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Gustavo Flores Chapacais

EFEITO DA SUPLEMENTAÇÃO COM VITAMINA D SOBRE A EXPRESSÃO DE PPAR-γ E VDR NO TECIDO ESPLÊNICO DE CAMUNDONGOS COM LÚPUS INDUZIDO POR PRISTANE

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RESUMO

Lúpus eritematoso sistêmico (LES) é uma doença autoimune crônica de caráter inflamatório sistêmico e de etiologia pouco conhecida, apresentando uma diversidade de órgãos e sistemas acometidos. Estão envolvidos no desencadeamento e desenvolvimento da doença fatores ambientais, genéticos e hormonais. O resultado das manifestações do LES é uma resposta imune exacerbada contra antígenos do próprio organismo, através da ação tanto dos mecanismos do sistema imune inato quanto do sistema imune adaptativo. Como consequência, são depositados, nos tecidos, imunocomplexos. Um dos principais processos envolvidos na patogenia da doença é a inflamação crônica, cuja resposta persistente é promovida pelo sistema imune indevidamente ativado, podendo desencadear perda de função dos órgãos afetados. Um dos tipos celulares envolvidos nesse processo são os macrófagos – células fagocíticas derivadas dos monócitos circulantes – que residem estrategicamente nos tecidos. Eles apresentam perfis distintos, M1 e M2, cujas funções são pró-inflamatória e anti-inflamatória, respectivamente. Para se estudar o LES, recorre-se a modelos animais. Um destes é o de lúpus induzido por pristane (PIL), um hidrocarboneto capaz de desencadear resposta autoimune em camundongos. Neste trabalho, procurou-se testar, em modelo PIL, o efeito da vitamina D (VD) sobre a expressão proteica de dois receptores nucleares (RNs) envolvidos na imunomodulação: receptor ativado por proliferadores de peroxissoma do tipo gama (PPAR-γ), presente em macrófagos M2, e receptor de vitamina D (VDR), presente em diversas células do sistema imune. Para isso, foram analisadas lâminas de imunohistoquímica, para os dois marcadores, em amostras de baço de 23 camundongos BALB/c fêmeas, divididos em três grupos: controle (CO), lúpus induzido por pristane (PIL) e PIL com suplementação de vitamina D (VD). Tanto para a expressão de PPAR-γ quanto para a de VDR, não houve diferença estatisticamente significativa entre os três grupos. Por outro lado, foi constatada correlação positiva entre as expressões de PPAR-γ e VDR no grupo CO (r=0,643) e correlação negativa no grupo VD (r=-0,071), embora os testes não tenham apresentado significância estatística. Isso pode ser explicado pelo fato de os dois RNs competirem pela ligação com outro RN, o RXRα, para a translocação para o núcleo da célula. Na presença de suplementação com VD, o VDR pode ter se sobreposto ao PPAR-γ nessa competição. Este é o primeiro estudo a avaliar a relação entre esses dois receptores no modelo PIL.

Palavras-chave: lúpus eritematoso sistêmico; PPAR gama; receptor de vitamina D; vitamina D.

ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with a systemic inflammatory nature and underknown etiology, affecting a diversity of organs and systems. Environmental, genetic and hormonal factors are involved in the triggering and development of the disease. The result of SLE is an exacerbated immune response against self-antigens, by the action of both the mechanisms of the innate immune system and the adaptive immune system. As a result, immune complexes are deposited in tissues. One of the main processes involved in the pathogenesis of the disease is chronic inflammation. Its persistent response is promoted by the improperly activated immune system, which can lead to organ damage. One of the cell types involved in this process are the macrophages – phagocytic cells derived from circulating monocytes – which reside strategically in tissues. They present distinct profiles, M1 and M2. Their functions are pro-inflammatory and anti-inflammatory, respectively. To study SLE, animal models are used. One of those is pristane-induced lupus (PIL), a hydrocarbon-based model capable of triggering an autoimmune response in mice. In this work, we tried to test, in a PIL model, the effect of vitamin D (VD) on the tissue expression of two nuclear receptors (NRs) involved in immunomodulation: peroxisome proliferator-activated receptor gamma (PPAR-γ), expressed in M2 macrophages, and vitamin D receptor (VDR), expressed in several immune cells. For this, immunohistochemistry slides were analyzed for the two markers in spleen samples from 23 female BALB/c mice, divided into three groups: control (CO), pristane-induced lupus (PIL) and PIL with vitamin D supplementation (VD). For both PPAR-γ and VDR tissue expressions, there was no statistically significant difference among the three groups. On the other hand, a positive correlation was observed between the tissue expressions of PPAR-γ and VDR in the CO group (r=0.643) and a negative correlation in the VD group (r=-0.071), although the tests have not showed statistical significancy. This can be explained by the fact that the two NRs compete for binding with another NR, RXRα, for translocation to the cell nucleus. In the presence of VD supplementation, VDR may have overlapped PPAR-γ in this competition. This is the first study to assess the nexus between these two receptors in the PIL model.

Keywords: systemic lupus erythematosus; PPAR gamma; vitamin D receptor; vitamin D.

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LISTA DE ABREVIATURAS E SIGLAS

ACR	*American College of Rheumatology*
ANA	Anticorpo antinuclear
AR	Artrite reumatoide
C1q	Complemento 1q
C3	Complemento 3
C4	Complemento 4
CD163	Receptor do complexo hemoglobina-haptoglobina
CD206	Receptor de manose tipo C
dsDNA	DNA de fita dupla
EBV	Vírus Epstein-Barr
EULAR	*European League Against Rheumatism*
IFN	Interferon
IFN-α	Interferon alfa
IFN-β	Interferon beta
IFN-γ	Interferon gama
IgG	Imunoglobulina G
IL-12	Interleucina 12
IL-1β	Interleucina 1 beta
IL-21	Interleucina 21
IL-23	Interleucina 23
IL-4	Interleucina 4
IL-6	Interleucina 6
IL-8	Interleucina 8
LECA	Lúpus eritematoso cutâneo agudo
LECS	Lúpus eritematoso subagudo
LED	Lúpus eritematoso discoide
LES	Lúpus eritematoso sistêmico
NET	Armadilha extracelular de neutrófilo
NOD	Proteína com domínio de ligação a nucleotídeos e oligomerização
NPSLE	Lúpus eritematoso sistêmico neuropsiquiátrico
PAMP	Padrão molecular associado a patógenos
PIL	Lúpus induzido por pristane

PPAR-γ	Receptor ativado por proliferadores de peroxissoma gama
PPRE	Elemento responsivo aos proliferadores de peroxissoma
RN	Receptor nuclear
RNP	Ribonucleoproteína
RXR	Receptor do retinoide X
SLICC	*Systemic Lupus International Collaborating Clinics*
Sm	Smith
ssDNA	DNA de fita simples
Th2	Linfócito T auxiliar do tipo 2
TLR	Receptor do tipo *toll*
TLR7	Receptor do tipo *toll* 7
TNF	Fator de necrose tumoral
UV	Radiação ultravioleta
VD	Vitamina D
VDR	Receptor de vitamina D
VDRE	Elemento responsivo à vitamina D
Ym1	Proteína tipo quitinase-3

SUMÁRIO

1 INTRODUÇÃO COMPREENSIVA

1.1 LÚPUS ERITEMATOSO SISTÊMICO

Lúpus eritematoso sistêmico (LES) é uma doença autoimune crônica de caráter inflamatório sistêmico e de etiologia pouco conhecida. É caracterizada pela heterogeneidade de manifestações, variando extensamente entre diferentes portadores (TRENTIN *et al*., 2021). Deste modo, a doença se apresenta como um desafio no cotidiano da prática clínica e nas atividades acadêmicas e de pesquisa.

Os dados de prevalência e incidência da doença variam geograficamente em escala mundial, tanto por conta de diferenças de desenhos de estudos realizados, quanto pelas diferenças étnicas das populações. Rees *et al.* (2017), em uma revisão sistemática sobre estudos epidemiológicos envolvendo o LES, mostraram que essa incidência é maior na América do Norte (23,2 para cada 100 mil pessoas) e menor no continente africano (0,3). Ainda, países europeus apresentaram menores taxas de incidência do que países asiáticos.

No Brasil, os estudos epidemiológicos sobre o lúpus são escassos. Pereira Vilar e Sato (2002) estimaram a incidência da doença em Natal/RN como sendo de 8,7 para cada 100 mil pessoas por ano, sendo de 14,1 para mulheres e 2,2 para homens. Em outro estudo brasileiro, dos 3038 indivíduos pesquisados, 7,2% receberam diagnóstico para alguma doença reumática após avaliação médica. A prevalência estimada para o LES foi de 0,098% (SENNA *et al.*, 2004).

A incidência de LES é maior em mulheres do que em homens, numa proporção média de 9:1 (PONS-ESTEL *et al.*, 2010), e acomete especialmente aquelas em idade fértil (TSOKOS, 2011). Uma das hipóteses para isso implica em fatores endócrinos possivelmente assumirem um papel no desenvolvimento da doença. A propósito, em 2005, estudo randomizado duplo-cego com placebo apresentou como resultado que a administração de estrógenos em mulheres com LES aumentava a atividade da doença (BUYON *et al.*, 2005).

Diferenças étnicas também são relevantes nas manifestações do LES. Pacientes de ascendência africana, ameríndia e asiática apresentam atividade de doença mais intensa e desenvolvem o lúpus em idades mais jovens. A frequência e diversidade de sintomas e a gravidade do dano aos órgãos afetados também variam de acordo com a etnia (GONZÁLEZ; TOLOZA; ALARCÓN, 2014).

Do mesmo modo, propõe-se que fatores ambientais também desempenhem papel importante no desencadeamento da doença. Como por exemplo, exposição à radiação

ultravioleta (UV), infecções – como pelo vírus Epstein-Barr (EBV) e outros patógenos –, uso de determinados fármacos – hidralazina, por exemplo – ou exposição a outras substâncias, como o tabaco (KAUL *et al.*, 2016) e a sílica (PARKS *et al.*, 2017).

