UNIVERSITAT DE BARCELONA

Synthesis and biological function of fucose in *Plasmodium falciparum*

Silvia Sanz Sender

Synthesis and biological function of fucose in *Plasmodium falciparum*

Sílvia Sanz Sender

Director: Luis Izquierdo

Línia de recerca: Salut Internacional

Programa de Doctorat en Medicina

ISGlobal & Universitat de Barcelona

ISGlobal Barcelona Institute for Global Health

UNIVERSITAT DE BARCELONA

A vosaltres, família

"Si vols arribar ràpid camina sola,
però si vols arribar lluny camina acompanyada"

Proverbi Africà

TABLE OF CONTENTS

LIST OF ABREVIATIONS

α-gal – α-galactose

AAL - *Aleuria aurantia* lectin

ACT – Artemisinin-based Combined Therapy

Alg – Asparagine linked glycosylation

AMA – 1 – Apical Membrane Antigen – 1

Asn – Asparagine

C – Carbon

CDPK3 – Calcium dependent protein kinase 3

CMP – Cytidine monophosphate

CQ – Chloroquine

CSA – Chondroitin Sulphate A

CSP – Circumsporozoite Protein

CTRP – Circumsporozoite and TRAP related protein

Cys – Cysteine

Dol–P – Dolichol-Phosphate

Dol-P-Man – Dolichol-phosphate-mannose

Dol-P-P-GlcNAc – dolichol pyrophosphate-N-acetylglucosamine

DPM1 - Dolichol-phosphate mannose polypeptide 1

DTT - Dichloro-diphenyl-trichloroethane

EBA 175 - Erythrocyte binding antigen 175

ECM - Extracellular Matrix

EEF – Exoerythrocytic Form

EGF – Epidermal Growth factor

ER – Endoplasmic Reticulum

EtN-P - Ethanolamine – Phospahte

Fru – Fructose

Fru6P – Fructose-6-phosphate

FS - GDP – L – Fucose synthase

Fuc – Fucose

GAG – Glycosaminoglycan

Gal – Galactose

GALE – UDP-Glucose 4-epimerase

GalNAc – *N*- acetylgalactosamine

Gal1P – Galactose-1-phosphate

GDP – Guanine diphosphate

GDP-Fuc – GDP-Fucose

GDP-Man – GDP-Mannose

GFPT – Glucosamine – fructose – 6 – phosphate aminotransferase

GK – Galactokinase

Glc – Glucose

Glc1P – Glucose-1-phosphate

Glc6P – Glucose-6-phosphate

GlcA – Glucoronic acid

GlcN - Glucosamine

GlcNAc – *N* – acetylglucosamine

GlcNAc1P – N-acetylglucosamine-1-phosphate

GlcNAc6P – N-acetylglucosamine-6-phosphate

GlcN6P – Glucosamine-6-phosphate

Gly - Glycine

GMD – GDP – mannose 4,6 – dehydratase

GNA – Glucosamine – phosphate N – acetyltransferase

GpA – Glycophorin A

GPI – Glycosylphosphatidylinositol

G6PD – Glucose – 6 – phosphate dehydrogenase

G6PI – Glucose-6-phosphate isomerase

H – Hydrogen

HA – Hyaluronic Acid

HK – Hexokinase

hpi – hours post invasion

HSPG – heparin sulphate proteoglycan

IPTi – Intermitent Preventive Treatment in infants

IPTp – Intermitent Preventive Treatment in pregnancy

IRS – Indoor Residual insecticide Sprays

KC – Kupffer Cell

KO – Knockout

LLIN – Long Lasting Insecticide-treated Nets

LLO – Lipid linked oligosaccharide

Man – Mannose

Man1P – Mannose-1-phosphate

Man6P – Mannose-6-phosphate

MPG – Mannose – 1 – Phosphate guanyltransferase

MPI – Mannose – 6 – phosphate isomerase

MSP – Merozoite Surface Protein

N - Nitrogen

O – Oxygen

OH – hydroxyl

OST - Olygosaccharyltransferase

P - Phosphate

PAGM – Phosphoacetylglucosamine mutase

PCR – Polymerase chain reaction

PfEMP1 – *Plasmodium falciparum* erythrocyte membrane protein 1

PGM – Phosphoglucomutase

PI – Phosphatidylinositol

PIESP1 – parasite-infected erythrocyte surface protein 1

PM – Peritrophic Matrix

PMM – Phosphomannomutase

PoFUT – Protein *o*-fucosyltransferase

PV – Parasitophorous Vacuole

RBC – Red Blood Cell

RDT – Rapid Diagnostic Test

S – Sulfur

Ser – Serine

Sia – Sialic Acid

SMC – Seasonal Malaria Chemoprevention

SP – Sulphadoxine Pyrimethamine

SPATR - Secreted Protein with Altered Thrombospondin Repeat domain

TLP – TRAP like Protein

Thr – Threonine

TRAMP - Thrombospondin-Related Apical Membrane Protein

TRAP – Thrombospondin Related Anonymous Protein

TREP – TRAP like protein

Trp - Tryptophan

TRSP – Thrombospondin-related sporozoite protein

TSR – Thrombospondin Type 1 Repeat

UAP – UDP – N – acetylglucosamine pyrophosphorylase

UDP – Uridine diphosphate

UDP-Gal – UDP-Galactose

UDP-Glc – UDP-Glucose

UDP-GlcNAc – UDP-N-acetylglucosamine

UEA-I – *Ulex europaeus* agglutinin I

UGP – UTP – glucose – 1 – phosphate urydiltransferase

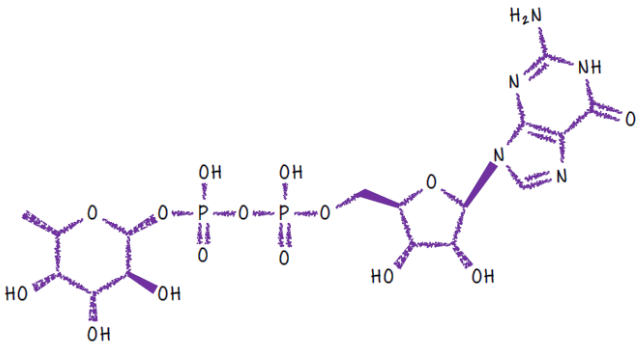
USP – UDP – sugar pyrophosphorylase

vWF-A - von Willebrand factor A

WHO – World Health Organization

Xyl – Xylose

INTRODUCTION



INTRODUCTION

1. Malaria

1.1. Malaria burden

Malaria kills a child in Africa every 2 minutes. Malaria is one of the most important parasitic diseases that affect humans, threatening almost half of the world population. It is caused by an apicomplexan protozoan parasite of the genus *Plasmodium* that is transmitted by female mosquitoes of the genus *Anopheles*. There are five different species of *Plasmodium* that cause malaria in humans: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*[1]. More than 400 species of *Anopheles* mosquitoes exist around the world but only 70 are capable of transmitting malaria. Due to their propensity of feeding in humans, abundance, longevity or a combination of all these factors, 40 different species have been described as dominant vector species[2,3].

Eliminate *P. falciparum* and *P. vivax* malaria became the most important challenge to control the disease because they are responsible for the majority of malaria cases. *P. falciparum* is present in tropical, subtropical and warm temperature regions, and it is the predominant species in Africa[4]. It is responsible for most malaria deaths and the severity is more acute[4,5]. *P. vivax* is more widely distributed than *P. falciparum*, however it carries a lower risk of fatal outcome, approximately only 1% of malaria deaths[4,6]. The lack of a robust and continuous culture technique of this parasite hinders the study of its biology, transmission and pathogenesis, even though some improvements have been done[7]. Moreover, *P. vivax* elimination is more challenging due to the relapse of the dormant liver-stage hypnozoites, a reservoir of infection[8].

Although there has been a significant decrease in the incidence of malaria since the beginning of the millennium, the disease still kills 1200 people a day. According to the latest estimates from the World Health Organization (WHO), there were 241 million new cases of malaria and 429.000 deaths worldwide in 2015. Globally, the African region accounted for most of the malaria cases (88%). Despite the situation is still dramatic, there has been a drastic decrease trend from 2000[6].

In areas where the transmission is high, children under five and women in their first pregnancy are more vulnerable to malaria than other population with acquired immunity. The continuous contact with *Plasmodium* parasites confers the so called acquired immunity, making the majority of adults refractory to the disease[9]. In low transmission areas, where this acquired immunity is lower, all ages are at risk of infection. Refugees are also very vulnerable due to the poor living conditions. Non-immune individuals going to endemic areas or travelers visiting tropical Africa are also very susceptible to the disease[10].

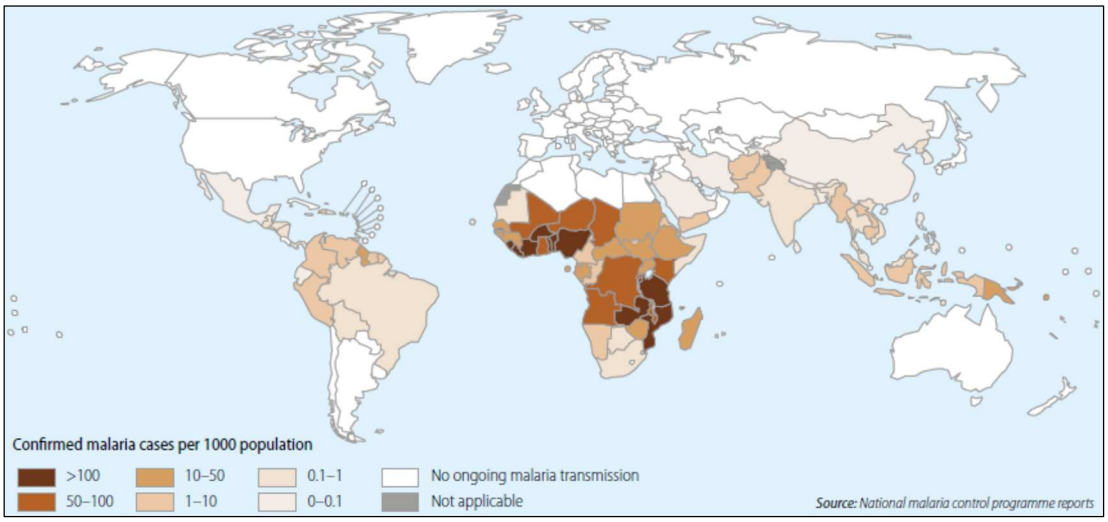


Figure 1. **Countries with outgoing transmission of malaria, 2013.** Reproduced from World Malaria Report, WHO, 2014[11].

1.2. Disease

Seven to fifteen days after the infected *Anopheles* bite, the initial symptoms of malaria may appear and the disease can be classified as uncomplicated or severe. When an infection with *Plasmodium* parasites occurs, it can lead to a very wide range of symptoms: from absent or very weak manifestations to a severe illness or even death.

Uncomplicated or mild malaria symptoms are nonspecific and can comprise general discomfort, headache and fever as well as mild anemia and a palpable spleen. When treated fast enough with antimalarial drugs, mortality of uncomplicated malaria patients is very low[1]. The principal manifestations of severe malaria are cerebral malaria, renal failure, severe jaundice and pulmonary edema. Severe malaria is dependent on transmission intensity, it occurs in young children in high malaria transmission areas, and in people of all ages in low transmission areas[12].

Malaria in pregnancy has an overwhelming effect on mother and baby's health and it is an important case of maternal and infant mortality and morbidity[13]. Natural acquired immunity decreases during pregnancy, particularly during first and second pregnancies[9]. In high transmission areas malaria in pregnancy is associated with spontaneous abortion, premature delivery, stillbirth, severe maternal anemia low birth weight, and neonatal death[14]. In low transmission areas the manifestations may include severe disease and mother or fetus death[15].

1.3. Control and elimination

A great number of countries are working towards malaria elimination[16]. In the last 15 years, the number of countries with less than 1.000 malaria cases has increased from 13 to 33 and the WHO European Region reported zero indigenous cases of malaria in 2015. Nevertheless, it is known that only 15 countries accounted for the 80% of the cases, being countries in sub-Saharan Africa the ones suffering a higher mortality and disease burden. The Democratic Republic of Congo and Nigeria together represent the 35% of the global total of estimated malaria deaths. A decrease in the incidence in these countries is urgently needed to improve the global progress towards malaria elimination[5].

Malaria can be prevented and treated using different cost-effective interventions. The main ones comprise vector control, chemoprevention, and appropriate case management (including diagnostic and treatment)[17].

1.3.1. Prevention

Vector control is an essential component of prevention. Its main objective is to diminish the vectorial capacity of local mosquito population. The vector control strategies with a higher impact comprise indoor residual insecticide sprays (IRSs) and long lasting insecticide-treated nets (LLINs). These tools contribute to malaria control and local malaria elimination in many malaria-endemic regions[18]. *Anopheles* resistance to existing pesticides is emerging, so there is a need of developing a broader range of insecticides with novel mechanisms of action[19,20]. Another key issue is the need for the development of new strategies to act on vector species not effectively targeted by currently used tools[21]. Nevertheless, the most difficult challenge to be faced is the design of new approaches to permanently reduce the high vectorial capacities of dominant malaria vectors in Sub-Saharan Africa[18]

Chemoprevention is the use of antimalarial drugs or medicines for prophylaxis and preventive treatment in risk groups. The objective of chemoprevention is to prevent malaria

infection by maintaining therapeutic drug levels in the blood. WHO currently recommends the Intermittent Preventive Treatment in pregnancy (IPTp), the Intermittent Preventive Treatment in infants (IPTi), and the Seasonal Malaria Chemoprevention (SMC) in endemic areas. IPTp consists in delivering Sulphadoxin-Pyrimethamin (SP) to all women in their first or second pregnancy in malaria endemic areas. IPTi (SP) is given to infants younger than 12 months and SMC (amodiaquine and SP) is recommended in malaria seasonal transmission areas to all children under 6 years[5].

The absence of financially lucrative, high-income markets makes the investment in malaria prevention, treatment, and vaccine development to massively depend on public and philanthropic funding[22]. Many vaccine trials have become unsuccessful for different reasons including antigen polymorphism, poor immunogenicity, and inadequate understanding of protein function in the parasite life cycle[23]. Nevertheless, effective vaccines would be an excellent tool towards malaria eradication. The malaria vaccine RTS,S/AS01, which provides a modest protection against clinical malaria, was recently licensed and approved for use by the European Medicine Agency[5]. WHO's Strategic Advisory Group of Experts on Immunization has recommended a large-scale pilot implementation of the malaria vaccine in parts of three to five countries[6].

1.3.2. Diagnostic

A prompt and reliable diagnosis is another important tool to effectively determine if someone is infected with malaria parasite, treat accordingly and, contribute to disease control. Sensitive and specific malaria diagnosis is required to significantly reduce morbidity and death. Giving malaria treatment specifically to those patients with confirmed presence of parasites will contribute to diminish the emergence and expansion of drug resistances.

Malaria Rapid Diagnostics Tests (RDT) detect specific antigens produced by malaria parasites that are present in blood. These kits are an alternative to diagnosis based on microscopy, especially in the places where good quality microscopy services are difficult to find. These two tools, RDTs and microscopy, are the first option for malaria diagnosis in the field, while the detection of parasites by molecular techniques (Polymerase Chain Reaction (PCR)) are only to be considered for epidemiological research and surveys[24].

1.3.3. Treatment

The first-line treatment for malaria has evolved with time considering new discoveries and the appearance of drug resistances. Chloroquine (CQ) was implemented by WHO as first-line treatment, but unfortunately the first resistance isolates were confirmed

at the beginning of the 1960's after treatment failures in Venezuela and Thailand at the late 50's[25]. After the emergence of CQ resistance, alternative drugs such as SP, mefloquine, amodiaquine and quinine were used as monotherapies, leading again to the emergence of resistant strains[26–28]. In 1972 Youyou Tu re-designed a protocol to obtain artemisinin from *Artemisa Annua*. This compound, by entering the parasite food vacuole, is able to damage the parasite hemoglobin catabolism and kill the parasite[29]. She received a Nobel Prize for this discovery in 2015. This component has become part of the current WHO treatment recommendation in endemic areas with the Artemisinin – based Combination Therapy (ACT)[30], to try to avoid the emergence of artemisinin resistance.

Combined therapies consist in a combination of artemisinin derivatives with longer-acting antimalarials that have a different mechanism of action[17]. Artemisinin derivatives clear parasite from the blood very fast and are also active against sexual stages[31], while the longer-acting antimalarial cleans remaining parasites and provides protection against the development of resistances to artemisinin derivatives. Unfortunately, resistance to artemisinin recently emerged in Western Cambodia[32,33] and it has been confirmed in four other countries: Lao People's Democratic republic, Myanmar, Thailand, and Vietnam. In most of these places, the patients still recover since they are treated with an ACT containing the partner drug that is still effective. But in Cambodia-Thailand border, *P. falciparum* has become resistant to almost all currently available antimalarial drugs, leading to a reversion of what has been achieved in the past years[34]. The emergence of new resistances stresses the need to find new drugs and therapies to overcome this situation.

2. *Plasmodium falciparum*

Malaria parasites were first described by Alphonse Laveran in 1880[35]. He started to follow the pigment (i.e. hemozoin) of malaria described by Meckel in 1847 and later by Virchow and Frerichs[35]. It had been observed that the spleen of malaria patients contained this malaria pigment so Laveran tried to identify it in fresh unstained blood of patients. He observed it in leucocytes and red blood cells (RBC). He realized that he was seeing different intraerythrocytic forms (Figure 2), which he first thought they were outside the erythrocytes. He concluded that he had found a parasitic protozoan, which he called *Oscillaria malariae*, that later was renamed as *Haemamoeba malariae*, the present *P. falciparum*[35]. Laveran was awarded the Nobel Prize for Medicine in 1907 for his discoveries[36].

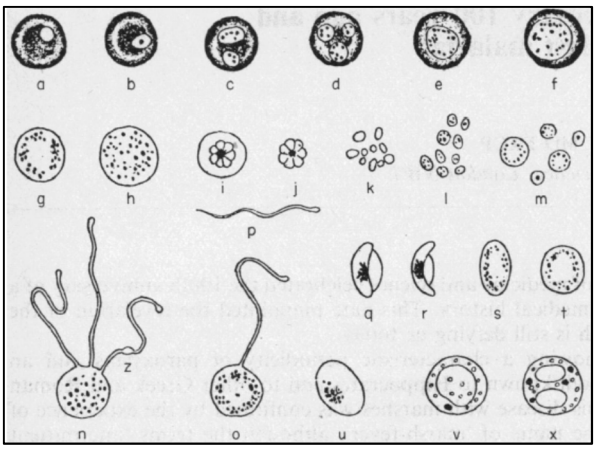


Figure 2. **Malaria parasites (*Haemamoeba malariae*) observed and drawn by Alphonse Laveran.** published in Comptes Rendus de l'Academie des Sciences, 24 October of 1881 (From Sergent & Sergent, 1929). Reproduced from Bruce-Chwatt, J Roy Soc Med, 1981[35].

It was in 1897 when William MacCallum, while studying the mosquito stages of avian malaria, found some flagellated structures that fused with non-motile bodies forming a vermicular, what we now know as ookinetes[37]. He suggested that he was observing sexual reproduction that should correlate in mammals and defined that flagellated forms were male gametes and non-motile forms corresponded to female gametes, being the vermicule the zygote[38]. Ronald Ross, during his time in India, discovered that avian malaria parasite *Plasmodium relictum* was transmitted by culicine mosquitoes and suggested that the same could be true for the human malaria parasite. However, it was not until 1898, when he moved to Sierra Leone, that he proved that human malaria parasites were certainly transmitted by anopheline mosquitoes[36].

In 1934 chloroquine (CQ) was discovered by the german scientist Hans Andersag, but only in 1943 was established as an effective and safe antimalarial. Three years after the CQ discovery, the insecticidal properties of Dichloro-diphenyl-trichloroethane (DTT) were observed by Paul Muller, who received a Nobel Prize in 1948. This finding pushed the WHO (created in 1948) to launch a malaria eradication campaign. It was so effective that between 1955 and 1972 many countries were certified as malaria-free by this organization. Within

this period, the first cases of malaria resistance against CQ were reported[25]. Between 1967 and 1981 the People's Republic of China initiated a program to find new drugs for malaria. This project contributed to the discovery of artemisinin and derivatives, for which Youyou Tu won a Nobel Prize in 2015[29]. Nowadays ACT is the recommended first line treatments.

The sequencing of *P. falciparum* 3D7 clone genome in 2002[39,40] and the later completion of other *Plasmodium* species sequencing[41,42] have opened the door to new opportunities for further studies leading to the discovery of new drugs and vaccines to fight malaria[43].

2.1. *P. falciparum* life cycle

P. falciparum has a complex life cycle that includes important stages in two different hosts: a mosquito and a human. The transmission between the human and the mosquito host also involves the transition between asexual and sexual forms of the parasites. *P. falciparum* life cycle starts when an infected female *Anopheles* mosquito inoculates sporozoites into the human host during a blood meal. The parasites migrate through the skin reaching the circulation and then the liver. In the liver, the sporozoites invade a hepatocyte and, after development inside the cell, a hepatic schizont is formed. After approximately a week the schizont bursts and thousands of merozoites are released into the blood circulation. These pre-erythrocytic merozoites invade RBCs and undergo repeated asexual multiplication cycles[44]. A small percentage of these parasites differentiate to sexual male and female gametocytes. These sexual forms will be uptaken by another female *Anopheles* mosquito when feeding on an infected human[45]. Gametocytes will go through fertilization and maturation in the mosquito midgut, forming an infective ookinete that migrates through the midgut wall to the hemocele evolving to an oocyst. Inside oocysts thousands of sporozoites are formed. After sporozoite maturation the oocyst breaks and sporozoites travel to the mosquito salivary glands where they attach getting ready to be injected into a human host again (Figure 3)[44,46].

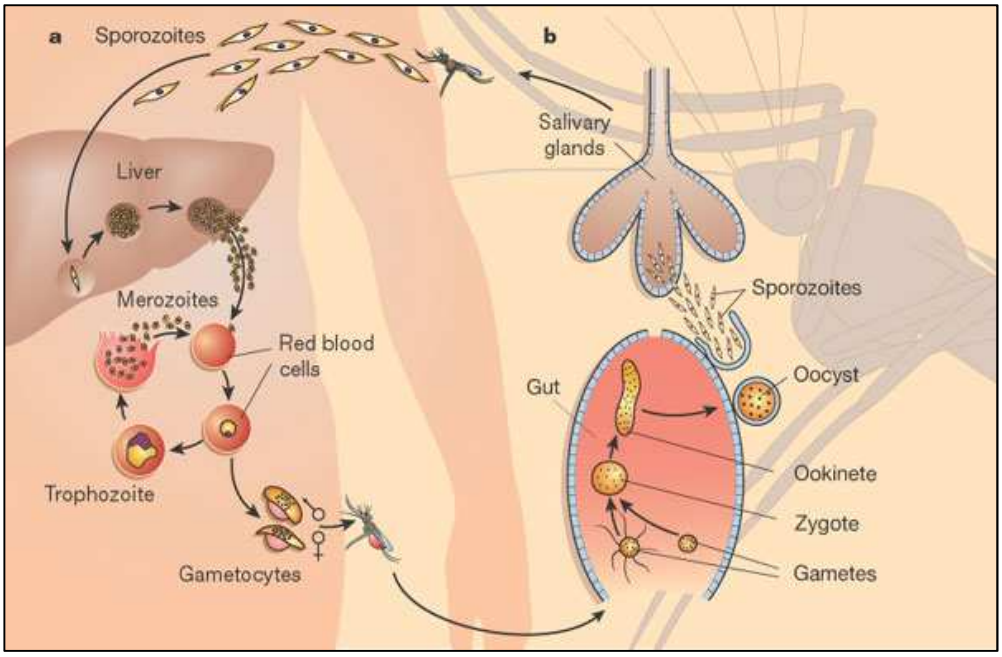


Figure 3. Life cycle of the parasite *Plasmodium falciparum*. (A) When a parasite-infected female mosquito feeds on a human, it injects sporozoites. These sporozoites travel to the liver, where they develop producing merozoites which will invade red blood cells. A subset of infected red blood cells develops into female and male gametocytes. (B) When a mosquito bites the infected human, it takes up blood containing gametocytes, which develop into gametes in the mosquito midgut. The gametes fuse in the insect's gut to form a zygote. The zygote in turn develops into the ookinete, which crosses the wall of the mosquito gut and forms a sporozoite-filled oocyst. When the oocyst bursts, mature sporozoites migrate to the mosquito's salivary glands, and the process begins again. Reproduced from Wirth, D.F., Nature, 2002[44].

2.1.1. From the mosquito to the liver

A female infected mosquito inoculates approximately 20 to 200 sporozoites into the human skin after a bite. The injected saliva also contains anticoagulants and vasodilators to facilitate blood meal and digestion. Most of the sporozoites are injected into the dermis and not directly to the blood circulation[47,48]. A great proportion of these sporozoites show a robust forward gliding motility in an apparently random direction at an average speed of 1-2 μm/s[49]. Sporozoites move until they find a blood vessel and reach the circulatory system. However, some sporozoites remain in the dermis retained by anti-sporozoite antibodies[48]. Some sporozoites can also reach the lymphatic vessels and be baited in the lymph nodes where most are phagocytized. Interestingly, a few of them are able to differentiate into exoerythrocytic stages in the lymph nodes[46,49].

Once sporozoites reach the circulatory system, they travel rapidly through the blood vessels and arrive to the liver sinusoids (Figure 4). Sporozoites target the liver very specifically and they can be found in the hepatocytes just a few minutes after entering the blood circulation[50]. This suggests the presence of a specific interaction between parasite

and host cell molecules. Sporozoites liver targeting is mediated by the binding of the Circumsporozoite Protein (CSP) to the Heparan Sulphate Proteoglycans (HSPG)[51].

Another important sporozoite surface protein, the Thrombospondin Related Anonymous Protein (TRAP), is also involved in target cell identification[52]. CSP and TRAP interplay with hepatocytes and, while CSP has an active role in the attachment, TRAP plays a role during internalization[53]. These two proteins recognize distinct cell-type specific surface proteoglycans, not only on hepatocytes but also on Kupffer cells (KCs) and on stellate cells, two cell subpopulations in the liver. It has been proposed that the proteoglycans from the Extracellular Matrix (ECM) of stellate cells are the principal candidates for sporozoite targeting in the liver sinusoid[51,54].

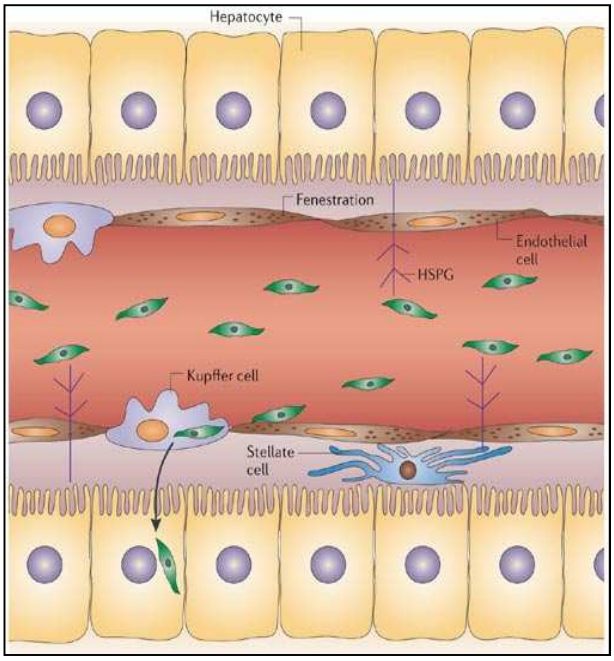


Figure 4. **Sporozoite arrest in the liver.** Once the sporozoites (green) reach the liver sinusoids, they glide over the endothelium and interact with (HSPGs) from hepatocyte and stellate cells. They then cross the sinusoidal layer possibly through Kupffer Cells (KC). Reproduced from Prudêncio *et al.*, Nat.Rev.Microbiol, 2006[46].

Sporozoites invade hepatocytes and then initiate the intrahepatocytic cycle (Figure 4). They cross the liver sinusoidal barrier targeting KC or endothelial cells[55]. In the liver they then migrate traversing various hepatocytes, finally reaching one for definitive invasion, forming the parasitophorous vacuole (PV) and starting replication[56]. Sporozoites use gliding motility to traverse liver (and dermis) cells. However, the mechanism that triggers the commitment to the invasion of the target hepatocyte is not clear[57]. *Plasmodium* parasites, as other Apicomplexa, use the so-called gliding motility for migration through tissues and host-cell invasion. Is a form of substrate-dependent locomotion, in which myosin is anchored to an internal membrane complex and linked to actin-associated adhesins, translocating it and leading the parasite forward. The actin – myosin motor that drives this type of motility is called glideosome[58].

Six to eight days after sporozoite invasion of the hepatocytes, thousands of new exoerythrocytic merozoites are generated[1]. Data describing this specific stage of the

parasite cycle is limited. The sporozoite usually lies close to the nucleus of the infected hepatocyte along the intrahepatocytic development. During the first 24 hours after invasion the sporozoite-specific organelles are dismantled. The parasite then goes to an schizogonic stage, growing to a larger size than the original hepatocyte and generating thousands of merozoites that will infect RBCs[59]. The last step of the liver stage consists in exiting the hepatocyte and reaching the bloodstream again, in a process also not very well understood. It has been reported that merozoites are released by the formation of merozoites-filled vesicles called merosomes. Merosomes plasma membrane has its origin on the hepatocyte cell membrane, making more difficult to be recognized by Dendritic or Kupffer cells and contributing to a final safe passage to the blood circulation[60].

2.1.2. Red blood cell invasion and sexual commitment

After being released from the merosome, merozoites recognize, attach, and enter the erythrocytes avoiding exposure to host antibodies[61]. This starts the asexual cycle of the parasite. The asexual blood stages of *Plasmodium* parasites are responsible for the symptoms of the disease. Merozoites, with an approximately size of 1.6 µm long and 1 µm wide, enclose all the machinery needed to eggress from the merosome and, in the case of intraerythrocytic merozoites, from the RBC. They also contain mediators to attach to a new erythrocyte, invade it, and restart multiplication (Figure 3). Merozoites have a polarized morphology and specialized apical organelles – rophtries, micronemes and dense granules – to effectively enter the RBC[62].

The first step during RBC invasion is the attachment of the merozoite to the erythrocyte through weak interactions. Different merozoite surface proteins (MSP) have been related to this initial process[63,64]. After this, the merozoite reorientates connecting the anterior apex towards the RBC membrane[61]. The next stage of the invasion process consists in the formation of a tight junction between the merozoite and the erythrocyte. This is a critical step, since it provides a firm support for the merozoite to drive into the RBC[65,66]. In the last step, proteins from the rophtries involved in the induction of the PV are injected in the RBC as the parasite glides into it. The PV is formed from the erythrocyte membrane forming the tight junction while the parasite enters the RBC[67,68] (Figure 5).

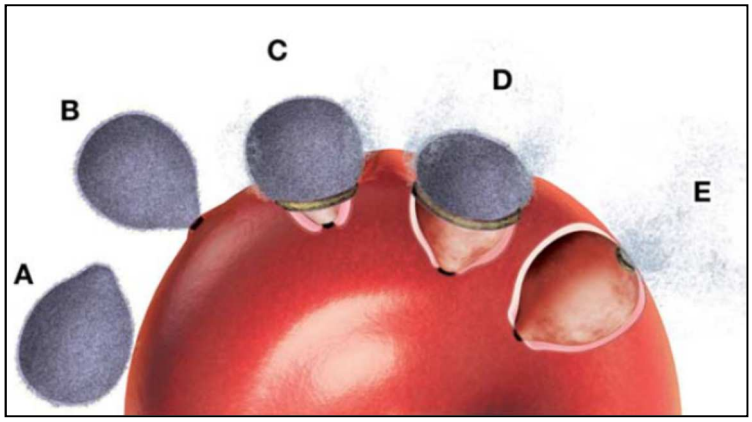


Figure 5. **Merozoite invasion of erythrocytes.** Invasion involves an initial "long distance" recognition of surface receptors (A) followed by a reorientation process whereby these low - affinity contacts are maintained. Once the apical end is adjacent to the erythrocyte surface (B), a tight junction is formed involving high-affinity ligand-receptor interactions. This thight junction then moves from the apical to posterior pole (C and D) by gliding motility. The surface coat is shed at the moving junction by a serine protease, or "sheddase". Upon reaching the posterior pole, adhesive proteins at the junction are also proteolytically removed (E), in a process that facilitates RBC membrane resealing. By this process, the parasite does not actually penetrate the membrane but invades it in a manner that creates a PV. Reproduced from Cowman *et al.*, Cell, 2006[61].

Once the merozoite has invaded the RBC, the asexual development requires approximately 48 hours in *P. falciparum*[69]. During this cycle the parasite undergoes three main morphological states: rings, trophozoites and schizonts (Figure 6)[70]. Through the process, the parasite reshapes the erythrocyte membrane by exporting a large number of proteins. The ring stage (0-24 hours post invasion (hpi)) is characterized by a thin discoidal or cup-shaped form. It has a thick cytoplasm edge where the principal parasite organelles can be found. The parasite feeds on a small disc at the surface of the parasite, the cytostome, and digests hemoglobin accumulating haemozoin crystals in the pigment or digestive vacuole[62]. When entering the trophozoite stage (24-36 hpi), the metabolic activity of the parasite increases and the trafficking of proteins to the RBC membrane intensifies[71–73]. The ingestion of RBC

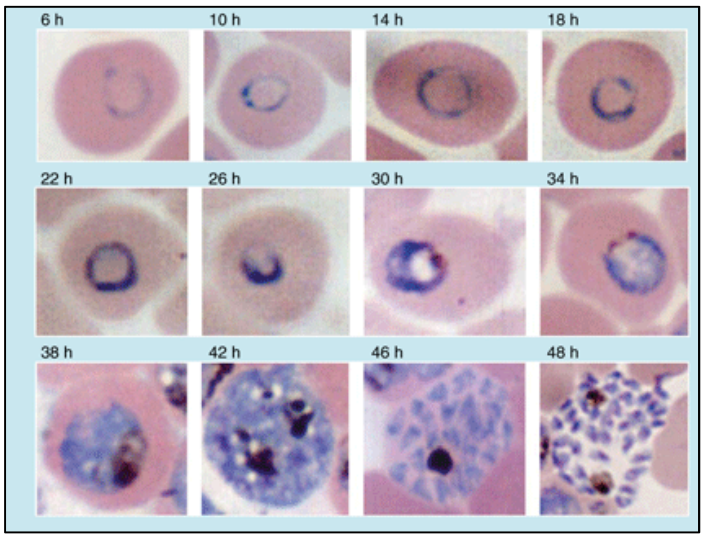


Figure 6. **Giemsa staining of intraerythrocytic blood stages of *P. falciparum* in culture**. Ring stages can be observed between 0 and 24 hours post invasion. Trophozoites occur between 24 and 36 hours and schizonts develop from 36 to 48 hours after invasion. Adapted from Radfar *et al.*, Nat Proc, 2009[69].

cytoplasm takes place until the final stages of the cycle, generating more haemozoin crystals deposited in the pigment vacuole. The schizont stage (36-48 hpi) starts after multiple nuclear division and results in the formation of about 16-32 merozoites. Each nuclei forms a merozoite that gets oriented closer to the RBC surface. The erythrocyte membrane ruptures and the merozoites egress being released into the bloodstream. They will be then ready to start the asexual cycle again invading a fresh erythrocyte[62].

A small proportion , approximately 1%[74], of the blood stage parasites differenciate into male and female sexual forms, the gametocytes, which are essential for parasite transmission to the mosquito vector. Gametocyte commitment occurs at a not yet defined point before schizont nuclear division, since merozoites derived from the same schizont become either all gametocytes or continue as asexual parasites[75]. *In vitro*, the exposure of parasites to different stress factors, such as high parasitemia or drug treatment, is correlated with an increment of commitment rate. However, how environment in natural infection triggers gametocytogenesis is unknown, although some studies indicate that commitment may be stochastic[45]. After gametocytogenesis starts, gametocyte development takes approximately 10 to 12 days. Gametocytes go through five different stages (I-V) that are distinct microscopically (Figure 7).

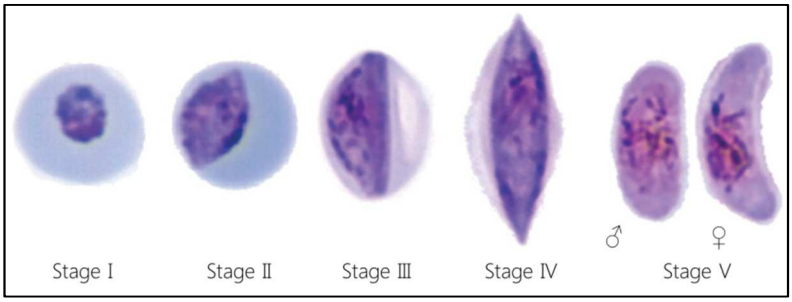


Figure 7. **The stages of gametocyte development in *P. falciparum*.** Development of a mature *P. falciparum* gametocyte occurs over 10 to 12 days and is divided into five morphologically distinct stages. Adapted from Josling *et al.*, Nat. Rev. Microbiol., 2015[45].

In *P. falciparum*, stage I gametocytes are very similar to young asexual trophozoites, while stage II start to elongate in a D shape. Stage III have slightly rounded ends whereas stage IV are thiner and have pointed ends. Stage V male and female can be distinguished from each other since female are more elongated and curved than the thicker males[45,76]. During their development, only early (stage I) and late (stage V) gametocytes are circulating in the peripheral blood whereas II-IV stage gametocytes are accumulated in the bone marrow to avoid clearance by the spleen[77]. Circulating stage V gametocytes are ready for uptake in a mosquito blood meal again.

Gametocytogenesis is an essential step in the parasite life cycle required for transmission from human to mosquito. Given the low number of asexual parasites that develop into gametocytes, sexual development is considered one of the critical bottlenecks in the parasite life cycle that can be targeted for transmission blocking therapies. The majority of antimalarials are not effective against mature gametocytes. Therefore, even if asexual parasites responsible for the malaria symptoms are killed, the gametocytes involved in transmission can remain in circulation over a month[78]. Primaquine is an approved antimalarial drug very active against mature gametocytes. However it can cause haemolytic anemia in people with glucose-6-phosphate dehydrogenase (G6PD) defficency, a highly prevalent deficit in malaria endemic areas[79,80]. New therapies affecting asexual and sexual blood parasites could greatly contribute to malaria eradication.

2.1.3. Parasite development in the mosquito vector

Stage V male and female gametocytes circulate through the peripheral blood and are taken up by a mosquito during a blood meal. Soon after ingestion, gametocytes are subjected to environmental changes such as a drop in temperature, pH increase, and the exposure to xanthurenic acid that cause the egression and maturation of gametocytes into male and female gametes in the mosquito midgut[81]. Male gametocytes undergo three different rounds of DNA replication producing eight flagellated sexually competent cells. The release of these 8 gametes is called exflagellation. After this process, fertilization takes place and female and male gametes fuse to form a diploid zygote, a proces that can occur within an hour.

After male and female nuclei fuse, the nucleus and microtubules elongate and the cell grows through a polarization process. This is essential for ookinete formation. Ookinetes are elongated and motile cells with specialized secretory organelles, the micronemes, that contain proteins involved in motility, cell traversal, and invasion. Ookinetes use gliding motility to leave the blood meal and penetrate through the peritrophic matrix (PM) that encloses the blood meal and acts as a physical barrier[82] (Figure 8). Ookinete maturation is required for chitinase activation, an enzyme needed to cross the PM. CDPK3 (Calcium dependent protein kinase 3) and CTRP (Circumsporozoite and TRAP related protein) are proteins involved in ookinete motility and infectivity. Their disruption generates mutants unable to invade the mosquito midgut epithelium or to produce oocysts[83,84]. After breaching the PM the ookinete penetrates the apical end of the midgut epithelium (Figure 8)[85]. Ookinete migration through the epithelial cell activates cell changes from a motile form to a sessile condition, starting transformation to oocyst[86].

Oocyst development is the only extracellular stage of the parasite life cycle where there is parasite multiplication. The parasite goes through a dozen nuclear divisions becoming a multinucleated parasite form, since cytokinesis does not take place in paralel with karyokinesis. The cytoplasm starts partitioning into sporoblasts finally resulting in a mature oocyst full of haploid sporozoites. During oocyst growth, a capsule that surrounds the forming sporozoites is made. This capsule is a bilayered structure with a wall derived from the mosquito basal lamina and an inner membrane covered with a dense coat of CSP, a protein that also covers oocyst and salivary gland sporozoite surfaces[87,88]. Oocyst development is fully completed after approximately 14 days and thousands of sporozoites bud asynchronously from oocysts's sporoblasts. Mature sporozoites are released in the hemocoel (Figure 8) and circulate through mosquito tissues. When sporozoites pass by the basal lamina of salivary glands they recognize mosquito receptors that allow salivary gland adhesion. Sporozoites cross this basal lamina and invade the salivary gland, being TRAP one of the key proteins for invasion and attachment[89]. After this process, the sporozoite finishes its development in the mosquito host and it is ready to be injected in the human dermis again[90].

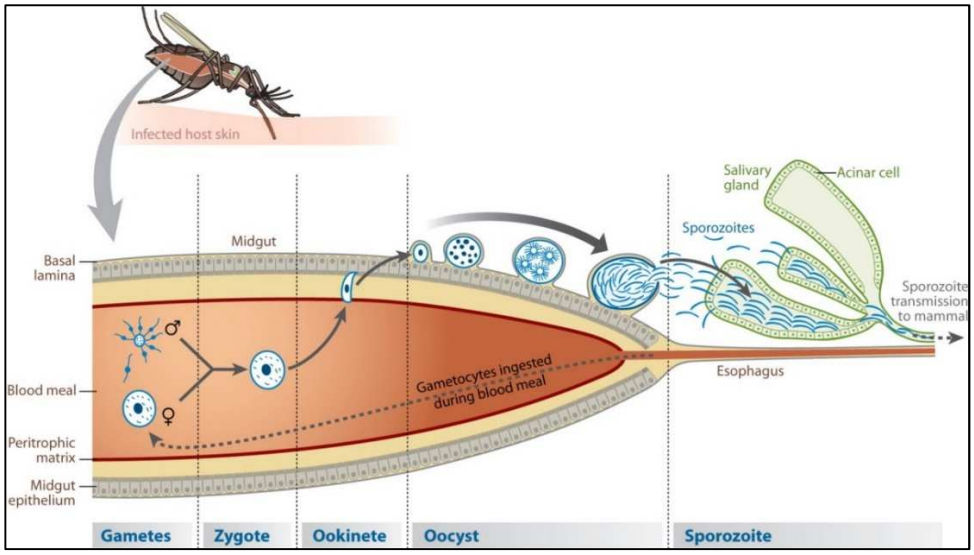


Figure 8. **Malaria parasite development in the mosquito vector.** *Plasmodium* gametocytes are ingested by *Anopheles* mosquitoes during a blood meal. Male and female gametes emerge from gametocytes and fertilization occurs to form the zygote. The zygote undergoes meiosis and transforms into the ookinete. Ookinetes first breach the peritrophic matrix surrounding the blood meal, followed by entry into the apical end of the midgut epithelium and traversal of several midgut epithelial cells. Ookinetes exit the midgut epithelium through the basal end and transform into sessile oocysts. The oocyst undergoes extensive growth and completes its development within 10–14 days, resulting in the production of thousands of sporozoites. Sporozoites egress from the oocyst and travel in the hemolymph before attaching to the basal side of the salivary gland acinar cells. Sporozoites traverse the acinar cells and enter the ducts of the salivary glands. The salivary gland sporozoites then wait to be transmitted to a mammalian host during the next blood meal. Adapted from Aly *et al.*, Annu. Rev. Microbiol, 2009[86].

3. Glycobiology

3.1. General aspects

Glycobiology is a broad field that comprises the study of any molecule containing carbohydrates as part of its structure. Therefore, it includes the study of carbohydrate metabolism and glycoconjugate (glycoprotein and glycolipid) structures. It has become a well – established area of study in the last two decades.

Carbohydrates (often called sugars) are molecules composed of carbon (C), hydrogen (H) and oxigen (O). Monosaccharides are the most basic examples of carbohydrates and are the basic building blocks from which all bigger carbohydrate chains are made. Monosaccharides are classified according to the number of carbon atoms in a molecule. Thus, pentoses are the monosaccharides that have 5 carbons, hexoses the ones that contain 6 carbons, and so on. Two monosaccharides with the same molecular formula may be different esterioisomers, due the chirality or asymmetry of their carbon atoms. Free monosacharides can exist in open or in ring form, but when forming part of a polysaccharide they are in the ring form. Glucose (Glc), N-acetylglucosamine (GlcNAc), galactose (Gal), N-acetylgalactosamine (GalNAc), mannose (Man) and fucose (Fuc) are some of the most common monosachharides (Figure 9). Linkage of different monosaccharides form the so-called oligosaccharides or polysaccharides. Monosaccharide residues within a carbohydrate may contain non-carbohydrate substituent moieties, such as phosphate, sulfate or other functional groups. Monosaccharides can be covalently bound through different linkages (α or β) to any of the several C positions on other monosaccharides in a carbohydrate chain. Therefore, polysaccharide chains present an enormous number of possible combinations. Nevertheless, not all the possible combinations have been described in nature[91]. Carbohydrates are important as energetic source, but they also play roles in other important biological processes including cell–cell adhesion, cell–matrix adhesion, cell–signaling, protein folding quality control and host–pathogen interactions, among others.

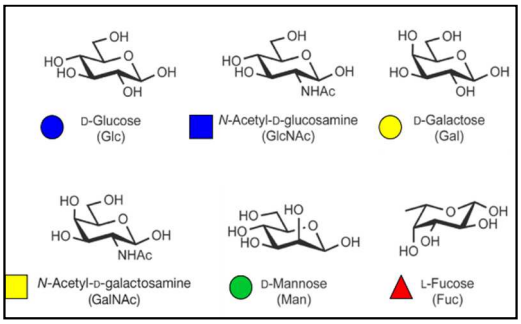


Figure 9. Ring and symbolic representations of common monosaccharides. Each symbol represents a specific monosaccharide. Glc, D-glucose; GlcNAc, N-acetyl-D-glucosamine; Gal, D-galactose; GalNAc, N-acetyl-D-galactosamine; Man, D-mannose; Fuc, L-fucose.

Glycoconjugates are defined as compounds that contain a saccharide (mono or polysaccharide) covalently linked to another type of molecules like proteins or lipids, forming glycoproteins or proteoglycans and glycolipids, respectively. In eukaryotic cells there are different types of glycans covalently attached to proteins or lipids, among which we find *N*–linked oligosaccharides, linked to asparagine (Asn) amino acids; *O*–linked oligosaccharides, associated to serine (Ser) or threonine (Thr) residues; glycosylphosphatidylinositol (GPI) molecules that anchor proteins in cell surfaces and consist of a glycan chain linked to phosphatidylinositol groups and; other types of glycosylation, as shown in Figure 10[92].

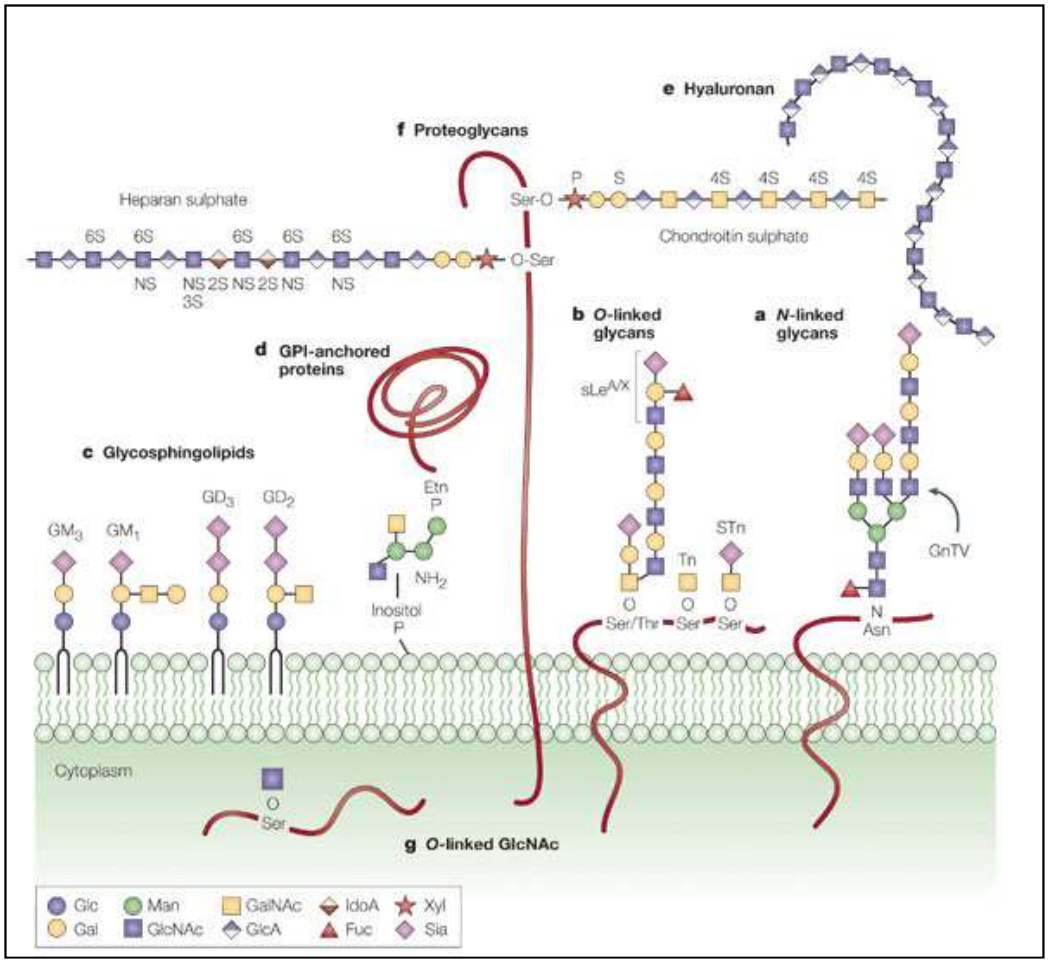


Figure 10. **Glycans present in higher eukaryotes. a)** *N*-linked glycans on glycoproteins are covalently bound to Asn. **b)** *O*-linked glycans are found covalently linked to Ser or Thr residues on glycoproteins and mucins. **c)** Glycosphingolipids consist on the lipid ceramide linked to one or more sugars. **d)** GPI-linked proteins are anchored to the membrane by a glycan covalently linked to phosphatidylinositol. **e)** Glycosaminoglycans can occur as free chains (as in hyaluronan case) or as covalent complexes with proteoglycan core proteins. **f)** Proteoglycan. **g)** Cytoplasmic or nuclear proteins contain *O*-GlcNAc. Glc, glucose; Gal, galactose; Man, mannose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; Fuc, fucose; Xyl, xylose; Sia, sialic acid. Adapted from Fuster *et al.*, Nat. Rev. Cancer, 2005[92].

The biological roles of glycans are very diverse. Some of them are essential for development, growth or survival of an organism, while alterations on others produce different syndromes or diseases[92,93]. Still, in many cases the function that they play at a cellular or whole organism level it is not known.

Protozoan parasites synthesize several glycoconjugates and glycan binding proteins (or lectins) for protection and to interact and respond to changes in their environment. Host–parasite interactions are crucial for parasite survival and many of these interactions take place through carbohydrate recognition[94]. Glycoconjugates coat parasite surfaces with carbohydrates that are generally different from the host ones. This glycocalyx, critical for parasite virulence and survival, masks proteins from the host immune system protecting the parasite against the host defenses[95]. The glycome is the complete set of glycosylations that an organism or cell produces at a certain time point. A parasite glycome description is an enormous challenge which, together with the determination of the biological significance of the different glycosylations, may contribute to the description of host parasite interactions in parasitic diseases[94]. Hence, the study of parasite glycobiology is a promising field that may lead to the identification of novel parasiticidal drugs for carbohydrate related targets or to the discovery of new vaccine candidates or diagnostic tools.

