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What's behind a sand fly bite? The profound effect of sand fly saliva on host hemostasis, inflammation and immunity

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Abstract

Sand flies are blood-feeding insects and vectors of the *Leishmania* parasite. For many years, saliva of these insects has represented a gold mine for the discovery of molecules with anti-hemostatic and immuno-modulatory activities. Furthermore, proteins in sand fly saliva have been shown to be a potential vaccine against leishmaniasis and also markers of vector exposure. A bottleneck to progress in these areas of research has been the identification of molecules responsible for the observed activities and properties of saliva. Over the past decade, rapid advances in transcriptomics and proteomics resulted in the completion of a number of sialomes (salivary gland transcriptomes) and the expression of several recombinant salivary proteins from different species of sand fly vectors. This review will provide readers with a comprehensive update of recent advances in the characterization of these salivary molecules and their biological activities and offer insights pertaining to their protective effect against leishmaniasis and their potential as markers of vector exposure.

Keywords

Sand flies; saliva; salivary protein; hemostasis; inflammation; immunity; leishmaniasis; vaccine; markers of exposure; auto-immune disease; transcriptomes; salivary gland

1. Introduction

Phlebotomine sand flies are blood feeders and vectors of *Leishmania* parasites, causative agents of the neglected disease leishmaniasis. Sand flies are found in tropical and temperate regions of the world where the majority of medically important vector species belong to the genus *Phlebotomus* in the Old World and to the genus *Lutzomyia* in the New World.

During the process of getting a blood meal, sand flies damage the skin of the host with their proboscis and provoke an immediate response by the host's hemostatic system to prevent blood loss. Sand flies have developed creative ways to circumvent this problem by

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producing pharmacologically active components in their saliva that can counteract the host's hemostatic system (Charlab et al., 1999; Ribeiro and Francischetti, 2003). Over two decades various groups have progressively investigated several aspects of sand fly saliva, exploring its composition and the biological activity of its salivary proteins and their potential use in biomedicine. In this review we will provide an account of the progress made in these different disciplines over the past decade.

2. Saliva in hemostasis, inflammation and immunity

The saliva of sand flies is composed of a limited number of secreted proteins (**Table 1)**, and in some cases nucleosides and nucleic acids (Ribeiro et al., 1999; Ribeiro and Modi, 2001). Approximately 20 to 40 abundant secreted proteins are visible on a SDS-PAGE gel (Anderson et al., 2006; Valenzuela et al., 2001a; Valenzuela et al., 2004), no study has yet confirmed the presence of secreted lipids or other components in the saliva of these insects (Charlab et al., 1999). Most salivary proteins are soluble and only a few nanograms or femtograms of each molecule are injected into the skin of the host. Despite the low complexity of sand fly saliva, the molecules it contains are potent and exert a profound effect on the host physiology at very low concentrations altering various important biological systems of the host as described below.

2.1. Hemostasis

One of the primary functions attributed to sand fly saliva (and to saliva of other blood-feeders) is to counteract host hemostasis by inhibiting the blood coagulation cascade, platelet aggregation and vasoconstriction (Ribeiro and Francischetti, 2003). Thus far, saliva of sand flies was shown to have a vasodilator (Ribeiro et al., 1999; Ribeiro and Modi, 2001; Ribeiro et al., 1989), an apyrase that works as an inhibitor of platelet aggregation by destroying the agonist adenosine diphosphate (APD) (Charlab et al., 1999; Hamasaki et al., 2009; Valenzuela et al., 2001b), an inhibitor of the blood coagulation cascade (Charlab et al., 1999) and an inhibitor of the classical pathway of the complement cascade (Cavalcante et al., 2003). These biological activities counteract the redundant and efficient hemostatic system consequently facilitating the acquisition of a blood meal by sand flies.

2.2. Immunomodulation

Though sand flies do not feed for a long time on their host, they depend mostly on the salivary anti-hemostatic components to acquire a blood meal. However, a number of immuno-modulatory activities have been reported from the saliva of sand flies (Andrade et al., 2007; Prates et al., 2012; Rohousova and Volf, 2006). The exact role of these immunomodulatory components in blood feeding still needs to be elucidated and may be linked prospectively to other anti-hemostatic activities. Nevertheless, their potential use in biomedicine makes them an attractive target as therapeutic molecules for inflammatory diseases. Furthermore, these immuno-modulatory activities may facilitate pathogen transmission and its establishment.

One of the first immuno-modulatory activities shown in sand fly saliva was the inhibitory effect of *Lutzomyia longipalpis* saliva on the activation of T cells and macrophages by

inhibiting the expression of Th1 type cytokines and inducing the expression of Th2 cytokines by activated macrophages (Hall and Titus, 1995; Soares et al., 1998; Theodos and Titus, 1993). Saliva of this sand fly was also shown to have a chemotactic activity for macrophages (Teixeira et al., 2005). Similarly, *Phlebotomus papatasi* saliva was shown to have the same effect on T cells down-regulating the production of Th1 cytokines and up-regulating the production of Th2 cytokines (Abdeladhim et al., 2011; Mbow et al., 1998; Rogers and Titus, 2003). Further work needs to be done in identifying the salivary proteins responsible for the inhibitory effect in T cells and the mechanism of this inhibition.

Dendritic cells (DC) are potent antigen presenting cells specialized in the initiation of the immune response by direct activation of naïve T lymphocytes and induction of their differentiation to specific subtypes. *P. papatasi* and *P. duboscqi* saliva inhibited the ability of DC to present antigens (Carregaro et al., 2008). Sand fly saliva induced the sequential production of prostaglandin E_2 (PGE_2) and IL-10 by DC. PGE_2 and IL-10 then acted on DC in an autocrine manner reducing the expression of MHC-II and CD86 co-stimulatory molecules on their surface (Carregaro et al., 2008).

Neutrophils are one of the first lines of defense against infection (Abi Abdallah and Denkers, 2012). It was recently reported that saliva of sand flies has an effect on the function of neutrophils. *Lu. longipalpis* saliva increased the apoptosis of neutrophils directly in a FasL-mediated caspase-dependent manner (Prates et al., 2011). On the other hand, as explained above the expression of PGE_2 and IL-10 by DC induce the inhibition of the expression of neutrophil chemotactic factors MIP-1α, TNF-α and Leukotriene B_4 and consequently diminishing neutrophil migration during specific antigen-induced inflammation. (Carregaro et al., 2008).

It was recently shown that *Lu. longipalpis* saliva induces the formation of lipid bodies and PGE_2 in macrophages via the ERK-1/2 and PKC-α signaling pathways (Araujo-Santos et al., 2010). These lipid bodies are cytoplasmic organelles involved in arachidonic acid metabolism that form eicosanoids in response to inflammatory stimuli (Araujo-Santos et al., 2010). The production of PGE_2 by *Lu. longipalpis* saliva will probably induces anti-inflammatory activities similar to those observed in *P. papatasi* saliva (Carregaro et al., 2008).

2.3. Effect of saliva on leishmaniasis

2.3.1. Exacer bating effect of saliva—The above-mentioned effects of sand fly saliva on the host's hemostatic and immune systems results in a different environment at the feeding site and this modified environment has been shown to favor the establishment of *Leishmania* parasites. In mice, co-injection of *Lu. longipalpis* or *P. papatasi* saliva with *Leishmania major* resulted in an exacerbated disease reflected by a larger ulcer that developed to a necrotic lesion compared to the injection of *L. major* alone (Belkaid et al., 1998; Theodos et al., 1991; Titus and Ribeiro, 1988). Similar results were observed with *L. braziliensis braziliensis* (Samuelson et al., 1991) or *L. mexicana amazonensis* (Theodos et al., 1991) parasites and saliva of *Lu. intermedia* and *Lu. longipalpis*, respectively.

