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Research Article

Sugar nucleotide quantification by liquid chromatography tandem mass spectrometry reveals a distinct profile in *Plasmodium falciparum* sexual stage parasites

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The obligate intracellular lifestyle of *Plasmodium falciparum* and the difficulties in obtaining sufficient amounts of biological material have hampered the study of specific metabolic pathways in the malaria parasite. Thus, for example, the pools of sugar nucleotides required to fuel glycosylation reactions have never been studied in-depth in well-synchronized asexual parasites or in other stages of its life cycle. These metabolites are of critical importance, especially considering the renewed interest in the presence of *N*-, *O*-, and other glycans in key parasite proteins. In this work, we adapted a liquid chromatography tandem mass spectrometry (LC-MS/MS) method based on the use of porous graphitic carbon (PGC) columns and MS-friendly solvents to quantify sugar nucleotides in the malaria parasite. We report the thorough quantification of the pools of these metabolites throughout the intraerythrocytic cycle of *P. falciparum*. The sensitivity of the method enabled, for the first time, the targeted analysis of these glycosylation precursors in gametocytes, the parasite sexual stages that are transmissible to the mosquito vector.

Introduction

Plasmodium falciparum is the causative agent of the deadliest form of human malaria and it is responsible for the majority of malaria-related deaths [1]. With approximately half of the world living at risk of malaria, the emergence of parasite resistance to the current last-line of antimalarials poses a serious threat to malaria control and elimination efforts [2]. The development of novel drugs is still limited by our incomplete understanding of the biology of the parasite [3]. The characterization of parasite metabolic pathways, new protein functions, post-translational modifications, and the identification of cognate receptors on human and vector host cells can ultimately guide the design of selective inhibitors to prevent disease and parasite transmission through the vector.

The frequency of glycosylation modifications of *P. falciparum* is generally assumed to be low in the blood stages of the parasite [4,5] except for the well-described glycosylphosphatidylinositol (GPI) anchors [6]. GPI anchors attach essential proteins, such as merozoite surface proteins 1 and 2 (MSP-1 and MSP-2) [7], to the parasite plasma membrane. Following parasite egress from the red blood cell, GPI anchors are also thought to induce proinflammatory cytokine responses, which are a major contributing factor to malaria pathogenesis [8]. Previous advances in the identification of new glycosylated structures both in the human [9] and mosquito stages [10], and the quantification of sugar nucleotides in the blood stages of parasite development [11] have renewed interest in the glycobiology of *P. falciparum*. Sugar nucleotides are high energy donors that feed the biosynthesis of glycoconjugates [12]. For instance, GDP-mannose (GDP-Man) and UDP-N-acetylglucosamine (UDP-GlcNAc) are required for

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the synthesis of GPI anchors in eukaryotes [13,14]. Similarly, UDP-GlcNAc is also involved in the synthesis of N-glycans, which in *P. falciparum* consist of one or two residues of N-acetyl-glucosamine (GlcNAc) [9,15]. As obligate glycosylation precursors, the quantification of sugar nucleotide pools provides strong evidence of the presence of cellular glycosylation reactions [11,16]. Furthermore, the ability to identify and quantify these metabolites remains an invaluable tool for functional analyses aimed at elucidating the mechanisms of sugar nucleotide biosynthesis [17–19]. However, a robust analytical method must take into consideration their highly polar nature, low *in vivo* concentration, and rapid turnover. Moreover, structurally analogous sugar nucleotides, such as UDP-glucose (UDP-Glc) and UDP-galactose (UDP-Gal), display similar chromatographic profiles, hindering an adequate resolution.

Sugar nucleotide pools of *P. falciparum* and other protozoan parasites have been previously quantified by ion-pair reversed-phase (RP) liquid chromatography-tandem mass spectrometry (LC-MS/MS) [11,16]. In this approach, the weak retention displayed by polar compounds under RP conditions is solved with the addition of ion-pairing solvents. Still, this strategy should be avoided when the instrumentation is intended to be employed in different applications, since trace amounts of ion-pair reagents cannot be fully removed [20]. Moreover, these reagents reduce sensitivity by partially suppressing electrospray ionization (ESI) [21,22]. In contrast with conventional columns used for RP chromatography, porous graphitic carbon (PGC) not only retains non-polar compounds but also exhibits a strong affinity towards polar analytes. Thus, PGC is an excellent choice for multipurpose MS instrumentation (such as those housed in a shared facility) since it does not require the addition of ion-pair reagents to achieve an efficient retention of sugar nucleotides [20,23]. Additionally, the complex retention mechanism displayed by PGC results in an increased selectivity with respect to other approaches; allowing the separation of structurally similar analytes [24].

