Immune dysfunction in obesity-associated hypertension and effects of vitamin D supplementation

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(3º ciclo de estudos)

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Integrity Declaration

I, Catarina Cecília Pinheiro Reis dos Santos, who sign below, student with registration number D1346 of the 3rd Cycle in Medicine of the Faculty of Health Sciences, declare to have developed this work and written this text in full compliance with the Integrity Code of the University of Beira Interior.

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University of Beira Interior, Covilhã 22/07 /2023

Dedication

Ao meu querido filho Francisco,

Que nenhum sonho fique por cumprir.

Acknowledgments

At the end of this task, I shall acknowledge to all that believed in this project. And they were a lot.

At first, Dr. Ernesto Rocha, as the great driver to begin this idea. His care and motivation were of outstanding importance for me, not only creating the conditions and resources that underpinned the project but also because of its constant attention and support. Since we work together, for almost 18 years, conveyed to me his interest and affection for the theme of hypertension.

I shall also acknowledge Professor Miguel Castelo Branco, who accepted to conduct me with the support of Professora Mafalda Fonseca. In their busy lives, they hosted a long lasting and arduous project, pointing out the best ways forward, with incommensurable patience. They were always there for my doubts, questions, failures, and anxieties. And they never said no.

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To all the medical and nurse staff of my department: for their continuous support and motivation through the last years. A special thanks for the multiple articles that they were always forwarding into my mailbox.

To my family, the "special ones". To my husband, shuttling frozen samples in the height of summer, to my parents, continuous sources of inspiration, to my sisters, fundamental parts of my life, but especially, to my 10 years old boy, Francisco. Never

complained, never bored, and not understanding everything, always supporting is mother.

Finally, a mention to Dr. Pedro Marques da Silva. Although no longer between us, it was a man I have the pleasure to meet and work with. An enthusiastic of all what concerns hypertension, a medical reference in our country and that taught me much of what I know in this field.

Thesis overview

The thesis here presented is organized in seven main chapters.

The first chapter entitled "General introduction and literature review" presents some of the most relevant data available at the date of thesis writing. The aim was to present a perspective on the evolution of the concepts associated with hypertension pathogenesis, specially, obesity-related hypertension, with a special focus on immune and metabolic dysregulation.

The second chapter presents the Aims and Objectives for the thesis development, also presenting the endpoints that underpinned the conduction of the clinical trial.

At the third chapter an extensive description of the Methodology is presented, describing trial conception, procedures employed, and all the Institutions and Laboratories involved.

At the fourth chapter the global results are presented. At this chapter, seemed more logical that results would be presented as a whole, being also added some unpublished results.

In the fifth chapter, an integrative discussion ensues along with concluding remarks (Chapter 6). In this chapter the discussion begins with the integration of cytometry and metabolomics data, followed by histology results, to finish with the main conclusions but also future investigation perspectives.

Finally, it is also presented some supplementary data, namely Official Entities approvals for the trial development.

Publications

- Santos C, Monteiro A, Rodrigues R, Ferreira C, Coutinho J, Filipe R, Ramos S, Castelo-Branco M, Fonseca M. In obese hypertensives cholecalciferol inhibits circulating TH17 cells but not macrophage infiltration on adipose tissue. Clin Immunol 2023 Feb; 247:109244.

- Santos C, Marques da Silva P. Hemodynamic patterns in obesity-associated hypertension. BMC Obes 2018 Apr 16:5:13.

- Santos C; Carvalho, R.; Fonseca, A.M.; Castelo Branco, M.; Alves, M., Jarak I. Standard Doses of Cholecalciferol Reduce Glucose and Increase Glutamine in Obesity-Related Hypertension: Results of a Randomized Trial. *Int. J. Mol. Sci. 2024, 25*(6), 3416.

Resumo

A hipertensão arterial (HTN) é uma doença crónica e de atingimento sistémico conhecida há várias décadas, mas com um prognóstico que pouco mudou desde então. A elevada morbilidade e mortalidade associadas a esta doença têm sido o fio condutor para o desenvolvimento de novas estratégias clínicas e terapêuticas, mas, mesmo com progressos científicos assinaláveis, o seu prognóstico continua reservado. Na última década, a evidência científica tem documentado que as células imunitárias também contribuem para a patogénese da HTN, sublinhando o seu papel no desenvolvimento de inflamação crónica e sustentada. Dada a estreita ligação entre a imunidade inata e adaptativa, não surpreende que a maioria das células imunitárias esteja implicada na patogénese das respostas hipertensivas. No caso da HTN associada à obesidade, uma epidemia crescente nos dias de hoje, temos também de considerar a contribuição do tecido adiposo enquanto órgão endócrino muito relevante, mas também este imunologicamente comprometido. Contrabalançar este ambiente inflamatório não é, por isso, uma tarefa fácil e para a qual apenas alguns imunossupressores demonstraram eficácia. No entanto, a sua aplicabilidade na prática clínica é limitada e devem ser procuradas outras alternativas. A vitamina D (VD) pode representar uma dessas opções, uma vez que possui reconhecidas propriedades imunomoduladoras, sendo já utilizada no contexto de várias doenças autoimunes como a diabetes mellitus tipo 1 ou a esclerose múltipla.

Com o objetivo de explorar a desregulação imunitária que acompanha a HTN associada à obesidade, e o papel imunoregulador da VD, desenvolvemos um ensaio clínico de iniciativa do investigador. Tratou-se de um estudo de fase 2, unicêntrico, aleatório, aberto e com duração de 24 semanas. Foram recrutados adultos com HTN associada à obesidade e deficiência de VD para receber a terapêutica habitual e 50 000 UI/semana de colecalciferol ou apenas a terapêutica habitual. Procedeu-se a colheita de amostras de sangue periférico e tecido adiposo abdominal subcutâneo nas semanas 0 e 24 com o objetivo de avaliar as diferenças nos respetivos perfis imunológicos, mas também no metaboloma sérico. Um grupo de controlo de normotensos com excesso de peso foi também avaliado no início do estudo. O objetivo primário foi a avaliação da variação percentual das células periféricas TCD4+, TCD8+, T reguladoras (Tregs) e T helper 17 (Th17). Foram definidos três endpoints secundários adicionais: variação das células Th1, T citotóxicas 1 (Tc1), Tc17 e monócitos no sangue periférico, variação do número de macrófagos perivasculares e não perivasculares, linfócitos TCD4+ e TCD8+ no tecido adiposo e, por último, as modificações no metaboloma sérico, com especial incidência para as variações na glutamina. Foram também monitorizadas as modificações nos valores médios dos índices de resistência à insulina, incluindo HbA1c e índice de adiposidade visceral.

O ensaio incluiu 36 participantes com o diagnóstico simultâneo de HTN e obesidade ou síndrome metabólica, sendo 18 aleatorizados para cada grupo. Apenas um doente não cumpriu a medicação do ensaio. Todas as análises foram efetuadas de acordo com o protocolo. Em comparação com os controlos normotensos, os voluntários hipertensos apresentaram percentagens mais elevadas de linfócitos T ($p= 0.016$), Tregs ($p = 0.014$) e monócitos não clássicos ($p < 0.001$). Na semana 24, as células Th17 aumentaram no grupo de controlo ($p = 0.017$), mas permaneceram estáveis no grupo da VD. As células Tregs reduziram para valores próximos aos documentados no grupo de normotensos ($p = 0.003$) e, em análise multivariada, apresentaram um aumento significativo do seu "loading" no conjunto das células da imunidade adaptativa (Eigenvalue 1.78; $p <0.001$). Não foram registadas alterações na percentagem das células TCD4+, TCD8+ ou monócitos periféricos. Na semana 0 observou-se no tecido adiposo uma correlação negativa entre os níveis de VD e os macrófagos perivasculares ($r = -0.387$; $p = 0.024$), que persistiu no grupo de controlo ($r = -0.528$, $p = 0.024$) na semana 24, mas não no grupo de tratamento com colecalciferol. Neste grupo documentou-se um aumento dos macrófagos não perivasculares ($p = 0.029$) na semana 24 e a estabilidade das restantes células identificadas. O metaboloma sérico dos doentes hipertensos, em comparação com o dos normotensos, caracterizou-se por uma diminuição global da expressão de vários aminoácidos, incluindo aminoácidos cetogénicos ($p<0.05$) e glucogénicos ($p<0.0001$), bem como aminoácidos aromáticos ($p<0.001$). Após a suplementação com colecalciferol, foram observados aumentos significativos nos níveis de glutamina ($p<0.001$) e histidina ($p<0.05$), enquanto outros aminoácidos permaneceram inalterados. A glicose ($p<0.05$) e o acetato ($p<0.05$) diminuíram após as 24 semanas no grupo sob colecalciferol e foram também observadas alterações na saturação de ácidos gordos, sugerindo um papel da VD lipossolúvel no metabolismo lipídico. Não foram observadas alterações nos valores médios dos índices de resistência à insulina. Também não foram registados eventos adversos graves em nenhum dos participantes.

Concluímos que a VD pode ser um fármaco útil e seguro no contexto da HTN associada à obesidade. A transição para um estado menos pró-inflamatório pode ser alcançada utilizando os esquemas posológicos habituais para a VD. O perfil imunológico periférico dos hipertensos contrastou significativamente com o dos normotensos, tendo sido identificados aumentos paradoxais tanto nas células pró-inflamatórias como nas anti-inflamatórias. A diferença estatisticamente significativa observada para as Tregs, à semelhança do que se verifica noutras doenças crónicas, aponta para uma regulação positiva como forma de reequilibrar o estado de inflamação crónica. Sob o efeito do

colecalciferol, a percentagem de TCD4+ e de TCD8+ periféricas não foi afetada, mas a expansão de Tregs e de células Th17 foi limitada, aproximando-se os seus valores das percentagens observadas para os controlos normotensos. As células Th1 aumentaram, mas esta alteração não está necessariamente associada a uma maior libertação de citocinas, e os monócitos não foram afetados pela VD.

Foi identificado um fenótipo metabólico único para os doentes hipertensos, que se caracteriza por níveis reduzidos de vários aminoácidos, tanto glucogénicos como cetogénicos, mas também de subprodutos do metabolismo da glicose, como o lactato e o acetato. Em conjunto, estas alterações sugerem um desequilíbrio a favor do aumento basal do catabolismo, contribuindo para baixos níveis de aminoácidos, mas também para o desvio da produção de energia para outras vias que não o ciclo de Krebs. Com a suplementação de VD, foram observadas modificações em alguns metabolitos, nomeadamente na glutamina e na histidina, compensando parcialmente as perturbações observadas basalmente. A glutamina é um substrato fundamental para a atividade das células imunitárias, e a biodisponibilidade aumentada pode possibilitar a maior utilização do ciclo de Krebs relativamente à glicólise aeróbia. Em combinação com a redução dos níveis de glicose, as alterações introduzidas no microambiente metabólico, poderão ter contribuído para menor utilização da glicólise e, consequentemente, para menor ativação e proliferação das células T.

Ao nível do tecido adiposo, a VD também documentou ter um papel imunomodulador relevante. A correlação negativa com os macrófagos perivasculares, que são importantes na libertação de substâncias vasoconstritoras, sugere que possa interferir com a migração de monócitos para o tecido adiposo perivascular. No tecido adiposo foram ainda observadas frequências aumentadas de macrófagos com distribuição não perivascular após tratamento com VD, mas não nos controlos, num quadro que parece reproduzir as modificações observadas após a cirurgia bariátrica, contribuindo o aumento dos macrófagos para uma maior fagocitose dos detritos celulares no tecido adiposo.

Os resultados obtidos neste ensaio não são completamente consensuais com outros já previamente publicados e há várias limitações que devem ser referidas, nomeadamente a dimensão da amostra ou as concentrações finais de VD no grupo de doentes tratados. Ainda assim, apresenta resultados relevantes quanto ao papel imunomodulador desta pró-hormona, sendo um dos poucos ensaios que estudou do ponto de imunológico e metabolómico os efeitos da VD em HTN, indo para além dos estudos experimentais bem controlados em laboratório e que não têm em conta a heterogeneidade da doença clínica. As suas principais conclusões corroboram os mecanismos de ação da VD, que

em conjunto com a terapêutica anti-hipertensora habitual, pode modular o perfil imunológico e metabolómico dos doentes com HTN associada à obesidade.

Palavras-chave

Hipertensão arterial; obesidade; síndroma metabólica; linfócitos T; células T helper 17; células T reguladoras; tecido adiposo; vitamina D; glutamina.

Abstract

Arterial hypertension (HTN) is a systemic and chronic disease known for several decades but with a prognosis that as changed little since then. The high morbidity and mortality associated with high blood pressure have always been the thread to the development of new strategies and therapeutics but, even with noticeable scientific progress, the prognosis of HTN remains poor. In the last decade, multiple evidence as documented that immune cells may also contribute to the pathogenesis of HTN, underlining its role in the development of chronic and sustained inflammation. Given the close link between the innate and adaptive immune systems, it was not surprising that most immune cells were found to be implicated in the pathogenesis of hypertensive responses. In the case of obesity-related HTN, a growing epidemic in the nowadays, we must also consider the contribution of an already unbalanced immune system, supported by a powerful but also immunologically unbalanced endocrine organ, which is the adipose tissue. Counterbalancing this stormy environment is a difficult task and for which only some immunosuppressives have demonstrated efficacy. However, their applicability in clinical practice is limited and other alternatives should be searched. Vitamin D (VD) may represent one of such alternatives, since it has recognized immunomodulatory properties, being already used in the context of several auto-immune diseases like type 1 diabetes mellitus or multiple sclerosis.

Aiming to explore the immune dysregulation that comes with obesity-related HTN, and the immunoregulatory role of VD in this setting, we developed an investigator-initiated trial. It was a phase-2, single-centre, randomised, open, 24-week study, where adults with obesity-associated HTN and VD deficiency were assigned to receive usual therapy plus 50 000 IU/week of cholecalciferol or usual therapy alone. Sampling of peripheral blood and subcutaneous abdominal adipose tissue ensued to evaluate for differences in immunological profiles. A control group of overweight normotensives was also evaluated at baseline. The primary endpoint was the percentual variation in peripheral TCD4$^+$, TCD8$^+$, T regulatory (Tregs), and T helper 17 (Th17) cells. Three additional secondary endpoints were defined: variation of peripheral T helper 1 (Th1), T cytotoxic 1 (Tc1) and Tc17 cells, and monocytes, variation in the number of adipose tissue perivascular and non-perivascular macrophages, TCD4$^+$ and TCD8$^+$ lymphocytes, and, lastly, the modifications in serum metabolome, with a special focus in glutamine. Modifications in the average values of insulin resistance indexes were also monitored and included HbA1c and visceral adiposity index.

The trial recruited 36 participants with the simultaneous diagnosis of HTN and obesity or metabolic syndrome, 18 randomised to each group. Only one patient was non-compliant with trial medication. All the analyses were performed per protocol. In comparison with normotensive controls, hypertensives presented higher percentages of T lymphocytes ($p = 0.016$), Tregs ($p = 0.014$), and non-classical monocytes ($p< 0.001$). At week 24, Th17 cells increased in control group ($p = 0.017$) but remained stable in cholecalciferol group. For Tregs, downregulation towards the values of normotensive controls was observed ($p = 0.003$), and in multivariate analysis, an increased loading in the setting of the cells of adaptive immunity observed (Eigenvalue 1.78, $p < 0.001$). No changes were documented for peripheral TCD4+, TCD8+ or monocytes. In adipose tissue, a baseline negative correlation between VD and perivascular macrophages was observed ($r = -0.387$, $p = 0.024$) that persisted in the control group ($r = -0.528$, $p = 0.024$) but not in the cholecalciferol group, which presented an increase in non-perivascular macrophages ($p = 0.029$) at week 24. Serum metabolome of hypertensives compared to normotensives presented an overall decreased expression of several amino acids, including ketogenic ($p< 0.05$) and glucogenic ($p< 0.0001$) amino acids as well as aromatic amino acids ($p < 0.001$). Following cholecalciferol supplementation, notable increases were observed in glutamine ($p< 0.001$) and histidine ($p< 0.05$) levels, while several other amino acids remaining unaffected. Glucose ($p< 0.05$) and acetate ($p< 0.05$) decreased after the 24 weeks in the group taking cholecalciferol and changes in the saturation of fatty acids were also observed, suggesting a role of liposoluble VD in lipid metabolism. No changes in the average values of insulin-resistance indexes were observed. No serious adverse events were reported for all the participants.

We concluded that VD may be a useful and safe drug in setting of obesity-related HTN. The shift towards a more anti-inflammatory state may be reached using standard doses of VD. Peripheral immune profile of hypertensives contrasted significantly with normotensives, being identified paradoxical increases in both pro- and anti-inflammatory cells. The statistically significant difference observed for Tregs, as seen in other chronic conditions, points to upregulation as a means of counterbalancing the inflammatory milieu. Under the effects of cholecalciferol, the percentage of TCD4+ and TCD8+ was unaffected but the unrestrained proliferation of Tregs and Th17 cells was limited, with the frequencies of some cells approaching those of normotensive controls. Th1 cells increased but this change is not necessarily linked to increased release of cytokines, and monocytes were unaffected by VD. A unique metabotype was identified for hypertensives, which is characterized by reduced levels of several amino acids, both gluco- and ketogenic, but also sub-products of glucose metabolism, like lactate and acetate. Together, these modifications suggest a high catabolic rate and protein wasting

but also routing of energy production outside of the Krebs cycle. With VD supplementation modifications were seen for some metabolites, most notably glutamine and histidine, partially offsetting the disturbances observed at baseline. Glutamine is a fundamental substrate to fuel immune cells activity, and increased availability may route immune cells to Krebs cycle instead of aerobic glycolysis. In combination with the reduction in glucose levels, this new microenvironment that surrounded immune cells could have dampen the fast energy production through glycolysis and, hence, attenuate T cell activation and proliferation.
At the level of the adipose tissue, VD also documented immunomodulatory potential. Correlating negatively with perivascular macrophages, important releasers of vasoconstrictors, increased levels in the peripheral blood may interfere with monocytes migration to perivascular adipose tissue. Above of all, increased frequencies of macrophages with non-perivascular distribution were seen after supplementation, but not in controls, in a picture that seems to mimic the modifications seen after bariatric surgery, and the increased cells contributing to enhanced phagocytosis of cellular debris in an involuting adipose tissue mass.

Although some of our findings are not in line with other reports and several limitations should be addressed, we think our trial represents an important effort of translation of results to the patient level, where heterogeneity and a multiple array of factors disrupt the well-controlled conditions of the laboratory. Above of all, this trial allowed us to gain a more comprehensive understanding of the immune mechanisms underlying HTN in a specific context and to explore more deeply the well-known immunomodulatory actions of VD.

Keywords

Arterial hypertension; obesity; metabolic syndrome; T lymphocytes; T helper 17 cells; regulatory T cells; adipose tissue; vitamin D; glutamine.

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List of Acronims

ACEI	Angiotensin converting enzyme inhibitors
AG	Active or cholecalciferol group
Ang II	Angiotension II
APC	Antigen-presenting cells
AT	Adipose tissue
ATP	Adenosine triphosphate
BMI	Body mass index
CG	Control group
CXCL12	Chemokine ligand C-X-C motif chemokine ligand 12
DC	Dendritic cells
DPP-4	Dipeptidyl peptidase 4
HTN	Arterial hypertension
IFN-γ	Interferon- γ
IL	Interleukin
JNK	c-Jun N-terminal kinases
KMO	Keiser-Meyer-Olkin
MCP-1	Monocyte chemoattractant protein-1
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NF-kb	Nuclear factor kappa B
NMR	Nuclear magnetic ressonance
NO	Nitric oxide
PB	Peripheral blood
PCA	Principal component analysis
PNS	Parasympathetic nervous system
PPAR-γ	Peroxissome proliferator activated receptor-γ
RAAS	Renin-angiotensin-aldosterone system
RANTES	Regulated upon Activation Normal T cell Expressed and Secreted
ROS	Reactive oxygen species
CNS	Central nervous system
SNS	Sympathetic nervous system
Tc1 cells	T cytotoxic 1 cells
TCA	Tricarboxylic acid cycle
Th1 cells	T helper 1 cells
Th17 cells	T helper 17 cells
Th2 cells	T helper 2 cells
TNF-α	Tumour necrosis factor-α
Tregs	T regulatory cells
VAI	Visceral adiposity index
VDR	Vitamin D receptor
WC	Waist circumference

Chapter 1- General introduction and literature review

1.1 – Background of obesity-related hypertension

Arterial hypertension (HTN) is a systemic and chronic disease known for several decades but with a prognosis that as changed little since then. The high morbidity and mortality associated with high blood pressure have always been the thread to the development of new strategies and therapeutics but, even with noticeable scientific progress, the prognosis of hypertension remains dark shaded.

There are reports describing the values of blood pressure in the pre-industrial era (1), averaging around 115/75 mmHg, but industrialization has moved this scenario to a new pattern of blood pressure regulation. Pollution (2), change in dietary patterns and exposition to multiple antigens in processed foods (3) as well as pandemics have brought a changing paradigm in the area of cardiovascular diseases.

The impact of energy excess, salt-overloaded fast food, high glycaemic exposure, saturated fats and a vast array of sweeteners, emulsifiers and additives has contributed to a change in the dietary consumption and, consequently, in the risk of cardiovascular diseases.

Reports from the beginning of the 20th century already document the clinical significance of hypertension (4), emphasising the need for blood pressure monitoring. The role of HTN in life expectancy in these early years of 1900 is well mirrored in the following sentence extracted from North Western Mutual Life Insurance Company: "The sphygmomanometer is indispensable in life insurance examinations, and the time is not far distant when all progressive life insurance companies will require its use in all examinations of applicants for life insurance." (4)

Since then, the accumulated evidence, mainly associated with excessive mortality, has led to changes in clinical practice, and by 1918 several reports already document that all insurance companies were performing systolic and diastolic blood pressures measurements by auscultation (4).

The following years, in simultaneous with improvements in scientific awareness and methodologies, brought a plentiful of information that began to be stratified according to gender, race, body size and overweight as well as other clinical conditions and comorbidities (4). And, in this way, the decades of 1960 and 1970 brought the first available large population-based studies (5). These reports, like nowadays, were not devoid of limitations, but are the first evidence of the need to study large numbers of patients to understand this evolving disease.

Interestingly, the association of increased body weight and high blood pressure was already mentioned in a report of 1925 (6), where obesity is described in terms of build groups, that is, average weight for each inch of height both for men and women and in different age groups. Following this early observations, subsequent studies documented the association of overweight, obesity and hypertension. In a search in PubMed using the terms "arterial hypertension" and "obesity" between 1938 and 1978 we find 1368 results, of which 109 were reviews (no systematic reviews documented) but only 6 clinical randomized trials. The National Health Examination Survey (NHES), started in 1960, was probably one of the most extensive collection of data on a wide range of health issues and already included information on blood pressure values in individuals aged 18-74 years old (7). Followed in 1971 by the National Health and Nutritional Examination Survey (NHANES I), this inquiry started to include data on nutritional status (7), specifically body mass index and other body measurements to evaluate, in a nationwide survey, the prevalence of malnutrition in certain specific population groups. From herein, accumulated evidence from decades of epidemiological surveillance have documented the central role of abdominal obesity and weight excess in the incidence and prevalence of hypertension (8, 9, 10), across several ethnicities (11) and in different parts of the world (10).

The epidemics of obesity (Figure 1) (11, 12), accompanying the increasing trend in the values of systolic blood pressure (SBP) is also well documented in a cross-sectional analysis from 1959 to 2008 of consecutive NHES and NHANES surveys (11), demonstrating a progressive increase in BMI over the last decades across black and white USA-based populations and independent of economic income.

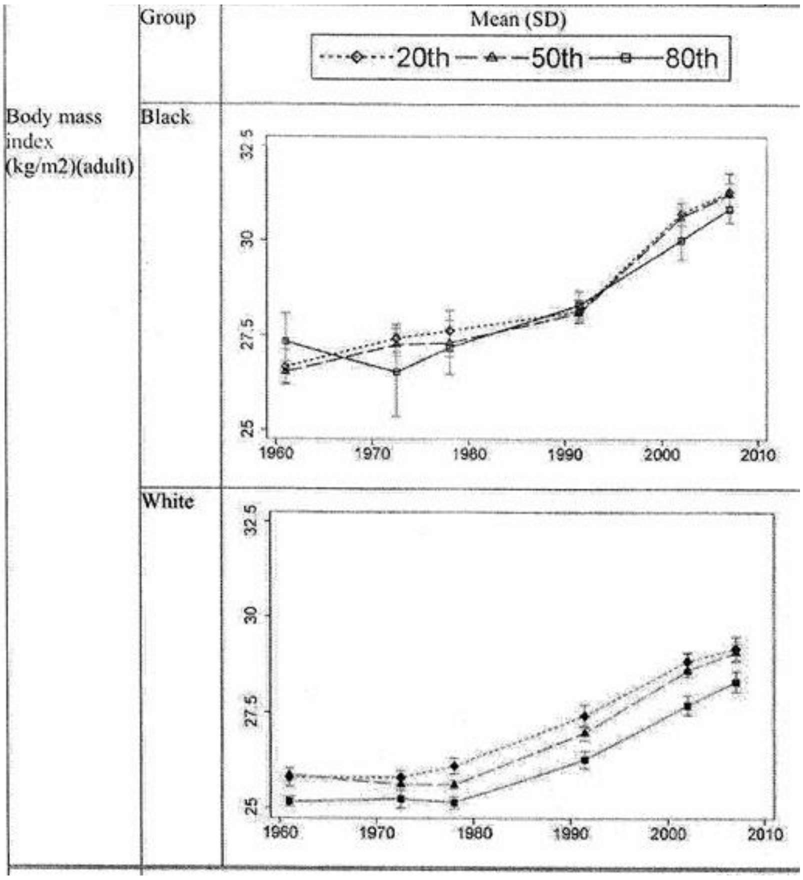


Figure 1: Evolution of body mass index by income percentile for adult individuals (1959-2008) (Data from National Health and Examination Survey). Adapted from (11).

Even if a fell in SBP was observed through these decades because of introduction and widespread utilization of anti-hypertensive medications, development of clinical guidelines and greater awareness of the disease, SBP remained higher in lower economic income populations, and perpetuating a cycle of social disadvantage and chronic disease (Figure 2).

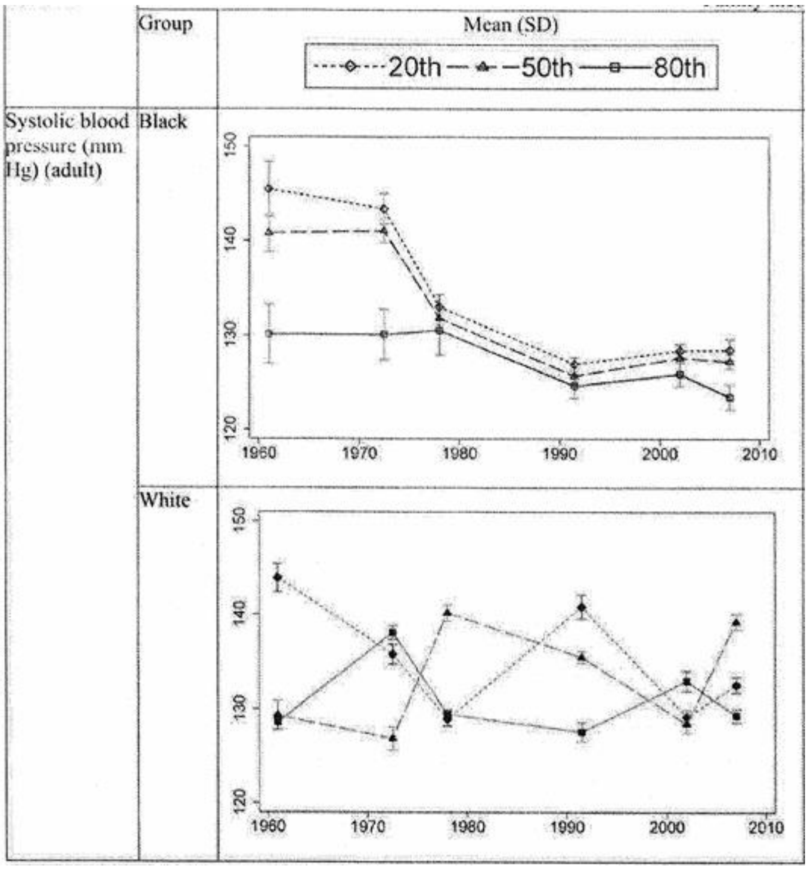


Figure 2: Evolution of systolic blood pressure by income percentile for adult individuals (1959-2008) (Data from National Health and Examination Survey). Adapted from (11).

The risk of hypertension with increasing BMI is also very clear from other cohorts like the Chicago Heart Association Detection Project (13), that in a follow-up period of 32 years of adults between 18-74 years recruited during the years of 1967 through 1973 documented an intertwined role of BMI and blood pressure in the increase of cardiovascular mortality (Figure 3).

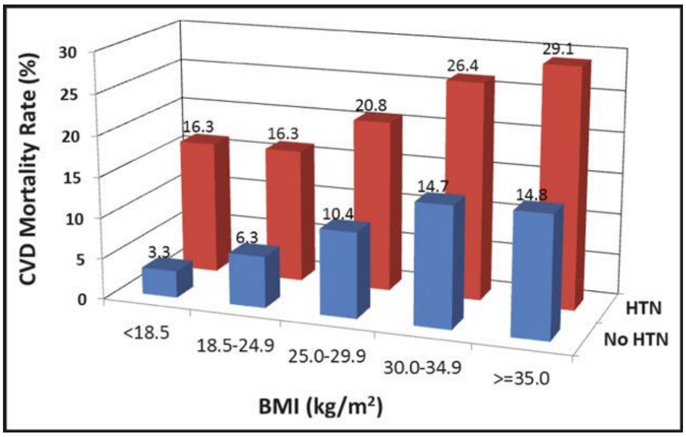
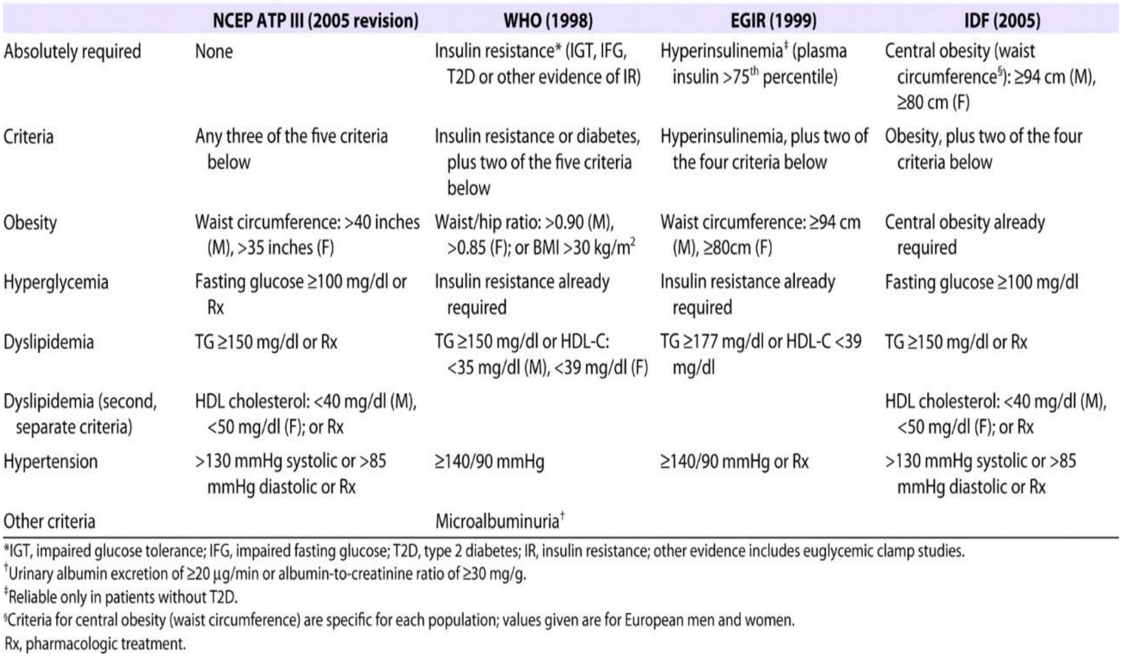


Figure 3: Trends in cardiovascular disease mortality (Data from the Chicago Heart Association Detection Project). From (13).

Data about European population appeared latter, around the 80's, but in general confirmed these previous observations in patients of Caucasian ancestry (14, 15), leading to the presentation by Gerald Reaven in 1988 of a concept that aimed to aggregate all these observations, and at that time called the X-syndrome (16), nowadays, metabolic syndrome. Although relatively recent, since its creation, this concept of metabolic syndrome was adopted by several scientific societies and suffered many adaptations with the intention of better reflecting the underlying pathophysiological mechanism, insulin resistance (17). Even if the criteria may change slightly according to the different Societies (Table 1), the combination of high blood pressure and excess body weight is consensual to all the accepted definitions, as the cornerstones of insulin resistance.

Table 1: Definitions of metabolic syndrome. From (17).



	NCEP ATP III (2005 revision)	WHO (1998)	EGIR (1999)	IDF (2005)
Absolutely required	None	Insulin resistance* (IGT, IFG, T2D or other evidence of IR)	Hyperinsulinemia‡ (plasma insulin >75th percentile)	Central obesity (waist circumference§): ≥94 cm (M), ≥80 cm (F)
Criteria	Any three of the five criteria below	Insulin resistance or diabetes, plus two of the five criteria below	Hyperinsulinemia, plus two of the four criteria below	Obesity, plus two of the four criteria below
Obesity	Waist circumference: >40 inches (M), >35 inches (F)	Waist/hip ratio: >0.90 (M), >0.85 (F); or BMI >30 kg/m^2	Waist circumference: ≥94 cm (M), ≥80cm (F)	Central obesity already required
Hyperglycemia	Fasting glucose ≥100 mg/dl or Rx	Insulin resistance already required	Insulin resistance already required	Fasting glucose ≥100 mg/dl
Dyslipidemia	TG ≥150 mg/dl or Rx	TG ≥150 mg/dl or HDL-C: <35 mg/dl (M), <39 mg/dl (F)	TG ≥177 mg/dl or HDL-C <39 mg/dl	TG ≥150 mg/dl or Rx
Dyslipidemia (second, separate criteria)	HDL cholesterol: <40 mg/dl (M), <50 mg/dl (F); or Rx			HDL cholesterol: <40 mg/dl (M), <50 mg/dl (F); or Rx
Hypertension	>130 mmHg systolic or >85 mmHg diastolic or Rx	≥140/90 mmHg	≥140/90 mmHg or Rx	>130 mmHg systolic or >85 mmHg diastolic or Rx
Other criteria		Microalbuminuria†		

*IGT, impaired glucose tolerance; IFG, impaired fasting glucose; T2D, type 2 diabetes; IR, insulin resistance; other evidence includes euglycemic clamp studies.

