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TESE DE DOUTORADO

BIOASSINATURAS DE VIAS INFLAMATÓRIAS NA LEISHMANIOSE TEGUMENTAR: UM OLHAR INTEGRADO

HAYNA MALTA SANTOS

Salvador - Bahia
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Tese apresentada ao Curso de Pós- Graduação em Patologia Humana para a obtenção do grau de Doutora.

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Coorientadora: Profa. Dra. Jaqueline França Costa

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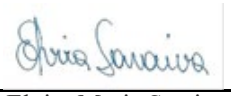

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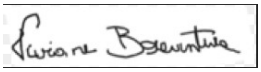

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SANTOS, Hayna Malta. **Bioassinaturas de vias inflamatórias na leishmaniose tegumentar: um olhar integrado**. 110 f.: il. Tese (Doutorado em Patologia Humana) – Universidade Federal da Bahia, Faculdade de Medicina, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2022.

RESUMO

INTRODUÇÃO: A Leishmaniose Tegumentar (LT) é uma doença parasitária que pode resultar em um amplo espectro de formas clínicas a depender de determinantes do hospedeiro e do parasito. Entender essas características nos diferentes desfechos clínicos é importante a fim de identificar novos alvos terapêuticos. Fármacos antimoniais pentavalentes, são a primeira classe de fármacos utilizadas durante o tratamento das leishmanioses. No entanto, cerca de 45% dos pacientes apresentam falha na terapia no Brasil. Nesse sentido, a identificação de potenciais biomarcadores de gravidade de doença ou controle do parasito pode favorecer o desenvolvimento de terapias direcionadas ao hospedeiro que podem incrementar o manejo clínico de casos graves/complicados. **OBJETIVO**: Neste estudo, avaliamos biomarcadores de gravidade de doença e falha terapêutica em pacientes com leishmaniose tegumentar e o papel das resolvinas na resistência à infecção por *Leishmania*. **RESULTADOS**: Nossos resultados demonstram que pacientes com Leishmaniose Cutânea Difusa apresentam uma ativação diferencial da via das poliaminas e aminoácidos quando comparados a Leishmaniose Cutânea Localizada ou Leishmaniose Cutânea Mucosa podendo ser utilizada como biomarcador de gravidade de doença. Além disso, encontramos uma bioassinatura influenciada por proteínas plasmáticas e mediadores lipídicos que prediz com alta acurácia a falha terapêutica na LT. Experimentos adicionais *in vitro* utilizando neutrófilos humanos revelaram que a RvD1 promove a replicação intracelular da *L. braziliensis,* no entanto, o mecanismo por trás desse efeito ainda precisa ser investigado. **CONCLUSÃO**: Os resultados sugerem que as vias de produção das poliaminas e mediadores lipídicos podem ser utilizados como biomarcadores de gravidade de doença e falha terapêutica e que a RvD1 favorece a resistência do parasito, podendo em conjunto servir como potencial estratégia terapêutica.

Palavras-chave: Leishmaniose tegumentar. Arginas. Poliaminas. *L. braziliensis.* Mediadores lipídicos. Resolvina D1.

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ABSTRACT

INTRODUCTION: Tegumentary leishmaniasis (TL) is a parasitic disease that can result in wide spectrum clinical manifestations depending on host and parasite determinants. Understanding these characteristics in different clinical outcomes is important to identify novel therapeutic targets. Pentavalent antimonials are the first-line drugs used to treat Leishmaniosis. However, occurrence of treatment failure in Brazil can be as high as 45%. In this context, identification of potential biomarkers of disease severity or parasite control may favor the development of host-oriented therapies that may increase the clinical management of severe/complicated cases. **AIM**: In this study, we evaluated biomarkers of disease severity and therapeutic failure in patients with tegumentary leishmaniasis and the role of resolving in the resistance to *Leishmania* infection. **RESULTS**: Our results show that patients with DCL present a differential activation of the polyamine and amino acid pathway when compared to LCL and MCL and this pathway can be used as a biomarker of disease severity. In addition, we found a biosignature influenced by plasma proteins and lipid mediators that accurately predicted treatment failure in LT. Additional *in vitro* experiments using human neutrophils revealed that RvD1 promotes intracellular replication of *L. braziliensis* however, the mechanism behind this effect still needs to be investigated. **CONCLUSION**: The results suggest that the production pathways of polyamines and lipid mediators can be used as biomarkers of disease severity and therapeutic failure and that RvD1 favors parasite resistance, and can together serve as a potential therapeutic strategy.

Keywords: Tegumentary leishmaniasis. Arginase. Polyamines. *L. braziliensis*. Lipid mediators. Resolvina D1.

LISTA DE FIGURAS

LISTA DE SIGLAS E ABREVIATURAS

DNA	Ácido Desoxirribonucléico
IFN-γ	Interferon gama
IL	Interleucina
LCD	Leishmaniose Cutânea Difusa
LCL	Leishmaniose Cutânea Localizada
LCM	Leishmaniose Cutânea Mucosa
LT	Leishmaniose Tegumentar
PGE	Prostaglandina E
LTB	Leucotrieno B
TGF- β	Fator transformante de crescimento β
Th	Célula T auxiliadora, do inglês "*T helper*"
TNF-α	Fator de necrose tumoral
COX	Ciclooxigenase
LO	Lipoxigenase
SPM	Mediadores especializados na pró-resolução DHA Ácido docosahexanóico
EPA	Ácido eicosapentanóico
RvD1	Resolvina D1
RvD2	Resolvina D2
iNOS	Óxido Nítrico Sintase induzida
NO	Óxido Nítrico
AA	Ácido araquidônico
PUFAs	Ácidos graxos poli-insaturados
GM-CSF	Fator estimulante da colônia granulócito-macrófago ARG1 Arginase 1
SpdS	Espermidina sintase
SpmS	Espermina sintase
ODC	Ornitina descarboxilase
nor-NOHA	Acetato de nω-Hydroxy-nor-L-arginina DFMO Difluorometilornitina
PLA	Fosfolipase A
HETE	Ácido hidroxieicosatetraenoico
PPAR	Receptor ativado por proliferador peroxisoma ROS Espécies reativas de oxigênio
MPO	Mieloperoxidase
NE	Elastase neutrofílica
LDH	Lactato desidrogenase

HO-1	Heme-oxigenase
SRM	Espermidina sintase
PAOX	Poliamina oxidase
SSAT	Espermidina/espermina N1-acetiltransferase 1 SMS Espermina sintase

SUMÁRIO

1 **INTRODUÇÃO**

1.1 CICLO BIOLÓGICO DO PARASITO

As Leishmanioses são doenças causadas por parasitas protozoários intracelulares pertencentes à ordem Kinetoplastidae, família Trypanosomatidae e ao gênero *Leishmania*, que acometem ao homem e diferentes espécies de animais silvestres e domésticos (AUWERA; VAN DER AUWERA; DUJARDIN, 2015; WHO, 2020). Esses parasitos caracterizam-se pela presença de um cinetoplasto, estrutura formada por ácido desoxirribonucleico (DNA) altamente compactado dentro da mitocôndria, estando relacionada ao fornecimento de energia para o batimento flagelar e consequente locomoção do parasito (MACNEILL, 2014) (SUNTER; GULL, 2017).

A *Leishmania* é um parasito que possui ciclo de vida digenético envolvendo uma forma promastigota (flagelada e infectante) no trato digestivo do hospedeiro invertebrado e a forma amastigota nos hospedeiros vertebrados (SUNTER; GULL, 2017). A transmissão natural da doença ocorre no momento do repasto sanguíneo, quando as formas promastigotas são inoculadas no tecido subcutâneo, por fêmeas de insetos hematófagos da subfamília Phlebotominae (Díptera, Psychodidae) denominados, genericamente, de flebótomos. No local da picada, essas formas flageladas são fagocitadas pelas células do sistema fagocítico do hospedeiro e transformam-se em amastigotas que se replicam no interior da célula hospedeira (HENARD et al., 2014). Após diversas multiplicações, os macrófagos ficam intensamente infectados e rompem-se, liberando as amastigotas que são rapidamente fagocitadas por novos macrófagos. O ciclo é completado quando os vetores, ao se alimentarem, ingerem sangue e células infectadas do hospedeiro vertebrado. No trato digestivo do vetor, ocorre o rompimento da membrana dos macrófagos e as amastigotas liberadas diferenciam-se na forma promastigota no intestino do vetor (GHAZANFAR; MALIK, 2016) (**Figura 1**).

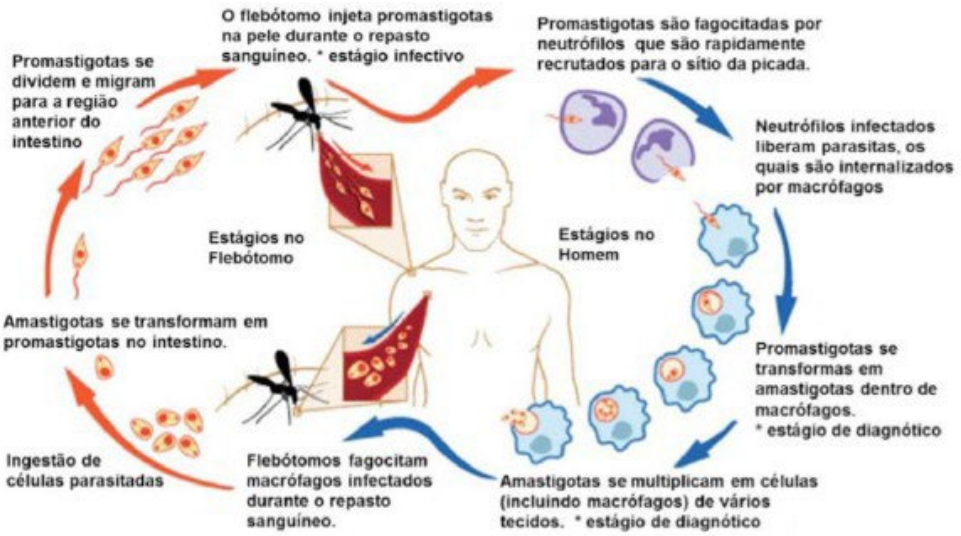

Figura 1- Ciclo biológico da *Leishmania sp.*
Fonte: adaptado (NATIONAL INSTITUTES OF ALLERGY AND INFECTIOUS DISEASES, [2022?])

Os neutrófilos são as primeiras células a migrar para o sítio da infecção, fagocitar e matar os parasitos pela geração do *burst* oxidativo. No entanto, algumas espécies de *Leishmania* são capazes de escapar dos mecanismos de defesa do neutrófilo usando essas células para infectar os macrófagos, seu hospedeiro final (REGLI et al., 2017). Nos macrófagos, o controle ou persistência do parasito irá depender do grau de ativação dessas células (PODINOVSKAIA; DESCOTEAUX, 2015).

Entender como os parasitas do gênero Leishmania são capazes de sobreviver na célula hospedeira favorecendo sua sobrevivência e replicação é extremamente importante para o desenvolvimento de terapias estratégicas contra a doença causada por esse parasito.

1.2 ASPECTOS GERAIS E EPIDEMIOLOGIA DA LEISHMANIOSE TEGUMENTAR

As Leishmanioses são endêmicas em diversos países, distribuídos pela África, Ásia, Europa, América Latina e no Mediterrâneo. Esta doença apresenta uma incidência estimada de 0,7 a 1 milhão de novos casos por ano. Além disso, estima-se que 350 milhões de pessoas estão em risco de contrair Leishmaniose (WHO, 2020). A Leishmaniose é uma doença multifatorial, caracterizada pela diversidade e complexidade da resposta do hospedeiro, agentes etiológicos e vetores, uma vez que pode ser causada por mais de 20 espécies de *Leishmania* e transmitida por aproximadamente 90 espécies de flebotomíneos vetores (AUWERA; VAN DER AUWERA; DUJARDIN, 2015; WHO, 2020). Ambos, espécies de *Leishmania* e vetores, podem ser classificadas de acordo com a distribuição geográfica das áreas endêmicas como espécies do Velho Mundo (Europa e Ásia), transmitidos por vetores do gênero Phlebotomus, e do Novo Mundo (Américas) transmitidos por vetores do gênero Lutzomyia (GONZÁLEZ et al., 2015)([BRASIL,

2007)(BURZA; CROFT; BOELAERT, 2019).

As leishmanioses se apresentam sob diversas formas clínicas, podendo ser divididas em: Leishmaniose Visceral (LV) ou calazar e Tegumentar (LT) (WHO, 2020). A LT é uma antropozoonose considerada uma das doenças mais negligenciadas do mundo e constitui-se um grave problema de saúde pública, atingindo diversos países, sendo que 95% dos casos se concentram nas Américas (principalmente Brasil e Peru), bacia do Mediterrâneo, Oriente Médio (principalmente Afeganistão, Paquistão, Síria e Arábia Saudita) e Ásia Central (WHO 2020) (**Figura 2**).

No Brasil, a distribuição da LT é alta, com registro de casos em todas as regiões brasileiras. Em 2018, foram registrados 16.4323 casos, distribuídos em 1.926 municípios brasileiros (WHO 2018). O crescente número de casos na última década vem sendo atribuído ao melhor diagnóstico e com o aumento de notificações. Além disso, acredita-se que esse cenário pode ser resultado de uma série de fatores de risco como o aumento do desmatamento, urbanização e controle inadequado do vetor (ORYAN; AKBARI, 2016).

A importância da LT reside não somente na sua alta distribuição geográfica e incidência, mas também na possibilidade de assumir manifestações clínicas distintas. Formas graves da doença podem apresentar lesões dermatológicas incapacitantes e desfigurantes, afetando o paciente psicologicamente, com reflexos no campo social e econômico e com graves repercussões na vida do indivíduo (GONTIJO; DE CARVALHO, 2003).

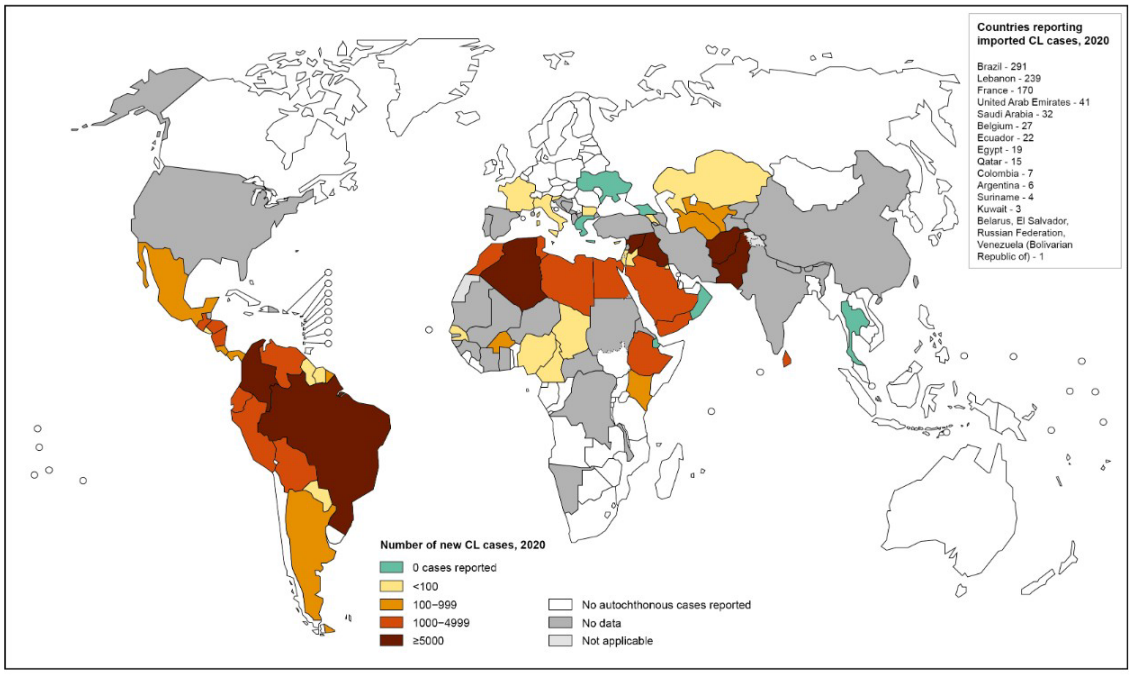

Figura 2 - Distribuição endêmica da Leishmaniose Tegumentar
Fonte: (WHO, 2020)

1.3 FORMAS CLÍNICAS E TRATAMENTO

A LT, forma cutânea da doença, pode manifestar-se em três formas clínicas principais: Leishmaniose Cutânea Localizada (LCL), Leishmaniose Mucocutânea (LCM) e a Leishmaniose Cutânea Difusa (LCD).

No espectro imunológico intermediário a forma mais frequente é a LCL que pode ser causada no Novo Mundo pelas espécies: *Leishmania braziliensis*, *Leishmania amazonensis* e *Leishmania guyanensis.* As lesões de pacientes com LCL são cutâneas, bem definidas por bordas elevadas, suportadas por uma eficiente resposta de células T, que geralmente favorece uma boa resposta à terapia antimonial tradicional, tendendo à cicatrização (BARRAL et al., 1995; BRASIL, 2007). Os cortes histológicos revelam um infiltrado inflamatório tecidual misto com poucos parasitos na lesão estabelecida. Além disso, foi demonstrado que pacientes com LCL apresentam um equilíbrio na resposta imune com aumento de transcritos dos genes inflamatórios (IFN-γ e TNF-α) e também de moléculas inibitórias (IL10, PD1 e PDL1) (CHRISTENSEN et al., 2017).

A LCM e LCD são as formas mais graves da doença encontrando-se em lados opostos no espectro imunológico (SCOTT; NOVAIS, 2016) (BURZA; CROFT; BOELAERT, 2018) (**Figura 3**). No pólo hiperérgico, a LCM caracteriza-se pela exacerbada reação imune fortemente associada com elevada produção de IFN-γ e TNF-α que muitas vezes levam a necrose do tecido mucoso oral, nasal, faríngea e laríngea e consequente escassez de parasitos na lesão (SILVEIRA et al., 2009). Estima-se que 3% dos casos de LT causados por *L. braziliensis* desenvolvam lesão cutânea mucosa (BOAVENTURA et al., 2009). No pólo anérgico, encontra-se a LCD, com lesões nodulares e refratariedade ao tratamento medicamentoso utilizado (GONTIJO; DE CARVALHO, 2003). Essas lesões apresentam grande quantidade de parasitas, baixos níveis de citocinas Th1 e altos títulos de anticorpos circulantes. Além disso, pacientes com LCD são fortemente marcados pela elevada produção de IL-4 e IL-10, enquanto os níveis dessas citocinas são encontradas em baixas concentrações nos pacientes com LCM (SILVEIRA et al., 2009) (FRANÇA-COSTA et al., 2015) (SCOTT; NOVAIS, 2016).

Outra importante manifestação clínica que muitas vezes pode ser confundida com a LCD é a Leishmaniose Disseminada (LD). Normalmente, esses pacientes apresentam numerosas lesões devido a rápida metástase do parasito que podem ser nodulares ou ulceradas (SCORZA; CARVALHO; WILSON, 2017). Além disso, em alguns casos podem ser observadas lesões na mucosa nasal semelhante as observadas da LCM (SCORZA; CARVALHO; WILSON, 2017). No espectro imune da doença a LD é caracteriza pela produção de citocinas Th1 como TNF-α e IFN-γ, no entanto, esses níveis são menores que os produzidos na LCL (SCORZA; CARVALHO; WILSON, 2017).

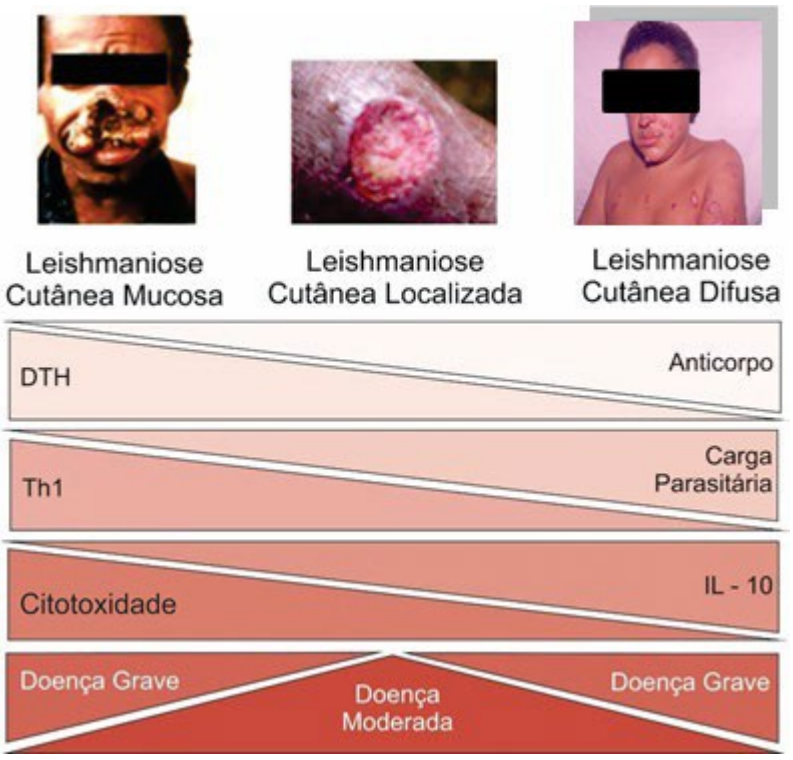

Figura 3 - Aspectos imunológicos observados na Leishmaniose Tegumentar. (Adaptado de: Manual de Vigilância Tegumentar Americana.
Fonte: (BRASIL, MINISTÉRIO DA SAÚDE, 2009; SCOTT; NOVAIS, 2016).

Fármacos antimoniais pentavalentes, como o glucantime, são a primeira classe de drogas utilizadas durante o tratamento das leishmanioses. Embora esses fármacos sejam utilizados em todas as formas clínicas de LT, seu uso traz algumas limitações e efeitos colaterais (JIRMANUS et al., 2012). Outros fármacos como a anfotericina B e outras pentamidinas são frequentemente utilizadas como tratamentos alternativos em pacientes com LT que apresentaram falha terapêutica ou não responderam ao tratamento com antimoniais (MEYERHOFF, 1999). A resistência aos fármacos, mesmo os de segunda escolha, apresenta- se como um problema durante o tratamento da doença (GUERIN et al., 2002).

Formas clínicas de leishmaniose cutânea causada por *L. braziliensis*, apresentam baixa taxa de cura após o tratamento com os antimoniais de primeira escolha quando comparado com outras espécies (JIRMANUS et al., 2012). Prates e colaboradores demonstraram que o tratamento com glucantime foi capaz de curar apenas 54% dos pacientes tratados, sendo necessária a identificação de novos fármacos ou estratégias terapêuticas (PRATES et al., 2017).

Em 2005 foi demonstrado que a combinação do GM-CSF com o glucantime foi eficiente em melhorar a resposta aos antimoniais nos pacientes refratários ao tratamento (ALMEIDA et al., 2005; PRATES et al., 2017). Ensaios clínicos controlados também mostraram a eficácia da miltefosina no tratamento das leishmanioses com taxa de cura de 71% (pacientes com LCL causada *L. guyanensis*) (CHRUSCIAK-TALHARI et al., 2011) e 75% (pacientes com LCL causada por *L. braziliensis*) (MACHADO et al., 2010) quando comparado ao tratamento tradicional. Embora a associação da miltefosina com GM-CSF tópico não tenha alterado a taxa de cura ou o tempo de cicatrização das lesões, o uso da miltefosina se mostrou eficaz, podendo ser

utilizada como terapia alternativa em pacientes refratários ou para prevenir o desenvolvimento de resistência aos fármacos tradicionais (MACHADO et al., 2020).

1.4 PAPEL DA VIA DA ARGINASE E POLIAMINAS NA INFECÇÃO POR LEISHMANIA

A ativação diferencial do macrófago pode influenciar fortemente o desfecho da infecção por *Leishmania*. Enquanto o controle da infecção está mais associado à resposta imune Th1, a predominância da resposta Th2 se relaciona a maior susceptibilidade à doença (PESSENDA; DA SILVA, 2020). Nesse contexto, duas enzimas que utilizam o aminoácido L- arginina como substrato (a arginase 1 (ARG1) e a óxido nítrico sintase (NOS)) representam dois possíveis caminhos para a ativação macrofágica e imune (WANASEN; SOONG, 2008).

A resposta protetora contra os parasitas é iniciada por células Th1. Na ativação macrofágica clássica induzida pelo IFN-γ e o TNF-α, a iNOS (óxido nítrico sintase) oxida a *L*-arginina em Óxido Nítrico (NO) e citrulina ativando mecanismos microbicidas da célula hospedeira (PESSENDA; DA SILVA, 2020). Por outro lado, na presença de IL-4, IL-10, IL- 13 e TGF-β, a L-arginina é preferencialmente metabolizada pela ARG1 produzindo poliaminas que favorecem o sucesso da infecção (WANASEN; SOONG, 2008).

Após a ativação da via das poliaminas pela ARG1 uma série de cátions alifáticos são gerados. Entre eles, a putrescina, a cadaverina, a espermidina e a espermina estão envolvidos em diversos processos biológicos essenciais como a regulação e expressão gênica, modulação da sinalização celular, estabilização de membranas e proliferação celular (HANDA; FATIMA; MATTOO, 2018)(PEGG, 2016). Esses metabólitos são convertidos a partir da L-arginina em L-ornitina e ureia pela ARG1 (MUXEL et al., 2018; PEGG, 2016). Em seguida a ornitina descarboxilase (ODC) catalisa a conversão de L-ornitina em putrescina (MUXEL et al., 2018). Essa por sua vez, participa de uma cascata de reações envolvendo várias enzimas como a espermidina sintase (SRM) e espermina sintase (SMS) que resulta na formação das poliaminas espermidina e espermina, respectivamente (MUXEL et al., 2018) (**Figura 4**). O aumento das poliaminas livres induz altos níveis de espermidina/espermina N1-acetiltransferase 1 (SSAT) que juntamente com a poliamina oxidase (PAOX) participa da interconversão das poliaminas (CASERO; MURRAY STEWART; PEGG, 2018). Essa via catabólica é importante no controle dos níveis de poliaminas livres uma vez que sua disponibilidade pode estar associada ao metabolismo celular, mas também a dieta alimentar e a produção intestinal por bactérias presentes na microbiota (MADEO et al., 2018) (CASERO; MURRAY STEWART; PEGG, 2018).

Trabalhos anteriores do grupo demonstram que a inibição da via da arginase é capaz de conter a carga parasitária em macrófagos humanos infectados *in vitro* por *L. amazonensis*. O tratamento com Nω-hydroxy-nor-arginina (nor-NOHA) e Difluorometilornitina (DFMO), inibidor da arginase e da ODC respectivamente, resultou no controle do parasito e na alteração do perfil de

citocinas produzidas no sobrenadante de culturas de macrófagos humanos infectados (FRANÇA-COSTA et al., 2015). Além disso, o trabalho mostrou que os níveis de ARG-1 se correlacionaram positivamente com o TGF-β e PGE2 no plasma de pacientes com LCD, podendo estar contribuindo com a ineficiente resposta imune observada nesses pacientes (FRANÇA-COSTA et al., 2015).

Além da importância das citocinas na ativação de macrófagos, seja ela a alternativa ou a clássica, vem sendo descrito que os mediadores lipídicos também são capazes de induzir essa ativação. Nesse contexto, destacam-se as prostaglandinas onde, a depender do modelo experimental, podem desempenhar papéis antagônicos durante a ativação macrofágica.

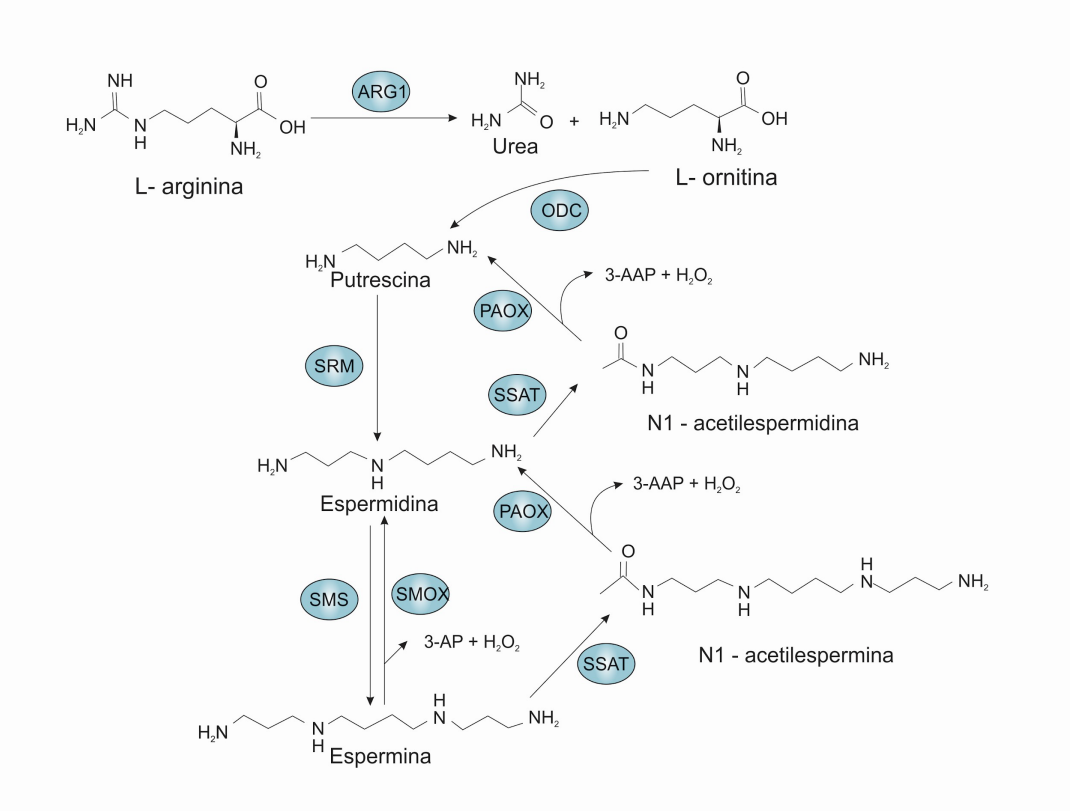

Figura 4 - Via biossintética das poliaminas
Fonte: Adaptado e traduzido de (CASERO; MURRAY STEWART; PEGG, 2018)

1.5 MEDIADORES LIPÍDICOS

1.5.1 **Mediadores lipídicos na resposta inflamatória: síntese e função**

Mediadores lipídicos são moléculas orgânicas de curta duração que são liberadas durante todas as etapas da resposta inflamatória e estão fortemente associadas com uma série de processos fisiológicos e patológicos incluindo câncer, cicatrização de lesões e doenças inflamatórias e infecciosas (TUNCER; BANERJEE, 2015).

A biossíntese dos mediadores lipídicos ocorre a partir da conversão dos ácidos graxos poli-insaturados (PUFAs) derivados dos fosfolipídios em: AA (ácido araquidônico), EPA (ácido eicosapentanóico) ou em DHA (ácido docosahexanóico) (SZEFEL; KRUSZEWSKI; SOBCZAK,

2015). A disponibilidade desses ácidos graxos depende da suplementação nutricional que altera a composição da membrana plasmática e da capacidade da fosfolipase A2 (PLA2) de retirá-los dos fosfolipídios de membrana. Após liberados pela PLA2, o AA, EPA e DHA podem ser então metabolizados pelas enzimas ciclooxigenase (COX) ou pela lipoxigenase (LO) em mediadores lipídicos biologicamente ativos (PETERS-GOLDEN; HENDERSON, 2007).

Os mediadores lipídicos sintetizados pelas vias da COX e LO incluem uma série de prostanóides, leucotrienos (LTs), epoxinas, lipoxinas e SPMs que são diferencialmente produzidas em diferentes tipos celulares por interações cooperativas entre enzimas (BOZZA et al., 2011). O AA é convertido em prostaglandinas (PGs) e tromboxano A2, quando metabolizado pela COX-1 e em LTs, lipoxinas e mediadores lipídicos imunoreguladores como os ácidos hidroxieicosatetraenoico (12-/15-HETE) quando metabolizado pela 5-LO e 12/15- LO (BIEREN, 2017). O EPA e DHA derivados enzimaticamente do ω - 3 (ω - 3 PUFAs), após sua biossíntese produzem as resolvinas da série E (RvE1) e as da série D (RvD1) respectivamente (SERHAN; CHIANG; DALLI, 2015) via ação das LO.

Finalmente, o citocromo P450 (CYP1A1 e CYP1B1) representa outra via que metaboliza os PUFAs e aumentam o repertório imune regulatório dos mediadores lipídicos (LEFÈVRE et al., 2015) (**Figura 5**).

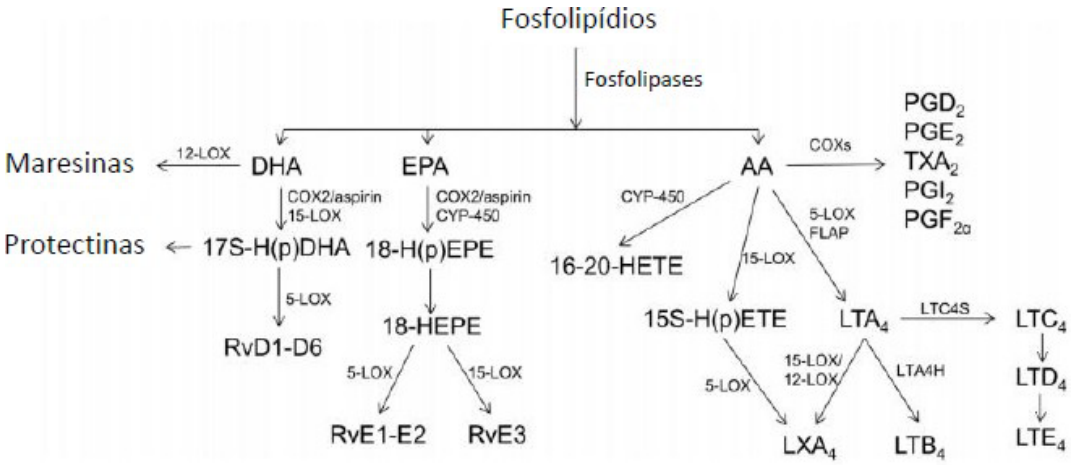

Figura 5 - Via de biossíntese dos mediadores lipídicos
Fonte: adaptado de (DE MACEDO et al., 2018).

