PAPEL DOS RECEPTORES TOLL-LIKE 2 E 4 E DO ESTADO NUTRICIONAL EM PACIENTES COM LEISHMANIOSE VISCERAL

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Dissertação apresentada ao Programa de Pós-Graduação em Doenças Tropicais da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista – UNESP, para obtenção do título de Mestre em Doenças Tropicais.

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Dissertação de Mestrado

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Epígrafe

A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.

(Arthur Schopenhauer)

Dedicatória

Dedico este estudo às pessoas mais
Importantes da minha vida:
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"Na convivência, o tempo não importa. Se for um minuto, uma hora, uma vida. O que importa é o que ficou deste minuto, desta hora...desta vida..."

(Mário Quintana)

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Resumo

A leishmaniose visceral (LV) é um problema emergente de saúde pública, sendo uma das principais doenças negligenciadas no Brasil e no mundo. É causada por *L. donovani*e *L.chagasi/infantum.* A resistência à infecção está associada à produção de citocinas de perfil Th1 e a suscetibilidade às citocinas do perfil Th2. Os receptores Toll-like (TLRs) da imunidade inata têm importante papel no reconhecimento microbiano e no desenvolvimento da resposta imune adaptativa. Além disso, o estado nutricional possui grande impacto na resposta imune. Desse modo, nosso objetivo foi avaliar o perfil bioquímico-nutricional, o envolvimento dos TLR2 e TLR4 na produção de citocinas e NO e relacionar as lipoproteínas com a resposta imune em pacientes com LV antes e após o tratamento. Foram estudados 13 pacientes com LV antes e após o tratamento e 16 indivíduos controles.A avaliação bioquímica-nutricional foi realizada através da impedância biolétrica e testes bioquímicos de colesterol total (CT), HDL, LDL, triglicerídeos (TG), glicose e proteínas totais e frações. A expressão de TLR2 e TLR4 em linfócitose monócitosfoi realizada através da citometria de fluxo. A produção das citocinas e NO em sobrenadantes de cultura de PBMCs estimuladas ou não com agonistas dos receptores TLR2 (PGN) e do TLR4 (LPS), foi avaliada através da técnica de CBA Flex e de kits comerciais. Pacientes pré-tratamento apresentaram menor ângulo de fase em relação aos controles, níveis diminuídos de CT, HDL, LDL e albumina e níveis aumentados de triglicerídeos em relação ao pós-tratamento e controles. Além disso, mostraram maior porcentagem de linfócitos expressando TLR2 e TLR4 e aumento da expressão destes receptores em monócitos. Quanto às citocinas, foi verificado aumento da produção de TNF-α, IL-10 e TGF-β enquanto os níveis de IFN-γ, IL-17 e NO foram menores. Os resultados também mostraram o envolvimento dos receptores TLR2 e TLR4 na produção do TNF-α, IL-10 e NO. Com o tratamento houve aumento dos níveis de CT, HDL, LDL e albumina e diminuição dos triglicerídeos. Não houve diferença em relação ao ângulo de fase. Mesmo com o tratamento ainda houve expressão de TLR2 e TLR4 em linfócitos e monócitos. Foi observado diminuiçãodos níveis de TNF-α, IL-10 e aumento de IFN-γ, IL-17 e NO. Níveis de TGF-β permaneceram aumentados. Tanto TLR2 quanto TLR4 estavam relacionados com a produção dessas citocinas, com exceção do TGF-β. Os resultados ainda mostram uma relação entre HDL, LDL, colesterol total e produção de IFN-γ, IL-17 e NO. Nossos resultados mostram que pacientes com LV apresentam alterações bioquímico-nutricionais que possivelmente interferiram na resposta imune. Os receptores TLR2 e TLR4 foram expressos em monócitos e linfócitos tanto não pré quanto no pós-tratamento e envolvidos na produção de mediadores pró e anti-inflamatórios. De forma geral, os resultados sugerem a recuperação do estado clínico-nutricional e imunológico após o tratamento.

Palavras chave:*Leishmaniose visceral, receptores Toll-like, citocinas, estado nutricional.*

Abstract

Visceral leishmaniasis (VL) is an emerging public health problem, being of the major neglected diseases in Brazil and worldwide. It is caused by *L. chagasi* and *L. donovani/infantum*. Resistance to infection is associated to production of Th1 cytokines and susceptibility to Th2 cytokine profile. The toll-like receptors (TLRs) of the innate immunity plays an important role in microbial recognition and development of the adaptive immune response. In addition, nutritional status has a great impact on the immune response. Our objective was to evaluate the biochemical and nutritional profile, the involvement of TLR2 and TLR4 in the production of cytokines and NO and relate the lipoproteins to the immune response in VL patients, before and after the treatment. Were studied 13 patients with LV before and after the treatment and 16 control subjects. Biochemical and nutritional evaluation was performed by bioelectrical impedance and biochemical assays of total cholesterol (TC), HDL, LDL, triglycerides (TG), glucose, and total protein and fractions. Expression of TLR2 and TLR4 in lymphocytes and monocytes was performed by flowcytometry. The production of cytokines and NO in culture supernatants of PBMCs stimulated or not with agonists of TLR2 (PGN) and TLR4 (LPS) receptors was evaluated by CBA Flex technique and commercial kits. Patients on pre-treatment had lower phase angle compared to controls, reduced levels of TC, HDL, LDL and albumin and increased triglycerid elevels in relation to post-treatment and controls. In addition, pre-treatment patients presented a higher percentage of lymphocytes expressing TLR2 and TLR4 and increased expression of these receptors on monocytes. Regarding the cytokines, was observed increased of the production of TNF-α, IL-10 and TGF-β, while production of IFN-γ, IL-17 and NO were lower. The results also demonstrated the involvement of TLR2 and TLR4 receptors on TNF-α, IL-10 and NO production. With treatment there was an increase in the levels of TC, HDL, LDL, and albumin and decreased of levels of triglycerides. There was no difference with respect to phase angle. Even with treatment, still was expression of TLR2 and TLR4 in lymphocytes and monocytes. Was observed a decreased levels of TNF-α, IL-10 and increased levels of IFN-γ, IL-17 and NO. TGF-β levels remained increased. Both TLR2 as TLR4 were related to the production of these cytokines, except the TGF-β. The results also show a relation between levels of HDL, LDL, total cholesterol and production of IFN-γ, IL-17 and NO. Our results show that patients with LV present biochemical and nutritional alterations that possibly interfered with the immune response. The TLR2 and TLR 4receptors are expressed on monocytes and lymphocytes both pre and post treatment, and were involved in the production of pro- and anti-inflammatory mediators. Overall, the results suggest the recovery of clinical nutritional and immune status after treatment.

Keywords: *Visceral leishmaniasis, Toll-like receptors, cytokines, nutritional status.*

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As leishmanioses são causadas por protozoários intracelulares obrigatórios que pertencem à ordem *Kinetoplastida*, família *Trypanosomatida* e ao gênero *Leishmania*.(1) Existem mais de 20 espécies de *Leishmania*, que podem ser transmitidas ao homem por aproximadamente 30 diferentes espécies de flebotomíneos.(2) A forma visceral, também chamada Kala Azar, é causada por protozoários do complexo *Leishmania donovani.*(3) É uma doença letal se não tratada apresentando como sintomas comprometimento do baço e fígado (hepatomegalia e esplenomegalia), febre, anemia, perda de peso, hiperglobulinemia e pancitopenia.(4) A leishmaniose visceral (LV) é uma zoonose, tendo o cão como principal reservatório animal nos centros urbanos, e raposas e outros mamíferos em ambientes silvestres.(5-7) O inseto vetor é o mosquito *Lutzomya longipalpis* que mede de 2 a 3 centímetros, e vive em ambientes peri-domésticos sendo amplamente distribuído em toda a América Latina.(8,9) Embora os fatores de risco para essa doença não são totalmente conhecidos, sabe-se que fator genético, desnutrição e presença de animais infectados no ambiente são grandes contribuintes para a manutenção do alto índice dessa doença.(10-12)

A LV é endêmica em 62 países, com 500.000 casos por ano e cerca de 200 milhões de pessoas vivendo em áreas de risco, sendo que 90% dos casos ocorrem no Brasil, Bangladesh, India, Nepal e Sudão.(13-15) Nos últimos dez anos, as regiões endêmicas da doença têm expandido juntamente com aumento do número de casos. No entanto, uma grande parcela de casos não é reportada e apenas 32 dos 88 países afetados consideram a leishmaniose uma doença de notificação compulsória. (16) No Brasil, a doença que era antigamente restrita às áreas rurais do nordeste, hoje já alcança os centros urbanos. A partir dos anos 90, as regiões Centro-oeste, Norte e Sudeste também começaram a fazer parte das estatísticas, e até 2011 ocorreram 3.894 casos no país e 262 óbitos. (17-19) No Estado de São Paulo, até 1998, os casos de LV eram somente oriundos de outras regiões endêmicas do país. No entanto, em 1999, houve o primeiro caso humano e desde então tem ocorrido uma expansão da área de transmissão da doença, principalmente em cidades situadas no Planalto Atlântico Paulista, onde a transmissão é exclusivamente urbana. (20) No período de 1999 a 2012 foram confirmados 2234 casos de LV no estado de São Paulo. (21,22) Na cidade de Bauru, um dos locais onde foram realizadas as coletas deste trabalho, a LV foi documentada pela primeira vez em cachorros em 2002 e em humanos em 2003, e mesmo com medidas preventivas os casos da doença continuaram a aparecer até os dias atuais. Bauru, além de possuir inúmeras espécies de *Lutzomya*, também apresenta áreas de florestas que estão constantemente sofrendo ação antrópica, facilitando a entrada de animais silvestres no ambiente urbano. (23) Em humanos foram reportados no ano de 2006,

72 casos e 4 mortes, em 2008, 79 casos e 9 mortes, em 2011, 30 casos e 3 mortes e em 2012, 40 casos e 2 mortes. [21,22]

Os antimoniais pentavalentes (SbV) têm sido usados como tratamento da LV desde os anos 40, no entanto, além de sua alta toxicidade também apresenta pobre absorção oral.[24] Os SbV são o tratamento de primeira escolha, embora certos mecanismos de ação ainda não são completamente entendidos e seu uso é contraindicado em alguns casos. [25,26] Alguns estudos mostram que SbV possui atividade leishmanicida, aumenta a produção dos reativos do oxigênio e elimina os parasitas por apoptose.[27-29] Na década passada foram registrados casos de resistência aos antimoniais e falhas terapêuticas. [30] A anfotericina B é usada desde os anos 60 como o medicamento de segunda escolha, o qual exibe uma taxa de cura de 90 a 95%. [31] É indicada também em casos de toxicidade ou resposta insatisfatória aos antimoniais, e é a primeira escolha para tratar pacientes grávidas e casos terminais da doença.[25] Formulações lipídicas da anfotericina B como a lipossomal, são a primeira escolha de tratamento indicada pelo Ministério da Saúde em certos casos como, crianças, pacientes acima de 50 anos ou aqueles diagnosticados com complicações cardíacas, doenças no fígado, rim ou positivos para a doença de Chagas.[32-35] As anfotericinas lipossomais são seletivamente absorvidas pelo retículo endoplasmático, e além de possuírem ação leishmanicida elevada, possuem menor toxicidade.[29,33-35] É a primeira escolha de tratamento em países endêmicos da Europa e em países desenvolvidos por apresentar taxa de cura de 100%, e ter um regime curto de no máximo cinco dias. [32-35]

O reconhecimento inicial de microrganismos é realizado pelo sistema imune inato através de receptores celulares nomeados receptores de reconhecimento padrão (PRRs). Esses receptores são aptos em reconhecer estruturas de patógenos, ausentes nas células dos mamíferos, chamadas de padrões moleculares associados aos patógenos (PAMPs). [36,37] Os PAMPs incluem proteínas, ácidos nucléicos, complexos de lipídeos e carboidratos sintetizados por microrganismos tais como, lipopolissacarídeos (LPS), ácidos teicóicos e oligossacarídeos ricos em manose encontrados em glicoproteínas.[38] Na superfície da *Leishmania,* os principais PAMPs que são reconhecidos pelo sistema imune incluem lipofosfoglicanos (LPG), glicoinositolfosfolipídios (GPIL) e gp63.[39-41] Os PRRs são expressos em certas linhagens celulares do hospedeiro, independente da memória imunológica. São capazes de reconhecer os PAMPs dos patógenos em qualquer estágio de seu ciclo de vida. Diferentes PRRs apresentam diferentes padrões de expressão capazes de reconhecer PAMPs específicos, ativando distintos percursos de sinais e levando a diferentes respostas imunológicas contra os microrganismos. [37]

Dentre os PRRs, se destacam os receptores Toll-like (TLR) ou receptores semelhantes ao Toll. Os receptores Toll fazem parte de uma família de receptores que foram primariamente identificados como um gene essencial para o desenvolvimento embriogênico da mosca *Drosophila,* e também relacionado com a resposta antifúngica do inseto.(42) A maioria dos mamíferos possui de 10 a 15 TLRs e diferentes membros dessa família de receptores reconhecem PAMPs de origem bacteriana, viral, fúngica ou de protozoários, e são capazes de iniciar a ativação da imunidade inata e, eventualmente, a imunidade adaptativa específica para os antígenos destes diferentes patógenos.(43,44) Desta forma, a ativação de TLRs pode regular, não apenas a fagocitose e atividade microbicida, mas também a liberação de citocinas e diferenciação de células dendríticas e outras células, capacitando o sistema imune inato a induzir a resposta imune adaptativa. (44)

Os TLRs são expressos em uma ampla variedade de células que incluem células dendríticas, macrófagos, monócitos, células B, células T, células NK, fibroblastos e células epiteliais.(37,41,44) Estão presentes na membrana extracelular ou em compartimentos intracelulares, como os endossomas.(37) São glicoproteínas que contém um domínio extracelular, constituído por repetições ricas em leucina e flanqueado por motivos ricos em cisteína, e um domínio intracelular, responsável pela sinalização, o qual é homólogo ao receptor de IL-1 (IL-1R), chamado receptor Toll/IL-1R (TIR).(46,47) A ligação dos TLRs com o patógeno ativa uma cascata de sinais que levam a transcrição de genes envolvidos na defesa do hospedeiro.(37) Geralmente, com exceção do TLR3, a sinalização do TLR ocorre através da proteína adaptadora MyD88, a qual possui um domínio C-terminal, que se liga ao receptor TIR, e um domínio de morte, N-terminal. Esse domínio interage com o domínio de morte da IRAK (proteína quinase serina/treonina), levando a sua fosforilação. IRAK forma um complexo com TRAF6 (fator 6 associado ao TNFR), ativa TAK-1 (membro da família MAP-3 quinase), que ativa quinases IκB. Fosforilação de IκB leva a degradação e translocação do fator NF-κB para o núcleo, para futura transcrição de genes de citocinas.(48-51) Os TLRs, particularmente o TLR3, ativa um percurso independente de MyD88através de TRIF.(52) TRIF associa-se com TRAF3 e TRAF6 através de sua porção N-terminal (TNFR). O domínio de morte de TNFR (TRADD) forma um complexo com proteína FAS (FADD) e leva a ubiquitinação de RIP1, e consequente ativação de NF-κB.(53) Para a sinalização dos TLR2 e TLR4, outra proteína adaptadora, TIRAP/Mal é requerida para recrutar MyD88.(54)

Na leishmaniose os receptores TLR2 e TLR4 têm sido estudados, no entanto, a grande maioria dos trabalhos se limita a modelos experimentais e *in vitro*. Estudo realizado

por Kropf et al. [55] mostrou que TLR4 está envolvido no controle da infecção com *L. major* já que camundongos TLR4 deficientes foram mais suscetíveis à infecção, enquanto que os animais TLR4 competentes apresentam aumento da expressão de iNOS e da produção de NO, resultando em maior destruição do parasita. Além disso, camundongos deficientes de TLR4 e de IL-12R não são capazes de controlar a replicação de *L. major*, desenvolvendo lesões mais severas e número elevado de parasitas, quando comparados com camundongos normais.[56] Pacientes com LV ativa após tratamento com miltefosine apresentam aumento da expressão de TLR9 e TLR4, acompanhado da produção elevada de IFN-γ, TNF-α e IL-12 e diminuição de IL-10 e TGF-β.[57]

O TLR2 está envolvido no reconhecimento da *L. major* e na indução da resposta imune.[58] Em macrófagos murinos estimulados com IFN-γ, o TLR2 atuou em sinergismo com o TLR3 no reconhecimento e fagocitose de promastigotas de *L. donovani*, e na produção de NO e TNF-α.[59] Camundongos deficientes de MyD88 foram mais suscetíveis à infecção com LPG de *L. major*, e apresentaram lesões mais prolongadas. No entanto, camundongos normais para MyD88 tiveram aumento da produção de IL-12p40 por macrófagos via MyD88 de TLR2.[40] Células dendríticas murinas $MyD88^{-/-}$, infectadas com *L. braziliensis*, apresentaram baixos níveis de ativação celular e produção de IL-12p40, que foram correlacionados com diminuição de linfócitos T CD4+ produtores de IFN-γ durante o curso da doença. Entretanto, em experimentos com camundongos $TLR2^{-/-}$, observou-se maior resistência à infecção e maior ativação de células dendríticas e linfócitos T quando comparado aos controles, com aumento de IL-12 e IFN-γ e diminuição de IL-10. Estes resultados demonstram que a MyD88 é essencial para geração de imunidade protetora, enquanto que o TLR2 parece ter um papel regulatório durante a infecção com *L. braziliensis.*[60]

LPGs purificadas de promastigotas de *L. major* estimularam células NK de indivíduos normais, levando a translocação nuclear do NF-κB, com aumento da produção de IFN-γ e TNF-α, bem como, da expressão gênica do RNA mensageiro do TLR2.[41] Células mononucleares de sangue periférico (PBMCs) de indivíduos normais mostraram-se capazes de reconhecer LPG de *Leishmania* através de TLR2, levando ao aumento da produção de citocinas inflamatórias e reativos do oxigênio.[61] Células THP-1 infectadas com *L. donovani* não apresentaram defeito na produção de citocinas pró e anti-inflamatórias. No entanto, quando estimuladas com ligantes de TLR2, ocorreu uma

supressão na produção e expressão de mRNA de IL-12, e aumento na produção e expressão de IL-10. [62]

Na LV ativa a resposta imune mediada por células encontra-se prejudicada, processo que pode ser revertido com o tratamento.[63] Infecções cutâneas experimentais apresentam uma resposta Th1/Th2 polarizada, com produção de IFN-γ e IL-2, que levam a proteção ao hospedeiro, ou produção de citocinas anti-inflamatórias como IL-10 e IL-4, que conferem suscetibilidade.[64] No entanto, na leishmaniose visceral aguda humana, esse aspecto não é evidente. Estudos sugerem que a resposta imune tenha um padrão Th1 concomitante com Th2. [65] Ainda, é mostrado que resposta Th1 é suprimida enquanto que a resposta Th2 é predominante, e após o tratamento há restauração do perfil Th1.[66,67]

Embora alguns aspectos da resposta imune na LV ainda não estão bem entendidos, sabe-se que o desenvolvimento da resposta imune adaptativa depende, dentre outros fatores, da produção de citocinas por células NK, células dendríticas e macrófagos, envolvidas na resposta imune inata.[68] A síntese de IFN-γ, TNF-α, IL-12 e NO são importantes para a proteção do hospedeiro e são induzidas por moléculas da *Leishmania*. Inicialmente, após a interação das células com receptores, inicia-se o processo de fagocitose da forma promastigota, e os macrófagos e células dendríticas liberam IL-12, o que estimula as células NK a secretarem principalmente o IFN-γ. Em combinação com esta citocina, a IL-12 induz a diferenciação e proliferação de linfócito T $CD4^+$ em perfil Th1, o qual produz mais IFN-γ. Macrófagos infectados secretam TNF-α, que age em sinergismo com o IFN-γ para ativar e induzir a atividade antiparasitária desta célula, que uma vez ativada, é capaz de produzir moléculas efetoras tóxicas aos parasitas, levando-os à destruição. [2,69,70]

O IFN-γ é considerado como a principal citocina responsável pela indução da atividade leishmanicida por macrófagos e induz a expressão da iNOS e produção de NO, atuando na eliminação dos parasitas e na resolução da infecção pela *Leishmania.*[71-73] Crianças infectadas com *L. chagasi*, vivendo em área endêmica, exibiram alta produção de IFN-γ quando PBMCs foram estimuladas com antígeno de *Leishmania,* e conseguiram controlar a infecção, enquanto que aquelas que apresentavam baixos níveis desta citocina progrediram para a doença.[74] Entretanto,foi observada uma baixa resposta proliferativa de linfócitos e de produção de IFN-γ contra o parasita *in vitro*, associada com alta carga parasitária. [75]

Em experimentos com células de pacientes com LV, a administração exógena de IL-12 induziu a produção de IFN-γ por PBMCs, restaurou a resposta proliferativa de linfócitos e diminuiu a apoptose espontânea ou induzida de PBMCs. Além disso, a IL-12 apresentou efeito regulatório na produção de IL-10 durante infecção, e a adição de IL-12 recombinante ou de anticorpos anti-IL-10, restaurou a produção de IFN-γ e a resposta linfoproliferativa nesses pacientes. (76) O TNF-α é produzido por macrófagos ativados, e age em conjunto com IFN-γ para exercer a atividade leishmanicida mediada pela produção de NO. (77) Pacientes com lesões cutâneas graves e aqueles com LV apresentam altos níveis de TNF-α. (78) Entretanto, em estudo realizado por Barral et al. (79) com LV foi verificado que embora tenha ocorrido elevada produção de IFN-γ e TNF-α, a infecção ainda persistiu.