†Urinary albumin excretion of ≥20 µg/min or albumin-to-creatinine ratio of ≥30 mg/g.

‡Reliable only in patients without T2D.

§Criteria for central obesity (waist circumference) are specific for each population; values given are for European men and women.

Rx, pharmacologic treatment.

And, in this way, the role of metabolic syndrome, in particular insulin resistance, in the pathophysiology of obesity-associated HTN became well established (18). Since then, several reports have documented the importance of this constellation of symptoms, more so when more criteria of the syndrome are present (17).

Despite these efforts, the overall improvement in prognosis has been hard to achieve and the more recent numbers related to hypertension mortality and morbidity remain high (19). Even if we can argue that the most recent guidelines for HTN definition both from the American and European Societies have lowered the threshold for HTN definition and, hence, increased the proportion of patients eligible for treatment (19), the fact is that the rates of reported cardiovascular mortality remain exceedingly high. It is postulated that the implementation of these new targets may further reduce 340 000 cardiovascular events and 156 000 deaths.

However, in their latest report from 2019 (published in December 2020), World Health Organisation continues to document ischemic heart disease, frequently secondary to long-term HTN, has the leading cause of mortality for noncommunicable diseases, being responsible for the largest increase in deaths in the last two decades (between 2000-2019) (20).

1.2 – Pathophysiology of obesity-related hypertension

Despite the noticeable advances in the field of HTN, the growing epidemics of obesity, metabolic syndrome, and insulin resistance, which runs hand by hand with HTN, makes this an unsolved and ongoing clinical challenge.

By the mid-1980s and with the greater availability of basic clinical research, remarkable progresses have been made, not the least of which was the discovery of several hormones involved in the dysregulation of blood pressure control (13).

Nowadays, it has become clear that obesity-associated HTN depends on the interaction of multiple stimulus, like genetic, epigenetic, and environmental factors but also from gestational history (Figure 4). This concept goes far beyond the simple and restrictive explanations that historically attributed high cardiac output and error measurements as the main potential mechanisms to explain raised blood pressure in the context of obesity.

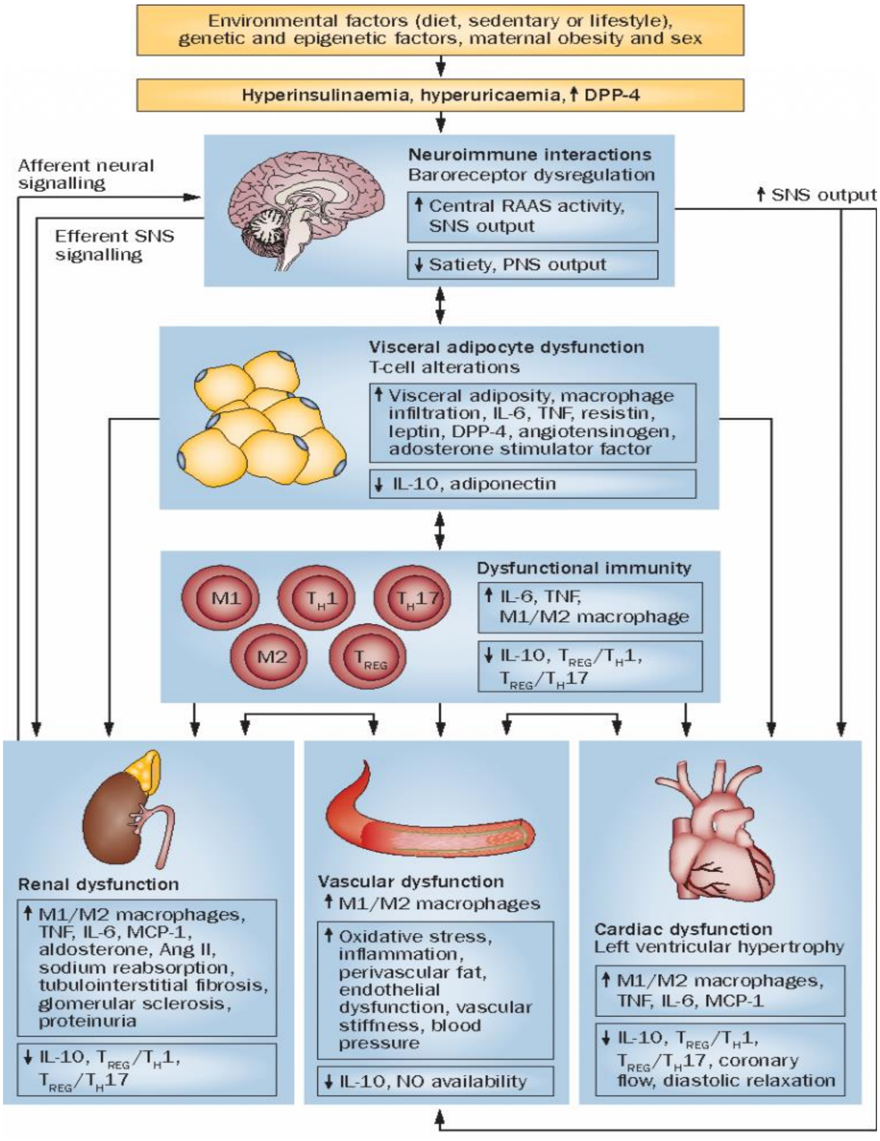


Figure 4: Obesity-associated HTN pathophysiology. From (21).

It is difficult to ascertain what is the first trigger but energy overload from high calories diets in the form of rapid absorption carbohydrates, saturated fats and sodium rich processed foods may constitute the first step for this vicious cycle. The overall energy positive balance induces a compensatory rise in the levels of insulin but also uric acid, especially in high fructose diets, that is further amplificated by the synergistic action of increased levels of the dipeptidyl peptidase 4 (DPP-4) (21). This exopeptidase rapidly degrades several gut derived hormones that modulate postprandial and long-term glucose metabolism (21). From herein, a set of multiple cascades became dysregulated, leading to short and long-term derangements in neuroendocrine communication, involving not only effector organs like the kidneys or the peripheral vasculature but also the central and autonomic nervous systems that can mediate the crosstalk between distant organs like the brain and the abdominal adipose tissue. Gut microbiota imbalance may also contribute to this dysregulation(22). A truly new and complex system of communication and biofeedback is engendered with an overall increase in tubular sodium reabsorption and pressure natriuresis compromise, increased peripheral vascular resistance and baroreflex dysfunction contributing to high blood pressure mediated by the activation of the sympathetic nervous system (SNS)   (21).

This activation is most pronounced in the sympathetic renal and skeletal muscle nerves, being only modest in other organs, or even normal in heart, and doesn´t explaining all the observations made in obesity-associated HTN (23). Other mediators, besides SNS, are involved and probably play a major role in the chronic and long-standing perpetuation of HTN in the context of obesity.

The adipose tissue (AT), mainly visceral AT in central obesity, is a well-recognized source of a countless number of cytokines and hormones that can also modulate the SNS but also central nervous system (23). AT is no longer considered an inert reservoir of fat depots but rather a genuinely endocrine organ, from where numerous mediators like leptin, adiponectin, resistin, apelin, visfatin, multiple cytokines and mediators are released (21, 24).

In the case of obesity-associated HTN, the role of leptin is especially relevant as hyperleptinemia resulting from dysfunctional adipocytes compromises the appetite mechanisms and hyperactivates the SNS. Even if the crossing of the blood-brain-barrier by leptin can reduce appetite, in obesity, like in diabetes or HTN, resistance to the anorexic effects of leptin is present and several factors, like hypertriglyceridemia, may compromise the transport of leptin to the central nucleus of the brain (23). In this way, hyperleptinemia and resistance to leptin action may result in long-term weight gain (Figure 5).

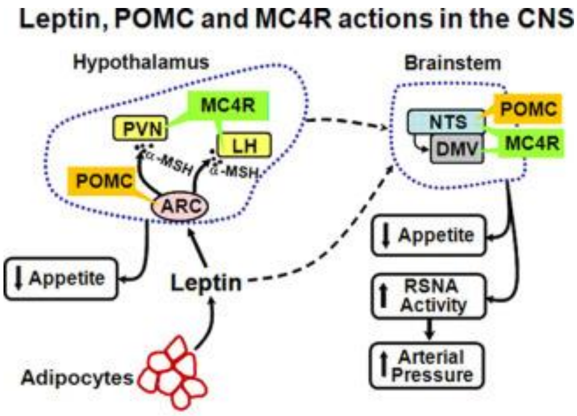


Figure 5: Appetite regulation mechanisms mediated by leptin. Adapted from (23). ARC: arcuate nucleus; LH: lateral hypothalamus; DMV: dorsal motor nucleus of the vagus; MSH: melanocyte-stimulating hormone; POMC: pro-opiomelanocortin neurons.

On the other hand, the increase in blood pressure mediated by leptin is insidious, taking days to weeks to be observed and is essentially mediated by the sympathetic renal nerves. A generalized vasoconstriction mediated by SNS is not observed but its effects can be antagonized by α- and β-adrenergic blockade, further supporting the role of SNS as the mediator between the brain and the kidney to increase blood pressure (23). Of interest is the fact that this leptin resistance is selective, since vasopressor responses mediated by SNS activity are maintained but the suppressant effects in appetite are attenuated, meaning that appetite is increased. This paradoxical selectivity is explained by the different locations of leptin receptors in CNS centres, leading to activation of different intracellular signalling cascades (25) (Figure 5).

Upon activation by leptin, the neurons that express pro-opiomelanocortin can release melanocyte-stimulating hormone that subsequently activates melanocortin 3 and melanocortin 4 receptors. At the level of arcuate nucleus in the hypothalamus in the CNS or in the nucleus solitary tract in the brainstem the effects of this melanocortin-mediated cascade are potentially different since the activation at the level of brainstem may be responsible for increased blood pressure. In physiological conditions, a positive energy balance increases melanocortin types 3 and 4 signalling, increasing energy expenditure and thermogenesis (25). However, in obesity the chronic activation of melanocortin receptors, specially type 4, may selectively increase sympathetic tonus,

and raise blood pressure, without interfering with appetite and food consumption, meaning that leptin is a sub-product of obesity necessary to induce SNS-mediated increases in blood pressure but by itself does not increase weight.
Strengthening these observations is the fact that about 5% of childhood obesity cases are explained by melanocortin type 4 receptor deficiency and the affected individuals are extremely obese but not hypertensive (23, 25). This divergent actions of the melanocortin cascade may justify the different effects at the level of appetite regulation, thermogenesis, and blood pressure in overweight and obese individuals (Figure 5).

In the same way, also hyperinsulinemia contributes to melanocortinergic dysregulation, but in this case via efferent stimulus to lumbar sympathetic nerves and making evident the importance of the axis phosphatidylinositol 3-kinase–melanocortin receptor 4 (PI3K-MC4R) (Figure 6). Excess adiposity also leads to the loss of the metabolic effects of both insulin and leptin, meaning that, once established, these mechanisms reprogramme the overall metabolism, being very difficult to revert.

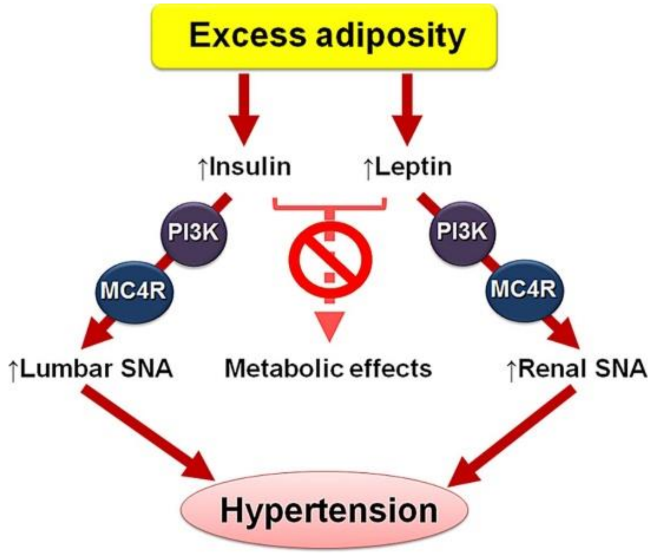


Figure 6: Actions of insulin and leptin in obesity-associated HTN. From (24).

Other hormones originating in the adipose tissue contribute to the pathogenesis of obesity-associated HTN. For example, adipocyte-derived aldosterone is involved not only in vascular function, acting in a paracrine way but also in long-term changes in AT vessel walls (24) in a manner similar to that observed for systemic aldosterone. The AT derived-aldosterone also contributes to the already increased concentration observed in peripheral plasma of obese patients and is the rational for the efficacy of aldosterone antagonists in this patient population.

Angiotensinogen can also be synthesized by adipocytes. It is not completely clear if angiotensin II synthesized by adipose tissue in obese patients increases systemic angiotensin II, as several other tissues also display local renin-angiotensin-aldosterone system (RAAS) proteins but ablation of angiotensinogen gene in adipose tissue significantly blunts blood pressure elevation (21).

The role of the several other hormones derived from AT also deserves mention, even if their synthesis is not exclusive from the adipocytes but being upregulated when obesity develops. This is the case of resistin, associated with cardiovascular diseases by means of inflammation promotion, endothelial dysfunction, and smooth muscle cell apoptosis (26) and also chemerin, an endogenous vasoconstrictor implicated in the reduction of nitric oxide bioavailability (27).

The discovery in the last two decades of this vast array of molecules, peptides and hormones has led to a more comprehensive perspective of the definitive role of the adipose tissue in the pathogenesis of obesity-associated HTN (27) (Figure 7).

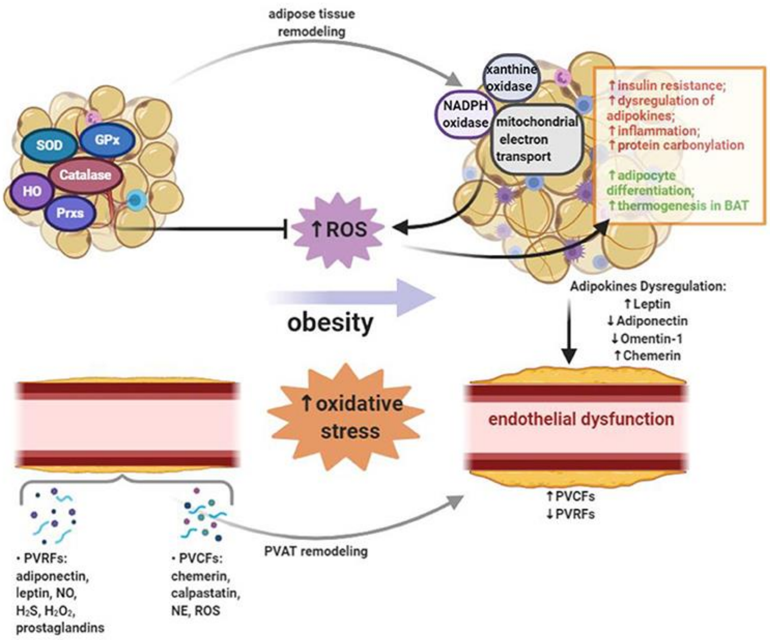


Figure 7: Adipose tissue adaptation to obesity. SOD: superoxide dismutase (SOD), GPx: glutathione peroxidases, HO: heme oxygenase, Prxs: peroxiredoxins ROS: reactive oxygen species, PVAT: perivascular adipose tissue (PVAT), PVRFs: PVAT-derived relaxing factors (PVRFs); PVCFs: PVAT-derived contracting factors. From (27).

The description of the effects of increased AT would not be complete without mentioning the role of fat depots at the organ level. Beyond the visceral and subcutaneous abdominal reservoirs, fat depots in vessels, epicardium, muscles, and kidneys also interfere with physiological hemodynamics (21). In the case of fat accumulation in the kidneys, several histological derangements may be observed, ranging from mesangial matrix expansion to collapsing segmental and focal glomerulonephritis, glomerulosclerosis, interstitial fibrosis and tubular atrophy, the latter's hallmarks of chronic kidney disease. At the functional level, there is worsening of medullary hypoxia, glomerular hyperfiltration, increased tubular sodium reabsorption and progressive proteinuria (21). As a result of these changes in glomerular hemodynamic and tubule-glomerular balance, the inappropriate hyperactivation of RAAS cannot dampen the glomerular hyperfiltration and perpetuates sodium reabsorption. The compensatory increase in sodium/glucose cotransporters at the level of the proximal convoluted tubule compensates for this increased glomerular hyperfiltration with sodium reabsorption but at the expenses of simultaneous glucose absorption, further amplifying the vicious cycle of hyperglycaemia and insulin resistance, which may be partially counterbalanced by gliflozins.

Moreover, other extrarenal factors can also participate in the intrarenal activation of RAAS like the SNS-stimulated release of renin, the extrarenal angiotensinogen that reaches the kidneys from other sources like the AT and the effects of free fatty acids in direct aldosterone production (13).

Perivascular adipose tissue depots are also known to release several hormones, some with relaxing properties but others with vasoconstrictor effects. More recently, even, these soluble factors were documented to extend to peripheral skeletal muscle arteries, and their modulation proved to interfere with arterial resistance in animal models of HTN, via K^+ channels (24).

In this way, several mechanisms promote a positive net sodium balance: neural mechanisms elicited by the SNS, hormonal mechanisms involving leptin, aldosterone, and insulin and renovascular mechanisms that rely in the vasoconstrictor properties of angiotensin II at the level of efferent arteriole. The pathophysiological consequences of this sodium overload lead to a shift in the pressure/natriuresis curve to the right, meaning that for attaining sodium and volume balance, higher blood pressures are necessary and blood pressure becomes very sensitive to the variations in sodium consumption (13).

This endless list of obesity-associated HTN related factors should also consider the role of obstructive sleep apnea. With a high prevalence among obese patients, the hyperactivation of SNS also underlines its contribution to hypertension (28) but also hypoxemia, sleep disruption and changes in intrathoracic pressures (28).

In this respect, the measurement of hemodynamic parameters, like cardiac index or systemic vascular resistance index, using impedance cardiography, may be a valuable tool for the management of obese hypertensives (29). Identification of major hemodynamic abnormalities may help shorten the time to achieve blood pressure control and, hence, contribute to global cardiovascular risk reduction. Since, invasive procedures are not adequate for ambulatory patients, non-invasive monitoring may be easily implemented, and previous studies have already shown the value of this technique in tailoring hypertension treatment (30).

In summary, the pathophysiology of obesity-associated HTN is a complex, but also ongoing process that is not explained by a single hormone or peptide, but rather a constellation of factors that result from the interaction of men with its surrounding environment. In supporting this evidence is the fact that neither beta- or alfa-blockers in monotherapy or in combination with diuretics can control HTN in most of the obese patients nor the development sympathetic renal denervation has proved to be superior to standard anti-hypertensive therapy to control HTN or solve the cases of resistant HTN. Given the constants changes and challenges that we continuously face, it is not surprising that the future years or decades will bring new players to this intricated network, that for nowadays is still unfinished.

1.3 - Immunological dysfunction in obesity- associated hypertension

Immunological dysfunction is one of the most promising new players in this network. It was not until the end of the first decade of the 21st century, when the first reports describing the immunological involvement in HTN were published (31). Since then, the knowledge about the role of immunological cells has grown rapidly but the definite transition for translational medicine and application in human trials still waits for more developments, as uncertainty prevails. The cells of the adaptive immunity, especially T cells (31), were the first assigned this role in HTN but subsequently all sets of lymphocytes were documented to be involved, including the cells of innate immunity. This initial work was focused on animal models of angiotensin-II (Ang-II) induced hypertension that documented that in the absence of T and B cells, infusion of Ang-II did not elicit a hypertensive response. However, this was re-established after adoptive transfer of the T cells and with a mechanism of action that mostly replicates the one observed for other clinical conditions (31). However, above of all, the most relevant aspect about the discovery of the role of immunological cells in HTN may be the fact that these mediators are the missing link between sympathetic activation, chronic inflammation, and end-organ disease. If the introduction of specific immunomodulators or immunosuppressors will change the course of the disease is still unknow but efforts to explore this new dimension in the field of HTN are welcome.

To better understand the action of the different cell subsets, we will review the published findings available for animal models and human data. Adaptive immunity seems to be the cornerstone (32, 33), with activated T cells infiltrating target organs involved in blood pressure control (34, 35), but also the adipose tissue (36, 37). In this regard, recent evidence documents that the kidney may be the primer site for immune activation (38). The panel of antigens, or better, neoantigens that trigger antigen presentation is not well characterized, but may result from endogenous protein oxidation and modification secondary to the exposure to the numerous environmental factors that surround the human milieu. The emergence of this neoantigens stimulates antigen-presenting cells (APC), like dendritic cells, preferentially in the kidney, that subsequently migrate to secondary lymphoid organs like the spleen or lymph nodes (38). Interestingly, Ang-II produced by the kidneys by itself can also activate APC's. Upon migration, there is activation of T cells that, intriguingly, return to the kidneys, making these organs a preferential location of immune cell enrichment.

In this way, the inflammation triggered by these immune cells result in SNS activation, looping again via efferent signals to the kidney and contributing to continued RAAS activation, renin release and tubular sodium reabsorption (Figure 8). This feedback mechanism, commonly referred as the reno-cerebral reflex (39), not entirely explained yet, but depending on afferent and efferent fibres of the SNS (40), clearly demonstrates, that immune cells actively participate in the pathogenesis of HTN (41).

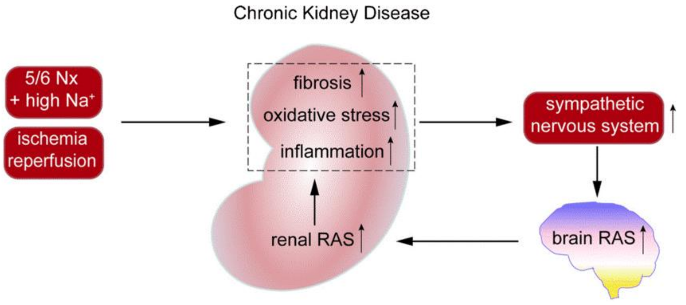


Figure 8: Reno-cerebral reflex. From (39).

Strengthening this theory is the fact, some studies (41), but not all (40), document that renal denervation inhibits T cell infiltration after Ang-II infusion but also dendritic cell maturation and, hence, immune activation (41). The contribution of the renal nerves for dendritic cell activation is essentially attributed to the efferent nerves to which is assigned, in these circumstances, the capacity to form neoantigens (38). The subsequent cascade of events that develops includes, among others, deregulated synthesis of cytokines, endothelial dysfunction, and increased angiogenesis, hyperactivation of the RAAS, adipose tissue remodelling, and insulin resistance (42, 43, 44, 45) (Figure 9).

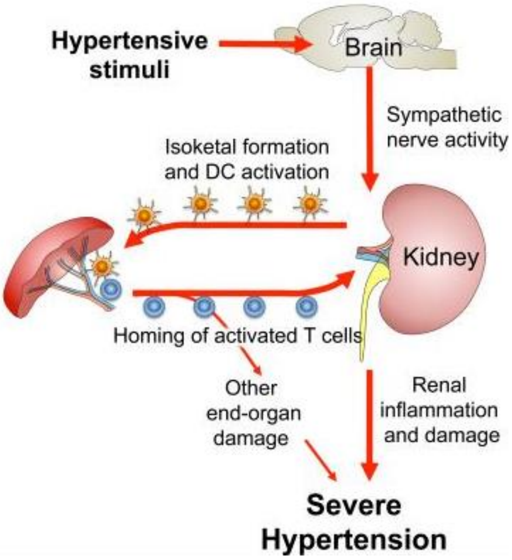


Figure 9: Integration of cerebral, kidney and immunological mechanisms in the pathogenesis of hypertension. Adapted from (41).

Common hallmarks of the immunological dysregulation involve not only the CD4^{+} compartment, with reduction of Tregs and upregulation of Th17 cells (46, 47), but also CD8^{+}-derived cells (48) and the cells of the innate immunity.

The CD4+ derived cells, including its all-major subsets, T helper(h)1, Th2, Th17 and T regulatory cells (Tregs) are all implicated in the several immune-mediated mechanisms of HTN. A skewed phenotype towards Th1 cells and downregulation of Th2 cells is usually described, with a parallel increase in IFN-γ release from Th1 cells. This pattern is usually described in Ang-II and deoxycorticosterone acetate (DOCA) salt induced hypertension (46) models. The DOCA-salt model is indeed a good model to replicate some of the changes observed in obesity induced hypertension. With a marked neurogenic component and dependent from a high-salt diet, it also reproduces some of the immunologic changes that are observed in HTN, like the increased T cells and their derived cytokines, in particular IFN-γ, interleukin-17 (IL-17) and tumour necrosis factor- α (TNF-α) (49). Even if obesity is missing, the neurohormonal components, specially the increased SNS activity, remains as one of the main features of this model.

Still regarding Th1 cells and its signature cytokine IFN-γ, which is also released from Tc1 – CD8+ derived cells, its role in HTN is commonly described after the observations in IFN -/- mice (46). Although not completely identical across the several animal models, uni-nephrectomised mice fed a high-salt diet but lacking INF-γ do not exhibit a hypertensive response to aldosterone or Ang-II perfusion but, paradoxically, develop severe cardiac end-organ disease (46). In line with this observations, other studies confirmed the same results regarding the absence of an immediate hypertensive response in the presence or absence of IFN-γ (50), but found a differential involvement in end-organ systems like the heart and the kidneys. In the absence of IFN-γ, cardiac hypertrophy and myocardial immune cell infiltration was prevented, and a reduced degree of fibrosis and remodelling were observed independent of mean arterial pressure. At the kidney level, no changes were seen at the glomerular level and glomerular filtration rate was preserved but proximal tubular cells increased angiotensinogen expression and conversion to Ang-II, which resulted in increased sodium and water reabsorption at the proximal but also at the distal tubules (46). All the major sodium transporters in the different tubule segments, including proximal and distal, may participate in this Ang-II driven sodium retention and thus contribute to HTN. It becomes evident from this data that although IFN-γ is not a primarily pro-hypertensive cytokine, contributes to local Ang-II production and amplification of hypertensive end-organ disease. The suppression of IFN-γ may thus improve pressure natriuresis and reduce local Ang-II synthesis, representing a potential therapeutic approach in hypertensive patients in which increased CD8+ T cells producing IFN-γ was already well documented (34).

For Th17 cells, the repertoire of literature that documents their involvement as one of the major players in the setting of HTN is even more consistent. In animal models, the frequency of Th 17 cells is increased after Ang-II perfusion and deletion of the gene of IL-17A blunts the response to hypertensive stimulus, endothelial dysfunction and reactive oxygen species (ROS) production (46). In the absence of IL-17A the migration of T cells to the vascular walls is greatly impaired but also of other proinflammatory cells, mainly monocytes and granulocytes, because of loss of this costimulatory signal to chemokines (46, 51). Salt seems to be a potent stimulator of IL-17A synthesis through specific kinases (salt-sensing kinase and glucocorticoid-regulated kinase 1) (51), and in this way linking salt to the immune-mediated mechanisms of HTN, but also a potent inhibitor of endothelial nitric oxide synthase and nitric oxide production. Interestingly, some of these findings were documented in the DOCA-salt hypertension models but scarce information is available from other animal models, specifically of obesity and HTN, like the Zucker rat. In humans there is also evidence of an increased pool of Th17 cells in the context of HTN (46, 52, 53) with a concomitant increase in IL-17A, although more evidence is needed. Clinical scenarios with documented increase in Th17 cells include pre-hypertension, diabetes mellitus associated with HTN, preeclampsia, and non-dipping HTN (53). Of note is also the fact that, besides salt, other factors may potentiate IL-17A secretion like transforming growth factor-β, Ang-II, endothelin and, importantly, vitamin D (VD) deficiency (53). The establishment of this pro-IL-17A factors may serve as a bridge to develop new therapeutic approaches that target not only IL-17A, like the already available monoclonal antibodies in use for patients with ankylosing spondylitis or psoriasis, but also their promoters, and having in mind that other cell types like γ/δ T cells, Tc 17 cells and some B cells can also release IL-17 (46).

Besides its role in the pathogenesis and maintenance of HTN, IL-17A producing cells can also be involved in end-organ damage, as collagen deposition is inhibited in the absence of T cells and, specifically, in IL-17A -/- mice. On the other hand, the effects of IL-17A over adipose tissue seem controversial as some studies point to no role in adipogenesis and insulin resistance (54) but others confirm that inhibition of IL-17A suppresses diet-induced obesity and enhances adipose-tissue browning and energy expenditure (55).

Another cytokine that is also very relevant for the pathogenesis of obesity induced HTN include tumoral necrosis factor alfa (TNF-α), which acts in a manner very similar to the one observed for IFN-γ. That is, not being a primarily pro-hypertensive cytokine, it is essential for HTN development. In experimental models, not only TNF-α null mice do not develop HTN as the neural activation for HTN becomes compromised in its absence (46). TNF-α interferes with endothelial nitric oxide (NO) synthase, severely compromising NO release to the vascular beds. In the kidney, for example, vasoconstriction ensues, counteracting NO action in the thick ascending limb of the loop of Henle and distal tubule in sodium excretion, and compromising blood flow in the renal medulla. T cells can produce TNF-α after Ang-II perfusion but other cell lineages, like monocytes, endothelial and neuronal cells can also release TNF-α. In this way, it becomes evident that not only adaptive immunity, but also innate immunity contributes to the deregulated cytokine synthesis. Furthermore, interleukin-6 (IL-6) synthetised by dendritic cells but also monocytes and macrophages, signals other immune cells, triggering immune activation of Th17 derived CD4+ cells and blood pressure elevation. IL-6 is also sensitive to Ang-II perfusion and increases sodium reabsorption in distal convoluted tubules through the epithelial sodium channels (56), an effect that is blocked by spironolactone treatment.

Meanwhile, the cytokines that may antagonize this inflammatory milieu are proportionally less, with the equilibrium between pro- and anti-inflammatory regulators consistently unbalanced, and clearly hanging down for the pro-inflammatory side. Interleukin-10 (IL-10), produced by Tregs, functions as a regulator in this inflammatory environment, with the potential to modulate endothelial dysfunction and ROS appearance. These protective functions can be reproduced in HTN in a manner very similar to the one observed for other cytokines, with animal models devoid of IL-10 having great susceptibility to damage induced by Ang-II and proportional increases of other cytokines, IL-6 to a greater extent but also TNF-α (46). In preeclampsia, IL-10 is also decreased and its administration in pregnant animals with DOCA-salt HTN re-establishes low blood pressure values. The extent to which the reduction in IL-10 and, consequently, its anti-inflammatory effects are attributed to a vast array of diseases is impressively extensive and ranges, but is not limited to, obesity (57), metabolic syndrome (58), chronic inflammatory diseases (59), neurological diseases (60) and neoplasms. With this scenario it is not unexpected that its properties have been extensively studied, with adoptive transfer in several animal models correcting or, at least, ameliorating some markers of end-organ disease, most notably inflammation but also fibrosis (61). But adoptive transfer of Tregs is not yet clinically feasible due to the limitations in obtaining enough cells and the possible alternative, administration of IL-2/IL-2Ab complex, is not tested in humans so far (61). IL-2 is a fundamental substrate for all the immune cells but specially for Tregs, and its efficacy is enhanced when it is complexed with antibodies, like anti-IL2 monoclonal antibody. In the absence of this antibody, Tregs are destroyed. In certain experimental conditions, the administration of IL-2/IL-2Ab complex can double or even triplicate Tregs numbers, relocate the cells in lymphoid organs like the spleen, and reduce the infiltration of pro-inflammatory cells in target organs, while promoting its recovery (61, 62).

In HTN, the best studied animal model regarding Tregs expansion is the Ang-II-infused mice, with data about obesity-associated HTN being more limited. Nonetheless, the spontaneously hypertensive rat, which is characterized by increased numbers of T CD4+, T CD8+ and Th17 cells also presents increased Tregs in females (63), specially at the kidney. This compensatory rise in renal Tregs is thought to be a response to the elevations in blood pressure, although its exact mechanism remains poorly defined. More recently, other authors also confirmed increased Tregs in female mice of Dahl salt-sensitive strain (64), a model not of primary obesity but instead concurrent obesity and HTN (65). Previously thought as a consequence of increased TGF-β (66), subsequent work documented that this increase promoted peripheral but not renal Tregs, being difficult to ascertain if peripheral Tregs are as relevant players in the pathogenesis of HTN as renal Tregs. In animal models of primary obesity, the role of Tregs is not so well understood, because in the most approximate models, like the obese Zucker rat or the Zucker diabetic fatty rat, generalized lymphocytopenia, compromised cellular immunity and amplification of innate cells is observed (67), findings that contrast with the more frequent studied models of Ang-II HTN. Besides, a great degree of uncoupling between insulin resistance, obesity and human and animal data exists, imposing cautious translation of results (48).

To add even more complexity to the role of Tregs in HTN, several reports document that in other chronic inflammatory conditions that may evolve or not with HTN, Tregs may be increased rather than decreased. This is the case of tuberculosis (68), sarcoidosis (69), chronic otitis media (70) or periodontal disease (71). In this setting, a fully differentiated phenotype is described but with marked instability or even capacity to promote disease (72). Chronic inflammatory diseases, more than autoimmune diseases, may be linked to this risk of pathological reshaping (73), meaning that the aim shouldn't be only the expansion of Tregs in hypertensive-challenged organs, but instead Tregs stability. In this circumstances, it is also very frequently described for Tregs the loss of suppressive capacity (74) and Th17 related functions, including IL-17A production (71).

Upregulation of Tregs was also observed in supra-ST myocardial infarction, sepsis, joint fluid in rheumatoid arthritis or in the adipose visceral tissue in obesity (73). Interestingly, in this last location, Tregs may facilitate obesity and insulin resistance due to an excessively immunosuppressed local environment that supresses not only immune cells but also adipocytes (75). Also in obesity, a small report describes increased levels of Treg's associated with morbid obesity and a skewed T cell phenotype to Th2 cells (76), pointing to the increased regulatory cells as a means of controlling inflammation and inducing self-tolerance, and emerging somehow as an escape mechanism.

Concerning innate immunity, the finding of increased monocytes in animal models of primary obesity is in line with human data that confirms their role not only in obesity (77) but also in HTN. Moreover, monocyte derived stimulation of T cells is essential to maintain a T-cell phenotype towards pro-inflammatory cells and a Th17 cell signature in type 2 diabetic patients (78).

Blocking of macrophage colony stimulating factor blunts the hypertensive response to Ang-II and arteriolar remodelling (79) and, in the same way, in the absence of monocytes there is reduced endothelial dysfunction and superoxide generation (80). To the cells of innate immunity, other properties are also recognized in the sodium balance. Some monocyte derived macrophages are localized to the skin, where they can sense interstitial osmolarity variations secondary to sodium gain and by means of tonicity-responsive enhancer binding proteins increases lymphatics density and sodium excretion (81).

