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Protein glycosylation in *Leishmania* spp.†

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Protein glycosylation is a co- and post-translational modification that, in *Leishmania* parasites, plays key roles in vector–parasite–vertebrate host interaction. In the mammalian host, *Leishmania* protein glycosylation is involved in virulence, host cell invasion, and immune evasion and modulation. The *Leishmania* glycocalyx is composed by a dense array of glycoconjugates including lipophosphoglycan, glycoinositolphospholipids, glycoproteins and proteophosphoglycans which varies in composition between *Leishmania* species and developmental stages. The current knowledge on *Leishmania* protein glycosylation is quite limited. The development of novel analytical tools to characterize the *Leishmania* glycoproteome and the expanding toolbox to modulate the parasite glycocode will help in deciphering the processes involved in *Leishmania*–host interaction. This review will recapitulate the current knowledge of *Leishmania* protein glycosylation, and glycan structures reported, and the potential application of mass spectrometry-based analysis for system-wide *Leishmania* glycoproteome and glycome analysis.

Introduction

Leishmania biology and life cycle

Leishmania spp. are unicellular protozoan parasites belonging to the *Trypanosomatidae* family, and are the etiological agents of leishmaniasis. Of the 53 known *Leishmania* species, 20 are pathogenic to humans.[1] Leishmaniasis is prevalent in the tropical, subtropical and Mediterranean basin affecting over 98 countries,[2] with over 350 million people at risk of infection.[1,3] The *dipteran* sandfly insects of the genus *Phlebotomus* and *Lutzomyia* of the Old and New Worlds, respectively, are the biological vectors of *Leishmania* parasites.[4] Organ transplant,[5,6] blood transfusion[7] and in rare cases congenital transmission[8,9] may also contribute to leishmaniasis transmission. The World Health Organization (WHO) estimates that 0.7–1 million new cases and 20 000–30 000 deaths are reported each year,[10] while current infection cases are estimated at 12 million. Leishmaniasis has varying clinical pathology and disease outcomes, depending on the infecting *Leishmania* spp. and host factors including the immune status.[11] Infected individuals can be asymptomatic or may present one of the two main clinical forms: cutaneous/tegumentary and visceral leishmaniasis, which account for approximately 0.7–1.2 and 0.2–0.4 million new cases each year, respectively.[12] Cutaneous leishmaniasis (CL) is further subdivided into localized disease, the most common form, characterized by localized ulcerative lesions at or near the site of the bite, and the mucocutaneous form, in which the parasites reach the mucosa of the upper respiratory tract affecting the nasal cavity and often the oral cavity.[13] Other rare forms of CL include disseminated leishmaniasis (DL)[14] and diffuse cutaneous leishmaniasis (DCL).[15,16] Visceral leishmaniasis occurs through the metastasis of infected cells from the bite site to visceral organs such as spleen, liver and bone marrow, causing hepatosplenomegaly, pancytopenia, thrombocytopenia, and anemia due to marrow suppression,[17] and is fatal if left untreated. Post-kalazar dermal leishmaniasis (PKDL) occurs in clinically cured patients of visceral leishmaniasis in East Africa and the Indian subcontinent, where *L. donovani* is the causative agent.[18] Clinical diagnosis is complicated and lacks specificity due to the broad clinical spectrum of leishmaniasis and closely related diseases, which usually co-exist in endemic regions.[19,20] Few chemotherapeutic agents exist, but are limited by high costs, toxicity, and treatment failures due to resistance.[21,22] As leishmaniasis is endemic mostly in developing countries, there is little interest by pharmaceutical companies and public health authorities to invest in drug and vaccine development, research, prevention, or control strategies.[23,24] Rightly, the inclusion of leishmaniasis as a neglected tropical disease (NTD) by WHO is commendable for it has allowed improved focus on surveillance, technical and financial support for control programs, monitoring disease trends, prevention, promotion of research and the use of safe and affordable drugs.[10]

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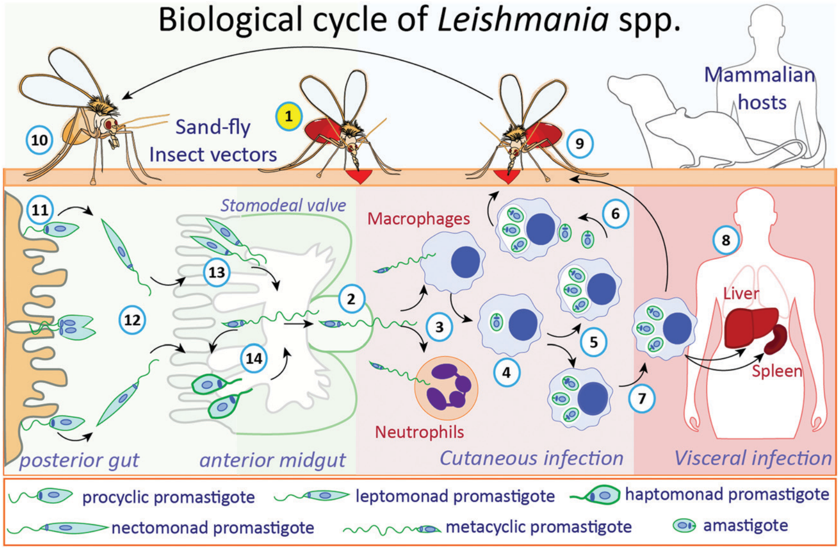


Fig. 1 The biological cycle of *Leishmania* spp. During a blood meal (1, in yellow circle), infected female phlebotomine insects regurgitate blood, transmitting, and releasing the non-replicative but infective metacyclic promastigotes (2) into the mammalian host. These flagellated forms are phagocytosed by macrophages and neutrophils (3). In macrophages, parasites transform into infective amastigotes (4), which multiply by binary fission (5), following the exit from ruptured cells and infect other neighbor cells (6). For *Leishmania* species associated with visceral infection, different organs can be reached, such as liver or spleen, by infected macrophages or released amastigotes (7), where parasites will replicate and establish the infection (8). When a phlebotomine insect takes a blood meal from an infected mammalian host (9), surrounded cells containing-amastigotes or released free amastigotes will be ingested. Inside the insect's gut (10), amastigotes will transform into procyclic promastigotes in the posterior gut (11), which will multiply and migrate (12) to the anterior midgut (stomodeal valve) of the insect (13) where they will differentiate into metacyclic infective forms, establishing the process called metacyclogenesis. Metacyclic promastigotes that are not regurgitated during the blood meal undergo re-differentiation into retro-leptomonad promastigotes, which replicate inside the vector, increasing the haptomonad promastigotes attached to the anterior sandfly midgut (14).

The life cycle of *Leishmania* parasites alternates between sandfly insect vectors and mammalian hosts, including humans (Fig. 1). During a blood meal, infected female phlebotomine insects transmit the non-replicative infective metacyclic promastigotes to the bite site in the host. This mobile and flagellated form is phagocytosed by immune cells, such as macrophages and neutrophils.[25,26] In the macrophages, the main *Leishmania* host cells, metacyclic promastigotes transform into amastigotes inside parasitophorous vacuoles, where they multiply and subsequently are released upon host cell rapture, infecting other cells.[27] When a phlebotomine insect takes a blood meal from an infected vertebrate host, cells containing amastigotes or free amastigotes are ingested. Inside the insect's gut, amastigotes transform into procyclic promastigotes, which multiply and migrate to the anterior midgut (stomodeal valve) of the insect where they undergo metacyclogenesis, differentiating into metacyclic infective forms, which are passed to a new mammalian host during a blood meal through regurgitation.[27] Metacyclic promastigotes that are not regurgitated during a blood meal may undergo re-differentiation into retroleptomonad promastigotes, which replicate inside the vector, increasing the haptomonad promastigotes attached to the sandfly anterior midgut.[28]

Chemotherapy of leishmaniasis

The chemotherapy for leishmaniasis counts on few drugs: pentavalent antimonials, pentamidine, amphotericin B and miltefosine (Table 1). Most of them are considerably toxic, have high cost and, except for miltefosine, must be administered by the parenteral route.[22] Despite their toxicity and narrow therapeutic window, pentavalent antimonials (meglumine antimoniate and sodium stibogluconate) are still the first line drugs for cutaneous leishmaniasis in most endemic countries and for visceral leishmaniasis in regions such as Latin America and East Africa.[22,29] The mechanism of action of pentavalent antimonials (SbV) is not completely understood. Some studies suggest that SbV acts as a prodrug that is reduced by the macrophage or by the amastigote to SbIII, more toxic and active against *Leishmania*.[30] Others support the direct involvement of SbV in parasite death.[31] SbV was shown to inhibit *Leishmania* type I DNA topoisomerase. It also forms complexes with ribonucleosides, and these complexes may either inhibit *Leishmania* purine transporters or be internalized and inhibit the purine salvage pathway. This process leads to the depletion of ATP and GTP (revised in ref. 31). The drug also affects the immune system. It was shown to damper the activity of protein tyrosine phosphatases, increasing cytokine responses, and to augment the phagocytic capacity of monocytes and neutrophils and the production of superoxide anion by phagocytes (revised in ref. 31). SbIII interacts with sulfhydryl-containing biomolecules such as thiols, peptides, proteins and enzymes. It affects parasite's redox equilibrium by two mechanisms: by interacting with trypanothione reductase, leading to accumulation of disulfide forms of glutathione and trypanothione, and by increasing the

Table 1 Drugs available for treating leishmaniasis and their main mechanisms of action

Drug	Mechanisms of action	Ref.
Pentavalent antimonials (SbV) (meglumine antimoniate and sodium stibogluconate)	Inhibition of type I DNA topoisomerase Inhibition of purine transporters or of purine salvage pathway, depleting ATP and GTP Increase in cytokine responses, in phagocytic capacity of monocytes and neutrophils and in the production of superoxide anion by phagocytes Interaction with trypanothione reductase, leading to accumulation of disulfide forms of glutathione and trypanothione Increase in the efflux of trypanothione and glutathione, increasing reactive oxygen species levels Induction of oligonucleosomal DNA fragmentation	31–33
Pentamidine	Decrease in ornithine decarboxylase and spermine synthase, leading to a decrease in polyamines Enlargement of mitochondria and disintegration of the kinetoplast structure Inhibition of phosphohydrolytic activity of nucleoside triphosphate diphosphohydrolase (NTPDase)	34–36
Amphotericin B (AmB)	Binding to ergosterol-related sterols, creating pores in the plasma membrane that lead to exchange of ions across the surface and consequent cell death Induction of oxidative stress	29
Miltefosine	Decrease in intracellular choline, affecting cell membrane composition Induction of mitochondrial depolarization, reduction in cytochrome-*c* oxidase activity and decrease of intracellular ATP levels, leading to cell death	22 and 29

efflux of trypanothione and glutathione, increasing the levels of reactive oxygen species (ROS).[32] Besides, SbIII induces parasite death by oligonucleosomal DNA fragmentation.[33]

Pentamidine interferes with the synthesis of polyamines by decreasing ornithine decarboxylase and spermine synthase. It also probably competes with polyamines for binding to nucleic acid, particularly kinetoplast DNA.[34] In fact, the drug induces changes in the kinetoplast and mitochondria, with enlargement of mitochondria and disintegration of the kinetoplast structure.[35] Besides, it was shown to inhibit phosphohydrolytic activity of nucleoside triphosphate diphosphohydrolase (NTPDase).[36]

