# UNIVERSIDADE FEDERAL DA BAHIA

## FACULDADE DE MEDICINA DA BAHIA

**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE**


# ANÁLISE DO POLIMORFISMO E DA EXPRESSÃO DE GENES DE REPARO TECIDUAL NA LEISHMANIOSE TEGUMENTAR AMERICANA CAUSADA PELA INFECÇÃO POR *LEISHMANIA BRAZILIENSIS*


**Lucas Frederico de Almeida**


**Tese de Doutorado**


**Salvador (Bahia), 2016**

A447 Almeida, Lucas Frederico de

Análise do polimorfismo e da expressão de genes de reparo tecidual na leishmaniose tegumentar americana causada pela infecção por *Leishmania braziliensis* / Lucas Frederico de Almeida. – Salvador, 2016.

VIII, 150 p.: il.

Orientadora: Profª. Drª. Léa Castellucci.

Tese (doutorado) – Universidade Federal da Bahia, Faculdade de Medicina da Bahia, Programa de Pós-graduação em Ciências da Saúde, Salvador, 2016.

1. Leishmania. 2. Cura de lesão. 3. FLI1. 4. COL1A1. 5. COL1A2. 6. MMP1. 7. Regulação epigenética. I. Castellucci, Léa. II. Universidade Federal da Bahia, Faculdade de Medicina da Bahia, Programa de Pós-graduação em Ciências da Saúde. III. Título.

CDU – 616.928.5

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Lucas Frederico de Almeida

Professora-orientadora : Léa Castellucci


Tese apresentada ao Colegiado do PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE, da Faculdade de Medicina da Universidade Federal da Bahia, como pré-requisito obrigatório para a obtenção do grau de Doutor em Ciências da Saúde.


Salvador (Bahia), 2016

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Dedico este trabalho à minha esposa Germana, aos meus pais Silvio e Conceição, às minhas irmãs Tatiana e Mirela, aos meus sobrinhos Clarisse, Eduardo, Letícia e Helena.

**FONTES DE FINANCIAMENTO**

National Institutes of Health – Grant AI 30639

Fundação de Amparo à Pesquisa do Estado da Bahia

**AGRADECIMENTO ESPECIAL**

À Doutora Léa Castellucci, agradeço especialmente pela orientação, paciência e pelos ensinamentos, sempre estimulando e respeitando os limites do outro. Pelo exemplo, capacidade e humildade, que vou sempre admirar e levar como modelo, minha eterna gratidão.

**AGRADECIMENTOS**

Ao Doutor Edgar Carvalho, pela oportunidade de desenvolver este trabalho no Serviço de Imunologia.

A Ednaldo Lago, companheiro essencial na área endêmica, pela solicitude com que pude contar durante a realização deste estudo.

A toda equipe do Serviço de Imunologia, de maneira especial, Andréa Magalhães, Joyce Oliveira, Jamile Leão, Nadja Lima, Anadilton da Hora, Tainã Lago, Marcos Braz, pelo carinho e pela convivência diária.

À equipe que dá suporte na área endêmica. Gostaria de registrar o nome de Luiz Henrique Guimarães, pela amizade e proximidade, e Juliana Silva e Viviane Andrade pela realização das biópsias.

Aos funcionários do Posto de Saúde de Corte de Pedra, por atenderem às solicitações do nosso grupo de maneira tão colaborativa.

Aos pacientes da área endêmica, por nos receberem em seus lares e colaborarem com a nossa pesquisa de forma tão generosa.

Aos professores do Programa de Pós-graduação em Ciências da Saúde da Universidade Federal de Bahia (PPgCS), pelos conhecimentos transmitidos.

Aos colegas do PPgCS, por tornarem esta jornada mais agradável e enriquecedora.

A todos, minha sincera gratidão.

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# I. RESUMO

Estudos prévios têm demonstrado um papel para os genes de cura de lesão na resolução das lesões cutâneas causadas por espécies de *Leishmania* em camundongos e humanos, incluindo o gene *FLI1* (*Friend leukemia vírus integration 1*). O alto grau de metilação de ilhas CpG na região promotora do gene *FLI1* é conhecido por tornar o gene transcricionalmente inativo. Redução da expressão de *FLI1* resulta na regulação positiva dos genes *COL1A1* (*collagen type I alpha 1*) e *COL1A2* (*collagen type I alpha 2*), e, inversamente, na regulação negativa do gene *MMP1* (*matrix metallopeptidase 1*). Ambos, colágeno do tipo 1 e metaloproteinase de matriz 1, desempenham um importante papel nas condições fisiológicas normais e patológicas de muitas doenças, e estão envolvidos no reparo de lesões. Adicionalmente, em estudo prévio, foi mostrada uma associação entre leishmaniose e um polimorfismo regulatório no gene *IL6* (*interleukin 6*), e dados na literatura sugerem uma interação funcional entre *IL6* e *FLI1*. Para entender melhor o papel desta via na leishmaniose, nós avaliamos o polimorfismo dos genes *COL1A1*, *COL1A2* e *MMP1* e sua possível associação com a leishmaniose tegumentar americana na população de Corte de Pedra, Bahia, assim como também avaliamos a expressão gênica de *COL1A1*, *COL1A2*, *MMP1*, *FLI1*, e o grau de metilação em ilhas CpG da região promotora deste último gene em biópsias de pele e em culturas de macrófagos infectados por *Leishmania braziliensis*. No estudo de associação genética, FBAT (*Family-based association tests*) mostrou uma forte associação entre os marcadores *COL1A1*_rs1061237 (P = 0,002) e *COL1A1*_rs2586488 (P = 0,027) e o fenótipo leishmaniose cutânea. O estudo com biópsias de pele revelou que a porcentagem de DNA metilado na região promotora de *FLI1* foi mais baixa (P = 0,001) em biópsias de lesão de leishmaniose cutânea comparadas com biópsias de pele normal. A expressão gênica de *FLI1* e de *COL1A1* não diferiu entre as biópsias de lesão e as biópsias de pele normal, enquanto a expressão de *COL1A2* foi menor (P = 0,033) e a expressão de *MMP1* foi maior (P = 0,0002) nas biópsias de lesão de leishmaniose cutânea. Por fim, a análise da expressão gênica em macrófagos infectados mostrou que a expressão de *FLI1* induzida pela infecção por *Leishmania braziliensis* teve um pico com 24 horas de infecção (P < 0,0001), e foi maior (P = 0,005) e teve um pico mais tardio (48 horas) na presença de IL-6. A expressão de genes de colágeno do tipo 1, *COL1A1* e *COL1A2*, foi baixa em macrófagos infectados, e não foi detectável em macrófagos tratados com IL-6. A expressão de *MMP1* foi fortemente induzida (P = 0,007) após infecção de macrófagos, mas não foi facilmente detectável em macrófagos tratados com IL-6 até 72 horas de infecção, quando o efeito da IL-6 na expressão de *FLI1* diminuiu. *MMP1* quebra o colágeno do tipo 1 intersticial, que é essencial para a migração de queratinócitos e o processo de re-epitelização. Porém, em lesões ativas de leishmaniose cutânea, baixos níveis de colágeno do tipo 1 junto com a exagerada expressão de *MMP1* indica que o *MMP1* está contribuindo para o dano tecidual em vez de levar ao reparo. Similarmente, foi observado que níveis exagerados de *MMP1* contribuem para a destruição do tecido e progressão para a doença na tuberculose, levando outros autores a destacar o *MMP1* como potencial alvo terapêutico. Nossos dados sugerem que a modulação desta via pode também ser relevante no tratamento da leishmaniose cutânea.

Palavras-chave: Leishmania; cura de lesão; FLI1; COL1A1; COL1A2; MMP1; regulação epigenética.

# II. OBJETIVOS

## II.1. PRINCIPAL

Avaliar o polimorfismo e a expressão de genes de reparo tecidual, bem como as interações epigenéticas entre genes previamente associados com a leishmaniose tegumentar americana no Brasil.

## II.2. SECUNDÁRIOS

1. Avaliar o polimorfismo dos genes *COL1A1*, *COL1A2* e *MMP1* e sua possível associação com a leishmaniose tegumentar americana na população de Corte de Pedra, Bahia;

2. Avaliar a expressão gênica de *FLI1* e de genes sob sua regulação (*COL1A1*, *COL1A2* e *MMP1*), assim como seu padrão de metilação, em biópsias de pele normal e de lesões de leishmaniose cutânea; e

3. Avaliar a expressão gênica de *FLI1* e de genes sob sua regulação (*COL1A1*, *COL1A2* e *MMP1*), assim como seu padrão de metilação, em macrófagos de pacientes com leishmaniose cutânea infectados por *Leishmania braziliensis*.

## III. INTRODUÇÃO

A leishmaniose tegumentar americana (LTA), causada por protozoários parasitas do gênero *Leishmania*, é um importante problema de saúde pública em muitos países em desenvolvimento. A infecção está associada com um amplo espectro de fenótipos clínicos, resultante dos fatores de risco ambientais e uma combinação da composição genética do parasita e do hospedeiro. Nos últimos anos, tem sido mostrado que polimorfismos em genes associados com cura de lesão e reparo tecidual são importantes fatores de risco para leishmaniose cutânea (LC) causada por *Leishmania braziliensis* (Castellucci et al., 2011, 2012). Assim, o gene *FLI1*, inicialmente identificado e mapeado como um gene que controla susceptibilidade a LC causada pela infecção por *Leishmania major* em camundongos (Sakthianandeswaren et al., 2005, 2010), foi também associado com desenvolvimento da doença em humanos expostos a *Leishmania braziliensis* no Brasil. Além disso, dados mostraram que polimorfismos em outros genes de cura de lesão ligados à via do *FLI1*, em particular *TGFB1*, *TGFBR2*, *CTGF* e *SMAD2/3/7*, são fatores de risco para LC.

O reparo de lesões cutâneas pode ser dividido em uma série de fases que se sobrepõem, incluindo a resposta inflamatória, formação de tecido de granulação, e, finalmente, o remodelamento da matriz (Li et al., 2007; Reinke & Sorg, 2012). As proteínas mais abundantes na matriz extracelular são os colágenos, que podem ser degradados através da ação de colagenases, tais como o MMP1. Redução da expressão de *FLI1* resulta na regulação positiva dos genes de colágeno do tipo 1, *COL1A1* e *COL1A2*, e, inversamente, na regulação negativa do gene *MMP1* (Nakerakanti et al., 2006), sugerindo que a supressão do gene *FLI1* esteja envolvida na ativação da resposta fibrótica. Ambos, colágeno tipo 1 e as

metaloproteinases de matriz, desempenham um importante papel nas condições fisiológicas normais e patológicas de muitas doenças (Imai et al., 2000; Alexakis et al., 2006; Wynn, 2008; Amalinei et al., 2010). Adicionalmente, em um estudo prévio foi observada uma associação entre leishmaniose causada por *Leishmania braziliensis* e um polimorfismo regulatório no promotor do gene *IL6* (Castellucci et al., 2006). Dados têm mostrado que esta citocina pode regular positivamente genes essenciais para a metilação epigenética do DNA, através da expressão de *FLI1*, o que sugere uma interação funcional entre os dois genes (Thaler et al., 2011).

Apesar da grande contribuição destes estudos para o melhor entendimento da patogênese da doença, menor atenção tem sido dada aos mecanismos de regulação da expressão destes genes. Um crescente número de evidências tem apontado a importância dos mecanismos epigenéticos, tais como a metilação do DNA, na regulação da expressão gênica. No caso do gene *FLI1*, esta ideia é suportada por dados que demonstram um alto grau de metilação em ilhas CpG deste gene na esclerose sistêmica, tornando o gene transcricionalmente inativo (Wang et al., 2006). Um estudo mais recente também identificou alteração na metilação do DNA em centenas de ilhas CpG seguida da infecção de macrófagos por *Leishmania donovani* (Marr et al., 2014). Este dado sugere que a *Leishmania* induz mudanças na célula hospedeira para favorecer a sua sobrevivência. Assim, o objetivo deste estudo foi entender o papel funcional do gene *FLI1* na leishmaniose, comparando o perfil de metilação em ilhas CpG da região promotora deste gene em biópsias de pele e em culturas de macrófagos, e correlacionar este padrão de metilação com a sua expressão gênica e também com a expressão de genes que estão sob a sua regulação (*COL1A1*, *COL1A2* e *MMP1*). Adicionalmente, nós também avaliamos o polimorfismo destes genes e sua possível associação com o desenvolvimento da LTA na população de Corte de Pedra, Bahia.

# IV. REVISÃO DA LITERATURA

## IV.1. ASPECTOS CLÍNICOS E EPIDEMIOLÓGICOS DAS LEISHMANIOSES

A leishmaniose constitui um grupo amplamente diverso de doenças parasitárias causadas por protozoários pertencentes ao gênero *Leishmania*, que são transmitidos ao hospedeiro vertebrado por flebotomíneos infectados. A infecção por *Leishmania spp.* resulta em um amplo espectro clínico que pode ser classificado em duas formas principais: a leishmaniose tegumentar, um termo que compreende as formas cutâneas com potencial para evoluir para formas mucosas secundárias, e a leishmaniose visceral (LV). A LV é principalmente causada por espécies do complexo *Leishmania donovani* (Desjeux et al., 2013), e está associada com episódios de febre, perda de peso, anemia, hepatomegalia, esplenomegalia, e, com o tempo, os casos não tratados podem ser fatais (Murray et al., 2005). A leishmaniose tegumentar, por sua vez, pode ser causada por múltiplas espécies, incluindo *Leishmania tropica*, *Leishmania major* e *Leishmania aethiopica* no Velho Mundo, e as espécies *Leishmania mexicana*, *Leishmania amazonensis*, *Leishmania panamensis*, *Leishmania peruviana*, *Leishamnia guyanensis* e *Leishmania braziliesis* no Novo Mundo (Murray et al., 2005). Assim, esta forma da doença pode ser classificada de acordo com a sua distribuição geográfica em Leishmaniose Tegumentar do Velho Mundo e Leishmaniose Tegumentar do Novo Mundo (ou Leishmaniose Tegumentar Americana – LTA). Dentro das manifestações de LTA, destacamos as formas LC e leishmaniose mucosa (LM). A LC é a forma mais comum da doença e começa como uma pápula no local da picada do flebotomíneo e se desenvolve em um nódulo ou uma úlcera que pode ou não sofrer

cura espontânea (Murray et al., 2005). A LM se desenvolve como uma complicação da LC, com parasitas disseminando para colonizar o trato das mucosas, e ocorre em 1-10% das infecções. Esta forma da doença se desenvolve 1-5 anos após a LC ter sido curada, mas em alguns casos coincide com lesões cutâneas ativas, e 90% dos pacientes possuem uma cicatriz cutânea precedente (Weigle & Saravia, 1996; Blum et al., 2004; Machado-Coelho et al., 2005). A LM começa com eritema e ulcerações nas narinas, seguindo para perfuração do septo nasal e lesões inflamatórias destrutivas. Estas lesões podem obstruir a faringe ou a laringe e levar a desfigurações marcantes (Franke et al., 1990).

Apesar de ser considerada uma doença negligenciada, um grande número de estudos tem sido gerado na taxonomia, epidemiologia, manifestações clínicas, diagnóstico e terapia das leishmanioses. Uma análise dos artigos publicados sobre a doença durante um período de 50 anos (1957-2007) mostrou um crescente número de publicações durante este período, com um pico de 1.226 trabalhos em 2006 (Al-Mutawakel et al., 2010). Porém, a despeito da importante contribuição destes trabalhos na dinâmica e melhor entendimento da imunopatogênese da doença, ainda não há uma vacina efetiva que garanta imunidade de longa duração, assim como uma queda na prevalência global. Em uma publicação recente, foi mostrado que as leishmanioses são responsáveis por mais de quatro milhões de anos perdidos por incapacidade (*DALYs - disability-adjusted life years*), e foi uma das poucas doenças que tiveram um aumento deste parâmetro entre 2005 e 2013 (GBD, 2015).

Além disso, existe uma gradativa quantidade de dados destacando a ideia de que o impacto na saúde da leishmaniose é grosseiramente subestimado devido a inúmeros fatores, como aumento da prevalência, casos não registrados e expansão de áreas endêmicas. A respeito disso, dados secundários são uteis para calcular a real carga da doença (Bern et al., 2008). De acordo com

um trabalho publicado recentemente, a leishmaniose é endêmica em 98 países (Alvar et al., 2012). Apesar disso, deve ser ressaltado que mais de 90% dos casos de LV ocorrem em apenas seis países: Índia, Bangladesh, Sudão, Brasil, Nepal e Etiópia. A leishmaniose tegumentar é mais amplamente distribuída. Os dez países com maior número de casos estimados de leishmaniose tegumentar, Afeganistão, Argélia, Colômbia, Brasil, Irã, Síria, Etiópia, Sudão, Costa Rica e Peru, são responsáveis por aproximadamente 70-75% da incidência global da doença (Alvar et al., 2012). Apesar do seu importante impacto na saúde pública, principalmente nos países em desenvolvimento, a leishmaniose ainda é fortemente ignorada nas discussões sobre as prioridades das doenças tropicais (Hotez et al., 2004, 2006).

## IV.2. LEISHMANIOSE NO BRASIL

Entre as dez doenças tropicais negligenciadas estabelecidas pela Organização Mundial de Saúde, nove estão presentes no Brasil, incluindo a leishmaniose (Lindoso & Lindoso, 2009). Neste cenário, o Brasil está entre os sete países que concentram 90% dos casos de leishmaniose (Alvar et al., 2012), e é o responsável pelas maiores taxas de incidência da doença nas Américas. Aqui no Brasil, é estimada uma incidência anual de 4.200 a 6.300 casos de LV, e 72.800 a 119.600 casos de LTA (Alvar et al., 2012).

Até a metade do século passado, a maioria dos casos estava concentrada nos estados de São Paulo, Paraná, Minas Gerais, Ceará e Pernambuco, e associada com desmatamento e novos assentamentos. Devido ao aumento das atividades rurais, a doença se espalhou para outras regiões, incluindo áreas metropolitanas

(Passos et al., 1993; Brandão-Filho et al., 1999). Em particular, áreas da região nordeste do Brasil, onde a *Leishmania braziliensis* é endêmica, têm visto um aumento na notificação de casos de LTA nas últimas décadas (Brandão-Filho et al., 1999; Oliveira et al., 2004), e epidemias recorrentes nesta região compreendem um componente cada vez mais importante na carga global da doença no Brasil (Broutet et al., 1994; Sousa et al., 1995).

A região sudeste do estado da Bahia, onde está localizada a vila de Corte de Pedra, é reconhecida como uma das mais importantes áreas de transmissão de *Leishmania braziliensis* no Brasil, e registra uma alta prevalência de casos de LTA. Entre 1988 e 2008, 12.424 indivíduos com lesões atribuídas a LTA visitaram o Posto de Saúde da região para avaliação clínica e tratamento (Jirmanus et al., 2012). Assim, pacientes desta área endêmica têm sido acompanhados no Posto de Saúde, que se tornou o centro de referência para o tratamento e diagnóstico da doença na região.

Durante as duas últimas décadas, os estudos conduzidos nesta área pela equipe médica e pelos pesquisadores do Serviço de Imunologia do Complexo Hospitalar Universitário Professor Edgard Santos da Universidade Federal da Bahia notaram um aumento do número de casos mais graves, uma diminuição da eficácia do antimonial pentavalente, mudanças na demografia dos pacientes atendidos (Turetz et al., 2002; Guimarães et al., 2009), e estabeleceram novos tipos de terapias para LTA (Lessa et al., 2001; Almeida et al., 2005; Machado et al., 2007, 2010). Os estudos realizados em Corte de Pedra também determinaram a incidência de LC e LM, e descreveram novas formas clínicas de LTA causadas pela infecção por *Leishmania braziliensis*, tais como a leishmaniose disseminada (LD) e a leishmaniose cutânea atípica (LCA) (Costa et al., 1986; Morgan et al., 2007; Guimarães et al., 2009).

A LD é uma forma emergente da doença causada pela infecção por *Leishmania braziliensis*, que se distingue por suas características clínicas e imunológicas exclusivas (Costa et al., 1986; Carvalho et al., 1994; Turetz et al., 2002). Esta forma da doença é caracterizada pela presença de um grande número de lesões cutâneas que abrange mais de uma área do corpo do paciente, tipos variados de lesões na pele (papulares, nodulares, ulceradas) e uma alta freqüência de envolvimento mucoso (Carvalho et al., 1994; Turetz et al., 2002). O paciente tende a apresentar uma lesão única inicial, geralmente nas extremidades, seguida da disseminação que pode envolver todo o corpo, por vezes, associada com febre e calafrios. A importância da LD é delineada pela gravidade da doença, terapêutica desafiadora pelo alto grau de refratariedade e aumento da prevalência. Enquanto em 1986 apenas 0,2% dos casos de infecção por *Leishmania braziliensis* desenvolveu leishmaniose disseminada, em 2000 este número subiu para 2% (Carvalho et al., 1994; Turetz et al., 2002). Estudos de epidemiologia molecular estão sendo realizados na área de Corte de Pedra objetivando a identificação de cepas emergentes que respondam por este aumento de prevalência. Recentemente, foi demonstrado que alguns polimorfismos de nucleotídeos simples (SNPs) e inserções-deleções (*indels*) do genoma de *Leishmania braziliensis* estão associados com esta forma da doença (Queiroz et al., 2012).

Embora raro, casos de LCA também estão emergindo na área endêmica de Corte de Pedra. Pacientes de LCA são definidos como indivíduos que não preenchem os critérios de LC, LM e LD e claramente não apresentam fatores predisponentes, como imunossupressão (Guimarães et al., 2009). Estes pacientes apresentam lesões atípicas de LC, tais como lesões vegetativas, verrucosas, crostosas e lupóides. Entre 2005 e 2006, 35 pacientes (2,5%) apresentaram manifestações atípicas da doença (Guimarães

et al., 2009). Estes pacientes eram predominantemente do sexo masculino, frequentemente apresentaram lesões na face e tinham altas taxas de falha ao tratamento quando comparados com pacientes com lesões clássicas de LTA atendidos no mesmo período (Guimarães et al., 2009).

Uma série de estudos conduzidos em Corte de Pedra nos últimos trinta anos tem contribuído notavelmente para o conhecimento da epidemiologia e da resposta imune de LTA (Carvalho et al., 2012; de Oliveira & Brodskyn, 2012). Durante a última década, o nosso grupo tem se concentrado em avaliar o papel de fatores genéticos do hospedeiro e do parasita nos desfechos de LTA, e, como consequência deste esforço, uma série de trabalhos foi publicada, fornecendo evidências de que cepas do parasita (Schriefer et al., 2004, 2009; Queiroz et al., 2012) e fatores genéticos do hospedeiro (Castellucci et al., 2006, 2010, 2011, 2012; Ramasawmy et al., 2010) desempenham um importante papel na susceptibilidade aos diferentes fenótipos clínicos de LTA.

## IV.3. FATORES GENÉTICOS DO HOSPEDEIRO NO DESENVOLVIMENTO DAS LEISHMANIOSES

Uma importante característica de muitas infecções humanas é que apenas uma proporção dos indivíduos expostos desenvolve a doença clínica. Por muito tempo, foi considerado que os fatores hereditários tinham um papel dominante nesta explicação interindividual na susceptibilidade. Por exemplo, a observação inicial de que os casos de tuberculose eram agrupados dentro das famílias levantou a impressão de que a tuberculose era uma doença hereditária. No entanto, a descoberta posterior de bactérias como

agentes de doença infecciosa e a demonstração experimental da natureza contagiosa das infecções concentrou atenção sobre os patógenos (Kaufmann, 2005), negligenciando a potencial importância dos fatores do hospedeiro.

Os primeiros dados sobre a influência da variação genética do hospedeiro na susceptibilidade a doenças infecciosas surgiram a partir de estudos com gêmeos e filhos adotados. Estudos com gêmeos mostraram um importante papel dos fatores genéticos do hospedeiro na susceptibilidade a doenças infecciosas, tais como tuberculose, hanseníase, poliomielite e hepatite B (Herndon & Jennings, 1951; Comstock, 1978; Lin et al., 1989; Kaufmann, 2005; Misch et al., 2010). No final da década de 80, um estudo marcante relatou que crianças adotadas tinham um risco significantemente aumentado de morte por doença infecciosa se um dos seus pais biológicos tivesse morrido prematuramente da infecção (Sorensen et al., 1988). Estudos em camundongos também ilustraram a potencial importância dos efeitos genéticos do hospedeiro, mostrando diferenças na carga bacteriana, resposta de citocinas e desfechos após infecção por bactérias e micobactérias entre as diferentes linhagens de camundongos (Gingles et al., 2001).

Na leishmaniose, estudos genéticos de LC e LV em camundongos identificaram vários loci envolvidos na patogênese da leishmaniose, destacando a natureza multigenética desta doença (DeTolla et al., 1980; Kirkpatrick & Farrell, 1982; Beebe et al., 1997, 1999; Roberts et al., 1997; Lipoldova et al., 2000; Badalova et al., 2002; Blackwell et al., 2003; Bucheton et al., 2003a, 2003b, 2007; Mohamed et al., 2003, 2004; Vladimirov et al., 2003; Baguet et al., 2004; Elso et al., 2004; Havelkova et al., 2006; Lipoldova & Demant, 2006; Jamieson et al., 2007; Jeronimo et al., 2007; Miller et al., 2007).

Em humanos, estudos de associação com genes candidatos são frequentemente utilizados nos estudos genéticos de LC. Por exemplo, polimorfismos nos genes *IL-4* e *IFN-γ* foram associados com susceptibilidade a LC em um grupo de iranianos expostos a *Leishmania major* (Kamali-Sarvestani et al., 2006). Análise de um polimorfismo na região promotora do gene *IL10* em indivíduos brasileiros sintomáticos e assintomáticos para LC causada por *Leishmania braziliensis* demonstrou uma associação entre o genótipo *IL10* -819 C/C, altos níveis desta citocina e gravidade das lesões. A IL-10 suprime a reposta inflamatória importante para a eliminação da *Leishmania*, e foi admitida a hipótese de que os altos níveis desta citocina em carreadores do genótipo C/C promovem a sobrevivência da *Leishmania* (Salhi et al., 2008).

A LM se desenvolve como uma complicação da LC com disseminação da *Leishmania* para as mucosas. Análise de polimorfismos do gene *TNF* em indivíduos venezuelanos sintomáticos e assintomáticos para LM expostos a *Leishmania braziliensis* identificou uma associação de polimorfismos dos genes *TNFA* e *TNFB* com susceptibilidade a doença (Cabrera et al., 1995). Avaliação do polimorfismo -174 C(G) do gene *IL6* em casos de LM da Bahia, onde *Leishmania braziliensis* é endêmica, mostrou uma forte associação entre a presença do alelo C e susceptibilidade a LM. Os genótipos C/C e C/G foram associados com redução dos níveis de IL-6. Foi admitida a hipótese que a baixa produção de IL-6 em carreadores do alelo C contribui para a capacidade reduzida de balancear as respostas Th1 e Th2 e controlar as manifestações patológicas destrutivas associadas com a doença (Castellucci et al., 2006).

Mais recentemente, a importância dos processos de cura de lesão nas formas cutâneas de leishmaniose também foi demonstrada a partir de estudos de mapeamento genético em camundongos (Sakthianandeswaren et al., 2005, 2009, 2010). Em particular,

mapeamento fino na região do cromossomo 9 em camundongos (cromossomo 11q24 em humanos) identificou o gene *Fli1* (*FLI1* em humanos) como novo gene candidato influenciando resistência a *Leishmania major* e uma resposta aumentada de cura de lesão (Sakthianandeswaren et al., 2010).

Recentemente, o nosso grupo de pesquisa, trabalhando com a hipótese de que genes mapeados em camundongos poderiam ser também importantes biomarcadores de doença humana, demonstrou que um polimorfismo neste gene está associado com LC causada por *Leishmania braziliensis*, com uma associação inversa observada para LM (Castellucci et al., 2011). De maneira interessante, como relatado anteriormente, nosso grupo de pesquisa também mostrou uma associação entre LM e o alelo C do polimorfismo -174 G/C de *IL6* (Castellucci et al., 2006). Foi recentemente demonstrado que a estimulação de *IL6* dependente de homocisteína regula positivamente genes essenciais para a metilação epigenética do DNA via expressão de *FLI1* (Thaler et al., 2011). Na outra direção, foi também visto que o Fli-1 é um regulador de IL-6 em um modelo murino de lúpus sistêmico eritematoso (Sato et al., 2014), indicando a existência de um possível mecanismos de *feedback loop* entre estes dois fatores. Isto sugere que, embora haja muitas funções imunológicas tanto para IL-6 quanto para FLI1, que poderiam ser responsáveis pela associação com LTA causada por *Leishmania braziliensis*, pode haver uma ligação funcional direta entre estes dois genes que medeia a susceptibilidade a doença por meio da resposta de cura de lesão e reparo tecidual.

Na resposta de cura de lesão, ambos, FLI1 (Nakerakanti et al., 2006) e IL-6 (Gressner et al., 2011), reprimem o CTGF, e todos os três genes interagem com a via de sinalização de TGFβ (Figura 1). Nos últimos anos, foi realizado um screening de genes envolvidos nesta via de sinalização, que mostrou que polimorfismos em outros genes de cura de lesão relacionados com a

função de FLI1, em particular *TGFB1*, *TGFBR2*, *CTGF* e *SMAD2/3/7*, são fatores de risco para o desenvolvimento de LC (Castellucci et al., 2012). Estes dados suportam a hipótese de que a regulação fisiológica normal do processo de cura de lesão é importante na resolução da LTA.