1.4 – The role of the adipose tissue

This integrated answer of adaptive and innate immunity is not naturally complete without the crosstalk with the adipose tissue (AT), even more when excess visceral AT tissue is present, as is the case of central obesity. Being a source of multiple cytokines derived from infiltrating immune cells but also chemokines, hormones, and ROS, it is evident that in the context of energy overload, AT plays an important role in the deregulation of metabolic homeostasis. Although macrophages are the most abundant resident immune cells, T and B lymphocytes also migrate to a growing AT that progressively loses its microscopic characteristics, with transforming adipocytes becoming enlarged and hypertrophic as result of lipid droplets accumulation. This slowly but progressive whitening of the AT compromises its ability for thermogenesis and energy expenditure that is characteristic of beige or brown tissues. However, even if macrophages are the most observed cell type, their infiltration in AT is almost always preceded by other cells, like CD8+ cells (82). In a lean state, adipose Tregs supresses the expression of monocyte chemotactic protein-1 and TNF-α, which limits macrophage migration and upregulates glucose transporter-4, promoting glucose uptake and utilization (37). Once again, peripheral Tregs may not mirror the observations that are made at the level of AT, since it is thought that AT Tregs do not originate from circulating Tregs, because of a distinct transcriptome, but rather from thymus after local antigen-presentation. In knockout peroxisome proliferator activated receptor-γ models a decrease in VAT Tregs is observed but not in the thymus and other lymphoid organs, suggesting that insulin-sensitizing agents may promote AT Tregs enrichment (83). Even if previously these drugs were available for clinical prescription, an unfavourable adverse effect profile set aside their routine use.

Another set of insulin sensitizing cells present in AT are Th2 cells, although present in significantly lower numbers than Tregs. Adoptive transfer of Th2 cells ameliorates markers of insulin sensitivity and may also promote AT Tregs accumulation.

Nevertheless, if over the years overnutrition develops, it will lead to a picture that in several ways mirrors the changes observed in the peripheral blood. Migration and establishment of CD4+ and CD8+ derived cells will ensue, disrupting the insulin sensitivity conditions. The usual culprits include the CD4+-derived Th1 and Th17 cells, CD8+ cells and innate derived cells, namely dendritic antigen-presenting cells, and monocyte-derived macrophages. Of interest is the fact that as soon as 2 weeks the inflammatory mediators of these cell subsets can be identified after a obesogenic stimulus like high-fat diet (84) in mice models. In the case of INF-γ produced by Th1 cells, mRNA could be identified 1 week after a high-fat diet (84), accompanied by increased levels of class II major histocompatibility complex proteins. In this regard, macrophage migration is also precociously stimulated by these cytokines, becoming resident immune cells that easily expand in the setting of continued exposure to obesogenic stimulus.

For IL-17A and Th17 cells the role in AT inflammation is less clear. Several reports document that IL-17A prevents adipogenesis and whitening of AT (54). In the setting of IL-17A deficiency and low-fat diet, fat mass accumulation is accelerated, but confounding factors like γδ T cells also having the ability to release IL-17A, raises the question of an unravelled dissociation in the roles of the different cells that produce IL-17 in the AT (37).

CD8+ T cells are usually regarded as primers for pro-inflammatory macrophage recruitment (82), generally for pro-inflammatory M1 macrophages, that are observed in average after 10-12 weeks of a high-fat diet initiation but even after weight loss there is continued CD8+ T cells infiltration (85). This process contributes to sustained inflammation, even if there is transition to low-energy and low-fat diet, because only in the absence of macrophages there is interruption of T CD8+ cells migration. This is probably one of the reasons why weight regain, sometimes soon after a period of weight reduction occurs, because there is continued influx of inflammatory cells to adipose tissue but also to the liver (85), compromising the negative energy balance.

Concerning macrophages, once established, the revised literature points to polarization towards a M1 or classically activated phenotype. There are not many therapeutic options to reverse this permanent translocation, and resident macrophages have even the ability to form complex structures surrounding adipocytes named crown-like structures, that correlate well with the degree of inflammation and insulin resistance (86).

During the expansion and remodelling of visceral AT the role of dendritic cells (DC) for antigen presentation is fundamental but co-stimulation during the lean or obese states may result in different outcomes (37). Besides, given the solid nature of the tissue, in contrast to peripheral blood, the linking between the innate and adaptive cells may be mediated by major histocompatibility complex class II proteins. In the lean state, CD103+ DC cells polarize CD4+ T lymphocytes to Treg development and supress inflammation. Atypical CD103- DC cells are also present in AT but in very low numbers. With increasing visceral fat both types of DC cells increase, the APC function is enhanced and the differentiation of Th17 cells is boosted (87).

Among the multiple metabolic and mechanical stressors that trigger the inflammation cascade in AT, potential candidates result mainly from the disrupted tissue architecture and insufficient vascularization of a rapidly growing tissue, resulting in hypoxia, crescent amounts of cellular debris from necrotizing adipocytes and an expanding extracellular matrix with ongoing fibrosis (36). Increased fat mass is also an important mechanical stressor, as the increased pressure is also transmitted to intra-abdominal organs like the kidney. On the other hand, increased pressures may also interfere with intestinal blood and lymphatic vessels permeability, allowing the enhanced absorption via chylomicrons or translocation of products from gut microbiota, like lipopolysaccharide. These antigens, together with, free fatty acids and carbohydrate metabolites may activate immune responses through toll-like receptors, also abundantly expressed in adipocytes. At the intracellular level, the JNK and NF-κB pathways lead to the release of multiple pro-inflammatory cytokines and chemokines, like TNF-α, INF-γ, IL-6, RANTES, CXCL12, that modulate the pattern of infiltrating immune cells, and reshape the phenotype of AT immune cells from Th2 to Th1 cells and from M2 to M1 macrophages (36). It is noteworthy that, the number of receptor macrophages may increase from 5% to 45% in adipocytes during the transition from a lean to an obese state and the signalling through the AT insulin receptors decreases dramatically.

Brown AT has a more limited distribution than white AT but seems to be more resistant to the inflammatory changes induced by obesity. “Browning” of white AT is more probable in subcutaneous than in visceral AT, which makes subcutaneous samples a good imprint of the changes induced by therapeutic interventions. The main limitation resides in the fact that subcutaneous AT has lower immune cell infiltration and, hence, lower levels of pro-inflammatory cytokines but in several aspects the changes that occur in visceral AT are mirrored in subcutaneous AT (36). Such anti-inflammatory approaches (usually by inactivation of receptor and signalling proteins) usually produce more intensive “browning” of white AT and thermogenesis capacity recovery, without significant changes in brown AT, strengthening its selective resistance to obesogenic inflammation.

A last note to perivascular AT, site of intense immune cell infiltration, specially of macrophages, at least in infra-diaphragmatic perivascular AT. It is documented that is able to respond to thermogenic cold challenges, ranging from beige to a brown (36), in a manner similar to the observed for non-perivascular AT with increased responsiveness to peroxisome proliferator-activated agonists. Obesity is regarded as an important stimulus that reduces the thermogenic efficiency of perivascular AT and its capacity to increase multilocular cells with increased mitochondrial content. The vascular contractile properties, partially mediated by perivascular macrophages, may also be hyperactivated in view of the increased content of AT vasoconstrictors, like ROS and pro-inflammatory cytokines and reduced availability of NO (27).

1.5 – Metabolic reprogramming of immune cells in obesity-related hypertension

In this environment of immune reshaping driven by insulin resistance, availability of the substrates that fuel immune cells to accompany cell expansion is fundamental, in a way that nutrients needs are achieved for this new configuration. In this way, the transition from lean to obese as well as from normotensive to hypertensive state should engender some kind of metabolic reprogramming to accomplish with greater energy expenditure and a shift from an anabolic protein-based metabolism to a catabolic lipid-overload metabolism. In this way, the availability of metabolites for immune cells, may affect cell activation or suppression, and hence the integrated answers of innate and adaptive immunity.

In resting T cells, maintenance of basal metabolism depends on ATP-derived energy from the tricarboxylic acid cycle (TCA). The catabolism of glucose-derived pyruvate, fatty acids and amino acids provides the source energy to non-activated T cells with low levels of glycolysis (88). These metabolic pathways were observed not only in mice but also in human T cells. However, after antigen presentation aerobic glycolysis and glutamine oxidation ensues, providing “fast fuels” to support the rapid proliferation. Interestingly, this dependence of glucose for activation is seen in TCD4+ and TCD8+ cells, although through different glucose transporters, but Tregs, for example, do not rely their activation in glucose uptake but instead in fatty acid oxidation (88, 89). The transition from a high energy TCA cycle-dependent to a lower energy pathway during activation is thought to be a means by which glycolytic intermediary substrates become available for a more rapid proliferation. Even if glycolysis it is not so efficient in terms of energy production, its speed and ability to produce new metabolites and modulate glycolytic enzymes, overcome its lower energetic efficiency. In Th1 and Th17 cells, upon activation there is also high glycolytic activity, but increased efflux of glucose-6-phosphate through the pentose-phosphate pathway to generate nucleotide precursors and NADPH. In line with this, several experimental models’ of glucose deprivation or glycolysis inhibition prevented T cell activation and promoted Tregs differentiation (88).

Likewise, fatty acid metabolism is implicated in T cell activation. With antigen presentation there is also a transition in the intracellular metabolism of fatty acids from low grade oxidation to increased synthesis. To support this increased production, glucose-derived pyruvate is incorporated in the TCA cycle to generate citrate that can be used for fatty acid production, which links glycolysis with increased fatty acid bioavailability. Fatty acids are required not only for Tregs metabolism but also for membrane phospholipids in rapidly dividing cells.

T cells also depend on glutamine for activation. Glutaminolysis yields glutamate and α-ketoglutarate that can enter TCA cycle to produce high quantities of ATP, but it also releases carbon to nucleotides and amino acids synthesis. Importantly, T cells express glutamate receptors that at the extracellular level promote migration, integrin-mediated adhesion to matrix proteins and proliferation (90) and, hence, a more efficient immune response. The importance of glutamine in cell proliferation is similarly documented in non-immune cells, like epithelial mammary cells, which is inhibited by vitamin D (VD) action on glutamine synthase (91). The effects of glutamine may be even more complex and depend on degree of cell differentiation. For example, in hematopoietic stem and immune cells, glutamine supports rapidly proliferation but in end-differentiated cells like cardiomyocytes, may serve as an energy and biomass source, improve cardiac oxygen consumption and reduce apoptosis (92). Some reports also a describe a role for glutamine in the stimulation of insulin secretion by providing increased bioavailability of citrate to the isocitrate dehydrogenase reaction in pancreatic islet cells (92).

However, at the patient level and in large scale trials, the effects of increased glutamate were associated with incident cardiovascular outcomes (93), which may reflect underutilization of this metabolite in the several metabolic pathways and plasma accumulation.

Effector memory T cells, which are the predominant cell type that infiltrates the kidney in chronic HTN (46), also distinguish from resting T cells because they don't rely in glutamine but instead in fatty acid synthesis and oxidation, somehow recycling fatty acids synthesized from citrate. They present a low rate of glycolysis from which citrate is derived but increased mitochondrial mass, which means that they can significantly increase intrinsic ATP production upon activation (88).

In contrast to effector cells, Tregs in the resting state do not depend in glycolysis but rather in fatty acid oxidation (88, 89). Deprivation of fatty acids but not of glutamine compromises the generation of new Tregs, a finding that is observed not only in mouse cells lines but also across human Tregs.

Innate immune cells metabolism, mostly monocyte-derived macrophages, may differ from that of T cells. M1 macrophages rely in glycolysis but, essentially, through the pentose-phosphate pathway, and glutamine anaplerosis is used for ATP and fatty acid synthesis to accomplish with the high metabolic activity. On the other hand, M2 macrophages use preferentially fatty acid oxidation rather than synthesis and glutamine breakdown, with a low rate of classical glycolysis (88). Other innate immune cells, like mature dendritic cells, depend on pentose-phosphate pathway and fatty acid synthesis in a fashion very similar to the observed for M1 macrophages. Of note, is also the dependence of immune cells on other amino acids like tryptophan, tyrosine and valine mostly involved in NAD+ generation, important for macrophage function, but also serine, alanine and threonine, amino acids of one-carbon metabolism, that are involved in nucleotide synthesis and redox balance through the folate and methionine cycles and arginine metabolism, which is involved in NO production (90).

In the setting of inflammation, immune cells undergo metabolic adaptation and reprogramming to accomplish with the new pathophysiological demands. One of the first modifications observed is aerobic glycolysis, in contrast to glycolysis, in which glucose degradation to lactate occurs even in the presence of hyperoxia (Warburg phenomenon). The advantage is high energy production for rapid proliferation and inflammatory mediator release and is used by almost all immune cells upon antigen activation (94). This process also involves the activation of mTOR receptors to induce glycolysis in T cells. Other stimulus like hypoxia or nutrients depletion, characteristics of inflammation, engender a series of metabolic adaptations to enhance pro-inflammatory immune cells performance. Under hypoxic conditions, T-cell receptor becomes heavily stimulated and induces transcription of hypoxia inducible factor-1α (HIF-1α), favouring aerobic glycolysis. Although the process is very complex at the molecular level, it is especially relevant for Th17 cells and macrophages, with HIF-1α being required for glycolysis and cell maturation. In other conditions, like severe glucose deprivation or increased ROS, immune cells are reprogrammed to a conserved catabolic pathway of serine/threonine kinases that sense energy signals in immune cells, converting their energy consumption from glucose to glutamine and, in more severe conditions, reduce fatty acid synthesis from intermediates of the TCA cycle (94). In summary, under inflammatory conditions, HIF factors promote the rapid activation of T cells, and the conserved catabolic pathway is reserved for extreme conditions to ensure energy production from alternative sources.

Other metabolites that are also relevant for the pathogenesis of HTN include, but are not limited to, branched chain, aromatic and alcoholic amino acids (95, 96). Although they can function as a source for immunometabolites and immunotransmitters, under a pro-inflammatory environment, their routing to other pathways may contribute to the progression of disease. Some nutrients may also elicit a re-routing of metabolic pathways in immune cells like VD but also vitamins A and C (97), rebalancing the equilibrium of the different adaptive immune cells, specially CD4+ derived cells but also improving the chemotactic and phagocytosis capacity of innate immune cells.

Overall, the impact of immunometabolism is quite clear in HTN and end-organ related diseases, like atherosclerosis, with metabolomic profiling of hypertensive patients yielding a vast array of metabolites that may modulate the fate of immune cells that are involved in the pathogenesis of the disease (88). Multiple serum metabolites have been identified, demonstrating their predictive ability not only for the diagnosis of HTN but also distinction of different hypertensive cohorts across different ethnicities (98). Amino acids, sub-products of glucose and lipid metabolism, sex steroids, bile acids, uric acid, creatine, and creatinine have all been implicated as predictors of HTN in human trials (98, 99, 100, 101).

The macrophage immunometabolism in the atherosclerotic plaque is a well-studied example of the effect of the different metabolic pathways in the fate of immune cells. The metabolic modulation that occurs in macrophages with the transition from a quiescent to activated state implies that a metabolic adjustment is performed to react to the external stimulus, like hypoxia, glucose, or increased lipoproteins (102). Monocytes that are recruited to the atherosclerotic plaques' present high glycolytic activity and mitochondrial oxidative phosphorylation, but priming to an M1 phenotype inside the plaque, for example, implies a modulation that diverges glucose metabolism to the pentose-phosphate pathway.

In summary, in obesity-associated HTN there is a complex interplay between neuro-hormonal, immunological and metabolomic factors. A shifting from the classical paradigm that goes beyond the genetic, hemodynamic, environmental, and dietary factors should, nowadays, include other components that until the last decade were unsuspected players in this dynamic framework. The inflammatory immune cell enriched environment implies availability of several substrates to cells pursue their routing to other metabolic pathways. The question that arises now, however, is still the same that was formulated several years ago: how to break the cycle?

1.6 – The immunomodulatory role of vitamin D

To keep up with the latest developments that have come up to light in the last years, a significant interest has emerged in the targeting of the immune and inflammatory components of HTN. Even if addressing all this issues at the patient-level may be complex, the introduction of several immune modulators to target cardiovascular disease is ongoing and the forthcoming years will bring more data on their efficacy and safety. In the particular context of HTN, the first reports come from small trials of autoimmune diseases where the use of immunosuppressants like mycophenolate mofetil or infliximab concomitantly reduced blood pressure in patients with autoimmune diseases (103). For some anti-hypertensive drugs an anti-inflammatory effect is also described, with a special emphasis in monocytes: angiotensin-converting enzyme inhibitors (ACEI) have been documented a inhibitory role in monocytes activation and macrophage infiltration in atherosclerotic plaque and for angiotensin-receptor antagonists a PPAR-γ agonistic function reported (103).

In animal models, a multitude of drugs have been tested, ranging from alkylating agents, inhibitors of innate immune response like anti-toll-like receptors, anti-TNF-α or etanercept and inhibitors of adaptive immune response (bortezomib, muromonab, rituximab). Although a reduction in blood pressure is observed in almost all cases, with concomitant reduction in several chemokines and inflammatory cytokines, the limitations for its application in the real world of HTN clinical practice are enormous (61). Increased infections, excessive immunosuppression, renal and cardiac toxicity are among, but not limited to, the most frequent adverse reactions, which greatly limits its clinical applicability, at least in the short-term.

In face of this, there is a clear need for other alternatives that simultaneously have immunosuppressive properties but, at the same time, a more favourable profile, making them attractive and reasonable options in the context of the daily practice. Vitamin D, administered as either cholecalciferol or calcifediol, is a pro-hormone with documented action on immunological cells (104, 105, 106, 107, 108) and extensive bioavailability at the level of the subcutaneous adipose tissue (109), may be an effective and safe option. In vitro, the effects of cholecalciferol extend over all the cells of the immune system (110) because of the ubiquitous expression of vitamin D receptor (VDR) (107, 111, 112). The active form of VD - $1,25(OH)_2D_3$, formed after a two-step hydroxylation, reduces the activity of dendritic cells, in particular antigen presentation, but also T cells proliferation and migration, while Tregs activity may be enhanced (113) (Figure 10). Immune cells can by themselves produce VD because of VDR expression and 1α-hydroxylase activity, leading to autonomous VD production that is described to act in an autocrine and paracrine fashion, modulating the activity of nearby immune cells (114).

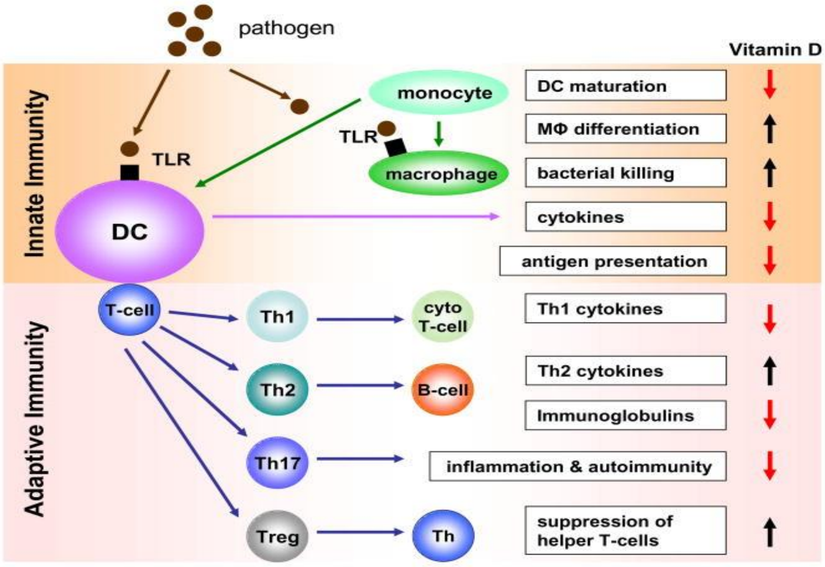


Figure 10: Effects of vitamin D over immune cells. From (115).

It is consensual that, at least in vitro, immune cells exposure to VD induces a transition from a Th1 to a Th2 phenotype, reduces Th17 cells and enhances Tregs proliferation (115). Although this favourable picture was documented in several experimental models of auto-immune disease like multiple sclerosis, type 1 diabetes mellitus or inflammatory bowel disease (114), translation into human data is not so straightforward (116). For example, in type 1 diabetes, numerous trials have reached mixed conclusions about the beneficial effects of VD supplementation: some have found a positive effect in terms of immunoregulation but in others the results were null or negative (117). However, some of these beneficial aspects of VD treatment may become evident only in the long-term, with the snapshots taken after short periods of supplementation not definitively mirroring the substantial extra skeletal benefits of VD.

In healthy patients the results of cholecalciferol supplementation are more readily visible, at least in terms of Tregs induction and increased suppressive capacity (118). Of course, it is not possible to describe its effects under a linear dose-response curve, given the importance of intra-individual characteristics and genetic polymorphisms. However, it also becomes clear that the pathophysiological factors that are in the basis of the multiple clinical conditions for which cholecalciferol was tested, somehow create a resistance to the VD-mediated immunological actions. To further complicate the interpretation of the results obtained by blood peripheral sampling, some reports describe fluctuating dynamic changes in some cell compartments, like in Tregs in experimental type 1 diabetes. In this condition, an increase in Treg's after initial strong inflammation is followed by a regulatory profile with capacity to control inflammation in a later phase of the disease (119). Chronic inflammation may also induce functional changes in immunological cells, like in long-term diabetes the possibility of Tregs acquiring the capacity to release IL-17, meaning that interpretation of results should always integrate data from several sources.

In what concerns HTN, and beyond immune cells, the active form of VD, calcitriol, is also a well-known inhibitor of RAAS (112). Absence of VDR signalling or inactivation of 1α-hydroxylase activity results in increased renin and Ang-II concentrations and activity, HTN and cardiac hypertrophy (120). VD insufficiency also promotes increased vascular sensibility to Ang-II, especially in obese patients, with a progressive increase in RAAS activity with reducing levels of VD (121). Being obese patients highly susceptible to VD insufficiency, because of increased storing of liposoluble VD in adipose tissue and, possibly, low sun exposure due to reduced physical activity, the sustained increases in RAAS activity will also contribute to chronic hypertension.

The question that arises now is how far we can reproduce the immunological and anti-hypertensive effects of cholecalciferol in a chronically inflamed, T-cell unbalanced and VD-prone deficient population, like the obese hypertensives. While reaching reversion of HTN end-organ disease or blood pressure lowering effects does not seem feasible in the short-term, a better characterization of the immune cells associated with this chronic condition as well as the metabolites that support them may be an additional step to deepen our knowledge in this complex disease. Further, evaluating the effects of VD replenishment in the context of a randomized trial may bring new and rigorous evidence to support the translation of its immunomodulatory effects into this new scenario.

Chapter 2 – Hypothesis and Aims

Hypothesis

In obese hypertensives cholecalciferol supplementation increases the percentage of peripheral Tregs and reduces peripheral Th17 cells and the number of macrophages in subcutaneous abdominal adipose tissue.

Aims

Primary aim

We developed a single centre, randomized, 24-week trial including overweight/obese hypertensives with the aim to compare the effects of cholecalciferol supplementation in one primary and three secondary endpoints:

Primary endpoint:

1.1 – Variation in the percentage of peripheral TCD4+, TCD8+, Tregs and Th17 cells

Secondary endpoints:

1.2 – Variation in the percentage of peripheral Th1, Tc1 and Tc17 cells and monocytes

1.3 – Variation in the concentrations of metabolites of glucose, amino acids and lipid pathways

1.4 – Variation in the number of subcutaneous abdominal adipose tissue TCD4+ and TCD8+ lymphocytes and perivascular and non-perivascular macrophages

Secondary aims

2.1 – Comparison of the percentages of peripheral immune cells of obese hypertensives and normotensive controls
2.2 – Comparison of serum metabolome of obese hypertensives and normotensive controls
2.3 – Comparison of insulin resistance indexes before and after cholecalciferol supplementation in obese hypertensives
2.4 – Correlation between adipose tissue and peripheral blood immune cells
2.5 – Correlation between adipose and peripheral blood immune cells and metabolic parameters
2.6 – Peripheral immune cell loadings before and after VD supplementation
2.7 – Effects of free VD over pro-inflammatory cells

Chapter 3 – Patients and Methods

Section 3.1: Trial design

Twenty-four week, single-centre, randomised, open, parallel-group, phase-2 clinical trial to explore the immunomodulatory effects of cholecalciferol in patients with essential HTN associated with obesity or metabolic syndrome.

Clinical trial registered at EudraCT in September 2015 with the number 2015-003910-26. Approval from National Data Protection Committee in June 2016. Submission to competent authorities in May 2017, being approved by INFARMED (National Agency for Medicines and Health Products) in July 2017 and by CEIC (National Ethics Committee) in December 2017. Registered in the National Registry of Clinical Trials with the number MD001501. Communication from Local Ethics Committee of Unidade Local de Saúde de Castelo Branco in March 2018.

Section 3.2: Sample size calculation

The variable used for sample size determination was Tregs percentage in blood samples of healthy volunteers, usually accepted between 4-9% but can vary between 2.1%- 11.7% in healthy donors (122). It is also relevant to note that the percentage of Tregs varies with age: 6.1% in cord blood, 7.2% in adults aged 20-25 years and 7.5% in adults over 60 years (123).

The value of the standard deviation for the distribution of Tregs is also very variable. Based on values obtained from healthy controls, the standard deviation is usually between 1.7% and 2% (124), but in some cases, it may be close to the median value for Treg distribution, i.e., close to 6%.

Considering 7% as an average percentage and the doubling of this value in the cholecalciferol group after the 24-week trial of supplementation as a clinically relevant result (increase in the percentage of Tregs from 7 to 14%) and assuming $\alpha=0.05$ and $\beta=0.2$ (or sample power of 80%) and standard deviation of 6, the expected sample size would be 24 individuals, or 12 patients/group.

If we consider the most frequent value of standard deviation, i.e., approximately 2, the size of each group would be approximately 2 patients, a clearly low value.

The calculations for determining the sample size were made based on the formula $N=2[(a+b)2\ \sigma2]/(\mu1-\mu2)$, where N is the size of each group, $\mu1$ the mean of the variable "percentage of Tregs in the active treatment group", $\mu2$ the mean of the variable "percentage of Tregs in the control group", $(\mu1-\mu2)$ the difference expected to be found, σ the value of the standard deviation, "a" the conventional multiplier for $\alpha=0.05$ and "b" the conventional multiplier for sample power of 80% (125).
Considering this numbers and anticipating a drop-out rate of 10% and 5% chance of spurious or missing data, we projected a trial sample size of 36 participants.

Section 3.3: Patients selection criteria

Hypertensive patients were selected from a routine HTN consultation according to age, diagnosis of essential hypertension, and the presence of obesity or metabolic syndrome. The selected age range was 45 to 65 years because of the greater prevalence of isolated systolic HTN above 65 years, which pathologically differs from obesity-associated HTN. As there is also a reduction in the number of T cells with ageing, narrowing the age span was important to reduce possible bias.

All the patients included in the trial had a diagnosis of arterial hypertension (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg) (126) and were treated with at least one anti-hypertensive medication. Participants with uncontrolled blood pressure after two measurements during the screening (systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 100 mmHg) were excluded. Obesity was defined according to the World Health Organization criteria as body mass index (BMI) ≥ 30 Kg/m^2 and metabolic syndrome according to the criteria of the International Diabetes Federation (17). The choice of the last criteria was based on a recognized increase in insulin-resistance in patients diagnosed with metabolic syndrome using these standards. For inclusion, all patients had to have serum VD levels between 5 (lower limit level for assay detection) and 20 ng/mL. The aim was to exclude patients with undetectable levels of VD but also patients with sufficient levels. The capacity to take oral medication and adhere to the therapeutic regimen was also an entry criterion, in addition to the use of contraception and a negative pregnancy test in premenopausal women.

Exclusion criteria included a concomitant diagnosis of auto-immune disease, previous or actual medication with immunosuppressive agents or antihistamines, concomitant diagnosis of sarcoidosis, and nephrocalcinosis or kidney stones because of augmented risk of hypercalcemia associated with treatment with cholecalciferol. Patients with acute or chronic infectious diseases or active neoplastic diseases were also excluded. Additional exclusion criteria were active smoking, serum calcium > 10.5 mg/dL, glomerular filtration rate < 60 mL/min/1.73m^2, current treatment with cholecalciferol before the beginning of the trial, cholecalciferol intolerance, or simultaneous participation in another trial.

All the participants were evaluated in the same centre (HTN consultation at Unidade Local de Saúde de Castelo Branco) by the same two physicians and nurses' team. The trial was conducted in observance of the principles laid down on the 2008 Declaration of Helsinki.

Section 3.4: Recruitment, screening, and randomization

A 12-week recruitment and screening period during the months of April to June 2018 was performed to evaluate inclusion and exclusion criteria and to collect blood to evaluate serum VD and calcium. Informed consent was also obtained for all the participants during this period. By the end of June 2018, fifty patients had been recruited to obtain the predefined sample size of 36 participants.

Stratified randomisation proceeded immediately according to gender (male or female) and diabetes diagnosis (yes or no) and was performed immediately before week 0. To avoid significant differences in the number of Tregs, since the female gender is usually associated with a greater percentage (63) and diabetes with a reduced number of Tregs (127), the number of female participants was the same in both groups and only type 2 diabetes patients were included. For allocation concealment, participants were divided into four blocks (diabetic men / non-diabetic men, diabetic women / non-diabetic women) and subsequently assigned in a 1:1 ratio after the creation of an internet-based random number sequence (from 1-9) for each block. Intervention assignment was undertaken by the sealed envelope method, with the first envelope opened being allocated to active treatment. Patients and staff were not blinded to the trial group assignments, but laboratory technicians were. All the samples were coded (one capital letter and two random numbers) and simultaneously sent to a laboratory outside the trial centre where they were analysed by independent technicians who ignored the allocation of participants and assigned codes.

The trial began in second week of July 2018, with all the participants beginning the trial in the same week, that from here in was called week 0. This week coincided with a summer month, making the influence of sun exposure the same for all the participants and maximizing pre-trial levels of VD. At this time point, demographic and clinical data were obtained, peripheral blood samples collected for blood chemistry and flow cytometry, subcutaneous adipose tissue biopsy performed, and cholecalciferol supplementation started in the active treatment group.

The trial included two more planned visits at weeks 16 and 24. At week 16, all the patients were assessed for adverse events, and serum VD levels repeated in the cholecalciferol group. Dose adjustments were performed per protocol according to dosing recommendations to the general population. Since the trial included only two more scheduled visits after week 0, and to avoid the risks of hypercalcemia, protocols including higher dosages of VD were not used. At the end of the trial, repeated measurements of blood chemistry, flow cytometry, and subcutaneous adipose tissue biopsy were repeated for both groups. Blood chemistry included HbA1c (measured by high-performance liquid chromatography on EDTA anti-coagulated blood samples in automated analyser ADAMS A1c HA-8160, Arkray, Inc.), total cholesterol, HDL cholesterol, and triglycerides (measured by endpoint colorimetric enzymatic assay by reflectance photometry, based on a cholesterol ester hydrolysis reaction, VITROS® MicroSlide technology, Ortho Clinical Diagnostics), with forward visceral adiposity index calculation (128). A complete leucogram was also obtained at weeks 0 and 24 to adjust the percentages of the different immune cells to the total leucocyte count.
VD determination included total VD and free VD: for total VD a competitive two-step chemiluminescent microparticle immunoassay was employed. The test uses magnetic beads coated with a mouse anti-fluorescein monoclonal antibody as its solid phase, a VD analogue conjugated with fluorescein as its competing ligand, and a mouse anti-

25(OH) vitamin D monoclonal antibody labelled with acridine ester as its conjugate. The sample was incubated with a release reagent, calibrated by two lyophilized materials with different concentrations of 25(OH) vitamin D, used to adjust a previously configured master curve, traceable to the NIST SRM 2972 standard, and controlled in two levels by materials with different concentrations of 25(OH) vitamin D, according to the instructions of the manufacturer (Atellica™ IM Vitamin D Total with reference number 10995719, manufactured by Siemens Healthcare Diagnostics Inc.).

Free VD determination was done using a competitive two-step ELISA immunoassay that uses microtiter plates coated with a mouse anti-25(OH) vitamin D2/D3 monoclonal antibody as its solid phase, biotinylated 25(OH) vitamin D as labelled ligand, peroxidase-conjugated streptavidin and TMB chromogenic substrate for measurement at 450nm, calibrated by five lyophilized materials with different concentrations of 25(OH) vitamin D in VD-depleted human serum and controlled in two levels by normal human serum and 25(OH) vitamin D-spiked human serum, also lyophilized, according to the instructions of the manufacturer (Free 25OH Vitamin D ELISA, catalogue number KAPF1991, manufactured by Future Diagnostic Solutions B.V. and distributed by DIASource ImmunoAssays® S.A, Louvain-la-Neuve, Belgium).
Clinical data included medical history, weight, height, blood pressure, waist circumference, and chronic ambulatory medication.

At week 0, a normotensive group, including 12 age-matched, non-diabetic, overweight but otherwise healthy participants, coming from routine consultations from the same centre, was also sampled for peripheral blood flow cytometry and serum metabolome to compare with obese hypertensives. In the case of serum metabolome, it was felt that an increased number of controls was necessary, and 5 additional controls were evaluated for plasma metabolites with the same characteristics.

In every visit, revision of ambulatory medication was performed to check for the introduction of calcium supplements or inadvertent VD prescription by an outside trial physician. Trial medication adherence was evaluated through the return of empty vials. Compliance with the remaining medication including anti-hypertensives was also checked.

Outcomes were evaluated at week 24 for both groups of obese participants, and no additional testing for the primary endpoint was done. Per protocol analyses were performed for both primary and secondary endpoints. Safety outcomes were monitored during the trial, and specifically, measurements of calcium and phosphorus were performed at weeks 16 and 24 for the cholecalciferol group (safety analyses).

Section 3.5: Cholecalciferol supplementation

Cholecalciferol was dispensed to trial participants as an oral solution of 25 000 IU/2.5 mL. Patients were instructed to keep experimental medication at room temperature, below 30°C, away from sunlight, and to take it at once during a principal meal. The dosing scheme for the participants with $5 < VD \leq 20$ ng/mL at week 0 was: $VD \leq 20$ ng/mL: 50 000 IU/week for 8 weeks followed by 25 000 IU every other week until week 16. Dosing of serum VD was repeated for all patients in the active treatment group at week 16. At this time point, new adjustments were performed as follows: $VD \leq 20$ ng/mL: 50 000 IU/week until week 24; $20 < VD \leq 30$ ng/mL: 25 000 IU every 4 weeks; $30 < VD \leq 50$ ng/mL: 25 000 IU every 6 weeks.

The following sections describe the methodologies used for each of the pre-specified aims and endpoints.

Section 3.6: Flow cytometry analysis of circulating immune cells

Evaluation of primary and secondary aims: To compare the percentages of adaptive and innate immune cells between normotensives and hypertensives, at baseline and after a 24-week period of cholecalciferol supplementation.