Macrófagos são capazes de controlar a infecção com *L. braziliensis* mesmo na ausência de células T em indivíduos com infecção subclínica. Já os indivíduos com infecção cutânea e mucocutânea mostraram altos níveis de citocinas inflamatórias, o que levou a ativação de células T e contribuiu para a patologia da doença. (80) Células T CD4+ e T CD8+ estão envolvidas na defesa contra a *Leishmania*, principalmente devido à produção de IFN-γ e da lise de células infectadas.(81,82) Camundongos BALB/c infectados com *L. donovani* foram capazes de controlar a infecção devido ao aumento de células T CD8+. (83) Pacientes com LV ativa que apresentam prejudicada resposta imune mediada por células possuem supressão de células T CD4+. (84) Em contraste, pacientes com LV ativa não são capazes de produzir IFN-γ por células T CD8+ e T CD4+, mas possuem altos níveis dessa citocina no plasma, resultado de outra fonte celular de IFN-γ. (85)

A suscetibilidade à infecção e a progressão da doença estão relacionadas com o direcionamento de uma resposta do tipo Th2, com produção de IL-4 e de outros componentes que desativam macrófagos, como a IL-10, TGF-β e prostaglandina E2.(72,73,86) A IL-10 além de desativar as funções dos macrófagos também suprime produção de IL-12 e de IFN-γ por células NK.(76,87) Indivíduos capazes de controlar a infecção apresentaram resposta Th1 específica com produção de IFN-γ e IL-12. Por outro lado, pacientes com doença ativa apresentaram altos níveis séricos de IL-10 e aumento do mRNA de IL-10 no tecido lesionado. (88) Em estudos realizados no Sudão e Índia em pacientes com LV em atividade foi detectada expressão elevada de mRNA de IL-10 na medula óssea, linfonodos, PBMCs e células esplênicas, que diminuiu após o tratamento.(89) A adição de IL-10 recombinante em macrófagos humanos inibiu a destruição, mediada por

NO, da *L. infantum*, *L. major* e *L. braziliensis*.[90] Aspirados de medula óssea de pacientes com LV ativa apresentaram alta expressão de mRNA de IL-10 e IFN-γ. No entanto, com a resolução da infecção os níveis de IL-10 diminuíram. [91]

Células T reguladoras (Tregs) são uma subpopulação de células T CD4+ que expressam o fator de transcrição Foxp3 (Forkhead Box P3) e CD25, além de produzirem citocinas como a IL-10. [92-94] Na infecção experimental com *L. major*, essas células foram as responsáveis por manter a sobrevivência do parasita nas lesões, controlando a expressão de mecanismos efetores. [93,95] Pacientes com LV apresentaram aumento de células Tregs na medula óssea com consequente aumento da produção de IL-10 e supressão de células T efetoras locais, o que contribuiu para a persistência do parasita e patologia. [96] Outro estudo mostrou que pacientes com LV ativa apresentam aumento de CD4+CD25+ em PBMCs, que diminuem após a cura. [97] No entanto, foi mostrado que células TCD4+CD25+foxp3+ não são a principal fonte de IL-10 no baço em pacientes com LV ativa, e que quando as células esplênicas são depletadas de CD25+ há aumento da produção de IL-10, sugerindo que na doença ativa, a fonte principal dessa citocina são as células TCD4+CD25-Foxp3-. [98] Camundongos BALB/c infectados com *L. donovani* apresentaram aumento de Tregs em linfonodos e baço, resultando em resposta imune efetora Th1 e Th2 deprimidas. TGF-β produzido por células T CD4+ CD25+ contribuiu para a imunossupressão e controlou a imunopatologia mediada pelo parasita. No entanto, a fonte de IL-10 não foram células T CD4+ CD25- foxp3-, o que sugere desenvolvimento de diferentes células T regulatórias em resposta ao parasita. [99]

O TGF-β possui funções imunes como aumento da quimiotaxia, supressão da resposta Th1 e Th2 e de algumas funções microbicidas dos macrófagos. [100,101] Essa citocina desenvolve um papel regulatório aumentando a replicação e virulência parasitária em macrófagos, permitindo que a *Leishmania* sobreviva no hospedeiro.[102, 103] Estudo mostrou que o parasita manipula o microambiente para sua sobrevivência, através da ativação de TGF-β latente na matriz extracelular.[104] Tratamento *in vivo* com anti-TGF-β promoveu a cura em infecção com *L. major* e aumentou a produção de NO por macrófagos.[105] Também foi responsável por aumento da patologia na infecção experimental com leishmaniose visceral. [106] Camundongos BALB/c infectados com *L. chagasi* apresentaram resposta imune Th1 prejudicada devido à produção de TGF-β por células T CD4+. [107]

A subpopulação de células Th17 vem ganhando importância na resposta contra a *Leishmania.* Em camundongos Balb\c infectados com *L. major* foi detectada alta produção de IL-17, acompanhada por gravidade da doença, destruição tecidual e grande carga parasitária. Camundongos BALB/c, deficientes de IL-17 desenvolveram menores lesões, sem acúmulo de CXCL2e presença de poucos neutrófilos. [108] Estudo realizado por Bacellar et al. [109] mostrou que tanto PBMCs quanto células T CD4+, presentes nas lesões de pacientes infectados com *L. braziliensis,* produzem altas quantidades de IL-17. Além disso, foi observado que esta citocina juntamente com TNF-α contribuiu para a patogênese da doença. Entretanto, em pacientes com Kala-zar a produção de IL-17 e IL-12 foram associadas com resistência à doença, sugerindo que essas citocinas desenvolveram papel complementar às citocinas de padrão Th1 protegendo contra a doença. [110] Estudo experimental com *L. donovani* mostrou que curdlan, um polissacarídeo com propriedades imunomoduladoras, é capaz de induzir citocinas de padrão Th17 como IL-17 e IL-23, o que contribuiu para a diminuição da carga parasitária. [111]

A LV e a desnutrição são consideradas como importantes problemas de saúde pública. Quando analisadas em conjunto, os índices epidemiológicos da LV e a prevalência de desnutrição são responsáveis por milhões de mortes anualmente.[112] O estado nutricional de indivíduos infectados com *Leishmania ssp*. apresenta envolvimento na evolução clínica da LV, especialmente em crianças menores que cinco anos, e a alta suscetibilidade à infecção, neste grupo de risco, tem sido relacionada com imaturidade imunológica e desnutrição, muito comum em áreas endêmicas de LV.[113,114]

A desnutrição é considerada como um dos principais determinantes na progressão, gravidade e aumento de mortalidade na leishmaniose, podendo ocorrer como consequência de uma deficiência energética (desnutrição proteico-calórica – DPC) ou de um micronutriente. [115,116] Todo processo infeccioso generalizado costuma ser acompanhado por hipercatabolismo, agravado por anorexia, resultando na perda, e na consequente depleção, das reservas corpóreas de nutrientes, maior demanda energética durante o processo infeccioso é acompanhada por grandes alterações no metabolismo do hospedeiro, que tem como finalidade prepará-lo no sentido de combater o agente agressor. [117]

Alguns estudos observaram que a LV afetou o estado nutricionaldos pacientes, resultando em perda de músculo e gordura, efeitos que possivelmente foram mediados pela IL-1 e/ou outros fatores produzidos por macrófagos infectados pela *L. donovani*.[118] Desnutrição, também, foi relacionada com intensa produção de prostaglandina E2 e

diminuição dos níveis de IL-10 e NO, após infecção com *L. donovani*. A estimulação de macrófagos de camundongos desnutridos e infectados com *L. donovani* com IFN-γ/LPS, levou a produção de baixos níveis de TNF-α, IL-10 e NO, quando comparados aos animais controles, contribuindo para a gravidade da doença. [(119)] Em modelos experimentais com *L. chagasi* a diminuição dos níveis séricos de glicose, albumina, globulina e proteínas totais em animais desnutridos, foram associadas com aumento da carga parasitária, não resposta à vacina e redução da produção de IFN-γ. Os resultados sugerem que a desnutrição afetou a resposta imune nestes modelos de infecção. [(120)] Outros estudos em pacientes com LV ativa também têm relatado diferenças no índice de massa corporal (IMC), glicose, triglicerídeos e lipoproteínas. [(121-123)]

Embora existam fortes evidências de que a imunidade ou suscetibilidade à LV estejam diretamente relacionadas ao estado nutricional do hospedeiro, os mecanismos imunológicos envolvidos na relação entre a desnutrição e o desenvolvimento de LV são múltiplos e ainda pouco explorados e entendidos. Além disso, apesar do conhecimento através de estudos experimentais, do envolvimento de mediadores pró e anti-inflamatórios na imunidade inata assim como dos TLRs, não se sabe ainda qual é a precisa interação entre eles em pacientes. Baseado na ausência destas informações, e na hipótese de que os TLRs estariam envolvidos diretamente na produção destes mediadores, acreditamos que o estudo do envolvimento dos receptores TLRs na produção de citocinas pró e anti-inflamatórias e do NO, além do conhecimento do perfil bioquímico-nutricional de pacientes com LV pré e pós-tratamento, permitirá a melhor compreensão dos mecanismos envolvidos na patogênese da doença.

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Capítulo I

Biochemical and nutritional evaluation of patients with visceral leishmaniasis before and after treatment with leishmanicidal drugs

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ABSTRACT

Introduction: Visceral leishmaniasis (VL) is caused by the intracellular protozoan *Leishmania donovani* complex. VL may be asymptomatic or progressive and is characterized by fever, anemia, weight loss and the enlargement of the spleen and liver. The nutritional status of the patients with VL is a major determinant of the progression, severity and mortality of the disease, as it affects the clinical progression of the disease. Changes in lipoproteins and plasma proteins may have major impacts in the host during infection. Thus, our goal was evaluate the serum total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, glucose, albumin, globulin and total protein levels, as well as the body composition, of VL patients before and after treatment. **Methods**: Nutritional evaluation was performed using the bioelectrical impedance (BIA) to assess body composition. Biochemical data on the serum total cholesterol, HDL, LDL, triglycerides, glucose, albumin, globulin and total protein were collected from the medical charts of the patients. **Results:**BIA indicated that both pre-treatment and post-treatment patients exhibited decreased phase angles compared to the controls, which is indicative of disease. Prior to treatment, the patients exhibited lower levels of total body water compared to the controls.Regarding the biochemical evaluation,patients with active VL exhibited lower levels of total cholesterol, HDL, LDL and albumin and higher triglyceride levels compared to patients after treatment and the controls. Treatment increased the levels of albumin and lipoproteins and decreased the triglyceride levels. **Conclusions:**Our results suggest that patients with active VL present biochemical and nutritional changes that are reversed by treatment.

Keywords: Visceral leishmaniasis. Nutritional status.Leishmanicidal drugs.

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INTRODUCTION

Leishmaniasis is caused by an obligatory intracellular parasite belonging to the genus *Leishmania*[1]. There are over 20 species of this parasite that can be transmitted to humans by approximately 30 different species of sandflies[2].Visceral leishmaniasis (VL), also known as kala-azar, is caused by the protozoa of the *Leishmania donovani* complex[3]. Although the risk factors for this disease are not fully understood, it is known that genetic factors, malnutrition and the presence of infected animals in the environment are major contributors to the high maintenance rate of this disease[4-6].

Regarding the treatment for VL, pentavalent antimonials are the first choice of treatment[7,8]. Amphotericin B can be used in cases of toxicity or in patients with unsatisfactory responses to the antimonials and is the first choice for treating pregnant patients and terminal cases of the disease[9,10]. However, according to the Ministry of Health, patients older than 50 years, patients with Chagas disease or patients diagnosed with kidney, cardiac or hepatic complications should be treated with lipid or colloidal formulations of amphotericin B[8].

Visceral leishmaniasishas a large clinical range, from asymptomatic infections and auto resolution to progressive visceral leishmaniasis, which is characterized by fever, hepatosplenomegaly, hypergammaglobulinemia and death if not treated properly and in time[11,12].Although the factors that lead to the development of the disease are still unclear, the complex interactions between the parasite, the immune response and the nutritional status may influence the host's response to *Leishmania* infection[4,13-16].

Visceral leishmaniasis and malnutrition are considered important public health problems and together are responsible for millions of deaths each year[5]. The nutritional status of the individuals infected with *Leishmania* spp. is involved in the clinical course of the disease and is a major determinant of the progression, severity and increased mortality of VL. Malnutrition may occur as a consequence of energy deficiency, such as protein-calorie malnutrition (PCM), or a micronutrient deficiency[17-20]. The infectious process is usually followed by hypercatabolism, which is aggravated by anorexia, resulting in the loss and the consequent depletion of the body's nutrient reserves, thereby causing great changes in the metabolism of the host[21].

Some studies have reported differences in body mass index (BMI), glucose, triglycerides and lipoproteins in patients with active VL[22-24].Studies performed on patients with VL showed high triglyceride levels and low high-density lipoprotein (HDL), low-density lipoprotein (LDL) and total cholesterol levels[25,26]. Additionally, VL patients

showed reduced serum cholesterol concentrations as a function of their splenic parasite burden[23,27]. Elevated levels of triglycerides and low levels of HDL were observed in canine VL[28]. Changes in lipoproteins have also been reported in children with active VL[29-31]. Lipoproteins have the ability to modulate the immune response. Low density lipoproteins, such as very low-density lipoprotein (VLDL) and LDL, can inhibit lymphocyte proliferation *in vitro*[32,33], and another study in VL patients found that lipoproteins altered the immune response and the pathogenesis of the disease by modulating cytokine production[25].

Changes in cholesterol levels can have a major impact on the host during infection. In addition to playing an important role in the maintenance of membrane fluidity, cholesterol is essential for the proper function of antigen presenting cells and is also a prognostic indicator of increased morbidity and mortality associated with pathological conditions[34-36]. This lipid is responsible for the formation of membrane rafts, which are essential for *Leishmania* entry[37-39]. In malnourished animals infected with *Leishmania chagasi*, decreases in serum albumin, globulin and total protein were observed, and these changes were associated with an increased parasite burden and a non-response to the vaccine[40].

Interest in body composition has grown substantially in the last few years due to the association between increased body fat and metabolic disorders, including cardiovascular diseases, diabetes, hypertension and dyslipidemia[41,42]. Bioelectrical impedance analysis (BIA) has been used to analyze body composition for more than 25 years. Additionally, BIA is a portable, noninvasive method to measure body composition that is practical, reproducible and relatively inexpensive. BIA can be repeated often and does not require patient cooperation[43,44].

Although some studies have reported changes in lipoproteins in patients with VL, studies concerning the serum total protein, albumin, globulin and BIA body measurements are absent in these patients, and there are few published papers that examine a wider range of nutritional status factors.Thus, our goal was to evaluate the serum total cholesterol, HDL, LDL, triglycerides, glucose, albumin, globulin and total protein, as well as body composition using BIA, in LV patients before and after treatment with leishmanicidal drugs.

METHODS

Patients

We evaluated 13 patients pre- and post-treatment at an interval of 18 months. The patients were of both genders and were 18 years or older. The age of the patients ranged from 18 to 53 years, and the patients were predominantly male (11 individuals).We excluded patients with other infectious or granulomatous diseases, as well as HIV positive patients and pregnant women. The patients were enrolled in the study when they contacted health services for diagnosis and treatment of the disease. The patients came from the State Hospital of Bauru, São Paulo and the Hospital of Marilia, Sao Paulo. We evaluated 16 healthy, age- and sex-matched subjects as controls. The age of the control subjects ranged from 21 to 58 years, and the group included 3 women and 13 men.

Diagnosis and treatment of visceral leishmaniasis

The diagnosis of visceral leishmaniasis was performed through the confirmation of the parasites in bone marrow aspirate smears. Patients were treated with N-methylglucamine (Glucantime), amphotericin B deoxycholate or amphotericin B liposome. Data were collected from the patients in the hospital where they were admitted prior to starting treatment for VL. The post-treatment period ranged from 1 to 3 months after the end of the drug regimen, and the patient data were included when the patients returned to the hospital for monitoring. However, two patients did not return to the hospital after the treatment. Post-treatment data were collected only from patients that were cured according to the serological and clinical tests.

Biochemical and nutritional analysis

The biochemical and nutritional evaluation was performed in patients before and after treatment and was performed once in the control subjects. BIA was used for body measurements in the control subjects and in patients pre-and post-treatment using a Biodynamics BIA, model 450 device (TBW). For this procedure, the patients and the control subjects laid down on a non-conductive surface, with their legs apart and their arms parallel and apart from the trunk. Immediately prior to the placement of the electrodes, the contact areas were cleaned with alcohol. An emitter electrode was placed near the metacarpo-phalanx of the dorsal surface of the right hand, and another was placed near the distal transverse arch of the upper surface of the right foot. An electrode detector was placed between the distal prominences of the radius and ulna of the right wrist, and another

was placed between the medial and lateral malleolus of the right ankle. The following data were acquired from the patients and control subjects: lean mass (%), body fat (%), body mass index (kg/m^2), phase angle (°), resistance (ohms), reactance (ohms) and total body water (L). In addition to the BIA, the patient's weight (kg) and height (m) were also recorded.

The analyses of the total cholesterol, HDL, LDL, triglycerides, glucose, total protein, albumin and globulin in the control subjects were performed in the Clinical Laboratory of the Faculty of Medicine of Botucatu (***Universidade Estadual****Paulista*-UNESP). The patient analyses were performed at the hospital where they were admitted, as these are routine tests for the treatment and follow-up of the disease. These determinations were performed using enzymatic, colorimetric and dry-chemistry methods. To evaluate these parameters, blood was collected from the patient in the morning after fasting for 12h, after no rigorous physical activity for the last 24h and after no alcohol consumption within the last 72h.

Statistical analysis

Non-continuous variables were analyzed using Fisher's test. Comparisons for the continuous variables were performed using an analysis of variance (ANOVA) with a repeated measures design between the pre- and post-treatment groups, followed by an adjusted Tukey's test if the distribution were normal. Otherwise, the same design was used to adjust a generalized linear model with gamma distribution, followed by a multiple comparison test (Wald type). Comparisons between the pre- and post-treatment groups and the control group were made using separate Student's t-tests (control vs. pre and control vs. post). All of the analyses were made using the Statistical Analysis System (SAS) software, v.9.3. The significance level was fixed at 5% or the correspondent p-value.

Ethical considerations

All enrolled patients and control subjects were fully informed of the study and signed the consent form. This study was approved by the Ethics in Research of the Faculty of Medicine of Botucatu, Bauru State Hospital and the Hospital of Marilia and is in accordance with the Declaration of Helsinki of 1964.

RESULTS

Evaluation of body measurements by bioelectrical impedance in patients pre- and post-treatment

There was a male predominance in the patients and control subjects in this study, and there was no significant difference in the mean age of the patients and the control subjects. The results obtained from the BIA analysis showed that there were no significant differences in the phase angle between the patients before and after treatment; however, the phase angle in the patients pre-treatment and post-treatment were significantly lower (p-value <0.05) than the phase angle of the control subjects. In relation to total body water, there were no significant differences between pre- and post-treatment or between post-treatment and the controls. However, patients with active VL showed a decreased (p-value <0.05) amount of total body water than the control subjects. No significant differences in weight (kg), height (m), body mass index (kg/m^2), percentage of lean mass, percentage fat, resistance (ohms) and reactance (ohms) were detected between the patients pre-treatment and post-treatment or between these two groups and the control subjects. However, even without significant differences between the pre- and post-treatment measurements or between these groups and the control group, the pre-treatment patients tended to have smaller reactance, BMI, fat percentage and weight values than the post-treatment patients and the controls. The percentage of lean mass tended to decrease with treatment and tended to be higher in the control subjects. Data are shown in **Table 1**.

TABLE 1 - Bioeletrical impedance analysis of patients with visceral leishmaniasis (difference estimates and 95% confidence intervals).

	Pre- vs post-treatment	Pre-treatment vs controls	Post-treatment vs controls
	Estimate (95% CI)	Estimate (95% CI)	Estimate (95% CI)
Weight (kg)	-5.662 (-14.35-3.03)	-10.84 (-22.25-0.567)	-5.184 (-16.47-6.1)
Height (m)	0.006 (-0.02-0.03)	-0.02 (-0.09-0.03)	-5.184 (-16.47-6.1)
Resistance (ohms)	4.59 (-61.33-70.51)	12.36 (-63.16-87.87)	16.95 (-68.17-102.07)
Reactance (ohms)	2.52 (-10.14-15.18)	9.124 (-0.671-18.91)	6.599 (-1.884-15.082)
Phase angle (º)	0.28 (-0.82-1.38)	1.5 (0.31-2.7)*	1.22 (0.17-2.27)*
BMI (kg/m^2)	2.03 (-1.61-5.67)	2.9 (-0.641-6.45)	0.86 (-2.89-4.63)
% Fat mass (%)	4.18 (-3.02-11.38)	4.6 (-2.24-11.45)	0.42 (-6.08-6.92)
% Lean mass	2.638 (-3.993-9.270)	1.965 (-5.818-9.749)	-0.195 (-6.970-6.580)
Total body water (L)	-2.57 (-8.787-3.633)	6.825 (0.417-13.23)*	4.73 (-1.944-11.40)

vs: versus ; CI: confidence interval; BMI: body mass index. *p value <0.05

Biochemical assessment of patients with visceral leishmaniasis before and after treatment

The pre-treatment patients showed significantly lower levels of total cholesterol, HDL and LDL compared to the post-treatment patients and control subjects (p-value <0.05). However, with exception of LDL, the treated patients also showed significantly lower levels of these variables when compared to the controls (p-value <0.05). Prior to treatment, patients exhibited significantly higher levels of triglycerides compared to post-treatment and the control subjects; even after treatment, these levels still remained elevated when compared to the controls (p-value <0.05). Pre-treatment patients also had lower albumin levels compared to the post-treatment and the control groups (p-value <0.05). However, the treated patients showed higher albumin levels than the controls (p-value <0.05). No differences were found in the levels of glucose, total protein and globulin between these groups. Data are shown in **Table 2**.