Os mediadores lipídicos são conhecidos como sinalizadores lipídicos envolvidos na modulação do sistema imune (DOYLE; SADLIER; GODSON, 2018) (BIEREN, 2017), além de serem importantes em processos patológicos (BOZZA et al., 2011). Esses mediadores também desempenham um importante papel em todos os estágios da resposta inflamatória, desde os eventos iniciais até a resolução da inflamação. Após lesão tecidual ou invasão de organismos, inicia-se uma sequência de eventos que caracterizam a resposta inflamatória aguda, que tem como objetivo o reparo tecidual ou eliminação dos invasores (SERHAN; CHIANG; DALLI, 2015).

A produção de mediadores pró-inflamatórios como as PGs e LTs gerados durante a fase aguda da resposta inflamatória, são responsáveis pela vasodilatação, aumento da permeabilidade

vascular e recrutamento celular de neutrófilos (SERHAN; CHIANG; DALLI, 2015). Essa fase inicial está associada à produção de diferentes mediadores lipídicos inflamatórios a depender do tipo de célula e substratos presentes (SUGIMOTO et al., 2016). Já nos estágios tardios, a fagocitose de neutrófilos apoptóticos pelos macrófagos recrutados para o sítio da lesão, induz uma reprogramação no exsudato com produção de mediadores especializados na pró-resolução que promovem a pausa na produção dos mediadores pró-inflamatórios e redução do influxo de células ao sítio inflamatório, ocasionando a resolução da inflamação (SUGIMOTO et al., 2016).

Ainda não é conhecido o exato momento em que ocorre essa reprogramação, no entanto sabe-se que essa mudança implica em modificações transcricionais e pós-translacionais que envolvem a PGE2 e a PGD2 (CROASDELL et al., 2015). A prostaglandina E2 tem quatro receptores prostanóide acoplado a proteína G: EP1, EP2, EP3 e EP4, que se ligam a PGE2 induzindo diferentes vias de sinalização e várias funções efetoras, refletindo as funções ubíquas de PGE2 (HATA; BREYER, 2004; KALINSKI, 2012; SUGIMOTO; NARUMIYA, 2007), dentre elas a estimulação na produção de IL-10, citocina anti-inflamatória que bloqueia a via da inflamação crônica (CROASDELL et al., 2015). Além disso, o PGD2 também pode ser convertido em subprodutos (PGJ2 e 15-deoxi-1(12,14) -PGs J2 (15-D-PGJ2)) que ativam a via do PPAR-γ e promovem a resolução (CROASDELL et al., 2015).

1.5.2 **Mediadores lipídicos especializados na resolução da inflamação**

Além do envolvimento dos eicosanóides em diferentes modelos de inflamação e doenças infecciosas, uma nova super-família de mediadores lipídicos derivados enzimaticamente do ω - 3 PUFAs, como o EPA e o DHA vem sendo estudada. Eles funcionam como SPMs (mediadores especializados na pró-resolução), marcando a transição da fase inicial da inflamação para a fase de resolução durante a resposta inflamatória (SERHAN; CHIANG; DALLI, 2015) (**Figura 6**). Esses SPMs incluem lipoxinas, resolvinas, protectinas e maresinas, e compreendem a nova família de autacóides com potente ação anti-inflamatória e de resolução (SERHAN; CHIANG; DALLI, 2015).

Os efeitos biológicos atribuídos aos SPMS estão altamente associados com suas propriedades anti-inflamatórias e imunomodulatória, que incluem a inibição da quimiotaxia e aderência dos leucócitos, aumento da fagocitose de bactérias e neutrófilos apoptóticos (**Figura 6**) (SERHAN; CHIANG; DALLI, 2018) e bloqueio na produção de citocinas como o TNF-α, IFN-γ e IL-6, ao mesmo tempo em que induz o aumento da expressão de IL-10 (SERHAN; CHIANG; DALLI, 2018; WANG et al., 2011) (CHIURCHIÙ et al., 2016) (CHENG; RONG, 2019).

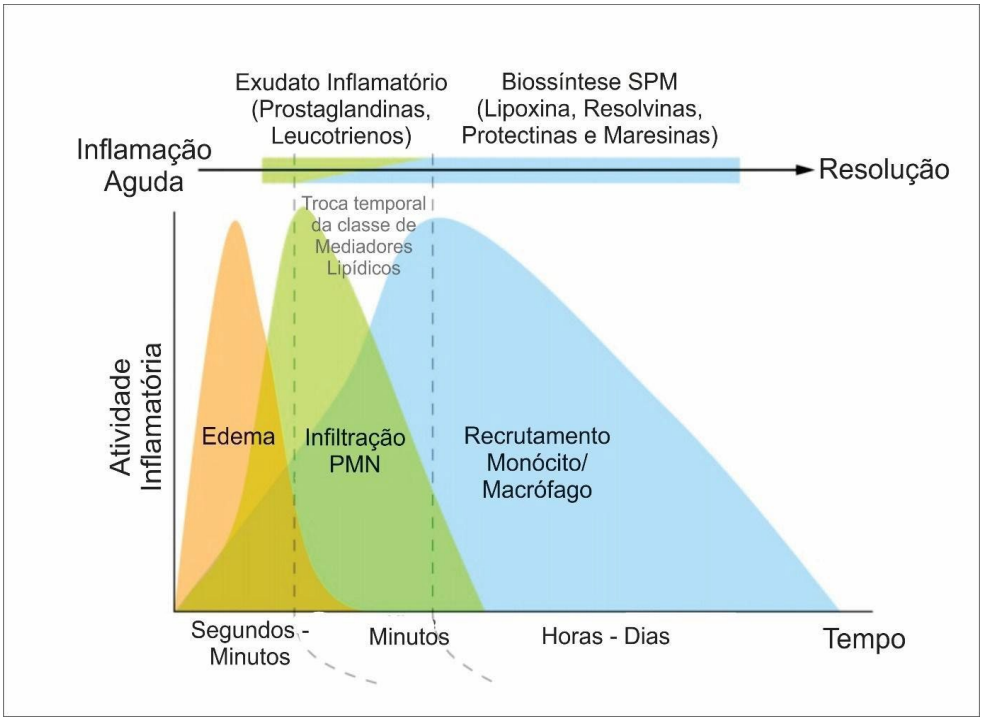

Figura 6 - Etapas da resposta inflamatória
Fonte: adaptado de (SERHAN; LEVY, 2018).

A atividade anti-inflamatória das resolvinas vem sendo descrita em vários modelos de doenças inflamatórias, incluindo doenças cardiovasculares, câncer, inflamação renal aguda, lesões pulmonares e infecções virais e bacterianas (SERHAN; CHIANG; DALLI, 2015). Durante infecções bacterianas, resolvinas da série D foram capazes de regular enzimas e citocinas inflamatórias após o tratamento (CROASDELL et al., 2016). Mais recentemente, foi demonstrado o papel anti-inflamatório da RvD1 em pacientes com doença de Chagas (OGATA et al., 2016) e com hanseníase (SILVA; BELISLE, 2018). Células mononucleares do sangue periférico (PBMCs) de pacientes, após tratadas com altas doses de RvD1 foram capazes de reduzir a produção de IFN-γ e a proliferação celular induzida pelos antígenos de *T. cruzi* (OGATA et al., 2016). Além disso, altos níveis plasmáticos de RvD1 foram associados à infecção por *Mycobacterium leprae* aumentando a susceptibilidade à infecção *(OGATA et al., 2016; SILVA; BELISLE, 2018)*. O aumento dos níveis de resolvina antes do tratamento, induz a fagocitoce e eferocitose do patógeno e seus antígenos além de inibir as respostas Th1 e Th17 favorecendo a sua sobrevivência (SILVA; BELISLE, 2018).

Em humanos as ações da RvD1 são mediadas via receptores acoplados a proteínas G como o ALX/FPR2 e o GPR32 (SANSBURY et al., 2020). Embora ainda não esteja completamente descrito na literatura os receptores e vias ativadas pelos SPM, sabe-se da importância do eixo RvD1 – ALX/FPR2 na resolução da inflamação, eferocitose e redução na produção de citocinas inflamatórias (SANSBURY et al., 2020).

1.5.3 **Mediadores lipídicos na LT**

O desequilíbrio entre as respostas imunes Th1 e Th2 vem se mostrando um grande paradigma no combate às infecções por organismos intracelulares (LÓPEZ-MUÑOZ et al., 2018). Por outro lado, a dicotomia na expressão dos mediadores lipídicos produzidos durante a infecção vem sendo associada ao perfil imune do hospedeiro e a progressão da doença. A PGE_2 suprime a resposta imune Th1 inibindo a produção de IFN-γ, TNF-α e IL-12 (KALINSKI, 2012) (LÓPEZ-MUÑOZ et al., 2018) ao mesmo tempo em que é capaz de induzir a produção de citocinas do perfil Th2, bem como IL-10, IL-4 e TGF- β (BARATELLI et al., 2010). Os produtos da via da 5-LO como o LTB_4, por sua vez, induzem o aumento da produção de mediadores pró-inflamatórios e óxido nítrico, importante no controle de infecções em diversos modelos (BROCK; PETERS-GOLDEN, 2007).

No contexto de infecção por *Leishmania,* essa dicotomia está implicada no controle, ou sucesso da infecção. Trabalhos anteriores do grupo mostraram que a distinta ativação da via dos eicosanóides está associada às diferentes formas clínicas de LT (FRANÇA-COSTA et al., 2016). Pacientes com LCL apresentam significativamente maiores níveis plasmáticos de PGE_2 enquanto pacientes com LCM exibiram altos níveis de LTB_4 (FRANÇA-COSTA et al., 2016). Além disso, pacientes com a forma mais grave da doença (LCD) apresentaram altos níveis plasmáticos de RvD1 (MALTA-SANTOS et al., 2017) assim como expressão de RNAm de enzimas envolvidas na síntese de PGE_2 (FRANÇA-COSTA et al., 2015). Um estudo prospectivo de pacientes com LV demonstrou que o tratamento anti-leishmania foi capaz de alterar os níveis de proteínas inflamatórias e mediadores lipídicos (ARAÚJO-SANTOS et al., 2017).

Em modelos de infecção *in vitro* por *Leishmania amazonensis* a secreção de PGE_2 vem sendo associada ao favorecimento da sobrevivência do parasito, enquanto que o aumento dos níveis de LTB_4 controla a carga parasitária em células hospedeiras infectadas (MORATO et al., 2014). Macrófagos infectados por *Leishmania major* demonstram alta expressão dos receptores EP1 e EP3, além de altos níveis de PGE_2 que modulam a resposta imune e o papel das células B (ARCANJO et al., 2017). Sob o efeito do PGE_2, essas células modulam a atividade fagocítica dos macrófagos pela liberação de IL-10 resultando na resistência da infecção (ARCANJO et al., 2017).

Trabalhos do grupo mostram a participação do LTB_4 em conter a infecção por *L. amazonensis* em neutrófilos humanos pela indução da produção de ROS e a ativação do NFκB levando a produção de LTB_4 via TLR2 (TAVARES et al., 2014). A inibição da 5-LO, via biossintética de produção do LTB_4, com o zileuton foi capaz de aumentar a carga parasitária de neutrófilos infectados, confirmando a importância desse mediador em controlar a infecção (TAVARES et al., 2014). O papel do neutrófilo durante a infecção vai depender da espécie do parasito envolvido. Durante infecções por *L. braziliensis,* essas células contribuem para o controle

(FALCÃO et al., 2015), no entanto quando infectados por *L. major,* observou-se que o parasito é capaz de subverter o sistema imune do hospedeiro modulando o perfil de eicosanoides e favorecendo a infecção (PLAGGE; LASKAY, 2017).

A infecção por *L. amazonensis* foi capaz de induzir altos níveis de RvD1 em macrófagos humanos infectados e a suplementação exógena desse mediador favoreceu a replicação do parasita (MALTA-SANTOS et al., 2017). De fato, tem sido descrita a habilidade de promastigotas modificarem o fenótipo macrofágico do hospedeiro produzindo PUFAs específicos que favorecem a sua sobrevivência (PALOQUE et al., 2019).

Embora trabalhos anteriores do nosso grupo tenham ampliado o conhecimento a respeito do papel dos eicosanóides na modulação da resposta imune e controle da infecção por *Leishmania*, o papel das resolvinas durante a infecção de neutrófilos ainda não foi estudado. Entender esses mecanismos, bem como as estratégias de evasão do parasito, são importantes para desenvolver fármacos capazes de contrabalançar a evasão da *Leishmania*.

Nesse estudo, identificamos potenciais biomarcadores de gravidade de doença e de falha terapêutica em pacientes com LT e examinamos o papel da RvD1 e seus efeitos anti-inflamatórios no contexto da infecção de neutrófilos por *L. braziliensis*.

2 **JUSTIFICATIVA**

A ativação da via da arginase e dos mediadores lipídicos desempenham um papel crucial durante a infecção por *Leishmania* (AFONSO et al., 2008) (FRANÇA-COSTA et al., 2015) (FRANÇA-COSTA et al., 2016) (MUXEL et al., 2017b). Trabalhos anteriores mostraram que a via biossintética das poliaminas é crucial para o crescimento e sobrevivência do parasita (MUXEL et al., 2017b). Além disso, altas concentrações de ARG1, ODC, PGE_2 e TGF-β foram encontradas no plasma de pacientes com LC (FRANÇA-COSTA et al., 2015). Por outro lado, também foi demonstrado pelo grupo que a distinta produção de eicosanóides (PGE e LTB) está envolvida no estabelecimento da infecção e com bioassinaturas inflamatórias das distintas formas de manifestações clínicas da LT (FRANÇA-COSTA et al., 2016). No entanto os referidos trabalhos não exploraram prospectivamente o perfil de ativação da via das poliaminas em pacientes com LT bem como a identificação de biomarcadores de falha terapêutica.

Fármacos antimoniais pentavalentes, como o glucantime, são a primeira classe de drogas utilizadas durante o tratamento das leishmanioses. No entanto, estudos têm mostrado que no Brasil cerca de 45% dos pacientes apresentam falha na terapia (MACHADO et al., 2010). Por ser uma doença associada à falha de uma resposta imune a *Leishmania*, a identificação de fatores chaves implicados no sucesso do estabelecimento da infecção se mostram como uma importante ferramenta para o controle da doença. Nesse sentido, a identificação de potenciais biomarcadores de gravidade de doença ou controle do parasito pode favorecer o desenvolvimento de terapias direcionadas ao hospedeiro que podem incrementar o manejo clínico de casos graves/complicados.

O papel dos mediadores especializados na resolução da inflamação, assim como seus mecanismos de ação vem sendo cada vez mais explorado no contexto da infecção. É fato que seu efeito vai depender de uma série de fatores, como por exemplo a espécie de parasito e tipo de célula envolvida. Por outro lado, até o presente momento não existem trabalhos que avaliem o efeito das resolvinas na infecção de neutrófilos por *Leishmania*.

Neutrófilos são as principais células encontradas em lesões de pacientes com LCL sendo cruciais na infecção devido a seu papel protetor. O influxo dessas células para o local afetado, assim como a fagocitose de agentes invasores pode ser regulado por mediadores de pró-resolução como as resolvinas. Trabalhos anteriores do grupo mostraram que a resolvina D1 favorece a replicação intracelular de *L. amazonensis* em macrófagos humanos pela indução de mecanismos antioxidantes (MALTA-SANTOS et al., 2017).

No presente estudo, investigamos a ativação da via das poliaminas e aminoácidos em pacientes com diferentes manifestações da LT e analisamos prospectivamente se proteínas plasmáticas e mediadores lipídicos poderiam ser utilizados como biomarcadores de falha terapêutica. Além disso, devido à associação das resolvinas com respostas supressoras e antioxidantes que favorecem a replicação do parasito, iremos buscar avaliar se a RvD1 está

envolvida no favorecimento da proliferação da *L. braziliensis*, principal agente etiológico da forma localizada da doença e se ela é capaz de alterar a habilidade dos neutrófilos em controlar a infecção.

Tendo em vista que este trabalho tem três abordagens, duas em relação a identificação de biomarcadores de gravidade de doença e falha terapêutica e outra do aspecto de resistência à infecção por Leishmania, achamos conveniente dividi-lo em três partes.

3 **PARTE I**

3.1 HIPÓTESE

A via das poliaminas é diferentemente ativada entre os pacientes das diversas formas clínicas da LT.

3.2 OBJETIVOS

3.2.1 **Objetivo geral**

Investigar o papel da via das poliaminas e sua ativação entre os pacientes com diferentes formas clínicas de LT.

3.2.2 **Objetivos específicos**

- Verificar os níveis plasmáticos de poliaminas e aminoácidos em indivíduos com LCD em comparação a LCL e LCM;
- Analisar a expressão de enzimas e transportadores da via das poliaminas em lesões de pacientes com as diferentes formas clínicas;
- Identificar o perfil de expressão gênica da via das poliaminas nas lesões de pacientes com LCD, LCL e LCM;
- Avaliar se a ativação dos genes da via das poliaminas se associa com a carga parasitária nas lesões de pacientes com LT;
- Verificar se a via das poliaminas contribui com os diferentes fenótipos da leishmaniose tegumentar.

4 **MANUSCRITO 1**

Esse trabalho investiga a ativação distinta da via das poliaminas e sua participação na patogênese da Leishmaniose Cutânea Difusa.

4.1 **DIFFERENTIAL EXPRESSION OF POLYAMINE BIOSYNTHETIC PATHWAYS IN SKIN LESIONS AND IN PLASMA REVEALS DISTINCT PROFILES IN DIFFUSE CUTANEOUS LEISHMANIASIS**

Resumo

Leishmaniose Tegumentar (LT) é uma doença parasitária que pode resultar em um amplo espectro de manifestações clínicas. É necessário entender determinantes do hospedeiro e do parasito para compreender os diferentes desfechos clínicos e identificar novos alvos terapêuticos. Trabalhos anteriores têm indicado que a via de biossíntese das poliaminas é fundamental para o crescimento e sobrevivência da *Leishmania*. Apesar de sua importância, a expressão da via não tem sido anteriormente investigada em pacientes com LT. Nesse estudo, realizamos uma análise exploratória utilizando ferramentas de Biologia de Sistemas para comparar as concentrações circulantes de poliaminas e aminoácidos, bem como a expressão gênica da via de poliaminas em lesões cutâneas de pacientes com as diferentes formas clínicas de LT. A LCD foi associada com maiores concentrações de aminoácidos, poliaminas e seus transportadores do que os pacientes com LCM ou LCL. Além disso, a expressão de RNA de genes relacionados às poliaminas em lesões de pacientes de duas coortes independentes, demonstrou que a ativação diferencial dessa via está associada com a carga parasitária e é eficaz em separar os espectros clínicos da LT. Esses resultados destacam um novo aspecto da imunopatogênese da LCD sugerindo que a via das poliaminas pode servir como uma potencial estratégia terapêutica para controlar a carga parasitária da doença.

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Differential expression Of polyamine biosynthetic pathways in skin lesions And in plasma reveals distinct profiles in diffuse cutaneous leishmaniasis

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Tegumentary leishmaniasis (TL) is a parasitic disease that can result in wide spectrum clinical manifestations. It is necessary to understand host and parasite determinants of clinical outcomes to identify novel therapeutic targets. Previous studies have indicated that the polyamine biosynthetic pathway is critical for *Leishmania* growth and survival. Despite its importance, expression of the such pathway has not been previously investigated in TL patients. We performed an exploratory analysis employing Systems Biology tools to compare circulating polyamines and amino acid concentration as well as polyamine pathway gene expression in cutaneous lesions patients presenting with distinct TL disease presentations. Diffuse cutaneous leishmaniasis (DCL) was associated with higher concentrations of amino acids, polyamines and its substrate transporters than mucosal cutaneous leishmaniasis or localized cutaneous leishmaniasis. In addition, the RNA expression of polyamine- related genes of patients lesions from two separate cohorts demonstrated that differential activation of this pathway is associated with parasite loads and able to discriminate the clinical spectrum of TL. Taken together, our findings highlight a new aspect of DCL immunopathogenesis indicating that the polyamine pathway may be explored as a novel therapeutic target to control disease burden.

Leishmania infection causes Tegumentary Leishmaniasis (TL), which exhibits a broad spectrum of clinical manifestations. Clinical forms vary from self-healing localized cutaneous leishmaniasis (LCL), with a moderate cell-mediated immune response, to more severe forms such as the hyper-inflammatory mucocutaneous leishma- niasis (MCL); both conditions are caused by *L. braziliensis*. A less common disease manifestation is the diffuse cutaneous leishmaniasis (DCL), which is caused by *L. amazonensis* and associated with immune anergy[1,2]. The differences observed between the distinct clinical forms of TL and its associated immune activation are described

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to be linked to the parasite load in lesion sites[3]. In MCL lesions, parasites are rarely detected whereas in DCL lesions heavily parasitized macrophages are usually observed[2]. We have previously shown high concentrations of arginase-1 (ARG1), ornithine decarboxylase (ODC), prostaglandin E2 (PGE2) and transforming growth factor β (TGF-β) in DCL patients[4], which could contribute to an ineffective immune response unable to hamper parasite replication. Although recent studies have shown that components of the polyamine biosynthetic pathway are linked to survival of *Leishmania sp.* inside macrophages in experimental settings[5,6] it is unknown whether there is a differential expression of such components in patients with distinct clinical forms of TL.

Among the metabolites from the polyamine pathway, putrescine, cadaverin, spermidine and spermine are aliphatic cations derived from amino acids such as l-arginine and lysine, with multiple functions which are essential for all living organisms[7]. Polyamines are critically involved in a diverse range of cellular processes such as regulation of gene expression and translation, modulation of cell signaling, membrane stabilization and cell proliferation[7,8]. These metabolites are synthesized in a reaction catalyzed by ARG1, which converts l-arginine to l-ornithine and urea[6]. Another enzyme, ODC, catalyzes l-ornithine conversion to putrescine[6]. Putrescine then participates in an intricate cascade of reactions involving several enzymes such as spermidine synthase (SpdS) and spermine synthase (SpmS), which results in formation of polyamines, spermidine and spermine, respectively[6]. Cadaverine, a polyamine poorly studied in humans, is derived from the amino acid lysine[9].

The uptake of l-arginine in macrophages infected with *Leishmania sp.* occurs via transporters from the cati- onic amino acid family (CAT)[10]. Hence, inhibition of the l-arginine transporter by melatonin reduces parasite burden by decreasing the production of polyamines[11]. We have previously demonstrated that treatment of *L. amazonensis* infected macrophages with arginase or ODC inhibitors leads to enhanced parasite clearance and dampened secretion of pro-inflammatory cytokines[4]. Indeed, different immune response profiles can influence l-arginine catabolism that, ultimately, result in resistance or susceptibility to *Leishmania* infection. l-arginine is catabolized by ARG1 in the presence of interleukin 4 (IL-4), IL-10, IL-13 and TGF-β, producing polyamines and collagen and enhancing *Leishmania* infection[12]. In converse, in the presence of pro-inflammatory mediators, such as interferon γ (IFNγ), tumor necrosis factor α (TNFα) and IL-12, the nitric oxide synthase 2 (iNOS/NOS2) will be preferentially activated, resulting in production of nitric oxide (NO) and citrulline[12,13]. Although NO alone is not sufficient to control infection, it can be further metabolized in reactive nitrogen and oxygen species, which are then involved in parasite killing[14,15]. Therefore, the profile of the host immune responses dictates differential activation of the polyamine biosynthetic pathway which strongly influences the outcome of *Leishmania* infection. In the present study, we examined in situ (in skin lesions) and systemic concentrations of enzymes and products from the polyamine pathway in patients with LCL, MCL and DCL. We identified a distinct biosigna- ture of DCL, with increased expression of polyamine enzymes and transporters in skin lesions and in plasma samples of DCL as compared to MCL and LCL. In addition, patients with DCL exhibited a distinct profile of gene expression in lesions. These findings suggest that the polyamine pathway contributes to disease phenotypes in tegumentary leishmaniasis.

Results

DCL patients exhibit high plasma levels of polyamines and amino acids. Initially we tested if there is a distinct systemic profile of plasma concentrations of arginase-1 (protein), amino acids and free poly- amines in patients with TL. We found that patients with DCL presented a distinct profile compared to LCL and MCL patients (Fig. 1A). We observed that the relative systemic concentrations of arginase-1, cadaverine and spermidine, but not of putrescine, were significantly higher in DCL, compared with either LCL, MCL patients or health controls (Fig. 1B, C and Table S1). Moreover, concentrations of ornithine and citrulline, but not of arginine, were significantly higher in DCL patients compared to LCL (Fig. 1B). Noteworthy, our analyses showed that, among the polyamines, cadaverine was the most abundant in DCL patients, relative to the other TL clinical forms (Fig. 1C).

Expression of genes from the polyamine pathways in lesion biopsy specimens from patients with DCL. To further characterize the polyamine pathway in DCL, LCL and MCL patient lesions, we evalu- ated mRNA transcripts for key molecules isolated from skin biopsies from these clinical forms using a pre- defined nanostring panel (Table 1). We observed a specific profile of gene expression that, when combined, could successfully discriminate the different clinical groups (Fig. 2A, B). Notably, patients with DCL exhibited substantial up regulation of all genes from the polyamine pathway, except *ODC*, compared with LCL or MCL patients (Fig. 2B). Among all genes examined, we found that the expression values of *CAT2A* (isoform encoded by *SLC7A2*), *ARG1* and *SMS* were significantly higher in DCL lesions compared to MCL lesions (Fig. 2C).

Transcriptomic analyses of tegumentary lesions from publicly available datasets validate the differential expression of the polyamine biosynthetic pathway in distinct clinical forms of leishmaniasis. To validate our findings on the polyamine pathway in TL, we re-analyzed the gene expres- sion data from an independent patient cohort, which was recently published[16]. This approach revealed that skin lesions from TL patients generally exhibit a distinct gene expression profile compared to normal skin from uninfected healthy endemic controls (Fig. 3A). Fold-difference analysis highlighted that DCL patients exhibited higher expression of *AMD1*, whereas *SLC7A2* was down modulated compared to health controls (Fig. 3A). Addi- tional investigation using a principal component analysis (PCA) model revealed that the overall gene expression profile of the transcripts evaluated here was able to effectively segregate the TL clinical forms (Fig. 3B, C). Such PCA model including expression values of all genes validated the results from the fold-difference analysis, with segregation of all clinical forms (Fig. 3B). Indeed, a discriminant analysis using ROC curves of such combination of genes resulted in high accuracy in distinguishing the clinical groups (Fig. 3C).

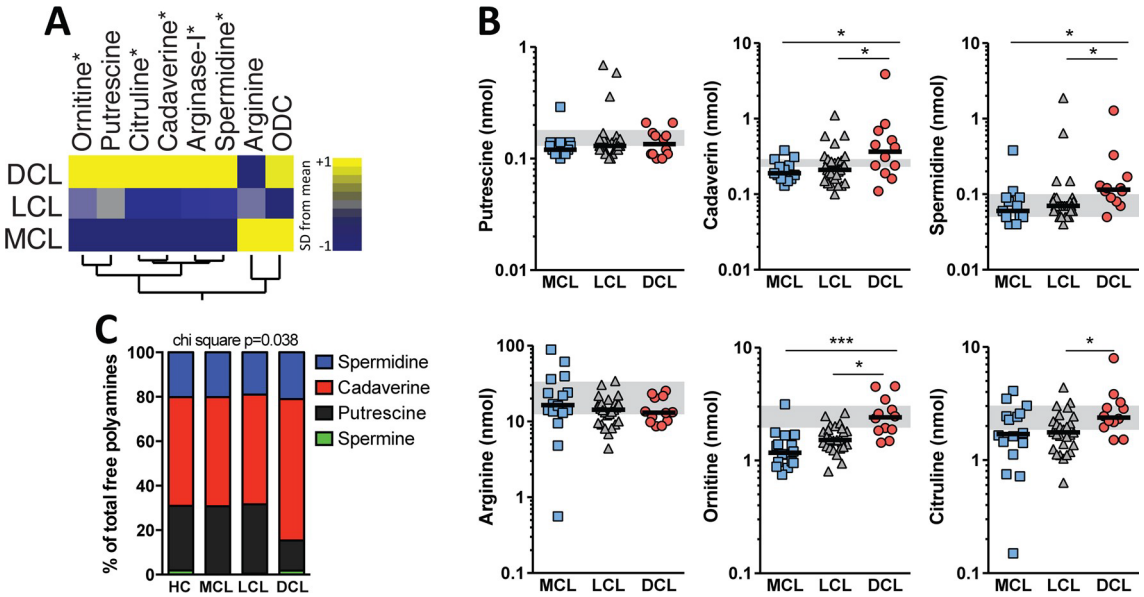

Figure 1. Plasma concentrations of amino acids and polyamines in patients with tegumentary leishmaniasis. Plasma levels of ARG1, polyamines: Putrescin, Cadaverine, Spermidine and amino acids: Arginine, Ornithine, Citrulline, were compared among patients with localized (LCL; n = 29), mucosal (MCL; n = 13) and diffuse (DCL) leishmaniasis as well as healthy controls (n = 43). (**A**) A hierarchical clustering analysis (Ward's method) was employed to show the amino acids and polyamines measured using a representative profile of geometric mean values (log2 -transformed) displayed for each clinical group. The color scale of the heatmap represents z-score by row. (**B**) Univariate analyzes with scatter plots of the comparisons are shown. Data were compared using the Kruskal–Wallis test with Dunn's multiple comparisons ad hoc test (*P<0.05, ***P<0.001). Lines represent median values. Grey bars represent the percentiles 25 and 75 from healthy controls (**C**) Frequency of the indicated polyamines in healthy controls or those with MCL, LCL and DCL was compared the total free polyamines using the chi square test.

Biomarker	MCL (n = 4)	LCL (n = 7)	DCL (n = 3)	*P* value	Post-test result
ARG1	−2.66 (−2.83; −2.46)	−2.26 (−2.9; −1.93)	−1.20 (−2.10; −0.34)	.04	C
ODC1	−0.69 (−0.81; −0.54)	−0.63 (−0.85; −0.32)	−0.88 (−1.37; 4.36)	.77	n.s
CAT2A	−2.66 (−2.84; −2.47)	−2.11 (−2.81; −1.71)	−1.05 (−2.03; −0.4)	.04	C
CAT2B	−2.26 (−2.67; −1.81)	−1.35 (−1.88; −1.06)	−1.31 (−2.10; −0.54)	.28	n.s
SAT1	0.91 (0.68; 1.14)	1.01 (0.83; 1.13)	1.26 (0.77; 1.62)	.68	n.s
SMS[a]	−0.69 (−0.71; −0.40)	−0.43 (−0.56; −0.26)	0.11 (−0.22; 0.74)	.03	C
SRM	−0.61 (−0.72; −0.40)	−0.23 (−0.38; −0.03)	0.19 (−0.55; 0.69)	.07	n.s
OAZ1	0.15 (0.11; 0.43)	0.48 (0.34; 1.04)	0.88 (0.44; 1.16)	.06	n.s
PAOX	−1.36 (−1.51; −1.25)	−1.41 (−1.93; −0.93)	−0.49 (−1.14; −0.07)	.11	n.s

Table 1. mRNA expression of polyamine synthetic pathways biomarkers in patient lesions with Tegumentary Leishmaniasis. Data represent expression normalized gene count over CD45 (log2). Data was analyzed using the Kruskal–Wallis test with Dunn's multiple comparisons ad hoc test. Comparisons with P value < .05:
LCL X MCL, LCL X DCL, [a] DCL X MCL; n.s. nonsignificant. *MCL* mucosal cutaneous leishmaniasis; *LCL* localized cutaneous leishmaniasis, *DCL* diffuse cutaneous leishmaniasis, *ARG1* Arginase 1, *ODC* ornithine decarboxylase, *CAT2A* cationic aminoacid transporter 2A, *CAT2B* cationic aminoacid transporter 2B, *SAT1* spermidine-spermine acetyl trabtransferase, *SMS* spermine synthase, *SRM* spermidine synthase, *OAZ1* ornithine antyenzime, *PAOX* peroxisomal oxidase.

