Published in final edited form as:

Expert Rev Proteomics. 2018 May ; 15(5): 371–390. doi:10.1080/14789450.2018.1468754.

Understanding *Leishmania* parasites through proteomics and implications for the clinic

Shyam Sundar^{*,1} and Bhawana Singh¹

¹Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221 005, UP, India.

Abstract

Introduction—*Leishmania* spp. are causative agents of leishmaniasis, a broad-spectrum neglected vector-borne disease. Genomic and transcriptional studies are not capable of solving intricate biological mysteries, leading to the emergence of proteomics, which can provide insights into the field of parasite biology and its interactions with the host.

Areas covered—The combination of genomics and informatics with high throughput proteomics may improve our understanding of parasite biology and pathogenesis. This review analyses the roles of diverse proteomic technologies that facilitate our understanding of global protein profiles and definition of parasite development, survival, virulence and drug resistance mechanisms for disease intervention. Additionally, recent innovations in proteomics have provided insights concerning the drawbacks associated with conventional chemotherapeutic approaches and *Leishmania* biology, host-parasite interactions and the development of new therapeutic approaches.

Expert commentary—With progressive breakthroughs in the foreseeable future, proteome profiles could provide target molecules for vaccine development and therapeutic intervention. Furthermore, proteomics, in combination with genomics and informatics, could facilitate the elimination of several diseases. Taken together, this review provides an outlook on developments in *Leishmania* proteomics and their clinical implications.

Keywords

leishmaniasis; virulence; vaccine; bioinformatics; proteomics

1. Introduction

The complicated scenario of neglected tropical diseases and number of deaths due to these diseases are major issues to be addressed in tropical and sub-tropical countries of the world [1, 2, 3]. Research for drug discovery and vaccine design requires an in depth and holistic

^{*}Corresponding Author: Dr. Shyam Sundar, Infectious Disease Research Laboratory, Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005.

Financial and competing interest disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

knowledge of parasite biology and host immunology. Indeed, there are major gaps in drug discovery and vaccine design, in addition to poor market investments and commercialization, which has led to the classification of leishmaniasis amongst the most neglected tropical diseases by the WHO, with an estimated risk to 350 million individuals and 12 million infected subjects and 0.9–1.6 million new cases per year in 98 countries in five continents: Asia, Africa, Europe, North America and South America [4, 5].

Despite the aforesaid shortcomings, completion of the human genome project has revolutionized biomedical research, as reported for Duchenne muscular dystrophy, retinoblastoma, cystic fibrosis, neurofibromatosis and different kinds of neoplastic disorders. It has further provided valuable insights into cell biology using different cyclic array sequencing strategies (ChIP-Seq, RNA-Seq, etc.) [6]. However, understanding of a complex biological system requires knowledge of the functional parts of the genome, its dynamics and the gene functionality, which has led to emergence of systems biology [7]. Likewise, mRNA-based studies do not capture information on translational and post-translational modifications, nor can they discern protein localization and interaction analyses, which are required to better understand gene functions. Therefore, knowledge about the proteins encoded by mRNAs is essential. Moreover, DNA-based conventional approaches do not provide answers for the putative genes in *Leishmania*, as half of the open reading frames (ORFs) have been annotated as hypothetical [8]. Proteins are the regulatory units of cellular functions, and advancements in proteomics would provide clues about unaddressed areas of the genome. As a result, proteomic studies have gained significant relevance with the advancements in large-scale technologies [9]. The last few decades have seen the emergence of high-throughput technologies in proteomics, providing a way to acquire a more detailed understanding of cellular processes compared to DNA-based studies. Recent innovations have provided accessibility to pathway analysis, compared to the conventional proteomic methodologies in which a single protein analysis is performed at a time. Indeed, proteomics has allowed comprehensive and integrated analyses of biological processes. Therefore, proteomic studies using different automated systems have contributed greatly to our understanding of disease mechanisms, identification of biomarkers for diagnosis, and characterization of molecules for drug design in different disease models. The history of proteomics dates back to the times of protein characterization and development of analytical tools for detecting thousands of proteins in one run; however, the term proteomics was coined in 1995 in an attempt to provide a snapshot of the physiological behaviour of the cell [10–13]. In the field of *Leishmania*, a plethora of research has provided valuable insights into the host-parasite interaction and identification of several key candidates for vaccine development [14, 15]. Indeed, proteomics has provided promising opportunities for overcoming the drawbacks associated with the genomic approach, with many untold challenges. This review aims to examine the contribution of proteomic technologies to our understanding of *Leishmania* pathogenesis and the implications of this knowledge for vaccine development, disease surveillance and in clinical settings for diagnosis, as well as therapeutics.

2. Cell biology of *Leishmania*

Leishmania is a digenetic parasite that shuttles between the flagellated promastigote form that resides in the midgut of the vector and an amastigote form within the phagolysosome of mammalian macrophages. Upon the bite of an infected sandfly, parasites are released into the blood stream, facilitating their colonization in macrophages in an amastigote form. The cycle continues when the sandfly bites the host during a blood meal and ingests infected macrophages.

These events drive the adaptive changes in the parasite from a free-living form within the poikilothermic vector to an obligate intracellular form in the homeothermic mammalian host. Acclimatization of the parasite includes morphological, physiological and biochemical modifications. Research on these changes has been supported by *in vitro* culture systems and further supported by genomics and transcriptomics to study the adaptive changes during differentiation. Some processes, such as trans-splicing and RNA editing, constitute the characteristic feature of protozoan parasite differentiation. The bite of the infected vector releases numerous substances that trigger the recruitment of neutrophils and subsequently macrophages to the skin. The parasite invades the neutrophils upon recognition, adhesion and invasion, which later serve as “Trojan horses” for parasites. Subsequently, macrophages play a vital role in the final establishment and amplification of infection [16]. This process of invasion also involves the parasite lipophosphoglycan and gp63 [17], which interacts with different complement receptors and fibronectin receptors to facilitate phagocytosis [18]. The parasite then moves into the parasitophorous vacuole, where it undergoes differentiation into the amastigote form and proliferates. Upon intense multiplication, the macrophage ruptures and releases parasites into the tissue, which then invade new macrophages and/or are ingested by sand flies upon the next blood meal [19].

Infection in the vector sandfly begins with the intake of an infected bloodmeal. When the sandfly bites, it pierces its mouthpart into the skin and forms a haemorrhagic pool that causes ingestion of blood with amastigote-infected macrophages [20]. The blood meal moves into the midgut for digestion, where amastigotes are clustered and form a membranous structure called a peritrophic matrix to surround the bloodmeal and prevent its digestion by digestive enzymes of the gut. The amastigote then differentiates into the replicative “procyclic promastigote” form, followed by rupture of the anterior portion of peritrophic matrix and release of promastigotes into the midgut epithelium. In the midgut, the parasite replicates *via* binary fission and attaches to microvilli of the epithelium through LPG [21, 22]. Upon detachment, the parasite moves towards the stomodeal valve in the anterior of the midgut where it continues to undergo replication. This process leads to the formation of the promastigote secretory gel (PSG), which serves as plug to obstruct the midgut and pharynx, and the parasite transforms into the infective metacyclic form [23]. The metacyclics then break the stomodeal valve, providing a way for movement of the metacyclics from the thoracic midgut [24]. This process leads to the next infection cycle upon sandfly bite and the release of metacyclics into the new mammalian host. The post-genomic era has greatly contributed to accessibility to different structural proteins and virulence factors in the parasite life cycle with therapeutic potential.

2. Scope of proteomics in understanding *Leishmania* biology

Omics technology is increasingly being used for investigations of disease phenotype, mechanism of action of drugs and parasite biology. This approach has undoubtedly refined the infrastructure of postgenomic research. Proteomics remains an essential armoury of biomedical research because proteins serve as one of the main functional units of the cell. DNA-based conventional molecular methods do not answer proteome-related queries because gene expression is regulated at transcriptional, translational and/or post-translational levels. Additionally, the dynamic nature of protein profiles accentuates the need to study protein expression as the most suited factor for understanding complex cellular behaviours. Structural mapping of genes/proteins remains the backbone for understanding gene and protein functions. Ongoing developments and improvements in proteomics technology have led to the emergence of several proteomic aspects, i.e., structural, functional and expression proteomics.

Structural proteomics addresses the systematic understanding of protein structures and their functions. With the advent of new technologies, structural proteomics has gained momentum and has served the biological/clinical sciences by providing the structural organization of vast arrays of proteins available in public databases using structural analyses. Structural proteomic methodologies have provided a platform for understanding the protein production by employing high-throughput cloning, expression from multiple vectors, core domain identification as well as the use of expression and detection tags [25]. Further improvements in structural proteomics have been introduced in the form of multidimensional-NMR, which uses isotope-enriched protein samples in combination with high-field spectroscopy; cryogenic probes and automated spectra assignments have facilitated the structural studies [26]. Reverse genetic approaches have complemented proteomics using different workflows [27]. CRISPR technology for proteomic analysis has broad applications for studying molecular components and the dynamic regulation of cellular processes [28]. Additionally, the use of cryo-EM represents another milestone in structural proteomic studies; the development of cryo-EM in combination with other tools has allowed the isolation of intact viral proteins and virions and has been recognized with a Nobel prize in 2017. Despite the aforesaid improvements, the major setback in structural studies remains in the form of efficient production of proteins, their expression, the crystallization of membrane proteins and the ability to correctly model structures based on homology templates. However, structural proteomics has served to validate functions assigned to proteins depending on the number and diversity of homologous sequences and extent to which these sequences are functionally characterized [29]. Therefore, it has provided an accurate and efficient method for understanding a pool of datasets for predicting biological functions.

Functional proteomics remains another emerging field with the aim of addressing the functional aspects of unknown proteins and their roles in cellular microenvironment. This aspect of proteomics answers queries related to spatial and temporal properties of molecular networks in the cell [30]. Additionally, functional proteomics has also revealed protein-protein interactions to understand molecular mechanisms and, therefore, biological functions [31]. The identification of protein-protein interactions relies solely on affinity-based methods and tagging systems, where protein of interest is expressed with a suitable tag to

identify its specific partner from the cellular extract using immunoproteomic tools [32], tandem affinity purification (TAP) tagging systems [33], etc. The entire complex is then isolated using an anti-tag system and immobilized on an agarose/sepharose matrix for functional proteomic studies [34]. Moreover, knowledge from interaction studies could potentially serve in depicting the cellular signalling pathways, giving rise to a new field of interaction proteomics [35]. It remains one of the important components of functional proteomics because many proteins exist as protein complexes, which ultimately determine the specific function of a protein. The protein complexes contribute to the information encoded by the gene, and therefore interactome analysis remains one of the challenges of the post-genomic era. The composition of protein complexes is not easily inferred by studying pairwise interactions; however, protein complex purification strategies in combination with protein identification by MS/MS serve as powerful tools for interactome studies [36], in addition to protein tagging strategies.

Another aspect of proteomics is the expression proteomics in which the up- or down-regulated protein levels are measured and their expression patterns determined in different disease states compared to the normal cell. This aspect serves as a comparative approach in biomedical research to identify up/down-regulated proteins during specific physiological conditions in turn, determining disease-associated markers or therapeutic targets [37, 38, 39]. Several platforms for expression proteomics use label-free and labelled approaches for protein quantification. In the label-free approach, proteins/peptides are quantified based on the precursor signal intensity or on spectral counting with mass analysers. They are spiked with internal standard to compensate for chromatographic drift during runs. Intact protein expression spectrometry is a label-free approach under development by the Food and Drug Administration Centre and 2DE, constituting another vital label-free approach. In the labelling approach, proteins/peptides are labelled with stable isotopes as in 2D-DIGE, isobaric labelling tandem mass tags, isobaric tags for relative and absolute quantification of quantification (iTRAQ), isotope coded affinity tags (ICAT), metal coded tags, N-terminal labelling and terminal amine isotopic labelling of substrates (TAILS). This comparative analysis approach has been further refined by the use of isotopes and fluorescent techniques, thus overcoming the drawbacks associated with labour-intensive in-gel staining protocols [40, 41, 42]. Therefore, protein identification, expression and characterization of interacting protein partners as well as their roles in regulating cellular functions sets the foundation for depicting signalling networks and interaction maps for understanding parasite biology and disease pathogenesis.

3. Proteomic technologies for understanding *Leishmania* biology

After the success of human genome sequencing, biologists are now shifting their focus towards the analysis of proteins, which remains an important regulatory unit of integration in diverse cellular events. Therefore, we have attempted to present a comprehensive view of different proteomic technologies (Table 1), bridging the gap between functional proteins and the genome of an organism and, thus, providing insights into disease pathophysiology for a better understanding of host-parasite interactions. Additionally, proteomics has also paved the way for screening new chemicals for potential therapeutic applications and disease intervention. Figure 1 comprehensively illustrates the use of different proteomic techniques/

platforms that have greatly added to proteomic studies for exploring new avenues in biomedical research.

Analysis of the parasite proteome in the search for new therapeutic and vaccine targets has remained a priority for the scientific community since the early 1970s and 1980s, leading to the establishment of one-dimensional electrophoresis and Edman sequencing for structural and molecular characteristics of proteins [43, 44]. Since 1975, two-dimensional electrophoresis (2DE) has improved, allowing the simultaneous determination of the molecular weight and isoelectric point of proteins [45]. These are amongst the most conventional techniques of choice for protein profiling. The 2-DE involves the polyacrylamide gel configuration, which allows quantitative as well as qualitative separation of proteins by using the cell lysate and its visualization by silver staining. The first proteome map for *Leishmania spp.* was prepared using this technique to identify virulence markers [46] and was later also used for species differentiation [47]. Nevertheless, this approach had limited accessibility to the diverse array of proteins and was biased towards the analysis of relatively abundant proteins; however, the resolving power of 2-D has placed it amongst the top choices for studying post-translational protein modifications. Later in the 1980s, it was combined with mass spectrometry (MS), in which the sample was ionized either by matrix-assisted laser ionization/desorption (MALDI) and electrospray ionization (ESI) [48, 49, 50]. MALDI-MS has been used to analyse simple mixtures of peptides, while ESI-MS uses an additional system of liquid chromatography, which is commonly used for the identification of proteins in complex samples [51] and can serve to provide clues regarding changes in protein expression in different pathological conditions. However, conventional 2-DE approaches have now been supplanted by quantitative mass spectrometric techniques, which have superior chemical selectivity that facilitates comparisons of two to ten samples in one experiment. Furthermore, quantitative mass spectrometry results in the separation of multiple protein extracts on the same 2-D gel, while pervasive concerns with conventional 2-DE limits its prospects in proteome research. Later, the concept of shotgun proteomics was developed, which uses a bottom-up proteomic technique for the identification of proteins using a coupled system of high-performance liquid chromatography (HPLC) with MS to study whole proteins in a complex biological sample such as serum, urine, etc. The inability of the one-dimensional separation methodology to resolve a complex mixture of proteins led to the advent of shotgun proteomics, a bottom-up approach for the characterization of proteins by analysis of peptides produced upon proteolysis, using MS and HPLC. This approach was further refined with the emergence of a multidimensional separation phenomenon (MudPIT) that included the coupling of two or more independent techniques (i.e., reverse-phase, ion exchange, size exclusion and affinity chromatography) for analysis of samples from complex biological systems [52]. The basic requirement during the experimental procedure remains the analysis of two or more physiological conditions within a biological system, which has led to the establishment of labelling techniques in which peptides labelled with radioisotopes are quantified by LC-MS/MS. These techniques were further categorized into iTRAQ, Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC) and Metal Affinity Chromatography (iMAC), which served to quantify proteins in addition to examining post-translational modifications in *Leishmania* [53, 54, 55, 56].