Blood flow cytometry was performed using freshly collected peripheral blood into tube containing ethylene-diamine-tetra acetic tripotassium acid (K3-EDTA) and stained with surface antigens with mouse anti-human antibodies in the subsequent 24 hours. For T cells identification, anti-CD3 with allophycocyanin was added (APC, clone OKT3, Biolegend, San Diego, CA, USA) and for the compartments of TCD4^{+} and CD8^{+} cells (phenotypically characterized as CD4+CD8- and CD4-CD8+, respectively) were added anti-CD4 with peridinin-chlorophyll-protein-Cyanine5.5 (PerCP-Cy5.5, clone RPA-T4, Biolegend, San Diego, CA, USA) and anti-CD8 with Fluorescein isothiocyanate (FITC, clone SK1, Biolegend, San Diego, CA, USA). In a second tube, the identification of Tregs was made based on the following phenotype: CD4^{+}CD25$^{+\mathrm{bright}}$CD127$^{-/+\mathrm{low}}$ expression, with anti-CD127 FITC (clone A019D5, Biolegend, San Diego, CA, USA**)**, anti-CD25 with phycoerythrin (PE, clone PC61, Becton Dickinson Biosciences, San Jose, CA, USA), CD4 PerCP-Cy5.5 and CD3 APC. To study the monocyte subsets, in a third tube was added anti-CD14 FITC (clone HCD14, Biolegend, San Diego, CA, USA), anti-CD16 PE (clone 3G8, Biolegend, San Diego, CA, USA) and anti-HLA-DR APC (clone L243, Biolegend, San Diego, CA, USA**)** allowing the identification of total monocytes and monocyte subsets: classical monocytes as CD14^{+} CD16^{-}, intermediate monocytes as CD14^{+} CD16^{+}, and non-classical monocytes as CD14$^{+/-}$ CD16^{+}.

After an incubation period of 15 minutes at room temperature in the dark, an erythrocyte lysis procedure was carried out and the samples were washed with PBS. The cells were resuspended in 500 μL of PBS (PBS; Gibco, Paisley, Scotland), and acquired in the flow cytometer. Results illustrate the percentage of positive cells within each subset.

A total of 500 μL of peripheral blood collected in heparin was diluted 1/1 (vol/vol) in RPMI-1640 medium (Gibco; Paisley, Scotland, UK), supplemented with 2 mM L-glutamine, and incubated at 37°C in a sterile environment with a 5% CO2, humid atmosphere for 4 hours in the presence of 10 μg/ml of Brefeldin A (Golgi plug Sigma, Saint Louis, MO, USA), 50 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma, Saint Louis, MO, USA) and 1 µg/ml of ionomycin (Boehringer Mannheim, Germany).

After the activation period, samples were aliquoted in two tubes (200 µL/tube) and stained for the surface antigens with mouse anti-human antibodies. All the tubes were stained with anti-CD3 APC, and each one with anti-CD4 PerCP-Cy5.5 and anti-CD8 PerCP-Cy5.5 (clone SK1, BD Pharmingen, San Diego, CA, USA) for 15 minutes in the dark, at room temperature and washed with PBS. According to the manufacturer's instructions, BD Cytofix/Cytoperm (Becton Dickinson Biosciences, San Jose, CA, USA), an intracytoplasmic permeabilization and staining protocol were followed. All the tubes were stained with IL-17 PE (clone N49-653, Becton Dickinson Biosciences, San Jose, CA, USA) and interferon-gamma (IFN-γ) with FITC (clone 4S.B3, Cyflow Sysmex Europe), allowing the identification of the Th17, Tc17, Th1, and Tc1 subsets. After washing twice with PBS, the cell pellet was resuspended in 500 µL of PBS and immediately acquired.

Data acquisition was performed in a FACSCalibur™ (BD) flow cytometer equipped with CellQuest acquisition software (BD). The number of events acquired was always above 0.5×10^6. The flow cytometry gating strategy is presented in Figure 12. For data analysis, Infinicyt (version 1.8) software (Cytognos SL, Salamanca, Spain) was used. Flow cytometry data analysis was performed at the Department of Clinical Pathology of Hospital and University Centre of Cova da Beira.

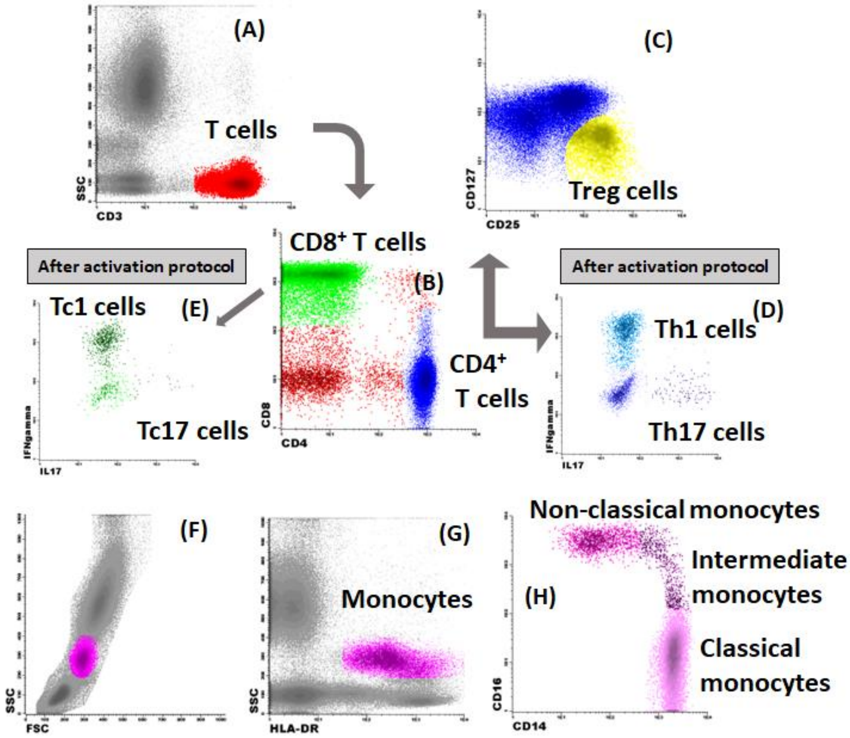


Figure 11: Flow cytometry gating strategy

Section 3.7: Metabolomic analysis

<u>Evaluation of primary and secondary aims</u>: to evaluate the differences in serum metabolome between normotensives and hypertensives, at baseline and after the 24-week supplementation period, including variations in the concentrations of metabolites of glucose, amino acids, and lipid pathways.

Nuclear magnetic resonance (NMR) based metabolomics approach was used to evaluate changes in the total metabolome of blood serum. Plasma samples obtained from both normotensive controls and obese hypertensive trial participants at weeks 0 and 24 were analyzed. The samples were thawed, homogenized using a vortex, and centrifuged (9200 rpm, 5 min). Only small metabolites were analyzed since large macromolecules like proteins and lipids are filtered out by applying 1D cpmg NMR experiment. The ^{1}H-NMR spectra of plasma samples were acquired and processed according to a protocol previously published (129). NMR spectra were acquired on a Varian Inova 600 MHz (14.1 T) spectrometer equipped with a 3 mm QXI probe with a z-water presaturation. Water saturation frequencies were optimized for each sample. Sodium fumarate (final concentration of 1 mM) was used as shimming reference. Chemical shifts were internally referenced to a glucose doublet at 5.23 ppm. Metabolite assignment was based on reference spectra available in public databases such as Human Metabolome Database (https://hmdb.ca). Additionally, 2D homonuclear TOCSY spectra were recorded to help with spectral assignment. Data matrices for multivariate analysis were created in AMIX-Viewer (version 3.9.15, BrukerBiospin, Rheinstetten) by integrating metabolite multiplets. Integrals were normalized by total integral area to account for matrix dilution effects and experimental conditions (130). The samples for metabolomic analyses were analysed at the Endocrine and Metabolic Research, UMIB - Unit for Multidisciplinary Research in Biomedicine, ICBAS - School of Medicine and Biomedical Sciences, University of Porto.

Section 3.8: Adipose tissue sampling and histological analysis

Evaluation of primary and secondary aims: To compare the percentages of adaptive and innate immune cells in subcutaneous abdominal adipose tissue at baseline and after the supplementation period and to correlate immune cells from adipose tissue and peripheral blood.

Subcutaneous adipose tissue was obtained using a skin needle biopsy with a 4 mm diameter (Kai Europe GmbH, Germany). The procedure was performed with the patient in dorsal decubitus in the left lower quadrant (LLQ). The skin was disinfected with povidone-iodine solution and a local anaesthetic with 2% lidocaine was applied. The sample was obtained after several rotation movements of the needle biopsy over the abdominal wall until a fragment with of 5 to 6 mm was detached from the remaining subcutaneous adipose tissue. The second biopsy was also performed in the LLQ but at different location. Fixation was performed with 3.7-4.0% formaldehyde (Panreac AppliCheam; ITW Reagents), processed in a tissue processor (Citadel 2000 model, Thermo, 2006) where the samples were dehydrated in ethanol in crescentic concentrations (70%, 95% and 99,9%). Samples were then treated with 99,9% ethanol and xylol, in similar proportions, prepared to diaphanization, which was performed with xylol followed by impregnation in paraffin.

After paraffin embedding in a Microm station (model EC 3501/2), samples were sectioned with 5 µm thickness (microtome HM340E, Microm, USA). For immunohistochemistry antibodies included CD3 (LN10) bond RTU primary antibody (PA0553), CD4 (4B12) bond RTU primary antibody (PA0427), CD8 (4B11) bond RTU primary antibody (PA0183) and CD68 (514H12) bond RTU primary antibody (PA0273). All antibodies were formol fixed or immunohistochemistry formalin paraffin embedded (Leica Biosystems, USA) and previously tested using tonsil as a positive control tissue.

Samples were treated with Trilogy (920P-07), Peroxidase block (925-05), HRP- HiDef 2-Step Polymer Detection Kit anti-mouse/anti-rabbit (954D-30) for amplification and detection and AB Substrate Kit (957D-40) to obtain a brown precipitate (all solutions from CellMarque, Sigma-Aldrich, USA).

Subsequent immune cell counting was performed after photographic record, registering the number of cells per 10 high power fields (hpf). The procedure was done by the same investigator, but counting was subsequently validated by another physician by random sampling of 20 histology slides from each antibody. For counting macrophages, the following methodology was applied: the total number of macrophages per patient in the two-time points considered resulted from the sum of macrophages with perivascular distribution plus macrophages associated with small adipocytes (< 70 µm) (131) and macrophages associated with big adipocytes (> 70 µm). Macrophages in these last two locations were collectively named non-perivascular macrophages. Crown-like structures, constituted mainly by macrophages surrounding adipocytes, were also counted.

Adipose tissue samples were prepared at the Health Sciences Faculty at University of Beira Interior and simultaneously observed at the Pathologic Anatomy Laboratory of this Faculty and at the Pathologic Anatomy Department of the Occidental Lisbon Hospital Centre by an experienced pathologist.

Section 3.9: Statistical analysis

Data are presented as mean ± standard deviation (SD). The Shapiro-Wilk test was applied to check for normality. For primary and secondary endpoints, univariate analysis was performed using Student's t-test with a 5% significance level, independent samples followed by paired-samples to observe for between and within-groups differences. Non-parametric tests were applied for the cells of adipose tissue because of non-normal distribution.

Forward multivariate analysis included principal component analysis (PCA) to evaluate the relationship of the different cells of adaptive immunity in the 2-time points considered (Weeks 0 and 24). From PCA, we obtained new loadings or components, which were not correlated as the original variables, and allowed us to better interpret and explain the latent structure observed between the original variables. A Kaiser-Meyer-Olkin (KMO) ≥ 0.5 was defined as the cut-off to pursue multivariate analysis and an Eigenvalue ≥ 1 to validate the new components. The graphical distribution of cells according to the PCA classification was also performed. Variables in the new components were selected according to the scores in the matrix component.

The effect of increasing values of free VD on the cells with inflammatory activity (T CD8+, Th17, Th1, Tc1, and classical monocytes) was evaluated using discriminant analysis. Trial participants were stratified according to the week 24 value of free VD (≤ 3 pg/mL, 3,1-5,9 pg/mL, ≥ 6 pg/mL) and differences in cell expression were explored. Statistically significant discriminant functions were identified whenever lambda of Wilk's p- value ≤ 0.05. For PCA and discriminant analysis, standardised scores were used to correct for dimension bias, since the average percentage of cells differs considerably according to the cell type.

Correlations between metabolic parameters (HbA1c, visceral adiposity index, total and HDL cholesterol and triglycerides), VD, and peripheral and adipose tissue immune cells are presented as exploratory endpoints. Correlations were performed using Pearson's correlation coefficient, after adjustment to the total number of leukocytes to correct for the variable numbers of cells between patients. A value of $p < 0.05$ was considered significant. IBM Statistics SPSS version 28.0 was used.

For metabolites differences, obtained normalized areas representative of metabolite concentrations in human serum were analysed by univariate and multivariate statistical analysis. Multivariate approach analyses all variables simultaneously and considers simultaneous relationship between variables (metabolite abundance expressed as normalized signal areas). Univariate and multivariate analysis were performed on online platform MetaboAnalyst 5.0 (www.metaboanalyst.ca) (132). Autoscaling of variables (metabolites) was applied for multivariate analysis. Principal component analysis (PCA) evaluated the initial data structure and was used to identify outliers and evaluate possible clustering. Next, supervised method, Orthogonal projection to latent structures discriminant analysis (OPLS-DA) was applied to pair-wise group comparisons to identify differentially expressed metabolites with discriminative potential. OPLS-DA models were cross-validated by permutation test using 100 permutations, and the permuted parameters R2Y and Q2 were used to assess the fitting validity and predictive abilities of the models (p values for comparison of each permuted parameter with the original classification >0.05). Models with similar values of R2 and Q2 (within 0.2-0.3 units), Q2>0.5 and validated by permutation test were considered as relevant. Metabolites with OPLS-DA VIP>1 were considered relevant to group separation. For univariate analysis non-parametric Wilcoxon rank-sum test with false discovery rate (p-value threshold 0.05) was used. For comparisons of active treatment group before and after 24 weeks of treatment, pairwise comparison was applied. Box-whisker plots based on medians and 95% confidence intervals were produced by GraphPad Prism (GraphPad Software Inc., California, USA).

Chapter 4 – Results

From July 2018 to January 2019, 36 participants underwent randomisation and 35 completed the trial (Figure 11). Each group comprised 18 participants, which were randomly assigned and received the intended treatment. All the patients were analysed per protocol for the primary outcome, except one patient in the cholecalciferol group because of non-compliance with the experimental product. Data relative to this patient was not included in the control group and analysis proceeded by the original groups.

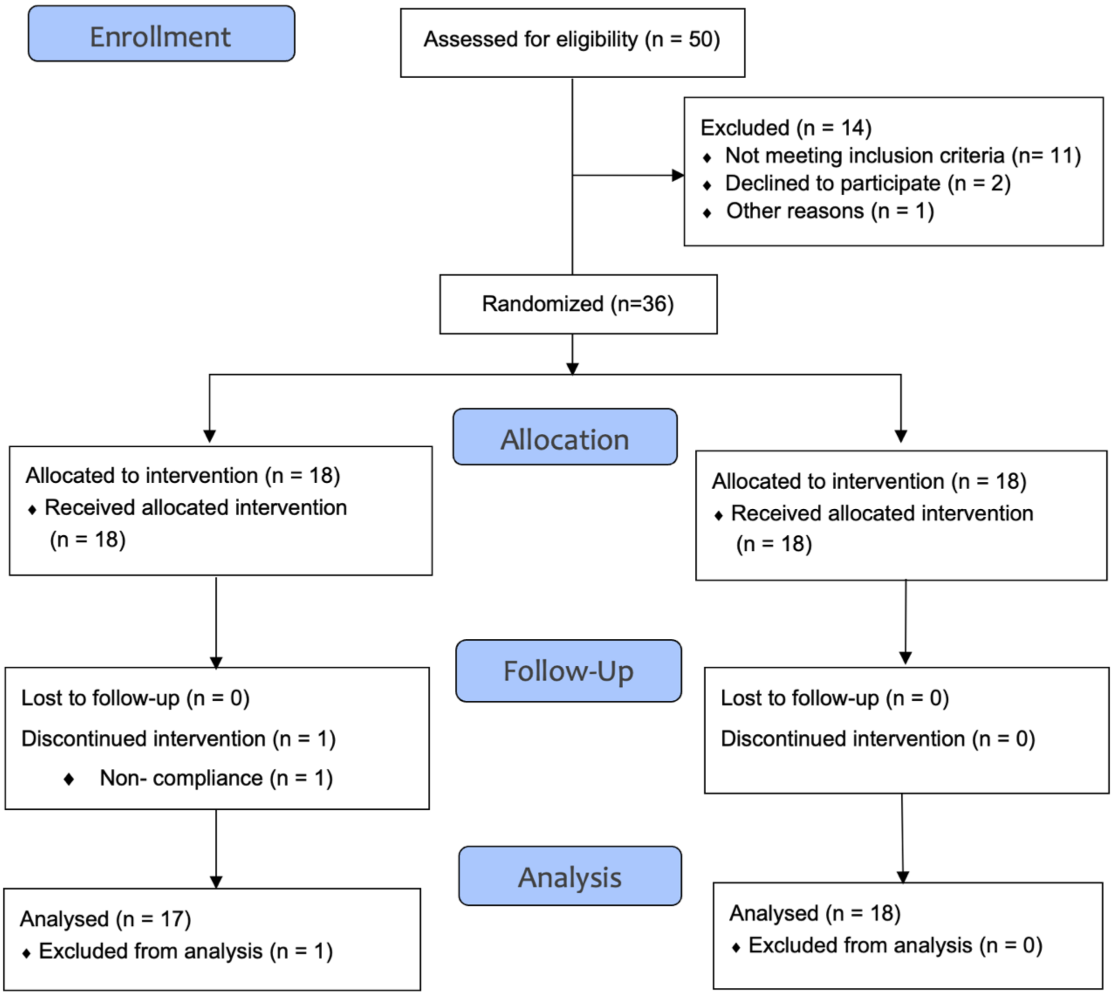


Figure 12: CONSORT flow chart diagram

Section 4.1: Trial participants and normotensive controls

Participants were all Caucasian, with an average age of 57.1 ± 5.5 years. The average BMI and waist circumferences were 34.5 ± 5.1 Kg/m^2 and 113.8 ± 10.7 cm, respectively. Eleven patients were diabetic. The distribution of patients according to the degree of obesity was 44.4%, 19.4%, and 16.6% with obesity class I, II, and III, respectively. Seven patients were overweight and fulfilled the criteria of metabolic syndrome.

All patients were on at least one antihypertensive drug and seven (19.4%) used four drugs for blood pressure control. Twenty-nine patients had some type of diuretic prescribed and 32 patients were on inhibitors of RAAS. The average blood pressure at the beginning of the trial for all the participants was 135.6 ± 18.2 mmHg for systolic and 81.9 ± 10.2 mmHg for diastolic blood pressure. Participants had an average HbA1c of 6 ± 0.9 % (6.5 ± 1.2 % for diabetics and 5.7 ± 0.5% for non-diabetics) and visceral adiposity index of 5.7 ± 3.8, a value consistent with severe adipose tissue dysfunction (128). All the patients ended the trial, with no losses to follow-up. In the cholecalciferol group, one participant abandoned experimental treatment but maintained scheduled visits. Baseline characteristics of control and cholecalciferol groups are presented in Table 2.

Table 2: Baseline characteristics of participants (average ±SD and 95% CI)

WEEK 0			
	Control group (N=18)	**Cholecalciferol group** (N=18)	***p*-value**
Vitamin D (ng/mL)	17.2 ± 5.6 (14.3 – 19.9)	14.4 ± 4.3 (12.2 – 16.7)	0.100
Free vitamin D (pg/mL)	6 ± 2.4 (4.8 -7.3)	5.2 ± 1.2 (4.6 - 5.8)	0.210
Visceral Adiposity Index	5.7 ± 4.1 (3.7 – 7.8)	5.6 ± 3.4 (3.7 - 6.8)	0.957
HbA1c (%)	5.8 ± 0.7 (5.5 – 6.2)	6.1± 1.1 (5.6 – 6.6)	0.416
BMI (Kg/m^2)	35.1 ± 5.9 (32.2 – 38.1)	34 ± 4.3 (31.2 – 36.2)	0.502
Waist circumference (cm)	117.7 ± 8.7 (113.4 – 122.1)	110 ± 11.3 (104.1 – 116.1)	0.026
Systolic BP (mmHg)	138.8 ± 19,8 (129 – 148)	131.3 ± 15 (124 – 139)	0.210
Diastolic BP (mmHg)	84.2 ± 9.1 (79 – 89)	79 ± 10.9 (75 – 85)	0.133
Total cholesterol (mg/dL)	164.2 ± 34.8 (146.8 – 181.5)	164.6 ± 27.5 (148.6 – 175.1)	0.966
HDL cholesterol (mg/dL)	47.4 ± 12.5 (41.1 – 53.6)	48.2 ± 12.3 (42.2 – 55.2)	0.842
Triglycerides (mg/dL)	137.7 ± 73.7 (101.1 – 174.4)	147.1 ± 63.1 (110.6 – 170)	0.684
Number of AH drugs	2.8 ± 1	2.3 ± 0.9	0.146

The normotensive controls were also evaluated at week 0, seven of which were women. The average BMI in these controls was 27.8 ± 4.1 Kg/m^2, a value lower than the observed in the trial participants, but nonetheless, in the range of overweight. Blood samples for flow cytometry were collected, but no additional analyses or follow-ups were performed.

Section 4.2: Immunological profile of normotensive controls and obese hypertensives

Obese hypertensives had an increased number of T lymphocytes in comparison with age-matched, healthy controls ($p = 0.016$) (Table 3). Although no differences in the proportion of $CD4^+$ and $CD8^+$ cells were observed, Tregs ($p = 0.014$) as well as Tc1 cells ($p < 0.001$) were significantly increased in obese hypertensives. The proportion of Th1 and Th17 cells was similar between groups, but Tc17 cells were significantly decreased ($p=0.004$). With respect to innate immunity, hypertensive patients had increased numbers of monocytes ($p = 0.008$), specifically non-classical monocytes ($p < 0.001$), while in contrast, classical monocytes were reduced ($p < 0.001$). In patients with class III obesity, these differences persisted and even if a trend towards reduced Tregs was observed, this was not statistically significant.

Table 3: Baseline PB immune profile of normotensive controls and obese hypertensives (average ± SD)

PB Immune cells (%)	**Healthy controls** (N = 12)	**Obese hypertensives** (N = 36)	***p*-value**
T lymphocytes	18.5 ± 5.8	22.6 ± 4.5	0.016
T $CD4^+$	62.2 ± 4.4	61.9 ±9.4	0.928
T $CD8^+$	34.2 ± 5.5	32.7 ± 9.6	0.514
Th17 cells	1.8 ± 0.55	2.03 ± 1.03	0.562
Tc 17 cells	2.13 ± 1.8	0.22 ± 0.25	0.004
Th1 cells	27.2 ± 6.46	29.37 ± 10.6	0.518
Tc1 cells	36.2 ± 7.5	61.2 ± 18.9	0.000
Treg cells	5.11 ± 1.07	6.5 ± 1.72	0.014
Monocytes	4.8 ± 1.37	6.8 ± 2.27	0.008
Classical monocytes	89.5 ± 3.2	84.03 ± 4.29	0.000
Intermediate monocytes	4.5 ± 1.95	5.02 ± 1.99	0.436
Non-classical monocytes	5.2 ± 2.24	10.9 ± 3.7	0.000

For obese hypertensives, no major differences between the two groups were documented, except for $CD8^+$ cells, which were marginally increased in the control group (Table 4). At baseline, no other differences between the two groups were documented.

Table 4: Average ± SD percentages of immunological cells at week 0.

PB Immune cells (%)	**Week 0**		
	Control group (N = 18)	**Cholecalciferol group** (N = 18)	*p-value*
T $CD4^+$	58.7 ± 10.1	63.8 ± 7.4	0.101
T $CD8^+$	35.7 ± 10.1	29.4 ± 8.1	0.052
Th17 cells	2.4 ± 1	1.8 ± 1	0.088
Tc17 (%)	0.2 ± 0.2	0.3 ± 0.28	0.481
Th1 cells	29.1 ± 9.2	29.5 ± 11.8	0.910
Tc1 (%)	64.9 ± 16.9	59.7 ± 20.5	0.483
Treg cells	6.7 ± 1.6	6.8 ± 1.6	0.887
Monocytes	7.3 ± 2.5	6.5 ± 2	0.296
Classical monocytes	84.7 ± 4.6	83.3 ± 3.9	0.353
Intermediate monocytes	4.9 ± 2.1	5.1 ± 1.9	0.892
Non-classical monocytes	10.3 ± 4.3	11.6 ± 3	0.315

Section 4.3: Change in serum vitamin D and free vitamin D

The mean ± SD of VD at the beginning of the trial for all the participants was 15.7 ± 5.1 ng/mL, and at week 24, was 15.5 ± 3.4 ng/mL for the control group and 26.9 ± 5.6 ng/mL for the active treatment group (Figure 13).

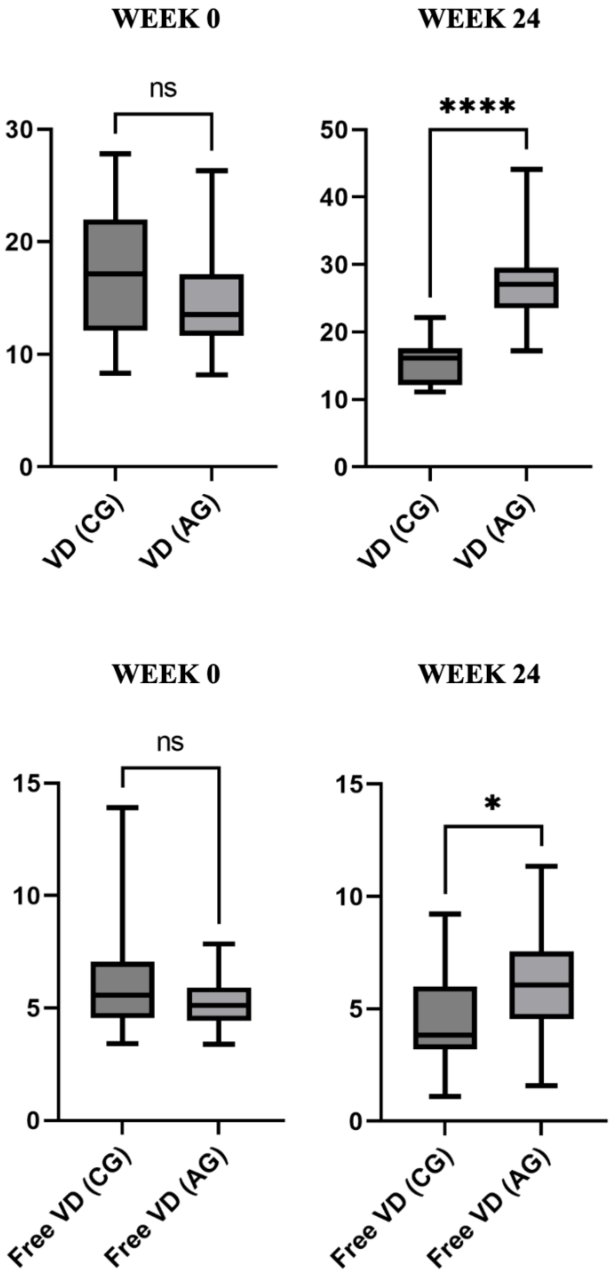


Figure 13: Variation of VD and free VD in control and active treatment groups between weeks 0 and 24 CG: control group; AG: active treatment or cholecalciferol group). Statistical significance obtained by univariate independent samples parametric t-test is denoted as: * p<0.05, **** p<0.001.

At week 16, after the high dose supplementation period, the average value of VD in the active treatment group rose to 39.4 ± 7.5 ng/mL. However, the transition to the maintenance dose resulted in a significant drop in VD levels that reached an average value of < 30 ng/mL at the end of the trial. Considering the difficulties in raising serum

VD levels in overweight and obese patients, the basal levels of VD almost doubled in the active treatment group at the end of the trial. In the case of free VD, we also observed an increase in the cholecalciferol group, from 5.2 ± 1.2 pg/mL to 6.2 ± 2.3 pg/mL ($p < 0.001$) (Figure 13). A reduction was seen in the control group, from 6 ± 2.4 pg/mL at week 0 to 4.6 ± 2.3 pg/mL at week 24 ($p < 0.001$). Although the widespread determination of free VD is not readily available in most laboratories and the cut-off levels are not precisely determined, according to the manufacturer's instructions levels above 5.8 pg/mL correspond to sufficiency or that said, equivalent to a level of VD > 30 ng/mL. Although the levels of free VD at week 0 were sufficient in the control group and not attaining that level in the cholecalciferol group, the difference was not statistically significant. At the end of the trial, even if serum total VD levels were low, treated patients had sufficient free VD.

Of interest was also the fact that, at baseline, women had lower levels of total VD ($p=0.048$) but equivalent concentrations of the free hormone (Figure 14), pointing to free VD as a better measure to evaluate reservoirs in the female gender.

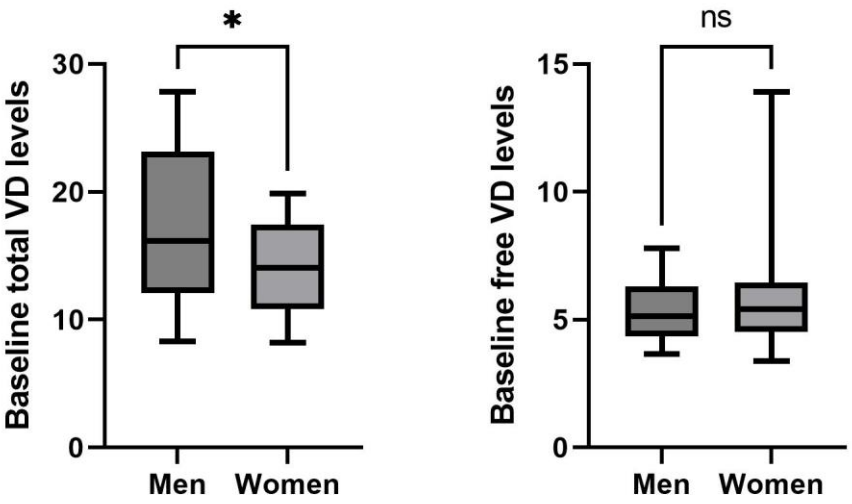


Figure 14: Baseline total and free serum VD levels by gender. Statistical significance obtained by univariate independent samples parametric t-test is denoted as: * $p<0.05$.

In the same way, in extreme forms of obesity (BMI > 40 Kg/m^2), lower values of total serum VD corresponded to almost equivalent free concentrations of VD (Figure 15).

The absence of statistical significance may be related to the small sample size. Nevertheless, having in mind that the free fraction of the hormone is the one that is available for binding to VDR, the clinical significance of free VD measurements should be explored.

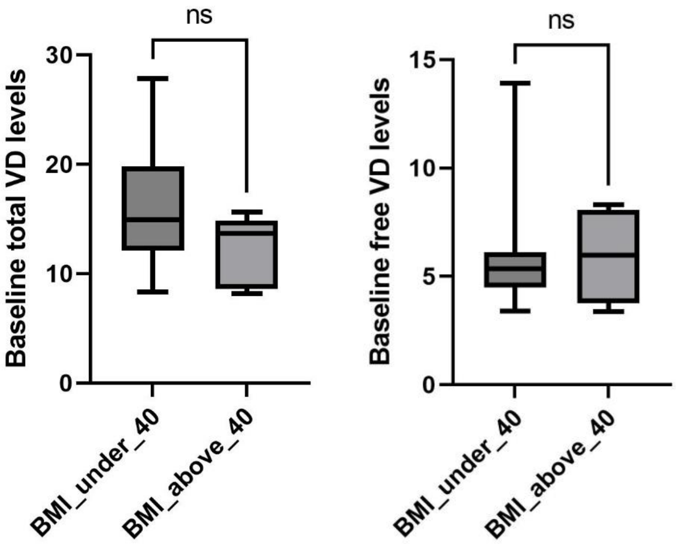


Figure 15: Baseline total and free serum VD levels by obesity grade (> 40 Kg/m^2). Statistical significance obtained by univariate independent samples parametric t-test: non-significant (ns).

On the other hand, a positive association between low levels of total VD (under 15 ng/mL) and the number of perivascular macrophages was observed (p=0.01) but the same observation was not true for a cut-off level of 5.8 pg/mL of free VD (Figure 16). What becomes apparent from these observations is that the total or bound VD (and, consequently, the fraction related to the levels of serum albumin) displays a set of associations that contrast significantly with the one made for free, unbound but readily available free VD.

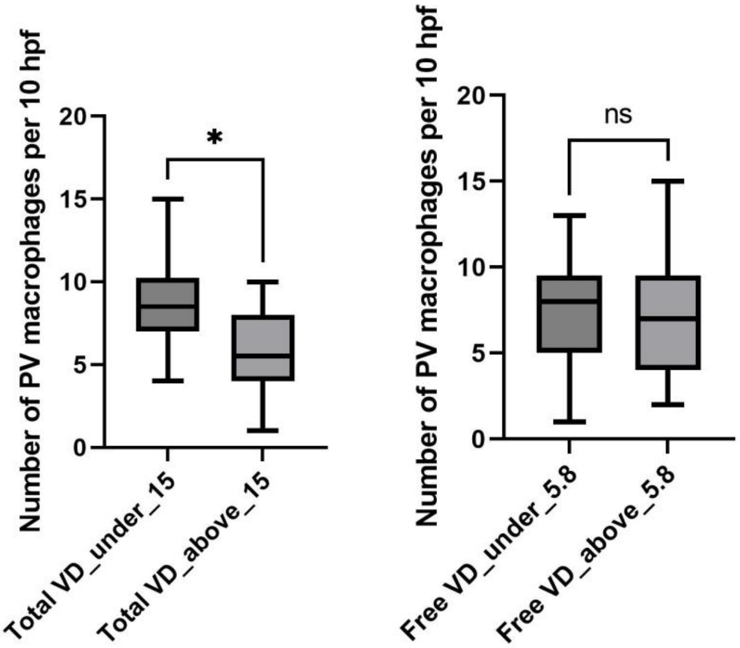


Figure 16: Number of perivascular macrophages according to baseline total and free VD levels. Statistical significance obtained by univariate independent samples parametric t-test is denoted as: * $p<0.05$.

Section 4.4: Variations of the cells of adaptive and innate immunity during the follow-up in the control group

We looked for statistically significant variations in the control group during the follow-up that would represent false positive cases, and not the effect of cholecalciferol if also observed in the active group. Immunological cells constitute dynamic populations in which variations in the percentage in the peripheral blood depend not only on potential therapeutic interventions but also on their own turnover and underlying pathophysiological conditions. Using pairwise comparisons, we observed a statistically significant increase in the percentage of Th17 cells ($p = 0.012$) and non-classical monocytes ($p = 0.023$). For the remainder of the cell lineages, no differences were identified. In this way, and looking at our sample population, a parallel increase in pro-inflammatory cells of both adaptive and innate immunity was observed during the follow-up in the control group. Tregs remained stable throughout the trial, albeit a trend towards increased values was observed, further distancing from normotensive controls. For Tc1 cells, a significant decrease was observed independent of variations of Th1 cells.