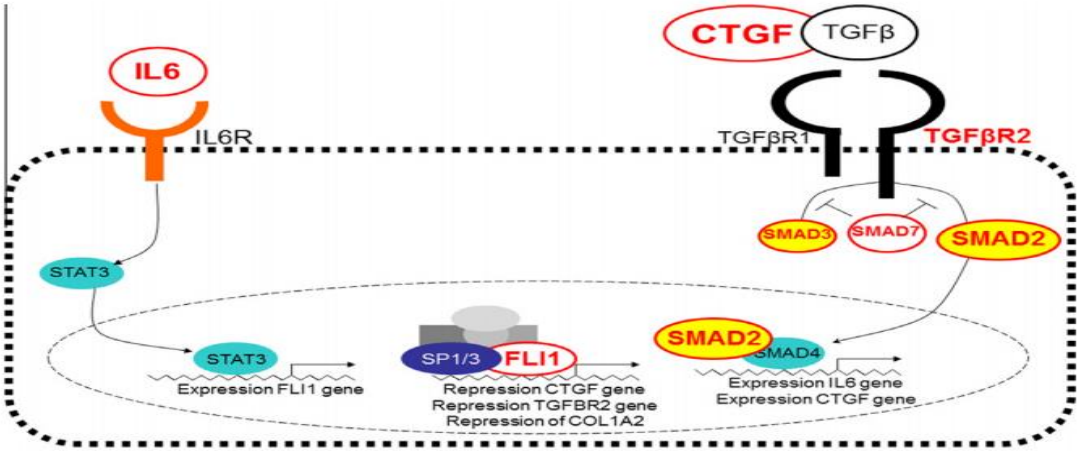


**Figura 1** Diagrama de genes que têm sido implicados na susceptibilidade a LC e LM causadas por *Leishmania braziliensis* na área de Corte de Pedra, mostrando o envolvimento e a interação com a via de TGF-β. Polimorfismos em genes anotados em letra vermelha foram associados com LC ou LM (retirado de Castellucci et al., 2012).

Adicionalmente, foi relatado que os níveis de expressão de *FLI1* estão sujeitos a regulação epigenética. Wang e colaboradores (2006) demonstraram que as ilhas CpG do promotor de *FLI1* permanecem não metiladas em fibroblastos normais, resultando em um gene transcricionalmente ativo, enquanto em pacientes com escleroderma a metilação nas mesmas ilhas CpG é significativamente aumentada, resultando em repressão da transcrição de *FLI1*. Dados preliminares do nosso grupo de pesquisa com linhagem celular THP-1 estimuladas com antígeno solúvel de *Leishmania* (SLA) também indicam mudanças no padrão de metilação do gene *FLI1*, consistente com a expressão reduzida

de FLI1 observada nos mesmos pontos de tempo (dados não publicados). O aumento concomitante na metilação do DNA na ilha CpG da região promotora do gene *FLI1* indica que a diminuição do nível de expressão tem uma origem epigenética, e é regulada negativamente seguindo estímulo com SLA. Nesse sentido, estudos de interações epigenéticas entre os genes que compartilham vias de ativação importantes na patogênese da leishmaniose irão esclarecer como certos mecanismos regulatórios estão ocorrendo no hospedeiro devido ao desafio com o parasita.

## IV.4. MECANISMOS DE REGULAÇÃO EPIGENÉTICA E SUA IMPORTÂNCIA NO DESENVOLVIMENTO DE DOENÇAS COMPLEXAS

Poucas áreas da biologia atraem tanta atenção e, ao mesmo tempo, ainda exigem tanta apresentação como o campo da epigenética. O termo “epigenética” foi usado pela primeira vez por Waddington para descrever o processo através do qual os genótipos dão origem aos fenótipos durante o desenvolvimento (Waddington, 1942). De um modo geral, epigenética se refere a mudanças na expressão gênica desencadeadas por um estímulo devido a processos independentes de alterações na sequencia de DNA. Alguns destes processos foram elucidados e incluem a metilação do DNA (Suzuki & Bird, 2008), modificações de histonas (Bannister & Kouzarides, 2011) e silenciamento do DNA por RNA não codificante (Storz, 2002).

A metilação do DNA, que compreende a adição de um grupo metil ao carbono 5 da citosina, principalmente em sítios de dinucleotídeos CpG, é o evento epigenético mais frequentemente

estudado. Dinucleotídeos CpG não estão distribuídos aleatoriamente no genoma. Em vez disto, eles estão mais concentrados em regiões pouco frequentes do DNA conhecidas como “ilhas CpG” (Ioshikhes & Zhang, 2000), mais da metade das quais está localizada em conhecidas regiões promotoras de genes (Weber et al., 2007). Geralmente, níveis elevados de metilação do DNA em regiões promotoras estão associados com a diminuição da expressão gênica e vice-versa, mas esta relação não é sempre desta forma (Jones et al., 2013).

Nos últimos anos, um grande número de trabalhos sobre o papel dos fenômenos epigenéticos na expressão gênica e no fenótipo trouxe enormes progressos em outros campos, como a epigenética do câncer (revisado em Esteller, 2007), graças, em parte, aos avanços significativos de tecnologias moleculares. Por outro lado, ainda sabemos relativamente pouco sobre a extensão e o significado da variação epigenética em interações patógeno-hospedeiro.

Interações patógeno-hospedeiro estão entre os sistemas mais plásticos e dinâmicos da natureza. Para lidar com as restrições seletivas impostas por seus hospedeiros, muitos patógenos desenvolveram níveis incríveis de plasticidade fenotípica em suas histórias evolutivas (Reece et al., 2009). Da mesma forma, o fenótipo do hospedeiro é rapidamente alterado pela presença de um patógeno (Poulin & Thomas, 2008). Além disso, adaptações entre hospedeiros e patógenos ocorrem frequentemente em períodos evolutivamente curtos de tempo para considerar o papel exclusivo das modificações genéticas, tais como polimorfismos e recombinações, como os mecanismos responsáveis por estas adaptações (Rando & Verstrepen, 2007). Neste sentido, modificações epigenéticas podem fornecer uma fonte alternativa de ação rápida, reversível, e prontamente disponível de variação fenotípica que pode ser diretamente regulada pelas pressões de

seleção do hospedeiro e do patógeno (Rando & Verstrepen, 2007; Bonduriansky & Day, 2009).

O campo de mudanças epigenéticas induzidas por microorganismos em células hospedeiras está começando a ser explorado (Minarovits, 2009; Gomez-Díaz et al., 2012; Lang et al., 2012). Recentemente, modificações epigenéticas induzidas por microrganismos em células hospedeiras surgiram como um mecanismo através do quais organismos patogênicos intracelulares, tais como vírus e bactérias, manipulam processos do hospedeiro para favorecer a sua sobrevivência intracelular (Minarovits, 2009; Gomez-Díaz et al., 2012; Silmon de Monerri & Kim, 2014). Alterações na metilação do DNA de macrófagos em resposta a protozoários intracelulares permanecem pouco estudadas e conhecidas, e a inibição permanente da resposta imune inata poderia ser explicada pelas alterações do epigenoma da célula hospedeira.

A *Leishmania* tem um ciclo de vida bastante complexo, geralmente alternando entre o inseto vetor e o hospedeiro mamífero. Dentro do hospedeiro mamífero, a *Leishmania* infecta os macrófagos, células que desempenham um papel crítico na regulação do sistema imune e na defesa do hospedeiro (Moradin & Descoteaux, 2012). Estes protozoários evoluíram para escapar dos mecanismos de defesa desta célula através da inibição da ativação dos macrófagos, o que permite a replicação do patógeno e sua sobrevivência (Gregory & Olivier, 2005; Dogra et al., 2007; Bhardwaj et al., 2010; Liu & Uzonna, 2012). Por exemplo, a ativação de moléculas e vias de sinalização essenciais, tais como PKC, JAK/STAT, MAPK, NF-kB, assim como o fator de transcrição AP-1, é desativada pela infecção por *Leishmania* (Shio et al., 2012). Em adição, moléculas como a SHP-1 são ativadas durante a infecção, causando a inativação de JAK2 nos macrófagos (Shio et al., 2012). Assim, a *Leishmania* evoluiu diversas estratégias para

inibir a ativação macrofágica, a capacidade de apresentar antígenos na sua superfície, assim como para interferir a comunicação dos macrófagos com outras células do sistema imune adaptativo (Shio et al., 2012).

Mais recentemente, um estudo identificou 443 sítios CpG com alterações na metilação correlacionadas com a infecção por *Leishmania donovani* (Marr et al., 2014). Muitos destes sítios CpG diferencialmente metilados são relacionados com genes cujas funções são modificadas durante a infecção por *Leishmania*. Isto inclui genes que codificam proteínas envolvidas em vias de sinalização, como a via de sinalização JAK/STAT (Shio et al., 2012), sinalização de cálcio (Olivier et al., 1992), sinalização MAPK (Shadab & Ali, 2011), sinalização Notch (Auderset et al., 2012) e sinalização mTOR (Jaramillo et al., 2011), assim como proteínas envolvidas na adesão celular, como a integrina-1 beta (Pinheiro et al., 2006), e proteínas envolvidas em alterações na fosforilação oxidativa do hospedeiro (Rabhi et al., 2012). Estes resultados fornecem forte suporte para um novo paradigma nas respostas hospedeiro-patógeno, onde, acerca da infecção, o patógeno induz mudanças epigenéticas no genoma da célula hospedeira, resultando na regulação negativa da imunidade inata, permitindo a sobrevivência do patógeno e sua replicação dentro do macrófago.

Finalmente, estas observações podem ter implicações significativas no que diz respeito à terapia, uma vez que a metilação é um processo epigenético reversível. Devido a esta reversibilidade, a regulação epigenética é teoricamente passível de intervenção (Verma & Srivastava, 2002). Assim, um melhor entendimento do papel da epigenética na patogênese da leishmaniose pode conduzir ao desenvolvimento de novas ferramentas terapêuticas.

## IV.5. REMODELAMENTO TECIDUAL NA LEISHMANIOSE TEGUMENTAR

A pele é o maior órgão do corpo humano. Ela forma uma barreira eficaz entre o corpo e o ambiente externo, e protege o corpo da desidratação e de outras agressões ambientais. Hospedeiros mamíferos estão em constante risco de infecção causada por agentes patogênicos, tais como vírus, bactérias, fungos e parasitas. A defesa do hospedeiro contra estes patógenos requer uma resposta inflamatória bem regulada e marcada pela migração de leucócitos para o local da infecção, destruição do microrganismo, resolução da inflamação, e, finalmente, cura das lesões e reparo da arquitetura tecidual. Em particular, quando ocorre uma lesão na pele, uma série de eventos dinâmicos começa imediatamente. O reparo de lesões cutâneas pode ser dividido em uma série de fases que se sobrepõem, incluindo a resposta inflamatória, formação de tecido de granulação, e o remodelamento da matriz (Li et al., 2007; Reinke & Sorg, 2012).

A pele é essencialmente composta por células, principalmente fibroblastos, células endoteliais e queratinócitos, e pela matriz extracelular. Diversos estudos têm mostrado que o remodelamento não regulado da matriz extracelular está associado com condições patológicas e pode exacerbar a progressão de doenças. Por exemplo, a deposição anormal de matriz extracelular é observada em câncer e fibrose (Frantz et al., 2010), e, por outro lado, sua degradação excessiva está ligada ao desenvolvimento de osteoartrite (Zhen & Cao, 2014).

Após ser inoculada na pele do hospedeiro mamífero, a forma promastigota de *Leishmania* é exposta ao microambiente da derme, permanecendo aí até o primeiro contato com o macrófago (Lira et al., 1997; McGwire et al., 2003) ou outra célula hospedeira, e,

assim, interage com diferentes componentes da matriz extracelular (Ghosh et al., 1999). Assim, um delicado balanço entre a patologia do tecido e o controle da infecção determina o desfecho clínico observado na leishmaniose tegumentar. Apesar da importância da resposta imune ter sido bem estabelecida na eliminação da infecção por *Leishmania* (Carvalho et al., 2012), a interação do parasita com a matriz extracelular e os mecanismos envolvidos no remodelamento tecidual ainda são pouco compreendidos.

Nos mamíferos, a matriz extracelular é composta por aproximadamente 300 proteínas, incluindo os colágenos (Hynes & Naba, 2012). Os colágenos compreendem o principal componente estrutural e a maior concentração de proteínas da matriz extracelular. Os dois tipos de colágeno predominantes na cura de lesões são os colágenos do tipo 1 e do tipo 3. Na pele normal, as fibras de colágeno são compostas por ambas, com o colágeno do tipo 3 compreendendo aproximadamente 20% do total (Wolfram et al., 2009). Esta razão aumenta de 20% para 50% nas fases iniciais da cura (Hayakawa et al., 1979), e retorna ao nível normal durante o processo de cicatrização. Assim, este resultado sugere um importante papel do aumento da quantidade de colágeno do tipo 1 em relação ao colágeno do tipo 3 durante o processo de reparo tecidual.

Recentemente, foi demonstrado que promastigotas de *Leishmania amazonensis* interagem com o colágeno do tipo 1, e esta interação resulta em grandes modificações na organização das fibras de colágeno (Petropolis et al., 2014). Ainda de acordo com este último estudo, os resultados apresentados sugerem que promastigotas de *Leishmania amazonensis* liberam proteases que remodelam o ambiente, facilitando a sua migração, e isto aumenta a chance das promastigotas interagirem ativamente com a matriz enquanto “procuram” sua célula hospedeira.

Entre as principais famílias de proteases está a família das metaloproteinases de matriz, que representam as enzimas mais significantes no remodelamento da matriz extracelular (Cawston & Young, 2010). A expressão de metaloproteinases de matriz em pele normal é muito baixa. Após uma lesão cutânea, porém, múltiplas metaloproteinases de matriz são produzidas durante o processo de cicatrização, incluindo a MMP-1 (Gill & Parks, 2008; Toriseva & Kahari, 2009). A MMP-1 é uma colagenase capaz de degradar o colágeno do tipo 1 (Visse & Nagase, 2003). Em resposta à lesão, a expressão de MMP-1 ocorre rapidamente, seguida por um decréscimo gradual, sendo não detectável no momento da re-epitelização completa (Saarialho-Kere et al., 1993).

Ambos, colágeno do tipo 1 e as metaloproteinases de matriz, desempenham um importante papel nas condições fisiológicas normais e patológicas de muitas doenças (Imai et al., 2000; Alexakis et al., 2006; Wynn, 2008; Amalinei et al., 2010). De maneira interessante, foi visto que as expressões dos genes das cadeias alfa 1 (*COL1A1*) e alfa 2 (*COL1A2*) de colágeno do tipo 1 e do gene de metaloproteinase de matriz 1 (MMP1) são mediadas através da regulação do gene *FLI1* (Nakerakanti et al., 2006), gene que interage com a via de TGF-β (*Transforming growth factor-β*). A maioria das células envolvidas na cura de lesões expressa TGF-β1, um fator de crescimento que promove a quimiotaxia de fibroblastos para o local da lesão e desempenha um papel crítico na proliferação de fibroblastos e produção de matriz extracelular (Chen & Davidson, 2005). O TGF-β1, apesar de induzir a expressão de MMP-2, MMP-9 e MMP-13 em fibroblastos, reduz a degradação de colágeno através da inibição da expressão de MMP-1 (Huang et al., 2012; Simon et al., 2012).

Junto com os dados prévios de associação genética do nosso grupo de pesquisa (Castellucci et al., 2011, 2012), estes trabalhos sugerem um importante papel desta via de sinalização na

patogênese da leishmaniose tegumentar americana. **Assim, a nossa hipótese é de que genes da via de sinalização de *FLI1* são importantes biomarcadores de LTA humana causada por *Leishmania braziliensis*.** Dadas evidências de que a *Leishmania* altera os padrões de metilação da célula hospedeira, regulando negativamente os mecanismos desta célula em uma possível tentativa de favorecer a sua sobrevivência intracelular (Marr et al., 2014), **também são admitidas as hipóteses de que a expressão de *FLI1* e de genes sob sua regulação (*COL1A1*, *COL1A2* e *MMP1*) sofrem alteração como resultado da infecção por *Leishmania braziliensis*, e de que a *Leishmania braziliensis* exerce a sua patogênese em parte por mudanças epigenéticas induzidas no DNA da célula hospedeira após a infecção.**

# V. CASUÍSTICA, MATERIAL E MÉTODOS

## V.1. ÁREA DE ESTUDO

Corte de Pedra, uma vila localizada na região sudeste do Estado da Bahia, pertence ao município de Presidente Tancredo Neves, cuja população é de aproximadamente 17.928 habitantes. A área endêmica de Corte de Pedra, no entanto, se estende muito além da vila, cobrindo 20 municípios em uma área total de aproximadamente 9.935 $km^2$ em torno do local onde um Posto de Saúde foi construído na década de 1980, e que se tornou o centro de referência para o diagnóstico e tratamento de leishmaniose na região. Atualmente, 430.347 pessoas estão distribuídas por estas cidades (IBGE), para as quais a principal atividade econômica é a agricultura de subsistência, principalmente o cultivo de cacau, cravo, guaraná, banana, café, pimenta e borracha. A área endêmica de Corte de Pedra é tipicamente uma área de floresta tropical que ao longo dos anos foi reduzida a áreas isoladas de floresta secundária, com as atividades agrícolas proporcionando a principal fonte de renda para a maioria dos seus habitantes. Deste modo, os hábitos ocupacionais e domésticos destes indivíduos, que envolvem o trabalho nas fazendas e casas construídas em clareiras abertas na mata, aumentaram a exposição à infecção por *Leishmania braziliensis*.

## V.2. COLETA DE AMOSTRAS PARA O ESTUDO DE ASSOCIAÇÃO GENÉTICA E EXTRAÇÃO DE DNA

Para os estudos de associação genética, dois grupos de famílias foram coletados durante dois períodos de estudo, 2000-2004 e 2008-2010, conforme descrito anteriormente (Castellucci et al., 2006, 2010, 2011, 2012). A coleta de amostras para o primeiro grupo foi baseada no recrutamento de casos índices de LM a partir de fichas clínicas do Posto de Saúde de Corte de Pedra, e busca ativa para identificar e coletar todos os outros membros da família, incluindo aqueles com LC ativa ou passada. Este primeiro recrutamento resultou em uma coleção de amostras de 168 famílias nucleares, com 250 casos de LC e 87 casos de LM. A coleta de amostras para o segundo grupo de famílias foi baseada principalmente em casos incidentes de LC ou LM apresentados no Posto de Saúde, com acompanhamento das famílias para coleta de pais e irmãos afetados, e irmãos não afetados se um ou ambos os pais estivessem ausentes. Esta coleção forneceu amostras de 157 famílias nucleares, com 402 casos de LC e 39 casos de LM, conforme mostrado na Quadro 1.

Sangue (8 mL) foi coletado através de punção venosa em tubos contendo citrato ácido dextrose (ACD) (Becton Dickinson) após assinatura do termo de consentimento livre e esclarecido. O DNA genômico foi obtido utilizando o método de *salting-out* com Proteinase K, como descrito por Sambrook e colaboradores (Sambrook et al., 1989), e estocado a -20°C até posterior genotipagem. A concentração e a integridade das amostras foram determinadas por medidas de densidade ótica (260 e 280 nm) utilizando o L-Quant (Loccus Biotecnologia).

## V.3. ESPECIMES DE BIÓPSIAS E EXTRAÇÃO DE DNA E RNA

Para o estudo de expressão dos genes *FLI1*, *COL1A1*, *COL1A2* e *MMP1* e do padrão de metilação de ilhas CpG da região promotora do gene *FLI1* em biópsias de pele normal e de lesões de leishmaniose cutânea, a coleta de amostras foi baseada no recrutamento de casos novos de leishmaniose cutânea apresentados no Posto de Saúde de Corte de Pedra, e que ainda não tinham iniciado o tratamento. As biópsias de pele normal foram obtidas dos mesmos pacientes de onde foram realizadas as biópsias de lesão de LC. Estas biópsias foram realizadas utilizando *punch* de 4 milímetros, e preservadas em RNAltater (Ambion) até posterior extração de DNA e RNA. Após intensa maceração mecânica, RNA e DNA foram extraídos para os estudos de expressão gênica e regulação epigenética respectivamente. O RNA foi extraído utilizando o PureLink RNA Mini Kit (Ambion), e a extração de DNA foi realizada por meio do QIAmp DNA Mini kit (Qiagen). Estas extrações seguiram precisamente os protocolos fornecidos pelos fabricantes. A concentração e a integridade de RNA e DNA foram determinadas utilizando medidas de densidade ótica (260 nm e 280 nm), e, finalmente, os ácidos nucléicos foram estocados a -70°C.

## V.4. CULTURA DE MACRÓFAGO E EXTRAÇÃO DE DNA E RNA

Para o estudo de expressão dos genes *FLI1*, *COL1A1*, *COL1A2* e *MMP1* e do padrão de metilação de ilhas CpG da região promotora do gene *FLI1* em macrófagos de pacientes com leishmaniose cutânea, a coleta de amostras também foi baseada no recrutamento de casos novos apresentados no Posto de Saúde de Corte de Pedra, e que ainda não tinham iniciado o tratamento.

Células mononucleares do sangue periférico (PBMCs) foram isoladas a partir de sangue venoso heparinizado (60 mL) pelo gradiente de centrifugação de Ficoll-Hypaque (GE Healthcare Bio-Sciences AB). Após duas lavagens com 0,9% de NaCl, estas células foram ressuspensas em RPMI-1640 (Gibco) suplementado com 10% de soro fetal bovino, 10 UI/mL de penicilina e 100 µg/mL de estreptomicina, e incubadas em placas de teflon por 6 dias a 37°C e 5% de $CO_2$ para maturação dos monócitos em macrófagos. Após este período de incubação, os macrófagos derivados de PBMC foram ajustados para 1 x $10^6$ células/mL e plaqueados em placas de 12 poços em duplicadas. Neste momento, algumas culturas foram tratadas com IL-6 humana recombinante (20 ng/mL) (Gibco). Posteriormente, as células foram incubadas por 24 horas para aderência das células na placa, e, após esta nova incubação, os poços foram lavados com HBSS para retirar as células que não aderiram.

As culturas de macrófagos derivados de PBMC foram infectadas por promastigotas vivas de *Leishmania braziliensis* na fase estacionária na razão de 5 parasitas para cada célula. Culturas não infectadas foram utilizadas como controle. Seguindo uma nova incubação de 2 horas, as culturas foram lavadas com HBSS para remoção de *Leishmania braziliensis* livres. Após cada ponto de tempo (3, 24, 48 e 72 horas) as células foram recuperadas para

posterior extração de RNA e DNA. As células utilizadas para extração de DNA foram recuperadas em solução de congelamento (50% soro fetal bovino, 40% RPMI-1640 e 10% DMSO) e estocadas e -70°C. A duplicada restante utilizada para extração de RNA foi recuperada em TRIzol (Invitrogen) e também estocada a -70°C. Por fim, RNA e DNA foram extraídos utilizando os mesmos kits descritos acima e utilizados para o estudo de biópsias.

## V.5. GENOTIPAGEM DE POLIMORFISMOS DE NUCLEOTÍDEOS SIMPLES

A genotipagem de polimorfismos de nucleotídeos simples (SNPs) foi realizada utilizando ensaios pré-desenhados de PCR qualitativo pelo método de TaqMan® (Life Technologies) para polimorfismos no genes *COL1A1* (rs1061237, rs2586488 e rs2075554), *COL1A2* (rs388625 e rs1177020) e *MMP1* (rs5854, 470747 e 7125062), como apresentado no Quadro 2. Os SNPs foram selecionados de forma pragmática com base em sua utilização prévia como *tagging* SNPs em outros estudos de associação (Metlapally et al., 2009; Erdei et al., 2013), disponibilidade de ensaios pré-desenhados de PCR qualitativo validados, e frequência alélica mínima (FAM) ≥ 0,15 para as populações Caucasiana (CEU) e Africana (Yri) do projeto HapMap. Estas duas populações de referência foram selecionados para simular ao máximo possível a mistura étnica da população da Bahia. O programa PECHECK (O'Connell & Weeks, 1998) foi utilizado para determinar inconsistências mendelianas dentro das famílias e os genótipos para estes indivíduos foram definidos como zero para as análises.

## V.6. EXPERIMENTOS DE EXPRESSÃO GÊNICA

Utilizando RNA previamente extraído de biópsias de pele e de culturas de macrófagos derivados de PBMC, as reações de transcrição reversa e a obtenção de DNA complementar (cDNA) foram realizadas utilizando o kit comercialmente disponível High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), seguindo precisamente as orientações do fabricante. A expressão gênica foi, então, avaliada pela técnica de PCR em tempo real, utilizando o equipamento 7500 Real Time PCR System (Applied Biosystems), a partir de reações preparadas como descrito a seguir: 2 µl de cDNA, 5 µl de TaqMan Universal Mastermix, 2,5 µl de água livre de DNAse-RNAse e 0,5 µl de ensaio em um volume final de 10 µl. Os ensaios contendo primers e sondas específicas para *FLI1* (Hs00956709_m1), *COL1A1* (Hs00164004_m1), *COL1A2* (Hs01028972_m1) e *MMP1* (Hs00899658_m1) foram pré-desenhados pela Applied Biosystems. As condições de amplificação obedeceram a seguinte ciclagem (I) 5 minutos a 50˚C, (II) 10 minutos a 95˚C, (III) 15 segundos a a 95˚C e (IV) 1 minuto a 60˚C, sendo os ciclos 3 e 4 repetidos 40 vezes. Todas as amostras foram utilizadas em duplicatas, e o resultado da expressão foi calculado pela média das duas duplicatas. O nível de mRNA foi normalizado para o nível de β-actina (Hs01060665_g1) correspondente para cada amostra a fim de controlar a variabilidade entre as amostras.

Por fim, a comparação dos padrões de expressão dos genes de interesse foi realizada utilizando o método de Ct comparativo, através da equação $2^{-\Delta\Delta Ct}$, onde ΔCt corresponde ao valor de Ct do gene alvo subtraído do Ct do gene endógeno, e ΔΔCt corresponde ao valor de ΔCt de cada condição subtraído da mediana do ΔCt do grupo controle (MEIO no caso dos macrófagos, e PELE NORMAL

no caso das biópsias), conforme descrito previamente (Livak & Schmittgen, 2001).

## V.7. EXPERIMENTOS DE METILAÇÃO

Para avaliação do padrão de metilação de ilhas CpG da região promotora do gene *FLI1* em biópsias de pele e em culturas de macrófagos derivados de PBMC, o DNA foi obtido como descrito em tópico anterior. A digestão do DNA foi realizada utilizando o EpiTect Mehtyl II DNA Restriction Kit (Qiagen). As reações eram preparadas utilizando as enzimas do kit (*Methylation-sensitive enzyme A* e *Methylation-dependent enzyme B*) conforme instruções do fabricante, e incubadas por 6 horas a 37˚C. Após esta incubação, as enzimas eram inativadas a 65˚C por 20 minutos e as amostras foram estocadas a -20˚C até a reação de PCR ocorrer. Seguindo esta etapa, os produtos da digestão foram utilizados para determinar a porcentagem de ilhas CpG da região promotora do gene *FLI1* metilada, utilizando a técnica de PCR em tempo real através de um ensaio comercialmente disponível pela Qiagen (EPHS102855-1A). As reações de PCR em tempo real foram estabelecidas para cada um dos produtos da digestão seguindo o protocolo fornecido pelo fabricante. A ciclagem foi realizada no equipamento 7500 Real Time PCR System (Applied Biosystems), obedecendo as seguintes condições: (I) 10 minutos a 95˚C, 3 ciclos de (II) 30 segundos a 99˚C, (III) 1 minuto a 72˚C, e 40 ciclos de (IV) 15 segundos a 97˚ (V) 1 minuto a 72˚C. A curva Melting foi utilizada de acordo com instruções do instrumento. Após o fim desta ciclagem, os valores de Ct foram exportados do software do instrumento para uma planilha de Microsoft Excel. Por fim, análise dos dados foi realizada utilizando o EpiTect Methyl II PCR Array

Microsoft Excel, disponível em www.sabiosciences.com/dna_methylation_data_analysis.php. Este planilha realiza automaticamente todos os cálculos baseados nos valores de Ct para determinar o status de metilação do DNA.

## V.8. ANÁLISE ESTATÍSTICA

Para avaliação do polimorfismo dos genes candidatos (*COL1A1*, *COL1A2* e *MMP1*) e sua possível associação com a leishmaniose tegumentar americana na população de Corte de Pedra, Bahia, testes de associação baseados em famílias (FBAT) foram conduzidos sob os modelos de herança aditivo, dominante ou genótipo, utilizando a bandeira –o para considerar ambos, filhos afetados e não afetados (Laird et al., 2000). Indivíduos com LM foram definidos como fenótipo desconhecido na análise do fenótipo LC isolado. O valor de P necessário para alcançar significância estatística, aplicando correção de Bonferroni para levar em consideração múltiplos testes, foi P = 0,006 (= 0,05/8 SNPs testados).

Aproximações de poder do teste de desequilíbrio de transmissão (TDT) (Knapp, 1999) foram utilizadas para determinar o poder das famílias para detectar associações genéticas, e o desequilíbrio de ligação (DL) entre os polimorfismos de nucleotídeos simples analisados foi determinado utilizando o Haploview v.4.2 (Barrett et al., 2005).

Para o estudo de expressão dos genes *FLI1*, *COL1A1*, *COL1A2* e *MMP1* e do padrão de metilação de ilhas CpG da região promotora do gene *FLI1* em biópsias de pele e em culturas de macrófagos derivados de PBMC, a significância estatística foi

avaliada por Mann-Whitney para comparação entre dois grupos ou pela análise de variância de Kruskall Wallis para comparação entre múltiplos grupos. Valores de P menores que 0,05 foram consideradas estatisticamente significantes, e, para todas estas análises estatísticas, o software GraphPad Prism 5 foi utilizado.

## V.9. APROVAÇÃO EM COMITÊ DE ÉTICA EM PESQUISA

O presente estudo conta com aprovação do Comitê de Ética em Pesquisa do Complexo Hospitalar Universitário Professor Edgard Santos da Universidade Federal da Bahia, sob o cadastro 22/2012, e da Comissão Nacional de Ética em Pesquisa (CONEP) - 1258513.1.000.5537. Todos os pacientes convidados a participar do estudo e que aceitaram o convite assinaram o Termo de Consentimento Livre e Esclarecido.

## VI. ARTIGOS

"Wound healing genes and susceptibility to cutaneous leishmaniasis in Brazil: role of *COL1A1*". *Infection, Genetics and Evolution* 30: 225-9, 2015.