TABLE 2 - Biochemical assessment of patients with visceral leishmaniasis (difference estimates and 95% confidence intervals).

	Pre- vs post-treatment	Pre-treatment vs controls	Post-treatment vs controls
	Estimate (95% CI)	Estimate (95% CI)	Estimate (95% CI)
Total cholesterol (mg/dL)	58.82 (11.14-66.5)*	94.27 (59.5-129)*	35.44 (0.33-70.55)*
HDL (mg/dL)	30.44 (22.26-38.62)*	46.8 (32.5-61.17)*	16.4 (1.38-31.41)*
LDL (mg/dL)	38.29 (14.01-62.57)*	62.55 (33.15-91.96)*	24.26 (-6.71-55.23)
Triglycerides (mg/dL)	0.0016 (0.00007-0.0032)*	0.001672 (0.000072-0.003272)*	0.005 (0.003105-0.007285)*
Glucose (mg/dL)	0 (0)	0.00068 (-0.000852-0.002212)	-0.00148 (-0.00061-0.00357)
Albumin (g/dL)	1.47 (0.99-1.95)*	0.9 (0.39-1.42)*	0.56 (0.12-0.99)*
Globulin (g/dL)	-1.04 (-2.52-0.44)	0.91 (-0.19-2.01)	0.13 (-0.77-1.03)
Total protein (g/dL)	0.38 (-0.88-1.64)	0.04 (-0.94-1.04)	0.43 (-0.29-1.15)

vs: versus; CI: confidence interval; LDL: low-density lipoprotein; HDL: high-density lipoprotein. *p value <0.05

DISCUSSION

According to our results, BIA demonstrated a decreased phase angle in patients before treatment and after treatment compared to the control subjects. The phase angle is formed when the electric current is stored across cell membranes and depends on the permeability of the membranes; this measurement is associated with tissue hydration and cellularity, as well as cell size and body cell mass[45].Thus, the phase angle is associated with cell balance[46]. The phase angle measurement can vary between 0° and 90°, and the reference values are between 4° and 10°[47]. The phase angle has been used as an indicator of the general state of health and nutrition in various diseases, as nutrition is interconnected with changes in membrane integrity and the balance of body fluids[46-48].

Our findings showed that both groups were within the reference values, however, the pre-treatment patients had lower phase angle values than the control group.

According to our results, phase angle in patients with infectious diseases and inflammation is lower than the reference values[47,49].Therefore, studies in patients co-infected with HIV and tuberculosis also exhibited low phase angles when compared to the control subjects[50]. Although no statistically significant difference was found, our results showed that treatment for VL tended to increase the phase angle. However, even after treatment, the phase angle was still lower than in the control subjects. A possible explanation could be that the patients in the study period had not yet fully recovered. Furthermore, we have to consider that the phase angle is positively associated with reactance, which in our study tended to be smaller in patients with active VL compared to the controls. While the phase angle is decreased, the reactance is low, which indicates the presence of a disease.

Reactance values inversely correlate with the percentage of lean body mass, which is highly conductive to electrical current, as resistance values directly correlate to the percentage of fat mass, which is poor electrical conductor[45]. In the present study, although no significant difference in weight was detected between the groups, the amount of lean mass tended to be higher in the pre-treatment patients, while the percentage of fat mass tended to be lower in these patients. Taking into account these results, the pre-treatment patients also exhibited normal BMI values, as malnutrition is considered a BMI below 18.5 kg/m^2. Contrary to our findings, one study reported that children with active VL had lower BMI values than healed children and uninfected children; additionally, children that breastfeed for longer period of time have asymptomatic VL, while children with lower birth weight are more likely to develop VL, which shows that nutritional status plays a

crucial role in the pathogenesis of visceral leishmaniasis[22]. Thus, we can conclude that patients with active VL showed no difference in weight compared to the post-treatment patients and the controls and, therefore displayed no difference in BMI, percentage of lean mass and fat mass.

According to our results, glucose levels did not differ between the groups. Unlike our results, previous studies have shown that experimental infection with *L. chagasi* leads to a reduction in glucose levels compared to control mice[24]. Concerning lipid levels, our results showed that pre-treatment patients had lower HDL, LDL and total cholesterol levels and higher levels of triglycerides compared to the post-treatment patients and the controls. However, it is worth noting that all groups were within the normal range for total cholesterol (reference values <200mg/dL) and LDL (reference values <130mg/dL). The HDL levels (reference values >35mg/dl) and triglycerides levels (reference value <150mg/dL) were below and higher than normal in patients before treatment, respectively. These results are in agreement with Soares et al.[25], who showed that patients with VL had high triglycerides levels and low HDL, LDL and total cholesterol levels. Other studies of visceral leishmaniasis and other infectious diseases, such as HIV and schistosomiasis, have reported the same lipid changes[28,51-54].

Our findings show that patients with active VL, which most likely have a high parasite load, have total cholesterol levels lower than the control group, and after treatment, these levels increase. In this sense, Ghosh et al.[23] showed that patients with VL have cholesterol concentrations that were inversely correlated with their splenic parasite load. Due to the high parasitic loads in the spleen, patients with active VL experience dysfunctions in this organ, which is responsible for cholesterol biosynthesis, thus increasing the morbidity of this disease[27]. Our results showed that treatment appeared to normalize the HDL and triglyceride levels and increase the LDL and total cholesterol levels.

The changes in the levels of lipoproteins can be directly related to the modulation of the immune response, as the plasma membrane, which is composed of lipids, is essential for antigen presentation and phagocytosis. Internalization involves interaction between the parasite and the plasma membrane of the cell host[36,55,56]. Low cholesterol levels in humans decrease the number of circulating lymphocytes, thus increasing the chances of mortality[23,34]. The decrease in lipoproteins may affect the immune response and the pathogenesis of the disease, as lipoproteins are related to the production of TNF-α, IL-6 and IL-10[25]. In addition to being targets for the immunomodulatory activity of lipoproteins, macrophages express lipoprotein receptors and are the host cells of the *Leishmania*

parasite[57]. However, the study demonstrated that macrophage-depleted lipids decreased their ability to interact with *Leishmania donovani* and internalize promastigotes by 45%, thus impairing the replication of the parasite. On the other hand, when depleted macrophages are treated with cholesterol, there is an increased chance that the parasites will destroy the cell membrane[39].

In relation to the plasma proteins, it was demonstrated in our study that patients with active VL have lower albumin levels compared to the post-treatment patients and the controls, and no differences were observed in the levels of total protein and globulin between the groups. Contrary to the findings of our study, Malafaia et al.[40] reported decreased globulin and total protein in mice infected with *L. chagasi* compared to uninfected mice. However, according to our results, animals infected with *L. chagasi* also exhibit a decrease in serum albumin, which is associated with increased parasite load, a negative response to the vaccine and reduced IFN-γ production[40].Another study reported that the albumin/globulin ratio was lower in VL patients compared to control subjects in endemic and non-endemic areas[58].

Our results show that patients with active VL were eutrophic, had a lower phase angle and exhibited changes in their albumin and lipoprotein levels. Treatment changed the biochemical frame and the nutritional status of the patients, tending to return to normal levels. The data presented in this study suggest an association between the biochemical and nutritional alterations and leishmaniasis. However, our study does not conclude that the changes were responsible for the worseningof the VL or if the infection led to the biochemical and nutritional changes, thus aggravating the clinical manifestations of VL. Thus, we are developing new studies to have a better understanding of the involvement of biochemical and nutritional parameters in the pathogenesis of LV.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Capítulo II

Role of TLR2 and TLR4 on cytokine production and nitric oxide in patients with visceral leishmaniasis before and after treatment with leishmanicidal drugs

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ABSTRACT

The recognition molecules derived from *Leishmania* is performed by different TLRs, mainly by TLR2 and TLR4, which trigger the production of pro- and anti-inflammatory mediators and activate the immune response. Were evaluated in13 VL patients before and after treatment the expression of TLR2 and TLR4 in CD3 + and CD14 + cells and production of cytokines by PBMCs stimulated or not with PGN (TLR2 agonist) and LPS (TLR4 agonist). In the active VL was increased of TLR2 and TLR4 on lymphocytes and monocytes, increase of TNF-α, IL-10, TGF-β and decrease of IFN-γ, IL-17 and NO. After treatment, there was still TLR2 and TLR4 expression, decreased of TNF-α and IL-10 levels, increased of IFN-γ, IL-17 and NO production, while TGF-β kept high. Before treatment TNF-α and NO production was associated with TLR2 and TLR4, while IL-10 was linked to TLR2. In the post-treatment, the two receptors are related to the production of TNF-α, IFN-γ, IL-10 and NO, whereas IL-17 production only TLR4. Our results suggest that in patients with LV the TLR2 and TLR4 participate in the modulation of cytokines and NO, contributing to the pathogenesis of LV on pre-treatmentand protective immune response after treatment.

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INTRODUCTION

Visceral leishmanisis (VL), also known as Kala Zar, is caused by obligate intracellular protozoa belonging to *Leishmania donovani* complex [1,2]. It is a lethal disease if not treated presenting symptoms such as hepatosplenomegaly, fever, anemia, weight loss, hypergammaglobulinemia and pancytopenia [3]. The VL is endemic in 62 countries and 90% of cases occur in Bangladesh, Brazil, India, Nepal and Sudan [4,5].

Regarding the treatment for VL, pentavalent antimonials are the first choice of treatment [6,7]. Amphotericin B can be used in cases of toxicity or in patients with unsatisfactory responses to the antimonials and is the first choice for treating pregnant patients and terminal cases of the disease [8,9]. However, according to the Ministry of Health, patients older than 50 years, patients with Chagas disease or patients diagnosed with kidney, cardiac or hepatic complications should be treated with lipid or colloidal formulations of amphotericin B [7].

The Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that mediate the recognition of microbicides structures and induce inflammatory and adaptative responses [10]. Several studies have shown that the recognition molecules derived from *Leishmania* is performed by different TLRs such as TLR2, TLR4 and TLR3 [11-14]. On the surface of *Leishmania*, the main pathogen associated molecular patterns (PAMPs) recognized by these receptors of the immune system are lipophosphoglycans (LPG), glicoinositol phospholipids (GIPL) and gp63 molecules [11,12,15]. For instance, *Leishmania major* LPG stimulated macrophages to secrete cytokines such as IL-12 and TNF-α by binding to TLR-2 [11]. Additionally, BALB/c infected with *L. chagasi* showed increased mRNA expression of TLR2 and TLR4 in the spleen which correlated with parasite load [16]. Murine macrophages stimulated with IFN-γ were able to recognize and phagocyte *L. donovani* with consequent production of TNF-α and NO via TLR2 and TLR3 [14]. Human NK cells stimulated with LPG of *L. major* showed increased expression of TLR2 mRNA and increased production of TNF-α and IFN-γ [12].TLR4 deficient mice are not able to contain the replication of *L. major* and developmore severe lesions when compared to controls [17]. Additionally, the involvement of TLRs in induction of nitric oxide (NO) and TNF-α has been reported [14].Accordingly, Kropf et al 2004 [13] reported on *in vitro* experiments the involvement of TLR4 in the control of *L. major* infection through increased expression of the iNOS.

The host protection during LV depends on the development of a Th1 response. IL-12 secreted by antigen-presenting cells activates CD4+ T lymphocytes and NK cells to

produce IFN-γ. Infected macrophages produce NO and also secrete TNF-α that acts in synergy with IFN-γ activating the microbicidal capacity of this cell [10,18,19]. On the other hand, susceptibility to infection is related to the development of a Th2-type response with IL-4 production and other components that deactivate macrophages such as IL-10 and TGF-β [20-22]. Up to the present, is not well established in leishmaniasis if IL-17 participates in defense mechanisms or whether it contributes to the pathogenesis of this infection [23]. BALB/c mice that do not produce IL-17 and infected with *L. major* developed smaller lesions compared to the controls animals [24]. However, other studies showed an association of this cytokine with inflammatory response and protection against VL [25,26].

Given these observations and because of the absence of information on the interaction between pro and anti-inflammatory mediators and TLRs in patients with VL, especially before and after treatment, studies evaluating these aspects may contribute to a better understanding of the relationship parasite/host in VL patients. This study evaluated the expression of TLR2 and TLR4 on monocytes and lymphocytes and the involvement of these receptors in the TNF-α, IFN-γ, IL-17, IL-10, TGF-β and NO production in patients with VL before and after treatment.

MATERIAL AND METHODS

Patients

We evaluated 13 patients pre- and post-treatment at an interval of 18 months. The patients were of both genders and were 18 years or older. The age of the patients ranged from 18 to 53 years, and the patients were predominantly male (11 individuals).We excluded patients with other infectious or granulomatous diseases, as well as HIV positive patients and pregnant women. The patients were enrolled in the study when they contacted health services for diagnosis and treatment of the disease. The patients came from the State Hospital of Bauru, São Paulo and the Hospital of Marilia, Sao Paulo. We evaluated 16 healthy, age- and sex-matched subjects as controls. The age of the control subjects ranged from 21 to 58 years, and the group included 3 women and 13 men.

Diagnosis, treatment of visceral leishmaniasis

The diagnosis of visceral leishmaniasis was performed through the confirmation of the parasites in bone marrow aspirate smears. Patients were treated with N-methylglucamine (Glucantime), amphotericin B deoxycholate or amphotericin B liposome. Data were collected from the patients in the hospital where they were admitted prior to starting treatment for VL. The post-treatment period ranged from 1 to 3 months after the end of the drug regimen, and the patient data were included when the patients returned to the hospital for monitoring. Post-treatment data were collected only from patients that were cured according to the serological and clinical tests.

Ethical considerations

All enrolled patients and control subjects were fully informed of the study and signed the consent form. This study was approved by the Ethics in Research of the Faculty of Medicine of Botucatu, Bauru State Hospital and the Hospital of Marilia and is in accordance with the Declaration of Helsinki of 1964.

Blood sample collection

Blood samples (25 ml) were collected from the forearm vein at one time point from controls and at two different time points from patients with VL; before and after treatment. Samples were collected in heparinized tubes and initially centrifuged at 450 g for 10 minutes. Plasma was stored and the remaining blood samples were used to obtain peripheral blood mononuclear cells (PBMCs) for later evaluation of the cytokines in culture supernatants and the expression of TLR2 and TLR4 on the cell surface using flow cytometry.

TLR2 and TLR4 cell surface expression

To evaluate the expression of TLR2 and TLR4 on monocytes and lymphocytes, 100 µl of whole blood of patients and control subjects were incubated 20 minutes in the dark in falcon tubes (Becton, Dickinson and Company) with a monoclonal anti-TLR4 antibody conjugated to PE (BioLegend, San Diego, CA, USA), anti-TLR2 antibody conjugated to FITC (BioLegend, San Diego, CA, USA), anti-CD3 antibody conjugated to PE-DY647 and anti-CD14 antibody conjugated to PE-DY647 (EXBIO, Vestec, Czech Republic). After incubation, the cells were washed with 450 µl of RBC lysis solution (Becton, Dickinson and Company) and incubated in the dark for 15 minutes, centrifuged for 5 minutes at 650 g and the supernatant was discarded. This procedure was performed

twice. Cells were suspended in 300 μl of electrolyte solution ISOTON II (Becton, Dickinson and Company). Analyses were performed using flow cytometry (FACSCalibur^{TM}, Becton and Dickinson Company) using CellQuest software (Becton, Dickinson and Company) for cell acquisition and analysis. Acquisition was standardized for 10,000 events per sample. Each test contained a control tube in which cells were incubated with isotopic control antibodies labeled with respective fluorochromes.

Harvesting mononuclear cells from peripheral blood

PBMCs were obtained by the Histopaque®(Sigma-Aldrich, St. Louis, MO, EUA)gradient separation method. The layer rich in lymphocytes and monocytes was aseptically removed and washed with RPMI-1640 (Sigma-Aldrich, St. Louis, MO, EUA)for 15 minutes at 450 g. The cells were then resuspended in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, EUA)supplemented with 2 mM L-glutamine, 40 μg/ml gentamicyn and 10% fetal bovine serum. Cell identification and viability analysis were performed by Turk and Tripan Blue dye, respectively. A $1x10^6$/ml cell concentration was then prepared for the described protocols.

Cell stimulation

The PBMCs (1×10^6 cells/ml) were added to 24-well plates in complete medium in the absence or presence of the TLR ligands according to the manufacturers recommendations (Invivogen; San Diego, CA): 5 μg/mlof peptydoglycan(PGN) from*Staphylococcus aureus*(TLR2 agonist) and 1 μg/ml of lipopolysaccharide (LPS) ultrapurified from *Escherichia coli* K12 (TLR4 agonist). PBMCs were incubated with agonists at 37 ºC in a 5% CO_2 environment for 24 hours. After incubation, supernatants were aspirated and aliquots were stored at – 80 ºC until analysis.

Cytokine production

The levels of the cytokines TNF-α, IFN-γ, IL-17, IL-10 and TGF-β were measured in the culturesupernatants by the CBA technique and analyzed using flow cytometryFACSCalibur^{TM} (Becton, Dickinson and Company) using CellQuest software (Becton, Dickinson and Company) according to the manufacturer's instructions.

NO production

NO production in the culture supernatants of PBMCs was performed by a commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Statical analysis

Non-continuous variables were analyzed by Fisher's exact test. Friedman test was used for analysis of asymmetric data between groups dependents. Nonparametric data between two dependents groups were analyzed by Wilcoxon test and between two groups independents by Mann-Whitney. Negative binomial regression was applied to count data with extra variation, since this type of distribution can estimate the variance of the Poisson regression cannot identify. After applied the negative binomial regression, the dependent variables were calculated by repeated measures and independent for comparison of means.All of the analyses were made using the Statistical Analysis System (SAS) software, v.9.3. The significance level was fixed at 5% or the correspondent p-value.

RESULTS

TLR2 and TLR4 expression on monocytes and lymphocytes

The expression and co-expression of TLR2 and TLR4 on monocyte and lymphocyte cell surfaces was evaluated by measuring the percentage of $CD3^+$and $CD14^+$cells positive for TLR2 and TLR4in the patients pre-treatment and post-treatment. Surface expression of TLR2 and TLR4 on lymphocytes showed that pre-treatment patients had significantly higher percentage of CD3+ cells expressing TLR2(23.83±20.91) and TLR4 (4.32±6.16) compared to post-treatment(4.59±4.45; 1,03±1,58), respectively ($p<0.05$). Percentage of CD3+ cells co-expressing these receptors was also significantly higher in patients pre-treatment (3.03±3.86) compared to post-treatment (0.76±1.69). Patients with active VL also showed higher percentages ($p<0.05$) of CD3+ cells expressing TLR2, TLR4 and co-expressing TLR2/TLR4 when compared to controls (0.62±0.86, 1.35±2.56 and 0.16±0.13 respectively), and even after treatment, patients also showed higher expression of TLR2 (4.59±4.45) and co-expression TLR2/TLR4 (0.76±1.69) than controls. No statistical differences were detected in the expression of TLR4 in the post-treatment in relation to controls (Figure 1).

Surface expression of TLR2 and TLR4 on monocytes showed that patients with active VL had lower percentage of CD14+ cells expressing TLR2 (90.93±10.73), TLR4 (29.37±29.79) and co-expressing TLR2/TLR4 (28.93±28.8) compared to controls subjects (98.75±1.52, 62.79±32.94 and 60.78±33.68), respectively. After treatment, patients still had lower percentages of CD14+ cells expressing TLR4 (22.6±8.94) and co-expressing TLR2/TLR4 (21.71±8.91) than controls. No significant differences in CD14+ cells expressing TLR2, TLR4 and co-expressing TLR2/TLR4 between pre- and post-treatment (Figure 1).

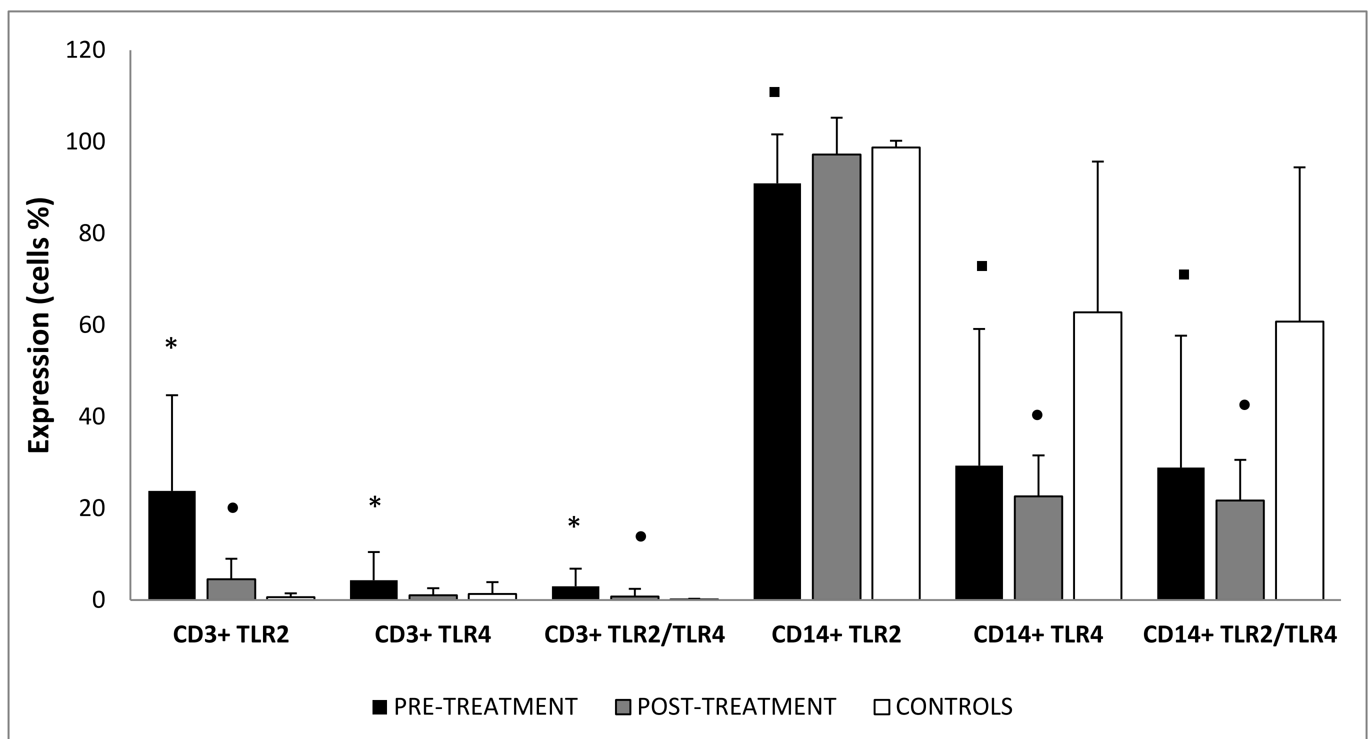

Figure 1.Mean percentages of $CD3^+$ and $CD14^+$ cells expressing TLR-2, TLR-4 and co-expressing TLR2 and TLR4 in whole blood from patients with visceral leishmaniasis pre-treatment, post-treatment and control subjects. Gating strategies to distinguish between cells populations analyzed by flow cytometry. FSC-SSC profile was used to distinguish total lymphocytes and monocytes. T lymphocytes and monocytes were gated according to light scatter profile and the expression of CD3 and CD14. The results are expressed as the mean and standard deviation and are representative of three independents replicates. *$p<0.05$ compared pre-treatment x post-treatment and controls; ▪ $p<0.05$ compared pre-treatment x controls and • $p<0.05$ compared post-treatment x controls.