We next used the same dataset described above to examine if the expression profile of the genes from the polyamine pathway was associated with parasite load in patient lesions. In fact, the estimated values of parasite load were correlated with: (i) the *Leishmania* genes (which included annotated genes and also putative genes) (Fig. 4A) and (ii) host genes from the polyamine biosynthetic pathway (Fig. 4B). Hierarchical cluster analysis of the *Leishmania* transcripts quantified by the RNAseq indicated that patients with DCL were the ones who displayed the highest levels of parasite transcripts (Fig. 4A). This approach revealed in Fig. 4A that expression values of all the *Leishmania* genes examined were positively correlated with parasite load in the group of patients with DCL, but not in those with LCL. Indeed, only histone H4 expression level displayed a positive correlation with the parasite load in the group of LCL patients whereas levels of histone H1 putative, 60S ribosomal protein

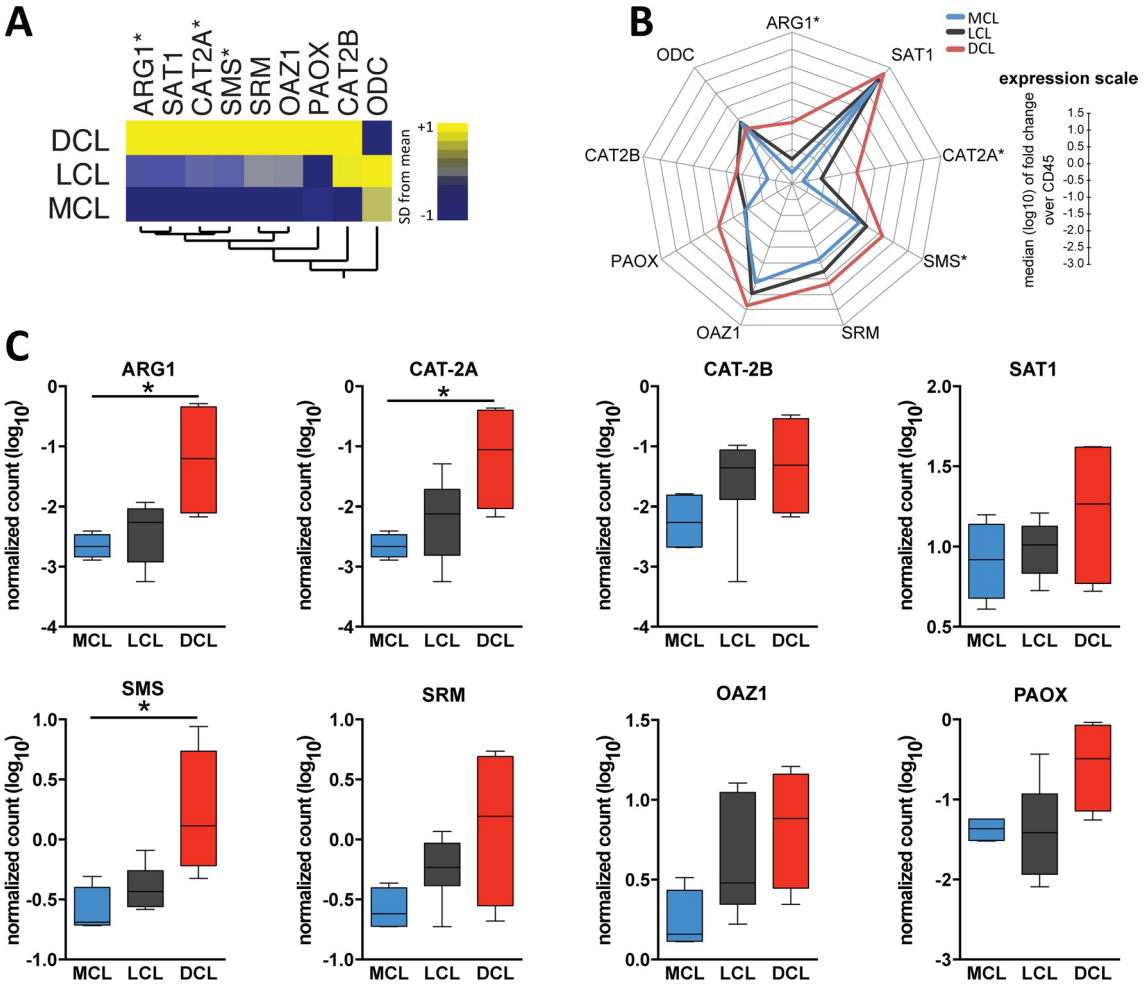

Figure 2. Differential expression of genes from polyamines pathways in lesions from patients with tegumentary leishmaniasis. Total RNA was extracted from lesion biopsy from patients with MCL (n = 4), LCL (n = 7) and those with DCL (n = 4). Indicated messenger RNA transcripts of host-specific cellular genes were quantified
by nCounter (Nanostring) and were normalized by pan-leukocyte gene CD45 to account for detection of immune infiltration into tissues. (**A**) A hierarchical clustering analysis (Ward's method) was employed to show the targeted genes of the polyamine pathway displaying a different profile for each clinical group. (**B**)
A representative profile of geometric mean values (log2 transformed), for indicated genes, was compared among MCL, LCL and DCL patients. Data were compared using the Kruskal–Wallis test with Dunn's multiple comparisons ad hoc test (*P < 0.05, **P < 0.01, ***P < 0.001). (**C**) Box- and-whisker plots of gene expression relative to CD45 are shown. Lines represent median values and interquartile ranges. Data were compared using the Mann–Whitney U test. *P < 0.05.

L39 putative, histone h1 like protein, and amino acid transporter (putative) were not statistically correlated and the remaining genes from the list were actually negatively correlated with parasite loads. Finally, we tested if the expression values of genes from the polyamine pathway could be associated with parasite load. First, the overall gene expression profile of the host polyamine pathway could separate the individuals from the distinct clinical groups (Fig. 4B), corroborating with the results from the PCA model (Fig. 3B). Our analyses also demonstrated that while expression values of several genes exhibited similar association profiles with parasite loads between DCL and LCL patient groups, such as *ARG1*, other targets displayed a divergent pattern, such as *NOS1* and *NOS3* (Fig. 4B). Intriguingly, the analyses of a publicly available transcriptome dataset of skin samples demonstrated that expression values of both *ARG1* and *ARG2* were higher in healthy controls than in LCL (Fig. 3A). In addition, the expression of these molecules was slightly lower, but not statistically significant in DCL patients compared to healthy controls. Furthermore, the transcriptome data also indicated that expression values of *ARG1* were inversely correlated with parasite loads in the lesions from both LCL and DCL whereas the those of *ARG2* were also negative correlated in LCL but only marginally directly correlated with parasite loads in DCL lesions (Fig. 4B).

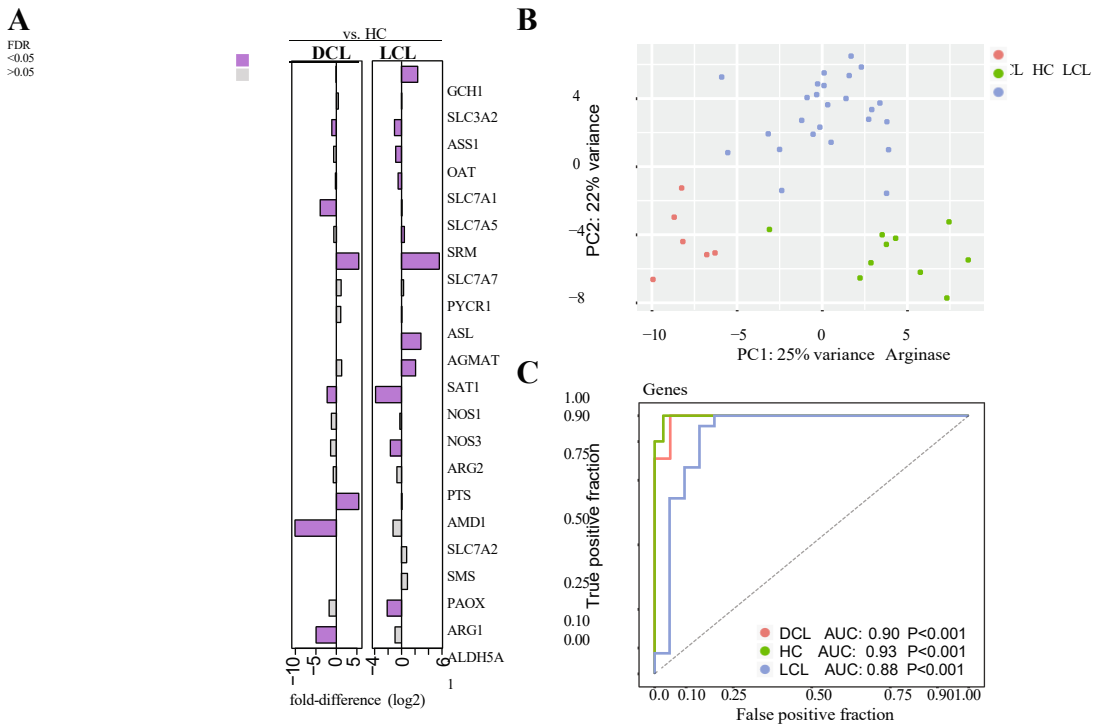

Figure 3. Tegumentary leishmaniasis patients display a unique profile of gene expression from the polyamine biosynthetic pathway. (**A**) Genes involved in the polyamine pathway were retrieved from the transcriptome dataset as described in "Methods" section and used in additional analyses to test whether its expression values were able to separate the distinct clinical groups. Fold-differences (DCL or LCL vs. healthy controls) were calculated and statistically significant differences are highlighted in colored bars. (**B**) A principal component analysis (PCA) model was employed to verify if the expression values of the genes from the polyamine biosynthetic pathway were able to classify the samples within the groups, regardless of the fact that some of these genes were not differentially expressed between the clinical groups. We used the normalized count table without the calculation of fold difference against the control group to input the PCA algorithm (see "Methods" section for details). (**C**) A Receiver Operator Characteristics (ROC) curve analysis was performed with these same gene expression values, to assess the sensibility and specificity of this classification. The ROC curve analysis used a multinomial model, in which the outcomes (HC, DCL and LCL) were binarized. Thus, this approach allowed us to compare the power of all the genes from polyamine biosynthetic pathway described above to discriminate between the following groups: (i) DCL vs. HC + LCL; (ii) HC vs. LCL + DCL; and (iii) LCL vs. HC + DCL. AUC: area under the curve.

Discussion

The polyamine biosynthetic pathway has been described as critical to promote *Leishmania* intracellular rep- lication inside host macrophages[6,18,19]. In this exploratory study, we performed a detailed investigation of the expression profiles of components of such pathway in skin lesions and in peripheral blood of patients presenting with distinct clinical forms within the spectrum of TL disease.

Our analyses demonstrated that DCL patients, compared with LCL and MCL, presented higher plasma concentration of polyamines (spermidine and cadaverin) and specific amino acids (ornithine and citruline), identifying a distinct biosignature that characterized this anergic form of TL. In mammalian cells, putrescine is the precursor for spermidine formation and probably for this reason its concentrations are usually low[18–20]. Indeed, we did not find any difference in the plasma concentrations of putrescine between the clinical forms, although spermidine concentrations were higher in DCL patients. We hypothesize that putrescine may be rapidly converted into other polyamines that promote *Leishmania* replication in DCL lesions. Interesting, cadaverin was the most abundant polyamine detected in plasma from the TL patients, and especially in those with DCL. Previous studies reported that cadaverin is induced to compensate significant decreases in concentrations of polyamines or their substrates[21]. Studies with *Escherichia coli* cell-free system showed that cadaverin has the same ability as putrescine and spermine in promoting protein synthesis[21]. The role of cadaverin in parasitic infections is still largely unknown.

Previous work from our group has shown that patients with DCL display elevated plasma protein concentra- tions of ARG1 and ODC[4]. These enzymes regulate arginine availability in through regulation of its precursor, ornithine[22]. Arginase is also involved in the urea cycle, in which it catalyzes interconversion of arginine-citrulline- ornithine[22]. l-arginine is considered a conditionally essential amino acid because even though it is synthesized in human body, it still needs dietary supplementation[23]. Interestingly, here we demonstrate that, although arginine

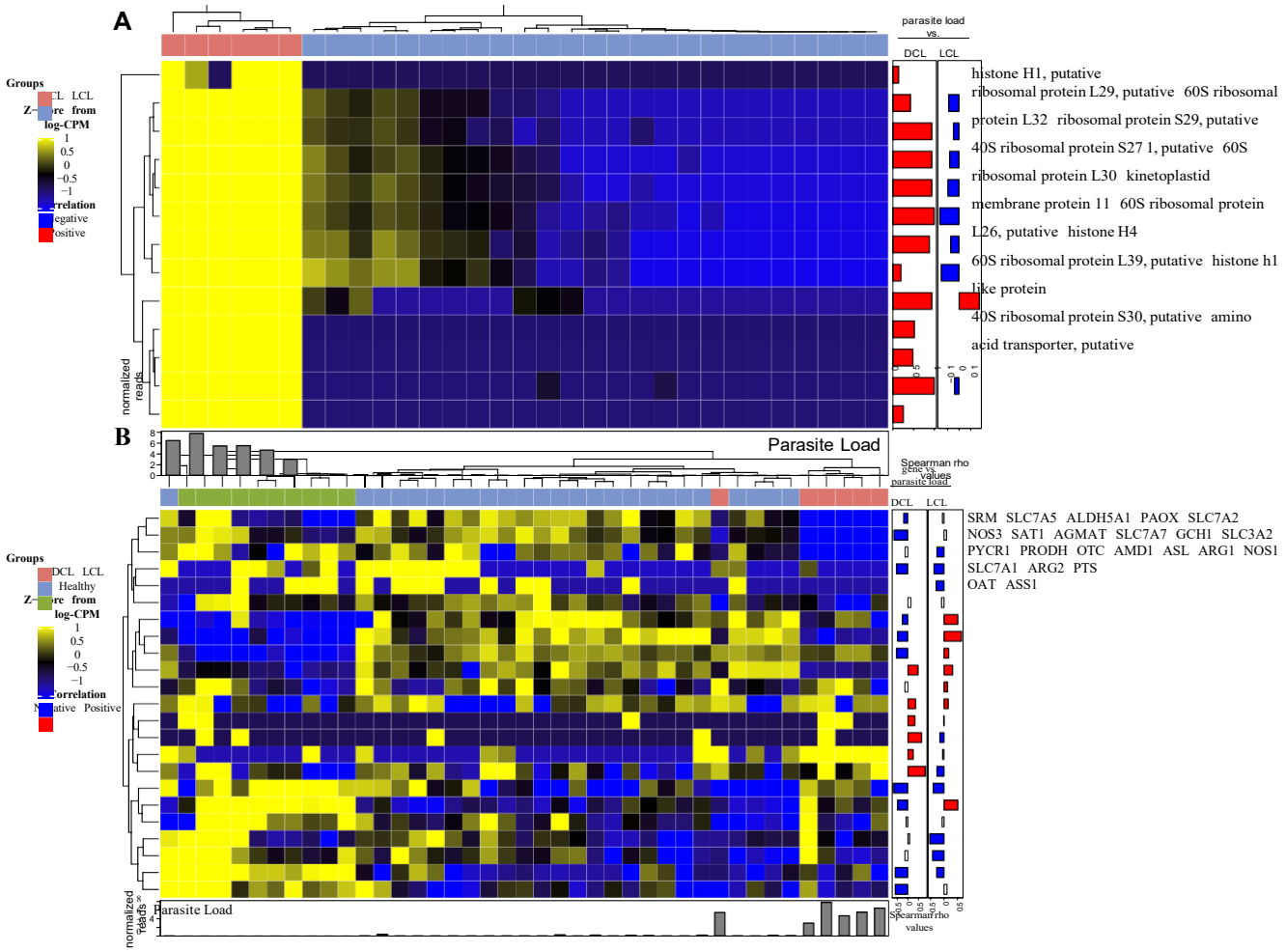

Figure 4. Association between activation of genes from the polyamine pathway and parasite transcripts. (**A**) A hierarchical clustering analysis (Ward's method) was employed to illustrate the overall expression profile of *Leishmania* genes in DCL and LCL lesion and its correlation with parasite loads (normalized leishmania transcripts as described in "Methods" section). Identifiers refer to putative functions (described in "Methods" section). (**B**) A similar statistical approach was used to evaluate the overall profile of genes of the polyamine biosynthetic pathway in TL patients vs. parasite loads. Correlations were tested using the Spearman rank. In (**A**) and (**B**), each column represents one patient. Spearman correlation rank coefficient (rho) values were displayed as bar plots, to demonstrate strength and directionality of the associations.

plasma concentrations were not different among the distinct TL clinical forms, DCL patients had high con- centrations of ornithine and citrulline. Although citrulline can be produced from arginine by iNOS/NOS2[22], our findings led us to suggest that in the context of DCL, this amino acid is potentially being interconverted by arginase, favoring the production of polyamines.

In recent years, there has been an increase in the number of studies describing the importance of amino acid transporters in the trypanosomatid metabolism and especially in *Leishmania*. Most amino acids are involved in osmotic control, metacyclogenesis, establishment of infection, regulation of autophagy and apoptosis, resistance to oxidative stress, and synthesis of polyamines[24]. Regarding the essential amino acids for the biosynthesis of polyamine, such as arginine and ornithine, *Leishmania* is auxotrophic for arginine and depends on uptake from the external environment through a specific transporter, amino acid permease 3 (AAP3)[17,25,26]. This means that the production of polyamines in the parasites is directly related to their ability to acquire such amino acids from the host cell[26]. In fact, *Leishmania sp*. has been described to benefit from host-derived polyamines and several studies have indicated that genetic or pharmacological suppression of parasite enzymes involved in polyam- ine pathways results in impairment of parasite growth and establishment of host cell infection[22,27,28,29]. More recently, it has been shown that during infection, *L. amazonensis* is able to alter the host metabolism, inducing polyamine production[30]. Although in our analyses we could not find a specific AAP3 gene, due to the lack of genome annotation, we retrieved the functional annotation in other *Leishmania* putative genes that are involved

amino acid transport (shown in Fig. 4 as amino acid transporter, putative). Our results demonstrated a higher expression of the parasite amino acid transporter (putative) in DCL lesions, which positively correlated with the total counts of parasite transcripts, reinforcing the hypothesis that higher expression of amino acid transporters by *Leishmania* may indeed favor parasite persistence during infection. It is possible that the systemic balance of polyamines and amino acids induced by infection could contribute to the persistence of the high parasite loads observed in DCL lesions. Our results are in agreement with this idea, although a formal confirmation by mechanistic studies is still necessary.

In the present study, we tested if there are differences in expression of the target genes of the polyamine path- way among different clinical forms in skin lesions. Our results demonstrated that among the expression values of *ARG1, CAT-2A* and *SMS* were higher in DCL patients. It has been shown that *CAT-2* expression values are increased by both *Leishmania* infection and l-arginine deprivation[10]. Therefore, we hypothesize that parasites from DCL patients positively regulate *CAT-2A* expression, thereby increasing polyamine production, which is important for their proliferation and maintenance of infection. In previous studies from our group and others, lesions from DCL patients were found to present greater expression of IL-4 and IL-10 transcripts in relation to TNF expression[4], whereas lesions from patients with LCL and MCL are characterized by high expression of IFNγ mRNA and absence of IL-4[3]. In addition, THP-1 macrophages infected with *L. donovani* are described to exhibit increased l-arginine uptake by CAT-2, augmented ARG1 activity and higher levels of spermidine, that correlated with increased concentrations of IL-10 and reduced concentrations of IL-12 and TNF-α[10]. Whether the increased expression of *ARG1* and *CAT-2A* in skin lesions from patients with DCL is associated with the immunosuppression warrants further investigation.

The analysis in patient lesions demonstrated that *ODC1* was not a part of the biosignature of the polyam- ine pathway in DCL skin lesions. ODC is the key enzyme implicated in polyamine biosynthesis required for in vitro intracellular proliferation in *Leishmania*-infected cells[19,31]. The intracellular concentration of polyamines is regulated by several mechanisms, including the synthesis, degradation and efflux/uptake by the polyamine transporters[6]. High concentrations of intracellular polyamines induce the expression of important enzymes involved in polyamine recycling: spermidine/spermineN1-acetyltransferase (SSAT) and peroxisomal n(1)-acetyl- spermine/spermidine oxidase (PAOX), associated to interconversion and degradation of polyamines. Another important step of the regulation of polyamine production is mediated by the fast turnover of ODC. When cellular polyamine levels are high, they induce the biosynthesis of an ODC inhibitor named ODC antizyme (OAZ), which prevents its dimerization and promotes ODC degradation, through the 26S proteasome[32,33]. OAZ is induced by an excess of polyamines (and has a fast turnover), and besides regulating the degradation of ODC, negatively regulates cellular polyamine transporters[34]. However, the proteins involved in polyamine transport and the exact mechanisms by which polyamines regulate their uptake in the mammalian cells are not well known. Cells in which the OAZ enzyme is expressed to high levels exhibit a marked reduction in polyamine uptake. Our data suggest that high expression of *OAZ* in the DCL lesion could be responsible for the degradation of ODC, justi- fying the low levels of this enzyme. However, the exact molecular and cellular mechanisms involved in *ODC* in lesions or systemic regulation in DCL patients remain to be explored.

Although the RNA transcripts for *CAT-2B, SSAT1, SRM, OAZ* and *PAOX* did not show statistical difference between the diverse TL clinical forms (Fig. 2C), we observed a distinct biosignature of the polyamine biosyn- thetic pathway in DCL individuals in the hierarchical cluster analysis, corroborating the idea that this metabolic pathway is indeed important in parasite proliferation and successful establishment of *Leishmania* infection. How- ever, a limitation of the present study was that we did not have available sample/data to directly test correlations between polyamine expression and parasite burden in the lesions. Thus, we employed an analytical approach to specifically test this hypothesis in a separate patient cohort.

An important contribution of our study is the validation analysis using an independent patient cohort recently published[16]. In this approach, we tested whether the transcriptomic profile also revealed differential gene expres- sion in the TL disease groups. Indeed, the transcriptional data from skin biopsies from DCL and LCL patients compared to normal skin revealed that the targets from the polyamine pathway are able to discriminate the distinct TL clinical groups. Interestingly, the *SLC7A2* gene was down modulated in DCL patients. Arginine transport is mediated by members of the solute carrier 7 family (SLC7), that is divided into two subfamilies: the cationic amino acid transporters (CATs; *SLC7A1-4* and *SLC7A14*) and L-type amino acids transporters (LATs: *SLC7A5-13*), also known as CATs[35]. Although expression of this gene diverges between the RNAseq and *n-counter* analyses, being higher in the first and lower in the latter, CAT2A and CAT2B are isoforms of the *SLC7A1* gene and by RNAseq technique, we could not identify such isoforms separately. According to our study, the Adeno- sylmethionine decarboxylase 1 (*AMD1*) up regulation in DCL patients may be relate to the high concentrations of spermidine found in these patients. AMD1 is an enzyme involved in biosynthesis of spermidine[36]. The impor- tance of this enzyme in tumorigenesis in gastric cancers associated with polyamine synthesis has been recently been demonstrated[36]. Of note, its role in parasitic diseases, including *Leishmania* infection, is still not clear.

The transcriptome data from skin lesions indicated that expression levels of *ARG1* and *ARG2* were not sta- tistically distinct between DCL patients and healthy controls. Further analyses also revealed a negative correla- tion between *ARG1* expression levels and parasite loads in both LCL and DCL whereas *ARG2* expression was negatively correlated in LCL but slightly positively correlated with parasite loads in DCL lesions. At first glance, such findings may contrast with the idea that arginase-1 is involved in the pathogenesis of DCL, as previously reported[4]. Our experiments in plasma revealed higher levels of arginase-1 in DCL patients compared to controls, and the nanostring experiment unfortunately did not include samples from healthy controls for comparisons. Although the results presented here clearly show a differential modulation of the polyamine pathway in distinct clinical forms of TL, additional studies are still warranted to investigate the direct participation of arginase-1 in cellular responses to *Leishmania* in skin lesions.

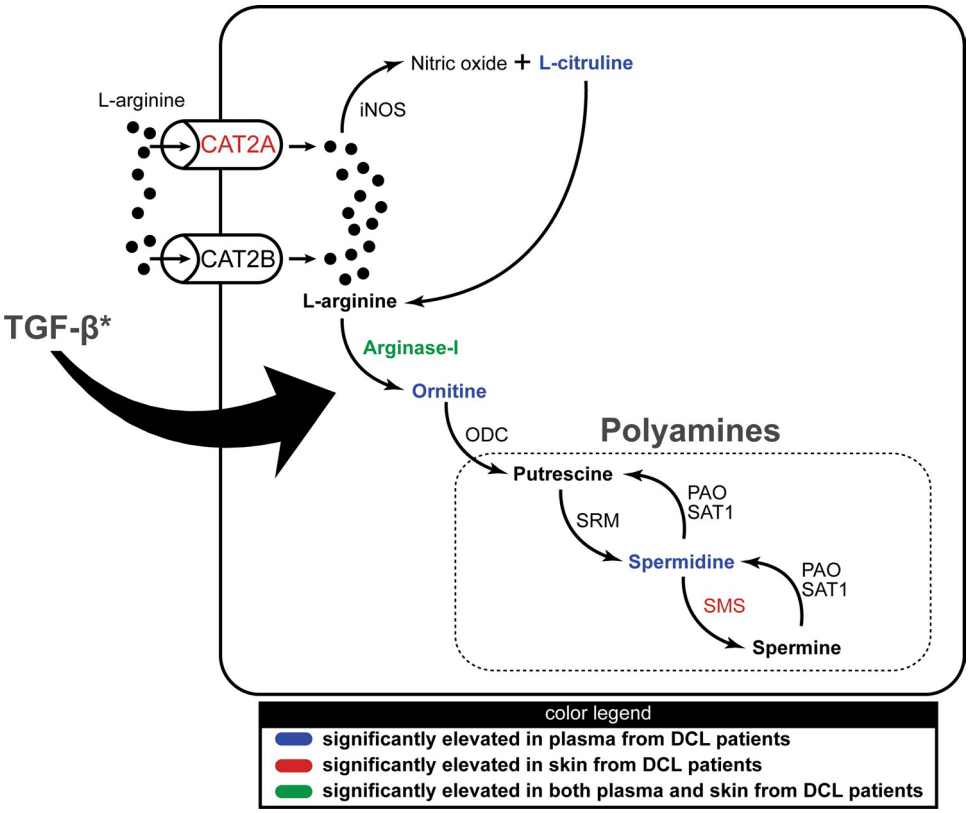

Figure 5. Polyamine pathway in patients with Diffuse Cutaneous Leishmaniasis. This illustration summarizes the data of Figs. 1 and 2. The figure shows the cascade of the polyamine biosynthesis and highlights the parameters which were statistically significant in plasma and/or skin lesions of Diffuse Cutaneous Leishmaniasis (DCL) patients compared to the other clinical forms of tegumentary leishmaniasis in our study settings.

The transcriptome analyses also revealed that the overall gene expression profile of the polyamine pathway is associated with the parasite loads and that such association is linked to the TL clinical presentation (in either LCL or DCL). Although the expression level of genes from the parasite should be proportional to transcriptionally estimated parasite load, it is known that post-transcriptional, epigenetic regulation can result in discrepancies between abundance of a given gene and number of parasites. The previous report on this transcriptome data has identified the 15 most highly and uniformity expressed parasite genes in each of the patients analyzed with DCL[16]. It is indeed expected that such expression levels of genes should directly follow the parasite loads. However, we detected an important difference in the profile of correlation between expression levels of *Leishmania* genes and parasite loads between the groups of DCL and LCL. This finding is surprising and reinforce the hypothesis of differential modulation of gene expression occurring in the parasites. Interestingly, among the genes that exhib- ited divergence in association of expression levels with parasite loads between DCL and LCL are those whose functions are related to amino acids transport. *Leishmania* putative genes of these transporters were shown here to be highly expressed in patients with DCL and the expression level was related to parasite loads in DCL but not in LCL, supporting the hypothesis that parasites may take advantage of an immunomodulated environment. Additional studies are necessary to test if the activation of the polyamine pathway is a cause or consequence of the higher parasite loads in DCL.

In conclusion, our findings indicate that the differential activation of the polyamine pathway characterizes DCL relative to other clinical forms of TL (summary of findings from the experiments performed here are illus- trated in Fig. 5) and open perspectives for future studies testing the manipulation of such pathway to reduce immunopathology in TL.

Methods

Ethics statement. This study was approved by the institutional review board from Instituto Gonçalo Moniz, Fundação Oswaldo Cruz. All clinical investigations were conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants or legal guardians, and all data analyzed were anonymized.

Study design. We performed a cross-section study of patients presenting with different clinical forms of TL as well as uninfected endemic controls. Social-demographic and clinical characteristics of all patients were

previously described[4,37]. DCL cases (n = 14) were obtained from a study performed between 1980 and 1990 in the state of Maranhão, Northeast of Brazil[4]. DCL diagnosis was based using previously described criteria[1,2]. In addition, we evaluated data from age- and sex-matched patients with MCL (n = 13) or LCL (n = 29) recruited at our reference clinic in Jiquiriçá, BA-Brazil, as previously described[37]. MCL and LCL individuals included in the present study were required to have no previous history of TL and to be treatment naïve. For plasma analyses, we included samples from 43 healthy controls (matched by age and sex to the TL groups) from the same region of endemicity and who had negative results of an anti-*Leishmania* delayed-type hypersensitivity test. Diagno- sis criteria for LCL and MCL were published previously[37] and were based on anti-*Leishmania* delayed-type hypersensitivity test, detection of anti-*Leishmania* antibody, or detection *of Leishmania* parasites in biopsy tissue specimens by either immunohistochemistry or qualitative polymerase chain reaction (PCR) assays.

Polyamine trofiles. The polyamine profiles and content were performed according to a previous study[38] Briefly, serum samples from patients with Leishmaniasis (60 µl) were mixed with cold 5% (v/v) perchloric acid at a ratio of 1:4 (v/v). Then, the samples were submitted to three cycles of freezing (− 20 °C) and thawing (at room temperature), prior to centrifugation at 11,000*g* for 10 min. The supernatant containing free polyamines was col- lected. Free polyamines were derivatized with dansylchloride (5 mg ml^{-1} in acetone), 0.05 mM diaminoheptane- DAH (internal standard) and saturated sodium carbonate. After 50 min incubation in the dark at 70 °C, the excess of dansylchloride was converted to dansylalanine by adding proline (100 mg ml^{-1}). After 30 min incuba- tion (room temperature), dansylated PAs were extracted with of toluene 1:1 (v/v), the organic phase containing the polyamines was collected. The toluene phase was evaporated with gaseous nitrogen (40 °C). Dansylated polyamines were resuspended in 100 µl of acetonitrile.

Polyamines were separated by high-performance liquid chromatography (HPLC, Shimadzu, Japan) using a C18 reversed-phase column (5 µm × 4.6 mm × 250 mm—Sulpelcosil, Supelco), as described previously[38]. Poly- amines were detected at 340 nm (excitation) and 510 nm (emission) wavelengths with an RF-20A fluorescence detector (Shimadzu). Peak areas and retention times were measured by comparison with standard known con- centrations of polyamines (Table S1).

Free amino acids profiles. The amino acid content was determined as previously described[39]. Serum sam- ples from patients with Leishmaniasis (60 µl) were extracted in 1.8 ml of 80% ethanol (v/v) and concentrated in 'speed vac'. Samples were resuspended in 0.6 ml Milli'Q and centrifuged at 20,000*g* for 10 min. The supernatant was filtered through a 20 µm membrane. Amino acids were derivatized before injection with o-phthalaldehyde and separated by HPLC (Shimadzu) on a C18 reverse phase column (as described above). The gradient program was developed as in[40], with a total running time of 55 min (including the time for column regeneration) at a flow rate of 1.0 ml min^{-1}, at 40 °C. Mobile phase A is a 0.1 M sodium acetate pH 7.2 and Mobile phase B is 100% methanol (MeOH). The proportion of mobile phase B (MeOH 100%) is as follows: 0–15 min, 14%; 15–20 min, 30%; 20–24 min, 35%; 24–26 min, 47%; 26–34 min, 50%; 34–38 min, 70%; 38–40 min, 100%; 40–45 min, 100%; 45–55 min, 14%; and 55 min, 14%. A fluorescence detector (Shimadzu, RF-20A), set at 250 nm excitation and 480 nm emission wavelengths, was used for detection and quantification. Peak areas and retention times were measured by comparison with standard known concentrations of amino acids.

Arginase-1 protein measurement in plasma. Plasma levels of arginase 1 (Hycult Biotech, Uden, the Netherlands) were measurement using enzyme-linked immunosorbent assay ELISA according to the manufac- turer's instructions.

nCounter analysis. Tissue samples from which we had high-quality messenger RNA (mRNA) were obtained from a subset of three patients with DCL, four patients with MCL and seven patients with LCL who were also matched for age and sex. Skin tissues were used from DCL and LCL whereas nasal mucosal samples were obtained from MCL patients as previously described[4,37]. Total RNA was extracted from cryopreserved lesion biopsy specimens, using Trizol reagent (Invitrogen, Carlsbad, California), with an additional purifica- tion step using RNeasy columns (Qiagen, Venlo, Netherlands). nCounter analysis (NanoString Technologies, Seattle, Washington) was performed based on direct molecular bar coding of target RNA transcripts and digital detection[41]. The chosen targets genes were: *ARG1* (Arginase 1), *ODC1* (Ornitine Decarboxylase 1), *SRM* (Sper- midine Syntahse), *SMS* (Spermine Synthase), *SAT1* (spermidine-spermine acetyl transferase), *PAOX* (Peroxiso- mal oxidase), *SLC7A2* (CAT2-cationic aminoacid transporter 2), *OAZ1* (Ornithine Decarboxylase antienzyme). To account for differences in leukocyte infiltration between patient lesions, data were normalized for CD45, which encodes the pan-leukocyte marker CD45, detectable at fentomolar range, as previously reported[41].

RNA-seq analysis. Data samples were downloaded from *bioproject PRJNA307599* and labeled according to the informed metadata (41 samples sequenced with *Illumina HiSeg 2000* of 10 controls, 8 early LCL biopsies 17 late LCL biopsies and 6 DCL biopsies with unspecified disease duration). For all samples, low-quality bases have been removed and adapters were trimmed using *Trimmomatic* V0.32[42]. After quality check, sequences were aligned to the human reference transcriptome (GRCh38.p13—https://www.ncbi.nlm.nih.gov/assembly/ GCF_000001405.26/ version), comprising both mRNA and miRNA transcripts, and the recent versions of *L. braziliensis* (MHOM/BR/75/M2903) and *L. major Fiendlin* transcriptomes obtained from the *TriTrypDB* data- base (www.tritrypdb.org), with *Salmon* (v0.8.2)[43]. After mapping, the *Salmon* output was converted with the *tximport* package to count table by R (3.5.3 version). We used the Reactome database (R-HSA-351202), to iden- tify the genes involved in the polyamine biosynthetic pathway. Using this approach, we retrieved 22 genes from the transcriptome dataset: *GCH1, SLC3A2, ASS1, OAT, SLC7A1, SLC7A5, SRM, SLC7A7, PYCR1, ASL, AGMAT,*

SAT1, NOS1, NOS3, ARG2, PTS, AMD1, SLC7A2, SMS, PAOX, ARG1 and *ALDH5A1* (as shown in Fig. 3A) and they were used in additional analyses to test whether its expression values were able to separate the distinct clinical groups. Differentially expressed genes (DEGs) were examined by *edgeR* package[44]. The parasite burden from RNAseq data was measured by normalizing the library size[45]. The gene expression values used were the TMM-normalized log CPM values. The correlation between the parasite burden and gene expression values (both human and leishmania) were performed with the *Hmisc* package. As only a few *Leishmania* genes have been functionally annotated by biological assays, we collapsed the genes by putative functions and used the most representative expressed genes (Table S2). This strategy was employed to avoid the synonymies between genes with the same function; such approach is commonly used in high throughput analysis (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3166942/pdf/1471-2105-12-322.pdf). Changes in gene expression were considered significant when statistical test values (false discovery rate [FDR] adjusted p-value) were lower than 0.05 and the fold-difference higher than ± 1.5. Principal Component Analysis (PCA) was performed using the TMM-nor- malized log-transformed CPM values with *plotPCA* function from *Deseq2* package[46] to verify if the expression values of the genes from the polyamine biosynthetic pathway were able to classify the samples within the groups, regardless of the fact that some of these genes were not differentially expressed between the clinical groups. The receiver operator characteristics (ROC) curves with area under the curve were measured and plotted with *pROC* package[47]. The ROC curve analysis was performed with the same gene expression values which were inputted in the PCA algorithm, to assess the sensibility and specificity of this classification. Moreover, the ROC curve analysis used a multinomial model, in which the outcomes (HC, DCL and LCL) were binarized. Thus, this approach allowed us to compare the power of all the genes from polyamine biosynthetic pathway described above to discriminate between the following groups: (i) DCL vs. HC + LCL; (ii) HC vs. LCL + DCL; and (iii) LCL vs. HC + DCL. Heatmaps of the polyamine pathway genes as well as of the Leishmania genes were plotted with the *Complexheatmap* package[48].

