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VITAMIN D DEFICIENCY AND RESISTANCE STATE

ASSOCIATION WITH CLINICAL AND IMMUNOLOGICAL DEREGULATION IN SYSTEMIC LUPUS ERYTHEMATOSUS

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Tese de Candidatura ao grau de Doutor em Ciências Médicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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PREFACE

The idea of this thesis was born many years ago. The clinical observation that supplementation with Vitamin D, for the prevention of metabolic bone disease, improve Systemic Lupus Erythematosus (SLE, Lupus) patients, has become almost a certainty for the “lupulogists”. This supplementation was done without clinical guidelines and without measuring the levels of Vitamin D. This empirical recognition happened before the explosion of randomised controlled trials and small studies on the effect of this hormone in human disease. Lupus was still a disease with large acknowledgement to achieve, still over treated, regardless of the fact that treating asymptomatic patients with steroids and immunosuppressors was inappropriate. Hydroxychloroquine was used as immunomodulator, but many clinicians ignored or dismissed it. At that time, Vitamin D was undervalued. The observations in the nineteenth and early twentieth centuries of its antimicrobial effects were forgotten with the advent of new antimicrobial molecules.

At the beginning of the XXI century, the world awakens to this hormone and begins an intense investigation that cuts across all areas of medicine. After all, it is a steroid hormone with a nuclear receptor, capable of activating or suppressing a myriad of genes and acting as a natural regulator of thousands of metabolic pathways and cytokines. In Lupus, despite the switch of using Vitamin D supplementation regularly, based in the theoretical concept of being an innocuous immunomodulator, there is still much to know regarding its effects in the disease and to define the best dosage. Additionally, much of the research has been carried out with scarse theoretical reflection and based on limited practical experience. The experience of working in a multidisciplinary unit with more than 3000 autoimmune diseases patients, led us to accumulate empirical observations of Vitamin D benefits. Nevertheless, basic questions about its mechanism of action and the best way to use it, came to prominence.

Thus, this project entitled “Vitamin D deficiency and resistance state - association with clinical and immunological deregulation in Systemic Lupus Erythematosus” was born. Its aim was to create a conceptual framework based on questions that arised over the past years and came from the accumulated experience of its use in hundreds of patients. Its structure was systematized through the creation of a theoretical concept of resistance to Vitamin D, based on clinical observation and supported by hundreds of articles; and trying to prove it in clinical practice. As a secondary aim, we intended to show that the Portuguese population is largely deficient in Vitamin D, as well as patients with SLE, and that Vitamin D is related to disease

susceptibility, severity, damage accrual and disease stabilization throughout their lives. We also intended to demonstrate that standard recommendations for metabolic bone disease are inappropriate to autoimmune diseases. Sustained serum levels and biomarkers of efficacy should individualize Vitamin D supplementation for Lupus patients. For this purpose, we have collected clinical, epidemiological, serological and DNA data from a cohort with more than 600 SLE patients.

We elaborated six hypotheses, answered with *in vivo* data and not *in vitro* studies, and we categorized new concepts tailored to the clinical practice. At the end of this project, more questions remained than at the beginning, but also the notion that many of the questions are innovative and perhaps give new insights on the treatment of SLE patients.

AGRADECIMENTOS

Uma tese é um trabalho académico que resulta de uma profunda reflexão de um tema, de um esforço individual, mas também de um extenso grupo de trabalho multidisciplinar, sem o qual nenhum trabalho de investigação se realiza.

Por isso, os meus sinceros agradecimentos:

Ao Centro Hospitalar do Porto - Hospital de Santo António (CHP-HSA), entidade que me permitiu ser médico, ser ensinado, ensinar e investigar.

Os trabalhos realizados no âmbito desta tese iniciaram-se na Consulta de Imunologia Clínica do CHP-HSA, na qual são seguidos os doentes com Lúpus. Agradeço aos doentes, familiares e dadores de sangue a colaboração nos trabalhos. Agradeço ao Diretor do Hospital, Dr. Sollari Allegro, e ao Diretor Clínico, Dr. Paulo Barbosa, que autorizaram e apoiaram a realização deste trabalho.

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SCIENTIFIC OUTPUTS

According to "Artigo 34º do Decreto-Lei nº 115/2013", results contained in the following published works were used for this thesis:

1. Published articles

Articles published in indexed journals

1. **António Marinho**, Cláudia Carvalho, Daniela Boleixa, Andreia Bettencourt, Bárbara Leal, Judite Guimarães, Esmeralda Neves, José Carlos Oliveira, Isabel Almeida, Fátima Farinha, Paulo P. Costa, Carlos Vasconcelos, Berta M. Silva. Vitamin D supplementation effects on FoxP3 expression in T cells and FoxP3+/IL-17A ratio and clinical course in systemic lupus erythematosus patients: a study in a Portuguese cohort. Immunol Res. 2016 Jul 16. [Epub ahead of print]. doi: 10.1007/s12026-016-8829-3.

2. Carvalho C, **Marinho A*,** Leal B, Bettencourt A, Boleixa D, Almeida I, Farinha F, Costa PP, Vasconcelos C, Silva BM. Association between vitamin D receptor (VDR) gene polymorphisms and systemic lupus erythematosus in Portuguese patients. Lupus. 2015 Jul;24(8):846-53. doi: 10.1177/0961203314566636. (Carvalho C and **Marinho A** contributed equally for this article).

3. Bettencourt A, Carvalho C, Leal B, Brás S, Lopes D, Martins da Silva A, Santos E, Torres T, Almeida I, Farinha F, Barbosa P, **Marinho A**, Selores M, Correia J, Vasconcelos C, Costa PP, da Silva BM. The Protective Role of HLA-DRB1*13 in Autoimmune Diseases. J Immunol Res. 2015; 2015:948723. doi: 10.1155/2015/948723.

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Articles accepted in indexed journals

1. **António Marinho**, Mariana Taveira, Carlos Vasconcelos. Topics on Vitamin D in Systemic Lupus Erythematosus: analysis of evidence and critical literature review. *Accepted in Immunology Research – special issue on SLE*.

Articles submitted in indexed Journals

1. Andreia Bettencourt, Daniela Boleixa, Júlia Reis, José Carlos Oliveira, Denisa Mendonça, Paulo Pinho Costa, Berta Martins da Silva, **António Marinho**, Ana Martins da Silva. Vitamin D levels in a healthy population from the North of Portugal. *Submitted in The Journal of Steroid Biochemistry and Molecular Biology 2016.*

Abstract publications

1. **Marinho, António J.**; Vasconcelos, Carlos; Farinha, Fátima; Rocha, Guilherme; Almeida, Isabel; Correia, João; Mendonça, Teresa; Barbosa, Paulo. Don't forget what natural SLE history is teaching us when establishing clinical-genetic correlations – Poster. 9th International Congress on Systemic Lupus Erythematosus June 24-27, 2010, Vancouver, Canada. Lupus. 2010 Jun;19 Suppl 1:1-185. doi: 10.1177/09612033100190010101.

2. Sá N, Ferreira B, Brandão M, **Marinho A**, Almeida I, Mendonça T, Correia J, Farinha F, Vasconcelos C. In-Hospital mortality review in lupus patients – Poster. 8th European Lupus Meeting. Oporto 2011. Lupus. 2011 Apr;20(4):339-437. doi: 10.1177/0961203311404766.

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   Contribuition: clinical evaluation of Lupus patients and database registry.

Major Works presented in Congress

Oral communications

1. António Marinho. Vitamin D supplementation effects on FoxP3 expression in T cells and FoxP3+/IL-17A ratio and clinical course in systemic lupus erythematosus patients: a study in a Portuguese cohort. XX Autoimmunity meeting. Leipzig 2016.

Posters

1. **Marinho A**, Carvalho C, Boleixa D, Bettencourt A, Leal B, Vasconcelos C, Costa PP, Silva BM. Vitamin D Receptor Polymorphisms and Cumulative Damage in Systemic Lupus Erythematosus Patients – Poster. "XX Reunião Annual do NEDAI 2014".

ABBREVIATIONS

$1,25(OH)_2D3$	Calcitriol
25(OH)D	Cholecalciferol
ACPA	Anti-Citrulinnated Protein Antibodies
AIDs	Autoimmune Diseases
ANA	Anti-Nuclear Antibodies
anti-La	Anti-La (SSb) Antibody
anti-RNP	Anti-Ribonucleoprotein Antibody
anti-Ro	Anti-Ro (SSa) Antibody
anti-Sm	Anti-Smith Antibody
APC	Antigen Presenting Cells
aPl	Anti-Phospholipid Antibodies
BAFF	B-cell Activating Factor
BCR	B-Cell Receptor
BILAG	British Isles Lupus Assessment Group
BMD	Bone Mineral Density
BMI	Body Mass Index
C	Complement
CBC	Complete Blood Count
CD	Clusters of Differentiation
CHP-HSA	Centro Hospitalar do Porto-Hospital de Santo António
CLASI	Cutaneous Lupus Erythematosus Disease Area ans Severity Index
CNS	Central Nervous System
CpG	5'-C-phosphate-G-3'
CRB	Complete Red Blood Cell Count
CRF	Chronic Renal Failure
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
CVD	Cardiovascular Disease
CYP2R1	25-hydroxylase
CYP27B1	1α-hydroxylase
CYP24A1	24-hydroxylase
DCs	Dendritic Cells
DERAA	HLA-DR Rheumatoid Arthritis Protective Epitope
DNA	Deoxyribonucleic Acid
dsDNA	Anti-double-strand DNA
ECLAM	European Consensus Lupus Activity Measurements
EULAR	European League Against Rheumatism
GFR	Glomerular Filtration Rate
ESR	Erythrocyte Sedimentation Rate
FcγR	Complement Factor Gamma Receptor Family
FoxP3	Forkhead FoxP3
GM-CSF	Granulocyte Macrophage–Colony Stimulating Factor
HAP	Haplotype
HLA	Human Leukocyte Antigen
hsCRP	high-sensitivity C-Reactive Protein
ICBAS-UP	Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto
ICs	Immunocomplexes
Ig	Immunoglobulin
IL	Interleukin
IL2Rβ	interleukin-2 Receptor subunit Beta
IL2RG	Interleukin-2 Receptor subunit Gamma
IFN	Interferon
IP-10	Interferon-Gamma Induced Protein 10
IRF	Interferon Regulatory Factor
IRF5-TNPO3	Interferon Regulatory Factor Transportin 3
ITGAM	Integrin Subunit Alpha M

ITP	Immune Thrombocytopenia
JmjC	Jumonji C
kDa	kiloDalton
LPS	Lipopolysaccharide
MDDCs	Monocyte Derived Dendritic Cells
mDCs	Myeloid Dendritic Cells
MG	Myasthenia Gravis
MHC	Major Histocompatibility Complex
miR	micro-RNA
mRNA	messenger RNA
MS	Multiple Sclerosis
NYHA	New York Heart Association
OAS-2	Oligoadenylate Synthetase 2
OR	Odds Ratio
PAP	Pap smear
PBMCs	Peripheral Blood Mononuclear Cells
pDCs	Peripheral Dendritic Cells
PCR	Polymerase Chain Reaction
PTH	Parathyroid Hormone
RA	Rheumatoid Arthritis
RCT	Randomized Controlled Trial
RNA	Ribonucleic Acid
RNPs	Ribonucleoproteins
RXR	Retinoid X Receptor
SELENA-SLEDAI	Safety of Estrogens in Lupus National Assessment–Systemic Lupus Erythematosus Disease Activity Index
SF-36	Short-Form 36
SLAM	Systemic Lupus Activity Measure
SLAMs	Signalling Lymphocyte Activation Molecules
SLE	Systemic Lupus Erythematosus
SLEDAI-2K	Systemic Lupus Erythematosus Disease Activity Index 2000
SLICC-ACR	Systemic Lupus International Collaborating Clinics Damage Index
SNPs	Single Nucleotide Polymorphisms
snoRNA	nucleolar RNA
SPH-2	Tyrosine Phosphatase 2
SSc	Systemic Sclerosis
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of Interferon Genes
Sγc	Soluble Form of Gamma Chain
SYk	Spleen Tyrosine Kinase
T2T	Treat to Target
TCR	T-Cell Receptor
TGFβ	Transforming Growth Factor Beta
Th	T-helper
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
Tregs	Regulatory T Cells
UIC	Unidade de Imunologia Clínica
UV	Ultraviolet
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element
XL9HAP3	XL9 Polymorphism Haplotype 3

ABSTRACT

The clinical practice in “autoimmunology”, challenged us to question the relation of Vitamin D and SLE disease. The aim of this thesis was to answer some of those questions, according to the following hypotheses:

Hypothesis 1: Vitamin D levels in our SLE patients are lower than in a healthy population with the same demographic characteristics (age and sex).
Hypothesis 2: Vitamin D status is related to SLE susceptibility.
Hypothesis 3: SLE severity is related to VDR Single Nucleotide Polymorphisms (SNPs).
Hypothesis 4: Vitamin D deficiency is associated with SLE severity.
Hypothesis 5: Supplementation with Vitamin D alters the clinical expression and immunological deregulation of SLE patients (T-CD4$^+$ cells expressing Forkhead FoxP3 (FoxP3) and T-CD4$^+$ cells producing interleukin (IL)-17A).
Hypothesis 6: Clinical and Immunological response to Vitamin D supplementation is limited by SLE disease activity.

To achieve those answers, the following studies were developed:

– Two epidemiologic studies in Portuguese SLE patients (n=124) and in healthy controls (n=198), in order to evaluate Vitamin D levels in both populations.

– An immunogenetic study with more than 1000 patients in order to prove the effect of different alleles of Major Histocompatibility Complex (MHC) class II to AIDs susceptibility.

– Two immunogenetic studies with 170 SLE patients (DNA databank):

- To correlate VDR polymorphisms with different disease aspects (activity, flares and damage accrual).
- To correlate baseline Vitamin D levels and mean levels (in a 10-year follow-up) with different disease aspects (activity, flares and damage accrual).

– Two interventional studies with Vitamin D supplementation were performed:

- To study the effect of Vitamin D supplementation (despite baseline levels) in 24 stable SLE patients, regarding disease severity, T-CD4+ cells expressing FoxP3 and T-CD4+ cells producing IL-17A.

- To study the effect of Vitamin D supplementation (despite baseline levels) in 4 severely active SLE patients, regarding Tregs, T-CD4+ cells producing IL-17A and miR-146a expression.

Concerning Vitamin D levels, in the winter period, 74.2% of the healthy studied population had a 25(OH)D concentration below 50.0 nmol/L compared with 22.8% in the summer period ($p<0.0001$). In the SLE group, 85% of the patients have Vitamin D deficiency. The mean Vitamin D levels were 48.9±27.2 and there were no significant differences between summer and winter ($p=0.117$). Comparing with the control group, a significant difference was only observed during the summer time ($p=0.0003$).

We documented that Human Leukocyte Allele (HLA)-DRB1*13 act as protective allele for four autoimmune diseases (AIDs): SLE (OR=0.58, $p=0.016$), Psoriasis and Psoriatic Arthritis (OR=0.621, $p=0.050$), Rheumatoid Arthritis (RA) (OR=0.58, $p=0.044$) and Systemic Sclerosis (SSc) (OR=0.42, $p=0.035$). HLA-DRB1*03 was found to be a risk factor for SLE (OR=2.49, p=4.2x10-5).

SLE damage, according to Systemic Lupus International Collaborating Clinics Damage Index (SLICC) values, was higher in SLE patients with FokI CT and TaqI TT VDR genotypes ($p=0.031$ and $p=0.046$ respectively).

We observed a higher number of flares in patients with low baseline Vitamin D levels ($p=0.045$). We also observed that patients with three or more flares had significant lower baseline Vitamin D levels ($p=0.004$).

The mean Vitamin D levels in the previous 10 years of disease in 68 patients were lower in patients with more severe flares, although not statistacally significant ($p=0.178$). However, if we consider two subgroups (patients with three or more and less than three severe flares), the difference is significant ($p=0.044$).

The FoxP3+/IL-17A ratio in patients with stable SLE after 6 months of Vitamin D supplementation was higher than that in the baseline ($p<0.001$), even in patients with normal 25(OH)D levels at baseline (≥75 nmol/L) ($p=0.043$).

Considering IL-17A T $CD4^+$ producing cells, Tregs and Tregs/IL-17A ratio before and after high dose Vitamin D supplementation, in patients with severely active disease, no enhancement of Tregs or Tregs/IL-17A was observed.

Finally, no significant differences were found in miR-146a expression, when comparing controls and SLE active patients, before and after Vitamin D supplementation.

In conclusion, Vitamin D levels are insufficient in at least two thirds of Portuguese healthy adult population and 85% of SLE patients. However, in these patients, mean levels are lower than in general population, only during the summer time. These results

allow us to conclude that hypovitaminosis D in SLE population may differ, from that observed in healthy population. This is probably due to deficient sun exposure during summer time, and not to a disease effect on Vitamin D metabolism.

We confirmed and described original genetic data in our SLE Portuguese population, that can allow new studies, which will be able to determine a Vitamin D effect in SLE susceptibility: HLA-DRB1*03 risk and the HLA-DRB1*13 protective effects in disease susceptibility. We should study the possibility of this effect to be mediated by presence of the canonical Vitamin D Response Element (VDRE) in HLA-DRB1 genes.

Specific VDR polymorphisms are related to a higher long-term cumulative damage in SLE patients. These effects seem to be unrelated to vitamin D levels. Lower baseline Vitamin D levels at SLE diagnosis (before supplementation) and mean Vitamin D levels in a 10-year follow-up, correlate negatively with disease severity (resulting in more aggressive SLE phenotypes).

Vitamin D supplementation seems to provide favourable immunological effects in patients with stable SLE; this effect is probably independent of the 25(OH)D patients' status, but not independent from SLE activity.

Finnaly, our results suggest that in Lupus patients, severe active disease may cause resistance to Vitamin D therapeutical effects.

RESUMO

A prática clínica em "autoimunologia", desafiou-nos a questionar a relação da Vitamina D com o Lúpus. O objetivo desta tese foi o de responder a algumas dessas perguntas, de acordo com as seguintes hipóteses:

Hipótese 1: Os níveis de Vitamina D nos nossos doentes com Lúpus são mais baixos do que em uma população saudável com as mesmas características demográficas (sexo e idade).
Hipótese 2: o nível de Vitamina D está relacionado com a susceptibilidade ao Lúpus.
Hipótese 3: A gravidade do Lúpus está relacionada com os polimorfismos do VDR.
Hipótese 4: A deficiência de Vitamina D está associada com a gravidade do Lúpus.
Hipótese 5: A suplementação com Vitamina D altera a expressão clínica e desregulação imunológica dos doentes com Lúpus (células T-CD4 + expressando FoxP3 e T-CD4+produtoras de IL -17).
Hipótese 6: A resposta clínica e imunológica à suplementação de Vitamina D é limitada pela atividade da doença lúpica.

Para conseguir essas respostas, os seguintes estudos foram desenvolvidos:

– Dois estudos epidemiológicos em doentes com Lúpus (n=124) e controlos saudáveis (n=198), de modo a avaliar os níveis de Vitamina D em ambas as populações.

– Um estudo imunogenético com mais de 1000 doentes, a fim de provar o efeito de diferentes alelos do Complexo Principal de Histocompatibilidade (MHC) da classe II para a susceptibilidade às doenças autoimunes.

– Dois estudos de imunogenética com 170 doentes com Lúpus (Banco de DNA).

- Para correlacionar os polimorfismos do VDR, com os diferentes aspectos da doença (actividade,flares e dano).
- Para correlacionar os níveis basais de Vitamina D e os seus níveis médios (em 10 anos de follow-up), com os diferentes aspectos da doença (actividade,flares e dano).

– Dois estudos intervencionais com suplementação de Vitamina D:

- Para estudar o efeito da suplementação de Vitamina D (independentemente dos níveis basais) em 24 doentes com Lúpus estável, em relação à gravidade da doença, células T-CD4 + expressando FoxP3 e T-CD4 + produtoras de IL-17A.
- Para estudar o efeito da suplementação de Vitamina D (independentemente dos níveis basais) em 4 doentes com Lúpus grave ativo, sobre as células T reguladoras, T-CD4 + produtoras de IL-17A e a expressão do miR-146a.

Relativamente aos níveis de Vitamina D, no período de inverno, 74,2% da população saudável estudada tinha uma concentração de 25(OH)D abaixo de 50,0 nmol/L, em comparação com 22,8% no período de verão (p <0,0001). Em doentes com Lúpus, 85% dos doentes tinham deficiência de Vitamina D. Os níveis médios de Vitamina D nos doentes com lúpus, foram 48,9 ± 27,2 e não houve diferenças significativas entre verão e inverno (p=0,117). Comparando com o grupo controle, uma diferença significativa foi observada somente durante o horário de verão (p=0,0003).

Foi documentado que o HLA-DRB1*13 era um alelo de protecção para quatro doenças autoimunes: Lúpus (OR=0,58, p=0,016), Psoríase e Artrite Psoriática (OR=0,621, p=0,050), Artrite Reumatóide (OR=0,58, p=0,044) e Esclerose Sistémica (OR=0,42, p=0,035). O HLA-DRB1*03 é um factor de risco para o desenvolvimento de Lúpus (OR=2,49, p=4.2x10-5).

De acordo com os valores do SLICC, o dano em doentes com lúpus foi maior nos que apresentavam os genótipos FokI CT e TaqI TT do VDR (p=0,031 e p=0,046, respectivamente).

Observou-se um maior número de flares em doentes com níveis basais de Vitamina D baixos (p = 0,045). Também foi observado que doentes com três ou mais flares tinham níveis de Vitamina D significativamente mais baixos do que os restantes (p=0,004).

Os níveis médios de Vitamina D nos últimos 10 anos de doença em 68 doentes foram menores em doentes com mais flares graves, embora não estatísticamente significativo (p=0,178). No entanto, se considerarmos dois subgrupos (doentes com três ou mais e menos do que três flares graves), a diferença é significativa (p=0,044).

A proporção FoxP3^{+}/IL-17A em doentes com Lúpus estável após 6 meses de suplementação com Vitamina D foi maior do que na linha de base (p<0,001), mesmo em doentes com níveis normais de 25(OH)D no início do estudo (≥75nmol/l) (p=0,043).

Em relação às células T produtoras de IL-17A, células T reguladoras e razão de Tregs/IL-17A, antes e após a suplementação de altas doses de Vitamina D, em doentes com doença severa ativa, nenhum aumento de Tregs e do ratio Tregs/IL-17A foi observado. Finalmente, não foram encontradas diferenças significativas na expressão de miR-146a, quando se comparam os controlos e doentes ativos com Lúpus, antes e após a suplementação de Vitamina D.

Em conclusão, os níveis de Vitamina D são insuficientes em, pelo menos, dois terços da população adulta saudável Portuguesa e 85% dos doentes com Lúpus. No entanto, nestes doentes, os níveis médios são inferiores à população em geral, apenas durante o tempo de verão. Estes resultados permitem concluir que a hipovitaminose D na população com lúpus pode ser diferente da observada na população saudável. Isto deve-se, provavelmente, à deficiente exposição solar durante o verão, e não devido a um efeito da doença sobre o metabolismo da Vitamina D.

Nós confirmamos e descrevemos dados genéticos originais na nossa população Portuguesa com Lúpus, que podem permitir novos estudos, capazes de determinar um efeito da Vitamina D na susceptibilidade ao Lúpus: efeito de risco do HLA-DRB1 * 03 e protector do HLA-DRB1 * 13 na susceptibilidade à doença. Devemos estudar a possibilidade de este efeito ser mediada pela presença de um “Vitamin D Responsive Element” (VDRE) funcional no gene HLA-DRB1.

Polimorfismos específicos do VDR estão relacionados a um maior dano cumulativo a longo prazo em doentes com Lúpus. Estes efeitos parecem não estar relacionados com níveis de Vitamina D. Os polimorfismos do VDR não se correlacionam com os flares e com envolvimento de órgãos específicos.

Níveis mais baixos de Vitamina D de base na data do diagnóstico de Lúpus (antes da suplementação) e os níveis médios de Vitamina D num follow-up de 10 anos, correlacionam-se negativamente com a severidade da doença (resultando em fenótipos lúpicos mais agressivos).

A suplementação de Vitamina D parece proporcionar efeitos imunológicos favoráveis em doentes com lúpus estável; este efeito é provavelmente independente dos níveis de 25(OH)D, mas não independentes da atividade do LES. Finalmente, os nossos resultados sugerem que em doentes com lúpus, a doença activa grave pode causar resistência aos efeitos terapêuticos da Vitamina D.

CHAPTER 1

INTRODUCTION

Vitamin D resistant state in SLE – the concept and state of the art

1.1. Systemic Lupus Erythematosus

This chapter introduces basic concepts on SLE and presents the evidence of the non-classical effects of Vitamin D on lupus.

Systemic lupus erythematosus is a systemic autoimmune disease with multi-organ inflammation (Carvalho et al., 2015a; Lauwerys et al., 2013), linked to loss of immune tolerance to self-antigens and the production of a diversity of autoantibodies. It mainly affects women of childbearing age. This disease has a female to male ratio in adults of approximately 9:1, a peak age to diagnosis between 15 and 44 years, and a negative impact on the quality of life, including reduced levels of employment and income. Despite phenotypic heterogeneity and the unpredictable disease evolution, a strong genetic and environmental contribution to the development of SLE is supported by broad evidence, including the reproducibility of the clinical picture in all continents (Morais & Isenberg, 2016). SLE is characterized by the presence of autoantibodies, primarily directed against chromatin and ribonucleic particle constituents (nucleosomes, single- and double-stranded deoxyribonucleic acid (DNA)) (Jiang, 2011; Lahita, Tsokos, Buyon, & Koike, 2011; Lauwerys, Ducreux, & Houssiau, 2014; Lauwerys et al., 2013), that may play a pathogenic role (Lauwerys et al., 2014). The hyperreactivity to these self-antigens leads to the formation of immune complexes that cause local inflammation and tissue damage (Mandal et al., 2014).

B cell regulation is important in the maintenance of immune balance due to the production of autoantibodies and their prolonged cell life. B cells from patients with SLE have been shown to present autoantigens, induce CD4+ T helper cells (Th1/Th2), inhibit Tregs, and secrete pro-inflammatory cytokines (Guerra, Vyse, & Cunninghame Graham, 2012).

Thus, SLE is a complex disease, involving diverse immune system mechanisms with multiorganic involvement and failure.

1.2. SLE – Classification, diagnosis, monitoring and treatment

1.2.1. SLE Classification and diagnosis

Considering the heterogeneity in clinical manifestations and multisystem involvement, SLE diagnosis as well as the classification criteria have been subject of debate over time. From a conceptual point of view, classification and diagnosis are not synonymous. Classification criteria apply to patients diagnosed with Lupus in order to recognize patterns that allow the comparison of the disease among centres anywhere in the world. Usually they correspond to signs, symptoms and laboratory data of high sensitivity and specificity. As such, some patients with these key data are used as a comparison reference for multicenter studies.

This means that experience is necessary in the diagnosis of SLE and is incorrect to support this conviction on classification criteria. For that purpose some examples are given.

Example 1: 19 years old white man, fever with 1 week of evolution, malar rash during febrile periods, arthralgia, cervical lymphadenopathy, myalgia, pharyngeal exudate and positive antinuclear antibodies (ANAs) 1/160 speckled pattern; absence of other autoantibodies.

In this case, the fact of being male, having an acute disease, rash during fever periods and having an autoantibody that has low specificity for SLE, points, most likely, to a viral disease. If we consider the 1997 and 2012 revised classification criteria, we could classify the patient as having SLE, with both of them.

Example 2: female, 18 years old, appears with petechiae, has 10,000 platelet/µl, and no other clinical finding. It has positive ANAs 1/1280 mottled pattern. There is no complement consumption and no other autoantibodies.

In this example, the patient has no classification criteria, but, with the analytical finding, age, sex, and high titers of ANAs (though not specific), many clinicians would give her the diagnosis of SLE.

Therefore, to a correct diagnosis of SLE, a set of epidemiological, clinical and laboratory findings that allow the patient to complete this syndrome is necessary. Table 1 shows the sensitivity and specificity of autoantibodies for the diagnosis of SLE.

Table 1 - Sensitivity and specificity of autontibodies to SLE diagnosis (adapted from (Aringer, Dorner, Leuchten, & Johnson, 2016)).

Autoantibody test	*Sensitivity estimate (based on refs 2–6, 23)*	*Specificity for SLE?*	*Other diseases*
ANA (HEp-2 IFLU)	98	No	Many
Anti-dsDNA	50	Yes (95%)	–
Anti-Histone	50	No	DIL, SSc, JIA
Anti-C1q	30	No	IC vasculitides[a]
Anti-Sm	10	Yes (99%)	–
Anti-Ro60	40	No	Sjögren's, CLE[a], SSc
Anti-SSB/La	20	No	Sjögren's
Anti-U1RNP	20	No	MCTD
Rheumatoid factors	20	No	RA, Sjögren's
Anti-Cardiolipin IgG	20	No[a]	Primary APS[a]
Anti-Cardiolipin IgM	10	No[a]	Primary APS[a]
Lupus anticoagulant	10	No[a]	Primary APS[a]

[a]The differential diagnoses can also be part of the SLE spectrum.
IFLU: immunofluorescence; DIL: drug-induced lupus; SSc: systemic sclerosis; JIA: juvenile idiopathic arthritis; IC: immune complex; CLE: cutaneous lupus erythematosus; MCTD: mixed connective tissue disease; RA: rheumatoid arthritis; APS: anti-phospholipid (Hughes') syndrome.

Therefore, in the present context, SLE remains a difficult clinical diagnosis based on experience and not in classification models.

Table 2 presents the fundamental data that distinguish classification criteria from diagnosis criteria.

Table 2 – Differences between diagnosis and classification criteria (adapted from (Aringer et al., 2016)).

	Diagnosis	*Classification*
Aim	Individual prognosis and therapy.	Homogenous groups for research purposes.
Information base	Theoretically unlimited information of various natures and sources.	Feasible, and therefore limited set of objective criteria only.
Sensitivity	Critical issue (will often limit access to therapy).	Low sensitivity narrows patient population, but is usually not critical.
Specificity	Diagnosis will be questioned if new information sheds doubt.	Critical issue (starting point classification cannot be corrected).

Finally, some considerations on SLE classification. SLE is a disease involving any organ or system and having different manifestations among patients, and even in the same patient along the disease. It was necessary to create a common language allowing the comparison between patients and disease patterns. This pressure increased because a large number of available drugs were failing their targets in clinical trials, and, this effect is due to the small number of patients involved in these trials and to the impossibility of comparing patients in real life. In 1982, the American College of Rheumatology criteria were published (Tan et al., 1982) and revaluated in 1997 (Hochberg, 1997). Tables 3 and 4 present the 1982 SLE classification criteria.

Table 3 - The 1982 revised criteria for classification of SLE (adapted from (Tan et al., 1982).

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
5. Arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Serositis	a) Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion *OR* b) Pericarditis--documented by ECG or rub or evidence of pericardial effusion
7. Renal disorder	a) Persistent proteinuria greater than 0.5 grams per day or grater than 3+ if quantitation not performed *OR* b) Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed
8. Neurologic disorder	a) Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance *OR* b) Psychosis--in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic disorder	a) Hemolytic anemia--with reticulocytosis *OR* b) Leukopenia--less than 4,000/mm<>3<> total on 2 or more occasions *OR* c) Lyphopenia--less than 1,500/mm<>3<> on 2 or more occasions *OR* d) Thrombocytopenia--less than 100,000/mm<>3<> in the absence of offending drugs

Table 4 - The 1982 revised criteria for classification of SLE (continuation of table 3) (adapted from (Tan et al., 1982).

Criterion	Definition
10. Immunologic disorder	a) Positive LE cell preparation *OR* b) Anti-DNA: antibody to native DNA in abnormal titer *OR* c) Anti-Sm: presence of antibody to Sm nuclear antigen *OR* d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test
11. Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome

* The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have systemic lupus erythematosus if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

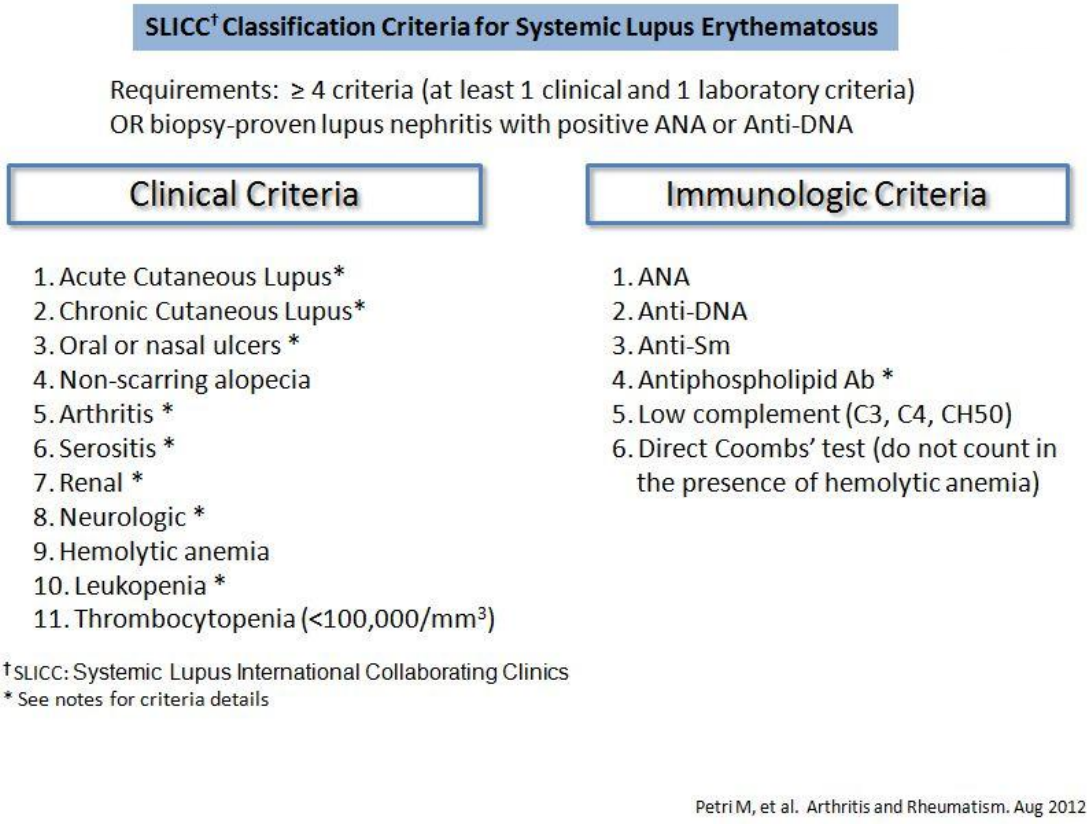
Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271---7. and Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus [letter]. Arthritis Rheum 1997;40:1725.

In these criteria, and for the first time, each clinical syndrome had its characteristics defined, increasing the ability to distinguish self-limiting findings and / or common findings with specific characteristics. As examples: the painless aphthosis and the non-erosive arthritis. However, the analytical characteristics are sparse, in particular, the lack of specific autoantibodies. On the other hand, all disease features are placed in the same "bag".

The clinical and analytical findings are not associated and it would make sense to create a group of well-defined analytical and clinical criteria, separated as clinical and analytical features. As an example, the use of complement factors, long used in clinical practice as SLE characteristic, is not a criteria feature in the 1982 and 1997 classification.

In 2012, following the needs identified above, Petri et al. (Petri et al., 2012) published a review of criteria that separate the clinical from the laboratory components of the disease (Table 5).

Table 5 - Classification criteria for SLE by Petri et al., 2012.



SLICC† Classification Criteria for Systemic Lupus Erythematosus

Requirements: ≥ 4 criteria (at least 1 clinical and 1 laboratory criteria)
OR biopsy-proven lupus nephritis with positive ANA or Anti-DNA

Clinical Criteria	Immunologic Criteria
1. Acute Cutaneous Lupus*	1. ANA
2. Chronic Cutaneous Lupus*	2. Anti-DNA
3. Oral or nasal ulcers *	3. Anti-Sm
4. Non-scarring alopecia	4. Antiphospholipid Ab *
5. Arthritis *	5. Low complement (C3, C4, CH50)
6. Serositis *	6. Direct Coombs' test (do not count in the presence of hemolytic anemia)
7. Renal *	
8. Neurologic *	
9. Hemolytic anemia	
10. Leukopenia *	
11. Thrombocytopenia (<100,000/mm^3)	

†SLICC: Systemic Lupus International Collaborating Clinics
* See notes for criteria details

Petri M, et al. Arthritis and Rheumatism. Aug 2012

Since then, many studies have compared the old criteria with the current ones. A study of the Iberian Peninsula evaluated 2055 patients (Ines et al., 2015) and concluded that the new criteria can classify patients early in the disease course. This means a trend towards diagnostic criteria. This is clearly an evolution and new criteria revisions are on the horizon in the next two years.

1.2.2. Lupus Monitoring

SLE monitoring is complex, as the disease is heterogeneous. Monitoring a chronic systemic disease comprises many factors: disease activity, disease damage, quality of life, drug monitoring and side effects.

European League Against Rheumatism (EULAR) published, in 2010, 10 general recommendations summarized in the following chapter.

1.2.2.1. Definitions (adapted from Mosca et al., 2010)

Monitoring involves observing and recording the evolution of the disease and anticipating outcomes based on knowledge of the disease. Monitoring is different from diagnosis, but may overlap with screening. Monitoring includes the observation and anticipation of complications of the disease, of comorbidity, and of side effects of the medication.

Active SLE refers to the presence of symptoms, signs, or abnormal investigations related to active inflammation and that indicate organ involvement that is likely to be reversible with therapy.

Definition of remission is the absence of relevant symptoms and signs of inflammatory activity in any organ or system. Remission implies the absence of immunosuppressive therapy, with the exception of hydroxychloroquine. Clinical remission on therapy defines “complete response”. Autoantibodies may be present, even when patients achieve clinical remission.

1.2.2.2. Minimal requirements for assessment of organ involvement in SLE

Table 6 - Minimal requirements for assessment of organ involvment in SLE (adapted from (Mosca et al., 2010).

Laboratory assessment	Erythrocyte Sedimentation Rate (ESR), Complete Red Blood Cell Count (CRP), Complete Blood Count (CBC), serum albumin, serum creatinine or Glomerular Filtration Rate (GFR), urinalysis, protein/creatinine ratio (or 24 h proteinuria), Complement (C) 3, C4
Autoantibody assessment	At baseline: ANA, Anti-double-strand DNA (anti-dsDNA), Anti-Ro (SSa) Antibody (anti-Ro), Anti-La (SSb) Antibody (anti-La), Anti-Ribonucleoprotein Antibody(anti-RNP), Anti-Smith Antibody (anti-Sm), Anti-Phospholipid Antibodies (aPL). Re-evaluation in previously negative patients: - Anti-phospholipid antibodies: prior to pregnancy, surgery, transplant, and oestrogen containing treatments, or in the presence of new neurologic or vascular event. - Anti-Ro and anti-La antibodies before pregnancy.
Joint involvement	Ask for the presence of arthralgias, assess joints for arthritis, if present perform a joint count
Mucocutaneous involvement	Mucocutaneous lesions should be characterized according to the existing classification systems (lupus specific, lupus non-specific, lupus mimickers or drug-related)
Kidney involvement	Protein/creatinine ratio (or 24 h proteinuria), urine microscopy, immunological tests (C3, C4, anti-dsDNA), blood pressure
CNS involvement	Focused history for neuropsychiatric symptoms (seizures, paraesthesia, numbness, weakness, headache, epilepsy, depression, etc.)
Pulmonary involvement	History: pleuritic chest pain, dyspnea New York Heart Association (NYHA), cough. Examination: pulmonary crackles/rales, pleural effusion
Heart involvement	History: Chest pain, dyspnea (NYHA), atherosclerosis risk factors Examination: peripheral oedema, arterial blood pressure, heart and carotid murmurs, heart rate
Eye assessment	Examination by an ophthalmologist or an optician
Vascular involvement	Inquire about Raynaud's, thrombotic risk factors, and intermittent claudication
Gastrointestinal tract	Ask about symptoms

1.2.2.3. Recommendations (adapted from Mosca et al., 2010)

Recommendation #1: Patient assessment

In addition to the standard of care of non-lupus patients of the same age and sex, the assessment of an SLE patient must include the evaluation of disease activity by a validated index at each visit and organ damage annually. Registry of general quality of life his done with patient history and/or by a 0 to 10 Visual Analogue Scale at each visit, comorbidities and drug toxicity.

In recent years, indices have been developed and validated to measure these parameters. The most frequently used activity indices are the British Isles Lupus Assessment Group (BILAG), the European Consensus Lupus Activity Measurements (ECLAM), the Systemic Lupus Activity Measure (SLAM), and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and revised versions as SLEDAI-2K and Safety of Estrogens in Lupus National Assessment–Systemic Lupus Erythematosus Disease Activity Index (SELENA-SLEDAI). The SLICC Damage Index was developed to assess irreversible damage in SLE patients occurring after disease onset. Recently, SLE-specific indices to assess patient's quality of life have been developed and validated as well, although the Short Form (SF-36) is the most widely used to assess quality of life.

Recommendation # 2: Cardiovascular risk factors

At baseline and during follow up at least once a year*:

- Assess smoking, vascular events (cerebral/cardiovascular), physical activity, oral contraceptives, hormonal therapies, and family history of cardiovascular disease.
- Blood tests: blood cholesterol and glucose.
- Examination: Blood pressure, BMI (and/or waist circumference).

*Some patients may need more frequent follow-up (i.e. patients on glucocorticoids).

Recommendation # 3: Other comorbidities

1. **Osteoporosis**

All SLE patients

- Should be assessed for adequate calcium and Vitamin D intake, regular exercise, and smoking habit,
- Should be screened and followed for osteoporosis according to existing guidelines
    - For postmenopausal women
    - For patients on glucocorticoids, or on any other medication that may reduce Bone Mineral Density (BMD)

2. **Cancer.** Cancer screening recommended according to the guidelines for the general population, including Papaniculau smears (PAP).

SLE patients' screening, according to the existing guidelines for postmenopausal women and/or for patients treated with medications that may affect bone mass.

Table 7 - Drugs that may affect bone mass.

Drugs that may affect bone mass
Glucocorticoids
Immunosuppressive drugs
- Cyclosporine
- Tacrolimus
- Methotrexate
Heparin
Thyroxine
Aromatase inhibitors
Long-term anticonvulsants (phenytoin, phenobarbital, carbamazepine, piramidone)
Gonadotropin releasing hormone antagonists
Proton pump inhibitors (omeprazole)
Loop diuretics
Antidepressants (particularly selective serotonin reuptake inhibitors)

Recommendation #4: Infection risk

 # 4.1 Screening

We recommend that patients to be screened for:

- Human Immunodeficiency virus based on patient's risk factors,
- Hepatitis C virus, hepatitis B virus based on patient's risk factors, particularly before immunosuppressive drugs including high dose glucocorticoids are started,
- Tuberculosis, according to local guidelines, especially before IS drugs including high dose glucocorticoids are started,
- Cytomegalovirus testing considered during treatment in selected patients.

 # 4.2 Vaccination

SLE patients are at high risk of infections and prevention his recommended. The administration of inactivated vaccines (especially influenza and pneumococcus), following the Centres for Disease Control guidelines for immunosuppressed patients, should be strongly considered in SLE patients on Immunosuppressive drugs, preferably administered when the SLE is inactive. For other vaccinations, an individual risk/benefit analysis his recommended.

 # 4.3 Monitoring

At follow up visits, continuous assessment of the risk of infection by taking into consideration the presence:

- Severe neutropenia (<500/mmc),
- Severe lymphopenia (<500/mmc),
- Low Immunoglobulin (Ig) G (<500mg/dL).

Recommendation #5: Frequency of assessments

Patients with no activity, no damage, and no comorbidity, are recommending assessments every 6 to 12 months. Preventive measures during this visits, are important to implement.

Recommendation #6: Laboratory assessment

#6.1 Laboratory assessments

We recommend to monitor the following autoantibodies and complement:

- At baseline: ANA, anti-dsDNA, anti-Ro, anti-La, anti-RNP, anti-Sm, anti-phospholipid, C3, C4.
- Re-evaluation in previously negative patients:
    - Anti-phospholipid antibodies: prior to pregnancy, surgery, transplant, and oestrogen containing treatments, or in the presence of a new neurologic or vascular event.
    - Anti-Ro and anti-La antibodies before pregnancy.
    - Anti-dsDNA/ low C3 or C4 may support evidence of disease activity / remission.

#6.2 other laboratory assessments

At 6-12 month intervals, patients with inactive disease should have:

- CBC
- ESR
- CRP
- Serum albumin
- Serum creatinine (or GFR)
- Urinalysis and urine protein/creatinine ratio

If a patient is on a specific drug treatment, monitoring for that drug is required as well.

Recommendation #7: Mucocutaneous involvement

Characterization of mucocutaneous lesions, according to the existing classification systems, as to whether they may be:

- Lupus erythematosus - specific.
- LE-nonspecific.
- LE mimickers.
- Drug-related.

Assessement of lesions, for activity and damage, with validated indices (i.e. Cutaneous Lupus Erythematosus Disease Area and Severity Index (CLASI)).

Recommendation #8: Kidney

- Patients with a persistently abnormal urinalysis or raised serum creatinine should have urine protein/creatinine ratio (or 24 h proteinuria), urine microscopy, renal ultrasound and be considered for referral for biopsy.

- Patients with established nephropathy should have protein/creatinine ratio (or 24 h proteinuria) and immunological tests (C3, C4, anti-dsDNA), urinary sediment microscopy and blood pressure at least every 3 months for the first 2-3 years.
- Patients with established chronic renal disease (GFR < 60 ml or stable proteinuria > 0.5 mg/24 hrs.) should be followed according to the National Kidney Foundation guidelines for chronic kidney disease.

Recommendation #9: Neuropsychiatric manifestations

- Monitoring of SLE patients for the presence of neuropsychological symptoms (seizures, paraesthesia, numbness, weakness, headache, epilepsy, depression, etc.) by focused history.
- Evaluating attention, concentration, word finding and memory difficulties may be used to assess cognitive impairment (i.e. by asking the patient about problems with multitasking, with household tasks, or memory). If there is a suspicion of any cognitive impairment, the patient should be assessed in further detail then.

Recommendation #10: Eye assessment

In patients treated with glucocorticoids or antimalarials, a baseline eye examination is recommended according to standard guidelines.

An eye examination during follow up is recommended:

- in selected patients taking glucocorticoids (high risk of glaucoma or cataracts),
- in patients on antimalarial drugs and
    - low risk -> no further testing is required until 5 years after baseline; after the first 5 years of treatment, eye assessment is recommended yearly,
    - High risk -> eye assessment is recommended yearly.

1.2.3. Lupus treatment: treat to target in SLE

Regarding SLE management, after steroid therapy in the 50ths and cyclophosphamide in the late 70ths, no other drug his approved. In the 21th century, Belimumab (anti-blys monoclonal antibody) was approved for severe SLE (Narain & Furie, 2016).

However, a long road of off-label drugs his used in SLE. Despite these drugs, a state of the art therapy is not yet a reality. However, in the last years, more important than drug therapy is the treatment strategy, which should target a low activity disease and a low long-term damage accrual. In this chapter, will be reviewed this new treatment strategies.

The concept of Treat to Target (T2T) has been successfully applied to many diseases including diabetes mellitus, hypertension and hypercholesterolemia, and more recently to rheumatoid arthritis (Smolen et al., 2010).

In 2014, a task force of specialists involved in the care of SLE patients developed key recommendations for T2T in SLE (van Vollenhoven et al., 2014). Prominent features of the T2T/SLE recommendations include early identification of lupus nephritis, targeting remission, minimizing exposure to corticosteroids, optimal management of co-existent antiphospholipid syndrome, prevention of long-term damage accrual and improving quality of life in SLE patients. The T2T/SLE recommendations will led to improved care for SLE patients and provide useful guidance for those involved in their clinical management. However, targeting some SLE manifestations are easier to quantify than others, such as lupus nephritis compared with central nervous system (CNS) manifestations, which may make T2T/SLE challenging. Table 8 summarizes T2T in SLE.

Table 8 - T2T recommendations in SLE (adapted fron Van Vollenhoven annals of Rheumatic diseases. April 17, 2014).

- The management of systemic lupus erythematosus (SLE) should be based on shared decisions between the informed patient and her/his physician(s).
- Treatment of SLE should aim at ensuring long-term survival, preventing organ damage, and optimising health-related quality-of-life, by controlling disease activity and minimising comorbidities and drug toxicity.
- The management of SLE requires an understanding of its many aspects and manifestations, which may have to be targeted in a multidisciplinary manner.
- Patients with SLE need regular long-term monitoring and review and/or adjustment of therapy.
- The treatment target of SLE should be remission of systemic symptoms and organ manifestations or, where remission cannot be reached, the lowest possible disease activity, measured by a validated lupus activity index and/or by organ-specific markers.
- Prevention of flares (especially severe flares) is a realistic target in SLE and should be a therapeutic goal.
- It is not recommended that the treatment in clinically asymptomatic patients be escalated based solely on stable or persistent serological activity.
- Since damage predicts subsequent damage and death, prevention of damage accrual should be a major therapeutic goal in SLE.
- Factors negatively influencing health-related quality of life (HRQOL), such as fatigue, pain and depression should be addressed, in addition to control of disease activity and prevention of damage.
- Early recognition and treatment of renal involvement in lupus patients is strongly recommended.
- For lupus nephritis, following induction therapy, at least 3 years of immunosuppressive maintenance treatment is recommended to optimise outcomes.
- Lupus maintenance treatment should aim for the lowest glucocorticoid dosage needed to control disease, and if possible, glucocorticoids should be withdrawn completely.
- Prevention and treatment of antiphospholipid syndrome (APS)-related morbidity should be a therapeutic goal in SLE; therapeutic recommendations do not differ from those in primary APS.
- Irrespective of the use of other treatments, serious consideration should be given to the use of antimalarials.
- Relevant therapies adjunctive to any immunomodulation should be considered to control comorbidity in SLE patients.

1.3. Etiopathogenesis

Intense research of the cellular and molecular processes involved in SLE, allowed the design of specific and efficient therapies. However, its pathogenesis is still unknown. Genetic factors, contribute to the risk of SLE, and environmental factors (such as tobacco, Vitamin D…) also play an important role. Some of these genetic and environmental factors interact with each other, increasing the disease's risk.