P. falciparum has different glycosylated proteins and lectins that play important roles in virulence of the parasite, attachment and invasion of the host cell. Among these, we find CSP, TRAP, erythrocyte binding antigen 175 (EBA 175) and *Pf*EMP1 (*P. falciparum* erythrocyte membrane protein 1)[96]. Some of the carbohydrate containing receptors on host cells that bind to parasite proteins comprise Glycophorin A (GpA), Glycosaminoglycans (GAGs), Chondroitin Sulfate A (CSA) and Hyaluronic Acid (HA). Describing these protein-glycan interactions could open new ways to target them and inhibit parasite development[97].

3.2. Glycosylation precursors and glycosyltransferases

Sugar nucleotides are activated forms of monosaccharides. These sugar nucleotide precursors are monosaccharides covalently linked to nucleotides and are used by specific glycosyltransferases as donors for glycosylation reactions. The most common sugar nucleotide donors present in eukaryotic cells are listed in Table 1. In general, two main metabolic routes for the biosynthesis of sugar nucleotides have been described. They can be synthesized by a salvage pathway that involves phosphorylation by a kinase and a further

pyrophosphorylase activation, or by a *de novo* route that consists in the bioconversion of an existing sugar or sugar nucleotide[91].

Table 1. **Activated sugar donors.** Adapted from Varki *et al.*, Essentials of glycobiology, 2009[91]

Sugar	Activated form
Glucose (Glc)	] UDP-sugar (Uridine diphosphate – sugar)
Galactose (Gal)	
N – acetylcglucosamine (GlcNAc)	
Glucuronic acid (GlcA)	
Xylose (Xyl)	
Mannose (Man)	] GDP-Sugar (Guanosine diphosphate – sugar)
Fucose (Fuc)	
Sialic acid (Sia)	] CMP-Sia (Cytidine monophosphate - Sia)

Glycosylation reactions consist in the transfer of the monosaccharide component of a sugar nucleotide to a specific acceptor. Glycosyltransferases are the enzymes responsible for the biosynthesis of glycans, by adding monosaccharides to specific positions on specific molecules[98]. Acceptors can be oligosaccharides, proteins (specific aminoacids), lipids or even DNA. The majority of the glycosyltransferase enzymes are located in the Endoplasmic Reticulum (ER) – Golgi pathway, since most of the glycosylation reactions take place in the cellular secretory pathway. Glycosyltransferases act sequentially and the product of a reaction acts as acceptor for the following one, generating linear and/or branched polysaccharides[91].

Identification and quantification of sugar nucleotides in the different *P. falciparum* stages could contribute to outline the parasite glycosylation profile and its glycome, since these precursors would be substrates for the parasite glycosyltransferases[94]. A brief explanation of the different sugar nucleotides present, or predicted to be present, in *P. falciparum* when this work was started is represented in Figure 11[99].

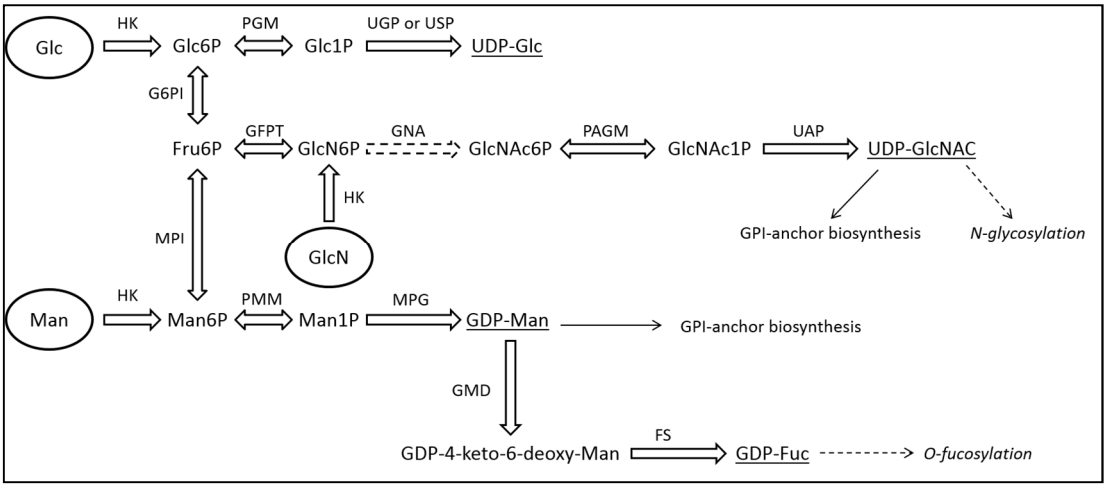


Figure 11. Sugar nucleotide biosynthesis pathways in *Plasmodium falciparum*. Activated sugars, used, or predicted to be used for glycoconjugate biosynthesis, are underlined. Carbohydrates, taken up from the medium or salvaged, are circled. Sugar nucleotide donor fates are indicated and they are in italics when glycosylations have not been proved to be present in the parasite. Discontinuous arrow depicts a predicted enzymatic activity that can not be identified in *P. falciparum* genome. HK, Hexokinase, EC 2.7.1.1; PGM, Phosphoglucomutase, EC 5.4.2.2; UGP, UTP–glucose–1–phosphate urydiltransferase EC 2.7.7.9; USP, UDP–sugar pyrophosphorylase, EC 2.7.7.64; G6PI, Glucose-6-phosphate isomerase; GFPT, Glucosamine–fructose–6–phosphate aminotransferase, EC 2.6.1.16; GNA, Glucosamine–phosphate N–acetyltransferase, EC 2.3.1.4; PAGM, Phosphoacetylglucosamine mutase, EC 5.4.2.3; UAP, UDP–N–acetylglucosamine pyrophosphorylase, EC 2.7.7.23; MPI, Mannose–6–phosphate isomerase, EC 5.3.1.8; PMM, Phosphomannomutase, EC 5.4.2.8; MPG, Mannose–1–Phosphate guanyltransferase, EC2.7.7.13; GMD, GDP–mannose 4,6–dehydratase, EC 4.2.1.47; FS, GDP– L– fucose synthase, EC 1.1.1.271. Glc, Glucose; Glc6P, Glucose-6-phosphate; Glc1P, Glucose-1-phosphate; UDP-Glc, UDP-Glucose; Fru6P, Fructose-6-phosphate; GlcN, Glucosamine; GlcN6P, Glucosamine-6-phosphate; GlcNAc6P, N-acetylglucosamine-6-phosphate; GlcNAc1P, N-acetylglucosamine-1-phosphate; UDP-GlcNAc, UDP-N-acetylglucosamine; Man, Mannose; Man6P, Mannose-6-phosphate; Man1P, Mannose-1-phosphate; GDP-Man, GDP-Mannose; GDP-Fuc, GDP-Fucose Adapted from Cova *et al.* Malar. J, 2015[99].

3.2.1. UDP–Glucose (UDP-Glc):

Considering the conserved enzymatic activities in *P. falciparum* genome, the synthesis of UDP–Glc from Glc-6-P is catalyzed by phosphoglucomutase (PGM) followed by UTP–glucose–1–P uridyltransferase (UGP). The fate of *P. falciparum* UDP-Glc has not been studied in depth. The first evidence of UDP-Glc incorporation was demonstrated by Couto *et al.* when the sugar nucleotide was used as a donor for a glycosylation reaction with two different ceramides[100]. Another possible function for this sugar nucleotide is the addition of a glucose residue on *O*–fucosylated Thrombospondin type I repeat (TSR) domains[101,102].

3.2.2. UDP–N–acetylglucosamine (UDP-GlcNAc):

UDP–GlcNAc is the sugar donor for all GlcNAc transferases. In *P. falciparum* two main ways of synthesizing UDP-GlcNAc have been described. The *de novo* pathway consists in the interconversion of Fru–6P to UDP–GlcNAc by the action of different enzymes, via the

so-called amino sugar pathway (Figure 11). All of them, except glucosamine–6-phosphate N–acetyltransferase (GNA), have been identified in the parasite genome. The salvage pathway for UDP-GlcNAc has been demonstrated and, through a hexokinase activity (HK), GlcN is salvaged and phosphorylated to GlcN – 6P. After the phosphorylation, GlcN – 6P follows the same route as in the *de novo* pathway[99,103]. In *P. falciparum*, UDP–GlcNAc is the sugar nucleotide donor in the GPI-anchor biosynthesis, predicted to be essential for parasite survival and infectivity. This sugar nucleotide is also used by *Alg* (Asparagine linked glycosylation) genes, involved in the *N*-glycosylation of *Plasmodium* parasites[104,105].

3.2.3. GDP–mannose (GDP-Man):

GDP-Man is the activated form of mannose. It can be synthesized by salvaging Man or via a *de novo* route, from Fru-6P (Figure 11). Both biosynthetic pathways are conserved in *P. falciparum*[106] genome. The salvage pathway requires a phosphorylation of a Man residue to Man-6P by a hexokinase and then follows the same route as the *de novo* pathway. The presence of the GDP-Man salvage pathway was demonstrated through the incorporation of [^{3}H]-Man into GPI-anchors in the blood stages of the parasite[103,107]. Man residues are present in parasite GPI-anchors[108], but remarkably mannose is not present in *P. falciparum N*–glycans[104,105]. This monosaccharide is also required for the *C*-mannosylation of tryptophan residues through a carbon-carbon bond, a modification recently described in *Plasmodium* sporozoites[102].

3.2.4. GDP–fucose (GDP-Fuc):

The activated form of fucose is the sugar nucleotide GDP-Fuc. The biosynthetic *de novo* pathway of this sugar nucleotide consists in the bioconversion of GDP-Man to GDP-Fuc through the actions of two different enzymes: a GDP-mannose 4,6-dehydratase (GMD) and a GDP-L-fucose synthase (FS). Homologues of this synthetic route are conserved in *P. falciparum* genome as well as in other *Plasmodium* species[106]. However, obvious candidates for the salvage pathway are absent in the parasite genome. The conservation of a protein *O*-fucosyltransferase 2 (PoFUT2) in the parasite genome suggests the usage of this sugar nucleotide in *P. falciparum*. Disruption in GDP-Fuc *de novo* biosynthetic pathway in *Trypanosoma brucei* resulted in growth arrest of the parasite, showing the importance of this precursor in other protozoan parasites[109].

3.3. Types of glycosylation in *P. falciparum*

In this section, we summarize the predicted glycosylations present in the malaria parasite.

3.3.1. GPI-anchors

GPI-anchors are glycolipid moieties that usually anchor proteins to the surface of cells. GPI-anchors are assembled through a sequential multistep pathway on a phosphatidylinositol lipid in the ER[110]. After the synthesis, GPIs can be covalently attached to the carboxyl terminus of proteins. The structure of the GPI-anchor core in higher eukaryotes consists of a phospahtidylinositol, a glycan generally comprising one glucosamine and three mannose residues, and a terminal phosphoethanolamine[110]. The *P. falciparum* GPI-anchor structure has been described and its glycan core sequence may have an extra Man residue, being (Manα1-2)Manα1-2Manα1-6Manα1-4GlcN (Figure 12)[107,108].

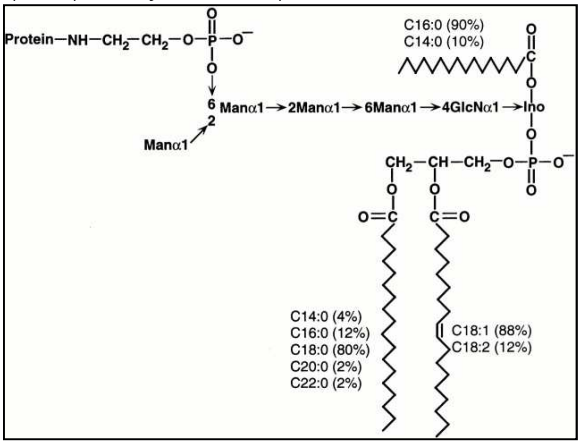


Figure 12. Proposed structure of *P. falciparum* GPI-anchors. The fatty acids and their molar proportions are indicated. Reproduced from Naik *et al.*, J Exp Med, 2000[108].

GPI-anchor synthesis is a complex process that starts in the cytoplasmic face of the ER and ends in the ER lumen (Figure 13)[99]. The first step of the process consists in the transfer of a GlcNAc residue from a UDP-GlcNAc to Phosphatidylinositol (PI) to give a GlcNAc-PI[111]. The responsible for this step is the PIG-A enzyme of which a homolog is found in *P. falciparum*[112]. After some steps, the GPI-anchor intermediate flips to the luminal face of the ER membrane. The next steps in the process consist in the addition of the different Man residues. They are added by four different GPI mannosyltransferases and using Dolichol-phosphate-mannose (Dol-P-Man) as sugar nucleotide donor. GDP-Man is required for the synthesis of Dol-P-Man. Genes for the addition of the first three Man can also be identified in the parasite genome, but no candidate for the fourth gene has been found[112]. The attachment of the proteins to *P. falciparum* GPI-anchor structure happens via the Ethanolamine – Phospahte (EtN-P) group present in the third Man residue[113].

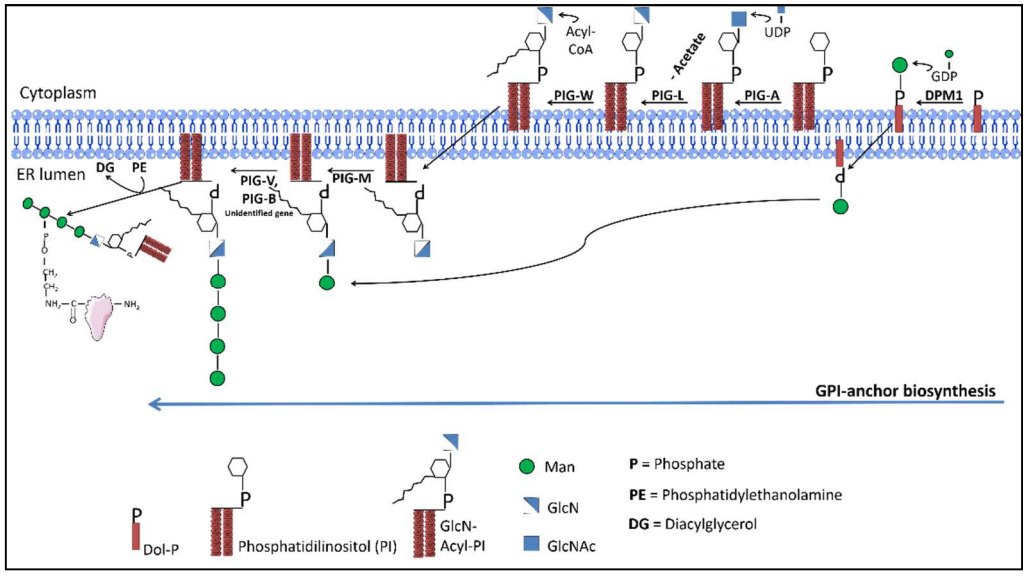


Figure 13. Biosynthetic scheme for GPI-anchor biosynthesis in *P. falciparum*. DPM1, Dolichol-phosphate mannosyltransferase polypeptide 1; PIG-A, phosphatidylinositol N-acetylglucosaminyl-transferase; PIG-L, N-acetylglucosaminyl phosphatidylinositol deacetylase; PIG-W, Inositol acyltransferase; PIG-M, GPI mannosyltransferase I; PIG-V, GPI mannosyltransferase II; PIG-B, GPI mannosyltransferase III; Unidentified gene, GPI mannosyltransferase IV. Man, Mannose; GlcN, Glucosamine, GlcNAc, N-acetylglucosamine. Adapted from Cova *et al.*, Malar J, 2015[99].

The proteins to be attached to the GPI-structure present a C-terminal signal peptide required for GPI-anchor replacement. The loss of this signal in GPI-anchored proteins, such as CSP, blocks GPI attachment and impedes the surface localization of the protein[114].

GPI-anchors are the main glycoconjugates described in the blood stages of *P. falciparum*[103,115] and anchor critical proteins in the parasite plasma membrane[115]. Different essential parasite proteins, such as MSP1 or MSP2 are GPI-anchored to the merozoite surface, strongly suggesting that the disruption of the GPI biosynthetic pathway in the parasite would inhibit parasite growth[115]. GPI-anchors are also present in gametocytes, ookinete and sporozoite parasite forms[115]. Proteins p25 and p28, two of the most important proteins in the surface of ookinetes, are anchored to the parasite membrane via a GPI-anchor[116]. In the sporozoite stage, CSP is also attached to the surface by a GPI-structure[117]. GPI-anchors have also been described as toxins able to induce cytokine expression in macrophages, being partially responsible for malaria associated pathogenesis[108,118]. Schofield *et al.* synthesized a synthetic malarial GPI-glycan showing that it was immunogenic in rodents, making it a candidate for an anti-toxin vaccine[119]. GPI's from other protozoan parasites as *T. brucei* and *T. cruzi* have similar pro-inflammatory properties[120,121].

3.3.2. *N*-glycosylation

The production of *N*–glycans requires a lipid-linked oligosaccharide (LLO) precursor that is covalently attached to a protein Asn residue through a *N*–glycosidic bond. LLO synthesis begins in the cytoplasmic face of the ER when a GlcNAc residue from UDP-GlcNAc is transferred to the lipid–like precursor Dolichol–P (Dol-P), forming dolichol pyrophosphate-N-Acetylglucosamine (Dol-P-P-GlcNAc). Once synthesized the LLO precursor, the olygosaccharyltransferase (OST) complex is responsible for its transferring *en bloc* to the protein in the ER[91].

N-glycosylation in *Plasmodium* was a controversial issue during the final decade of last century since different studies showed diverging results. Dieckmann *et al.* showed that *P. falciparum* asexual blood stages had little or no capacity of producing *N*-glycans[122]. Kimura *et al*. described that *N*-glycans represented approximately 70% of carbohydrate during blood stages[123], whereas Gowda *et al.* stated that *P. falciparum* had *N*-linked carbohydrates but its presence was minor in relation with GPI-anchor structures[103]. The availability of *P. falciparum* genome[39] enabled the identification of genes involved in the *N*-glycosylation pathway in the parasite. Bioinformatic analysis showed that *Apicomplexans* lack some of the *Alg* genes, the glycosyltransferases responsible for *N*-glycan synthesis. *P. falciparum* genome has conserved genes encoding for Alg7, Alg13 and Alg14 as well as Stt3, the catalytic subunit of the OST complex responsible for the transfer of the *N*-glycan precursor to the protein sequon (Figure 14)[99,105,124]. In 2010 Bushkin *et al.*[104] showed indirect evidences of the presence of short *N*-glycan carbohydrates in *P. falciparum* blood stages[104].

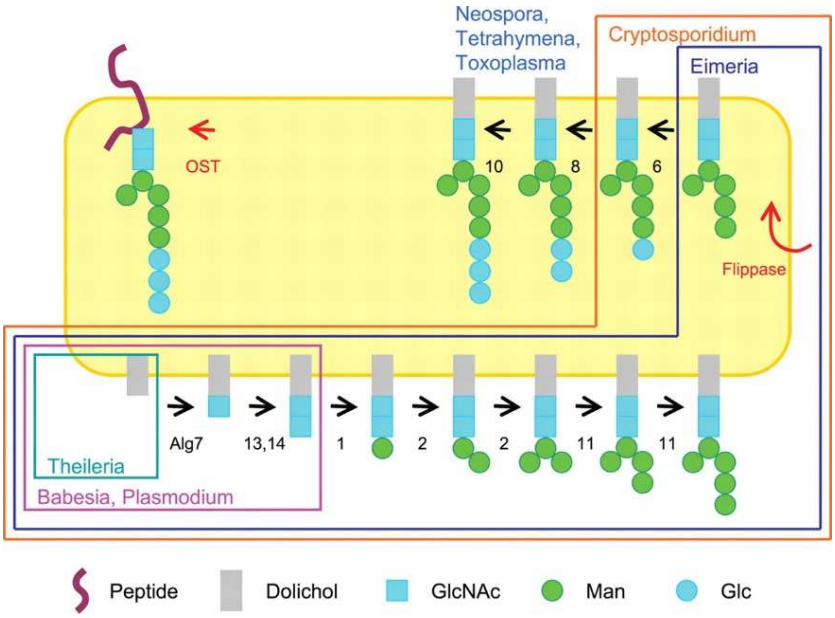


Figure 14. ***N*-glycan precursor in *P. falciparum* and other apicomplexan parasites.** *P. falciparum* genome lacks Alg enzymes adding Man and Glc to the N-glycan LLO precursors. The precursor is predicted to contain just two GlcNAc sugars ($GlcNAc_2$). Reproduced from Bushkin *et al.*, Eukaryotic cell, 2010[104].

In higher eukaryotes, protein *N*–glycosylation plays a crucial role in protein quality control, since this glycosylation is important for protein folding[125,126]. It is also important for protein stability, solubility and could be relevant for antigenicity and the antibody recognition of modified glycoproteins. However, *N*-glycans have not yet been completely described in *Plasmodium* species. The biological significance of the short length of *N*-glycans in *P. falciparum* has not been elucidated yet. It may be important to ascertain whether this short saccharide chains trigger a specific immune response in the human host, since these glycans are not expected to be present in the human glycome[99].

3.3.3. *C*-mannosylation

C-mannosylation consists in the addition of a Man residue into tryptophan amino acid residues. The precursor of this reaction is a Dol-P-Man, synthesized from GDP-Man. The C-1 atom of a single mannose residue is linked to C-2 atom of the indole group of a tryptophan (W) amino acid. This is a C-C bond, different from the C-O or C-N bonds common in *O*- or *N*-glycosylations, respectively. The consensus sequence of the acceptor peptide was first defined as WXXW, where the first W residue is *C*-mannosylated[127]. However, later studies established that the consensus sequence was WXXWXXWXXC (where C corresponds with cysteine) and all the tryptophans could be glycosylated[128]. This glycosylation is usually found in proteins containing TSR domains[129–131]. Several proteins in *Plasmodium* have this domain and the modification has been recently demonstrated[102]. The enzyme responsible for this reaction is DPY19 a mannosyltransferase recently identified in *Caenhorabditis elegans*[129] and mammals[130]. A homolog of this gene can be found in all *Plasmodius spp.* genomes[99]. The biological significance of this posttranslational modification remains to be investigated.

3.3.4. *O*-glycosylation

O–linked glycosylation consists in the covalent link of a sugar to the hydroxyl (OH) group of Ser or Thr residues by an *O*-glycosidic bond. In higher eukaryotes the most abundant *O*-glycosylation type is initiated with the attachment of a GalNAc residue to the protein, that can be further extended generating glycoconjugates called mucins[91]. Nevertheless, other types of *O*-glycosylation also take place in eukaryotic cells, including *O*-fucosylation, *O*-xylosylation, *O*-mannosylation, *O*-GlcNAc, *O*-galactosylation and *O*-glucosylation[91].

Despite some controversial studies, *O*-glycosylation had never been clearly described in *P. falciparum* (besides *O*-fucosylation, see below). The first reports on *P. falciparum* *O*-glycosylation described the presence of *O*-glycosylated proteins in *P. falciparum* bloodstages[132]. After this, Dieckmann-Schuppert *et al.*[133] published an article describing the synthesis of *O*-GlcNAc by malaria parasites. However, the parasite seems to lack *O*-GalNAc glycans (mucin type-like)[133]. This is also supported by *in silico* studies indicating the absence of a GalNAc transferase activity in *Plasmodium* [134]. More recent works from Perez-Cervera *et al.*[135] showed that *P. falciparum*, as well as *Toxoplasma gondii*, had *O*-GlcNAcylated proteins. The authors were able to identify *T. gondii* *O*-GlcNAc transferase, but did not find it in *P. falciparum*[135].

3.3.4.1. *O*-fucosylation

The conservation of homolog genes for the GDP-Fuc *de novo* biosynthetic pathway in *P. falciparum* (i.e GMD and FS, Figure 11) suggests the presence of fucosylated glycans in the parasite. The *O*-fucosylation modification consists in the linkage of a Fuc from a GDP-Fuc donor to a OH group of a Ser or Thr residue. *O*-fucosylation is found on Epidermal Growth Factor (EGF) and/or on TSR domains in other organisms (Figure 15). Both EGF and TSR domains are *O*-fucosylated by PoFUT enzymes. These enzymes are able to differentiate and modify completely folded structures, suggesting that they are related with in protein folding quality control [136]. EGF *O*-fucosylation is mediated by PoFUT1, an enzyme that localizes in the ER [137]. This post-translational modification can be extended with 2 or 3 monosaccharide residues in mammals and other organisms and plays an important role in development[138]. PoFUT2 is the enzyme responsible for the *O*-fucosylation of TSR domains. It can also be extended with a glucose residue[136]

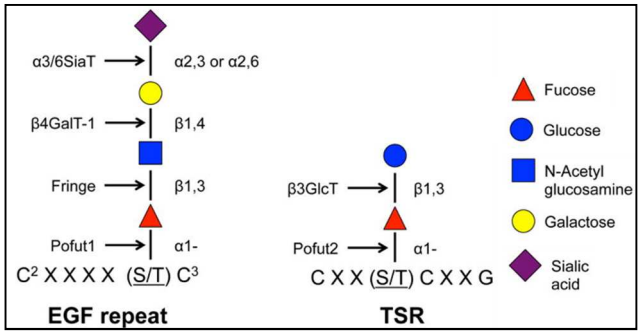


Figure 15. Two distinct *O*-fucosylation pathways. EGF repeats and TSRs are *O*-fucosylated by PoFUT1 and PoFUT2, respectively. Adapted from Vasudevan *et al.*, Glycoconjugate Journal, 2014[136].

Plasmodium species contain several TSR domain-containing proteins, that are putative acceptors of PoFUT2-mediated *O*-glycosylation. TSR domains are small (~60 aa residues) cysteine rich motifs with 3 conserved disulfide bonds (Figure 16). They were first

described in the multi-domain extracellular matrix protein thrombospondin 1[139]. In higher eukaryotes, these domains have different important functions such as the regulation of extracellular matrix organization and of cell-cell interactions[140]. In 2002 the crystal structure of the TSR domain was described, showing that they are folded as three antiparallel strands: the strand A is irregular, while strands B and C have β-sheet structure[141] (Figure 16). There are two main groups of TSR containing proteins depending on the distribution of the disulfide bonds. The first group has the top bond linking the third and the fourth cysteine (numbered according the order of cysteine appearance in the motifs, as proposed by Huwiler *et al.*[142]), so the connectivity is C_1-C_5, C_2-C_6, C_3-C_4. The top bond in the second group is between cysteine 1 and cysteine 4, so the connectivity between disulfide bonds is C_1-C_4, C_2-C_5, C_3-C_6[142] (Figure 16B). These domains are conserved in evolution and can be found in different organisms, including humans, *Plasmodium* parasites, *Drosophila*, *C. elegans*, etc.

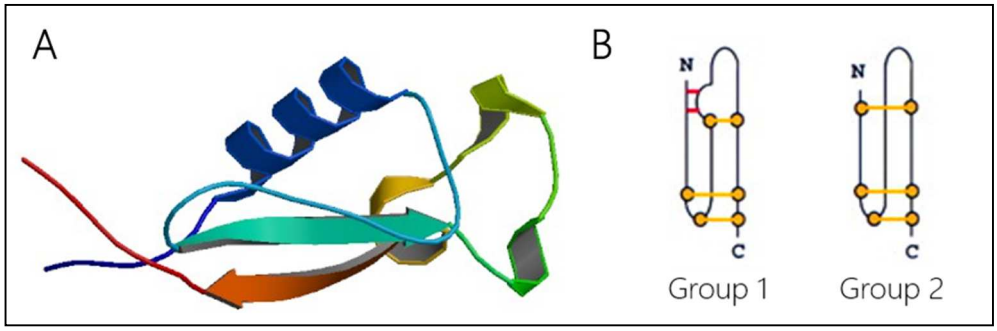


Figure 16. **TSR domain and scheme of TSR groups 1 and 2. (A)** TSR domain of *P. falciparum* CSP protein. Dark blue represents chain A and, light blue and orange are B and C β-sheet structures. Reproduced from Protein Data Bank. **(B)** Scheme of TSR groups 1 and 2. The prototypical disulfide bond patterns are represented as yellow lines. Adapted from Tan *et al.*, Journal of Cell Biology, 2002[141].

TSR-domain *O*-fucosylation is carried out by PoFUT2[143] using GDP-Fuc as a donor on $CX_2(S/T)CX_2G$[144,145] consensus sequences. PoFUT2 is only able to modify properly folded TSR domains substrate, suggesting the ability to distinguish between folded and unfolded structures[143]. *In vitro* assays have shown a decrease in secretion of ADAMTS13, a target protein of PoFUT2, after silencing PoFUT2 or reducing cellular GDP-Fuc levels[145,146]. Furthermore, PoFUT2 knockout (KO) mice show embryonic lethality[147]. The crystallization of PoFUT2 has recently helped to define in depth aspects about the proteins substrate recognition, specificity and catalytic activity[148,149]. The *O*-Fuc residues on TSR domains can be further elongated by the addition of a Glc residue. The enzyme responsible of this glucosylation is a β-1,3 Glucosyltransferase[101]. In higher eukaryotes, mutations on this enzyme lead to the Peter Plus Syndrome, a congenital disorder of glycosylation

characterized by eye anterior chamber defects, disproportionate short stature and developmental delay among other symptoms[101].

TSR domains are present in different *Plasmodium* proteins expressed along the life cycle of the parasite, being putative acceptors of *O*-fucosylation modifications. Among these, the proteins more studied are MTRAP, CTRP, CSP and TRAP, of which a brief description is included below. Other parasite proteins with functions less well known, such as SPATR, TRAMP, TREP or TLP, also contain TSR domains.

- Merozoite TRAP (MTRAP) (PF3D7_1028700)

MTRAP is a protein from the TRAP family, a surface associated family protein conserved across *Apicomplexans* and related with gliding motility[150], a substrate-dependent locomotion mechanism. MTRAP is a micronemal protein expressed in *Plasmodium* blood sexual and asexual stages with a cytoplasmic tail that binds to aldolase *in vitro*. It is released and processed during erythrocyte invasion[151]. The initial failure to knock out MTRAP suggested that this protein played an essential role in asexual blood stages parasite development [151]. Surprisingly, a very recent study showed that MTRAP could be disrupted in asexual parasites but was essential for gametocyte egress from erythrocytes[152]. The KO mutants generated in the study confirmed that the protein had no effect on asexual parasite growth *in vitro* or *in vivo*[152]. MTRAP, unlike other TSR domain containing proteins described below, does not conserve the O-fucosylation consensus amino acids (CXX(S/T)CXXG), showing a modified sequence CDKWGEWSECKDGRMHRKVLNCPFIKEEQECDVNNEMAED

- Circumsporozoite and TRAP Related Protein (CTRP) (PF3D7_0315200)

CTRP gene encodes for a 2114 amino acid protein that contains a transmembrane domain, a short cytoplasmic segment, 6 repeats of von Willebrand factor A (vWF-A) domains and 7 TSR domains[153]. It is expressed in *Plasmodium* ookinetes and localizes in the micronemes, being involved in the invasion of the mosquito midgut epithelium[84]. CTRP KO abolished oocyst production, despite the formation of mature ookinetes. The KO cell lines were unable to migrate through the mosquito midgut and produce oocysts[96]. Further studies narrowed CTRP domains' function, suggesting that the essential part of the protein were the 6 vWF-A domains. The elimination of these domains lead to the production of a non-infectious parasite line, while the disruption of the 7 TSR domain did not seem to significantly affect oocyst development [154]. The importance of the conservation of these TSR domains and their putative glycosylation remains unknown

- Circumsporozoite Protein (CSP) (PF3D7_0304600)

CSP is a highly conserved protein among *Plasmodium* species. Its structure consists in a N-terminal region that contains the so-called Region I domain, a central repeat region and the C-terminal region that contains a TSR domain and a GPI-anchor signal. The licensed RTS,S malaria vaccine is based on a fragment of this protein, containing the repeat region and the TSR domain. CSP is first detected in oocysts and then forms a very dense coat in the sporozoite surface[155]. It plays a critical role during mosquito salivary gland invasion but also during human hepatocyte invasion[156]. Coppi *et al.*[157] proposed a model describing CSP function during sporozoite migration from oocysts to the host hepatocytes. It was shown that CSP has one adhesive conformation, in which the TSR is exposed, and a non-adhesive conformation, in which the protein N-terminus masks the TSR domain. TSR exposure occurs during sporozoite development in the oocysts and also during hepatocyte invasion. Throughout sporozoite migration, from the hemolymph to the mosquito salivary glands and from the skin to the liver, the TSR domain is masked, making it less accessible to antibodies. Once the sporozoite gets in contact with hepatocytes, a proteolytic cleavage in protein's Region I is the signal for hepatocyte invasion[157]. CSP is essential for parasite development[157], and during the preparation of this thesis, the *O*-fucosylation and further glucose elongation of CSP was reported[102].

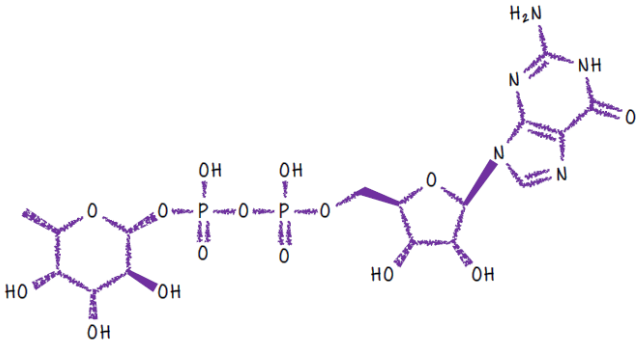
- Thrompospondine Related Anonymous Protein (TRAP) (PF3D7_1335900)

TRAP is a critical parasite protein involved in sporozoite invasion and motility. It consists of a region with a vWF-A domain, a TSR domain, a transmembrane domain and a cytoplasmic tail domain. It localizes in the sporozoite surface and in the micronemes[158,159]. It has been shown that TRAP binds to hepatocytes through the TSR and vWF-A domains[160]. The TSR domain binds to heparin sulfate proteoglycans (HSPG) on the hepatocyte surface, whereas vWF-A binds to heparin with a higher affinity than the TSR domain[159]. TRAP has two key roles during the hepatocyte invasion process. Initially, when the parasite gets in contact with the host cell, TRAP is released to the anterior surface of the sporozoite being able to interact with hepatocyte HSPG. After that, TRAP migrates and translocates its contact zone to the posterior end of the sporozoite, driving the parasite inside the hepatocyte cytoplasm[159,161]. Deletion of TRAP, the TSR domain and/or of the vWF-A domain lead to different parasite lines with less sporozoite gliding motility, less salivary gland invasion and fewer sporozoite infectivity, underlining the importance of this protein[162,163]. The *C*-mannosylation and *O*-fucosylation of the TSR domain present in TRAP was reported by Swearingen *et al*[102].

- Other TSR domain – containing proteins

Other TSR domain-containing proteins are expressed through the life stages of the parasite. Among these we can find: SPATR (PF3D7_0212600), TRAMP (PF3D7_1218000), TLP (PF3D7_0616500), TREP (PF3D7_1442600) and TRSP (PF3D7_0104000). SPATR is a protein expressed along the parasite life cycle that has been described as a target for liver cell-invasion inhibitory antibodies[164]. TRAMP is expressed during the blood stages of the parasite but its function in RBC invasion remains unknown[165], even though a recent study suggests its essential role in this process[166]. TLP, TREP and TRSP are expressed in sporozoite stages. TLP has been suggested to play a role in sporozoite migration from the skin to the liver[167], while TRSP KO parasites showed a deficiency in hepatocyte cell entry[168]. TREP is related with gliding motility and salivary gland infection[169], but more in depth studies regarding all these proteins need to be done. Among all these proteins the ones that are expressed during the blood stages of the parasite do not conserve the consensus sequence mentioned before.

HYPOTHESIS & OBJECTIVES



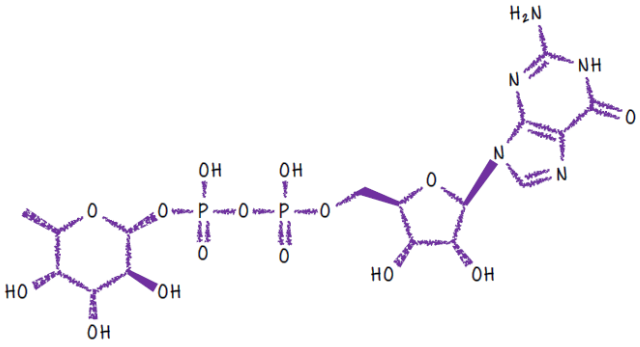
HYPOTHESIS AND OBJECTIVES

Malaria is a global health problem caused by a protozoan parasite of the genus *Plasmodium* transmitted by female *Anopheles* mosquitoes. Almost half of the world population is at risk of infection and Africa is the region where most of the cases and deaths occur. Glycobiology is the study of carbohydrate-related processes including its metabolism, glycan structures and biological function. Glycans are key players in cell-adhesion and in host-pathogen interactions. Hence parasite glycobiology may open new research lines leading to discovery of new selective parasiticidal drugs or vaccine candidates through the identification of parasite novel essential targets. Until recently, GPI-anchors were the only glycan structures well described in *Plasmodium* parasites. However, recent studies confirm the presence of truncated *N*-glycosylated structures and other glycosylations in the parasites[102,104,170].

According to the glycans described in other *Plasmodium* studies, the presence of GDP-Fuc was not expected. However, the genes for the GDP-Fuc *de novo* biosynthetic pathway were conserved in *P. falciparum* genome, as well as in the other *Plasmodium* species sequenced. Moreover, in other protozoan parasites, such as *T. brucei*, GDP-Fuc was described as an essential sugar nucleotide[109]. Furthermore, *Plasmodium* also conserves a putative protein *O*-fucosyltransferase 2, an enzyme that is responsible for the addition of fucose on TSR domains. These domains are present in key *Plasmodium* proteins in different stages of the parasite. Based in all this information we hypothesize that ***P. falciparum*** has an active *O*-fucosylation mechanism. To explore this hypothesis, the detailed objectives of this PhD thesis are:

- To identify and quantify the pools of sugar nucleotides present in *P. falciparum* blood stages.
- To confirm *Pf*GMD and *Pf*FS enzymatic activity, the responsible enzymes for GDP-Fuc synthesis.
- To describe the biological role of *Pf*GMD and *Pf*FS, by creating null mutant parasites of these enzymes and studying its phenotype.
- To determine the function of *P. falciparum* PoFUT2 by creating a null mutant and characterizing its phenotype through all the stages of the parasite life cycle.

RESULTS



RESULTS

1. Article 1

"Biosynthesis of GDP-fucose and other sugar nucleotides in the blood stages of *Plasmodium falciparum*"

Sílvia Sanz, Giulia Bandini, Diego Ospina, Maria Bernabeu, Karina Mariño, Carmen Fernández-Becerra, and Luis Izquierdo

The Journal of Biological Chemistry, 288 (2013) 16506-16517

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 23, pp. 16506–16517, June 7, 2013

Biosynthesis of GDP-fucose and Other Sugar Nucleotides in the Blood Stages of *Plasmodium falciparum*[*]

Received for publication, November 26, 2012, and in revised form, April 11, 2013 Published, JBC Papers in Press, April 24, 2013, DOI 10.1074/jbc.M112.439828

Sílvia Sanz[‡], Giulia Bandini[§1], Diego Ospina[‡], Maria Bernabeu[‡], Karina Mariño[§2], Carmen Fernández-Becerra[‡], and Luis Izquierdo[‡3]

From the [‡]Barcelona Centre for International Health Research, Hospital Clínic-Universitat de Barcelona, CEK, 1a Planta, Rosselló 149-153, 08036, Barcelona, Spain and the [§]College of Life Sciences, University of Dundee, Division of Biological Chemistry and Drug Discovery, Wellcome Trust Biocentre, Dundee DD15EH, Scotland, United Kingdom

Background: GDP-fucose and other sugar nucleotide biosynthetic pathways are conserved in the *P. falciparum* genome.
Results: These pathways are active in the intraerythrocytic life cycle of the parasite.
Conclusion: The parasite biosynthesizes GDP-fucose and other sugar nucleotides not related to the glycosylphosphatidylinositol structures
Significance: Their presence strongly suggests that they are involved in the biosynthesis of glycans not yet characterized.

Carbohydrate structures play important roles in many biological processes, including cell adhesion, cell-cell communication, and host-pathogen interactions. Sugar nucleotides are activated forms of sugars used by the cell as donors for most glycosylation reactions. Using a liquid chromatography-tandem mass spectrometry-based method, we identified and quantified the pools of UDP-glucose, UDP-galactose, UDP-*N*-acetylglucosamine, GDP-mannose, and GDP-fucose in *Plasmodium falciparum* intraerythrocytic life stages. We assembled these data with the *in silico* functional reconstruction of the parasite metabolic pathways obtained from the *P. falciparum* annotated genome, exposing new active biosynthetic routes crucial for further glycosylation reactions. Fucose is a sugar present in glycoconjugates often associated with recognition and adhesion events. Thus, the GDP-fucose precursor is essential in a wide variety of organisms. *P. falciparum* presents homologues of GDP-mannose 4,6-dehydratase and GDP-L-fucose synthase enzymes that are active *in vitro*, indicating that most GDP-fucose is formed by a *de novo* pathway that involves the bioconversion of GDP-mannose. Homologues for enzymes involved in a fucose salvage pathway are apparently absent in the *P. falciparum* genome. This is in agreement with *in vivo* metabolic labeling experiments showing that fucose is not significantly incorporated by the parasite. Fluorescence microscopy of epitope-tagged versions of *P. falciparum* GDP-mannose 4,6-dehydratase and GDP-L-fucose synthase expressed in transgenic 3D7 parasites shows that these enzymes localize in the cytoplasm of *P. falciparum* during the intraerythrocytic developmental cycle. Although the function of fucose in the parasite is not known, the presence of GDP-fucose suggests that the metabolite may be used for further fucosylation reactions.

Malaria is a global health problem caused by protozoan parasites of the genus *Plasmodium* and transmitted by the *Anopheles* mosquito. Approximately half of the world's population is at risk of infection, and there are around 200 million cases annually, leading to more than half a million deaths each year (1). Most of the people who die are children living in resource-poor countries in sub-Saharan Africa. Among the five *Plasmodium* species that cause malaria in humans (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*), *P. falciparum* is the most deadly and is responsible for the majority of the malaria-linked deaths in sub-Saharan Africa (1). Chemotherapy is one of the central strategies for malaria treatment, but unfortunately, drug resistance to commonly used antimalarial drugs has spread very rapidly (2, 3). The implementation of new approaches to prevent and fight malaria has greatly benefited from the sequencing of the parasite genome (4) and the development of improved tools for functional genomics (5–8). However, this area of research remains greatly limited by our incomplete knowledge of parasite biology (9). Therefore, it is critical to promote research efforts that probe the basic biochemistry and cell biology of *Plasmodium* with the aim of characterizing essential proteins, metabolic pathways, and other processes that could be suitable for intervention and to validate new candidate drug targets.

Glycosylphosphatidylinositol (GPI)[4] anchors (10) represent the major carbohydrate modification described in *P. falciparum* cell surface proteins (11). Several of these GPI-anchored glycoproteins are essential for parasite invasion and virulence (12), and GPI anchors are generated via a complex synthetic pathway (13). Recently, Bushkin *et al.* (14) presented new evidence demonstrating the existence of functional *N*-glycosyla-

* This work was supported by Spanish Ministry of Economy and Competitiveness Grant SAF2010-21069 (to L. I.).
[1] Present address: Dept. of Molecular and Cell Biology, Boston University Goldman School of Dental Medicine, Boston, MA 02118.
[2] Present address: Laboratorio de Glicómica Funcional y Molecular, Instituto de Biología y Medicina Experimental (IBYME), Ciudad de Buenos Aires C1428ADN, Argentina.
[3] Recipient of a travel fellowship from the Catalan Government (Research and Universities Commission) and support from the Ramón y Cajal Program and Grant SAF2010–21069 (Spanish Ministry of Economy and Competitiveness). To whom correspondence should be addressed. E-mail: luis.izquierdo@cresib.cat.

[4] The abbreviations used are: GPI, glycosylphosphatidylinositol; Man, mannose; Fuc, fucose; TSR, thrombospondin type 1 repeat.

tion in the intraerythrocytic stages of *P. falciparum*. In general, protozoan parasite *N*-glycosylation patterns are atypical of those described in higher eukaryotes (15, 16). In *P. falciparum*, the secondary loss of enzymes related to the biosynthesis of *N*-glycosylation precursors and the quality control of glycoprotein folding in the endoplasmic reticulum (17, 18) result in a very unusual *N*-glycosylation (14) that, if essential, could be therapeutically exploitable. To our knowledge, despite some controversy (19–24), *O*-glycans have never been unequivocally described in *P. falciparum*. Thus, although there have been significant efforts to understand the glycobiology of the parasite, several questions on this topic remain unanswered.

Sugar nucleotides, the essential intermediates in carbohydrate metabolism and glycoconjugate biosynthesis, are activated forms of sugars produced by the cell as donor precursors for most of the glycosylation reactions. They are formed in two main ways: by a salvage pathway involving "activation" of the sugar using a kinase and a pyrophosphorylase or by a *de novo* pathway involving the bioconversion of an existing sugar or sugar nucleotide. Specific *P. falciparum* metabolic databases (25), based on the parasite genome sequence, predict the conservation of the *de novo* biosynthetic pathways for UDP-*N*-acetyl-glucosamine, GDP-mannose, GDP-fucose, and UDP-glucose. Most of the predicted open reading frames (ORFs) that encode for the enzymes involved in these pathways are also present in other *Plasmodium* species (*P. vivax*, *P. knowlesi*, *Plasmodium chabaudi*, *Plasmodium yoelii*, and *Plasmodium berghei*) (26), suggesting that they are conserved and encode for proteins playing important roles in the parasite. Indeed, UDP-*N*-acetyl-glucosamine and GDP-mannose (through its product dolichol-phosphomannose) are essential donor substrates for the biosynthesis of GPI structures (27). Because sugar nucleotides are the "basic building blocks" of glycoconjugates, the conservation of their biosynthetic metabolic routes strongly suggests the existence of further downstream glycosylation reactions in which these metabolites are involved (*i.e.* glycan biosynthesis).

In this work, we identify and quantify the sugar nucleotides present in different stages of the intraerythrocytic life cycle of *P. falciparum* using a liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assay, and we present the results in the context of the functional metabolic pathways reconstructed from the genome of the parasite. We demonstrate that *P. falciparum* is unable to take up significant amounts of tritiated fucose from the culture media. Furthermore, we show that the putative GDP-fucose biosynthesis enzymes are functional *in vitro*, by expressing them and detecting GDP-fucose production. Endogenous forms of these enzymes localize to the cytosol of *P. falciparum*. We show that the genes encoding these enzymes are expressed at low abundance, and their levels are modulated throughout the red blood cell stages of the parasite. Finally, we also demonstrate that the parasite expresses a protein *o*-fucosyltransferase homolog, and schizont extracts incorporate tritiated GDP-fucose.

EXPERIMENTAL PROCEDURES

Parasites and Parasite Culture—*P. falciparum* 3D7 (obtained from MR4-ATCC) parasites were cultured with human erythrocytes (3–4% hematocrit) in RPMI medium (Sigma) supplemented with 10% AB^+ human serum, incubated at 37 °C in an atmosphere of 92% N_2, 3% O_2, and 5% CO_2 using standard methods (28). For sugar nucleotide analysis and labeling experiments, parasites were grown in RPMI medium supplemented with 0.5% Albumax II (Invitrogen) to avoid possible variations due to the uses of different human serum batches. Parasite growth was monitored by counting the infected erythrocytes in Giemsa-stained thin blood smears under light microscopy.

Sugar Nucleotide Analysis—Osmotic lysis of red blood cells of sorbitol-synchronized cultures at different stages of the intraerythrocytic life cycle (rings, trophozoites, and schizonts, approximately 18, 35, and 44 h postinvasion, respectively) and 10–12% parasitemia was performed by resuspending erythrocyte pellet twice in 60 volumes of cold erythrocyte lysis buffer (10× stock solution: 0.15 M NH_4Cl, 0.1 M $KHCO_3$, 0.01 M EDTA). Non-infected red blood cells were lysed and included as controls in every analysis to discard noise due to the detection of sugar nucleotides carried away from the erythrocytes. The suspension was incubated on ice until lysis was completed (~10 min) (29). Pellets were washed three times with cold phosphate-buffered saline (PBS), and sugar nucleotide analysis was performed as described elsewhere (30). Briefly, parasite pellets were lysed in 70% ethanol in the presence of 20 pmol of GDP-glucose internal standard, and sugar nucleotides were extracted using Envi-Carb columns (31). Sugar nucleotides were then analyzed using LC-MS/MS, using multiple-reaction monitoring for detection. HPLC conditions were adapted from Ref. 31, and acetonitrile was added postcolumn to produce stable electrospray ionization. The peak areas for each sugar nucleotide, along with their empirically determined molar relative response factors and the known amount of internal GDP-glucose, were used to quantify sugar nucleotides. Analyses were performed on three different sugar nucleotide extracts.

Metabolic Labeling of Parasite with Tritiated Sugars—Parasite cultures at 10–12% parasitemia were synchronized to the ring state and then washed and resuspended in RPMI medium supplemented with Albumax II. Cells were incubated at 3–4% hematocrit for 16–18 h, until the trophozoite stage. The parasites were then washed with glucose-free RPMI supplemented with Albumax II and 20 mM fructose, and they were metabolically labeled with ^{3}H-sugars (50 μCi/ml) for 1 or 4 h, adapting the conditions used in previous work (23, 32, 33). Triplicates of each condition were included, and controls were "mock-labeled" for a few seconds using medium at 4 °C supplemented with an excess (100-fold) of the unlabeled (cold) sugar. Labeled cultures and controls were washed three times with PBS at 4 °C and treated with saponin to release parasites. Free parasites were washed again three times with PBS at 4 °C and lysed, and the radioactivity was measured by liquid scintillation counting.

To calculate the amount of sugar incorporated into the glycoprotein and glycolipid compartments, after the labeling, free parasites were further lysed, and the proteins were precipitated with 10% trichloroacetic acid and filtered (glycoproteins), or the glycolipids were extracted with organic solvents (glycolipids) (34).

Expression and Purification of GDP-fucose Biosynthesis Genes in Escherichia coli—Codon harmonization was used for the production of heterologously expressed *P. falciparum* GDP-mannose 4,6-dehydratase (*Pf*GMD, gene ID PF3D7_0813800) and GDP-L-fucose synthase (*Pf*FS, gene ID PF3D7_1014000) in *E. coli* (35). Codon-harmonized genes (Genscript) were cloned into the pGEX 6P expression vector (GE Healthcare) and expressed in BL21 (DE3) *E. coli* cells. 3-Liter cultures were grown to an $A_{600\ nm}$ of 0.6 and induced overnight at 18 °C with 100 μM isopropyl-β-D-thiogalactopyranoside. Cells were harvested and lysed in PBS including 0.1 mg/ml lysozyme in the presence of Complete protease inhibitor mixture (Roche Applied Science). Lysis was ensured by sonication (Misonix Microson XL 2000). 0.5% Triton X-100 was added, and, after a 30-min incubation at 4 °C, the lysate was clarified by centrifugation at 12,000 × *g* for 20 min, and the supernatant was added to 1 ml of PBS-washed GST beads (GE Healthcare) and incubated overnight at 4 °C. The beads were collected in chromatography columns, washed with >10 bead volumes of PBS before three elutions of 1 ml with 10 mM glutathione in 50 mM Tris-HCl, pH 8. The eluates were pooled, diafiltered with PBS, and concentrated in 10,000 nominal molecular weight limit centrifugal filter units (Amicon, Millipore).

