Plasmodium falciparum malaria: proteomic studies

Malária por Plasmodium falciparum: estudos proteômicos

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ABSTRACT

Despite advances in treatment and campaigns for prevention and control of malaria on the various continents where it is still rampant, this disease remains significantly relevant to the contemporary world. *Plasmodium falciparum* is the organism that is mainly responsible for severe malaria, which is characterized by disturbances in different organs and systems, with possibly fatal outcomes. Although incipient, proteomic studies of malaria have yielded favorable prospects for elucidating the biological aspects of *Plasmodium* as well as the pathophysiological, diagnostic, prophylactic, and therapeutic mechanisms of the disease. Thus, the aim of the present article is to present a brief review of the applications of proteomic analysis in *P. falciparum* malaria.

Keywords: Proteome; Malaria; *Plasmodium falciparum*

INTRODUCTION

Advances in treatment, prophylaxis, and control of malaria around the world have been substantial, yet they have not been sufficient to change the current outlook for the disease significantly. In fact, malaria is still the parasitosis with the greatest impact on the planet, figuring as the fifth cause of death from infectious diseases worldwide.(1,2) There are different outcomes of the clinical evolution, and severe malaria is one of them.

Severe malaria is usually caused by *Plasmodium falciparum* and is characterized by disturbances in multiple organs and systems, such as metabolic acidosis, involvement of the central nervous system, severe anemia, shock, disseminated intravascular coagulation, pulmonary dysfunction, liver disorder, hypoglycemia, and renal insufficiency. This clinical picture is pathophysiologically similar to bacterial sepsis,(3) which requires treatment in an intensive care unit (ICU) to avoid progression to death.(4) Transferring a patient with suspected severe malaria to the ICU allows the early detection and proper management of complications that might culminate in a fatal outcome.(5-7)

Due to peculiarities in the clinical management of these complications, the integration between intensive care doctors and specialists in tropical medicine/infectious diseases facilitates a more favorable outcome in affected patients.(6) Consequently, the intensive care doctor must know the epidemiological, clinical, diagnostic, and therapeutic concepts behind malaria, within the context of the so-called "intensive care for infection".(8)

Despite studies directed at new drugs and the development of more effective support measures, the application of scales that support the diagnosis and predict the prognosis, and the use of methods for early detection of complications from the infection, the risk of death from severe malaria remains high; moreover, in many case studies, the lethality has been determined to be greater than 50%.[5-7,9] Thus, the biological approach to the interaction between *P. falciparum* and *Homo sapiens sapiens* might support the development of new models of scientific research, providing a beneficial opportunity for further understanding this infectious condition.[10,11] Therefore, proteomic analysis – a methodology that aims to outline the functional units of a cell, its proteins, and its complex network of interactions and signaling pathways in a given underlying disease and in a physiological condition[12] - has the potential to clarify the pathophysiological, diagnostic, prophylactic, and therapeutic aspects of infectious diseases,[13] including severe malaria.[14,15] The improvement of proteomic studies could elucidate the molecular mechanisms involved in the development of the disease, allowing (1) the identification of changes in the expression of proteins related to intracellular and intercellular signaling pathways, (2) the development of biomarkers for early diagnosis and prognostic prediction, and (3) the establishment of new therapies.[16,17]

Consequently, the aim of the present article is to present a brief review of the current applications of proteomic analysis in *P. falciparum* malaria.

METHODS

For the literature review, the following sources were accessed: PubMed (U.S. National Library of Medicine), SciELO (Scientific Electronic Library Online), Lilacs (Literatura Latino-Americana e do Caribe em Ciências da Saúde/Latin-American and Caribbean Health Science Literature), and Cochrane. The search terms were defined based on Health Science Descriptors (DeCS) from two search strategies: (1) *malaria* + *proteome* and (2) *Plasmodium falciparum* + *proteome*. The search returned 551 citations (Table 1) that were published within the last 10 years in Spanish, English, or Portuguese. Twelve original articles were selected from the total.

Table 1 - Number of articles obtained from the literature search

Search strategy	Accessed database			
	Cochrane	Lilacs	PubMed	SciELO
Strategy 1: malaria + proteome	60	1	157	1
Strategy 2: *Plasmodium falciparum* + proteome	197	1	133	1

The eligibility criterion was the application of proteomic techniques for studying *P. falciparum* malaria, focusing on the etiological, pathogenic, diagnostic, therapeutic, and prophylactic aspects. Table 2 presents a synthesis of the 12 studies that met this criterion. In addition to the manuscripts described, 23 other texts were accessed, including review articles obtained using the search strategy and texts previously known by the authors, which were not necessarily related to proteomic analysis of *P. falciparum* malaria but were useful for contextualization of the research question.