AIDs are chronic conditions initiated by the loss of immunological tolerance to self-antigens. This heterogeneous group of disorders presents common genetic risk factors and shares several pathophysiological mechanisms leading to overlapping clinical manifestations targeting specific organs or multiple organ systems (Shoenfeld et al., 2008). Although they exhibit contrasting epidemiological features and clinical manifestations, there is evidence that they share similar immunogenetic mechanisms (Anaya, Gomez, & Castiblanco, 2006). Underlying these diverse clinical phenotypes may exist, a dysregulated immune system with an enhanced capacity to respond against self. The fact that AIDs share several clinical signs and symptoms (i.e. sub phenotypes), physiopathological mechanisms and genetic factors have been called autoimmune tautology and indicates that they have a common origin (Anaya, 2010).

The function of the immune system is to defend the organism against foreign pathogens and self-altered elements. To complete these purpose, selected T and B-lymphocytes and innate immune cell, activated by pathogen or damage associated molecular patterns, work together. These cells orchestrate a precise immune response through tightly regulated cell-cell interactions and secretion of cytokines and other inflammatory mediators. The body's defense against foreign pathogens must occur without causing unnecessary harm to self. To accomplish this, the elimination of the majority of self-reactive T and B-lymphocytes, in the thymus and bone marrow, through a process denominated negative selection his done. However, this process is imperfect, albeit purposely, and self-reactive lymphocytes that escape into the periphery must be kept under control by an array of peripheral tolerance mechanisms (Sprent & Kishimoto, 2001). When the balance of the effector and regulatory parts of an immune response is disturbed, self-reactive T and B cells become activated and promote autoimmunity (Bluestone, 2011).Discussion of regulatory cells and mechanisms his done, later on this chapter.

Environmental factors combined with genetic and hormonal characteristics have been associated with SLE phenotype and with disease progression (J. Choi, Kim, & Craft, 2012; Kunz, 2013).

Vitamin D is one of the environmental factors likely related to SLE pathogenesis and its deficiency appears to be associated with immunomodulatory abnormalities in this disease (Schneider, Dos Santos, Santos, da Silva Chakr, & Monticielo, 2014). Later on in this chapter, will be reviewd the Vitamin D effect in the immune system, AIDs and SLE.

1.3.1. SLE and Immune Response

One or more mechanisms of B-cell tolerance are lost in SLE, allowing the production of ANAs by plasma cells (Figure 1).

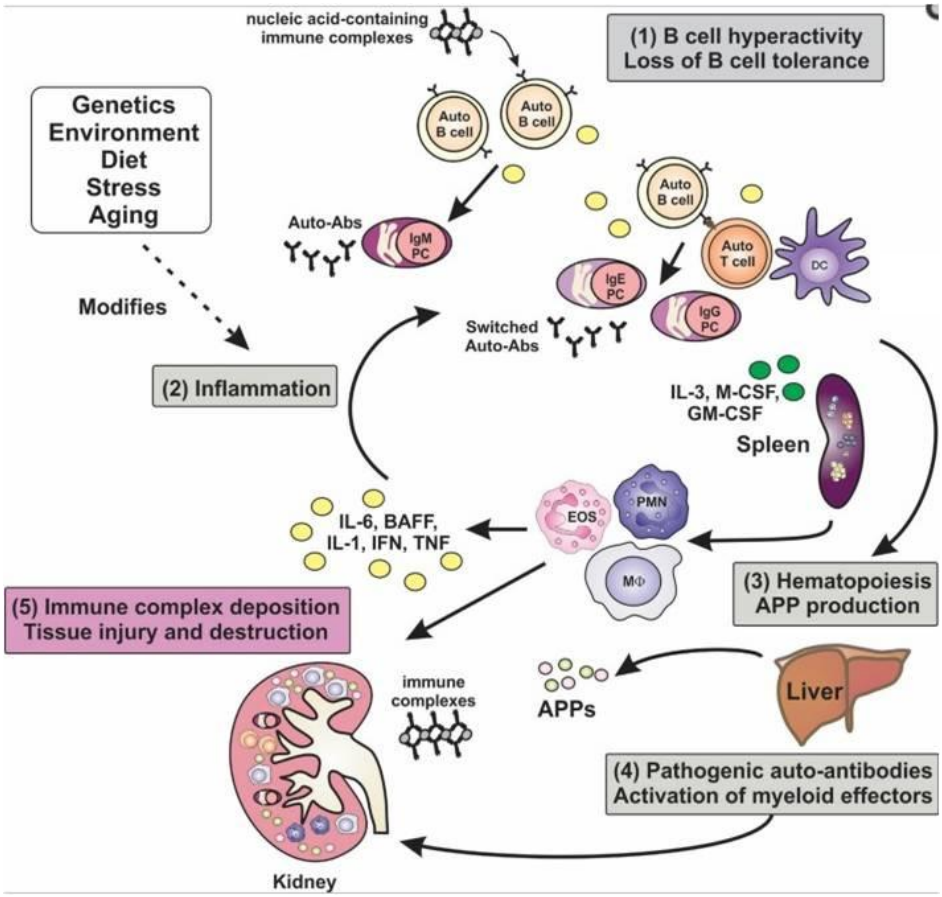


Figure 1 - Production of ANAs by plasma cells (adapted from (Gottschalk, Tsantikos, & Hibbs, 2015)).

Up to 90% of SLE patients have elevated titters of serum ANAs, up to 9.4 years and on average 3.3 years prior to clinical onset of SLE (Figure 2) (Arbuckle et al., 2003).

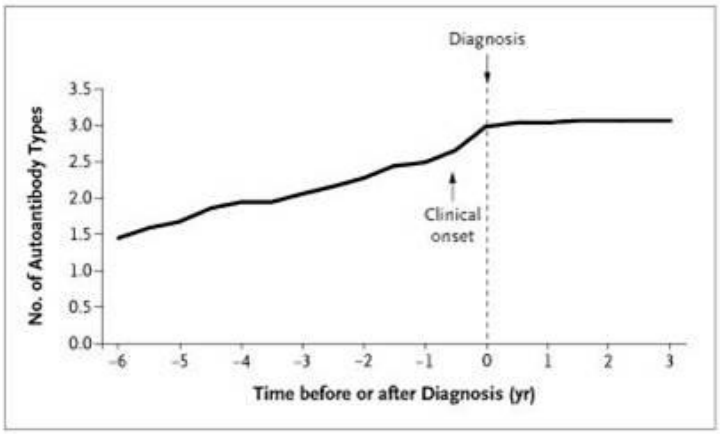


Figure 2 - Cummulative antibodies in SLE (adapted from Arbuckle et al., 2003).

The temporal delay between autoantibody development and disease onset coupled with incomplete penetrance of ANA-mediated disease suggests that pathogenesis is conditional upon other factors, such as antigen availability, a pre-established inflammatory environment, and T-cell-mediated antibody isotope switching. Inadequate apoptosis can expose nuclear antigens allowing ANAs to extensively bind and form Immunocomplexes (ICs). Such ICs can deposit in organ and vessels resulting in activation of the alternative complement pathway and recruitment of proinflammatory macrophages and DCs via chemotactic signalling which upregulate inflammatory cytokine production and activate autoreactive T-cell subsets through antigen presentation and costimulation (Gottschalk et al., 2015).

Endosomal Toll-Like Receptors (TLR)-7 and TLR-9 in activated B cells, plasmacytoid Dendritic Cells (DCs), and macrophages can respond to internalized self ICs containing nucleic acids, which can contribute to the initiation and perpetuation of the inflammatory cascade (Clancy, Markham, & Buyon, 2016).

Disease-related autoantibodies in SLE focus on particular targets, including DNA-containing antigens, such as dsDNA, and ribonucleic acid (RNA)-containing antigens, such as Sm/RNP. A variety of in vitro studies in mouse cells show that RNA- and DNA-containing ICs, activate TLR7 and - 9, respectively, through B-Cell Receptor (BCR) - mediated internalization in B cells or through Complement Fraction Gamma Receptor Family (FcγR)-mediated internalization in DCs. TLR engagement in B cells increases BCR signalling and antibody production, whereas, in peripheral DCs (pDCs), TLR

induces interferon (IFN)-α production, which causes myeloid DCs (mDCs) to release BAFF and further activates autoreactive B cells (Nundel et al., 2015).

Additionally, upregulated TLR7 and TLR9 messenger RNA (mRNA) expression has been reported in Peripheral Blood Mononuclear Cells (PBMCs) from SLE patients, and the levels correlate with the expression of IFN-α (Wu, Tang, & Zuo, 2015).

Regarding T-Cells, an aberrant T-Cell Receptor (TCR) signalling, has been reported, which leads to their hyperresponsiveness. There were describe alterations in the expression of TCRζ (zeta), the activation of intracellular Spleen Tyrosine Kinase (Syk), calcium signalling, and various other kinase pathways. Decreased in the expression levels of TCRζ, exists in the majority of patients with SLE. The downregulation of TCRζ expression and activity is, in contrast, with the hyperresponsiveness of T cells. However, it has been described that, in T cells of these patients, the more potent FCγR can replace the TCRζ (Yoshimoto, Setoyama, Tsuzaka, Abe, & Takeuchi, 2010). Furthermore, the inhibitory function of Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4) is impaired in T cells of lupus patients (Jury et al., 2010). Normally, CTLA-4 is associated with the Tyrosine Phosphatase 2 (SHP-2), which dephosphorylates TCRζ, thereby disrupting TCR signalling (Barreto et al., 2009). Due to the decreased expression of TCRζ in patients with SLE, the regulatory function of CTLA-4 may be impaired. Finally, it has been suggested that impaired TCRζ signalling interferes with T cell selection processes in the thymus, which results in increased numbers of autoreactive T cells; however, this phenomenon is not enough to induce autoimmunity (Hwang et al., 2012).

1.3.1.1. Tregs and Th17 in SLE

Regulatory T cells are specialized suppressor cells (Lahita et al., 2011; Mocanu, Oboroceanu, & Zugun-Eloae, 2013), with the phenotype CD4+FOXP3+CD25highCD127low (Lahita et al., 2011), that have the capacity to regulate the intensity and quality of the immune response (Mocanu, Oboroceanu, & Zugun-Eloae, 2013). They actively suppress effector cells, including those associated with autoimmune diseases (Jiang, 2011), thereby establishing and maintaining immunological self-tolerance (Daniel & von Boehmer, 2011).

Some studies suggest that Treg cells are deficient in number and function in several autoimmune diseases including SLE (Sawla, Hossain, Hahn, & Singh, 2012). However, there are also reports of an enrichment of this cell type (Lin et al., 2007; Suarez, Lopez, Gomez, & Gutierrez, 2006). These contradictory observations may be either due to the lack of a well-defined specific Treg marker in humans or to the

heterogeneity of SLE phenotypes (Sawla et al., 2012). Concerning Treg population, the CD25 *per se,* was the initial marker, which could explain the reported contradictory results. The fact that FoxP3-expressing cells loses CD25 expression and consequently his suppressive functionality, can explain the disfunction of these cells (Bonelli et al., 2009; Valencia, Yarboro, Illei, & Lipsky, 2007).

Besides Th1 and Th2, identification of a third subset of CD4+ effector Th cells named Th17 was reported. These cells, because of its unique ability to produce IL-17 (IL-17A and IL-17F), play a critical role in the recruitment, activation and migration of neutrophils (Jiang, 2011). Beyond their protective role in the clearance of extracellular pathogens, its major role seems to be their involvement in the induction and maintenance of chronic inflammatory processes (Bansal, Henriquez, Sumar, & Patel, 2012; Jiang, 2011).

The hypothesis that certain forms of autoimmunity may result from a conversion of Treg cells into a Th17 cell phenotype, has been suggested by some studies (Eisenstein & Williams, 2009).

In SLE patients' sera, the IL-17 levels are abnormally high when compared to healthy individuals. IL-17 is also produced by neutrophils, Innate Lymphoid Cells, and other T cell types including CD4+, CD8+, double negative (CD4-CD8-) and TCR-γδ (Jiang, 2011). This phenomenon could promote the autoimmune process by increasing the activation of immune cells itself. Release of IL-17 by infiltrating T cells in specific organs may also contribute to local tissue injury by instigating the inflammatory response (Jiang, 2011).

Th17 and SLE

The Signal Transducer and Activator of Transcription (STAT) 3 are overexpressed and hyperactivated in T cells from SLE patients. STAT3 plays a central role in T cell differentiation into Th17 and T follicular helper cells, two subsets that orchestrate autoimmune responses in SLE. Moreover, STAT3 is important in chemokine-mediated T cell migration. Expression of STAT3 is increased in T cells in SLE, whereas inhibition of STAT3 leads to decreased T cell migration and delayed onset of autoimmunity in lupus prone mice (Edwards, Mizui, & Kyttaris, 2015).

Increased IL-17 production in Lupus, may also be explained by overactive costimulation via Signalling Lymphocyte Activation Molecules (SLAMs). Evidence suggests a crucial role for SLAMs in the expression of autoimmunity. SLAM Family (SLAMF) 3 and SLAMF6 expression is increased on the surface of SLE T cells compared with normal cells.

SLAM coengagement with CD3 under Th17 polarizing conditions results in increased IL-17 production. On the other hand, naïve and memory CD4+ T cells produce more IL-17 in response to SLAMs costimulation as compared with CD28 costimulation. Engagement of SLAMF3 and SLAMF6 along with antigen (Ag)-mediated CD3/TCR stimulation represents an important source of IL-17 production. Disruption of this interaction with decoy receptors or blocking antibodies should mitigate disease expression in SLE and other autoimmune conditions (Chatterjee et al., 2012).

IL-17A, a member of IL-17 family, amplifies the immune response by inducing the local production of chemokines and cytokines, recruiting neutrophils and monocytes, augmenting the production of autoantibodies, and aggravating the inflammation and damage of target organs such as the kidney in SLE (D. Li et al., 2015).

Regulatory T-cells in SLE

Above, we defined the nature and properties of regulatory T-Cells. Regarding the suppressive function of Tregs in SLE, different explanations have been published. Several reports demonstrate that the suppressive function of Tregs in SLE is impaired (Sawla et al., 2012). Other reports claim that the suppressive function of Tregs in SLE is not impaired, but that autoreactive effector T cells in SLE are less susceptible to suppression by Tregs (Mercadante & Lorenz, 2016). However, it has also been demonstrated that Tregs from healthy controls are able to suppress effector T cells of patients with SLE (Valencia et al., 2007). Taken together, Tregs seem to play a role in the pathogenesis of SLE.

Disturbed balance between Tregs and Th 17 Cells

In SLE has been suggested, a disturbed balance between Th17 cells and Tregs. However, we only understand partially, the mechanisms underlying alterations in numbers and/or function of Th17 and Tregs in SLE, but may involve the overall cytokine milieu.

For the development of Th17 cells, the combined action of Transforming Growth Factor Beta (TGFβ) and IL-6 is required, whereas Treg development depends on IL-2 and TGFβ (X. Li et al., 2011). In SLE increased in IFN type I has been suggested, and it also promotes the development of Th17 cells, and an Interferon alfa monoclonal antibody is a nearby promising therapy (Khamashta et al., 2016). It has been shown that IFN-α triggers the production of IL-6 by mDCs, whereas IL-6 is required for the development of Th17 cells (Biswas, Aggarwal, Levesque, Maers, & Ramani, 2015). However, IFN-α impairs the suppressive function of Tregs in SLE. Importantly, in the

presence of IFN-α producing DCs, Tregs of SLE patients as well as Tregs of healthy controls were not capable to suppress T effector cells (Becker, Bopp, & Steinbrink, 2013). In summary, immune response involving SLE pathogenesis is complex and includes defective removal of opsonized immune complexes and a complex cytokine milieu favouring B and T autoreactive cells as well as defective function or number of regulatory cells.

1.3.2. Genetic factors and Immune Response

Genetics and epigenetic studies have led to the exploration of the pathogenesis of SLE. Identification of differentially expressed genes and their regulatory mechanism(s) at whole-genome level may contribute to a comprehensive understanding of the development of SLE.

Most of the genes identified as susceptibility risk factors for SLE are involved in the pathways of innate and adaptive immune system (Teruel & Alarcon-Riquelme, 2016). Numerous studies reported an association with susceptibility, phenotype and even with monogenic rare presentations. This chapter is divided in immune system components and summarizes the most important genetic findings in this subject.

1.3.2.1. Innate immune system

Complement

It is well known that complement deficiency, primary or secondary (anti-c1q antibodies for example), is related to disease susceptibility and phenotype (Orbai et al., 2015). There is also a relation of anti-c1q antibodies to lupus nephritis (Orbai et al., 2015) and C2 deficiency to cutaneous lupus and arthritis (Macedo & Isaac, 2016). Impaired “cleaning” of nucleic acid-containing auto-antigens is a very important mechanism for the development of autoantibodies, and the cumulative effect of new autoantibodies along many years is important to disease beginning (Macedo & Isaac, 2016). Figure 3 presents the normal mechanism of efficient complement-mediated opsonisation of ICs.

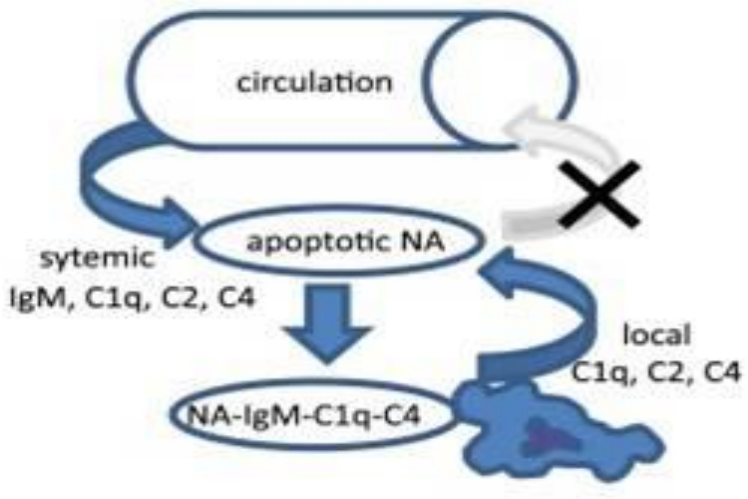


Figure 3 - Normal opsonisation of Nucleic acids (NA) (adapted from (Prechl et al., 2016).

Integrin Subunit Alpha M (ITGAM) gene is clearly associated with the disease in all populations (Teruel & Alarcon-Riquelme, 2016). The Gapaid consortium studied the genetic evidence for altered complement system functionality in SLE. This consortium genetic analysis revealed that the non-synonymous variant rs1143679 in complement receptor type 3 is associated with an increased production of anti-dsDNA IgG antibodies. They also found out that secondary complement deficiency in SLE does not impair opsonisation of nucleic acid-containing autoantigens. It is the dysfunction of the receptor which recognizes complement opsonized ICs that promote the development of class-switched autoantibodies targeting nucleic acids (Figure 4) (Prechl et al., 2016).

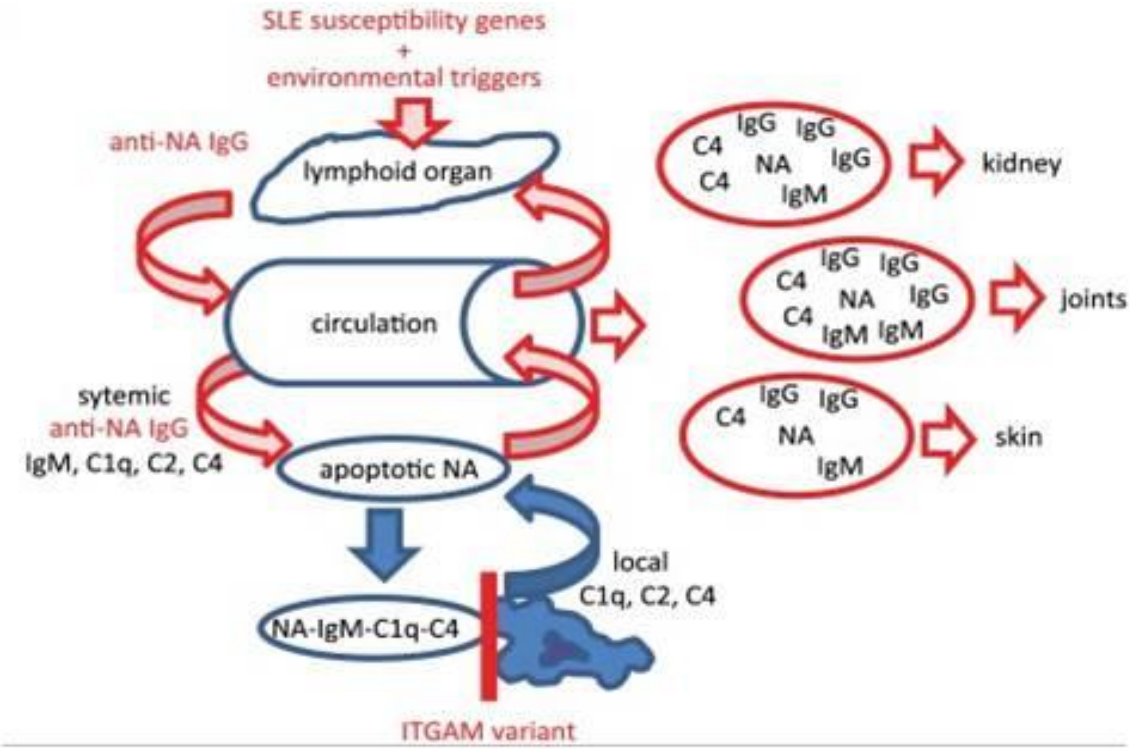


Figure 4 - Mechanism of impaired opsonisation in SLE (adapted from (Prechl et al., 2016).

One of the main recent findings is that opsonisation is not impaired in SLE but complement receptors SNPs are important, and some of them have impaired efficacy in recognizing opsonized ICs.

Mononuclear cells and interferon signature

Increased surface expression of class II molecules is an essential event in the maturation of DCs, in that higher surface expression is crucial to the increased capacity of mature DCs to effectively present antigens to naïve T cells (Banchereau & Steinman, 1998).

The HLA class II genes, especially HLADRB1*03:01 and HLA-DRB1*15:01 alleles are consistently associated with SLE susceptibility (Bettencourt et al., 2015; Vasconcelos et al., 2009). However, several genes within the HLA region contribute independently to SLE genetic susceptibility. Most interestingly has been the targeted next-generation sequencing of the MHC region, where a haplotype with regulatory polymorphisms was associated with changes in gene expression of the HLA class II molecules (Raj et al., 2016). Individuals homozygous for XL9 Polymorphism Haplotype 3 (XL9 HAP3) (risk) variations have more than two-fold higher surface expression for HLA-DR and DQ molecules at baseline which increases to four (4)-fold after stimulation

with TLR-ligand on monocyte-derived DCs than haplotype (HAP) 1 (protective) homozygote's (Raj et al., 2016).

Among non-HLA genes, the Interferon Regulatory Factor 5 Transportin 3 (IRF5-TNPO3) region shows the strongest and most consistent association signals with odds ratios (ORs) of 2.0 in all populations studied. IRF5 is a transcription factor involved both in the type I interferon and the toll-like receptor signalling pathways. In fact, a recent study in Hispanics with enriched Native American ancestry revealed IRF5 as the major locus in SLE, whereas the HLA association was secondary (Alarcon-Riquelme et al., 2016). DNA debris with aberrant methylation, like virus, can activate TLR7, 8 and 9 and stimulate intracellular pathways that conclude in IRF5 activation and finally Interferon-I secretion (Ouyang et al., 2007).

This may explain why pathway analysis on the differential genes in SLE revealed significant enrichment in interferon signalling and toll-like receptor signalling pathways with consequent elevated serum levels of inflammatory cytokines, including IL-17A, Interferon-Gamma Induced Protein 10 (IP)-10, bFGF, TNF-α, IL-6, IL-15, Granulocyte Macrophage–Colony Stimulating Factor (GM-CSF), IL-1RA, IL-5, and IL-12p70 (Zhu et al., 2016).

1.3.2.2. Adaptive immune system

T-Cells

T cells regulate the adaptive immune response and present themselves with an altered function in autoimmunity.

The mRNA analysis identified new genes related to T cell dysfunction and confirmed induction of interferon signature genes; including Oligoadenylate Synthetase 2 (OAS2) which was showed to be specific to SLE autoimmunity (Bradley, Suarez-Fueyo, Moss, Kyttaris, & Tsokos, 2015).

Among highly-expressed and significantly upregulated genes was Interleukin-2 Receptor subunit Gamma (IL-2RG), encoding CD132, the common subunit of receptors for IL-2, -4, -7, -9, -15 and -21 (Bradley et al., 2015).

Originally identified as the third subunit of the high-affinity IL-2 receptor complex, the common γ-chain also acts as a non-redundant receptor subunit for a series of other cytokines, collectively known as γ-chain family cytokines. Ɣ-chain plays essential role in T cell development and differentiation, so that understanding the molecular basis of its signalling and regulation is a critical issue in T cell immunology. Unlike most other cytokine receptors, the γ-chain receptor is thought to be constitutively expressed and limited in its function to the assembly of high-affinity cytokine receptors. Recent studies

reported a series of findings that refute γ-chain as a simple housekeeping gene, and unveiled it as a new regulatory molecule in activation and differentiation of T-cells. Cytokine-independent binding of γ-chain to other cytokine receptor subunits suggested a pre-association model of γ-chain with proprietary cytokine receptors. In addition, identification of a γ-chain splice isoform revealed expression of Soluble Form of Gamma Chain (Sγc). Sγc directly interacted with surface IL-2R subunit Beta (IL2Rβ) to suppress IL-2 signalling and to promote pro-inflammatory Th17 cell differentiation. As a result, endogenously produced Sγc exacerbated autoimmune inflammatory disease, while the removal of endogenous Sγc significantly ameliorated disease outcome. These data provide new insights into the role of both membrane and soluble γ-chain in cytokine signalling, and open new venues to interfere and modulate γ-chain signalling during immune activation. These unexpected discoveries further underscore the perspective that γ-chain biology remains a largely uncharted territory that invites further exploration (Waickman et al., 2016). Figure 5 explains this activation.

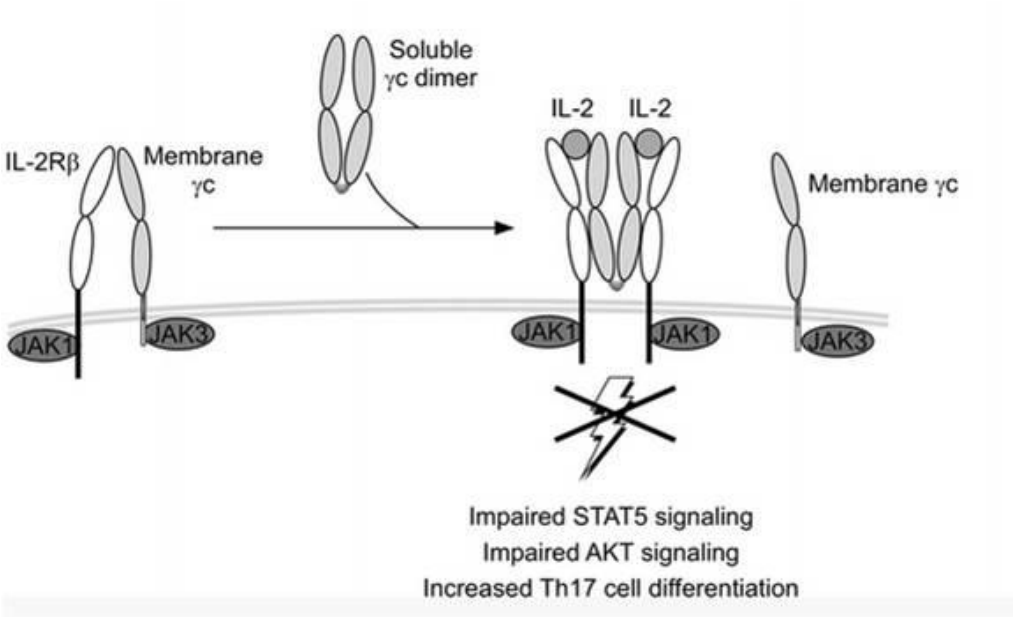


Figure 5 - Th17 activation via Syc (adapted from (Waickman, Park, & Park, 2016).

Recently, a defined subset of T helper cells, Th9 cells has been identified by the potent production of IL-9. The critical role of IL-9 in promoting cellular and humoral immune responses makes it an important focus of potential therapeutic interventions. IL-9 is a 28-30 Kilo Dalton (kDa) monomeric glycosylated polypeptide belonging to the IL-7/IL-9 family of proteins that bind to a composite receptor consisting of the private receptor IL-9R and IL2RG (Ciccia et al., 2016).

B-Cells

B-Cell survival is also crucial in SLE disease. B-Cell activating Factor (BAFF) is a cytokine critical for development and proper selection of B cells, and the targeting of BAFF has emerged as a successful treatment strategy for SLE (Hahn, 2013). Previous reports have suggested that BAFF expression is directly induced by type I IFNs, but the precise mechanism for this remains unknown. In fact, it seems that IRFs control the expression of BAFF. IRF1 and IRF2 were identified as positive regulators of BAFF transcription and IRF4 and IRF8 as potent repressors. These data suggest that type I IFN blockade in SLE patients will lead to downregulation of BAFF and a consequential reduction of autoreactive B cell clones and autoantibodies (Sjostrand et al., 2016).

There is a progressive selection against ANA+ B cells as they mature from transitional to naïve to CD27+IgD- and CD27+IgD+ memory cells in both healthy subjects and SLE patients; however, SLE patients failed to anergize ANA+ naïve B cells to the same extent as healthy individuals. It has also been shown that anergy induction is restored in SLE patients treated with belimumab, an inhibitor of BAFF (Malkiel et al., 2016).

In summary, for individual patients a unique profound combination of genetic factors modulating innate and adaptive immune responses is necessary to induce SLE (Figure 6).

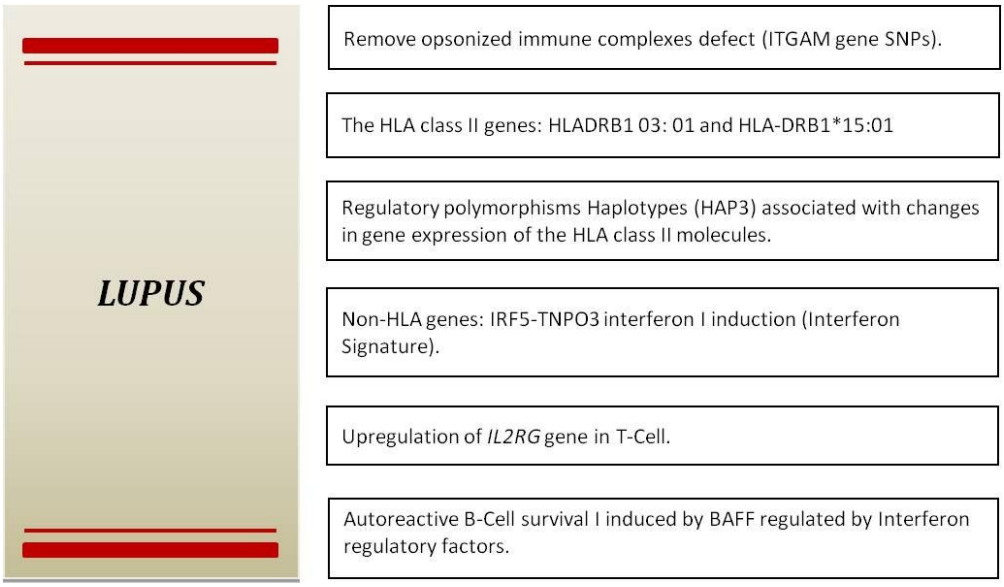


Figure 6 - Genetic influence in SLE susceptibility

Various environmental exposures have been implicated in the induction, acceleration or promotion of flares in SLE. Nevertheless, there is no definitive data

quantifying the relative contributions of these environmental risk factors to disease development, onset or flare.

Among the environmental agents studied in SLE (M. Y. Choi, Barber, Barber, Clarke, & Fritzler, 2016), the followings are described:

- Diet and microbiota.
- Pesticides, chemical and industrial exposures.
- Cigarette smoking, alcohol consumption.
- Drugs and vaccines.
- Ultraviolet radiation.

Diet and microbiota

The capacity of the intestinal microbiota to shape immune responses outside the intestine will be highlighted with examples from one potent immunomodulatory species; the unculturable, Gram-positive, spore-forming segmented filamentous bacteria, which are influenced by a single micronutrient, vitamin A. These bacteria support the development of autoimmune arthritis. In this model, the immunologic phenotypes induced by these microorganisms have been linked to excessive Th17 cell responses. However, cellular and molecular mechanisms by which intestinal commensals influence autoimmune responses at distal sites remain poorly understood.

In some instances, progress has been made in defining specific molecules that mediate immunomodulatory effects. Oral administration of the capsular polysaccharide A of the human gut commensal *Bacteroides fragilis* protects against experimental autoimmune encephalomyelitis in mice, via conversion of naïve $CD4^+$ T cells into IL-10-producing Treg cells.

The evidence that the intestinal microbiota is involved in the development of SLE is less clear than for other autoimmune diseases. More recent discoveries of an important role for TLR7/9 in the pathogenesis of lupus suggest that bacterial or viral commensal triggers might also contribute to SLE pathogenesis.

In addition, several dietary manipulations can alter the course of SLE, which may be partly mediated by effects on the gut microbiota. Dietary interventions or factors, such as polyunsaturated fatty acids, vitamins A, D, and E, and phytoestrogens also lead to improved outcome in animal models of SLE, mostly via reduction in proteinuria and glomerulonephritis (Vieira, Pagovich, & Kriegel, 2014).

On the other hand, the gut microbiota interacts not only with the host but also with other organisms and environmental factors. Exogenous viruses and the virome – the genomes of all the viruses that inhabit a host – are interacting with the gut microbiota. The common “IFN-α signature” in the peripheral blood of SLE patients suggests a potential viral trigger of flares. While exogenous viruses have been implicated as potential contributors (e.g. Epstein-Barr virus), a chronic trigger within the gastrointestinal virome or endogenous retroviruses seems at least as likely, especially since retroelements have been shown to drive autoimmunity (Vieira et al., 2014).

Pesticides, chemical and industrial exposures

Very large, population-based studies reported increased SLE patients death or hospitalization in association with high level of silica and other ambiental toxics, including mining machine operators, miners and quarry workers, other construction workers, glass ceramic and tile workers and chimney sweepers (Parks & De Roos, 2014).

Data on pesticides are inconsistent and no specific pesticide or product has been identified as a likely causative agent. The most convincing data on specific pesticides come from animal studies of organochlorine insecticides; the fact that most of these pesticides are banned or highly restricted diminishes the opportunities for continued research of these compounds (Parks & De Roos, 2014).

Mineral oils are broadly classified mixtures of alkanes and cyclic paraffins, typically by-products of petroleum distillation or production from crude oil. Mineral oils have diverse industrial pesticides, chemical and industrial exposures related to SLE (Parks & De Roos, 2014).

Studies have shown elevated risk of SLE associated with self-reported exposure to mercury. Endotoxins are another important class of immune adjuvants, present in organic dusts across a variety of industries. An association with SLE is suggested by one large study showing SLE mortality was associated with work as a textile machine operator (Parks & De Roos, 2014).

Cigarette smoking, alcohol consumption

It is well known that current smokers have more serious cutaneous manifestations than former or never smokers. It is also likely that smoking decreases the efficacy of antimalarials in patients with cutaneous lupus erythematosus (Takvorian, Merola, & Costenbader, 2014).

Smoking has also been linked with more severe SLE with larger organ system involvement. Among patients with lupus nephritis, those who smoked had accelerated development of end-stage renal disease. Smoking is also associated with higher odds of thrombotic events and avascular necrosis (Takvorian et al., 2014).

On the other hand, alcohol consumption in moderate doses may have a protective effect against the development of SLE, although this is still debated (Takvorian et al., 2014).

Drugs and vaccines

Drug-induced SLE is also a recognized entity. However, drug induced SLE is usually a mild disease, recovering after drugs withdraw. Despite this information, some patients can develop severe lupus. Many drugs have been proposed but only few were definitively associated with SLE. Table 9, summarizes this information (Araujo-Fernandez et al., 2014).

Table 9 – Drugs that can induce SLE (adapted from (Araujo-Fernandez, Ahijon-Lana, & Isenberg, 2014).

	Definite	*Possible*	*Suggested*
High risk	Hydralazine Procainamide		
Moderate risk	Quinidine Isoniazid	Sulfadiazine	
Low risk	Methyldopa Chlorpromazine	Carbamazepine Propylthiouracil Penicillamine	Captopril
Very low risk	Minocycline	Ethosuximide Phenytoin Primidone Trimethadione Valproate Dipheylhydantoin Zonisamide Methimazol Atenolol Timolol Pindolol Oxprenolol Propranolol Labetalol Acebutolol Metoprolol Hydrochlorothiazide Terbinafine Lovastatin Simvastatin Fluvastatin Pravastatin Atorvastatin Fluorouracil Interferon α	Gold salts Penicillin, Streptomycin, Tetracycline, Ciprofloxacin, Rifampicin, Nitrofurantoin Cefuroxime, Cefepime Phenylbutazon Estrogens, Oral contraceptives Danazol. Lithium Para-aminosalicylic acid Ibuprofen Diclofenac, Benoxaprofen, Mesalazine Reserpine Griseofulvin Clonidine Hydroxiurea Interferons (others than IFN alpha) Gemfibrosil Allopurinol Quinine Minoxidil Calcium channel blockers Enalapril, Lisinopril Amiodarone, Spironolactone Psoralen Interleukin-2 Clobazam, Clozapine Tocainide Etanercept Infliximab Adalimumab, Certolizumab pegol Zafirlukast Ticlopidine Bupropion Omeprazol, Esomerazol

The vaccines-autoimmunity interplay is very similar to the established association between infections and autoimmunity. Infectious agents can cause or trigger autoimmunity through several mechanisms such as molecular mimicry, polyclonal activation, bystander activation and the presence of superantigens. Vaccines, as well as infections, activate immune-mediated mechanisms that can induce protective immunity. SLE induced by vaccination is not very well recognized and many vaccines are recommended to overpass the risk of immunosuppressive therapy. Immune Thrombocytopenia (ITP) is the most studied model and most probably related to vaccination (Rinaldi, Perricone, Ortega-Hernandez, Perricone, & Shoenfeld, 2014).

Ultraviolet radiation

While the link between Ultraviolet (UV) radiation exposure and SLE exacerbation is more firmly well established, only few studies suggest an association between UV radiation and the development of incident SLE. Given that photosensitivity due to SLE could be present for several years before diagnosis, this association may be due to reverse causation.

One of the major unanswered questions related to a potential role of UV radiation in the development of SLE is when the relevant susceptibility window for UV-B exposure happens: in utero, at birth, in childhood, adolescence or adulthood. It is also not known whether UV-B exposure acts as an instantaneous hazard, triggering SLE onset very soon after exposure, or whether SLE risk is more related to cumulative lifetime exposure. Accurate assessment and quantification of individual exposure to UV radiation is critical for understanding its potential role in the etiology of SLE. However, studies to date have largely relied on subject recall or occupational categories to quantify past solar UV radiation exposure (Barbhaiya & Costenbader, 2014).

On the other hand, Vitamin D, mainly synthetized after sun exposure, will be one of the main important environmental factors to SLE susceptibility and severity. Thus, this is a paradox. Vitamin D will be addressed later because is one of the main links between genetic and environment.

1.4. Vitamin D

1.4.1. The Physiology of Vitamin D

Vitamin D is unique among vitamins, since it works as a hormone and can be synthesized on the skin, as a result of exposure to sunlight. It is well known the Vitamin D role on the phosphorus and calcium metabolism regulation, ensuring the normal bone mineralization, among other functions (Alves et al., 2013). This hormone is responsible for the maintenance of calcium serum levels by promoting calcium and phosphorus absorption from the intestine and calcium bone reabsorption ("Declaração Portuguesa da Vitamina D," 2009).

Vitamin D is acquired both through nutritional means (10-20%) and by the cutaneous synthesis under the action of sunlight. Dietary sources of Vitamin D are scarce, but the main ones are fish oils and fortified food products (dairy and bread products) that exist in some countries (USA and Northern Europe). In Portugal, several vitamin supplements containing Vitamin D exist in the market. However, the main source of Vitamin D results from the cutaneous synthesis after sun exposure and is dependent on various factors such as the geographical area latitude, season, time of day, the body surface exposed to the sun and exposure duration, use of sunscreens, skin pigmentation, obesity and age ("Declaração Portuguesa da Vitamina D," 2009). Vitamin D3 or cholecalciferol, after formation in the skin, and Vitamin D2 or D3, from dietary sources, are hydroxylated in the liver, resulting in the formation of 25-hydroxyvitamin D or 25(OH)D, the main circulating form. 25(OH)D subsequently undergoes hydroxylation in the kidney to generate the biologically active, dihydroxylated form of Vitamin D, calcitriol or $1,25(OH)_2D$, which acts through specific Vitamin D receptors to regulate not only calcium metabolism, but also differentiation and division of several cell types (Mithal et al., 2009). The identification of Vitamin D receptors in immune system cells and the discovery that DCs can produce the metabolically active form of Vitamin D have led to the suggestion that Vitamin D is an immune modulator (Carvalho et al., 2015b). Recent evidence correlate insufficient Vitamin D levels with an increased risk of developing other non-bone-related disorders: cardiovascular diseases, hypertension, malignant neoplasia, type I diabetes mellitus, MS, dementia, RA and infectious disease (Alves et al., 2013; Cutolo, 2009; "Declaração Portuguesa da Vitamina D," 2009; Pludowski et al., 2013; L. Silva et al., 2010).

The high prevalence of inadequate Vitamin D is nowadays seen as a public health problem affecting several countries in Europe and the USA, particularly those at risk for developing osteoporosis and its consequences ("Declaração Portuguesa da

Vitamina D," 2009). In chronic Vitamin D deficiency, secondary hyperparathyroidism occurs, with a consequent increase in bone turnover, loss of bone mass and increased risk of low impact fractures. This vitamin has also relevant extra-osseous functions, namely in muscle function and balance, and activation of receptors located in these tissues allows the improvement of these parameters ("Declaração Portuguesa da Vitamina D," 2009; L. Silva et al., 2010).

1.4.2. Vitamin D deficiency

The environmental, hormonal, genetic and nutritional conditions will influence the serum levels of Vitamin D. Vitamin D deficiency screening is accomplished through measurement of 25(OH)D, which is the best index for assessing Vitamin D store in the body ("Declaração Portuguesa da Vitamina D," 2009), due to its greater half-life comparing with the metabolically active form. Routinely only at-risk populations should be screened, including the elderly, institutionalized, pregnant women and post-menopausal women (increased risk of fractures) (Alves et al., 2013).

Much debate has taken place over the definition of Vitamin D deficiency. Most agree that a 25(OH)D concentration <50 nmol/L, or 20 ng/mL, is an indication of Vitamin D deficiency, whereas a 25(OH)D concentration of 51–74 nmol/L, or 21–29 ng/mL, is considered to indicate insufficiency; concentrations >75 nmol/L, or 30 ng/mL, are considered to be adequate (Dawson-Hughes et al., 2005; Holick, 2007, 2009; Souberbielle et al., 2010). The optimal serum 25(OH)D levels are those for which calcium absorption is optimized, Parathyroid Hormone (PTH) levels are reduced and the greatest benefit to the bone and muscle function are obtained. Currently levels above 75 nmol/L (30 ng/mL) are recommended.

Several studies have described inadequacy of Vitamin D levels all over the Europe, although the Vitamin D status within different European countries shows a high variation (Cashman et al., 2016; Pludowski et al., 2014; Quraishi, Camargo, & Manson, 2016). 25(OH)D levels lower than 25 nmol/L were found in 2% to 30% of adults, but this percentage may increase to 75% or more in older individuals in institutions. An international study, the European Action on Nutrition and Health-Survey on independent older subjects showed mean serum 25(OH)D levels of 20 to 30 nmol/L in Southern European centers and 40 to 50 nmol/L in Northern Europe. The strong positive correlation between serum 25(OH)D and latitude in this survey was much unexpected because UV irradiation is more effective in Southern than in Northern European countries (McKenna, 1992; Mithal et al., 2009; van der Wielen et al., 1995).

This inadequacy was also observed for younger subjects (Braegger et al., 2013; Gonzalez-Gross et al., 2012; Pludowski et al., 2014) and for pregnant women (Eggemoen et al., 2016; O'Riordan, Kiely, Higgins, & Cashman, 2008).

In fact, when Northern European countries are considered, mean values of 25(OH)D range from 45±35 nmol/L in men to 47±34nmol/L in women in studies from Finland (Lamberg-Allardt, Outila, Karkkainen, Rita, & Valsta, 2001), and are dependent on season (82.5±34.1 nmol/L in July-August vs 36.3±30.5 nmol/L in January-February) ($p<0.001$) (Savolainen, Maenpaa, Alhava, & Kettunen, 1980). In Danish population, mean values are about 55.5 nmol/L (Christiansen, Christensen, McNair, Nielsen, & Madsbad, 1982). In the same way, Norwegians have constant values during winter (50-55 nmol/L), being different from July to September ($p<0.0006$) (Vik, Try, & Stromme, 1980). Regarding different age groups, younger adults have mean values of 63.0 nmol/L (30.3-95.5), while active elderly living at home have lower levels (46.8 nmol/L (18.4-110.6)) (Aksnes, Rodland, Odegaard, Bakke, & Aarskog, 1989). In United Kingdom, a study has demonstrated that 29% of the population was 25(OH)D profoundly deficient (<25 nmol/L) and 32% insufficient (25-50 nmol/L) (Hayden, Sandle, & Berry, 2015). In Ireland, this issue was subject of further studies, which have demonstrated that 40 to 51% of the population has 25(OH)D insufficiency (<50 nmol/L) (Cashman et al., 2013; T. Hill et al., 2005; Lardner, Fitzgibbon, Wilson, Griffin, & Mulkerrin, 2011), reaching 58% in winter (O'Sullivan, Nic Suibhne, Cox, Healy, & O'Morain, 2008). Also, 7 to 10% has 25(OH)D deficiency (<25 nmol/L) (T. R. Hill et al., 2006; Lardner et al., 2011) and in the elderly this percentage rises to 79% (McKenna, Freaney, Meade, & Muldowney, 1985). Overall, 75.6% of the Irish population had inadequate levels of 25(OH)D (<75 nmol/L) (Cashman et al., 2013).

Considering Southern Europe, some groups are interested in learning more about the prevalence of the 25(OH)D in those populations. Three studies from Italy demonstrated hypovitaminosis D. In postmenopausal women (n=570; 41-80 years) mean values of serum 25(OH)D were 18.3±8.3 ng/mL (45.75±20.75 nmol/L) and 28% of the subjects presented hypovitaminosis D (<30 nmol/L), with a significant ($p<0.001$) seasonal variation (Bettica, Bevilacqua, Vago, & Norbiato, 1999). This evidence was confirmed few years later in a larger cohort (n=700; 60-80 years), where values of 25(OH)D lower than 5 ng/mL (12.5 nmol/L) were found in 27% of the women and lower than 12 ng/mL (30 nmol/L) in 76% (Isaia et al., 2003). In an Italian Single-Center Study, with 478 subjects with diagnosis of acute myocardial infarction, it was possible to observe a high prevalence of Vitamin D deficiency among these subjects. Mean serum 25(OH)D levels was 14.5 (7.8-22.7) ng/mL [36.25 (19.5-56.75) nmol/L], with 324 (67.78%) of the subjects presenting deficiency (≤20 ng/mL) and 107 (22.38%)

insufficiency (21-30 ng/mL) (Aleksova et al., 2015). Mean serum 25(OH)D was about 25 nmol/L in breastfed children and about 30 nmol/L in their mothers in Greece (Challa et al., 2005). In Greek adolescents, serum 25(OH)D was lower than 25 nmol/L in 47% during winter (Lapatsanis et al., 2005). Very low levels were also found in Spanish elderly and institutionalized persons, confirming the prevalence of poor Vitamin D status in this social group (Gonzalez-Clemente et al., 1999; Larrosa et al., 2001; Perez-Llamas et al., 2008; Quesada, Jans, Benito, Jimenez, & Bouillon, 1989; Vaqueiro, Bare, Anton, Andreu, & Gimeno, 2006), as well as in postmenopausal women (Aguado et al., 2000; Mezquita-Raya et al., 2001; Quesada-Gomez et al., 2013) and children (Docio et al., 1998).

Studies in other European countries were also published. The SUVIMAX study in French adult men and women between 35 and 65 years showed a mean serum 25(OH)D of 43 nmol/L in the North and 94 nmol/L in the Southwest of France (Chapuy et al., 1997). In this study, the correlation between serum 25(OH)D and latitude was negative as expected. In the Netherlands, the Longitudinal Ageing Study in Amsterdam showed a serum 25(OH)D lower than 25 nmol/L in 8% of men and 14% of women, and lower than 50 nmol/L in 45% of men and 56% of women (Snijder et al., 2005). Similar data were found in the population-based Hoorn study (van Dam et al., 2007) and the Swiss MONICA project (Burnand et al., 1992). A study of Swiss elderly persons living in nursing homes observed that 90% of the elderly women had serum 25(OH)D levels below 50 nmol/L compared to 57% in non-institutionalized elderly women (Krieg, Cornuz, Jacquet, Thiebaud, & Burckhardt, 1998). In Belgium, the mean serum 25(OH)D levels of 126 subjects (21-65 years) was 19.4±7.7 ng/mL, where 34% of the population exhibited insufficiency (<15 ng/mL, or 37.5 nmol/L), predominantly males (MacFarlane et al., 2004).

In Portugal, the prevalence of inadequate Vitamin D levels is not known yet, since there are no epidemiological studies of this prevalence in a national healthy adult population, but it is expected that the Portuguese population follows the same trend of other European countries. However, several studies in specific hospital populations, and pediatric healthy population, have been published.

In 2005, 1456 adult women (18-92 years) from Porto were studied. Data about dietary habits were obtained, through a semi-quantitative questionnaire, validated for the Portuguese population, and compared with the daily reference values recommended by the European Union. The proportion of women with calcium ingestion below the recommendations varied from 41% (<30 years) to 58.1% (≥70 years); ingestion of inadequate values of Vitamin D varied from 70,5% (<30 years) to 96% (60-

69 years), showing a trend to increase linearly with age ($p<0.001$) (Lucas, Costa, & Barros, 2005).

Few years later (2009), a study in a healthy pediatric population from Porto was published. A group of 45 children (33F, 12M; 2.5-16 years) was evaluated in winter and spring of 2007/2008. None of them was had been on supplementation after the first year of life. Values above 100 nmol/L were considered optimal, 75-100 nmol/L sufficient, 50-74 nmol/L relative insufficient and <50 nmol/L deficient. Nine (20%) had optimal levels and 24 (53%) had sufficient levels, while 6 (13%) and 6 (13%) presented relative insufficiency and deficiency, respectively. Thus, a lack of Vitamin D was found in 26% of the studied population during the months with less sunlight. According to these cut off values, 80% of the children did not achieve optimal levels (Monteiro, 2009).

