UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

AVALIAÇÃO DA SUPLEMENTAÇÃO DE VITAMINA D NO DESENVOLVIMENTO E EVOLUÇÃO DE LÚPUS ERITEMATOSO SISTÊMICO EM MODELO EXPERIMENTAL

EDUARDA CORREA FREITAS

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EDUARDA CORREA FREITAS

Orientador: Prof. Dr. Odirlei André Monticielo

Tese apresentada como requisito parcial para obtenção de Doutor em Medicina: Ciências Médicas, da Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Medicina: Ciências Médicas.

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“É preciso ter o caos em si mesmo para ser capaz de dar à luz uma estrela dançante.”

Friedrich Nietzsche

Dedico à minha mãe, ao meu irmão, a minha avó materna, especialmente, ao meu companheiro, pelo apoio, compreensão e carinho destinados a mim durante a realização desta tese de doutorado.

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À Universidade Federal do Rio Grande do Sul, especialmente ao programa de Pós-Graduação em Ciências Médicas pela oportunidade.

SUMÁRIO

LISTA DE ABREVIATURAS

$1,25(OH)_2D$	1,25-dihidroxivitamina D
$24,25(OH)_2D$	24,25-dihidroxivitamina D
25(OH)D	25-hidroxivitamina D
7-DHC	7-Deidrocolesterol
ACR	*American College of Rheumatology*
BAFF	*B-cell activating factor* (Fator de ativação de células B)
BANK1	*B cell scaffold protein with ankyrin repeats*
BLK	*B lymphoid kinase* (Cinase dos linfócitos B)
BLys	*B lymphocyte stimulator* (Estimulador de linfócitos B)
CMV	Citomegalovírus
CYP24A1	*Cytochrome P450 family 24 subfamily A member 1*
CYP27B1	*Cytochrome P450 family 27 subfamily B member 1*
CYP2R1	*Cytochrome P450 family 2 subfamily R member 1*
DCV	Doença cardiovascular
DHEA	Dehidroepiandrosterona
DNA	Ácido desoxirribonucleico
dsDNA	*Double stranded* DNA (DNA de cadeia dupla)
EBV	Epstein–Barr vírus
FcγRIIA	*Fc gamma receptor IIA*
FcγRIIIA	*Fc gamma receptor IIIA*
FcγRIIIB	*Fc gamma receptor IIIB*
GWAS	*Genome-wide association study* (Estudos de associação global do genoma humano)
HLA	*Human leukocyte antigen* (Antígeno leucocitário humano)
IC	Imunocomplexo
IFN-α	Interferon-alfa
IFN-γ	Interferon-gama
IL-2	Interleucina-2
IL-6	Interleucina-6
IRAK1	*Interleukin 1 receptor associated kinase 1*
IRF5	*Interferon regulatory factor 5*

ITGAM	*Integrin alpha M*
LES	Lúpus Eritematoso Sistêmico
LYN	*Src family of protein tyrosine kinase*
MBL	*Mannose-binding lectin* (Lectina ligadora da manose)
MECP2	*Methyl CpG binding protein 2*
MHC	*Major histocompatibility complex* (Complexo maior de histocompatibilidade)
NK	*Natural Killer* (Exterminadora natural)
OX40L	OX40 ligante
PCR	Proteína C-reativa
PDCD1	*Programmed cell death 1*
PTH	Paratormônio
PTPN22	*Protein tyrosine phosphatase non-receptor 22*
RNA	Ácido ribonucleico
RXR	Receptor X Retinóico
SLICC	*Systemic Lupus International Collaborating Clinics*
SPP1	*Secreted phosphoprotein 1*
STAT4	*Signal transducer and activator of transcription 4*
Tfh	Linfócitos T auxiliares foliculares
Th	Linfócitos T auxiliares
TLR	*Toll-like receptors*
TNF-α	Fator de Necrose Tumoral-alfa
TREX1	*Three-prime repair exonuclease*
UVB	Raios ultravioleta B
VDBP	Proteína ligadora da vitamina D
VDR	Receptor de vitamina D
VDRE	Elementos de resposta à vitamina D

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RESUMO

INTRODUÇÃO: O Lúpus eritematoso sistêmico (LES) é uma doença inflamatória multifatorial e autoimune, caracterizada pela produção de autoanticorpos, formação e deposição de imunocomplexos (IC), inflamação em diversos órgãos e dano tecidual. O estudo de diferentes modelos animais proporcionou uma melhor compreensão desta doença. O modelo de lúpus induzido por pristane representa um modelo adequado para estudar fatores que podem influenciar a indução e/ou progressão do LES, incluindo fatores genéticos. A vitamina D, é um destes fatores, e exerce efeitos imunomodulatórios nas células do sistema imune, dentre elas linfócitos T, linfócitos B e células dendríticas. Desta forma a suplementação de vitamina D pode interagir minimizando os sintomas do lúpus.

OBJETIVO: Avaliar o desenvolvimento e evolução de LES após suplementação de vitamina D em modelo experimental de lúpus induzido por pristane.

METODOLOGIA: O modelo experimental foi induzido com uma injeção intraperitoneal contendo 500ul de pristane em camundongos BALB/c fêmeas de 8-12 semanas de idade. Os animais foram divididos em três grupos: grupo controle (CO), grupo lúpus induzido por pristane (PIL) e grupo lúpus induzido por pristane suplementado com vitamina D (VD). Após a indução do modelo, os animais do grupo VD foram tratados com vitamina D através de injeção subcutânea contendo 100ul de Calcijex [2ug/kg/animal] diluído em PBS-Tween 20 em dias alternados durante 180 dias. Nós tempos 0, 60, 120 e 180 dias após a indução foi avaliado escore clínico articular, nocicepção por Von Frey e o edema articular por pletismômetro. No dia 150 após a indução do modelo foi coletado urina em gaiola metabólica para dosagem de proteína na urina. No dia 180 após a indução os animais foram submetidos à eutanásia. No mesmo tempo, foi coletado soro para dosagem de citocinas inflamatorias, e tecido articular e renal para análise histológica.

RESULTADOS: Os animais do grupo PIL apresentaram artrite e lesão renal, caracterizada pelo aumento dos níveis de proteinuria, deposição de IC e hipercelularidade mesangial glomerular. Além disso, os animais do grupo PIL demonstraram níveis aumentados de IL-6, TNF-α e IFN-γ no soro. No presente estudo, nós observamos que o tratamento com vitamina D melhorou a artrite através da redução da incidência de artrite e da redução de escore clínico articular e edema

das patas posteriores, mas não foi capaz de influenciar na lesão renal. O tratamento com vitamina D não reduziu níveis de proteinuria, hipercelularidade mesangial glomerular e deposição de IgG e IgM no tecido renal. A suplementação de vitamina D não alterou os níveis séricos das citocinas IL-6, TNF-α, IL-2 e IL-4, mas reduzir os níveis de séricos de IFN-γ.

CONCLUSÃO: Neste estudo demonstrou-se que a vitamina D foi capaz de modular os sintomas clínicos e histopatológicos da artrite, mas não alterou o curso clínico e histopatológico da doença renal, apesar de ter modificado o perfil de citocinas. Estes resultados confirmam que o papel da vitamina D pode ser diferente dependendo do sítio de ativação, o que poderia explicar diferentes respostas de acordo com o fenótipo clínico. Ainda é necessário explorar a concentração de dose adequada e segura, o tempo de tratamento e a influência da vitamina D nas diferentes bases moleculares no LES.

PALAVRAS-CHAVE

Lúpus Eritematoso Sistêmico, vitamina D, 1,25-hidroxivitamina D, receptor da vitamina D, lúpus induzido por pristane.

ABSTRACT

INTRODUCTION: Systemic lupus erythematosus (SLE) is a multifactorial and autoimmune inflammatory disease, characterized by the production of autoantibodies, formation and deposition of immunocomplexes, inflammation in various organs and tissue damage. The study of different murine models has provided a better understanding of these autoimmune phenomena. Pristane-induced lupus model represents a suitable model for studying factors that may influence the induction and/or progression of SLE, including genetic factors. Vitamin D is one of these factors and exerts immunomodulatory effects on the cells of the immune system, among them T lymphocytes, B lymphocytes and dendritic cells.

OBJECTIVE: To evaluate the development and evolution of SLE after vitamin D supplementation in an experimental model of lupus induced by pristane.

METHODOLOGY: The experimental model was induced with an intraperitoneal injection containing 500ul pristane in 8-12 week old female BALB/c mice. The animals were divided into three groups: control group (CO), pristane-induced lupus group (PIL) and pristane-induced lupus group supplemented with vitamin D (VD). After induction of the model, the animals of the VD group were treated with vitamin D by subcutaneous injection containing 100ul of Calcijex [2ug / kg / animal] diluted in PBS-Tween 20 on alternate days for 180 days. At 0, 60, 120 and 180 days after induction we evaluated the arthritis clinical score, nociception and hind paws edema. On day 150 after the induction of the model urine was collected in metabolic cage for protein dosage in the urine. At day 180 after induction, the animals were euthanized. At the same time, serum was collected for the dosage of inflammatory cytokines and renal and articular tissue for histological analysis.

RESULTS: PIL group showed arthritis and kidney injury, characterized by increased proteinuria, glomerular mesangial expansion and inflammation. Moreover, PIL model showed increased levels of IL-6, TNF-α and IFN-γ in serum. We observed that treatment with vitamin D improved arthritis through reduced of incidence and arthritis clinical score and edema, but does not influenced renal injury. Treatment with vitamin D was not able to reduce proteinuria levels, decrease mesangial hypercellularity or IgG and IgM deposition in the kidney. Vitamin D supplementation did not alter IL-6, TNF-α, IL-2 and IL-4 cytokine levels, but we observed a reduce IFN-γ levels.

CONCLUSION: In this study, it was demonstrated that vitamin D was able to modulate the arthritis clinical and histopathological symptoms, but did not alter the clinical and histopathological course of renal disease, although it modified the cytokine profile. These results support that the role of vitamin D may be different depending on the acting site, which could explain different responses according clinical phenotype. Therefore, further investigations are still necessary to explore the adequate and safe dose concentration, the timing of treatment and the influence of vitamin D on the different molecular bases in SLE.

KEYWORDS

Systemic lupus erythematous; Vitamin D; 1,25- hydroxyvitamin D; Pristane – induced lupus.

1. INTRODUÇÃO

O Lúpus eritematoso sistêmico (LES) é uma doença autoimune caracterizada pela produção de autoanticorpos, formação e deposição de imunocomplexos (IC), inflamação em diversos órgãos (pele, articulações, coração, pulmões, sangue, rins e cérebro) e dano tecidual. É estimada uma incidência de 1-22 casos para cada 100.000 pessoas por ano, acometendo mais mulheres na idade fértil (1). A etiologia do LES é multifatorial e permanece ainda pouco conhecida, porém sabe-se da importante participação de fatores hormonais, ambientais, genéticos e imunológicos para o surgimento da doença. Inúmeros genes têm sido relacionados com o surgimento do LES, incluindo o gene *VDR* (do inglês *vitamin D receptor* - receptor de vitamina D) que sintetiza o receptor de vitamina D.

O VDR é um receptor nuclear, pertencente à família dos receptores esteroides de classe 2, semelhante aos receptores do ácido retinoico e do hormônio tireoestimulante (2). A 1,25-dihidroxivitamina D ($1,25(OH)_2D$), forma ativa da vitamina D, liga-se ao VDR e determina uma resposta genômica através da regulação da transcrição de alguns genes. As principais etapas envolvidas no controle da transcrição genética incluem: a ligação da $1,25(OH)_2D$ ao VDR, heterodimerização com o receptor X retinoico (RXR), ligação deste heterodímero a sequências específicas do DNA (do inglês *deoxyribonucleic acid* - ácido desoxirribonucleico), conhecidas como elementos de resposta à vitamina D (do inglês *vitamin D response elements* - VDRE) localizados no DNA, nas regiões promotoras dos genes que são ativados pela vitamina D e recrutamento de outras proteínas nucleares para dentro do complexo transcricional (3).

Diversos autores descreveram o VDR não somente em tecidos clássicos como ossos, rins e intestino, mas também em outros locais, tais como células do sistema imune, musculatura lisa e esquelética, pele, cérebro e fígado (4). Além da distribuição quase universal do VDR, algumas células (queratinócitos, monócitos, ossos e placenta) expressam a enzima 1α-hidroxilase que produz a forma ativada da vitamina D *in situ* (2,5).

As funções biológicas da vitamina D são mediadas pelo VDR. A função clássica da $1,25(OH)_2D$ esta relacionada a regulação da homeostase do cálcio, sendo responsável por incrementar a reabsorção de cálcio e fósforo nos rins. Além

dos efeitos no metabolismo do cálcio, a $1,25(OH)_2D$ está envolvida na regulação do magnésio, liberação de insulina pelo pâncreas, secreção de prolactina pela hipófise, inibição da síntese de renina, aumento da contratilidade miocárdica, manutenção da musculatura esquelética e depuração de creatinina endógena (6–10). Ainda a $1,25(OH)_2D$ interfere de forma direta ou indireta no controle de mais de 200 genes envolvidos na regulação do ciclo celular, diferenciação, apoptose e angiogênese, podendo determinar diminuição da proliferação de células normais ou neoplásicas (11).

A $1,25(OH)_2D$ também exerce efeitos imunomodulatórios nas células do sistema imune, dentre elas linfócitos T, linfócitos B e células dendríticas (12,13), onde se destacam a diminuição da produção de interleucina-2 (IL-2), do interferon-gama (IFN-γ) e do fator de necrose tumoral-alfa (do inglês*, tumor necrosis factor-alpha* -TNF-α). Cada uma destas células expressa VDR e 1α-hidroxilase, podendo produzir $1,25(OH)_2D$ localmente. Seus efeitos parácrinos/autócrinos dependem da adequada concentração da 25(OH)D, o que faz da deficiência de 25(OH)D um fator crucial no funcionamento do sistema imune (14). Há estudos com polimorfismos em pacientes cuja influência se dá através de alterações envolvendo a imunidade inata, os quais estão associados com níveis elevados de interferon-alfa (IFN-α), uma importante citocina envolvida na fisiopatogenia do LES (15–18).

Devido à heterogenidade da doença e as diferenças interpessoais quanto aos efeitos da vitamina D no organismo em humanos, causados por polimorfismos descritos principalmente no gene *VDR*, estudos experimentais podem ser uma boa alternativa para avaliar a modulação do VDR após a suplementação com $1,25(OH)_2D$. Os modelos animais mais estudados em LES são os espontâneos, principalmente (NZBXW)F1 e MRL*lpr*. Porém o modelo (NZBXW)F1 diverge em alguns aspectos do LES humano, como na ausência da produção de autoanticorpos contra o RNA (do inglês *Ribonucleic acid* – Ácido ribonucleico) (19). Enquanto que as alterações fisiopatológicas do modelo de LES induzido por pristane são semelhantes às encontradas em pacientes (aumentando os níveis de IFN-α/β, produção de autoanticorpos em altos níveis, glomerulonefrite por deposição de IC e hipercelularidade mesangial) (20).

Até o presente momento existem 5 estudos com suplementação de vitamina D em modelo animal de LES. O primeiro trabalho publicado, em 1992 com camundongos MRL/*lpr*, demonstrou que a suplementação com 1,25$(OH)_2$D atenuou algumas manifestações do LES, como ausência das lesões cutâneas e redução da proteinúria (21). Em 2000, um estudo brasileiro realizado em camundongos (NZBXW)F1, observou piores achados histopatológicos nas biópsias renais do grupo que recebeu a suplementação de colecalciferol (vitamina D3), o que sugere que a vitamina D pode ter agravado a doença (22). Arab e colaboradores realizaram um estudo com suplementação de 1,25$(OH)_2$D em modelo de lúpus induzido por cromatina ativada, onde os animais apresentaram redução na proteinúria, aumento da expressão de TGF-β e Foxp3, diminuição da expressão de interleucina-6 (IL-6) e interleucina-10 (IL-10) e aumento de células T regulatórias (23). Em 2016, um estudo submeteu o modelo de MRL/*lpr* a uma dieta deficiente em vitamina D, e os animais apresentaram baixos níveis de 25(OH)D, mas essa deficiência não foi capaz de alterar os níveis de anti-dsDNA (do inglês *double stranded DNA* - DNA de cadeia dupla), IgG total e proteinúria, a histologia renal ou o depósito de IC (IgG e C3) no rim (24). O último trabalho, publicado em 2017, suplementou 1,25$(OH)_2$D em modelo de MRL/*lpr* por via oral. Os animais apresentaram redução das úlceras cutâneas e injúria renal e aumento dos níveis de linfócitos CD4+ quando comparados com o grupo MRL/*lpr* sem suplementação (25). Estes trabalhos realizaram a suplementação de vitamina D através de diferentes vias de tratamento, concentrações do ativo e moléculas da vitamina D. Desta forma, mais estudos são necessários para determinar o papel da vitamina D no LES.

2. REVISÃO SISTEMÁTICA DA LITERATURA

2.1 ESTRATÉGIAS PARA LOCALIZAR E SELECIONAR AS INFORMAÇÕES

A estratégia de busca envolveu as seguintes bases de dados: EMBASE, PubMed e LILACS. O período da pesquisa foi de 1962 a 2018. Foram realizadas buscas através da lista de temas e suas combinações: “vitamin D”, “vitamin D receptor”, “lupus nephitis”, “systemic lupus erythematosus”, “vitamin D response element”, “vitamin D-binding protein”, “vitamin D deficiency”, “25-Hydroxyvitamin D”, “1,25-dihydroxyvitamin D”.

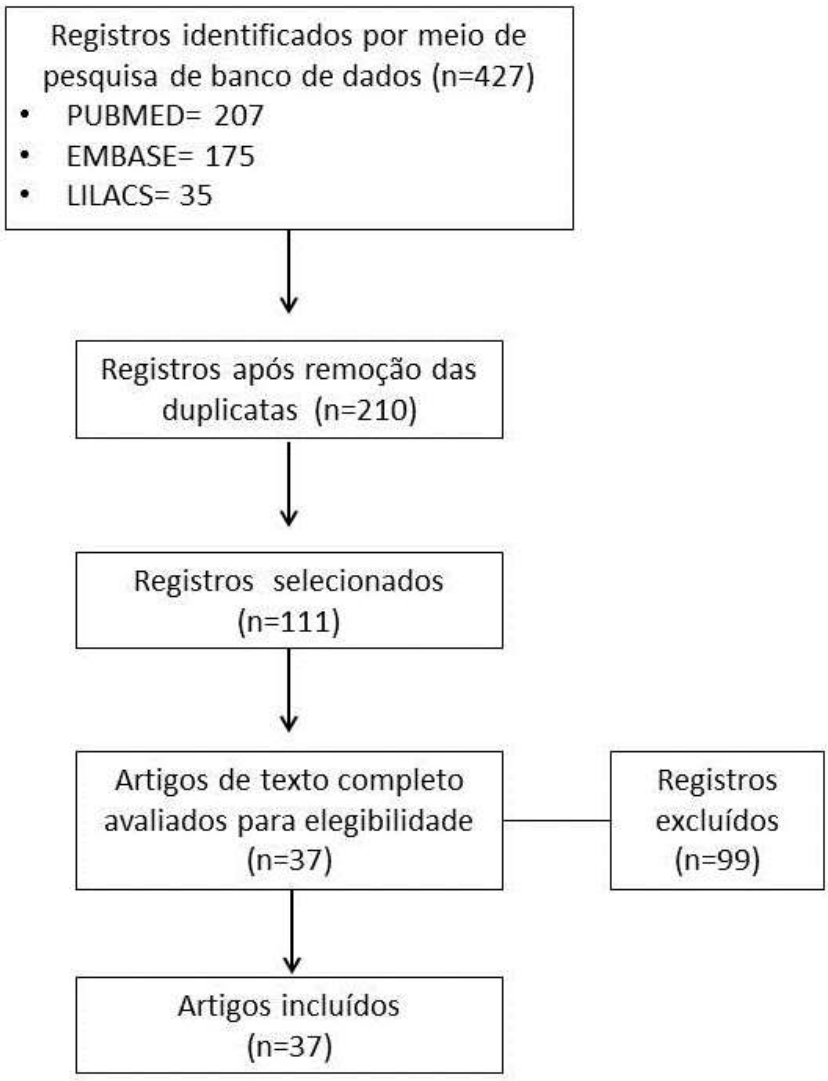

Figura 1. Modelo esquemático da estratégia de busca de informações. *Critérios de exclusão dos artigos: tema não relacionado aos objetivos da pesquisa; artigos não disponíveis na íntegra; artigos não disponíveis em inglês e/ou português.

2.2 LÚPUS ERITEMATOSO SISTÊMICO

2.2.1 Conceito de LES

O LES é uma doença autoimune multissistêmica complexa, caracterizada por amplo espectro de anormalidades clínicas, laboratoriais e imunológicas, com curso e desfecho variáveis. É uma doença caracterizada pela produção exacerbada de autoanticorpos direcionados particularmente ao dsDNA e a pequenas proteínas nucleares de ligação ao RNA. O LES possui um envolvimento multissistêmico que afeta comumente os sistemas cutâneo, renal, musculoesquelético e hematopoiético. A etiologia ainda é pouco conhecida, mas sabe-se da participação de fatores genéticos, imunológicos, hormonais e ambientais. As características clínicas são polimórficas e a evolução costuma ser crônica, com períodos de exacerbação e remissão. A doença pode cursar com sintomas constitucionais, artrite, serosite, nefrite, vasculite, miosite, manifestações mucocutâneas, hemocitopenias imunológicas, diversos quadros neuropsiquiátricos, hiperatividade reticuloendotelial e pneumonite. Diante desta apresentação heterogênea da doença foram desenvolvidos critérios de classificação para facilitar as pesquisas. Desta forma, convencionou-se realizar seu diagnóstico através de achados clínicos e laboratoriais, conforme os critérios de classificação propostos pelo *American College of Rheumatology* (ACR) em 1982 (26) e revisados em 1997 (27). No entanto, ao longo do tempo, surgiu a discussão sobre itens redundantes/ausentes, e em 2012, o grupo *Systemic Lupus International Collaborating Clinics* (SLICC) elaborou novos critérios de classificação onde se devem observar quatro ou mais sintomas para o diagnóstico de LES, sendo no mínimo 1 critério clínico e 1 critério laboratorial/imunológico (28).