Several online 2-DE derived databases have further assisted in building the foundation of proteome mapping through data mining and management. One of the major advances in this technique has been the use of an immobilized pH gradient (IPG) matrix [57, 58], a modification of this system using zoom-gels, in which fractionation of the sample into narrow pH ranges with low resolution is followed by high-resolution separation by 2-D PAGE. Another modification of 2-D PAGE is differential in-gel electrophoresis (DIGE) [59], in which differences in protein profiles of cells in different functional states are examined by labelling the samples with different fluorescent dyes. DIGE from VL sera has provided important clues concerning the prognosis of VL [60]. The 2-DE-based protein array has also been reported to provide a framework for inter-species profiling of *Leishmania* proteins [61]. The major shortcoming associated with conventional 2-DE is the under representation of basic proteins [62], especially in the context of intracellular pathogens that have been predicted to bear larger arrays of basic proteomes than their free-living counterparts [63, 64]. There is evidence for liquid phase isoelectric focusing (IEF) prior to 2-D gel separation with increased resolution of basic proteins but no clear identification of proteins [65], which led to the emergence of free-flow electrophoresis (FFE) that was later miniaturized approximately 1994. FFE has offered possibilities due to its rapid separation protocol and requirement for low sample volumes (in the microliter range). This technique employs electrophoretic separation of analytes by flowing through a planar flow channel and electric field perpendicular to the flow that diverts the analytes based on their mobility. This combination of IEF-FFE and 2-DE-based high-resolution separation technology has paved the way for the discovery of novel basic proteins from *Leishmania* [66] that could be of therapeutic importance. These microfluidic FFE systems and their different modifications, viz. free-flow zone electrophoresis (FFZE), free-flow IEF (FFIEF), free-flow ITP (FFITP), and free-flow field-step electrophoresis (FFFSE), might find applications in so-called lab-on-a-chip devices for real-time monitoring and separation applications. Cup-loading at the anode has been another useful tool for basic proteome mapping [67]. The limited throughput associated with 2-DE has led to difficulties in large scale comparative protein expression assays, resulting in the introduction of 3-dimensional geometry gel electrophoresis in which a large number of samples are analysed simultaneously. IEF followed by the use of several IPG strips arrayed on the surface of a 3-D gel body and the application of electro-kinetic field-mediated transfer to the gel ensures constant thermal conditions that provide data concerning the comparability of separation patterns. This format uses a laser-based fluorescence detection system for Cy3 dyes, and images are acquired, processed and recorded as a stack of 3-D images by the digital camera, thus offering wide range of applications [68]. The use of fluorescent dyes (Cy and Sypro Ruby dyes) has added quantification feature in 2-DE for analysing quantitative differences among the spots on the 2-D gel [68, 69, 70].

Low-abundance proteins have been investigated using conventional cellular pre-fractionation methods [71], while gel-free mass spectrometry [53, 72] has further served in the discovery of new proteins. Mass spectroscopy (MS) has also been added to the foundation stone for proteome mapping. Proteomic studies have gained momentum with high-quality proteome maps of *Leishmania*, in combination with MS and bioinformatics tools, enabling the identification of some novel drug targets, virulence factors, and vaccine antigens for use in

disease control as well as in understanding drug resistance in *Leishmania* parasites. The most common approach to separate complex protein mixtures is by 2-DE, and the proteins are generally separated based on net charge by IEF in the first dimension followed by second-dimension separation based on their molecular weight using standard SDS-PAGE. Gels are stained using CBB, silver nitrate, or fluorescent dyes to identify protein spots.

Quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE), a recent variant of native gel electrophoresis for separating proteins based on their isoelectric points, is broadly used for the isolation of metalloproteins [73]. QPNC-PAGE, when combined with biological mass and NMR spectrometry, provides clues concerning the metabolism of metal cofactors in biological systems [74].

When considering protein-protein interactions and functional links between different proteins in regulating cellular functions, the yeast two hybrid system is appropriate, a conventional approach for identifying interacting proteins [76] in which the gene sequence coding for a particular protein of interest is fused to a transcription factor binding domain and expressed as a fusion protein in yeast [77]. In contrast, assessment of the interactions of multiple proteins in a multi-protein complex has been made feasible by immunoaffinity methods using the glutathione S-transferase tag in the fusion protein [78]. This approach has been used to study human spliceosome multi-protein assembly [79], while computational biology homology [80] and non-homology [81]-based approaches are used to study protein interactions among eukaryotic proteins based on sequence and functional similarities. Thus, conventional proteomic tools and high-throughput platforms have provided an avenue for exploring parasite biology in combination with different genomic and bioinformatic tools to extensively examine the pathophysiological causes of disease.

4. Modulation of the *Leishmania* proteome profile

Leishmaniasis involves alterations in protein level expression upon infection; therefore, studying changes in protein profiles during disease manifestation provides clues for protein signatures of disease and validation for disease intervention. Hence proteomics tools can be used to identify protein markers to screen for their utility in developing clinical trials or for diagnosis. In contrast, proteomics also provides potent chemicals for T-cell activation, as reported for Mycobacterium infection [82, 83].

The genome sequencing of *L. donovani* in 2011 [84] has further provided clues for understanding the role of the parasite-host-vector trio in determining the pathogenesis of disease. Thus, annotated genome sequencing has provided the landmark for the identification of proteins that regulate different developmental stages of the parasite. In this section, we have provided an understanding regarding how the aforesaid proteomic technologies have facilitated our understanding of parasite biology and host-parasite interactions.

4.1. Developmental regulation of the proteome profile in *Leishmania*

The combination of genomic tools along with proteomics has shed light on the different life-stages of the parasite and its developmental regulation. Host-parasite interactions continue to

be crucial determinants of disease pathogenesis and have been widely studied for centuries. Although proteomic applications remain a challenge in diverse areas of pathogen biology and provide huge datasets, current knowledge has provided a good degree of understanding regarding the mechanism of molecular pathogenesis [85, 86, 87] and further paved the way for research on parasite and host proteomics defining the concept of “parasitoproteomics” to investigate global proteins of parasite and host during cross-talk. The vital question in this context continues to be the specificity of host responses during parasite infections. We will individually examine the effects of each factor on disease pathogenesis.

4.1.1. Proteomics of sandfly salivary components in disease pathogenesis—

Blood-feeding arthropods are the vectors for several debilitating diseases, including malaria, leishmaniasis, chagas’ disease, and trypanosomiasis. The salivary component has been documented to enhance the virulence of the causative agent due to the presence of a diverse array of pharmacological substances in their saliva, which facilitates disease pathology. Insights into vector biology has documented the disease exacerbatory effects of salivary components on *L. major* infection [88] and Lyme disease [89], which has fuelled research examining immunogenic salivary components of vectors, especially in case of *Leishmania* infection [90, 91] where salivary components enhance parasite transmission by altering the innate and acquired arms of immunity [92, 93]. Results from functional transcriptomics [94, 95] have laid the foundation for analyses of genus and species-specific proteins that assist in building up the repertoire of salivary proteins for the development of vector-based vaccines. Proteomics has further answered several questions by identifying almost all immunogenic salivary proteins in 1-dimensional gels and by western blotting [96, 97, 98, 99, 100, 101]. The combination of cDNA sequencing, proteomics and computational biology has revealed the secreted proteins from salivary gland of *Lutzomyia longipalpis*, leading to a better understanding of the host immune response to salivary proteins in the context of parasite infection and providing clues for the identification of exposure markers and targets for vaccine development [102].

Salivary protein profiling studies using 2-DE, western blotting and MALDI-TOF/TOF has established the first proteomic map of antigenic *P. perniciosus* salivary components [103] and *P. argentipes* [104]. These proteomic datasets have suggested an increased flux in protein content with a huge number of hypothetical as well as known proteins depending on the age of the sandfly. The utility of proteomic tools has also served to identify species specific salivary antigens for the development of vector-based vaccines [104]. In contrast, MALDI-TOF has further assisted epidemiological surveillance of the vector, its identification [105, 106] and knowledge about the infection status [107].

4.1.2. Proteomics of sandfly feeding behaviour and parasite differentiation—

Leishmania employs myriad adaptation strategies to ensure its survival and transmission. The initial blood meal is followed by migration of the parasite into the midgut of the vector, where it gradually slows down its replication and differentiates into the elongated promastigote form. The early phase of survival within the midgut involves encounters with an array of proteases-trypsin, chymotrypsin and aminopeptidases [108, 109]. Another evasion strategy of the parasite is the presence of protease inhibitors, which have been suggested to be weapons used for protection against vertebrate macrophage and insect

midgut proteases [110, 111]. The most important phase in the host-parasite interaction is the attachment of the parasite in the midgut of the sandfly via lipophosphoglycan (LPG) [22, 112] and the flagellar proteins FLAG1/SMP-1 [113]. Although LPG is essential for adhesion to the midgut, it does not constitute the basis for the permissivity of the vector, which actually depends on the glycosylation status of glycoproteins in the midgut [114]. Recent evidence from functional annotation and protein profiling datasets from *Anopheles culicifacies* in a malaria model suggests a differential behaviour of blood-feeding arthropods, providing insight into the physiology of blood feeding and pathogen transmission [115].

Differentiation is a highly regulated process with a dominance of post-transcriptional mechanisms over transcriptional control; thus, proteomic approaches are essential for understanding the stage-specific protein profile. In this context, research on *L. mexicana* proteins by 2-DE and CapLC-QTOF MS has provided clues about stage-specific subsets of proteins, which has improved our understanding of host-parasite interactions and parasite infectivity [116]. Proteogenomic approaches have further revealed 22,322 proteins from two different life stages of *L. donovani* using a homology-based search system available for three *Leishmania* species (*L. infantum*, *L. braziliensis* and *L. major*). This finding suggests the utility of proteomic technologies as a weapon for biological predictions, even for organisms with unsequenced genomes [117]. Similarly, proteogenomic approaches have provided insight into the proteome map, genome annotation and N-terminal acetylated peptides of several genes from *L. major* [118]. Proteome mapping has further enhanced our understanding regarding the life stages of the parasites, promastigotes and axenic amastigotes. It has served to identify putative developmentally regulated proteins, changes in metabolic profiles, stress tolerance, cell cycle, cytoskeleton, phosphorylation status and parasite virulence [119, 120]. However, initial studies using quantitative proteomic approaches (isotope coded affinity tags) in combination with DNA oligonucleotide genome microarray and mRNA expression profiles led to better characterizations of stage-specific proteins in promastigotes and amastigotes [72]. The molecular basis of differentiation was further assessed in *L. donovani* by metabolic labelling examining protein synthesis patterns and protein levels [121].

Recent reports for phosphoproteomic analyses have revealed differences in the phosphorylation status between different stages and identified phosphorylation motifs [68, 122]. These findings have been further validated by detailed assessments of the kinetics of protein phosphorylation during differentiation by iTRAQ affinity tagging [123]. Quantitative phosphoproteomics in combination with systems biology has revealed the role of chaperones tethered to protein kinases in parasite viability [69]. Further quantitative proteomics analysis of *L. donovani* have provided clues regarding the differential expression of proteins related to nutrient acquisition, energy metabolism, cell motility, signalling during parasite differentiation and survival [56].

4.1.3. Role of proteomic tools for understanding the cell biology of

Leishmania—Since infectious and parasitic agents remain the major cause of chronic diseases in developing countries, molecular studies are still the first choice for understanding host-parasite interactions. Although the rate of successful infection is determined by the

virulence of the parasite and immune competency of the host, immune pathology, in turn, is determined by secreted, intracellular and surface molecules. However, proteomic tools have also paved a holistic way for understanding parasite biology at organelle level, with the aim of obtaining a precise understanding of host-parasite interactions and assisting in the development of new drug targets.

First-generation proteomic tools have provided a way to understand post-translational modifications in response to parasite invasion, but understanding parasite proteomics remains a hotspot for expediting disease pathogenesis. In this context, *Leishmania*-associated surface coat molecules have been of interest. However, these molecules also constitute the major fraction of the parasite secretome, as evidenced by the N-terminal secretion signal containing proteins that include surface proteins, e.g., proteophosphoglycan [124].

Proteome analysis of the cell membrane has remained a daunting task because of difficulties associated with their isolation and solubilization. However, detergent extraction has been the choice for extraction of metabolic enzymes, transporters and virulence factors associated with membrane fractions, in addition to the differentiation of virulent and avirulent parasites [116, 125, 126]. Recently, the combination of FFE with 1-DE and mass spectrometry [127] has refined the search for enriched fractions of proteins, including transmembrane fractions. The characterization of *L. infantum* membrane antigens in Mediterranean VL has served as an important serological marker for the disease, identified using 2DE and mass spectrometry (liquid chromatography-mass spectrometry/mass spectrometry), an important candidate for diagnostic implications [128]. Quantitative mass spectrometry using iTRAQ has identified differences in the membrane fractions of promastigotes and amastigotes in *L. infantum* and *L. mexicana* with a potential role in host-parasite interactions and parasite survival [129], providing clues to target these molecules for therapeutic advantages.

However, proteomic patterns of subcellular fractions have been analysed by high-resolution micro sequencing, while 2-D gel analysis-based organelle fingerprints have been prepared and made available in the public domain. In this context, the proteomic composition of phagosomes from macrophages and nucleoli has been evaluated. For parasite sub cellular proteomics, microsomes are an important target organelle for proteomic analysis, for which early analysis by combining sonication and serial centrifugation for protein characterization has led to the identification of GP63, GP46, trypanothione peroxidase and cytosolic nucleoside diphosphate kinase, Rab protein and HSP 60 [130]. Proteomic coverage of cytosolic and intracellular proteins has been achieved with great precision by prefractionation with digitonin [131]. Tandem mass spectrometry based proteomic analysis with electron microscopy has provided evidence for the most stable and abundant 45S ribonucleoprotein complex associated with the mitochondrial ribosome from *L. tarentola* [132], which is known to be essential for normal mitochondrial translation in trypanosomatids [133]. Sub-cellular fractionation offers advantages for proteomic research and has been applicable for gel-free protein separation techniques as isotope-coded and biotinylated affinity tags in different experimental models. Proteomic analyses in cell free systems developed in single-tube translation systems have led to the analysis of proteins

from *L. tarentola*, which later formed the basis for understanding the functional roles of proteins from *Plasmodium falciparum* and mammals [134].