PROTEOMIC APPROACH OF SEVERE MALARIA

Several studies have demonstrated the role of the proteomic approach in the elucidation of the etiological, pathogenic, diagnostic, and therapeutic aspects of severe malaria. The main information that was obtained from the accessed articles (Table 2) was organized into the following sections: (1) *Plasmodium falciparum*, (2) pathogenesis, (3) diagnosis, (4) treatment, and (5) prevention/vaccine development.

Plasmodium falciparum

The lifecycle of *P. falciparum* is extraordinarily complex and involves the expression of specialized proteins when the protist is present in different hosts - *H. sapiens sapiens* and mosquitoes of the genus *Anopheles* - for (i) intracellular and/or extracellular survival, (ii) penetration into the different cell types, and (iii) evasion of the immune response of the hosts. Intervention strategies, including vaccines and drugs, are more effective if focused at specific stages of the microorganism's lifecycle and/or the microorganism's proteins that are expressed during these stages.[17] The decoding of the genome of *P. falciparum* in 2002 provided a basis for proteomic studies focused on the pathogen. In that same year, Florens et al.[18] published the results of employing proteomic analysis of the lifecycle of the etiologic agent. A multidimensional protein identification technology (MudPIT), which combines high performance liquid chromatography and mass spectrometry, was used. The proteome of *P. falciparum* sporozoites (the infectious form that is injected into humans by the mosquito), merozoites (the erythrocyte-invading stage), trophozoites (the multiplication stage inside erythrocytes), and gametocytes (the sexual stage) were studied. Of the 2,415 proteins that were identified, 46% were detected in all four stages of the *Plasmodium* lifecycle. Of the total number of proteins that were identified, 49% were found exclusively in sporozoites, demonstrating that this stage is

Table 2 - Summary of the main articles that were accessed

Study	Technique used	Comments
Florens et al.[18]	DNA microarrays for studying the differential gene expression in *P. falciparum* blood stages.	More than 2,400 proteins involved in four evolutionary stages of *P. falciparum* were identified.
Lasonder et al.[19]	Mass spectrometry of selected stages of the *P. falciparum* evolutionary cycle.	In total, 1,289 proteins were identified, of which 714 were in the asexual stages. Some of the proteins had potential for the development of immunization strategies.
Gelhaus et al.[20]	Two-dimensional electrophoresis and mass spectrometry.	Several proteins from two evolutionary stages of the protist, schizont, and merozoite were isolated. The findings might contribute to the development of drugs with higher efficacies.
Khan et al.[21]	Mass spectrometry.	Differences between male and female gametocytes were identified, which would enable the development of strategies for controlling the transmission of the disease.
Lal et al.[23]	Proteomic analysis of ookinete micronemes by one-dimensional electrophoresis, liquid chromatography, and mass spectrometry.	This study was the first investigation into proteomic analysis of the protist organelles, which could be the focus of pathophysiological studies and therapeutic development.
Torrentino-Madamet et al.[24]	Combined analyses of transcriptomes and proteomes for studying the metabolic adaptation of *P. falciparum* to different oxygen pressures during its intraerythrocytic cycle.	Hyperoxia activates the antioxidant defense systems in protozoa to preserve the integrity of their cellular structures. This information is useful for the development of more highly effective control and therapeutic strategies.
Fontaine et al.[25]	2D-DIGE.	Proteins of erythrocytes infected by *P. falciparum* were detected.
Makanga et al.[31]	*In vitro* isolation and culture of strains of chloroquine-resistant *P. falciparum* K1 using conventional methods.	The proteomic analysis showed that artemether and lumefantrine induced opposite effects on the main glycolytic enzymes. These findings demonstrate the power of this approach for determining the pleomorphic mechanisms of drug action.
Le Roch et al.[32]	Analysis of the transcriptome and proteome to assess the response of *P. falciparum* to T4 during the intraerythrocytic cycle of the protozoan.	The study revealed a significant reduction in the level of *P. falciparum* choline/ethanolamine phosphotransferase, which is an enzyme involved in the final stage of phosphatidylcholine synthesis. These findings contribute to the understanding of the mechanisms involved in the increasing resistance of the protozoan to antimalarial drugs.
Jensen et al.[33]	*In silico* evaluation of *P. falciparum* and human host protein-protein interactions.	In total, 293 *P. falciparum* proteins were found. Six of these proteins are already being studied for the treatment of malaria and may potentially be employed as therapeutic targets, based on their ability to inhibit the active compounds that were analyzed.
Briolant et al.[34]	2D-DIGE and iTRAQ.	Certain changes in *P. falciparum* after the use of doxycycline, which had not been previously described, were identified by the proteomic analysis.
Doolan et al.[35]	Protein microarrays to elucidate the antibodies' profile after infection and vaccination.	The results of the study demonstrate that the antibody profiles are different after exposure to infection and vaccination, creating prospects for future investigations to elucidate the molecular basis of immunity in *P. falciparum* malaria for the development of vaccines.