Embora se entenda que fatores ambientais sejam imprescindíveis na patogenia da doença, sabe-se que fatores genéticos também influenciam na suscetibilidade ao LES. A hereditariedade atribuída ao lúpus é alta – maior que 66% – e o índice de ocorrência da doença entre gêmeos monozigóticos varia entre 24 e 56%, enquanto gêmeos dizigóticos possuem uma concordância de 2 a 5% (DENG; TSAO, 2010; JAMES, 2014). A influência genética sobre a doença envolve especialmente processos como degradação/metilação do DNA; vias da imunidade inata envolvendo interferon (IFN) e receptores do tipo *toll* (TLRs, na sigla em inglês para *toll-like receptors*); função, sinalização e desenvolvimento de linfócitos, neutrófilos e monócitos; apresentação antigênica e; produção de autoanticorpos (JAMES, 2014).

O resultado das manifestações do LES é uma resposta imune exacerbada contra antígenos do próprio organismo, através da ação tanto dos mecanismos do sistema imune inato quanto do sistema imune adaptativo, com produção excessiva de citocinas pró-inflamatórias, como interferon do tipo I e interleucina (IL)-6. Como consequência, há promoção da diferenciação de linfócitos B produtores de autoanticorpos e perda de tolerância, levando a um processo de inflamação crônica acentuada (TSOKOS *et al.*, 2016).

Como efeito, são depositados, nos tecidos, imunocomplexos compostos pelos autoanticorpos e seus respectivos antígenos. Os principais autoanticorpos presentes na patogenia do LES são categorizados de acordo com seu alvo: DNA e proteínas associadas, RNA e proteínas associadas e β2-glicoproteína 1 associada a fosfolipídios. Especificamente, os mais importantes autoanticorpos encontrados em amostras de soro de pacientes com lúpus são o anti-dsDNA (DNA dupla-fita) e o anti-Sm (Smith) (KAUL *et al.*, 2016), sendo altamente específicos para esta doença, ao passo que anticorpos anti-Ro, anti-La e anti-RNPs (ribonucleoproteínas) estão presentes também em outras doenças reumáticas imunomediadas (GORDON *et al.*, 2018).

Os autoantígenos que se tornam disponíveis para o desenvolvimento da doença são provenientes da apoptose de células, cujos detritos liberados – em especial, restos nucleares – não são devidamente removidos pelos mecanismos próprios para isso, quais sejam, os que envolvem células fagocíticas, como macrófagos e células dendríticas. Em pessoas saudáveis, não se espera que esses antígenos próprios desencadeiem resposta imunológica, mas em pacientes com LES o mecanismo de limpeza apresenta falhas que antecedem o

desenvolvimento da doença crônica (JAMES, 2014). Isso ocorre especialmente em função da ativação inadequada de TLRs (TSOKOS *et al.*, 2016), levando à sinalização de células B autorreativas (MA *et al.*, 2019).

Consequentemente, a diversidade de órgãos e sistemas afetados pelas manifestações da doença é variada, mas os mais acometidos são a pele, os rins, as articulações, o sangue e o sistema nervoso. O paciente também pode apresentar anormalidades nos fenótipos e funções não só de células da linhagem mieloide (como os fagócitos mencionados anteriormente) como também nas de linhagem linfoide (plasmócitos e linfócitos T e B); desbalanço entre citocinas e quimiocinas pró e anti-inflamatórias; heterogeneidade na frequência e funcionalidade de linfócitos reguladores e; heterogeneidade de autoanticorpos (DÖRNER; FURIE, 2019).

Em 1982, o *American College of Rheumatology* (ACR) definiu 11 critérios para auxiliar no diagnóstico do lúpus, os quais foram revisados pelo próprio ACR em 1997 e, em 2012, pelo *Systemic Lupus International Collaborating Clinics* (SLICC). De acordo com os critérios, o paciente precisa ter quatro ou mais dos sintomas definidos, sendo pelo menos um critério clínico e um critério laboratorial ou imunológico (TAN *et al.*, 1982; PETRI *et al.*, 2012).

Contudo, em 2019 a EULAR (*European League Against Rheumatism*) e o ACR realizaram uma nova revisão dos critérios para LES, propondo como obrigatório que o paciente tenha apresentado pelo menos uma vez anticorpo antinuclear (ANA) positivo. Além disso, critérios adicionais foram agrupados em “clínicos” e “imunológicos”, com pesos aferidos de 2 a 10. Pacientes com uma pontuação maior do que 10 atendem à classificação (ARINGER *et al.*, 2019). A lista de manifestações encontra-se na tabela 1.

Tabela 1 – Critérios diagnósticos para o LES, segundo a EULAR e o ACR

Domínio Clínico	Item	Pontuação
Constitutivo	Febre	2
Hematológico	Leucopenia	3
	Trombocitopenia	4
	Hemólise autoimune	4
Neuropsiquiátrico	Delírio	2
	Psicose	3
	Convulsão	5
Mucocutâneo	Alopecia	2
	Úlceras orais	2
	LECS/LED	4
	LECA	6

Seroso	Efusão pericárdica ou pleural	5
	Pericardite aguda	6
Musculoesquelético	Envolvimento articular	6
Renal	Proteinúria >0,5g/24h	4
	Biópsia renal classe II ou V de nefrite lúpica	8
	Biópsia renal classe III ou IV de nefrite lúpica	10
Domínio Imunológico	**Item**	**Pontuação**
Anticorpos antifosfolipídio	Antifosfolipídio	2
Complemento	C3 ou C4 baixo	3
	C3 e C4 baixos	4
Anticorpos positivos específicos do LES	Anti-Sm	6
	Anti-dsDNA	6

Fonte: Aringer *et al*. (2019). O paciente atende aos critérios diagnósticos de lúpus eritematoso sistêmico ao somar mais de 10 pontos, desde que também tenha apresentado anticorpo antinuclear (ANA) positivo pelo menos uma vez na sua história clínica. LECS: lúpus eritematoso subagudo; LED: lúpus eritematoso discoide; LECA: lúpus eritematoso cutâneo agudo; C3: complemento 3; C4: complemento 4; Sm: Smith; dsDNA: DNA de fita dupla.

O tratamento para o LES leva diversos aspectos em consideração, como o perfil de órgãos atingidos, o nível de atividade da doença e a extensão dos danos (DÖRNER; FURIE, 2019). As principais classes farmacológicas utilizadas são os anti-inflamatórios esteroidais (prednisona, dexametasona e metilprednisolona) e não-esteroidais (ibuprofeno e diclofenaco, por exemplo), agentes antimaláricos (cloroquina e hidroxicloroquina), imunossupressores (azatioprina, ciclosporina, micofenolato de mofetil, metotrexato, ciclofosfamida) e moléculas imunobiológicas (belimumabe e rituximabe, por exemplo).

Além disso, pacientes são orientados a manter controle do peso corporal e estimulados a manter uma rotina de atividade física. Para prevenir ou manejar comorbidades, podem ser utilizados antiagregantes plaquetários, suplementos de cálcio, vitamina D, ácido fólico, vitamina B12, bisfosfonados, anti-hipertensivos e anti-diabéticos (KUHN *et al.*, 2015).

Em geral, o tratamento utilizado no LES é indicado para a redução da ativação imune e diminuição do processo inflamatório e redução de danos, conduzindo o paciente a um estado de remissão de doença.

1.2 INFLAMAÇÃO

A inflamação é um processo promovido pelo sistema imune na defesa do organismo contra invasões e outros potenciais danos. Envolve uma série de alterações moleculares, celulares e fisiológicas. Em geral, é uma resposta que ocorre em contextos de infecção ou hipersensitividade (FUNES *et al.*, 2018). Assim, um processo inflamatório fisiológico envolve a migração de plasma e células (leucócitos) da corrente sanguínea para o local afetado, desencadeado por receptores TLR e de proteínas com domínio de ligação a nucleotídeos e oligomerização (NOD, *nucleotide-binding oligomerization-domain protein*), localizados principalmente em células fagocíticas, como macrófagos e células dendríticas, residentes dos tecidos afetados. Com isso, após o processo ser reconhecido por estas, passa-se à produção de mediadores inflamatórios que ativarão o endotélio dos vasos sanguíneos, permitindo a entrada de células granulosas para o tecido alvo. Estas, principalmente neutrófilos, são responsáveis pela secreção de substâncias tóxicas ao agente invasor, como as espécies reativas de oxigênio e nitrogênio, permitindo a posterior limpeza do tecido (MEDZHITOV, 2008; FULLERTON; GILROY, 2016).

Entretanto, um ambiente de inflamação persistente pode ser danoso, como no caso de uma doença como o lúpus em que esse processo mantém um estado permanente de prejuízo aos tecidos e órgãos acometidos. Dito isto, para que a inflamação seja um processo restrito no tempo e exerça sua função fisiológica, precisa se limitar a um conjunto de estágios, quais sejam, iniciação, inflamação, resolução e restauração tecidual (FUNES *et al.*, 2018).

A resolução da inflamação diz respeito a uma mudança no perfil fenotípico da função de células do sistema imune adaptativo, com o objetivo de promover reparo e equilíbrio, prevenindo a inflamação crônica e dano permanente (CROASDELL *et al.*, 2015). É um processo ativo classicamente definido como “o momento entre o auge do influxo das células inflamatórias para o tecido e a restauração da homeostase funcional” (FULLERTON; GILROY, 2016, p. 552).