In this work we used a PGC-based LC-MS/MS technique [20] to identify and quantify the sugar nucleotide pools in tightly synchronized *P. falciparum* parasites. Different stages of intraerythrocytic development were analyzed, resulting in greater precision in the estimates of availability or abundance of these obligate donors for glycosylation reactions. The sensitivity of the analytical method allowed us to compare the sugar nucleotide content of gametocytes, which are intraerythrocytic, non-proliferative single parasite forms, with the multi-nucleated erythrocytic schizonts containing between 12–18 merozoites. The *P. falciparum* gametocyte makes up less than 1% of the entire parasite biomass in an infected individual, retains a unique, falciform shape, sequesters in the hematopoietic system of human bone marrow, and does not egress from its red blood cell home until it is inside the mosquito midgut [25,26]. Given this known biology, previous work suggested that gametocytes entered a quiescent phase associated with low metabolic activity [27]. Thus, it was thought that sugar nucleotide biosynthesis in gametocytes would be lower (or even absent) in comparison with the more dynamic asexual blood stages. Nevertheless, our results seem to show that in fact the converse is true, in agreement with a previous study that indicates significant metabolic activity in *P. falciparum* gametocytes [28].

Experimental

Chemicals

All reagents, standards, and solvents used for chromatography (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A), unless otherwise specified. Ammonia and absolute ethanol were purchased from Merck (Darmstadt, Germany). High purity water was prepared with a Millipore Milli-Q Plus system (Millipore, Bedford, MA, U.S.A.). Standard stock solutions of sugar nucleotides were prepared in water at 2 μM and stored at −80°C prior to use. Further dilutions were prepared in 25% acetonitrile + formate buffer (0.1% formic acid adjusted to pH 9 with ammonia).

P. falciparum culture and synchronization

P. falciparum asexual stages were cultured in an atmosphere of 92% N_2, 3% O_2, and 5% CO_2 with B+ human erythrocytes at 2–4% hematocrit in RPMI medium supplemented with 0.5% Albumax II. Human erythrocytes were purchased from the Banc de Sang i Teixits (Catalonia, Spain), after approval from the Comitè Ètic Investigació Clínica Hospital Clínic de Barcelona. For the analysis of asexual blood stages, *P. falciparum* strain 3D7 (BEI Resources/MR4/American Type Culture Collection, Manassas, VA, U.S.A.) was tightly synchronized to a 5-hour window by combining Percoll purification of schizonts [29], followed by sorbitol lysis 5 h later [30]. To reduce biological variation, a single culture was split and parasites were harvested at 18, 25, 32, 35, and 37 ± 2.5 h post-invasion.

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Gametocytogenesis

The previously described 3D7-B subclone E5 [31] was cultured in RPMI medium supplemented with 10% human serum for gametocyte production. Sorbitol synchronized rings (day 0) were treated with 50 mM N-acetyl glucosamine (GlcNAc) [32] that was maintained for the remainder of the experiment. On day 10, asexual late stages were removed by sorbitol lysis and gametocytes (mostly stage IV) were enriched from uninfected red blood cells (uRBCs) and residual ring stages by magnetic cell sorting using MACS columns (Miltenyi Biotech, Germany).

