**UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO
DEPARTAMENTO DE CLÍNICA MÉDICA**

SEBASTIAN ANDRES VERNAL CARRANZA

*Proteínas salivares de insetos hematófagos na patogênese do pênfigo foliáceo em amostragem
brasileira*

RIBEIRÃO PRETO

2019

SEBASTIAN ANDRES VERNAL CARRANZA

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Tese apresentada à Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo para a obtenção do título de Doutor em Ciência Médicas; área de concentração: Clínica Médica; opção: Investigação Clínica. Subprojeto VIII que compõe o projeto temático "Relação dos fatores imunogenéticos e ambientais na etiopatogênese dos pênfigos em amostragem brasileira" FAPESP N.º 2010/51729-2.

Orientadora: Prof^a. Dr^a. Ana Maria Ferreira Roselino

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VERNAL, S. Proteínas salivares de insetos hematófagos na patogênese do pênfigo foliáceo em amostragem brasileira

Tese apresentada à Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo para a obtenção do título de Doutor em Ciências Médicas do Programa de Clínica Médica. Área de concentração: Investigação Clínica.

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Dedico este trabalho à meu amor, minha esposa; à meu maior presente da vida, minha filha; à meu suporte nesse mundo, meus pais e irmãos; e especialmente aos que me permitiram estar onde estou, meus avós.

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“Los días más felices son aquellos que nos hacen más sabios”

Gabriel Mistral

RESUMO

VERNAL, S. **Proteínas salivares de insetos hematófagos na patogênese do pênfigo foliáceo em amostragem brasileira**, 2019, 159f. Tese (Doutorado) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 2019.

Introdução. Os pênfigos representam um grupo de dermatoses bolhosas autoimunes caracterizados pela produção de autoanticorpos contra as desmogleínas (Dsg). O pênfigo foliáceo (PF) decorre da produção de autoanticorpos contra Dsg1. No Brasil, o PF é descrito em focos geográficos definidos: áreas rurais próximas de bacias hidrográficas. Considerando essa distribuição, tem-se aventado a hipótese de que fatores ambientais, aliados à associação de alelos *HLA*, tenham papel na sua endemicidade, interrogando-se a participação de insetos hematófagos na etiopatogênese do PF.

Objetivos. Verificar a participação das proteínas salivares de insetos hematófagos na etiopatogênese do PF em amostragem da região nordeste do estado de São Paulo (NESP), Brasil. Como objetivos específicos: (i) documentar a relação epidemiológica (fator de risco/doença) das principais espécies de insetos antropofílicos com a distribuição geográfica dos casos de PF no NESP; (ii) avaliar a resposta imune humoral IgG em pacientes com PF e controles contra extratos proteicos salivares (EPS) de *Aedes aegypti*, *Nyssomyia neivai* e *Simulium nigrimanum*, e contra as proteínas salivares maxadilan (expressa em *Lutzomyia longipalpis* e *N. neivai*) e LJM11 (expressa em *Lu. longipalpis*), e correlacionar essas respostas com os títulos anti-Dsg1; (iii) quantificar o IgE total e anti-IgE contra EPS dos insetos citados, em pacientes com PF e controles, e (iv) descrever o sialoma de *N. neivai*. **Métodos.** Os dados dos casos de PF e das principais espécies hematófagas do NESP foram registrados em mapas de distribuição espacial (*ArcGIS v10.2*). Para a detecção de anticorpos séricos (IgG e IgE) contra os EPS realizou-se ELISA *in-house* seguida de immunoblotting (IB). A dosagem de IgE total foi realizada pelo kit ImmunoCAPTM Total IgE segundo as instruções da fabricante. Para a detecção de anticorpos séricos (IgG) contra maxadilan e LJM11 também usou-se ELISA *in-house*. A quantificação de anticorpos anti-Dsg1 foi feita por ELISA comercial (MBL, Japão). A descrição do sialoma foi realizada com tecnologia *Illumina RNA seq*. A análise estatística para cada objetivo foi feita com testes não paramétricos. **Resultados.**

Houve confirmação entre a distribuição geográfica dos insetos hematófagos *S. nigrimanum* e *Nyssomyia neivai* com os casos de PF registrados no NESP, confirmando elo epidemiológico. Pacientes com PF apresentaram maiores títulos de anticorpos IgG contra EPS de *S. nigrimanum* ($P<0.001$) e de *N. neivai* ($P<0.050$), comparados aos controles, porém sem diferença em relação ao EPS de *A. aegypti* ($P=0.469$). Os pacientes com PF apresentaram maiores níveis de IgE total em comparação aos controles ($P<0.001$). Títulos de anticorpos contra EPS de *S. nigrimanum* e *N. neivai* correlacionaram-se com os títulos de anti-Dsg1 no grupo PF ($r=0.384$, $P=0.039$ e $r=0.416$, $P=0.022$, respectivamente). Títulos de anticorpos IgE contra EPS de *A. aegypti*, *S. nigrimanum* e *N. neivai* correlacionaram-se com títulos de IgE total no grupo PF; e maiores títulos IgE contra EPS de *N. neivai* e *A. aegypti* foram observados no grupo PF, em comparação aos controles ($P<0.001$ ambos). Anticorpo IgE contra EPS de *N. neivai* evidenciaram uma correlação positiva com os títulos de anti-Dsg1 no grupo PF ($P=0.077$). Frações proteicas de EPS foram reconhecidas pelos pacientes com PF e controles no IB, sendo que PF reconheceram principalmente frações proteicas

menores de 60kDa do EPS *S. nigrimanum* e entre 5-10kDa do EPS de *N. neivai*. Ainda, maiores títulos de IgG anti-maxadilan, mas não anti-LJM11, foram identificados no grupo PF comparado aos controles ($P<0.001$). Títulos de IgG anti-maxadilan correlacionaram-se com títulos de IgG anti-ESP de *N. neivai*, assim como com anti-Dsg1 no grupo PF. **Conclusão.** Um *link* epidemiológico entre a distribuição dos casos de PF e das espécies *S. nigrimanum* e *N. neivai* foi confirmado no NESP. A exposição à picada desses insetos foi confirmada pela produção de anticorpos da classe IgG e IgE, com reconhecimento de várias frações proteicas do EPS, sugerindo a participação de diversos peptídeos salivares de insetos hematófagos na etiopatogênese do PF. Ainda, a correlação dos anticorpos anti-ESP e anti-maxadilan com anti-Dsg1 mostra possível mimetismo molecular, e participação das proteínas salivares na patogênese do PF no NESP.

Palavras chave: Pênfigo foliáceo; Desmogleína; Diptera; Psychodidae; Simuliidae; Culicidae

ABSTRACT

VERNAL, S. **Salivary proteins of hematophagous insects in the pathogenesis of pemphigus foliaceus in a Brazilian sample**, 2019, 159p. Doctoral Thesis - Ribeirão Preto Medical School, University of São Paulo, 2019

Introduction. Pemphigus encompasses a group of autoimmune bullous dermatoses characterized by the production of autoantibodies against desmogleins (Dsg). Pemphigus foliaceus (PF) results from the production of autoantibodies against Dsg1. In Brazil, PF is described in well-defined geographic foci: rural areas near watersheds. Considering this distribution, it has been hypothesized that environmental factors, allied to the association of *HLA* alleles, play a role in PF endemicity, questioning the participation of hematophagous insects in the PF pathogenesis. **Main objective.** To verify the participation of salivary proteins of hematophagous insects in the PF etiopathogenesis in a sample from northeastern São Paulo state (NSPS), Brazil. As specific objectives: (i) document the epidemiological relationship (risk factor/disease) of the main species of anthropophilic insects with the geographic distribution of PF cases in NSPS; (ii) to evaluate the humoral IgG immune response in patients with PF and controls against *Aedes aegypti*, *Nyssomyia neivai* and *Simulium nigrimanum* salivary gland extracts (SGE), and against maxadilan salivary proteins (expressed in *Lutzomyia longipalpis* and *N. neivai*) and LJM11 (expressed in *Lu. longipalpis*), and correlate these responses with anti-Dsg1 titers; (iii) quantify the total IgE and anti-IgE against SGE of the cited insects, in patients with PF and controls, and (iv) describe *N. neivai* sialoma. **Methods.** Data from the PF cases and the main hematophagous species of NSPS were recorded in spatial distribution maps (*ArcGIS* v10.2). For the detection of serum antibodies (IgG and IgE) against SGE, in-house ELISA was performed followed by immunoblotting (IB). Total IgE dosing was performed by the ImmunoCAPTM Total IgE kit according to the manufacturer's instructions. Serum antibodies (IgG) against maxadilan and LJM11 were also used in-house ELISA. Quantification of anti-Dsg1 autoantibodies was done by commercial ELISA (MBL, Japan). The description of the sialoma was performed using *Illumina RNAseq*. Statistical analysis for each objective was performed with nonparametric tests. **Results.** There was confirmation between the geographic distribution of the hematophagous insects *S. nigrimanum* and *Nyssomyia neivai* with the cases of PF registered in NSPS, confirming the epidemiological link. Patients with PF had higher IgG antibodies titers against *S. nigrimanum* ($P<0.001$) and *N. neivai* ($P<0.050$) SGE compared to controls, but no difference from *A. aegypti* SGE ($P=0.469$). Patients with PF had higher total IgE levels compared to controls ($P<0.001$). Antibody titers against *S. nigrimanum* and *N. neivai* correlated with anti-Dsg1 titers in the PF group ($r=0.384$, $P=0.039$; and $r=0.416$, $P=0.022$, respectively). IgE antibodies titers against SGE from *A. aegypti*, *S. nigrimanum* and *N. neivai* correlated with total IgE titers in the PF group; and higher SGE IgE titers from *N. neivai* and *A. aegypti* were observed in the PF group compared to controls ($P<0.001$ both). IgE antibody against *N. neivai* SGE showed a positive correlation with anti-Dsg1 titers in the PF group ($P=0.077$). SGE protein fractions were recognized by patients with PF and controls in IB assay, and PF mainly recognized protein fractions smaller than 60kDa from SGE from *S. nigrimanum* and between 5-10kDa from *N. neivai* SGE. Further, higher anti-maxadilan but not anti-LJM11 IgG titers were identified in the PF group compared to controls ($P<0.001$). Anti-maxadilan IgG titers correlated with IgG anti-SGE from *N. neivai* titers as well as anti-Dsg1 in PF group. **Conclusion.** An epidemiological link between the distribution of PF cases and *S. nigrimanum* and *N. neivai* species was confirmed in NSPS. Exposure to the bite of these insects was confirmed by the production of IgG and IgE class antibodies, with recognition of several SGE protein fractions, suggesting the participation of several salivary peptides of hematophagous insects in PF etiopathogenesis. Moreover, the correlation of anti-SGE and anti-

maxadilan antibodies with anti-Dsg1 shows possible molecular mimicry, and participation of salivary proteins in the pathogenesis of PF in NSPS.

Keywords: *Pemphigus foliaceus*; *Desmogleina*; *Diptera*; *Psychodidae*; *Simuliidae*; *Culicidae*

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Fonte: QIAN et al., 2016 44

LISTA DE ABREVIATURAS

<i>A. aegypti</i>	<i>Aedes aegypti</i>
Dsc	Desmocolina
Dsg	Desmogleína
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
<i>HLA</i>	<i>Human leukocyte antigen</i>
IgE	Imunoglobulina classe E
IgG	Imunoglobulina classe G
IgG ₁	Imunoglobulina classe G subtipo 1
IgG ₄	Imunoglobulina classe G subtipo 4
IL	Interleucina
<i>Lu. longipalpis</i>	<i>Lutzomyia longipalpis</i>
<i>L. (L.)</i>	<i>Leishmania Complexo Leishmania</i>
LTA	Leishmaniose Tegumentar Americana
LV	Leishmaniose Visceral
<i>L. (V.)</i>	<i>Leishmania Complexo Viannia</i>
<i>MHC</i>	<i>Major Histocompatibility Complex</i>
<i>N. neivai</i>	<i>Nyssomyia neivai</i>
PB	Penfigóide bolhoso
PF	Pênfigo foliáceo
PV	Pênfigo Vulgar
<i>S. nigrimanum</i>	<i>Simulium nigrimanum</i>
<i>S. vittatum</i>	<i>Simulium vittatum</i>
Th ₂	Linfócito T <i>helper</i> 2

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1. INTRODUÇÃO

Os pênfígos se referem a um grupo de dermatoses bolhosas autoimunes, caracterizados pelo acometimento da pele e/ou das mucosas devido à produção de autoanticorpos contra proteínas desmosomais. A ligação dos autoanticorpos IgG no complexo desmosomal conduz à perda da adesão dos queratinócitos, processo conhecido como acantólise, e consequentemente à formação de bolhas intraepiteliais (HAMMERS; STANLEY, 2016).

Há duas formas clássicas de pênfigo: pênfigo vulgar (PV) e pênfigo foliáceo (PF), os quais apresentam autoanticorpos contra diferentes antígenos, afetando assim diferentes níveis da epiderme. O PF se caracteriza imunologicamente pela produção de autoanticorpos contra a Desmogleína (Dsg) 1, manifestando-se histologicamente por bolha superficial, abaixo da camada córnea - acantólise subcórnea. Por outro lado, o PV se caracteriza imunologicamente pela produção de autoanticorpos contra a Dsg3, proteína distribuída mais abundantemente nas camadas profundas da epiderme, com formação de bolha acima da camada basal - acantólise suprabasal. O PV também pode apresentar produção simultânea de autoanticorpos contra Dsg1 e Dsg3, acarretando envolvimento mucocutâneo (KASPERKIEWICZ et al., 2017).

O PF subdivide-se em dois tipos: [a] a forma clássica não endêmica: “de Cazenave”; e [b] uma forma endêmica - Pênfigo Foliáceo Endêmico, também conhecido no Brasil como “Fogo Selvagem”. Ambos diferem quanto à epidemiologia: enquanto o pênfigo de Cazenave é esporádico ao redor do mundo e afeta principalmente a idade adulta, a forma endêmica se apresenta de maneira peculiarmente concentrada em focos geográficos definidos e incide majoritariamente nos adultos jovens (ALPSOY; AKMAN-KARAKAS; UZUN, 2015; MEYER et al., 2010).

A patogênese do PF é complexa e multifatorial, envolvendo fatores genéticos, imunológicos e ambientais (ABREU-VELEZ et al., 2010; AOKI, et al, 2015; QIAN et al., 2016). Considerando os fatores genéticos, alelos *HLA* de classes I e II estão sabidamente envolvidos na patogênese dos pênfígos, sendo claramente identificados os alelos e haplótipos de suscetibilidade/proteção ao desenvolvimento do PF (BROCHADO et al., 2016; FRANCO BROCHADO et al., 2016). Em contraparte, os fatores imunológicos ainda não são totalmente esclarecidos. Acredita-se que a ligação dos autoanticorpos IgG às Dsgs ativaria uma série de mecanismos sinérgicos no processo de acantólise, os quais estariam caracterizados por: [a] inibição direta da transinteração homofílica Dsg-Dsg, gerando perda da ligação entre elas; [b] internalização das Dsgs, diminuindo sua expressão na superfície dos queratinócitos; e [c] ativação de diferentes vias de sinalização,

enfraquecendo a adesão celular (HAMMERS; STANLEY, 2016; KASPERKIEWICZ et al., 2017). Já, os fatores ambientais envolvidos na etiopatogênese do PF continuam sendo investigados. Fatores ambientais, como infecções virais (MACHADO et al., 2017), exposição a contaminantes hídricos e/ou agrícolas (CELERE et al., 2017; LA SERRA et al., 2019), doenças infecto-parasitárias (DIAZ et al., 2004; SOUSA, et al., 2012; WALSH et al., 2017) e picadas de insetos (EATON et al., 1998; LOMBARDI, et al., 1992; WAMBIER et al., 2017) têm sido propostos como potenciais iniciadores da resposta autoimune no PF.

A possível implicação de que insetos vetores estejam relacionados à etiopatogênese do PF vem sendo mencionada desde 1940 (ARANHA-CAMPOS, 1942; VIEIRA, 1940). A própria endemicidade da doença e a existência de focos geograficamente definidos, principalmente em áreas rurais próximas de fontes hidrográficas, demonstra a importância dos fatores ambientais (ABREU-VELEZ et al., 2010; AOKI, et al., 2015; DIAZ, et al., 1989). Desde os primeiros estudos sobre PF endêmico no Brasil, levantou-se a suspeita da participação de insetos antropofílicos na gênese da doença, particularmente *Simulium nigrimanum* (DIAZ et al., 1989; EATON et al., 1998; LOMBARDI et al., 1992). Inicialmente, considerou-se a possibilidade destes insetos atuarem como vetores de infecções particularmente virais. Ao longo do tempo, os estudos afastaram a origem viral e, com o conhecimento dos autoanticorpos anti-Dsg1, formulou-se a hipótese de mimetismo antigênico, isto é, admite-se a possibilidade dos Simulídeos, ou mesmo outros insetos, ao picarem o indivíduo, introduzirem através da saliva antígenos que despertariam a formação de anticorpos que reagiriam cruzadamente com a Dsg1 (LOMBARDI et al., 1992). Assim sendo, exposições repetidas ao antígeno em pacientes cronicamente expostos à picada de insetos desencadeariam uma resposta autoimune em indivíduos geneticamente predispostos (AOKI et al., 2015; QIAN et al., 2016).

Embora *S. nigrimanum* tenha sido por muitos anos o principal "suspeito" na etiopatogênese do PF, nas últimas duas décadas os flebotomíneos surgem como um novo fator ambiental associado ao PF no Brasil (ROSELINO et al., 2001). Mantendo o conceito de que proteínas salivares produziram reação cruzada com a Dsg1, e já considerando os avanços da biologia molecular com a consequente caracterização dos sialomas (transcriptomas das glândulas salivares) de insetos hematófagos de importância médica, dois peptídeos salivares emergem como potenciais candidatos: Maxadilan (ROSELINO et al., 2001) e LJM11 (QIAN et al., 2012), duas proteínas altamente imunogênicas presentes na saliva do flebotomíneo *Lutzomyia longipalpis* (LERNER et al., 1991; VALENZUELA et al., 2004).

O propósito desse estudo, constituindo o subprojeto VIII do projeto temático FAPESP 2010/51729-2, visou avaliar os fatores ambientais envolvidos na etiopatogênese do PF em amostra brasileira, focando na eventual participação das proteínas salivares dos insetos hematófagos presentes na região de estudo - a região nordeste do estado de São Paulo, Brasil.

2. REVISÃO BIBLIOGRÁFICA

2.1. CONCEITOS GERAIS

2.1.1 O DESMOSSOMO

O desmossomo (do grego “*desmos*”=ligação, e “*somatos*”=corpo) é uma estrutura celular especializada responsável pela adesão célula-célula. Os desmossomos se encontram localizados de maneira aleatória na membrana plasmática dos queratinócitos – as células diferenciadas do tecido epitelial – sendo constituídos por: [a] um domínio intracelular que outorga ancoragem na célula mãe, e por [b] um domínio extracelular que permite a união com a célula vizinha. O sistema de ancoragem está principalmente constituído por proteínas da família “armadilha”, as quais proporcionam acoplamento adequado com os filamentos de queratina da célula de origem. Assim, os filamentos se ligam às Desmoplaquinas, que por sua vez se conectam à estrutura circular citoplasmática constituída por Placofilinas e Placoglobinas, uma em cada célula. A partir dessas placas, surgem duas glicoproteínas da família das caderinas, que atravessam a membrana plasmática e atingem o espaço extracelular alcançando as caderinas da célula vizinha. Tanto Desmogleínas (Dsg) como Desmocollinas (Dsc), caracterizam-se por formar enlaces rígidos, cálcio dependentes, através de interações heterofílicas com seus pares contralaterais (**Figura 1**) (KITAJIMA, 2014; KOTTKE et al., 2006).

2.1.2 OS PÊNFIGOS

O termo pênfigo (do grego “*pemphix*”=bolha) refere-se a um grupo heterogêneo de dermatoses bolhosas crônicas autoimunes que podem afetar tanto a pele como membranas mucosas. Os pênfigos se caracterizam imunologicamente pela produção de autoanticorpos principalmente da classe IgG contra proteínas do desmossomo; histopatologicamente pela presença de acantólise intraepidérmica secundária ao depósito dos autoanticorpos nas Dsg; e clinicamente pela formação de lesões bolhosas na pele e/ou mucosas (KASPERKIEWICZ et al., 2017; POLLMANN et al., 2018).

Os pênfigos podem ser divididos em seis tipos: pênfigo vulgar (PV), pênfigo foliáceo (PF), pênfigo vegetante, pênfigo eritematoso, pênfigo paraneoplásico e pênfigo induzido por drogas

(RUOCCO et al., 2013), sendo os mais comuns (chamados de clássicos) o PV e o PF (AMAGAI et al., 2015). Nos pênfigos clássicos, os autoanticorpos são da classe IgG – geralmente do subtipo IgG₄ na atividade da doença – contra componentes extracelulares do desmossomo, principalmente as Dsgs. O PV caracteriza-se imunologicamente pela produção de autoanticorpos contra a Dsg3 ou de maneira simultânea contra Dsg1 e Dsg3, tendo como manifestação acometimentos mucoso e mucocutâneo. Por outro lado, o PF se caracteriza imunologicamente pela produção de autoanticorpos contra a Dsg1, levando a manifestações exclusivamente cutâneas (KASPERKIEWICZ et al., 2017).

Figura 1. Estrutura microscópica e representação esquemática do Desmossomo. Microscopia eletrônica evidenciando o Desmossomo seguido de representação esquemática dos diferentes componentes envolvidos na adesão celular. Dsg=Desmogleínas; Dsc=Desmocollinas; KIF=filamentos intermediários de queratina. Fonte: KOTTKE et al., 2006.

Embora apresentado em todo o mundo, a prevalência dos pênfigos é influenciada pela localização geográfica e etnia. A incidência anual dos pênfigos varia de país a país, de 0,76 a 16,1 casos por milhão de habitantes por ano, sendo o PV o mais comum dos pênfigos na maioria das populações (AMAGAI et al., 2015; ALCAIDE-MARTIN et al., 2010; FEMIANO, 2007; JOLY et al., 2011; KRIDIN et al., 2017; MEYER et al., 2010). As maiores taxas de PV foram documentadas em populações com ascendência judaica (*judeus Ashkenazi*) - sudeste da Europa e o Oriente Médio. No entanto, na África do Norte, Tunísia e América do Sul, a prevalência de PF é superior ao PV

(AMAGAI et al., 2015; CHACÓN et al., 2012; JOLY et al., 2011; KRIDIN et al., 2017; MEYER et al., 2010).

2.2. O PÊNFIGO FOLIÁCEO ENDÊMICO

O PF é subdividido em [a] PF clássico (também chamado de pênfigo “de Cazenave”) e [b] PF endêmico (conhecido no Brasil como “Fogo selvagem”). Ambas formas de PF compartilham a mesma imunopatogênese, caracterizada pela presença de autoanticorpos anti-Dsg1, mas diferem em seus padrões epidemiológicos e de distribuição (ABREU-VELEZ et al., 2010; CHACÓN et al., 2012). Enquanto o PF clássico é observado ao redor do mundo esporadicamente sem evidência de agrupamentos geográficos, sua forma endêmica tem sido descrita principalmente na América Latina, com focos geográficos claramente identificados no Brasil (DIAZ et al., 1989) (**Figura 2**), Colômbia (ABREU-VELEZ et al., 2003), Peru (ORTEGA-LOAYZA et al., 2013), Paraguai (ALDAMA et al., 1996) e Venezuela (GONZÁLEZ et al., 2006); e também em alguns países do norte da África (SALEH, 2015), com aglomerados definidos na Tunísia (BASTUJI-GARIN et al., 1995).

2.2.1 EPIDEMIOLOGIA DO PÊNFIGO FOLIÁCEO ENDÊMICO

Em algumas regiões endêmicas de ameríndios, no Estado do Mato Grosso do Sul, a prevalência de PF varia entre 1,3% e 3% (FRIEDMAN et al., 1995; HANS-FILHO et al., 1996). Assim, o Brasil apresenta a maior incidência de PF em todo o mundo (25-35 casos/milhão de habitantes/ano) (CHAGAS et al., 2005; DIAZ et al., 1989), seguida pela da Tunísia (6,7 casos/milhão de habitantes/ano) (BASTUJI-GARIN et al., 1995).

Atualmente, os principais focos da doença estão localizados nos estados de Goiás, Distrito Federal, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Paraná e São Paulo. No Estado de São Paulo, os municípios de Franca e Ribeirão Preto, foram os que apresentaram a maior prevalência de PF (CELERE et al., 2017; CHIOSSI et al., 2001; ABRÉU-VELEZ et al., 2010). Embora seja aceita a alta prevalência do PF no Brasil, uma série histórica de 21 anos confirmou o aumento da incidência do PV, ultrapassando a incidência do PF desde 1998 (GONÇALVES et al., 2011). Além deste aumento na incidência do PV, também há descrição de mudanças nos padrões

epidemiológicos, semelhantes ao PF, configurando uma nova variante de PV endêmico (CELERE, et al., 2017; CHACÓN et al., 2012; ROCHA-ALVAREZ et al., 2007).

Figura 2. Focos de Pênfigo Foliáceo endêmico no Estado de São Paulo. Representação esquemática do estado de São Paulo identificando os casos de pênfigo foliáceo endêmico (pontos pretos). Observa-se a distribuição geográfica (próximos de bacias hidrográficas) e em conglomerados principalmente na cidade de Franca, Ribeirão Preto e Araraquara. Fonte: DIAZ et al., 1989.

2.3. ETIOPATOGENESE DO PÊNFIGO FOLIÁCEO ENDÊMICO

A etiopatogênese do PF ainda não é totalmente esclarecida, porém tem sido amplamente estudada nos últimos anos, envolvendo fatores genéticos, imunológicos e ambientais (ABREU-VELEZ et al., 2010; AOKI, et al, 2015; QIAN et al., 2016).

2.3.1 FATORES GENÉTICOS

Igualmente a outras doenças autoimunes, o PF tem sido associado a genes do complexo maior de histocompatibilidade (MHC – *Major Histocompatibility Complex*). O MHC corresponde a um segmento genômico localizado na região 6p21.3 no braço curto do cromossomo 6, o qual codifica glicoproteínas de membrana especializadas na apresentação antigênica e resposta imune.