2.2.2 Epidemiologia do LES

As taxas de incidência globais para o LES variam de cerca de 0,3 a 23,7 casos para cada 100.000 pessoas por ano (29,30). Enquanto as taxas de prevalência variam de 6,5 a 178 casos para cada 100.000 pessoas (31). A taxa de incidência e prevalência em crianças (<16 anos) é consideravelmente menor. A taxa de incidência anual de LES em crianças foi relatada como inferior a 1 caso para

cada 100.000 pessoas em estudos da Europa e da América do Norte (32). Estudos epidemiológicos envolvendo o LES são complexos devido à diversidade de apresentações clínicas da doença, dependência de critérios de classificação para definição do diagnóstico e baixa frequência na população. As variabilidades genéticas entre os povos e o local onde é conduzido o estudo influenciam nos resultados epidemiológicos referentes à doença.

A prevalência do LES na população norte-americana é de cerca de 70 a 178 casos para cada 100.000 pessoas e estimativas de incidência variam aproximadamente de 3,7 a 7,4 casos para cada 100.000 pessoas por ano (33). Até recentemente, pouco se sabia sobre a epidemiologia do LES entre a população árabe no mundo e no Oriente Médio. Em 2015, uma incidência de 2,1 vezes maior de LES entre os árabes-americanos em comparação com caucasianos não-árabes e afro-americanos foi descrita em sudeste de Michigan (34). Al Dhanhani e colaboradores estudaram a incidência e prevalência de LES nos Emirados Árabes Unidos (35). A incidência observada neste estudo foi de 8,6 casos para cada 100.000 pessoas por ano e a prevalência 103 casos para cada 100.000 pessoas.

Em uma revisão sistemática de estudos epidemiológicos da incidência e prevalência mundial de LES (36), as maiores estimativas de incidência e prevalência de LES foram na América do Norte (23,2 casos para cada 100.000 pessoas por ano e 241 casos para cada 100.000 pessoas, respectivamente). As menores incidências de LES foram relatadas na África e na Ucrânia (0,3 casos para cada 100.000 pessoas por ano) (29,37), e a menor prevalência foi no norte da Austrália (0 casos em uma amostra de 847 pessoas). Em geral, os países europeus tiveram menor incidência de LES, enquanto a Ásia, a Australásia e as Américas tiveram maior incidência.

No Brasil, um estudo no nordeste brasileiro estimou uma incidência de LES em torno de 8,7 casos para cada 100.000 pessoas por ano. Nas mulheres esta estimativa foi de 14 casos para cada 100.000 pessoas por ano e nos homens foi de 2,2 casos para cada 100.000 pessoas por ano. O pico de incidência ocorreu em mulheres entre 35 e 39 anos, com 32,7 casos para cada 100.000 mulheres por ano (38). Em 2004, outro estudo estimou a prevalência de doenças reumáticas em Montes Carlos. Dentre os pacientes diagnosticados com doenças reumáticas

somente 3 (1,4%) foram diagnosticados com lúpus e a prevalência nesta amostra foi de 0,098% para o lúpus (39).

O LES é mais comumente visto em mulheres durante a idade reprodutiva (40), para todos os grupos étnicos, com uma taxa nove vezes maior que a dos homens (9:1), e aquelas de ascendência afro-americana e asiática manifestam doença mais grave do que aquelas de ancestralidade europeia (41). A proporção entre os sexos pode variar de 3:1 (42) a 15:1 (43). Em crianças, esta razão é de 3:1; em adultos jovens chega a 15:1 e nos indivíduos com maior idade, novamente tende a ser menor, em torno de 8:1. Observa-se uma maior incidência em mulheres em todas as faixas etárias, embora as proporções sejam menores em ambos os extremos de idade. Este fato pode estar relacionado tanto ao cromossomo X duplo quanto às diferenças nos níveis de estrogênio, que modulam as respostas imunológicas (44–46). As curvas de prevalência por idade possuem uma distribuição semelhante à dos dados de incidência (36).

É bem conhecido que existem diferenças nas variantes de risco de doenças autoimunes em diferentes populações. A prevalência de LES varia substancialmente por ancestralidade étnica. Nos estudos que relatam diferenças entre grupos étnicos (30,47–49), as taxas de incidência são 3 a 5 vezes maior de LES nos afro-americanos comparado aos indivíduos com ascendência europeia (50–52). Os grupos étnicos asiáticos e hispânicos possuem taxas de incidências intermediárias entre os grupos étnicos citados anteriormente (53–55). Semelhante aos dados de incidência, os grupos étnicos negros possuem maior prevalência de LES, os grupos brancos, menor prevalência, e os asiáticos e hispânicos, intermediários, tanto para homens quanto para mulheres (36).

A sobrevida nos pacientes com LES tem melhorado muito nos últimos anos. Na década de 50, a sobrevida média em cinco anos era de 50% (56). De 1975 a 1990, a sobrevida em 10 anos aumentou de 64 para 87%. Ainda mais melhorias ocorreram recentemente de 1990 a 2004, quando a sobrevida de 20 anos aumentou para 78% (57). Nos dias atuais, taxas de sobrevida de 5, 10 e 15 anos foram relatadas como estando na faixa de 96, 93 e 76%, respectivamente (58–60). Vários fatores contribuíram para isso, principalmente melhoria da conscientização e classificação da doença, levando a um diagnóstico precoce, o melhor entendimento

da sua fisiopatologia e as melhores condições de tratamento, bem como melhorias no tratamento da hipertensão, infecção e insuficiência renal, algumas das principais comorbidades que esses pacientes apresentam (59,61).

As doenças cardiovasculares (DCV), as infecções e a doença ativa são as principais causas de morte no LES. Os pacientes com LES apresentam taxas de mortalidade duas a cinco vezes maiores que a população geral (62). Um padrão bimodal de mortalidade tem sido reconhecido no LES desde meados da década de 1970 (63), nos primeiros anos da doença, as principais causas de morte são infecções graves devido à imunossupressão ou morte por complicações da doença ativa, como nefrite lúpica e lúpus neuropsiquiátrico; enquanto as causas de morte tardias incluem danos há longo prazo do LES, complicações do tratamento e as DCV (57,64). Lee e colaboradores realizaram um estudo de meta-análise e examinaram as taxas de mortalidade padrão no LES. O risco de mortalidade foi significativamente maior para doença renal, DCV e infecção, mas não devido a câncer (65). A doença renal ocorre em até 60% dos pacientes com LES e continua sendo uma causa predominante de morbidade e mortalidade no LES (66).

No Brasil, um estudo publicado em 2017, caracterizou as causas de mortalidade no lúpus de 2002-2011. A taxa de mortalidade no LES no Brasil foi de 4,76 mortes para cada 105 habitantes, sendo maior nas regiões Centro-Oeste, Norte e Sudeste do que no país como um todo. Os distúrbios do sistema musculoesquelético e do tecido conjuntivo foram mencionados como a causa subjacente de morte em 77,5% dos casos; também foram observadas doenças do sistema circulatório e infecciosas e parasitárias, embora em menor frequência (67).

2.2.3 Etiologia e patogênese do LES

A etiologia e a patogênese do LES são complexas e permanecem ainda pouco conhecidas, mas envolvem múltiplos fatores, especialmente genéticos, imunológicos, hormonais e ambientais (68). O dano tecidual e o acometimento de órgãos e sistemas podem estar relacionados à presença de autoanticorpos e IC. No LES, observa-se ativação da imunidade inata e adaptativa, com a participação de receptores do tipo Toll (do inglês *Toll-like receptors* – TLR) 7 e 9 no reconhecimento de autoantígenos derivados do RNA e DNA, respectivamente, provenientes de

células apoptóticas, e o envolvimento de células B e T ativadas a partir da interação com estes autoantígenos (69,70). A resposta imune anormal que permite a persistência de células B e T patogênicas, também aumenta o processamento de autoantígenos pelas células apresentadoras de antígenos, ocasiona hiperativação linfocitária e determina falha nos mecanismos imunorregulatórios que poderiam interromper este processo.

A predisposição ao LES tem sido relacionada à herança de determinados genes e não segue um modelo monoalélico. Estudos demonstram que gêmeos monozigóticos possuem um risco 10 vezes maior de desenvolver LES do que gêmeos dizigóticos ou irmãos não gêmeos, e em estudos observacionais a presença de múltiplos casos de LES nas famílias (71–74). Aproximadamente, de 5 a 12% dos familiares de pacientes com LES poderão ter a doença, o que representa um risco 100 vezes maior do que a população geral (75). Deficiências geneticamente herdadas de componentes do complemento (C1q, C4A e B e C2) e a mutação do gene que sintetiza a exonuclease 3'-5' envolvida no metabolismo e depuração do DNA (do inglês *Three-prime repair exonuclease* - TREX1), apesar de raramente encontradas, são fatores genéticos fortemente associados com o desenvolvimento do LES (76–79). O encontro de outras doenças autoimunes em familiares de pacientes com LES e sua associação com alguns distúrbios geneticamente determinados, também reforçam a importância da predisposição genética para o surgimento da doença. A combinação de fatores genéticos, tanto a presença de genes de suscetibilidade, quanto à ausência de genes de proteção, determina risco suficiente para o desencadeamento da doença. Estes dados, juntamente com os achados em estudos de associação global do genoma humano (do inglês *Genome-wide association study* - GWAS), implica um forte *background* genético para o LES (80,81).

Os GWAS têm sido bem sucedidos na identificação de novos *loci* que contribuem para a suscetibilidade genética do LES. A contribuição isolada de cada um deles no desenvolvimento da doença é pequena, geralmente com risco inferior a 2 ou 3 vezes (82). Através destes trabalhos, começou-se a elucidar a complexa arquitetura genética do LES, e coletivamente, esses estudos identificaram e confirmaram aproximadamente 90 *loci* que contribuem para esta patogênese. Esses dados destacam a importância de várias vias imunológicas incluindo aquelas que

envolvem a ativação e função dos linfócitos, a depuração do complexo imunológico, a resposta imune inata e as resposta imune adaptativa (68,83). Dentre os alelos do complexo maior de histocompatibilidade (do inglês *Major histocompatibility complex* - MHC), sabe-se que a região do antígeno leucocitário humano (do inglês *Human leukocyte antigen* - HLA), mais especificamente DR2, DR3 e DR8, contribuem para o risco de LES e outras doenças autoimunes desde a década de 1970 (84–87). Polimorfismos genéticos de suscetibilidade podem influenciar a depuração de IC e células apoptóticas através da alteração de componentes do sistema complemento, como os descritos anteriormente (C1q, C4A e B e C2) e também outros, tais como a lectina ligadora da manose (do inglês *Mannose-binding lectin* - MBL), receptores Fc gama IIA (do inglês *Fc gamma receptor IIA* - FcγRIIA), IIIA (do inglês *Fc gamma receptor IIIA* - FcγRIIIA), IIIB (do inglês *Fc gamma receptor IIIB* - FcγRIIIB), proteína C-reativa (PCR) e integrina alfa M (do inglês *Integrin alpha M* - ITGAM) (81,88–91). Há variantes alélicas encontradas nos genes PTPN22 (do inglês *Protein tyrosine phosphatase non-receptor 22*), OX40L (OX40 ligante, também conhecida como glicoproteína 34 kDa), PDCD1 (do inglês *Programmed cell death 1*), BANK1 (do inglês *B cell scaffold protein with ankyrin repeats*), LYN (do inglês *Src family of protein tyrosine kinase*) e BLK (do inglês *B lymphoid kinase* – cinase dos linfócitos B) que afetam a sinalização de linfócitos, resultando em mudanças na ativação, supressão e sobrevida das células B e T (92–98). Há estudos com polimorfismos cuja influência se dá através de alterações envolvendo a imunidade inata, dentre eles, os que são encontrados nos genes IRF5 (do inglês *Interferon regulatory factor 5*), STAT4 (do inglês *Signal transducer and activator of transcription 4*), IRAK1 (do inglês *Interleukin 1 receptor associated kinase 1*) e SPP1 (do inglês *Secreted phosphoprotein 1*), os quais estão associados com níveis elevados de IFN-α, uma importante citocina envolvida na fisiopatogenia do LES (99–101). O alelo B do polimorfismo Bsm1 do gene *VDR* mostrou significante associação com LES em pacientes asiáticos (102–104). Todos estes marcadores genéticos são encontrados de maneira muito variada em diferentes populações, especialmente os que se relacionam com o MHC, sendo alguns mais ou menos frequentes, de acordo com a etnia. A influência genética destes fatores potencialmente pode alterar a regulação imune, a degradação de proteínas, o transporte de peptídeos através da membrana celular, a cascata do complemento, o funcionamento do sistema reticuloendotelial, a

produção de imunoglobulinas, a apoptose e a produção e liberação de hormônios (105).

Os hormônios têm função imunorregulatória e alterações em suas concentrações podem ter papel na patogênese do LES, influenciando na incidência e no curso clínico da doença (44). O estrogênio estimula timócitos, linfócitos T CD8+ e CD4+, linfócitos B, macrófagos, a liberação de citocinas, a expressão de moléculas do HLA e moléculas de adesão endotelial, além de reduzir a apoptose de linfócitos B ativados (106,107). Os androgênios evidenciam efeito imunossupressor (108). O desequilíbrio entre os níveis de estrogênio e androgênio parece influenciar a resposta imune. Em uma meta-análise de estudos clínicos, observaram-se níveis mais baixos de testosterona e dehidroepiandrosterona (DHEA) e níveis mais altos de estradiol e prolactina em mulheres com LES (109). Isto justificaria, em parte, o maior número de casos de LES em mulheres na idade reprodutiva e em indivíduos com síndrome de Klinefelter (47,XXY) e a menor prevalência da doença em pacientes com síndrome de Turner (45,X) (110,111). Estas duas síndromes suscitam a possibilidade de o risco para desenvolver LES estar relacionado à existência de dose genética cumulativa, visto que há pelo menos três genes predisponentes ao LES localizados no cromossomo X (IRAK1, TLR7 e MECP2 – do inglês *Methyl CpG binding protein 2*) (112). Alguns estudos mostraram associação entre o risco de desenvolver LES com a menarca precoce, o uso exógeno de hormônios (incluindo contraceptivos orais e terapia de reposição hormonal) e a menopausa cirúrgica (113,114). Um estudo de coorte prospectivo com mulheres do *Nurse's Health Study* demonstrou que uma maior exposição estrogênica, decorrente de menarca precoce ou do uso de estrogênio exógeno através de contraceptivos orais ou terapia de reposição hormonal na pós-menopausa, aumentou em 1,5 a 2,5 vezes o risco do desenvolvimento do LES (113). Em contraste, a progesterona parece reduzir o risco de LES ao contrabalançar os efeitos do estrogênio, o que sugere que o equilíbrio entre o estrogênio e a progesterona pode determinar a expressão da doença (115). A progesterona diminui a proliferação de células T e aumenta o número de células CD8+ (116). A prolactina encontra-se elevada em pacientes com LES de ambos os sexos (117). Os níveis de prolactina estão correlacionados diretamente com a atividade clínica e sorológica da doença (118). Ocorre também aumento de doença tireoidiana autoimune em pacientes com LES, o que altera os níveis dos hormônios

tireoidianos e do hormônio tireoestimulante (119). A vitamina D apresenta efeitos imunorregulatórios em diversas células do sistema imune, especialmente linfócitos T, linfócitos B e células dendríticas (120,121). Alguns estudos têm demonstrado associação entre baixos níveis de vitamina D e LES, sugerindo sua participação na etiopatogenia desta doença (122).

Anormalidades na regulação do sistema imune, incluindo quebra inicial da autotolerância, reconhecimento de autoantígenos, ativação de linfócitos T e B, secreção de citocinas, proliferação e diferenciação de linfócitos B e produção de autoanticorpos, são características de pacientes com LES (123). Defeitos na morte celular, nos mecanismos de fagocitose e depuração ineficaz de material apoptótico, restos celulares e de IC são achados que colaboram para perpetuação da autoimunidade no LES (124).

Plasmócitos produtores de autoanticorpos estão persistentemente sendo ativados por uma molécula chamada de fator de ativação de células B (do inglês *B-cell activating fator* – BAFF), também conhecida como BLys (do inglês *B lymphocyte stimulator* – estimulador de linfócitos B) ou CD257, no qual encontra-se elevada em pacientes com LES (125,126). Outras anormalidades também podem ser destacadas, dentre elas: a diminuição do número dos linfócitos T supressores e citotóxicos (127); deficiência qualitativa e quantitativa de células T regulatórias (128); aumento dos linfócitos T auxiliares foliculares (do inglês *Follicular helper T cells* - Tfh) e linfócitos T auxiliares (do inglês *T helper* - Th) CD4+ (129,130); ativação policlonal de linfócitos B e defeitos na tolerância destes linfócitos (131); aumento do microquimerismo fetal, provendo antígenos para o sistema imune (132); elevação dos níveis circulantes de IFN-α e aumento da expressão da transcrição do RNA indutor de IFN-α pelas células mononucleares (133,134). A sinalização anormal dos TLR7 e 9, levando ao reconhecimento de autoantígenos contendo RNA e DNA, respectivamente, aliada ao aumento de células B de memória e plasmócitos expressando TLR9, apontam uma possível ativação de linfócitos B diretamente através do sistema imune inato, independente de células T, o que tem sido relacionado com predisposição e modulação do quadro clínico de pacientes com LES (135,136).

Vários fatores ambientais têm sido associados ao desenvolvimento do LES. Mecanismos que ligam exposições ambientais e LES incluem modificações epigenéticas, aumento do estresse oxidativo, da inflamação sistêmica, das citocinas inflamatórias e efeitos hormonais (137). O *National Institute of Environmental Health Science Expert Panel* revisou e avaliou a influência de fatores ambientais sobre a autoimunidade e associou um aumento no desenvolvimento do LES com a exposição à sílica e o tabagismo (138). Infecções causadas por vírus, dentre eles o vírus Epstein-Barr (do inglês *Epstein–Barr vírus* – EBV), poderiam contribuir para o desenvolvimento de autoimunidade, principalmente por mimetismo molecular (139). Outros vírus, como Citomegalovírus (CMV), Parvovírus B19 e alguns retrovírus, também estão relacionados (140). Radiação ultravioleta B (UVB) pode estimular os queratinócitos a expressarem antígenos nucleares em sua superfície e aumentar a secreção de citocinas que estimulam linfócitos B à produção de autoanticorpos (141). Medicações, como procainamida, hidralazina e isoniazida podem desencadear lúpus induzido por droga, que é caracterizado pelo surgimento de manifestações clínicas e laboratoriais após exposição a estes fármacos, seguido por desaparecimento do quadro com a suspensão dos mesmos (Figura 1).

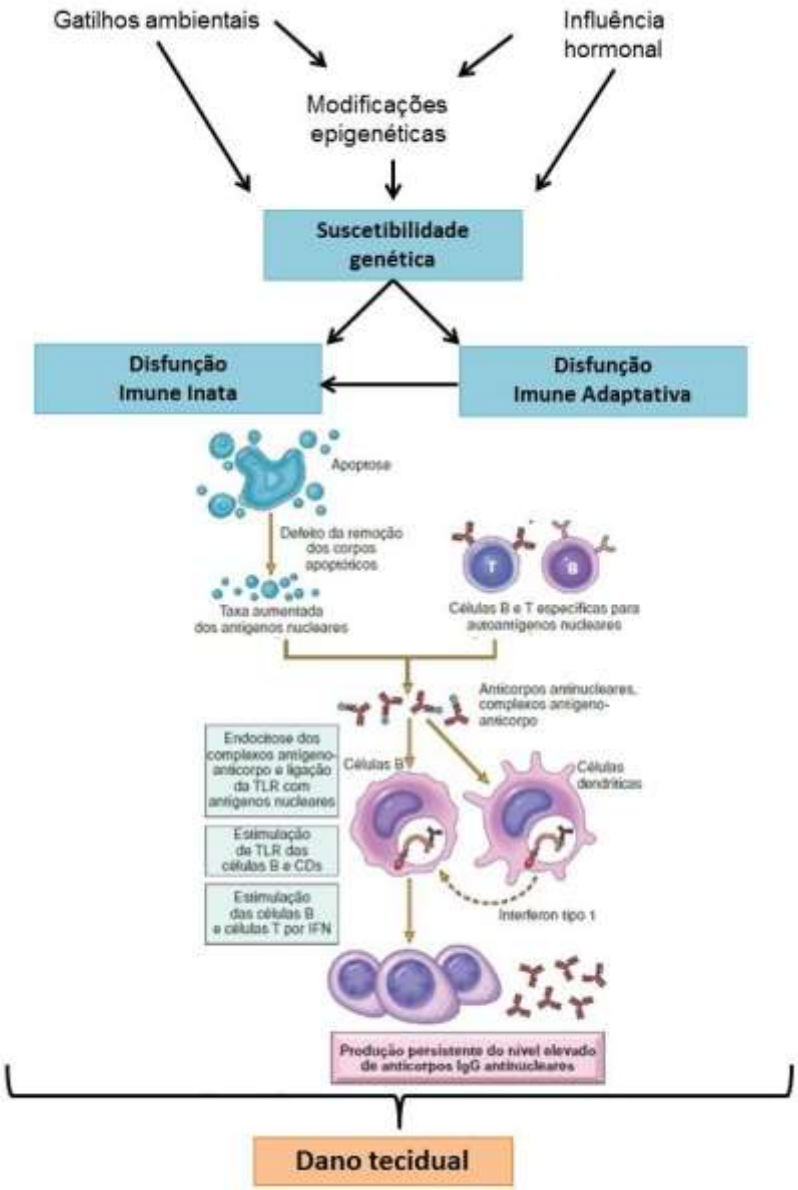

Figura 2. Resumo do envolvimento de múltiplos fatores, especialmente genéticos, imunológicos, hormonais e ambientais, presentes na etiologia e a patogênese do LES. Fonte: Robbins Patologia Básica (9ª Edição), 2013.

