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PfHPRT: A New Biomarker Candidate of Acute *Plasmodium falciparum* Infection

Marie L. Thézénas,[†,▽] Honglei Huang,[†,▽] Madi Njie,[‡] Abhinay Ramaprasad,[†,§] Davis C. Nwakanma,[‡] Roman Fischer,[†] Katalin Digleria,[∥] Michael Walther,[⊥] David J. Conway,[#] Benedikt M. Kessler,[†] and Climent Casals-Pascual*[,†]

[†]Wellcome Trust Centre for Human Genetics and Henry Wellcome Building for Molecular Physiology, Nuffield Department of Medicine, University of Oxford, Oxford OX3 7BN, U. K.

[‡]Malaria Programme, MRC Unit, Banjul, The Gambia

[§]King Abdulla University of Science and Technology, Saudi Arabia

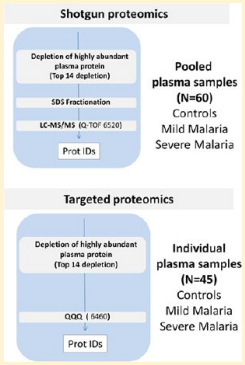
[∥]Weatherall Institute of Molecular Medicine, Nuffield Department of Medicine, University of Oxford, Oxford, U. K.

[⊥]National Institutes of Health, National Institute of Allergy and Infectious Diseases, Rockville, Maryland, United States

[#]London School of Hygiene and Tropical Medicine, London, U. K.

Supporting Information

ABSTRACT: *Plasmodium falciparum* is a protozoan parasite that causes human malaria. This parasitic infection accounts for approximately 655 000 deaths each year worldwide. Most deaths could be prevented by diagnosing and treating malaria promptly. To date, few parasite proteins have been developed into rapid diagnostic tools. We have combined a shotgun and a targeted proteomic strategy to characterize the plasma proteome of Gambian children with severe malaria (SM), mild malaria, and convalescent controls in search of new candidate biomarkers. Here we report four *P. falciparum* proteins with a high level of confidence in SM patients, namely, PF10_0121 (hypoxanthine phosphoribosyltransferase, pHPRT), PF11_0208 (phosphoglycerate mutase, pPGM), PF13_0141 (lactate dehydrogenase, pLDH), and PF14_0425 (fructose bisphosphate aldolase, pFBPA). We have optimized selected reaction monitoring (SRM) assays to quantify these proteins in individual patients. All *P. falciparum* proteins were higher in SM compared with mild cases or control subjects. SRM-based measurements correlated markedly with clinical anemia (low blood hemoglobin concentration), and pLDH and pFBPA were significantly correlated with higher *P. falciparum* parasitemia. These findings suggest that pHPRT is a promising biomarker to diagnose *P. falciparum* malaria infection. The diagnostic performance of this marker should be validated prospectively.



KEYWORDS: *Plasmodium falciparum, malaria, biomarker, hypoxanthine phosphoribosyltransferase, pHPRT, shotgun proteomics, SRM*

INTRODUCTION

Human malaria is a life-threatening disease caused by *Plasmodium* parasites that accounts for approximately 655 000 deaths yearly, primarily in African children.[1] Five *Plasmodium* species cause human malaria, but the majority of deaths are attributable to *Plasmodium falciparum*. Since most deaths in children admitted to hospital with severe malaria occur within the first 24 h, it is critical to diagnose and treat malaria patients promptly.[2]

The standard method to diagnose malaria infection requires the visualization of the parasite in blood using light microscopy. However, laboratory facilities to diagnose malaria are not available in many resource-poor endemic settings, and the administration of antimalarials is often based on nonspecific clinical symptoms.[3] Treatment allocation based on clinical presentation results in overdiagnosis of malaria and thus in the overuse of antimalarial treatment, which contributes to the increasing resistance of *P. falciparum* to available drugs.[4,5] Consequently, the World Health Organization (WHO) recommends that all suspected malaria cases should be confirmed with a parasite-based diagnostic assay. A number of commercially available rapid diagnostic tests (RDTs) based on lateral flow immunochromatographic assays (dipsticks) that detect *P. falciparum* proteins have been developed and implemented with variable success.[6] RDTs are relatively inexpensive and simple to use, and results can be obtained in approximately 20 min. The most widely available RDTs used in malaria detect histidine-rich protein 2 (HRP2), lactate dehydrogenase (pLDH) and aldolase (pFBPA).[7] However, there is a need for additional markers to develop more accurate tests for more effective treatment.

The eukaryotic genome of *P. falciparum* contains approximately 5300 genes, and a large proportion of these are

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Table 1. Population Description

		severe malaria	mild malaria	convalescent malaria	*p*-value[a]
patients	number	20	20	20	
age (years)	mean (SD)	3.61 (0.99)	3.65 (1.11)	3.63 (1.03)	0.88
hemoglobin (g/dL)	mean (SD)	8.7 (2.05)	10.8 (1.6)	11.4 (1.42)	0.001
parasite density (parasites/ μL)	median (IQR)	418 710 (205 000−662 340)	228 060 (89 280−384 300)	0	0.13

[a]Severe versus mild malaria cases (*t*-test: age and hemoglobin; Mann−Whitney: parasite density).

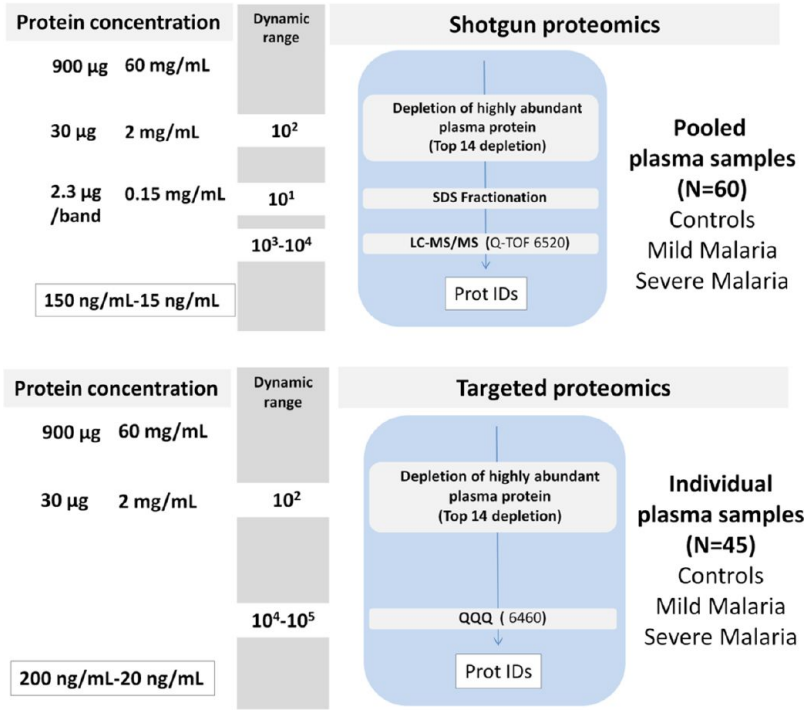

Figure 1. Study outline. Description of the methodological workflow indicating the limits and the dynamic range of plasma proteins quantified using shotgun and targeted proteomics.