2D-DIGE - two-dimensional differential gel electrophoresis; iTRAQ - isobaric tags for relative and absolute quantification.

the most distinct. Sporozoites share an average of 25% of their proteins with any other stage. By comparison, 20 to 33% of merozoite, trophozoite, and gametocyte proteins are exclusive, and these stages share from 39 to 56% of their peptides. Therefore, only 152 proteins (6%) are common among the four stages. These proteins relate to basic cellular functions (ribosomal proteins, transcription factors, histones, and cytoskeletal proteins).[18]

Concurrently, Lasonder et al.[19] published a study on the different stages of the biological cycle of *P. falciparum* in which liquid chromatography combined with mass spectrometry was used. The analysis yielded 1,289 proteins, from which 714 were identified in the asexual blood stage, 931 were identified in gametocytes, and 645 were identified in gametes. Two previous studies provided information on the biology of the microorganism's sexual stages, which include conserved peptides and secreted and membrane-associated, stage-specific proteins. Using a subset of these proteins, it is possible to understand further their role in intercellular interactions and thus to obtain funding for the development of a potential malaria vaccine.[19]

In 2005, Gelhaus et al.[20] obtained the first protein maps from a two-dimensional gel of proteomes of merozoites and schizonts of *P. falciparum*, which was a pioneering study on the use of two-dimensional electrophoresis and mass spectrometry, important techniques in contemporary proteomic analysis. The investigation established a new strategy for identifying plasmodial proteins, which is a key aspect in the search for vaccines and new drugs. Nevertheless, there was significant contamination with host proteins, which required the development of more effective protocols for separation, extraction, and analyses for independently evaluating the proteome of the etiologic agent.

The individual study of the different evolutionary stages and their subproteomes became necessary, as it would in any study with microorganisms that have several stages of development, as mentioned above. This fact decreases the number of spots per gel, facilitating the identification of the referred proteins. Khan et al.[21] developed an efficient method of separating and purifying male and female gametocytes to study their

respective proteomes. The male gametocyte proteome had 36% (236 of 650) male-specific proteins, while the female gametocyte proteome had 19% (101 of 541) female-specific proteins. These forms share only 69 proteins, which emphasizes the different characteristics of the evolutionary forms. Of all the analyzed stages of the *P. falciparum* lifecycle, male gametocytes have the most distinctive proteome, containing many proteins involved in flagellar motility and in rapid replication of the genome. The sexual development that enables transmission of the protist produces surface proteins used in the sexual stages (such as gametes and zygotes), which are attractive molecules to target for the development of strategies to prevent transmission.[21]

Pathogenesis

Severe *P. falciparum* malaria can be pathophysiologically characterized as a type of sepsis,[3,22] involving elements from the systemic inflammatory response. Thus, the characterization of invasive organelles of *P. falciparum* (via one-dimensional electrophoresis, liquid chromatography, and mass spectrometry) has yielded the ookinete microneme proteome,[23] with favorable prospects for the pathophysiological elucidation of severe *P. falciparum* malaria. In the study of Lal et al.,[23] the most abundant protein constituents were chitinase, circumsporozoite- and thrombospondin-related proteins, HSP70, disulfide isomerase, and ookinete-secreted adhesive proteins. The proteomic analysis revealed 345 proteins, including M1 aminopeptidase and disulfide isomerase, which are known targets for drug development.[23,24]

Therefore, the analysis of subproteomes allows for increased accuracy in identifying proteins from a particular organelle of interest. This analysis minimizes the complexity of protein maps, facilitating the study of proteins contained in the gel and allowing the analysis of life forms that are more infective and/or more harmful to *H. sapiens sapiens*. In addition, unveiling the protein map of these forms enables the investigation of new strategies for (i) control, by developing vaccines that prevent infection by inhibiting pathogen evolution, and (ii) treatment, by interfering in certain vital processes of the protist, which highlights the need to distinguish between protozoan and human proteins.[25]