Amphotericin B (AmB) was first employed as an antifungal agent and later used to treat leishmaniasis due to its ability to bind to ergosterol-related sterols, the main membrane sterols in both organisms (revised in ref. 29). Its interaction with these lipids creates a pore, allowing the exchange of ions across the surface and consequent cell death. AmB also induces oxidative stress.[29]

Miltefosine binds to the plasma membrane and is internalized either by the endocytic pathway or by complex formed by the miltefosine transporter (MT) and its non-catalytic subunit Ros3 (revised in ref. 22). This complex is responsible for phosphocholine accumulation, and by decreasing intracellular choline, the drug inhibits phospholipid metabolism and phosphatidylcholine and phosphatidylethanolamine synthesis, affecting cell membrane composition (revised in ref. 22). Miltefosine treatment also induces mitochondrial depolarization, a reduction in cytochrome-*c* oxidase activity and a decrease of intracellular ATP levels, leading to cell death.[22,29]

Treatment failure has been reported for all drugs mentioned. Numerous facts account for failure, including parasite resistance to the drug and host factors. Combination therapies employing two or more drugs with different mechanisms of action may reduce the development of resistance.[29] Besides, new drugs must be tested. Although there have been some advances in the treatment of visceral leishmaniasis and some novel compounds are currently in pre-clinical and clinical phases for this disease form, there were few advances in drug research and development for cutaneous leishmaniasis, the most common disease form.[37]

The role of glycans in *Leishmania* biology

Glycans may occur as free oligosaccharides or as simple (*e.g.* monosaccharides such as the addition of *O*-linked GlcNAc to nucleocytoplasmic proteins) or complex (oligosaccharides such as complex and/or hybrid *N*-linked glycans) structures covalently attached to different macromolecules such as proteins and lipids to form glycoproteins, proteoglycans or proteophosphoglycans, and glycolipids, respectively.[38] During the intricate life cycle of *Leishmania* spp. between the invertebrate insect vector and vertebrate hosts, glycoconjugates play crucial roles in the interaction and survival of the parasite. The surface membrane of *Leishmania* spp. is covered by a dense layer of glycoconjugates collectively termed glycocalyx, which play different roles in the parasites' survival, infectivity, virulence and establishment of disease. The *Leishmania* spp. surface membrane glycoconjugates include lipophosphoglycans (LPGs), glycoinositolphospholipids (GIPLs), glycoproteins and proteophosphoglycans (PPGs), and seminal reviews and studies have been published describing their molecular and functional characterization.[39–55] These *Leishmania* spp. glycoconjugate structures are been summarized in Fig. 2.

Glycosyl-phosphatidylinositols (GPIs) anchor LPGs, cell membrane glycoproteins, proteophosphoglycans (PPGs) and glycoinositolphospholipids (GIPLs) to the plasma membrane.[43] GPI anchors are ubiquitously conserved in other protozoan and higher eukaryotic cells, and have the general structure composed of a conserved backbone of ethanolamine-phosphate-Manα1-2Manα1-6Manα1-4GlcNα1-6myo-inositol.[43] GIPLs are the free (unattached to phosphoglycans or proteins), low molecular

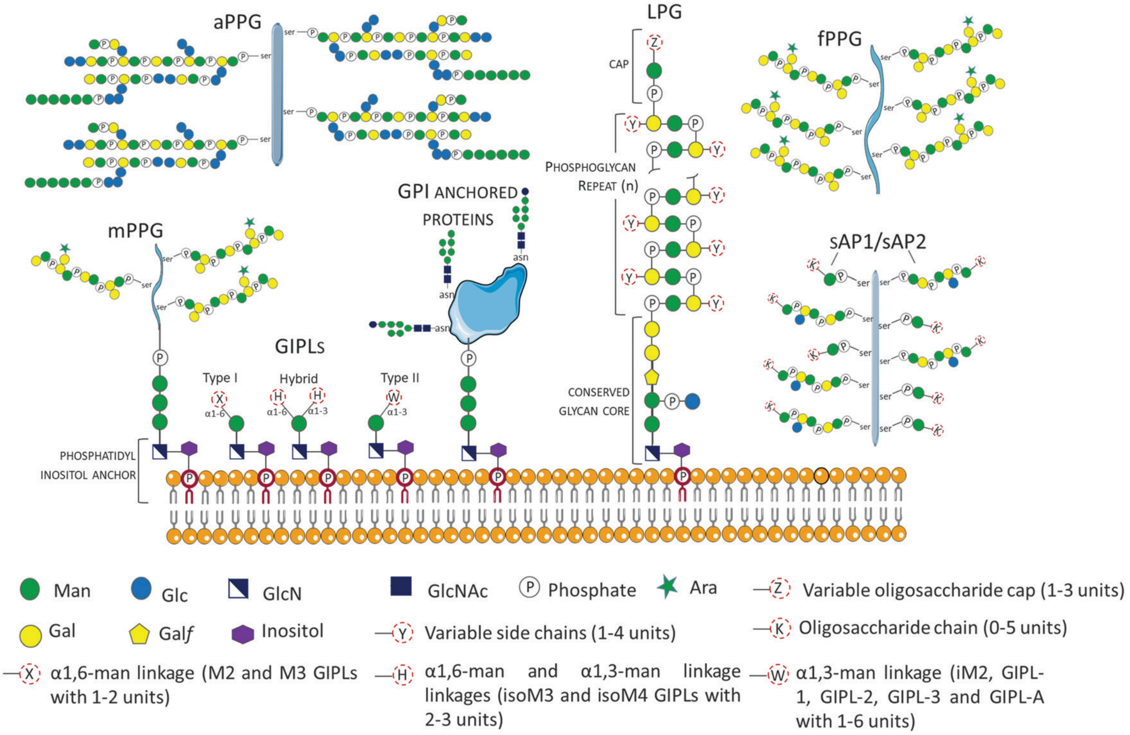


Fig. 2 Schematic representation of glycoconjugates in *Leishmania* spp. The glycoconjugates include the GPI anchored lipophosphoglycans (LPGs)[39,40,43,55] and membrane proteins (*e.g.* gp63),[57] and the free low molecular weight glycoinositolphospholipids (GIPLs).[41–43,56,58] Proteophosphoglycans (PPGs)[45,46,57,59,60] predominantly expressed in promastigote life stage can be bound to the membrane (mPPGs), or they can be secreted (sAPs and fPPGs). A structurally different aPPG is predominantly expressed in the amastigote life stage.

weight GPIs, with a conserved Manα1-4GlcN linked to an alkylacylglycerol through a phosphatidylinositol (PI) residue.[41,43,53,56,57] GIPLs are the most abundant glycoconjugates in both promastigote and amastigote life stages,[42,43] and show species and stage-specific modulation.[42,53,56,58,61] Based on monosaccharide substitutions in glycan moiety, GIPLs are classified in three types[43,56,57] (Fig. 2). Type I GIPLs have α1,6-mannose linked to Manα1-4GlcN, and are structurally similar to protein GPI anchors. Type II GIPL glycan moiety is structurally related to LPG with α1,3-mannose residue linked to Manα1-4GlcN. Hybrid-type GILPs have both α1,6-mannose and α1,3-mannose linked to the Manα1-4GlcN motif.[43,44] GIPLs play important roles in macrophage infectivity,[62] and modulation of the innate immune system by inhibition of cytokines and nitrite production.[53] In *L. mexicana*, GIPLs were shown to be essential for growth.[49,63]

In *Leishmania* spp. promastigote life stage, LPGs are predominantly expressed on the flagellar and cell surface.[39,64,65] The general structure of the LPG molecule is composed of terminating oligosaccharide cap structures, a repeating phosphorylated saccharide region attached to a lyso-alkyl phosphatidylinositol (lysoalkyl-PI) lipid anchor through a phosphorylated hexasaccharide glycan core (Fig. 2).[39,40,66] The role of LPG in host–parasite interaction has been extensively reported to be an important virulence factor with different roles depending on the *Leishmania* species.[67,68] *Leishmania* spp. promastigote stage surface membrane is predominantly covered by species-specific and growth stage specific lipophosphoglycans (LPGs).[39,41,66] *L. major* amasigotes express a structurally, biochemically and antigenically distinct LPG from the promastigote life stage parasites.[69,70] LPG from *L. donovani* was shown to be a potent inhibitor of PKC activity *in vitro*.[71] During infection, PKC is involved in the generation of macrophage's oxidative burst, which leads to the destruction of invading microbes. In infections with *L. mexicana*, LPG differentially regulates PKCa in macrophages derived from BALB/c or C57BL/6 mice by inhibiting or stimulating their activities, respectively.[72] Promastigote LPGs from *L. amazonensis* play an immune modulatory role by the induction of neutrophil extracellular traps (NETs).[73] Inside the macrophage, LPG inhibits maturation of the phagosome at the early stage of infection,[74] allowing the parasite's survival within the vacuole until their differentiation into amastigotes. In addition, *L. amazonensis* LPG has been demonstrated to induce the activation of the promotor for protein kinase R (PKR), whose expression favors infection by the induction of IFN-1.[75] LPG also plays a vital role in the parasite–vector interaction. Indeed, LPG has been shown to be involved in the protection of promastigotes from the insect's digestive system, as demonstrated by the inability of *L. donovani* LPG2 mutants deficient in LPG and other phosphoglycans to survive in the hydrolytic environment of the insect midgut.[76] Besides, LPG has been demonstrated to be involved in the binding of promastigotes to epithelial cells in the insect midgut, preventing the parasites from being excreted with the blood meal.[76,77] Structural and functional details of LPG have been extensively reviewed.[39,54,76,78]

Among *Leishmania* spp. glycoconjugates with protein components include glycoproteins and proteophosphoglycans (PPGs). Protein glycosylation is a co- and post-translational modification in which glycan macromolecules are covalently attached to specific amino acid residues. Depending on the amino acid residue attachment of the glycan moiety, two major classes of protein glycosylation have been reported; *N*-linked glycosylation and *O*-linked glycosylation where the glycans are attached to asparagine (Asn) and serine (Ser)/threonine (Thr) amino acid side chains, respectively.[79] In *Leishmania* parasites, *N*-linked glycosylation has been associated with virulence, immune evasion, host–cell interaction (including, adhesion and invasion), host immune modulation and structural integrity of proteins.[80–85] The highly *O*-glycosylated proteophosphoglycans play crucial roles in the parasites' complement activation, inhibition of lysis by serum, and prevention of opsonization of amastigote life stages.[86] In addition, *O*-GlcNAc, a unique type of protein *O*-glycosylation of nucleocytoplasmic proteins, was described in *Leishmania* spp. gp96/92,[87] and has been proposed to exert glycan-dependent signaling similar to protein phosphorylation such as transcriptional activation, nuclear transport and degradation of proteins.[88,89]