Additionally, intracellular organisms also release a plethora of metabolites, enzymes and virulence factors constituting the secretome, which interferes with host cell signalling and modulates immune responses; thus, they constitute a key determinant for disease pathogenesis. Evidence from previous studies has validated the export of virulence factors into the host cell with the identification of elongation factor-1 α in conditioned medium from *Leishmania*. It activates SHP-1 in macrophages and thus deactivates effector functions in macrophages [135], instigating research on secretome analysis. However, the advent of sophisticated proteomic tools along with *in silico* modelling approaches has led to the understanding that protein secretion remains a heterogeneous process with several non-classical secretory pathways, e.g., the identification of exosome-mediated vesicle-based secretion systems [136]. These exosomes are an integral part of the parasite life cycle and are formed by invagination of the endocytic compartment. They have immunomodulatory properties and effects on the disease severity by serving as virulence factors associated with vector transmission [137]. Secretome analysis by the quantitative approach has also revealed that proteins that are actively secreted by the parasite differ from those associated with the cell body of the parasite [136]. However, this study had a limitation of contamination from serum proteins and degraded products, which were removed using serum-free medium with peptidase inhibitor and analysed by LC-MS/MS. The field of secretome analysis is a key avenue in parasite proteomics employing 2DE and mass spectrometry methods for analysing the soluble protein content of the microsomal fraction from *Leishmania* [130].

The secreted proteins also have a diversity of roles during the establishment of infection [138], involving alterations in host macrophage microbicidal signalling [139, 140]. Although secretome-based studies have suggested many key targets, these studies have limitations associated with intramacrophagic studies. The exoproteome studies from *in vitro* culture systems have paved the way towards proteomic insights into parasite biology. However, these studies are focused on stationary-phase metacyclics with a good degree of immunogenicity in mice and canine models [141, 142, 143]. LaPSA-38S and LiPSA-50S are recently discovered proteins from the excretory/secretory fractions of promastigote and amastigote stages with immunodominant influence [144] and potential vaccine candidacy [145].

The exoproteome of some *Leishmania* species has revealed important insights into host–parasite interactions and led the way for novel disease interventions. Studies of the *L. infantum* exoproteome revealed its molecular profile by a shotgun proteomic approach in which 102 proteins were identified and their relative abundance index (emPAI) shown for the complex nature of the parasite exoproteome. Several molecules in particular were involved in nucleotide metabolism and antioxidant activity, which were later tested for immunogenicity using sera from carriers of differing clinical forms of canine visceral leishmaniasis (CVL). Therefore, this approach's utility in discriminating symptomatic animals from those exhibiting other clinical forms of the disease has opened up new research avenues related to the treatment, prognosis and diagnosis of CVL [146].

4.2. Systems biology-based analysis of *Leishmania* proteomics

In the current post genomic era, bioinformatics has taken centre stage in biomedical fields in reference to data storage, management, retrieval and visualization, which is meant to cater to research by facilitating understanding of the complexities inherent to the development of new drug targets via computational tools. Currently, modelling and simulations for biomedical research require the establishment of a strong database and its curation for dataset management. However, many databases in the public domain, such as GenBank, EMBL, and UniProt, along with mathematical packages, such as MATLAB and Bioconductor, have helped to strengthen our understanding of parasite biology [147, 148]. In contrast, immunoproteomic approaches have further offered possibilities for the identification of diagnostic markers, vaccine candidates and/or drug targets.

Bioinformatics tools have enabled researchers to extract biological information from organisms and understand protein functions (Table 2). Technical difficulties associated with 2DE experiments, time-consuming procedures, a limited number of biological replicates analysed per reaction, limitations associated with the analysis of highly hydrophobic and acidic/basic proteins and limited automation are other reasons for the use of bioinformatics-based approaches, which have helped to establish complete proteome maps for cells and organelles under normal and pathological conditions. Public proteomic data repositories have provided avenues for peptide identification, biomarker discovery and systems biology. The large volume of data in these repositories are easily managed and may actually require the combined efforts of many laboratories, and a few main repositories for data mining and visualization include the Proteomics IDentification database (PRIDE) [149], the Global Proteome Machine database (GPMDB) [150], PeptideAtlas [151], Human Proteinpedia [152, 153], SwedCAD [154], and PepSeeker [155], among others, with different aims and functionalities. The HUPO Proteomics Standards Initiative (PSI) (<http://psidev.info/>) continues to strive to provide a single format for MS spectrum analysis, e.g., mzData and mzXML have been replaced with single mzML 1.0. In contrast, quantitative proteomic approaches, such as SILAC, iTRAQ and ICAT [156], are the most demanding techniques in the current scenario with the limited datasets available in public repositories because of the lack of formats for data capture, storage and exchange. Furthermore, best practices for protein quantification have not been established. The integration of proteomic data with other types of biological data provide a better understanding of systems biology.

Proteins do not function in their native states but require interactions with other proteins. Therefore, the analysis requires an understanding of the protein networks and their importance in the biological system. Understanding the topological metrics using an *in silico* approach has further paved the way for exploring proteins in detail with the help of software such as PSIMAP [157] and PlasmID [158]. A similar protein interaction and network studies have been reported for *L. major* using an *in silico* approach with iPfam, PEIMAP and PSIMAP [159]. Proteomics studies in combination with biological network analysis have shed light on the protein profiles of CL, thus highlighting the mechanism of tissue damage in lesions of CL [160]. Bioinformatics approaches have also assisted in identifying novel targets for immunodiagnosis in tegumentary, VL and canine VL by characterizing B-cell epitopes that are conserved in *Leishmania* [161, 162]. Similarly, epitope prediction has

further unveiled *Leishmania*-derived cysteine protease as the B-cell epitope, thus paving the way for designing peptide-based vaccine [163]. Moreover, epitope prediction studies of the C-terminus of cysteine proteases have provided clues for their role in parasite survival [164].

Computational analysis in combination with immunoproteomic approaches has led to the screening and identification of new drug targets based on their localization and biological functions [165]. *In silico* approaches have also been proven to be efficacious in determining the serological significance of leishmanial antigens with tandem repeat domains [166]. A combination of immunoproteomic approaches along with *in silico* predictions led to the search for new drug targets according to their localization and biological functions [165].

Another arm of bioinformatics to discover novel antigens continues to be the reverse vaccinology approach. Previous studies have reported the integration of the predictions from B- and T-cell epitopes, protein-protein interactions networks and metabolic pathways revealing protective proteins [167]. Furthermore, reverse vaccinology application in *L. infantum* has demonstrated its potential in discovering immunogenic antigens based on their differential binding affinities for MHC class I and class II instead of culturing the whole organism [168].

4.3. *Leishmania* and drug resistance proteomics

Proteomic technologies complement genomics and transcriptome profiling studies in terms of the detailed data on the nature of the final gene product. Proteomics have been widely used to generate a proteome map providing an overall picture of gene expression at a given point in time. Several proteomic tools have provided an in-depth understanding of biological procedures and have been used to answer specific research questions. In this context, quantitative proteomics and protein profiles have provided clues regarding proteomic responses to drug resistance, habitat and life-cycle-related processes. The differentially expressed proteins in the resistant phenotypes display an innately regulated gene expression programme as part of an adaptive strategy to the environment. Little information had been reported about the proteomic aspects of drug resistance, until the first report in 2003, in which high-resolution mass spectrometry-based proteome analysis identified several landmark proteins, including trypanothine reductase and pteridine reductase associated with drug resistance mechanisms [64]. Recent reports of meglumine antimoniate resistance in anthroponotic CL have shown an association with over or under-expression of activated protein kinase C receptor (LACK), α -tubulin, prostaglandin f2- α synthase, protein disulphide isomerase, vesicular transport protein and many unknown proteins. These findings demonstrate the utility of proteomics in demarcating sensitive and resistant isolates [169].

Protein phosphorylation is a frequently studied process for understanding regulatory patterns of cellular events. Comparative phosphoproteomic analysis by 2D-DIGE from antimony-resistant and susceptible lines of *L. braziliensis* have revealed stress response proteins and chaperones associated with antimony treatment. Altered patterns of antioxidants, RNA/DNA processing, metabolic processes and protein biosynthesis have been observed in antimony-resistant isolates. These findings have provided an understanding of the role of proteomics in biochemical signalling associated with drug resistant and/or susceptible phenotypes [170].

The 2D-DIGE quantitative proteomics has further identified mitochondrial HSP70 from miltefosine-resistant and sensitive isolates of *L. donovani*, which is associated with resistant isolates and the stress response [171] and involvement of eukaryotic elongation factor 4A(euIF4A) in miltefosine resistance [172]. Another study has reported increased argininosuccinate synthetase and decreased levels of KMP-11 associated with antimony-resistant isolates [172]. An increasing body of evidence suggests that mitochondrial-induced cell death is the basic mechanism of action in antimonial and miltefosine-based treatments. Therefore, differential protein analysis of mitochondrial fractions from resistant and susceptible strains has provided clues regarding the active involvement of mitochondria in antimony resistance [173].

In addition to the role of the mitochondrial proteome in drug resistance, proteomic mapping has recently identified 60S ribosomal L23 (60sRL23) protein associated with SAG resistance in *L. donovani*. This resistance has been further extended to miltefosine and paromomycin, where this protein reduces drug pressure by increasing the proliferation of parasites [174]. Cysteine and leucine-rich proteins are another target characterized by bioinformatics, immunoblotting and immunolocalization analyses in resistant isolates of *L. donovani*, which acts by promoting parasite survival in macrophages [175]. Another quantitative proteomics tool, SILAC in combination with next-generation sequencing, has been used to characterize the marker of antimony resistance, MRPA, in *L. infantum* in addition to the involvement of chromosome number variations, specific gene amplifications and SNPs as key determinants of antimony resistance [176].

Miltefosine resistance is being further examined by the 2D-DIGE and MALDI-TOF/TOF approach in miltefosine-resistant and sensitive isolates of *L. infantum* for which proteins associated with redox homeostasis, stress response, protection against apoptosis and drug translocation were up-regulated and thereby provided evidence of the multifactorial nature of drug resistance [177]. Quantitative proteomics by SILAC has also been revealed for signatures associated with amphotericin B resistance in cytoplasmic and membrane-enriched fractions of isolates. Furthermore, altered profiles of metabolic enzymes, redox pathways, transcription and translation processes has been reported in resistant isolates [178]. Global proteomic analysis of paromomycin-resistant isolates, similar to amphotericin B-resistant isolates, have altered transcriptional and translational pathways in addition to intracellular survival and vesicular transport-associated proteins [54].

Similarly, proteomic profiles of sitamaquine-resistant isolates suggested for altered profiles of sterol and phospholipid metabolisms have been shown to be related to alterations in phosphatidylethanolamine-N-methyl-transferase and choline kinase activities, leading to decreased cholesterol uptake and ergosterol biosynthesis [179].

4.4. Application of proteomic studies in understanding disease pathogenesis and clinical importance

Proteins constitute the basic unit of the functional diversity of cells and the key for cellular regulatory processes. Since genomics has been proven to be inadequate for predicting the structural, functional and dynamic properties of proteins, proteomics has emerged, which

constitutes a large-scale analysis of protein structure, function and differential regulation during normal physiological and diseased states.

Additionally, it aims to correlate the structural and functional properties of proteins with biological processes, providing new horizons for clinical research in prognostics, diagnostics and therapeutics and, ultimately, allowing the development of individualized therapy. This section addresses the application of proteomic technology in understanding disease biology. Clinical applications of proteomics date back to the times when the term was coined, utilizing agarose gels, zones and boundaries of electrophoresis for proteins. The electrophoretic techniques have formed the backbone for the diagnosis of haemoglobinopathies [180]. Subsequently, capillary electrophoresis was introduced for the diagnosis of different diseases [181, 182]. Later, with the advent of new technology, a flood of data was obtained in a short time and provided different options for the development of other proteome-based approaches for diagnosis.

Immunoproteomic approaches have led to the discovery of several markers from stationary-phase promastigote and amastigote forms with serodiagnostic potential [183, 184]. Quantitative proteomics has further added to the diagnostic value by providing an enormous amount of data for the selection of epitopes and potential protein candidates for diagnosis and vaccine development [185]. Additionally, the membrane fraction proteins have also been identified to have diagnostic potential for *L. infantum* [128]. P32 antigen from *L. infantum* has been identified as a diagnostic marker and is recognized by 95% of Mediterranean VL sera but not by sera of CL patients; therefore, it is a candidate for discriminating patients with different clinical forms [186]. In contrast, glycoproteomics has remained the most interesting subfield of proteomics in the identification of diagnostic/prognostic markers and understanding parasite survival in the hostile environment of the host. In the context of early diagnosis, MALDI-TOF-MS-based proteome analysis of serum components has provided promising results. Serum proteomics (MALDI/SELDI-TOF) has provided clues for the analysis of multiple peptides and signals in a single mass spectrum [187].

Serum proteomic fingerprinting has improved the diagnosis of several infectious diseases [188, 189]; it could also contribute to the diagnosis of VL. The revolution in the field of protein microarrays in the form of analytical microarray, reverse-phase microarray as well as functional microarray, has brought the lab on a chip and has the potential to revolutionize biomedical research. Functional protein microarrays have facilitated biomarker identification and high-throughput characterization of monoclonal antibodies for use in identifying biomarkers for several cancers. Single-cell analyses have further paved the way for understanding immune responses in a cell-specific manner using microfluidic, mass and multiparametric flow cytometry.

Comparative proteomic studies of plasma proteins from VL cases have revealed their utility in understanding alterations in host responses, pathogenesis-associated biomarkers, therapeutic monitoring and the establishment of protein-protein interaction networks [190]. However, the aforementioned research provides only a glimpse of the role of proteomics in diagnostics and a plethora of research is involved in proteomics for the diagnosis of various diseases. Quantitative proteomics has further offered chances for clinical analysis of serum

and plasma using antibodies and nanoflow liquid chromatography and tandem MS [191, 192]. Urine-based proteome mapping constitutes another aspect of non-invasive samples for diagnosis [193]. Although mass spectroscopy-based LC-MS/MS and MS/MS datasets have multiple utilities; however, these techniques are limited because they overlook some sequences and post-translational modifications that could be of relevance in establishing the proteome-based diagnostic and prognostic models and for understanding disease progression and pathogenesis.

The absence of efficient chemotherapeutic options calls for the development of effective vaccine candidates for leishmaniasis. The main goal of vaccine development remains the identification of candidate molecules to raise protective immune responses. Recent efforts in the field of systems biology, proteomics and bioinformatics have led to the emergence of T- and B-cell epitopes as vaccine targets. In this context, a fraction of soluble *L. donovani* promastigote protein induced a T_H1 response in humans and hamsters, enriched in T_H1 stimulatory cells, T-cell stimulatory proteins, and virulence factors, among others [194], with significant levels of cellular immune response. Other immunostimulatory proteins identified by one-dimensional gel electrophoresis and MALDI-TOF-MS included HSP-70, elongation factor-2, p45, HSP-83, aldolase, triose phosphate isomerase, and aldolase. Therefore, the development of a successful subunit vaccine with multiple immunostimulatory proteins [195] using proteomic approaches has been of great use.

There is burgeoning interest in proteomics by pharmaceutical industries for the discovery of novel drug targets, which remains urgent due to alarmingly higher numbers of drug resistant cases. The use of proteomics for drug development had been a great priority. There is evidence for the application of functional proteomics in target identification from specific signalling pathways, and activity-based screening provides clues for the development of inhibitors of specific target molecules for disease intervention. In contrast, biochips have been the landmark in drug development, serving as a weapon for the efficient evaluation of compounds for specificity and selectivity with respect to binding targets. Proteomics has also served to increase the efficiency of clinical trials by providing relevant markers for the efficacy and safety of investigational drugs.

