made possible follow up throughout the cycle of endometrium changes [7]. Uterine receptivity is regulated by a number of factors including uterine perfusion, and better yet endometrial perfusion. Differences between infertile and fertile women uterine perfusion have been reported. It has been suggested that impaired uterine and endometrial perfusion may be the cause of failure. In ART cycles blood flow resistance in uterine artery and in the endometrial territory has been reported to be a predictive indicator of implantation. However using this method is still controversial in clinical applications, and many studies reported a small sample size or a single one moment observation. From a clinical point of view some objective parameters must be obtained in order to ascertain the likelihood of an on going pregnancy in ART cycles, preferably in a non-invasive and cost efficient way [8]. Many studies have been conducted on the hemodynamic changes of utero-ovarian arteries during ART cycles. For optimizing the results of ART it is critical to decide the best timing for embryo transfer. With the introduction of high-resolution transvaginal probes, the non-invasive, accessible transvaginal sonography made it particularly suitable for serial follow up throughout the stimulated cycle. The aim of this prospective study is to further evaluate the capability of serial measurement of subendometrial fluxometry doppler flow as a non-invasive procedure to determine endometrial receptivity [9–11].

## Material and methods

Prospective case control study of 169 women in ART cycles. All data collected and informed written consent was obtain according to the Ethics Committee of our Institution.

Only subjects with viable good grade embryos for transfer (double embryo transfer on day 3 of development) were selected. All subjects have been in a short protocol regimen with antagonist for ovarian stimulation using gonadotropins. All used recombinant human gonadochorionic hormone (rhCG) for induction of ovulation 36 h prior to oocyte pick up. Demographics data was collected for all patients and serial ultrasound analysis (uterine artery fluxometry and subendometrial blood blow) was performed using the same protocol for all participating subjects. Colour Doppler signals are measured in uterine artery and their ascending branches located in the outer third of the myometrium. The impedance of blood flow through the uterine arteries may be expressed as the pulsatility index (PI), unit less and angle independent, and the resistance index (RI), unit less and angle dependent. The PI is measured from the flow velocity waveforms as the systolic peak velocity minus end-diastolic velocity divided by the mean. It can be classified as low (0.00–1.99), medium (2.00–2.99) and high (over 3.0). The RI is calculated as the ratio of peak systolic flow velocity minus end-diastolic velocity divided by peak systolic velocity, ranging from 0.0 to 1.0. Subendometrial blood flow represents vessels irrigating endometrium within 10 mm of the lateral endometrium border [12–15]. During ovarian controlled stimulation serial ultrasound exams were performed and serum estradiol levels obtained for all participants. Uterine and subendometrial resistance and pulsatility index was obtained with 2D sagittal uterine view with power coloured doppler in all evaluations (Basal moment – Day 2 or 3 of women menstrual cycle and prior to begin of ovarian controlled stimulation; at Day 6, Day 8 and Day 10 after the begin of ovarian controlled stimulation; at Trigger day; and at Embryo Transfer). Blood flow evaluations were performed in the morning to avoid fluctuation due to circadian rhythm of uterine artery blood flow [16–18].

At Day 12 after embryo transfer, human gonadochorionic sub-unit B serum levels were obtained, and groups were set: positive results (for values over 5 International Units - IU) and negative results (for values under 5 IU). All data collected was analysed between these two set groups and compared.

All data was analysed using SPSS (Statistical Package for Social Sciences) version 25.0. Results are presented as mean values with standard deviation (SD). Comparisons between means among the study groups were performed using independent samples t-Test. A Value of $p < 0.05$ was considered statistically significant.

The authors do not report any conflict of interest.

The study protocol has been approved by the Ethics Committee of our Institution (CHCB 22/2017), in accordance with the relevant guidelines and regulations.

## Results

Uterine artery resistency and pulsatility index, as well as subendometrial blood flow resistance and pulsatility index was obtained in all 169 cycles using 2D power doppler transvaginal ultrasound in continuous observations. Demographics characteristics and ART parameters are shown in Table 1. Women were divided into two groups depending on the value of hCG at Day 12 after

**Table 1** Demographics and assisted reproductive technology cycle parameters between two groups. (Implantation, $N = 46$ and Non-Implantation, $N = 123$). Descriptive statistics between two groups. Mean values with standard deviation (SD)

| | Non-Implantation $N = 123$ (72.8%) | Implantation $N = 46$ (27.2%) | t-Test $p$ value |
|---|---|---|---|
| Female Age (in years) | 34.94 ± 4.03 (19–39) | 34.28 ± 3.35 (25–39) | 0.290 |
| Male Age (in years) | 36.14 ± 4.76 (22–46) | 37.19 ± 5.91 (29–62) | 0.832 |
| Time of Infertility (in months) | 54.46 ± 33.82 (12–204) | 60.22 ± 38.49 (14–192) | 0.375 |
| Type of Infertility: | | | 0.297 |
| Primary | 95/123 (77.2%) | 38/46 (82.6%) | |
| Secondary | 28/123 (22.8%) | 8/46 (17.4%) | |
| AntiMullerian hormone (pg/mL) | 2.45 ± 2.45 (0.09–16.65) | 2.62 ± 2.46 (0.04–13.56) | 0.679 |
| Antral follicle count | 8.43 ± 5.07 (2–40) | 8.63 ± 3.74 (2–20) | 0.801 |
| Total dose of gonadotropins (in International Units) | 2500.81 ± 812.19 (300–4500) | 2508.15 ± 757.91 (450–4500) | 0.956 |
| Progesterone levels at Trigger day (ng/mL) | 0.88 ± 0.44 (0.01–2.20) | 0.78 ± 0.47 (0.01–2.10) | 0.188 |
| Number of collected Oocytes | 8.25 ± 5.14 (2–22) | 10.50 ± 5.20 (2–23) | 0.140 |
| Metaphase II Oocytes | 6.57 ± 4.22 (2–17) | 7.06 ± 4.77 (2–21) | 0.150 |
| Number of day 3 embryos | 3.18 ± 2.40 (2–12) | 3.84 ± 2.65 (2–12) | 0.120 |
| Number of blastocyst for vitrification | 0.65 ± 1.51 (0–6) | 0.86 ± 1.71 (0–9) | 0.200 |

embryo transfer: 123 in the negative group – non-implantation group (72.8%) and 46 on the positive group – implantation group (27.2%). There were no statistical difference between two set groups in terms of demographics and ART parameters.

Uterine artery blood flow showed no statistical difference between two groups at baseline, both for resistance and pulsatility index. Statistical difference between two groups is shown after day 6 of ovarian controlled stimulation for both parameters in analysis (Table 2 and Fig. 1).

We can see that both resistance and pulsatility index increase its values slightly until trigger day with hCG. The results are however; always lower for the implantation group. (trigger day with hCG 0.93 ± 0.10 on the non-implantation group versus 0.88 ± 0.09 for the implantation group with $p$ value of 0.011 in the resistance index and 1.48 ± 0.38 versus 1.18 ± 0.27 with $p$ value of 0.001 for the pulsatility index). After trigger day values tend to return to previously observed during controlled ovarian stimulation.

**Table 2** Ultrasound parameters between two groups. (Uterine artery resistance index and uterine artery pulsatility index) at baseline, at day 6, 8 and 10 after controlled ovarian stimulation, at trigger day and at embryo transfer day. Mean values with standard deviation (SD). rhCG – recombinant Human chorionic gonadotropin

| | Non-Implantation $N = 123$ (72.8%) | Implantation $N = 46$ (27.2%) | t Test $p$ value |
|---|---|---|---|
| Uterine Resistance Index (Ut RI) | | | |
| Basal | 0.97 ± 0.16 | 0.92 ± 0.12 | 0.1 |
| Day 6 | 1.01 ± 0.15 | 0.94 ± 0.11 | **0.04** |
| Day 8 | 1.09 ± 0.14 | 0.97 ± 0.12 | **0.001** |
| Day 10 | 1.19 ± 0.16 | 1.07 ± 0.16 | **0.001** |
| Trigger Day with rhCG | 0.93 ± 0.10 | 0.88 ± 0.09 | **0.011** |
| Embryo Transfer Day | 1.12 ± 0.12 | 1.02 ± 0.09 | **0.001** |
| Uterine Pulsatility Index (Ut PI) | | | |
| Basal | 1.46 ± 0.51 | 1.33 ± 0.34 | 0,06 |
| Day 6 | 1.64 ± 0.45 | 1.47 ± 0.40 | **0,023** |
| Day 8 | 1.74 ± 0.47 | 1.44 ± 0.44 | **0.001** |
| Day 10 | 1.87 ± 0.43 | 1.51 ± 0.37 | **0.001** |
| Trigger Day with rhCG | 1.48 ± 0.38 | 1.18 ± 0.27 | **0.001** |
| Embryo Transfer Day | 1.91 ± 0.54 | 1.49 ± 0.42 | **0.001** |

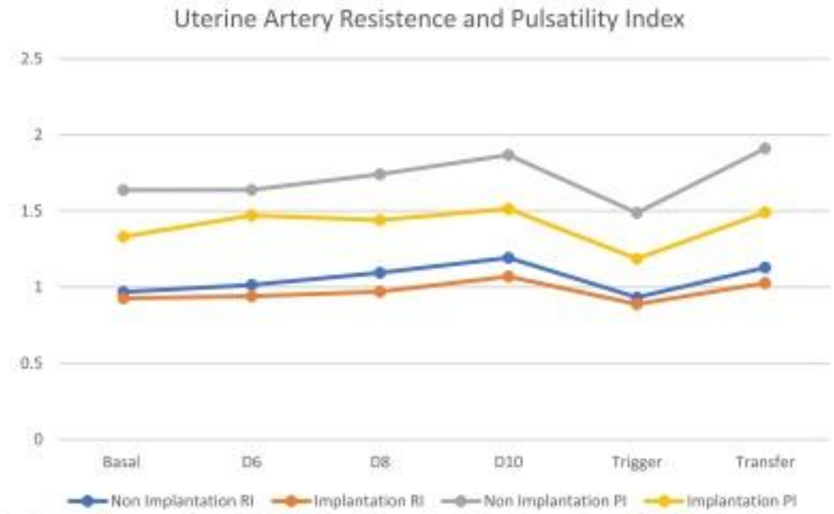


**Fig. 1** Serial Uterine Artery Resistance and Pulsatility index flow (Mean values). RI – Resistance Index; PI – Pulsatility Index

Subendometrial blood flow analysis (resistance and pulsatility index) showed no statistical difference between two groups at baseline, with increasing values for both groups during controlled ovarian stimulation. During that period and also at trigger, and at embryo transfer day, there was statistical difference between two groups with lower scores for the implantation Group (resistance index of 0.78 ± 0.16 versus 0.65 ± 0.12 with $p$ value of 0.001 and pulsatility index of 0.95 ± 0.14 versus 0.83 ± 0.14 with $p$ value of 0.001 for non-implantation versus implantation group at trigger day respectively) (Table 3 and Fig. 2).

The ratio between subendometrial blood flow and uterine artery fluxometry showed no statistical difference between both groups at baseline for the resistance index. Statistical difference between two groups was set after controlled ovarian stimulation, trigger day with hCG and at embryo transfer for the resistance Index. However, in terms of pulsatility index, no statistical difference was met between the two groups except for the trigger day with hCG and at embryo transfer day (0.72 ± 0.12 versus 0.68 ± 0.11 with $p$ value of 0.01and 0.66 ± 0.12 versus 0.70 ± 0.08 with $p$ value of 0.018, for the non-implantation versus implantation group respectively) (Fig. 3).