In the same year, 233 prevalent hemodialysis (HD) patients (107F, 116M; 27% diabetics and 32% hypertensive; mean age=62.7±15.3 years; mean HD time=42.9±39.3 months) from the Hospital of Vila Franca de Xira were investigated. Almost half (47%) of the patients were taking active forms of Vitamin D. Deficiency was considered for values below 15 ng/mL, insufficiency for values below 30 ng/mL and normal values above 30 ng/mL. Serum levels of 25(OH)D were found to be low (21.6±12.2 ng/mL) and negatively correlated with age, diabetes mellitus, C-reactive protein and vascular calcifications; levels of 25(OH)D were positively correlated with $1,25(OH)_2D3$ and albumin. These results suggested that lower levels of 25(OH)D are a cardiovascular risk marker in HD patients (Matias et al., 2009).

Since Vitamin D deficiency is highly prevalent in chronic kidney disease (due to inhibition of renal 1α-hydroxylase), 158 HD patients (84F, 74M; mean age=62.8±14.8 years), 25% with diabetes and 34% with hypertension, with a 1-year follow-up, were studied in Hospital of Vila Franca de Xira. Baseline 25(OH)D and $1,25(OH)_2D$ levels were measured twice (end of winter and of summer, respectively) and at the end of the study, after 6 months of supplementation. At baseline, both 25(OH)D and $1,25(OH)_2D$ serum levels were low and positively correlated ($p<0.001$). The authors observed that 80% of the patients had 25(OH)D below sufficiency values (<30 ng/mL), and there was an increase in serum 25(OH)D and $1,25(OH)_2D$ after supplementation. Oral cholecalciferol supplementation in HD patients seems to be an easy and cost-effective therapeutic measure; it allows reduction of Vitamin D deficiency, a better control of mineral metabolism with less use of active Vitamin D, attenuation of inflammation, and possibly improvement of cardiac dysfunction (Matias et al., 2010).

In 42 adult individuals (39F, 3M; mean age=64±10.2 years (35-85 years)) admitted to the Orthopedic Service of Hospital de São João (Porto) with wrist fragility fracture, serum 25(OH)D levels were quantified between July and October 2009. 25(OH)D mean value was 25.6 ng/mL. Only 26.2% of these patients had levels above 30 ng/mL, whilst 45.2% had insufficiency (20-29 ng/mL), 23.8% had deficiency (8-20 ng/mL) and 4.8% had severe deficiency (<8 ng/mL). The values are similar to those registered in the Nordic countries. The findings reveal worrying factors that should alert to specificities of the Portuguese elderly: high rate of hyperparathyroidism, nutritional deficits reported to their youth, and high rate of untreated early menopause (L. Silva et al., 2010).

It has been described that low levels of Vitamin D are associated with CVD, including myocardial infarction. In a case-study, a male patient with 70 years and myocardial infarction was observed. He had vitiligo diagnosed for 20 years and clinical instructions to avoid sun exposure, which led to hypovitaminosis D. Serum 25(OH)D levels were 12 ng/mL (optimal, 30-80 ng/mL). Thus, an association between myocardial infarction, hypovitaminosis D and vitiligo was established (Nunes & Martins, 2010).

In another pediatric study, 73 children (37F, 36M), aged 12 months to 17 years, from the outpatient clinic of Centro Hospitalar do Porto, were studied. The study occurred between March 2008 and July 2010. The children were divided in pre-school age (12 months to 5 years; 23.3% (17/73)) and school age (6 to 17 years; 76.7% (56/73)). Normal 25(OH)D levels (>75 nmol/L) were observed in 17.8% of the children (11% with optimal values, >100nmol/L; and 6.8% with sufficient values, 75-100 nmol/L). On the other hand, 82.2% presented lack of Vitamin D (42.5% with relative insufficiency, 50-74 nmol/L; and 39.7% with deficiency, <50 nmol/L). Gender, residential area, BMI and season were not related to 25(OH)D levels. It was observed that children in school age had higher Vitamin D deficiency (p=0.013), thus establishing a correlation with age (Rocha, 2012).

Another study, a cohort of 122 healthy children and adolescents (5-18 years) from Porto evaluated in the pediatric outpatient clinic during the winter and spring of 2011/2012. Vitamin D status was observed to be of insufficiency (≥20 and <30 ng/mL) in 92.5% of the cases, from which 47.8% presented deficiency (≥10 and <20 ng/mL) and 6% severe deficiency (<10 ng/mL). Only 7.5% of the sample had an adequate status (≥30 ng/mL) (Ferreira et al., 2012).

In a group of 123 patients (64F, 59M; mean age 71±17.2 years) admitted to an internal medicine ward in Coimbra, it was observed a high prevalence (67.5%) of hypovitaminosis D (<20 ng/mL), 25.2% of which had moderate hypovitaminosis D (10-

20 ng/mL). Differences between ages were found: patients with severe hypovitaminosis D (<10 ng/mL) were older (p=0.027) (Santiago et al., 2012).

From the outpatient clinic of Rheumatology in Hospital de Ponte de Lima, a group of 39 patients with Juvenile Idiopathic Arthritis (28F, 11M; mean age=17.8 years (4-28 years); disease duration=9.5±5.1 years) was studied. Serum 25(OH)D levels between 20 and 30 ng/mL were considered as insufficiency and levels below 20 ng/mL as deficiency. Low levels of 25(OH)D were observed in 74.4% (29/39) of the patients, 46.2% (18/39) with insufficiency and 28.2% (11/39) with deficiency (Peixoto, Teixeira, Costa, & Araújo, 2013).

Between September and December 2010, 25(OH)D levels were measured in 2071 patients (61.3% F, 38.7% M; mean age=54 years) at Hospitais Universitários de Coimbra laboratory. The majority of the requisitions were from the internal medicine ward (38.2%) and endocrinology service (21.3%). Values below 30 ng/mL were observed in 88.1% of the evaluated patients, and 65% of whole cohort had values below 20 ng/mL (mean 11.3 ng/mL). PTH levels were increased in 55.3% of the requests (mean=221.3 pg/mL). The findings showed a high prevalence of Vitamin D deficiency (Alves et al., 2013).

In Lisbon, from 2012 to January 2014, plasma samples from 518 HIV-infected patients (197F, 321M; mean age 46±11 years) were collected to determine 25(OH)D levels. Levels below 20 ng/mL were considered as deficiency, 20-30 ng/mL as insufficiency and above 30 ng/mL as optimal. The 25(OH)D levels median was 20 ng/mL (4.1 to 99.7 ng/mL) with differences between winter (median 16.7 ng/mL) and summer (median 24.9 ng/mL) (p<0.0001). Levels below 30 ng/mL were present in 80.1% of the patients (30.9% insufficiency and 49.2% deficiency). An association between antiretroviral therapy regimens with Efavirenz and low levels of 25(OH)D was found (Boura et al., 2014).

In 2015, at Guimarães hospital, 76 patients (55F, 21M; mean age 33.8±10.2 years), with inflammatory bowel disease, were enrolled, 19 of which with ulcerative colitis and 57 with Crohn's disease. Mean serum 25(OH)D levels were low (26.0±10.0 ng/mL), but in Crohn's patient's levels were lower than in those with ulcerative colitis (24.6±8.0 ng/mL vs. 30.0±12.5 ng/mL; p=0.032). Sixty-eight percent of all patients had insufficient levels of Vitamin D (≥20 ng/mL and <30 ng/mL) and Vitamin D deficiency (<20 ng/mL) was found in 30% of the patients (Castro et al., 2015).

In a hospital-based population study from Braga, 5439 25(OH)D measurements were performed between 6 June 2012 and 13 November 2014, corresponding to 3257 different individuals. Women were the majority fraction, 55% (2.992/5439), with median age of 64 years. Deficiency (≤20 ng/mL) was observed in 60% of the cases,

insufficiency (21-29 ng/mL) in 20.7% and only 18.9% had sufficient levels (≥30 ng/mL). It was observed a negative correlation between age and Vitamin D levels ($p<0.001$; $r=-0.054$) and no differences between genders were observed. Vitamin D levels were variable throughout the year ($p<0.001$): higher in summer (July to September; 14-31 ng/mL), followed by autumn (October to December; 11-27 ng/mL), spring (April to June; 10-25 ng/mL) and winter (January to March; 7-20 ng/mL). During summer, sufficient levels of Vitamin D were present in 27.8% of the cases, while this was observed in only 9.2% during winter (Santos, Fernandes, & Garcia, 2015).

1.4.3. Vitamin D and Autoimmune Diseases

The importance of Vitamin D in several autoimmune disorders has been reported and Vitamin D deficiency has been associated with the pathogenesis and severity of MS, RA, SSc and SLE, among others (Cutolo, 2009). In SLE patients, serum Vitamin D levels seem to correlate inversely with SLEDAI scores (Mandal et al., 2014).

It has been found that one of the consequences of $1,25(OH)_2D3$ on the immune response is the stimulation of innate immunity and suppression of adaptive immunity (Hart & Gorman, 2013). Studies on the immunomodulatory properties of $1,25(OH)_2D3$ confirmed the inhibition of Th1 cell development via an inhibition of IL-12 production by Antigen Presenting Cells (APCs). Further work documented its ability to drive $CD4^+$T lymphocytes to a Th2 phenotype with a reduction in Th1 type activity (Bansal et al., 2012), by increasing the production of IL-5 and IL-10. Vitamin D, indirectly, reduces the production of IFNγ (Guillot, Semerano, Saidenberg-Kermanac'h, Falgarone, & Boissier, 2010). It also affects B cells causing induction of B cell apoptosis, inhibition of B cell proliferation, and generation of memory B cells, plasma cell differentiation and immunoglobulin production/secretion (Antico, Tampoia, Tozzoli, & Bizzaro, 2012; Baeke, Takiishi, Korf, Gysemans, & Mathieu, 2010; Guillot et al., 2010; D. Kamen & Aranow, 2008). In human epidermal and dermal cells, it was demonstrated that $1,25(OH)_2D3$ modulates regulatory T cell numbers and their suppressive abilities through DCs (Hart & Gorman, 2013). The effect of Vitamin D on DCs include the differentiation of monocytes into immature dendritic cells, the maturation of dendritic cells and dendritic cell survival (D. Kamen & Aranow, 2008).

Local Vitamin D metabolism allows immune cells to modulate immune responses in an independent way when regulation is required, but the optimization of this autocrine and/or paracrine circuit is strictly dependent of the circulating 25(OH)D availability. The levels of circulating 25(OH)D needed to meet the requirements of Vitamin D sufficiency are still a matter of debate, especially in light of the non-classical

effects of Vitamin D (Baeke et al., 2010). These evidences justify the motivation to consider Vitamin D supplementation as an immunomodulatory intervention in SLE.

The FOXP3 gene has a VDRE in its promoter region, being important for its cellular expression (Kang et al., 2012). The immunomodulatory effect assessed by the imbalance of FoxP3+/IL-17A CD4+ T lymphocytes is widely recognized (Astry, Venkatesha, & Moudgil, 2015).

1.5. Vitamin D and SLE: the evidence

1.5.1. Vitamin D and SLE susceptibility

Vitamin D insufficiency may be an important factor in SLE susceptibility during early life, based on Vitamin D effect in autorreactive T-lymphocytes central deletion (Cocco et al., 2012; Ramagopalan et al., 2009). We can look for autoimmune susceptibility as an “early-life” condition in which Vitamin D insufficiency is also a determining factor for an autorreactive profile. VDREs have been found in the promoter region of the MS associated allele HLA-DRB1*15:01 (Cocco et al., 2012; Ramagopalan et al., 2009), suggesting that with low Vitamin D availability, VDREs are incapable of inducing DRB1*15:01 expression, allowing early life autorreactive T-cells to escape central thymic deletion. These findings have been found in MS; in SLE, no data is available.

We thus postulate that the preventive role of Vitamin D in SLE (and other autoimmune diseases) could be limited in time, during the development of the immune system. Later on, its role should be essentially a modulating one.

With these ideas in mind, retrospective genetic susceptibility studies, that do not have controlled Vitamin D levels in childhood, may yeld no valid results. The susceptibility studies related with polymorphisms of Vitamin D receptor are such an example, with different susceptibility profiles in different populations, but without controlled Vitamin D levels in the studied population (Mao & Huang, 2014; Xiong, He, Zeng, Zhang, & Hu, 2014). In this chapter, we will review the evidence of Vitamin D effect on SLE.

Usually, patients with established SLE lack Vitamin D. The reduced sun exposure, the use of sunscreens, the gut malabsorption (example: secondary to drugs such as corticosteroids), among others, are all significant factors for this steroid hormone insufficiency (Holick, 2012). However, sun exposure, the most important factor for Vitamin D precursor synthesis at the skin level, is also one of the strong activators of SLE flares. This is thought to result from the UV wavelengths of sunlight

potentiating the stimulator of interferon genes (STING)-dependent activation of the IRF3 in response to cytosolic DNA and cyclic dinucleotides in keratinocytes and other human cells (Kemp, Lindsey-Boltz, & Sancar, 2015).

This paradoxical phenomenon, being well recognized, is also ignored in the supplementation strategies, which are based on the general population guidelines. SLE patients need treatment strategies based on serum levels of 25(OH)D and not standard supplementation strategies. Serum levels of 30 ng/ml are validated for metabolic bone disease but not for immunological effects (Antico et al., 2012).

1.5.2. The emerging role of Vitamin D in immunomodulation

Vitamin D has a nuclear receptor, the VDR, which is present in all immune system cells. Many key genes involved in immune regulation have VDREs (Kang et al., 2012), implying a Vitamin D effect in immune modulation. This Vitamin exercises its role, both, at the innate immunity and adaptive immunity levels. During immune response, it modulates the activation of DCs and their ability to present antigens, and polarizes the adaptive immune response to a Th2 response (Holick, 2007). Finally, it is essential for the expression of the FoxP3, via VDRE present in the promoter region of its gene and therefore essential to the normal expression of regulatory T cells (Kang et al., 2012).

In view of these properties, an inflammatory profile in patients with Vitamin D deficiency must be considered. However, the benefit and efficacy of Vitamin D supplementation are very heterogeneous among patients and diseases. Many questions about dosage and patients' optimization are still in debate.

1.5.2.1. Vitamin D and APC in SLE

Lerman et al. studied the ability of $1,25(OH)_2D3$ to affect human monocyte phenotype. This phenotype was assessed by incubating cells with sera from 15 patients with SLE and from 5 healthy volunteers. Addition of $1,25(OH)_2D3$ resulted in significant reductions in the expression of MHC Class II, CD40, and CD86 and increases in expression of CD14 in both types of sera. Overall, $1,25(OH)_2D3$ limited human APC activation via IFNα-induced and independent mechanisms. $1,25(OH)_2D3$ inhibited APC activation by SLE sera, suggesting that it may be possible for $1,25(OH)_2D3$ to reduce the immunostimulatory effects of the SLE milieu by interfering with the soluble cytokine mediators in the sera of these patients (Lerman, Burnham, & Behrens, 2011).

Ben-Zvi et al. studied 19 SLE patients stratified by 25(OH)D. There were no differences between circulating DCs number and phenotype. Monocyte Derived

Dendritic Cells (MDDCs) of the patients were normally responsive to the regulatory effects of Vitamin D in vitro as evidenced by decreased activation in response to Lipopolysaccharide (LPS) stimulation in the presence of $1,25(OH)_2D3$. Additionally, Vitamin D supplementation reduced expression of IFN-α regulated genes by healthy donors and SLE MDDCs in response to factors in activating SLE plasma (Ben-Zvi et al., 2010).

Wu HJ et al. showed that "tolerogenic" DCs are a potential cell-based therapy in SLE by treating monocyte-derived DCs from SLE patients and healthy subjects with combination of $1,25(OH)_2D3$ and dexamethasone followed by LPS-induced maturation. Lupus activated DCs were found to acquire semi-mature phenotype that remained maturation-resistant to immunostimulants and was also found to acquire enhancing Regulatory-T Cells phenotype (H. J. Wu et al., 2015).

Finally, Cynthia Aranow et al. published a Randomized Control Trial (RCT) trying to prove the effect of Vitamin D supplementation in interferon signature. The studied failed the primary outcome (Aranow et al., 2015).

We learned with Aranow's RCT that we still do not know the *in vivo* behaviour of this effect on interferon signature and human studies with tolerogenic DCs in SLE are not yet available. We also do not know if the effect on APC is identical in the various body compartments with different cytokine expression, for example in lupus nephritis, which can be the only manifestation of SLE. On the other hand, we do not know the role of MHC Class II down-regulation in APC in patients with only positive antinuclear antibodies, in serological active quiescent Lupus or during severe flares. It is necessary to replicate the effect *in vivo* and to know in which stage of the disease this adjustment brings more benefit. DCs induced tolerance may be essential in the early stages of the disease and less important in severe flares.

1.5.2.2. Vitamin D, Regulatory T-Cells and T-Helper 17 Cells

Terrier et al. showed the safety and efficacy of 25(OH)D high doses supplementation on restoration of regulatory and effector T cell balance and B cell homeostasis in 24 SLE patients (Terrier et al., 2012). However, several limitations were found in this study. The time established for new flares (6 months) is too short to evaluate this effect. On the other hand, there is no control group with evaluation of regulatory T cells, Th17 cells and IL-17A expression in a healthy population. We only know that Vitamin D changes the profile of immune deregulation, but its meaning is completely unknown. We also do not know if numerical changes correct functional

changes (Ohl & Tenbrock, 2015). However, one can say that Vitamin D high doses are a safe therapy.

Viviane de Souza et al., in a small open-label study from Brazil, also found that Vitamin D deficiency was more prevalent in patients with SLE and was associated with higher levels of IL-6 (de Souza et al., 2014).

Banica LM et al. showed in vitro that rapamicin and Vitamin D together were able to induce regulatory properties in $CD4^+$ T-Cells. However, this sustained effect was observed only in the rapamycin group (Banica et al., 2014).

Piantoni S et al. also showed an enhancement of Treg cells and the production of Th2 cytokines after a long-term of monthly treatment with Vitamin D in SLE patients. These numeric differences were only significant after 2 years of treatment (Piantoni et al., 2015).

Wahono CS et al. and Drozdenko et al. observed that the effect of Vitamin D therapy was mainly on IL-17A cytokine expression and less on regulatory T-Cells (Drozdenko, Heine, & Worm, 2014; Wahono et al., 2014).

In summary, we found a universal trend to Vitamin D immune regulation based on regulatory T-Cell properties and IL-17A expression. However, we need controlled trials with a large number of SLE patients, controlled methodology, especially functional studies with micro-RNA of inducible regulatory T-Cell genes and time dependent studies. Maja Vukić et al. made the first trials with this concept. They studied 71 patients from the VitDmet study (ClinicalTrials.gov Identifier: NCT01479933) and 10 from VitDbol (ClinicalTrials.gov Identifier: NCT02063334). In an interventional prospective study, they correlated the changes in mRNA expression with serum 25(OH)D levels in primary human cells. In peripheral blood mononuclear cells, direct transcriptional effects were observed on selected VDR target genes, such as an up to 2.1-fold increase, after one day only of supplementation onset. This allows us to infer that both long-term and short-term Vitamin D supplementation studies allow monitoring of the Vitamin D responsiveness in human individuals and represent a new kind of human *in vivo* investigations about Vitamin D in humans (Vukic et al., 2015).

1.5.3. Vitamin D Inflammation and atherosclerosis

Vascular protective effects of Vitamin D have been postulated due to modulation of inflammatory cytokines. However, the effects of Vitamin D supplementation on inflammatory cytokines in trials have been inconsistent.

Petri's investigation group studied 200 patients enrolled in the Lupus Atherosclerosis Prevention Study and found that 25(OH)D was not associated with any

measure of subclinical atherosclerosis. 25(OH)D deficiency was associated with higher high sensitivity (hs)CRP at baseline, but did not predict a change in hsCRP over 2 years (Kiani, Fang, Magder, & Petri, 2013).

However, in pediatric Lupus patients enrolled in the Atherosclerosis Prevention in Pediatric Lupus Erythematosus substudy (Robinson, Tangpricha, Yow, Gurion, McComsey, et al., 2014; Robinson, Tangpricha, Yow, Gurion, Schanberg, et al., 2014) the same data were not consistent with the adults' study. They also found that Vitamin D deficiency is independently associated with elevated hsCRP, a marker of inflammation that predicts cardiovascular disease risk. Although, association is not proof of causation and they did not study the impact of Vitamin D supplementation in the follow-up of these patients' hsCRP. By chance, the same study found that Vitamin D status is a determinant of atorvastatin effect on carotid intima medial thickening progression rate in children with lupus at 3 years, and it was significant.

Lertratanakul et al. reported a retrospective study from a large international inception cohort; they studied the risk of cardiovascular (CV) event incidence. Patients in the higher quartiles of 25(OH)D were less likely to have hypertension and hyperlipidemia and were more likely to have lower C-reactive protein levels and lower SLEDAI 2K scores at baseline when compared with the first quartile. Vitamin D levels were not associated with CV event incidence; however, hazard ratios for CV event incidence decreased with successively higher quartiles (Lertratanakul et al., 2014).

Reynolds et al. studied 75 SLE patients in a cross-sectional retrospective study to correlate Vitamin D levels with disease and markers of subclinical vascular disease. Their results were significant and correlated Vitamin D deficiency with increased aortic stiffness in SLE, independent of cardiovascular disease (CVD) risk factors and insulin resistance. They concluded that Increased inflammatory disease activity may be the mechanism by which Vitamin D deficiency mediates vascular stiffness in this patients' group (Reynolds et al., 2012). Sabio et al. showed the same results in a RCT (Sabio et al., 2015).

Reynolds et al. also made a prospective study with Vitamin D deficient SLE patients and described the effectiveness of calcitriol to restore angiogenic capacity of SLE myeloid angiogenic cells and to positively modulate the paracrine regulation of endothelial nitric oxide synthase (Reynolds et al., 2016).

Abou-Raya et al. have done a randomized placebo-controlled trial with 206 patients showing that high dose cholecalciferol was effective in disease activity control and in the improvement of hemostatic and inflammatory markers: IL-1, IL-6, IL-18, Tumour Necrosis Factor (TNF-α), Von Willebrand factor and fibrinogen (Abou-Raya, Abou-Raya, & Helmii, 2013).

In essence, there is enough evidence to affirm that Vitamin D deficiency parallels hsCRP as an inflammatory biomarker for atherosclerosis and this effect is independent of insulin resistance. This observation could be because the effect is more significant on endothelium dysfunction rather than an improvement of metabolic syndrome. SLE, like RA, is an independent risk factor for accelerated atherosclerosis and Vitamin D deficiency may be one modifying risk factor.

The different data between the adult and pediatric patients are explained by different study designs, by the lack of a follow-up on the hsCRP after Vitamin D supplementation and by the fact that the adult study screening of the carotid intima medial thickening progression rate was not an association study with statins.

It would be rather important to repeat the analysis of both groups with the statin effect and following-up the hsCRP in pediatric patients.

1.5.4. Vitamin D resistant state and SLE

SLE could be a conceptual form of resistance to Vitamin D effect. This resistant state has multiple steps including the epigenetic mechanisms (also regulation of metabolic pathways), gene regulation in different cell types and Vitamin D receptor polymorphisms (the transcriptome).

The following summarizes the concept of resistance:

1.5.4.1 Epigenetic mechanisms

Epigenetic mechanisms play a crucial role in regulating gene expression as we showed above with the VDRE in non-coding sequences of MHC Class II (Cocco et al., 2012; Ramagopalan et al., 2009). The main mechanisms involve methylation of DNA and covalent modifications of histones by methylation, acetylation, phosphorylation, or ubiquitination. Mainly DNA methyltransferases and ten-eleven translocation proteins, while a plethora of enzymes, such as histone acetyltransferases, histone deacetylases, histone methyltransferases, perform modifications in DNA methylation and histone demethylases regulate covalent histone modifications.

In many diseases, such as cancer, the epigenetic regulatory system is often disturbed. Vitamin D interacts with the epigenome on multiple levels. Firstly, critical genes in the Vitamin D signalling system, such as those coding for VDR and the enzymes 25-hydroxylase (CYP2R1), 1α-hydroxylase (CYP27B1), and 24-hydroxylase (CYP24A1) have large 5'-C-phosphate-G-3' (CpG) islands in their promoter regions and therefore can be silenced by DNA methylation. Secondly, VDR protein physically interacts with coactivator and corepressor proteins, which in turn are in contact with

chromatin modifiers, such as histone acetyltransferases, histone deacetylases, histone methyltransferases, and histone demethylases, and with chromatin remodelers. Thirdly, a number of genes encoding for chromatin modifiers and remodelers, such as histone demethylases of the Jumonji C-domain containing proteins and lysine-specific demethylase families, are primary targets of VDR and its ligands. Finally, there is evidence that certain VDR ligands (1,25-dihydroxyvitamin D3) have DNA demethylating effects (Fetahu, Hobaus, & Kallay, 2014).

This is the first resistance mechanism. SLE, like cancer, is a state of DNA hypermethylation. The disease itself can mute key enzymes of its metabolism and create a state of 25(OH)D deficiency. Conversely, the “DNA demethylating” effect can be important to the maintenance of key enzymes and their target genes, maintaining immune system homeostasis. Once again, the replacement of cholecalciferol is not established and is likely to need very high levels or greater affinity analogues to overpass this resistance. This particular field lacks evidence. Other resistance mechanism will be addressed in the chapter dedicated to VDR.

1.5.4.2 VDR related mechanisms

Vitamin D acts via binding to an intranuclear receptor, VDR, present in target tissues and cells. This receptor belongs to the superfamily of transacting transcriptional regulatory factors (Kostner et al., 2009). In humans, VDR gene is located on chromosome 12q13.1, extends over 100 kb and includes eight protein-coding exons, six untranslated exons, eight introns and two promoter regions (de Azevedo Silva et al., 2013).

VDR function in transcriptomic era

Genome-wide overview does not involve additional signal transduction steps, as they are known for hydrophilic signalling molecules, such as peptide hormones, growth factors and cytokines. VDR shares the main structural characteristics of nuclear receptors, which is a highly conserved DNA-binding domain and a structurally conserved ligand binding domain VDRs. DNA-binding domain specifically contacts the hexameric consensus sequence RGKTSA (R=A or G, K=G or T, S=C or G) within the major groove of genomic DNA. However, like most other transcription factors, VDR uses a partner DNA-binding protein, in order to bind efficiently to its target sites. More than 20 years ago, this heterodimeric partner turned out to be the nuclear receptor Retinoid X Receptor (RXR). Steric constraints of the dimerizing DNA-binding domains

of VDR and RXR determine the optimal binding site of the VDR-RXR complex as a direct repeat of two hexameric nuclear receptor binding motifs spaced by three nucleotides (DR3). Within VDRs ligand-binding domain, a network of some 40 mostly non-polar amino acids forms a ligand-binding pocket in which $1,25(OH)_2D3$ and its synthetic analogues are specifically fixed with high affinity. This ligand binding process induces a conformational change in the surface of VDRs ligand-binding domain, which results in a significant change of VDRs protein-protein interaction profile: from a suprepressor it is turned into an activator (Carlberg, 2014).

The genome-wide location of VDR is an essential information for understanding the pleiotropic physiological action of $1,25(OH)_2D3$.

Probably, B cells have accessible VDR binding sites. Nevertheless, it can be questioned whether the regulation of a few hundred primary VDR target genes per tissue requires a far higher number of high quality genomic VDR binding sites. For the 23,409 non-overlapping, VDR binding sites a full 75% of them are observed only in one cell type. These unique VDR binding sites may be the mediators of cell-type specific actions of the receptor and its ligand. In fact, on the level of VDR target gene expression, as measured by microarrays, it is already known that in most tissues a rather different set of genes respond to stimulation with $1,25(OH)_2D3$. On the other hand, VDR locations that overlap between two or more tissues represent independent confirmations of the validity of a VDR binding site. Moreover, genomic regions that are recognized in multiple cell types by VDR may have a more generalized, and therefore likely higher, impact on the physiological actions of the receptor and its ligand than the cell type specific sites (Tuoresmaki, Vaisanen, Neme, Heikkinen, & Carlberg, 2014).

In turn, and in light of the renewed understanding of the distribution of transcriptional binding patterns and the diversity of RNA species, it is also timely to consider how these relationships illuminate VDR function (Long, Sucheston-Campbell, & Campbell, 2015).

Hossein-nezhad et al. published a RCT showing that Vitamin D supplementation caused at least a 1.5-fold change in the expression of 291 genes that are involved in apoptosis, immune function, transcriptional regulation, and epigenetic modification, response to stress, cell cycle activity and differentiation. They identified 66 genes, that were most significantly affected by the subjects' Vitamin D status, i.e. those who were Vitamin D deficient with 25(OH)D of 16.2±4.2 ng/ml compared to adults who had a 25(OH)D of 27.5±8.4 ng/ml at baseline. Of these 66 genes, 17 genes whose expression significantly changed after Vitamin D3 supplementation, in both deficient and insufficient/sufficient groups, were found to have novel VDREs (Hossein-nezhad, Spira, & Holick, 2013).

The VDR Transcriptome regulation

With the emergence of comprehensive transcriptomic approaches including microarray technologies, an understanding was developed concerning the VDR transcriptome in different cell types and treatment conditions. In many ways, these studies also highlighted the heterogeneity of VDR actions. This heterogeneity may in part reflect experimental conditions (e.g., growth conditions, ligand exposures) with very different cell lines, but also genuine tissue-specific differences of co-factor expression that alter the amplitude and periodicity of VDR transcriptional actions. These transcriptomic direct actions can regulate cell cycle, activate key enzymes (like kinases), perform chromatin remodelling and have event demethylation effects, very important in cancer and autoimmune diseases as showed above (Long et al., 2015).

VDR Regulation of Non-Coding RNA Species

Many researchers have considered roles for non-coding RNA in the regulation of cell function and have begun to examine the interplay between the at least 20 different types of different non-coding RNA. Many of these RNA species are gene regulatory RNA and include microRNA (miRNA) and long noncoding RNA (long crane), whereas others are involved in the post-transcriptional modification of RNA, for example small nucleolar RNA (snoRNA). The data is still sparse, but non-coding sequences regulation is an important mechanism regarding Vitamin D/VDR interaction and immune regulation. An important example is the VDREs in non-coding sequences of MHC-class II (Cocco et al., 2012). It is likely that the increased application and integration of microarray and next generation sequencing approaches will identify the key networks downstream of the VDR transcriptome, pertaining to both protein coding and non-coding RNA (Long et al., 2015).

The Repertoire of VDR-Protein Interactions and Genomic Binding Sites

Microarray studies showed that VDR combines with other proteins in a network of interactions, quite likely in a cell type specific manner, to participate in diverse gene regulatory networks. It remains to be established how targeted this is. The variation observed in both the type and position of binding sites for the VDR, depending on cell phenotype and disease state, suggests it is a directed process. At the very least, this will establish a paradigm for hypothesis testing, regarding what directs the VDR to bind and participate in gene transcription. The specificity of VDR signalling may arise due to integration with other, perhaps more dominant, transcription factors (Carlberg, 2014).

Again, for other nuclear receptors, the concept has emerged that receptor binding is guided by the actions of more dominant, so-called pioneer, factors including the

Forkhead family members. However, efforts to define the major pioneer factors for the VDR have proven to be less consistent between the different VDR microarray studies. This may reflect the biology of the VDR, given its existance in the nucleus both in the presence and absence of ligand, such that a single pioneer dominant factor is not as deterministic (Long et al., 2015).

Genetic Variation in VDR Binding – The VDR Polymorphisms

Four common SNPs in the VDR gene have been extensively investigated: FokI C>T (rs2228570), BsmI A>G (rs1544410), ApaI G>T (rs7975232), and TaqI C>T (rs731236). BsmI and ApaI SNPs are both located in intron 8, and the TaqI is a silent SNP in exon 9. Although three of these polymorphisms do not produce any structural change on the VDR protein, they are in strong linkage disequilibrium. On the other hand, the T allele of the FokI SNP creates an alternative ATG initiation codon in exon 2, leading to a VDR protein that is three amino acids longer, suggesting a potential functional consequence (Yang, Leung, Adamopoulos, & Gershwin, 2013). The combination of this data allows the use of phenotype-driven data to identify what VDR binding sites, and interactions, are important.

There are few studies regarding transcriptomic alterations in real life patients and all with indirect results and no microarrays essays for microRNA or others.

Barry EL et al. investigated whether 41 candidate SNPs in Vitamin D and calcium pathway genes (Vitamin D Binding Protein, 7-dehydrocholesterol reductase, CYP2R1, CYP27B1, CYP24A1, VDR, and calcium-sensing receptor) are associated with 25(OH)D or modify the increase in 25(OH)D from Vitamin D3 supplementation. They studied 1878 healthy persons and showed that the increase in 25(OH)D due to Vitamin D3 supplementation was modified by genotypes at rs10766197 near CYP2R1, rs6013897 near CYP24A1, and rs7968585 near VDR (Barry et al., 2014).

Monticielo et al. studied 195 SLE patients and 201 healthy controls and, according to genotype distribution, 25(OH)D concentrations were significantly higher in patients carrying the FokI f/f genotype in comparison with patients carrying the F/F genotype (31.6±14.1 ng/ml vs. 23.0±9.2 ng/ml, p=0.004), reinforcing its role in the functional activity of VDR (Monticielo, Brenol, et al., 2012).

In summary, a number of resistance mechanisms can be postulated with this overview. One of the potential Vitamin D resistance mechanisms in SLE is the fact that different cells require different concentrations of active hormone, and we do not know the targets nor the concentrations required for each cells at the various stages of the disease.

On the other hand, there seems to be 1000–10,000 genomic VDR binding sites per cell type. This is far more than the number of primary 1,25$(OH)_2$D3 target genes, which is in the order of 100-500 per tissue. This indicates that some genes are controlled by more than one VDR binding site, i.e., they may have a higher potential to be regulated by 1,25$(OH)_2$D3 rather than target genes with only one active VDR locus.

Finally, the VDR receptor has polymorphisms, which interfere with their conformation, as the FokI. Furthermore, the remaining key enzymes in the synthesis of 25(OH)D, as well as its carrier (the VDR binding-protein) also have polymorphisms, which alter its enzyme activity and transport. As such, the state of resistance to Vitamin D is complex and the analysis of the VDR polymorphisms only evaluates one of the key steps of the problem.

1.5.5. Vitamin D in SLE: real practice

Many studies address Vitamin D therapy in real practice. There are some considerations that we have made, which are important for real practice:

- Can Vitamin D supplementation prevent SLE flares?
- Can Vitamin D levels predict flares?
- What is the optimal dosage in SLE?
- Is there a window of opportunity?

There are a large number of studies addressing this issue, but, the evidence is sparse.

Birmingham et al. made a study with 46 SLE patients from the specimen bank and database of the Ohio SLE Study. They studied the Vitamin D seasonal variation levels and their correlation with flares. The major finding of this study was that in non-African-Americans SLE patients, there was a highly significant decrease in 25-hydroxyvitamin D serum levels at the time of flare, for those flares occurring during low daytime sun exposure season. This was observed in both non-renal and renal flares (Birmingham et al., 2012).

Schoindre Y et al. prospectively studied the relationship between 25(OH)D levels and SLEDAI score, in 170 SLE patients, and assessed the role of Vitamin D in predicting SLE flare-ups. There was no association between baseline 25(OH)D levels and relapse-free survival rate (Schoindre et al., 2014).

Finally, Susmita Roy et al. published a complex paper, developing a general kinetic model in an attempt to capture the role of Vitamin D in immunomodulatory

responses. This kinetic model, developed using the ideas of chemical network theory, leads to a system of nine coupled equations that were solved both by direct and by stochastic (Gillespie) methods. They found that although Vitamin D plays a negligible role in the initial immune response, it exerts a profound influence in the long term, especially in helping the system to achieve a new, stable steady state. They also found that the optimal Vitamin D level lies in the 50–100 nmol/L range, where both pathogen and effector T-cell levels remain at reasonably low risk range (Roy, Shrinivas, & Bagchi, 2014).

This is a difficult issue. Despite the general idea that low Vitamin D levels are related to higher disease activity, proofs are needed in RCT. It seems that SLE flares can relate to large variations of Vitamin D, but, the hormone levels themselves cannot predict flares. This statement does not exclude that Vitamin D basal therapy, at least in stable SLE patients, cannot prevent flares. Finally, in theory, immunomodulation can be achieved with optimal levels between 75-100 nmol/L, however it is shown that optimal individual levels need to be tailored.

This important medical problem leads to the question of whether an insight into the genome-and transcriptome-wide actions of VDR and 1,25$(OH)_2$D3 is helpful in a more accurate evaluation of the human individual's responsiveness to, and needing for, Vitamin D. Interestingly, only for a subset of individuals, significant correlations between the up-regulation of both genes and the intervention-induced raise in serum 25(OH)D concentrations were obtained. This suggests that, on a molecular level, not all study participants benefited from the Vitamin D3 supplementation because they had already reached their individual optimal Vitamin D status before the start of the intervention, or they carry a genetic polymorphism making them less responsive to Vitamin D3, or other undefined reasons.

CHAPTER 2

HYPOTHESES, AIMS AND THESIS DESIGN

2.1. Thesis framework

This thesis was designed based on a cohort of patients with systemic autoimmune diseases that currently has between 3,000 and 4,000 patients in active follow-up. The Unidade de Imunologia Clínica (UIC) of the Centro Hospitalar do Porto (CHP) is a unique multidisciplinary service in Portugal, as it covers the whole are of what is now considered clinical immunology (primary immunodeficiencies, secondary immunodeficiencies, autoinflammatory syndromes and AIDs). UIC is an ambulatory service in essence, providing consulting services to hospitalizations and offering a multidisciplinary approach to difficult clinical cases.

The personal contact with about 1000 patients with systemic autoimmune diseases was important to recognize a widespread symptomatic Vitamin D deficiency. Many findings such as fatigue, myalgia and arthralgia are common aspects in SLE but many patients improve solely with Vitamin D replacement. We also found that replenish Vitamin D guided by serum levels was by far more effective than empirical prescription and it was usual to need doses greater than 5,000 IU of cholecalciferol per day for effective treatment.

Several issues were raised and addressed in this thesis. First, structured Portuguese studies to give a problem overview could not be found and secondly there was a lack of real-life data on patients with SLE. There are many reasons for patients with lupus to be potentially deficient in Vitamin D. Available data miss a lot of information: the effect of seasons of the year, sun exposure, diet variations, food supplements and latitude. Without these data, it is difficult to define whether this deficiency is multifactorial and substantially different from that found in the healthy population. This knowledge is crucial because no one could answer the question whether lack of Vitamin D is the cause or consequence of the disease.

Then the question of whether Vitamin D was related to SLE susceptibility has surfaced. Many theoretical models were generated, but the one developed for MS, in which VDRE in noncoding sequences in the HLA-DRB1*15 plays a potential role in disease protection, interested us particularly.

Finally, two equally fundamental questions were placed: what is the role of Vitamin D and its receptor in SLE progression and if Vitamin D-based therapeutic strategies will be equally effective in patients with stable as well as severe active disease.

The hypotheses, aims, material and methods to answer these questions will be presented.

2.2. Hypotheses

Patients with SLE have significant Vitamin D insufficiency, which leads to an increased risk of disease flares, damage accrual and long-term cardiovascular disease.

This dysfunction is likely due, at least in part, to failure of “immunomodulatory” effects of Vitamin D.

The hypotheses of this work are:

Hypothesis 1: Vitamin D levels in our SLE patients are lower than in a healthy population with the same demographic characteristics (age and sex).
Hypothesis 2: Vitamin D status is related to SLE susceptibility.
Hypothesis 3: SLE severity is related to VDR SNPs
Hypothesis 4: Vitamin D deficiency is associated with SLE severity.
Hypothesis 5: Supplementation with Vitamin D alters the clinical expression and immunological deregulation of SLE patients (T-$CD4^+$ cells expressing FoxP3 and T-$CD4^+$ cells producing IL-17A).
Hypothesis 6: Clinical and Immunological response to Vitamin D supplementation is limited by SLE disease activity.

2.3. Aims

With respect to the first hypothesis is to:

- Develop two epidemiological studies in Portuguese SLE patients and healthy controls to prove deficiency of Vitamin D in both populations and their different characteristics.

With respect to the second hypothesis is to:

- Develop a retrospective longitudinal/ genetic study with more than 1000 patients proving a protective effect of different alleles of MHC class II to AID susceptibility, in an attempt to theorize on VDRE in MHC class II genes as being such as important as in MS.

With respect to third and fourth hypotheses is to:

- Develop two retrospective longitudinal/ immunogenetic studies with 170 SLE patients (DNA databank) from a well-characterized cohort, to correlate VDR

polymorphisms and longitudinal and baseline levels of Vitamin D with disease different aspects (activity, flares and damage accrual).

With respect to the fifth and sixth hypotheses is to:

- Develop two interventional studies with Vitamin D supplementation:
  - To study the effect of Vitamin D supplementation (despite baseline levels) in 24 stable SLE patients, regarding disease severity, T-CD4$^+$ cells expressing FoxP3 and T-CD4$^+$ cells producing IL-17A.
  - To study the effect of Vitamin D supplementation (despite baseline levels) in 4 severely SLE patients, regarding Tregs, T-CD4$^+$ cells producing IL-17A and miR-146a expression.

2.4. Thesis Design

The design of the thesis was based in a large national cohort with more than 3,000 patients with AIDs and SLE patients of UIC from Centro Hospitalar do Porto. In order to prove our hypotheses and answer the questions raised, we have made two epidemiological studies, three retrospective/ immunogenetic studies and finally two interventional studies. The table above shows the distribution of these studies (table 10).

Table 10 - Thesis design.

Hypothesis 1 Vitamin D levels in our SLE patients are lower than in a healthy population with the same demographic characteristics (age and sex).	Vitamin D levels in a healthy population from the North of Portugal. **(198 healthy blood donors)** Vitamin D levels in a SLE population from the North of Portugal. **(124 SLE patients)**
Hypothesis 2 Vitamin D status is related to SLE susceptibility.	The Protective Role of HLA-DRB1*13 in Autoimmune Diseases. **(1228 AIDs patients)**
Hypothesis 3 SLE severity is related to VDR SNPs. **Hypothesis 4** Vitamin D deficiency is associated with SLE severity.	Association between Vitamin D Receptor (VDR) gene polymorphisms and Systemic Lupus Erythematosus in Portuguese Patients. **(170 SLE patients)** Relation of Vitamin D levels with SLE number of severe flares. **(112 SLE patients)**
Hypothesis 5 Supplementation with vitamin D alters the clinical expression and immunological deregulation of SLE patients. **Hypothesis 6** Clinical and Immunological response to Vitamin D supplementation is limited by SLE disease activity.	Vitamin D supplementation effects on FoxP3 expression in T cells and FoxP3+/IL-17A ratio and clinical course in systemic lupus erythematosus patients: a study in a Portuguese cohort. **(24 SLE patients)** Effect of high doses cholecalciferol during SLE flares in miR-146a expression, regulatory T-Cells expression and IL-17A expression. **(4 SLE patients)**

CHAPTER 3

MATERIAL AND METHODS

3.1. Subjects

Patients

A population of one thousand and two hundred twenty-eight (1228) patients that had collected DNA in our databank, with six autoimmune different diseases, including 213 SLE patients, were selected, by convenience, from the cohorts of the Neurology, Dermatology and Clinical Immunology of Centro Hospitalar do Porto - Hospital de Santo António for HLA genetic studies.

Regarding Vitamin D studies in lupus patients, diagnosed according to the 1997 and 2012 revised ACR criteria for SLE, they were selected, again by convenience, from the described cohort:

One hundred and twenty-four (124) SLE patients participated in Vitamin D levels epidemiologic studies,

One hundred and seventy (170) SLE patients with at least five years of disease, was selected for VDR polymorphisms study,

Concerning Vitamin D relation with disease severity, one hundred and twelve (112) patients were selected, from the described database of 170 SLE patients, and Twenty-four (24) stable and four (4) severely active SLE patients, were recruited for the interventional studies of Vitamin D in SLE patients.

Healthy controls

Healthy unrelated individuals were recruited from the same region of SLE patients, with a total of 674 analyses.

Two hundred (200) blood donors voluntarily participated in the Vitamin D levels epidemiologic study.

Two hundred and eighty-two (282) individuals were used as control group for HLA genetic study.

On hundred and ninety-two (192), were used as controls for VDR polymorphisms studies.

3.2. Data collection

SLE disease characterization

Disease severity was assessed using number of affected organs, number of severe flares (defined by the SELENA-SLEDAI flare composite) and the need of steroids or immunosuppressive therapy during disease duration (assumed as those who still were on these therapeutics at the time of data collection). Disease activity was

assessed by the SLEDAI 2-K and by the BILAG score. Cumulative damage was evaluated with the SLICC/ACR Damage Index. Flares were defined by the SELENA-SLEDAI flare composite index.

Clinical manifestations (kidney involvement, Central Nervous System involvement, Peripheral Nervous System involvement, articular, malar rash, photosensitivity, dsDNA, leukopenia, lymphopenia, pericarditis, pleuritis, lung involvement, myositis, ascitis, serositis, haematological and skin involvements) were established using a standard protocol accordingly to ACR glossaries for SLE classification criteria and EULAR recommendations for neuropsiquiatric, nephritis and SLE management.

Considering kidney involvement, we took into account the response to treatment: complete response, partial response, no-response, chronic renal failure (CRF) and CRF in dialysis.

Questionnaires

A questionnaire about age, gender, weight, height, ethnicity, nationality, place of birth, occupation, sun exposure, sunscreens use, eating habits, smoking habits, physical activity, diagnosed pathologies, use of medicines and food supplements, was answered during blood donation, by healthy controls selected for the Vitamin D epidemiological studies.

3.3. Laboratory assessments

Vitamin D measurement

Blood was collected in Vacuette® Z Serum Clot Activator tubes for the measurement of PTH and in Vacuette® Z Serum Separator Clot Activator tubes for the other measurements. Serum was obtained by centrifugation and stored in several aliquots at -20°C until analysed. Serum 25(OH)D was chosen as a reliable marker of individual Vitamin D status as it reflects Vitamin D obtained from food sources and cutaneous synthesis, and it is not prone to diurnal variation.

Serum 25(OH)D was measured using an electro-chemiluminescence binding assay (ECLIA) for the *in vitro* determination of total 25-hydroxyvitamin D (Elecsys® Vitamin D total, Cobas, Roche©). The reference range for 25(OH)D was >75 nmol/L (measurement range: 7.50-175 nmol/L). The serum PTH concentration was assessed using an electro-chemiluminescence assay with 15-65 pg/mL as a reference range. Serum total calcium, phosphate and creatinine concentrations were measured by routine laboratory methods in a Cobas Integra 800.

DNA Samples and Genotyping

Peripheral blood samples (10 mL) were collected in EDTA. Genomic DNA was obtained from Proteinase-K–treated peripheral blood leukocytes by using a Salting-Out procedure.

HLA-DRB1 genotyping

DNA was amplified using polymerase chain reaction and sequence-specific primers. Primers and probes were validated by the Twelfth International Histocompatibility Workshop. Polymerase Chain Reaction (PCR) products were visualized under ultraviolet light after running in a 1.5% agarose gel containing ethidium bromide.

VDR Genotyping

The single nucleotide polymorphisms (SNP) were genotyped using pre-designed TaqMan® allelic discrimination assays from Applied Biosystems (Foster City, CA, USA) in a Rotor Gene 6000 Real-Time PCR machine (Corbett Life Science).

Genotyping of the BsmI (rs1544410), ApaI (rs7975232), TaqI (rs731236) and FokI (rs2228570) genetic variants, located within the 12q12-14 (VDR gene) region, was carried out.

Flow Cytometry

Peripheral blood lymphocyte's subsets, including T, B, NK, $TCD4^{+}FoxP3^{+}$, $TCD4^{+}IL\text{-}17A^{+}$ were analysed by flow cytometry, in a Coulter Epics XL-MCL® cytometer. Cells counts (cells/µL) and proportions (%) were established from fresh blood samples using different protocols and monoclonal antibodies (mAbs), conjugated to fluorescein (FITC), phycoerythrin (PE), Phycoerythrin-Texas Red (ECD) and Phycoerythrin Cyanin 5.1 (PC5).

T ($CD3^{+}/CD4^{+}$ and $CD3^{+}/CD8^{+}$), B ($CD19^{+}$) and NK ($CD56^{+}$) lymphocytes were analysed using a Beckman Coulter standard protocol with the following mAbs: anti-CD45 FITC (clone B3821F4A), anti-CD3 PC5 (clone UCHT1), anti-CD4 RD1 (clone SFCI12T4D11), anti-CD8 ECD (clone SFCI21Thy2D3), anti-CD19 ECD (clone J4.119) and anti-CD56 FITC (clone N901/NKH-1); all from Beckman Coulter, Fullerton, California, USA).

Effector $CD4^{+}$ T cells producing IL-17 were quantified after four hour stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomicin in the presence of brefeldin A. Cells were fixed and permeabilized using the IntraPrep Permeabilization Reagent (Beckman Coulter) buffer system and stained with anti-CD4 PC5 (clone 13B8.2;

Immunotech, Marseille, France) and anti-IL-17 PE (clone eBio64DEC17; eBioscience Inc, San Diego, CA).

$CD4^+FoxP3^+$ T cells were quantified after cell fixation and permeabilization using the mAbs anti-CD4 PC5 (clone 13B8.2; Beckman Coulter - IOTest; Marseille, France), anti-CD25 FITC (clone B1.49.9; Immunotech, Marseille, France, only for severely active SLE interventional study), and anti-FoxP3 PE (clone PCH10; eBioscience Inc, San Diego, CA), according to the manufacturer's staining protocol.