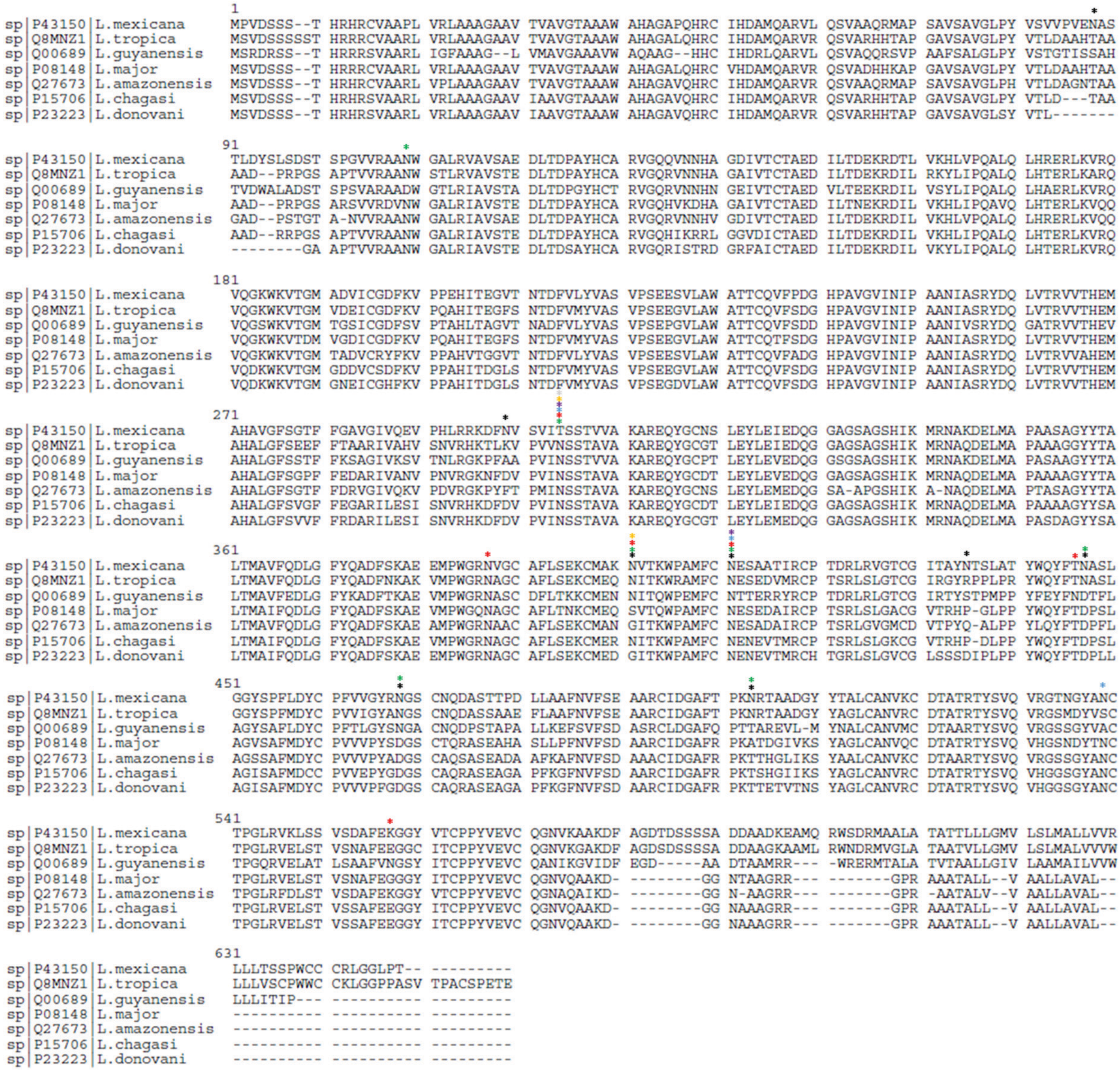
One of the most studied *Leishmania* glycoproteins is gp63, also known as leishmanolysin, a 60–66 kDa zinc-metalloprotease recognized as a major surface antigen and the most abundant surface protein in promastigotes[90–92] and also an important virulence factor.[93] Gp63, a predominant *N*-glycosylated surface protein is present in both promastigote and amastigote stages, and confers protection from phagolysosomal degradation in the host macrophages.[94,95] In addition, gp63 is involved in the attachment of promastigotes to macrophages,[83,84,96] and in the cleavage of C3b to iC3b, avoiding parasite lysis by the complement cascade.[97] The generated iC3b acts in the opsonization of the parasite, crucial for internalization by the macrophage receptor CR3.[97,98] Fibronectin-like properties of GP63 have also been demonstrated, with studies showing the interaction of gp63 with β1 integrins, receptors for fibronectin.[96,99,100] Moreover, gp63 mediates the degradation of components of the extracellular matrix or subcutaneous tissue.[101]

In Uniprot database,[102] gp63 protein from different *Leishmania* spp. show varying numbers of *N*-linked glycosylation sites: *L. mexicana* (P43150) has 8 sites, *L. tropica* (Q8MNZ1) has 7, *L. guyanensis* (Q00689) has 6, *L. major* (P08148) has 3, *L. amazonensis* (Q27673) and *L. chagasi* (P15706) have 2 sites each, and *L. donovani* (P23223) has 1 sites, indicating a species-specific *N*-linked glycosylation pattern. Based on sequence alignment analysis (Fig. 3), shared and specie-specific *N*-linked glycosylation sites were identified. All *Leishmania* spp. gp63 amino acid sequences, with the exception of *L. mexicana*, have a conserved *N*-glycosite at position 304 suggesting a conserved role. At position 401, *L. mexicana*, *L. tropica*, *L. guyanensis* and *L. chagasi* gp63 share a conserved *N*-linked glycosylation site. At position 411, *L. mexicana*, *L. tropica*, *L. guyanensis*, *L. major* and *L. amazonensis* gp63 glycoprotein share a conserved *N*-glycosylation site, which is absent for *L. chagasi* and *L. donovani*. The glycosylation sites on gp63 glycoprotein indicate that *Leishmania* parasites have shaped their protein glycosylation sites in a species-specific manner.

Three *N*-glycosylation sites were reported on *L. major* gp63 protein by gene transfection into gp63 deficient *L. amazonensis* promastigotes and characterization by site-specific mutagenesis.[94] Structural analysis of released gp63 *N*-linked glycans from *L. amazonensis* revealed 4 major biantennary *N*-linked oligomannose structures, with no evidence of hybrid or complex *N*-glycan structures.[103] As shown in Table S1 (ESI†), there is species-specific *N*-linked protein glycosylation on gp63, as evidenced in GlyConnect.[104] In *L. donovani* and *L. major* promastigote life stage, 2 *N*-linked oligosaccharides structures ($Man_6GlcNAc_2$ and $GlcMan_6GlcNAc_2$) were reported by Funk and colleagues, who showed that the glycosylation was not consistent between the two species in the amastigote life stage, with *L. donovani* lacking *N*-linked glycans, indicating a specie and life stage-specific protein *N*-linked glycosylation.[105]

Tunicamycin, a nucleoside analog inhibitor of GlcNAc-1-P-transferase (ALG7, Fig. 4) which transfers *N*-acetylglucosamine-1-phosphate to dolichol monophosphate,[106] has been used by different research groups to establish the functions of protein *N*-linked glycosylation in *Leishmania* spp. *L. chagasi* promastigotes expressing deglycosylated gp63 surface proteins from tunicamycin-resistant population showed that deglycosylated form of gp63 was proteolytically inactive compared to the glycosylated form.[107] This strain was able to bind to the receptor for the iC3b fragment of complement, CR3, but not to the mannose receptor indicating a selective *N*-linked glycan-dependent binding.[107] On the contrary, treatment of *L. major* promastigotes with tunicamycin and the deglycosylation of gp63 from *L. major* and *L. amazonensis* promastigotes demonstrated that deglycosylation did not interfere with the proteolytic activity of this membrane protease,[108] suggesting that gp63 folding, function, signaling to the plasma membrane, and resistance to proteolysis were not dependent on its *N*-linked glycosylation.[108,109] These conflicting results could arise from differences in degrees of deglycosylation of gp63,[94] differences between *Leishmania* species, enzymatic assays, and the limited site-specific knowledge on gp63 glycosylation modulation upon tunicamycin and/or glycosidase treatment.

Proteophosphoglycans (PPGs) are modified by phosphoglycosylation of phosphoglycan chains through the unusual Manα1-PO(4)-Ser linkage.[46] Filamentous (fPPG), membrane (mPPG) and secreted acid phosphatases (SAP) are primarily synthesized by the promasigote life stage, while the non-filamentous (aPPG) is secreted in the amastigote life stage.[45] In the sandfly, PPGs play important roles in parasite–vector interaction, including insect gut colonization by conferring resistance to insect digestive enzymes.[110,111] fPPGs aid in the efficient transmission of the parasites to the mammalian hosts through plug formation.[112] In the mammalian host, PPGs increase macrophage recruitment to the site of the bite, augmenting the activity of arginase generating polyamides involved in the growth of the parasite, which reduces L-arginine for the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS).[113] Inside the macrophages, secreted amastigote PPGs play a key role in the enhancement of the maturation of parasitophorous vacuoles.[59,114]



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                            1
sp|P43150|L.mexicana       MPVDSSS--T HRHRCVAAPL VRLAAAGAAV TVAVGTAAAW AHAGAPQHRC IHDAMQARVL QSVAAQRMAP SAVSAVGLPY VSVVPVENAS
sp|Q8MNZ1|L.tropica        MSVDSSSSST HRRRCVAARL VRLAAAGAAV TVAVGTAAAW AHAGALQHRC IHDAMQARVR QSVARHHTAP GAVSAVGLPY VTLDAAHTAA
sp|Q00689|L.guyanensis     MSRDRSS--T HRRRSVAARL IGFAAAG--L VMAVGAAAVW AQAAG--HHC IHDRLQARVL QSVAQQRSVP AAFSALGLPY VSTGTISSAH
sp|P08148|L.major          MSVDSSS--T HRRRCVAARL VRLAAAGAAV TVAVGTAAAW AHAGALQHRC VHDAMQARVR QSVADHHKAP GAVSAVGLPY VTLDAAHTAA
sp|Q27673|L.amazonensis    MSVDSSS--T HRHRCVAARL VPLAAAGAAV TVAVGTAAAW AHAGAVQHRC IHDAMQARVR QSVAAQRMAP SAVSAVGLPH VTLDAGNTAA
sp|P15706|L.chagasi        MSVDSSS--T HRHRSVAARL VRLAAAGAAV IAAVGTAAAW AHAGAVQHRC IHDAMQARVR QSVARHHTAP GAVSAVGLPY VTLD---TAA
sp|P23223|L.donovani       MSVDSSS--T HRHRSVAARL VRLAAAGAAV IAAVGTAAAW AHAGAVQHRC IHDAMQARVR QSVARHHTAP GAVSAVGLSY VTL-------