2.3 MODELOS EXPERIMENTAIS DE LÚPUS

O alto grau de complexidade e heterogeneidade da apresentação clínica no LES, aliada às limitações inerentes à pesquisa clínica, dificultam a investigação da etiologia dessa doença diretamente nos pacientes. Os modelos animais tem sido valiosos para investigar os mecanismos celulares e genéticos do LES, bem como na identificação e validação de alvos terapêuticos. Desta forma, os modelos experimentais de LES têm contribuído para entender os mecanismos celulares, de

sinalização e metabólicos que contribuem para a doença, e como o direcionamento dessas vias pode fornecer alvos terapêuticos (142).

Os modelos experimentais de LES podem ser divididos basicamente em espontâneo e induzido. Cada modelo compartilha subconjuntos específicos de atributos da doença observada em humanos, o que fornece aos pesquisadores uma ferramenta para adequá-los às suas necessidades específicas. No entanto, nenhum deles representa completamente todo o espectro clínico encontrado em pacientes com LES (Tabela 1) (143).

Tabela 1. Modelos animais de LES.

Modelo animal	Assinatura de IFN	Anti-DNA	Anti-Sm/RNP	Manifestações Clínicas	Critérios de LES*
NZB/W F1	Fraca	Sim	Não	ANAs, NL grave	3
MRL/*lpr*	Ausente	Sim	Sim	ANAs, NL grave, artrite, erupções cutâneas	6
B6/*lpr*	Ausente	Não	Não	ANAs	1
BXSB male	N/A	Sim	Não	ANAs, NL grave	3
Pristane	Forte	Sim	Sim	ANAs, NL, artrite, HAD, anemia, serosite	8

N/A: Não disponível; ANAs: anticorpos antinucleares; NL: nefrite lúpica; HAD: hemorragia alveolar difusa.
*Número de critérios do SLICC para classificação do LES (em humanos são necessários quatro critérios para definir o lúpus com 95% de certeza, pelo menos um critério clínico e um laboratorial). Fonte: Tabela adaptada de Zhuang e colaboradores, 2015 (143).

2.3.1 Modelo de lúpus induzido por pristane

O pristane é um alcano isoprenoide encontrado em alta concentração no óleo mineral. Uma injeção intraperitoneal de pristane é um método padrão para obtenção de um fluido ascítico enriquecido em anticorpos monoclonais. Dentre os anticorpos produzidos no modelo de lúpus induzido por pristane em BALB/c podemos encontrar anti-ribonucleoproteína, anti-DNA e anti-histona. Os animais submetidos à injeção de pristane também apresentam deposição de IC no rim, causando uma glomerulonefrite (144).

O modelo de lúpus induzido por pristane também é impulsionado por uma forte resposta do IFN-I, e este modelo é bem adequado para investigar a assinatura

de IFN-I presente em muitos pacientes com LES, mas mais fraca ou ausente em outros modelos animais (143). Este modelo também é útil para testar o impacto de um gene específico no desenvolvimento do lúpus.

Assim como observado no LES em humanos, o modelo de lúpus induzido por pristane é mais grave nas fêmeas do que nos machos (145). Ainda, dependendo da linhagem de camundongo utilizado na indução do modelo de lúpus induzido por pristane são observadas diferentes manifestações clínicas, o que ilustra o papel das interações gene/ambiente na suscetibilidade da doença (146). Uma explanação mais aprofundada deste modelo experimental esta descrita no Artigo 1 apresentado neste trabalho.

2.4 VITAMINA D

2.4.1 Considerações gerais

As vitaminas são compostos orgânicos essenciais para o metabolismo dos seres vivos. A vitamina D compreende compostos lipossolúveis, e existe em duas isoformas principais, de origem vegetal (vitamina D2 ou ergosterol) ou animal (vitamina D3 ou colecalciferol), responsáveis principalmente pela manutenção do equilíbrio do metabolismo ósseo (147,148). Ambas as vitaminas D2 e D3 são metabolizadas pela mesma via e produzem metabólitos ativos com efeitos biológicos equivalentes (Figura 2) (149). A vitamina D pode ser encontrada em alguns alimentos e suplementos alimentares, porém sua maior fonte é proveniente da síntese cutânea, a partir da adequada exposição à radiação UVB da luz solar. Apenas cerca de 20% das necessidades corporais diárias são supridas pela alimentação, fato que a torna diferente da maioria das demais vitaminas que geralmente precisam ser adquiridas através da dieta (150). Poucos alimentos contêm ou são enriquecidos, naturalmente, com vitamina D, dentre eles as principais fontes de vitamina D3 são o óleo de fígado de peixe, peixes gordurosos (atum e salmão) e a gema do ovo, enquanto que a vitamina D2 é derivada da ingestão de fontes de fungos, como cogumelos e leveduras. A vitamina D2 é uma molécula com 28 carbonos e pode ser manufaturada através da irradiação UVB do ergosterol encontrado em fungos (151). A vitamina D3 apresenta 27 carbonos e pode ser obtida através da irradiação UVB do 7-deidrocolesterol (7-DHC) proveniente da lanolina (152). Diante destas particularidades envolvendo a biodisponibilidade da

vitamina D nos seres humanos, entende-se o crescente interesse em pesquisas envolvendo este esteroide, visto que estudos em diferentes populações têm mostrado uma alta prevalência de hipovitaminose D e a implicação clínica deste achado ainda continua pouco entendida (153).

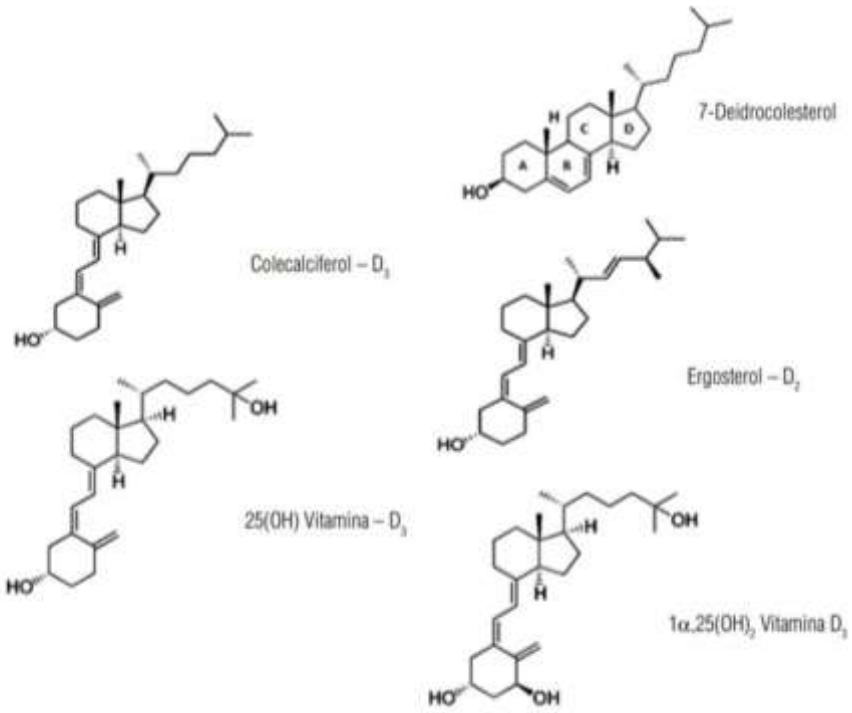

Figura 3. Estrutura química dos precursores e metabólitos da vitamina D. 7-deidrocolesterol (pró-vitamina D3); colecalciferol (Vitamina D3); ergosterol (Vitamina D2); 25-hidroxivitamina D [25(OH)D ou calcidiol]; 1α,25-diidroxivitamina D [1α,25(OH)2D ou calcitriol]. Fonte: de Castro LCG, 2011 (149).

A vitamina D tem um importante papel no metabolismo ósseo, incluindo absorção de cálcio e fósforo. A descoberta de que a maioria das células expressa o VDR, e algumas possuem também maquinaria enzimática para produzir formas ativas da vitamina D, gerou grande interesse nos seus potenciais efeitos biológicos, visto que há evidências da influência desta vitamina na patogenia de doenças autoimunes, neoplásicas, osteometabólicas, infecciosas, cardiovasculares e metabólicas (153). A mais alta expressão do gene *VDR* é encontrada em tecidos metabólicos, como intestino, rins e ossos, mas níveis baixos a moderados do VDR podem ser observados em mais da metade dos cerca de 400 tecidos e tipos de células que formam o corpo humano (154). Estudos sugerem que a vitamina D

apresenta efeitos regulatórios em aproximadamente 900 genes (155). A identificação do VDR nas células responsáveis pela resposta imune, como as células mononucleares, dendríticas e apresentadoras de antígeno, e também nos linfócitos B e T, sugere que a vitamina D possa exercer atividade imunorregulatória (121,156). No sistema imune, a vitamina D promove diferenciação e regulação de monócitos, linfócitos e células exterminadoras naturais (do inglês, *natural killer* - NK), além de interferir na secreção de quase todas as citocinas envolvidas no sistema imune. Entre os efeitos imunomoduladores, destacam-se a diminuição da produção de IL-2, do IFN-γ e TNF-α, inibição da expressão de IL-6 e inibição da secreção e produção de autoanticorpos pelos linfócitos B (153,157).

Há grandes diferenças interpessoais quanto aos efeitos da vitamina D no organismo e, parte desta variação, deve-se a alterações nas sequências de DNA que sintetizam importantes proteínas envolvidas no metabolismo e na ação da vitamina D nas células-alvo. Um exemplo disto seria a mutação deletéria no gene *VDR* que causa raquitismo por resistência a 1,25$(OH)_2$D. Outras variações genéticas mais sutis envolvem a presença de alguns polimorfismos no gene *VDR*, porém com consequências funcionais ainda pouco conhecidas (158,159).

2.4.2 Metabolismo da vitamina D

Nos seres humanos, a pele é o único sítio capaz de produzir vitamina D. A pró-vitamina D (7-DHC) é produzida na derme e epiderme e, sob ação da radiação UVB com comprimento de onda entre 280 e 315 nm, sofre conjugação de pontes de hidrogênio nos carbonos C5 e C7, dando origem a pré-vitamina D. Esta, após aproximadamente 24 horas, forma homodímeros, transformando-se em vitamina D (160,161).

A vitamina D proveniente da dieta é absorvida no intestino delgado e, juntamente com a vitamina D endógena, transportada até o fígado para ser metabolizada. O transporte ocorre principalmente através da proteína ligadora da vitamina D (do inglês *vitamin D-binding protein* - VDBP) e, em menor proporção, com a albumina (162). A enzima hepática 25-hidroxilase (CYP2R1 – do inglês *Cytochrome P450 family 2 subfamily R member 1*) incorpora um radical hidroxila na posição 25 da molécula da vitamina D, originando a forma 25-hidroxivitamina D

(25(OH)D), também conhecida como calcidiol (163). Por manter níveis séricos mais estáveis, é a forma usada para dosar a vitamina D do paciente. O fígado é o reservatório usual da vitamina D. O tecido adiposo também pode atuar como reservatório. Além do fígado, outros tecidos, como pele, intestino e rins são capazes de promover a 25-hidroxilação da vitamina D, porém em menor proporção (159). A 25(OH)D circula ligada à VDBP até o rim, onde sofre nova hidroxilação, resultado da atividade das enzimas 1α-hidroxilase (CYP27B1 – do inglês *Cytochrome P450 family 27 subfamily B member 1*) ou 24-hidroxilase (CYP24A1 – do inglês *Cytochrome P450 family 24 subfamily A member 1*) (164). Os produtos são, respectivamente, a $1,25(OH)_2D$ (calcitriol), a forma mais ativa e a 24,25-dihidroxivitamina D ($24,25(OH)_2D$), um metabólito inativo hidrossolúvel, conhecido também como ácido calcitroico que é excretado na bile. A concentração plasmática da $1,25(OH)_2D$ é regulada a partir dos níveis de 25(OH)D e da atividade das enzimas 1α-hidroxilase e 24-hidroxilase. A enzima 1α-hidroxilase é regulada pelo paratormônio (PTH), pela concentração de fósforo e pelos níveis séricos de $1,25(OH)_2D$ (165). O aumento do PTH estimulam a enzima a sintetizar $1,25(OH)_2D$, enquanto a $1,25(OH)_2D$ exerce estímulo negativo sobre esta enzima (165,166). Ambas, $1,25(OH)_2D$ e 25(OH)D são degradadas, em parte, pela enzima 24-hidroxilase, através da síntese do ácido calcitroico (2). Os níveis séricos da $1,25(OH)_2D$, ao contrário da 25(OH)D, são fortemente controlados por mecanismos de retroalimentação, com níveis séricos bastante variados e meia-vida de cerca de 6 horas (162). A 1α-hidroxilase também esta presente nos tecidos extrarrenal (pulmão, próstata, cérebro, placenta) e nas células do sistema imune, o que torna possível a produção local da forma ativa da vitamina D, que passa a ter efeito parácrino e autócrino (167). É importante salientar que a 1α-hidroxilase extrarrenal é regulada diferentemente da resposta vista ao PTH, cálcio e fósforo séricos (168). Particularmente, esta 1α-hidroxilase não é estimulada pelo PTH e, consequentemente, a produção de $1,25(OH)_2D$ passa a depender das concentrações do substrato, ou seja, a 25(OH)D, o que pode determinar maior impacto na função da vitamina D nestes locais em casos de hipovitaminose D (14,167). Por outro lado, alguns estudos sugerem um mecanismo de autorregulação negativo exercido pela $1,25(OH)_2D$ sobre a síntese do seu precursor via inibição da 25-hidroxilase (169,170).

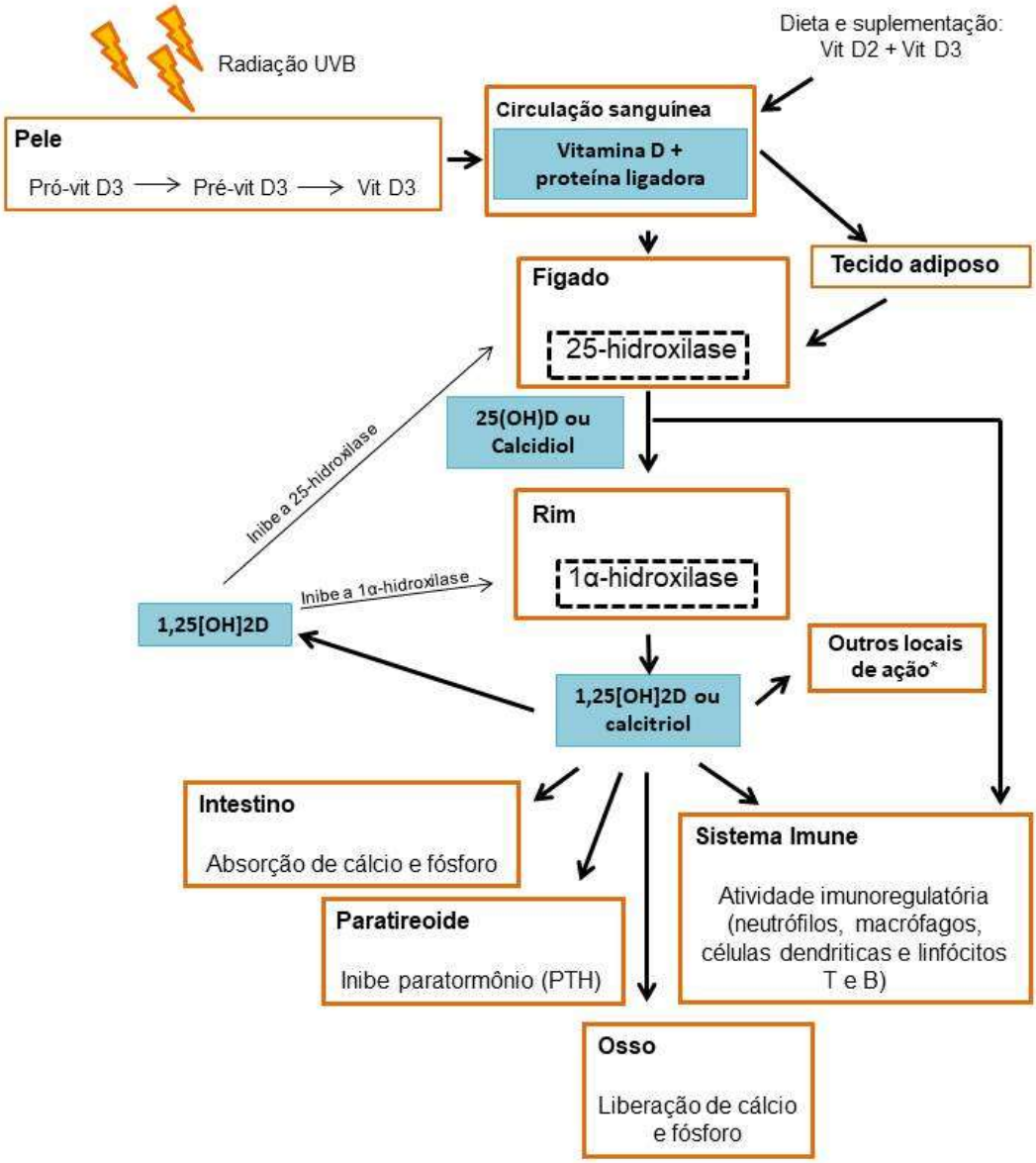

Figura 4. Metabolismo da vitamina D e principais efeitos biológicos. * Trato reprodutivo (útero, ovário, placenta, testículos e próstata), mamas, ilhotas pancreáticas, hipófise, tireóide, córtex adrenal, musculatura lisa e esquelética, coração, pele, cérebro.

2.4.3 Níveis séricos e deficiência de vitamina D

A vitamina D pode ser quantificada a partir da verificação da concentração da 25(OH)D, que representa sua forma circulante em maior quantidade, com meia-vida de aproximadamente três semanas. A $1,25(OH)_2D$ não costuma ser usada para avaliação da concentração da vitamina D, devido a sua curta meia-vida e sua baixa

concentração, cerca de 1000 vezes inferior à da 25(OH)D (171). Além disso, no caso de deficiência de vitamina D, existe um aumento compensatório na secreção do PTH, o que estimula o rim a aumentar a produção de $1,25(OH)_2D$. Deste modo, quando ocorre deficiência de vitamina D e queda nos níveis de 25(OH)D, a concentração sérica de $1,25(OH)_2D$ se mantém dentro dos níveis normais ou mesmo elevados (172).

Não existe consenso sobre a concentração sérica da 25(OH)D. Há uma concordância de que os níveis séricos de vitamina D sejam mantidos dentro de uma faixa que não induza o aumento do PTH (150,173). A deficiência de vitamina D é definida pela maioria dos autores como níveis séricos de 25(OH)D inferiores a 50 nmol/l (20 ng/ml) (174–177). Níveis séricos entre 50 e 75 nmol/l (20 e 30 ng/ml) seriam indicativos de insuficiência relativa de 25(OH)D e níveis iguais ou maiores que 75 nmol/l (30 ng/ml) poderiam ser considerados como estado suficiente de vitamina D (162,177). Valores abaixo de 25 nmol/l (10 ng/ml) indicariam níveis criticamente baixos (178,179). Intoxicação associada com hipervitaminose D poderia ser vista com níveis de 25(OH)D superiores a que 374 nmol/l (150 ng/ml) (180).

As concentrações plasmáticas ideais de 25(OH)D foram definidas, como visto anteriormente, a partir da sua influência sobre os níveis séricos do PTH, o que as tornam clinicamente relevantes para a manutenção da homeostase do cálcio. Entretanto, as concentrações ideais de 25(OH)D necessárias para o bom funcionamento do sistema imunológico ainda não foram definidas e podem ser distintas daqueles valores que atualmente são conhecidos (181). Além disso, estudos demonstram que alguns polimorfismos genéticos envolvendo a VDBP, enzimas envolvidas no seu metabolismo, e o próprio VDR podem determinar variações nos níveis séricos da 25(OH)D e tornam ainda mais complexa a definição da concentração ideal de vitamina D, a qual pode ser diferente para cada indivíduo (182). Há inúmeras causas para haver deficiência de vitamina D, dentre elas, redução da síntese cutânea e da absorção intestinal, além de doenças herdadas ou adquiridas do seu metabolismo ou que interfiram na responsividade à vitamina D (166).

2.4.4 Receptor de vitamina D

O VDR é um receptor nuclear de 50 kDa (183), pertencente à família dos receptores esteroides de classe 2, semelhante aos receptores do ácido retinoico e do hormônio tireoestimulante. Seu gene de 75kb esta situado no braço longo do cromossomo 12 (159). A estrutura de VDR consiste em um domínio N-terminal, para ligação com o DNA, uma região de charneira flexível e um domínio de ligação com o ligante, $1,25(OH)_2D$ (183). Além do clássico VDR nuclear, acredita-se que exista um VDR de membrana responsável por ações rápidas da vitamina D (184).