With increasing drug resistance, there has been interest in identifying and characterizing proteins for the development of adjunct therapies to reduce drug resistance. Proteome mapping has provided insights into drug resistance mechanisms and target proteins from trypanosomatids as a marker of drug resistance. Post-translational protein modifications play an important role in the regulation of protein functions. In, addition, to better exploit proteomic approaches to understand resistance mechanisms, resistant mutants of *L. major* were generated to study methotrexate resistance using a comparative proteomic approach described by Drummelsmith *et al.*, constituting the first proteomic study of the study of mechanism of drug resistance. These findings provided a basic skeleton for establishing the proteomic aspects of parasite biology [64, 196]. Similarly, antimony, amphotericin B, sitamaquine and paramomycin resistance has been widely studied, as discussed in an earlier section.

In recent years, the concept of chemical proteomics has emerged, wherein small-molecule probes are used for tagging proteins in a functionally dependent manner to discover new drug targets [197, 198]. Plasma/serum proteomics have been revisited with the recent improvements in MS [199, 200, 201, 202], providing another venture for understanding disease pathogenesis. However, plasma proteome analysis remains one of the most challenging tasks, and it has been rekindled with immunodepletion and peptide fractionation methods for gaining insight into the plasma proteome [203, 204, 205]. Multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) constitutes another mass spectroscopic technique for analyses of plasma proteins with high sensitivity for the detection of attomole concentrations of target peptides [206]. Recently, the plasma proteome profiling pipeline was developed using a single-run shotgun proteomic workflow with the ability to quantitatively analyse hundreds of plasma proteomes from small volumes of blood from finger pricks [207]. It provides an opportunity to explore new avenues for host-parasite interactions and novel targets with therapeutic potential.

4.5. Current issues and challenges in *Leishmania* proteomics

In the post-genomic era, proteomics has been the most demanding field in recent decades for identifying effective biomarkers because of the wide applicability of mass spectrometry to biological systems. However, proteomics has undergone major revolution by the discovery of protein chips, affinity and multidimensional fractionation, which have assisted in elucidating disease pathogenesis, drug targets, post-translational modifications, and diagnosis and prognosis markers. Comparative proteomic approaches enable the scientific community to draw correlations between different proteins, their interactions and post-translational modifications produced during disease, therapeutic cure or disease remission.

Despite these major advances in proteomic technologies, various issues and challenges remain unaddressed, which could be attributed to the complexities of biological samples, physical and chemical characteristics, post-translational modifications and the dynamic nature of the proteome. The major challenges in proteomics include the cumbersome process of the experimental design, including the selection of quality reagents, optimization, limitations associated with multiplex proteomic assays and lack of technologies when working with clinical samples. In the context of *Leishmania*, the daunting task of purification of amastigotes in sufficient quantities further complicates reproducible proteomic analyses. However, proteomic studies have been underestimated in the past decade in terms of research for effective biomarkers due to limitations related to connecting biomarker discovery with well-established clinical research methodologies. Therefore, proteomic studies to date have lacunae in reproducibility and accuracy for the detection of proteins in biological specimens. Additionally, alternative splicing has the capability of producing multiple versions of a protein, with an extreme example of a gene encoding 38000 proteins [208], mutations and more than 200 post-translational modifications, further complicating studies of the protein repertoire. Thus, on average, three different proteins are encoded by each gene. These pitfalls highlight the need for a better understanding of strategies for proteomic studies to design quality biomarkers.

With the advent of high-throughput technology platforms, it is anticipated that information from proteomic datasets will become widespread and complex. In this context, there is an urgent need to develop a universal standard for data representation, storage and management with a minimum level of annotation to facilitate analysis and exchange of proteomic datasets between different groups to build a robust analysis tool. This exchange of information and reanalysis by different research groups will serve to overcome the current challenge of variability and reproducibility in proteomic research.

Another key issue regarding proteomic research continues to be the integration of different datasets, since any dataset in research is of limited use until it is integrated with the vast knowledge from genomic and proteomic datasets and disease, leading to the concept of “intelligent data”, wherein object-oriented programming is designed to address complex proteomic issues [209]. The improvements in current database concepts can be implemented by integrating the transcriptomic and proteomic information with the cellular regulatory pathways [210], metabolomics, protein interaction studies and network analyses. Since current databases provide limited information concerning the analysis of particular proteins or interactions between a limited number of proteins, a combination of results from transcriptomic and proteomic studies with pathway and network analyses will provide a better understanding for developing and testing hypotheses [211, 212, 213]. However, further efforts are still required to integrate functional genomics and proteomic datasets.

Expert commentary

High-throughput proteomic platforms have established the importance of proteomics for identifying proteins that are expressed during different developmental stages of the parasite, subcellular proteome, secretome and post-translational modifications. The sensitivity and applicability of proteomics have improved our understanding of many aspects of this biological system. However, a comprehensive approach is required to understand complex biological processes since single approaches (2DE, MS, protein chip/microarray) alone do not provide answers to different facets of host-parasite interactions. Recent improvements, including the use of fluorescent dyes in 2DE, have expanded the utility of expression proteomics with good reproducibility and accuracy. Several modifications in mass spectrometry have made it one of the favourite options for structural proteomic studies. In studies of host responses upon encountering pathogen, international initiatives by the human proteome organization (HUPO), including the Proteome Standard Initiative (PSI), are serving as strong pillars for the standardization, storage and processing of large proteome datasets, thereby facilitating data interpretation and integration to generate hypotheses to understand host-parasite interactions. Plasma/serum proteome mapping has been another landmark in proteomics leading to a better scope for exploring disease biomarkers. Experimental approaches for determining protein interactions along with computational analyses provide the framework for studying cellular processes. Functional analysis and interaction proteomics have further consolidated protein and/or peptide analyses to understand protein functions in the regulation of cellular behaviour.

Furthermore, proteomic studies are complemented by genomic approaches to gain insights into entire proteome maps, providing clues for disease pathogenesis and drug resistance and

action. In contrast, subcellular proteomic analysis serves to identify novel protein targets, while analyses of post-translational modifications provide an in depth understanding of host-parasite interactions and pathogenesis mechanisms, thus unveiling candidate pathways that could be targeted therapeutically in VL.

Functional protein microarrays are now thought to serve as versatile tools for high-throughput biological applications; however, they are still in their infancy. Recent progress in mass spectrometry has provided accessibility to the global profiling of post-translational protein modifications using a shotgun approach. Other modifications, such as reverse-phase or analytical microarrays, microfluidics, and multiparametric and mass flow cytometry, can further accelerate biomedical research.

Single-cell protein analysis with advanced microfluid platforms represents another avenue for understanding disease immunobiology at the single-cell level. However, the widespread use of single-cell analysis requires the support of modelling and informatics tools that can integrate protein datasets with other omics tools. Thus, proteomics remains important to depict parasite biology, disease pathogenesis and immune responses. Analyses of subproteomes, exoproteomes and post-translational modifications have unveiled several structural as well as functional aspects of infection. Chip/microarray-based proteomic platforms have potentially raised concerns for identifying novel protein targets for therapeutic intervention, while proteome mapping and/or their combination with other post-genomics have contributed to our understanding of several untouched aspects of host-parasite interactions, disease pathogenesis and drug resistance mechanisms. The advent of newer proteomic tools will serve to establish the framework for disease diagnostics, treatment and vaccine development.

Five-year view

Leishmania undergoes profound changes in gene expression upon transmission from the vector into the host and upon their differentiation from the metacyclic to the amastigote form to survive the hostile intracellular environment of the host macrophage.

‘Omics’ tools have provided a strong platform for understanding the global genome and proteome profile during the entire life stages of the parasite. The combined analysis of the proteome maps along with the metabolomic profiles may provide a key for understanding the host-parasite interactions and depict the cellular pathways involved in pathogenesis, thus establishing the framework for network analysis. New experimental approaches for proteomic analysis along with computational methods have also strengthened biomedical research, leading to the development of cell interaction maps to understand cellular processes. In contrast, microfabrication and microfluidic technologies have promoted the miniaturization of protein modules, and the integration of current strategies with high-throughput techniques have opened avenues for large-scale analysis. SELDI-TOF [214] and LC-MS [215] have been shown to be efficient ways to complement 2DE to screen spotted proteins that serve as diagnostic tools and identify therapeutic targets. Several platforms, together with bioinformatic and genomic approaches, have provided a new interface to better understand parasite biology and its interaction with the host. Distinct databases and

bioinformatics approaches have identified proteins associated with parasite viability and infectivity, while subcellular proteomic studies and post-translational modifications have provided insights into new candidate pathways of therapeutic importance.

High-throughput approaches, including protein, peptide and small molecule microarrays, have expanded the horizon for future research. The cocktail of these high-throughput platforms with *in silico* approaches to understand protein interactions can be integrated with other information to decipher host-vector-parasite crosstalk. Further advances in proteomic methodologies, such as iTRAQ and SILAC, have been established as milestones to improve functional approaches in terms of their utility and accessibility, thus compiling the entire story of disease development.

Therefore, proteomics, with its huge armoury, has generated a backbone for advanced research in biological science by providing a vast array of datasets for understanding parasite biology, vector biology and host-parasite interactions, which could serve humanity by providing tools for diagnosis, prognosis and drug development with the aim of eliminating leishmaniasis.

Acknowledgments

This work was supported by the NIAID, NIH grant number: 2U19 AI074321, TMRC project.

References

* Of interest

** of considerable interest

1. Das A, Karthick M, Dwivedi S, et al. Epidemiologic Correlates of Mortality among Symptomatic Visceral Leishmaniasis Cases: Findings from Situation Assessment in High Endemic Foci in India. *PLoS Negl Trop Dis*. 2016 Nov.10(11):e0005150. PubMed PMID: 27870870; PubMed Central PMCID: PMC5117587. doi: 10.1371/journal.pntd.0005150 [PubMed: 27870870]
2. Martins-Melo FR, Lima Mda S, Ramos AN Jr, et al. Mortality and case fatality due to visceral leishmaniasis in Brazil: a nationwide analysis of epidemiology, trends and spatial patterns. *PLoS One*. 2014; 9(4):e93770. PubMed PMID: 24699517; PubMed Central PMCID: PMC3974809. doi: 10.1371/journal.pone.0093770 [PubMed: 24699517]
3. Adam GK, Ali KM, Abdella YH, et al. Trend in cumulative cases and mortality rate among visceral leishmaniasis patients in Eastern Sudan: a 14-year registry, 2002–2015. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases*. 2016 Oct.51:81–84. PubMed PMID: 27596686. DOI: 10.1016/j.ijid.2016.08.021 [PubMed: 27596686]
4. Alvar J, Velez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*. 2012; 7(5):e35671. PubMed PMID: 22693548; PubMed Central PMCID: PMC3365071. doi: 10.1371/journal.pone.0035671 [PubMed: 22693548]
5. Organization WH. [cited 2016 Mar 04] Leishmaniasis: situation and trends. Available from: http://www.who.int/gho/neglected_diseases/leishmaniasis/en
6. Shendure J, Lieberman Aiden E. The expanding scope of DNA sequencing. *Nat Biotechnol*. 2012 Nov; 30(11):1084–94. PubMed PMID: 23138308; PubMed Central PMCID: PMC4149750. DOI: 10.1038/nbt.2421 [PubMed: 23138308]
7. Ideker T, Galitski T, Hood L. A new approach to decoding life: systems biology. *Annual review of genomics and human genetics*. 2001; 2:343–72. PubMed PMID: 11701654. DOI: 10.1146/annurev.genom.2.1.343

8. Myler PJ, Stuart KD. Recent developments from the Leishmania genome project. *Current opinion in microbiology*. 2000 Aug; 3(4):412–6. PubMed PMID: 10972503. [PubMed: 10972503]
9. Chambers G, Lawrie L, Cash P, et al. Proteomics: a new approach to the study of disease. *The Journal of pathology*. 2000 Nov; 192(3):280–8. PubMed PMID: 11054709. DOI: 10.1002/1096-9896(200011)192:3<p:AID-PATH748>3.0.CO;2-L [PubMed: 11054709]
10. Wilkins, M., Williams, KL., Appel, RD., et al. *Proteome research: new frontiers in functional genomics*. Springer Science & Business Media; 2013.
11. Persidis A. Proteomics. *Nat Biotechnol*. 1998 Apr; 16(4):393–4. PubMed PMID: 9555734. DOI: 10.1038/nbt0498-393 [PubMed: 9555734]
12. Page MJ, Amess B, Rohlf C, et al. Proteomics: a major new technology for the drug discovery process. *Drug discovery today*. 1999 Feb; 4(2):55–62. PubMed PMID: 10234157. [PubMed: 10234157]
13. Wilkins MR, Sanchez J-C, Gooley AA, et al. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnology and genetic engineering reviews*. 1996; 13(1):19–50. [PubMed: 8948108]
14. Singh B, Sundar S. Leishmaniasis: vaccine candidates and perspectives. *Vaccine*. 2012; 30(26): 3834–3842. [PubMed: 22475861]
15. Sundar S, Singh B. Identifying vaccine targets for anti-leishmanial vaccine development. *Expert review of vaccines*. 2014; 13(4):489–505. [PubMed: 24606556]
16. Peters NC, Egen JG, Secundino N, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science*. 2008 Aug 15; 321(5891):970–4. PubMed PMID: 18703742; PubMed Central PMCID: PMC2606057. DOI: 10.1126/science.1159194 [PubMed: 18703742]
17. Russell DG, Wilhelm H. The involvement of the major surface glycoprotein (gp63) of *Leishmania* promastigotes in attachment to macrophages. *Journal of immunology*. 1986 Apr 1; 136(7):2613–20. PubMed PMID: 3950420.
18. Ueno N, Wilson ME. Receptor-mediated phagocytosis of *Leishmania*: implications for intracellular survival. *Trends Parasitol*. 2012 Aug; 28(8):335–44. PubMed PMID: 22726697; PubMed Central PMCID: PMC3399048. DOI: 10.1016/j.pt.2012.05.002 [PubMed: 22726697]
19. Stuart K, Brun R, Croft S, et al. Kinetoplastids: related protozoan pathogens, different diseases. *The Journal of clinical investigation*. 2008 Apr; 118(4):1301–10. PubMed PMID: 18382742; PubMed Central PMCID: PMC2276762. DOI: 10.1172/JCI33945 [PubMed: 18382742]
20. Ribeiro JM. Blood-feeding arthropods: live syringes or invertebrate pharmacologists? *Infectious agents and disease*. 1995 Sep; 4(3):143–52. PubMed PMID: 8548192. [PubMed: 8548192]
21. Secundino NF, Eger-Mangrich I, Braga EM, et al. *Lutzomyia longipalpis* peritrophic matrix: formation, structure, and chemical composition. *Journal of medical entomology*. 2005 Nov; 42(6): 928–38. PubMed PMID: 16465730. [PubMed: 16465730]
22. Pimenta PF, Turco SJ, McConville MJ, et al. Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. *Science*. 1992 Jun 26; 256(5065):1812–5. PubMed PMID: 1615326. [PubMed: 1615326]
23. Rogers ME. The role of leishmania proteophosphoglycans in sand fly transmission and infection of the Mammalian host. *Frontiers in microbiology*. 2012; 3:223. PubMed PMID: 22754550; PubMed Central PMCID: PMC3384971. doi: 10.3389/fmicb.2012.00223 [PubMed: 22754550]
24. Schlein Y, Jacobson RL, Messer G. *Leishmania* infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite. *Proc Natl Acad Sci U S A*. 1992 Oct 15; 89(20):9944–8. PubMed PMID: 1409724; PubMed Central PMCID: PMC50250. [PubMed: 1409724]
25. Edwards AM, Arrowsmith CH, Christendat D, et al. Protein production: feeding the crystallographers and NMR spectroscopists. *Nature Structural & Molecular Biology*. 2000; 7:970–972.
26. Montelione GT, Zheng D, Huang YJ, et al. Protein NMR spectroscopy in structural genomics. *Nature structural biology*. 2000 Nov; 7(Suppl):982–5. PubMed PMID: 11104006. DOI: 10.1038/80768 [PubMed: 11104006]