expressed during blood stage infection.[8] Initial studies describing the stage-specific proteome of *P. falciparum* 3D7 isolate showed that more than half of the parasite proteins identified were annotated as hypothetical proteins.[9] Similarly, the proteome of *P. falciparum* NF54 isolate using high accuracy mass spectrometry reported 1289 parasite proteins.[10]

Using a standard shotgun proteomic approach (LC−MS/MS), we have recently identified a group of *P. falciparum* proteins in plasma from patients with severe malaria in Gambian children. We have used a targeted proteomic approach using selected reaction monitoring (SRM) to validate these parasite proteins in plasma from individual patients.

MATERIALS AND METHODS

Clinical Study

This study was conducted as part of a case-control study on severe malaria in the Gambia from 2005 to 2008. Patients were enrolled after written informed consent was obtained from the parents or guardians. The study design and population have been described elsewhere.[11]

All patients received standard care provided by the health center staff in accordance with the Gambian Government Treatment Guidelines. The patients were followed up at home 7 days after admission. The study was reviewed and approved by the Joint Gambian Government/MRC Ethics Committee and the Ethics Committee of the London School of Hygiene & Tropical Medicine (London, U.K.).

Mild malaria was defined as an episode of fever (temperature > 37.5 °C) within the last 48 h with more than 5000 parasites/μL detected by slide microscopy. Severe malaria (SM) was defined using modified WHO criteria. Eight of the 20 cases (40%) presented with coma in the absence of hypoglycemia or hypovolemia, with the coma lasting at least for 2 h (cerebral malaria), 8 cases (40%) presented with severe anemia and 7 cases (35%) with respiratory distress. Four cases (20%) presented with more than one SM syndrome, and 2 cases were admitted to hospital (complicated malaria) but did not meet criteria for specific SM syndromes.

Sample processing and storage were performed according to standard operating procedures at the MRC Laboratories in the Gambia, which follow good clinical and laboratory practice (GCLP) regulations. Blood samples were processed within 2 h of collection. Plasma was separated and stored at −80 °C. The plasma proteome of children with SM (n = 20) was compared with that of children in the same age range with a mild malaria infection on admission (n = 20) (Table 1). The proteomic profiles were also obtained from age-matched patients 28 days after successful treatment (n = 20). The shotgun discovery phase was performed on samples collected during the malaria season 2005−2006, and the SRM based validation assays were conducted on an independent set of samples collected in the

2007−2008 malaria season. The study outline is described in Figure 1.

Shotgun Proteomics

Plasma depletion of highly abundant proteins. Antibody affinity depletion was performed using an Agilent Human top 14 Multiple Affinity Removal System following manufacturer's instructions. Briefly, plasma samples were centrifuged at 10000*g* for 10 min. Samples were then diluted 8 times in buffer A (Agilent Technologies, U.K.) and filtered through a 0.22 μm spin filter (Agilent Technologies, U.K.) for 2 min at 16000*g*. For each run, 120 μL of diluted plasma were injected into the Agilent Human 14 MARS Column (4.6 × 100 mm) coupled with a 1200 Series HPLC (Agilent Technologies, U.K.). Protein depletion was carried out using the following 48 min isocratic elution: 100% buffer A for 20 min at 0.125 mL/min, and then 2.5 min at 0.7 mL/min followed by 10 min of 100% buffer B (Agilent Technologies, U.K.) at 0.7 mL/min and 15.5 min of buffer A for the column equilibration. The flow-through was collected between 12 and 21 min with buffer A. Bound proteins were eluted between 26 and 30 min with buffer B. Each sample was injected 10 times in order to obtain sufficient quantity of proteins for analysis.

Trichloroacetic Acid/Deoxycholate (TCA/DOC) Protein Precipitation and Quantitation. In order to concentrate and desalt depleted plasma samples, TCA/DOC precipitation was performed as previously described.[12] Briefly, sodium deoxycholate (final concentration: 125 μg/mL) was added to the samples and then vortexed for 15 min at room temperature (RT) before the addition of trichloroacetic acid (final concentration 6%). The samples were centrifuged for 10 min at 12000*g* at 4 °C. Ice cold acetone was added to wash the pellets, and samples were centrifuged again at 12000*g* for 5 min at 4 °C. The supernatants were discarded, and the dried pellets were resuspended in 50 μL of 6 M Urea in 100 mM Tris HCl (pH 7.8). Pierce BCA protein assay (Thermo Scientific, Basingstoke, U.K.) was used for protein quantitation following manufacturer's instructions.

SDS PAGE Electrophoresis

Equal amounts of proteins were separated onto a criterion XT Bis-Tris gel 4−12% using XT MES running buffer (Biorad, U.K.). Samples were loaded according to protein quantitation and run at 25 mA/gel in 1X SDS running buffer. After separation of the proteins, the gels were stained with Instant Blue (Expedeon Ltd., Harston, U.K.) for 10 min and transferred in distilled water for direct use or stored at 4 °C overnight.

In-Gel Digestion

Gel bands were cut using a sterile scalpel, further cut into 1−2 mm^2 gel pieces, and transferred to a 1.5 mL tube. The gel was covered in 200 μL of destaining solution (50% methanol, 5% acetic acid in Milli-Q-H_2O) and shaken at RT overnight. The destaining solution was changed regularly until all blue color had been removed. The gel pieces were dehydrated using 200 μL of acetonitrile for 5 min at RT and dried in a vacuum centrifuge for 2−3 min. Then 30 μL of 10 mM dithiothreitol (DTT) buffer was added to reduce for 30 min at RT before 30 μL of 50 mM iodoacetamide buffer was added to alkylate at RT for 30 min. Excess solution was removed before 200 μL of acetonitrile was added to dehydrate the gel pieces for 5 min at RT until white coloration appeared. The excess acetonitrile was removed, and the gel was further dried in a vacuum centrifuge for 2−3 min (or until completely dry). The trypsin reagent was prepared by adding 1 mL of ice-cold 50 mM ammonium bicarbonate to 20 μg of trypsin (final concentration 20 ng/μL) and keeping the solution on ice. Then a 30 μL trypsin solution (Promega) was added to the sample to rehydrate the gel pieces on ice for 10 min with gentle mixing. Gel pieces were spun down for 30 s, and the excess trypsin solution was removed. Five microliters of 50 mM ammonium bicarbonate were added to the gel pieces, and the digestion was carried out overnight at 37 °C with gentle mixing. Fifty millimolar ammonium bicarbonate, 450 μL of extraction buffer 1 (50% acetonitrile, 5% formic acid in Milli-Q-H_2O), and 50 μL of extraction buffer 2 (85% acetonitrile, 5% formic acid in Milli-Q-H_2O) were sequentially added to the gel pieces for 10 min incubation and transferred into the same 1.5 mL tube. After the last incubation in the extraction buffer, the sample was completely dried in a vacuum centrifuge and then resuspended in 20−40 μL of buffer A (98% Milli-Q-H_2O, 2% acetonitrile, 0.1% formic acid).