A $1,25(OH)_2D$ liga-se ao VDR nuclear e determina uma resposta genômica através da regulação da transcrição de alguns genes. As principais etapas envolvidas no controle da transcrição genética incluem a ligação da $1,25(OH)_2D$ ao VDR, heterodimerização com o RXR, ligação deste heterodímero a sequências específicas do DNA, também conhecidas como VDRE localizados no DNA, nas regiões promotoras dos genes que são ativados pela vitamina D e recrutamento de outras proteínas nucleares para dentro do complexo transcricional (figura 5) (3,183). Acredita-se que a $1,25(OH)_2D$ possa exercer ações genômicas e não genômicas rápidas interagindo com o VDR. As atividades não genômicas rápidas são mediadas pelo VDR de membrana que também é ativado por $1,25(OH)_2D$, o que resulta na abertura dos canais de cálcio dependentes de voltagem ou na ativação de segundos mensageiros, como por exemplo a proteína quinase C (185).

O VDR foi descoberto inicialmente nos tecidos envolvidos na regulação da homeostase do cálcio e fosfato (intestino, ossos, rins e paratireoides) (186). Além dos tecidos clássicos, o VDR também está presente em outros locais, tais como células do sistema imune, trato reprodutivo (útero, ovário, placenta, testículo e próstata), mamas, sistema endocrinológico (ilhotas pancreáticas, hipófise, tireoide, paratireoide e córtex adrenal), musculatura lisa e esquelética, coração, pele, cérebro e fígado (187). Além da distribuição quase universal do VDR, algumas células (queratinócitos, monócitos, ósseas e placentárias) expressam a enzima 1α-hidroxilase que produz a forma ativa da vitamina D *in situ*, podendo ocorrer efeito parácrino e autócrino deste hormônio (2,5).

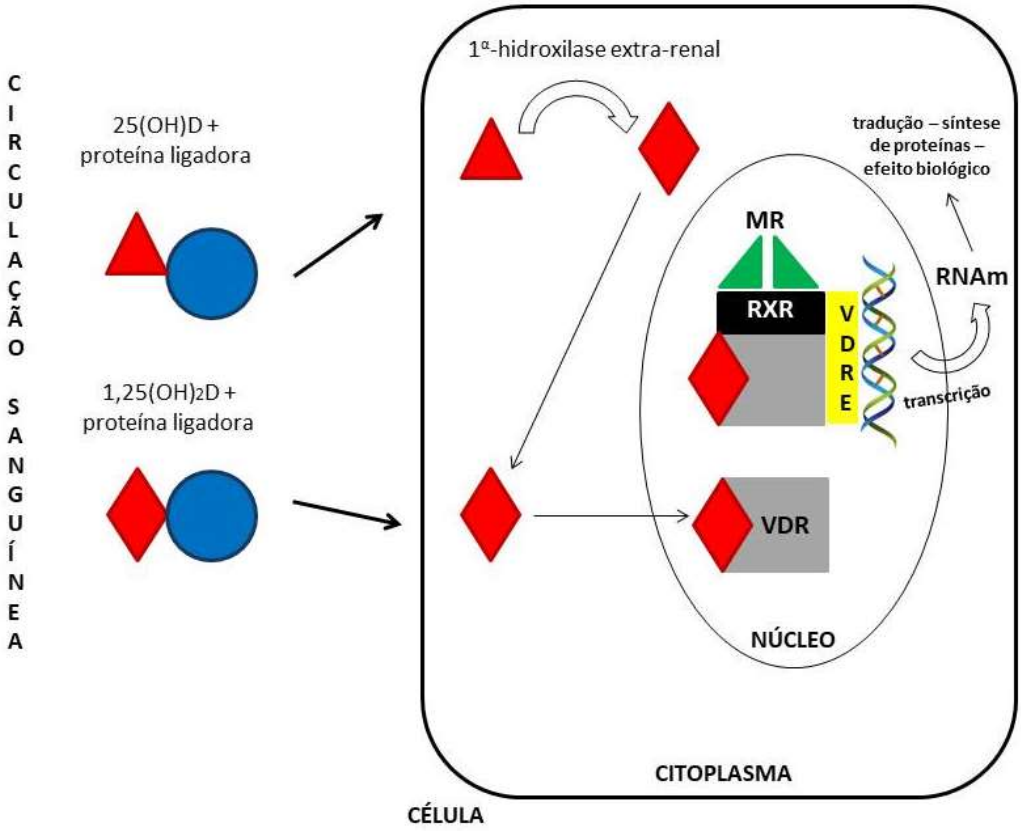

Figura 5. Mecanismo de ação da vitamina D. Abreviações: MR (molécula reguladora); mRNA (ácido ribonucleico mensageiro); RXR (receptor X retinóico); VDR (receptor da vitamina D); VDRE (elementos de resposta a vitamina D).

2.4.5 Efeitos fisiológicos da vitamina D

A função clássica da $1,25(OH)_2D$ relaciona-se com a regulação da homeostase do cálcio. No duodeno, estimula o transporte ativo de cálcio para a corrente circulatória. A absorção de fósforo também é aumentada sob ação deste hormônio. Há participação na manutenção da massa óssea, permitindo a mineralização normal do osso e atuando na maturação do colágeno e da matriz (188). Atua de forma sinérgica com o PTH na ativação e maturação de osteoclastos, resultando na mobilização do cálcio do osso para a circulação. Nos rins, é responsável por incrementar a reabsorção de cálcio e fósforo. Além dos efeitos no metabolismo do cálcio, a $1,25(OH)_2D$ está envolvida na regulação do magnésio, liberação de insulina pelo pâncreas, secreção de prolactina pela hipófise, inibição da síntese de renina, aumento da contratilidade miocárdica, manutenção da musculatura esquelética e depuração de creatinina endógena (6–10).

A 1,25$(OH)_2$D interfere de forma direta ou indireta no controle de mais de 200 genes envolvidos na regulação do ciclo celular, diferenciação, apoptose e angiogênese, podendo determinar diminuição da proliferação de células normais ou neoplásicas (189). Estudos com animais evidenciaram que a 1,25$(OH)_2$D pode suprimir o crescimento tumoral por indução da apoptose, inibição da angiogênese e redução da atividade invasiva das células cancerígenas (190). Na pele, atua de forma parácrina, inibindo a proliferação de queratinócitos e fibroblastos (162).

Vários efeitos imunomodulatórios também têm sido atribuídos a 1,25$(OH)_2$D. O sistema imune inato atua como primeira barreira na defesa contra invasão de micro-organismos e a presença de alguns peptídeos antimicrobianos tem sido um fator importante neste contexto. Em humanos, pode ser encontrada a catelicidina, um peptídeo antimicrobiano produzido por macrófagos, monócitos e queratinócitos, com grande atividade contra bactérias, micobactérias, vírus e fungos (191,192). A vitamina D ativada parece estimular a produção de catelicidina por estas células (193). Monócitos e macrófagos expostos a lipopolissacarídeos bacterianos ou à presença do *Mycobacterium tuberculosis* ativam o gene *VDR* e 1α-hidroxilase, levando ao aumento local da 1,25$(OH)_2$D e do seu receptor, o que eleva a produção de catelicidina. Esta resposta frente aos micro-organismos parece ser atenuada em indivíduos que apresentam deficiência de 25(OH)D (194,195).

Como descrito anteriormente, a vitamina D tem efeitos conhecidos sobre diversas células do sistema imune, dentre elas linfócitos T CD4+ e CD8+, linfócitos B, células dendríticas e macrófagos (181). Cada uma destas células expressa VDR e 1α-hidroxilase, podendo produzir 1,25$(OH)_2$D localmente. Seus efeitos parácrinos e autócrinos dependem da adequada concentração da 25(OH)D, o que faz da deficiência de vitamina D um fator crucial no funcionamento do sistema imune (14). Nos linfócitos podem ser verificadas as seguintes alterações relacionadas à vitamina D: supressão do receptor de célula T, alteração no perfil de citocinas (diminuição de IFN-γ, IL-22, IL-2 e TNF-α e aumento de TGF-β, IL-4, IL-5 e IL-10) e troca do fenótipo Th1 para Th2, com maior tolerância imunológica; supressão das células Th17 envolvidas na autoimunidade e redução da produção de IL-17; supressão da produção de IL-6 e IL-12 e IL-23; estímulo à atividade de células T regulatórias na supressão da proliferação de células T (196–205); inibição da produção de imunoglobulinas e diminuição e interrupção da diferenciação de células B (206–209).

Todos estes efeitos salientam o importante papel da vitamina D na resposta das células B e T, no processo inflamatório e na produção de autoanticorpos, o que potencialmente pode resultar em dano tecidual. Nas células dendríticas, os efeitos da vitamina D talvez sejam os mais relevantes para o fenômeno da autoimunidade, devido ao papel protetor e mantenedor da tolerância imunológica desempenhado por estas células, visto que a $1,25(OH)_2D$, atuando na sua maturação, diferenciação e migração, estimula a produção de células dendríticas mais tolerantes (210–216). Nós macrófagos, a $1,25(OH)_2D$ aumenta a maturação de monócitos em macrófagos, mas reduz simultaneamente a sua capacidade de apresentar antígenos para as células T, diminuindo a expressão do MHC de classe II e diminui as citocinas IL-1β, IL-6, TNF-α e aumenta IL-10 (217–221).

2.5 VITAMINA D E LÚPUS

A vitamina D exerce inúmeras ações sobre o sistema imunológico e várias descobertas têm sido feitas a respeito da sua influência na etiopatogenia de algumas doenças autoimunes. Do ponto de vista das doenças autoimunes, o papel mais importante da vitamina D é sua capacidade de regular negativamente os mecanismos relacionados imunidade e induzir tolerância imunológica, bem como um efeito anti-inflamatório (222).

Em 1979, surgiu a primeira descrição que sugeriu a associação entre deficiência de vitamina D e LES em humanos. Foi um estudo com doze adolescentes lúpicas usuárias de glicocorticoides que evidenciou baixos níveis de $1,25(OH)_2D$ em 7 delas (223). A literatura relata uma prevalência de insuficiência de vitamina D (entre 20 e 30 ng/ml) entre 38-96% em pacientes com LES e a prevalência de deficiência de vitamina D (menor que 20 ng/ml) entre 8 e 30% (178). Os baixos níveis de vitamina D nos pacientes com LES estão associados à exposição solar reduzida devido à fotossensibilidade, ao uso de fotoproteção, a alteração do metabolismo renal de vitamina D, entre outras (224). Os estudos demonstram uma associação entre baixa concentração de vitamina D e a atividade da doença no LES (225). Existem diversos trabalhos publicados na literatura avaliando níveis de vitamina D em pacientes com LES (226).

Há evidências de que a vitamina D reduz a chance de desenvolver doença autoimune em modelos murinos experimentais de artrite reumatoide, encefalite e lúpus (21,227,228). Visto que esta tese avaliou os efeitos da suplementação de vitamina D em modelo de lúpus induzido por pristane, os trabalhos discutidos a seguir são sobre os efeitos desta suplementação em modelos experimentais de lúpus.

Em 1992, foi publicado por Lemire e colaboradores o primeiro trabalho avaliando a influência da suplementação de vitamina D em modelo experimental de lúpus. Este trabalho utilizou o modelo espontâneo de MRL/*lpr*. Os animais foram suplementados com $1,25(OH)_2D$ na concentração de 0,1 e 0,15 pg por via intraperitoneal em dias alternados. Dentre os principais resultados, a suplementação com $1,25(OH)_2D$ inibiu completamente o aparecimento de lesões cutâneas e reduziu a proteinúria. Apesar destes resultados positivos, a redução na linfodenopatia, o retardo no início do aparecimento de proteinúria e a redução estatisticamente significativa nos níveis de anti-ssDNA não foram observados nestes animais (21).

Em um estudo brasileiro, realizado em 2000, Vaisberg e colaboradores utilizaram camundongos (NZBxW) F1, geneticamente predispostos a desenvolver lúpus, e suplementaram estes animais com colecalciferol nas concentrações de 3 e 10 ug por via intraperitoneal 1 vez por semana. Os animais apresentaram piores achados histopatológicos na biópsia renal no grupo que recebeu suplementação de vitamina D, sugerindo que a vitamina D pudesse ter atuado como fator agravante da doença (22). Estes dados não foram reproduzidos em outros estudos.

Após 15 anos sem atualizações sobre a suplementação de vitamina D e modelos de lúpus, Arab e colaboradores utilizaram o modelo de lúpus induzido por cromatina ativada em camundongos BALB/c e suplementaram estes animais com $1,25(OH)_2D$ na concentração de 50 ng por via oral diariamente. Neste estudo foi utilizado dois protocolos de tratamento: (a) um protocolo preventivo (início da suplementação 2 semanas antes da indução do modelo) e (b) um protocolo de tratamento (início do tratamento 2 semanas após o estabelecimento da doença). Tanto a suplementação preventiva quanto a suplementação como tratamento não foram capaz de reduzir os níveis de anti-dsDNA ou alterar os níveis de proteinúria, mas observou-se um aumento na expressão de Foxp3 e TGF-β e uma redução de

IL-6 e IL-10. Este trabalho também avaliou o efeito da suplementação nas células Treg. A porcentagem de células Treg no grupo que recebeu tratamento preventivo aumentou significativamente (23).

Em 2016, um trabalho avaliou os efeitos de uma dieta deficiente em vitamina D exposta aos camundongos MRL/*lpr* durante 6 semanas. Após este período os animais apresentaram baixos níveis séricos de 25(OH)D, confirmando a deficiência em vitamina D. Apesar dos níveis séricos de vitamina D baixos, os animais não apresentaram nenhum anormalidade quanto ao peso corporal, alopecia, nos níveis séricos de anti-dsDNA e IgG total e no dano renal (proteinúria, histologia renal e depósito de IgG e C3 no rim) (24).

Em 2017, Ding e colaboradores exploraram os efeitos da $1,25(OH)_2D$ no modelo de MRL/*lpr*. A suplementação de $1,25(OH)_2D$ ocorreu na concentração de 5ug/kg através da via oral diariamente. A suplementação demonstrou um efeito protetivo, reduzindo as úlceras cutâneas e o dano renal (25). A tabela 2 apresenta resumo dos trabalhos publicados até o momento entre vitamina D e modelo experimental de lúpus.

Tabela 2. Modelos experimentais de lúpus e suplementação de vitamina D.

Modelo experimental	Forma da vitamina D	Concentração	Via de administração	Frequência	Resultados principais	Referências
Espontâneo						
	1,25(OH)2D	0,1 e 0,15 pg	intraperitoneal	Dias alternados	Inibição das lesões de pele Redução da proteinúria	Lemire e colaboradores, 1992 (21)
MRL/*lpr*	Dieta deficiente em vitamina D	N/A	N/A	N/A	Redução nos níveis de 25(OH)D Sem alterações nos níveis de anti-dsDNA, IgG total, proteinúria, histologia renal e depósito de IgG e C3.	Reynolds e colaboradores, 2016 (24)
	1,25(OH)2D	5ug/kg	oral	Diariamente	Redução das úlceras cutâneas Redução do dano renal	Ding e colaboradores, 2017 (25)
(NZBxW)F1	Colecalciferol	3 e 10 ug	intraperitoneal	Semanalmente	Piora da histologia renal	Vaisberg e colaboradores, 2000 (22)
Induzido						

Cromatina ativada	1,25(OH)2D	50 ng	oral	Diariamente	Aumento de Foxp3 e TGF-β Redução de IL-6 e IL-10	Arab e colaboradores, 2015 (23)

3. MARCO CONCEITUAL

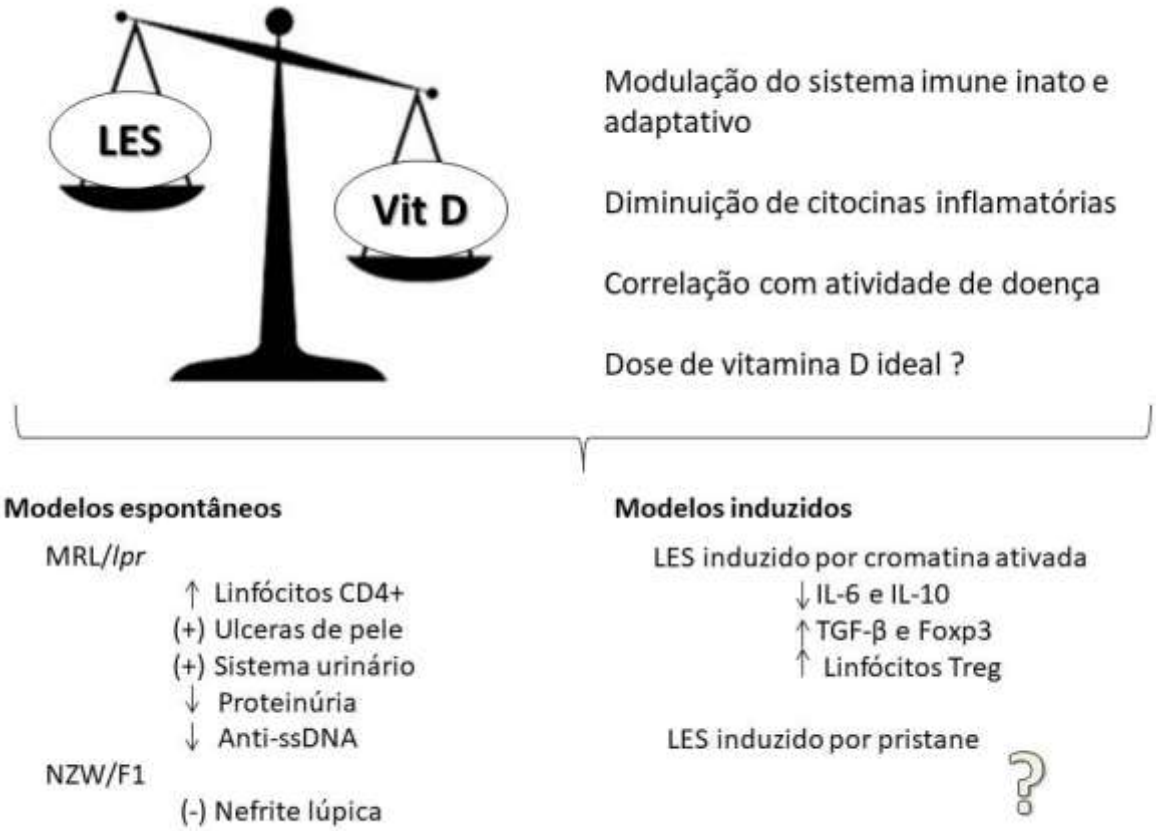

Figura 6. Marco teórico do presente trabalho. ssDNA: DNA de cadeia simples; (+): melhora; (-): piora;

4. JUSTIFICATIVA

O uso da suplementação de vitamina D em pacientes com doenças autoimunes é uma recomendação comum. Contudo ainda são controversos os estudos que observam as mudanças no processo crônico dos LES, bem como quais os mecanismos de imunomodulação da vitamina D. Devido à heterogenidade da doença e as diferenças interpessoais quanto aos efeitos da vitamina D no organismo em humanos, estudos experimentais podem $1,25(OH)_2D$.

5. OBJETIVOS

5.1 OBJETIVO GERAL

Avaliar o desenvolvimento e evolução de LES após suplementação de vitamina D em modelo experimental de lúpus induzido por pristane.

5.2 OBJETIVOS ESPECÍFICOS

1. Estudar a ação da suplementação de vitamina D nas manifestações clínicas do LES induzido por pristane através de:

   a. Avaliação de escore clínico articular;
   b. Avaliação de nocicepção articular;
   c. Avaliação de edema articular;

2. Estudar a ação da suplementação de vitamina D nas manifestações laboratoriais do LES induzido por pristane através de:

   a. Deposição de IgM e IgG nos rins (glomérulo);
   b. Quantificação de mediadores inflamatórios sistêmicos;

3. Determinar os padrões histopatológicos no rim e na articulação;

6. REFERÊNCIAS DA REVISÃO SISTEMÁTICA DA LITERATURA

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7. ARTIGO 1

Pristane-induced lupus: considerations on this experimental model

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REVIEW ARTICLE

Pristane-induced lupus: considerations on this experimental model

Eduarda Correa Freitas[1] · Mayara Souza de Oliveira[1] · Odirlei André Monticielo[1]

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Abstract Systemic lupus erythematosus (SLE) is a multifactorial, autoimmune inflammatory disease with pleomorphic clinical manifestations involving different organs and tissues. The etiology of this disease has been associated with a dysfunctional response of B and T lymphocytes against environmental stimuli in individuals genetically susceptible to SLE, which determines an immune response against different autoantigens and, consequently, tissue damage. The study of different murine models has provided a better understanding of these autoimmune phenomena. This review primarily focuses on that has been learned from the pristane-induced lupus (PIL) model and how this model can be used to supplement recent advances in understanding the pathogenesis of SLE. We also consider both current and future therapies for this disease. The PubMed, SciELO, and Embase databases were searched for relevant articles published from 1950 to 2016. PIL has been shown to be a useful tool for understanding the multiple mechanisms involved in systemic autoimmunity. In addition, it can be considered an efficient model to evaluate the environmental contributions and interferon signatures present in patients with SLE.