Também há etapas envolvidas na resolução, abrangendo primeiramente o cessar da secreção de citocinas que sinalizam para o recrutamento das células inflamatórias. Em seguida, os neutrófilos anteriormente recrutados aos tecidos devem sofrer apoptose e há mudança no perfil dos macrófagos, sendo removidos por células residentes – processo conhecido como “eferocitose”. Por fim, é promovida a secreção de moléculas anti-inflamatórias e pró-resolução, abrindo caminho para o reparo e cicatrização do tecido lesado (SERHAN; CHIANG; VAN DYKE, 2008; CROASDELL, 2015; PERRETTI, 2017).

A inflamação crônica ou persistente ocorre quando os mecanismos de resolução são frustrados e o sistema imune não é capaz de combater adequadamente o agente etiológico. Isso se torna especialmente relevante quando a etiologia da inflamação é um processo autoimune e a origem é a ativação da própria imunidade. Deste modo, a inflamação crônica é um processo originado pelo persistente recrutamento de células de defesa para o tecido e a consequente secreção contínua de moléculas pró-inflamatórias. Isso incide em um ambiente de dano tecidual incessante (MEIROW; BANIYASH, 2017).

Recentemente, alternativamente ao uso de substâncias anti-inflamatórias, tem se investido em estudos sobre moléculas pró-resolução. Entre as mais proeminentes, estão as resolvinas, a adenosina e alguns mediadores gasosos, todas atuantes como autacoides. Há também as moléculas proteicas, como melanocortinas, anexina A1 e galectinas (PERRETTI e *et al.*, 2017), além das moléculas lipídicas derivadas do ácido araquidônico conhecidas como lipoxinas, membros da classe dos eicosanoides (SERHAN; CHIANG; VAN DYKE, 2008). A função das moléculas pró-resolução é a de inibir o influxo de mais neutrófilos para os tecidos e promover a ação de macrófagos na fagocitose de restos apoptóticos e microrganismos combatidos (*ibid*.).

No LES, o processo inflamatório envolvido implica a ativação do sistema complemento e o recrutamento de células mieloides e linfoides para os tecidos em que há formação dos imunocomplexos. Contudo, modelos animais demonstram particularidades nesse processo a depender do órgão analisado. Importante sublinhar também a importância da secreção de citocinas de caráter pró-inflamatório como IFNs do tipo I, IL-6, IL-12, IL-21 e IL-23. Essas moléculas medeiam a inflamação ao sinalizar para mudanças de função em células residentes dos tecidos e o recrutamento das células dos sistemas imune inato e adaptativo (KAUL *et al.*, 2016). Também há participação de IL-8 e IL-1β na sinalização da morte celular específica dos neutrófilos, a "NETose", apoptose mediada por armadilhas extracelulares de neutrófilos (em inglês, *neutrophil extracellular traps - NETs*) (PODOLSKA *et al.*, 2015).

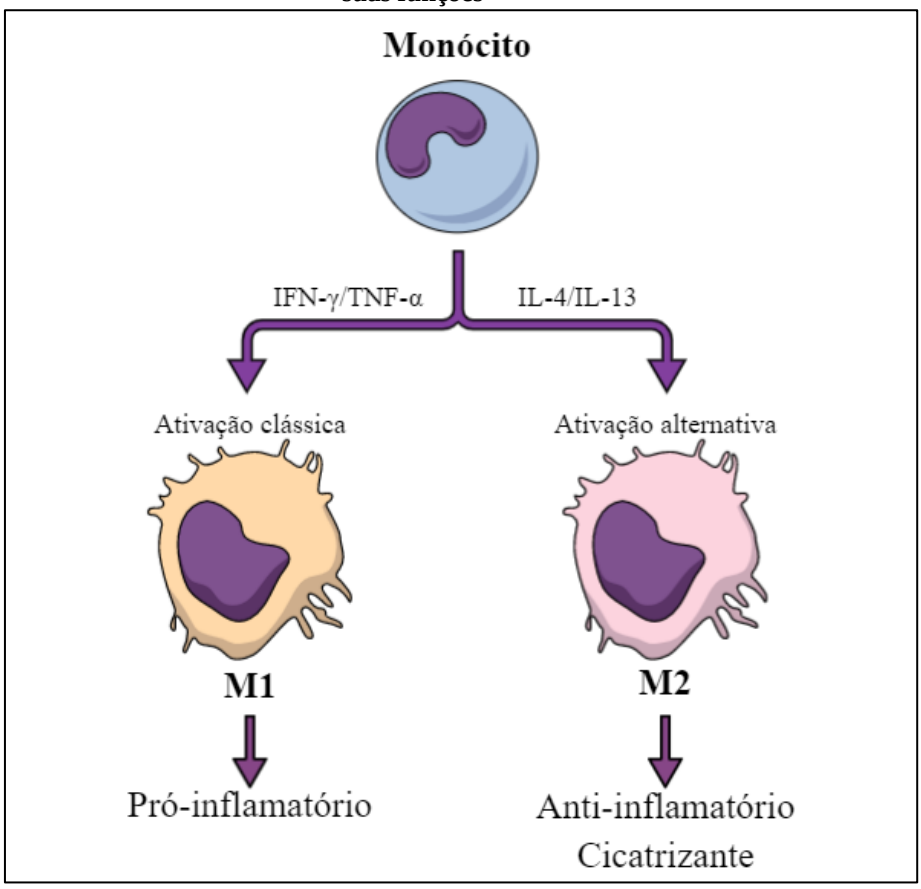
1.3 MACRÓFAGOS

Os macrófagos são células que compõem o sistema imune inato, habitando tecidos específicos, sendo recrutados durante processos inflamatórios ou atuando como residentes estratégicos para os mecanismos de defesa contra invasores. Entre suas principais funções

estão a fagocitose de patógenos, limpeza de resquícios de células apoptóticas, apresentação de antígenos a outras células imunes – como os linfócitos – e a secreção de citocinas e quimiocinas. Essas células são derivadas dos monócitos circulantes na corrente sanguínea, produzidos pela medula óssea, que ficam disponíveis para se deslocarem a tecidos comprometidos. Então, passam a se diferenciar em macrófagos (KATSIARI; LIOSSIS; SFIKAKIS, 2010), os quais apresentam morfologias diferentes a depender do tecido em que residem.

Macrófagos também apresentam perfis distintos, sendo os mais elucidados, em resumo, conhecidos como M1 e M2. Por sua vez, estes são classificados de acordo com o tipo de resposta exercida. Moléculas e citocinas excretadas durante uma infecção ou processo inflamatório, como padrões moleculares associados a patógenos (PAMPs, *pathogen-associated molecular patterns*) e interferon gama (IFN-γ), assim como fator de necrose tumoral (TNF, *tumor necrosis factor*) e ativadores de TLRs, induzem a ativação clássica de macrófagos, levando ao perfil M1. Por outro lado, a produção de IL-4 por linfócitos auxiliares Th2 (*helper* do tipo 2) induz a ativação alternativa – perfil M2 (Figura 1). Deste modo, sabe-se que perfis M1 estão envolvidos em contextos de infiltração tecidual e inflamação crônica, como na esclerose múltipla e no lúpus eritematoso sistêmico, enquanto o perfil M2 está envolvido na resolução da inflamação aguda via mecanismos de limpeza tecidual (MILLS *et al.*, 2000; SZANTO *et al.*, 2010; JABLONSKI, 2015).

Figura 1 – Esquema ilustrando o processo de polarização dos perfis de macrófagos M1 e M2 e suas funções



Fonte: Autoria própria, baseado em Funes *et al.* (2018). IFN-γ: interferon gama; TNF-α: fator de necrose tumoral alfa; IL-4: interleucina 4; IL-13: interleucina 13.

Para a investigação dos perfis de macrófagos em amostras biológicas, utilizam-se marcadores imunológicos baseados em anticorpos, particularmente aqueles que são específicos para o perfil que se quer observar. Para discriminar macrófagos do tipo M2, os marcadores mais utilizados são Arginase-1, Ym1 (proteína tipo quitinase-3), CD163 (receptor do complexo hemoglobina-haptoglobina) e CD206 (receptor de manose tipo C). Os dois últimos são encontrados em macrófagos de humanos e de roedores; em contrapartida, os demais são específicos de macrófagos de murinos (RÖSZER, 2015).

Outro marcador importante para a distinção de macrófagos ativados alternativamente são os receptores ativados por proliferadores de peroxissoma do tipo gama (PPAR-γ), moléculas nucleares conhecidas pelo seu envolvimento na expressão de genes implicados no metabolismo de ácidos graxos. Elas são notoriamente expressas em macrófagos estimulados por IL-4 (ODEGAARD *et al.*, 2007), mas também são encontradas em adipócitos (JANANI; RANJITHA KUMARI, 2015), plaquetas e linfócitos (CROASDELL *et al.*, 2015). Têm a função de regular a expressão gênica em resposta aos elementos responsivos aos

proliferadores de peroxissoma (PPREs, *peroxisome proliferator response elements*) (ALIMIRAH *et al.*, 2012).

Moléculas agonistas de PPAR-γ são utilizadas no tratamento de diabetes resistente à insulina e possuem propriedades imunorregulatórias e anti-inflamatórias, tendo sido testadas em modelos de doenças inflamatórias como o LES (APRAHAMIAN *et al.*, 2014). Além disso, uma deleção de PPAR-γ em macrófagos de camundongos NZBWF1 com nefrite autoimune contribuiu para a produção de anticorpos antinucleares e glomerulonefrite (RÖSZER *et al.*, 2011).

No LES, o processo de polarização dos macrófagos exerce um papel importante, uma vez que a atividade da doença induz a ativação clássica dessas células, tendo os macrófagos M1 como participantes do contexto inflamatório envolvido (MA *et al.*, 2019). A respeito disso, uma análise comparativa dos padrões genéticos de pacientes com LES ativo ou inativo mostrou diferenças na expressão gênica relacionada aos diferentes perfis de macrófagos. Naqueles em que a doença estava ativa, a expressão de genes associados ao perfil M1 era mais proeminente, enquanto em pacientes sem atividade da doença houve maior expressão de genes M2 (LABONTE *et al.*, 2018). Adicionalmente, um estudo realizado por Li *et al.* (2015) constatou que roedores com síndrome tipo lúpus, ao receberem transplante de macrófagos M2, tiveram atividade da doença reduzida. Isso demonstra o potencial de se estimular a polarização alternativa de macrófagos como terapia para o LES.