RNA Samples and Quantification

Total RNA, including small RNAs, was extracted using a miRNeasy Mini Kit with a Qiacube (QIAGEN, Courtaboeuf, France) according to the manufacturer's instruction. RNA concentration, purity, and quality were assessed using the NanoDrop® ND-1000 (Thermo Fisher Scientific, Massachusetts, EUA). MiR-146a expression was analysed, using TaqMan microRNA pre-designed assay as recommended by manufacturer (Applied Biosystems, Inc., Foster City, CA). The assay includes two steps: generation of cDNA followed by TaqMan real time PCR. Briefly, total RNA was reverse-transcribed, using TaqMan miRNA Reverse Transcription Kit following the manufacturer's protocol (Applied Biosystems, Inc., Foster City, CA). TaqMan miRNA assay included specific RT Primers and TaqMan Probes to quantify mature hsa-miR-146a (P/N 4373132). For normalization, hsa-miR-RNU48 (P/N 4373883) was used. All reactions were incubated at 95°C for 10 min followed by 50 cycles of 92°C for 15 sec followed by 72°C for 20 sec. in ROTOR GENE 6000 (Corbett Life Science). Melt curve analysis was performed at the end of each qPCR run. No-template and no-reverse transcriptase controls were included. A threshold cycle (CT) was observed in the exponential phase amplification, and quantification of relative expression levels was performed, using standard curve for target genes and endogenous control. An efficiency of 90-100% of PCR was only taken into consideration and mean ΔCT±SE (CT value of target gene-CT value of normalizer) for each target was calculated as described previously (Dorak, 2006; Williams et al., 2007). Therefore, higher ΔCT indicates lower expression of target gene.

3.4. Studies design

In this section, we present the studies design, which is explained in table 11.

Table 11 - Studies design.

Ref.	Type of Study	Name of Study	Year of data collection	Design of study	N	Objective	Outcome Measured	Methods of Hypothesis Testing
1	EPIDEMIOLOGICAL STUDIES	Vitamin D levels in a healthy population from the North of Portugal	Summer 2015 and winter 2016	Prevalence study	198 healthy controls	Prevalence of Vitamin D deficiency in non-supplemented healthy population in different seasons of the year.	Vitamin D levels in winter and summer time	Kolmogorov-Smirnov test, Student's t-test or one-way ANOVA (continuous variables) and χ2 test (dichotomous variables). Pearson's or Spearman's correlation coefficients. Multiple linear regression analysis. A p-value < 0.05 statistically significant. Statistical analyses: Statistical Package for the Social Sciences software (version 23, IBM SPSS Statistics, NY, USA).
2		Vitamin D levels in a SLE population from the North of Portugal	2015	Prevalence case–control study	124 SLE patients and 198 healthy controls	Prevalence of Vitamin D deficiency in non-supplemented SLE patients, in different seasons of the year, comparing with healthy controls.	Vitamin D levels in winter and summer time	Kolmogorov-Smirnov test, Student's t-test or one-way ANOVA (continuous variables) and χ2 test (dichotomous variables). Pearson's or Spearman's correlation coefficients. A p-value < 0.05 statistically significant. Statistical analyses: Statistical Package for the Social Sciences software (version 23, IBM SPSS Statistics, NY, USA).
1	IMMUNOGENETIC STUDIES	The Protective Role of HLA-DRB1 (*) 13 in Autoimmune Diseases.	2015	Retrospective cohort study	1228 patients with AIDs and 281 healthy controls	HLA-DRB1 genes association with AIDs risk and protection.	HLA-DRB1 alleles frequency	Stepwise logistic regression on an allelic level. ORs obtained in a multivariable logistic regression analysis adjusted for all the other genes. A p-value < 0.05 statistically significant. Statistical analyses: Statistical Package for the Social Sciences software (version 23, IBM SPSS Statistics, NY, USA).
2		Association between Vitamin D Receptor (VDR) gene polymorphisms and systemic lupus erythematosus in Portuguese patients.	2014	Retrospective cohort study	170 SLE patients and 192 healthy patients	Correlation of VDR SNPs to SLE severity and damage.	Number of severe flares, number of involved organs, concomitant therapy, SLICC, VDR polymorphisms.	Allele and genotype frequencies obtained by direct counting. Chi-square test, Mann-Whitney test. Uni and then multivariate analysis (logistic regression with the significant variables in univariate analysis). Odds Ratio calculated with a binary logistic regression. Statistical analyses: Statistical Package for the Social Sciences software (version 23, IBM SPSS Statistics, NY, USA).
3		Relation of Vitamin D levels with SLE number of severe flares.	2016	Retrospective cohort study	112 SLE patients	Correlation of baseline and mean Vitamin D levels during 10 years with SLE severity.	Number of severe flares, Vitamin D levels.	Kolmogorov-Smirnov test, Student's t-test or one-way ANOVA (continuous variables) and χ2 test (dichotomous variables). Pearson's or Spearman's correlation coefficients. A p-value < 0.05 statistically significant. Statistical analyses: Statistical Package for the Social Sciences software (version 23, IBM SPSS Statistics, NY, USA).
1	INTERVENTIONAL STUDIES	Vitamin D supplementation effects on FoxP3 expression in T cells and FoxP3+/IL-17A ratio and clinical course in systemic lupus erythematosus patients: a study in a Portuguese cohort.	2013	Interventional cross-sectional	24 SLE patients	To evaluate the effect of a 6 - month escalating dose of Vitamin D supplementation on FoxP3 TCD4+ cells and IL-17A TCD4+ cells expression, and FoxP3+/IL-17A ratio	Vitamin D levels, SLEDAI, SLICC, FoxP3 TCD4+ cells, IL-17A TCD4+ cells expression, and FoxP3+/IL-17A ratio	Wilcoxon signed-rank test. Statistical analyses: Statistical Package for the Social Sciences software (version 23, IBM SPSS Statistics, NY, USA).
2		Effect of high dose cholecalciferol during SLE flares in miR-146a expression, regulatory T-Cells and IL-17A expression.	2016	Interventional cross-sectional	4 SLE patients	To evaluate the effect of a 3-week high dose Vitamin D supplementation in severely active SLE patients, on FoxP3 TCD4+ cells, IL-17A TCD4+ cells, FoxP3+/IL-17A ratio and miR-146a expression.	Vitamin D levels, SLEDAI, BILAG, FoxP3 TCD4+ cells, IL-17A TCD4+ cells expression, and FoxP3+/IL-17A ratio. and miR-146a expression.	Relative expression values were calculated using the 2-ΔΔCt method. Differences in ΔCt were evaluated using two-tailed Student's t-test. Statistical analyses: Statistical Package for the Social Sciences software (version 23, IBM SPSS Statistics, NY, USA).

3.5. Statistical Analyses

In our studies, continuous data were checked for normality using the Kolmogorov-Smirnov test and natural logarithm (ln) transformations were used for skewed variables previously to the statistical analysis. Differences between groups were tested using the Student's t-test or one-way ANOVA (continuous variables) and $\chi 2$ test (dichotomous variables). Pearson's or Spearman's correlation coefficients were calculated to test relationships between continuous variables.

Multiple linear regression analysis was used to consider potential determinants of 25(OH)D levels (dependent variable). The following independent variables: age and BMI (as continuous variables), season and gender (as categorical variables) were included in the model.

A stepwise logistic regression on an allelic level was applied, using forward selection which involves starting with all HLA-DRB1 alleles, testing the deletion of each allele using a chosen model comparison criterion, deleting the allele (if any) that improves the model the most by being deleted, and repeating this process until no further improvement is possible. It should be noted that odds ratios (ORs) obtained in a multivariable logistic regression analysis are adjusted for all the other genes included in the model, and therefore differ from those obtained when a given gene is compared with all other genes, in order to identify the HLA-DRB1 genes contributing to the six different AIDs.

Allele and genotype frequencies were obtained by direct counting, for VDR polymorphisms study. Chi-square test was used to determine the differences of genotypic and allelic frequencies between patients and control groups. The same test was also conducted for the study of the association of the different genotypes or alleles with SLE clinical symptoms. Clinical and laboratory features associated with SLE were analysed by both uni and then multivariate analysis (logistic regression with the significant variables in univariate analysis). Mann-Whitney test was used to compare SLICC distributions and Odds Ratio was calculated with a binary logistic regression. The prevalence of genotypes in healthy controls was examined for deviation from Hardy–Weinberg equilibrium by using exact Chi-square test.

Paired Wilcoxon Signed Ranked Test, because the data did not follow a Gaussian distribution, was used to compare Vitamin D levels taken at baseline (before Vitamin D supplementation, D0) with those taken at the end of the studies (M6 and D21). The miR-146a relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. Differences in ΔCt were evaluated using two-tailed Student's t-test (severely active SLE patients study).

A p-value below 0.05 was considered to be statistically significant. Statistical analyses were performed using Statistical Package for the Social Sciences software (version 23, IBM SPSS Statistics, NY, USA).

3.6. Ethical approval

Written informed consent was obtained for each volunteer, and the study was approved by the Ethics Committee of Centro Hospitalar do Porto, according to Declaration of Helsinki. Studies using DNA databank and data storage, were approved by National Commission of Data Security

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CHAPTER 4

RESULTS AND CONCLUSIONS

4.1. EPIDEMIOLOGIC STUDIES

4.1.1. Vitamin D levels in a healthy population from the North of Portugal

(Submitted to The Journal of Steroid Biochemistry and Molecular Biology 2016)

The study was conducted in Porto (~41° N), in July and August 2015 (summer time) and April 2016 (winter time). The average age of these individuals was 43.1±12.1 years. Subjects were stratified in three groups according to age. Two men were excluded because they were under multivitamin supplementation. In order to analyse the influence of BMI on the 25(OH)D levels, the subjects were categorized into two groups based on BMI values: BMI<30 kg/m2 (non-obese) or BMI≥30 kg/m2 (obese).

The characteristics of the study population are shown in Table1. Approximately 48% were women, and the mean age (±SD) of the study population was 43.1±12.1 years. No statistically significant differences were observed between genders. The frequency of obesity was significantly higher in this population compared with the general Portuguese population (22.7% vs. 14.2%, p=0.001, OR=1.77, 95%CI=1.26-2.50) (do Carmo et al., 2008).

The mean serum 25(OH)D concentration was 55.4±23.4 nmol/L in all participants (median 50.9 nmol/L) with no significant differences between men and women (57.4±23.9 vs. 53.3±22.8 nmol/L; p=0.219). Fifty women (52.6%) and 45 men (43.7%) were deficient in 25(OH)D (Table 12).

No statistically significant correlation was found between age and 25(OH)D levels (p=0.349). When subjects were categorized in groups according to age (Figure 7), no differences in 25(OH)D levels between the three groups (p=0.311) were observed.

Table 12 - Characteristics of the study population.

Characteristics	Total (n=198)	Women (n=95)	Men (n=103)
Sociodemographics			
Age, years, mean ± SD	43.1±12.1	41.9±12.5	44.2±11.7
Season			
Summer, n (%)	101 (51.1)	44 (46.3)	57 (55.3)
Winter, n (%)	97 (49.0)	51 (53.7)	46 (44.7)
BMI, mean ± SD	27.0±4.3	26.9±4.4	27.2±4.2
Laboratory measurements			
PTH levels (pg/mL), mean ± SD	44.9±14.7	45.6±12.6	44.3±16.5
Creatinine levels (mg/dL), mean ± SD	0.8±0.2	0.8±0.1	0.8±0.2
Total calcium levels (mmol/L), mean ± SD	2.4±0.1	2.4±0.1	2.4±0.1
Phosphorus levels (mmol/L), mean ± SD	1.0±0.2	1.0±0.2	1.0±0.2
25(OH)D levels (nmol/L)			
Mean ± SD	55.4±23.4	53.3±22.8	57.4±23.9
<50 nmol/L (deficiency), n (%)	95 (48.0)	50 (52.6)	45 (43.7)
50-75 nmol/L (insufficiency), n (%)	60 (30.3)	27 (28.4)	33 (32.0)
>75 nmol/L (optimal), n (%)	43 (21.7)	18 (18.9)	25 (24.3)

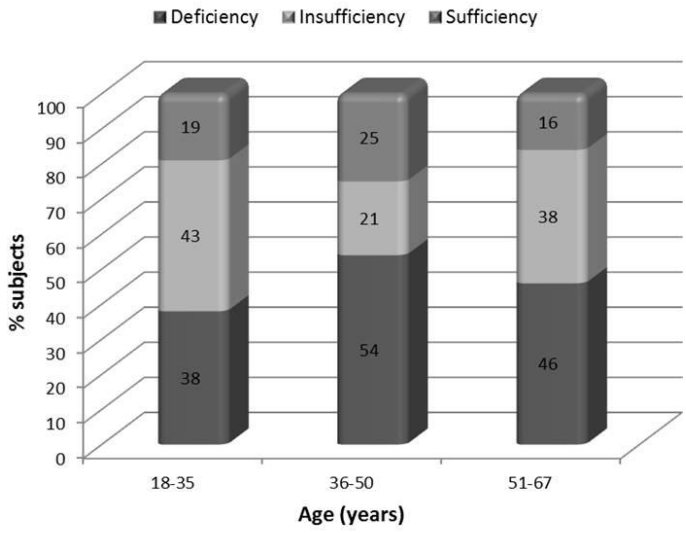

Figure 7 - Prevalence of Vitamin D deficiency and insuficiency by age.

BMI was negatively correlated with 25(OH)D levels (p=0.001, r=-0.237) (Figure 9). In conformity, 25(OH)D levels were significantly lower in obese compared to non-obese subjects (46.6±17.6 vs. 57.7±24.2 nmol/L, p=0.012) (Figure 8).

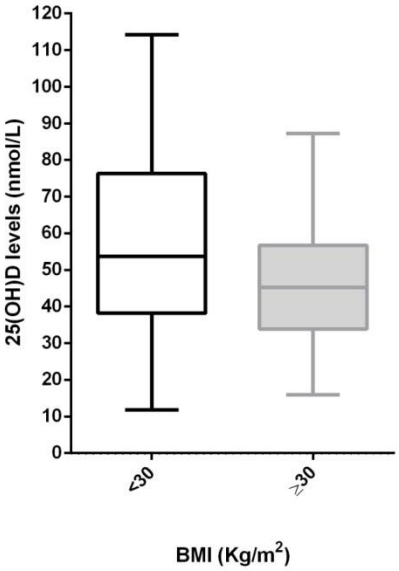

Figure 8 - Comparison of serum 25(OH)D levels between obese (BMI≥30) and non-obese (BMI<30) individuals.

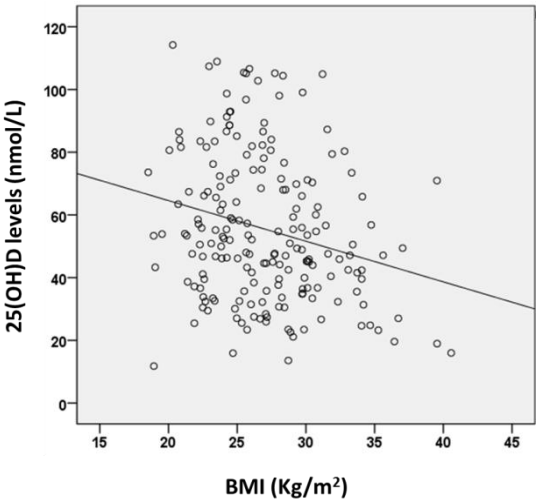

Figure 9 - Correlation between 25(OH)D and BMI.

In the winter period, 74.2% of the studied population had a 25(OH)D concentration below 50.0 nmol/L compared with 22.8% in the summer period ($p<0.0001$). Only five individuals (5.2%) presented optimal levels of 25(OH)D in winter, and 38 (37.6%) in summer (Table 13).

Table 13 – Differences in 25(OH)D concentrations according to season.

25(OH)D levels (nmol/L)	Winter (n=97)	Summer (n=101)	p
Mean ± SD	42.2±16.9	68.2±21.5	<0.0001
<50 nmol/L (deficiency), n (%)	72 (74.2)	23 (22.8)	
50-75 nmol/L (insufficiency), n (%)	20 (20.6)	40 (39.6)	<0.0001
>75 nmol/L (optimal), n (%)	5 (5.2)	38 (37.6)	

In multiple linear regression analysis, controlling for age and gender, significant associations between 25(OH)D levels and season and BMI were found. Winter and higher BMI were significantly associated with lower serum 25(OH)D levels (Table 14).

Table 14 - Results of a multiple linear regression analysis on determinants of 25(OH)D levels.

Variable	B	SE	p
Intercept	4.224	0.189	<0.0001
Age	-0.002	0.002	0.499
Season	0.482	0.054	<0.0001
Gender	-0.40	0.054	0.456
Body Mass Index (kg/m²)	-0.17	0.007	0.010
Corr .r^2=0.341			

4.1.2. Vitamin D levels in a SLE population from the North of Portugal

(Unpublished results)

The study was conducted in Porto (~41° N), for the last 10 years. Subjects were stratified in two groups according to winter and summer Vitamin D levels. Data were compared with 198 healthy blood donors. Patients taking Vitamin D supplements were excluded.

The characteristics of the study population are shown in Table 15. In SLE patients, 85% had Vitamin D deficiency. The mean Vitamin D levels were 48.9±27.2 and there were no significant differences between summer and winter (p=0.117). Comparing with the control group, a significant difference was only observed during the summer time (p=0.0003).

Table 15 - Vitamin D levels in SLE patients *vs.* Control Population.

	SLE Patients (n=124)			**Control Population (n=198)**			**p**	
25(OH)D levels (nmol/L)	**Total**	**Winter (n=54)**	**Summer (n=70)**	**Total**	**Winter (n=97)**	**Summer (n=101)**	**Winter**	**Summer**
Mean ± SD	48.9±27.2	43.0±21.4	53.5±30.2	55.4±23.4	42.2±16.9	68.2±21.5	0.799	0.0003
<50 nmol/L (deficiency), n (%)	72 (58.1)	38 (70.4)	34 (48.6)	95 (48.0)	72 (74.2)	23 (22.8)		
50-75 nmol/L (insufficiency), n (%)	34 (27.4)	11 (20.4)	23 (32.8)	60 (30.3)	20 (20.6)	40 (39.6)	0.621	0.001
>75 nmol/L (optimal), n (%)	18 (14.5)	5 (9.2)	13 (18.6)	43 (21.7)	5 (5.2)	38 (37.6)		

Conclusions

Hypothesis 1: *Vitamin D levels in our SLE patients are lower than in a healthy population with the same demographic characteristics (age and sex).*

- Vitamin D is insufficient in at least two thirds of Portuguese healthy adult population and 85% of SLE patients.

- SLE patients' mean levels are lower than in general population, but only during the summertime.

- Hypovitaminosis D in SLE population may differ from the observed in healthy population, due to deficient sun exposure during summer time, and not due to the disease effect on Vitamin D metabolism.

4.2. IMMUNOGENETIC STUDIES

4.2.1. The Protective Role of HLA-DRB1*13 in Autoimmune Diseases

(J Immunol Res. 2015; 2015:948723. doi: 10.1155/2015/948723)

The HLA-DRB1 frequencies in all the patients were compared with the ones of a control group consisting of 282 unrelated individuals without disease and with the same geographic origin: Portugal. With this methodology, different types of association between alleles and AIDs were found (Table 16). These included three risk alleles for two or more AIDs, two protective alleles for two or more AIDs and three risk alleles for a particular AID.

HLA-DRB1*13 was a protective allele for four AIDs: SLE (OR=0.58, p=0.016), Psoriasis and Psoriatic Arthritis (OR=0.621, p=0.050), RA (OR=0.58, p=0.044) and SSc (OR=0.42, p=0.035).

There was one risk allele associated with three AIDs. HLA-DRB1*03 was found to be a risk factor for SLE (OR=2.49, p=4.2x10-5), MS (OR=1.81, p=0.003) and MG (OR=2.98, p=6.1x10-5). There were two risk alleles associated with two AIDs: HLA-DRB1*08 were positively associated with MS (OR=1.73, p=0.033) and SSc (OR=3.01, p=0.004) and HLA-DRB1*01 was found to be a risk factor for RA (OR=1.79, p=0.017) and SSc (OR=2.28, p=0.006).

HLA-DRB1*09 was negatively associated with SLE (OR=0.18, p=0.027) and MS (OR=0.22, p=0.004).

Three risk disease-specific alleles were found. HLA-DRB1*04 for RA (OR=2.81, p=6x10-6), HLA-DRB1*07 for Psoriasis and Psoriatic Arthritis (OR=1.79, p=0.006) and HLA-DRB1*15 for MS (OR=2.17, p=2x10-5).

Considering the total of AIDs, HLA-DRB1*03 frequencies were significantly higher (p=0.026, OR=1.49) in the total of patients compared with controls; conversely HLA-DRB1*13 (p=0.003, OR=0.64) and HLA-DRB1*09 (p=0.001, OR=0.31) frequencies were significantly lower.

Table 16 - Associations between HLA class II and six AIDs: SLE, Psoriasis (Ps) and Psoriatic Arthritis (PsA), RA, SSc, MS, and MG.

	Controls (n=282)	**SLE (n=213)**	**Ps+PsA (n=166)**	**RA (n=153)**	**SSc (n=67)**	**MS (n=536)**	**MG (n=93)**	**TOTAL (n=1228)**
HLA-DRB1*01	66 (23.4%)	40 (18.8%)	39 (23.5%)	50 (32.7%) OR=1.79 p=0.017	28 (41.8%) OR=2.28 p=0.006	100 (18.7%)	23 (24.7%)	280 (22.8%)
HLA-DRB1*03	44 (15.6%)	73 (34.3%) OR=2.49 $p=4.2X10^{-5}$	25 (15.1%)	28 (18.3%)	11 (16.4%)	123 (22.9%) OR= 1.81 p=0.003	33 (35.5%) OR=2.98 $p=6.1X10^{-5}$	293 (23.9%) OR=1.51 p=0.022
HLA-DRB1*04	69 (24.5%)	42 (19.7%)	46 (27.7%)	73 (47.7%) OR=2.81 $p=6X10^{-6}$	13 (19.4%)	128 (23.9%)	23 (24.7%)	325 (26.5%)
HLA-DRB1*07	72 (25.5%)	55 (25.8%)	66 (39.8%) OR=1.79 p=0.006	38 (24.8%)	14 (20.9%)	147 (27.4%)	23 (24.7%)	343 (27.9%)
HLA-DRB1*08	24 (8.5%)	21 (10.0%)	10 (6.0%)	3 (2.0%) OR=0.24 p=0.026	15 (22.4%) OR=3.01 p=0.004	65 (12.1%) OR=1.73 p=0.033	7 (7.5%)	121 (9.9%)
HLA-DRB1*09	14 (5.0%)	2 (1.0%) OR=0.18 p=0.027	5 (3.0%)	0 (0.0%)	3 (4.5%)	5 (1.0%) OR=0.22 p=0.004	2 (2.2%)	17 (1.4%) OR=0.23 $p=1x10^{-4}$
HLA-DRB1*13	84 (29.8%)	39 (18.3%) OR=0.58 p=0.016	32 (19.3%) OR=0.62 p=0.050	25 (16.3%) OR=0.58 p=0.044	8 (11.9%) OR=0.42 p=0.035	124 (23.1%)	17 (18.3%)	245 (20.0%) OR=0.58 p=0.004
HLA-DRB1*15	56 (19.9%)	55 (25.8%)	22 (13.3%)	17 (11.1%)	12 (17.9%)	175 (32.7%) OR=2.17 $p=2X10^{-5}$	15 (16.1%)	296 (24.1%)

4.2.2. Association between VDR gene polymorphisms and Systemic Lupus Erythematosus in Portuguese Patients.

(Lupus. 2015 Jul;24(8):846-53.doi:10.1177/0961203314566636)

The demographic and clinical features of the 170 (15 M and 155 F) patients with SLE are presented in Table 17. Patients with less than 5 years of disease where not considered.

The average age of the SLE patients was 45 ± 13.4 years and the mean disease duration was 14.5 ± 6.5 (7 – 43) years. The subjects without known autoimmune disease had an average age of 47 ± 12.8 years.

Phenotypic and genotypic frequencies for VDR SNPs were in Hardy-Weinberg equilibrium.

No statistically significant differences were observed between frequencies of the VDR polymorphisms in the patients group when compared with controls as shown in Table 18.

The relationship between VDR polymorphisms and disease expression was analysed in patients, with complete clinical data compiled, according to the clinical manifestations listed in table 17 and malar rash, photosensitivity, leukopenia, lymphopenia, pericarditis, pleuritis, lung involvement, myositis, ascitis, serositis as well as SLICC scores, number of affected organs, number of severe flares and the need of steroids or immunosuppressive therapy during disease duration. No associations were found between VDR SNPs and all the previously referred clinical features, except with skin involvement where the ApaI TC genotype frequency was higher in the patient group (p=0.038).

No significant differences were observed in number of affected organs, number of severe flares and the probability of long-term need of steroids or immunosuppressive therapy during disease duration.

The only exception was the association of different polymorphisms with damage accrual, namely, the significantly higher frequencies of VDR genotypes TaqI TT and FokI CT found in patients with SLICC>0 (Table 19).

SLICC values were higher in SLE patients with FokI CT and TaqI TT genotypes (p=0.031 and p=0.046 respectively, Figure 10).

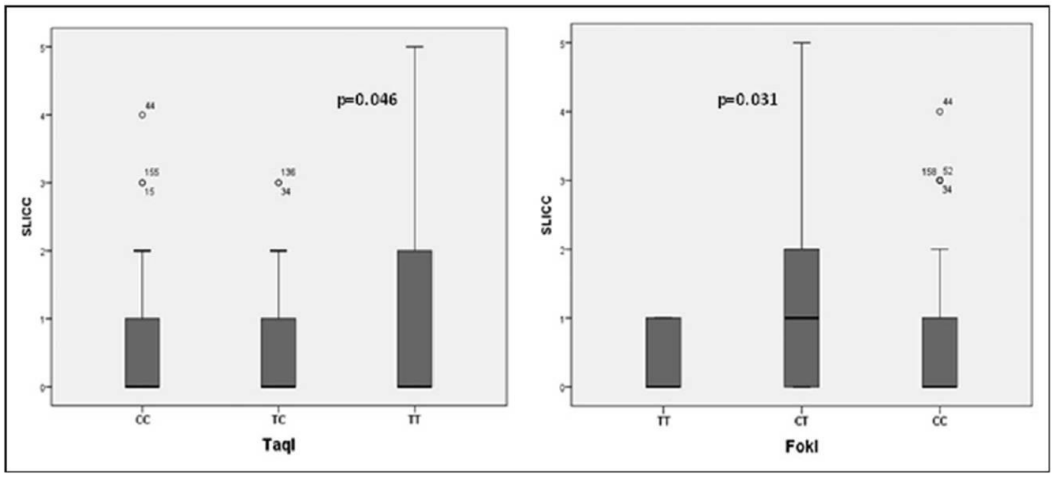

Figure 10 - Box-whisker plots of SLICC distribution according to FokI and TaqI genotype, in SLE patients. SLICC: Systemic Lupus International Collaborative Clinics; SLE: systemic lupus erythematosus.

Table 17 – Demographic and clinical/laboratory features of SLE patients.

Patients Features	n / n total	(%)
Male	15/170	(9)
Female	155/170	(91)
Age (years)	45 (±13.4)	
> 10 years of disease	118/170	(69)
ANA+	170/170	(100)
ANTI ds-DNA	90/170	(56)
Haematological abnormalities	101/170	(59)
Arthritis	120/170	(71)
Skin involvement	140/170	(82)
Nephritis	48/170	(28)
Type III	4/48	(8)
Type IV	31/48	(65)
Type V	8/48	(17)
Type VI	2/48	(4)
NS involvement	13/170	(8)
Immunosuppressors/steroids *	161/170	(95)
Severe Flares	147/170	(86)
>3 affected organs or systems	42/170	(25)

* Patients with immunosuppressors or steroids at the time of clinical evaluation.

Table 18 - Genotype and phenotype frequencies of VDR polymorphisms in SLE patients and Control Population.

Polymorphism	SLE patients n (%)		Control Population n (%)		Odds Ratio (95% CI)		p-value
rs1544410 (BsmI)	161		186				
Allele							
A	105	(65.2%)	131	(70.4%)	0.89	(0.65-1.22)	0.47
G	133	(82.6%)	148	(79.6%)	1.06	(0.79-1.44)	0.68
Genotype							
AA	28	(17.4%)	38	(20.4%)	0.11	(0.10-1.36)	0.09
AG	77	(47.8%)	93	(50.0%)	0.59	(0.18-1.88)	0.37
GG	56	(34.8%)	55	(29.6%)	1		
rs7975232 (ApaI)	167		187				
Allele							
T	132	(79.0%)	159	(85.0%)	0.88	(0.65-1.19)	0.42
G	117	(70.1%)	129	(68.9%)	1.02	(0.75-1.40)	0.88
Genotype							
TT	50	(29.9%)	58	(31.0%)	0.97	(0.36-2.57)	0.95
TG	82	(49.1%)	101	(54.0%)	0.78	(0.37-1.63)	0.5
GG	35	(21.0%)	28	(15.0%)	1		
rs731236 (TaqI)	169		179				
Allele							
C	107	(63.3%)	127	(70.9%)	0.84	(0.61-1.15)	0.29
T	142	(84.0%)	145	(81.0%)	1.06	(0.79-1.44)	0.69
Genotype							
CC	27	(16.0%)	34	(19.0%)	5.6	(0.46-67.9)	0.17
CT	80	(47.3%)	93	(52.0%)	1.39	(0.43-4.51)	0.58
TT	62	(36.7%)	52	(29.1%)	1		
rs2228570 (FokI)	164		192				
Allele							
T	85	(51.8%)	102	(53.1%)	0.97	(0.69-1.35)	0.84
C	147	(88.0%)	174	(90.6%)	0.98	(0.73-1.32)	0.89
Genotype							
TT	17	(10.4%)	18	(9.4%)	1.08	(0.50-2.31)	0.85
CT	68	(41.5%)	84	(43.8%)	0.86	(0.54-1.37)	0.53
CC	79	(48.2%)	90	(46.9%)	1		

Table 19 - Genotype and phenotype frequencies of VDR polymorphisms in SLE patients with SLICC=0 and SLICC>0.

	VDR polymorphism	SLICC=0 n (%)		SLICC>0 n (%)	
	rs731236 (TaqI)	98		63	
Allele	C	68	(69.4%)	34	(54.0%)
	T	81	(82.7%)	55	(87.3%)
	CC	17	(17.3%)	8	(12.7%)
Genotype	CT	51	(52.0%)	26	(41.3%)
	TT	30	(30.6%)	29	(46.0%)
	rs2228570 (FokI)	93		63	
Allele	T	44	(47.3%)	39	(61.9%)
	C	81	(87.1%)	58	(92.1%)
	TT	12	(13.3%)	5	(7.9%)
Genotype	CT	32	(35.6%)	34	(54.0%)
	CC	40	(51.1%)	24	(38.1%)

4.2.3. Relation of Vitamin D levels with SLE number of severe flares

(Unpublished results)

For this study, we correlated the baseline Vitamin D levels with severe flare number and with patients with three or more and less than three severe flares. We also correlated severe flares with mean Vitamin D value in the last 10-years follow-up.

We compared baseline Vitamin D levels of 112 SLE patients with the number of severe flares.

We observed a higher number of flares in patients with low baseline Vitamin D levels (p=0.045) (Figure 11). We also observed that patients with three or more flares have significant lower baseline Vitamin D levels (p=0.004) (table 20).

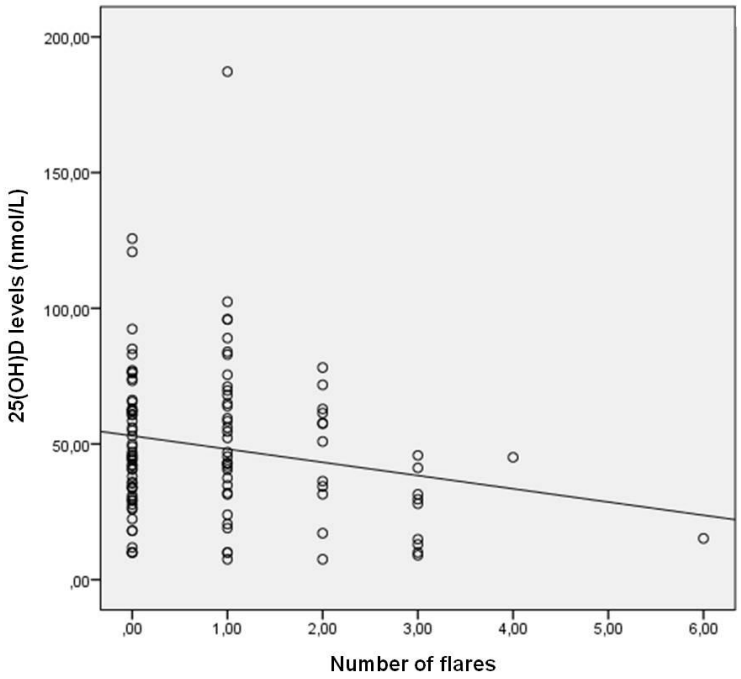

Figure 11 - Inverse correlation between baseline 25(OH)D levels and number of severe flares (p=0.045).

Table 20 - Relation between less than and more or equal to three severe flares and baseline 25(OH)D levels.

	<3 Flares (n=101)	≥3 Flares (n=11)	p
Baseline 25(OH)D levels (nmol/L)	**51.34 ± 28.24**	**25.74±14.09**	**0.004**

The mean Vitamin D levels in the previous 10 years of disease in 68 patients, were lower in patients with higher number of severe flares, although not significant (p=0.178) (Figure 12). However, if we categorized them in two subgroups (patients with three or more and less than three severe flares), the difference is significant (p=0.044) (table 21).

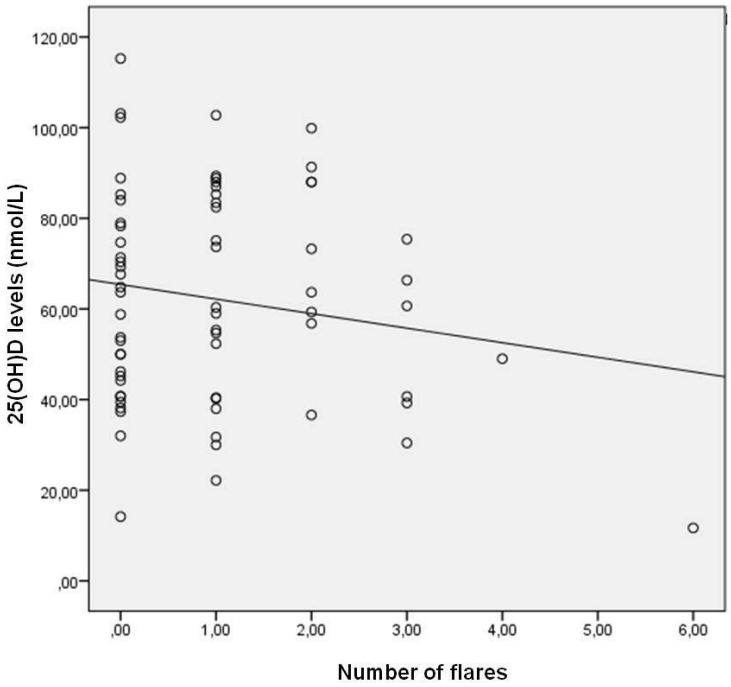

Figure 12 - Inverse correlation between mean 25(OH)D levels over the last 10 years and number of severe flares (p=0.178).

Table 21 - Relation between less than and more or equal to 3 severe flares and mean 25(OH)D levels over the past 10 years.

	<3 Flares (n=60)	≥3 Flares (n=8)	p
Mean 25(OH)D levels (nmol/L)	64.3 ± 23.0	46.6±20.6	0.044

Conclusions

Hypothesis 2: *Vitamin D status is related to SLE susceptibility.*

- We cannot answer this question with this study. However, we confirm genetic data that can allow new studies able to determine Vitamin D effect in SLE susceptibility.

- We confirmed the HLA-DRB1*03 risk and the HLA-DRB1*13 protective effect to SLE. The possibility of this effect to be mediated by the canonical VDRE presence in HLA-DRB1 genes should be explored. Low Vitamin D levels in mothers during pregnancy and in early childhood could enhance this effect.

- The effect of HLA-DRB1 alleles (*01 and *13 in Lupus) should be studied in different latitudes to prove that optimal sun exposure (and Vitamin D levels) could be one of the causes of lower incidence of AIDs.

Conclusions

Hypothesis 3: *SLE severity is related to VDR SNPs*.

- VDR polymorphisms (TaqI and FokI variants) are related to damage accrual in SLE patients.

- These effects seem to be unrelated to Vitamin D levels.

- VDR polymorphisms are not related to flares or specific organ involvement.

Conclusions

Hypothesis 4: *Vitamin D deficiency is associated with SLE severity*.

- Baseline Vitamin D levels at SLE diagnosis (before supplementation) correlate with the number of severe flares and specifically in patients with three or more severe flares during follow-up.

- Mean Vitamin D levels in a 10-year follow-up of SLE patients are related to the number severe flares (p= 0.178), but especially in those who had three or more severe flares (p=0.044).

- Vitamin D levels at the beginning of the disease and the Vitamin D burden during disease are related to the number of severe flares, leading to phenotypes that are more aggressive.

4.3. INTERVENTIONAL STUDIES

4.3.1. Vitamin D supplementation effects on FoxP3 expression in T cells and FoxP3$^+$/IL-17A ratio and clinical course in systemic lupus erythematosus patients: a study in a Portuguese cohort.

(Immunol Res. 2016 Jul 16. [Epub ahead of print]. doi: 10.1007/s12026-016-8829-3)

Patients were recruited between 1 November 2012 and 31 January 2013. By protocol, all these patients should have more than 5 years of disease duration, and should be in a stable phase of disease and without major flares for at least 1 year. Stability was defined as no new major organ involvement in the last year independently of the SLEDAI-2K score and no changes in steroid dose or immunosuppressive therapy in the last 12 months. Baseline 25(OH)D serum level was measured. Hypovitaminosis D was defined as serum 25(OH)D <75 nmol/L and patients were selected irrespectively of their baseline 25(OH)D levels.

Demographic, clinical and laboratorial features of SLE patients at baseline are described in Table 22. Serum 25(OH)D levels significantly increased under Vitamin D supplementation from 59.32 ± 29.59 nmol/L at Day 0 to 80.39 ± 24.57 nmol/L at Month 3 (p=0.030) and to 85.25 ± 30.92 nmol/L at Month 6 (p=0.001) (Figure 13).

Treatment was safe, with no significant increase of serum phosphorus or calcium levels. Serum calcium significantly decreased from 2.34 ± 0.10 mmol/L at Day 0 to 2.27 ± 0.10 mmol/L at Month 6 (p=0.026) but all in the normal range.

Disease activity, assessed by SLEDAI-2K, significantly decreased from 2.75 ± 4.76 at Day 0 to 1.67 ± 2.79 at Month 6 (p=0.026) (Figure 14).

Serum anti-dsDNA levels (evaluated by immunofluorescence) remained stable during follow-up, while C3 complement fraction significantly decreased from 101.5 ± 23.57 mg/dL at Day 0 to 95.46 ± 21.71 mg/dL at Month 6 (p=0.013), but no new hypocomplementemia occurred (reference values: 88-201 mg/dL).

None of the patients required modification of the prednisone and immunosuppressive dosage or initiation of new immunosuppressive agents. We did not observe SLE flares during the 6 months' follow-up period.

The impact of Vitamin D supplementation on the proportions of $CD3^+$ T cells, $CD4^+$ and $CD8^+$ T cells is shown in Figure 15.

The mean proportions at baseline were 80.05 ± 5.64% for $CD3^+$ T cells (Figure 15A), 47.8 ± 7.58% for $CD4^+$ T cells (Figure 15B), and 30.56 ± 7.73% for $CD8^+$ T cells (Figure 15C). At Month 6, the proportion of $CD3^+$ T cells, $CD4^+$ and $CD8^+$ T cells remained stable.

The impact of Vitamin D supplementation on $CD3^+CD4^+FoxP3^+$ T cells was evaluated. The percentage of $CD4^+FoxP3^+$ cells at baseline was 7.27 ± 5.92%. The percentage of $CD4^+FoxP3^+$ cells was increased at 11.42 ± 6.54% at Month 6 ($p<0.001$) (Figure 16A). A decrease was observed in $CD4^+$IL-17A from 3.89 ± 1.76% at Day 0 to 2.82 ± 1.03% at Month 6 ($p=0.001$) after Vitamin D supplementation (Figure 16B).

Accordingly, the $FoxP3^+$/IL-17A ratio in patients with SLE after 6 months of Vitamin D supplementation was higher than that in the baseline ($p<0.001$) (Figure 17).

Considering patients with 25(OH)D levels at baseline ≥75 nmol/L, the increase of $FoxP3^+$/IL-17A ratio observed for the entire group after 6 months of Vitamin D supplementation was also valid ($p=0.043$) (Figure 18).

The relationship between $CD4^+FoxP3^+$ and $CD4^+$IL-17A cells and 25(OH)D levels, serum calcium, serum phosphorus and serum PTH levels was also analysed. No associations were found between these features and $CD4^+FoxP3^+$ and $CD4^+$IL-17A proportions.

No significant differences were observed for the other laboratory and clinical parameters, such as SLICC score.

Table 22 – Demographic, clinical and laboratorial features of SLE patients at baseline. All patients completed the 6 months' follow-up period.

Characteristics	N=24
Epidemiology	
Age, years, mean ± SD	47 ± 11
Female gender, n (%)	23 (95.83%)
Previous SLE manifestations	
Skin, n (%)	19 (79,2%)
Joints, n (%)	18 (75%)
Serositis, n (%)	4 (16.7%)
Kidney, n (%)	7 (29.2%)
Nervous system, n (%)	3 (12.5%)
Flares, n (%)	10 (41.7%)
Flares, mean (range)	0.66 (0-3)
SLEDAI/SLICC and analytical studies	
SLEDAI at Day 0, mean (range)	2.71 (0-21)
SLICC at Day 0, mean (range)	1.04 (0-5)
Calcium levels, mean (range), mmol/L	2.34 (2.12-2.52)
Phosphorus levels, mean (range), mmol/L	1.02 (0.65-1.4)
C3 levels at Day 0, mean (range), mg/dL	101.5 (51-147)
C4 levels at Day 0, mean (range), mg/dL	16.5 (6-35)
Anti-dsDNA levels at Day 0, mean (range), IU/mL	34.15 (0.2-302)
% $CD3^+$ levels at Day 0, mean (range)	80.05 (69.1-90.5)
% $CD3^+CD4^+$ levels at Day 0, mean (range)	47.8 (39-62.5)
% $CD3^+CD8^+$ levels at Day 0, mean (range)	30.55 (18.2-44.9)
%T $CD4^+$ $FoxP3^+$ at Day 0, mean (range)	7.27 (0.48-15.57)
%T $CD4^+IL17^+$ at Day 0, mean (range)	3.89 (1.19-8.58)
%T $CD4^+$ $FoxP3^+$/%T $CD4^+$ $IL17^+$ ratio at Day 0, mean (range)	2.23 (0.16-8.9)
25-hydroxyvitamin D levels	
Mean ± SD, nmol/L	59.3 ± 29.6
25(OH)D ≤ 25 nmol/L, n (%)	2 (8.3%)
25 < 25(OH)D ≤ 50 nmol/L, n (%)	8 (33.3%)
50 < 25(OH)D ≤ 75 nmol/L, n (%)	7 (29.2%)
25(OH)D > 75 nmol/L, n (%)	7 (29.2%)
Associated treatments*	
Prednisone, n (%)	15 (62.5%)
Prednisone <10 mg	11
Prednisone 10-20 mg	3
Prednisone >20 mg	1
Hydroxychloroquine, n (%)	17 (70.8%)
Azathioprine, n (%)	2 (8.3%)
Mycophenolate mofetil, n (%)	3 (12.5%)

*At the time of the study.

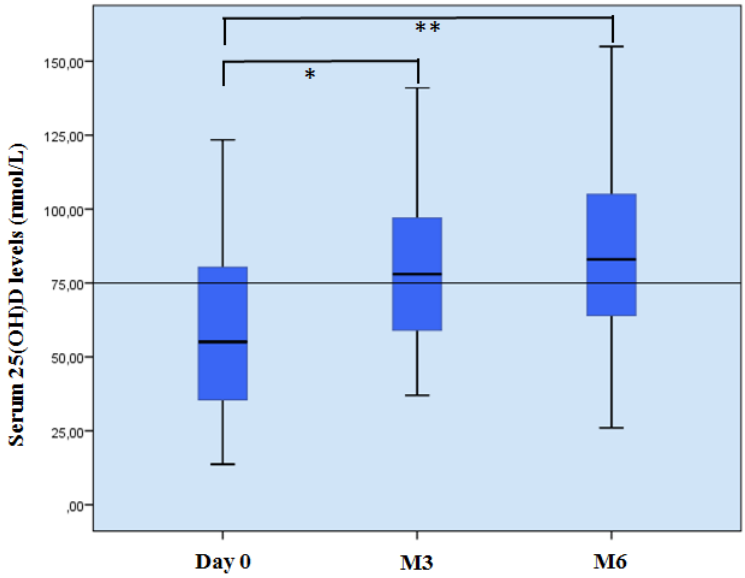

Figure 13 - Time evolution of serum 25(OH)D levels (M3 – month 3; M6 – month 6); *p<0.05, **p<0.01.

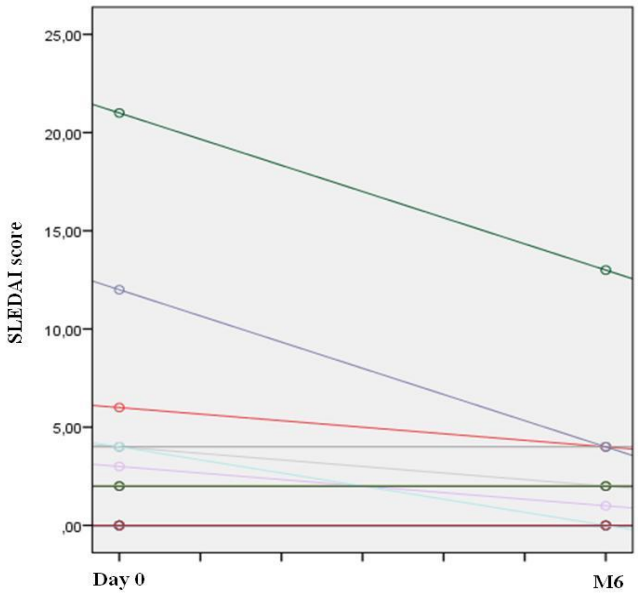

Figure 14 - Disease activity assessed by SLEDAI-2K scores.

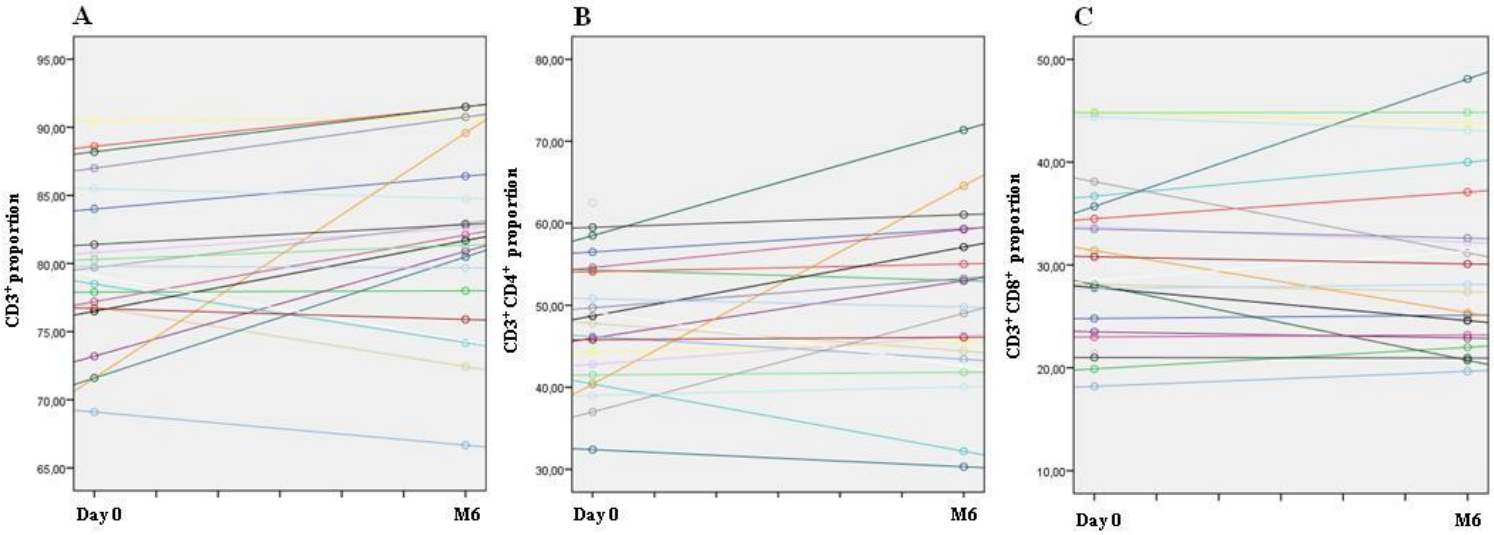

Figure 15 - Evolution of peripheral blood lymphocytes proportions with Vitamin D supplementation. $CD3^+$ (A), $CD3^+CD4^+$ (B) and $CD3^+CD8^+$ (C) proportions at Day 0 and M6.

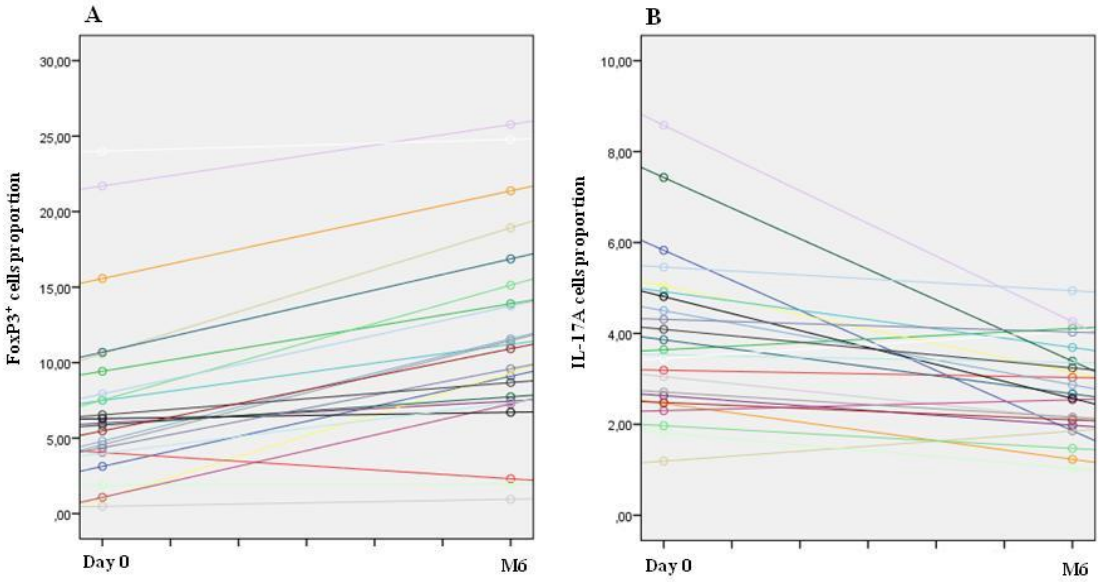

Figure 16 - Variation of the proportions of $CD4^+FoxP3^+$ T cells (A) and $CD4^+$IL-17A T cells (B) after Vitamin D supplementation.

