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HEPARAN SULFATE AND CHONDROITIN SULFATE: GENERAL ASPECTS OF THEIR PARTICIPATION DURING *Plasmodium falciparum* DEVELOPMENT

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ABSTRACT

Background: Malaria is one of the most important infectious diseases worldwide due to its high morbidity and mortality rates every year in tropical countries. Despite efforts in malaria research, several mechanisms underlying host-parasite interactions remain unclear, which is a big obstacle for the management and control of malaria.

Recently, numerous studies have attempted to provide a better understanding of the physiopathological mechanisms to assist in the design of new drugs, vaccines and transmission blocking agents. These research topics have indicated that glycans are key molecules in the life cycle of the malarial parasites.

The aim of this review is to highlight the relevance of glycans for the development and transmission of *Plasmodium* and to use that information as a valuable research tool to fight malaria.

Because glycans play roles in parasite invasion and interactions with the mosquito host, both of which are part of "parasite development", this review seeks to specify the role of glycans in parasite development.

Methods: This review was mainly based on research articles published between 1985 and 2015 that were obtained from the PubMed and Embase databases. The keywords used in this search were sulfated glycans, malaria, *Anopheles* and *Plasmodium*.

Conclusions: Sulfated glycoconjugates are intimately linked to the development, transmission and survival of *Plasmodium* in the intermediate and definitive hosts. A better understanding of the role of sulfated glycoconjugates in malaria infection would permit the development of new therapeutic strategies and the design of strategies to inhibit parasite transmission.

Key words: sulfated glycans, malaria, mosquitoes, *Plasmodium*.

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SULFATO DE HEPARÁN Y SULFATO DE CONDROITINA: ASPECTOS GENERALES DE SU PARTICIPACIÓN DURANTE EL DESARROLLO DE *Plasmodium falciparum*

RESUMEN

La malaria es considerada una de las enfermedades infecciosas de mayor importancia alrededor del mundo debido a la alta morbimortalidad que causa cada año en países tropicales. A pesar de los esfuerzos de investigación en malaria, muchos de los mecanismos que entrañan las interacciones hospedero-parásito aún no son claros, lo que constituye un gran obstáculo en el manejo y control de la malaria.

Numerosos estudios se han llevado a cabo en los últimos años en busca de una mejor comprensión de los mecanismos fisiopatológicos, diseño de nuevas drogas, diseño de una vacuna y bloqueo de la transmisión. En todos estos temas de investigación, un elemento común son los glicanos como moléculas clave en el ciclo de vida de los parásitos de la malaria.

El objetivo de esta revisión es mostrar como los glicanos se necesitan para el desarrollo y la transmisión de *Plasmodium* y como esta información resulta ser una valiosa herramienta en la investigación para combatir la malaria.

Métodos: La presente revisión se basó principalmente en artículos originales publicados entre 1985 y 2015, obtenidos de las bases de datos PubMed y EmBase. La búsqueda fue hecha en inglés y se usaron las palabras clave: glicanos sulfatados, malaria, *Anopheles* y *Plasmodium*.

Conclusión: Los glicoconjugados sulfatados están íntimamente vinculados al desarrollo, la transmisión y la supervivencia de *Plasmodium,* tanto en el hospedero intermediario como en el hospedero definitivo. Una mejor comprensión del rol de los glicoconjugados sulfatados en la infección malárica permitiría el desarrollo de nuevas alternativas terapéuticas, así como el diseño de estrategias para inhibir la transmisión.

Palabras clave: glicanos sulfatados, malaria, mosquitos, *Plasmodium.*

INTRODUCTION

Malaria is an important infectious disease that has a huge impact on the health of millions of people who inhabit the tropical and subtropical zones of the planet. The treatment and eradication of malaria entail an enormous effort by research organizations, which are always seeking new strategies to achieve a better understanding of the disease. The malaria problem can be approached from different aspects (drugs resistance, some physiopathological aspects and transmission). Many research studies have been dedicated to the design of new drugs to combat the disease based on knowledge of the metabolic pathways of the parasite, which has revealed several enzymes as molecular targets. However, the results have not represented definitive successes in the solution to this problem (1, 2).

Some studies have addressed the problem from a different perspective and sought to understand the biology of the malaria parasites. This approach also represents a great challenge because *Plasmodium falciparum* is not a conventional parasite. Instead, *P. falciparum* diverges significantly from other eukaryotic organisms and has developed a battery of genes (*var* genes) that permit diverse antigenic repertoire to permanently evade the host immune response (3, 4).