                          91
sp|P43150|L.mexicana       TLDYSLSDST SPGVVRAANW GALRVAVSAE DLTDPAYHCA RVGQQVNNHA GDIVTCTAED ILTDEKRDTL VKHLVPQALQ LHRERLKVRQ
sp|Q8MNZ1|L.tropica        AAD--PRPGS APTVVRAANW STLRVAVSTE DLTDPAYHCA RVGQRVNNHA GAIVTCTAED ILTDEKRDIL RKYLIPQALQ LHTERLKARQ
sp|Q00689|L.guyanensis     TVDWALADST SPSVARAADW GTLRIAVSTA DLTDPGYHCT RVGQRVNNHN GEIVTCTAED VLTEEKRDIL VSYLIPQALQ LHAERLKVRQ
sp|P08148|L.major          AAD--PRPGS ARSVVRDVNW GALRIAVSTE DLTDPAYHCA RVGQHVKDHA GAIVTCTAED ILTNEKRDIL VKHLIPQAVQ LHTERLKVQQ
sp|Q27673|L.amazonensis    GAD--PSTGT A-NVVRAANW GALRIAVSAE DLTDPAYHCA RVGQRVNNHV GDIVTCTAED ILTDEKRDIL VKHLVPQALQ LHRERLKVQQ
sp|P15706|L.chagasi        AAD--RRPGS APTVVRAANW GALRIAVSTE DLTDPAYHCA RVGQHIKRRL GGVDICTAED ILTDEKRDIL VKHLIPQALQ LHTERLKVRQ
sp|P23223|L.donovani       --------GA APTVVRAANW GALRIAVSTE DLTDSAYHCA RVGQRISTRD GRFAICTAED ILTDEKRDIL VKYLIPQALQ LHTERLKVRQ

                         181
sp|P43150|L.mexicana       VQGKWKVTGM ADVICGDFKV PPEHITEGVT NTDFVLYVAS VPSEESVLAW ATTCQVFPDG HPAVGVINIP AANIASRYDQ LVTRVVTHEM
sp|Q8MNZ1|L.tropica        VQGKWKVTGM VDEICGDFKV PQAHITEGFS NTDFVMYVAS VPSEEGVLAW ATTCQVFSDG HPAVGVINIP AANIASRYDQ LVTRVVTHEM
sp|Q00689|L.guyanensis     VQGSWKVTGM TGSICGDFSV PTAHLTAGVT NADFVLYVAS VPSEPGVLAW ATTCQVFSDD HPAVGVINIP AANIVSRYDQ GATRVVTHEV
sp|P08148|L.major          VQGKWKVTDM VGDICGDFKV PQAHITEGFS NTDFVMYVAS VPSEEGVLAW ATTCQTFSDG HPAVGVINIP AANIASRYDQ LVTRVVTHEM
sp|Q27673|L.amazonensis    VQGKWKVTGM TADVCRYFKV PPAHVTGGVT NTDFVLYVAS VPSEESVLAW ATTCQVFADG HPAVGVINIP AANIASRYDQ LVTRVVAHEM
sp|P15706|L.chagasi        VQDKWKVTGM GDDVCSDFKV PPAHITDGLS NTDFVMYVAS VPSEEGVLAW ATTCQVFSDG HPAVGVINIP AANIASRYDQ LVTRVVTHEM
sp|P23223|L.donovani       VQDKWKVTGM GNEICGHFKV PPAHITDGLS NTDFVMYVAS VPSEGDVLAW ATTCQVFSDG HPAVGVINIP AANIASRYDQ LVTRVVTHEM

                         271
sp|P43150|L.mexicana       AHAVGFSGTF FGAVGIVQEV PHLRRKDFNV SVITSSTVVA KAREQYGCNS LEYLEIEDQG GAGSAGSHIK MRNAKDELMA PAASAGYYTA
sp|Q8MNZ1|L.tropica        AHALGFSEEF FTAARIVAHV SNVRHKTLKV PVVNSSTAVA KAREQYGCGT LEYLEIEDQG GAGSAGSHIK MRNAQDELMA PAAAGGYYTA
sp|Q00689|L.guyanensis     AHALGFSSTF FKSAGIVKSV TNLRGKPFAA PVINSSTVVA KAREQYGCPT LEYLEVEDQG GSGSAGSHIK MRNAKDELMA PASAAGYYTA
sp|P08148|L.major          AHALGFSGPF FEDARIVANV PNVRGKNFDV PVINSSTAVA KAREQYGCDT LEYLEVEDQG GAGSAGSHIK MRNAQDELMA PAAAAGYYTA
sp|Q27673|L.amazonensis    AHALGFSGTF FDRVGIVQKV PDVRGKPYFT PMINSSTAVA KAREQYGCNS LEYLEMEDQG SA-APGSHIK A-NAQDELMA PTASAGYYTA
sp|P15706|L.chagasi        AHALGFSVGF FEGARILESI SNVRHKDFDV PVINSSTAVA KAREQYGCDT LEYLEIEDQG GAGSAGSHIK MRNAQDELMA PAAAAGYYSA
sp|P23223|L.donovani       AHALGFSVVF FRDARILESI SNVRHKDFDV PVINSSTAVA KAREQYGCGT LEYLEMEDQG GAGSAGSHIK MRNAQDELMA PASDAGYYSA

                         361
sp|P43150|L.mexicana       LTMAVFQDLG FYQADFSKAE EMPWGRNVGC AFLSEKCMAK NVTKWPAMFC NESAATIRCP TDRLRVGTCG ITAYNTSLAT YWQYFTNASL
sp|Q8MNZ1|L.tropica        LTMAVFQDLG FYQADFNKAK VMPWGRNAGC AFLSEKCMEQ NITKWRAMFC NESEDVMRCP TSRLSLGTCG IRGYRPPLPR YWQYFTNASL
sp|Q00689|L.guyanensis     LTMAVFEDLG FYKADFTKAE VMPWGRNASC DFLTKKCMEN NITQWPEMFC NTTERRYRCP TDRLRLGTCG IRTYSTPMPP YFEYFNDTFL
sp|P08148|L.major          LTMAIFQDLG FYQADFSKAE VMPWGQNAGC AFLTNKCMEQ SVTQWPAMFC NESEDAIRCP TSRLSLGACG VTRHP-GLPP YWQYFTDPSL
sp|Q27673|L.amazonensis    LTMAVFQDLG FYQADFSKAE AMPWGRNAAC AFLSEKCMAN GITKWPAMFC NESADAIRCP TSRLGVGMCD VTPYQ-ALPP YLQYFTDPFL
sp|P15706|L.chagasi        LTMAIFQDLG FYQADFSKAE VMPWGRNAGC AFLSEKCMER NITKWPAMFC NENEVTMRCP TSRLSLGKCG VTRHP-DLPP YWQYFTDPSL
sp|P23223|L.donovani       LTMAIFQDLG FYQADFSKAE EMPWGRNAGC AFLSEKCMED GITKWPAMFC NENEVTMRCH TGRLSLGVCG LSSSDIPLPP YWQYFTDPLL

                         451
sp|P43150|L.mexicana       GGYSPFLDYC PFVVGYRNGS CNQDASTTPD LLAAFNVFSE AARCIDGAFT PKNRTAADGY YTALCANVKC DTATRTYSVQ VRGTNGYANC
sp|Q8MNZ1|L.tropica        GGYSPFMDYC PVVIGYANGS CNQDASSAAE FLAAFNVFSE AARCIDGAFT PKNRTAADGY YAGLCANVRC DTATRTYSVQ VRGSMDYVSC
sp|Q00689|L.guyanensis     AGYSAFLDYC PFTLGYSNGA CNQDPSTAPA LLKEFSVFSD ASRCLDGAFQ PTTAREVL-M YNALCANVMC DTAARTYSVQ VRGSSGYVAC
sp|P08148|L.major          AGVSAFMDYC PVVVPYSDGS CTQRASEAHA SLLPFNVFSD AARCIDGAFR PKATDGIVKS YAGLCANVQC DTATRTYSVQ VHGSNDYTNC
sp|Q27673|L.amazonensis    AGSSAFMDYC PVVVPYADGS CAQSASEADA AFKAFNVFSD AAACIDGAFR PKTTHGLIKS YAALCANVKC DTAARTYSVQ VRGSSGYANC
sp|P15706|L.chagasi        AGISAFMDCC PVVEPYGDGS CAQRASEAGA PFKGFNVFSD AARCIDGAFR PKTSHGIIKS YAGLCANVRC DTATRTYSVQ VHGGSGYANC
sp|P23223|L.donovani       AGISAFMDYC PVVVPFGDGS CAQRASEAGA PFKGFNVFSD AARCIDGAFR PKTTETVTNS YAGLCANVRC DTATRTYSVQ VHGGSGYANC

                         541
sp|P43150|L.mexicana       TPGLRVKLSS VSDAFEKGGY VTCPPYVEVC QGNVKAAKDF AGDTDSSSSA DDAADKEAMQ RWSDRMAALA TATTLLLGMV LSLMALLVVR
sp|Q8MNZ1|L.tropica        TPGLRVELST VSNAFEEGGC ITCPPYVEVC QGNVKGAKDF AGDSDSSSSA DDAAGKAAML RWNDRMVGLA TAATVLLGMV LSLMALVVVW
sp|Q00689|L.guyanensis     TPGQRVELAT LSAAFVNGSY ITCPPYVEVC QANIKGVIDF EGD-----AA DTAAMRR--- -WRERMTALA TVTAALLGIV LAAMAILVVW
sp|P08148|L.major          TPGLRVELST VSNAFEGGGY ITCPPYVEVC QGNVQAAKD- --------GG NTAAGRR--- -------GPR AAATALL--V AALLAVAL--
sp|Q27673|L.amazonensis    TPGLRFDLST VSDAFEKGGY VTCPPYVEVC QGNAQAIKD- --------GG N-AAGRR--- -------GPR -AATALV--V AALLAVAL--
sp|P15706|L.chagasi        TPGLRVELST VSSAFEEGGY ITCPPYVEVC QGNVQAAKD- --------GG NAAAGRR--- -------GPR AAATALL--V AALLAVAL--
sp|P23223|L.donovani       TPGLRVELST VSSAFEEGGY ITCPPYVEVC QGNVQAAKD- --------GG NAAAGRR--- -------GPR AAATALL--V AALLAVAL--

                         631
sp|P43150|L.mexicana       LLLTSSPWCC CRLGGLPT-- ---------
sp|Q8MNZ1|L.tropica        LLLVSCPWWC CKLGGPPASV TPACSPETE
sp|Q00689|L.guyanensis     LLLITIP--- ---------- ---------
sp|P08148|L.major          ---------- ---------- ---------
sp|Q27673|L.amazonensis    ---------- ---------- ---------
sp|P15706|L.chagasi        ---------- ---------- ---------
sp|P23223|L.donovani       ---------- ---------- ---------
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Fig. 3 Sequence alignment analysis of gp63 from different *Leishmania* spp. highlighting conserved and species-specific *N*-glycosites (N[X]S/T/C, where X is any amino acid except P). The *N*-glycosylation sites for *L. mexicana* (*), *L. tropica* (*), *L. guyanensis* (*), *L. major* (*), *L. amazonensis* (*), *L. chagasi* (*) and *L. donovani* (*) are highlighted.

In this review, protein *N*- and *O*-linked glycosylation in *Leishmania* spp. will be presented, with a focus on the biosynthetic pathways, cellular functions and analytical tools to characterize the glycoconjugates. At present, there is a paucity of system-wide glycoproteome and/or glycome studies on LC-MS/MS-based approaches in *Leishmania* parasites. An in-depth understanding of protein glycosylation would offer unprecedented opportunities for the identification and/or development of next generation glycoprotein-based therapeutics and/or in the uncovering of putative novel drug target candidates. Indeed, glycopeptide-based therapeutics have been developed and used in the treatment of metabolic disorders, development of antivirals, and the shaping of vaccines for viruses and cancer (recently reviewed[115]). Unusual and/or unique features between the parasite and mammalian host glycosylation pathways are highlighted here. Moreover, we have used literature mining to assemble the *Leishmania* spp. glycoproteins described so far.