Nos seres humanos, são denominados de antígeno leucocitário humano (*HLA – Human Leukocyte antigen*) e apresentam extenso polimorfismo, sendo cada variante denominada como alelo (KELLY et al., 2019).

Os genes codificados pelo MHC podem ser classificados em três classes de acordo com sua estrutura e função. As classes de *HLA* mais estudadas no PF são: classe I - *HLA-A, B e C*; e as de classe II - *HLA-DR, DQ e DP* (BROCHADO et al., 2016; KASPERKIEWICZ et al., 2017). Tanto na Tunísia como no Brasil, os alelos relacionados à susceptibilidade ou proteção para o desenvolvimento de PF tem sido claramente identificados (ABIDA et al., 2009; BROCHADO et al., 2016).

No Brasil, os alelos *HLA-DRB1*01:01, *01:02, *01:03, *04:04, *04:06, *04:10, *14:06 e *16:01* têm sido associados à suscetibilidade ao PF, enquanto o alelo *HLA-DQB1*02:01* tem sido associado à proteção ao PF (BROCHADO et al., 2016). Há poucos estudos que associam pênfigos aos alelos *HLA* de classe I e a significância desses achados ainda não está totalmente esclarecida (SINHA, 2011). No Sudeste do Brasil, os genes *HLA* de classe I - *HLA-A* 11/33 e -B *14* foram associados à suscetibilidade, e *HLA-A*02* à proteção ao PF. Os haplótipos associados à suscetibilidade ao PF foram *HLA-DRB1*04-DQAI*03-DQB1*04, -DRB1*01-DQAI*01-DQB1*05 e -DRB1*16-DQAI*01-DQB1*05* enquanto o *-DRB1*11-DQAI*05-DQB1*03* à proteção ao PF (FRANCO BROCHADO et al., 2016; BROCHADO et al., 2016).

2.3.2 FATORES IMUNOLÓGICOS E MECANISMOS DE ACANTÓLISE

As Dsgs (do grego “*desmos*”=nó e “*glein*”=semelhante à cola) são um grupo de proteínas pertencentes à família das caderinas, responsáveis pela ligação extra-citoplasmática do desmossomo. Há quatro isoformas de Dsgs: Dsg1, Dsg2, Dsg3 e Dsg4. A Dsg2 é expressa em todos os tecidos que têm desmossomos, incluindo epitélios simples e miocárdio, enquanto a expressão de Dsg1 e Dsg3 está restrita ao epitélio escamoso estratificado. A Dsg1, proteína de 160kDa, localiza-se preferencialmente nas camadas mais superficiais da epiderme (KASPERKIEWICZ et al., 2017; POLLMANN et al., 2018)

As Dsgs são expressas como pró-peptídeos, isto é, como proteínas precursoras (inativas) que sofrem modificação pós-transducional. Logo da sua maturação, com cerca de 26 resíduos, as Dsgs são clivadas na superfície da membrana celular pela porção N-terminal, que promove a interação intercelular, seguida de uma hélice de transmembrana para finalmente se unir à placa desmossômica

citoplasmática pela porção C-terminal. Assim, o segmento extracelular apresenta cinco domínios (EC1 a EC5) (**Figura 3**) (KITAJIMA, 2014; KOTTKE et al., 2006; YOKOUCHI et al., 2009).

Figura 3. Maduração e clivagem das Desmogleínas. De esquerda à direita: a expressão do pro-peptídeo (precursor da Desmogleína 1 com os domínios extracelulares EC1 – EC5) seguido da maturação e pós clivagem no desmossomo. Fonte: YOKOUCHI et al., 2009.

As extremidades amino-terminais das Dsgs expressam epítomos importantes para a patogenicidade do PF. Sabe-se que o soro de pacientes com PF na fase pré-clínica reconhece epítomos na porção terminal COOH do domínio EC5 da Dsg1. O início da fase clínica da doença está associado ao surgimento de anticorpos específicos para epítomos das porções terminais NH2 dos domínios EC1 e EC2. Quando há remissão da doença, nota-se aumento da dosagem de autoanticorpos que reconhecem o domínio EC5 da Dsg1, sugerindo que a variação na expressão desses epítomos possa estar relacionada aos episódios de remissão e recorrência da doença. Assim, a soroconversão de indivíduo saudável para doente seria caracterizada pela modificação do perfil das imunoglobulinas - de IgG contra o epítomo EC5 na fase pré-clínica para IgG₄ reconhecendo os domínios EC1 e EC2 - fenômeno denominado de *epitope spreading* (**Figura 4**) (HANS-FILHO et al., 1996; QIAN et al., 2009; LI et al., 2003).

Para comprovar a patogenicidade na mudança desse perfil sorológico, anticorpos anti-Dsg1 da classe IgG₁ foram transferidos passivamente para camundongos recém-nascidos, evidenciando não haver desenvolvimento do PF; ao contrário, a transferência passiva de anti-Dsg IgG₄ levou ao desenvolvimento de acantólise subcórnea (CULTON et al., 2008).

Nos pênfigos, pode ocorrer discordância entre os perfis clínicos e sorológicos em cerca de um terço dos casos (SARDANA et al., 2013). Esta observação, e o conhecimento de que a presença de autoanticorpos anti-Dsg1 e anti-Dsg3 não resulta em completa dissolução do epitélio, sugere que

outros fatores contribuem para o desenvolvimento do pênfigo. Nos últimos anos há ênfase no papel de antígenos desmossomais diferentes da Dsg como causadores de pênfigos, aumentando consideravelmente o número de novas moléculas estudadas. Alguns exemplos são as Desmoplaquinas, receptores de acetilcolina dos queratinócitos, Dscs, colágeno XVII, além de outras moléculas relacionadas à alteração celular que resultam em acantólise (CIRILLO, 2013).

Figura 4 – Etiopatogênese do pênfigo foliáceo endêmico. Representação esquemática do queratinócito com destaque na Desmogleína 1 incluindo os domínios extracelulares EC1 – EC5. Anticorpos do subtipo IgG₁ (nos patogênicos – representados em laranja) contra o domínio EC5. Observa-se a mudança no perfil sorológico devido a fatores genéticos (alelos *HLA* de susceptibilidade) e ambiente, levando a produção de autoanticorpos do tipo IgG₄ (patogênicos – representados em vermelho) contra o domínio EC1. Fonte: HANS-FILHO et al., 2018

Outros dados que suportam o envolvimento de outros fatores na patogênese do pênfigo são as mudanças nos padrões epidemiológicos e clínicos do PF. Têm sido descritos anticorpos circulantes anti-Dsg1 em indivíduos saudáveis - em familiares de indivíduos acometidos pelo PF ou em moradores de áreas endêmicas. Há casos de transição de fenótipos de PF para PV e vice-versa, com expressão de anticorpos anti-Dsg1 no PV, e anti-Dsg3 no PF, e fenótipo de PV em área endêmica para PF (ROCHA-ÁLVAREZ et al., 2007). Estes dados, aliados ao aumento progressivo dos níveis de anticorpos anti-Dsg1 de acordo com a proximidade do foco endêmico de PF, suportam a hipótese da associação de um agente ambiental na patogênese da doença (WARREN et al, 2000; DIAZ et al, 2008).

Os mecanismos da acantólise nos pênfigos ainda permanecem desconhecidos. Inicialmente, a perda da tolerância à Dsg1 é estabelecida por ocasião da exposição imunológica do peptídeo (ou parte dele) aos linfócitos Th₂ pelas células apresentadoras de antígenos (células de Langerhans e células dendríticas), mediado por alelos *HLA* relacionados à susceptibilidade da doença, e

consequentemente na produção de autoanticorpos pelos linfócitos B (HEUPEL, 2008; KASPERKIEWICZ et al., 2017; POLLMANN et al., 2018; SHARMA; MAO; PAYNE, 2007).

Estudos com modelos experimentais em ratos, culturas de queratinócitos humanos e biópsias de pele de pacientes com pênfigo sugerem que a ligação dos autoanticorpos nas Dsgs podem gerar acantólise por diversos mecanismos sinérgicos. O primeiro deles seria devido à inibição direta da união Dsg-Dsg por bloqueio mecânico da transinteração homofílica (“*steric hindrance*”) gerando perda da ligação entre elas. Apesar disso, esse mecanismo não explicaria totalmente o processo de acantólise, considerando que a dissociação do enlace Dsg-Dsg pode ocorrer sem bloquear a transinteração homofílica da Dsg1 (**Figura 5**) (HEUPEL, 2008; KASPERKIEWICZ et al., 2017; POLLMANN et al., 2018; SHARMA et al., 2007).

Figura 5. Mecanismo de acantólise nos pênfigos. Acima à direita observa-se a estrutura normal do desmossomo. Em seguida mostra acima à direita e embaixo os três mecanismos que explicariam o processo de acantólise, incluindo o bloqueio mecânico (*steric hindrance*), a depleção de Desmogleínas devido a fenômenos de agrupação e internalização, e a ativação de vias de sinalização que contribuiriam com o enfraquecimento dos enlaces. Fonte: KASPERKIEWICZ et al., 2017.

Assim, o mecanismo de acantólise por *steric hindrance* passou a ser complementado por uma nova hipótese, a qual se baseia na capacidade dos autoanticorpos anti-Dsg1 agruparem e redistribuírem a Dsg1 no desmossomo. A formação de cluster e a internalização das Dsgs levariam à depleção das caderinas no desmossomo, evitaria a incorporação de novas Dsgs sendo produzidas,

dificultando a adesão celular pela falta da expressão de Dsg1 na superfície dos queratinócitos (HAMMERS; STANLEY, 2016; KASPERKIEWICZ et al., 2017; SPINDLER et al., 2014).

Além destes dois mecanismos, numerosas moléculas de sinalização e vias metabólicas têm sido implicadas na acantólise nos pênfigos: p38 *mitogen-activated protein kinase* e seu efector; *Epidermal growth factor receptor*; RHO GTPases; c-MYC; fosfolipase C e sua forma ativada fosfoquinase C, e caspases. Entretanto, nenhum evento de sinalização é suficiente para induzir acantólise epidérmica/ porém contribuem no enfraquecimento da adesão celular (HAMMERS; STANLEY, 2016; KASPERKIEWICZ et al., 2017; SPINDLER et al., 2014).

2.3.3 FATORES AMBIENTAIS

Fatores genéticos isolados não são suficientes para causar os pênfigos, e desencadeantes ambientais parecem ter papel na indução e/ou exacerbação da doença (MARTINELLI et al., 2016). Os fatores ambientais envolvidos na etiopatogênese do PF continuam sendo investigados. Do ponto de vista imunológico, a maioria dos agentes que estimulam o sistema imune pode atuar como gatilhos à autoimunidade, tais como alterações hormonais, medicamentos, doenças, alimentos, estresse emocional, gestação, entre outros (TAVAKOLPOUR, 2017)

No sudeste brasileiro, os fatores ambientais associados ao PF têm sido amplamente estudados, incluindo infecções virais (MACHADO et al., 2017), estresse (MATIAS; ROSELINO, 2013), exposição a contaminantes hídricos e/ou agrícolas (CELERE et al., 2017; LA SERRA et al., 2019), doenças infecto-parasitárias (DIAZ et al., 2004; SOUSA, et al., 2012; WALSH et al., 2017) e picadas de insetos (EATON et al., 1998; LOMBARDI, et al., 1992; WAMBIER et al., 2017). Todos têm sido propostos como potenciais desencadeantes da resposta autoimune no PF.

A expansão das fronteiras urbanas com a consequente invasão das florestas (principalmente devido ao agronegócio da cana e às plantações de café no estado de São Paulo) apresentou novo estágio de convivência com a fauna local anteriormente confinada fora das cidades. Há maior exposição às picadas de insetos hematófagos, levando ao aumento de inúmeras doenças anteriormente restritas às atividades rurais (por exemplo, leishmaniose, uma doença transmitida por picada de flebotomíneos) (TOLEZANO, 1994). É nesse cenário que os focos geográficos dos casos de PF começam a aparecer, especialmente bacias hidrográficas ao redor, levando a suspeitar que um ambiente local possa estar envolvido na etiopatogênese do PF (AOKI et al., 2015).

2.4. ALERGIA E AUTOIMUNIDADE

Anticorpos da classe IgE têm sido tradicionalmente relacionados a reações alérgicas por hipersensibilidade tipo 1. Nos últimos anos, a detecção de anticorpos IgE antinucleares na artrite reumatoide e no lúpus eritematoso sistêmico permitiram a hipótese de que os fenômenos alérgicos poderiam ter papel fisiopatológico na autoimunidade (ETTINGER et al., 2017; MURO, 2001). Tanto a alergia como a autoimunidade são resultados de disfunção do sistema imunológico. Embora os processos patológicos sejam diferentes, existem evidências de que as duas anormalidades poderiam compartilhar um processo fisiopatológico em comum (ROTTEM, 2002). Assim, condições alérgicas e autoimunes podem coincidir no mesmo paciente. Por exemplo, crianças com artrite reumatoide e doença celíaca têm maior incidência de asma (KERO et al., 2001; SIMPSON et al., 2002), e anticorpos IgE de pacientes com dermatite atópica podem reagir contra proteínas humanas (VALENTA et al., 1996; VALENTA et al., 2000). Outro mecanismo fisiopatológico em comum é que os alérgenos que a IgE visa em pacientes atópicos podem ser exógenos (alérgenos) ou endógenos (autoantígenos) (ETTINGER et al., 2017; MITTERMANN et al., 2004; VALENTA et al., 1997), além de vários outros alérgenos que exibem epítomos compartilhados com proteínas próprias do ser humano, por exemplo, infecções fúngicas ou pólen (CRAMERI et al., 1996; VALENTA et al., 1991).

Em relação às dermatoses bolhosas autoimunes, estudos com penfigóide bolhoso (PB) mostraram autoanticorpos elevados da classe IgE com papel fisiopatológico; de fato, uma revisão sistemática recente confirma que, quanto mais altos os níveis séricos de autoanticorpos IgE contra os alvos patogênicos do PB (BP180 e BP230), mais grave será a manifestação do PB (SANIKLIDOU et al., 2018). Da mesma forma, anticorpos IgE anti-Dsg3 no PV se correlacionam diretamente com autoanticorpos patogênicos IgG₄ contra Dsg3, e se associam intimamente com a atividade da doença (NAGEL et al., 2010).

Outra ligação direta entre alergia e autoimunidade são os anticorpos envolvidos em ambos processos, IgE e IgG₄, sendo esse último o principal anticorpo envolvido na patogênese das dermatoses bolhosas autoimunes, incluindo o PF (EVANGELISTA et al., 2018; QIAN et al., 2016). Reações alérgicas estão caracterizadas pela formação de anticorpos da classe IgE, mas com uma resposta paralela caracterizada pela formação de anticorpos IgG₄ com o intuito de bloquear a resposta inflamatória induzida pelos alérgenos (ETTINGER et al., 2017; ROTTEM, 2002). Esse fato pode ser comprovado em estudos de imunoterapia, demonstrando valores séricos recíprocos de

anticorpos IgE e IgG₄, isto é, os níveis de IgE diminuíram à medida que os níveis de IgG₄ aumentaram durante os meses após a imunoterapia (TRAMPERT et al., 2018). O papel da IgG₄ na hipersensibilidade tem sido amplamente estudado na última década. Hipotetizou-se que IgG₄ atuaria como bloqueador da resposta alérgica por três mecanismos: [I] competição com IgE por locais antigênicos nos alérgenos, [II] ligação de receptores Fc em células imunes (macrófagos, células B, neutrófilos, mastócitos e basófilos), desencadeando eventos imunomoduladores, e [III] comportando-se como anticorpos anti-idiotípicos, isto é, agindo contra o próprio IgE (**Figura 6**) (KONECZNY, 2018; LARCHE, 2007; STRAIT et al., 2006; TRAMPERT et al., 2018).

Figura 6. Papel dos anticorpos IgG₄ na alergia e hipersensibilidade. Representação esquemática dos mecanismos pelos quais os anticorpos IgG₄ participariam na resposta alérgica e de hipersensibilidade. Fonte: KONECZNY, 2018

A troca de classe supracitada estaria determinada por uma resposta Th₂, sendo mediada principalmente pelas citocinas IL-4 e IL-13. No entanto, a estimulação antigênica crônica induz a secreção de IL-10, na tentativa de diminuir a inflamação provocada pelos alérgenos, porém que altera o equilíbrio da troca de classe em direção à IgG₄ (JEANNIN et al., 1998). Desde o ponto de vista de hipersensibilidade, a intervenção de IL-10 apresenta um resultado benéfico ao paciente,

mas em indivíduos geneticamente susceptíveis, esse processo pode desencadear uma resposta IgG mais robusta, levando à produção de IgG₄ com atividade patogênica (**Figura 7**). Assim, é possível que um alérgeno ambiental desencadeie a resposta de IgE no PF, e os anticorpos direcionados contra esse antígeno externo reajam de maneira cruzada com a Dsg1 (QIAN et al., 2016).

Figura 7. Evolução da resposta alérgica durante exposição crônica. Em fase aguda, o estímulo alérgico leva a uma resposta Th₂ com uma produção de anticorpos maioritariamente do tipo IgE (acima). A exposição crônica dos alérgenos estimularia uma modulação por IL-10 visando uma redução dos efeitos imunológicos associados à hipersensibilidade associado a uma troca de classe para anticorpos do tipo IgG₄. Fonte: TRAMPERT et al., 2018.

2.4.1 PÊNFIGO FOLIÁCEO E IMUNOGLOBULINA E

Considerando a endemicidade do PF em um contexto de países tropicais em vias de desenvolvimento (principalmente América Latina e o norte da África) (BASTUJI-GARIN et al., 1995; DIAZ et al., 1989), a presença de anticorpos IgE no PF foi atribuída a uma potencial infestação por parasitas intestinais (BRUNS et al., 1978). A potencial relação da alergia e autoimunidade nos últimos anos levou à hipótese de que altos níveis de IgE anti-Dsg1 em pacientes com PF poderia ser melhor explicado por uma exposição contínua a um antígeno (ou antígenos) sensibilizantes de origem ambiental, presente nas regiões em que esses pacientes vivem, que por infecções infecto-parasitárias *per se* (QIAN et al., 2016).

Na última década, estudos revelaram que pacientes com PF de áreas endêmicas do Brasil apresentavam níveis significativamente mais altos de anticorpos IgE anti-Dsg1 em comparação com pacientes com PF (“de Cazenave”) procedentes dos Estados Unidos e Japão. Os níveis de IgE anti-Dsg1 nesses pacientes brasileiros com PF estariam intimamente correlacionados aos autoanticorpos

patológicos da classe IgG₄ anti-Dsg1 (QIAN et al., 2011). Interessantemente, os níveis de IgE anti-Dsg1 de pacientes saudáveis moradores de áreas endêmicas também foram significativamente mais altos em comparação com paciente saudáveis que moravam em áreas não endêmicas fora do Brasil, sugerindo que existe um fator ambiental presente exclusivamente em regiões com alta incidência de PF (QIAN et al., 2011). Além disso, os títulos de IgE anti-Dsg1 de pacientes com PF aumentavam significativamente na transição da fase pré-clínica para a fase clínica da doença (QIAN et al., 2015).

2.5 INSETOS HEMATÓFAGOS E O PÊNFIGO FOLIÁCEO ENDÊMICO

Em 1940 Vieira descreveu o comportamento geográfico do PF no estado de São Paulo, mostrando vários focos claramente definidos, especialmente próximos aos rios e principalmente na região nordeste do estado. Vieira também apontou um vínculo epidemiológico entre *S. nigrimanum* (um inseto hematófago da família das moscas), e a endemicidade do PF (VIEIRA, 1940). Posteriormente, a relação epidemiológica entre borrachudos e o PF também foi confirmada em outros focos endêmicos, sendo evidenciada a presença de diferentes espécies de borrachudos incluindo *S. nigrimanum* (EATON et al., 1998). Além disso, as picadas de borrachudos também foram consideradas como um fator de risco para o desenvolvimento de PF (razão de risco de 4,7) em uma reserva indígena do Mato Grosso do Sul (LOMBARDI et al., 1992).

2.5.1 PROTEÍNAS SALIVARES DE INSETOS HEMATÓFAGOS E DESMOGLEÍNA 1

Doenças infecto-parasitárias são associadas à patogênese do PF. Oncocercose, Leishmaniose e a Doença de Chagas estão sendo relacionadas ao PF endêmico tanto no Brasil como na Tunísia. Os pacientes portadores dessas condições infecciosas também exibem autoanticorpos circulantes contra Dsg1 (DIAZ et al., 2004; KALLEL-SELLAMI et al., 2007; WALSH et al., 2017; ZARAA et al., 2012). Curiosamente, as três condições infecciosas supracitadas são transmitidas por insetos hematófagos vetores: Simulídeos, Flebotomíneos e Triatomíneos, respectivamente. Ressalta-se que os títulos de IgG anti-Dsg1 nessas três condições parasitárias são significativamente mais altas em comparação com outras infecções, tais como Blastomicose e Hanseníase (doenças infecciosas não vetoriais) (DIAZ et al., 2004), o que sugere que os componentes salivares de seus respectivos vetores poderiam ter participação na etiopatogênese do PF.

No estado do Mato Grosso do Sul, pacientes com PF endêmico possuem anticorpos da classe IgE e IgG₄ contra antígenos (extrato proteico total) da glândula salivar do flebotômico *Lu. longipalpis*. Os níveis de IgE e IgG₄ contra esse extrato salivar se correlacionam significativamente com os autoanticorpos IgG₄ anti-Dsg1, sugerindo um fenômeno de “*cross reaction*” durante exposição crônica alérgica contra a picada desse inseto (QIAN et al., 2012).

2.6 SIMULÍDEOS (*BLACKFLIES*)

2.6.1 CONCEITOS GERAIS DOS SIMULÍDEOS

Pertencentes à família Simuliidae, os borrachudos ocupam a trigésima posição em número de espécies dentre as 130 famílias de *Diptera* (ADLER, 2004). De acordo com a última contagem, aproximadamente 2.151 espécies válidas estão descritas no planeta (ADLER, CROSSKEY, 2014), o que representa aproximadamente 2% do total de espécies de Dípteras descritas. Sua abundância, ampla distribuição geográfica, importância médica e econômica e, principalmente, as picadas nos humanos lhes garantem um privilegiado reconhecimento. Os borrachudos adultos são diurnos, ambos os sexos alimentam-se de néctar de plantas e flores, e somente as fêmeas de algumas espécies antropofílicas alimentam-se de sangue humano (ADLER, 2004; COSCARÓN; COSCARÓN-ÁRIAS, 2007).

2.6.2 IMPORTÂNCIA MÉDICA DOS SIMULÍDEOS

As fêmeas adultas de simulídeos estão entre os insetos hematófagos mais incômodos ao ser humano e outros animais. São de importância médica e veterinária por seu potencial como vetores de várias doenças transmitidas pelo carreamento de diversos agentes etiológicos, tais como, bactérias, helmintos, protozoários e vírus. Algumas espécies são incriminadas como vetores de *Onchocerca volvulus* e *Mansonella ozzardi*, agentes patogênicos da oncocercose (“cegueira de rio”) e mansonelose, respectivamente (SHELLEY et al., 1976; SHELLEY et al., 1997; SHELLEY; COSCARÓN, 2001; MARTINS et al., 2010; REIDPATH et al. 2011).

No estado do Pará, Pinheiro et al. (1974) sugerem que a “Síndrome Hemorrágica de Altamira” (uma doença endêmica, caracterizada por púrpura petequial, equimoses e sufusões hemorrágicas, além de sangramento da mucosa oral e gastrointestinal) seja uma virose transmitida

por simúlideos. Atualmente, considera-se o mecanismo do desencadeamento da síndrome desconhecido, porém, possivelmente relacionada à hipersensibilidade às substâncias contidas na saliva dos “piuns” (nome dos borrachudos no Pará) (PINHEIRO et al., 1974).

S. vittatum tem sido incriminado como vetor de vesículo vírus, *New Jersey Virus*, agente etiológico de dermatoses bolhosas em cavalos, porcos e vacas (ADLER et al., 2004).

2.6.3 DISTRIBUIÇÃO DOS SIMULÍDEOS NO ESTADO DE SÃO PAULO.

No Brasil, há registro de 91 espécies, sendo que 55 dessas espécies encontram-se no estado de São Paulo (PEPINELLI, 2003). Não existem estudos de organizações oficiais governamentais em relação à distribuição dos borrachudos no Brasil (sem importância na saúde pública). No entanto, no ambiente acadêmico foi possível evidenciar a distribuição do *S. nigrimanum* na tese de doutorado do Dr. Mateus Pepinelli, Universidade de Campinas, São Paulo. Embora não existisse uma coleção sistemática envolvendo todos os municípios do estado, foi possível evidenciar o georreferenciamento desse inseto principalmente na região nordeste do estado de São Paulo, coincidentemente onde se localizam os principais focos geográficos de PF (**Figura 8**) (PEPINELLI, 2003).

28-*Simulium nigrimanum*

Figura 8. Georreferenciamento da distribuição do *Simulium nigrimanum* no estado de São Paulo, Brasil. Locais de coleta positiva (pontos pretos) do borrachudo *Simulium nigrimanum* no estado de São Paulo, Sudeste Brasileiro. Fonte: PEPINELLI, 2003.

2.6.4 PROTEÍNAS SALIVARES (SIALOTRANSCRIPTOMA) DO *SIMULIUM NIGRIMANUM*

A identificação de possíveis antígenos salivares de Simúlideos com a capacidade de gerar o fenômeno de mimetismo molecular foi descrita pela análise do sialotranscritoma de *S. nigrimanum*. Proteínas salivares do *S. nigrimanum* - denominadas “*Collagen-like*” - evidenciaram semelhanças

significativas com as Dsgs, porém essas proteínas ainda não foram testadas para avaliar possível reação cruzada com a Dsg1 (RIBEIRO et al., 2010). Com o mesmo intuito supracitado, considerando-se os títulos elevados de anti-Dsg1 na Doença de Chagas (DIAZ et al., 2004), o mesmo grupo também analisou a glândula salivar do *Triatoma matogrossensis*. A descrição do sialotranscritoma desta última espécie não revelou semelhança significativa com as Dsgs (ASSUMPÇÃO et al., 2012).