During its lifecycle, *P. falciparum* is exposed to different environmental conditions, particularly variations in O_2 pressure in the vertebrate host. In addition, the microorganism is exposed to 21% O_2 levels in the salivary glands of *Anopheles*.[24] This variation in the O_2 pressure results in metabolic changes in the protozoa, which are essential for its survival under hypoxic and hyperoxic conditions and which minimize the production of reactive oxygen species (ROS), an important requirement for the organism to remain infective. The elucidation of this phenomenon by proteomic analysis revealed increased levels of heat-shock proteins and decreased levels of glycolytic enzymes. It is noteworthy that some of these events reflect post-transcriptional modifications during the response to hyperoxia.[24] These results suggest that, under hyperoxic conditions, the antioxidant response systems of *P. falciparum* are activated to preserve the integrity of its cellular structures. In addition, the environmental restrictions appear to induce an energetic adaptation of the protist's metabolism.

Diagnosis

The rapid growth and use of proteomic technologies have resulted in an exponential increase of the description of proteins, which have been suggested as potential biomarkers for specific diseases. Currently, there is increased emphasis on developing methods to direct and measure the absolute quantity of proteins and specific peptides in complex proteomic samples.[26] In recent decades, many of the efforts related to proteomic analyses have been directed toward the design of experiments and the development of technologies that can characterize the proteome as much as possible. Once the tools for a complete proteome analysis became available, much of the interest turned to the evaluation of biological fluids and tissues, aiming to find new biomarkers of diseases, including malaria. The general premise was simple: to identify as many proteins as possible in a specific biofluid obtained from infected patients and to compare these proteins to those obtained from healthy individuals. The identification of more than 1,000 proteins in a sample of serum or plasma can take several days per sample.[27] Such production has limited the number of samples analyzed per study to rarely more than 10. This lack of statistical power is one reason the progress in validation has been so poor, which creates a lack of confidence in most potential biomarkers reported by the literature and low credulity for others. Despite advances in technology, the number of studies that describe biomarkers using proteomic identification most likely will remain lower compared with investigations of techniques such as mRNA microarray or genome sequencing.[26]

One of the crucial aspects for the success of studies of biological systems is the controlled disturbance of the system to obtain quantitative measurements for each component of the disturbance. Consequently, gene expression analysis using microarrays has been the most widely employed strategy in biological systems for storing a collection of data. DNA arrays and their associated protocols can provide many gene transcripts from the studied system in a precise and reproducible manner. Thus, a technique that is accurate for the absolute or relative quantification of all relevant proteins is necessary for proteomic analysis.[28]

A new course in targeted proteomics is emerging to provide a solution for this problem.[29] This approach provides extensive information from a quantitative and qualitative perspective. By programming the instrument to collect data from detectable ions, similarly to expressed sequence tag (EST) sequencing in genomics, targeted proteomics begins with a list of specific elements that will be marked, such as during microarray experiments for transcriptomics. The mass spectrometer is set to monitor single signals from targets that are specified prior to the assays. This step not only results in increased sensitivity but also ensures that these targets can be measured by several runs. [16] Thus, using targeted proteomics, it is possible to obtain absolute quantification of the target peptides and, therefore, of the target protein. The injection of known concentrations of reference peptides or synthetic proteins into the sample to be analyzed is the most common practice. Such reference peptides are usually isotopically labeled heavy forms.[30] This method might be the proteomic tool that is used in the near future to diagnose morbid conditions such as *P. falciparum* malaria quickly and accurately. Furthermore, in addition to diagnosis, the method may be useful to evaluate the disease progression by accurately quantifying specific proteins and by determining the disease stage based on the increase or decrease in the analyzed substance.

Treatment

Proteomic studies also show great potential for evaluating the mechanisms of action of antimalarial drugs and for revealing the biochemical pathways by which these drugs act. In 2005, Makanga et al.[31] analyzed the effects of two antimalarial drugs (artemether and lumefantrine) separately on the proteome of *P. falciparum*. Both drugs induced profound changes in the proteome of the microorganism. However, the pattern of change in the proteome was specific for each antimicrobial used. Both drugs induced opposite effects on the main glycolytic enzymes and had a similar influence on the expression of stress-response proteins. These results demonstrate the scope of the proteomic approach to study the mechanisms of pharmacological action.[31]