Data analysis. Median values with interquartile ranges (IQRs) were used as measures of central tendency and dispersion. For expression assays, Mann–Whitney *U* test was used to compare the groups. Plasma values were compared using Kruskal–Wallis test with the Dunn's multiple comparisons test. Unsupervised 2-way hier- archical cluster analyses (Ward's method) with bootstrap were used to test whether patients with DCL, MCL and LCL can be grouped separately on the basis of simultaneous quantification from their plasma and lesion poly- amine biosynthetic pathway profile. Dendrograms represent Euclidean distance. Differences with p value < 0.05 were considered statistically significant.

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Author contributions

Designed experiments: H.M.S., J.F.C., A.M., S.M., A.B., E.J.S.F., B.B.A, L.F.W., V.M.B. Performed experiments: H.M.S., J.F.C., A.M., A.Q., K.F., S.M., R.K., J.V.W. Wrote MS: H.M.S., J.F.C., K.F., S.M., J.V.W., V.B., B.B.A, L.F.W.,
V.M.B. Supervised experiments: A.B., J.C., E.J.S.F., B.B.A, L.F.W., V.M.B. All authors approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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5 **PARTE II**

5.1 HIPÓTESE

O balanço na produção de mediadores lipídicos em pacientes com LC causada por *L. braziliensis* é distinto entre os pacientes que respondem ao tratamento tradicional ou nos que apresentam falha terapêutica.

5.2 OBJETIVOS

5.2.1 **Objetivo geral**

Investigar prospectivamente a produção de mediadores lipídicos em pacientes com LC causada por *L. braziliensis* responsivos ao tratamento tradicional ou na falha terapêutica.

5.2.2 **Objetivos específicos**

- Fazer o acompanhamento clínico de pacientes responsivos ao tratamento tradicional ou na falha terapêutica;
- Avaliar prospectivamente os níveis plasmáticos de citocinas inflamatórias;
- Avaliar prospectivamente os níveis plasmáticos sistêmicos de mediadores lipídicos derivados do ácido araquidônico (prostaglandinas, leucotrienos e tromboxano) e mediadores lipídicos de pró-resolução (lipoxinas, resolvinas, maresinas e protectinas);
- Testar se os níveis plasmáticos de mediadores lipídicos correlacionam- se com dados clínicos e citocinas inflamatórias como possíveis marcadores de prognóstico e de gravidade de doença;
- Identificar *in situ* marcadores moleculares (DNA, RNA e proteína) de enzimas envolvidas na produção de mediadores lipídicos.

6 **MANUSCRITO 2**

Esse trabalho investiga o perfil da bioassinatura de citocinas plasmáticas e mediadores lipídicos em pacientes que apresentaram falha terapêutica.

6.1 **MULTI-OMICS ANALYSES OF PLASMA CYTOKINES, LIPIDOMICS AND TRANSCRIPTOMICS DISTINGUISH TREATMENT OUTCOMES IN CUTANEOUS LEISHMANIASIS.**

Resumo

A infecção por *Leishmania braziliensis* geralmente resulta na Leishmaniose Cutânea (LC). Foi demonstrado que o aumento da resistência à droga na LC está associado à falha terapêutica. A identificação de preditores de desfechos clínicos é necessária para otimizar o atendimento ao paciente. Aqui, nós realizamos um estudo prospectivo de caso controle em que os níveis plasmáticos de citocinas e mediadores lipídicos foram avaliados em diferentes tempos durante a terapia anti-leishmania em pacientes com LC no Brasil. Análises multidimensionais foram empregadas para descrever a combinação de biomarcadores hábil em predizer e caracterizar a falha terapêutica. Nós encontramos uma bioassinatura influenciada principalmente por níveis plasmáticos de mediadores lipídicos que predizem com acurácia a falha terapêutica. Além disso, análises de transcriptoma em dados disponíveis publicamente revelaram que os níveis de expressão dos genes relacionados ao metabolismo lipídico medido em lesões cutâneas poderiam distinguir os desfechos clínicos na LC. Assim, a ativação de vias ligadas a biossíntese lipídica prevê a falha terapêutica em LC, podendo ser explorados como alvos terapêuticos.

Esse artigo foi publicado no periódico internacional i*Science* (Fator de Impacto = 4,4) e recebeu destaque em uma coleção especial sobre doenças tropicais negligenciadas (https://www.cell.com/cp/collections-world-neglected-tropical-diseases-day-2022). Halta-Santos H, Fukutani KF, Sorgi CA, Queiroz ATL, Nardini V, Silva J, Lago A, Carvalho LP, Machado PLR, Bozza PT, França-Costa J, Faccioli LH, Carvalho EM, Andrade BB, Borges VM. Multi-omic Analyses of Plasma Cytokines, Lipidomics, and Transcriptomics Distinguish Treatment Outcomes in Cutaneous Leishmaniasis. IScience. 2020 Nov 23; 23(12):101840. Doi: 10.1016/j.isci.2020.101840. PMID: 33313489; PMCID: PMC7721649.

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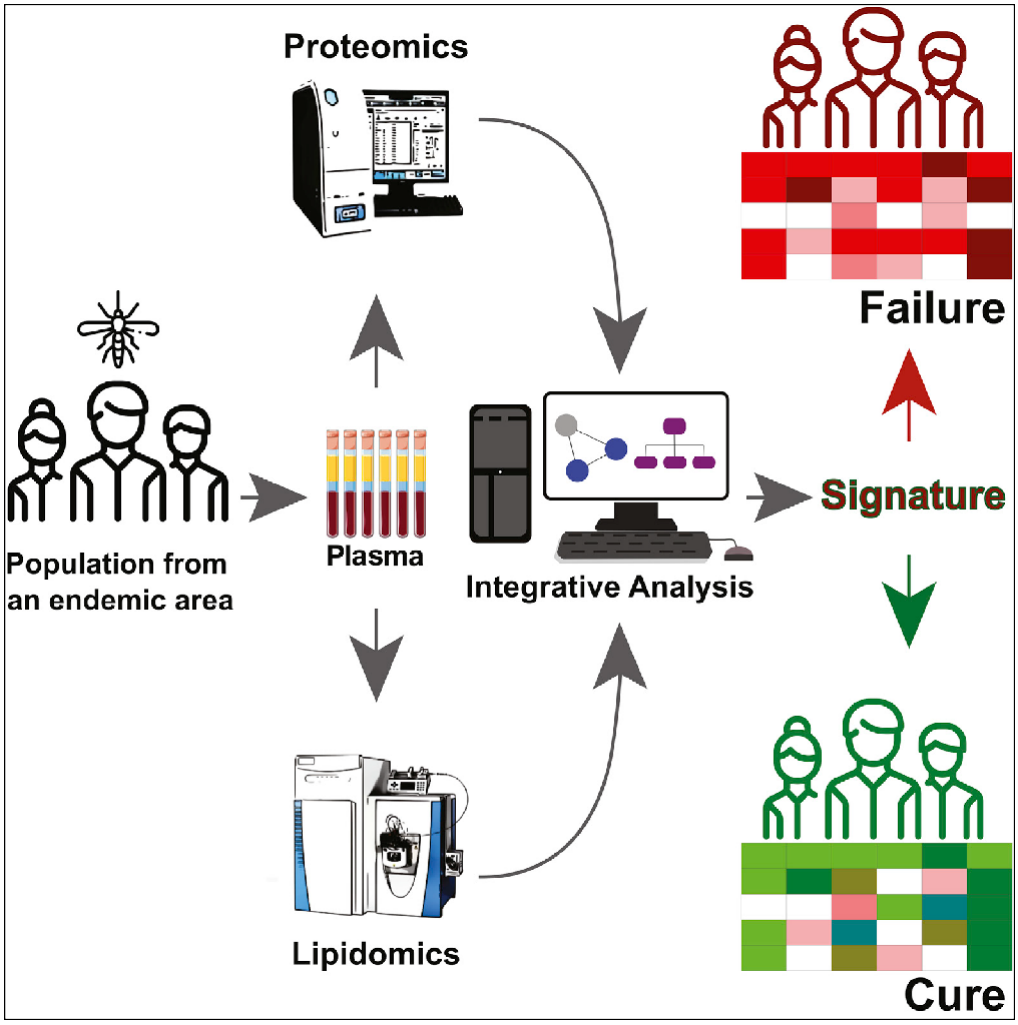

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HIGHLIGHTS

Plasma markers were tested to predict outcomes of patients with cutaneous leishmaniasis

Patients who failed treatment exhibited distinction in biomarker correlation networks

A biosignature of treatment failure included plasma cytokines and lipid mediators

Levels of eotaxin, TGF-b, and 11-HETE could be used to predict outcomes

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Multi-omic Analyses of Plasma Cytokines, Lipidomics, and Transcriptomics Distinguish Treatment Outcomes in Cutaneous Leishmaniasis

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SUMMARY

Leishmania braziliensis infection frequently results in cutaneous leishmaniasis (CL). An increase in incidence of drug-resistant CL leading to treatment failure has been reported. Identification of reliable predictors of treatment outcomes is necessary to optimize patient care. Here, we performed a prospective case- control study in which plasma levels of cytokines and lipid mediators were assessed at different time points during antileishmanial therapy in patients with CL from Brazil. Multidimensional analyses were employed to describe a combina- tion of biomarkers able to predict and characterize treatment failure. We found a biosignature influenced mainly by plasma levels of lipid mediators that accurately predicted treatment failure. Furthermore, transcriptomic analysis of a publicly available data set revealed that expression levels of genes related to lipid meta- bolism measured in skin lesions could distinguish treatment outcomes in CL. Thus, activation of pathways linked to lipid biosynthesis predicts treatment failure in CL. The biomarkers identified may be further explored as therapeutic targets.

INTRODUCTION

Leishmaniasis is a group of diseases caused by *Leishmania spp* parasites. The World Health Organization (WHO) considers leishmaniasis a serious public health concern (World Health Organization, 2018), with a worldwide incidence reaching as high as 1,2 million new cases every year (Alvar et al., 2012). Individuals in- fected with *Leishmania* can develop a wide spectrum of clinical manifestations, ranging from localized cuta- neous disease (cutaneous leishmaniasis [CL]) to a chronic systemic illness named visceral leishmaniasis (VL) (Dutra et al., 2011). The determinants of disease outcomes are described to involve factors directly linked to parasite species, as well as those associated with the host immune system (Dutra et al., 2011). Brazil is a major endemic region for both cutaneous and visceral leishmaniasis, with a recent geographical spread of disease transmission and increased detection of cases in more urbanized areas (Bustamante et al., 2009; Costa, 2008; Desjeux, 2001; Nascimento et al., 2008). Within this country, *Leishmania braziliensis* ac- counts for the vast majority of the CL cases (Scorza et al., 2017). This parasite species has been associated with development of different clinical forms such as localized, mucosal, and disseminated leishmaniasis (Queiroz et al., 2012; Scorza et al., 2017), highlighting its contribution to the high burden of this disease. Although there are many different pathophysiologic mechanisms underlying the progression of distinct clinical forms of leishmaniasis, the treatment options are few, with no significant recent advances in the field that have led to implementation of new therapies (Uliana et al., 2018).

Pentavalent antimonials (Sb^v) are the first-line drugs used to treat leishmaniasis in Brazil and other countries (World Health Organization, 2018). Other medications, such as amphotericin B, pentamidine, and miltefo- sine, are often used as alternative treatment options in patients who have failed Sb^v therapy or relapsed (Uliana et al., 2018). Treatment failure is reflected by persistence of open ulcers without re-epithelization, whereas relapse is defined as the reactivation of lesions once the therapy is terminated (Ponte-Sucre et al., 2017). In Brazil, studies have shown that occurrence of treatment failure in CL can be as high as 45% (Ma- chado et al., 2010; Prates et al., 2017). Factors that may underlie this high incidence of unfavorable

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outcomes are not fully understood. Early identification of patients at high risk of treatment failure can lead to optimization of therapeutic regimens and potential reduction of drug resistance. In fact, a recent study has demonstrated that expression levels of genes related to cytolytic and IL-1 pathways, as well as increased counts of parasite transcripts in skin lesions, are able to predict treatment failure in patients with CL (Amorim et al., 2019). However, such study was focused only on transcriptomic analysis and no eval- uation of protein or lipid mediators in similar setting has been performed.

We have previously described that different clinical forms of CL are associated with distinct activation of the eicosanoid pathway (Franc¸a-Costa et al., 2016). Briefly, among the distinct disease presentations related to CL, patients with localized cutaneous leishmaniasis (LCL) exhibit higher levels of prostaglandin E_2 (PGE_2), whereas those with mucosal cutaneous leishmaniasis (MCL) display augmented levels of leukotriene B_4 (LTB_4) in plasma (Franc¸a-Costa et al., 2016). Furthermore, a prospective cohort study of patients with VL demonstrated that this disease presentation is associated with heightened levels of both inflammatory pro- teins and lipid mediators, which significantly diminish after antileishmanial treatment (Arau´jo-Santos et al., 2017). Whether a prospective change in biomarker signatures, especially in those composed by lipid me- diators, among patients with CL undergoing treatment relates to risk of unfavorable outcomes has not been previously described.

Here, we employed systems biology analyses to prospectively examine whether simultaneous assessment of plasma levels of inflammatory proteins and lipid mediators could identify biomarkers able to predict and characterize treatment failure in patients with CL from an endemic region in Brazil. Our findings identified a biosignature highly influenced by unique expression of lipid mediators which is able to accurately predict treatment failure. Such findings, if validated in other settings, may be useful for predicting therapeutic out- comes in CL. In future studies, the molecules identified here as part of the biomarker signature could be explored as potential targets in a host-directed therapy focused on reducing odds of treatment failure.

RESULTS

Patient Characteristics

A total of 63 patients with CL were included in the study. The median age of the study population was 27 years old (interquartile range [IQR]: 19–33), with the majority of the study participants being men (71%). The groups of patients stratified according to treatment outcomes (cure vs. failure) were similar with regard to age and sex (Table 1). In addition, the median disease duration was also similar between the groups (p = 0.8, Table 1). At pre-treatment, patients who further experienced treatment failure often presented with increased number of lesions than those who were further cured (Figure S1).

A Unique Profile of Plasma Cytokines and Chemokines Characterizes Patients Who Fail Antileishmanial Treatment

Cryopreserved plasma samples were used for measurements of several biomarkers. The overall design of the analytical plan is described in Figure S2. We prospectively examined changes in plasma concentrations of cytokines, chemokines, and growth factors in patients with CL undergoing antileishmanial treatment. We compared plasma measurements in treatment-na¨ıve patients (day 0) and after treatment (day 60). To do that, we first built a heatmap inputting log-transformed and z-score normalized data on the mean concen- tration value for each biomarker calculated for each clinical group and time point. An unsupervised

Parameter	Cure	Failure	p-value
N	31	32	
Male, no (%)	21 (67.7%)	24 (75%)	0.5
Age, years	25 (13–52)	27 (16–56)	0.8
Disease duration, days	32 (21–90)	33 (15–70)	0.8

Table 1. Clinical and Epidemiological Characteristics for Patients with Cutaneous Leishmaniasis that Showed Cure or Therapeutic Failure After Antileishmanial Treatment The variables age and disease duration are presented as median values with range (minimum and maximum values), whereas sex and the number of active lesions were plotted as frequencies.

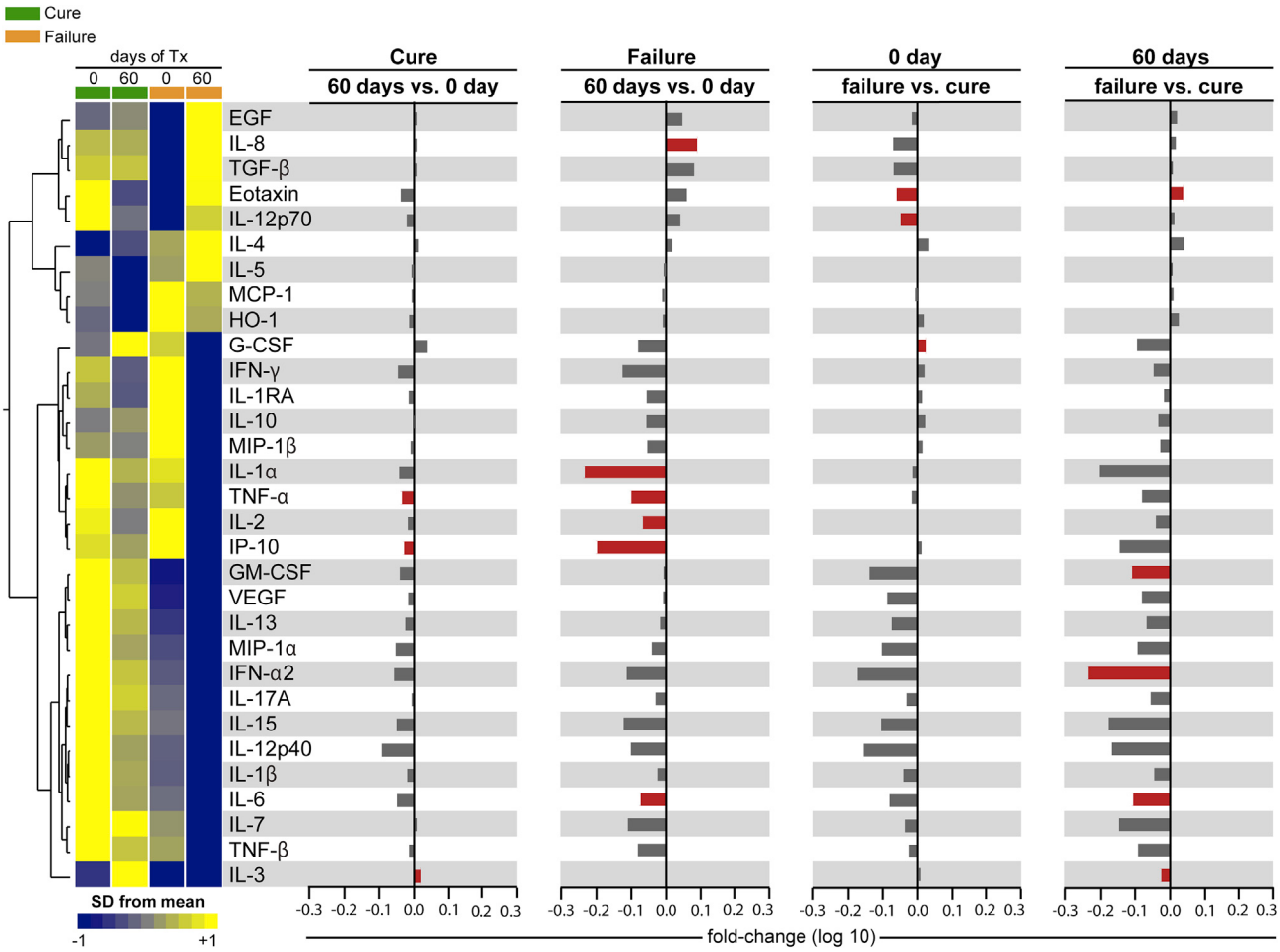

Figure 1. Patients Who Failed Therapy Exhibit a Distinct Profile of Inflammatory Proteins in Plasma Left panel Data on mean plasma concentration of each indicated marker per patient group and time point were log-transformed and *Z* score normalized, and a heatmap was used to illustrate trends in data variation. A hierarchical cluster analysis (Ward's method with 100X bootstrap) was used to group the biomarkers with similar distribution between clinical groups and time points. Dendrograms represent Euclidean distance. Rigth panel Fold differences between indicated means were calculated, and log10 values were plotted. Differences between day 60 and day 0 within each clinical group were examined using the Wilcoxon matched paired test. Comparisons between the groups of treatment failure and cure at the indicated time points were performed using the Mann-Whitney *U* test. Red bars indicate mediators that were significantly different between groups.

hierarchical clustering (Ward's method) was therefore used to test whether biomarkers could be grouped based on similarity in their profile of expression between the clinical groups. By comparing the two clinical groups, we observed that patients with CL who experienced treatment failure exhibited a unique bio- signature characterized by a distinct expression profile of inflammatory cytokines in plasma in both study time points (Figure 1, left panel). Furthermore, we calculated fold differences in concentration values of the cytokines and growth factors between the time points within each clinical group and also between the clin- ical groups in each time point, as depicted in Figure 1 (right panel). This approach was used to summarize large numbers of comparisons. Details on the distribution of the individual values are shown in Figure 2 (comparisons between time points) and Figure S3 (between the clinical groups at each time point). In the group of patients that were successfully treated, median values of TNF-a and IP-10 substantially decreased whereas, those of IL-3 significantly increased at day 60 (Figure 2). Patients who failed therapy exhibited a significant reduction in concentrations of IL-1a, TNF-a, IL-2, IP-10, and IL-6 with increased levels of IL-8 after therapy (Figure 2). When the two clinical groups were compared at each time point, we observed that, before therapy initiation, patients who would experience treatment failure displayed lower levels of eotaxin and of IL-12p70 and increased concentrations of G-CSF compared to those in individuals who were successfully treated (Figures 1 and S3). At day 60, treatment failure was associated with.

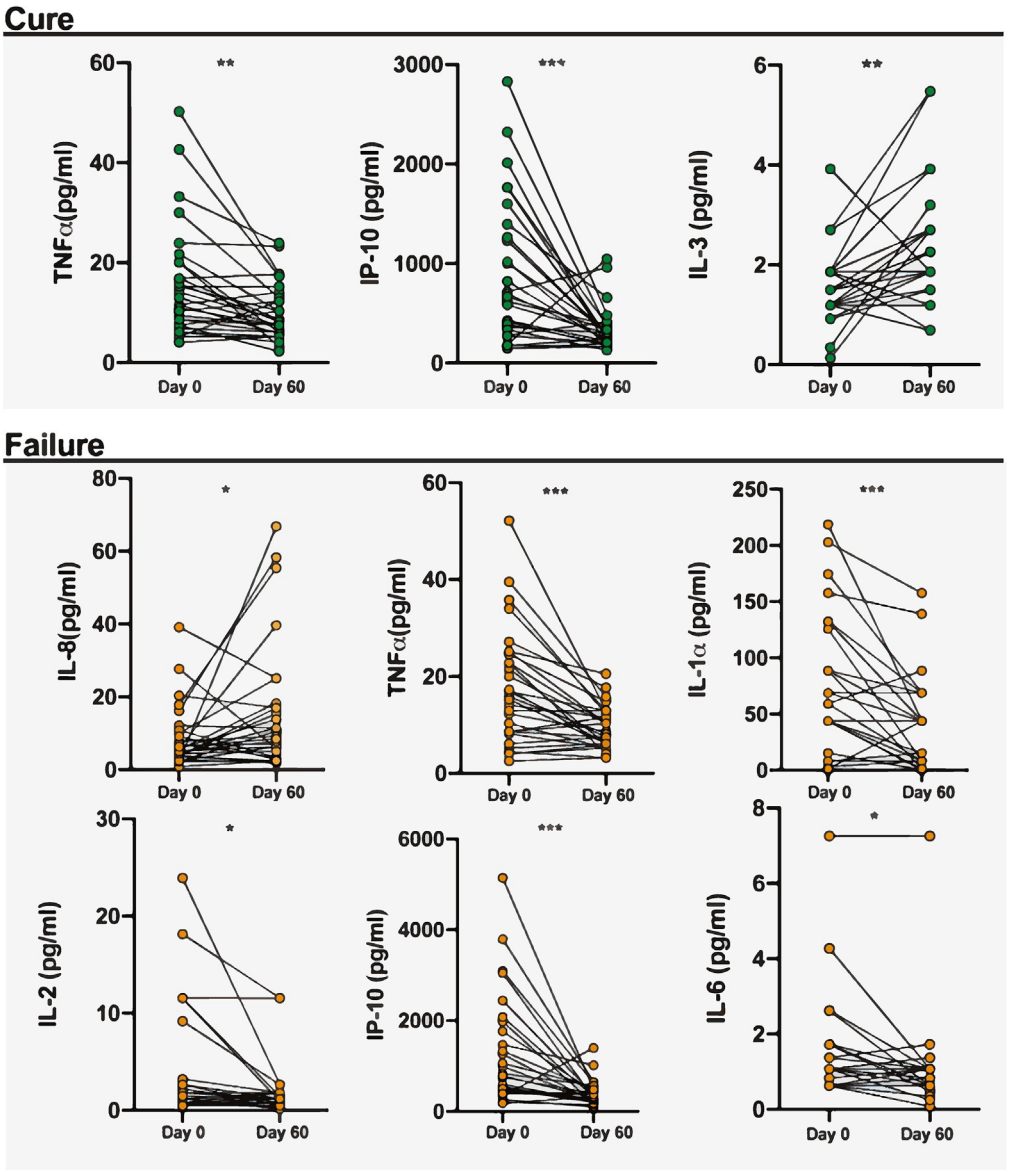

Figure 2. Inflammatory Proteins in Plasma of Patients with Cutaneous Leishmaniasis According To Treatment Outcome

Parameters that displayed statistically significant differences between the time points were tested using the Wilcoxon matched pairs. *p % 0.01; **p % 0.001; ***p % 0.0001.

heightened levels of eotaxin and diminished concentrations of GM-CSF, IFN-a2, IL-6, and of IL-3 (Figures 1 and S3).

Abundance of Lipid Mediators in Patients with Cutaneous Leishmaniasis according to Treatment Outcomes

To gain insights into the association between a specific lipid profile and the treatment outcome of the study population, two sets of analyses were performed. First, we prospectively assessed abundance levels of lipid mediators in plasma and performed hierarchical clustering analysis. This analysis is useful because it con- siders the representation of each given lipid individually in the total amount of measurable lipids detected in the lipidomics assay. The lipidomics was able to detect lipid mediators from both the inflammatory and resolution pathways, as well metabolites from the cyclooxygenase and lipoxygenase biosynthetic pathways

(Figure 3). Irrespective of the study group or time point, the most abundant lipid mediators in the popula- tion were LTB_4, 5-HETE, 5-oxo-HETE, 12-HETE, 11-HETE, PGE_2, and 15-HETE. In addition, the hierarchical clustering revealed that the overall abundance profile of the lipid mediators was distinct between the groups of patients who cured and those who failed treatment (Figure 3A), suggesting that there were dif- ferences in relative concentration of several lipids relative to all those measure in the lipidomics. Such dif- ference persisted at day 60 of follow-up. A second analysis of the same lipidomics data set was performed, now considering the raw concentration values in plasma. Using this approach, we found that among all the lipid mediators quantified, plasma concentrations of LTB_4, 5-oxo-HETE, 12-oxo-HETE, 12-HETE, 11-HETE, and of 15-HETE were all significantly reduced at day 60 compared to pre-treatment levels in patients who were successfully treated but not in those from the treatment failure group (Figure 3B).

Correlation Networks between Plasma Proteins and Lipid Mediators

Next, we performed network analyses based on Spearman correlations to evaluate the relationship be- tween lipid mediators and inflammatory proteins in plasma of the subgroups of patients at different time points. The analytical steps leading to design of the correlation networks are illustrated in Figure S2. This kind of analysis is used in systems biology to define statistical relationships between molecules that may suggest regulation or even direct molecular interaction. This approach allowed us to visualize the qual- ity (whether a correlation is positive or negative, indicated in the network by the color of the connecting line) and strength (the thickness of the connecting line being proportional to the Spearman rank coefficient rho value) of the associations in a given network. In such analytical setting, the number of connections (e.g. statistically significant correlations) infers how coordinated a biological process is. Comparing networks thus allow us to estimate the degree or regulation in a given clinical condition. In the context of this study, we observed that, before treatment commencement, there were already major differences in the correla- tion profiles, which involved bot number and directionality (e.g. positive or negative relationships) of sig- nificant correlations (defined here as p-value <0.05 after adjustment for multiple comparisons; Figure 4). In the group of patients who failed treatment later, there were a significantly higher number of relevant cor- relations than that from the patients who were successfully treated (Figure 4). Moreover, the vast majority of the statistically significant correlations detected in the treatment failure group were composed by positive associations, whereas a higher number of negative correlations were found in those who were further cured. At this time point, GCSF was the most relevant marker exhibiting positive relationships, whereas MIP-1b and TGF-b were the parameters with the highest number of negative correlations in the group of patients who were further cured. In the group who experienced treatment failure, several markers ex- hibited similar number of correlations, with no clear predominance of any specific parameter. The few negative relationships observed in the group of treatment failure at the study enrollment were between AA and IL-1RA and between EPA and IL-1b or IL-2 (Figure 4).

The findings described above indicated that even before initiation of antileishmanial treatment, patients who are prone to fail therapy already exhibit a distinct profile of associations between concentrations of plasma lipid mediators and inflammatory proteins. Strikingly, such difference in the correlation profiles was even more dramatic at day 60 of follow-up, when patients who were cured exhibited a predominance of negative relationships, whereas those who failed treatment persisted with several positive interactions (Figure 4). In the group constituted by those who were successfully treated at day 60, the most relevant markers were eotaxin, IFNa2, and TGF-b, and the only positive correlation found was between EGF and TXB_2. On the converse, patients with treatment failure exhibited TGF-b, and eotaxin as the most relevant nodes with negative correlations and TNF-b as the most significant marker with positive correlations in the network (Figure 4).

The networks were further explored in more details using node analyses. In such an analytical approach, the markers of a given network are ranked according to the number of connections (e.g. statistically significant correlations) that it is involved with. Each marker is represented by a node in the network. Highly connected markers are thought to be relevant in the regulation of the biological process underlying the network pro- file. Here, this analysis revealed the top 15 markers in each network that were highly connected. The top highly connected markers were different between the groups and time points. While the biomarker profile network at day 0 was dominated by lipid mediators in the patients who were cured (and this lipid mediator profile remained predominant after treatment), in patients who failed therapy we observed that cytokines represented the most predominant nodes in the networks. Interestingly, after treatment, this highlighted profile has changed (Figure 4, right panels).

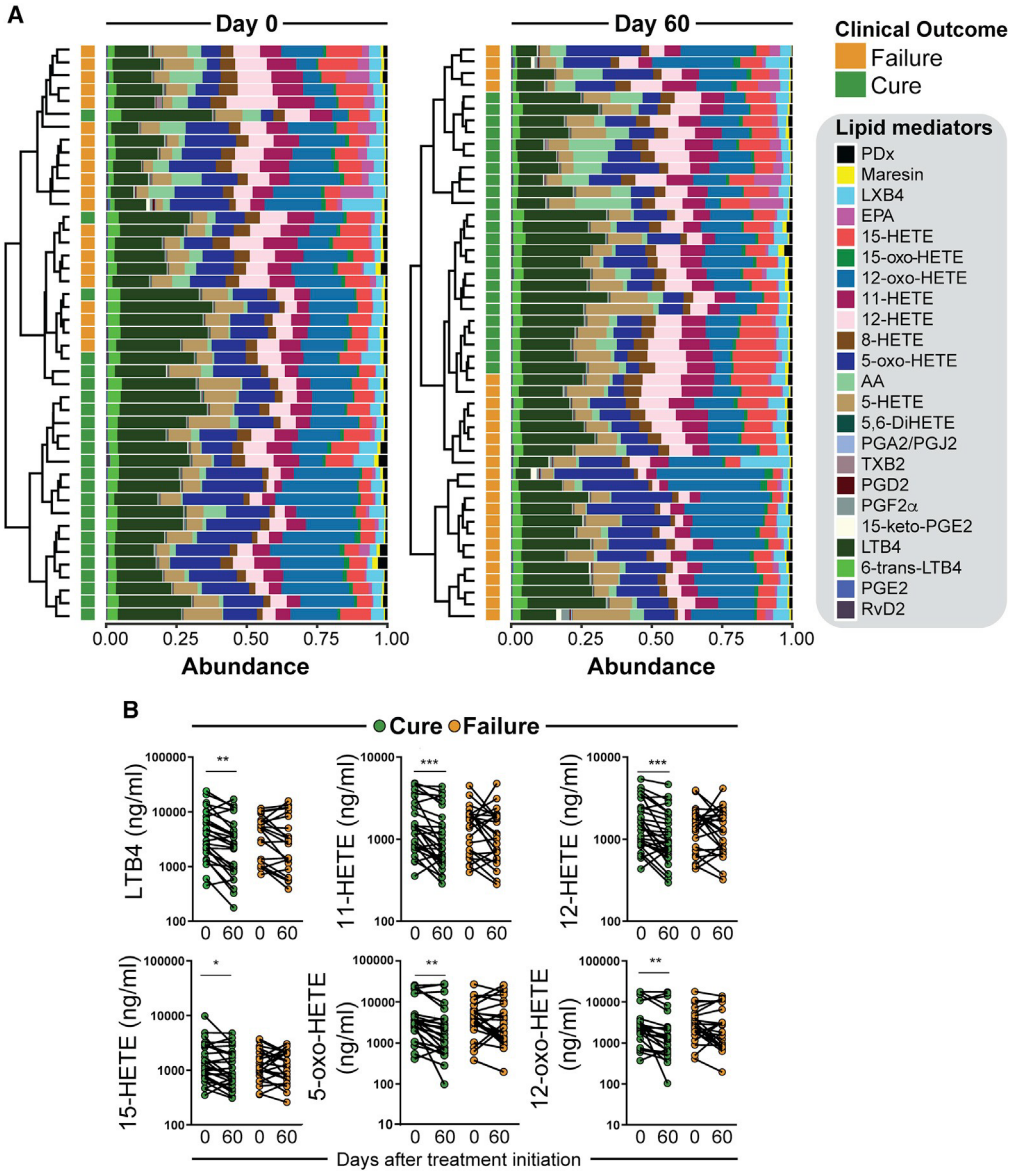

Figure 3. Abundance of lipid mediators in plasma identifies a distinct profile that characterizes patients who failed antileishmanial treatment

(A) Abundance of each lipid mediator was calculated according to Methods. Hierarchical clustering (Ward's method with 100X bootstrap) was performed to test whether the overall profile of lipid mediator abundance could group the patients who failed treatment from those who cured at indicated time points.