2.7 FLEBOTOMÍNEOS (*SANDFLIES*)

2.7.1 CONCEITOS GERAIS DOS FLEBOTOMÍNEOS

Os flebotomíneos são insetos pequenos que pertencem à ordem *Diptera* (mesmo grupo das moscas, mosquitos, borrachudos e maruins), família *Psychodidae*, sub-família *Phlebotominae*. No Brasil, esses insetos podem ser conhecidos por diferentes nomes, de acordo com a sua ocorrência geográfica, como *tatuquira*, *mosquito palha*, *asa dura*, *asa branca*, *cangalhinha*, *birigui*, *anjinho*, entre outros. São conhecidas cerca de 900 espécies no mundo, das quais 470 estão distribuídas no continente Americano (GALATI, 2003), e 69 têm sido descritas no Estado de São Paulo (SHIMABUKURO, GALATI, 2010), sendo o gênero *Lutzomyia* responsável pela transmissão da Leishmaniose. Só as fêmeas sugam o sangue, necessário para completar seu ciclo reprodutivo. Sua picada costuma ser dolorosa, e pode transmitir várias espécies de *Leishmania*, tanto em florestas quanto em ambientes modificados, incluindo áreas urbanizadas ou só com vegetação arbustiva (SUCEN, 2015^a).

2.7.2 IMPORTÂNCIA MÉDICA DOS FLEBOTOMÍNEOS

Existem registros de Leishmaniose Tegumentar Americana (LTA) no estado de São Paulo desde 1884. A expansão das plantações de café durante o século XIX provocou aumento da transmissão da LTA, produto do desmatamento que continuou crescendo durante o início do século XX, chegando até a região noroeste do estado, pela construção de ferrovias para o transporte de grãos de café para a costa (TOLEZANO, 1994). Depois dos anos 50's, a LTA tornou-se endêmica na região sul do estado e sua transmissão deixou de ser influenciada pelos fatores ambientais do

desmatamento, passando a ser dependente principalmente da adaptação de insetos vetores no ambiente humano e de animais de criação (CAMARGO-NEVES et al., 2002).

L. (V.) braziliensis é o principal agente etiológico da LTA no estado de SP, a qual é transmitida por 5 espécies de flebotomíneos: *Nyssomyia intermedia*, *Nyssomyia neivai*, *Nyssomyia whitmani*, *Migonemyia migonei* e *Pintomyia pessoai* (MARCONDES, 1996; CAMARGO-NEVES et al., 2002; ANDRADE-FILHO et al., 2007). *L. (L.) amazonensis* também tem sido descrita no nordeste de São Paulo (MEDEIROS et al., 2008), porém o único vetor reconhecido para esta espécie é *Bichromomyia flaviscutellata*, o qual tem sido reportado apenas na costa sul do estado de SP (GOMES et al., 1990).

2.6.3 DISTRIBUIÇÃO DOS FLEBOTOMÍNEOS NO ESTADO DE SÃO PAULO

Em 2010, Shimabukuro et al. reuniram todos os dados de distribuição de flebotomíneos do estado de SP entre os anos 1943 e 2009 (publicações e registros da SUCEN) e foram contrastados com os casos de LTA notificados. Descreveu-se a presença de pelo menos uma das 5 espécies relacionadas à transmissão da LTA em 183 (28,4%) dos 645 municípios do estado. Para os demais municípios, não havia informação disponível, e não existem estudos públicos que reportem a presença de flebotomíneos; a maioria destes municípios se encontra no centro e norte do estado (SHIMABUKURO et al., 2010).

Nyssomyia intermedia e *Nyssomyia neivai* foram as espécies descritas com maior frequência, principalmente na região sul do estado onde existe maior prevalência de LTA (**Figura 9**).

Figura 9. Distribuição de *Nyssomyia neivai* em relação aos casos reportados de Leishmaniose Tegumentar Americana no Estado de São Paulo. Representação esquemática dos municípios do estado de São Paulo. Em tonalidades de amarelo até vermelho (baixa até alta respectivamente) mostram a incidência de Leishmaniose Tegumentar Americana. Municípios riscados revelam a presença de *Nyssomyia neivai*. Fonte: SHIMABUKURO et al., 2010.

A forma sistêmica da infecção por leishmania - Leishmaniose Visceral (LV) - vem ocorrendo no estado de São Paulo em municípios situados na região oeste. A espécie de flebotomíneo envolvida na transmissão é *Lutzomyia longipalpis*, a qual foi registrada pela primeira vez no município de Araçatuba, em 1997 (CAMARGO-NEVES et al., 1999). Camargo-Neves et al. realizaram, em 2003, revisão epidemiológica de LV no estado de SP, verificando a expansão da doença e o aumento do número de casos em todos os municípios onde a doença havia sido detectada (**Figura 10**) (CAMARGO-NEVES, et al, 2003).

Figura 10. Distribuição de *Lutzomyia longipalpis* no Estado de São Paulo. Representação esquemática dos municípios do estado de São Paulo. Em tonalidades de cinza mostra-se a pesquisa entomológica positiva, negativa ou não pesquisa de *Lutzomyia longipalpis*. Fonte: CAMARGO-NEVES et al, 2003.

2.6.4 PROTEÍNAS SALIVARES (SIALOTRANSCRIPTOMA) DE *LUTZOMYIA LONGIPALPIS*

Em 2001, constatou-se que soros de pacientes com PF apresentavam maiores títulos de anticorpos contra Maxadilan – proteína salivar de *Lu. longipalpis* - comparados aos indivíduos saudáveis moradores da mesma região endêmica (ROSELINO et al., 2001).

Considerando a reação cruzada entre anticorpos IgE e IgG₄ contra o extrato salivar de *Lu. longipalpis*, Qian et al. realizaram testes adicionais utilizando três proteínas altamente imunogênicas do sialoma de *Lu. Longipalpis*: LJM11, LJM17 e LJM143 (QIAN et al., 2012; VALENZUELA et al., 2004). Curiosamente, Maxadilan, a proteína mais imunogênica do extrato salivar, não foi utilizada nesses testes. Esses experimentos evidenciaram que os anticorpos monoclonais IgG₄ anti-Dsg1, isolados de pacientes com PF, reagiram de maneira cruzada aos antígenos Dsg1 e LJM11. Além disso, camundongos imunizados contra LJM11, mas não contra

LJM17 e LJM143, desenvolveram intensa resposta humoral à Dsg1 humana. Todas essas evidências sugerem fortemente a existência de um fenômeno de “*antigenic mimicry*” entre LJM11 e Dsg1 (**Figura 11**) (QIAN et al., 2012).

Figura 11. Hipótese sobre a reatividade cruzada entre antígenos ambientais e a etiopatogênese do pênfigo foliáceo endêmico. A exposição ao estímulo ambiental (nesse caso a proteína salivar LJM11) levaria inicialmente a uma resposta por hipersensibilidade à produção de anticorpo IgE anti-LJM11 os quais levariam um reação cruzada por mimetismo antigênico à produção de anticorpos IgE anti-Desmogleína 1. A exposição crônica levaria a uma estimulação de IL-10 e consequentemente a uma troca do perfil de anticorpos para IgG₄ (patogênicos) contra Desmogleína 1. Fonte: QIAN et al., 2016.

2.7 CULICÍDEOS

Assim como os flebotomíneos, esses insetos pertencem à ordem *Diptera*, família *Culicidae*. O gênero *Aedes* é responsável pela transmissão da Dengue, sendo incriminadas várias espécies do subgênero *Stegomyia* (*Aedes aegypti*, *Aedes albopictus* e *Aedes polynesiensis*), nas quais o vírus foi encontrado *in natura* (BRAGA et al., 2007). A espécie *A. aegypti* tem distribuição mundial encontrando-se em geral entre as latitudes 35° Norte e 35° Sul, que correspondem à isoterma de inverno de 10°C (GUZMAN; HARRIS, 2015). É um mosquito adaptado ao ambiente urbano e utiliza os recipientes mais frequentes no domicílio ou peridomicílio, tanques de armazenamento de água e vasilhames temporários como potes, barris, pneumáticos usados, etc., para o desenvolvimento de sua fase larvária (BRAGA et al., 2007; WHO, 2009).

Nas Américas, embora amplamente disseminado, até o momento, não há evidências de que *Aedes albopictus* seja eficiente transmissor da dengue. No entanto, *A. aegypti*, responsável pela transmissão dessa doença desde a década 50 no Brasil, apresentou sucessivos registros de reintrodução no nosso país, estabelecendo-se definitivamente ao final do século XX (TEIXEIRA et

al., 2009). Atualmente, essas espécies estão presentes em 3.592 (*A. aegypti*) e 1.533 (*Aedes albopictus*) municípios brasileiros (HONÓRIO, LOURENÇO-DE OLIVEIRA, 2001). No estado de São Paulo, a infestação teve início na década dos anos 80. Em 1995, foi registrada a presença desses vetores em 626 municípios, quando 415 estavam infestados por *A. aegypti* (GLASSER et al., 2000).

A ocorrência de sucessivas epidemias de dengue em todo o mundo e no Brasil, em particular, vem sendo objeto de inquietação por parte de técnicos, pesquisadores e da sociedade em geral, com aumento da morbidade e letalidade (WHO, 2009).

Na literatura médica, não existem registros que relacionem a picada de *A. aegypti* com a etiopatogênese do PF. Porém, *Dipteros*, incluindo *Simuliidae* e *Psychodidae*, possuem certas famílias de proteínas salivares em comum, especificamente a família *D7/odorant-binding protein*, que auxiliam nos processos de vasoconstrição, antiplaquetário e inflamatório (JABLONKA, 2019; VALENZUELA et al., 2002). Do mesmo modo, uma proteína salivar imunogênica, conhecida como antígeno *30kDa/Aegyptin* presente no *A. Aegypti*, com função antiplaquetária, também foi descrita no sialotranscriptoma de *S. nigrimanum*, demonstrando a única família de proteínas salivares que descreve origem comum entre *Blackflies* e *Mosquitos* (JABLONKA, 2019; RIBEIRO et al., 2010; GRIMALDI, ENGEL, 2005).

2.8 INSETOS HEMATÓFAGOS E A ENDEMICIDADE DO PÊNFIGO FOLIÁCEO ENDÊMICO NO NORDESTE DO ESTADO DE SÃO PAULO

Os pênfegos têm sido extensivamente estudados ao nível molecular, em especial suas características genéticas e imunológicas, porém, a etiopatogênese ainda se encontra incompreendida. O papel dos fatores ambientais é crucial no estudo dos pênfegos, pois na forma endêmica do PF em alguns países, ainda é desconhecido, suscitando vasto campo para pesquisa.

Embora *S. nigrimanum* seja o Simulídeo antropofílico mais citado na literatura médica em relação à etiopatogênese do PF (AOKI et al., 2004; DIAZ et al., 1989; EATON et al., 1998, LOMBARDI et al., 1992), outras espécies de Simulídeos antropofílicos estão distribuídas nas regiões onde existem registros de pênfegos, como *Simulium pertinax* e *Simulium incrustatum* (EATON et al., 1998). Porém, a distribuição destas duas últimas espécies não tem sido descrita na região nordeste do Estado de São Paulo.

Lu. longipalpis é sem dúvida o flebotomíneo que tem sido mais estudado no desenvolvimento do PF. Os componentes anticoagulantes, antiplaquetários, anestésicos e

vasodiladores da saliva destes insetos, além da ação imunomoduladora, têm sido alvo de amplo estudo, demonstrando-se nos últimos anos estreita relação da proteína salivar LJM11 com a etiopatogênese do PF (QIAN et al., 2016). Por outro lado, a proteína Maxadilan, descrita inicialmente em *Lu. longipalpis* e posteriormente descrita em *N. neivai* (AIRES et al., 2017), também tem sido relacionada em pacientes com PF.

Apesar do *A. aegypti* não ter sido alvo de estudo em relação ao desencadeamento do PF, no estado de São Paulo existe uma relação epidemiológica importante entre a presença de *A. aegypti* e sua expansão geográfica ao longo da região nordeste do estado (SUCEN, 2015^b). No Estado de São Paulo registrou-se, em 2007, a mais elevada incidência de dengue desde o início da transmissão no Estado, sendo de 219,8/100 mil habitantes (FERREIRA et al., 2009), o que acompanha o aumento nos casos de PF registrados no Hospital das Clínicas-FMRP-USP nas últimas décadas (CELERE et al., 2017; GONÇALVES et al., 2011)

A importância da identificação de um provável mimetismo antigênico molecular entre peptídeos salivares de insetos hematófagos, amplamente distribuídos em regiões endêmicas para PF, e componentes epidérmicos humanos, e sua possível participação na etiopatogênese dessa doença leva ao objetivo principal deste estudo.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Verificar a participação das proteínas salivares de insetos hematófagos na etiopatogênese do PF em amostragem da região nordeste do estado de São Paulo (NESP), Brasil.

3.2 OBJETIVOS ESPECÍFICOS

- 3.2.1 Mapear a distribuição das principais espécies antropofílicas de simulídeos e flebotomíneos em relação aos casos de PF descritos na região NESP: relação epidemiológica (presença de inseto/doença) (manuscrito 1 - publicado).
- 3.2.2 Quantificar a resposta imune humoral IgG e IgE em amostras séricas de pacientes com PF e controles contra antígenos salivares dos insetos hematófagos distribuídos na região NESP: *A. aegypti*, *N. neivai* e *S. nigrimanum* (ensaio ELISA *in-house*) (manuscrito 2 – submetido).
- 3.2.3 Identificar quais frações proteicas salivares dos insetos *N. neivai* e *S. nigrimanum* são reconhecidas pelos anticorpos dos pacientes com PF e controles (IB) (manuscrito 3 – em submissão).
- 3.2.4 Quantificar a resposta imune humoral IgG em amostras séricas de pacientes com PF e controles contra as duas principais proteínas salivares de *Lu. longipalpis* relacionadas à etiopatogênese do PF na literatura médica: Maxadilan e LJM11 (ensaio ELISA *in-house*) (manuscrito 4 - submetido).
- 3.2.5 Descrever o sialotranscriptoma de *N. neivai* (manuscrito 5 – em submetido).

5. RESULTADOS

Os resultados dessa Tese de Doutorado serão apresentados em formato de manuscrito, sendo que o primeiro deles já foi publicado em jornal internacional no ano 2017. De maneira complementar, outros resultados obtidos, porém ainda não incorporados nos manuscritos supracitados submetidos para publicação, serão apresentados em formato regular de acordo com estrutura da tese e serão abordados de forma global na seção de discussão.

5.1 ARTIGOS PUBLICADOS.

5.1.1 Insights into the epidemiological link between biting flies and pemphigus foliaceus in southeastern Brazil. *Jornal*: ACTA TROPICA. *Fator de impacto*: 2,629. *Formato*: Original article

Insights into the epidemiological link between biting flies and pemphigus foliaceus in southeastern Brazil, *Acta Tropica*, 2017;176:455-462

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Insights into the epidemiological link between biting flies and pemphigus foliaceus in southeastern Brazil

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ABSTRACT

Background: Black fly and sandfly bites are related to the endemicity of pemphigus foliaceus (PF); however, an immune reaction against the salivary proteins from these flies still requires confirmation in the case of PF patients living in southeastern Brazil.

Purpose: To georeference the distribution of Simuliidae (Diptera: Simuliidae) and Phlebotominae (Diptera: Psychodidae) and of PF cases in the northeastern region of São Paulo State, and to assess the humoral immune response against salivary gland extracts (SGEs) from biting flies in PF patients, relatives, and neighbours.

Methods: PF patients' medical information recorded between 1965 and 2014 were obtained from the database of the University Hospital. Data on the distribution of fly species were collected from scientific reports and epidemiological databases. Spatial maps relating the distribution of biting flies with PF cases were plotted. Serum IgG antibodies against the SGEs from *Simulium nigrimanum*, *Nyssomyia neivai*, and *Aedes aegypti* (as control) were determined by ELISA.

Results: Two hundred and eighty-five PF cases were distributed in 60 municipalities with a prevalence of 57.5 per million inhabitants, revealing well-defined geographical clusters. *S. nigrimanum* and *N. neivai* specimens were registered in eight (13.3%) and 26 (43.3%) of these municipalities, respectively. PF patients, and their relatives presented higher levels of IgG against the SGEs of *S. nigrimanum* and *N. neivai* ($P < 0.001$ for both), but not against the SGE from *A. aegypti* ($P = 0.115$ and $P = 0.552$, respectively), as compared to controls. IgG against the SGEs from *S. nigrimanum* and *N. neivai* but not against the SGE from *A. aegypti* correlated with levels of anti-Desmoglein 1 in PF patients ($r = 0.3848$, $P = 0.039$; and $r = 0.416$, $P = 0.022$, respectively).

Conclusion: An epidemiological link between biting flies and PF in southeastern Brazil is proposed, implying a possible role of the salivary proteins from these flies in PF etiopathogenesis.

1. Introduction

Pemphigus foliaceus (PF) is an autoimmune bullous disease caused by autoantibodies against desmoglein (DSG) 1. It is subdivided into classic sporadic worldwide Cazenave's pemphigus and endemic pemphigus (known as *Fogo Selvagem* in Brazil). Although the pathogenesis of PF remains unclear, genetic and environmental factors have been implicated in the susceptibility to this disease (Abréu-Vélez et al., 2010;

Brochado et al., 2016).

Historically, PF cases have been recorded in rural areas of Brazil and other South American countries such as Colombia, Peru, Paraguay, and Venezuela (Abréu-Vélez et al., 2010; Diaz et al., 1989). That insect bites might be associated with PF has been pointed out since 1940 (Vieira, 1940), when an epidemiological link between *Simulium nigrimanum* (Macquart, 1838) and the endemicity of PF was hypothesized in São Paulo State, southeastern Brazil (Vieira, 1940). PF remains endemic in

Abbreviations: ATL, American Tegumentary Leishmaniasis; DSG, Desmoglein; NRSP, northeastern region of São Paulo State; SGE, salivary gland extract; PF, Pemphigus Foliaceus; VL, Visceral Leishmaniasis

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the northeastern region of São Paulo State (Abréu-Vélez et al., 2010; Diaz et al., 1989; Roselino and Almeida, 1995). *S. nigrimanum* bites have been considered a risk factor for the development of PF in an Amerindian reserve in Mato Grosso do Sul State, Brazil (Aoki et al., 2004; Hans-Filho et al., 1996; Eaton et al., 1998; Lombardi et al., 1992). Two other widespread voracious black fly species—*Simulium pertinax* (Kollar, 1882) and *Simulium incrustatum* (Lutz, 1910)—have also been linked to the endemicity of PF in this same reserve (Eaton et al., 1998).

The relation between *S. nigrimanum* and other hematophagous insects with PF has been not explored enough in southeastern Brazil. Higher levels of serum IgG against maxadilan —a highly immunogenic salivary protein described in *Lutzomyia longipalpis* (Lutz and Neiva, 1912), a vector of visceral leishmaniasis (VL) (Lerner et al., 1991)—have been determined in PF patients as compared to controls living in a southeastern Brazilian region where PF is endemic (Roselino et al., 2001). There is also evidence that LJM11, another highly immunogenic protein from *Lu. longipalpis*, cross-reacts with antibodies against DSG1 (Qian et al., 2012). Zarea et al. (Zarea et al., 2012) have also reported the recognition of the salivary protein from *Phlebotomus papatasi* (Scopoli, 1786) by patients' sera in a Tunisian region where PF is endemic.

Nyssomyia intermedia (Lutz & Neiva, 1912) and *Nyssomyia neivai* (Pinto, 1926) (= syn. *Lutzomyia intermedia*) are the main vectors of *L. (V.) braziliensis* in São Paulo State. Moreover, *N. neivai* has been identified in research collections done systematically in several municipalities of the northeastern region of São Paulo State (Andrade Filho et al., 2007). *Lu. longipalpis* is mainly distributed in the western region of São Paulo State, but it has not been related to VL in the northeastern region of this state (Casanova et al., 2015).

Another important group of biting insect—Culicidae—has never been associated with PF; however, 23 (76%) out of 30 PF patients have reported mosquito bites in a Brazilian Amerindian reserve (Lombardi et al., 1992). Mosquitoes are vectors of various pathogens, including the Dengue and the Zika viruses, and their populations have been also reported in areas of São Paulo State where PF is endemic (Glasser and de Castro Gomes, 2000).

Considering these previous studies, the relation between the distribution of black flies and sandflies with the endemicity of PF in southeastern Brazil requires confirmation. Furthermore, assessment of PF patients exposed to bites of these flies is mandatory to establish an epidemiological link. The present study aims (i) to compare the spatial distribution of black flies (Diptera: Simuliidae) and Phlebotominae sandflies (Diptera: Psychodidae) with PF cases georeferenced in the northeastern region of São Paulo State; and (ii) to demonstrate that serum samples from PF patients recognize salivary gland extracts (SGEs) from *S. nigrimanum* (Simuliidae), *N. neivai* (Phlebotominae), and *Aedes aegypti* (Linnaeus, 1762) (Culicidae) populations from the studied region.

2. Materials and methods

2.1. Mapping and spatial distribution

2.1.1. Type of study

This is an observational study with a retrospective cohort analysis

2.1.2. PF cases

Medical records of PF cases were collected from the database of the University Hospital of the Ribeirão Preto Medical School, University of São Paulo, Brazil. Records concerned patients who resided in the northeastern region of São Paulo State (hereafter referred as NRSP) at the onset of PF symptoms between 1965 and 2014. The clinical diagnosis of PF was confirmed by acantholytic bullae on the histopathological examination, positive IgG fluorescence around keratinocytes in skin samples, and presence of serum autoantibodies against DSG1 as revealed by ELISA (MBL, Nagoya, Japan).

2.1.3. Study area

NRSP, southeastern Brazil, has a tropical climate and is located between 19°52' and 22°51'S and 46°16' and 49°20'W. The region comprises 125 municipalities that cover an area of 51,661 km². This region represents about 21% of the total area of the state and was inhabited by 5,156,660 people in 2016 (IBGE, 2017).

2.1.4. Data on black flies

Reports of *S. nigrimanum* collections in NRSP have been registered since the beginning of the last century (Vieira, 1940). Most data were obtained from Dr Mateus Pepinelli doctoral thesis, in which he reports collecting specimens from 151 streams and rivers across São Paulo State, of which 26 were situated in 25 NRSP municipalities. Collections were accomplished between 2002 and 2005 (Pepinelli, 2008).

2.1.5. Data on sandflies

The distribution of sandflies was obtained from epidemiological data deposited at the database of the Health Secretariat of São Paulo State since 1940, obtained mainly via the VL and American tegumentary leishmaniasis (ATL) surveillance programs. Entomological collections were performed in locations with known or suspected human cases of ATL and in municipalities with suspected or confirmed cases of human and canine VL; a minimum of four dwellings were sampled (Casanova et al., 2015; Casanova et al., 2014; Secretary of Health of São Paulo State, 2011; Secretary of Health of São Paulo State, 2008).

2.1.6. Spatial maps

The scale for map construction was 1:3,266,000 kilometres (0–130.7 latitude/longitude projection). Cases were plotted against the number of patients in each municipality of NRSP. Black fly and sandfly records were also plotted per municipality.

2.1.7. Nomenclature

Nomenclature of insect species follows Galati and Adler & Crosskey's guidelines (Adler and Crosskey, 2015; Galati, 2003). Abbreviation of generic names for sandflies follows Marcondes' guidelines (Marcondes, 2007).

2.2. Assessment of exposure to bites

2.2.1. Type of study

This is a transversal study

2.2.2. Samples of insects

Given their pest status and wide distribution, *S. nigrimanum* and *N. neivai* were included as the most representative simuliid and phlebotomine, respectively, of the target region. *A. aegypti*—another biting fly that is widely distributed in NRSP (Glasser and de Castro Gomes, 2000) but which has not been related to PF—was included as a control.

2.2.2.1. *Simulium nigrimanum*. Adult females were collected in *Ribeirão Corrente* river, in the municipality of *Jardínópolis*, NRSP (20°30'30.04"S, 47°33'04.34"W) during oviposition, while flying, or while biting humans. Aquatic life stages were collected for additional identification, following Hamada & Pepinelli methods (Hamada and Pepinelli, 2004).

2.2.2.2. *Nyssomyia neivai*. Adult female samples were obtained from a laboratorial colony supervised by Dr Mara Pinto, State University of São Paulo, Araraquara, Brazil.

2.2.2.3. *Aedes aegypti*. Adult female samples were obtained from a laboratorial colony supervised by Dr Anderson Sá-Nunes, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil.

Fig. 1. Spatial distribution of pemphigus foliaceus cases in northeastern São Paulo State, southeastern Brazil, between 1965 and 2014. Well-defined pemphigus foliaceus clusters: municipalities of (1) São Joaquim da Barra; (2) Franca; (3) Batatais; (4) Jardinópolis; (5) Ribeirão Preto; and (6) Santa Rita do Passa Quatro. *Figure modified from Celere et al., 2017.

2.2.3. Salivary gland extracts (SGEs)

Salivary glands were dissected and stored in phosphate-buffered saline (PBS, pH 7.4). Salivary protein extracts were properly characterized in SDS gel (Supplementary Fig. 1S). Immediately before use, the SGEs were disrupted by ultrasonication and centrifuged at 14,000g; the resulting supernatant –SGE– was used in this study (Bizzarro et al., 2013; Souza et al., 2010).

2.2.4. In-house ELISA

To assess exposure to bites, an in-house ELISA assay was standardized with the SGE from each species of insect. Briefly, 300 ng of SGE diluted in PBS was incubated in 96-well microplates (Corning, Sigma-Aldrich, St. Louis, USA) overnight and blocked with PBS/Tween/0.005%/BSA 1% for an hour. After three washings, the patients' sera (1:100, in duplicate) were monitored with secondary antibody 1:10,000 (anti-human IgG, Abcam, Cambridge, USA). Then, the wells were washed four times, and 100 μ L of chromogenic substrate (3, 3', 5, 5'-Tetramethylbenzidine-TMB) was added. After 15-min incubation at room temperature (in the dark), 50 μ L of stop solution (0.2 M H_2SO_4) was added. The Optical Density (OD) values were determined with an ELISA plate reader (Biochrom Asys Expert Plus Microplate Reader, Cambourne, Cambridge, UK) fitted with a 450-nm absorbance filter. In-house ELISA was performed by a blinded technician, in duplicate, on the same day; the same reagents were used to avoid plate variability. The experiments were performed twice. In-house ELISA was not considered as a diagnostic tool for PF. Hence, instead of positive/negative results being presented, in-house ELISA was used only as a comparative tool to assess the humoral response to SGEs. In-house ELISA was validated by using serum samples from female rabbits before and after immunization with each SGEs (Supplementary Material and Methods).