In this study the intraobserver reliability was 0.96. In addition, because the same operator performed all measurements, in this study there was no interobserver variability.

## Discussion

Endometrial receptivity in ART cycle has always been a challenge for physicians that need real time data in order to make better treatment options [12–15]. Vaginal 2D power doppler ultrasound is a non-invasive and a relative inexpensive tool at clinician's disposal [16]. Single analysis of endometrial pattern at trigger day has been the most used, with contradictory findings. Also sample size of many studies led to conflicting results and further investigation in this area has been postponed with the advent of other technologies. In this study we aimed to address these issues in a wholesome way with several observations over time with a good sample size in order to obtain further data and better knowledge of the working process underlying endometrial receptivity.

Uterine artery fluxometry (resistance and pulsatility index) showed with statistical difference lower values in the implantation group in comparison to the non-implantation group. We could also monitor increasingly higher levels during controlled ovarian stimulation for both parameters. These findings may relate to the hormonal status during ovarian controlled stimulation and the effects of higher serum estradiol. A significant decrease of all parameters for both groups was observed on the trigger day with rhCG, with the recovery of fluxometry parameters at embryo transfer day. The decrease of both resistance and pulsatility flow may be associated with the rhCG effect on vascularization, due to its up-regulation effect on vascular endothelial growth factors.

Subendometrial blood flow displayed a similar pattern with comparable values at baseline and increasingly higher values during stimulation and also a significant decrease after rhCG administration and recovery to previous values at embryo transfer. In a similar pattern values were statistical similar at baseline, and significantly different between the two groups afterwards.

**Table 3** Ultrasound parameters between two groups. (Subendometrial resistance index, subendometrial pulsatility index, subendometrial / uterine artery resistance index ratio and subendometrial / uterine artery pulsatility index ratio) at baseline, at day 6, 8 and 10 after controlled ovarian stimulation, at trigger day and at embryo transfer day. Mean values with standard deviation (SD). SE/Ut – Subendometrial / Uterine Arteries ratio; rhCG – recombinant Human chorionic gonadotropin

| | Non Implantation $N = 123$ (72.8%) | Implantation $N = 46$ (27.2%) | t Test $p$ value |
|---|---|---|---|
| Basal | | | |
| Subendometrial Resistance Index | 0.77 ± 0.17 | 0.71 ± 0.17 | 0.82 |
| Subendometrial Pulsatility Index | 1.16 ± 0.25 | 1.01 ± 0.26 | 0.1 |
| SE/Ut RI ratio | 0.80 ± 0.09 | 0.76 ± 0.12 | 0.6 |
| SE/Ut PI ratio | 0.73 ± 0.12 | 0.77 ± 0.11 | 0.117 |
| Day 6 after Controlled Ovarian Stimulation | | | |
| Subendometrial Resistance Index | 0.84 ± 0.17 | 0.73 ± 0.14 | **0.001** |
| Subendometrial Pulsatility Index | 1.14 ± 0.20 | 0.98 ± 0.22 | **0.001** |
| SE/Ut RI ratio | 0.82 ± 0.10 | 0.77 ± 0.09 | **0.04** |
| SE/Ut PI ratio | 0.72 ± 0.14 | 0.68 ± 0.12 | 0.132 |
| Day 8 after Controlled Ovarian Stimulation | | | |
| Subendometrial Resistance Index | 0.95 ± 0.16 | 0.79 ± 0.18 | **0.001** |
| Subendometrial Pulsatility Index | 1.24 ± 0.20 | 1.03 ± 0.25 | **0.001** |
| SE/Ut RI ratio | 0.87 ± 0.13 | 0.81 ± 0.12 | **0.04** |
| SE/Ut PI ratio | 0.75 ± 0.15 | 0.73 ± 0.10 | 0.615 |
| Day 10 after Controlled Ovarian Stimulation | | | |
| Subendometrial Resistance Index | 1.04 ± 0.718 | 0.88 ± 0.19 | **0.001** |
| Subendometrial Pulsatility Index | 1.32 ± 0.23 | 1.12 ± 0.31 | **0.001** |
| SE/Ut RI ratio | 0.88 ± 0.11 | 0.82 ± 0.08 | **0.04** |
| SE/Ut PI ratio | 0.72 ± 0.10 | 0.75 ± 0.11 | 0.251 |
| Trigger Day with rhCG | | | |
| Subendometrial Resistance Index | 0.78 ± 0.16 | 0.65 ± 0.12 | **0.001** |
| Subendometrial Pulsatility Index | 0.95 ± 0.14 | 0.83 ± 0.14 | **0.001** |
| SE/Ut RI ratio | 0.84 ± 0.14 | 0.73 ± 0.09 | **0.01** |
| SE/Ut PI ratio | 0.72 ± 0.12 | 0.68 ± 0.11 | **0.01** |
| Embryo Transfer Day | | | |
| Subendometrial Resistance Index | 0.99 ± 0.15 | 0.87 ± 0.12 | **0.001** |
| Subendometrial Pulsatility Index | 1.19 ± 0.17 | 1.07 ± 0.20 | **0.001** |
| SE/Ut RI ratio | 0.84 ± 0.15 | 0.74 ± 0.10 | **0.001** |
| SE/Ut PI ratio | 0.66 ± 0.12 | 0.70 ± 0.08 | **0.018** |

The ratio obtained between subendometrial flow and uterine artery fluxometry parameters showed, for all parameters (resistance and pulsatility) and for both groups that values in the subendometrial compartment were sustained and lower in comparison to the uterine artery flow (values always under 1). This means that subendometrial territory has lower resistance to blood flow allowing further and privileged vascularization. We could also note that in terms of resistance index the values on the implantation group were lower with statistical difference between the two sets. In terms of pulsatility, values were also lower in comparison to uterine artery fluxometry, but no significant pattern was met. Since we are dealing with a reason between two values (pulsatility index from subendometrial flow and uterine artery), with increasingly higher values until trigger day with rhCG, followed by a significant drop and recovery afterwards at embryo transfer day, this might be an explanation to the observed pattern [17].

We could not refrain to uphold expectation of these results as they show a serial of values, demonstrating a certain pattern of evolution that one should expect from a transforming living tissue and its natural

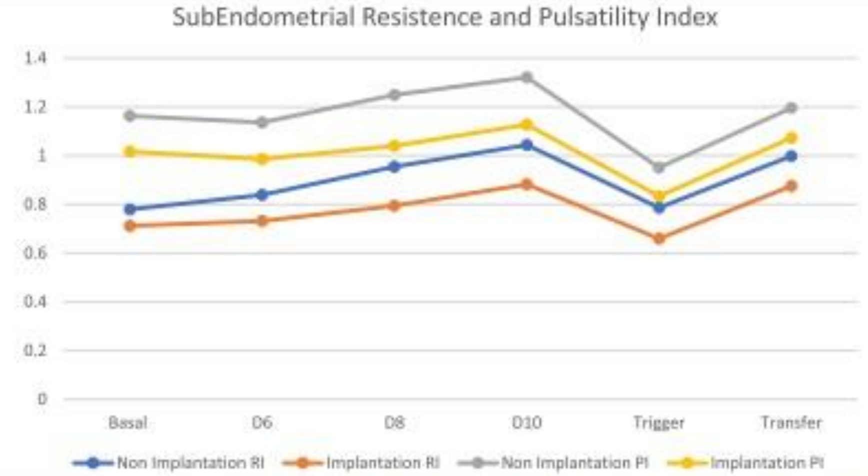


**Fig. 2** Serial Subendometrial artery Resistance and Pulsatility index flow (Mean values). RI – Resistance Index; PI – Pulsatility Index

adaptations in need to further assist on the complex binding process of implantation.

## Conclusions

Endometrial receptivity plays an important role in the successful outcome in ART cycles. Much has improved over recent years in the area of embryo transfer, and embryo cultures. Yet the underlying mechanism that results in failure of implantation of a good quality embryo on a supposed receptive endometrium is still unclear. Implantation window is the most critical period of time in human reproduction. In a clinical point of view, practitioners need to have some objective measurements to determine the probability for a healthy pregnancy.

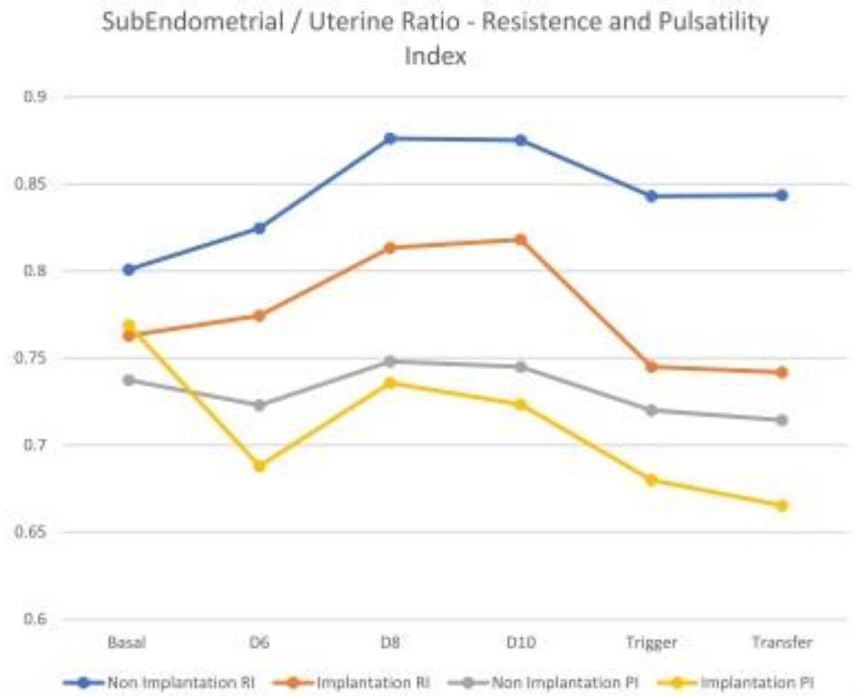


**Fig. 3** Serial Subendometrial / Uterine Arteries Ratio – Resistance and Pulsatility index flow (Mean values) RI – Resistance Index; PI – Pulsatility Index

Many techniques have been developed but results are still controversial, or in some cases proven to be too invasive and lacking reliability especially in women with irregular menstrual cycles.

The continuous evolution of endometrium makes it difficult to establish a pattern that might be useful in identifying a receptive endometrium.

Ultrasound developments have been able to clarify and make aware more information about the morphokynetics of this tissue and its changes throughout the cycle. Better understanding of the role that makes an endometrium receptive may be the key in solving these issues, providing a diagnostic tool that will enhance ART cycles and elective embryo transfers more effective in producing better outcomes.

This study showed that endometrial 2D power doppler analysis may identify a receptive endometrium as soon as day 6 of ovarian stimulation. Uterine artery fluxometry and subendometrial blood flow as single evaluation parameters, or in combination as a ratio show a clear continuous mechanism that enables endometrium to become receptive to a healthy embryo. In this way clinicians may be made aware of this possibility and further enhance its procedures with better knowledge weather or not to perform embryo transfer on that given cycle.

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#### Authors' contributions
RSM, AHO and JMO are responsible for the study design. RSM has been the principal investigator and the principal collector of data. RSM has been responsible for data analysis. DVO, AHO and JMO have been responsible for reviewing the article for publication. All authors read and approved the final manuscript.