Table 5: Pairwise comparisons of control group at weeks 0 and 24 (average ±SD)

PB Immune cells (%)	**Week 0** (N=18)	**Week 24** (N=18)	**p value**
T CD4$^+$	58.7 ± 10.1	57.2 ± 10.8	0.129
T CD8$^+$	35.7 ± 10.2	37.3 ± 10.9	0.116
Th17 cells	2.4 ± 1.1	2.9 ± 1.3	0.012
Tc17	0.18 ± 0.2	0.22 ± 0.3	0.649
Th1 cells	29.1 ± 9.2	31.1 ± 9.5	0.313
Tc1	64.9 ± 16.9	75.8 ± 13.7	0.000
Treg cells	6.3 ± 1.8	6.8 ± 1.8	0.168
Monocytes	7.3 ± 2.5	6.5 ± 2	0.061
Classical monocytes	84.7 ± 4.6	83.5 ± 7.7	0.422
Intermediate monocytes	4.9 ± 2.1	5.4 ± 3.6	0.554
Non-classical monocytes	10.3 ± 4.3	12.3 ± 5	0.023

Section 4.5: Primary endpoint – Variation in the percentage of peripheral TCD4+, TCD8+, Tregs and Th17 cells

Following the same statistical approach as described in the previous section for the control group, we performed first pairwise comparisons for cholecalciferol group between weeks 0 and 24 (for practical purposes all the cells studied in the present trial are presented in Table 6, even if some were included only in secondary endpoints). From the analysis of Table 6, changes observed for cholecalciferol group do not match with the one's observed for the control, pointing to some effect of cholecalciferol in the observed variations. While, once again, it is not expectable that all patients present changes in the same direction, it is noticeable that Tregs continued to increase in the control group, but a significant decrease was observed in the treated patients, meaning that opposite variations had occurred in both groups.

Table 6: Pairwise comparisons of cholecalciferol group at weeks 0 and 24 (average ± SD)

PB Immune cells (%)	Week 0 (N=17)	Week 24 (N=17)	p value
T CD4$^+$	65.4 ± 7.3	63.2 ± 7.5	0.078
T CD8$^+$	29.5 ± 8.1	31.6 ± 9.4	0.082
Th17 cells	1.75 ± 0.95	1.88 ± 0.79	0.366
Tc17	0.25 ± 0.28	0.09 ± 0.07	0.023
Th1 cells	29.5 ± 11.7	32.6 ± 14.1	0.014
Tc1	59.6 ± 20.5	70.1 ± 16.5	0.000
Treg cells	6.8 ± 1.7	6.1 ± 1.5	0.003
Monocytes	6.7 ± 1.9	5.7 ± 1.3	0.018
Classical monocytes	83.4 ± 4	82.7 ± 5	0.539
Intermediate monocytes	5.1 ± 1.9	4.2 ± 1.3	0.149
Non-classical monocytes	11.6 ± 3.1	13.1 ± 4.3	0.068

Noteworthy, were also the differences in the lineage of Th17 cells. A significant increase followed in the control group during the 24-week period, that was not observed in the cholecalciferol group, that additionally presented a decrease in Tc 17 cells. For Th1-derived cells, including Th1 and Tc1 cells, also opposite changes were observed, with a decrease of Tc1 cells in the control group and an unexpected increase of Th1 cells in the cholecalciferol group (p=0.014).

Since, at baseline, the two groups were homogeneous in terms of immune cells percentages (Table 4), we also proceeded with independent samples testing to check for differences between the two groups at week 24.

Table 7: Average ± SD percentages of immunological cells at week 24

PB Immune cells (%)	**Week 24**		
	Control group (N = 18)	Cholecalciferol group (N = 17)	*p-value*
T CD4$^+$	57.3 ± 10.8	62.1 ± 8.6	0.149
T CD8$^+$	37.4 ± 10.9	31.6 ± 9.4	0.104
Th17 cells	2.7 ± 1.2	1.8 ± 0.8	0.017
Tc17 (%)	0.18 ± 0.27	0.09 ± 0.06	0.225
Th1 cells	30.4 ± 9.3	35.5 ± 18.1	0.296
Tc1 (%)	75.1 ± 11.9	71.5 ± 16.9	0.476
Treg cells	6.8 ± 1.8	5.9 ± 1.7	0.126
Monocytes	6.5 ± 2	5.7 ± 1.3	0.215
Classical monocytes	83.5 ± 7.8	82.7 ± 5	0.731
Intermediate monocytes	5.4 ± 3.6	4.3 ± 1.3	0.219
Non-classical monocytes	12.3 ± 5	13.1 ± 4.3	0.594

No significant changes were observed in the TCD4$^+$ and TCD8$^+$ compartments after the 24-week period. At baseline, the numbers of TCD8$^+$ cells were, to some extent, greater in the control group, but this difference disappeared at the end of the trial. Within groups, Tregs decreased and Th17 cells were stable. However, when comparing with the control group, for Th17 cells, a statistically significant difference was observed ($p = 0.017$, estimated effect size -0.854, 95% CI: -1.542, -0.154), with treated patients presenting fewer Th17 cells than the parent cohort. Considering that in the control group an increase was observed, these results may suggest that cholecalciferol may have halted the increase in Th17 cells.

For Tregs, a non-significant reduction was observed between active and control groups (Table 7). However, at the level of within-group comparisons, this decrease was significant in the cholecalciferol group (p = 0.03, estimated effect size 0.934, 95% CI: 0.312, 1.534) and occurred inversely to that observed in the controls.

The following figures present the pairwise variations for each of the cells defined in primary endpoint and the trend variation for all the patients between the two time points considered. Since the average values do not accurately reflect the range interval and amplitude dispersion for a certain variable, this graphical presentation also allows for a better understanding of the range of values obtained for each variable.

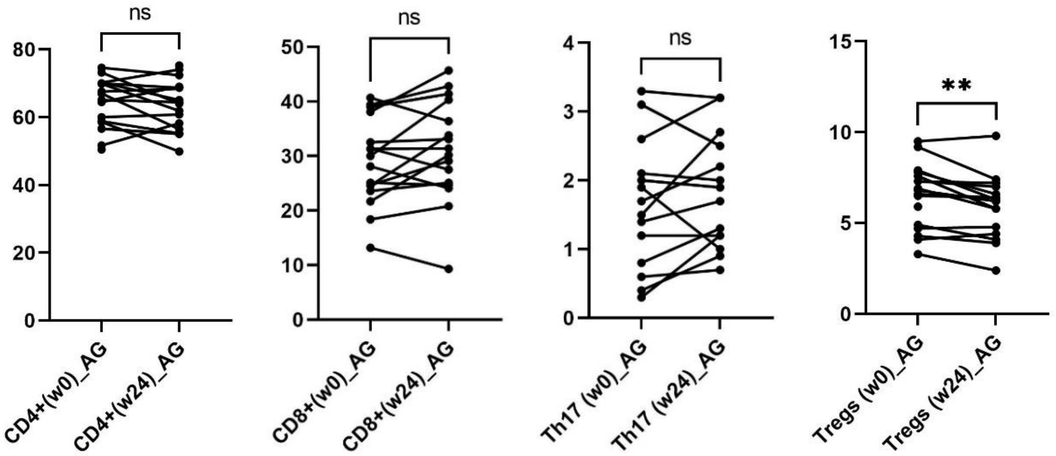


Figure 17: Pairwise comparisons for primary endpoint immune cells. Statistical significance obtained by univariate paired samples parametric t-test is denoted as: ** p < 0.01.

Overall, these results point to no effect of cholecalciferol in $TCD4^+$ and $TCD8^+$ cells at the levels of serum VD attained. Tregs decreased over the follow-up period, approximating from values of age-matched, overweight, and normotensive controls. This effect seems to be independent of the time point of the year since, in the control group, no decrease in Tregs was observed during the winter months. The stability of Th17 cells in the cholecalciferol group accounted for the statistically significant difference observed between groups, since Th17 cells increased in the control group.

Section 4.6: Secondary endpoint – Variation in the percentage of peripheral Th1, Tc1 and Tc17 cells and monocytes

Pairwise comparisons for control and cholecalciferol groups documented decreased Tc1 (p < 0.001, estimated effect size -1.443, 95% CI: -2.139, -0.723) and increased Th1 (p = 0.014, estimated effect size -0.672, 95% CI: -1.192, -0.135) and Tc1 percentages (p < 0.001, estimated effect size -1.443, 95% CI: -2.139, -0.723), respectively (Tables 5 and 6).

Between-groups, however, these differences were not significant and Th1 and Tc1 cells remained stable during the follow-up in comparison to the control group (Table 7). For Tc1 cells the absence of a significant difference suggests that the observed variations cancelled each other. Given the intertwined role of Th1 and Tc1 cells in cytotoxicity, this effect was not expected but seems to be potentiated by cholecalciferol. The trend of variation between weeks 0 and 24, as well as the range of distribution of the values obtained, is shown in Figure 18.

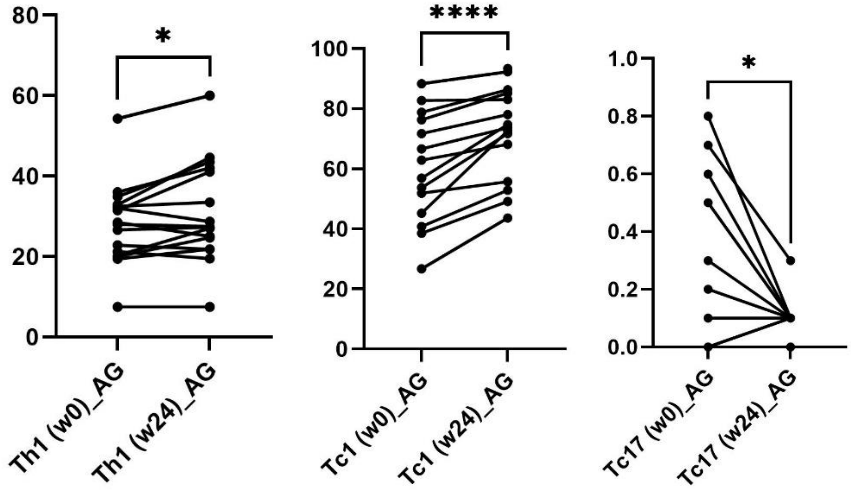


Figure 18: Pairwise comparisons for Th1, Tc1 and Tc17 cells. Statistical significance obtained by univariate paired samples parametric t-test is denoted as: * p < 0.05 and **** p < 0.001.

Between groups, the average values of Tc17 cells non-significantly decreased in the active treatment group, accompanying the variation observed for Th17 cells. In pairwise comparison, this reduction was statistically significant, and further skewing of the levels of Tc17 cells from the control group was observed (p = 0.023, estimated effect size 0.631, 95% CI: 0.084, 1.161).

For total monocytes as well as for the different sub-classes of monocytes, we observed stability during the follow-up in comparison to the control group (Table 7). Even if some statistically significant differences were observed in pairwise comparisons (Figure 19), parallel variations occurred in both groups.

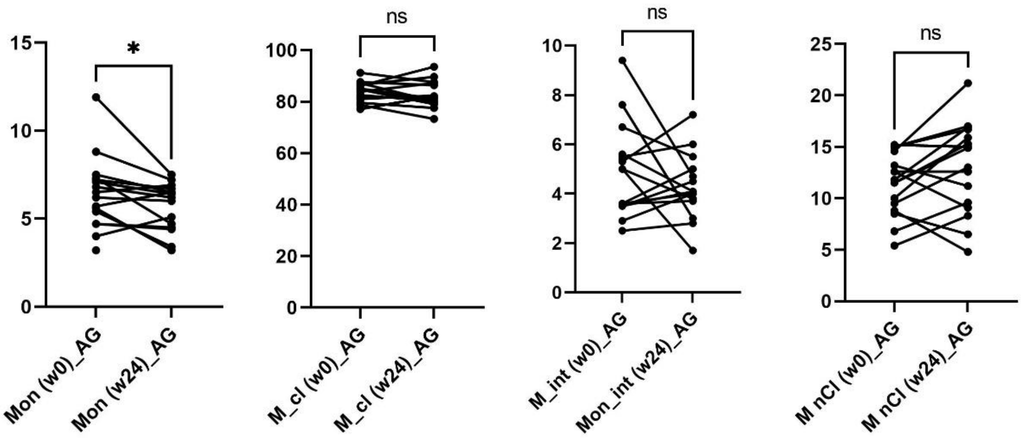


Figure 19: Pairwise comparisons for total monocytes and its derived subsets. Statistical significance obtained by univariate paired samples parametric t-test is denoted as * $p<0.05$.

The trend for total monocytes in both groups at week 24 was a decrease, albeit reaching statistical significance only in the cholecalciferol group. Similar trends were observed for non-classical monocytes, pointing to no effect of cholecalciferol in these cell compartment, usually described as very sensitive to VD modulation. Of note, however, is the fact that the increase in non-classical monocytes was more pronounced in the control group (almost reaching statistical significance), leading use to speculate of unrestrained proliferation to compensate progressive inflammation in the control group, that was partially modulated by VD.

Section 4.7: Serum metabolome of normotensive controls and obese hypertensives

Clustering tendencies and possible outliers in metabolome profiles collected at different time points were evaluated by the principal component analysis (PCA). Since for the hypertensive trial participants -VD control group (VD CG) and VD active group (VD AG) no differences in metabolic profiles were found at time 0, they were treated as one group (Hyper0). Comparison of the metabolic signatures of normotensive controls (Normo0) and hypertensive patients (Hyper0) collected before VD therapy, revealed clustering tendencies according to the tested groups. Although a certain overlap can be observed in the PCA model, good group separation between Normo0 controls and Hyper0 patients indicates metabolome differences (Figure 20).

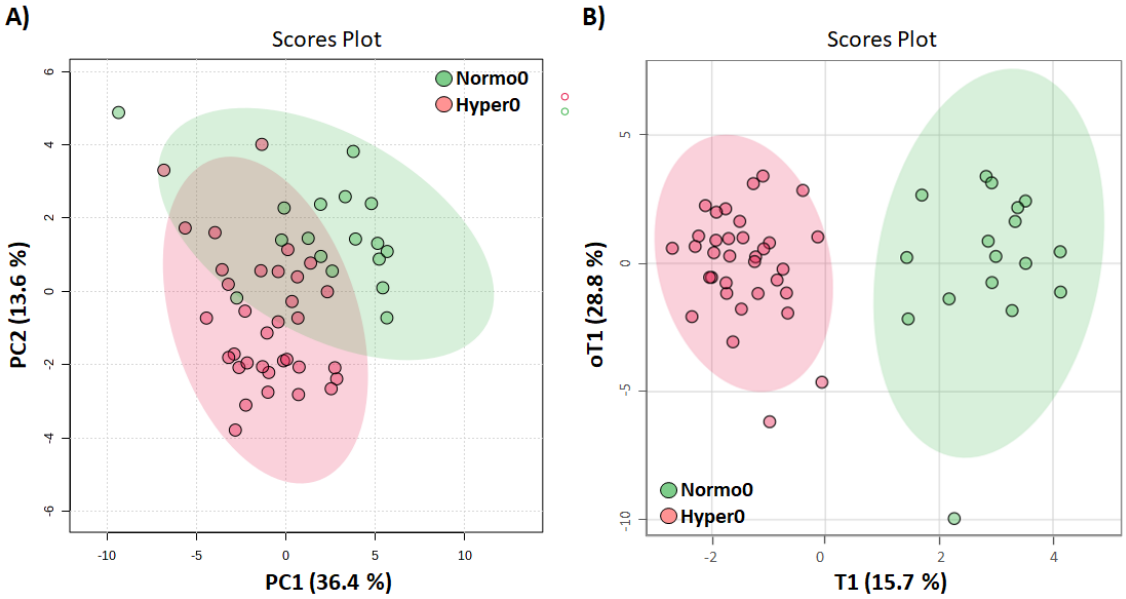


Figure 20: Blood metabolome of normotensive controls (Normo0, n=17) and hypertension patients (Hyper0, n=34) before VD therapy. A) PCA scores scatter plot of blood serum 1D cpmg NMR spectra. Principal components PC1 and PC2 and related explained variability (%) are indicated on axes x and y, respectively; B) OPLS-DA scores scatter plot of blood serum 1D cpmg NMR spectra. The first predictive (T1) and the first orthogonal (oT1) components, and the respective explained between- and within-group variabilities (%) are indicated on axes x and y. The model is described by 1 predictive and 3 orthogonal components, with the total explained variation of $R^2X=0.58$ and $R^2Y=0.89$, and the goodness of prediction (Q^2) of 0.73. Each point in the scatter plots represents a single patient as a function of all analysed metabolites. 95% confidence intervals are represented by ellipses.

Further statistical models were applied to investigate these differences. OPLS-DA model used to increase group separation and identify differentially expressed metabolites demonstrates that the two groups are separated without overlap (Figure 20B), with high goodness of fit and predictability confirmed by the model validation based on random permutation (Figure 21).

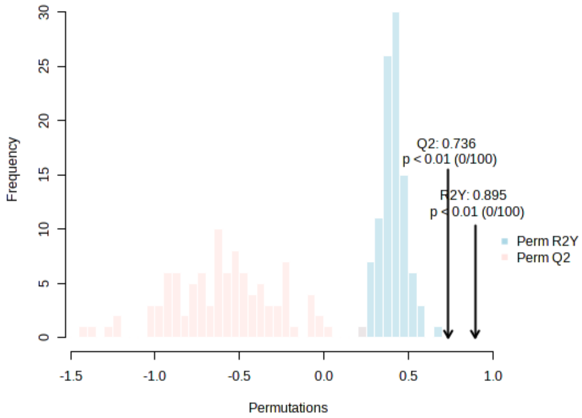


Figure 21: The performance of the Normo0 vs Hyper0 OPLS-DA model was evaluated by the permutation test (n=100). High values of predictive parameter Q^2 and goodness of fit R^2Y (>0.5) as well as $p<0.05$ (difference between original and permutated parameters) indicate robust and reliable predictive model.

Variable importance to projection (VIPs) were extracted for each variable and those with VIP>1 and univariate $p<0.05$ considered as overexpressed metabolites that contribute to the difference between the groups (Figure 22).

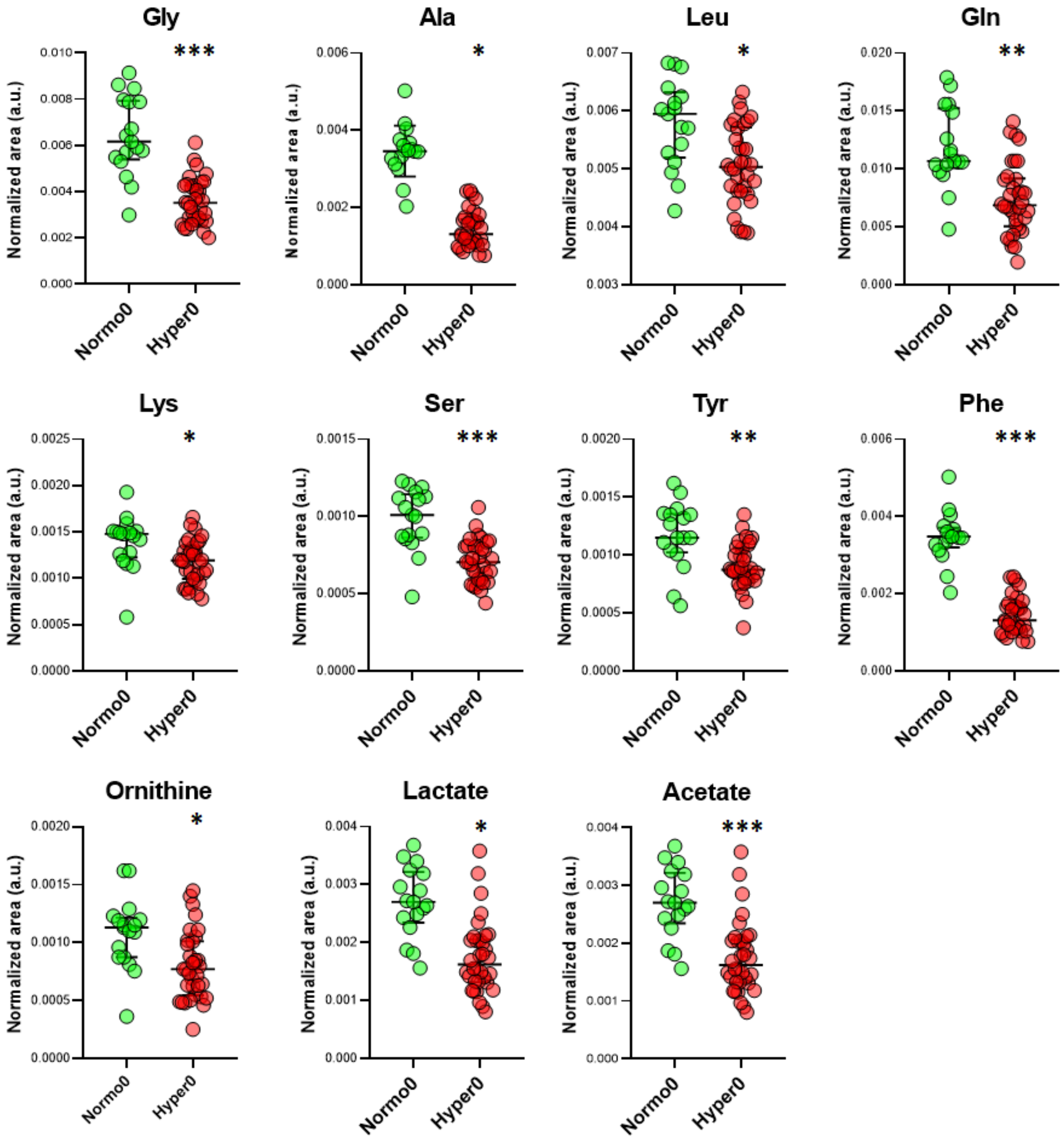


Figure 22: Differentially expressed metabolites between normotensive (Normo0, n=17) and hypertensive patients (Hyper0, n=34) before VD therapy. The differentially expressed metabolites between the 2 groups were selected based on the OPLS-DA VIP values (>1) and univariate analysis (p<0.05). Medians with interquartile range are presented. Statistical significance obtained by univariate non-parametric test are denoted as: * p<0.05, ** p<0.001, *** p<0.0001.

Differences in various classes of metabolites were characteristic for HTN, including amino acids and glucose metabolism (lactate and acetate). Overall, decreased expression of several amino acids, including leucine, glutamine, lysine, alanine, glycine, and the aromatic amino acids phenylalanine and tyrosine was documented in hypertensives. Additional metabolites were identified as differentially expressed only by univariate analysis and include, amongst others, glucose (Figure 23), an expected finding given the prevalence of diabetic patients in the cohort of trial participants. Nevertheless, the average glycated haemoglobin levels were within the recommended target of <7% and the highest value observed amongst all the participants was 6.6%.

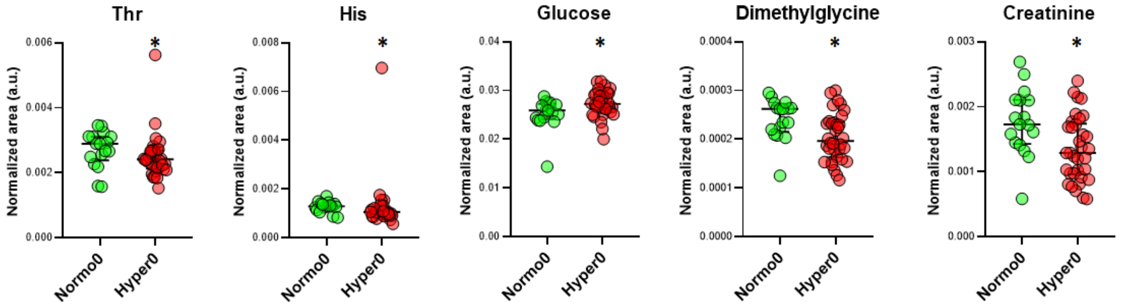


Figure 23: Differentially expressed metabolites between normotensive (Normo0, n=17) and hypertensive patients (Hyper0, n=34) before VD therapy (T0). Medians with interquartile range are presented. Statistical significance obtained by univariate non-parametric test are denoted as: * p<0.05, ** p<0.001, *** p<0.0001.

Section 4.8: Secondary endpoint – Variation in the concentrations of metabolites of glucose, amino acids, and lipid pathways

To investigate the possible effects of VD therapy we performed pairwise comparisons of hypertensive participants before and after cholecalciferol supplementation. Thirty-one metabolites were analysed and included in the PCA model. Due to spectral artefacts and compromised spectral quality, 16 paired values were included in the analysis (with the remaining 2 pairs being excluded). As can be seen in the Figure 24, high degree of overlap exists between weeks 0 and 24. Although OPLS-DA score scatter plot (Figure 24) potentiates group separation, considerable group overlap is still visible suggesting significant similarities in the metabolome signature before and after supplementation with cholecalciferol.

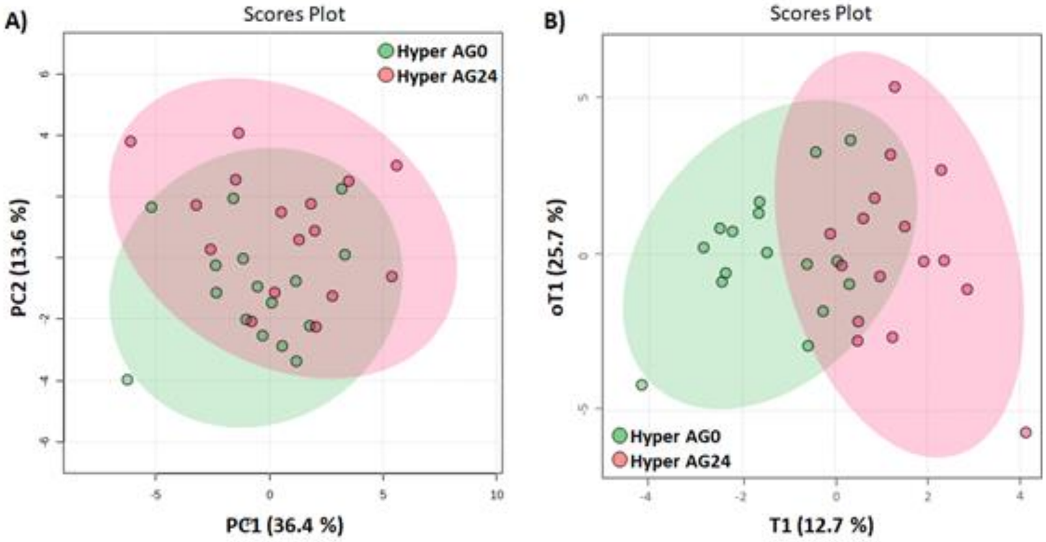


Figure 24: Comparison of blood metabolome between hypertensive active therapy group before the therapy (Hyper AG0, n=16) and after 24 weeks of VD treatment (Hyper AG24, n=16). A) PCA scores scatter plot of blood serum 1D cpmg NMR spectra. Principal components PC1 and PC2, and related explained variability (%) are indicated on axes x and y, respectively; B) OPLS-DA scores scatter plot of blood serum 1D cpmg NMR spectra. The first predictive (T1) and the first orthogonal (oT1) components, and the respective explained variability (%) are indicated on axes x and y. The model is described by 1 predictive and 1 orthogonal component, with the total explained variation of $R^2X=0.38$ and $R^2Y=0.59$, and the goodness of prediction (Q^2) of 0.28. Each point in the scatter plots represents a single patient as a function of all analysed metabolites. 95% confidence intervals are represented by ellipses.

Model validation reveals low accuracy of the model R^2 parameters (<0.6) and low predictive ability of the model ($Q^2<0.3$) (Figure 25). However, despite the modest explanatory and predictive capacity of the model, it was not overfitted and we considered it for identification of metabolites responsible for metabolome changes induced by cholecalciferol supplementation.

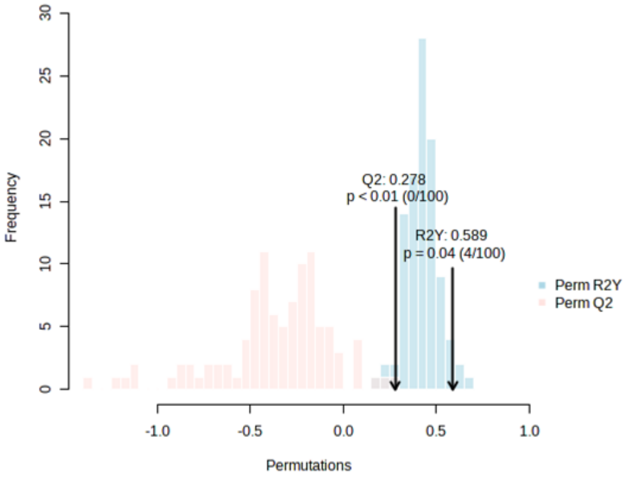


Figure 25: The performance of the HyperAG0 vs HyperAG24 OPLS-DA model was evaluated by the permutation test (n=100). Low values of predictive parameter Q^2 (<0.3) and goodness of fit R^2Y (<0.6) indicate low quality model. Comparison of original and permuted parameters ($p<0.05$) indicates the model performance is adequate and the model is not overfitted.

OPLS-DA VIP values (VIP>1) combined with the paired univariate analysis provided insight into metabolic modulation by VD (Figure 26). As can be seen, various classes of metabolites were affected by VD therapy, including amino acids, as well as glucose and lipid metabolism.

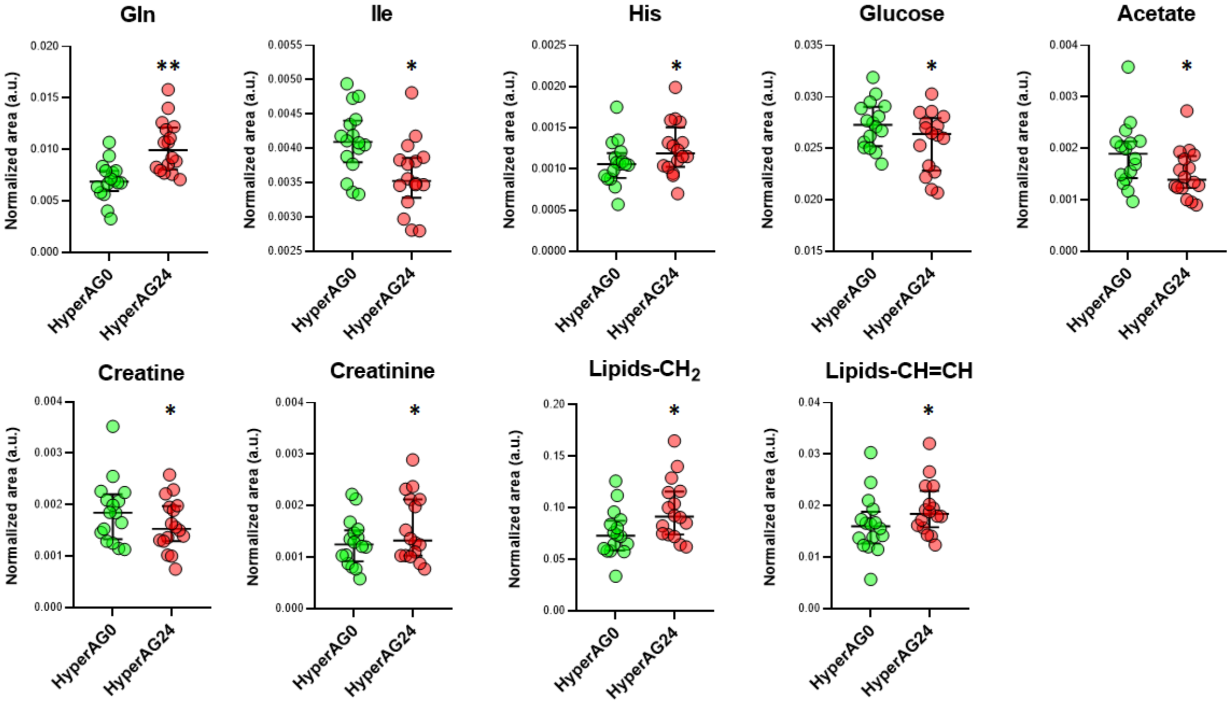


Figure 26: Metabolome changes induced by VD therapy: differentially expressed metabolites between hypertensive patients before (HyperAG0, n=16) and after VD therapy (HyperAG24, n=16). The differentially expressed metabolites between the 2 groups were selected based on the OPLS-DA VIP values (>1) and paired univariate analysis ($p<0.05$). Medians with interquartile range are presented. Statistical significance obtained by univariate non-parametric test are denoted as: * $p<0.05$, ** $p<0.001$, *** $p<0.0001$.

One of the hallmarks of HTN identified by the analysis of this specific patient cohort, was systemic decrease in several blood amino acids. After 24-week exposure to cholecalciferol, this trend was reverted for some. The most notable changes were observed for Gln levels which increased after the treatment and approached the levels observed in normotensive patients (Figure 26). Histidine also increased but the relative weight of other glucogenic amino acids like serine and threonine, which was high at week 0, decreased in the follow-up in univariate analysis (Figure 27).

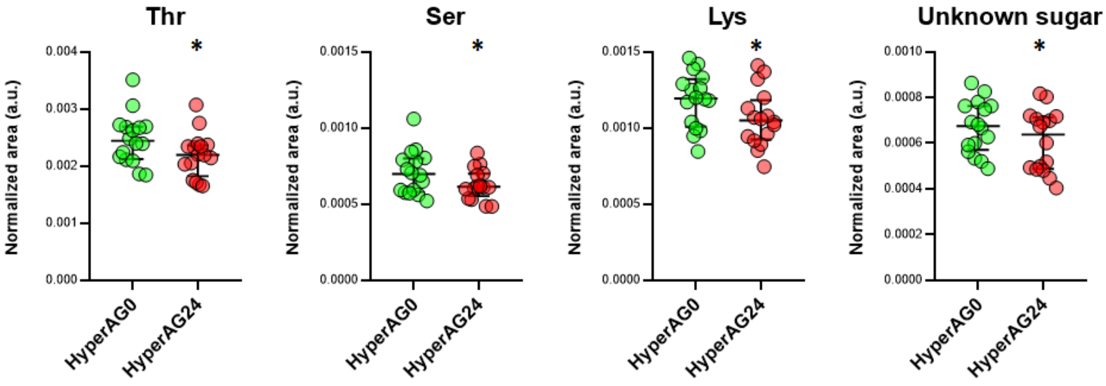


Figure 27: Differentially expressed metabolites in hypertensive patients before (HyperAG0, n=16) and after VD therapy (HyperAG24, n=16). Medians with interquartile range are presented. Statistical significance obtained by paired univariate non-parametric test are denoted as: * $p<0.05$, ** $p<0.001$, *** $p<0.0001$.