Infection, Genetics and Evolution 30 (2015) 225–229

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Short communication

# Wound healing genes and susceptibility to cutaneous leishmaniasis in Brazil: Role of *COL1A1*

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ARTICLE INFO

*Article history:*
Received 23 October 2014
Received in revised form 22 December 2014
Accepted 27 December 2014
Available online 3 January 2015

*Keywords:*
COL1A1
Gene analysis
Cutaneous leishmaniasis
Wound healing

ABSTRACT

Previous studies have demonstrated a role for wound healing genes in resolution of cutaneous lesions caused by *Leishmania spp.* in both mice and humans, including the gene *FLI1* encoding Friend leukemia virus integration 1. Reduction of Fli1 expression in mice has been shown to result in up-regulation of collagen type I alpha 1 (*Col1a1*) and alpha 2 (*Col1a2*) genes and, conversely, in down-regulation of the matrix metalloproteinase 1 (*Mmp1*) gene, suggesting that *Fli1* suppression is involved in activation of the profibrotic gene program. Here we examined single nucleotide polymorphisms (SNPs) in these genes as risk factors for cutaneous (CL) and mucosal leishmaniasis (ML), and leishmaniasis *per se*, caused by *L. braziliensis* in humans. SNPs were genotyped in 168 nuclear families (250 CL; 87 ML cases) and replicated in 157 families (402 CL; 39 ML cases). Family-based association tests (FBAT) showed the strongest association between SNPs rs1061237 (combined $P = 0.002$) and rs2586488 (combined $P = 0.027$) at *COL1A1* and CL disease. This contributes to our further understanding of the role of wound healing in the resolution of CL disease, providing potential for therapies modulating COL1A1 via drugs acting on FLI1.



## 1. Introduction

American tegumentary leishmaniasis (ACL), caused by protozoan parasites of the genus *Leishmania*, is a major health problem in many low income countries. The infection is associated with a broad spectrum of clinical phenotypes, resulting from both environmental risk factors and a combination of the host's immune and genetic background. In the last few years we have shown that polymorphisms at genes associated with wound healing and tissue repair are important risk factors for cutaneous leishmaniasis (CL) caused by *Leishmania braziliensis* (Castellucci et al., 2012, 2011). Thus, the *FLI1* gene, initially identified and mapped as a gene controlling susceptibility to CL caused by *L. major* infection in mice (Sakthianandeswaren et al., 2010, 2005), was also associated with development of CL in humans exposed to *L. braziliensis* in Brazil. In addition, our data showed that polymorphisms in other wound healing genes related to *FLI1* function, in particular genes (*TGFB1*, *TGFBR2*, *CTGF*, *SMAD2|3|7*) encoding proteins in the transforming growth factor β (TGF-β) signaling pathway, were risk factors for CL. These data support the hypothesis that the normal physiological regulation of the wound healing process is important in the cure of ACL. Of interest, protective alleles for CL at these loci were often acting as risk alleles for mucosal leishmaniasis (ML) caused by *L. braziliensis* in the same population. This likely reflects the exaggerated pro-inflammatory response associated with ML disease, compared to the measured tumor necrosis factor and interferon-γ responses required to cure CL lesions.

Reduction of Fli1 expression in mice has been shown to result in up-regulation of collagen type I alpha 1 (*Col1a1*) and alpha 2 (Col1a2) genes and, conversely, in down-regulation of the matrix metalloproteinase 1 (*Mmp1*) gene, suggesting that *Fli1* suppression is involved in activation of the profibrotic gene program (Nakerakanti et al., 2006). Both type I collagens and matrix metalloproteinases play an important role in the normal physiological and pathological conditions of many diseases (Alexakis et al., 2006; Amalinei et al., 2010; Imai et al., 2000; Wynn, 2008). Considering the role of these genes in the wound healing response, together with our previous data showing genetic association of their regulator gene *FLI1* with CL in families from Brazil, we

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http://dx.doi.org/10.1016/j.meegid.2014.12.034
1567-1348/

extended our analysis of the *FLI1* pathway to determine whether polymorphisms at *COL1A1*, *COL1A2* or *MMP1* genes could also be involved in the outcome of ACL.

## 2. Materials and methods

### 2.1. Study site, diagnosis and sample collection

Our genetic studies are conducted in a region of rural rain forest, Corte de Pedra, Bahia, Brazil, where *L. braziliensis* is endemic. For host genetic association studies, two family-based cohorts were collected during two study periods, 2000–2004 and 2008–2010, as reported previously along with details of epidemiology and clinical phenotypes of disease (Castellucci et al., 2011, 2010, 2006). Sample collection for the first cohort was based on ascertainment of index cases of ML from medical records of the Corte de Pedra Public Health Post, and active follow-up to identify and collect all other family members, including those with current or past CL disease. This provided DNA samples (Table 1) from 168 nuclear families that contain 250 CL cases and 87 ML cases. Sample collection for the second cohort was based primarily on incident cases of CL or ML presenting to the health post, with family follow-up to acquire samples from parents and affected siblings, and unaffected siblings if one or both parents were missing. This provided DNA samples (Table 1) from 157 nuclear families that contain 402 CL cases and 39 ML cases. The characteristics of the two cohorts have been described in detail elsewhere (Castellucci et al., 2011), including diagnostic criteria for ML and CL disease.

### 2.2. Sample collection and DNA extraction

Blood (8 ml) was taken by venipuncture and collected into dodecyl citrate acid (DCA)-containing vacutainers (Becton Dickinson). Genomic DNA was prepared using the proteinase K and salting-out method (Sambrook et al., 1989).

### 2.3. Genotyping

Genotyping was performed using pre-designed Taqman® qPCR assays (Life Technologies) for polymorphisms at *COL1A1* (rs1061237, rs2586488, rs2075554), *COL1A2* (rs388625, rs11770203) and *MMP1* (rs5854, rs470747, rs7125062) as presented in Table 2. SNPs were selected pragmatically on the basis of prior use as tagging SNPs in other disease association studies (Erdei et al., 2013; Metlapally et al., 2009), availability of validated predesigned Taqman® qPCR assays, and MAF ⩾ 0.15 for both CEU (Caucasian) and YRI (African) HapMap populations. These two reference populations were selected to mimic as closely as possible ethnic admixture in the population of Bahia. Analysis of linkage disequilibrium using Haploview v4.2 (Barrett et al., 2005) for SNPs with a MAF ⩾ 0.15 for the CEU HapMap populations showed that these SNPs tagged >90% of the *COL1A1* gene at $D' > 0.67$ and ~30% cover at $r^2 > 0.58$, >90% of *COL1A2* at $D' > 0.56$ and <20% cover at $r^2 > 0.58$, and >90% of *MMP1* at $D' > 0.65$ and ~30% cover at $r^2 > 0.70$. All SNPs were in Hardy Weinberg Equilibrium in genetically unrelated founders of the families (data not shown). Missingness (i.e., failure to score on Taqman assays) ranged from 0.80% (8/1008 individuals available for genotyping) to 2.5% (25/1008) across the 8 SNPs. PEDCHECK (O'Connell and Weeks, 1998) was used to determine Mendelian inconsistencies within families and genotypes for these individuals were set to zero for analysis.

### 2.4. Statistical analyses

Linkage disequilibrium (LD) was determined using Haploview v4.2 (Barrett et al., 2005). Family-based Association Tests (FBAT) were carried out under additive, dominant or genotype inheritance models using the –o flag to take both unaffected and affected offspring into account (Laird et al., 2000). ML patients were set to unknown phenotype in the analysis of the CL alone phenotype. Nominal *P*-values are presented throughout, i.e., without correction for multiple testing. The *P*-value needed to achieve statistical significance by applying a strict Bonferroni correction to take multiple testing into account was $P = 0.006$ (=0.05/8 SNPs tested). Transmission disequilibrium test power approximations (Knapp, 1999) were used to determine power of the families to detect genetic associations.

## 3. Results

### 3.1. Characteristics of the samples and power to detect association

Table 1 provides details of the samples used in this study. Sample collections made during two different time periods, 2000–2004 and 2008–2010, were well-matched geographically (Castellucci et al., 2012) and demographically (Table 1). Table 2 provides details of SNPs genotyped, and demonstrates that all SNPs were at a MAF ⩾0.25 in this Brazilian population. The *P*-value needed to achieve statistical significance taking multiple testing into account is $P = 0.006$ (=0.05/8 SNPs tested). TDT power approximations (Knapp, 1999) showed that the first (250 CL cases) and second (402 CL cases) family datasets had 68% and 89% power, respectively, to detect an odds ratio ⩾1.5 at $P = 0.01$ for markers with MAF ⩾0.3. The combined 652 trios had 98.7% power to detect association for the same effect size, *P*-value and MAF. Similarly, for leishmaniasis *per se* (i.e., CL + ML), power estimations for 337 pri-

**Table 1**
Characteristics of collections made during the primary (2000–2004) and secondary (2008–2010) sampling periods.

| | Primary sample period | | | Secondary sample period | | |
|---|---|---|---|---|---|---|
| | CL[a] | ML[a] | Leishmaniasis[a] *per se* | CL[a] | ML[a] | Leishmaniasis[a] *per se* |
| No. cases | 250 | 87 | 337 | 402 | 39 | 441 |
| Males | 128 | 60 | 188 | 219 | 24 | 243 |
| Females | 122 | 27 | 149 | 183 | 15 | 198 |
| Age at disease | | | | | | |
| Mean | 19.1 | 30.3 | 22.4 | 21.5 | 26.6 | 21.9 |
| 95% CI | 17.1–21.2 | 25.8–34.7 | 20.3–24.4 | 20.1–22.9 | 20.7–32.4 | 20.6–23.3 |
| No. nuclear families | 168 | – | 168 | 157 | – | 157 |
| Total *N* in families/trios | 767 | – | 767 | 764 | – | 764 |

[a] Unpaired *t* tests with Welch's correction for unequal variances show that there are no significant differences in age range for CL primary vs secondary, ML primary vs secondary, and Leishmaniasis *per se* primary vs secondary.

**Table 2**
Information on the single nucleotide polymorphisms genotyped for *COL1A1*, *COL1A2* and *MMP1* in the Brazilian population.

| Gene/Marker | Location | Physical position (Chr:bp) | Alleles[a] | MAF[b] | Caucasian | African |
|---|---|---|---|---|---|---|
| COL1A2_rs388625 | Intron | 7:94396612 | A/G | 0.48 | 0.48 | 0.42 |
| COL1A2_rs11770203 | Intron | 7:94402145 | G/T | 0.26 | 0.33 | 0.18 |
| MMP1_rs5854 | 3′UTR | 11:102790143 | A/G | 0.25 | 0.35 | 0.19 |
| MMP1_rs470747 | Intron | 11:102790864 | A/G | 0.34 | 0.35 | 0.35 |
| MMP1_rs7125062 | Intron | 11:102792772 | C/T | 0.33 | 0.27 | 0.35 |
| COL1A1_rs1061237 | 3′UTR | 17:50185414 | C/T | 0.30 | 0.31 | 0.42 |
| COL1A1_rs2586488 | Intron | 17:50188065 | A/G | 0.37 | 0.37 | 0.42 |
| COL1A1_rs2075554 | Intron | 17:50196948 | C/T | 0.26 | 0.19 | 0.26 |

[a] Major > minor alleles for this Brazilian population.
[b] Minor allele frequencies (MAF) for this Brazilian population, and for Caucasian (CEU) and African (YRI) populations as indicated.

mary, 441 replication, and 778 combined trios had 91.9%, 91.9%, and 99.6% power, respectively. Power to detect associations with ML disease was low (≤25%) even in the combined dataset.

### 3.2. Association tests for SNPs at COL1A1, COL1A2 and MMP1 as candidate genes for CL disease

No associations were observed between CL disease and SNPs at *COL1A2* or *MMP1* (data not shown) used in this study. This does not rule out the possibility that associations could be uncovered given more dense SNP coverage of these two genes. Results of the *COL1A1* FBAT analysis for the CL clinical phenotype in the primary (Families 101–168), replication (Families 169–325) and combined data set are presented in Table 3. Analysis under an additive model was sufficient to explain the data. SNP rs1061237 showed a trend for association ($P = 0.058$) in the smaller primary family sample, achieved association at nominal $P = 0.018$ in the replication family sample, and attained a *P*-value ($P = 0.002$) in the combined analysis that withstands application of a strict correction factor for the 8 SNPs genotyped. SNP rs2586488 also provided evidence (nominal $P < 0.05$) for association in the larger replication sample and the combined analysis. This is consistent with quite strong LD ($D'$ 0.78; $r^2$ 0.55) between these two SNPs (Fig. 1). In contrast, there was no evidence for associations between CL disease and SNP rs2075554 and CL disease (Table 3), or of LD between this SNP and the two associated SNPs (Fig. 1).

### 3.3. COL1A1 SNPs and CL versus ML disease

No associations were observed between ML disease, or leishmaniasis *per se*, and SNPs at *COL1A2* or *MMP1* (data not shown). The *COL1A1* FBAT analysis for the ML clinical phenotype, and leishmaniasis *per se* (CL + ML disease) in the primary (Families 101–168), replication (Families 169–325) and combined data set are presented in Supplementary Tables 1 and 2, respectively. Of interest, a trend for association (nominal $P = 0.079$) between rs2586488 and ML disease in the replication families (Supplementary Table 1) showed the minor allele as the risk allele, whereas for CL disease (Table 1) the major allele was the risk allele, reminiscent of earlier findings with other wound healing genes (Castellucci et al., 2012, 2011). This contributed to reduction in the strength of associations seen at both rs1061237 and rs2586488 in the analysis of CL + ML as leishmaniasis *per se* (Supplementary Table 2).

## 4. Discussion

Here we provide evidence for association between polymorphic variants at *COL1A1* and susceptibility to CL disease caused by *L.*

**Table 3**
Results of Family-based Association Tests (FBAT) using an additive model of inheritance for associations between tag SNPs at COL1A1 (see Table 2) and CL caused by *L. braziliensis* in Brazil.

| Gene/Marker | Allele | Allele frequency | No. families | Var(S) | *Z* | *P* |
|---|---|---|---|---|---|---|
| *Families 101–168* | | | | | | |
| COL1A1_rs1061237 | C | 0.64 | 64 | 8.07 | 1.90 | 0.058 |
| COL1A1_rs1061237 | T | 0.36 | 64 | 8.07 | −1.90 | 0.058 |
| COL1A1_rs2586488 | G | 0.59 | 64 | 8.70 | 0.74 | 0.462 |
| COL1A1_rs2586488 | A | 0.41 | 64 | 8.70 | −0.74 | 0.462 |
| COL1A1_rs2075554 | C | 0.74 | 56 | 6.14 | 0.87 | 0.382 |
| COL1A1_rs2075554 | T | 0.26 | 56 | 6.14 | −0.87 | 0.382 |
| *Families 169–325* | | | | | | |
| COL1A1_rs1061237 | C | 0.66 | 93 | 6.72 | 2.36 | **0.018** |
| COL1A1_rs1061237 | T | 0.34 | 93 | 6.72 | −2.36 | **0.018** |
| COL1A1_rs2586488 | G | 0.64 | 88 | 5.77 | 2.30 | **0.021** |
| COL1A1_rs2586488 | A | 0.36 | 88 | 5.77 | −2.30 | **0.021** |
| COL1A1_rs2075554 | C | 0.75 | 78 | 6.44 | 0.61 | 0.542 |
| COL1A1_rs2075554 | T | 0.25 | 78 | 6.44 | −0.61 | 0.542 |
| *Combined analysis* | | | | | | |
| COL1A1_rs1061237 | C | 0.66 | 157 | 14.84 | 3.07 | **0.002** |
| COL1A1_rs1061237 | T | 0.34 | 157 | 14.84 | −3.07 | **0.002** |
| COL1A1_rs2586488 | G | 0.63 | 152 | 14.66 | 2.21 | **0.027** |
| COL1A1_rs2586488 | A | 0.37 | 152 | 14.66 | −2.21 | **0.027** |
| COL1A1_rs2075554 | C | 0.75 | 134 | 12.61 | 1.08 | 0.279 |
| COL1A1_rs2075554 | T | 0.25 | 134 | 12.61 | −1.08 | 0.279 |

No. families = number of families informative for the FBAT analysis; V(S) is the variance.
A positive *Z*-score indicates association with disease; a negative *Z*-score indicates the non-associated or protective allele.
Bold indicates nominal $P < 0.05$.

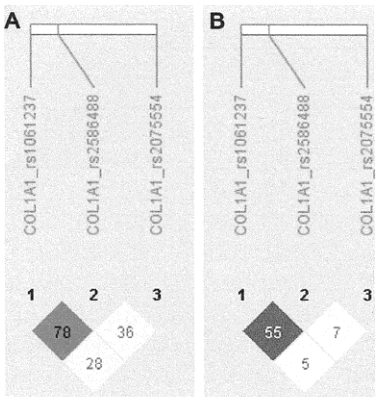


**Fig. 1.** LD patterns for D′ (A) and $r^2$ (B) were determined in Haploview software v4.2 (Barrett et al., 2005) for the 3 *COL1A1* SNPs used in the association analyses. D′ values and confidence levels (LOD) are represented as red for D′ = 1, LOD > 2 (none present); shades of pink for varying D′, LOD < 0.2; white for D′ < 1, LOD < 2 (none present). $r^2$ values are represented white for $r^2 = 0$, with intermediate values for $0 < r^2 < 1$ indicated by shades of grey. The numbers within the squares represent the D′ or $r^2$ scores for pairwise LD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

*braziliensis* in Brazil. This adds to our previous demonstration of roles for wound healing genes in determining the outcome of CL disease, supporting prior murine studies that had highlighted a role for wound healing pathways in the resolution of cutaneous forms of leishmaniasis (Sakthianandeswaren et al., 2005, 2009).

Collagen type I is a predominant extracellular matrix component of the fibrotic lesion. Extracellular matrix remodeling is a complex and tightly regulated process that occurs during wound repair. In many pathological conditions the balance between extracellular matrix synthesis and degradation is disrupted, leading to abnormal remodeling (Border et al., 1996; Curran and Murray, 1999; Forget et al., 1999; Friedman, 1993; Malemud and Goldberg, 1999; Trojanowska et al., 1998; Vincenti et al., 1994). Leishmaniasis wound repair depends on a balanced immune response, as well as the cooperation of matrix elements and collagens. In addition, previous work suggests that type I collagen remodeling by promastigotes could play an important role during the infection process (Larsen et al., 2006; Petropolis et al., 2014; Stamenkovic, 2003). Even though the importance of the immunological response has been well established in the elimination of *Leishmania* infection and healing of resulting lesions, the mechanisms involved in skin damage and ulcer resolution needs to be better understood. For example, it needs to be clarified what happens after infection if the promastigotes pass through the dermis extracellular matrix, remaining there until the first contact with macrophages or other potential host cells (Lira et al., 1997; McGwire et al., 2003). Results presented here provide support for a role for polymorphism at *COL1A1*, which lies within the *FLI1* network, in determining the outcome of *L. braziliensis* infection. This is consistent with our previous demonstration (Castellucci et al., 2011) of association between SNPs at *FLI1* that are in strong linkage disequilibrium with functional elements known to influence FLI1 expression through epigenetic (CpG motifs) and enhancer activities. Epigenetic repression of the *FLI1* gene is associated with enhanced *COL1A1* expression (Wang et al., 2006), and there is a complex interplay between FLI1 and the TGF-β signaling pathway in regulating collagen deposition and fibrosis during the wound healing process. Further functional work will be required to evaluate the mechanisms of expression of these genes either in biopsies or cells from patients infected by *L. braziliensis* to determine their potential as therapeutic targets.

In brief, our data contribute to the dissection of an important pathway associated with development of CL, identifying host molecular biomarkers for this disease. This contributes to our further understanding of the role of wound healing in the resolution of CL disease, providing potential for therapies modulating COL1A1 via drugs acting on FLI1.

## Authors' contributions

LC, LA and JO carried out the field collection and preparation of the samples. LC supervised the laboratory work and statistical analyses. LA performed the genotyping. LA and LC undertook the statistical analyses, interpretation of the data, and preparation of the draft manuscript. LHG participated in the field collection of data. EMC helped conceive the study, initial selection of cases from the health post, and provided the logistical support to make the study possible. JMB participated in the design of the study, conceived the specific hypothesis to be tested, made the final interpretation of the data, and finalized preparation of the manuscript. All authors read and approved the final manuscript.

## Funding

This work was supported by the NIH Grant AI 3639 and CNPq/INCT.

## Acknowledgments

We acknowledge Ednaldo Lago for the support in the field work in Brazil.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.12.034.

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“Host genetic factors in American cutaneous leishmaniasis: a critical appraisal of studies conducted in an endemic area of Brazil”. *Memórias do Instituto Oswaldo Cruz* 109: 279-88, 2014.

# Host genetic factors in American cutaneous leishmaniasis: a critical appraisal of studies conducted in an endemic area of Brazil

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*American cutaneous leishmaniasis (ACL) is a vector-transmitted infectious disease with an estimated 1.5 million new cases per year. In Brazil, ACL represents a significant public health problem, with approximately 30,000 new reported cases annually, representing an incidence of 18.5 cases per 100,000 inhabitants. Corte de Pedra is in a region endemic for ACL in the state of Bahia (BA), northeastern Brazil, with 500-1,300 patients treated annually. Over the last decade, population and family-based candidate gene studies were conducted in Corte de Pedra, founded on previous knowledge from studies on mice and humans. Notwithstanding limitations related to sample size and power, these studies contribute important genetic biomarkers that identify novel pathways of disease pathogenesis and possible new therapeutic targets. The present paper is a narrative review about ACL immunogenetics in BA, highlighting in particular the interacting roles of the wound healing gene* FLI1 *with interleukin-6 and genes* SMAD2 *and* SMAD3 *of the transforming growth factor beta signalling pathway. This research highlights the need for well-powered genetic and functional studies on* Leishmania braziliensis *infection as essential to define and validate the role of host genes in determining resistance/susceptibility regarding this disease.*

Key words: genetic biomarkers - American cutaneous leishmaniasis - wound healing genes

American cutaneous leishmaniasis (ACL) is a complex, multifactorial disease that results from environmental factors such as parasite polymorphism, phlebotomine sandfly components, as well as the host's immune and genetic background. In northeastern Brazil, the endemic area of Corte de Pedra registers the highest incidence of ACL in the state of Bahia (BA). While the incidence of CL in BA varies from 1.5-3.2 per 10,000, the incidence in Corte de Pedra varies from 15-35 per 10,000. The predominant causative species is *Leishmania braziliensis*, which in most cases leads to CL, characterised by one or more ulcers with raised borders, most frequently located on the upper and lower extremities, but also on the head, face and trunk (Barral-Netto et al. 1997). Although CL is a self-limiting disease, approximately 3-5% of subjects infected with *L. braziliensis* will eventually develop mucosal leishmaniasis (ML) or disseminated leishmaniasis (DL), considered now an emerging form of the disease in the area. Fig. 1 demonstrates these different clinical phenotypes, highlighting the sometimes disfiguring nature of the disease and the need to understand the variable disease pathology.

doi: 10.1590/0074-0276140028
Financial support: NIH (AI 30639)
LC was supported by NIH/FIC 1 D43 TW007127-01 (UK).
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Received 23 January 2014
Accepted 7 April 2014

A number of studies on ACL conducted in Corte de Pedra in the past 30 years have contributed enormously to the knowledge of ACL epidemiology and immune response (Carvalho et al. 2012, de Oliveira & Brodskyn 2012). Particularly in the last decade, a number of studies evaluating both parasite and host polymorphisms have demonstrated that genetic factors are associated to different clinical forms, revealing relevant biomarkers to understanding the disease pathogenesis (Schriefer et al. 2004, Castellucci et al. 2006, 2010, 2011, 2012, Ramasawmy et al. 2010, Queiroz et al. 2012). Here we present a narrative review of host genetic studies of ACL conducted in Corte de Pedra over the last decade. Although there are a number of studies evaluating candidate genes in ACL (Table I), no genome-wide association studies have so far been reported that would provide a comprehensive map of genetic risk factors for this disease. This is in contrast to host genetic analysis of visceral leishmaniasis (VL), for which a well-powered genome-wide association study was recently reported (Fakiola et al. 2013). Here we will focus on genetic susceptibility to ACL, beginning with the demonstration of familial aggregation of ACL disease in Corte de Pedra that led to analysis of specific candidate genes arising both from our knowledge of immune responses to human *L. braziliensis* infection, and through consideration of wound healing genes that was inspired initially by studies in mice (Sakthianandeswaren et al. 2005, 2009, 2010). These data are further discussed in relation to studies of genetic susceptibility to CL in other geographic regions, as summarised along with all published (Barbier et al. 1987, Lara et al. 1991, Petzl-Erler et al. 1991, El-Mogy

et al. 1993, Cabrera et al. 1995, Karplus et al. 2002, Olivo-Diaz et al. 2004, Castellucci et al. 2006, 2010, 2011, 2012, Kamali-Sarvestani et al. 2006, Matos et al. 2007, Salhi et al. 2008, Ajdary et al. 2010, 2011, Ramasawmy et al. 2010, Samaranayake et al. 2010, Oliveira et al. 2011, Fernández-Figueroa et al. 2012, Covas et al. 2013) data on susceptibility to CL in Table I. One factor that affects interpretation of all of these studies is the issue of sample size and power, which we will return to in our concluding remarks.

*The endemic site of Corte de Pedra* - Corte de Pedra, a village located in the southwestern region of BA, belongs to the municipality of Presidente Tancredo Neves, whose population is approximately 17,928 inhabitants (source: Brazilian Institute of Geography and Statistics). The endemic area of Corte de Pedra, however, extends far beyond the village, covering 20 municipalities in a total area of approximately 9,935 km$^2$ around the site where a Health Post was established in 1980s as a reference centre for the treatment of leishmaniasis in the region. Currently, 430,347 people are distributed across these towns, for which the main economic activity is subsistence farming, particularly the cultivation of cocoa, cloves, guarana, banana, coffee, black pepper and rubber. The endemic area of Corte de Pedra is typically an area of rainforest that over the years has been reduced to isolated areas of secondary forest with agricultural activities providing the main source of income for the majority of its inhabitants. The occupational and domestic habits of these individuals, which involve work on farms and homes built in clearings in the woods, have increased the population's exposure to *L. braziliensis* infection. From 2007-2012, 7,093 cases of ACL were recorded in the region, with 6,747 (95%) cases of CL, 138 (2%) cases of ML and 208 cases (3%) cases of DL.

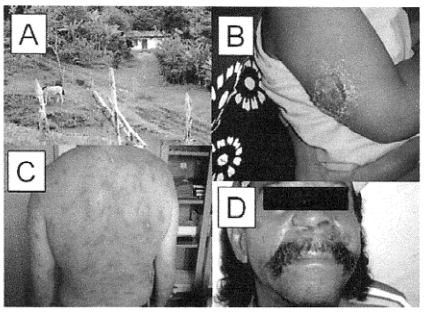


Fig. 1: the study area and spectrum of clinical disease caused by *Leishmania braziliensis* infection in Corte de Pedra, state of Bahia, Brazil. A: typical house and farm area; B: typical cutaneous leishmaniasis lesion characterised by granulomatous background and elevated borders; C: disseminated leishmaniasis, a form of disease that is increasing in the study area; D: mucosal leishmaniasis characterised by infiltrated ulcers that can cause extensive destruction of the nasal septum, columella and the upper lip.

*A familial aggregation study* - It is well known that the clinical outcome of parasitic infections is influenced by the complex interaction of parasite strain, host genetics and environmental factors. Leishmaniasis, in particular, has a broad clinical spectrum associated with variable profiles of immune response and different *Leishmania* species (Cabrera et al. 1995, Alcais et al. 1997, Ribeiro-de-Jesus et al. 1998). Previous studies have described familial clustering of VL and CL (Alcais et al. 1997, Blackwell et al. 1997, Jeronimo et al. 2000). Given that ML is a rare phenotype associated with a vigorous inflammatory response to parasite antigens (Bacellar et al. 2002), we conducted a study to address the hypothesis that familial clustering of ML would occur in the endemic area of Corte de Pedra. The study was a reconstructed cohort, a hybrid between a case-control and a retrospective cohort study. All members of 30 ML and 30 neighbourhood control families were assessed for history of exposure, as assessed by positive delayed type hypersensitivity (DTH) response and/or current or past disease confirmed from medical records or by clinical examination for presence of a scar in association with a positive DTH response. First-degree relatives of index cases were compared with those of index controls (Castellucci et al. 2005). There were significant differences between the frequencies of CL (37% vs. 20%) and ML (5% vs. 0%) when comparing case families and control families, respectively. Additionally, families with two cases of ML had a higher frequency (29.6%) of DTH-positive individuals than control families (9.4%). In this way we documented familial aggregation of CL and ML in a region where *L. braziliensis* is highly endemic. Although shared environment reflecting the rate of exposure to sandflies, the number of parasites inoculated by the infected sandflies, pre-existing immune responses to sandfly saliva products and variation between isolates of *L. braziliensis* (Grimaldi Jr & Tesh 1993, Gillespie et al. 2000) could contribute to this familial aggregation, our data favoured the hypothesis that genetic background could be influencing a higher rate of infection and/or a propensity to develop or retain a positive skin test in family members. This was supported by our failure to detect differences between ML and neighbourhood control families for environmental factors evaluated in our study area. At the same time, other studies were already documenting (Table I) host genetic factors influencing the immune response and clinical outcome of leishmaniasis in mice and humans (Blackwell et al. 1997, Blackwell 1998). Based on these findings, we conducted a number of candidate gene studies in order to identify polymorphic markers associated with ACL in the Corte de Pedra population.

*Analysis of candidate immune response genes* - The first series of candidate gene studies undertaken in our study area were based on analysis of candidate immune response genes informed by our knowledge of the immunopathology of disease. These studies were initially based on a case-control study design, where possible supported by family-based analysis to control for ethnic admixture. Both cohorts were geographically and demographically equivalent. Table II describes the structure of case-control and family sample sets used as a resource for these candidate gene studies.