2.2.5. Serum samples

Thirty serum samples from PF patients were compiled randomly from the Pemphigus Bank of the Laboratory of Dermatology at the University Hospital of the Ribeirão Preto Medical School, University of

São Paulo, Brazil. All the patients presented active clinical disease with the presence of Nikolsky's sign at the moment serum was collected. The control groups consisted of 30 healthy relatives of PF patients and 30 healthy neighbours of PF patients' living in the same studied region and without any clinical manifestation of PF. They comprised first-degree family members and accompanying neighbour on the occasion of the outpatient appointment.

2.2.6. Statistical analysis

Parametric model assumptions were assessed by means of a normal plot. Demographic and clinical data from patients and controls were compared by non-parametric tests as appropriate. Kruskal–Wallis followed by Dunn's test was employed to compare ELISA OD values amongst the groups. The Spearman's test was used to correlate the levels of antibodies against SGEs and anti-DSG1 as well as the levels of antibodies and time of disease in the PF group. Significance was set at $\alpha = 5\%$. GraphPad Prism 7 was used for the statistical analysis (GraphPad software Inc., La Jolla, CA, USA).

2.2.7. Ethics

This study was approved by the local Human Research Ethics Committee (#866.027/2014), in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association; it was also approved by the Animal Research Ethics Committee (#78/2015).

3. Results

3.1. Georeferencing analysis

The georeferenced maps illustrate the distribution of 285 PF cases in 60 (48%) out of 125 municipalities whose prevalence comprised 57.5/1,000,000 inhabitants in NRSP. Six municipalities concentrated ten or more PF cases (Fig. 1).

Concerning the distribution of anthropophilic Simuliidae species, the compiled data refer to specimens collected in 25 (20%) out of 125

Distribution of the main Black flies in northeastern Sao Paulo State, southeastern Brazil

Distribution of the main Sandflies in northeastern Sao Paulo State, southeastern Brazil

Fig. 2. Spatial distribution of black fly and sandfly species in the northeastern region of São Paulo State, southeastern Brazil – (A) *Simulium nigritum*, (B) *Simulium pertinax*, (C) *Simulium incrustatum*, (D) *Nyssomyia neivai*, (E) *Nyssomyia whitmani*, (F) *Lutzomyia longipalpis*, (G) *Pintomyia pessoai*, (H) *Pintomyia fischeri*, and (I) *Migonemyia migonei*.

municipalities. Fig. 2A–C shows that Simuliidae was distributed around the main PF focus. *S. nigritum* was recorded in 11 (44%) out of 25 municipalities (Fig. 2A), including eight (72.7%) municipalities where PF cases were registered. One of these municipalities corresponded to a well-defined PF geographical cluster (municipality of Franca, number 2,

Fig. 1). *S. pertinax* was distributed in nine (36%) out of 25 municipalities; PF cases occurred in five (55.5%) of them (Fig. 2B). *S. incrustatum* was detected in 12 (48%) out of 25 municipalities; PF cases were registered in eight (66.6%) of them (Fig. 2C). It is noteworthy that *S. nigritum*, *S. pertinax* and *S. incrustatum* have been found inhabiting

streams of the same municipality area in three cases, and that PF was reported in two of these municipalities (Cajuru and São Carlos municipalities).

Regarding the distribution of sandflies (Fig. 2D–I), the compiled data refer to specimens collected in 76 (60.8%) out of 125 municipalities. *N. neivai* was recorded in 55 (44%) municipalities (Fig. 2D). PF cases occurred in 26 (47.2%) out of these 55 municipalities. The presence of *N. neivai* in four out of six well-defined PF clusters is worthy of note. Comparison of the spatial distribution of *N. neivai* with the spatial distribution of other studied sandflies revealed that this species was the most widely distributed in NRSP, followed by *Nyssomyia whitmani* (Antunes & Coutinho, 1939) (Fig. 2E), reported in 33 (26.4%) out of 125 municipalities, 13 (39.4%) of which had reported PF cases.

Lu. longipalpis was investigated in 44 (35.2%) out of 125 municipalities. There were positive records in nine (20.5%) out of 44 municipalities, which represented less than 8% of the total number of municipalities in the region. The distribution of *Lu. longipalpis* did not match the occurrence of any well-defined PF cluster (Fig. 2F).

We also plotted the distribution of other sandfly species—*Pintomyia pessoai* (Coutinho & Barreto, 1940), *Pintomyia fischeri* (Pinto, 1926), and *Migonemyia migonei* (França, 1920) (Fig. 2G–I).

3.2. Immunological study

We tested serum samples obtained from 30 PF patients (median age = 47 years, 18 females), 30 PF patients' healthy first-degree relatives (median age = 46 years, 23 females), and 30 PF patients' healthy neighbours (median age = 55 years, 12 females) as controls living in the same area where PF is endemic. Table 1 summarizes the clinical and the laboratorial data (for more details please check Supplementary Table 1S and Supplementary Table 2S). No significant differences were found among groups related to gender ($P = 0.117$). There were no significant differences between PF patients as compared to relatives and neighbours in terms of gender, but healthy neighbours were older than healthy relatives ($P = 0.029$). The median time of PF evolution was nine years (p25th 2 years, p75th 18 years), and most of the patients (83.3%) were being treated for PF at the moment serum was collected. The PF patients that had not started immunosuppressive treatment or who had been without treatment for at least 60 days at the moment serum was collected tended to have higher values of anti-DSG1 as compared to PF patients under treatment ($P = 0.065$) (data not shown).

Fig. 3 shows the detection of IgG against the SGE from each insect. There were no statistical differences amongst the PF patients, their

Table 1

Demographic and clinical data from pemphigus foliaceus (PF) patients, PF patients' healthy relatives, and PF patients' healthy neighbours.

		PF patients	Healthy relatives	Healthy neighbours	P-value
Gender	Female	18 (60%)	23 (76.7%)	12 (40%)	0.117
	Male	12 (40%)	7 (23.3%)	18 (60%)	
Age (years) median (p25/p75)		47 (31/57)	46 ^a (33/56)	55 ^b (45/59)	0.029 ^{a,b}
Duration of disease (years) median (p25/p75)		9 (2/18)	N.A.	N.A.	N.A.
Treatment of serum collection data	Yes	25 (83.3%)	N.A.	N.A.	N.A.
	No	5 (16.7%)			
Activity of disease on the day of serum collection	Yes	30 (100%)	N.A.	N.A.	N.A.
	No	0 (0%)			

PF: Pemphigus Foliaceus; p25: percentile 25th; p75: percentile 75th; N.A.: Not Applicable.

relatives, and their neighbours for the SGE from *A. aegypti* ($P = 0.320$). On the other hand, PF patients presented higher levels of IgG antibodies against the SGEs from *S. nigrimanum* and *N. neivai* as compared to PF patients' neighbours ($P < 0.001$ for both SGEs), but not as compared to PF patients' relatives ($P = 0.115$ and $P = 0.552$, respectively). Higher levels of antibodies against the SGEs from *S. nigrimanum* and *N. neivai* were also found in PF patients' relatives as compared to PF patients' neighbours ($P < 0.001$). Levels of IgG against the SGEs from *S. nigrimanum* and *N. neivai* but not against the SGE from *A. aegypti* were significantly higher in PF patients who had not started immunosuppressive treatment or without treatment for at least 60 days as compared to patients under treatment ($P = 0.007$, $P = 0.019$, and $P = 0.327$, respectively) (data not shown).

Levels of anti-DSG1 antibodies correlated positively with antibodies against the SGEs from *S. nigrimanum* and *N. neivai* ($r = 0.3277$, $P < 0.001$; and $r = 0.3958$, $P < 0.001$, respectively), but not with antibodies against the SGE from *A. aegypti* ($r = -0.1515$, $P = 0.154$) (Fig. 4A). Antibodies against the SGEs from *S. nigrimanum* and *N. neivai* also correlated positively with anti-DSG1 in PF patients ($r = 0.3848$, $P = 0.039$; and $r = 0.416$, $P = 0.022$ respectively) (Fig. 4B). There was no correlation between the levels of antibodies against DSG1 and the SGE from each insect with the duration of the disease in PF patients ($P > 0.05$ in all cases) (data not shown).

4. Discussion

The expansion of urban boundaries has been accompanied by increased occurrence of diseases (e.g., leishmaniasis) that had been previously restricted to rural areas (Tolezano, 1994), a phenomenon that seems to apply for our study. Population growth associated with expansion of agricultural production has resulted in urban invasion of forested regions. This scenario represents a new stage of human cohabitation with the local fauna, which had been previously confined to places outside the cities (Tolezano, 1994). Historically, PF was distributed in rural areas and in areas close to rivers, which reinforces the hypothesis that Simuliidae and/or Phlebotominae might be associated with PF (Abréu-Vélez et al., 2010; Aoki et al., 2004; Chiossi and Roselino, 2001; Diaz et al., 1989; Eaton et al., 1998; Roselino and Almeida, 1995).

The present study shows coincidental georeferenced maps of anthropophilic Simuliidae and Phlebotominae species and PF cases and proposes an epidemiological link between them (Abréu-Vélez et al., 2010; Chiossi and Roselino, 2001; Diaz et al., 1989; Roselino and Almeida, 1995). Spatial geographical distribution maps of Simuliidae, Phlebotominae, and PF cases overlap 34 municipalities. The epidemiological link between *S. nigrimanum* and PF, described over 70 years ago in the municipality of Franca (Vieira, 1940), a well-defined focus of PF characterized as number 2 in Fig. 1, represents the first attempt to establish a relationship between Simuliidae and PF. The present study confirms that PF is still prevalent in Franca, as well as anthropophilic Simuliidae species. Unfortunately, we have not been able to collect Simuliidae species in the other well-defined focus of PF (municipalities numbered 1 and 3–6 in Fig. 1). However, the surrounding cities surveyed present populations of anthropophilic black fly species reported herein, so it is expected that *S. nigrimanum* populations inhabit streams and rivers in some of these municipalities.

Although *S. nigrimanum* has been more frequently associated with PF, other anthropophilic species like *S. pertinax* and *S. incrustatum* are widely distributed in regions with reported PF cases. Previous studies have also mentioned that *S. pertinax* and *S. incrustatum* are present in the Limão Verde indigenous reserve, a region where Brazilian PF is significantly endemic (Eaton et al., 1998).

Simuliidae species have been related to PF on the basis of two main hypotheses: (i) black fly species could carry a virus, or (ii) salivary proteins may mimic DSG (Abréu-Vélez et al., 2010; Aoki et al., 2004; Chiossi and Roselino, 2001; Roselino and Almeida, 1995). Ribeiro et al.

Fig. 3. In-house ELISA employing salivary gland extracts (SGEs) from *Aedes aegypti*, *Simulium nigritanum*, and *Nyssomyia neivai* to evaluate the levels of IgG antibody in pemphigus foliaceus (PF) patients, PF patients' relatives, and PF patients' neighbours (* $P < 0.001$).

Fig. 4. A – Spearman's rank correlation between IgG anti-desmoglein 1 antibodies (U/mL) and IgG antibodies (OD value) against the salivary gland extract (SGEs) of *Aedes aegypti*, *Simulium nigritanum*, and *Nyssomyia neivai*; (left) in the three groups (pemphigus foliaceus (PF) patients; PF patients' relatives and PF patients' neighbours); (right) in PF patients group. B – Spearman's rank correlation coefficient among IgG antibodies (OD value) against the SGEs from *Aedes aegypti*, *Simulium nigritanum*, and *Nyssomyia neivai* in the three groups (pemphigus foliaceus (PF) patients; PF patients' relatives and PF patients' neighbours). Fig. 4B shows the correlation amongst the levels of antibodies against the SGEs from *S. nigritanum*, *N. neivai*, and *A. aegypti*. The levels of antibodies against the SGEs from *S. nigritanum* and *N. neivai* were positively correlated ($r = 0.7205$, $P < 0.001$). However, the correlation of the levels of antibodies against the SGEs from *S. nigritanum* and *N. neivai* with the levels of antibodies against the SGE from *A. aegypti* was not significant ($r = -0.1841$, $P = 0.082$; and $r = 0.003$, $P = 0.970$, respectively) (Fig. 4B).

have recently compared the peptide sequence of the *S. nigritanum* sialotranscriptome with the peptide sequence of DSG to identify some candidate proteins that could induce anti-DSG cross-reactivity (Ribeiro et al., 2010). Although these authors found some similarity, no cross-reactivity with salivary peptide has been proven so far. The

sialotranscriptome analysis of other Simuliidae species is desirable because salivary proteins shared by the three species identified in NRSP could be recognized. Moreover, this analysis could uncover a putative black fly salivary antigenic mimicry with DSG. The positive correlation between anti-*S. nigritanum* SGE with anti-DSG1 in PF patients suggests

a possible cross-reactivity between antigens; notwithstanding, further studies are necessary to prove this possible mimicry and to find the precise salivary peptide that is involved in the process.

The involvement of the sandfly *Lu. longipalpis* in PF has also been studied. This sandfly is an important vector of *Leishmania infantum* *chagasi* (Chagas & Cunha, 1937), a parasite that causes VL in Brazil. Many reports have described the transmission of VL in some regions of São Paulo State (Casanova et al., 2015; Secretary of Health of Sao Paulo State, 2011). In the NRSP municipalities, *Lu. longipalpis* occurred without association with PF clusters (Fig. 2F). Roselino et al. (2001) (Roselino et al., 2001) have shown that sera collected from PF patients exhibit higher levels of IgG antibodies against maxadilan—a salivary protein from *Lu. Longipalpis*—as compared to healthy controls living in the same endemic area. There is also evidence that the LJM11 salivary peptide from *Lu. longipalpis* cross-reacts with antibodies against DSG1 (Qian et al., 2012). Furthermore, IgE antibodies against LJM11 have been proposed to trigger PF in some Amerindian populations living in endemic regions (Qian et al., 2015).

Nevertheless, *Lu. longipalpis* is poorly distributed in the NRSP. Establishing an epidemiological link between LJM11 and PF in the NRSP would be ground-breaking. For this purpose, a LJM11-simile salivary peptide could be expressed in other biting fly species that inhabit NRSP, such as *N. neivai*, a vector of ATL in the studied region (Casanova et al., 2015). Recently, Ribeiro and our group have described the *N. neivai* sialotranscriptome (data not published – available in GenBank NCBI-NIH, USA (term: *Nyssomyia neivai*)), and surprisingly we did not find a LJM11-simile peptide, but 16 putative sequences of maxadilan-simile were described for *N. neivai*.

On the basis of the production of antibodies against the SGEs from *S. nigrimanum* and *N. neivai* but not against the SGE from *A. aegypti*, we have demonstrated that PF patients, PF patients' healthy relatives, and PF patients' neighbours living in the same area were exposed to bites by these flies, which has helped us to establish the epidemiological link. Remarkably, higher levels of IgG antibodies against the SGEs from *S. nigrimanum* and *N. neivai* but not against the SGE from *A. aegypti* have been detected in PF patients as compared to PF patients' neighbours. These results have led us to hypothesize that specific salivary gland proteins from black flies and sandflies, but not from mosquitoes possibly participate in PF. To our knowledge, no other study has dealt with exposure to *S. nigrimanum* bite or with the humoral response against its SGE.

The increased humoral response against the SGEs from *S. nigrimanum* and *N. neivai* in PF patients' relatives deserves attention. Genetic factors could be involved in this humoral response. In fact, three out of 30 PF patients' relatives but no PF patients' neighbours presented positive anti-DSG1 or antibodies against the SGEs from *S. nigrimanum* and *N. neivai* (data not shown); however, none of them have developed clinical PF yet. Further determination of the HLA susceptibility/protection profile of PF patients' relatives is necessary to compare it to the HLA profile of PF patients (Brochado et al., 2016).

Finally, it is important to discuss the positive correlation between the antibodies against the SGEs from *S. nigrimanum* and *N. neivai* in the three groups (PF patients; PF patients' relatives and PF patients' neighbours). This correlation shows that exposure to the bites of both species is possible because they are similarly distributed in NRSP. Nevertheless, the hypothesis of cross-reactivity between their salivary gland peptides should be carefully analysed. Although the two incriminated species are not closely related (Wiegmann et al., 2011), similar salivary proteins with similar antigen effects might be playing a role on PF pathogenesis.

4.1. Limitations of the study

Because the data reported here have been compiled from a third-level hospital, the number of PF cases could be underestimated due to lack of notification of pemphigus in Brazil and to possible treatment of

patients at secondary healthcare level. Hence, we believe that the prevalence of PF in southeastern Brazil could be even higher.

Fig. 2 is a representative distribution of nine species of flies in NRSP; however, their distribution is obviously dynamic and might change over time. Ecological, epidemiological, and demographic transformations in NRSP could determine variations on the distribution of the different studied species in this region. Regarding the aim of this study, maps have been plotted for pedagogical and research purposes regarding PF cases for further selection of the most representative black fly and sandfly species in NRSP. Further ecological studies may provide fly population variations over the years.

No immunosuppressed PF patients presented higher levels of antibodies against the SGEs from *S. nigrimanum* and *N. neivai* or a tendency to have higher levels of anti-DSG1 as compared to patients under treatment. Therefore, immunosuppression treatment of PF patients could be underestimating the levels of anti-SGE IgG levels because the levels of anti-DSG1 decreased upon immunosuppression. Repeating the assays with PF patients that have never received any treatment is desirable.

5. Conclusion

PF cases and the spatial distribution of *S. nigrimanum* and *N. neivai* biting flies have shown defined geographical clusters in NRSP. Increased levels of IgG antibodies against the SGEs from *S. nigrimanum* and *N. neivai* in PF patients confirm exposure to fly bites, suggesting an epidemiological link between flies and PF in southeastern Brazil.

Summary

An epidemiological link between biting flies and Pemphigus Foliaceus (PF) in Brazil is proposed, implying a possible role of biting fly salivary gland proteins in PF etiopathogenesis.

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Conflict of interest

The authors have no conflicts of interest to declare.

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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.actatropica.2017.09.015>.

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Supplementary Materials and Methods

To assess in-house ELISA performance, we immunized three female rabbits with each salivary glands extract (SGE) respectively.

- *Rabbit immunization against salivary gland extract (SGE).* Briefly, SGE (40 μ g/mL) were diluted in PBS to a final volume of 2mL, distributed into 500 μ L aliquots. On the first day, 500 μ L of Complete Freund's adjuvant (Sigma-Aldrich®, Saint Louis, MO, USA) was mixed with an aliquot of SGE/PBS, and the rabbit was injected in each hip. Nine days later, another dose was injected in the foreleg and shoulder of the rabbit. On day 20th, a third dose was injected in four subcutaneous points. Immunization was confirmed by counterimmunoelectrophoresis on 1% agarose gel to confront rabbit serum before and after immunization (positive pole) against each SGE respectively (negative pole). Runs were accomplished in sodium barbital buffer at an electric current of 30 mA, for 60 min, and then agarose gel was stained with Coomassie Blue (Sigma-Aldrich®, Saint Louis, MO, USA).
- *ELISA dose-curve analysis.* In-house ELISA was standardizing comparing pre- and post-immunize rabbit sera. Briefly, 11 serial dilutions of each SGE (10,000ng to 9,37ng) were pre-incubated overnight in 96-well micro-plates (Costar®), and then blocked for 1h with PBS1X/Tween 0.005%/BSA 1%. Rabbit' sera pre- and post-immunization were diluted 1:50 and 1:100 in PBS1X/Tween 0.005%/BSA 1%, followed by two serial dilution of secondary antibody (anti-rabbit IgG, Abcam®) (1:5,000 and 1:10,000 for both primary antibody dilutions). Rabbit' sera were pipetted in duplicate. Lecture was done at 450nm. Best performance was obtained with 300ng of SGE for three insects, primary antibody dilution of 1:100 and secondary antibody 1:10,000. Positive (post immunization rabbit) and negative (pre immunization rabbit) values were as follow: 2.049/0.136 (OD), 2.321/0.182 (OD), and 2.154/0.172 (OD) for *Ae. aegypti*, *S. nigrimanum* and *Ny. neivai* micro-plaques, respectively.

SUPPLEMENTARY TABLE 1S

Patient	Sex	Age at serum collection (years)	Time of disease (Years)	PF activity at serum collection	Treatment at serum collection	ELISA anti-DSG1 (U/mL)	ELISA anti-SGE <i>S. nigritanum</i> (OD)	ELISA anti-SGE <i>N. neivai</i> (OD)	ELISA anti-SGE <i>A. aegypti</i> (OD)
PF1	F	25	9	Yes	Yes	106,340	1,192	0,605	0,161
PF2	F	59	<1	Yes	Yes	92,850	1,251	0,331	0,160
PF3	M	28	<1	Yes	Virgin	193,039	1,202	1,736	0,367
PF4	F	58	49	Yes	Yes	56,679	0,552	0,314	0,212
PF5	M	32	12	Yes	Virgin	146,284	1,404	1,398	0,191
PF6	M	39	23	Yes	No*	134,425	0,605	0,456	0,343
PF7	M	31	22	Yes	Yes	147,319	0,597	0,411	0,215
PF8	F	17	<1	Yes	Yes	103,857	0,536	0,538	0,222
PF9	M	56	11	Yes	Yes	182,408	0,515	0,393	0,287
PF10	F	52	22	Yes	Yes	20,151	1,219	0,868	0,108
PF11	M	34	20	Yes	Yes	154,264	0,608	0,539	0,393
PF12	F	73	5	Yes	Yes	43,472	0,494	0,358	0,415
PF13	F	37	2	Yes	Yes	29,952	0,369	0,383	0,420
PF14	F	55	2	Yes	Yes	108,307	0,628	1,330	0,342
PF15	M	71	10	Yes	Yes	171,166	0,679	0,439	0,080
PF16	F	48	17	Yes	Yes	100,958	1,244	1,888	0,262
PF17	M	17	8	Yes	Yes	85,543	0,574	0,375	0,864
PF18	M	18	<1	Yes	Yes	26,570	0,594	0,318	0,415
PF19	M	66	3	Yes	Yes	106,763	0,729	0,477	0,265
PF20	F	73	3	Yes	Yes	180,637	0,594	0,570	0,187
PF21	M	17	<1	Yes	Virgin	166,879	1,274	0,758	0,903
PF22	F	57	4	Yes	Yes	234,522	0,649	0,371	0,251
PF23	M	62	23	Yes	Yes	256,561	0,690	0,547	0,440
PF24	F	33	1	Yes	Virgin	269,809	1,589	1,561	0,286
PF25	F	36	11	Yes	Yes	188,408	1,034	0,985	0,397
PF26	F	49	<1	Yes	Yes	168,384	0,665	0,729	0,291
PF27	F	54	9	Yes	Yes	97,643	0,495	0,526	0,195
PF28	F	38	9	Yes	Yes	40,236	0,547	0,656	0,317
PF29	F	46	18	Yes	Yes	163,289	0,734	0,534	0,120
PF30	F	49	35	Yes	Yes	16,959	0,487	0,310	0,273

* Without immunosuppressor treatment for more than 60 days. PF: Pemphigus Foliaceus; F: Female; M: Male; DSG1: Desmoglein 1; SGE: Salivary Gland Extract; OD: Optical Density

SUPPLEMENTARY TABLE 2S

Patient	Sex	Age at collection	ELISA anti-DSG1 (U/mL)	ELISA anti-SGE <i>S. nigrimanum</i> (OD)	ELISA anti-SGE <i>N. neivai</i> (OD)	ELISA anti-SGE <i>A. aegypti</i> (OD)
Relative 1	M	14	1,012	1,356	1,090	0,448
Relative 2	F	48	0,935	0,804	0,694	0,304
Relative 3	F	47	0,389	0,502	0,414	0,759
Relative 4	F	§	2,784	0,562	0,497	0,548
Relative 5	M	42	89,658	0,652	0,541	0,244
Relative 6	F	56	37,868	0,867	0,663	1,207
Relative 7	F	46	1,114	0,451	0,280	0,316
Relative 8	F	64	0,080	0,573	0,667	0,200
Relative 9	F	§	1,591	0,481	1,545	0,363
Relative 10	F	39	3,421	0,597	0,743	0,520
Relative 11	F	38	2,068	0,704	0,344	0,207
Relative 12	F	42	0,000	0,887	0,202	0,204
Relative 13	F	60	0,796	0,738	1,152	0,452
Relative 14	M	59	1,671	0,451	0,304	0,273
Relative 15	F	57	1,962	0,821	0,312	0,204
Relative 16	F	32	23,707	0,658	0,595	0,244
Relative 17	F	18	0,160	0,442	0,423	0,370
Relative 18	M	31	1,597	0,488	0,264	0,417
Relative 19	M	46	0,121	0,709	0,507	0,499
Relative 20	F	57	0,604	0,899	0,356	0,176
Relative 21	F	38	11,353	0,489	0,255	0,179
Relative 22	M	38	3,694	0,508	0,377	0,216
Relative 23	F	29	0,764	0,408	0,218	0,205
Relative 24	M	18	0,127	0,564	0,260	0,111
Relative 25	F	49	0,764	0,826	1,214	0,319
Relative 26	F	57	1,054	0,535	0,148	0,273
Relative 27	F	15	0,000	0,215	0,190	0,953
Relative 28	F	81	5,219	0,272	0,125	0,342
Relative 29	F	41	1,661	0,927	1,299	0,200
Relative 30	F	35	2,510	0,685	1,160	0,290
Neighbour 1	M	58	5,330	0,384	0,293	0,532
Neighbour 2	M	18	3,500	0,350	0,189	1,022
Neighbour 3	M	§	2,653	0,341	0,488	0,238
Neighbour 4	F	45	1,129	0,459	0,683	0,421

Neighbour 5	M	65	11,947	0,095	0,196	0,294
Neighbour 6	F	54	1,129	0,150	0,017	0,267
Neighbour 7	F	53	0,753	0,150	0,013	0,781
Neighbour 8	F	60	0,719	0,534	0,387	0,356
Neighbour 9	F	66	0,240	0,377	0,145	0,432
Neighbour 10	M	44	0,160	0,096	0,091	0,230
Neighbour 11	F	44	0,399	0,188	0,192	0,469
Neighbour 12	M	59	1,283	1,155	0,777	0,535
Neighbour 13	M	59	0,302	0,848	0,904	0,487
Neighbour 14	M	56	1,811	0,559	0,026	0,228
Neighbour 15	F	67	0,000	0,582	0,127	0,405
Neighbour 16	F	57	2,077	0,474	0,213	0,245
Neighbour 17	F	51	1,656	0,438	0,449	0,258
Neighbour 18	M	44	0,127	0,094	0,139	0,196
Neighbour 19	F	26	5,350	0,321	0,078	0,354
Neighbour 20	F	59	0,000	0,335	0,001	1,718
Neighbour 21	M	49	0,382	0,753	0,354	0,251
Neighbour 22	M	37	3,513	0,744	0,561	0,110
Neighbour 23	M	40	1,756	0,259	0,309	0,208
Neighbour 24	M	62	2,357	0,438	0,194	0,376
Neighbour 25	M	71	15,993	0,412	0,395	0,303
Neighbour 26	0	51	0,505	0,531	0,180	0,056
Neighbour 27	M	60	2,990	0,625	0,522	0,458
Neighbour 28	M	65	1,495	0,267	0,107	0,352
Neighbour 29	M	52	1,163	1,084	0,915	0,362
Neighbour 30	M	55	1,827	0,195	0,108	0,760

§ Missing value; F: Female; M: Male; DSG1: Desmoglein 1; SGE: Salivary Gland Extract; OD: Optical Density

Supplementary Figure 1S. Electrophoresis of salivary glands extracts (SGE) in polyacrylamide 4-15% gel (BioRad). MW: Molecular Weight – Precision Plus protein TM dual-color (BioRad); 1: SGE of *Simulium nigrimanum*; 2: SGE of *Nyssomyia neivai*; 3: SGE of *Aedes aegypti*.