Ketogenic amino acids remained stable during the trial, with changes being observed only by univariate analysis for lysine (Figure 27), and the weight in the OPLS-DA model overlapped with the values of week 0.

At the same time, levels of glucose decreased in VD treated patients. A decrease was also observed in acetate which is at the crossroad of glucose and lipid metabolism and links them to the oxidative phosphorylation via the Krebs cycle. Incomplete suppression of macromolecule signals by of 1D cpmg NMR sequence permitted the analysis of blood lipids. Although finer distinguishing between the lipid species present in blood was not possible under the experimental conditions, we were able to observe changes in aliphatic chain methylene's (CH_2, 1.25 ppm) and olefinic hydrogens (CH=CH, 5.3 ppm) suggesting changes in the length and saturation of fatty acids. Lack of changes in the signals corresponding to terminal CH_3 groups of fatty acids (0.85 ppm) indicate that the total lipid levels remained unchanged. While it is not possible to fully characterize the affected lipid species, the statistically significant increase observed may suggest some role of the liposoluble VD in lipid metabolism. Further changes include perturbations in creatine metabolism, specifically a statistically significant increase in creatinine after VD suggesting increased muscle mass in obese hypertensives.

When compared with normotensive controls, perturbed amino acid levels remain the main distinction between hypertensive and normotensive subjects, even after long-term VD treatment of hypertensives (Figure 28).

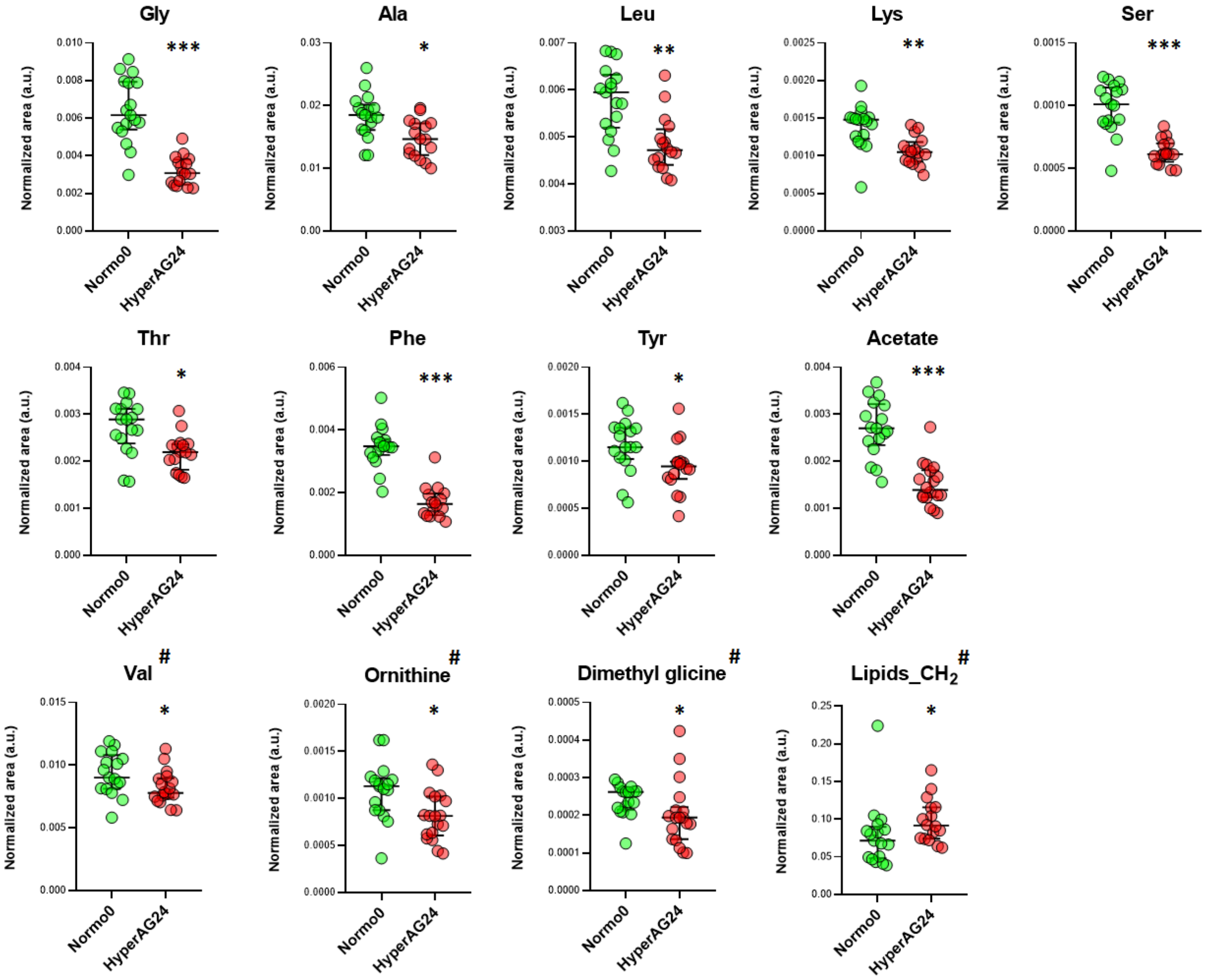


Figure 28: Differentially expressed metabolites between normotensive patients (Normo0, n=17) and hypertensive patients after VD therapy (HyperAG24, n=16). The differentially expressed metabolites between the two groups were selected based on the OPLS-DA VIP values (>1) and univariate analysis ($p<0.05$). Medians with interquartile range are presented. Statistical significance obtained by univariate non-parametric test are denoted as: * $p<0.05$, ** $p<0.001$, *** $p<0.0001$. $^{\#}$ Statistical reference observed only by univariate analysis.

In parallel, in the hypertensive control group (HyperCG24) no significant changes were documented at week 24. Combination of complete overlap in the PCA and overfitted OPLS-DA model suggested that blood metabolome did not suffer any changes during the study (data not shown). Given that physiological variations are constantly occurring, this control group allows us to track changes that would be secondary to the normal physiological processes rather than to VD treatment. In our trial no modifications were seen in the follow-up for the control hypertensive patients (HyperCG), further supporting the role of VD as modulator of the several metabolic pathways discussed.

Section 4.9: Secondary endpoint – Variation in the number of subcutaneous abdominal adipose tissue TCD4+ and TCD8+ lymphocytes and perivascular and non-perivascular macrophages

Representative images of the different immune cells in the adipose tissue are presented in Figure 29. Only two samples of adipose tissue were discarded because of insufficient material to analyse.

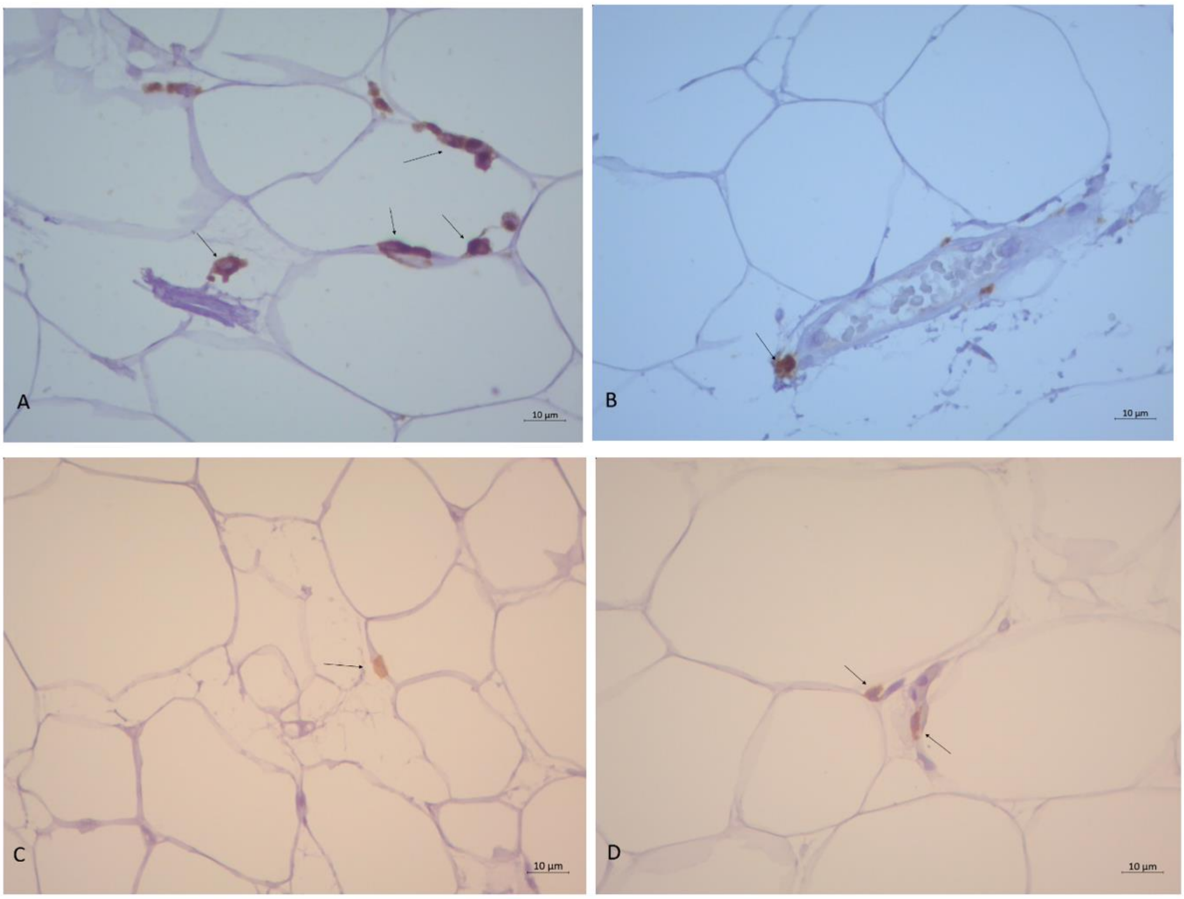


Figure 29: Representative images of adipose tissue cells (x40): macrophages with non-perivascular distribution (A) and perivascular distribution (B), AT Ly TCD4^{+} (C) and AT Ly TCD8^{+} lymphocytes (D).

At baseline, no differences in the number of macrophages and T lymphocytes were observed between active and control groups (Figure 30).

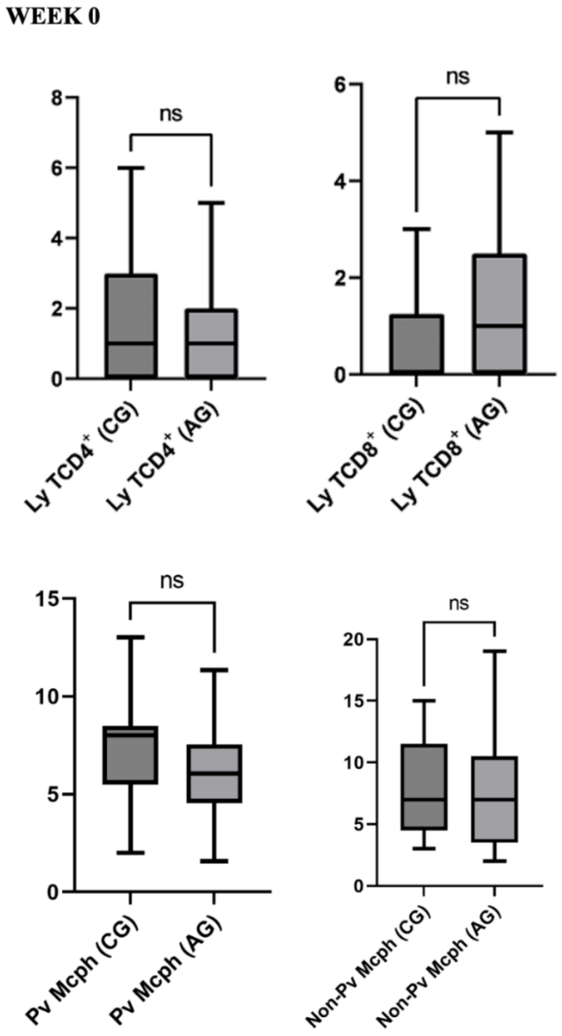


Figure 30: Number of adipose resident immune tissue cells per 10 hpf at week 0 in control and cholecalciferol groups. Ly TCD4+: AT TCD4+ lymphocytes; Ly TCD8+: AT TCD8+ lymphocytes; Pv Mcph: perivascular macrophages; Non-Pv Mcph: non-perivascular macrophages. Statistical significance obtained by univariate independent samples parametric t-test: non-significant (ns).

A positive correlation was identified for tissue TCD4$^+$ and TCD8$^+$ lymphocytes ($r = 0.558$, $p < 0.01$) but not for macrophages and tissue T lymphocytes. Macrophages associated with small adipocytes correlated positively with tissue CD4$^+$ lymphocytes ($r = 0.482$, $p = 0.005$).

At week 24, repetition of subcutaneous abdominal tissue biopsy yielded an increased number of non-perivascular macrophages in the cholecalciferol group ($p = 0.029$) (Figure 31) at the expense of macrophages associated with big adipocytes ($p = 0.04$). At this time point, statistically significant correlations were observed for AT TCD4$^+$ (r =

0.523, p = 0.026) and TCD8^{+} lymphocytes (r = 0.637, p = 0.004) and visceral adiposity index, as well as for tissue TCD8^{+} and HDL cholesterol (r = -0.511, p = 0.030).

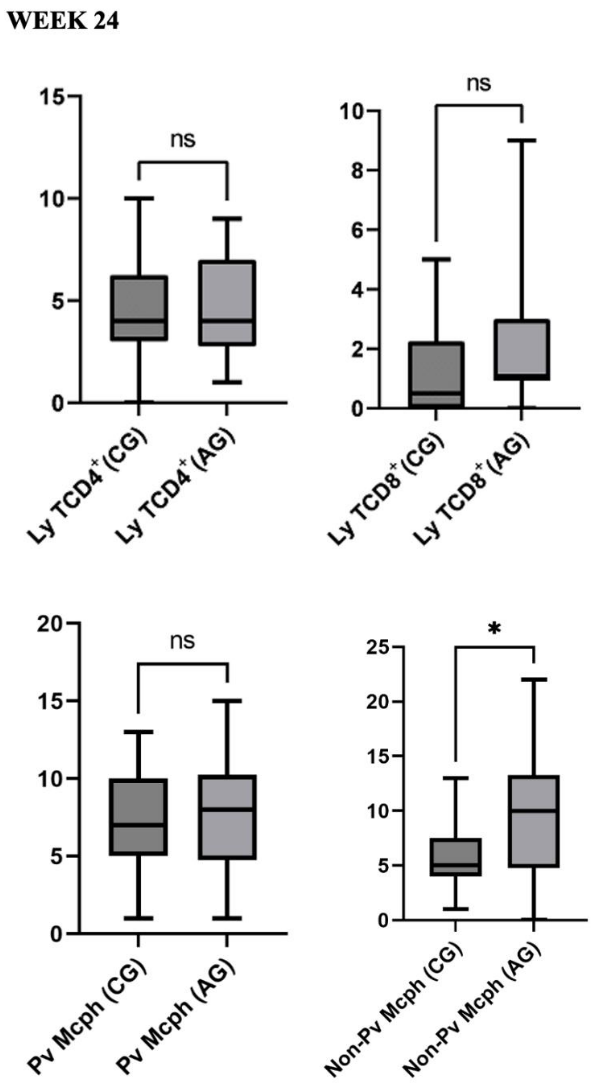


Figure 31: Number of adipose resident immune tissue cells at week 24 in control and cholecalciferol groups per 10 hpf. Statistical significance obtained by univariate independent samples non-parametric t-test is denoted as * $p<0.05$.

In pairwise testing, in a similar approach to the one employed with peripheral immune cells, we observed an inconsistent pattern in the variation of AT CD8+cells (Figure 32). Adipose tissue sampling identified more resident cells in some patients, but a reduced number per 10 hpf was also documented. From Figure 32 is evident, however, that the range interval for AT TCD8+ lymphocytes is greater after supplementation.

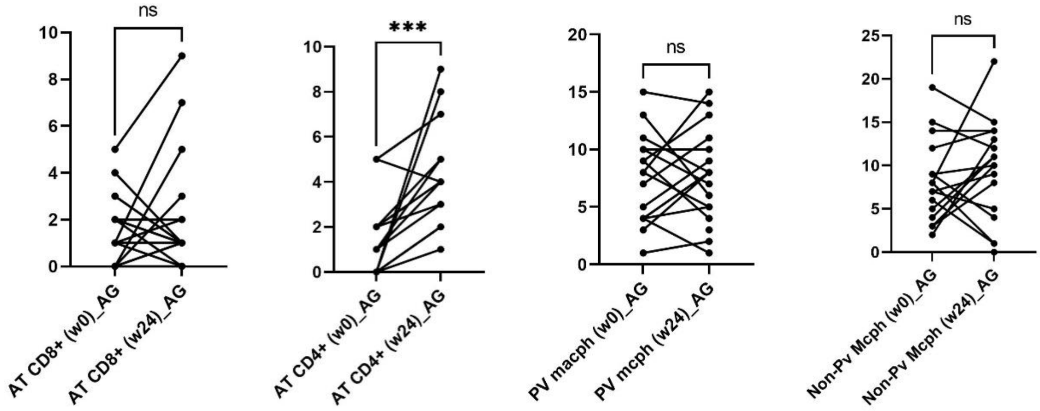


Figure 32: Pairwise comparisons of adipose resident immune cells (dots express the numbers of cells per 10 hpf). Statistical significance obtained by univariate paired samples parametric t-test is denoted as *** p<0.01.

For AT TCD4+ cells a significant increase (p=0.005) was observed during the follow-up. This suggests continued migration of lymphocytes into the subcutaneous AT that was not inhibited by VD, since the average number of AT CD4+ lymphocytes in these patients were not significantly different from controls.

The number of perivascular macrophages remained constant under the effect of cholecalciferol (Figure 32) and at 6 months post-supplementation no differences existed between controls and treated participants (Figure 31). For perivascular macrophages there was not a big discrepancy in the range interval in the two time points.

In the case of non-perivascular macrophages, we observed an increasing tendency in their numbers between times 0 and 24 (Figure 32) that resulted in a significant difference when compared to control patients.

Also at baseline, we documented a negative correlation between perivascular macrophages and total serum VD (r = -0.387, p = 0.024), but no association was seen for free VD.

In the control group, no differences between perivascular and non-perivascular macrophages at times 0 and 24 were documented but the number of crown-like structures correlated with peripheral T CD4$^+$ (r = -0.496, p = 0.036) and T CD8$^+$ cells (r = 0.571, p = 0.013). The correlation between total VD and perivascular macrophages persisted at week 24 for the control group (r = -0.528, p = 0.024), but not for treated patients, once again suggesting some kind of modulation that was induced by VD.

Section 4.10: Comparison of insulin resistance indexes before and after cholecalciferol supplementation in obese hypertensives

Baseline visceral adiposity index was high and remained severe after cholecalciferol supplementation without significant variations in the trial period (Figure 33). This index reflects the degree of adipose tissue dysfunction and was not ameliorated by cholecalciferol. Although it is not widely used, it is a marker specific for visceral abdominal tissue derived insulin resistance. In this regard, it is interesting to observe that VAI correlated with non-perivascular (r=0.560; p=0.015) but not with perivascular macrophages (r=0.09; p=0.730), and in this setting, with macrophages associated with small adipocytes (r=0.670; p=0.002). For HbA1c (Figure 34) no changes were also documented.

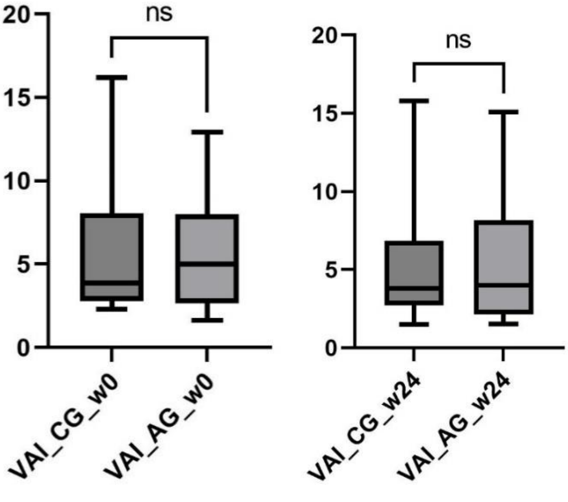


Figure 33: Variation of visceral adiposity index between weeks 0 and 24 in control and cholecalciferol groups. Statistical significance obtained by univariate independent samples non-parametric t-test: non-significant (ns).

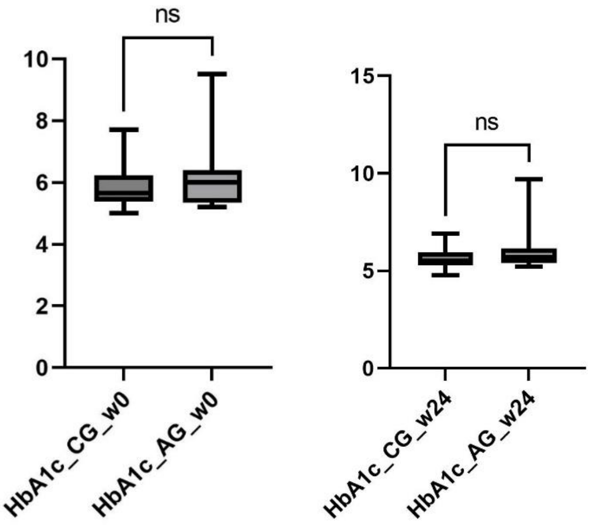


Figure 34: change in HbA1c between weeks 0 and 24 in control and cholecalciferol groups. Statistical significance obtained by univariate independent samples parametric t-test: non-significant (ns).

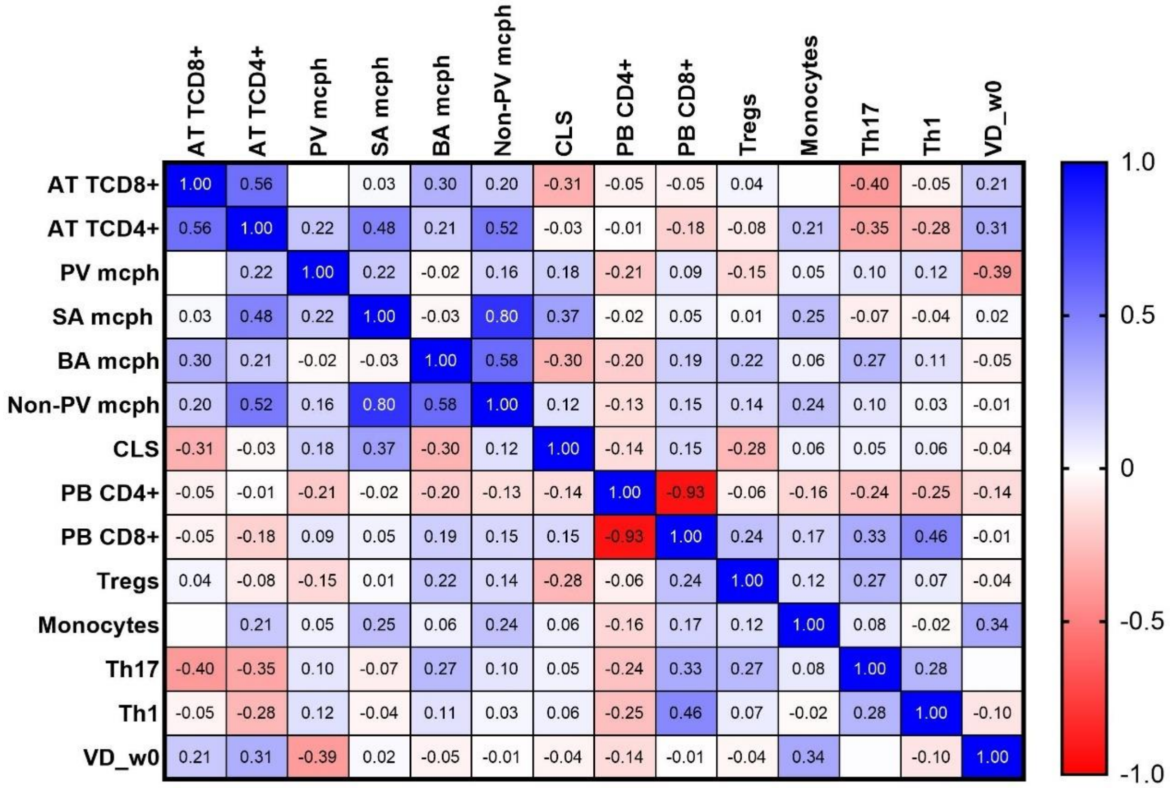
Section 4.11: Correlation between subcutaneous adipose tissue and peripheral blood immune cells

Trends or associations between the different immune cells in peripheral blood and AT were investigated using Pearson correlation coefficient. For better visualization, results are presented as heatmaps.

At baseline (Table 8) and including all the trial participants (N=36) that at initiation had serum VD < 20 ng/mL, significant correlations were seen for AT TCD4+ and AT CD8+ cells (r=0.560; p=0.0006). This contrasts with the significant but negative correlation that is documented in peripheral blood (r=-0.930; p<0.0001), suggesting a skewed phenotype of AT CD4+ compartment to essentially pro-inflammatory cells. There was a significant correlation between AT TCD4+ cells and non-perivascular macrophages (r=0.520; p=0.002), especially small adipocyte associated macrophages (r=0.480; p=0.004). In accordance with previous studies, significant associations between peripheral and adipose immune cells were not documented, except for Th17 cells and AT TCD8+ (r=-0.400; p=0.029), suggesting a dissociation between peripheral and AT resident pro-inflammatory cells. The correlation with AT CD4+ did not reach statistical significance (p=0.063).

Of note was the negative correlation between serum VD levels and perivascular macrophages (r=-0.387; p=0.024), an association not documented for other cell types and suggesting that the proximity with blood vessels may reduce monocytes migration to the AT.

Table 8: Matrix correlation between VD, AT tissue and PB immune cells at baseline (N=36).

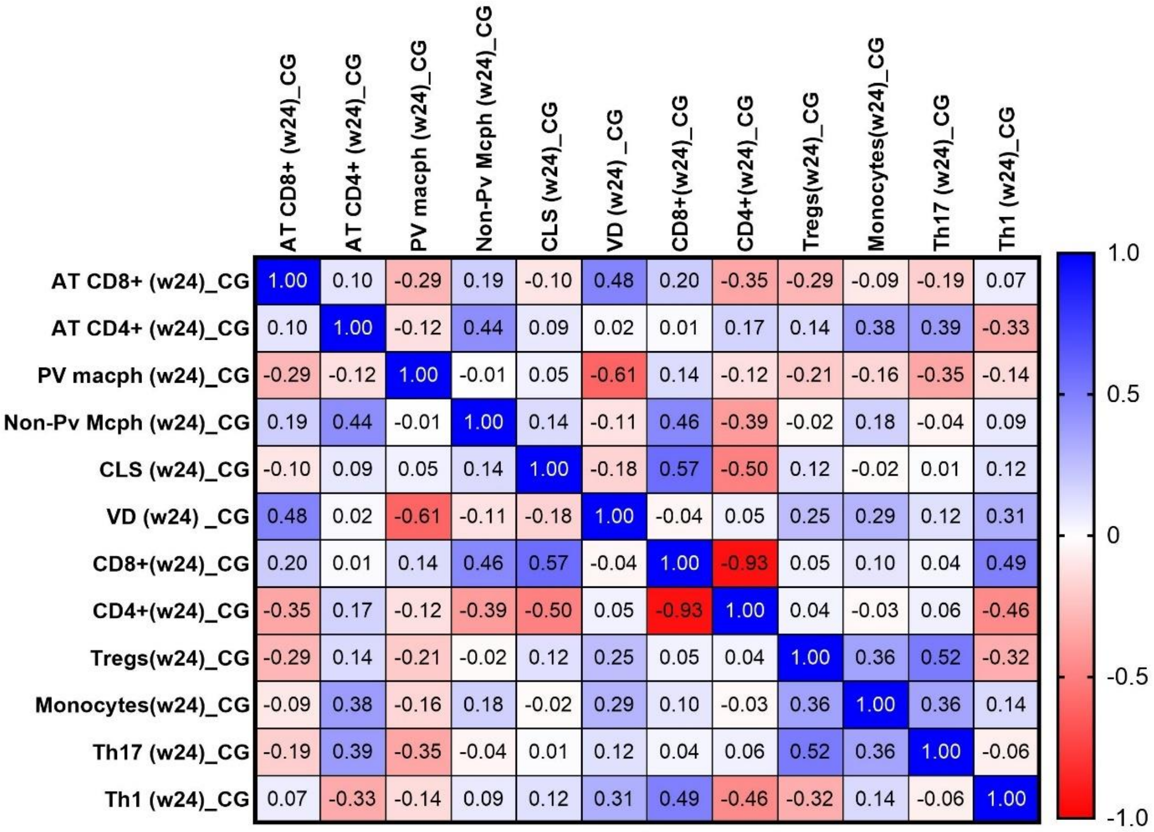


	AT TCD8+	AT TCD4+	PV mcph	SA mcph	BA mcph	Non-PV mcph	CLS	PB CD4+	PB CD8+	Tregs	Monocytes	Th17	Th1	VD_w0
AT TCD8+	1.00	0.56		0.03	0.30	0.20	-0.31	-0.05	-0.05	0.04		-0.40	-0.05	0.21
AT TCD4+	0.56	1.00	0.22	0.48	0.21	0.52	-0.03	-0.01	-0.18	-0.08	0.21	-0.35	-0.28	0.31
PV mcph		0.22	1.00	0.22	-0.02	0.16	0.18	-0.21	0.09	-0.15	0.05	0.10	0.12	-0.39
SA mcph	0.03	0.48	0.22	1.00	-0.03	0.80	0.37	-0.02	0.05	0.01	0.25	-0.07	-0.04	0.02
BA mcph	0.30	0.21	-0.02	-0.03	1.00	0.58	-0.30	-0.20	0.19	0.22	0.06	0.27	0.11	-0.05
Non-PV mcph	0.20	0.52	0.16	0.80	0.58	1.00	0.12	-0.13	0.15	0.14	0.24	0.10	0.03	-0.01
CLS	-0.31	-0.03	0.18	0.37	-0.30	0.12	1.00	-0.14	0.15	-0.28	0.06	0.05	0.06	-0.04
PB CD4+	-0.05	-0.01	-0.21	-0.02	-0.20	-0.13	-0.14	1.00	-0.93	-0.06	-0.16	-0.24	-0.25	-0.14
PB CD8+	-0.05	-0.18	0.09	0.05	0.19	0.15	0.15	-0.93	1.00	0.24	0.17	0.33	0.46	-0.01
Tregs	0.04	-0.08	-0.15	0.01	0.22	0.14	-0.28	-0.06	0.24	1.00	0.12	0.27	0.07	-0.04
Monocytes		0.21	0.05	0.25	0.06	0.24	0.06	-0.16	0.17	0.12	1.00	0.08	-0.02	0.34
Th17	-0.40	-0.35	0.10	-0.07	0.27	0.10	0.05	-0.24	0.33	0.27	0.08	1.00	0.28	
Th1	-0.05	-0.28	0.12	-0.04	0.11	0.03	0.06	-0.25	0.46	0.07	-0.02	0.28	1.00	-0.10
VD_w0	0.21	0.31	-0.39	0.02	-0.05	-0.01	-0.04	-0.14	-0.01	-0.04	0.34		-0.10	1.00

Repetition of the subcutaneous abdominal biopsy in the control group documented loss of the correlation between AT TCD8+ and AT TCD4+ cells, but, on the other hand, an overlapping picture concerning other associations, like AT CD4+ cells and non-perivascular macrophages (r=0.440; p=0.077) but also VD and perivascular macrophages (r=-0.610; p=0.008) (Table 9).

Interestingly CLSs' correlated with peripheral TCD4+ (p=0,036) and TCD8+ cells (p=0.013), with a pattern that mostly resembles their actions in peripheral blood and AT, respectively.

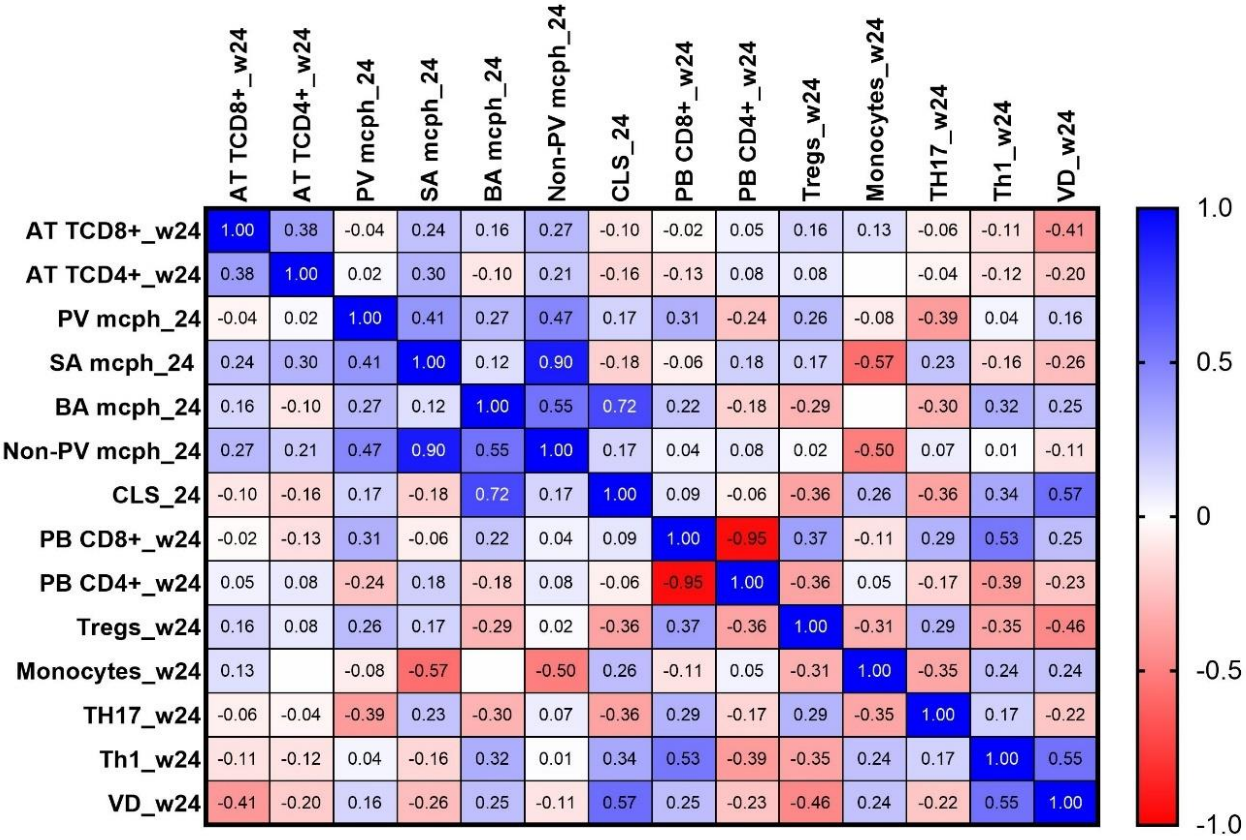
Table 9: Matrix correlation between VD, AT tissue and PB immune cells at week 24 for control group (N=18).