TABLE I

Summary of published genetic association studies for cutaneous leishmaniasis

| Papers reporting significant linkage or association | | | | | | |
|---|---|---|---|---|---|---|
| Candidate gene | Population | Phenotype | Sample size | Reported result | Reference | PMID |
| MHC region (class I-III) | | | | | | |
| Cw7 | SE Asian Hmong | CL | NA | p = 0.01 | Barbier et al. (1987) | 3478848 |
| A28; Bw22 | Venezuelan | LCL | 24 families | p = 0.0018; 0.0122 | Lara et al. (1991) | 2022495 |
| Bw22 | Venezuelan | LCL | Ca = 26; Co = 26 | RR = 12.5; p = 0.048 | Lara et al. (1991) | 2022495 |
| DQw8 | Venezuelan | LCL | 24 families | p = 0.0364 | Lara et al. (1991) | 2022495 |
| DQw3 | Venezuelan | LCL | Ca = 26; Co = 26 | RR = 4.25; p = 0.036 | Lara et al. (1991) | 2022495 |
| DR2 | Brazilian | MCL | Ca = 43; Co = 111 | RR = 0.07; p = 0.004 | Petzl-Erler et al. (1991) | 1783572 |
| DQw3 | Brazilian | MCL | Ca = 43; Co = 111 | RR = 4.2; p = 0.006 | Petzl-Erler et al. (1991) | 1783572 |
| DR2; DR7/DRw9 | Venezuelan | CL | Ca = 49; Co = 43 | p < 0.05 | Cabrera et al. (1995) | 7595196 |
| LTA | Venezuelan | MCL | Ca = 25; Co = 43 | RR = 7.5; p < 0.001 | Cabrera et al. (1995) | 7595196 |
| TNF (-308) | Venezuelan | MCL | Ca = 25; Co = 43 | RR = 3.5; p < 0.05 | Cabrera et al. (1995) | 7595196 |
| DRB1*0407; DPA1*0401; DPB1*0101 | Mexican mestizos | LCL | Ca = 65; Co = 100 | OR = 2.92, 10.07, 5.99 | Olivo-Diaz et al. (2004) | 15041165 |
| DPB1*0401; DR2 | Mexican mestizos | LCL | Ca = 65; Co = 100 | OR = 0.38, 0.14 | Olivo-Diaz et al. (2004) | 15041165 |
| Non-MHC candidate genes | | | | | | |
| *IL6*-174 | Brazilian | ML | Ca = 60; Co = 180 | OR = 2.29 (1.40-3.77); p = 0.001 | Castellucci et al. (2006) | 16845637 |
| IFNG+874 | Iranian | Chronic CL | Ca = 58; Co = 688 | $\chi^2$ = 12.53; p = 0.0019 | Kamali-Sarvestani et al. (2006) | 16950634 |
| *IL-4*-590 | Iranian | LCL | Ca = 201; Co = 92 | $\chi^2$ = 8.64; p = 0.003 | Kamali-Sarvestani et al. (2006) | 16950634 |
| *IL-10*-819 | Brazilian | CL | 30 families | OR = 2.5 (1.12-5.7); p = 0.003 | Salhi et al. (2008) | 18424735 |
| CXCR1 rs2854386 | Brazilian | CL | Ca = 60; Co = 60 | OR = 2.38 (1.23-4.57); p = 0.009 | Castellucci et al. (2010) | 20089160 |
| CXCR1 rs2854386 | Brazilian | ML | 104 families | p = 0.046 | Castellucci et al. (2010) | 20089160 |
| SLC11A1 rs17235416 | Brazilian | CL | 104 families | p = 0.011 | Castellucci et al. (2010) | 20089160 |
| CCL2-2518 | Brazilian | ML | Ca = 67; Co = 120 | OR = 4.40 (1.42-13.65); p = 0.010 | Ramasawmy et al. (2010) | 20430117 |
| FLI1 rs7930515 | Brazilian | CL | 209 transmissions | OR = 1.62 (1.26-2.09); p = 1.8 x $10^{-4}$ | Castellucci et al. (2011) | 21633373 |
| TLR4 Asp299Gly | Iranian | Chronic CL | Ca = 22; Co = 75 | OR = 25.3 (5.2-115.6); p < 0.001 | Ajdary et al. (2011) | 21056683 |
| TLR4 Asp299Gly | Iranian | Acute CL | Ca = 61; Co = 75 | OR = 8.03 (1.7-37.7); p = 0.006 | Ajdary et al. (2011) | 21056683 |
| TLR4 Thr399Ile | Iranian | Chronic CL | Ca = 22; Co = 75 | p < 0.001 | Ajdary et al. (2011) | 21056683 |
| TLR4 Thr399Ile | Iranian | Acute CL | Ca = 61; Co = 75 | p = 0.016 | Ajdary et al. (2011) | 21056683 |
| CTGF rs6918698 | Brazilian | CL | 271 transmissions | OR = 1.67 (1.10-2.54); p = 0.016 | Castellucci et al. (2012) | 22554650 |
| FLII rs2071242 | Brazilian | CL | 268 transmissions | OR = 1.60 (1.14-2.24); p = 0.005 | Castellucci et al. (2012) | 22554650 |
| TGFBR2 rs1962859 | Brazilian | CL | 295 transmissions | OR = 1.50 (1.12-1.99); p = 0.005 | Castellucci et al. (2012) | 22554650 |
| SMAD2 rs1792658 | Brazilian | CL | 210 transmissions | OR = 1.57 (1.04-2.38); p = 0.03 | Castellucci et al. (2012) | 22554650 |
| SMAD7 rs4464148 | Brazilian | CL | 278 transmissions | OR = 2.80 (1.00-7.87); p = 0.05 | Castellucci et al. (2012) | 22554650 |
| SMAD3 rs1465841 | Brazilian | ML | 52 transmissions | OR = 2.15 (1.13-4.07); p = 0.018 | Castellucci et al. (2012) | 22554650 |
| SMAD7 rs2337107 | Brazilian | ML | 50 transmissions | OR = 3.70 (1.27-10.7); p = 0.016 | Castellucci et al. (2012) | 22554650 |
| *IL-1β*-511 | Mexican mestizos | LCL | Ca = 58; Co = 123 | OR = 3.23 (1.2-8.7); p = 0.0167 | Fernández-Figueroa et al. (2012) | 22629474 |
| MIF-173 | Brazilian | CL | Ca = 110; Co = 682 | OR = 1.79 (1.15-2.78); p = 0.008 | Covas et al. (2013) | 23068083 |

↳

Papers reporting no significant linkage or association

| Candidate gene | Population | Phenotype | Sample size | Reported result | Reference | PMID |
|---|---|---|---|---|---|---|
| MHC region (class I-III) | | | | | | |
| TNF rs1800629 | Sri Lankan | CL | Ca = 200; Co = 200 | NS | Samaranayake et al. (2010) | 20214763 |
| LTA rs909253 | Sri Lankan | CL | Ca = 200; Co = 200 | NS | Samaranayake et al. (2010) | 20214763 |
| Non-MHC candidate genes | | | | | | |
| IFNG+874 | Brazilian | CL | Ca = 136; Co = 609 | NS | Matos et al. (2007) | 17456233 |
| SLC11A1 rs2276631 | Sri Lankan | CL | Ca = 200; Co = 200 | NS | Samaranayake et al. (2010) | 20214763 |
| SLC11A1 rs3731865 | Sri Lankan | CL | Ca = 200; Co = 200 | NS | Samaranayake et al. (2010) | 20214763 |
| SLC11A1 rs17235409 | Sri Lankan | CL | Ca = 200; Co = 200 | NS | Samaranayake et al. (2010) | 20214763 |
| TLR2 Arg753Gln | Iranian | CL | Ca = 84; Co = 120 | NS | Ajdary et al. (2010) | 20388552 |
| TLR2 Arg677Trp | Iranian | CL | Ca = 84; Co = 120 | NS | Ajdary et al. (2010) | 20388552 |
| FcγRIIA-H/R131 | Brazilian | CL | Ca = 88; Co = 98 | NS | Oliveira et al. (2011) | 21324097 |

PubMed search term: leishmaniasis and susceptibility not drug; field: text word; limits: humans. Human leukocyte antigen notation is as reported in the original papers and has not been updated to current nomenclature as resolution is influenced by the typing method employed at the time. Ca: cases; CL: cutaneous leishmaniasis; Co: controls; DCL: diffuse CL; IFN: interferon; IL: interleukin; LCL: localised CL; LTA: leishmaniose tegumentar americana; MCL: mucocutaneous leishmaniasis; MHC: major histocompatibility complex; ML: mucosal leishmaniasis; NA: not available; NS: not significant; OR: odds ratio; PMID: PubMed identifier; RR: relative risk; Th: T helper; TNF: tumour necrosis factor.

*Interleukin (IL)-6* - ML is a severe disease that normally follows localised CL. Immune pathology is created by a strong pro-inflammatory response with high levels of tumour necrosis factor (TNF) and failure of type 2 cytokines to regulate this response. IL-6 down-regulates T helper (Th) cell type 1 differentiation and drives Th2 cell differentiation. Previous studies have shown that pre-treatment with recombinant human IL-6 inhibits interferon (IFN)-γ and TNF mediated activation of human macrophages for killing of *L. amazonensis* (Hatzigeorgiou et al. 1993) and IL-6 has been shown to down regulate the expression of TNF membrane receptors (Bermudez et al. 1992). We evaluated (Castellucci et al. 2006) the functional *IL6*-174 bp G/C promoter polymorphism, a single nucleotide polymorphism (SNP) associated with pro-inflammatory diseases and IL-6 regulation (Fishman et al. 1998, Bidwell et al. 1999, Terry et al. 2000). In addition, IL-6 levels were measured in macrophages with or without stimulation with soluble *Leishmania* antigen (SLA) from *L. braziliensis*. Our data (Castellucci et al. 2006) provide both population-based [odds ratio (OR) = 2.29, 95% confidence intervals (CI) = 1.40-3.77, p = 0.001] and family-based ($z = 4.3$, $p = 1.5 \times 10^{-5}$) evidence for an association between the C allele of the -174 bp SNP at *IL6* and susceptibility to ML. The family-based analysis was important in confirming that the association was not due to population substructure that might have differed between case and control groups. In addition, we found that the C allele was associated with reduced baseline expression of IL-6 in unstimulated macrophages and in macrophages stimulated with SLA. There are inconsistencies among studies concerning the role of the *IL6*-174 bp G/C polymorphism, both in terms of which is the disease-associated allele, and when attempting to determine whether different genotypes are functionally associated with the production of differing IL-6 levels. The fact that IL-6 has many pleiotropic effects in regulating both type 1 and type 2 immune response pathways (Diehl & Rincon 2002), plus the complexities of the immunopathogenesis of these different diseases (Rincon et al. 1997, Diehl et al. 2000), might explain such differences. Besides, it is important to bear in mind that the -174 bp SNP is not the sole polymorphic determinant of differential and cell type-specific promoter activity driving *IL6* gene transcription (Fishman et al. 1998, Terry et al. 2000). In relation to our own study, as macrophages are the primary site of infection, we hypothesise that low IL-6 production in carriers of the C allele may contribute to a reduced capacity to induce Th2 cell differentiation and regulate the activity of $CD4^+$ Th1 cell-generated cytokines (such as IFN-γ and TNF) that contribute to the destructive pathological manifestations associated with ML.

*CCL2/MCP1* - There are several reports for the putative roles of the *CCL2*-encoded monocyte chemoattractant protein-1 (MCP-1) in leishmaniasis from infection studies in vitro (Ritter & Moll 2000, Bhattacharyya et al. 2002) as well as by analysis of human (Ritter et al. 1996) and murine (de Moura et al. 2005) lesions. Previous studies have variably demonstrated increased risk or protection from pulmonary tuberculosis associated

with single SNP variants and/or different haplotypes created by promoter region SNPs at -362 bp and at -2,518 bp (Flores-Villanueva et al. 2005, Thye et al. 2009, Intemann et al. 2011). One of these studies (Flores-Villanueva et al. 2005) further showed that tuberculosis patients carrying the G allele for the SNP at -2,518 bp had the highest plasma levels of MCP-1 and the lowest plasma levels of IL-12p40, which was therefore interpreted as a secondary effect of MCP-1 in impairing the Th1 immune response against *Mycobacterium tuberculosis*. We also demonstrated (Ramasawmy et al. 2010) that the G allele at the regulatory *CCL2* -2,518 bp promoter is a risk factor for ML using our population-based (OR = 4.4, 95% CI = 1.42-13.65, p = 0.01) and family-based (z = 2.68, p = 0.007) samples (Table II) from Corte de Pedra. A number of studies suggest a link between the leishmanicidal capacity of MCP-1 and lesion healing. Previous work has demonstrated that MCP-1 enhances the cytotoxic response *via* induction of reactive oxygen intermediates by infected macrophages (Ritter & Moll 2000, Bhattacharyya et al. 2002). Moreover, in patients with self-healing CL, high levels of MCP-1 were detected in infected skin whereas, in the non-healing lesions of diffuse CL, MCP-1 expression was much lower with a predominance of another CC chemokine, CCL3 or macrophage inflammatory protein 1-α (MIP-1α) (Ritter et al. 1996). In addition, it was demonstrated that the chemokines MCP-1, MIP-1α and CXCL1 were expressed in ears and draining lymph nodes of mice infected in the ear with *L. braziliensis* (de Moura et al. 2005). Our results suggest that high levels of MCP-1 appear to exacerbate ML disease. In contrast to previous data (Flores-Villanueva et al. 2005), plasma levels of IL-12p40 and IL-12p70 did not differ significantly between our *CCL2* -2,518 bp genotype groups. We also observed higher MCP-1 levels in the supernatants of macrophages from GG compared to AA genotypes both in un-stimulated as well as SLA and LPS stimulated cultures. Our data support the alternative view that the proinflammatory capacity of MCP-1 in recruiting host monocytes could provide both the environment for parasite replication and for tissue damage and lesion development. This could be due to a direct effect of MCP-1 in bringing fresh monocytes to the site of infection and/or to downstream events regulated by MCP-1 in macrophages and other cells.

*CXCR1 and SLC11A1* - It has been hypothesised (Peters & Sacks 2009) that differences in the ability of macrophages and dendritic cells from different inbred mouse strains to respond to apoptotic vs. necrotic polymorphonuclear leukocytes (PMN), arising during the wound healing response to an infected sandfly bite, determines disease progression. The arrival and maintenance of infiltrating cells at bite sites is thought to be mediated by sandfly derived factors that either mimic a tissue damage signal or activate chemokine/chemokine receptor pathways (Teixeira et al. 2005a, b, 2006). Ex-

TABLE II

Characteristics of collections made during the primary (2000-2004) and secondary (2008-2010) sampling periods (A) and demographic data of the case-control groups (B)

| | Primary sample period | | | Secondary sample period | | |
|---|---|---|---|---|---|---|
| A | CL | ML | Leishmaniasis *per se* | CL | ML | Leishmaniasis *per se* |
| Cases (n) | 250 | 87 | 337 | 402 | 39 | 441 |
| Males | 128 | 60 | 188 | 219 | 24 | 243 |
| Females | 122 | 27 | 149 | 183 | 15 | 198 |
| Age at disease (years) | | | | | | |
| Mean | 19.1 | 30.3 | 22.4 | 21.5 | 26.6 | 21.9 |
| 95% confidence interval | 17.1-21.2 | 25.8-34.3 | 20.3-24.4 | 20.1-22.9 | 20.7-32.4 | 20.6-23.3 |
| Nuclear families (n) | - | - | 168 | - | - | 157 |
| Total families/trios (n) | - | - | 767 | - | - | 764 |

| B | ML | CL | Unaffected control | DTH+ |
|---|---|---|---|---|
| Individuals (n) | 60 | 60 | 60 | 60 |
| Age range (years) | 11-69 | 10-80 | 11-75 | 12-75 |
| Mean age (years) ± SD | 40 ± 17.1 | 41 ± 17.8 | 40 ± 18.0 | 38 ± 18.0 |
| Males:females | 47:13 | 47:13 | 47:13 | 47:13 |
| Mean time residing in study area ± SD | 27 ± 16.9 | 31 ± 18.2 | 29 ± 17.4 | 32 ± 17.7 |
| Farm as main occupation (%) | 80 | 70 | 68 | 75 |

CL: cutaneous leishmaniasis; DTH: delayed type hypersensitivity; ML: mucosal leishmaniasis; SD: standard deviation.

pression patterns for chemokines have been associated with the evolution of large and small lesions in mice following *L. braziliensis* infection, influenced by both the strain of parasite (Teixeira et al. 2005b) and the mouse genetic background (Teixeira et al. 2005a). One way to look at the interplay between PMN and macrophages in disease progression in humans is to determine whether polymorphisms at genes that regulate their infiltration or function are associated with different clinical phenotypes following infection with *Leishmania* spp. *CXCR1* (IL8RA) and *CXCR2* (IL8RB) are genes encoding receptors for chemokines that attract PMN to inflammatory sites. They lie on human chromosome 2q25 230-260 kb upstream of *SLC11A1*, a gene that regulates macrophage activation and resistance to VL (Blackwell et al. 2001). In our studies (Castellucci et al. 2010), we showed an association between ACL and polymorphic variants at the *CXCR1*, specifically at SNP rs2854386 for both population-based (OR = 2.38, 95% CI = 1.23-4.57, p = 0.009) and family-based (z = 2.00, p = 0.045). Of interest, the common C allele (presumed to be the functional variant) was associated with CL, whereas the rare G allele was associated with ML (z = 2.00, p = 0.046). This suggested that, whereas high numbers of PMN might be detrimental in the context of CL disease, they may have an important positive role to play in preventing ML disease. In addition, in the family-based study CL was associated (z = 2.55, p = 0.011) with a 3' insertion/deletion polymorphism at *SLC11A1*, a gene primarily known for its role in the regulation of macrophage activation. The association is also of interest in relation to the putative role of this molecule in regulating expression of secretory leukocyte protease inhibitor and hence affecting the wound healing response (Thuraisingam et al. 2006). Differences in lesion development have not been observed following subcutaneous needle injection of either *Leishmania major* (Alexander & Blackwell 1986) or *Leishmania mexicana* (Roberts et al. 1989) into *Slc11a1* congenic mice, suggesting that the genetic influence of *SLC11A1* on susceptibility to CL following natural infection in humans might be mediated by the effect on the wound healing response to the sandfly bite. This means that the mechanism by which *SLC11A1* influences CL disease may be different to its influence on VL in mice following intravenous needle injection (Bradley & Kirkley 1977) or in natural infection of dogs (Sanchez-Robert et al. 2005, 2008) and humans (Bucheton et al. 2003, Mohamed et al. 2004). Our data supports roles for both *CXCR1* and *SLC11A1* in determining the outcome of *L. braziliensis* infection, providing interesting insight into the possible roles of PMN and macrophages in ACL.

*The wound healing gene hypothesis: studies inspired by mice* - Our observations on the possible role of wound healing genes in response to sandfly delivered parasites were not the first to suggest a possible role for wound healing genes in CL susceptibility. Indeed, our interpretation was based largely on the seminal mapping studies of susceptibility to CL carried out in mice (Sakthianandeswaren et al. 2005, 2009, 2010), which inspired us to look for the possible role of these and other wound healing genes in susceptibility to ACL in Corte de Pedra.

*FLI1* - Fine mapping in the region of chromosome 9 in mice (chromosome 11q24 in humans) identified Friend leukaemia virus integration 1 (*Fli1*) (*FLI1* in humans) as a novel candidate influencing both resistance to *L. major* and an enhanced wound healing response (Sakthianandeswaren et al. 2010). To determine whether polymorphisms at *FLI1* were important in human disease, SNPs that tagged the first two major linkage disequilibrium blocks and the proximal promoter of the *FLI1* gene were analysed in 325 endemic *L. braziliensis* families (Castellucci et al. 2011). The proximal promoter region of *FLI1* contains a functional GAn microsatellite, as well as a CpG island that spans the proximal promoter region and the 5′ region of intron. Using robust case-pseudocontrol conditional logistic regression analysis of discovery (OR = 1.65, 95% CI = 1.18-2.29, p = 0.003) and replication (OR = 1.60, 95% CI = 1.10-2.33, p = 0.014) family-based cohorts, we demonstrated association between *FLI1* (rs7930515; $P_{combined} = 1.8 \times 10^{-4}$) and susceptibility to CL caused by *L. braziliensis* (Castellucci et al. 2011). In the murine study, resistance to *L. major* correlated with a wound-healing response that presented in congenic resistant mice as a large population of fibroblasts and an organised and abundant deposition of collagen bundles in the absence of inflammatory cells (Sakthianandeswaren et al. 2005). Recent studies have shown an association between enhanced type I collagen expression and epigenetic repression of the *FLI1* gene (Wang et al. 2006). As reviewed above, our group also reported an association between ML and the C allele at the *IL6*-174 bp G/C promoter polymorphism (Castellucci et al. 2006), which determines low levels of IL-6 release from macrophages. Homocysteine dependent stimulation of IL-6 has recently been reported (Thaler et al. 2011) to upregulate genes essential for epigenetic DNA methylation *via* expression of *FLI1*. Homocysteine increases the CpG methylation status (and hence represses gene expression) of the CpG-rich proximal promoter of the lysyl oxidase (*LOX*) gene (Thaler et al. 2011), an extra-cellular copper enzyme that initiates the cross-linking of collagens and elastins. Inhibition of IL-6 reverses this repression. Regulation of collagen expression and organisation may thus involve epigenetic regulation at both *FLI1* and *LOX* genes, consistent with the presence of the CpG island across the region of the functional *FLI1* promoter elements. This suggests that, although there are many immune-related functions for both IL-6 and *FLI1* that could account for association with CL caused by *L. braziliensis*, there may be a direct functional link between these two genes that mediates resistance or susceptibility to infection through the wound-healing response. This, in turn, might provide novel therapeutic opportunities.

*Transforming growth factor β (TGFβ) signalling pathway* - IL-6 is known to increase expression of FLI1 (Thaler et al. 2011). In the wound healing response, both FLI1 (Nakerakanti et al. 2006) and IL-6 (Gressner et al. 2011) repress connective tissue growth factor (CTGF) and all three genes interact with the TGFβ pathway. We therefore interrogated further the possible roles of wound healing pathways in cutaneous forms of leishmaniasis caused by *L. braziliensis* by looking for genetic associations with

polymorphisms in other genes through interaction with FLI1 and the TGFβ signalling pathway (Castellucci et al. 2012). Robust case-pseudocontrol conditional logistic regression analysis showed associations between CL and SNPs at *CTGF* (rs6918698, OR = 1.67, 95% CI = 1.10-2.54, p = 0.016), *TGFBR2* (rs1962859, OR = 1.50, 95% CI = 1.12-1.99, p = 0.005), *SMAD2* (rs1792658, OR = 1.57, 95% CI = 1.04-2.38, p = 0.03), *SMAD7* (rs4464148, OR = 2.80, 95% CI = 1.00-7.87, p = 0.05) and *FLII* (rs2071242, OR = 1.60, 95% CI = 1.14-2.24, p = 0.005) and between ML and SNPs at *SMAD3* (rs1465841, OR = 2.15, 95% CI = 1.13-4.07, p = 0.018) and *SMAD7* (rs2337107, OR = 3.70, 95% CI = 1.27-10.7, p = 0.016). There is a complex interplay between FLI1 and the TGFb signalling pathway in regulating collagen deposition and fibrosis during the wound healing process. In looking for genetic associations that might throw light on how those genes are influencing the wound healing processes important in CL vs. ML disease caused by *L. braziliensis*, our results indicate that CTGF regulated *via* the SMAD2 arm of the TGFβ signalling pathway is required for wound healing in CL disease. In contrast, ML disease was associated with polymorphism in *SMAD3*, suggesting that alternative regulation of gene expression *via* the TGFβ signalling pathway may lead to ML disease. Fig. 2 provides a model for how polymorphisms at genes regulating the different signalling pathways might influence CL and ML disease. Further functional data will be required to determine what the downstream events following signalling *via* SMAD3 in ML compared to signalling *via* SMAD2 for CL disease might be. Additionally, both forms of disease were influenced by polymorphisms in the negative regulator *SMAD7* that blocks the TGFβ pathway upstream of both SMAD2 and SMAD3 emphasising the relevance of TGFβ signalling on ACL.

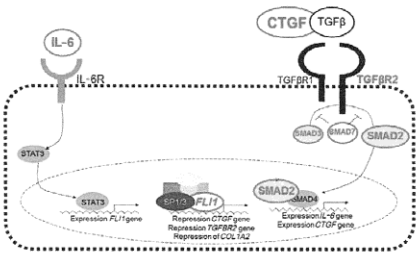


Fig. 2: diagram of genes that have been implicated in susceptibility to cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML) disease caused by *Leishmania braziliensis* in the area of Corte de Pedra, state of Bahia, Brazil, showing involvement of, and interaction with, the transforming growth factor β (TGFβ) pathway. Polymorphisms in genes annotated in red lettering have been associated with CL or ML disease. Turquoise circles indicate the pathway through which interleukin (IL)-6 influences SMAD4 *via* FLI1. SP1/3 are transcription factors that influence FLI1 expression. CTGF: connective tissue growth factor. Source: Castellucci et al. (2012).

*Leishmania* infection is associated with a broad spectrum of clinical phenotypes. *L. braziliensis*, in particular, causes debilitating and disfiguring CL, ML and DL that generally take a long time to heal. For over 50 years, pentavalent antimony ($Sb^v$) given by the intramuscular or intravenous route remained the first-line drug for the treatment of ACL. This therapy can cause toxic side effects and is difficult to administer in poor rural areas (Machado et al. 2010). In Corte de Pedra, cure rates after $Sb^v$ therapy are becoming increasingly lower and vary from 50-90% (Romero et al. 2001, Unger et al. 2009). In light of this, identifying important pathways/mechanisms of disease can lead to new therapeutic targets and more efficient intervention strategies that aim to increase adherence to treatment in areas with limited access to health services. Genetic studies in humans provide a potentially powerful route to understanding novel pathways of disease pathogenesis that could provide new chemotherapeutic targets.

Whilst broadly driven by parasite species, many studies have implicated host genetics in determining the outcome of infection within each species (El-Safi et al. 2006, Lipoldova & Demant 2006, Blackwell et al. 2009, Sakthianandeswaren et al. 2009). Nevertheless, the only definitive study carried out in humans to date was the recent genome-wide association study on VL (Fakiola et al. 2013), which demonstrated that polymorphisms within the DRB1-DQA1 class II region of human leukocyte antigen were the only SNPs to attain genome-wide significance. Remarkably, this finding crossed the epidemiological divide of parasite species (*Leishmania donovani* and *Leishmania chagasi*) and geography (Indian and Brazil) and has important implications for the development of molecularly defined vaccines. While candidate gene studies (Table II) have implicated a broader array of genes in susceptibility to CL, these are compromised by lack of power and failure to obtain replication within and between populations. Large well-powered genome-wide studies with replication will be required to evaluate the real significance of these findings. It is of interest, nevertheless, that our studies of ACL have provided evidence in support of important roles for immune response genes involved in wound healing, which are underpinned by initial genetic studies in murine models of disease. These wound healing genes may provide novel therapeutic opportunities in ACL, not the least because there may already be great interest in the same genes as therapeutic targets for other skin disorders. For example, the use of imatinib mesylate has been proposed for treatment of systemic sclerosis (Asano 2010, Asano et al. 2010), an autoimmune disorder similarly resulting from immune activation, fibrosis development and damage of small blood vessels, in which FLI1 is down regulated through an epigenetic mechanism (Asano et al. 2010). Imatinib mesylate reverses the expression levels of FLI1. Similar opportunities might apply in the case of other genes that we have demonstrated are associated with the spectrum of ACL disease. Work is in progress to analyse expression levels of FLI1 and other wound healing genes in tissue biopsies from *L. braziliensis* patients to determine

their potential as therapeutic targets, along with plans to undertake well-powered genome-wide association studies to validate our genetic findings for this important tropical infectious disease.

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"Analysis of expression of wound healing gene *FLI1*, *COL1A1*, *COL1A2* and *MMP1* in American cutaneous leishmaniasis caused by *Leishmania braziliensis* infection". PLOS Neglected Tropical Diseases [Submetido, *vide* Normas de Publicação no **ANEXO 1** e carta ao Editor no **ANEXO 2**].

# Analysis of expression of wound healing genes *FLI1*, *COL1A1*, *COL1A2* and *MMP1* in American cutaneous leishmaniasis caused by *Leishmania braziliensis* infection

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Abstract

Genetic risk factors contributing to clinical outcome following *Leishmania braziliensis* infection include polymorphisms at *IL6* (interleukin 6; IL-6), *FLI1* (Friend leukemia virus integration 1) and *COL1A1* (collagen type I alpha 1). Low FLI1 expression results in up-regulation of type I collagens and down-regulation of matrix metalloproteinase 1 (MMP1). Heavy methylation of CpG islands in the *FLI1* promoter is known to render *FLI1* transcriptionally inactive. To understand the role of this pathway in cutaneous leishmaniasis we examined FLI1 promoter-specific DNA methylation and expression, together with expression COL1A1, COL1A2 and MMP1, in lesion biopsies *ex vivo* and in *L. braziliensis* infected macrophages *in vitro*. Percent methylation of FLI1 was lower (P=0.001) in lesion biopsies compared to normal skin. FLI1 and COL1A1 expression did not differ between lesion and normal skin, whereas COL1A2 was reduced (P=0.033) and MMP1 enhanced (P=0.0002). FLI1 expression induced by infection *in vitro* peaked at 24 hours (P<0.0001), and was higher (P=0.005) and peaked later (48 hours) in the presence of IL-6. Expression of type I collagens was low in infected macrophages, and undetectable in macrophages treated with IL-6. MMP1 was strongly induced (P=0.007) following infection of macrophages, but was not readily detectable in IL-6 treated macrophages until 72 hours post infection when the effect of IL-6 on FLI1 expression had waned. MMP1 breaks down interstitial type I collagens, which is essential

for keratinocyte migration and re-epithelialization. However, in active cutaneous leishmaniasis lesions, low levels of type I collagen together with exaggerated levels of MMP1 indicate that MMP1 is mediating tissue damage rather than repair. Exaggerated MMP1 levels have similarly been observed to contribute to tissue destruction and disease progression in tuberculosis, leading others to highlight it as a potential therapeutic target. Our data suggest that therapeutic modulation of MMP1 might also be relevant in treatment of cutaneous leishmaniasis. (300 words; maximum 300 words allowed)

## Author Summary

The single-celled parasite known as *Leishmania braziliensis* causes cutaneous lesions that take a long time to heal even with current treatments available. The severity of disease differs between individuals. We showed previously that some of this variation is due to genetic pre-disposition. We found that the genes influencing the disease outcome belonged to a pathway of genes that are normally involved in wound healing. In the present study we look in more detail at how these genes are expressed in tissue taken from the lesion, and in cells infected with the parasite in culture dishes. We find that one gene in particular, whose normal role is to help in tissue repair, has very exaggerated expression that is associated with tissue damage rather than repair. We therefore propose that the enzyme encoded by this gene could be a potential therapeutic target for the treatment of this disease.