Mass Spectrometry Analysis

LC−MS/MS of peptides was performed using a Q-TOF 6520 (Agilent Technologies) equipped with a nanoLC-chip cube. The HPLC consisted of a nanoflow analytical and a capillary loading pump (Agilent 1200 series). Peptides were enriched and separated via nano-LC (0.075 × 150 mm, packed with Zorbax 300SB-C18, 5 μm material, 300 Å pore size) integrated in the HPLC Chip (G4240−62001). For each mass spectrometry experiment, peptides were loaded onto the enrichment column with 100% solvent A (2% acetonitrile with 0.1% formic acid). A two-step gradient generated at a flow rate of 0.6 μL/min was used for peptide elution. This included a linear gradient from 5 to 40% buffer B (95% acetonitrile with 0.1% formic acid) over 45 min followed by a sharp increase to 100% B within 10 min. The total run time, including column reconditioning, was 60 min. Full scan was acquired over a range of m/z 400−1700 at 5 spectra per sec and MS/MS over m/z 50−1700 at 2 spectra per sec selecting the six most abundant doubly or triply charged precursor ions per cycle. Spectra were deconvoluted and analyzed using Mass Hunter (v. 4.0) and Spectrum Mill software (Agilent). Auto-MS/MS was performed with a total cycle time of 3.3 s. Selected precursor masses were excluded for 0.9 min in order to avoid repeated sequencing.

MS/MS spectra generated above were extracted from the raw data in mzXML file format using Agilent trapper (version 4.3.0 Aug 2009) as conversion tool. The identification of proteins was performed using mzXML files with the central proteomics facilities pipeline (CPFP_OXFORD, version 1.3.0). A database including both *Plasmodium falciparum* 3D7 proteins (20 405 entries from UniProtKB/TrEMBL) and *Homo sapiens* (25 899 (reviewed) entries from UniProtKB) in FASTA format was constructed and uploaded into the CPFP for data analysis. This pipeline combines data from three search engines (Mascot, OMSSA and X!tandem k-score).[13] The search was carried out using the following parameters. Trypsin was the enzyme used for the digestion of the proteins, and only one missed cleavage was allowed. The accepted tolerance for the precursor was 50 ppm and 0.1 Da for the fragment. The search encompassed 1+, 2+ and 3+ charge state, fixed modification for cysteine carbamidomethyl and variable modification for asparagine and glutamine deamidation, and methionine oxidation. All trypsin fragments were added to an exclusion list. False discovery rate was calculated by peptide/proteinprophet or estimated empirically from decoy hits; identified proteins were filtered

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to an estimated 1% FDR.[13] Protein analysis of shotgun data was qualitative as parasite proteins were only identified in SM patients and not in MM or controls.

In order to confirm parasite protein identification, plasma samples from independent set of severe malaria patients were prepared as described above and analyzed in a Nano HPLC-LTQ Orbitrap Velos mass spectrometry (Thermo Scientific, USA). Orbitrap Velos has high resolution and high mass accuracy with fast scan speed and can identify parasite protein with high sequence coverage. Briefly, digested peptides were separated on a Proxeon Easy-nLC unit (Bruker Daltonik, Germany) using 100 μm-inner diameter × 15 cm C18 column with 90 min gradient of 2−42% solvent B (solvent A: 100% H_2O, 0.1% FA; solvent B: 100% ACN, 0.1% FA) at a flow rate 300 nL/min. MS scan was done on the Orbitrap with mass range: 300−1600 (m/z) and mass resolution: 60 000, analyzing top 10 peaks with exclusion list of 100 and a dynamic exclusion duration of 20 s. Fragmentation was recorded in CID mode in the Iontrap at collision energy 35 V with resolution 15 000 at m/z 400. The poly-dimethylcyclosiloxane ions generated in the electrospray process from ambient air (m/z = 445.120025) were used for internal recalibration in real time. Two technical replicates per sample were run in positive mode using 1 μg of peptides per sample per run. MS/MS spectra were extracted from raw files by Proteome Wizard MSConvert (Thermo) using the 200 most intense peaks in each spectrum and converted into MGF-format peak lists. The database searching for protein identification and quantitation was processed as described above.

Selective Reaction Monitoring (SRM) Validation Assays

Sample Preparation. Twelve microliters of plasma samples from 48 individual patients were delipidated and depleted from the 14 most abundant proteins (MARS Human-14, Agilent Technologies), precipitated, and quantified following the same protocol used for shotgun experiments.

Peptide Selection and Synthesis. For each protein, 3−5 proteotypic peptides (PTP) were selected on the basis of the compilation of the shotgun data from Q-TOF and LTQ-Velos Orbitrap on severe malaria cases in addition to bioinformatic prediction.[14] On the basis of the y-series fragment ions, we included 3−5 transitions for each of the two main charge states for each peptide. The predicted PTPs were synthesized (unpurified) with isotopically labeled amino acids either with a C-term Arg ($^{13}C_6$ $^{15}N_4$) or a C-term Lys ($^{13}C_6$ $^{15}N_2$) using SPOT synthesis (JPT Peptide Technology, Germany) and used as a reference to develop the corresponding SRM assays. The mass difference between the endogenous and the heavy labeled peptides for the 1+ fragment was either 8 or 10 Da. The stable isotope standard peptides were added prior to protein digestion and used as internal standards.

Purified heavy labeled peptides were synthesized on an APEX396 solid phase synthesizer (AAPPTec, Louisville, KY, USA). A 9-fluorenylmethyloxycarbonyl (Fmoc) strategy was used in the synthesis of the peptides. Initially, the first Fmoc amino acid was attached to an insoluble support resin via an acid labile linker. Deprotection of the Fmoc group was accomplished by treatment of the amino acid with 20% piperidine in dimethylformamide (DMF). The second Fmoc amino acid was coupled utilizing in situ activation of the amino acid to the growing peptide chain. After the desired peptide is synthesized, the resin bound peptide was deprotected and detached from the solid support via trifluoroacetic acid (TFA) cleavage. Peptides were precipitated with ether, dried and analyzed on a reversed phase HPLC column and MALDI-TOF mass spectrometer (Ultraflex, Bruker Daltonics, Germany).

