UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
DEPARTAMENTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

MARIA ANDREZA BEZERRA CORREIA

AVALIAÇÃO DO PERFIL DE CITOCINAS SÉRICAS E EFEITO IMUNOMODULADOR *IN VITRO* DE UM NOVO DERIVADO TIAZOLIDÍNICO LPSF/JB-20 EM PACIENTES PORTADORES DE ESPONDILITE ANQUILOSANTE

Recife
2020

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como requisito parcial para a obtenção do título de Doutor em Ciências biológicas.

Área de concentração: Biologia química para a saúde.

Orientadora: Profª. Dra. Maira Galdino da Rocha Pitta.

Coorientadoras: Profª. Dra. Claudia Dinis Lopes Marques;

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Recife

2020

Catalogação na Fonte:
Bibliotecário Bruno Márcio Gouveia, CRB-4/1788

Correia, Maria Andreza Bezerra
Avaliação do perfil de ocitocinas séricas e efeito imunomodulador *in vitro* de um novo derivado tiazolidínico LPSF/JB-20 em pacientes portadores de espondilite anquilosante / Maria Andreza Bezerra Correia. - 2020.

113 f. : il.

Orientadora: Profa. Dra. Maira Galdino da Rocha Pitta.
Coorientadoras: Profa. Dra. Claudia Dinis Lopes Marques, Dra. Marina Galdino da Rocha Pitta.
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-graduação em Ciências Biológicas, Recife, 2020.
Inclui referências.

1. Doença crônica. 2. Espondilite. 3. Farmacologia. I. Pitta, Maira Galdino da Rocha (orientadora). II. Marques, Claudia Dinis Lopes (coorientadora). III. Pitta, Marina Galdino da Rocha. IV. Título.

616.7 CDD (22.ed.) UFPE/CB-2021-069

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Aprovada em: 31 / 08 / 2020.

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A todos que contribuíram direta ou indiretamente para sua execução

Dedico

AGRADECIMENTOS

Ao Todo que a tudo permeia e se manifesta através de todos nós como pai, filho e espírito santo. E a toda proteção Divina e livramento que nos é dada a cada segundo em nossas vidas. À minha família que acreditou em mim e me ajudou na realização do trabalho. Agradeço ao Professor Dr. Ivan da Rocha Pitta por me permitir realizar este projeto. À Professora Dra. Maira da Rocha Pitta por sua atenção e orientação para a execução do projeto. Às coorientadoras, professora Dra. Claudia Marques e a Dra. Marina Pitta pelo supor para a execução do projeto. À Dra. Nara Dornelas por seu carinho e apoio nas coletas. Aos professores Moacyr Rêgo e Michelly Pereira por todo apoio e atenção. A todos os integrantes do LINAT que me receberam e me ensinaram e ensinam todos os dias. Em especial a Thiago Lins que ensinou as primeiras metodologias do laboratório com muito cuidado, atenção e dedicação, à Mayara Souza por me acolher e receber como amiga, à Flaviana Soares por sua dedicação e apreço a todos. A todos os pós-doutorandos que se dedicam e mantém o laboratório. A todos os funcionários da Universidade Federal de Pernambuco e em especial a secretária do programa a Sra. Adenilda e ao Sr. Paulo do PPGIT. Agradecimentos finais a todos que fizeram e fazem parte da coordenação do Programa da pós-graduação, Prof. Dr. Ranilso de Souza Bezerra, Profa. Dra. Maria Tereza dos Santos Correia, Prof. Dr. Valdir de Queiroz Balbino, Profa. Dra. Márcia Vanusa da Silva e Prof. Dr. Antônio Carlos de Freita. Ao apoio financeiro do Conselho Nacional de Desenvolvimento científico e tecnológico (CNPq) e à Universidade Federal de Pernambuco (UFPE) ao qual me orgulho fazer parte.

...seja princesa, ou seja, lavadeira pra ir mais alto vai ter que suar...

música: a natureza das coisas compositor: José Accioly Cavalcante Neto)

RESUMO

A espondilite anquilosante (EA) é uma doença crônica de caráter inflamatório que ao longo do tempo pode levar ao desenvolvimento de sindesmófitos e anquilose da coluna. Os tratamentos atuais são capazes de melhorar os sintomas, mas não impedem o dano estrutural, sobretudo em pacientes com atraso no diagnóstico. Os mecanismos de remodelação óssea são complexos e pesquisas vêm sendo realizadas com o intuito de esclarecer melhor a doença e encontrar novos tratamentos para os pacientes. Estudos com pacientes EA identificaram que o PPARγ pode atuar como um mediador provável dos efeitos das citocinas durante a inflamação e no processo de remodelação óssea. Os derivados tiazolidínicos (TZDs) vêm sendo estudados por atuar como agonistas PPARγ. Diante desse contexto nosso trabalho se propõe avaliar o perfil de citocinas no soro e no sobrenadante de cultura de PBMC de pacientes e voluntários sadios para investigar a resposta ao tratamento *in vitro* de novos derivados tiazolidínicos, o LPSF/JB-20. As citocinas IL-1β, IL-6, IL-17A, IL-18BP, IL-18 total, IL-23, OSM, sOSMR e sGP130 presentes no soro de 130 pacientes EA e 130 voluntários sadios foram avaliadas por kit comerciais de ELISA *sanduíche*. A atividade imunomoduladora do TZD foi avaliada no sobrenadante de cultura de PBMC estimulada com fitohemaglotinina-M e a dosagem das citocinas IL-6, IL-10, IL-17A, IFN-γ, TNF-α foram avaliadas. Ensaios de diferenciação adipogênica e modelagem molecular foram avaliadas para determinar o mecanismo de ação ao PPARγ. Os resultados mostram que a expressão de sOSMR, sGP130 e IL-18BP estava significativamente reduzida e a de IL-18 elevada, comparado com voluntários sadios. A expressão da OSM foi detectada através de Wester blotting e sua expressão foi significadamente maior comparado aos voluntários sadios. As demais citocinas avaliadas, não apresentaram expressão no soro da população de estudo. O *docking* molecular mostrou que em termos de afinidade predita, o composto LPSF/JB-20 obteve uma melhor pontuação que a rosiglitazona, ao se ligar no PPARγ. O derivado tiazolidínico apresentou baixa citotoxicidade em PBMC de voluntários sadios além de apresentar ação imunomoduladora de forma significativa das citocinas IL-6, IL-10, IL-17A, IFN-γ, TNF-α. Na diferenciação adipogênica o LPSF/JB-20 apresentou uma baixa acumulação do corante nas células transformadas de adipócitos derivadas da

maturação em meio diferenciado na linhagem 3T3-L1, em comparação com a rosiglitazona, indicando que o tratamento com o LPSF/JB-20 pode atuar de forma independente de PPARγ. Dessa forma, o presente estudo demonstrou que pacientes com EA apresentam um perfil diferenciado de produção de sOSMR, sGP130, IL-18 e IL-18BP em comparação com indivíduos saudáveis. O tratamento da doença, para reverter o processo de calcificação impróprio, ainda é um desafio, mas a avaliação dos efeitos *in vitro* de novas moléculas fornecem subsídios para o futuro desenvolvimento de ensaios clínicos.

Palavras-chave: Inflamação; Espondiloartrites axial; Oncostatina M; tiazolidinedionas.

ABSTRACT

Ankylosing spondylitis (AS) is a chronic disease of an inflammatory character that over time can lead to the development of syndesmophytes and ankylosis of the spine. Current treatments are able to improve symptoms, but do not prevent structural damage, especially in patients with delayed diagnosis. The mechanisms of bone remodeling are complex and research has been carried out in order to better clarify the disease and find new treatments for patients. Studies with AS patients have identified that PPARγ may act as a probable mediator of the effects of cytokines during inflammation and in the bone remodeling process. Thiazolidine derivatives (TZDs) have been studied for acting as PPARγ agonists. In this context, our work proposes to evaluate the cytokine profile in serum and in the PBMC culture supernatant of healthy patients and volunteers to investigate the response to the in vitro treatment of new thiazolidine derivatives, the LPSF / JB-20. The cytokines IL-1β, IL-6, IL-17A, IL-18BP, total IL-18, IL-23, OSM, sOSMR and sGP130 present in the serum of 130 EA patients and 130 healthy volunteers were evaluated by ELISA commercial kits sandwich. The immunomodulatory activity of TZD was evaluated in the PBMC culture supernatant stimulated with phytohemagglotinin-M and the dosage of cytokines IL-6, IL-10, IL-17A, IFN-γ, TNF-α were evaluated. Adipogenic differentiation and molecular modeling assays were evaluated to determine the mechanism of action for PPARγ. The results show that the expression of sOSMR, sGP130 and IL-18BP was significantly reduced and that of IL-18 elevated, compared with healthy volunteers. OSM expression was detected through Wester blotting and its expression was significantly higher compared to healthy volunteers. The other cytokines evaluated did not show any expression in the serum of the study population. Molecular docking showed that in terms of predicted affinity, the compound LPSF / JB-20 obtained a better score than rosiglitazone, when binding to PPARγ. The thiazolidine derivative showed low cytotoxicity in PBMC of healthy volunteers, in addition to having a significant immunomodulatory action of the cytokines IL-6, IL-10, IL-17A, IFN-γ, TNF-α. In adipogenic differentiation, LPSF / JB-20 showed a low accumulation of dye in transformed cells from adipocytes derived from maturation in differentiated media in the 3T3-L1 strain, in comparison with rosiglitazone, indicating that treatment with LPSF / JB-20 may act independently of PPARγ. Thus, the present study demonstrated that patients with AS have a

differentiated production profile of sOSMR, sGP130, IL-18 and IL-18BP compared to healthy individuals. The treatment of the disease, to reverse the improper calcification process, is still a challenge, but the evaluation of the in vitro effects of new molecules provide subsidies for the future development of clinical trials.

Keywords: Inflammation. Axial spondyloarthritis; Oncostatin M; thiazolidinediones.

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LISTA DE SIGLAS

AINE	ANTI-INFLAMATÓRIOS NÃO-ESTERÓIDES
anti-TNF	BLOQUEADORES DO FATOR DE NECROSE TUMORAL
ASDAS	ESCORE DE ATIVIDADE DA DOENÇA
ASAS	SOCIEDADE INTERNACIONAL DE AVALIAÇÃO DAS ESPONDILOARTRITES
BASDAI	ÍNDICE DE ATIVIDADE DA DOENÇA ESPONDILITE ANQUILOSANTE
BASFI	ÍNDICE FUNCIONAL DA ESPONDILITE ANQUILOSANTE
BASMI	ÍNDICE DE METROLOGIA DE ESPONDILITE ANQUILOSANTE
EA	ESPONDILITE ANQUILOSANTE
EpA	ESPONDILOARTRITE
HLA-B27	ANTÍGENO LEUCOCITÁRIO HUMANO B 27
IL	INTERLEUCINA
JAK	JANUS KINASE
LIF	FATOR INIBIDOR DE LEUCEMIA
LIFR	RECEPTOR DO FATOR INIBIDOR DE LEUCEMIA
MHC	COMPLEXO PRINCIPAL DE HISTOCOMPATIBILIDADE
MTT	BROMETO DE 3-[4,5-DIMETIL-TIAZOL-2-IL]-2,5-DIFENILTETRAZÓLIO
NF-kB	FATOR DE TRANSCRIÇÃO NUCLEAR KAPPA B
OSM	ONCOSTATINA M
OSMR	RECEPTOR DA ONCOSTATINA M
PBMC	CÉLULAS MONONUCLEADAS DO SANGUE PERIFÉRICO

PCR	PROTEÍNA C REATIVA
PMA	ACETATO MIRISTATO DE FORBOL
PPAR	RECEPTORES PROLIFERADORES DE PEROXISSOMA
RANKL	RECEPTOR ATIVADOR DO FATOR NUCLEAR KAPPA B
RPMI-1640	MEIO DE CULTURA PARA CÉLULAS E TECIDOS
sGP130	RECEPTOR SOLÚVEL DA GLICOPROTEÍNA 130
SFB	SORO FETAL BOLVINO
sOSMR	RECEPTOR SOLÚVEL DA ONCOSTATINA M
SSZ	SULFASALAZINA
TCLE	TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO
TNF-α	FACTOR DE NECROSE TUMORAL ALFA
TZD	DERIVADOS TIAZOLIDÍNICOS
VS	VOLUNTÁRIOS SADIOS

SUMÁRIO

1 INTRODUÇÃO

A espondilite anquilosante (EA) é uma doença inflamatória crônica, caracterizada pelo comprometimento do esqueleto axial e das articulações sacroilíacas (BRAUN; SIEPER, 2007). O estado inflamatório persistente desencadeia o dano estrutural, que em maior ou menor grau, leva ao desenvolvimento de formações ósseas nos locais de inflamação na coluna, originando os chamados sindesmófitos. Esta formação óssea ao longo do tempo pode desencadear a anquilose da coluna (RAMIRO, et al., 2015).

O tratamento farmacológico da EA inclui anti-inflamatórios não-esteroidais (AINEs), sulfassalazina (SSZ), metotrexato (MTX), agentes inibidores do fator de necrose tumoral (anti-TNF) e antagonistas de IL-17 (BABAIE et al., 2018; BRAUN et al., 2011; WARD et al., 2016). O objetivo principal do tratamento é a melhora dos sintomas, com redução da dor e supressão da inflamação (GOPALARATHINAM, et al., 2017). O tratamento com anti-TNF tem se mostrado eficaz para o controle dos sintomas (HENDERSON; DAVIS, 2006) e no retardo da progressão radiográfica (MAGREY; KHAN, 2017).

A interação de fatores genéticos e ambientais desencadeiam um processo inflamatório que se inicia com a produção de IL-23, que irá estimular as vias Th17 e Th22, levado a uma produção de citocinas pro-inflamatórias como: IL-17, IL-22 e TNF-α (BABAIE et al., 2018). A expressão destas citocinas através da ativação das células T desencadeiam o aumento da sinalização Wingless (Wnt) e da proliferação óssea (CORR, 2014) o que leva ao processo de calcificação que origina os sindesmófitos. Os mecanismos de remodelação óssea na EA são complexos e consistem na perca e neoformação óssea (VAN MECHELEN et al., 2017).

Outras citocinas, como as da família da IL-6 estão intimamente envolvidas na fisiopatologia de doenças inflamatórias como as espondiloartrites (SIMS; WALSH, 2010). A citocina oncostatina M (OSM), faz parte da família da IL-6 e possui a capacidade de modular o comportamento celular estromal em numerosos tecidos e órgãos, além de participar de processos homeostáticos (WEST; OWENS; HEGAZY, 2018). A sinalização de OSM via seu receptor OSMR/gp130 induz a produção de RANKL e a formação de osteoclastos e diminui a expressão de antagonistas da

sinalização Wnt, como esclerostina em osteócitos, sugerindo que essa citocina pode mediar à formação óssea aumentada (WALKER et al., 2010).

Lencel e colaboradores (2011) identificaram que os receptores ativados por proliferadores de peroxissoma gama (PPARγ) pode atuar como um mediador provável dos efeitos das citocinas em EA. Os autores sugeriram que este importante achado merece consideração para futuras pesquisas sobre os mecanismos do PPARγ na EA, tanto na parte da inflamação quanto no processo de ossificação ectópica (LENCEL et al., 2011).

Os derivados tiazolidínicos vêm sendo estudados por atuarem como agonistas de PPARγ para o controle da inflamação (ESCHER; WAHLI, 2000; SCHUPP; LAZAR, 2010). O PPARγ é expresso principalmente no tecido adiposo e vem sendo estudado por ser um alvo para a diminuição da obesidade e aumento da densidade óssea (MCCANN, et al., 2019). Em pacientes EA já se sabe que o estresse mecânico pode desencadear o processo de ossificação e que células adipogênicas que sofreram estresse mecânico apresentam uma diminuição da expressão de PPARγ e o aumento da expressão de marcadores de inicialização da osteogênese (SANTISH, 2017).

Novos direcionamentos são necessários para desvendar os mecanismos envolvidos com a doença, não completamente esclarecidos. Dentro desde contexto, nos propomos a avaliar o perfil de citocinas no soro de pacientes com EA e avaliar o potencial terapêutico e anti-inflamatório de uma série de derivados tiazolidínicos (LPSF/JB-2, LPSF/JB-3, LPSF/JB-4, LPSF/JB-5, LPSF/JB-6, LPSF/JB-7, LPSF/JB-8, LPSF/JB-9, LPSF/JB-11, LPSF/JB-12, LPSF/JB-13, LPSF/JB-14, LPSF/JB-20) sintetizados pelo laboratório de planejamento e síntese de fármacos (LPSF) e sua ação no PPARγ em PBMCs de pacientes portadores de EA e voluntários sadios. Uma vez que, a busca por novos alvos terapêuticos para inibir a formação óssea se faz necessário (GONZÁLEZ-CHÁVEZ; QUIÑONEZ-FLORES; PACHECO-TENA, 2016).

1.1 OBJETIVO

1.1.1 Objetivo geral

- Avaliação da atividade imunomoduladora dos derivados tiazolidínicos em células mononucleares do sangue periférico (PBMC) e o perfil de citocinas no soro de pacientes com diagnóstico de espondilite anquilosante (EA).

1.1.2 Objetivos específicos

- Descrever as características clínicas dos pacientes diagnosticados com EA;
- Avaliar as citocinas IL-1β, IL-6, IL-17A, IL-18, IL-23, OSM, sOSMR, sGP130 no soro dos pacientes EA e voluntários sadios;
- Realizar o *doking* molecular dos derivados tiazolidínicos LPSF/JB-2, LPSF/JB-3, LPSF/JB-4, LPSF/JB-5, LPSF/JB-6, LPSF/JB-7, LPSF/JB-8, LPSF/JB-9, LPSF/JB-11, LPSF/JB-12, LPSF/JB-13, LPSF/JB-14, LPSF/JB-20 no receptor ativado por proliferadores de peroxissoma gamma (PPARγ);
- Avaliar a citotoxicidade dos derivados tiazolidínicos;
- Avaliar os efeitos dos derivados tiazolidínicos na expressão das citocinas IL-1β, IL-6, IL-10, IL-17A, TNF-α, lFN-γ em PBMC de pacientes EA e voluntários sadios;
- Verificar o mecanismo de ação dos derivados tiazolidínicos no PPARγ.

2 REFERENCIAL TEÓRICO

2.1 A ESPONDILITE ANQUILOSANTE

2.1.1 Definição e epidemiologia

O termo espondiloartrite engloba um grupo de doenças inflamatórias (espondilite anquilosante, artrite psoriásica, artrite reativa e artrite da doença inflamatória intestinal), que compartilham características genéticas, radiográficas e clínicas acompanhadas de manifestações extra-articulares (SIEPER et al., 2009).

A EA é a uma doença inflamatória crônica que afeta primariamente o esqueleto axial. O estado inflamatório persistente desencadeia o dano estrutural, que em maior ou menor grau, leva ao desenvolvimento de formações ósseas nos locais de inflamação na coluna (sindesmófitos), que ao longo do tempo, pode desencadear a anquilose da coluna (STOLWIJK et al., 2015).

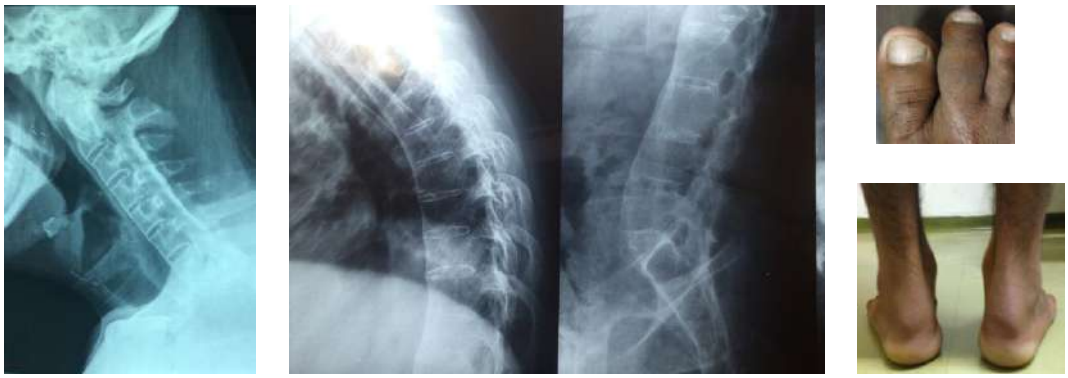


Figura 1. Anquilose cervical, espondilite, dactilite e entesite. Fonte: HC

A doença acomete principalmente homens jovens. A proporção entre homens e mulheres é de 3,4:1 no mundo. A prevalência média estimada para cada 10.000 pessoas nos continentes é de 23,8 na Europa; 16,7 na Ásia, 31,9 na América do Norte, 10,2 na América latina e 7,4 na África (DEAN et al., 2014).

2.1.2 Manifestações clínicas e avaliação

As manifestações clínicas mais frequentes são dor lombar inflamatória, artrite periférica variável e inflamação nos pontos de ligação entre o tendão e o osso que causa a entesite. Além dessas manifestações articulares podem apresentar sintomas extra-articulares, particularmente uveíte, doença inflamatória intestinal e psoríase (SIEPER, et al., 2002; SIEPER et al., 2015)

Para o diagnóstico de EA são avaliados os pacientes com relato de dor lombar inflamatória por mais de 3 meses e idade de início da doença até 45 anos. O critério clínico mais utilizado é o de Nova York modificado que considera um critério clínico e o radiográfico (LINDEN; VALKENBURG; CATS, 1984). Além do critério de Nova York atualmente o grupo ASAS (*Assessment of spondyloarthritis international society*) publicou critérios classificatórios para espondiloartrites axial e periférica, o que permite um diagnóstico mais precoce das espondiloartrites, ainda que não apareçam alterações radiográficas no raio-x (SIEPER et al., 2016).