## Introduction

Leishmaniasis is a disease with a wide spectrum of clinical presentations, ranging from self-limiting lesions to severe mucosal disease. The magnitude of disease severity results from environmental factors such as parasite polymorphism, phlebotomine sandfly components, as well as the host's immune and genetic background. In particular, a number of studies (reviewed [1,2,3]) have reported the role of host genetic factors in regulating the clinical disease outcome of *Leishmania braziliensis* infection in humans. The importance of the wound healing processes in cutaneous forms of leishmaniasis has also been demonstrated from studies mapping murine susceptibility genes [4,5,6]. In particular, fine mapping in the region of Chromosome 9 in mice (Chromosome 11q24 in humans) identified *Fli1* (Friend leukemia virus integration 1; *FLI1* in humans) as a novel candidate influencing both resistance to *L. major* and an enhanced wound healing response [4]. Recently we demonstrated [7] that polymorphism at *FLI1* is associated with cutaneous leishmaniasis (CL) caused by *L. braziliensis* in humans, with an inverse association (i.e. association with opposite alleles) observed for mucosal leishmaniasis (ML) disease. This was interesting in relation to our previous demonstration that the C allele at the *IL6*-174 G/C promoter polymorphism, which determines low levels of IL-6 release from macrophages, was a risk factor for ML disease [8]. IL-6 is known to increase expression of *FLI1* [9]. In addition,

we found evidence for association between polymorphic variants at collagen type I alpha 1 (*COL1A1*) gene and susceptibility to cutaneous leishmaniasis caused by *L. braziliensis* in Brazil [10], supporting our series of studies [2,7,11] on the potential role of wound healing genes in leishmaniasis. Reduction of FLI1 expression in human fibroblasts has been shown to result in up-regulation of *COL1A1* and collagen type I alpha 2 (*COL1A2*) at the mRNA level, and in down-regulation of the matrix metalloproteinase 1 (*MMP1*) gene, suggesting that FLI1 suppression is involved in activation of the profibrotic gene program [12].

Despite the contribution of these studies to better understand the pathogenesis of the disease, less attention has been given to the mechanisms that regulate expression of these genes. A growing body of evidence points towards the importance of epigenetic mechanisms, including chromatin modifications and DNA methylation, as key mechanisms in the regulation of gene expression. In the case of FLI, this is supported by data demonstrating heavy methylation of the CpG islands in the *FLI1* promoter region in systemic sclerosis fibroblasts and skin biopsy specimens [13]. This renders *FLI1* transcriptionally inactive, resulting in augmented collagen synthesis [13]. Data are also emerging on microbe-induced epigenetic changes in host cells [14,15,16]. Intracellular pathogens, including viruses and bacteria, manipulate host processes to favor their intracellular survival

[14,15,17]. A recent study also identified changes in DNA methylation at hundreds of CpG islands following infection of macrophages with live *L. donovani* [18]. The data suggest that Leishmania induces, either directly or indirectly, changes in the host cell genome that result in down-regulation of innate immunity favoring survival and replication of the parasite. The aim of the present study was to understand the functional role of FLI1 in CL by comparing the DNA methylation profile of the *FLI1* gene in normal and lesion skin biopsies from patients with CL, as well as in infected and uninfected macrophages, and to correlate the methylation patterns with the gene expression of *FLI1* and wound healing genes (*COL1A1*, *COL1A2* and *MMP1*) downstream of *FLI1*.

## Methods

### Ethical statement

The study was conducted according to the principles specified in the Declaration of Helsinki and under local ethical guidelines. The study was approved by the institutional review board of the Federal University of Bahia (CEP-UFBA 22/2012) and the Brazilian National Ethical Committee (CONEP: 1258513.1.000.5537). All patients provided written informed consent for the collection of samples and subsequent analysis. Parents or guardians provided informed consent for patients less than 18 years of age. Patients between 12 and 17 years of age provided written assent.

## Study site, diagnosis and sample collection

The study was conducted in the rural area of Corte de Pedra, Bahia, Brazil, where *L. braziliensis* is endemic. The endemic area of Corte de Pedra is characterized by isolated sites of secondary forest with agricultural activities providing the main source of income for the majority of its inhabitants. The work on farms and homes built in clearings in the woods has increased the population's exposure to *L. braziliensis* infection over the last decades [19]. Participants of the present study include CL patients recruited at the health post in Corte de Pedra, the reference center for disease treatment of twenty municipalities. As in our previous studies [7,8,11,20] CL is defined as the presence of a single chronic ulcerative lesion at a skin site without evidence of mucosal involvement, without evidence of dissemination to 10 or more sites, and confirmed by detection of parasites or two of the three following criteria: positive delayed-type hypersensitivity test, PCR positive to *L. braziliensis*, and a histopathology suggestive of leishmaniasis. All cases in the current study also responded to the standard anti-leishmanial therapy subsequent to samples being taken.

## Biopsy specimens and DNA/RNA extraction

Biopsies of CL lesions (taken from the active leading edge at the lesion border) and normal skin (taken from a site remote to the

lesion, usually another limb) from the same patient were taken using a 4-mm punch and tissues preserved in RNAlater Solution (Ambion) until the extraction of RNA and DNA. After intensive mechanical maceration, RNA and DNA were extracted for the gene expression and epigenetic regulation studies, respectively. RNA was extracted by using the PureLink RNA Mini Kit (Ambion) and DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen). The protocols recommended by the kit manufacturers were followed precisely. Both, RNA and DNA concentration and integrity were determined by spectrophotometric optical density measurement (260 and 280 nm) and samples stored at -70°C.

### Macrophages cultures and DNA/RNA extraction

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Hypaque (GE Healthcare Bio-Sciences AB) gradient centrifugation. After washing three times in 0.9% NaCl, PBMC were resuspended in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum, 10 IU/mL penicillin and 100 µg/mL streptomycin, and incubated for 6 days at 37°C in 5% CO2. Following this incubation period, PBMC-derived macrophages were adjusted to $1x10^6$ cells/mL and placed on 12 wells plates in duplicates in the presence or absence of recombinant human IL-6 (20 ng/mL) (Gibco). The cultures were incubated for 24 hours for cell adhesion, and washed in HBSS to remove non-adherent cells.

PBMC-derived macrophage cultures were infected with *L. braziliensis* stationary phase promastigotes at a 5:1 ratio. Uninfected macrophages were used as controls. Infected macrophages were incubated for 2 hours, after which time remaining extracellular parasites were removed by washing in HBSS. At each time point (3, 24, 48 and 72 hours after infection), cells were harvested for DNA and RNA extraction. Cells used for DNA extraction were harvested in 50% fetal bovine serum, 40% RPMI-1640 (Gibco) and 10% DMSO and maintained at -70°C. The remaining cells to be used for matched RNA extraction were harvested in TRIzol Reagent (Invitrogen) and also stored at -70°C until use. DNA and RNA were extracted using the same kits and protocols described above.

### Gene expression of FLI1, COL1A1, COL1A2 and MMP1

Reverse transcription reactions were performed using the commercially available High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), starting from 10µl of total RNA and using the MultiScribe Reverse Transcriptase enzyme, according to the manufacturer's instructions. PCR cycling was carried out using the GeneAmp PCR System 9700 as follows: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, finally cooling at 4°C. Assays containing specific primers and probes for *FLI1* (Hs00956709_m1), *COL1A1* (Hs00164004_m1), *COL1A2* (Hs01028972_m1) and *MMP1* (Hs00899658_m1) genes were pre-

designed by Applied Biosystems®. The mRNA levels within each sample were normalized to the level of the house-keeping gene Beta-actin (Hs01060665_g1). The quantitative RT-PCR (qRT-PCR) individual reactions were prepared from 2 µl of cDNA, 0.5 µl of each assay, 5 µl of TaqMan Universal Mastermix® and 2.5 µl of RNase-DNase free water in a final volume of 10 µl. All samples were run in duplicate and the output level reported as the average of the two samples. Amplification conditions included: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute on the 7500 Real Time PCR System (Applied Biosystems). The data were analyzed by comparing the threshold cycle (Ct), according to the equation $2^{-\Delta\Delta CT}$ where ΔCT is the Ct value of the target gene subtracted from the Ct of the endogenous house-keeping gene, and ΔΔCT is the ΔCT value of each individual less the median ΔCT of the control group.

### Methylation of FLI1 gene

Analysis of DNA methylation was performed using an EpiTect Methyl II PCR assay specific for the *FLI1* promoter CpG island (Reference EPHS102855-1A; Qiagen). This assay is based on the differential cleavage of methylated versus unmethylated genomic DNA when using methylation sensitive or dependent restriction enzymes. Briefly, prior to the PCR reactions the DNAs were digested using the EpiTect Methyl II DNA Restriction Kit (Qiagen) according to the manufacturer's instructions. The percentage of

DNA that is fully methylated or fully unmethylated is determined by quantitative PCR (q-PCR) of digestion products. The q-PCR was set up by preparing individual reactions for each of the digestions following the protocols provided by the assay. Cycling was performed by the 7500 Real Time PCR System programmed according to the manufacturer's instructions. After the cycling program has completed, the Ct values were exported from the instrument software to a Microsoft Excel spreadsheet. The data were analyzed using the EpiTect Methyl II PCR Array Microsoft Excel template, available at www.sabiosciences.com/dna_methylation_data_analysis.php. This template automatically performs all ΔCt based calculations from the raw threshold cycle (Ct) values to determine gene specific DNA methylation status. The Excel template normalizes the Ct values of both digests against mock digestion values to calculate and report the percentage of the DNA that is methylated and unmethylated.

### Statistical analysis

Statistical significance was assessed by Mann-Whitney for comparison between two groups and Kruskall Wallis one way analysis of variance for comparison of multiple groups. $P<0.05$ were considered statistical significant results. For all statistical analyses the GraphPad Prism5 software was utilized.

## Results

## FLI1-specific methylated DNA is decreased in lesion biopsies relative to normal skin biopsies

Previous studies have demonstrated heavy methylation of the CpG islands in the promoter of the FLI1 gene in skin biopsies from patients with systemic sclerosis [13]. To determine whether methylation in the FLI1 promoter is similarly altered in lesions associated with CL caused by *L. braziliensis* infection, we first compared methylation at the *FLI1* promoter region in DNA derived from CL lesion biopsies *versus* biopsies from distant normal skin (Fig 1). While the percentage of FLI1 promoter-specific methylated DNA in biopsies was low (<10%), our results show that normal skin biopsies presented a higher percentage of methylated DNA (P=0.001) compared to CL biopsies (Fig 1). This suggests that pathology associated with CL results in de-methylation of CpGs in the *FLI1* promoter, opening up potential regulatory regions upstream of the *FLI1* transcription start site.

## Analysis of expression of FLI1 and downstream genes in skin biopsies

To determine whether CL pathogenesis *in vivo* is also accompanied by altered expression of *FLI1* and downstream genes (*COL1A1*, *COL1A2* and *MMP1*) under its control, mRNA levels were compared between biopsies from CL lesions and paired samples from normal skin distant to the lesion (Fig 2). There were no significant

differences in mRNA expression of FLI1 (Fig 2A) or COL1A1 (Fig 2B) between biopsies of CL and normal skin. However, expression of COL1A2 (Fig 2C; P=0.033) was significantly lower in biopsies of CL lesions, whereas expression of MMP1 (Fig 2D; P=0.0002) was significantly higher in CL lesions, relative to normal skin.

### *L. braziliensis* infection does not alter the FLI1 methylation profile in PBMC-derived macrophages

To determine to what extent the reduction in methylation of the FLI1 promoter observed *ex vivo* in CL lesion samples was due to direct effects of the parasite on its preferred host cell, we also evaluated the percentage of *FLI1* methylated DNA in PBMC-derived macrophages at 0, 3, 24, 28 and 72 hours following infection with *L. braziliensis*. Although there was a trend for increased methylation in the FLI-specific CpG with time after infection (Fig 3), this did not achieve statistical significance whether analyzed across different time points (Fig 3A CT samples; Krukal-Wallis $P$=0.816), or when data were pooled to compare uninfected and infected macrophages across all time points (Fig 3B; Mann-Whitney $P$=0.471 for uninfected *versus* infected). The results showed high individual variability across donors (N=9) for the percentage of FLI1-specific methylated DNA.

Since we have observed that polymorphisms at both *FLI1* and *IL6* are genetic risk factors for CL disease [7,8], and IL-6 is known to increase expression of FLI1 [9], we next looked to see whether

the presence of IL-6 in the infected PBMC-derived macrophage cultures would influence percent methylation of CpGs in the FLI1 promoter. Again, whilst there was a trend for lower FLI-specific methylation across all time points with IL-6 present in the cultures (Fig 3), none of the differences between IL-6-treated and untreated macrophages achieved statistical significance.

Overall the results of FLI1-specific DNA methylation observed in macrophages in culture (trend for increased methylation with infection) did not support the *ex vivo* observations comparing CL lesions with normal skin biopsies (decreased methylation with infection), suggesting a more complex wound healing process involving other cell types and/or the presence of IL-6 might be at play *in vivo*.

## IL-6 enhances FLI1 expression in *L. braziliensis* infected PBMC-derived macrophages

Overall, the percentage of FLI1-specific methylated DNA in lesions and macrophages was low, especially in the presence of IL-6 in macrophages *in vitro*, suggesting that potential regulatory regions upstream of the *FLI1* transcription start site are open to regulated expression of FLI1. To determine whether *L. braziliensis* infection of macrophages directly influences FLI1 expression, we next examined its expression in PBMC-derived macrophages infected *in vitro* in the presence and absence of IL-6. Fig 4A shows that the percent of donors for whom FLI1 expression

was measurable by qRT-PCR declined with time in culture, an effect that was more marked in the presence of IL-6. This could reflect loss of cells due to death or apoptosis [21] in culture, although equivalent amounts of mRNA were used in all assays and specific gene expression was measured relative to time-point specific measurements of the endogenous beta-actin control. For those donors for whom FLI1 mRNA was detectable (Fig 4B), expression peaked at 24 hours ($P<0.0001$ 24 hr CT *versus* 3 hr CT) in the absence of IL-6, whereas FLI1 expression in the presence of IL-6 peaked at 48 hours and was significantly higher in IL-6 treated *versus* untreated (=CT) at 24 ($P=0.05$), 48 ($P=0.005$) and 72 ($P=0.04$) hours after infection. Hence, our data are consistent both with our expectation that FLI1 would be open to regulated expression due to low CpG methylation in its promoter, and that IL-6 would enhance FLI1 expression.

### Expression of the FLI1-regulated genes *COL1A1*, *COL1A2* and *MMP1* in *L. braziliensis* infected PBMC-derived macrophages

FLI1 is known to regulate expression of downstream wound healing genes. Specifically, if FLI1 expression is reduced COL1A1 and COL2A1 mRNA expression goes up whereas MMP1 mRNA expression goes down [12]. Conversely, if FLI1 expression is high, we expect COL1A1 and COL2A1 to be low, and MMP1 to be high. Consistent with this prediction, we found that the percent of donors whose macrophages expressed COL1A1 (Fig 5A) and

COL2A1 (Fig 5B) was low compared to MMP1 (Fig 6A), an observation also reflected in levels of COL1A1- (Fig 5C) and COL2A1- (Fig 5D) compared to MMP1- (Fig 6B) specific mRNA in donors with levels detectable by qRT-PCR. COL1A1-specific mRNA was up-regulated with *L. braziliensis* infection, peaking at 24 hr post infection ($P$=0.011 uninfected *versus* 24 hr CT; $P$=0.028 3 hr CT versus 24 hr CT), but was undetectable in the presence of IL-6 which drove higher levels of FLI1 expression. Expression of COL1A2 was low for all treatments. Of interest, while MMP1-specfic mRNA levels were highly up-regulated with infection (Fig 4B), peaking at 24 hours post infection ($P$=0.007 uninfected *versus* 24 hr CT; $P$=0.016 3 hr CT versus 24 hr CT), it was low in the presence of Il-6 (and therefore high FLI1 expression) rising only at 72 hours post infection when FLI1 expression declined (see Fig 4).

## Discussion

Previous studies have demonstrated a role for wound healing genes in resolution of cutaneous lesions caused by *Leishmania spp.*, including the *Fli1/FLI1* gene in both mice [4] and humans [7]. Reduction of *FLI1* expression in human fibroblasts has been shown to result in up-regulation of *COL1A1* and *COL1A2* genes and down-regulation of the *MMP1* gene, suggesting that *FLI1* suppression is involved in activation of the profibrotic gene program [12]. Moreover, studies in scleroderma fibroblasts and in skin biopsies from patients with systemic sclerosis has demonstrated that the

CpG island in the *FLI1* promoter region is heavily methylated, indicating that enhanced expression of type I collagen genes is associated with epigenetic repression of FLI1 [13]. Conversely, here we find a low percentage of FLI1-specific methylated DNA in lesion biopsies from CL patients. Although FLI1 expression itself did not differ significantly between normal skin and CL lesion biopsies, the reduced methylation of the FLI1 promoter was associated with a concomitant decrease in COL1A2 expression and highly exaggerated expression of MMP1 compared to normal skin biopsies. The pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α), which characterizes human CL caused by *L. braziliensis* [22,23], could also be contributing to exaggerated expression of matrix metalloproteinases [24]. TNF-α is known to accelerate degradation of type I collagens by MMP-1 leading to their gradual loss in human skin [25]. Our results are also consistent with recently published expression profiling results in which MMP1 was amongst the 10 most highly induced genes, with a 36-fold higher level of expression in biopsies from CL lesions caused by *L. braziliensis* compared to normal skin [26].

The situation in the CL lesion is complex, with many cell types contributing to tissue injury and ultimately tissue repair and wound healing. Our biopsy samples were taken when patients presented with active lesions, before treatment had commenced. At this stage lesions are characterized by tissue damage and ulceration. MMP1 is a matrix metalloproteinase that breaks down

interstitial type I collagens [27]. Normally in injured human skin, re-epithelialization occurs when keratinocytes migrate along type I collagen, but this can only occur when MMP1 is present to cleave type I collagens and loosen the tight contacts keratinocytes establish with the dermal matrix [28]. The low levels of type I collagen, particularly of COL1A2, in CL lesions, together with very high levels of MMP1, suggest that the enzyme is mediating tissue damage rather than repair. Such exaggerated expression of MMP1 has been observed to contribute to tissue destruction and disease progression in a number of other clinical contexts, including progression of rheumatoid arthritis [29], chronic obstructive pulmonary disease, acute respiratory distress syndrome, sarcoidosis, and tuberculosis [30].

Given the complexity of cellular responses occurring in CL lesion biopsies, we were interested to determine to what extent our observations on expression of FLI1 and its downstream genes was due to a direct effect of the parasite on macrophage function. Of interest, while methylation of CpGs in the FLI1 promoter was reduced in lesion biopsies compared to normal skin, there was a trend towards a higher percentage of methylation in infected compared to uninfected macrophages. The percentage of methylated DNA was lowest in the presence of IL-6. Overall, we concluded that the FLI1 promoter was largely unmethylated, thus making it available for transcription factor binding and regulated gene expression. Infection of macrophages with *L. braziliensis* did result in an increased expression of FLI1, which was dramatically

increased up to 48 hours post infection in the presence of IL-6. Concomitant with this we observed low levels of induction of COL1A1 24 hours post infection, but neither type I collagen was expressed in the presence of IL-6 and high levels of FLI1. However, as we observed in CL lesions, there was an exaggerated expression of MMP1 in infected macrophages. Thus it seems likely that high levels of MMP1 associated with CL pathogenesis may be contributed to by a direct effect of the parasite on macrophage function. This is similar to the situation in tuberculosis infection, where exaggerated MMP1 responses are observed in infected macrophages [31], leading to tissue destruction and highlighting MMP1 inhibition as a potential therapeutic target [30].

In summary, our work suggests that the exaggerated *MMP1* gene expression may play a key role in tissue destruction presented in CL, and that this expression is probably regulated by FLI1 but also by other factors such as TNF-α. Thus, our data points to the possibility that modulation of this pathway through therapeutic intervention might be relevant in the treatment and lesion resolution of CL. This alternative would be welcome in severe and less responsive forms to current the treatment available.

## Legends to figures

**Fig 1. Percentage FLI1 promoter-specific DNA methylation in lesion compared to normal skin biopsies.** Analysis of DNA methylation was performed using an EpiTect Methyl II PCR assay specific for the *FLI1* promoter CpG island (Reference EPHS102855-1A; Qiagen). Following real-time PCR, the data are expressed as percentage of methylated DNA. Results are plotted using Tukey Box and Whiskers plots. The band within the box shows the second quartile or median value. The P-value is for the non-parametric Mann-Whitney test comparing data for normal skin biopsies with cutaneous leishmaniasis (CL) lesion biopsies.

**Fig 2. Relative gene expression for (A) FLI1, (B) COL1A1, (C) COL1A2 and (D) MMP1 in lesion compared to normal skin biopsies.** Gene-specific mRNA levels were measured in biopsies using quantitative RT-PCR. Data are individual $2^{-\Delta\Delta CT}$ comparing relative expression in normal skin and lesion biopsies from the same individual. Bars are for median values and interquartile range.

**Fig 3. Percentage FLI1 promoter-specific DNA methylation in macrophages infected with *L. braziliensis*.** Analysis of DNA methylation was performed using an EpiTect Methyl II PCR assay specific for the *FLI1* promoter CpG island (Reference EPHS102855-1A; Qiagen). Following real-time PCR, the data are expressed as percentage of methylated DNA. DNA methylation was determined in uninfected macrophages, in macrophages infected

(designated CT for untreated with IL-6) for 3, 24, 48 and 72 hours in culture, in uninfected cells treated with IL-6, and in infected cells treated with IL-6. Results are plotted using Tukey Box and Whiskers plots. The band within the box shows the second quartile or median value. No significant differences in percentage methylation were observed either by (A) comparing across all time points, or (b) by pooling data for all infection time points.

**Fig 4. Relative gene expression for FLI1 in macrophages infected with *L. braziliensis*.** Gene-specific mRNA levels were measured in biopsies using quantitative RT-PCR. (A) shows the percent of donors for whom FLI1 expression was detectable by qRT-PCR. (B) Shows $2^{-\Delta\Delta CT}$ values for relative expression for donors where expression was detectable by qRT-PCT, in uninfected macrophages, in macrophages infected (designated CT for untreated with IL-6) for 3, 24, 48 and 72 hours in culture, in uninfected cells treated with IL-6, and in infected cells treated with IL-6. Results are plotted using Tukey Box and Whiskers plots. The band within the box shows the second quartile or median value. The P-values are for the non-parametric Mann-Whitney test comparing for pairwise comparisons as indicated.

**Fig 5. Relative gene expression for COL1A1 and COL1A2 in macrophages infected with *L. braziliensis*.** Gene-specific mRNA levels were measured in biopsies using quantitative RT-PCR. (A) and (B) show the percent of donors for whom COL1A1 and COL1A2 expression was detectable by qRT-PCR, respectively. (C)

and (D) show $2^{-\Delta\Delta CT}$ values for relative expression for donors where COL1A1 (C) and COL1A2 (D) expression was detectable by qRT-PCT, in uninfected macrophages, in macrophages infected (designated CT for untreated with IL-6) for 3, 24, 48 and 72 hours in culture, in uninfected cells treated with IL-6, and in infected cells treated with IL-6. Results are plotted using Tukey Box and Whiskers plots. The band within the box shows the second quartile or median value. The P-values are for the non-parametric Mann-Whitney test comparing for pairwise comparisons as indicated.

**Fig 6. Relative gene expression for MMP1 in macrophages infected with *L. braziliensis*.** Gene-specific mRNA levels were measured in biopsies using quantitative RT-PCR. (A) shows the percent of donors for whom MMP1 expression was detectable by qRT-PCR. (B) Shows $2^{-\Delta\Delta CT}$ values for relative expression for donors where expression was detectable by qRT-PCT, in uninfected macrophages, in macrophages infected (designated CT for untreated with IL-6) for 3, 24, 48 and 72 hours in culture, in uninfected cells treated with IL-6, and in infected cells treated with IL-6. Results are plotted using Tukey Box and Whiskers plots. The band within the box shows the second quartile or median value. The P-values are for the non-parametric Mann-Whitney test comparing for pairwise comparisons as indicated.

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Figure 1

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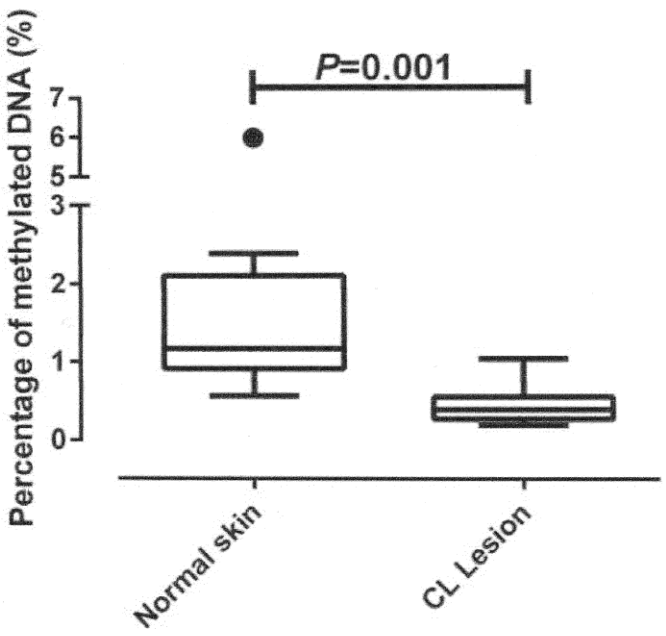

Figure 2

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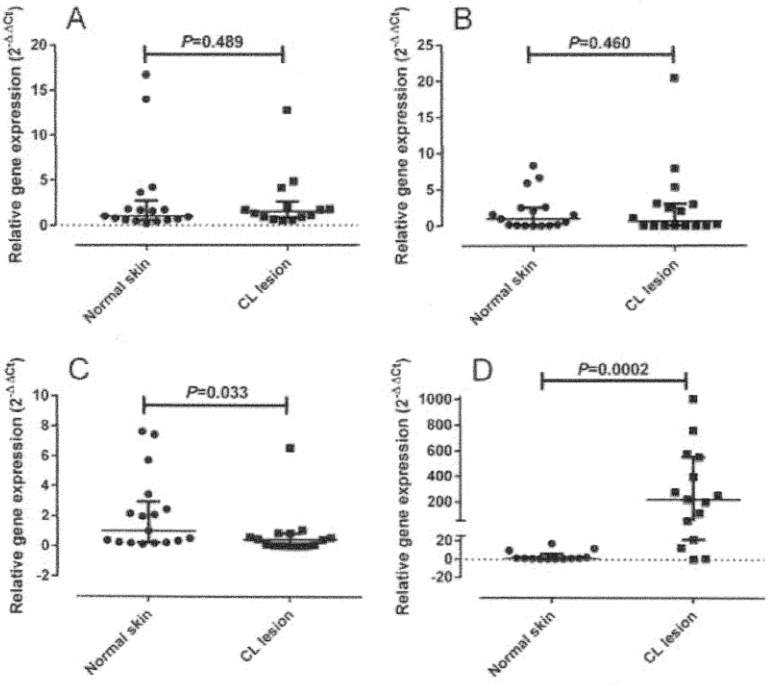

Figure 3

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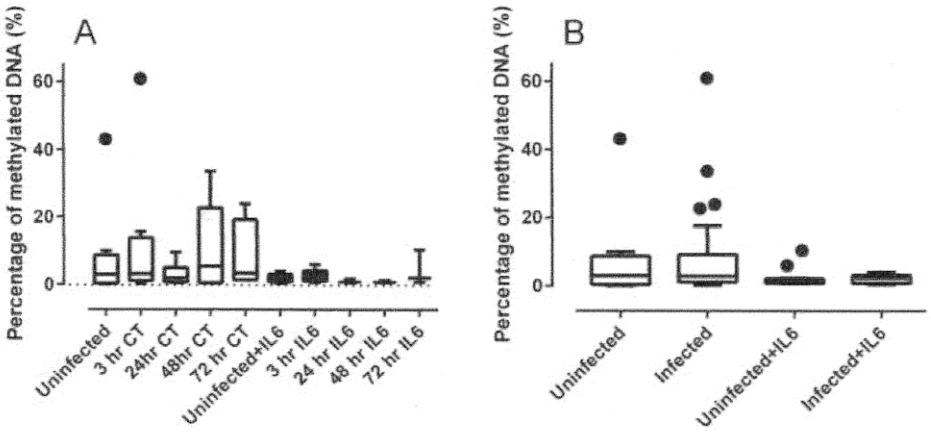

Figure 4

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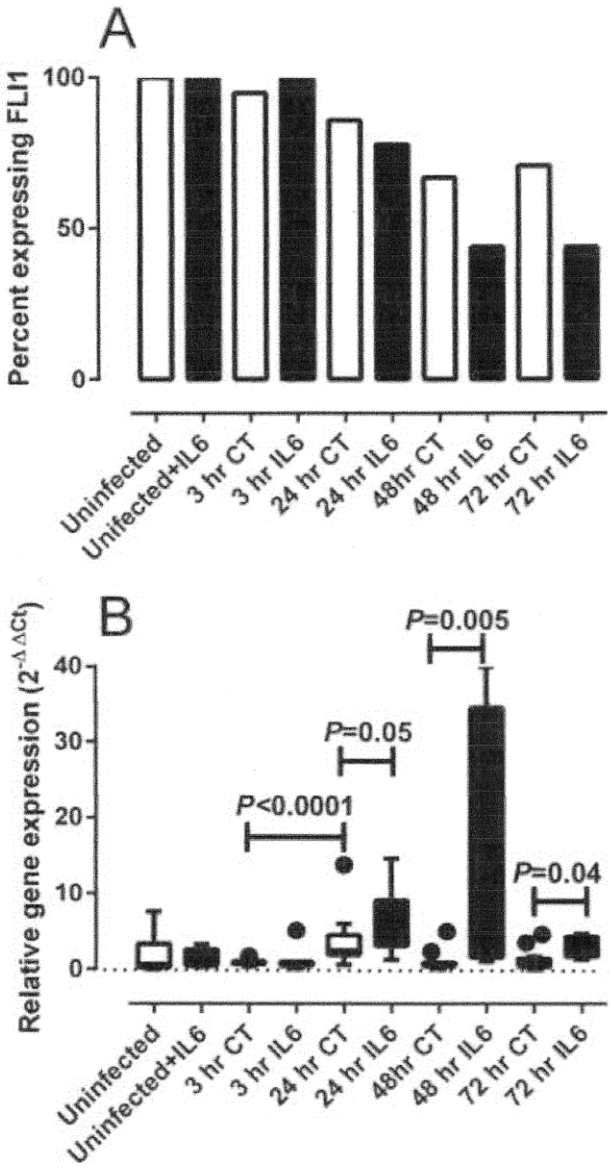