1.4 MODELO ANIMAL DE LÚPUS INDUZIDO POR PRISTANE

Modelos animais são excelentes para uma melhor compreensão dos processos envolvidos nas doenças. São essenciais para o estudo de uma doença complexa como o LES, em que diferentes tecidos e sistemas são acometidos, os quais são de difícil acesso para estudo em pacientes, mas que podem ser acessados em animais menores. Ainda, modelos animais são essenciais para se testar potenciais intervenções terapêuticas.

Modelos da doença que utilizam roedores podem ser divididos em duas grandes categorias: aqueles em que as manifestações se desenvolvem espontaneamente, devido a alterações genéticas já mapeadas no genoma das linhagens utilizadas, ou modelos em que a doença se manifesta a partir da indução por uma substância específica (LI; TITOV; MOREL, 2017). Um destes modelos é o de lúpus induzido por pristane (PIL, sigla em inglês para *pristane-induced lupus*).

O pristane é um hidrocarboneto alcano isoprenoide que, ao ser injetado intraperitonealmente em camundongos da linhagem BALB/c, ocasiona um processo inflamatório. Por ser uma substância oleosa, o pristane interage com a membrana das células do peritônio, ocasionando lipogranulomas e rompimento celular, o que libera detritos a permanecerem disponíveis para a ação do sistema imune. Com isso, são produzidos autoanticorpos antinucleares circulantes – em especial, imunoglobulinas G (IgG) contra DNA, cromatina, Sm e RNPs (REEVES *et al.*, 2009) – e formam-se imunocomplexos nos tecidos, como é observado na patogenia do LES em pacientes humanos. Igualmente, há produção excessiva de moléculas pró-inflamatórias IFN-α e β (FREITAS; DE OLIVEIRA; MONTICIELO, 2017) e outras citocinas, tais quais IL-6 e IL-12, derivadas de macrófagos (SHAHEEN *et al.*, 1999).

A ativação dos macrófagos neste modelo ocorre pela mediação da molécula do complemento 1q (C1q) após a interação dos fagócitos residentes do peritônio com o pristane injetado. Com a liberação de citocinas e quimiocinas pelas células que engolfam o alcano, há sinalização para o acúmulo de monócitos de perfil pró-inflamatório na região. Estes, por sua vez, passam a produzir IFN-I, conduzindo às manifestações autoimunes. Assim como em pacientes, o processo inflamatório se dá via sinalização de TLR, especificamente o de tipo 7 (TLR7) (CARLUCCI *et al.*, 2016).

A ação inflamatória da indução por essa substância possibilita uma síndrome crônica semelhante ao lúpus, causando nefrite, serosite e artrite. Nos animais BALB/c, o desenvolvimento das manifestações é lento, exigindo desenhos experimentais de meses de duração (CORREA FREITAS *et al.*, 2019). Assim como em humanos, no modelo PIL a doença é mais preponderante em fêmeas do que em machos (SMITH *et al.*, 2007).

Uma grande vantagem do modelo PIL em comparação a outros modelos está no fato de apresentar mais sinais e sintomas da doença humana, promovendo, nos animais, oito dos onze critérios SLICC (FREITAS; DE OLIVEIRA; MONTICIELO, 2017), como se ilustra no quadro 1. Além disso, o grupo de estudos em Lúpus Experimental do Laboratório de Doenças Autoimunes tem estudado a possibilidade de o modelo promover a síndrome neuropsiquiátrica (*neuropsychiatric systemic lupus erythematosus*, NPSLE) encontrada em pacientes com LES (KARNOPP *et al.*, 2022), possibilitando se atestar ainda maior similaridade com as manifestações observadas.

Quadro 1 – Manifestações observadas no modelo PIL

Tipo	Manifestação
Clínica	Proteinúria
	Serosite
	Artrite
	Glomerulonefrite
	Capilarite pulmonar hemorrágica
	Anemia
	Déficits de memória e de aprendizado
	Locomoção reduzida
Molecular e Bioquímica	Expressão de TNF-α, IFN-α, IFN-β, IFN-γ, IL-6, IL-12
	Autoanticorpos anti-dsDNA, anti-ssDNA, anti-Sm/RNP, anti-ribosomal P, ANA

Fonte: Karnopp *et al*. (2020). TNF-α: fator de necrose tumoral alfa; IFN-α: interferon alfa; IFN-β: interferon beta; IFN-γ: interferon gama; IL-6: interleucina 6; IL-12: interleucina 12; dsDNA: DNA de fita dupla; ssDNA: DNA de fita simples; Sm: Smith; RNP: ribonucleoproteína; ANA: anticorpo antinuclear.

1.5 VITAMINA D

A vitamina D (VD) é uma molécula utilizada pelo metabolismo humano para desempenhar diversas funções. É conhecida em suas isoformas ergosterol (D2, adquirida via dieta) e colecalciferol (D3, metabolizada pelo organismo a partir da exposição à luz ultravioleta B, como a proveniente dos raios solares). Sua função mais conhecida é sua participação no processo de reabsorção óssea (CHRISTAKOS *et al.*, 2015; ANDERSON, 2017) e no metabolismo do cálcio e do fósforo (SCOLLETTA *et al.*, 2013). Como agente imunorregulatória, acredita-se que a forma ativa da VD, calcitriol, tenha uma ação promotora das funções do sistema imune inato e inibitória do sistema imune adaptativo (IRURETAGOYENA *et al.*, 2015).

Relativamente a isso, células dendríticas têm a função de apresentadoras de antígeno para linfócitos T, assim como de prevenir reação autoimune por parte destes. Esses fagócitos são importantes alvos da VD, pois esta tem ação de inibir a diferenciação de células dendríticas, tornando-as mais tolerantes (*ibid.*). Além disso, a VD atua suprimindo atividade imune humoral e celular, regulando a proliferação, diferenciação e produção de

imunoglobulinas pelos linfócitos B, uma vez que estes expressam o receptor para vitamina D (VDR) (SCOLLETTA *et al.*, 2013; IRURETAGOYENA *et al.*, 2015).

O VDR é um fator de transcrição da superfamília de receptores nucleares (RNs), da qual fazem parte os PPAR e o receptor do retinoide X (RXR, *retinoid X receptor*). Está localizado na membrana nuclear de vários tipos celulares, sendo responsável por modular a atividade de genes alvo, ao se ligar aos elementos responsivos à vitamina D (VDREs, sigla em inglês para *vitamin D response elements*) (ALIMIRAH *et al.*, 2012). Os agonistas do VDR possuem a capacidade de inibir expressão de citocinas pró-inflamatórias e promover a secreção de moléculas com efeito anti-inflamatório sobre células residentes dos tecidos (SCOLLETTA *et al.*, 2013).

Este receptor pode se ligar ao RXR e, possivelmente, ao PPAR-γ para formar heterodímeros. Entretanto, VDR e PPAR-γ possuem atuações divergentes na adipogênese, embora reste definir como essa relação se comporta em outras situações (ALIMIRAH *et al.*, 2012), como nos processos inflamatórios e imunológicos implicados em doenças como o LES. Ambos os receptores são alvos da molécula de VD (WANG *et al.*, 2019).

Outro aspecto notável sobre a atuação desses receptores na modulação da inflamação envolve sua capacidade de se ligar ao complexo NF-κB para reprimir a expressão de genes alvo desse fator de transcrição (ADORINI *et al.*, 2007; CROASDELL *et al.*, 2015). Além disso, Wang *et al.* (2019) foram capazes de demonstrar uma relação de *feedback* positivo entre VDR e PPAR-γ quando este é estimulado por agonistas, utilizando modelo animal de artrite reumatoide (AR). Essa relação positiva permitiu a recuperação da função autofágica na AR, inibindo a resposta inflamatória e o dano articular.

Tanto VDR quanto PPAR-γ são fatores de transcrição de grande interesse na avaliação da capacidade de a VD contribuir para a mudança do tipo de resposta imune e inflamatória no LES. Isso pode envolver especialmente os diferentes perfis de ativação de macrófagos.

2 JUSTIFICATIVA

O lúpus eritematoso sistêmico é uma doença de etiologia pouco conhecida, mas significativamente incapacitante. Afeta a qualidade de vida dos pacientes acometidos, inclusive no que concerne à empregabilidade, manutenção da renda e prejuízos psicológicos, levando à baixa autoestima e falta de confiança. Sendo assim, investir em estudos sobre o LES aproxima a comunidade científica e médica de elucidar diversos aspectos desconhecidos de sua etiologia e promover melhores alternativas de manejo da doença e sua terapêutica.

Além disso, por ser uma doença de caráter inflamatório multissistêmico, a investigação de processos envolvendo fagocitose, inflamação e imunomediação contribui para o desenvolvimento de novas alternativas terapêuticas. Deste modo, este trabalho é pioneiro ao investigar a relação entre dois receptores intimamente envolvidos na resolução da inflamação – VDR e PPAR-γ – e sua possível ação sobre a polarização de macrófagos, quando estimulados pela vitamina D, em modelo animal de lúpus induzido por pristane.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o efeito da suplementação por vitamina D sobre a expressão proteica em tecido esplênico de camundongos com lúpus induzido por pristane.

3.2 OBJETIVOS ESPECÍFICOS

Avaliar:

a) expressão proteica de PPAR-γ;

b) expressão proteica de VDR;

c) correlação entre a expressão proteica de PPAR-γ e a expressão proteica de VDR.

4 ARTIGO CIENTÍFICO

O presente trabalho foi escrito em forma de artigo científico de acordo com as normas de publicação da revista *Clinical Rheumatology*.