2.3.2. Effect of adaptive immunity to saliva—There is a significant body of literature demonstrating that exposure to saliva induces an immune response in animals that mostly confers protection against leishmaniasis. The first line of evidence was obtained when animals immunized with *P. papatasi* saliva (Belkaid et al., 1998) or previously exposed to bites of uninfected *P. papatasi* (Kamhawi et al., 2000) were protected against *L. major* infection initiated by needle challenge or infected sand flies, respectively. Protection from leishmaniasis conferred by previous exposure to sand fly saliva or to bites was later reported for other sand fly and parasite species (Teixeira et al., 2014). The protective effect of sand fly saliva has been attributed to either the neutralization of the exacerbative effect of saliva (Belkaid et al., 1998; Morris et al., 2001) or to the induction of a Th1 cellular immune response in a form of a delayed-type hypersensitivity response (DTH) that is detrimental for the establishment of the *Leishmania* parasite (Kamhawi et al., 2000).

Although the majority of studies reproduced the protective effect of previous exposure to saliva, for the New World sand fly *Lu. intermedia* it resulted in exacerbation of the disease (de Moura et al., 2013; de Moura et al., 2010; Weinkopff et al., 2014). Mice pre-exposed to *Lu. intermedia* salivary gland extract (SGE) and challenged with *L. braziliensis* plus SGE showed a significant decrease in CXCL10 expression paralleled by an increase in IL-10 expression (de Moura et al., 2010).

3. From saliva and activities to transcriptomes and characterization of the bioactive molecules

Pharmacological and immuno-modulatory activities were described in the saliva of sand flies but only one protein with attributable activities was identified; the salivary vasodilator and immunomodulator protein maxadilan (Lerner et al., 1991). This remained the only characterized molecule from the saliva of a sand fly despite the diverse activities reported from several vector species. Three salivary proteins with biological activity, an apyrase, a hyalorunidase and a 5’ nucleotidase, and 5 other proteins of unknown function were later on identified from *Lu. longipalpis* by PCR subtraction technology (Charlab et al., 1999). Considering that about 30 phlebotomine species transmit leishmaniasis (Desjeux, 2004) having only partial information on the salivary transcripts present of *P. papatasi* and *Lu. longipalpis* (Charlab et al., 1999; Valenzuela et al., 2001a) reflected the paucity of our knowledge of sand fly saliva up to that point. At the turn of the century a new technology hit the vector biology field, the transcripomics revolution, where salivary glands transcriptomes from different blood-feeding arthropods including the salivary gland transcriptome of *Lu. longipalpis* were completed (Ribeiro and Francischetti, 2003; Valenzuela et al., 2004). This was followed, by the completion of salivary gland transcriptomes from relevant sand fly vectors including the salivary transcriptomes from *P. ariasi* (Oliveira et al., 2006), *P. perniciosus*, *P. argentipes* (Anderson et al., 2006), *P. duboscqi* (Kato et al., 2006), *P. arabicus* (Hostomska et al., 2009), *P. tobbi*, *P. sergenti* (Rohousova et al., 2012), *P. papatasi* (Abdeladhim et al., 2012), *Lu. ayacuchensis* (Kato et al., 2013), *Lu. intermedia* (de Moura et al., 2013) and *P. orientalis* (Vlkova et al., 2014). It is important to note that the majority of currently available transcriptomes are from sand flies of the genus *Phlebotomus* and more salivary transcriptomes of sand flies from the Americas are need (**Figure 1**).

Transcriptomic analysis of different species from the Old World and the few species from New World are providing insights into the nature of the array of molecules present in sand fly saliva. Fifteen salivary gland cDNA libraries representing twelve different sand fly species resulted in the identification of distinct families of secreted proteins (**Table 2**). Some of the salivary proteins in some sand fly species have multiple members, probably due to gene duplication events, forming a large family of proteins (**Table 3**). Examples include the small odorant binding family of salivary proteins (SP15 like) in *P. duboscqi* (Kato et al., 2006) and *P. papatasi* (Abdeladhim et al., 2012) and the C-type lectin-like proteins in *Lu. longipalpis* (Valenzuela et al., 2004). Most of the listed protein families in Table 2 and Table 3 are found in all sand fly species studied so far; others are specific only to New World species and only few appear to be specific for Old World sand flies. The information gathered by these salivary gland transcriptomes represents a significant advancement compared to the few molecules known prior to the era of transcriptomics.

4. From transcriptomics to functional genomics: assigning activities to molecules

The vector biology field as well as many other fields faced a new challenge: the post-genomics era. For sand flies, it is better stated as the post-transcriptomics era since there is no genome of sand flies available yet. Despite the identification of many salivary transcripts we still faced the challenge of how to connect this information to biological activities previously described from saliva of these insects (**Table 2**) and how to utilize this gigantic source of new information. Bioinformatic approaches helped this transition by providing the means to organize and annotate newly described transcripts towards the identification of proteins with predicted function; for “unknown” proteins the challenge remains.

4.1. Deciphering the function of unknown salivary proteins

There are a number of salivary proteins in the trancriptomes of sand flies with no homologies to other proteins in accessible data banks; therefore, their function is difficult to predict. Various strategies such as expression of recombinant proteins in bacteria or mammalian cells, DNA vaccination and reverse antigen screening were developed to identify the function of these proteins. These approaches have resulted in the discovery of new functions, and new properties of salivary molecules including their potential as markers of exposure and as vaccines against leishmaniasis.

4.1.1. Salivary proteins with anti-hemostatic properties

a. The yellow family of salivary proteins, biogenic amine binding proteins: For many years the function of yellow proteins in sand flies, namely LJM11, LJM17 and LJM111, was unknown. This is one of the most abundant families of salivary proteins and it is found in all sand fly transcriptomes. Recently, it was demonstrated that all three members of the yellow family of proteins from saliva of the sand fly *Lu. longipalpis* bind biogenic amines, including serotonin, catecholamines, and histamine (Xu et al., 2011). Biogenic amines are a group of pro-hemostatic and pro-inflammatory mediators that potentially obstruct feeding. The crystal structure of LJM11 was solved and represented the first structure described for any sand fly salivary protein up to date (Xu et al., 2011). LJM11 has a six bladed-propeller

fold and each blade consists of a four-stranded sheet. This structure forms a central channel that binds biogenic amines (Xu et al., 2011). LJM11 and LJM111 share a similar structure the only difference being the surface charge distribution; LJM11 has a positive surface charge while LJM111 has a more neutral surface charge (Xu et al., 2011). The amino acids responsible for the binding to biogenic amines were identified (Xu et al., 2011) and are conserved in yellow related proteins from other sand fly species as recently reported for *P. papatasi* (Abdeladhim et al., 2012). This suggests that sand fly yellow proteins may also share this biological activity.