To date, many parasite proteins have been described (5, 6), and their participation has been documented in various events that are important for the survival of the parasite, such as invasion and adherence. However, recent glycobiology studies have revealed the importance of sulfated glycoconjugates and their relationship with malaria (7, 8).

Current investigations aim to demonstrate that these compounds are critical for *Plasmodium* development during different stages of the parasite's life cycle in both the definitive host (the mosquito) and the intermediate host (the vertebrate). Additionally, these compounds are necessary for the invasion and adhesion processes (9–12).

This review aims to highlight the importance of sulfated glycoconjugates (glycosaminoglycans (GAGs) and proteoglycans) during the development of *Plasmodium* in both the mosquito and the vertebrate host and thereby motivate researchers to undertake studies regarding this topic. These studies will aid in the development of a better understanding of the pathophysiology of malaria infection and consequently contribute to the development of new strategies to control this infection.

Plasmodium life cycle

The *Plasmodium* life cycle is complex and alternates between a definitive host (mosquito) where the sexual phase occurs and the intermediate host (a vertebrate) where the asexual phase occurs. The life cycle starts when a female *Anopheles*-infected mosquito releases sporozoites into the bloodstream of a human. This form of the parasite invades the liver. At this stage, the parasite adopts an intracellular lifestyle and multiplies into hundreds of invasive forms called merozoites. These merozoites invade erythrocytes and give rise to the pre-erythrocytic and exo-erythrocytic stages. The merozoites maintain the intra-erythrocytic cycle by invading non-infected red cells. A small proportion of the merozoites grow inside the erythrocytes into either a female or male gametocyte, which will be consumed in a blood meal by a female mosquito. After a process called exflagellation, the male gametocytes convert into flagellated forms called microgametes, which fertilize the macrogametes, resulting in a zygote. This parasite stage progresses to an ookinete. After penetrating the mosquito gut wall, the oocyst appears and multiplies by an asexual mechanism into hundreds of motile forms called sporozoites that reach the salivary glands. The sporozoites will be released during a new blood meal by the female mosquito, and the cycle starts again to guarantee parasite transmission (13).

Sulfated glycoconjugates in living organisms

GAGs are the most abundant hetero-polysaccharides in organisms and are arranged in long branched chains that consist of repeating disaccharide units, including N-acetylglucosamine (GlcNA) or N-acetylgalactosamine (GalNAc) and glucuronate or iduronate. Sulfated glycans exhibit a high density of negative electrical charges due to the presence of many sulfate groups ($-SO_4^{-2}$), which confer different properties depending on their amount and location (14,15). These molecules are abundant in the extracellular matrix, where they behave as molecular mechanical shock absorbers due to their hygroscopic nature and low compressibility (15); additionally, these molecules are found on the surface of every cell in the organism and form part of the glycocalyx, which creates a permissive microenvironment for functions such as cellular differentiation, adhesion and physiological and pathological signaling events (16,17). Glycoconjugates are quintessential communication molecules for these functions (18).

Glycoconjugates, ABO blood group, *Plasmodium falciparum* and severity

In the evolutionary history of *Plasmodium falciparum* and humans, the parasite first appeared approximately 200 million years ago, whereas *Homo sapiens* appeared only 200,000 years ago. Between 100,000 and 40,000 years ago, the first human group migrations from Africa to Oceania, Europe and Asia occurred. The ABO blood group polymorphisms that developed under pressure by *Plasmodium falciparum* were carried with these individuals (19). When the

ABO group appeared in the human population, the parasite selected individuals with blood group O because this group conferred a survival advantage for the human population that was not present in the A, B and AB blood groups (20). Studies in 2007 found that the severity of malaria appeared to be related to the A, B or AB blood groups, whereas a minority of complications occurred in individuals in the O blood group (19,20). Studies of the geographical distribution of blood group polymorphisms in areas where malaria is endemic are just beginning to emerge (20,21).

The above finding seems to be related to the fact that blood groups are classified according to the antigens present on erythrocytes. These antigens correspond to chains of monosaccharides (GalNAc or D-galactose) that carry sialic acid in the terminal positions of the glycan. Individuals in the O blood group lack surface antigens, which may explain why the invasion process is minimal in patients in this blood group; *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) presents domains that recognize surface antigens during the initiation of the adhesion and invasion processes (20).