5.2 ARTIGOS SUBMETIDOS

5.2.1 Relationship between IgE and salivary proteins from biting flies in pemphigus foliaceus.

Jornal: Arquivos of Dermatological Research. *Fator de impacto*: 2,309. *Formato*: Letter to the Editor

Relationship between IgE and salivary proteins from biting flies in pemphigus foliaceus

S. Vernal; M. Pepinelli; W. Oliveira; T. Goulart; M. Pinto; A. Sá-Nunes; A.M. Roselino

Archives of Dermatological Research

Relationship between IgE and salivary proteins from biting flies in pemphigus foliaceus --Manuscript Draft--

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Title. Relationship between IgE and salivary proteins from biting flies in pemphigus foliaceus

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To the editor,

Well-defined geographical clusters of pemphigus foliaceus (PF) cases in Southeastern Brazil disclosed a prevalence of 57.5 patients per million people [1]. Historically, PF occurrence in Brazil has been recorded in rural areas; presence of biting flies might be associated with this condition [2,3]. Recently, we have demonstrated a geographical link between blood-sucking insects and PF cases diagnosed in this Brazilian endemic region [1]. Production of IgG antibodies against salivary gland extracts (SGEs) from the black fly *Simulium nigrimanum* and the sand fly *Nyssomyia neivai*, but not against SGE from the mosquito *Aedes aegypti*, by PF patients and their relatives (PFR) confirmed an epidemiological link between PF and biting flies. Moreover, IgG antibodies against SGEs from *S. nigrimanum* and *N. neivai*, but not SGE from *A. aegypti*, correlated with IgG anti-desmoglein 1 (Dsg1) autoantibodies in the PF group, which suggested an antigenic cross-reactivity [1].

A previous study reported that Brazilian PF patients had higher anti-Dsg1 IgE titres compared to healthy controls (HC), and that Brazilian controls exhibited higher anti-Dsg1 IgE titres compared to American and Japanese controls, which suggested that Brazilian PF patients and controls share some environmental antigens that raise IgE antibody production [4]. Another study provided strong evidence that antibodies against LJM11 (a highly immunogenic salivary peptide from the sand fly *Lutzomyia longipalpis*) cross-reacted with Dsg1 peptide [3]. In fact, it has been proposed that anti-LJM11 IgE could underlie PF triggering in some Amerindians living in PF endemic region [5].

In this study, we aimed to evaluate total serum IgE and IgE antibodies production against SGEs from *A. aegypti*, *N. neivai*, and *S. nigrimanum* in PF, PFR, and HC groups

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4 living in the same Brazilian PF endemic area. We also present the correlation between total
5
6 IgE titres and IgE antibody titres production against each SGE.
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9 Serum samples from 30 PF patients, 30 PFR, and 30 HC were used, as approved by
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11 the Local Ethics Committee on Human Research. For total IgE determination, 50µL of
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13 serum sample was prepared by following the manufacturer's recommendations
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15 (ImmunoCAPTM Total IgE, Thermo-Fisher, USA) and read in Phadia100 laboratory system
16
17 (Thermo-Fisher, USA). IgE antibodies specific for SGEs were evaluated by ELISA, as
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19 described previously [1]; replacement with secondary murine monoclonal antibody anti-
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21 human-IgE conjugated with HRP was carried out (Abcam, UK).
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26 Compared to PFR and HC, total IgE levels in the serum were higher in PF patients
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28 (Kruskall-Wallis followed by Dunn's test: $P<0.001$) (**Figure 1A**). In fact, 28 (93.3%) of 30
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30 PF patients displayed IgE frequencies above the cut-off value (100kU/L) as compared to 10
31
32 (33.3%) PFR and 14 (46.6%) HC (chi-square test: $P<0.001$). Most PF patients (83.3%)
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34 were under treatment at the moment of serum collection. PF patients who had not started
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36 immunosuppressive drugs or had been without treatment for at least 60-days at the moment
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38 of serum collection tended to have higher total IgE levels as compared to PF patients under
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40 treatment (Mann-Whitney test: $P=0.095$) (**Figure 1B**).
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45 Anti-SGE IgE was present in the three studied groups, but PF patients exhibited
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47 higher IgE titres against *A. aegypti* and *N. neivai* SGEs as compared to PFR and HC
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49 ($P<0.001$). Regarding anti-*S. nigrimanum* SGE IgE, PF patients had higher titres than PFR
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51 ($P<0.001$), but similar titres to HC ($P=0.219$) (**Fig 1C**). In the three groups, total IgE levels
52
53 strongly correlated with IgE antibodies against SGEs from *A. aegypti*, *N. neivai*, and *S.*
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55 *nigrimanum* (Spearman test: $r=0.848$; $r=0.852$ and $r=0.740$, respectively, $P<0.001$ for all)
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(Fig 2A). In PF patients, anti-*N. neivai* SGE IgE titres were higher as compared to anti-*A. aegypti* and anti-*S. nigrimanum* SGE IgE titres ($P<0.001$ for both) (data not shown).

IgG (data extracted from Vernal *et al.* [1]) and IgE autoantibody levels against SGEs from *N. neivai* and *S. nigrimanum* ($r=0.438$, $P=0.015$ and $r=0.419$, $P=0.021$, respectively), but not against SGE from *A. aegypti*, correlated positively in PF patients. Anti-SGE IgG and IgE did not correlate significantly in PFR and HC for any of the studied SGEs (data not shown).

In PF patients, anti-*A. aegypti* and anti-*S. nigrimanum* SGE IgE did not correlate with anti-Dsg1 titres, but anti-*N. neivai* SGE IgE and anti-Dsg1 IgG titres presented a slightly positive correlation in PF patients ($r=0.327$; $P=0.077$) (Fig 2B). Total IgE levels did not correlate with anti-Dsg1 IgG in PF patients ($r=-0.256$; $P=0.179$) (data not shown).

High IgE production has been traditionally linked to intestinal parasitic helminth infestations related to allergic type-1 hypersensitivity reactions [6]. High IgE values in PF patients suggested that IgE production was due to another hypersensitivity response because PF patients assisted at our service are usually treated with antiparasitic drugs before immunosuppressant therapeutics is initiated, and no atopy signs were detected in these patients.

We found a positive correlation between IgG and IgE antibodies against SGEs from *S. nigrimanum* and *N. neivai*, but not against SGE from *A. aegypti*, which supported that PF patients presented a hypersensitivity immune response to the biting flies. Patients had reported the presence of biting flies in their living areas [2]. Although we have not demonstrated a cause-effect relationship between biting flies and PF triggering, we have reported a case of pemphigus vulgaris following myriads of *Simulium* sp. bites [7].

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4 IgE and IgG responses usually correlate significantly [8]. Even though we were not
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6 able to assess anti-Dsg1 IgE, we could expect that anti-Dsg1 IgE and IgG titres correlate
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8 positively, as reported previously by Diaz *et al.* in a Brazilian PF population [4].
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11 Recently, IgE has also been linked to autoimmune diseases: IgE antinuclear
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13 antibodies have been detected in patients with rheumatic arthritis, systemic lupus, and even
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15 bullous pemphigoid [9]. In contrast to IgG, IgE generally has much higher cross-reactivity,
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17 which is mainly explained by recognition of carbohydrate-containing epitopes; however, its
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19 involvement in self-antigen recognition remains unclear [9].
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23 In conclusion, high total IgE levels correlate with IgE antibodies against *A. aegypti*,
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25 *N. neivai*, and *S. nigrimanum* SGEs, reinforcing a hypersensitivity immune response in PF
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27 patients. Therefore, successive insect bites – specially sand fly bites – may act as a
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29 repetitive antigenic stimulus for individuals living in PF endemic regions and as a
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31 triggering and maintenance environmental autoimmunity factor in individuals that carry
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33 susceptibility alleles for PF [1].
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Author contributions.

Conceptualization, methodology: SV, AMR. Formal analysis: SV, MPe, WO, TG, MPi, AS, AMR. Funding acquisition: AS, AMR. Investigation, resources: SV, MP, WO, TG, MP, AS, AMR. Supervision: MPe, MPi, AS, AMR. Writing and reviewing manuscript: SV, MPe, WO, TG, MPi, AS, AMR.

Conflict of interested.

All authors declared: none

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4 **Ethical approval.**
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6 All procedures performed in studies involving human participants were in
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9 accordance with the ethical standards of the research committee University Hospital of the
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Ribeirão Preto Medical School of the University of São Paulo and with the 1964 Helsinki
declaration and its later amendments or comparable ethical standards.

18 **Informed consent.**
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Informed consent was obtained from all individual participants included in the
study.

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Figure legends

Figure 1. **A** – Total IgE determination (kU/L) ImmunoCAP™ Total IgE in the studied groups. Cut-off = 100 kU/L. **B** – Total IgE determination (kU/L) ImmunoCAP™ Total IgE in the PF group: without versus under treatment. Cut-off = 100 kU/L. **C** – Anti-IgE ELISA (OD – optical density) against salivary gland extracts (SGEs) from *Aedes aegypti*, *Nyssomyia neivai*, and *Simulium nigrimanum* in the studied groups.

PF = pemphigus foliaceus. ** = $P < 0.05$, *** = $P < 0.001$

Figure 2. **A** – Correlation between IgE antibodies against SGE (OD) and total IgE among three groups. **B** – Correlation between IgE antibodies against salivary gland extract (OD – optical density) and anti-Desmoglein 1 IgG (U/mL) in the PF group. IgE antibodies (OD) against salivary gland extracts (SGEs) from *Aedes aegypti* (A), *Nyssomyia neivai* (B), and *Simulium nigrimanum* (C)

Figure 1

[Click here to access/download;Figure;Fig_1.jpg](#)

Figure 2

[Click here to access/download;Figure;Fig_2.jpg](#)

5.2.2 Patients with pemphigus foliaceus recognized several salivary proteins from biting flies in Southeastern Brazil: anew hypothesis to explain its endemicity. *Jornal: Transactions of the Royal Society of Tropical Medicine and Hygiene*. Fator de impacto: 2,065. Formato: Concise Communication.

Exposure to salivary proteins of biting flies may be related to Brazilian pemphigus foliaceus etiopathogenesis in genetically susceptible individuals

S. Vernal; M. Pepinelli; W. Oliveira; T. Goulart; M. Pinto; A.M. Roselino

Patients with pemphigus foliaceus recognized several salivary proteins from biting flies in Southeastern Brazil: a new hypothesis to explain its endemicity

Running title: Biting flies and pemphigus foliaceus

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Background

Pemphigus foliaceus (PF) is an autoimmune bullous disease immunologically characterized by the production of autoantibodies against desmoglein (Dsg) 1. PF is subdivided into classic sporadic worldwide Cazenave's pemphigus and endemic pemphigus (known in Brazil as *Fogo Selvagem*). Both PF forms share the same immune-pathogenesis, but differ in their epidemiological and distribution patterns.¹ Endemic PF etiopathogenesis remains unclear; notwithstanding, a combination of genetic and environmental factors has been implicated in its triggering.^{2,3}

Several environmental factors have been related to PF including virus infection, contaminated soil and/or water, agricultural additives, parasitic infections, and insects biting^{2,4-7} In the forties, Vieira⁸ described the geographical behavior of PF in *São Paulo* state, southeastern Brazil, showing several well-defined foci especially near to rivers and mainly in the northeastern region. Vieira also pointed out an epidemiological link between *Simulium nigrimanum* (Macquart, 1838) and the endemicity of PF. Later Diaz *et al.* confirmed *Simulium* sp. bites have been considered a risk factor for the development of PF (odds ratio=4.7) in an Amerindian reserve in *Mato Grosso do Sul* State, Brazil.⁹

Despite *S. nigrimanum* was for many years the main implicated insect on PF etiopathogenesis, in the last two decades sand flies species arisen as a new environmental factor triggering PF autoimmunity in genetic susceptible individuals, suggesting that some of their salivary glands components may lead to a mimetic antigenicity cross-reacting with Dsg1.¹⁰⁻¹³ In 2001, Roselino *et al.* reported the first approach to a sand fly–PF link, showing higher titres of IgG antibodies against maxadilan – one of the most immunogenic salivary proteins described in *Lutzomyia longipalpis* (Lutz and Neiva, 1912) – in PF patients compared to healthy controls (HC) living in northeastern *São Paulo* State (NSSP).¹⁰ In 2012, Diaz *et al.* evidenced that LJM11 – another highly immunogenic protein from *Lu. longipalpis* – had similarity with Dsg1 peptide.¹¹ In the same way, Zaraa *et al.*

reported the recognition of five salivary proteins of 12, 15, 21, 30 and 36kDa from the sand fly *Phlebotomus papatasi* (Scopoli, 1786) by the sera of PF Tunisian patients where PF is also endemic.¹³

Nyssomyia intermedia (Lutz & Neiva, 1912) and *Nyssomyia neivai* (Pinto, 1926) are the main vectors of tegumentary leishmaniasis (TL) in *São Paulo* State. However, only *N. neivai* has been identified in research collections done systematically in NSSP.¹⁴ *Lu. longipalpis* has been documented in *São Paulo* state after the sixties, at least forty-years after the first publication of PF geographical foci in this region, and so far it has not been related to PF clusters.^{2,15}

Focusing in southeastern Brazil PF endemic foci, PF patients and their relatives (PFR) presented higher titres of IgG antibodies against salivary gland extract (SGE) of *S. nigrimanum* and *N. neivai*, but not against the mosquito *Aedes aegypti* when compared to healthy controls (HC) living in that region.² Moreover, IgG antibodies against SGE from *S. nigrimanum* and *N. neivai*, but not from *A. aegypti*, correlated with IgG anti-Dsg1 autoantibodies in PF patients.²

Considering these results, the aimed of this study is to reveal further SGE recognition using immunoblotting (IB) assay, and to describe the demographic and clinical profile of those patients with the higher titres against *S. nigrimanum* and *N. neivai* SGEs in NSSP. Following our results and using the compiled data available in the literature, we proposed an adjustment in flies-PF etiopathogenic link hypothesis.

Methods

Study Population

Patients were assisted at the University Hospital of *Ribeirão Preto* Medical School, University of São Paulo, Brazil, which is the main reference institution for the diagnosis and treatment of pemphigus in NSSP. The clinical diagnosis of PF was confirmed by acantholytic bullae on the histopathological examination, positive IgG fluorescence around keratinocytes in skin samples, and presence of serum autoantibodies against Dsg1 using commercial ELISA (MBL, Nagoya, Japan).

As we reported previously² we tested 30 PF patients' serum samples randomly selected from the Pemphigus Bank, Laboratory of Dermatology, *Ribeirão Preto* Medical School, with active clinical disease at the time of serum collection (presence of Nikolsky's sign). Sera were tested in ELISAs coated with SGE from *A. aegypti*, *N. neivai* and *S. nigriumanum*. The control groups consisted of sera randomly selected from 30 PFR and 30 HC living in the PF endemic region and without any autoimmune diseases.

Now, we selected sera samples presenting the highest ELISA IgG titres anti-*S. nigriumanum* and *N. neivai* SGEs results for immunoblotting (IB) assays as follow:

- Twenty-five serum samples with highest anti-SGE titres from *S. nigriumanum* including: 11 PF patients and 14 controls (nine PFR and five HC). Demographic and clinical data of PF, PFR and HC are available in Supplementary Table 1S, 2S and 3S respectively.
- Twenty serum samples with highest anti-SGE titres from *N. neivai*, including 9 PF patients and 11 controls (eight PFR and three HC). Besides, considering *N. neivai* as the main vector of TL in NSSP, we tested three serum samples from TL patients to compare the recognition patterns on IB assay. Demographic and clinical data of PF, PFR and HC are available in Supplementary Table 4S, 5S and 6S respectively.

Patients were subscribed as 1Sn to 25Sn from the highest value and progressive decreased titres on ELISA against SGE from *S. nigrimanum*, and 1Ny to 20Ny from the highest value and progressive decreased titres on ELISA against SGE from *N. neivai*.

Insect samplings, salivary glands extract and SDS-PAGE

Samples collections, salivary glands dissection and extraction, and SDS-PAGE characterization of the extractions were performed as previously described.²

Immunoblotting assay

Immunoblotting was carried out by transferring the SGE protein (5µg/well [2,5µg/µL]) to a 0.45-nm nitrocellulose membrane (Biorad®, USA), followed by blocking in 3% skimmed milk (BioRad®, USA) at room temperature (RT) for 1h. Strips were incubated with serum samples diluted 1:20 in TBS containing 3% skimmed milk at 4°C, overnight. After washing with Tween 20-TBS for 5min three times and with TBS for 5min once, the strips were incubated in HRP-anti-human IgG (BioRad®, USA) 1:2000 at RT for 2h. After four more washings, the reaction was developed by adding Color-Plus HRP (BioRad®, USA).

Ethics

The study complies with the 1975 Declaration of Helsinki, as revised in 2013 (Declaration of Helsinki World Medical Association, 2014), and was approved by the Local Committee for Ethics in Human Research #12248/2010.

Results

Immunoblot using Simulium nigrimanum SGE

PF and controls mainly recognized a ~250kDa peptide from *S. nigrimanum* SGE (blue square). Recognition of ~75kDa peptide (red square) was more clearly defined in PF patients (7/11) and PFR (5/9), and weaker recognized by HC (2/5). Salivary peptides smaller than 60kDa were mainly recognized by PF (8/11) and some PFR (2/5), but not in HC (0/5) (green square) (**Figure 1A**).

Immunoblot using Nyssomyia neivai SGE

PF and controls mainly recognized peptides between 50kDa and 75kDa (red square) from *N. neivai* SGE. Recognition of peptides between 5kDa and 10kDa (green square) was more clearly defined in PF (6/9) patients and PFR (4/8), and weaker by HC (1/3). Interestingly, the three TL patients shared similar recognition patterns, showing well-defined peptides between 50kDa and 75kDa (red square) and between 5kDa to 10kDa (green square) (**Figure 1B**).

Discussion

Despite the decreased PF diagnosis in NSSP, especially at seventies and eighties, new cases of PF have been diagnosed over the last decades, with maintenance of its prevalence.⁴ The expansion of urban boundaries, migrations movements to Brazil and forest invasion by sugar cane agribusiness and coffee plantations presents a new stage of cohabitation with local fauna previously confined outside the cities, especially relate to the greater exposure to flies' bites, leading to diseases previously restricted to rural activities (e.g. leishmaniasis, a disease

transmitted by a sandfly bite).¹⁶ It is in this scenario that geographical foci of PF cases began to appear, especially surrounding watersheds, leading to suspect that a local setting may be involved in PF etiopathogenesis.

Regarding our previously epidemiological link confirmation between biting flies and geographical foci of PF cases in NSPS,² we now detail the humoral response of these PF patients and endemic HC to SGEs from two widely distributed biting flies' using IB assays. We complemented our results with a demographic and clinical description of each patient analyzed. Interestingly, most of Brazilian PF data came from studies in Amerindians population, a very homogenous and isolated sample;¹⁷⁻¹⁹ here we study a mixed population sample including Caucasian, Black and Mullatos, but any Amerindian patient, expanding our understanding of susceptibility and protection ethnicity factors involved in PF etiopathogenesis.

Our results invigorate that flies salivary proteins seem to contribute to PF etiopathogenesis in genetically susceptible individuals. *HLA* susceptibility/protection alleles seems to decide if individuals with continuous exposure to flies' bites may induce an autoimmunity trigger to IgG pathogenic autoantibodies against Dsg1. Self-antigen reaction could be explained by: (a) salivary peptides introduced into the host's skin while acquiring a blood meal triggering an antigen cross-reaction with Dsg1; (b) local skin inflammatory reactions due to flies' bites may produce an anomalous Dsg1 exposure (damaged) leading to an aberrant Dsg1 presentation to immune cells.

In last decades, this first hypothesis has gained scientific support by the description of a sandfly salivary protein – LJM11 – which cross-reacted Dsg1 autoantibodies.¹¹ Furthermore, it has been proposed that anti-LJM11 IgE could underlie PF triggering in some Amerindian populations living in PF endemic regions.¹² Nevertheless, three main points play against this proposal: (1) LJM11 has been only described in New World sandflies, specifically *L. longipalpis*, then it can not explain PF endemic foci in the Old World regions (ex. Tunisia);^{13,20} (2) Even considering the New World, *L. longipalpis* is not widely distributed in some Brazilian PF endemic foci as we recently confirmed;^{2,15} (3) Others sandflies distributed in those PF endemic regions do not exhibit LJM11

peptide in their sialotranscriptomes (ex. *N. neivai*, *P. papatasi*).²¹ Even more, biologically is implausible that just one peptide of one insect may explain the endemicity of PF worldwide (**Figure 2**).

Thus, the second hypothesis emerges as a more plausible explanation for PF endemicity in some world's regions. Despite each local fly evidences a specific high immunogenic salivary peptides, during the bite the fly also introduces other various pharmacological components of its saliva (vasodilatation, platelet anti-aggregation, anesthetic, etc.) modulating the host's immune system.²² Chronic exposure to biting flies leads to a hypersensitivity type-1 response including IgG and IgE antibodies recruitment to the bite-site.²³ Population settled around watersheds is daily bitten by endemic flies.⁹ The continuous submission to local immunogenic peptides into the skin plus the local allergic response by the host may induce to an immunocompromised district including an abnormal Dsg1 exposure and subsequently an irregular dendritic cells presentation in genetically susceptible individuals, losing the immune-tolerance to Dsg1. This hypothesis is supported by: (1) PF patients recognized salivary gland extracts from their respective endemic region (ex. PF Tunisian patients evidenced antibodies against *P. papatasi*; (2)^{2,11,13} Brazilian Amerindian populations exhibited antibodies and Dsg1 cross-reaction with more than one salivary protein of *L. longipalpis* (LJM11 and LJM17);^{11,24} (3) Elevated total IgE antibodies have been described in PF patients, with a hypersensitive type-1 response to biting flies and IgE anti-Dsg1 antibodies;^{12,23,25} and (4) vector-borne parasitic infections (ex. Leishmaniasis and Onchocerciasis) also demonstrated circulation of IgG anti-Dsg1;^{7,26} (5) Healthy controls living in the endemic regions also evidenced anti-Dsg1 IgG circulated antibodies;²⁷⁻³⁰ and (6) other cadherin family proteins as Dsg2 and/or Dsc1-3.³⁰ Thereby, our findings reinforce that more than one salivary protein of biting flies is involved in PF etiopathogenesis, and not from only a single fly species.

Maxadilan is a 7-kDa peptide present in *L. longipalpis* salivary gland; however, a Maxadilan-simile has also been described in *N. neivai* sialome.³¹ Maxadilan was the first molecule to be identified in the sandfly saliva and it is recognized for its powerful vasodilator effect.³² Inoculation of Max in experimental animals exacerbates

Leishmania infection to the same degree as the whole salivary gland.³³ JAV08462.1 (putative Maxadilan *N. neivai*) is closely related to Maxadilan peptide, suggestion that pharmacological properties, including vasodilation, may be conserved in *N. neivai* (GenBank NCBI accessed). Description of anti-Maxadilan antibodies in the TL patients and healthy controls living in Southeastern Brazil confirms previous exposure to *N. neivai* sandfly bites and suggests natural and chronic exposure to this vector.³¹ Here we used three TL patients as controls in IB assays against *N. neivai* SGE. Interestingly, PF patients and PF relatives showed a very similar IB recognition patterns to these three TL patients, leaving in evidence well-defined bands between 50kDa and 75kDa and ~7kDa. Our findings confirmed the results by Roselino *et al.* in 2001, when it was proposed the participation of sandflies biting, specifically the most immunogenic peptides as Maxadilan, might play a role in PF etiopathogenesis.¹⁰

Although *S. nigrimanum* and *N. neivai* are two species far away in evolution time line, they converge in Diptera (Nematocera) family.³⁴ IgG antibodies from these two insects positively correlated in PF patients,² hypothesizing that their SGE could have some salivary proteins with similar antigen and/or pharmacological effects, modulating alike the host's immune response. In the nineties, Amerindians PF patients were questioned about insects as risk environmental factor; curiously, there were not description of sandflies but *S. nigrimanum* and other blackflies (ex. *Simulium pertinax*) were described.³⁵ Flies from the Diptera family (true flies) are widely spread worldwide,³⁶ but they especially prevalent in tropical conditions were most of the PF foci have been observed, including north region of South America (Venezuela, Colombia, Peru, Paraguay and Brazil) and Africa (Tunisia).^{2,13,15,36-38} The Diptera larvae can be herbivores, scavengers, decomposers, predators or parasites, with the consumption of decaying organic matter being one of the most prevalent feeding behaviors;³⁹ thus, its population density is concentrated near to watersheds as PF cases also grouped.