Effect of Vitamin D supplementation on splenic tissue expression of Peroxisome Proliferator-Activated Receptor Gamma and Vitamin D Receptor in a pristane-induced lupus model

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Abstract

Introduction/objectives Chronic inflammation has a pivotal role in the pathogenesis of systemic lupus erythematosus (SLE). It is a persistent response promoted by the immune system and can lead to organ damage. Vitamin D (VD) has been investigated for its role in immunomodulation. The primary target of VD is the Vitamin D Receptor (VDR), a member of the superfamily of nuclear receptors (NRs) along with another important receptor thought to offer immunomodulatory properties: the Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ). Animal models such as the pristane-induced lupus (PIL) model in mice present as good alternatives for the objective of investigating SLE pathogenesis and potential treatment targets. Our study aims to investigate the effect of VD supplementation on the splenic tissue expression of PPAR-γ and VDR in the PIL model. ***Methods*** Female BALB/c mice were divided into three different groups: controls (CO, *n*=7), PIL (*n*=9) and PIL supplemented with VD (VD, *n*=7). Immunohistochemistry was performed for the quantification of PPAR-γ and VDR tissue expressions. ***Results*** No statistically significant differences were observed in the tissue expression for either PPAR-γ or VDR among the three groups. A positive correlation between PPAR-γ and VDR was observed in the CO group (*r*=0.643), while a negative correlation was observed in the VD group (*r*=-0.071). ***Conclusion*** Based on the results, we hypothesize that, stimulated by VD supplementation, VDR overlapped PPAR-γ for the heteromerization with RXR, which led to the negative correlation observed in the VD group.

Key words: PPAR-γ, VDR, PIL, vitamin D, spleen.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory and autoimmune disease. It is characterized by heterogeneous presentations regarding the manifestations, symptoms and affected organs and systems, varying among different patients [1]. The disease involves an exacerbated immune response against self-antigens by the action of mechanisms from both the innate immunity and the adaptive immunity. In healthy individuals the available self-antigens do not represent a threat to homeostasis, but in SLE patients there is an impaired clearance system. This cleaning system is expected to be conducted by innate immune cells such as macrophages and dendritic cells, which somehow present defectively in the disease [2–4].

Pro-inflammatory cytokines such as type 1 interferons (IFN) and types 6, 12, 21 and 23 interleukins (ILs) exert a primary role in SLE pathogenesis. In response to the action by those molecules, B lymphocytes are capable of differentiate themselves into plasmocytes that will produce and secrete self-antibodies into the blood circulation. The result of that is loss of tolerance, immune complexes accumulation on tissues and chronic inflammation [3,5].

Chronic inflammation is defined as the process in which acute inflammation persists and does not follow the usual physiologic path it is expected. In this case, the resolution phase of inflammation is not reached, persevering into tissue damage and the eventual loss of organ function [6]. This is especially relevant in a context where the activation of inflammation comes specifically from an autoimmune response, which is the case in a disease such as SLE. The inflammation involved in SLE implies the complement activation and the influx of immune cells into the tissues [5].

With the aim of searching for new molecules potentially worthwhile into action against inflammation, vitamin D (VD) has gained attention due to its immunoregulatory properties. This molecule promotes innate immunity functions and suppresses adaptive immunity response by inhibiting dendritic cells differentiation and by regulating immunoglobulins (Ig) production by B lymphocytes [7]. The role of VD in animal models of SLE is controversial, with studies showing either beneficial outcomes in skin, arthritic and renal manifestations [8–11] or dismissive ones, especially regarding nephritis [8,10,11].

The primary target of VD is the Vitamin D Receptor (VDR), which is a transcription factor found in several cellular types including lymphocytes and macrophages. It forms the superfamily of nuclear receptors (NRs) along with other molecules such as the Retinoid X Receptors (RXRs), Retinoic Acid Receptors (RARs), Peroxisome Proliferator-Activated Receptors (PPARs) and more [12]. A prominent receptor in the studies of inflammation is

PPAR gamma (PPAR-γ), especially because it is expressed in macrophages and dendritic cells presenting anti-inflammatory profiles [13,14].

Animal models have been used with the purpose of studying the SLE pathogenesis and possible treatments. The most successful one, due to its capacity of mimicking the majority of Systemic Lupus International Collaborating Clinics (SLICC) criteria, is pristane-induced lupus (PIL) [15]. This model is based on the intraperitoneal (i. p.) injection of pristane – a hydrocarbon – in mice. The consequence of this injection is an inflammatory process in the peritoneum, and ascites, leading to cell disruption and the availability of cellular debris in the circulation. Thereafter, there is production of antinuclear antibodies (ANAs) against nuclear components of the cell [16–18], as seen in the human disease.

In this study, we intended to investigate if VD supplementation influences the tissue expression of PPAR-γ and VDR in the spleen of PIL mice, as well as if there is a possible link between these two receptors in response to the treatment.

2. Materials and Methods

2.1. Ethics

The Animal Ethics Committee of Hospital de Clínicas de Porto Alegre has approved the study project under the number 2018-0603. Biological samples used in this study were obtained from a previous study (n. 2017-0011). The experiments were performed in accordance with the guidelines by the National Institutes of Health (NIH, Bethesda, MD, USA) and current Brazilian legislation regarding animal experimentation.

2.2. Animals and experimental design

Twenty-three (23) female BALB/c mice aged 8-12 weeks were used. Animals were randomly divided into three groups: control (CO, n=7), pristane-induced lupus (PIL, n=9) and pristane-induced lupus supplemented with vitamin D (VD, n=7). CO group received a single i.p. injection with 500 µL of a 0.9% saline solution whilst the PIL and VD groups received 500 µL of pristane oil (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich, St. Louis, MO, USA) [16]. During the induction procedure, mice were anesthetized with isoflurane 10% (Abbott Laboratórios do Brasil Ltda., São Paulo, SP, Brazil) and 90% oxygen.

Aditionally, mice from the VD group received subcutaneous (s.c.) injections with 100 µL of Calcijex (Abbott Labs, Chicago, IL, USA), which contains 2 µg/kg/day of calcitriol (1,25-[OH]), in phosphate-buffered saline (PBS)-Tween 20. This treatment was performed

every two days beginning at the day of induction and finishing at the end of experimentation (day 180). Simultaneously, groups CO and PIL received s.c. injections with 100 µL of PBS-Tween 20.

2.3. Biological samples

Six months after induction with pristane, animals were euthanized by inhalation of isoflurane. The spleens were collected, immersed in 10% buffered formalin for fixation for 24 hours at room temperature and then embedded in paraffin for storage.

2.4. Immunohistochemical analysis of PPAR-γ and VDR in the spleen

PPAR-γ and VDR tissue expression in the spleen was assessed by immunohistochemistry (IHC). The samples were deparaffinized and sliced before stained with rabbit polyclonal antibodies for PPAR-γ (1:300 dilution, pH 6, SAB4502262, Sigma-Aldrich, St. Louis, MO, USA) or VDR (1:500 dilution, pH 9, ab3508, Abcam, Cambridge, UK). Slides were incubated with 10 mM 6.0 pH citrate buffer for 20 minutes and heated in water bath at 95ºC for 10 minutes. Then, slides were immersed in a 5% H_2O_2-methanol solution for endogenous peroxidase blocking. Likewise, in order to block the binding of nonspecific antigen in the tissue, slides were pre-incubated for 20 minutes with 5% skim milk plus PBS. Polyclonal antibody (anti-PPAR-γ or anti-VDR) was suspended in PBD and 0.1% bovine serum albumin (BSA, CAS 9048-46-8, INLAB Confiança, São Paulo, SP, Brazil) and incubated at 4ºC overnight. Peroxidase-conjugated goat anti-rabbit IgG (1:200, AP123P, Millipore, Burlington, MA, USA), which was used as the secondary antibody, was incubated at room temperature for 1,5 hours. The chromogen solution used for immunoreactivity visualization was 3,3'-diaminobenzidine (DAB, K3468, Agilent Dako, Santa Clara, CA, USA). Prior to slides mounting, counterstaining with Mayer's hematoxylin was performed for cells nuclei observation. Images were captured using an Olympus BX51 (Tokyo, Japan) microscope and a camera. For each slide, ten photos of the splenic tissue were randomly captured, at a final magnification of 400x. DAB final color intensity was quantified and calculated using ImageJ (NIH) software.

2.5. Statistical analysis

Statistical analyses and graphics were performed using GraphPad Prism 8 (San Diego, CA, USA). All results are presented as mean ± standard deviation. Kruskal-Wallis and

Spearman's correlation were the statistical tests used in this study, assuming a 5% risk ($p \leq 0.05$).

3. Results

Spleen samples utilized in this study were obtained in the context of a previous study conducted by our same group of researchers, which demonstrated that mice with PIL developed a lupus-like syndrome. Clinical manifestations such as arthritis and glomerulonephritis, as well as immunologic manifestations such as high levels of inflammatory cytokines (IL-6, TNF-α and IFN-γ) in the serum, were observed. Plus, it was demonstrated that vitamin D is capable of ameliorate arthritis [10]. Also, another study from our group used brain samples and demonstrated a link between IgG infiltration and VDR expression in the hippocampus [19].

In the present study, immunohistochemistry analyses did not demonstrate statistically significant differences among the three groups, neither with PPAR-γ antibody expression (Fig. 1) nor with VDR (Fig. 2). The correlation between PPAR-γ and VDR tissue expressions was positive for the CO group (r=0.643) and negative for the VD group (r=-0.071), although neither of the tests showed statistical significancy (Fig. 3a and 3c). There was no correlation in the PIL group (Fig. 3b).