Heparan sulfate and chondroit in sulfate mediate *Plasmodium falciparum* invasion and adhesion

Glycans such as heparan sulfate (HS) and chondroitin sulfate (CS) have been identified to play roles during the *Plasmodium falciparum* adhesion and invasion processes. HS has been found in endothelial cells, on the surface of erythrocytes and on the surface of sporozoites (14,17). CS is expressed in numerous cells in the body, although a variety of this glycan that serves as a *Plasmodium* parasite receptor occurs exclusively in syncytiotrophoblast cells in pregnant women (21).

Formation of the invasive phenotype

Following inoculation of sporozoites into the skin or bloodstream of vertebrates by the infected mosquito, the parasites migrate to the liver and establish this organ as an obligatory pathway for *Plasmodium* spp. However, why the parasite follows this route is unknown (22, 23).

Studies in which sporozoites are cultured have been performed in the following cell lines: Chinese hamster ovary cells (CHO), endothelial cells (HBMVEC), dermal fibroblasts (MDF) and liver cells (Hepa1-6); the degrees of surface HS sulfation in these cell lines are known. The experiments consisted of altering the concentration of sulfate in the HS molecule prior to sporozoite culture. Additionally, soluble heparin assays were performed at different concentrations to verify the degree of inhibition of sporozoite invasion in the sulfated cells.

The results demonstrated that the degree of HS sulfation present in the hepatocytes was greater than the degree of HS sulfation on other cells in the body and that the concentration of sulfate played a determinant role in the development of an invasive phenotype of the parasite by preparing the parasite for the subsequent invasion of erythrocytes. The sporozoites only migrated through cells with low HS sulfation and only invaded cells with highly sulfated HS. However, soluble heparin proved not to be a competitive inhibitor of cell invasion by the sporozoites (24).

Rosette formation and sequestration

In malaria, one event that greatly contributes to the development of complications is the phenomenon of cytoadherence (17), which involves sequestration (i.e., the adhesion of the parasitized erythrocytes to the endothelium of the microvasculature of various organs) and rosetting or the formation of rosettes, which consists of the adhesion of parasitized erythrocytes to other healthy erythrocytes or other blood cells. Both events lead to obstruction of the microvasculature and trigger a state of tissue hypoxia and eventually organ failure (7, 8).

The adherence of parasitized erythrocytes involves several molecules, including PfEMP1, which is exported by *Plasmodium* parasites to the surface of the erythrocyte and presents several domains that adhere to molecules present on the host cell surface, such as intercellular adhesion molecule 1 (ICAM1), CD36 and HS and CS proteoglycans (25, 26). However, studies conducted by Fried and Duffy in 1996 showed that CS exclusively participated in the sequestration of erythrocytes parasitized by *Plasmodium falciparum* in the placenta (27). Another study by Valiyaveettil et al. in 2001 suggested that hyaluronic acid (HA), which is a non-sulfated glycan, was a potential receptor for parasitized red blood cells (pRBCs) in the placenta (28).These results were confirmed by Beeson et al. (29). Different types of CS exist; only chondroitin sulfate A (CSA) and chondroitin-4-sulfate (C4S) are responsible for sequestration in the placenta because these forms are expressed in the placental extracellular matrix and syncytiotrophoblast cells (21, 27, 30). These events are intimately related to the pathophysiology and complications of the disease. There is evidence of a DBLX-2 domain in the *Plasmodium falciparum var* 2CSA protein that constitutes the CSA binding site; thus, *var* 2CSA is a strong vaccine candidate (9).

In vitro assays conducted with different HS and CS glycans and modified heparin (with variations in the degree of sulfation and the position of the sulfate groups) have shown that the formation of rosettes can be reversed in cultured laboratory strains (FCR3S1) and wild isolates when HS and CS glycans are introduced into the cultures. However, wild isolates exhibit some adhesion preferences (31) (i.e., there is a delicate regulation within this interaction, which makes some strains more adherent than others depending on the subtle variations present in the distribution and concentration of sulfated glycans on vertebrate cells (32)). Another important potential role of sulfated glycans such as CSA and HS is their regulatory activity on placental inflammation as a result of the parasite presence (33).

Heparan sulfate and chondroitin sulfate in the mosquito-parasite interaction

The parasite passes through two tissue types during its development in the mosquito vector. The first obligatory pathway involves binding of the ookinetes to the basement membrane of the mosquito's intestinal epithelium, followed by invasion by the sporozoites to the salivary glands.