Endemic PF regions also shared some interested geo-demographical features. Additionally to environmental conditions, the European immigrants introduced a genetic patterns and mixed populations previously unexposed to tropical local fauna. The largest influx of European immigrants to Brazil occurred in the

late 19th and early 20th centuries.⁴⁰ According to the *Memorial do Imigrante* statistics data, Brazil attracted nearly 5 million immigrants between 1870 and 1953.⁴¹ These immigrants were divided in two groups: a part of them was sent to Southern Brazil to work as small farmers; however, the biggest part of the immigrants was sent to Southeast Brazil to work in the coffee plantations.^{40,41} It is exactly in this geographical region where the first cases and endemic foci of PF were described at the beginning of the nineteenth century. Similar situation was noted in Tunisia: from the late 19th century to after World War II, Tunisia was home to large populations of French and Italians,⁴² a population virgin to local flies continuous bite exposure.

Conclusion

Brazil represents a perfect scenario to the autoimmunity diseases' triangle: genetics, environment and inflammation. Genetic profile (*HLA* I and II) has been well identified in Southeastern Brazilian population, exhibited specific susceptibility alleles to PF. In this same population, we already confirm the epidemiological link between PF clusters and biting flies geographical distribution. In this report we now confirmed the genetic-environmental link in patients genetically susceptible exposed to biting flies (black- and sandflies). Moreover, we proposed a more plausible explanation for PF endemicity in some world's regions, which included a continuous inflammation by biting flies followed by an immunological local unbalance and losing self-antigen intolerance to Dsg1. Further studies are necessary to confirm our hypothesis.

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Conflict of interest

The authors state that they do not have any conflict of interest, and that they agree with the publication.

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Figures' legends

Figure 1. Anti-IgG human Immunoblot assay: **A** – Twenty-two serum samples selected with the higher titres on ELISA IgG anti-salivary glands extract of *Simulium nigrimanum*: numbered as 1 to 22 (from the highest value and progressive decrease titers). This sample includes: 11 *Pemphigus foliaceus* patients (left set), and 14 controls: nine *Pemphigus foliaceus* relatives (middle set) and five healthy controls (right set). **B** – Twenty serum samples selected with the higher titres on ELISA IgG anti-salivary glands extract of *Nyssomyia neivai*, numbered as 1 to 20 (from the highest value and progressive decrease titers). This sample includes: nine *Pemphigus foliaceus* patients (left set), and 11 controls: eight *Pemphigus foliaceus* relatives (middle set) and three healthy controls (right set). **C** – Three serum samples of tegumentary leishmaniasis patients.

Figure 2. Two hypotheses trying to explain the trigger of Desmoglein-1 self-antigen reaction. **A** – Salivary peptides introduced into the host's skin while acquiring a blood meal triggering a antigen that cross-react with Desmoglein-1. **B** – Local skin inflammatory chronic reaction due to flies' bites may produce an anomalous Desmoglein-1 exposure (damaged) leading to an aberrant presentation to immune cells.

Supplementary Tables' legends

Table 1S. Pemphigus foliaceus patients with the higher IgG antibodies titres against salivary glands extracts from *Simulium nigrimanum*.

Table 2S. Pemphigus foliaceus relatives with the higher IgG antibodies titres against salivary glands extracts from *Simulium nigrimanum*

Table 3S. Healthy endemic controls with the higher IgG antibodies titres against salivary glands extracts from *Simulium nigrimanum*

Table 4S. Pemphigus foliaceus patients with the higher IgG antibodies titres against salivary glands extracts from *Nyssomyia neivai*.

Table 5S. Pemphigus foliaceus relatives with the higher IgG antibodies titres against salivary glands extracts from *Nyssomyia neivai*

Table 6S. Pemphigus foliaceus neighbors with the higher IgG antibodies titres against salivary glands extracts from *Nyssomyia neivai*

Figure 1. Anti-IgG human Immunoblot assay

Figure 2. Two hypotheses trying to explain the trigger of Desmoglein-1 self-antigen reaction

Table 1S. Pemphigus foliaceus patients with the highest IgG antibodies titres against *Simulium nigrimanum* SGE

Pemphigus foliaceus patients											
	1Sn (3Ny)	2Sn (5Ny)	4Sn (17Ny)	5Sn	6Sn (1Ny)	7Sn (15Ny)	8Sn (2Ny)	9Sn	12Sn (12Ny)	24Sn	24Sn
Anti-SGE <i>S. nigrimanum</i> (OD)	1.589	1.404	1.274	1.251	1.244	1.219	1.202	1.192	1.034	0.734	0.734
Anti-SGE <i>Ny. Neivai</i> (OD)	1.561	1.398	0.759	0.331	1.888	0.868	1.736	0.605	0.985	0.534	0.534
Anti-Dsg1 (U/ml)	269.8	146.2	166.8	92.8	100.9	20.1	193.0	106.3	188.4	163.2	163.2
Age (years)	33	32	17	59	48	52	28	25	36	46	46
Sex	F	M	M	F	F	F	M	F	M	F	F
Ethnic	B	C	C	C	Mu	C	C	C	C	C	C
Duration of disease (years)	1	>1	0.5	<0.5	>1	>1	<0.5	>1	>1	>1	>1
Treatment	Virgin	Virgin	Virgin	Yes	Yes	Yes	Virgin	Yes	Yes	Yes	Yes

Sn: *Simulium Nigrimanum*. **Ny:** *Nyssomyia neivai*. **SGE:** salivary gland extracts; **OD:** optical density; **Dsg1:** desmoglein 1; **M:** male; **F:** Female; **C:** Caucasian; **B:** Black; **Mu:** Mulatto; **PF:** pemphigus foliaceus.

Table 2S. Pemphigus foliaceus relatives with the higher IgG antibodies titres against salivary glands extracts from *Simulium nigrimanum*

Pemphigus Foliaceus relatives									
	3Sn (11Ny)	13Sn (7Ny)	14Sn	15Sn	16Sn	18Sn (8Ny)	19Sn	20Sn (20Ny)	23Sn (10Ny)
Anti-SGE <i>S. nigrimanum</i> (OD)	1.356	0.927	0.899	0.887	0.867	0.826	0.821	0.804	0.738
Anti-SGE <i>Ny. Neivai</i> (OD)	1.090	1.299	0.356	0.202	0.663	1.214	0.312	0.694	1.152
Anti-Dsg1 (U/ml)	1.0	1.6	0.6	0.0	37.8	0.7	1.9	0.9	0.7
Age (years)	17	41	57	42	60	49	57	48	60
Sex	M	F	F	F	F	F	F	F	F
Ethnic	N.A.	C	C	N.A.	N.A.	C	Mu	N.A.	C
Relation with PF case	Son	Sister	Mother	Mother	Mother	Sister	Sister	Sister	Mother

SGE: salivary gland extracts; **OD:** optical density; **Dsg1:** desmoglein 1; **M:** male; **F:** Female; **C:** Caucasian; **Mu:** Mulatto; **PF:** pemphigus foliaceus; **N.A.:** not available.

Table 3S. Healthy endemic controls with the higher IgG antibodies titres against salivary glands extracts from *Simulium nigrimanum*

Healthy endemic controls					
	10Sn (17Ny)	11Sn (10Ny)	17Sn (11Ny)	21Sn	22Sn
Anti-SGE <i>S. nigrimanum</i> (OD)	1.155	1.084	0.848	0.753	0.744
Anti-SGE <i>N. Neivai</i> (OD)	0.777	0.915	0.904	0.354	0.561
Anti-Dsg1 (U/ml)	1.2	1.1	0.3	0.3	3.5
Age (years)	59	52	59	49	37
Gender	M	M	M	M	M
Ethnic	N.A.	Mu	Mu	Mu	N.A.

SGE: salivary gland extracts; **OD:** optical density; **Dsg1:** desmoglein 1; **M:** male; **Mu:** Mulatto; **PF:** pemphigus foliaceus; **N.A.:** not available.

Table 4S. Pemphigus foliaceus patients with the higher IgG antibodies titres against salivary glands extracts from *Nyssomyia neivai*

Pemphigus Foliaceus patients									
	1Ny (6Sn)	2Ny (8Sn)	3Ny (1Sn)	5Ny (2Sn)	6Ny	12Ny (12Sn)	15Ny (7Sn)	17Ny (4Sn)	19Ny
Anti-SGE <i>N. neivai</i> (OD)	1.888	1.736	1.561	1.398	1.330	0.985	0.868	0.758	0.729
Anti-SGE <i>S. nigrimanum</i> (OD)	1.244	1.202	1.589	1.404	0.628	1.034	1.219	1.274	0.665
Anti-Dsg1 (U/ml)	100.9	193.0	269.8	146.2	108.3	188.4	20.1	166.8	168.3
Age (years)	48	28	33	39	55	36	52	17	49
Gender	F	M	F	M	F	F	F	M	F
Ethnic	M	C	B	C	C	C	C	C	C
Duration of disease (years)	>1	<0.5	1	>1	>1	>1	>1	0.5	1
Treatment	Yes	Virgin	Virgin	Virgin	Yes	Yes	Yes	Virgin	Yes

Sn: *Simulium Nigrimanum*. **Ny:** *Nyssomyia neivai*. **SGE:** salivary gland extracts; **OD:** optical density; **Dsg1:** desmoglein 1; **M:** male; **F:** Female; **C** Caucasian; **B:** Black; **Mu:** Mulatto; **PF:** pemphigus foliaceus

Table 5S. Pemphigus foliaceus relatives with the higher IgG antibodies titres against salivary glands extracts from *Nyssomyia neivai*

Pemphigus foliaceus relatives								
	4N	7N (13S)	8N (18S)	9N	10N (23S)	11N (3S)	18N	20N (20S)
Anti-SGE <i>Ny. neivai</i> (OD)	1.545	1.299	1.214	1.160	1.152	1.090	0.743	0.694
Anti-SGE <i>S. nigrimanum</i> (OD)	0.481	0.927	0.826	0.685	0.738	1.356	0.597	0.804
Anti-Dsg1 (U/ml)	1.591	1.661	0.764	2.510	0.796	1.012	3.421	0.935
Age (years)	N.A.	41	49	35	60	14	39	48
Gender	Female	Female	Female	Female	Female	Male	Female	Female
Ethnic	C	C	C	C	C	N.A	N.A.	C
Relation with PF case	Daughter	Sister	Sister	Mother	Mother	Son	Sister	Sister

Sn: *Simulium Nigrimanum*. **Ny:** *Nyssomyia neivai*. **SGE:** salivary gland extracts; **OD:** optical density; **Dsg1:** desmoglein 1; **M:** male; **F:** Female; **C:** Caucasian; **B:** Black; **Mu:** Mulatto; **PF:** pemphigus foliaceus

Table 6S. Pemphigus foliaceus neighbors with the higher IgG antibodies titres against salivary glands extracts from *Nyssomyia neivai*

Pemphigus Foliaceus neighbors			
	10Ny (11Sn)	11Ny	17Ny (10Sn)
Anti-SGE <i>N. neivai</i> (OD)	0.915	0.904	0.777
Anti-SGE <i>S. nigrimanum</i> (OD)	1.084	0.848	1.155
Anti-Dsg1 (U/ml)	1.163	0.302	1.283
Age (years)	52	59	59
Gender	F	M	M
Ethnic	Mu	Mu	N.A.

Sn: *Simulium Nigrimanum*. **Ny:** *Nyssomyia neivai*. **SGE:** salivary gland extracts; **OD:** optical density; **Dsg1:** desmoglein 1; **M:** male; **F:** Female; **Mu:** Mulatto

5.2.3 Pemphigus foliaceus and sandflies biting: Assessing the humoral immune response to Maxadilan and LJM11 salivary proteins. *Fator de impacto*: 6,767. *Formato*: Original article.

Brazilian pemphigus foliaceus and sandflies: Maxadilan or LJM11?

Sebastian Vernal; Natália A. de Paula, Valdés Bollela, Ethan Lerner, Ana Maria Roselino

Title

Pemphigus foliaceus and sandflies biting: Assessing the humoral immune response to Maxadilan and LJM11 salivary proteins

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To the Editor,

In 2001, Roselino *et al.*¹ reported the first link between salivary proteins from sandflies and pemphigus foliaceus (PF) relationship, showing higher titres of IgG antibodies against Maxadilan - one of the most immunogenic salivary proteins described in *Lutzomyia longipalpis*, in PF patients compared to controls living in a PF endemic Brazilian region. In 2012, Qian *et al.*² evidenced in PF patients antibodies against LJM11 - another highly immunogenic salivary protein from *Lu. longipalpis*, and demonstrated that LJM11 cross-reacted with desmoglein (Dsg) 1 peptide.

Here, we evaluated the production of IgG antibodies against Maxadilan and LJM11 in PF patients and controls living in a well-known Brazilian endemic region for PF in the northeastern São Paulo state (NSPS).³ Serum samples from 60 PF patients and 160 controls were randomly selected after the Local Ethics Committee on Human Research approval. Control groups comprehended 50 PF relatives (PFR), 70 healthy controls (HC), 20 patients with visceral leishmaniasis (VL) and 20 with tegumentary leishmaniasis (TL).

Commercial ELISA (MBL, Japan) detected anti-Dsg1 antibodies in those samples. Recombinant Maxadilan (rMax) was kindly provided by Dr. Lerner, and rLJM11 commercially produced by WatsonBio LLC, USA (**Figure 1A**). Since TL and VL patients have been certainly bitten by sand flies, to standardize the in-house ELISA for rMax and rLJM11, sera samples from 5 TL patients with known high anti-rMax titres as reported previously by Aires *et al.*⁴; and from 5 VL patients were tested. As control samples, 5 HC with no medical records of leishmania infection were used. Experiments were repeated twice as described previously.³ After that, ELISA was performed with all studied sera samples.

Considering anti-rMax results, VL and TL patients exhibited similar and the highest titres. VL presented higher titres compared to PF, PFR and HC groups ($P=0.0039$, 0.0008 , and <0.0001 , respectively) (**Figure 1B**). On the other hand, TL titres were higher than HC titres ($P<0.0001$); however, there was no significance when compared to PF and PFR. Moreover, PF titres were higher than HC ($P=0.0071$), but similar to PFR. Focusing on anti-rLJM11 results, VL group showed higher titres when compared to the other groups ($P<0.0001$ for all) (**Figure 1B**). Anti-rMax titres were positively correlated with rLJM11 titres exclusively in VL group (Spearman test: $r=0.766$; $P<0.0001$) (data not shown).

Considering all values of the studied groups, anti-rMax but not anti-rLJM11 correlated with anti-Dsg1 titres ($r=0.226$, $P\text{-value}=0.0022$ and $r=-0.030$, $P\text{-value}=0.6870$, respectively) (data not shown). Considering the titres of PF group, anti-rMax but not anti-rLJM11 correlated with anti-Dsg1 titres (**Figure 1C**).

Endemic PF foci in NSPS revealed a prevalence of 57.5 cases per million inhabitants.³ The HLA profile in this population was also clearly identified.⁵ Recently, an epidemiological link of PF with biting flies was confirmed by geographical distributions maps followed by the biting exposure confirmation through to humoral response assessment against salivary glands extracts (SGE) from the most widely distributed flies species, *Nyssomyia neivai* and *Simulium nigrimanum*.³

N. neivai, a vector of TL, but not *Lu. longipalpis*, a vector of VL, has been systematically collected in NSPS.³ Moreover, we confirmed a Maxadilan-simile cDNA sequence in *N. neivai*,⁴ followed by the description of the *N. neivai* sialome, revealing a Maxadilan-simile peptide (accession number JAV08642.1 – data in submission) which represents the closest relationship with the Maxadilan powerful vasodilator, suggesting that *N. neivai* salivary protein might have preserved the pharmacological properties. Notwithstanding, no similar peptides to LJM11 were evidenced.⁶ These data support why PF patients living in NSPS produce IgG antibodies against rMax but not against rLJM11.

Recently, we reported antibodies against SGE from *N. neivai* in 30 PF, 30 PFR and 30 HC groups. Using these data compiled from Vernal et al.³ allowed us to demonstrate a strong positive correlation with anti-rMax ($r=0.799$, $P<0.0001$), and a fragile correlation with anti-rLJM11 ($r=0.207$, $P=0.0501$) (**Figure 1D**). These results suggest that even LJM11 peptide was not demonstrated in *N. neivai* sialome, there is still some cross-reactivity amongst LJM11 and other salivary proteins present in the *N. neivai* SGE.

Zaraa et al.⁷ reported the recognition of salivary proteins from the sandfly *Phlebotomus papatasi* by PF sera in a Tunisian PF endemic region. We also recently described the recognition of more than one salivary protein from black- and sandflies by PF patients (data in submission).⁸ Thus, our results support the hypothesis that not a specific salivary protein may be involved in PF etiopathogenesis. The triggering factor to the PF autoimmune process in genetically susceptible individuals could be better explained by a continuous stimulus of highly immunogenic pool of salivary proteins from biting flies rather than a single specific protein.

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Figure Legends

Figure 1. A – SDS-Page (4-15%, *Bio-Rad*, USA) stained by *Comassie blue*. Black arrow: rLJM11 protein (43kDa). Blue arrow: rMaxadilan protein (~6.5kDa). **B** – In house ELISA results of IgG antibodies against rMax and rLJM11 in pemphigus foliaceus (PF) patients, PF relatives, healthy controls (HC), and in visceral leishmaniasis (VL) and tegumentary leishmaniasis (TL) patients. **C** – Correlation between IgG antibodies against rMax and rLJM11 and IgG autoantibodies against desmoglein 1. **D** – Correlation between IgG antibodies against rMax and rLJM11 and IgG antibodies against salivary gland extract (SGE) from *Nyssomyia neivai* (data compiled from Vernal *et al.*³).

5.2.4 An insight into the Sialome of *Nyssomyia neivai* (Pinto 1926): a sandfly vector in Brazilian endemic area for Tegumentary Leishmaniasis and Pemphigus foliaceus (Fogo selvagem). *Jornal: Parasite and Vectors* *Fator de impacto:* 2,564 *Formato:* Original article.

An insight into the Sialome of *Nyssomyia neivai* (Pinto 1926): a sandfly vector in Brazilian endemic area for Tegumentary Leishmaniasis and Pemphigus foliaceus (*Fogo selvagem*)
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Title

An insight into the Sialome of *Nyssomyia neivai* (Pinto 1926): a sandfly vector in Brazilian endemic area for Tegumentary Leishmaniasis and *Pemphigus foliaceus* (*Fogo selvagem*)

Running title

Nyssomyia neivai sialotranscriptome

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INTRODUCTION

Leishmaniasis encompasses a broad spectrum of diseases caused by an obligate intramacrophage protozoan belonging to the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), that is predominantly in tropical and subtropical regions. The parasite is transmitted by the bite of sandfly vectors (Diptera: Psychodidae: Phlebotominae), which inoculate the promastigote forms of *Leishmania* into the host's skin while acquiring a blood meal.^{1,2} During this process, the phlebotomine injects the parasite with saliva.² The pharmacological activities of the saliva assist in blood feeding and cell functions, modulating the host's immune system.^{3,4}

Leishmania (Viannia) braziliensis (Viana 1911) and *Leishmania (Leishmania) amazonensis* (Lainson & Shaw 1972) are the main species involved in both the cutaneous and mucocutaneous forms of Tegumentary Leishmaniasis (TL) in Brazil.⁵ Autochthonous cases of TL have been reported in the northeastern São Paulo state (NSPS), Southeastern Brazil. Both sand flies: *Nyssomyia intermedia* (Lutz & Neiva 1912) (syn=*Lutzomyia intermedia*) and *Nyssomyia neivai* (Pinto 1926), are the main vectors of *L. (V.) braziliensis* in São Paulo state; however, only *N. neivai* has been recognized in systematic research collections in NSPS cities.⁶

Pemphigus foliaceus (PF) is an autoimmune bullous disease caused by autoantibodies against desmoglein (Dsg) 1. It is subdivided into classic sporadic worldwide Cazenave's pemphigus and endemic pemphigus (known in Brazil as *Fogo Selvagem*). Although the pathogenesis of PF remains unclear, genetic and environmental factors have been implicated in the susceptibility to this disease.^{7,8} Interestingly, the salivary proteins of sandflies have been related to pemphigus etiopathogenesis. Higher levels of serum IgG against maxadilan, a highly immunogenic salivary protein described in *Lutzomyia longipalpis* (Lutz and Neiva 1912), a vector of visceral leishmaniasis (VL), have been determined in PF patients as compared to healthy controls living in the same

endemic region.⁹ There is also evidence that LJM11, another immunogenic protein from *Lu. longipalpis*, cross-reacts with antibodies against Desmoglein (Dsg) 1, and it has been proposed as the antigen that triggers PF diagnosed in Amerindians living in *Mato Grosso* state, Brazil.¹⁰ Of note, *Lu. longipalpis* is not widely distributed in NSPS.^{8,11}

The profile of salivary components has been defined in nine Old World Phlebotomus species;¹²⁻¹⁹ notwithstanding, the sialotranscriptome of the New World sandflies have been documented only in four species: *Bichromomyia olmeca* (Vargas and Diaz-Najera 1959) (syn=*Nyssomyia olmeca*), *Lutzomyia ayacuchensis* (Caceres and Galati 1988), *Lu. longipalpis* and *N. intermedia*.²⁰⁻²³ Considering the vector-based vaccines for *Leishmania* sp. infection, and to assess a possible candidate protein that might be the trigger of anti-Dsg1 autoantibodies in Brazilian endemic PF in NSPS, we describe the sialome of *N. neivai* and to compare it to: *N. intermedia*²¹ (the main vector of *Leishmania* (V.) *braziliensis* in the coastal SP state), and *Lu. longipalpis*²² (the vector of VL in SP state), pemphigus etiopathogenesis.^{25,26}

METHODS

Collection and maintenance of sand flies

Sand flies were collected in Santa Eudóxia, SP state - Brazil (along the edges of the *Mogi Guaçu* River) on the wall of a house, using a manual aspirator between 18:00hs and 23:00hs. In the laboratory, the sand flies were maintained in cages covered with voile (30cm³) at 26 ±1°C, 80–90% humidity, and a 12:12 (L:D) photoperiod. Salivary glands were extracted as described previously.²⁷

Salivary gland transportation

Two hundred pairs of salivary gland tissue samples were submitted in 1mL RNA Later each to the North Carolina State Genomic Sciences Laboratory (Raleigh, NC, USA) for RNA extraction, Illumina library preparation, and sequencing. The salivary glands tissue samples exportation was approved by the United States Department of Agriculture – Veterinary Permit under the ID number #130339.

Salivary gland RNA extraction

Prior to extraction, salivary glands were pelleted by the addition of 1mL PBS in a benchtop centrifuge at 5000x g for 10m to remove the RNA Later. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, MD, USA) following the manufacturer's protocol for purification of total RNA from animal tissue. Briefly, Qiagen RLT buffer with β -Mercaptoethanol (β -ME) was added to the tissue samples and samples were homogenized using a Qiagen TissueLyser with 5mm stainless steel beads (Qiagen). Samples were then purified with provided RNeasy spin columns. Total RNA was then assessed for purity and size integrity using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Chip (Agilent Technologies, CA, USA). Purification of messenger RNA (mRNA) was performed using oligo-dT beads provided in the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, MA, USA). Complementary DNA (cDNA) libraries for Illumina sequencing were constructed using the NEBNext Ultra Directional RNA Library Prep Kit (NEB) and NEBNext Multiplex Oligos for Illumina (NEB) using the manufacturer-specified protocol.

Sialome library

Briefly, the mRNA was chemically fragmented and primed with random Oligos for first strand cDNA synthesis. Second strand cDNA synthesis was then carried out with dUTPs to preserve strand orientation information. The double-stranded cDNA was then purified, end repaired and “a-tailed” for adaptor ligation. Following ligation, the samples were selected for a final library size (adapters included) of 400-550bp using sequential AMPure XP bead isolation (Beckman Coulter, USA). Library enrichment was performed and specific indexes for each sample were added during the protocol-specified PCR amplification. The amplified library fragments were purified and checked for quality and final concentration using an Agilent 2200 Tapestation (D1000 chip, Agilent Technologies, USA) combined with a Qubit fluorometer (Thermo-Fisher, MA, USA). The final quantified libraries were pooled in equimolar amounts for clustering and sequencing on an Illumina HiSeq 2500 DNA sequencer, utilizing a 125bp single-end cycle sequencing kit (Illumina, CA, USA). The software package Real Time Analysis (RTA), was used to generate raw bcl, or base call files, which were then de-multiplexed by sample into fastq files using bcl2fastq Conversion Software v2.17 (Illumina, CA, USA).

Bioinformatics

Custom bioinformatic analysis were describe elsewhere ²⁸. Succinctly, low quality reads were trimmed from Fastq files (<20) and contaminating adapter primer sequences removed. De novo assembly from reads was a result of Abyss (using k parameters from 21 to 91 in 5-fold increments)²⁹ and SOAP de novo-trans³⁰ assemblers. The fasta files were combined and further assembled using an iterative blast and CAP3 pipeline as previously described³¹. Coding sequences (CDs) were extracted based on the existence of a signal peptide in the longer open reading frame (ORF) and by similarities to other proteins found in the Refseq invertebrate database from the

National Center for Biotechnology Information (NCBI), proteins from “diptera[organism]” deposited at NCBI’s Genbank and from SwissProt. Contigs containing an open reading frame and/any similarity to sequences in the chosen databases were selected for further analysis. Reads for each library were mapped on the deducted CDs using blastn with a word size of 25, 1 gap allowed and 95% identity or better required. Up to five matches were allowed if and only if the scores were the same as the largest score. Mapping of the reads was also included in the Excel spreadsheet. Values of the Reads Per Kilobase of transcript, per Million mapped reads (RPKM)³² for each coding sequence were also mapped to a spreadsheet. Automated annotation of proteins was based on a vocabulary of nearly 350 words found in matches to various databases, including Swissprot, Gene Ontology, KOG, Pfam, and SMART, Refseq-invertebrates and the diptera subset of the GenBank sequences obtained by querying diptera[organism] and retrieving all protein sequences. Raw reads were deposited on the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under bioproject ID PRJNA359206. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession SAMN0618923.