Figure 5

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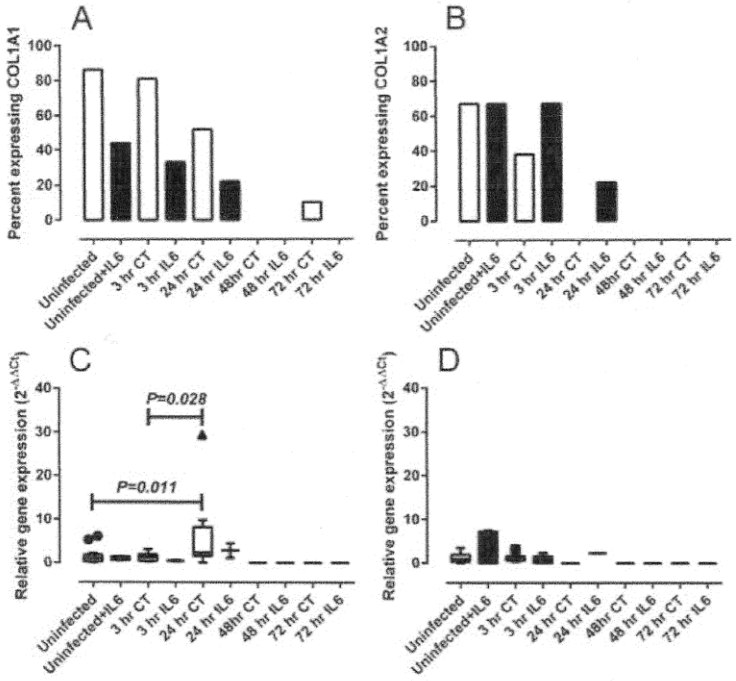

Figure 6

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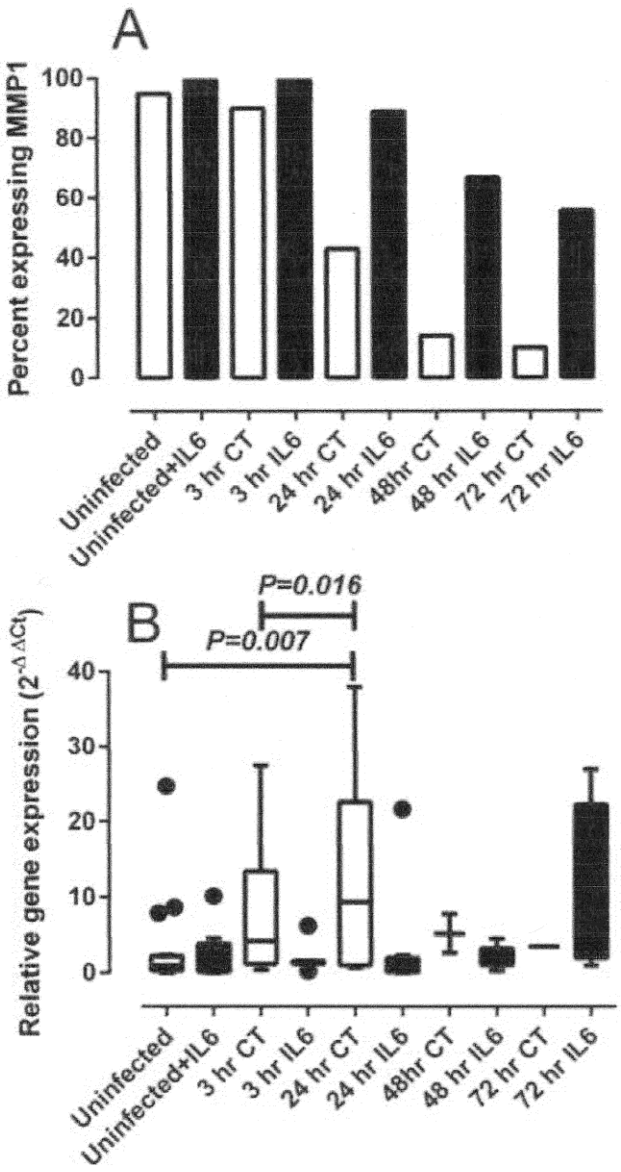

# VII. RESULTADOS GERAIS

## VII.1. CARACTERÍSTICAS DA COLEÇÃO DE FAMÍLIAS E PODER PARA DETECTAR ASSOCIAÇÃO GENÉTICA

O Quadro 1 fornece detalhes das amostras utilizadas neste estudo para avaliação do polimorfismo dos genes candidatos (*COL1A1*, *COL1A2* e *MMP1*) e sua possível associação com a leishmaniose tegumentar americana na população de Corte de Pedra, Bahia. As coletas de amostras realizadas durante os dois períodos diferentes de tempo, 2000-2004 e 2008-2010, foram equivalentes geograficamente (Castellucci et al., 2012) e demograficamente (Quadro 1). O Quadro 2 fornece detalhes dos SNPs genotipados, e demonstra que todos os SNPs tiveram uma FAM ≥ 0,25 na população estudada. Todos os SNPs estavam em Equilíbrio de Hardy Weinberg. Falha no procedimento de genotipagem variou de 0,80% (8/1008 indivíduos) a 2,5% (25/1008) entre os 8 SNPs genotipados. Aproximações do poder de TDT (Knapp, 1999) mostraram que o primeiro (250 casos de LC) e segundo (402 casos de LC) grupos de famílias tiveram 68% e 89% de poder, respectivamente, para detectar uma *odds ratio* ≥ 1,5 em um valor de P = 0,01 para os SNPs com FAM ≥ 0,3. Os 652 trios combinados tiveram 98,7% de poder para detectar associação para o mesmo tamanho de efeito, valor de P e FAM. Similarmente, para leishmaniose *per se* (LC + LM), estimativas de poder para os 337 trios do primeiro grupo de famílias, 441 trios do segundo grupo de famílias e 778 trios da análise combinada (primeiro + segundo grupos de famílias) foi de 91,9%, 91,9% e 99,6%, respectivamente. O poder para detectar associações com LM foi menor (≤ 25%) mesmo na análise combinada.

**Quadro 1**

Características das amostras coletadas durante o primeiro (2000-2004) e segundo (2008-2010) períodos para os estudos de associação genética.

| | Primeiro grupo de famílias (2000-2004) | | | Segundo grupo de famílias (2008-2010) | | |
|---|---|---|---|---|---|---|
| | LC | LM | Leishmaniose *per se* | LC | LM | Leishmaniose *per se* |
| Número de casos | 250 | 87 | 337 | 402 | 39 | 441 |
| Homens | 128 | 60 | 188 | 219 | 24 | 243 |
| Mulheres | 122 | 27 | 149 | 183 | 15 | 198 |
| Idade | | | | | | |
| Média | 19,1 | 30,3 | 22,4 | 21,5 | 26,6 | 21,9 |
| IC 95% | 17,1-21,2 | 25,8-34,7 | 20,3-24,4 | 20,1-22,9 | 20,7-32,4 | 20,6-23,3 |
| Número de famílias nucleares | | | 168 | | | 157 |
| *N* total nas famílias/trios | | | 767 | | | 764 |

**Quadro 2**

Informações sobre os polimorfismos de nucleotídeos simples genotipados para *COL1A1*, *COL1A2* e *MMP1* na população de Corte de Pedra.

| Gene/SNP | Localização | Posição física (Cro/pb) | Alelos | FAM (BA) | Caucasianos (CEU) | Africanos (YRI) |
|---|---|---|---|---|---|---|
| COL1A2_rs388625 | Íntron | 7:94396612 | A/G | 0,48 | 0,48 | 0,42 |
| COL1A2_rs11770203 | Íntron | 7:94402145 | G/T | 0,26 | 0,33 | 0,18 |
| MMP1_rs5854 | 3'UTR | 11:102790143 | A/G | 0,25 | 0,35 | 0,19 |
| MMP1_rs470747 | Íntron | 11:102790864 | A/G | 0,34 | 0,35 | 0,35 |
| MMP1_rs7125062 | Íntron | 11:102792772 | C/T | 0,33 | 0,27 | 0,35 |
| COL1A1_rs1061237 | 3'UTR | 17:50185414 | C/T | 0,30 | 0,31 | 0,42 |
| COL1A1_rs2586488 | Íntron | 17:50188065 | A/G | 0,37 | 0,37 | 0,42 |
| COL1A1_rs2075554 | Íntron | 17:50196948 | C/T | 0,26 | 0,19 | 0,26 |

## VII.2. AVALIAÇÃO DA ASSOCIAÇÃO ENTRE OS SNPs DE *COL1A1*, *COL1A2* E *MMP1* E O FENÓTIPO LEISHMANIOSE CUTÂNEA

Nenhuma associação foi observada entre LC e os SNPs dos genes *COL1A2* e *MMP1* utilizados neste estudo. Os resultados da análise de FBAT entre os SNPs de *COL1A1* e LC estão apresentados no Quadro 3. Análises sob o modelo aditivo foram suficientes para explicar os resultados. O SNP rs1061237 mostrou uma tendência de associação (P = 0,058) no primeiro grupo de famílias, que possui o menor número de casos, e alcançou uma associação significante (P = 0,018) no segundo grupo de famílias, associação confirmada na análise combinada (P = 0,002), que resistiu a aplicação de um fator de correção rigoroso para os 8 SNPs genotipados. O SNP rs2586488 também mostrou evidência de associação (P < 0,05) no segundo grupo de famílias e na análise combinada. Isto é consistente com o forte DL (D' 0,78; $r^2$ 0,55) entre este dois SNPs, como mostrado na Figura 2. Em contraste, não houve nenhuma evidência de associação entre LC e o SNP rs2075554. Em adição, não há DL entre este SNP e os outros dois associados com a doença.

**Quadro 3**

Resultados do teste de associação baseado em família utilizando modelo aditivo de herança entre os SNPs de *COL1A1* e a LC causada por *Leishmania braziliensis*.

| Gene/SNP | Alelo | Frequência alélica | Número de famílias | Var(S) | Z | P |
|---|---|---|---|---|---|---|
| Primeiro grupo de famílias | | | | | | |
| COL1A1_rs1061237 | C | 0,64 | 64 | 8,07 | 1,90 | 0,058 |
| COL1A1_rs1061237 | T | 0,36 | 64 | 8,07 | -1,90 | 0,058 |
| COL1A1_rs2586488 | G | 0,59 | 64 | 8,70 | 0,74 | 0,462 |
| COL1A1_rs2586488 | A | 0,41 | 64 | 8,70 | -0,74 | 0,462 |
| COL1A1_rs2075554 | C | 0,74 | 56 | 6,14 | 0,87 | 0,382 |
| COL1A1_rs2075554 | T | 0,26 | 56 | 6,14 | -0,87 | 0,382 |
| Segundo grupo de famílias | | | | | | |
| COL1A1_rs1061237 | C | 0,66 | 93 | 6,72 | 2,36 | 0,018 |
| COL1A1_rs1061237 | T | 0,34 | 93 | 6,72 | -2,36 | 0,018 |
| COL1A1_rs2586488 | G | 0,64 | 88 | 5,77 | 2,30 | 0,021 |
| COL1A1_rs2586488 | A | 0,36 | 88 | 5,77 | -2,30 | 0,021 |
| COL1A1_rs2075554 | C | 0,75 | 78 | 6,44 | 0,61 | 0,542 |
| COL1A1_rs2075554 | T | 0,25 | 78 | 6,44 | -0,61 | 0,542 |
| Análise combinada | | | | | | |
| COL1A1_rs1061237 | C | 0,66 | 157 | 14,84 | 3,07 | 0,002 |
| COL1A1_rs1061237 | T | 0,34 | 157 | 14,84 | -3,07 | 0,002 |
| COL1A1_rs2586488 | G | 0,63 | 152 | 14,66 | 2,21 | 0,027 |
| COL1A1_rs2586488 | A | 0,37 | 152 | 14,66 | -2,21 | 0,027 |
| COL1A1_rs2075554 | C | 0,75 | 134 | 12,61 | 1,08 | 0,279 |
| COL1A1_rs2075554 | T | 0,25 | 134 | 12,61 | -1,08 | 0,279 |

Var(S) significa variância. Valor de Z positivo indica associação com a doença. Valor de Z negativo indica alelo protetor.

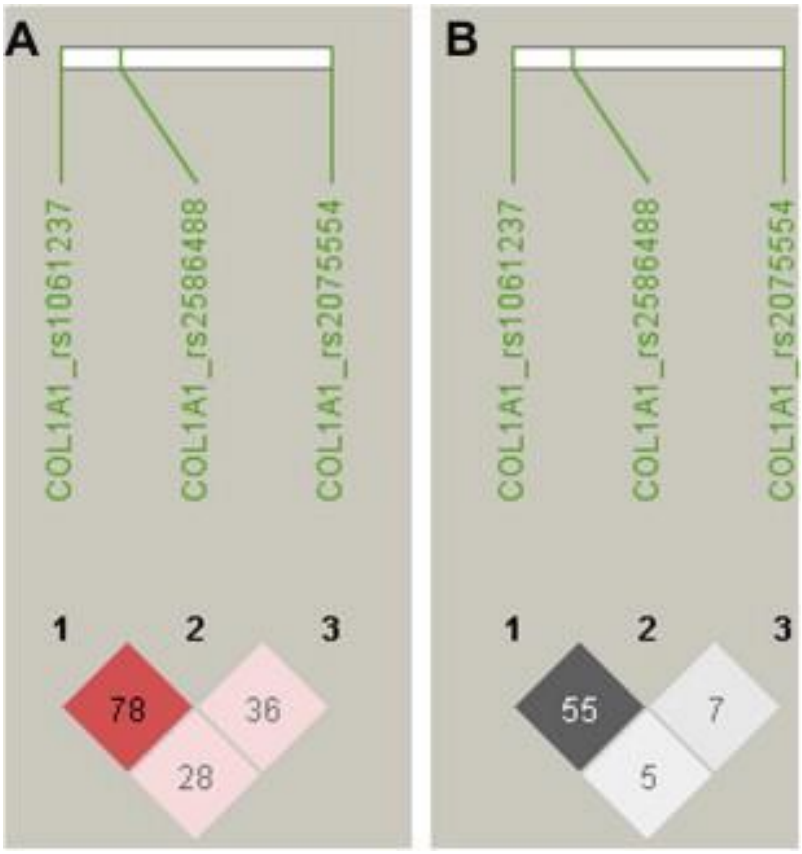


**Figura 2** Padrões de desequilíbrio de ligação (DL) para D' (A) e $r^2$ (B) para os 3 SNPs de *COL1A1* utilizados nas análises de associação. Os números dentro dos quadrados representam os valores de D' e $r^2$ para o DL, e mostrou DL de moderado a forte entre os SNPs rs1061237 e rs2586488.

## VII.3. AVALIAÇÃO DA ASSOCIAÇÃO ENTRE OS SNPs DE *COL1A1*, *COL1A2* E *MMP1* E OS FENÓTIPOS LEISHMANIOSE MUCOSA E LEISHMANIOSE *PER SE*

Novamente, nenhuma associação foi observada entre LM, ou leishmaniose *per se*, e os SNPs dos genes *COL1A2* e *MMP1*. Por sua vez, a análise de FBAT entre os SNPs de *COL1A1* e LM e leishmaniose *per se* é apresentada nos Quadros 4 e 5 respectivamente. De interessante, uma tendência para associação (P = 0,079) entre o SNP rs2586488 e LM no segundo grupo de famílias mostrou o alelo mais raro como alelo de risco (Quadro 4), enquanto para LC (Quadro 3) o alelo mais comum foi o alelo de risco, assim como ocorreu anteriormente com outros genes de cura de lesão previamente analisados (Castellucci et al., 2011, 2012).

Com relação ao fenótipo leishmaniose *per se*, formado pela reunião dos casos de LC e LM, os resultados encontrados foram semelhantes aos resultados encontrados para o fenótipo leishmaniose cutânea. Nesta análise, mais uma vez os SNPs rs1061237 e rs2586488 mostram associação no segundo grupo de famílias e na análise combinada (Quadro 5). Porém, esta associação foi um pouco mais fraca, provavelmente influenciada pela presença dos casos de LM.

**Quadro 4**

Resultados do teste de associação baseado em família utilizando modelo aditivo de herança entre os SNPs de *COL1A1* e a LM causada por *Leishmania braziliensis*.

| Gene/SNP | Alelo | Frequência alélica | Número de famílias | Var(S) | Z | P |
|---|---|---|---|---|---|---|
| Primeiro grupo de famílias | | | | | | |
| COL1A1_rs1061237 | C | 0,64 | 62 | 12,26 | 0,17 | 0,863 |
| COL1A1_rs1061237 | T | 0,36 | 62 | 12,26 | -0,17 | 0,863 |
| COL1A1_rs2586488 | A | 0,41 | 62 | 13,69 | -0,11 | 0,911 |
| COL1A1_rs2586488 | G | 0,59 | 62 | 13,69 | 0,11 | 0,911 |
| COL1A1_rs2075554 | C | 0,74 | 55 | 10,00 | -0,38 | 0,707 |
| COL1A1_rs2075554 | T | 0,26 | 55 | 10,00 | 0,38 | 0,707 |
| Segundo grupo de famílias | | | | | | |
| COL1A1_rs1061237 | C | 0,66 | 101 | 5,69 | -0,73 | 0,464 |
| COL1A1_rs1061237 | T | 0,34 | 101 | 5,69 | 0,73 | 0,464 |
| COL1A1_rs2586488 | A | 0,36 | 96 | 6,11 | 1,76 | 0,079 |
| COL1A1_rs2586488 | G | 0,64 | 96 | 6,11 | -1,76 | 0,079 |
| COL1A1_rs2075554 | C | 0,75 | 81 | 4,57 | -0,60 | 0,551 |
| COL1A1_rs2075554 | T | 0,25 | 81 | 4,57 | 0,60 | 0,551 |
| Análise combinada | | | | | | |
| COL1A1_rs1061237 | C | 0,66 | 163 | 18,23 | -0,48 | 0,633 |
| COL1A1_rs1061237 | T | 0,34 | 163 | 18,23 | 0,48 | 0,633 |
| COL1A1_rs2586488 | A | 0,37 | 158 | 20,13 | 1,07 | 0,285 |
| COL1A1_rs2586488 | G | 0,63 | 158 | 20,13 | -1,07 | 0,285 |
| COL1A1_rs2075554 | C | 0,75 | 136 | 14,96 | -0,80 | 0,423 |
| COL1A1_rs2075554 | T | 0,25 | 136 | 14,96 | 0,80 | 0,423 |

Var(S) significa variância. Valor de Z positivo indica associação com a doença. Valor de Z negativo indica alelo protetor.

**Quadro 5**

Resultados do teste de associação baseado em família utilizando modelo aditivo de herança entre os SNPs de *COL1A1* e a leishmaniose *per se* (LC + LM) causada por *Leishmania braziliensis*.

| Gene/SNP | Alelo | Frequência alélica | Número de famílias | Var(S) | Z | P |
|---|---|---|---|---|---|---|
| Primeiro grupo de famílias | | | | | | |
| COL1A1_rs1061237 | C | 0,64 | 56 | 8,70 | 1,952 | 0,051 |
| COL1A1_rs1061237 | T | 0,36 | 56 | 8,70 | -1,952 | 0,051 |
| COL1A1_rs2586488 | A | 0,41 | 57 | 9,57 | -0,75 | 0,453 |
| COL1A1_rs2586488 | G | 0,59 | 57 | 9,57 | 0,75 | 0,453 |
| COL1A1_rs2075554 | C | 0,74 | 53 | 6,76 | 0,842 | 0,400 |
| COL1A1_rs2075554 | T | 0,26 | 53 | 6,76 | -0,842 | 0,400 |
| Segundo grupo de famílias | | | | | | |
| COL1A1_rs1061237 | C | 0,66 | 104 | 7,32 | 1,988 | 0,047 |
| COL1A1_rs1061237 | T | 0,34 | 104 | 7,32 | -1,988 | 0,047 |
| COL1A1_rs2586488 | A | 0,36 | 99 | 6,48 | -1,92 | 0,055 |
| COL1A1_rs2586488 | G | 0,64 | 99 | 6,48 | 1,92 | 0,055 |
| COL1A1_rs2075554 | C | 0,75 | 83 | 7,04 | 0,276 | 0,783 |
| COL1A1_rs2075554 | T | 0,25 | 83 | 7,04 | -0,276 | 0,783 |
| Análise combinada | | | | | | |
| COL1A1_rs1061237 | C | 0,66 | 160 | 16,04 | 2,834 | 0,005 |
| COL1A1_rs1061237 | T | 0,34 | 160 | 16,04 | -2,834 | 0,005 |
| COL1A1_rs2586488 | A | 0,37 | 156 | 16,12 | -1,883 | 0,060 |
| COL1A1_rs2586488 | G | 0,63 | 156 | 16,12 | 1,883 | 0,060 |
| COL1A1_rs2075554 | C | 0,75 | 136 | 13,80 | 0,797 | 0,425 |
| COL1A1_rs2075554 | T | 0,25 | 136 | 13,80 | -0,797 | 0,425 |

Var(S) significa variância. Valor de Z positivo indica associação com a doença. Valor de Z negativo indica alelo protetor.

## VII.4. A METILAÇÃO DO GENE *FLI1* É DIMINUÍDA EM BIÓPSIAS DE LESÕES DE LEISHMANIOSE CUTÂNEA

Estudos prévios têm demonstrado um alto grau de metilação de ilhas CpG na região promotora do gene *FLI1* em biópsias de pele de pacientes com esclerose sistêmica (Wang et al., 2006). Para determinar se o padrão de metilação na região promotora de *FLI1* é similarmente alterado em lesões associadas com leishmaniose cutânea causada pela infecção por *Leishmania braziliensis*, inicialmente nós comparamos a metilação da região promotora de *FLI1* em DNA obtido de biópsias de lesões de leishmaniose cutânea *versus* biópsias de pele normal (Figura 3). Embora a porcentagem de DNA metilado na região promotora de *FLI1* tenha sido baixa (< 10%), nossos resultados mostram que biópsias de pele normal apresentaram uma maior porcentagem de DNA metilado (P = 0,001) comparadas com as biópsias de lesão de leishmaniose cutânea. Isto sugere que a patologia associada com leishmaniose cutânea resulta na não metilação de ilhas CpG na região promotora do gene *FLI1*, tornando abertas potenciais regiões regulatórias à jusante o sítio de início de transcrição do gene.

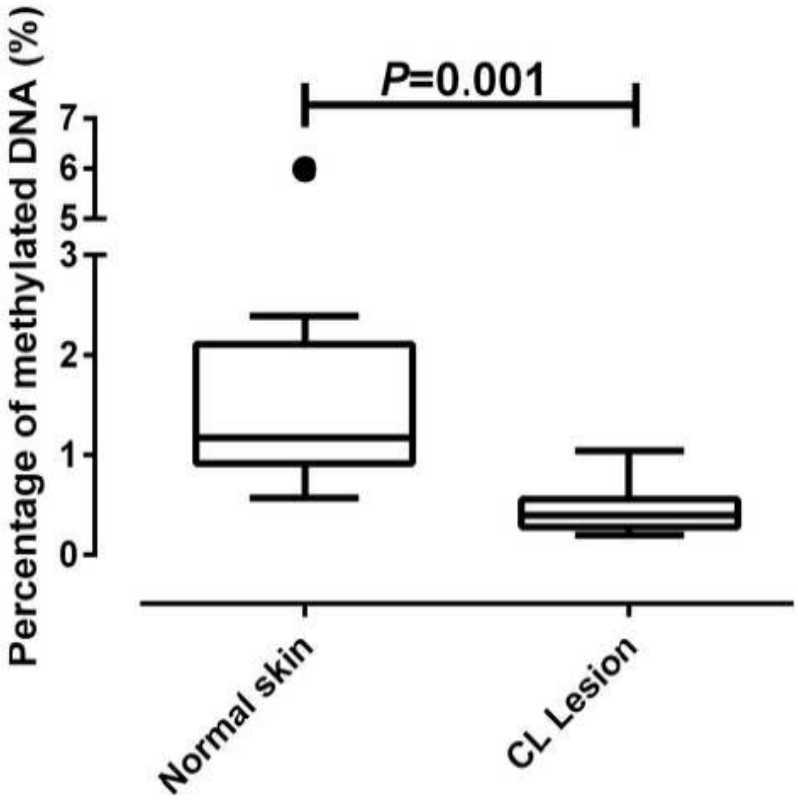


**Figura 3** Porcentagem de DNA metilado na região promotora do gene FLI1 em biópsias de lesão de leishmaniose cutânea comparadas com biópsias de pele normal. Os resultados estão representados utilizando Tukey Box e Whiskers plots. A faixa dentro das caixas representa o segundo quartil ou valor da mediana. O valor de P é para o teste não paramétrico de Mann Whitney comparando os dados de biópsias de pele normal com biópsias de lesão de leishmaniose cutânea.

## VII.5. ANÁLISE DA EXPRESSÃO DE *FLI1* E DE GENES SOB A SUA REGULAÇÃO EM BIÓPSIAS DE PELE

Para determinar se a patogênese da leishmaniose cutânea *in vivo* é também acompanhada pela alteração da expressão gênica de *FLI1* e de genes que estão sob a sua regulação (*COL1A1*, *COL1A2* e *MMP1*), os níveis de mRNA foram comparados entre biópsias de lesões de leishmaniose cutânea e amostras pareadas a partir biópsias de pele normal distante da lesão (Figura 4). Não houve diferença significante na expressão de mRNA para *FLI1* (Figura 4A) e *COL1A1* (Figura 4B) entre as biópsias de lesão de leishmaniose cutânea e as biópsias de pele normal. Porém, a expressão de *COL1A2* (Figura 4C; P = 0,033) foi significantemente mais baixa nas biópsias de lesão de leishmaniose cutânea, enquanto a expressão de *MMP1* (Figura 4D; P = 0,0002) foi significantemente maior nas biópsias de lesão de leishmaniose cutânea, comparadas com as biópsias de pele normal.

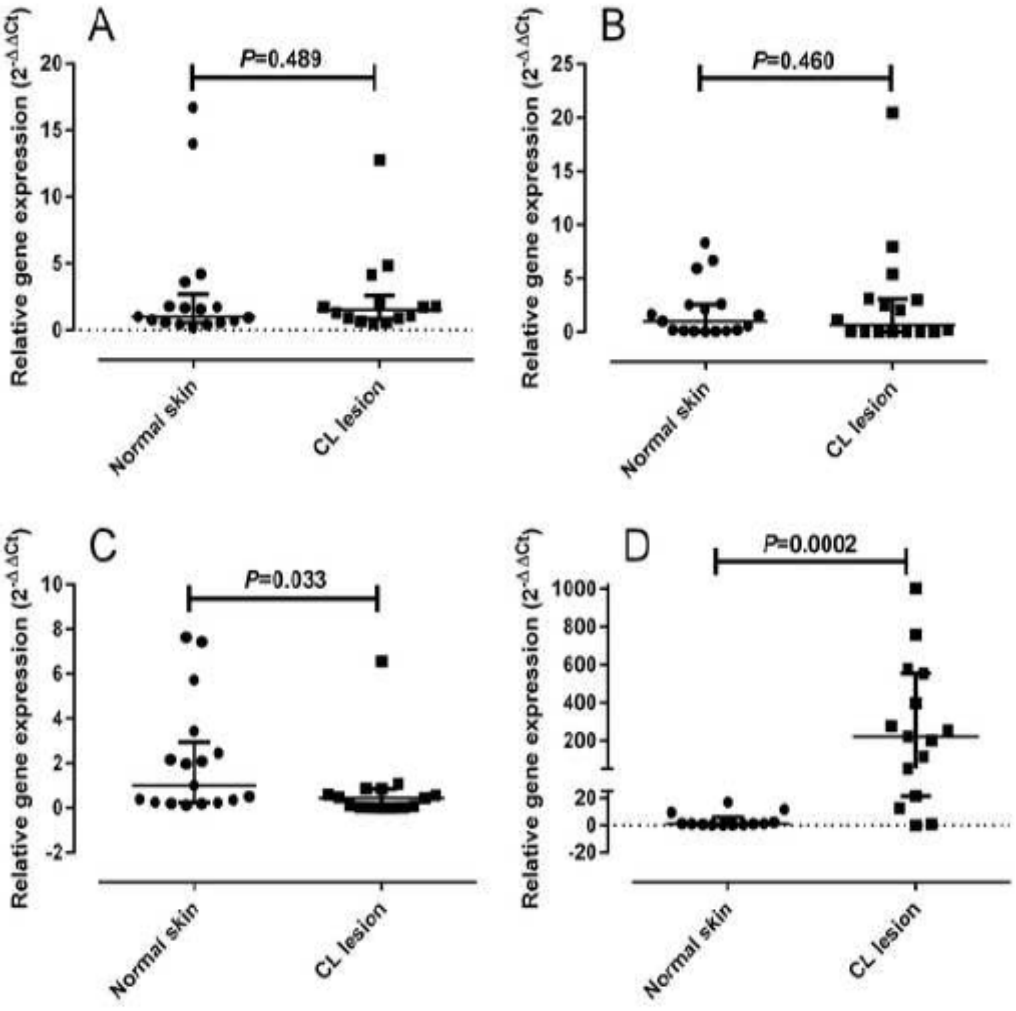


**Figura 4** Expressão gênica relativa de (A) *FLI1*, (B) *COL1A1*, (C) *COL1A2* e (D) *MMP1* em biópsias de lesão de leishmaniose cutânea comparadas com biópsias de pele normal. As barras representam os valores de mediana e o intervalo interquartil.

## VII.6. A INFECÇÃO POR *LEISHMANIA BRAZILIENSIS* NÃO ALTERA O PADRÃO DE METILAÇÃO DO GENE *FLI1* EM MACRÓFAGOS DERIVADOS DE PBMC

Para determinar em que medida a redução do grau de metilação na região promotora do gene *FLI1* observada *ex vivo* em biópsias de lesão de leishmaniose cutânea deveu-se a efeitos diretos do parasita na sua célula hospedeira preferida, nós também avaliamos a porcentagem de DNA metilado na região promotora de *FLI1* em macrófagos derivados de PBMC após 0, 3, 24, 48 e 72 horas de infecção por *Leishmania braziliensis*. Apesar de ter sido observada uma tendência para o aumento da metilação de ilhas CpG na região promotora de *FLI1* com o tempo após a infecção (Figura 5), esta observação não alcançou significância estatística, seja analisada em diferentes pontos de tempo (Figura 5A amostras CT; Kruskal Wallis P = 0,816), ou quando os dados foram reunidos para comparar macrófagos infectados e não infectados em todos os períodos de tempo (Figura 5B; Mann Whitney P = 0,471 para infectados *versus* não infectados).