Para o acompanhamento clínico dos pacientes são utilizados escores compostos de atividade: BASDAI (Bath *AS Disease Activity Index*) e o ASDAS (*Ankylosing Spondylitis Disease Activity Score*). Ambos utilizam a escala visual analógica de dor (EVA) com pontuação de 0 a 10 (0 = bom; 10 = ruim) (SCHEFTE; HETLAND, 2010). Também são utilizados índices compostos para avaliar a função (BASFI - *Bath AS Functional Index*) e mobilidade (BASMI - *Bath AS Metrology Index*) (ZOCHLING, 2011).

2.1.3 Tratamento

Os principais objetivos do tratamento são aliviar a dor, a rigidez e a fadiga, além de preservar a postura adequada e a função física e psicossocial (ZOCHLING, 2011). O tratamento farmacológico da EA inclui anti-inflamatórios não-esteroidais (AINEs) e modificadores das doenças reumáticas como sulfassalazina (SSZ), metotrexato (MTX) e agentes inibidores do fator de necrose tumoral (anti-TNF) (BRAUN et al., 2011; WARD et al., 2016).

O tratamento combina modalidades terapêuticas farmacológicas e não farmacológicas, de forma individualizada, utilizando-se de uma estratégia de decisão

compartilhada entre o médico e o paciente. Pacientes apresentando sintomas característicos de agravo da doença, com índices ASDAS maior que 2,1 ou BASDAI maior que 4, com falha ao tratamento com AINE, devem receber tratamento com imunobiológicos, sendo recomendado o anti-TNF como primeira linha. No caso de falha após 12 semanas ou toxicidade, pode ser feita a troca por um segundo anti-TNF ou para o inibidor de IL-17 (VAN DER HEIJDE et al., 2017).

2.2 FISIOPATOLOGIA DA ESPONDILITE ANQUILOSANTE

2.2.1 Pré-disposição genética do HLA-B27

O risco genético à EA está atribuído ao complexo principal de histocompatibilidade (MHC) codificador do alelo de classe 1, o HLA-B27. A associação do HLA-B27 e a doença são fontes de questionamentos e pesquisas desde 1970 (BREWERTON et al., 1973). O HLA-B27 está presente em 80-90% dos pacientes com EA. No entanto, apenas uma pequena proporção de pessoas na população em geral que abriga o HLA-B27 (de 5–6%), e em geral pessoas brancas, desenvolvem EA (DOUGADOS; BAETEN, 2011).

Dentre todas as associações genéticas de doenças humanas, o HLA-B27 constitui uma das mais fortes variantes genéticas de herdabilidade. Vários estudos de regiões não HLA tentam entender seu mecanismo de ação. Existe de fato uma forte associação do HLA-B27 com a doença, no entanto de 15 a 20% dos pacientes são HLA-B27 negativos e apenas 5% dos pacientes apresentam um acometimento mais grave da doença. (BROWN et al., 1997). Estes dados indicam que outros fatores de riscos estão envolvidos na doença e possam ser decisivos para o desenvolvimento da doença em pacientes HLA-B27 negativos (BROWN, 2018; LIN, 2018).

Este e outros achados levam a crê que a EA é uma doença complexa e provavelmente possui a interações de múltiplos genes suscetíveis, além de polimorfismos de nucleotídeo único (SNPs) e fatores ambientais, tais como estilo de vida, dieta, álcool e exercícios físicos que podem alterar o equilíbrio do indivíduo (LIN, 2018; HERN; BIDICHANDANI, 2004).

2.2.2 A imunologia do processo inflamatório

A diversidade é uma das características essenciais do sistema imunológico. Esta variação do sistema imunológico foi abordada por Liston e Goris (2018) que trouxeram estudos sobre a origem e diversidade do sistema imune. Eles descreveram que cerca de 50% destas variações do sistema imunológico é decorrente de efeitos ambientais, que inclui idade, sexo, infecções anteriores, vacinação e hábitos de saúde. Entre 30 a 40% são decorrentes de fatores genéticos e entre 10 a 20% de fatores indeterminados (LISTON; GORIS, 2018).

Na busca constante do sistema vivo em manter a homeostase do meio, esta resposta imune possui um papel crucial de atuação. A principal função fisiológica do sistema imunológico é manter a defesa contra microrganismos. No entanto, substâncias estranhas não infectantes também podem desencadear respostas imunológicas. A imunidade inata possui sistemas naturais de ação, e a imunidade adaptativa responde a infecções adquiridas e adapta-se a elas gerando uma resposta imunológica. O principal componente da imunidade adaptativa são os linfócitos que possuem o papel de secretar anticorpos (ABBAS, A. K.; LICHTMAN, A. H.; PILLAI, S. H. I. V, 2012).

O sistema imunológico por possuir várias células, mediadores secretados e marcadores, possui a capacidade de controlar e regular as respostas imunes e a inflamação. Todos os fatores que perturbam esta regulação podem descontrolar o equilíbrio interno e, com isso, podem causar inflamação crônica (REZAIEMANESH et al., 2018). Até a presente data os mecanismos precisos envolvidos na etiologia do EA não foram totalmente compreendidos. Sabe-se que a inflamação crônica tem um papel crítico na patogênese da doença e contribuições de fatores genéticos e ambientais podem ser responsáveis pela inflamação (SIEPER, et al., 2002).

Várias células imunes mediadoras e marcadores desempenham um papel importante em EA superativando seu sistema imune. As células imunes envolvidas no desencadeamento, iniciação, desenvolvimento e regulação são oriundas da imunidade inata (células dendríticas, macrófagos e células natural killer) e das células imunes adaptativas (células T auxiliares, células T reguladoras, TCD8 + e células B). Um esquema ilustrativo está demonstrado na figura 1 (REZAIEMANESH et al., 2018).

Atualmente as evidências vêm correlacionando a associação genômica a outros fatores como o receptor da interleucina 23 (IL-23R) (REZAIEMANESH et al., 2018) e ao eixo das citocinas IL-23 e IL-17 na patogênese das Espondiloartrites (GASTON, 2017). Há fortes evidências de que o HLA-B27 opera em EA interagindo aberrantemente com as células de rolamento do receptor do tipo imunoglobulina das células natural killer para conduzir a produção da citocinas chave como a IL-17 (BROWN, 2018).

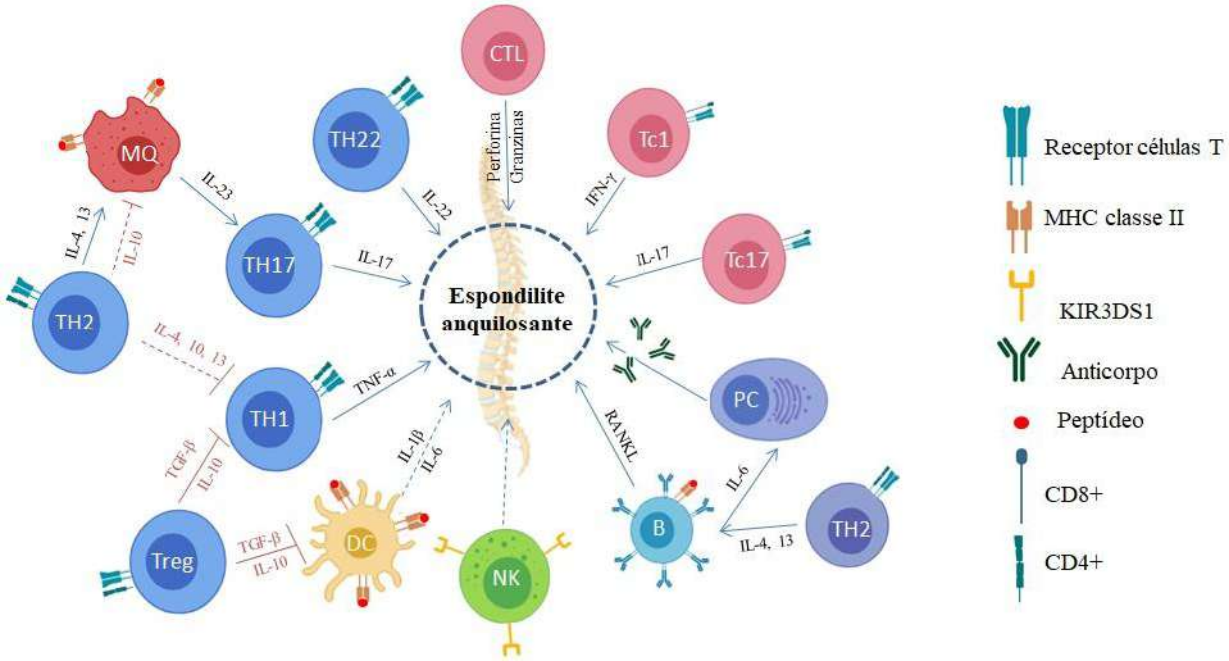


Figura 2. Células do sistema imune que participam dos mecanismos celulares de desencadeamento, iniciação, desenvolvimento e regulação na EA. B: célula B; CTL: linfócito T citotóxico; CD: célula dendrítica; IFNy: interferon-gama; IL: interleucina; KIR3DS1: receptor semelhante a imunoglobulina de células natural killer, três domínios de Ig e cauda citoplasmática curta 1; MQ: macrófago; NK: célula natural killer; PC: célula plasmática; TC: célula T citotóxico; TH: células T auxiliar; TNF-α: fator de necrose tumoral alfa.

Fonte: REZAIEMANESH, A., et al. 2018. Traduzido e adaptado.

A via IL-23/IL-17 participa da imunopatofisiologia da EA e esforços vêm sendo realizados com o intuito de estudá-las. A interação de fatores genéticos e epigenéticos, especialmente das células Th17 e Th22 em conjunto com vários tipos de estresse (mecânico, microbiota e ambientais) levam a uma produção de citocinas pró-inflamatórias como, IL-17, IL-22, TNF- α e IL-23 (figura 2) (BABAIE et al., 2018).

Concentrações séricas de IL-23 e IL-17 foram encontradas em altos níveis em pacientes com EA (CHEN et al., 2012). Além disso, estudos relataram que macrófagos de pacientes com EA produzem altos níveis de IL-23 em reação ao lipopolissacarídeo (ZENG; LINDSTROM; SMITH, 2011). A existência de células IL 17+ também foram relatadas em articulações facetarias de pacientes EA (APPEL, et al., 2011).

Conjuntamente a estes achados, cada vez mais evidências clínicas e moleculares sugerem que a inflamação exerce influência significativa na renovação óssea e atualmente a crescente compreensão da fisiologia óssea indica que os fatores envolvidos na inflamação estão ligados aos processos de remodelação óssea. Principalmente através das citocinas pró-inflamatória e outros mediadores imunológicos e fatores de transcrição descobertos mais recentemente (GINALDI; MARTINIS, 2016).

2.2.3 Mecanismos de remodelação ósseo

Na remodelação óssea as células progenitoras se diferenciam e a modelagem óssea ocorre com uma renovação contínua do esqueleto por osteoclastos que media a reabsorção óssea e os osteoblastos que atuam na formação óssea. Os osteócitos são células mecano-sensíveis e orquestram o ciclo de remodelação óssea. A modelagem óssea nas espondiloartrites é um processo associado à doença, no qual a formação de osso novo está ocorrendo fora das bordas originais do esqueleto (LORIES; SCHETT, 2012).

Essa formação óssea desordenada causa um distúrbio esquelético que afeta a coluna e as articulações sacrilíacas. Este fenômeno é considerado complexo, pois nele ocorre a perda e neoformação óssea simultaneamente ocasionando a morbidade da doença (VAN MECHELEN et al., 2017). Esta calcificação ocorre nos ligamentos dos tendões aos ossos (enteses) e recebem o nome de sindesmófitos. Patologicamente existem três estágios com evidências radiográficas e patológicas no processo de inflamação e formação dos sindesmófitos: inflamação aguda, entesite fibrocartilagínea, que leva a erosão, e uma entesopatia ossificante que causa anquilose das articulações sacrilíacas e discos intervertebrais (MAGREY; KHAN, 2017).

A atual hipótese sobre o processo de perda e neoformação óssea se baseia na predisposição genética ao HLA-B27, IL-23R, PGER4 associado a fatores ambientais como, cigarro, infecções e mecanismos de estresse que podem desencadear a ativação de células T do sistema imune. As células T ativadas produzem citocinas como, IL-17, IL-22 e TNF-α que podem participar dos processos de osteoclastogênese e osteoproliferação (MAGREY; KHAN, 2017).

Estudos com camundongos vêm mostrando que a simultânea erosão e neoformação óssea podem ser impulsionadas por citocinas Th17 (IL-17 e IL-22). Nesse processo, a participação da IL-17 pode promover a erosão óssea através da regulação positiva do receptor ativador do fator nuclear kappa B (RANKL), um regulador chave da osteoclastogênese, promovendo erosão por regular os osteoclastos. Já a IL-22, regulada pela IL-23, pode está participando do processo de formação óssea (RAYCHAUDHURI; SAXENA; RAYCHAUDHURI, 2015).

Vias de sinalização e crescimento, como proteínas morfogenéticas ósseas, Wnt e Hedgehogs (BMP) também foram identificadas como fatores moleculares para a formação óssea, mas a relação entre inflamação e ativação destas vias continua a ser debatido. Li e colaboradores (2018) relatam que a ativação das vias Wnt/β-catenina canônica e Wnt / PKCδ não canônica são necessária para a neoformação óssea induzida por inflamação (LI et al., 2018). Estudos em animais mostram que o controle prolongado da inflamação parece ser necessário para evitar a anquilose (VAN MECHELEN et al., 2017; LORIES; SCHETT, 2012).

2.2.4 OSM/OSMR no processo de ossificação

A oncostatina M (OSM) é produzida pela medula óssea, macrófagos, células T, osteoblastos e osteócitos (SIMS; QUINN, 2014). Entre os vários processos fisiológicos de controle, a OSM atua na osteogênese e hematopoiese nos ossos e articulações aumentando a diferenciação de osteoblastos, RANKL e osteoclastos e diminuindo a medula gordurosa relacionada com a idade. Os efeitos patológicos da OSM nos ossos estão relacionados com a ossificação heterotrópica e fibrose medular, além da junção, destruição e degradação de cartilagens (WEST; OWENS; HEGAZY, 2018). Sua principal ação é a diferenciação e indução da fase aguda

através da sinalização JAK1/STAT3 (HERMANNS, 2015; O'SHEA; GADINA; SIEGEL, 2019).

A OSM tem como receptor LIFRβ, formando o complexo receptor tipo I (LIFRβ/GP130) e o receptor OSMRβ ou complexo receptor tipo II (OSMRβ/GP130). A expressão de LIFR geralmente não está associada a processos patológicos, diferentemente da OSMR, que tem sido associada a papéis críticos em estudos que avaliaram a OSM. O receptor OSM apresenta-se em duas formas distintas, uma ligada à membrana, sem a região intracelular (CHEN et al., 2008), e outra OSMR solúvel, a sOSMR (DIVEU et al., 2006).

Este receptor faz parte de uma superfamília denominada de receptores de citocinas tipo I ou receptores hematopoiéticos. O OSMR faz parte do tipo I por possuir em sua estrutura básica quatro hélices α antiparalelas com duas conexões de laço longo e curto. Na membrana celular, o OSMR possui resíduos de cisteína bem conservados (Trp-Ser-X-Trp-Ser - onde X indica qualquer aminoácido) e repetições semelhantes à fibronectina nos seus domínios extracelulares. Esse receptor possui um único domínio transmembrana e domínios citoplasmáticos divergentes. Dentro da porção citoplasmática, são encontrados dois segmentos, denominados de motivos de Caixa 1 e Caixa 2. O domínio proximal da membrana liga as Janus quinases (O'SHEA; GADINA; SIEGEL, 2019).

As citocinas da família IL-6 estão intimamente envolvidas em distúrbios esqueléticos localizados, incluindo artropatias como: artrite reumatóide e espondiloartrite e das doenças periodontais (SIMS; WALSH, 2010). Em pacientes com Espondiloartrite axial, foram encontrados no soro fatores solúveis circulantes como: OSM, IL-6, IL-17A e TNFα. Estes fatores são capazes de estimular a resposta de monócitos saudáveis a níveis séricos relativamente baixos de RANKL, induzindo a expressão de RANK e aumentando seu potencial osteoclastogênico. Neste estudo os autores sugerem que o RANKL produzido localmente nos pacientes EpA axial podem induzir os osteoclastos (KORKOSZ et al., 2018).

OSM modula o comportamento celular estromal em numerosos tecidos e órgãos (WEST; OWENS; HEGAZY, 2018) e participa de processos homeostáticos e patológicos, bem como da regulação da renovação óssea fisiológica do corpo. A sinalização via OSM via OSMR induz a produção de RANKL e a formação de

osteoclastos e diminui a expressão de antagonistas da sinalização Wnt, como esclerostina em osteócitos, sugerindo que essas citocinas medeiam à formação óssea aumentada (WALKER et al., 2010).

2.2.5 IL-18 na espondilite anquilosante

Um estudo pioneiro de 2010 observou o aumento da concentração sérica de IL-18 em pacientes com EA, em comparação com controles, e constataram que a atividade da doença e os tratamentos aplicados independem para seu aumento (SARI et al., 2010). Níveis significativamente maiores de IL-18 foram encontrados em pacientes do sexo masculino com sindesmófitos em comparação com aqueles sem sindesmófitos (HUANG et al., 2012). E um estudo com 81 pacientes EA, 76 com artrite psoriática e 34 com a síndrome de SAPHO também constataram seu aumento e encontraram correlação positiva com os parâmetros clínicos PCR, VHS e colesterol total (PRZEPIERA-BĘDZAK; FISCHER; BRZOSKO, 2015).

A IL-18 é estruturalmente semelhante aos membros da superfamília da citocina IL-1, especialmente IL-1β e IL-33. O precursor inativo da IL-18 (pro-IL-18) permanece armazenado no espaço intracelular e é clivado e processado pela caspase-1 na sua forma ativa e posteriormente liberada no meio extracelular, podendo também se ligar a membrana (BELLORA et al., 2012). A pro-IL-18 é expressa constitutivamente através de uma gama de diferentes tipos de células e tecidos e exibe um perfil imunoestimulador distinto (KAPLANSKI, 2018).

A atividade da IL-18 é regulada através da atividade da proteína de ligação a IL-18 (IL-18BP) que liga preferencialmente a IL-18 livre restringindo a sua capacidade para interagir com a IL-18R. Seu único receptor heterodímero é formado pela cadeia IL-18Rα, altamente expressa, e pela cadeia acessória IL-18Rβ, que necessita da indução de uma sinalização interna de fatores pró-inflamatórios, como a IL-12, para que haja sua expressão na superfície celular (KAPLANSKI, 2018).

A atividade da IL-18 tem sido extensivamente caracterizada na patogênese de várias doenças inflamatórias crônicas e seus altos níveis séricos livres descritos tornam a IL-18 como um mediador da inflamação no contexto da doença (HERNANDEZ-SANTANA et al., 2019). Recentemente novas abordagens

terapêuticas vêm se expandindo com o tratamento com a IL-18BP recombinante (CANNA et al., 2014).

2.3 NOVOS TRATAMENTOS

Os pacientes portadores de EA fazem uso de tratamento farmacológico convencional (AINEs, sulfassalazina, metotrexato) e imunobiológicos (BABAIE et al., 2018; BRAUN et al., 2011; WARD et al., 2016). Os imunobiológicos possibilitaram uma melhora no tratamento dos pacientes, e o uso prolongado de anti-TNF e do inibidor IL-17 vem mostrando ser eficaz no na redução da velocidade radiográfica e da progressão em EA (MAGREY; KHAN, 2017), mas não é capaz de inibir o dano estrutural causado por fraturas vertebrais radiográficas em EA (MAAS, et al.; 2016). Além disso, os imunobiológicos requerem um elaborado processo biotecnológico e trazem altos custos para o Sistema Único de Saúde (GAVA et al., 2010).

2.3.1 Derivados tiazolidínicos e PPARγ

Atualmente a busca por novas moléculas naturais e sintéticas vem revolucionando a pesquisa clínica e trazendo vários benefícios. Dentre elas, novas moléculas vêm sendo estudadas pela capacidade de atuar como agonista dos receptores ativados por proliferadores de peroxissoma (PPARs) (MASSARO et al., 2016; WANG et al., 2017).

Esta nova geração de agonistas de PPARs vem pavimentando novos campos de aplicação para doenças metabólicas com componente inflamatório subjacente (BOUGARNE et al., 2018) e os derivados tiazolidínicos vêm sendo estudados por atuarem como agonistas de PPAR para o controle da inflamação (ESCHER; WAHLI, 2000; SCHUPP; LAZAR, 2010). No entanto mais estudos são necessários para comprovar sua relevância fisiológica nos processos inflamatórios (BOUGARNE et al., 2018).

Os PPARs são uma classe de receptores nucleares que atuam como fatores de transcrição ligando ou desligando a síntese de genes específicos no núcleo celular. Três proteínas fazem parte dos PPARs: PPARα, PPARβ, PPARγ. Para que

possam atuar formam um heterodímero com o seu respectivo receptor do ácido 9-cis retinóico (RXR), formando um complexo PPAR:RXR. Este complexo para ser ativado precisa da liberação de um co-repressor (feita pela atividade deacetilase da histona) e do recrutamento de um ligante co-ativador (que possui atividade acetiltransferase). Após ativado o complexo PPAR:RXR se liga de forma dependente ao PPAREs (elementos responsivos aos proliferadores de peroxissoma) que estão situados em sítios específicos de cada gene. Nesta etapa a ação de agonistas irá mudar as conformações estruturais alterando a estabilidade e criando um sítio de ligação para o recrutamento de coativadores transcricionais que irão aumentar a transcrição gênica (TAVARES; HIRATA; HIRATA, 2007).