A critical interaction between the ookinetes and intestinal cells is mediated by GAGs, including CS, which are located on the basement membrane proteoglycans of the mosquito's intestinal epithelium. The importance of this interaction has been demonstrated in *Anopheles gambiae,* where 95% inhibition of ookinete invasion of the middle intestine has been shown (34). Additionally, other studies have suggested that the parasite uses not only the GAGs of CS but also of HS to locate the target organs and for development during different life cycle stages in the vector and the vertebrate host (Fig. 1) (10, 11).

Whether the composition and quantity of the GAGs are the same in cells from different *Anopheles* species with different vectorial conditions of natural populations and how these differences influence malaria transmission are unknown. The answer to this question will allow the determination of key aspects of the development of strategies aimed at vector control and decreasing malaria transmission. Vaccine design was thought to represent a good strategy to block malaria transmission. In 2015, the *Anopheles* monoclonal (mAb) anti-aminopeptidase-N antibody (An APN1) 4H5B7 was shown to recognize AnAPN1, which is the *Plasmodium* ookinete receptor in the mosquito midgut (35). The identity of the GAGs involved in the interaction are unknown at present, and their identification will contribute to vaccine design. The possible GAGs involved in this interaction need to be identified to contribute to vaccine development.

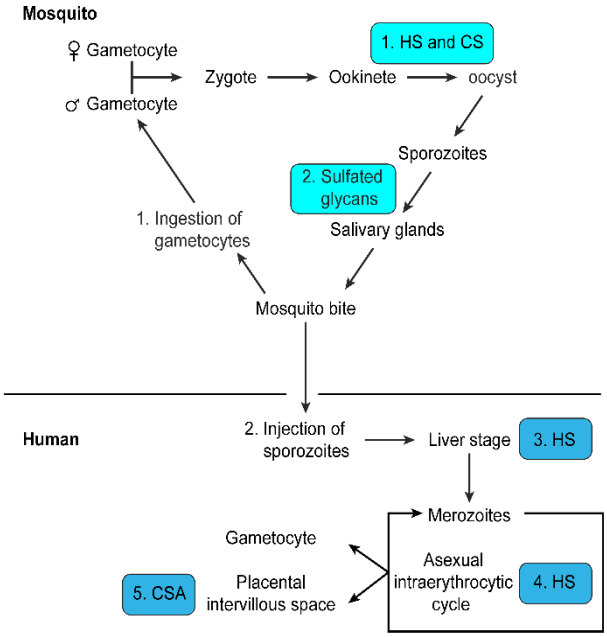


Figure 1. Function of glycans in the life cycle of *Plasmodium spp.*

Figure captions
Fig. 1 Function of glycans in the *Plasmodium* spp. life cycle in the definitive host (mosquito) and the intermediate host (vertebrate/human). 1. Heparan sulfate (HS) and chondroitin sulfate (CS) are necessary for the maturation of the ookinete to the oocyst. 2. Sulfated glycoconjugates mediate the arrival of sporozoites into the mosquito salivary glands. 3. HS induces the hepatic stages of *Plasmodium* spp. that initiate the formation of an invasive phenotype capable of infecting red blood cells (RBCs). 4. HS mediates rosette formation and the adherence of the parasitized erythrocytes to the endothelium of the microvasculature of deep organs. 5. Chondroitin sulfate A (CSA) is the principal mediator of the adherence and sequestration of parasitized maternal RBCs in the intervillous space of the placenta during placental malaria.

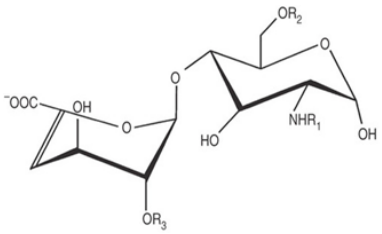
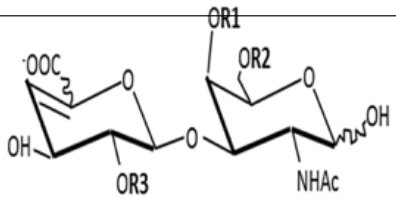
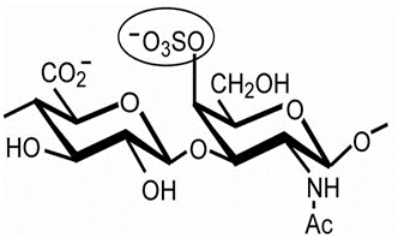
Glycosylation enzymes in *Plasmodium falciparum*

In 2002, the genome of *Plasmodium falciparum* was sequenced, and the parasite was found to lack glycosylation enzymes (3). However, in 2007, a study conducted by Landoni et al. (36) revealed that the parasite performed sulfo glycosphingolipid synthesis in three different stages. In addition to requiring glycosylation enzymes for their synthesis, these compounds are important for stabilizing the membrane of the parasite and may be involved in the invasion phenomenon. Additionally, a different sulfo glycosphingolipid biosynthesis process was found for each of the three intraerythrocytic stages (ring, trophozoite and schizont), suggesting the possibility of different levels of sulfation throughout the parasite's life cycle. Another event that has been reported in the intraerythrocytic stages is the synthesis of glycophosphatidylinositol, for which the parasite requires the glycosylation enzyme dolichol phosphate mannose (37).