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#### Availability of data and materials
Encrypted non-disclosure data available at Open Science Framework database for peer review purpose only. Project name Physical Biomarkers in Endometrial Receptivity with access link: https://osf.io/hr25m/?view_only=8d5f6dcb8b25420bbd9188382163e7d7

#### Ethics approval and consent to participate
The study has been approved by the Ethics Committee of our Institution (CHCB 22/2017). Oral and written consent was obtained for all willing participants prior to registering for this study. Patient Informed consent to participate in this study CHCB 22/2017.
The authors have consented for publication.

#### Competing interests
The authors declare that they have no competing interests.

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## Publisher's Note

# Appendix 2

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RESEARCH ARTICLE Open Access

## Continuous endometrial volumetric analysis for endometrial receptivity assessment on assisted reproductive technology cycles

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**Abstract**

**Background:** Human implantation is a complex process requiring synchrony between a healthy embryo and a functionally competent or receptive endometrium. In order to assess endometrial receptivity in Assisted Reproductive Technology (ART) cycles serial evaluation of endometrial volumetric analysis may have a predictive value on a positive outcome.

**Methods:** Serial 3D transvaginal ultrasound performed in women on ART cycle to evaluate embryo implantation predictors. Prospective case control study of 169 subjects were assessed. Endometrial pattern, thickness, volume and adjusted endometrial volume (ratio between endometrial volume and uterine volume) was performed to all subjects on a continuous process from baseline, during controlled ovarian stimulation, trigger day with human chorionic gonadotropin hormone (hCG) and at embryo transfer day.

**Results:** Demographics and ART procedures and scores, was similar between the two groups. Endometrial morphology also showed no difference between the two groups. Endometrial volume and adjusted endometrial volume was significantly higher in the positive group as soon as day 6 of ovarian controlled stimulation.

**Conclusions:** Serial 3D endometrial volume and adjusted endometrial volumes provides a predicting clinical tool enhancing elective embryo transfers in fresh ART cycle. Thus providing a non-invasive continuous technique for endometrial receptivity assessment that reflects endometrial changes during ART procedures.

**Keywords:** Endometrial receptivity, Assisted reproductive technology, Endometrial volume, Adjusted endometrial volume, Embryo implantation

### Background

Successful assisted reproductive technology cycles outcome depends on the intricate interplay between embryo quality and endometrial receptivity. Endometrium is a dynamic tissue that grows, differentiates and suffers regression throughout the menstrual cycle in response to hormonal regulation to prepare the uterus for embryo implantation [1]. Endometrium is a highly dynamic tissue undergoing physiological changes in response to ovarian steroid hormones. It has been proven that the supraphysiological hormonal levels as in the ART cycle has a harmful effect on endometrial receptivity. Endometrial characteristics compatible with a successful pregnancy have proven to be difficult to be properly assessed. Adequate endometrial development seems to be important for implantation given that previous studies have shown an association between abnormal glandular or vascular development and defective placentation disorders. The window of implantation (WOI) is defined as a short period of

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time while the endometrium is receptive to the embryo [2, 3].

Diagnosis of endometrial receptivity (ER) has posed a challenge and so far, most available tests have been subjective and lack accuracy and a predictive value [4].

The use of transcriptomic signature of the WOI by microarray technology is possible, however it demands an endometrial biopsy [5]. This requires an invasive procedure and it has an associated cost. Also, in women with irregular cycles it may not prove to be cost-efficient [6].

Ultrasound can asses changes in the endometrium during stimulated cycles. It also has minimal inter-observer and intra-observer variability. Monitoring of both the endometrial and ovarian response to ovarian stimulation on ART cycles with transvaginal ultrasound has become an important predictor of the success of ART. Published studies have conflicting results on this subject, but the common feature in all, is the lack of continuity on endometrial assessment [7]. The use of high-resolution transvaginal probes makes it possible to follow endometrium changes throughout the cycle [8]. From a clinical point of view some objective parameters must be obtained in order to ascertain the likelihood of an ongoing pregnancy in ART cycles, preferably in a non-invasive and cost-efficient way [9]. Some published work has recently proven a pattern of hemodynamic changes in utero-ovarian arteries during ART cycles with predictive value on endometrial receptivity [10]. Hou et al. have also confirmed the possibility of non-invasive prediction of success in ART cycles, with serial assessments of the echogenicity pattern transformation, after human recombinant gonadrotropin hormone [11]. The aim of this prospective study is to further evaluate the capability of serial and continuous evaluation of biophysical markers as a non-invasive procedure to determine endometrial receptivity [12, 13].

## Methods

Prospective cohort study of 169 women in ART cycles. All infertile couples submitted to ART treatment at our institution were included from January 2017 to December 2018 (2 Year period). Canceled treatments prior to oocyte pickup; cycles with missing or erroneous data; and cycles with elective single embryo transfer were excluded. (Fig. 1.)

The primary data source for this study was the local databases routinely used in the participating centre in ongoing treatments. The data output was anonymized in the extraction for statistical treatment purposes. All data collected and written informed consent was obtained according to the Ethics Committee of our Institution.

Subjects with double viable good grade embryo transfer on day 3 of embryo development were included. All subjects have been in a short protocol regimen with antagonist for ovarian controlled stimulation using gonadotropins. All subjects were submitted to recombinant human chorionic gonadotropin hormone (rhCG) for induction of ovulation 36 h prior to oocyte pick up.

Ultasonographic protocol for serial ultrasound analysis (endometrial morphology, endometrial thickness, endometrial volume and uterine volume) was performed.

During ovarian controlled stimulation serial ultrasound exams were performed, using 3D transvaginal probe.

Ultrasonographic markers were obtained in all evaluations (Basal moment – day 2 or 3 of women menstrual cycle and prior to onset of ovarian controlled stimulation; at day 6, day 8 and day 10 after initiating ovarian controlled stimulation; at Trigger day with recombinant human gonadochorionic hormone; and at embryo transfer day).

Endometrial morphology was based on the two grade system by *Sher et al.* [14]: non-multilayered homogeneous hyperechogenic or iso-echogenic endometrium compared with the myometrium and multilayered triple-line pattern, 'halo pattern' with an outer peripheral layer of denser echogenicity and a central sonolucent area.


**Fig. 1** Study Population and Exclusion Criteria

Endometrial thinckness was obtained in milimeters (mm) on the long axis or sagittal plane, with the entirely of the endometrial lining through and endocervical canal in view. The measurement was taken of the thickest echogenic area from one basal endometrial interface across the endometrial canal to the other basal surface.

Endometrial volume calculation by 3D ultrasound presented as voxels and geometric information of surfaces in a 3D dataset. The results obtained are then converted to mililitres. Adjusted Endometrial volume was also obtained as a ratio between endometrial volume calculated on 3D analysis and uterine volume based on 3D volumetric acquisitions which then generated an estimated uterine volume (also in mililiters). Adjusted endometrial volume deflects the potential difference in uterine volume from each single individual.

At day 12 after successful embryo transfer, human gonadochorionic sub-unit B serum levels were obtained, and groups were set: positive results (for values over 5 International Units - IU) and negative results (for values under 5 IU). For the aim of our study the positive cases were afterwards assessed and classified with positive clinical pregnancy by the evidence of at least one viable foetus by ultrasound performed 2 weeks after the positive biochemical result.

All data collected was analysed between these two set groups and compared.

Data was analysed in Excel 2019 (Microsoft Corp, Redmond, WA) and IBM SPSS statistics v25 (IBM Corp. Armonk, NY). Continuous variables were analysed with Levene's test (equality of variances) and visual assessment of the histogram (normality).

For analysis of parametric continuous variables, a t-student test for independent samples was used. Chi-square and Fisher's exact tests were used to analyse associations between categorical variables. Endometrial thickness, endometrial volume and adjusted endometrial volume were analysed using analysis of variance for repeated measurement data.

Value of $p < .05$ was considered statistically significant.

The authors do not report any conflict of interest.

The study protocol has been approved by the Ethics Committee of our Institution (CHCB 22/2017), in accordance with the relevant guidelines and regulations. This study has been conducted in accordance with legal and regulatory requirements, as well as follow generally accepted research practices described in International Conference Harmonisation (ICH) guidelines, Good Clinical Practices (GCP) and the Declaration of Helsinki.

## Results

Clear morphology and volume of the endometrium was obtained in all 169 cycles using 3D transvaginal ultrasound in continuous serial observations. Demographics characteristics and ART parameters are shown in Table 1.

Women were divided into two groups depending on the value of hCG at Day 12 after embryo transfer and ultrasound confirmation of clinical pregnancy: 123 on the negative group (72.8%) and 46 on the positive group (27.2%).

There were no statistical difference between the two set groups in terms of demographics and ART parameters: mean age of female partner or mean age of male

**Table 1** Demographics and ART parameters between two Groups

| | Negative Group $N = 123$ (72.8%) | Positive Group $N = 46$ (27.2%) | t-Test $p$ value |
|---|---|---|---|
| Female Age (in years) | 34.94 ± 4.03 (19–39) | 34.28 ± 3.35 (25–39) | 0.290 |
| Male Age (in years) | 36.14 ± 4.76 (22–46) | 37.19 ± 5.91 (29–62) | 0.832 |
| Time of Infertility (in months) | 54.46 ± 33.82 (12–204) | 60.22 ± 38.49 (14–192) | 0.375 |
| Type of Infertility: | | | |
| • Primary | 95/123 (77.2%) | 38/46 (82.6%) | 0.297 |
| • Secondary | 28/123 (22.8%) | 8/46 (17.4%) | |
| Antimullerian hormone (pg/mL) | 2.45 ± 2.45 (0.09–16.65) | 2.62 ± 2.46 (0.04–13.56) | 0.679 |
| Antral follicle count | 8.43 ± 5.07 (2–40) | 8.63 ± 3.74 (2–20) | 0.801 |
| Total dose of gonadotropins (in International Units) | 2500.81 ± 812.19 (300–4500) | 2508.15 ± 757.91 (450–4500) | 0.956 |
| Progesterone levels at Trigger day (ng/mL) | 0.88 ± 0.44 (0.01–2.20) | 0.78 ± 0.47 (0.01–2.10) | 0.188 |
| Number of collected Oocytes | 8.25 ± 5.14 (2–22) | 10.50 ± 5.20 (2–23) | 0.140 |
| Metaphase II Oocytes | 6.57 ± 4.22 (2–17) | 7.06 ± 4.77 (2–21) | 0.150 |
| Number of day 3 embryos | 3.18 ± 2.40 (2–12) | 3.84 ± 2.65 (2–12) | 0.120 |
| Number of blastocyst for vitrification | 0.65 ± 1.51 (0–6) | 0.86 ± 1.71 (0–9) | 0.200 |

(Positive Group, $N = 46$ and Negative Group, $N = 123$)
Descriptive statistics between two Groups. Mean values with standard deviation (SD)

partner, duration and type of infertility, total drug dose used for ovarian stimulation, overall median number of harvested oocytes per cycle defined as the total number of oocytes harvested during oocyte pick up procedure, rate of collected metaphase II (MII) oocytes. Also, the mean number of cleaved embryos at day 3 of embryo development, and mean number of blastocysts for cryopreservation showed no significant statistical difference between the two set groups.

Endometrial morphology showed no statistically significant difference between the two set groups.

Endometrial thickness on a single 2D sagittal profile showed no statistical difference at baseline and at Day 6 after ovarian controlled stimulation. Statistically significant difference was only met at a later phase of ovarian controlled stimulation – at day 8 and on following evaluations.

Uterine Volume was comparable between the two Groups with no statistical difference between the two. (Table 2).