Na inflamação estes receptores desempenham uma ação importantes na resposta imune devido à capacidade de inibir a expressão de citocinas inflamatórias e estimular a diferenciação de células imunes em fenótipos anti-inflamatórios, além de ativar o fator de transcrição nuclear kappa B (NF-kB) (TAVARES; HIRATA; HIRATA, 2007). Estudos com pacientes EA identificaram que o PPARγ pode atuar como um mediador provável dos efeitos das citocinas. Estes achados merecem consideração para futuras pesquisas sobre os mecanismos do PPARγ na EA, tanto na parte de inflamação quanto no processo de ossificação ectópica (LENCEL et al., 2011).

A classe de moléculas tiazolidínicas é famosa pela sua atividade antiglicêmica e apresenta na sua estrutura química um anel pentamérico heterocíclico com um enxofre na posição 1 e um nitrogênio na posição 3, podendo conter carbonilas na posição 2 e/ou 4.

Uma variedade de tiazolidinadionas foram estudadas em 1982 com a finalidade de investigar a sua propriedade anti-hiperglicemica. Após vários estudos se observou que o núcleo tiazolidina-2,4-diona era responsável pela ação farmacológica. Comercialmente estão disponíveis no mercado a Roziglitazona e o Pioglitazona sendo indicados para o tratamento de Diabetes Mellitus tipo 2 (JAIN; VORA; RAMAA, 2013). Atualmente vários estudos apontam que os derivados tiazolidínicos apresentam um amplo espectro de atividade biológica, como: antimicrobiana (GOUVEIA et al., 2009; TUNCBILEK; ALTANLAR, 2006), anti-diabética (MURUGAN; ANBAZHAGAN; SRIMAN NARAYANAN, 2009; PATTAN et

al., 2005), anti-obesidade (KIM et al., 2012), anti-inflamatória (YOUSSEF et al., 2010), antioxidante (BOZDAĞ-DÜNDAR et al., 2009), antiproliferativa e antitumoral (PATIL et al., 2010). Além de ser uma alternativa aos ani-inflamatórios e ter um baixo custo em comparação aos imunobiológicos que são utilizados atualmente.

3 MÉTODO

3.1 TIPO DE ESTUDO

O presente estudo foi desenvolvido em duas etapas. A primeira consiste em um estudo descritivo transversal, com componente analítico, para avaliar o perfil de citocinas na população de pacientes diagnosticados com EA. A segunda etapa consistiu em um estudo do tipo experimental translacional para avaliar o derivado tiazolidínico LPSF/JB-20 e sua possível ação em atuar via PPAR-γ.

3.2 LOCAL E PERÍODO DO ESTUDO

O estudo foi realizado de março de 2016 à março de 2020. A etapa de seleção e avaliação clínica dos pacientes foi realizada no ambulatório de Reumatologia do HC-UFPE e os procedimentos experimentais realizados no Laboratório de Imunomodulação e Novas Abordagens Terapêuticas (LINAT) do Núcleo de Pesquisa em Inovação Terapêutica (NUPIT-SG) da UFPE.

3.3 POPULAÇÃO DE ESTUDO

3.3.1 Recrutamento dos pacientes EA

Foram selecionados e avaliados 130 pacientes com EA segundo os critérios de inclusão e exclusão.

Critérios de inclusão:

- idade acima de 18 anos;
- consentimento em participar do estudo e assinar o Termo de Consentimento Livre e Esclarecido (TCLE);
- diagnóstico de EA de acordo com critérios de Nova Iorque modificados (LINDEN, VALKENBURG, CATS, 1984);
- acompanhamento regular no ambulatório de reumatologia do HC-UFPE.

Critérios de exclusão:

. não consentimento em participar do estudo;
. diagnóstico prévio ou atual de outras doenças reumatológicas ou neoplasias;
. gestantes;
. presença de infecção aguda ou crônica no momento da avaliação;
. impossibilidade de coleta de sangue periférico.

3.3.2 Recrutamento dos voluntários saudáveis

O grupo controle formado por 130 voluntários saudáveis, pareados por sexo e idade, foram escolhidos aleatoriamente no HC-UFPE (acompanhantes dos pacientes ou funcionários do hospital). Esses indivíduos assinaram o TCLE e foram submetidos a uma entrevista clínica com o pesquisador principal. Foram excluídos casos com diagnóstico conhecido de doença reumática, imunodeficiências, infecção aguda ou crônica ou uso de tratamento imunossupressor e/ou corticoide e/ou derivado tiazolidínico.

3.3.2 Definição das variáveis clínicas

Para caracterização do comprometimento clínico dos pacientes foram utilizadas as seguintes avaliações:

<u>Avaliação farmacoterapêutica:</u>

. tempo dos sintomas da doença;
. idade do diagnóstico;
. familiares com a doença;
. outras doenças diagnosticadas;
. tabagismo;
. medicamentos em uso.

<u>Avaliações diagnósticas:</u>

. resultados laboratoriais (HLA-B27, PCR, VHS);
. sintomas extra-articulares presentes no momento da coleta (uveíte, psoríase, doença inflamatória intestinal);

. variáveis clínicas relacionadas à atividade da doença (dor lombar inflamatória, entesite, dactilite, artrite periférica);
. EVA- do examinador e paciente.

Para avaliar a atividade da doença foram aplicados os critérios de acompanhamento médico, o BASDAI. Para calcular foi utilizada a calculadora para aplicativos móveis da Sociedade Internacional de Avaliação das espondiloartrites que utiliza as recomendações ASAS/EULAR para o tratamento das espondiloartrites/espondiloartrites axial. O item da duração da rigidez matinal é estipulado de acordo com o tempo (em minutos) relatado pelo paciente, sendo 2 horas o intervalo máximo equivalendo à nota 10 (muito grave).

3.4 CONSIDERAÇÕES ÉTICAS CONFORME A RESOLUÇÃO 466/12 DO CNS

As coletas foram realizadas após aprovação pelo comitê de ética em pesquisa do centro de ciências biológicas da Universidade Federal de Pernambuco (ANEXO A). Para cada participante foi realizada a leitura, assinatura e entrega do TCLE, (APÊNDICE A). Os participantes da pesquisa foram devidamente informados e qualquer dúvida foi esclarecida no momento ou posteriormente pelos contatos descritos na cópia que ficou no poder deles. As coletas de 10-15 ml de sangue foram realizadas por profissionais treinados, competentes e orientados para reduzir os riscos. Os dados coletados nesta pesquisa, provenientes dos resultados das amostras coletadas foram devidamente armazenadas em arquivos digitais em computadores e impressos nos cadernos de resultados sob responsabilidade do pesquisador responsável e pelo Laboratório de Imunomodulação e Novas Abordagens Terapêuticas (LINAT).

3.5 COLETA SANGUÍNEA

A coleta do sangue foi realizada aos pacientes diagnosticados com EA em atendimento ao Hospital das Clínicas de Pernambuco. Foram coletadas amostras de sangue periférico por profissional competente através de punção venosa. Foi coletado o volume de 9 mL de sangue periférico no tubo seco e 9 mL em tubo heparinizado. Voluntários sadios também foram recrutados e pareados por sexo e

idade para posterior análise dos resultados. As amostras foram encaminhadas ao LINAT/UFPE. O processamento das amostras foi realizado através de centrifugação a 3000 RPM por 10 minutos para a obtenção do soro. As amostras de soro foram devidamente identificadas e armazenadas a -80º C para posterior utilização. O sangue do tubo contendo heparina foi utilizado para obtenção das PBMCs.

3.6 COMPOSTO AVALIADO

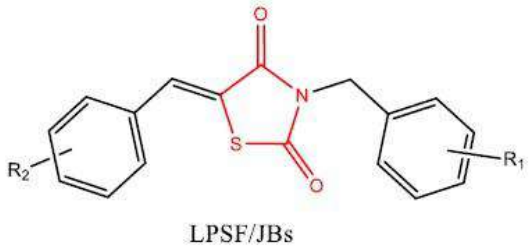
Para avaliar a atividade imunomoduladora das citocinas IL-6, IL-17A, TNF-α, IFN-γ foi utilizado o derivado tiazolidínico da série 3-(3,5-dimetilbenzil)tiazolidina-2,4-diona, codificados como LPSF/JB-20, sintetizados pelo Laboratório de Planejamento e Síntese de fármacos (LPSF), demonstrados na tabela 1.

Tabela 1. Propriedades físico-químicas dos derivados tiazolidínicos LPSF/JBs

JBs	Nome químico	Fórmula molecular	Peso molecular
20	5-((5-bromo-1H-indol-3-il)metileno)-3-(3,5-dimetilbenzil)tiazolidina-2,4-diona	C21H17BrN2O2S	441.34 g/mol

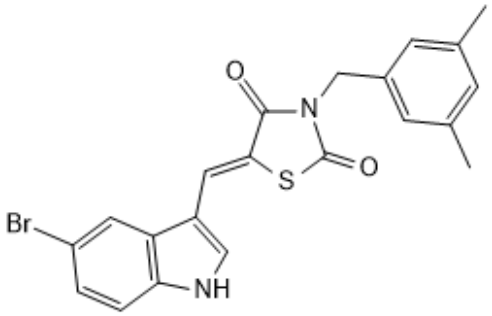
Fonte: BRANCO JÚNIOR, 2017.

Para as sínteses foram utilizadas várias rotas reacionais e as análises estruturais foram realizadas por: Ressonância Magnética Nuclear (RMN) de Hidrogênio e Carbono, Espectrofotometria de Infravermelho (IV), Cromatografia Gasosa acoplada ao Espectrômetro de Massas (GC-MS) e Cromatografia Líquida de Alta Eficiência (CLAE). Na estrutura geral dos derivados tiazolidínicos, ao grupo farmacofórico (em vermelho) foram adicionados substituintes em ambos os lados (figura 3) de acordo com a estrutura de cada molécula (figura 4).



LPSF/JBs

Figura 3. Estrutura química básica dos derivados tiazolidínico 3,5-diaril substituídos (LPSF/JBs)



JB-20

Figura 4. Estrutura química do derivado tiazolidínico 3,5-diaril substituídos (LPSF/JB-20). Fonte: BRANCO JÚNIOR, 2017.

3.6.1 Modelagem molecular

A estrutura tridimensional do composto LPSF/JB-20, incluindo seus isômeros E e Z foram desenhadas utilizando-se o programa SPARTAN 08' (*SPARTAN, 2008*) e as estruturas otimizadas aplicando-se o método RM1 (*ROCHA, et al., 2006).* A análise de docking foi realizada no ligante PPARγ (siga: PDB:5YCP) no site http://www.rcsb.org, a qual possui o composto rosiglitazona (siga: BRL) como ligante co-cristalizado. O sítio ativo foi definido como todos os átomos presente no raio de 6,0 Å a partir da posição onde o ligante co-cristalizado BRL se encontra. O programa GOLD 5.6 (*Gold software, 2018*) foi utilizado para a realização dos cálculos de docking, utilizando a função de pontuação ChemPLP. Os cálculos de docking envolvendo o composto LPSF/JB-20 levaram em consideração os graus de

liberdade do receptor com o intuito de simular o efeito de ajuste induzido, permitindo a flexibilização dos seguintes aminoácidos: PHE282, CYS285, GLN286, ARG288, HIS323, TYR327, PHE363, LYS367, HIS449 e TYR473. O programa BINANA (*DURRANT; MCCAMMON, 2011*) foi utilizado para mapear as interações intermoleculares das soluções de docking, utilizando as configurações padrão do programa. As figuras foram geradas utilizando-se o programa Pymol (*DELANO, 2002*).

3.7 CULTURA DE PBMC

As células mononucleares de sangue periférico (PBMC) dos pacientes e controles foram obtidas através da técnica de centrifugação Ficoll-Paque PLUS (GE Healthcare) à 450g por 45 minutos, aceleração 4, freio 0. Após a formação do anel de PBMCs, as mesmas foram removidas e lavadas duas vezes com tampão fosfato-salino (PBS) 1x nas seguintes condições 350g por 20 minutos, aceleração 6, freio 4 na primeira lavagem; 350g por 15 minutos, aceleração 6, freio 4 na segunda lavagem.

Terminadas as lavagens, o sobrenadante foi descartado e o precipitado de células foi ressuspendido em meio de cultivo RPMI-1640 (Gibco) suplementado com L-Glutamina, 10% de soro fetal bovino (SFB) (Gibco), 10 mM de HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Gibco) e 200 U/mL de Penicilina/Estreptomicina (Gibco). Posteriormente, as células foram contadas em câmara de Neubauer e plaqueadas segundo as concentrações necessárias.

3.7.1 Ensaio de citotoxicidade

O ensaio de citotoxicidade MTT (3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina) (MOSMANN, 1983) foi realizado para avaliar o derivado tiazolidínico em estudo. As PBMCs de 3 voluntários sadios foram avaliadas nas doses de 10 e 75µM em triplicata em placas de 96 poços contendo $3x10^5$ células por poço no volume de 150 µL de meio suplementado. Em seguida foram incubadas por 48 horas em estufa umidificada a 37ºC e 5% de CO_2. Após este período, foram adicionados 20 µL de

MTT (5 mg/mL), incubadas por 3 horas e mantidas as condições de incubação. Depois foi adicionado dodecil sulfato de sódio (SDS) a 20% para a diluição dos cristais de Formazan. Após aguardadas 24 horas a absorbância foi aferida em um leitor de microplacas (Biotek EL808) a 570nm.

Para expressar a porcentagem de células metabolicamente ativas, foram calculadas as viabilidades celulares de cada uma das doses testadas no software Microsoft Office Excel. A concentração requerida para inibir 50% da viabilidade das células (IC 50) foi calculada utilizando o software de estatística Origin v. 8,0.

3.7.2 Mecanismos de ação PPARγ

Avaliação da diferenciação adipogênica

Para avaliar a possível atividade agonista no PPARγ o derivado tiazolidínico LPSF/JB-20 foi avaliado na indução de diferenciação adipogência. Para isso foram utilizadas as células da linhagem de fibroblastos pluripotentes murinos 3T3-L1 (Banco de células do Rio de Janeiro), mantidas em no meio de cultivo DMEM (Gibco) suplementado com soro fetal bovino (SFB) a 10% (Gibco).

Para o protocolo de diferenciação, as células foram cultivadas em placas de 24 poços na concentração de $2x10^4$ células por poço. Após alcançar uma confluência de aproximadamente 80%, foi adicionado o meio de indução de diferenciação, composto por DMEM suplementado com 10% de SFB e 10µg/ml de insulina, 0,5 Mm de 3-isobutil1-metilxantina (Sigma) e 1 µM de dexametasona (Sigma), por 72 horas.

Para a avaliação do derivado tiazolidínico aos poços com o meio de diferenciação foi adicionado o LPSF/JB-20 na dose de 50µM (baseado em testes realizados anteriormente). Após as 72 horas o meio de diferenciação foi substituído por meio de manutenção (DMEM suplementado com 10% SFB e 10µg/mL de insulina) a cada 48h, até completar 7 dias e as células estarem com a completa diferenciação.

A avaliação da diferenciação adipogênica foi realizada com a coloração Oilred-O (Sigma) 0,5% (500mg do Oilred-O em 100mL de isopropanol) nas células diferenciadas na placa de 24 poços. Ao término do protocolo de indução, o meio de

cultura foi removido, as células foram lavadas com PBS 1x e fixadas com formalina a 10% por 15 minutos em temperatura ambiente. Em seguida, os poços foram lavados com água destilada (2 vezes) por 1 minuto. As células foram coradas por 15 minutos com o Oilred-O (diluído 2:3 com água). O corante foi retirado e as células foram lavadas (3 vezes) por um minuto. Em seguida, as células foram lavadas com isopropanol 50%, e em seguida água destilada (1 vez).

As células coradas aderidas na placa foram observadas e no microscópio óptico para visualização do acúmulo de lipídeos corados em vermelho. O experimento foi realizado em triplicata.

3.7 DOSAGENS DE CITOCINAS PELO ENSAIO DE IMUNOABSORÇÃO ENZIMÁTICA (ELISA)

As citocinas presentes no sobrenadante de cultura e no soro dos pacientes e dos controles foram medidas por ELISA *sanduíche* humano, seguindo as instruções recomendadas pelos fornecedores (tabela 2). Os soros foram identificados e estocados no refrigerador a -80ºC para posterior utilização. No soro dos pacientes foram avaliadas as seguintes citocinas: IL-1β, IL-6, IL-17A, IL-18BPa, IL-18 total, IL-23, OSM, sOSMR e sGP130. No sobrenadante das culturas de PBMC foram avaliadas: IL-6, IL-10, IL-17A, TNF-α, IFN-γ. A leitura da absorbância foi realizada a 450/570nm em espectrofotômetro devidamente calibrado.

3.7.1 Análise estatística

A análise dos resultados foi obtida através do programa GraphPad Prism, versão 6.0, programa estatístico (San Diego, CA). A média e o intervalo interquartílico (IQR) da expressão das citocinas foram medidas nos grupos avaliados. A normalidade das amostras foi verificada com o teste D'Agostino. A diferença nos níveis séricos entre os grupos foi avaliada pelo teste t de Student ou pelo teste U de Mann-Whitney. O coeficiente de correlação de postos de Spearman foi usado para examinar a relação entre duas variáveis contínuas. Foram consideradas a correlação (R2): $0< R2 \leq 0.35$, fraca; $0.35 < R2 \leq 0.67$ moderada e

entre 0.67 < R2≤1 forte correlação. O valor de p <0,05 foi considerado estatisticamente significante.

Tabela 2. Limites de detecção das citocinas dosadas e empresas dos Kits

Citocina	Marca do kit	Limite de detecção
IL-6	BD Biosciences	4,69 – 300 pg/mL
IL-1β	BD Biosciences	3,90 – 500 pg/mL
IL-17A	Ebioscience	3,90 – 500 pg/mL
IL-18BPa	R&D	6000-46,8 pg/ml
IL-18 total	R&D	750-5,8 pg/ml
IL-23	Ebioscience	15,63 – 2000 pg/ml
IFN-γ	BD Biosciences	4,69 – 600 pg/mL
TNF-α	BD Biosciences	7,82 – 1000 pg/mL
OSM	Invitrogen	62,5 – 8000 pg/mL
sOSMR	Invitrogen	156,25 – 20000 pg/mL
sGP130	R&D	39,06-5000 pg/ml

4 RESULTADOS

4.1 ARTIGO 1 - SOLUBLE ONCONSTATIN M RECEPTOR (sOSMR) AND GLYCOPROTEIN 130 (sGP130) UNDEREXPRESSION IN PATIENTS WITH ANKYLOSING SPONDYLITIS

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Objective: The objective of the present study was to evaluate the serum levels Oncostatin M (OSM), soluble oncostatin M receptor (sOSMR) and soluble glycoprotein 130 (sGP130) in patients with ankylosing spondylitis (AS), and the possible associations and correlations with clinical parameters of the disease.

Methods. Serum levels of OSM, sOSMR and sGP130 were evaluated in one hundred and thirty AS patients and one hundred and thirty healthy individuals by immunoassay (ELISA) human *sandwich*. The serum of seven patients and seven healthy volunteers were used to detect OSM expression by West blotting.

Results. Serum sOSMR levels were significantly lower (p<0,0001) in AS patients (mean of 180,6 ng/mL) compared to healthy volunteers (mean of 404,7 ng/mL). Serum sGP130 levels were it as also lower (p<0,0001) in patients (mean of 184,6 ng/mL) compared to healthy volunteers (mean of 231,8 ng/mL). When the AS group was stratified by disease activity, a statistically significant difference was observed between sOSMR levels in patients with low BASDAI when compared to controls. Statistical difference was also observed among and patients with low and high disease activity. Patients who used anti-TNF, had reduced sOSMR expression (p=0,0004) in comparison with disease-modifying anti-rheumatic drugs (DMARDs). Differently, patients who used anti-TNF had significantly sGP130 elevated levels and had reduced serum levels with DMARDs. The expression of OSM was undetectable in human *sandwich* ELISA but significantly elevated by West blotting.

Conclusion: Serum levels of OSM are elevated and sOSMR and sGP130 are underexpression in patients with AS. These results suggest that, despite the control of clinical activity, the neutralization of the OSM generated by its sOSMR antagonists and sGP130 can be improved and can eventually become a potential therapeutic target in AS.

INTRODUCTION

The oncostatin M axis is involved in several homeostatic processes and, in pathological situations (WEST; OWENS; HEGAZY, 2018) being intimately involved in localized skeletal disorders impacting the pathological bone remodeling evidenced in periodontal disse, rheumatoid arthritis, osteoarthritis and spondyloarthropathies (SIMS ; WALSH, 2010). The pathological effects of OSM on bones are related to heterotropic ossification and spinal cord fibrosis, in addition to cartilage junction, destruction and degradation (WEST; OWENS; HEGAZY, 2018). Its main action is the differentiation and induction of the acute phase through the JAK1/STAT3 signaling (HERMANNS, 2015; O'SHEA; GADINA; SIEGEL, 2019).

OSM is produced by bone marrow, macrophages, T cells, osteoblasts and osteocytes (SIMS; QUINN, 2014). The IL-6 family of which the OSM is part shares the common subunit of the GP130 signaling receiver in the formation of its multimeric receivers (DIVEU, et al., 2006). The LIFR receptor or type I receptor complex (LIFRβ/gp130) is activated by OSM and LIF and the OSMR receptor or type II receptor complex (OSMRβ/gp130) and activated only by OSM (GEARING et al., 1992; MOSLEY et al. ., 1996; ADRIAN-SEGARRA, et al., 2018; WEST et al., 2019).