Uma vez que nós observamos que polimorfismos em *FLI1* e *IL6* são fatores genéticos de risco para leishmaniose (Castellucci et al., 2006, 2011), e que a IL-6 é conhecida por aumentar a expressão gênica de *FLI1* (Thaler et al., 2011), nós, a seguir, avaliamos se a presença de IL-6 em culturas de macrófagos derivados de PBMC infectados influenciaria a porcentagem de ilhas CpG metiladas na região promotora do gene *FLI1*. Novamente, enquanto houve uma tendência para uma menor metilação do gene *FLI1* em todos os tempos com a presença de IL-6 nas culturas (Figura 5), nenhuma das diferenças entre macrófagos tratados e não tratados com IL-6 alcançou significância estatística.

Em geral, os resultados de metilação de ilhas CpG na região promotora do gene *FLI1* observados em culturas de macrófagos (tendência para o aumento da metilação com o tempo de infecção) não suportaram os dados *ex vivo* comparando biópsias de lesão de leishmaniose cutânea e biópsias de pele normal (metilação diminuída com a infecção), sugerindo um processo de reparo tecidual mais complexo envolvendo outros tipos celulares e/ou que a presença de IL-6 pode estar envolvida *in vivo*.

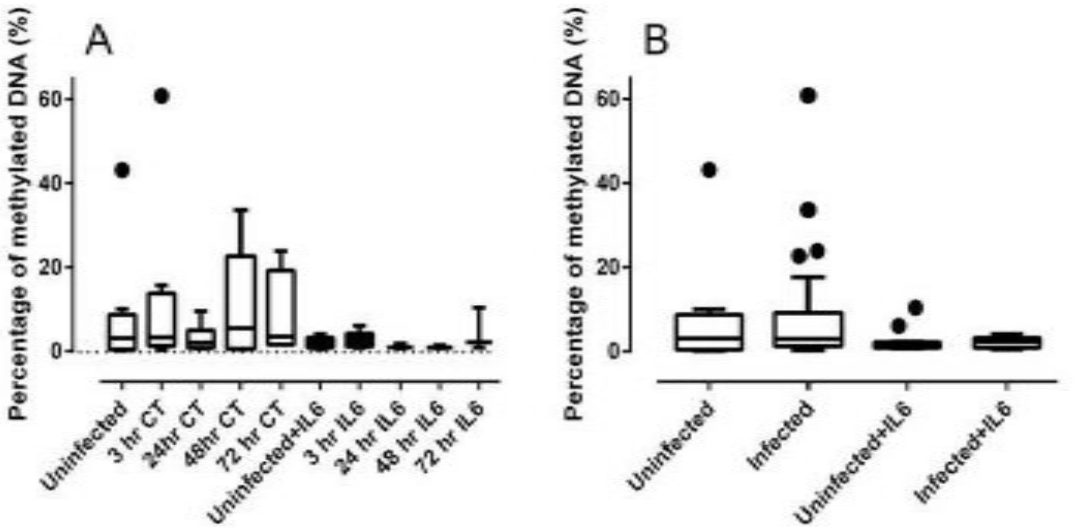


**Figura 5** Porcentagem de DNA metilado na região promotora do gene *FLI1* em macrófagos infectados por *Leishmania braziliensis*. A metilação do DNA foi determinada em macrófagos não infectados, macrófagos infectados (nomeados CT para macrófagos não tratados com IL-6) por 3, 24, 48 e 72 horas, macrófagos não infectados tratados com IL-6, e macrófagos infectados e tratados com IL-6. Os resultados estão representados utilizando Tukey Box e Whiskers plots. A faixa dentro das caixas representa o segundo quartil ou valor da mediana.

## VII.7. IL-6 AUMENTA A EXPRESSÃO GÊNICA DE *FLI1* EM MACRÓGAGOS DERIVADOS DE PBMC INFECTADOS POR *LEISHMANIA BRAZILIENSIS*

Em geral, a porcentagem de DNA metilado na região promotora de *FLI1* em lesões de leishmaniose cutânea e em culturas de macrófagos foi baixa, especialmente na presença de IL-6 em culturas de macrófagos *in vitro*, sugerindo que regiões regulatórias potenciais à jusante o sítio de início de transcrição do gene *FLI1* estão abertas para regulação da expressão do gene. Para determinar se a infecção de macrófagos por *Leishmania braziliensis* diretamente influencia a expressão gênica de *FLI1*, a seguir nós avaliamos a sua expressão em macrófagos derivados de PBMC infectados *in vitro* na presença e ausência de IL-6. A Figura 6A mostra que a porcentagem de doadores para os quais a expressão de *FLI1* foi detectada por qRT-PCR diminuiu com o tempo de infecção, um efeito que foi mais evidente na presença de IL-6. Isto pode refletir a perda de células por morte ou apoptose (Getti et al., 2008), apesar de quantidades equivalentes de mRNA terem sido utilizadas em todos os ensaios e a expressão gênica ter sido calculada relativa à quantidade específica do controle endógeno beta-actina para cada ponto de tempo. Para os doadores em que a expressão de *FLI1* foi detectada (Figura 6B), a expressão de *FLI1* atingiu um pico após 24 horas de infecção ($P < 0,0001$ 24hr CT *versus* 3hr CT) na ausência de IL-6, enquanto a expressão de *FLI1* na presença de IL-6 atingiu um pico após 48 horas de infecção e foi significantemente mais alta em culturas tratadas com IL-6 *versus* culturas não tratadas após 24 ($P = 0.05$), 48 ($P = 0,005$) e 72 horas de infecção ($P = 0,04$). Assim, nossos dados são consistentes com a nossa expectativa de que o gene *FLI1* estaria aberto devido a baixa metilação de ilhas CpG na sua região promotora, e que a IL-6 aumentaria a expressão de *FLI1*.

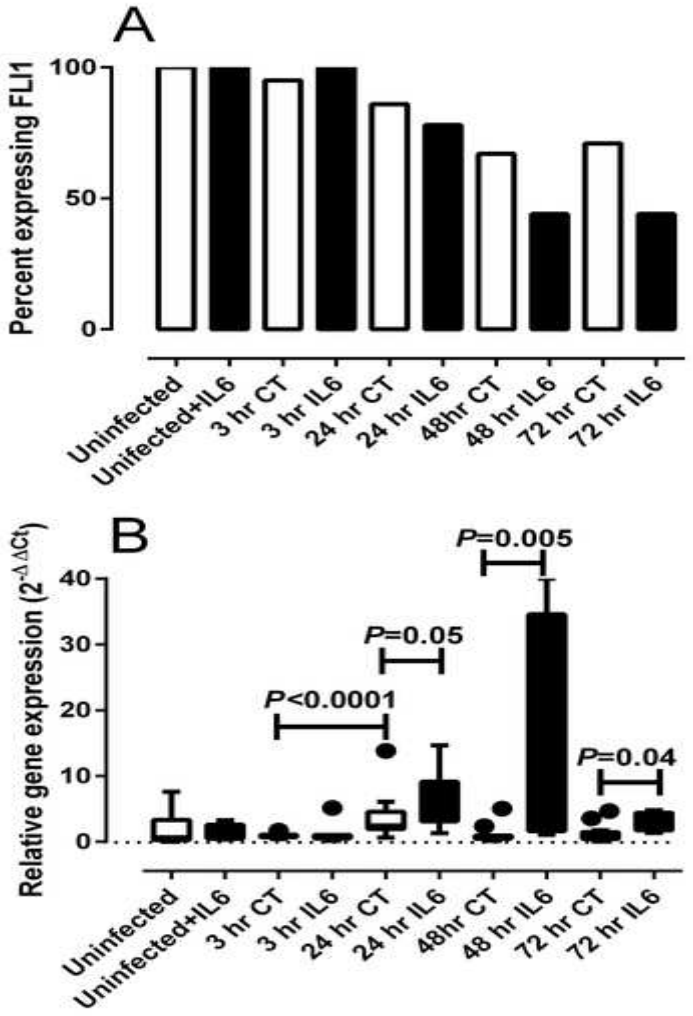


**Figura 6** Expressão gênica relativa de *FLI1* em macrófagos infectados por *Leishmania braziliensis*. (A) mostra a porcentagem de doadores para os quais a expressão de *FLI1* foi detectável por qRT-PCR. (B) mostra os valores de $2^{-\Delta\Delta Ct}$ de expressão gênica relativa para doadores tiveram expressão detectável por qRT-PCR em macrófagos não infectados, macrófagos infectados (nomeados CT para macrófagos não tratados com IL-6) por 3, 24, 48 e 72 horas, macrófagos não infectados tratados com IL-6, e macrófagos infectados e tratados com IL-6. Os resultados estão representados utilizando Tukey Box e Whiskers plots. A faixa dentro das caixas representa o segundo quartil ou valor da mediana. O valor de P é para o teste não paramétrico de Mann Whitney para comparações de pares como indicado.

## VII.8. EXPRESSÃO DE GENES REGULADOS POR *FLI1*, *COL1A1*, *COL1A2* e *MMP1*, EM MACRÓFAGOS DERIVADOS DE PBMC INFECTADOS POR *LEISHMANIA BRAZILIENSIS*

*FLI1* é conhecido por regular a expressão de outros genes envolvidos no processo de cura de lesão. Especificamente, se a expressão gênica de *FLI1* é reduzida, a expressão de *COL1A1* e *COL1A2* aumenta, enquanto a expressão de mRNA de *MMP1* diminui (Nakerakanti et al., 2006). Por outro lado, se a expressão de *FLI1* é alta, é esperado que as expressões de *COL1A1* e *COL1A2* sejam baixas, e a expressão de *MMP1* seja alta. Consistente com esta predição, nós encontramos que a porcentagem de doadores que expressaram *COL1A1* (Figura 7A) e *COL1A2* (Figura 7B) em culturas de macrófagos foi baixa comparada com a porcentagem de doadores que expressaram *MMP1* (Figura 8A), uma observação também refletida nos níveis específicos de mRNA de *COL1A1* (Figura 7C) e *COL1A2* (Figura 7D) comparado com os níveis de *MMP1* (Figura 8B) em doadores com expressões detectáveis por qRT-PCR. mRNA específico de *COL1A1* foi regulado positivamente pela infecção por *Leishmania braziliensis*, com um pico após 24 horas de infecção (P = 0,011 não infectado *versus* 24hr CT; P = 0,028 3hr CT *versus* 24hr CT), mas não foi detectável na presença de IL-6, que aumentou os níveis de expressão de *FLI1*. A expressão de *COL1A2* foi baixa em todos os tratamentos. De maneira interessante, enquanto os níveis específicos de mRNA de *MMP1* foram regulados positivamente com a infecção (Figura 8B), com um pico após 24 horas de infecção (P = 0,007 não infectado *versus* 24 hr CT; P = 0,016 3 hr CT *versus* 24 hr CT), a sua expressão foi baixa na presença de IL-6 (e, portanto, alta expressão de FLI1), aumentando somente após 72 horas de infecção, quando a expressão de FLI1 diminuiu (ver Figura 6).

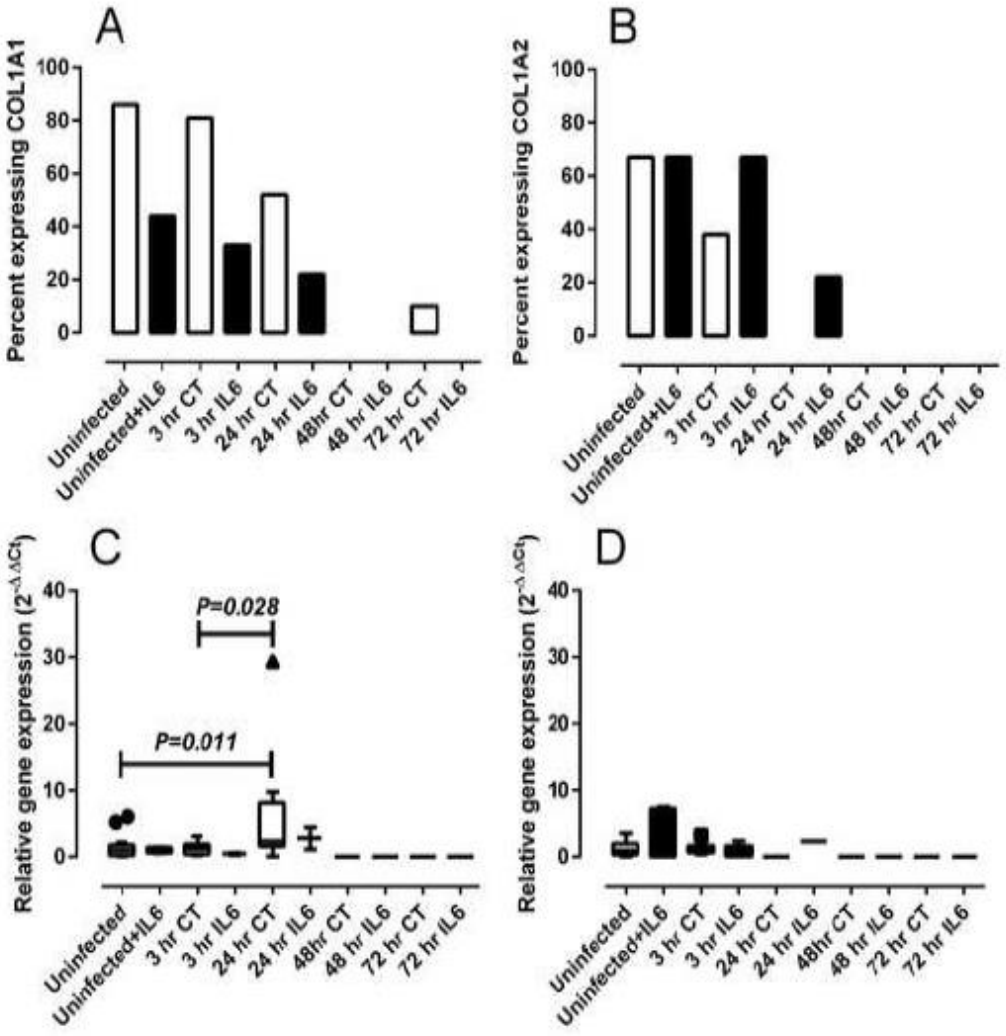


**Figura 7** Expressão gênica relativa de *COL1A1* e *COL1A2* em macrófagos infectados por *Leishmania braziliensis*. (A) e (B) mostram a porcentagem de doadores para os quais as expressões de *COL1A1* e *COL1A2* foram detectáveis por qRT-PCR respectivamente. (C) e (D) mostram os valores de $2^{-\Delta\Delta Ct}$ de expressão gênica relativa para doadores tiveram expressão de *COL1A1* (C) e *COL1A2* (D) detectáveis por qRT-PCR em macrófagos não infectados, macrófagos infectados (nomeados CT para macrófagos não tratados com IL-6) por 3, 24, 48 e 72 horas, macrófagos não infectados tratados com IL-6, e macrófagos infectados e tratados com IL-6. Os resultados estão representados utilizando Tukey Box e Whiskers plots. A faixa dentro das caixas representa o segundo quartil ou valor da mediana. O valor de P é para o teste não paramétrico de Mann Whitney para comparações de pares como indicado.

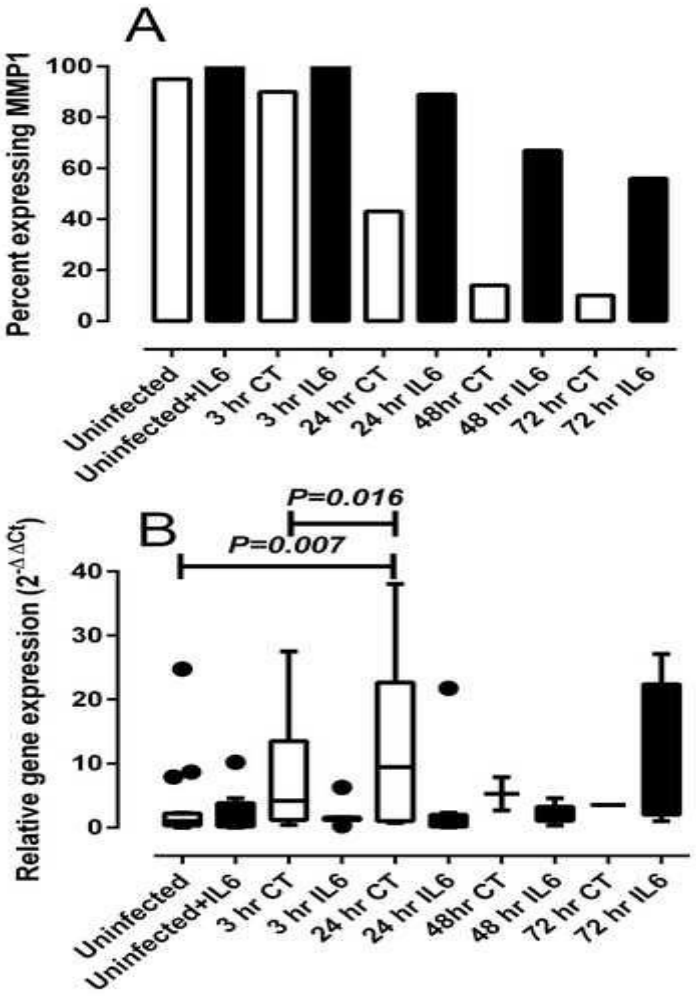


**Figura 8** Expressão gênica relativa de *MMP1* em macrófagos infectados por *Leishmania braziliensis*. (A) mostra a porcentagem de doadores para os quais a expressão de *MMP1* foi detectável por qRT-PCR. (B) mostra os valores de $2^{-\Delta\Delta Ct}$ de expressão gênica relativa para doadores tiveram expressão detectável por qRT-PCR em macrófagos não infectados, macrófagos infectados (nomeados CT para macrófagos não tratados com IL-6) por 3, 24, 48 e 72 horas, macrófagos não infectados tratados com IL-6, e macrófagos infectados e tratados com IL-6. Os resultados estão representados utilizando Tukey Box e Whiskers plots. A faixa dentro das caixas representa o segundo quartil ou valor da mediana. O valor de P é para o teste não paramétrico de Mann Whitney para comparações de pares como indicado.

# VIII. DISCUSSÃO

Estudos de associação genética constituem uma poderosa ferramenta utilizada para avaliar importantes vias envolvidas na patogênese de diversas doenças. Ao longo dos últimos anos, dados gerados utilizando esta abordagem contribuíram para o entendimento da susceptibilidade às formas cutânea e mucosa de leishmaniose causadas por *Leishmania braziliensis*. Estes estudos descrevem biomarcadores relacionados à resposta imune e ao dano tecidual na leishmaniose (Castellucci et al., 2006, 2010, 2011, 2012). No presente estudo, nós fornecemos evidências de associação entre variantes polimórficas do gene *COL1A1* e leishmaniose cutânea causada por *Leishmania braziliensis*. Este dado se junta a estudos prévios sobre o papel dos genes de cura de lesão no desfecho da doença (Castellucci et al., 2011, 2012), reforçando os estudos murinos anteriores que destacaram o papel da via de cicatrização na resolução das formas cutâneas da leishmaniose (Sakthianandeswaren et al., 2005, 2009). Nenhuma associação foi observada entre LTA e os SNPs dos genes *COL1A2* e *MMP1* utilizados neste estudo. Porém, isto não exclui a possibilidade de que associações poderiam ter sido observadas se outros SNPs de cobertura mais densa destes dois genes fossem avaliados.

O colágeno do tipo 1 é um importante componente da matriz extracelular na lesão fibrótica. O remodelamento da matriz extracelular é um processo complexo e altamente regulado que ocorre durante o reparo tecidual. Em muitas condições patológicas o balanço entre a síntese e a degradação desta matriz é rompido, levando a um remodelamento anormal (Friedman, 1993; Vincenti et al., 1994; Border et al., 1996; Trojanowska et al., 1998; Curran and Murray, 1999; Forget et al., 1999; Malemud & Goldberg, 1999). O

reparo tecidual na leishmaniose depende de uma resposta imune balanceada, assim como da cooperação entre elementos da matriz e colágenos. Adicionalmente, estudos prévios sugerem que o remodelamento do colágeno do tipo 1 por promastigotas pode desempenhar um importante papel durante o processo de infecção (Stamenkovic, 2003; Larsen et al., 2006; Petropolis et al., 2014).

Apesar da importância da resposta imunológica ter sido bem estabelecida na eliminação da infecção, os mecanismos envolvidos no dano tecidual e resolução das úlceras precisam ser melhores compreendidos. Por exemplo, precisa ser esclarecido o que acontece após a infecção se a promastigota de *Leishmania* passa através da matriz extracelular da derme, permanecendo aí até o primeiro contato com macrófago ou outra célula hospedeira (Lira et al., 1997; McGwire et al., 2003). Os resultados do estudo de associação genética apresentados aqui fornecem suporte para o papel de variantes polimórficas de *COL1A1*, gene que está sob a regulação de *FLI1*. Isto é consistente com nossa demonstração prévia (Castellucci et al., 2011) de associação entre SNPs de *FLI1* que estão em forte desequilíbrio de ligação com elementos funcionais conhecidos por influenciar a expressão deste gene através de atividades epigenéticas.

Foi demonstrado previamente que a redução da expressão gênica de *FLI1* em fibroblastos humanos resulta na regulação positiva dos genes *COL1A1* e *COL1A2* e na regulação negativa do gene *MMP1*, sugerindo que a supressão do *FLI1* está envolvida na ativação da resposta fibrótica (Nakerakanti et al., 2006). Além disso, estudos em fibroblastos esclerodérmicos e em biópsias de pele de pacientes com esclerose sistêmica têm demonstrado que ilhas CpG da região promotora do gene *FLI1* são altamente metiladas, indicando que a expressão aumentada de genes relacionados ao colágeno do tipo 1 está associada com a repressão epigenética do gene *FLI1* (Wang et al., 2006). Por outro lado, no

presente estudo, nós encontramos uma baixa porcentagem de DNA metilado na região promotora do gene *FLI1* em biópsias de pele de pacientes com leishmaniose cutânea. Apesar da expressão gênica de *FLI1* não ter sido estatisticamente diferente entres biópsias de lesão de leishmaniose cutânea e biópsias de pele normal, a metilação reduzida da região promotora do gene *FLI1* foi associada com uma concomitante redução da expressão gênica de *COL1A2* e com uma exagerada expressão gênica de *MMP1* comparada com as biópsias de pele normal. A citocina pró-inflamatória TNF-α, que caracteriza a leishmaniose cutânea humana causada por *Leishmania braziliensis* (Castes et al., 1993; Ribeiro-de-Jesus et al., 1998), também pode estar contribuindo para a expressão exagerada de metaloproteinases de matriz (Lehmann et al., 2005). TNF-α é conhecido por acelerar a degradação do colágeno do tipo 1 pela ação de MMP1, levando a uma perda gradual de colágeno na pele humana (Agren et al., 2015). Nossos resultados também são consistentes com dados de perfil de expressão recentemente publicados, e que mostram que *MMP1* está entre os 10 genes mais fortemente induzidos, com uma expressão 36 vezes maior nas biópsias de lesão de leishmaniose cutânea causada por *Leishmania braziliensis* comparadas com biópsias de pele normal (Novais et al., 2015).

A situação na lesão de leishmaniose cutânea é complexa, com muitos tipos celulares contribuindo para a lesão tecidual e, por fim, no reparo tecidual e cura das lesões. Nossas amostras de biópsias foram obtidas quando os pacientes apresentavam lesões ativas, e antes de iniciarem o tratamento. Neste estágio, as lesões são caracterizadas por dano tecidual e ulceração. MMP1 é uma metaloproteinase de matriz que quebra o colágeno do tipo 1 intersticial (Brinckerhoff & Matrisian, 2002). Normalmente, na pele humana ferida, a re-epitelização ocorre quando queratinócitos migram ao longo do colágeno do tipo 1, mas este processo pode

apenas acontecer quando MMP1 está presente para clivar o colágeno do tipo 1 e "afrouxar" o estreito contato que os queratinóticos estabelecem com a matriz dermal (Rohani & Parks, 2015). Os baixos níveis de expressão de colágeno do tipo 1, particularmente de *COL1A2*, nas lesões de leishmaniose cutânea, junto com altos níveis de expressão de *MMP1*, sugerem que esta protease está contribuindo para o dano tecidual em vez de contribuir para o processo de reparo. Tem sido observado que tal expressão exagerada de *MMP1* contribui para a destruição tecidual e progressão para a doença em outros contextos clínicos, incluindo progressão para artrite reumatoide (Xu et al., 2015), doença pulmonar obstrutiva crônica, síndrome de insuficiência respiratória aguda, sarcoidose e tuberculose (Elkington & Friedland, 2006).

Dada a complexidade da resposta celular em biópsias de lesão de leishmaniose cutânea, nós ficamos interessados em determinar em que medida nossas observações acerca da expressão gênica de *FLI1* e de genes que estão sob a sua regulação deveram-se a efeitos diretos do parasita na função macrofágica. De maneira interessante, enquanto a metilação de ilhas CpG na região promotora do gene *FLI1* foi reduzida em biópsias de lesão comparadas com biópsias de pele normal, houve uma tendência para uma maior porcentagem de metilação em macrófagos infectados comparados com macrófagos não infectados. A porcentagem de DNA metilado foi menor na presença de IL-6. Em geral, nós concluímos que a região promotora do gene *FLI1* foi amplamente não metilada, assim, tornando-a aberta para a ligação de fatores de transcrição. A infecção por *Leishmania braziliensis* resultou no aumento da expressão gênica de *FLI1*, que foi dramaticamente aumentada até 48 horas de infecção na presença de IL-6. Concomitante com isto, nós observamos baixos níveis de indução de *COL1A1* com 24 horas de infecção, porém nenhum gene de colágeno do tipo 1 foi expresso na presença de IL-6 e altos níveis de FLI1. Assim como nós

observamos em lesões de leishmaniose cutânea, houve uma exagerada expressão gênica de *MMP1* em macrófagos infectados. Assim, parece provável que altos níveis de *MMP1* associados com a patogênese da leishmaniose cutânea podem contribuir para um efeito direto do parasita da função do macrófago. Isto é semelhante ao que acontece na tuberculose, onde a resposta exagerada de *MMP1* é observada em macrófagos infectados (Elkington et al., 2005), levando a destruição tecidual e destacando a inibição do MMP1 como um potencial alvo terapêutico (Elkington & Friedland, 2006).

Em síntese, nosso trabalho sugere que a expressão gênica exagerada de *MMP1* pode desempenhar um papel chave na destruição tecidual observada na leishmaniose cutânea, e que esta expressão é provavelmente regulada por *FLI1*, mas também por outros fatores tais como o TNF-α. Assim, nosso dado aponta para a possibilidade de que a modulação desta via de sinalização através de intervenção terapêutica pode ser relevante para o tratamento e resolução da leishmaniose cutânea. Esta alternativa pode ser bem-vinda em formas clínicas mais graves e menos responsivas ao tratamento atual disponível.

# IX. PROPOSTAS DE ESTUDO

## IX.1. OBJETIVO

O principal objetivo desta proposta de estudo é revelar fatores genéticos de risco para leishmaniose tegumentar americana causada por *Leishmania braziliensis* no Brasil, através da realização de um estudo de associação genômica ampla, e relacioná-los com vias funcionais para explicar a patogênese da doença.

## IX.2. JUSTIFICATIVA

Uma série de estudos conduzidos em Corte de Pedra nos últimos trinta anos tem contribuído notavelmente para o conhecimento da epidemiologia e da resposta imune da LTA (Carvalho et al., 2012; de Oliveira & Brodskyn, 2012). Especialmente durante a última década, o nosso grupo tem se concentrado em avaliar o papel de fatores genéticos do hospedeiro nos desfechos da LTA, e, como consequência deste esforço, uma série de trabalhos foi publicada, fornecendo evidências de que o hospedeiro (Castellucci et al., 2006, 2010, 2011, 2012; Ramasawmy et al., 2010; Almeida et al., 2015) desempenha um importante papel na susceptibilidade aos diferentes fenótipos clínicos de LTA.

Adicionalmente, uma série de estudos avaliando o papel da genética do hospedeiro no desfecho da infecção tem implicado em uma vasta gama de genes envolvidos na susceptibilidade a

leishmaniose tegumentar (Barbier et al., 1987; Lara et al., 1991; Petzl-Erler et al., 1991; Cabrera et al., 1995; Olivo-Diaz et al., 2004; Kamali-Sarvestani et al., 2006; Salhi et al., 2008; Ajdary et al., 2011; Fernandez-Figueroa et al., 2012; de Jesus Fernandes Covas et al., 2013). Porém, estes estudos são limitados pela falta de poder e falha em obter replicação entre diferentes populações. Estudos de GWAS (*Genome Wide Association Study*) mais amplos e poderosos são ferramentas ouro para o mapeamento de biomarcadores relevantes que poderiam fornecer novas perspectivas no campo da farmacogenômica. Estudos deste tipo são necessários para avaliar a real significância e validar os nossos achados genéticos prévios para esta importante doença infecciosa tropical. No entanto, ainda não há relato de estudos desta natureza na LTA.

Utilizando esta abordagem para a leishmaniose visceral (LV), foi demonstrada evidência de que polimorfismos na região de classe II do complexo HLA (*Human Leukocyte Antigen*) são os principais determinantes genéticos nesta forma da doença (Fakiola et al., 2013). Este resultado foi replicado em três coortes independentes da Índia e do Brasil. Assim, o principal objetivo desta proposta é determinar se existe a mesma evidência sobre a importância da região de classe II do HLA no desfecho da LTA, e/ou se outros importantes fatores genéticos de risco estão envolvidos no desenvolvimento da LTA. Para alcançar este objetivo, o presente estudo é proposto para revelar fatores genéticos de risco para LTA causada por *Leishmania braziliensis* através da realização de um GWAS de acordo com o que se segue:

(1) Realizar um GWAS de estágio 1 de descoberta, genotipando 547.644 polimorfismos de nucleotídeos simples (SNPs) em 1.123 casos de LTA e 1.120 controles sadios utilizando o Illumina HumanCoreExome beadchip, imputando contra um painel de referência, e realizando análises de associação utilizando modelos mistos para controlar parentesco e estrutura da população;

(2) Realizar um GWAS de estágio 2 de replicação, genotipando 547.644 SNPs em um segundo set de DNAs de 1.000 casos de LTA e 1.000 controles sadios, utilizando o Illumina HumanCoreExome beadchip, imputando contra um painel de referência, e utilizando modelos mistos para controlar o parentesco e a estrutura da população em uma análise de associação;

(3) Realizar a montagem de um banco de amostras e um perfil imunológico detalhado durante a coleta prospectiva de um grupo de amostras do GWAS de estágio 2 de replicação;

(4) Combinar os dados dos dois estágios de GWAS em uma meta-análise para fornecer uma evidência formal de replicação;

(5) Realizar validação funcional das vias de genes que atuam como fatores de risco para a doença, utilizando dados e amostras coletados durante o segundo estágio de coleta de amostras, incluindo perfil transcricional de biópsias de pele de pacientes com genótipos de risco.