Endometrial Volume and Adjusted Endometrial Volume showed statistical difference from Day 6 after Ovarian controlled stimulation (Table 3 and Figs. 2 and 3). Consistently higher values were seen, for both ultrasonographic markers on the positive group. In terms of endometrial volume, the positive group had statistically significant higher values in all observations except at the basal moment prior to ovarian controlled stimulation (2.77 ± 0.63 vs 2.52 ± 0.71 with $p$ value of 0.54). Similar findings were noted on adjusted endometrial volume (Table 3).

By comparing the difference between two consecutive measurements (endometrial growth rate) for endometrial and adjusted endometrial volumes, and the overall difference between the final value and the initial basal measurement we were able to note with statistical difference that values were higher on the positive group on initial phases of endometrial development and in the overall assessment (Table 4 and Fig. 4).

In terms of endometrial volume assessment, a cut off ≥5 mL in the prediction of endometrial receptivity was used with good sensitivity (85%) and low specificity (69%) in a group application; in individual setting it had a good predictive negative value (90.1%) and low predictive positive value (81.1%), with a diagnostic accuracy of 75%.

The degree of agreement was assessed by calculation of Kappa (κ) statistics. κ is a statistical parameter of agreement that does not require any assumption of the correct diagnosis, expressed through a coefficient ranging between − 1.0 and + 1.0. Perfect agreement corresponds to a coefficient of 1.0, a value of zero indicates agreement no better than that expected by chance, and negative values indicate agreement worse than that expected by chance. There is no absolute way to interpret the values between 0 and 1. As a guideline Landis and Koch indicated that values of < 0.20 suggest poor agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 good agreement and > 0.81 excellent agreement. In this study the intra-observer reliability was 0.96.

In addition, because all measurements were performed by the same operator in this study there was no inter-observer variability.

## Discussion

In this study we aimed to assess endometrial evolution in order to ascertain a plausible predictive non-invasive

**Table 2** Ultrasound parameters between two Groups - Endometrial morphology and Endometrial thickness at baseline, at day 6, 8 and 10 after controlled ovarian stimulation, at trigger day and at embryo transfer day

| | | **Negative Group** $N = 123$ **(72.8%)** | **Positive Group** $N = 46$ **(27.2%)** | ***p* Value** |
|---|---|---|---|---|
| Basal | Endometrial Morphology (ML/NM) | 0% / 100% | 0% / 100% | NS |
| | Endometrial Thickness (in mm) | 4.32 ± 0,72 | 4.22 ± 0,51 | 0.387 |
| Day 6 after Controlled Ovarian Stimulation | Endometrial Morphology (ML/NM) | 78.9% / 21.1% | 93.5% / 6.5% | 0.15 |
| | Endometrial Thickness (in mm) | 6.32 ± 0.96 | 6.28 ± 0.75 | 0.827 |
| Day 8 after Controlled Ovarian Stimulation | Endometrial Morphology (ML/NM) | 100% / 0% | 100% / 0% | NS |
| | Endometrial Thickness (in mm) | 7.47 ± 0.80 | 7.96 ± 0.79 | **0.01** |
| Day 10 after Controlled Ovarian Stimulation | Endometrial Morphology (ML/NM) | 100% / 0% | 100% / 0% | NS |
| | Endometrial Thickness (in mm) | 8.01 ± 1.04 | 8.61 ± 0.98 | **0.01** |
| Trigger Day with rhCG | Endometrial Morphology (ML/NM) | 100% / 0% | 100% / 0% | NS |
| | Endometrial Thickness (in mm) | 8.53 ± 1.32 | 9.59 ± 1.44 | **0.001** |
| Embryo Transfer Day | Endometrial Morphology (ML/NM) | 4.1% / 95.9% | 4.3% / 95.7% | 0.613 |
| | Endometrial Thickness (in mm) | 9.06 ± 1.30 | 10.15 ± 1.35 | **0.001** |

Ratios in percentages (%) and mean values with standard deviation (SD). *NM* Non multi-layered endometrium; *ML* Multi-layered endometrium; *rhCG* recombinant human chorionic gonadotropin; *NS* No statistical analysis performed

**Table 3** Ultrasound parameters between two groups - Endometrial volume and adjusted endometrial volume at baseline, at day 6, 8 and 10 after controlled ovarian stimulation, at trigger day and at embryo transfer day

| | | Negative Group $N = 123$ (72.8%) | Positive Group $N = 46$ (27.2%) | t-Test *p* Value |
|---|---|---|---|---|
| Basal | Endometrial Volume (in mm3) | 2.52 ± 0.71 | 2.77 ± 0.63 | 0.54 |
| | Adjusted Endometrial Volume | 4.60 ± 1.42 | 5.51 ± 1.28 | 0.21 |
| Day 6 after Controlled Ovarian Stimulation | Endometrial Volume (in mm3) | 3.08 ± 0.66 | 3.33 ± 0.57 | **0.024** |
| | Adjusted Endometrial Volume | 5.63 ± 1.50 | 6.67 ± 1.38 | **0.001** |
| Day 8 after Controlled Ovarian Stimulation | Endometrial Volume (in mm3) | 3.90 ± 0.94 | 4.40 ± 0.71 | **0.002** |
| | Adjusted Endometrial Volume | 7.28 ± 2.67 | 8.98 ± 2.47 | **0.001** |
| Day 10 after Controlled Ovarian Stimulation | Endometrial Volume (in mm3) | 4.12 ± 1.01 | 4.91 ± 0.82 | **0.001** |
| | Adjusted Endometrial Volume | 7.60 ± 2.54 | 9.99 ± 2.61 | **0.001** |
| Trigger Day with rhCG | Endometrial Volume (in mm3) | 4.52 ± 1.00 | 5.33 ± 0.76 | **0.001** |
| | Adjusted Endometrial Volume | 8.30 ± 2.52 | 10.76 ± 2.62 | **0.001** |
| Embryo Transfer Day | Endometrial Volume (in mm3) | 4.84 ± 1.01 | 5.59 ± 0.77 | **0.001** |
| | Adjusted Endometrial Volume | 8.32 ± 2.58 | 10.83 ± 2.73 | **0.001** |

Ratios in percentages (%) and mean values with standard deviation (SD). *rhCG* recombinant human chorionic gonadotropin

diagnostic tool for clinicians to better understand endometrium changes.

The pregnancy potential of good quality embryos is still not high on ART cycles, even with the progress in the programs of ovarian stimulation, ART technique and embryo development and culture. Implantation is still strongly reliable on the cross talk between a healthy good quality embryo and the receptive endometrium.

Although several parameters have been used to assess the pregnancy rate in ART cycles, there is still some controversy about its efficacy, and underlying mechanisms in endometrial receptivity [15–18]. Vaginal 3D ultrasound is a non-invasive and an inexpensive tool at clinician's disposal [19]. The process of endometrial transformation from proliferative phase to secretory phase under the steroids hormonal influence, called endometrial decidualization is a set goal for optimal implantation. The cyclic changes of endometrium are regulated by ovarian hormones and its receptors, and endometrial luteal phase development may alter in ART cycles due to supraphysiological hormone levels.

Contradictory findings is often reported in single analysis of endometrial pattern at trigger day.

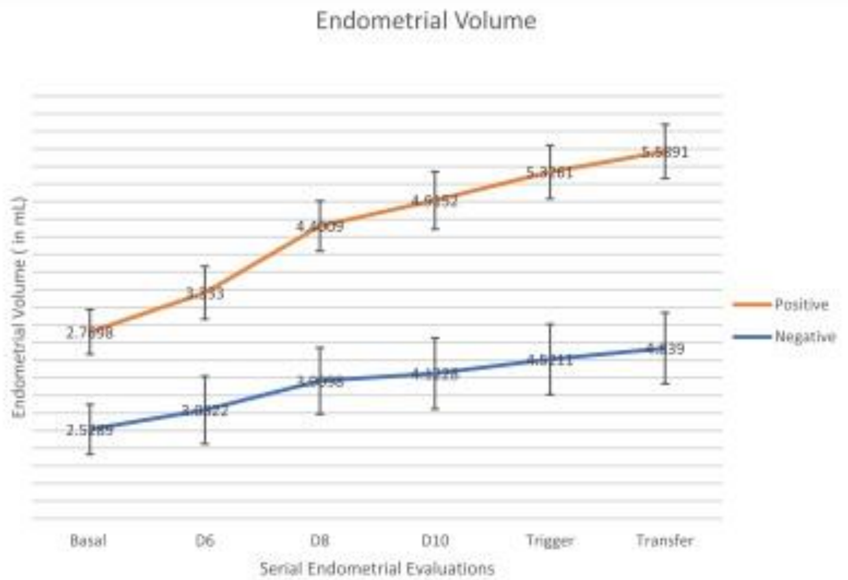


**Fig. 2** Continuous endometrial volume analysis (Mean values with Standard Deviation)

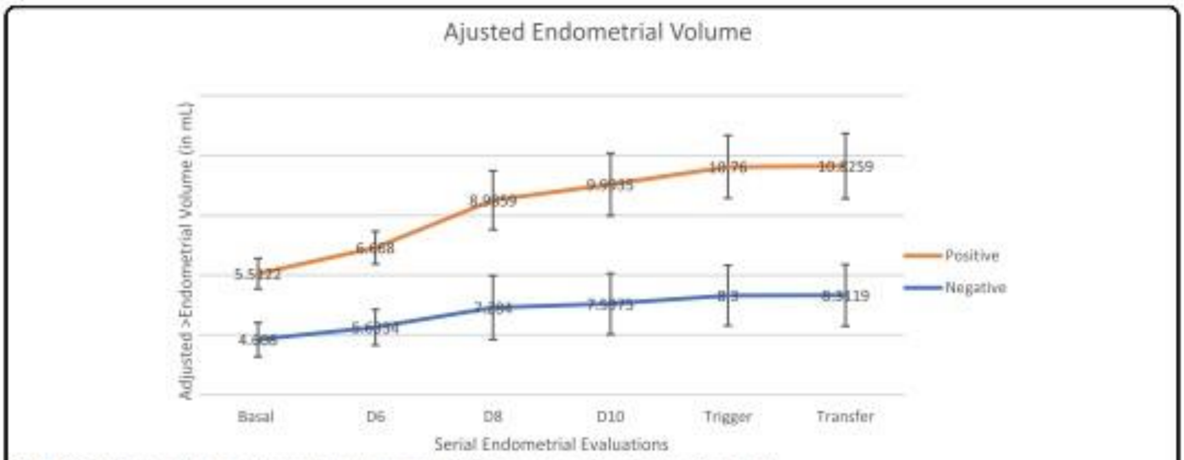


**Fig. 3** Continuous adjusted endometrial volume analysis. (Mean values with Standard Deviation)

Recent studies (*Silva Martins, R.* et al.) have proven that perhaps serial evaluations provide better understanding rather than a single scoop at a pre-determined phase of the process. It has been proven that in terms of angiogenesis that there is a certain pattern of evolution. This relates to what one should expect from a transforming living tissue and its natural adaptations on the complex binding process of implantation.

The main purpose of this study was to further evaluate potential ultrasonographic markers that might be evaluated in the continuous changes that endometrium goes through during an ART cycle. The possibility to use non-invasive techniques to determine endometrial receptivity would allow better clinical judgment. This way on the clinical point of view, the decision to transfer a fresh embryo on that same ART cycle or postpone it for a deferred transfer with better endometrial conditions is possible. This non-invasive tool to predict endometrial receptivity will improve clinical setting and allow better understanding of endometrial receptivity. This may be the way to optimize and achieve greater results in ART cycles.