(B) Individual parameters that displayed statistically significant differences between the time points tested by Wilcoxon matched pairs test (after log10 transformation) are shown. *p % 0.01; **p % 0.001; ***p % 0.0001.

To test whether the differences in the correlation profiles observed before therapy initiation could be used to predict treatment failure, we employed a discriminant analysis based on canonical correlations (Manabe et al., 2019). The canonical correlation analysis uses the correlation profiles between the markers of a given network, rather than the raw concentration values of each marker, to calculate the prediction performance. In the present study, this analysis was employed to perform a proof of concept that the correlation profiles could be used to characterize and/or predict antileishmanial treatment outcomes. In this approach, we tested three distinct models: one inputting only the inflammatory proteins, a second model inputting data on lipid mediators, and a third model including data on both inflammatory proteins and lipid medi- ators. Receiver operator characteristics (ROC) curve analysis demonstrated that all the predictive models

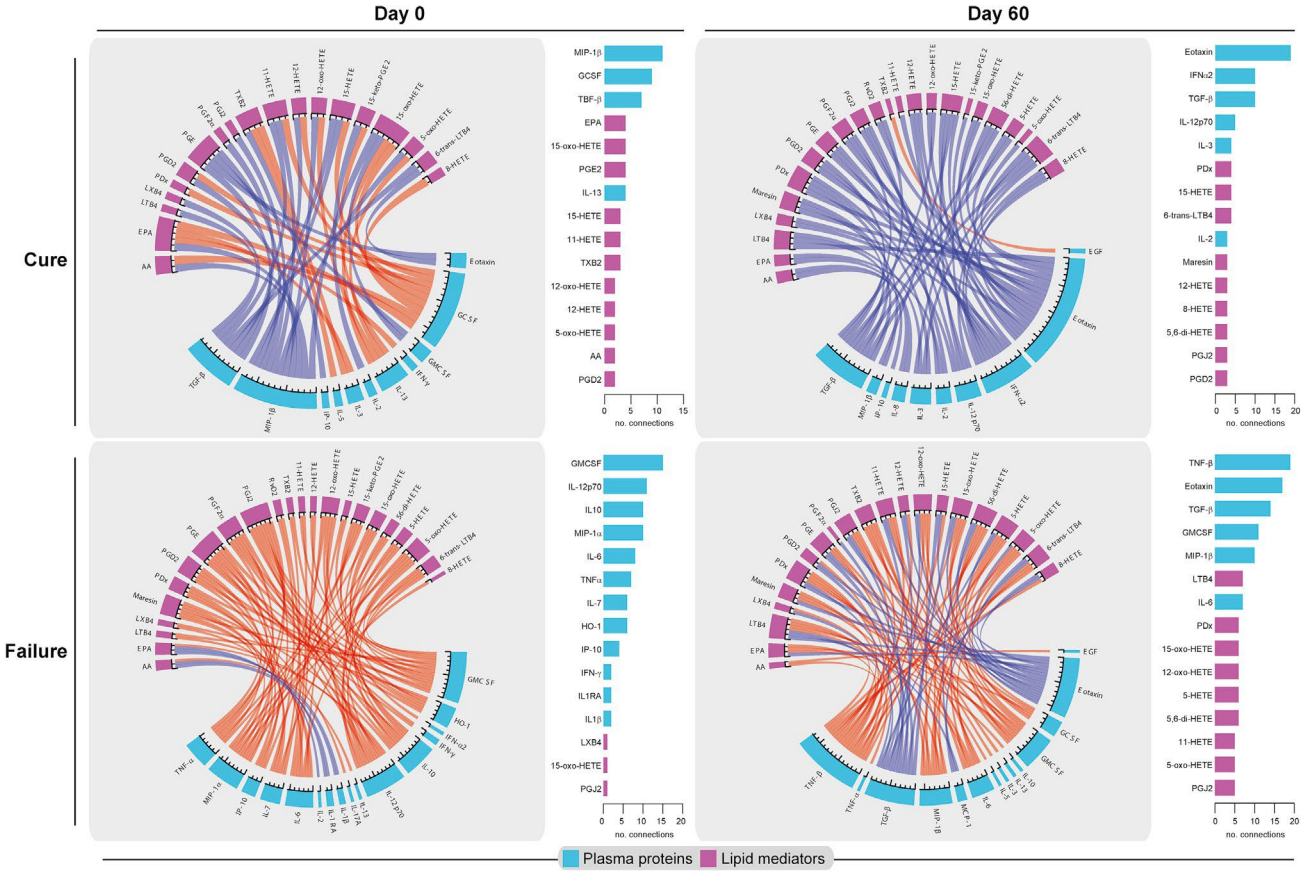

Figure 4. Network analysis of correlations between plasma proteins and lipid mediators in patients undergoing leishmaniasis treatment Correlations were built using Spearman correlation matrices. Each bar represents a different parameter. The length of each bar is proportional to the number of significant correlations. The connecting lines represent statistically significant correlations ($p < 0.05$ after adjustment for multiple comparisons using the Holm-Bonferroni's method). Red connecting lines represent positive correlations, whereas blue lines infer negative correlations. The thickness of the connecting lines is proportional to the Spearman correlation rank coefficient (rho) value. Markers that did not exhibit statistically significant correlations are not shown. Node analyses shows the top 15 markers highly connected in each network.

were able to discriminate treatment outcomes with high accuracy, with gain in power when data on both proteins and lipid mediators were considered (Figure 5).

Multi-omic Factor Analysis Defined a Signature Enriched in Lipid Mediators that Predicts Treatment Failure

To identify the main factors that were contributing to the prediction of treatment failure in the canonical correlation analysis, we developed a factor analysis integrating the two data sets, one including plasma levels of cytokines and a second including lipidomic measurements, using the Multi-Omic Factor Analysis (MOFA) tool as previously described (Argelaguet et al., 2018). This approach is also focused on the corre- lation profiles between the biomarkers measured. MOFA generates different combinations of either pro- teins and/or lipid mediators that compose factors (''latent factors'') which correlations more robustly ac- count for discrimination between the clinical groups. The analysis revealed that the latent factor 1 (LF1) was the element that contributed the most for the distinction between treatment cure and failure (Fig- ure 6A). In addition, the lipidomic correlation profile had more relevance in explaining the variance of the LF1 than did the profile of plasma proteins (Figure 6A). Finally, we plotted the loading scores of the LF1 to identify the most relevant lipid mediators and plasma proteins which correlation profiles could explain the distinction of the treatment outcomes. We found that TNF-b was the most important plasma protein (Figure 6B), whereas 12-oxo-HETE, 5-oxo-HETE, and LTB_4 were the top loading parameters in the lipid mediator component of LF1 (Figure 6C).

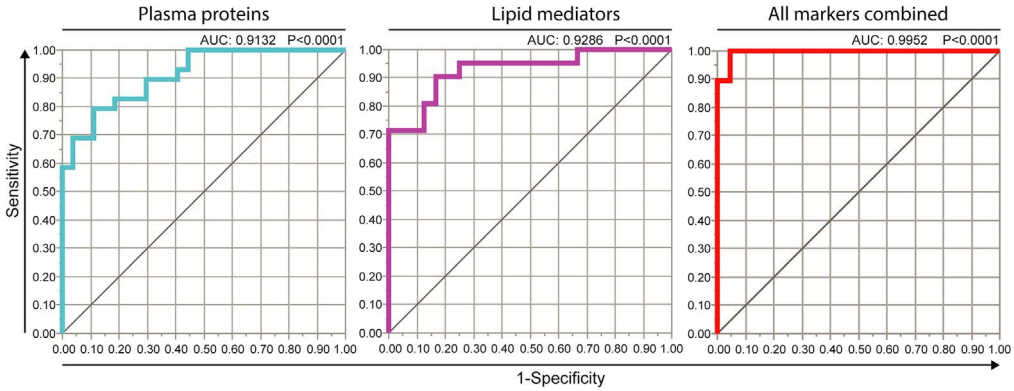

Figure 5. Canonical discriminant analysis of plasma proteins and lipid metiator measures before therapy initiation predicts treatment failure

Receiver operator characteristics (ROC) curve analysis of indicated models inputting data on plasma proteins, lipid mediators, or both, was performed to test power to distinguish treatment cure from failure in patients with leishmaniasis before initiation of antileishmanial therapy.

Machine Learning Decision Tree Using Data from Lipidomics and Proteomics Predicts Treatment Outcomes

The results so far indicated that correlation profiles are so distinct between the groups of patients with CL and different treatment outcomes that could be used in a predictive model. To address the question on whether there is a reasonable format to test prediction using concentration values of biomarkers, rather than correlation profiles, we have employed a stepwise approach using machine learning decision tree (Figure S4). A machine learning conditional tree inference model incorporating values of all the parameters from both Luminex and lipidomics assays assessed at the study baseline (day 0) was designed to answer two main issues: (i) to identify a combination of biomarkers that could best identify treatment failure cases;
(ii) to establish cut-off values of the markers that could be used to differentiate between treatment failure and cure. This approach identified three significant splits in the decision tree, including circulating concen- trations of eotaxin, 11-HETE, and TGF-b (Figure S4). The results indicate that a rational, stepwise assess- ment of these three parameters could be used to help identifying patients with high risk of treatment failure.

Analyses from Publicity Available Data Sets Validate the Involvement of the Lipid Biosynthetic Pathway in Discriminating Different Treatment Outcomes in Patients with CL

To investigate the importance of the lipid biosynthetic pathway in patients with treatment failure, we re- analyzed data from a RNAseq experiment recently published (Amorim et al., 2019), from an independent patient cohort of the same endemic region. This analysis included transcriptomes of specimens obtained from skin lesion biopsies collected prior to initiation of antileishmanial treatment. We focused the analysis on genes which were associated with lipid biosynthetic pathway and observed that patients with CL ex- hibited a completely distinct gene expression profile compared to uninfected healthy controls (HCs) which was able to separate 2 different hierarchical clusters (Figure 7A). Surprisingly, the gene expression profile of the genes related to the lipid pathway was not related to lesion size and parasitic load. Although this approach failed to completely segregate clinical outcomes based on treatment outcomes, we observed that there were two gene clusters which exhibited opposite expression profiles between patients with CL and HCs (Figure 7A). In addition, a principal component analysis (PCA) model including expression values of all genes included in the hierarchical analysis also demonstrated a complete segregation to pa- tients with CL and controls and partial segregation between the CL subgroups with distinct clinical out- comes (Figure 7B). Lastly, a discriminant analysis using ROC curves of such combination of genes resulted in high accuracy in distinguishing *Leishmania* infection and clinical outcomes (Figure 7C).

DISCUSSION

Incidence of treatment failure among patients with CL has been increasing in recent decades (Ponte-Sucre et al., 2017). The identification of markers that can predict treatment outcomes in CL is important not only

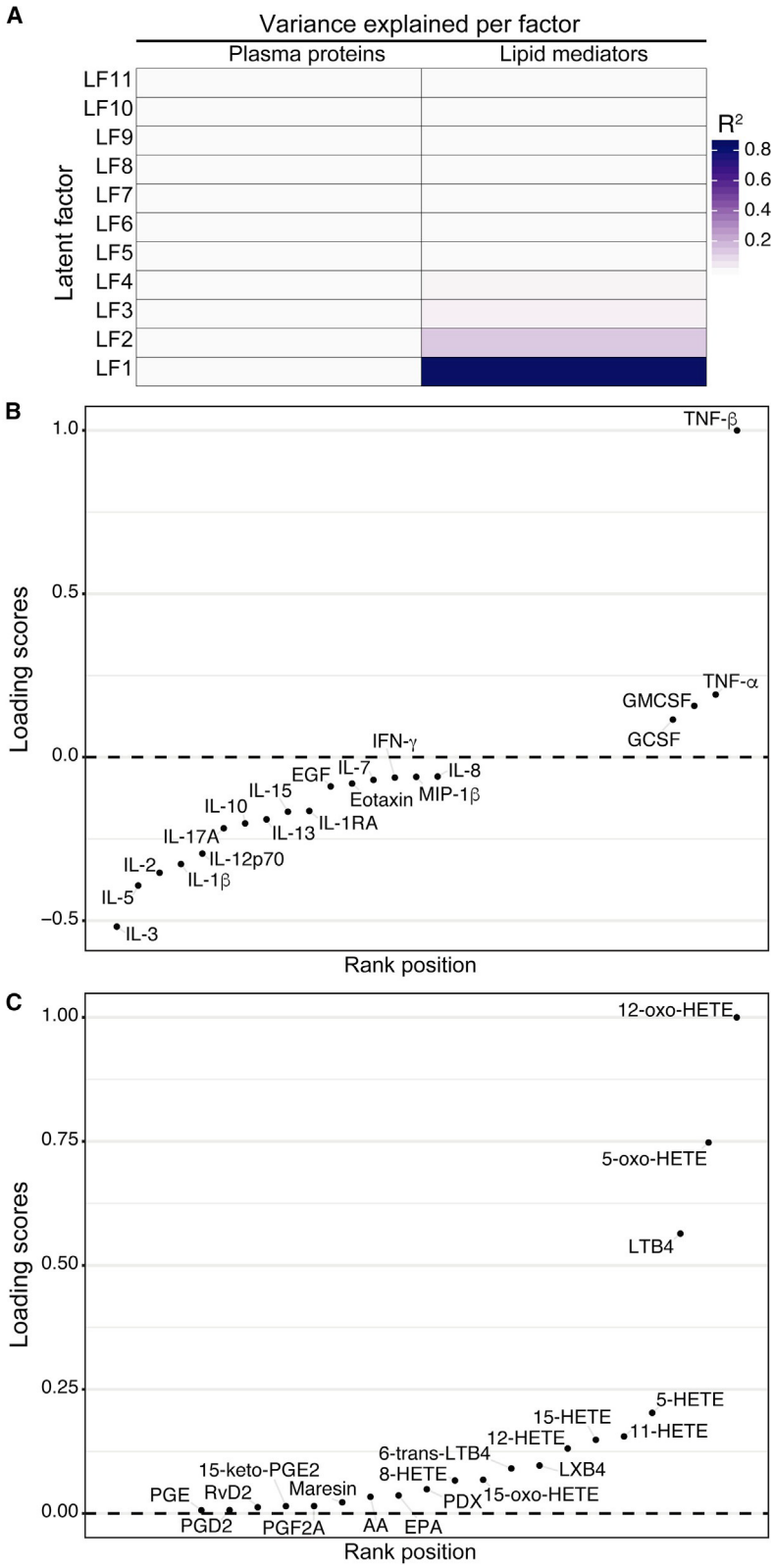
A
Variance explained per factor
Plasma proteins
Lipid mediators
Latent factor
LF11
LF10
LF9
LF8
LF7
LF6
LF5
LF4
LF3
LF2
LF1
R2
0.8
0.6
0.4
0.2
B
Loading scores
1.0
0.5
0.0
−0.5
TNF-β
TNF-α
GMCSF
GCSF
IFN-γ
EGF
IL-7
IL-8
IL-15
Eotaxin
MIP-1β
IL-10
IL-1RA
IL-13
IL-17A
IL-12p70
IL-2
IL-1β
IL-5
IL-3
Rank position
C
Loading scores
1.00
0.75
0.50
0.25
0.00
12-oxo-HETE
5-oxo-HETE
LTB4
5-HETE
15-HETE
11-HETE
12-HETE
6-trans-LTB4
8-HETE
LXB4
15-keto-PGE2
PGE
RvD2
Maresin
PDX
15-oxo-HETE
PGD2
PGF2A
AA
EPA
Rank position

Figure 6. Multi-Omics Factor Analysis Identified Latent Factors Able To Predict Treatment Outcomes in Patients with Tegumentary Leishmaniasis

(A–C) (A) Samples with paired data on plasma cytokines and lipid mediators were analyzed using MOFA as described in Methods. MOFA summarized the protein and lipid mediator data in 11 latent factors (LFs) with different associations (evaluated using the proportion of total variance explained, R^2) with the protein data set, the lipid mediator data set, or both. Each latent factor was inputted as a principal component in a PCA algorithm. Loading scores of the most relevant factor (LF1) in the data set of proteins (B) or lipid mediators (C) were plotted to quantify the contribution of each parameters to the LF1 final score. The most relevant markers are illustrated by the ones with the highest loading score values. PCA, principal component analysis.

to identify patients at higher risk of failure but also to better understand the mechanisms underlying such conditions. In the present study, we show that patients with CL who failed therapy exhibit a very distinct expression profile of plasma proteins and lipid mediators in peripheral blood. Interestingly, the changes are present even before the commencement of therapy, and our analyses reveal that these differences may predict the patients who will experience treatment failure. Furthermore, the results indicate that phar- macological intervention of the specific pathways identified here may serve as adjunct therapy to antileish- manial treatment to optimize clinical management.

The inflammatory response during the CL is characterized by high levels of circulating Th1 lymphocytes, cytokines, and chemokines (Franc¸a-Costa et al., 2015; Ribeiro-de-Jesus et al., 1998) that significantly reduce after treatment (Brito et al., 2014). Our results indicated that circulating levels of TNF-a and IP- 10 substantially reduced at day 60 of therapy compared to that detected at pre-treatment in the group of patients that were successfully treated. Patients who failed therapy exhibited a significant reduction in TNF-a, IP-10, and also in IL-2, IL-1a, and IL-6 at day 60. Interestingly, at day 60, low levels of GM-CSF, IFN-a2, IL-6, and IL-3 were observed in patients who failed therapy, compared to those from patients who cured. The observed profile in treatment failure made us hypothesize that significant reductions on cytokine levels in blood after onset of antileishmanial therapy may be associated with impaired capac- ity to eliminate the parasite. Indeed, levels of GM-CSF, which is a marker that has been shown to pro- mote protection against *Leishmania* infection (Carvalho et al., 2019), were reduced in patients who failed therapy.

Although several studies have demonstrated the role of cytokines in the pathogenesis of CL and its contri- bution to treatment outcomes, the potential difference in lipid mediator expression profiles and their me- tabolites that may contribute to treatment failure is still unknown. Lipid mediators are important modula- tors of inflammation, being involved in both the initiation and resolution of the inflammatory response (Serhan et al., 2015). While inflammatory lipids such prostaglandins , leukotrienes , and hydroxyeicosate- traenoic acids are derived from arachidonic acid (AA), the specialized pro-resolving mediators (SPMs), including resolvins and maresins, are derived mostly from the eicosapentaenoic acid (EPA) and the doco- sahexaenoic acid (DHA) (Serhan et al., 2015). We have previously demonstrated important roles of different lipid mediators produced by both AA and DHA pathways in determining the distinct clinical forms of leish- maniasis (Arau´jo-Santos et al., 2017; Franc¸a-Costa et al., 2016; Malta-Santos et al., 2017), highlighting the importance of such mediators as biomarkers of cutaneous and visceral leishmaniasis.

In this study, we show the characterization of the abundance profile of lipid mediators in blood of patients with CL undergoing therapy and tested associations with treatment outcomes. We observed that the most abundant lipid mediators were AA derived, such as LTB_4, 5-HETE, 5-oxo-HETE, 12-HETE, 11-HETE, PGE_2, and 15-HETE. Strikingly, the analysis of lipid mediator abundance revealed that patients who failed treat- ment exhibited a slight but distinct profile from those who were successfully treated even before the initi- ation of therapy. Thus, the overall composition of lipid mediators in plasma of patients with CL is able to characterize treatment failure. Of note, we and others have demonstrated the balance between PGE_2 and LTB_4, two of the most abundant mediators described here, as a critical factor determining clinical man- ifestations and/or outcomes in leishmaniasis (Franc¸a-Costa et al., 2016), as well as in other diseases such as tuberculosis (Mayer-Barber et al., 2014; Shivakoti et al., 2019; Sorgi et al., 2020)and malaria (Abreu-Filho et al., 2019). Interesting, with exception of PGE_2 and 5-HETE, the most abundant inflammatory mediators significantly reduced their levels in patients who were successfully treated (Figure 3B) Supplementary Infor- mation, suggesting that the levels of such mediators may be reflecting the degree of immune activation and infection control.

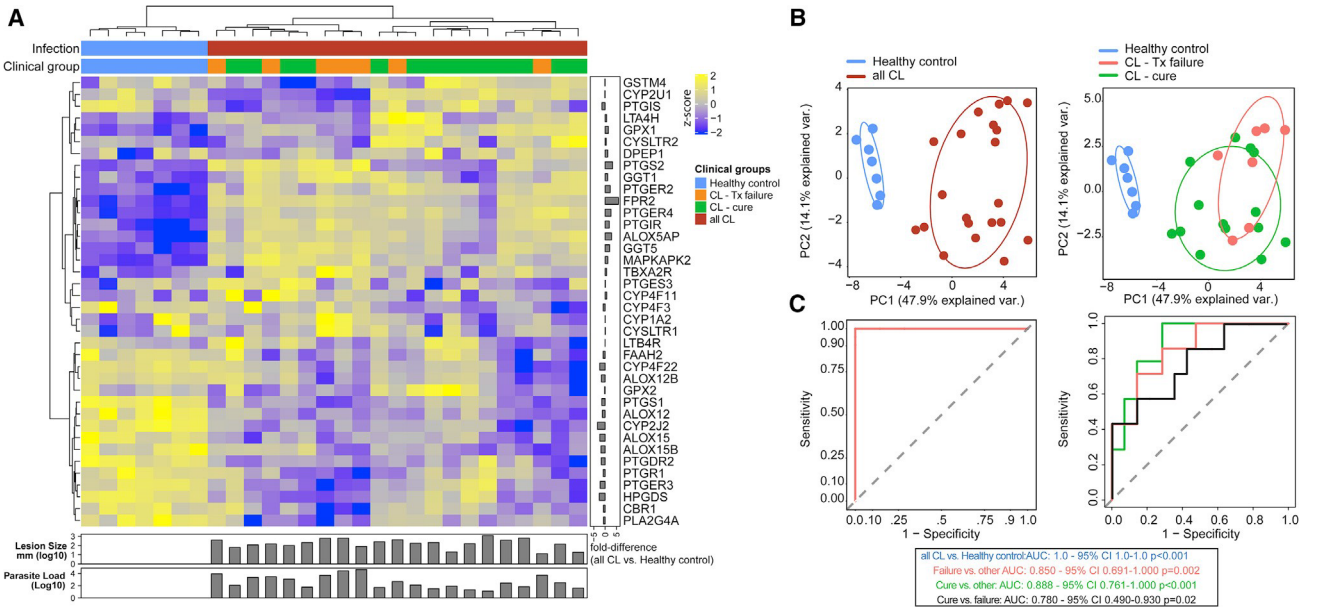

Figure 7. Patients with Cutaneous Leishmaniasis Display a Distinct Profile of Gene Expression from the Lipid Biosynthetic Pathway

(A) A hierarchical clustering analysis (Ward's method) was employed to illustrate the overall profile of genes of the lipid biosynthetic pathway in patients with CL who cured or failed therapy. Each column represents one patient.

(B) A principal component analysis (PCA) model was employed to test whether combination of the genes evaluated could cluster patients with CL separately from controls and the clinical outcomes. A vector analysis was utilized to illustrate the influence of each gene in the distribution of the data of the PCA model.

(C) Lipid gene expression significantly discriminated patients with CL to HCs (area under the curve (AUC) receiving operating characteristic [ROC] curve, 1) and the cure or failure (0.88 and 0.85, respectively).

Our Spearman correlation network analyses of plasma proteins and lipid mediators revealed that there are significant differences in the relationships between plasma levels of the biomarkers that can characterize the distinct clinical outcomes and time points. In addition, the top highly connected markers also were different between the groups and time points, indicating that there is a change in regulation of systemic inflammation induced by treatment, which was distinct between patients who failed and those who were successfully treated. Before treatment, patients who cured exhibited a relative balance hallmarked by a similar number of positive and negative correlations, involving G-CSF and MIP-1b/TGF-b, respectively, with AA-derived metabolites. In general, treatment implementation was associated with reduction in the number of significant interactions, completely changing the network profiles. Thus, at day 60, the most important markers were eotaxin, IFNa2, and TGF-b. Although MIP-1b and eotaxin are chemokines released by monocytes and eosinophil in response to *Leishmania* infection, their marked production is associated with an intense inflammatory response (Matte and Olivier, 2002). TGF-b, another important marker involved in the suppression of immune response favoring *Leishmania* infection (Barral-Netto et al., 1992), was down modulated in patients who were cured throughout the follow-up. Taken together, these data suggest that in patients who were successfully treated, there is a downmodulation of chemokines and mediators involved in persistent inflammation, leading to diminished immune activation, favoring skin healing.

The correlation profile in patients who failed therapy was predominantly marked by positive interactions and the only negative interactions involved IL-1b and IL-1RA. Interestingly, IL-1b is known to be involved in the CL pathogenesis and has been shown to associate with treatment failure (Zamboni and Sacks, 2019). More recently, a transcriptional signature including *IL1B* has been reported to predict clinical out- comes in CL (Amorim et al., 2019). In our study, we found that other cytokines could also characterize treat- ment outcomes. Interestingly, after treatment implementation in individuals who further experienced treat- ment failure, TGF-b and eotaxin remained the most relevant markers exhibiting negative connections in the network, whereas TNF-b was the parameter exhibiting the highest number of positive correlations. Poly- morphism in the TNF locus, which includes genes encoding TNF-a and TNF-b, is associated with increased susceptibility to infection with either *L. braziliensis* (Cabrera et al., 1995) or *L. infantum* (Karplus et al., 2002). Our results argue that in patients who failed the conventional treatment, there is a potential persistent

interaction between plasma markers. Additional studies are needed to test if polymorphisms in genes of the cytokines reported here could result in increased risk of treatment failure.

Finally, the integrative analysis of protein with lipidomic profiles revealed that the lipid mediators are a ma- jor component able to discriminate treatment outcomes. A recently published study used the similar me- tabolomic approach to identify predictive biomarkers of the treatment outcome in patients with CL caused by *L. viannia* (Vargas et al., 2019). As noted in present study, drug exposure was able to modulate the meta- bolic products, and this change was associated with immune response and outcome of treatment (Vargas et al., 2019). Here, we demonstrated that among the lipids, the 12-oxo-HETE, 5-oxo-HETE, and LTB_4 stood out as the most robust biomarkers in the predictive model employed. To the best of our knowledge, there are no previously reported studies on describing concentration of oxo-HETEs lipids in CL. Future studies using *in vitro* systems and animal models are warranted to directly elucidate and describe the role to HETEs and oxo-HETEs in CL pathogenesis.

Many of the relevant findings on the present study were based on correlation profiles between biomarker concentrations in plasma. Such an approach is widely used, but it has limited application in clinical practice. In order to come up with a strategy that could be eventually employed in a clinical setting, we built a step- wise approach to predict treatment failure, which was based on assessment of concentration values rather in correlation profiles. This analysis used machine learning to build a decision tree. The results indicate that eotaxin, 11-HETE, and TGF-b levels measured at pre-treatment could be used in sequence to identify in- dividuals who will fail treatment, in case of development of an easily assessable point of care.

The analysis of a publicly available transcriptome data set was an important contribution to the study, due to the fact that reinforced the idea that links expression of genes related to the lipid biosynthesis and odds of antileishmanial treatment failure in an independent cohort (Amorim et al., 2019). Such lipid-related signature was not influenced by lesion size or parasite load. In addition, results presented here are the first formal demonstration that gene expression of targets from the lipid mediator pathway can accurately iden- tify patients with CL. The transcriptional data from normal skin biopsies compared to patients with CL re- vealed that the gene expression values of targets from the lipid biosynthetic pathway are able to identify samples from *Leishmania*-infected patients, as well as to reliably predict treatment outcomes. Interesting, genes involved in the synthesis of LTs and HETEs (*alox5*), as well PGs (*ptgs2*) and their inflammatory recep- tors (*ptger2*, *ptger4*, and *ptgir*), presented upregulated values in all patients with CL. These observations are consistent with our findings from plasma lipidomics, where the most abundant lipid mediators were products of the enzymatic activity of the proteins encoded by those genes found to be upregulated in skin lesions from patients with CL. Regardless, our data reveal that a biosignature enriched in lipid medi- ators is able to reliably predict treatment outcomes in CL, and the markers contributing to such signature may serve as targets in a potential host-directed therapy.

Limitations of the Study

Our study has some limitations. The number of patients was relatively low, although it was similar to other previously published investigations. In addition, patients from only one clinical site, from a single endemic area, were investigated, and thus, larger studies recruiting patients from more diverse clinical and epide- miologic settings are necessary to validate our findings. We performed validation analyses of the hypoth- esis associating lipid mediator pathways and treatment outcomes, and a more stringent approach measuring the biomarkers in a distinct cohort will be necessary to ultimately confirm the results.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Bruno B. Andrade (bruno.andrade@fiocruz.br).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Original data sample for Figure 6 in the paper is available GSE127831 (Amorim et al., 2019).

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101840.

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AUTHOR CONTRIBUTIONS

Conceptualization: H.M.S., C.A.S., V.N., P.T.B., J.F.C., B.B.A, and V.M.B. Methodology: H.M.S., C.A.S., and V.N. Software, Formal analysis, Validation, and Data Curation: K.F.F. and A.T.L.Q. Clinical support: J.S., A.L., L.P.C., P.L.R.M., and E.M.C. Writing: H.M.S., C.A.S., P.L.R.M., L.H.F., E.M.C., B.B.A, and V.M.B. Super- vision: B.B.A and V.M.B. All authors approved the final manuscript.

DECLARATION OF INTEREST

The author(s) declare no competing interests.

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Supplemental Information

Multi-omic Analyses of Plasma Cytokines, Lipidomics, and Transcriptomics Distinguish Treatment Outcomes in Cutaneous Leishmaniasis

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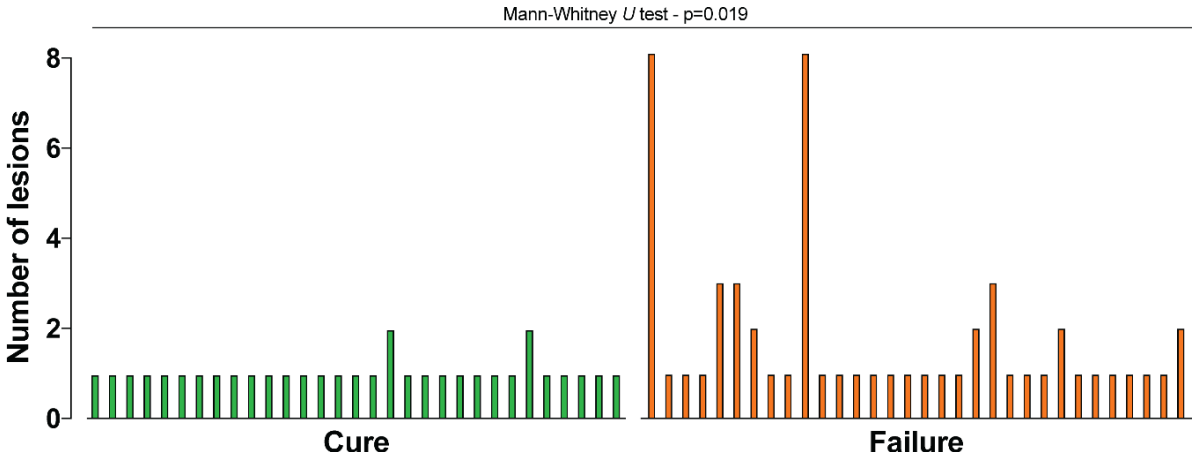

Figure S1. Number of lesions in cutaneous leishmaniasis patients, related to table 1. Number of active lesions per each study participant at study enrollment was plotted in histograms stratified by treatment outcomes. Distribution of values were analyzed using the Mann Whitney *U* test.