In relation to the mean of fluorescence intensity, no significant differences were detected in TLR2 and TLR4 expression in CD3+ cells between pre-treatment patients compared to post-treatment patients and controls(Figure 2A and2B). However, after treatment, the fluorescence of TLR4 in CD3+ cells (203.67 ± 150.77) was higher when compared to controls subjects (86.61 ± 77.03) (Figure 2B). Regarding of CD14+ cells, pre-treatment patients showed increased of fluorescence intensity of TLR2(483 ± 619) and TLR4 (311.5 ± 575.25) significantly higher than post-treatment patients (94.06 ± 46.19 e 111.43 ± 60.33) and controls subjects (53.28 ± 11.85 and 59.02 ± 22.28) (Figures2C e 2D). After treatment, fluorescence of TLR2 (94.06 ± 46.19) and TLR4 (111.43 ± 60.33) in CD14+ cellswas even higher when compared to controls (Figures 2B, 2C e 2D).

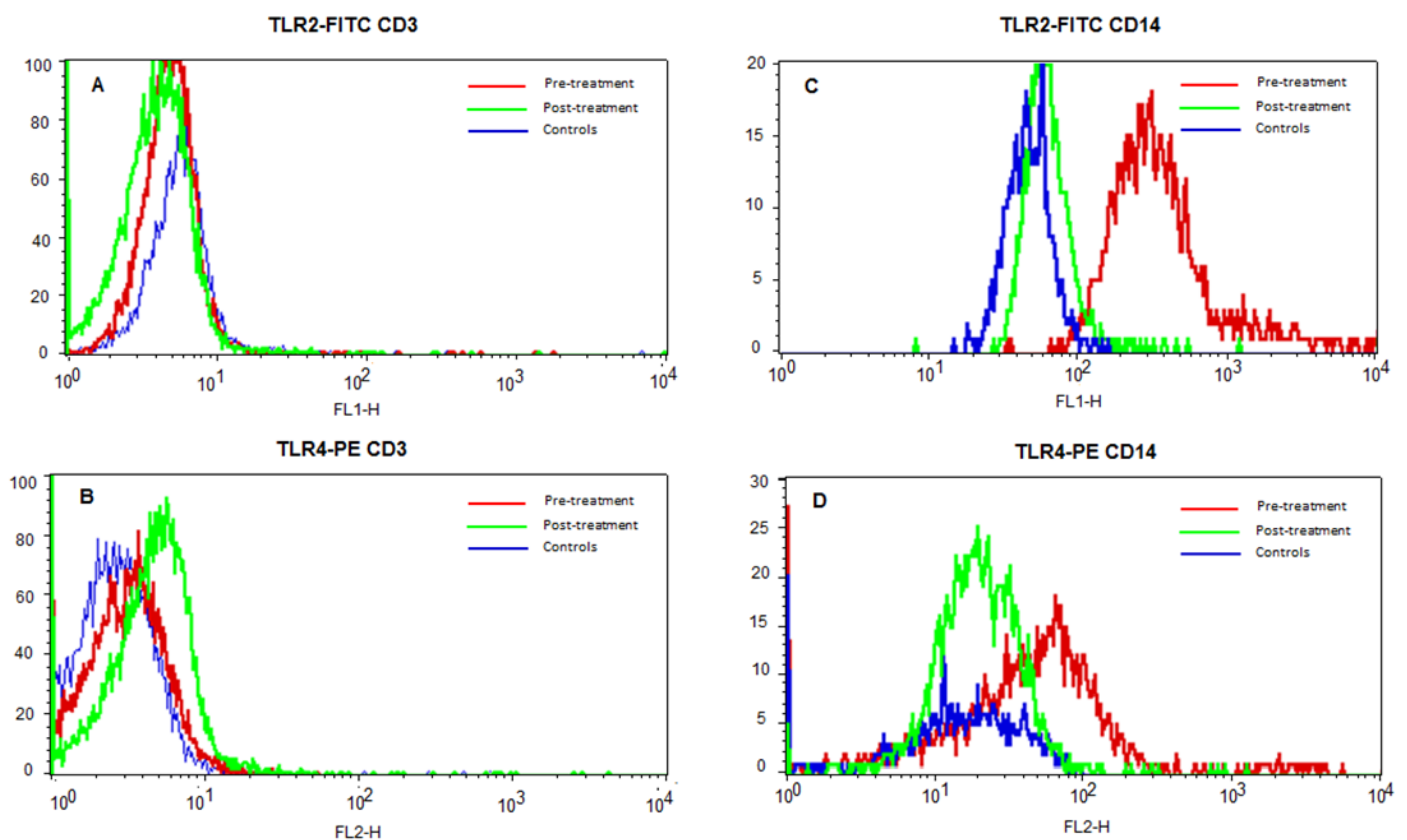

Figure 2.Representative histograms plots of TLR2 and TLR4 expression in CD3+ and CD14+ cells are shown in figures A - D, being red histograms represents the pre-treatment patients, green histograms represents the post-treatment patients and blue histograms represents the controls subjects. The expression of TLR2 and TLR4 in T lymphocytes (A, B) and monocytes (C, D), respectively.

Production of cytokines in patients with VL

The production of cytokines by PBMCs in culture supernatant of VL patients before and after treatment was observed. In relation to TNF-α (Fig. 3A), patients with active LV produced significantly higher levels (p= 0.02) compared to post-treatment patients. No significant differences were found in TNF-α production between pre-treatment and post-treatment patients compared to control subjects. As for IFN-γ (Figure 3B) and IL-17 (Figure 3C), patients with active LV produced significantly lower levels (p <0.001) compared to post-treatment patients and controls. No significant differences were observed in IFN-γ and IL-17 production between post-treatment and control subjects.

Regarding the production of IL-10 (Figure 4A) patients pre-treatment produced significantly higher levels (p<0.001) when compared to patients after treatment and control individuals. No significant differences were detected in IL-10 production between post-treatment patients and controls. In relation to TGF-β (Figure 4B), no difference was observed in the levels of this cytokine between patients before and after treatment. However, pre- and post-treatment patients produced significantly higher levels (p<0.001 and p=0.01, respectively) of TGF-β compared to control subjects.

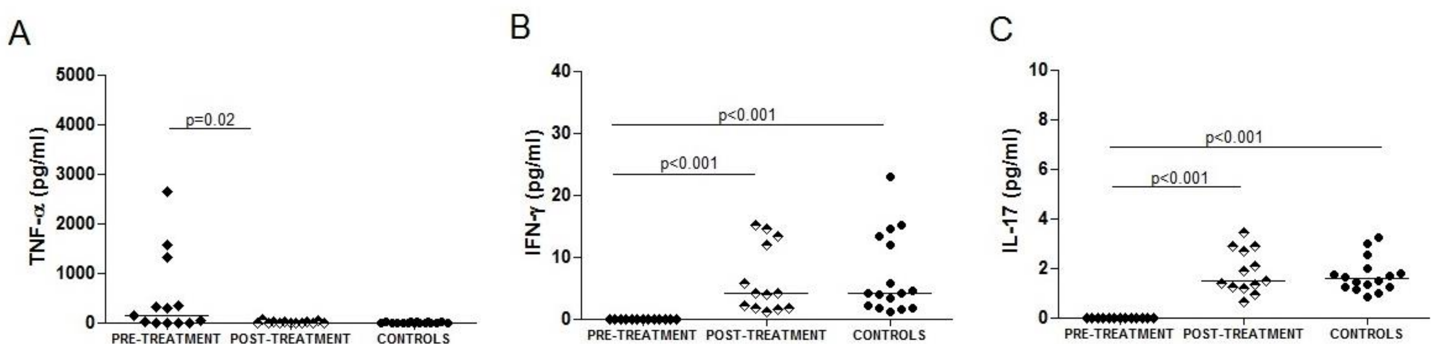

Figure 3. Levels of TNF-α (A) IFN-γ (B) and IL-17 in culture supernatants of PBMCs (1x10^{6} cells/ml) from patients with VL before and after treatment. The cytokine levels were measured by CBA. Each dot represents a different patient and each bar represents the median. Data are representative of three independents replicates.

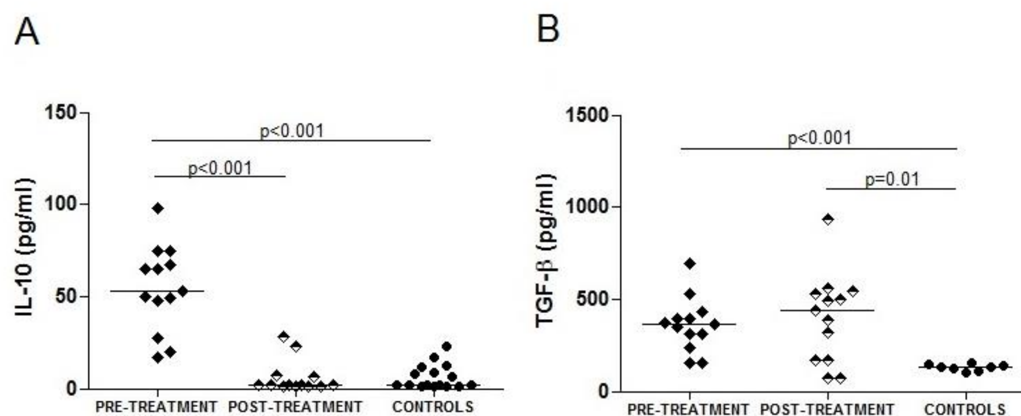

Figure 4.Levels of IL-10 (A) and TGF-β (B) in culture supernatants of PBMCs ($1x10^6$ cells/ml) from patients with VL before and after treatment. The cytokine levels were measured by CBA. Each dot represents a different patient and each bar represents the median. Data are representative of three independents replicates.

Role of TLR2 and TLR4 in cytokine production

Other purpose of this study was to evaluate the role of TLRs in Th1, Th2 and Th17 cytokine production through stimulation with TLR2 (PGN) and TLR4 (LPS) agonists. Regarding TNF-α, unstimulated PBMCs produced significantly lower levels ($p<0.05$) compared to cells stimulated with LPS, PGN and LPS+PGN in patients pre-treatment, post-treatment and controls subjects (Figure 5A).

Regarding the production of IFN-γ, no significant differences were found between PBMCs stimulated or not with agonists in patients with active VL. However, unstimulated PBMCs of patients after treatment and controls subjects produced significantly lower levels ($p<0.05$) this cytokine compared to cells stimulated with LPS, PGN and LPS+PGN (Figure 5B).

The results concerning to IL-17 (Figure 5C), no significant differences were detected in the production of IL-17 in cells stimulated or not with agonists in pre-treatment patients. In patients after treatment, PBMCs unstimulated and stimulated with
PGN produced significantly lower levels of IL-17 ($p<0.05$) compared to PBMCs stimulated with LPS and LPS+PGN. PBMCs from controls subjects stimulated with LPS, PGN and LPS+PGN produced higher amounts of this cytokine compared to cells unstimulated.

PBMCs unstimulated and stimulated by LPS in pre-treatment patients showed lower levels of IL-10 ($p<0.05$) than the cells stimulated with PGN and LPS+PGN. In post-

treatment patients and controls, PBMCs significantly increased ($p<0.05$) IL-10 production when stimulated with all agonists. Additionally, in controls subjects, stimulation with LPS+PGN produced larger amounts ($p<0.05$) compared to stimulation with LPS (Figure 6A).

Regarding TGF-β (Figure 6B) PBMCs stimulated or not with agonists showed no significant differences in production of this cytokine in patients pre- and post-treatment. However, in healthy subjects, stimulation with LPS and LPS+PGN increased production of TGF-β compared to unstimulated cells.

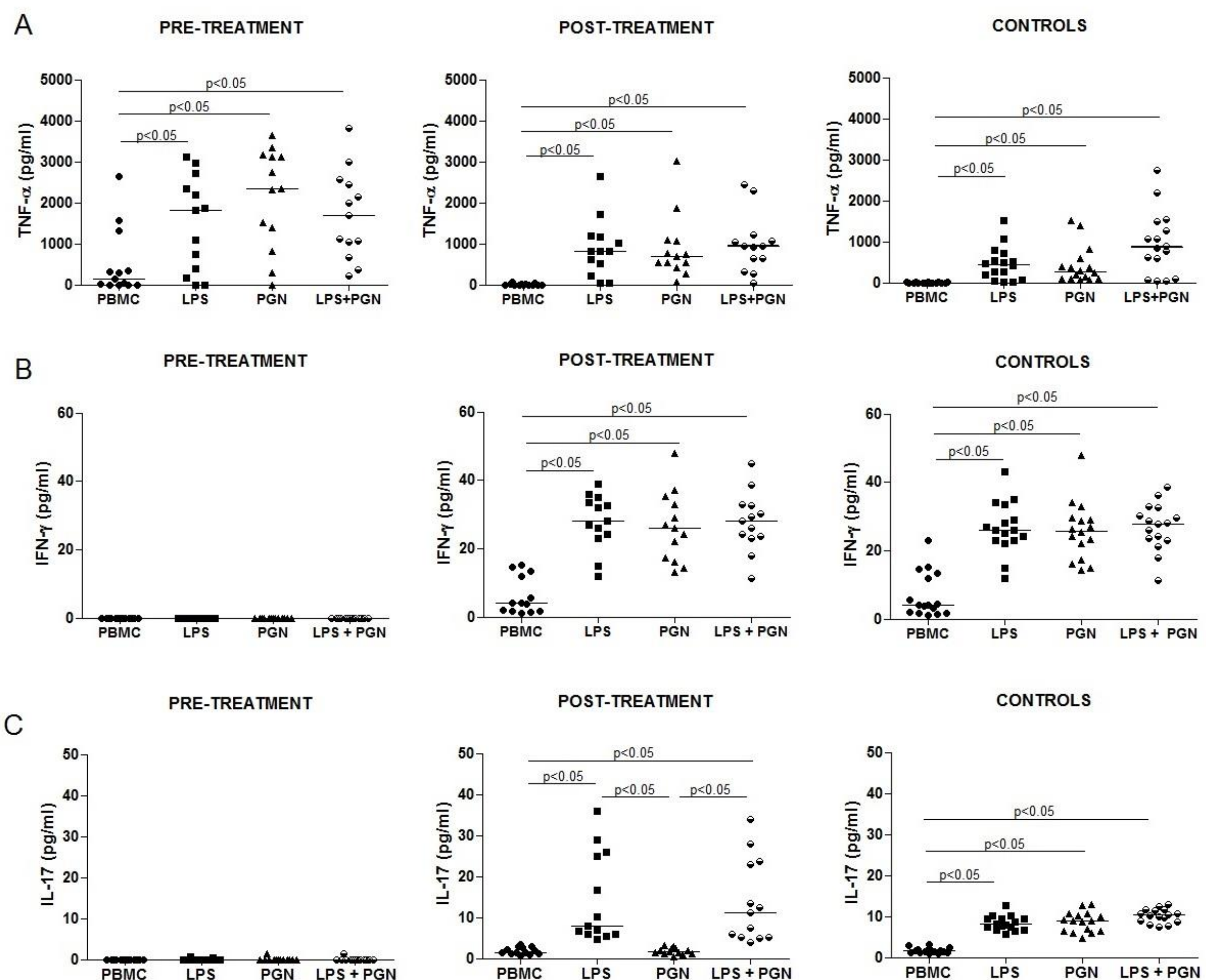

Figure 5.Levels of TNF-α (A), IFN-γ (B) and IL-17 (C) in culture supernatants of PBMCs ($1x10^6$ cells/ml) stimulated or not with PGN (5μg/ml) and LPS (1 μg/ml) for 24 hours in patients with VL before and after treatment. The cytokines levels were measured by CBA. Each point represents a patient and the bar represents the median. Data are representative of three independents replicates.

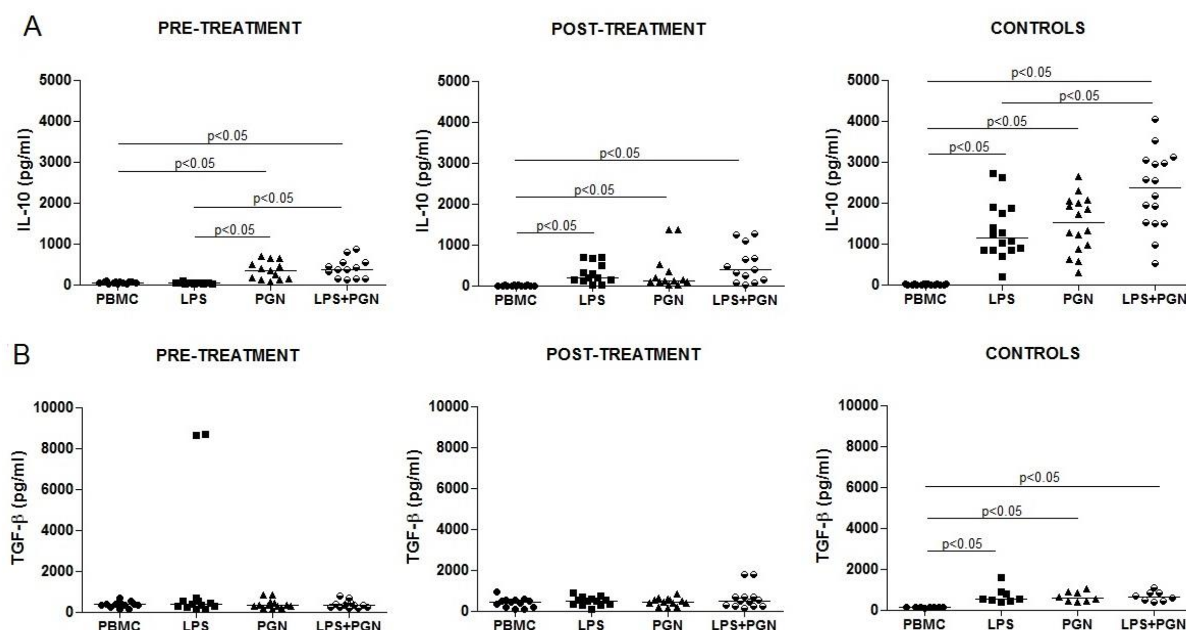

Figure 6.Levels of IL-10 (A) and TGF-β (B) in culture supernatants of PBMCs ($1x10^6$ cells/ml) stimulated or not with PGN (5µg/ml) and LPS (1 µg/ml) for 24 hours in patients with VL before and after treatment. The cytokines levels were measured by CBA. Each point represents a patient and the bar represents the median. Data are representative of three independents replicates.

Role of TLR2 and TLR4 in NO production

NO production was measured in culture supernatants of PBMCs from patients with LV before and after treatment. Our results show that cells of pre-treatment patients produce significantly lower levels ($p=0.04$) of NO compared to post-treatment patients. No significant differences were observed in NO production by PBMCs from patients pre- and post-treatment and controls subjects (Figure 7).

The involvement of TLRs in NO production was verified by stimulation with TLR2 (PGN) and TLR4 (LPS) agonists. It was found that stimulation with LPS, PGN and LPS+PGN significantly increased ($p<0.05$) levels of NO compared to cells unstimulated in pre- and post-treatment patients and controls (Figure 8).