Peptide SRM Standard Curve and Linear Range. The peptides were weighed with high precision scale, solubilized in 10% ACN and 0.5% FA, and dried in a speed vacuum centrifuge. Serial dilutions from a stock concentration of 23 ng/μL were produced for the peptides GILAADESTQTIK and VLVPNGVIK in 2% ACN and 0.1% FA.

For SRM assays, 2−3 peptides and 3−5 transitions per peptide were used to target the 4 proteins detected in the shotgun. Depleted plasma samples were spiked with a mixture of stable isotope standard (SIS) peptides in equimolar amount to get 109.1 fmol of each SIS peptide and 1 μg of plasma proteins in 1 μL. The mixture was reduced with 200 mM DTT in 100 mM Tris buffer, pH 7.8 for 30 min, alkylated with 200 mM iodoacetamide in 100 mM Tris buffer, pH 7.8 for 30 min followed by second 30 min incubation with DTT. The proteins were digested with sequencing grade modified trypsin (Promega) at 37 °C overnight using an enzyme to substrate ratio of 1:20. The digestion was stopped by adding 5% formic acid. Samples were desalted by Sep-Pak Light C18 cartridge (Waters), dried by speed vacuum, and resuspended in buffer A (2% ACN, 0.1% FA) to get a final concentration of 1 μg/μL for plasma sample and 109.1 fmol/μL for each heavy-labeled peptide. One microliter was injected on the Agilent 6460 Triple Quadrupole instrument coupled to an Agilent 1200 HPLC system equipped with a Chip-cube (Agilent Technologies).

Two separate SRM methods with 45 or 47 transitions each were used to obtain cycle times at 1.8 s and dwell-times at 35 ms. The full width at half height was 0.3 min. The optimal collision energy (CE) used for SRM assays was derived from empirical data of SIS peptide fragmentation in a 6520 Q-TOF instrument (Agilent Technologies). Q-TOF MS/MS data from SIS peptides was searched against a FASTA file that included (1) a concatenated sequence of all synthetic peptides, (2) *P. falciparum* 3D7, and (3) *Homo sapiens* proteomes to search MS/MS data.

Samples were analyzed on an Agilent 1200 Series HPLC-Chip (C18 chip with 160 nL trap column) using a 0.3 μL/min flow rate and a 60 min gradient (as for the shotgun experiment). Briefly, we used a linear gradient from 3 to 40% buffer B (95% ACN, 0.1% FA) for 46.67 min, followed by a sharp increase to 100% within 4 min, kept for 5.33 min followed by a linear gradient from 100 to 3% for 4 min to finally equilibrate the system for 9 min with 3% buffer B.

SRM Data Analysis

SRM data were analyzed using Skyline software (v. 1.2).[15] Relative quantitation was done using the area ratio (light/heavy), and for each peptide, only the 3 most intense transitions were considered. For each selected peptides, the most intense peak was used as quantifier, and the others were used as qualifiers to validate the ratio and the retention time of the peptide measured. Retention times and peak selection were initially performed automatically by the software and validated by inspection of individual chromatograms, and selection was based on the retention time obtained with the heavy labeled peptides. The result was an alignment of the endogenous with the heavy labeled peptides. In those cases where transitions for endogenous peptides were not well resolved (e.g., retention time mismatches), the analysis was discontinued.

Protein sequence alignment was performed using Clustal Omega and visualized in ClustalX v2.1.[16,17] The statistical analysis was done with GraphPad Prism 5 (GraphPad Software Inc., USA) and Stata 11 (StataCorp LP, USA).

The data associated with this manuscript may be downloaded from ProteomeCommons.org Tranche using the following hash: g2U4/T8lrkmHEk2ccjkb7CsFzu0+QP+nFeM/Mk26roLhy8sw54o9a1iSDPFqYtnCnpAyZ1SSaZeAImUIvBVfJzFQS0oAAAAAAA7cow==.

RESULTS

Identification of *P. falciparum* Proteins Using Plasma Shotgun Proteomics

We used pooled plasma samples from Gambian children with severe malaria (N = 20), mild malaria (N = 20), and convalescent controls (28 days after treatment) (N = 20) to identify *P. falciparum* proteins associated with disease severity (Table 1). With a shotgun proteomic strategy, we identified 8905 unique peptides corresponding to 322 proteins (calculated "a posteriori" FDR of 2.33%) in 78 LC−MS/MS of in-gel digested plasma samples (13 fractions per group were run in duplicates). Of these, 257 host proteins were identified in children with severe malaria, 188 in children with mild malaria, and 203 in convalescent cases. In severe malaria cases, 65 *P. falciparum* proteins were identified (Table S3, Supporting Information). Four of these proteins were unequivocally assigned to *P. falciparum* (Table 2), namely, PF11_0208 (pPGM), PF10_0121 (pHPRT), PF14_0425 (pFBPA) and PF13_0141 (pLDH) in severe malaria samples but not in mild or convalescent samples. The identification of *P. falciparum* proteins in the shotgun experiment was based on at least 2 unique parasite peptides.

Selected Reaction Monitoring Assays to Quantify *P. falciparum* Proteins

To validate and quantify *P. falciparum* proteins identified in the shotgun experiment, we used SRM assays. We initially selected 12 proteotypic peptides, 3 for each of the parasite proteins identified (pPGM, pHPRT, pFBPA and pLDH) (Table 2). SRM-based quantification was performed only for those cases where both the heavy-labeled and the endogenous peptide consistently showed identical retention times and at least 3 transitions (n = 45). These criteria were met by 5 proteotypic peptides (2 for pFBPA and one for each of the other proteins).

The transitions detected for each peptide are shown in Figure 2. The SIS and the endogenous peptides were eluted at the same retention time. In the majority of cases, the intensity of the precursor was higher for the SIS peptide (with the exception of TTTQDVGFLTVR). However, despite lower intensity, the number of transitions detected with endogenous peptides was higher than those of spiked peptides. Peptides were detected for all the proteins and in most cases analyzed. The area of the transition with the highest intensity (quantifier) of the endogenous peptide (light) and the corresponding area of the heavy-labeled peptide (spiked) were used to calculate the ratios to perform relative quantitation of the peptides in individual samples. A total of 167 SRM measurements were performed in individual plasma samples (41 in controls, 62 in mild and 64 in severe malaria cases). pPGM was measured with FTGWTDVPLSEK peptide and could be quantified in 36 patients, pHPRT with VLVPNGVIK in 28 patients, pFBPA with GILAADESTQTIK and TTTQDVGFLTVR in 33 and 36 patients, respectively, and pLDH with IIGLGGVLDTSR in 34 patients.