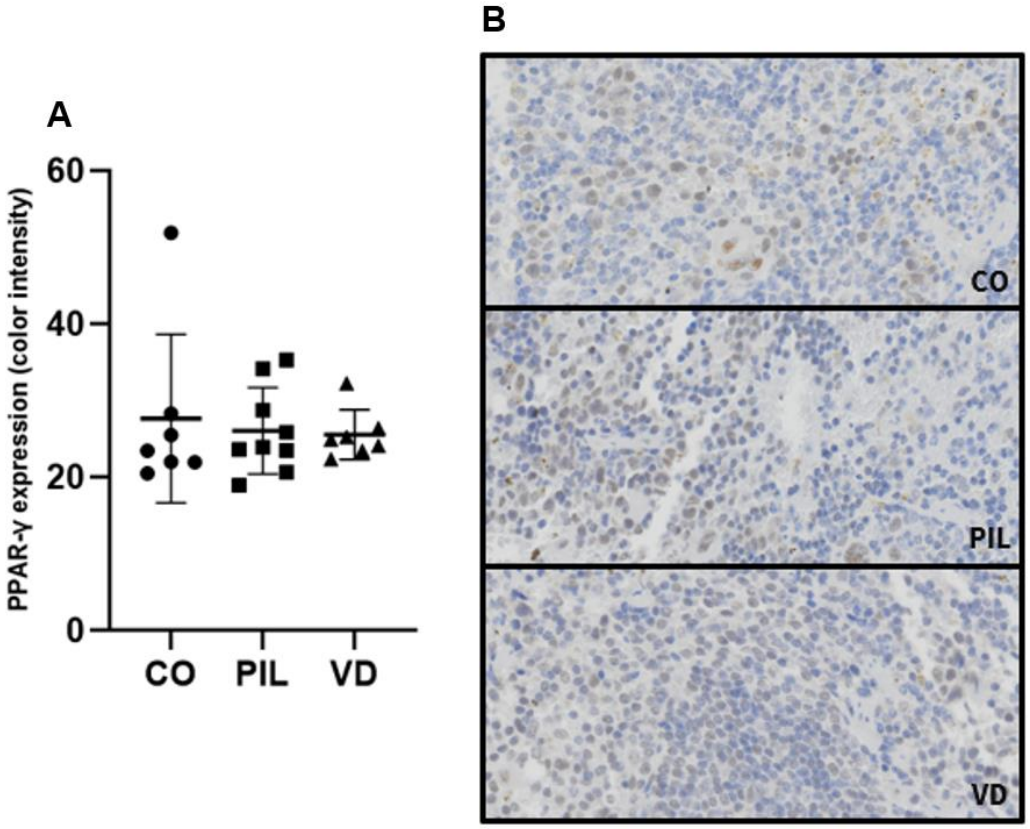

Fig. 1: Expression of Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) in the spleen of BALB/c mice. a) PPAR-γ expression (color intensity); values are expressed as means ± SD. b) PPAR-γ expression (DAB final color intensity) was observed under optical microscopy with 400x maximization and analyzed with ImageJ software. CO, controls; PIL, pristane-induced lupus; VD, pristane-induced lupus supplemented with vitamin D.

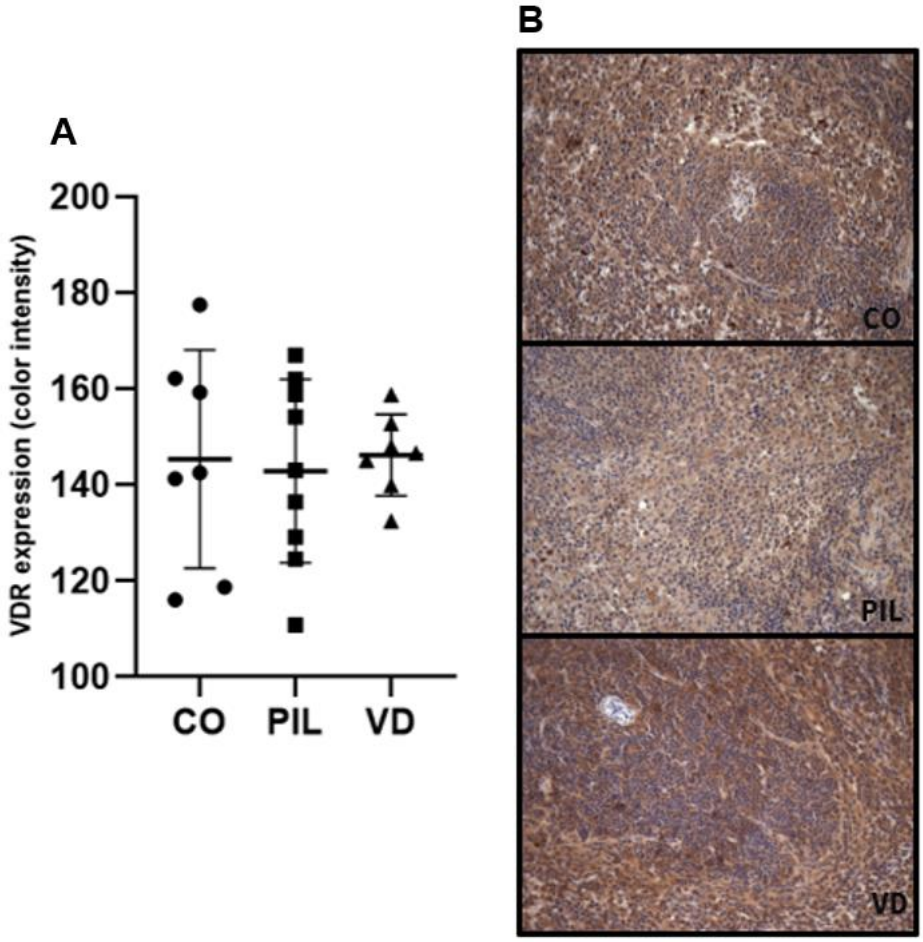

Fig. 2: Expression of Vitamin D Receptor (VDR) in the spleen of BALB/c mice. a) VDR expression (color intensity); values are expressed as means ± SD. b) VDR expression (DAB final color intensity) was observed under optical microscopy with 400x maximization and analyzed with ImageJ software. CO, controls; PIL, pristane-induced lupus; VD, pristane-induced lupus supplemented with vitamin D.

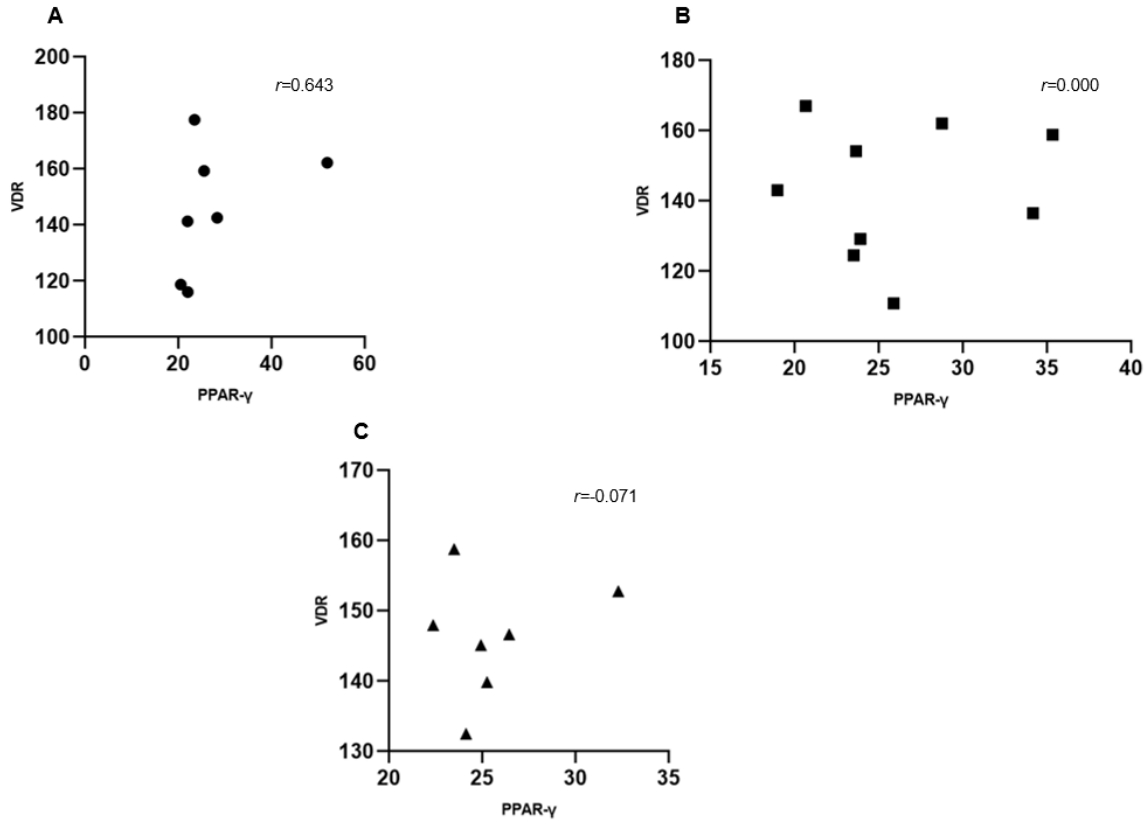

Fig. 3: Correlation between Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) expression and Vitamin D Receptor (VDR) expression in the spleen of BALB/c mice. a) Correlation within CO group; r=0.643; b) Correlation within PIL group; r=0.000; c) Correlation within VD group; r=-0.071. Spearman's correlation test.

4. Discussion

This study was not able to show differences among the three groups regarding the tissue expressions of PPAR-γ and VDR in the spleen of mice with pristane-induced lupus supplemented with vitamin D or a statistically significant correlation between the two markers. To the best of our knowledge, this is the first work to assess the nexus between these two NRs in this animal model of SLE.

Once inflammation is the main process involved in the impairment of organ function in patients with SLE, studying such occurrence is indispensable on the path to better understand this disease. Thus, both PPAR-γ and VDR seem to exert a role in the control of inflammation [20–25], which makes the study of their crosstalk a potent target.