Keywords Animal model · Lupus · Pristane · Pristane-induced lupus · Systemic lupus erythematosus

✉ Odirlei André Monticielo
omonticielo@yahoo.com.br

[1] Laboratory of Autoimmune Diseases, Division of Rheumatology, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2350, room 645, Porto Alegre 90035-003, Brazil

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Introduction

Systemic lupus erythematosus (SLE) is characterized by multisystem inflammation and the loss of tolerance of T and B lymphocytes to host antigens. The etiology of SLE is still poorly known and is considered multifactorial, involving genetic, hormonal, and environmental aspects. Patients with this disease have various clinical symptoms including renal disease, non-erosive arthritis, serositis, hematological and respiratory manifestations, as well as the production of antinuclear antibodies (ANA). The genetic profile and clinical and laboratory changes of SLE can be studied in experimental models. Animal models of SLE induced in healthy mouse strains by exposure to hydrocarbon oils, such as pristane, have facilitated research into this disease by providing insight into the role of environmental factors that may predispose to SLE [1–3]. Furthermore, they allow study of the initial events that lead to a break in tolerance in the absence of genetic defects, and provide a better understanding of the cellular mechanisms involved in SLE development and progression. In this review, we will discuss what has been learned from the pristane-induced lupus (PIL) model and how this model can be used to supplement recent advances in understanding the pathogenesis of SLE.

Materials and methods

The PubMed, SciELO, and Embase databases were searched for articles published from 1950 to 2016, using the following terms and combinations thereof: "pristane-induced lupus," "tetramethylpentadecane-induced lupus," "pristane-treated mice," "murine lupus," and "hydrocarbon oil pristane." Articles in Portuguese, Spanish, and English were included in this review.

Pristane

Numerous chemicals and drugs have been identified as capable of triggering the production of autoantibodies or inducing a syndrome similar to SLE [4, 5]. However, none of them reproduce completely the spectrum of autoantibodies observed in human SLE. Drugs such as procainamide, hydralazine, quinidine, chlorpromazine, methyldopa, and isoniazid, which act on gene expression, induce a highly restricted and directed response against chromatin antigens (ssDNA, histones) [5, 6]. Pristane, acting differently from the above, is capable of inducing in mice a wide range of autoantibodies specific to or associated with SLE [7–10].

Pristane, also known as hydrocarbon oil (2,6,10,14-tetramethylpentadecane, TMPD), is an isoprenoid alkane. In nature, this oil can be found in small amounts in vegetables [11], in the liver of some sharks [12], and as a byproduct of petroleum distillation [11]. Mice administered pristane into the abdominal cavity develop an ascitic fluid enriched with monoclonal antibodies, local chronic inflammation (lipogranulomas), and a rheumatoid-like erosive arthritis [13], as well as autoantibodies and clinical manifestations similar to those of SLE [7, 14].

The mechanisms by which pristane induces a breakdown in tolerance and intracellular targets become antigenic remain to be defined. Pristane is a membrane-activating compound that interacts with the phospholipid bilayer. It has a cytotoxic effect dependent on concentration and cell lineage, although the mechanism of this cytotoxicity also remains unknown [15]. Apoptosis may explain how autoantigens become available to the immune system [16]. One study demonstrated that pristane induces programmed cell death both in lymphoid cell lines and in peritoneal exudate cells of mice in vitro and in vivo. This suggests that pristane-induced apoptosis provides a sufficient autoantigen substrate for immune tolerance to be broken, causing an immune disorder linked to overproduction of interferon alpha and beta (IFN-α and β), which consequently leads to the development of an autoimmunity similar to SLE [17].

Cytokine production

The production of inflammatory cytokines plays an important role in PIL. IFN-α, β and γ, interleukin-6 (IL-6), and interleukin-12 (IL-12) stimulate the formation of autoantibodies in this model. Animals deficient in the production of these cytokines are not able to produce autoantibodies [18, 19]. In the mouse PIL model, IFN-γ deficiency has been shown to have a protective effect on renal disease and production of autoantibodies [20]. The role of IFN will be described in more detail in the next sections, due to its importance in this model. BALB/c IL-6$^{-/-}$ mice do not produce anti-ssDNA, anti-dsDNA, or anti-chromatin antibodies, but continue to produce anti-RNP/Sm and anti-Su (Fig. 1). In the same study, production of anti-dsDNA antibodies in BALB/c IL-6$^{+/+}$ occurred 5 months after intraperitoneal injection of pristane, well after the onset of nephritis, suggesting that this antibody is not responsible for the induction of renal disease. These results suggest that induction of anti-DNA and anti-chromatin antibodies in mice treated with pristane is strictly dependent on IL-6, whereas the induction of anti-RNP/Sm and anti-Su autoantibodies is not [21]. Anti-RNP/Sm antibodies are associated with IL-12 production. IL-12$^{-/-}$ mice exposed to PIL do not develop anti-RNP antibodies or nephritis [22]. In conjunction with interleukin-18 (IL-18), IL-12 promotes differentiation of naive T cells into Th1 cells. IL-12 is produced primarily by antigen-presenting cells (APCs), such as macrophages and dendritic cells. These mice have a relative defect, but are not entirely devoid of Th1 responses [23]. In the absence of IL-12, IFN production can be induced by IL-18 signaling [24], although this process is believed to require the presence of other cytokines, such as interleukin-2 (IL-2) [25]. These studies demonstrate that the production of autoantibodies can be induced by different cytokine pathways that contribute to pathogenesis.

The role of interferon in pristane-induced lupus

IFN is an antiviral cytokine that plays an important role in SLE. Interferon type I (IFN-I) is composed of IFN-α and β subunits, which bind to the same receptor (IFNAR). Through microarray and quantitative PCR techniques in peripheral blood, it was observed that two-thirds of adults and nearly all children with SLE exhibit overexpression of IFN-I and interferon-stimulated genes (ISGs) [26–28]. Also known as "IFN signature," this phenomenon is closely associated with disease activity, lupus nephritis, and autoantibody production [28–31].

Pristane-treated mice exhibit a robust IFN signature [32]. The ectopic lymphoid tissue formed in PIL increases the expression of ISGs [33]. In IFNAR$^{-/-}$ mice, anti-DNA, anti-

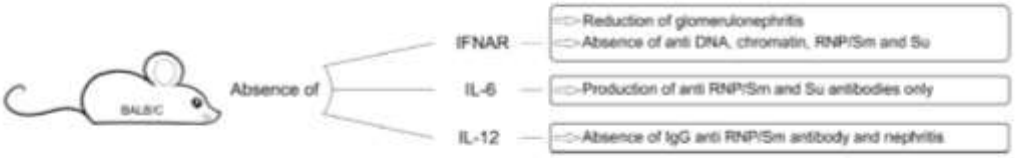

Fig. 1 Cytokines that can modify the production of autoantibodies and clinical expression in pristane-induced lupus

chromatin, anti-RNP, anti-Sm, and anti-Su antibodies are not produced and glomerulonephritis is definitely reduced, demonstrating that IFN-I plays an important role in the pathogenesis of PIL [18, 34]. Although autoantibody production develops around the third or fourth month after induction with pristane, IFN-I production is already detectable as early as 2 weeks after induction [35].

Dendritic cells are the main source of IFN-I production in healthy individuals and in patients with SLE, although their role may be limited in the PIL model [36]. In PIL, Ly6C^{hi} monocytes are the cell type responsible for IFN-I production. In response to intraperitoneal injection of pristane, these cells accumulate in the inflamed peritoneum, where they are triggered to synthesize IFN. Normally absent in the peritoneum, these cells are attracted through CCL2, and represent about 30% of the peritoneal exudate 2 weeks after pristane injection, suggesting that monocytes play an important role in the interferonopathy observed in the PIL model [37].

The mechanism for IFN-I overproduction in SLE cells is known to utilize various innate receptors in response to pathogen-associated molecules [38]. The toll-like receptors 7 (TLR7), 8 (TLR8), and 9 (TLR9) have received considerable attention because of their ability to recognize endogenous nucleic acids [39–41]. TLR7 and TLR9 are expressed intracellularly in dendritic cells, macrophages, and B cells [42, 43], within an endosomal compartment, and trigger IFN-I secretion via the myeloid differentiating factor 88 (MyD88) protein signaling pathway. Indeed, experiments with TLR knockout mice have revealed that production of IFN-I in PIL occurs via the TLR7-MyD88 pathway [44]. There is no production of anti-RNP, anti-Sm, and anti-Su antibodies or accumulation of Ly6C^{hi} monocytes and development of glomerulonephritis in TLR7$^{-/-}$ mice [45]. The Ly6C^{hi} monocytes of the peritoneal cavity express high levels of TLR7 and are considered the main source of IFN-I production. TLR8 is not associated with IFN-I production in humans or in mice, possibly because dendritic cells and B cells do not express this receptor [46].

The activation mechanism of TLR7 in the PIL model is still undefined. As the chemical structure of pristane is different from that of TLR7 ligands, this compound cannot directly activate the receptor [44]. It is possible that pristane increases the effects of TLR7 ligands, such as the endogenous U1 RNA Sm and RNP antigen. Furthermore, when incorporated into the cell membrane, pristane can modify the endosomal site, providing access to TLR7 [47]. However, neither TLR7 localization nor phagocytosis is altered by pristane [44]. Pristane also lacks the ability to increase TLR7 expression.

In SLE, an increase in apoptotic and necrotic cells is believed to result in the formation of immunocomplexes (ICs) formed by autoantibodies and autoantigens containing DNA and RNA [48]. In vitro, the Fcγ receptors (FcγR) of dendritic cells have been shown to mediate transport of DNA- or RNA-containing ICs into endosomes, allowing the activation of TLR7, TLR8, and TLR9 by these internalized endogenous nucleic acids [49, 50]. Thus, the production of autoantibodies against autoantigens containing RNA (U1 snRNP) is a prerequisite for the production of IFN-I. However, in the PIL model, IFN-I production precedes the appearance of anti-dsDNA, anti-RNP, or anti-Sm autoantibodies. FcγR$^{-/-}$ animals are able to produce autoantibodies and IFN, thus excluding the role of ICs in initial IFN generation [44, 51].

TLR9$^{-/-}$ BALB/c mice injected intraperitoneally with pristane develop more severe autoimmunity than do their TLR-sufficient cohorts. Early indications include an increased accumulation of TLR7-expressing Ly6C^{hi} inflammatory monocytes at the site of injection, upregulation of ISGs expression in the peritoneal cavity, and an increased production of myeloid lineage precursors (common myeloid progenitors and granulocyte myeloid precursors) in the bone marrow. These mice also develop higher autoantibody titers against RNA, neutrophil cytoplasmic antigens, and myeloperoxidase than do pristane-injected wild-type (WT) BALB/c mice, as well as a marked increase in glomerular IgG deposition and infiltrating granulocytes, much more severe glomerulonephritis, and a reduced lifespan. The BALB/c pristane model recapitulates other TLR7-driven spontaneous models of SLE and is negatively regulated by TLR9 [52].

However, recent research has also suggested that opsonization of dead cells by C3 and IgM in PIL is involved in the pathogenesis of the IFN signature. The data imply that complement receptor-mediated phagocytosis of dead cells opsonized by natural IgM and complement generates IFN-I and other proinflammatory cytokines in PIL. Like C3-deficient mice, C4-deficient lupus patients do not exhibit an IFN signature. This novel pathway, which likely involves the early classical complement cascade, is essential for the IFN signature in PIL and also appears to be relevant in human SLE [53]. Pristane-primed macrophages from C3-deficient mice did not exhibit impaired cytokine production. In contrast, C1q-deficient pristane-primed resident peritoneal macrophages secreted significantly less CCL3, CCL2, CXCL1, and IL-6 when stimulated in vitro with a TLR7 ligand. Furthermore, C1q$^{-/-}$ mice developed lower titers of circulating antibodies and milder arthritis compared with controls. These findings demonstrate that C1q deficiency impairs TLR7-dependent chemokine production by pristane-primed peritoneal macrophages and suggest that C1q, and not C3, is involved in the handling of pristane by phagocytic cells, which is required to trigger disease in this model [54].

Patients with SLE present decreased expression of an estrogen-regulated microRNA, miR-302d, in their monocytes. Its target is the interferon regulatory factor 9 (IRF9), a critical component of the transcriptional complex that regulates the expression of ISGs. Thus, with reduced miR-302d expression, IRF9 levels increase, as does the expression of ISGs. In the PIL model, transfection of miR-302d has a protective effect

against pristane-induced inflammation, suggesting that modulation of miR-302d levels may be protective in SLE. Thus, these findings classify miR-302d as a key regulator of IFN-I-directed gene expression, underscoring the importance of non-coding RNA in the regulation of the IFN pathway both in the PIL model and in patients [55].

In summary, the literature demonstrates that pristane may mimic human SLE by causing synergistic abnormalities in interferon production along with defective clearance of apoptotic cells and overactive B cell signaling. IFN production is essential for development of the disease. PIL may be a good model for studying dysregulation of this cytokine.

Lymphoid neogenesis and autoantibody production

The production of autoantibodies is a central event in the pathogenesis of SLE [56]. BALB/c, SJL/J, and C57BL/6 mice injected intraperitoneally with pristane develop SLE-specific autoantibodies, including anti-dsDNA, anti-ssDNA, anti-Sm, anti-RNP, and anti-ribosomal P [7, 14, 57, 58]. Antibody production after pristane injection was first described by Satoh et al. in 1995 [59]. Pristane also causes polyclonal hypergammaglobulinemia, which stimulates the production of cytokines. Both the production of antinuclear antibodies and hypergammaglobulinemia are characteristics of human SLE [11], as are the production of antibodies against type II collagen and the presence of rheumatoid factor [60].

In BALB/c mice, a single intraperitoneal injection of 0.5 ml pristane is able to stimulate the production of autoantibodies against the RNA component of U1 small nuclear ribonucleoproteins via TLR7-driven IFN-I production [61]. The increased TLR7 expression may contribute to B cell hyperactivity and autoantibody production in SLE [62]. PIL features an expanded population of B cells with a switched memory-like phenotype and hyperresponsiveness to synthetic TLR7 ligands and apoptotic cells, probably resulting from increased TLR7 expression due to IFN-I production [63]. Also, a build-up of dead cells in lupus tissues may help maintain high serum levels of anti-RNP/Sm autoantibodies [63]. Production of Su autoantigens persists in 50–90% of animals 4–6 months after injection, and production of anti-dsDNA for even longer, between 6 and 10 months [7, 14]. Titers of anti-Su and anti-snRNP/Sm are present in this model at levels as high as 1:25,000–1:250,000 (ELISA). This level of autoantibody production resembles that found in spontaneous autoimmune diseases [59].

Recently, a role for caspase-1 in murine lupus was described, indicating an involvement of inflammasomes in the development of SLE. $Nlrp3^{R258W}$ mice with PIL were observed to have higher mortality than WT mice following pristane injection. Furthermore, anti-dsDNA and total IgG levels were increased in the serum of $Nlrp3^{R258W}$ mice compared with those of WT mice. These data indicate that $Nlrp3^{R258W}$ mutant mice exhibited enhanced autoimmune responses after pristane treatment [64]. Severe glomerular renal damage, characterized by hypercellularity, mesangial expansion, crescent formation, and interstitial mononuclear cell infiltration, was also observed. In PIL, a lack of caspase-1 does not alter the recruitment of inflammatory cells into the peritoneal cavity or change the formation of lipogranulomas, which are considered a nidus of chronic inflammatory mediators for disease development [65]. In caspase-$1^{-/-}$ mice, anti-dsDNA and anti-RNP autoantibody production is attenuated, as is hypergammaglobulinemia. These mice mount intact immune responses, but do not develop an expanded marginal zone B cell population in response to pristane [66]. This may be one explanation for reduced autoantibody production in these mice [66]. Furthermore, levels of circulating inflammatory cytokines, such as IL-6 and IL-17, were lower in control and PIL caspase-$1^{-/-}$ mice, suggesting an overall reduced inflammatory phenotype [65].

Disease induction and production of autoantibodies in the PIL model are independent of exogenous organisms, such as viral, bacterial, and parasitic agents. Experiments with BALB/c mice free of exogenous organisms and treated with pristane showed chronic peritoneal inflammation with lipogranuloma formation, cytokine production, hepatosplenomegaly, and hypergammaglobulinemia similar to those observed in conventionally housed animals. This indicates that stimulation by exogenous agents is not necessary for this inflammatory process to occur [67]. Regarding the origin of the autoantibodies, the literature describes that BALB/$c^{nu/nu}$ (nude) mice [68] or mice deficient in T cell receptors (C57BL6 TcR$\beta^{-/-}$, TcR$\delta^{-/-}$) [69] do not develop IgG or IgM anti-snRNP/Sm/Su autoantibodies after administration of pristane, but produce rheumatoid factor (IgM), which is independent of T lymphocytes [70]. This demonstrates that production of these antibodies occurs through a T cell-dependent immune response, similar to that observed in patients with SLE [68].

Lipogranulomas are inflammatory lesions resembling germinal centers that arise in response to the presence of pristane in the peritoneal cavity, and represent an example of lymphoid neogenesis [33]. This formation of ectopic lymphoid tissue at sites of inflammation [71] is associated with the production of autoantibodies [72]. Ectopic lymphoid tissue resembles secondary lymphoid tissue. It often exhibits B cell, T cell, and dendritic cell zones. The organization of this tissue occurs through the presence of CCL19, CCL21, CXCL12, and CXCL13 lymphoid chemokines. These lymphoid tissues form when the body cannot clear a pathogen, and are also common in autoimmune diseases [72]. Cytokines produced in ectopic lymphoid tissue may play an important role in the production of autoantibodies [33, 73]. Indeed, lipogranulomas exhibit proliferation and interaction of T and B cells [69], and may be a site of antibody production by B cells.

Clinical manifestations of pristane-induced autoimmune disease

Epidemiological studies suggest that occupational exposure to mineral oil or petroleum residue is associated with rheumatoid arthritis (RA) and SLE [74, 75]. Since the first description of PIL in mice, substantial progress has been made in characterizing the relevant immunobiological events. In addition to pristane, other compounds such as incomplete Freund's adjuvant (IFA) and squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexane) have been reported to induce lupus-related anti-nRNP/Sm and anti-Su autoantibodies in non-autoimmune BALB/c mice. Induction of these autoantibodies appeared to be associated with the hydrocarbon's ability to induce IL-12, IL-6, and TNF-alpha, suggesting a relationship with adjuvanticity. Thus, the potential of hydrocarbon oils to induce autoimmunity has implications for the use of oil adjuvants in basic research [76].

Animals subjected to intraperitoneal injection of pristane develop clinical manifestations such as arthritis [60], glomerulonephritis with immunoglobulin and complement deposition, pulmonary capillaritis, anemia, and autoantibody production (Fig. 2). Many of these manifestations are cytokine-driven. As in human SLE, they develop primarily in females, at an approximate female-to-male ratio of 9:1 [77].

Regarding arthritis, BALB/c mice developed synovial hyperplasia, periostitis, and marginal erosions reminiscent of RA [13, 60]. Arthritis in patients with lupus is generally not erosive, although erosions similar to those of RA may develop in some cases. The overlapping characteristics of both autoimmune diseases are known as *rhupus*. The nature of joint disease in animals suggests an arthritis similar to that found in this syndrome [11].

PIL is one of the few inducible models that can progress to glomerulonephritis. Glomerulonephritis is induced in about one-third of BALB/c mice following intraperitoneal administration of pristane, a frequency similar to that of nephritis in humans with SLE [78]. The inflammatory process in PIL nephritis is mediated by the interaction between ICs containing IgG and myeloid effector cells, monocytes/macrophages, with proteinuria beginning 4–6 months after pristane injection [14, 79, 80]. Mice deficient of IL-6 [21] and IL-12 [22] are highly resistant to induction of renal disease. Monocyte influx also appears to play an important role in the pathogenesis of lupus nephritis in humans and mice [81]. Several chemokines involved in recruitment of monocytes are products of IFN, for example, IFN-α and β induce CCL2. The decrease in glomerular cell production in response to immune complexes could modulate the severity of renal disease in IFN-α and β knockout mice.

Despite the important role of IFN-I in the development of several clinical manifestations of this model, the anemia present in PIL animals is TNF-α dependent and IFN-I independent. The bone marrow of animals administered pristane intraperitoneally exhibits high levels of TNF-α, an abnormality also present in patients with SLE [82].

Pristane administration to apolipoprotein E (apoE) knockout C57BL/6 mice led to the development of an experimental model of lupus with atherosclerosis. The animals presented had poor spirit, less activity, obvious hair loss, splenomegaly, and renomegaly. Also, levels of ANA, anti-dsDNA, and anti-Sm antibodies were significantly higher. The same study also evaluated expression of TLRs, and found that pristane induced abnormally high expression of TLR2 and TLR4 in the aorta and TLR2, TLR4, TLR7, and TLR9 in the kidney [83].