	AT CD8+ (w24)_CG	AT CD4+ (w24)_CG	PV macph (w24)_CG	Non-Pv Mcph (w24)_CG	CLS (w24)_CG	VD (w24) _CG	CD8+(w24)_CG	CD4+(w24)_CG	Tregs(w24)_CG	Monocytes(w24)_CG	Th17 (w24)_CG	Th1 (w24)_CG
AT CD8+ (w24)_CG	1.00	0.10	-0.29	0.19	-0.10	0.48	0.20	-0.35	-0.29	-0.09	-0.19	0.07
AT CD4+ (w24)_CG	0.10	1.00	-0.12	0.44	0.09	0.02	0.01	0.17	0.14	0.38	0.39	-0.33
PV macph (w24)_CG	-0.29	-0.12	1.00	-0.01	0.05	-0.61	0.14	-0.12	-0.21	-0.16	-0.35	-0.14
Non-Pv Mcph (w24)_CG	0.19	0.44	-0.01	1.00	0.14	-0.11	0.46	-0.39	-0.02	0.18	-0.04	0.09
CLS (w24)_CG	-0.10	0.09	0.05	0.14	1.00	-0.18	0.57	-0.50	0.12	-0.02	0.01	0.12
VD (w24) _CG	0.48	0.02	-0.61	-0.11	-0.18	1.00	-0.04	0.05	0.25	0.29	0.12	0.31
CD8+(w24)_CG	0.20	0.01	0.14	0.46	0.57	-0.04	1.00	-0.93	0.05	0.10	0.04	0.49
CD4+(w24)_CG	-0.35	0.17	-0.12	-0.39	-0.50	0.05	-0.93	1.00	0.04	-0.03	0.06	-0.46
Tregs(w24)_CG	-0.29	0.14	-0.21	-0.02	0.12	0.25	0.05	0.04	1.00	0.36	0.52	-0.32
Monocytes(w24)_CG	-0.09	0.38	-0.16	0.18	-0.02	0.29	0.10	-0.03	0.36	1.00	0.36	0.14
Th17 (w24)_CG	-0.19	0.39	-0.35	-0.04	0.01	0.12	0.04	0.06	0.52	0.36	1.00	-0.06
Th1 (w24)_CG	0.07	-0.33	-0.14	0.09	0.12	0.31	0.49	-0.46	-0.32	0.14	-0.06	1.00

At week 24, in VD subjected patients it was also not evident the correlation between AT TCD8+ and AT TCD4+ observed at baseline (Table 10). However, a significant correlation between perivascular and non-perivascular macrophages was now present (r=0.470; p=0.05). The baseline association between peripheral Th17 cells and AT immune cells was now displaced to innate immunity cells, specifically total monocytes, which correlated with non-perivascular macrophages (r=-0.500; p=0.046), in particular, with small adipocyte associated macrophages (r=-0.570; p=0.022). Noteworthy, was also the negative correlation at this time moment between VD and Tregs (r=-0.460; p=0.061), which we think may be in line with the results documented by us of increased Tregs in a pro-inflammatory environment to rebalance the multiple derangements of other inflammatory cascades. VD also correlated with CLS's (r=0.570; p=0.014) (Table 10) and we speculate that increased reservoirs of liposoluble VD may promote the aggregation of scattered macrophages in AT, rendering this pro-inflammatory clusters as a more localized site of inflammation.

Table 10: Matrix correlation between VD, AT tissue and PB immune cells at week 24 for cholecalciferol group (N=17).



	AT TCD8+_w24	AT TCD4+_w24	PV mcph_24	SA mcph_24	BA mcph_24	Non-PV mcph_24	CLS_24	PB CD8+_w24	PB CD4+_w24	Tregs_w24	Monocytes_w24	TH17_w24	Th1_w24	VD_w24
AT TCD8+_w24	1.00	0.38	-0.04	0.24	0.16	0.27	-0.10	-0.02	0.05	0.16	0.13	-0.06	-0.11	-0.41
AT TCD4+_w24	0.38	1.00	0.02	0.30	-0.10	0.21	-0.16	-0.13	0.08	0.08		-0.04	-0.12	-0.20
PV mcph_24	-0.04	0.02	1.00	0.41	0.27	0.47	0.17	0.31	-0.24	0.26	-0.08	-0.39	0.04	0.16
SA mcph_24	0.24	0.30	0.41	1.00	0.12	0.90	-0.18	-0.06	0.18	0.17	-0.57	0.23	-0.16	-0.26
BA mcph_24	0.16	-0.10	0.27	0.12	1.00	0.55	0.72	0.22	-0.18	-0.29		-0.30	0.32	0.25
Non-PV mcph_24	0.27	0.21	0.47	0.90	0.55	1.00	0.17	0.04	0.08	0.02	-0.50	0.07	0.01	-0.11
CLS_24	-0.10	-0.16	0.17	-0.18	0.72	0.17	1.00	0.09	-0.06	-0.36	0.26	-0.36	0.34	0.57
PB CD8+_w24	-0.02	-0.13	0.31	-0.06	0.22	0.04	0.09	1.00	-0.95	0.37	-0.11	0.29	0.53	0.25
PB CD4+_w24	0.05	0.08	-0.24	0.18	-0.18	0.08	-0.06	-0.95	1.00	-0.36	0.05	-0.17	-0.39	-0.23
Tregs_w24	0.16	0.08	0.26	0.17	-0.29	0.02	-0.36	0.37	-0.36	1.00	-0.31	0.29	-0.35	-0.46
Monocytes_w24	0.13		-0.08	-0.57		-0.50	0.26	-0.11	0.05	-0.31	1.00	-0.35	0.24	0.24
TH17_w24	-0.06	-0.04	-0.39	0.23	-0.30	0.07	-0.36	0.29	-0.17	0.29	-0.35	1.00	0.17	-0.22
Th1_w24	-0.11	-0.12	0.04	-0.16	0.32	0.01	0.34	0.53	-0.39	-0.35	0.24	0.17	1.00	0.55
VD_w24	-0.41	-0.20	0.16	-0.26	0.25	-0.11	0.57	0.25	-0.23	-0.46	0.24	-0.22	0.55	1.00

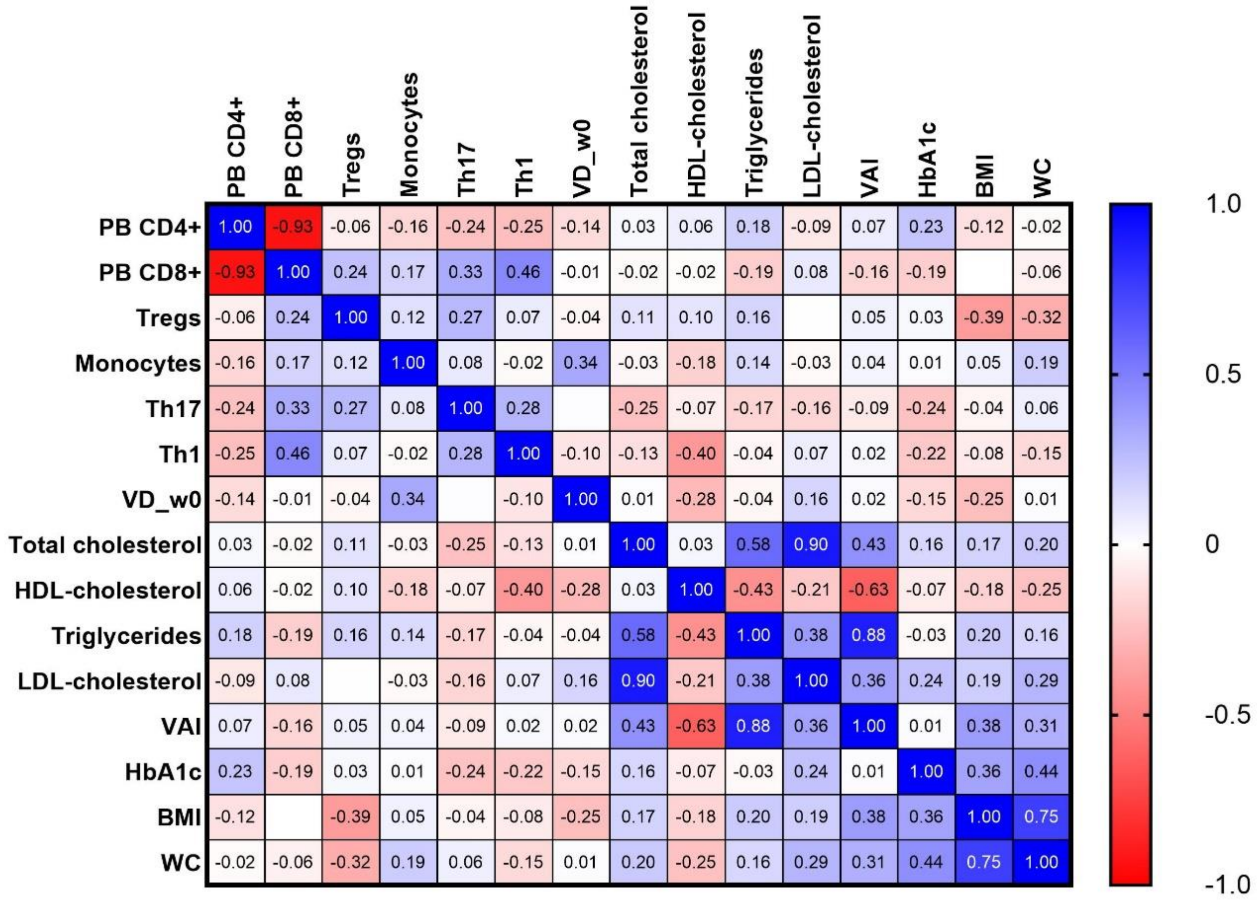
Section 4.12: Correlation between adipose tissue and peripheral blood immune cells and metabolic parameters

Consistent with our previous observations, also in AT a shortage of statistically significant correlations was documented between immune cells and parameters of carbohydrate and lipid metabolism at baseline. Although AT and peripheral blood are tightly linked, the adaptions seen in one compartment do not necessarily mirror in the other. Besides the correlations observed with immune cells in AT described previously, no significant associations were seen with the parameters of lipid metabolism, except for macrophages associated with small adipocytes and triglycerides (r=0.430; p=0.011).

Concerning peripheral blood immune cells and metabolic parameters, more associations were documented than in AT, in line with the results of other studies, probably because it is a more accessible and easier to study sample and more data is available for comparison.

Relevant correlations observed by us included Tregs and BMI (r=-0.390; p=0.019) and Tregs and waist circumference (r=-0.320; p=0.056), both negative, and demonstrating the effects of increasing BMI in the Tregs frequency and percentage (Table 11). Although obese hypertensives had more Tregs than their normotensive counterparts, it also becomes clear that with increasing BMI and waist circumference, Tregs do decrease. Several confounding factors may affect our interpretation, and forward comparisons may be limited because of patients' characteristics, namely simultaneity of HTN and obesity diagnosis. As a whole, however, the strength of the correlation was stronger for BMI than for waist circumference, implicating BMI as a solid predictor for Treg reduction. For the remainder cells of the adaptive immunity, we documented a negative correlation between Th1 cells and HDL-cholesterol (r=-0.400; p=0.030), with more correlations with lipid parameters probably being missed because of concomitant lipid-lowering medication. Finally, monocytes and VD also correlated (r=0.340; p=0.047) but the same association for the different monocytes subsets was not observed. Other significant correlations between waist circumference, BMI, visceral adiposity index, HbA1c as well as between other immunological cells are signalled according to colour intensity and the respective value of Pearson coefficient correlation but were, to some extent, expected (Table 11).

Table 11: Matrix correlation between PB immune cells and metabolic parameters at baseline for all the participants.



	PB CD4+	PB CD8+	Tregs	Monocytes	Th17	Th1	VD_w0	Total cholesterol	HDL-cholesterol	Triglycerides	LDL-cholesterol	VAI	HbA1c	BMI	WC
PB CD4+	1.00	-0.93	-0.06	-0.16	-0.24	-0.25	-0.14	0.03	0.06	0.18	-0.09	0.07	0.23	-0.12	-0.02
PB CD8+	-0.93	1.00	0.24	0.17	0.33	0.46	-0.01	-0.02	-0.02	-0.19	0.08	-0.16	-0.19		-0.06
Tregs	-0.06	0.24	1.00	0.12	0.27	0.07	-0.04	0.11	0.10	0.16		0.05	0.03	-0.39	-0.32
Monocytes	-0.16	0.17	0.12	1.00	0.08	-0.02	0.34	-0.03	-0.18	0.14	-0.03	0.04	0.01	0.05	0.19
Th17	-0.24	0.33	0.27	0.08	1.00	0.28		-0.25	-0.07	-0.17	-0.16	-0.09	-0.24	-0.04	0.06
Th1	-0.25	0.46	0.07	-0.02	0.28	1.00	-0.10	-0.13	-0.40	-0.04	0.07	0.02	-0.22	-0.08	-0.15
VD_w0	-0.14	-0.01	-0.04	0.34		-0.10	1.00	0.01	-0.28	-0.04	0.16	0.02	-0.15	-0.25	0.01
Total cholesterol	0.03	-0.02	0.11	-0.03	-0.25	-0.13	0.01	1.00	0.03	0.58	0.90	0.43	0.16	0.17	0.20
HDL-cholesterol	0.06	-0.02	0.10	-0.18	-0.07	-0.40	-0.28	0.03	1.00	-0.43	-0.21	-0.63	-0.07	-0.18	-0.25
Triglycerides	0.18	-0.19	0.16	0.14	-0.17	-0.04	-0.04	0.58	-0.43	1.00	0.38	0.88	-0.03	0.20	0.16
LDL-cholesterol	-0.09	0.08		-0.03	-0.16	0.07	0.16	0.90	-0.21	0.38	1.00	0.36	0.24	0.19	0.29
VAI	0.07	-0.16	0.05	0.04	-0.09	0.02	0.02	0.43	-0.63	0.88	0.36	1.00	0.01	0.38	0.31
HbA1c	0.23	-0.19	0.03	0.01	-0.24	-0.22	-0.15	0.16	-0.07	-0.03	0.24	0.01	1.00	0.36	0.44
BMI	-0.12		-0.39	0.05	-0.04	-0.08	-0.25	0.17	-0.18	0.20	0.19	0.38	0.36	1.00	0.75
WC	-0.02	-0.06	-0.32	0.19	0.06	-0.15	0.01	0.20	-0.25	0.16	0.29	0.31	0.44	0.75	1.00

Section 4.13: Immune cells loadings before and after cholecalciferol supplementation

The latent relationship between the cells of adaptive immunity in the 2-time points considered was evaluated using PCA. Since all the cell lineages are highly correlated, and for some, similar variations were observed between groups, PCA allowed us to analyse their interrelationship before and after cholecalciferol supplementation. PCA of the cells of normotensive controls was not studied due to the small sample size producing an invalid model to perform factor analysis (KMO 0.291; p = 0.297).

Considering the cells of adaptive immunity, we found in obese hypertensives a distribution comprising two components that were designated “pro-inflammatory” and explaining 44.7% of variance and “anti-inflammatory” explaining only 18.2% of variance (KMO = 0.605; $p < 0.01$) (Table 12). The “pro-inflammatory component” included high and positive factorial loads for Th1 and Tc1 and negative loads for TCD4$^+$ and Tc17 cells. The second component, named “anti-inflammatory” comprised TCD4$^+$, TCD8$^+$ and Tregs with high factorial loads, but T CD4$^+$ cells with a negative value, unveiling an unexpected high correlation between TCD8$^+$ and Tregs. Th17 cells did not significantly load in any of the two components, meaning that the association with either the pro- or anti-inflammatory cells was not strong at the baseline.

Table 12: PCA at baseline for all the trial participants.

Week 0	Obese hypertensives (N = 36)	
	Loadings	
	Pro-inflammatory component	Anti-inflammatory component
Eigenvalue	3.13	1.27
CD4+	-0.556	**-0.664**
CD8+	0.547	**0.733**
Th17	0.145	0.479
Treg	-0.255	**0.776**
Th1	**0.826**	-0.017
Tc1	**0.908**	0.097
Tc17	-0.531	-0.323
% cumulative variance	44.7%	18.2%
	62.9%	
KMO	0.605	
p-value	<0.001	

After cholecalciferol, the structural relationship of the cells of adaptive immunity changed, being now identified three principal components (Table 13). The first very similar to the one documented in controls, explaining a comparable proportion of variance (43.7%) and comprising mostly proinflammatory cells. A second component in which Tregs presented the highest factorial load, but now explaining 25.4% of variance and a third new component in which Th17 cells appeared with high loading. In contrast, at week 24, the distribution of the cells in the control group was very similar to the one observed at week 0, being again identified 2 components with a distribution that largely mimics the one observed at the beginning of the trial and, additionally, high factorial loads for Tregs and Th17 cells, supporting a high correlation between these two variables (Figure 35). The communalities were high for all the variables included, meaning that a great proportion of the information of the original variables was extracted in this new rearrangement.

Table 13: PCA at week 24 for control and cholecalciferol groups.

Week 24	Control group		Cholecalciferol group		
	Loadings		Loadings		
	Pro-inflammatory component	Anti-inflammatory Component	Pro-inflammatory Component 1	Anti-inflammatory Component	Pro-inflammatory Component 2
Eigenvalue	3.32	1.72	3.06	1.78	1.08
CD4+	**0.769**	-0.526	**-0.822**	0.277	0.463
CD8+	**-0.761**	**0.602**	**0.913**	-0.221	-0.308
Th17	0.417	**0.704**	0.500	-0.112	**0.789**
Treg	0.501	**0.700**	0.228	**-0.852**	0.046
Th1	**-0.678**	0.142	**0.770**	0.581	0.018
Tc1	**-0.805**	-0.183	**0.774**	0.477	0.220
Tc17	**0.782**	0.214	-0.231	0.593	-0.311
% cumulative variance	47.3%	24.6%	43.7%	25.4%	15.4%
	71.9%		84.5%		
KMO	0.599		0.512		
p-value	<0.001		<0.001		

The splitting between Tregs and Th17 observed in the treatment group at week 24 contrasts with the strong association observed in the control group, suggesting a role for cholecalciferol in this new configuration (Figure 36). Moreover, we also observed that Th1 and Th17 cells separated well in all the analyses performed, supporting different pathways contributing to inflammation and pathogenicity.

The cells of the innate immunity did not change their loads or interrelationship in the time frame considered in none of the groups. It was noticeable the opposite loads of classical and non-classical monocytes in all the time points and groups tested. Loadings and PCA graphics are not presented because KMO was <0.5 for all the groups producing an invalid model to perform factor analysis.

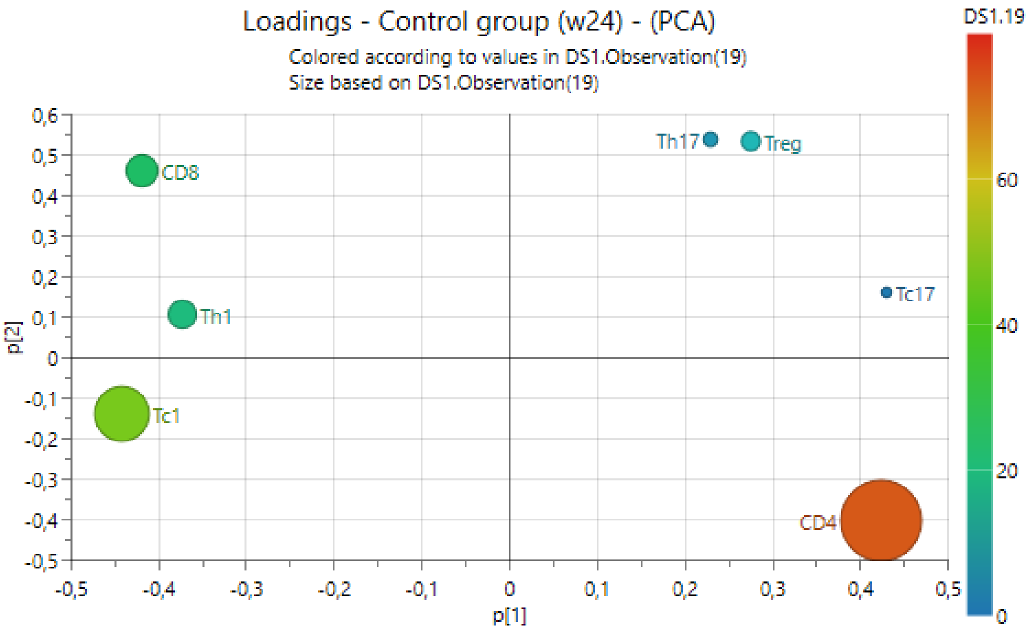


Figure 35: PCA at week 24 for control group.

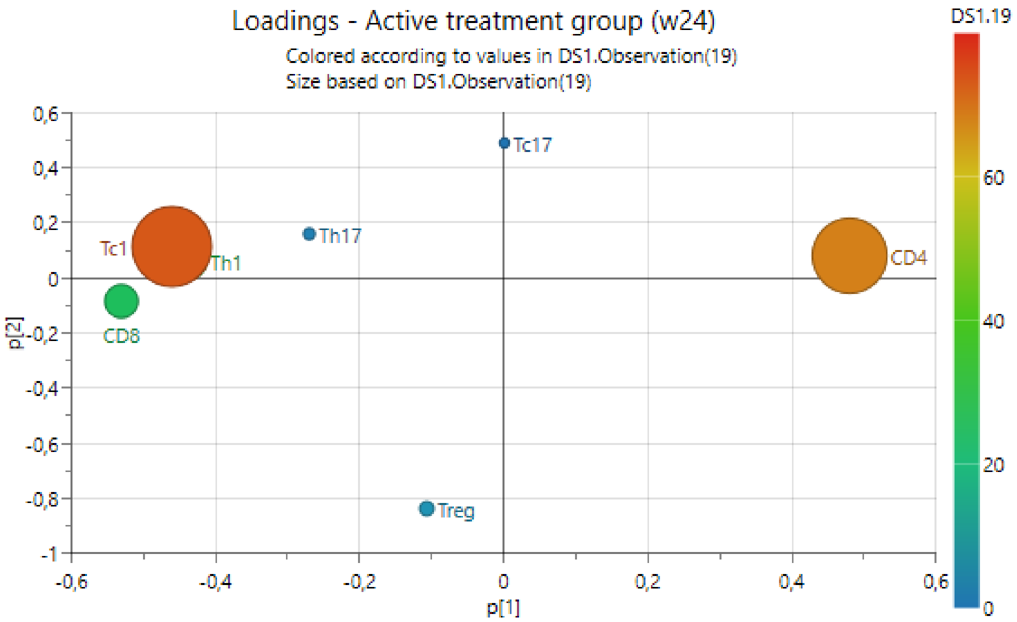


Figure 36: PCA at week 24 for cholecalciferol group.

Section 4.14: Effects of increasing free VD over pro-inflammatory cells

We found that the higher levels of free VD (≥6 ng/mL) significantly separated patients from those who presented lower levels, being Th17 and classical monocytes the variables with greatest standardised coefficients (discriminant scores 1.164 and –0.639, respectively; lambda of Wilk's 0.514; p = 0.036), and hence, better discriminant power. These results are in line with the decrease in the total percentage of classical monocytes in pairwise comparisons in the treatment group, supporting a role of cholecalciferol in the cells of innate immunity.

Section 4.15: Safety endpoints

No serious adverse events were observed during the trial. None of the participants reported adverse events secondary to cholecalciferol or complications from venepuncture in the several time points considered. Subcutaneous abdominal biopsy resulted in no major complications or serious adverse events. Local pain or hematoma was resolved with analgesics and ice. No cardiovascular events were reported or worsening of blood pressure control. All the participants maintained the usual anti-hypertensive scheme, and none required any new anti-hypertensive addition. Hypercalcemia and hyperphosphatemia were not observed in any of the participants and cholecalciferol dose reduction was not necessary.

Chapter 5 – Discussion

The aim of this trial was the study of non-traditional pathophysiological factors associated with long-term HTN. In the last years, experimental medicine has brought to our knowledge a plentiful of new information's that may challenge our approach to this disease. However, translation into clinical practice still waits for more data that supports the forward step, that will probably include in HTN therapeutics a set of new drugs, like immunomodulators.

To a more comprehensive understanding of these new triggers in HTN, we studied peripheral blood and AT immune cells, complemented with the analysis of the metabolic microenvironment that surrounds them, and support their immunological activity. The sampling of accessible AT, rarely done in human studies, allowed a better understanding of end-organ involvement by immune cells, its correlation with peripheral blood findings and evaluation of the therapeutic trial intervention. It is worth noting that, despite having included hypertensive patients, the intervention proposed in the trial was not designed to explore any anti-hypertensive effects but rather broaden our understanding with the use of a drug with recognized immunomodulatory role. In an area where human data is still scarce and not completely consensual, clinical trials urge to provide more scientific evidence and expertise in the field of immunology in HTN. Searching in PubMed by May 2023 with the terms "hypertension" and "immune cells" retrieves 765 results, of which only 4 are randomized clinical trials. In this way, exploring the immunomodulatory properties of several drugs available seems rationale as, at the same time, more data is becoming available about the contribution of the different immune cells to HTN.

Given the pathophysiology of the disease, the choice of a drug or combination of drugs, at a first glance, looks relatively easy, because of the availability of multiple drugs that selectively can block several steps in the process of immunological activation and replication (61). We choose cholecalciferol because of multiple reasons: documented effects *in vitro* and *in vivo* over all the cells of the immune system, favourable adverse effect profile, low cost, accessibility, and perception by the community of the benefits of VD, which may have prompted a better acceptance of participants in trial enrolment. The choice of other commonly used immunomodulators like mycophenolate, rituximab or bortezomib, would necessarily be limited by ethical restrictions, adverse events and costs in a trial that already faced a recruitment challenge.

The definition of endpoints was based in the available literature at the time when protocol was written, by 2015. The effects of VD were well described in several diseases, however, mainly, auto-immune diseases, and not specifically chronic inflammatory conditions. The ubiquitous expression of VDR as well as the widespread 1α-hydroxylase activity were all in favour of the immunological effects of VD, especially in pluripotent cells (113). So, we hypothesized that VD supplementation could booster anti-inflammatory cells expansion, while reducing the frequency of pro-inflammatory Th17 cells, as seen in many other conditions (104, 105, 115, 118). Since AT mirrors in several aspects the changes occurring in peripheral blood, sampling of AT could also provide a picture of the ongoing changes after therapeutic intervention.

For the primary endpoint, however, a decrease in Tregs and Th17 cells frequency in the AG was observed in comparison to pre-treatment values. These changes were accompanied by an increased number of macrophages in the subcutaneous AT, also an unexpected variation in the time frame considered. Comparative analysis with published literature (107, 108, 133), which reports a positive effect of cholecalciferol on Tregs number and function, was not observed in this patient population. In many other fields, with a marked auto-immune component, like type 1 diabetes mellitus or multiple sclerosis, the immunomodulatory effects of cholecalciferol have been explored, with variable supplementation schemes, but neutral or positive results in terms of Tregs induction (107). While the straightforward comparison between HTN and auto-immune diseases is not possible, and so the extrapolation of results, we speculate that the high Tregs plasticity may lead to a different routing and stability in auto-immune and chronic inflammatory diseases. In an environment of chronic low-grade inflammation, like obesity-associated HTN, a state of high immune regulation, characterised by increased Tregs, may coexist with pro-inflammatory responses. This inflammation-experienced Tregs distinguish from resting and activated Tregs but, importantly, can reverse many of this activation induced changes (134). In this way, the genetic and epigenetic changes induced by chronic inflammation potentiate Treg function that sharply increases their suppressive functions. However, most of these changes are transient and largely reversible, unless T cell exhaustion is reached (134). This last process is not characteristic of inflammatory conditions but rather of chronic viral infections that lead to T cell hyporesponsiveness. Triggered by inflammatory signals, Tregs route their transcriptome to functionally active and suppressive cells.

However, over time, this increased immunoregulatory function is progressively lost, which is balanced by the acquisition of immunological memory by this Tregs (134). The transition between an inflammation-challenged to a resting memory state demonstrates the high degree of plasticity of Tregs and may be a plausible mechanism to explain the variations observed in our trial, since that in the resting state Tregs may migrate to lymphoid but, preferentially, non-lymphoid-organs, reducing their frequencies in peripheral blood (134). The importance of this mechanism also lies in the fact that the transition to a resting state avoids excessive immunoregulation and suppression and probably explains the increased infectious risk seen in some chronic conditions (77).

In the case of sterile inflammation, as is the case of HTN, obesity but also atherosclerosis, the role of Tregs may also depend on tissue location. In the AT, for example, not only Tregs are phenotypically different from the ones observed in lymphoid organs, as their immunosuppressive ability may contribute to damage rather than reparation and healing (75). At peripheral tissues, their dependence in IL-2 is reduced and other cytokines and co-stimulators like IL-7, IL-33 or serotonin become able to activate and maintain Tregs. Other tissue specific characteristics, like microbiota and TGF-β availability, are also relevant and may contribute to a specific pattern of Treg differentiation (135).

Our trial was not designed to distinguish the different Treg subsets or changes in their suppressive capacity in blood or peripheral tissues. However, it showed that at sufficient levels VD can significantly modulate Tregs leading to a transition from a strong association with TCD8+ cells as seen at baseline, to a new arrangement where Tregs separated from not only from TCD8+ but also from Th17 cells. Of utmost importance is the fact that in the control group and in the same circumstances except VD, these correlations persisted, or even heightened, as was the case of Tregs and Th17 cells. This intriguing association between Tregs, TCD8+ but also Th17 cells, with opposite loads for TCD4+ lymphocytes, suggests that the aforementioned pathological reshaping may, in fact, occur in obesity-associated HTN. Although functional tests were not performed and forward arguments are not available, the fact that the frequencies of Tregs approached those of normotensives is already a clue pointing to a favourable effect of VD.

The decrease in Th17 cells was the most noticeable effect observed in our trial and, in this case, in line with several other reports (111, 136). Th17 cells are very sensitive to changes in VD, which inhibits their activity and differentiation (108). This fact is quite evident when, at the end of the trial, Th17 cells clearly separated not only from Tregs, but also from the pro-inflammatory cells Th1 and Tc1 in the cholecalciferol group. This new component, in which Th17 cells present the highest load, distinguishes from the predominantly cytotoxic Th1 and Tc1 cells. In discriminant analysis, Th17 cells were also the cell type that demonstrated the more pronounced effect with the increase in free VD. Moreover, the stability of Th17 cells in the cholecalciferol group was linked to a significant decrease in Tc17 cells, which are well-recognized promoters of Th17 cell pathogenicity (137).

VD can blunt several cytokines and transcription factors associated with Th17 cells, being usually increased in obese adults and diabetics (52), although that was not observed in our trial. The biology of Th17 cells is, however, far more complex than simple variation in cell frequencies or the concentrations of its derived interleukins, collectively named IL-17 but including different isoforms, like IL-17A, IL-17F, IL-22 and IL-23 (138). Subject to great heterogeneity and plasticity, Th17 cells but also Th17-like cells, some originating from previous Tregs after loss of Foxp3+ in conditions of great pathogenicity, may confound our interpretation (72). Routine techniques for the distinction of Th17 from Th17-like cells are not straightforward and considering that an increase in their percentage was observed in the follow-up in the control group, we cannot exclude that in HTN, like in other chronic conditions, conversion of Tregs to Th17 cells does not occur.

However, the effects of VD are systemic and not restricted to one or another compartment, and at this time moment matters to know if the changes observed for immune cells somehow were accompanied by variations in the serum metabolites. Although several metabolites of different pathways changed significantly with VD, two caught our attention: glucose and glutamine. Essential substrates for cell proliferation and energy production through the Krebs cycle, an overall decrease in glucose and an opposite variation in glutamine were characteristic for VD patients. Glucose is the preferential source for energy production in rapidly proliferating T cells, and Th17 cells do rely in aerobic glycolysis for activation. Tregs also use glucose and glutamine but preferentially oxidize fatty acids. The reduction in glucose bioavailability may ameliorate the inflammatory milieu because glucose and its deleterious effects at the endothelial level are reduced, but also a compromise of a more generalized immune cell activation may ensue, given the lower substrate availability for T cell proliferation. Indeed, the inhibition of glycolysis was documented to dampen several immune cell responses (88) and, although gene expression studies are lacking in our trial, it was remarkable that at the metabolome level a significant difference was documented for glucose, that was not reflected in insulin resistance indexes. Probably, the use of more sensitive biomarkers of glucose metabolism in several negative VD trials, would have identified a glucose lowering effect of VD that the current measures cannot detect.

The increase in glutamine levels also favour the anabolic effects of supplementation. At baseline, the reduction in comparison to normotensives contrasts with the significant increase seen after cholecalciferol. As previously described, glutamine is a non-essential amino acid but the most abundant in humans. Induction of increased reservoirs may be one important mean to counteract the increased catabolism seen in many chronic conditions, like obesity-related HTN. Besides its importance as a source of energy through glutaminolysis and precursor for nucleotide synthesis (92, 139), improvement of muscular mass in sarcopenic obesity is an important intervention in a chronic patient.

HTN and obesity, insulin resistance and chronic dysmetabolism may all lead to reduced glutamine availability, well documented in peripheral blood, and probably the reflex of reduced amino acid pools in the liver and skeletal muscle. Although we cannot ascertain if reduced glutamine synthesis or increased glutaminolysis prevailed, the net balance is a shift toward reduced levels of this nitrogen source for nucleotides and amino sugar synthesis (139). In the long term, this chronic glutamine deficiency may act synergistically with other factors to promote immune dysregulation, since glycolysis and glutaminolysis interrelate to guarantee the adequate substrate levels for immune cell function. The observations made by us are in accordance with the ones made by other authors, with normotensives and hypertensives presenting significant differences in metabotype expression (98), with a special emphasis for the generalized reduction in the different classes of amino acids seen in our work.