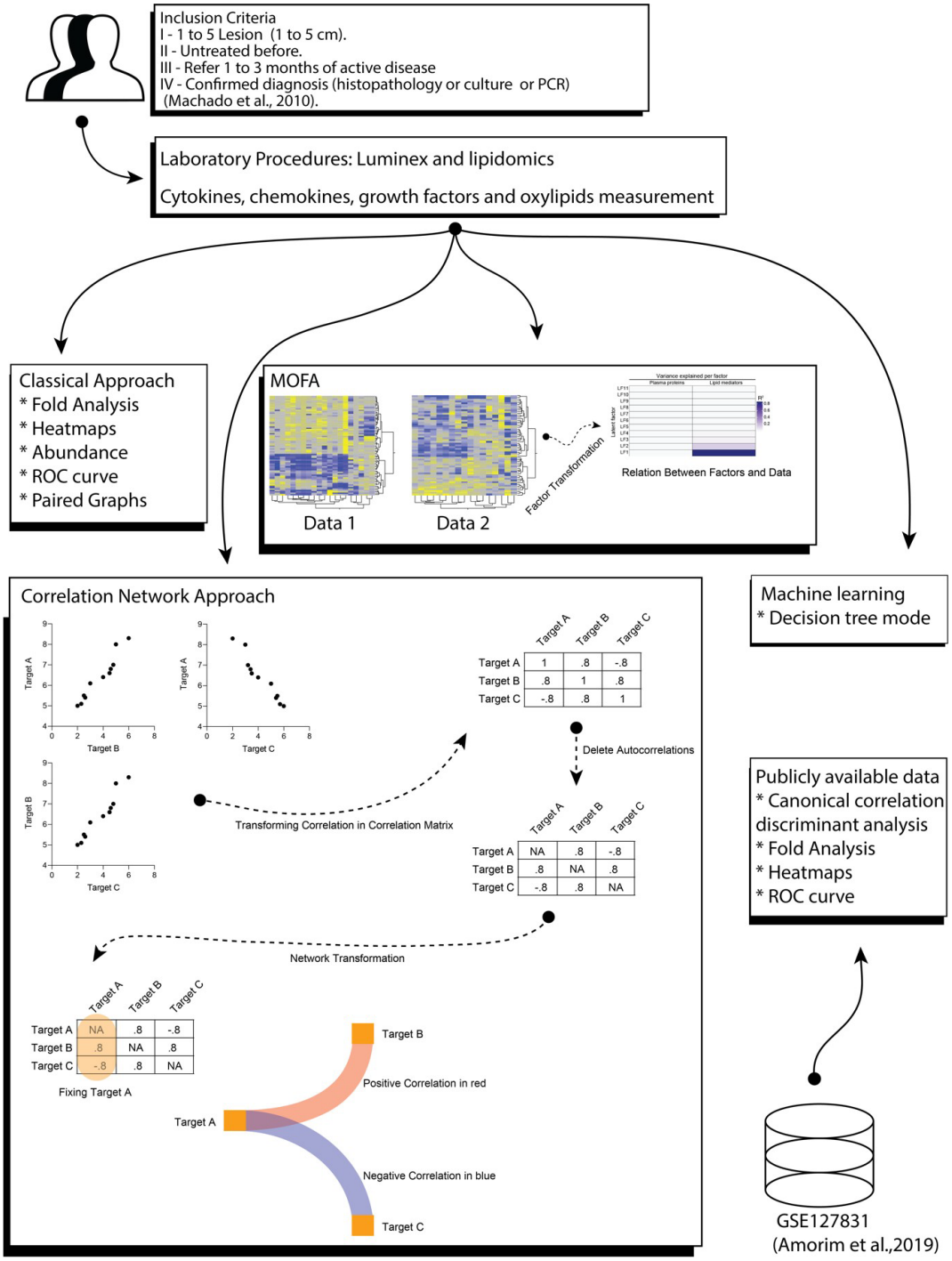

Figure S2. Outline of the analysis plan, related to all figures. Cryopreserved EDTA plasma samples were used for the omics assays. Cytokines and growth factors were measured using a Luminex assay whereas oxylipids were quantified using lipidomics as described in Methods. Analyses were divided in two main portions. In the first batch of analyses, classical analytical approach, which included calculations of fold-differences, design of heatmaps and abundance color maps, estimation of accuracy in predicting treatment outcomes by using Receiver Operator Characteristics (ROC) curves and dynamic changes using paired analysis. In addition, Correlation matrices were calculated and used to build networks with the objective of describing the overall profile of relationships between plasma lipids and proteins in each clinical group and study timepoint. Data from Luminex and lipidomics were also integrated in a Multi-Omic Factor Analysis (MOFA) to identify major contributor of the regulatory networks driving the distinctions between the clinical groups that could predict treatment outcomes. Furthermore, a machine learning strategy using decision trees was designed to identify combination of markers with their respective cut-off values which were able to predict treatment failure. Finally, Transcriptome data from a publicly available dataset was used to validate the hypothesis that gene expression values of targets from the lipid biosynthesis pathway could be used to identify characterize patients with leishmaniasis who developed different treatment outcomes.

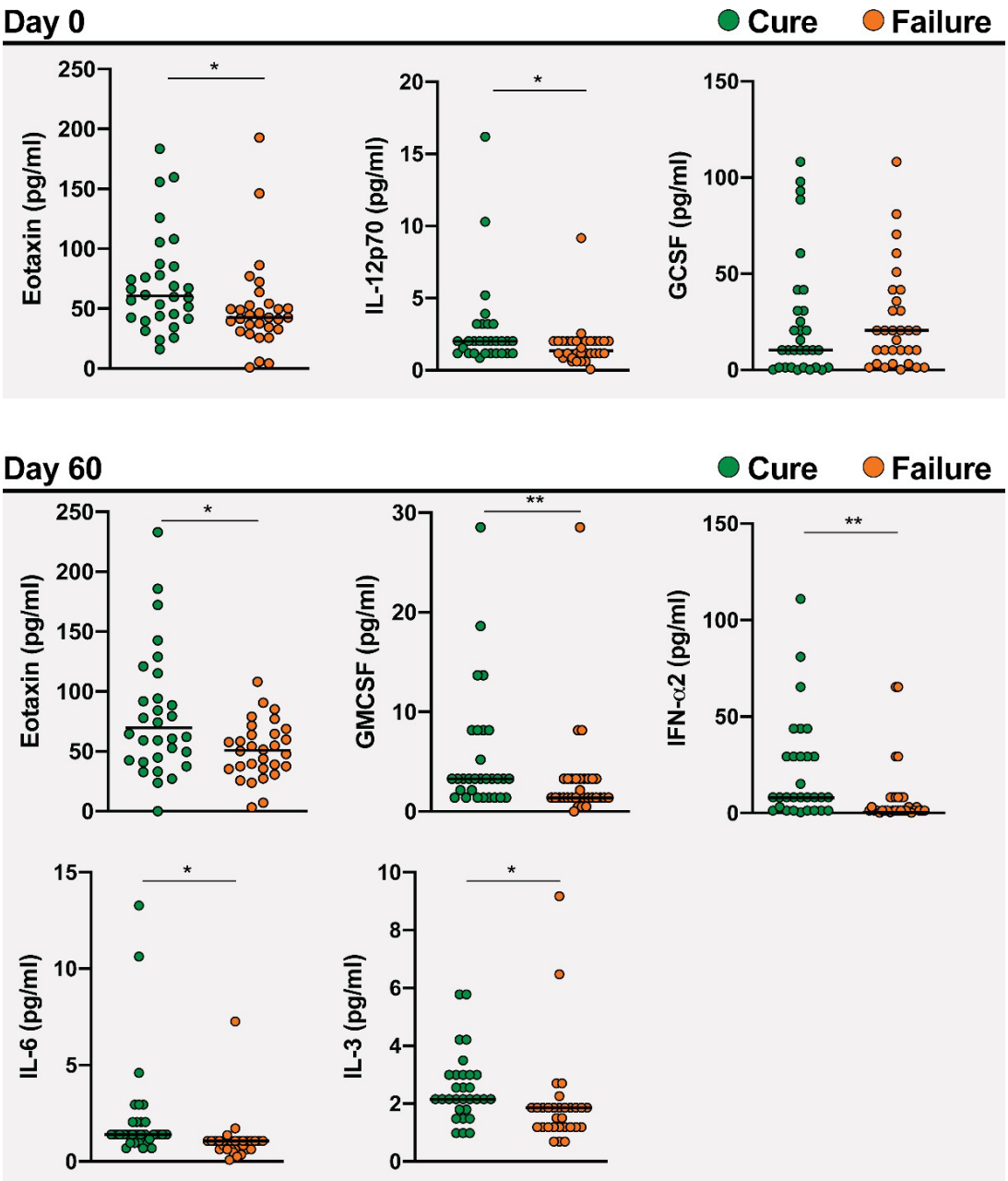

Figure S3. Inflammatory proteins in plasma of cutaneous leishmaniasis patients according to timepoint and treatment outcome, related to Figure 1. Parameters that displayed statistically significant differences between the study groups at each timepoint were tested using the Mann-Whitney *U* test. *$P \leq 0.01$; **$P \leq 0.001$.

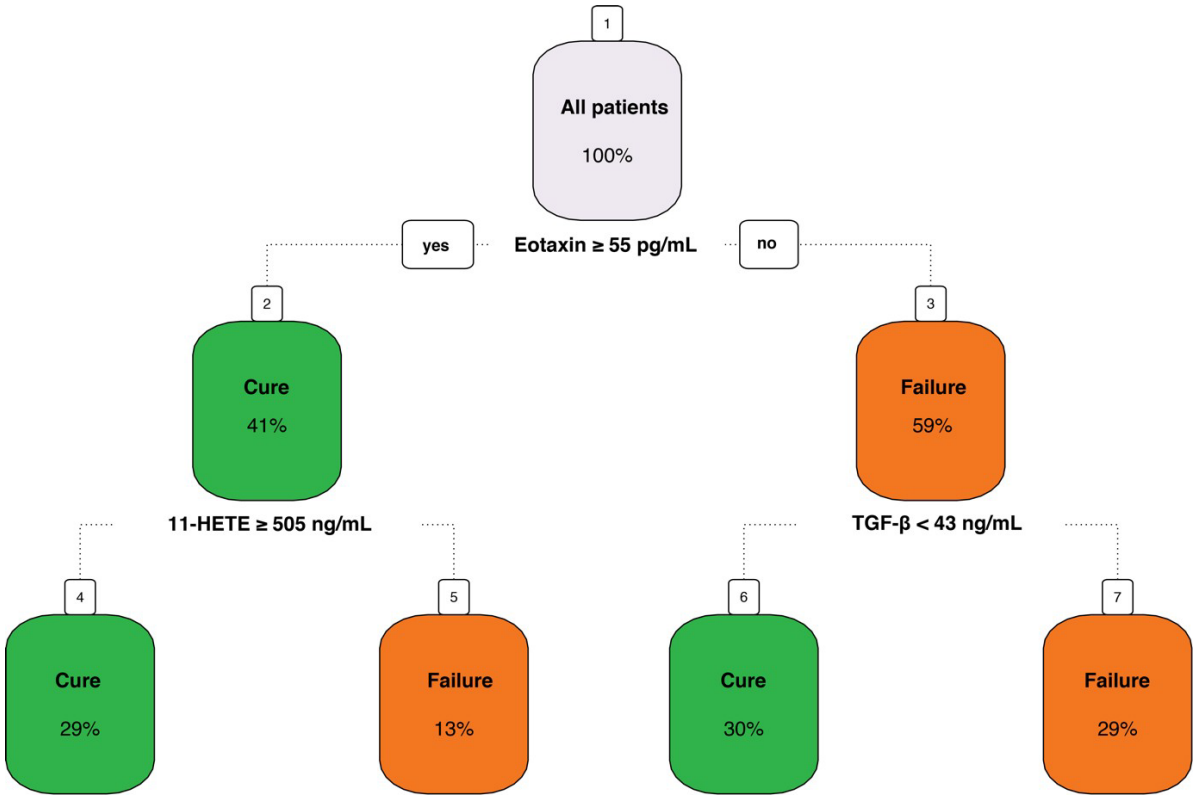

Figure S4. Machine learning decision tree model to predict treatment failure using pre- treatment levels of plasma biomarkers, related to Figure 3. All markers from both the Luminex and the lipidomic assays were included in the model. Measurements from study baseline (day 0) were considered. The p-value of the combined performance was 0.001. The area under the curve (AUC) of the receiver operator characteristics (ROC) curve was 0.7 (95% confidence interval: 0.6-0.8).

Transparent Methods Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Ethics Committee of the Hospital Universitário Prof. Edgard Santos of the University Federal da Bahia (number 2.471.184). Written informed consent was obtained from all participants or legal guardians, and all data analyzed were anonymized.

Clinical study design

A prospective case control study was performed in eligible patients that spontaneously sought medical treatment at a referral center in Corte de Pedra, Brazil, an endemic area for CL caused by *L. braziliensis*. The present study was nested with a clinical investigation in which all patients were evaluated at days 0, 15, 30, 60, 90 and 210 after recruitment, however blood specimens were available only at day 0 (pre-treatment) and day 60 (for monitoring of biochemical parameters). All patients were treated with intravenous 20 mg Sb^V/kg for 20 days. Patients were followed up for 210 days to define the main outcomes, cure and treatment failure. EDTA plasma samples were cryopreserved at -80ºC for the laboratory assessments. Clinical, epidemiological and therapeutic outcome data were captured in standardized clinical report forms by trained physicians who are also part of the research team (the authors P.R.L.M. and E.M.C.). The major aim of the study was to describe biomarkers able to predict treatment failure. To estimate the total sample size for a study power greater than 90% with a Type 1 error of less than 5% and considering the incidence of treatment failure of 40% (Machado et al., 2010) and loss to follow up of 30% (for whatever reason), the sample calculation revealed that we would need to recruit a total of 60 treatment-naïve CL patients. We recruited a total of 63 patients, with individuals 32 developing treatment failure.

Patients

Inclusion criteria were: (i) to present with one to five ulcerated lesions with sizes varying between 1 and 5 cm in diameter; (ii) to be anti-*Leishmania* treatment naïve; (iii) to refer 1 to 3 months of active disease; and (iv) to have a confirmed diagnosis by positive identification of amastigotes in histopathological examination, positive *L. braziliensis* culture or positive polymerase chain reaction for *L. braziliensis*, as previously described (Machado et al., 2010). Exclusion criteria were: (i) pregnant or breastfeeding women; childbearing-age women unwilling to adhere to contraceptive measures during treatment and until 2 months after the end of treatment; (ii) previous history of leishmaniasis treatment; (iii) malnutrition; (iv) referred

or confirmed concomitant diseases such as cardiac, pulmonary, hepatic, cancer, tuberculosis, Hansen disease, malaria, HIV/AIDS or any other infectious disease; (v) laboratory evidence of chronic liver or kidney disease (Machado et al., 2018).

Outcome definition

Treatment outcomes were reported as the following: cure was defined by complete re-epithelization of lesion(s) and absence of infiltration whereas treatment failure denoted persistence of ulceration at up to 60 days after the end of treatment. Patients who failed treatment received an additional cycle of Sb^{v} or Amphotericin B.

Cytokines, chemokines and growth factors measurement

Plasma levels of EGF (epidermal growth factor), Eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-α2, IFN-γ, interleukin (IL)-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, interferon inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, tumor necrosis factor (TNF)-α, TNF-β and vascular endothelial growth factor (VEGF) were measured in cryopreserved EDTA plasma samples using a commercially available Luminex kit (Merck, Darmstadt, Germany) according to the manufacturer instructions. Levels of transforming growth factor β (TGF-β) and heme oxygenase-1 (HO-1) were measured using enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, Minnesota).

Oxylipids extraction

Oxylipids extraction was performed from plasma samples using the SPE (Solid Phase Extraction) method according to a previously described protocol (Machado et al., 2010; Sorgi et al., 2018). In brief, each plasma sample (150 μL) was spiked with internal deuterated standard (IS) solution (Cayman Chemical, Ann Arbor, Michigan) before being extracted. The samples were then submitted to protein precipitation with 1.5 mL of methanol/acetonitrile (1:1, v/v) at 4 °C, which was left to denature overnight. Furthermore, plasma samples were centrifuged for at 800x *g* for 10 min at 4 °C. The denatured proteins were quantified using the Bradford protein assay (Sigma-Aldrich, St. Louis, MO) to normalize the lipid concentration for each sample, and the resulting supernatants were diluted with Milli-Q water to decrease the organic solvent to a maximum concentration of 10-15 %. For the SPE extraction protocol, the cartridge (Hypersep C18-500 mg, 3 mL, Thermo Scientific, Bellefonte, Pennsylvania) was washed with 4 mL of MeOH and equilibrated with 4 mL of H_2O using an extraction manifold (Waters, Milford,

Connecticut). After loading the diluted samples, the cartridges were again flushed with 4 mL of Milli-Q water to remove hydrophilic impurities. The analytes were eluted with 1 mL of MeOH. The solvent was removed in vacuum (Concentrator Plus, Eppendorf, Hamburg, Germany) at room temperature and re-dissolved in 50 µL of MeOH/H_2O (7:3, *v/v*) for LC-MS/MS analysis.

Oxylipids identification and quantification

After lipid extraction, specimens were transferred to autosampler vials and 10 µL of each sample were injected on the TripleTOF® 5600+ Target Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) system (Sciex, Foster City, California), as previously described (Sorgi et al., 2018). The method employed a High-Performance Liquid Chromatography (HPLC) system (Nexera X2, Shimadzu, Kyoto, Japan) using an Ascentis Express C18 column (Supelco, St. Louis, Missouri) with the following specifications: 100 × 4.6 mm and particle size of 2.7 µm. Elution was conducted under a binary gradient system with Phase A constituted by H_2O/ACN/acetic acid (69.98:30:0.02, *v/v/v*) at pH 5.8, and Phase B composed by ACN/isopropanol (70:30, *v/v*). Gradient elution was carried out for 25 min at a flow rate of 0.6 mL.min−1. An electrospray ionization (ESI) source in the negative ion mode was used for high-resolution multiple-reaction monitoring (MRMHR) scanning. The mass range of the product ion from the experiments varied from 50 to 700 m/z; the dwell time was 10 ms at a mass resolution of 35,000. Additional instrumental parameters were: nebulizer gas (GS1), 50 psi; turbo-gas (GS2), 50 psi; curtain gas (CUR), 25 psi; electrospray voltage (ISVF), −4.0 kV; and turbo ion spray source temperature, 550°C. Data acquisitions were performed using AnalystTM Software (Sciex, Foster, California). Data processing proceeded through multiple steps, including filtering, feature detection, alignment, and normalization. The PeakView 2.1 (Sciex, Foster, California) software was used for identification of the lipid species and MultiQuant™ (Sciex, Foster, California) software was utilized for quantitative analysis. The final oxylipids concentration in plasma samples was normalized by protein concentration.

Statistical analysis

Median and interquartile ranges (IQR) were used as measured of central tendency and dispersion, respectively. Percentage was used to describe categorical variables such as sex and number of active lesions. Continuous variables were compared using the Mann–Whitney *U* test (between cure and failure groups at each time point) or the Wilcoxon matched pairs test (the

same patient group between two different timepoints). The Fisher's exact test was used to compare frequencies. In some analyses, data on each biomarker was log-transformed and z-score normalized to build heatmaps to illustrate overall trends of data variation between the study groups. A hierarchical cluster analyses (Ward's method) were used to group the biomarkers with similar distribution between clinical groups and time points. In such analyses, dendrograms represent Euclidean distance. In addition, abundance of each lipid mediator was calculated as the following: the concentrations of all lipid mediators detected were summed and considered 100% abundance of lipid mediators in plasma, as previously described (Shivakoti et al., 2019). Then, abundance of the total lipid mediators was calculated for each individual marker relative to the total value considered 100% abundance (Shivakoti et al., 2019). Moreover, a second hierarchical clustering analysis was performed to test whether the overall profile of lipid mediator abundance could group the patients who failed treatment from those who were cured at indicated timepoints. In further analyses, a machine-learning based conditional tree including the values of all the biomarkers measured at study baseline (day 0) was designed to identify the best biomarker or combination of markers that were able to discriminate treatment outcomes. All analyses were pre-specified. To account for multiple measurements, the p-values were adjusted using the Holm-Bonferroni's method. Differences with adjusted p-values < 0.05 were considered statistically significant. All data is available in supplementary data.

Network analysis

Profiles of correlations between inflammatory proteins and lipid mediators at different timepoints in the groups of patients with distinct clinical outcomes were examined using network analysis of the Spearman correlation matrices (with 100X bootstrap). Only statistically significant correlations (adjusted p values < 0.05) were included in the network visualization (lines represent statistically significant correlations). This model is based in quality of correlations (whether a correlation is positive or negative). *Circos* plots were used to illustrate the networks as previously reported (Vinhaes et al., 2019). In additional analysis, number of correlations were quantified per each node (marker) in patients stratified based on timepoint and treatment outcome.

Canonical correlation discriminant analysis

The discriminant analysis model using sparse canonical correlations (canonical correlation analysis, CCA) was employed to test if different combinations of plasma and/or lipid mediators

measured at pre-treatment could distinguish patients who failed anti-*Leishmania* treatment from those who were cured. The CCA algorithm was chosen because many variables were studied. This approach reduces dimensionality for two co-dependent data sets (biomarker profile and baseline characteristics profile, which were sex and age) simultaneously so that the discrimination of the clinical endpoints (treatment failure or cure) represents a combination of variables that are maximally correlated. Thus, trends of correlations between parameters in different clinical groups rather than their respective distribution within each group are the key components driving the discrimination outcome. In our CCA algorithm, simplified and adapted from previously reported investigations of biomarkers for diagnosis of infectious diseases (Mayer-Barber et al., 2014), linear regression graphs represent coefficients from different combinations of plasma factors and baseline characteristics. In addition, investigating statistical relationships between the markers rather than just concentrations allow us to infer about regulatory immune networks (Mayer-Barber et al., 2014). The overall accuracy of each canonical model was tested using C-statistics, with Receiver Operator Characteristics (ROC) curves resulting in calculation of area under the curves (AUC), sensitivity and specificity using the pROC package of the R software as previously described (Manabe et al., 2019; Robin et al., 2011).

Multi-Omics factor analysis

Multi-omics factor analysis (MOFA) enables to analyze biological multidimensional data, ranging from genome, transcriptome, proteome, lipidome and metabolome, integrating all these layers across a more comprehensive result (Argelaguet et al., 2018). The MOFA model used here integrated data from plasma inflammatory proteins and lipidomic analysis, defined by several parameters: (i) the logical scale and paired samples were selected by the functions #scaleViews set as “FALSE” and #removeIncompleteSamples set as “TRUE”; (ii) the number of factors was selected by default with #likelihood = “gaussian” (log2 transformed) and #sparsity set as “TRUE”; (iii) the #tolerance was selected as recommend = 0.01. After selecting all the parameters and preparing the datasets, we used as an input the corrected bath effect count table of the plasma proteins and lipid mediators obtained as the formal analytical merged dataset. All the data were paired by study, individual and platform. Two models of principal component analyses (PCA) were used to identify which plasma proteins and lipid mediators were contributing to separation of the different study groups based on treatment outcomes. In all analyses, a p-value < 0.05 after the 5% FDR adjustment was considered statistically significant.

Decision tree analysis

The metabolite abundance values were used as input to perform a supervised machine-learning approach, based on the outcome definition. Thus, a decision tree algorithm was applied to identify the minimal variable (metabolite) set which exhibit the higher classification power to describe the groups using the rpart package.

Transcriptomic analyses of skin lesions from publicly available datasets

Data samples were downloaded from NCBI GEO: GSE127831 (Amorim et al., 2019) and labeled according to the informed metadata (21 samples infected to *L. braziliensis* before treatment sequenced with *Illumina NexSeq 500,* and 7 uninfected endemic controls). Changes in gene expression levels were considered significant when statistical test values (FDR adjusted p-value) were lower than 0.05 and the fold-difference higher than ±1.5. A heatmap of including expression values of genes identified by our group as being part of lipid biosynthetic pathways was plotted using the *Complexheatmap* package (Gu et al., 2016). A PCA algorithm was performed using cpm log-transformed data of the indicated genes using the *plotPCA* function from *Deseq2* package from R software (Gu et al., 2016; Love et al., 2014).

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7 **PARTE III**

7.1 HIPÓTESE

Resolvina D1 favorece a sobrevivência da *L. braziliensis* em neutrófilos humanos *in vitro*.

7.2 OBJETIVOS

7.2.1 **Objetivo geral**

Avaliar o papel da RvD1 na infecção de neutrófilos humanos por *L. braziliensis in vitro.*

7.2.2 **Objetivos específicos**

- Testar se a suplementação de RvD1 em culturas axênicas de promastigotas de *L. braziliensis* interfere no crescimento do parasito;
- Avaliar a carga parasitária e os mediadores produzidos no sobrenadante de neutrófilos humanos infectados por *L. braziliensis* na presença de RvD1 e inibidores da via;
- Investigar o papel do receptor de RvD1 FPR2/ALX durante a infecção de Leishmania;
- Identificar os mecanismos de ação das resolvinas durante a infecção por *L. braziliensis*.

7.3 RESUMO

Os neutrófilos são as primeiras células da linha de defesa contra patógenos invasores e são rapidamente recrutados durante a infecção por *Leishmania*. Trabalhos anteriores têm demonstrado a importância da síntese dos eicosanóides pelos neutrófilos durante a infecção por *L. braziliensis.* Mais recentemente, o papel dos mediadores lipídicos de pró-resolução durante os processos inflamatórios e patológicos foi associado às suas ações anti-inflamatórias e imunomoduladoras em vários modelos experimentais. Dentre eles, as RvD1 possuem atividades que incluem a inibição da quimiotaxia de leucócitos, bloqueio na produção de citocinas pró-inflamatórias e aumento na expressão de mediadores regulatórios e de resolução. Nesse estudo, avaliamos o papel das resolvinas durante a infecção de neutrófilos por *L. braziliensis.* Observamos que o tratamento com RvD1 promove a replicação intracelular do parasito, e modula a produção de IL-6, IL-10 e de mediadores lipídicos, no entanto os mecanismos envolvidos nesse efeito ainda precisam ser melhor investigados.

8 **METODOLOGIA CULTURA DE PARASITOS**

Nos experimentos *in vitro* foram utilizados isolados de cepas de *L. braziliensis* BA 788 (MHOM/BR/01/BA788) disponíveis no banco de cepas mantido no Laboratório de Imunologia e Biomarcadores, Fiocruz/BA. As culturas foram mantidas em meio Schneider completo contendo 10% de soro bovino fetal e 2 mM de L-glutamina, penicilina 100 U/ml e 100 mg/ml de estreptomicina, pH 7,2 a 25°C, até atingirem a fase estacionária de crescimento (máximo de 5 passagens).

8.1 TRATAMENTOS

Para avaliar o efeito biológico das RvD1 (Cayman Chemical) e do inibidor da 15-LO1 (Cayman Chemical) nas culturas de parasitos, promastigotas na fase estacionária ($2x10^5$/ml) foram incubadas com meio completo sozinho ou com os compostos nas concentrações de 10, 50 e 100 nM. As culturas foram incubadas por 4 dias a 24°C e o efeito desses compostos na viabilidade do parasito foi avaliado pela contagem direta dos promastigotas utilizando câmera de Neubauer.

8.2 ENSAIOS DE INFECÇÃO

O sangue humano de voluntários saudáveis foi obtido do Hemocentro do estado da Bahia (HEMOBa). Neutrófilos humanos foram isolados por gradiente de separação utilizando meio polimorfonuclear (PMN) de acordo com instruções do fabricante (AxisShieldPoc AS, Oslo, Norway). Neutrófilos foram coletados e lavados três vezes com salina a 4°C por 10 min a 1300 RPM. As células foram plaqueadas $5x10^5$ por poço, com meio RPMI-1640 suplementado com 1% de Nutridoma-SP, 2 mM L-glutamina, 100 U/ml penicilina, e 100 µg/ml estreptomicina. Os neutrófilos foram infectados com promastigotas estacionárias de *L. braziliensis* (cinco parasitos por célula) na presença ou ausência das substâncias (RvD1, inibidor da 15-LO1 e BOC). Em alguns casos, os neutrófilos foram pré-tratados por 15 min com Boc - Phe - Leu - Phe - Leu – Phe, antagonista do receptor FPR2/ALX e lavados 1 vez antes da infecção e adição da RvD1. As culturas foram incubadas por 3 h, 37°C, 5% CO2. Após 3 h de incubação, os neutrófilos infectados foram lavados três vezes com salina (centrifugados a 1,300 rpm por 10 min a 4°C) e o sobrenadante foi guardado para dosagem posterior de citocinas, proteínas inflamatórias, mediadores lipídicos e da enzima heme oxigenase 1 (HO-1). Em seguida, foi adicionado 200 µl de meio Schneider suplementado com 10% de soro bovino fetal, 1% l- glutamine (2 mM), penicilina (100 U/ml), e estreptomicina (100 µg/ml) e as culturas permaneceram à 23°C por 24 h. Finalmente, a carga parasitária foi avaliada através da

recuperação e contagem dos promastigotas extracelulares viáveis contados em câmaras de Neubauer.

8.3 MICROSCOPIA ELETRÔNICA DE TRANSMISSÃO

Neutrófilos infectados e tratados com RvD1 (3h) foram fixados em solução contendo 0.1 M de cacodilato. Posteriormente, foram pós-fixadas em tetróxido de ósmio a 1% e 0,08% de ferricianeto de potássio. A desidratação foi feita em séries crescentes de concentração de acetona (70, 80, 90, and 100∘ GL) e incorporadas por resina Polybed (Polysciences Inc., USA). Cortes ultrafinos foram montados em grades de malha de 300, corados com acetato de uranila a 5% e então observados em microscópio eletrônico de transmissão Zeiss EM 109 onde foram realizados os registros de imagens representativas na plataforma de microscopia eletrônica do IGM/ FIOCRUZ-Ba.

8.4 AVALIAÇÃO DA ATIVIDADE NEUTROFÍLICA

Após 3h de infecção, o sobrenadante das culturas de neutrófilos infectados na presença ou ausência de RvD1 foi coletado para avaliar a atividade enzimática da mieloperoxidase (MPO) e elastase neutrofílica (NE) utilizando o substrato enzimático específico para cada enzima de acordo com o protocolo anteriormente descrito (TAVARES et al., 2016). Os resultados foram obtidos por espectofotômetro pela detecção da densidade óptica ideal para cada ensaio.

8.5 ENSAIO DE TOXICIDADE

A dosagem da atividade enzimática da lactato desidrogenase (LDH) citoplasmática foi realizada em neutrófilos tratados com diferentes concentrações do inibidor da 15LO e em neutrófilos infectados e tratados com RvD1. Após o tempo de infecção, as culturas foram centrifugadas para obtenção de sobrenadantes livres de células e medida da atividade da LDH utilizando o kit LDH Cytotoxicity Detection Assay (Roche). A atividade total do LDH foi determinada pela lise das células com 1% de Triton X-100. A porcentagem de LDH liberado foi calculado da seguinte forma: [(LDH da amostra - LDH Blank) * 100]/ LDH total.

8.6 IMUNOENSAIOS

Concentrações de EGF (fator de crescimento epidérmico), eotaxina, G-CSF (fator estimulante de colônia), GM-CSF (fator estimulante de colônia granulócito-macrófago), IFN-α2, IFN-γ, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, IP-10 (proteína indutível de interferon), MCP-1 (proteína quimioatraente de monócito), MIP-1α (proteína inflamatória de macrófago), MIP-1β, TNF-α, TNF-β e VEGF (fator de crescimento endotelial vascular) foram mensuradas no sobrenadante de neutrófilos infectados e tratados utilizando kit de Luminex (Merck, Darmstadt, Germany) de acordo com instruções do fabricante. Níveis de PGE2 e LTB4 foram avaliados utilizando kit de EIA (Cayman).

8.7 ANÁLISES ESTATÍSTICAS

Médias e desvio padrão foram utilizados nos experimentos *in vitro*. As diferenças entre os grupos foram calculadas utilizando o teste Kruskal-Wallis com as comparações múltiplas de Dunn ou pós testes de tendências lineares (mais de 2 grupos). As diferenças com os valores $p<0,05$ foram consideradas estatisticamente significativas. Análises de cluster hierárquicos (método de Ward) com bootstrap foram usadas para testar se os grupos de neutrófilos (não infectados, infectados por *L. braziliensis* e tratados com RvD1 poderiam ser agrupados separadamente com base na quantificação simultânea de seu perfil de citocinas e quimiocinas inflamatórias no sobrenadante.

9 **RESULTADOS PRELIMINARES**

9.1 RVD1 AUMENTA A CARGA PARASITÁRIA EM NEUTRÓFILOS INFECTADOS POR *L. BRAZILIENSIS*

Inicialmente nós avaliamos como a RvD1 poderia interferir na viabilidade da *L. braziliensis* dentro de neutrófilos. Nós encontramos que a suplementação exógena com resolvina nas culturas de neutrófilos infectados resultou no aumento da viabilidade do parasito quando comparado com as culturas não tratadas (**Figura 1A**). Análises dos neutrófilos infectados por microscopia eletrônica de transmissão mostraram a habilidade dessas células em controlar a infecção. Nesse grupo, podemos notar parasitas internalizados degenerados com desorganização citoplasmática e vacuolização (**Figura 1B**). No entanto, quando tratados com RvD1, observamos que os neutrófilos permaneceram com o citoplasma bem preservado e os parasitos viáveis dentro dos vacúolos parasitóforos (**Figura 1C**).