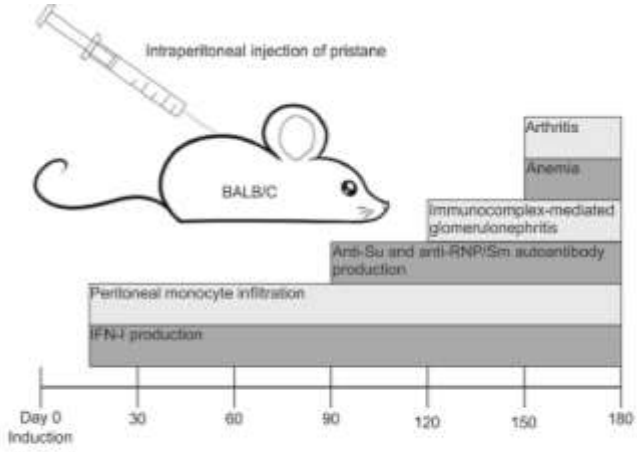

Fig. 2 Clinical manifestations in BALB/c over the trial period

Diffuse alveolar hemorrhage (DAH) is not present in the PIL model in BALB/c mice, but occurs, in a manner similar to that seen in human patients, when this model is developed in C57BL/6 and C57BL/10 mice. Although only 3% of SLE patients develop DAH, this is a significant problem associated with > 50% mortality, and its cause is unknown [84, 85]. In PIL, approximately half of animals die during the experiment [79, 86]. These animals develop pulmonary capillaritis with a perivascular infiltrate of macrophages, neutrophils, lymphocytes, and eosinophils and deposition of ICs, with moderate-to-severe alveolar inflammation [79, 86]. After intraperitoneal injection, pristane migrates to the lung, causing cell death, small-vessel vasculitis, and alveolar hemorrhage similar to that seen in DAH in humans. Anti-neutrophil cytoplasmic antibodies (ANCAs) are absent [79]. The recruitment of macrophages and neutrophils precedes hemorrhage, starting 3 days after pristane injection and peaking at 2 weeks [87]. Furthermore, DAH is independent of MyD88, TLR7, Fcγ receptor, Fas, and T cells, but immunoglobulin-deficient mice are resistant [87]. B cell-deficient animals do not develop DAH [87], possibly because of the lack of production of immunoglobulins or other B cell functions. DAH is also absent in $C3^{-/-}$ and $CD18^{-/-}$ mice [88]. Thus, DAH in PIL is mediated by IgM, C3, and CD18, a component of the C3b receptors CR3 and CR4 [88]. With regard to the underlying mechanism of DAH, pristane was also detected in the lungs of treated $C3^{-/-}$ mice, but not untreated $C3^{-/-}$ mice, indicating that C3 is not necessary for migration of pristane from the peritoneum to lung [88]. As noted earlier, peritoneal pristane injection also causes bone marrow inflammation [82], and pristane was detected in the bone marrow of PIL mice, but not in bone marrow of untreated mice, which suggests that the oil was widely dispersed following IP injection. Examination of lung tissue by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay revealed dead cells in pristane-treated mice but not in untreated controls. Dead cells also accumulate in the bone marrow of PIL mice [82], which suggests that pristane might be cytotoxic. Taken together, these data indicate that pristane migrates from the peritoneum to the lungs and other tissues, where it may cause death of certain cell types. Opsonization of these dead cells by IgM and C3 may promote pulmonary inflammation, as also seen in the peritoneum [53]. Lung interstitial macrophages and epithelial cells are anti-inflammatory and secrete IL-10. Although alveolar macrophages are normally anti-inflammatory, when activated via TLRs, IL-10R signal transduction is inhibited and they become proinflammatory [89, 90]. Thus, IL-10 is a crucial regulator of lung inflammation. $IL\text{-}10^{-/-}$ mice had significantly increased mortality from DAH. TLR-activated genes are targeted by IL-10 [91], and pristane induces proinflammatory cytokine production via TLR7 [44]. Unexpectedly, $MyD88^{-/-}$ and $TRIF^{-/-}$ mice developed DAH at a frequency similar to that of WT mice [88]. In summary, induction of DAH is independent of TLR, inflammasomes, and inducible nitric oxide; its mortality is increased in IL-10-deficient mice; and pristane treatment decreases IL-10 receptor expression in monocytes and STAT-3 phosphorylation in lung macrophages [88]. Similar to IFN production in PIL, ischemia-reperfusion injury in mice is mediated by the early classical complement cascade and natural IgM. Thus, the pathogenesis of DAH involves opsonization of dead cells by natural IgM and complement followed by complement receptor-mediated lung inflammation. The disease is macrophage-dependent, and IL-10 is protective. It follows that complement inhibition and/or macrophage-targeted therapies may reduce mortality in lupus-associated DAH.

Relevance of animal models to human SLE

Animal models are advantageous when they reproduce all or some clinical features of the disease in humans. Such models exist for SLE and have contributed significantly to our understanding of its pathogenesis.

SLE in animals may occur spontaneously or be induced. Induction of lupus in animals can be accomplished by a variety of methods, such as genetic manipulation (expression or suppression), autoimmune serum or lymphocyte-lymphocyte injection, dendritic cell vaccination with apoptotic debris, immunization with antigens such as protein complex DNA and RNA, or by hydrocarbons such as pristane [7, 20]. Unlike other autoimmune and inflammatory experimental models, PIL most closely resembles human SLE.

In BALB/c mice, PIL induces mild glomerulonephritis [14], arthritis [13, 60], and the production of several autoantibodies characteristic of SLE, including anti-dsDNA and anti-Sm [7]. However, in this model, animals are not genetically prone to developing the disease as are human patients with SLE. Thus, this model does not provide insight into the genetic abnormalities involved in SLE. Nevertheless, overproduction of IFN-I, which is a core feature of SLE pathogenesis, is present in this model. Moreover, PIL is useful for examining the role of environmental triggers involved in the disease [7, 14]. It is possible to assume that the model induction pathways may be relevant for SLE patients [92, 93]. In addition to BALB/c, almost all other strains of mice are susceptible to pristane induction to varying extents, with production of autoantibodies and other manifestations similar to those of human SLE [58], corroborating the importance of environmental factors in the pathophysiology of this disease.

Table 1 Major studies using the pristane-induced lupus model

Author (Reference)	Objective	Treatment	Summary	Main treatment effects
Zhou L et al. 2010 [98]	To investigate the effect of melatonin on environmental-related SLE	Melatonin	Female BALB/c mice (age 2 months) were divided into 6 groups (n = 10 animals per group): normal control, PIL, prednisone 5 mg/kg, melatonin 0.01 mg/kg, melatonin 0.1 mg/kg, and melatonin 1.0 mg/kg; daily intragastric treatment with onset after disease induction.	Delayed production of anti-ssDNA and histone IgM antibodies; Decreased IL-6 and IL-13; Increased IL-2; Greater kidney damage
Minhas U et al. 2012 [99]	To investigate the therapeutic effect of *Withania somnifera* pure root powder on pristane-induced lupus in BALB/c mice	*Withania somnifera*	Female BALB/c mice (age 3–4 months) were divided into 6 groups (n = 8 animals per group): normal control, PIL, indomethacin 3 mg/kg treatment, *Withania somnifera* 500 mg/kg, *Withania somnifera* 1000 mg/kg, or 2% gum acacia; daily oral treatment starting 1 month after disease induction.	Reduction of lipogranulomas; Reduction of IL-6 and TNF-α levels in serum and ascitic fluid; Inhibitory effect on proteinuria; Decreased nephritis; Decreased inflammatory markers
Wang Z et al. 2014 [101]	To evaluate the preventive effects of resveratrol on pristane-induced lupus	Resveratrol	Female BALB/c mice (age 2–3 months) were divided into 4 groups (n = 10 animals per group): normal control, PIL, resveratrol 50 mg/kg, and resveratrol 75 mg/kg; oral treatment in daily diet starting on day 2 after disease induction.	Inhibitory effect on proteinuria; Significant reduction in glomerular lesions; Decreased IgG and IgM deposition in the kidney
Li M et al. 2015 [102]	To investigate the potential therapeutic effect of A20 on renal inflammation in pristane-induced lupus	A20	Three months after pristane injection, female BALB/c mice (age 6–8 weeks) were randomized into 3 groups and injected with 1.0×10^9 plaque forming units (PFU) of adenovirus-A20, control adenovirus or PBS (100 μl, n = 6–8 per group) i.p.	Decreased proinflammatory cytokine production; Reduction in anti-dsDNA and anti-nRNP levels in serum; Inhibition of lupus-related renal injury
Bender A et al. 2016 [104]	To determine the therapeutic efficacy of Btk inhibition in two mouse lupus models driven by TLR7 activation and type I interferon	M7583 (Btk inhibitor)	Starting 2 months after pristane injection, female DBA/1 mice (age 11–12 weeks) were fed chow formulated with M7583 at a concentration of 25 mg compound/kg chow.	Reduction in clinical signs of arthritis; Reduction of anti-dsDNA, anti-histone, and anti-Ro/SSA, but not anti-Sm/RNP antibody levels
He Y et al. 2016 [106]	To investigate the potential therapeutic effect of MSL in SLE and explore the underlying mechanisms	Methyl salicylate 2-O-β-d-lactoside (MSL)	Female BALB/c mice (age 7–8 weeks), 45 days after PIL induction, were randomly divided into 5 groups: PIL, low-dose MSL (200 mg/kg), medium-dose MSL (400 mg/kg), high-dose MSL (800 mg/kg), or prednisone 5 mg/kg; doses were administered orally once daily.	Reduction in DNA autoantibody titers; Total IgG concentrations in lupus mice were significantly lower at months 4–6; Reduction in IL-6 levels on day 60 after induction; On day 180, IL-17A levels were not significantly reduced.
Lin Y et al. 2017 [107]	To investigate the effects of SAA in pristane-induced lupus in BALB/c mice	Salvianolic acid A (SAA)	60 female BALB/c mice were randomly divided into five equal groups: control, model, SAA, prednisone, or aspirin (n = 12 per group). Mice in the control and model groups were given saline each day by gavage, while mice in the SAA, prednisone and aspirin groups were administered SAA (5 mg/kg/d), prednisone (5 mg/kg/d) or aspirin	Reduction in anti-Sm autoantibody titers; Inhibition of IKK, IκB, and NFκB phosphorylation in renal tissue

Table 1 (continued)

Author (Reference)	Objective	Treatment	Summary	Main treatment effects
			(300 mg/kg/d) by gavage, respectively. Treatment began 1 month after pristane injection.	
Mihaylova N et al. 2017 [109]	To examine the possibility of suppressing autoreactive B and T cells with a monoclonal antibody against ANX A1 in murine pristane-induced lupus	Anti-ANX A1	Female BALB/c mice (age 8 weeks) were randomized into 3 groups ($n = 10$ each). Pristane-injected mice were immunized every 6 days with 200 ng/mouse of anti-ANX A1 antibody i.p., while the control group of pristane-injected animals was treated with PBS.	Decreased expression of T cell activation markers; Decreased number of anti-dsDNA IgG antibody-secreting plasma cells; Attenuated lupus symptoms in pristane-injected mice

Treatments in pristane-induced lupus

Animal models have been used not only to improve knowledge of the mechanisms involved in SLE, but also to test potential therapies. In addition to assessing possible therapeutic targets, animal models are indispensable before clinical trials. The following section summarizes the main therapeutic studies performed with the PIL model (Table 1). Most of these treatments are preventive.

Researchers tested the regulatory effect of melatonin at concentrations of 0.01, 0.1, and 1.0 mg/kg/daily for 6 months via intragastric administration. Melatonin slowed the increase in anti-ssDNA and histone IgM antibody levels, decreased IL-6 and IL-13, and increased IL-2 production in the splenocyte supernatant of pristane-treated mice. In addition, melatonin decreased the renal damage caused by pristane. These results suggest that melatonin has a beneficial effect on PIL through cytokine regulation [94].

In 2012, the therapeutic effect of the herb *Withania somnifera* in PIL was tested. Treatment with root powder at concentrations of 500 and 1000 mg/kg was administered orally once daily, starting 1 month after disease induction. Animals treated with the 1000 mg/kg concentration exhibited reduced lipogranuloma formation compared to the untreated disease group. Furthermore, treatment was associated with a reduction in IL-6 and TNF-α levels in both serum and ascitic fluid, and was shown to have a potent inhibitory effect on proteinuria, nephritis, and inflammatory markers. However, the production of autoantibodies in serum appeared to be unchanged in both groups treated with *W. somnifera*, demonstrating the same pattern of nuclear fluorescence [95].

Resveratrol (3,5,4-trihydroxystilbene) is a natural antimicrobial compound found in various plants and fruits [96]. It has anti-inflammatory and immunoregulatory properties and was recently tested in the PIL model. In this study, resveratrol was added to the animals' diet at concentrations of 50 and 75 mg/kg and administered for 7 months. The results obtained with resveratrol treatment included decreased proteinuria, immunoglobulin deposition in the kidney, glomerulonephritis, and serum levels of IgG1 and IgG2a treatment. At the end of the experiment period, IFN-α levels in mice in the resveratrol groups were lower than those of control mice, but the difference was not statistically significant. This suggests that resveratrol has protective effects in murine PIL and may represent a novel approach for the treatment of SLE [97].

Considering the development of new therapies for the control of systemic inflammation in patients with SLE, treatment with A20 has been proposed [98]. A20, also known as tumor necrosis factor alpha-induced protein 3 (TNFAIP3), is an anti-inflammatory factor induced by TNF [99]. A20 overexpression significantly mitigated pristane-induced systemic inflammation and renal injury in mice. The therapeutic effect of A20 may be associated with inhibition of the NLRP3 inflammasome and NFκB activation in macrophages. Because of this dual inhibitory effect, A20 may be a promising new candidate for the treatment of SLE [98].

Likewise, Bender et al. found that Bruton's tyrosine kinase (Btk) inhibition treats TLR7/IFN-driven murine lupus [100]. Btk is expressed in a variety of immune cells, and previous work has demonstrated that blocking this protein is a promising strategy for treating autoimmune diseases. In PIL, Btk inhibition suppressed arthritis, but neither autoantibodies nor the IFN gene signature was significantly affected, suggesting efficacy was mediated through inhibition of Fc receptors [100].

Methyl salicylate 2-O-β-d-lactoside (MSL) is a novel salicylic acid analogue, extracted from the traditional Chinese herbal medicine *Gaultheria yunnanensis* that has been widely used for treatment of swelling and various inflammatory responses in the southern regions of the People's Republic of China [101]. In PIL, MSL was found to antagonize the increasing levels of antibodies and cytokines, suppressing joint swelling, and having an inhibitory effect on arthritis-like symptoms. It also significantly decreased the

spleen index and expression of inflammatory markers, and protected the kidneys of PIL mice from injury by inhibiting expression of inflammatory cytokines and reducing IgG and C3 immunocomplex deposition [102].

Lin Y et al. demonstrated that treatment with salvianolic acid A (SAA), isolated from the dried roots of *Salvia miltiorrhiza* Bunge, alleviates renal injury in PIL. The NFκB pathway may be implicated. SAA treatment caused a significant reduction in the level of anti-Sm autoantibodies (including IgG and IgM) and reduced total IgG levels. SAA inhibited phosphorylation of IKK, IκB, and NFκB in renal tissues, possibly accounting for its renoprotective effects [103].

Studies have also suggested anti-annexin A1 (ANX A1) monoclonal antibodies as a potential therapy. Annexin A1 (ANX A1) is a member of the annexin superfamily which, in the presence of Ca^{2+}, binds acid phospholipids with high affinity [104]. The administration of anti-ANX A1 monoclonal antibody resulted in inhibition of T cell activation and proliferation, suppression of IgG anti-dsDNA antibody-secreting plasma cells and proteinuria, decreased disease activity, and prolonged survival compared to control animals [105].

Conclusion

SLE is a complex disease that involves several immune dysfunctions. Experimental models have provided useful insight into its etiology and pathogenesis. The PIL model in particular has shed light on the role of environmental factors that may predispose to development of SLE. In addition, this model is associated with excess production of IFN-I and expression of ISGs and, thus, may be a good tool for studying the dysregulation of this cytokine observed in SLE patients.

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Compliance with ethical standards

Disclosure None.

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8. ARTIGO 2

Vitamin D supplementation ameliorates arthritis but does not alleviates renal injury in pristane-induced lupus model

(Manuscrito submetido para publicação no periódico *Autoimmunity*)

Vitamin D supplementation ameliorates arthritis but does not alleviates renal injury in pristane-induced lupus model

Eduarda Correa Freitas[1,2,5], Thaís Evelyn Karnopp[1,2,5], Jordana Miranda de Souza Silva[1,2,5], Rafaela Cavalheiro do Espírito Santo[1,2,5], Thales Hein da Rosa[1,5], Mayara Souza de Oliveira[4], Fabiany da Costa Gonçalves[1], Francine Hehn de Oliveira[3] , Pedro Guilherme Schaefer[3] and Odirlei André Monticielo[1,5].

[1] Laboratory of Autoimmune Diseases, Division of Rheumatology, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil.

[2] Post-Graduate Program in Medical Sciences, School of Medicine, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[3] Department of Surgical Pathology, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[4] Post-Graduate Program in Medical Sciences: Endocrinology, School of Medicine, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[5] Division of Rheumatology, Department of Internal Medicine, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Corresponding author:

Eduarda Correa Freitas

Programa de Pós-Graduação em Medicina: Ciências Médicas – UFRGS

Laboratory of Autoimmune Diseases, Division of Rheumatology - HCPA

Rua Ramiro Barcelos, 2350

ZIP Code 90035-003 - Porto Alegre, Brazil

Telephone: +55 51 3359 8340

Email: eduarda.freitas.hcpa@gmail.com

ABSTRACT

Systemic lupus erythematosus (SLE) is a multifactorial and autoimmune inflammatory disease with pleomorphic clinical manifestations involving different organs and tissues. The study of different murine models has provided a better understanding of these autoimmune phenomena. Pristane-induced lupus represents a suitable model to study factors that could influence the induction and/or progression of SLE, including genetic factors. The objective of the present study was to evaluate the development and evolution of SLE after vitamin D supplementation in PIL model. Here, we evaluated the effects of vitamin D supplementation in model of pristane-induced SLE in female BALB/c mice. The animals were randomly divided into three groups: control group (CO), pristane-induced lupus group (PIL) and pristane-induced lupus group plus vitamin D (VD). Lupus was induced in PIL and VD groups using pristane. PIL group showed arthritis and kidney injury, characterized by increased proteinuria, glomerular mesangial expansion and inflammation. Moreover, PIL model showed increased levels of IL-6, TNF-α and IFN-γ in serum. We observed that treatment with vitamin D improved arthritis through reduced of incidence and arthritis clinical score and edema, but does not influenced renal injury. Treatment with vitamin D was not able to reduce proteinuria levels, decrease mesangial hypercellularity or IgG and IgM deposition in the kidney. Vitamin D supplementation did not alter IL-6, TNF-α, IL-2 and IL-4 cytokine levels, but reduce IFN-γ levels. These results support that the role of vitamin D may be different depending on acting site, what could explain different responses according clinical phenotype. Therefore, further investigations of vitamin D are needed to explore the supplement dosage, timing, and the molecular basis in SLE.

KEYWORD

Lupus, animal model, systemic lupus erythematosus, pristane-induced lupus, vitamin D, vitamin D receptor

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a chronic, multisystem, inflammatory and autoimmune disease, characterized by the production of autoantibodies and tissue damage. An incidence of 1-22 cases is estimated for every 100,000 people per year, affecting more women of childbearing age (1). Etiology of SLE is multifactorial and remains poorly understood. However, it is well known that hormonal, environmental, genetic and immunological factors contribute to the disease onset (2). Several genes have been linked to the onset of SLE, including the *VDR* (vitamin D receptor) that synthesizes the vitamin D receptor (3).

Experimental models have shown to be advantageous when reproducing clinical features of the disease in humans. Such models contribute significantly to the understanding of the SLE pathogenesis. Moreover, PIL model is useful for examining the role of environmental triggers involved in the disease (4,5). PIL model develop clinical manifestations of SLE, including arthritis, immune complex-mediated glomerulonephritis and vasculitis, as well as increases in autoantibodies (6,7), including anti-dsDNA and anti-Sm (4) and overproduction of IFN-I, which is an important feature of SLE pathogenesis. The PIL model replicates many phenotypic and functional abnormalities of human SLE and proved to be very useful in identifying putative pathogenic mechanisms and environmental triggers of this disease (6). Pristane is an isoprenoid alkane found at high concentrations in mineral oil and is widely used as a sensitizer for increasing the yield of mice ascites. Phagocytosis of pristane by macrophages induces the production of cytokines and the formation of lipogranuloma on mesenteric and other peritoneal surfaces (8).

Animal model studies have demonstrated a relationship between vitamin D supplementation/deficiency and SLE symptoms. However, there are conflicts in the literature about this association. Female MRL/lpr mice that received 0.1–0.15 μg vitamin D3 intraperitoneally showed a reduced degree of serum single stranded-DNA antibodies and proteinuria (9). On the contrary, female NZB/W mice injected intraperitoneally 3 or 10 μg vitamin D3 demonstrated renal histopathology worsening

(10). In this context, we conducted a study to evaluate the development and evolution of SLE after vitamin D supplementation in the PIL model.

MATERIALS AND METHODS

Animals and ethics

Female BALB/c mice that were 8 weeks-old were obtained from the Universidade Federal de Pelotas (Pelotas, RS, Brazil). All mice were housed in the Animal Experimentation Unit at Hospital de Clínicas de Porto Alegre (HCPA) and maintained in standard 12h light/dark cycle, with controlled temperature (22±2°C) and given water and food *ad libitum*. Mice were adapted to the new environmental conditions for 2 weeks. The present study was approved by the Animal Ethics Committee of HCPA (number 17-0011) and was conducted in accordance with National Institute of Health guidelines.

Sample size and experimental design

The sample size calculation was made considering 80% sample power, alpha error 5% and to detect a 30% reduction in the mononuclear cell infiltrate in the kidney. Since there is a chance of error in the intraperitoneal (i.p.) in the induction of the model and early death of the animals due to ascites were added 2 animals per pristane induction group. Twenty-eight female BALB/c mice were randomly divided into the following three groups: (1) Control group (CO; n=8); (2) Pristane-induced lupus group (PIL; n=10); and (3) Pristane-induced lupus treated for six months with vitamin D group (VD; n=10). CO group received a single i.p. injection with 500 µl 0,9% saline solution and the PIL and VD groups received a single i.p. injection with 500 µl pristane oil (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich, MO, USA), according to Satoh et al. (5). During the procedures, mice were anaesthetized with isoflurane 10% (Abbott Laboratório do Brasil Ltda.,Brazil) and 90% of oxygen. Throughout the experiment, we had some losses of animals: 1 animal from the control group (accident), 1 animal from the PIL group (accident) and 3 animals from the vitamin d group (1 animal died in the induction of the model and 2 animals died from intestinal intussusception).