Metabolite extraction and pre-analytical purification

Harvested parasites were released by osmotic lysis in 60 volumes of cold erythrocyte lysis buffer (from a 10× stock solution consisting of 0.15 M NH_4Cl, 0.1 M $KHCO_3$, 0.01 M EDTA) and incubated in ice for 10 min [33]. As a control, 4×10^9 uRBCs were subjected to the same treatment. Host red blood cell material was washed three times with ice-cold phosphate-buffered saline and released parasites were lysed with 500 µls of −20°C 70% ethanol and spiked with 20 pmols of internal standard GDP-glucose (GDP-Glc). Cell debris was removed by centrifugation (20 000 × **g** for 10 min at 4°C) and supernatants were dried under nitrogen or in a Savant™ SPD 1010 SpeedVac™ concentrator (Thermo Scientific) at room temperature. For comparison purposes, some samples were further processed to reduce complexity. Briefly, lipids were extracted by n-butanol:water partition, and sugar nucleotides enriched by solid phase enrichment (SPE) with Supelclean™ Envi™-Carb columns (Supelco), as described previously [16,34]. Extracted metabolites were finally resuspended in 80–100 µls of 25% acetonitrile + 0.1% formic acid adjusted to pH 9 with ammonia. 10 µls were injected for LC-MS/MS analysis.

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

The LC-MS/MS method was carried out on an Agilent 1290 Infinity LC System equipped with a Hypercarb PGC column (5 µm, 2.1 × 100 mm; ThermoFisher Scientific) coupled by ESI to a QTRAP 6500 (AB Sciex). The column was maintained at 60°C and samples (10 µl volume) were injected at a constant flow rate of 150 µl/min. The binary mobile phase was composed of 0.1% formic acid adjusted to pH 9 with ammonia (A) and acetonitrile (B). The LC run started with a 30 min gradient from 2% acetonitrile to 15%, ramping linearly to 50% at 42 minutes and followed by a 1 min gradient to 100%, which was maintained for 2 min. Before running the next sample, the column was re-equilibrated by returning to the initial conditions of 2% acetonitrile for 35 minutes (Supplementary Figure S1). The mass spectrometer was operated in the negative ion mode with the following settings: ion spray voltage: −4200 V, declustering potential: −80 V, entrance potential: −10 V, source temperature: 600°C. Parent and fragment ions were detected in multiple reaction monitoring (MRM) mode for mass transitions described in Table 1. Peak identities were verified by the analysis of

Table 1. Retention times, limits of detection, and quantification and linearity of standard calibration curves

Analyte	MRM transition (m/z)[1]	tR (min)	tR intra-day SD (min)[2]	tR inter-day SD (min)[3]	LoD (nM)[4]	LoQ (nM)[5]	R^2 [6]
UDP-Gal	565 → 323	20.38	1.57	2.21	0.30	1.02	0.9968
UDP-Glc	565 → 323	21.68	1.51	3.44	0.44	1.48	0.9987
UDP-GlcNAc	606 → 385	22.40	1.54	3.11	0.77	2.56	0.9973
GDP-Man	604 → 424	31.18	1.94	2.95	0.77	2.56	0.9966
GDP-Glc	604 → 362	33.57	1.22	2.57	0.20	0.68	1.0000
GDP-Fuc	588 → 442	34.21	1.04	2.90	0.25	0.84	0.9985

[1]The major product ion for every sugar nucleotide corresponds to its nucleotidic moiety, since ion fragmentation takes place around the unstable bond between the sugar and the nucleotide (i.e. at the sugar-1-phosphate or pyrophosphate linkages) [16].
[2]Intra-day standard deviation of retention times ($n = 12$ measurements).
[3]Inter-day standard deviation of retention times ($n = 3$ measurements).
[4]Limit of detection (LoD) was calculated at a signal-to-noise ratio (S/N) of 3.
[5]Limit of quantification (LoQ) was calculated at a signal-to-noise ratio (S/N) of 10.
[6]Correlation coefficients of the known amount of commercial sugar nucleotides in the calibration curve and the analyte MS signal relative to the GDP-glucose internal standard.