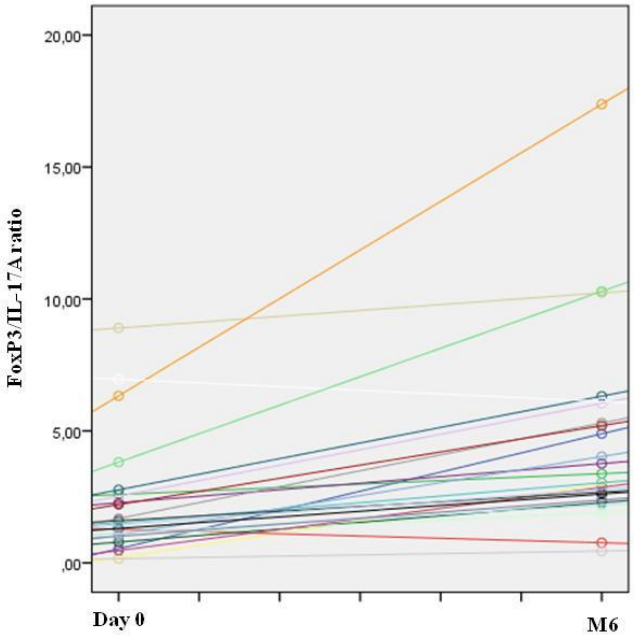

Figure 17 - Variation of FoxP3^{+}/IL-17A ratio after Vitamin D supplementation.

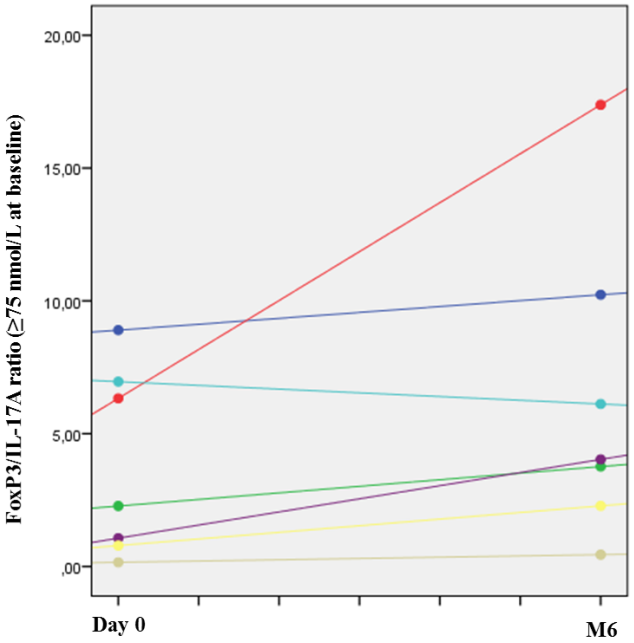

Figure 18 - Variation of FoxP3^{+}/IL-17A ratio in patients group with ≥75 nmol/L 25(OH)D levels (at baseline) after Vitamin D supplementation.

4.3.2. Effect of high dose cholecalciferol during SLE flares in miR-146a expression, regulatory T-Cells and IL-17A expression.

(Unpublished results)

The characteristics of the studied patients and Vitamin D levels before and after high-dose supplementation are presented in table 23.

Four (4) SLE patients with severely active disease were selected. Severely active disease was defined as having a SLEDAI2-K score > 6 and a least one BILAG A for organ or system.

Regarding Vitamin D levels, even with Vitamin D high-doses, no significant differences were observed before and after supplementation.

Table 23 - Baseline disease characteristics and vitamin D levels before and after supplementation.

	Patient 1	*Patient 2*	*Patient 3*	*Patient 4*
BILAG	A: skin, arthritis.	A: skin, arthritis.	A: skin, B: arthritis, D: renal.	A: lung
SLEDAI	8	10	12	8
Baseline 25(OH)D (nmol/L)	66	110	138	108
25(OH)D dose (IU)	100.000/ week x3	50.000/ week x3	50.000/ week x3	100.000/ week x3
25(OH)D after supplementation (nmol/L)	84	131	129	130

Considering IL-17A T $CD4^+$ producing cells, Tregs and Tregs/IL-17A ratio before and after supplementation, no enhancement of Tregs or Tregs/IL-17A was observed (table 24).

Table 24 - $CD25^+FoxP3^+CD4^+$ T-cells and $IL\text{-}17A^+CD4^+$ T-cells proportions in 4 SLE patients after Vitamin D therapy.

	Baseline			**After Vitamin D Shot**			**p**
Patients	**%$CD25^+$ $FoxP3^+CD4^+$**	**%$IL\text{-}17A^+$ $CD4^+$**	**Ratio (Treg/IL-17A)**	**%$CD25^+$ $FoxP3^+$ $CD4^+$**	**%$IL\text{-}17A^+$ $CD4^+$**	**Ratio (Treg/IL-17A)**	**Ratio**
1	13.88	5.31	2.61	9.41	4.29	2.19	0.054
2	8.88	2.02	4.40	5.11	2.37	2.16	
3	8.46	5.87	1.44	1.51	5.57	0.27	
4	5.04	3.4	1.48	1.79	3.17	0.56	

No significant differences were found in miR-146a expression between SLE patients (before and after Vitamin D supplementation) and controls (Figure 19).

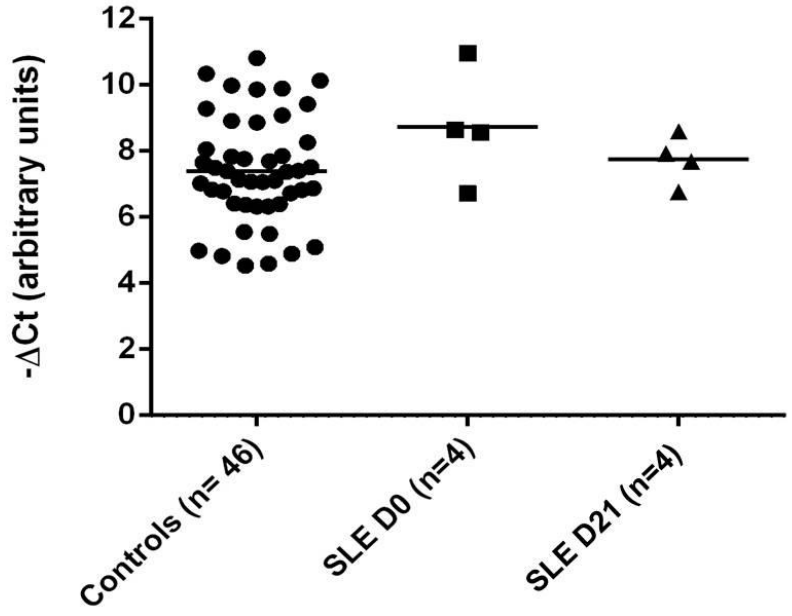

Figure 19 – miR-146a expression before and after Vitamin D supplementation.

Conclusions

Hypothesis 5: *Supplementation with Vitamin D alters the clinical expression and immunological deregulation of SLE patients (T-CD4$^+$ cells expressing FoxP3 and T-CD4$^+$ cells producing IL-17A).*

- Vitamin D supplementation seems to provide favourable immunological effects in SLE patients with stable and low disease activity;
- This effect is probably independent of the 25(OH)D patient's status.

Conclusions

Hypothesis 6: *Clinical and Immunological response to Vitamin D supplementation is limited by SLE disease activity*.

- Severe SLE activity may cause resistance to Vitamin D therapeutical effects, including enhancing of Vitamin D levels and immunogenetic effects.

CHAPTER 5

GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1. Epidemiologic studies

5.1.1. Discussion of hypothesis 1: *Vitamin D levels in our SLE patients are lower than in a healthy population with the same demographic characteristics (age and sex).*

The first issue of this thesis was to prove that healthy Portuguese population is Vitamin D deficient.

There is ongoing debate related to the optimal levels of 25(OH)D, but all available evidences suggest that children and adults should maintain a 25(OH)D blood level above 50 nmol/L to prevent rickets and osteomalacia, respectively. However, to maximize Vitamin D effect on calcium, bone and muscle metabolism, the 25(OH)D blood level should be above 75 nmol/L. Numerous epidemiological studies have suggested that a 25(OH)D blood level above 75 nmol/L may have additional health benefits in reducing the risk of common cancers, autoimmune diseases, type 2 diabetes, cardiovascular disease, and infectious diseases (Grober, Spitz, Reichrath, Kisters, & Holick, 2013).

Deficient Vitamin D status of the general population in the USA, Canada, Asia Pacific, Middle East, Africa and across Europe (Hilger et al., 2014) is well documented, being more prevalent in winter, in women, in older age groups, in individuals with dark skin, and at higher latitudes (Nair & Maseeh, 2012).

To the best of our knowledge, we conducted the first epidemiologic study in a Portuguese healthy population. In our study we also observed that almost half of the healthy non-supplemented individuals presented significant Vitamin D deficiency, reaching 74% during the winter period, confirming the well-known seasonal fluctuation in 25(OH)D concentrations. No association between Vitamin D levels and gender was observed, although women presented slightly lower levels of 25(OH)D. Moreover, we did not find any association between Vitamin D status and age. These observations are in line with a recent study suggesting the widespread hypovitaminosis D across Europe, even in countries with abundant sunlight, and with prevalence rates meeting the criteria of a pandemic (Cashman et al., 2016).

Concerning SLE patients, although the common idea that patients with lupus have more Vitamin D deficiency than the normal population, this is only evident in the summer time. Theoretically, SLE severe disease activity would block the effect of the key enzymes for Vitamin D metabolism (D. L. Kamen, 2010). However, our data shows that this effect may not be important, as Vitamin D levels in SLE population during winter time are similar to those observed in the control population. Therefore, our

results suggest that Vitamin D deficiency in patients with lupus may be only related to the performance of solar eviction and not to SLE disease activity.

This is the first time that this approach was accomplished in patients with SLE. Literature data, though extensive and covering the dates or times of the year on which blood collections are made, have important limitations. These limitations are related to study methodologies, being restricted to the study of seasonal hypovitaminosis D effect in flares rate or to Vitamin D deficiency prevalence in non-supplemented SLE patients without a healthy controlled population (Dall'Ara et al., 2015; Munoz-Ortego, Torrente-Segarra, Prieto-Alhambra, Salman-Monte, & Carbonell-Abello, 2012). In summary, to study the prevalence of Vitamin D deficiency in SLE patients, we need to compare them with healthy controls and it is obvious the concomitant need of a non-supplemented SLE cohort.

The negative association of Vitamin D status with obesity is well documented in different studies (Peterson, Tosh, & Belenchia, 2014; Vogt, Baumert, Peters, Thorand, & Scragg, 2016). Concerning our study with healthy blood donors, an inverse correlation was observed between 25(OH)D levels and BMI. This is probably due to the decreased bioavailability of Vitamin D from cutaneous and dietary sources as a result of its sequestration in body fat compartments (Pourshahidi, 2015).

In conclusion, season and BMI are predictors for Vitamin D deficiency in healthy population. As expected, season is also a predictor for lower levels in lupus patients. More significant is the fact that 74% of healthy adults are Vitamin D deficient and that this effect is similar in SLE patients. The fact that levels are identical in wintertime, points to that the main cause of Vitamin D deficiency is not SLE severity.

The strengths of our epidemiologic studies comprise a detailed questionnaire documenting demographic data, blood sampling taking place through summer as well as winter times and a selection of a Vitamin D non-supplemented patients and controls. On the other hand, although the questionnaire in healthy population included data about sun exposure, sunscreens use, eating and smoking habits, physical activity, diagnosed pathologies, and use of medicines and/or food supplements, these parameters were only used to exclude confounding factors that could bias our results, and were not used in the analysis of the results, as that was beyond the scope of the study.

These new data allowed the understanding that hypovitaminosis D is essentially iatrogenic in SLE patients and Vitamin D should be measured and given as supplement, not only to patients but also to populations at risk for AIDs.

5.2. Immunogenetic studies

5.2.1. Discussion of hypothesis 2: *Vitamin D status is related to SLE susceptibility*.

No SLE cohort exists in the world with extensive data on Vitamin D levels since the diagnosis. For this reason, studies relating SLE susceptibility and low Vitamin D levels are not available. On the other hand, it seemed challenging for many investigators the study of the importance of VDR polymorphisms in disease susceptibility (Mao & Huang, 2014; Xiong et al., 2014).

Many studies have been published in different populations, but with inconsistent data and weak statistical correlations (Mao & Huang, 2014; Xiong et al., 2014). However, these populations need controlled Vitamin D levels since childhood, in order to remove the hypovitaminosis D effect in disease susceptibility. Why this need? Because Vitamin D seems essential in central clonal selection, as described in the "introduction" section of this thesis (Ramagopalan et al., 2009).

In our study, different, already described, associations of HLA-DRB1 alleles with different AIDs were observed. We confirmed the positive associations of HLA-DRB1*03, HLA-DRB1*08 and HLA-DRB1*15 with MS in a large population of Portuguese MS patients (Cree, 2014; A. M. Silva et al., 2007) and SLE was positively associated with HLA-DRB1*03 and negatively associated with HLA-DRB1*09 and HLA-DRB1*13. These observations are also important in other Rheumatic diseases.

When AIDs were considered, the frequency of HLA-DRB1*03 was significantly overrepresented in the total of patients. This allele is associated with a wide range of AIDs (Bilbao et al., 2003), has low affinity for CLIP (class II-associated invariant chain peptide) and may not require HLA-DM to ensure peptide presentation, avoiding efficient peptide selection and allowing the binding of low stability peptides (Collado, Guitart, Ciudad, Alvarez, & Jaraquemada, 2013). Our observations suggest that the HLA-DRB1*13 allele may confer protection for AIDs. HLA-DRB1*13 is a high frequency allele in the general population and its frequency in this sample of the Portuguese population is similar to the ones observed worldwide (Bruno & Lima, 2013). This sustains that the lower frequency of HLA-DRB1*13 in every individual AIDs groups is not secondary to the deviations granted by the concurrent positive associations.

When the data obtained from previous studies are taken into consideration, HLA-DRB1*13 seems to be an almost universal protective allele for RA (Jun et al., 2007; Oka et al., 2014; Ucar et al., 2012; van der Helm-van Mil et al., 2005; van der Woude et al., 2010). Recently, this allele was also described to be protective in SLE in the Japanese population (Furukawa et al., 2014). Subtle structural differences in the HLA

molecule have functional implications at the protein level. Specific amino acid patterns at the peptide binding cleft are involved in disease susceptibility, such as the well known shared epitope first described in the RA susceptibility alleles HLA-DRB1*01 and HLA-DRB1*04 (Michou et al., 2006). In a recent study, Van Heemst and collaborators identified (citrullinated) vinculin, present in the joints of Anti-Citrulinnated Protein Antibodies $(ACPA)^+$ RA patients, as an autoantigen targeted by ACPA and $CD4^+$ T cells. These T cells recognize an epitope with the core sequence HLA-DR Rheumatoid Arthritis Protective Epitope (DERAA), which is also found in many microbes and in protective HLA-DRB1*13 molecules, presented by predisposing HLA-DQ molecules. Intriguingly, DERAA-directed T cells are not detected in HLA-DRB1*13^+ donors, indicating that the DERAA epitope from HLA-DRB1*13 mediates (thymic) tolerance in these donors, thus explaining the protective effects associated with HLA-DRB1*13. The authors suggest that subjects HLA-DRB1*13^+ will present the HLA-DRB1*13-derived DERAA-peptide in the thymus, leading to tolerization of the DERAA-reactive T cell response (van Heemst et al., 2015). The negative association we describe here supports the idea that the HLA-DRB1*13 allele, possibly by its specific structural features, may as well influence disease status by conferring resistance to AIDs. The protective effect of HLA-DRB1*13 could be explained by a more proficient antigen presentation by these molecules, favouring an appropriate immune response (Diepolder et al., 1998; Ramezani et al., 2008). As a result, negative selection and development of HLA-DR-driven autoreactive regulatory T cells are promoted (Tsai & Santamaria, 2013).

A different model would relate HLA molecules with the presence of specific endophenotypes indirectly associated with autoimmunity. Fesel C. suggested in 2012 that the HLA genotype may primarily influence the general activation state of CD4 T-cells, instead of disease outcome (Fesel et al., 2012). The apparent protective effect of HLA-DRB1*13 could also be explained by this effect. Curiously, several reports have suggested an association of HLA-DRB1*13 and/or HLA-DQB1*06 with slow disease progression in human immunodeficiency virus-infected individuals, meaning that among HIV controllers there is an association between the presence of certain class II HLA alleles and strong CD4 T-cell responses (Ferre et al., 2010; Malhotra et al., 2001).

Low sunlight exposure is postulated to be the major latitude-linked component in MS risk (Acheson, Bachrach, & Wright, 1960). The HLA-DRB1*0301 allele, like the HLA-DRB1*1501 allele, harbours a putative VDRE in its promoter region (Ramagopalan et al., 2009). Alleles without the putative VDRE were associated with disease resistance. These parallel results suggest an influence of UV light and Vitamin

D on HLA-DRB1 gene expression and presentation to $CD4^+$ T lymphocytes of peptides relevant to Type 1 Diabetes and MS etiology. The nature of the peptides as well as the timing and outcome of the presentation event are unknown. These could be related to thymic tolerance or peripheral T-cell responses to peptides from infectious agents (Hayes et al., 2015).

The genetic data obtained in this work cannot allow us to assume any relationship between SLE susceptibility and Vitamin D. However, we found the same risk allele HLA-DRB1*03 for SLE, which has the functional canonical VDRE in its noncoding sequence, and the same protective HLA-DRB1*13, which has a mutant non-effective VDRE. The answer to our question needs studies out of this thesis scope, but it would be interesting to study the mother-child effect of Vitamin D deficiency and relate them to this risk and protective alleles. Does this effect may explain the low incidence of autoimmune diseases in the African population? May the phenotypic change that is seen in this population when they migrate to other latitudes be explained by this effect? These questions should be answered with maternal and child population-based studies in different world latitudes. This seems to be the way Vitamin D could be related to SLE susceptibility.

The association of UV radiation and Vitamin D with autoimmune disease risk is strong and consistent, shows a dose–response relationship, is temporally plausible and appears to be universal with respect to genotypes, dietary and smoking habits, and exposure to infectious and commensal organisms. Thus, it is reasonable to suggest that *Vitamin D is probably the environmental factor with the greatest influence on the emergence of an autoimmune disease phenotype given a disease-susceptible genotype.* What remains to be done is to rigorously test this correlation in humans experimentally, and to uncover plausible biological mechanisms that coexist with known facts of autoimmune disease.

5.2.2. Discussion of Hypotheses 3 and 4

Hypothesis 3: *SLE severity is related to VDR Polymorphisms*.
Hypothesis 4: *Vitamin D deficiency is associated with SLE severity.*

These studies, to the best of our knowledge, established, for the first time, an association between VDR polymorphisms (TaqI and FokI) and a higher long-term cumulative damage in SLE patients.

The Vitamin D receptor is a pleiotropic gene associated with multiple autoimmune diseases. ApaI, BsmI and TaqI polymorphisms do not affect directly VDR protein

structure, but differences in stability and/or translation efficiency of the RNA have been reported. The FokI polymorphism seems to have consequences for both VDR protein structure and transcriptional activity (van Etten et al., 2007). Thus, VDR gene polymorphisms can modify the immunomodulatory action of Vitamin D and may have an effect on the clinical manifestations of SLE, namely autoantibody production (Ritterhouse et al., 2011).

Four previous studies in Asian populations reported a positive association between BsmI and FokI VDR polymorphisms and susceptibility to SLE (Huang, Wu, Wu, & Tsai, 2002; Luo et al., 2012; Luo et al., 2011; Ozaki et al., 2000). It has also been observed that VDR polymorphisms may influence clinical manifestations. Concerning nephropathy, an association with the bb genotype of BsmI was described in a Japanese cohort by Ozaki *et al.* On a Han Chinese population, Luo *et al.* associated this nephropathy with the B allele of the same polymorphism. Emerah *et al.* replicated this result in an Egyptian female population, observing that AA (ApaI) and FF (FokI) genotypes were also risk factors for the development of nephropathy in SLE patients. In a study of Polish females, Mostowska *et al.* compared patients with and without renal symptoms and observed that the FF and Ff genotypes of FokI had higher frequencies in patients with clinical manifestations. Additional research associated VDR polymorphisms with other SLE features such as serositis, ANA, anti-dsDNA, anti-Sm and anti-histone antibodies (Luo et al., 2012; Luo et al., 2011). In other studies, no association between VDR polymorphisms and SLE was reported (Abbasi et al., 2010; Kaleta et al., 2013; Monticielo, Teixeira Tde, Chies, Brenol, & Xavier, 2012; Sakulpipatsin et al., 2006).

In summary, the association between Vitamin D receptor polymorphisms and SLE has been extensively studied, but the results described are widely dissonant so far. The comparison of data present in the literature is difficult, due either to study design or ethnic differences. Up to date, the role of VDR polymorphisms in this disease remains uncertain and limited by the lack of controlled Vitamin D populations.

These studies suggest a role for VDR gene polymorphisms in SLE long-term outcome. A positive association was found between VDR polymorphisms and SLE damage accrual. The presence of CT genotype of FokI and TT genotype of TaqI seems to confer risk for a worse prognosis, being a risk factor for a higher long-term cumulative damage. Several hypotheses may explain these results.

It is known that VDR polymorphisms may alter VDR expression, transcription and function, and thus these modifications may interfere with Vitamin D activity. In fact, Vitamin D has an important role in the control of inflammatory responses with a switch from Th1 to Th2 cytokine profile, enhancing Th17 pathway via transcriptional

modulation of interleukin-17A, as well as facilitating an induction of regulatory T cells (Joshi et al., 2011; Peelen et al., 2011).

Additionally, VDR polymorphisms may also be in linkage disequilibrium within HLA-DRB1 alleles that are central players in several autoimmune diseases (Israni, Goswami, Kumar, & Rani, 2009).

Furthermore, VDR signalling may influence the transcriptional activity of genes involved in the pathophysiological mechanisms of autoimmune diseases. The promoter region of HLA-DRB1 locus has a Vitamin D responsive element that controls/regulates HLA-DRB1 expression (Israni et al., 2009; Ramagopalan et al., 2009). Binding of $1,25(OH)_2D3$ originates conformational changes in VDR, which promote its hetero-dimerization with RXR and translocation of this complex into the nucleus. The RXR-VDR heterodimer binds to the Vitamin D responsive elements in promoter regions of $1,25(OH)_2D3$ responsive genes, such as HLA-DRB1 gene. It has been described that Vitamin D deficiency in early childhood may lead to lower HLA-DRB1 expression, impairing thymic selection and promoting autoimmune disease development (Israni et al., 2009).

Despite the positive association observed between CT genotype of FokI and TT genotype of TaqI and SLE damage accrual, there is a lack of clear association with the number of affected organs, number of severe flares and pharmacological therapy. This can be explained by the small number of individuals in each subgroup, nevertheless the main explanation is that damage in SLE is multifactorial and not only related to disease activity itself. For example, a more inflammatory profile could be found in Lupus patients, which could explain a worse long-term prognosis. This is not necessarily related to disease activity, but with other factors, as already described in larger cohorts, like cardiovascular disease (Perez-Hernandez et al., 2016).

These results provide a support to the hypothesis that Vitamin D polymorphisms are associated with SLE disease activity, although the number of subjects studied was relatively small. Therefore, this finding requires independent replication in larger cohorts (Liberman, 2007).

Furthermore, we did not find a correlation between VDR polymorphisms and low Vitamin D levels at disease onset, neither with Vitamin D average levels over 10 years. Moreover, the effect found on disease damage with diverse VDR SNPs was unrelated to Vitamin D levels (data not presented).

In the same cohort, we also analysed baseline Vitamin D levels and correlated them with the number of severe flares. We observed a significant inverse correlation between these two variables, specifically in patients with three or more severe flares.

Finally, we studied patients with available mean Vitamin D levels in the previous 10 years of disease and correlated them with the number of severe flares. Once again, we observed a negative correlation but it was not significant; on the other hand, statistical significance was obtained for patients with ≥3 severe flares.

In conclusion, in Lupus patients with VDR risk genotypes, Vitamin D supplementation could be a useful strategy for prevention of long-term cumulative damage, compensating VDR dysfunction. Vitamin D daily dose should be adapted to the concept of "Vitamin D Resistant State" and the optimal dose should be tailored for each patient. Individual genotype cannot be changed; however, Vitamin D deficiency can be searched and addressed accordingly.

We propose that patients with Vitamin D deficiency, and heterozygotes for FokI and TT for TaqI polymorphisms of VDR, have more severe disease and damage.

5.3. Interventional studies

5.3.1. Discussion of hypotheses 5 and 6.

Hypothesis 5: *Supplementation with Vitamin D alters the clinical expression and immunological deregulation of SLE patients (T-CD4$^+$ cells expressing FoxP3 and T-CD4$^+$ cells producing IL-17A.*

Hypothesis 6: *Clinical and Immunological response to Vitamin D supplementation is limited by SLE disease activity*.

In these studies, for the first time, we assessed the safety, the clinical and immunological effects of Vitamin D supplementation in patients with SLE in a Portuguese population. We addressed 24 stable patients, with stable therapy, and supplemented them during 6 months guided by levels and aiming more than >75 nmol/L. The protocol comprised the registry of flares, new damage and, more important, the FoxP3 expression and IL-17A in T CD4$^+$ cells. We also created a concept of FoxP3/IL-17A ratio to use as a biomarker of immunologic imbalance. We confirmed the high frequency of hypovitaminosis D in SLE patients (70.8%) already described in some studies, and we have also demonstrated that Vitamin D supplementation significantly increased serum 25(OH)D levels, at 3 and 6 months after the beginning of the study. Furthermore, it was shown that this therapy was safe, since no alterations on phosphorus or calcium levels and no side effects were reported.

After 6 months of Vitamin D supplementation, it was observed an increase of FoxP3 expression in $CD4^+$ T cells and a decrease in IL-17A cells. The $FoxP3^+$/IL-17A ratio was found to be significantly higher at the end of the treatment. This effect was also observed in patients with elevated Vitamin D levels at baseline, showing that immunological effects were unrelated to the standard cut-offs used for metabolic bone disease. These results suggest that Vitamin D supplementation may improve $FoxP3^+$/IL-17A ratio (and indirectly the Treg/Th17 ratio), an effect described for the first time in SLE patients. Vitamin D supplementation was shown to be a safe and efficient treatment to improve clinical and immunological stability in SLE patients, as well as for flare prevention. Since none of the patients exhibited complications resulting from Vitamin D supplementation, perhaps the administered doses could be increased, maintaining the safety profile and leading to even better results.

MicroRNAs (miRNA) have gene expression regulatory roles in innate immunity (Chan, Ceribelli, & Satoh, 2013). The miR-146a, an NFkB regulated transcript, is activated during LPS stimulation of monocytes and is critical in endotoxin tolerance to prevent cellular overstimulation when in excess of the TLR4 ligand. So, miR-146a act as a negative regulator of innate immunity (Ma, Becker Buscaglia, Barker, & Li, 2011). Interestingly, miR-146a has been reported to be upregulated in PBMCs and synovial tissue in rheumatoid arthritis patients, but reported to be downregulated in PBMCs of SLE patients (Qu et al., 2015). Further analysis showed that underexpression of miR-146a negatively correlated with clinical disease activity and with IFN scores in patients with SLE (Tang et al., 2009).

Of note, miR-146a directly repressed the downstream transactivation of type I IFN. At the molecular level, miR-146a targeted IFN regulatory factor 5 and STAT-1 (Saba, Sorensen, & Booth, 2014). The ability of Vitamin D to regulate miRs and their emerging relationship have been proposed through several experimental and clinical approaches; however, the implications of their impact in inflammatory responses have only been studied in *in vitro* models (Arboleda & Urcuqui-Inchima, 2016). The aim of this study was to determine the Vitamin D effect in miR-146a expression as well as in T $CD4^+CD25^+FoxP3^+$ (Treg) and T$CD4^+$ IL-17A producing cells, in active SLE.

Furthermore, we have treated 4 patients with severely active SLE (with clinical SLEDAI >6 and at least one BILAG A for an organ or system) with high doses of Vitamin D. These patients had previous supplementation and only one had a mild deficiency. It was shown here that even with high dose therapy there was no effect on Vitamin D levels. Two patients were treated with 50,000 IU of cholecalciferol for 3 weeks and the remaining 2 with 100,000 IU weekly for 3 weeks. Interestingly, it was not demonstrated a significant increase in Vitamin D levels in these patients and much less

a change (decrease) in miR-146a expression, which has already been described in other studies. The miR-146a expression is described to be low in SLE and its expression down-regulates IFN-α production (Ma et al., 2011). We also did not found any enhancement in $CD4^+CD25^+FoxP3^+$ T-cells and $CD4^+IL\text{-}17A^+$T-cells ratio (Treg/IL-17A ratio), as it was observed in the stable SLE patients study.

It was shown earlier that disease activity may not be important in patients' Vitamin D status, but in this open-label study, we found that disease activity may prevent its increase and hence truncate its immunomodulatory effect. As such, it appears to be resistance to treatment with Vitamin D (even without hypovitaminosis) and this is associated with very active disease. However, this effect does not seem to occur in stable patients and low activity SLE patients.

In conclusion, these studies demonstrated that Vitamin D supplementation seems to provide favourable immunological effects in patients with SLE, independently of the 25(OH)D patients' status. Nevertheless, these results should be interpreted with caution since previous studies evaluating the supplementation of 25(OH)D in SLE disease have had inconsistent results.

It is important to consider every existing variable and their specific outcomes in each patient. Using the $FoxP3^+$/IL-17A ratio, it may be possible to tailor Vitamin D therapy for each patient. An individualized therapy should be undertaken, since some patients will need very high doses of supplementation whereas others will need only modest doses to achieve the same outcome. In these stable patients we showed, indirectly, that the general guidelines (>75 nmol/L) are probably not enough to achieve a better immunological tolerance state.

Finally, we are closing the gate of the resistant state to Vitamin D in SLE. It seems that cholecalciferol regulatory effects are dependent of SLE disease activity, and there seems to be a window of opportunity to supplement these patients with higher doses of Vitamin D. During severe flares, a resistant state to Vitamin D supplementation and benefits may exist.

5.4. Main Conclusions

Table 25 - Main conclusions.

MAIN CONCLUSIONS
Hypovitaminosis D in SLE population may differ from the observed in healthy population due to deficient sun exposure during summer time, and not due to the disease effect on Vitamin D metabolism.
Confirmation of HLA-DRB1*03 risk and HLA-DRB1*13 protective effect to SLE. The possibility of this effect to be mediated by the canonical VDRE presence in HLA-DRB1 genes should be explored.
VDR polymorphisms (TaqI and FokI variants) are related to damage accrual in SLE patients.
Vitamin D levels at the beginning of the disease and the vitamin D burden during disease are related to more aggressive phenotypes.
Vitamin D supplementation seems to provide favourable immunological effects in SLE patients with stable and low disease activity; independent of baseline vitamin D levels.
Severe SLE activity may cause resistance to Vitamin D therapeutical effects, including enhancing of Vitamin D levels and immunogenetic effects.

5.5. Future directions

Theoretically, Vitamin D should have relevant effects on musculoskeletal health, immunity, autoimmunity, cardiovascular disease, cancer, fertility, pregnancy, dementia and even mortality. However, many of these effects are theoretical and it is necessary to demonstrate these *in vivo* cellular effects, detected *in vitro*, in a large group of patients followed in a systematic and controlled manner. These studies are needed to identify the optimal dose of Vitamin D for each patient, based on their clinical, genetic and physiopathological state. On a quick observation of the factors that modulate the response to Vitamin D, it would be tempting to use very high doses, because they seem safe and could surpass any therapeutic resistance. However, secure scientific evidence only applies to calcium metabolism, and other deleterious effects of the hormone when used massively are essentially unknown.

As such, the great challenge is to recognize all factors involved in the modulation of Vitamin D, such as transcriptomic and epigenetic factors, polymorphisms of various enzymes, key receptors of its metabolism, specific cellular targets and the expression of cytokines. This is the only way we can develop tailored therapies for each disease and each patient using an individualized treatment plan.

- Vitamin D levels in SLE patients are needed to be addressed further. It is necessary to study the difference between summer and winter in a larger cohort and in different populations, which could allow us to confirm that SLE activity is not a determinant for lower Vitamin D levels.

- We should study further VDRE in protective and risk HLA alleles, the effect of different latitudes and start to study mothers and newborns.

- VDR polymorphisms can be biomarkers for damage in SLE and their identification in these patients should be studied.

- Vitamin D supplementation may have a window of opportunity for immunomodulation, during SLE low disease activity phases.

- We need new guidelines for Vitamin D supplementation in SLE.

- An RCT study should be done using better Vitamin D analogues or in combination with immunosuppression during severe disease flares.

CHAPTER 6

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REFERENCES

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APPENDICES

Appendix 1

Topics on Vitamin D in Systemic Lupus Erythematosus: analysis of evidence and critical literature review

Topics on Vitamin D in Systemic Lupus Erythematosus: analysis of evidence and critical literature review.

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Key Words: Vitamin D, Lupus, inflammation; Randomized controlled trials; Vitamin D receptor single nucleotide polymorphisms; vitamin D response elements; Major Histocompatibility Complex genes; antigen presenting cells; regulatory T-Cells; T helper 17 Cells; epigenome; transcriptome.

Abstract

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with multi-organ inflammation, linked to the loss of immune tolerance to self-antigens and the production of a diversity of autoantibodies. The phenotype and progression of SLE have been linked to a combination of environmental, genetic and hormonal factors. One such environmental factor is Vitamin D, a vital hormone with well-established effects on mineral metabolism, skeletal health and effects on cardiovascular system. The purpose of this article is to make the analysis of evidence and literature review of the pleomorphic effects of Vitamin D in SLE. The article is structured in topics of interest based in the authors' opinion and summarizes the evidence of studies and trials of Vitamin D in SLE.

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with multi-organ inflammation, linked to the loss of immune tolerance to self-antigens and the production of a diversity of autoantibodies.
In adults, the female to male ratio, is approximately 9:1, the peak age of SLE diagnosis is between 15 and 44 years, affecting mainly women of childbearing age [1]. There is evidence of strong genetic contribution to the development of SLE: familiar prevalence was found to be 5.6% if one first degree relative is affected; disease concordance in dizygotic twins is 2-5% and in monozygotic twins 29-57% [2].
The phenotype and progression of SLE have been linked to a combination of environmental, genetic and hormonal factors [1, 3]. One such environmental factor is Vitamin D, a vital hormone with well-established effects on mineral metabolism, skeletal health and effects on cardiovascular system [4;5] The major source of vitamin D, at least in lower latitudes is sun exposure. Vitamin D is also available in some foods and supplements.

The identification of vitamin D receptors in immune system cells and the discovery that dendritic cells can produce the metabolically active form of vitamin D, 1,25-dihydroxyvitamin D3 (calcitriol), have led to the suggestion that vitamin D is an immune modulator and also anti-infective agent [6,7,8,9] This can explain why vitamin D deficiency-related symptoms, such as fatigue, are frequently observed in patients with SLE [10]. Furthermore, SLE patients may be at increased risk of low vitamin D status as a result of both photosensitivity, with consequent sun avoidance, as well as chronic use of medications which interfere with vitamin D metabolism [11, 12]. Moreover, the existence of Vitamin D response elements in key promoter regions of HLA-DRB1 [13], polymorphisms in key enzymes and receptors of vitamin D metabolism, can explain why patients have different clinical responses during supplementation [14]. Vitamin D deficiency is categorized in different classifications, but it can be classified as optimal, insufficiency/inadequacy and deficiency (supplement 1) [15].

The purpose of this article is to make the analysis of evidence and literature review of the pleomorphic effects of Vitamin D in SLE. The article is structured in topics of interest based in the authors' opinion. In appendix 2 we present a summary of the trials and studies with Vitamin D intervention in SLE.

1 - Vitamin D and SLE susceptibility

Vitamin D insufficiency may be an important factor in SLE susceptibility during early life, based on Vitamin's D effect in auto-reactive T-lymphocytes central deletion [13, 14]. We can look for autoimmune susceptibility as an "early-life" condition in which vitamin D insufficiency is also a determining factor for an auto-reactive profile. Vitamin D response elements (VDREs) have been found in the promoter region of the Multiple Sclerosis (MS) associated allele HLA-DRB1*15:01 [13, 14] suggesting that with low vitamin D availability, VDREs are incapable of inducing *15:01 expression allowing early life auto-reactive T-cells to escape central thymic deletion. These findings have been found in MS, however in SLE that should also occur.

In our epigenetic data we genotyped for HLA-DRB1 locus 1228 patients with autoimmune diseases (AIDs): 213 with Systemic Lupus Erythematosus (SLE), 166 with Psoriasis or Psoriatic Arthritis (Ps + PsA), 153 with Rheumatoid Arthritis (RA), 67 with Systemic Sclerosis (SSc), 536 with Multiple Sclerosis (MS), and 93 with Myasthenia Gravis (MG) and 282 unrelated controls. We confirmed previously established associations of HLA-DRB1(∗)15 (OR = 2.17) and HLA-DRB1(∗)03 (OR = 1.81) alleles with MS, HLA-DRB1(∗)03 with SLE (OR = 2.49), HLA-DRB1(∗)01 (OR = 1.79) and HLA-DRB1(∗)04 (OR = 2.81) with RA, HLA-DRB1(∗)07 with Ps + PsA (OR = 1.79), HLA-DRB1(∗)01 (OR = 2.28) and HLA-DRB1(∗)08 (OR = 3.01) with SSc, and HLA-DRB1(∗)03 with MG (OR = 2.98). We further observed a consistent negative association of HLA-DRB1 (∗) 13 allele with SLE, Ps + PsA, RA, and SSc (18.3%, 19.3%, 16.3%, and 11.9%, resp., versus 29.8% in controls). HLA-DRB1 (∗) 13 frequency in the AIDs group was 20.0% (OR = 0.58). Although different alleles were associated with particular AIDs, the same allele, HLA-DRB1 (∗) 13, was underrepresented in all of the six diseases analyzed. This observation suggests that this allele may confer protection for AIDs, particularly for systemic and rheumatic disease.

The protective effect of HLA-DRB1 (∗) 13 could be explained by a more proficient antigen presentation of these molecules, favoring efficient clonal deletion during thymic selection [16]. However, an association with VDRE is yet to be proven.

We thus postulate that vitamin D preventive role in SLE (and other autoimmune diseases) could be limited in time, during the development of the immune system. Later on, its role should be essentially a modulating one.

With these ideas in mind, retrospective epigenetic susceptibility studies, that do not have controlled Vitamin D population levels in childhood, have little scientific validity. The susceptibility studies related with polymorphisms of vitamin D receptor are such an example, with different susceptibility profiles in different populations, but without controlled vitamin D levels in the studied population [17, 18].

2 - SLE and Vitamin D a paradoxical phenomenon

Usually, patients with established SLE lack vitamin D. The reduced sun exposure, the use of sunscreens, the gut malabsorption (example: secondary to drugs such as corticosteroids), among others, are all significant factors for this steroid hormone insufficiency [19]. However, sun exposure, the most important factor for vitamin D precursor synthesis at the skin level, is also one of the strong activators of SLE flares, possibly because the ultraviolet (UV) wavelengths of sunlight potentiates the stimulator of interferon genes (STING)-dependent activation of the immune signaling transcription factor interferon regulatory factor 3 (IRF3) in response to cytosolic DNA and cyclic dinucleotides in keratinocytes and other human cells [20].

This paradoxical phenomenon, being well recognized, is also ignored in the supplementation strategies, which are based on the general population guidelines. SLE patients need treatment strategies based on serum levels of 25-hydroxyvitamin D and not standard supplementation strategies, even though serum levels of 30 ng/ml are validated to metabolic bone disease but not for immunological effects [21].

3 - The emerging role of vitamin D in immunomodulation

Vitamin D is a steroid hormone with a nuclear receptor, the Vitamin D receptor (VDR). This receptor is present in all immune system cells. Many key genes to immune regulation have VDREs [22]. So, beyond its classical function in calcium metabolism, it has an essential role in immune modulation. This Vitamin exercises its role both at the innate immunity and adaptive immunity levels. The hormone has a modulator role in the activation of dendritic cells and their ability to present antigens. Moreover, it polarizes the adaptive immune response to a Th2 response [4]. Finally, it is essential to the expression of the transcription factor forkhead box P3 (FoxP3), via VDRE present in the promoter region of his gene and therefore essential to the normal expression of regulatory T cells [21].

Taking this in consideration, it seems logical to have an inflammatory profile in patients with vitamin D deficiency. However, the benefit and efficacy of Vitamin D supplementation are very heterogeneous among different patients and different diseases. Many questions about dosage and patients' optimization are still in debate.

3.1 Vitamin D and Antigen Presenting Cells (APC) in SLE

Lerman et al. studied the ability of 1,25-dihydroxyvitamin D3 to affect human monocyte phenotype. This phenotype was assessed by incubating cells with sera from 15 patients with SLE and from 5 healthy volunteers. Addition of 1,25-dihydroxyvitamin D3 resulted in significant reductions in the expression of MHC Class II, CD40, and CD86 and increases in expression of CD14 in both types of sera. Overall, 1,25-dihydroxyvitamin D3 limited human APC activation via IFNα-induced and independent mechanisms. 1,25-dihydroxyvitamin D3 inhibited APC activation by SLE sera, suggesting that it may be possible for 1,25-dihydroxyvitamin D3 to reduce the immunostimulatory effects of the SLE milieu by interfering with the soluble cytokine mediators in the sera of SLE patients [23].

Ben-Zvi et al. studied 19 SLE patients stratified by 25-hydroxyvitamin D There were no differences between circulating Dendritic Cells (DC) number and phenotype. Monocyte-derived DCs (MDDCs) of SLE patients were normally responsive to the regulatory effects of vitamin D *in vitro* as evidenced by decreased activation in response to LPS stimulation in the presence of 1,25-dihydroxyvitamin D3. Additionally, vitamin D supplementation reduced expression of interferon-alfa regulated genes by healthy donors and SLE MDDCs in response to factors in activating SLE plasma [7].

Wu HJ et al. showed that "tolerogenic" dendritic cells (DCs) are a potential cell-based therapy in SLE by treating monocyte-derived DCs from SLE patients and healthy subjects with combination of 1,25-dihydroxyvitamin D3 and dexamethasone followed by lipopolysaccharide-induced maturation. Lupus activated DCs were found to acquire semi-mature phenotype that remained maturation-resistant to immunostimulants and was also found to acquire enhancing Regulatory-T Cells phenotype [24].

Finally, Cynthia Aranow, et al. published a Randomized Controlled Trial (RCT) trying to prove the effect of vitamin D supplementation in interferon signature. The studied failed the primary outcome [25].

We learned from Aranow's RCT that we still do not know in vivo behavior of this effect on interferon signature and human studies with tolerogenic dendritic cells in SLE are not yet available. We also do not know if the effect on APC is identical in the various body compartments with different cytokine expression, for example in lupus nephritis, which can be the only manifestation of SLE. On the other hand, we do not know the role of MHC Class II down-regulation in APC in patients with only positive antinuclear antibodies, in serological active quiescent Lupus (SAQLs) or during severe flares. It is necessary to replicate the effect in vivo and to know in which stage of the disease this adjustment brings more benefit. Dendritic cells' induced tolerance may be essential in the early stages of the disease and less important during severe flares.

3.2 – Vitamin D, Regulatory T-Cells and T-Helper 17 Cells

Terrier et al. showed the safety and efficacy of 25-hydroxyvitamin D high doses supplementation on restoration of regulatory and effector T cell balance and B cell homeostasis in 24 SLE patients [26]. However, several limitations were found in this study. The time established for new flares (6 months) is too short to evaluate this effect. On the other hand, there is no control group with evaluation of regulatory T cells, TH 17 cells and IL17A expression in a healthy population. We only know that vitamin D changes the profile of immune dysregulation, but its meaning is completely unknown. We also do not know if numerical changes correct functional changes [27]. However, one can say that vitamin D high doses are a safe therapy.

Viviane de Souza et al. in a small open-label study from Brazil also found that vitamin D deficiency was more prevalent in patients with SLE and was associated with higher levels of interleukine-6 (IL-6) [28].

Banica LM et al. showed in vitro that rapamicin and vitamin D together were able to induce regulatory properties in CD4+ T-Cells. However, this sustained effect was observed only in rapamycin group [29].

Piantoni S et al. also showed an enhancement of T-reg cells and the production of Th2 cytokines after a long-term of monthly treatment with vitamin D in SLE patients. These numeric differences were only significant after 2 years of treatment [30].

Wahono CS et al. and *Drozdenko et al.* showed that the effect of vitamin D therapy was mainly on IL17-A cytokine expression and less on regulatory T-Cells [31, 32].

In our data from Oporto, we obtained the same findings. However, Forkhead box P3 (FOXP3) expression can also be enhanced in patients who are not vitamin D deficient, in part because, may be, the reference value of vitamin D in autoimmune diseases, 75nmol/L (30ng/mL), is not the ideal one. [33].

In summary, we found a universal trend to vitamin D immune regulation based on regulatory T-Cell properties and IL17A expression. However, we need controlled trials with a large number of SLE patients, controlled methodology, especially functional studies with micro-RNA of inducible regulatory T-Cell genes and time dependent studies. Maja Vukić et al. made the first trials with this concept. They studied 71 patients from the VitDmet study (ClinicalTrials.gov Identifier: NCT01479933) and 10 from VitDbol (ClinicalTrials.gov Identifier: NCT02063334). In an interventional prospective study, they correlated the changes in mRNA expression with serum 25(OH) D3 level in primary human cells. In peripheral blood

mononuclear cells, direct transcriptional effects were observed on selected VDR target genes, such as an up to 2.1-fold increase, after one day only of supplementation onset, showing that both long-term and short-term vitamin D3 supplementation studies allow monitoring of the vitamin D responsiveness of human individuals and represent new types of human in vivo vitamin D3 investigations [34].

4 - Vitamin D Inflammation and atherosclerosis

Vascular protective effects of vitamin D have been postulated due to modulation of inflammatory cytokines. However, the effects of vitamin D supplementation on inflammatory cytokines in trials have been inconsistent.

Petri et al. studied 200 patients enrolled in the Lupus Atherosclerosis Prevention Study and found 25-hydroxyvitamin D was not associated with any measure of subclinical atherosclerosis. 25-hydroxyvitamin D deficiency was associated with higher high sensitive C-reactive protein (hsCRP) at baseline, but did not predict a change in hsCRP over 2 years [35].

However, in pediatric Lupus patients enrolled in the Atherosclerosis Prevention in Pediatric Lupus Erythematosus (APPLE) substudy [36, 37] the same data were not consistent with the adult's study. They also found that vitamin D deficiency is independently associated with elevated hsCRP, a marker of inflammation that predicts cardiovascular disease risk. Although association is not proof of causation and they did not study the impact of vitamin D supplementation in hsCRP during the follow-up of these patients. By chance, the same study found that Vitamin D status is a determinant of atorvastatin effect on carotid intima medial thickening progression rate in children with lupus at 3 years, and it was significant.

Lertratanakul et al. reported a retrospective study from a large international inception cohort; they studied the risk of cardiovascular (CV) event incidence. Patients in the higher quartiles of 25-Hydroxivitamin D were less likely to have hypertension and hyperlipidemia and were more likely to have lower C-reactive protein levels and lower Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI 2000) scores at baseline when compared with the first quartile. Vitamin D levels were not independently associated with CV event incidence; however, hazard ratios for CVD event incidence decreased with successively higher quartiles [38].

Reynolds et al. studied 75 SLE patients in a cross-sectional retrospective study to correlate Vitamin D levels with disease and markers of subclinical vascular disease. Their results were significant and correlate Vitamin D deficiency with increased aortic stiffness in SLE, independent of CVD risk factors and insulin resistance. They concluded that increased inflammatory disease activity may be the mechanism by which vitamin D deficiency mediates vascular stiffness in this patient group [39]. Sabio et al. showed the same results in a RCT [40].

Reynolds et al. also made a prospective study with vitamin D deficient SLE patients and described the effectiveness of calcitriol to restore angiogenic capacity of SLE myeloid angiogenic cells and to positively modulate the paracrine regulation of endothelial nitric oxide synthase [41].

Abou-Raya et al have done a randomized placebo-controlled trial with 206 patients showing that high dose cholecalciferol was effective in disease activity control and in the improvement of hemostatic and inflammatory markers: Il-1, Il-6, IL18, TNF alfa, Von Willebrand factor and fibrinogen [42].

In essence, there is enough evidence to say that vitamin D deficiency parallels hsCRP as an inflammatory biomarker for atherosclerosis and this effect is independent of insulin resistance. This observation could be because the effect is more significant on endothelium dysfunction rather than an improvement of metabolic syndrome. SLE, like RA, is an independent risk factor for atherosclerosis and vitamin D deficiency may be one modifying risk factor.

The different data between the adult and pediatric patients are explained by different study designs, by the lack of a follow-up on the hsCRP after Vitamin D supplementation and by the fact that the adult study screening of the carotid intima medial thickening progression rate was not an association study with statins.

It would be rather important to repeat the analysis of both groups with the statin effect and following-up the hsCRP in pediatric patients.

]he recognition of SLE as risk factor for cardiovascular disease is very important in clinical practice and, like Rheumatoid Arthritis, a standard practice of correcting traditional and non-traditional risk factors is imperative **[43].**

5 – Vitamin D resistant state and SLE

SLE could be a conceptual form of resistance to vitamin D effect. This resistant state has multiple steps including the epigenetic mechanisms (also regulation of metabolic pathways), gene regulation in different cell types and vitamin D receptor polymorphisms (the transcriptome).
The following summarizes the concept of resistance:

a) Epigenetic mechanisms

Epigenetic mechanisms play a crucial role in regulating gene expression as we showed above with the VDRE elements in non-coding sequences of MHC Class II [13, 14]. The main mechanisms involve methylation of DNA and covalent modifications of histones by methylation, acetylation, phosphorylation, or ubiquitination. Modifications in DNA methylation are performed mainly by DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) proteins, while a plethora of enzymes, such as histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs) regulate covalent histone modifications.