In our study endometrial morphology proven not to be useful and no significant difference was found between the two groups. Also 2-D endometrial thickness showed no difference at early stages of ovarian controlled stimulation, but significant difference could be seen after day 8 of stimulation. These findings are compatible to the ones provided by the literature. The main reason for such may be the fact that subjective tools produce conflicting results and therefore are not able to provide an accurate diagnostic tool for endometrial receptivity assessment.

Endometrial volume and adjusted endometrial volume proven to be more effective with differences shown since early stages of ovarian controlled stimulation. Both groups were similar at baseline but as soon as controlled ovarian stimulation started, the differences between the ones with a positive outcome and the negative group were clearly met.

We have also been able to show differences between the two groups in terms of endometrial and adjusted endometrial volume in early stages of endometrial development under the influence of controlled ovarian stimulation. Higher volumes were seen in the positive controls, but the changes were more evident in early stages (especially between day 6 and day 8 of ovarian

**Table 4** Endometrial and Adjusted Endometrial volume Growth rate (difference between two consecutive continuous evaluations, and Overall difference between final and first evaluation)

| **Endometrial Volume Growth Rate** | | | | | | |
|---|---|---|---|---|---|---|
| | **GR1** | **GR2** | **GR3** | **GR4** | **GR5** | **Overall GR** |
| Negative EV | 0,5533 | 0,8275 | 0,4357 | 0,3626 | 0,2861 | 24,654 |
| Positive EV | 0,5632 | 10,657 | 0,5686 | 0,4108 | 0,326 | 29,367 |
| p Value | 1,34 | **0,01** | 0,74 | 0,987 | 0,567 | **0,01** |
| **Adjusted Endometrial Volume Growth Rate** | | | | | | |
| | GR1 | GR2 | GR3 | GR4 | GR5 | Overall GR |
| Negative AdjEV | 0,0102 | 0,165 | 0,077 | 0,0063 | 0,005 | 0,0459 |
| Positive Adj EV | 0,0115 | 0,231 | 0,112 | 0,0083 | 0,007 | 0,061 |
| p Value | 1,43 | **0,01** | 0,53 | 0,873 | 0,678 | **0,01** |

*GR1* Growth rate 1 (difference between Basal Moment and Day 6 after controlled ovarian stimulation) GR2 – Growth rate 2 (difference between Day 8 and Day 6 after controlled ovarian stimulation) GR3 – Growth rate 3 (difference between Day 10 and Day 8 after controlled ovarian stimulation) GR4 – Growth rate 4 (difference between Trigger with hCG day and Day 10 after controlled ovarian stimulation) GR5 – Growth rate 5 (difference between Transfer Day and Trigger with hCG day) Overall GR – Overall Growth day (difference between embryo transfer day and Basal moment) Negative EV – Negative Group of Endometrial Volume; Positive EV – Positive Group of Endometrial Volume; *Negative AdjEV* Negative Group of Adjusted Endometrial Volume; *Positive AdjEV* Positive Group of Adjusted Endometrial Volume. Value of $p < .05$ considered statistically significant

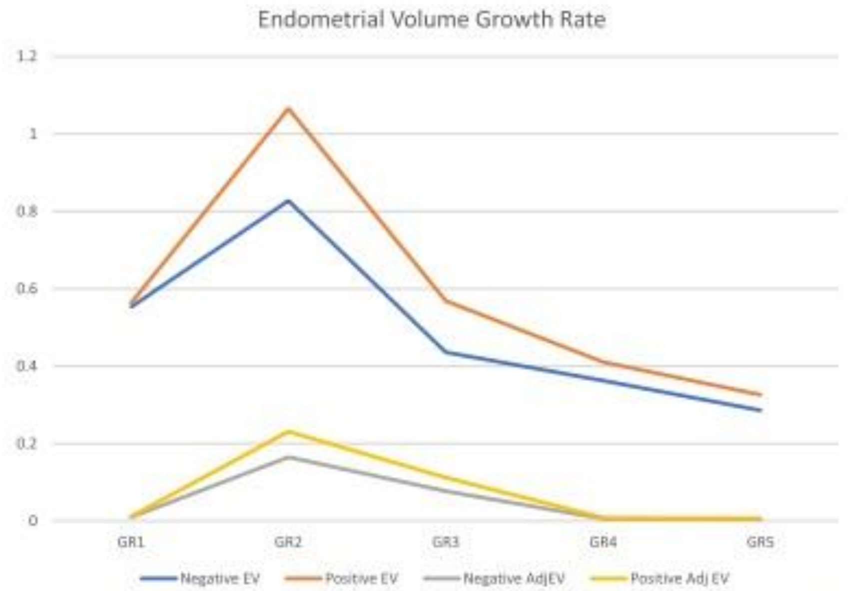


**Fig. 4** Endometrial and Adjusted Endometrial Volume Growth Rate (difference between two consecutive measurements). Negative EV – Negative Group of Endometrial Volume; Positive EV – Positive Group of Endometrial Volume; Negative AdjEV – Negative Group of Adjusted Endometrial Volume; Positive AdjEV – Positive Group of Adjusted Endometrial Volume

controlled stimulation). This is also corroborated by the fact that growth rate was statistically higher on the positive group, but the higher difference was met in early stages of endometrial development (between day 6 and day 8). The supraphysiological environment produced by the controlled ovarian stimulation may have leading role in such. Our study showed that serial continuous endometrial volume was significantly higher on the positive group, whereas studies from Kupesic et al., and Wu et al., [20, 21] have conflicting results in the assessment of endometrial receptivity. The difference can possibly be explained by the fact of serial continuous evaluations better reflect endometrial changes, rather than predetermined single scoop analysis.

The use of a cut off ≥5 mL in endometrial volume in prediction of endometrial receptivity had a good sensitivity and low specificity and may be used as a good test to exclude success. The fact that we can have a tool that indicates a non-optimal endometrium aids in the decision process of postponing embryo transfer for a more suitable and receptive endometrium.

The possibility of real time non-invasive continuous assessment of the endometrium further induces clinicians to better medical decisions. The current study demonstrated that it is possible to evaluate endometrial morphologic parameters, serial endometrial volume and adjusted serial endometrial volume in the coronal plane in accordance with published method by Mercer et al. [22]

All findings may prove to be a useful management tool for clinicians in order to establish a diagnostic tool for better decision making in selective embryo transfers.

Nevertheless, one must always be cautious that artefacts during 3D analysis may occur due to 2D imaging process, patient motion during rendering of images and artefacts due to operator choice in the selection of which part of the volume to display [23].

We can highlight as the major strength of this study the awareness to a controversial topic, with a different uptake on the question of non-invasive methods to assess endometrial receptivity. This new methodology of continuous or better yet consecutive serial evaluations ascertain the discriminatory value of volumetric endometrial ultrasound assessment.

Also, the fact of prospective continuous assessment using the same protocol for all subjects can be pointed out as a strength of this study.

The limited number of subjects of our sample can be deemed as a limitation or weakness to this study. Still, this new methodological approach can now be used in a larger setting to provide further information and knowledge to sustain the information already seen.

This study provides a different uptake on the question of non-invasive methods to assess endometrial receptivity. We have successfully been able to obtain limited data concerning a preferential pathway on endometrial volumetry. However larger studies should be carried out to further sustain our findings.

## Conclusions

Many have been published regarding endometrial receptivity. Still many questions remain without an answer. The possibility to accurately predict implantation is one of the most challenging ones. Progress in embryo transfer and cultures, still relies on the uncertainty of embryo implantation even in the presence of what appears to be a receptive endometrium.

Some techniques have been developed but the results are still controversial, invasive and lacking reliability.

Endometrium is a living, adaptative transformative tissue. Understanding this ability to transform, and the continuity of the process may allow better knowledge of its ability to allow the implantation process of a good quality embryo.

Ultrasonographic advances in the past decades, provides a useful tool to evaluate the morphokynetics of this transformative tissue.

Information of what makes an endometrium receptive may be the key in solving these issues, providing a diagnostic tool that will enhance ART cycles and elective embryo transfers. This will result in more effective transfers and better ART outcomes. Also the possibility to determine in real time endometrial receptivity will shorten the time to birth lapse, thus improving quality of life for infertile couples.

This study showed that endometrial 3D volume analysis as well as adjusted 3D endometrium volume may identify a receptive endometrium as soon as day 6 of ovarian controlled stimulation, and also the results show a high accuracy in detecting the non-receptive endometrium (with a high Predictive Negative Value of 90.1%). In this way clinicians may be made aware of this possibility, and further enhance its procedures with better knowledge weather or not to perform embryo transfer on that given cycle.

### Abbreviations

ART : Assisted Reproductive Technology; CHCB: Centro Hospitalar Cova da Beira; ER: Endometrial Receptivity; ERA: Endometrial Receptivity Array; GCP: Good Clinical Practices; hCG: human chorionic gonadotropin; ICH: International Conference Harmonisation; IU: International Units; ML: Multi-layered; NM: Non Multi-layered; SD : Standard Deviation; WOI: Window of Implantation; 2D: Two Dimensions; 3D : Three Dimensions

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### Authors' contributions

RSM, AHO and JMO are responsible for conception and study design. RSM has been the principal investigator and the principal responsible for acquisition and analysis of data. RSM has been responsible for data interpretation. DVO, AHO and JMO have been responsible for reviewing the article for publication. All authors have read and approved the manuscript, and can be personally accountable for the contributions and can ensure that questions related to the accuracy and integrity of any part of the work.

### Funding

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### Availability of data and materials

Encrypted non-disclosure data available at Open Science Framework database for peer review purpose only. Project name Physical Biomarkers in Endometrial Receptivity with access link: https://osf.io/hr25m/?view_only=8d5f6dcb8b25420bbd9188382163e7d7

### Ethics approval and consent to participate

The study has been approved by the Ethics Committee of Centro Hospitalar Universitário Cova da Beira (study committee reference number: CHCB 22/2017). Oral and written consent was obtained for all willing participants prior to registering for this study. Patient Informed consent to participate in this study CHCB 22/2017.

### Consent for publication

Not Applicable.

### Competing interests

The authors do not report any conflict of interest.

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## Publisher's Note

# Appendix 3

Silva Martins et al. BMC Pregnancy and Childbirth (2021) 21:184
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BMC Pregnancy and Childbirth

RESEARCH ARTICLE Open Access

# The predictive value of serial serum estradiol and serial endometrial volume on endometrial receptivity on assisted reproductive technology cycles

R. Silva Martins[1,2*], A. Helio Oliani[1], D. Vaz Oliani[1] and J. Martinez de Oliveira[1,2]

**Abstract**

**Background:** Diagnosis of endometrial receptivity is still unclear and conflicting. Despite advances in embryo development during assisted reproductive technologies (ART) cycles, the intricate process of implantation is still matter for debate and research.

**Materials and methods:** Prospective case control of 169 subjects during ovarian controlled stimulation for ART. Endometrial receptivity assessment to predict clinical pregnancy with serial continuous biochemical (serum estradiol) and biophysical (endometrial volume and adjusted endometrial volume) parameters were used. Both parameters were compared between negative and positive outcome in terms of clinical pregnancy.

**Results:** No statistical difference was noted between the two groups in terms of demographics and ART procedures and scores. Serum estradiol was significantly higher in the positive group from day 8 after ovarian controlled stimulation. Endometrial volume and adjusted endometrial volume were significantly higher in the positive group as soon as day 6 of ovarian controlled stimulation.