b. Lufaxin-like molecules and Lufaxin (LJL143), anticoagulants of sand fly saliva: The anticoagulant from sand flies has remained elusive for many years. The activity was identified but the corresponding protein was not confirmed (Charlab et al., 1999). A C-type lectin-like protein from *Lu. longipalpis* co-eluted with the anticoagulant activity leading to it's identification as the anticoagulant. However, this initial characterization could not be confirmed due to the lack of the activity of the recombinant protein. Furthermore, the same anticoagulant activity was also present in *P. papatasi* and *P. duboscqi* saliva that do not contain the C-type lectin family of proteins arguing against it being the molecule responsible for the salivary anticoagulant activity. Only recently expression and testing of a number of recombinant salivary proteins from the *Lu. longipalpis* salivary gland transcriptome identify LJL143 as the protein responsible for the anticoagulant activity. This 32 kDa protein was consequently renamed Lufaxin (*Lutzomyia* Factor Xa inhibitor) (Collin et al., 2012). Importantly, homologues of the transcript coding for this protein are present in all sand flies studied so far suggesting that these Lufaxin-like proteins are the anticoagulants of all New world and Old world sand flies (Abdeladhim et al., 2012; Anderson et al., 2006; de Moura et al., 2013; Hostomska et al., 2009; Kato et al., 2006; Kato et al., 2013; Martin-Martin et al., 2013; Oliveira et al., 2006; Valenzuela et al., 2004; Vlkova et al., 2014).

Lufaxin is a novel protein with no similarities to any other protein in accessible databases and it was shown to be a potent and specific inhibitor of the coagulation Factor Xa (FXa). This coagulation factor (FXa) is one of the most important proteases in the blood coagulation cascade where both extrinsic and intrinsic pathways converge leading to prothrombinase assembly with subsequent thrombin generation and fibrin formation. Importantly, Lufaxin was also shown to be of great consequence in inflammation. FXa activates receptor PAR1 or PAR2 in different cell types enabling and promoting inflammation and immune modulation. Lufaxin was shown to act on PAR receptors inhibiting the activity of FXa and to attenuate inflammation and prevent arterial thrombosis in a mouse model (Collin et al., 2012).

c. The SP15-like odorant binding family of proteins, contact activation inhibitors: Transcriptomic analysis from other sand fly species revealed that this family of proteins is common and abundant (**Table 2**). Phylogenetic studies show that these proteins have some resemblance to small odorant binding proteins in insects (Abdeladhim et al., 2012), however, the biological activity of this family of proteins remained elusive for many years. Recently, PdSP15 from saliva of the sand fly P. duboscqi, a member of this family of proteins, was shown to inhibit the activation of FXII and FXI, as well as the cleavage of FXI

by FXIIa or thrombin, and to inhibit other anionic surface-mediated reactions (Alvarenga et al., 2013). The relevance of these findings is that anionic surfaces help to stabilize complexes of the blood coagulation cascade such as FXIIa with FXI and also complexes involved in inflammatory processes such as high molecular weight kininogen, prekallikrein, thrombin and FXI.

This family of proteins may prevent downstream effects of mast cell activation caused by the bite of the sand fly including the formation of bradykinin, an inducer of pain. The targeting of anionic surfaces rather than specific proteins by this family of proteins suggests they may inhibit other processes of hemostasis and inflammation and may have some therapeutic uses that need further exploration.

d. LJL138 or Lundep, an endonuclease: LJL138 is a salivary protein present in the transcriptome of *Lu. longipalpis* and encodes an endonuclease, however, for a long time the predicted activity of this protein was never tested or confirmed (Valenzuela et al., 2004). Renamed Lundep, the activity of this protein was recently established as a potent endonuclease (**Table 2)** (Chagas et al., 2014). This protein contains the NUC-motif indicative of nonspecific double and single stranded DNA and RNA endonuclease activity.

The DNase activity of Lundep may contribute to the anti-thrombotic and anti-inflammatory functions of *Lu. longipalpis* saliva by hydrolyzing the DNA scaffold of neutrophil extracellular traps (NETs) at the biting site. Lundep may assist blood-meal intake by lowering the local viscosity induced by the release of host DNA at the injury site caused by the bite of the insect when taking a blood meal. In fact, anti-Lundep antibodies significantly decreased the feeding success of female *Lu. longipalpis* flies in passively immunized mice (Chagas et al., 2014).

4.1.2. Salivary proteins and nucleosides with anti-inflammatory activity

a. LJM111, a potent anti-inflammatory molecule: In addition to binding biogenic amines (Xu et al.), LJM111, was shown to have another function (**Table 2**). LJM111 (but not LJM11 or LJM17) was characterized as a potent anti-inflammatory molecule. Recombinant LJM111 was shown to inhibit IL-17, TNF-α and IFN-γ production by leukocytes obtained from lymph node suspension following *in vitro* stimulation with methylated bovine serum albumin (mBSA). LJM111 also inhibited neutrophil migration in a dose dependent manner following mBSA-challenge of Ovalbumin (OVA)-immunized mice used as a model of OVA-induced neutrophil migration (Grespan et al., 2012). Furthermore, LJM111 reduced the hypernociception (pain) in a model of arthritis and inhibited the production of pro-inflammatory molecules consequently reducing *in vivo* neutrophil recruitment.

DCs that emigrate to inflamed joints produce pro-inflammatory mediators that support expansion and differentiation of Th1 and/or Th17 cells, which play a pathologic role in arthritis (Carregaro et al., 2011). LJM111 affected the maturation of DCs leading to increased IL-10 production and reduced synthesis of TNF-α (Grespan et al., 2012).

b. Nucleosides: Sand fly saliva triggers an inflammatory response characterized by a cellular influx and suppression of hemostatic and immune mechanisms. The saliva of *P.*

papatasi contains large amounts of adenosine and 5′-AMP. These two non-proteic components were shown to block antigen presentation by DCs by inhibiting TNF-α and IL-12p40, increasing IL-10 production and interfering with Th17 cell activation consequently suppressing the inflammatory immune response (Carregaro et al., 2011).

4.1.3. Direct effects of isolated sand fly salivary components on disease

a. Effects of Hyaluronidase on leishmaniasis: Hyaluronidase activity was originally described in saliva of the sand fly *Lu. longipalpis* (Charlab et al., 1999) and was later shown to be present in the saliva of Old World sand flies (**Table 2**) (Cerna et al., 2002; Rohousova et al., 2012; Vlkova et al., 2014; Volfova et al., 2008). The salivary hyaluronidase plays an important role in blood meal acquisition, by degrading hyaluronan (HA), abundant in host skin, and probably increasing tissue permeability for other salivary components (Volfova et al., 2008). Using commercially available hyaluronidase, Volfova et al. demonstrated that the activity of this enzyme facilitated transmission and establishment of *L. major* parasites (Volfova et al., 2008). Importantly, fragments of HA were shown to have immunomodulatory properties; they affect maturation and migration of dendritic cells, induction of iNOS, chemokine secretion by macrophages and proliferation of activated T cells (Volfova et al., 2008).

b. Impact of Lundep on leishmaniasis: As an endonuclease, the activity of Lundep had a direct impact on the development of *Leishmania* parasites. *Leishmania* parasites evade killing by neutrophils either by blocking the oxidative burst and entering a non-lytic compartment unable to fuse with lysosomes or by resisting the leishmanicidal activity of parasite-induced NETs (Gabriel et al., 2010). Chagas et al. demonstrated that Lundep can effectively facilitate the survival of *L. major* parasites (Chagas et al., 2014). Survival of *Leishmania* in neutrophils has been reported as a mechanism for silent uptake by macrophages favoring establishment of *Leishmania* infections (Peters et al., 2008).