The full-length OSMRβ has two described variants, a short form present in the membrane without the intracellular region (CHEN et al., 2008) and a soluble form called sOSMR (DIVEU et al., 2006; KAUSAR et al., 2011). The sOSMR is generated by alternative splicing, leading to a premature stop codon that generates a truncated receptor protein causing sOSMR to act as an antagonist, thus blocking OSM-mediated signaling through the transmembrane receptor complex (DIVEU et al., 2006). Soluble sGP130 and sLIFR are known to neutralize responses to cytokines of

the IL-6 family (NARAZAKI et al., 1993; ZHANG et al., 1998) and that sOSMR and sGP130 act together by blocking OSM signaling (DIVEU et al., 2006).

AS is a chronic inflammatory disease that has a marked feature of bone neoformation occurring outside the original borders of the axial skeleton (TAM, et al., 2010; LORIES; SCHETT, 2012; BRAUN; BARALIAKOS, 2011). These calcifications occurs in the ligaments of the tendons to the bones (entheses) and are called syndesmophytes. This disordered bone formation causes a skeletal disorder that affects the sacriliac spine and joints (MAGREY; KHAN, 2017). This phenomenon is considered complex, as it involves bone loss and new formation simultaneously causing the disease morbidity (VAN MECHELEN et al., 2017).

Until now, the expression of sOSMR and sGP130 had not been investigated in AS. In view of this question, we evaluated the circulating levels of OSM and soluble receptors sOSMR and sGP130 in the serum of AS patients and evaluated their possible association and correlation with the parameters of the disease.

MATERIALS AND METHODS

Patients and controls

Patients diagnosed with AS (n = 130) according to the modified New York criteria (VAN DER LINDEN; VALKENBURG, 1984) and healthy control (HC) (n = 130) matched by sex and age group were recruited at the Hospital of Clinics at the Federal University of Pernambuco and included in this study. Among the 130 patients with AS, the majority were male (71.5%; n = 93), with a mean age of 43.1 (± 12) and diagnosis time of 11.8 (± 8.3) years and 37 (28.5%) females with a mean age of 43.9 (± 10) and diagnosis time of 12.7 (± 8.7) years. one hundred and thirty healthy volunteers from the local community matched for sex and age were also included in the present study. Of the healthy male volunteers, the average age was 43.6 (± 12.2) and women with an average of 43.9 (± 12.1). Patients and volunteers with a previous or current diagnosis of other rheumatic diseases or neoplasm were excluded from the study; smokers, pregnant women or presence of acute or chronic infection at the time of assessment.

The AS disease activity score was calculated using the Disease Activity Index (BASDAI- Bath AS Disease Activity Index) (VAN DER LINDEN; VALKENBURG; CATS, 1984). The BASDAI average was 2.9 (± 2.02). In this study, patients in activity with BASDAI ≥4 were considered. Table 1 summarizes the clinical and demographic characteristics of patients with AS.

The study protocol was approved by the research ethics committee of Universidade Federal de Pernambuco, CAAE: 55261116.2.0000.5208, according to the Declaration of Helsinki, and informed consent was obtained from all participants.

Enzyme-linked immunosorbent assay (ELISA)

Peripheral venous blood samples were obtained from all subjects in tubes without anticoagulant. Subsequently, the serum was separated by centrifugation and stored at -80 °C until use. Serum levels of OSM, sOSMR and sGP130 were measured in AS patients and healthy controls by ELISA using specific kits (R&D Systems, Minneapolis, USA and Invitrogen, Carlsbad, Califórnia, EUA), according to the manufacturer's protocol. The lower detection limits of the assays were 15.2 pg/mL for OSM and 156.25 pg/mL for sOSMR and sGP130.

Western Blotting Assay

The volume equivalent to 50ug of total proteins (amount of protein per well) was denatured and subjected to gel electrophoresis in the presence of (SDS-PAGE). After electrophoretic separation, the proteins were transferred to a PVDF membrane and subsequently blocked with TBS / BSA solution and incubated with primary antibody (Rabbit IgG polyclonal antibody/ P13725) (BOSTER) and secondary antibodies, following the respective time intervals and washes. Albumin expression was used as load control (WANG, et al., 2018). The signal was detected using the chemiluminescent ECL substrate and the chemiluminescence detected by ChemiDoc XRS + System (Bio-Rad).

Statistical analysis

Data were analyzed using the GraphPad Prism, version 6.0, statistical program (San Diego, CA). The results are expressed as the median [interquartile (range–IQR)]. The normality of the samples was verified with the D'Agostino test. The difference in serum levels between groups was evaluated by Student's t test or Mann-Whitney U test Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. We considered correlation (R2) strength as follows: $0<R2 \leq 0.35$ is weak correlation; $0.35<R2 \leq 0.67$ is moderate correlation; $0.67<R2 \leq 1$ is strong correlation. Continuous variables are expressed as median and interquartile range. P value<0.05 was considered statistically significant.

RESULTS

Serum levels of OSM, sOSMR and sGP130 in patients with ankylosing spondylitis and healthy volunteers

Serum OSM levels were undetectable in all patients with AS and HC with the Elisa assay. Through the Western Blotting trial, we confirmed a significant increase in OSM expression in patients compared to controls (figure 1). The serum levels of sOSMR [median (IQR (25-75 percentile)] [171.1 (63.06-224.7) ng / mL] and sGP130 [163.9 (143.1-231.8) ng / mL] were significantly lower in patients with AS when compared to healthy controls [409.7 (323.9-480.0) ng / mL]; [284.3 (253.1-318.9) ng / mL], both with (p <0.0001) (Figure 2). We did not obtain any difference regarding demographic data.

Associations and correlations between sOSMR and sGP130 levels in disease activity in patients with ankylosing spondylitis

When the AS group was stratified by disease activity, a statistically significant difference was observed in the association between sOSMR levels in patients with low activity (BASDAI <4) (157.8 [47.82- 192.2] ng/mL) when compared to high activity (BASDAI ≥4) (198.5 [120.1-396.5] ng/mL), (p <0.002), and significantly lower than controls (p <0.0001). The same was not observed with the sG130 (Figure 3). No

clinical correlations were found between the expression of sOSMR and sGP130 with BASDAI disease activity.

Associations between sOSMR and sGP130 levels in the treatment of patients with ankylosing spondylitis

When patients were stratified by treatment, the sOSMR levels of untreated patients [175.1 (61.49-190.6] ng/mL)] were similar to those using anti-TNF alone [137.5 (29, 89-176.8) ng/mL] and IL-17 inhibitor [121.3 (278.5-230.3) ng / mL], however, there was a significant decrease compared to controls (p<0,0001). Patients who used only DMARDs [195.2 (118.2-372.1) ng/mL] showed a significant increase (p=0.0004) compared to patients treated with anti-TNF [137.5 (29.89 -176.8) ng/ml]. Patients using NSAIDs [216.5 (155.9-390.1) ng/mL], had significantly higher levels (p=0.01) of sOSMR when compared to patients without treatment [175.1 (61.49-190.6] ng/mL)]. Serum levels of sGP130, patients without treatment [153.9 (132.4-206.1) ng/mL] showed similar levels of patients using DMARDs [144.2 (134.6-169.3) ng/mL] and NSAIDs [153.5 (139.3-23.8) ng/mL], and a significant increase in patients using anti-TNF [220.5 (157.7-223.8) ng/mL], and IL-17 inhibitor [220.2 (172.8-236.5) ng/mL].

DISCUSSION

In our study we observed an increase in OSM expression, and a decrease in soluble sOSMR and sGP130 receptors circulating in AS patients in relation to HC and we observed their correlation with BASDAI disease activity and time of diagnosis. Until now, the evaluation of these soluble receptors and their interaction with disease comorbidities have not been investigated.

Circulating serum OSM is not observed under normal conditions, but high levels are being detected in many chronic inflammatory conditions, especially those involving fibrosis (HERGOVITS et a., 2017; STAWSKI; TROJANOWSKA, 2019). Are also found in the synovial fluid of patients with rheumatoid arthritis, but not in osteoarthritis (OKAMOTO et al., 1997; HUI et al., 1997; MANICOURT, et al., 2000).

This effect can significantly exacerbate IL-1 and TNF-α activity, resulting in inflammation and destruction of cartilage (CAWSTON, et al., 2005, LE GOFF et al., 2014).

OSM has been studied in osteoclastogenesis process. One *in vitro* study conducted by Korkosz collaborators (2018) in Poland assessed the serum osteoclastogenic potential of 27 AS patients compared to healthy controls. This study investigated the levels of osteoclastogenesis mediating factors, soluble RANKL, M-CSF, OPG and the OSM, IL-6, IL-17, TNF cytokines in sera from patients with axial spondyloarthritis. The serum of 11 patients with axial spondyloarthritis and 10 controls were cultured in vitro in the presence of monocytes obtained from healthy individuals. The study found that the serum levels of soluble RANKL seen in axial spondyloarthritis are significantly lower compared to healthy blood donors. It showed that the sera of patients with axial spondyloarthritis contain in the circulation soluble factors, such as IL-6, OSM, IL-17A, TNFα and that these factors influence the differentiation of healthy monocytes in relation to the osteoclast lineage. Median OSM values were 11.39 in patients and were not detected in controls.

We couldn't detect OSM by the Elisa assay, so we propose to evaluate using Western Blotting. Two studies that evaluated the OSM in the serum of patients with systemic sclerosis reported that the expression of this cytokine in the serum must be less than the capacity of Elisa's commercial kits (HASEGAWA, et al., 1998; HASEGAWA, et al., 1999). In our study, we observed a significant increase in the expression of the OSM protein in patients' serum compared to healthy volunteers using Western Blotting.

Circulating sOSMR levels are commonly detected in healthy individuals with an average value of approximately 80 to 200 ng/mL, similar to that reported for sGP130 (DIVEU et al., 2006; NARAZAKI et al., 1993; ZHANG et al., 1998). Unlike what is observed, for example, of circulating TNF-α that is expressed in the picogram range in healthy individuals (NARAZAKI et al., 1993; NIKOLAUS et al., 2018).

In our study, we observed that the expression of sOSMR and sGP130 is decreased in relation to the HC and that the OSM found high in relation to the controls. OSM is able to upregulate its own soluble receptor and the presence of small concentrations such as 12.5 ng / mL of OSM are able to increase sOSMR

secretion (DIVEU et al., 2006). For the antagonistic action of sOSMR to occur initially, it is necessary to connect to sGP130 and later both to OSM, and with this the reduction of signaling of receptors I (GP130 / LIFR) and II (GP130 / OSMR). It is important to note that sOSMR can also bind to the soluble IL-31 receptor (soluble IL-31R) and neutralize the action of IL-31 to its IL-31R receptor (DIVEU et al., 2006). The increase in serum IL-31 levels has already been described in AS because it is associated with a decrease in structural damage in patients with early axial spondyloarthritis, but which is also associated with low bone mineral density (ROSINE, et al., 2018). What can generate something very peculiar, because in our study we found that patients with low BASDAI have a significant decrease in the expression of sOSMR in relation to those with high BASDAI. And this increase can be due to the increased expression of OSM in patients with high BASDAI and, as a consequence, influencing the IL-31 pathway, increasing the structural damage in AS.

In the literature, studies with OSM are based on the use of live models that employ animals that are disabled for OSMR (WEST, et al., 2018). A study using the OSM (-, -) and OSMR (-, -) models showed greater expression of OSM and new bone reduction, subsequently reducing osteoblasts. This study stated that bone formation is mediated by OSMR and STAT3 signaling (WALKER et al., 2010; GUIHARD, 2012). Studies show that mice with OSMR deficiency exhibit less osteoclasts and less bone formation. Rats without CT-1, IL-11R and OSMR exhibit reduced bone formation (PLOTKIN, AGUILAR-PEREZ, BIVI, 2019). Which leads us to assume that the low expression of sOSMR in AS serum, compared to healthy control patients, found in our study, may be implicated in the bone remodeling process.

The understanding of the molecular mechanisms that lead to new bone formation in AS has significantly improved, but is still incomplete. We already know that the factors involved in inflammation are linked to bone remodeling processes (GINALDI; MARTINIS, 2016). In addition, pro-inflammatory cytokines involved in inflammation can affect osteoclastic and osteoblastic activity, leading to systemic bone loss (SHAW; GRAVALLESE, 2016; WALSH, 2009).

Regarding treatments, we observed that patients not treated or treated only with immunobiologicals showed a significant reduction in the expression of sOSMR in relation to other treatments and controls, unlike those who use DMARDs or NSAIDs

that had sOSMR expression similar to the volunteers. In contrast to that observed with sGP130, which showed greater expression in patients using immunobiologicals. DMARDs and NSAIDs are part of the first line of drugs used by patients. However, many patients do not respond to conversational treatment, requiring the use of biological drugs. And many patients respond better to combined biological and conventional treatment (BRAUN et al., 2011).

CONCLUSION

In summary, these results showed that the expression of sOSMR and sGP130 is reduced in patients with AS compared to HC, and serum sOSMR levels are lower in patients with low disease activity (BASDAI <4). These results suggest that, despite the control of clinical activity, the active OSM / OSMR pathway can mediate bone remodeling and the soluble receptors sGP130 and sOSMR antagonists of OSM are of fundamental importance to neutralize its action. These findings suggest that the expression control of soluble sOSMR / sGP130 receptors may eventually become a potential therapeutic target in spondyloarthritis.

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FIGURES

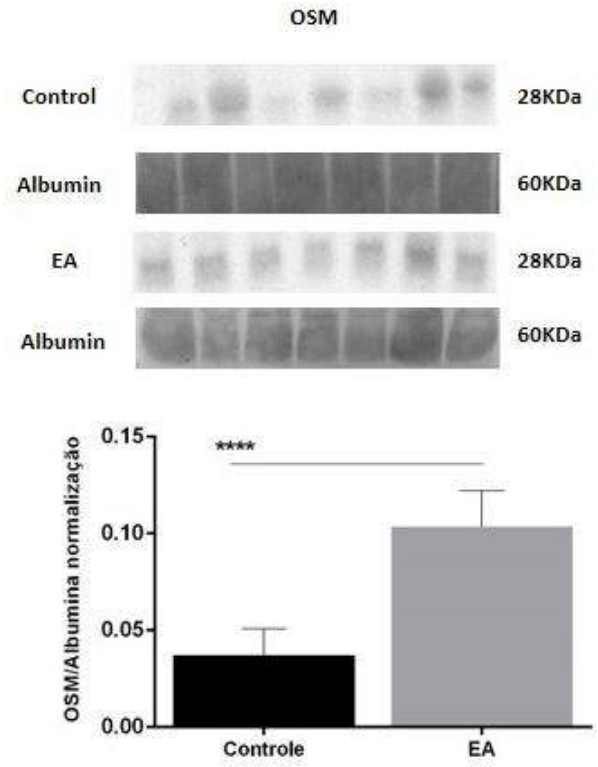

Figure 1: Serum OSM levels in ankylosing spondylitis patients (AS; n=7) compared with healthy controls (HC; n=7)

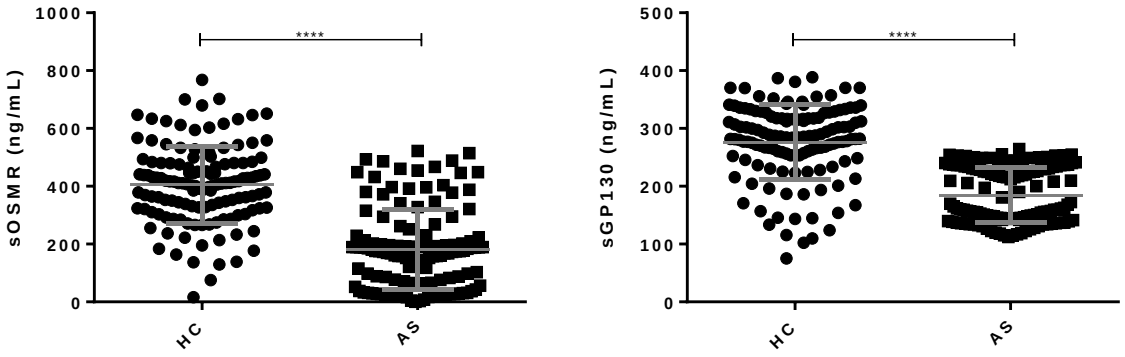

Figure 2: Serum sOSMR and sGP130 levels in ankylosing spondylitis patients (AS; n=130) compared with healthy controls (HC; n=130) (CI=95%; Mann Whitney test)

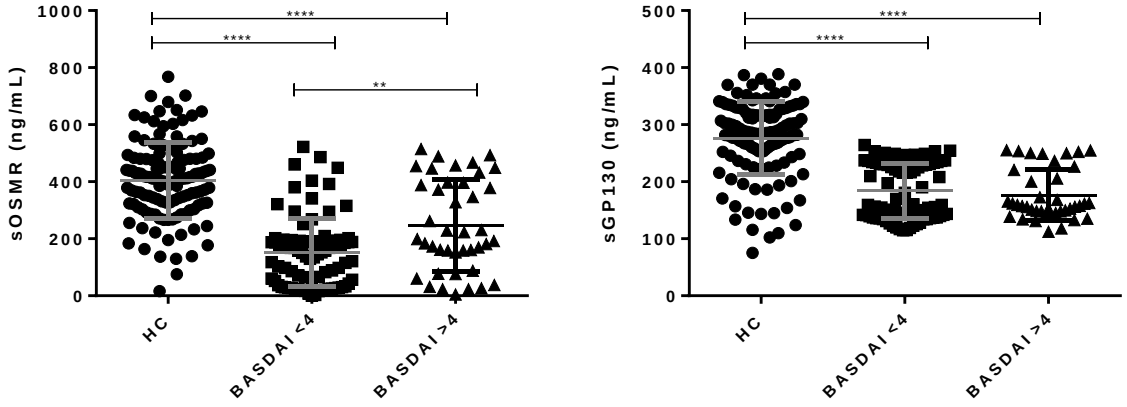

Figure 3: Serum sOSMR and sGP130 levels in ankylosing spondylitis patients with low disease activity (BASDAI <4; n=89), high disease activity (BASDAI≥4; n=41) and healthy controls (HC; n=130) (CI=95%; Mann Whitney test)

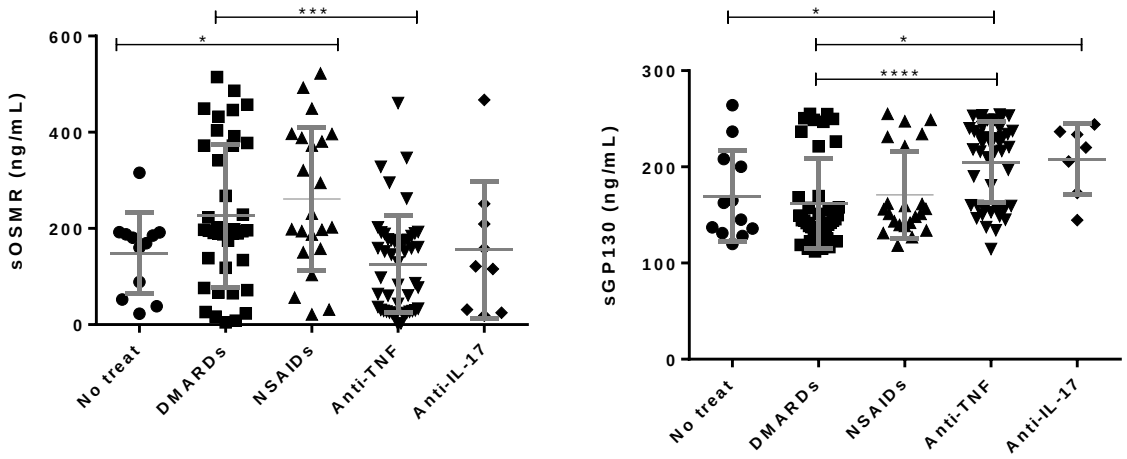

Figure 4: Serum sOSMR levels in ankylosing spondylitis patients comparing healthy controls (n=130) and ankylosing spondylitis treatment: no treat = no treatment (n=12); DMARDs= disease-modifying antirheumatic drugs (n=40); NSAID= no steroidal anti-inflammatory drugs alone (n=22); anti-TNF=tumor necrosis factor inhibitor (n=45); Anti-IL-17= Interleukin 17A inhibitor (n=7) (CI=95%; Mann Whitney test)

TABLES

Table 1. Clinical and laboratory characteristic of the patients with ankylosing spondylitis.

Characteristics	AS (n=130)	HC (n=130)
Males; n (%)	93 (71.5)	93 (71.5)
Female; n (%)	37 (28.5)	37 (28.5)
Males age (years; mean ± SD)	43.2 (±12)	43.6 (±12.2)
Female age (years; mean ± SD)	43,9 (±10)	43.8 (±12.1)
Smoking, n (%)	6/130 (4.6)	0/130 (0)
Median of disease duration (years; mean ± SD)	12.1 (±8.2)	-
BASDAI (median ± SD)	2,85 (±1.46)	-
BASDAI elevated ≥4 (N; %)	5 (±1.46)	-
Extra-articular symptoms present at the time of collection:		
uveitis, n (%)	3/130 (2.3)	
psoriasis, n (%)	1/130 (0.76)	
IBD, n (%)	1/130 (0.76)	
Clinical variables related to disease activity:		
dactylite, n (%)	1/130 (0.76)	
peripheral arthritis, n (%)	17/130 (13)	
enthesitis, n (%)	32/130 (24.6)	
low back pain, n (%)	89/130 (68.4)	
Treatment:		
NSAIDs, n (%)	24/72 (18.4)	

sulfasalazine, n (%)	39/130 (30)
methotrexate, n (%)	12/130 (9.2)
infliximab, n (%)	16/130 (12.3)
etanercept, n (%)	6/130 (4.6)
adalimumab, n (%)	35/130 (26.9)
golimumab, n (%)	1/130 (0.76)
secuquinumabe, n (%)	9/130 (6.9)
Statin, n (%)	10/130 (7.6)
antihypertensive, n (%)	13/130 (10)
Corticosteroid, n (%)	2/130 (1.53)
Antidiabetic, n (%)	2/130 (1.53)
proton pump inhibitors, n (%)	6 /130 (4.6)
antidepressant, n (%)	4/130 (3)
beta-blocker, n (%)	3/130 (2.3)
antirheumatic, n (%)	4/130 (3)
no treatment, n (%)	12/130 (9.2)

Abbreviations: AS, ankylosing spondylitis; HC, healthy controls; n, sample number; IBD, inflammatory bowel disease; NSAIDs, Nonsteroidal anti-inflammatory drugs; SSZ, sulfasalazine.