Protein glycosylation landmarks in *Leishmania* spp.

The surface coat of *L. tarentolae* promastigote stage was detected by Strauss in 1971 using electron microscopy in the presence of rabbit antiserum.[116] In 1973, lectin agglutination assays with concanavalin A and phytohemagglutinin-P (PHA-P) specific for terminal α-D-glucose/α-D-mannose and *N*-acetyl-D-galactosamine sugar moieties, respectively, on trypsinized and non-trypsinized promastigote samples confirmed that the surface of *L. donovani* promastigotes was decorated by sugar molecules.[117] Pre-treatment of *Leishmania* parasites with dextranase and α-amylase enzymes showed reduced agglutination by both Con A and PHA-P lectins, confirming that the

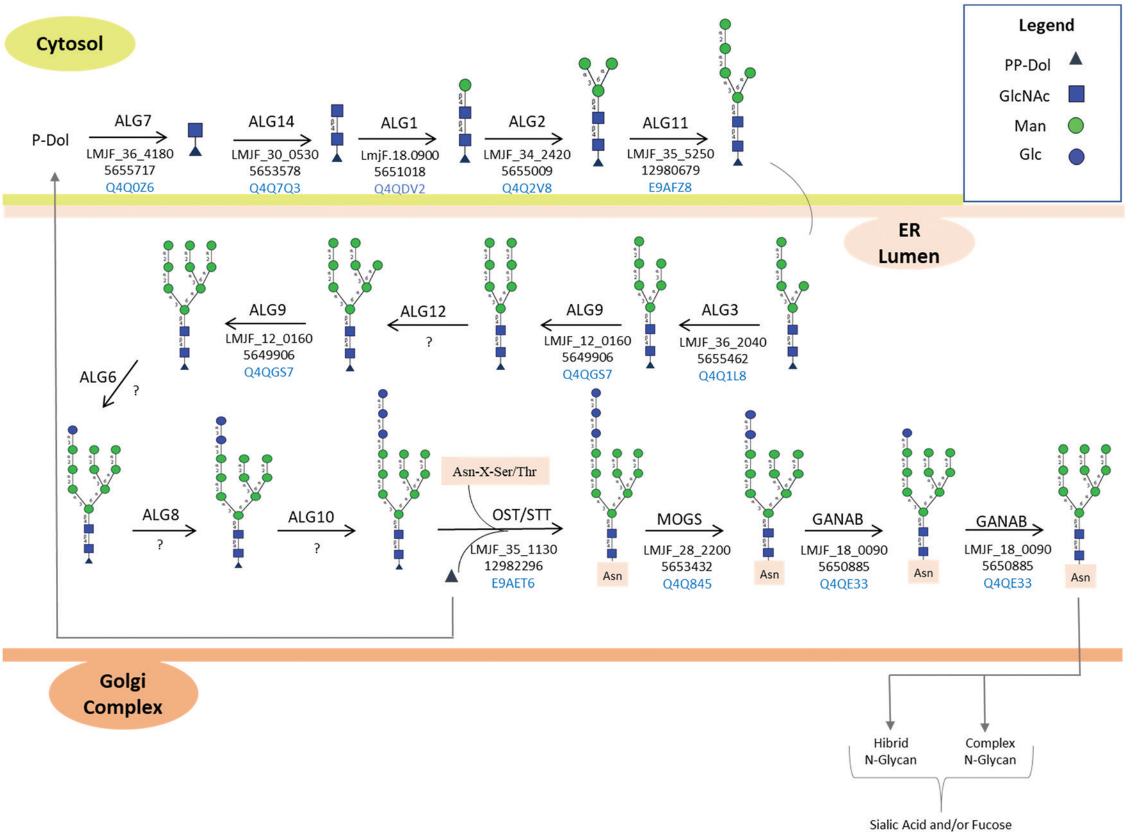

Fig. 4 Protein *N*-glycosylation biosynthesis in *Leishmania* spp. according to KEGG pathway. *L. major*, was used as a model to illustrate the *N*-glycan biosynthetic pathway. The spaces filled by "?" indicate enzymes not mapped in the *Leishmania* spp. genome. Unusual biosynthetic and structural features of *N*-linked protein glycosylation in *Leishmania* spp. are reported in Box 1. ER: endoplasmic reticulum.

polysaccharides contained D-glucose like units with α-1,6 and α-1,4 glyosidic linkages.[117] During the same year, Dwyer and colleagues studied the surface coats of *L. donovani* in the promastigote and amastigote stages by cytochemical techniques coupled to light and electron microscopy.[118] Using alcian blue-lanthanum nitrate and ruthenium dyes which precipitate and stain acid mucopolysaccharides, they were able to stain the parasites' surface coats and established that the cell surface (and flagellar tip for promastigotes) were covered with acid polysaccharide moieties in the different stages of development.

A year later, lectin agglutination assays on *L. braziliensis* promastigote and amastigote stages were carried out to determine the terminal sugar moieties on the polysaccharide chains on the parasite's surface in the different life stages.[119] Agglutination reactions with Con A specifically recognized by α-D-glucose and α-D-mannose terminal sugars and *Ricinus communis* agglutinin (RCA) recognized by α-D-galactose terminal sugars confirmed the presence of polysaccharide complexes with glucose/mannose and/or galactose terminal residues in both life stages.[120] Wheat germ agglutinin (WGA) specific for *N*-acetylglucosamine and sialic acid, soybean agglutinin (SBA), and PHA-P specific for *N*-acetyl-D-galactosamine were negative in both promastigote and amastigote stages, suggesting that the polysaccharides attached to the surface of the parasites lacked these terminal sugar moieties on their cell surfaces, or their presence in undetectable amounts. Interestingly, RCA agglutination was negative for non-infective promastigotes with high passage numbers in cultures, suggesting loss of or processing of D-galactose terminal residues of the carbohydrate chains. Lectin agglutination assays of surface radioiodinated proteins extracted from *L. tropica* promastigotes using detergents showed that concanavalin A agglutinin, but not RCA, WGA, SBA and UEA (Ulex europaeus agglutinin I), agglutinated with the surface components, suggesting the presence of surface glycoproteins with mannose and/or glucose residues.[121] Taken together, these early studies using lectin agglutination assays and glycan-specific stains demonstrated that protein glycosylation is species and strain-specific and modulated during *Leishmania* spp. life stages.

In 1981, Chang demonstrated *L. donovani* surface glycoproteins' role in macrophage binding by pretreatment of promastigotes with enzymes which cleaved off sialic acids (neuraminidase), mannoses (α-mannosidase), GlcNAc (α-*N*-acetylglucosaminidase) and glucose (β-glucosidase) moieties from the parasites surface, which led to a reduction in the binding of the parasites to hamster peritoneal macrophages.[81] In 1984, Parodi and Martin-Berrientos studied the glycoprotein assembly in *Leishmania* parasites looking at the lipid-linked oligosaccharide (LLO) molecules transferred to asparagine (Asn) residues during *N*-glycosylation in *L. mexicana*.[122] They showed that, as opposed to most eukaryotic systems which transfer $Glc_3Man_9GlcNAc_2$ from the carrier lipid to a nascent polypeptide at the asparagine side chain,[123] *L. mexicana* LLO carrier was attached to $Man_6GlcNAc_2$ oligosaccharide chain which migrated equally to a $Man_6GlcNAc_2$ standard by paper chromatography. In addition, paper chromatography analysis of glycoproteins labeled with [U-^{14}C]-glucose showed that processing of the transferred $Man_6GlcNAc_2$ occurred, with a glucose moiety being transiently added to yield $GlcMan_6GlcNAc_2$, but the glycan structure on mature glycoproteins lacks glucose.[122] During this study, no complex and hybrid-type glycan structures were detected.

In 1984, functional studies by Dagger and colleagues on *N*-linked protein glycosylation inhibition using tunicamycin revealed the involvement of protein *N*-linked glycosylation in growth and cellular morphology in *L. braziliensis* promastigotes[124] and macrophage infection by *L. donovani* in 1985 by Nolan and Farrell.[125] Studies on *L. amazonensis* showed growth inhibition of promastigotes in the presence of tunicamycin.[126] In 1987, Lovalace and Gottlieb demonstrated the effect of *N*-linked glycoprotein inhibition using tunicamycin in the growth of *L. donovani* promastigotes, and its role in secreted acid phosphatase activity,[127] which did not interfere with the secretion of the enzyme. Later that year, Bates and Dwyer[128] arrived at the same conclusion by showing the presence of *N*-linked glycans on mature acid phosphatase in *L. donovani* promastigotes using tunicamycin and *N*-glycosidase F treatment, and that the glycosylation of the enzyme did not inhibit its secretion and processing. In 1988, Bates and colleagues applied lectin binding assays using agarose-conjugated lectin beads on secreted proteins from metabolically labeled *L. donovani* promastigotes cultured *in vitro* in RE-III medium lacking bovine serum albumin.[129] Out of 40 electrophoretically distinct bands corresponding to secreted proteins, half were modified with carbohydrate moieties with terminal mannose residues, as demonstrated by positive binding to concanavalin A and *Lens culinaris* lectins.[129] The relevance of protein *N*-glycosylation as a biochemical basis for *Leishmania* virulence was demonstrated by Kink and Chan in 1988[93] who showed decreased virulence of *L. amazonensis* of tunicamycin treated promastigotes as compared to tunicamycin-resistant parasites consistent with wild type parasites.

O-Linked fucosylated glycoconjugates in *Leishmania* parasites have been reported by Guo and colleagues working on *L. major*.[130] Using cryo-electron microscopy coupled with lectin binding using biotinylated Ulex europaeus agglutinin I (UEA-I) specific for *O*-fucose,[131] they demonstrated that fucose was present in various cellular compartments including the endoplasmic reticulum (ER), cytosol, mitochondria, Golgi apparatus and parasite surface.[130] Non-permeabilized cells bound to fucose-specific fluorescent UEA-I, as confirmed by flow cytometry, albeit with lower reactivity compared to mutant parasites ectopically overexpressing genes coding for enzymes which catalyze the synthesis of GDP-fucose.[130] At least five genes involved in the fucosylation/arabinosylation have been mapped in the *Leishmania* genome. This study shed new light on the importance of GDP-fucose and fucosylated glycoconjugates for the viability of *L. major*.

The reported glycans and the methodologies used in their characterization are summarized in Table 2.

N-Linked glycosylation biosynthesis in *Leishmania* spp.