Vitamin D supplementation was administrated from the day of disease induction to the euthanasia. VD group mice received a subcutaneous injection of

Calcijex (11) (Abbott Labs, Chicago, Ill) containing 2 µg/kg/day of calcitriol (1,25-[OH]2-D3) in PBS-Tween 20 buffer (12) every two days. CO and PIL groups received subcutaneous injections with PBS-Tween 20 buffer on the same days. Six months after pristane induction, animals were killed and specimens including blood, spleen, liver, tibiotarsal joint and kidney were collected. The illustration of the experimental design is shown in figure 1.

Body and organ weight measurement

The animals were observed weekly for body weight measurements. At the end of the experimental period, mice were killed by cervical dislocation, and weight of organs such as spleen, liver, tibiotarsal joint and kidney was measured.

Evaluation of articular nociception

Nociception was evaluated before induction of the experimental model and on days 60, 120 and 180 after induction of the model. Nociception was assessed as Oliveira et al (13). The nociceptive mechanical threshold from the hinds paws were measured by the electronic Von Frey method (electronic Von Frey, Insight Equipamentos Ltda, Ribeirão Preto, SP, Brazil). Mice were placed in acrylic cages (12 x 20 x 17 cm) with wire grid floors in a quiet room 15-30 minutes before testing for environmental adaptation. The test consisted of evoking a hind paw flexion reflex with a handheld force transducer adapted with a tip. The investigator was trained to apply the tip in the plantar region with a gradual increase in pressure. The stimulus was automatically discontinued, and its intensity was recorded, in grams (g), when the paw was withdrawn.

Measurement of articular edema

Edema was evaluated before induction of the experimental model and on days 60, 120 and 180 after induction of the model. Hind paw edema volume was measured using a plethysmometer (Insight Ltda., Ribeirão Preto, Brazil). Briefly, this equipment is a small cylinder filled with a buffer connected to a device capable to measure the total fluid volume, the hind paw of the animal was immersed into the cylinder and the total volume added was then measured. The difference between the final volume minus the initial volume results to the paw total volume.

Determination of arthritis severity score

Mice were examined for the onset and severity of arthritis every 2 months after pristane injection. For scoring of arthritis severity, a previously published scoring system was used as follows: score scale of 0–3, where 0 = normal paw, 1 = slight swelling or erythema of the wrist/ankle joint or footpad, 2 = moderate swelling and erythema of the wrist/ankle joint or footpad, and 3 = severe swelling and erythema of the paw (14). The scores for individual limbs were summed to obtain a total clinical arthritis severity score of 12 per animal. The incidence of arthritis was determined as the percentage of mice that had developed redness or swelling in at least 1 paw.

Histological analysis of joint morphology

Hind paws were collected to confirm the development of arthritis by histological analysis with hematoxylin and eosin (HE) staining. The hind paws of the BALB/c animals were dissected and immersed in 10% buffered formalin for fixation for up to 3 days. Then, the joints were decalcified in 10% nitric acid for 24h. These tissues were dehydrated and embedded in paraffin blocks. Slices 6μm thick were arranged on microscope slides. Histopathological scoring was performed using a semi-quantitative score to evaluate individual joints and assess arthritis severity. For synovial inflammation, five high-power magnification fields were scored for the percentage of infiltrating mononuclear cells as follows: 0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%); for synovial hyperplasia: 0, absent; 1, mild (5–10 layers); 2, moderate (11–50 layers); 3, severe (>20 layers); for extension of pannus formation based on the reader's impression: 0, absent; 1, mild; 2, moderate; 3, severe; for cartilage erosion, that is, the percentage of the cartilage surface that was eroded: 0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%); and for bone erosion: 0, none; 1, minor erosion(s) observed only at high-power magnification fields; 2, moderate erosion(s) observed at low magnification; 3, severe transcortical erosion(s) (15).

Quantification of urinary protein

On day 150 after pristane induction, the animals were placed in individual metabolic cages for urine collection for a period of 12h. Urine samples were analyzed using urine test strips Sensi 10 Sensitive (Cral, Brazil) to measure total protein level. The results were expressed as mg/dL.

Kidney histopathological evaluation

After killing, one kidney from each animal was removed and fixed for 24h in 10% buffered formalin prior to paraffin embedding and sectioned at 3µm thickness in the transversal plane containing the renal long axis. Slides were stained using HE, according to standard procedures.

HE stained sections were observed with a light microscopy (Olympus, Germany) at a final magnification of 400x. The glomerular cellularity was quantified by counting the total cell nuclei per glomerulus on HE-stained slides. At least 30 glomeruli/slides were assessed, and the results were expressed as number of nuclei per glomerulus. All measurements and analyses were performed in a blind fashion and using an image analyzer (Image Pro Premier 9.1, Media Cybernetics Inc., Rockville USA).

Detection of IgG and IgM in renal tissue by immunofluorescence

Immune complex deposition in kidneys was examined by direct immunofluorescence using protocol described earlier (16). The other kidney from each animal was removed and immediately frozen in dry ice and stored in -80°C freezer. Kidney was embedded in OCT (optimal cutting temperature compound) and frozen in a cryostat at −20°C. Kidney was cut into sections of 5 mm thickness to detect IgG and IgM by immunofluorescence analyses. Frozen sections were stained for IgG and IgM with goat polyclonal antibody anti-mouse IgG-FITC (1:100 dilution; Abcam – ab97022, USA) or goat polyclonal anti-mouse IgM-Alexa Fluor 647 (1:200 dilution; Abcam – ab150123, USA) antibody. All antibodies were incubated with PBS-tween 20 containing 2% BSA in a humid incubator for 24 hours at 4°C. Sections were washed three times for 5 min using PBS-tween 20. Fluorescence intensity was scanned and quantified by ImageJ software.

Quantification of serum cytokines

Blood sample was collected by cardiac puncture at the end of the experiment (180 days), prior to euthanasia of the animals. Blood was obtained in vacutainer tubes (BD Biosciences, San Diego, CA); serum was prepared within 45 minutes of collection and stored at −80˚C for later analysis. Cytokines levels (Interleukin 2 (IL-2), IL-4, IL-6, interferon-gama (IFN-γ) and tumor necrosis factor-alpha (TNF-α)) were

analyzed using a ProcartaPlex High Sensitivity mouse multiplex assay, according to the manufacturer's instructions. Events were counted on a Luminex® 200™ system (Luminex, Austin, TX, USA) and cytokine concentrations in the samples were determined using a standard curve for each cytokine. All results were obtained in duplicate.

Statistical analysis

All group results are expressed as mean ± standard deviation (SD). Clinical scores were analyzed using the non-parametric Mann-Whitney U test and histological scores were assessed using the one-way analysis of variance (ANOVA) test. Other data were compared using the Student's t-test or one-way ANOVA. P values <0.05 were considered statistically significant. Statistical analysis was performed using SPSS version 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Body weight was measured and recorded weekly. All groups showed a body weight gain over the experimental period in relation to time 0 ($p<0.0001$). PIL group presented the greatest weight gain in relation to the other groups after 30 days. VD group gained weight in a similar manner to the CO group until 90 days (3.67 ± 1.26g vs. 3.64± 1.28g). After this time the VD group increased significantly the body weight, equaling the PIL group at the end of the experimental period (Figure 2). Furthermore, vitamin D supplementation did not reduce the size and the number of lipogranuloma in the peritoneal cavity of mice in comparison with the PIL group (data not show).

Spleen, liver and kidney weight are summarized in Table 1. PIL animals present a higher spleen (0.177 ± 0.036g vs. 0.114 ± 0.011g; $p<0.01$) and liver (1.306 ± 0.110g vs. 1.157 ± 0.132g; $p<0.05$) weight in relation to the CO group. However, there was no difference in relation to the weight of the kidney among the groups. Vitamin D supplementation was not able to affectthe spleen and liver weight.

A single injection of pristane oil into the peritoneal cavity induces an erosive arthritis in BALB/c mice (17). The incidence of arthritis has increased over time. We observed an incidence of 85% in the PIL group and 43% in the VD group at the end of the experiment (Figure 3A). After 180 days of pristane oil injection, the mean clinical arthritis severity score was significantly reduced in VD mice compared to PIL

mice (Figure 3B). These results indicate that vitamin D supplementation protects against arthritis development in mice, and reduces the severity of arthritis in mice. However, there was no statistically significant difference in relation to joint pain (Figure 3C). Interestingly, PIL group had significantly higher hind paw edema volumes than CO and VD groups, observed after 180 days of immunization (Figure 3D). Synovial hyperplasia, severe leukocyte infiltration, pannus formation, and cartilage erosions were present in the joints of PIL, whereas the joints of VD mice were largely spared, showing marked reductions in the extent of synovitis and severity of joint erosion (Figure 3E). The histopathologic arthritis severity scores were significantly lower in VD mice than in PIL mice (Table 2).

After 150 days of observation, total protein in the urine of PIL mice was significantly increased compared with the CO mice ($p<0.01$). However, compared to PIL mice, VD mice showed no difference in proteinuria in the same experimental period (Figure 4A). Therefore, vitamin D appears to be unable to prevent the development of proteinuria in this PIL model.

In HE staining, pathologic kidneys features were detected through the assessment of glomerular hypercellularity. PIL mice showed significant increase of glomerular cellularity after 6 months induction compared to CO mice. The treatment of vitamin D was not unable to affect significantly the glomerular hypercellularity. These observations are summarized in Figure 4B.

Immunocomplex deposits are a primary cause of lupus nephritis. In our study, the complex deposits were detected by an immunofluorescence assay. Kidneys from the PIL mice showed the presence of immune-complex deposits in the glomeruli (Figure 5). However, vitamin D supplementation did not show any effect on immune-complex deposition. Further quantitative analysis using fluorescence intensity supported the results (Figure 5BC).

We assessed serum cytokine levels to evaluate the role of vitamin D supplementation in immunomodulation (Figure 6). Levels of IL-6, TNF-α and IFN-γ were elevated in PIL group ($p<0.05$) in response to pristane-induced inflammation when compared to the CO group.. Interestingly, vitamin D supplementation was able

to dramatically decrease serum IFN-γ levels. There is no significant diference in the levels of IL-2 and IL-4 in all groups.

DISCUSSION

SLE therapy includes corticosteroids and immunosuppressants, with varying success and usually several side effects (18). For this reason, new therapeutic strategies continue to be investigated and dietary supplements and nutritional therapy may be considered as promising therapeutic strategies for SLE patients. The PIL represents a suitable model for studying the effects of the factors other than the genetic factors which could influence the induction and/or on the progression of SLE (19). To assess the clinical symptoms and SLE disease development we demonstrated increase of IL-6, TNF-α and IFN-γ serum levels, arthritis, IgG and IgM deposition in kidney and proteinuria in PIL group. Vitamin D supplementation showed a significant improve in arthritis but did not influence on renal disease in this model.

Many reports suggested that cytokines are considered to have a critical role in SLE progression (20,21). Pro-inflammatory cytokines were related to immune dysregulation and tissue damage (20,22). IL-6, TNF-α and IFN-γ participate the differentiation of Th17 cells (23). In the present study, the levels of IL-6, TNF-α and IFN-γ in the serum were significantly increased in PIL group, corroborating with literature (24,25).

During innate immune responses IFN-γ is produced by NK and NKT cells as well as macrophages and DCs (26,27) . In adaptive immunity it is produced by CD8+ T cells in the control of infection, and by the CD4+ Th1 subset (28). SLE model universally display high IFN levels, thus providing excellent animal models to investigate this matter (29). In both the murine model and in the patients with SLE, vitamin D deficiency was associated with increased expression of ISGs. IFN-γ is one of the major mediators of several autoimmune disorders and administration of this cytokine accelerates disease progression in both mice and human lupus. Our results show high IFN-γ serum levels in PIL model, while the vitamin D supplementation influenced the production of IFN-γ in 180 days. In fact the dominance of Th1 or Th2 cytokines in SLE has been controversial until now (30). However, most studies have demonstrated that the Th1 response may play a major role in the onset and progression of SLE. Interestingly, vitamin D and its receptor were found in significant

concentrations in the T lymphocyte and macrophage populations, suggesting that the vitamin D plays an important role involved in immunomodulation on Th1 or Th2 phenotype (31).

In most studies, levels of TNF-α were found to correlate with SLE disease and lupus nephritis activity, and anti-TNF treatment showed a curative effect in a few SLE patients (32,33). Researchers also reported that the loss of immune tolerance and the production of autoantibodies in SLE patients were mediated through the TNF-α signaling pathway (34). In addition, studies have revealed that elevated serum levels of TNF-α are positively associated with disease activity as well as renal involvement in SLE patients (35,36).

Higher IL-6 levels in the urine have already been correlated with active renal inflammation and pathology (37,38). IL-6 has a range of biological activities on various target cells that plays an important role in immune regulation and inflammation. Data from several studies suggest that IL-6 plays a critical role in the B cell hyperactivity and immunopathology of human or murine models of SLE, induction of IgG production (39), and may have a direct role in mediating tissue damage (40). Consistent with previous report, we have observed a marked increase in levels of IL-6 in the PIL model (41). These results were similar to the findings of previous studies which have shown the role of increased levels of IL-6 in the pathogenesis of SLE in MRL/lpr mice (42–44).

A report demonstrated that IL-6 and TNF-α can influence the permeability of the glomerular basement membrane and alter glomerular filtration (45). In the current study we showed that vitamin D supplementation neither alleviated the renal injury of SLE nor reduced IL-6 nor TNF-α levels in serum. The vitamin D supplementation did not inhibit IL-6 secretion, and there by not suppress B-cell hyperactivity and autoantibodies production. Nevertheless, reduction of IL-6 have already been previously reported (9,46,47).

Although the essential mechanism of lipogranuloma formation remains not established yet, proinflammatory cytokines, for example IL-6, play a remarkable role in lymphoid neogenesis (lipogranuloma) and autoimmunity initiation (48,49). The recruitment of inflammatory cells in response to different chemokines and cytokines

triggered by pristane injection plays a crucial role in the development of this unique structure. Lipogranulomas are regarded as a form of ectopic lymphoid tissue, i.e., tertiary lymphoid tissue (48). Furthermore, pristane is found to activate the TLR7-MyD88 pathway on monocytes, thus upregulating IFN-γ production (6). Our results showed that vitamin D supplementation not suppressed lipogranuloma development after 180 days. These data are consistent with non-decrease in serum IL-6 levels after vitamin D treatment.

Several studies indicated that the production of different autoantibodies is dependant and controlled by different cytokine pathways (50). The intact immune system provides a balance between regulatory Th1 and Th2 cytokines; however, in the case of autoimmune diseases, such as SLE, an imbalanced immune response reflects in abnormal levels of some of them (30). The severity and mechanism of pristane-induced lupus differ from one of the lupus-prone strains and cannot be strictly balanced during the treatment. The exact combination of cytokines involved in disease progression still remains undefined, as measured cytokine levels vary greatly at the different stages of the disease and differ from healthy controls (51).

Few SLE models are characterized by arthritis, and PIL represents a inducible SLE-associated arthritis in previously healthy mice. In the model studied, joint evaluation is usually not reported by the authors. The reason may be that arthritis is erosive, which makes it more alike to rheumatoid arthritis (RA) than that observed in a SLE patient (6). As a difference from RA, the inflammatory synovial infiltrate in PIL model is dominated by granulocytes; this contrasts with arthritis in CIA mice as well as in human RA, in which macrophages are the cells that prevail (52–54). Our findings demonstrate that vitamin D treatment significantly attenuates the development of arthritis in PIL model. This was evidenced by the observed reduction in clinical scores and synovial inflammation in the VD animals. The reduction of synovial hyperplasia into the joint cavity, damage to the articular cartilage and bone erosion were observed. It has been shown that 1,25 (OH)2D3, active form of vitamin D, inhibited the maturation of dendritic cells (DCs) and the activation of Th1 cells in SLE patients (55). The ability of 1,25(OH)2D3 to suppress inflammation has been linked to its capacity to regulate DC and T-cell functions.

There are several studies that show a relationship between low levels of vitamin D and chronic pain (56). Despite the results beneficial on arthritis developed by animal model, we did not observe improvement in nociceptive mechanical threshold. The analysis of nociceptive by electronic Von Frey method may have been a bias of this result. The values obtained by this technique are from the middle area of the plantar region of the hind paws and a more exacerbated edema was observed in the large joints of the animals, wrists and ankles, both PIL group and VD group.

One of the most common clinical feature of SLE is glomerulonephritis, which can be seen in up to 60% of all SLE patients (57). Lupus nephritis is thought to involve glomerular inflammation induced by immune complexes and complement deposition (16). This type of nephritis is detected by the presence of biomarkers for kidney damage such as proteinuria. IgG autoantibodies induced by pristane i.p. injection can promote kidney injury (6). Autoantibodies are directly targeted to a variety of nuclear components including dsDNA, single stranded DNA, chromatin, Sm, U1 small nuclear RNP (U1snRNP), Su and ribosomal P. A single dose of pristane leads to autoantibodies against the U1snRNP, RNP, Sm and Su antigens in 50-90% of these mice (5,58). Our results demonstrate an accumulation of IgG and IgM deposits in the glomeruli in PIL model. In addition, renal injury involves the release of pathogenic mediators which worsen glomerular mesangial damage, such as inflammatory cytokines (41). Moreover, glomerular morphological injury is a potent inducer for IL-6 generation (59). The inflammatory infiltration in the glomeruli, a prominent feature in both human and mice glomerulonephritis, induces the secretion of inflammatory cytokines and chemokines that contribute to the apoptosis of both glomerular mesangial cells (60–62). The PIL model display an imbalance of cytokine network with increasing level of IL-6 that, in turn, induces B cells to secret more antibodies and promote the development and progression of SLE nephritis (63).

We have shown no decreased amount of proteinuria, in IgG and IgM deposition or suppression the proliferation of glomerular mesangial cells after vitamin D supplementation. In contrast, Lemire et al., showed a remarkable reduction of proteinuria after administration of 1,25-dihydroxyvitamin D3 in MRL/lpr mice (9). In addition, Deluca et al. observed the preventive effects of 1,25-dihydroxyvitamin D3 on the proteinuria and pathologic renal disease in the same model (64).

There were some limitations in this study. First, in this study we chose vitamin D supplementation through subcutaneous injections, whereas generally vitamin D treatment occurs through the i.p. via. We chose to treat the animals subcutaneously via, because the experimental model is induced by an i.p. injection of 500ul pristane. Different routes of administration may have different absorption from treatments. Second, significant renal tissue lesions are the last to be manifested in this model and our study included an experimental period of 180 days after induction. A longer experimentation period (240 to 360 days after induction) could lead to more severe renal damage. Third, in this study we had some losses of animals throughout the experimental period, which reduced our sample. Some analyzes, such as cytokine levels, had a lot of variation, leading to a result with no statistical significance.

In summary, vitamin D supplementation had protective effect on arthritis but did not influence kidney disease in a pristane-induced lupus model. These results support that the role of vitamin D may be different depending on acting site, what could explain different responses according clinical phenotype. Vitamin D effect on inflammatory pathways is still under investigation and not fully elucidated. Therefore, further investigations of vitamin D are needed to explore the supplement dosage, timing, and the molecular basis in SLE.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The present study was approved by the Animal Ethics Committee of Hospital de Clínicas de Porto Alegre (HCPA, Porto Alegre, RS, Brazil) and was conducted in accordance with National Institutes of Health guidelines.

DISCLOSURE STATEMENT

The authors declare that they have no competing interests.

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Table 1. Organs weight of control (CO), pristane-induced lupus (PIL) and pristane-induced lupus + vitamin D (VD) mices.

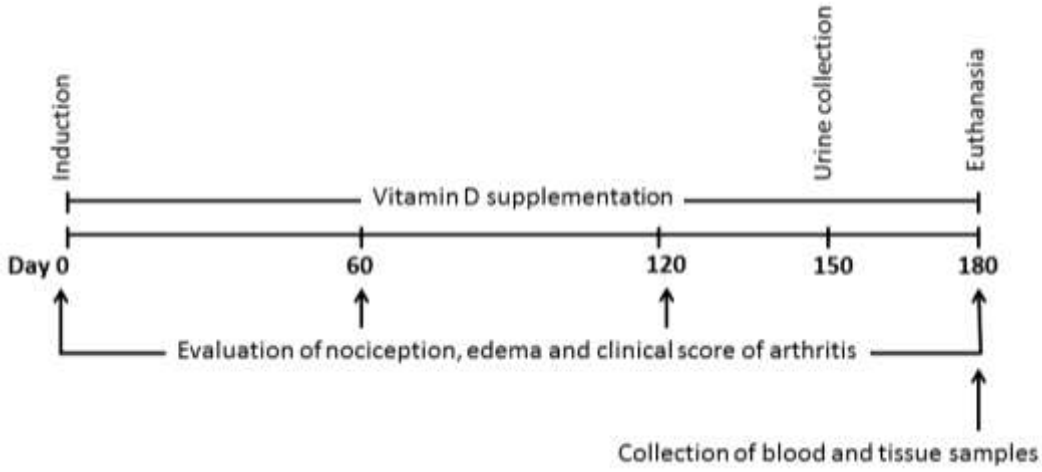
	CO group (n=7)	PIL group (n=9)	VD group (n=7)
Spleen (g)	0.114 ± 0.011	0.177 ± 0.036**	0.163 ± 0.024***
Liver (g)	1.157 ± 0.132	1.306 ± 0.110*	1.428 ± 0.394
Kidney (g)	0.163 ± 0.016	0.169 ± 0.013	0.176 ± 0.019

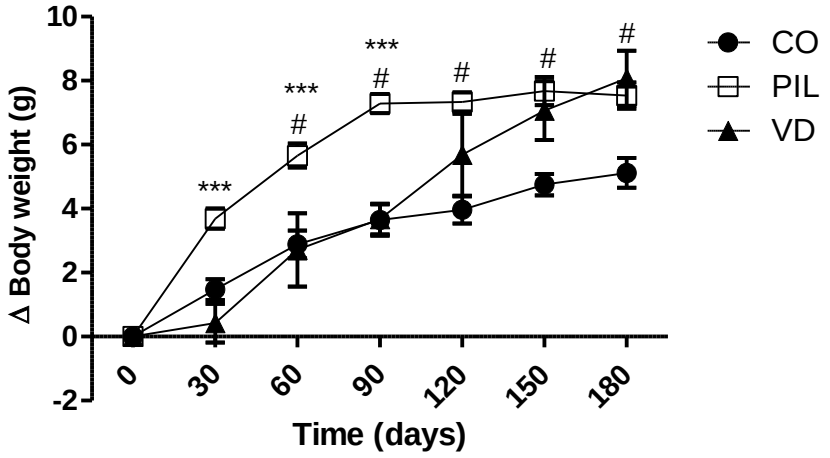
Values are expressed in mean ± SD. * $p<0.05$ vs. CO group; ** $p<0.01$ vs. CO group; *** $p<0.001$ vs. CO group.