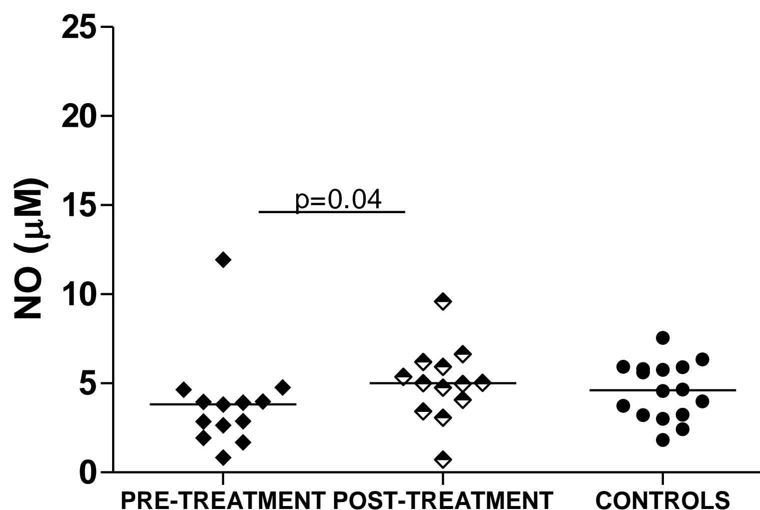

Figure 7.Levels of NO in culture supernatants of PBMCs (1x10^6 cells/ml) from patients with VL before and after treatment. The NO levels were measured by commercial kit according to the manufacturer's instructions. Each dot represents a different patient and each bar represents the median. The data are representative of three independents replicates.

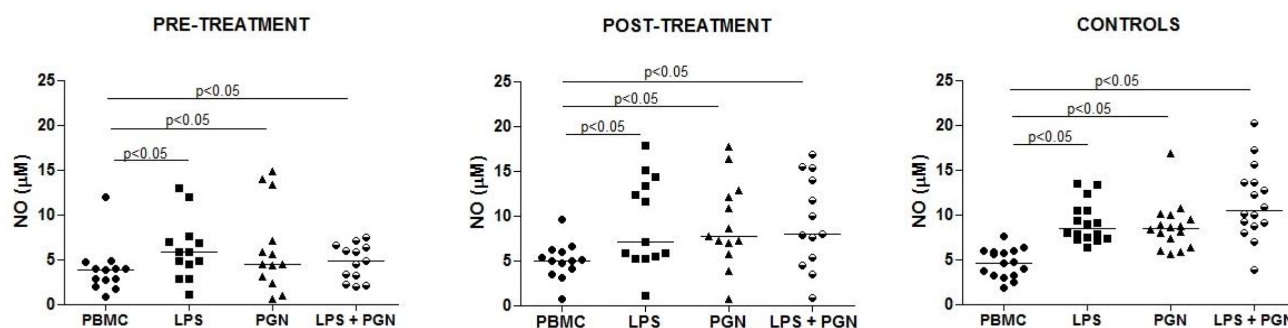

Figure 8.Levels of NO in culture supernatants of PBMCs (1x10^6 cells/ml) stimulated or not with PGN (5μg/ml) and LPS (1 μg/ml) for 24 hours in patients with VL before and after treatment. The cytokines levels were measured by commercial kit according to the manufacturer's instructions. Each point represents a patient and the bar represents the median. Data are representative of three independents replicates.

DISCUSSION

As we know, this is the first study that evaluates the role of TLR2 and TLR4 receptors in the cytokines and NO production of VL patients before and after treatment with leishmanicidal drugs. In cells labeled with anti-CD3 and anti-CD14, both lymphocytes and monocytes populations from pre- and post-treatment presented TLR2 and TLR4 expression. These results are in agreement with those reported by other studies [13,14,16] demonstrating that TLR2 and TLR4 are involved in *Leishmania*recognition. Patients in pre-treatment showed higher percentage of CD3+ T cells expressing TLR4 and mainly TLR2 compared to post-treatment and controls. After treatment, although patients have expressed a lower percentage of TLR2 and co-expression of TLR2/TLR4, these values were still higher than the control group. In addition, was observed a decrease of T CD3+ cells expressing TLR4 in treated group, however the mean of fluorescence intensity was higher than the controls, indicating a decrease in the number of cells expressing this receptor, TLR4 expression was increased on these cells. These results agree with others studies that showed increased expression of TLR mRNA in CD4+ and CD8+ lymphocytes of patients with pleural and pulmonar tuberculosis [27-29]. TLRs play an important role in the immune response, and their ligands on lymphocytes can modulate the immune function of these cells. TLRs expressed on lymphocytes may act as costimulatory receptors for the TCR with increased proliferation and/or cytokine production [30].

The ligands of TLRs in macrophages have different effects such as to induce the cytokine production and the expression of MHC II molecules [31]. Experimental study with *L. donovani* showed that macrophages express TLR2, which is responsible for the NO and TNF-α production [14]. The TLR4 is required for the control of infection with *Leishmania* because in mice $TLR4^{-/-}$ had promoted the parasites persistence in macrophages [32]. Regarding expression of TLR2 and TLR4 on monocytes, we showed that patients with active VL presented the lower percentage of cells expressing TLR2 and TLR4 compared to controls. However, the mean fluorescence intensity of these receptors on monocytes was increased in patients pre- and post-treatment compared to controls, indicating that few cells are able to express large quantities of these receptors. After treatment of patients, the TLR2 expression on monocytes was similar to controls, although the fluorescence intensity was higher in those treated. Our results indicate a possible role of TLR2 and TLR4 in the mechanisms of pathogenicity or parasite control, due its involvement in the induction or inhibition of pro- and anti-inflammatory mediators in patients before or after treatment with leishmanicidal drugs.

We hypothesized that occurred modulation of cytokines and NO in patients before and after treatment. The mechanism responsible for cytokine and NO production probably involves TLRs. Parasite components can lead to an activation of TLR2 and TLR4 receptors, resulting in the cytokines production [10]. Our results show that patients with active VL produce high levels of TNF-α, values that decrease after treatment, which is in agreement with some studies [33-35] and unlike others, that have not showed difference in TNF-α production between pre-treated patients and control subjects [36-38]. Furthermore, our results show that this cytokine production in those treated and untreated is related to the TLR2 and TLR4 expression. Similarly, hepatitis C patients showed an increased of TNF-α production in vitro when PBMCs were stimulated with receptors agonists [39]. In *Leishmania* infection, TLR2 and TLR4 receptors are involved in the production of TNF-α contribuing to the cure of disease [14,40].

In active LV, TNF-α is involved in host protection, and it is produced by activated macrophages and CD4+ Th1 lymphocytes, acting synergistically with IFN-γ to activate microbicidal functions of macrophages and thus, eliminate the parasites [18,21,41,42]. On the other hand, excess of TNF-α can be deleterious to the host, causing tissue injury, inflammation and some symptoms [43,44]. Furthermore, it has been considered as a marker of LV cure [45]. In our study, despite the high value of TNF-α found in patients with active LV, we observed that this cytokine was not protective at this phase and could be involved in the pathogenesis of disease. Because the treatment reduces TNF-α levels, we suggest that at lower levels, this cytokine could be involved in host protection through the stimulation of parasite destruction mechanisms via the receptors TLR2 e TLR4.

IFN-γ plays an essential role in leishmanicidal activity mediated by macrophages, whose act in parasite clearance and consequently to the resolution of infection [20,21,46]. Our study showed that PBMCs from pre-treated patients had lower production of IFN-γ compared to post-treatment and controls, and after the treatment these levels significantly increased. Children from endemic areas that had low production of IFN-γ, progressed to the disease [47]. These results agree with other studies that associated the low production of IFN-γ with a high parasite load [48]. Moreover, children infected with *L. chagasi* were able to control the infection when presented high production of IFN-γ [47]. Contrary to our results, Duthie et al. [49] found that patients with active VL had high levels of this cytokine, with a decrease of them after the treatment.

When we evaluated the involvement of TLR2 and TLR4 relative to the production of IFN-γ, the low production of this cytokine was not related to TLR2 and TLR4 receptors in PBMCs of pre-treated patients, suggesting the involvement of other receptors in this IFN-

γ production regarding the active LV.However, when the cells of patients after treatment were stimulated with LPS and PGN, the IFN-γ levels were significantly increased, demonstrating an association between IFN-γ production and TLR2 and 4 expression. One study about VL patients treated with miltefosine showed increased TLR4 expression in PBMCs and a consequent production of IFN-γ and iNOS [40]. Another study showed high levels of IFN-γ and TNF-α following the increased expression of TLR-2 mRNA [12]. Our results suggest that the increasedlevels of IFN-γ after treatment seem to have been sufficient to activate a protective response involving TLR2 and TLR4 receptors.

In our study, patients with active LV presented lower IL-17 production compared with those controls and in post-treatment. Th17 cells are involved in the development of inflammatory and autoimmune diseases, and are also involved in the protection against some intracellular pathogens [50-52], including *leishmania* [26]. However, the exact role of Th17 cells in individuals with leismaniasis, mainly during anti-leishmanicidal treatment, is not very clear. In experimental infection with *L. major*, high levels of this cytokine were associated with tissue destruction and inflammation [24]. In patients infected with *L. braziliensis,* cells present in the lesions produced high amounts of IL-17 which were associated with the pathogenesis of disease [23]. On the other hand, our data showed that high levels of this cytokine were obtained after treatment, probable decreasing the parasite load and suggesting that IL-17 is involved in infection control. According to our results, also a previous experimental study showed that the treatment with a polysaccharide that has immunomodulatory properties was able to induce the production of cytokines such as IL-17 and IL-23, which are correlated with the decrease in parasite load [53].

Our results showed that low IL-17 production in the pre-treated group was not related to TLR2 and TLR4 receptors, however after treatment, we observed an association between TLR-4 and IL-17, due the PBMC stimulation with LPS that increased this cytokine production. This is the first study showing an association of human IL-17 in the evolution of VL infection. In agreement with our study, an experimental model using *L. chagasi* also showed this IL-17/TLR-4 correlation [16]. Furthermore, it was demonstrated in other infections and comorbidities the interaction between the TLR-4 and IL-17 [54,55].After all, our findings showed a low IL-17 levels in patients without treatment, but usually these values tended to be increased after the treatment and pathogen killing [53]. Taken together, we suggest that a reduced Th17 response could be associated with the clinical manifestation of leishmaniasis and this cell subtype might be involved in protection, rather than disease pathogenesis.

NO is produced during active leishmaniasis and has the role of holding the parasites and prevent disease [56-59]. However, according to our results, NO production in patients pre-treated was low, which increased after treatment. Consistent with this, Kumar et al [60] found that monocytes from LV patients before treatment produced low levels of NO, but in the end of the treatment, levels of this cytokine were increased. Furthermore, large amounts of NO were reported in the supernatants of murine macrophages infected with *L. chagasi* and stimulated with IFN-γ; these results reflect the importance of NO in leishmanicidal activity [61]. Thus, we suggest that in the pre-treatment, the parasite activate some suppressor mechanisms through TLR receptors, so inhibiting NO production. After the treatment there was an increase in IFN-γ levels, occurring also increased levels of NO via TLR2 and TLR4 receptors. Other studies have shown that NO production is involving with the interaction with TLR2 and TLR4 receptors [14,62].

In leishmaniasis, similar to other infections, IL-10 and TGF-β have been identified as important cytokines involved in homeostatic mechanisms and limiting tissue damage caused by extensive inflammation [63-67]. Nevertheless, these cytokines also favor the pathogen persistence, disabling macrophages functions and suppressing the production of IL-12, IFN-γ, TNF-α e NO [68-74]. In visceral leishmaniasis, IL-10 has been detected in the serum, lymph nodes and the spleen [75-78]. Our results show high levels of IL-10 in patients with active VL, which is in agreement with other studies [78,79]. However our results suggest that, at the pre-treatment phase, IL-10 levels were associated with TLR2 expression and similarly, study performed by Chandra et al [80] showed that monocytes humans infected with *L. donovani* were able to modulate the immune response through TLR-2 and IL-10. In active LV, Although TLR4 seems not have participated in IL-10 production, macrophages infected with *L. amazonensis* and *L. major* increased this cytokine production when were stimulated with LPS [81].In pre-treatment, lower levels of IFN-γ and IL-17 were produced and after treatment, the decreased levels of IL-10 were associated with the TLR2 and TLR4 expression and increased levels of IFN, IL-17 and NO. This phenomenon suggested that IL-10 in pre-treatment played a role regulatory, inhibiting a protective response.

To TGF-β, studies showed its involvement together to IL-6, in the IL-17 induction, which may also be related to the suppression of protective mechanisms [82-84]. In our study, high TGF-β levels were detected at the pre- and post-treatment and they are associated with low levels of IFN-γ, IL-17 and NO in pre-treated patients and high levels of these cytokines and NO in the post-treatment. According to our results, we suggest that TGF-β production was associated with immunosuppressive mechanisms in the active

disease, and after treatment it's contributed to the increased of IL-17. BALB/c mice infected with *L. chagasi* exhibit impaired Th1 immune response due to increased production of TGF-β by CD4 + T cells [84]. Furthermore, our study showed that the production of TGF-β did not involve TLR2 and TLR4, suggesting that other receptors may have acted in this production.According to Bhattacharyya et al [85], TGF-β production was associated with TLR4 receptor.

CONCLUSIONS

Finally, our study showed in active LV a low production of IFN-γ, IL-17 and NO and an increased in TNF-α, IL-10 and TGF-β levels. After treatment, we observed infection control and levels of IFN-γ, IL-17, TNF-α, IL-10 and NO close to normal levels. In addition, our results indicated in pre-treatment the involvement of TLR2 and TLR4 receptors in the production of TNF-α and NO, and of TLR2 receptor in the production of IL-10. After treatment, are involved TLR2 and TLR4 receptors in the production of TNF-α, IFN-γ, IL-10, NO and TLR4 receptor in the production of IL-17. These data suggest that parasites interacted with TLR2 e TLR4, leading to modulation of cytokines and NO in both groups of LV patients. Furthermore, we suggest that probably the TLR2 and TLR4 receptors acted mainly on immunopathogenesis of active LV and, with the treatment, they participated in the induction of protective mechanisms.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Capítulo III

Lipoproteínas e resposta imune inflamatória em pacientes com leishmaniose visceral

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RESUMO

A leishmaniose visceral (LV) é causada por protozoários que pertencem ao complexo *Leishmania donovani*. É uma doença letal se não tratada, sendo responsável por 500.000 novos casos anualmente. Alterações nas lipoproteínas como HDL, LDL, colesterol e triglicerídeos têm sido relatadas na LV e parecem influenciar a resposta imune do hospedeiro. A resposta imune contra LV é ativada quando receptores Toll-like (TLRs) entram em contato com o parasita e ativam a produção de mecanismos efetores como TNF-α e IFN-γ e produção de NO. Em relação a IL-17 os resultados ainda são controversos e mostram que pode participar tanto da resposta inflamatória protetora como pode estar relacionada com gravidade da doença. No entanto, ainda não é completamente entendida na LV a relação entre lipoproteínas e resposta imune, principalmente no pré e pós-tratamento de pacientes. Desta forma nosso objetivo foi avaliar em pacientes com LV pré e pós-tratamento a relação entre lipoproteínas, TLRs, citocinas inflamatórias e NO. Foram avaliados 13 pacientes com LV antes e após o tratamento e 16 indivíduos controles. Níveis séricos de LDL, HDL, colesterol total e triglicerídeo foram avaliados por testes bioquímicos. A expressão de TLR2 e TLR4 em monócitos e linfócitos foi realizada através de citometria de fluxo. Sobrenadantes de PBMCs foram avaliadas quanto à produção de TNF-α, IFN-γ, IL-17 e NO. Pacientes pré-tratamento apresentaram menores níveis de HDL, LDL e colesterol total e aumento de triglicerídeos. Também foi relatada maior porcentagem de linfócitos expressando TLR2 e TLR4 e maior expressão desses receptores por monócitos. Níveis de TNF-α foram elevados e de IFN-γ e IL-17 diminuídos. Após o tratamento houve aumento de HDL, LDL e colesterol total e diminuição de triglicerídeos. Níveis de LDL se igualaram com os dos controles. Monócitos e linfócitos continuaram expressando TLR2 e TLR4. Produção por PBMCs de TNF-α diminuiu e de IL-17 e IFN-γ aumentou, chegando aos valores normais. Os resultados mostram que houve associação das lipoproteínas (colesterol, HDL e LDL) com os receptores TLR (no pós-tratamento) e com IFN-γ, IL-17 e NO (no pré e pós-tratamento). Esses resultados sugerem o possível envolvimento das lipoproteínas na resposta imune protetora auxiliando na eliminação da infecção e cura dos pacientes.

Artigo a ser enviado para publicação

INTRODUÇÃO

A leishmaniose visceral (LV) é causada por protozoários intracelulares obrigatórios pertencentes ao complexo *Leishmania donovani*.[1] É uma das principais doenças negligenciadas no mundo acometendo cerca de 500.000 pessoas por ano, sendo que 90% dos casos ocorrem no Brasil, Bangladesh, Índia, Nepal e Sudão.[2,3]

Tanto a infecção quanto inflamação induz uma resposta de fase aguda com consequentes alterações nos lipídeos e metabolismo de lipoproteínas.[4] As lipoproteínas são partículas compostas por triglicerídeos (TG), colesterol, fosfolipídios e vitaminas lipossolúveis.[5] Na LV já foram observadas alterações lipídicas, tais como hipocolesterolemia, com redução dos níveis de LDL (lipoproteína de baixa densidade), HDL (lipoproteína de alta densidade) e colesterol total (CT).[6] Crianças com LV apresentaram diminuição dos níveis de HDL e aumento de CT e TG.[7] As lipoproteínas plasmáticas parecem ter um papel importante na resposta imune.[8] Os lipids rafts são domínios de membrana formados por colesterol e outros lipídeos.[9,10] Esses domínios são importantes para ativação de linfócitos T, pois neles se localizam as moléculas de MHC-II, importantes para apresentação de antígeno.[11-16] Desse modo, diminuição dos níveis de colesterol podem prejudicar os lipids rafts e consequentemente prejudica a ativação da resposta imune.[17-19] Os TLRs, após serem ativados, se movem para os lipids rafts. Nesse sentido, foi visto que integridade dos lipids rafts é essencial para ativação de resposta imune por LPS.[20]

Interessantemente, alguns estudos tem demonstrado o envolvimento das lipoproteínas como VLDL e LDL na proteção contra alguns vírus como o tagavírus e o rabdonovírus e do colesterol na infecção causada por *Mycobactéria, Plasmodium falciparum, Clamydia trachomatis, Listéria monocytogenes, L. donovani e L. infantum.*[4,21-26] Em estudo realizado com *Schistosoma* foi visto que o LDL se liga à superfície do parasita, ativa monócitos e assim há a produção de reativos do oxigênio. Esses reativos oxidam o LDL (oxLDL) o que permite sua endocitose por monócitos. Remoção de oxLDL expõe o parasita ao ataque de monócitos e outras células.[4,27,28] Além disso, outros estudos têm sugerido o envolvimento das lipoproteínas não só na apresentação de antígenos aos linfócitos como também na produção de citocinas e óxido nítrico (NO).[29,30]

Durante o curso da LV são desencadeados vários mecanismos imunológicos no hospedeiro. A resposta imune mediada por células encontra-se prejudicada na LV ativa, mas esse processo pode ser revertido com o tratamento. [31] Na resposta imune contra LV humana, estudos mostram o envolvimento de um padrão Th1 concomitante com Th2 sendo

que, na doença ativa, o perfil Th1 é suprimido enquanto o Th2 é predominante e, após o tratamento, há restauração do perfil Th1. [32-34] Na infecção com *Leishmania* a produção de citocinas inflamatórias contribui para a defesa do hospedeiro. O TNF-α produzido age em sinergismo com IFN-γ ativando mecanismos efetores e eliminando os parasitas. [35,36] No entanto, excesso de mediadores inflamatórios pode ser deletério para o hospedeiro. [37,38] Atualmente tem sido estudo o envolvimento do padrão de resposta Th17 na LV, embora os resultados ainda sejam bastante controversos. Altos níveis dessa citocina têm sido relacionados com gravidade da doença em camundongos. [39] Entretanto, em pacientes com Kala-zar observou-se associação entre a produção de IL-17 e resistência à doença, sugerindo que essa citocina desenvolve um papel complementar às citocinas de padrão Th1, protegendo contra a LV.[40] Além disso, tem sido demonstrado o envolvimento dos receptores Toll-like (TLR) no desencadeamento e direcionamento da resposta imune na leishmaniose através da produção de mediadores pró e anti-inflamatórios.[41-44] O TLR4 está envolvido no controle da infecção contra *Leishmania* através do aumento da expressão de iNOS e da produção de NO com consequente destruição do parasita. [45] Outros autores mostraram, ainda, que o TLR2 está envolvido no reconhecimento do parasita e na indução da resposta imune. [46]

Embora existam estudos que mostrem a ocorrência de alterações nas lipoproteínas e sua possível modulação na infecção causada pela *Leishmania*, a relação entre perfil lipídico e a resposta imune inflamatória em pacientes principalmente na doença ativa e após o tratamento ainda é pouco explorada e entendida. Neste sentido baseado na ausência destas informações e para o melhor entendimento dos mecanismos envolvidos na patogênese da doença, nosso objetivo foi avaliar em pacientes pré e pós-tratamento a relação entre lipoproteínas, expressão de TLRs, produção de citocinas inflamatórias e NO.

MATERIAIS E MÉTODOS

Pacientes

Foram avaliados 13 pacientes com LV antes e após o tratamento em um intervalo de tempo de 18 meses. Os pacientes eram de ambos os sexos com idade superior à 18 anos. A média de idade foi de 18 a 53 anos, com predominância do sexo masculino (11 indivíduos). Foram excluídos pacientes com outras infecções ou doenças granulomatosas bem como sorologia positiva para HIV e pacientes grávidas. Os pacientes foram arrolados no estudo quando contataram o serviço de saúde para diagnóstico e tratamento da doença. Os pacientes eram provenientes do Hospital Estadual de Bauru, São Paulo e Hospital das

Clínicas de Marília, São Paulo. Foram avaliados 16 indivíduos saudáveis, com idade e sexo pareados com os pacientes. A idade dos controles variou de 21 a 58 anos com predominância do sexo masculino (13 indivíduos).