N-Linked protein glycosylation in eukaryotes involves the covalent attachment of sugar molecules to asparagine residues within the canonical sequon (AsnXxxSer/Thr/Cys; where Xxx $\neq$ Pro). Unlike gene transcription and protein translation, protein

Table 2 Reported glycans and the methodologies applied in different *Leishmania* spp. in different life stages

Glycan type	*Leishmania* sp.	Glycans	Methodology	Ref.
N-Glycan	*L. donovani* promastigotes	Terminal α-1,4 and α-1,6-glucan linked D-glucose; *N*-acetyl-D-galactosamine	Lectin agglutination assays (ConA and PHA-P lectins); inhibition assays	117
	L. braziliensis promastigote and amastigotes	Terminal α-D-mannose and/or α-D-glucose and α-D-galactose	Lectin agglutination assays (ConA and RCA); inhibition assays with MAM, glucose and D galactose	119
	L. tropica	Terminal α-D-mannose residues	Lectin agglutination assays on radioiodinated proteins (ConA); inhibition assays	121
	L. mexicana promastigotes	Man6GlcNAc2; GlcMan6NAc2 (transient)	Paper chromatography oligosaccharides released by Endo H treatment of proteins from [U-^{14}C] glucose labelled cells	122
	L. donovani promastigotes	Secreted soluble glycoproteins with terminal mannose and galactose (some linked subterminally to *N*-acetyl galactosamine residues *i.e.* Gal-β(1–3)-GalNAc)	Lectin affinity chromatography (ConA+, LCA, PNA, RCA); inhibition assays	129
	L. mexicana amazonensis	Glc1Man6GlcNAc2 Man6GlcNAc2 Man5GlcNAc2 Man4GlcNAc2	High resolution gel-permeation chromatography, exoglycosidase digestion, acetolysis fragmentation and methylation analysis	103
O-Glycan	*L. major*	*O*-GlcNAc on gp96/92	Lectin affinity chromatography (WGA+), transfer of radiolabelled [3H]UDP-galactose to terminal GlcNAc; anhydrous hydrogen fluoride deglycosylation, β elimination, β-galactosidase treatment	87
	L. donovani	Fucoglycoconjugates	ITRAQ LC-MS/MS	158
	L. major	Fucoglycoconjugates	Cryo-electron mcroscopy; lectin binding assays (UEA I)	130

glycosylation undergoes a non-template driven biosynthesis pathway, which is determined by the expression and activity of the glycosylation machinery enzymes, the nature of the proteins undergoing glycosylation, the availability of sugar donors and the cellular metabolism.[132] The *en bloc* transfer of oligosaccharides from a lipid-linked oligosaccharide (LLO) donor to asparagine side chains of nascent proteins in the ER and the subsequent processing of the attached oligosaccharide in the ER and Golgi apparatus is a conserved and well-orchestrated process catalyzed by finely tuned biosynthetic enzymes. Glycosyltransferases, α-glycosidases, and α-mannosidases in the ER and *cis*-Golgi transfer or cleave off glycan residues. Most of these transmembrane enzymes are located in the ER and Golgi apparatus.[133] Four types of mature *N*-glycans exist in higher eukaryotes depending on their structure: high mannose, hybrid, complex, and paucimannose types. The high mannose, hybrid, and complex types share a common core termed pentasaccharide/trimannosylchitobiose core composed of Manα1,3(Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAcβ (M3).[134] In particular, high mannose *N*-glycans contain only mannose attached to the core. Complex *N*-glycans have all mannose residues after the core removed by enzymatic processing and replaced to form antenna structures. Hybrid *N*-glycans are incompletely processed and have at least one antenna containing only mannose residues.[135] Paucimannosidic structures contain a monosaccharide composition less than or equal to the *N*-glycan trimannosylchitobiose core, *i.e.*, $Man_{1-3}Fuc_{0-1}GlcNAc_2$.[136] Paucimannose glycans have been reported in several eukaryotes ranging from plants, protists, and vertebrates.[136] A deep glycoprotein-centric analysis of *Trypanosoma cruzi* following enrichment of glycopeptides revealed that $Man_3GlcNAc_2$ was present in the epimastigote and trypomastigote life stages.[137] *T. brucei* transferrin receptor and class 2 variant surface glycoproteins have been identified with the $Man_3GlcNAc_2$ paucimannose structure.[138,139] In *Leishmania*, high mannose ($Man_{5-6}GlcNAc_2$) and an unusual terminally glucosylated glycan structure ($GlcMan_6GlcNAc_2$) have been reported.[103,122] $Man_4GlcNAc_2$, also identified in *Leishmania* mature proteins, is of unknown biosynthetic origin.[136] No report on paucimannose structures on *Leishmania* glycoproteins is available in the literature.

N-Linked glycosylation biosynthetic process begins in the cytosolic side of the ER membrane, where glycosyltransferases add sugar molecules to a growing LLO chain.[140] The *N*-glycan biosynthetic pathway for *L. major* is shown in Fig. 4, according to KEGG pathway.[141] Trypanosomatid dolichol chain is shorter with 10–12 isoprene units compared to mammalian LLO (18–21), plant and fungi (15–16) dolichol.[142] The lipid-linked oligosaccharide (LLO) carrier, known as dolichol (Dol, a polyprenol lipid), already phosphorylated to dolichyl diphosphate (Dol-PP),[143] acts as the acceptor molecule for *N*-acetylglucosamine phosphate through a reaction catalyzed by the enzyme UDP-*N*-acetylglucosamine-dolichyl-phosphate *N*-acetylglucosaminephosphotransferase to form *N*-acetyl-D-glucosaminyldiphosphodolichol. This enzyme is coded by the NAGT gene, and its inhibition by treatment of *Leishmania* parasites using tunicamycin,[107] corroborates the expression of this enzyme in *Leishmania* parasites.

β-1,4-*N*-Acetylglucosaminyltransferase adds the second *N*-acetyl glucosamine to the growing LLO chain, to form *N*,*N*′-chitobiosyldiphosphodolichol [$(GlcNAc)_2$(PP-Dol)]. Dolichyl-P-Man:GDP-ManGlcNAc$_2$-PP-dolichyl beta-1,4-mannosyltransferase adds a mannose sugar to the chitobiosyldiphosphodolicol to form beta-D-mannosyldiacetylchitobiosyldiphosphodolichol [(Man)$(GlcNAc)_2$(PP-Dol)]. A second mannosyltransferase enzyme, termed dolichyl-P-Man:GDP-Man1GlcNAc2-PP-dolichyl alpha-1,3-mannosyltransferase (alpha-1,3/alpha-1,6-mannosyltransferase) adds the second and third mannose sugars to the growing LLO chain, resulting in alpha-D-mannosyl-beta-D-mannosyldiacetylchitobiosyldiphosphodolichol $(Man)_2(GlcNAc)_2$(PP-Dol) and $(Man)_3(GlcNAc)_2$(PP-Dol), respectively. Alpha-1,2-mannosyltransferase adds the fourth and fifth mannose sugar molecules, forming $(Man)_4(GlcNAc)_2$(PP-Dol) and $(Man)_5(GlcNAc)_2$(PP-Dol), respectively. Dolichyl-P-Man:GDP-Man5GlcNAc2-PP-dolichyl alpha-1,3-mannosyltransferase adds the sixth mannose sugar to form $(Man)_6(GlcNAc)_2$(PP-Dol). Paper chromatography analysis of dolichol-P-P bound oligosaccharides isolated from *L. mexicana* after incubation with radiolabelled glucose ([U-^{14}C] glucose) did not show glucose incorporation into the oligomannose chain, while the largest oligomannose structure identified on the dolichol-P-P was $(Man)_6(GlcNAc)_2$.[122] The terminal Man (and Glc in mammals) are essential in the correct folding of the newly glycosylated proteins and ensure glycoprotein quality control in the ER.[132] The transfer of unglucosylated oligosaccharides in *Leishmania* parasites is an unusual feature in trypanosomatids compared to the canonical biosynthesis of *N*-linked glycosylation of higher animals, fungi and plants, which occurs in the ER lumen.

Oligosaccharyltransferase (OST) enzyme (dolichyl-diphosphooligosaccharide–protein glycosyltransferase) catalyzes the *en bloc* transfer of the oligosaccharide from the PP-Dol to an asparagine side chain of the acceptor protein to form $(Man)_6(GlcNAc)_2$(Asn).[122] Kinetoplastids encode a single-subunit OST enzyme, while higher eukaryotes encode OSTs with different complexities varying among species. STT3 is the catalytic subunit, and in *Trypanosoma brucei* and *L. major*, a single subunit is encoded[144,145] from several copies of the gene. In the parasitic protozoa *L. major*, four STT3 paralogues, but no homologs to the other OSTase subunits are encoded in the genome.[146]

Subsequently, a glucose molecule is transiently transferred from UDP-Glc to the newly glycosylated protein to form $(Glc)(Man)_6(GlcNAc)_2$ by a glucose transferase enzyme.[122] The addition of a glucose residue to $(Man)_6(GlcNAc)_2$(Asn) to form $(Glc)(Man)_6(GlcNAc)_2$Asn has been proposed to protect the newly glycosylated proteins from α-mannosidase hydrolysis of the proteins during transit and modification in the ER. Analysis of *L. mexicana* parasites after a 2 h incubation with radiolabeled glucose [U-^{14}C] resulted in glycoproteins with $Man_6GlcNAc_2$, suggesting that the added glucose is transiently removed after its addition in the ER.[122] However, the presence of $(Glc)(Man)_6(GlcNAc)_2$ sugars on the main cell surface glycoprotein (gp63) suggests that, even though the glucose is susceptible to glucosidase II enzyme activity, the addition of the glucose molecule (glucosylation) is not necessarily transient.[103]

Glucosylation of smaller molecular weight oligomannose structures was not identified, suggesting that the addition of a glucose moiety was specific and restricted to $(Man)_6(GlcNAc)_2$ substrate.[103] The predominant oligosaccharides identified in *L. major* promastigote and amastigote stages after the enzymatic release of gp63 were $(Glc)(Man)_6(GlcNAc)_2$ and $(Man)_6(GlcNAc)_2$,[108] Table S1 (ESI†). The binding of human mannose-binding protein (MBP) to live promastigotes of *L. major* and *L. mexicana*, and to purified LPG immobilized on microwells using I-labelled MBP also indicated the presence of molecules with terminal mannose.[147] GIPLs have also been demonstrated to bind to MBP, indicating the presence of terminal mannose sugars.

As noted before, hybrid, complex and paucimannose-type glycan glasses have not been characterized in *Leishmania* spp.[103] In addition, high mannose-type glycans with only one glucose molecule and 4–6 mannoses [$(Glc)(Man)_6(GlcNAc)_2$ and $(Man)_{4–6}(GlcNAc)_2$] have been characterized on mature *Leishmania* spp. proteins.[103] Genome mapping of *Leishmania* spp. enzymes involved in *N*-linked protein glycosylation is shown in Fig. 4. Based on KEGG PATHWAY database,[141] glycans comprised of up to $(Glc)_{1–3}(Man)_9(GlcNAc)_2$ are reported. However, no evidence of bi and triglucosylated high mannose together with hybrid, complex and paucimannose glycan structures are reported in the literature so far.