GDP-fucose Biosynthesis Assay—Assay conditions were adapted from elsewhere (36–38). Reactions were performed in 25 μl (final volume) of 100 mM MOPS, pH 7.0, 100 mM NaCl, 10 mM dithiothreitol, 5 mM EDTA, 1 mM GDP-mannose (Sigma), 0.4 mM NADPH, and 1 mM NADP. The reaction was started by adding 1.5 mg/ml of the recombinant *Pf*GMD enzyme, and the reaction was left to proceed for 3 h at 37 °C. Recombinant *Pf*FS (1.5 mg/ml) was then added, and the concentration of NADPH was adjusted to 1.5 mM. For experiments without the *Pf*FS enzyme, only NADPH was added at this point. Reactions were stopped after 2 h at 37 °C by heating to 100 °C for 2 min, and then the samples were filtered to remove insoluble material, and sugar nucleotides were analyzed by HPLC (31), including standards for every HPLC series of experiments.

For LC-MS/MS analysis, HPLC conditions were adapted from Ref. 39, using a porous graphitic carbon column (Hypercarb, 100 × 2.1 mm, 5-μm particle size, Thermo Scientific) and MS-compatible mobile phases. Starting buffer was 0.1% formic acid, brought to pH 9.0 with ammonia, followed by a 36-min gradient from 10 to 50% acetonitrile at a flow rate of 100 μl/min. Detection was performed by negative mode electrospray ionization-MS on an API3000 triple quadrupole LC-MS/MS mass spectrometer (PE-Sciex) with a declustering potential of −50 V, focusing potential of −300 V, collision energy of 30 V, and source temperature of 375 °C.

Cloning and Expression of Epitope-tagged Versions of PfGMD and PfFS in P. falciparum—Gene *PfGMD* was amplified from *P. falciparum* genomic DNA using primer pARL1F-GMD-(KpnI) (CGCGGTACCATGCGAGTTGCTTTAATC) and pARL1R-GMD(PstI) (CGCCTGCAGTTGCTTTTTACCCATTT). *PfFS* was amplified from *P. falciparum* cDNA using primer pARL1F-FS(KpnI) (CGCGGTACCATGACACGAATTTGTCTTGTAACTG) and pARL1R-FS(PstI) (CGCCTGCAGTTTTCTTACATTTTTGTATTCGTCAATAAACCA). *P. falciparum* genes were cloned in the KpnI-PstI site of transfection vector pARL1a-3HA (40) under the control of the *pfcrt* promoter region (41). *P. falciparum* were transfected as described previously (41). Briefly, 150 μg of each plasmid was used to electroporate (310 V, 950 millifarads) 200 μl of infected red blood cells at >5% parasitemia, synchronized for ring stage parasites. Transfected parasites were selected on 2 nM of WR99210 drug, and resistant parasites appeared in culture from 25 to 35 days after drug application.

Indirect Immunofluorescence Assays—Cultured *P. falciparum* transgenic lines were washed in PBS and then fixed with 4% EM grade paraformaldehyde and 0.075% EM grade glutaraldehyde in PBS (42). Fixed cells were permeabilized with 0.1% Triton X-100 in PBS and blocked for 1 h at room temperature in 3% PBS-bovine serum albumin (PBS-BSA). Samples were incubated overnight with primary antibody (rat anti-HA (1:10; Roche Applied Science) or rabbit anti-HSP70 (1:50; StressMarq)) (43) diluted in 3% PBS-BSA, followed by a 1-h incubation with secondary antibody (anti-rat conjugated with Alexa Fluor 488 or anti-rabbit conjugated with Alexa Fluor 594 (1:200, Invitrogen)) diluted in 3% PBS-BSA. Nuclei were stained for 1 h with 4′,6-diaminido-2-phenylindole (DAPI; 2 mg/ml diluted in PBS) during the secondary antibody incubation. Confocal microscopy was performed using a laser scanning confocal microscope (TCS-SP5; Leica Microsystems) at the microscopy scientific and technical services facility of the Universitat de Barcelona.

RNA Preparation and Quantitative Real-time PCR—Tight synchronization of parasites was achieved by Percoll purification of schizonts followed by sorbitol lysis 5 h later, to obtain a population of a defined age window of 0–5 h postinvasion. RNA was purified using the TRIzol method at the ring, trophozoite, and schizont stage, and cDNA was synthesized by reverse transcription performed using random hexaprimers and SuperScript-III reverse transcriptase (RT; Invitrogen) according to the manufacturer's instructions and including controls without RT. All quantitative PCRs were performed using PowerSYBR Green Master Mix (Invitrogen), and expression values were calculated using the relative standard curve method. Results are expressed in arbitrary units, relative to a genomic DNA standard curve, and were normalized against seryl-tRNA synthetase (PF3D7_0717700). The primers used were GGTGATTGCTCAAAGGCAAAA and TTCATAAACGAGCTGGGTAAATGTAT for *PfGMD*; CTTGTATTTTCCCTGTAAATTGTTCTCTAC and GATGGTATAACGTGCGCATTTTC for *PfFS*; TTTTTTAAAGTGATAGACAGGGTTAT and AAAAAAGTCCCCTAAATATACATCCT for *PfPoFUT2*; and AAGTAGCAGGTCATCGTGGTT and TTCGGCACATTCTTCCATAA for seryl-tRNA synthetase.

Preparation of P. falciparum Cell Extracts and [^{3}H]GDP-fucose Incorporation Assays—Percoll-purified infected red blood cells (36–42 h postinvasion) grown at 10–12% parasitemia were subjected to saponin lysis and washed three times with PBS. The cell pellet was placed on ice and homogenized for 1 h (at 10^9 cells/ml) with 1% Nonidet P-40, 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 10 mM $MnCl_2$, 5 mM $MgCl_2$, 1× protease inhibitor mixture (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 μg/ml N^{α}-*p*-tosyl-L-lysine chloromethyl ketone. Inactivated extracts were boiled for 20 min

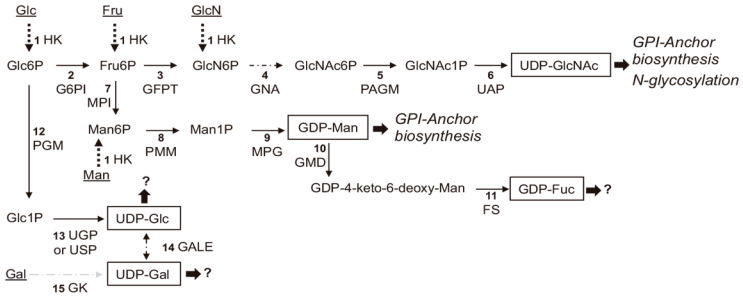


FIGURE 1. **Sugar nucleotide biosynthetic pathways identified in the genome of *P. falciparum*.** The *numbers* refer to the enzymes and known or candidate genes described in Table 1. Predicted known fates of sugar nucleotide donors according to the glycoconjugates described in *P. falciparum* are in *italic type* (or marked with a *question mark* if the fate is unknown). Sugar nucleotides identified in this study are *boxed*. *Dotted lines* indicate confirmed salvage pathways, and sugars, taken up from the medium, are *underlined*. The *discontinuous arrow* (step 4) represents the glucosamine-phosphate *N*-acetyltransferase activity (EC 2.3.1.4) for which a candidate gene is not yet identified. *HK*, hexokinase; *G6PI*, glucose-6-phosphate isomerase; *GFPT*, glucosamine-fructose-6-phosphate aminotransferase; *GNA*, glucosamine-phosphate *N*-acetyltransferase; *PAGM*, phosphoacetylglucosamine mutase; *UAP*, UDP-*N*-acetylglucosamine pyrophosphorylase; *MPI*, mannose-1-phosphate isomerase; *PMM*, phosphomannomutase; *MPG*, mannose-1-phosphate guanyltransferase; *GMD*, GDP-mannose 4,6-dehydratase; *FS*, GDP-L-fucose synthase; *PGM*, phosphoglucomutase; *UGP*, UTP-glucose-1-phosphate uridylyltransferase; *GALE*, UDP-glucose 4-epimerase; *GK*, galactokinase. The suggested pathway for the biosynthesis of UDP-Gal through the activity of the UDP-sugar pyrophosphorylase enzyme is indicated with a *gray discontinuous arrow* (see "Discussion").

with 3 M urea, 0.1 M dithiothreitol, and 5 mM EDTA. 2 μCi of [^{3}H]GDP-fucose (American Radiochemical Chemicals, Inc.) was added to the extract, and the reaction was incubated at 37 °C for 1 h. Proteins were TCA-precipitated and washed three times with cold 100% acetone, and tritium incorporated into the TCA pellets was measured by scintillation counting.

RESULTS

Sugar Nucleotide Biosynthetic Pathways Predicted in P. falciparum—Based on the current knowledge of *P. falciparum* sugar biochemistry (11, 14, 23, 32) and the predicted metabolic pathways (25), we have reconstructed the sugar nucleotide biosynthetic routes present in *P. falciparum* (Fig. 1). The enzymes and genes involved in these metabolic routes are shown in Table 1. Bioinformatic analysis suggests that sugar nucleotides are made by conventional eukaryotic *de novo* routes from glucose 6-phosphate, although other salvage pathways also exist (Fig. 1). Conserved pathways for the biosynthesis of UDP-GlcNAc and GDP-mannose agree well with the monosaccharide content of known *P. falciparum* glycoconjugates (*i.e.* GPI anchors and *N*-glycans). However, the conservation of UDP-glucose and GDP-fucose biosynthetic pathways (Fig. 1) does not correspond with any previous description of fucose and/or glucose-containing glycans in the parasite. Because sugar nucleotides are obligate donors for glycosylation reactions mediated by glycosyltransferases, the presence of these metabolic routes in the parasite genome prompted us to identify the sugar nucleotide pools present in the blood stages of the parasite to assess the activation state of these pathways.

Identification and Quantification of Sugar Nucleotide Pools in P. falciparum Blood Stages—Commercially available sugar nucleotides were used as standards to identify and quantify the products of the conserved sugar nucleotide metabolic pathways (25) using the 3D7 strain of *P. falciparum*. Variable levels of UDP-galactopyranose, UDP-Glc, UDP-GlcNAc, GDP-mannose (GDP-Man), and GDP-fucose (GDP-Fuc) pools were detected through the different blood stages (*i.e.* rings, trophozoites, and schizonts) of the parasite (Fig. 2 and Table 2). The sugar nucleotide levels were, in general, 10–100-fold lower than those of other extracellular protozoan parasites. However, in the case of GDP-fucose, levels detected were comparable (Table 2) (30). To exclude the possibility of contamination from host cell material, non-infected osmotically lysed erythrocyte ghosts were analyzed and included as negative controls. The results were at least 1 order of magnitude lower than in the parasite analysis for every sugar nucleotide (Fig. 2 and Table 2). The sugar nucleotides detected in the parasite agree well with the monosaccharide contents of known glycoconjugates in *P. falciparum* consisting of glucosamine (derived from GlcNAc) and Man residues present in GPI anchors and *N*-glycans (11, 14). Furthermore, the detection of UDP-GlcNAc, UDP-Glc, GDP-Man, and GDP-Fuc is consistent with the presence of known or candidate sugar nucleotide biosynthetic enzymes encoded in the genome of *P. falciparum* (25). We were surprised to find that pools of UDP-galactopyranose can be identified at the different blood stages of the parasites, although no apparent candidates for UDP-glucose-4′-epimerase can be detected in the genome of the parasite. The possible metabolic route for the biosynthesis of this sugar nucleotide is discussed below.

Metabolic Labeling of Parasites with Tritiated Sugars—To assess the presence of carbohydrate salvage routes in the parasite *P. falciparum*-infected erythrocytes, cultures were metabolically labeled with tritiated Man or Fuc in medium containing 20 mM D-fructose (32, 34, 44, 45). Whereas [^{3}H]Man was incorporated as described previously (23), [^{3}H]Fuc was not significantly taken up by the parasite (Fig. 3*A*), contributing to less than 15% of the average pool amount, and we did not detect a significant incorporation into its glycoproteins or glycolipids (Fig. 3*B*). This indicates that the main source of GDP-Fuc for *P. falciparum* intraerythrocytic life stages is through the bio-

TABLE 1
Enzymes and genes involved in *P. falciparum* sugar nucleotide biosynthesis

Step no.[a]	Enzyme name	Enzyme no.	Putative *P. falciparum* homologues[b]
1	Hexokinase (HK)	EC 2.7.1.1	PF3D7_0624000
2	Glucose-6-phosphate isomerase (G6PI)	EC 5.3.1.9	PF3D7_1436000[c]
3	Glucosamine-fructose-6-phosphate aminotransferase (GFPT)	EC 2.6.1.16	PF3D7_1025100
4	Glucosamine-phosphate *N*-acetyltransferase (GNA)	EC 2.3.1.4	No gene identified[d]
5	Phosphoacetylglucosamine mutase (PAGM)	EC 5.4.2.3	PF3D7_1130000
6	UDP-*N*-acetylglucosamine pyrophosphorylase (UAP)	EC 2.7.7.23	PF3D7_1343600
7	Mannose-6-phosphate isomerase (MPI)	EC 5.3.1.8	PF3D7_0801800
8	Phosphomannomutase (PMM)	EC 5.4.2.8	PF3D7_1017400
9	Mannose-1-phosphate guanyltransferase (MPG)	EC 2.7.7.13	PF3D7_1420900
10	GDP-mannose 4,6-dehydratase (GMD)	EC 4.2.1.47	**PF3D7_0813800**
11	GDP-L-fucose synthase (FS)	EC 1.1.1.271	**PF3D7_1014000**
12	Phosphoglucomutase (PGM)	EC 5.4.2.2	PF3D7_1012500
13	UTP-glucose-1-phosphate uridylyltransferase (UGP) or UDP-sugar pyrophosphorylase (USP)	EC 2.7.7.9 or EC 2.7.7.64	PF3D7_0517500
14	UDP-glucose 4-epimerase (GALE)	EC 5.1.3.2	No gene identified
15	Galactokinase (GK)	EC 2.7.1.6	No gene identified

[a] Step numbers refer to those shown in Fig. 1.
[b] Gene ID numbers underlined are as identified and annotated in the *P. falciparum* genome (25, 46), and those in boldface type and underlined have been functionally characterized in this paper.
[c] Glucose-6-phosphate isomerase (EC 5.3.1.9) enzyme, PF3D7_1436000 has been crystallized, but to our knowledge its activity has not been published (77).
[d] *P. falciparum* is thought to process GlcN6P to GlcNAc6P (step 4, Fig. 1) based on the radiolabeling of the parasite with [^{3}H]GlcN (22, 23).

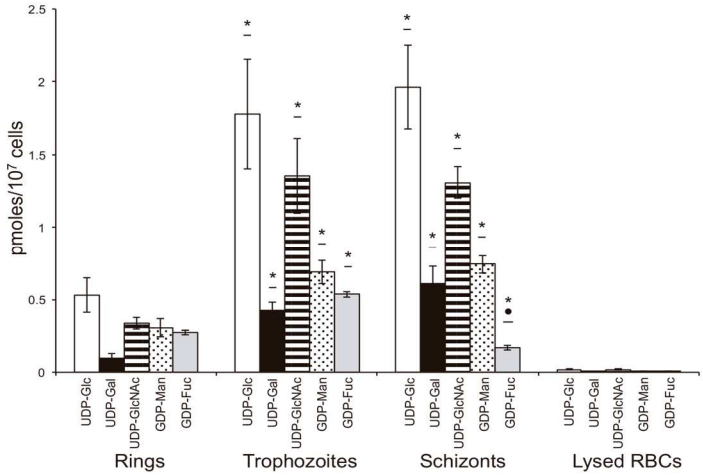


FIGURE 2. **Specific sugar nucleotide levels in different blood stages of *P. falciparum*.** Values of UDP-Glc (*open bars*), UDP-Gal (*black solid bars*), UDP-GlcNAc (*striped bars*), GDP-Man (*dotted bars*), and GDP-Fuc (*gray solid bars*) indicate the average of three different extractions. *Error bars*, S.D. The *last set of bars* represent the levels of sugar nucleotides measured on osmotically lysed non-infected red blood cells (*RBCs*; used as control). Values are indicated in pmol/10^7 cells. Analyses were performed in triplicate, and mean values ± S.D. are shown. Statistically significant differences (one-way analysis of variance Tukey's post-test) between stages are shown with an *asterisk* (*, $p < 0.05$, rings *versus* trophozoites or schizonts) or a *dot* (●, $p < 0.05$, trophozoites *versus* schizonts).

TABLE 2
Sugar nucleotide levels in blood stages of *P. falciparum*

Sugar nucleotide	Stage			Lysed red blood cells[a,b]
	Rings[a]	Trophozoites[a]	Schizonts[a]	
UDP-Glc	0.53 ± 0.12	1.78 ± 0.38	1.96 ± 0.29	0.02
UDP-Gal	0.09 ± 0.04	0.42 ± 0.06	0.61 ± 0.12	<0.01
UDP-GlcNAc	0.34 ± 0.04	1.35 ± 0.26	1.31 ± 0.11	0.02
GDP-Man	0.31 ± 0.06	0.69 ± 0.08	0.74 ± 0.06	<0.01
GDP-Fuc	0.27 ± 0.01	0.53 ± 0.02	0.17 ± 0.02	<0.01

[a] Amounts are indicated in pmol/10^7 cells.
[b] S.D. values are at least 1 order of magnitude smaller than the calculated amount and are not included.

conversion of an existing sugar/sugar nucleotide, which is consistent with the apparent absence of genes encoding enzymes involved in the GDP-fucose salvage pathway in the *P. falciparum* genome (25). Thus, the *de novo* GDP-Fuc synthetic route from GDP-Man, which depends on GMD and FS (Fig. 1 and Table 1), is the most feasible pathway for the biosynthesis of this sugar nucleotide in *P. falciparum*.

Similarly, metabolic labeling with [^{3}H]GlcNAc showed that parasites were unable to take up this sugar from the media (Fig. 3*C*). This, together with the labeling of the parasite with [^{3}H]GlcN, strongly suggests that the sequence of intermediates in the *P. falciparum* UDP-GlcNAc biosynthetic pathway is GlcN6P → GlcNAc6P → GlcNAc1P → UDP-GlcNAc.

P. falciparum GDP-Fuc Biosynthetic Genes Are Active in Vitro—Both PfGMD and PfFS genes are identified and annotated in the *P. falciparum* genome (25, 46). The predicted *PfGMD* (PF3D7_0813800) protein product is assigned to EC

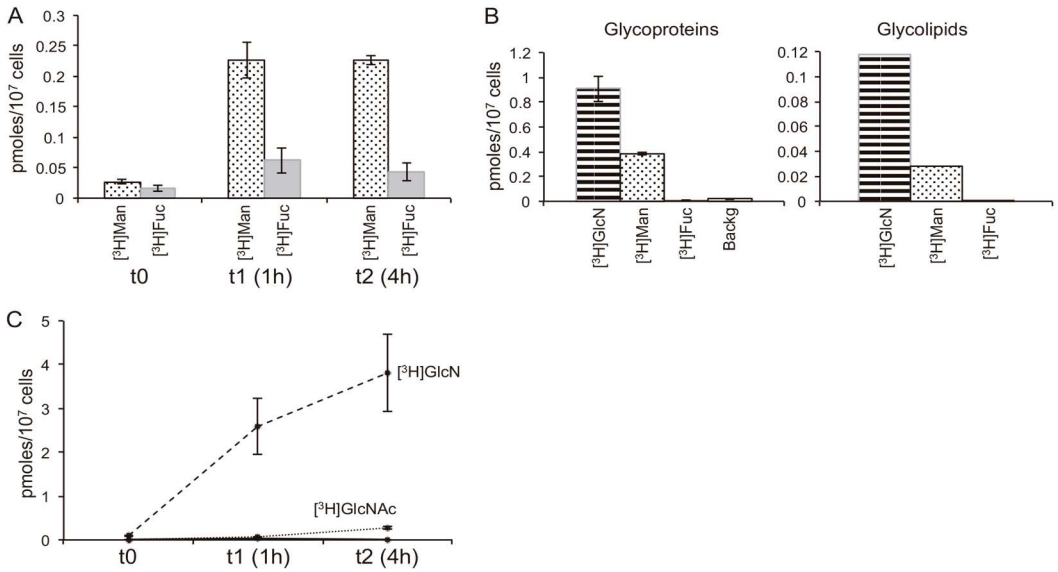


FIGURE 3. **[^{3}H]Sugars incorporated by *P. falciparum* trophozoites.** *A*, amounts of mannose (*dotted bars*) and fucose (*gray solid bars*) indicate the average of three different determinations at different times. *Error bars*, S.D. Values are indicated in pmol of sugar/10^7 cells. t_0 controls were mock-labeled for a few seconds using medium at 4 °C supplemented with an excess (100-fold) of the unlabeled (cold) sugar. *B*, amount of sugar incorporated to the glycoproteins (*left*; determinations performed in triplicate; *error bars*, S.D.) or glycolipids (*right*) of *P. falciparum*. ^{3}H incorporated is indicated in pmol of sugar/10^7 cells. *C*, [^{3}H]GlcN (*dashed line*) and [^{3}H]GlcNAc (dotted line) incorporated by *P. falciparum* trophozoites. L-Glc, which is not incorporated by the parasite, is included as a negative control of incorporation (*solid line*). Determinations were performed in triplicate at three different times, and mean values ± S.D. are shown. t_0 controls were generated as in *A*.

4.2.1.47 and presents 48.1% sequence identity and 61.4% similarity to its human counterpart. *PfFS* (PF3D7_1014000) encodes a protein assigned to EC 1.1.1.271 that has a 35.7% sequence identity and 57.1% similarity to its human homolog.

The presence of putative *PfGMD* and *PfFS* genes, together with the detection of GDP-Fuc in *P. falciparum* intraerythrocytic stages, suggests that the protein products of these genes would be active GDP-Fuc biosynthetic enzymes. To assess this, we expressed both *PfGMD* and *PfFS* genes in *E. coli* with the addition of N-terminal GST tags. In both cases, soluble proteins could be purified by GST affinity chromatography (Fig. 4*A*). The enzymes were assayed as described under "Experimental Procedures," and the sugar nucleotides produced were analyzed either by LC-MS/MS or by reverse phase ion pairing chromatography. When both enzymes were added to the reaction, GDP-Fuc was readily detected (Fig. 4*B*, *top panels*). Both enzymes are required for the production of GDP-Fuc, because in the presence of *Pf*FS only, there was no turnover of GDP-Man (not shown), and with only *Pf*GMD, a novel GDP-deoxyhexose with a different retention time was generated (Fig. 4*B*, *middle panels*). The production of a novel GDP-deoxyhexose by a GMD enzyme in the absence of an FS enzyme has been reported before (36, 47). In addition, in the presence of *Pf*GMD alone, we detected also a product at 586 *m*/*z*, which coincides with the molecular weight of GDP-4-keto-6-deoxy mannose. Although there are no commercial standards available to analyze the transition of GDP-4-keto-6-deoxy mannose for identification, the fragmentation of this novel product produced a major product ion at *m*/*z* 442 corresponding to a $[GDP\text{-}H]^-$, strongly resembling the fragmentation of GDP-deoxyhexoses (Fig. 4*B*, *bottom panels*) (30, 36).

To assess the effect of time and GDP-Man and *Pf*GMD/*Pf*FS concentration on the production of GDP-Fuc, we performed different activity assays where sugar nucleotides were measured by reverse phase HPLC. GDP-Fuc production was dependent on time and the initial concentration of GDP-Man (Fig. 4*C*). Interestingly, at the *Pf*GMD and GDP-Man concentrations tested (1.5 mg/ml and 1 mM, respectively), *Pf*FS did not become rate-limiting at 1 mg/ml, whereas decreasing the initial concentration of *Pf*GMD to 1 mg/ml reduced the relative yield of GDP-Fuc to a 70%.

PfGMD and PfFS Are Expressed during P. falciparum Intraerythrocytic Life Cycle and Their Protein Products Localize in the Cytosol of the Parasite—Real-time quantitative RT-PCR analysis of *PfGMD* and *PfFS* RNA transcripts was performed at the different asexual stages of *P. falciparum* (rings, trophozoites, and schizonts), and expression values were calculated using the relative standard curve method. In both cases, the highest expression levels were found at the schizont phase (Fig. 5), in agreement with what had been observed previously in other studies and with what is annotated in *P. falciparum* metabolic databases (25, 48, 49). Nevertheless, transcription of both

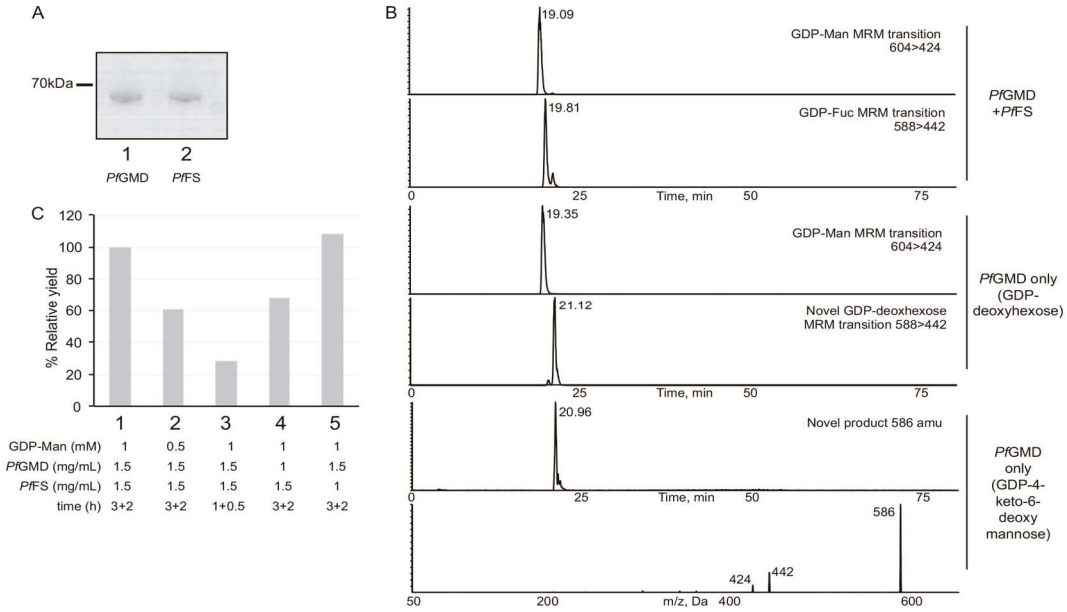


FIGURE 4. **Activity of expressed *Pf*GMD and *Pf*FS.** *A*, Coomassie Blue-stained SDS-polyacrylamide gel of purified, GST-tagged, recombinantly expressed *Pf*GMD (*lane 1*) and *Pf*FS (*lane 2*) proteins. *B*, the purified recombinant enzymes were assayed using GDP-Man as a substrate. Sugar nucleotides were analyzed by multiple-reaction monitoring LC-MS/MS. Multiple-reaction monitoring (*MRM*) transitions are indicated in the *panels*. *C*, effect of time and GDP-Man and enzyme concentration on the yield of GDP-Fuc by *Pf*GMD and *Pf*FS. Conditions tested are: 1 mM GDP-Man, 3 h of 1.5 mg/ml *Pf*GMD, and 2 h of 1.5 mg/ml *Pf*FS (*1*) (control); 0.5 mM GDP-Man, 3 h of 1.5 mg/ml *Pf*GMD, and 2 h of 1.5 mg/ml *Pf*FS (*2*); 1 mM GDP-Man, 1 h of 1.5 mg/ml *Pf*GMD, and 0.5 h of 1.5 mg/ml *Pf*FS (*3*); 1 mM GDP-Man, 3 h of 1 mg/ml *Pf*GMD, and 2 h of 1 mg/ml *Pf*FS (*4*); and 1 mM GDP-Man, 3 h of 1.5 mg/ml *Pf*GMD, and 2 h of 1 mg/ml *Pf*FS (*5*).

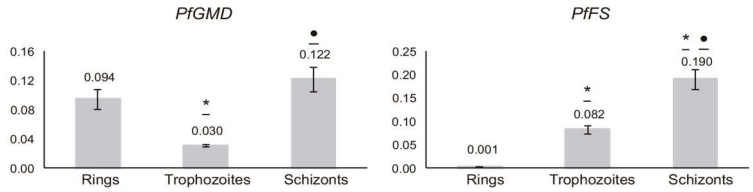


FIGURE 5. **Real-time quantitative RT-PCR analysis of *PfGMD* (*left*) and *PfFS* (*right*) at different stages of *P. falciparum* intraerythrocytic cycle.** Results are expressed in arbitrary units and normalized against seryl-tRNA synthetase. Data are representative of two independent RNA extractions per stage, assayed in triplicate. Statistically significant differences (one-way analysis of variance Tukey's post-test) between stages are shown with an *asterisk* (*, $p < 0.05$, rings *versus* trophozoites or schizonts) or a *dot* (●, $p < 0.05$, trophozoites *versus* schizonts).

genes through the whole intraerythrocytic cycle is ~10-fold lower than that of the seryl-tRNA synthetase housekeeping gene expression taken as a reference (48), with much lower *PfFS* levels in the ring stage (Fig. 5).

To assess the subcellular location of *Pf*GMD and *Pf*FS proteins, HA-tagged versions of the genes were transiently expressed under the control of the *crt* promoter using the pARL1a-3HA expression vector (41). Immunofluorescence microscopy using anti-HA antibodies produced a typical cytosolic distribution in the different stages of the intraerythrocytic life cycle (Fig. 6). To confirm this, we also stained the cells using anti-HSP70 antibody, a commonly used cytosolic marker (43). The *merged image* shows co-localization, indicating that the expressed HA-tagged *Pf*GMD and *Pf*FS are localized in the cytosol of *P. falciparum* through the different stages of the parasite asexual life cycle.

P. falciparum Schizonts Express a Protein o-Fucosyltransferase (PoFUT2) Homolog and Incorporate [^{3}H]GDP-Fuc in a Cell-free Assay—The *P. falciparum* genome presents a homolog of PoFUT2 that, in other organisms, is involved in the *O*-fucosylation of thrombospondin type 1 repeat (TSR) domains (50). Because this gene (PF3D7_0909200) is the most suitable candidate to encode for a fucosyltransferase activity, we performed real-time quantitative RT-PCR analysis of *PfPoFUT2* at the different blood stages of *P. falciparum*. Despite the low levels, a peak of expression was observed at the schizont phase of

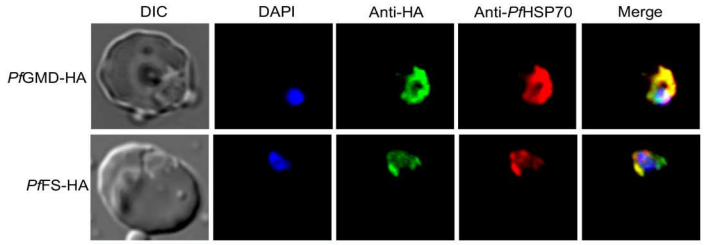


FIGURE 6. **Subcellular localization of *Pf*GMD and *Pf*FS expressed in *P. falciparum*.** *Pf*GMD-HA-expressing and *Pf*FS-HA-expressing trophozoites were labeled with anti-HA (*green*), anti-*Pf*HSP70 (*red*), and DAPI for nuclear staining (*blue*). The *first column* represents the differential interference contrast (*DIC*), and *last column* represents the merge of HA and *Pf*HSP70 signals. Similar co-localization was observed in other stages of the intraerythrocytic life cycle (not shown).

the parasite (Fig. 7*A*) coinciding with the maximum expression of *PfGMD* and *PfFS* genes. Furthermore, *P. falciparum* schizont extracts incorporated [^{3}H]GDP-Fuc, strongly suggesting that fucosylation processes are active in the late stages of the asexual life cycle of the parasite (Fig. 7*B*).

DISCUSSION

Carbohydrate structures that decorate the surface of cells play important roles in the biology of host-pathogen interactions. In the particular case of the malaria parasite, glycan structures associated with the parasite itself appear to be limited to GPI anchors (11, 51) and the recently described *N*-glycans (14). This seems to confirm the general trend of *Plasmodium* parasites to reduce several metabolic and biosynthetic pathways and reflects the process of evolving toward a parasitic niche (52). However, the parasite genome encodes orthologues for enzymes involved in the biosynthesis of sugar nucleotides not related to GPI anchor or *N*-glycan structures, which prompted us to survey the sugar nucleotide pools present in the asexual life stages of *P. falciparum*. Qualitatively, the sugar nucleotide content of *P. falciparum* obtained by LC-electrospray ionization-MS/MS agrees well with the presence of known or candidate sugar nucleotide biosynthesis enzymes (Fig. 1) (25), with the exception of the presence of a significant pool of UDP-Gal (see below). Quantitatively, the sugar nucleotide amounts observed are consistent with the increased metabolic activity of mature stage parasites (53, 54). The analysis performed, together with the information available in annotated *P. falciparum* metabolic databases (25), enabled us to thoroughly verify the presence of active sugar nucleotide metabolic routes during the parasite blood stages and, in some cases, add new evidence to support the annotations of genes likely to be involved in sugar nucleotide biosynthesis. The presence and conservation of these sugar nucleotide metabolic pathways in several *Plasmodium* genomes (46), together with the detection of their final products, are strong arguments to presume the existence of further downstream reactions in which these metabolites are involved and expose new active metabolic pathways accessible for the exploration of their biological function.

The identification of UDP-Gal is somewhat puzzling. The *P. falciparum* genome lacks candidate genes for a UDP-glucose 4-epimerase (EC 5.1.3.2) that can produce UDP-galactose via the epimerization of UDP-glucose or a galactose-1-phosphate uridylyl transferase activity (EC 2.7.7.12) (25). Thus, two main activities can be proposed for the production of this sugar nucleotide. Either an enzyme with UTP-glucose-1-phosphate uridylyltransferase activity (EC 2.7.7.9) presents a weak galactose-1-phosphate uridylyltransferase activity (EC 2.7.7.10), as described in mammals (55, 56), or a broad substrate range UDP-sugar pyrophosphorylase (EC 2.7.7.64) is present in the genome of *P. falciparum*, as described in plants or *Leishmania major* (57–61). UDP-sugar pyrophosphorylase is an enzyme that can nonspecifically utilize UTP and glucose 1-phosphate or galactose 1-phosphate to produce UDP-glucose or UDP-galactose and pyrophosphate. Interestingly, searches with functionally characterized UDP-sugar pyrophosphorylase orthologues from plants and *L. major* identify a match, PF3D7_0517500, with more than 30% similarity and an *e* value of $<10^{-55}$. However, although galactose competes for *P. falciparum* PfHT1 hexose permease (45) and its incorporation into the parasite galactolipids has been reported (62, 63), there is not a clear galactokinase candidate in the *P. falciparum* genome, and tritiated galactose is not significantly incorporated into the parasite proteins (23) or into the α-galactose moieties of digalactosyl diglycerides (64).

Bioinformatic analysis (25) and our own work suggest that UDP-GlcNAc, the direct donor for all GlcNAc transferases, is made in *P. falciparum* by the conventional eukaryotic *de novo* route from glucose 6-phosphate (Fig. 1), although a salvage pathway also exists, via the action of hexokinase (GlcN → GlcN-6-P) (22, 23). With more than 30% similarity and *e* values of $<10^{-20}$ to plant and human UDP-GlcNAc diphosphorylases (EC 2.7.7.23), PF3D7_1343600 is the most suitable candidate gene to encode this activity in the *P. falciparum* genome (Fig. 1 and Table 1), although redundant activity from PF3D7_0517500 might be expected. The lack of a candidate for a gene encoding for UDP-glucose 4-epimerase (EC 5.1.3.2) that could present a UDP-GlcNAc 4-epimerase activity (EC 5.1.3.7) to convert UDP-GlcNAc into UDP-GalNAc indicates that the parasite probably does not produce UDP-*N*-acetyl galactosamine, in agreement with the inability of the parasite to synthesize GalNAc and the absence of mucin-type *O*-glycosylation in *P. falciparum* (63, 65).

The main question regarding the UDP-GlcNAc biosynthetic pathway is the identity of a gene encoding for the glucosamine-phosphate *N*-acetyltransferase activity (EC 2.3.1.4 (25)). The identification of pools of UDP-GlcNAc in the parasite (Fig. 2),

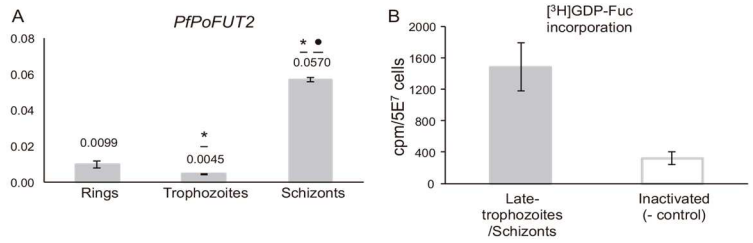


FIGURE 7. ***P. falciparum* late blood stages express *PfPoFUT2* and incorporate [^{3}H] GDP-fucose.** *A*, real-time quantitative RT-PCR results are expressed in arbitrary units and normalized against seryl-tRNA synthetase. Data are representative of two independent RNA extractions per stage, assayed in triplicate. Statistically significant differences (one-way analysis of variance Tukey's post-test) between stages are shown with an *asterisk* (*, $p < 0.05$, rings *versus* trophozoites or schizonts) or a *dot* (●, $p < 0.05$, trophozoites *versus* schizonts). *B*, detergent homogenates of late trophozoite/schizont stage parasites were incubated with [^{3}H]GDP-fucose before (*gray solid bar*) and after inactivation (*open bar*). The experiment was repeated two times on different days, and [^{3}H]GDP-fucose incorporation was assayed in triplicate. Data are expressed as mean values ± S.D. (*error bars*).

along with the labeling of GPI-anchored proteins using tritiated glucosamine (Fig. 3*C*) (23, 63), strongly suggests that the UDP-GlcNAc metabolic route (25) (Fig. 1) is active in *P. falciparum*. Most likely the UDP-GlcNAc *de novo* pathway from glucose is the most important *in vivo* for the parasite, because glucosamine is not an abundant free sugar in the mammalian or insect host. Because the glucosamine moiety, derived from GlcNAc by de-*N*-acetylation (27), is present in GPI structures that serve as membrane anchors for many important surface antigens of the parasite invasive stages (12, 66), the inability to identify a candidate gene encoding for the glucosamine-phosphate *N*-acetyltransferase activity is very intriguing because this pathway is potentially targetable for selective anti-malarial drug design.

The presence of the fucose donor GDP-Fuc in the intraerythrocytic stages of *P. falciparum* is not surprising, because the parasite contains homologues of the enzymes involved in the biosynthesis of this precursor from GDP-Man. We have shown that *Pf*GMD and *Pf*FS are active *in vitro* (Fig. 4), and they are expressed through the parasite's asexual life cycle (Fig. 5) (67). The GDP-Fuc pools in *P. falciparum* are, on average (Table 2), comparable with the pools of other protozoan parasites, such as trypanosomatids (30), including *T. brucei*, for which this metabolite is essential (36). The apparent lack of agreement with the amount of the GDP-Fuc pool in the schizont stage of the parasite when the *PfFS* gene is more expressed may be due to the specific demand of the metabolite at that stage. Because sugar nucleotides are donors for glycosylation reactions, the rates of turnover and the amount of the pools detected may change, reflecting their utilization. The localization of both enzymes in the cytoplasmic compartment (Fig. 6), as aldolase and other enzymes involved in the metabolism of carbohydrates (68), agrees well with the identification of a putative GDP-Fuc transporter in the *P. falciparum* endoplasmic reticulum/Golgi apparatus (69), where *N*- and *O*-glycosylation processes occur. The location and function of a putative fucose-containing glycan in *P. falciparum* remain enigmatic, and biosynthetic labeling with tritiated fucose is not feasible, because there is no prominent GDP-Fuc salvage pathway in the parasite, and/or hexose transporters do not efficiently take up fucose (Fig. 3). However, the labeling of parasite extracts after incubation with modified precursors opens a door to the identification of putative fucose-containing glycoconjugates (Fig. 7*B*). Interestingly, the C-terminal region of *P. falciparum* circumsporozoite surface protein (CS), in which the RTS,S malaria vaccine is based (70), and other proteins of the parasite (71) contain TSR domains. TSR domains are generally *O*-fucosylated in higher eukaryotes by PoFUT2, and the fucose residue can be further modified by the addition of β1–3 glucose (72–74). The C-terminal region of CS, containing the TSR domain, was recently expressed in HEK293T cells and structurally analyzed, showing the presence of a fucose and a glucose residue (75, 76). The expression of PoFUT2 in *P. falciparum* (Fig. 7*A*) and its conservation in other *Plasmodium* species raise the possibility of the presence of a mechanism of *O*-fucosylation of CS and other TSR-containing proteins in the parasite.

In summary, in this work, we have reported the first evidence of the presence of sugar nucleotides in the blood stages of *P. falciparum*, and we have described the active metabolic routes involved in their biosynthesis. In addition, we have characterized the *de novo* route of GDP-Fuc, a metabolite that may probably be involved in the biosynthesis of novel fucosylated glycans not yet described in the malaria parasite.

Acknowledgments—We thank Hernando A. del Portillo for support, reagents, and helpful scientific discussions throughout this project. We thank Mike Ferguson for helpful discussions and for access to the LC-MS/MS system to measure sugar nucleotides. We are grateful to I. H. Hallyburton, J. A. Nunes, M. Ramírez, and CCiTUB (Scientific and Technological Centers Universitat de Barcelona) for technical support, advice, and assistance. We also thank L. Martín-Jaular, A. Cortés, N. Rovira-Graells, and M. Llinás for useful suggestions and comments on the manuscript.

REFERENCES

1. World Health Organization (2011) *World Malaria Report 2011*, World Health Organization, Geneva
2. Price, R. N., Douglas, N. M., and Anstey, N. M. (2009) New developments in *Plasmodium vivax* malaria. Severe disease and the rise of chloroquine resistance. *Curr. Opin. Infect. Dis.* **22,** 430–435
3. Guinovart, C., Navia, M. M., Tanner, M., and Alonso, P. L. (2006) Malaria. Burden of disease. *Curr. Mol. Med.* **6,** 137–140
4. Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W.,

Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M. S., Nene, V., Shallom, S. J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W., Fraser, C. M., and Barrell, B. (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419,** 498–511

5. Sakata, T., and Winzeler, E. A. (2007) Genomics, systems biology, and drug development for infectious diseases. *Mol. Biosyst.* **3,** 841–848
6. Meissner, M., Agop-Nersesian, C., and Sullivan, W. J., Jr. (2007) Molecular tools for analysis of gene function in parasitic microorganisms. *Appl. Microbiol. Biotechnol.* **75,** 963–975
7. Ekland, E. H., and Fidock, D. A. (2007) Advances in understanding the genetic basis of antimalarial drug resistance. *Curr. Opin. Microbiol.* **10,** 363–370
8. Kooij, T. W., Janse, C. J., and Waters, A. P. (2006) Plasmodium post-genomics. Better the bug you know? *Nat. Rev. Microbiol.* **4,** 344–357
9. Greenwood, B. M., Fidock, D. A., Kyle, D. E., Kappe, S. H., Alonso, P. L., Collins, F. H., and Duffy, P. E. (2008) Malaria. Progress, perils, and prospects for eradication. *J. Clin. Invest.* **118,** 1266–1276
10. Ferguson, M. A., Homans, S. W., Dwek, R. A., and Rademacher, T. W. (1988) Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. *Science* **239,** 753–759
11. von Itzstein, M., Plebanski, M., Cooke, B. M., and Coppel, R. L. (2008) Hot, sweet and sticky. The glycobiology of *Plasmodium falciparum*. *Trends Parasitol.* **24,** 210–218
12. Sanders, P. R., Kats, L. M., Drew, D. R., O'Donnell, R. A., O'Neill, M., Maier, A. G., Coppel, R. L., and Crabb, B. S. (2006) A set of glycosylphosphatidyl inositol-anchored membrane proteins of *Plasmodium falciparum* is refractory to genetic deletion. *Infect. Immun.* **74,** 4330–4338
13. Delorenzi, M., Sexton, A., Shams-Eldin, H., Schwarz, R. T., Speed, T., and Schofield, L. (2002) Genes for glycosylphosphatidylinositol toxin biosynthesis in *Plasmodium falciparum*. *Infect. Immun.* **70,** 4510–4522
14. Bushkin, G. G., Ratner, D. M., Cui, J., Banerjee, S., Duraisingh, M. T., Jennings, C. V., Dvorin, J. D., Gubbels, M. J., Robertson, S. D., Steffen, M., O'Keefe, B. R., Robbins, P. W., and Samuelson, J. (2010) Suggestive evidence for Darwinian selection against asparagine-linked glycans of *Plasmodium falciparum* and *Toxoplasma gondii*. *Eukaryot. Cell* **9,** 228–241
15. Izquierdo, L., Atrih, A., Rodrigues, J. A., Jones, D. C., and Ferguson, M. A. (2009) *Trypanosoma brucei* UDP-glucose:glycoprotein glucosyltransferase has unusual substrate specificity and protects the parasite from stress. *Eukaryot. Cell* **8,** 230–240
16. Izquierdo, L., Schulz, B. L., Rodrigues, J. A., Güther, M. L., Procter, J. B., Barton, G. J., Aebi, M., and Ferguson, M. A. (2009) Distinct donor and acceptor specificities of *Trypanosoma brucei* oligosaccharyltransferases. *EMBO J.* **28,** 2650–2661
17. Banerjee, S., Vishwanath, P., Cui, J., Kelleher, D. J., Gilmore, R., Robbins, P. W., and Samuelson, J. (2007) The evolution of *N*-glycan-dependent endoplasmic reticulum quality control factors for glycoprotein folding and degradation. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 11676–11681
18. Samuelson, J., Banerjee, S., Magnelli, P., Cui, J., Kelleher, D. J., Gilmore, R., and Robbins, P. W. (2005) The diversity of dolichol-linked precursors to Asn-linked glycans probably results from secondary loss of sets of glycosyltransferases. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 1548–1553
19. Dayal-Drager, R., Hoessli, D. C., Decrind, C., Del Guidice, G., Lambert, P. H., and Nasir-ud-Din (1991) Presence of *O*-glycosylated glycoproteins in the *Plasmodium falciparum* parasite. *Carbohydr. Res.* **209,** c5–c8
20. Nasir-ud-Din, Drager-Dayal, R., Decrind, C., Hu, B. H., Del Giudice, G., and Hoessli, D. (1992) *Plasmodium falciparum* synthesizes *O*-glycosylated glycoproteins containing *O*-linked *N*-acetylglucosamine. *Biochem. Int.* **27,** 55–64
21. Nasir-un-Din, Hassan, M., Qazi, M. H., Fayyazuddin, Sinaldi, G., Hoessli, D., and Walker-Nasir, E. (1992) *Plasmodium falciparum* synthesizes 43000 daltons protein containing *O*-linked glucosamine. *Biochem. Soc. Trans.* **20,** 388S
22. Dieckmann-Schuppert, A., Bender, S., Odenthal-Schnittler, M., Bause, E., and Schwarz, R. T. (1992) Apparent lack of *N*-glycosylation in the asexual intraerythrocytic stage of *Plasmodium falciparum*. *Eur. J. Biochem.* **205,** 815–825
23. Gowda, D. C., Gupta, P., and Davidson, E. A. (1997) Glycosylphosphatidylinositol anchors represent the major carbohydrate modification in proteins of intraerythrocytic stage *Plasmodium falciparum*. *J. Biol. Chem.* **272,** 6428–6439
24. Kimura, E. A., Couto, A. S., Peres, V. J., Casal, O. L., and Katzin, A. M. (1996) *N*-Linked glycoproteins are related to schizogony of the intraerythrocytic stage in *Plasmodium falciparum*. *J. Biol. Chem.* **271,** 14452–14461
25. Ginsburg, H. (2006) Progress in *in silico* functional genomics. The malaria metabolic pathways database. *Trends Parasitol.* **22,** 238–240
26. Stoeckert, C. J., Jr., Fischer, S., Kissinger, J. C., Heiges, M., Aurrecoechea, C., Gajria, B., and Roos, D. S. (2006) PlasmoDB v5. New looks, new genomes. *Trends Parasitol.* **22,** 543–546
27. Ferguson, M. A. (1999) The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. *J. Cell Sci.* **112,** 2799–2809
28. Trager, W., and Jensen, J. B. (1976) Human malaria parasites in continuous culture. *Science* **193,** 673–675
29. Di Girolamo, F., Raggi, C., Birago, C., Pizzi, E., Lalle, M., Picci, L., Pace, T., Bachi, A., de Jong, J., Janse, C. J., Waters, A. P., Sargiacomo, M., and Ponzi, M. (2008) Plasmodium lipid rafts contain proteins implicated in vesicular trafficking and signalling as well as members of the PIR superfamily, potentially implicated in host immune system interactions. *Proteomics* **8,** 2500–2513
30. Turnock, D. C., and Ferguson, M. A. (2007) Sugar nucleotide pools of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*. *Eukaryot. Cell* **6,** 1450–1463
31. Räbinä, J., Mäki, M., Savilahti, E. M., Järvinen, N., Penttilä, L., and Renkonen, R. (2001) Analysis of nucleotide sugars from cell lysates by ion-pair solid-phase extraction and reversed-phase high-performance liquid chromatography. *Glycoconj. J.* **18,** 799–805
32. Gerold, P., Dieckmann-Schuppert, A., and Schwarz, R. T. (1994) Glycosylphosphatidylinositols synthesized by asexual erythrocytic stages of the malarial parasite, *Plasmodium falciparum*. Candidates for plasmodial glycosylphosphatidylinositol membrane anchor precursors and pathogenicity factors. *J. Biol. Chem.* **269,** 2597–2606
33. Azzouz, N., de Macedo, C. S., Ferguson, M. A., Smith, T. K., and Schwarz, R. T. (2005) Mannosamine can replace glucosamine in glycosylphosphatidylinositols of *Plasmodium falciparum in vitro*. *Mol. Biochem. Parasitol.* **142,** 12–24
34. Azzouz, N., Gerold, P., and Schwarz, R. T. (2008) Metabolic labeling and structural analysis of glycosylphosphatidylinositols from parasitic protozoa. *Methods Mol. Biol.* **446,** 183–198
35. Williams, M., Sprenger, J., Human, E., Al-Karadaghi, S., Persson, L., Louw, A. I., and Birkholtz, L. M. (2011) Biochemical characterisation and novel classification of monofunctional *S*-adenosylmethionine decarboxylase of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **180,** 17–26
36. Turnock, D. C., Izquierdo, L., and Ferguson, M. A. (2007) The *de novo* synthesis of GDP-fucose is essential for flagellar adhesion and cell growth in *Trypanosoma brucei*. *J. Biol. Chem.* **282,** 28853–28863
37. Rhomberg, S., Fuchsluger, C., Rendić, D., Paschinger, K., Jantsch, V., Kosma, P., and Wilson, I. B. (2006) Reconstitution in vitro of the GDP-fucose biosynthetic pathways of *Caenorhabditis elegans* and *Drosophila melanogaster*. *FEBS J.* **273,** 2244–2256
38. Sullivan, F. X., Kumar, R., Kriz, R., Stahl, M., Xu, G. Y., Rouse, J., Chang, X. J., Boodhoo, A., Potvin, B., and Cumming, D. A. (1998) Molecular cloning of human GDP-mannose 4,6-dehydratase and reconstitution of GDP-fucose biosynthesis *in vitro*. *J. Biol. Chem.* **273,** 8193–8202
39. Pabst, M., Grass, J., Fischl, R., Léonard, R., Jin, C., Hinterkörner, G., Borth, N., and Altmann, F. (2010) Nucleotide and nucleotide sugar analysis by liquid chromatography-electrospray ionization-mass spectrometry on surface-conditioned porous graphitic carbon. *Anal. Chem.* **82,** 9782–9788
40. Bernabeu, M., Lopez, F. J., Ferrer, M., Martin-Jaular, L., Razaname, A., Corradin, G., Maier, A. G., Del Portillo, H. A., and Fernandez-Becerra, C. (2012) Functional analysis of *Plasmodium vivax* VIR proteins reveals dif-