Some technical difficulties must still be overcome to identify the glycosylation enzymes in *Plasmodium*. This process requires very sensitive analytical methods and the choice of a synchronous culture in a mature stage

(trophozoites) because this stage is "where instead of that" that *Plasmodium* exhibits most of its proteins. Experimental strategies must be designed to eliminate the possibility of contamination by host molecules because *Plasmodium* is an obligate intracellular parasite. To date, a portion of the saccharide complex-synthesis capacity of the parasite has been outlined. Furthermore, the parasite contains lectins that enable interactions with both the vector and the intermediate host (8) (Table 1).

Table 1. Structure, role, receptor and function of sulfated glycans during the infection by *Plasmodium falciparum*.

Sulfated Glycan	Rol during infection	Chemical structure	Receptor in the parasite
Heparan sulfate (HS)	- Adhesion in endothelium and rosetting -Development of an invasive phenotype in the liver - Motility and invasion in the mosquito midgut and salivary glands *Maturation of ooquinetes		PfEMP-1 protein (DBL-α domain) – human infection/ CSP and TRAP proteins in mosquito infection
Chondroitin sulfate (CS)	- Motility and invasion in the mosquito midgut and salivary glands *Maturation of ookinete		CSP and TRAP proteins – only in mosquito infection
Chondroitin sulfate A (CSA)	-Sequester in the syncytiotrophoblast *Regulation of the placental inflamation		VAR2CSA protein - only in placental infection

*Information non-confirmed yet.

Sulfated glycans as antimalarial therapies

Sulfated GAGs, such as heparin, CSA and HS, have been proposed as potential antimalarial therapies because these glycans can inhibit adhesion phenomena in pRBCs when soluble (38). Heparin was used as a treatment for severe malaria years ago but was abandoned due to its potent anticoagulant action and adverse effects, such as intracranial bleeding (8).

Recent studies have shown that depolymerized heparin that lacks anticoagulant activity can maintain its ability to function as an inhibitor

for pRBC sequestration and rosette disruption not only *in vitro* but also *in vivo* in fresh parasite isolates (38).

An important property of heparin is its ability to penetrate pRBCs. Furthermore, heparin can easily be conjugated to drug-containing nanovessels while preserving its antimalarial effect. These ideas may provide a new cost-effective pharmacotherapy in the socioeconomic context of malaria (39).

Based on the above findings, heparin still remains at the spearhead of GAGs with antimalarial activity and may be considered a targeting molecule for the localized delivery of drugs to *Plasmodium*-infected cells. However, further research is required in that particular field of study (39, 40).

CONCLUSIONS

Sulfated glycoconjugates are linked to the development, transmission and survival of *Plasmodium* in its intermediate and definitive hosts. A better understanding of the role of sulfated glycoconjugates in malaria infection will permit the identification of new therapeutic strategies for individuals who suffer from the disease and its complications and facilitate blocking of parasite transmission and/or successful infection in humans. The mechanism used by the parasite to sense the sulfate concentration in both HS and CS in different tissues in mosquitoes and humans and to accomplish the adhesion and invasion processes must be determined.

List of abbreviations
Glc-Nac: N-acetylglucosamine
Gal-Nac: N-acetylgalactosamine
PfEMP-1: *Plasmodium falciparum* erythrocyte membrane protein 1
pRBCs: parasitized red blood cells
HS: heparan sulfate
CS: chondroitin sulfate
CSA: chondroitin sulfate A
ICAM-1: intercellular adhesion molecule 1

Conflicts of Interest
The authors declare they have no conflicts of interest.

Authors' contributions statement
Idea conception: E. Garrido and L.C. Burgos; acquisition of data: E. Garrido and L. C. Burgos; analysis and interpretation: E. Garrido; drafting: E. Garrido and L. Rocha. All authors subsequently read, revised and approved the manuscript.

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