O-Linked glycan biosynthesis

While *N*-glycans are transferred *en bloc* from pre-synthesized oligosaccharide precursors to newly synthesized polypeptide chains, stepwise addition of individual monosaccharide units to the hydroxyl group of a serine/threonine amino acid side chain defines *O*-glycosylation.[148] *O*-Glycans vary depending on the connecting sugar. Generally, the term *O*-glycosylation refers to mucin-type *O*-glycosylation, which is the modification of proteins at Ser/Thr hydroxyl group by GalNAc monosaccharides with α-linkages.[149] This modification involves the transfer of *N*-acetylgalactsamine (GalNAc) from UDP-GalNAc to a serine or threonine amino acid residue to form (GalNAc)(Ser/Thr). Mannose-type *O*-glycosylation (*O*-mannosylation) is another type of protein *O*-glycosylation whose initial step involves the transfer of a mannose sugar from mannose-P-dolichol to a Ser/Thr residue by the enzyme dolichyl-phosphate-mannose-protein mannosyltransferase to form (Man) (Ser/Thr).[150] Mucin and mannan-type protein *O*-glycosylation have not been evidenced in *Leishmania* spp.[46]

Another type of *O*-glycosylation, termed phosphoglycosylation, involves the addition of phosphoglycans to a polypeptide at a serine residue forming a phosphodiester bond with the sequence R-Manα1-PO_4-Ser.[151,152] Proteophosphoglycan biosynthesis and functional characterization have been reported in *Leishmania* spp.[47,153] and reviewed elsewhere,[46] and their structures are summarized in Fig. 2.

O-GlcNAc modification, also known as cytosolic *O*-glycosylation, involves the attachment of *O*-linked GlcNAc sugars to Ser/Thr hydroxyl groups, forming (GlcNAc)(Ser/Thr).[154] Despite the fact that pathways that utilize UDP-GlcNAc have not been described in *Leishmania* spp.,[155,156] two cytosolic glycoproteins, gp96/92 were described by Handman and colleagues in 1993.[87] The *O*-GlcNac modified proteins were detected by WGA lectin affinity chromatography in various *Leishmania* species and their corresponding gene and transcript characterized by southern and northern blotting techniques, respectively.[87] The gene coding for the protein was termed 1–3B, a 3 kb single-copy gene, while the transcripts for this protein were 7.5 and 4.0 kb, respectively. The *O*-linked *N*-acetylglucosamine glycoprotein was expressed in both promastigote, and amastigote stages of the *Leishmania* species assayed but varied in molecular weight between the species.[87] In addition, these proteins shared the peptide backbone and appeared to be highly conserved in *Leishmania*, *Leptomonas*, and *Crithidia* protozoa, suggesting a vital conserved function. This glycoprotein lacked any *N*-linked glycosylation, as confirmed by the lack of effect after digestion with *N*-glycanase or treatment with tunicamycin. The cytosolic water-soluble pg96/92 glycoprotein contained unusual glycan composition of glucosamine, as confirmed by WGA lectin binding characteristics.

Protein *O*-fucosylation is a protein modification which occurs within consensus sequences on serine or threonine amino acid residues, and is catalyzed by protein *O*-fucosyltransferases.[157] The directly linked fucose attached to the modified protein can be elongated by other glycans.[157] In *Leishmania* spp., *O*-fucosylated glycoconjugates were first described by Rosenzweig and colleagues in 2008 by employing iTRAQ-LC-MS/MS on *L. donovani* clone 1SR from promastigote and amastigote life stages.[158] Four fucosylated proteins were identified in this study from the soluble protein fraction: chaperonin HSP60, HSP70-related protein 1, β-tubulin and kinesin k39, putative.

Sialylated glycoproteins in *Leishmania* spp.

The sialic acid molecule is a 9 carbon polyhydroxyl amino keto sugar of *N*- and *O*-substituted derivatives of neuraminic acid (2-keto-3-deoxy-5-acetamido-D-*glycero*-D-galacto-nonulosonic acid), a monosaccharide commonly referred to as *N*-acetylneuraminic acid (Neu5Ac). Sialic acids (Sias) are terminally located on oligosaccharide chains, which decorate cell membranes and secreted macromolecules (*e.g.*, serum glycoproteins). Commonly occurring derivatives of sialic acids include Neu5Ac, Neu5Gc, and the *O*-acetylated sialoglycoconjugates, which are acetylated at C-7/8/9 to form *N*-acetyl 7/8/9 *O*-acetylneuraminic acid, respectively.[159,160] Over 50 different derivatives of *N*-acetyl neuraminic acid molecules[161] occur naturally in a diverse range of living organisms from the Animalia kingdom, ranging from microbes (viruses, pathogenic bacteria, and protozoa), higher invertebrates (starfish) and vertebrates (from echinoderms to higher vertebrates).[160] Microorganisms usually have a different pathway from eukaryotes for the *de novo* biosynthesis of sialic acids (*e.g.*, *E. coli* K1 and *Neisseria meningitides*),[162] but some microbes utilize truncated sialylation pathways (*e.g.*, *Neisseria gonorrhoeae*)[161] for the biosynthesis of sialic acids. Many biological and structural functions have been attributed to sialic acids in different cells and tissues. In higher eukaryotes, sialic acids decorate virtually all cell-surface glycans and serum glycoconjugates terminally,[159] where they contribute to the structural integrity of cell membranes, recognition functions by receptor molecules and in the masking of recognition sites by target molecules ligands.[163]

Leishmania spp. and *T. cruzi* protozoan parasites lack the endogenous sialic acid biosynthetic pathways.[164,165] *T. cruzi* incorporates sialic acid sugar molecules on their surface glycans by scavenging from host cell glycoconjugates by the function of *trans*-sialidase enzymes (a modified α(2,3) sialidase) through a glycosyl-transfer reaction. Other trypanosomatids, including *T. brucei* and *T. congelense*, also express the *trans*-sialidase enzyme.[166] *Trypanosoma rangeli* and *T. vivax* express a sialidase enzyme without the *trans*-sialylation function, while *Leishmania* parasites do not code for either the sialidase or *trans*-sialidase enzymes.[166] However, sialic acids have been demonstrated on the glycosylated surface membrane (glycoproteins and lipophosphoglycans) in *L. donovani* promastigotes and amastigote life stages,[166] and have been implicated in parasite–host interaction, infectivity and immune evasion.[164,167] The presence of sialic acids in *L. donovani* promastigotes has also been demonstrated by Chava *et al.*[168] by employing fluorometric HPLC, GC-MS, lectin binding and ELISA to identify and characterize different sialic acid derivatives and their linkages transferred from the culture medium to the promastigote surface membrane. 9-*O*-Acetylated sialic acids and α2-3 and α2-6 linked sialoglycans were reported.[168] *L. major* lacks the *trans*-sialidase enzyme, and the subsequent α2-3 linked sialic acids, which are the products of the *trans*-sialidase enzyme. Transgenic *L. major* expressing recombinant TS from *T. cruzi* was shown to be more virulent in genetically susceptible mice *in vivo*, suggesting that the expression of *T. cruzi* TS conferred virulence to *L. major*.[169] The interaction of *L. donovani* sialic acids and siglecs (sialic acid-specific receptors on haematopoetic cells) has shown that sialic acids play a key role in their cell–cell interaction, adherence, virulence, proliferation of amastigotes in macrophage phagolysosomes, and immune evasion.[170] A total of 14 siglecs have been reported on mammalian immune cells.[171] The interaction between sialic acids and siglec 1 enhances adherence to macrophages and their subsequent uptake by the cells through phagocytosis. Siglec 5 interaction with Sias leads to an upregulation of an acid phosphatase expressed by the host cells (SHP-1), downregulation of MAPKs (p38, ERK, and JNK), PI3K/Akt pathways followed by the reduced translocation of p65 subunit of NF-κβ to the nucleus from cytosol in the downstream signaling pathways leading to suppression of effector functions of innate immune response.[170] See reviews on trypanosomatid sialoglycosylation.[168,172,173]

Box 1. Unusual features of *Leishmania* glycosylation

i. *Leishmania* parasites, as all other trypanosomatids, synthesize a truncated dolicholpyrophosphate-linked precursor ($Man_6GlcNAc_2$) compared to the common LLO for most eukaryotes, $Glc_3Man_9GlcNAc_2$. In addition, trypanosomatids transfer unglucosylated oligosaccharides from LLOs to nascent proteins.[142]

ii. After the transfer of $Man_6GlcNAc_2$ to protein chains, minor trimming occurs in *Leishmania* parasites, with the most common modification being restricted to the addition of unusual α1-3 glucosyl residues.[103,105,174]

iii. UDP-galactopyranose mutase (UGM) and galactofuranosyl transferases (GalfTs), enzymes in the biosynthesis of galactofuranose pathway and attachment to cell surfaces, respectively, lack mammalian homologs, and have been proposed as promising drug targets.[175]

iv. Sialic acid derivatives Neu5Gc and 9-*O*-acetylated Neu5Gc could be candidates for sialoglycotherapeutics.[176]

Advances in mass spectrometry-based analysis of protein glycosylation in *Leishmania*

Carbohydrate moieties attached to proteins or lipids have received increased attention with respect to their structure and function. This has been facilitated by the speed and sensitivity of improved and novel analytical methodologies.[177] The complexity of glycan structures and their subsequent conformations present both unprecedented opportunities and challenges in the study of their structures, biosynthesis, and functions.[177] Glycoproteomics, defined as the system-wide site-specific characterization of protein glycosylation,[177] faces many analytical challenges that have made it very difficult to comprehensively perform the structural, biosynthesis and regulation studies of the glycoproteome.[132] These challenges include the multiple layers of structural diversity that form a spectrum of the chemically similar repertoire of glycans present on variant synthesized glycoproteins (termed glycoforms).[178] Glycomics, defined as the study of the complete set of glycans and glycoconjugates synthesized by a cell or organism in different conditions(glycome),[179] also faces enormous challenges arising from the complexity of sugar molecules expressed in a cell or organism.[38] Attempts to address these challenges are being sought by new advances in mass spectrometry, genetics, and cell biology studies.[38]

Proteomic approaches (the large scale characterization of proteins in a cell line, tissue and/or organism to gain a global and integrated overview as opposed to the individual information of all the proteins in a cell, tissue or organism[180]) have been utilized in the study of *Leishmania* parasite and leishmaniasis, and their applications to *Leishmania* biology and clinical implications recently reviewed.[181–183] These large scale characterization techniques utilize mass-spectrometry based approaches. The large scale study of proteins, glycoproteins, and glycans using mass spectrometry-based tools, both on the parasite and the host in response to infection, offers unprecedented opportunities to uncover novel drug/vaccine candidates and to help elucidate mechanisms of infection, parasite survival, disease diagnosis, and biomarkers. We reviewed mass spectrometry-based studies on *Leishmania* parasites with a strict focus on the system-wide analysis of *Leishmania* glycoproteins and glycans by LC-MS/MS.