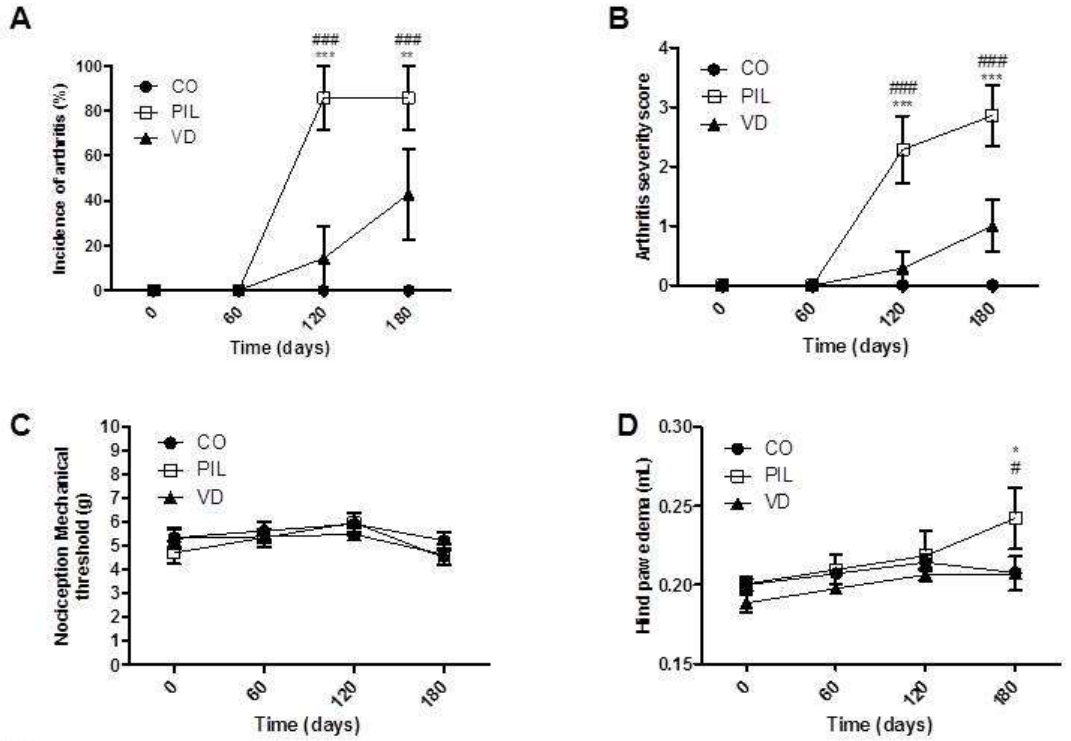
Table 2. Histopathology parameters of ankle joints from mice in the control (CO), pristane-induced lupus (PIL) and pristane-induced lupus with vitamin D (VD) groups.

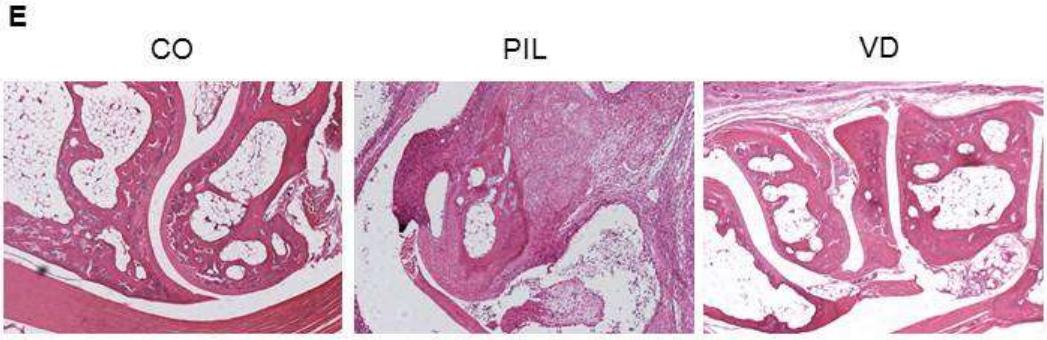
	CO group (n=7)	PIL group (n=9)	VD group (n=7)
Inflammatory infiltration	0 (0,0)	3 (2,3)*	2 (0,3)
Synovial hyperplasia	0 (0,0)	2 (2,3)*	0 (0,1)#
Pannus extension	0 (0,0)	3 (2,3)*	1.5 (0,3)
Cartilage erosion	0 (0,0)	3 (2,3)*	1 (0,2)#
Bone erosion	0 (0,0)	2 (2,2)*	0 (0,1)#

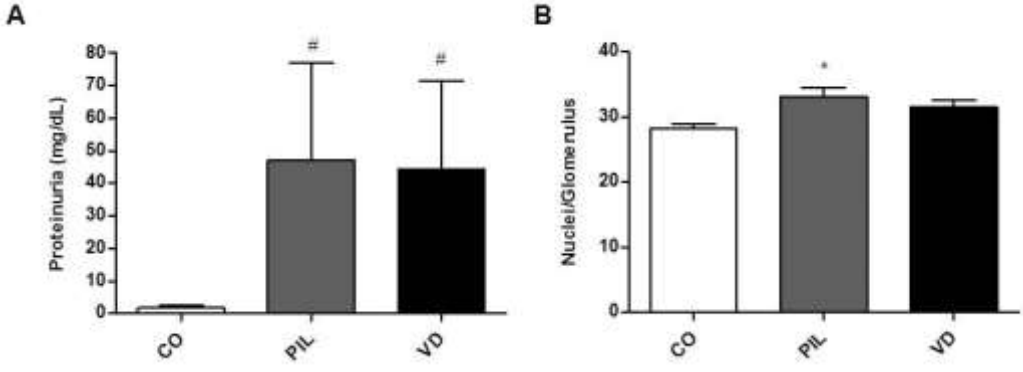
Values are the median (25th-75th percentile). Statistical analysis between groups was performed using chi-square analysis. * $p<0.05$ vs. CO group; # $p<0.05$ vs. PIL group;

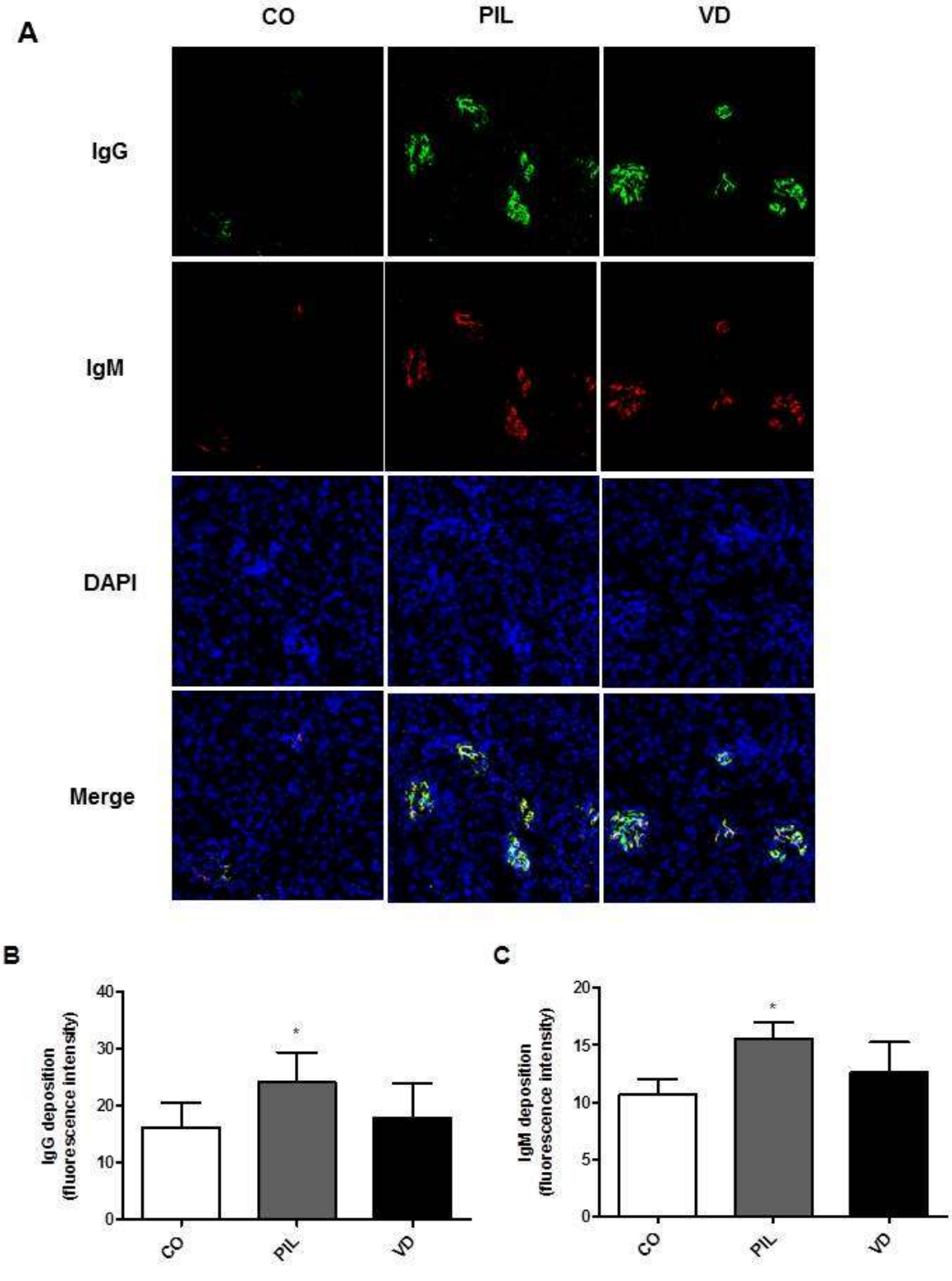

Induction
Urine collection
Euthanasia
Vitamin D supplementation
Day 0
60
120
150
180
Evaluation of nociception, edema and clinical score of arthritis
Collection of blood and tissue samples


Δ Body weight (g)
Time (days)
CO
PIL
VD
***
#


A
Incidence of arthritis (%)
CO
PIL
VD
###
***
###
**
0
60
120
180
Time (days)
B
Arthritis severity score
CO
PIL
VD
###
***
###
***
0
60
120
180
Time (days)
C
Nociception Mechanical threshold (g)
CO
PIL
VD
0
60
120
180
Time (days)
D
Hind paw edema (mL)
CO
PIL
VD
*
#
0
60
120
180
Time (days)


E
CO
PIL
VD


A
Proteinuria (mg/dL)
#
#
CO
PIL
VD
B
Nuclei/Glomerulus
*
CO
PIL
VD


A
CO
PIL
VD
IgG
IgM
DAPI
Merge
B
IgG deposition (fluorescence intensity)
40
30
20
10
0
*
CO
PIL
VD
C
IgM deposition (fluorescence intensity)
20
15
10
5
0
*
CO
PIL
VD

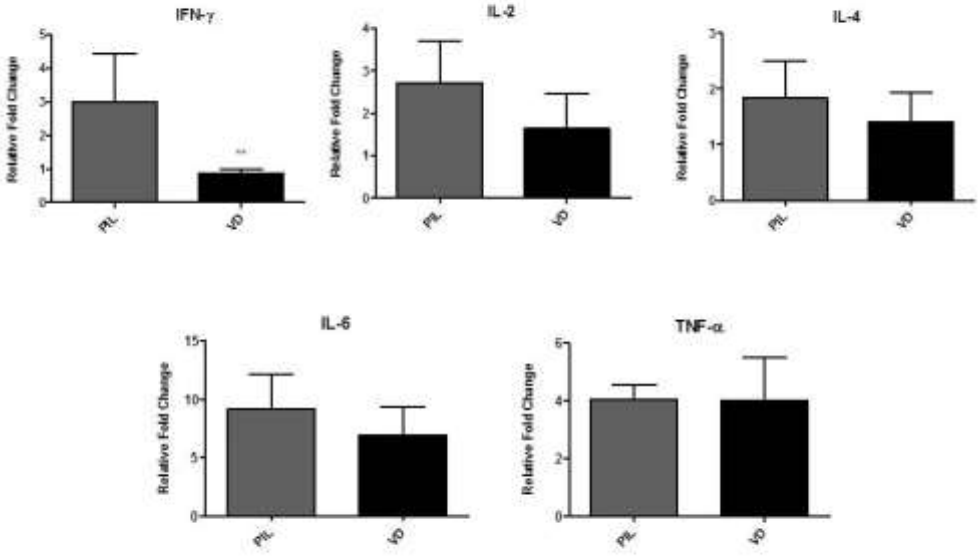


Figure 1. Illustration of experimental design.

Figure 2. Delta body weight of control (CO), pristane-induced lupus (PIL) and pristane-induced lupus + vitamin D (VD) animals. *** PIL vs. VD: p<0.001 and #CO vs. PIL: p<0.05. Data are shown as the mean ± SD.

Figure 3. Clinical analysis evaluated at 0, 60, 120 and 180 days after induction and joint histology in HE. (A) Incidence of arthritis, calculated as the percentage of mice that developed redness or swelling in at least 1 paw among all mice in each group. (B) Mean clinical arthritis severity score. The arthritis severity score (scale of 0–3) evaluated the severity of erythema/swelling in the wrist or ankle. (C) Nociceptive mechanical threshold from the hind paw measured by the electronic Von Frey method, recorded in grams (g). (D) Hind paw edema volume measured in plethysmometer, recorded in milliliters (mL). (E) Representative histopathology of ankle joint in control (CO), pristane-induced lupus (PIL) and pristane-induced lupus treated for six months with vitamin D (VD) groups at 180 day after immunization. Results are the mean ± SD. #CO vs. PIL: P < 0.05. ###CO vs. PIL: P < 0.001.*PIL vs. VD: P < 0.05. **PIL vs. VD: P < 0.01. ***PIL vs. VD: P < 0.001, by two-away analysis of variance (ANOVA) followed by Tukey's post hoc test.

Figure 4. The graph shows proteinuria levels at 150 days after pristane induction (A) and summarizes the glomerular cellularity evaluation of control (CO), pristane-

induced lupus (PIL) and pristane-induced lupus with vitamin D (VD) groups. Results are the mean ± SD. *vs. CO: $p<0.05$; #vs. CO: $p<0.01$, by one-away analysis of variance (ANOVA) followed by Tukey's post hoc test.

Figure 5. Deposition of total IgG and IgM in kidneys of BALB/c mice in each group was observed under fluorescence microscopy (x200) (A). Fluorescence intensity of total IgG (B) and IgM (C) in kidneys of BALB/c mice in each group was analyzed and calculated by ImageJ. Data represent mean ± SD. *vs. CO = $P<0.05$.

Figure 6. Effect of vitamin D supplementation on cytokines. Cytokine concentrations were determined using the Luminex multiplex assay and are presented as fold change relative to CO group. Control group mean values for each cytokine were set at value of 1. Values are expressed as mean ± SD; Statistical analysis between groups was performed using One-away ANOVA. ** $p<0.01$ vs. PIL group; (PIL) pristane-induced lupus and (VD) pristane-induced lupus with vitamin D.

9. CONDIDERAÇÕES FINAIS

Neste estudo foi avaliada a influência da suplementação de vitamina D no desenvolvimento e evolução de LES em modelo experimental induzido por pristane. Demonstrou-se que a vitamina D foi capaz de modular os sintomas clínicos e histopatológicos da artrite, mas não alterou o curso clínico e histopatológico da doença renal, apesar de ter modificado o perfil de citocinas.

A suplementação com vitamina D [2ug/kg] foi capaz de reduzir a incidência da artrite e retardou as manifestações clínicas, diminuindo o escore de atividade articular e o edema nas patas posteriores. Entretanto, não foram observadas alterações na nocicepção articular dos animais. A vitamina D não alterou o curso da doença renal neste modelo experimental, visto que não diminuiu os níveis de proteinuria, não reduziu a proliferação mesangial glomerular e não teve influência na deposição de IC no tecido renal.

Os animais com lúpus induzido por pristane apresentaram níveis elevados de IL-6, TNF-α e IFN-ɣ, o que corrobora com os dados obtidos na literatura. A suplementação com vitamina D não alterou os níveis de IL-6 e TNF-α, mas reduziu drasticamente os níveis de IFN-ɣ. Os níveis de IL-2 e IL-4 não foram alterados. O IFN-γ é um dos principais mediadores de distúrbios autoimunes e esta correlacionado com a progressão da doença tanto em camundongos quanto em lúpus humano.

Estes resultados confirmam que o papel da suplementação de vitamina D é depende do sítio de atuação, o que poderia explicar diferentes respostas de acordo com o fenótipo clínico. Estes achados seriam explicados parcialmente pela diferença na expressão e ativação do receptor da vitamina D nas células e nos tecidos. Adicionalmente, deve-se levar em consideração que os mecanismos fisiopatogênicos também podem ser distintos de acordo com o sistema envolvido, o que de certa forma interferiria mais ou menos na influencia da suplementação de vitamina D. Ainda é necessário explorar a concentração de dose adequada e segura, o tempo de tratamento e a influência da vitamina D nas diferentes bases moleculares no LES.

10. PERSPECTIVAS FUTURAS

Este estudo implementou e padronizou o modelo de lúpus induzido por pristane no Laboratório de Doenças Autoimunes (LABDAI) do Centro de Pesquisa Experimental (CPE) vinculado ao Serviço de Reumatologia do Hospital de Clínicas de Porto Alegre (HCPA). A partir disto, teve origem uma linha de pesquisa em lúpus experimental.

Na continuidade deste trabalho, pretendemos investigar:

- A composição corporal (tecido muscular e adiposo) no modelo de lúpus induzido por pristane;
- A influencia da vitamina D combinada com exercício físico no desenvolvimento e evolução do lúpus induzido por pristane;
- O papel da vitamina D no dano do DNA no hipocampo dos animais com lúpus induzido por pristane;
- A possibilidade deste modelo experimental permitir o estudo de manifestações neuropsiquiátricas do LES;
- A interação entre os receptores nucleares, VDR e PPAR-ɣ, na polarização de macrófagos M1/M2 no modelo de lúpus induzido por pristane.

11. CONSIDERAÇÕES GERAIS

A presente tese de doutorado é fruto do trabalho realizado no Laboratório de Doenças Autoimunes no Centro de Pesquisa Experimental vinculado ao Serviço de Reumatologia do Hospital de Clínicas de Porto Alegre. A participação de professores, médicos contratados, veterinários, outros funcionários contratados, alunos e bolsistas de iniciação científica foi fundamental para a idealização e realização desta pesquisa.

Durante o programa de pós-graduação que iniciou em janeiro de 2015, o autor participou dos seguintes publicações:

1. Cavalheiro R, Miranda J, Silva DS, Oliveira V, Teixeira N, Vinicius P, et al. Individualized moderate aerobic exercise improves physical capacity and prevents weight loss in collagen-induced arthritis. Int J Clin Exp Med. 2016;9(11):22696–703.

2. Freitas EC, de Oliveira MS, Monticielo OA. Pristane-induced lupus: considerations on this experimental model. Clin Rheumatol. 2017;36(11):2403–14.

3. Alabarse PVG, Lora PS, Silva JMS, Santo RCE, Freitas EC, de Oliveira MS, et al. Collagen-induced arthritis as an animal model of rheumatoid cachexia. J Cachexia Sarcopenia Muscle. 2018;9(3):603–12.

4. Silva JM de S, Alabarse PVG, Teixeira V de ON, Freitas EC, de Oliveira FH, Chakr RM da S, et al. Muscle wasting in osteoarthritis model induced by anterior cruciate ligament transection. PLoS One. 2018;13(4):e0196682.

12. ANEXO 1 – CONSORT 2010 GUIDELINE

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item N°	Checklist item	Reported on page N°
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	12
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	16
	2b	Specific objectives or hypotheses	46
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	83
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	83
Participants	4a	Eligibility criteria for participants	83
	4b	Settings and locations where the data were collected	83
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	83
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	83
	6b	Any changes to trial outcomes after the trial commenced, with reasons	83
Sample size	7a	How sample size was determined	83
	7b	When applicable, explanation of any interim analyses and stopping guidelines	83
Randomisation:			

Sequence generation	8a	Method used to generate the random allocation sequence	N/A
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	N/A
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	N/A
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	N/A
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	83
	11b	If relevant, description of the similarity of interventions	83
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	83
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	83
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	83
	13b	For each group, losses and exclusions after randomisation, together with reasons	83
Recruitment	14a	Dates defining the periods of recruitment and follow-up	N/A
	14b	Why the trial ended or was stopped	N/A
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	N/A
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	N/A
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	N/A

	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	N/A
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	N/A
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	97
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	97
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	97
Other information			
Registration	23	Registration number and name of trial registry	N/A
Protocol	24	Where the full trial protocol can be accessed, if available	N/A
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	98

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.