4.1.4. Sand fly salivary proteins in auto-immunity—Pemphigus foliaceus (PF) is an auto-immune disease targeting the skin. It is mediated by pathogenic IgG4 anti-epidermal autoantibodies against desmoglein 1 (Dsg1) causing epidermal cell detachment that leads to blister formation (Diaz et al., 2004). Dsg1 is a calcium-binding transmembrane glycoprotein component of epithelial cells (Wheeler et al., 1991). In Brazil, endemic areas of PF, known as Fogo Selvagen, overlap with leishmaniasis endemic areas (Diaz et al., 2004). Similar observations were made in Tunisia where the prevalence of PF was higher in foci of zoonotic cutaneous leishmaniasis (Kallel Sellami et al., 2007). The association between PF and leishmaniasis was attributed to a cross-reaction between anti-Dsg1 auto-antibodies and antibodies to sand fly salivary proteins. Antibodies from PF patients recognized salivary gland antigens from *Lu. longipalpis* and *P. papatasi* (Qian et al., 2012; Zaraa et al., 2012) and anti-Dsg1 antibodies were more prevalent in visceral leishmaniasis patients in Tunisia (Kallel Sellami et al., 2007). Further investigation demonstrated that LJM11, a yellow protein from *Lu. longipalpis*, is the target of auto-antibodies in Brazilian PF patients (Qian et al., 2012), while from *P. papatasi* five proteins of 12, 15, 30, 21 and 36kDa proteins where recognized by pemphigus patients sera (Zaraa et al., 2012). This suggests that antibodies made against LJM11 may recognize Dsg1 triggering auto-antibody production. There is no

sequence similarity between Dsg1 and LJM11 and apparently conformational mimotopes may be responsible for the cross-reactivity in these two molecules (Qian et al., 2012). Further studies are needed to identify these structural motifs in LJM11 that mimics the structure of dsg1.

5. Practical applications ascribed to sand fly salivary proteins

5.1. Salivary proteins as markers of exposure

The antigenic nature of certain salivary proteins provides an opportunity for their use as indicators of host-vector contact. The uniqueness of sand fly salivary proteins, many being specific only to sand flies or to a particular species, makes them ideal as markers of exposure to vectors species and important epidemiological tools to measure of the risk of contracting leishmaniasis and for vector surveillance and control programs (Andrade et al., 2007; Barral et al., 2000; Vlkova et al., 2011). Importantly, the use of sand fly salivary proteins as markers of exposure offer several advantages over the use of total saliva promising increased specificity and the ability to produce large quantities of proteins in a reproducible manner.

5.1.1. LJM11 and LJM17 as markers for exposure to *Lu. longipalpis*—The yellow proteins LJM11 and LJM17 from saliva of *Lu. longipalpis* were recognized by sera from humans living in endemic areas of VL in Brazil (Souza et al., 2010; Teixeira et al., 2010). Sera from dogs and foxes, important reservoirs of *L. infantum*, recognized LJM17; LJM11 was only immunogenic in humans and dogs (Teixeira et al., 2010). Importantly, LJM17 and LJM11 showed little cross-reactivity with salivary proteins of *Lu. intermedia*, a sympatric vector of CL in large parts of Brazil (Souza et al., 2010; Teixeira et al., 2010). Encouragingly, in a large cohort of over 1000 subjects from a VL endemic area in Brazil, the combined use of LJM11 and LJM17 displayed a similar sensitivity as the use of total saliva validating the promise of these antigens as specific markers of exposure to *Lu. longipalpis* saliva (Souza et al., 2010). These two molecules were also used successfully to monitor seroconversion in chicken that can be used as sentinels to reflect the presence of *Lu. longipalpis* in peri-domiciliary parts within VL endemic regions (**Table 4)** (Soares et al., 2013).

5.1.2. PpSP32 as a marker for exposure to *P. papatasi*—PpSP32 was the immunodominant target of the antibody response in humans naturally exposed to *P. papatasi* saliva in Tunisia (Marzouki et al., 2012). Forty two sera positive to whole salivary gland extract and 24 negative controls were tested against recombinant PpSP32. Sixty four percent of saliva-exposed donors showed a positive correlation between antibodies response against saliva and rPpSP32 (**Table 4**). Despite the promising humoral response to PpSP32, its specificity needs to be verified, at least with regards to major sympatric vectors, to establish its usefulness as a good marker of exposure to *P. papatasi*, the vector of *L. major* in this country (Marzouki et al., 2012).

5.1.3. SP01B, SP01 and SP03B from *P. perniciosus* saliva as markers of exposure—The apyrases rSP01B and rSP01 and the yellow protein rSP03B from saliva of *P. perniciosus*, a principal vector of *L. infantum* in the Mediterranean Basin, show promise

as markers of canine exposure to the vector and for estimating the risk of canine leishmaniasis in the western Mediterranean area (**Table 4**) (Drahota et al., 2014). Sera from three dogs repeatedly exposed to *P. perniciosus* bites specifically recognized the 3 molecules listed above. Additionally, a strong positive correlation was observed in the antibody titers measured against whole salivary extract and these molecule, suggesting they can be good markers of exposure against *P. perniciosus* saliva (Drahota et al., 2014). More work is needed to establish the specificity of these proteins against other vectors present in the same areas.

5.2. Salivary proteins as vaccines against leishmaniasis

To date several molecules from various vector species of sand flies, some with and others without any known activity, have demonstrated immunogenicity and protective efficacy against cutaneous and visceral leishmaniasis (**Table 5**). Following is an updated account of the salivary vaccine candidates identified thus far:

5.2.1. PpSP15—PpSP15, (**Table 2**), was the first salivary protein to be identified as a potential vaccine (Oliveira et al., 2008; Valenzuela et al., 2001a). Named after its molecular weight, PpSP15 was identified by SDS-PAGE separation of salivary gland proteins from *P. papatasi* (Valenzuela et al., 2001a). The proteins were grouped into three fractions and only the one containing PpSP15 protected mice against *L. major* infection. Immunization of mice with PpSP15 plasmid induced a delayed-type hypersensitivity response (DTH) that was correlated to protection (Valenzuela et al., 2001a). The authors also demonstrated that PpSP15-specific protection was cell-mediated and antibody-independent. This seminal work established that DTH-induction in immunized animals is the hallmark of protective salivary proteins. Seven years later, Oliveira et al. validated the protective effect of PpSP15 against *L. major* in mice (**Table 5**) (Oliveira et al., 2008). Furthermore, in addition to the induction of a DTH, PpSP15-immunized mice expressed IFN-γ and IL-12 2h after bites and showed an accelerated development of a *Leishmania*-specific immunity (Oliveira et al., 2008). Importantly, the DTH-inducing salivary protein PpSP44 produced a Th2 response in immunized mice that exacerbated *L. major* infections (Oliveira et al., 2008). From thereon, only Th1-biased DTH-inducing salivary proteins were considered as vaccine candidates for leishmaniasis.

5.2.2. Maxadilan—Morris et al. demonstrated that mice vaccinated with maxadilan, the vasodilator in *Lu. longipalpis* saliva, developed both cellular immunity and antibodies against this salivary protein that protected animals against *L. major* infection. The authors proposed that maxadilan had an exacerbatory effect on *L. major* infection that was neutralized by protective anti-maxadilan antibodies (**Table 5**) (Morris et al., 2001). Maxadilan, a protein of 6.8 kDa, is absent from *Phlebotomus* species and has only been described from some *Lutzomyia* vectors thus far (**Table 2**) (de Moura et al., 2013; Valenzuela et al., 2004).