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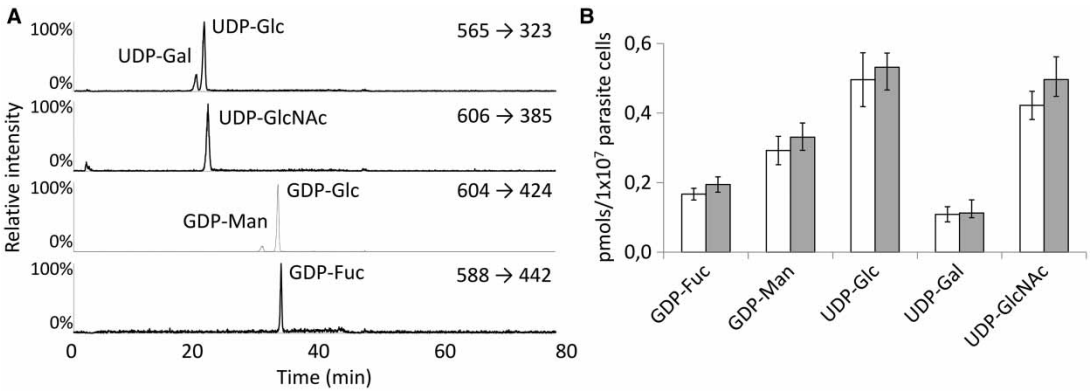

Figure 1. Development of the PGC LC-MS/MS quantification method for sugar nucleotides analysis.
(**A**) Representative extracted ion chromatograms (XIC) of targeted sugar nucleotides from an extract of *P. falciparum* 3D7 trophozoites (30 ± 2.5 hpi). (**B**) Sugar nucleotide pools of 7.3×10^7 *P. falciparum* 3D7 trophozoites (30 ± 2.5 hpi) analyzed by PGC LC-MS/MS before (white bars) or after (grey bars) a pre-analytical purification consisting of butan-1-ol delipidation and solid phase enrichment. Values of each sugar nucleotide indicate the average of three different extractions. Error bars display S.D. GDP-Fuc, GDP-fucose; GDP-Glc, GDP-glucose; GDP-Man, GDP-mannose; UDP-Gal, UDP-galactose; UDP-Glc, UDP-glucose; UDP-GlcNAc, UDP-N-acetylglucosamine.

commercial sugar nucleotide standards and selected areas were integrated with the Analyst® 1.6.2 software. Sugar nucleotides were quantified by correlating the known amount of commercial sugar nucleotides in the calibration curve and the ratio between metabolite and internal standard MS signals.

Results

Validation of a PGC chromatography LC-MS/MS method for the quantification of sugar nucleotides in asexual *P. falciparum* blood stages

Commercial sugar nucleotide standards were subjected to chromatographic separation on a PGC column and identified by their characteristic elution times when MRM failed to select between analyzed compounds as in the case of stereoisomers, such as UDP-Glc and UDP-Gal or GDP-Man and GDP-Glc (Figure 1A). All of the standards could be well resolved to baseline. Linearity, limits of detection, and quantification and intra-day and inter-day variability of retention times are included in Table 1. Previously described methods involved a pre-analytical purification step consisting of butan-1-ol [16] delipidation and SPE [16,20,34] to reduce sample complexity. To assess whether the use of this sample preparation step could be circumvented in intraerythrocytic stages, 7.3×10^7 *P. falciparum* 3D7 trophozoites (30 ± 2.5 hpi) were analyzed with or without delipidation and SPE. Sugar nucleotide levels displayed no significant difference between both sample treatments (Figure 1B), allowing a faster and less laborious method. Furthermore, the background signal for each MRM transition remained unaltered and thus the low limits of quantification observed using standards were maintained (Supplementary Table S1). Remarkably, under these conditions we did not observe any decrease in the chromatographic performance of the column (i.e. peak resolution of the analytes) in more than five different runs with several samples over a period of more than a year.

Quantifying sugar nucleotide levels at different stages of *P. falciparum* asexual intraerythrocytic development

In its human host, *P. falciparum* goes through a cycle of repeated invasion of erythrocytes, exhibiting a synchrony duration of approximately 48 h. In culture, *P. falciparum* grown in culture loses synchronicity quickly, making it more difficult to analyze and compare sugar nucleotide levels across specific intraerythrocytic developmental stages. Different synchronization methods, based on selective lysis [30] or physical separation of specific stages [29] generate synchronization windows as wide as the duration of that stage, which is not optimal to thoroughly describe the regulation of specific metabolites throughout the parasite intraerythrocytic life cycle. Higher degrees of synchrony can be achieved by successive combination of different methods, such as Percoll