As for the limitations imposed to this work, we cite the small sample size and the fact that this study was not designed for the purpose of evaluating these specific markers in the spleen, once it is derived from another study, led by Freitas and colleagues [10]. Moreover,

paraffin-stored samples do not allow the use of certain techniques such as flow cytometry and it makes more difficult to perform immunofluorescence tests.

Although the correlation between PPAR-γ and VDR within CO and VD groups was statistically dismissive, some hypotheses brought to surface amidst the course of this study are worth mentioning. Within the CO group, PPAR-γ and VDR tend to follow a similar path of expression without external stimuli (i. e. supplementation with VD). On the other hand, we had a group supplemented with VD in which VDR tissue expression tends to increase while PPAR-γ expression decreases.

A handful of studies show a crosstalk between these two receptors [12,26,27] and some show positive feedback within this relation [28]. Furthermore, it is known that both PPAR-γ and VDR can form heterodimers by binding to RXRα [12], another NR. Still, a study conducted by Fadel and colleagues showed *in vitro* evidence that different NRs acting as partners to RXRα compete for this ligand, with different levels of affinity to it. Nevertheless, when a specific agonist to one of the receptors is used, the cognate receptor is the preferred one for RXRα binding [29]. That said, we hypothesize that, in our model, when mice were supplemented with VD, VDR overlapped PPAR-γ in the competing for the RXRα binding.

Another study, in which the researchers treated PIL mice with retinoic acid (RA), observed the expansion of dendritic cells subsets implicated in the promotion of systemic inflammation in this model [30]. The retinoic acid receptor (RAR) is a strongly affine ligand to RXRα [29], so RA might have had a downregulating action towards receptors thought to promote positive immunoregulation, such as PPAR-γ and VDR, by making its receptor compete for RXRα against the other NRs. The lack of RXRα binding availability incapacitates NRs of translocating into the nucleus [29] to exert their roles as transcription factors.

Lastly, VDR agonists such as VD present as a potential treatment against SLE inflammation. Hence, we highlight the importance of further investigations about the crosstalk between different NRs in the PIL model, especially those that compete for the RXRα ligand, as well as their agonists.

Conflicts of interest: none.

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5 CONCLUSÕES E PERSPECTIVAS

Os resultados do estudo não demonstraram efeito estatisticamente significativo da suplementação com vitamina D sobre a expressão proteica de PPAR-γ e VDR no baço de camundongos com lúpus induzido por pristane, no comparativo entre os três grupos estudados. Por outro lado, observou-se correlação positiva entre as expressões dos dois marcadores no grupo controle (CO) e correlação negativa no grupo suplementado com vitamina D (VD). O reduzido tamanho amostral e a falta de um desenho de pesquisa específicos para os objetivos propostos podem ter sido fatores implicados na falta de resultados significativos estatisticamente.

A linha de pesquisa na qual o trabalho se inclui é pioneira, tendo em vista este ter sido o primeiro estudo a avaliar a relação entre esses dois receptores nucleares no modelo PIL, mostrando-se promissor para a melhor compreensão do processo inflamatório envolvido no LES. Como perspectivas futuras, há a intenção de expandir a experimentação, estudando também aspectos da polarização de macrófagos no mesmo tecido, para avaliar se a vitamina D contribui para a diferenciação dessas células em perfil anti-inflamatório (M2).

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ANEXO A – NORMAS DE PUBLICAÇÃO DA REVISTA *CLINICAL RHEUMATOLOGY*

As normas de publicação da *Clinical Rheumatology* foram extraídas da página da revista na *web* (https://www.springer.com/journal/10067/submission-guidelines) e estão transcritas abaixo:

Submission guidelines

Instructions for Authors

Clinical Rheumatology endorses complete and transparent reporting of biomedical and clinical research. Depending on the study, we recommended the authors to adhere to the relevant EQUATOR Network reporting guidelines when preparing their manuscript.

Types of papers

Original Research articles: word limit 4000 words, 45 references, no more than 6 figures/tables; abstracts must be structured as specified below. After the abstract, include key-points (min. of 1, max. of 4) presented in brief sentences, highlighting the contributions of the paper.

Please note: **It is mandatory that abstracts for Original Research articles adopt the below structure (150-250 words):**• Introduction / objectives – specify the context of the problem (optional), the research aim, question or purpose, and the tested hypothesis.• Method – specify the design of the study, selection criteria of patients, main outcome variable and statistical approach used to test the primary hypothesis.• Results – summarise the main results, present the results that support the hypothesis stated in Introduction providing results in numbers, not just p-values or interpretations, and other relevant results (optional).• Conclusions – summarise what the findings might imply, and the implications and recommendations for future research; conclusions should be coherent with the objectives and the results presented in the abstract.

Review articles: Provide accessible, authoritative, and balanced overviews of a field or topic. Abstract (unstructured, 150-250 words, please avoid using abbreviations and references); 4-6 keywords (please note that the word count refers to individual MeSH words

representing the main content of the article); up to 4 key-points; up to 100 references; up to 5 Tables/figures, not including supplementary material. Word limit: 5,000 words.

Brief report 2000 word limit, 25 references, no more than four figures; include key points (minimum of 1, maximum of 4) presented in short sentences, highlighting the contributions of the article. Each manuscript must be formatted as an original article, including a structured abstract.

Clinical Image: Clinical Images are a brief clinical report describing a unique image. All Clinical Images must have patient consent for publication, and the copyright form must be sent with your submission to Clinical Rheumatology, including:• Title page• Structured text including two sections: Presentation (case narrative); Discussion (brief, concise messages).• References (up to 5)• Figure (up to 1, not including supplementary material). Make sure that the figure complies with the image quality standards described in the Clinical Rheumatology instructions for authors section (artwork).• Word count: Up to 300. Provide a figure footnote that is not redundant with the main text.

Letters of Biomedical and Clinical Research: word limit 650 words, 10 references, 2 tables or figures and a discussion; summaries and supplementary material should not be included. Should present advances in highly focused, original clinical and biomedical research that is easily replicable.

Perspectives in Rheumatology: word limit 3500 words, 40 references, no more than 3 figures. Should share a clinical, methodological, scientific, or ethical point of view regarding provocative or hot topics emerging in the clinical practice of rheumatology. Unstructured abstract.

Case Based Review: a case report of extreme clinical interest incorporating a mini review in an area of new knowledge. Word limit 3500 words, 50 references, no more than 5 figures.

Letters to editor: up to 600 words

Editorial: up to 1500 words

Please note:

Case Reports are no longer accepted in the journal.

Translated questionnaires can only be included in *Clinical Rheumatology* articles if permission to use the questionnaire has been granted by the copyright holder.

Clinical Rheumatology prioritizes **systematic reviews, with or without meta-analysis, including scoping reviews**, wherever possible and appropriate. Please follow the

'Review article' guidelines. Articles should be critical, in-depth, literature-based reviews. In addition, authors must include the completed PRISMA flow chart as a cited figure as well as uploading a supplementary file of the most recent version of the appropriate PRISMA checklist for the type of systematic review, with or without meta-analysis, that was conducted. A list of current PRISMA checklists can be found here: http://prisma-statement.org/. *Clinical Rheumatology* also strongly encourages prospective registration of systematic reviews in a registry database (PROSPERO, Open Science Framework, etc.). Please note that because PROSPERO does not currently allow for the registration of scoping reviews, an alternative registry such as Open Science Framework should be considered. *Clinical Rheumatology* will also consider for publication protocols for systematic reviews, with or without meta-analysis. Prior to submission, these should be registered in a registry database such as PROSPERO, Open Science Framework, etc., as well as including as a supplementary file the PRISMA protocol checklist (PRISMA-P). If previous systematic reviews, with or without meta-analysis exist on the topic of interest, a strong case should be made for why another systematic review is needed.

Use of any writing or editing services should be noted in the Acknowledgements section.

To ensure the integrity of the reporting of patient-centered trials, authors **must register prospective clinical trials** (phase II to IV trials) in suitable publicly available repositories. For example www.clinicaltrials.gov or any of the primary registries that participate in the WHO International Clinical Trials Registry Platform. **The trial registration number (TRN) and date of registration should be included as the last line of the manuscript abstract.** For clinical trials that have not been registered prospectively, authors are encouraged to register retrospectively to ensure the complete publication of all results.

Manuscript Submission

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

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Please follow the hyperlink “Submit manuscript” and upload all of your manuscript files following the instructions given on the screen.

Source Files

Please ensure you provide all relevant editable source files at every submission and revision. Failing to submit a complete set of editable source files will result in your article not being considered for review. For your manuscript text please always submit in common word processing formats such as .docx or LaTeX.

Conflict of Interest Disclosure Form

The manuscript must be accompanied by the “Conflict of Interest Disclosure Form”. The form can be downloaded here.

Title Page

Title Page

Please make sure your title page contains the following information.

Title

The title should be concise and informative.

Author information

The name(s) of the author(s)

The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country

A clear indication and an active e-mail address of the corresponding author

If available, the 16-digit ORCID of the author(s)

If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

For life science journals only (when applicable)

Trial registration number and date of registration for prospectively registered trials

Trial registration number and date of registration, followed by “retrospectively registered”, for retrospectively registered trials

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Statements and Declarations

The following statements should be included under the heading "Statements and Declarations" for inclusion in the published paper. Please note that submissions that do not include relevant declarations will be returned as incomplete.

Competing Interests: Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Please refer to “Competing Interests and Funding” below for more information on how to complete this section.

Please see the relevant sections in the submission guidelines for further information as well as various examples of wording. Please revise/customize the sample statements according to your own needs.

Specific Remark for Original Research articles

It is mandatory that abstracts for Original Research articles adopt the below structure:

Introduction / objectives – specify the context of the problem (optional), the research aim, question or purpose, and the tested hypothesis.