5.2.3. Identification of vaccine candidates in the post-transcriptomic era—Based on information from the salivary transcriptomes, plasmids encoding the most abundant secreted salivary proteins, irrespective of whether their identity and function were

known, were prioritized for screening. Classical vaccinology was used to identify salivary vaccine candidates in rodents where vaccination of groups of mice or hamsters with different vaccine candidates is feasible. On the other hand, identification of vaccines in larger and more relevant animal models such dogs represented a challenge due to the cost and logistics of such an endeavor. This led to the development of reverse antigen screening (RAS) where large animals were exposed to bites of a particular vector species, thus becoming immune to the native salivary proteins secreted into the host. Exposed animals were then injected with the salivary plasmids at marked location in the animals (usually belly or thigh) along with appropriate controls (Collin et al., 2009). The plasmids that induced a DTH were further tested as recombinant proteins to validate their immunogenicity. Furthermore, biopsies were taken from the DTH site to determine the cytokine environment and therefore, the molecules that induced a Th1-biased DTH

5.2.4 Vaccine candidates identified by classical vaccinology (forward immunology)

a. LJM19: LJM19, a 10.7 kDa protein from saliva of *Lu. longipalpis* (**Table 3**), induced a pronounced Th1 cellular immune response in immunized hamsters characterized by an increase in IFN-γ expression in the absence of IL-10 or TGF-β (Gomes et al., 2008). Hamsters immunized with LJM19 and challenged with *L. chagasi* and *L. braziliensis* in the presence of *Lu. longipalpis* saliva were protected from visceral and cutaneous leishmaniasis, respectively (**Table 5**) (Gomes et al., 2008; Tavares et al., 2011). Thus far, LJM19 has only been identified from some *Lutzomyia* vector species (de Moura et al., 2013; Kato et al., 2013; Valenzuela et al., 2004).

b. LJM11: LJM11, is a 43 kDa salivary protein from *Lu. longipalpis* that belongs to the yellow family of proteins (**Table 2**) (Valenzuela et al., 2004). LJM11 was shown to be a DTH-inducing protein that conferred protection against cutaneous leishmaniasis in a mouse model of infection (Gomes et al., 2012a; Xu et al., 2011). The protective effect was related to a DTH response mediated by $CD4^+$ T cells producing IFN-γ, or both IFN-γ and TNF-α, at the bite site of LJM11-immunized mice 48h after challenge with infected sand flies (**Table 5**) (Gomes et al., 2012a).

c. Linb-11: Linb-11, a small salivary protein of 4.5 kDa, was identified from saliva of *Lu. intermedia*, another New World sand fly vector species (de Moura et al., 2013). Mice immunized with Linb-11 were protected against a challenge with *L. braziliensis* parasite injected together with *Lu. intermedia* saliva (**Table 5**). Linb-11, found only in *Lu. intermedia* sand flies so far, was shown to induce a transient cellular immune response in BALB/c mice that correlated with an early predominance of IFN-γ-secreting over IL-4- and IL-10-secreting CD4+ T cells (Mbow et al., 1998).

5.2.5. Vaccine candidates identified by reverse antigen screening (RAS)

a. LJM17: LJM17 is a protein of 45kDa from the saliva of *Lu. longipalpis* (**Table 2**) that was shown to induce a strong DTH response in dogs previously exposed to uninfected sand flies bites. Immunized dogs were primed with the plasmid coding for this protein and boosted with a LJM17 recombinant protein (rLJM17) (Collin et al., 2009). PBMC from

immunized dogs produced a high amount of IFN-γ upon *in vitro* stimulation with rLJM17, confirming the Th1 profile of the generated immune response. Additionally, immunized dogs showed a strong predominantly IgG2 humoral immune response to LJM17 (**Table 5**). Biopsies taken from the DTH-site of bite 48h after exposing these dogs to uninfected sand flies showed a cellular infiltrate dominated by CD3 T cells and macrophages. These infiltrated cells expressed high levels of IFN-γ and IL-12 corroborating the result obtained from PBMC stimulation (Collin et al., 2009). Importantly, in an *in vitro* killing assay, autologous T cells from immunized dogs significantly reduced the percent of infected macrophages as well as the number of amastigotes per cell (Collin et al., 2009).

b. Lufaxin (LJL143): The immune response seen in dogs immunized with LJM17 was also observed in dogs immunized with LJL143 (Lufaxin) (Collin et al., 2009). PBMC from LJL143-immunized dogs produced a high amount of IFN-γ upon *in vitro* stimulation with recombinant LJL143 and showed a strong IgG2-biased humoral response (**Table 5**). Interestingly, the immune response at the site of bite differed depending on the number of flies used in the challenge. Following exposure to bites of 20 sand flies, TGF-β was the dominant cytokine induced with a low expression of IFN-γ, IL-12 and IL-4. In contrast, a high level of IFN-γ and a low level of IL-4 were induced when these dogs were exposed to bites from 5 sand flies (Collin et al., 2009).

5.2.6 Vaccines combining *Leishmania* antigens and sand fly salivary proteins

—Up to date, there is no vaccine against human leishmaniasis despite the large portfolio of promising Leishmania antigens. Recent data from vaccination with sand fly salivary antigens suggest that immunity to sand fly saliva may promote a specific and protective immunity to Leishmania (Gomes et al., 2012a; Oliveira et al., 2008). These findings may suggest that a combination of a sand fly salivary protein with a Leishmania antigen may produce a more robust and protective immune response. Some groups have tested combinations of sand fly saliva or salivary proteins with Leishmania parasites with promising results. In the study by Aguiar-Soares et al., a vaccine composed of *L. braziliensis* antigens adjuvanted with saponin and *Lu. longipalpis* salivary gland extract (LBSapSal vaccine) was tested in dogs. This combination vaccine elicited both anti-*Leishmania* and anti-saliva humoral and cellular immune responses and resulted in a reduction of the splenic parasite load in immunized dogs as compared to control groups (Aguiar-Soares et al., 2014). Recently, a vaccine combination of a single sand fly salivary protein (PpSP15) with live non-pathogenic *L. tarentolae* expressing the cysteine proteases A and B resulted in a better immunity and better protection against cutaneous leishmananiasis than animals vaccinated with PpSP15 alone or with non-pathogenic *L. tarentolae* expressing the cysteine proteases A and B without the salivary protein (Zahedifard et al., 2014). It is important to note that this increase in protection was only observed when animals were first primed with PpSP15 DNA and then boosted with PpSP15 DNA and live CPA/CPB/EGFP-expressing recombinant *L. tarontolae* (Zahedifard et al., 2014). Apparently the priming with the salivary is important since on a separate study animals vaccinated simultaneously with KMP11 and the salivary protein LJM19 showed no improvement in protective efficacy over the KMP11 or LJM19 vaccines alone (da Silva et al., 2011). These studies suggest that priming with a sand fly

salivary protein may be required for a vaccine that envision a combination of a sand fly salivary protein and a *Leishmania* antigen.