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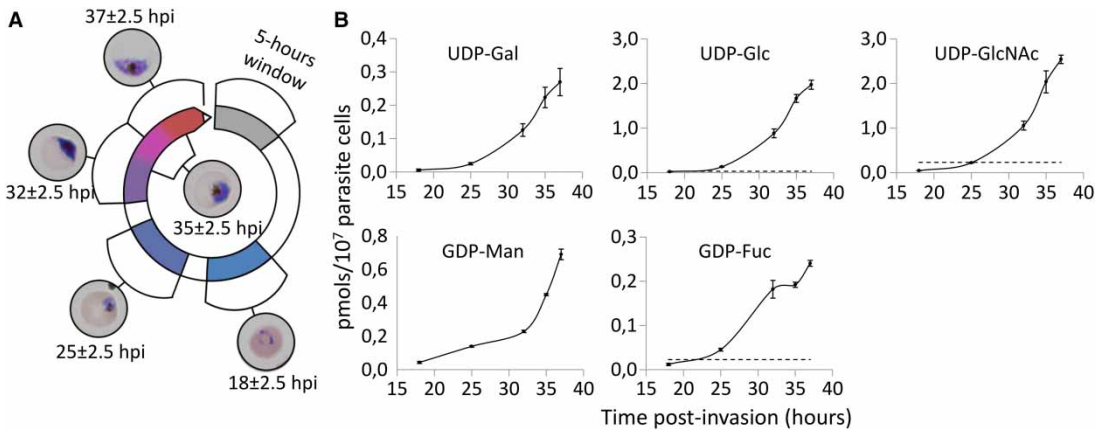

Figure 2. Sugar nucleotide pools of *P. falciparum* asexual blood stages.

(**A**) Schematic representation of the synchronization strategy and sampling time-points (hpi, hours post-invasion).

(**B**) Quantification of sugar nucleotides from extracts of *P. falciparum* 3D7 tightly synchronized asexual blood stages. Values are the average of three extractions at different time-points. Error bars display S.D. Uninfected red blood cells were lysed as a negative control and were used to estimate the maximum contamination threshold expected from host red blood cells (dashed line) according to the amount of red blood cells in each sample (except for non-quantifiable analytes with a signal-to-noise ratio <10, i.e. UDP-Gal and GDP-Man).

purification followed by sorbitol lysis [35]. However, these procedures require significant amounts of culture reagents or a reduction in the quantity of parasites in culture, making the analysis of low-abundant metabolites more difficult. The low quantification limits (0.8–2.6 nM; Table 1) achieved by this LC-MS/MS method prompted us to analyze tightly synchronized parasite cultures (0–5-h window) with ~$1–4 \times 10^8$ parasites cells. These analyses resulted in a comprehensive quantification of the sugar nucleotide levels along the *in vitro* asexual intraerythrocytic development of *P. falciparum* (Figure 2B).

One of the main issues when analyzing the intraerythrocytic stages of malaria and other intracellular parasites is the extent of host-cell contamination. Human red blood cells (RBCs) contain pools of sugar nucleotides (Supplementary Figure S2A) and the LC-MS/MS method could detect significant amounts of these metabolites carried over from the parasite host cells. To assess the degree of this contamination, we analyzed the sugar nucleotides detected in lysed uRBCs and established a contamination threshold that may be carried over from the host cell (see dashed lines in Figure 2B). Only GDP-Fuc, UDP-Glc, and UDP-GlcNAc were detected above the limit of quantification when high numbers of uRBCs (~4×10^9) were lysed (Supplementary Figure S2B). Nevertheless, even in that case, the background levels were negligible when compared with sugar nucleotides detected in parasites in the trophozoite stages and later (from 32 ± 2.5 hpi, see Figure 2B).