Phylogenetic analysis

For multiple sequence alignment and phylogenetic analysis PkanSILK, PkanYLW, PkanSP15 had their predicted signal peptide signal (SignalIP-5.0 sever [49]) removed and resulting protein sequence entered into a Basic Local Alignment Search tool (BLAST) against nr and tsa_nr databases. Homolog sequences were included if E-value was below 1-10 and belonged to the sand fly genera, with the exception of PkanYLW where a homologue from drosophila was used to root the tree. Multiple sequence alignment and identity/similarity matrix were constructed on MacVector (Version 15.5.3) with MUSCLE using PAM 200 profile. In order to determine the best method for estimating Maximum

Likelihood phylogenetic trees, the “Find best protein Models” feature of MEGA7[44] was utilized. Through this feature, it was determined that the best method for modeling phylogeny for PkanSILK was JTT+G+I, PkanYLW was WAG+G and PkanSP15 was WAG+G+I. For Gaps/Missing data treatment, a partial deletion option was utilized. Finally, the reliability of the trees were tested, by bootstrap method (N=1000). Nodes with <70% reliability were removed.

RESULTS AND DISCUSSION

cDNA library of Nyssomyia neivai salivary gland

cDNA library was constructed from salivary glands of *N. neivai* females dissected one day after emergence. From this cDNA library 1,302,396 high quality reads were assembled in 1200 contigs (**Table 1**). Contigs were classified in five categories namely: secreted, housekeeping, transposable elements, viral and unknown. Remarkably, the secreted proteins category comprised 41.2% of the contigs and 92.4% of the number of reads. All previous salivary transcriptomes were based in low output cDNA library sequencing, nevertheless a high abundance of transcripts encoding secreted proteins were also reported.^{13-16,19,21,33,34} Our data further validates the specialization of the salivary gland machinery and the specificity of the material obtained with the sand fly dissection. The housekeeping category had 33.3% of the clusters and 3.2% of the total sequences. The category of “unknowns” comprised 24.6% of the cluster and 4.3% of the sequences. Finally, Viral products and transposable elements included less than 1% of the families (0.3% and 0.8% respectively) and less than 0.1% of total sequences.

➤ *Housekeeping and Unknow proteins sequences*

The 399 clusters (comprising 42,119 sequences) attributed to Housekeeping genes expressed in the salivary glands of *N. neivai* were further divided into 22 subgroups according to their function. Two sets were associated with (a) protein synthesis machinery (22 contigs), including translation, ribosomal structure and biogenesis, and (b) metabolism (94 contigs), a pattern also

observed in other sialotranscriptomes. Proteins with unknown function (295 contigs comprising 4.3% of reads) were classified as “unknown”.

➤ *Secreted proteins sequences*

The putative secreted salivary proteins of *N. neivai* were classified into 35 main protein families (**Table 2**). The most abundant transcripts were SP13-15 protein family (34.4%), followed by C-type lectins (15.9%), Maxadilan (15.6%), ML domain salivary proteins (5.8%), Yellow protein family (5.1%), Apyrase (1.5 %) and 10-kDa family (3.4%). The following paragraphs describe the most important families in detail, focusing on protein family characteristics, possible function, biochemical, immune-modulatory, and antigenic properties, and phylogenetic analysis in context with related proteins from other Brazilian sandflies, *S. nigrimanum*'s sialome and desmogleins proteins.

I. SP13-15 family

SP15 family was described for the first time in the sialome of *Phlebotomus perniciosus* (Newstead 1911), and was named after 15-kDa salivary protein of *Phlebotomus papatasi* (Scopoli 1786) (PpSP15: AF335487).¹⁵ Thus far, SP15-like proteins have only been reported in sandflies and not in any other Diptera; Alvarenga *et al.*, 2013,³⁵ demonstrated that SP15 from *Phlebotomus duboscqi* (Neveu-Lemaire 1906) inhibit anionic surface-mediated reactions suggesting a role in anticoagulation, inhibiting the activation of FXII and FXI, and anti-inflammatory processes.

It has been suggested that SP15-like proteins were derived from an ancestral odorant-binding protein and were closely related to short D7 proteins.^{12,15} The SP15 family is an abundant salivary family among other species of sandflies, and in *N. neivai* is the most abundant salivary protein with 34.4% of the RPKM belonging to the SP13-15 family.

PpSP15-like proteins were reported as promising anti-*Leishmania* vaccine candidates. Immunization of mice with *P. papatasi* SP15 protein conferred partial protection against *Leishmania* (*Leishmania*) *major* (Yakimoff and Schokhor 1914) infection;³³ furthermore, a DNA vaccine containing the PpSP15 cDNA provided the same protection.³³ ParSP03 (AAX56359), a PpSP15-like protein from *Phlebotomus ariasi* (Tonnoir 1921), elicited similar delayed type hypersensitivity and humoral immune responses upon DNA vaccination.¹⁶ Recently, BALB/c mice immunized to PsSP19 (HM56964), a protein member of the SP15 family from *Phlebotomus sergenti* (Parrot 1917), acts as an adjuvant to accelerate the cell-mediated immune response to co-administered *Leishmania* antigens, providing protection against *Leishmania* (*Leishmania*) *tropica* (Wright 1903) infection.³⁶

Phylogenetic analysis of *N. neivai* SP13-15 family grouped these proteins in a New World sandflies cluster next to *B. olmeca*, *Lu. ayacuchensis* and *N. intermedia* proteins, and to the only one SP-15 family protein described in *Lu. longipalpis* so far, SL1²² (AAD32197.1) (**Figure 1A**). *N. neivai* SP-15 proteins were align to *N. intermedia* and *Lu. longipalpis* proteins of the same cluster tree (**Figure 1B**). SL1 from *Lu. longipalpis* is closely related to *Lu. ayacuchensis* SP-15 proteins.²⁰ Despite, *N. neivai* SP13-15 family proteins shared a 47 to 56% of identity and 67 to 79% of similarity to SL1.

JAV08231 shared 90% identity and 92% similarity with Linb-8 (AFP99232.1) of *N. intermedia*. Interestingly, BALB/c immunization with DNA plasmids encoding Linb-8 induced the highest humoral immune response against *N. intermedia* salivary gland homogenate (SGH), even greater than Linb-7 (AFP99230.1), another SP-15 protein also tested.²¹ Linb-7-immunized mice induce a strong humoral response leading to a sustained local inflammatory process, which may exacerbate *Leishmania* sp. infection by *L. (V). braziliensis*.²¹ On the other hand, Linb-11 (AFP99228.1), a 4.5kDA molecule from the SP-13 family, induces low humoral, and controlled cellular immune responses in Linb-11-immunized mice, resulting in a better *L. (V). braziliensis* infection control;²¹ notwithstanding, no

significant alignments were found between Linb-11 and *N. neivai* salivary proteins. Regarding *N. neivai* and *N. intermedia* are the two vector of *L. (V). braziliensis* in Southeastern Brazil, similarities between JAV08231 and Linb-8 may be interested target to a vector-based vaccine for TL in Brazil.

II. ***C-type lectins family and Lufaxin-like proteins***

In animals, protein-carbohydrate interactions serve multiple functions in the immune system. C-type lectins family is component of the innate immune response, pathogen neutralization through the complement pathway, and adaptive immune response.³⁷ Homologous proteins with molecular weight of 16.2–16.5 kDa have been identified from *Lu. longipalpis* saliva.²² This putative domain may function as a Ca^{+2} -dependent carbohydrate-binding pocket involved in extracellular matrix organization, pathogen recognition and cell-to-cell interactions.³⁷ The exactly role of this proteins in sandflies remains elusive.

At the end of the last century, the anticoagulant activity of *Lu. longipalpis* salivary proteins was attributed to a C-type lectin-like protein.³⁸ Years later, expression and testing of recombinant salivary proteins from *Lu. longipalpis* identified a 32-kDa protein responsible for the anticoagulant activity named as Lufaxin (Lutzomyia Factor Xa inhibitor)³⁹ (AAS05319.1). Others Lufaxin-like proteins have also been described in New world and Old world sand flies, suggesting a preserved anticoagulation property.^{13-16,19,21,33}

Linb-17 (AFP99233.1) from *N. intermedia* sialome²¹ shared a 53% identity and 69% similarity with Lufaxin, but *N. neivai* salivary proteins do not shared any significant alignment with both. Only another C-type lectin-like protein from *N. intermedia*, Linb-22 (AFP99236.1), shared some similarities with two *N. neivai* proteins, JAV08581.1 and JAV08563.1 (56 and 57% similarity respectively)

(Figure 2A and 2B); notwithstanding, the pharmacological properties and a supposed anticoagulation activity are still unknown.

III. **Maxadilan-like family**

Maxadilan (AAA29288.1) is a 7-kDa peptide present in the salivary gland of the sandfly *Lu. longipalpis*. Max was the first molecule to be identified in the sandfly saliva,⁴⁰ and it is recognized for its powerful vasodilator effect. Inoculation of Maxadilan in experimental animals exacerbates Leishmania infection to the same degree as the whole salivary gland.⁴¹ This peptide can drive a Th1 response to Th2, to up-regulate IL-10 and TGF- β production, and it suppresses IL-12p40, TNF- α , and NO production.^{42,43}

In animal models, mice vaccinated with maxadilan become markedly protected against Leishmania infection, producing not only anti-maxadilan antibodies, but also immunized CD4+ T cells against maxadilan, to generate IFN- γ and induce NO production.⁴¹ Notoriously, immunization against maxadilan also inhibits correct blood meal acquisition by sandflies, so immunization could help to block the vector reproduction process.⁴⁴ Nonetheless, maxadilan is not free of polymorphism; its aminoacids substitution is around 23%, and some aminoacids sequences are not conserved.^{45,46}

Maxadilan-like proteins have never been identified in Old World Phlebotomus species.^{13-16,19,21,33} Phlebotomus sandflies except for *P. duboscqi*, lack of adenosine deaminase (ADA) that hydrolyze adenosine and adenosine monophosphate (AMP)^{14-16,19}, but contain large amounts of adenosine and AMP^{47,48}. In contrast, *Lu. longipalpis* has ADA and lacks adenosine and AMP in the saliva.^{22,38} Unexpectedly, neither ADA nor maxadilan were identified in *Lu. ayacuchensis*. Thus, with those exceptions, in general salivary vasodilators of sandflies are considered to be adenosine and AMP in Phlebotomus complex, and maxadilan in Lutzomyia complex.

Linb-147 (JK846521) from *N. intermedia* showed a relatively low match to maxadilan, provided for only 34% identity and 70% similarity over a stretch of 50 amino acids. Moreover, this sequence is scarcely present in *N. intermedia* sialome, only one transcript was identified in the present cDNA library as compared to 30 transcripts of maxadilan present in *Lu. longipalpis* sialome.^{21,22}

JAV08642.1 present in *N. neivai* sialome is closely related to Maxadilan (**Figure 3A**), evidenced a match 2e-05 providing 47% of identity and 68% similarity over a stretch of 49 amino acids (**Figure 3B**). In contrast to the other two New Worlds sandflies' sialomes, *Lu. ayacuchensis* and *N. intermedia*, JAV08642.1 represent the closest relationship with this powerful vasodilator, suggesting that *N. neivai* may have preserved this pharmacological properties.⁴⁹ Furthermore, regarding *Lu. longipalpis* as vector of VL and *N. neivai* as vector of TL in the same endemic Brazilian regions,^{6,50} Maxadilan-like proteins could bring new insight in a common vaccine for VL and TL.

The novel families 8-kDa, 6-kDa and 5-kDa only described in *N. intermedia* sialome until now,²¹ they were now grouped next to *N. neivai* peptides; but their functions and properties remain unknown.

IV. ML domain peptide family

The MD-2-related lipid-recognition (ML) domain is implicated in lipid-mediated membrane binding mechanisms with a critical role in the execution and regulation of many cellular processes, including cell signaling and membrane trafficking.⁵¹ The ML domain from the SMART database also indicates proteins involved in innate immunity.⁵²

This family is relatively common in tick sialomes;⁵² but it had only been described in *N. intermedia* sialome so far and not another sandfly transcriptomes.²¹ *N. neivai* salivary peptides now

join these family proteins of *N. intermedia* in two main clusters (**Figure 4A**), expanding the initial 5 sequences reported (**Figure 4B**).

V. **Yellow protein family**

Yellow-related proteins are abundantly expressed in salivary glands of sandflies.^{13-16,19,21,33,34} Phlebotomine yellow-related proteins are characterized by the presence of major royal jelly protein domain (MRJP). Originally, MRJP proteins were described from honeybee larval jelly, making up to 90% of the protein content.⁵³ Sequences related to MRJP proteins were described in *Drosophila*, where it is related to cuticle pigmentation and, when mutated, it produced a yellow phenotype and thus named Yellow proteins.⁵⁴

In bloodsucking Diptera, salivary yellow-related proteins have only been described in sand flies (all sand fly species studied to date)^{13-16,19,21,33,34}, and some black flies.⁵⁵ Regarding the black fly of the gender *Simulium*, there are no descriptions of yellow-related proteins in the *Simulium vittatum* (Zetterstedt 1838) and *S. nigricornis* sialomes.^{24,56}

The proteins of this family are immunogenic and host antibody responses to this protein can be a potential marker for sandfly exposure in experimentally bitten mice and dogs, as well as naturally exposed dogs, humans, and foxes.⁵⁷ *Lu. longipalpis* proteins, LJM11, LJM111 and LJM17, act as high affinity binders of pro-inflammatory biogenic amines such as serotonin, catecholamines and histamine, suggesting that the proteins play a role for the reduction of inflammation during sand fly blood-feeding.⁵⁸

A combination of recombinant LJM17 (AAD32198.1) and LJM11 (AAS05318.1) successfully substituted *Lu. longipalpis* whole SGH in probing sera of individuals for vector exposure.²⁷ Yellow proteins are also under consideration for anti-Leishmania vector-based vaccines. LJM17 from *L.*

longipalpis elicited leishmanicidal Th1 cytokines in immunized dogs,^{59,60} and LJM11 protected laboratory animals against *L. (L.) infantum* (Nicolle 1908), *L. (L.) major* and *L. (V.) braziliensis*.^{58,61,62}

In contrast, mice immunized with *P. papatasi* yellow-related proteins PpSP42 or PpSP44 (AAL11052 and AAL11051, respectively) elicited Th2 cytokines and exacerbated *L. (L.) major* infection.⁶³ Other yellow-related proteins from *P. papatasi*, specifically PPTSP36 (AGE83101.1), induce a strong Th1 response constituting potential vaccine candidates against leishmaniasis.⁶⁴ It remains to be elucidated whether the protection induced by yellow-related proteins is related to particular protein immunogenicity, to sandfly species, or to the vector-Leishmania host combination, as all of these factors can contribute to vaccine efficacy. New approaches using novel vaccine techniques consisting in a single dose of plasmid followed by two doses of recombinant Canarypoxvirus expressing *Lu. longipalpis* yellow-related salivary proteins are a promised strategy to control Leishmania infection.⁶⁰

N. intermedia sialome exhibited two yellow-related proteins with significant alignments to LJM11 and LJM17, Linb-30 (AFP99277.1) and Linb-21 (AFP99235.1) respectively.²¹ LayS118 (BAM69185.1) from *Lu. ayacuchensis* salivary gland also evidenced match with LJM17 (identity 68%, similarity 81%) and LJM11 (identity 54%, similarity 72%). In the same way, *N. neivai* sialome positioned yellow-related protein in the New World sandflies cluster (**Figure 5A**); grouped next Linb-21 other two proteins, JAV07958.1 and JAV07968.1) closed related to LJM17 (61-62% identity and 78-79% similarity), but not as LayS118. Also highlights the proximity of JAV07960.1 and Linb-30 (identity 99% similarity 99%); however, Linb-30 has a more significant alignment to LJM11 from *Lu. longipalpis* (identity 79% and similarity 88% against 72% identity and 84% similarity when compared to JAV07960.1) (**Figure 5B**).

VI. *Apyrase family*

Apyrases are nucleoside triphosphate-diphosphohydrolases present in a variety of organisms. Apyrase appears to be a universal enzyme used to prevent blood coagulation by diverse hematophagous animals such as bloodsucking leeches, ticks, triatomine bugs, fleas, and mosquitoes. This enzyme hydrolyses both ATP and ADP to AMP, thus destroying an important physiologic stimulus of platelet aggregation released from damaged tissues and blood cells.⁶⁵

In the saliva of blood sucking arthropods, apyrases function to hydrolyze ADP in a Ca^{+2} -dependent manner and inhibit ADP-induced platelet aggregation to facilitate blood feeding.⁶⁵ The apyrase from sand flies belongs to the Cimex family of apyrases³³ and is very distinct from the 59 nucleotidase family of proteins found in mosquitoes.⁶⁶

Due the Apyrase property to induce a strong Th1 response, it has also been target of study as promising vaccine candidates against leishmaniasis.⁶⁴

Linb-45 (AFP99246.1) showed 66% identity with *L. longipalpis* salivary apyrase (AAD33513.1) and with *Lu. ayacuchensis* apyrase (BAM69098.1).

➤ ***Simulium nigrimanum*, a endemic blackfly related to pemphigus foliaceus in northeastern São Paulo state**

Historically, PF was distributed in rural areas and surrounding watersheds, which reinforces the hypothesis that insects bites might be associated to PF.^{8,25} Recently, the epidemiological link between *N. neivai* and *S. nigrimanum* and PF was confirmed in NSPS by spatial geographical distribution maps, where PF foci overlap with these insects distribution.⁸

In 2010, Ribeiro *et al.*²⁴ compared the peptide sequence of the *S. nigrimanum* sialotranscriptome with the peptide sequence of Dsg to identify some candidate proteins that could

induce anti-Dsg1 cross-reactivity: the *collagen-like* family. Although these authors found some similarity, no cross-reactivity with salivary peptide has been proven so far.

Focusing in NSPS PF endemic foci, PF patients and their relatives presented higher titres of IgG antibodies against SGH of both, *S. nigritanum* and *N. neivai*, but not against mosquito *Aedes aegypti* (Linnaeus, 1762) (used as endemic vector control) when compared to their healthy controls living in the same endemic area.⁸ Vernal *et al.*, also described a positive correlation between the antibodies against SGH from *S. nigritanum* and *N. neivai* in the three studied groups: PF patients, PF patients' relatives and healthy endemic controls.⁸ This correlation shows that exposure to the bites of both species is possible because they are similarly distributed in NSPS, but also similar antigen effects between both flies may be involved in PF etiopathogenesis. Although *S. nigritanum* and *N. neivai* are two species far away in evolution time line, they converge in Diptera family (Nematocera order).⁶⁷ Host's hemostasis represents a barrier to efficient vectors' blood feeding. Considering this feeding mode evolved independently in several insect orders and families, the salivary composition among insects is typical of a convergent evolutionary scenario due to the vertebrate host immune pressure over the salivary products.^{68,69} For example, *Simplagin* (ACZ28269) from *S. nigritanum*, and Aegyptin from mosquitos have low similarity at amino acid level; notwithstanding, they retained the same biological function: platelet aggregation inhibition.⁷⁰ Thus, despite no significant alignment between *S. nigritanum* and *N. neivai* salivary proteins were found, similar pharmacological properties and/or conformation mimotopes and/or antigenic effects may not be discharge as responsible for the immune-tolerance loss to Dsg1 in genetically susceptible individuals. Further studies are necessary to assess this hypothesis.

➤ ***Nyssomyia neivai*, flies and Desmoglein-1**

Since 2001,⁹ sandflies' salivary proteins have been related to endemic PF etiopathogenesis. Firstly reported in Brazilian endemic PF foci in NSPS, and later also described in Tunisia,⁷¹ the hypothesis that exposure to flies' bites may induce an autoimmunity trigger to IgG pathogenic autoantibodies against Dsg1 in genetically susceptible individuals is gained value in the last years. Self-antigen reaction could be explained by salivary peptides introduced into the host's skin while acquiring a blood meal triggering an antigen cross-reaction with Dsg1. Thus, some sandflies proteins have been proposed as the trigger: Maxadilan, LJM11 and recently LJM17, all three described in *Lu. longipalpis* sialome. Nevertheless, this hypothesis does not explain PF endemicity in the Old World and even in Brazilian regions where *Lu. longipalpis* is not widely distributed.

The similarity of some *N. neivai* salivary proteins to Maxadilan, represented by JAV08642.1, and/or to LJM11, represented by JAV07960.1, may reinforce this hypothesis; however, sera from individuals exposed to *N. intermedia* bites did not recognize the yellow proteins from *Lu. longipalpis*, and this lack of recognition maybe due to some of the differences observed in the amino acid sequence of these two proteins.²¹ Thus, it would be expected that *N. neivai*'s salivary proteins might also have this lack of recognition.

Not a single salivary protein of *N. neivai*, neither Maxadilan nor LJM11 from *Lu. longipalpis* have a linear sequence match by BLAST with Dsg1 peptide (AAC83817.1). Interestingly, an anticoagulant protein of *Lu. longipalpis* saliva (AAD33512.1) has a 73% identity and 72% similarity on 11 amino acids (e6.3) when compared Dsg1 peptide from *Homo sapiens*. Further studies are required to assess the characteristic of this match.

Regarding that IgG antibodies against the salivary proteins extract from *N. neivai* positive correlated with IgG anti-Dsg1 in PF patients,⁸ we may expect that not a single peptide is acting as an independent antigen, and probably more than one protein is involved together in PF etiopathogenesis as reported Zraa *et al.* in Tunisia, where endemic PF patients recognizes 12, 15, 30, 21 and 36 kDa

proteins from the SGH of *P. papatasi*.⁷¹ Pharmacological properties and conformational mimotopes of these proteins are necessary to better understand.

CONCLUSION

Leishmaniasis is still a frequent and neglected disease in Brazil. Our results increase valuable data related to New World Phlebotominae salivary proteins, complementing the findings reported in *Lu. longipalpis* and *N. intermedia* sialomes. The complete knowledge of these three main species of sandflies widely distributed in Brazil will bring new insights into the host-vector-parasite relationship of *L. (L.) infantum* and *L. (V.) braziliensis* infections and exposed interested targets looking for a vector-based vaccine.

Despite not matches between *N. neivai* and Dsg1 were found, the salivary proteins sequences deposited in the *GenBank*, will serve as reference to a possible antigenic peptide candidate to be the trigger of anti-Dsg1 autoantibodies production in Brazilian endemic pemphigus.