**Conclusions:** Continuous serum estradiol and 3D endometrial volume and adjusted endometrial volumes may reflect endometrial changes during ART procedures and provide a useful real time tool for clinicians in predicting endometrial receptivity.

**Keywords:** Endometrial receptivity, Serum estradiol, Endometrial volume, Adjusted endometrial volume, Embryo implantation

## Main text

### Introduction

Since the introduction of assisted conception, many techniques have been developed to improve ovarian stimulation, oocyte retrieval, and embryo culture, but despite this, more than 70% of apparently normal embryos transferred fail to implant. Endometrial receptivity is still a controversial topic and the mechanism to which leads the normal endometrium to become receptive to a healthy embryo unclear. Endometrial differentiation, embryo development and foremost embryo-endometrium interaction leading to implantation requires synchronous and continuous dialogue between these two important components. The physiological and biochemical steps which will allow endometrium to become receptive remains

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unclear, poorly understood and matter for many discussion and study.

The impact of serum estradiol levels in assisted reproductive techniques (ART), has been debated for over 25 years with conflicting results about the effect of supraphysiological levels of estradiol during controlled ovarian stimulation. Some studies showed a negative impact, while others showed a positive impact in ART outcome. The majority showed no impact [1–5]. High levels of estradiol and its influence on embryonic implantation, and embryonic development is still controversial and unclear. Many has been published regarding the deleterious effects of supraphysiological levels of estradiol on ovarian stimulation for ART procedures. Also, questions have been raised concerning high levels of estradiol and the impairment caused by it on endometrial receptivity. Ovarian hyperstimulation syndrome (OHSS) is an iatrogenic complication of assisted reproduction technology. Estrogen, estradiol, prolactin, histamine and prostaglandins have all been implicated in OHSS.

A metanalysis was conducted in 2019 and no quality evidence was found to support or refute the value of estradiol levels on the day of hCG administration, as a predictor of pregnancy in ART cycles [6]. Conflicting results may relate to the difference between the way the trials were conducted, the difference of stimulation protocol, number of embryos transferred, and the definition of outcome in terms of pregnancy rates.

According to Paulson (2011) the supraphysiological elevation of serum estradiol compromises endometrial receptivity [7]. This elevation plays a definite role in embryo implantation which is claimed to be dose dependent [8]. As a result of multiple follicle maturation, the rise of serum estradiol to supraphysiological levels, alters endometrial receptivity by morphological and biochemical changes produced against this tissue (Simon et al.) [9, 10]. Mirkin et al., 2005 reports that elevated serum estradiol levels have a negative impact on endometrial receptivity especially in fresh embryo transfer cycles [11].

Still clinical trials, contrast with these claims with conflicting results. By Sharara & McClamrock 1999, no significant impact on implantation rates were reported due to the supraphysiological levels of estradiol [12].

In a similar way, ultrasonographic parameters have been attempted to understand endometrial receptivity. Still the results of all these features remain uncertain and also conflicting. Ultrasound can assess endometrium changes during a stimulated ART cycle, in a non-invasive manner. Monitoring both follicle development and endometrium during an ART cycle is normal clinical procedure. Many published studies have conflicting results on this subject but the common feature in all, is the lack of continuity on the endometrial assessment [13–16].

The primary objective of this study is to assess both parameters (endometrial volume and serum estradiol), not only in a single scope pre-determined moment but with a serial prospective continuous evaluation. Endometrium is a responsive tissue that has to undergo serial transformations as a result of the ovarian stimulation. The main goal is to determine the changes and follow up the way both serum estradiol and ultrasound parameters influence this process.

## Material and methods

Prospective case control study of 169 women in ART cycles of diagnosed infertile couples on ART treatment at our institution during a 2-year period (from January 2017 to December 2018). Subjects with canceled treatments prior to oocyte pickup, or with donate gametes, and cryopreserved oocytes were excluded from this study. Also cycles with erroneous or missing date were excluded. Written informed consent was obtained according to the Ethics Committee of our Institution. Inclusion criteria included subjects that had morphologically two good grade cleaved (day 3 of embryo development) embryos for transfer. Only Grade A and B cleaved embryos were included in this selection according to the morphocynetic analysis using ASEBIR embryo assessment criteria. All subjects were submitted to short protocol regimen with antagonists for ovarian controlled stimulation with gonadotropins and were given recombinant human chorionic gonadotropin hormone (rhCG) for induction of ovulation 36 h prior to oocyte pick up.

Demographics and ART parameters were collected for all patients.

Continuous serial serum estradiol levels obtained and ultrasound analysis were performed using the same protocol for all participating subjects, from basal moment (prior to ovarian controlled stimulation) and throughout ovarian controlled stimulation.

Serum levels of estradiol were measured by chemiluminescent enzyme immunoassays (IMMULITE; Diagnostic Products, Los Angeles, CA, USA). Inter-assay and intra-assay coefficients of variation were 9.8 and 9.4%.

Women were scanned by 3D ultrasound using a transvaginal 7.5 MHz transducer (General Electric – GE Volusson E6, USA TM) by a single operator. Sagital midline 2D longitudinal cross section of the uterus was obtained and a volume box was superimposed on the scan image. The volume was captured through the automatic sweep of the transducer. Endometrial volume calculation by 3D ultrasound presented as voxels and geometric information of surfaces in a 3D dataset. The results obtained are then converted to millilitres.

Adjusted Endometrial volume was also obtained as a ratio between endometrial volume calculated on 3D analysis and uterine volume based on 3D volumetric acquisitions which then generated an estimated uterine volume (also in millilitres).

Positive outcome was considered for subjects with positive pregnancy test 12 days after successful embryo transfer and consequently ultrasonographic confirmation of pregnancy with the presence of gestational sac with at least one embryo with positive fetal heart activity.

Data was analysed in Excel 2019 (Microsoft Corp, Redmond, WA) and IBM SPSS statistics v25 (IBM Corp. Armonk, NY).

Continuous variables were analysed with Levene's test (equality of variances) and visual assessment of the histogram (normality). Results were expressed as mean ± SD, frequency, and percentages. For statistical differences between the means of two groups, t-student for independent samples was applied. Categorical characteristics of patients were compared with $\chi 2$ test.

Value of $p < .05$ was considered statistically significant.

Receiver operating characteristic (ROC) curve analysis and comparison of area under curves (AUC) were performed to determine cut-off values of Estradiol, Endometrial Volume and Adjusted Endometrial Volume for the prediction of positive outcome. Investigation of the predictive capability of each logistic model by means of the area under the ROC curve. This curve measures the accuracy of biomarkers to predict positive outcome in endometrial receptivity displayed by the relationship between sensitivity (true positive rate, y-axis) and 1-specificity (false positive rate, x-axis) across the possible threshold values considered. Values of AUC equal to 1 indicate a perfect test, values of AUC higher than 0.9 demonstrate high accuracy and values between 0.7 and 0.9 reflect moderate accuracy of the test.

The authors do not report any conflict of interest.

The study protocol was approved by the Ethics Committee of our Institution (CHCB 22/2017), in accordance with the relevant guidelines and regulations. The study was developed under the research practices described in International Conference Harmonisation (ICH) guidelines, Good Clinical Practices (GCP) and the Declaration of Helsinki.

This study integrates part of a major protocol study and methodological used shares similitude to previous published article by Silva Martins, R [17].

## Results

Subjects were divided into two groups depending on the value of hCG at Day 12 after embryo transfer and ultrasonographic confirmation of clinical pregnancy: 123 on the negative group (72.8%) and 46 on the positive group (27.2%). Demographics characteristics and ART parameters of the 169 subjects are shown in Table 1 and no statistical difference between the two set groups in terms of demographics and ART parameters was met, especially in terms of overall median number of harvested oocytes per cycle

**Table 1** Demographics and ART parameters between two Groups

| | **Negative Group** *N* = 123 (72.8%) | **Positive Group** *N* = 46 (27.2%) | **t-Test** *p* **value** |
|---|---|---|---|
| Female Age (in years) | 34.94 ± 4.03 (19–39) | 34.28 ± 3.35 (25–39) | 0.290 |
| Male Age (in years) | 36.14 ± 4.76 (22–46) | 37.19 ± 5.91 (29–62) | 0.832 |
| Time of Infertility (in months) | 54.46 ± 33.82 (12–204) | 60.22 ± 38.49 (14–192) | 0.375 |
| Type of Infertility: | | | |
| • Primary | 95/123 (77.2%) | 38/46 (82.6%) | 0.297 |
| • Secondary | 28/123 (22.8%) | 8/46 (17.4%) | |
| Male Associated Infertility | 28.2% | 31.1% | 0.654 |
| Body Mass Index (Kg/cm2) | 25.8 ± 3.2 | 26.1 ± 3.4 | 0.765 |
| Antimullerian hormone (pg/mL) | 2.45 ± 2.45 (0.09–16.65) | 2.62 ± 2.46 (0.04–13.56) | 0.679 |
| Antral follicle count | 8.43 ± 5.07 (2–40) | 8.63 ± 3.74 (2–20) | 0.801 |
| Total dose of gonadotropins (in International Units) | 2500.81 ± 812.19 (300–4500) | 2508.15 ± 757.91 (450–4500) | 0.956 |
| Progesterone levels at Trigger day (ng/mL) | 0.88 ± 0.44 (0.01–2.20) | 0.78 ± 0.47 (0.01–2.10) | 0.188 |
| Number of collected Oocytes | 8.25 ± 5.14 (2–22) | 10.50 ± 5.20 (2–23) | 0.140 |
| Metaphase II Oocytes | 6.57 ± 4.22 (2–17) | 7.06 ± 4.77 (2–21) | 0.150 |
| Number of day 3 embryos | 3.18 ± 2.40 (2–12) | 3.84 ± 2.65 (2–12) | 0.120 |
| Number of blastocysts for vitrification | 0.65 ± 1.51 (0–6) | 0.86 ± 1.71 (0–9) | 0.200 |

(Positive Group, *N* = 46 and Negative Group, *N* = 123)
Descriptive statistics between two Groups. Mean values with standard deviation (SD)

**Table 2** Serum estradiol measurements between two Groups (in picograms per mililiter – pg/mL)

| | Serum Estradiol Levels (pg/mL) Negative Group $N = 123$ (72.8%) | Serum Estradiol Levels (pg/mL) Positive Group $N = 46$ (27.2%) | t-Test p Value |
|---|---|---|---|
| Basal | 10.47 ± 2.89 | 10.39 ± 2.87 | 0.727 |
| Day 6 after Controlled Ovarian Stimulation | 305.10 ± 130.49 | 381.65 ± 122.46 | 0.055 |
| Day 8 after Controlled Ovarian Stimulation | 840.68 ± 230.49 | 896.32 ± 222.46 | **0.034** |
| Day 10 after Controlled Ovarian Stimulation | 1652.56 ± 607.90 | 1848.65 ± 599.42 | **0.01** |
| Trigger Day with rhCG | 1746.76 ± 654.34 | 2214.65 ± 612.34 | **0.01** |

(Positive Group, $N = 46$ and Negative Group, $N = 123$)
Mean values with standard deviation (SD)

defined as the total number of oocytes harvested during oocyte pick up procedure, and rate of collected metaphase II (MII) oocytes. Also, the mean number of cleaved embryos at day 3 of embryo development, and mean number of blastocysts for cryopreservation showed no significant statistical difference between the two set groups.

Serum estradiol levels on basal (prior to ovarian controlled stimulation) was similar between the two groups. Significantly higher levels were noted on the positive outcome group, but it only met statistical significance at Day 8 after ovarian controlled stimulation. (Table 2).