27. Bauer M, Ueffing M. Reverse genetics for proteomics: from proteomic discovery to scientific content. *Journal of neural transmission*. 2006 Aug; 113(8):1033–40. PubMed PMID: 16835688. DOI: 10.1007/s00702-006-0516-4 [PubMed: 16835688]
28. Waldrip ZJ, Byrum SD, Storey AJ, et al. A CRISPR-based approach for proteomic analysis of a single genomic locus. *Epigenetics*. 2014 Sep; 9(9):1207–11. PubMed PMID: 25147920; PubMed Central PMCID: PMC4169012. DOI: 10.4161/epi.29919 [PubMed: 25147920]
29. Norin M, Sundstrom M. Structural proteomics: developments in structure-to-function predictions. *Trends Biotechnol*. 2002 Feb; 20(2):79–84. PubMed PMID: 11814598. [PubMed: 11814598]
30. Godovac-Zimmermann J, Brown LR. Perspectives for mass spectrometry and functional proteomics. *Mass Spectrometry Reviews*. 2001; 20(1):1–57. [PubMed: 11223909]
- 31*. Gavin A-C, Bösch M, Krause R, et al. Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature*. 2002; 415(6868):141–147. Used tandem affinity purification (TAP) and MS for the first time for characterization of proteins from multiple protein complexes. [PubMed: 11805826]
32. Cho S-Y, Park S-G, Lee D-H, et al. Protein-protein interaction networks: from interactions to networks. *BMB Reports*. 2004; 37(1):45–52.
33. Puig O, Caspari F, Rigaut G, et al. The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods*. 2001; 24(3):218–229. [PubMed: 11403571]
34. Terpe K. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Applied microbiology and biotechnology*. 2003; 60(5):523–533. [PubMed: 12536251]
35. Monti M, Orrù S, Pagnozzi D, et al. Interaction proteomics. *Bioscience reports*. 2005; 25(1–2):45–56. [PubMed: 16222419]
36. Rigaut G, Shevchenko A, Rutz B, et al. A generic protein purification method for protein complex characterization and proteome exploration. *Nature biotechnology*. 1999; 17(10):1030–1032.
37. Soldes OS, Kuick RD, Thompson IA 2nd, et al. Differential expression of Hsp27 in normal oesophagus, Barrett's metaplasia and oesophageal adenocarcinomas. *British journal of cancer*. 1999 Feb; 79(3–4):595–603. PubMed PMID: 10027336; PubMed Central PMCID: PMC2362445. DOI: 10.1038/sj.bjc.6690094 [PubMed: 10027336]
38. Banks RE, Dunn MJ, Hochstrasser DF, et al. Proteomics: new perspectives, new biomedical opportunities. *Lancet*. 2000 Nov 18; 356(9243):1749–56. PubMed PMID: 11095271. DOI: 10.1016/S0140-6736(00)03214-1 [PubMed: 11095271]
39. Dunn MJ. Studying heart disease using the proteomic approach. *Drug discovery today*. 2000 Feb; 5(2):76–84. PubMed PMID: 10652458. [PubMed: 10652458]
40. Gygi SP, Rist B, Gerber SA, et al. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol*. 1999 Oct; 17(10):994–9. PubMed PMID: 10504701. DOI: 10.1038/13690 [PubMed: 10504701]
41. Zhou H, Ranish JA, Watts JD, et al. Quantitative proteome analysis by solid-phase isotope tagging and mass spectrometry. *Nat Biotechnol*. 2002 May; 20(5):512–5. PubMed PMID: 11981568. DOI: 10.1038/nbt0502-512 [PubMed: 11981568]
- 42*. Alban A, David SO, Bjorkesten L, et al. A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics*. 2003 Jan; 3(1):36–44. PubMed PMID: 12548632. Defines the concept of 2-dimensional difference gel electrophoresis as a technique of comparative proteomic studies. DOI: 10.1002/pmic.200390006 [PubMed: 12548632]
43. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature*. 1970; 227(5259):680–685. [PubMed: 5432063]
44. Matsudaira P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *Journal of Biological Chemistry*. 1987; 262(21):10035–10038. [PubMed: 3611052]
45. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. *Journal of biological chemistry*. 1975; 250(10):4007–4021. [PubMed: 236308]

46. Handman E, Mitchell G, Goding J. Identification and characterization of protein antigens of *Leishmania tropica* isolates. *The Journal of Immunology*. 1981; 126(2):508–512. [PubMed: 7451987]
47. Saravia N, Gemmell M, Nance S, et al. Two-dimensional electrophoresis used to differentiate the causal agents of American tegumentary leishmaniasis. *Clinical chemistry*. 1984; 30(12):2048–2052. [PubMed: 6499179]
48. Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Analytical chemistry*. 1988; 60(20):2299–2301. [PubMed: 3239801]
49. Tanaka K, Waki H, Ido Y, et al. Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid communications in mass spectrometry*. 1988; 2(8):151–153.
50. Fenn JB, Mann M, Meng CK, et al. Electrospray ionization for mass spectrometry of large biomolecules. *Science*. 1989 Oct 06; 246(4926):64–71. PubMed PMID: 2675315. [PubMed: 2675315]
51. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003; 422(6928):198–207. [PubMed: 12634793]
52. Domon B, Aebersold R. Mass spectrometry and protein analysis. *Science*. 2006; 312(5771):212–217. [PubMed: 16614208]
53. Rosenzweig D, Smith D, Oppendoes F, et al. Retooling *Leishmania* metabolism: from sand fly gut to human macrophage. *The FASEB journal*. 2008; 22(2):590–602. [PubMed: 17884972]
54. Chawla B, Jhingran A, Panigrahi A, et al. Paromomycin affects translation and vesicle-mediated trafficking as revealed by proteomics of paromomycin-susceptible-resistant *Leishmania donovani*. *PloS one*. 2011; 6(10):e26660. [PubMed: 22046323]
- 55*. Hem S, Gherardini PF, Hourdel V, et al. Identification of *Leishmania*-specific protein phosphorylation sites by LC-ESI-MS/MS and comparative genomics analyses. *Proteomics*. 2010; 10(21):3868–3883. Employs phosphoproteomics approach to study parasite biology and its differences with host biology and role of post-translational modification in parasite survival. [PubMed: 20960452]
56. Biyani N, Madhubala R. Quantitative proteomic profiling of the promastigotes and the intracellular amastigotes of *Leishmania donovani* isolates identifies novel proteins having a role in *Leishmania* differentiation and intracellular survival. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*. 2012; 1824(12):1342–1350. [PubMed: 22850196]
57. Skoda U, Faßbender L, Händler C, et al. Application of immobilized pH gradient isoelectric focusing to forensic hemogenetics: A survey on a three year experience with the transferrin (TF) and alpha 1-antitrypsin (PI) systems. *Electrophoresis*. 1988; 9(9):606–609. [PubMed: 3266595]
58. Righetti PG. Isoelectric focusing in immobilized pH gradients. *Journal of Chromatography A*. 1984; 300:165–224.
- 59*. Tannu NS, Hemby SE. Two-dimensional fluorescence difference gel electrophoresis for comparative proteomics profiling. *Nature protocols*. 2006; 1(4):1732–1742. Utilizes fluorescent probe labelling for 2-DE which assisted in reducing spot pattern variability thus, providing accurate, simple and reproducible method for protein analysis. [PubMed: 17487156]
- 60*. Rukmangadachar LA, Kataria J, Hariprasad G, et al. Two-dimensional difference gel electrophoresis (DIGE) analysis of sera from visceral leishmaniasis patients. *Clinical proteomics*. 2011; 8(1):4. Provides clues for sera analysis based on 2-dimensional differential gel electrophoresis to identify prognostic marker. [PubMed: 21906353]
61. Reynolds K, Fang C, Xiadong C, et al. Comparative two-dimensional gel electrophoresis maps for promastigotes of *Leishmania amazonensis*, L major. *Brazilian J Infec Dis*. 2006; 10(1):1–6.
62. Görg A, Drews O, Lück C, et al. 2-DE with IPGs. *Electrophoresis*. 2009; 30(S1):S122–S132. [PubMed: 19441019]
63. Kiraga J, Mackiewicz P, Mackiewicz D, et al. The relationships between the isoelectric point and: length of proteins, taxonomy and ecology of organisms. *BMC genomics*. 2007; 8(1):163. [PubMed: 17565672]

64. Drummelsmith J, Brochu V, Girard I, et al. Proteome mapping of the protozoan parasite *Leishmania* and application to the study of drug targets and resistance mechanisms. *Molecular & Cellular Proteomics*. 2003; 2(3):146–155. [PubMed: 12644573]
65. Brobey RK, Soong L. Establishing a liquid-phase IEF in combination with 2-DE for the analysis of *Leishmania* proteins. *Proteomics*. 2007; 7(1):116–120. [PubMed: 17124718]
66. Brotherton M-C, Racine G, Ouellette M. Separation of Basic Proteins from *Leishmania* Using a Combination of Free Flow Electrophoresis (FFE) and 2D Electrophoresis (2-DE) Under Basic Conditions. *Parasite Genomics Protocols*. 2015:247–259.
67. Drews O, Reil G, Parlar H, et al. Setting up standards and a reference map for the alkaline proteome of the Gram-positive bacterium *Lactococcus lactis*. *Proteomics*. 2004; 4(5):1293–1304. [PubMed: 15188396]
68. Morales MA, Watanabe R, Laurent C, et al. Phosphoproteomic analysis of *Leishmania donovani* pro- and amastigote stages. *Proteomics*. 2008; 8(2):350–363. [PubMed: 18203260]
69. Morales MA, Watanabe R, Dacher M, et al. Phosphoproteome dynamics reveal heat-shock protein complexes specific to the *Leishmania donovani* infectious stage. *Proceedings of the National Academy of Sciences*. 2010; 107(18):8381–8386.
70. Pescher P, Blisnick T, Bastin P, et al. Quantitative proteome profiling informs on phenotypic traits that adapt *Leishmania donovani* for axenic and intracellular proliferation. *Cellular microbiology*. 2011; 13(7):978–991. [PubMed: 21501362]
71. Righetti PG, Castagna A, Antonoli P, et al. Prefractionation techniques in proteome analysis: the mining tools of the third millennium. *Electrophoresis*. 2005; 26(2):297–319. [PubMed: 15657944]
72. Leifso K, Cohen-Freue G, Dogra N, et al. Genomic and proteomic expression analysis of *Leishmania* promastigote and amastigote life stages: the *Leishmania* genome is constitutively expressed. *Molecular and biochemical parasitology*. 2007; 152(1):35–46. [PubMed: 17188763]
73. Kastenholtz B. Quantitative Preparative Native Continuous Polyacrylamide Gel Electrophoresis (QPNC-PAGE). 2008
74. Kastenholtz B. Important contributions of a new quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE) procedure for elucidating metal cofactor metabolisms in protein-misfolding diseases—a theory. *Protein and peptide letters*. 2006; 13(5):503–508. [PubMed: 16800806]
75. Lemkin PF, Lester EP. Database and search techniques for two-dimensional gel protein data: A comparison of paradigms for exploratory data analysis and prospects for biological modeling. *Electrophoresis*. 1989; 10(2):122–140. [PubMed: 2731513]
76. Naaby-Hansen S, Waterfield MD, Cramer R. Proteomics—post-genomic cartography to understand gene function. *Trends in pharmacological sciences*. 2001 Jul; 22(7):376–84. PubMed PMID: 11431033. [PubMed: 11431033]
77. Stephens DJ, Banting G. The use of yeast two-hybrid screens in studies of protein:protein interactions involved in trafficking. *Traffic*. 2000 Oct; 1(10):763–8. PubMed PMID: 11208066. [PubMed: 11208066]
78. Pandey A, Mann M. Proteomics to study genes and genomes. *Nature*. 2000 Jun 15; 405(6788):837–46. PubMed PMID: 10866210. DOI: 10.1038/35015709 [PubMed: 10866210]
- 79*. Neubauer G, King A, Rappsilber J, et al. Mass spectrometry and EST-database searching allows characterization of the multi-protein spliceosome complex. *Nature genetics*. 1998 Sep; 20(1):46–50. PubMed PMID: 9731529. Reports the characterization of mammalian multi-protein complex of spliceosome by expressed sequence tag (EST) database and mass spectroscopy. DOI: 10.1038/1700 [PubMed: 9731529]
80. Bock JR, Gough DA. Predicting protein–protein interactions from primary structure. *Bioinformatics*. 2001 May; 17(5):455–60. PubMed PMID: 11331240. [PubMed: 11331240]
81. Eisenberg D, Marcotte EM, Xenarios I, et al. Protein function in the post-genomic era. *Nature*. 2000 Jun 15; 405(6788):823–6. PubMed PMID: 10866208. DOI: 10.1038/35015694 [PubMed: 10866208]
82. Sinha S, Arora S, Kosalai K, et al. Proteome analysis of the plasma membrane of *Mycobacterium tuberculosis*. *Comparative and functional genomics*. 2002; 3(6):470–483. [PubMed: 18629250]