Table 2. Parasite Proteins Identified in the Shotgun Proteomic Study of Plasma Samples from Gambian Children with Severe Malaria[a]

protein	peptides charge/sequence	probability	no. of instances
pLDH	2 LISDAELEAIFDR	0.9998	2
	2 NLGDVVLFDIVK	0.9997	2
	2 IIGLGGVLDTSR	0.9724	1
	3 DVNAHIVGAHGNK	0.9685	2
	Max sequence coverage (%): 15.8		
PGM	2 HYGSLQGLNK	0.9999	2
	3 **FTGWTDVPLSEK**GEEEAIAAGK	0.9999	4
	3 TADLLHVPVVK	0.9999	4
	3 HGESTWNKENK	0.9995	2
	3 VLPFWFDHIAPDILANKK	0.9995	2
	2 AICTAWNVLK	0.9975	1
	2 KYGEEQVK	0.9815	1
	4 VLPFWFDHIAPDILANKK	0.9171	1
	2 YGEEQVK	0.0618	1
	Max sequence coverage (%): 49.2		
FBPA	2 ALQASVLNTWQGK	0.9999	4
	2 FISGAILFEETLFQK	0.9999	4
	2 KLPADVAEELATTAQK	0.9999	3
	2 **TTTQDVGFLTVR**	0.9999	5
	2 YASICQQNR	0.9999	5
	3 KLPADVAEELATTAQK	0.9999	4
	3 GKPTDLSIHETAWGLAR	0.9993	4
	2 LENTIENR	0.999	4
	2 **GILAADESTQTIKK**	0.9972	1
	2 DLLFGTK	0.9945	4
	2 STQGLDGLAER	0.9894	1
	2 VLSCVFK	0.9063	1
	3 YKGGAGGENAGASLYEK	0.8826	2
	3 LVPIVEPEILADGPHSIEVCAVVTQK	0.7831	2
	3 GLVNIPCTDEEKSTQGLDGLAER	0.7808	2
	Max sequence coverage (%): 49.9		
HPRT	2 GFFTALLK	0.9999	2
	2 HVLIVEDIIDTGK	0.9999	2
	3 IHNYSAVETSKPLFGEHYVR	0.9999	2
	2 VLVPNGVIK	0.9994	2
	2 DLDHCCLVNDEGK	0.9985	1
	3 DLDHCCLVNDEGK	0.9984	2
	2 LAYDIKK	0.9902	1
	Max sequence coverage (%): 30.3		

[a]In bold, peptides selected for quantification.

The uniqueness criterion used for PTP selection of *P. falciparum* proteins was based on differences with host protein sequence (*Homo sapiens*) but not with other parasite species that may cause human malaria. The sequence alignment indicates that peptides selected to quantify *P. falciparum* PGM, HPRT and FBPA (TTTQDVGFLTVR) were species specific. The peptide measured for pLDH was identical in all human malaria species (Figure 3).

P. falciparum Protein Validation in Plasma and Clinical Correlates

Using SRM, the parasite peptides were detected mostly in plasma from severe malaria cases but were also found in plasma samples from mild malaria and convalescent cases. The SRM assays for pFBPA indicated a significantly higher concentration

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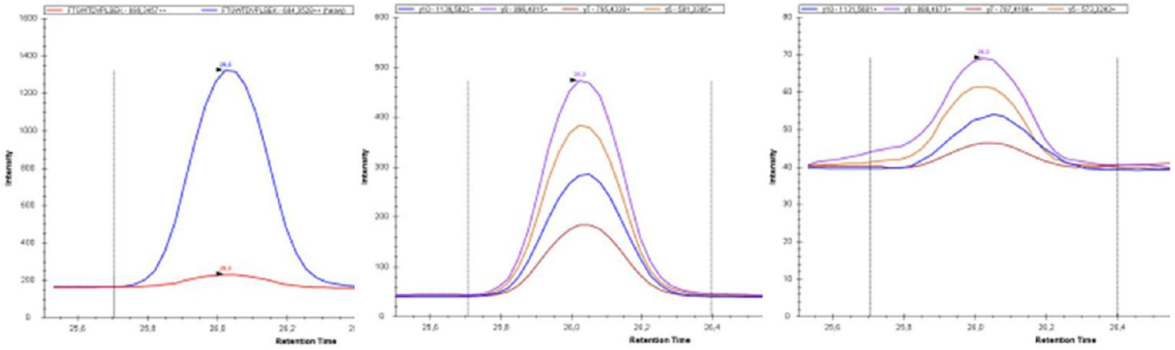
(a) **PF11_0208 Phosphoglycerate Mutase**

Percent Coverage: 49.2%

MTTYTLVLLR HGESTWNKEN KFTGWTDVPL SEKGEEEAIA AGKYLKEKNF KFDVVYTSVL KRAICTAWNV LKTADLLHVP
VVKTWRLNER HYGSLQGLNK SETAKKYGEE QVKIWRRSYD IPPPKLDKED NRWPGHNVVY KNVPKDALPF TECLKDTVER
VLPFWFDHIA PDILANKKVM VAAHGNSLRG LVKHLDNLSE ADVLELNIPT GVPLVYELDE NLKPIKHYYL LDSEELKKKM
DEVANQGKAK

PF11_0208 FTGWTDVPLSEK



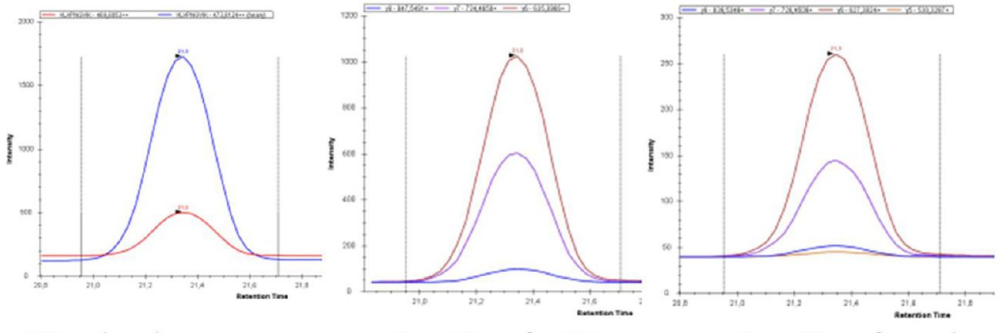
SIS and endogenous peptides | Transitions for SIS peptides | Transitions for endogenous peptides