Endometrial Volume and Adjusted Endometrial Volume showed statistical difference from Day 6 after Ovarian controlled stimulation (Table 3). Consistently higher values were seen for both of these biophysical markers on the positive group. (Fig. 1).

Receiver operating characteristic (ROC) curve analysis and comparison of area under curves (AUC) were performed for Estradiol, Endometrial Volume and Adjusted Endometrial Volume on the prediction of positive outcome with sensitivity (true positive rate, y-axis) and 1-specificity (false positive rate, x-axis). Values of 0,701; 0, 723 and 0,756 were obtained respectively for the examined parameters. (Fig. 2).

In this study the intra-observer reliability was 0.96. In addition, because all measurements were performed by the same operator in this study there was no inter-observer variability.

**Table 3** Ultrasound parameters between two groups – Endometrial thickness, volume and adjusted endometrial volume at baseline, at day 6, 8 and 10 after controlled ovarian stimulation, at trigger day and at embryo transfer day

| | | Negative Group $N = 123$ (72.8%) | Positive Group $N = 46$ (27.2%) | t-Test p Value |
|---|---|---|---|---|
| Basal | Endometrial Thickness (mm) | 4.32 ± 0.72 | 4.22 ± 0.51 | 0.387 |
| | Endometrial Volume ($mm^3$) | 2.52 ± 0.71 | 2.77 ± 0.63 | 0.54 |
| | Adjusted Endometrial Volume | 4.60 ± 1.42 | 5.51 ± 1.28 | 0.21 |
| Day 6 after Controlled Ovarian Stimulation | Endometrial Thickness (mm) | 6.32 ± 0.96 | 6.28 ± 0.75 | 0.827 |
| | Endometrial Volume ($mm^3$) | 3.08 ± 0.66 | 3.33 ± 0.57 | **0.024** |
| | Adjusted Endometrial Volume | 5.63 ± 1.50 | 6.67 ± 1.38 | **0.001** |
| Day 8 after Controlled Ovarian Stimulation | Endometrial Thickness (mm) | 7.47 ± 0.80 | 7.96 ± 0.79 | **0.01** |
| | Endometrial Volume ($mm^3$) | 3.90 ± 0.94 | 4.40 ± 0.71 | **0.002** |
| | Adjusted Endometrial Volume | 7.28 ± 2.67 | 8.98 ± 2.47 | **0.001** |
| Day 10 after Controlled Ovarian Stimulation | Endometrial Thickness (mm) | 8.01 ± 1.04 | 8.61 ± 0.98 | **0.01** |
| | Endometrial Volume ($mm^3$) | 4.12 ± 1.01 | 4.91 ± 0.82 | **0.001** |
| | Adjusted Endometrial Volume | 7.60 ± 2.54 | 9.99 ± 2.61 | **0.001** |
| Trigger Day with rhCG | Endometrial Thickness (mm) | 8.53 ± 1.32 | 9.59 ± 1.44 | **0.001** |
| | Endometrial Volume ($mm^3$) | 4.52 ± 1.00 | 5.33 ± 0.76 | **0.001** |
| | Adjusted Endometrial Volume | 8.30 ± 2.52 | 10.76 ± 2.62 | **0.001** |
| Embryo Transfer Day | Endometrial Thickness (mm) | 9.06 ± 1.30 | 10.15 ± 1.35 | **0.001** |
| | Endometrial Volume ($mm^3$) | 4.84 ± 1.01 | 5.59 ± 0.77 | **0.001** |
| | Adjusted Endometrial Volume | 8.32 ± 2.58 | 10.83 ± 2.73 | **0.001** |

Ratios in percentages (%) and mean values with standard deviation (SD). *rhCG* recombinant human chorionic gonadotropin

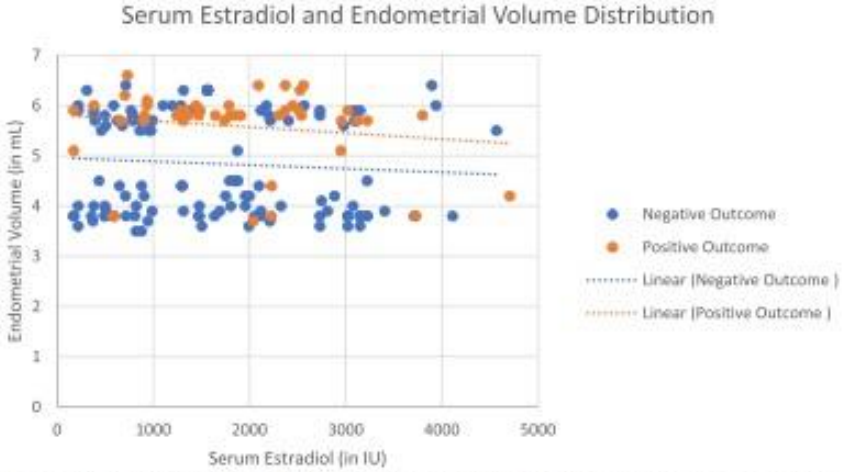


**Fig. 1** Scatter-plot chart for Serum Estradiol levels and Endometrial volume distribution on trigger day with hCG are displayed. (in blue the negative outcome and in orange the positive outcomes)

## Discussion

The process of endometrial transformation from proliferative phase to secretory phase under the steroids hormonal influence, called endometrial decidualization is a set goal for optimal implantation. Cyclic changes of endometrium are regulated by ovarian hormones [18]. This pattern may alter due to the supraphysiological hormonal levels during ART cycles.

Single analysis of endometrial pattern at trigger day has been the most used, with contradictory findings.

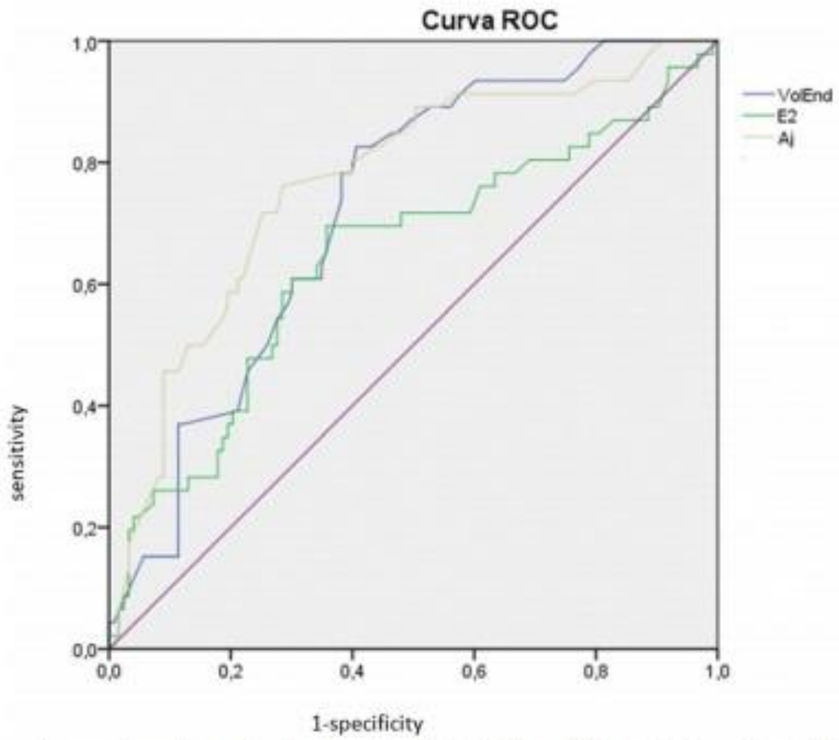


**Fig. 2** The ROC area under curves for prediction of positive outcome. Vol End – Endometrial Volume; E2 – Serum Estradiol; Aj – Adjusted Endometrial Volume

Recent studies (*Silva Martins, R.* et al.) have proven that perhaps serial evaluations provide better understanding rather than a single scoop at a pre-determined phase of the process [17, 19]. The main purpose of this study was to assess both parameters not only in a single scope pre-determined moment but with a serial prospective continuous evaluation.

Conflicting results have been published, but this new methodological approach to endometrial assessment during ART procedures, may shine a new light in better understating the way endometrium transforms and becomes receptive for successful embryo implantation. Serial evaluations, of both biochemical and biophysical parameters, better translate endometrium transformations and may be a base for the understating of endometrial receptivity [20, 21].

Endometrial volume and adjusted endometrial volume proven to be more effective with differences shown since the beginning of ovarian controlled stimulation. Both groups were similar at baseline but as soon as controlled ovarian stimulation started, the differences between those with a positive outcome and those without were clearly met.

In a similar way the rise of serum estradiol was significantly higher in the positive outcome group, despite the fact that the number of oocytes on pick up, mature oocytes and number of cleaved and blastocysts was similar between both groups. This reflects the effects that serum estradiol has directly on endometrium in later phases of its development prior to embryo implantation.

Serum estradiol and endometrial volume, may prove to be a useful management tool for clinicians in order to establish yet another diagnostic tool for better decision making in selective embryo transfer. This new methodology uptake, and a new perspective of endometrial analysis is certainly the strongest factor of this study, as well as the number of continuous serial evaluations on the same patient, throughout the ovarian controlled stimulation and its effects on endometrium. Still in our work, in line with previous publications positive outcomes in term of clinical pregnancies were met for values of serum estradiol at trigger day bellow 3000 pg/mL. This can be explained by the policy of tailor made controlled ovarian stimulation protocol, in mild stimulation to avoid potential OHSS.

We could not refrain to uphold expectation of these results as they show a serial of values, demonstrating a certain pattern of evolution on a transforming living tissue and its natural adaptations to a complex and yet unknown process. Also, the number of subjects in this study constitutes a limitation and further larger studies must be carried out in order to certainly establish this promising results.

## Conclusions

The underlying mechanism that results in failure of implantation of a good quality embryo on a supposed receptive endometrium is still unclear. Endometrial receptivity is still up to this date a controversial subject and a hot topic for discussion and analysis.

Most published studies present conflicting results and are still controversial. The adaptative changes and continuous evolution of the endometrium makes it difficult to establish a normative pattern of development in way to provide useful information regarding its receptiveness, in real time and in a personalized individualized setting.

This study showed that endometrial volumetry may identify a receptive endometrium as soon as day 6 of ovarian controlled stimulation. Serum Estradiol also showed some predicting value, with statistical significance. Nevertheless, the difference noted between groups did not affect the number of oocytes in oocyte retrieval, nor the number of mature oocytes. Also, the number of cleaved embryos and blastocysts was similar in both groups. This shows that serum estadiol has potential effects on endometrium development in later phase of its development. In this way clinicians may be made aware of this possibility and further enhance its procedures with better knowledge weather or not to perform embryo transfer on that given cycle.

### Abbreviations

ART: Assisted reproductive technology; CHCB: Centro Hospitalar Cova da Beira; ER: Endometrial receptivity; ERA: Endometrial receptivity array; GCP: Good clinical practices; hCG: human chorionic gonadotropin; ICH: International Conference Harmonisation; IU: International Units; SD : Standard deviation; WOI: Window of implantation; 2D: Two dimensions; 3D: Three dimensions

### Acknowledgements

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### Authors' contributions

RSM, AHO and JMO are responsible for conception and study design. RSM has been the principal investigator and the principal responsible for acquisition and analysis of data. RSM has been responsible for data interpretation. DVO, AHO and JMO have been responsible for reviewing the article for publication. All authors have read and approved the manuscript and can be personally accountable for the contributions and can ensure that questions related to the accuracy and integrity of any part of the work.