5.3 Vector-transmitted models of infection

Several studies highlighted the significance of vector-transmitted models of infection in the assessment of *Leishmania* vaccines. In contrast to needle-initiated infection, vector-transmission of *L. major* caused a persistent infiltration of neutrophils to the site of bite that promoted parasite establishment (Peters et al., 2008). Vector-transmission by *Lu. longipalpis* also enhanced *L. mexicana* infection, attributed to regurgitation of the promastigote secretory gel alongside parasites (Rogers et al., 2004). Moreover, the modulatory properties of saliva have been associated with exacerbation of *L. major* infection (Gomes and Oliveira, 2012). Recently, vector-transmission of *L. infantum* and *L. donovani* by bites of *Lu. longipalpis* succeeded causing fatal visceralizing infections in hamsters and reflected disease characteristics similar to those observed in nature (Aslan et al., 2013). Taken together, it is not surprising that the enhanced virulence of vector-transmission abrogated the protection observed following needle challenge of vaccinated animals (Peters et al., 2009) or led to a different outcome to that observed following needle challenge (Rogers et al., 2006). Today, vector-transmission is considered the golden standard for testing *Leishmania* vaccines (Gomes et al., 2012a; Gomes et al., 2012b; Peters et al., 2012) and the closest experimental model to mimic what occurs naturally.

For salivary vaccines, vector-transmitted models are doubly consequential. Apart from testing the vaccine against a stringent challenge, they permit the evaluation of the adaptive immune response to the native salivary vaccine candidate injected at the site of bite. Moreover, the protein will be injected in physiological quantities and assessed in the context of the other salivary proteins present in saliva and other vector-derived factors co-injected during infected vector-bites. Promisingly, vector-challenge of animals previously exposed to saliva or immunized with salivary vaccines demonstrated the powerful and long-term protection of mice against leishmaniasis (Gomes et al., 2012a; Kamhawi et al., 2000; Teixeira et al., 2014).

5.4. From rodent models to humans

The selection of vaccine candidates against leishmaniasis, including salivary proteins, has been traditionally undertaken in rodent models of infection. A leishmaniasis vaccine needs to induce a robust T cell immunity that depends on recognition of specific epitopes by major histocompatibility complex molecules. With that in mind, the immunogenicity and protective effects of an antigen in rodents may not necessarily be observed in humans. The variability of the immune response in different hosts was exemplified in by studies of the salivary vaccine candidate LJM19. Immunization of hamsters with LJM19 protected against fatal progressive VL but was non-immunogenic in dogs (Collin et al., 2009; Gomes et al., 2008). The danger of choosing the wrong antigen as a vaccine can be overcome by testing their immunogenicity in target hosts; for leishmaniasis vaccines, these are either dogs or humans.

To date only one study reported the immunogenicity of salivary proteins in dogs. For the rest, the focus remained on the effect of total saliva on target species. In dogs, immunization with both LJL143 and LJM17 induced an *in vivo* Th1-biased DTH, IFN-γ-production by PBMC and *in vitro* killing of *Leishmania* parasites by T cells obtained from immunized animals (Collin et al., 2009). Recently, dogs immunized with a combination vaccine composed of *L. braziliensis* antigen adjuvanted with saponin and *Lu. longipalpis* saliva developed *Leishmania*-specific T cells and exhibited a reduced splenic parasite load compared to controls (Aguiar-Soares et al., 2014).

In human studies, volunteers experimentally or naturally exposed to sand fly bites developed a distinct immune response to saliva (Abdeladhim et al., 2011; Oliveira et al., 2013; Vinhas et al., 2007). In one study in Tunisia, PBMC obtained from individuals living in a CL endemic area, and stimulated with saliva of *P. papatasi* induced activation of IL-10-producing CD8+ T cells and IFN-γ-producing CD4+ T cells when the effect of IL-10 was blocked (Abdeladhim et al., 2011).In another study, cellular immune response to sand fly salivary proteins was persistent for one year. In this study, PBMC from human volunteers experimentally exposed to *Lu. longipalpis* bites displayed increased frequency of $CD4^+$ and $CD8^+$ T lymphocytes expressing $CD25^+$ with an increase in the secretion of IFN-γ and IL-10 upon stimulation with sand fly saliva (Vinhas et al., 2007). In another study, subjects from Mali and naturally exposed to *P. duboscqi* bites were challenged by bites of three uninfected sand flies and these individuals produced a Th1-biased DTH response at the bite site (Oliveira et al., 2013). This immune response observed in these individuals is the same response observed in rodents and is the hallmark of a protective immune response against leishmaniasis induced by sand fly salivary proteins (Gomes et al., 2012a). It is important to note that the DTH response to sand fly saliva observed in these individuals was maintained to mid-life in 48% of tested subjects (Oliveira et al., 2013), relevant to the consideration of salivary proteins as leishmaniasis vaccines. Interestingly, PBMC from a larger cohort in the same study area exhibited a varied immune response to saliva where around 25% of individuals produced a Th1 response to sand lfy salivary proteins, 25% to a Th2 immune response and the rest of the individuals showed a mixed Th1/Th2 profile. This suggest that not all individuals respond in the same way to sand fly salivary proteins and these differences may account for the different outcomes of leishmaniasis in a population that is constantly bitten by sand flies.

Despite of the limited number of reports, it is evident that sand fly saliva is immunogenic in humans. Further studies are needed to identify distinct salivary proteins from saliva of vectors that are immunogenic in targeted species for consideration as components of *Leishmania* vaccines.

5.5 Perspectives on salivary proteins as vaccines

There are no human vaccines against any form of leishmaniasis to date. The immunogenicity of certain salivary proteins and their capacity to confer powerful protection in various animal models argues strongly for their inclusion in *Leishmania* vaccines. However, a potential challenge to salivary proteins as vaccines is that some may have functions that affect human physiology such LJL143, an anti-coagulant, or maxadilan, a

potent vasodilator. In large animals this did not represent a problem since dogs immunized with LJL143 or with LJM17 were shown to be safe and with no adverse reactions even after a robust immunization protocol (Collin et al., 2009).

The specificity of salivary proteins also poses an obvious restriction to their use as vaccines particularly since different vector species transmit the same *Leishmania* species in several parts of the world. As such, a salivary vaccine candidate should ideally be conserved across vector species or alternately target major vectors such *P. argentipes* , the only vector of *L. donovani* in Nepal, India, Bengladesh and Sri Lanka (Lane et al., 1990; Picado et al., 2010); *P. orientalis* the main vector of *L. donovani* in Sudan, Ethiopia and Kenya (Elnaiem, 2011); and *Lu. longipalpis*, the main vector of *L. infantum chagasi* in Latin America. Together, these regions contain over 90% of global VL cases (http://www.who.int/gho/neglected_diseases/leishmaniasis/en/). Though salivary proteins may be specific to a sand fly species, several studies have demonstrated that for the most part these molecules are conserved across the distribution range of a species. *P. duboscqi* transcriptomes of flies originating from Kenya and Mali in East Africa and West Africa, respectively, showed a high degree of conservancy (Kato et al., 2006) while analysis of PpSP15 from *P. papatasi* field-caught sand flies from Sudan and from colonies originating from Egypt, Jordan, Israel and Saudi Arabia showed no significant polymorphisms (Elnaiem et al., 2005). Another recent study of the apyrases of *P. ariasi* collected from different regions in southwest Europe and northeast Africa also found no variation (Mahamdallie, 2012).