(b) **PF10_0121: Hypoxanthine phosphoribosyltransferase**

Percent Coverage: 30.3%

MPIPNNPGAG ENAFDPVFVK DDDGYDLDSF MIPAHYKKYL TKVLVPNGVI KNRIEKLAYD IKKVYNNEEF HILCLLKGSR
GFFTALLKHL SRIHNYSAVE TSKPLFGEHY VRVKSYCNDQ STGTLEIVSE DLSCLKGKHV LIVEDIIDTG KTLVKFCEYL
KKFEIKTVAI ACLFIKRTPL WNGFKADFVG FSIPDHFVVG YSLDYNEIFR DLDHCCLVND EGKKKYKATS L

PF10_0121 VLVPNGVIK



SIS and endogenous peptides | Transitions for SIS peptides | Transitions for endogenous peptides

Figure 2. continued

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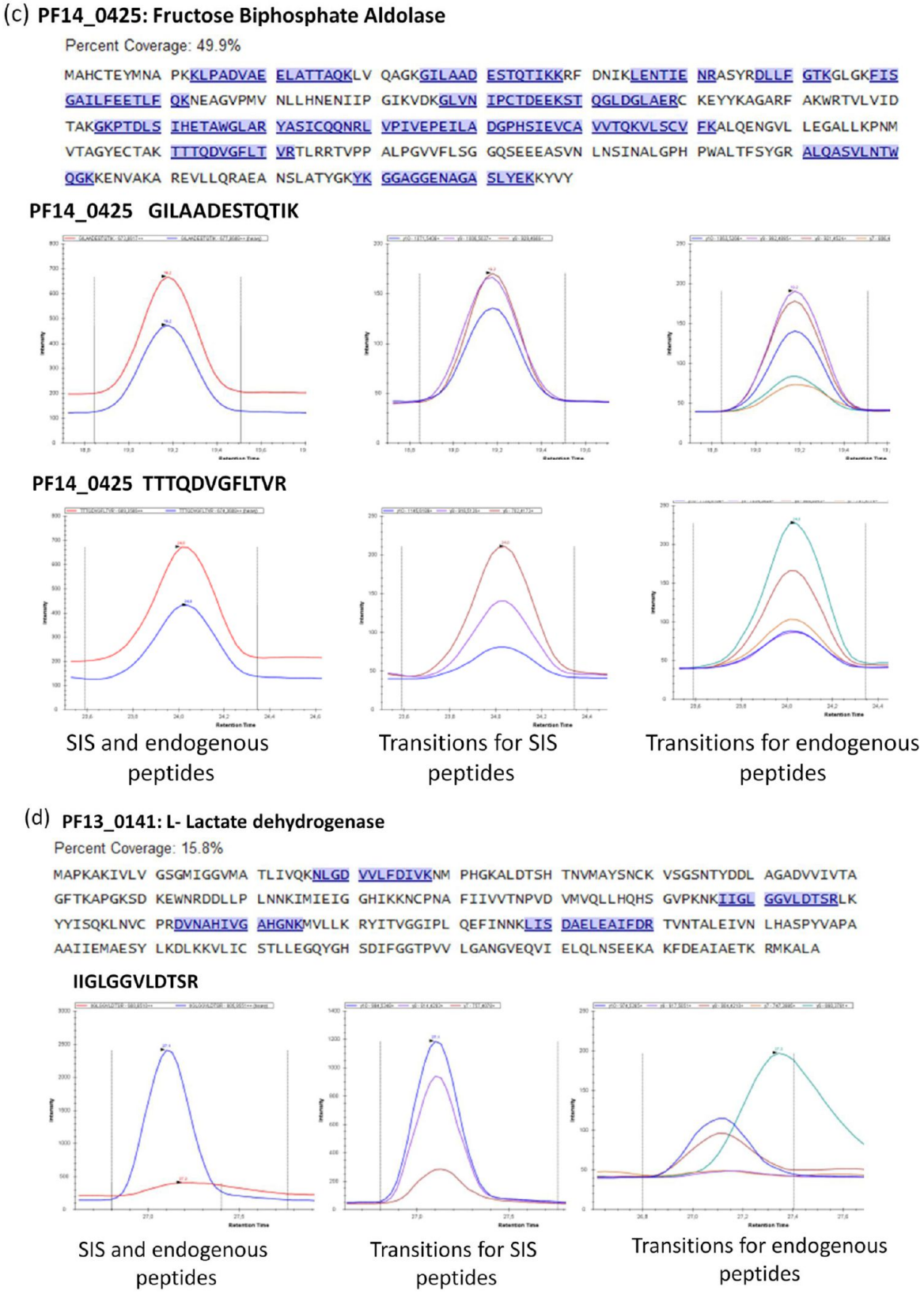

Figure 2. *P. falciparum* proteins identified in shotgun analysis of 20 pooled plasma samples from Gambian children with severe malaria. The top panel shows protein sequence coverage and peptide sequences used to identify parasite proteins with high confidence (highlighted in purple). The lower panel shows a representative optimized SRM assay for selected proteotypic peptides. The left panel shows the relative abundance of heavy-labeled stable isotopic peptides (SIS) versus endogenous peptides in plasma. The middle panel shows intensity of the transitions measured for SIS peptides in plasma, and the right panel shows the intensity of endogenous transitions measured in the plasma of a representative individual.