In many diseases, such as cancer, the epigenetic regulatory system is often disturbed. Vitamin D interacts with the epigenome on multiple levels. Firstly, critical genes in the vitamin D signaling system, such as those coding for vitamin D receptor (VDR) and the enzymes 25-hydroxylase (CYP2R1), 1α-hydroxylase (CYP27B1), and 24-hydroxylase (CYP24A1) have large CpG islands in their promoter regions and therefore can be silenced by DNA methylation. Secondly, VDR protein physically interacts with coactivator and corepressor proteins, which in turn are in contact with chromatin modifiers, such as HATs, HDACs, HMTs, and with chromatin remodelers. Thirdly, a number of genes encoding for chromatin modifiers and remodelers, such as HDMs of the Jumonji C (JmjC)-domain containing proteins and lysine-specific demethylase (LSD) families are primary targets of VDR and its ligands. Finally, there is evidence that certain VDR ligands 1, 25-dihydroxyvitamin D3 have DNA demethylating effects [44].

This is the first resistance mechanism. SLE, like cancer, is a state of DNA hypermetilation. The disease itself can mute key enzymes of its metabolism and create a state of deficiency of 25-hydroxyvitamin D. Conversely, its "DNA demethylating" effect can be important to the maintenance of key enzymes and their target genes, maintaining immune system homeostasis. Once again the replacement of cholecalciferol is not established and is likely to need very high levels or greater affinity analogs to overpass this resistance. This particular field lacks evidence.

b) VDR function in transcriptomic era

Vitamin D acts via binding to an intranuclear receptor, VDR, present in target tissues and cells. This receptor belongs to the superfamily of transacting transcriptional regulatory factors. In humans, VDR gene is located on chromosome 12q13.1, extends over 100 kb and includes eight protein-coding exons, six untranslated exons, eight introns and two promoter regions [45].

Genome-wide overview does not involve additional signal transduction steps, as they are known for hydrophilic signaling molecules, such as peptide hormones, growth factors and cytokines. VDR shares the main structural characteristics of nuclear receptors, which is a highly conserved DNA-binding domain and a structurally conserved ligand binding domain VDRs. DNA-binding

domain specifically contacts the hexameric consensus sequence RGKTSA (R = A or G, K = G or T, S = C or G) within the major groove of genomic DNA. However, like most other transcription factors, VDR uses a partner DNA-binding protein, in order to bind efficiently to its target sites. More than 20 years ago, this heterodimeric partner turned out to be the nuclear receptor retinoid X receptor (RXR). Steric constraints of the dimerizing DNA-binding domains of VDR and RXR determine the optimal binding site of the VDR-RXR complex as a direct repeat of two hexameric nuclear receptor binding motifs spaced by three nucleotides (DR3). Within VDRs ligand-binding domain, a network of some 40 mostly non-polar aminoacids forms a ligand-binding pocket in which 1, 25-dihydroxyvitamin D3 and its synthetic analogs are specifically fixed with high affinity. This ligand binding process induces a conformational change to the surface of VDRs ligand-binding domain, which results in a significant change of VDRs protein-protein interaction profile: it transforms from a repressor to an activator [46]. The genome-wide location of VDR has essential information for understanding the pleiotropic physiological action of 1, 25-dihydroxyvitamin D3.

Probably B cells have accessible VDR binding sites. Nevertheless, it can be questioned whether the regulation of a few hundred primary VDR target genes per tissue requires a far higher number of high quality genomic VDR binding sites. For the total of 23,409 non-overlapping VDR binding sites, full 75% of them are observed only in one cell type. These unique VDR binding sites may be the mediators of cell-type specific actions of the receptor and its ligand. In fact, on the level of VDR target gene expression, as measured by microarrays, it is already known that in most tissues a rather different set of genes respond to stimulation with 1, 25-dihydroxyvitamin D3. On the other hand, VDR locations that overlap between two or more tissues represent independent confirmations of the validity of a VDR binding site. Moreover, genomic regions that are recognized in multiple cell types by VDR may have a more generalized, and therefore likely higher, impact on the physiological actions of the receptor and its ligand than the cell type specific sites [46,47].

In turn, and in light of the renewed understanding of the distribution of transcriptional binding patterns and the diversity of RNA species, it is also timely to consider how these relationships illuminate VDR function [48].

Arash et al. published a RCT showing that vitamin D supplementation caused at least a 1.5-fold change in the expression of 291 genes that are involved in apoptosis, immune function, transcriptional regulation, and epigenetic modification, response to stress, cell cycle activity and differentiation. They identified 66 genes that were most significantly affected by the subjects' vitamin D status, comparing those who were vitamin D deficient with 25(OH)D of 16.2±4.2 ng/ml to those who had a 25(OH)D of 27.5±8.4 ng/ml at baseline. Of these 66 genes, 17 genes whose expression significantly changed after

vitamin D3 supplementation in both deficient and insufficient/sufficient groups were found to have novel VDREs [49].

- The VDR Transcriptome regulation

With the emergence of comprehensive transcriptomic approaches including microarray technologies, an understanding was developed concerning the VDR transcriptome in different cell types and treatment conditions. In many ways, these studies also highlighted the heterogeneity of VDR actions. This heterogeneity may in part reflect experimental conditions (e.g., growth conditions, ligand exposures) with very different cell lines, but also genuine tissue-specific differences of co-factor expression that alter the amplitude and periodicity of VDR transcriptional actions. These transcriptomic direct actions can regulate cell cycle, activate key enzymes (like kinases), chromatin remodeling and event demethylation effects very important in cancer and autoimmune diseases as showed above [45].

- VDR Regulation of Non-Coding RNA Species

Many researchers have considered roles for non-coding RNA in the regulation of cell function and have begun to examine the interplay between at least 20 different types of different non-coding RNA. Many of these RNA species are gene regulatory RNAs and include microRNA (miRNA) and long noncoding RNA (long crane), whereas others are involved in the post-transcriptional modification of RNA, for example small nucleolar RNA (snoRNA). The data is still sparse, but non-coding sequences regulation is an important mechanism regarding vitamin D/VDR interaction and immune regulation. An important example is the presence of VDRE elements in non-coding sequences of MHC-Class II [13]. It is likely that the increased application and integration of microarray and next generation sequencing approaches will identify the key networks downstream of the VDR transcriptome, pertaining to both protein coding and non-coding RNA [50].

- The Repertoire of VDR-Protein Interactions and Genomic Binding Sites

New microarray studies showed that VDR combines with other proteins in a network of interactions, quite likely in a cell type specific manner, to participate in diverse gene regulatory networks. It remains to be established how targeted this is. The variation observed in both the type and position of binding sites for the VDR, depending on cell phenotype and disease state, suggests it is directed, and at least will establish a paradigm for hypothesis testing concerning what directs the VDR to bind and participate in gene transcription. The specificity of VDR signaling may arise due to integration with other perhaps more dominant transcription factors.

Again, for other nuclear receptor the concept has emerged that receptor binding is guided by the actions of more dominant so-called pioneer factors

including the Forkhead (FKH) family members. However, efforts to define the major pioneer factors for the VDR have proven to be less consistent between the different VDR microarray studies and may reflect the biology of the VDR, given its existence in the nucleus both in the presence and absence of ligand, such that a single dominant pioneer factor is not so deterministic [51].

- Genetic Variation in VDR Binding – The VDR Polymorphisms

Four common single nucleotide polymorphisms (SNPs) in the VDR gene have been extensively investigated: FokI C>T (rs2228570), BsmI A>G (rs1544410), ApaI G>T (rs7975232), and TaqI C>T (rs731236). BsmI and ApaI SNPs are both located in intron 8, and the TaqI is a silent SNP in exon 9. Although these 3 polymorphisms do not produce any structural change on the VDR protein, they are in strong linkage disequilibrium. On the other hand, the T allele of the FokI SNP creates an alternative ATG initiation codon in exon 2, leading to a VDR protein that is three amino acids longer, suggesting a potential functional consequence [41]. The combination of this data allows the use of phenotype-driven data to identify what VDR binding sites, and interactions, are important. In our own study with 170 SLE patients and 192 controls we found a positive association between CT genotype of FokI and TT genotype of TaqI and SLE accrual damage. Nevertheless, there is a lack of a clear association with the other analysed factors (number of affected organs, number of severe flares, and pharmacological therapy). This can be explained because damage in SLE is multifactorial and SNPs studies are more likely to give us the disease's burden rather than the disease's susceptibility [52].

There are few studies regarding transcriptomic in real life patients and all of them show indirect results and no microarrays essays for microRNA or others.

Barry et al. investigated whether 41 candidate single nucleotide polymorphisms (SNPs) in vitamin D and calcium pathway genes (GC, DHCR7, CYP2R1, CY P27B1, CYP24A1, VDR, and CASR) are associated with 25-hydroxyvitamin D or modify the increase in 25-hydroxyvitamin D from vitamin D3 supplementation. They studied 1878 healthy persons and showed that the increase in 25-hydroxyvitamin D due to vitamin D3 supplementation was modified by genotypes at rs10766197 near CYP2R1, rs6013897 near CYP24A1, and rs7968585 near VDR [53].

Monticielo et al. studied 195 SLE patients and 201 healthy controls and according to genotype distribution, 25-hydrovitamin D concentrations were significantly higher in patients carrying the FokI f/f genotype in comparison with patients carrying the F/F genotype (31.6 ± 14.1 ng/ml versus 23.0 ± 9.2 ng/ml, $p = 0.004$), reinforcing its role in the functional activity of VDR [54].

In summary, a number of resistance mechanisms could be postulated with this overview.

One of the potential vitamin D resistance mechanism in SLE is the fact that different cells require different concentrations of active hormone, and we do not know the targets nor the concentrations required for each target at the various stages of the disease.

On the other hand, there seems to be 1000–10,000 genomic VDR binding sites per cell type. This is far more than the number of primary 1, 25-dihydroxyvitamin D3 target genes, which is in the order of 100-500 per tissue. This indicates that some genes are controlled by more than one VDR binding site, i.e., they may have a higher potential to be regulated by 1,25-dihydroxyvitamin D3 rather than target genes with only one active VDR locus.

This important medical problem leads to the question, whether an insight into the genome- and transcriptome-wide actions of VDR and 1, 25-dihydroxyvitamin D3 help in a more accurate evaluation of the human individual's responsiveness to, and needs for, vitamin D. Interestingly, only for a subset of individuals, significant correlations between the up-regulation of both genes and the intervention-induced raise in serum 25- **hydroxyvitamin D** ***concentrations were obtained. This suggests that, on a molecular level, not all study participants benefited from the vitamin D3 supplementation because they had already reached their individual optimal vitamin D status before the start of the intervention, or they carry a genetic polymorphism making them less responsive to vitamin D3 or other undefined reasons.***

Finally, the VDR receptor has polymorphisms which interfere with their conformation, as the FokI as we showed in our data. Yet, the remaining key enzymes in the synthesis of **25-hydroxyvitamin D,** ***as well as its carrier (the VDR binding-protein) also have polymorphisms which alter its enzyme activity and transport. So, the state of resistance to vitamin D is complex and the evaluation of the VDR polymorphisms only evaluates one of the key steps of the problem.***

6 – Vitamin D in SLE real practice

Many studies and revisions address Vitamin D therapy in real practice [55].There are some considerations that we have made which are important for real practice:

- Can Vitamin D supplementation prevent SLE flares?
- Can Vitamin D levels predict flares?
- Are Vitamin D related to SLE specific clinical features?
- What is the optimal dosage in SLE?
- Is there a window of opportunity?

There a large number of studies addressing this issue, however the evidence is sparse and some reviews have been made [56]. We present the two more important studies addressing this issue.

Birmingham et al. made a study with 46 SLE patients from the specimen bank and database of the Ohio SLE Study. They studied the vitamin D seasonal variation levels and their correlation with flares. The major finding of this study was that in non-African-Americans SLE patients, there was a highly significant decrease in 25-Hydroxivitamin D serum levels at the time of flare, for those flares occurring during low daytime sun exposure season. This was observed in both nonrenal and renal flares [57].

Schoindre Y et al. prospectively studied the relationship between 25-hydroxivitamin D levels and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score, in 170 SLE patients and assessed the role of vitamin D in predicting SLE flare-ups. There was no association between baseline 25-Hydroxivitamin D levels and relapse-free rate [58].

Susmita Roy et al. published a complex paper, developing a general kinetic model in an attempt to capture the role of Vitamin-D in immunomodulatory responses. This kinetic model, developed using the ideas of chemical network theory, leads to a system of nine coupled equations that were solved both by direct and by stochastic (Gillespie) methods. They found that although Vitamin D plays a negligible role in the initial immune response, it exerts a profound influence in the long term, especially in helping the system to achieve a new, stable steady state. They also founded that the optimal Vitamin-D level lies in the 50–100 moll/lit range, where both pathogen and effector T-cell levels remain at reasonably low risk range [59].

Finally some SLE specific clinical features and SLE associated diseases are being related do hypovitaminosis D, like immune cytopenias [60],neuropathy and Lynphoma in Sjögren's Syndrome [61], Hashimoto's disease [62] and others.

This is a difficult to prove issue. Despite the general idea that low vitamin D levels are related to higher disease activity, proves are needed in RCT. It seems that large variations of vitamin D can be related to SLE flares, but Vitamin D levels themselves cannot predict flares. This statement thus not exclude that vitamin D basal therapy, at least in stable SLE patients, cannot prevent flares, and we may have a window of opportunity to immune modulation. Finally, in theory, immunomodulation can be achieved with optimal levels between 75-100 moll/l, however it is shown that optimal individual levels have to be tailored.

Future directions

Theoretically Vitamin D should have relevant effects on musculoskeletal health, immunity, autoimmunity, cardiovascular disease, cancer, fertility, pregnancy, dementia and even mortality [63]. However, many of these effects are theoretical and it is necessary to demonstrate *in vivo* cellular effects, detected *in vitro*, in a large group of patients followed-up in a systematic and controlled manner. These studies are needed to identify the optimal dose of vitamin D for each patient, based on their clinical, genetic and physiopathological state. On a quick observation of the factors that modulate the response to vitamin D, it would be tempting to use very high doses, because they seem safe and could surpass any therapeutic resistance. However, secure scientific evidence only applies to calcium metabolism, being clearly unknown its deleterious effects on other actions of the hormone when used massively.

As such, the big challenge is to recognize all factors involved in the modulation of vitamin D, such as transcriptomic and epigenetic factors, polymorphisms of various enzymes, key receptors of its metabolism, specific cellular targets and the expression of cytokines. This is the only way we can develop tailored therapies for each disease and each patient using an individualized treatment plan.

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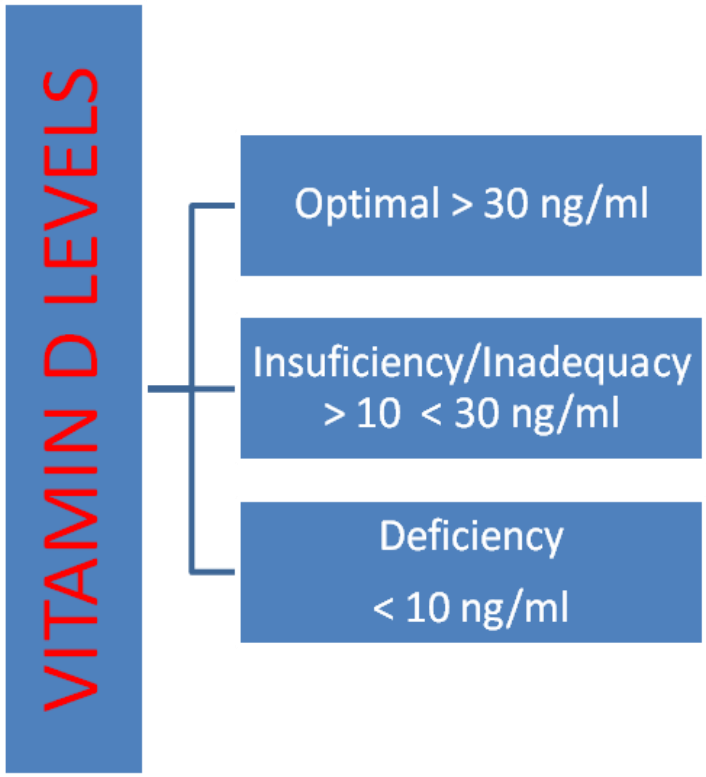

Supplement 1: classification of Vitamin D levels.
Adapted from: *Neil Binkley et al., Endocrinol Metab Clin North Am. Endocrinol Metab Clin North Am. 2010 June; 39(2): 287-contents*

Appendix 2

Vitamin D effect on SLE (review table)

Appendix 2 - Vitamin D effect on SLE (review table).

Ref.	Exposition	First Author	Name of Study	Year of publication	Language	Design of study	Level of Evidence	N	Objective	Outcome Measured	Methods of Hypothesis Testing	Results
16	**Vitamin D autoimmunity and SLE susceptibility**	. Bettencourt A. et al.	The Protective Role of HLA-DRB1 ( ) 13 in Autoimmune Diseases.	2015	English	Retrospective cohort studie	4	1228 patients with AIDs and 281 healthy controls	HLA-DRB1 genes assotiation with AIDs risk and protection.	HLA alleles frequency	Stepwise logistic regression on an allelic level. ORs obtained in a multivariable logistic regression analysis are adjusted for all the other genes included in the model and therefore differ from those obtained when a given gene is compared with all other genes. .	Associations of HLA-DRB1( )15 (OR = 2.17) and HLA-DRB1( )03 (OR = 1.81) alleles with MS, HLA-DRB1( )03 with SLE (OR = 2.49), HLA-DRB1( )01 (OR = 1.79) and HLA-DRB1( )04 (OR = 2.81) with RA, HLA-DRB1( )07 with Ps + PsA (OR = 1.79), HLA-DRB1( )01 (OR = 2.28) and HLA-DRB1( )08 (OR = 3.01) with SSc, and HLA-DRB1( )03 with MG (OR = 2.98). Consistent negative association of HLA-DRB1 ( ) 13 allele with SLE, Ps + PsA, RA, and SSc (18.3%, 19.3%, 16.3%, and 11.9%, resp., versus 29.8% in controls). HLA-DRB1 ( ) 13 frequency in the AIDs group was 20.0% (OR = 0.58).
22	**The emerging role of vitamin D in immunomodulation**	Kang SW et al.	1,25-Dihyroxyvitamin D3 promotes FOXP3 expression via binding to vitamin D response elements in its conserved noncoding sequence region	2012	English	Intervention in human cells stimulation in vitro.	4	Unknown	To prove the existence of a VDRE in the noncoding sequence region of FOX P3	Identification of putative VDREs in the human FOXP3 non coding sequence region.	A paired t-test	Identified three putative VDREs in the non coding sequence region +1714 to +2554 of the FOXP3 gene.
23	**Vitamin D and Antigen Presenting Cells in SLE**	Lerman et al.	1,25 dihydroxyvitamin D3 limits monocyte maturation in lupus sera.	2011	English	Intervention in human cells stimulation in vitro.	4	5 Healthy donnors and 15 Pediatric SLE	To examine whether treatment with 1,25-dihydroxyvitaminD(3) affects human APC maturation in vitro under multiple cytokine milieus.	Monocyte maturation under vitamin D and different cytokines millieu.	Student's t-tests . The non-parametric Spearman correlation coefficient.	Was demonstrated that 1,25(OH)2D3 has a broad ability to limit monocyte maturation, serving to limit maturation of cells cultured in IFNα, GM-CSF/IL-4, and even in a cytokine-free environment. We made the novel observation that 1,25(OH)2D3 retains this immunomodulatory capacity in the presence of SLE sera.
7		Ilan Ben-Zvi	The Impact of Vitamin D on Dendritic Cell Function in Patients with Systemic Lupus Erythematosus	2010	English	Intervention in human cells stimulation in vitro.	4	198 SLE patients	To observe if Monocyte-derived DCs of SLE patients were normally responsive to vitamin D in vitro	Monocyte-derived DCs activation with LPS stimulation under vitamin D presence	Pearson product moment correlation, Spearman rank order correlation. Chi-square analysis-of-contingency tables. Non-parametric Mann-Whitney tests.	Severe 25-D deficiency presetn in a substantial percentage of SLE patients and demonstrate an inverse correlation with disease activity. vitamin D supplementation will contribute to restore immune homeostasis in SLE patients through its inhibitory effects on DC maturation and activation.
24		Wu HJ.	Alternatively activated dendritic cells derived from systemic lupus erythematosus patients have tolerogenic phenotype and function.	2015	English	Intervention in human cells stimulation in vitro.	4	SLE patients and healthy controls	To generate alternatively activated DCs by treating monocyte-derived DCs from patients with SLE and healthy subjects with combination of vitD3 and dexamethasone followed by lipopolysaccharide-induced maturation.	Generation of alternatively activated DCs and tolerogenic effects	the non-parametric Spearman correlation coefficient.	Alternative activated DCs from SLE patients with quiescent disease by treating peripheral monocytes in vitro with vitD3 and dexamethasone followed by LPS-induced maturation have superior tolerogenic effects than those treated with Vitamin D alone.
25		Aranow C et al.	Randomized, Double-Blind, Placebo-Controlled Trial of the Effect of Vitamin D3 on the Interferon Signature in Patients With Systemic Lupus Erythematosus.	2015	English	Randomized, Double-Blind, Placebo-Controlled Trial	2	57 SLE patients	To investigate the effects of vitamin D supplementation on the IFN signature in patients with SLE.	3 IFN response genes. SLEDAI. Vitamin D levels.	Fisher's Exact statistics or Cochran-Mantel-Haenzsel (CMH) statistics used to compare categorical outcomes among treatment groups. Pearson's $\chi 2$ statistics used to evaluate relationships between 25(OH)D repletion status and categorical outcomes or baseline characteristics. Analysis of variance (ANOVA) or analysis of covariance (ANCOVA) models were used for continuous outcomes.	Vitamin D3 supplementation up to 4,000 IU daily was safe and well-tolerated but failed to diminish the IFN signature in vitamin D-deficient SLE patients. Higher 25(OH)D levels sustained for a longer duration may be required to affect immunologic outcomes.

Appendix 2 (cont.)

Ref.	Exposition	First Author	Name of Study	Year of publication	Language	Design of study	Level of Evidence	N	Objective	Outcome Measured	Methods of Hypothesis Testing	Results
26	**Vitamin D, Regulatory T-Cells and T-Helper 17 Cells**	Terrier et al.	Restoration of regulatory and effector T cell balance and B cell homeostasis in systemic lupus erythematosus patients through vitamin D supplementation.	2012	English	Prospective intervencional	4	20 SLE patients	To evaluate the safety and the immunological effects of vitamin D supplementation in 20 SLE patients with hypovitaminosis D	SLEDAI, Tregs, TH17, B-Cells	Wilcoxon signed-rank test.	Vitamin D was well tolerated and induced a preferential increase of naïve CD4+ T cells, an increase of regulatory T cells and a decrease of effector Th1 and Th17 cells. Vitamin D also induced a decrease of memory B cells and anti-DNA antibodies.
28		Viviane de sousa et al.	Association of hypovitaminosis D with Systemic Lupus Erythematosus and inflammation.	2014	English	Cross-sectional study	4	45 SLE patients and 25 controls	To evaluate the association between vitamin D insufficiency with SLE and inflammatory markers.	Vitamina D levels,High sensitivity C reactive protein interleukin-6 (IL-6).Urinay sediment (AE proteinuria and creatinine clearance in 24-hour urine,serum anti-double stranded DNA.	The Shapiro-Wilk test,Mann-Whitney U test, chi-square test, pearson's correlation coefficient.	The prevalence of 25(OH)D insufficiency was 55% in SLE patients and 8% in the controls participants (p = 0.001). The median of 25(OH)D was lower in patients than in controls. Patients with insufficient 25(OH)D had higher levels of IL-6 and higher prevalence of hematuria.There was no correlation between vitamin D and SLEDAI or lupus nephritis.
29		Banica LM et al.	Dysregulation of anergy-related factors involved in regulatory T cells defects in Systemic Lupus Erythematosus patients: Rapamycin and Vitamin D efficacy in restoring regulatory T cells.	2014	English	Intervention in human cells stimulation in vitro.	4	SLE patients and controls	To explore the expression of anergy-related factors in CD4+ T cells in relation to Tregs frequency in SLE patients and to identify strategies to redress these defects.	Casitas B-cell lymphoma b and 'gene related to anergy in lymphocytes' proteins were analyzed in (PBMCs). Growth response factors (egr)2 and egr3 messenger RNAs. CD4+ T cells Tregs caractherization and expansion.	Pearson product moment correlation, Spearman rank order correlation. Chi-square analysis-of-contingency tables. Non-parametric Mann-Whitney tests.	Experimental activation of CD4+ T cells in the presence of IL-2 and Rapamicyn or VitD induced the expansion of SLE Tregs. However, on long-term, only Rapamicyn exposure of SLE CD4+ T cells yielded high numbers of Tregs with sustained suppressive activity.
30		Piantoni et al.	Phenotype modifications of T-cells and their shift toward a Th2 response in patients with systemic lupus erythematosus supplemented with different monthly regimens of vitamin D.	2015	English	Prospective intervencional	4	34 patients with systemic lupus erythematosus (SLE) were randomly enrolled in a two-year prospective study.	Effect of different vitaminD regimens in T-Cell subtypes.	T-Cell subtypes by flow cytometry and vitamin D levels	Shapiro-Wilk test . Wilcoxon signed-rank test, Mann-Whitney test. Chi-square test	After a long-term of monthly treatment with vitamin D in SLE patients, an enhancement of T-reg cells and the production of Th2 cytokines should be expected.

Ref.	Exposition	First Author	Name of Study	Year of publication	Language	Design of study	Level of Evidence	N	Objective	Outcome Measured	Methods of Hypothesis Testing	Results
31	Vitamin D, Regulatory T-Cells and T-Helper 17 Cells	Wahono et al.	.Effects of 1,25(OH)2D3 in immune response regulation of systemic lupus erithematosus (SLE) patient with hypovitamin D.	2014	English	Prospective intervencional	4	5 SLE patients and controls	To investigate the effects of calcitriol on dendritic cells maturation and Th17 and Treg cells activation in SLE patients with hypovitamin D.	Vitamin D levels, dendritic cell maturation (the percentage of CD40, CD86, and HLA-DR expression), amount of Th17 and Treg cells; Cytokines production of IL-12, IL-17A, and TGF-β.	Paired t-test.	The study concluded that 1,25(OH)2D3 inhibited dendritic cells maturation and Th17 cells activation in SLE patients. The 1,25(OH)2D3 increased Treg cells but not significant.
32		Drozdenko G. et al.	Effects of 1,25(OH)2D3 in immune response regulation of systemic lupus erithematosus (SLE) patient with hypovitamin D	2014	English	Prospective interventional	4	25 SLE patients for vitamin D supplementation and controls without treatment	To asses whether oral vitamin D supplementation leads to a systemic modulation of the phenotype of circulating lymphocyte and whether a defined serum 25-hydroxyvitamin D (25(OH)D) concentration can be related to the effects on lymphocytes.	Vitamin D levels and circulating B and T Cell phenotype before and after supplementation.	Paired t-test.	Following cholecalciferol intake, the frequencies of circulating CD38 expressing B cells were significantly increased and IFN-γ+ , and/or IL-17+ CD4+ T helper cells were decreased. These data indicate that increasing 25(OH)D serum concentrations are associated with an increased expression of CD38 on B cells and a decreased T-cell-dependent proinflammatory cytokine production.
33		Marinho A. et	Vitamin D supplementation effects on FoxP3 expression in T cells and FoxP3+/IL-17A ratio and clinical course in systemic lupus erythematosus patients: a study in a Portuguese cohort.	2016	English	Prospective interventional	4	24 SLE patients	The aim of this study was to evaluate the effect of vitamin D supplementation on FoxP3 expression and IL-17A-producing T cells, through FoxP3+/IL-17A ratio	Vitamina D levels, FoxP3+ and Il17A expression after supplementation.	Wilcoxon signed-rank test	Serum 25(OH)D levels significantly increased under vitamin D supplementation ($p = 0.001$). The FoxP3+/IL-17A ratio in SLE patients after 6 months of vitamin D supplementation was higher than that in the baseline ($p < 0.001$). The favourable effect was also observed in patients with baseline Vitamin D > 75 nmol/l.
34		Maja Vukić et al.	Relevance of Vitamin D Receptor Target Genes for Monitoring the Vitamin D Responsiveness of Primary Human Cells	2015	English	Prospective intervencional	4	71 from the VitDmet study and 10 VitDbol study.	The correlations between the changes in mRNA expression in relation to serum 25(OH)D3 level in primary human cells	mRNA expression from selected VDR target genes.	The network analysis is based on the fit, measured by r2, of the changes in mRNA expression/clinical parameter in relation to 25(OH)D3 serum level variations, for the participants not removed.	In Peripheral blood mononuclear cells was observed direct transcriptional effects on the selected VDR target genes, such as an up to 2.1-fold increase already one day after supplementation onset. Both long-term and short-term vitamin D3 supplementation studies allow monitoring the vitamin D responsiveness of human individuals and represent new types of human in vivo vitamin D3 investigations.

Appendix 2 (cont.)

Ref.	Exposition	First Author	Name of Study	Year of publication	Language	Design of study	Level of Evidence	N	Objective	Outcome Measured	Methods of Hypothesis Testing	Results
35	**Vitamin D Inflammation and atherosclerosis**	Petri et al.	Vitamin D deficiency does not predict progression of coronary artery calcium, carotid intima media thickness or high-sensitivity C-reactive protein in systemic lupus erythematosus.	2013	English	Prospective Cohort	2	154 SLE patients	To determined whether levels of vitamin D at baseline were associated with subclinical measures of atherosclerosis, or with changes in subclinical measures over 2 years.	Baseline and follow-up data [including coronary artery calcium , carotid intima-media thickness (IMT), 25-hydroxy vitamin D and high-sensitivity CRP levels] were available for 154 patients.	Pearson chi-square tests and analysis of variance (ANOVA).	25(OH)D was not associated with any measure of subclinical atherosclerosis. 25(OH)D deficiency was associated with higher hsCRP at baseline, but did not predict a change in hsCRP over 2 years.
33		Angela Byun Robinson et al.	Vitamin D status is a determinant of atorvastatin effect on carotid intima medial thickening progression rate in children with lupus: an Atherosclerosis Prevention in Pediatric Lupus Erythematosus (APPLE) substudy.	2014	English	Randomized controle trial	2	201 SLE patients	Carotid intima medial thickness (CIMT) measurements and other existing data from the Atherosclerosis Prevention in Pediatric Lupus Erythematosus (APPLE) trial use to assess interactions between serum 25-hydroxyvitamin D, atorvastatin randomisation and CIMT progression rate.	Progression of CIMT wth vitamin D and atorvastatin group and placebo group	The primary efficacy analysis for APPLE compared rates of mean-mean common CIMT progression between treatment groups based on a test of two-way interaction between treatment group and time in a longitudinal linear mixed effects model under data missing at random assumptions.	There was significant interaction between baseline vitamin D deficiency and atorvastatin randomisation in 3-year progression of mean-max CIMT. In four out of six carotid segments, there was a greater decrease in mean-max CIMT progression rate in subjects who were treated with atorvastatin compared with placebo if they had baseline serum 25(OH)D levels ≥20 ng/mL.Subjects with serum 25(OH)D ≥20 ng/mL had less mean-max CIMT progression following 3 years of atorvastatin treatment.
36		Robinson AB et al.	Vitamin D deficiency is common and associated with increased C-reactive protein in children and young adults with lupus: an Atherosclerosis Prevention in Pediatric Lupus Erythematosus substudy.	2014	English	Randomized controle trial	2	201 SLE patients	Assessment of associations between 25-hydroxyvitamin D [25(OH)D] and measures of systemic lupus erythematosus (SLE) disease activity and cardiovascular risk.	vitamin D levels <20, race, season, latitude, disease duration, disease activity, high-sensitivity C-reactive protein, proteinuria, fasting lipids and CIMT.	A multivariable linear regression model was constructed with the outcome serum 25(OH)D level at baseline and the list of selected predictors.	In multivariable modelling, vitamin D deficiency was associated with age, latitude, season, minority status, proteinuria and hsCRP.
38		Apinya Lertratanakul et al.	25-Hydroxyvitamin D and Cardiovascular Disease in Patients With Systemic Lupus Erythematosus: Data From a Large International Inception Cohort.	2014	English	Restrospective inception cohort	4	890 SLE patients	to estimate the associations of 25(OH)D levels with Cardiovascular risk factors and to determine whether low baseline 25(OH)D levels predict future Cardiovascular events.	SLE activity and damage assessments, CV risk factors and events, medications, laboratory assessments of 25(OH)D levels, and inflammatory markers.	Multiple logistic and Cox regressions were used to estimate the associations of baseline 25(OH)D levels with baseline CV risk factors and CVD events.	Patients in the higher quartiles of 25(OH)D were less likely to have hypertension and hyperlipidemia and were more likely to have lower C-reactive protein levels and lower Systemic Lupus Erythematosus Disease Activity Index 2000 scores at baseline when compared with the first quartile. Vitamin D levels were not independently associated with CVD event incidence; however, hazard ratios for CVD event incidence decreased with successively higher quartiles.

Appendix 2 (cont.)

Ref.	Exposition	First Author	Name of Study	Year of publication	Language	Design of study	Level of Evidence	N	Objective	Outcome Measured	Methods of Hypothesis Testing	Results
39	**Vitamin D Inflammation and atherosclerosis**	JA Reynolds et al.	25-Hydroxyvitamin D deficiency is associated with increased aortic stiffness in patients with systemic lupus erythematosus	2011	English	Retrospective Cross sectional	4	Women with SLE = 75	To determine the relationship between serum vitamin D and markers of subclinical cardiovascular disease in patients with SLE.	Vitamin D levels.Insulin resistance calculated using the homeostatic model assessment method. Vascular cell adhesion molecules (VCAM-1, E-selectin). Aortic pulse wave velocity	Non-parametric tests (Mann–Whitney U-test). Linear and logistic regression models used to investigate linear associations between 25(OH)D and markers of subclinical CVD. Logistic regression was used to analyse binomial variables.	Vitamin D deficiency is associated with increased aortic stiffness in SLE, independent of CVD risk factors and insulin resistance. Increased inflammatory disease activity may be the mechanism by which vitamin D deficiency mediates vascular stiffness in this patient group.
40		Sabio JM et al.	Association between low 25-hydroxyvitamin D, insulin resistance and arterial stiffness in nondiabetic women with systemic lupus erythematosus.	2014	English	Cohort nested cross-sectional study	4	106 SLE patients and 101 match controls.	To examine if there is an association between low levels of 25-hydroxyvitamin D (25(OH)D) and insulin resistance (IR) in nondiabetic women with systemic lupus erythematosus (SLE) and to evaluate its impact on arterial stiffness.	25(OH)D, insulin, insulin resistance measured, homocysteine, fibrinogen, characteristics of SLE, medications and pulse-wave velocity.	Data presented as interquartile range and percentages. Mann-Whitney's test or Student's t-test.Two-tailed Fisher's exact test, OR, 95% CI. Linear regression analysis to determine relationship between 25(OH)D concentrations and PWV and other clinical and analytical variables. A multiple backward stepwise linear regression analysis to identify factors independently associated with PWV in women with SLE.	Low 25(OH)D levels were found to be associated with increased IR in nondiabetic women with SLE independently of BMI. Low 25(OH)D levels, but not IR, could be associated with increased arterial stiffness in these patients.
41		Reynalds JA et al.	Vitamin D improves endothelial dysfunction and restores myeloid angiogenic cell function via reduced CXCL-10 expression in systemic lupus erythematosus.	2016	English	Open-label case-control study	4	40	To evaluate if CXCL-10 (IP-10) reduced expression by vitamin D may be important, has anti-angiogenic functions in an in vitro Human Umbilical Vein Endothelial Cell (HUVEC) mode	Improvement in endothelial function correlated with increase in serum 25(OH)D concentrations independently of disease activity	Patient-level data were compared using the Mann-Whitney U test (and Spearman's correlation), whilst data from in vitro models were compared using either a two-tailed t test or paired t test as appropriate.	Effectiveness of calcitriol to restore SLE Myeloid angiogenic cells (MACs) phenotype, augment the angiogenic capacity of MACs and to positively modulate the paracrine regulation of endothelial nitric oxide synthase.
42		Abou-Raya A et al.	The effect of vitamin D supplementation on inflammatory and hemostatic markers and disease activity in patients with systemic lupus erythematosus: a randomized placebo-controlled trial.	2013	English	Randomized controlled trial	2	SLE (n = 267)	vitamin D status in patients with SLE and determined alterations in inflammatory and hemostatic markers and disease activity before and after vitamin D supplementation.	Assessment of alterations in levels of proinflammatory cytokines and hemostatic markers, and SLEDAI after 12 months of colecalciferol supplementation.	Means ± SD, there were used intent-to-treat method, Student's t-test, and chi-square test or Fisher's exact test. Spearman's rank correlation coefficient. Multivariate regression was used to adjust for potential codeterminants of vitamin D. Multivariate linear regression used when 25(OH)D was a continuous variable and multivariate logistic regression used when 25(OH)D was a categorical variable. levels, 95% CI; P values.	At 12 months of therapy, tsignificant improvement in levels of inflammatory and hemostatic markers as well as SLEDAI in the treatment group compared to the placebo group.

Appendix 2 (cont.)

Ref.	Exposition	First Author	Name of Study	Year of publication	Language	Design of study	Level of Evidence	N	Objective	Outcome Measured	Methods of Hypothesis Testing	Results
49	Vitamin D resistant state and SLE	Arash Hossein-nezhad et. al.	Influence of Vitamin D Status and Vitamin D3 Supplementation on Genome Wide Expression of White Blood Cells: A Randomized Double-Blind Clinical Trial.	2013	English	Randomized Clinical Trial	2	8 Healthy adults	To determine the effect of vitamin D status and subsequent vitamin D supplementation on broad gene expression in healthy adults.	Comparing gene expression in two groups (deficient vs. insufficient or sufficient) to study the effect of vitamin D status on broad gene expression.	To identify the differentially expressed genes before versus after supplementation and between two kinds of supplementation (400 IUs or 2000 IUs), a 2-way ANOVA in the linear model was applied.	Vitamin D supplementation caused at least a 1.5 fold change in the expression of 291 genes that are involved in apoptosis, immune function, transcriptional regulation, epigenetic modification, response to stress, cell cycle activity and differentiation. 66 genes were identified, that were most significantly affected by the subjects' vitamin D status. Of these 66 genes, 17 genes whose expression significantly changed after vitamin D3 supplementation in both deficient and insufficient/sufficient groups were found to have novel VDREs.
52		Marinho et al.	Association between vitamin D receptor (VDR) gene polymorphisms and systemic lupus erythematosus in Portuguese patients.	2015	English	Retrospective study	4	170 SLE patients and 192 healthy patients	Correlation of VDR SNPs to SLE severity and damage.	SLEDAI, Flares, SLICC, VDR SNPs.	Chi-square test, Mann-Whitney test, multivariate analysis.	VDR (TaqI and FokI variants) SNPs are related to a higher long-term cumulative damage in SLE patients, but not to severe flares.
53		Barry EL	Genetic variants in CYP2R1, CYP24A1, and VDR modify the efficacy of vitamin D3 supplementation for increasing serum 25-hydroxyvitamin D levels in a randomized controlled trial.	2014	English	Randomized controled trial	2	1787 healthy non-Hispanic white participants aged 45-75 years.	To investigated whether 41 candidate SNPs in vitamin D and calcium pathway genes (GC, DHCR7, CYP2R1, CYP27B1, CYP24A1, VDR, and CASR) are associated with [25(OH)D] or modify the increase in [25(OH)D] from vitamin D3 supplementation.	Genotype main effects and interactions with vitamin D3 treatment.	Multiple Linear regression	The increase in [25(OH)D] attributable to vitamin D3 supplementation may vary according to common genetic differences in vitamin D 25-hydroxylase (CYP2R1), 24-hydroxylase (CYP24A1), and the vitamin D receptor (VDR) genes. These findings have implications for achieving optimal vitamin D status and potentially for vitamin D-related health outcomes.
54		Monticiel o AO et.al.	The role of BsmI and FokI vitamin D receptor gene polymorphisms and serum 25-hydroxyvitamin D in Brazilian patients with systemic lupus erythematosus.	2012	English	Retrospective study	4	195 SLE patients and 201 Healthy controls.	To investigate the influence of BsmI and FokI VDR SNPs on susceptibility to SLE. In addition, 25-hydroxyvitamin D was measured in SLE patients to evaluate associations with VDR SNPs and clinical and laboratory expressions of disease.	Vitamina D levels, VDR SNPs genotyping.	Chi-square test, Mann-Whitney test, multivariate analysis.	According to genotype distribution, 25(OH)D concentrations were significantly higher in patients carrying the FokI f/f genotype compared with patients carrying the F/F genotype (31.6 ± 14.1 ng/ml versus 23.0 ± 9.2 ng/ml, p = 0.004).

Ref.	Exposition	First Author	Name of Study	Year of publication	Language	Design of study	Level of Evidence	N	Objective	Outcome Measured	Methods of Hypothesis Testing	Results
57	Vitamin D in SLE real practice	Birmingham DJ	Evidence that abnormally large seasonal declines in vitamin D status may trigger SLE flare in non-African Americans	2012	English	Database nested Case - Control	4	Specimen bank and database of the Ohio SLE Study (OSS). 46 patients.	To inestigate the Influence of low daylight months on 25-hydroxyvitamin D levels and Flares . Influence of race and daylight status on SLE flare rates.	Vitamin D levels. SLE flare rates in white and Afro-Americans.	Mixed effects models	The major finding of this study was that in non-AA SLE patients, there was a highly significant decrease in 25(OH)D serum levels at the time of flare for those flares occurring during LDM. This was observed in both nonrenal and renal flares.
58		Schoindre Y et al.	Lower vitamin D levels are associated with higher systemic lupus erythematosus activity, but not predictive of disease flare-up.	2014	English	Prospective Cohort	4	170 SLE patients	To assess the role of vitamin D in predicting SLE flare-ups.	SLEDAI and Vitamin D levels	Univariate analysis with a linear regression model for quantitative variables and with Mann–Whitney tests for qualitative variables. Variables with univariate p value <0.2 were included in a multivariate stepwise linear regression model.	There was no association between baseline 25(OH)D levels and relapse-free survival rate.

Articles included in the Literature Review: trials and studies with Vitamin D intervention. SLE: Systematic Lupus Erythematosus; NCP: Non-calcified coronary plaque; RR: Relative Risk; OR: Odds Ratio; SD: standard deviation; CAD: coronary artery disease; 95% CI: 95% confidence interval; ESR: erythrocyte sedimentation rate; AIDs: Autoimmune diseases. MS: multiple sclerosis, SLE: lupus; RA: Rheumatoid arthritis; PsA: Psoriatic arthritis; Ps: psorisis; MG: Miastenia Gravis; SSc: Systemic Sclerosis; VDRE: Vitamin D response element. FOXP3: Forkhead box P3; APC: Antigen Presenting Cells; DCs: Dendritic Cells; LPS: Lypopolisacharide; OD: Odds Ratio; Tregs: regulatory T-Cells; PMBCs: peripheral blood mononuclear cells; VDR: Vitamin D receptor; SPNs: single nucleotide polymorphisms; IFN α: alpha interferon; GM-CSF: Granulocyte macrophage colony-stimulating factor; SLEDAI: Systemic Lupus Erythmatosus Disease Activity Index; SLICC: Systemic Lupus International Collaborating Clinics.

Appendix 3

Vitamin D levels in a healthy population from the North of Portugal

Vitamin D levels in a healthy population from the North of Portugal

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Abstract

Vitamin D *status* in human populations has become a matter of great concern, in the wake of a multitude of published works that document widespread Vitamin D deficiency across Europe, even in countries with abundant sunlight. In Portugal there are no measures of 25-hydroxy vitamin D – 25(OH)D – levels in the general adult population. The purpose of this study was to measure 25(OH)D levels in a healthy population cohort and investigate the possible association with season and selected demographic and laboratory measurements.

A cohort of 198 participants (18-67 years) living in the north of Portugal, Porto, conducted in July and August 2015 (summer time) and April 2016 (winter time) was studied to evaluate serum 25(OH)D levels. Sociodemographic characteristics (age, sex and body mass index) and season of the year were taken into account as possible 25(OH)D levels codeterminants.

In the whole group, the mean level of serum 25(OH)D was 55.4±23.4 nmol/L, with 48% of the population presenting levels compatible with vitamin D deficiency (below 50 nmol/L). In the winter period, this value reaches 74%. No statistically significant differences were observed between genders (57.4±23.9 vs. 53.3±22.8 nmol/L, p=0.219) as well as no statistically significant correlation was found between age and 25(OH)D levels (p=0.349). As expected higher levels of 25(OH)D were observed in summer than in winter (68.2±21.5 *vs*. 42.2±16.9 nmol/L; p<0.0001). Serum 25(OH)D levels were significantly lower in obese compared to non-obese subjects (46.6±17.6 vs. 57.7±24.2 nmol/L, p=0.012).

Vitamin D deficiency is prevalent in this area, affecting almost half of the population. Body mass index and season are predictors for lower vitamin D levels and deficiency *status*. An effective strategy to prevent Vitamin D deficiency and insufficiency should be envisaged in our population.

Key words: Portugal; 25(OH)D levels; Vitamin D deficiency; healthy adult population.

1. Introduction

Vitamin D is unique among vitamins, since it works as a hormone and can be synthesized on the skin as a result of exposure to sunlight. It is acquired both through nutrition (10-20%) and by cutaneous synthesis under the action of sunlight [1]. Dietary sources of vitamin D include fish oils and, in some countries (USA and Northern Europe) fortified food products (dairy and bread products). In Portugal, vitamin supplements containing vitamin D exist in the market. However, the main source of vitamin D results from cutaneous synthesis on sun exposure and is dependent on various factors such as the geographical area latitude, season, time of day, the exposed body surface and exposure duration, use of sunscreens, skin pigmentation, obesity and age [2].

Vitamin D_3 or cholecalciferol, after formation in the skin, and vitamin D_2 or D_3, from dietary sources, are hydroxylated in the liver, resulting in the formation of 25-hydroxyvitamin D [25(OH)D], the main circulating form. This form subsequently undergoes hydroxylation in the kidney to generate the biologically active, dihydroxylated form of vitamin D, calcitriol or 1,25$(OH)_2$D, which acts through specific vitamin D receptors [1]. The vitamin D role on the maintenance of calcium serum levels, by promoting calcium and phosphorus absorption from the intestine and calcium bone reabsorption, is well known [3]. Recent evidences correlate insufficient vitamin D levels with an increased risk of developing other non-bone-related disorders: cardiovascular diseases, hypertension, malignant neoplasia, type I diabetes mellitus, multiple sclerosis, dementia, rheumatoid arthritis, and infectious disease [2-4].The identification of vitamin D receptors in immune system cells and the discovery that dendritic cells can produce the metabolically active form of vitamin D have led to the suggestion that vitamin D is also an immune modulator [5].

The high prevalence of inadequate vitamin D is nowadays seen as a public health problem affecting several countries in Europe and the USA, particularly in those people at risk for osteoporosis and its consequences [2]. Vitamin D deficiency screening is accomplished through measurement of 25(OH)D, which is the best index for assessing vitamin D reserve in the body [2], due to its greater half-life comparing with the metabolically active form. Only at-risk populations are routinely tracked for Vitamin D deficiency, including the elderly, the institutionalized, pregnant women and post-menopausal women (increased risk of fractures) [3]. Much debate has taken place over the definition of vitamin D deficiency. Most agree that a 25(OH)D concentration <50nmol/L, or 20 ng/mL, is an indication of vitamin D deficiency, whereas a 25(OH)D concentration of 51–74 nmol/L, or 21–29 ng/mL, is considered to indicate insufficiency;

concentrations >75 nmol/L, or 30 ng/mL, are considered to be adequate [6-9]. The optimal serum 25(OH)D levels are those for which calcium absorption is optimized, parathyroid hormone (PTH) levels reduced and the greatest benefit to the bone and muscle function are obtained; currently levels above 75 nmol/L (30 ng/mL) are recommended.

Several studies have described inadequacy of vitamin D levels all over the Europe, although vitamin D *status* within different European countries shows a high variation [10-12]. In Portugal, the prevalence of vitamin D deficiency is unknown because there are no epidemiological studies in adult healthy individuals; however, several studies in healthy pediatric populations and in specific hospital populations have been published [3, 13-22]. In 2009 a study in a healthy pediatric population from Porto was published. A group of 45 children (33F, 12M; 2.5-16 years) were evaluated in winter and spring. None of them was supplemented after the first year of life. Values above 100 nmol/L were considered optimal, 75-100 nmol/L sufficient, 50-74 nmol/L relative insufficient and <50 nmol/L deficient. A lack of vitamin D was found in 26% of the studied population during the months with less sunlight. According to these cut off values, 80% of the children did not achieve optimal levels [23]. In another pediatric study, 73 children (37F, 36M), aged 12 months to 17 years, from the outpatient clinic of Centro Hospitalar do Porto, were studied. The study occurred between March 2008 and July 2010. The children were divided in to pre-school age (12 months to 5 years; 23.3% (17/73)) and school age (6 to 17 years; 76.7% (56/73)). Normal 25(OH)D levels (>75 nmol/L) were observed in 17.8% of the children (11% with optimal values, >100nmol/L; and 6.8% with sufficient values, 75-100 nmol/L). On the other hand, 82.2% had low vitamin D levels (42.5% with relative insufficiency, 50-74 nmol/L; and 39.7% with deficiency, <50 nmol/L). Gender, residential area, BMI and season were not related to 25(OH)D levels. It was observed that school age children had higher vitamin D deficiency (p=0.013), thus establishing a relation with age [24]. Another cohort of 122 healthy children and adolescents (5-18 years) from Porto was studied. They were observed in the pediatric outpatient clinic during the winter and spring of 2011/2012. Vitamin D *status* was observed to be insufficiency (≥20 and <30 ng/mL) in 92.5% of the cases, from which 47.8% presented deficiency (≥10 and <20 ng/mL) and 6% severe deficiency (<10 ng/mL). Only 7.5% of the sample had an adequate Vitamin D *status* (≥30 ng/mL) [25].

As already stated, vitamin D *status* is often studied in specific groups that have increased risk of vitamin D deficiency or osteoporosis, such as hospitalized or elderly people. In such groups, confounding of variables makes it difficult to translate findings

to the general population [26]. Thus, the aim of the current study is to evaluate Vitamin D *status* in non-supplemented healthy adults living in Porto, north of Portugal.