### Funding

No Grant support on this study.

### Availability of data and materials

Encrypted non-disclosure data available at Open Science Framework database for peer review purpose only. Project name Physical Biomarkers in Endometrial Receptivity with access link: https://osf.io/hr25m/?view_only=8d5f6dcb8b25420bbd9188382163e7d7

## Declarations

### Ethics approval and consent to participate

The study has been approved by the Ethics Committee of Centro Hospitalar Universitário Cova da Beira (study committee reference number: CHCB 22/2017). Oral and written consent was obtained for all willing participants prior to registering for this study. Patient Informed consent to participate in this study CHCB 22/2017.

**Consent for publication**
Not Applicable.

**Competing interests.**
The authors do not report any conflict of interest.

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## Publisher's Note

# Appendix 4



The between group difference in serum progesterone levels was pronounced +23-29d post ET (patients 50mg/d: 1.82±1.5; n=22; patients 30mg/d: 2.22±2.09; n=18;), +30-36d post ET (patients 50 mg/d: 5.07±3.03; n=18; patients 30 mg/d: 4.77±3.32; n=20;) and reached its peak 37-43d post ET (patients 50mg/d: 8.37±2.73; n=20; patients 30mg/d: 9.35±3.99; n=17;). On average, serum progesterone levels were reduced by 16±11% from +16-22d - 37-43d post ET.

**Limitations, reasons for caution:** Differences in serum progesterone levels do not reach statistical significance yet due to insufficiently large group sizes. Moreover, the observed differences are small and close to the ELISA quantification limits.

**Wider implications of the findings:** Modulating the progestogen exposure of the early conceptus by exogenous administration or by creating multiple corpora lutea via ovarian stimulation may influence placental growth and activity, which may have far-reaching consequences on fetal growth and well being.

**Trial registration number:** NCT03507673

### P-401 Serial Endometrial Volume Assessment as a predictor of Endometrial Receptivity in Assisted Reproductive Technology Cycles

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**Study question:** Investigate the ultrasonographic value of sequenced endometrial volume analysis in ART cycles as a indicator of endometrial receptivity and embryo implantation.

**Summary answer:** Adjusted endometrial volume, and the pattern of increment volume in a continuous analysis showed significant impact in predicting endometrial receptivity and embryo successful implantation.

**What is known already:** There is no controversy regarding the importance of precise and specific endometrial maturational development in allowing implantation following assisted reproduction treatment. Adequate proliferation and differentiation during the proliferative phase must be followed by timely secretory changes during luteal phase with stroll decidualization. However there has been many conflicting results in regards to the role of endometrial assessment by ultrasonographic parameters. Although ultrasonographic parameters of endometrial receptivity have a strong negative value in setting some minimum criteria, their prognostic value for implantation following embryo transfer is yet scarce.

**Study design, size, duration:** Prospective longitudinal study. Continuous, sequenced ultrasonographic analysis from basal point prior to ovarian controlled stimulation to continuous analysis throughout duration of treatment, trigger day with hCG and embryo transfer. From initial 200 recruited subjects, complete full evaluation of 169 subjects that completed treatment and had a successful embryo transfer.

**Participants/materials, setting, methods:** Short term antagonist ovarian controlled stimulation to all participants. Assessment of Endometrial Thickness, Endometrial pattern, Endometrial Volume and Adjusted Endometrial Volume ( in ratio with Uterine Volume for each participant ). Excluded patients with clinical risk for ovarian hyper stimulation syndrome and those without viable good qualitiy day 3 double embryos for transfer. Evaluation of remaining 169 subjects complete and sequenced in time.

**Main results and the role of chance:** There was no statistical difference between groups with and without successful implantation in demographics, cause of infertility, duration and total dose of gonadotropins and treatment course used. Statistical significant difference was found in terms of Endometrial volume between the group with successful implantation versus the one without implantation, not at base line but after day 10 of controlled ovarian stimulation (Mean value at base line , day 6 and day 8 after controlled ovarian stimulation for the non implantation group of 2,52; 3.08 and 3,91 respectively versus 2,77; 3,33 and 4,40 on the implantation group and Mean value at Day 10 and embryo transfer day for non implantation group of 4,12 and 4,83 versus 4,91 and 5,59 on the implantation group). The continuous analysis of endometrial patterns showed statistical difference between the two groups after day 10 of the ovarian controlled stimulation with p values <0.001. With adjusted volume scores the difference between two groups is shown and sustained in all moments except at base line (Mean values at baseline for non implantation versus implantation group 4,60 versus 5,53 and Mean Values of 5,63; 7,23 ; 7,59 and 8,31 versus 6,70; 9,05; 9,92 and 10,77 with p value <0.001).

**Limitations, reasons for caution:** Differences in ultrasonographic examination techniques do exist, but the use of a rigid protocol and single operator in all evaluations reduces the possibility of conflicting evaluations. The use of high resolution and 3-D analysis further reduces the possibility of inaccurate evaluation.

**Wider implications of the findings:** The presence of a receptive endometrium is a major factor in deterring implantation and success in reproductive medicine. The possibility of a non invasive ready to use ultrasonographic parameter would allow predicting more accurately the outcome.

**Trial registration number:** NONE

### P-402 Chromosomal testing at the blastocyst stage reduces miscarriages, aneuploid gestations and provides similar cumulative live birth rate per cycle: a large public health-care provider experience.

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[1]*Humanitas Research Hospital, Department of Gynecology- Division of Gynecology and Reproductive Medicine- Fertility Center, Rozzano MI, Italy*

[2]*Igenomix Italy, Igenomix, Marostica, Italy*

**Study question:** Is aneuploidy testing at blastocyst stage effective in reducing miscarriages, aneuploid gestations and time-to pregnancy in advanced reproductive age (ARA) women?

**Summary answer:** Preimplantation genetic testing for aneuploidies (PGT-A) at the blastocyst stage is effective in reducing miscarriages, aneuploid gestations and time to pregnancy in selected IVF populations

**What is known already:** PGT-A allows the detection of chromosomal abnormalities in preimplantation embryos. However, it is known to be challenging to quantify the benefits that such program brings to the patients treatment outcome. Particularly, long-term clinical data are lacking from single, large IVF centres where inclusion/exclusion criteria for accessing treatment options (i.e., PGT, blastocyst transfer) are standardized for all patients. In this study, we combined laboratory and clinical outcomes, preimplantation and prenatal testing genetic results with detailed effects on pregnancy and neonatal follow-up from a large, public healthcare provider without commercial interest in genetic analysis

**Study design, size, duration:** Observational cohort study performed between January 2015 and May 2017 at an Academic tertiary care healthcare provider. Couples with female age >38 were counselled for PGT-A before ovarian stimulation. Consenting couples dropped out from PGT-A if <4 fertilized eggs were available after ICSI. In these cases, day3 embryo transfers were performed (DROP-OUT group). All non-consenting couples were included as controls (Standard IVF group). Laboratory, clinical, follow-up and genetic analysis outcomes were compared across all groups.

**Participants/materials, setting, methods:** This study compares data from groups: PGT-A (273 couples, 312 cycles), DROP-OUT (101-couples, 106-cycles) and Standard IVF (2,168-couples, 2,905-cycles). Confounders-adjusted multivariate analysis was used to compare main outcome measures: miscarriage rate, aneuploid gestation rate, live-birth rate per cycle (LBR) with one year minimum follow-up. Kaplan-Meier and Cox regression were used to compare time-to-pregnancy(TTP) in PGT-A and Standard IVF using as primary outcome the cumulative live birth rate in function of number of transfers required

**Main results and the role of chance:** Mean female age was higher (P<0.001) in DROP-OUT (41.13 ± 3.03 vs 40.38 ± 3.33 in PGT-A and 39.72 ± 2.37 in Standard-IVF), BMI, infertility duration, FSH and AMH levels, oocyte maturation rate, fertilization and cleavage rates were similar across groups (NS). Euploidy rate in PGT-A group was 56% (306/542). In total, 188 (100% blastocysts), 117 (cleavage-stage) and 3573 (32.1% blastocyst stage) embryo transfers were performed in PGT-A, DROPOUT, Standard-IVF, respectively. PGT-A group showed significantly superior transfer clinical outcomes (P<0.01). Particularly, ongoing implantation (43.1% PGT-A, 18.4% Standard IVF; 10.8% DROPOUT), miscarriage (8.6% PGT-A, 32.0% Standard-IVF; 50% DROPOUT), ascertained

# Appendix 5

Dear Renato Silva Martins ,

We are pleased to advise you that the COGI Organizing Committe has **accepted** your abstract entitled

**ENDOMETRIAL VOLUME AND ADJUSTED ENDOMETRIAL VOLUME ASSESSMENT OF ENDOMETRIAL RECEPTIVITY IN ASSISTED REPRODUCTIVE TECHNOLOGY CYCLES**

With reference number: 625

as an **ePoster presentation** for **the 27th World Congress on Controversies in Obstetrics, Gynecology and Infertility (COGI) to be held in Paris, France, November 21 – 23, 2019**.
See below your abstract details for review.

Details regarding how to upload your ePoster will be sent at a later date.

# Appendix 6

Dear Renato Silva Martins ,

We are pleased to advise you that the COGI Organizing Committe has **accepted** your abstract entitled

**BIOPHYSICAL ULTRASONOGRAPHIC MARKERS ASSESSMENT OF ENDOMETRIAL RECEPTIVITY IN ASSISTED REPRODUCTIVE TECHNOLOGY CYCLES**

With reference number: 626

as an **oral presentation** for the **27th World Congress on Controversies in Obstetrics, Gynecology and Infertility (COGI)** to be held in **Paris, France, November 21 – 23, 2019.**
See below your abstract details for review.

Details as to the exact session and time of your presentation will be sent at a later stage.

COGI

All About Women's Health

Certificate of Attendance

This is to certify that

Renato Silva Martins

presenter of the oral presentation

BIOPHYSICAL ULTRASONOGRAPHIC MARKERS ASSESSMENT OF ENDOMETRIAL RECEPTIVITY IN ASSISTED REPRODUCTIVE TECHNOLOGY CYCLES

has attended

The 27th World Congress on Controversies in Obstetrics, Gynecology & Infertility (COGI)

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# Appendix 7

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*Gynecology 2020*

*Young Scientists Forum*

*Prof/Dr/Mr/Ms.* **Renato Alessandre Silva Martins**

*Centro Hospitalar Universitário Cova da Beira EPE, Portugal*

*for presenting the oral entitled*

***Subendometrial resistence and pulsatility index assessment of endometrial receptivity in assisted reproductive technology cycles***

*at the European Gynecology and Obstetrics Congress*
*held during February 17-18, 2020 in Paris, France*

The award has been attributed in recognition of research paper quality, novelty and significance.

**Alfred Shibata**
Scripps Research Institution, USA

**Natalia Artymuk**
Kemerovo State Medical University, Russian Federation

# Appendix 8

CERTIFICADO DE ATRIBUIÇÃO DE PRÉMIO LABORATÓRIO DA SPMR

A Sociedade Portuguesa de Medicina da Reprodução certifica que o

**PRÉMIO CLÍNICO SPMR 2020** foi atribuído a

**RENATO SILVA MARTINS**

Pela sua participação, como primeiro co-autor, no trabalho intitulado

**"Characterization of the infertile couple and first IVF's results in public fertility centers of Portuguese Northern and Central regions – a five year cohort study"**

A Direção da SPMR