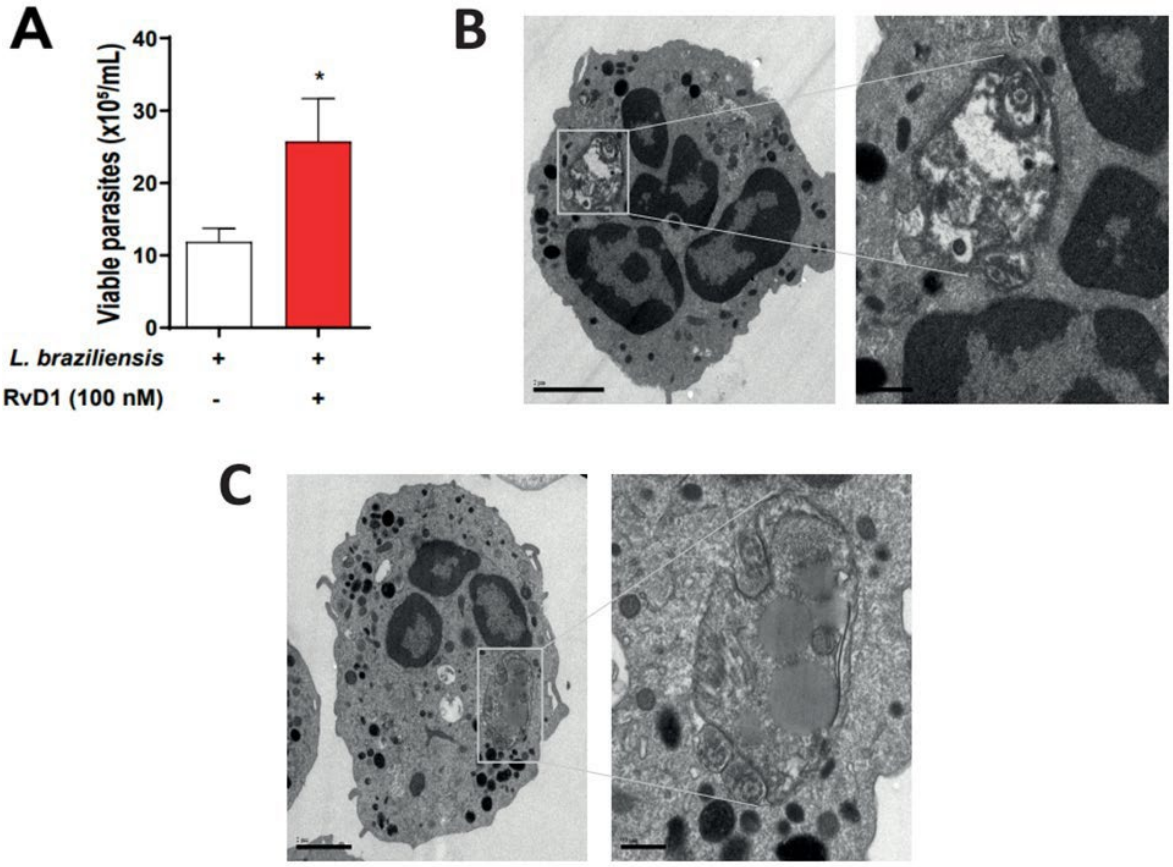

Figura 1. Efeito da suplementação de RvD1 em culturas de neutrófilos infectados com *L. braziliensis.* Neutrófilos isolados e infectados foram cultivados com meio ou RvD1 (100nm). (A) Contagem de parasitos viáveis foi realizada como descrito na metodologia. Dados representam a média de um experimento reprodutivo que foi repetido 4 vezes com 5 doadores. Asteriscos indicam as diferenças estatísticas utilizando o teste Mann- Whitney. *P<0,05 (B) Neutrófilo infectado com *L. braziliensis* mostrando desorganização citoplasmática, vacuolização e estruturas do parasito destruídas. (C) Neutrófilos infectados e tratados com RvD1 mostrando estruturas do parasito preservadas como os microtúbulos e corpúsculos lipídicos. Amplificações de imagem mostram a barra de escala representativa de 2 µm ou 0,5 µm.

9.2 RVD1 NÃO ALTERA A ATIVAÇÃO NEUTROFÍLICA DURANTE A INFECÇÃO POR *L. BRAZILIENSIS*

A liberação de enzimas neutrofílicas foi avaliada no sobrenadante das culturas de neutrófilos infectados. A infecção por *L. braziliensis* foi capaz de induzir a liberação significante de MPO (**Figura 2A**), mas não alterou a produção da elastase neutrofílica (**Figura 2B**). O tratamento com RvD1 não alterou a liberação de ambas enzimas quando comparado com o grupo controle não tratado (**Figura 2**). Além disso, a suplementação com RvD1 não alterou os níveis de LDH em neutrófilos infectados ou não, indicando ausência de dano celular pela perda da integridade de membrana (**Figura 2C**).

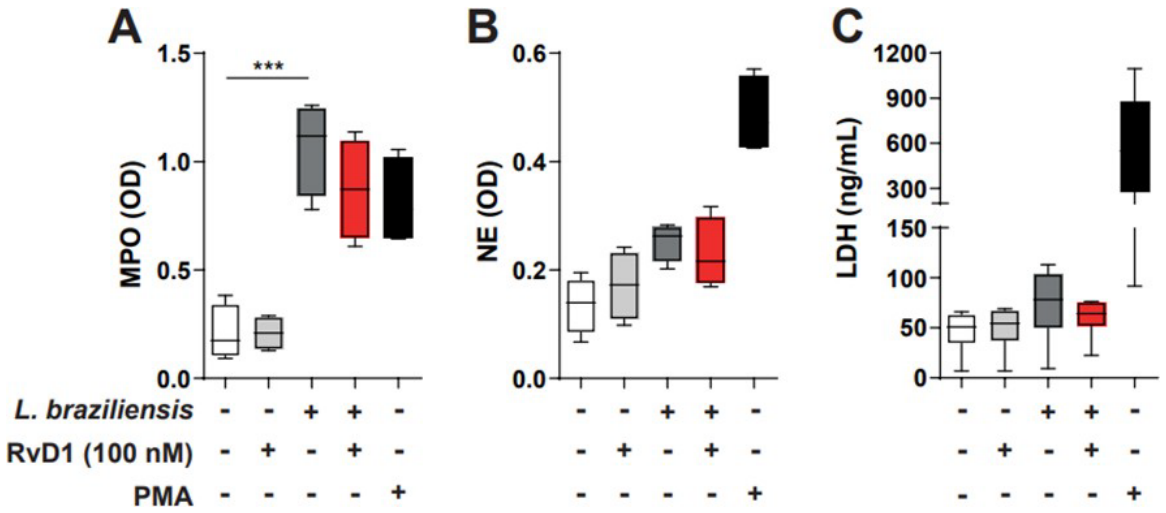

Figura 2. RvD1 não promove a ativação de neutrófilos humanos. Foi utilizado o sobrenadante de neutrófilos não infectados e infectados para avaliar a indução de MPO (A) e NE (B) utilizando ensaios de atividade enzimática e os níveis de LDH (C) conforme descrito na metodologia. O PMA (100 nM) foi utilizado como controle positivo de ativação de neutrófilos. Dados são representados por mediana de um experimento reprodutivo que foi repetido 4 vezes com 5 doadores. Asteriscos indicam as diferenças estatísticas utilizando o teste Kruskal–Wallis com pós- teste de Dunn's. ***$p < 0.001$.

9.3 RVD1 AUMENTA OS NÍVEIS DE IL-6 E IL-10 NO SOBRENADANTE DE NEUTRÓFILOS HUMANOS INFECTADOS

O sobrenadante dos neutrófilos infectados e tratados com RvD1 foi utilizado para dosar vários biomarcadores inflamatórios. Aqui nós avaliamos as alterações nos perfis de concentrações de citocinas, quimiocinas e fatores de crescimento produzidos pelos neutrófilos durante a infecção. Ao comparar os três grupos, observamos que o grupo não infectado apresenta uma bioassinatura única caracterizada por um perfil de expressão distinto de citocinas inflamatórias no sobrenadante e que a infecção por *L. braziliensis* altera completamente esse perfil. Em ambos os grupos, infectado e infectado com RvD1, os níveis de MC-SF, IL-8, IL-9, IL-12p70, Basic FGF e IL-4 aumentaram significativamente em relação ao grupo não infectado.

Além disso, o tratamento com RvD1 aumentou significativamente os níveis de IL-6 e IL-10 quando comparado ao não infectado (**Figura 3**). Por fim, o agrupamento hierárquico revelou 2 clusters que exibiram perfis de expressão opostos entre os grupos avaliados.

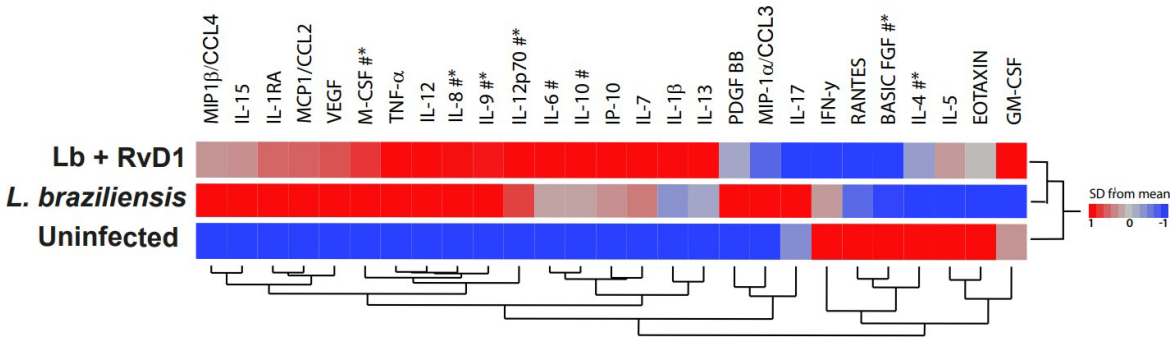

Figura 3- Neutrófilos infectados exibem um perfil distinto de citocinas inflamatórias no sobrenadante. Foi utilizado o sobrenadante de neutrófilos não infectados, infectados e tratados com RvD1 para avaliar a produção de citocinas e quimiocinas conforme descrito na metodologia. Os dados sobre a concentração média de cada marcador indicado por grupo de condições foram transformados em log e o escore Z normalizado, e um mapa de calor foi usado para ilustrar tendências na variação de dados. Uma análise de cluster hierárquica (método de Ward) foi usada para agrupar as citocinas com distribuição semelhante entre as condições. Os dendrogramas representam a distância euclidiana. Diferenças estatísticas foram calculadas utilizando o teste Kruskal–Wallis com pós-teste de Dunn's entre oS grupos (# Usn x Lb+RvD1; * Usn x *L.b*), $p < 0.01$.

9.4 O TRATAMENTO FARMACOLÓGICO COM O INIBIDOR DA 15-LO REDUZ A VIABILIDADE DA INFECÇÃO EM NEUTRÓFILOS

Para melhor entender a associação da RvD1 com o aumento da replicação da *L. braziliensis,* tratamos as culturas de neutrófilos infectados com o inibidor da 15-LO, enzima chave envolvida na produção de resolvina D1. O tratamento com o inibidor da lipoxigenase 15 resultou na redução significativa da viabilidade do parasito quando comparado com o grupo tratado com RvD1 (**Figura 4A**). Diante desses dados, fomos avaliar se o inibidor era tóxico para os neutrófilos. Para isso, tratamos as células com crescentes concentrações do inibidor da lipoxigenase por 3 horas e dosamos os níveis de LDH. O tratamento com o fármaco não alterou a liberação de LDH quando comparado com o grupo não tratado (**Figura 4B**). Além disso, nossos dados revelam que o tratamento com o inibidor foi capaz de reverter o efeito induzido pela RvD1, inibindo significativamente os níveis de IL-6 e IL-10 (**Figura 4 C e D**).

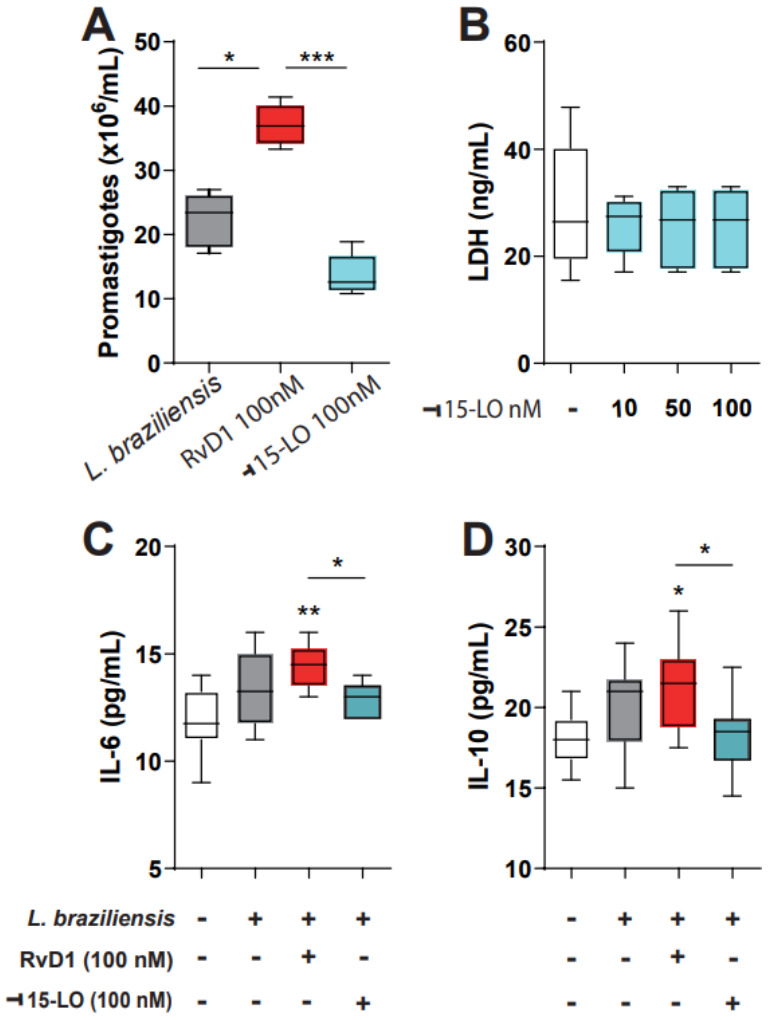

Figura 4 - Efeito do inibidor da 15-LO em culturas de neutrófilos humanos. (A) Neutrófilos isolados e infectados foram cultivados com meio, RvD1 (100 nM) e com inibidor da 15-LO (100 nM). A contagem de parasitos viáveis foi realizada como descrito na metodologia. (B) O sobrenadante de culturas de neutrófilos não infectados e tratados com diferentes concentrações do inibidor foi utilizado para avaliar os níveis de LDH (lactato desidrogenase), (C) IL-6 e (D) IL-10. Dados representam a mediana de um experimento independente que foi repetido 4 vezes. Asteriscos indicam as diferenças estatísticas utilizando o teste Kruskal–Wallis com pós-teste de Dunn's. *p< 0.01, **p<0.001, ***p<0.0001.

9.5 O RECEPTOR FPR2/ALX DESEMPENHA UM PAPEL ATIVO NA INFECÇÃO DE NEUTRÓFILOS

Sabe-se que os efeitos induzidos pela RvD1 ocorrem via ativação de receptores acoplados a proteínas G como o FPR2/ALX (CHIANG; SERHAN, 2020). A fim de esclarecer e aprofundar nossas descobertas, analisamos o envolvimento do receptor FPR2/ALX nos efeitos da RvD1 durante a infecção de neutrófilos por *L. braziliensis*. Para nossa surpresa, observamos que a inibição do receptor FPR2/ALX com seu antagonista BOC, foi capaz de aumentar significativamente a carga parasitária nas concentrações de 50 e 100 uM (**Figura 5A**). O mesmo foi observado quando as células foram pré-tratadas com o BOC 15 minutos antes da infecção (**Figura 5B**).

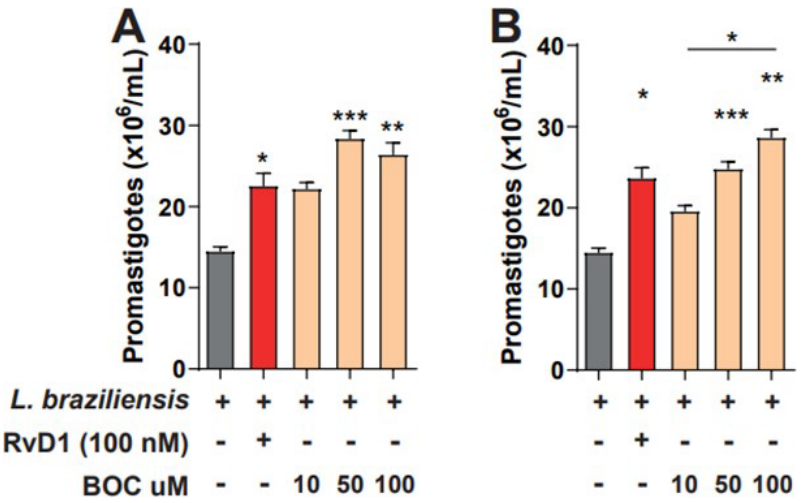

Figura 5 - Efeito do BOC durante a infecção por *L. braziliensis* em neutrófilos. Neutrófilos isolados e infectados foram cultivados com meio, RvD1 (100nm) e BOC (10, 50 e 100uM). (A) Os compostos foram adicionados no momento da infecção. A contagem de parasitos viáveis foi realizada como descrito na metodologia. (B) Neutrófilos tratados com BOC receberam pré-tratamento por 15 minutos antes da infecção com *L. braziliensis.* Dados são representados por média e desvio padrão. Asteriscos indicam as diferenças estatísticas utilizando o teste Kruskal–Wallis com pós-teste de Dunn's. *p< 0.01, **p<0.001, ***p<0.0001.

No entanto, quando adicionamos RvD1 após o pré-tratamento com BOC observamos uma redução significativa da carga parasitária em relação aos grupos sem o pré-tratamento (**Figura 6**).

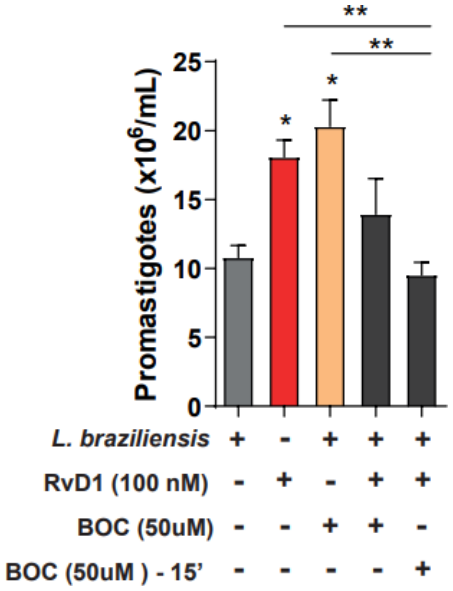

Figura 6 - Pré-tratamento com BOC reverte o efeito da RvD1 durante a infecção por *L. braziliensis* em neutrófilos. Neutrófilos isolados e infectados foram cultivados com meio, RvD1 (100nm) e BOC (50uM). (A) Pré-tratamento com BOC reduz a carga parasitária induzida pela RvD1. Dados são representados por média e desvio padrão. Asteriscos indicam as diferenças estatísticas utilizando o teste Kruskal–Wallis com pós-teste de Dunn's. *p< 0.01, **p<0.001.

9.6 RVD1 REDUZ OS NÍVEIS DE MEDIADORES LIPÍDICOS

Está bem estabelecido que o equilíbrio entre LTB4 e PGE2 pode influenciar o resultado da infecção por Leishmania (CHAVES; CANETTI; COUTINHO-SILVA, 2016). Para avaliar se a RvD1 poderia estar modulando a produção dos mediadores lipídicos nós dosamos os níveis de PGE2 e LTB4 no sobrenadante de neutrófilos infectados e tratados com RvD1 e BOC. Nossos dados revelam que o tratamento bom BOC foi capaz de aumentar os níveis de LTB4 quando comparado com o grupo infectado (**Figura 7A**) e os níveis de PGE2 quando comparado com o grupo infectado e tratado com RvD1 (**Figura 7B**). Para definir a relação desses mediadores no microambiente da infecção, calculamos a razão entre eles nas condições avaliadas. O tratamento com RvD1 foi capaz de reduzir significativamente seus níveis nas culturas de neutrófilos infectadas ou não (**Figura 7C**). Juntos, esses dados mostram a importância dos mediadores lipídicos no controle da infecção e que o desequilíbrio na proporção PGE/LTB4 induzida pela RvD1 pode estar contribuindo com o favorecimento da infecção por *L. braziliensis* em neutrófilos.

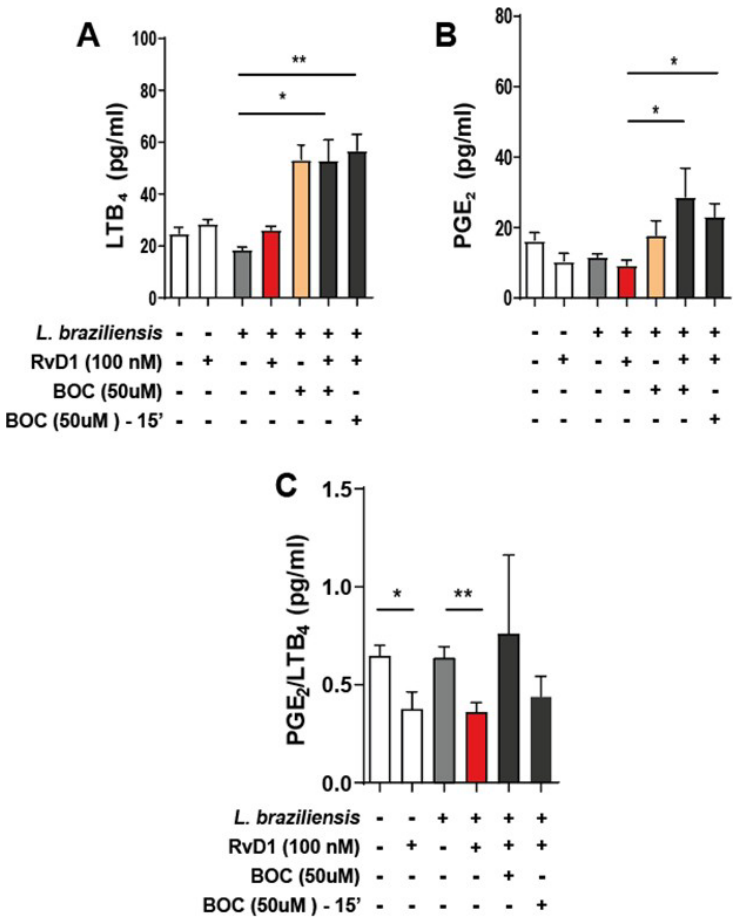

Figura 7. Tratamento com BOC aumenta a produção de mediadores lipídicos durante a infecção por *L. braziliensis* em neutrófilos. Neutrófilos isolados e infectados foram cultivados com meio, RvD1 (100nm) e BOC (50uM). (A) Tratamento e pré-tratamento com BOC aumenta a produção de LTB4 e (B) PGE durante a infecção. (C) Relação entre a produção de PGE2 e LTB4 no sobrenadante de neutrófilos não infectados e infectados e tratados com RvD1 e BOC. Dados são representados por média e desvio padrão. Asteriscos indicam as diferenças estatísticas utilizando o teste Kruskal–Wallis com pós-teste de Dunn's. *p< 0.01, **p<0.001.

9.7 EFEITO DA RVD1 E DO INIBIDOR DA 15-LO EM CULTURAS AXÊNICAS DE *L. BRAZILIENSIS*

Para avaliar se a RvD1 poderia exercer algum efeito sobre o crescimento *in vitro*, tratamos culturas axênicas de *L. braziliensis* com doses crescentes de RvD1. Observamos que a suplementação nas culturas de promastigotas não altera a curva de crescimento do parasito (**Figura 8A**). No entanto, a suplementação com o inibidor da 15-LO resultou na morte do parasito de forma dose dependente (**Figura 8B**). A suplementação com RvD1 na concentração de uso (100 nM) não foi capaz de reverter esse efeito (**Figura 8C**).

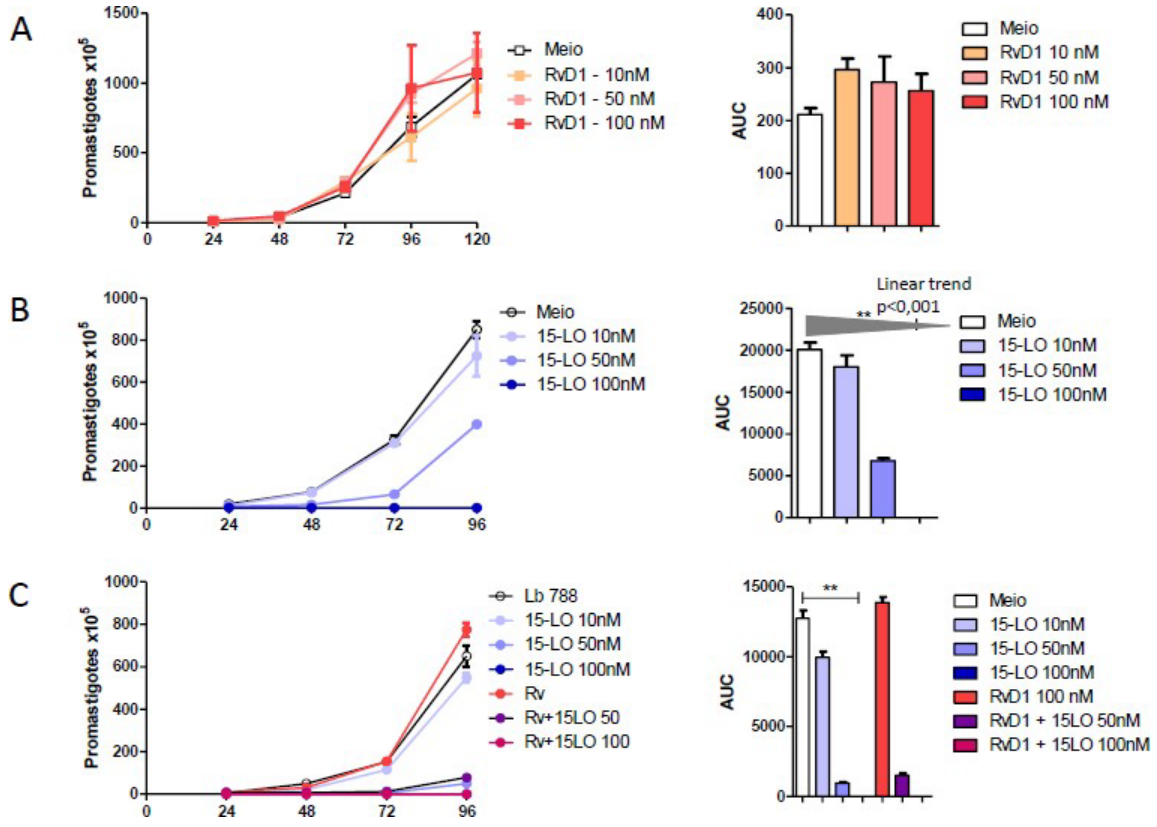

Figura 8 - Efeito da RvD1 e do inibidor da 15-LO em culturas axênicas de *L. braziliensis*. Os parasitos foram incubados por 4 dias com meio ou com as concentrações indicadas de RvD1 (A) e do inibidor da lipoxigenase 15 (B). Em C acrescentamos a suplementação conjunta do inibidor com a RvD1. O número de parasitos viáveis foi avaliado pela contagem direta na câmara de Neubauer. Cada ponto representa a média com desvio. Os dados representam um de pelo menos três ensaios independentes que foram realizados em triplicata para cada condição.
**P<0.001.

10 **CONCLUSÕES PRELIMINARES E PERSPECTIVAS**

Dados da literatura, bem como resultados prévios obtidos pelo grupo em ensaios *in vitro* em macrófagos infectados por *L. amazonensis* (MALTA-SANTOS et al., 2017), mostram que as Resolvinas modulam a produção de mediadores anti-inflamatórios e de resolução durante a infecção, favorecendo a sobrevivência e replicação do parasito. Nossa hipótese neste trabalho é de que a Resolvina (RvD1) favorece o aumento da carga parasitária em neutrófilos infectados com *L. braziliensis*, principal agente etiológico da forma clínica de leishmaniose tegumentar no Brasil.

Nossos dados corroboram esta hipótese, uma vez que mostram que o tratamento com RvD1 aumentou a viabilidade do parasito, os quais permanecem intactos dentro dos neutrófilos como observado nas imagens de micrografias eletrônicas confirmando os dados descritos na literatura (FALCÃO et al., 2015). Entretanto, a RvD1 não alterou a ativação neutrofílica nem a produção de ROS, mecanismos importantes no controle da infecção em neutrófilos. Por outro lado, o tratamento com RvD1 foi capaz de induzir altos níveis de IL-6 e IL-10 durante infecção, citocinas inflamatórias associadas à susceptibilidade e sucesso da infecção por *Leishmania* (CASTELLUCCI et al., 2014; GOLLOB; VIANA; DUTRA, 2014). Esta resposta inflamatória inicial pode estar sendo balanceada pela ativação de vias que incluem mecanismos anti-oxidativos deflagrados pelo tratamento com a RvD1 que protegem a *L. braziliensis* dos mecanismos microbicidas de neutrófilos. Entretanto esta possibilidade ainda precisa ser investigada.

A inibição de enzimas da via dos mediadores lipídicos têm sido amplamente utilizada para controlar respostas inflamatórias (MEDEIROS et al., 2012), bem como a carga parasitária de infecções por *Leishmania (AFONSO et al., 2008)* (TAVARES et al., 2016) (MALTA- SANTOS et al., 2017). Em nosso modelo experimental *in vitro*, demonstramos que o tratamento com inibidor da 15-LO (15 lipoxigenase), resultou no controle da viabilidade intracelular do parasita. Embora o inibidor utilizado tenha sido capaz de matar o parasito, controlar a carga da infecção e reduzir os níveis de IL-6 e IL-10 induzidos pela RvD1, ele não é um inibidor específico da síntese de resolvina, podendo inibir também outros mediadores lipídicos produzidos via 15-LO como as lipoxinas. Logo, estudo futuros avaliando se de fato a inibição farmacológica da 15-LO refletiu nos níveis de RvD1 durante a infecção de neutrófilos são necessários a fim de utilizar essa ferramenta como possível estratégia terapêutica para reduzir a carga da infecção por *L. braziliensis* em neutrófilos.

Finalmente nossos dados demonstram que o antagonismo do receptor FPR2/ALX reverte os efeitos da RvD1 reduzindo significativamente a carga parasitária dos neutrófilos infectados, sugerindo que a RvD1 exerce sua ação pela interação com esse receptor.

Adicionalmente a inibição do receptor resultou no aumento significativo nos níveis de PGE2 e LTB4 enquanto que o tratamento com RvD1 foi capaz de modular a proporção dos mediadores lipídicos favorecendo assim a infecção por *L. braziliensis* em neutrófilos.

Curiosamente, o tratamento de culturas axênicas de promastigotas com RvD1 sintética não foi capaz de alterar a curva de crescimento do parasito, enquanto que o tratamento com o inibidor da 15-LO resultou no controle do crescimento de forma irreversível. Nesse contexto, nossos dados sugerem que enquanto o efeito biológico das resolvinas em nosso modelo experimental depende da função dos neutrófilos e não do estágio promastigota do parasito, enquanto que o inibidor da via das resolvinas pode atuar tanto no parasito como na célula hospedeira controlando a infecção.

Como perspectiva para o trabalho, pretendemos avaliar a dosagem de mediadores como a arginase e o TGF-β assim como a via antioxidante no sobrenadante de neutrófilos infectados com *L. braziliensis* em presença de RvD1. Além disso, iremos investigar com mais acurácia o efeito desse mediador na infecção pelo bloqueio de seus receptores e os mecanismos que a RvD1 pode estar modulando na célula hospedeira.

11 **DISCUSSÃO**

Embora o número de estudos a respeito da LT tenha crescido significativamente nos últimos 20 anos, ainda há lacunas importantes a respeito da imunopatogênese da doença e do tratamento, cuja falha terapêutica tem chegado a cerca de 55% (CUTANEOUS LEISHMANIASIS - SEARCH RESULTS - PUBMED, [s.d.]). Essa situação se agrava ainda mais em relação às formas clínicas polares da doença que devido a sua baixa incidência, possui limitações do desenho experimental de estudos diversos.

Para o desenvolvimento de novas terapias, a identificação de marcadores biológicos é indispensável. Biomarcadores robustos permitem mensurar fatores importantes durante os processos patológicos desde antes do início da terapia até o seu desfecho clínico (WALLIS et al., 2013). Dessa forma, seu estudo vem sendo bastante utilizado na identificação de subpopulações de risco, confirmação de diagnósticos, gravidade de doença e predição de desfecho clínico (WALLIS et al., 2013) (FERNANDES et al., 2019).

Na Leishmaniose os fatores que determinam a gravidade da doença assim como a falha terapêutica ainda permanecem sem justificativa definida, no entanto, sabe-se que estão associados a combinação de características imunológicas e genéticas do hospedeiro e de fatores do parasito (SCOTT; NOVAIS, 2016) (MOKNI, 2019). Nesta tese, investigamos possíveis biomarcadores de gravidade de doença e falha terapêutica da leishmaniose tegumentar e os mecanismos deflagrados pelos mediadores lipídicos de pró-resolução durante a infecção de neutrófilos por *L. braziliensis*, principal agente etiológico no Brasil desta forma clínica da doença.

Inicialmente buscamos avaliar o perfil de ativação da via das poliaminas como um fator do hospedeiro que poderia estar contribuindo com a gravidade e imunopatogênese da LCD (manuscrito 1). Estudos das vias de biossíntese da arginase, poliaminas e prostaglandinas vem sendo amplamente relacionados à proliferação do parasito e consequente sucesso da infecção por Leishmania (WANASEN et al., 2007) (FRANÇA-COSTA et al., 2015). Trabalhos anteriores do grupo mostraram que os altos níveis de Arg1, ODC, PGE_2 e TGF-ß, poderiam contribuir com a ineficiência de montar uma resposta imune eficaz na LCD (FRANÇA-COSTA et al., 2015). No entanto, dados sobre a relação dos componentes da via das poliaminas com a imunopatogênese da forma clínica difusa ainda não eram conhecidos.

Nossos dados mostram que os pacientes com LCD possuem uma bioassinatura distinta com elevada expressão de enzimas e transportadores da via das poliaminas nas lesões e no plasma quando comparado com LCM e LCL. Além disso, a expressão de RNA dos genes relacionados à via tanto em lesões cutâneas como em estudo transcriptômico de validação, demonstrou uma ativação diferencial que foi associada a carga parasitária em LCD e a

capacidade de distinguir o espectro clínico da LT.

Um dos mecanismos que sustentam a associação da arginase com a susceptibilidade e manutenção da infecção por *Leishmania sp.* é a indução de poliaminas a partir da L-arginina (CALDWELL et al., 2018) (PESSENDA; DA SILVA, 2020). Os altos níveis plasmáticos de poliaminas e aminoácidos nos pacientes com LCD, quando comparado com LCM e LCL, nos permite considerá-los como potenciais biomarcadores para a gravidade da leishmaniose. Nesse contexto, a putrescina, cadaverina, espermina e espermidina vêm sendo descritas como biomarcadores inflamatórios associados à gravidade de doenças de Parkinson (SAIKI et al., 2019) e diversos modelos de câncer (PROVENZANO et al., 2019) (YU et al., 2015). Elevados níveis plasmáticos de poliaminas foram encontrados em pacientes com Parkinson (SAIKI et al., 2019) e lúpus (KIM et al., 2018). A diferencial expressão dos genes que regulam a biossíntese e catabolismo da via das poliaminas também foi observado em análises de transcriptoma, onde pacientes com Alzheimer apresentaram maior expressão de SAT1, SMOX e PAOX (MAHAJAN et al., 2020). A diferença na expressão das poliaminas observada no plasma e lesões de pacientes com LT poderia estar sendo induzida pelo parasito ou ser consequência do background do hospedeiro, como demonstrado para a arginase (AOKI et al., 2017).