2. Subjects and Methods

2.1 Subjects

The study was conducted in Porto (~41° N), in July and August 2015 (summer time) and April 2016 (winter time). Two hundred healthy blood donors voluntarily participated in this study. Two men were excluded, because they were under multivitamin supplementation. The average age of these individuals was 43.1±12.1 years. Subjects were stratified in three groups according to age [27].

A questionnaire about age, gender, weight, height, ethnicity, nationality, place of birth, occupation, sun exposure, sunscreens use, eating habits, smoking habits, physical activity, diagnosed pathologies, use of medicines and food supplements, was answered during blood donation. In order to analyze the influence of BMI on the 25(OH)D levels, the subjects were divided into two groups based on BMI values: BMI<30 kg/m^2 (non-obese) or BMI≥30 kg/m^2 (obese). Written informed consent was obtained for each volunteer, and the study was approved by the Ethics Committee of Centro Hospitalar do Porto, according to Declaration of Helsinki.

2.2 Laboratory Measurements

Blood was collected in Vacuette® Z Serum Clot Activator tubes for the measurement of PTH and in Vacuette® Z Serum Separator Clot Activator tubes for the other measurements. Serum was obtained by centrifugation and stored in several aliquots at -20°C until analyzed. Serum 25(OH)D was chosen as a reliable marker of individual vitamin D *status* as it reflects vitamin D obtained from food sources and cutaneous synthesis, and it is not prone to diurnal variation.

Serum 25(OH)D was measured using an electro-chemiluminescence binding assay (ECLIA) for the in-vitro determination of total 25-hydroxyvitamin D (Elecsys® Vitamin D total, Cobas, Roche©). The reference range for 25(OH)D was >75 nmol/L (measurement range: 7.50-175 nmol/L). The serum PTH concentration was assessed using an electro-chemiluminescence assay with 15-65 pg/mL as a reference range. Serum total calcium, phosphate and creatinine concentrations were measured by routine laboratory methods in a Cobas Integra 800.

2.3 Statistical Analyses

Continuous data were checked for normality using the Kolmogorov-Smirnov test and natural logarithm (ln) transformations were used for skewed variables previous to the statistical analysis. Differences between groups were tested using the Student's t-test or one-way ANOVA (continuous variables) and χ^2 test (dichotomous variables). Pearson's or Spearman's correlation coefficients were calculated to test relationships between continuous variables.

Multiple linear regression analysis was used to consider potential determinants of 25(OH)D levels (dependent variable). The following independent variables: age and BMI (as continuous variables), season and gender (as categorical variables) were included in the model.

A *p*-value below 0.05 was considered to be statistically significant. Statistical analyses were performed using *Statistical Package for the Social Sciences* software (version 23, IBM SPSS Statistics, NY, USA).

3. Results

The characteristics of the study population are shown in Table1. Approximately 48% were women, and the mean age (±SD) of the study population was 43.1±12.1 years. No statistically significant differences were observed between genders. The frequency of obesity was significantly higher in this population compared with the general Portuguese population (22.7% vs. 14.2%, p=0.001, OR=1.77, 95%CI=1.26-2.50) [28].

The mean serum 25(OH)D concentration was 55.4±23.4 nmol/L in all participants (median 50.9 nmol/L) with no significant differences between men and women (57.4±23.9 vs. 53.3±22.8 nmol/L; p=0.219). Fifty women (52.6%) and 45 men (43.7%) were deficient in 25(OH)D but the gender difference was not statistically significant (Table 1).

No statistically significant correlation was found between age and 25(OH)D levels (p=0.349). When subjects were categorized in groups according to age (Figure 1), no differences in 25(OH)D levels between the 3 groups (p=0.311) were found.

Table 1 - Characteristics of the study population.

Characteristics	Total (n=198)	Women (n=95)	Men (n=103)
Sociodemographics			
Age, years, mean ± SD	43.1±12.1	41.9±12.5	44.2±11.7
Season			
Summer, n (%)	101 (51.1)	44 (46.3)	57 (55.3)
Winter, n (%)	97 (49.0)	51 (53.7)	46 (44.7)
BMI, mean ± SD	27.0±4.3	26.9±4.4	27.2±4.2
Laboratory measurements			
PTH levels (pg/mL), mean ± SD	44.9±14.7	45.6±12.6	44.3±16.5
Creatinine levels (mg/dL), mean ± SD	0.8±0.2	0.8±0.1	0.8±0.2
Total calcium levels (mmol/L), mean ± SD	2.4±0.1	2.4±0.1	2.4±0.1
Phosphorus levels (mmol/L), mean ± SD	1.0±0.2	1.0±0.2	1.0±0.2
25(OH)D levels (nmol/L)			
Mean ± SD	55.4±23.4	53.3±22.8	57.4±23.9
<50 nmol/L (deficiency), n (%)	95 (48.0)	50 (52.6)	45 (43.7)
50-75 nmol/L (insufficiency), n (%)	60 (30.3)	27 (28.4)	33 (32.0)
>75 nmol/L (optimal), n (%)	43 (21.7)	18 (18.9)	25 (24.3)

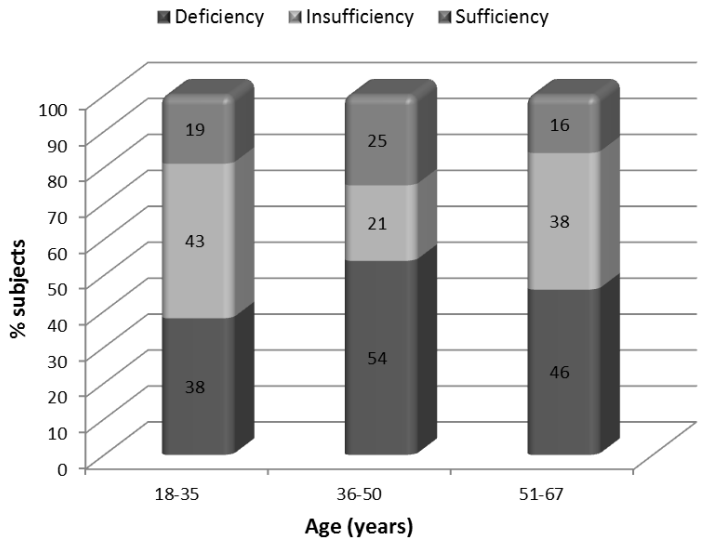

Figure 1 – Prevalence of Vitamin D deficiency and insuficiency by age.

Body mass index was negatively correlated with 25(OH)D levels (p=0.001, r=-0.237) (Figure 2). In conformity, 25(OH)D levels were significantly lower in obese compared to non-obese subjects (46.6±17.6 vs. 57.7±24.2 nmol/L, p=0.012) (Figure 3).

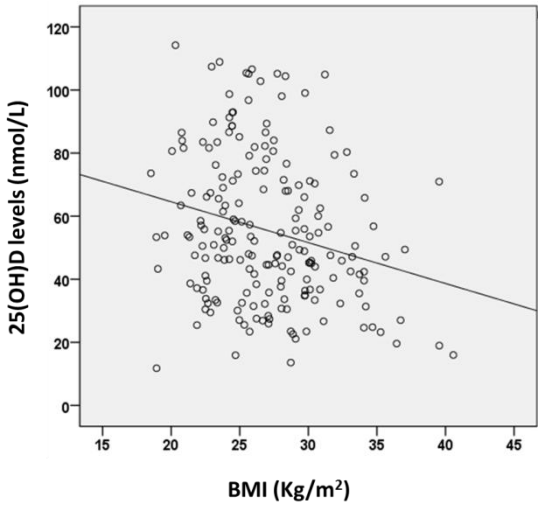

Figure 2 –Correlation between 25(OH)D and BMI.

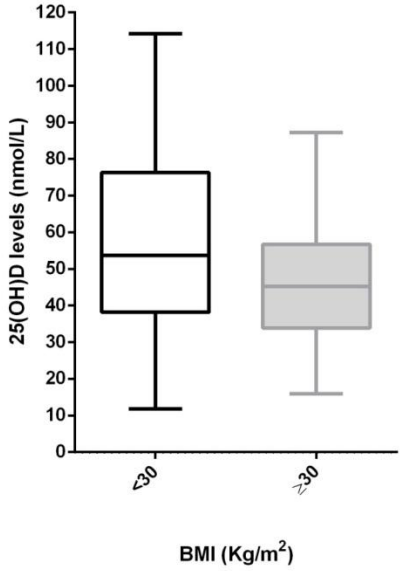

Figure 3- Comparison of serum 25 (OH)D levels between obese (BMI≥30) and non-obese (BMI<30) individuals.

In the winter period, 74.2% of the studied population had a 25(OH)D concentration below 50.0 nmol/L compared with 22.8% in the summer period (p<0.0001). Only 5 individuals (5.2%) presented optimal levels of 25(OH)D in winter, and 38 (37.6%) in summer (Table 2).

Table 2 – Differences in 25(OH)D concentration according to season.

25(OH)D levels (nmol/L)	**Winter (n=97)**	**Summer (n=101)**	**p**
Mean ± SD	42.2±16.9	68.2±21.5	<0.0001
<50 nmol/L (deficiency), n (%)	72 (74.2)	23 (22.8)	
50-75 nmol/L (insufficiency), n (%)	20 (20.6)	40 (39.6)	<0.0001
>75 nmol/L (optimal), n (%)	5 (5.2)	38 (37.6)	

In multiple linear regression analysis, controlling for age and gender, significant associations between 25(OH)D levels and season and BMI were found. Winter and higher BMI were significantly associated with lower serum 25(OH)D levels (Table 3).

Table 3 – Results of a multiple linear regression analysis on determinants of 25(OH)D levels

Variable	B	SE	p
Intercept	4.224	0.189	<0.0001
Age	-0.002	0.002	0.499
Season	0.482	0.054	<0.0001
Gender	-0.40	0.054	0.456
Body Mass Index (kg/m^2)	-0.17	0.007	0.010
Corr .r^2=0.341			

4. Discussion

There are many studies on vitamin D *status* of the general population in the USA, Canada, Asia Pacific, Middle East, Africa and across Europe [29], but to the best of our knowledge this is the first conducted in a Portuguese healthy adult population.

Globally, vitamin D deficiency is more prevalent in winter, women, older age groups, individuals with darker skin, and higher latitudes [7, 30, 31]. In the present study, the frequency of 25(OH)D deficiency was significantly higher in winter, confirming the well-known seasonal fluctuation in 25(OH)D concentration. No association between Vitamin D levels and gender was observed in our study, although women presented slightly lower levels of 25(OH)D but the difference was not statistically significant. It has been established that the ageing skin produces less vitamin D [32]. However, in our study, we did not find any association between Vitamin D *status* with age.

The negative association of vitamin D *status* with obesity is well documented in different studies [33]. This is probably due to the decreased bioavailability of vitamin D from cutaneous and dietary sources because of its sequestration in body fat compartments [34]. Our observations confirm the association of Vitamin D *status* with BMI. Furthermore, an inverse correlation between vitamin D *status* and BMI was found. There is ongoing debate related to the optimal levels of 25(OH)D. All available evidence suggest that children and adults should maintain a blood level of 25(OH)D above 50 nmol/L to prevent rickets and osteomalacia, respectively. However, to maximize vitamin D effect on calcium, bone, and muscle metabolism, the 25(OH)D blood level should be above 75 nmol/L. Numerous epidemiological studies have suggested that a 25(OH)D blood level above 75 nmol/L may have additional health

benefits in reducing the risk of common cancers, autoimmune diseases, type 2 diabetes, cardiovascular disease, and infectious diseases [35].

In this study we observed that almost half of the studied population presented serum 25(OH)D values suggestive of vitamin D deficiency, reaching 74% in winter. This observation is in line with a recent study that suggests that Vitamin D deficiency is widespread across Europe, even in countries with abundant sunlight, and at prevalence rates that meet the criteria of a pandemic [10]. An effective strategy to prevent Vitamin D deficiency and insufficiency should be envisaged. For specific high-risk groups use of vitamin D supplements would be an effective measure. For the general population fortification of widely used foods could be considered, especially in winter.

5. Conclusions

The present study analyzed serum Vitamin D levels in healthy adults between 18-67 years of age. BMI and season are predictors for lower vitamin D levels and deficiency *status* in this population. The strengths of this work comprise a detailed questionnaire documenting demographic data and blood sampling taking place through summer as well as wintertime. On the other hand, although the questionnaire included data about sun exposure, sunscreens use, eating habits, smoking habits, physical activity, diagnosed pathologies, and use of medicines and/or food supplements, these parameters were only used to exclude confounding factors that could bias our results, and were not used in the analysis of the results, as that was beyond the aim of the present study.

Conflict of interest

The authors have declared no conflict of interests.

Funding source

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QUESTIONÁRIO – POPULAÇÃO CONTROLO NORTE

IDENTIFICAÇÃO (INICIAIS):_____

SEXO: F ☐ M ☐ DATA DE NASCIMENTO: ☐☐-☐☐-☐☐☐☐

NATURALIDADE:_____NACIONALIDADE:_____

DATA DE COLHEITA: ☐☐-☐☐-☐☐☐☐ Nº REGISTO : ☐☐-☐☐☐ Nº PC: ☐☐☐

- HISTÓRIA FAMILIAR -

1. NATURALIDADE DOS FAMILIARES DIRECTOS

	PAI	MÃE	AVÓ PATERNOS	AVÔ PATERNOS	AVÓ MATERNOS	AVÔ MATERNOS	BISAVÓS
TRÁS-OS MONTES	☐	☐	☐	☐	☐	☐	☐
DOURO LITORAL	☐	☐	☐	☐	☐	☐	☐
MINHO	☐	☐	☐	☐	☐	☐	☐
BEIRA LITORAL	☐	☐	☐	☐	☐	☐	☐
BEIRA-ALTA	☐	☐	☐	☐	☐	☐	☐
BEIRA-BAIXA	☐	☐	☐	☐	☐	☐	☐
ESTREMADURA	☐	☐	☐	☐	☐	☐	☐
RIBATEJO	☐	☐	☐	☐	☐	☐	☐
ALTO ALENTEJO	☐	☐	☐	☐	☐	☐	☐
BAIXO ALENTEJO	☐	☐	☐	☐	☐	☐	☐
ALGARVE	☐	☐	☐	☐	☐	☐	☐
MADEIRA	☐	☐	☐	☐	☐	☐	☐
AÇORES	☐	☐	☐	☐	☐	☐	☐
OUTROS	☐	☐	☐	☐	☐	☐	☐

COMENTÁRIOS:

_____

_____

_____

_____

2. TIPO DE AFECÇÃO DOS FAMILIARES

	PAI	MÃE	AVÓ PATERNOS	AVÔ PATERNOS	AVÓ MATERNOS	AVÔ MATERNOS	BISAVÓS
AFECÇÕES OCULARES	☐	☐	☐	☐	☐	☐	☐
ALTERAÇÕES DO SONO	☐	☐	☐	☐	☐	☐	☐
AFECÇÕES ARTICULARES	☐	☐	☐	☐	☐	☐	☐
ESPONDILOARTROPATIA	☐	☐	☐	☐	☐	☐	☐
ARTRITE REMATÓIDE	☐	☐	☐	☐	☐	☐	☐
BEHÇET	☐	☐	☐	☐	☐	☐	☐
PSORÍASE	☐	☐	☐	☐	☐	☐	☐
LUPUS	☐	☐	☐	☐	☐	☐	☐
ESCLERODERMIA	☐	☐	☐	☐	☐	☐	☐
MIASTENIA GRAVIS	☐	☐	☐	☐	☐	☐	☐
EPILEPSIA	☐	☐	☐	☐	☐	☐	☐
ESCLEROSE MULTIPLA	☐	☐	☐	☐	☐	☐	☐
SJÖGREN	☐	☐	☐	☐	☐	☐	☐
CANCRO	☐	☐	☐	☐	☐	☐	☐
OUTRA	☐	☐	☐	☐	☐	☐	☐

COMENTARIO:

_____

_____

_____

_____

ESTE QUESTIONÁRIO É OPCIONAL. OS CAMPOS QUE NÃO FOREM PREENCHIDOS DEVERÃO SER ASSINALADOS DE FORMA INEQUÍVOCA.

TODA A INFORMAÇÃO CONSTANTE É CONFIDENCIAL E PODERÁ APENAS SER UTILIZADA PARA GESTÃO DAS DÁDIVAS DE SANGUE E ESTUDOS RELACCIONADOS

TODAS AS INFORMAÇÕES SERÃO SALVAGUARDADAS DE ACORDO COM A DECLARAÇÃO DE HELSÍNQUIA

Elegível?

☐ ***SIM***

☐ ***NÃO***

IDENTIFICAÇÃO (INICIAIS):_____

SEXO: F ☐ M ☐ DATA DE NASCIMENTO: ☐☐-☐☐-☐☐☐☐

NATURALIDADE: NACIONALIDADE:

PROFISSÃO:

LOCAL DE TRABALHO:
Fechado, aberto, com janelas e exposição solar ou não

ESCOLARIDADE (NÃO INCLUI NOVAS OPORTUNIDADES):

CO-MORBILIDADES: _____

_____

PESO: ALTURA: IMC:

Data de Resposta ao Inquérito:

Exposição solar

- ❖ O (a) Sr (a) expõe-se ao sol?

1. Sim
2. Não

Se SIM, qual a frequência?

	Verão	Inverno
1 a 2 vezes na semana		
3 a 4 vezes na semana		
5 a 6 vezes na semana		
7 vezes na semana		

Qual a duração da exposição solar?

	Verão	Inverno
Até 15 minutos		
Mais de 15 e menos de 30 minutos		
De 30 a 60 minutos		
Mais de 60 minutos		

- Usa protetor solar?

1. Sim
2. Não

Se SIM, qual é a frequência?

	Verão	Inverno
1 a 2 vezes na semana		
3 a 4 vezes na semana		
5 a 6 vezes na semana		
7 vezes na semana		

- Qual o fator de proteção solar (FPS)?

1. 15
2. 30
3. 50
4. > 50

- Qual o local de aplicação?

1. Rosto
2. Braços
3. Rosto e braços

Hábitos tabágicos:

- Atuais (SIM/NÃO) _____; Se sim, indique a idade de inicio_____

Quantidade dia (média) _____

- Passados (SIM/NÃO) _____; Se sim, indique a idade de inicio_____

Quantidade dia (media) _____

Data STOP tabaco_____

Hábitos alimentares

- Habitualmente consome peixe?
  1. Sim
  2. Não

Quantidade e tipo de peixe que consome

	Ocasionalmente	1x mês	2-3x mês	1-2x semana	3-4x semana	5-6x semana	1x dia	2-3x dia
Solha/Bacalhau								
Salmão/Truta								
Salmão enlatado								
Cavala/carapau/chicharro								
Atum enlatado água/óleo								
Sardinhas em óleo/tomate								
Outro _____ Ex: enguias								

- Come carne regularmente?

1. Sim
2. Não

Qantidade e tipo de carne que consome

	Ocasionalmente	1x mês	2-3x mês	1-2x semana	3-4x semana	5-6x semana	1x dia	2-3x dia
Salsicha/porco								
Porco								
Cordeiro								
Fígado								
Vaca								
Frango								
Outro _____								

❖ Habitualmente consome ovos (estrelados/cozidos/omoletes)?

1. Sim
2. Não

❖ Se SIM qual a frequência?

1. 1x mês
2. 2-3x mês
3. 1-2x semana
4. 3-4x semana
5. 5-6x semana
6. 1x dia
7. 2-3x dia

❖ Consome habitualmente sumos ou outros alimentos fortificados em Vitamina D?

1. Sim
2. Não

Especificar (nome do produto; data de inicio dos suplementos): _____

❖ Toma suplementos alimentares (Vitaminas, óleo de fígado de bacalhau)?

1. Sim
2. Não

Especificar (nome do produto; data de inicio dos suplementos): _____

<u>Hábitos farmacológicos:</u>

Appendix 4

The Protective Role of HLA-DRB1*13 in Autoimmune Diseases

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Research Article

The Protective Role of HLA-DRB1*13 in Autoimmune Diseases

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Autoimmune diseases (AIDs) are characterized by a multifactorial aetiology and a complex genetic background, with the MHC region playing a major role. We genotyped for HLA-DRB1 locus 1228 patients with AIDs-213 with Systemic Lupus Erythematosus (SLE), 166 with Psoriasis or Psoriatic Arthritis (Ps + PsA), 153 with Rheumatoid Arthritis (RA), 67 with Systemic Sclerosis (SSc), 536 with Multiple Sclerosis (MS), and 93 with Myasthenia Gravis (MG) and 282 unrelated controls. We confirmed previously established associations of HLA-DRB1*15 (OR = 2.17) and HLA-DRB1*03 (OR = 1.81) alleles with MS, HLA-DRB1*03 with SLE (OR = 2.49), HLA-DRB1*01 (OR = 1.79) and HLA-DRB1*04 (OR = 2.81) with RA, HLA-DRB1*07 with Ps + PsA (OR = 1.79), HLA-DRB1*01 (OR = 2.28) and HLA-DRB1*08 (OR = 3.01) with SSc, and HLA-DRB1*03 with MG (OR = 2.98). We further observed a consistent negative association of HLA-DRB1*13 allele with SLE, Ps + PsA, RA, and SSc (18.3%, 19.3%, 16.3%, and 11.9%, resp., versus 29.8% in controls). HLA-DRB1*13 frequency in the AIDs group was 20.0% (OR = 0.58). Although different alleles were associated with particular AIDs, the same allele, HLA-DRB1*13, was underrepresented in all of the six diseases analysed. This observation suggests that this allele may confer protection for AIDs, particularly for systemic and rheumatic disease. The protective effect of HLA-DRB1*13 could be explained by a more proficient antigen presentation by these molecules, favouring efficient clonal deletion during thymic selection.

1. Introduction

Autoimmune diseases (AIDs) are chronic disorders originated by the loss of immunological tolerance to self-antigens. This heterogeneous group of conditions present common genetic risk factors and share several pathophysiological mechanisms leading to overlapping clinical manifestations targeting specific organs or multiple organ systems [1]. There is evidence that they share similar immunogenetic mechanisms, even though they exhibit varying epidemiological features and clinical manifestations [2, 3]. Underlying these diverse clinical phenotypes is a deregulated immune system

with an enriched ability to respond against self-tissues. The fact that AIDs share several clinical signs and symptoms (i.e., subphenotypes) and also share physiopathological mechanisms and genetic factors has been called autoimmune tautology and indicates that they may have a common origin [4].

The immune system is in charge of the defence against external pathogens. For this purpose, T and B lymphocytes are responsible for the immune response through regulated cell-cell interactions and secretion of cytokines, chemokines, and other inflammatory mediators. This defence against external pathogens must occur without causing unnecessary harm to self. To achieve this delicate balance, the majority of self-reactive T and B lymphocytes are destroyed in the thymus and bone marrow through negative selection [5]. Nevertheless, this process is far from perfect, and self-reactive lymphocytes escape into the periphery. Consequently, peripheral tolerance mechanisms are necessary to keep these self-reactive cells in check [6]. Activated self-reactive T and B cells promote autoimmunity when the effector and regulatory balance of the immune response is disturbed [7].

Major histocompatibility complex (MHC) molecules are widely distributed surface membrane glycoproteins that present antigenic peptides to T cell receptors (TCRs). Developing thymocytes encounter a highly heterogeneous repertoire of self (endogenous) peptide-MHC (pMHC) complexes on thymic epithelial cells, the main thymus antigen presenting cells. The affinity/avidity with which these thymocyte TCRs bind self pMHC determines if it is destined to perish or if it will survive [8]. In this way, a repertoire of peripheral T cells that is essentially self-tolerant is generated [6, 9, 10].

Several hypotheses have been put forward to explain how MHC polymorphisms influence autoimmunity risk or protection. They must do so, somehow, by shaping the central or peripheral T cell repertoires toward autoimmune resistance or proclivity [8]. A protective MHC profile could achieve this by the selection of a T cell repertoire with diminished pathogenicity [11]. On the other hand, protective MHC molecules may keep autoimmunity in check by favouring the negative selection of particular self-reactive T cells [12–14].

The functional basis of the association between specific HLA alleles and development of AIDs can be classically explained by two possible etiopathogenic models [15].

The molecular mimicry hypothesis proposes that certain HLA alleles are more efficient in presenting pathogen epitopes that share structural features with self-peptides to mature T cells. Once the response to the pathogen is initiated the self-antigen is also recognized and disease ensues.

Central selection failure proposes that certain HLA alleles are less efficient at presenting self-peptides to developing T cells in the thymus, so negative selection fails.

A different hypothesis proposes that different alleles can modulate the immunologic profile of an individual, through antigen-independent mechanisms, resulting in either promoting a higher autoimmune predisposition or, in opposition, a more efficient immune regulation. Given the consistent association of HLA-DRB1 alleles with different autoimmune diseases (Table 1), we explored the idea that the same HLA-DRB1 alleles could be influencing several different autoimmune diseases. To this end we compared the immunogenetic profile in different AIDs. This study includes four autoimmune systemic diseases, namely, Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA), Psoriasis or Psoriatic Arthritis (Ps + PsA), and Systemic Sclerosis (SSc). Patients with Multiple Sclerosis (MS) and Myasthenia Gravis (MG) were also included.

2. Patients and Methods

2.1. Patients and Controls. A total of 1228 patients with AIDs, 213 patients with SLE and 153 patients with RA diagnosed according to the American College of Rheumatology (ACR) criteria, 166 patients with Ps + PsA, 67 with SSc, 536 with definitive diagnosis of MS according to the revised McDonald criteria, and 93 with MG, were recruited from the Neurology and Medicine Outpatient Clinic of Centro Hospitalar do Porto-Hospital de Santo António (CHP-HSA). The HLA-DRB1 frequencies of patients were compared with the ones of a control group consisting of 282 unrelated individuals without disease and from the same geographic origin (north of Portugal).

2.2. HLA-DRB1 Genotyping. Peripheral blood samples (10 mL) were collected in EDTA. Genomic DNA was obtained from proteinase-K–treated peripheral blood leukocytes by using a Salting-Out procedure [27]. Low-resolution genotyping for HLA–DRB1 locus (i.e., 2-digit HLA nomenclature) was performed using polymerase chain reaction and sequence-specific primers (PCR-SSP), based on methods previously described [28]. In order to produce PCR-SSP reactions able to detect and discriminate each of the known HLA-DRB1 genes, primers were designed using sequence alignments comprising all HLA-DRB1 variants and were validated by the Twelfth International Histocompatibility Workshop. PCR products were visualized under ultraviolet light after running in a 1.5% agarose gel containing ethidium bromide.

2.3. Statistical Analysis. To identify the HLA-DRB1 genes contributing to the six different AIDs, we applied stepwise logistic regression on an allelic level, using forward selection which involves starting with no variables in the model, testing the addition of each variable using a chosen model comparison criterion, adding the variable (if any) that improves the model the most, and repeating this process until none improves the model. It should be noted that odds ratios (ORs) obtained in a multivariable logistic regression analysis are adjusted for all the other genes included in the model and therefore differ from those obtained when a given gene is compared with all other genes. The data were analysed using IBM SPSS 20 statistical software.

3. Results

A total of 1228 cases and 282 controls were analysed and different types of association between alleles and AIDs were found (Table 2). These included three risk alleles for two or more AIDs, two protective alleles for two or more AIDs, and three risk alleles for a particular AID.

Table 1: HLA-DRB1 alleles associated with SLE, Ps + PsA, RA, SSc, MS, and MG.

Autoimmune disease	HLA-DRB1 associated allele		References
	Susceptibility	Protection	
Systemic Lupus Erythematosus (SLE)	HLA-DRB1*03 HLA-DRB1*08 HLA-DRB1*15	HLA-DRB1*09 HLA-DRB1*13	[16–18]
Psoriasis or Psoriatic Arthritis (Ps + PsA)	HLA-DRB1*07	—	[19, 20]
Rheumatoid Arthritis (RA)	HLA-DRB1*01 HLA-DRB1*04 HLA-DRB1*10	HLA-DRB1*13	[21]
Systemic Sclerosis (SSc)	HLA-DRB1*01 HLA-DRB1*08 HLA-DRB1*11	HLA-DRB1*07 HLA-DRB1*15	[22, 23]
Multiple Sclerosis (MS)	HLA-DRB1*03 HLA-DRB1*08 HLA-DRB1*15	HLA-DRB1*10 HLA-DRB1*14	[24, 25]
Myasthenia Gravis (MG)	HLA-DRB1*03	—	[26]

Table 2: Associations between HLA class II and six AIDs: SLE, Ps + PsA, RA, SSc, MS, and MG.

	Controls ($n = 282$)	SLE ($n = 213$)	Ps + PsA ($n = 166$)	RA ($n = 153$)	SSc ($n = 67$)	MS ($n = 536$)	MG ($n = 93$)	Total ($n = 1228$)
HLA-DRB1*01	**66 (23.4%)**	40 (18.8%)	39 (23.5%)	**50 (32.7%) OR = 1.79 p = 0.017**	**28 (41.8%) OR = 2.28 p = 0.006**	100 (18.7%)	23 (24.7%)	280 (22.8%)
HLA-DRB1*03	**44 (15.6%)**	**73 (34.3%) OR = 2.49 $p = 4.2 \times 10^{-5}$**	25 (15.1%)	28 (18.3%)	11 (16.4%)	**123 (22.9%) OR = 1.81 p = 0.003**	**33 (35.5%) OR = 2.98 $p = 6.1 \times 10^{-5}$**	**293 (23.9%) OR = 1.51 p = 0.022**
HLA-DRB1*04	**69 (24.5%)**	42 (19.7%)	46 (27.7%)	**73 (47.7%) OR = 2.81 $p = 6 \times 10^{-6}$**	13 (19.4%)	128 (23.9%)	23 (24.7%)	325 (26.5%)
HLA-DRB1*07	**72 (25.5%)**	55 (25.8%)	**66 (39.8%) OR = 1.79 p = 0.006**	38 (24.8%)	14 (20.9%)	147 (27.4%)	23 (24.7%)	343 (27.9%)
HLA-DRB1*08	**24 (8.5%)**	21 (10.0%)	10 (6.0%)	**3 (2.0%) OR = 0.24 p = 0.026**	**15 (22.4%) OR = 3.01 p = 0.004**	**65 (12.1%) OR = 1.73 p = 0.033**	7 (7.5%)	121 (9.9%)
HLA-DRB1*09	**14 (5.0%)**	**2 (1.0%) OR = 0.18 p = 0.027**	5 (3.0%)	**0 (0.0%)* OR = 0.95 p = 0.003**	3 (4.5%)	**5 (1.0%) OR = 0.22 p = 0.004**	2 (2.2%)	**17 (1.4%) OR = 0.23 $p = 1 \times 10^{-4}$**
HLA-DRB1*13	**84 (29.8%)**	**39 (18.3%) OR = 0.58 p = 0.016**	**32 (19.3%) OR = 0.62 p = 0.050**	**25 (16.3%) OR = 0.58 p = 0.044**	**8 (11.9%) OR = 0.42 p = 0.035**	124 (23.1%)	17 (18.3%)	**245 (20.0%) OR = 0.58 p = 0.004**
HLA-DRB1*15	**56 (19.9%)**	55 (25.8%)	22 (13.3%)	17 (11.1%)	12 (17.9%)	**175 (32.7%) OR = 2.17 $p = 2 \times 10^{-5}$**	15 (16.1%)	296 (24.1%)

AIDs: autoimmune diseases; SLE: Systemic Lupus Erythematosus; Ps + PsA: Psoriasis or Psoriatic Arthritis; RA: Rheumatoid Arthritis; SSc: Systemic Sclerosis; MS: Multiple Sclerosis; MG: Myasthenia Gravis. *Fisher's exact test was used to calculate this value.

HLA-DRB1*13 was a protective allele for four AIDs: SLE (18.3% versus 29.8%, $p = 0.016$, OR = 0.58, and 95% CI = 0.37–0.90), Ps + PsA (19.3% versus 29.8%, $p = 0.050$, OR = 0.621, and 95% CI = 0.39–1.00), RA (16.3% versus 29.8%, $p = 0.044$, OR = 0.58, and 95% CI = 0.34–0.98), and SSc (11.9% versus 29.8%, $p = 0.035$, OR = 0.42, and 95% CI = 0.19–0.94).

There was a specific risk allele associated with three AIDs. HLA-DRB1*03 was found to be a risk factor for SLE (34.3% versus 15.6%, $p = 4.2 \times 10^{-5}$, OR = 2.49, and 95% CI = 1.61–3.86), MS (22.9% versus 15.6%, $p = 0.003$, OR = 1.81, and 95% CI = 1.23–2.67), and MG (35.5% versus 15.6%, $p = 6.1 \times 10^{-5}$, OR = 2.98, and 95% CI = 1.75–5.07). There were

two risk alleles associated with two AIDs: HLA-DRB1*08 was positively associated with MS (12.1% versus 8.5%, $p = 0.033$, OR = 1.73, and 95% CI = 1.05–2.87) and SSc (22.4% versus 8.5%, $p = 0.004$, OR = 3.01, and 95% CI = 1.43–6.31) and HLA-DRB1*01 was found to be a risk factor for RA (32.7% versus 23.4%, $p = 0.017$, OR = 1.79, and 95% CI = 1.11–2.88) and SSc (41.8% versus 23.4%, $p = 0.006$, OR = 2.28, and 95% CI = 1.27–4.09).

HLA-DRB1*09 was negatively associated with SLE (1.0% versus 5.0%, $p = 0.027$, OR = 0.18, and 95% CI = 0.04–0.83), MS (1.0% versus 5.0%, $p = 0.004$, OR = 0.22, and 95% CI = 0.08–0.63), and RA (0.0% versus 1.0%, $p = 0.003$, OR = 0.95, and 95% CI = 0.93–0.98).

Three risk disease-specific alleles were found: HLA-DRB1*04 for RA (47.7% versus 24.5%, $p = 6\times10^{-6}$, OR = 2.81, and 95% CI = 1.79–4.39), HLA-DRB1*07 for Ps + PsA (39.8% versus 25.5%, $p = 0.006$, OR = 1.79, and 95% CI = 1.18–2.72), and HLA-DRB1*15 for MS (32.7% versus 19.9%, $p = 2\times10^{-5}$, OR = 2.17, and 95% CI = 1.53–3.10).

Considering AIDs as a group, HLA-DRB1*03 frequency was significantly higher (23.9% versus 15.6%, $p = 0.022$, OR = 1.51, and 95% CI = 1.0–2.15) compared with controls; conversely HLA-DRB1*13 (20.0% versus 29.8%, $p = 0.004$, OR = 0.58, and 95% CI = 0.43–0.79) and HLA-DRB1*09 (1.4% versus 5.0%, $p = 1\times10^{-4}$, OR = 0.23, and 95% CI = 0.11–0.49) frequencies were significantly lower.

4. Discussion

Through a systematic review of published works, Cruz-Tapias and collaborators, in 2012, identified some common HLA class II alleles that contribute to susceptibility to AIDs in Latin Americans [3]. The present study is, to date and to the best of our knowledge, the only one that addresses the hypothesis that a HLA-DRB1 allele could influence different autoimmune diseases, using a new cohort, encompassing six different autoimmune diseases.

In this study we observed associations of different HLA-DRB1 alleles with several AIDs. We confirmed positive and negative associations in MS [24, 25], SLE [16–18], Ps + PsA [19, 20], RA [21], SSc [22, 23], and MG [26], previously reported in our or other populations.

When AIDs studied were considered as a group, HLA-DRB1*03 allele was significantly overrepresented, as already described [29]. It has been shown that this allele has low affinity for CLIP (class II-associated invariant chain peptide) and may not require HLA-DM to ensure peptide presentation, preventing efficient peptide selection and allowing the binding of low stability peptides [30]. Concerning the observed negative association with HLA-DRB1*09, we think that this is likely a spurious association, as this is a rare allele and the frequency found in controls is, for some reason, double the one reported for the Portuguese population [31].

Our observations suggest that the presence of HLA-DRB1*13 allele may confer protection for AIDs. HLA-DRB1*13 is a high frequency allele in the general population both in Portugal [31] and worldwide. Our results confirm that the lower frequency of HLA-DRB1*13 in every individual AIDs group is not secondary to the deviations granted by the concurrent positive associations. When the data obtained from previous studies are taken into consideration, the HLA-DRB1*13 allele seems to be a universal protective allele for RA. It was reported as protective against RA in Asian [32, 33], Turkish [34], and several European populations [35–37]. Recently this allele was also described to be protective in SLE in the Japanese population [18] and for ANCA-associated vasculitis in the Dutch population [38].

Subtle structural differences in the HLA molecule have functional implications at the protein level. Specific amino acid patterns at the peptide binding cleft are involved in disease susceptibility, such as the well-known shared epitope first described in the RA susceptibility alleles HLA-DRB1*01 and HLA-DRB1*04 [37, 39]. Similar to the shared epitope classification of susceptibility alleles, protective HLA-DRB1 alleles have been categorized according to several models. One of the most accepted classifications proposes that protection against RA is conferred by the DERAA sequence at positions 70–74 of the HLA-DRB1 allele [40]. Other models suggest that protection is conferred by an aspartic acid at position 70 (D70 allele) [41] or an isoleucine at position 67 (I67 allele) of the HLA-DRB1 molecule. Because it was unclear which HLA-DRB1 alleles were protective a meta-analysis was performed involving four European populations with >2,700 patients and >3,000 control subjects. The objective was to investigate exhaustively which HLA-DRB1 alleles were associated with protection against RA [36]. Interestingly, this study showed that the protective effect attributed to DERAA and D70 was no longer present after the exclusion of HLA-DRB1*13. The authors concluded that this evidence indicates that HLA-DRB1*13 rather than DERAA, D70, or I67 is associated with protection [36]. In a recent study van Heemst and collaborators identify citrullinated vinculin, present in the joints of $ACPA^+$ RA patients, as an autoantigen targeted by ACPA and $CD4^+$ T cells. These T cells recognize an epitope with the core sequence DERAA, which is also found in many microbes and in protective HLA-DRB1*13 molecules, presented by predisposing HLA-DQ molecules. Intriguingly, DERAA-directed T cells were not detected in HLA-DRB1*13^+ donors, indicating that the DERAA epitope from HLA-DRB1*13 could mediate thymic tolerance in these donors and explain the protective effects associated with HLA-DRB1*13. They suggest that subjects born with HLA-DRB1*13 will present the HLA-DRB1*13-derived DERAA-peptide in the thymus, leading to tolerization of the DERAA-reactive T cell response [42]. The negative association we describe here supports the idea that the HLA-DRB1*13 allele, possibly by its specific structural features, may as well confer resistance to several other AIDs. The protective effect of HLA-DRB1*13 could be explained by a more proficient antigen presentation by these molecules [43, 44], favouring an efficient thymic selection. As a result, negative selection and development of DR-driven autoreactive regulatory T cells are promoted [8].

A different model would relate HLA molecules with the presence of specific endophenotypes indirectly associated with autoimmunity. Other studies of our group suggest that the HLA genotype may primarily influence the general activation state of CD4 T cells [45]. The protective effect

of HLA-DRB1*13 could also be explained by this effect. Curiously, several reports have suggested an association of HLA-DRB1*13 and/or HLA-DQB1*06 with slow disease progression in human immunodeficiency virus (HIV) infected individuals, meaning that among HIV controllers there is an association between the presence of certain class II HLA alleles and strong CD4 T cell responses [46, 47].

Although different alleles are associated with particular AIDs, the same allele, HLA-DRB1*13, was underrepresented in all six diseases. This difference is statistically significant for the four rheumatic diseases studied. This observation suggests that this allele confers protection to AIDs in general and particularly to rheumatic diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Appendix 5

Association between Vitamin D receptor (VDR) gene polymorphisms and systemic lupus erythematosus in Portuguese patients

Association between Vitamin D receptor (VDR) gene polymorphisms and Systemic Lupus Erythematosus in Portuguese Patients

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Keywords: SLE, VDR gene, Vitamin D, SNPs

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Abstract

SLE (Systemic Lupus Erythematosus) is a chronic autoimmune disease of unknown origin, in which both genetic and environmental factors are involved. One of such environmental factors is vitamin D, a vital hormone that plays specific functions in the immune system homeostasis, acting through a nuclear receptor (VDR) expressed in all immune cells.

The aim of this study was to determine the possible association between VDR gene polymorphisms (BsmI, ApaI, TaqI e FokI) and SLE susceptibility and severity, in a cohort of Lupus patients from the North of Portugal.

One hundred and seventy patients with SLE (diagnosed according the American College of Rheumatology [ACR] criteria) were studied. Patients and 192 ethnicity-matched controls were genotyped for BsmI (rs1544410), ApaI (rs7975232), TaqI (rs731236) and FokI (rs2228570) polymorphisms by TaqMan allelic discrimination assay.

SLE patients with the CT genotype of FokI polymorphism have a higher SLICC value (p=0.031). The same result was observed for the group of patients with the TT genotype of TaqI polymorphism (p=0.046). No differences were observed in VDR genotypic frequencies between patients and controls. We also observed that the other analysed clinical features were not influenced by VDR polymorphisms.

Our study confirms a possible role of VDR gene polymorphisms in SLE. A positive association was found between VDR polymorphisms and SLE severity (chronic damage). The presence of CT genotype of FokI and TT genotype of TaqI seems to confer a worse prognosis and may constitute a risk factor to a higher long term cumulative damage in SLE patients.

Introduction

Systemic lupus erythematosus (SLE or lupus) is a systemic autoimmune disease with multi-organ inflammation, linked to loss of immune tolerance to self-antigens and the production of a diversity of autoantibodies. It affects mainly women of childbearing age[1]. The female to male ratio in adults is approximately 9:1 and the peak age of SLE diagnosis is between 15 and 44 years. Despite phenotypic heterogeneity, a strong genetic contribution to the development of SLE is supported by numerous evidences. Familial prevalence was found to be 5.6% if one first degree relative is affected. Disease concordance in dizygotic twins is 2–5%, and in monozygotic twins 29–57%.[2] These evidences from epidemiologic data were the trigger for the study of the genetic basis of the disease.

Candidate gene case–control studies are frequently used to calculate whether a genetic marker is present at a higher frequency among patients with a certain disease than in ethnically-matched healthy control individuals. The first genetic association described for SLE based in this methodology was with the HLA region at chromosome 6p21.3, which encodes over 200 genes, many of them with known immunological role. Candidate genes are chosen on the basis of their functional importance to disease pathogenesis.

Nevertheless, these evidences suggest that although genes are important, they are not the only explanation, and environmental factors are presumed to play an important role in disease development.

The phenotype and progression of SLE have been linked with a combination of environmental, genetic and hormonal factors[1,3]. One such environmental factor is vitamin D, a vital hormone with well-established effects on mineral metabolism, skeletal health, and more recently recognized profound effects on cardiovascular system[4]. The major source of vitamin D is sun exposure, but it is also available in some foods and supplements.

The identification of vitamin D receptors on cells of the immune system and the discovery that dendritic cells can produce the metabolically active form of vitamin D, 1,25-dihydroxyvitamin D_3

(calcitriol), have led to the suggestion that vitamin D is an immune modulator[5,6]. This can explain why vitamin D deficiency-related symptoms such as fatigue are frequently observed in those with SLE[7]. Furthermore, it is postulated that SLE patients may be at increased risk of low vitamin D status as a result of both photosensitivity, with consequent sun avoidance, as well as chronic use of medications prescribed for SLE management, which interfere with vitamin D metabolism[8,9]. In fact, research has not established whether low vitamin D levels are a contributing factor to the development of SLE or a consequence of the disease itself. If this correlation reflects a protective effect of vitamin D, considering the genetic susceptibility factors, the risk to develop SLE should be lower among individuals carrying genetic variants that predict high 25(OH)D levels and SLE patients with higher 25(OH)D levels should presumably have lower SLE disease activity.

Vitamin D acts via binding to an intranuclear receptor, Vitamin D Receptor (VDR), present in target tissues and cells. This receptor belongs to the superfamily of transacting transcriptional regulatory factors [10]. In humans, VDR gene is located on chromosome 12q13.1, extents over 100 kb and includes eight protein-coding exons, six untranslated exons, eight introns and two promoter regions[11]. Four common single nucleotide polymorphisms (SNPs) in the VDR gene have been extensively investigated: FokI C>T (rs2228570), BsmI A>G (rs1544410), ApaI G>T (rs7975232), and TaqI C>T (rs731236). BsmI and ApaI SNPs are both located in intron 8, and the TaqI is a silent SNP in exon 9. Although these 3 polymorphisms do not produce any structural change on the VDR protein, they are in strong linkage disequilibrium. On the other hand, the T allele of the FokI SNP creates an alternative ATG initiation codon in exon 2, leading to a VDR protein that is three amino acids longer, suggesting a potential functional consequence[12].

The purpose of this study was to evaluate the possible association between these common VDR gene polymorphisms and SLE susceptibility and severity in a Northern Portuguese population.

Material and Methods

Subjects

The study population consisted of 170 (15 M and 155 F) sequential unrelated SLE patients and 192 (79 M and 105 F) healthy individuals recruited from the same region of Portugal, representing the general population in this area. These subjects without known autoimmune disease have an average age of 47 ± 12.8 years.

SLE patients (diagnosed according to the 1997 and 2012 revised ACR criteria for SLE)[13] were recruited from the outpatient clinic database of the Clinical Immunology Unit from Centro Hospitalar do Porto (Portugal). The average age of the SLE patients was 45 ± 13.4 years and the mean disease duration was 14.5 ± 6.5 (7 – 43) years. Patients with less than 5 years of disease where not considered.

Disease severity was assessed using number of affected organs, number of severe flares (defined by the SELENA-SLEDAI flare composite)[14,15] and the need of steroids or immunosuppressive therapy during disease duration (assumed as those who still have these therapeutics at the time of data collection). Cumulative damage was evaluated with the Systemic Lupus International Collaborative Clinics/American College of Rheumatology (SLICC/ACR) Damage Index [16].

Clinical manifestations (kidney involvement, Central Nervous System (CNS) involvement, Peripheral Nervous System involvement (PNS), articular, serositis, haematological and skin involvement) were established using a standard protocol accordingly to ACR glossaries for SLE classification criteria and EULAR recommendations for neuropsiquiatric, nephritis and SLE management.[17-19]

Ethical approval was obtained from the research ethics committee of Centro Hospitalar do Porto and written informed consent for DNA analysis was obtained from all subjects.

DNA Samples and Genotyping

Genomic DNA was extracted from peripheral white blood cells following standard procedures. The single nucleotide polymorphisms were genotyped using pre-designed TaqMan® allelic discrimination assays from Applied Biosystems (Foster City, CA, USA) in a Rotor Gene 6000 Real-Time PCR machine (Corbett Life Science).

Genotyping of the BsmI (rs1544410), ApaI (rs7975232), TaqI (rs731236) and FokI (rs2228570) genetic variants, located within the 12q12-14 (VDR gene) region, was carried out.

Statistical analysis

Allele and genotype frequencies were obtained by direct counting. Chi-square test was used to determine the differences of genotypic and allelic frequencies between patients and control groups. The same test was also conducted for the study of the association of the different genotypes or alleles with SLE clinical symptoms. A p-value <0.05 was considered to be statistically significant. Clinical and laboratory features associated with SLE were analysed both by uni and then multivariate analysis (logistic regression with the significant variables in univariate analysis). Mann-Whitney test was used to compare SLICC distributions and Odds Ratio was calculated with a binary logistic regression. The prevalence of genotypes in healthy controls was examined for deviation from Hardy–Weinberg equilibrium by using exact Chi-square test. IBM SPSS Statistics v.20 software was used for analyses.

Results

The demographic and clinical features of the 170 patients with SLE are presented in Table 1.

Table 1: Demographic, clinical/laboratory features of SLE patients

Patients Features		
	n / n total	**(%)**
Male	15/ 170	(9)
Female	155 / 170	(91)
Age (years)	45 (±13.4)	
> 10 years of disease	118 / 170	(69)
ANA+	170 / 170	(100)
ANTI ds-DNA	90 / 170	(56)
Hematological abnormalities	101 / 170	(59)
Arthritis	120 / 170	(71)
Skin involvement	140 / 170	(82)
Nephritis	47 / 170	(28)
NS involvement	13 / 170	(8)
Immunosuppressors/steroids *	161 / 170	(95)
Severe Flares	147 / 170	(86)
>3 affected organs or systems	42 / 170	(25)

* Patients with immunosuppressors or steroids at the time of clinical evaluation.

Phenotypic and genotypic frequencies for VDR SNPs were in Hardy-Weinberg equilibrium.

No statistically significant differences were observed between frequencies of the VDR polymorphisms in the patients group when compared with controls as shown in Table 2.

Table 2: Genotype and phenotype frequencies of VDR polymorphisms in SLE patients and Control Population.

Polymorphism	SLE patients n (%)		Control Population n (%)		Odds Ratio (95% CI)		p-value
rs1544410 (BsmI)	161		186				
Allele							
A	105	(65.2%)	131	(70.4%)	0.89	(0.65-1.22)	0.47
G	133	(82.6%)	148	(79.6%)	1.06	(0.79-1.44)	0.68
Genotype							
AA	28	(17.4%)	38	(20.4%)	0.11	(0.10-1.36)	0.09
AG	77	(47.8%)	93	(50.0%)	0.59	(0.18-1.88)	0.37
GG	56	(34.8%)	55	(29.6%)	1		
rs7975232 (ApaI)	167		187				
Allele							
T	132	(79.0%)	159	(85.0%)	0.88	(0.65-1.19)	0.42
G	117	(70.1%)	129	(68.9%)	1.02	(0.75-1.40)	0.88
Genotype							
TT	50	(29.9%)	58	(31.0%)	0.97	(0.36-2.57)	0.95
TG	82	(49.1%)	101	(54.0%)	0.78	(0.37-1.63)	0.5
GG	35	(21.0%)	28	(15.0%)	1		
rs731236 (TaqI)	169		179				
Allele							
C	107	(63.3%)	127	(70.9%)	0.84	(0.61-1.15)	0.29
T	142	(84.0%)	145	(81.0%)	1.06	(0.79-1.44)	0.69
Genotype							
CC	27	(16.0%)	34	(19.0%)	5.6	(0.46-67.9)	0.17
CT	80	(47.3%)	93	(52.0%)	1.39	(0.43-4.51)	0.58
TT	62	(36.7%)	52	(29.1%)	1		
rs2228570 (FokI)	164		192				
Allele							
T	85	(51.8%)	102	(53.1%)	0.97	(0.69-1.35)	0.84
C	147	(88.0%)	174	(90.6%)	0.98	(0.73-1.32)	0.89
Genotype							
TT	17	(10.4%)	18	(9.4%)	1.08	(0.50-2.31)	0.85
CT	68	(41.5%)	84	(43.8%)	0.86	(0.54-1.37)	0.53
CC	79	(48.2%)	90	(46.9)	1		

The relationship between VDR polymorphisms and disease expression was analysed in patients, with complete clinical data compiled, according to the clinical manifestations listed in table 1, as well as SLICC scores, number of affected organs, number of severe FLARES and the need of steroids or immunosuppressive therapy during disease duration. Association of different polymorphisms with accrual damage was noted, namely, the frequencies of VDR genotypes TaqI TT and Fok I CT were significantly higher in patients with SLICC>0.