Diagnostico e tratamento da LV

O diagnóstico da LV foi realizado através da confirmação de parasitas em aspirados de medula óssea. Pacientes foram tratados com Glucantime®, anfotericina B ou anfotericina B liposomal. Dados e amostras foram coletados dos pacientes no hospital antes de iniciarem o tratamento para LV. No período pós-tratamento, que variou de 1 a 3 meses após o final do regime do medicamento, as amostras e dados dos pacientes foram coletados quando os mesmos voltaram para o retorno e acompanhamento no próprio hospital. Todos os pacientes avaliados no pós-tratamento foram considerados curados de acordo com testes sorológicos e clínicos.

Análise bioquímica

Nos pacientes antes e após o tratamento, as análises de colesterol total, HDL, LDL e triglicerídeos foram realizadas no próprio hospital onde estavam admitidos. A análise desses parâmetros faz parte da rotina do hospital para tratamento e acompanhamento da doença. Nos indivíduos controles a análise desses parâmetros foi realizada no Laboratório Clínico da Faculdade de Medicina de Botucatu (Universidade Estadual Paulista – UNESP). Essas determinações foram realizadas usando métodos enzimáticos, colorimétricos e química seca. Para avaliação desses dados foram coletados 5 ml de sangue dos pacientes e indivíduos controles pela manhã, após jejum de 12 horas, sem a prática de atividade física rigorosa nas últimas 24 horas e sem consumo de álcool por 72 horas.

Ética

Todos os pacientes e indivíduos controles foram informados do estudo e assinaram o termo de Consentimento Livre e Esclarecido. Este estudo foi aprovado pelos Comitês de Ética em Pesquisa da Faculdade de Medicina de Botucatu, do Hospital Estadual de Bauru, Hospital das Clínicas de Marília e estão de acordo com a Declaração de Helsinki de 1964.

Coleta de sangue

Para a realização dos parâmetros imunológicos foram coletados 25 ml de sangue heparinizado em dois momentos dos pacientes, antes e após o tratamento, e uma única vez dos indivíduos controles.

Expressão de TLR2 e TLR4 em monócitos e linfócitos

Para avaliar a expressão de TLR2 e TLR4 em monócitos e linfócitos, 100 µl de sangue total dos pacientes e indivíduos controles foram incubados 20 minutos no escuro em tubos falcon (Becton, Dickinson and Company) com anticorpo monoclonal anti-CD3 humano conjugado com PE-DY647 (EXBIO, Vestec, República Tcheca), anticorpo monoclonal anti-CD14 humano conjugado com PE-DY647 (EXBIO, Vestec, República Tcheca), anticorpo anti-TLR4 humano conjugado com FITC (Biolegend, San Diego, CA, EUA) e de anticorpo anti-TLR2 humano conjugado com PE (Biolegend, San Diego, CA, EUA). Após a incubação, as células foram lavadas com 450 µl de solução de lise de hemácias (Becton, Dickinson and Company), incubadas no escuro por 15 minutos, centrifugadas por 5 minutos a 1500 rpm e o sobrenadante foi descartado. Esse procedimento foi realizado duas vezes. Células foram suspendidas em 300 µl de solução eletrolítica ISOTON II (Becton, Dickinson e Company). Para cada teste havia um tubo controle no qual as células foram incubadas com anticorpos controles isotípicos marcados com os respectivos fluorocromos dos testes: PE mouse IgG2a, κ isotype Ctrl FC (Biologend, San Diego, CA, EUA), FITC mouse IgG2a, κ isotype Ctrl FC) e conjugado com PE-DY647 (EXBIO, Vestec, República Tcheca). Os tubos foram analisados em citômetro de fluxo modelo FACSCALIBUR ™ (Becton, Dickinson and Company) usando o programa "Cell Quest" (Becton, Dickinson and Company) para aquisição e análise celular.

Obtenção e cultura de células mononucleares totais do sangue periférico

As células mononucleares do sangue periférico (PBMCs) dos pacientes e controles foram obtidas por meio da separação em gradiente de Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO, EUA). O anel rico em linfócitos e monócitos foi lavado, inicialmente, com meio de cultura RPMI 1640 (Sigma-Aldrich, St. Louis, MO, EUA) e centrifugados por 15 minutos a 1500 rpm. Após esse período, as células foram suspensas com meio de cultura RPMI suplementado com 2 mM de L-glutamina, 40 ug/ml de gentamicina (Sigma-Aldrich, St. Louis, MO, EUA) e 10% de soro bovino fetal. A identificação e viabilidade das células foram realizadas através de contagem com Turk e azul de Tripan (alíquotas de 50 µl da suspensão celular com 50µl da solução do corante a 5% foram incubadas a 37°C durante 10 minutos). A seguir, a concentração celular foi acertada para $1x10^6$ células/ml. As células mononucleares totais ($1x10^6$/ml), foram incubadas a 37 °C, em tensão de 5% de CO_2 por 24 horas. Após esse período, os sobrenadantes foram aspirados, sendo que as alíquotas deste

material foram conservadas a -80 ^{o}C até o momento de sua utilização para dosagem das citocinas e NO.

Dosagem das citocinas e NO

As citocinas TNF-α, IFN-γ, IL-17 e NO foram dosados nos sobrenadantes de cultura de PBMCs. Dosagem de citocinas foi realizada através da técnica de CBA Flex (Becton, Dickinson e Company) e dosagem do NO através de kit comercial (Cayman Chemical Company, Ann Arbor, MI, USA), ambos de acordo com as indicações do fabricante.

Análise estatística

Varívaies não contínuas foram analisadas por teste exato de Fisher. Comparações para varíaveis contínuas do perfil nutricional foi usado análise de variância (ANOVA) com teste de medidas repetidas entre pré e pós-tratamento, e ajustados com teste de Tukey se a distribuição fosse normal. O mesmo desenho foi usado para ajustar modelo linear com distribuição gamma, seguido por teste de múltiplas comparações. Comparações entre pré e pós-tratamento com o grupo controle foram feitas usando teste t de Student. Para análise da expressão de TLRs foi usada regressão binomial negativa foi aplicada para analisar dados com extra variação. Após aplicada a regressão binomial, variáveis dependentes foram calculadas por medidas repetidas e independentes por comparação de médias. Para dosagem das citocinas foi usado Teste Friedman para análise de dados assimétricos entre grupos dependentes. Dados não paramétricos entre dois grupos dependentes foram analisados por teste de Wilconxon e entre dois grupos independentes por teste de Mann-Whitney. Todas as análises foram realizadas usando software SAS (Statitical Analysis System) versão 9.3. Níveis significantes foram fixados a 5% ou valor-p correspondente.

RESULTADOS

Avaliação bioquímica em pacientes com LV antes e após o tratamento

Dos pacientes incluídos no estudo observou-se a predominância do sexo masculino, com 84.7% antes do tratamento e 81.9% após o tratamento. Não foi observada diferença significativa na média de idade entre pacientes (36.3±11.59) e indivíduos controles (35.97±10.96).

Em pacientes com LV ativa, os níveis de colesterol total (105.1 ± 31.36), HDL (8.6 ± 8.88) e LDL (55.3 ± 24.95) apresentaram níveis significativamente menores ($p<0.05$) em

relação ao pós-tratamento (164.0 ± 24.83; 39 ± 7.89; 93.6 ± 25.0) e indivíduos controles (199.4 ± 43.85; 55.4 ± 19.4; 117.9 ± 37.7) respectivamente. No entanto, com exceção do LDL, pacientes tratados ainda mostraram níveis menores ($p<0.05$) dessas variáveis quando comparados com os controles. Quanto aos triglicerídeos, pacientes com LV ativa (216.1 ± 57.93) apresentaram níveis significativamente maiores ($p<0.05$) em relação ao pós-tratamento (158.8 ± 72.2) e controles (87.0 ± 14.26). Após o tratamento, esses níveis ainda permaneceram significativamente aumentados ($p<0.05$) em relação aos controles (Figura 1).

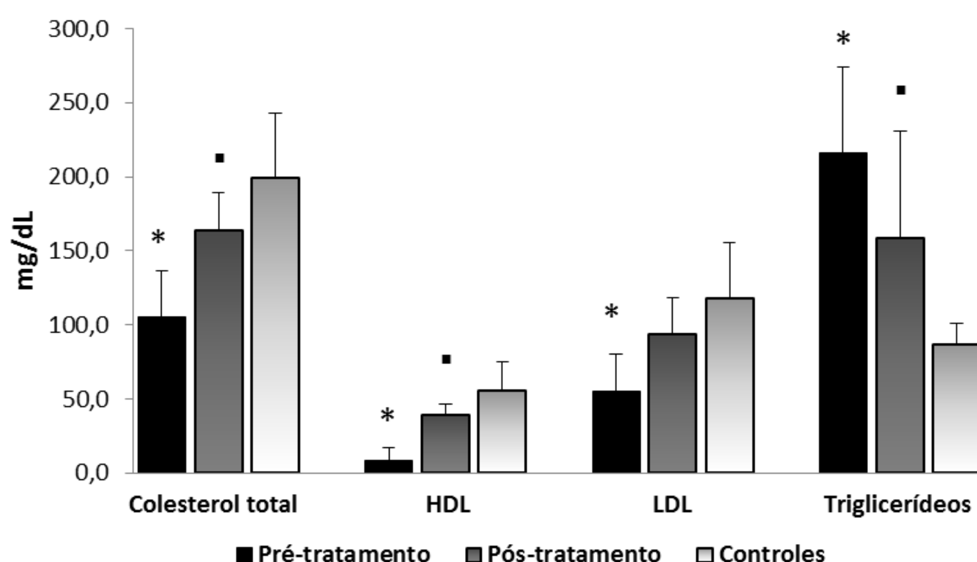

Figura 1. Níveis séricos de colesterol total, HDL, LDL, triglicerídeos e glicose em pacientes pré-tratamento, pós-tratamento e indivíduos controles. Resultados são expressos em média ± DP. * $p<0,05$ pré-tratamento x pós-tratamento e controles; ▪ pós-tratamento x controles.

Expressão de TLR2 e TLR4 em células CD3+ e CD14+

Os resultados referentes à expressão dos receptores TLR2 e TLR4 em células T CD3+ T mostraram que pacientes pré-tratamento apresentaram porcentagem significativamente maior de células CD3+ expressando TLR2 (23.83 ± 20.91) e TLR4 (4.32 ± 6.16) na superfície celular em relação ao pós-tratamento (4.59 ± 4.45 e 1.03 ± 1.58) respectivamente ($p<0.05$). Porcentagem de células CD3+ coexpressando TLR2/TLR4 também foi significativamente maior em pacientes pré-tratamento (3.03 ± 3.86) em relação ao pós-tratamento (0.76 ± 1.69). Pacientes com LV ativa também tinham maiores porcentagens ($p<0.05$) de células CD3+ expressando TLR2, TLR4 e coexpressão TLR2/TLR4 quando comparados aos controles (0.62 ± 0.86, 1.35 ± 2.56 e 0.16 ± 0.13)

respectivamente, e mesmo após o tratamento, pacientes ainda apresentaram maior expressão de TLR2 (4.59 ± 4.45) e coexpressão TLR2/TLR4 (0.76 ± 1.69) em relação aos controles. Não foram detectadas diferenças estatísticas na expressão de TLR4 entre pós-tratamento em relação aos controles (Tabela 1).

A análise da expressão dos receptores TLR2 e TLR4 em células CD14+ mostram que pacientes com LV ativa apresentaram menores porcentagens dessas células expressando TLR2 (90.93 ± 10.73), TLR4 (29.37 ± 29.79) e coexpressão TLR2/TLR4 (28.93 ± 28.8) em relação aos indivíduos controles (98.75 ± 1.52, 62.79 ± 32.94 e 60.78 ± 33.68) respectivamente. Após serem submetidos ao tratamento, os pacientes ainda apresentaram menores porcentagens de células CD14+ expressando TLR4 (22.6 ± 8.94) e coexpressão TLR2/TLR4 (21.71 ± 8.91) em relação aos controles. Não foram detectadas diferenças significativas de células CD14+ expressando TLR2, TLR4 e coexpressão TLR2/TLR4 entre pré e pós-tratamento (Tabela 1).

Também foi analisada a média de intensidade de fluorescência dos receptores TLR2 e TLR4 em células CD3+ e CD14+. Pacientes pré-tratamento apresentaram média de intensidade de fluorescência do TLR2 (483.0 ± 619.0) e TLR4 (311.5 ± 575.25) significativamente maior em células CD14+, em relação ao pós-tratamento (94.06 ± 46.19 e 111.43 ± 60.33) e indivíduos controles (53.28 ± 11.85 e 59.02 ± 22.28) (Figuras 2A e 2B). Não foram detectadas diferenças significativas na média de intensidade de fluorescência de TLR2 e TLR4 em CD3+ (Figura 2C e 2D) no pré-tratamento, em relação ao pós-tratamento e controles. Após o tratamento, a fluorescência do TLR4 em células CD3+ (203.67 ± 150.77) e do TLR2 (94.06 ± 46.19) e TLR4 (111.43 ± 60.33) em células CD14+ foi maior em relação aos controles (Figuras 2A, 2B e 2D).

Tabela 1. Porcentagem de células CD3+ e CD14+ expressando TLR2, TLR4 e coexpressando TLR2/TLR4 em pacientes com leishmaniose visceral antes e após o tratamento.

		Pacientes pré-tratamento (n=13)	**Pacientes pós-tratamento (n=13)**	**Controles (n=16)**
CD3+	**TLR2**	23.83 ± 20.91 [a b]	4.59 ± 4.45 [c]	0.62 ± 0.86
	TLR4	4.32 ± 6.16 [a b]	1.03 ± 1.58	1.35 ± 2.56
	TLR2/TLR4	3.03 ± 3.86 [a b]	0.76 ± 1.69 [c]	0.16 ± 0.13
CD14+	**TLR2**	90.93 ± 10.73 [b]	97.27 ± 8.02	98.75 ± 1.52
	TLR4	29.37 ± 29.79 [b]	22.63 ± 8.94 [c]	62.79 ± 32.94
	TLR2/TLR4	28.93 ± 28.8 [b]	21.71 ± 8.91 [c]	60.78 ± 33.68

a $p<0,05$ pré-tratamento x pós-tratamento, b $p<0,05$ pré-tratamento x controles, c $p<0,05$ pós-tratamento x controles. Resultados são expressos em média ± DP.

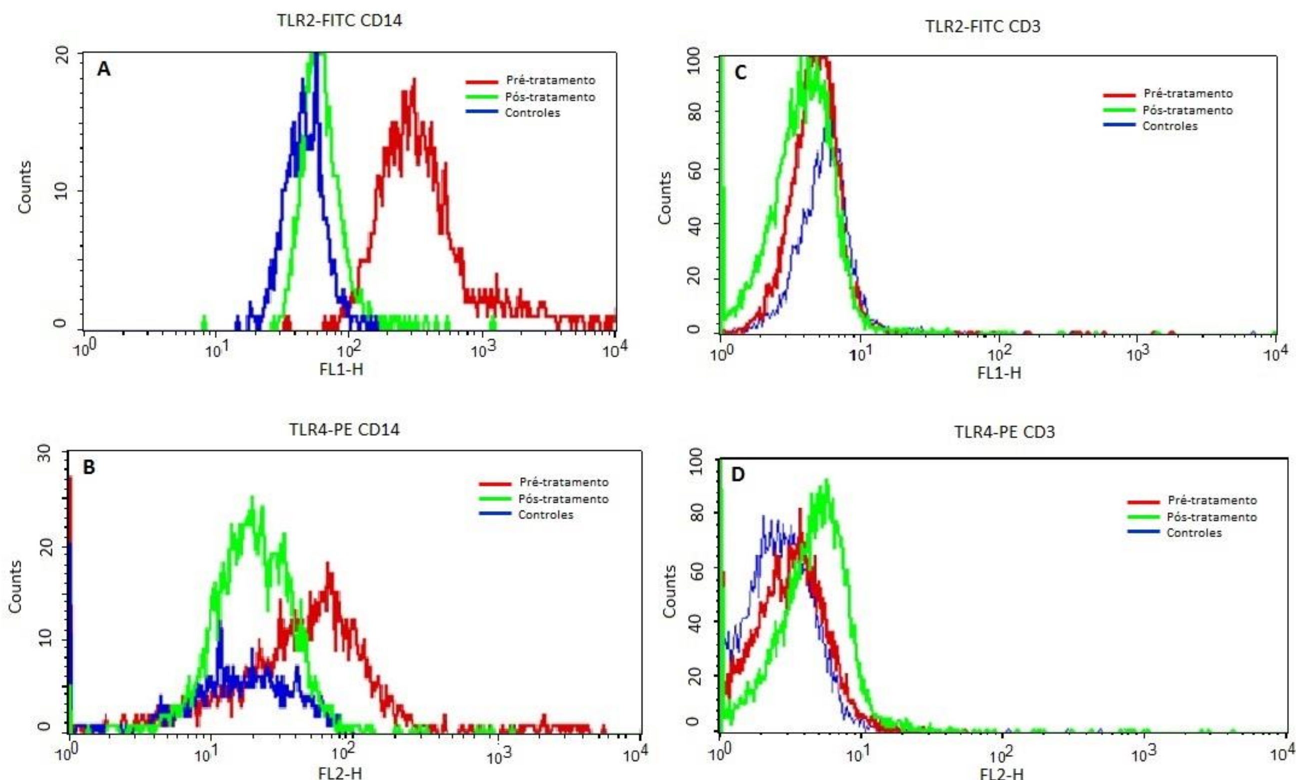

Figura 2. Histogramas representativos da expressão de TLR2 e TLR4 em células CD3+ e CD14+ são mostrados nas figuras de A – D, sendo que os histogramas vermelhos representam pacientes pré-tratamento, histogramas verdes representam os pacientes pós-tratamento e histogramas azuis representam os indivíduos controles. A expressão de TLR2 e TLR4 em monócitos está na figura A e B e expressão de TLR2 e TLR4 em linfócitos nas figuras C e D.

Produção de citocinas e do NO por PBMCs de pacientes com leishmaniose visceral antes e após o tratamento

A produção das citocinas por PBMCs em sobrenadante de cultura de pacientes com LV antes e após o tratamento foi verificada. Em relação ao TNF-α (Figura 3A), pacientes com LV ativa produziram níveis significativamente maiores ($p=0.02$) em relação aos pacientes pós-tratamento. Não foram detectadas diferenças significativas entre pré-tratamento e pós-tratamento em relação aos indivíduos controles. Quanto ao IFN-γ (Figura 3B) e IL-17 (Figura 3C) pacientes com LV ativa produziram níveis significativamente menores ($p<0.001$) destas citocinas em relação ao pós-tratamento e controles. Não foram verificadas diferenças significativas na produção de IFN-γ e IL-17 entre pós-tratamento e indivíduos controles.

A produção de NO também foi avaliada em sobrenadante de cultura de PBMCs de pacientes com LV antes e após o tratamento. Nossos resultados mostram que células de

pacientes pré-tratamento produzem níveis significativamente menores (p=0.04) de NO em relação ao pós-tratamento. Não foram observadas diferenças significativas na produção de NO por PBMCs entre pacientes pré e pós-tratamento e os indivíduos controles (Fig. 4).

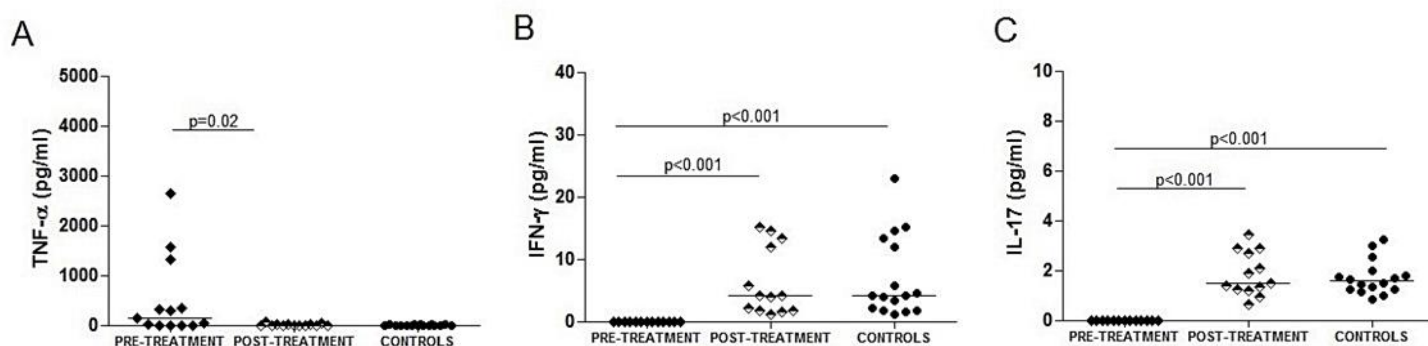

Figura 3. Níveis de TNF-α (A), IFN-γ (B) e IL-17 em sobrenadante de cultura de PBMCs ($1x10^6$ cells/ml) de pacientes com leishmaniose visceral antes e após o tratamento. Os níveis de citocinas foram determinados por CBA. Cada ponto representa um paciente diferente e cada barra representa a mediana. Os dados são representativos de 3 replicatas diferentes.