Recentemente, estudos *in vitro* mostraram que a putrescina e espermidina são essenciais para a proliferação e viabilidade da *Leishmania donovani.* Parasitos *knockouts* para ODC e SRM apresentaram baixas taxas de crescimento (PERDEH et al., 2020). Embora sejam cruciais para o parasito, esses não podem sintetizar o aminoácido precursor das poliaminas, a L-arginina, sendo necessário regular sua captação de células do hospedeiro por receptores específicos (MUXEL et al., 2017a) (AOKI et al., 2017). Além disso, vem sendo descrito que a *Leishmanis sp.* é capaz de alterar o metabolismo do hospedeiro regulando a expressão gênica transcricional e pós-transcricional para induzir o aumento na produção de poliaminas (MUXEL et al., 2019). Analisando a expressão de RNAm nas lesões de pacientes com LCD foram detectadas diferenças para a ARG1, CAT-2A e SMS quando comparado com a forma clínica mucosa que apresenta poucos parasitos. Tem sido demonstrado em infecções *in vivo* que a alta atividade da ARG1 correlaciona-se positivamente com a alta carga parasitária nos sítios de infecção (PESSENDA; DA SILVA, 2020). Nossos achados reforçam a ideia de que o balanço sistêmico de poliaminas e aminoácidos induzidos pela infecção podem contribuir com a alta carga parasitária e alta expressão de transportadores de aminoácidos observados na lesão dos pacientes com LCD.

Os efeitos imunomoduladores desempenhados pela via da arginase são associados a polarização de macrófagos para um perfil M2 que favorece a sobrevivência da Leishmania (PESSENDA; DA SILVA, 2020). Demonstrou-se que a alta atividade da arginase coincide com a ativação ineficiente de células T na LT (ABEBE et al., 2012). Em células tumorais, a inibição da COX-2 resultou na inibição da expressão da ARG1 assim como na resposta tumoral induzida

pelas células T (OCHOA et al., 2007). Nessas células os altos níveis de PGE_2 foram capazes de induzir rapidamente a expressão de ARG1 (OCHOA et al., 2007). Mais recente foi demonstrado que a arginase e a COX-2 são co-produzidas pelas células do infiltrado inflamatório e seus produtos (poliaminas e prostaglandinas) são importantes na cicatrização de lesões (ABD EL-ALEEM et al., 2019). Na LCD os elevados níveis de PGE_2 e TGF-ß também agem em sinergia com a arginase-1 suprimindo a resposta imunológica, enquanto que em LCL a correlação negativa da arginase com o TNF e IL-12 provê uma ativação adequada do sistema imune (FRANÇA-COSTA et al., 2015). O distinto perfil de ativação da via da arginase, poliaminas e mediadores lipídicos entre os pacientes com LT enfatiza que há de fato uma associação entre essas vias metabólicas e a resposta imune resultando no desfecho clínico das leishmanioses cutâneas.

Curiosamente, a infecção por *L. braziliensis* isolada de pacientes refratários ao tratamento antimonial produziu lesões graves em camundongos BALB/c, estando associada a alta expressão de ARG1 e IL-4 (COSTA et al., 2011). Nesses animais foram encontrados maior quantidade de neutrófilos e macrófagos do que nos animais que curaram (COSTA et al., 2011). Além disso, as cepas isoladas de pacientes refratários apresentaram resistência ao óxido nítrico devido a superexpressão de proteínas que atuam contra o estresse oxidativo (GIUDICE et al., 2007). Embora na fase ativa da doença a arginase possa ser utilizada como biomarcador de gravidade e este cenário seja altamente consistente com o desfecho clínico da LT, análises prospectivas futuras são necessárias para avaliar se os pacientes que falham a terapia tradicional apresentam elevados níveis plasmáticos de arginase.

Tendo em vista a importância do uso de marcadores biológicos para indicar gravidade e progressão de doença, assim como explorar novos alvos terapêuticos, buscamos identificar prospectivamente no manuscrito 2 biomarcadores que fossem capazes de predizer a falha terapêutica na leishmaniose cutânea. A identificação de citocinas e mediadores lipídicos como marcadores de falha nos permite selecionar quais pacientes apresentam o maior risco de não responderem ao tratamento antes mesmo da sua conclusão e entender os mecanismos por trás dessas condições.

A resposta inflamatória na LCL é caracterizada pela presença de altos níveis circulantes de linfócitos Th1, citocinas e quimiocinas que reduzem após o tratamento (BRITO et al., 2014). Esse cenário é um indicativo de resposta eficaz ao tratamento e cura. Embora o tratamento também tenha reduzido os níveis plasmáticos de TNF-α, IP-10, IL-2, IL-1a, e IL-6 nos pacientes que falharam a terapia, esses pacientes apresentaram uma redução significativa nos níveis de GM-CSF, IFN-a2, IL-6, e IL-3 quando comparado com os pacientes que curaram, indicando que a incapacidade de eliminar o parasito pode estar associada a esse perfil plasmático.

Além das citocinas, os mediadores lipídicos, importantes moduladores da resposta

inflamatória, também apresentaram diferenças importantes entre os desfechos clínicos. Os mediadores lipídicos produzidos pelas vias do AA ou DHA desempenham importantes papéis em determinar diferentes formas clínicas da leishmaniose (ARAÚJO-SANTOS et al., 2017) (FRANÇA-COSTA et al., 2016) (MALTA-SANTOS et al., 2017). De fato, quando comparamos a abundância dos oxilipídeos verificamos um perfil distinto nos pacientes que falharam comparado com os que curaram. Curiosamente, os mediadores mais abundantes são produtos da via do AA e a maioria reduziu significativamente seus níveis nos pacientes tratados com sucesso, sugerindo que seus níveis em conjunto com as citocinas podem estar refletindo o grau de ativação imune e controle da infecção. De acordo com essa ideia, análises de curva ROC mostraram que a combinação dos marcadores (proteínas inflamatórias e mediadores lipídicos) separa os desfechos clínicos com alta acurácia.

Citocinas e mediadores lipídicos regulam diversos tipos de respostas imunes que se envolvem em intrincadas redes regulatórias (BIEREN, 2017). Muitas vezes a regulação de síntese dos marcadores, assim como suas correlações, são negligenciadas em estudos imunológicos deixando de lado essa visão integrada e resultando em um conhecimento incompleto dos mecanismos imunológicos que controlam as funções *in vivo.* Para verificar a intensidade e qualidade das relações entre as proteínas plasmáticas e os mediadores lipídicos utilizamos análises de correlação nos subgrupos de pacientes. As análises de rede mostraram diferenças significativas entre os marcadores biológicos que caracterizam o desfecho clínico e o tempo de tratamento. Além disso, a identificação dos marcadores mais conectados em cada perfil confirma que existe uma regulação sistêmica inflamatória induzida pelo tratamento que é distinta entre os pacientes responsivos ou não. Nos pacientes que curaram, observamos um equilíbrio entre correlações positivas e negativas que foi completamente alterado após o início do tratamento. Por outro lado, o perfil de correlações nos pacientes que falharam foi predominantemente marcado por correlações positivas que continuaram prevalecendo após o tratamento. Enquanto o perfil de biomarcadores no dia 0 foi predominado pelos mediadores lipídicos, e permaneceu após o tratamento nos pacientes que curaram, nos que falharam observamos a predominância das citocinas e o tratamento alterou esse perfil. De fato, a exposição a medicamentos é capaz de modular produtos metabólicos alterando a resposta imune e desfechos clínicos (VARGAS et al., 2019).

A construção de um modelo de árvore de decisão indicou que quando medidos antes do tratamento, os níveis de eotaxina, 11-HETE e TGF-ß poderiam ser usados em sequência para identificar indivíduos que irão falhar no tratamento, desenvolvendo facilmente um ponto de corte. Essa análise permite separar os grupos com o menor número possível de marcadores com uma máxima precisão e vem sendo frequentemente utilizada para o diagnóstico de HIV (VERHOFSTEDE et al., 2017), tuberculose (DUTTA et al., 2020) e diabetes (KUMAR et al., 2019). Além disso, foi observado altos níveis plasmáticos de TGF-ß (FRANÇA-COSTA et al.,

2015) e alta expressão de CCL11 em células dendríticas de pacientes com LCD (DÍAZ; ZERPA; TAPIA, 2013), ambos associados à ativação da resposta TH2 e favorecimento da infecção. Sendo assim, é possível monitorar o estado clínico dos pacientes por medições de biomarcadores plasmáticos no sangue, através de uma forma rápida e menos invasiva, que pode ser útil no prognóstico e escolha do tratamento mais adequado.

Embora a assinatura relacionada ao perfil lipídico não tenha se relacionado com a carga parasitária ou tamanho de lesão como observado para a via das poliaminas, análises integrativas e de transcriptoma revelaram que o perfil lipídico é o principal componente capaz de separar com alta acurácia o desfecho clínico e a infecção por Leishmania, respectivamente. Uma observação importante nas análises de dados públicos foi a super regulação dos genes envolvidos na síntese de prostaglandinas (ptgs2) assim como seus receptores inflamatórios (ptger2, ptger4, and ptgir). A análise de dados de uma coorte independente confirmam nossos achados de lipidômica, onde tanto os níveis plasmáticos como a expressão de genes da via estavam aumentados nos pacientes com LC.

De fato, trabalhos anteriores do grupo vinham demonstrando que as diferenças na imunopatogênese da LCL e LCM poderiam resultar do desequilíbrio entre LTs e PGs (FRANÇA-COSTA et al., 2016). Além disso, pacientes com a forma anérgica da doença apresentaram alta expressão dos receptores EP2 e EP4 nas lesões que se relacionam com os altos níveis plasmáticos de arginase (FRANÇA-COSTA et al., 2015). Esse dado destaca mais uma vez a possível associação entre as vias dos lipídios e da arginase com o desfecho clínico.

Estudos baseados em lipidômica revelaram a importância dos lipídios de pró-resolução na patogênese da tuberculose-diabetes (SHIVAKOTI et al., 2020). No contexto das leishmanioses foi demonstrado altos níveis plasmáticos de RvD1 em pacientes com a forma visceral (ARAÚJO-SANTOS et al., 2017) e com a forma cutânea difusa quando comparado com o controle saudável (MALTA-SANTOS et al., 2017). Além disso, trabalhos anteriores do grupo mostraram que a RvD1 foi capaz de induzir mecanismos antioxidativos pela ativação da HO-1 em macrófagos infectados com *L. amazonensis* tornando essas células mais permissivas à infecção e proliferação do parasito (MALTA-SANTOS et al., 2017). Aqui nós demonstramos que o tratamento com RvD1 em culturas de neutrófilos infectados por *L. braziliensis* favoreceu o sucesso da infecção.

As resolvinas D1 regulam o influxo de neutrófilos e aumentam a fagocitose de agentes invasores para promover o remodelamento tecidual e retorno à homeostasia (SERHAN; LEVY, 2018) (ABDOLMALEKI et al., 2020). Em lesões de paciente com LCL a identificação de neutrófilos em cortes histológicos vem sendo utilizado com padrão no diagnóstico (HANDLER et al., 2015). Em modelos experimentais os neutrófilos são cruciais para o controle da infecção e sua ausência acarreta no aumento da carga parasitária de *L. braziliensis* (NOVAIS et al., 2009). Embora os parasitos cresçam e dividam-se preferencialmente em macrófagos, os neutrófilos são

cruciais na infecção podendo exercer papéis protetores ou permissivos durante a infecção (HANDLER et al., 2015). Além disso, muitos estudos têm mostrado uma associação entre o aumento no recrutamento de neutrófilos e a severidade/cronicidade na LC (CARDOSO et al., 2019). Nossos dados ampliam o conhecimento atual do papel das resolvinas durante a infecção de neutrófilos tendo em vista a importância dessas células para a imunidade inata.

Diante disso, avaliamos se os mecanismos ativados durante a exposição de RvD1 e o aumento da carga parasitária estariam relacionados com a subversão da ativação dos neutrófilos. Está bem descrito na literatura que durante a infecção por *Leishmania,* ocorre a liberação de enzimas presentes nos grânulos neutrofílicos como o MPO e NE (TAVARES et al., 2014) (Falcão et al. 2015) (QUINTELA-CARVALHO et al., 2017). Aqui verificou-se que a infecção por *L. braziliensis* desencadeou a produção de MPO pelos neutrófilos, mas a suplementação com RvD1 sintética nas culturas falhou em amplificar esse efeito. Esse resultado sugere que a RvD1 pode afetar outro mecanismo que favorece a suscetibilidade da replicação do parasita. Recentemente foi demonstrado que pacientes infectados por *Leishmania* apresentam elevados níveis de IL-6 e IL-10 e esse aumento foi respectivamente associado a gravidade da doença e a inibição da atividade leishmanicida da célula, facilitando a replicação do parasito e desenvolvimento da doença (FRANÇA et al., 2020). Além disso, o IL-6 é um importante regulador do tráfico de neutrófilos durante a resposta inflamatória na infecção por *T. bruci* (CALJON et al., 2018). Nossos dados revelam que o tratamento com RvD1 foi capaz de modular a produção de ambas citocinas o que poderia estar favorecendo a proliferação da *L. braziliensis* dentro dos neutrófilos.

Esther Titos e colaboradores demonstraram que tanto a RvD1, como seu precursor (DHA) foram capazes de induzir fortemente a expressão da arginase-1 enquanto reduzia a produção de IFN-$_\gamma$ e citocinas do perfil Th1 durante a inflamação crônica no tecido adiposo (TITOS et al., 2011). Além disso, neutrófilos que expressam ARG1 são hábeis em suprimir a resposta de células T durante as leishmanioses, além de ser induzida pelo TGF-ß (PESSENDA; DA SILVA, 2020). Estudos futuros são necessários para avaliar se as resolvinas são capazes de aumentar os níveis de outras moléculas relacionadas ao favorecimento do crescimento do parasito como o TGF-ß e a arginase e se a inibição da via da arginase e poliaminas com o Nor-NOHA e DFMO respectivamente é capaz de reverter o efeito das resolvinas.

Sabe-se que após ativar os receptores ALX/FPR2 e GPR32 as resolvinas deflagram uma série de mecanismos intracelulares como supressão do cálcio citosólico e redução na ativação das MAPK-APK2 (LÓPEZ-MUÑOZ et al., 2018). A inibição desses receptores com seus antagonistas resultou na inibição do efeito protetor induzido pelas resolvinas em lesões pulmonares induzidas pelo LPS (WANG et al., 2011). No contexto da infecção por *Leishmania*, ainda não se sabe qual receptor está sendo positivamente regulado pela RvD1. Nossos dados mostram que o pré-tratamento com o antagonista do ALX/FPR2 resultou na reversão do efeito

induzido pela Resolvina D1, reduzindo a carga parasitária. Além disso, o tratamento com BOC foi capaz de aumentar significativamente os níveis de LTB4, eicosanoide envolvido no controle da infecção por *Leishmania (TAVARES et al., 2014)* (CHAVES; CANETTI; COUTINHO-SILVA, 2016). Experimentos futuros são necessários para avaliar se o GPR32, outro receptor que as resolvinas tem afinidade, participa na modulação dos efeitos induzidos pelas resolvinas ou se a infecção induz sua expressão na célula hospedeira. A descoberta de receptores específicos para RvD1 abre novas perspectivas como estratégia terapêutica, uma vez que a utilização de inibidores seletivos ou agonistas desses receptores poderiam reduzir os efeitos induzidos pela RvD1 dentro da célula, como o aumento da carga parasitária, bem como poderiam modular a resposta imune da célula hospedeira.

Dessa forma, nossos dados demonstram que moléculas da via da arginase e dos mediadores lipídicos estão envolvidas no favorecimento da infecção por *Leishmania*. Experimentos e investigações adicionais são necessários para elucidar os mecanismos deflagrados por essa via e como esses podem ser explorados como novos alvos para a intervenção terapêutica no tratamento da Leishmanios

12 **PRINCIPAIS ACHADOS DA TESE**

- Pacientes com LCD apresentam elevados níveis plasmáticos de poliaminas e aminoácidos, assim como de enzimas expressas nas lesões quando comparado com as outras formas clínicas;
- A diferencial ativação da via das poliaminas caracteriza os pacientes com LCD em relação às outras formas clínicas da LT;
- Marcadores plasmáticos podem ser utilizados para predizer desfechos clínicos na LT;
- Pacientes que falharam ao tratamento apresentaram uma bioassinatura distinta incluindo proteínas plasmáticas e mediadores lipídicos;
- A via das poliaminas e dos mediadores lipídicos podem ser utilizadas biomarcadores de gravidade de doença e falha terapêutica;
- Poliaminas e mediadores lipídicos podem ser utilizados para definir estágios clínicos da doença, estimar prognóstico e como novos alvos terapêuticos para LT;
- Resolvinas favorecem a infecção por *L. braziliensis* em neutrófilos.

13 **CONCLUSÃO**

Em conjunto, os resultados aqui apresentados nos permitem concluir que a arginase/poliaminas e os mediadores lipídicos estão associados à patogênese da LT podendo ser utilizados como biomarcadores de gravidade e falha terapêutica e que as resolvinas favorecem a persistência do parasito.

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Anexo A - Relacionados a tese

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SCIENTIFIC REPORTS

OPEN

Resolvin D1 drives establishment of *Leishmania amazonensis* infection

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Previous studies have indicated that the balance between different eicosanoids reflect the intensity of the inflammatory profile in patients with tegumentary leishmaniasis. More recently, pro-resolution lipid mediators have been shown to play critical roles in dampening pathological inflammatory processes to reestablish homeostasis in a diverse range of experimental settings. Among these lipid mediator, resolvins from D series have been described as potent anti-inflammatory and immunomodulatory mediators, and its activities include inhibition of leukocyte chemotaxis and blockage production of proinflammatory cytokines, while increasing the expression of regulatory mediators. Whether resolvins play significant roles in establishment and persistence of *Leishmania* infection is currently unknown. We addressed this question in the current study by assessing circulating levels of D-series resolvins in tegumentary leishmaniasis patients presenting with localized or diffuse disease. We found heightened expression of resolvin D1 in diffuse cutaneous leishmaniasis which was correlated with expression profile of biomarkers associated with disease pathogenesis. Additional *in vitro* experiments using primary human macrophages indicated that resolvin D1 may promote intracellular *Leishmania amazonensis* replication through a mechanism associated with induction of heme oxygenase-1. These results suggest that targeting resolvin D1 could serve as potential strategy for host directed therapy in diffuse cutaneous leishmaniasis.

Resolvins are oxygenated lipid mediators derived from ω-3 polyunsaturated fatty acids that have been associated with resolution of acute inflammation and restoration of tissue homeostasis[1]. The majority of the studies involving resolvins has focused on those from the D series, represented mainly by resolvin D1 (RvD1) and D2 (RvD2)[1]. The biological effects attributed to resolvins have been linked to its anti-inflammatory and immunomodulatory properties, which include inhibition of leukocyte chemotaxis, blocking production of pro-inflammatory cytokines, while increasing the expression of anti-inflammatory mediators such as heme oxygenase 1 (HO-1)[2,3]. The anti-inflammatory activity of resolvins has been extensively described in several inflammatory disease models, including cardiovascular diseases, cancer, acute kidney and lung injuries and metabolic inflammation in adipose tissue[1,3]. More recently, resolvins have also been implicated in host protective responses during viral and bacterial infections[1]. Although lipid mediator levels in plasma have been reported in patients with tegumentary leishmaniasis[4], it is still unknown whether resolvins associate with disease pathogenesis driven by *Leishmania* infection.

Tegumentary leishmaniasis is a disease caused by *Leishmania* parasites and exhibits a spectrum of clinical manifestations associated with the balance between parasite replication and immune-mediated inflammatory destruction of the skin tissue[5]. In the most common clinical form, named localized cutaneous leishmaniasis (LCL), single self-healing skin ulcers are usually observed. In this setting, a modest infiltration of macrophages is detected, with very few parasites, due moderate inflammation and cell-mediated immune responses[5]. A rare clinical form, diffuse cutaneous leishmaniasis (DCL), is characterized by the numerous nonulcerated nodular lesions with heavily parasitized macrophages[6]. Patients with DCL lack protective cell-mediated immunity and are reported to present

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The Journal of Infectious Diseases
BRIEF REPORT

Differential Expression of the Eicosanoid Pathway in Patients With Localized or Mucosal Cutaneous Leishmaniasis

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Unfettered inflammation is thought to play critical role in the development of different clinical forms of tegumentary leishmaniasis. Eicosanoids are potent mediators of inflammation and tightly associated with modulation of immune responses. In this cross-sectional exploratory study, we addressed whether targets from the eicosanoid biosynthetic pathway, assessed by multiplexed expression assays in lesion biopsy and plasma specimens, could highlight a distinct biosignature in patients with mucocutaneous leishmaniasis (MCL) or localized cutaneous leishmaniasis (LCL). Differences in immunopathogenesis between MCL and LCL may result from an imbalance between prostaglandins and leukotrienes, which may serve as targets for future host-directed therapies.

Keywords. tegumentary leishmaniasis; inflammation; eicosanoids; prostaglandin; leukotrienes; biomarkers.

Tegumentary leishmaniasis is a vector-borne disease caused by *Leishmania* parasites and exhibits a wide spectrum of clinical presentations. The most common clinical form of the disease caused by *Leishmania braziliensis* is localized cutaneous leishmaniasis (LCL), characterized by ulcerated dermal lesions, which usually heal spontaneously [1]. A more severe form of this disease, mucocutaneous leishmaniasis (MCL), is observed in 3% of individuals with LCL [2]. Patients with MCL usually present with severe and progressive destruction of nasopharyngeal and/or laryngeal structures [2]. MCL lesions exhibit intense inflammation and tissue damage and, counterintuitively, very few parasites. Necrosis of mucosal tissue is associated with a strong T-cell–mediated response, reflected by an exacerbated delayed-type hypersensitivity (DTH) reaction to *Leishmania* antigens [3]. Possible mechanisms linked to increased disease severity in MCL are still unknown, but the lack of immune modulation leading to uncontrolled inflammation seems to be critically involved [3].

Eicosanoids have been described to regulate key aspects of the host immune responses during *Leishmania* infection [4]. Prostaglandin E_2 (PGE_2) has been shown to enhance parasite survival, whereas increased leukotriene B_4 (LTB_4) production leads to enhanced intracellular parasite killing by infected host cells [5, 6]. These findings suggest that the balance between prostanoids and leukotrienes may directly affect the capacity of the host to control *Leishmania* infection. However, whether this dichotomy in the expression of eicosanoids is relevant in the context of MCL remains unknown.

We performed a cross-sectional exploratory study in patients with MCL and those with LCL from an area of endemicity in Brazil, assessing circulating levels, as well as in situ RNA expression of mediators from the eicosanoid pathway. We identified a distinct biosignature of MCL, with a hallmark of decreased expression of enzymes and receptors of prostaglandins, compared with LCL. Moreover, plasma levels of PGE_2 and LTB_4 indicated that patients with MCL are prone to skew the eicosanoid balance toward leukotrienes, whereas individuals with LCL exhibit an enriched prostanoid signature. These distinct expression profiles have potential implications for the understanding of tegumentary leishmaniasis pathogenesis, which can lead to development of new host-directed therapies targeting the eicosanoid pathway.

PATIENTS AND METHODS

This study was approved by the institutional review board from Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (number 136/2007). All clinical investigations were conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants or legal guardians, and all data analyzed were anonymized.

The present study assessed age- and sex-matched patients with MCL (n = 13; male to female ratio, 1.4; mean age [± standard deviation {SD}], 59 ± 17 years) and those with LCL (n = 29; male to female ratio, 1.9; mean age [±SD], 34 ± 15 years) recruited at our reference clinic in Jiquiriçá, Brazil. The 2 groups were not significantly different with respect to age ($P = .894$) or sex distribution ($P = .921$). Individuals included in the present study were required to have no previous diagnosis of tegumentary leishmaniasis and to be treatment naive. For plasma analyses, we included samples from 43 healthy controls

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MAJOR ARTICLE

Arginase I, Polyamine, and Prostaglandin E_2 Pathways Suppress the Inflammatory Response and Contribute to Diffuse Cutaneous Leishmaniasis

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Diffuse cutaneous leishmaniasis (DCL) is a rare clinical manifestation of tegumentary leishmaniasis. The molecular mechanisms underlying DCL pathogenesis remain unclear, and there is no efficient treatment available. This study investigated the systemic and in situ expression of the inflammatory response that might contribute to suppression in DCL. The plasma levels of arginase I, ornithine decarboxylase (ODC), transforming growth factor β (TGF-β), and prostaglandin E_2 (PGE_2) were higher in patients with DCL, compared with patients with localized cutaneous leishmaniasis (LCL) or with controls from an area of endemicity. In situ transcriptomic analyses reinforced the association between arginase I expression and enzymes involved in prostaglandin and polyamine synthesis. Immunohistochemistry confirmed that arginase I, ODC, and cyclooxygenase2 expression was higher in lesion biopsy specimens from patients with DCL than in those from patients with LCL. Inhibition of arginase I or ODC abrogates *L. amazonensis* replication in infected human macrophages. Our data implicate arginase I, ODC, PGE_2, and TGF-β in the failure to mount an efficient immune response and suggest perspectives in the development of new strategies for therapeutic intervention for patients with DCL.

Keywords. *Leishmania amazonensis*; diffuse cutaneous leishmaniasis; arginase I; ornithine decarboxylase; prostaglandin E_2; TGF-β

Cutaneous leishmaniasis exhibits a wide spectrum of clinical manifestations varying from self-healing localized cutaneous leishmaniasis (LCL) with a moderate cell-mediated immune response to diffuse cutaneous leishmaniasis (DCL) [1]. DCL is distinct from disseminated cutaneous leishmaniasis [2] and is characterized by the presence of several nonulcerated nodular skin lesions, the predominance of highly parasitized macrophages in the lesions, an absent or modest in vitro antileishmanial antigen cellular immune response, a negative delayed-type hypersensitivity (DTH) response, and resistance to antiparasite therapy [3]. The molecular mechanisms underlying DCL pathogenesis remain unclear, and there is no efficient treatment available.

In patients with DCL, antiinflammatory cytokines are abundant in lesions and in restimulated peripheral blood mononuclear cells (PBMCs), whereas proinflammatory cytokines and chemokines are absent or present at low levels [1]. However, the mechanisms responsible for this imbalance are not yet understood.

The arginase I pathway is emerging as a critical mechanism of immune regulation in *Leishmania* infection [4]

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Anexo B - Artigos publicados em colaboração durante o período de doutorado

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Tamoxifen and meglumine antimoniate combined therapy in cutaneous leishmaniasis patients: a randomised trial

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Abstract OBJECTIVES There is a clear need for new strategies of leishmaniasis treatment. This work was conducted to evaluate the efficacy of the co-administration of tamoxifen and meglumine antimoniate (Sb^V) in a phase II pilot clinical trial in localised cutaneous leishmaniasis patients.
METHODS A randomised controlled pilot clinical trial was conducted to evaluate the efficacy and safety of oral (40 mg/day for 20 days) or topical tamoxifen (0.1% tamoxifen citrate for 20 days) combined with meglumine antimoniate (20 mg Sb^V/kg/day for 20 days) *vs.* a standard Sb^V protocol (20 mg/kg/day for 20 days) for the treatment of cutaneous leishmaniasis. Primary outcome was complete epithelisation of the lesion 6 months after the end of treatment. Secondary outcomes were lesion healing 2 months after the end of treatment and frequency and severity of adverse events.
RESULTS A total of 38 subjects were included in the trial, 15 were treated with standard Sb^V and 23 with the combination of tamoxifen and Sb^V. Of the patients treated with the co-administration scheme, 12 received tamoxifen orally and 11 were treated with topical tamoxifen. Tamoxifen administered by the oral or topical routes was well tolerated. Cure rates 6 months after the end of treatment per intention to treat were 40% in the group treated with the standard Sb^V scheme, and 36.4% and 58%, respectively, for groups treated with Sb^V plus topical or oral tamoxifen.
CONCLUSIONS In the doses and schemes used in this study, co-administration of oral tamoxifen and Sb^V resulted in higher cure rates in comparison with the standard scheme of treatment, although not to statistically significant levels.

keywords cutaneous leishmaniasis, treatment, pentavalent antimonials, tamoxifen, topical, oral

Introduction

Leishmaniasis, an insectborne disease endemic in tropical and subtropical areas of the world, affects 0.9–1.6 million people yearly [1]. The diversity of clinical presentations correlates with parasite species but also with immune status of the host. Brazil is endemic for both visceral and tegumentary leishmaniasis, and *Leishmania (Viannia) braziliensis* is the most frequent parasite found in Brazilian patients with the tegumentary forms of leishmaniasis [2, 3].

Leishmaniasis treatment is based on a few available drugs, most of which with a poor safety profile. Pentavalent antimonials (Sb^V) were the first-line class of drugs used globally until parasite resistance was detected in India [4]. Since then, WHO recommendations replaced Sb^V with amphotericin B or miltefosine as first-line drugs [5]. Nevertheless, in some countries, such as Brazil, Sb^V remains the main stem of leishmaniasis chemotherapy [6]. However, studies have shown that the efficacy of antimonial treatment for localised cutaneous leishmaniasis (CL) can be as low as 50% in some areas [7–9].

Globally, there is a consensus that new strategies and alternatives for treating leishmaniasis are needed. We have described the antileishmanial activity of tamoxifen, a selective oestrogen modulator, and demonstrated, in animal models of visceral and cutaneous leishmaniasis, an efficacy equivalent or superior to Sb^V [10–12]. We have also shown that tamoxifen and Sb^V, when combined, presented additive properties and that tamoxifen was effective through topical administration [12]. Based on these findings, we conducted a pilot clinical trial in localised CL patients, to test whether the combination of

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Article

Heightened Plasma Levels of Transforming Growth Factor Beta (TGF-β) and Increased Degree of Systemic Biochemical Perturbation Characterizes Hepatic Steatosis in Overweight Pediatric Patients: A Cross-Sectional Study

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Abstract: Nonalcoholic Fatty Liver Disease (NAFLD) is a common cause of chronic liver disease in childhood and strongly associated with obesity. Routine biochemical non-invasive tests remain with low accuracy for diagnosis of NAFLD. We performed a cross-sectional study to examine potential associations between anthropometric and biochemical parameters, specially TGF-β, a prognosis marker for hepatic steatosis (HS). Between May and October 2019, seventy-two overweight adolescents were enrolled, of which 36 had hepatic steatosis. Hepatic, lipidic and glycemic profiles, and levels of vitamin D, ferritin and TGF-β were analyzed. Hierarchical cluster and a discriminant model using canonical correlations were employed to depict the overall expression profile of biochemical markers and the biochemical degree of perturbation. Median values of alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GGT), and TGF-β were higher in the adolescents with HS. Values of body mass index (BMI)/age and ALT, but not of TGF-β, were gradually increased proportionally to augmentation of steatosis severity. In a multivariate analysis, TGF-β plasma concentrations were associated with occurrence of hepatic steatosis independent of other covariates. Discriminant analysis confirmed that TGF-β concentrations can identify HS cases. Our data reveal that HS patients exhibit a distinct biosignature of biochemical parameters and imply TGF-β as an important biomarker to evaluate risk of steatosis development.

Keywords: non-alcoholic fatty liver disease; pediatric obesity; transforming growth factor beta; systemic biochemical perturbation; transaminases; cross-sectional studies

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RISK6, a 6-gene transcriptomic signature of TB disease risk, diagnosis and treatment response

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Improved tuberculosis diagnostics and tools for monitoring treatment response are urgently needed. We developed a robust and simple, PCR-based host-blood transcriptomic signature, RISK6, for multiple applications: identifying individuals at risk of incident disease, as a screening test for subclinical or clinical tuberculosis, and for monitoring tuberculosis treatment. RISK6 utility was validated by blind prediction using quantitative real-time (qRT) PCR in seven independent cohorts. Prognostic performance significantly exceeded that of previous signatures discovered in the same cohort. Performance for diagnosing subclinical and clinical disease in HIV-uninfected and HIV-infected persons, assessed by area under the receiver-operating characteristic curve, exceeded 85%. As a screening test for tuberculosis, the sensitivity at 90% specificity met or approached the benchmarks set out in World Health Organization target product profiles for non-sputum-based tests. RISK6 scores correlated with lung immunopathology activity, measured by positron emission tomography, and tracked treatment response, demonstrating utility as treatment response biomarker, while predicting treatment failure prior to treatment initiation. Performance of the test in capillary blood samples collected by finger-prick was noninferior to venous blood collected in PAXgene tubes. These results support incorporation of RISK6 into rapid, capillary blood-based point-of-care PCR devices for prospective assessment in field studies.

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Prevalence and Clinical Profiling of Dysglycemia and HIV Infection in Persons With Pulmonary Tuberculosis in Brazil

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Background: There are scarce data on the prevalence and disease presentation of HIV in patients with tuberculosis (TB) and dysglycemia (diabetes [DM] and prediabetes [PDM]), especially in TB-endemic countries.

Methods: We assessed the baseline epidemiological and clinical characteristics of patients with culture-confirmed pulmonary TB, enrolled in a multicenter prospective cohort in Brazil (RePORT-Brazil) during 2015–2019. Dysglycemia was defined by elevated glycated hemoglobin and stratified as PDM or DM. Additionally, we used data from TB cases obtained through the Brazilian National Notifiable Diseases Information System (SINAN), during 2015–2019. In SINAN, diagnosis of diabetes was based on self-report. Logistic regression models were performed to test independent associations between HIV, dysglycemia status, and other baseline characteristics in both cohorts.

Results: In the RePORT-Brazil cohort, the prevalence of DM and of PDM was 23.7 and 37.8%, respectively. Furthermore, the prevalence of HIV was 21.4% in the group of persons with TB-dysglycemia and 20.5% in that of patients with TBDM. In the SINAN cohort, the prevalence of DM was 9.2%, and among the TBDM group the prevalence of HIV was 4.1%. Logistic regressions demonstrated that aging was independently

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