Table 3: Genotype and phenotype frequencies of VDR polymorphisms TaqI and FokI in SLE patients with SLICC=0 and SLICC>0.

	SLE Patients	
Polymorphism	SLICC=0 n (%)	SLICC>0 n (%)
rs731236 (TaqI)	98	63
Allele		
C	68 (69.4%)	34 (54.0%)
T	81 (82.7%)	55 (87.3%)
Genotype		
CC	17 (17.3%)	8 (12.7%)
CT	51 (52.0%)	26 (41.3%)
TT	30 (30.6%)	29 (46.0%)
rs2228570 (FokI)	93	63
Allele		
T	44 (47.3%)	39 (61.9%)
C	81 (87.1%)	58 (92.1%)
Genotype		
TT	12 (13.3%)	5 (7.9%)
CT	32 (35.6%)	34 (54.0%)
CC	40 (51.1%)	24 (38.1%)

SLICC values were higher in SLE patients with FokI CT and TaqI TT genotypes (p=0.031 and p=0.046 respectively, Figure 1).

No significant differences were observed in number of affected organs, number of severe flares and the probability of long term need of steroids or immunosuppressive therapy during disease duration.

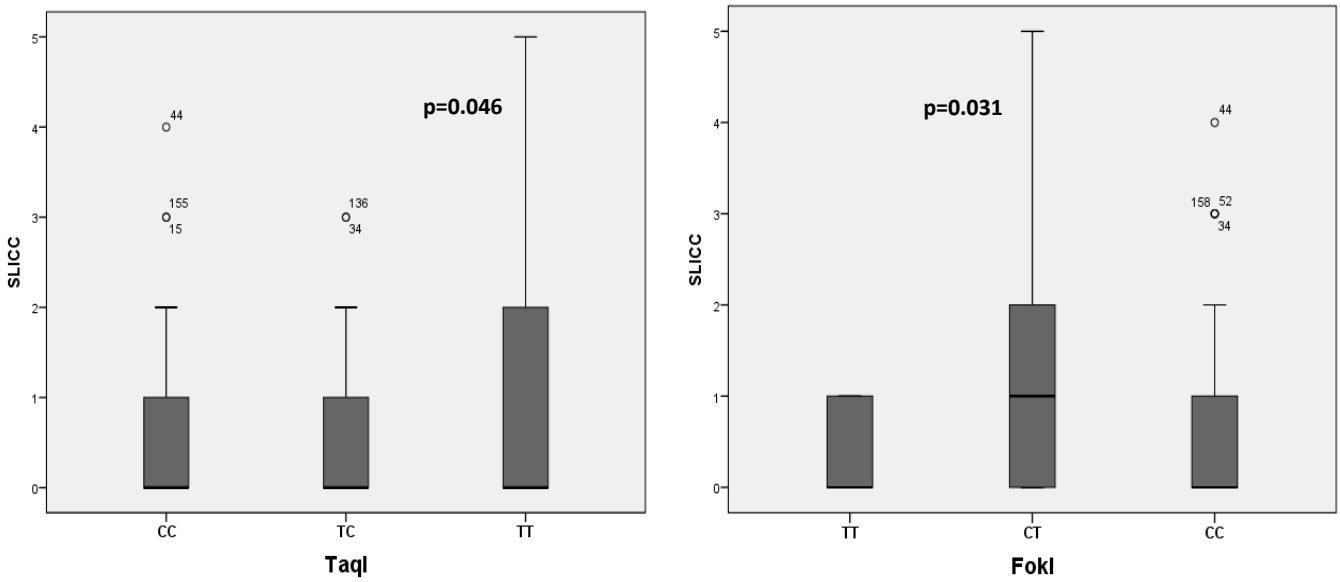

Figure 1: Box-whisker plots of SLICC distribution according to FokI and TaqI genotype, in SLE patients.

Discussion

To the best of our knowledge, this is the first study to establish an association between VDR (TaqI and FokI variants) SNPs and a higher long-term cumulative damage in SLE patients.

The concept that Vitamin D exerts immunomodulatory effects and therefore could play a role in the course of autoimmune diseases came from the last decade, when the perspective on how vitamin D influences human health was significantly altered[20]. Several epidemiological studies have shown that vitamin D deficiency could contribute to the risk of autoimmune diseases like systemic lupus erythematosus[4,21,22].

The main metabolic effect of $1,25(OH)_2D_3$, which is mediated through its interaction with vitamin D receptors, is promoting the intestinal absorption and renal resorption of calcium in order to increase its circulating levels. However, the discovery that vitamin D receptor is expressed in multiple immune cell types, such as monocytes, dendritic cells, and activated T cells, widens

vitamin D role, to modulating immunological functions, of potential relevance in the susceptibility and development of autoimmune diseases, such as multiple sclerosis[23], rheumatoid arthritis [24,25] and type 1 diabetes mellitus[26].

The Vitamin D receptor is a pleiotropic gene associated with multiple autoimmune diseases. ApaI, BsmI and TaqI polymorphisms do not affect directly VDR protein structure, but differences in stability and/or translation efficiency of the RNA have been reported. The FokI polymorphism seems to have consequences for both VDR protein structure and transcriptional activity[27]. Thus, VDR gene polymorphisms can modify the immunomodulatory action of vitamin D and may have an effect on the clinical manifestations of SLE, namely autoantibody production[28].

Four previous studies in Asian populations reported a positive association between BsmI and FokI VDR polymorphisms and susceptibility to SLE[29-32]. It has also been observed that VDR SNPs may influence clinical manifestations. Concerning nephropathy, an association with the bb genotype of BsmI was described in a Japanese cohort by Ozaki *et al*.. On a Han Chinese population, Luo *et al.* associated this nephropathy with the B allele of the same polymorphism[33]. Emerah *et al.* replicated this result in an Egyptian female population, observing that AA (ApaI) and FF (FokI) genotypes were also risk factors for the development of nephropathy in SLE patients[34]. In a study of Polish females, Mostowska *et al.* compared patients with and without renal symptoms and observed that the FF and Ff genotypes of FokI had higher frequencies in patients with clinical manifestations[35]. Additional research associated VDR SNPs with other SLE features such as serositis, ANA, anti-dsDNA, anti-Sm and anti-histone antibodies[31,32]. In contrast, in other studies, no association between VDR polymorphisms and SLE was reported[30,32,36-39].

In summary, the association between Vitamin D Receptor polymorphisms and SLE has been extensively studied, but the results described so far are widely dissonant. The comparison of data present in the literature is mostly difficult due to study design and ethnic differences in the several studies. Up to date, the role of VDR polymorphisms in this disease remains uncertain.

The present study is the first to suggest a role for VDR gene polymorphisms in SLE long term outcome. A positive association was found between VDR polymorphisms and SLE accrual damage. The presence of CT genotype of FokI and TT genotype of TaqI seems to confer a risk to a worse prognosis being a risk factor for a higher long-term cumulative damage. Several hypotheses may explain these results.

It is known that VDR polymorphisms may alter VDR expression (FokI), transcription (BsmI) and function. These modifications may interfere with vitamin D activity. In fact, vitamin D has an important role in the control of inflammatory responses with a switch from Th1 to Th2 cytokine profile, enhancing Th17 pathway via transcriptional modulation of interleukin-17A, as well as induction of regulatory T cells[40,41].

Additionally, VDR polymorphisms may also be in linkage disequilibrium with HLA-DRB1 alleles that are central players in several autoimmune diseases[42].

Furthermore, VDR signalling may influence the transcriptional activity of genes involved in the pathophysiological mechanisms of autoimmune diseases. The promoter region of HLA-DRB1 locus has a vitamin D responsive element (VDRE) that controls/regulates HLA-DRB1 expression[42,43]. Binding of $1,25(OH)_2D_3$ originates conformational changes in VDR which promote its hetero-dimerization with Retinoid X Receptor (RXR) and translocation of this complex into the nucleus. The RXR-VDR heterodimer binds to the vitamin D_3 responsive elements in promoter regions of $1,25(OH)_2D_3$ responsive genes, such as HLA-DRB1 gene. It has been described that vitamin D deficiency in early childhood may lead to lower HLA-DRB1 expression, impairing thymic selection and promoting autoimmune disease development[42].

In our study, although the positive association found between CT genotype of FokI and TT genotype of TaqI and SLE accrual damage, there is a lack of clear association with the other analysed factors (number of affected organs, number of severe FLARES, and pharmacological therapy). This can be explained by the small number of individuals in each subgroup, nevertheless

the main explanation is that damage in SLE is multifactorial and not only related to disease activity itself [44].

For example, a more inflammatory profile could be found in Lupus patients, which could explain a worse long term prognosis, not necessarily related to disease activity but also associated with other factors like cardiovascular disease, as has been already described in larger cohorts[45].

The results of this study provide a support to the hypothesis that vitamin D polymorphisms are associated with SLE disease activity, although the number of subjects studied was relatively small. Therefore, this finding requires independently replication in larger cohorts.

In conclusion, in Lupus patients with the risk VDR genotypes, vitamin D supplementation could be a useful strategy for prevention of long term cumulative damage, compensating for a deficiency in VDR. Vitamin D daily dose should be adapted to the concept of "Vitamin D Resistant State" [46]and the optimal dose should be tailored for each patient.

Individual genotype cannot be changed however vitamin D deficiency can be searched and addressed.

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Appendix 6

Vitamin D supplementation effects on FoxP3 expression in T cells and FoxP3+/IL-17A ratio and clinical course in systemic lupus erythematosus patients: a study in a Portuguese cohort

Vitamin D supplementation effects on FoxP3 expression in T cells and FoxP3^{+}/IL-17A ratio and clinical course in systemic lupus erythematosus patients: a study in a Portuguese cohort

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Abstract

Background: Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with multi-organ inflammation, linked to loss of immune tolerance to self-antigens and the production of a diversity of autoantibodies, with a negative impact on the patients' quality of life.

Regulatory T cells have been reported as deficient in number and function in SLE patients. However, some authors also described an enrichment of this cell type. The hypothesis that certain forms of autoimmunity may result from a conversion of Treg cells into a Th17 cell phenotype, has been suggested by some studies. In fact, in SLE patients' sera, the IL-17 levels were observed as abnormally high when compared with healthy individuals.

Environmental factors, such as vitamin D, that is considered a potential anti-inflammatory agent, combined with genetic and hormonal characteristics have been associated with SLE phenotype and with disease progression.

Objectives: The aim of this study was to evaluate the effect of vitamin D supplementation on FoxP3 expression and IL-17A producing T cells, through $FoxP3^{+}$/IL-17A ratio. Additionally, disease evolution, serum vitamin D levels, serum autoantibodies levels and calcium metabolism (to assure safety) were also studied.

Methods: We assessed 24 phenotypically well characterized SLE patients. All patients were screened before vitamin D supplementation and 3 and 6 months after the beginning of this treatment. Peripheral blood lymphocyte's subsets were analysed by flow cytometry.

Results: Serum 25(OH)D levels significantly increased under vitamin D supplementation ($p=0.001$). The $FoxP3^{+}$/IL-17A ratio in SLE patients after 6 months of vitamin D supplementation was higher than that in the baseline ($p<0.001$).

Conclusions: In conclusion, this study demonstrated that vitamin D supplementation provided favourable immunological and clinical impact in SLE.

Keywords: SLE, Vitamin D, FoxP3 T cells, IL-17A T cells

Introduction

Systemic lupus erythematosus (SLE or lupus) is a systemic autoimmune disease with multi-organ inflammation [1, 2], linked to loss of immune tolerance to self-antigens and the production of a diversity of autoantibodies. It mainly affects women of childbearing age. This disease has a female to male ratio in adults of approximately 9:1, a peak age of diagnosis between 15 and 44 years, and a known negative impact on the quality of life, including reduced levels of employment and income. Despite phenotypic heterogeneity and an unpredictable disease evolution, a strong genetic and environmental contribution to the development of SLE is supported by broad evidence. The autoantibodies are primarily directed against chromatin and ribonuclear particle constituents (nucleosomes, single- and double-stranded DNA (dsDNA), RNPs) [2-5], and play a pathogenic role [3]. The hyperreactivity to these self-antigens leads to the formation of immune complexes that cause local inflammation and tissue damage [6].

With the production of autoantibodies and prolonged cell life, B cell regulation is important in the maintenance of immune balance. B cells from patients with SLE have been shown to present autoantigens, induce $CD4^+$ T helper cells (Th1/Th2), inhibit regulatory T cells (Tregs), and secrete pro-inflammatory cytokines [7].

Regulatory T cells are specialized suppressor cells [5, 8], with the phenotype $CD4^+FOXP3^+CD25^{high}CD127^{low}$ [9], that have the capacity to regulate the intensity and quality of the immune response [10]. They actively suppress effector cells, including those associated with autoimmune diseases [4], thereby establishing and maintaining immunological self-tolerance [11].

Some studies suggest that regulatory T cells are deficient in number and function in several autoimmune diseases including SLE [12]. However, an enrichment of this cell type has also been reported [13, 14]. These contradictory observations may be either due to the lack of a well-defined specific Treg marker in humans or to the heterogeneity of SLE phenotypes [12]. Concerning Tregs

markers, this population was initially characterized by CD25 alone, which could explain the reported contradictory results. Dysfunction of these cells could also be explained by the fact that FOXP3-expressing cells lose CD25 expression and consequently their suppressive functionality [15, 16].

Besides Th1 and Th2, a third subset of $CD4^+$ effector Th cells was identified, named Th17 because of its unique ability to produce IL-17 (IL-17A and IL-17F). These cells play a critical role in the recruitment, activation and migration of neutrophils [4]. Beyond their protective role in the clearance of extracellular pathogens, a major role of Th17 lymphocytes seems to be their involvement in the induction and maintenance of chronic inflammatory processes [4, 17].

The hypothesis that certain forms of autoimmunity may result from a conversion of Treg cells into a Th17 cell phenotype, has been suggested by some studies [18].

In SLE patients' sera, the IL-17 levels are abnormally high when compared with healthy individuals. IL-17 is also produced by neutrophils, Innate Lymphoid Cells (ILC), and other T cell types including $CD4^+$, $CD8^+$, double negative ($CD4^-CD8^-$) and TCR-γδ [4]. This phenomenon could promote the autoimmune process by increasing the activation of immune cells itself. Release of IL-17 by infiltrating T cells in specific organs may also contribute to local tissue injury by instigating the inflammatory response [4].

Environmental factors combined with genetic and hormonal characteristics have been associated with SLE phenotype and with disease progression [19, 20].

Vitamin D is one of the environmental factors likely related to SLE pathogenesis and its deficiency appears to be associated with immunomodulatory abnormalities in this disease [21].

The identification of vitamin D receptors in immune system cells and the discovery that dendritic cells can produce the metabolically active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$; calcitriol), have led to the suggestion that vitamin D is an immune modulator [1]. The importance of vitamin D in several autoimmune disorders has been reported and vitamin D deficiency has been associated with the pathogenesis and severity of multiple sclerosis (MS),

rheumatoid arthritis (RA), systemic sclerosis (SSc) and SLE, among others [22]. In SLE patients, serum vitamin D3 levels seem to correlate inversely with SLEDAI scores [6].

It has been found that one of the consequences of $1,25(OH)_2D_3$ on the immune response is the stimulation of innate immunity and suppression of adaptive immunity [23]. Studies on the immunomodulatory properties of $1,25(OH)_2D_3$ confirmed the inhibition of Th1 cell development via an inhibition of IL-12 production by antigen-presenting cells. Further work documented its ability to drive CD4 T lymphocytes to a Th2 phenotype with a reduction in Th1 type activity [17], by increasing the production of IL-5 and IL-10.Vitamin D indirectly, reduces the production of IFNγ [24]. It also affects B cells causing induction of B cell apoptosis, inhibition of B cell proliferation, and generation of memory B cells, plasma cell differentiation and immunoglobulin production/secretion [24-27]. In human epidermal and dermal cells, it was demonstrated that $1,25(OH)_2D_3$ modulates regulatory T cell numbers and their suppressive abilities through dendritic cells [23]. The effect of vitamin D on dendritic cells include the differentiation of monocytes into immature dendritic cells, the maturation of dendritic cells and dendritic cell survival [26].

Local vitamin D metabolism allows immune cells to modulate immune responses in an independent way when regulation is required, but the optimization of this autocrine and/or paracrine circuit is strictly dependent of the circulating 25(OH)D (the calcitriol precursor) availability. The levels of circulating 25(OH)D needed to meet the requirements of vitamin D sufficiency are still a matter of debate, especially in light of the non-classical effects of vitamin D [27]. These evidences justify the motivation to consider vitamin D supplementation as an immunomodulatory intervention in SLE.

The FOXP3 gene has a vitamin D receptor element (VDRE) in its promoter region, being important for its cellular expression [28]. The immunomodulatory effect assessed by the imbalance of $FoxP3^+$/IL-17A $CD4^+$ T lymphocytes is widely recognized [29]. However, it is not known if this immune imbalance can be represented by a ratio between these two cell populations. This ratio can

be calculated by dividing the percentage of total lymphocytes that are $CD4^+$ and express FoxP3 by the percentage of total lymphocytes that are $CD4^+$ and synthesize IL-17A.

In this study, using a well phenotypically characterized patients' cohort and a well-defined therapeutic intervention, the authors investigate if the $FoxP3^+$/IL-17A ratio and disease activity is modified after vitamin D supplementation (cholecalciferol). For this purpose, we evaluated the effect of vitamin D supplementation on $CD4^+$ FoxP3 expression and $CD4^+$ IL-17A producing T cells. Additionally, disease evolution, serum vitamin D levels, serum autoantibodies levels and calcium metabolism (to assure safety) were studied.

Therefore, whether the $FoxP3^+$/IL-17A ratio has a positive effect on SLE disease activity is the question addressed in this study.

Material and Methods

Subjects

The study population consisted of SLE patients recruited between 1 November 2012 and 31 January 2013. SLE patients (diagnosed according to the 1997 [30] and 2012 [31] revised ACR criteria for SLE) were selected from the outpatient clinic database of the Clinical Immunology Unit from Centro Hospitalar do Porto (North of Portugal). By protocol all these patients should have more than 5 years of disease, and should be in a stable phase of disease and without major flares for at least 1 year. Stability was defined as no new major organ involvement in the last year independently of the SLEDAI-2K score and no changes in steroid dose or immunosuppressive therapy in the last 12 months. Baseline 25(OH)D serum level was measured. Hypovitaminosis D was defined as serum 25(OH)D < 75 nmol/L and patients were selected irrespectively of their baseline 25(OH)D levels.

Ethical approval was obtained from the research ethics committee of Centro Hospitalar do Porto and written informed consent for all analysis was obtained from all subjects.

Study Design

A prospective cross-sectional study with 6 months follow-up evaluations of patients with a dose escalating protocol of vitamin D supplementation was done. Safety of high dose vitamin D supplementation was also monitored (increase of serum phosphorus or calcium).

We assessed 24 SLE patients for eligibility (1 man and 23 women). The clinical characterization of SLE patients comprised: 1) Evaluation of the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) [32]; 2) Evaluation of the Systemic Lupus International Collaborating Clinics/American College Rheumatology (ACR) Damage Index (SLICC-SDI) [33]; 3) Flare evaluation according to modified SLE Flare Index (SFI) [34-36]; 4) Cumulative organ involvement (specific data and treatment); 5) Previous and present immunosuppressive therapy and hydroxicloroquine.

All patients were screened before vitamin D supplementation (Day 0 or D0), and 3 and 6 months (M3 and M6) after the beginning of this treatment.

At D0, before vitamin D supplementation, relevant data were compiled, namely: baseline SLEDAI-2K, baseline SLICC-SDI, disease duration, concomitant therapy (including steroid doses), previous SLE manifestations (including cumulative organ involvement and number of disease flares), baseline 25(OH)D serum levels, baseline %TCD4$^+$FoxP3$^+$/%TCD4$^+$IL-17A$^+$ ratio (FoxP3$^+$/IL-17A ratio), baseline autoantibodies levels, complement (C3 and C4) as required for SLEDAI-2K, serum calcium, serum phosphorus and serum PTH (parathyroid hormone) levels.

At M3: SLEDAI-2K, concomitant therapy (including variations on steroid doses) , flare evaluation, 25(OH)D serum levels, autoantibodies levels and complement levels (C3 and C4) as required for SLEDAI-2K, serum calcium, serum phosphorus and serum PTH levels.

At M6: SLEDAI-2K, SLICC, concomitant therapy (including variations on steroid doses), flare evaluation, 25(OH)D serum levels and %TCD4$^+$FoxP3$^+$/%TCD4$^+$IL-17A$^+$ ratio (FoxP3$^+$/IL-

17A ratio). Serum autoantibodies levels, complement (C3 and C4) as required for SLEDAI-2K, serum calcium, serum phosphorus and serum PTH levels.

Supplementation protocol

Baseline (Day 0)

Vitamin D <50 nmol/l – 50,000 UI cholecalciferol/ week /8 weeks. Then 2,000U/day.

Vitamin D >75 nmol/L – 2,000 UI/day.

Vitamin D >50 nmol/L and <75 nmol/L – 4,000 UI/day/8 weeks, then 2,000 UI/day.

3 months Follow-up (M3)

<50 nmol/L – as the baseline.

>50 nmol/L and <75 nmol/L – duplicates the baseline dose per 8 Weeks, then 4,000/day.

>75 nmol/L and <125 nmol/L – same dose.

>125 nmol/L – 50% reduction.

Vitamin D assessment

25(OH)D protocol: vitamin D total assay for the Elecsys analysers and Cobas modular platforms - Roche®.

Flow Cytometry

Peripheral blood lymphocyte's subsets, including T, B, NK, $TCD4^{+}FoxP3^{+}$, $TCD4^{+}IL\text{-}17A^{+}$ were analysed by flow cytometry, in a Coulter Epics XL-MCL® cytometer. Cells counts (cells/μL) and proportions (%) were established from fresh blood samples using different protocols and monoclonal antibodies (mAbs), conjugated to fluorescein (FITC), phycoerythrin (PE), Phycoerythrin-Texas Red (ECD) and Phycoerythrin Cyanin 5.1 (PC5).

T ($CD3^{+}/CD4^{+}$ and $CD3^{+}/CD8^{+}$), B ($CD19^{+}$) and NK ($CD56^{+}$) lymphocytes were analysed using a Beckman Coulter standard protocol with the following mAbs: anti-CD45 FITC (clone B3821F4A), anti-CD3 PC5 (clone UCHT1), anti-CD4 RD1 (clone SFCI12T4D11), anti-CD8 ECD

(clone SFCI21Thy2D3), anti-CD19 ECD (clone J4.119) and anti-CD56 FITC (clone N901/NKH-1); all from Beckman Coulter, Fullerton, California, USA).

Effector $CD4^+$ T cells producing IL-17 were quantified after for 4 hour stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomicin in the presence of brefeldin A. Cells were fixed and permeabilized using the IntraPrep Permeabilization Reagent (Beckman Coulter) buffer system and stained with anti-CD4 PC5 (clone 13B8.2; Immunotech, Marseille, France) and anti-IL-17 PE (clone eBio64DEC17; eBioscience Inc, San Diego, CA).

$CD4^+FoxP3^+$ T cells were quantified after cell fixation and permeabilization using the mAbs anti-CD4 PC5 (clone 13B8.2; Beckman Coulter - IOTest; Marseille, France), and anti-FoxP3 PE (clone PCH10; eBioscience Inc, San Diego, CA), according to the manufacturer's staining protocol.

Statistical analysis

We compared measures taken at baseline (before vitamin D supplementation, D0) with those taken at M6, using the non-parametric paired Wilcoxon Signed Ranked Test, because the data did not follow a Gaussian distribution. Significance level was set at $\alpha=0.05$. Statistical analysis was performed using the SPSS v.22.

Results

Demographic, clinical and laboratorial features of SLE patients at baseline are described in

Serum 25(OH)D levels significantly increased under vitamin D supplementation from 59.32 ± 29.59 nmol/L at Day 0 to 80.39 ± 24.57 nmol/L at Month 3 (p=0.030) and to 85.25 ± 30.92 nmol/L at Month 6 (p=0.001) (Figure 1).

Treatment was safe, with no significant increase of serum phosphorus or calcium. Serum calcium significantly decreased from 2.34 ± 0.10 mmol/L at Day 0 to 2.27 ± 0.10 mmol/L at Month 6 (p=0.026) but all in the normal range (Figure 2).

Disease activity, assessed by SLEDAI-2K, significantly decreased from 2.75 ± 4.76 at Day 0 to 1.67 ± 2.79 at Month 6 (p=0.026) (Figure 3).

Serum anti-dsDNA levels (evaluated by immunofluorescence) remained stable during follow-up, while C3 complement fraction significantly decreased from 101.5 ± 23.57 mg/dL at Day 0 to 95.46 ± 21.71 mg/dL at Month 6 (p=0.013) (Figure 4), but no new hypocomplementemia occurred (reference values: 88-201 mg/dL).

None of the patients required modification of the prednisone and immunosuppressive dosage or initiation of new immunosuppressive agents. We did not observe SLE flares during the 6 months follow-up period.

The impact of vitamin D supplementation on the proportions of $CD3^+$ T cells, $CD4^+$ and $CD8^+$ T cells is shown in Figure 5.

The mean proportions at baseline were 80.05 ± 5.64% for $CD3^+$ T cells (Figure 5A), 47.8 ± 7.58% for $CD4^+$ T cells (Figure 5B), and 30.56 ± 7.73% for $CD8^+$ T cells (Figure 5C). At Month 6, the proportion of $CD3^+$ T cells, $CD4^+$ and $CD8^+$ T cells remained stable.

The impact of vitamin D supplementation on $CD3^+CD4^+FoxP3^+$ T cells was evaluated. The percentage of $CD4^+FoxP3^+$ cells at baseline was 7.27 ± 5.92%. The percentage of $CD4^+FoxP3^+$ cells was increased at 11.42 ± 6.54% at Month 6 ($p<0.001$) (Figure 6A). A decrease was observed in $CD4^+$IL-17A from 3.89 ± 1.76% at Day 0 to 2.82 ± 1.03% at Month 6 ($p=0.001$) after vitamin D supplementation (Figure 6B).

Accordingly, the $FoxP3^+$/IL-17A ratio in patients with SLE after 6 months of vitamin D supplementation was higher than that in the baseline ($p<0.001$) (Figure 7).

Considering patients with 25(OH)D levels at baseline ≥75 nmol/L, the increase of $FoxP3^+$/IL-17A ratio observed for the entire group after 6 months of vitamin D supplementation was also valid ($p=0.043$) (Figure 8).

The relationship between $CD4^+FoxP3^+$ and $CD4^+$IL-17A cells and 25(OH)D levels, serum calcium, serum phosphorus and serum PTH levels was also analysed. No associations were found between these features and $CD4^+FoxP3^+$ and $CD4^+$IL-17A proportions.

No significant differences were observed for the other laboratory and clinical parameters, such as SLICC score.

Discussion

In this study, we assessed for the first time the safety, the clinical and immunological effects of vitamin D supplementation in patients with SLE in a Portuguese population.

Vitamin D deficiency is now recognized as a pandemic [37] and strong evidence exist that vitamin D deficiency can contribute to a raised risk for autoimmune diseases [38-41]. The definition of vitamin D insufficiency (25-50 nmol/L) and deficiency (<25 nmol/L) varies between studies and the degree of hypovitaminosis D varies from region to region. While 25(OH)D levels below 75 nmol/L are common worldwide, levels below 25 nmol/L that constitute frank vitamin D deficiency are most commonly seen in groups at risk, in particular, with autoimmune diseases [42], such as MS [43], RA, type 1 diabetes mellitus and SLE [6, 44].

In SLE, some studies have demonstrated an association between higher disease activity and low serum levels of vitamin D, but the results are controversial. Nevertheless, vitamin D supplementation is currently recommended as a treatment for some of these patients. However, data about the effects of its administration in immune regulation in SLE is still missing [45].

To date, only one study has assessed the *in vivo* benefit of vitamin D supplementation in SLE, but only in vitamin D deficient patients [46]. In our study we included patients with 25(OH) vitamin D > 75 nmol/L.

We confirmed the high frequency of hypovitaminosis D in SLE patients (70.8%) described in some studies, and we also demonstrated that vitamin D supplementation significantly increase serum 25(OH)D levels, at 3 and 6 months after the beginning of the study. Furthermore, we could show that this therapy was safe, since no alterations on phosphorus or calcium levels and no side effects were reported.

It is well known that T cells initiate and sustain the secretion of antibodies by B cells. The high degree of hyper mutation in SLE-associated autoantibodies demonstrates the T cell dependency of autoantibodies development in these patients. SLE is also associated with pathologically altered immune responses, with hyperactive B cells playing an important role in its pathogenesis. In this context, SLE is a T and B cell-dependent disease, associated with rather functional deficiency of regulatory T cells [47], an increase in T helper lymphocytes producing IL-17 (Th17 cells) [48, 49] and an increased expression of IFN-inducible genes [50]. The

immunomodulatory properties of vitamin D have been under increased scrutiny in the last years. $1,25(OH)_2D_3$ was shown to inhibit Th17 responses, probably owing to its capacity to inhibit IL-23 production [46], and to induce the differentiation and/or expansion of $FoxP3^+$ Tregs and an increased expression of CTLA-4 [46].

In our study, after 6 months of vitamin D supplementation, we observed an increase of FoxP3 expression in $CD4^+$ T cells and a decrease in $CD4^+$IL-17A. The $FoxP3^+$/IL-17A ratio was found to be significantly higher at the end of the treatment when compared with D0. This effect was also observed in patients with elevated vitamin D levels at baseline, showing that immunological effects were unrelated to the standard cut-off used for metabolic bone disease. It is well known that the imbalance between Th17 and Treg cells results in inflammation and/or autoimmunity. The shared requirement of cytokines in iTreg and Th17 cell differentiation naturally leads to the hypothesis that an imbalance between these two cell types may lead to tissue inflammation. While Th17 cells promote inflammation and autoimmunity, Treg cells modulate the function of effector T cells, preventing this phenomenon. A reestablishment of this balance may be achieved by decreasing Th17 function and differentiation, and by the increase of Treg cells number and function. This hypothesis offers numerous potential pharmacologic targets for immunomodulation. The inflammatory imbalance might be modified to restore immune homeostasis, resulting in therapeutic benefit. An intriguing alternative approach involves pharmacologically altering iTreg and Th17 cell differentiation or expansion, using cytokines, cytokine inhibitors, and small molecule inhibitors of key signalling pathways [18].

Our results suggested that vitamin D supplementation improves the Treg/Th17 ratio, an effect described for the first time in SLE patients, of real benefit, as shown by the effective decrease of the SLEDAI scores. Vitamin D supplementation was shown to be a safe and an efficient treatment for improving stable SLE patients' condition and flare prevention. Since none of the patients exhibit complications resulting from vitamin D supplementation, perhaps the administered doses could be increased, maintaining the safety and leading to even better results.

In spite of displaying optimal circulating vitamin D levels, the ability to metabolize vitamin D may vary between individuals' genetics and may therefore contribute to the risk of developing immune abnormalities. These situations are illustrated by the presence of certain gene polymorphisms in the vitamin D metabolizing enzymes [27], such as CYP2R1 (hydroxylates vitamin D3 to 25(OH)D in the liver) or CYP27B1(activated by PTH and hydroxylates 25(OH)D to $1,25(OH)_2D_3$ in the kidney) [51]. Even if all these steps are properly functioning, the vitamin D active metabolite ($1,25(OH)_2D_3$) must be recognized, bound and activated by its receptor (VDR). Polymorphisms in the VDR gene may also play a role in this mechanism [1].

The main limitation of this study is the lack of an adequate Portuguese control population with known vitamin D levels.

In conclusion, this study demonstrated that vitamin D supplementation seems to provide favourable immunological effects in patients with SLE, independently of the 25(OH)D patients status. We observed a decrease of IL-17A producing T cells and an increase in FoxP3 expression, confirming the relationship between vitamin D status and immunological balance. Nevertheless, these results should be interpreted with caution since previous studies evaluating the supplementation of 25(OH)D in SLE disease have had inconsistent results.

It is important to consider all the existing variables and their specific outcomes in each patient. Therapy should be individualized, to take into account all the existing factors and differences between patients. Using the $FoxP3^+$/IL-17A ratio it may be possible to tailor vitamin D therapy for each patient. An individualized therapy should be undertaken since some patients will need very high doses of supplementation whereas others will need only modest doses to achieve the same outcome.

Finally, concerning vitamin D therapy safety, screening to monitor PTH levels will be only required if hypocalcaemia occurs.

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Table 1. All patients completed the 6 months follow-up period.

Table 1 – Demographic, clinical and laboratorial features of SLE patients at baseline

Characteristics	**N=24**
Epidemiology	
Age, years, mean ± SD	47 ± 11
Female gender, n (%)	23 (95.83%)
Previous SLE manifestations	
Skin, n (%)	19 (79,2%)
Joints, n (%)	18 (75%)
Serositis, n (%)	4 (16.7%)
Kidney, n (%)	7 (29.2%)
Nervous system, n (%)	3 (12.5%)
Flares, n (%)	10 (41.7%)
Flares, mean (range)	0.66 (0-3)
SLEDAI/SLICC and analytical studies	
SLEDAI at Day 0, mean (range)	2.71 (0-21)
SLICC at Day 0, mean (range)	1.04 (0-5)
Calcium levels, mean (range), mmol/L	2.34 (2.12-2.52)
Phosphorus levels, mean (range), mmol/L	1.02 (0.65-1.4)
C3 levels at Day 0, mean (range), mg/dL	101.5 (51-147)
C4 levels at Day 0, mean (range), mg/dL	16.5 (6-35)
Anti-dsDNA levels at Day 0, mean (range), IU/mL	34.15 (0.2-302)
% $CD3^+$ levels at Day 0, mean (range)	80.05 (69.1-90.5)
% $CD3^+CD4^+$ levels at Day 0, mean (range)	47.8 (39-62.5)
% $CD3^+CD8^+$ levels at Day 0, mean (range)	30.55 (18.2-44.9)
%T $CD4^+$ $FoxP3^+$ at Day 0, mean (range)	7.27 (0.48-15.57)
%T $CD4^+IL17^+$ at Day 0, mean (range)	3.89 (1.19-8.58)
%T $CD4^+$ $FoxP3^+$/%T $CD4^+$ $IL17^+$ ratio at Day 0, mean (range)	2.23 (0.16-8.9)
25-hydroxyvitamin D levels	
Mean ± SD, nmol/L	59.3 ± 29.6
25(OH)D ≤ 25 nmol/L, n (%)	2 (8.3%)
25 < 25(OH)D ≤ 50 nmol/L, n (%)	8 (33.3%)
50 < 25(OH)D ≤ 75 nmol/L, n (%)	7 (29.2%)
25(OH)D > 75 nmol/L, n (%)	7 (29.2%)
Associated treatments*	
Prednisone, n (%)	15 (62.5%)
Prednisone <10 mg	11
Prednisone 10-20 mg	3
Prednisone >20 mg	1
Hydroxychloroquine, n (%)	17 (70.8%)
Azathioprine, n (%)	2 (8.3%)
Mycophenolate mofetil, n (%)	3 (12.5%)

*At the time of the study.

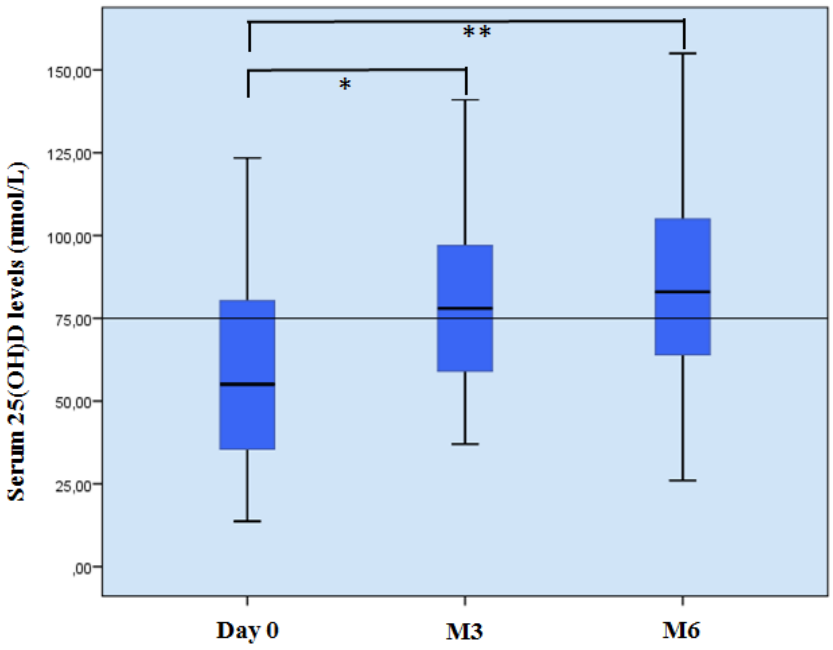

Figure 1 –Time evolution of serum 25(OH)D levels (M3 – month 3; M6 – month 6); *p<0.05, **p<0.01.

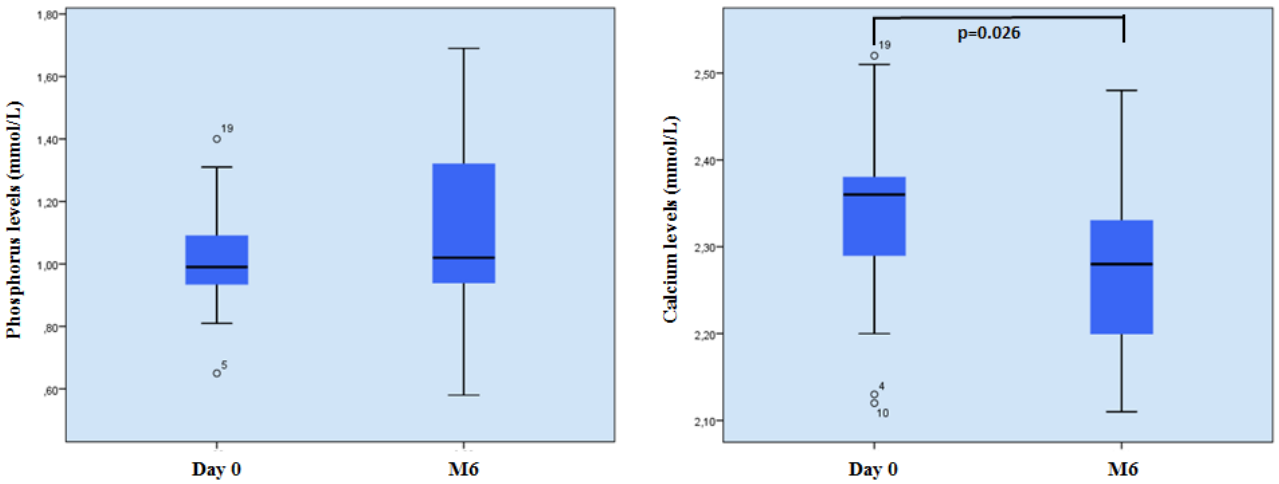

Figure 2 – Time evolution of phosphorus (p=ns) and calcium levels.

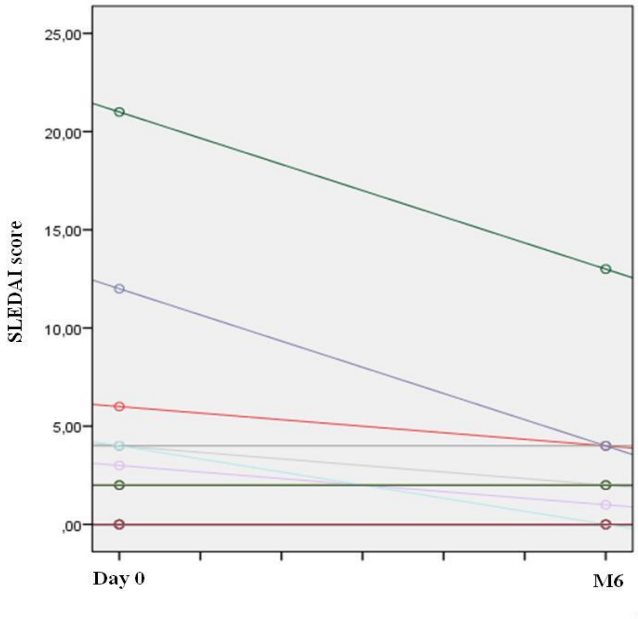

Figure 3 – Disease activity assessed by SLEDAI-2K scores.

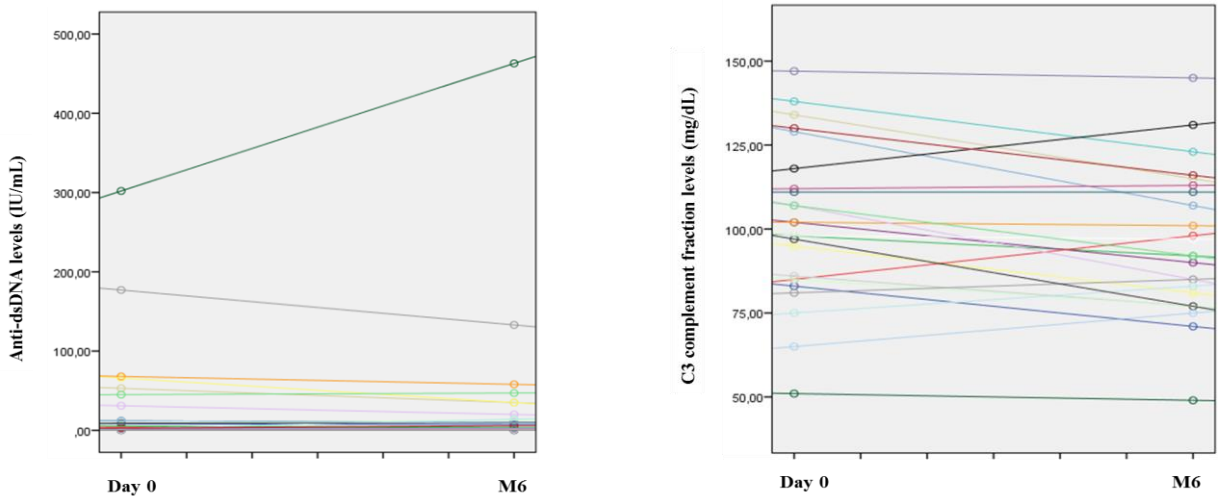

Figure 4 – Time evolution of anti-dsDNA (p=ns) and C3 complement fraction levels.

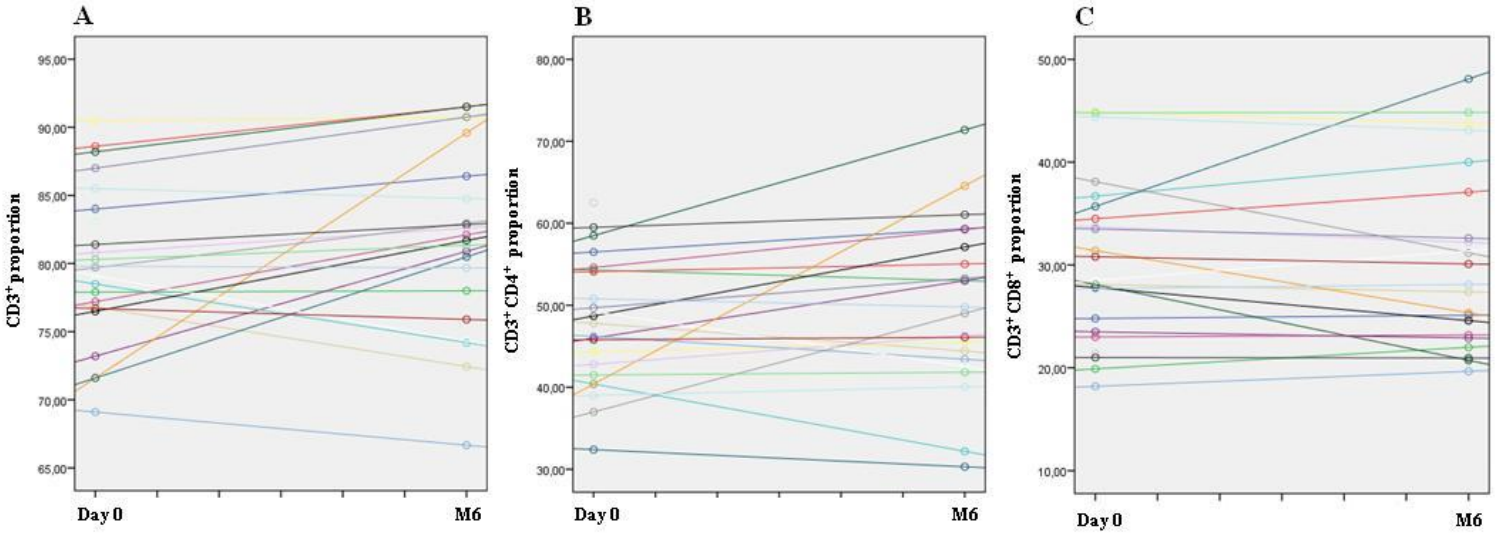

Figure 5 – Evolution of peripheral blood lymphocytes proportions with vitamin D supplementation. $CD3^+$ (A), $CD3^+CD4^+$ (B) and $CD3^+CD8^+$ (C) proportions at Day 0 and M6.

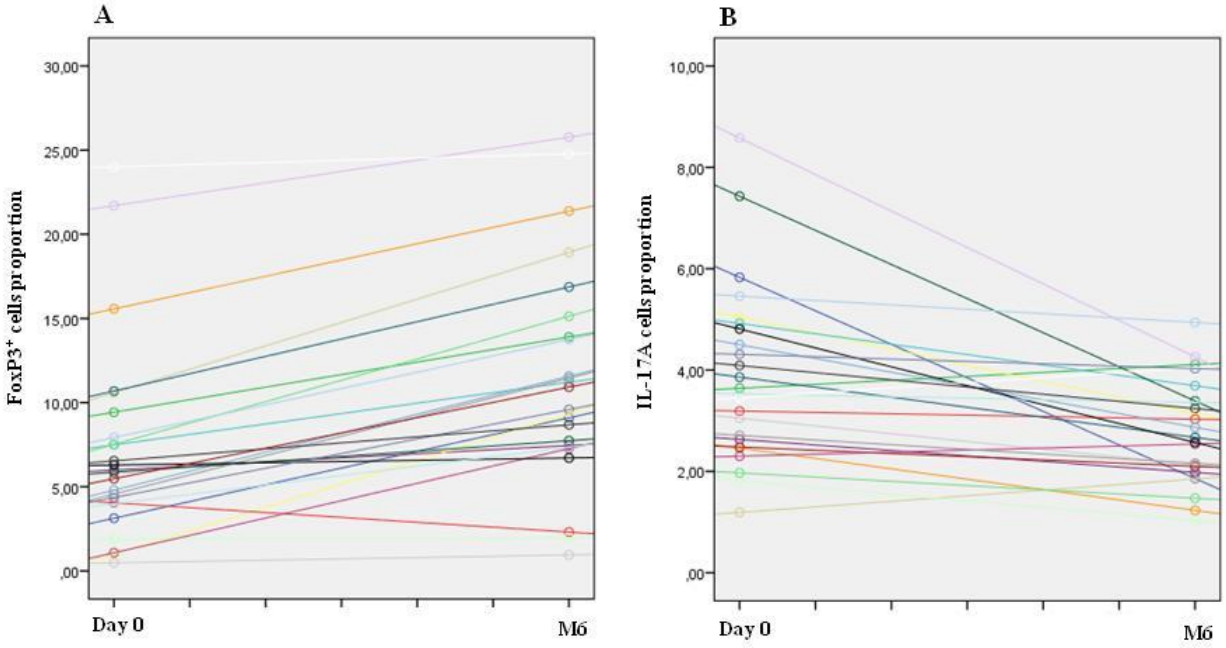

Figure 6 – Variation of the proportions of $CD4^+FoxP3^+$ T cells (A) and $CD4^+$IL-17A T cells (B) after vitamin D supplementation.

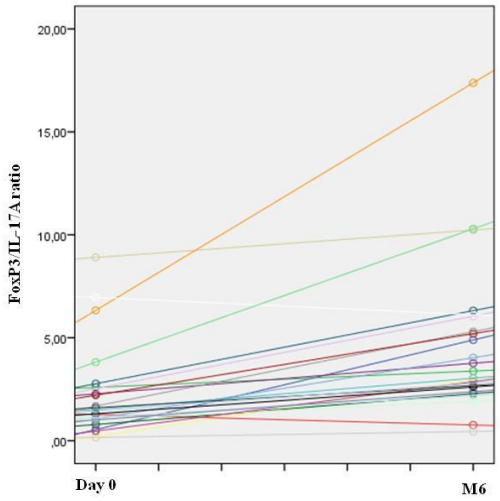

Figure 7 – Variation of FoxP3^{+}/IL-17A ratio after vitamin D supplementation.

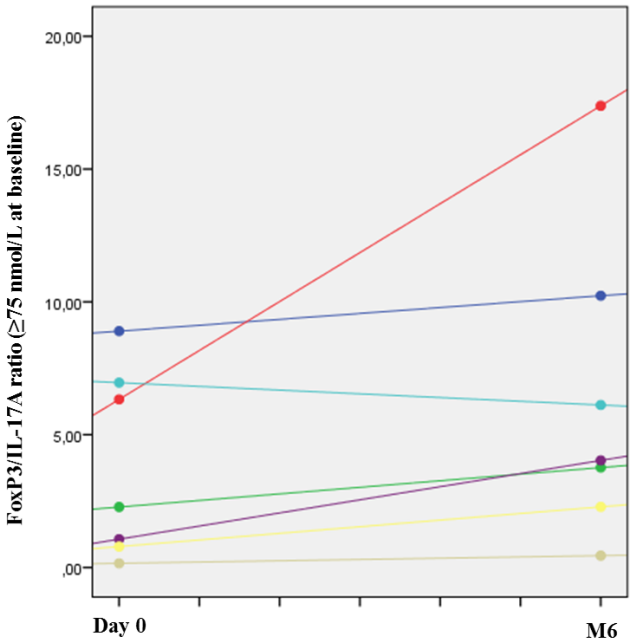

Figure 8 – Variation of FoxP3^{+}/IL-17A ratio in patients group with ≥75 nmol/L 25(OH)D levels (at baseline) after vitamin D supplementation.