4.2 ARTIGO 2 - PPARγ agonist activity and decreased cytokine excretion of new thiazolidine-2,4-dione (LPSF/JB-20) in human lymphocytes of patients with ankylosing spondylitis

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ABSTRACT

Ankylosing spondylitis (AS) is a chronic inflammatory disease characterized by ossification that can lead to bone fusion in the spine. Thiazolidine derivatives (TZD) are a class of PPAR transcription factor agonist drugs that have been synthesized in order to reach not only PPARs, but also other pathways of adipogenesis and osteogenesis. In view of the need for new treatments that can treat inflammation and the bone remodeling process, we propose to evaluate the immunomodulatory activity of a new TZD, in peripheral blood mononucleated cells (PBMC) from AS patients and volunteers to assess its possible action via PPARγ. The new 5-((5-bromo-1H-indol-3-yl)methylene)-3-(3,5-dimethylbenzyl)thiazolidine-2,4-dione (LPSF/JB-20) was synthesized and the effect of was evaluated on the culture supernant of PBMCs of 30 EA and 13 healthy volunteers and cytokines were measured by the sandwich immunoassay (ELISA). In addition, we evaluated the toxicity and adipogenic differentiation assay to ascertain its possible action via PPARγ. *In silico* analyzes were performed to investigate the binding of the compound to PPARγ. Our results indicated that LPSF / JB-20 is not toxic to PBMCs and that its action is able to significantly decrease the expression of IFN-γ, IL-6, IL-17, IL-10 and TNF-α in comparison with the conditions stimulated with PHA-M at a dose of 50μM ($p < 0,0001$; 0,009; 0,0001; 0,0001; 0,01) respectively, but not IL-1β. The expression of these cytokines is known for their role in the inflammation process in AS. The control of inflammation can contribute to the reduction of signs and symptoms of patients and act in the process of calcification. We conclude that the compound LPSF/JB-20 is capable of acting on inflammation induced by PHA-M and it decreased the expression of IFN-γ, IL-6, IL-17, IL-10 and that this action may not be correlated with the PPARγ transcription factor.

Keywords: thiazolidine derivatives, receptors activated by peroxisome proliferators, phytohemagglutinin-M, spondyloarthropathies

1 INTRODUCTION

Ankylosing spondylitis (AS) is a chronic inflammatory disease, characterized mainly by impairment of the axial skeleton and sacroiliac joints (BRAUN; SIEPER, 2007). The persistent inflammatory state generated through pro-inflammatory cytokines and other immune mediators has a significant influence on bone remodeling (GINALDI; MARTINIS, 2016). Can cause severe chronic pain and in more advanced cases fusion of the spine (ZHU, et al., 2019; POPITZ, 1997) Immunobiologicals made it possible to improve the treatment of patients. They are able to decrease bone mineral density, but not to prevent structural damage caused by radiographic vertebral fractures in AS (BEEK, et al, 2019; MAGREY; KHAN, 2017; MAAS et al., 2016, LENCEL, et al, 2011).

Thiazolidine derivatives (TZDs) are a class of drugs that have been studied for a long time (LALLOYER et al., 2010; GALE et al., 2001; SOHDA, et al., 1982). Varied substituents are added to the thiazolidine-2,4-dione nucleus and provide this molecule with a broad spectrum of biological activities (JAIN; VORA; RAMAA, et al., 2013). Especially those that have substitutions in the methylene group and in the TZD -NH, which target a range of enzymes involved in various pathological conditions, such as diabetes, hyperlipidemia, infectious diseases, inflammation and cancer (GUPTA; JOSHI; RAMAA, 2019).

TZDs are known for their action on receptors activated by peroxisome proliferators (PPARs) and for being potent synthetic agonists of PPAR-γ (SCHUPP; LAZAR, 2010; ESCHER; WAHLI, 2000). And by repressing the expression of the nuclear factor kB (NF-kB) signaling pathway independently by negative feedback (YKI-JARVINEN, 2009; REGINATO; LAZAR, 1999). PPARs are nuclear receptors that act together with retinoid X receptors (RXRs) and can control the expression of genes involved in adipogenesis and inflammation (AHMADIAN et al., 2013).

Three isoforms of the PPAR nuclear receptor are known: PPAR-α, PPAR-γ and PPAR-β / δ. The ligands act to control the expression of different genes. In the

absence of a linker, the PPAR / RXR heterodimer is linked to co-repressors, which prevent their binding to elements responsive to peroxisome proliferators (PPRE). When induced by a natural or synthetic ligand, such as TZDs, they can trigger changes in structural conformation and recruit transcriptional coactivators causing the PPAR: RXR complex to bind to PPAREs in a dependent way. The PPAREs are located in specific sites of each gene, increasing their gene transcription (ZANG, 2015). This increase in gene expression leads to a decrease in the expression of inflammatory cytokines (SCHUPP; LAZAR, 2010; ESCHER; WAHLI, 2000). What makes it essential to study the action of TZDs to identify the role of PPAR as a probable mediator of cytokines, as well as in the ectopic ossification process in AS (LENCEL et al., 2011).

Given the above, we aim to investigate the immunomodulatory activity of a new thiazolidine derivative 5-((5-bromo-1H-indol-3-il)metileno)-3-(3,5-dimetilbenzil) tiazolidina-2,4-diona, the LPSF / JB-20 and its PPARγ agonist activity in peripheral blood mononuclear cells (PBMC) from EA patients activated with PHA-M.

MATERIALS AND METHODS

Study population

The study included 30 AS patients (20 men, mean age 40.1 ± 8.8 years, and 10 females 40.8 ± 9.6) classified according to modified New York criteria (LINDEN; VALKENBURG; CATS, 1984). All in attendance at the Hospital das Clínicas (UFPE) Pernambuco / Brasil. The AS disease activity score was calculated using the Disease Activity Index (BASDAI- *Bath AS Disease Activity Index*) (van der LINDEN; VALKENBURG; CATS, 1984). Patients with hypertension and osteoarthritis were included in the study. In this study, patients with low activity (BASDAI <4) and high activity (BASDAI ≥ 4) were considered. Healthy controls (HC), members of the local community, had no known disease diagnosis and did not use medication.

The study was approved by the Ethics Committee of Universidade Federal de Pernambuco (CEP/CCS/UFPE: 55.261.116.2.0000.5208), and was conducted

according to the 1964 Helsinki Declaration. Informed consent was obtained from all individual participants included in the study.

The thiazolidine derivative 5-((5-bromo-1H-indol-3-yl)methylene)-3-(3,5-dimethylbenzyl)thiazolidine-2,4-dione (LPSF/JB-20)

The LPSF / JB-20 was synthesized and provided by Laboratório de Planejamento e Síntese de Fármacos Lab (LPSF) to perform the tests. The compound was weighed and dissolved initially in dimethylsulfoxide (DMSO) and later in culture medium and serum under aseptic conditions to obtain the treatment concentrations.

Isolation and cultivation of PBMC

PBMCs were isolated from the peripheral blood of patients EA (n = 30) and HC (n = 13) by the centrifugation technique Ficoll-Paque PLUS (GE Healthcare, Pittsburgh, PA, USA). After separation, cells were counted and cultured ($1x10^6$ cells/ mL) in 24-well plates in RPMI-1640 (Gibco) culture medium supplemented with L-Glutamine, 10% fetal bovine serum (FBS) (Gibco), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Gibco) and 200 U/mL penicillin/streptomycin (Gibco). After plating the cells, they were stimulated with 1% phytohemagglutinin M (Gibco) in the presence or absence of LPSF / JB-20 at concentrations of 10, 25 and 50 µM, determined by previous tests, and grown in a humid atmosphere of 5% CO_2 at 37 ºC. After 48 hours, the culture supernatant was collected for cytokine measurements.

Cytotoxicity assays

The MTT assay was used to assess the cytotoxicity of the thiazolidine derivative in HC PBMCs (n = 3) (MOSMANN et al.,1983). The cells were plated in 96-well plates ($3x10^5$ cells / well). Subsequently, the cells were subjected to concentrations of 10 and 75 µM for 48 hours at 37ºC and 5% CO_2. After that period, the cells were subjected to 20µL (5mg / mL) of MTT reagent (3-(4,5-dimetiltiazol-2yl)-

2,5-difenil brometo de tetrazolina) por 3 horas. Then 130 µL of 20% sodium dodecyl sulfate (SDS) was added for the dilution of Formazan crystals. Absorbance was measured after 24 hours at 570 nm using a microplate reader (Elx808, Biotek, EUA). The experiments were carried out in triplicate. The concentration required to inhibit 50% of cell viability (IC50) relative to the DMSO vehicle (0.1%) was calculated using the Origin v. 8.0.

Quantification of cytokines

The levels of IFN-γ, IL-6, IL-17, IL-10, TNF-α and IL-1β in the PBMC culture supernatant stimulated by PHA-M and treated with LPSF/JB-20 were measured by enzyme immunoassay *sandwich* adsorbent (ELISA) according to manufacturers' recommendations (BD Biosciences, San Jose, CA, USA) for IFN-γ, IL-6, IL-10 and TNF-α; (Invitrogen, Carlsbad, Califórnia, EUA) for IL-17A e IL-1β). The lower detection limits of the assays were 9,37pg/mL for IFN-γ and IL-6; 15,62pg/mL for IL-10 and 7,81pg/mL for IL-17, TNF-α and IL-1β. The absorbance reading was performed at 450/570nm in a microplate reader (Biotek EL808).

Adipogenic differentiation assay

Murine pluripotent fibroblast lineage cells 3T3-L1 ($2x10^4$ cell/mL) were maintained in a 24-well plate and DMEM culture medium (Gibco) supplemented with 10% FBS (Gibco) to evaluate the possible LPSF / JB-20 agonist activity in PPARγ. After reaching an approximate confluence of 80%, the cells were differentiated into pre-adipposites with differentiation induction medium, composed of DMEM supplemented with 10% FBS and 10µg / ml of insulin, 0.5 Mm of 3-isobutyl-methylxanthine (Sigma) and 1µM dexamethasone (Sigma), for 72 hours. LPSF / JB-20 (50µM) and rosiglitazone (100µM) were added to the differentiated medium. Subsequently, the cells were maintained in maintenance medium (DMEM supplemented with 10% FBS and 10 µg/mL insulin) until complete differentiation. The evaluation of lipid accumulation was performed with 0.5% Oilred-O (Sigma) staining. First, the cells were washed with PBS1x and fixed with 10% formalin for 15 minutes, then washed with distilled water for the addition of the dye for 15 minutes. Afterwards, the cells were washed with distilled water and 50% isopropanol and

observed under an optical microscope to visualize the accumulation of red stained lipids.

Molecular modeling

The three-dimensional structure of the LPSF/ JB-20 compound, including its E and Z isomers, were designed using the SPARTAN 08 'program (SPARTAN, 2008) and the optimized structures using the RM1 method (ROCHA, et al., 2006). The docking analysis was performed on the PPARγ ligand (follow: PDB: 5YCP) on the website http://www.rcsb.org, which has the compound rosiglitazone (follow: BRL) as a co-crystallized ligand. The active site was defined as all the atoms present in the 6.0 Å radius from the position where the co-crystallized ligand BRL is found. The GOLD 5.6 program (Gold software, 2018) was used to perform docking calculations, using the ChemPLP scoring function. The docking calculations involving the compound LPSF/ JB-20 took into account the degrees of freedom of the receiver in order to simulate the induced adjustment effect, allowing the flexibility of the following amino acids:PHE282, CYS285, GLN286, ARG288, HIS323, TYR327, PHE363, LYS367, HIS449 e TYR473. O programa BINANA (*DURRANT; MCCAMMON, 2011*) was used to map the intermolecular interactions of docking solutions, using the program's default settings. The figures were generated using the Pymol program (DELANO, 2002).

Statistical analysis

GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) was used for data analysis. The results of the continuous variables were expressed by mean/standard deviation (Mean ± SD) or median/interquartile range (Median ± IQR). Significant differences between the treatments were calculated by Wilcoxon’s signed rank test. Differences were considered significant when $p < 0.05$.

RESULTS

Organic Synthesis

The compound 5-((5-bromo-1*H*-indol-3-yl)methylene)-3-(3,5-dimethylbenzyl) thiazolidine-2,4-dione (LPSF/JB-20) was synthesized throughout three reaction steps, as showed in figure 1. The thiazolidine nucleus was obtained by cyclization reaction between equimolar quantities of thiourea and 2-chloroacetic acid in aqueous medium (a). Then, the thiazolidine-2,4-dione was used as limiting reagent in second-order nucleophilic substitution reaction (Sn2) with 3,5-dimethylbenzyl bromide in basic medium (b) to obtain the 3-(3,5-dimethylbenzyl)thiazolidine-2,4-dione (LPSF/JB-1) intermediary. The last step was carried out through a Knoevenagel condensation reaction between the LPSF/JB-1 intermediary and the 5-bromo-1*H*-indole-3-carbaldehyde (c), forming the LPSF/JB-20 thiazolidine derivative. All the worked compounds presented purity above 98%.

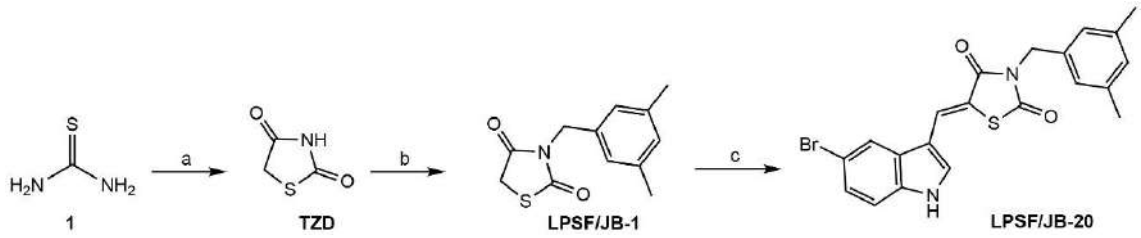


Figure 1. Synthesis route of LPSF/JB-20. (a) 2-chloroacetic acid (1eq), H_2O, 90°C, 24h; (b) KOH (1eq), 3,5-dimethylbenzila bromide (1,3eq), 60°C, 2h; (c) 5-bromo-1*H*-indole-3-carbaldehyde (1eq), ammonium acetate (2eq), acetic acid, 110°C, 4h.

The reaction was monitored by thin-layer chromatography (TLC) using EM silica gel 60 F254, visualized using UV light. The purity was assessed using the High-Performance Liquid Chromatography LC-20AT model (Shimadzu). The H^1NMR spectra were recorded on an Ascend III 300 MHz (Bruker) spectrometer at 20°C. The chemical shifts (δ, ppm) were assigned based on the internal standard signal of tetramethylsilane (TMS) in DMSOd6 or CDCl3 (δ = 0 ppm). The C^{13}NMR spectra were recorded by de same model machine, but using 75 MHz radiofrequency. The melting point was obtained through the automatic method using capillary tubes in a Buchi melting point M-565 apparatus. The infrared sign was recorder using KBr discs in spectrophotometer IR Prestige-21 model by Fourier transform (Shimadzu).

3-(3,5-dimethylbenzyl)thiazolidine-2,4-dione (LPSF/JB-1)

Thiazolidine-2,4-dione (42,68mM) was solubilized in acetonitrile (ACN). Laterally, the equimolar quantity of potassium hydroxide was solubilized in methanol (MeOH). The solutions were mixed and kept at room temperature and stirring to obtain the transition salt. Then, 3,5-dimethylbenzyl bromide (55,49mM) was solubilized in acetonitrile and added to the mixture. The reaction mixture was heated to 60°C for 2h. Afterward, the solvent was removed using a rotatory evaporator. The crystals were solubilized in ethanol and recrystallized at 60°C. After that, the crystals were filtrated and washed with ethanol. White solid. $C_{12}H_{13}NO_2S$. MW: 235.30; MP: 106.8°C. Yield: 60%. Purity: >99%. IR (cm^{-1}; KBr): 1751, 1683, 1377, 1147, 852, 789, 663. H^1NMR (300 MHz, DMSO-d6): δ = 2.23 (s, 6H, methyl groups), 4.27 (s, 2H, thiazolidine), 4.58 (s, 2H, methylene), 6.85 (s, 2H, benzilic ring), 6.91 (s, 1H, benzilic ring). ^{13}C NMR (300MHz, DMSO-d_6): δ = 20,83 (CH_3), 44.23 (CH_2), 125,29 (CH), 129,06 (CH), 135,47 (C4), 137,57 (C4), 171,9 (C=O), 172,29 (C=O). MS *m/z* (%): $(M)^+$ 234.95 (83), calculated 235.30.

5-((5-bromo-1*H*-indol-3-yl)methylene)-3-(3,5-dimethylbenzyl)thiazolidine-2,4-dione (LPSF/JB-20)

Equimolar quantities of LPSF/JB-1 intermediate and 5-bromo-1*H*-indole-3-carbaldehyde were solubilized in acetic acid with ammonium acetate (2eq.) as promoter. The solution was heated to 110°C for 4h. Then, the solution was cooled to obtain the crystals. The compounds were purified with successive ethanol washes. Yellow solid. $C_{21}H_{17}BrN_2O_2S$. MW: 441.34. MP: 240°C. Yield: 30.9%. Purity: >99%. IR (cm^{-1}; KBr): 3363, 1751, 1661, 1603, 1371, 1061, 882, 795, 697. H^1NMR: 2.33 (s, 6H, -CH_3); 4.74 (s, 2H, methylene); 6.90 (s 3H, benzilic ring); 7.38 (d, 1H, J = 9Hz, indole); 7.47 (d,1H, J = 9Hz, indole,); 7.84, (s, 1H, -CH=C), 8.19 (s, 1H, indole), 8.22 (s, 1H, indole), 12.32 (s, 1H, -NH). ^{13}C NMR (300MHz, DMSO-d_6): δ = 20,84 (CH_3), 44,41 (CH_2), 110,13 (C), 113,87 (C), 114,30 (C), 114,38 (C), 121,20 (CH), 125,20 (CH), 125,70 (CH), 125,88 (CH), 128,33 (C4), 129, 12 (CH), 130,15 (CH), 134,96 (C4), 135,65 (C), 137,67 (C), 165,28 (C=O), 167,95 (C=O).

Clinical differences of patients and disease activity

Of the patients included in the study (N = 30) the duration of the disease was (mean years ± standard deviation) 10.37 ± 7.92. No patient had extra-articular symptoms present at the time of collection, such as uveitis, psoriasis, dactitilitis or inflammatory bowel disease. The total mean disease activity was 2.8 ± 1.94. Other demographic clinical data of the patients are described in Table 1. We assessed the correlation separately from patients who had high composite BASDAI activity scores from those below. We did not observe statistical differences between clinical variables and BASDAI disease activity.