ferent subcellular localizations and cytoadherence to the ICAM-1 endothelial receptor. *Cell Microbiol.* **14,** 386–400
41. Crabb, B. S., Rug, M., Gilberger, T. W., Thompson, J. K., Triglia, T., Maier, A. G., and Cowman, A. F. (2004) Transfection of the human malaria parasite *Plasmodium falciparum*. *Methods Mol. Biol.* **270,** 263–276
42. Tonkin, C. J., van Dooren, G. G., Spurck, T. P., Struck, N. S., Good, R. T., Handman, E., Cowman, A. F., and McFadden, G. I. (2004) Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. *Mol. Biochem. Parasitol.* **137,** 13–21
43. Pesce, E. R., Acharya, P., Tatu, U., Nicoll, W. S., Shonhai, A., Hoppe, H. C., and Blatch, G. L. (2008) The *Plasmodium falciparum* heat shock protein 40, Pfj4, associates with heat shock protein 70 and shows similar heat induction and localisation patterns. *Int. J. Biochem. Cell Biol.* **40,** 2914–2926
44. Geary, T. G., Divo, A. A., Bonanni, L. C., and Jensen, J. B. (1985) Nutritional requirements of *Plasmodium falciparum* in culture. III. Further observations on essential nutrients and antimetabolites. *J. Protozool.* **32,** 608–613
45. Woodrow, C. J., Burchmore, R. J., and Krishna, S. (2000) Hexose permeation pathways in *Plasmodium falciparum*-infected erythrocytes. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 9931–9936
46. Aurrecoechea, C., Brestelli, J., Brunk, B. P., Dommer, J., Fischer, S., Gajria, B., Gao, X., Gingle, A., Grant, G., Harb, O. S., Heiges, M., Innamorato, F., Iodice, J., Kissinger, J. C., Kraemer, E., Li, W., Miller, J. A., Nayak, V., Pennington, C., Pinney, D. F., Roos, D. S., Ross, C., Stoeckert, C. J., Jr., Treatman, C., and Wang, H. (2009) PlasmoDB. A functional genomic database for malaria parasites. *Nucleic Acids Res.* **37,** D539–D543
47. Tonetti, M., Zanardi, D., Gurnon, J. R., Fruscione, F., Armirotti, A., Damonte, G., Sturla, L., De Flora, A., and Van Etten, J. L. (2003) *Paramecium bursaria* Chlorella virus 1 encodes two enzymes involved in the biosynthesis of GDP-L-fucose and GDP-D-rhamnose. *J. Biol. Chem.* **278,** 21559–21565
48. Bozdech, Z., Llinás, M., Pulliam, B. L., Wong, E. D., Zhu, J., and DeRisi, J. L. (2003) The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. *PLoS Biol.* **1,** E5
49. Rovira-Graells, N., Gupta, A. P., Planet, E., Crowley, V. M., Mok, S., Ribas de Pouplana, L., Preiser, P. R., Bozdech, Z., and Cortés, A. (2012) Transcriptional variation in the malaria parasite *Plasmodium falciparum*. *Genome Res.* **22,** 925–938
50. Luo, Y., Koles, K., Vorndam, W., Haltiwanger, R. S., and Panin, V. M. (2006) Protein *O*-fucosyltransferase 2 adds *O*-fucose to thrombospondin type 1 repeats. *J. Biol. Chem.* **281,** 9393–9399
51. Mendonça-Previato, L., Todeschini, A. R., Heise, N., and Previato, J. O. (2005) Protozoan parasite-specific carbohydrate structures. *Curr. Opin. Struct. Biol.* **15,** 499–505
52. Olszewski, K. L., and Llinás, M. (2011) Central carbon metabolism of *Plasmodium* parasites. *Mol. Biochem. Parasitol.* **175,** 95–103
53. Rosenthal, P. J. (2002) Hydrolysis of erythrocyte proteins by proteases of malaria parasites. *Curr. Opin. Hematol.* **9,** 140–145
54. Roth, E., Jr. (1990) *Plasmodium falciparum* carbohydrate metabolism. A connection between host cell and parasite. *Blood Cells* **16,** 453–460; discussion 461–456
55. Isselbacher, K. J. (1958) A mammalian uridinediphosphate galactose pyrophosphorylase. *J. Biol. Chem.* **232,** 429–444
56. Leslie, N., Yager, C., Reynolds, R., and Segal, S. (2005) UDP-galactose pyrophosphorylase in mice with galactose-1-phosphate uridyltransferase deficiency. *Mol. Genet. Metab.* **85,** 21–27
57. Damerow, S., Lamerz, A. C., Haselhorst, T., Führing, J., Zarnovican, P., von Itzstein, M., and Routier, F. H. (2010) *Leishmania* UDP-sugar pyrophosphorylase. The missing link in galactose salvage? *J. Biol. Chem.* **285,** 878–887
58. Dickmanns, A., Damerow, S., Neumann, P., Schulz, E. C., Lamerz, A. C., Routier, F. H., and Ficner, R. (2011) Structural basis for the broad substrate range of the UDP-sugar pyrophosphorylase from *Leishmania major*. *J. Mol. Biol.* **405,** 461–478
59. Lamerz, A. C., Damerow, S., Kleczka, B., Wiese, M., van Zandbergen, G., Lamerz, J., Wenzel, A., Hsu, F. F., Turk, J., Beverley, S. M., and Routier, F. H. (2010) Deletion of UDP-glucose pyrophosphorylase reveals a UDP-glucose independent UDP-galactose salvage pathway in *Leishmania major*. *Glycobiology* **20,** 872–882
60. Litterer, L. A., Schnurr, J. A., Plaisance, K. L., Storey, K. K., Gronwald, J. W., and Somers, D. A. (2006) Characterization and expression of *Arabidopsis* UDP-sugar pyrophosphorylase. *Plant Physiol. Biochem.* **44,** 171–180
61. Kotake, T., Hojo, S., Yamaguchi, D., Aohara, T., Konishi, T., and Tsumuraya, Y. (2007) Properties and physiological functions of UDP-sugar pyrophosphorylase in *Arabidopsis*. *Biosci. Biotechnol. Biochem.* **71,** 761–771
62. Maréchal, E., Azzouz, N., de Macedo, C. S., Block, M. A., Feagin, J. E., Schwarz, R. T., and Joyard, J. (2002) Synthesis of chloroplast galactolipids in apicomplexan parasites. *Eukaryot. Cell* **1,** 653–656
63. Dieckmann-Schuppert, A., Bender, S., Holder, A. A., Haldar, K., and Schwarz, R. T. (1992) Labeling and initial characterization of polar lipids in cultures of *Plasmodium falciparum*. *Parasitol. Res.* **78,** 416–422
64. Ramasamy, R., and Field, M. C. (2012) Terminal galactosylation of glycoconjugates in *Plasmodium falciparum* asexual blood stages and *Trypanosoma brucei* bloodstream trypomastigotes. *Exp. Parasitol.* **130,** 314–320
65. Dieckmann-Schuppert, A., Bause, E., and Schwarz, R. T. (1993) Studies on *O*-glycans of *Plasmodium falciparum*-infected human erythrocytes. Evidence for *O*-GlcNAc and *O*-GlcNAc-transferase in malaria parasites. *Eur. J. Biochem.* **216,** 779–788
66. Gilson, P. R., Nebl, T., Vukcevic, D., Moritz, R. L., Sargeant, T., Speed, T. P., Schofield, L., and Crabb, B. S. (2006) Identification and stoichiometry of glycosylphosphatidylinositol-anchored membrane proteins of the human malaria parasite *Plasmodium falciparum*. *Mol. Cell Proteomics* **5,** 1286–1299
67. Otto, T. D., Wilinski, D., Assefa, S., Keane, T. M., Sarry, L. R., Böhme, U., Lemieux, J., Barrell, B., Pain, A., Berriman, M., Newbold, C., and Llinás, M. (2010) New insights into the blood-stage transcriptome of *Plasmodium falciparum* using RNA-Seq. *Mol. Microbiol.* **76,** 12–24
68. Bhowmick, I. P., Kumar, N., Sharma, S., Coppens, I., and Jarori, G. K. (2009) *Plasmodium falciparum* enolase. Stage-specific expression and sub-cellular localization. *Malaria J.* **8,** 179
69. Martin, R. E., Ginsburg, H., and Kirk, K. (2009) Membrane transport proteins of the malaria parasite. *Mol. Microbiol.* **74,** 519–528
70. Agnandji, S. T., Lell, B., Soulanoudjingar, S. S., Fernandes, J. F., Abossolo, B. P., Conzelmann, C., Methogo, B. G., Doucka, Y., Flamen, A., Mordmüller, B., Issifou, S., Kremsner, P. G., Sacarlal, J., Aide, P., Lanaspa, M., Aponte, J. J., Nhamuave, A., Quelhas, D., Bassat, Q., Mandjate, S., Macete, E., Alonso, P., Abdulla, S., Salim, N., Juma, O., Shomari, M., Shubis, K., Machera, F., Hamad, A. S., Minja, R., Mtoro, A., Sykes, A., Ahmed, S., Urassa, A. M., Ali, A. M., Mwangoka, G., Tanner, M., Tinto, H., D'Alessandro, U., Sorgho, H., Valea, I., Tahita, M. C., Kaboré, W., Ouédraogo, S., Sandrine, Y., Guiguemdé, R. T., Ouédraogo, J. B., Hamel, M. J., Kariuki, S., Odero, C., Oneko, M., Otieno, K., Awino, N., Omoto, J., Williamson, J., Muturi-Kioi, V., Laserson, K. F., Slutsker, L., Otieno, W., Otieno, L., Nekoye, O., Gondi, S., Otieno, A., Ogutu, B., Wasuna, R., Owira, V., Jones, D., Onyango, A. A., Njuguna, P., Chilengi, R., Akoo, P., Kerubo, C., Gitaka, J., Maingi, C., Lang, T., Olotu, A., Tsofa, B., Bejon, P., Peshu, N., Marsh, K., Owusu-Agyei, S., Asante, K. P., Osei-Kwakye, K., Boahen, O., Ayamba, S., Kayan, K., Owusu-Ofori, R., Dosoo, D., Asante, I., Adjei, G., Chandramohan, D., Greenwood, B., Lusingu, J., Gesase, S., Malabeja, A., Abdul, O., Kilavo, H., Mahende, C., Liheluka, E., Lemnge, M., Theander, T., Drakeley, C., Ansong, D., Agbenyega, T., Adjei, S., Boateng, H. O., Rettig, T., Bawa, J., Sylverken, J., Sambian, D., Agyekum, A., Owusu, L., Martinson, F., Hoffman, I., Mvalo, T., Kamthunzi, P., Nkomo, R., Msika, A., Jumbe, A., Chome, N., Nyakuipa, D., Chintedza, J., Ballou, W. R., Bruls, M., Cohen, J., Guerra, Y., Jongert, E., Lapierre, D., Leach, A., Lievens, M., Ofori-Anyinam, O., Vekemans, J., Carter, T., Leboulleux, D., Loucq, C., Radford, A., Savarese, B., Schellenberg, D., Sillman, M., Vansadia, P., and RTS,S Clinical Trials Partnership (2011) First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. *N. Engl. J. Med.* **365,** 1863–1875
71. Morahan, B. J., Wang, L., and Coppel, R. L. (2009) No TRAP, no invasion. *Trends Parasitol.* **25,** 77–84
72. Tan, K., Duquette, M., Liu, J. H., Dong, Y., Zhang, R., Joachimiak, A.,

Lawler, J., and Wang, J. H. (2002) Crystal structure of the TSP-1 type 1 repeats. A novel layered fold and its biological implication. *J. Cell Biol.* **159,** 373–382
73. Hofsteenge, J., Huwiler, K. G., Macek, B., Hess, D., Lawler, J., Mosher, D. F., and Peter-Katalinic, J. (2001) *C*-Mannosylation and *O*-fucosylation of the thrombospondin type 1 module. *J. Biol. Chem.* **276,** 6485–6498
74. Kozma, K., Keusch, J. J., Hegemann, B., Luther, K. B., Klein, D., Hess, D., Haltiwanger, R. S., and Hofsteenge, J. (2006) Identification and characterization of abeta1,3-glucosyltransferase that synthesizes the Glc-β1,3-Fuc disaccharide on thrombospondin type 1 repeats. *J. Biol. Chem.* **281,** 36742–36751
75. Doud, M. B., Koksal, A. C., Mi, L. Z., Song, G., Lu, C., and Springer, T. A. (2012) Unexpected fold in the circumsporozoite protein target of malaria vaccines. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 7817–7822
76. Song, G., Koksal, A. C., Lu, C., and Springer, T. A. (2012) Shape change in the receptor for gliding motility in *Plasmodium* sporozoites. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 21420–21425
77. Aoki, K., Tanaka, N., Kusakabe, Y., Fukumi, C., Haga, A., Nakanishi, M., Kitade, Y., and Nakamura, K. T. (2010) Crystallization and preliminary x-ray crystallographic study of phosphoglucose isomerase from *Plasmodium falciparum*. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **66,** 333–336

2. Article 2

"The disruption of GDP-fucose de novo biosynthesis suggests the presence of a novel fucose – containing glycoconjugate in *Plasmodium* asexual blood stages"

Sílvia Sanz, Borja López-Gutiérrez, Giulia Bandini, Sebastian Damerow, Sabrina Absalon, Rhoel R. Dinglasan, John Samuelson and Luis Izquierdo

Scientific Reports, 6 (2016), srep37230

SCIENTIFIC REPORTS

OPEN

Received: 21 July 2016
Accepted: 03 October 2016
Published: 16 November 2016

The disruption of GDP-fucose de novo biosynthesis suggests the presence of a novel fucose-containing glycoconjugate in *Plasmodium* asexual blood stages

Sílvia Sanz[1,*], Borja López-Gutiérrez[1,*], Giulia Bandini[2], Sebastian Damerow[3], Sabrina Absalon[4], Rhoel R. Dinglasan[5], John Samuelson[2] & Luis Izquierdo[1]

Glycosylation is an important posttranslational protein modification in all eukaryotes. Besides glycosylphosphatidylinositol (GPI) anchors and *N*-glycosylation, *O*-fucosylation has been recently reported in key sporozoite proteins of the malaria parasite. Previous analyses showed the presence of GDP-fucose (GDP-Fuc), the precursor for all fucosylation reactions, in the blood stages of *Plasmodium falciparum*. The GDP-Fuc *de novo* pathway, which requires the action of GDP-mannose 4,6-dehydratase (GMD) and GDP-L-fucose synthase (FS), is conserved in the parasite genome, but the importance of fucose metabolism for the parasite is unknown. To functionally characterize the pathway we generated a *PfGMD* mutant and analyzed its phenotype. Although the labelling by the fucose-binding *Ulex europaeus* agglutinin I (UEA-I) was completely abrogated, GDP-Fuc was still detected in the mutant. This unexpected result suggests the presence of an alternative mechanism for maintaining GDP-Fuc in the parasite. Furthermore, *PfGMD* null mutant exhibited normal growth and invasion rates, revealing that the GDP-Fuc *de novo* metabolic pathway is not essential for the development in culture of the malaria parasite during the asexual blood stages. Nonetheless, the function of this metabolic route and the GDP-Fuc pool that is generated during this stage may be important for gametocytogenesis and sporogonic development in the mosquito.

Protozoan parasites of the genus *Plasmodium* are the causative agents of human malaria, the most lethal being *Plasmodium falciparum*. Malaria is a global disease that presents symptoms ranging from fever and headaches to seizures, coma and death. Despite all the efforts to control malaria worldwide, 200 million malaria cases that led to more than half a million malaria deaths were reported in 2013[1]. Chemotherapy remains the most important tool for malaria control, although there are several vaccine candidates currently under evaluation[2] and one of them has received regulatory approval[3].

The life cycle of the parasite is highly complex, including development in two divergent hosts and in different tissue compartments. *Plasmodium* is transmitted to humans by the bite of infected female *Anopheles* mosquitoes. During a blood meal, sporozoites from the mosquito salivary glands are injected to humans and initiate infection in the liver. Parasites are then released from the liver into the bloodstream, where they invade red blood cells and reproduce asexually. A fraction (1–2%) of the parasites released from infected red blood cells develop into sexual

[1]ISGlobal, Barcelona Ctr. Int. Health Res. (CRESIB), Hospital Clínic - Universitat de Barcelona, Barcelona, Spain. [2]Department of Molecular and Cell Biology, Boston University Goldman School of Dental Medicine, 72 East Concord St, Boston, MA 02118, USA. [3]Division of Biological Chemistry and Drug Discovery, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom. [4]Division of Infectious Diseases, Boston Children's Hospital and Harvard Medical School, Boston MA 02115, USA. [5]The University of Florida Emerging Pathogens Institute, Department of Infectious Diseases & Pathology, Gainesville FL 32611, USA. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to L.I. (email: luis.izquierdo@isglobal.org)

stage gametocytes and are picked up by female *Anopheles* to initiate the sporogonic life cycle, resulting in the generation of sporozoite infection of mosquito salivary glands[4].

Cell-surface glycoconjugates are important mediators of host-pathogen interactions for many microbes, including protozoan parasites[5]. Glycosylphosphatidylinositol (GPI) anchors are the major glycosylated molecules on the surface of the *Plasmodium* parasite[6,7]. Several GPI-anchored glycoproteins are essential for parasite invasion and virulence[8] and parasite-derived GPIs, free or associated with protein, induce pro-inflammatory responses that contribute to the pathogenesis of malaria[9,10]. The presence of *N*-glycosylation of *P. falciparum* proteins has been a controversial issue for years[11,12] that was recently solved by the characterization of very short *N*-glycans composed of one or two residues of *N*-acetylglucosamine (GlcNAc)[13,14]. However, the extent of protein *N*-glycosylation in the blood stages of the parasite seems to be rather low[15]. Furthermore, the existence of *O*-glycosylation and *C*-mannosylation in the parasite has been recently reported for the sporozoite stages[16].

Sugar nucleotides are activated forms of sugars composed of a monosaccharide and a nucleotide moiety. These molecules are formed by either a salvage pathway, involving "activation" of the sugar using a kinase and a pyrophosphorylase, or by a *de novo* pathway, involving the bioconversion of an existing sugar or sugar nucleotide. These precursors feed the biosynthesis of glycans by acting as donors for glycosylation reactions[17]. We recently analysed the sugar nucleotide pool in asexuals and found that *P. falciparum* synthesises five different sugar nucleotides including GDP-fucose (GDP-Fuc) or UDP-galactose (UDP-Gal), both of which are not involved in the biosynthesis of the aforementioned GPI-anchors or *N*-glycans[18]. Therefore, the presence of these metabolites suggests that they may be involved in the biosynthesis of glycans not yet characterised in the parasite[19]. For example UDP-Gal may be required for the synthesis of the α-galactosyl epitopes recently identified in sporozoites[20], which seem to be absent in the parasite asexual blood stages[21]. Similarly, GDP-Fuc could be involved in the posttranslational modification of thrombospondin type I repeat (TSR) domains present in several key proteins of the malaria parasite[16,22].

In this work we analysed the functional role of the GDP-Fuc *de novo* biosynthetic pathway in the blood stages of *P. falciparum* by creating null mutants for GDP-mannose 4,6-dehydratase (GMD) and GDP-L-fucose synthase (FS). This metabolic route is not essential for the survival of the parasite in culture. Therefore we analysed the role of GDP-Fuc *de novo* biosynthesis by characterizing the phenotype of the null mutants *in vitro*.

Materials and Methods

Transfection constructs. To disrupt *Pf*GMD we generated a transfection construct based in the pHH1plasmid. pHH1-*Pf*GMD consisted of a ~500 bp sequence, from the start of the gene and without the first 2 nucleotides (AT), cloned into pHH1inv, a plasmid derived from pHH1 that excludes both promoter and termination sequences[23,24]. As a result of a single crossover homologous recombination event a disrupted copy of *GMD* and a copy without the first 2 nucleotides would be generated in parasites (Fig. 1A). The PCR product amplified using primers CGCGGTACCGCGAGTTGCTTTAATCTTCG and CACCCGCGGCCGCTTATATGATTCTCTATAATTTATTG was cloned into KpnI/NotI restriction sites (underlined) of plasmid pHH1inv.

To confirm our results, we also disrupted *Pf*FS using a pCC1-based construct. pCC1-*Pf*FS consisted of two fragments of ~1 kb and ~800 bp, respectively, from different regions of the *Pf*FS locus. F1 (nucleotides from −492 to +526 of the *Pf*FS locus) was amplified using primers ATCCCGCGGGTGTATAATATGTTGGTATAC and ATCACTAGTGTTAATGGTAGAGAACAATTTAC and cloned into SacII/SpeI restriction sites (underlined) of pCC1 plasmid[25]. Similarly, F2 (nucleotides 830 to 1584 of *Pf*FS locus) was amplified using primers AATCCCATGGACGAATGTATATCTTTTGGG and CTACCTAGGGATCTAATATCTTTATGAGATG and cloned into NcoI/AvrII restriction sites (underlined) of the same plasmid. The construct generated would integrate into *FS* disrupting it by double crossover homologous recombination (Supplementary Figure S1A)[25].

Southern blotting. Genomic DNA (gDNA) was isolated by Phenol/Chloroform method from ~600 µl of red blood cells infected with >5% late trophozoites/schizonts. Two µg of 3D7- and 3D7ΔGMD-gDNA were digested with EcoRI and HindIII in separate reactions and probed with ^{32}P (Perkin Elmer)-labelled *Pf*GMD. Similarly, 2 µg of 3D7-, SRΔFS- and 3D7ΔFS gDNA were digested with KpnI and HindIII or EcoRI and probed with ^{32}P labelled F1 or F2 probes.

Parasite culture and transfection. *P. falciparum* 3D7 (obtained from MR4-ATCC) parasites were cultured with human B^+ erythrocytes (2–4% hematocrit) in RPMI medium (Sigma) supplemented with 10% AB^+ human serum or 0.5% Albumax II, incubated at 37 °C in an atmosphere of 92% N_2, 3% O_2 and 5% CO_2 using standard methods[26]. Human erythrocytes and serum were purchased from the Banc de Sang i Teixits (Catalonia, Spain), after approval from the Comitè Ètic Investigació Clínica Hospital Clínic de Barcelona. Parasite growth was monitored by counting the infected erythrocytes in Giemsa-stain blood smears by light microscopy. *P. falciparum* 3D7 parasites were transfected as described previously[23]. Briefly, 150 µg of each plasmid was used to electroporate (310 V, 950 millifarads) 200 µl of infected red blood cells at >5% parasitemia, synchronised for ring stage parasites. Transfected parasites were selected on 2 nM of WR99210 drug, and resistant parasites appeared in culture from 25 to 35 days after drug application. After the appearance of resistant parasites drug cycling with WR99210 was started. Clonal parasite lines were then derived from WR99210 resistant populations by limiting dilution. To calculate growth curves, tightly synchronised parasites were adjusted to 0.5% and measured by FACS. After 48 h and 96 h, parasitemia was again determined by FACS using SYTO 11 as previously described[27].

Heat-shock experiments. To measure survival to heat-shock[28] parasites were sorbitol-synchronised and parasitemia adjusted to 1%. Heat shock was performed 22 h after sorbitol treatment by transferring cultures to an

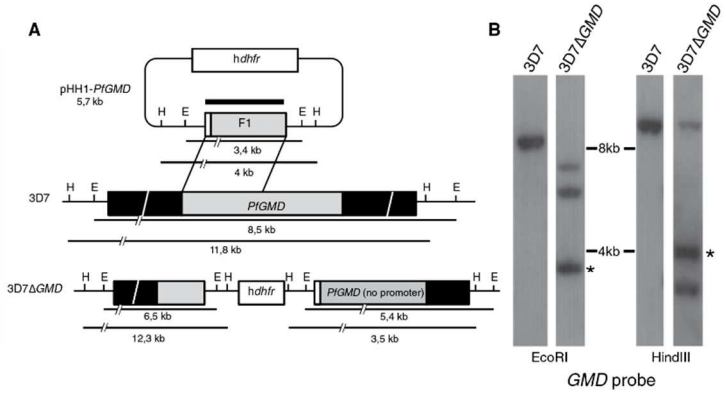


Figure 1. *PfGMD* transfection construct and integration events. (A) Schematic representation of the transfection plasmid (pHH1-*Pf*GMD) used to target and disrupt *GMD* gene in *P. falciparum* 3D7 parasites (3D7) and the expected recombination event (3D7Δ*GMD*). The small white box in front of F1 fragment represents the absence of the first two 'AT' nucleotides in the start codon. The black boxes represent upstream and downstream DNA sequence flanking the *PfGMD* gene locus. The position of HindIII (H) and EcoRI (E) restriction sites, the position of *GMD* probe (thick black line) and the predicted length of the restriction fragments (thin black lines) are shown. **(B)** Southern Blot analysis of EcoRI and HindIII digested genomic DNA from 3D7 and 3D7Δ*GMD* parasites. Hybridisation of *GMD* probe to digested DNA from 3D7Δ*GMD* revealed restriction fragment sizes consistent with the disruption of *PfGMD* by the single recombination of the plasmid. *Matches with the expected size of the episomic plasmid copy. The panel shows a cropped blot and full-length blot is shown in Supplementary data (Supplementary Fig. S3).

incubator at 41.5 °C for 3 h. Control cultures were maintained in a 37 °C incubator all the time. Parasitemia was determined by FACS at the next generation when all the schizonts had burst. Resistance to temperature stress was calculated by measuring the percentage of growth relative to identical cultures not subjected to heat-shock.

Sugar nucleotide analysis. We synchronised the asexual cultures with sorbitol (or by combining Percoll and sorbitol treatments) and performed osmotic lysis of the trophozoite-infected red cells at ~35 h post-invasion and 10–12% parasitemia. Briefly, infected and uninfected erythrocyte pellets were resuspended twice in 60 volumes of erythrocyte lysis buffer (10× stock solution: 0.15 M NH_4Cl, 0.1 M $KHCO_3$, 0.01 M EDTA) and then incubated on ice for ~10 mins until lysis was completed (31). Pellets, consisting of free parasites and/or erythrocyte ghosts, were washed three times with cold phosphate-buffered saline (PBS), lysed in 70% ethanol in the presence of 20 pmols of GDP-glucose (GDP-Glc) internal standard, and sugar nucleotides were extracted using Envi-Carb (Supelco) columns[29,30]. Sugar nucleotides were then analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS) and multiple-reaction monitoring (MRM) using a TSQ Quantiva Triple Quadrupole Mass Spectrometer (Thermo Scientific) or a QTRAP 6500 System (Sciex). A C18 reversed-phase column was used for sugar nucleotide separation with a gradient from 0.5% to 4% acetonitrile in 20 mM triethylammonium acetate buffer over 20 min at a flow rate of 25 µl/min. Sugar nucleotides were identified by their diagnostic MRM transitions[18]. The peak areas for each sugar nucleotide, along with their empirically determined molar relative response factors and the known amount of internal GDP-Glc, were used to quantify sugar nucleotides. Analyses were performed on three different sugar nucleotide extracts[30].

Uptake assays using tritiated sugars and GDP-Fuc. 3D7 and *PfGMD* null mutant parasite cultures at 9–12% parasitemia were synchronized to the ring state and then washed and resuspended in RPMI medium supplemented with Albumax II. Cells were incubated at 3–4% hematocrit for 16–18 h, until the trophozoite stage. Cultures were then treated with saponin to release parasites and washed with glucose-free RPMI supplemented with Albumax II and 20 mM fructose. Free parasites were metabolically labeled with ^{3}H-fucose (50 µCi/ml) and GDP-[^{3}H]Fuc (30 µCi/ml) for 2 h, adapting the conditions used in previous works[31]. The same experiments were also repeated with infected RBCs not treated with saponin[32]. ^{3}H-glucosamine and ^{3}H-L-glucose were used as positive and negative control, respectively[32,33]. Triplicates of each condition, and duplicates in the case of GDP-[^{3}H] Fuc, were included. Labeled parasites were washed three times with PBS at 4 °C and lysed, and the radioactivity was measured by liquid scintillation counting.

Fluorescence microscopy. Cultured wild type and mutant *P. falciparum* lines were washed in PBS and fixed in 4% PFA and 0.075% glutaraldehyde in PBS overnight at 4 °C. Fixed cells were washed, permeabilised in

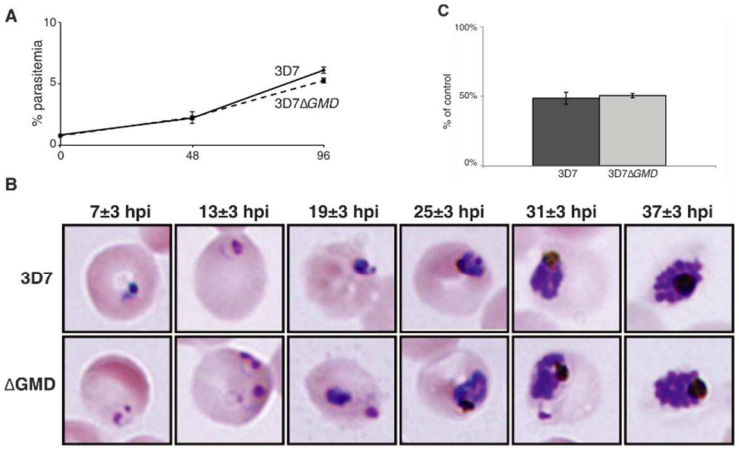


Figure 2. ***P. falciparum*** **3D7Δ*GMD* grow at comparable rates to 3D7 control cell lines.** (**A**) Synchronous ring-stage 3D7Δ*GMD* and 3D7 growth was monitored over two complete life cycles (96h) by flow cytometry. (**B**) Intraerythrocytic development (x1000 magnification) of wild-type (3D7) and *Pf*GMD (ΔGMD) null mutants of *P. falciparum* 3D7 strain. Time indicates hours post-invasion of tightly (5 hours window) synchronised *in vitro* cultures by means of a combination of Percoll and sorbitol treatments. (**C**) Inhibition of 3D7 (dark grey bar) and 3D7Δ*GMD* (light grey bar) growth by a 3-h heat-shock at 41.5 °C. Values are the average of three independent replicas, with standard deviation, and represent percentage of growth relative to identical cultures not subjected to heat-shock. Heat-shock was performed when parasites were at the trophozoite stage and parasitemia was measured by FACS at the next generation. Statistical analysis, performed by applying one-way analysis of variance Tukey's post-test, showed no significant differences.

0.1% TX-100 in 1xPBS for 5 min at RT, washed again and then blocked in 3% BSA in 1xPBS overnight at 4 °C. All washes were performed in 0.5% BSA in 1xPBS by pelleting cells for 2 min at 800 g. Samples were incubated in solution 1 h at RT with ~10 μg/ml *Ulex europaeus* agglutinin I (UEA-I) and 5 μg/ml *Griffonia simplicifolia* lectin II (GSL-II) conjugated to either Alexa Fluor488 or 594 (ThermoFisher Scientific). For the sugar inhibition experiments, the lectin solution was pre-incubated with 0.2 M methyl-α-fucopyranoside (αMeFuc) for 30 min at RT before being added to the cells. Nuclei were stained with 2 μg/ml DAPI (4,6-diaminido-2-phenylindole) for 15 min at RT following lectin incubation. Cells were washed in 0.5% BSA in 1xPBS, mounted using Vectashield (Vector Labs) and examined by deconvolving fluorescence microscopy using an Olympus IX70 microscope. Images were collected at 0.2-μm optical sections, deconvolved using SoftWoRx (Applied Precision) and further processed with Fiji[34]. Data is presented as a projection of the entire stack.

Results

Generation of *Pf*GMD (3D7Δ*GMD*) and *Pf*FS (3D7Δ*FS*) null mutants. To investigate the role of GDP-Fuc, we generated null mutants of the enzymes in the *de novo* biosynthetic pathway for this sugar nucleotide. For *Pf*GMD we used a targeting construct (pHH1-*Pf*GMD), which truncated the gene by single homologous recombination (Fig. 1A)[23,24]. In addition to the gene disruption, the single recombination event removed the first two nucleotides (AT) at the *Pf*GMD start codon. Thus, any translation products generated would be expected to yield aberrant or non-functional proteins. Southern blot analysis of restriction enzyme-digested DNA using a *Pf*GMD probe showed that the gene was mutated as intended (Fig. 1B). The isolation of 3D7Δ*GMD* mutants indicated that the gene was not essential. To confirm our results, we also disrupted the *Pf*FS locus using a double-crossover recombination approach based on a pCC1-targeting construct (pCC1-*Pf*FS) (Supplementary Fig. S1A)[25]. The disruption of both genes in the GDP-Fuc *de novo* pathway allow us to conclude that this metabolic route is not required for the survival of the blood stages of the parasite in culture.

***Pf*GMD null mutants did not exhibit a growth phenotype *in vitro*.** 3D7Δ*GMD* null mutant parasites showed growth rates similar to the 3D7 wild type strain (Fig. 2A), indicating that the GDP-Fuc *de novo* biosynthetic pathway is not necessary during *in vitro* blood stage development. In line with the above, we did not observe patent changes in cell size and morphology at the trophozoite stage between wild type and the mutant cell line (Fig. 2B).

Given that we did not observe a reduced growth phenotype for 3D7Δ*GMD* null mutants under *in vitro* conditions, we hypothesized that parasite survival may be impacted under stressful conditions. Glycosyltransferase

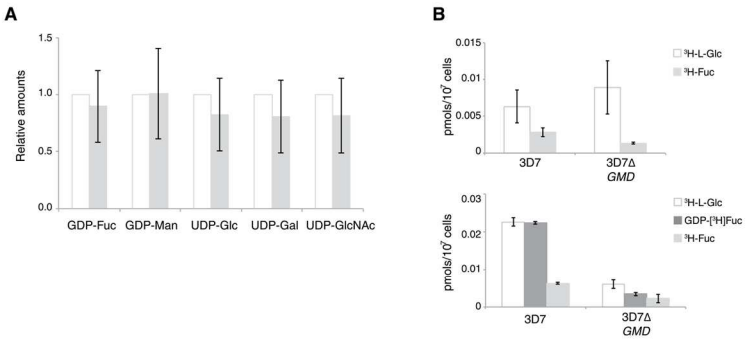


Figure 3. A pool of GDP-Fuc is detected in 3D7Δ*GMD* mutants. (**A**) The levels of GDP-Fuc and other sugar nucleotides are quantified in wild type (3D7; white bars) and 3D7Δ*GMD* mutants (grey bars). Values are shown as relative amounts to wild type sugar nucleotide levels and indicate the average variation ± S.D. of five different analyses. Analyses of every single experiment were always performed in triplicate. (**B**) Picomols of free ^{3}H-fucose (light grey bars) and GDP-[^{3}H]Fuc (dark grey bars) incorporated by wild type (3D7) and 3D7Δ*GMD* mutant parasites in culture (top) or after saponin treatment to release parasites (bottom). ^{3}H-L-glucose (white bars) is included for comparison purposes, as it is incorporated through equilibrative (not active) transport mechanisms[45]. A control of ^{3}H-glucosamine, incorporated by parasites[15,32], was also used in every experiment (not shown).

activity and sugar nucleotides donors have long been linked to protein folding and thermotolerance in many organisms, including different parasites[35–37]. It has been suggested that Protein *O*-fucosyltransferase 2 (PoFUT2), of which a homolog is conserved and expressed in the *P. falciparum* genome[18], may be involved in a novel quality control mechanism for the proper folding of TSR-domain containing proteins in the endoplasmic reticulum (ER)[37,38]. Proteins with TSR-domains have been shown to be expressed in asexual stages and to be involved in host-cell invasion[39,40]. To assess if abrogation of the GDP-Fuc *de novo* pathway could alter PoFUT2 function in the parasite, we studied the ability of 3D7Δ*GMD* mutant to cope with conditions likely to lead to the accumulation of misfolded TSR-containing proteins in the ER by measuring parasite growth after a 3 h heat shock at 41.5 °C. We did not observe any significant difference in resistance to heat shock in the mutant cell line compared to wild type, as judged by growth rate after temperature stress (Fig. 2C). Similar results were also observed for 3D7Δ*FS* cell line (Supplementary Fig. S1C).

GDP-Fuc is still detected in the parasite after disruption of the GDP-Fuc *de novo* pathway. To analyse the effect of *PfGMD* mutagenesis on parasite GDP-Fuc levels, sugar nucleotides from trophozoites were extracted, separated by reverse phase HPLC and quantified by multiple reaction monitoring tandem mass spectrometry using an internal standard (GDP-Glc) not present in *P. falciparum* blood stages[30]. We performed several biological replicates of the analyses, anticipating certain variability of the dynamic pools of sugar nucleotides. Nevertheless, the presence of GDP-Fuc in the mutant cell line was observed in all the experiments (Fig. 3A and Supplementary Table SI). Furthermore, the ratios between GDP-Fuc and other sugar nucleotides were similar throughout the different analyses, suggesting that there was not a specific reduction of the GDP-Fuc levels.

The detection of GDP-Fuc in the mutants strongly suggests the presence of an alternative pathway in the parasite for the generation of this sugar nucleotide. In order to identify an alternative mechanism of fucose transport and activation in *PfGMD* null mutants, we used tritiated sugar to perform uptake assays in infected RBCs or saponin-released parasites. However, no specific incorporation was observed in the *PfGMD* mutant cell line using either free fucose or GDP-Fuc (Fig. 3B).

Fucose-binding lectin *Ulex europaeus* agglutinin 1 (UEA-I) labelling is abrogated in parasites with a disrupted GDP-Fuc *de novo* pathway. UEA-I, which has affinity for α-linked-fucose, binds to red blood cells (RBCs) membranes and also to wild type parasite surfaces (Fig. 4A). The UEA-I binding is absent in *PfGMD* null mutant parasites, suggesting that the mutation affects lectin binding to the surface of the parasite. On the contrary *Griffonia simplicifolia* lectin II (GSL-II) labelling, a GlcNAc-specific lectin which binds to *P. falciparum N*-glycans on the surface of the parasite and co-localises with UEA-I in wild type cells, is not affected in mutants (Fig. 4B)[13]. Therefore, GSL-II and UEA-I bind likely to different glycosylated structures on the plasma membrane of the parasite, but only UEA-I labelling is affected in mutants. UEA-I labelling specificity was confirmed by binding inhibition after lectin preincubation with αMeFuc (Supplementary Fig. S2).

Discussion

Glycoconjugate biosynthesis requires the activation of monosaccharides to nucleotide sugars. These precursors are used by different glycosyltransferases for diverse glycosylation reactions[41]. Only five sugar nucleotides

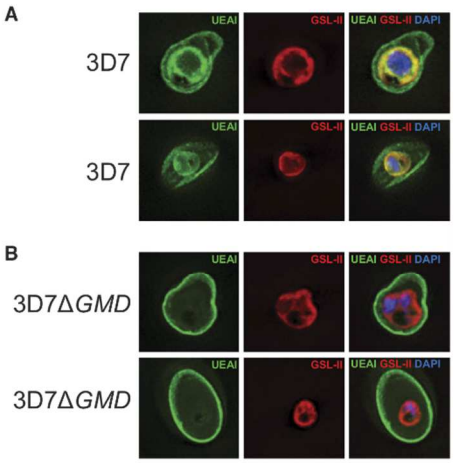


Figure 4. UEA-I labelling is abrogated in 3D7Δ*GMD* mutants. (**A**) Deconvolving micrographs of *P. falciparum* infected RBCs show a specific UEA-I labelling (green) on the surface of wild type parasites and host RBCs. GSL-II also binds to the surface of parasites[13] (**B**) UEA-I labelling disappears in 3D7Δ*GMD* mutant surface (bottom panels) whereas GSL-II binding is not altered.

have been identified in the blood stages of *P. falciparum*: UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-Gal, UDP-glucose (UDP-Glc), GDP-mannose (GDP-Man) and GDP-Fuc[18]. *P. falciparum* genome encodes and expresses homologues of GMD and FS, the enzymes responsible for the *de novo* biosynthesis of GDP-Fuc from GDP-Man[42]. Tritiated fucose is not taken-up significantly from the media by the parasite during the intraerythrocytic life cycle, which is consistent with the apparent absence of genes encoding for the GDP-Fuc salvage pathway in the parasite genome[18,43,44]. Altogether, this suggested that most, if not all, of the GDP-Fuc present is formed by the bioconversion of GDP-Man[18]. Surprisingly, the detection of GDP-Fuc in *PfGMD* mutants (Fig. 3A and Supplementary Table SI) implies that a substantial alternative source of GDP-Fuc is still present and active in the parasite. Our analyses with null mutant cell lines did not reveal the existence of an evident route for the incorporation of free fucose or GDP-Fuc (Fig. 3B). Nevertheless, it has to be considered that human erythrocytes contain significant levels of fucosylated proteins and their putative degradation may release monosaccharides that might be activated by the parasite. Furthermore, red blood cells also have a significant pool of GDP-Fuc (data not shown) which might be scavenged by the parasite during the intraerythrocytic life cycle. In this sense, previous studies had suggested that *P. falciparum* may obtain some sugars and/or other metabolites through passive mechanisms during the intraerythrocytic life cycle[45,46].

A considerable variability in sugar nucleotide levels between different biological replicates was observed, independently of the synchronization method used (Fig. 3A). This disparity is possibly derived from inter-assay variability, differences on the sources of RBCs for *in vitro* culture or even fluctuations of the dynamic pools of sugar nucleotides in parasites. Nevertheless, GDP-Fuc was always detected and there was not a specific reduction of this sugar nucleotide in comparison to the rest of the pools, suggesting that the GDP-Fuc *de novo* pathway may not represent a significant contribution to the levels of GDP-Fuc in the blood stages of the parasite. Furthermore, mutant cell lines did not present alterations in their growth *in vitro* (Fig. 2A and Supplementary Fig. S1B). This also seems to be the case in *P. berghei* Δ*GMD* and Δ*FS* mutants[47]. Further studies may help to completely dissect the impact of the disruption of the GDP-Fuc *de novo* route in other stages of the parasite life cycle.

Nevertheless, fluorescence microscopy assays using AlexaFluor488-labelled UEA-I and performed on detergent-permeabilised and parasitised erythrocytes showed a striking reduction in lectin binding to the parasite membrane in *PfGMD* null mutant (Fig. 4B). Despite the presence of GDP-Fuc in the mutant, this reduction in UEA-I binding to a fucose glycotope might somehow be related to the disruption of a source of GDP-Fuc, namely the *de novo* pathway. If this metabolic route contributes even in part to the metabolite pool, mutants could be reacting to a marginal decrease of GDP-Fuc by shutting down the biosynthesis of fucose-containing glycans dispensable for *in vitro* growth (Fig. 4B). Examples of glycan biosynthesis cessation as adaptation to a lower availability of precursors have been reported recently[48].

The decrease of UEA-I binding strongly suggests the presence of a fucose-containing glycan on the surface of *P. falciparum* blood stages. Despite several attempts, we were not successful in reproducing the observed differences by Western blot (not shown) perhaps indicating that UEA-I binds to a glycolipid in the parasites. UEA-I is best known for its ability to agglutinate human erythrocytes by binding to the α-1,2-fucose-contaning

H-antigen[49]. Only erythrocytes of the rare Bombay phenotype, which are completely devoid of such epitopes due to an inactivated mutation in the α-1,2-fucosyltransferase gene (FUT1), are not agglutinated[50,51]. The reduction in UEA-I binding in mutant parasites indicates that a fucosyl transferase may be involved in the synthesis of a UEA-I-glycotope on the surface of the parasite.

No putative α-1,2-fucosyltransferase can be identified in the *P. falciparum* genome by bioinformatic searches, however a homolog of PoFUT2 (PF3D7_0909200) is present. PoFUT2 is involved in the *O*-fucosylation of TSR-domains in other organisms[38]. Peptides belonging to this putative *Pf*PoFUT2 have been detected in the sporozoite stages of the parasite[52], when large amounts of TSR-containing proteins such as the circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP) are expressed and posttranslationally modified[16]. Hence, there is the possibility that the *de novo* GDP-Fuc disruption results in a more striking phenotype during sporozoite development in the mosquito, potentially affecting both salivary gland and hepatocyte invasion. *P. falciparum* PoFUT2 gene is also upregulated during the schizont stages of the intraerythrocytic life cycle[18] and therefore it may be involved in the fucosylation of other TSR-domain containing proteins required for host-cell invasion in these stages[16,39,40,53]. Although *Pf*PoFUT2 might be the fucosyltransferase involved in the biosynthesis of the UEA-I glycotope, there is also the possibility that other GDP-Fuc-dependent transferases are present and active throughout the blood stages of *P. falciparum*. Further work is being carried out at present to characterise this glycan modification and the glycosyl transferase/s involved in its biosynthesis.

References

1. WHO. World Malaria Report 2014. *WHO* (2016).
2. Schwartz, L., Brown, G. V., Genton, B. & Moorthy, V. S. A review of malaria vaccine clinical projects based on the WHO rainbow table. *Malar. J.* **11,** 11 (2012).
3. RTS,S Clinical Trials Partnership *et al.* Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. *Lancet (London, England)* **386,** 31–45 (2015).
4. Kooij, T. W., Janse, C. J. & Waters, A. P. Plasmodium post-genomics: better the bug you know? *Nat Rev Microbiol* **4,** 344–357 (2006).
5. Rodrigues, J. A. *et al.* Parasite glycobiology: a bittersweet symphony. *PLoS Pathog.* **11,** e1005169 (2015).
6. McConville, M. J. & Ferguson, M. A. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.* **294** (Pt 2), 305–324 (1993).
7. von Itzstein, M., Plebanski, M., Cooke, B. M. & Coppel, R. L. Hot, sweet and sticky: the glycobiology of Plasmodium falciparum. *Trends Parasitol* **24,** 210–218 (2008).
8. Sanders, P. R. *et al.* A set of glycosylphosphatidyl inositol-anchored membrane proteins of Plasmodium falciparum is refractory to genetic deletion. *Infect Immun* **74,** 4330–4338 (2006).
9. Schofield, L., Hewitt, M. C., Evans, K., Siomos, M.-A. & Seeberger, P. H. Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. *Nature* **418,** 785–789 (2002).
10. Krishnegowda, G. *et al.* Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of Plasmodium falciparum: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. *J. Biol. Chem.* **280,** 8606–8616 (2005).
11. Gowda, D. & Davidson, E. Protein glycosylation in the malaria parasite. *Parasitol. Today* **15,** 147–152 (1999).
12. Kimura, E. A., Couto, A. S., Peres, V. J., Casal, O. L. & Katzin, A. M. N-linked glycoproteins are related to schizogony of the intraerythrocytic stage in Plasmodium falciparum. *J Biol Chem* **271,** 14452–14461 (1996).
13. Bushkin, G. G. *et al.* Suggestive evidence for darwinian selection against asparagine-linked glycans of Plasmodium falciparum and Toxoplasma gondii. *Eukaryot Cell* **9,** 228–241 (2010).
14. Samuelson, J. & Robbins, P. W. Effects of N-glycan precursor length diversity on quality control of protein folding and on protein glycosylation. *Semin. Cell Dev. Biol.* **41,** 121–128 (2014).
15. Gowda, D. C., Gupta, P. & Davidson, E. A. Glycosylphosphatidylinositol anchors represent the major carbohydrate modification in proteins of intraerythrocytic stage Plasmodium falciparum. *J Biol Chem* **272,** 6428–6439 (1997).
16. Swearingen, K. E. *et al.* Interrogating the Plasmodium sporozoite surface: identification of surface-exposed proteins and demonstration of glycosylation on CSP and TRAP by mass spectrometry-based proteomics. *PLoS Pathog.* **12,** e1005606 (2016).
17. Caputto, R., Leloir, L. F., Cardini, C. E. & Paladini, A. C. Isolation of the coenzyme of the galactose phosphate-glucose phosphate transformation. *J. Biol. Chem.* **184,** 333–350 (1950).
18. Sanz, S. *et al.* Biosynthesis of GDP-fucose and other sugar nucleotides in the blood stages of Plasmodium falciparum. *J. Biol. Chem.* **288,** 16506–16517 (2013).
19. Cova, M., Rodrigues, J. A., Smith, T. K. & Izquierdo, L. Sugar activation and glycosylation in Plasmodium. *Malar. J.* **14,** 427 (2015).
20. Yilmaz, B. *et al.* Gut microbiota elicits a protective immune response against malaria transmission. *Cell* **159,** 1277–1289 (2014).
21. Ramasamy, R. & Field, M. C. Terminal galactosylation of glycoconjugates in Plasmodium falciparum asexual blood stages and Trypanosoma brucei bloodstream trypomastigotes. *Exp. Parasitol.* **130,** 314–320 (2012).
22. Doud, M. B. *et al.* Unexpected fold in the circumsporozoite protein target of malaria vaccines. *Proc. Natl. Acad. Sci. USA*. **109,** 7817–7822 (2012).
23. Crabb, B. S. *et al.* Transfection of the human malaria parasite Plasmodium falciparum. *Methods Mol Biol* **270,** 263–276 (2004).
24. Cortes, A. *et al.* Epigenetic silencing of Plasmodium falciparum genes linked to erythrocyte invasion. *PLoS Pathog* **3,** e107 (2007).
25. Maier, A. G., Braks, J. A., Waters, A. P. & Cowman, A. F. Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in Plasmodium falciparum for targeted gene deletion by double crossover recombination. *Mol Biochem Parasitol* **150,** 118–121 (2006).
26. Trager, W. & Jensen, J. B. Human malaria parasites in continuous culture. *Science* **193,** 673–675 (1976).
27. Urbán, P., Estelrich, J., Cortés, A. & Fernàndez-Busquets, X. A nanovector with complete discrimination for targeted delivery to Plasmodium falciparum-infected versus non-infected red blood cells *in vitro*. *J. Control. Release* **151,** 202–211 (2011).
28. Rovira-Graells, N. *et al.* Transcriptional variation in the malaria parasite Plasmodium falciparum. *Genome Res* **22,** 925–938 (2012).
29. Rabina, J. *et al.* Analysis of nucleotide sugars from cell lysates by ion-pair solid-phase extraction and reversed-phase high-performance liquid chromatography. *Glycoconj J* **18,** 799–805 (2001).
30. Turnock, D. C. & Ferguson, M. A. Sugar nucleotide pools of Trypanosoma brucei, Trypanosoma cruzi, and Leishmania major. *Eukaryot Cell* **6,** 1450–1463 (2007).
31. Wang, P., Wang, Q., Sims, P. F. G. & Hyde, J. E. Characterisation of exogenous folate transport in Plasmodium falciparum. *Mol. Biochem. Parasitol.* **154,** 40–51 (2007).
32. Gerold, P., Dieckmann-Schuppert, A. & Schwarz, R. T. Glycosylphosphatidylinositols synthesized by asexual erythrocytic stages of the malarial parasite, Plasmodium falciparum. Candidates for plasmodial glycosylphosphatidylinositol membrane anchor precursors and pathogenicity factors. *J Biol Chem* **269,** 2597–2606 (1994).