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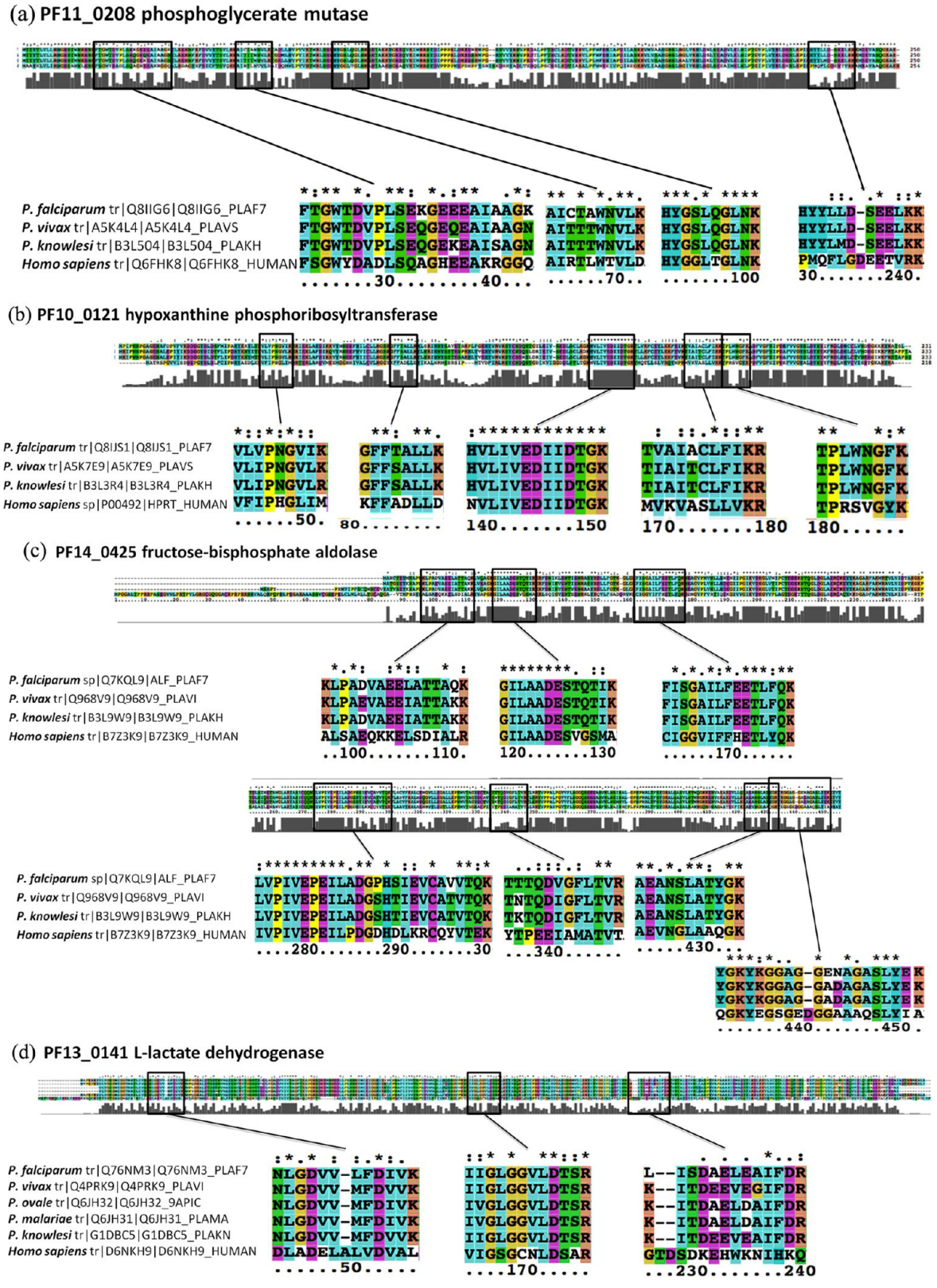

Figure 3. Sequence alignment of *Plasmodium falciparum* proteins identified in shotgun experiments and selected proteotypic peptides and corresponding protein sequences in *Homo sapiens*. Data show protein alignment for parasites that may cause human malaria (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*) and *Homo sapiens*.

of this protein in SM cases as compared to MM cases. A similar trend was observed for pHPRT and pLDH. This association was of borderline significance in pHPRT (P = 0.08) and not significant for pLDH (P = 0.17). pPGM was higher in acute malaria cases compared with convalescent controls, but we did not observe any differences between SM and MM cases (Figure 4).

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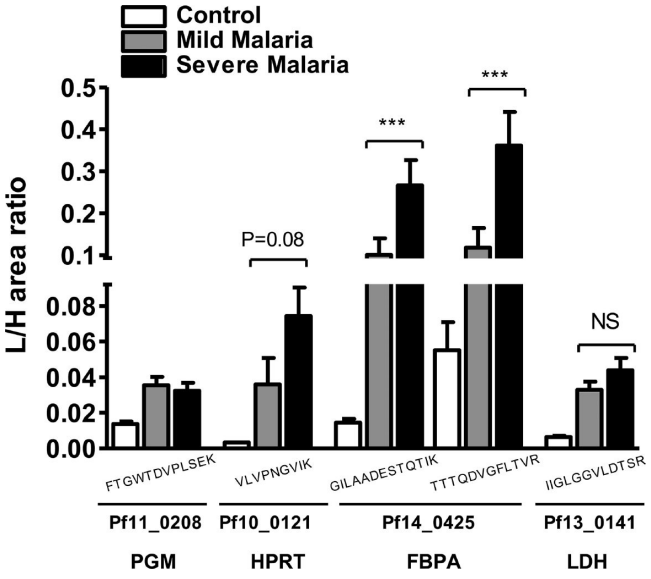

Figure 4. Quantification of *P. falciparum* proteotypic peptides (PTPs) using SRM assays. The bars indicate the mean ratio and standard error of severe (black) and mild cases (gray) and convalescent control samples (28 days after infection, white) calculated for 5 PTPs derived from 4 *P. falciparum* proteins, PGM, HPRT, FBPA, and LDH. Abundance of PTPs was measured by comparing the area of light peptide peaks (endogenous) to the area of heavy labeled peptide peaks. Independent *t*-test was used to test for statistically significant differences between severe and mild cases. *** $P < 0.001$, and NS: nonsignificant.

We reasoned that the differences observed in the quantity of *P. falciparum* protein detected with SRM assays in MM cases and SM cases were likely to be explained by differences in the number of parasites in blood in these patients or by the release of antigen proteins following *P. falciparum* infected erythrocyte rupture. We therefore correlated (Spearman's rho) the light-to-heavy ratio of individual assays with the *P. falciparum* parasite density measured by light microscopy and blood hemoglobin (a measure of erythrocyte destruction) in these patients. Indeed, we found that pFBPA and pLDH significantly correlated with parasite density. However, we did not observe a significant correlation with pPGM or pHPRT. Interestingly, hemoglobin concentration markedly correlated with peptide quantification in all SRM assays with the exception of pPGM (Figure 5).

DISCUSSION

A fast and reliable system to diagnose human malaria remains a major diagnostic challenge in most developing countries. We analyzed plasma samples from 60 children with severe or mild malaria and convalescent controls using a shotgun proteomic strategy and identified four *P. falciparum* proteins only in the severe malaria cases. These proteins were then validated in individual samples using a targeted proteomic strategy. The concentration of pPGM, pHPRT, pFBPA and pLDH measured by SRM assays indicated a higher concentration of these proteins in patients with *P. falciparum* malaria than in controls (Figure 4). MS-based quantification of the parasite proteins pLDH and pFBPA showed a significant correlation with parasite density and hemoglobin concentration in these patients. pHPRT did show a good correlation with anemia and the lowest concentration 28-days after treatment (Figure 5). The use of pHPRT as parasite-based diagnostic to identify malaria patients should be further investigated and validated prospectively.