Table 1. Clinical characteristics of AS

Characteristics	AS = 30
Men; *N* (%) Age (years)	20 (75)
Mean ± *SD* (range)	40.15 ± 8.81 (25-58)
Female; *N* (%) Age (years)	10 (25)
Mean ± *SD* (range)	40.8 ± 9.65 (29-57)
Disease duration Men (years) Mean ± *SD* (range)	11.15 ± 6.87 (1-29)
Disease duration Female (years) Mean ± *SD* (range)	8.8 ± 9.16 (1-33)
BASDAI <4 Mediana (range)	1.7 (0-3.8)
BASDAI ≥4 Mediana (range)	4.8 (4.0-7.4)
Clinical manifestations related to disease activity:	
peripheral arthritis *N* (%)	7 (23.3)
Enthesitis *N* (%)	4 (13.3)
inflammatory low back pain *N* (%)	20 (66.6)
Other comorbidities not associated with the disease:	
arterial hypertension *N* (%)	7 (23.3)
Arthrosis *N* (%)	2 (6.6)
Diabetis	2 (6.6)
Treatments:	
Adalimumab *N* (%)	9 (30)

Secuquinumab *N* (%)	7 (23.3)
Etanercept *N* (%)	1 (3.3)
infliximab *N* (%)	3 (9.9)
Antibiotic *N* (%)	1 (3.3)
Hypertensive *N* (%)	5 (16.6)
Sulfasalazine *N* (%)	4 (13.3)
Antidiabetic *N* (%)	2 (6.6)
Proton pump inhibitors *N* (%)	2 (6.6)
Statins	2 (6.6)
No use of medication	3 (9.9)

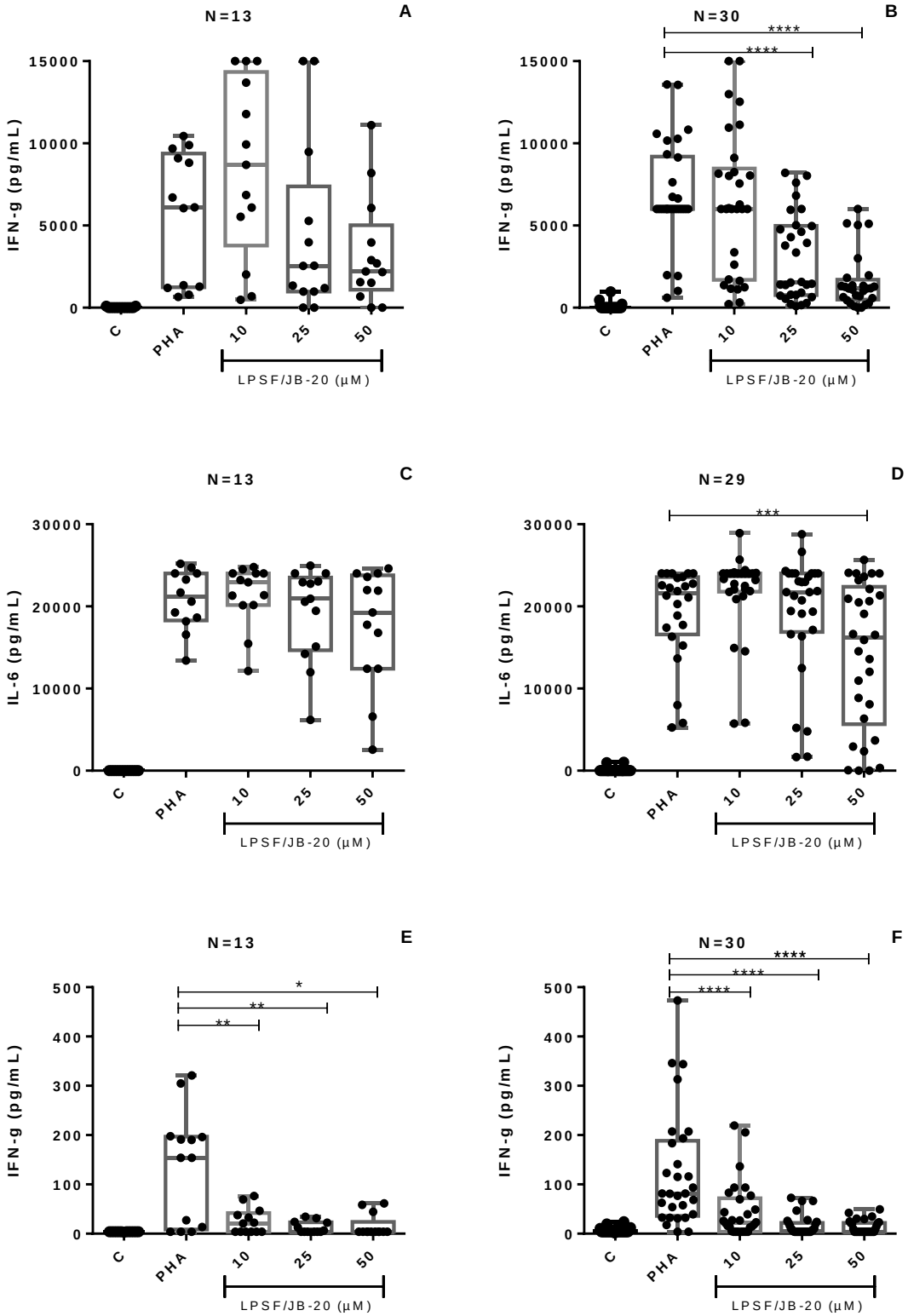
N, number of patients; *SD*, standard deviation

Cytotoxic activity of LPSF / JB-20

Cell viability was assessed after exposure to the LPSF / JB-20 thiazolidine derivative. The compound showed viability of 95.57 ± 7.67 at 10 µM and 78.50 ± 12.21 for a 75 µM dose. The compound has no cytotoxic activity in the concentrations evaluated in PBMCs of healthy volunteers.

LPSF / JB-20 inhibited the expression of cytokines produced in the PBMCs of healthy patients and volunteers

In evaluating the effect of LPSF/ JB-20 on PBMC of EA patients and HC, we observed a significant decrease in the levels of IFN-γ and IL-10 at the concentration of 25µM (0.0008; 0.001) and IFN-γ, IL -6, IL-10 and TNF-α at a concentration of 50µM ($p < 0.0001$; 0.009; <0.0001; 0.01), respectively, in AS patients when compared to conditions stimulated with PHA-M. At concentrations of 25 and 50 µM for IL-10 (0.01; 0.02) respectively, and 10 and 50 µM for IL-17 (0.003; 0.03) respectively, in the cells of the HC. IL-17 expression was significantly decreased at all concentrations in AS (<0.0001) and at concentrations of 10 and 50 µM in HC (0.003; 0.03) respectively. However, our results showed that the action of the compound was dependent on the disease, as it showed a greater decrease in the excretion of cytokines in relation to HC. The results are shown in figure 2.


A
N=13
IFN-g (pg/mL)
15000
10000
5000
0
C
PHA
10
25
50
LPSF/JB-20 (µM)
B
N=30
****
****
IFN-g (pg/mL)
15000
10000
5000
0
C
PHA
10
25
50
LPSF/JB-20 (µM)
C
N=13
IL-6 (pg/mL)
30000
20000
10000
0
C
PHA
10
25
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LPSF/JB-20 (µM)
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IL-6 (pg/mL)
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LPSF/JB-20 (µM)
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IFN-g (pg/mL)
500
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IFN-g (pg/mL)
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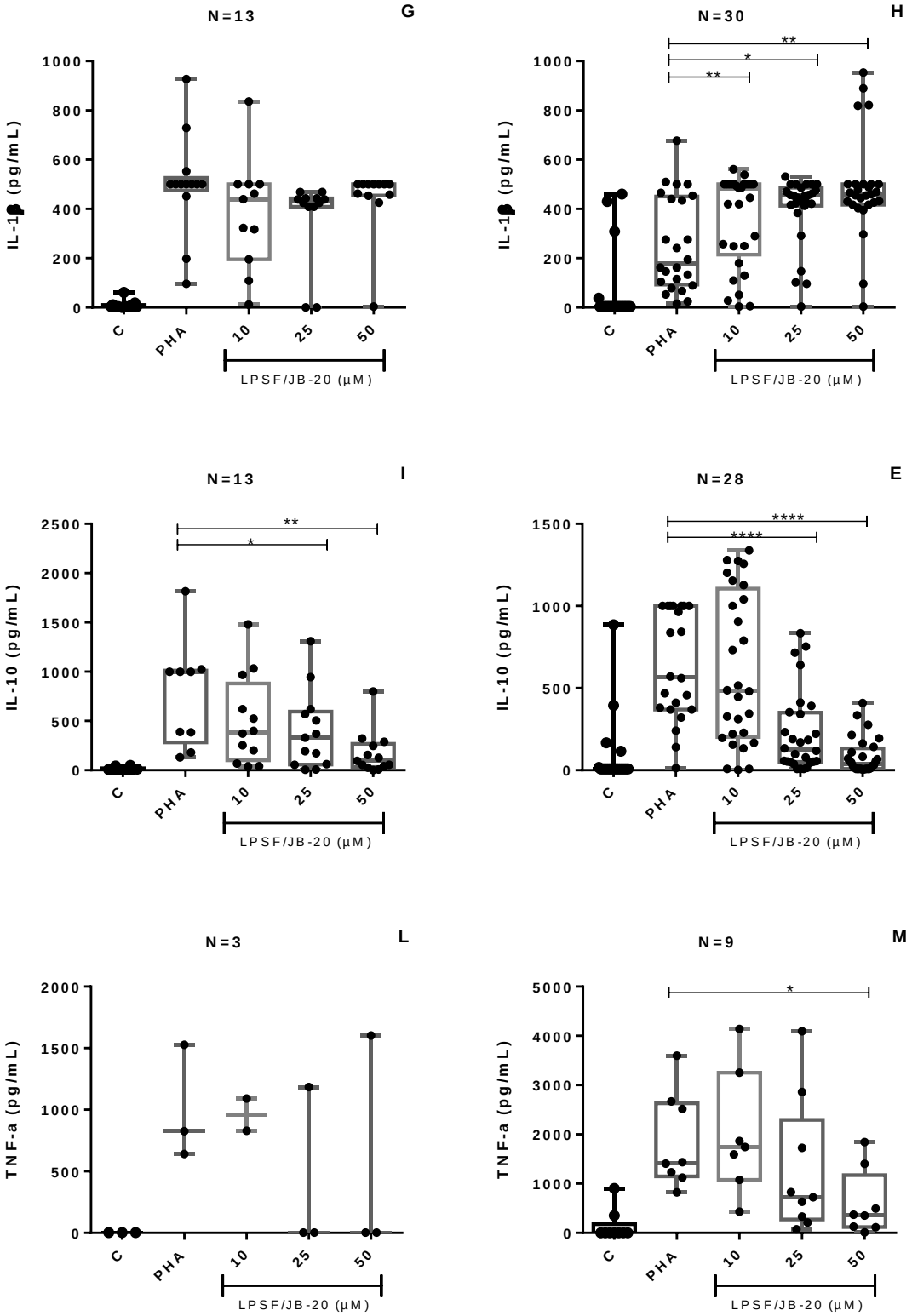

Figure 2- Levels of IFN-γ, IL-6, IL-17, IL-1β, IL-10 and TNF-α cytokines secreted by the PBMCs of healthy volunteers (A, C, E, G, I, L) and patients AS (B, D, F, H, J, M) treated with different concentrations of LPSF / JB-20 under stimulation with PHA-M

Adipogenic differentiation assay

To assess the activity of LPSF / JB-20 and its possible mediation via PPARγ, we induced adipogenic differentiation in pre-adipocyte cells. To this end, the 3T3-L1 mouse fibroblast cells were incubated with LPSF / JB-20 and with the synthetic ligand of PPARγ (rosiglitazone) for 7 days. Microscopy showed substantial time-dependent accumulation of cytosolic oil droplets induced by rosiglitazone and low accumulation with LPSF / JB-20 in these cells. Demonstrated in figure 3.

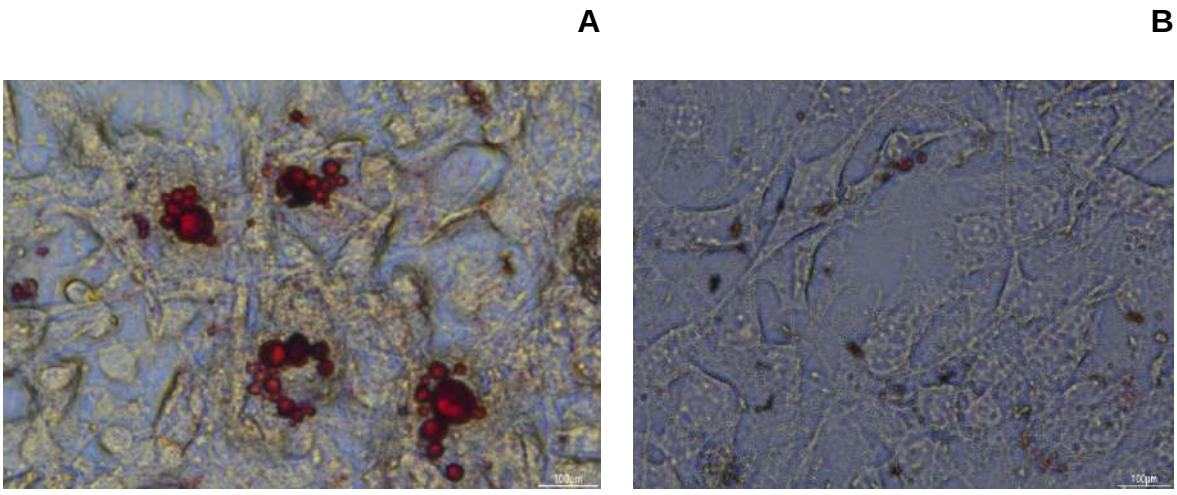

Figure 3- The 3T3-L1 pre-adipocytes were incubated with LPSF / JB-20 and rosiglitazone for 7 days. Light microscopy showed a substantial time-dependent accumulation of oil droplets in the cytosol stained by Oil red-O in cells treated with rosiglitazone (a) and a low accumulation in cells treated with LPSF / JB-20 (b)

Molecular modeling

In terms of affinity predicted by the scoring function (ChemPLP), the LPSF/JB-20-Z thiazolidine derivative scored (81.00) and rosiglitazone (76.73). Rosiglitazone (Figure 4A) performed a hydrogen bond with the SER289 residue with a distance (donor-acceptor) of 2.8 Å, and hydrophobic contacts with the ILE281, GLY284, CYS285, LEU330, VAL339, ILE341, LEU353, PHE363 residues, MET364 and HIS449. Similarly, the compound LPSF / JB20-Z (Figure 4B) also bonded hydrogen with the same residue as Rosiglitazone (SER289) with a distance of 2.5 Å, two pi-pi stackings with residues PHE282 and HIS449, both T-shaped, and hydrophobic

contacts with residues CYS285, GLN286, ARG288, ILE326, TYR327, LEU330, LEU333, ILE341, PHE363 and LEU469.

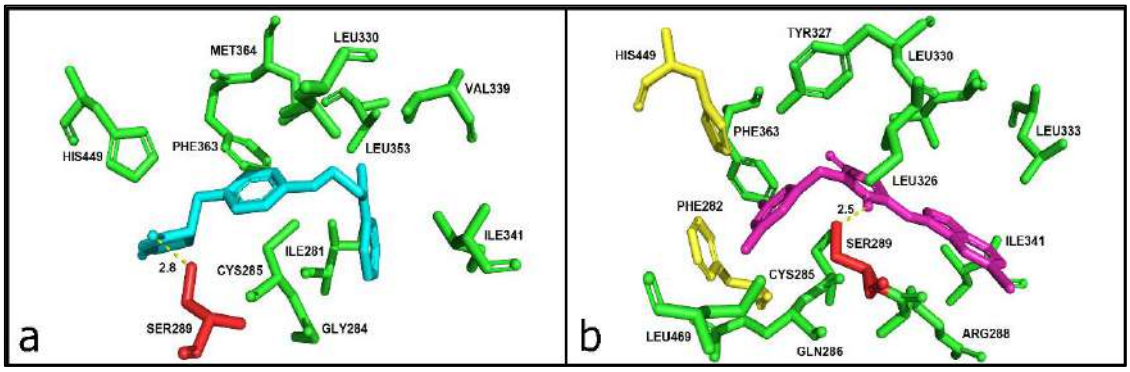

Figure 4- The intermolecular interactions between the PPARγ active site and the compounds. (a)rosiglitazone (cyan), (b) LPSF / JB20-Z (magenta)

DISCUSSION

In this study we evaluated LPSF/JB-20, a molecule with an action potential on the activation of the PPARγ transcription factor. We evaluated its cytotoxicity, and the molecule showed no toxicity at the doses studied. The expression of the cytokines IFN-γ, IL-6, IL-10, IL-17, IL-1β and TNF-α in PBMC estimated by PHA-M were evaluated and we found that LPSF/JB-20 has a potent action on decreased expression of these cytokines, except for IL-1β. The mechanism of action of the thiazolidine derivative was evaluated through adipogenic differentiation and its binding to the PPARγ receptor through molecular *doking*.

For the evaluation of immunomodulation, we used the inflammatory stimulus PHA-M for its ability to activate and selective non-specific proliferation of T cells in the presence of monocytes (CEUPPENS, et al. 1988; POTTER; MOORE, 1975). And with that, produce appropriate cytokines to mount the immune response (WATTRANG; PALM; WAGNER, 2012; ZHANG; ZHAO; HE, 2011). In our study, PHA-M effectively stimulated the expression of IFN-γ, IL-6, IL-10, IL-17, IL-1β and TNF-α in the PBMCs of healthy patients and volunteers. And the LPSF / JB-20 thiazolidine derivative was able to significantly decrease the expression IFN-γ, IL-6, IL-10, IL-17 and TNF-α. PBMCs of AS patients with elevated BASDAI had previously been evaluated when stimulated with PHA in previous studies compared to healthy

volunteers. And in these trials, they observed a significant increase in TNF-α and IL-1β, and suggested that these pro-inflammatory cytokines may play an important role during the active inflammation of these patients (CHOU, et al., 2007). In another study, the production of TNF-α, IL-1β and IL-10 was compared in response to PHA stimulation in PBMC of AS patients compared to healthy controls. And they found that IL-1β levels were significantly higher in patients with AS than in controls. However, they did not observe differences in median levels of TNF-α or IL-10 (VAZQUEZ-DEL, et al, 2002). In our study, we observed an increase in the expression of these cytokines after stimulation with PHA-M, however the compound LPSF/JB-20 was unable to decrease the expression of IL-1β, but significantly reduced IL-17.

Suppression of the excretion of cytokines involved in the pathogenesis of AS using the LPSF/JB-20 thiazolidine derivative may have benefits in reducing inflammation. And the effect that the compound is having on the secretion of cytokines can be independent of PPARγ. In the adipogenic differentiation assay, it showed that LPSF/JB-20 may not act in a similar way to rosiglitazone, which may suggest that its action is related to a partial activity of PPARγ, which leads us to believe that it may be an agonist. partial or act independently of PPARγ. Partial agonist TZDs have been reported in the literature and referenced for their ability to improve side effects and also increase the therapeutic value for different diseases such as cancer, inflammation and cardiovascular diseases (MIRZA; ALTHAGAFI; SHAMSHAD, 2019). An assay performed with a PPAR indole-thiazolidiene pan ligand nicknamed LYSO-7 in its action triggered by neutrophils activated by the G protein coupled receptor was able to activate PPAR gamma and beta and blocked NF-kB controlling the activity of neutrophils that is the target of medications and thereby treat unwanted inflammations (SANTIN et al., 2018).

In the literature we find a series of TZDs that were designed using the PPARγ crystallized active site. The molecules that showed the best ChemPLP scores were synthesized and subsequently tested (SAWANT, et al., 2018). The in vitro tests of this series of TZDs demonstrated that the compound maintained antidiabetic activity comparable to the drug pioglitazone, which is also a standard PPARγ agonist drug (QUINTANILLA RODRIGUEZ; CORREA, 2020). This was due to the fact that TZDs present substitution of non-polar substituents that remove electrons in the fourth position of the pyrimidine backbone and contain hydrogen bond acceptor in the

nitrogen of the thiazolidine nucleus, thereby causing the PPARγ receptor to be inhibited (SAWANT, et al., 2018). Computer simulations have also been applied to evaluate a single molecule. Molecular docking simulation was also performed on a new thiazolidine compound, GQ-11, and in this study they demonstrated that the compound has a partial / double agonist action of PPARα/γ. The in vivo tests of this study using mice showed that GQ-11 has antidiabetic effects by acting on LDL receptors (LDLr - /-) (SILVA et al., 2019). In our study, we can see that in terms of affinity predicted by the scoring function (ChemPLP), the LPSF/JB-20-Z thiazolidine derivative obtained better scores than rosiglitazone. The higher the score, the greater the affinity for the action site. Regarding the molecular doc, it is observed that the compounds that performed hydrogen bonding are among the docking solutions that showed a better affinity predicted by PPARγ than rosiglitazone. This behavior was expected since, typically, hydrogen bonds are among the strongest intermolecular interactions that a compound (drug candidate) can perform at the active site of the biological target, allowing greater stability of these compounds when compared to the molecules that do not perform this type of interaction. We observed that rosiglitazone made a hydrogen bond with the SER289 residue and other contacts with other residues. Similarly, the LPSF/JB20-Z compound also carried out hydrogen bonding with the same residue as rosiglitazone, however two pi-pi stackings bonds with residues PHE282 and HIS449, both T-shaped, in addition to hydrophobic contacts with other residues . Which may be why the docking solution for this compound scored slightly better than rosiglitazone in terms of predicted affinity (ChemPLP).

It is not known for sure whether bone loss reported in the literature generated by TZDs can be beneficial for AS patients. Treatment with TZDs can lead to a reduction in bone formation, however, it can increase the risk of fractures, since the inhibited Wnt signaling in progenitor cells promotes adipogenesis while osteogenesis is reduced (GUSTAFSON, ELIASSON, SMITH, 2010). In addition, it is not known whether the LPSF/JB-20 thiazolidine derivative will be able to act via Wnt. Further studies are needed to evaluate the mechanisms of action of the LPSF/JB-20.

CONCLUSION

Our results showed that the new thiazolidine-2,4-dione LPSF/JB-20 is a promising molecule for acting on inflammation induced by PHA-M and decreasing the

secretion of IFN-γ, IL-6, IL-10, IL-17 and TNF-α in PBMC of patients and HC. The control of inflammation can contribute to the reduction of signs and symptoms of patients and act in the process of calcification. However, more results are needed to ascertain the mechanisms by which the molecule is acting and its binding to the PPARγ receptor.

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5 CONCLUSÃO

- A expressão de sOSMR e sGP130 é reduzida no soro pacientes com EA em comparação com voluntários sadios;
- A baixa expressão de sOSMR está significativamente associada a baixa atividade da doença;
- O composto LPSF/JB-20 é uma molécula que não mostrou citotoxicidade nas doses de 10 e 75µM em PBMC de voluntários sadios;
- O LPSF/JB-20 é uma molécula promissora capaz de atuar na inflamação induzida por PHA-M e diminuir a expressão de IFN-γ, IL-6, IL-10, IL-17 e TNF-α em PBMC de pacientes e voluntários sadios;
- O ensaio de diferenciação adipogênica mostrou que o LPSF/JB-20 pode ser um agonista parcial ou atuar de forma dependente do PPARγ;
- Observamos que em termos de afinidade predita pela função de pontuação (ChemPLP), o derivado tiazolidínico LPSF/JB-20-Z obtive melhor pontuações que a rosiglitazona confirma a ligação do composto ao receptor PPARγ.