33. Woodrow, C. J., Penny, J. I. & Krishna, S. Intraerythrocytic Plasmodium falciparum expresses a high affinity facilitative hexose transporter. *J. Biol. Chem.* **274,** 7272–7277 (1999).
34. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9,** 676–682 (2012).
35. Izquierdo, L., Atrih, A., Rodrigues, J. A., Jones, D. C. & Ferguson, M. A. Trypanosoma brucei UDP-glucose:glycoprotein glucosyltransferase has unusual substrate specificity and protects the parasite from stress. *Eukaryot Cell* **8,** 230–240 (2009).
36. Naderer, T., Wee, E. & McConville, M. J. Role of hexosamine biosynthesis in Leishmania growth and virulence. *Mol. Microbiol.* **69,** 858–869 (2008).
37. Vasudevan, D. & Haltiwanger, R. S. Novel roles for O-linked glycans in protein folding. *Glycoconj. J.* **31,** 417–426 (2014).
38. Luo, Y., Koles, K., Vorndam, W., Haltiwanger, R. S. & Panin, V. M. Protein O-fucosyltransferase 2 adds O-fucose to thrombospondin type 1 repeats. *J Biol Chem* **281,** 9393–9399 (2006).
39. Chattopadhyay, R. *et al.* PfSPATR, a Plasmodium falciparum protein containing an altered thrombospondin type I repeat domain is expressed at several stages of the parasite life cycle and is the target of inhibitory antibodies. *J. Biol. Chem.* **278,** 25977–25981 (2003).
40. Thompson, J. *et al.* PTRAMP; a conserved Plasmodium thrombospondin-related apical merozoite protein. *Mol. Biochem. Parasitol.* **134,** 225–232 (2004).
41. Coutinho, P. M., Deleury, E., Davies, G. J. & Henrissat, B. An evolving hierarchical family classification for glycosyltransferases. *J Mol Biol* **328,** 307–317 (2003).
42. Becker, D. J. & Lowe, J. B. Fucose: biosynthesis and biological function in mammals. *Glycobiology* **13,** 41R–53R (2003).
43. Ginsburg, H. Progress in in silico functional genomics: the malaria Metabolic Pathways database. *Trends Parasitol* **22,** 238–240 (2006).
44. Aurrecoechea, C. *et al.* PlasmoDB: a functional genomic database for malaria parasites. *Nucleic Acids Res* **37,** D539–D543 (2009).
45. Kirk, K., Horner, H. A. & Kirk, J. Glucose uptake in Plasmodium falciparum-infected erythrocytes is an equilibrative not an active process. *Mol. Biochem. Parasitol.* **82,** 195–205 (1996).
46. Downie, M. J., Saliba, K. J., Howitt, S. M., Broer, S. & Kirk, K. Transport of nucleosides across the Plasmodium falciparum parasite plasma membrane has characteristics of PfENT1. *Mol. Microbiol.* **60,** 738–748 (2006).
47. Gomes, A. R. *et al.* A genome-scale vector resource enables high-throughput reverse genetic screening in a malaria parasite. *Cell Host Microbe* **17,** 404–413 (2015).
48. Damerow, S. *et al.* Depletion of UDP-glucose and UDP-galactose using a degron system leads to growth cessation of leishmania major. *PLoS Negl. Trop. Dis.* **9,** e0004205 (2015).
49. Matsumoto, I. & Osawa, T. Purification and characterization of an anti-H(O) phytohemagglutinin of Ulex europeus. *Biochim. Biophys. Acta - Protein Struct.* **194,** 180–189 (1969).
50. Kelly, R. J. *et al.* Molecular basis for H blood group deficiency in Bombay (Oh) and para-Bombay individuals. *Proc. Natl. Acad. Sci.* **91,** 5843–5847 (1994).
51. Le Pendu, J., Cartron, J. P., Lemieux, R. U. & Oriol, R. The presence of at least two different H-blood-group-related beta-D-gal alpha-2-L-fucosyltransferases in human serum and the genetics of blood group H substances. *Am. J. Hum. Genet.* **37,** 749–760 (1985).
52. Lindner, S. E. *et al.* Total and putative surface proteomics of malaria parasite salivary gland sporozoites. *Mol. Cell. Proteomics* **12,** 1127–1143 (2013).
53. Uchime, O. *et al.* Analysis of the conformation and function of the Plasmodium falciparum merozoite proteins MTRAP and PTRAMP. *Eukaryot. Cell* **11,** 615–625 (2012).

Acknowledgements

LI thanks funding from Spanish Ministry of Economy (grants SAF2010-21069 and SAF2013-43656-R, supporting SS and BL-G) and from National Institutes of Health (grant 1R21AI115063-01, currently supporting SS, RRD and LI). LI is a member of the GlycoPar-EU FP7 funded Marie Curie Initial Training Network (GA 608295). The authors thank Hernando A. del Portillo and Carmen Fernández Becerra for support, reagents and helpful discussions throughout this project. We thank Mike Ferguson for helpful discussions and access to LC-MS/MS systems to measure sugar nucleotides. We are also grateful to M. Ramírez, M. Bernabeu, A. Cortés and CCiTUB (Scientific and Technological Centers Universitat de Barcelona) for technical support, advice, assistance and useful suggestions. This work was funded by the Spanish Ministry of Economy (grants SAF2010-21069 and SAF2013-43656-R) and National Institutes of Health (grant 1R21AI115063-01).

Author Contributions

S.S. and L.I. conceived the work. G.B., J.S. and S.A. devised and conducted immunofluorescence experiments. S.D., B.L.-G. and L.I. performed sugar nucleotide analysis. S.S., L.I. and R.R.D. outlined the document and all authors contributed to the writing and review of this manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Sanz, S. *et al.* The disruption of GDP-fucose de novo biosynthesis suggests the presence of a novel fucose-containing glycoconjugate in *Plasmodium* asexual blood stages. *Sci. Rep.* **6**, 37230; doi: 10.1038/srep37230 (2016).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

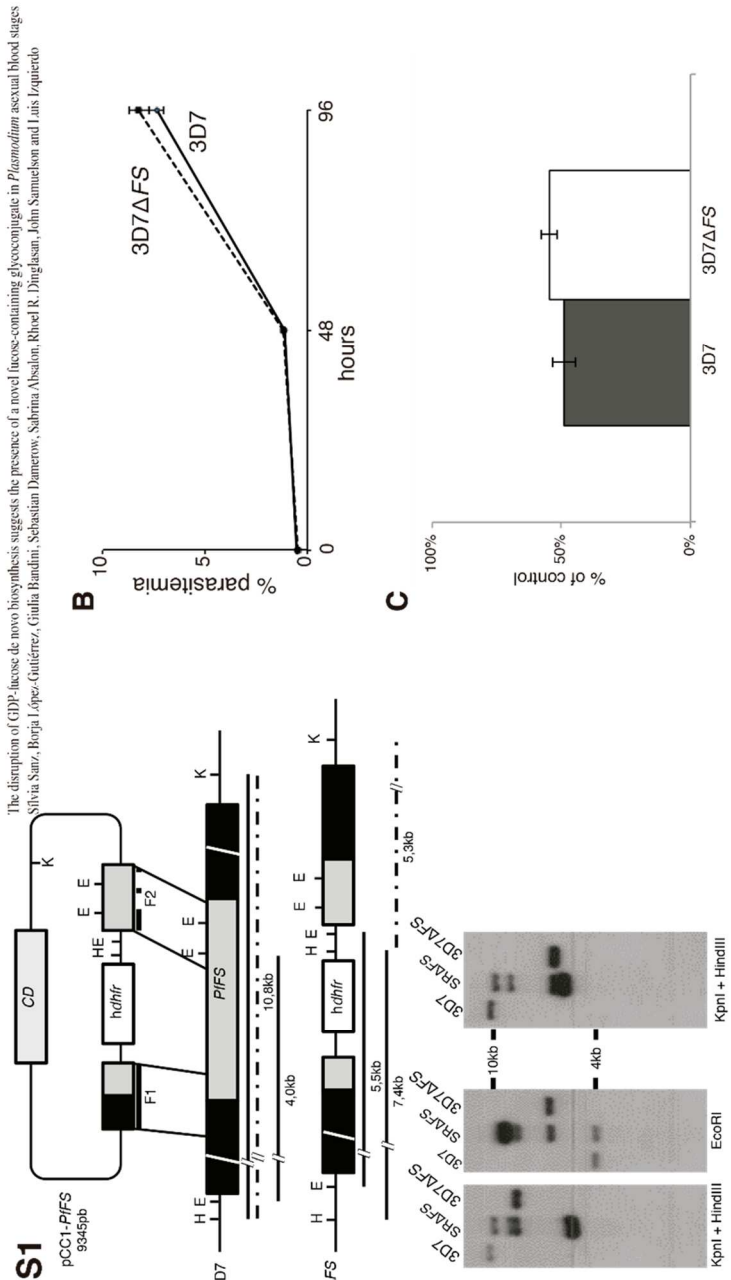


Fig. S1. *P. falciparum* 3D7Δ*FS* mutant generation and phenotyping. (A) (Top) Schematic representation of the transfection plasmid (pCC1-*PfFS*) used to target and disrupt FS gene in *P. falciparum* 3D7 parasites (3D7) and the expected double crossover recombination events (3D7Δ*FS*). The black boxes represent upstream and downstream DNA sequence flanking the *PfFS* gene locus. The position of HindIII (H), KpnI (K) and EcoRI (E) restriction sites, the predicted length of restriction fragments and the position of F1 and F2 probes (black and dotted line respectively) are shown. (Bottom) Southern Blot analysis of EcoRI and KpnI+HindIII digested genomic DNA from 3D7, SRΔFS (single recombinant parasites) and 3D7ΔFS parasites. SR refers to Single recombinant parasites, parasites before negative selection with 5-Fluorocytosine. Hybridisation of F1 and F2 probes to digested DNA from 3D7Δ*FS* parasites revealed restriction fragment sizes consistent with disruption of *PfFS* by integration of the hdhfr drug-resistance cassette. (B) Synchronous ring-stage 3D7Δ*FS* and 3D7 growth was monitored over two complete life cycles (96h) by flow cytometry. (C) Inhibition of 3D7 (dark grey bar) and 3D7Δ*FS* (white bar) growth by a 3-h heat-shock at 41.5oC. Values are the average of three independent replicas, with standard deviation, and represent percentage of growth relative to identical cultures not subjected to heat-shock. Statistical analysis showed no significant differences between cell lines.

The disruption of GDP-fucose de novo biosynthesis suggests the presence of a novel fucose-containing glycoconjugate in *Plasmodium* asexual blood stages

Sílvia Sanz, Borja López-Gutiérrez, Giulia Bandini, Sebastian Damerow, Sabrina Absalon, Rhoel R. Dinglasan, John Samuelson and Luis Izquierdo

Supplementary TABLE SI

Sugar nucleotide levels in 3D7 (wild type) and 3D7 Δ *GMD* cell lines, measured at the trophozoite stage in two different sets of experiments

Sugar nucleotide [a]	Experiment 1			Experiment 2		
	3D7	3D7Δ*GMD*	Lysed RBCs	3D7	3D7 Δ*GMD*	Lysed RBCs
UDP-Glc	1.87 ± 0.09	0.57 ± 0.02	0.006 ± 0.002	3.07 ± 0.09	3.00 ± 0.06	0.002 ± 0.00 [c]
UDP-Gal	0.39 ± 0.02	0.12 ± 0.01	0.003 ± 0.001	0.71 ± 0.04	0.69 ± 0.03	NQ [b]
UDP-GlcNAc	2.27 ± 0.09	0.66 ± 0.02	0.048 ± 0.016	2.85 ± 0.04	2.88 ± 0.08	0.008 ± 0.002
GDP-Man	0.51 ± 0.01	0.22 ± 0.00 [c]	NQ [b]	1.17 ± 0.02	1.61 ± 0.04	NQ [b]
GDP-Fuc	0.50 ± 0.01	0.22 ± 0.00 [c]	NQ [b]	0.32 ± 0.01	0.32 ± 0.01	NQ [b]

[a] Amounts are indicated in pmoles/10^7 cells

[b] Not Quantified (signal to noise ratio < 10)

[c] SD at least two orders of magnitude below the calculated amount

Fig. S2

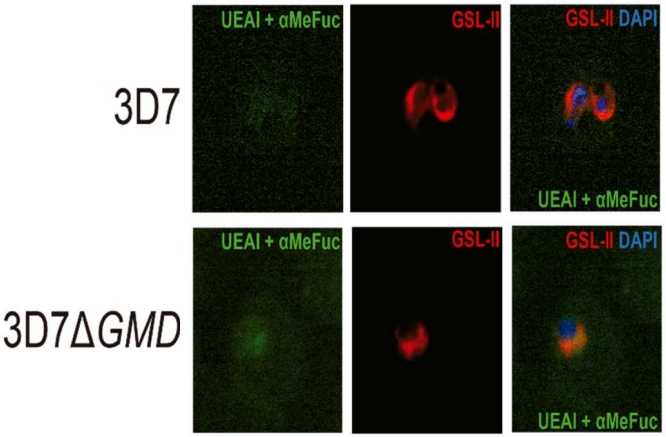


Fig. S2. Deconvolving micrographs of wild type (3D7) and mutant (3D7Δ*GMD*) *P. falciparum* infected RBCs labelled with α MeFuc preincubated UEA-I lectin. Cultured wild type and mutant *P. falciparum* lines were washed in PBS and smeared in glass slides. After drying, the smears were fixed in 4% PFA in PBS for 20 min ar RT. Fixed cells were washed, permeabilised in 0.1% TX-100 in 1xPBS for 5 min at RT, washed again and then blocked in 3% BSA in 1xPBS overnight at 4°C before incubation with UEA-I and GSL-II lectins and DAPI-staining. Preparations were mounted using Vectashield and examined on a Zeiss AXIO inverted microscope with Colibri LED and Hamamutsu Orca-R2 CCD camera. Images were collected at 0.2-µm optical sections and deconvolved using Zen. Images show single z slices.

Fig. S3

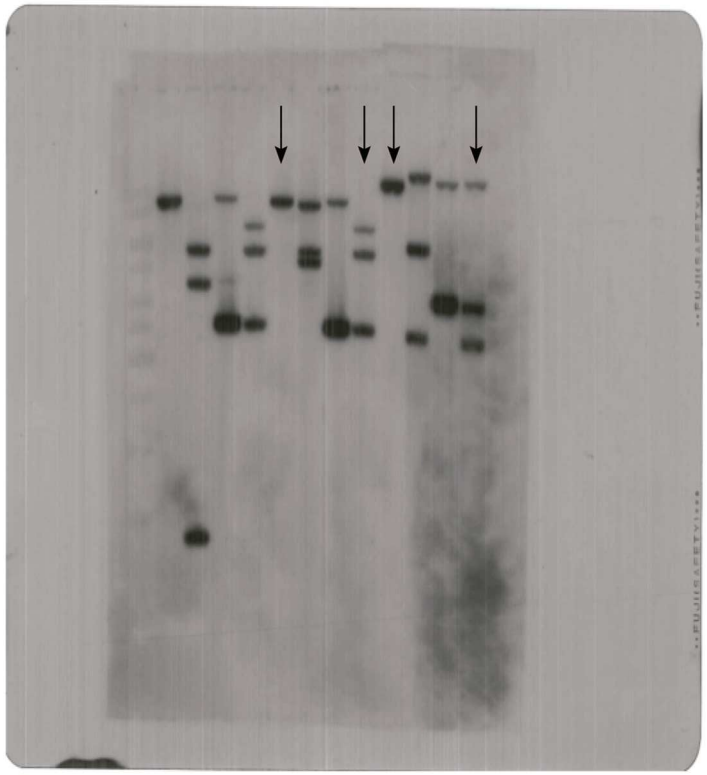


Fig. S3. Full-length Shouthern blot showing the lanes used for Fig. 1B. Arrow marks indicate the selected lanes used to illustrate the generation of 3D7Δ*GMD* cell line. Other lanes include either the use of extra restriction enzymes (not included in Fig. 1B), parasite cell lines where the recombination did not work or cell lines not related to this study

3. Unpublished work

"*Plasmodium* parasites with a disrupted *O*-fucosylation machinery are able to complete the human and rodent parasite life cycle."

Sílvia Sanz, Eleonora Aquilini, Rebecca Tweedell, Garima Verma, Tim Hamerly, Bernadette Hritzo, Abhai Tripathi, Marta Machado, Miguel Prudêncio, João Rodrigues, Rhoel R. Dinglasan, Luis Izquierdo

Preparing for submission

In collaboration with Johns Hopkins University and University of Florida

Plasmodium parasites with a disrupted *O*-fucosylation machinery are able to complete the human and rodent parasite life cycle

Authors

Silvia Sanz[1,2,3], Eleonora Aquilini[4], Rebecca Tweedell[2,3], Garima Verma[2,3], Tim Hamerly[2,3], Bernadette Hritzo[2], Abhai Tripathi[2], Marta Machado[4], Miguel Prudêncio[4], João Rodrigues[4], Rhoel Dinglasan[2,3,*], Luis Izquierdo[1,*].

Affiliations

[1] ISGlobal, Barcelona Ctr. Int. Health Res. (CRESIB), Hospital Clínic - Universitat de Barcelona, Barcelona, Spain

[2]Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

[3]The University of Florida Emerging Pathogens Institute, Department of Infectious Diseases & Pathology, Gainesville, FL, USA

[4]Instituto de Medicina Molecular, Unidade de Malária, Universidade de Lisboa, 1649–028 Lisboa, Portugal

[*] Corresponding authors

Abstract

Thrombospondin type I repeats (TSR) domains are commonly *O*-fucosylated by the protein-*O*-fucosyltransferase 2 (PoFUT2) and this modification is required for optimal folding and secretion of TSR-containing proteins. The human malaria parasite *Plasmodium falciparum* expresses proteins containing TSR domains that play essential roles in mosquito and human host invasion processes during the parasite's blood and transmission stages. A PoFUT2 homolog is conserved and expressed by *P. falciparum* and GDP-fucose, the *O*-fucosylation reaction donor, is actively synthesized and incorporated by the parasite in cell free assays. The proteomic detection of the *O*-fucosylation machinery in salivary gland sporozoites, toghether with the observed modification of Thrombospondin related anonymous protein (TRAP) or Ciscumsporozoite surface protein (CSP), strongly suggests the conservation of a PoFUT2 mediated *O*-fucosylation mechanism in *Plasmodium*. Here, we have generated a PoFUT2 null-mutant in malaria parasites and, by phenotyping the mutant throughout the complete life cycle of human and rodent parasites, we show that PoFUT2 disruption does not affect the growth of the blood and mosquito stages of the parasite. This suggests that the *O*-fucosylation of TSR-domain is not essential for parasitic development under laboratory conditions.

Introduction

Malaria is one of the most important human parasitic diseases, causing approximately 214 million new cases and more than 400000 deaths every year[1]. It is caused by a protozoan apicomplexan parasite of the genus *Plasmodium*, being *P. falciparum* the deadliest species. Malaria symptoms comprise general discomfort, fever and, chills. In more severe cases, malaria can cause breathing problems, organ failure, coma and, eventually death[2]. Chemotherapy is the main tool for malaria control, albeit some vaccine candidates are currently under evaluation and one of them has already received regulatory approval[3,4]. Parasites are transmitted by female *Anopheles* mosquitoes. After the bite of an infected mosquito, motile sporozoites are injected into the human dermis from where they travel through blood vessels to the liver and infect hepatocytes. One week later, the infected hepatocyte ruptures and releases merozoites that reach the blood circulation and invade

erythrocytes initiating the asexual reproduction cycle that causes the malaria symptoms. A small percentage of blood stage parasites become sexually committed cells, the gametocytes that are taken up during a mosquito bloodmeal. Once in the mosquito midgut, gametocytes turn into gametes, fertilization takes place and zygotes are formed. These zygotes develop into motile ookinetes that traverse the mosquito midgut wall to form oocysts. Every oocyst carries more than 10000 sporozoites that invade the mosquito salivary glands and are ready to infect another human host[5].

Thrombospondin type 1 repeat (TSR) domains are small (50-60 amino acid residues) cysteine-knot motifs with 3 conserved disulfide bonds that play important roles in cell adhesion and motility[6,7]. *Plasmodium* parasites express several TSR domain-containing proteins throughout the different stages of their life cycle that are critical for host-cell recognition, motility and invasion (Fig. 1)[8]. These proteins include Circumsporozoite Protein (CSP), Thrombospondin Related Anonymous Protein (TRAP), Circumsporozoite and TRAP-related protein (CTRP) and Merozoite TRAP (MTRAP) protein and all of them play important roles in sporozoite, ookinete and blood stages[9-12]. Antibodies against these proteins have been shown to inhibit invasion and halt parasite life cycle progression[13,14]. Therefore, they are important vaccine targets[15]. Indeed, the RTS,S licensed malaria vaccine is based in CSP, a GPI-anchored sporozoite protein that presents a TSR domain in the C-terminus[4].

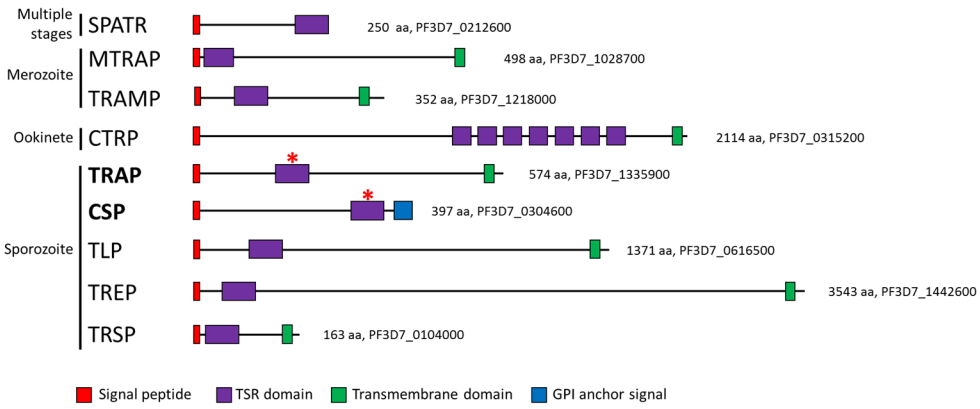


Figure 1. Schematic representation of *Plasmodium* proteins containing TSR domains. Red boxes represent secretion signals and purple boxes represent TSR domains. Predicted transmembrane domains are shown as green boxes and the GPI anchoring signal as a blue box. SPATR, Secreted Protein with Altered Thrombospondin Repeat Domain; MTRAP, Merozoite TRAP; TRAMP, Thrombospondin-Related Apical Membrane Protein; CTRP, Circumsporozoite and TRAP Related Protein; TRAP, Thrombospondin-Related Anonymous Protein; CSP, Circumsporozoite Protein; TLP, TRAP Like Protein; TREP, TRAP-like protein; TRSP, Thrombospondin-Related Sporozoite Protein. Proteins in boldface type (CSP and TRAP) are glycosylated in their TSR domains, marked with a red asterisk[21]. Adapted from Morahan *et al.*, Trends Parasitol, 2009[8].

It has been shown that TSR domains are *O*-fucosylated by protein *O*-fucosyltransferase 2 (PoFUT2)[16,17] and the fucose can be further elongated with a glucose residue, generating an *O*-linked disaccharide[18]. This modification is important for the secretion of TSR-domain containing proteins[19,20]. A recent report demonstrated that CSP and TRAP TSR-domains are also *O*-fucosylated in the sporozoite stages of the malaria parasite[21]. A homolog of PoFUT2 is conserved in all the *Plasmodium* species sequenced[22], and the parasite synthesizes the GDP-fucose precursor required for *O*-fucosylation[23,24], indicating that PoFUT2 could also mediate CSP and TRAP modification in the malaria parasite. Furthermore, this strongly suggests that this post-translational modification may be also present in other parasite TSR-containing proteins. This prompted us to create PoFUT2 null-mutants in *P. falciparum* and *P. berghei* and to characterize their phenotypes throughout the blood and transmission stages. In spite of the relevance of TSR domain-containing proteins for host-cell invasion and motility, *O*-fucosylation is not essential for the survival of the parasite throughout its life cycle. Nevertheless, this and other post-translational modifications of malaria vaccine antigens could be critical for vaccine design since glycans may alter specific epitopes affecting antibody recognition.

Materials and Methods

Mice and Ethics statement for animal experimentation

BALB/c and C57BL/6 mice (6–8 weeks of age) were purchased from Charles River and housed in the rodent facility of Instituto de Medicina Molecular (Lisbon, Portugal). All animal experiments were approved by the Portuguese official veterinary department for welfare licensing and the Instituto de Medicina Molecular Animal Ethics Committee. All animal experiments were performed in strict compliance to the guidelines of the institution´s animal ethics committee and the Federation of European Laboratory Animal Science Associations (FELASA).

P. falciparum transfection construct

To disrupt *Pf*PoFUT2 we generated a transfection construct based in the pCC1-plasmid[26]. pCC1-*Pf*PoFUT2 consisted of two fragments of ~850bp and ~960bp, respectively, from

different regions of the *Pf*PoFUT2 locus. F1 (nucleotides from -361 to +479 of the *Pf*PoFUT2 locus) and F2 (nucleotides from 1363 to 630 after of *Pf*PoFUT2 locus) fragments were amplified using In-Fusion HD Cloning Kit to SacII/SpeI and NcoI/AvrII restriction sites respectively, with the following primers: p3 (CAATGGCCCCTTTCCGCGG TCTTATGTCTTATTCTCATTTTGCTT) and p4 (AGATCTTCGGACTAGT CTTTTTGTAGCTGCAAGGGGG) for fragment F1 and p5 (ATCGATAACTCCATGG TGAGCAATGGATTTGTACAAGGT) and p6 (CAGGCGCCAGCCTAGG TCAAGTGCAAGGGTTCTTTT) for fragment F2. The construct generated would integrate into *PoFUT2* locus, disrupting it by double crossover homologous recombination (Fig. 2A).

P. falciparum asexual parasite culture and transfection

P. falciparum NF54 (a kind gift of Teun Bousema, Radboud University Nijmegen Medical Centre) parasites were cultured with human B^+ erythrocytes (2-4% hematocrit) in RPMI medium (Sigma) supplemented with 10% AB^+ human serum or 0.5% Albumax II, incubated at 37ºC in an atmosphere of 92% N_2, 3% O_2 and 5% CO_2 using standard methods[42]. Human erythrocytes and serum were purchased from the Banc de Sang i Teixits (Catalonia, Spain), after approval from the Comitè Ètic Investigació Clínica Hospital Clínic de Barcelona. Parasite growth was monitored by counting the infected erythrocytes in Giemsa-stained blood smears by light microscopy. *P. falciparum* NF54 parasites were transfected by schizont nucleofection as described previously[43,44]. Briefly, 60 µg of plasmid were transfected using P3 Primary cell 4D Nucleofector X-Kit (Lonza) program FP158 to 3.3 x 10^7 Percoll synchronized schizonts. To select transfected parasites 2nM of WR99210 were added to the culture 24h after transfection. After the appearance of resistant parasites, 2 on/off drug cycling with WR22910 were started followed by negative selection with 5-Fluorocytosine to select double recombinants. Integrant clonal parasites were obtained by limiting dilution. To calculate growth curves, tightly synchronized parasites were adjusted to 0.5% and measured by FACS. After 48 h and 96 h, parasitemia was again determined by FACS using SYTO 11 as previously described[45,46].

P. berghei cloning, transfection and PCR genotyping

Transfection experiments were performed on *P. berghei* ANKA strain 2.34 parasites according to the described protocol[47]. The *PoFut2* knockout vector was obtained from the PlasmoGEM resource[48] with the design number PbGEM-283938 (see http://plasmogem.sanger.ac.uk for details of vector design). The final knockout construct was digested with NotI to release the fragment for transfection (Fig. 2C). The pyrimethamine-resistant parasite population containing the correct genomic integration substituting *PoFut2* gene (*Pb*ΔPoFUT2) was cloned by injecting one parasite per mouse (BALB/c male mice, 6–8 weeks of age). Primers used to check the integration of *hu-dhfr* cassette and conveying resistance to pyrimethamine are p1 (CATACTAGCCATTTTATGTG), p2 (CTTTGGTGACAGATACTAC), p3 (AGCACCACGGGGGAAGGACT), p4 (ATGCAAAAACGTCTTCCCTT) and p5 (TCGAGCAACGATAAAATGCCT).

P. falciparum southern blotting and PCR analysis

Genomic DNA (gDNA) was isolated by Phenol/Chloroform method from ~600 µliters of red blood cells (RBC) infected with >5% late trophozoites/schizonts. Two µg of NF54-, SRΔPoFUT2- and NF54ΔPoFUT2-gDNA (four different clones) were digested with HindIII and SpeI or NdeI in separate reactions and probed with ^{32}P (Perkin Elmer)-labelled F1. PCR with gDNA of NF54, SRΔPoFUT2 and NF54ΔPoFUT2 (four different clones) was performed using primers p1 (TCTTGTACTTCCACCTTGGTGTT) and p2 (CATTCTTCGAAAGGTAAAATAAGAAAA) to detect a 231bp fragment of the interior of *Pf*PoFUT2 gene.

P. falciparum gametocyte cultures and mosquito infection

NF54 and NF54ΔPoFUT2 were diluted to 0.5% mixed stage asexual parasites and 4% hematocrit in complete culture medium in six-well plates. The plates were transferred to a 37°C incubator and cultured using the candle jar method[42]. The medium was exchanged daily from day 1 to day 17, to allow for gametocyte maturation from stage I through stage V. Standard membrane feeding assays (SMFA) were performed on day 15-18 post-culture initiation. Approximately 60 female *An. stephensi* or *An. gambiae* mosquitoes were distributed into pint-sized cups and starved of sugar and water for ~12 hours prior to feeding.

P. falciparum NF54 and NF54ΔPoFUT2 gametocyte cultures were pelleted and diluted to 0.03% or 0.3% gametocytemia with human blood at 50% hematocrit. Blood was washed with RPMI media and brought to 50% hematocrit with, heat-inactivated AB serum. Gametocytemic blood was kept at 37 °C until feeding. 250 to 300µL of gametocytemic blood were dispensed into a water-jacketed membrane feeder at 37 °C, and mosquitoes were allowed to feed for a minimum of 45 minutes. After blood feeding, non-blood-fed mosquitoes were removed. Blood fed mosquitoes were kept at 26°C, 70% humidity and, 12-hour light:dark cycle. They were provided a 10% sucrose solution for energy.

P. berghei mosquito infections

An. stephensi mosquitoes were bred at the insectary of the Instituto de Medicina Molecular (IMM). For mosquito infection, female BALB/c mice were intraperitoneally injected with *P. berghei* wild type and *Pb*ΔPoFUT2 mutant lines. After 3 to 5 days post infection the number of exflagellation events were determined with a Zeiss light microscope and a counting grid. If >1 exflagellation events per field of view were observed, mice were anaesthetized with a mixture of 10% ketamine and 2% xylazin in PBS (100 µL per 20 g mouse body weight i.p.) and fed to *An. stephensi* mosquitoes. Unfed mosquitoes were removed and fed mosquitoes were maintained at 19–22 °C and in 50–80 % relative humidity. Mosquitoes were used 10–23 days post infection for further experiments.

Oocyst counting and imaging

For *P. falciparum* oocyst counting, eight to ten days after the feeding, mosquito midguts were dissected and stained with 0.2% mercurochrome in water for 9 minutes. Midguts were placed on a slide with a drop of PBS, overlaid with a coverslip, and examined for oocysts counting using brightfield microscopy at 200x total magnification. Each midgut was imaged with Progress Capture Pro software to measure oocyst diameter, ensuring oocyst in all planes were visible. *P. berghei* infected midguts were collected and stained with 0.5 % mercurochrome, 10 days after infection. Oocysts were counted to determine the intensity of infection (number of oocysts per midgut). For statistical analysis, unpaired t-test was run using Graphpad.

Sporozoite purification and counting

On day 14 post-feeding, 30 mosquitoes, infected with each *P. falciparum* line (NF54 and NF54ΔPoFUT2) were dissected to obtain salivary glands. Each pair of salivary glands was kept in 100 µL of PBS in a 1.5 ml tube. The tubes were spun down at 1,200xg, for 3 minutes at room temperature. The salivary gland pellet was gently crushed in the 1.5 ml tube and vortexed for 3 seconds to resuspend the salivary gland contents in PBS. The tubes were spun down again at 1,200xg for 3 minutes at room temperature. Sporozoites were counted blindly on a hemocytometer by averaging 2 fields. Unpaired t-test was run using GraphPad. *P. berghei* sporozoites (*Pb*WT and *Pb*ΔPoFUT2) were collected on 21-24 days post infection from infected *An. stephensi* females bred at Instituto de Medicina Molecular. Salivary glands were dissected and kept in non-supplemented RPMI medium at 4ºC. After dissection, salivary glands were smashed with a pestle in a microcentrifuge tube. To eliminate mosquito debries and isolate the sporozoites, smashed samples were filtered on a 70 µm strainer. Sporozoites were then counted in a Neubauer chamber using an Olympus CKX41 inverted microscope.

P. berghei sporozoite infectivity to mice

For transmission experiments naïve C57BL/6 mice were exposed to 10 infected *P. berghei* wild type or *Pb*ΔPoFUT2 mosquitoes for 30 minutes; 3 mice were used per parasite clone. Parasitemias were followed daily by counting Giemsa smears from a drop of tail blood. Animals were monitored daily for clinical signs of cerebral malaria (CM) (head deviation, convulsions, ataxia and paraplegia).

Results

Generation of *Pf*PoFUT2 and *Pb*PoFUT2 null mutants

Several key *Plasmodium* proteins that play important roles along the different stages of the parasite developmental cycle contain TSR domains (Fig. 1). These domains are *O*-fucosylated by the glycosyltransferase PoFUT2[25], of which a homolog is found in the parasite genome. To assess the biological role of PoFUT2 through the parasite life cycle, we knocked out this gene in human and rodent malaria parasite species, *P. falciparum* and *P. berghei*, respectively. *P. falciparum* PoFUT2 (*Pf*PoFUT2) was disrupted by double crossover recombination using a

targeting construct (pCC1-*Pf*PoFUT2)[26] that replaced the gene with a *hu-dhfr* selection cassette (Fig. 2A) in *P. falciparum* NF54 line, which produces viable gametocytes. A similar approach was used to ablate *P. berghei* PoFUT2 (*Pb*PoFUT2), using PbGEM-283938 plasmid (Fig. 2C) kindly provided by PlasmoGEM[27]. After cloning *hu-dhfr* resistant parasites by limiting dilution, PCR or Southern Blot analyses confirmed PoFUT2 disruption in both *Plasmodium* species (Fig. 2B and 2D) and *P. falciparum* and *P. berghei* clones were selected for further phenotyping studies.

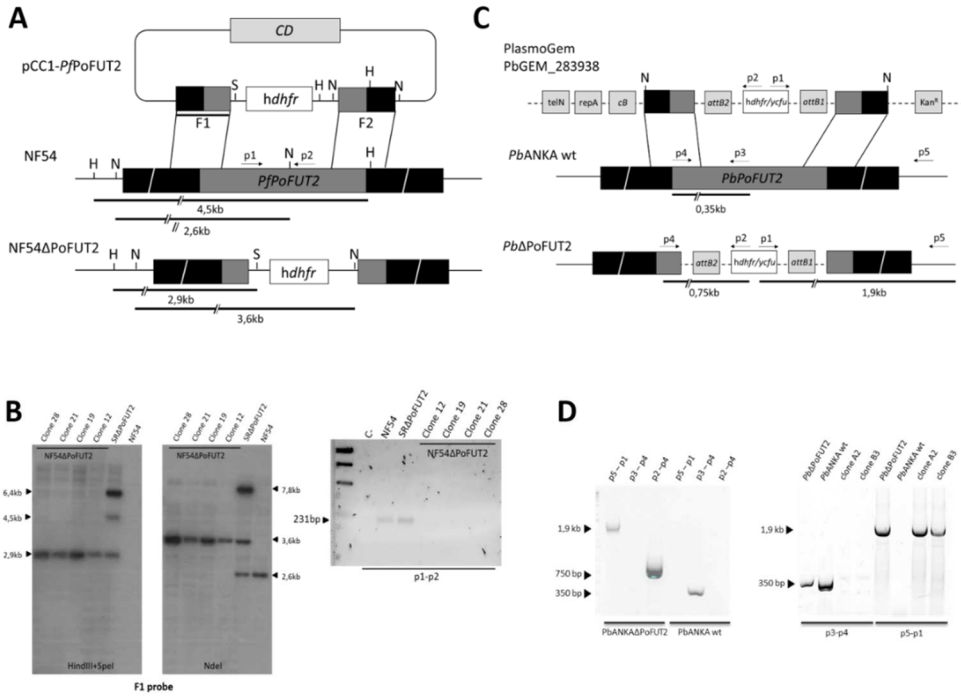


Figure 2. PoFUT2 transfection constructs and integration events in human and rodent malaria parasites. (A) Scheme of the transfection plasmid (pCC1-*Pf*PoFUT2) used to target and disrupt *Pf*PoFUT2 in *P.falciparum* NF54 parasites (NF54) and the expected double crossover recombination event (NF54ΔPoFUT2). Black boxes represent upstream and downstream DNA sequence flanking the *PfPoFUT2* gene locus. Position of HindIII (H), NdeI (N) and SpeI (S), position of F1 probe (thick black line), predicted length of the resistance fragment (thick black lines) and position of p1 and p2 primers are shown. **(B)** Southern blot of HindIII+SpeI and NdeI digested genomic DNA from NF54, SRΔPoFUT2 and NF54ΔPoFUT2 parasite clones. SR refers to Single recombinant parasites before negative selection with 5-Fluorocytosine. Hybridisation of F1 probe to digested DNA from NF54ΔPoFUT2 clones revealed restriction fragment sizes consistent with the replacement of a fragment of *PfPoFUT2* by integration of the *hu-dhfr* drug-resistance cassette (left panel). PCR using as template genomic DNA of NF54, SRΔPoFUT2 and different NF54ΔPoFUT2 clones and primers p1 and p2 (right panel). The absence of a band in NF54ΔPoFUT2 clone lanes reflects the replacement of *PfPoFUT2*. **(C)** Scheme of the PlasmoGem transfection plasmid (PbGEM-283938) used to disrupt *Pb*PoFUT2 in *P. berghei ANKA* parasites and the expected double crossover recombination events (*Pb*ΔPoFUT2). Black boxes represent upstream and downstream DNA sequence flanking the *PbPoFUT2* gene locus. Position of NotI (N), predicted length of PCR fragments (black lines) and position of primers p1, p2, p3, p4 and p5 is shown. TelN refers to Telomerase, RepA to Helicase, *cB* to arabinose inducible origin and Kan^R to the Kanamycin resistance cassette. *att*B1 and *att*B2 are the recombination sequences for the Gateway technology. **(D)** Genotype analysis of *P. berghei* transfectants (left panel) and *P. berghei* clones (right panel). PCRs on the genomic DNAs of *Pb*ANKA *wt* and *Pb*ΔPoFUT2 clones were done using primers p5 and p1, p3 and p4 and p4 and p2 for integration, wild type locus and cassette presence respectively. The presence of the integration band and the absence of the *wt* locus band in *Pb*ΔPoFUT2 parasites reflect that the replacement of the gene has taken place.

PoFUT2 null mutants do not show significant growth defects in *P. falciparum* or *P. berghei* asexual blood stages

PoFUT2 ablation demonstrates that the gene is not required for growth in the asexual blood stages of the parasite in culture or in a rodent model of infection. It has been suggested that proteins with TSR domains expressed in the *Plasmodium* asexual blood stages, such as the thrombospondin-related apical merozoite protein (TRAMP)[28,29], are involved in host cell invasion[8]. Thus, PoFUT2 absence might alter the secretion or function of these proteins affecting parasite's growth. Nevertheless, NF54ΔPoFUT2 knock out parasites exhibited growth rates similar to those of NF54 wild type strains (Fig. 3). Furthermore, the disruption of *Pb*PoFUT2 in *P. berghei* ANKA strain (PBANKA_0810400) also confirmed the non-essentiality of the protein in a malaria rodent model (data not shown).

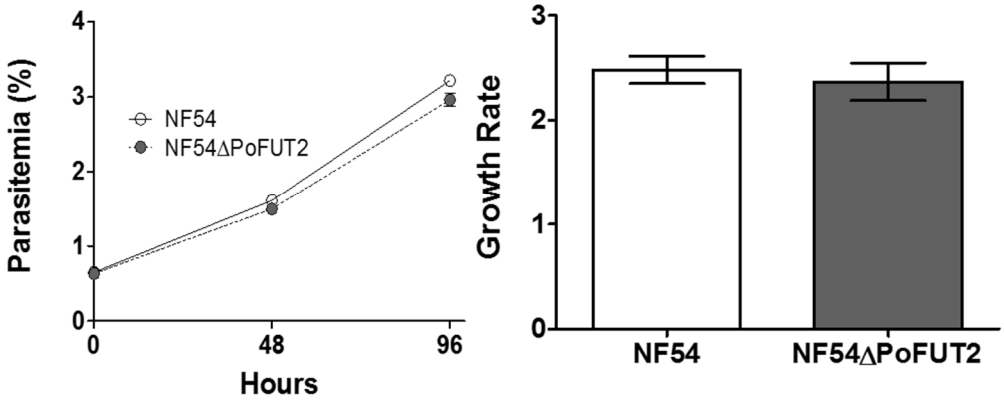


Figure 3. *P. falciparum* blood stages do not show any significant difference. (A) Synchronous ring stage NF54 (white) and NF54ΔPoFUT2 (grey) growth was monitored over two complete life cycles (96h) by flow cytometry using SYTO 11 as previously described (left panel). Growth rate of NF54 (white) and NF54ΔPoFUT2 (grey) is represented (right panel)

PoFUT2 null mutant parasites infect mosquitoes and exhibit normal oocyst production

To test whether PoFUT2 is affecting sexual stage development of malaria parasites, *An. gambiae* and/or *An. stephensi* mosquitoes were blood fed on gametocytemic human blood or on mice infected with wild type or PoFUT2 null mutant parasites. We did not observe significant differences in the parasite's ability to generate gametocytes (data not shown) and

to infect mosquitoes, suggesting that merozoite TRAP (MTRAP) protein, an essential protein for gamete egress from the RBC, is not affected by PoFUT2 disruption[152].

Circumsporozoite and TRAP-related protein (CTRP), an ookinete protein containing 7 TSR domains, is essential for mosquito infection (Fig. 1)[30]. Thus, we analyzed infected mosquito midguts to assess whether PoFUT2 ablation could alter oocyst development in *P. falciparum* or *P. berghei*. 8-10 days after blood fed with infected blood, mosquitoes were dissected to evaluate the presence of oocyst in midguts. No significant differences were appreciated (Fig. 4A and 4B), strongly suggesting that the invasion of the midgut epithelium was not affected in *P. berghei* or *P. falciparum* PoFUT2 mutants. Furthermore, the diameter and mean size of *P. falciparum* oocysts was similar in wild type and mutant parasites (Supplementary Fig. S1). Therefore, PoFUT2 disruption did not affect oocysts' maturation in the mosquito midgut.

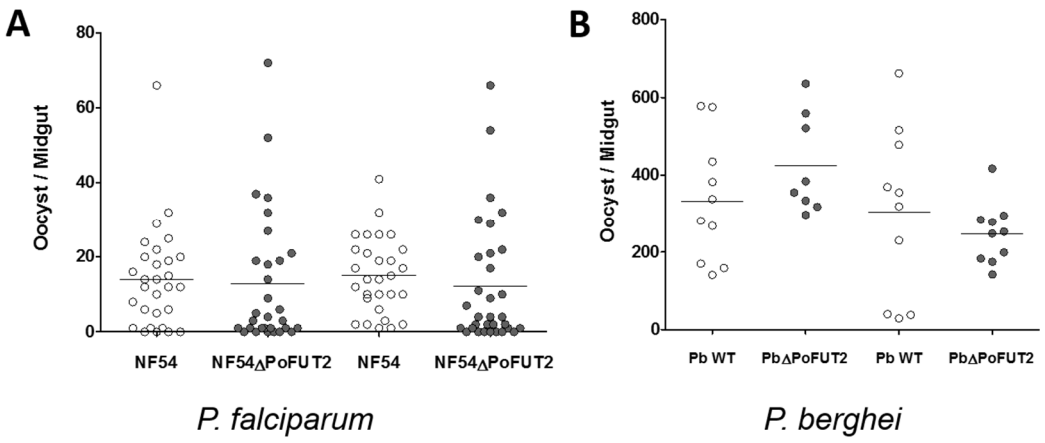


Figure 4. Oocyst production is similar in wild type and mutant parasite lines in both *Plasmodium* specie. (A) 30 *An. gambiae* female mosquitoes were fed at 0,03% gametocytemia. NF54 (white) and NF54Δ PoFUT2 (grey) oocyst number per midgut were counted 8 to 10 days post blood feed. Mean of the oocyst per midgut of wild type and mutant is represented. Two representative experiments are showed. Statistical analysis (unpaired t-test, 95% CI) did not show any significant difference (p value: 0,7609 and 0,4262 respectively) **(B)** 10 *An. stephensi* female mosquitoes were fed on BALB/c mice. *Pb*WT (white) and *Pb*ΔPoFUT2 (grey) oocyst number per midgut were counted 10 days post blood feed. Mean of the oocyst per midgut of wild type and mutant is represented. Two representative experiments are showed. Statistical analysis (unpaired t-test, 95% CI) did not show any significant difference (p value: 0.2026 and 0.4553 respectively).

PoFUT2 null mutant sporozoites are able to colonize *Anopheles* salivary glands and to re-infect mice

We also analyzed the number of sporozoites per pair of salivary glands after mosquito infection. *Anopheles* mosquitoes carrying mutant or wild type parasites showed similar numbers of salivary glands sporozoites in both *P. falciparum* and *P. berghei* species (Fig. 5A and 5B). Thus, salivary gland infection takes place normally in the absence of PoFUT2. Remarkably, *P. berghei* PoFUT2 null mutant sporozoites were able to infect C57BL/6 mice by direct bite-back (Fig. 5C). This result showed that *Pb*PoFUT2 mutants are fully infectious and able to go through hepatocyte infection and liver stage development. This was also confirmed by *in vitro* hepatic infection and cell traversal activity experiments performed using *P. berghei* PoFUT2 mutant parasites (Supplementary Fig. S2A and B). Interestingly CSP, the major sporozoite surface protein, was present in the sporozoite surface of *P. falciparum* parasites upon PoFUT2 deletion, suggesting that CSP secretion is not significantly altered in absence of PoFUT2 (Supplementary Fig. S3).

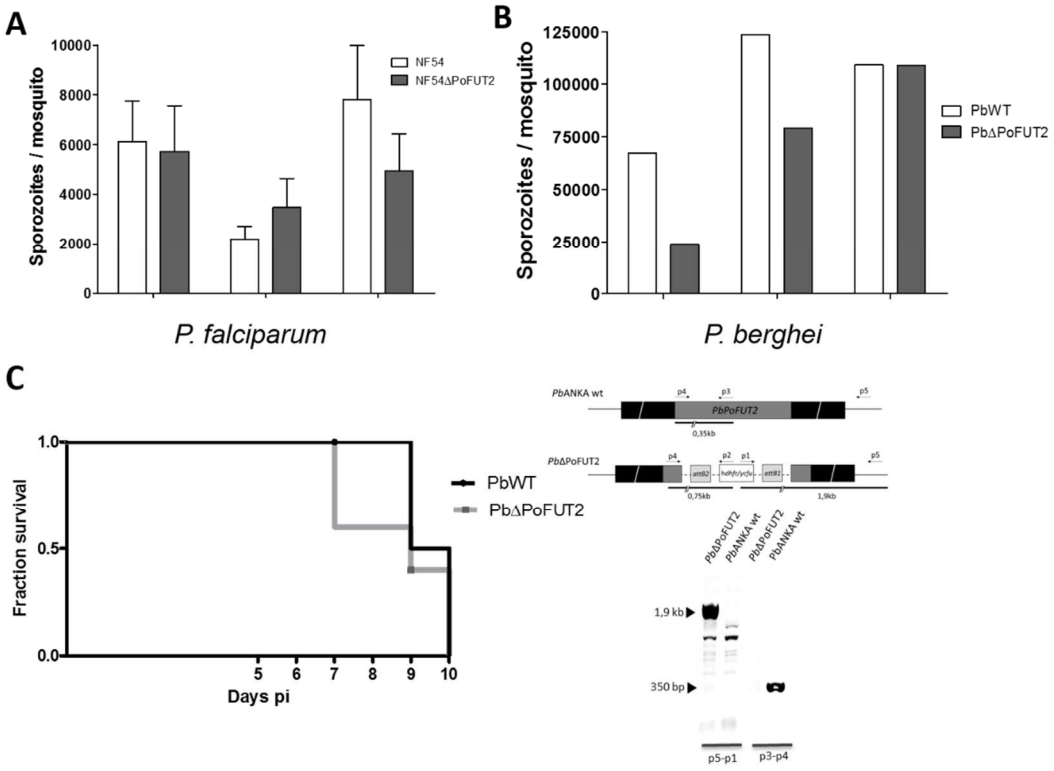


Figure 5. Sporozoites per salivary gland numbers are comparable between wild type and null mutant parasites. (A) NF54 (white) and NF54Δ PoFUT2 (grey) sporozoites per mosquito were counted 14 days post blood feed. Mean and Standard Error of Mean (SEM) of sporozites per mosquito is represented. Three representative experiments are shown. Statistical analysis (unpaired t-test, 95% CI) showed no significant difference (p value 0,8705; 0,3154 and 0,2862 respectively) between NF54 and NF54ΔPoFUT. **(B)** 21-24 days post infection *Pb*WT (white) and *Pb*ΔPoFUT2 sporozoites were dissected, pooled and counted using hemocytometer. Number of sporozoites per mosquito of three independent experiments is represented. **(C)** Comparision of mice survival bitten by 10 *Pb*WT or *Pb*ΔPoFUT2 infected mosquitoes. Animals were monitored daily for clinical signs of cerebral malaria (CM) (head deviation, convulsions, ataxia and paraplegia) and all of them developed CM around the same time. No significant differences were observed between the two groups (left panel). Scheme of *Pb*WT PoFUT2 locus and *Pb*ΔPoFUT2 deleted locus and genotype analysis of *P. berghei* parasites after the mouse bite-back (right panel, top). PCRs on the genomic DNAs of *Pb*ANKA *wt* and *Pb*ΔPoFUT2 after bite back were done using primers p5 and p1 and p3 and p4 for integration and wild type locus presence. The presence of the integration band and the absence of the *wt* locus band in the *Pb*ΔPoFUT2 parasites reflect that PoFUT2 disruption was maintained in mutant parasites after bite back infection (right panel, bottom).

Discussion

O-fucosylation, the process of adding a fucose residue to the -OH side chains of serines or threonines in TSR cysteine-rich domains[25,31], is mediated by PoFUT2 *O*-fucosyltransferase[32]. The *O*-fucosylation of *P. falciparum* CSP and TRAP sporozoite proteins has been recently demonstrated[21]. Considering the relevance of these and other TSR domain-containing proteins for *Plasmodium* invasion and motility[8], we created a PoFUT2 null-mutant in human and rodent parasites to completely assess the effect of PoFUT2 disruption throughout the parasite life cycle. The isolation of PoFUT2 null mutants both in *P. falciparum* and *P. berghei* species revealed that the protein is not necessary for parasite development. The ability of null mutants to complete the entire passage through the mosquito and to infect hepatocytes, suggests that the absence of PoFUT2 does not affect the function of key TSR-containing proteins such as CTRP, TRAP or CSP. Interestingly, a recent genome-wide screen performed in *Toxoplasma gondii* parasites showed that PoFUT2 was not among the genes conferring a more significant fitness cost to parasite tachyzoites during human fibroblast infection[33]. Therefore, *O*-fucosylation of TSR-domains seems to be dispensable for parasite growth under laboratory conditions.