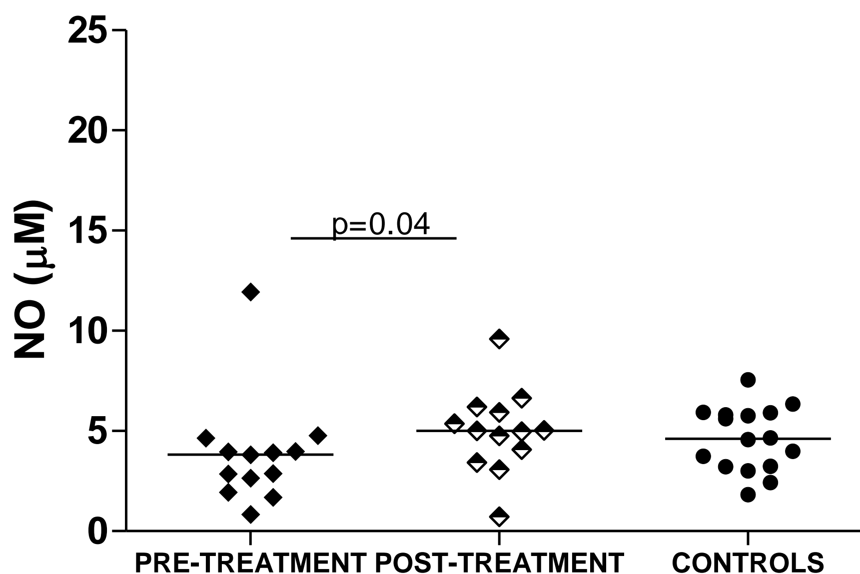

Figura 4. Níveis de NO em sobrenadante de cultura de PBMCs ($1x10^6$ cells/ml) de pacientes com leishmaniose visceral antes e após o tratamento. Os níveis de citocinas foram determinados por CBA. Cada ponto representa um paciente diferente e cada barra representa a mediana e os dados são representativos de 3 replicatas independentes.

DISCUSSÃO

Alterações lipídicas e no metabolismo de lipoproteínas tem sido observadas durante os processos infecciosos e inflamatórios. [4] Neste sentido, na leishmaniose já foi detectada a ocorrência dessas alterações que podem ter ocorrido devido a vários fatores, entre eles a indução da resposta de fase aguda. [6,7] Nossos resultados mostram que pacientes com a forma ativa da doença apresentam alterações no perfil lipídico e das lipoproteínas, com diminuição de HDL, LDL e colesterol total e aumento dos níveis de triglicerídeos. Estes resultados estão de acordo com os obtidos em outros estudos realizados com pacientes com LV que detectaram as mesmas alterações. [47-50]

Além disso, outros fatores podem também alterar as lipoproteínas, como no caso da diminuição de HDL no soro, que pode ter ocorrido devido ao seu sequestro no baço e fígado, órgãos onde o parasita se acumula. [48,51] Imunoglobulinas também podem formar imuno complexos entre HDL e anticorpos, acelerando a degradação de HDL e contribuindo para a diminuição dos seus níveis. [51] O aumento de triglicerídeos no soro pode ser devido à diminuição da atividade da lipase hepática, o que resulta na eliminação lenta de VLDL (lipoproteína de muito baixa densidade), a qual é responsável pela conversão de LDL.[52] O fígado é o principal local onde há a biossíntese de colesterol. Desse modo, disfunções hepáticas, que podem ocorrer devido à presença dos parasitas, podem ser a causa da diminuição do colesterol. [53] Pacientes com LV ativa apresentaram alterações lipídicas e provavelmente alta carga parasitária. Após serem submetidos ao tratamento, foi observado que os níveis de HDL, LDL, colesterol e triglicerídeos estavam próximos ao normal. Neste sentido, Ghosh *et al* [54] verificaram que pacientes com LV ativa apresentam concentrações de colesterol inversamente correlacionadas com a carga parasitária no baço.

A diminuição das lipoproteínas pode ainda ter um impacto na resposta imune. O colesterol presente na membrana plasmática das células, além de manter a membrana fluídica também formam os lipids rafts. [9,10,55,56] Nos lipids rafts são expressos MHC-II e receptores TLRs, os quais participam da apresentação de antígeno, fagocitose e consequentemente ativação de células T específicas.[11-16,57,58] Macrófagos, além de serem alvos para a atividade imunomoduladora das lipoproteínas por possuírem receptores para elas, são as células hospedeiras de *Leishmania* e depleção de colesterol dessas células prejudica, portanto, a ativação da resposta imune. [59-61] Neste sentido, estudo realizado em pacientes com LV mostrou que baixos níveis de colesterol resultam em menores quantidades de linfócitos T CD4+ e T CD8+ circulantes. [53] Além disso, lipossomas de

colesterol aumentam a produção de TNF-α, IL-6 e IL-10 e conferiram proteção ao hospedeiro com LV. [47,62]

Além do colesterol, o HDL também é importante no desenvolvimento da resposta imune. Estudo mostrou que na infecção com *Leishmania*, o TLF, uma porção do HDL, que se acumula nos fagolisossomos, é capaz de inibir a infecção por prejudicar diretamente o parasita.[63] O LPS interage com TLR4 através da LBP (proteína ligada ao LPS) e ativa a produção de citocinas inflamatórias por macrófagos.[64] O HDL é a lipoproteína que se liga preferencialmente á LBP.[65] Camundongos ausentes de ApoA (lipoproteína do HDL) apresentam aumento da mortalidade por choque séptico causado por LPS, pois essa lipoproteína é responsável por eliminar o LPS do fígado.[66] Por outro lado foi visto que o HDL também possui um papel regulatório durante uma infecção. A ApoA reduz a expressão de TLR4 na superfície celular por diminuir o colesterol dos lipids rafts. Desse modo, menor expressão desse receptor diminui a resposta inflamatória por LPS, protegendo contra o choque séptico.[67,68] Durante infecção há aumento da oxidação do LDL.[69] LDL oxidado (oxLDL) regula a expressão de receptores scavenger em macrófagos e aumenta a capacidade de fagocitose de bactérias Gram positivas e negativas.[69,70]

Diante destes estudos que mostram a influência das lipoproteínas na resposta imune, e a partir da detecção de alterações nas lipoproteínas em nossos pacientes, avaliamos a resposta inflamatória nesses indivíduos. Inicialmente, achamos importante verificar se a ligação do parasita às células do hospedeiro poderia ocorrer via os receptores TLR2 e TLR4. Os resultados mostraram que pacientes antes do tratamento apresentam aumento da porcentagem de linfócitos expressando TLR2 e TLR4 e aumento da expressão desses dois receptores em monócitos em relação ao pós-tratamento. Mesmo após o tratamento, esses dois subtipos celulares continuaram expressando TLR2 e TLR4, sugerindo que na LV ativa e mesmo após o tratamento esses receptores provavelmente participam do reconhecimento do parasita e ativação da resposta imune. De acordo com nossos resultados, estudos na LV e com outras doenças infecciosas também mostram aumento da expressão de TLR2 e TLR4.[71-75] Na infecção com *L. major* e *L. donovani*, o TLR2 foi responsável por ativação da resposta imune inata e produção de TNF-α e NO.[43,76] Quanto ao TLR4, foi visto que na infecção experimental com *L. major* esse receptor estava relacionado com proteção do hospedeiro já que ausência desse receptor resultou em lesões mais graves e com elevada carga parasitária.[42,45] Estudos têm demonstrado o envolvimento modulador do colesterol na função de alguns receptores de membrana.[77] De acordo com Lu et al [78] a expressão do TLR4 e produção de IL-8

induzida pelo *H. pylori* foi dependente do colesterol. De forma contrária nossos resultados não sugerem o envolvimento das lipoproteínas na expressão dos receptores em pacientes com a doença ativa, por outro lado observamos que no pós-tratamento o aumento das lipoproteínas podem ter influenciado a expressão dos TLRs. Entretanto, o mecanismo envolvido nesse processo precisa ser explorado.

Nossos resultados mostram que na LV ativa os pacientes apresentam alta produção de TNF-α, que atingem níveis normais após o tratamento, achados que concordam com os obtidos em outros estudos.[(38,79-81)] Na VL ativa, TNF-α está envolvido com a proteção do hospedeiro, sendo produzido por macrófagos ativados e linfócitos T CD4+ Th1, agindo sinergicamente com IFN-γ para ativar funções microbicidas dos macrófagos e então eliminar os parasitas. [(82-85)] Por outro lado, excesso de TNF-α pode ser deletério para o hospedeiro, causando injúria tecidual, inflamação e alguns sintomas.[(37,38)] De acordo com outros estudos, nossos resultados ainda permitem sugerir o possível envolvimento dos TLRs na produção desta citocina.[(43,86)] Alguns estudos tem demonstrado o envolvimento das lipoproteínas na produção do TNF-α. Na infecção *in vitro* de macrófagos com *Leishmania* ocorreu a síntese de TNF-α, IL-6 e IL-10 mesmo na presença de concentrações subfisiológicas de lipoproteínas séricas.[(44,87-90)] Estes achados discordam de outros estudos que mostraram que a infecção de macrófagos humanos com *L. infantum*, na ausência de concentrações fisiológicas de lipoproteínas, não induz a produção dessas citocinas.[(47)] Nossos resultados mostram que no pré-tratamento foi detectada produção elevada de TNF-α apesar dos baixos níveis de LDL, HDL e colesterol, sendo detectado o inverso após o tratamento e diminuição da carga parasitária, o que sugere o não envolvimento destas lipoproteínas na produção do TNF-α. Além disso, podemos sugerir que na LV ativa provavelmente esta citocina não foi protetora, podendo estar envolvida na patogênese da doença, enquanto que após o tratamento a redução destes níveis pode estar relacionada com os mecanismos protetores.

Neste estudo, pacientes pré-tratamento produziram quantidades menores de IFN-γ e IL-17 em relação ao pós-tratamento e controles, que aumentaram após o tratamento. Estudos mostraram que baixa produção destas citocinas está relacionada com a progressão da doença e aumento da carga parasitária.[(40,91-93)] Neste sentido, crianças infectadas com *L. chagasi* conseguiram controlar a infecção por exibirem alta produção de IFN-γ quando PBMCs foram estimuladas com antígeno de *Leishmania*.[(91)] Em relação a IL-17, nossos resultados mostram que essa citocina aumentou após o tratamento, provavelmente diminuindo a carga parasitária e sugerindo que está envolvida no controle da infecção. De acordo com isso, estudo mostrou que tratamento com um polissacarídeo de propriedades

imunomoduladoras, foi capaz de induzir a produção de citocinas como IL-17 e IL-23, o qual foi correlacionado com diminuição da carga parasitária.[93] Além disso, outros estudos verificaram o envolvimento dos TLR2 e TLR4 na produção dessa citocina. [74,86,95]

Neste estudo ainda constatamos que os níveis de NO foram mais baixos em pacientes com LV ativa em relação ao pós-tratamento, o que está de acordo com outro estudo que verificou níveis diminuídos em relação ao pós-tratamento.[96] Após o tratamento e diminuição da carga parasitária observamos aumento dos níveis do NO, sugerindo seu envolvimento na destruição do parasita. Além disso, também tem sido observado o envolvimento dos TLR2 e TLR4 na produção de NO.[43,97] Nossos resultados mostram que houve uma associação entre as lipoproteínas e NO tanto no pré quanto no pós-tratamento, sugerindo uma possível influência delas na produção deste metabólito. De acordo com estes resultados foi mostrado que o LDL e LDL oxidado pode induzir a maior expressão de iNOS e produção de NO por macrófagos.[98] Lipossomas de colesterol também conferiram proteção na infecção com *L. donovani*, induzindo geração de reativos do oxigênio e auxiliando na eliminação do parasita.[30]

Em resumo, nossos resultados mostraram que a baixa produção de IFN-γ, IL-17 e NO foi relacionada com baixos níveis de colesterol, LDL e HDL nos pacientes com leishmaniose ativa, enquanto que no pós-tratamento a expressão aumentada do TLRs, juntamente com a elevação dos níveis de IFN-γ, IL-17 e NO foi associada com o aumento das lipoproteínas. Esses resultados sugerem que as lipoproteínas poderiam estar envolvidas no desenvolvimento e manutenção da resposta imune em pacientes com LV ativa e no pós-tratamento com a cura dos pacientes. Futuros estudos serão conduzidos para confirmar o envolvimento das lipoproteínas na resposta imune em pacientes com LV.

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Conclusão

-Pacientes com LV pré-tratamento se mostraram eutróficos, no entanto com alterações bioquímico-nutricionais como ângulo de fase diminuído e menores níveis séricos de colesterol total, HDL, LDL e albumina e aumento de triglicerídeos. O tratamento não alterou o ângulo de fase, mas aumentou os níveis de HDL, LDL, colesterol total e albumina e diminuiu os níveis de triglicerídeos, deixando esses parâmetros próximos aos valores normais.

-Pacientes com LV pré-tratamento apresentaram aumento da expressão de TLR2 e TLR4 em linfócitos e monócitos, aumento da produção de TNF-α, IL-10 e TGF-β e menor produção de IFN-γ, IL-17 e NO. Foi verificado também que TLR2 e TLR4 estavam envolvidos com a produção de TNF-α e NO, e o TLR2 com a produção de IL-10. Com o tratamento, monócitos e linfócitos continuaram expressando os dois receptores, enquanto os valores de TNF-α, IFN-γ, IL-17, IL-10 e NO foram próximos aos normais. No pós-tratamento tanto TLR2 quanto TLR4 foram envolvidos com a produção de IFN-γ, TNF-α, IL-10 e NO, e o TLR4 com a produção de IL-17.

-Pacientes com LV pré-tratamento apresentaram baixos níveis de colesterol total, HDL e LDL que se relacionaram com baixos níveis de IFN-γ, IL-17 e NO. Após o tratamento, tanto a expressão elevada dos TLRs, quanto os altos níveis de IFN-γ, IL-17 e do NO, se relacionaram com o aumento das lipoproteínas.

Anexos

Anexo 01

Protocolo de avaliação clínico-nutricional

Nome:____

Telefone:__________________

Data de Nascimento:________ Idade:___________ Sexo:__________Profissão:_________

Naturalidade:______________Procedência:____

Escolaridade:_________________________

Tabagismo:_________________________Etilismo:_________________________________

Antecedentes familiares:____

Antecedentes pessoais:____

Avaliação 1

DATA	PESO	ALTURA	IMC	CB	DCB	DCT	DCSE
DCSI	CA	CQ	RCQ				
BIA	Resistência	Reactância	AF				
CT	HDL	TG	Glicose	Pt. totais	Albumina	Globulina	

Avaliação 2

DATA	PESO	ALTURA	IMC	CB	DCB	DCT	DCSE
DCSI	CA	CQ	RCQ				
BIA	Resistência	Reactância	AF				
CT	HDL	TG	Glicose	Pt. totais	Albumina	Globulina	

Anexo 02

UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA
Departamento de Doenças TropicaiseDiagnóstico por Imagem
BOTUCATU/SP – CP 584 – CEP. 18618-970

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO EM TRABALHO CIENTÍFICO

A leishmaniose visceral é uma doença causada por um protozoário o qual é transmitido pela picada de um mosquito do gênero *Lutzomya sp*. No sangue, aparecem níveis aumentados de algumas substâncias quando a doença está em atividade, que diminuem à medida que o tratamento vai fazendo efeito. Essas substâncias estão ligadas à reação do sistema de defesa da pessoa que está com a doença. Se a reação do sistema imune for muito intensa, poderá haver alguns danos nas células. O estado nutricional do paciente também pode estar ligado ao desenvolvimento da leishmaniose visceral.

No Hospital das Clínicas da UNESP, será realizada a pesquisa, denominada "**Papel dos receptores TLR-2 e TLR-4, do estado nutricional em pacientes com leishmaniose visceral**" na qual serão dosadas essas substâncias. Desse modo, estamos convidando você, sem nenhuma obrigação, a participar de nossa pesquisa e doar 25 ml de sangue antes e após o tratamento para essa finalidade, o qual será colhido por profissional devidamente habilitado e com material estéril. O sangue doado será utilizado nessa pesquisa e comparado na mesma com o de pessoas do grupo controle (não possuem a doença). Antes da coleta será feita uma avaliação nutricional e antropométrica (medidas do corpo) e será aplicado um questionário, sendo que esses procedimentos levarão cerca de 20 minutos. A quantidade de sangue coletada será suficiente para a pesquisa, mas, caso sobre algum material, este será estocado e poderá ser usado em projetos futuros, lembrando que se isso ocorrer, um novo Termo de Consentimento Livre e Esclarecido será encaminhado, podendo você aceitar ou não participar.

Tendo sido satisfatoriamente informado (a) sobre o projeto acima, sob a responsabilidade da Bióloga Mariana Gatto e da Bióloga Sueli Aparecida Calvi, do Departamento de Doenças Tropicais e Diagnóstico por Imagem, declaro que concordo em participar do mesmo. Estou ciente de que os resultados dos exames serão utilizados somente pelas pesquisadoras, que manterão sigilo sobre minha identidade, e que as mesmas estão disponíveis para responder a quaisquer perguntas. Sei ainda, que **poderei retirar este consentimento a qualquer tempo, sem que isso resulte em prejuízo para meu seguimento, atual ou futuro, em qualquer dependência do Hospital das Clínicas da UNESP.** Em caso de dúvida adicional, sei que poderei entrar em contato com o Comitê de Ética em Pesquisa da Faculdade de Medicina de Botucatu, através do fone: 3880-1608.

Este documento após aprovação do CEP será elaborado em 2 vias, sendo uma entregue ao sujeito da pesquisa e outra será mantida em arquivo pelo pesquisador.

Declaro que o presente projeto de pesquisa foi explicado em detalhes ao Sr(a)_______________
____.

Botucatu, ___ de _______________20____.

Assinatura do paciente Assinatura da pesquisadora

Mariana Gatto CRBio 72.708
Distrito de Rubião Junior
Departamento de Doenças Tropicais e Diagnóstico por Imagem
Botucatu – SP
Cel: 98116-7952Tel: 3880-1641

Anexo 03

UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA
Departamento de Doenças Tropicais e Diagnóstico por Imagem
BOTUCATU/SP – CP 584 – CEP. 18618-970

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO EM TRABALHO CIENTÍFICO – GRUPO CONTROLE

A leishmaniose visceral é uma doença causada por um protozoário o qual é transmitido pela picada de um mosquito do gênero *Lutzomya sp*. No sangue, aparecem níveis aumentados de algumas substâncias quando a doença está em atividade, que diminuem à medida que o tratamento vai fazendo efeito. Essas substâncias estão ligadas à reação do sistema de defesa da pessoa que está com a doença. Se a reação do sistema imune for muito intensa, poderá haver alguns danos nas células. O estado nutricional do paciente também pode estar ligado ao desenvolvimento da leishmaniose visceral.

No Hospital das Clínicas da UNESP, será realizada a pesquisa, denominada "**Papel dos receptores TLR-2 e TLR-4, do estado nutricional em pacientes com leishmaniose visceral**", na qual serão dosadas essas substâncias. Desse modo, estamos convidando você, sem nenhuma obrigação, a participar de nossa pesquisa como grupo controle e doar 25 ml de sangue em um único momento para essa finalidade, o qual será colhido por profissional devidamente habilitado e com material estéril. O sangue doado será utilizado nessa pesquisa e comparado na mesma com o de pessoas que possuem a doença. A quantidade de sangue coletada será suficiente para a pesquisa, mas, caso sobre algum material, este será estocado e poderá ser usado em projetos futuros, lembrando que se isso ocorrer, um novo Termo de Consentimento Livre e Esclarecido será encaminhado, podendo você aceitar ou não participar.

Tendo sido satisfatoriamente informado (a) sobre o projeto acima, sob a responsabilidade da Bióloga Mariana Gatto e da Bióloga Sueli Aparecida Calvi, do Departamento de Doenças Tropicais e Diagnóstico por Imagem, declaro que concordo em participar do mesmo. Estou ciente de que os resultados dos exames serão utilizados somente pelas pesquisadoras, que manterão sigilo sobre minha identidade, e que as mesmas estão disponíveis para responder a quaisquer perguntas. Sei ainda, que **poderei retirar este consentimento a qualquer tempo, sem que isso resulte em prejuízo para meu seguimento, atual ou futuro, em qualquer dependência do Hospital das Clínicas da UNESP.** Em caso de dúvida adicional, sei que poderei entrar em contato com o Comitê de Ética em Pesquisa da Faculdade de Medicina de Botucatu, através do fone: 3880-1608.

Este documento após aprovação do CEP será elaborado em 2 vias, sendo uma entregue ao sujeito da pesquisa e outra será mantida em arquivo pelo pesquisador.

Declaro que o presente projeto de pesquisa foi explicado em detalhes ao Sr(a) _____________ ____.

Botucatu, ___ de _______________20____.

Assinatura do paciente   Assinatura da pesquisadora

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Anexo 04

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Registrado no Ministério da Saúde
em 30 de abril de 1997

Botucatu, 14 de Março de 2011. Of. 87/11-CEP

Ilustríssima Senhora
Profª. Drª Sueli Aparecida Calvi
Departamento de Doenças Tropicais e Diagnóstico por Imagem da
Faculdade de Medicina de Botucatu

Prezada Drª. Sueli,

De ordem do Senhor Coordenador deste CEP, informo que Projeto de Pesquisa **(Protocolo CEP 3801-2011)** Papel dps receptores TLR-2 e TLR-4 e do estado nutricional em pacientes com leishmaniose viscerol, a ser conduzido por Mariana Gatto, orientada por Vossa Senhoria, com a colaboração de Carlos Magno Caselo Branco Fortaleza, Eliana Peresi, Fabiane Valentini Francisqueti, Fernanda de Nuzzi Dias, Karen Ingrid Tasca, Larissa Ragozo Cardoso de Oliveira, Paulo Camara Marques Pereira, recebeu do relator **parecer favorável** aprovado em reunião de 14 de março de 2011.

Situação do Projeto: APROVADO. Ao final da execução deste Projeto, apresentar ao CEP **"Relatório Final de Atividades"**.

Atenciosamente,

Alberto Santos Capelluppi
Secretário do CEP

Anexo 05

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Declaração

Declaro que tenho ciência e autorizo, o desenvolvimento da Pesquisa "Papel dos receptores TLR-2 e TLR-4 e do estado nutricional em pacientes com leishmaniose visceral", a ser conduzida pela Sra Mariana Gatto orientada pela Dra Sueli Aparecida Calvi, junto a esta Entidade, após aprovação do CEP.