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APÊNDICE A – ARTIGO SUBMETIDO NA REVISTA ADVANCES IN RHEUMATOLOGY

Elevated serum levels of IL-17 during disease activity of ankylosing spondylitis: a systematic review with meta-analysis

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ABSTRACT

The IL-23/IL-17 pathway participates in the immunopathophysiology of ankylosing spondylitis concentrations of serum IL-17 have been found at high levels in patients with AS. The role of serum IL-17 in AS was investigated through a meta-analysis undertaken to examine the correlation between AS disease activity and serum levels of IL-17 (interleukin-17) in AS compared to healthy controls and AS patients before and during treatment. Searches were performed in PubMed, ScienceDirect, Cochrane, and Lilacs databases for pertinent case-control studies using with the descriptors “Spondylitis, Ankylosing” and “Interleukin-17” in November 2019. Cross sectional or case–control studies were included. Expression in relation to healthy controls and correlation of IL-17 with BASDAI were plotted using Review Manager 5.3 software. Quality assessment of each eligible study used the Newcastle-Ottawa Scale (NOS). Thirteen case-control studies were selected for this meta-analysis and contained a pooled total of 752 AS patients and 607 healthy controls. Our main result revealed strikingly higher levels of serum IL-17 in AS patients, compared to healthy controls. Pooled mean difference 14.59, pooled risk ratios (RRs) with 95% confidence intervals (CIs) 7.73, 21.45; $P < 0.00001$. Serum IL-17 is highly expressed in serum of patients with AS and is related to disease activity. The treatment in use significantly influenced IL-17; however, we did not observe a significant difference in the expression of IL-17 in the treatment of patients taking anti-TNF, proving that it does not interfere in this pathway. Trial registration: PROSPERO CRD42018110427.

Keywords: interleukin 17; ankylosing spondylitis; meta-analysis; systematic review

Background

Ankylosing spondylitis (AS) is a chronic systemic disease that has undefined etiology with a strong genetic predisposition [1], characterized by involvement of the axial skeleton, especially of the sacroiliac joints [2]. The disease usually begins in young adults up to 45 years of age. Delayed diagnosis is common and is usually due to failure of non-rheumatologist physicians to recognize the disease, which increases the chances of disease progression [3]. The diagnosis used is based on the modified New York criteria of 1984 [4].

Current treatments aim to reduce symptoms, maintain spine flexibility and normal posture, and reduce limitations and complications while maintaining work ability [5]. The choice of treatment is based on remission or low disease activity accompanied by laboratory tests [6]. Non-steroidal anti-inflammatory drugs (NSAIDs), conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs), biological DMARDs (bDMARDs), and physical activities are recommended [7].

Anti-TNFs play a key role in blocking axial spondyloarthritis (SpA), significantly reducing inflammation and bone destruction; however, treatment does not disrupt bone formation after initiation [3]. Even with advances in the pathophysiology of the disease and treatments, the disease continues to be a significant challenge in terms of the inflammatory process and the radiographic progression. Current findings indicate participation in the IL-23/IL-17 pathway in the pathophysiology of SpA [8-11]. Genetic studies have shown the association between interleukin-23 receptor polymorphism and increased γ/δ T-cell expression associated with increased secretion of IL-17 [12]. The IL-23/IL-23R complex in predisposed patients appears to induce the activation of signal transduction and transcription, with consequent proliferation and terminal differentiation of Th17 cells, resulting in the production of IL-17 [9,13-15], TNF-alpha and other proinflammatory cytokines, and

chemokines [16]. The IL-23/IL-17 axis is emerging as an important inflammatory pathway [3, 17].

However, the literature diverges as to the expression of IL-17 in serum of EA patients and this may be related to the treatment used by the patient. Hence, we undertook a meta-analysis to investigate how the disease activity and influence of the treatments used is related to the expression of IL-17 in the serum of EA patients.

Methods

Our report adheres to the Meta-analysis of Observational Studies in Epidemiology (MOOSE) Statements [18]. Its protocol was registered in the database of International Prospective Register of Systematic Reviews, accessible under the protocol number CRD42018110427.

Eligibility criteria

All enrolled studies satisfied the following criteria: (1) all AS patients conformed to the New York clinical criteria for AS [19]; (2) was a cross-sectional or case–control study; (3) reported the correlation between serum IL-17 levels and AS; (4) included AS patients as case group and healthy controls as control group and/or patients who are not active; and (5) contained sufficient information on country, publication year, sample size, gender, IL-17 detection methods, serum IL-17 levels. The exclusion criteria were: (1) inconsistent diagnostic criteria for AS; (2) not case-control studies; and (3) incomplete original data.

Literature retrieval and data collection

The following electronic databases were consulted: Pubmed, ScienceDirect, Cochrane, Lilacs published until November 2019. The basic research strategy was developed for PubMed and modified as required for other databases. We used the health descriptors available in Descriptors in Health Sciences and Medical Subject Heading. The basic research strategy

included “Spondylitis, Ankylosing” and “Interleukin-17” [see Additional file 1]. There was no language restriction from inception through 17 November 2019. References to selected articles were reviewed to identify all relevant studies. A manual search of references was carried out in relevant journals and congresses in the area.

Data collection

Relevant data were independently extracted by two reviewers, and disagreement was resolved by a third reviewer. The following data were collected from each study: first author, year of publication, country of the population studied, numbers of cases and controls, diagnosis of AS, duration of disease, analysis method, medicines in use, serum IL-17 levels, as well as levels and correlation coefficients (CORs) between IL-17 and disease activity (erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI).

Quality assessment

The Newcastle–Ottawa Scale (NOS) was performed to blindly assess the methodological quality of the case–control studies by two reviewers. Some discrepancies were solved by discussion with the third author. The scale consists of nine items that cover three dimensions: 1) patient selection (four items); 2) comparability of the two study arms (two items); and 3) assessment of outcome (three items). A point is awarded for each item satisfied by the study. The total score for a single study ranges from 0 to 9. A study was considered to have high quality if scored >6 points. The graph was made according to the model provided in RevMan software.

Statistical analysis

The difference in serum IL-17 levels between the case and control groups and patients who were not active was compared by mean difference (MD) with 95% confidence intervals (95%

CI), and the correlation between serum IL-23 level and disease activity in AS was performed by CORs with 95% CI. The significance of clustered MDs and CORs was determined by the Z test and P <0.05 was considered statistically significant. Cochran's Q-statistic was considered significant with P < 0.05 and I2 tests were applied to determine heterogeneity. We quantified the effect of heterogeneity by using a recently developed measurement, namely, I2 = 100% × (Q-df)/Q, with values of 25%, 50%, and 75% indicating low, moderate, and high heterogeneity, respectively. Publication bias was evaluated by funnel plot to ensure reliability of the results. The parameters of enrolled studies were mean ± SD. The interquartile was calculated for the studies that did not have the standard deviation. All statistical analyses were carried out in Review Manager (RevMan) software (Cochrane collaboration).

RESULTS

Eligible studies selected for meta-analysis of serum IL-17 levels and AS

The systematic review conducted in November 2019 of studies with IL-17 cytokine in serum of AS patients found 13,472 articles in Pubmed, ScienceDirect, Cochrane, and Lilacs databases (Figure 1). After the removal of duplicates, 12,498 articles remained. By reading titles and abstracts, the list was reduced to 69 articles. After analyzing the texts in full, 13 were selected for the meta-analysis, according to the inclusion criteria previously established.

The selected studies were published between the years 2008 and 2019. The thirteen studies selected for meta-analysis contained a combined total of 1359 patients with AS and 746 healthy controls. Sample sizes in the studies ranged from 23 to 143 AS patients. Three studies were conducted in the Chinese population and the other four studies were conducted in Caucasian populations, with two from Brazil, and with one each from Mexico, Iran, Russia, Bulgaria, Poland, Korea, and France. All were published in English language excerpt the study from Russia. The main characteristics of the studies are presented in Table 1.

Two studies did not describe whether patients were using medications and the type used. In general, all patients use conventional therapy. Some used anti-TNF and had the values before and after use. In three of the studies, patients did not use any medication.

Meta-analysis results for serum level of IL-17

Of the 13 articles selected, only 8 found statistical significance (P <0.05) of patients' IL-17 expression in relation to healthy controls. A total of 3 articles reported the correlation of IL-17 with disease activity index (BASDAI). Our meta-analysis observed the existence of heterogeneity in the 13 studies published in the random effect model used (I2 = 99%, p = 0.00001). As a main result, we observed that IL-17 expression is higher in patients than in controls (MD = 9.75, 95% CI = 6.43 ~ 13.07, P <0.00001) (Figure 2). Nine studies found a correlation of BASDAI disease activity with serum levels of IL-17 (MD = 14.59, 95% CI = 7.73 ~ 21.45, P <0.00001) (Figure 3).

We evaluated studies with patients with and without activity (MD = 0.37, 95% CI = -2.19 ~ 2.93, P = 0.87, I2 = 0%) (Figure 5A) and before and after treatment (MD = 46; 95% CI = -15.79 ~ 32.71, P = 0.02, I2 = 74%) (Figure 5B).

When the treatments were analyzed, the IL-17 expression of the untreated patients was higher than those treated with conventional drugs, with the average difference of MD= 23.70 , 95% CI = 4.60 ~ 42.79, P = 0.02 and MD= 10.24, 95% CI = 3.32 ~ 17.17, P = 0.004, respectively. Patients using IL-17 did not show significance in relation to controls (Table 2).

Table 2. Correlation ship of IL-17 levels and disease activity in AS.

Treatments	Eligible studies	Participants	MD (95%CIs)	p-value	Heterogeneity test	Effect model
Untreated	3	385	23.70 (4.60, 42.79)	**0.02**	P< 0.00001, I^2 =98%	R

Anti-TNF	3	217	15.84 (-7.90, 39.58)	0.19	P< 0.00001, I^2 =99%	R
Conventional	3	228	10.24 (3.32, 17.17)	**0.004**	P< 0.00001, I^2 =99%	R

MD: mean difference; R: random effect model. Bold data means the results were statistically significant (P <0.05).

Sensitivity analysis and risk of publication bias

Only four studies had a weight lower than 7%. Funnel plots were not symmetrically distributed, indicating bias. The risk of bias in the studies was performed in the RevMan program and according to the Newcastle-Ottawa Scale (NOS) criteria (Figure 4)

DISCUSSION

In this study, we undertook a meta-analysis based approach to investigate the significance of elevated serum levels of IL-17 in AS development and the influence of conventional and anti-TNF drugs on cytokine expression. The serum IL-17 values in AS expression diverge greatly between articles. The studies showed that serum levels of IL-17 in AS patients were significantly higher than those of healthy controls. However, many case-control studies performed in different countries obtained conflicting results. This can be related to high heterogeneity index between studies.

Our main results found serum levels of IL-17 strikingly higher in AS patients than healthy controls, indicating that the cytokine play a prominent role in AS pathogenesis. The subgroup analysis based on disease activity also demonstrated that serum IL-17 has significant association with elevated BASDAI in AS, demonstrating that IL-17 is correlated with disease activity.

Recent studies have investigate the IL-23/IL-17 pathway and its influence on the pathogenesis of AS [27, 32-33]. IL-17 is secreted by specialized Th17 subset of CD4+ T cells

and is involved in host defense mechanisms against pathogens by inducing synthesis and secretion of pro-inflammatory molecules from fibroblasts, endothelial cells, and epithelial cells, including chemokines, antimicrobial peptides and matrix metalloproteinases [34-36].

To investigate the contribution of treatment in influencing serum levels of IL-17, subgroup analyses were conducted with untreated patients, with conventional medication and anti-TNF. Our results indicated that mean difference of serum expression of interleukin IL-17 was significantly influenced by the treatment in use, which was highe in untreated patients than controls. Among patients who use conversational therapy, the expression was also significantly higher than controls. No statistical significance was observed for serum expression of anti-TNF-treated patients compared to controls. We also evaluated the influence before and after anti-TNF treatment and the studies do not exhibit any heterogeneity for the impact of the treatments on the outcome, which contributes with the results previously discussed.

Our results were similar to a study performed by Milanez and collaborators (2016), which investigated long-term influence of anti-TNF drugs in IL-23/IL-17 axis at 12 and 24-months of TNF blockade in plasma. They found a strong correlation between IL-23 and IL-17A and ASDAS/PCR after anti-TNF therapy, and concluded that the IL-23/IL-17 axis is not influenced by TNF blockade in AS patients despite clinical and inflammation improvements and NSAID intake [28].

The availability of new biological products targeting the IL-17/IL-23 axis has shown promising results in reducing the rate of radiographic progression in AS [37]. IL-17 antagonists secukinumab, ixekizumab, and brodalumab blocking the Th17 pathway by suppressing IL-17 act directly or through inhibition of Th17 cell differentiation [38]. Secukinumab is the first non-TNF alpha inhibitor agent licensed for AS. The studies point

towards an efficacious role of IL-17A inhibition strategies targeting AS pathogenesis in a fundamental way with a good safety profile [39].

It is very important to resolve the inconsistencies to increase the credibility of the meta-analysis conclusion. Limitations of the present meta-analysis must be acknowledged. First, evaluating only the cytokine of interest may substitute its contribution to the pathogenesis of the disease. Second, the treatment response or before and after treatment were carried out in a limited number of articles. Finally, many studies do not describe the treatments used by the patients or do not describe the mean concentration of the cytokines. Nevertheless, this is the first meta-analysis that identified the association of serum IL-17 level with AS and disease activity before and during treatment.

CONCLUSIONS

In summary, our investigated was undertaken to correlate between AS disease activity and serum levels of IL-17 in AS patients compared to healthy controls and AS patients before and during treatment. This meta-analysis reveals that IL-17 is highly expressed in serum of patients with AS, and its amplitude is positively related to disease activity. IL-17 was significantly influenced by the treatment in use; however, the lack of significant difference in the expression of IL-17 in the treatment of patients taking anti-TNF proves that it does not interfere in this pathway. This clinical discovery provides implications for practice and research. Further studies are needed that include case-control trials and large population plus describe the medications that patients were using.

List of abbreviations

Anti-TNF: Tumor necrosis factor blockers; AS: Ankylosing Spondylitis; ASDAS: Ankylosing Spondylitis Disease Activity Score; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; bDMARDs: biological DMARDs; CC: correlation coefficients; CD4+: positive T helper lymphocytes; CI: confidence intervals; csDMARDs: conventional synthetic disease-modifying anti-rheumatic drugs; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IL-17: Interleukin-17; IL-23: Interleukin-23; MOOSE: Meta-analysis of Observational Studies in Epidemiology; NSAIDs: Non-steroidal anti-inflammatory drugs; NOS: Newcastle-Ottawa Scale; PROSPERO: Prospective Register of Systematic Reviews; RR: risk ratios; SpA: axial spondyloarthritis; TH17: TNF-α: Tumor necrosis factor alpha

Acknowledgements

Not applicable.

Authors' contributions

Relevant data were independently extracted by TULL and NGC, and disagreement was resolved by MABC. The Newcastle–Ottawa Scale was performed by MABC and MCP, discrepancies were solved by discussion by MGRP. All of the authors provided critical review, relevant edits, and feedback to direct content during multiple rounds of review. In addition, all authors have read and approved the final version of this manuscript.

Funding

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) grant for undergraduate student 140230/2016-5 and Instituto Nacional de Ciência e Tecnologia para Inovação Farmacêutica (INCT-if).

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Not applicable.

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Figures

Figure 1 Flow diagram of the study selection process. After excluding duplicate publication, unclear indicators, unmatched purposes, review, letters and editorial, thirteen articles were included in the final analysis.

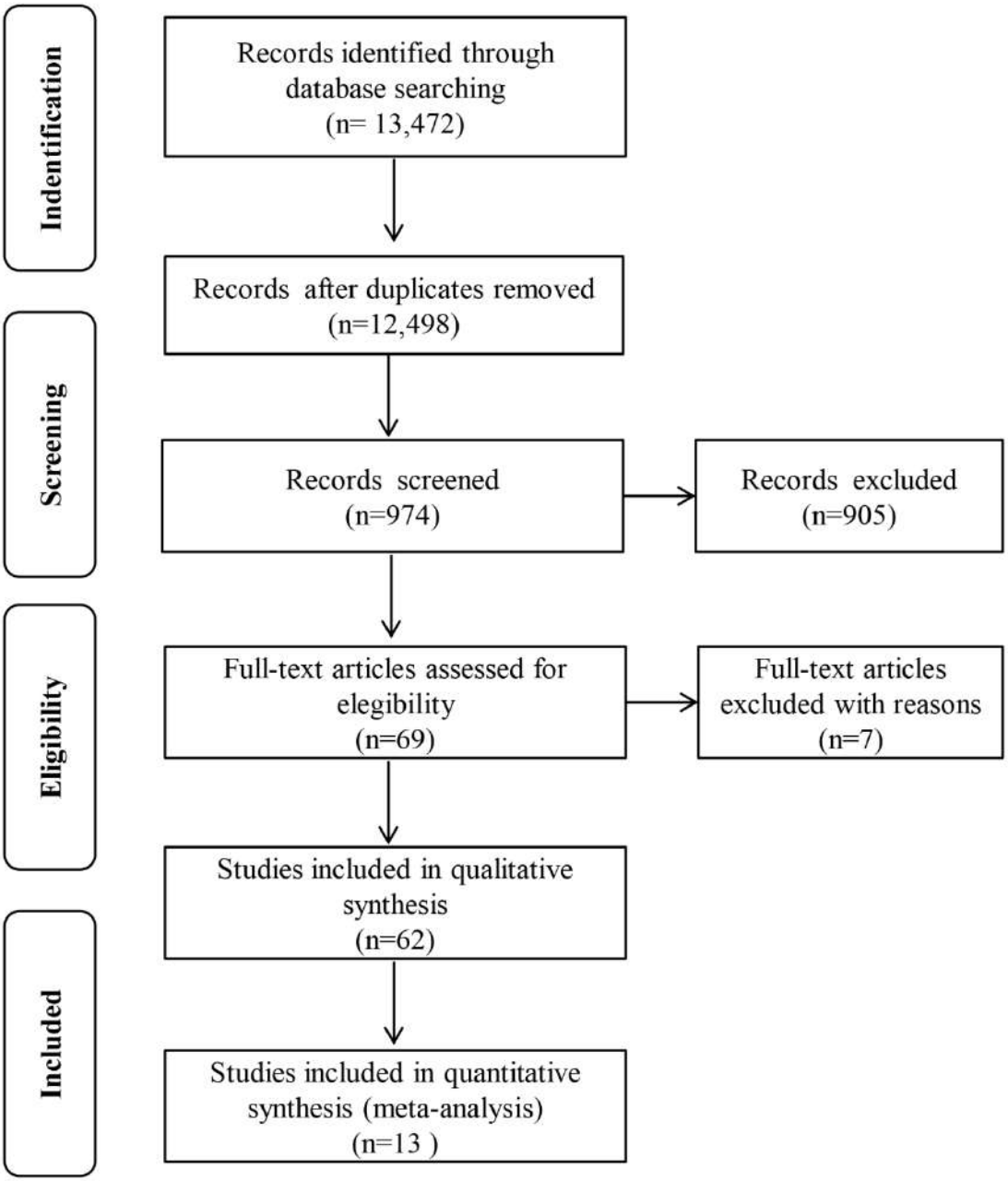

Figure 2 Detailed risk of bias results using the Newcastle-Ottawa Scale for Assessing Quality for observational studies. Plot of the criteria required by Newcastle-Ottawa Scale for Assessing Quality to assess the quality of selected articles

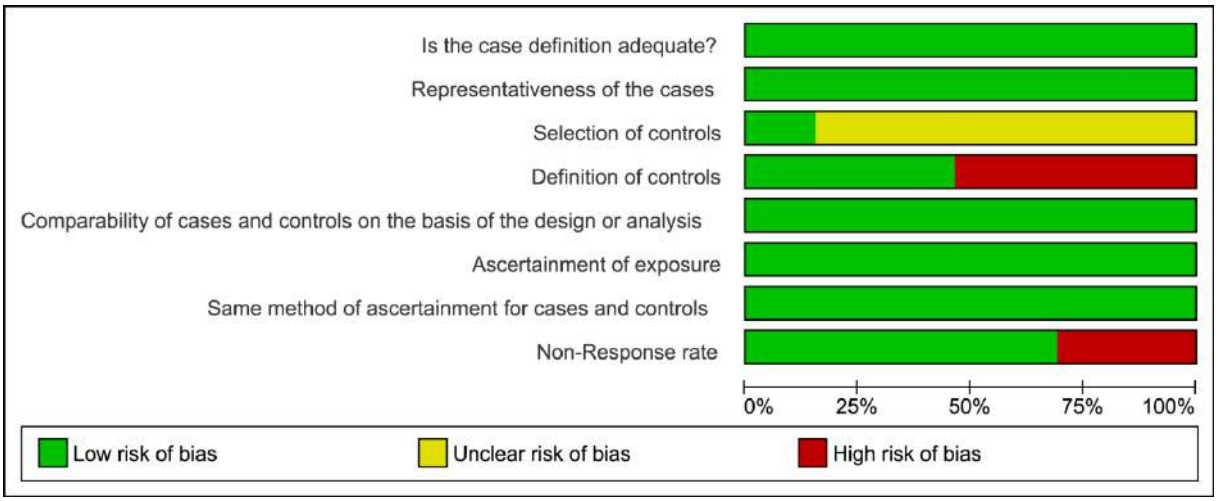

Figure 3 Meta-analysis for the association between serum IL-17 levels AS and control. Forest plots IL-17 serum levels in patients AS and controls.