Other cells of the adaptive immunity were also affected by VD treatment although at the end of the trial their values did not significantly separate from controls. Of note, is the case of Th1 cells after cholecalciferol. These cells are important promoters of inflammation and cytotoxicity, and the role of IFN-γ is very well documented in the pathogenesis of HTN. On the other hand, the scientific evidence documenting the suppressive effects of VD over Th1 cells is also extensive (115). However, more recently, data performed in naïve human T CD4+ cells demonstrated that VD promoted Th1 cell's differentiation but not IFN-γ production (140). The question is what factors may contribute to this paradoxical results in the setting of chronic inflammation. The microenvironment that triggers cell activation should be taken into account, since it is in inflammatory conditions, like tuberculosis, and not auto-immune diseases that this response was described (140). Apparently, VD through its genomic actions can block IFN-γ gene transcription but not commitment of TCD4+ cells to Th1 phenotype, leading to a dissociation between cells frequency and their related cytokines. Besides, in other experimental conditions, VD is also described to act in an autocrine manner in immune cells, leading to downregulation of IFN-γ production, that progresses initially as co-production of low levels of IFN-γ and IL-10 and then only IL-10 release, mostly resembling a Tc2 phenotype (141). This process was described by the authors as a progressive shutting down or switch off from the inflammatory programme of Th1 cells, demonstrating again that immune cells present great plasticity and ability to reshape and adapt to several stimulus. We also speculate if the different observations made by the above-mentioned authors do not correspond to distinct phases in the process of Th1 reshaping. It would also be interesting to study how these changes in cytokine release correlate with Th1 frequencies in peripheral blood. In our trial, we cannot speculate about none of these mechanisms and only conclude that under the effect of VD no anti-proliferative effect was observed. At the metabolomic level, Th1 cells share many of the pathways used by Th17 cells for energy production, mostly glycolysis and glutaminolysis. Reduction of glucose may engender a reduced rate of activation and IFN-γ synthesis, independent of cells frequency.

Tc1 followed a variation pattern mostly resembling that of Th1 cells, although in this case with statistical significance only within groups. This highly cytotoxic cell derived from TCD8+ cells fuels inflammatory processes through the release of IFN-γ, TNF-α, granzyme and perforin (142). No changes were seen in the total percentage of TCD8+

cells, that could justify this increase but the same is true for the control group. Considering the trends, and in the absence of measurement of cytokines that relate to their functional activity, we hypothesize that VD did not contribute to enhancement of Tc1 cells function, which are especially relevant for the elimination of infected macrophages (142). However, what stands out in all the measurements throughout the trial is the high loadings between Th1 and Tc1 cells, denoting a high and intertwined correlation, unaffected by VD treatment or reduced glucose availability. Considering their differential origin from TCD4+ and TCD8+ cells, respectively, and an apparent distancing from Th17 cells, these results may point to multiple and different pro-inflammatory mechanisms underlying the pathogenesis of obesity-related HTN. On one hand, a cytotoxic-based predominant component, unaffected by standard doses of cholecalciferol and other Th17-dependent that responds to VD supplementation. The synergistic action of both mechanisms is described in other conditions, that mostly resemble the immune imbalance described for HTN (143), and stressing the role of each of the components in the initiation and promotion of inflammation progression. Besides, these cells may mutually interact to facilitate migration and replication of each other, potentiating inflammation but also auto-immunity (144). Usually, IFN-γ is linked to pathogen recognition and antigen presentation, activation of microbicidal effector functions, macrophage activation and leucocyte trafficking (145). On the contrary, Th17 cell's main function is the mediation of inflammation, especially in the setting of chronic autoimmune diseases. Through TNF-α, IL-1β, IL-6 and several chemokines to attract neutrophils and macrophages, Th17 cells participate in the defence from many infectious diseases but, importantly, amplify inflammation in the setting of autoimmune diseases. The blocking of one or the another of the main cytokines produced by these cells has already proved to blunt HTN development, and we think our trial may add new information in this regard, as VD downregulated Th17 cells.

In the same way, Tc17 cells also decreased in the follow-up accompanying the variation observed for Th17 cells. However, this downregulation may not necessarily be linked to Th17 cells, as the differentiation into one or another phenotype depends on different transcription factors. Besides, the argument that low levels of glucose may have compromised Tc17 development does not apply in these circumstances because this cell subset is not affected by glucose restriction (146). Whether downregulation of Tc17 cells is linked to Th17 cells is not therefore clear, but overall Tc17 cells may contribute to increased inflammation in different contexts: in cancer, Tc17 cells may mediate the crosstalk between monocytes activated by dying tumour cells and Th17 cells. In multiple sclerosis, the role of Tc17 in inflammation is not clear, but one distinctive feature seems to be the great instability, presenting great plasticity and rapidly changing their frequencies in response to various stimulus (147). This might explain the pronounced reduction observed for Tc17 cells in the setting of VD supplementation, but also the discrepancy observed between normo- and hypertensives at baseline. Under inflammatory conditions, like obesity-related HTN, the stability of Tc17 cells may be compromised due to the multiple microenvironmental derangements.

In summary, although the percentages of peripheral TCD4+ and TCD8+ cells remained unchanged after cholecalciferol, their derived subsets did not, largely contributing to the differences observed after treatment, and confirming VD as a relevant immunonutrient. Immunonutrition is an unexplored domain in HTN but gains special relevance if we consider that not only the disease depends on dietary factors but also obesity underlines a severely compromised nutritional state. Rebalancing nutritional support in the setting of HTN and identification of nutrients with potential to modulate compromised immune responses may represent an important strategy to enhance the anti-inflammatory effect that is also recognized to most of the anti-hypertensive medications (148). The list of potential immunonutrients is large (149), but interestingly, not only VD is included but also glutamine is one of most the important and recurrently mentioned immunonutrients. It can be argued that multiple trials have evaluated the effects of glutamine supplementation with negative results but, once again, the effects of absorbed and metabolized glutamine may not be the same of the endogenously synthesized amino acid. So, if VD can induce a state of increased immunoregulation, as reflected by new interrelationships between the pro-inflammatory cells, we hypothesize that glutamine may be one of its mediators.

Others, like histidine, which also increased after VD, may also be an important mediator in this task of rebalancing the inflammatory and pro-inflammatory factors. As an immunonutrient, VD may engender this new phenotype, where not only cytokines, ROS and glutathione but also fatty acids, antioxidants, hormones and pro-hormones are affected (149). However, searching for these beneficial aspects is elusive in the short-term. This modulatory activity may be visible only several years after supplementation and the degree of end-organ reversibility may be limited. We also speculate that in the absence of more robust data, supplementation with VD at the time of anti-hypertensive initiation would be a rather better strategy, than introduction only when well-established cardiovascular disease is in place.

We think the inclusion of a control group of normotensive patients, although overweight, shed light on many aspects of trial results, leading to a more comprehensive interpretation of the obtained data. Physiological variations are constantly occurring, and immune cells but also metabolites respond to these triggers rapid and efficiently. So, there is not a right picture, but a picture taken at that moment, and only the sum of the information taken in different time points can reproduce more exactly the ongoing microenvironment. Evaluating the differences between normotensive controls and hypertensives showed in our trial that much work remains to be done. Not only the detailed characterization obtained by us does not completely match with the reports of other authors, as new and intriguing differences were documented. We shall highlight the discrepancies documented for Tregs, which replicated the results of only one study in morbidly obese patients (76), which may not truly reflect the population studied by us, but also the increase in non-classical monocytes. These changes simultaneously affect adaptive and innate immunity, somehow suggesting that they may contribute to a more sustained and controlled inflammation in the long-term.

At the same time, the generalized downregulation of amino acids of several classes and sub-products of glucose metabolism may also signal the absence of a proliferating limiting effect with overt consumption of several substrates. The non-polar amino acids glycine, alanine and phenylalanine are especially relevant in glutathione synthesis, but also collagen and creatine, and are accordingly reduced in the hypertensive cohort. The reduction of serine in comparison to normotensives also deserves attention because it may result from increased consumption for cell proliferation (90). The reduced availability of this one-carbon metabolism amino acid reduces T cell proliferation and expansion, but also macrophage mediated inflammation (97). It is also relevant to note that serine, and in parallel the interconvertible glycine, are precursors of membrane phospholipids and can also be used as an energy source to gluconeogenesis (97). The reduction of these amino acids may then be an additional factor for insulin resistance, because of compromised glycogen utilization. In this regard, it is interesting to note that after VD some free fatty acids increased, pointing to increased availability for membrane phospholipid synthesis, albeit increased serine was not shown.

A final note to monocytes, for which clear variations under the effect of VD were not statistically significant. Indeed, similar trends were observed between control and cholecalciferol groups, being the most notable aspect, the differences documented only with normotensives. We do not attribute to VD the downregulation observed for total monocytes, because in the control group the same change was observed, being the sample size probably the factor that explains the absence of difference in this last group. In what concerns innate cells metabolism, is also difficult to point if any the observations made may correlate with monocyte's biology. These pathways are well described for macrophages but not so well for monocytes. In the case of dendritic cells, for example, the basal metabolism relies in oxidative phosphorylation and only upon activation is the uptake of glucose increased (97). Protein-energy wasting contrasts with obesity in what relates monocytes frequencies. In conditions of poor nutrition, monocytes are usually decreased as well as their derived-cytokines. However, in obesity other reports confirm increased total and non-classical monocytes (77), like documented by us, with the skewed phenotype underlining increased risk of infections and cancer because of increased immunoregulation.

Finally, the question that arises is how the peripheral blood findings may correlate with the changes documented in the AT. The increase in the number of infiltrating macrophages in the samples of subcutaneous AT was an unexpected finding, moreover the same increase was not shown in the control group. Several aspects concerning technical issues may be argued to explain these differences. Care was taken during sampling and biopsies were not obtained from the same exact sites of the first biopsy, to avoid increased immune cell infiltration secondary to healing. The staining of immune cells was always done using the same antibodies clones and the procedure done by the same technician throughout the trial. The chosen quadrant was the left or right inferior quadrant, aiming for a distancing between the first and second biopsies of at least 3 to 4 cm. It is well-known that visceral AT encompasses great number of immune cells, but it is not accessible by means of percutaneous procedures. The subcutaneous AT sampling showed that immune cells also play a relevant role in the pathophysiology of obesity-associated HTN. Even in the absence of samples from normotensives (as obtained for flow cytometry and metabolomics), sampling of hypertensive controls gave us the opportunity to validate the results obtained in the cholecalciferol group. The classification used by us to distinguish the different macrophages according to their location was previously used by other authors (131). In general, big adipocytes represent large cells with capacity to store great quantities of lipid droplets and may represent a marker of severe AT dysfunction. It is thought they are associated with a greater degree of insulin resistance and dysmetabolism as well as increased risk of diabetes development (131). In the subcutaneous AT, the same associations were described and confirmed the worst prognosis associated with adipocyte hypertrophy. For small adipocytes a poorer prognosis is described in what relates to the risk of insulin resistance and diabetes but with a different mechanism, essentially explained by the incapacity to expand and accommodate a greater fat storage (131). In this setting, both big and small adipocytes may be linked to dysmetabolism but by different mechanisms, and essentially a worst prognosis is attributed to big adipocytes.

Resident AT macrophages are tightly connected to these adipocytes, leading us to hypothesize if their pro-inflammatory activity may not also reflect this differential contribution to insulin resistance. In terms of frequency no changes were expected for the macrophages with this location, as non-perivascular macrophages are resident cells. However, perivascular macrophages display a very close contact with endothelium and reduced migration from blood to the perivascular surroundings would be reasonable considering the immunomodulatory effects of VD. Moreover, the baseline finding of a negative correlation between VD and perivascular macrophages, lead us to hypothesize that increased hormone values could inhibit the migration to the AT. However, the number of perivascular macrophages was not reduced after trial intervention. Instead, an increase was observed for non-perivascular macrophages, in a way that mostly resembled the changes observed in the AT after bariatric surgery (85, 150, 151). This finding is not unanimous across all the studies as some authors have found an improvement in white AT function (152, 153). However, the effects of cholecalciferol in AT were studied in very few human trials, with inconsistent results in terms of immunoregulation. While *in vitro* incubation of AT samples from obese patients with VD reduced the expression of monocyte chemoattractant protein-1, IL-6, and IL-8, *in vivo*, the same treatment didn't produce any anti-inflammatory effects (154). The same authors also documented no changes in subcutaneous and visceral AT samples, even if at the end of the trial the average values of VD were above 110 nmol/L (155). While our trial adds new data about the effects of cholecalciferol in AT, more studies to evaluate immune cell kinetics with sampling at different time points are needed, as the numbers and type of resident cell populations change with time.

In simultaneous with the increase in non-perivascular AT macrophages, there was loss of the correlation between VD and perivascular macrophages. Although in steady-state these cells are involved in the regulation of vascular permeability and scavenging of blood-borne pathogens (156), their function in diseased states is greatly compromised and depends on their actual location (157). Descriptions of the function of perivascular macrophages at the level of brain, lungs, skin or kidneys are all in favour of a pro-inflammatory (156) and pro-hypertensive role (158). A neuro-immune-adipose crosstalk triggered by adrenaline release from perivascular AT macrophages, but also neuronal sources is also described affecting blood pressure control (158). In this way, the basal correlation between VD and perivascular AT macrophages exactly points to this modulatory role of VD, although for the levels we reached an inhibitory effect over perivascular macrophages was not shown.

The intertwined association between AT lymphocytes was not reproduced for AT macrophages. However, for AT macrophages associated with small adipocytes, a positive association with AT TCD4$^+$ lymphocytes were present, not being clear, however, if this correlation may suggest a less inflammatory role for the macrophages in this location. The distinction between M1 and M2 macrophages, as well as of the different AT TCD4+ subsets would be helpful to understand their roles and associated cytokine profiles. For example, in several reports using AT human samples, Foxp3 gene expression was increased in both visceral and subcutaneous AT (159) and correlated, in most cohorts, with BMI and weight. Interestingly, the expression of Foxp3 in subcutaneous AT did not significantly differ from the observed in visceral AT, meaning that sampling subcutaneous AT is not inferior to visceral AT. Once again, these results challenge the classical paradigm, mostly described in mice models, of a generalized downregulation of Tregs contributing to AT dysfunction (159). Indeed, some authors even attribute to AT Tregs a role in the progression of insulin resistance (75). In our trial only TCD4+ identification was done, but in other reports different techniques were applied for Tregs staining, which may additionally confound our interpretation, since Foxp3 may also be expressed by non-Treg cells. However, it seems clear from the reported literature that the increase in immunoregulatory cells in conditions with recognized inflammatory background is not unusual, as documented not only by increased Tregs in PB but also in AT.

The way the changes in AT may relate to the modifications seen in PB metabolomics is not completely clear. More data would be necessary as well as the measurement of several mediators. Although macrophages rely on glucose for activation, the changes in PB do not necessarily match the metabolites concentrations in subcutaneous AT, where multiple metabolic reactions may have already occurred, as well as the differential uptake of metabolites by adipocytes and immune cells.

Several correlations were present among the studied immune cells and metabolic indexes that demonstrate that an integrated overview, rather than a compartmentalized interpretation of the results should ensue. Even in the setting of a small sample size, we should emphasize the strong association between AT TCD4+ and AT TCD8+, clearly contrasting with the same correlation seen in peripheral blood and leading us to speculate of a skewed AT TCD4+ phenotype. In the same way, the baseline dissociation between Th17 cells and AT TC8+ cells, also suggests that although obesity-related HTN should be regarded as a whole and ongoing process, the mechanisms underlying inflammation in peripheral blood and AT do not necessarily match in the different organs involved. Several other authors have already reported that AT resident immune cells may not necessarily originate from peripheral blood migration but rather from lymphoid organs (160) and only in the presence of severe AT dysfunction a correlation between markers like CLSs' and peripheral TCD4+ and TCD8+ cells was documented. Finally, the negative association between Th1 cells and HDL cholesterol is also of note and probably unveiling the action of these cells in the pathogenesis of the atherosclerotic plaque.

Several limitations should be addressed to our trial: aiming to explore different aspects of obesity-related HTN, it was not possible to explore more deeply other components related to immunological dysfunction. Those included the measurement of the multiple cytokines synthesized by the different cells studied. Technical aspects and limited resources meant that we based the immunological evaluation only in the determination of the cell's frequencies. Dosing of some of the cytokines associated with the different cells studied, would have provided more broad and in-depth information, even if the immunological reshaping that we suspect underlines this clinical condition could also have confounded our interpretation (72, 73).

The sample size is also an important limitation. Determination of sample size was based on the projected variation in the percentage of Tregs and for this endpoint the trial included the number of participants necessary for evaluation. Obviously, more data would produce more consistent results to distinguish physiological from non-physiological, VD-induced changes. Even if a control group was followed for the all the trial length, the well-recognized dynamics of immune cells but also the constant modifications in small serum metabolites would better be evaluated if more blood samplings have been performed. However, more frequent sampling was limited by the technical complexity associated with these determinations, its costs, and the need for dedicated human resources which were not available.

Finally, the low levels of VD attained are also an important limitation. Measurements at week 16 documented higher average levels and a good rate of correction of VD, leading to a transition from a high dosing scheme to a maintenance dose according to the predefined protocol. Although at the end of the trial all the patients had higher serum VD, the average concentrations were within the sub-optimal range. Several reports relate that the immunological effects of VD do not dependent only in the serum levels (161), because of autocrine synthesis, but higher levels would probably have led to more sustained improvements in the baseline immunological and metabolic profile.

Chapter 6 – Conclusions

The conduction of the current trial allowed the study of new pathophysiological factors implicated in the pathogenesis of obesity-related HTN and, at the same time, explore the immunomodulatory role of cholecalciferol. HTN is a disease that has been increasingly known for several years, but with a prognosis that is still far from desirable, and which represents an important cause of morbidity in the 21st century. The last decade has brought a new shed of light into its pathogenesis, implicating the immune system as an important player in the intricated and endless list of factors that contribute to HTN development.

With this trial, we documented that not only HTN is associated with a significant imbalance in the cells of adaptive and innate immunity, as VD supplementation can have an important immunomodulatory role over some T cell subsets. In this condition, serum metabolome is also affected to a great extent and generalized reduction in several amino acids, as well as sub-products of glucose metabolism was documented. These modifications suggest a high catabolic rate and energy production based on aerobic glycolysis given the reduced availability of acetate. These changes can fuel immune cell activation.

At the level of the adipose tissue, organ of major involvement in obesity-related HTN, a disproportionate infiltration of macrophages relative to AT TCD4+ and AT TCD8+ cells was documented, confirming the findings of other reports but also the value of the subcutaneous AT sampling. AT TCD4+ and AT TCD8+ correlated strongly but, intriguingly, in an inverse fashion to that observed in peripheral blood. A paucity of associations was seen for AT and peripheral blood immune cells, suggesting a dissociation in the immune-inflammatory responses taking place. A strong to moderate correlation between VD and perivascular macrophages emphasized the value of this hormone in the regulation of monocytes migration to AT.

The trial of VD resulted in a significant increase of both total and free VD, although to sub-optimal levels, which may have impaired its' full immunomodulatory role.

Under the effects of VD changes were observed for the great majority of the pre-specified endpoints:

1. Th17 cells kept stable in relation to the control group, but Tregs downregulated in comparison to pre-trial percentages. At week 24, Th17 cells clearly separated from Tregs, which contrasted with the findings seen for the control group.
2. The remainder cells did not change in relation to the control group, but within groups Th1 cells and Tc1 cells upregulated from weeks 0 to 24.
3. Monocytes and its different subclasses did not change significantly regarding the control group.
4. Serum metabolites were affected by VD treatment although a reversion of the low levels of amino acids was not documented. Glutamine and histidine, which are major sources for nucleotide synthesis and immune cells metabolism were upregulated.
5. Increases in non-specific free fatty acids were also documented under the effects of VD.
6. The number of perivascular macrophages remained constant throughout the trial but not of non-perivascular macrophages, which increased, mostly resembling the effects of obesogenic memory.
7. AT TCD4+ and AT CD8+ frequencies were constant throughout the trial and no variations were documented under the effect of VD.
8. Significant differences in peripheral immunological profiles and serum metabolomes were seen in comparison to normotensive controls.
9. Insulin resistance indexes remained unchanged and were not affected by standard doses of VD.
10. Th17 cells were the immunologic cells that were most affected by VD.

The study of the crossroad of HTN and immunometabolism is only beginning. Although VD is a well-known drug and has been extensively studied in the field of cardiovascular diseases, the focus in hard endpoints may have led to some loss of enthusiasm in its therapeutic potential. Immune cell dysregulation underling HTN should, therefore, deserve our attention, more even that nowadays the available scientific data do confirm the involvement of the immune system in the development of the disease.

The transition from evidence to clinical practice will have to wait some years more. The introduction of immunosuppressors does not seem a promising strategy due to multiple reasons but other recent therapeutic advances could change the paradigm and move forward the prognosis of a long-lasting disease. Until then, cholecalciferol is a very reasonable option to modulate the multiple imbalances that characterize this disease.

Stepping upwards, with development of larger trials can unravel an important immunomodulatory role for an affordable and safe drug. A transition from a strategy mainly focused on the blood pressure lowering effects to an approach aimed, instead, to explore its anti-inflammatory role, may result in a better usage of cholecalciferol in extra-skeletal health.

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Supplementary Data

S1- Deliberation from Comissão de Ética para a Investigação Clínica (CEIC)

S2- Deliberation of CEIC to the request of substantial change

S3- Deliberation from Comissão Nacional de Proteção de Dados

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6200-085 - Castelo Branco
Portugal

Vossa ref.: 01/2015

Vosso Fax: 272000269

Data: 11-12-2017

Nossa ref.: MAP / MAP / OF / 2017 / 13981 / 20170666

Assunto: **Ensaio Clínico com o Nº EudraCT 2015-003910-26 e código CEIC 20170666**

Exmos. Senhores,

Em resposta ao V. pedido de parecer para realização do Ensaio Clínico supramencionado, informa-se V. Exa. que ao abrigo do artigo 16.º da Lei n.º 21/2014 de 16 de abril, na sua redação atual, a CEIC, em Sessão Plenária no dia 11-12-2017, deliberou emitir parecer favorável conforme documento em anexo.

Mais se informa que este parecer só se torna exequível após receção e aprovação por esta Comissão dos contratos definitivos, devidamente assinados pelas partes, os quais deverão respeitar na íntegra a versão submetida e aprovada pela CEIC, caso tal ainda não se tenha verificado.

A data do início da realização do Ensaio deverá ser comunicada a esta Comissão ao abrigo das alíneas b) e f), do artigo 36.º da Lei nº 21/2014, de 16 de abril, na sua redação atual.

Mais se declara que os pareceres emitidos por esta Comissão obedecem aos princípios e requisitos consagrados na Declaração de Helsínquia e nas Normas de Boas Práticas Clínicas, tal como previsto na Diretiva 2001/20/CE do Parlamento Europeu e do Conselho de 4 de abril de 2001 e no artigo 37.º do Decreto-Lei n.º 102/2007 de 2 de abril respetivamente.

Em caso de resposta a este documento sugerimos a indicação da nossa referência (MAP / MAP / OF / 2017 / 13981 / 20170666) e a data (11-12-2017) do mesmo.

Com os melhores cumprimentos,

A COMISSÃO DE ÉTICA PARA A INVESTIGAÇÃO CLÍNICA

MRibeiro
MARIA ALEXANDRA RIBEIRO
Vice-Presidente
CEIC

REPÚBLICA PORTUGUESA
SAÚDE

SNS SERVIÇO NACIONAL DE SAÚDE

ceic

APRECIAÇÃO E VOTAÇÃO DO PARECER

Deliberação nº: 2017-RP-17-02

Data da reunião: 11-12-2017

EudraCT: 2015-003910-26

Código CEIC: 20170666

Protocolo: 01/2015

Protocolo/Versão: Protocolo - Código do Protocolo: 01/15, Version: 3.0, 15/11/2017;

Consentimento Informado/Versão: Consentimento Informado - CONSENTIMENTO INFORMADO, LIVRE E ESCLARECIDO - Parte A, Versão 2 - 15 de Novembro de 2017 + FOLHETO INFORMATIVO, Versão 2 - 15 de Novembro de 2017 ;

Brochura do Investigador/versão: Brochura do Investigador - N/A;

A Comissão de Ética para a Investigação Clínica – CEIC, ao abrigo do disposto no Ponto 2, alínea d) do artigo 35º com base nos elementos referidos no nº6 do artigo 16º da Lei 21 / 2014 de 16 de abril, na sua redação atual, em reunião realizada nesta data, apreciou a fundamentação do perito relator sobre o pedido de parecer para a realização de Ensaio Clínico acima referenciado.

O processo foi votado pelos Membros da CEIC presentes:
Presidente Prof. Dr. Alexandre Quintanilha
Vice - Presidente Prof. Dra. Maria Alexandra Ribeiro
Prof. Dr. Alexandre Mendonça, Prof. Dra. Ana Espada de Sousa, Dr. António Lourenço, Prof. Dra. Emília Monteiro, Dra. Fátima Vaz, Prof. Dr. Filipe Almeida, Prof. Dr. Henrique Barros, Dra. Isabel Esperança Rodrigues, Dr. João Branco, Prof. Dr. João Lavinha, Dr. Jorge Penedo, Dra. Manuela Escumalha, Dra. Maria do Rosário Zincke dos Reis Dra. Maria Isabel Santana , Prof. Dra. Maria José Santos, Prof. Dr. Miguel Castelo-Branco Craveiro Sousa , Dr. Nuno Miranda, Prof. Dr. Pedro Manuel Póvoa, Dra. Rosa Fragoso, Dra. Teresa Carneiro

Resultado da Votação:

Parecer favorável à realização do ensaio

A deliberação foi aprovada por maioria com 0 votos contra e 1 abstenções

Data: 11-12-2017

A COMISSÃO DE ÉTICA PARA A INVESTIGAÇÃO CLÍNICA

MARibeiro
MARIA ALEXANDRA RIBEIRO
Vice-Presidente
CEIC

Deliberação nº 2017-RP-17-02 referente ao processo com o nº EudraCT 2015-003910-26 e nº CEIC 20170666

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Comissão de Ética para a Investigação Clínica
National Ethics Committee for Clinical Research

Exmo(a). Sr(a).:
Catarina Santos
Núcleo de Estudos de Hipertensão da Beira Interior
Serviço de Nefrologia, Unidade Local de Saúde de Castelo Branco, E.P.E.
6200-085 - Castelo Branco
Portugal

Vossa ref.: 01/2015

~~Vosso Fax: 272000269~~

Data: 16-11-2018

Nossa ref.: APC / MAP / OF / 2018 / 12128 / 20180888

Assunto: **Ensaio Clínico com o Nº EudraCT 2015-003910-26**
Alteração Substancial com o Código CEIC 20180888

Exmos. Senhores,

Em resposta ao V. pedido de parecer para alteração substancial ao Ensaio Clínico supra mencionado informa-se que o pedido foi considerado válido, pelo que o prazo para a emissão do parecer deverá ser contado nos termos do nº 3 do artigo 18º da Lei n.º 21/2014 de 16 de abril, na sua redação atual.

Em caso de resposta a este documento sugerimos a indicação da nossa referência (APC / MAP / OF / 2018 / 12128 / 20180888) e a data (16-11-2018) do mesmo.

Com os melhores cumprimentos,

O GABINETE DE APOIO DA COMISSÃO DE ÉTICA PARA A INVESTIGAÇÃO CLÍNICA

AnaPaulaCampos

Ana Paula Campos
Gabinete de Apoio da CEIC

COMISSÃO NACIONAL
DE PROTECÇÃO DE DADOS

AUTORIZAÇÃO N.º 5069 /2016

I. Pedido

Núcleo de Estudos de Hipertensão da Beira Interior notificou à Comissão Nacional de Protecção de Dados (CNPD) um tratamento de dados pessoais com a finalidade de elaborar o estudo intitulado "Disfunção imunológica na hipertensão arterial associada a obesidade e síndroma metabólica e efeitos da suplementação com vitamina D".

O estudo em causa tem como objetivo comparar a percentagem de linfócitos (...) no soro dos doentes com hipertensão arterial associada a obesidade e/ou síndroma metabólica antes e após 6 meses de suplementação com colecalciferol.

A população alvo do estudo serão pacientes, com idades compreendidas entre os 45 e os 65 anos, de ambos os sexos, com diagnóstico de hipertensão arterial associada a obesidade e/ou síndroma metabólica.

Os dados serão recolhidos num "caderno de recolha de dados" no qual não há identificação nominal do titular, sendo aposto um código. A chave desta codificação só pode ser conhecida da equipa de investigação.

As categorias de dados pessoais tratados são as seguintes: N.º de participante, idade, raça, peso, altura, perímetro abdominal, pressão arterial. História clínica: averiguar história de diabetes mellitus, hipertensão arterial, obesidade, síndroma metabólica e exclusão de doenças imunológicas, inflamatórias ou infecciosas crónicas. Doseamento de variáveis analíticas: cálcio, fósforo, hemoglobina A1c, colesterol total, colesterol-HDL, triglicéridos, vitamina D, percentagem de linfócitos T no sangue periférico e de macrófagos e linfócitos T no tecido adiposo abdominal. Análise de metabolitos no sangue periférico por análise metabolómica. Análise de dados de bioimpedância torácica.

II. Apreciação

A CNPD já se pronunciou na sua Deliberação n.º 1704/2015 sobre o enquadramento legal, os fundamentos de legitimidade, os princípios orientadores para o correto cumprimento da Lei 67/98, de 26 de outubro, com as alterações da Lei n.º 103/2015, de 24 de agosto (LPDP), bem como as condições gerais aplicáveis ao tratamento de dados pessoais para a finalidade de estudos de investigação na área da saúde.

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No caso em apreço, a notificação enquadra-se no âmbito tipificado pela referida Deliberação.
A informação tratada é recolhida de forma lícita (art.º 5.º, n.º1, alínea a), da LPDP) para finalidades determinadas, explícitas e legítimas (cf. alínea b) do mesmo artigo) e a informação recolhida não é excessiva.
O fundamento de legitimidade é o consentimento expresso e escrito do titular (cf. artigo 7.º, n.º 2, da LPDP).
Os titulares dos dados, previamente ao consentimento (que deve ser informado, livre, expresso e específico), deverão ser informados sobre a natureza, o alcance, as consequências e os riscos do estudo, bem como o direito de se retirar do mesmo, sem quaisquer consequências (cf. alínea j) do artigo 2.º da Lei n.º 21/2014, de 16 de abril).

No que respeita à recolha do dado raça o Promotor apresentou a seguinte justificação: «A hipertensão arterial e a síndroma metabólica são duas entidades clínicas cuja prevalência varia de acordo com a raça do doente.
Em relação à hipertensão arterial sabe-se que apresenta prevalência mais alta em indivíduos de raça negra comparativamente com indivíduos de raça caucasiana. Para além disso, os indivíduos de raça negra apresentam, em regra, hipertensão arterial de mais difícil controlo e também maior número de casos de hipertensão resistente. Do ponto de vista clínico, a indicação para a prescrição de classes de fármacos anti-hipertensores é também ajustada de acordo com a raça, pois indivíduos de raça negra podem beneficiar mais da prescrição de um diurético que os indivíduos de raça caucasiana. Neste sentido, a informação em relação à raça é importante para ajuste e optimização terapêutica nos casos de hipertensão arterial não controlada assim como para estratificação do risco cardiovascular.
A síndroma metabólica é igualmente uma entidade clínica cuja prevalência varia com a raça ou ascendência do doente. Dados publicados em revistas científicas indexadas documentam maior prevalência e incidência de síndroma metabólica em indivíduos de ascendência latino-americana e indiana e na raça negra».
Tendo em conta o teor da justificação a, entende a CNPD que é legítima a recolha e tratamento do dado raça.

III. Decisão

Assim, tendo em atenção o disposto nas disposições combinadas dos artigos 28.º, n.º1, alínea a), e 30.º da LPDP, e as condições e limites fixados na referida Deliberação, que se dão aqui por reproduzidos e que fundamentam esta decisão, autoriza-se o tratamento de dados pessoais nos seguintes termos:

Responsável pelo tratamento: Núcleo de Estudos de Hipertensão da Beira Interior;

Finalidade: estudo intitulado "Disfunção imunológica na hipertensão arterial associada a obesidade e síndroma metabólica e efeitos da suplementação com vitamina D";

Categoria de Dados pessoais tratados: n.º de participante, idade, raça, peso, altura, perímetro abdominal, pressão arterial. História clínica: averiguar história de diabetes mellitus, hipertensão arterial, obesidade, síndroma metabólica e exclusão de doenças imunológicas, inflamatórias ou infecciosas crónicas. Doseamento de variáveis analíticas: cálcio, fósforo, hemoglobina A1c, colesterol total, colesterol-HDL, triglicéridos, vitamina D, percentagem de linfócitos T no sangue periférico e de macrófagos e linfócitos T no tecido adiposo abdominal. Análise de metabolitos no sangue periférico por análise metabolómica. Análise de dados de bioimpedância torácica;

Entidades a quem podem ser comunicados: Não há;

Formas de exercício do direito de acesso e retificação: Junto do médico assistente;

Interconexões de tratamentos: Não há;

Transferência de dados para países terceiros: Não há;

Prazo de conservação: o código do titular deve ser destruído no prazo de 5 anos após o fim do estudo.

Dos termos e condições fixados na Deliberação n.º 1704/2015 e na presente Autorização decorrem obrigações que o responsável deve cumprir. Deve, igualmente, dar conhecimento dessas condições a todos os intervenientes no circuito de informação.

Lisboa, 31 de maio de 2016

Filipa Calvão (Presidente)

Laypersons abstract

Arterial hypertension, a well-known disease for decades, still remains a major cause of cardiovascular morbidity and mortality. The prognosis has changed little in the last years, and new and unexpected risk factors are constantly being discovered, that contribute to the development of this risky disease. One of these new factors is the involvement of the immune system, which is typically regarded as a defence only system. Some of the cells that compose the immune system may contribute to hypertension initiation, as they identify excess lipids or high-salt consumption as stimulus to trigger an aggressive and inflammatory response. Abrogating these immune responses is, therefore, very relevant to attenuate the damage induced by the immune system.

In this regard, we developed a clinical trial to evaluate the effects of vitamin D, a recognized immunonutrient, that is, a nutrient with the capacity to modulate and interfere with the different cells that are activated during the course of hypertension.

For this purpose, we recruited 36 obese hypertensives under chronic anti-hypertensive medication that were divided in two groups, one performing cholecalciferol and the other only the usual blood pressure lowering medication. Patients were followed for a period of 24 weeks and blood and abdominal adipose tissue samples collected before and after cholecalciferol supplementation. The primary aim was the evaluation of peripheral immune cells after treatment with vitamin D, as well as its effects in the immune cells that are also encountered in the adipose tissue. We also intended to evaluate the changes in small lipids, proteins and glucose molecules that circulate in blood.

After the 24-week trial we documented that vitamin D had the ability to interfere with several immune cells in the blood, most notably pro-inflammatory cells, and induced changes that brought the immune cell profile of hypertensives closer to that of healthy people. Immune cells in the adipose tissue also changed, and increased macrophages, a special immune cell type involved in the cleaning of spurious materials, also increased. Meanwhile, several metabolites in blood were also affected by vitamin D treatment, with a final balance favouring decreased concentrations of glucose and improvement in the availability of substrates to cell replication and maintenance. No differences were seen in the rate of blood pressure control.

Overall, a positive, anti-inflammatory, and anti-catabolic effect was observed with vitamin D, a safe and accessible drug. Routine vitamin D prescription in obese hypertensives promoted changes in immune cells and in the metabolites that fuel them that, in the long-term, may impact the overall prognosis.