Besides mediating important interactions between pathogens and their hosts[34], glycans are also involved in protein folding and secretion[35,36]. PoFUT2 endoplasmic reticulum (ER)

localization, its ability to distinguish between folded and unfolded structures and its importance for protein secretion, indicates that this glycosyltransferase plays a role in the folding, quality control and efficient secretion of TSR-contaning proteins[19,25]. Thus, PoFUT2-mediated TSR domain *O*-fucosylation may be contributing to adequate protein secretion under specific conditions, such as those that generate ER-stress[37], as it is the case of other ER-residing chaperones and glycosyltransferases[38]. Further experiments should help to ascertain the significance of *O*-fucosylation for the adequate secretion of critical TSR domain-containing proteins under ER-stress conditions, in a parasite particularly sensitive to this pressure[39].

CSP is the main immunodominant protein antigen in the sporozoite surface. This protein contains a glycosylated[21] C-terminal TSR-domain and it is essential for the sporozoite journey from mosquito midgut to the vertebrate host hepatocyte target[10]. The licensed RTS,S malaria vaccine is based in a fragment of CSP, containing part of the protein sequence repeats, the region III and the TSR domain. In the vertebrate host, CSP region I is cleaved, unmasking the TSR domain and exposing it to the host circulatory system[10]. Several studies emphasize the importance of knowing the structure of vaccine candidates due to its potential impact in antibody recognition[40,41]. Thus, the glycosylation of CSP and other key parasite antigens, may be important for the generation of an effective immune response against the parasite. The confirmation that *Plasmodium* PoFUT2 is involved in TSR-domain *O*-fucosylation may pave the way for the *in vitro* production of glycosylated antigens inducing more specific and protective immune responses.

Acknowledgements

Authors thank funding from National Institutes of Health (grant 1R21AI115063-01, currently supporting SS, RRD and LI). LI is a member of the GlycoPar-EU FP7 funded Marie Curie Initial Training Network (GA 608295). SS thanks funding from Boehringer Ingelheim Fonds and EMBO (ASTF 598 – 2015) for supporting exchanges. The authors thank M. Ramírez, B. López-Gutiérrez and M. Cova for technical support, advice, assistance and useful suggestions.

Declarations of interest

The authors have declared that no conflict of interest exists

Funding information

This work was funded by National Institutes of Health (grant 1R21AI115063-01)

Author contribution statement

SS, RRD and LI conceived the work. SS and EA knocked out and genotyped *P. falciparum* and *P. berghei* lines respectively. SS, RT, AT, BH and GV conducted the oocysts and sporozoite experiments of *P. falciparum* strains. EA and MM were responsible for *P. berghei* experiments. SS, LI and RRD outlined the document and all authors contributed to the writing and review of this manuscript.

Supplementary Data

Supplementary Figures

Fig S1

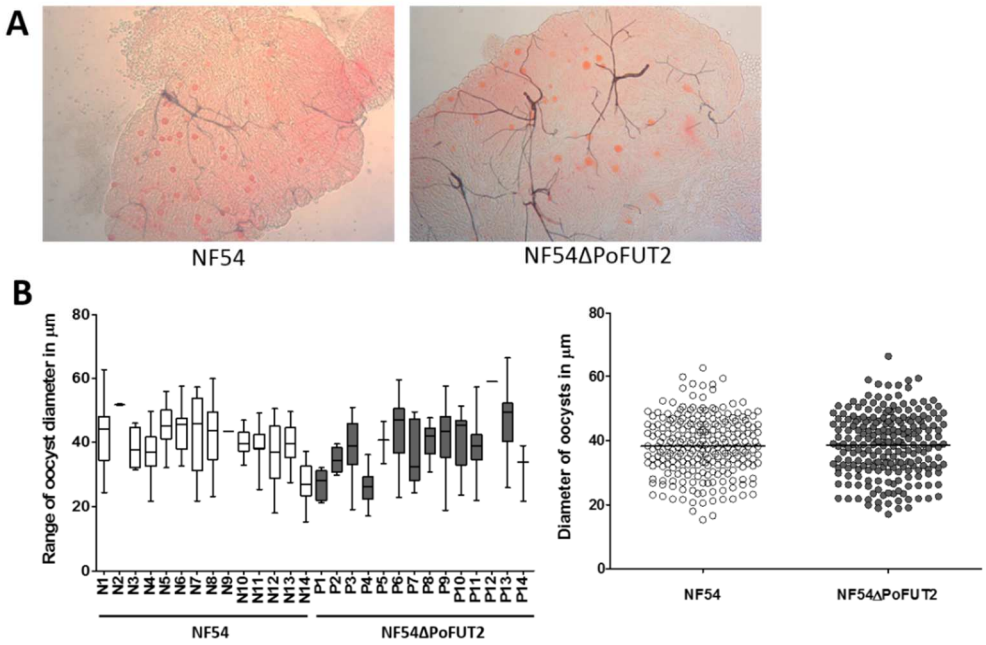


Fig S1. *P. falciparum* NF54 and NF54ΔPoFUT2 oocysts are similar in size. (A) *A. gambiae* female mosquitoes were fed at 0,03% gametocytemia and oocysts were visualized by mercurochrome staining of mosquito midguts 8-10 days after mosquito feeding. **(B)** The range of oocyst diameter (µm) (left panel) and the mean size (µm) (right panel) of the NF54 (white) and NF54ΔPoFUT2 (grey) oocyst is represented. Statistical analysis (unpaired t-test, 95% CI) showed that oocysts are not significantly different (pvalue 0,6897).

Fig. S2

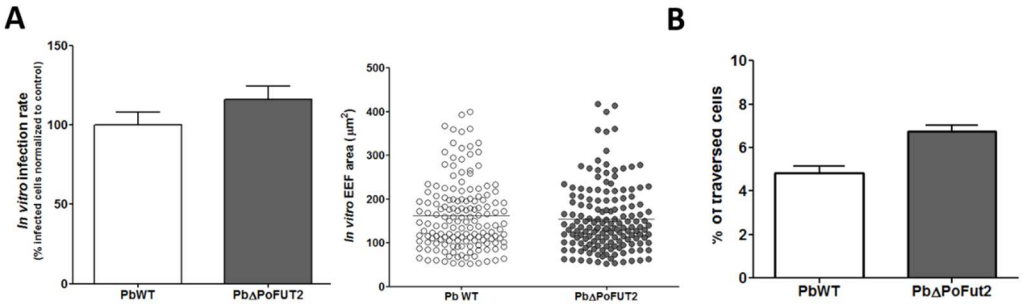


Fig. S2. Characterization of *Pb*WT and *Pb*ΔPoFUT2 sporozoites. (A) *In vitro* hepatic infection was performed and percentage of infected cells normalized to wild type is represented. Statistical analysis (Mann Whitney test) did not present any significant difference (p value: 0,1716) (left panel). Area of exoerythrocytic forms (EEF) was measured and statistical analysis (unpaired t-test, 95% CI) did not show any significant difference. (p value: 0,3931) (right panel). **(B)** Cell traversal activity was measured using *Huh7* cells and percentage of traversed cells is represented. Statistical analysis (Mann Whitney test) show that traversal activity of wild type (white) and mutant (grey) parasites is similar (pvalue: 0,1000)

Fig. S3

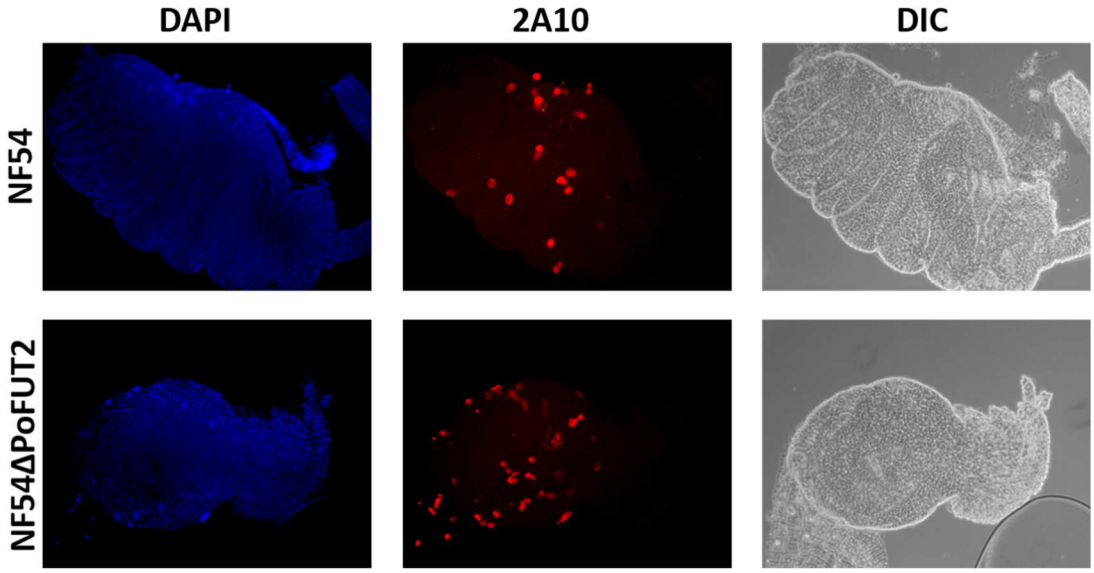


Fig. S3. CSP protein is present in the wild type and mutant parasites even upon *Pf*PoFUT2 deletion. *An. gambiae m*idguts infected with NF54 and NF54ΔPoFUT2 were dissected 8-10 days after blood feed and probed with anti-PfCSP antibodies and an Alexa, stained with DAPI, and imaged at 10X. Presence of CSP revelead that the secretion of the protein is not altered in the absence of *Pf*PoFUT.

Supplementary materials and methods

Midgut immunofluorescence assay

An. stephensi midguts were dissected 12 days post-infection and fixed in 4% paraformaldehyde for one hour on ice or overnight at 4°C. Midguts were incubated with 3% Bovine Serum Albumin (BSA) and 0.1% Triton-X-100 to block and permeabilize for one hour at room temperature or overnight at 4°C. Samples were washed in 1X Phosphate Buffered Saline (PBS) and incubated overnight at 4°C in a 1:1000 dilution of 0.5 mg/ml 2A10 mouse anti-*Pf*CSP, in 3% BSA. Following incubation with the primary antibody, midguts were washed in 1X PBS and incubated for one hour with a 1:1000 dilution of the 2 mg/ml Alexa Fluor goat anti-mouse 594 antibody in 3% BSA while being protected from light. After secondary antibody incubation, midguts were washed in 1X PBS, deposited on a slide in a drop of ProLong diamond Antifade Reagent with DAPI, and covered with a 22x22 mm coverslip. Slides were viewed and imaged using a Nikon Upright E800 and images were edited using the GNU Image Manipulation Program (GIMP).

Human hepatoma cell culture, cell traversal activity an *in vitro* infection

Huh7 cells, a human hepatoma cell line, were cultured in RPMI 1640 medium supplemented with 10% v/v FBS, 0.1 mM non-essential amino acids, 50 µg/ml penicillin/streptomycin, 2 mM glutamine and 1 mM HEPES (final concentrations), pH 7 and maintained at 37°C with 5% CO2

The traversing activity of the sporozoite was examined using a standard cell-wounding and membrane repair assay[49]. *Huh7* cells ($1.0x10^4$ per well) were seeded in 96-well plates the day before infection. Sporozoites were added to cells for 2 h in the presence of 0.5 mg/ml FITC-labeled dextran. Cells were collected for flow cytometry analysis at 48 hours post infection (hpi) and analyzed on a BD LSR Fortessa flow cytometer with the DIVA software (version 6.2). Analysis was carried out using the FlowJo software (version 6.4.7, FlowJo).

For *in vitro* hepatic infections, cells were seeded on glass coverslips in 24 well plates the day before infection. Sporozoite addition was followed by centrifugation at 1800xg for 5 min. 48 hpi, cells were fixed for 20 min at RT and incubated with permeabilization/blocking solution (0.1% v/v Triton X-100, 1% w/v BSA in 1x PBS) for 30 min at room temperature. Parasites were stained with an anti-UIS4 antibody (dilution 1:1000) for 1 h at room temperature, washed three times and further incubated with anti-mouse AlexaFluor 488 secondary antibody (1:400 dilution) in presence of Hoeschst (1:1000 dilution) for nuclear staining. Coverslips were mounted on microscope slides with Fluoromount (SouthernBiotech). Widefield images for size determination were acquired in a Zeiss Axiovert 200M microscope. Images were processed with ImageJ software (version 1.47).

References

1. World Health Organization. World Malaria Report 2015. World Health 243 (2015).
2. White, N. J. *et al*. Malaria. *Lancet*. **383**, 723–735 (2014).
3. Schwartz, L., Brown, G. V, Genton, B. & Moorthy, V. S. A review of malaria vaccine clinical projects based on the WHO rainbow table. *Malar. J*. **11** (2012).
4. RTS,S Clinical Trials Partnership. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. *Lancet*. **386**, 31–45 (2015).
5. Ménard, R. *et al*. Looking under the skin: the first steps in malarial infection and immunity. *Nat. Rev. Microbiol*. **11**, 701–12 (2013).
6. Adams, J. C. & Tucker, R. P. The thrombospondin type 1 repeat (TSR) superfamily: diverse proteins with related roles in neuronal development. *Dev. Dyn*. **218**, 280–99 (2000).
7. Tan, K. *et al*. Crystal structure of the TSP-1 type 1 repeats: a novel layered fold and its biological implication. *J. Cell Biol*. **159**, 373–82 (2002).
8. Morahan, B. J., Wang, L. & Coppel, R. L. No TRAP, no invasion. *Trends Parasitol*. **25**, 77–84 (2009).
9. Mathias, D. K. *et al*. A Small Molecule Glycosaminoglycan Mimetic Blocks *Plasmodium* Invasion of the Mosquito Midgut. *PLoS Pathog*. **9**, e1003757 (2013).
10. Coppi, A. *et al*. The malaria circumsporozoite protein has two functional domains, each with distinct roles as sporozoites journey from mosquito to mammalian host. *J. Exp. Med*. **208**, 341–56 (2011).
11. Bargieri, D. Y. *et al*. *Plasmodium* Merozoite TRAP Family Protein Is Essential for Vacuole Membrane Disruption and Gamete Egress from Erythrocytes. *Cell Host Microbe*. **20**, 618–630 (2016).

12. Wengelnik, K. *et al*. The A-domain and the thrombospondin-related motif of *Plasmodium falciparum* TRAP are implicated in the invasion process of mosquito salivary glands. *EMBO J*. **18**, 5195–204 (1999).

13. Li, F. *et al*. *Plasmodium* ookinete-secreted proteins secreted through a common micronemal pathway are targets of blocking malaria transmission. *J. Biol. Chem*. **279**, 26635–26644 (2004).

14. Chattopadhyay, R. *et al*. PfSPATR, a *Plasmodium falciparum* protein containing an altered thrombospondin type I repeat domain is expressed at several stages of the parasite life cycle and is the target of inhibitory antibodies. *J. Biol. Chem*. **278**, 25977–25981 (2003).

15. Moorthy, V. S. *et al*. A randomised, double-blind, controlled vaccine efficacy trial of DNA/MVA ME-TRAP against malaria infection in Gambian adults. *PLoS Med*. **1**, e33 (2004).

16. Luo, Y., Nita-Lazar, A. & Haltiwanger, R. S. Two distinct pathways for O-fucosylation of epidermal growth factor-like or thrombospondin type 1 repeats. *J. Biol. Chem*. **281**, 9385–92 (2006).

17. Leonhard-Melief, C. & Haltiwanger, R. S. O-Fucosylation of thrombospondin type 1 repeats. *Methods Enzymol*. **480**, 401–416 (2010).

18. Kozma, K. *et al*. Identification and characterization of abeta1,3-glucosyltransferase that synthesizes the Glc-beta1,3-Fuc disaccharide on thrombospondin type 1 repeats. *J. Biol. Chem*. **281**, 36742–51 (2006).

19. Ricketts, L. M., Dlugosz, M., Luther, K. B., Haltiwanger, R. S. & Majerus, E. M. O-fucosylation is required for ADAMTS13 secretion. *J. Biol. Chem*. **282**, 17014–23 (2007).

20. Wang, L. W. *et al*. O-fucosylation of thrombospondin type 1 repeats in ADAMTS-like-1/punctin-1 regulates secretion: implications for the ADAMTS superfamily. *J. Biol. Chem*. **282**, 17024–31 (2007).

21. Swearingen, K. E. *et al*. Interrogating the *Plasmodium* Sporozoite Surface: Identification of Surface-Exposed Proteins and Demonstration of Glycosylation on CSP and TRAP by Mass Spectrometry-Based Proteomics. *PLoS Pathog*. **12**, e1005606 (2016).

22. Aurrecoechea, C. *et al*. PlasmoDB: a functional genomic database for malaria parasites. *Nucleic Acids Res*. **37**, D539–D543 (2009).

23. Sanz, S. *et al*. Biosynthesis of GDP-fucose and Other Sugar Nucleotides in the Blood Stages of *Plasmodium falciparum*. *J. Biol. Chem*. **288**, 16506–17 (2013).

24. Cova, M., Rodrigues, J. A., Smith, T. K. & Izquierdo, L. Sugar activation and glycosylation in *Plasmodium*. *Malar. J*. **14**, 427 (2015).

25. Vasudevan, D. & Haltiwanger, R. S. Novel roles for O-linked glycans in protein folding. *Glycoconj. J*. **31**, 417–426 (2014).

26. Maier, A. G., Braks, J. A., Waters, A. P. & Cowman, A. F. Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in *Plasmodium falciparum* for targeted gene deletion by double crossover recombination. *Mol Biochem Parasitol*. **150**, 118–121 (2006)

27. Gomes, A. R. *et al*. A genome-scale vector resource enables high-throughput reverse genetic screening in a malaria parasite. *Cell Host Microbe*. **17**, 404–13 (2015).

28. Thompson, J. *et al*. PTRAMP; a conserved *Plasmodium* thrombospondin-related apical merozoite protein. *Mol. Biochem. Parasitol*. **134**, 225–232 (2004).

29. Green, J. L., Hinds, L., Grainger, M., Knuepfer, E. & Holder, A. A. *Plasmodium* thrombospondin related apical merozoite protein (PTRAMP) is shed from the surface of merozoites by PfSUB2 upon invasion of erythrocytes. *Mol. Biochem. Parasitol*. **150**, 114–7 (2006).

30. Dessens, J. T. *et al*. CTRP is essential for mosquito infection by malaria ookinetes. *EMBO J*. **18**, 6221–6227 (1999)

31. Leonhard-Melief, C. & Haltiwanger, R. S. O-Fucosylation of thrombospondin type 1 repeats. *Methods Enzymol*. **480**, 401–416 (2010)

32. Luo, Y., Koles, K., Vorndam, W., Haltiwanger, R. S. & Panin, V. M. Protein O-Fucosyltransferase 2 Adds O-Fucose to Thrombospondin Type 1 Repeats. *J. Biol. Chem*. **281**, 9393–9399 (2006).

33. Sidik, S. M. *et al*. A Genome-wide CRISPR Screen in *Toxoplasma* Identifies Essential a*picomplexan* Genes. *Cell*. **166**, 1423–1435.e12 (2016).

34. Rodrigues, J. A. *et al*. Parasite Glycobiology: A Bittersweet Symphony. *PLoS Pathog*. **11**, e1005169 (2015).

35. Trombetta, E. S. & Parodi, A. J. Quality Control and Protein Folding in the Secretory Pathway. *Annu. Rev. Cell Dev. Biol*. **19**, 649–676 (2003).

36. Samuelson, J. & Robbins, P. W. Effects of N-glycan precursor length diversity on quality control of protein folding and on protein glycosylation. *Semin. Cell Dev. Biol*. **41**, 121–8 (2015).

37. Rutkowski, D. T. & Kaufman, R. J. A trip to the ER: coping with stress. *Trends Cell Biol*. **14**, 20–8 (2004).

38. Izquierdo, L., Atrih, A., Rodrigues, J. A., Jones, D. C. & Ferguson, M. A. J. *Trypanosoma brucei* UDP-glucose:glycoprotein glucosyltransferase has unusual substrate specificity and protects the parasite from stress. *Eukaryot. Cell*. **8**, 230–40 (2009).

39. Chaubey, S., Grover, M. & Tatu, U. Endoplasmic reticulum stress triggers gametocytogenesis in the malaria parasite. *J. Biol. Chem*. **289**, 16662–74 (2014).

40. Doud, M. B. *et al*. Unexpected fold in the circumsporozoite protein target of malaria vaccines. *Proc. Natl. Acad. Sci*. **109**, 7817–7822 (2012).

41. Ringe, R. P. *et al*. Cleavage strongly influences whether soluble HIV-1 envelope glycoprotein trimers adopt a native-like conformation. *Proc. Natl. Acad. Sci*. **110**, 18256–18261 (2013).

42. Trager, W. & Jensen, J. B. Human Malaria Parasites in Continuous Culture. *J. Parasitol*. **91**, 484–486 (2005).

43. Moon, R. W. *et al*. Adaptation of the genetically tractable malaria pathogen *Plasmodium knowlesi* to continuous culture in human erythrocytes. *Proc. Natl. Acad. Sci*. **110**, 531–536 (2013).

44. Collins, C. R. *et al*. Robust inducible Cre recombinase activity in the human malaria parasite *Plasmodium falciparum* enables efficient gene deletion within a single asexual erythrocytic growth cycle. *Mol. Microbiol*. **88**, 687–701 (2013).

45. Urbán, P., Estelrich, J., Cortés, A. & Fernàndez-Busquets, X. A nanovector with complete discrimination for targeted delivery to *Plasmodium falciparum*-infected versus non-infected red blood cells in vitro. *J. Control. Release* **151**, 202–11 (2011).

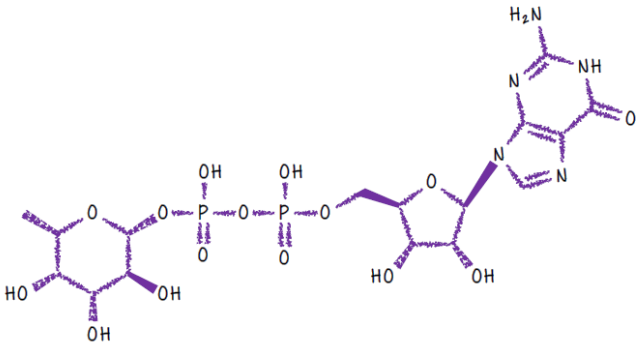
46. Rovira-Graells, N. *et al*. Transcriptional variation in the malaria parasite *Plasmodium falciparum*. *Genome Res*. **22**, 925–938 (2012).

47. Janse, C. J., Ramesar, J. & Waters, A. P. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. *Nat. Protoc*. **1**, 346–56 (2006).

48. Pfander, C. *et al*. A scalable pipeline for highly effective genetic modification of a malaria parasite. *Nat. Methods*. **8**, 1078–82 (2011).

49. Mota, M. M. *et al*. Migration of *Plasmodium* Sporozoites Through Cells Before Infection. *Science*. **291**, 141–144 (2001).

DISCUSSION



DISCUSSION

Malaria is one of the major global health problems affecting humans in developing countries. Important progresses towards malaria control and elimination have been achieved in the last decade, but much more efforts are needed to advance towards the goal of malaria eradication[6]. Despite the recent approval and recommendation of the RTS,S vaccine[171], chemotherapy is still the main strategy for malaria treatment. Unfortunately, the emergence of drug resistances threatens malaria control and elimination goals. Therefore, more research is urgently needed to complete our biochemical and cellular *Plasmodium* knowledge with the aim to describe novel parasite mechanisms amenable for intervention. Glycobiology is the study of carbohydrate and glycoconjugate related processes. Glycans are often related with host-pathogen interactions and cell-cell adhesion and communication mechanisms. Describing the glycobiology of *Plasmodium* and its relevance for the parasite life cycle (through all the different stages) may open new avenues for the identification of new vaccine and/or drug candidate targets.

Sugar nucleotides in *P. falciparum*

Previous *Plasmodium* glycobiology works hadn't elucidated the presence of fucose in the malaria parasite[97,103,104,172,173]. In this thesis, I describe the synthesis of GDP-Fuc and other sugar nucleotides in *P. falciparum*, shedding more light into the relevance of these precursors in the parasite. Thus, my first job reported evidence of the presence of different sugar nucleotides in the blood stages of *P. falciparum*[174], including GDP-Fuc (Figure 17). These findings improved the description of the metabolic pathways involved in the synthesis of these precursors.

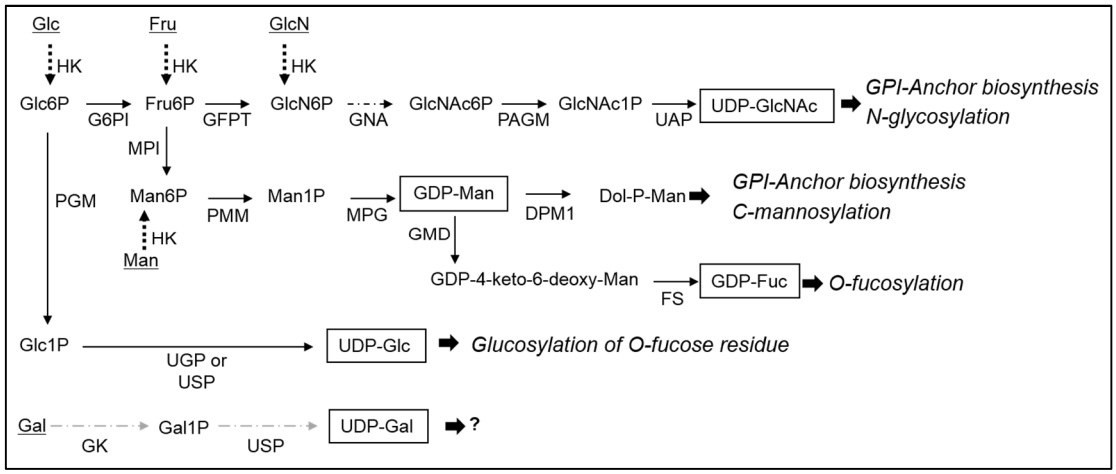


Figure 17. Sugar nucleotide biosynthetic pathways identified in the genome of *P. falciparum*. Predicted known fates of sugar nucleotide donors according to the glycoconjugates described in *P. falciparum* are in italics (or marked with a question mark if the fate is unknown). Activated sugars, used for glycoconjugate biosynthesis are boxed. Dotted lines indicate confirmed salvage pathways and sugars, take up from the medium, are underlined. GNA is represented with a discontinue black arrow because it can not be identified in the parasite genome The suggested pathway for the biosynthesis of UDP-Gal through the activity of the UDP-sugar pyrophosphorylase enzyme is indicated with a grey discontinuous arrow. Abbreviations: HK, hexokinase; G6PI, glucose 6-phosphate isomerase; GFPT, glucosamine-fructose-6-phosphate aminotransferase; GNA, glucosamine-phosphate N-acetyltransferase; PAGM, phosphoacetylglucosamine mutase; UAP, UDP-N-acetylglucosamine pyrophosphorylase; MPI, mannose-1-phosphate isomerase; PMM, phosphomannomutase; MPG, mannose-1-phosphate guanyltransferase; DPM1, Dolichol-phosphate-mannosyltransferase polypeptide 1; GMD, GDP-mannose 4,6-dehydratase; FS, GDP-L-fucose synthase; PGM, phosphoglucomutase; UGP, UTP-glucose-1-phosphate uridylyltransferase; USP, UDP-sugar pyrophosphorylase; GALE, UDP-glucose 4-epimerase; GK, galactokinase.

In this regard, the identification of UDP-Gal in the asexual blood stages of *P. falciparum* did not correlate with the apparent lack of an enzymatic activity responsible for the synthesis of this sugar nucleotide (Figure 17). UDP-Glc 4-epimerase (GALE)[175], involved in the synthesis of UDP-Gal through the epimerization of UDP-Glc, which is present in many organisms, including *T. brucei* and in *T. cruzi*, cannot be identified in *Plasmodium* genomes[99]. A homolog of UTP-glucose 1-phosphate uridyltransferase (UGP) (PF3D7_0517500) is annotated in *P. falciparum* genome. Other organisms, such as the protozoan parasite *Leishmania major*, present a similar activity, UDP-sugar pyrophosphorylase (USP) enzyme[176] with a broader substrate range than UGP. USP can use Gal-1P, Glc-1P or other sugars (i.e. D-xylopyranose-1P, UDP-β-L-arabinopyranose, or UDP-α-D-galacturonate)[177,178]. Thus, a possible way of UDP-Gal synthesis in *P. falciparum* would be through PF3D7_0517500, that shows significant similarities to USP enzymes. Further works are currently being performed to characterize this enzymatic activity (Cova *et al.* in preparation).

UDP-galactose:β-galactoside-α1-3-galactosyltransferase (*α1,3GT*) was inactivated in humans 28 milion years ago, possibly as a result from a selective pressure exerted by a pathogen[179]. This enabled the generation of a strong immune reactivity against these α-galactose (α-gal) epitopes[179]. The fate of the UDP-Gal pool in *P. falciparum* species is not known, but some studies suggested the presence of α-galactosylations in the blood stages of the parasite. These works also showed higher titers of anti-α-gal antibodies in sera from patients in malaria endemic areas[180,181]. However, later studies did not manage to identify α-gal-containing glycans in the asexual blood stages of the parasite[182,183]. Nevertheless, Yilmaz *et al*. recently reported indirect evidence of the presence of an α-gal containing glycan in the surface of *Plasmodium* sporozoites from different species, by the binding of an α-gal specific lectin (i.e. *Bandeiraea* (*Griffonia*) *simplicfolia*-1 isolectin IB_4). The authors also showed that the immune response against these glycans conferred malaria immune protection, associated with anti-α-gal antibodies[170]. The presence of a UDP-Gal pool in the

parasite suggests that this precursor may contribute to the biosynthesis of gal-containing glycans, despite an α-galactosyltransferase has not been described in the parasite genome.

Our data also demonstrated the expected presence of UDP-GlcNAc in the blood stages of the parasite[174]. There are two main metabolic routes described for the synthesis of UDP-GlcNAc: via a *de novo* pathway from Fru-6P or via a salvage pathway using a hexokinase, usually fed by GlcN. However, both routes require a glucosamine-6-phosphate N-acetyltransferase (GNA) activity (Figure 17), an enzyme that cannot be identified in *Plasmodium* genome, although it has been completely characterized in other eukaryotes, such as *S. cerevisae*[184], *T. brucei*[185], *Aspergillus fumigatus*[186] and many others. The presence of an active aminosugar pathway indicates that a GNA activity is also present in *P. falciparum*[107,108]. UDP-GlcNAc feeds the GPI-anchor biosynthesis, and it is also required for *N*-glycan synthesis in *Plasmodium*[104]. Therefore, the biosynthesis of this sugar nucleotide is an important process in the parasite, since these glycosylations are possibly critical for parasite survival and infectivity[114]. Our lab is currently working to elucidate the activity of *Plasmodium* GNA as well as in other apicomplexan parasites (Cova *et al*., in preparation).

GDP-Fuc in *P. falciparum*

P. falciparum genome encodes for homolog genes of the GDP-Fuc *de novo* biosynthetic pathway: GMD (GDP–mannose 4,6–dehydratase) and FS (GDP– L– fucose synthase) (Figure 17). It also lacks homolog genes for the fucose salvage pathway, consistent with the inability of the parasite for taking up [^{3}H]-Fuc from the media[174]. The finding of a GDP-Fuc pool in the asexual stage parasites agreed with the maintenance of GMD and FS genes and the demonstration of their enzymatic activity *in vitro*. Both enzymes localize in the cytoplasm and are expressed, at relative low levels, along the intraerythrocytic asexual life cycle of the parasite. The incorporation of [^{3}H]-GDP-Fuc by parasite extracts in cell-free assays suggested the presence of fucosyltransferase activities and of fucosylated conjugates in the blood stages. The presence of this putative fucosylation was indirectly proved in my next work[187]. The targeted disruption of GMD, responsible for GDP-Fuc synthesis, reduced *Ulex europaeus* agglutinin I (UEA-I) fucose-specific binding in the parasite surface. However, surprisingly, GMD disruption did not seem to significantly affect to the levels of GDP-Fuc present in the cell. This suggested the existence of an alternative route for the generation of this sugar nucleotide pool by the parasite, although we were not able to demonstrate a specific incorporation of [^{3}H]-Fuc or [^{3}H]-GDP-Fuc in mutants. Different studies have described passive transportation of monosaccharides, aminoacids and other molecules in *P. falciparum*[188,189], thus the parasite might be using equillibrative transportation mechanisms to obtain GDP-Fuc from the GDP-Fuc pools present in the RBC. Nevertheless,

even though the GDP-Fuc pools in the null mutant parasites were comparable with parental wild type parasites, GMD disrupted parasites showed the aforementioned clear decrease of UEA-I binding. Therefore, although the GDP-Fuc *de novo* biosynthetic pathway may not be the only source of this sugar nucleotide, its disruption seems to be shutting down the synthesis of a fucosylated glycoconjugate, without any other obvious fitness cost for the parasite.

Fucose metabolism is essential in other protozoan parasites, such as *T. brucei*. A conditional mutant of *Tb*GMD showed a decrease in GDP-Fuc, indicating that the *de novo* pathway was the only source of GDP-Fuc in this organism. Mutants went through growth arrest in both life cycle stages (bloodstream and procyclic forms), but the authors were unable to identify any Fuc-containing glycans present in *T. brucei*[109]. *T. gondii*, as *T. brucei*, does not have a GDP-Fuc salvage pathway using the *de novo* biosynthetic route to generate GDP-Fuc. In a recent paper, Bandini *et al*. suggested that this pathway may be essential or very deleterious for the parasite. The authors were capable of showing a binding of *Aleuria aurantia* lectin (AAL, a fucose specific lectin) in punctuate structures in the nuclei, close to the nuclear pore complex[190]. Nevertheless, in *P. falciparum* GDP-Fuc *de novo* metabolism is not essential for the asexual blood stages of the parasite[187]. However, GMD and/or FS may be important for other stages of the parasite. Both enzymes are detected in proteomic studies performed with *P. falciparum* sporozoites[191].

The presumed presence of a fucosylated glycoconjugate in the surface of *P. falciparum* parasites would require the action of a fucosyltransferase[187]. However, the only fucosyltransferase candidate identified in the parasite genome is PoFUT2 (PF3D7_0909200), which shows gene expression in the mature stages of the intraerythrocytic asexual parasite cycle[174].Proteomic analyses of highly purified salivary gland sporozoites from *P. falciparum* and *P. yoelii* detected this *O*-fucosyltransferase[191]. PoFUT2 is responsible for the *O*-fucosylation of TSR motifs, small cysteine-containing domains that have been related with several functions such as cell adhesion, the regulation of the extracellular matrix or cell-cell communication[136,140]. Remarkably, several key *P. falciparum* proteins contain TSR domains[161] and some of these proteins have been associated with motility and host cell invasion, a critical process for the parasite survival and propagation. Very recently, the *O*-fucosylation of two of these proteins, CSP and TRAP, has been demonstrated[102]. Taken together, this strongly suggests the conservation of an *O*-fucosylation mechanism in the parasite. In order to assess its relevance, we decided to generate a PoFUT2 KO and study its phenotype. The viability of *P. falciparum* and *P. berghei* blood stage PoFUT2 mutants revealed that the *O*-fucosylation machinery is not essential for parasite development. Furthermore, PoFUT2 KO

capacity to complete the entire parasite life cycle through the mosquito stages and to infect hepatocytes, clearly indicates that the lack of PoFUT2 is not significantly affecting key roles of TSR domain-containing proteins. In order to confirm the function of PoFUT2 in the parasite, we are currently assessing the glycosylation state of known fucosylated proteins, such as CSP or TRAP, by targeted proteomic approaches in mutant cell lines. This will further confirm whether PoFUT2 is the responsible for this posttranslational modification.

PoFUT2 in protein folding and quality control

Glycans do not only play important roles in cell-cell interaction and communication but are also involved in protein folding and quality control processes[192]. In higher eukaryotes *N*-glycans are recognized by ER chaperones that assist in the proper folding of the nascent proteins[91]. Samuelson *et al*. and others have described the effects of *N*-glycans during protein quality control in different organisms[193]. PoFUT1 is another ER localizing *O*-fucosyltransferase that differentiates folded and unfolded EGF domains. In different works it has been described that PoFUT1 has a chaperone like activity besides the fucosyltransferase activity[194,195] that contributes to the appropriate folding of EGF-containing proteins. Both, the chaperone and the transferase activities are independent[196]. PoFUT2 and *O*-fucosylation may also be involved in comparable quality control mechanisms in the parasite. PoFUT2, which is an ER localizing protein, only fucosylates properly folded TSR domains, indicating that, as PoFUT1, it distinguishes between folded and unfolded domains[143]. Moreover, different studies have shown that inhibiting *O*-fucosylation results in protein secretion defects *in vitro*[145] and that the elimination of PoFUT2 in mice produces embryonic lethality due to severe defects during the gastrulation process[147]. These results suggest a role of PoFUT2 in quality control folding and secretion of TSR containing proteins[136]. The publication of PoFUT2 crystal structure[148] and the aforementioned evidences relating PoFUT2 absence with secretion defects agree with a model in which PoFUT2 is involved in TSR folding (Figure 18). TSR domain cysteines need to be correctly paired for a proper domain folding. An unfolded TSR will be in equilibrium with different folding intermediates, with the cysteines paired incorrectly. PoFUT2 will only recognize and fucosylate the substrate when cysteines are partially but correctly paired (C_1-C_5 and C_3-C_4 or C_1-C_5 and C_2-C_6). This would shift the equilibrium to the fully folded fucosylated TSR domain (Figure 18). This model suggests that the fucosyltransferase activity and the chaperone activity are not independent[136].

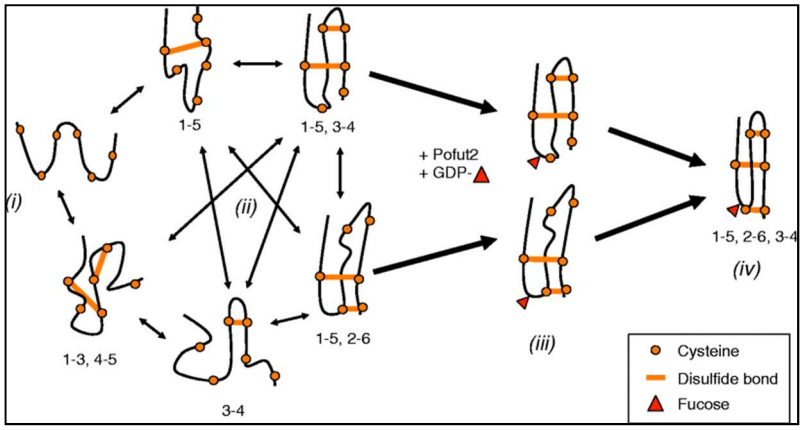


Figure 18.**PoFUT2 role in TSR domain folding.** The TSR domain (i) may exist as many different folding intermediates (ii) in equilibrium. When cysteines are partially but correctly paired, PoFUT2 recognizes it as a substrate and fucosylates it (iii). This drives the folding reaction to the formation of a fully folded TSR domain (iv). Reproduced from Vasudevan *et al.*, Glycoconj. J, 2014[136].

Unlike what has been described in other cell types or organisms[147,197], deletion of *Plasmodium* PoFUT2 appears to not affect significantly the folding and/or secretion of *Plasmodium* TSR domain-containing proteins in standard laboratory conditions, judging by the ability of the parasite to complete its life cycle. However, *O*-fucosylation may still be important for protein quality control when the parasites are under different ER stress conditions[136], as it is the case for other glycosyltransferases involved in similar quality control processes[198,199]. Therefore, more experiments regarding this issue may help to determine the role of *Plasmodium* PoFUT2 during protein folding and quality control.

O-Fuc on TSR domains can be further elongated with a glucose residue by a β1,3-glucosyltransferase[101]. It has been proposed that this enzyme, responsible for the glucose elongation in humans, also mediates a quality control mechanism for recognizing, modifying and stabilizing properly folded TSR domains[93]. Deficiency in this enzyme is responsible for the human Peter Plus Syndrome, an autosomal recessive disorder characterized by eye and developmental defects[200]. A homolog of this gene is not annotated in *P. falciparum* genome, but BLAST analysis with the human enzyme identified a putative parasitic protein with 31% identity and 50% similarity[102,201]. Previous studies indicate that this protein (parasite-infected erythrocyte surface protein 1 or PIESP1; (PF3D7_0310400)), is present in the surface of infected RBCs, which is challenging to associate with a predicted glucosyltransferase activity[201]. As mentioned before, it has been reported that *P. falciparum* CSP and TRAP are *O*-fucosylated and further glucosylated[102], but the role of this disaccharide has not yet been elucidated and further experiments are needed to address this issue.

Fucosylation of TSR domain-containing proteins

CSP is a highly-expressed protein and the main immunodominant antigen in the surface of sporozoites. It is essential for the sporozoite journey from the mosquito midgut to human hepatocytes[155,157]. The licensed RTS,S malaria vaccine is based in a fragment of CSP that contains the Repeat region and CSP's TSR domain (Figure 19)[202].

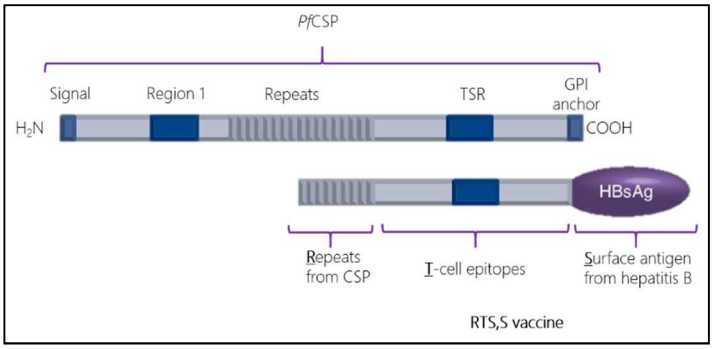


Figure 19. **RTS,S malaria vaccine.** It derives from *Pf*CSP protein and HBsAg. HBsAg, hepatitis B surface antigen; *Pf*CSP, *P. falciparum* CSP. Adapted from Regules *et al.*, Expert review of Vaccines, 2011[202].

A study from Coppi *et al.*[157] proposed a model for CSP processing and function throughout the sporozoite migration from the mosquito midgut oocyst to the mammalian host liver[157]. The authors showed that CSP has two different conformations: one in which the N-terminal masks the TSR domain and an adhesive arrangement where the TSR domain is exposed. Interestingly the TSR domain exposition occurs during oocyst development in the mosquito and during hepatocyte invasion[157].

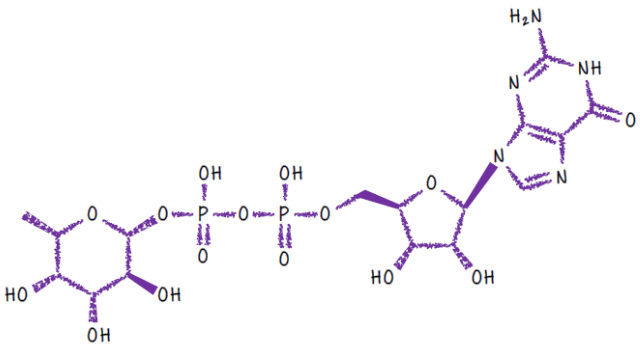
TRAP is another important sporozoite protein that has an essential role during parasite gliding motility and hepatocyte infection. Recently it was also confirmed that this protein is not only *O*-fucosylated but also *C*-mannosylated[102]. This was the first time that this posttranslational modification was reported in a parasite protein. GDP-Man, present in *P. falciparum*[174], can be further transformed to Dol-P-Man by the action of a Dolichol-phosphate mannose polypeptide 1 (DPM1), an enzyme annotated in the parasite genome[203]. Dol-P-Man acts as donor for the transference of Man residues during GPI-anchor biosynthesis, but it is also the donor for the *C*-mannosylation modification[204,205]. The enzyme responsible for this posttranslational reaction is a *C*-mannosyltransferase, that was first described in *C. elegans* as DPY-19[129]. A putative homolog enzyme is present (based on identity (26%) and similarity (47%)) in *P. falciparum* genome. This annotated gene contains DPY-19-like predicted transmembrane domains and it is expressed in *Plasmodium* salivary gland sporozoites[191]. Similar to *O*-fucosylation, *C*-mannosylation seems to play a role in

protein secretion[130], however, more research regarding the function and activity of the enzyme and the biological relevance of parasite *C*-mannosylation is required.

Several studies highlight the importance of the protein structure of vaccine candidates due to its impact on antibody response and recognition[206–208]. CSP and TRAP are both used in malaria vaccine development and both present TSR domains[209]. The presence of *O*-fucose and/or other posttranslational modifications on the TSR domains may alter their immunogenicity. Not only the presence of the sugar residue, but also the protein folding, possibly mediated by the action of PoFUT2, could be important for eliciting a more precise and effective immune response. Therefore, testing the affinity of anti-CSP or anti-TRAP antibodies against glycosylated or unglycosylated versions of CSP and/or TRAP would contribute to clarify the role of these posttranslational modifications present in key parasite antigens.

To sum up, in this work we have been able to identify, for the first time, the different pools of sugar nucleotides present in the parasite, comprising GDP-Man, GDP-Fuc, UDP-Gal, UDP-Glc and UDP-GlcNAc (Figure 17). We have also characterized the enzymes responsible for the biosynthesis of GDP-Fuc and showed preliminary evidences indicating the presence of a Fuc-containing glycan in the surface of the intraerythrocytic asexual stages of the parasite life cycle. Furthermore, we have contributed to the characterization of the *O*-fucosyltransferase that may be responsible for the addition of fucose in important *Plasmodium* proteins, such as CSP or TRAP. Nevertheless, several challenges concerning the glycobiology of *P. falciparum* remain to be addressed. Besides obtaining evidence of the glycosylation status of *O*-fucosylated proteins in PoFUT2 mutant parasites, the description of the *O*-fucose function in key parasite proteins and the characterization of other possible glycan modifications and glycosyltransferases in the parasite are important issues to be solved.

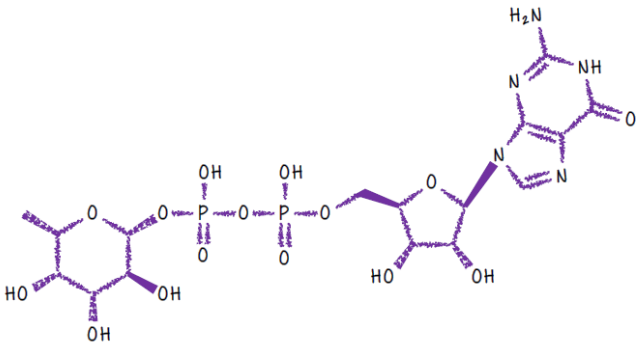
CONCLUSIONS



CONCLUSIONS

- *Plasmodium falciparum* intraerythrocytic stages contain five different sugar nucleotides that are developmentally regulated. These sugar nucleotides are: UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, GDP-mannose and GDP-fucose.
- The parasites conserve the enzymes responsible for the synthesis of GDP-Fuc (*i.e.* GMD and FS). They localize in the cytoplasm of the asexual blood stages of the parasite, are active *in vitro* and are expressed along the intraerythrocytic cycle.
- Homologs of the GDP-Fuc salvage pathway are not conserved in the parasite and it does not incorporate tritiated Fuc from the media.
- After the disruption of GMD or FS genes, the pools of GDP-Fuc present in mutant parasites are comparable with the pools of the parental line. Thus, an alternative pathway not yet described for GDP-Fuc synthesis may be present.
- A fucose-containing glycan is present in *P. falciparum* according to the binding of a fucose-specific lectin. This binding is abrogated in GMD mutant parasites.
- GMD or FS disruption does not have any significant consequence regarding the growth, cell size and morphology of the parasite in culture. Therefore, the GDP-Fuc *de novo* pathway is not essential during asexual blood stages
- *P. falciparum* expresses a protein *O*-fucosyltransferase (PoFUT2), but its disruption does not affect the growth of parasite blood stages
- Truncation of PoFUT2, which may be the responsible for the fucosylation of *Plasmodium* key proteins, does not affect parasite development through the transmission stages.

ANNEX



ANNEX

1. Summarized catalan version

INTRODUCCIÓ

Malària

La malària mata un infant a l'Àfrica cada dos minuts. La malària és una de les malalties parasitàries més importants que afecta als humans. Aproximadament la meitat de la població mundial es troba en risc d'infecció. Està causada per un paràsit protozou del gènere *Plasmodium* i es transmet mitjançant una femella de mosquit del gènere *Anopheles*. Hi ha 5 espècies de *Plasmodium* diferents que poden infectar humans: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* i *P. knowlesi*[1]. Existeixen més de 400 espècies de mosquit *Anopheles* però només 70 són capaces de transmetre la malària i d'entre aquestes només 40 són les denominades espècies dominants[2,3]. *P. falciparum* i *P. vivax* són les dues espècies de paràsit responsables de la majoria de casos de malària. *P. falciparum* és l'espècie predominant a l'Àfrica i la responsable de la majoria de morts per malària[4,5]; mentre que *P. vivax* està més àmpliament distribuïda però el risc de mort per aquesta espècie és menor[4,6].

D'acord amb l'Organització Mundial de la Salut (OMS), l'any 2015 va haver-hi 241 milions de casos nous i 429.000 morts causades per la malària, tot i aquesta dramàtica situació la incidència s'ha reduït de manera important des de l'any 2000[6]. Àfrica és la regió on es troben la majoria de casos de malària (88%), sent els infants de menys de 5 anys i les dones embarassades les persones amb més risc de patir la malaltia.

Entre set i quinze dies després de la picada d'un mosquit *Anopheles* infectat, els primers símptomes de la malaltia poden aparèixer i es classifiquen en malària no complicada o malària severa. La infecció amb el paràsit dóna lloc a un ampli rang de símptomes: des de manifestacions absents o molt lleus fins a la mort. La malària durant l'embaràs té efectes greus sobre la salut de la mare i del nadó, i és un cas important de mortalitat i morbiditat materno-infantil.

Un elevat nombre de països està treballant per assolir l'eliminació de la malària[16]. La malària pot prevenir-se i tractar-se fent servir diferents intervencions cost-efectives. Les

principals comprenen: el control del vector, quimioprevenció i gestió apropiada dels casos de malària (incloent diagnòstic i tractament)[17].

El control del vector és un component essencial de la prevenció i el seu principal objectiu és disminuir la capacitat vectorial de la població local de mosquits. Pel que fa la prevenció, les estratègies per controlar els mosquits amb més impacte són l'ús de mosquiteres tractades amb insecticida de llarga durada i l'acció d'insecticides residuals a l'interior dels habitatges[18]. La quimioprevenció consisteix en el subministrament de medicaments antimalàrics a grups de risc com poden ser les dones embarassades o bé els infants[5]. El desenvolupament d'una vacuna eficaç seria una eina excel·lent per a l'eradicació de la malària. La vacuna RTS,S, que ofereix protecció modesta contra la malària clínica va ser aprovada recentment per l'Agència Europea del Medicament i l'OMS n'ha recomanat la implementació pilot a gran escala a diferents àrees d'entre 3 i 5 països[6].

Una diagnosis a temps i ben feta és una molt bona eina per a identificar si una persona està infectada pel paràsit de la malària i tractar-la de manera adequada i així evitar l'aparició i propagació de resistències. Els tests de diagnosis ràpids (RDT per les seves sigles en anglès) detecten antígens específics produïts pel paràsits i que estan presents a la sang.