In recent years, a significant increase in resistance to antimalarial drugs has been reported. Choline analogues, such as bis-thiazolium T4, represent a new class of compounds with potent action against *P. falciparum* clones that are sensitive and resistant to other drugs. Although T4 and its analogues interfere with the protist's lipid metabolism, the exact mechanism for this action remains unknown.[32] Proteomic analyses of *P. falciparum* were performed to characterize the global response to this drug during the intraerythrocytic cycle of the microorganism, and they showed significant reduction in the levels of choline/ethanolamine phosphotransferase (PfCEPT), which is an enzyme involved in the final step of the synthesis of phosphatidylcholine (PC). This effect was also supported by metabolic studies, which demonstrated significant changes caused by the compound in the synthesis of PC from choline and ethanolamine.[32] The enzyme glycogen synthase kinase (GSK), which can inhibit the growth of wild-type (3D7) and multidrug-resistant strains (2D2) of *P. falciparum,* was targeted by the proteomic analysis to identify weak links in the proteome of *P. falciparum*. Of the 4,645 active compounds of GSK, 293 proteins of the protist were identified, six of which showed potential for therapeutic use.[33] Similarly, Briolant et al.[34] investigated the mechanism of action of doxycycline on the schizont form of *P. falciparum* using proteomic techniques. Additional two-dimensional differential gel electrophoresis (2D-DIGE) assays and isobaric tags for relative and absolute quantification (iTRAQ) were used to compare the protein expression from samples that were either treated or not treated with doxycycline. After treatment with doxycycline, 32 and 40 proteins of *P. falciparum* had deregulated expression levels as evidenced by 2D-DIGE and iTRAQ, respectively. Although the deregulation of these proteins has been previously determined via treatments with other drugs, numerous changes in the protein content appear to be specific to treatment with doxycycline, effects that may disturb the apicoplast metabolism.[34]

Prevention - vaccine development

The protein microarray technique was used by Doolan et al.[35] to elucidate the profile of antibodies

that develop after natural or experimental malarial infection or after immunization with attenuated organisms. Thus, immunoreactive antigens of interest to the development of vaccines or for other applications have been identified. A protein microarray uses a matrix in which different protein molecules or specific DNA sequences are separately fixed and ordered, forming a microscopic matrix. The technique offers a multiple approach to map protein-protein interactions, to identify the substrates of enzymes, to elucidate the activation of transcription factors, or to detect the targets of biologically active small molecules. Thus, the expression vectors for 250 proteins of *P. falciparum* were produced by PCR/cloning, after which the proteins were expressed individually with over 90% efficiency in *Escherichia coli* and were printed directly, without purification, on microarray slides. Protein microarrays were probed with human serum from one of the four groups that differed in the immune response to the protozoa. In total, (i) 72 highly reactive *P. falciparum* antigens and (ii) immunoreactivity-associated proteomic features were identified. A particularly important result regards the antibody profiles, which were different for each group of donors. Information obtained in these analyses may facilitate the identification of antigens for the development of a vaccine, which can be undertaken concomitantly with the investigation of the molecular bases of *P. falciparum* immunity[35], as well as the immunological mechanisms triggered in the host response.[36]

CLOSING REMARKS

P. falciparum malaria is one example of a disease that justifies the focus of intensive care doctors on infectious diseases. The knowledge base regarding malaria is directed toward exploring the biological aspects of the vector, the protist, the host, and the relationship that is established between these organisms - with the ultimate aim of delineating prophylactic, diagnostic, and therapeutic strategies.

Although still in the preliminary stage, proteomic studies represent a promising tool that, in the future, may offer resources for improving the care of patients with *P. falciparum* malaria or those persons at risk of developing the disease.

RESUMO

A despeito dos avanços no tratamento e das campanhas de prevenção e de controle da malária nos distintos continentes nos quais a moléstia grassa, a entidade mórbida permanece com significativa relevância no mundo contemporâneo. O *Plasmodium falciparum* é o grande responsável pela malária grave, caracterizada por distúrbios em diferentes órgãos e sistemas, com possibilidade de evolução ao óbito. Embora incipientes, os estudos proteômicos na malária têm trazido boas perspectivas para melhor compreensão dos aspectos biológicos do *Plasmodium*, assim como dos mecanismos fisiopatológicos, diagnósticos, terapêuticos e profiláticos da enfermidade. Desse modo, o objetivo do presente artigo é apresentar uma breve revisão das aplicações da análise proteômica na malária por *P. falciparum*.

Descritores: Proteoma; Malária; *Plasmodium falciparum*

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