83. Sinha S, Kosalai K, Arora S, et al. Immunogenic membrane-associated proteins of Mycobacterium tuberculosis revealed by proteomics. *Microbiology*. 2005; 151(7):2411–2419. [PubMed: 16000731]
84. Downing T, Imamura H, Decuypere S, et al. Whole genome sequencing of multiple Leishmania donovani clinical isolates provides insights into population structure and mechanisms of drug resistance. *Genome research*. 2011 Dec; 21(12):2143–56. PubMed PMID: 22038251; PubMed Central PMCID: PMC3227103. DOI: 10.1101/gr.123430.111 [PubMed: 22038251]
85. MOURA H, OSPINA M, WOOLFITT AR, et al. Analysis of Four Human Microsporidian Isolates by MALDI-TOF Mass Spectrometry. *Journal of Eukaryotic Microbiology*. 2003; 50(3):156–163. [PubMed: 12836871]
86. Vierstraete E, Verleyen P, Baggerman G, et al. A proteomic approach for the analysis of instantly released wound and immune proteins in Drosophila melanogaster hemolymph. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101(2):470–475. [PubMed: 14707262]
87. Levy F, Bulet P, Ehret-Sabatier L. Proteomic analysis of the systemic immune response of Drosophila. *Molecular & Cellular Proteomics*. 2004; 3(2):156–166. [PubMed: 14645501]
88. Ockenfels B, Michael E, McDowell MA. Meta-analysis of the effects of insect vector saliva on host immune responses and infection of vector-transmitted pathogens: a focus on leishmaniasis. *PLoS Negl Trop Dis*. 2014 Oct.8(10):e3197. PubMed PMID: 25275509; PubMed Central PMCID: PMC4183472. doi: 10.1371/journal.pntd.0003197 [PubMed: 25275509]
89. Scholl DC, Embers ME, Caskey JR, et al. Immunomodulatory effects of tick saliva on dermal cells exposed to Borrelia burgdorferi, the agent of Lyme disease. *Parasit Vectors*. 2016 Jul 08.9(1):394. PubMed PMID: 27391120; PubMed Central PMCID: PMC4938952. doi: 10.1186/s13071-016-1638-7 [PubMed: 27391120]
- 90*. Titus RG, Ribeiro JM. Salivary gland lysates from the sand fly Lutzomyia longipalpis enhance Leishmania infectivity. *Science*. 1988; 239(4845):1306–1309. Established the role of salivary componetns in the enhancing the infectivity of parasite. [PubMed: 3344436]
91. Samuelson J, Lerner E, Tesh R, et al. A mouse model of Leishmania braziliensis braziliensis infection produced by coinjection with sand fly saliva. *The Journal of experimental medicine*. 1991; 173(1):49–54. [PubMed: 1985126]
92. Rogers KA, Titus RG. Immunomodulatory effects of Maxadilan and Phlebotomus papatasi sand fly salivary gland lysates on human primary *in vitro* immune responses. *Parasite immunology*. 2003; 25(3):127–134. [PubMed: 12911520]
93. Rohoušová I, Volf P. Sand fly saliva: effects on host immune response and Leishmania transmission. *Folia parasitologica*. 2006; 53(3):161. [PubMed: 17120496]
94. Oliveira F, Anderson JM, Kamhawi S, et al. Comparative salivary gland transcriptomics of sandfly vectors of visceral leishmaniasis. 2006
95. Hostomská J, Volfová V, Mu J, et al. Analysis of salivary transcripts and antigens of the sand fly Phlebotomus arabicus. *BMC genomics*. 2009; 10(1):282. [PubMed: 19555500]
96. Valenzuela JG, Belkaid Y, Garfield MK, et al. Toward a defined anti-leishmania vaccine targeting vector antigens. *Journal of Experimental Medicine*. 2001; 194(3):331–342. [PubMed: 11489952]
97. Volf P, Rohoušová I. Species-specific antigens in salivary glands of phlebotomine sandflies. *Parasitology*. 2001; 122(01):37–41. [PubMed: 11197762]
98. Bahia D, Gontijo NF, León IR, et al. Antibodies from dogs with canine visceral leishmaniasis recognise two proteins from the saliva of Lutzomyia longipalpis. *Parasitology research*. 2007; 100(3):449–454. [PubMed: 17058112]
99. Drahota J, Lipoldova M, Volf P, et al. Specificity of anti-saliva immune response in mice repeatedly bitten by Phlebotomus sergenti. *Parasite immunology*. 2009; 31(12):766–770. [PubMed: 19891614]
100. Marzouki S, Ahmed MB, Boussoffara T, et al. Characterization of the antibody response to the saliva of Phlebotomus papatasi in people living in endemic areas of cutaneous leishmaniasis. *The American journal of tropical medicine and hygiene*. 2011; 84(5):653–661. [PubMed: 21540371]

101. Vlkova M, Rohousova I, Drahota J, et al. Canine antibody response to *Phlebotomus perniciosus* bites negatively correlates with the risk of *Leishmania infantum* transmission. *PLoS Negl Trop Dis*. 2011; 5(10):e1344. [PubMed: 22022626]
102. Valenzuela JG, Garfield M, Rowton ED, et al. Identification of the most abundant secreted proteins from the salivary glands of the sand fly *Lutzomyia longipalpis*, vector of *Leishmania chagasi*. *Journal of Experimental Biology*. 2004; 207(21):3717–3729. [PubMed: 15371479]
103. Martín-Martín I, Molina R, Jiménez M. An insight into the *Phlebotomus perniciosus* saliva by a proteomic approach. *Acta tropica*. 2012; 123(1):22–30. [PubMed: 22445778]
104. Martín-Martín I, Molina R, Jiménez M. Identifying salivary antigens of *Phlebotomus argentipes* by a 2DE approach. *Acta tropica*. 2013; 126(3):229–239. [PubMed: 23422341]
105. Mathis A, Depaquit J, Dvořák V, et al. Identification of phlebotomine sand flies using one MALDI-TOF MS reference database and two mass spectrometer systems. *Parasites & vectors*. 2015; 8(1):266. [PubMed: 25957576]
106. Lafri I, Almeras L, Bitam I, et al. Identification of Algerian field-caught phlebotomine sand fly vectors by MALDI-TOF MS. *PLoS Negl Trop Dis*. 2016; 10(1):e0004351. [PubMed: 26771833]
107. Laroche M, Almeras L, Pecchi E, et al. MALDI-TOF MS as an innovative tool for detection of *Plasmodium* parasites in *Anopheles* mosquitoes. *Malaria Journal*. 2017; 16(1):5. [PubMed: 28049524]
108. Dillon R, Lane P. Bloodmeal digestion in the midgut of *Phlebotomus papatasi* and *Phlebotomus langeroni*. *Medical and veterinary entomology*. 1993; 7(3):225–232. [PubMed: 8369557]
109. Telleria EL, de Araújo APO, Secundino NF, et al. Trypsin-like serine proteases in *Lutzomyia longipalpis*-expression, activity and possible modulation by *Leishmania infantum* chagasi. *PLoS One*. 2010; 5(5):e10697. [PubMed: 20502532]
110. Eschenlauer SC, Faria MS, Morrison LS, et al. Influence of parasite encoded inhibitors of serine peptidases in early infection of macrophages with *Leishmania major*. *Cellular microbiology*. 2009; 11(1):106–120. [PubMed: 19016791]
111. Morrison LS, Goundry A, Faria MS, et al. Ecotin-like serine peptidase inhibitor ISP1 of *Leishmania major* plays a role in flagellar pocket dynamics and promastigote differentiation. *Cellular microbiology*. 2012; 14(8):1271–1286. [PubMed: 22486816]
112. Dobson DE, Kamhawi S, Lawyer P, et al. *Leishmania major* survival in selective *Phlebotomus papatasi* sand fly vector requires a specific SCG-encoded lipophosphoglycan galactosylation pattern. *PLoS pathogens*. 2010 Nov 11; 6(11):e1001185. PubMed PMID: 21085609; PubMed Central PMCID: PMC2978724. doi: 10.1371/journal.ppat.1001185 [PubMed: 21085609]
113. Di-Blasi T, Lobo AR, Nascimento LM, et al. The Flagellar protein FLAG1/SMP1 is a candidate for *Leishmania*-sand fly interaction. *Vector-Borne and Zoonotic Diseases*. 2015; 15(3):202–209. [PubMed: 25793476]
114. Myskova J, Svobodova M, Beverley SM, et al. A lipophosphoglycan-independent development of *Leishmania* in permissive sand flies. *Microbes and infection*. 2007; 9(3):317–324. [PubMed: 17307009]
115. Rawal R, Vijay S, Kadian K, et al. Towards a Proteomic Catalogue and Differential Annotation of Salivary Gland Proteins in Blood Fed Malaria Vector *Anopheles culicifacies* by Mass Spectrometry. *PloS one*. 2016; 11(9):e0161870. [PubMed: 27602567]
116. Nugent PG, Karsani SA, Wait R, et al. Proteomic analysis of *Leishmania mexicana* differentiation. *Molecular and biochemical parasitology*. 2004; 136(1):51–62. [PubMed: 15138067]
117. Pawar H, Sahasrabudhe NA, Renuse S, et al. A proteogenomic approach to map the proteome of an unsequenced pathogen-*Leishmania donovani*. *Proteomics*. 2012; 12(6):832–844. [PubMed: 22539434]
118. Pawar H, Renuse S, Khobragade SN, et al. Neglected tropical diseases and omics science: proteogenomics analysis of the promastigote stage of *Leishmania major* parasite. *Omics: a journal of integrative biology*. 2014; 18(8):499–512. [PubMed: 24937402]
119. Walker J, Vasquez J-J, Gomez MA, et al. Identification of developmentally-regulated proteins in *Leishmania panamensis* by proteome profiling of promastigotes and axenic amastigotes. *Molecular and biochemical parasitology*. 2006; 147(1):64–73. [PubMed: 16530278]

120. Bente M, Harder S, Wiesgigl M, et al. Developmentally induced changes of the proteome in the protozoan parasite *Leishmania donovani*. *Proteomics*. 2003; 3(9):1811–1829. [PubMed: 12973740]
121. Thiel M, Bruchhaus I. Comparative proteome analysis of *Leishmania donovani* at different stages of transformation from promastigotes to amastigotes. *Medical microbiology and immunology*. 2001; 190(1):33–36. [PubMed: 11770106]
122. Tsigankov P, Gherardini PF, Helmer-Citterich M, et al. Phosphoproteomic analysis of differentiating *Leishmania* parasites reveals a unique stage-specific phosphorylation motif. *Journal of proteome research*. 2013; 12(7):3405–3412. [PubMed: 23688256]
123. Tsigankov P, Gherardini PF, Helmer-Citterich M, et al. Regulation dynamics of *Leishmania* differentiation: deconvoluting signals and identifying phosphorylation trends. *Molecular & Cellular Proteomics*. 2014; 13(7):1787–1799. [PubMed: 24741111]
124. Foth B, Piani A, Curtis JM, et al. *Leishmania* major proteophosphoglycans exist as membrane-bound and soluble forms and localise to the cell membrane, the flagellar pocket and the lysosome. *International journal for parasitology*. 2002; 32(14):1701–1708. [PubMed: 12464416]
125. Yao C, Li Y, Donelson JE, et al. Proteomic examination of *Leishmania chagasi* plasma membrane proteins: contrast between avirulent and virulent (metacyclic) parasite forms. *PROTEOMICS-Clinical Applications*. 2010; 4(1):4–16. [PubMed: 21137013]
126. Mojtahedi Z, Clos J, Kamali-Sarvestani E. *Leishmania* major: identification of developmentally regulated proteins in procyclic and metacyclic promastigotes. *Experimental parasitology*. 2008; 119(3):422–429. [PubMed: 18486941]
127. Brotherton M-C, Racine G, Ouameur AA, et al. Analysis of membrane-enriched and high molecular weight proteins in *Leishmania infantum* promastigotes and axenic amastigotes. *Journal of proteome research*. 2012; 11(8):3974–3985. [PubMed: 22716046]
128. Kamoun-Essghaier S, Guizani I, Strub JM, et al. Proteomic approach for characterization of immunodominant membrane-associated 30-to 36-kilodalton fraction antigens of *Leishmania infantum* promastigotes, reacting with sera from mediterranean visceral leishmaniasis patients. *Clinical and diagnostic laboratory immunology*. 2005; 12(2):310–320. [PubMed: 15699427]
- 129*. Lynn MA, Marr AK, McMaster WR. Differential quantitative proteomic profiling of *Leishmania infantum* and *Leishmania mexicana* density gradient separated membranous fractions. *Journal of proteomics*. 2013; 82:179–192. Provides information on the differences in the membrane composition of *Leishmania* causing cutaneous and visceral forms of disease to understand disease pathogenesis. [PubMed: 23466312]
130. de Oliveira AH, Ruiz JC, Cruz AK, et al. Subproteomic analysis of soluble proteins of the microsomal fraction from two *Leishmania* species. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*. 2006; 1(3):300–308.
131. Foucher AL, Papadopoulou B, Ouellette M. Prefractionation by Digitonin Extraction Increases Representation of the Cytosolic and Intracellular Proteome of *Leishmania infantum*. *Journal of proteome research*. 2006; 5(7):1741–1750. [PubMed: 16823982]
132. Maslov DA, Spremulli LL, Sharma MR, et al. Proteomics and electron microscopic characterization of the unusual mitochondrial ribosome-related 45S complex in *Leishmania tarentolae*. *Molecular and biochemical parasitology*. 2007; 152(2):203–212. [PubMed: 17292489]
133. Ridlon L, Škodová I, Pan S, et al. The importance of the 45 S ribosomal small subunit-related complex for mitochondrial translation in *Trypanosoma brucei*. *Journal of Biological Chemistry*. 2013; 288(46):32963–32978. [PubMed: 24089529]
134. Kovtun O, Mureev S, Johnston W, et al. Towards the construction of expressed proteomes using a *Leishmania tarentolae* based cell-free expression system. *PLOS one*. 2010; 5(12):e14388. [PubMed: 21203562]
135. Nandan D, Yi T, Lopez M, et al. *Leishmania* EF-1 α activates the Src homology 2 domain containing tyrosine phosphatase SHP-1 leading to macrophage deactivation. *Journal of Biological Chemistry*. 2002; 277(51):50190–50197. [PubMed: 12384497]
136. Silverman JM, Chan SK, Robinson DP, et al. Proteomic analysis of the secretome of *Leishmania donovani*. *Genome biology*. 2008; 9(2):R35. [PubMed: 18282296]