Declaro que conheço, cumprirei e farei cumprir os Requisitos da Resolução 196/96 e suas complementares e como esta instituição tem condições para o desenvolvimento deste Projeto, autorizo sua execução.

Por ser verdade, firmo a presente.

Bauru, 01 de Março de 2011.

Roberto Minoru Tani Inoue
Vice Presidente da Comissão Científica

Anexo 06

SECRETARIA DE DESENVOLVIMENTO ECONÔMICO, CIÊNCIA E TECNOLOGIA

FACULDADE DE MEDICINA DE MARÍLIA
Comitê de Ética em Pesquisa Envolvendo Seres Humanos

Marília, 23 de Maio de 2012

Ilmo(ª) Sr.(ª)
Mariana Gatto
Marília/SP

O Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Faculdade de Medicina de Marília, recebeu o protocolo de estudo nº 473/12, intitulado: "Papel dos Receptores Toll-Like 2 e 4, do Estado Nutricional e Alterações Genotóxicas em Pacientes com Leishmaniose Visceral", foi considerado **APROVADO "Ad Referendum"** após responder a pendência apontada em Reunião Ordinária – 23/04/2012, aceito de acordo com a Resolução 196/96 e suas Complementares do Conselho Nacional de Saúde.

Sendo só para o momento, reiteramos protestos de consideração e apreço.

Atenciosamente,

Prof. Dr. Valdeir Fagundes de Queiroz
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Major Article

Biochemical and nutritional evaluation of patients with visceral leishmaniasis before and after treatment with leishmanicidal drugs

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ABSTRACT

Introduction: Visceral leishmaniasis (VL) is caused by the intracellular protozoan *Leishmania donovani* complex. VL may be asymptomatic or progressive and is characterized by fever, anemia, weight loss and the enlargement of the spleen and liver. The nutritional status of the patients with VL is a major determinant of the progression, severity and mortality of the disease, as it affects the clinical progression of the disease. Changes in lipoproteins and plasma proteins may have major impacts in the host during infection. Thus, our goal was evaluate the serum total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, glucose, albumin, globulin and total protein levels, as well as the body composition, of VL patients before and after treatment. **Methods**: Nutritional evaluation was performed using the bioelectrical impedance analysis (BIA) to assess body composition. Biochemical data on the serum total cholesterol, HDL, LDL, triglycerides, glucose, albumin, globulin and total protein were collected from the medical charts of the patients. **Results:** BIA indicated that both pre-treatment and post-treatment patients exhibited decreased phase angles compared to the controls, which is indicative of disease. Prior to treatment, the patients exhibited lower levels of total body water compared to the controls. Regarding the biochemical evaluation, patients with active VL exhibited lower levels of total cholesterol, HDL, LDL and albumin and higher triglyceride levels compared to patients after treatment and the controls. Treatment increased the levels of albumin and lipoproteins and decreased the triglyceride levels. **Conclusions:** Our results suggest that patients with active VL present biochemical and nutritional changes that are reversed by treatment.

Keywords: Visceral leishmaniasis. Nutritional status. Leishmanicidal drugs.

INTRODUCTION

Leishmaniasis is caused by an obligatory intracellular parasite belonging to the genus *Leishmania*[1]. There are over 20 species of this parasite that can be transmitted to humans by approximately 30 different species of sandflies[2]. Visceral leishmaniasis (VL), also known as kala-azar, is caused by the protozoa of the *Leishmania donovani* complex[3]. Although the risk factors for this disease are not fully understood, it is known that genetic factors, malnutrition and the presence of infected animals in the environment are major contributors to the high maintenance rate of this disease[4-6].

Regarding the treatment for VL, pentavalent antimonials are the first choice of treatment[7,8]. Amphotericin B can be used in cases of toxicity or in patients with unsatisfactory responses to the antimonials and is the first choice for treating pregnant patients and terminal cases of the disease[9,10]. However, according to the Ministry of Health, patients older than 50 years, patients with Chagas disease or patients diagnosed with kidney, cardiac or hepatic complications should be treated with lipid or colloidal formulations of amphotericin B[8].

Visceral leishmaniasis has a large clinical range, from asymptomatic infections and auto resolution to progressive visceral leishmaniasis, which is characterized by fever, hepatosplenomegaly, hypergammaglobulinemia and death if not treated properly and in time[11,12]. Although the factors that lead to the development of the disease are still unclear, the complex interactions between the parasite, the immune response and the nutritional status may influence the host's response to *Leishmania* infection[4,13-16].

Visceral leishmaniasis and malnutrition are considered important public health problems and together are responsible for millions of deaths each year[5]. The nutritional status of the individuals infected with *Leishmania* spp. is involved in the clinical course of the disease and is a major determinant of the progression, severity and increased mortality of VL. Malnutrition may occur as a consequence of energy deficiency,

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such as protein-calorie malnutrition (PCM), or a micronutrient deficiency[17-20]. The infectious process is usually followed by hypercatabolism, which is aggravated by anorexia, resulting in the loss and the consequent depletion of the body's nutrient reserves, thereby causing great changes in the metabolism of the host[21].

Some studies have reported differences in body mass index (BMI), glucose, triglycerides and lipoproteins in patients with active VL[22-24]. Studies performed on patients with VL showed high triglyceride levels and low high-density lipoprotein (HDL), low-density lipoprotein (LDL) and total cholesterol levels[25,26]. Additionally, VL patients showed reduced serum cholesterol concentrations as a function of their splenic parasite burden[23,27]. Elevated levels of triglycerides and low levels of HDL were observed in canine VL[28]. Changes in lipoproteins have also been reported in children with active VL[29-31]. Lipoproteins have the ability to modulate the immune response. Low density lipoproteins, such as very low-density lipoprotein (VLDL) and LDL, can inhibit lymphocyte proliferation *in vitro*[32,33], and another study in VL patients found that lipoproteins altered the immune response and the pathogenesis of the disease by modulating cytokine production[25].

Changes in cholesterol levels can have a major impact on the host during infection. In addition to playing an important role in the maintenance of membrane fluidity, cholesterol is essential for the proper function of antigen presenting cells and is also a prognostic indicator of increased morbidity and mortality associated with pathological conditions[34-36]. This lipid is responsible for the formation of membrane rafts, which are essential for *Leishmania* entry[37-39]. In malnourished animals infected with *Leishmania chagasi*, decreases in serum albumin, globulin and total protein were observed, and these changes were associated with an increased parasite burden and a non-response to the vaccine[40].

Interest in body composition has grown substantially in the last few years due to the association between increased body fat and metabolic disorders, including cardiovascular diseases, diabetes, hypertension and dyslipidemia[41,42]. Bioelectrical impedance analysis (BIA) has been used to analyze body composition for more than 25 years. Additionally, BIA is a portable, noninvasive method to measure body composition that is practical, reproducible and relatively inexpensive. BIA can be repeated often and does not require patient cooperation[43,44].

Although some studies have reported changes in lipoproteins in patients with VL, studies concerning the serum total protein, albumin, globulin and BIA body measurements are absent in these patients, and there are few published papers that examine a wider range of nutritional status factors. Thus, our goal was to evaluate the serum total cholesterol, HDL, LDL, triglycerides, glucose, albumin, globulin and total protein, as well as body composition using BIA, in LV patients before and after treatment with leishmanicidal drugs.

METHODS

Patients

We evaluated 13 patients pre- and post-treatment at an interval of 18 months. The patients were of both genders and were 18 years or older. The age of the patients ranged from 18 to 53 years, and the patients were predominantly male (11 individuals). We excluded patients with other infectious or granulomatous diseases, as well as HIV positive patients and pregnant women. The patients were enrolled in the study when they contacted health services for diagnosis and treatment of the disease. The patients came from the State Hospital of Bauru, State of São Paulo and the Hospital of Marilia, State of Sao Paulo. We evaluated 16 healthy, age- and sex-matched subjects as controls. The age of the control subjects ranged from 21 to 58 years, and the group included 3 women and 13 men.

Diagnosis and treatment of visceral leishmaniasis

The diagnosis of visceral leishmaniasis was performed through the confirmation of the parasites in bone marrow aspirate smears. Patients were treated with N-methylglucamine (Glucantime), amphotericin B deoxycholate or amphotericin B liposome. Data were collected from the patients in the hospital where they were admitted prior to starting treatment for VL. The post-treatment period ranged from 1 to 3 months after the end of the drug regimen, and the patient data were included when the patients returned to the hospital for monitoring. However, two patients did not return to the hospital after the treatment. Post-treatment data were collected only from patients that were cured according to the serological and clinical tests.

Biochemical and nutritional analysis

The biochemical and nutritional evaluation was performed in patients before and after treatment and was performed once in the control subjects. BIA was used for body measurements in the control subjects and in patients pre-and post-treatment using a Biodynamics BIA, model 450 device (TBW). For this procedure, the patients and the control subjects laid down on a non-conductive surface, with their legs apart and their arms parallel and apart from the trunk. Immediately prior to the placement of the electrodes, the contact areas were cleaned with alcohol. An emitter electrode was placed near the metacarpo-phalanx of the dorsal surface of the right hand, and another was placed near the distal transverse arch of the upper surface of the right foot. An electrode detector was placed between the distal prominences of the radius and ulna of the right wrist, and another was placed between the medial and lateral malleolus of the right ankle. The following data were acquired from the patients and control subjects: lean mass (%), body fat (%), body mass index (kg/m^2), phase angle (°), resistance (ohms), reactance (ohms) and total body water (L). In addition to the BIA, the patient's weight (kg) and height (m) were also recorded.

The analyses of the total cholesterol, HDL, LDL, triglycerides, glucose, total protein, albumin and globulin in the control subjects were performed in the Clinical Laboratory of the Faculty of Medicine of Botucatu (*Universidade Estadual Paulista* - UNESP). The patient analyses were performed at the hospital where they were admitted, as these are routine tests for the treatment and follow-up of the disease. These determinations were performed using enzymatic, colorimetric and dry-chemistry methods. To evaluate these parameters, blood was collected from the patient in the morning after fasting for 12h, after no rigorous physical activity for the last 24h and after no alcohol consumption within the last 72h.

Statistical analysis

Non-continuous variables were analyzed using Fisher's test. Comparisons for the continuous variables were performed using an analysis of variance (ANOVA) with a repeated measures design between the pre- and post-treatment groups, followed by an adjusted Tukey's test if the distribution were normal. Otherwise, the same design was used to adjust a generalized linear model with gamma distribution, followed by a multiple comparison test (Wald type). Comparisons between the pre- and post-treatment groups and the control group were made using separate Student's t-tests (control vs. pre and control vs. post). All of the analyses were made using the Statistical Analysis System (SAS) software, v.9.3. The significance level was fixed at 5% or the correspondent p-value.

Ethical considerations

All enrolled patients and control subjects were fully informed of the study and signed the consent form. This study was approved by the Ethics in Research of the Faculty of Medicine of Botucatu, Bauru State Hospital and the Hospital of Marilia and is in accordance with the Declaration of Helsinki of 1964.

RESULTS

Evaluation of body measurements by bioelectrical impedance analysis in patients pre- and post-treatment

There was a male predominance in the patients and control subjects in this study, and there was no significant difference in the mean age of the patients and the control subjects. The results obtained from the BIA analysis showed that there were no significant differences in the phase angle between the patients before and after treatment; however, the phase angle in the patients pre-treatment and post-treatment were significantly lower (p-value <0.05) than the phase angle of the control subjects. In relation to total body water, there were no significant differences between pre- and post-treatment or between post-treatment and the controls. However, patients with active VL showed a decreased (p-value <0.05) amount of total body water than the control subjects. No significant differences in weight (kg), height (m), body mass index (kg/m^2), percentage of lean mass, percentage fat, resistance (ohms) and reactance (ohms) were detected between the patients pre-treatment and post-treatment or between these two groups and the control subjects. However, even without significant differences between the pre- and post-treatment measurements or between these groups and the control group, the pre-treatment patients tended to have smaller reactance, BMI, fat percentage and weight values than the post-treatment patients and the controls. The percentage of lean mass tended to decrease with treatment and tended to be higher in the control subjects. Data are shown in **Table 1**.

Biochemical assessment of patients with visceral leishmaniasis before and after treatment

The pre-treatment patients showed significantly lower levels of total cholesterol, HDL and LDL compared to the post-treatment patients and control subjects (p-value <0.05). However, with exception of LDL, the treated patients also showed significantly lower levels of these variables when compared to the controls (p-value <0.05). Prior to treatment, patients exhibited significantly higher levels of triglycerides compared to post-treatment and the control subjects; even after treatment, these levels still remained elevated when compared to the controls (p-value <0.05). Pre-treatment patients also had lower albumin levels compared to the post-treatment and the control groups (p-value <0.05). However, the treated patients showed higher albumin levels than the controls (p-value <0.05). No differences were found in the levels of glucose, total protein and globulin between these groups. Data are shown in **Table 2**.

TABLE 1 - Bioeletrical impedance analysis of patients with visceral leishmaniasis (difference estimates and 95% confidence intervals).

	Pre- vs post-treatment estimate (95% CI)	Pre-treatment vs controls estimate (95% CI)	Post-treatment vs controls estimate (95% CI)
Weight (kg)	-5.662 (-14.35-3.03)	-10.84 (-22.25-0.567)	-5.184 (-16.47-6.1)
Height (m)	0.006 (-0.02-0.03)	-0.02 (-0.09-0.03)	-5.184 (-16.47-6.1)
Resistance (ohms)	4.59 (-61.33-70.51)	12.36 (-63.16-87.87)	16.95 (-68.17-102.07)
Reactance (ohms)	2.52 (-10.14-15.18)	9.124 (-0.671-18.91)	6.599 (-1.884-15.082)
Phase angle (º)	0.28 (-0.82-1.38)	1.5 (0.31-2.7)*	1.22 (0.17-2.27)*
BMI (kg/m^2)	2.03 (-1.61-5.67)	2.9 (-0.641-6.45)	0.86 (-2.89-4.63)
Fat mass (%)	4.18 (-3.02-11.38)	4.6 (-2.24-11.45)	0.42 (-6.08-6.92)
Lean mass (%)	2.638 (-3.993-9.270)	1.965 (-5.818-9.749)	-0.195 (-6.970-6.580)
Total body water (L)	-2.57 (-8.787-3.633)	6.825 (0.417-13.23)*	4.73 (-1.944-11.40)

vs: versus ; CI: confidence interval; BMI: body mass index. *p value <0.05.

TABLE 2 - Biochemical assessment of patients with visceral leishmaniasis (difference estimates and 95% confidence intervals).

	Pre- vs post-treatment estimate (95% CI)	Pre-treatment vs controls estimate (95% CI)	Post-treatment vs controls estimate (95% CI)
Total cholesterol (mg/dL)	58.82 (11.14-66.5)*	94.27 (59.5-129)*	35.44 (0.33-70.55)*
HDL (mg/dL)	30.44 (22.26-38.62)*	46.8 (32.5-61.17)*	16.4 (1.38-31.41)*
LDL (mg/dL)	38.29 (14.01-62.57)*	62.55 (33.15-91.96)*	24.26 (-6.71-55.23)
Triglycerides (mg/dL)	0.0016 (0.00007-0.0032)*	0.001672 (0.000072-0.003272)*	0.005 (0.003105-0.007285)*
Glucose (mg/dL)	0 (0)	0.00068 (-0.000852-0.002212)	-0.00148 (-0.00061-0.00357)
Albumin (g/dL)	1.47 (0.99-1.95)*	0.9 (0.39-1.42)*	0.56 (0.12-0.99)*
Globulin (g/dL)	-1.04 (-2.52-0.44)	0.91 (-0.19-2.01)	0.13 (-0.77-1.03)
Total protein (g/dL)	0.38 (-0.88-1.64)	0.04 (-0.94-1.04)	0.43 (-0.29-1.15)

vs: versus; CI: confidence interval; LDL: low-density lipoprotein; HDL: high-density lipoprotein. *p value <0.05

DISCUSSION

According to our results, BIA demonstrated a decreased phase angle in patients before treatment and after treatment compared to the control subjects. The phase angle is formed when the electric current is stored across cell membranes and depends on the permeability of the membranes; this measurement is associated with tissue hydration and cellularity, as well as cell size and body cell mass[45]. Thus, the phase angle is associated with cell balance[46]. The phase angle measurement can vary between 0° and 90°, and the reference values are between 4° and 10°[47]. The phase angle has been used as an indicator of the general state of health and nutrition in various diseases, as nutrition is interconnected with changes in membrane integrity and the balance of body fluids[46-48]. Our findings showed that both groups were within the reference values, however, the pre-treatment patients had lower phase angle values than the control group. According to our results, phase angle in patients with infectious diseases and inflammation is lower than the reference values[47,49]. Therefore, studies in patients co-infected with human immunodeficiency virus (HIV) and tuberculosis also exhibited low phase angles when compared to the control subjects[50]. Although no statistically significant difference was found, our results showed that treatment for VL tended to increase the phase angle. However, even after treatment, the phase angle was still lower than in the control subjects. A possible explanation could be that the patients in the study period had not yet fully recovered. Furthermore, we have to consider that the phase angle is positively associated with reactance, which in our study tended to be smaller in patients with active VL compared to the controls. While the phase angle is decreased, the reactance is low, which indicates the presence of a disease. Reactance values inversely correlate with the percentage of lean body mass, which is highly conductive to electrical current, as resistance values directly correlate to the percentage of fat mass, which is poor electrical conductor[45]. In the present study, although no significant difference in weight was detected between the groups, the amount of lean mass tended to be higher in the pre-treatment patients, while the percentage of fat mass tended to be lower in these patients. Taking into account these results, the pre-treatment patients also exhibited normal BMI values, as malnutrition is considered a BMI below 18.5kg/m^2. Contrary to our findings, one study reported that children with active VL had lower BMI values than healed children and uninfected children; additionally, children that breastfeed for longer period of time have asymptomatic VL, while children with lower birth weight are more likely to develop VL, which shows that nutritional status plays a crucial role in the pathogenesis of visceral leishmaniasis[22]. Thus, we can conclude that patients with active VL showed no difference in weight compared to the post-treatment patients and the controls and, therefore displayed no difference in BMI, percentage of lean mass and fat mass.

According to our results, glucose levels did not differ between the groups. Unlike our results, previous studies have shown that experimental infection with *L. chagasi* leads to a reduction in glucose levels compared to control mice[24]. Concerning lipid levels, our results showed that pre-treatment patients had lower HDL, LDL and total cholesterol levels and higher levels of triglycerides compared to the post-treatment patients and the controls. However, it is worth noting that all groups were within the normal range for total cholesterol (reference values <200mg/dL) and LDL (reference values <130mg/dL). The HDL levels (reference values >35mg/dl) and triglycerides levels (reference value <150mg/dL) were below and higher than normal in patients before treatment, respectively. These results are in agreement with Soares et al.[25], who showed that patients with VL had high triglycerides levels and low HDL, LDL and total cholesterol levels. Other studies of visceral leishmaniasis and other infectious diseases, such as HIV and schistosomiasis, have reported the same lipid changes[28,51-54]. Our findings show that patients with active VL, which most likely have a high parasite load, have total cholesterol levels lower than the control group, and after treatment, these levels increase. In this sense, Ghosh et al.[23] showed that patients with VL have cholesterol concentrations that were inversely correlated with

their splenic parasite load. Due to the high parasitic loads in the spleen, patients with active VL experience dysfunctions in this organ, which is responsible for cholesterol biosynthesis, thus increasing the morbidity of this disease[27]. Our results showed that treatment appeared to normalize the HDL and triglyceride levels and increase the LDL and total cholesterol levels.

The changes in the levels of lipoproteins can be directly related to the modulation of the immune response, as the plasma membrane, which is composed of lipids, is essential for antigen presentation and phagocytosis. Internalization involves interaction between the parasite and the plasma membrane of the cell host[36,55,56]. Low cholesterol levels in humans decrease the number of circulating lymphocytes, thus increasing the chances of mortality[23,34]. The decrease in lipoproteins may affect the immune response and the pathogenesis of the disease, as lipoproteins are related to the production of TNF-α, IL-6 and IL-10[25]. In addition to being targets for the immunomodulatory activity of lipoproteins, macrophages express lipoprotein receptors and are the host cells of the *Leishmania* parasite[57]. However, the study demonstrated that macrophage-depleted lipids decreased their ability to interact with *Leishmania donovani* and internalize promastigotes by 45%, thus impairing the replication of the parasite. On the other hand, when depleted macrophages are treated with cholesterol, there is an increased chance that the parasites will destroy the cell membrane[39].

In relation to the plasma proteins, it was demonstrated in our study that patients with active VL have lower albumin levels compared to the post-treatment patients and the controls, and no differences were observed in the levels of total protein and globulin between the groups. Contrary to the findings of our study, Malafaia et al.[40] reported decreased globulin and total protein in mice infected with *L. chagasi* compared to uninfected mice. However, according to our results, animals infected with *L. chagasi* also exhibit a decrease in serum albumin, which is associated with increased parasite load, a negative response to the vaccine and reduced IFN-γ production[40]. Another study reported that the albumin/globulin ratio was lower in VL patients compared to control subjects in endemic and non-endemic areas[58].

Our results show that patients with active VL were eutrophic, had a lower phase angle and exhibited changes in their albumin and lipoprotein levels. Treatment changed the biochemical frame and the nutritional status of the patients, tending to return to normal levels. The data presented in this study suggest an association between the biochemical and nutritional alterations and leishmaniasis. However, our study does not conclude that the changes were responsible for the worsening of the VL or if the infection led to the biochemical and nutritional changes, thus aggravating the clinical manifestations of VL. Thus, we are developing new studies to have a better understanding of the involvement of biochemical and nutritional parameters in the pathogenesis of LV.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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