Method – specify the design of the study, selection criteria of patients, main outcome variable and statistical approach used to test the primary hypothesis.

Results – summarise the main results, present the results that support the hypothesis stated in Introduction providing results in numbers, not just p-values or interpretations, and other relevant results (optional).

Conclusions – summarise what the findings might imply, and the implications and recommendations for future research; conclusions should be coherent with the objectives and the results presented in the abstract.

Text

Text Formatting

Manuscripts should be submitted in Word.

The text of a research paper should be divided into Introduction, Materials and Methods, Results, Discussion, Acknowledgements, Conflict of Interest, and References.

Materials and Methods must include statement of Human and Animal Rights.

Use a normal, plain font (e.g., 10-point Times Roman) for text.

Use italics for emphasis.

Use the automatic page numbering function to number the pages.

Do not use field functions.

Use tab stops or other commands for indents, not the space bar.

Use the table function, not spreadsheets, to make tables.

Use the equation editor or MathType for equations.

Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX. We recommend using Springer Nature's LaTeX template.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

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Always use footnotes instead of endnotes.

Acknowledgments and Funding Information

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full. In addition, please provide the funding information in a separate step of the submission process in the peer review system. Funder names should preferably be selected from the standardized list you will see during submission. If the funding institution you need is not listed, it can be entered

as free text. Funding information will be published as searchable metadata for the accepted article, whereas acknowledgements are published within the paper.

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Please always use internationally accepted signs and symbols for units (SI units).

Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

References

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].

2. This result was later contradicted by Becker and Seligman [5].

3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

The entries in the list should be numbered consecutively.

If available, please always include DOIs as full DOI links in your reference list (e.g. “https://doi.org/abc”).

Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

ISSN.org LTWA

If you are unsure, please use the full journal title.

Authors preparing their manuscript in LaTeX can use the bibliography style file sn-basic.bst which is included in the Springer Nature Article Template.

Tables

All tables are to be numbered using Arabic numerals.

Tables should always be cited in text in consecutive numerical order.

For each table, please supply a table caption (title) explaining the components of the table.

Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.

Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork

For the best quality final product, it is highly recommended that you submit all of your artwork – photographs, line drawings, etc. – in an electronic format. Your art will then be produced to the highest standards with the greatest accuracy to detail. The published work will directly reflect the quality of the artwork provided.

Electronic Figure Submission

Supply all figures electronically.

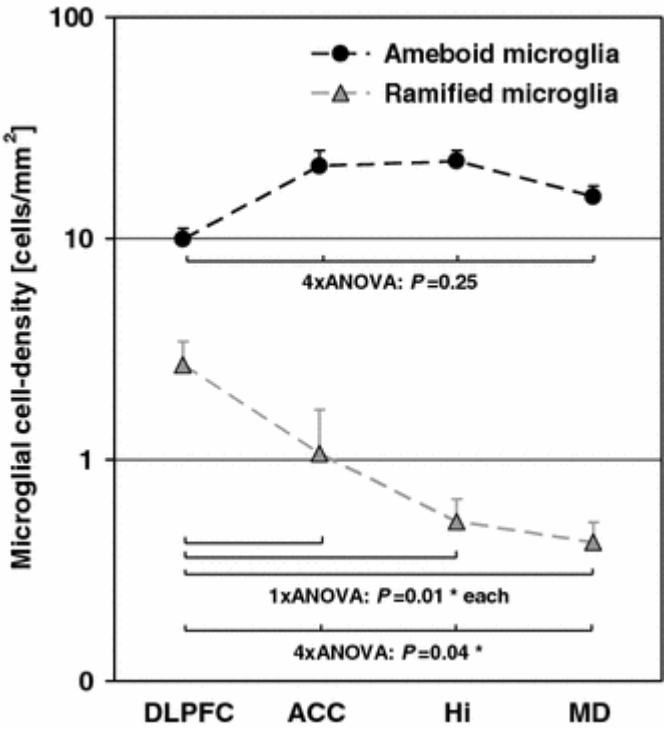
Indicate what graphics program was used to create the artwork.

For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MS Office files are also acceptable.

Vector graphics containing fonts must have the fonts embedded in the files.

Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art



Definition: Black and white graphic with no shading.

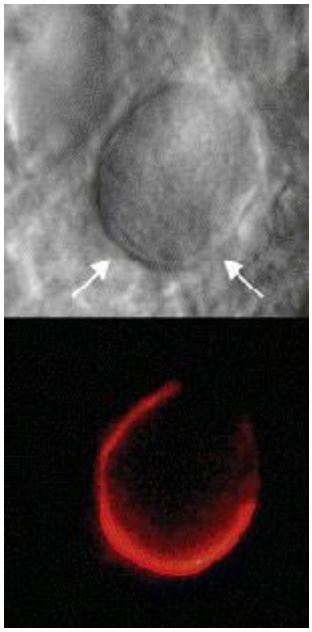
Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.

All lines should be at least 0.1 mm (0.3 pt) wide.

Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.

Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art



Definition: Photographs, drawings, or paintings with fine shading, etc.

If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.

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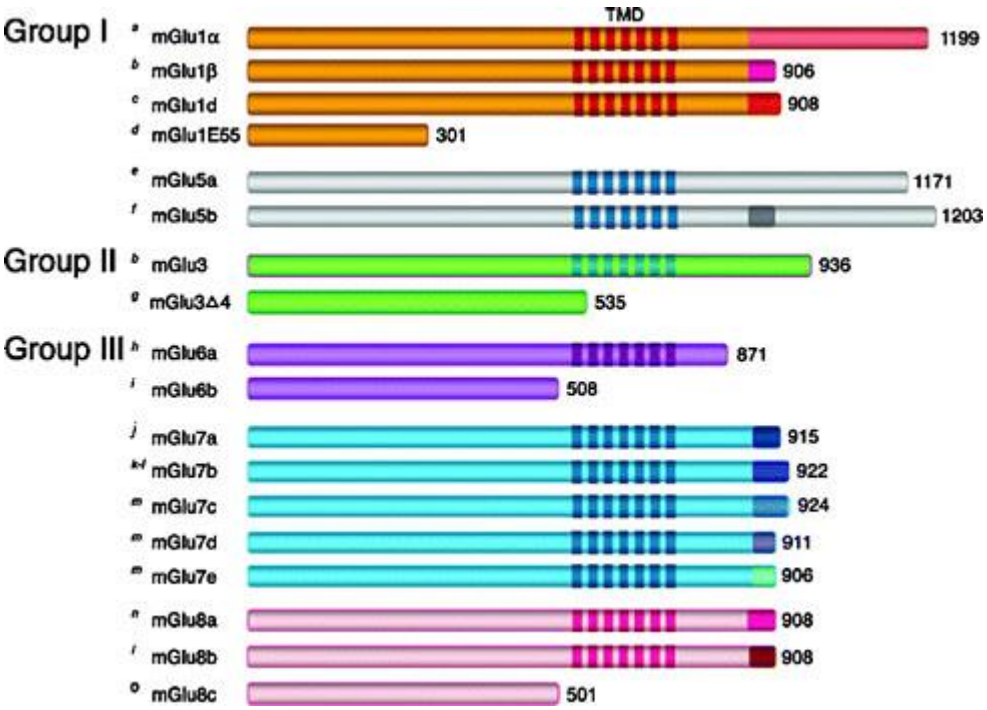

Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.

Combination artwork should have a minimum resolution of 600 dpi.

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Color art is free of charge for print and online publication.

Color illustrations should be submitted as RGB.

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To add lettering, it is best to use Helvetica or Arial (sans serif fonts).

Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).

Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.

Avoid effects such as shading, outline letters, etc.

Do not include titles or captions within your illustrations.

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Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.

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No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.

Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.

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When preparing your figures, size figures to fit in the column width.

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Patterns are used instead of or in addition to colors for conveying information (color-blind users would then be able to distinguish the visual elements)

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Springer accepts electronic multimedia files (animations, movies, audio, etc.) and other supplementary files to be published online along with an article or a book chapter. This feature can add dimension to the author's article, as certain information cannot be printed or is more convenient in electronic form.

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Supply all supplementary material in standard file formats.

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Text and Presentations

Submit your material in PDF format; .doc or .ppt files are not suitable for long-term viability.

A collection of figures may also be combined in a PDF file.

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Spreadsheets should be submitted as .csv or .xlsx files (MS Excel).

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Specialized format such as .pdb (chemical), .wrl (VRML), .nb (Mathematica notebook), and .tex can also be supplied.

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It is possible to collect multiple files in a .zip or .gz file.

Numbering

If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.

Refer to the supplementary files as “Online Resource”, e.g., "... as shown in the animation (Online Resource 3)", “... additional data are given in Online Resource 4”.

Name the files consecutively, e.g. “ESM_3.mpg”, “ESM_4.pdf”.

Captions

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Accessibility

In order to give people of all abilities and disabilities access to the content of your supplementary files, please make sure that

The manuscript contains a descriptive caption for each supplementary material

Video files do not contain anything that flashes more than three times per second (so that users prone to seizures caused by such effects are not put at risk)

Integrity of research and reporting

Ethical standards

Manuscripts submitted for publication must contain a statement to the effect that all human and animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

It should also be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted.

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The submitted work should be original and should not have been published elsewhere in any form or language (partially or in full), unless the new work concerns an expansion of previous work. (Please provide transparency on the re-use of material to avoid the concerns about text-recycling (‘self-plagiarism’).

A single study should not be split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time (i.e. ‘salami-slicing/publishing’).

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Results should be presented clearly, honestly, and without fabrication, falsification or inappropriate data manipulation (including image based manipulation). Authors should adhere to discipline-specific rules for acquiring, selecting and processing data.

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Research articles and non-research articles (e.g. Opinion, Review, and Commentary articles) must cite appropriate and relevant literature in support of the claims made. Excessive and inappropriate self-citation or coordinated efforts among several authors to collectively self-cite is strongly discouraged.

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