Sugar nucleotide levels in sexual gametocytes of *P. falciparum*

After establishing infection, less than 1% of intraerythrocytic *P. falciparum* differentiate to gametocytes [36]. Gametocytogenesis is an essential process for parasite transmission, since gametocytes are the only stages that can fertilize and establish the sporogonic cycle in the anopheline mosquito vector. A metabolomic study revealed that despite entering a non-proliferative state, gametocytes exhibit increased levels of glucose utilization and high energy requirements [28], indicating significant metabolic activity in these life stages. The gametocytes are known to harbor many transcripts that while in the human host are rapidly translated into proteins following gametogenesis in the mosquito, which occurs within a 10–15 min time period, followed by macrogamete fertilization to form the mosquito-infective ookinete stage. Fertilized zygotes and motile ookinetes express two major GPI-anchored proteins, P25 and P28 [37]. We hypothesize that sugar nucleotide pools are ramped-up and stored in preparation for gametogenesis inside the mosquito vector. To date, the pools of sugar nucleotides in gametocytes have not been analyzed. The sensitivity of the HPLC-MS/MS method designed enabled the robust analysis of samples containing less than 1×10^8 *P. falciparum* gametocytes (see Supplementary Table S2). We observed that some of the sugar nucleotides detected in this life stage were present at much higher levels than in the asexual blood stages (Figure 3B).

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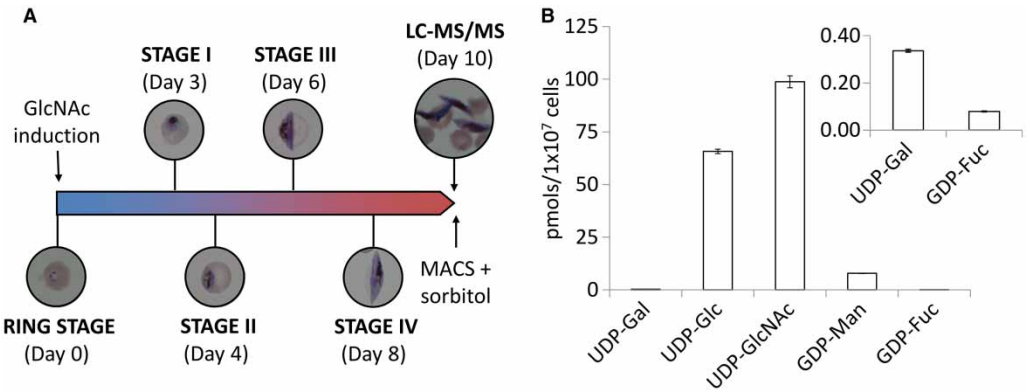

Figure 3. Sugar nucleotide pools of *P. falciparum* gametocytes.

(**A**) Schematic representation of gametocyte induction and sampling time-point. (**B**) Quantification of sugar nucleotides from an extract of *P. falciparum* 3D7B E5 subclone. Values are the average of three technical replicates. Error bars display S.D. GlcNAc, N-acetyl glucosamine; LC-MS/MS, liquid chromatography tandem mass spectrometry; MACS, Magnetic-Activated Cell Sorting.

Discussion

In this work we used an LC-ESI-MS/MS-based methodology for the quantification of sugar nucleotides in the intraerythrocytic stages of *P. falciparum* using an ion trap instrument (QTRAP 6500 AB Sciex), a PGC column, and an MS-compatible solvent system [20]. The low limit of detection for the targeted analytes, between 0.3–0.8 nM, allows a reduction in the number of parasites required for analysis (see Supplementary Table S2). This is critical when working with organisms such as *P. falciparum*, which pose significant problems in obtaining sufficient amounts of parasites that are tightly synchronized or from specific life stages (i.e. gametocytes or ookinetes). The analysis showed (1) a dramatic increase in the pools of different sugar nucleotides in the mature stages of the intraerythrocytic asexual development of the parasite, and (2) remarkably higher levels of these metabolites in stage IV gametocytes. We anticipate leveraging the sensitivity of this method to analyze sugar nucleotides in other important parasite life stages, such as the intrahepatic stages of *P. falciparum*, which are less amenable for large-scale purification.