El tractament de la malària ha evolucionat amb el temps en funció de nous descobriments i de l'aparaició de resistències. La cloroquina va ser el primer tractament implementat per la OMS, però desafortunadament van sorgir resistències[25]. Després d'aquest entrebanc, medicaments alternatius com la sulfadoxina-pirimetamina, la mefloquina, l'amodiaquina i la quinina es van utilitzar com a monoteràpies, fet que va portar a l'aparició de soques resistents. El tractament de primera línia recomanat ara per l'OMS consisteix en una teràpia combinada d'artemisinina (ACT)[30]. Aquest tractament consisteix en utilitzar un derivat de l'artemisinina amb un antimalàric d'acció prolongada amb un mode d'acció diferent, per tal d'evitar l'aparició de resistències[17]. Tot i això, ja s'han confirmat regions al món on pacients han desenvolupat resistència als derivats de l'artemisinina, accentuant la necessitat de trobar nous fàrmacs[32,33].

Plasmodium falciparum

El paràsit de la malària va ser descrit per primera vegada per Alphonse Laveran l'any 1880[35] i va ser guardonat pels seus descobriments amb el premi Nobel l'any 1907. L'any 1897 en William MacCallum va descriure la reproducció sexual del paràsit mentre estudiava la malària aviària[37]. I no va ser fins el 1898 que Ronald Ross va descobrir que el paràsit de la malària humana es transmetia mitjançant mosquits anofelins[36].

L'any 1934 es va descobrir la cloroquina, que més tard es va establir com a antimalàric effectiu i segur. Uns anys més tard es van observar les propietats insecticides del DTT. Tots aquests descobriments van fer que l'OMS es decidís per impulsar una campanya per a l'eradicació de la malària. Va ser molt efectiva i entre els anys 1955 i 1972 molts països van ser certificats com a lliures de malària. Durant aquest període però, també van aparèixer les primeres resistències contra la cloroquina[25]. La República de la Xina va promoure un programa per a trobar fàrmacs contra la malària i d'aquí en va sortir el descobriment de l'artemisinina i els seus derivats, pel qual Youyou Tu va guanyar un premi Nobel l'any 2015[29]. Les teràpies combinades d'artemisinina són ara els tractaments de primera línia recomanats. L'any 2002 es va publicar la seqüenciació del genoma de la soca 3D7 de *P. falciparum*[39,40], i una mica més tard la de la resta de genomes d'altres espècies de *Plasmodium*. Aquests fets van obrir la porta a noves oportunitats d'estudi que lideressin el descobriment de nous fàrmacs i vacunes per lluitar contra la malària.

P. falciparum té un cicle de vida complex que inclou estadis en dos hostes diferents: un mosquit i un humà. La transmissió entre humans i mosquits requereix la transició entre formes asexuals i sexuals dels paràsit. El cicle del paràsit comença quan una femella de mosquit *Anopheles* infectada inocula esporozoïts a l'hoste humà durant una ingesta de sang. Els paràsits migren a través de la pell fins que arriben al sistema circulatori i després al fetge. A l'interior del fetge els esporozoïts envaeixen un hepatòcit i al seu interior es desenvolupa i multiplica formant un esquizont hepàtic. Després d'aproximadament una setmana l'esquizont es trenca i allibera milers de merozoïts a la circulació sanguínia. Aquests merozoïts pre-eritrocítics envaeixen eritròcits i comencen diferents cicles de reproducció asexual. Durant el cicle asexual del paràsit, aquest passa per a diferents estadis anomenats: rings o anells, trofozoïts i esquizonts. Un petit percentatge d'aquests paràsits es diferencien en gametòcits mascle i femella. Aquestes formes sexuals son ingerides per una altra femella de mosquit *Anopheles* quan va a buscar aliment en un humà infectat. Els gametòcits maduren fins a gàmetes que fertilitzaran i maduraran a l'intestí del mosquit, formant un ooquinet infectiu que migra a través de la paret de l'intestí fins arribar a l'hemocel on el paràsit haurà evolucionat fins a oocist. A l'interior de l'oocist es formen milers d'esporozoïts que, després de la seva maduració, seran alliberats i viatjaran fins a les glàndules salivals del mosquit. Allà romandran llestos fins que tornin a ser injectats a un hoste humà i torni a iniciar-se el cicle[44–46].

Glicobiologia

La glicobiologia és un camp d'estudi molt ampli que comprèn l'estudi de qualsevol molècula que conté un carbohidrat a la seva estructura. Inclou l'estudi del metabolisme de carbohidrats i les estructures glicoconjugades (glicoproteïnes i glicolípids). Els monosacàrids són les unitats bàsiques dels carbohidrats i amb la unió de diferents monosacàrids es formen els oligosacàrids o polisacàrids. Les cadenes de polisacàrids presenten una enorme possibilitat de combinacions, però tot i així, no totes estan presents a la natura[91]. Els carbohidrats són una font important d'energia, però també tenen altres funcions biològiques importants com pot ser l'adhesió cèl·lula-cèl·lula, l'adhesió cèl·lula-matriu, control de qualitat de proteïnes i interaccions hoste-patògen, entre d'altres. Els glicoconjugats es defineixen com compostos que contenen una part sacarídica (mono o polisacarídica) unida covalentment a altres tipus de molècules com proteïnes o lípids, formant d'aquesta manera glicoproteïnes o glicolípids respectivament. Les funcions biològiques dels diferents glicans són molt diverses. Alguns d'ells són essencials pel desenvolupament, creixement o supervivència d'un organisme, mentre que alteracions en altres poden produir síndromes i malalties[92,93]. En altres casos, no es coneix la funció que desenvolupen a nivell cel·lular o a nivell d'organisme.

Els paràsits protozous sintetitzen diferents glicoconjugats o proteïnes d'unió a glicans (lectines) que fan servir per a protegir-se i per interactuar i respondre a canvis en el seu ambient. Les interaccions hoste-patogen són crucials per la supervivència del paràsit i moltes d'aquestes es donen a través del reconeixement de carbohidrats[94]. El glicoma és el conjunt complet de glicosilacions que un organisme o cèl·lula produeix en un moment determinat. La descripció del glicoma del paràsit és un repte important que, juntament amb la determinació de la importància biològica de les diferents glicosilacions pot contribuir a la descripció de les interaccions hoste-patògen en malalties parasitàries[94]. *P. falciparum* conté diferents proteïnes glicosilades i lectines que tenen papers importants en la virulència del paràsit, la unió i la invasió de la cèl·lula hoste[96,97].

Els sucres nucleòtides són formes activades de monosacàrids. Aquests sucres nucleòtids són els donadors de les reaccions de glicosilació, que estan mediades per les glicosiltransferases. Existeixen dues vies principals de biosíntesi de sucres nucleòtids. Una via *de novo* que consisteix en la bioconversió d'un sucre o d'un sucre nucleòtid ja existent i una via de *salvaging* que inclou la fosforilació i una següent pirofosforilació del sucre[91]. Les reaccions de glicosilació consisteixen en la transferència de la part sacarídica d'un sucre nucleòtid a un acceptor específic mitjançant l'acció de glicosiltransferases[98]. Els acceptors específics poden ser oligosacàrids, proteïnes, lípids o fins i tot DNA. La majoria de les

glicosiltransferases es troben a la via reticle endoplasmàtic (RE) – aparell de Golgi, ja que moltes reaccions es donen en la via de secreció cel·lular[91]. La identificació i quantificació dels sucres nucleòtids presents a *P. falciparum* pot contribuir a la definició del perfil de glicosilació i el glicoma del paràsit.

A continuació defineixo algunes de les glicosilacions presents o que, a partir d'altres estudis, podrien estar presents en el paràsit.

- Àncores de glicosilfosfatidil inositol (GPI):

Les àncores de GPI són una estructura glicolipídica que normalment ancora proteïnes a la superfície. Es sintetitzen a través d'una via de diferents passos seqüencials i després s'uneixen covalentment a l'extrem *C*-terminal de les proteïnes[110]. La part glicosídica de les àncores de GPI a *P. falciparum* ha estat descrita i consisteix en: (Manα1-2)Manα1-2Manα1-6Manα1-4GlcN[107,108]. La síntesi de les àncores GPI és un procés complex que comença a la cara citoplasmàtica del RE i durant la síntesi donarà la volta fins al lumen del RE[99]. Els sucres nucleòtids involucrats en la síntesi d'aquesta glicosilació són la GDP-manosa (GDP-Man) i la UDP-N-acetilglucosamina (UDP-GlcNAc). Les àncores de GPI són els principals glicoconjugats descrits en els estadis intraeritrocítics del paràsit i s'encarreguen d'ancorar proteïnes clau a la membrana plasmàtica del paràsit[103,115]. Estan també presents en tots els estadis del paràsit: gametòcits, ooquinets i esporozoïts[115]. La proteïna CSP, essencial per als paràsits i utilitzada a la vacuna contra la malària, està unida a la superfície del paràsit a través d'una ancora de GPI[117]. Les àncores de GPI també s'han descrit com toxines que són capaces d'induir l'expressió de citoquines[118].

- *N*-glicosilacions

La producció de *N*-glicans requereixen la presència d'un precursor oligosacàrid unit a lípids (LLO), que s'unirà al residu d'asparagina d'una proteïna mitjançant un enllaç *N*-glicosídic. Una vegada sintetitzat el precursor LLO, el complex oligosacariltransferasa (complex OST) serà l'encarregat de transferir el LLO en bloc a la proteïna al RE[91]. La presència de *N*-glicosilacions a *P. falciparum* ha sigut controvertida durant temps, ja que resultats de diverses investigacions eren bastant diferents[103,122,123]. El genoma del paràsit conserva pocs dels gens responsables de dur a terme *N*-glicosilacions, fet que concorda amb la presència de *N*-glicans més curts que en altres espècies, només contenen dos residus de GlcNAC[104]. En eucariotes superiors les *N*-glicosilacions tenen un paper crucial en el plegament de proteïnes i també en l'estabilitat de proteïnes, solubilitat. I també poden ser importants per a l'antigenicitat i el reconeixement d'anticossos[125,126]. En canvi, les *N*-glicosilacions a *P. falciparum* no s'han descrit completament i cal definir quina és la

importància biològica de la mida d'aquest tipus de modificació en el paràsit. Podrien desencadenar respostes immunes específiques ja que aquest tipus de glicoconjugats no es troba present en el glicoma humà[99]

- *C*-manosilacions

La *C*-manosilació consisteix en l'addició d'un residu de manosa en un aminoàcid triptòfan. El precursor d'aquesta reacció és la Dolichol-fosfat-manosa (Dol-P-Man). La particularitat d'aquesta glicosilació és el tipus d'enllaç glicosídic que es forma, ja que es dona entre dos àtoms de carboni, mentre que en els altres tipus de glicosilació es dóna entre un àtom de carboni i un àtom de nitrògen o oxigen[91]. Aquesta glicosilació es sol trobar en proteïnes que contenen dominis *Thrombospondin type 1 repeats* (dominis TSR)[129–131], un domini que està present en diverses proteïnes de *Plasmodium*. L'enzim responsable d'aquesta reacció és la manosiltransferas DPY19 i un homòleg d'aquest gen es troba conservat a tots els genomes de *Plasmodium*. La importància biològica d'aquesta modificació postraduccional cal que sigui estudiada.

- *O*-glicosilacions

Les *O*-glicosilacions consisteixen en la unió covalent d'un sucre al grup hidroxil (OH) d'una serina o d'una treonina mitjançant un enllaç *O*-glicosídic. En eucariotes superiors el tipus d'*O*-glicosilació més abundant consisteix en la unió d'un residu de N-acetilgalactosamina (GalNAc), que després s'allarga amb altres sucres i genera les anomenades mucines[91]. Tot i això existeixen altres tipus d'*O*-glicosilació en cèl·lules eucariotes com per exemple l'*O*-fucosilació, la *O*-manosilació o *O*-galactosilació entre d'altres[91]. Malgrat alguns estudis controvertits, l'*O*-glicosilació no s'ha descrit mai clarament al paràsit (excepte l'*O*-fucosilació, veure a continuació).

La conservació de gens homòlegs per la via de síntesi *de novo* de la GDP-Fuc (GMD i FS) suggereixen la presència de glicans fucosilats en el paràsit. La *O*-fucosilació consisteix en l'addició de una fucosa provinent de la GDP-Fuc a un grup OH d'un residu de serina o treonina. La *O*-fucosilació es troba en dominis *Epidermal Growth Factor* (EGF) i en dominis TSR. Tot dos motius estan fucosilats a través d'enzims PoFUT (proteïna o-fucosiltransferasa). Aquests enzims són capaços de distingir entre dominis ben plegats o no ben plegats, suggerint que tenen un paper important en mecanismes de plegament i de control de qualitat[136]. PoFUT2 és l'enzim encarregat de fucosilar els dominis TSR[136]. Aquests dominis es troben presents a diferents proteïnes de *Plasmodium* que són possibles acceptors d'aquesta *O*-glicosilació. Els dominis TSR són motius petits rics en cisteïnes i que conserven 3 ponts disulfur. Aquests dominis estan relacionats amb diferents funcions importants com

per exemple la regulació de la matriu extracel·lular i les interaccions cèl·lula-cèl·lula[145]. La fucosilació d'aquests dominis es dona en la seqüènca consens $CX_2(S/T)CX_2G$[144,145] (On C és cisteïna, X pot ser qualsevol aminoàcid, S correspon a la Serina, T a treonina i G a glicina). PoFUT2 pot fucosilar només dominis que están correctament plegats, suggerint l'habilitat de la proteïna per reconèixer les dues formes (ben plegada o no ben plegada) del domini TSR[143]. El silenciament de PoFUT2 o la reducció del sucre nucleòtid donador (GDP-Fuc) resulta en problemas de secreció de proteïnes, i knockouts de PoFUT2 en ratolí tenen conseqüències letals[145–147]. Els residus d'*O*-Fuc dels dominis TSR es poden allargar amb un residu de glucosa mitjançant una β-1,3 glicosiltransferasa[101].

Els dominis TSR estan presents en diferents proteïnes del paràsit que s'expressen al llarg de tot el cicle del paràsit. Entre aquestes trobem: MTRAP, CTRP, CSP i TRAP, que es descriuen a continuació. Altres proteïnes del paràsit que contenen dominis TSR són SPATR, TRAMP, TREP o TLP, però aquestes han estat menys estudiades.

Merozoite TRAP (MTRAP): és una proteïna de la família TRAP relacionada amb *gliding motility*, un mecanisme de transport que depen de substrat[150]. Està expressada als estadis sanguinis tant sexuals com asexuals del paràsit. S'ha descrit que té un paper important en la sortida del paràsit sexual o gàmeta de l'interior dels eritròcits[152].

Circumsporozoite and TRAP Related Protein (CTRP): és una proteïna expressada en els ooquinets de *Plasmodium* i que es localitza en un dels orgànuls del paràsit, les micronemes[153]. S'ha descrit el seu paper en la invasió de l'epiteli de l'intestí del mosquit.[84]

Circumsporozoite Protein (CSP): és una proteïna molt conservada en totes les espècies de *Plasmodium*. S'expressa en els esporozoïts del paràsit i es troba ancorada a la superfície mitjançant una àncora de GPI[155]. Té un paper essencial en la invasió de les glàndules salivals del mosquit així com la invasió dels hepatòcits[157]. S'ha descrit recentment que el domini TSR d'aquesta proteïna està fucosilat[102].

Thrompospondine Related Anonymous Protein (TRAP): és una proteïna essencial involucrada en la invasió i motilitat dels esporozoïts[158]. L'*O*-fucosilació i *C*-manosilació d'aquesta proteïna s'ha descrit recentment[102].

HIPÒTESI I OBJECTIUS

L'estudi de la glicobiologia de paràsits pot obrir noves línies de recerca que portin al descobriment de nous fàrmacs antiparasítics selectius o a candidats a vacunes, a través de la identificació de noves dianes essencials per als paràsits. Tenint en compte els glicoconjugats descrits en altres estudis fets amb *Plasmodium*, la presència de GDP-Fuc no s'esperava. Malgrat això, els gens de la ruta metabòlica per a la síntesi *de novo* de GDP-Fuc a *P. falciparum* estan conservats al seu genoma i al de totes les espècies de *Plasmodium* seqüenciades. A més a més, els paràsits també conserven en el genoma una putativa proteïna *O*-fucosyltransferasa (PoFUT2), que és la responsable de l'addició de fucosa a dominis TSR. Aquests dominis estan presents en proteïnes de *Plasmodium* que tenen funcions clau en diferents estadis del paràsit. Tenint en compte tota aquesta informació la nostra hipòtesi és que ***P. falciparum*** té un mecanisme de *O*-fucosilació actiu. Per explorar aquesta hipòtesi, els objectius d'aquesta tesis doctoral són:

- Identificar i quantificar el contingut de sucres nucleòtids present en els estadis intraeritrocítics de *P. falciparum*
- Confirmar l'activitat enzimàtica *in vitro* de *Pf*GMD i *Pf*FS, els enzims responsables de la síntesi de GDP-Fuc en els estadis asexuals del paràsit.
- Descriure la importància biològica de *Pf*GMD i *Pf*FS, tot creant paràsits mutants sense aquests enzims i estudiant el seu fenotip
- Determinar la funció de la proteïna *O*-fucosyltransferasa 2 (PoFUT2) de *P. falciparum* creant mutants complets per a aquest gen i caracteritzant el fenotip a tots els estadis del cicle de vida del paràsit.

RESULTATS

Article 1: "Biosíntesi de GDP-fucosa i altres sucres nucleòtids als estadis intraeritrocítics de *Plasmodium falciparum*"

Antecedents:

Les àncores de GPI representen la major part de modificacions de carbohidrats que s'han descrit a la superfície de *P. falciparum*. Moltes de les proteïnes que estan unides a aquestes àncores són essencials per a la invasió i la virulència del paràsit. Recenment s'ha descrit la presència de *N*-glicosilacions als estadis intraeritrocítics del paràsit. Fins ara l'existència d'*O*-glicosilacions en el paràsits no ha estat demostrada. Els sucres nucleòtids són formes activades dels sucres i que han estat produïdes per la cèl·lula. Són intermediaris essencials per a les reaccions de glicosilació. Poden sintetitzar-se mitjançant una via *de novo* o bé mitjançant una via de rescat. En el genoma de *P. falciparum*, així com en el de la resta d'espècies de *Plasmodium* seqüenciades es poden predir els gens implicats en la síntesi *de novo* de la UDP-N-acetilglucosamina, GDP-manosa, GDP-fucosa i UDP-glucosa. Aquesta idea suggereix que el seu manteniment es deu a la importància biològica d'aquestes vies i també l'existència de glicosilacions on aquests sucres nucleòtids són els donadors

Objectiu:

Tenint en compte la falta d'estudis en aquest camp volem quantificar el contingut de sucres nucleòtids present en el paràsit i caracteritzar els enzims responsables de la síntesi *de novo* de la GDP-Fuc anotats en el genoma de *Plasmodium*

Resultats:

- Mitjançant l'espectrometria de masses, hem identificat i quantificat, per primera vegada, el contingut de sucres nucleòtids present al paràsit, que són: UDP-galactopiranosa, UDP-glucosa, UDP-N-acetilglucosamina, GDP-manosa i GDP-fucosa
- El paràsit no és capaç d'incorporar fucosa tritiada ([^{3}H]-Fuc) del medi, indicant que la principal font de GDP-Fuc del paràsit és mitjançant la síntesi *de novo*
- Els gens encarregats de la síntesi de GDP-Fuc, que són GMD i FS, s'expressen al llarg de tot el cicle asexual del paràsit, són actius *in vitro* i es localitzen al citoplasma del paràsit.
- Els esquizonts del paràsit expressen una proteïna homòloga a la proteina O-fucosiltransferasa 2 (PoFUT2). A més a més, extractes d'esquizonts de *P. falciparum* incorporen GDP-Fuc tritiada.

Conclusions:

El paràsit *P. falciparum* conté diferents sucres nucleòtids que es corresponen amb el manteniment en el genoma de vies metabòliques encarregades de la seva síntesi. La presència de GDP-fucosa, la demostració de l'activitat *in vitro* dels enzims encarregats de sintetitzar-la així com l'expressió d'una fucosiltransferasa suggereixen la presència d'un glicà que conté fucosa.

Article 2: "La disrupció de la síntesi *de novo* de GDP-fucosa suggereix la presència d'un nou glicoconjugat que conté fucosa en els estadis asexuals de *Plasmodium*"

Antecedents:

Els glicoconjugats a la superfície cel·lular són mediadors important de les interaccions hoste-patògen. La presència d'un *pool* de GDP-fucosa i l'expressió d'una fucosiltransferasa suggereixen que aquest sucre nucleòtid pot estar implicat en la modificació post traduccional dels dominis TSR, dominis presents en proteïnes clau pel desenvolupament del paràsit de la malària. Recentment s'ha descrit la presència d'*O*-glicosilacions i *C*-manosilacions als esporozoïts del paràsits.

Objectiu:

Mitjançant la disrupció dels gens implicats en la biosíntesi *de novo* de GDP-fucosa (GMD i/o FS) volem caracteritzar i descriure la importància biològica d'aquesta via metabòlica.

Resultats:

- La disrupció de GMD i FS és possible als estadis asexuals del paràsit i aquesta no afecta el creixement *in vitro* ni la morfologia i mida del paràsit.
- El contingut de GDP-fucosa, així com el dels altres sucres nucleòtids no es veu significativament afectat a causa de la disrupció de GMD i FS.
- La síntesi d'un glicà que conté fucosa s'atura en els paràsits que tenen els gens GMD i FS disruptats. Això queda demostrat per la disminució del marcatge de la lectina d'unió a fucosa *Ulex europaeus* agglutinin 1 (UEAI) a la superfície del paràsit.

Conclusió:

La possibilitat de truncar els gens responsables de la síntesi *de novo* de GDP-fucosa ens permet concloure que aquesta via metabòlica no és essencial per al paràsit. El manteniment del contingut de sucres nucleòtids en els paràsits mutants suggereix que hi ha una via alternativa d'obtenció de GDP-fucosa que encara no ha estat descrita. Tot i això la disrupció d'aquesta via metabòlica sí que atura la síntesi del glicà que conté fucosa i que es troba a la superfície del paràsit.

Treball no publicat: Paràsits *Plasmodium* sense la maquinària d'*O*-fucosilació són capaços de completar el cicle de vida en humans i ratolins.

Antecedents:

Els dominis TSR són dominis petits (entre 50 i 60 aminoàcids) rics en cisteïnes i que contenen 3 ponts disulfurs conservats. Tenen papers importants en l'adhesió cel·lular i en la motilitat. Els paràsits del gènere *Plasmodium* expressen, al llarg de tot el cicle, diferents proteïnes amb dominis TSR; proteïnes amb papers claus en el reconeixement hoste-cèl·lula, en motilitat i en invasió. Entre aquestes proteïnes trobem la *Circumsporozoite protein* (CSP), la *Thrombospondin Related Anonymus protein* (TRAP) entre d'altres. S'ha desmostrat que anticossos contra aquestes proteïnes inhibeixen la invasió i paren la progressió del cicle del paràsit. A més a més, són importants candidats a vacunes. De fet la vacuna contra la malària amb llicència i recomenada per la OMS, RTS,S es basa en la proteïna CSP, que conté un domini TSR a l'extrem C-terminal.

S'ha demostrat que els dominis TSR són *O*-fucosilats per la proteïna PoFUT2 i que a més a més es poden allargar amb un residu de glucosa, generant un disacàrid *O*-linkat. Aquesta modificació és important per la secreció de proteïnes que contenen aquests dominis. Un estudi recent ha demostrat que les proteïnes CSP i TRAP estan ambdues fucosilades en els seus dominis TSR. El genoma de *P. falciparum* conserva un homòleg de la proteïna PoFUT2, el paràsit sintetitza GDP-fucosa, inidicant que PoFUT2 pot ser l'encarregada de dur a terme aquesta modificació post-traduccional.

Objectius:

Crear mutants que no continguin la proteïna PoFUT2 en el paràsit de la malària humana i el model de ratolí, *P. falciparum* i *P. berghei* respectivament.

Caracteritzar els mutants resultats al llarg de tot el cicle del paràsit, tant en els estadis sanguinis com en els estadis que es desenvolupen en els mosquits.

Resultats:

- La creació dels paràsits mutants de la proteïna PoFUT2 ha estat possible en el paràsit de la malària humana i en el del model de ratolí. Això indica que aquest gen no és essencial per al desenvolupament del paràsit.
- Els paràsits mutants no presenten cap tipus d'afectació en quant al creixement en els estadis asexuals intraeritrocítics.

- Les línies del paràsit que no contenen la proteïna encarregada de dur a terme la fucosilació són capaces d'infectar mosquits i la producció dels oocists és normal comparada amb la soca no mutant.
- Els esporzoïts de les soques de paràsit mutant poden infectar les glàndules salivals del mosquit i a més són capaces de re-infectar els ratolins.

Conclusió:

La disrupció del gen PoFUT2 no té un efecte obvi sobre el *fitness* del paràsit, ja que tant els estadis asexuals com els estadis que es desenvolupen en el mosquit creixen de manera comparable. L'efecte de la no presència de residus de fucosa en proteïnes essencials per al paràsit cal que s'estudiï amb més profunditat, ja que podria tenir un paper en la inducció de respostes immunes més específiques i més protectores.

DISCUSSIÓ

La malària és un dels principals problemes de salut que afecta als humans en països en desenvolupament. Cal seguir fent esforços per tal de poder arribar a l'objectiu d'eradicar la malària. La glicobiologia és l'estudi dels procesos relacionats amb els carbohidrats i els glicoconjugats. Descriure la glicobiologia del paràsit causant de la malària i la seva importancia pel cicle d'aquest, obrirà noves portes per a la identificació de noves dianes terapèutiques i per a vacunes.

Sucres nucleòtids a *P. falciparum*.

Estudis previs no havien descrit la presencia de fucosa en el paràsit de la malària[97,103,104,172,173]. En aquesta tesi es descriu la presencia de GDP-Fuc i d'altres sucres nucleòtids presents en el paràsit, avessant més llum a la importancia d'aquests precursors en el paràsit. Per tant, el meu primer treball descriu evidències de la presencia de diferents sucres nucleòtids en els estadis sanguinis de *P. falciparum*, incloent GDP-Fuc[174]. Aquesta troballa ha permès millorar la descripció de les vies metabòliques involucrades en la síntesi d'aquests precursors.

La identificació de UDP-galactosa (UDP-Gal) no es corresponia amb la falta aparent de l'activitat enzimàtica responsable de la síntesi d'aquest sucre nucleòtid. L'enzim UDP-Glc 4-epimerasa (GALE)[175], involucrat en la síntesi de UDP-Gal a través de l'epimerització de UDP-Glc, no es pot identificar en el genoma del paràsit mentre que si que el trobem en altres paràsits[99]. Un homòleg de l'enzim UTP-glucosa 1-fosfat uridiltransferasa (UGP) (PF3D7_0517500) està anotat al genoma del paràsit. Altres paràsits com *L. major* presenten una activitat similar amb un rang més ampli de substrats (enzim UDP-sucre pirofosforilasa (USP))[116], podent fer servir altres sucres[177,178]. Per tant una possible via de síntesi de UDP-Gal a *P. falciparum* seria a través de PF3D7_0517500, que presenta similaritats amb l'enzim USP. Altres treballs s'estan realitzant per tal de caracteritzar aquesta activitat enzimática (Cova *et al.*, en preparació). El destí del *pool* de UDP-Gal a *P. falciparum* no es coneix, però alguns estudis han suggerit la presencia de α-galactosilacions als estadis interaeritrocítics del paràsit[181,183]. Els estudis més recents descriuen evidències indirectes de la presencia d'α-galactosilacions i a més a més també desmostren que respostes immunes en contra d'aquests glicans confereixen protecció contra la malària[170].

Les nostres dades també demostren l'esperada presència de UDP-GlcNAc als estadis sanguinis del paràsit[174]. Hi ha dues vies de síntesi de UDP-GlcNAc: una via *de novo* i una de *salvaging*. Les dues vies necessiten però, l'activitat glucosamina-6-fosfat N-

acetiltransferasa (GNA), un enzim que no s'ha pogut identificar a *P. falciparum* tot i que s'ha descrit completament a altres organismes[184–186]. La presencia del sucre nucleòtid indica que aquesta activitat ha d'estar present en el paràsit. UDP-GlcNAc és donador a les reaccions de síntesi d'àncores GPI i en les de *N*-glicosilació[104]. Per tant la síntesi d'aquest sucre nucleòtid és un proces important pel paràsit ja que aquestes glicosilacions són essencials per aquest[114]. Al nostre laboratori s'està treballant per esbrinar l'activitat GNA de *Plasmodium*, així com la d'altres paràsits apicomplexos (Cova *et al.*, en preparació).

GDP-Fuc a *P. falciparum*

El genoma de *P. falciparum* codifica els enzims necessaris per a la síntesi *de novo* de GDP-fucosa. A més a més no conté gens homòlegs per a la via de *salvaging*. La descripció del *pool* de GDP-Fuc en el paràsit concorda amb el manteniment dels enzims i la desmostració de la seva activitat *in vitro*[174]. La incorporació de [^{3}H]-GDP-Fuc en assaigs lliures de cèl·lula en extractes de paràsits suggereixen la presencia d'una activitat fucosiltransferasa i de gliconconjugats fucosilats. La presència d'aquest conjugat es va demostrar de manera indirecta en el meu següent treball[187]. La disrupció de GMD va reduir la unió d'una lectina específica a fucosa. Però, de manera sorprenent, el truncament d'aquest gen no va afectar de manera significativa el *pool* de GDP-Fuc present a la cèl·lula, suggerint una via altrernativa de generació d'aquest sucre nucleòtid, tot i que no vam ser capaços de demostrar-ho mitjançant la incorporació de Fuc o GDP-Fuc tritiada. S'han descrits mecanismes de difusió passiva en el paràsit, essent una possibilitat per a l'obtenció de GDP-Fuc[188,189]. Tot i que la via de síntesi de GDP-Fuc *de novo* no sembla ser la única font d'aquest sucre nucleòtid, la seva disrpució sembla esta apagant la síntesi de glicoconjugats fucosilats, però sense cap altra cost obvi pel paràsit.

El metabolisme de la fucosa ha resultat ser essencial en altres paràsits protozous com *T. brucei*. Un mutant condicional de GMD en aquest paràsit va fer disminuir la quantitat de GDP-Fuc a l'organisme i els paràsits van deixar de crèixer. Tot i això els autors no van ser capaços de trobar el gliconjugat[109]. *T. gondii*, així com *T. brucei* tampoc té via de *salvaging* de GDP-Fuc. En un article recent, Bandini *et al.* van sugerir que la via *de novo* és esencial pel paràsit[190]. Tot i això, la via *de novo* de la GDP-Fuc a *P. falciparum* no és essencial pels estadis asexuals del paràsit[187], però pot ser que ho sigui en altres estadis que no hem pogut estudiar.

La presumpta presència d'un glicoconjugat fucosilat a la superficie del paràsit necesita de l'activitat fucosiltransferasa[187]. L'únic candidat identificat en el genoma del paràsit és PoFUT2 (PF3D7_0909200), que s'expressa en els estadis madurs del cicle

intraeritrocític del paràsit[174]. A més a més, anàlisis proteòmiques d'esporozoïts de *P. falciparum* van detectar aquesta fucosiltransferasa[191]. PoFUT2 és l'enzim responsable de la *O*-fucosilació dels dominis TSR, presents en diverses proteïnes del paràsit[161]. Algunes d'aquestes proteïnes que contenen aquest domini tenen papers importants i s'han definit com a essencials per al paràsit. Recentment s'ha descrit l'*O*-fucosilació de dues d'aquestes proteïnes, CSP i TRAP[102]. Tota aquesta informació, suggereix la presencia d'un mecanisme d'*O*-fucosilació. Per tal d'avaluar-ne la seva importància vam generar un KO de PoFUT2 per estudiar-ne el fenotip. La viabilitat dels KO de PoFUT2 en *P. falciparum* i en *P. berghei* van revelar que la maquinària d'*O*-fucosilació no és essencial pel desenvolupament del paràsit. A més a més els mutants van ser capaços de completar tot el cicle a través dels mosquits i d'infectar hepatòcits, indicant que la manca de PoFUT2 no afecta significativament als papers claus que tenen les proteïnes que contenen dominis TSR. Per tal de confirmar la funció de PoFUT2 al paràsit, estem investigant, mitjançant mètodes proteòmics, l'estat de glicosilació de proteïnes que es coneix que están fucosilades, com CSP o TRAP.

PoFUT2 en el plegament de proteïnes i en el control de qualitat

Els glicans no només tenen un paper important en les interaccions cèl·lula-cèlula, sinó que també estan involucrats en el plegament de proteïnes i en control de qualitat[192]. En eucariotes superiors, els *N*-glicans són reconeguts per chaperones del RE que ajuden en el correcte plegament de proteïnes que s'estan formant[91]. PoFUT1, que fucosila dominis EGF i es troba al RE, pot discernir entre dominis ben plegats o mal plegats. S'ha descrit que la PoFUT1 té activitat chaperona a més a més d'acitivitat fucosiltransferasa[194,195], i que aquestes dues activitats són independents l'una de l'altre[196]. PoFUT2, que també es localitza al RE pot tenir algun paper similar en mecanismes de control de qualitat. Diferents estudis han demostrat que la manca de PoFUT2 resulta en problemes de secreció de proteïnes[145] i el KO d'aquesta proteïna en ratolins no és viable[147]. Totes aquestes informacions suggereixen un paper en el correcte plegament i secreció de proteïnes i en control de qualitat[136]. Un model sobre el funcionament de PoFUT2 descriu el mecanisme pel qual aquest enzim actua sobre les proteïnes[136]. Un domini TSR desplegat està en equilibri amb diferents intermediaris de plegament, amb les cisteïnes emparellades de manera incorrecta. PoFUT2 només reconeixerà i fucosilarà, aquells intermediaris que estiguin correctament aparellats (C_1-C_5 i C_3-C_4 o C_1-C_5 i C_2-C_6), desplaçant l'equilibri cap a la forma totalment plegada i fucosilada del domini TSR. Aquest model suggereix que l'activitat fucosiltransferasa i l'activitat chaperona no són independents[136]. Els nostres resultats demostren que la disrupció de PoFUT2 no afecta el plegament i secreció de proteïnes de

Plasmodium que contenen dominis TSR en condicions de laboratori. Tot i això l'*O*-fucosilació pot ser important pel control de qualitat quan els paràsits es troben sota diferents tipus d'estrés del RE, com és el cas de glicosiltransferases involucrades en sistemes de control de qualitat de proteïnes similars[198,199]. Per tant, més experiments al voltant d'aquesta idea podrien ajudar a determinar el rol de PoFUT2 durant el plegament i control de qualitat de proteïnes.

Els residu d'*O*-Fuc als dominis TSR poden ser allargats mitjançant l'activitat d'una β1,3-glicosiltransferasa[101]. Aquest enzim, responsable de l'elongació amb glucosa en humans, també intervé en un mecanisme de control de qualitat, i deficiències en aquest enzim causen diferents síndromes en humans[200]. Homòlegs d'aquest gen no estan anotats al genoma de *Plasmodium*, pero anàlisis BLAST amb l'enzim humà van identificar una possible proteïna present a la superfície dels eritròcits infectats (parasite-infected erythrocyte surface protein 1 or PIESP1; (PF3D7_0310400))[101,201]. S'ha demostrat, que proteïnes del paràsit amb aquests dominis es troben fucosilades i també glicosilades[102], però el rol d'aquest disacàrid a *Plasmodium* no s'ha descrit encara.

Fucosilació de proteïnes que contenen dominis TSR

CSP és una proteïna altament conservada i expressada i és el principal antígen immunodominant a la superficie del paràsit. És una proteïna essencial per la migració de l'esporozoït des de l'intestí del mosquit fins a l'hepatòcit humà[155,157]. A més a més, la vacuna de la malària RTS,S es basa en un fragment que conté el domini TSR d'aquesta proteïna[202]. Un estudi de Coppi *et al.* va proposar un model pel processament i la funció d'aquesta proteïna[157]. Els autors mostren que CSP té dues conformacions: una en que l'extrem N-terminal emmascara el domini TSR i una conformació adhesiva on el domini TSR està exposat. Justament l'exposició del domini TSR ocorre durant el desenvolupament de l'oocist al mosquit i durant la invasió de l'hepatòcit[157].

TRAP és un altra proteïna important de l'esporozoït que té un paper essencial per a la motilitat del paràsit i la infecció de les cèl·lules del fetge. S'ha confirmat que aquesta proteïna a més d'estar fucosilada, també està *C*-manosilada[102], era la primera vegada que es describia aquest tipus de glicosilació en proteïnes del paràsit. Un posible enzim responsable de l'activitat manosiltransferasa està present en el genoma del paràsit, però calen més estudis per desxifrar l'activitat d'aquest enzim i la importancia biológica de la modificació postraduccional.

Diferents estudis resalten la importància de coneixer l'estructura dels candidats a vacunes, degut a l'efecte que té aquesta en la resposta i reconeixement d'anticossos[206–208].

CSP i TRAP es fan servir en el desenvolupament de vacunes per a la malària i les dues presenten dominis TSR[209]. La presència d'*O*-fucosilacions i altres modificacions en els dominis TSR poden alterar la immunogenicitat. Per tant, avaluar l'afinitat d'anticossos anti-CSP o anti-TRAP contra versions de CSP o TRAP glicosilades o no glicosilades pot clarificar el rol d'aquestes modificacions en antígens claus del paràsit.

Per resumir, en aquest treball hem pogut identificar per primera vegada, els diferents *pools* de sucres nucleòtids presents en el paràsit. També hem pogut caracteritzar els enzims responsables de la biosíntesi de GDP-Fuc i mostrar evidències preliminars que indiquen la presència d'un glicà que conté fucosa a la superfície dels estadis sanguinis del paràsit. A més a més, hem contribuit a la caracterització de la *O*-fucosiltransferasa que pot ser responsable de l'addició dels residus de fucosa als dominis TSR de proteïnes importants com poden ser TRAP o CSP. Tot i això queden reptes importants relacionats amb la glicobiologia del paràsit. A més d'obtenir evidències de l'estat de glicosilació dels mutants de PoFUT2; la descripció de la funció de l'*O*-fucosa en proteïnes clau i la caracterització d'altres possibles glicans i glicosiltransferases del paràsit són conceptes importants a resoldre.

CONCLUSIONS

- Els estadis intraeritrocítics de *Plasmodium falciparum* contenen cinc sucres nucleòtids diferents: UDP-glucosa, UDP-galactosa, UDP-*N*-acetylglucosamina, GDP-manosa, i GDP-fucosa
- Els paràsits conserven els enzims responsables de la síntesi de GDP-fucosa (GMD i FS). Es localitzen al citoplasma dels estadis asexuals del paràsits, són actius *in vitro* i s'expressen al llarg de tot el cicle intraeritrocític.
- Homòlegs de la via de *salvaging* de GDP-fucosa no estan conservats en el genoma del paràsit i aquest no incorpora Fuc del medi.
- Tot i la disrupció dels gens GMD i/o FS, el *pool* de GDP-Fuc present en els paràsits mutants són comparables amb el contingut de GDP-Fuc de la soca salvatge. Per tant, una via alternativa encara no descrita deu estar present en el paràsit.
- Un glicà que conté fucosa és present a *P. falciparum* tenint en compte la unió d'una lectina d'unió específica a fucosa. Aquesta unió desapareix en els mutants de GMD.
- La disrupció de GMD i FS no té cap conseqüència significativa pel creixement, la mida i la morfologia del paràsit en cultiu. Per tant, la via de síntesi *de novo* de la GDP-Fuc no és essencial durant els estadis sanguinis del paràsit.
- *P. falciparum* expressa una proteïna *O*-fucosiltransferasa (PoFUT2) durant els estadis sanguinis del paràsit. La seva disrupció no afecta el creixement dels estadis intraeritrocítics
- El truncament de PoFUT2, l'enzim que podria ser el responsable de la fucosilació de proteïnes clau de *Plasmodium*, no altera el desenvolupament del paràsit durant els estadis de transmissió

2. Other contributions

"Evaluation of a chemiluminescent enzyme-linked immunosorbent assay for the diagnosis of *Trypanosoma cruzi* infection in a nonendemic setting"

Luis Izquierdo, Alexandre Ferreira Marques, Montserrat Gállego, Sílvia Sanz, Sílvia Tebar, Cristina Riera, Llorenç Quintó, Edelweiss Aldasoro, Igor C Almeida, Joaquim Gascon

Mem Inst Oswaldo Cruz, 108 (2013) 928-931

Evaluation of a chemiluminescent enzyme-linked immunosorbent assay for the diagnosis of *Trypanosoma cruzi* infection in a nonendemic setting

Luis Izquierdo[1]/+, Alexandre Ferreira Marques[2], Montserrat Gállego[3], Sílvia Sanz[1], Sílvia Tebar[3], Cristina Riera[3], Llorenç Quintó[1], Edelweiss Aldasoro[1], Igor C Almeida[2], Joaquim Gascon[1]

[1]Hospital Clinic, Barcelona Centre for International Health Research [3]Laboratorio de Parasitología, Facultad de Farmacia, Universitat de Barcelona, Barcelona, Spain [2]Border Biomedical Research Center, University of Texas at El Paso, El Paso, Texas, USA

The disappearance of lytic, protective antibodies (Abs) from the serum of patients with Chagas disease is accepted as a reliable indicator of parasitological cure. The efficiency of a chemiluminescent enzyme-linked immunosorbent assay based on a purified, trypomastigote-derived glycosylphosphatidylinositol-anchored mucin antigen for the serologic detection of lytic Abs against Trypanosoma cruzi *was evaluated in a nonendemic setting using a panel of 92 positive and 58 negative human sera. The technique proved to be highly sensitive [100%; 95% confidence interval (CI) = 96-100] and specific (98.3%; 95% CI = 90.7-99.7), with a kappa score of 0.99. Therefore, this assay can be used to detect active* T. cruzi *infection and to monitor trypanosomicidal treatment.*

Key words: Chagas disease - lytic anti-α-Gal antibodies - *Trypanosoma cruzi*

American trypanosomiasis, or Chagas disease, is a chronic infection caused by the protozoan parasite *Trypanosoma cruzi*. The disease can be transmitted by triatomine insect vectors, blood transfusion, organ transplantation, tainted foods and fluids and transplacentally. The acute phase of Chagas disease lasts six-eight weeks during which certain patients have fever, lymphadenopathy, splenomegaly and/or oedema, though most cases are asymptomatic or oligosymptomatic. In the event that the acute phase ends and the parasite is not completely eliminated, *T. cruzi* infection passes into a clinically silent chronic phase designated as the indeterminate clinical form. Although most infected individuals remain asymptomatic for life, 20-35% of patients develop severe chronic Chagas disease over a period of 10-30 years that is characterised by cardiac and/or gastrointestinal tract disorders. During the chronic phase of the disease, the levels of circulating parasite are far below the threshold of microscopic detection and the diagnosis is therefore primarily based on conventional serology.

Due to migration trends, there are millions of people from Chagas disease-endemic countries now living in North America, Europe, Australia and Japan, including thousands of people with *T. cruzi* infection. Congenital, transfusion-associated and/or transplant-associated transmission has been documented in the United States of America, Spain, Canada and Switzerland (Gascon et al. 2010). In the present paper, we describe the use of a chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) for the serodiagnosis of chagasic infection (Almeida et al. 1997) in our facilities at the Barcelona Centre for International Health Research. The test is based on the reactivity of serum with glycosylphosphatidylinositol (GPI)-anchored mucins purified from mammalian cell culture-derived trypomastigotes (tGPI-mucins) of the Y strain (Almeida et al. 1994). These antigens are specifically recognised by protective chagasic anti-α-galactosyl antibodies (anti-α-Gal Abs) and can be used to diagnose active disease because the Abs elicit both complement-dependent and complement-independent lysis of infective parasite stages, which revert after *T. cruzi* elimination (Almeida et al. 1991, 1993, 1997, Andrade et al. 1996, 2004, Pereira-Chioccola et al. 2000). Therefore, the assay combines the use of a highly specific antigen from the infective (trypomastigote) form of the parasite with a highly sensitive procedure for the detection of positive reactions and allows the detection of lytic anti-α-Gal Abs in the serum of Chagas disease patients (Andrade et al. 1996, 2004, Almeida et al. 1997, de Marchi et al. 2011).

tGPI-mucins were purified by solvent extraction and hydrophobic interaction chromatography, as previously described (Almeida et al. 1994). CL-ELISA was performed as previously described (Almeida et al. 1997). Briefly, purified tGPI-mucins (50 µL at 0.32 pg/µL) in 50 mM carbonate-bicarbonate buffer (pH 9.5) were immobilised onto white, opaque 96-well CL-ELISA microplate wells (Nunc, Roskilde, Denmark). After an overnight incubation at 4ºC, free microplate binding sites were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.4. The plates were

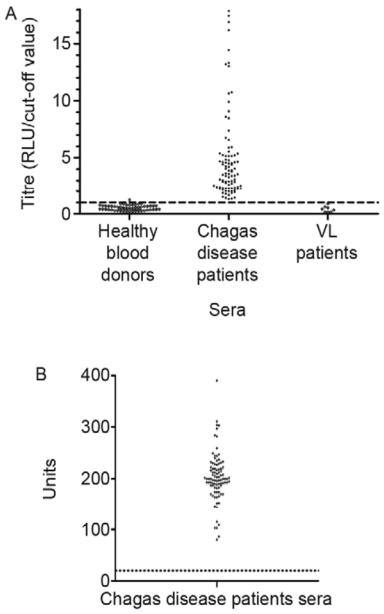
doi: 10.1590/0074-0276130112
Financial support: NIH (R01AI070655-A5, R01AI070655-A5S1, 5G12RR008124-16A1, 5G12RR008124-16A1S1, G12MD007592), AGAUR (2009SGR385) (to CRESIB)
LI and AFM contributed equally to this work.
+ Corresponding author: luis.izquierdo@cresib.cat
Received 22 June 2012
Accepted 19 June 2013

online | memorias.ioc.fiocruz.br

incubated with 50 µL human serum at a 1:800 dilution in PBS containing 1% BSA (PBS-BSA). The plates were then sequentially incubated with 50 µL biotinylated anti-human immunoglobulin G (1:2,000 dilution) (Amersham, GE Healthcare Life Sciences, Buckinghamshire, UK) in PBS-BSA and 50 µL streptavidin-horseradish peroxidase conjugate (1:1,000 dilution) (Amersham, GE Healthcare Life Sciences) in PBS-BSA. All the incubation steps were performed at 37ºC for 1 h. The plates were washed four times with PBS containing 0.05% Tween 20 after each incubation period. The reaction was developed with 50 µL enhanced CL reagent (Pierce, Thermo Scientific) diluted 1:10 in 50 mM carbonate-bicarbonate buffer (pH 9.5). The chemiluminescence measurements, expressed as relative luminescence units (RLUs), were performed in a multi-mode microplate reader (Synergy HT, Biotek). In each plate, we included a positive control (pool of 10 sera of confirmed chronic Chagas disease patients), a negative control (pool of 10 healthy donor sera from Chagas disease-endemic areas) and a background control (incubation buffer with no sera added), which were assayed in triplicate. Statistically valid cut-off values for the CL-ELISA results were calculated by defining the upper prediction limit, which was expressed as the standard deviation (SD) multiplied by a factor according to the number of negative controls and confidence level (99.5%) and calculated based on the Student *t* distribution (Frey et al. 1998). Thus, for each plate in which three negative controls were included (pool of 10 healthy donor sera), the cut-off values were established as the negative control mean plus 11.46 times the SD [cut-off value = (negative control mean) + 11.46 x (SD)] (Frey et al. 1998). The titre of each CL-ELISA is defined as the ratio of the tested serum's RLU value to the cut-off value. A serum sample was considered positive when its titre was equal to or higher than 1.0 and negative when the titre was equal or less than 0.9. Inconclusive or doubtful results showed titres above 0.9 or below 1.0 (Almeida et al. 1997). All the sera were tested in duplicate and the reported results are the means of two simultaneous determinations.

Negative serum samples from blood donors (n = 58) were obtained from the Catalonian Blood Bank and were assayed for the presence of *T. cruzi* Abs using two commercially available Chagas Ab tests: ID-PaGIA (DiaMed, Cressier sur Morat, Switzerland) and Chagas Bioelisa Assay (Biokit, Lliçà d'Amunt, Spain) (Piron et al. 2008). Negative results were confirmed with an ELISA using crude antigen from *T. cruzi* epimastigotes (TC-Maracay strain) (Riera et al. 2009). Positive samples (n = 92) were obtained from the serum collection of the Laboratory of Parasitology, Faculty of Pharmacy, University of Barcelona. The samples were tested for *T. cruzi* using a specific polymerase chain reaction assay and two ELISAs: Chagas Bioelisa Assay and the technique using crude antigen from *T. cruzi* (Riera et al. 2009). Eighty-two of the positive patients (89.1%) presented the indeterminate form of chronic Chagas disease and the other 10 subjects had cardiac and/or digestive manifestations. The serum samples were obtained before benznidazole treatment and, in all cases, both positive and negative donors signed an informed consent form approved by the Institutional Review Board of the Hospital Clinic of Barcelona.

The substantial number of inconclusive diagnoses of Chagas disease shows the need for a valid and widely accepted standard diagnostic procedure (Reithinger et al. 2009). As the available diagnostic procedures vary in sensitivity, the use of two diagnostic tests is generally recommended to confirm clinical suspicion. Because the samples used were previously confirmed as positive or negative by at least two different tests, the diagnostic performance characteristics of CL-ELISA using tGPI-mucins were calculated by employing those previous determinations as a reference. The measured end points were the number of true-positive (TP), true-negative (TN), false-positive (FP) and false-negative (FN) samples. Sensitivity was calculated as TP/(TP+FN) and specificity was calculated as TN/(TN+FP). The positive predictive value (PPV), i.e., the proportion of patients with positive results who were correctly diagnosed, was calculated as TP/(TP+FP). The negative predictive value (NPV), which is the proportion of patients with negative results who were correctly diagnosed, was calculated as TN/(TN+FN). The test efficiency, which is the proportion of all tests that gave correct results, was defined as



Reactivity of the chemiluminescent enzyme-linked immunosorbent assay (ELISA) using *Trypanosoma cruzi* glycosylphosphatidylinositol-mucins. The titres of sera of healthy blood donors (n = 58), chronic Chagas disease patients (n = 92) and patients with visceral leishmaniasis (VL) (n = 8) are indicated. Dotted line: cut-off value equivalent to titre = 1 (A). Reactivity of the positive sera in an ELISA using crude antigen from *T. cruzi* epimastigotes. Dotted line: cut-off value established at 20 units (B) (492 nm ratio between samples and calibrator pool). RLU: relative luminescence unit. Source: Riera et al. (2009).

(TP+TN)/(number of all tests). Cohen's kappa coefficient, which reflects the concordance between different tests, was calculated as follows: (Ao-Ae)/(1-Ae), with Ao being the observed agreement and Ae the agreement expected by chance.

Fifty-seven of 58 negative serum samples were negative by CL-ELISA. Only one healthy blood donor serum sample showed a titre immediately above the cut-off line (titre = 1.2) and was thus considered positive in the assay. Of the 92 positive sera analysed, 92 were positive by our CL-ELISA (A in Figure). Therefore, the sensitivity and specificity of this technique in detecting Chagas disease were 100% [95% confidence interval (CI) = 96-100] and 98.3% (95% CI = 90.7-99.7), respectively. PPV and NPV were 98.9% and 100%, respectively, and the percentage of sera correctly classified was 99.3%. The kappa test score was 0.99 (95% CI = 0.96-1.00). Thus, as previously reported (Almeida et al. 1997, de Marchi et al. 2011), CL-ELISA using tGPI-mucins has a high efficiency and discriminatory capacity. Importantly, the assay also shows a very high level of agreement with classic serology and other Chagas disease diagnostic tests. Only two (2.2%) of the 92 positive samples correctly tested by CL-ELISA showed a titre below 1.5, which might be considered too close to the assay cut-off to produce negative results when repeated. However, in those two cases, the replicates were always positive and presented values above the cut-off.