No geral, a presente proposta prevê o primeiro estudo com poder adequado para identificar os fatores genéticos de risco para LTA. Genes e vias identificados como fatores de risco para LTA podem proporcionar uma nova compreensão da patogênese da doença, promovendo novas pesquisas na identificação de novos alvos terapêuticos contra esta infecção parasitária debilitante, e, consequentemente, ajudando a reduzir a carga da doença em populações endêmicas.

Se a mesma evidência sobre a importância da região de classe II do HLA for observada para LTA, assim como foi observada previamente para LV, estudos funcionais adicionais podem ter grandes implicações no desenvolvimento de vacinas entre as diferentes espécies de *Leishmania*. Por outro lado, se outros genes ou vias forem descobertos, eles podem formar a espinha dorsal de futuros estudos, investigando o potencial terapêutico de drogas

conhecidas que influenciam estas vias, ou fornecer a base para a descoberta de novas intervenções. Mais amplamente, os estudos propostos podem ter impacto em significativos avanços no entendimento de doenças humanas complexas em geral. Frequentemente, os mesmos genes e vias podem desempenhar um papel em diferentes patologias e na cura de lesões. Portanto, os resultados deste estudo podem ser relevantes não só no campo da leishmaniose, mas também em áreas mais amplas de investigação de doenças imunes e infecciosas.

## X. CONCLUSÕES

1. Foi observada uma associação significante entre LC e SNPs do gene *COL1A1* na população estudada, reforçando a importância da via de *FLI1* na patogênese da doença.

2. A expressão de *COL1A2* foi significantemente maior nas biópsias de pele normal, ao passo que a expressão de *MMP1* foi significantemente maior nas biópsias de LC, padrão que pode ser explicado pela diferença no perfil de metilação das ilhas CpG da região promotora do gene regulador *FLI1* entre as duas condições.

3. A expressão gênica *in vitro* de *FLI1* induzida pela infecção por *Leishmania braziliensis*e foi maior e teve um pico mais tardio na presença de IL-6.

4. A expressão de genes de colágeno do tipo 1, *COL1A1* e *COL1A2*, foi baixa em macrófagos infectados, e não foi detectável em macrófagos tratados com IL-6.

5. A expressão de *MMP1* foi fortemente induzida após infecção de macrófagos por *Leishmania braziliensis*.

6. Nossos dados sugerem que baixos níveis de colágeno do tipo 1 junto com a exagerada expressão de *MMP1* está contribuindo para o dano tecidual. Assim, a modulação desta via pode ser relevante no tratamento da leishmaniose cutânea.

## XI. SUMMARY

Previous studies have shown a role for wound healing genes in the resolution of skin lesions caused by *Leishmania* species in mice and humans, including *FLI1* gene (Friend leukemia virus integration 1). Heavy methylation of CpG islands in the *FLI1* promoter is known to render *FLI1* transcriptionally inactive. Low FLI1 expression results in up-regulation of type I collagens and down-regulation of matrix metalloproteinase 1 (*MMP1*). Both, type 1 collagen and matrix metalloproteinase 1, play an important role in normal physiological and pathological conditions from many diseases, and are involved in wound healing process. Interestingly, we have shown that the C allele at the IL6-174 G/C promoter polymorphism, which determines low levels of IL-6 release from macrophages, was a risk factor for mucosal leishmaniasis disease. IL-6 is known to increase expression of *FLI1*. To understand the role of this pathway in american cutaneous leishmaniasis, we evaluated the polymorphism of *COL1A1*, *COL1A2* and *MMP1* genes and its possible association with tegumentary leishmaniasis in the population of Corte de Pedra, Bahia, and also examined FLI1 promoter-specific DNA methylation and expression, together with expression COL1A1, COL1A2 and MMP1, in lesion biopsies *ex vivo* and in *Leishmania braziliensis* infected macrophages *in vitro*. Family-based association tests (FBAT) showed the strongest association between SNPs rs1061237 (combined P = 0.002) and rs2586488 (combined P = 0.027) at *COL1A1* and cutaneous leishmaniasis disease. Percent methylation of FLI1 was lower (P = 0.001) in lesion biopsies compared to normal skin. FLI1 and COL1A1 expression did not differ between lesion and normal skin, whereas COL1A2 was reduced (P = 0.0 33) and MMP1 enhanced (P = 0.0002). FLI1 expression induced by infection *in vitro* peaked at 24 hours (P < 0.0001), and was higher (P = 0.005) and peaked later (48 hours) in the presence of IL-6. Expression of type I collagens was low in infected macrophages, and undetectable in macrophages treated with IL-6. MMP1 was strongly induced (P = 0.007) following infection of macrophages, but was not readily detectable in IL-6 treated macrophages until 72 hours post infection when the effect of IL-6 on FLI1 expression had waned. MMP1 breaks down interstitial type I collagens, which is essential for keratinocyte migration and re-epithelialization. However, in active cutaneous leishmaniasis lesions, low levels of type I collagen together with exaggerated levels of MMP1 indicate that MMP1 is mediating tissue damage rather than repair. Exaggerated MMP1 levels have similarly been observed to contribute to tissue destruction and disease progression in tuberculosis, leading others to highlight it as a potential therapeutic target. Our data suggest that therapeutic modulation of MMP1 might also be relevant in treatment of cutaneous leishmaniasis.

Keyword: Leishmania; wound healing; FLI1; COL1A1; COL1A2; MMP1; epigenetic regulation.

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# XIII. ANEXOS

## XIII.1. NORMAS DE PUBLICAÇÃO

# Submission Guidelines

*PLOS Neglected Tropical Diseases* publishes original research articles of importance to the NTDs community and the wider health community. We will consider manuscripts of any length; we encourage the submission of both substantial full-length bodies of work and shorter manuscripts that report novel findings that might be based on a more limited range of experiments.

The writing style should be concise and accessible, avoiding jargon so that the paper is understandable for readers outside a specialty or those whose first language is not English. Editors will make suggestions for how to achieve this, as well as suggestions for cuts or additions that could be made to the article to strengthen the argument. Our aim is to make the editorial process rigorous and consistent, but not intrusive or overbearing. Authors are encouraged to use their own voice and to decide how best to present their ideas, results, and conclusions.

*PLOS Neglected Tropical Diseases* is committed to the highest ethical standards in medical research. Accordingly, we ask authors to provide specific information regarding ethical treatment of research participants, patient consent, patient privacy, protocols, authorship, and competing interests. We also ask that reports of certain specific types of studies adhere to generally accepted standards. Our requirements are based on the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, issued by the International Committee for Medical Journal Editors.

## Style and Format

| | |
|---|---|
| **File format** | Manuscript files can be in the following formats: DOC, DOCX, RTF, or PDF. Microsoft Word documents should not be locked or protected.<br><br>LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines. |
| **Length** | Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information.<br><br>We encourage you to present and discuss your findings concisely. |
| **Font** | Use any standard font and a standard font size. |
| **Headings** | Limit manuscript sections and sub-sections to 3 heading levels. Make |

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|---|---|
| | sure heading levels are clearly indicated in the manuscript text. |
| **Layout** | Manuscript text should be double-spaced.<br><br>Do not format text in multiple columns. |
| **Page and line numbers** | Include page numbers and line numbers in the manuscript file. |
| **Footnotes** | Footnotes are not permitted. If your manuscript contains footnotes, move the information into the main text or the reference list, depending on the content. |
| **Language** | Manuscripts must be submitted in English.<br><br>You may submit translations of the manuscript or abstract as supporting information. Read the supporting information guidelines. |
| **Abbreviations** | Define abbreviations upon first appearance in the text.<br><br>Do not use non-standard abbreviations unless they appear at least three times in the text.<br><br>Keep abbreviations to a minimum. |
| **Reference style** | PLOS uses "Vancouver" style, as outlined in the ICMJE sample references.<br><br>See reference formatting examples and additional instructions below. |
| **Equations** | We recommend using MathType for display and inline equations, as it will provide the most reliable outcome. If this is not possible, Equation Editor is acceptable.<br><br>Avoid using MathType or Equation Editor to insert single variables (e.g., "$a^2 + b^2 = c^2$"), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values.<br><br>Do not use MathType or Equation Editor for only a portion of an equation. Rather, ensure that the entire equation is included. Avoid "hybrid" inline or display equations, in which part is text and part is MathType, or part is MathType and part is Equation Editor. |
| **Nomenclature** | Use correct and established nomenclature wherever possible. |

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| *Units of measurement* | Use SI units. If you do not use these exclusively, provi in parentheses after each value. Read more about SI ur |
| *Drugs* | Provide the Recommended International Non-Proprieta (rINN). |
| *Species names* | Write in italics (e.g., *Homo sapiens*). Write out in full t species, both in the title of the manuscript and at the fi an organism in a paper. After first mention, the first let |

| | | |
|---|---|---|
| | | genus name followed by the full species name may be used (e.g., *H. sapiens*). |
| | *Genes, mutations, genotypes, and alleles* | Write in italics. Use the recommended name by consulting the appropriate genetic nomenclature database (e.g., HUGO for human genes). It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman typeface (e.g., v-fes, c-MYC). |

# Manuscript Organization

Most manuscripts should be organized as follows. Instructions for each element appear below.

- Title
- Authors and Affiliations
- Abstract
- Author Summary
- Introduction
- Methods
- Results
- Discussion
- Acknowledgments
- References
- Supporting information Captions

Uniformity in format facilitates the experience of readers and users of the journal. To provide flexibility, however, the Results and Discussion can be combined into one Results/Discussion section.

## Other elements

- Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately.
- Tables are inserted immediately after the first paragraph in which they are cited.
- Supporting information files are uploaded separately.

Please refer to our downloadable sample files to make sure that your submission meets our formatting requirements:

- Download sample title, author list, and affiliations page (PDF)
- Download full manuscript sample (PDF)

# Parts of a Submission

## Title

Include a full title and a short title for the manuscript.

| Title | Length | Guidelines | Examples |
|---|---|---|---|
| **Full title** | 250 characters | Specific, descriptive, concise, and comprehensible to readers outside the field | Impact of Cigarette Smoke Exposure on Innate Immunity: A *Caenorhabditis elegans* Model<br><br>Solar Drinking Water Disinfection (SODIS) to Reduce Childhood Diarrhoea in Rural Bolivia: A Cluster-Randomized, Controlled Trial |
| **Short title** | 70 characters | State the topic of the study | Cigarette Smoke Exposure and Innate Immunity<br><br>SODIS and Childhood Diarrhoea |

Titles should be written in title case (all words capitalized except articles, prepositions, and conjunctions). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

## Author list

### Who belongs on the author list

All authors must meet the criteria for authorship as outlined in the authorship policy. Read the policy.

Those who contributed to the work but do not meet the criteria for authorship can be mentioned in the Acknowledgments.Read more about Acknowledgments.

### Author names and affiliations

Enter author names on the title page of the manuscript and in the online submission system.

On the title page, write author names in the following order:

- First name (or initials, if used)
- Middle name (or initials, if used)
- Last name (surname, family name)

Each author on the list must have an affiliation. The affiliation includes department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country.

If an author has multiple affiliations, enter all affiliations on the title page only. In the submission system, enter only the preferred or primary affiliation.

Author names will be published exactly as they appear in the manuscript file. Please double-check the information carefully to make sure it is correct.

### Corresponding author

One corresponding author should be designated in the submission system as well as on the title page.

One corresponding author should be designated in the submission system. However, this does not restrict the number of corresponding authors that may be listed on the article in the event of publication. Whoever is designated as a corresponding author on the title page of the manuscript file will be listed as such upon publication.

Include an email address for each corresponding author listed on the title page of the manuscript.

### Consortia and group authorship

If a manuscript is submitted on behalf of a consortium or group, include the consortium or group name in the author list, and include the full list of members in the Acknowledgments or in a supporting information file.

## Cover letter

Upload a cover letter as a separate file in the online system.

The cover letter should address the following questions:

- Why is this manuscript suitable for publication in *PLOS Neglected Tropical Diseases*?
- Why will your study inspire the NTDs community, and how will it drive the understanding of NTD pathobiology, epidemiology, prevention, treatment, control, or policy?

If your study addresses an infection that is outside our detailed scope, you must first send a presubmission inquiry indicating why you consider the infection to be a neglected tropical disease.

## Title page

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

Download sample title, author list, and affiliations page (PDF)

## Abstract

The Abstract comes after the title page in the manuscript file. The abstract text is also entered in a separate field in the submission system.

The Abstract succinctly introduces the paper. It should not exceed 250–300 words. It should mention the techniques used without going into methodological detail and summarize the most important results with important numerical results given.

The Abstract is conceptually divided into the following three sections with these headings: Background, Methodology/Principal Findings, and Conclusions/Significance.

Do not include any citations in the Abstract. Avoid specialist abbreviations.

## Author Summary

We ask that all authors of research articles include a 150- to 200-word non-technical summary of the work, immediately following the Abstract. Subject to editorial review and author revision, this short text is published with all research articles as a highlighted text box.

Distinct from the scientific abstract, the Author Summary should highlight where the work fits in a broader context of life science knowledge and why these findings are important to an audience that includes both scientists and non-scientists. Ideally aimed to a level of understanding of an undergraduate student, the significance of the work should be presented simply, objectively, and without exaggeration.

Authors should avoid the use of acronyms and complex scientific terms and write the author summary using the first-person voice. Authors may benefit from consulting with a science writer or press officer to ensure that they effectively communicate their findings to a general audience.

### Example Author Summaries

Pseudogenization of a Sweet-Receptor Gene Accounts for Cats' Indifference toward Sugar

A Hybrid Photoreceptor Expressing Both Rod and Cone Genes in a Mouse Model of Enhanced S-Cone Syndrome

Life in Hot Carbon Monoxide: The Complete Genome Sequence of Carboxydothermus hydrogenoformans Z-2901

## Introduction

The Introduction should put the focus of the manuscript into a broader context. As you compose the Introduction, think of readers who are not experts in this field. Include a brief review of the key literature. If there are relevant controversies or disagreements in the field, they should be mentioned so that a non-expert reader can delve into these issues further. The Introduction should conclude with a brief statement of the overall aim of the experiments and a comment about whether that aim was achieved.

## Methods

This section should provide enough detail for reproduction of the findings. Protocols for new methods should be included, but well-established protocols may simply be referenced. Detailed methodology or supporting information relevant to the methodology can be published on our web site.

This section should also include a section with descriptions of any statistical methods employed. These should conform to thecriteria outlined by the Uniform Requirements, as follows:

*Describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to judge its appropriateness for the study and to verify the reported results. When possible, quantify findings and present them with appropriate indicators of measurement error or uncertainty (such as confidence intervals). Avoid relying solely on statistical hypothesis testing, such as P values, which fail to convey important information about effect size and precision of estimates. References for the design of the study and statistical methods should be to standard works when possible (with pages stated). Define statistical terms, abbreviations, and most symbols. Specify the statistical software package(s) and versions used. Distinguish prespecified from exploratory analyses, including subgroup analyses.*

## Results

The Results section should include all relevant positive and negative findings. The section may be divided into subsections, each with a concise subheading. The Results section should be written in past tense.

PLOS journals require authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception.

Large data sets, including raw data, may be deposited in an appropriate public repository. See our list of recommended repositories.

For smaller data sets and certain data types, authors may provide their data within supporting information files accompanying the manuscript. Authors should take care to maximize the accessibility and reusability of the data by selecting a file format from which data can be efficiently extracted (for example, spreadsheets or flat files should be provided rather than PDFs when providing tabulated data).

For more information on how best to provide data, read our policy on data availability. PLOS does not accept references to "data not shown."

As outlined in the Uniform Requirements, authors that present statistical data in the Results section should do the following:

*Give numeric results not only as derivatives (for example, percentages) but also as the absolute numbers from which the derivatives were calculated, and specify the statistical significance attached to them, if any. Restrict tables and figures to those needed to explain the argument of the paper and to assess supporting data. Use graphs as an alternative to tables with many entries; do not duplicate data in graphs and tables. Avoid nontechnical uses of technical terms in statistics, such as "random" (which implies a randomizing device), "normal," "significant," "correlations," and "sample."*

## Discussion

The Discussion should be concise and tightly argued. It should start with a brief summary of the main findings. It should include paragraphs on the generalizability, clinical relevance, strengths, and limitations of your study.

You may wish to discuss the following points also:

- How do the conclusions affect the existing knowledge in the field?
- How can future research build on these observations and what are the key experiments that must be done?

**Copyediting manuscripts**

Please note that accepted manuscripts are not subject to detailed copyediting. Therefore, please carefully review your manuscript, paying special attention to spelling, punctuation, and grammar, as well as scientific content.

Authors who believe their manuscripts would benefit from in-depth professional copyediting are encouraged to use language-editing and copyediting services, such as the ones offered below (in alphabetical order):

- American Journal Experts
- Asia Science Editing
- Bioedit Ltd
- BiomEditor
- BioScience Writers
- Blue Pencil Science
- Boston BioEdit
- Carpe Diem Biomedical Writing and Editing
- English Manager Science Editing
- International Science Editing

- Life Science Publishing
- Online English
- Professional Editing Services
- Scienceditors.com
- SciTechEdit International
- Scitext Cambridge
- Scribendi
- Squirrel Scribe
- Stallard Scientific Editing
- Write Science Right

PLOS neither endorses nor takes responsibility for contracting with any of these individuals/companies, but we do recognize the value of the services they provide.

## Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

Do not include funding sources in the Acknowledgments or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the online submission system.

## References

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on pre-print servers, if the manuscript is submitted to a journal and also publicly available as a pre-print

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”). Instead, include those data as supplementary material or deposit the data in a publicly available database.
- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., “We used the techniques developed by our colleagues [19] to analyze the data”). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts or author summaries.

Make sure the parts of the manuscript are in the correct order *before* ordering the citations.

**Formatting references**

Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial.

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the "Vancouver" style. Example formats are listed below. Additional examples are in the ICMJE sample references.

A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.

Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

| Source | Format |
|---|---|
| **Published articles** | Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (*Ailuropoda melanoleuca*). Genet Mol Res. 2011;10: 1576-1588.<br><br>Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005<br><br>*Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers.* |
| **Accepted, unpublished articles** | Same as published articles, but substitute "In press" for page numbers or DOI. |
| **Web sites or online articles** | Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available: http://www.globalizationandhealth.com/content/1/1/14. |
| **Books** | Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992. |
| **Book chapters** | Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28. |

| Source | Format |
|---|---|
| **Deposited articles (preprints, e-prints, or arXiv)** | Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available: arXiv:1403.3301v1. Accessed 17 March 2014. |
| **Published media (print or online newspapers and magazine articles)** | Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html. Accessed 17 March 2014. |
| **New media (blogs, web sites, or other written works)** | Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/. |
| **Masters' theses or doctoral dissertations** | Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available: http://cumincad.scix.net/cgi-bin/works/Show?2e09 |
| **Databases and repositories (Figshare, arXiv)** | Roberts SB. QPX Genome Browser Feature Tracks; 2013. Database: figshare [Internet]. Accessed: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214. |
| **Multimedia (videos, movies, or TV shows)** | Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM. |

## Supporting Information

Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 10 MB in size.

Authors may use almost any description as the item name for a supporting information file as long as it contains an “S” and number. For example, “S1 Appendix” and “S2 Appendix,” “S1 Table” and “S2 Table,” and so forth.

Supporting information files are published exactly as provided, and are not copyedited.

### Supporting information captions

List supporting information captions at the end of the manuscript file. Do not submit captions in a separate file.

The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

**Example caption**

**S1 Text. Title is strongly recommended.** Legend is optional.

### In-text citations

We recommend that you cite supporting information in the manuscript text, but this is not a requirement. If you cite supporting information in the text, citations do not need to be in numerical order.

Read the supporting information guidelines for more details about submitting supporting information and multimedia files.

## Figures and tables

### Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file.

Cite figures in ascending numeric order upon first appearance in the manuscript file.

Read the guidelines for figures.

### Figure captions

Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

- A figure label with Arabic numerals, and "Figure" abbreviated to "Fig" (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of "Fig 1" must refer to a figure file named "Fig1.tif").
- A concise, descriptive title

The caption may also include a legend as needed.

Read more about figure captions.

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Cite tables in ascending numeric order upon first appearance in the manuscript file.

Place each table in your manuscript file directly after the paragraph in which it is first cited (read order). Do not submit your tables in separate files.

Tables require a label (e.g., “Table 1”) and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

Read the guidelines for tables.

## Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

Read our policy on data availability.

Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

See our list of recommended repositories.

To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include Dryad and FlowRepository. Please contact data@plos.org to make recommendations for further partnerships.

Instructions for PLOS submissions with data deposited in an integration partner repository:

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.
- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.

If you have any questions, please email us.

## Accession numbers

All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories.

Accession numbers (and version numbers, if appropriate) should be provided in the Data Availability Statement. Accession numbers or a citation to the DOI should also be provided when the data set is mentioned within the manuscript.

In some cases authors may not be able to obtain accession numbers of DOIs until the manuscript is accepted; in these cases, the authors must provide these numbers at acceptance. In all other cases, these numbers must be provided at submission.

### Identifiers

As much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene
- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem

Identifiers should be provided in parentheses after the entity on first use.

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Striking images should not contain potentially identifying images of people. Read our policy on identifying information.

# Additional Information Requested at Submission

## Funding statement

This section should describe sources of funding that have supported the work. Please include relevant grant numbers and the URL of any funder's web site. Please also include this sentence: “The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.” If this statement is not correct, you must describe the role of any sponsors or funders, and amend the aforementioned sentence as needed.

Read our policy on disclosure of funding sources.

## Competing interests

The corresponding author is asked at submission to declare, on behalf of all authors, whether there are any financial, personal, or professional interests that could be construed to have influenced the work.

Any relevant competing interests of authors must be available to editors and reviewers during the review process and will be stated in published articles.

Read our policy on competing interests.

## Prior publication

When submitting a manuscript, all authors are asked to indicate that they have not submitted a similar manuscript for publication elsewhere. If related work has been submitted elsewhere, then a copy must be included with the manuscript submitted to PLOS. Reviewers will be asked to comment on the overlap between related submissions.

## Reviewer and editor suggestions

We ask authors to suggest suitable editors and at least four potential reviewers when submitting their manuscript. Bear in mind any potential competing interests when making these suggestions. It is not appropriate to suggest recent collaborators or other researchers at your institution. See our policy on competing interests for more information.

# Guidelines for Specific Study Types

## Human and animal research

All research involving humans and animals must have been approved by the authors' institutional review board or equivalent committee(s), and that board must be named by the authors in the manuscript. For research involving human participants, informed consent must have been obtained (or the reason for lack of consent explained, e.g. the data were analyzed anonymously) and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. It must be stated in the Methods section of the paper whether informed consent was written or oral. If informed consent was oral, it must be stated in the paper: (a) why written consent could not be obtained, (b) that the IRB approved the use of oral consent, and (c) how oral consent was documented.

Authors should be able to submit, upon request, a statement from the research ethics committee or institutional review board indicating approval of the research. We also encourage authors to submit a sample of a patient consent form, and may require submission on particular occasions.

All animal work must have been conducted according to relevant national and international guidelines. In accordance with the recommendations of the Weatherall report, *The use of non-human primates in research* (PDF), we specifically require authors to include details of animal welfare and steps taken to ameliorate suffering in all work involving non-human primates. The institution that approved the study must be named, and it must be stated in the paper that the study was conducted adhering to the institution's guidelines for animal husbandry.

### Patient privacy and informed consent for publication

Our human participant policy conforms to the Uniform Requirements of the International Committee of Medical Journal Editors:

*Patients have a right to privacy that should not be infringed without informed consent. Identifying information should not be published in written descriptions, photographs, and pedigrees unless the information is essential for scientific purposes and the patient (or parent or guardian) gives written informed consent for publication. Informed consent for this purpose requires that the patient be shown the manuscript to be published. Complete anonymity is difficult to achieve, and informed consent for publication should be obtained if there is any doubt. If data are changed to protect anonymity, authors should provide assurance that alterations of the data do not distort scientific meaning. When informed consent has been obtained it should be indicated in the published article.*

For papers that include identifying information, or potentially identifying information, authors must download the *Consent Form for Publication in a PLOS Journal* from our web site, which the patient, parent, or guardian must

sign once they have read the paper and been informed about the terms of the PLOS content license.

Once authors have obtained the signed consent form, it should be filed securely in the patient's case notes and the manuscript submitted to PLOS should include this statement indicating that specific consent for publication was obtained: “The patients in this manuscript have given written informed consent (as outlined in the PLOS consent form) to publication of their case details.”

**Download the PLOS consent form:**

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## Clinical trials

We follow the World Health Organization’s (WHO) definition of a clinical trial:

*A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.*

*PLOS Neglected Tropical Diseases* requires that all trials be registered and, as of August 13, 2013, supports the position of the AllTrials.net Initiative that trials that are registered after the trial commences or retrospectively will be considered (see the blog post for more details). For all trials, authors are asked to provide the trial registration information and to register their trial in an approved registry (the WHO's list of approved registries is listed here). For trials that were registered after the trial began or retrospectively, authors are asked to provide the following information:

- The trial registration information (or indicate that registration is in process)
- The reason for late registration, explained within the Methods section
- A statement in which all authors affirm that any trials on the same or a related drug or intervention they're involved in are registered, and provide (either as part of the statement or in the supplementary information) links to the published versions of the trials or the registration numbers. This statement will be published in the Methods section.

The editors reserve the right to inform authors' institutions or ethics committees about unregistered trials that have been carried out. Authors will also be asked to submit an accurate summary of the trial's results to the relevant registry (if there is such a mechanism) within a year of study completion or at the time of publication, whichever is the earliest.

Authors of trials must adhere to the CONSORT reporting guidelines appropriate to their trial design. Please check the CONSORT statement web site for information on the appropriate guidelines for specific trial types. Before the paper can undergo peer review, authors must: 1) provide in the manuscript the trial registry, trial registration number, and IRB, and 2) provide a copy of the trial protocol (or a link to an open access version of the protocol) and a completed CONSORT checklist as supporting files (these documents will also be published alongside the paper, if accepted). The CONSORT flow diagram must be included as Figure 1. Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and PLOS reserves the right to request a copy of the patient consent form. Information on statistical methods or participants beyond what is indicated in the CONSORT statement should be reported in the Methods section.

PLOS supports the public disclosure of all clinical trial results, as mandated, for example, by the FDA Amendments Act, 2007. For trials in registries that permit posting of trial results, *PLOS Neglected Tropical Diseases* requires that an accurate summary of the trial's results be submitted to the relevant registry (if there is such a mechanism) within a year of study completion or at the time of publication, whichever is the earliest.

## Systematic reviews and meta-analyses

Reports of systematic reviews and meta-analyses must adhere to the PRISMA Statement or alternative guidelines appropriate to the study design, and include the completed checklist and flow diagram to accompany the main text. Authors must complete the appropriate reporting checklist not only with page references, but also with sufficient text excerpted from the manuscript to explain how they accomplished all applicable items.

Download blank templates of the checklist and flow diagram from the EQUATOR web site.

Abstracts should follow PRISMA for Abstracts, using the PLOS abstract format. Authors must also state within the Methods section of their paper whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information.

The journal supports the prospective registration of systematic reviews. Authors whose systematic review was prospectively registered (e.g., in a registry such as PROSPERO) should provide the registry number in their abstract. Registry details and protocols will be made available to editors and reviewers, and included with the paper if the report is ultimately published.

## Diagnostic studies

Reports of studies of diagnostic accuracy must adhere to the STARD requirements or alternative guidelines appropriate to the study design (see the EQUATOR web site) and include a completed checklist as supporting information. Authors must complete the appropriate reporting checklist not only with page references, but also with sufficient text excerpted from the manuscript to explain how they addressed all applicable items.

### Observational studies

For observational studies, including case control, cohort, and cross-sectional studies, authors must adhere to the STROBE Statement or alternative guidelines appropriate to the study design (see the EQUATOR web site) and include a completed checklist as supporting information. Authors must complete the appropriate reporting checklist not only with page references, but also with sufficient text excerpted from the manuscript to explain how they addressed all applicable items.

For observational studies, authors are required to clearly specify (a) What specific hypotheses the researchers intended to test, and the analytical methods by which they planned to test them; (b) What analyses they actually performed; and (c) When reported analyses differ from those that were planned, authors must provide transparent explanations for differences that affect the reliability of the study's results.

If a prospective analysis plan (from the study's funding proposal, IRB or other ethics committee submission, study protocol, or other planning document written before analyzing the data) was used in designing an observational study, authors must include the relevant prospectively written document with the manuscript submission for access by editors and reviewers and eventual publication alongside the accepted paper. If no prospectively written document exists, authors should explain how and when they determined the analyses being reported.

### Microarray experiments

Reports of microarray experiments must conform to the MIAME guidelines, and the data from the experiments must be deposited in a publicly accessible database.

## Other Article Types

If you are submitting content other than a research article, read the guidelines for other article types.

# XIII.2. CARTA AO EDITOR

Cover Letter

Dear Editors

**Re: Analysis of expression of wound healing genes *FLI1*, *COL1A1*, *COL1A2* and *MMP1* in American cutaneous leishmaniasis caused by *Leishmania braziliensis* infection**

We would like you to consider the above paper for publication in *PLOS Neglected Tropical Diseases*. The paper builds on our previous studies showing a role for polymorphisms in wound healing genes in determining the clinical outcome of cutaneous leishmaniasis caused by *Leishmania braziliensis* in Brazil. Here we have taken the work forward in endeavouring to understand the mechanisms by which these genes regulate disease outcome. In particular, we looked at whether epigenetic regulation is important for FLI1 expression, and demonstrated that FLI1 promoter-specific CpGs are largely unmethylated in lesion biopsies, and in macrophages infected with *L. braziliensis*. This leads to down-regulation of type I collagens, and exaggerated expression of metalloprotease MMP1, indicated dysregulation of the pathway causing tissue damage rather than tissue repair. We suggest, therefore, that MMP1 provides a good therapeutic target of treatment of cutaneous leishmaniasis.
Overall we believe that our study provides novel and interesting insight into the pathogenesis of VL disease. We hope that you will consider this paper worthy of review for publication in *PLOS Neglected Tropical Diseases*.

The authors declare no conflict of interest.

With thanks

Jenefer M. Blackwell (for the authors) and Léa Castellucci (corresponding author for the publication)