137. Atayde VD, Aslan H, Townsend S, et al. Exosome Secretion by the Parasitic Protozoan *Leishmania* within the Sand Fly Midgut. *Cell reports*. 2015 Nov 3; 13(5):957–67. PubMed PMID: 26565909; PubMed Central PMCID: PMC4644496. DOI: 10.1016/j.celrep.2015.09.058 [PubMed: 26565909]
138. Rittig M, Bogdan C. *Leishmania*-host-cell interaction: complexities and alternative views. *Parasitology Today*. 2000; 16(7):292–297. [PubMed: 10858648]
139. De Almeida M, Vilhena V, Barral A, et al. Leishmanial infection: analysis of its first steps. A review. *Memorias do Instituto Oswaldo Cruz*. 2003; 98(7):861–870. [PubMed: 14762510]
140. Gregory D, Olivier M. Subversion of host cell signalling by the protozoan parasite *Leishmania*. *Parasitology*. 2005; 130(S1):S27–S35. [PubMed: 16281989]
141. Tonui WK, Mejia JS, Hochberg L, et al. Immunization with *Leishmania* major exogenous antigens protects susceptible BALB/c mice against challenge infection with *L. major*. *Infection and immunity*. 2004; 72(10):5654–5661. [PubMed: 15385463]
142. Tonui WK, Titus RG. Cross-protection against *Leishmania donovani* but not *L. braziliensis* caused by vaccination with *L. major* soluble promastigote exogenous antigens in BALB/c mice. *The American journal of tropical medicine and hygiene*. 2007; 76(3):579–584. [PubMed: 17360887]
143. Lemesre J-L, Holzmuller P, Gonçalves RB, et al. Long-lasting protection against canine visceral leishmaniasis using the LiESAp-MDP vaccine in endemic areas of France: double-blind randomised efficacy field trial. *Vaccine*. 2007; 25(21):4223–4234. [PubMed: 17395339]
144. Bras-Gonçalves R, Petitdidier E, Pagniez J, et al. Identification, characterization of new *Leishmania* promastigote surface antigens, LaPSA-38S LiPSA-50S as major immunodominant excreted/secreted components of *L. amazonensis*, *L. infantum*. *Infection, Genetics and Evolution*. 2014; 24:1–14.
- 145*. Petitdidier E, Pagniez J, Papierok G, et al. Recombinant forms of *Leishmania amazonensis* excreted/secreted promastigote surface antigen (PSA) induce protective immune responses in dogs. *PLoS Negl Trop Dis*. 2016; 10(5):e0004614. Importance of parasite secretome in disease pathology/protection. [PubMed: 27223609]
- 146**. Braga MS, Neves LX, Campos JM, et al. Shotgun proteomics to unravel the complexity of the *Leishmania infantum* exoproteome and the relative abundance of its constituents. *Molecular and biochemical parasitology*. 2014; 195(1):43–53. Revealed the complex composition of parasite exoproteome which served to demarcate the asymptomatics from the clinical cases in animal model. [PubMed: 25017697]
147. Sun H, Palaniswamy SK, Pohar TT, et al. MPromDb: an integrated resource for annotation and visualization of mammalian gene promoters and ChIP-chip experimental data. *Nucleic acids research*. 2006; 34(suppl 1):D98–D103. [PubMed: 16381984]
148. Yamamoto YY, Obokata J. PPDB: a plant promoter database. *Nucleic acids research*. 2007; 36(suppl_1):D977–D981. [PubMed: 17947329]
149. Jones P, Côté RG, Martens L, et al. PRIDE: a public repository of protein and peptide identifications for the proteomics community. *Nucleic acids research*. 2006; 34(suppl 1):D659–D663. [PubMed: 16381953]
150. Craig R, Cortens JP, Beavis RC. Open source system for analyzing, validating, and storing protein identification data. *Journal of proteome research*. 2004; 3(6):1234–1242. [PubMed: 15595733]
151. Desiere F, Deutsch EW, King NL, et al. The peptideatlas project. *Nucleic acids research*. 2006; 34(suppl 1):D655–D658. [PubMed: 16381952]
152. Mathivanan S, Ahmed M, Ahn NG, et al. Human Proteinpedia enables sharing of human protein data. *Nature biotechnology*. 2008; 26(2):164–167.
153. Zhang Y, Zhang Y, Adachi J, et al. MAPU: Max-Planck Unified database of organellar, cellular, tissue and body fluid proteomes. *Nucleic acids research*. 2006; 35(suppl_1):D771–D779. [PubMed: 17090601]
154. Fälth M, Savitski MM, Nielsen ML, et al. SwedCAD, a database of annotated high-mass accuracy MS/MS spectra of tryptic peptides. *Journal of proteome research*. 2007; 6(10):4063–4067. [PubMed: 17711326]

155. McLaughlin T, Siepen JA, Selley J, et al. PepSeeker: a database of proteome peptide identifications for investigating fragmentation patterns. *Nucleic acids research*. 2006; 34(suppl 1):D649–D654. [PubMed: 16381951]
156. Megger DA, Bracht T, Meyer HE, et al. Label-free quantification in clinical proteomics. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*. 2013; 1834(8):1581–1590. [PubMed: 23567906]
157. Park D, Lee S, Bolser D, et al. Comparative interactomics analysis of protein family interaction networks using PSIMAP (protein structural interactome map). *Bioinformatics*. 2005; 21(15): 3234–3240. [PubMed: 15914543]
158. Rao A, Yeleswarapu SJ, Raghavendra G, Plasmol D, A P, et al. falciparum Information Discovery Tool. In silico biology. 2009; 9(4):195–202. [PubMed: 20109149]
159. Flórez AF, Park D, Bhak J, et al. Protein network prediction and topological analysis in *Leishmania major* as a tool for drug target selection. *BMC bioinformatics*. 2010; 11(1):484. [PubMed: 20875130]
160. da Silva Santos C, Attarha S, Saini RK, et al. Proteome profiling of human cutaneous leishmaniasis lesion. *Journal of Investigative Dermatology*. 2015; 135(2):400–410. [PubMed: 25207817]
161. Menezes-Souza D, de Oliveira Mendes TA, de Souza Gomes M, et al. Epitope mapping of the HSP83 1 protein of *Leishmania braziliensis* discloses novel targets for immunodiagnosis of tegumentary and visceral clinical forms of leishmaniasis. *Clinical and Vaccine Immunology*. 2014; 21(7):949–959. [PubMed: 24807053]
162. Menezes-Souza D, de Oliveira Mendes TA, Nagem RAP, et al. Mapping B-cell epitopes for the peroxidoxin of *Leishmania (Viannia) braziliensis* and its potential for the clinical diagnosis of tegumentary and visceral leishmaniasis. *PloS one*. 2014; 9(6):e99216. [PubMed: 24921246]
163. Saffari B, Mohabatkar H. Computational Analysis of Cysteine Proteases (Clan CA, Family C1) of *Leishmania major* to Find Potential Epitopic Regions. *Genomics, proteomics & bioinformatics*. 2009; 7(3):87–95.
164. Pereira BA, Silva FS, Rebello KM, et al. In silico predicted epitopes from the COOH-terminal extension of cysteine proteinase B inducing distinct immune responses during *Leishmania (Leishmania) amazonensis* experimental murine infection. *BMC immunology*. 2011; 12(1):44. [PubMed: 21824434]
165. Chávez-Fumagalli MA, Schneider MS, Lage DP, et al. An *in silico* functional annotation screening of potential drug targets derived from *Leishmania* spp hypothetical proteins identified by immunoproteomics. *Experimental Parasitology*. 2017; 176:66–74. [PubMed: 28327439]
166. Goto Y, Coler RN, Reed SG. Bioinformatic identification of tandem repeat antigens of the *Leishmania donovani* complex. *Infection and immunity*. 2007; 75(2):846–851. [PubMed: 17088350]
167. Brito RC, Guimarães FG, Velloso JP, et al. Immunoinformatics Features Linked to *Leishmania* Vaccine Development: Data Integration of Experimental and In Silico Studies. *International journal of molecular sciences*. 2017; 18(2):371.
168. John L, John GJ, Kholia T. A reverse vaccinology approach for the identification of potential vaccine candidates from *Leishmania* spp. *Applied biochemistry and biotechnology*. 2012; 167(5): 1340–1350. [PubMed: 22434357]
169. Zarean M, Maraghi S, Hajjarian H, et al. Comparison of Proteome Profiling of Two Sensitive and Resistant Field Iranian Isolates of *Leishmania major* to Glucantime® by 2-Dimensional Electrophoresis. *Iranian journal of parasitology*. 2015; 10(1):19. [PubMed: 25904942]
170. Moreira, DdS, Pescher, P., Laurent, C., et al. Phosphoproteomic analysis of wild-type and antimony-resistant *Leishmania braziliensis* lines by 2D-DIGE technology. *Proteomics*. 2015; 15(17):2999–3019. [PubMed: 25959087]
171. Vacchina P, Norris-Mullins B, Carlson E, et al. A mitochondrial HSP70 (HSPA9B) is linked to miltefosine resistance and stress response in *Leishmania donovani*. *Parasites & Vectors*. 2016; 9(1):621. [PubMed: 27906059]

172. Johnson R, Campbell-Bright S, Ralph H, et al. Proteomic analysis of miltefosine-resistant *Leishmania* reveals the possible involvement of eukaryotic initiation factor 4A (eIF4A). *Int J Antimicrobial Agents*. 2008; 31:581–592.
- 173**. Vincent IM, Racine G, Légaré D, et al. Mitochondrial proteomics of antimony and miltefosine resistant *Leishmania infantum*. *Proteomes*. 2015; 3(4):328–346. First report of the involvement of organellar proteomics in antimony and miltefosine resistance. [PubMed: 28248274]
174. Das S, Shah P, Baharia RK, et al. Over-expression of 60s ribosomal L23a is associated with cellular proliferation in SAG resistant clinical isolates of *Leishmania donovani*. *PLoS Negl Trop Dis*. 2013; 7(12):e2527. [PubMed: 24340105]
175. Das S, Shah P, Tandon R, et al. Over-expression of cysteine leucine rich protein is related to SAG resistance in clinical isolates of *Leishmania donovani*. *PLoS Negl Trop Dis*. 2015; 9(8):e0003992. [PubMed: 26295340]
176. Brotherton M-C, Bourassa S, Leprohon P, et al. Proteomic and genomic analyses of antimony resistant *Leishmania infantum* mutant. *PLoS One*. 2013; 8(11):e81899. [PubMed: 24312377]
177. Carnielli JB, de Andrade HM, Pires SF, et al. Proteomic analysis of the soluble proteomes of miltefosine-sensitive and-resistant *Leishmania infantum* chagasi isolates obtained from Brazilian patients with different treatment outcomes. *Journal of proteomics*. 2014; 108:198–208. [PubMed: 24874972]
178. Brotherton M-C, Bourassa S, Légaré D, et al. Quantitative proteomic analysis of amphotericin B resistance in *Leishmania infantum*. *International Journal for Parasitology: Drugs and Drug Resistance*. 2014; 4(2):126–132. [PubMed: 25057462]
179. Imbert L, Cojean S, Libong D, et al. Sitamaquine-resistance in *Leishmania donovani* affects drug accumulation and lipid metabolism. *Biomedicine & Pharmacotherapy*. 2014; 68(7):893–897. [PubMed: 25201056]
180. ROBINSON A, Robson M, Harrison AP, et al. A new technique for differentiation of hemoglobin. *Journal of laboratory and clinical medicine*. 1957; 50(5):745–52. [PubMed: 13476032]
181. Elgstoen KB, Jellum E. Capillary electrophoresis for diagnosis of metabolic disease. *Electrophoresis*. 1997; 18(10):1857–1860. [PubMed: 9372280]
182. Schmerr MJ, Cutlip RC, Jenny A. Capillary isoelectric focusing of the scrapie prion protein. *Journal of Chromatography A*. 1998; 802(1):135–141. [PubMed: 9588016]
183. Duarte MC, Pimenta DC, Menezes-Souza D, et al. Proteins selected in *Leishmania* (Viannia) *braziliensis* by an immunoproteomic approach with potential serodiagnosis applications for tegumentary leishmaniasis. *Clinical and Vaccine Immunology*. 2015; 22(11):1187–1196. [PubMed: 26376929]
184. Lima B, Pires S, Fialho L, et al. A proteomic road to acquire an accurate serological diagnosis for human tegumentary leishmaniasis. *Journal of proteomics*. 2017; 151:174–181. [PubMed: 27262223]
185. Costa MM, Andrade HM, Bartholomeu DC, et al. Analysis of *Leishmania chagasi* by 2-D difference gel electrophoresis (2-D DIGE) and immunoproteomic: identification of novel candidate antigens for diagnostic tests and vaccine. *Journal of proteome research*. 2011; 10(5): 2172–2184. [PubMed: 21355625]
186. Tebourski F, El Gaied A, Louzir H, et al. Identification of an immunodominant 32-kilodalton membrane protein of *Leishmania donovani* infantum promastigotes suitable for specific diagnosis of Mediterranean visceral leishmaniasis. *Journal of clinical microbiology*. 1994; 32(10):2474–2480. [PubMed: 7814485]
187. Agranoff D, Fernandez-Reyes D, Papadopoulos MC, et al. Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. *The Lancet*. 2006; 368(9540):1012–1021.
188. Papadopoulos MC, Abel PM, Agranoff D, et al. A novel and accurate diagnostic test for human African trypanosomiasis. *The Lancet*. 2004; 363(9418):1358–1363.
189. Hettick JM, Kashon ML, Simpson JP, et al. Proteomic profiling of intact mycobacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Analytical chemistry*. 2004; 76(19):5769–5776. [PubMed: 15456297]

190. Bag AK, Saha S, Sundar S, et al. Comparative proteomics and glycoproteomics of plasma proteins in Indian visceral leishmaniasis. *Proteome science*. 2014; 12(1):48. [PubMed: 25276097]
191. Petricoin EF, Zoon KC, Kohn EC, et al. Clinical proteomics: translating benchside promise into bedside reality. *Nature reviews Drug discovery*. 2002; 1(9):683–695. [PubMed: 12209149]
192. Zhang Y, Fonslow BR, Shan B, et al. Protein analysis by shotgun/bottom-up proteomics. *Chemical reviews*. 2013; 113(4):2343–2394. [PubMed: 23438204]
193. Pieper R, Gatlin CL, McGrath AM, et al. Characterization of the human urinary proteome: A method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. *Proteomics*. 2004; 4(4):1159–1174. [PubMed: 15048996]
194. Gupta SK, Sisodia BS, Sinha S, et al. Proteomic approach for identification and characterization of novel immunostimulatory proteins from soluble antigens of *Leishmania donovani* promastigotes. *Proteomics*. 2007; 7(5):816–823. [PubMed: 17295358]
195. Tripathi P, Gupta S, Sinha S, et al. Prophylactic Efficacy of High-Molecular-Weight Antigenic Fractions of a Recent Clinical Isolate of *Leishmania donovani* Against Visceral Leishmaniasis. *Scandinavian journal of immunology*. 2008; 68(5):492–501. [PubMed: 18803606]
196. Drummelsmith J, Girard I, Trudel N, et al. Differential protein expression analysis of *Leishmania* major reveals novel roles for methionine adenosyltransferase and S-adenosylmethionine in methotrexate resistance. *The Journal of biological chemistry*. 2004 Aug 6; 279(32):33273–80. PubMed PMID: 15190060. DOI: 10.1074/jbc.M405183200 [PubMed: 15190060]
197. Jeffery DA, Bogoy M. Chemical proteomics and its application to drug discovery. *Current opinion in biotechnology*. 2003 Feb; 14(1):87–95. PubMed PMID: 12566007. [PubMed: 12566007]
198. Cravatt BF, Wright AT, Kozarich JW. Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annual review of biochemistry*. 2008; 77:383–414. PubMed PMID: 18366325. DOI: 10.1146/annurev.biochem.75.101304.124125
199. Cox J, Neuhauser N, Michalski A, et al. Andromeda: a peptide search engine integrated into the MaxQuant environment. *Journal of proteome research*. 2011; 10(4):1794–1805. [PubMed: 21254760]
200. Muñoz J, Heck AJ. From the human genome to the human proteome. *Angewandte Chemie International Edition*. 2014; 53(41):10864–10866. [PubMed: 25079383]
201. Geiger T, Cox J, Mann M. Proteomics on an Orbitrap benchtop mass spectrometer using all-ion fragmentation. *Molecular & Cellular Proteomics*. 2010; 9(10):2252–2261. [PubMed: 20610777]
202. Geiger T, Cox J, Mann M. Proteomics on an Orbitrap benchtop mass spectrometer using all-ion fragmentation. *Molecular & cellular proteomics : MCP*. 2010 Oct; 9(10):2252–61. PubMed PMID: 20610777; PubMed Central PMCID: PMC2953918. DOI: 10.1074/mcp.M110.001537 [PubMed: 20610777]
203. Addona TA, Shi X, Keshishian H, et al. A pipeline that integrates the discovery and verification of plasma protein biomarkers reveals candidate markers for cardiovascular disease. *Nature biotechnology*. 2011; 29(7):635–643.
204. Cao Z, Tang H-Y, Wang H, et al. Systematic comparison of fractionation methods for in-depth analysis of plasma proteomes. *Journal of proteome research*. 2012; 11(6):3090–3100. [PubMed: 22536952]
205. Paczesny S, Braun TM, Levine JE, et al. Elafin is a biomarker of graft-versus-host disease of the skin. *Science translational medicine*. 2010; 2(13):13ra2–13ra2.
206. Kuzyk MA, Parker CE, Domanski D, et al. Development of MRM-based assays for the absolute quantitation of plasma proteins. *Methods in molecular biology*. 2013; 1023:53–82. PubMed PMID: 23765619. DOI: 10.1007/978-1-4614-7209-4_4 [PubMed: 23765619]
207. Geyer PE, Kulak NA, Pichler G, et al. Plasma proteome profiling to assess human health and disease. *Cell systems*. 2016; 2(3):185–195. [PubMed: 27135364]
208. Schmucker D, Clemens JC, Shu H, et al. *Drosophila* Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity. *Cell*. 2000 Jun 9; 101(6):671–84. PubMed PMID: 10892653. [PubMed: 10892653]