Of note, several of our studies have demonstrated that immunity to salivary proteins initiate a rapid immune response following an infective bite that seems to set the scene for the development of a robust *Leishmania*-specific immunity with minimal pathology (Gomes et al., 2012a; Oliveira et al., 2008). If driving a protective immunity against the parasites is achievable in the context of a vaccine it will diminish the biggest limitation faced by these molecules as vaccines.

6. Conclusion

The study of arthropod saliva exploded in the last decade facilitated by the arrival of the era of transcriptomics and proteomics. For sand flies, the discovery of new salivary proteins, elucidating new activities and functions and establishing the immunogenicity of some in various mammals reflects the diverse ways by which these molecules influence our condition. The advent of more powerful techniques such us deep sequencing and other functional genomic approaches may yet reveal that we are only seeing the tip of the iceberg, or for vector biologists, just the tip of the proboscis.

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Highlights

- Review of advances of sand fly saliva research
- Role of saliva in host hemostasis
- Role of saliva in host inflammation and immunity
- Transcriptomes of sand fly salivary glands
- Post-genomic approaches
- Sand fly salivary proteins as vaccines
- Sand fly salivary proteins as markers of exposure

Figure 1.

Presentation of the sand flies saliva published transcriptomes from different region of the world (Background map from: http://commons.wikimedia.org/wiki/File:World_map_blank_without_borders.svg)

*In progress

Table 1

Sand fly salivary gland transcriptomes.

Sand fly salivary gland transcriptomes	Parasites associated with sand fly species	Disease caused by parasites	Number of high quality sequences per transcriptome	Number of full-length secreted proteins per transcriptome	References of completed sand fly transcriptomes
Lu. longipalpis	***L. infantum chagasi***	VL	550	35	(Valenzuela et al., 2004)
Lu. intermedia	***L. braziliensis***	CL/MCL	1395	40	(De Moura et al., 2013)
Lu. ayacuchensis	***L. mexicana, L. peruviana***	CL	768	64	(Kato et al., 2013)
P. papatasi **Tunisia**	***L. major***	CL	1603	49	(Abdeladhim et al., 2012)
P. duboscqi **Mali**	***L. major***	CL	988	38	(Kato et al., 2006)
P. duboscqi **Kenya**	***L. major***	CL	924	32	(Kato et al., 2006)
P. sergenti	***L. tropica, L. aethiopica***	CL	853	25	(Rohousova et al., 2012)
P. arabicus	***L. tropica***	CL	985	34	(Hostomska et al., 2009)
P. argentipes	***L. donovani***	VL	603	23	(Anderson et al., 2006)
P. ariasi	***L. infantum***	VL	538	24	(Oliveira et al., 2006)
P. perniciosus	***L. infantum***	VL	535	20	(Anderson et al., 2006)
P. perniciosus **Madrid Spain**	***L. infantum***	VL	--	15	(Martin et al., 2013)
P. tobbi	***L. infantum***	VL/CL	997	32	(Rohousova et al., 2012)
P. orientalis **Addis Zemen**	***L. donovani***	VL	835	25	(Vlkova et al., 2014)
P. orientalis **Melka Werer**	***L. donovani***	VL	749	26	(Vlkova et al., 2014)

L., *Leishmania*; CL, cutaneous leishmaniasis; MCL, muco-cutaneous leishmaniasis; VL, visceral leishmaniasis.

Table 2

ith known biological activity

	Biological activities of salivary proteins (molecular weight)									
ontact eparin ;	Biogenic amine binding proteins	Anti-coagulant, Inhibitor of Factor Xa	Ecto ADPase, inhibitor of platelet aggregation	DNAse activity	Degradation of Hyaluronan. hydrolyzis of chondroitin sulfates	Purine metabolism hydrolysis of adenosine	Vasodilator and inhibitor of platelet aggregation	Nucleotidase	Vasodilator	Anti-inflammatory/anti-arthritis
binding) - lile a)	Yellow Protein (~ 45kDa)	Lufaxin/ Lufaxin like (~32 kDa)	Apyrase (‘~36 kDa)	Endonuclease (~44 kDa)	Hyaluronidase (~42 kDa)	Adenosine Deaminase (~56 kDa)	Adenosine	5’ Nucleotidase (~61 kDa)	Maxadilan peptide (6 kDa)	LJM111
	LJM11[*], **LJM17**[*], **LJM111**[*] (Assumpcao et al., 2011)	**LJL143 (Lufaxin)**[*] (Collin et al., 2012)	**LuloAPY**[*] (Charlab et al., 1999)	**LJL138 (Lundep)**[*] (Chagas et al., 2014)	**LuloHYA**[*] (Cerna et al., 2002; Charlab et al., 1999; Rohousova et al., 2012)[**]	**ADA**[*] (Charlab et a, 2001)		**Lulo5NUC**[*] (Charlab et al., 1999)	**Maxadilan**[*] (Lerner et al., 1992)	**LJM111**[*] (Grespan et al, 2012)
8, 59	**Linb-21**	**Linb-17**	**Linb-35**	**Linb-46**	**Linb-54**				**Linb-147**	
48-72	**LayS22-24, 117, 118**	**LayS120-132**	**LayS8-14, 16-21**	**LayS147**						
-15	**PPTSP42, 44**	**PPTSP34**[**] (Collin et al., 2012)	**PPTSP36**[**] (Ribeiro et al., 1989b)		[**] (Cerna et al., 2002)	[**] (Ribeiro et al., 1999 ; harlab et al., 1999 ; Carregaro et al., 2011)	**Adenosine and 5’-AMP**[**] (Ribeiro et al 1999)			
6-07, 12, 57-58, varenga 3)	**PduM10, 35**	**PduM04-05**[**] (Collin et al., 2012)	**PduM38-39**[*] (Hamasaki et al., 2009)		[**] (Cerna et al., 2002)	**PduM73**				
0-42, 49, 110[*] ., 2013)	**PduK 04-06, 86**	**PduK70**[**] (Collin et al., 2012)	**PduK50**[*] (Hamasaki et al., 2009)		[**] (Cerna et al., 2002)	**PduK60**				
5, 54-55	**PsSP18-20, 22, 26**	**PsSP49**	**PsSP40-42**		[**] (Cerna et al., 2002; Rohousova, 2012)					
3-64, 93	**PabSP26, 53**	**PabSP34, 32**	**PabSP39, 40-41**	**PabSP49**	**PabSP72**[**] (Rohousova et al., 2012)					