TABLES

Table 1. Classification of transcripts originating from the sialotranscriptome of *Nyssomyia neivai*

Class	# of contigs	% of contigs	RPKM	% RPKM
Secreted	494	41.2	1,203,951	92.4
Housekeeping	399	33.3	42,119	3.2
Unknown	295	24.6	56,243	4.3
Viral products	3	0.3	31	0.0
Transposable elements	9	0.8	52	0.0
Total	1,200	100	1,302,396	100

Table 2. Classification of secreted proteins originating from the sialotranscriptome of *Nyssomyia neivai*

Putative Secreted proteins		# of contigs	% of contigs	RPKM	% RPKM
Enzymes	Proteases	53	10.7	1186	0.1
	Endonuclease	4	0.8	4927	0.4
	Hyaluronidase	7	1.4	6887	0.6
	Lipases*	8	1.6	53	0.0
	Alpha amylase*	1	0.2	2	0.0
	Apyrase	3	0.6	18214	1.5
	5'nucleotidase	4	0.8	7537	0.6
	Adenosine deaminase	2	0.4	2923	0.2
Other ubiquitous protein families	Protease inhibitors	7	1.4	48	0.0
	Antigen 5 protein	6	1.2	47405	3.9
	Antimicrobial peptides	8	1.6	781	0.1
Small molecule binding proteins	JH/PB/OBP	36	7.3	1393	0.1
	Hormones	3	0.6	29	0.0
	C type lectins	20	4.0	190860	15.9
	Galectin	1	0.2	3	0.0
	ML domain salivary protein	13	2.6	69404	5.8
	Lipocalins	3	0.6	147	0.0
	Yellow protein family	13	2.6	61755	5.1
	Mucins	22	4.5	13174	1.1
	Diptera conserved salivary secreted peptide	59	11.9	3523	0.3
Hematophagous Nematocera specific families	D7 family	4	0.8	26832	2.2
Sand fly specific families	Maxadilan	16	3.2	187866	15.6
	56.6 kDa salivary protein	3	0.6	1029	0.1
	Gly rich salivary protein	6	1.2	808	0.1
	Toxin-like peptide	12	2.4	24330	2.0
	SP13-15 protein family	18	3.6	414587	34.4
	10 kDa family	3	0.6	40594	3.4
	14.2\ kDa family	2	0.4	8955	0.7
	15.5 kDa family	1	0.2	13	0.0
	30 kDa family	2	0.4	16470	1.4
	32.4 salivary protein - Lufaxin	2	0.4	7913	0.7
	34 kDa family	1	0.2	6850	0.6
	38.8 kDa family	1	0.2	1958	0.2
	Nyssomyia specific	1	0.2	3602	0.3
	Hypothetical secreted protein	149	30.2	31893	2.6
TOTAL		494	100.0	1203951	

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Figures legends

Figure 1. A – Tree of SP13-15 protein family of *Nyssomyia neivai*. **B** – Multiple alignments of SP13-15 from *Nyssomyia neivai* with *Nyssomyia intermedia* and *Lutzomyia longipalpis* SP15 proteins

JAV08233.1	1	-----VLVTIFLLVIQSR AEHP EKKCIQELGR TQESCITHCQYQHYGFTDENYRITKKHMEKFRDVLIVYKSVPLSDKS	74
JAV08232.1	1	-----VLVTIFLLVIQSR AEHP EKKCIAELGR TQESCITHCQYQHYGFTDENYRITKKHMEKFRDVLIEYKSVPLSDKS	74
AFP99232.1	1	MKYL CFVFI TIFFLVQHCHAGHP EKKCIQELGR TQESCITHCQYQHYGFTDENYRITKKHMEKFRDVLIEYKSVPLSDKS	80
JAV08231.1	1	-----VLVTIFLLVIQSR AGHP EKKCIAELGR TQESCITHCQYQHYGFTDENYRITKKHMEKFRDVLIEYKSVPLSDKS	74
JAV08229.1	1	-----ELGKTQSSCILHCEYNHYGFTDENYRITKKHMEKFRDVLIEYRSVPLSDKS	51
AAD32197.1	1	MNHL CFII IALFFLVQQSLAEHP EKKCIRELAR TDENCILHCTYSYGFVDKNFRIAKKHVQKFKKILVTFDVAPKKEKK	80
AFP99239.1	1	MKYLCCILISIFLLVNHTRGESPV RKCVR EKARTQLICMTQCKYNYGFTDEDSNITEKH MENFRDVLVKYGAVPSSDQA	80
JAV08239.1	1	MKYLCCILISIFLLVNHTRGESPV RKCVR EKARTQLICMTQCKYNYGFTDEDSNITEKH MENFRDVLVKYGAVSSDQA	80
JAV08235.1	1	MKYSVFVLAIFYLIKRSRANHPQQYCIDLAET EESC IQHCRFSYGF TNDKFQITKKHIEKFRDILLEFNAVPSKKN	80
JAV08230.1	1	-----VRKCVREKARTQLICMTQCKYNYGFTDEDSNITEKH MENFRDVLIKYGAVPSSDQT	57
JAV08233.1	75	KLFGHIRACGDKVNAKKPKSTEDKCMKIIEYYRCVVDGKLLSWNRYANAVIQYDKTINV	133
JAV08232.1	75	KIFGHIRACGDKVNAKKPKSTEDKCMKIIEYYRCVVDGKLLSWNRYANAVIQYDKTINV	133
AFP99232.1	81	KLFGHIRACGDKVNAKKPKSTQDKCMKIIEYYRCVVDGKLLTWNRYANAMIQYDKTINV	139
JAV08231.1	75	KLFGHIRAWGDKVNSKKPKSTQDKCMKIIEYYRCVVDGKFLTWNRYANAVIQYDK-VNS[26]	158
JAV08229.1	52	KLFGHIRACGDRANAKKPKSTEDKCMKIIEYYRCVVDGKLLSWNRYANAMIQYDKTINV	110
AAD32197.1	81	KLLEHIEACADSANADQPQTKDEKCTKINKYYRCVVDGKILPWNSYADAIKFDKTLNV	139
AFP99239.1	81	KIFDHIKACGQANAKNPQNT EEKCKLTKYYKCIVDNKTLTFSKYVQAVIKHDKTLNV	139
JAV08239.1	81	KIFDHIKACGQANAKNPQST EEKCKLTKYYKCIVDNKTLTFSKYVHAVIKHDKTLNV	139
JAV08235.1	81	QLFNHIKKCADKVNLSKSKDKSEKCMKILTYSRCVANGKTVSEHNVYTAILAHDKRINV	139
JAV08230.1	58	KIFDHIKACGQANAKNPQNT EEKCKLTKYYKCIVDNKTLTFSKYVQAVIKHDKTLNV	116

Figure 2. A – Tree of C-type lectin protein family of *Nyssomyia neivai*. **B** – Multiple alignments of C-type lectin from *Nyssomyia neivai* with *Nyssomyia intermedia* and *Lutzomyia longipalpis* C-type lectin proteins

AFP99236.1	1	MVKLFLT--FFVVSFL-TMCTAQNIVVRKLpSGKTIYISKTYMSWYEGLDfCNRKGMslVSIANDQENRQLSNVLQKilP	77
JAV08581.1	1	MLFRFVT--VLFLSYI-SFCQGELNAEYEL-TGKSIYISKIKKNWFDSDYCKNNGYELATIENEQEDSEIINALNK--E	74
JAV08563.1	1	MGFRIIA--AFLLCYI-AVCQADFQKKQIL-SGKTIVFVSKMKKNWFDADYCLNKGYTLATVKSAKENGELTNVLKT--M	74
JAV08584.1	1	MIVRFA--iILVLTYL-SFAHADFNPEHKL-TGKTILVSKIRKNWFDALDYCKNKGMTLATVGRGRRENRELTNALRN--S	74
JAV08565.1	1	NVFKEELkiILFLTYL-SFAHADFNPEHKL-TGKTILVSKIRKNWFDALDYCKNKGMTLATVGRGRRENRELTNALRN--S	76
JAV08561.1	1	MGFRIIA--AFLLCYI-ALCQADFQKKQNL-SGKTIVFVSKMKKNWFDADYCLNKGYTLATVKSAKENGELTNVLKT--M	74
JAV08591.1	1	MGR LIVTfILFILIPFT-QKCRGIDTILYRV-ADKILYMGRSQLDWISAVDECQRLNMV LASATSVEDN----NALTQflR	74
JAV08562.1	1	NVFKEELkiILFLTYL-SFAHADFNPEHKL-TGKTILVSKIRKNWFDALDYCKNKGMTLATVGRGRRENRELTNALRN--S	76
JAV08559.1	1	MTIRLLT--LLFLVYIfNLSYGNLNTGEHV-TGKTIYVSKIKKSWKDAQDYCSKNGYNLATIKSKKEQYEILIIITKw--	75
AFP99236.1	78	EHEVHCWIGGYKfQdeNgQNSMRWINGGRKV[1]----YTNFAGGEPNNDK--EYCLEMYRQY-RGDTARWNDRYCTDR	148
JAV08581.1	75	KPDSQIWIIGGFRHP--N-EHPFRWVINFKKI DSTVYTNWQPKQPNYSKNEELCLEYWNYPA-KSNIFKWNDRKCALE	147
JAV08563.1	75	PVATHTWIGGIRHP--Q-ENNFRWEDTQKQI DSTVYTNWEQGEPNNGRGVEFCIEYWNDVT-KKIEWKWNNDCKKE	147
JAV08584.1	75	PAATHHWIGGIRHP--N-ENFYRWIHNNNKI ETTGYTNWQGEPNFGRGIELCMEYWNDPK-KKIEWKWNNDCKQE	147
JAV08565.1	77	PVATHHWIGGIRHP--N-ENFYRWIHNNNKI ETTGYTNWQGEPNFGRGIELCMEYWNDPT-KKIEWKWNNDNCRLE	149
JAV08561.1	75	PVATHTWIGGIRHP--Q-ENNFRWEDTQKQI DSTVYTNWEQGEPNNGRGVEFCIEYWNDAT-KKIEWKWNNDCKKR	147
JAV08591.1	75	PRALMAWIGGYNYQedG-Q--WRWIEDGQLF N---YTNWSPGQPTTNNSTKNCLQLL-----RVSSGKWSVDSCVSK	140
JAV08562.1	77	PVATHHWIGGIRHL--N-ENFWRWIHNNNKI ETTGYTNWQGEPNFGRGIELCMEYWNDPTxxxEWKWNNDNDCRL-	149
JAV08559.1	76	TMAQHIWVGGYKNL--N-GDHLIWINDGKPI[4]TQTTFSNWASGKPNNSNKDEMCMELDfSKafHSP-----	141
AFP99236.1	149	HPFVCEKKC	157
JAV08581.1	148	QIFICENRN[28]	184
JAV08563.1	148	QIFVCEKRE	156
JAV08584.1	148	QIFVCEKRD	156
JAV08565.1	150	QVFVCEKRD	158
JAV08561.1	148	TDF-CV---	152
JAV08591.1	141	KKFVCELVK[2]	151
JAV08562.1		-----	
JAV08559.1		-----	

Figure 3. A – Tree of Maxadilan-simile protein family of *Nyssomyia neivai*. **B** – Multiple alignments of Maxadilan-simile from *Nyssomyia neivai* with *Nyssomyia intermedia* and *Lutzomyia longipalpis* Maxadilan-like proteins

AAA29288.1	1	MKQILLISLVVVLAVFAFNVA[9]	RKAIDDCQKQAHH	SNVLQTSVQTTATFTSMDTSQLPGNsVFKECMKQKKKE[5]	86
JAV08462.1	1	MKNFILLLLAFALCIAYSNA[7]	ATILRECSQEANS	KNIHQIQVQTTATFTSMTTDQLPGH--FKKCVTERWKG[2]	79
JAV08466.1	1	MKKFVLVFLAVALILCNEVE	AFSLPDCIKDENK	KMITQRQVHSTSTFSKLTEDDLAKK-VLANCLSKLASG	71
JAV08468.1	1	MKKFVLVFLAALILCNEIE	AFSLTDCIKEENK	KMIKKRQVHSTSTFSKLTEDELAKE-ALANCLNKLASG	71
JAV08471.1	1	MKKFVLVFLAVALILCNEIE	AFSLRDCINEENK	KMMEKRQVHSTSTFSKLTEDELSKK-ALAICLNRLGSG	71
AFP99237.1	1	MKKFVLVFLAVALILCNEVE	AFSLRDCVNDENK	KMIAQRQAHSTSTFSKLTEDELSKK-ALAICLNRLGSG	71
JAV08472.1	1	MKKFVLVFLAVALILCNEIE	AFSLRDCINEENK	KMMEKRQVHSTSTFSKLTEDDLSKQ-ALAICLNRLGSG	71
JAV08467.1	1	MKKFVLVFLAVALILCNEVE	AFSLRDCINDENK	KMMAQRQVHSTSTFSKLTEDDLSKQ-VLAICLSRLGSG	71
AFP99245.1	1	MRIGLILLFAAVLVFCDALP[5]	TQIGQPC EHNIVK[7]	KSKAKDAIAGLFTKAKSALKDVLKK-AKDKVKEKVAQH[5]	88

Figure 4. A – Tree of ML-domain protein family of *Nyssomyia neivai*. **B** – Multiple alignments of ML-domain from *Nyssomyia neivai* with *Nyssomyia intermedia* and *Lutzomyia longipalpis* ML-domain proteins

AFP99241.1	1	MIRYILLIAFFAISAFGQ-E---YKLYKCG--DIPLPNKLKIDQC---PRNKP--CSNIIDGQDA--VISGVLHFNIKH-	66
JAV08582.1	1	MIRYILLIAFFAISAFGQ-E---YKLFKCG--DIPLPYKLKIDQC---PRNKP--CSNIIDGQDA--VISGVLHFNIKH-	66
JAV08585.1	1	MILYIFVITLLPL-AFAS-EDDKYFLRSC-E--GVKMPSGFSINQC---PKDKDYICSGILKGTTA--VIDVNLKFKIDK-	70
AFP99248.1	1	MILYIFVITLLPL-AFAS-EDDKYFLRSC-E--GVKMPSGFSINQC---PKDKDYICSGILKGTTA--VIDVNLKFKIDK-	70
JAV08586.1	1	MILYIFVITLLPL-AFAS-EDDKYFLRSC-E--GVKMPSGFSINQC---PKDKDYICSGILKGTTA--VIDVNLKFKIDK-	70
AFP99259.1	1	MIRNFILLIFIPTFTFGFWYPDTEYAKSCFqpGIAIPMELIVNGC-----TSKENC-RIEGKDA--VLNINLEYKLRQ-	71
JAV08580.1	1	MIRYFLIALIPATVFATLWYRPFVWVNCK--GVNGPLDISVDEC-----PPYNICYRIWKSDTKsARVHVNFKFSLDR-	72
JAV08568.1	1	MIRYFLVLTLLPLT-FAGFWYRMWWEYDCP--GYKRPIDYSLDEC-----KPYKQCSGIARQGDI-AIINVNYTTINVENg	71
AFP99264.1	1	MIRYFLIALIPATVFATLWYRPFVWVNCK--GVNGPLDISVDEC-----PPYNICYRIWKSDTKsARVHVNFKFSLDR-	72
JAV08567.1	1	MIRYFLVLTLLPLT-FAGFWYRMWWEYDCP--GYKRPIDYSLDEC-----KPYKQCSGIARQGDI-AIINVNYTTINVENg	71
JAV08554.1	1	MIRYFLIALIPATVFATLWYRPFVWVNCK--GVNGPLDISVDEC-----PPYNICYRIWKSDTKsARVHVNFKFSLDR-	72
JAV08576.1	1	-FRYFFLICLVPQVFGAWWWHDMGTTNCK--SIDSPLTVTVDKcgetASSTAIRCNNIFDSNGM-AKMKISFRFKIDH-	75
JAV08588.1	1	MFRYFFLICLVPQVFGAWWWHDMGTTNCK--SIDSPLTVTVDKcgetASSTAIRCNNIFDSNGM-AKMKISFRFKIDH-	76
AFP99241.1	67	KTGILPVTANVQR[1]--NGQSETITIAgDAC--NLLTK-SR-CLIQPLGHEVKLPLRVKN-VKKGEKLTFSITIRS-A	136
JAV08582.1	67	KTGILPVTANVQR[1]--NGQSETITIAgDAC--NLLTK-SR-CLIQPLGHEVKLPLRVKN-VNKGEKLTFSITIRS-A	136
JAV08585.1	71	ELTKLPISADLIR--NGKSEVIPLPH-NAC--KGFYK-EK-CPLKAGTHEITLPLLIKD-VKKGERLHVGVGIGDYG	139
AFP99248.1	71	ELTKLPISADLIR--NGKSEVIPLPH-NAC--KGFYK-EK-CPLKAGTHEITLPLLIKD-VKKGERLHVGVGIGDYG	139
JAV08586.1	71	ELIKLPISADLIR--NGKSEVIPLPH-NAC--KGFYK-EK-CPLKAGTHEITLPLLIKD-VKKGERLHVGVGIGDYG	139
AFP99259.1	72	DVGKLIKISAHHS--NGKETEILHDG---CdGKGPVKIAW-CPSKYGTyKLPPQLRVKN-VKKGENLTVYLRIRDKN	141
JAV08580.1	73	EYTKLPISTDYR YSDGKVQLLPET-DAC----GTFIK--CPLKPGTHTIKVPVTIRDkVEWGEFLYVGLHINGTS	142
JAV08568.1	72	PFTKLPISAKLMR YPSGKIETILPPH-DACHKKNYNLN--CPLQNGKHSCLKLRMQK-VKHGEFIKVGLHINNPS	144
AFP99264.1	73	EYTKLPISTDYR YSDGKVIQLPET-DAC----GTFIK--CPLKPGTHTIKVPVTIRDkVEWGEFVYVGLHINGTS	142
JAV08567.1	72	PFTKLPISAKLMR YPSGKIETILPPH-DACHKKNYNLN--CPLQNGKHSCLKLRMQK-VKPGEFIKVGLHINNPS	144
JAV08554.1	73	EYTKLPISTDYR YSDGKVIQLPET-DAC----GTFIK--CPLKPGTHTIKVPVTIRD-----	124
JAV08576.1	76	EFKNLPVLANYET[4]TPAYRTKLNIPE--DACqnREFYNLKEsCPLKPGIYTIHFPLEIPGrYS-NEWLQVGLGVFDET	153
JAV08588.1	77	EFKNLPVLANYET[4]TPAYRTKLNIPE--DACqnREFYNLKEsCPLKPGIYTIHFPLEIPGrYS-NEWLQVGLGVFDET	154

Figure 5. A – Tree of Yellow-related protein family of *Nyssomyia neivai*. **B** – Multiple alignments of Yellow-related protein from *Nyssomyia neivai* with *Nyssomyia intermedia* and *Lutzomyia longipalpis* Yellow-related protein

AAS05318.1	228	DYGGKEYEFKAGIFGITLGDRDSEGNRPAYYLAGSAIKVYSVNTKELKQKGGKLNPELLGNRGKYNDALAYDPKTKVI	307
JAV07960.1	228	NHNDKEYTYNAGIFGITLGDRDSNGNRPAYYLAGSSIKVYSVNTKELKARGTTLPQLLGNRGKLSDAIALAYDPKTKVI	307
ABB00904.1	228	DHDGQQYEYAGIFGITLGERDNEGNRQAYYLVASSTKLHSINTKELKQKGSKNANYLGD RGESTDAIGLVYDPKTKTI	307
BAM69185.1	229	HLREQA--TYKVGLFGITLGDRDKQGNRPAYYIAGSSTKVYSVNTKELKTGGSLNP TLHGDRGPHTDAVALAYDPGHKVI	307
JAV07968.1	229	HFNNQSDKYKVGLFGITLGDRDNAGNRPAYYIAGSSTKVYSVNTKQLKTGTTLPKLHGDRGKHTDAIALAYDSVHKVI	308
AFP99235.1	229	HFNNQSDKYKVGLFGITLGDRDNAGNRPAYYIAGSSTKVYSVNTKQLKTGTTLPKLHGDRGKHTDAIALAYDSVHKVI	308
AAD32198.1	232	YSGEQMKYKVGLFGIALGDRDEMGRPACIYAGSSTKVYSVNTKELKTENGQLNPQLHGDRGKYTDAIALAYDPEHKVL	311
JAV07958.1	214	HFNNQSDKYKVGLFGITLGDRDNAGNRPAYYIAGSSTKVYSVNTKQLKTGTTLPKLHGDRGKHTDAIALAYDSVHKVI	293
AFP99277.1	16	YT--KXYTYNAGIFGITLGDRDSNGNRPAYYLAGSSIKVYSVNTKELKARGTTLPQLLGNRGKLSDAIALAYDPKTKVI	93
AAS05318.1	308	FFAEANTQVSCWNTqKMPLRMKNTDVVYTSSRFVFGTDISVDSKGGWLFMSNGFPPI---RKSEKFKYDFPRYRLMRI	384
JAV07960.1	308	FFAEANTQVSCWNTqKMPLRMANTDVIYTSRHFIFGTDISVDSNSTLWFMSNGFPPI---RNAHKFKYEDPRYRLFVK-	383
ABB00904.1	308	FFVESNSKRVSCWNT-QETLNKDKIDVIYHNADFSFGTDISIDSQDNLWFLANGLPPL---ENSDKFVFTKPRYQIFKV-	382
BAM69185.1	308	FFAESDTRQVSCWHV-QTELKPENTDVIYSYARFIFGTDISVDKKGTLWFMSNGYPPI---KDAEKLKFYDRKIRLMRV-	382
JAV07968.1	309	FFAESDTRQVSCWHV-DMELKPENTDVIYSYARFIFGTDISIDSENYLWFMSNGYPPI---NDAEKLKFDNRKIRLMRV-	383
AFP99235.1	309	FFAESDTRQVSCWHV-DMELKPENTDVIYSYARFIFGTDISIDSENYLWFLSNGYPPI---NDAEKLKFDNRKIRLMRV-	383
AAD32198.1	312	YFAESDSRQVSCWNV-NMELKPDNTDVIFSSARFTFGTDILVDSKGMWIMANGHPPVedqEKIWKMRFVNRKIRIMKV-	389
JAV07958.1	294	FFAESDTRQVSCWHV-DMELKPENTDVIYSYARFIFGTDISIDSENYLWFMSNGYPPI---NDAEKLKFDNRKIRLMRV-	368
AFP99277.1	94	FFAEANTQVSCWNTqKMPLRMANTDVIYTSRHFIFGTDISVDSNSTLWFMSNGFPPI---RNAHKFKYEDPRYRLFVK-	169
AAS05318.1	385	DTQEAIAGTACDMNA-----	399
JAV07960.1	384	DTRAAIAGTNCEIKS-----	398
ABB00904.1	383	NIQEAIAGTKCEKNL-----	397
BAM69185.1	383	NTYNVLPYSKCNPDKGPQGIPV	405
JAV07968.1	384	ATERVVKYAKCNPYKAPPSIPV	406
AFP99235.1	384	ATERVVKYAKCNPYKAPPSIPV	406
AAD32198.1	390	DTERVFKYSRCNPYKPPKEIEV	412
JAV07958.1	369	ATERVVKYAKCNPYKAPPSIPV	391
AFP99277.1	170	DTRAAIAGTNCEIKS-----	184

Conclusão

7. CONCLUSÃO

Brasil representa um cenário perfeito para a tríade das doenças autoimunes: genética, inflamação e meio ambiente. O perfil genético (*HLA* de classe I e II) foi bem identificado na população do sudeste brasileiro, exibindo alelos de suscetibilidade específicos para o desenvolvimento do PF. Nesta mesma população, através dos resultados da presente tese, confirmamos a relação epidemiológica (fator de risco/doença = inseto/PF) entre os aglomerados de PF e a distribuição geográfica das principais espécies de insetos antropofílicos. Da mesma forma, também confirmamos que os pacientes com PF do sudeste brasileiro foram expostos as picadas desses insetos, desenvolvendo anticorpos da classe IgG contra o extrato proteico salivar total, contra as proteínas salivares mais imunogênicas desses extratos e o reconhecimento de mais de uma fração proteica desses extratos salivares. Paralelamente também observamos a resposta de hipersensibilidade tipo 1 mediada por anticorpos da classe IgE contra os antígenos salivares supracitados. De maneira complementar, analisamos o sialoma de uma das principais espécies de flebotomíneos relacionadas com o PF. De maneira global, considerando o estado da arte e nossos resultados, finalizamos essa tese com uma nova proposta para explicar a endemicidade do PF incluindo a participação das picadas desses insetos hematófagos: *S. nigrimanum* e *N. neivai*.

7.1 PROSPECTIVAS

Nossa proposta abre portas para novas pesquisas, expandindo os horizontes no entendimento do PF endêmico, focando em uma concepção inédita e inovadora que servirá de inspiração para novos estudos com o fim de confirmar ou excluir nossa hipótese.

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ANEXO 1 - Protocolo de extração de glândulas salivares de *Aedes aegypti* do Instituto de Imunologia, Universidade de São Paulo. Gentilmente fornecido pelo Prof. Dr. Anderson Sá-Nunes.

PROTOCOLO DE EXTRAÇÃO DE GLÂNDULAS SALIVARES

Materiais utilizados

- Isopor com gelo.
- Placa de petri com papel filtro para colocar os mosquitos.
- Placa de petri para colocar álcool 70%.
- Duas pinças entomológicas.
- Estilete.
- Lâmina de vidro 5x5 cm.
- Pipeta Pasteur.
- Eppendorf com 100µL de PBS, Tubo Falcon de 15 mL com PBS.
- PBS 1X.

Preparo para extração da glândula salivar do mosquito *Aedes aegypti*

1. Colocar os mosquitos na geladeira, pois a baixa temperatura faz com que eles fiquem “adormecidos” e permite que os mesmos sejam manipulados para posterior extração da glândula salivar.
2. Enquanto isso, ligar o Estereomicroscópio conforme orientação do POP 00008 (Protocolo de uso da Lupa);
3. No isopor com gelo colocar uma placa de petri com um papel filtro dentro, um tubo Falcon contendo PBS 1X para coleta junto com uma pipeta Pauster de plástico e um eppendorf contendo 100µL de PBS;
4. Na bancada deixar duas pinças, o estilete, a placa de petri com álcool 70% (para submergir os mosquitos antes da coleta), uma folha de papel toalha umedecida com água destilada (para descartar os mosquitos após a coleta e realizar a contagem dos mesmos);
5. Retirar os mosquitos da geladeira e colocá-los na placa de petri dentro do isopor com gelo.

Procedimentos para extração

- I. Posicionar uma lâmina no centro da lupa e pingar uma gota de PBS;
- II. Com uma pinça, retirar um mosquito da placa de petri e passar no álcool 70%; com a outra pinça retirar as patas;
- III. Deitar o mosquito sobre a lâmina (dentro da gota de PBS), acertar o foco da imagem e retirar a cabeça do mosquito com uma pinça.
- IV. Deixar essa pinça de lado e segurar o estilete num ângulo de mais ou menos 20° em relação ao mosquito e pressioná-lo devagar sobre seu tórax.
- V. Assim que localizar a glândula, distanciá-la do mosquito utilizando a ponta do estilete.
- VI. Retirar o mosquito da lâmina e deixá-lo sobre o papel umedecido para posterior contagem.
- VII. Com a ajuda do estilete, coletar a glândula que ficou na lâmina e colocá-la dentro do eppendorf contendo PBS.
- VIII. Armazenar no freezer -80°C.

ANEXO 2 - Protocolo de extração proteica de epiderme do *Institute of Cutaneous Cell Biology, Kurume University*. Gentilmente fornecido pelo Prof. Dr. Takashi Hashimoto.

EPIDERMAL PROTEIN EXTRACTION

Preparation of epidermal and dermal extract of EDTA separated normal human skin

- **EDTA separation**

* All experiments should be done on the plastic dish cooled on ice.

1. Human foreskins keep at 4°C until used (use in the same day). Prepuce skin (3 pieces ~5x5cm).
2. Remove dermis as much as possible.
3. Wash foreskin with cold PBS.
4. Add the strips in 500ml of PBS containing 2mM EDTA and 1mM PMSF (500mM EDTA = 2ml + 100mM PMSF = 5ml).
5. Keep at 4°C for 24h.
6. Change into a new buffer (see step 4) and keep at 4°C for another 24h.
7. Remove epidermis and collect it.

- **Preparation of epidermal extracts.**

1. Extraction buffer:

✓ 0.01M Tris-HCl (pH 6.8)	1M Tris-HCl	50uL
✓ 1.5% SDS	20% SDS	375uL
✓ 5% 2-mercaptoethanol		250uL
✓ H ₂ O		4105uL
+		
✓ 2mM PMSF	100mM PMSF	100uL
✓ 2mM EDTA	500mM EDTA	20uL
✓ 4 proteinase inhibitor		100uL
i. Antipain/Leupeptin 100X concentrated in H ₂ O.		
ii. Chymotrypsin/Pepstatin 100X in DMSO		

* Also can be used commercial proteinase inhibitor pool (100uL).

2. Add epidermis into extraction buffer (about 2 mL for 1 foreskin). Homogenize completely by homogenizer on ice for 30m.
 3. Boll for 5 min.
 4. Centrifuged at 14,000rpm for 30m
- Aliquots (50uL) and keep at -80°C.

ANEXO 3 – Parecer do Comitê de Ética em Pesquisa da Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Mimetismo molecular de peptídeos salivares de insetos hematófagos com peptídeos epidérmicos na patogênese dos pênfigos

Pesquisador: Sebastian Vernal

Área Temática:

Versão: 2

CAAE: 35829314.0.0000.5440

Instituição Proponente: Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da USP -

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 866.027

Data da Relatoria: 30/10/2014

Apresentação do Projeto:

Trata-se de projeto de pesquisa, nível Mestrado, a ser desenvolvido por Sebastian Vernal mestrando no programa da Clínica Médica da FMRP - USP, sobre orientação da Profa. Dra. Ana Maria Roselino.