	AS			Control				Mean Difference	Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	IV, Random, 95% CI
Chen 2012	66.03	17.8	49	31.37	8.4	25	6.6%	34.66 [28.69, 40.63]	
Fattahi 2018	18	1.9	30	10.7	1.1	15	8.3%	7.30 [6.42, 8.18]	
Gaydukova 2017	28.4	14.4	30	2.4	2.1	20	6.9%	26.00 [20.77, 31.23]	
Han 2011	13.8	6.55	89	5.26	2.26	178	8.2%	8.54 [7.14, 9.94]	
Inova 2016	11.96	25.6	77	3.422	2.06	37	6.7%	8.54 [2.78, 14.29]	
Limon-Camacho 2012	25	3.2	39	3.3	0.2	20	8.3%	21.70 [20.69, 22.71]	
Madej 2015	3.84	6.66	49	0.01	0.01	19	8.1%	3.83 [1.97, 5.69]	
Mei 2011	10.57	4.7	50	8.72	2.39	43	8.2%	1.85 [0.36, 3.34]	
Milanez 2016	1.62	1.42	86	1.57	0.83	47	8.3%	0.05 [-0.33, 0.43]	
Rabelo 2018	6.2	0.88	32	6.23	0.71	32	8.3%	-0.03 [-0.42, 0.36]	
Sohn 2018	1.6	0.59	55	1.7	1.11	26	8.3%	-0.10 [-0.55, 0.35]	
Sveaas 2015	45	8.88	143	46	10.37	124	8.0%	-1.00 [-3.33, 1.33]	
Wendling 2008	60.47	18.89	23	32	1.54	21	5.7%	28.47 [20.72, 36.22]	
Total (95% CI)			**752**			**607**	**100.0%**	**9.75 [6.43, 13.07]**	

Heterogeneity: Tau² = 34.41; Chi² = 2226.87, df = 12 (P < 0.00001); I² = 99%
Test for overall effect: Z = 5.76 (P < 0.00001)

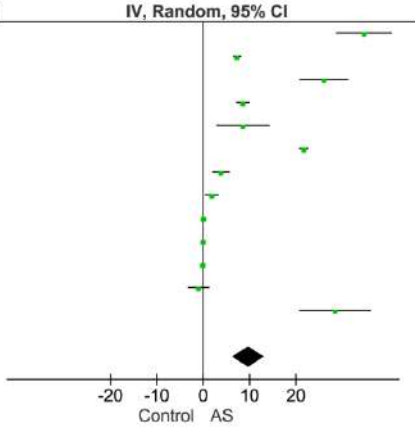

Figure 4 Meta-analysis for the correlation with disease activity.

Forest plots IL-17 serum levels correlation with disease activity Bath AS Disease Activity Index (BASDAI)

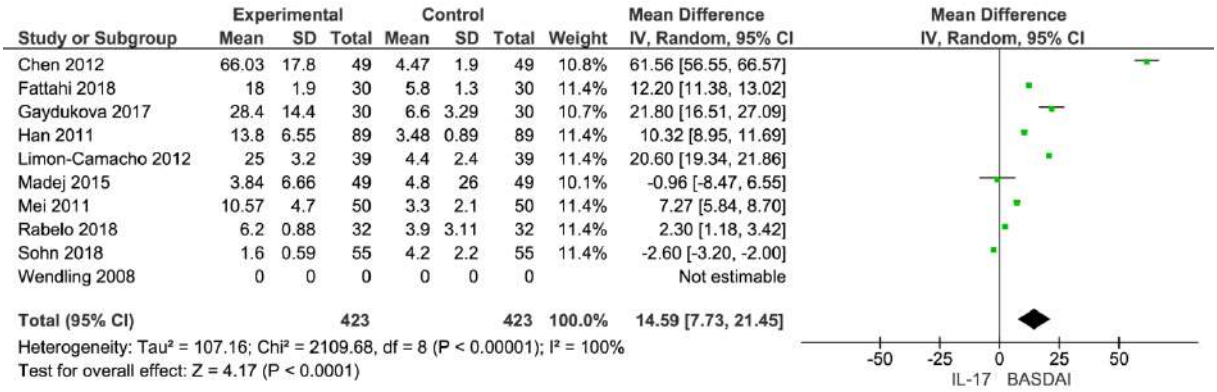

Figure 5 Forest plots IL-17 serum levels in patients AS.

A: between AS and active/inactive; B: before and after treatment with anti-TNF

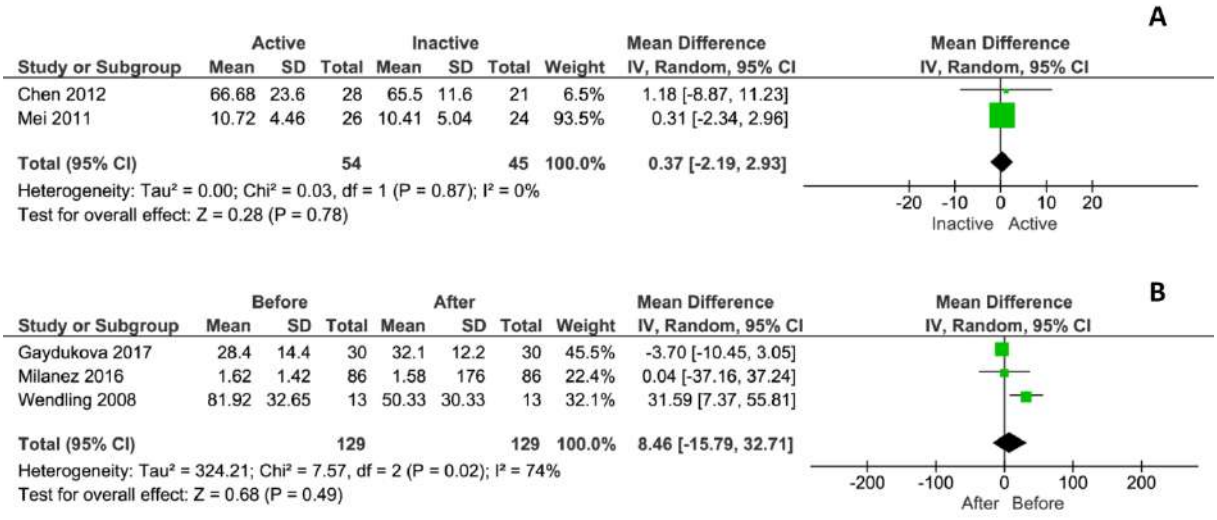

Table 1. Characteristics of the studies included in the meta-analysis.

Studies	Country	Type of research	Ethnicity	CASE							CONTROL		
				N	Gender (M/F)	Mean/SD age (years)	Duration of disease (years)	BASDAI mean/SD	C-reactive protein (mg/L)	ESR mm/h	N	Gender (M/F)	Mean age (years)
Chen et al. [20]	China	Case-control	Asian	49	43/6	39.0 ± 12.3	NA	4.47 ± 1.9	15.08 ± 13.2	25.35 ± 24.0	25	NA	NA
Fattahi et al. [21]	Iran	Case-control	Caucasians	30	22/8	22/8	11.8 ± 8.6	5.8 ± 1.3	NA	NA	15	11/4	11/4
Gaydukova et al. [22]	Russia	Case-control	Caucasians	30	22/8	38.35 ± 9.19	11.4 ± 9.6	6.6 ± 3.29	12.3 ± 3.9	19.3 ± 6.7	20	12/8	40.1 ± 7.7
Han et al. [23]	China	Case-control	Asian	89	89/0	38.27 ± 9.70	NA	3.48 ± 0.89	32.81 ± 17.21	42.23 ± 20.94	178	178/0	39.67 ± 7.1
Inova et al. [24]	Bulgaria	Case-control	Caucasians	77	59/18	38 ± 10	12 ± 8	NA	24.29 ± 2.89	30 ± 22	48	37/11	39 ± 11
Limon-Camacho et al. [25]	Mexico	Case-control	Caucasians	39	38/8	32 ± 13	17 ± 2	4.4 ± 2.4	14 ± 3.2	NA	20	NA	32 ± 8
Madej et al. [26]	Poland	Case-control	Caucasians	49	35/14	40,6 ± 13,4	NA	4,8 ± 2,6	24,6 ± 36	30 ± 24	19	NA	40,4 ± 10,4
Mei et al. [27]	China	Case-control	Asian	50	41/9	28.1 ± 8.9	NA	3.3 ± 2.1	22.5 ± 8.2	39.1 ± 11.6	43	35/8	25.3 ± 6.7
Milanez et al. [28]	Brazil	Case-control	Caucasians	47	35/11	38.0 ± 11.1	10.8 ± 4.5	NA	29.71 ± 39.56	29.70 ± 23.32	47	NA	NA
Rabelo et al. [29]	Brazil	Case-control	Caucasians	32	19/13	46.9 ± 10.7	18 (10–31)	3.9 ± 2.1–6.3	NA	NA	32	19/13	NA
Sohn et al. [30]	Korea	Case-control	Asian	55	55/0	37.8 ± 10.8	6.48 ± 4.3	4.2 ± 2.2	0.26 (0.08-0.7)	15 ± 17.77	26	26/0	35.6 ± 6.8
Sveaas et al. [31]	Norway	Case-control	Caucasians	143	88/55	49.3 ± 11.0	23.7 ± 11.3	NA	3 (1, 10)	15 ± 3.7	124	74/50	53.2 ± 11.3
Wendling et al. [32]	France	Case-control	Caucasians	23	18/05	39.9 ± 2.1	10.1 ± 1.3	44.1 ± 4.1	22.3 ±4.7	27.8 ± 5.3	21	NA	41.2 ± 2.7

Legends: M/F, male/female; NA, not available; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; BASDAI, Bath AS Disease

Additional Files

Additional file 1 - The basic research strategy used the in electronic databases

"Spondylitis, Ankylosing"[Mesh] OR (Spondyloarthritis Ankylopoietica) OR (Ankylosing Spondylarthritis) OR (Ankylosing Spondylarthritides) OR (Spondylarthritides, Ankylosing) OR (Spondylarthritis, Ankylosing) OR (Ankylosing Spondylitis) OR (Spondylarthritis Ankylopoietica) OR (Bechterew Disease) OR (Bechterew's Disease) OR (Bechterews Disease) OR (Marie-Struempell Disease) OR (Marie Struempell Disease) OR (Rheumatoid Spondylitis) OR (Spondylitis, Rheumatoid) OR (Spondylitis Ankylopoietica) OR (Ankylosing Spondyloarthritis) OR (Ankylosing Spondyloarthritides) OR (Spondyloarthritides, Ankylosing) OR (Spondyloarthritis, Ankylosing) OR (axial spondyloarthritis) OR (spondyloarthritis axial) AND "Interleukin-17"[Mesh] OR (Interleukin 17) OR (IL-17) OR (Interleukin-17ª) OR (Interleukin 17A) OR (IL-17A)”

APÊNDICE B- TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

(PARA MAIORES DE 18 ANOS OU EMANCIPADOS - Resolução 466/12)

Convidamos o (a) Sr. (a) para participar como voluntário (a) da pesquisa "**Avaliação da atividade imunomoduladora de derivados oxazolidínicos em células mononucleares de pacientes com Espondilite Anquilosante"**, que está sob a responsabilidade da pesquisadora **Maria Andreza Bezerra Correia**, email: marandrezabcorreia@yahoo.com.br, fone:98817-3200, CPF: 041.471.294-39, endereço: Universidade Federal de Pernambuco, Av. Prof. Moraes Rego S/N - Cidade Universitária, CEP: 50.670-901; sob a orientação do Prof. Dr. Ivan da Rocha Pitta, email: irpitta@gmail.com, fone: 2126-8347, e co-orientação de Dra. Claudia Diniz Lopes Marques, email: claudia.reumatologia@gmail.com, fone: 2126-3575.

Este Termo de Consentimento pode conter alguns tópicos que o/a senhor/a não entenda. Caso haja alguma dúvida, pergunte à pessoa a quem está lhe entrevistando, para que o/a senhor/a esteja bem esclarecido (a) sobre tudo que está respondendo. Após ser esclarecido (a) sobre as informações a seguir, caso aceite em fazer parte do estudo, rubrique as folhas e assine ao final deste documento, que está em duas vias. Uma delas é sua e a outra é do pesquisador responsável. Em caso de recusa o (a) Sr. (a) não será penalizado (a) de forma alguma. Também garantimos que o (a) Senhor (a) tem o direito de retirar o consentimento da sua participação em qualquer fase da pesquisa, sem qualquer penalidade.

A busca por novas moléculas visando tratar inflamações crônicas é necessária, pois é crescente o número de pacientes portadores de Espondilite anquilosante não-respondedores aos tratamentos atuais, acarretando em comprometendo da funcionalidade e mobilidade corporal. Para isso iremos avaliar o comportamento das células sanguíneas de pacientes portadores de Espondilite Anquilosante e voluntários sadios utilizando moléculas denominadas de oxazolidínas e armazenamento do soro para realização de análises necessárias para a obtenção dos resultados.

Portanto, nós solicitamos a sua colaboração e participação para esta pesquisa. Para este estudo, precisamos coletar algumas células do seu sangue. A coleta de sangue é feita no braço, e a quantidade coletada é equivalente a duas colheres de sopa (10-15 ml). Antes de iniciar a coleta, nós limparemos o seu braço com álcool, e todo material usado na coleta é descartável. A coleta será feita por profissionais treinados, competentes e orientados para reduzir os riscos. Os riscos envolvidos nesse projeto se referem à coleta de sangue, o constrangimento durante a coleta dos dados e o tempo de espera para atendimento. A coleta de sangue pode ser um pouco desconfortável e o braço apresentar um pequeno hematoma, que é uma área arroxeada no local da coleta. Todos os riscos serão minimizados, pois os procedimentos serão realizados por profissional habilitado e orientado para reduzir os riscos. O benefício deste estudo não é individual. Através dos resultados será possível uma melhor compreensão da fisiopatologia da doença e da investigação de moléculas visando possíveis tratamentos de inflamações crônicas de pacientes diagnosticados com EA e com isso reduzir o comprometendo da funcionalidade e

mobilidade corporal.

Os resultados deste estudo serão apresentados em congressos, conferências ou em revistas médicas, mas não aparecerá seu nome. Suas informações nunca serão divulgadas, permanecerão confidenciais. Os dados coletados nesta pesquisa, provenientes dos resultados das amostras coletadas ficarão devidamente armazenadas em arquivos digitais em computadores ou impressos em pastas de arquivos sob responsabilidade da pesquisadora responsável **Maria Andreza Bezerra Correia**, no Núcleo de Pesquisa em Inovação Terapêutica Suely Galdino NUPIT-SG localizado no 1º andar do Prédio da Diretoria de Inovação e Empreendedorismo - DINE (ao lado do Dept. de Educação Física) cujo endereço é Universidade Federal de Pernambuco, Av. Prof. Moraes Rego S/N - Cidade Universitária, CEP: 50.670-901 Recife – PE, por um período mínimo 5 anos.

Nada lhe será pago e nem será cobrado para participar desta pesquisa, pois a aceitação voluntária, mas fica também garantida a indenização em casos de danos, comprovadamer decorrentes da participação na pesquisa, conforme decisão judicial ou extra-judicial. Se hou necessidade, as despesas para a sua participação serão assumidas pelos pesquisado (ressarcimento de transporte e alimentação).

Em caso de dúvidas relacionadas aos aspectos éticos deste estudo, você poderá consultar Comitê de Ética em Pesquisa Envolvendo Seres Humanos da UFPE no endereço: (Avenida Engenharia s/n – 1º Andar, sala 4 - Cidade Universitária, Recife-PE, CEP: 50740-600, Tel.: (8 2126.8588 – e-mail: cepccs@ufpe.br).

_____

(Assinatura do pesquisador)

CONSENTIMENTO DA PARTICIPAÇÃO DA PESSOA COMO VOLUNTÁRIO (A)

Eu, _____, CPF _____, abai assinado, após a leitura (ou a escuta da leitura) deste documento e de ter tido a oportunidade conversar e ter esclarecido as minhas dúvidas com o pesquisador responsável, concordo e participar do estudo, "**Avaliação da atividade imunomoduladora de derivados oxazolidínic em células mononucleares de pacientes com Espondilite Anquilosante"**, como voluntário (a Fui devidamente informado (a) e esclarecido (a) pelo (a) pesquisador (a) sobre a pesquisa, procedimentos nela envolvidos, assim como os possíveis riscos e benefícios decorrentes de minh participação. Foi-me garantido que posso retirar o meu consentimento a qualquer momento, se que isto leve a qualquer penalidade (ou interrupção de meu acompanhament assistência/tratamento).

Local e data _____ Assinatura do participante: _____

Presenciamos a solicitação de consentimento, esclarecimentos sobre a pesquisa e o aceite do voluntário em participar. (02 testemunhas não ligadas à equipe de pesquisadores):

Nome:	**Nome:**
Assinatura:	**Assinatura:**

APÊNDICE C – FICHA CLÍNICA

N° HC_____ N° Prontuário_____ EA_____

1-DADOS DOS PACIENTES

Nome: _____ DN:____/___/_____

Idade:_____

Sexo: □F □M Naturalidade:_____ Telefones:_____

2-AVALIAÇÃO FARMACOTERAPÊUTICA

Tempo dos sintomas da doença _____Idade do diagnóstico _____Familiares com a doença_____

Possui outras doenças diagnosticadas: ☐ sim ☐ não Qual?_____

Tabagismo: Ativo ( ) Inativo ( ) Tempo:_____ Carga tabágica (maços/dia x anos):_____

Medicamentos em uso			
Droga	**Dose atual**	**Tempo (meses)**	**Uso no dia da coleta**
Sulfassalazina			
Metotrexato			
Cetoprofeno			

3- AVALIAÇÕES DIAGNÓSTICAS

Resultados laboratoriais		
Exame	**Data do exame**	**Resultado**
HLA- B27		
Proteína C reativa		
VHS		

Sintomas extra-articulares presentes no momento da coleta		
Uveíte S/N	Psoríase S/N	DII- Doença Inflamatória Intestinal S/N

Variáveis clínicas relacionadas à atividade da doença					
Dor lombar inflamatória S/N	Entesite S/N	Dactilite S/N	Artrite periférica S/N	EVA-Examinador	EVA-paciente

ASDAS	**Definição**	**Resultado**
☐ Dor axial	Nota para dor no pescoço, costas ou quadril que você tem sentido durante a última semana? (0=não, 10 muito grave)	
☐ Dor periférica/edema	Nota para dor/edema em outras articulações, que você tem sentido durante a última semana? (0=não, 10 muito grave)	
☐ Duração da rigidez matinal	Quanto tempo dura sua rigidez matinal, desde que você se levanta, durante a última semana (0=não, 10 muito grave)	
☐ Avaliação global do paciente	Quão ativo tem estado sua doença, em média, durante a última semana? (0=não, 10 muito grave)	
☐ PCR	Quando o valor da PCR está abaixo do limite de detecção ou > 2mg/L (0,2mg/dl), o valor constante da PCR de 2 mg/l (0,2 mg/dl) deve ser utilizado	

ANEXO A- APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA DA UFPE

Comitê de Ética em Pesquisa Envolvendo Seres Humanos CEP - CCS - UFPE

UNIVERSIDADE FEDERAL DE PERNAMBUCO CENTRO DE CIÊNCIAS DA SAÚDE / UFPE-

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação da atividade imunomoduladora de derivados oxazolidínicos em células mononucleares de pacientes com Espondilite Anquilosante

Pesquisador: Maria Andreza Bezerra Correia

Área Temática:

Versão: 2

CAAE: 55261116.2.0000.5208

Instituição Proponente: CENTRO DE CIÊNCIAS BIOLÓGICIAS

Patrocinador Principal: FUNDACAO DE APOIO INST AO DESENV CIENT E TECNOLOGICO

DADOS DO PARECER

Número do Parecer: 1.619.469

Apresentação do Projeto:

O projeto busca avaliar a atividade imunomoduladora dos derivados oxazolidínicos LPSF/NB-2, LPSF/NB-3 e LPSF/NB-5 e sua influência em receptores ativados por peroxissoma PPAR-, COX-2, receptor órfão do ácido retinóico RORt e nas citocinas IL-1, IL-6, IL-17, IL-23, IFN- e inibidores do fator de necrose tumoral TNF- em células mononucleares do sangue periférico de pacientes diagnosticados com Espondilite Anquilosante (EA). Uma vez que, a busca por novas moléculas visando tratar a inflamação em EA se faz necessária por conta dos pacientes não-respondedores aos tratamentos atuais. Os tratamentos anti-TNF têm sido desenvolvidos e se mostraram eficazes para o controle dos sintomas, no entanto, o bloqueio de TNF- não inibi o dano estrutural causado por fraturas vertebrais radiográficas em EA.Os derivados oxazolidínicos sintetizados pelo laboratório de planejamento e síntese de fármacos (LPSF/NB-2, LPSF/NB-3 e LPSF/NB-5) não apresentaram citotoxicidade e trouxeram resultados promissores modulando a resposta de PPAR- e RORt, além de reduzir os níveis de citocinas anti e pró-inflamatórias em trabalhos preliminares realizados pelo laboratório em voluntários sadios.

Objetivo da Pesquisa:

Avaliar a atividade imunomoduladora de derivados oxazolidínicos em células mononucleares do

ANEXO B- APROVAÇÃO REGISTRO PROSPERO

PROSPERO Registration message [110427]

De: CRD-REGISTER (irss505@york.ac.uk)

Para: marandrezabcorreia@yahoo.com.br

Data: sexta-feira, 9 de novembro de 2018 11:17 BRST

Dear Ms Correia,

Thank you for submitting details of your systematic review "Elevated serum levels of IL-17 during disease activity and treatment of ankylosing spondylitis: a systematic review with meta-analysis" to the PROSPERO register. We are pleased to confirm that the record will be published on our website within the next hour.

Your registration number is: CRD42018110427

You are free to update the record at any time, all submitted changes will be displayed as the latest version with previous versions available to public view. Please also give brief details of the key changes in the Revision notes facility. You can log in to PROSPERO and access your records at https://www.crd.york.ac.uk/PROSPERO

Comments and feedback on your experience of registering with PROSPERO are welcome at: crd-register@york.ac.uk

Best wishes for the successful completion of your review.

Yours sincerely,

PROSPERO Administrator
Centre for Reviews and Dissemination
University of York
York YO10 5DD
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e: CRD-register@york.ac.uk
www.york.ac.uk/inst/crd

PROSPERO is funded by the National Institute for Health Research and produced by CRD, which is an academic department of the University of York.

Email disclaimer: https://www.york.ac.uk/docs/disclaimer/email.htm