Due to the well-known cross-reactivity of chagasic sera with other organisms, mainly the closely related members of the Trypanosomatidae family, eight serum samples from patients affected with visceral leishmaniasis (VL) were tested using CL-ELISA; the results were negative in all of these cases (Figure). Thus, as previously reported in several studies, tGPI-mucins do not cross-react with sera from patients with VL or cutaneous leishmaniasis (Almeida et al. 1993, 1997, de Marchi et al. 2011). Therefore, this test could definitely be used in geographic regions in which leishmaniasis is endemic, such as Spain.

As previously described, the tGPI-mucin CL-ELISA test shows very high sensitivity and specificity values (Almeida et al. 1997). These values are comparable to those achieved using *T. cruzi* crude lysates (Riera et al. 2009), as observed in Figure (B), for mixtures of certain specific parasite recombinant antigens (Umezawa et al. 2003, Hernández et al. 2010, Longhi et al. 2012) and for other sensitive and specific confirmatory tests for Chagas disease, such as the trypomastigote excreted-secreted antigen blotting test (Umezawa et al. 1996). The high stability of tGPI-mucin antigen gives us the opportunity to use this unconventional technique in a nonendemic setting without any significant decrease in reactivity. In addition, it has been reported elsewhere that, together with other criteria, a tGPI-mucin antigenic preparation may be used as a criterion for cure in treated patients (Almeida et al. 1993, Andrade et al. 1996, 2004), as the assay can replace the in vitro determination of parasite lysis. This concept adds value to the use of the technique in our facilities.

It is widely accepted that conventional serologic assays should be the basis for the evaluation of drug efficacy against *T. cruzi* because the only possible reason for a negative result would be the disappearance of the stimulus for specific Ab production: the parasite. However, after successful treatment, the results of conventional serologic assays remain positive for years or even decades during the chronic phase of Chagas disease, for reasons that are not yet well understood. These reasons most likely involve recurrent, nonspecific polyclonal activation and thus nonspecific, low-titre Abs (Almeida et al. 1993, 1997, Travassos & Almeida 1993, Andrade et al. 1996, 2004, Rassi & Luquetti 2003). The Ab-dependent lysis of infective *T. cruzi* stages by patient serum is a more reliable indicator of ongoing active infection and detects lytic Abs in which the immunodominant epitopes present on the surface of living trypomastigotes are recognised (Travassos & Almeida 1993, Krettli 2009). Nevertheless, complement-mediated and complement-independent trypanolytic assays are not routine methods because these assays require the careful management of living infective trypomastigotes. Thus, the disappearance of specific lytic Abs has been advocated as a suitable indicator of parasitological cure (Krettli et al. 1982, Almeida et al. 1991, 1993, Galvão et al. 1993). A complete correlation (100%) has been reported between chagasic serum lytic activity and the recognition of the purified trypomastigote surface glycoprotein 160 in an immunoassay (Krettli 2009).

The present report shows an evaluation of the sensitivity and specificity of CL-ELISA using tGPI-mucins for the serodiagnosis of Chagas disease in a panel of human sera at our facilities. This assay has been previously employed to evaluate the efficacy of a 60-day course of benznidazole treatment in children with early chronic *T. cruzi* infection (Andrade et al. 2004) and to diagnose active infection in samples from blood banks in South America (Almeida et al. 1997, de Marchi et al. 2011). We propose the use of CL-ELISA with purified tGPI-mucins or synthetic α-Gal-containing glycans recognised by the lytic anti-α-Gal Abs (Almeida et al. 1994) in further research studies to assess the effectiveness of Chagas disease chemotherapy in both endemic and nonendemic settings.

ACKNOWLEDGEMENTS

To Hernando A del Portillo, for support, reagents and helpful scientific discussions throughout this work, to Ruth Aguilar, for helpful technical discussions, and to the Biomolecule Analysis Core Facility at the Border Biomedical Research Center, UTEP.

REFERENCES

Almeida IC, Covas DT, Soussumi LM, Travassos LR 1997. A highly sensitive and specific chemiluminescent enzyme-linked immunosorbent assay for diagnosis of active *Trypanosoma cruzi* infection. *Transfusion 37*: 850-857.

Almeida IC, Ferguson MA, Schenkman S, Travassos LR 1994. Lytic anti-alpha-galactosyl antibodies from patients with chronic Chagas disease recognize novel O-linked oligosaccharides on mucin-like glycosyl-phosphatidylinositol-anchored glycoproteins of *Trypanosoma cruzi*. *Biochem J 304*: 793-802.

Almeida IC, Krautz GM, Krettli AU, Travassos LR 1993. Glycoconjugates of *Trypanosoma cruzi*: a 74 kD antigen of trypomastigotes specifically reacts with lytic anti-alpha-galactosyl antibodies from patients with chronic Chagas disease. *J Clin Lab Anal 7*: 307-316.

Almeida IC, Milani SR, Gorin PA, Travassos LR 1991. Complement-mediated lysis of *Trypanosoma cruzi* trypomastigotes by human anti-alpha-galactosyl antibodies. *J Immunol 146*: 2394-2400.

Andrade ALSS, Martelli CM, Oliveira RM, Silva SA, Aires AI, Soussumi LM, Covas DT, Silva LS, Andrade JG, Travassos LR, Almeida IC 2004. Short report: benznidazole efficacy among *Trypanosoma cruzi*-infected adolescents after a six-year follow-up. *Am J Trop Med Hyg 71*: 594-597.

Andrade ALSS, Zicker F, de Oliveira RM, Silva SA, Luquetti A, Travassos LR, Almeida IC, de Andrade SS, de Andrade JG, Martelli CM 1996. Randomised trial of efficacy of benznidazole in treatment of early *Trypanosoma cruzi* infection. *Lancet 348*: 1407-1413.

de Marchi CR, Di Noia JM, Frasch AC, Amato Neto V, Almeida IC, Buscaglia CA 2011. Evaluation of a recombinant *Trypanosoma cruzi* mucin-like antigen for serodiagnosis of Chagas disease. *Clin Vaccine Immunol 18*: 1850-1855.

Frey A, Di Canzio J, Zurakowski D 1998. A statistically defined end-point titer determination method for immunoassays. *J Immunol Methods 221*: 35-41.

Galvão LM, Nunes RM, Cançado JR, Brener Z, Krettli AU 1993. Lytic antibody titre as a means of assessing cure after treatment of Chagas disease: a 10 years follow-up study. *Trans R Soc Trop Med Hyg 87*: 220-223.

Gascon J, Bern C, Pinazo MJ 2010. Chagas disease in Spain, the United States and other non-endemic countries. *Acta Trop 115*: 22-27.

Hernández P, Heimann M, Riera C, Solano M, Santalla J, Luquetti AO, Beck E 2010. Highly effective serodiagnosis for Chagas disease. *Clin Vaccine Immunol 17*: 1598-1604.

Krettli AU 2009. The utility of anti-trypomastigote lytic antibodies for determining cure of *Trypanosoma cruzi* infections in treated patients: an overview and perspectives. *Mem Inst Oswaldo Cruz 104* (Suppl. I): 142-151.

Krettli AU, Cancado JR, Brener Z 1982. Effect of specific chemotherapy on the levels of lytic antibodies in Chagas disease. *Trans R Soc Trop Med Hyg 76*: 334-340.

Longhi SA, Brandariz SB, Lafon SO, Niborski LL, Luquetti AO, Schijman AG, Levin MJ, Gómez KA 2012. Evaluation of in-house ELISA using *Trypanosoma cruzi* lysate and recombinant antigens for diagnosis of Chagas disease and discrimination of its clinical forms. *Clin Vaccine Immunol 17*: 1598-1604.

Pereira-Chioccola VL, Acosta-Serrano A, de Almeida IC, Ferguson MA, Souto-Padron T, Rodrigues MM, Travassos LR, Schenkman S 2000. Mucin-like molecules form a negatively charged coat that protects *Trypanosoma cruzi* trypomastigotes from killing by human anti-alpha-galactosyl antibodies. *J Cell Sci 113*: 1299-1307.

Piron M, Vergés M, Muñoz J, Casamitjana N, Sanz S, Maymó RM, Hernández JM, Puig L, Portús M, Gascon J, Sauleda S 2008. Seroprevalence of *Trypanosoma cruzi* infection in at-risk blood donors in Catalonia (Spain). *Transfusion 48*: 1862-1868.

Rassi A, Luquetti AO 2003. Specific treatment for *Trypanosoma cruzi* infection (Chagas disease). In KM Tyler, MA Miles (eds.), *American trypanosomiasis*, Springer, New York, p. 117-125.

Reithinger R, Tarleton RL, Urbina JA, Kitron U, Gurtler RE 2009. Eliminating Chagas disease: challenges and a roadmap. *BMJ 338*: b1283.

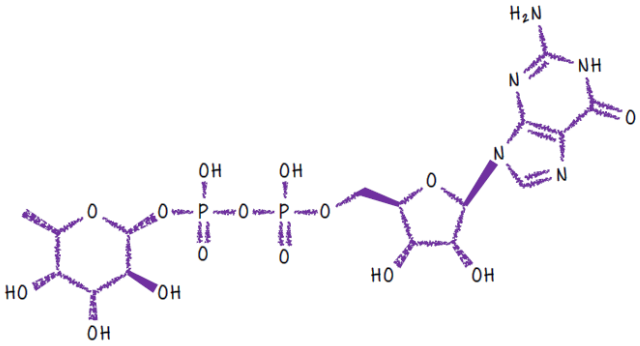
Riera C, Vergés M, López-Chejade P, Piron M, Gascon J, Fisa R, Gállego M, Portús M 2009. Desarrollo y evaluación de una técnica ELISA con antígeno crudo de *Trypanosoma cruzi* para el diagnóstico de la enfermedad de Chagas. *Enferm Emerg 11*: 22-29.

Travassos LR, Almeida IC 1993. Carbohydrate immunity in American trypanosomiasis. *Springer Semin Immunopathol 15*: 183-204.

Umezawa ES, Bastos SF, Coura JR, Levin MJ, Gonzalez A, Rangel-Aldao R, Zingales B, Luquetti AO, da Silveira JF 2003. An improved serodiagnostic test for Chagas disease employing a mixture of *Trypanosoma cruzi* recombinant antigens. *Transfusion 43*: 91-97.

Umezawa ES, Nascimento MS, Kesper Jr N, Coura JR, Borges-Pereira J, Junqueira AC, Camargo ME 1996. Immunoblot assay using excreted-secreted antigens of *Trypanosoma cruzi* in serodiagnosis of congenital, acute and chronic Chagas disease. *J Clin Microbiol 34*: 2143-2147.

BIBLIOGRAPHY



BIBLIOGRAPHY

1. White, N. J. *et al.* Malaria. *Lancet* **383,** 723–735 (2014).
2. Sinka, M. E. *et al.* A global map of dominant malaria vectors. *Parasit. Vectors* **5,** 69 (2012).
3. Hay, S. I. *et al.* Developing global maps of the dominant anopheles vectors of human malaria. *PLoS Med.* **7,** 1–6 (2010).
4. Carter, R. & Mendis, K. N. Evolutionary and Historical Aspects of the Burden of Malaria Evolutionary and Historical Aspects of the Burden of Malaria. *Clin. Microbiol. Rev* **15,** 564–594 (2002).
5. WHO | World Malaria Report 2015. *WHO* (2015).
6. WHO | World Malaria Report 2016. *WHO* (2016).
7. Roobsoong, W. *et al.* Improvement of culture conditions for long-term in vitro culture of *Plasmodium vivax*. *Malar. J.* **14,** 297 (2015).
8. Meibalan, E. & Marti, M. Biology of Malaria Transmission. *Cold Spring Harb. Perspect. Med.* **7,** a025452 (2016).
9. Doolan, D. L., Dobaño, C. & Baird, J. K. Acquired immunity to malaria. *Clin. Microbiol. Rev.* **22,** 13–36, Table of Contents (2009).
10. Garcia, L. S. Malaria. *Clin. Lab. Med.* **30,** 93–129 (2010).
11. WHO | World Malaria Report 2014. *WHO* (2014).
12. Olliaro, P. Mortality Associated with Severe *Plasmodium falciparum* Malaria Increases with Age. *Clin. Infect. Dis.* **47,** 158–160 (2008).
13. Menéndez, C., Ferenchick, E., Roman, E., Bardají, A. & Mangiaterra, V. Malaria in pregnancy: Challenges for control and the need for urgent action. *Lancet Glob. Heal.* **3,** e433–e434 (2015).
14. Schantz-Dunn, J. & Nour, N. M. Malaria and pregnancy: a global health perspective. *Rev. Obstet. Gynecol.* **2,** 186–92 (2009).
15. Lagerberg, R. E. Malaria in Pregnancy: A Literature Review. *J. Midwifery Womens. Health* **53,** 209–215 (2008).
16. WHO. Eliminating Malaria. (2015).
17. WHO. Treatment of Severe Malaria. *Guidel. Treat. Malar.* 71–88 (2015).

18. malERA Consultative Group on Vector Control. A research agenda for malaria eradication: vector control. *PLoS Med.* **8,** 1–8 (2011).

19. Ranson, H. *et al.* Insecticide resistance in *Anopheles gambiae*: data from the first year of a multi-country study highlight the extent of the problem. *Malar. J.* **8,** 299 (2009).

20. Glunt, K. D. *et al.* Long-lasting insecticidal nets no longer effectively kill the highly resistant *Anopheles funestus* of southern Mozambique. *Malar. J.* **14,** 298 (2015).

21. Terenius, O., Marinotti, O., Sieglaff, D. & James, A. A. Molecular genetic manipulation of vector mosquitoes. *Cell Host Microbe* **4,** 417–423 (2008).

22. Birkett, A. J. Status of vaccine research and development of vaccines for malaria. *Vaccine* **34,** 2915–2920 (2016).

23. Langhorne, J. & Duffy, P. E. Expanding the antimalarial toolkit: Targeting host-parasite interactions. *J. Exp. Med.* **213,** 143–153 (2016).

24. WHO. Recommended selection criteria for procurement of malaria rapid diagnostic tests. 16 (2016).

25. Nájera, J. A., González-Silva, M. & Alonso, P. L. Some lessons for the future from the global malaria eradication programme (1955-1969). *PLoS Med.* **8,** (2011).

26. Cowman, A. F., Galatis, D. & Thompson, J. K. Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the pfmdr1 gene and cross-resistance to halofantrine and quinine. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1143–1147 (1994).

27. Jelinek, T., Schelbert, P., Löscher, T. & Eichenlaub, D. Quinine resistant *falciparum* malaria acquired in east Africa. *Trop. Med. Parasitol.* **46,** 38–40 (1995).

28. Sá, J. M. *et al.* Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 18883–18889 (2009).

29. Chen, W.-J. Honoring antiparasitics: The 2015 Nobel Prize in Physiology or Medicine. *Biomed. J.* **39,** 93–97 (2016).

30. Wells, T. N. C., van Huijsduijnen, R. H. & Van Voorhis, W. C. Malaria medicines: a glass half full? *Nat. Rev. Drug Discov.* **14,** 424–442 (2015).

31. Eastman, R. T. & Fidock, D. a. Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. *Nat. Rev. Microbiol.* **7,** 864–874 (2009).

32. Noedl, H. *et al.* Evidence of Artemisinin-resistant malaria in Western Cambodia. *N. Engl. J. Med.* **359,** 2619–2620 (2008).

33. Dondorp, A. M. *et al.* Artemisinin Resistance in *Plasmodium falciparum* Malaria. *N. Engl. J. Med.* **361,** 455–467 (2009).

34. World Health Organization. Strategy for Malaria Elimination in the Greater Mekong Subregion (2015-2030). *J. Chem. Inf. Model.* **53,** 1689–1699 (2013).

35. Bruce-Chwatt, L. J. Alphonse Laveran's discovery 100 years ago and today's global fight against malaria. *J Roy Soc Med* **74,** 531–536 (1981).

36. Cox, F. E. History of the discovery of the malaria parasites and their vectors. *Parasit. Vectors* **3,** 5 (2010).

37. MacCallum, W. G. On the flagellated form of the malarial parasite. *Lancet* **150,** 1240–1241 (1897).

38. MacCallum, W. G. On the haematozoan infections of birds. *J. Exp. Med.* **3,** 117–136 (1898).

39. Gardner, M. J. *et al.* Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419,** 498–511 (2002).

40. Hall, N. *et al.* Sequence of *Plasmodium falciparum* chromosomes 1, 3-9 and 13. *Nature* **419,** 527–531 (2002).

41. Carlton, J. M. *et al.* Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature* **455,** 757–763 (2008).

42. Carlton, J. M. *et al.* Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* **419,** 512–519 (2002).

43. Winzeler, E. A. Malaria research in the post-genomic era. *Nature* **455,** 751–756 (2008).

44. Wirth, D. F. Biological revelations. *Nature* **419,** 495–496 (2002).

45. Josling, G. A. & Llinás, M. Sexual development in *Plasmodium* parasites: knowing when it's time to commit. *Nat. Rev. Microbiol.* **13,** 573–587 (2015).

46. Prudêncio, M., Rodriguez, A. & Mota, M. M. The silent path to thousands of merozoites: the *Plasmodium liver stage*. *Nat. Rev. Microbiol.* **4,** 849–856 (2006).

47. Sidjanski, S. & Vanderberg, J. P. Delayed migration of *Plasmodium* sporozoites from the mosquito bite site to the blood. *Am. J. Trop. Med. Hyg.* **57,** 426–429 (1997).

48. Vanderberg, J. P. & Frevert, U. Intravital microscopy demonstrating antibody-mediated immobilisation of *Plasmodium berghei* sporozoites injected into skin by mosquitoes. *Int. J. Parasitol.* **34,** 991–996 (2004).

49. Amino, R. *et al.* Quantitative imaging of *Plasmodium* transmission from mosquito to

mammal. *Nat. Med.* **12,** 220–224 (2006).

50. Sinnis, P. The malaria sporozoite's journey into the liver. *Infect. Agents Dis.* **5,** 182–189 (1996).

51. Pradel, G., Garapaty, S. & Frevert, U. Proteoglycans mediate malaria sporozoite targeting to the liver. *Mol. Microbiol.* **45,** 637–651 (2002).

52. Frevert, U., Usynin, I., Baer, K. & Klotz, C. *Plasmodium* sporozoite passage across the sinusoidal cell layer. *Subcell. Biochem.* **47,** 182–197 (2008).

53. Matuschewski, K. *et al. Plasmodium* sporozoite invasion into insect and mammalian cells is directed by the same dual binding system. *EMBO J.* **21,** 1597–1606 (2002).

54. Pradel, G., Garapaty, S. & Frevert, U. Kupffer and stellate cell proteoglycans mediate malaria sporozoite targeting to the liver. *Comp. Hepatol.* **3,** S47 (2004).

55. Tavares, J. *et al.* Role of host cell traversal by the malaria sporozoite during liver infection. *J. Exp. Med.* **210,** 905–915 (2013).

56. Silvie, O. *et al.* Hepatocyte CD81 is required for *Plasmodium falciparum* and *Plasmodium yoelii* sporozoite infectivity. *Nat. Med.* **9,** 93–96 (2003).

57. Govindasamy, K. *et al.* Invasion of hepatocytes by *Plasmodium* sporozoites requires cGMP-dependent protein kinase and calcium dependent protein kinase 4. *Mol. Microbiol.* **102,** 349–363 (2016).

58. Heintzelman, M. B. Gliding motility in apicomplexan parasites. *Semin. Cell Dev. Biol.* **46,** 135–142 (2015).

59. Frevert, U. Sneaking in through the back entrance: the biology of malaria liver stages. *Trends Parasitol.* **20,** 417–424 (2004).

60. Sturm, A. *et al.* Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* **313,** 1287–1290 (2006).

61. Cowman, A. F. & Crabb, B. S. Invasion of red blood cells by malaria parasites. *Cell* **124,** 755–766 (2006).

62. Bannister, L. H., Hopkins, J. M., Fowler, R. E., Krishna, S. & Mitchell, G. H. A brief illustrated guide to the ultrastructure of *Plasmodium falciparum* asexual blood stages. *Parasitol. Today* **16,** 427–433 (2000).

63. Kadekoppala, M. & Holder, A. A. Merozoite surface proteins of the malaria parasite: The MSP1 complex and the MSP7 family. *Int. J. Parasitol.* **40,** 1155–1161 (2010).

64. Goel, V. K. *et al.* Band 3 is a host receptor binding merozoite surface protein 1 during the Plasmodium falciparum invasion of erythrocytes. *Proc. Natl. Acad. Sci. U. S. A.*

100, 5164–5169 (2003).

65. Srinivasan, P. *et al.* Binding of *Plasmodium* merozoite proteins RON2 and AMA1 triggers commitment to invasion. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 13275–13280 (2011).

66. Wright, G. J. & Rayner, J. C. *Plasmodium falciparum* Erythrocyte Invasion: Combining Function with Immune Evasion. *PLoS Pathog.* **10,** 1–7 (2014).

67. Suss-Toby, E., Zimmerberg, J. & Ward, G. E. *Toxoplasma* invasion: the parasitophorous vacuole is formed from host cell plasma membrane and pinches off via a fission pore. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8413–8418 (1996).

68. Murphy, S. C. *et al.* Erythrocyte detergent-resistant membrane proteins: Their characterization and selective uptake during malarial infection. *Blood* **103,** 1920–1928 (2004).

69. Radfar, A. *et al.* Synchronous culture of *Plasmodium falciparum* at high parasitemia levels. *Nat. Protoc.* **4,** 1828–1844 (2009).

70. Maier, A. G., Cooke, B. M., Cowman, A. F. & Tilley, L. Malaria parasite proteins that remodel the host erythrocyte. *Nat. Rev. Microbiol.* **7,** 341–354 (2009).

71. Goldberg, D. E. & Cowman, A. F. Moving in and renovating: exporting proteins from *Plasmodium* into host erythrocytes. *Nat. Rev. Microbiol.* **8,** 617–621 (2010).

72. Grüring, C. *et al.* Development and host cell modifications of *Plasmodium falciparum* blood stages in four dimensions. *Nat. Commun.* **2,** 1–11 (2011).

73. Sherling, E. S. & van Ooij, C. Host cell remodeling by pathogens: the exomembrane system in *Plasmodium* -infected erythrocytes. *FEMS Microbiol. Rev.* **40,** 701–721 (2016).

74. Dixon, M. W. A., Dearnley, M. K., Hanssen, E., Gilberger, T. & Tilley, L. Shape-shifting gametocytes: how and why does *P. falciparum* go banana-shaped? *Trends Parasitol.* **28,** 471–478 (2012).

75. Bruce, M. C., Alano, P., Duthie, S. & Carter, R. Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development. *Parasitology* **100,** 191–200 (1990).

76. Baker, D. A. Malaria gametocytogenesis. *Mol. Biochem. Parasitol.* **172,** 57–65 (2010).

77. Joice, R. *et al. Plasmodium falciparum* transmission stages accumulate in the human bone marrow. *Sci. Transl. Med.* **6,** 244re5 (2014).

78. Bousema, T. *et al.* Revisiting the circulation time of *Plasmodium falciparum* gametocytes: molecular detection methods to estimate the duration of gametocyte

carriage and the effect of gametocytocidal drugs. *Malar. J.* **9,** 136 (2010).

79. Butterworth, A. S., Skinner-Adams, T. S., Gardiner, D. L. & Trenholme, K. R. *Plasmodium falciparum* gametocytes: with a view to a kill. *Parasitology* **140,** 1718–1734 (2013).

80. Howes, R. E. *et al.* G6PD deficiency prevalence and estimates of affected populations in malaria endemic countries: a geostatistical model-based map. *PLoS Med.* **9,** e1001339 (2012).

81. Billker, O. *et al.* Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. *Nature* **392,** 289–292 (1998).

82. Shahabuddin, M. *et al. Plasmodium* ookinete development in the mosquito midgut: a case of reciprocal manipulation. *Parasitology* **116,** S83–S93 (1998).

83. Ishino, T., Orito, Y., Chinzei, Y. & Yuda, M. A calcium-dependent protein kinase regulates *Plasmodium* ookinete access to the midgut epithelial cell. *Mol. Microbiol.* **59,** 1175–1184 (2006).

84. Dessens, J. T. *et al.* CTRP is essential for mosquito infection by malaria ookinetes. *EMBO J.* **18,** 6221–6227 (1999).

85. Limviroj, W., Yano, K., Yuda, M., Ando, K. & Chinzei, Y. Immuno-electron microscopic observation of *Plasmodium berghei* CTRP localization in the midgut of the vector mosquito Anopheles stephensi. *J. Parasitol.* **88,** 664–672 (2002).

86. Aly, A. S. I., Vaughan, A. M. & Kappe, S. H. I. Malaria Parasite Development in the Mosquito and Infection of the Mammalian Host. *Annu. Rev. Microbiol.* **63,** 195–221 (2009).

87. Vlachou, D., Schlegelmilch, T., Runn, E., Mendes, A. & Kafatos, F. C. The developmental migration of *Plasmodium* in mosquitoes. *Curr. Opin. Genet. Dev.* **16,** 384–391 (2006).

88. Hamilton, A. J., Davies, C. S. & Sinden, R. E. Expression of circumsporozoite proteins revealed in situ in the mosquito stages of *Plasmodium berghei* by the Lowicryl-immunogold technique. *Parasitology* **96,** 273 (1988).

89. Kappe, S. *et al.* Conservation of a Gliding Motility and Cell Invasion Machinery in *Apicomplexan* Parasites. *J. Cell Biol.* **147,** 937–944 (1999).

90. Matuschewski, K. Getting infectious: Formation and maturation of *Plasmodium* sporozoites in the *Anopheles* vector. *Cellular Microbiology* **8,** 1547–1556 (2006).

91. Varki, A. *et al. Essentials of Glycobiology*. (Cold Spring Harbor Laboratory Press, 2009).

92. Fuster, M. M. & Esko, J. D. The sweet and sour of cancer: glycans as novel therapeutic targets. *Nat. Rev. Cancer* **5,** 526–542 (2005).

93. Vasudevan, D., Takeuchi, H., Johar, S. S., Majerus, E. & Haltiwanger, R. S. Peters Plus Syndrome Mutations Disrupt a Noncanonical ER Quality-Control Mechanism. *Curr. Biol.* **25,** 286–295 (2015).

94. Rodrigues, J. A. *et al.* Parasite Glycobiology: A Bittersweet Symphony. *PLoS Pathog.* **11,** e1005169 (2015).

95. Ferguson, M. A. The surface glycoconjugates of trypanosomatid parasites. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **352,** 1295–1302 (1997).

96. Templeton, T. J., Kaslow, D. C. & Fidock, D. A. Developmental arrest of the human malaria parasite *Plasmodium falciparum* within the mosquito midgut via CTRP gene disruption. *Mol. Microbiol.* **36,** 1–9 (2000).

97. von Itzstein, M., Plebanski, M., Cooke, B. M. & Coppel, R. L. Hot, sweet and sticky: the glycobiology of *Plasmodium falciparum*. *Trends Parasitol.* **24,** 210–218 (2008).

98. Lairson, L. L., Henrissat, B., Davies, G. J. & Withers, S. G. Glycosyltransferases: structures, functions, and mechanisms. *Annu. Rev. Biochem.* **77,** 521–555 (2008).

99. Cova, M., Rodrigues, J. A., Smith, T. K. & Izquierdo, L. Sugar activation and glycosylation in *Plasmodium*. *Malar. J.* **14,** 427 (2015).

100. Couto, A. S. *et al.* Glycosphingolipids in *Plasmodium falciparum*. Presence of an active glucosylceramide synthase. *Eur. J. Biochem.* **271,** 2204–2214 (2004).

101. Kozma, K. *et al.* Identification and characterization of abeta1,3-glucosyltransferase that synthesizes the Glc-beta1,3-Fuc disaccharide on thrombospondin type 1 repeats. *J. Biol. Chem.* **281,** 36742–36751 (2006).

102. Swearingen, K. E. *et al.* Interrogating the *Plasmodium* Sporozoite Surface: Identification of Surface-Exposed Proteins and Demonstration of Glycosylation on CSP and TRAP by Mass Spectrometry-Based Proteomics. *PLoS Pathog.* **12,** e1005606 (2016).

103. Gowda, D. C., Gupta, P. & Davidson, E. A. Glycosylphosphatidylinositol Anchors Represent the Major Carbohydrate Modification in Proteins of Intraerythrocytic Stage *Plasmodium falciparum*. *J. Biol. Chem.* **272,** 6428–6439 (1997).

104. Bushkin, G. G. *et al.* Suggestive evidence for Darwinian Selection against asparagine-linked glycans of *Plasmodium falciparum* and *Toxoplasma gondii*. *Eukaryot. Cell* **9,** 228–241 (2010).

105. Samuelson, J. *et al.* The diversity of dolichol-linked precursors to Asn-linked glycans

likely results from secondary loss of sets of glycosyltransferases. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 1548–1553 (2005).

106. Ginsburg, H. Progress in in silico functional genomics: the malaria Metabolic Pathways database. *Trends Parasitol.* **22,** 238–240 (2006).

107. Gerold, P., Dieckmann-Schuppert, A. & Schwarz, R. T. Glycosylphosphatidylinositols synthesized by asexual erythrocytic stages of the malarial parasite, *Plasmodium falciparum*. Candidates for plasmodial glycosylphosphatidylinositol membrane anchor precursors and pathogenicity factors. *J. Biol. Chem.* **269,** 2597–2606 (1994).

108. Naik, R. S. *et al.* Glycosylphosphatidylinositol anchors of *Plasmodium falciparum*: molecular characterization and naturally elicited antibody response that may provide immunity to malaria pathogenesis. *J. Exp. Med.* **192,** 1563–1576 (2000).

109. Turnock, D. C., Izquierdo, L. & Ferguson, M. A. J. The de novo synthesis of GDP-fucose is essential for flagellar adhesion and cell growth in *Trypanosoma brucei*. *J. Biol. Chem.* **282,** 28853–28863 (2007).

110. Kinoshita, T. Glycosylphosphatidylinositol (GPI) Anchors: Biochemistry and Cell Biology: Introduction to a Thematic Review Series. *J. Lipid Res.* **57,** 4–5 (2016).

111. Smith, T. K. *et al.* Substrate Specificity of the *Plasmodium falciparum* Glycosylphosphatidylinositol Biosynthetic Pathway and Inhibition by Species-Specific Suicide Substrates. *Biochemistry* **41,** 12395–12406 (2002).

112. Delorenzi, M. *et al.* Genes for glycosylphosphatidylinositol toxin biosynthesis in *Plasmodium falciparum*. *Infect. Immun.* **70,** 4510–4522 (2002).

113. Ferguson, M. A. J., Duszenko, M., Lamont, G. S., Overaths, P. & Cross, G. A. M. Biosynthesis of *Trypanosoma brucei* Variant Surface Glycoproteins. N-glycosylation and addition of a phosphatidylinositol membrane anchor. *J. Biol. Chem.* **261,** 356–362 (1986).

114. Wang, Q., Fujioka, H. & Nussenzweig, V. Mutational analysis of the GPI-anchor addition sequence from the circumsporazoite protein of *Plasmodium*. *Cell. Microbiol.* **7,** 1616–1626 (2005).

115. Gilson, P. R. *et al.* Identification and stoichiometry of Glycosylphosphatidylinositol-anchored membrane proteins of the human malaria parasite *Plasmodium falciparum*. *Mol. Cell. proteomics MCP* **5,** 1286–1299 (2006).

116. del Carmen Rodriguez, M. *et al.* Characterisation and expression of Pbs25, a sexual and sporogonic stage specific protein of *Plasmodium berghei*. *Mol. Biochem. Parasitol.* **110,** 147–159 (2000).

117. Ozaki, L. S., Svec, P., Nussenzweig, R. S., Nussenzweig, V. & Godson, G. N. Structure

of the *Plasmodium knowlesi* gene coding for the circumsporozoite protein. *Cell* **34,** 815–822 (1983).

118. Schofield, B. L. & Hackett, F. Signal Transduction in Host Cells by a Glycosylphosphatidyllnositol Toxin of Malaria Parasites. *J. Exp. Med.* **177,** 145–153 (1993).

119. Schofield, L., Hewitt, M. C., Evans, K., Siomos, M.-A. & Seeberger, P. H. Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. *Nature* **418,** 785–789 (2002).

120. Tachado, S. D. & Schofield, L. Glycosylphosphatidylinositol toxin of *Trypanosoma brucei* regulates IL-1 alpha and TNF-alpha expression in macrophages by protein tyrosine kinase mediated signal transduction. *Biochem. Biophys. Res. Commun.* **205,** 984–991 (1994).

121. Almeida, I. C. *et al.* Highly purified glycosylphosphatidylinositols from *Trypanosoma cruzi* are potent proinflammatory agents. *EMBO J.* **19,** 1476–1485 (2000).

122. Dieckmann-Schuppert, A., Bender, S., Odenthal-Schnittler, M., Bause, E. & Schwarz, R. T. Apparent lack of N-glycosylation in the asexual intraerythrocytic stage of *Plasmodium falciparum*. *Eur. J. Biochem.* **205,** 815–825 (1992).

123. Kimura, E. A., Couto, A. S., Peres, V. J., Casal, O. L. & Katzin, A. M. N-linked glycoproteins are related to schizogony of the intraerythrocytic stage in *Plasmodium falciparum*. *J. Biol. Chem.* **271,** 14452–14461 (1996).

124. Kelleher, D. J. & Gilmore, R. An evolving view of the eukaryotic oligosaccharyltransferase. *Glycobiology* **16,** 47R–62R (2006).

125. Xu, C. & Ng, D. T. W. Glycosylation-directed quality control of protein folding. *Nat. Rev. Mol. Cell Biol.* **16,** 742–752 (2015).

126. Shental-Bechor, D. & Levy, Y. Effect of glycosylation on protein folding: a close look at thermodynamic stabilization. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 8256–8261 (2008).

127. Hofsteenge, J. *et al.* New type of linkage between a carbohydrate and a protein: C-glycosylation of a specific tryptophan residue in human RNase U. *Biochemistry* **33,** 13524–13530 (1994).

128. Julenius, K. NetCGlyc 1.0: prediction of mammalian C-mannosylation sites. *Glycobiology* **17,** 868–876 (2007).

129. Buettner, F. F. R., Ashikov, A., Tiemann, B., Lehle, L. & Bakker, H. *C. elegans* DPY-19 Is a C-Mannosyltransferase Glycosylating Thrombospondin Repeats. *Mol. Cell* **50,** 295–302 (2013).

130. Shcherbakova, A., Tiemann, B., Buettner, F. F. R. & Bakker, H. Distinct C-

mannosylation of netrin receptor thrombospondin type 1 repeats by mammalian DPY19L1 and DPY19L3. *Proc. Natl. Acad. Sci. U. S. A.* 201613165 (2017).

131. Gonzalez de Peredo, A. *et al.* C-mannosylation and O-fucosylation of thrombospondin type 1 repeats. *Mol. Cell. Proteomics* **1,** 11–8 (2002).

132. Dayal-Drager, R. *et al.* Presence of O-glycosylated glycoproteins in the *Plasmodium falciparum* parasite. *Carbohydr. Res.* **209,** C5–C8 (1991).

133. Dieckmann-Schuppert, A., Bause, E. & Schwarz, R. T. Studies on O-glycans of *Plasmodium falciparum*-infected human erythrocytes Evidence for O-GlcNAc and O-GlcNAc-transferase in malaria parasites. *Eur. J. Biochem.* **216,** 779–788 (1993).

134. Templeton, T. J. *et al.* Comparative Analysis of *Apicomplexa* and Genomic Diversity in Eukaryotes. *Genome Res.* **14,** 1686–1695 (2004).

135. Perez-Cervera, Y. *et al.* Direct evidence of O-GlcNAcylation in the apicomplexan *Toxoplasma gondii*: a biochemical and bioinformatic study. *Amino Acids* **40,** 847–856 (2011).

136. Vasudevan, D. & Haltiwanger, R. S. Novel roles for O-linked glycans in protein folding. *Glycoconj. J.* **31,** 417–426 (2014).

137. Shi, S. & Stanley, P. Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 5234–5239 (2003).

138. Okajima, T. & Irvine, K. D. Regulation of notch signaling by O-linked fucose. *Cell* **111,** 893–904 (2002).

139. Lawler, J. & Hynes, R. O. The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. *J. Cell Biol.* **103,** 1635–1648 (1986).

140. Tucker, R. P. The thrombospondin type 1 repeat superfamily. *Int. J. Biochem. Cell Biol.* **36,** 969–974 (2004).

141. Tan, K. *et al.* Crystal structure of the TSP-1 type 1 repeats: a novel layered fold and its biological implication. *J. Cell Biol.* **159,** 373–382 (2002).

142. Huwiler, K. G., Vestling, M. M., Annis, D. S. & Mosher, D. F. Biophysical Characterization, Including Disulfide Bond Assignments, of the Anti-Angiogenic Type 1 Domains of Human Thrombospondin-1. *Biochemistry* **41,** 14329–14339 (2002).

143. Luo, Y., Nita-Lazar, A. & Haltiwanger, R. S. Two distinct pathways for O-fucosylation of epidermal growth factor-like or thrombospondin type 1 repeats. *J. Biol. Chem.* **281,** 9385–9392 (2006).

144. Luo, Y., Koles, K., Vorndam, W., Haltiwanger, R. S. & Panin, V. M. Protein O-

Fucosyltransferase 2 Adds O-Fucose to Thrombospondin Type 1 Repeats. *J. Biol. Chem.* **281,** 9393–9399 (2006).

145. Ricketts, L. M., Dlugosz, M., Luther, K. B., Haltiwanger, R. S. & Majerus, E. M. O-fucosylation is required for ADAMTS13 secretion. *J. Biol. Chem.* **282,** 17014–17023 (2007).

146. Wang, L. W. *et al.* O-fucosylation of thrombospondin type 1 repeats in ADAMTS-like-1/punctin-1 regulates secretion: implications for the ADAMTS superfamily. *J. Biol. Chem.* **282,** 17024–17031 (2007).

147. Du, J. *et al.* O-fucosylation of thrombospondin type 1 repeats restricts epithelial to mesenchymal transition (EMT) and maintains epiblast pluripotency during mouse gastrulation. *Dev. Biol.* **346,** 25–38 (2010).

148. Chen, C.-I. *et al.* Structure of human POFUT2: insights into thrombospondin type 1 repeat fold and O-fucosylation. *EMBO J.* **31,** 3183–97 (2012).

149. Valero-González, J. *et al.* A proactive role of water molecules in acceptor recognition by protein O-fucosyltransferase 2. *Nat. Chem. Biol.* **12,** 240–246 (2016).

150. Bargieri, D. *et al.* Host Cell Invasion by *Apicomplexan* Parasites: The Junction Conundrum. *PLoS Pathog.* **10,** e1004273 (2014).

151. Baum, J. *et al.* A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. *J. Biol. Chem.* **281,** 5197–5208 (2006).

152. Bargieri, D. Y. *et al. Plasmodium* Merozoite TRAP Family Protein Is Essential for Vacuole Membrane Disruption and Gamete Egress from Erythrocytes. *Cell Host Microbe* **20,** 618–630 (2016).

153. Trottein, F., Triglia, T. & Cowman, A. F. Molecular cloning of a gene from *Plasmodium falciparum* that codes for a protein sharing motifs found in adhesive molecules from mammals and *Plasmodia*. *Mol. Biochem. Parasitol.* **74,** 129–141 (1995).

154. Ramakrishnan, C. *et al.* Vital functions of the malarial ookinete protein, CTRP, reside in the A domains. *Int. J. Parasitol.* **41,** 1029–1039 (2011).

155. Sinnis, P. & Nardin, E. Sporozoite Antigens: Biology and Immunology of the Circumsporozoite Protein and Thrombospondin-Related Anonymous Protein. *Chem Immunol* **80,** 70–96 (2002).

156. Kappe, S. H. I., Buscaglia, C. A. & Nussenzweig, V. *Plasmodium* sporozoite molecular cell biology. *Annu. Rev. Cell Dev. Biol.* **20,** 29–59 (2004).

157. Coppi, A. *et al.* The malaria circumsporozoite protein has two functional domains,

each with distinct roles as sporozoites journey from mosquito to mammalian host. *J. Exp. Med.* **208,** 341–356 (2011).

158. Rogers, W. *et al.* Characterization of *Plasmodium falciparum* sporozoite surface protein 2. *Med. Sci. J. C. I. Nat.* **89,** 9176–9180 (1992).

159. Pihlajamaa, T. *et al.* Structure of *Plasmodium falciparum* TRAP (thrombospondin-related anonymous protein) A domain highlights distinct features in apicomplexan von Willebrand factor A homologues. *Biochem. J.* **450,** 469–476 (2013).

160. Müller, H. M. *et al.* Thrombospondin related anonymous protein (TRAP) of *Plasmodium falciparum* binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes. *EMBO J.* **12,** 2881–2889 (1993).

161. Morahan, B. J., Wang, L. & Coppel, R. L. No TRAP, no invasion. *Trends Parasitol.* **25,** 77–84 (2009).

162. Wengelnik, K. *et al.* The A-domain and the thrombospondin-related motif of *Plasmodium falciparum* TRAP are implicated in the invasion process of mosquito salivary glands. *EMBO J.* **18,** 5195–5204 (1999).

163. Sultan, A. A. *et al.* TRAP Is Necessary for Gliding Motility and Infectivity of *Plasmodium* Sporozoites. *Cell* **90,** 511–522 (1997).

164. Chattopadhyay, R. *et al.* PfSPATR, a *Plasmodium falciparum* protein containing an altered thrombospondin type I repeat domain is expressed at several stages of the parasite life cycle and is the target of inhibitory antibodies. *J. Biol. Chem.* **278,** 25977–25981 (2003).

165. Thompson, J. *et al.* PTRAMP; a conserved *Plasmodium* thrombospondin-related apical merozoite protein. *Mol. Biochem. Parasitol.* **134,** 225–232 (2004).

166. Siddiqui, F. A. *et al.* A thrombospondin structural repeat containing rhoptry protein from *Plasmodium falciparum* mediates erythrocyte invasion. *Cell. Microbiol.* **15,** 1341–1356 (2013).

167. Moreira, C. K. *et al.* The *Plasmodium* TRAP/MIC2 family member, TRAP-Like Protein (TLP), is involved in tissue traversal by sporozoites. *Cell. Microbiol.* **10,** 1505–1516 (2008).

168. Labaied, M., Camargo, N. & Kappe, S. H. I. Depletion of the *Plasmodium berghei* thrombospondin-related sporozoite protein reveals a role in host cell entry by sporozoites. *Mol. Biochem. Parasitol.* **153,** 158–166 (2007).

169. Steinbuechel, M. & Matuschewski, K. Role for the *Plasmodium* sporozoite-specific transmembrane protein S6 in parasite motility and efficient malaria transmission.

Cell. Microbiol. **11,** 279–288 (2009).

170. Yilmaz, B. *et al.* Gut Microbiota Elicits a Protective Immune Response against Malaria Transmission. *Cell* **159,** 1277–1289 (2014).

171. WHO. Malaria vaccine: WHO position paper. *Wkly. Epidemiol. Rec.* **91,** 33–52 (2016).

172. Macedo, C. S. de, Schwarz, R. T., Todeschini, A. R., Previato, J. O. & Mendonça-Previato, L. Overlooked post-translational modifications of proteins in *Plasmodium falciparum*: N- and O-glycosylation -- a review. *Mem. Inst. Oswaldo Cruz* **105,** 949–956 (2010).

173. Gowda, D. C. & Davidson, E. A. Protein glycosylation in the malaria parasite. *Parasitol. Today* **15,** 147–152 (1999).

174. Sanz, S. *et al.* Biosynthesis of GDP-fucose and Other Sugar Nucleotides in the Blood Stages of *Plasmodium falciparum*. *J. Biol. Chem.* **288,** 16506–16517 (2013).

175. Roper, J. R., Guther, M. L. S., Milne, K. G. & Ferguson, M. A. J. Galactose metabolism is essential for the African sleeping sickness parasite *Trypanosoma brucei*. *Proc. Natl. Acad. Sci.* **99,** 5884–5889 (2002).

176. Damerow, S. *et al. Leishmania major* UDP-sugar pyrophosphorylase salvages galactose for glycoconjugate biosynthesis. *Int. J. Parasitol.* **45,** 783–790 (2015).

177. Damerow, S. *et al. Leishmania* UDP-sugar pyrophosphorylase: the missing link in galactose salvage? *J. Biol. Chem.* **285,** 878–887 (2010).

178. Dickmanns, A. *et al.* Structural Basis for the Broad Substrate Range of the UDP-Sugar Pyrophosphorylase from *Leishmania major*. *J. Mol. Biol.* **405,** 461–478 (2011).

179. Galili, U. & Swanson, K. Gene sequences suggest inactivation of alpha-1,3-galactosyltransferase in catarrhines after the divergence of apes from monkeys. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7401–7404 (1991).

180. Ramasamy, R. & Reese, R. T. Terminal galactose residues and the antigenicity of *Plasmodium falciparum* glycoproteins. *Mol. Biochem. Parasitol.* **19,** 91–101 (1986).

181. Ravindran, B., Satapathy, A. K. & Das, M. K. Naturally-occurring anti-alpha-galactosyl antibodies in human *Plasmodium falciparum* infections--a possible role for autoantibodies in malaria. *Immunol. Lett.* **19,** 137–141 (1988).

182. Botte, C. Y. *et al.* Atypical lipid composition in the purified relict plastid (apicoplast) of malaria parasites. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 7506–7511 (2013).

183. Ramasamy, R. & Field, M. C. Terminal galactosylation of glycoconjugates in *Plasmodium falciparum* asexual blood stages and *Trypanosoma brucei* bloodstream trypomastigotes. *Exp. Parasitol.* **130,** 314–320 (2012).

184. Mio, T., Yamada-Okabe, T., Arisawa, M. & Yamada-Okabe, H. *Saccharomyces cerevisiae* GNA1, an essential gene encoding a novel acetyltransferase involved in UDP-N-acetylglucosamine synthesis. *J. Biol. Chem.* **274,** 424–429 (1999).

185. Mariño, K. *et al.* Characterization, localization, essentiality, and high-resolution crystal structure of glucosamine 6-phosphate N-acetyltransferase from *Trypanosoma brucei*. *Eukaryot. Cell* **10,** 985–997 (2011).

186. Hurtado-Guerrero, R. *et al.* Structural and kinetic differences between human and *Aspergillus fumigatus* D-glucosamine-6-phosphate N-acetyltransferase. *Biochem. J.* **415,** 217–223 (2008).

187. Sanz, S. *et al.* The disruption of GDP-fucose de novo biosynthesis suggests the presence of a novel fucose-containing glycoconjugate in *Plasmodium* asexual blood stages. *Sci. Rep.* **6,** 37230 (2016).

188. Kirk, K., Horner, H. A. & Kirk, J. Glucose uptake in *Plasmodium falciparum*-infected erythrocytes is an equilibrative not an active process. *Mol. Biochem. Parasitol.* **82,** 195–205 (1996).

189. Downie, M. J., Saliba, K. J., Howitt, S. M., Bröer, S. & Kirk, K. Transport of nucleosides across the *Plasmodium falciparum* parasite plasma membrane has characteristics of PfENT1. *Mol. Microbiol.* **60,** 738–748 (2006).

190. Bandini, G. *et al.* O-fucosylated glycoproteins form assemblies in close proximity to the nuclear pore complexes of *Toxoplasma gondii*. *Proc. Natl. Acad. Sci. U. S. A.* **113,** 11567–11572 (2016).

191. Lindner, S. E. *et al.* Total and putative surface proteomics of malaria parasite salivary gland sporozoites. *Mol. Cell. Proteomics* **12,** 1127–1143 (2013).

192. Trombetta, E. S. & Parodi, A. J. Quality Control and Protein Folding in the Secretory Pathway. *Annu. Rev. Cell Dev. Biol.* **19,** 649–676 (2003).

193. Samuelson, J. & Robbins, P. W. Effects of N-glycan precursor length diversity on quality control of protein folding and on protein glycosylation. *Semin. Cell Dev. Biol.* **41,** 121–128 (2015).

194. Sasamura, T. *et al.* The O-fucosyltransferase O-fut1 is an extracellular component that is essential for the constitutive endocytic trafficking of Notch in Drosophila. *Development* **134,** 1347–1356 (2007).

195. Okajima, T., Xu, A., Lei, L. & Irvine, K. D. Chaperone Activity of Protein O-Fucosyltransferase 1 Promotes Notch Receptor Folding. *Science (80-.).* **307,** 1599–1603 (2005).

196. Okajima, T., Reddy, B., Matsuda, T. & Irvine, K. D. Contributions of chaperone and

glycosyltransferase activities of O-fucosyltransferase 1 to Notch signaling. *BMC Biol.* **6,** 1 (2008).

197. Benz, B. A. *et al.* Genetic and biochemical evidence that gastrulation defects in Pofut2 mutants result from defects in ADAMTS9 secretion. *Dev. Biol.* **416,** 111–122 (2016).

198. Fanchiotti, S., Fernández, F., D'Alessio, C. & Parodi, A. J. The UDP-Glc:Glycoprotein glucosyltransferase is essential for *Schizosaccharomyces pombe* viability under conditions of extreme endoplasmic reticulum stress. *J. Cell Biol.* **143,** 625–635 (1998).

199. Izquierdo, L., Atrih, A., Rodrigues, J. A., Jones, D. C. & Ferguson, M. A. J. Trypanosoma brucei UDP-glucose:glycoprotein glucosyltransferase has unusual substrate specificity and protects the parasite from stress. *Eukaryot. Cell* **8,** 230–240 (2009).

200. Hess, D., Keusch, J. J., Oberstein, S. A. L., Hennekam, R. C. M. & Hofsteenge, J. Peters Plus syndrome is a new congenital disorder of glycosylation and involves defective Omicron-glycosylation of thrombospondin type 1 repeats. *J. Biol. Chem.* **283,** 7354–7360 (2008).

201. Florens, L. *et al.* Proteomics approach reveals novel proteins on the surface of malaria-infected erythrocytes. *Mol. Biochem. Parasitol.* **135,** 1–11 (2004).

202. Regules, J. A., Cummings, J. F. & Ockenhouse, C. F. The RTS,S vaccine candidate for malaria. *Expert Rev. Vaccines* **10,** 589–599 (2011).

203. Shams-Eldin, H. *et al. Plasmodium falciparum* dolichol phosphate mannose synthase represents a novel clade. *Biochem. Biophys. Res. Commun.* **370,** 388–393 (2008).

204. Doucey, M.-A., Hess, D., Cacan, R. & Hofsteenge, J. Protein C-Mannosylation Is Enzyme-catalysed and Uses Dolichyl-Phosphate-Mannose as a Precursor. *Mol. Biol. Cell* **9,** 291–300 (1998).

205. Kinoshita, T., Ohishi, K. & Takeda, J. GPI-anchor synthesis in mammalian cells: genes, their products, and a deficiency. *J. Biochem.* **122,** 251–257 (1997).

206. Doud, M. B. *et al.* Unexpected fold in the circumsporozoite protein target of malaria vaccines. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 7817–7822 (2012).

207. Herrera, R. *et al.* Reversible Conformational Change in the *Plasmodium falciparum* Circumsporozoite Protein Masks Its Adhesion Domains. *Infect. Immun.* **83,** 3771–3780 (2015).

208. Ringe, R. P. *et al.* Cleavage strongly influences whether soluble HIV-1 envelope glycoprotein trimers adopt a native-like conformation. *Proc. Natl. Acad. Sci.* **110,** 18256–18261 (2013).

209. Schwartz, L., Brown, G. V, Genton, B. & Moorthy, V. S. A review of malaria vaccine clinical projects based on the WHO rainbow table. *Malar. J.* **11,** 11 (2012).