As previously observed [11], the availability of sugar nucleotides in the ring stages seems to be scarce (Figure 2B), suggesting that glycosylation reactions might occur later in the parasite cycle. However, it could also be argued that the high demand of these metabolites during the initial stages keeps the pools at a lower level. Throughout asexual development, there is a significant increase in parasite size within the red blood cell [38]. Its rate of multiplication, with 10 to 20 merozoites produced every 48 h, involves high metabolic and biosynthetic activity. The parasite assembles a variety of glycoconjugates, mostly free GPIs or glycoinositol phospholipids (GIPLs), and GPI-anchored proteins, several of which are essential for invasion [6,7,39]. Regardless of the presence of N-glycans [9] and other uncharacterized glycosylations [19], GPI biosynthesis requires UDP-GlcNAc and GDP-Man donors [40]. Accordingly, the pools of these metabolites (Figure 2B), but also other sugar nucleotides, expand exponentially along the asexual life cycle of the parasite, either reflecting an increased biosynthesis to fuel the demand of UDP-GlcNAc and GDP-Man during the mature stages of the asexual life cycle; or indicating a decrease in the need of these metabolites, due to a decline of glycoconjugate synthesis at the final stages. Data on gene expression of UDP-N-acetylglucosamine pyrophosphorylase and mannose-1-phosphate guanyltransferase might elucidate this matter, but their RNA transcripts are decreased and increased, respectively, towards the end of the life cycle [41].

The levels of GDP-fucose (GDP-Fuc) are steady between 32 ± 2.5 and 35 ± 2.5 h post-invasion suggesting the possible extra consumption of this metabolite by the parasite glycosylation machinery during this time period (Figure 2B). Interestingly, *P. falciparum* is known to express a Protein O-fucosyltransferase 2 (PoFUT2) homolog, a GDP-Fuc-dependent O-fucosyltransferase that fucosylates thrombospondin type 1 repeat (TSR) domains in other organisms [42]. Accordingly, the transcription of *Pf*PoFUT2 and potential O-fucose acceptors, the TSR-containing proteins MTRAP and TRAMP, increase during that same period [41].

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Finally, gametocytes present significantly higher levels of sugar nucleotides than the asexual blood stages (Figure 3B). The literature suggests that the cell volume of gametocytes and asexual schizonts is similar [38]. Therefore, the observed levels of UDP-GlcNAc, UDP-Glc, and GDP-Man above one order of magnitude in comparison with asexual parasites shows a much higher concentration and availability of these metabolites in gametocytes (i.e. 65.7, 98.8, and 7.8 pmols/10^7 parasite cells for UDP-Glc, UDP-GlcNAc and GDP-Man). Indeed, the levels of UDP-GlcNAc, UDP-Glc, and GDP-Man in gametocytes are comparable to the extracellular forms of other parasites, such as *Trypanosoma brucei*, *Trypanosoma cruzi*, or *Leishmania major* [16] that present more varied and profuse glycosylation patterns [43]. This could reflect a distinct glycosylation status in *P. falciparum* gametocytes in contrast with asexual blood stages and supports the contention that the gametocytes are preparing for an extracellular existence in the mosquito. Gametocytes egress from the red blood cell and become extracellular gametes in the mosquito midgut, where they could make use of the pools accumulated in the gametocyte stage. Thus, glycosylation could be more relevant in these extracellular forms and possibly also in other mosquito stages (such as oocysts and sporozoites). Further studies on the glycosylations present on the surface of *P. falciparum* extracellular stages will help to cast light on this issue.

Abbreviations

ESI, electrospray ionization; GDP, Guanosine diphosphate; GDP-Fuc, GDP-fucose; GDP-Glc, GDP-glucose; GDP-Man, GDP-mannose; GIPL, glycoinositol phospholipid; GPI, glycosylphosphatidylinositol; hpi, hours post invasion; LC-MS/MS, liquid chromatography tandem mass spectrometry; MACS, Magnetic-Activated Cell Sorting; MRM, multiple reaction monitoring; MSP-1, merozoite surface protein 1; MSP-2, merozoite surface protein 2; PGC, porous graphitic carbon; RP, reversed phase; RPMI, Roswell Park Memorial Institute; SPE, solid phase enrichment; TSR, thrombospondin type 1 repeat; UDP, Uridine diphosphate; UDP-Gal, UDP-galactose; UDP-Glc, UDP-glucose; UDP-GlcNAc, UDP-N-acetylglucosamine; uRBC, uninfected red blood cell.

Author contribution

B.L.-G. collected samples and carried out sugar nucleotide analysis. B.L.-G. and L.I. conceived the work, designed the method, and performed data analysis. B.L.-G., L.I., and R.R.D. outlined the document and contributed to the writing and review of this manuscript.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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