Glycoproteomic and glycomic-centric studies that have utilized mass spectrometry techniques to characterize *Leishmania* parasites or clinical samples from patients diagnosed with leishmaniasis to gain an in-depth understanding of how these parasites survive and interact with their vectors or hosts, and/or changes during the course of an infection are few, and highlighted herein. At present, two studies have been reported on the modulation of protein glycosylation in *Leishmania* infected patients looking at the host glycosylation. Bag and colleagues analyzed plasma proteins from healthy, and *L. donovani* infected (VL) patients to elucidate disease-associated alterations and host response to infection.[184] Two-dimensional fluorescent difference gel electrophoresis combined with MALDI-TOF MS allowed the detection of 39 differentially expressed spots assigned to 10 proteins with different heterogeneity. Alpha-1-antitrypsin, alpha-1-B glycoprotein, and amyloid-A1 precursor, fibrinogen gamma-B chain precursor were upregulated, while vitamin-D binding protein, alipoprotein-A-I, and transthyretin down-regulated. Multi-lectin affinity purification of glycoproteins in VL, healthy endemic and non-endemic controls revealed the up-regulation of μ_1-B glycoprotein and haptoglobin precursor, allele-1,2. No information on the glycosylation sites and glycan composition was described. Protein–protein interaction analysis of differentially regulated proteins/glycoproteins revealed biological associations pointing to cellular mechanisms employed by the parasites to cause disease, from the regulation of innate immune response to inhibition of proteases.[184]

The second glycoproteome-centric study was focused on immunoglobulin G Fc changes during infection with *L. chagasi*.[185] The antibodies were affinity-purified from serum or plasma on protein G monolithic plates and digested with trypsin. The resulting glycopeptides were purified and desalted by reverse-phase solid-phase extraction and analyzed by MALDI-TOF MS. *N*-Glycosylation of antibodies from VL patients were profoundly altered as compared to antibodies collected from uninfected individuals in endemic and non-endemic regions. Fc galactosylation, sialylation, and bisection of IgG1 and IgG2 and 3 Fc regions in VL patients was lower while fucosylation were upregulated compared to asymptomatic individuals and controls. Moreover, IgG1 and IgG2 and -3 Fc galactosylation and sialylation was negatively correlated with clinical severity. Treatment of VL patients with pentavalent antimony and/or amphotericin B changed IgG Fc *N*-glycosylation.[185] Taken together, these results offer new insights into the regulatory role of antibodies in immune responses elicited during *Leishmania* spp. parasite infection.

A study by Rosenzweig *et al.*, utilized ITRAQ LC-MS/MS to characterize the post translational modifications of *L. donovani* promastigote and amastigote life stages.[158] In addition to the previously mentioned fucosylated proteins identified in this study, they were able to identify one asparagine-linked hexosylated peptide.

An example of analytical approaches to characterize glycoproteins and glycans in *Leishmania* spp. are summarized in Fig. 5. Mass spectrometry-based analysis of intact glycopeptides involves the enzymatic hydrolysis of the parasites protein lysate using trypsin or a combination of different proteolytic enzymes (step 1–4). Glycopeptide enrichment strategies such as HILIC, TiO_2, lectins and others allow the separation of a glycopeptide enriched fraction (step 5). Glycopeptides can be analyzed directly (step 6) or treated with PNGase F to cleave the sugar moiety and generate a *N*-linked glycosylation site signature (step 7 and 8) before mass spectrometry analysis using dedicated chromatographic and glycopeptide fragmentation strategies (step 9–12). Intact glycopeptides can be identified

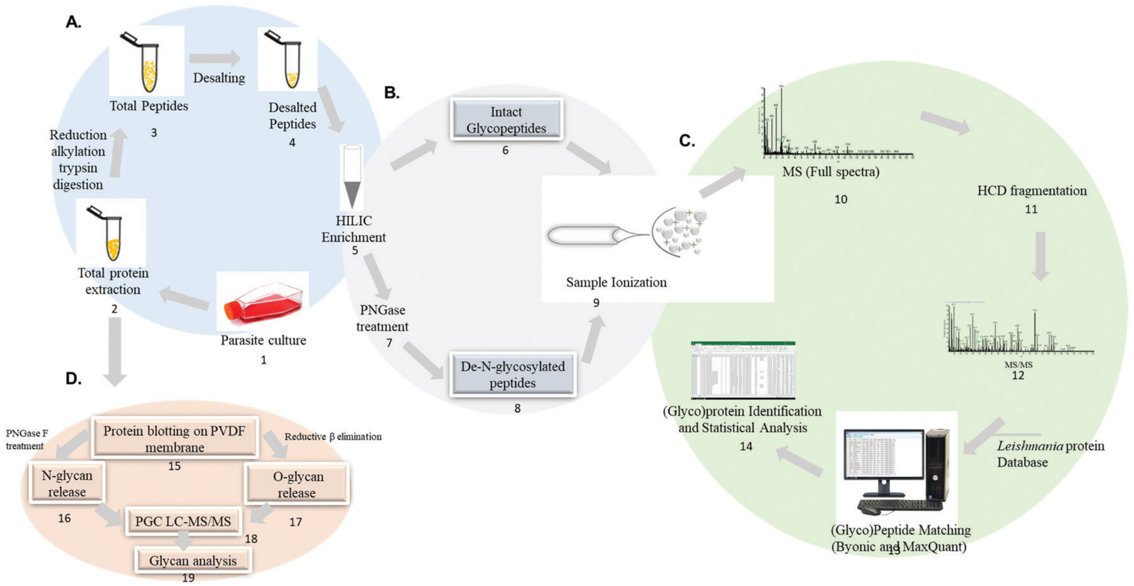

Fig. 5 Mass spectrometry-based analytical workflow envisioned for *Leishmania* spp. large-scale glycoproteome, deglycoproteome and glycome analysis. Proteins are extracted from parasite cell cultures, clinical isolates or biological fluids and digested into peptides (1–4). Glycopeptides are enriched from a complex mixture using specific affinity strategies (5). Intact glycopeptides can be directly analyzed by LC-MS/MS (6) or deglycosylated using PNGaseA/F and the formerly glycosylated peptides are analyzed by LC-MS/MS (7–8) followed by data and bioinformatic analysis (9–14). The analysis of intact glycopeptides and formerly glycosylated peptides will elucidate the glycoproteome and deglycoproteome, respectively. Extracted proteins can be alternatively blotted on PVDF membrane and subjected to enzymatic and chemical release of *N* and *O*-glycans (15–17). The released glycans are subsequently analyzed by PGC LC-MS/MS to uncover the glycome (18–19).

in specific software such as Byonic, pGlyco, Mascot, Protein Prospector, sugar QB and others.[137,186–193] Intact glycopeptide quantification can be performed by matching spectral features using MaxQuant and Proteome Discoverer (step 13–14).[177,193,194] The identification of *N*-linked glycosylation sites in PNGase F-treated glycopeptides can be identified by the conversion of asparagine to aspartic acid signature during peptide sequencing.[195–197]

In particular, the large scale identification of *N*-linked glycosylation sites allows to define the deglycoproteome of a biological system. The most common strategy to identify and quantify *N*-linked glycosylation sites in large scale involves: (1) a protein or peptide-centric enrichment strategy, (2) followed by peptide: *N*-glycosidase A or F (PNGase A/F) cleavage and (3) mass spectrometry analysis. Glycoproteins and/or glycopeptides can be enriched from a complex mixture using different affinity strategies such as hydrophilic interaction chromatography (HILIC), titanium dioxide (TiO_2), graphite, hydrazide chemistry, boronic acid and lectins.[197–203] Subsequently, PNGase A/F, a glycosylasparaginase, is used to cleave GlcNAc-Asn linkage releasing *N*-linked oligosaccharides and converting the asparagine to aspartic acid.[204] The formerly-glycosylated peptides are analyzed by LC-MS/MS and the data are searched including asparagine deamidation as variable modification. The asparagine to aspartic acid conversion is monitored during MS analysis due to a mass increment of $\Delta M = 0.9840$ Da. It should be noted that asparagine to (iso)aspartic acid conversion can be derived from chemical deamidation which can happen spontaneously during sample preparation.[205–207] To improve the confidence in the *N*-linked glycosylation site assignment, the enzymatic release of oligosaccharides by PNGaseF/A is performed in heavy-oxygen water ($H_2{}^{18}O$). Under these conditions, the incorporation of $H_2{}^{18}O$ will lead to a mass increment of $\Delta M = 2.9890$ Da on the corresponding asparagine residue reducing the false *N*-linked glycosylation site assignment.[208] The deglycoproteome strategy has improved the current knowledge on *N*-linked glycosylation sites in different biological systems and constitutes a useful technique to be applied to *Leishmania* spp.

The technological advances in the field of glycomics have helped improve deciphering the complexity and heterogeneity of the glycome. One approach for glycomic analysis relies on protein blotting on PVDF membrane, *N* and *O*-linked glycan release and PGC LC-MS/MS analysis for structural characterization (step 15–19).[209,210] Other analytical approaches for glycan release and MS analysis have been reported.[211–216] Glycan-centric approach is not able to provide information such as the carrier protein and the site-specific attachment of each glycan.[177] Thus, glycoproteomics offer more information regarding the protein carriers, glycan attachment sites and the structure, and occupancy of the glycan.[132]

Conclusion

The first efforts in characterizing *Leishmania* protein glycosylation have been important to build up the current knowledge on these biomolecules. However, protein glycosylation in *Leishmania* still deserves more investigation. The protein and glycan diversity of this parasite has been associated with specific species and developmental stages. Moreover, functional studies have highlighted the importance of these biomolecules in host–*Leishmania* interaction. Unusual glycan features represent valuable therapeutic, diagnostic, and vaccine opportunities for this disease. A detailed characterization of *Leishmania* spp. glycoproteome is needed and will benefit from advanced analytical LC-MS and computational strategies. We foresee the importance of studying the glycoproteome changes in: (1) different *Leishmania* species, (2) along the parasite life cycle, (3) parasite clinical isolates (4) the invertebrate vector during infection, (5) non-human hosts, and (6) patients' tissue biopsies and biofluids.

As shown in Fig. 3, *Leishmania* parasites have shaped their protein glycosylation sites in a species-specific manner. Knowing the glycan macro- and micro-heterogeneity for the different *Leishmania* species will help direct functional studies for each protein and species. Moreover, it will be possible to correlate the glycan profile of a certain species with the clinical phenotype.

The glycosylation profiles of *Leishmania* life stages shown in Fig. 1 can modulate parasite–host interaction. Due to that, measuring the glycosylation enzymes expression and activity, activated sugars availability, substrate concentration and the metabolic status of the parasites will offer a complete picture of the biosynthetic steps regulating the glycome of each life stage.

Since different cellular and phenotypic differences have been found between laboratory cultured *Leishmania* parasites and clinical isolates, evaluation of their protein glycosylation profiles will be important to understand the biology of this parasite to validate biological, diagnostic and therapeutic findings obtained in the laboratory cell cultures.

While this review has been focused on the parasite glycosylation, a comprehensive characterization of the hosts during infection is important. Development of alternative vector control strategies based on modulation of its glycosylation profile might impact vector competence and disease transmission. Moreover, the development of vaccines and chemotherapeutic treatments based on glycans can help in in veterinary and human medicine, supporting the concept of 'one health' to eradicate the disease. Deep characterization of protein glycosylation profiles of biopsies and biofluids will help in improving leishmaniasis disease diagnosis and prognosis, allowing a better stratification of the patients to deliver a proper therapeutic solution with the aim of developing personalized therapy.

Due to that, the elucidation of the hidden complexity of the *Leishmania* and host protein glycosylation will allow researchers to tailor more specific and less toxic chemotherapies and more sensitive and accurate diagnostic tools, calling attention to the importance of glycans in parasite biology and its applications in disease control.

Conflicts of interest

There are no conflicts to declare.

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