209. Hancock WS, Wu SL, Stanley RR, et al. Publishing large proteome datasets: scientific policy meets emerging technologies. *Trends in biotechnology*. 2002; 20(12):s39–s44. [PubMed: 12570159]
210. Tucker CL, Gera JF, Uetz P. Towards an understanding of complex protein networks. *Trends in cell biology*. 2001; 11(3):102–106. [PubMed: 11306254]
211. Eilbeck, K.Brass, A.Paton, NW., et al., editors. ISMB. 1999. INTERACT: an object oriented protein-protein interaction database.
212. Basik M, Mousses S, Trent J. Integration of genomic technologies for accelerated cancer drug development. *Biotechniques*. 2003; 35(3):580–593. [PubMed: 14513563]
213. Hegde PS, White IR, Debouck C. Interplay of transcriptomics and proteomics. *Current opinion in biotechnology*. 2003; 14(6):647–651. [PubMed: 14662396]
214. Bischoff R, Luider TM. Methodological advances in the discovery of protein and peptide disease markers. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences*. 2004 Apr 15; 803(1):27–40. PubMed PMID: 15025996. DOI: 10.1016/j.jchromb.2003.09.004 [PubMed: 15025996]
215. Brand S, Hahner S, Ketterlinus R. Protein profiling and identification in com-plex biological samples using LC-MALDI. *Drug Plus Int*. 2005
216. Worldwide protein Data Bank [internet]. Worldwide protein Data Bank Foundation; Available from: <http://www wwptdb.org/> [cited 2018 Mar 3]
217. Dynaomics [internet]. University of Washington: Daggett group; Available from: <http://www.dynaomics.org/> [cited 2018 Mar 3]
218. ModBase [internet]. Department of Bioengineering and Therapeutic Sciences and California Institute for Quantitative Biomedical Research, Mission Bay Campus, Byers Hall. University of California: Ben Webb; Available from: <http://salilab.org/mod> [cited 2018 Mar 3]
219. OPM database[internet]. University of Michigan,MI,USA: National Science Foundation; Available from:<http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/> [cited 2018 Mar 3]
220. SCOP2 [internet]. Cambridge,UK: MRC, Laboratory of Molecular Biology; Available from:<http://scop2.mrc-lmb.cam.ac.uk/> [cited 2018 Mar 3]
221. SWISS-MODEL[internet]. Switzerland: Biozentrum, University of Basel,The Centre for Molecular Life Sciences; Available from: <https://swissmodel.expasy.org/repository/> [cited 2018 Mar 3]
222. TOPSAN-TOPSAN [internet]. California: The Joint Centre for Structural Genomics; Available from: <http://www.topsan.org/> [cited 2018 Mar 3]
223. TrEMBL/SWISS-PROT [internet]. Cambridge and Geneva: EMBL-EBI and SIB; Available from: <http://www.mrc-lmb.cam.ac.uk/genomes/madanm/pres/swiss2.htm> [cited 2018 Mar 3]
224. UniProt [internet]. Bethesda: NIH; Available from: <http://www.uniprot.org/> [cited 2018 Mar 3]
225. Plasma proteome database [internet]. Bangalore: Institute of Bioinformatics; Available from: <http://www.plasmaproteomedatabase.org/> [cited 2018 Mar4]
226. Yale protein expression database [internet]. New Haven: Yale School of Medicine and W.M. Keck Foundation; Available from: <http://yped.med.yale.edu/repository/> [cited 2018 Mar4]
227. MOPED [internet]. Washington: Seattle Children's Research Institute; Available from: <http://moped.proteinspire.org> [cited 2018 Mar4]
228. Expression Atlas [internet]. Hinxton: EMBL-EBI; Available from: <https://omictools.com/expression-atlas-tool> [cited 2018 Mar4]
229. Kahn Dynamic Proteomics database [internet]. Rehovot,Israel: Weizmann Institute of Science; Available from: <http://www.weizmann.ac.il/mcb/UriAlon/DynamProt> [cited 2018 Mar4]
230. Human Proteinpedia [internet]. Baltimore and Bangalore: John Hopkins University and Institute of Bioinformatics; Available from: <http://www.humanproteinpedia.org/> [cited 2018 Mar 4]
231. PRIDE[internet]. Hinxton: European Bioinformatics Institute; Available from: <http://www.ebi.ac.uk/pride/archive/> [cited 2018 Mar 4]
232. PaxDb[internet]. Zurich: SIB and University of Zurich; Available from: <http://pax-db.org/> [cited 2018 Mar 4]

233. Human Protein Atlas [internet]. Stockholm: Knut and Alice Wallenberg Foundation; Available from: <http://proteinatlas.org/> [cited 2018 Mar 4]
234. DIP, Database of Interacting Proteins [internet]. Los Angeles: University of California; Available from: <http://dip.doe-mbi.ucla.edu/dip/Main.cgi> [cited 2018 Mar 4]
235. Human protein interaction database [internet]. Incheon: Biocomputing lab, Inha University; Available from: <http://www.hpid.org> [cited 2018 Mar 4]
236. [cited 2018 Mar 4] Open Proteome Database [internet]. Available from: <http://bioinformatics.icmb.utexas.edu/OPD/>
237. SBEAMS [internet]. Washington: Institute for System Biology; Available from: <http://www.sbeams.org/> [cited 2018 Mar 4]
238. HUPO-PSI [internet]. Vancouver, Canada: Human Proteome Organization; Available from: <http://www.hupo.org/> [cited 2018 Mar 4]
239. SWISS-2DPAGE [internet]. Geneva and Lausanne: Central Clinical Chemistry Laboratory of Geneva University Hospital and SIB; Available from: <http://www.expasy.ch/ch2d/> [cited 2018 Mar 4]
240. BioGRID [internet]. Bethesda: NIH; Available from: <http://thebiogrid.org> [cited 2018 Mar 4]
241. The biomolecular interaction network database [internet]. Toronto and Singapore: The Blueprint Initiative of Mount Sinai and The Blueprint Initiative Asia; Available from: <http://www.bind.ca> [cited 2018 Mar 4]
242. IntAct, molecular interaction database [internet]. Hinxton: EMBL-EBI; Available from: <http://www.ebi.ac.uk/intact> [cited 2018 Mar 4]
243. Molecular INTeraction database [internet]. Rome: Elixir Core Data Resource; Available from: <http://mint.bio.uniroma2.it/mint> [cited 2018 Mar 4]
244. Grant T, Grigorieff N. Measuring the optimal exposure for single particle cryo-EM using a 2.6 Å reconstruction of rotavirus VP6. *Elife*. 2015 May 29;4:e06980. PubMed PMID: 26023829; PubMed Central PMCID: PMC4471936. doi: 10.7554/eLife.06980 [PubMed: 26023829]

Key issues

- There remains an urgent need for the identification of less abundant protein from amastigotes, as few have been identified, and several are modulated during infection.
- Host-parasite interactions as well as protein profiles vary from species to species and tissue/cell of interest, which must be addressed.
- The establishment of a data integrating system for collecting queries and navigating multiple databases remains a challenge for constructing species-specific proteome maps.
- Systems biology approaches lack functional annotation and severely lack proteomic coverage of a considerable number of parasite genes.
- Although ‘Omics’ has provided global expression data for the parasite, the absence of a publicly accessible metabolomic database for parasites calls for improvement in systems biology approaches for understanding parasite biology.
- The dynamic nature of parasite exoproteome attributes impacts the virulence of the parasite and plays an active role in host–parasite interactions.
- There remains an urgent need to develop reproducible methodologies to analyse the proteome, subcellular proteome and exoproteome, which will provide an added advantage for disease diagnosis.
- ‘Interactome’ and post-translational modification studies provide an approach for understanding the regulatory role of protein-protein interactions in determining disease outcome and deciphering the molecular conversation governing the host-parasite interaction and drug resistance mechanisms.
- Single-cell analysis has been boosted with the use of microfluidic systems and multiparametric flow cytometry, which serve to open new avenues for proteomic analyses to overcome the challenges inherent to single-cell protein analyses.

Figure 1.

An outline of published literature using different proteomic technologies over a decade.

Table 1

A glimpse of different techniques for proteomic studies

Study year	Proteomic technique	Study reported	Reference
1970	One-dimensional electrophoresis	Bacteriophage T4	[43]
1987	Edman sequencing		[44]
1975	2-DE		[45]
1988–89	2DE+MALDI/ESI		[48, 49, 50]
2014	CRISPR-ChAP-MS	<i>Saccharomyces</i>	[28]
2015	Cryo EM	Rotavirus	[244]
2006	2D-DIGE		[59]
2006	Shotgun proteomics		[52]
2010	LC-MS/MS	<i>Leishmania</i>	[55]
2008	iTRAQ	<i>Leishmania</i>	[53]
2011	SILAC	<i>Leishmania</i>	[54]
2010	iMAC	<i>Leishmania</i>	[55]
2015	FFE and FFZE	<i>Leishmania</i>	[66]
2004	FF-IEF,FF-ITP, FFFSE	<i>Leishmania</i>	[67]
2008	QPNC-PAGE	<i>Lactococcus lactis</i>	[73]
2006	QPNC-PAGE+NMR		[74]
2015	MALDI-TOF MS	Phelbotomine sandflies	[105]
2004	CapLC-QTOF	<i>Leishmania</i>	[116]
2001	Yeast two hybrid system		[76]
2000	Immunoaffinity methods		[78]
2001	Homology		[80]
2000	Non-homology		[81]

Table 2

Bioinformatics tools for proteomic analysis and their URLs

Type of study	Database	Description	Web address	References
Structural and Functional proteomics	Protein Data Bank (PDB)	Predicts 3-D structure of protein	http://www.wwpdb.org/	[216]
	Dynaeomics	Describes motion in protein	http://www.dynaeomics.org/	[217]
	ModBase	Database of annotated comparative protein structure	http://salilab.org/mod	[218]
	OPM	Spatial position of membrane protein structure in lipid bilayer	http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/	[219]
	SCOP2	Structure and evolutionary relationship between proteins	http://scop2.mrc-lmb.cam.ac.uk/scop/	[220]
	SWISS-MODEL	Annotated protein model prepared by homology modelling	https://swissmodel.expasy.org/repository/	[221]
	TOPSAN	Collects, shares and distribute information about protein 3-D structure	http://www.topsan.org/	[222]
	TrEMBL/Swiss-Prot	Provides protein sequence information, structure and function	http://www.mrc-lmb.cam.ac.uk/genomes/madanm/pres/swiss2.htm	[223]
	UniProt	Provides protein sequence and functional information combining data from TrEMBL, SwissProt, UniParc and UniRef	http://www.uniprot.org/	[224]
	Plasma Proteome Database	Provides quantitative and qualitative information on proteins in serum and plasma	http://www.plasmaproteomedatabase.org/	[225]
Expression proteomics	Yale protein expression database	Storage, retrieval and integrated analysis of data from such techniques as DIGE, iTRAQ, LC-MS/MS, SILAC.	http://yped.med.yale.edu/repository/	[226]
	MOPED	Provides protein expression information, linked with several pathway databases	http://moped.proteinspire.org	[227]

Type of study	Database	Description	Web address	References
	Expression Atlas	Information on gene and protein expression, selected microarray and RNA sequencing studies	https://omictools.com/expression-atlas-tool	[228]
	The Kahn Dynamic Proteomics Database	Provides information on amount and position of protein in living human cell, links to GeneCards, Ensembl, InterPro, Entrez, UniProt	http://www.weizmann.ac.il/mcb/UriAlon/DynamProt	[229]
	Human Proteinpedia	Provides information of protein expression in cell lines, tissues, subcellular localization and enzyme-substrate relationship	http://www.humanproteinpedia.org/	[230]
	PRIDE	Information on protein/peptide identification, post-translational modifications with spectral evidence	http://www.ebi.ac.uk/pride/archive/	[231]
	Pax-DB	Integrated resource of protein expression	http://pax-db.org/	[232]
	Human Protein Atlas	Information on tissue restricted expression of human proteome and transcriptome	http://proteinatlas.org/	[233]
Interaction proteomics	Database of interacting proteins (DIP)	Catalogues experimentally determined interaction between proteins	http://dip.doe-mbi.ucla.edu/dip/Main.cgi	[234]
	Human Protein Interaction Database (HPID)	Provides information about human protein interactions, pre-computed by statistical tools, integrates data from BIND, DIP and HPRD	http://www.hpid.org	[235]
	Open Proteomics Database	Studies large scale organization of protein and how these are associated with system, pathways, and networks	http://bioinformatics.icmb.utexas.edu/OPD/	[236]
	Systems Biology Experiment Analysis Management System (SBEAMS)	Provides one platform for collection, storage and management of data from proteomics, microarray and immunohistochemistry	http://www.sbeams.org/	[237]
	HUPO/HUPO-PSI	Provides information on data representation	http://www.hupo.org/ http://psidev.sourceforge.net/ http://psidev.sf.net/	[238]

Type of study	Database	Description	Web address	References
Author Manuscript		in proteomics and interactomics for data exchange and verification		
	Swiss-2DPAGE	Provides data on proteins identified by various 2DE and SDS-PAGE	http://www.expasy.ch/ch2d/	[239]
	BioGRID	Interrogates protein function, interactions and analysis of global interaction properties	http://thebiogrid.org	[240]
	BIND	Provides data on interaction, chemical reaction conformation and post-translational modifications	http://www.bind.ca	[241]
	IntAct	Provides toolkit for storage, presentation and analysis of interactions	http://www.ebi.ac.uk/intact	[242]
	MINT	Catalogues protein interaction information, and functional interactions including enzymatic modifications	http://mint.bio.uniroma2.it/mint	[243]