	Biological activities of salivary proteins (molecular weight)								
	Inhibitor of contact activation, heparin binding	**Biogenic amine binding proteins**	**Anti-coagulant, Inhibitor of Factor Xa**	**Ecto ADPase, inhibitor of platelet aggregation**	**DNAse activity**	**Degradation of Hyaluronan. hydrolyzis of chondroitin sulfates**	**Purine metabolism hydrolysis of adenosine**	**Vasodilator and inhibitor of platelet aggregation**	**Nucleotidase**
Sand fly salivary transcriptomes	**Small odorant binding protein (OBP) - lile (~15 kDa)**	**Yellow Protein (~ 45kDa)**	**Lufaxin/ Lufaxin like (~32 kDa)**	**Apyrase (‘~36 kDa)**	**Endonuclease (~44 kDa)**	**Hyaluronidase (~42 kDa)**	**Adenosine Deaminase (~56 kDa)**	**Adenosine**	**5’ Nucleotidase (~61 kDa)**
***P. argentipes* (Anderson et al., 2006)**	**PagSP01, 02, 07, 12, 13**	**PagSP04**	**PagSP09**	**PagSP03**** (Ribeiro et al., 1989b)	**PagSP11**	** (Rohousova et al., 2012)		**Adenosine and 5’-AMP**** (Ribeiro et al 1999)	
***P. ariasi* (Oliveira et al., 2006)**	**ParSP03, 06, 08**	**ParSP04, 04B**	**ParSP09**	**ParSP01**	**ParSP10**				
***P. perniciosus* (Anderson et al., 2006)**	**PpeSP02, 09, 11**	**PpeSP03, 03B**	**PpeSP06**	**PpeSP01, 01B**** (Ribeiro et al., 1989b)	**PpeSP32**	** (Rohousova et al., 2012)			
***P. perniciosus* Madrid Spain (Martin et al., 2013)**	**SP02, 09, 11**	**SP03B**	**SP06**	**SP01,01B**** (Ribeiro et al., 1989b)		** (Rohousova et al., 2012)			
***P. tobbi* (Rohousova et al., 2012)**	**PtSP9, 17-18, 23, 31-32**	**PtSP37-38**	**PtSP66**	**PtSP4, 10**		**PtSP125**** (Rohousova et al., 2012)			
***P. orientalis* Addis Zemen (Vlkova et al., 2014)**	**PorASP28, 31, 37, 61, 64**	**PorASP2, 4**		**PorASP11, 14-15**** (Volkova et al., 2014)	**PorASP139**	**PorASP112**** (Volkova et al., 2014)			
***P. orientalis* Melka Werer (Vlkova et al., 2014)**	**PorMSP12, 74-75, 90, 96**	**PorMSP23-24**	**PorMSP78**	**PorMS P3-4**** (Volkova et al., 2014)	**PorMSP101**	**PorMSP108**** (Volkova et al., 2014)			

* Activity or function of a salivary protein detected in purified protein

** Activity detected in whole salivary extract (reference where the function or activity was described).

Table 3

teins with unknown function

Families of salivary proteins												
gen 5 kDa)	Large OBP/D7 (~28 kDa)	Silk related/ Collagen binding (~32 kDa)	C-type lectin (~15 kDa)	RGD-containing family of proteins (~5 kDa)	10 kDa family (~10 kDa)	16 kDa protein (~16 kDa)	(Glutathione S transferase like proteins) (~27 kDa)	11.5 kDa protein (~11 kDa)	ML domain peptide family (~14 kDa)	Small toxin-like proteins (~7 kDa)	37 kDa protein (~37 kDa)	9 kDa protein (~9 kDa)
L34	LJL11	LJL04	LJL91, 15, 18 LJM10, 06 LJS142	LJL35	LJM19 LJS192, 169			LJL17			LJM78	LJS201
b-13	Linb-26, 42		Linb-14, 15, 22, 48	Linb-1,2,11,36	Linb-19, 38, 44				Linb-29, 37, 55, 58, 33	Linb-40, 41, 43, 60, 52, 53, 88		
73-81	LayS95-103	LayS83-93	LayS120-LayS132	LayS44-47		LayS128, 129, 130-132		M8D12 (BAM69094)				LayS6, 7, 142
SP29	PPTSP28a-28c, 30	PPTSP32				PPTSP14.3					PPTSP56	
M48	PduM01, 29, 46-47, 74	PduM33-34, 72, 87									PduK84	
68, 107	PduK34-35, 69, 78, 103	PduK45-46, 83										
SP52	PsSP4-7	PsSP44										
bSP5	PabSP20, 54, 59	PabSP29-31				PabSP63, 64	PabSP11-15					
SP05	PagSP10, 25	PagSP06				PagSP73						
SP05	ParSP07, 12, 16, 84	ParSP02					ParSP25				ParSP17	

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Sand fly salivary transcriptomes (Reference)	Families of salivary proteins									
	Antigen 5 (~30 kDa)	Large OBP/D7 (~28 kDa)	Silk related/ Collagen binding (~32 kDa)	C-type lectin (~15 kDa)	RGD-containing family of proteins (~5 kDa)	10 kDa family (~10 kDa)	16 kDa protein (~16 kDa)	(Glutathione S transferase like proteins) (~27 kDa)	11.5 kDa protein (~11 kDa)	ML domain peptide family (~14 kDa)
P. perniciosus (Anderson et al., 2006)	PpeSP07	PpeSP04, 04B, 10	PpeSP05							
P. perniciosus Madrid Spain (Martin et al., 2013)		SP04, 04B								
P. tobbi (Rohousova et al., 2012)	PtSP77-79	PtSP42, 44, 54, 56-58, 60	PtSP27-29					PtSP73, 75, 76		
P. orientalis Addis Zemen (Vlkova et al., 2014)	PorASP74, 76	PorASP46, 48, 122	PorASP86				PorASP150	PorASP106		
P. orientalis Melka Werer (Vlkova et al., 2014)	PorMSP6, 8	PorMSP28, 38, 43, 67	PorMSP15				PorMSP162	PorMSP65		

Table 4

Sand fly salivary proteins characterized as markers of vector exposure.

Sand fly species	Marker of exposure	Species tested	Type of Leishmaniasis	Reference
Lu. longipalpis	LJM11	Humans, dogs, chicken	VL	(Teixeira et al., 2010)
Lu. longipalpis	LJM17	Humans, dogs, foxes, chicken	VL	(Teixeira et al., 2010)
P. papatasi	PpSP32	Humans	CL	(Marzouki et al., 2012)
P. perniciosus	SP01B	Dogs	VL	(Drahota et al., 2014)
P. perniciosus	SP01	Dogs	VL	(Drahota et al., 2014)
P. perniciosus	SP03B	Dogs	VL	(Drahota et al., 2014)

VL: Visceral Leishmaniasis; CL: Cutaneous Leishmaniasis

Table 5

Salivary vaccine candidates from different sand flies species.

Sand fly species	Vaccine candidate	Protect against	Nature of protective immune response	Model	Family of the protein or protein	Function
P. papatasi	**PpSP15**	***L. major*** (Valenzuela et al., 2001a; Oliveira et al., 2008)	Th1, DTH	Mice	OBP/SL1	Inhibitor of contact activation
Lu. longipalpis	**Maxadilan**	***L. major*** (Morris et al., 2001)	Th1, Humoral immune response	Mice	Maxadilan	Vasodilatator
Lu. longipalpis	**LJM19**	***L. infantum chagasi, L. braziliensis*** (Regis et al., 2008 ; Tavares et al., 2011)	Th1	Hamsters		
Lu. longipalpis	**LJM11**	***L. major*** (Xu et al., 2011; Regis et al., 2012a)	Th1, DTH	Mice	Yellow	Serotonin binding
Lu. intermedia	**Linb-11**	***L. brazileinsis*** (DeMoura et al., 2013)	Th1	Mice		
Lu. longipalpis	**LJM17**	***L. infantum chagasi*** (Collin et al., 2009)	Th1, DTH, IgG2	Dogs	Yellow	Serotonin binding
Lu. longipalpis	**LJL143**	***L. infantum chagasi*** (Collin et al., 2009)	Th1, DTH, IgG2	Dogs	Lufaxin	Anti-coagulant

L., *Leishmania*; Th1, T helper type 1 immune response; DTH, delayed-type hypersensitivity; IgG2, Immunoglobulin G class 2.