The use of RDTs to identify patients that should receive malaria treatment is an important area of research, and new antigen targets are required to deal with the limitations of existing tests. Currently, most RDTs available to diagnose malaria cases are based on the detection of three parasite proteins, namely, HRP2, LDH and aldolase (nonspecies specific).[18] PfHRP2 tests can detect >40−100 parasites/μL and pLDH >100−200 parasites/μL in approximately 20 min at an approximate cost ranging from $1.20 to 13.50 USD. However, these tests have limitations. For example, the PfHRP2 antigen may persist in blood after treatment with antimalarials up to 33 days, which invalidates the use of this test to monitor treatment success.[19] Similarly, the identification of PfHRP-2 in current and recent infections limits the prognostic value of PfHRP-2 in malaria-endemic areas with high transmission intensity.[20] Moreover, parasite populations with deletions in the histidine rich repeat region of the *hrp2* gene have been documented.[21] pLDH does not persist in blood, but *P. falciparum* gametocytes, the sexual stage of malaria parasites that does not cause acute infection, produce pLDH, and this may produce a positive test despite clearance of the asexual parasite forms that have caused the acute infection. In comparison, an experienced microscopist may detect infection densities as low as 5−10 parasites/μL in approximately the same time, distinguish parasite species, and identify sexual from asexual forms at 10% of the cost of an RDT test (<0.50 USD). We have identified four proteins in a plasma shotgun proteomic study: two of these proteins (pFBPA and pLDH) corroborate the utility of commercially available RDTs, whereas pHPRT and pPGM have not been reported previously. Peptides derived from pPGM and pLDH were conserved across *Plasmodium* species but not shared with host proteins.

MS-based quantitation methods using plasma from patients is limited by the many practical difficulties imposed by the high complexity of the plasma proteome. Indeed, its use as a source of diagnostic information has advantages and limitations. First, plasma contains tissue leakage proteins that can be used to diagnose and monitor treatment. Also, blood plasma is the most frequently sampled clinical specimen, and thus relevant findings in plasma can be readily validated and incorporated into clinical practice. On the other hand, the concentration of molecules that define the plasma proteome ranges from highly abundant proteins such as albumin (mg/mL) to less abundant cytokines and tissue leakage proteins (ng to pg/mL). Therefore, the biggest challenge in this study was to identify peptides/proteins across this wide dynamic range (~10 orders of magnitude).[22] Previous studies in children with severe malaria indicate that the median concentration of *P. falciparum* HRP-2 in plasma was 63 ng/mL in Kenyan children[23] and 286 ng/mL in Gambian children.[11] In this study we have measured comparable levels of parasite proteins in plasma using SRM assays (pHPRT, Figure S1, Supporting Information). The identification/quantification of low abundance proteins (at mid-to-low ng/mL concentration) using MS-based platforms requires additional depletion fractionation strategies, particularly in plasma samples where proteins have to be quantified across 10 orders of magnitude. In this context, the depletion of the most abundant plasma proteins (top-14 depletion) and sample fractionation using SDS-PAGE was sufficient to identify parasite proteins with a high degree of confidence in severe malaria cases. However, with this method we could not identify

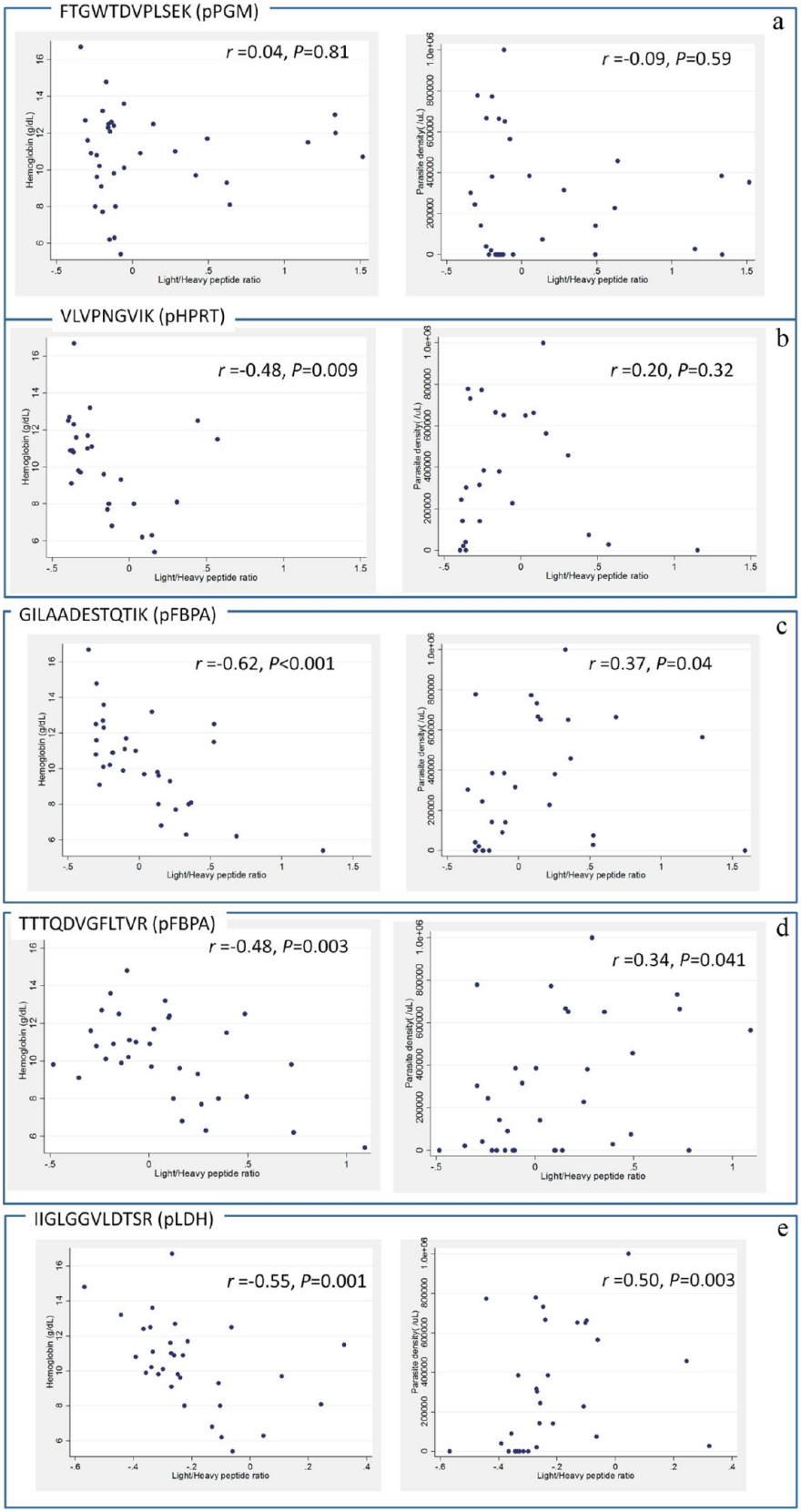

Figure 5. Correlation of *P. falciparum* parasite density (the number of parasites/μL of blood) and hemoglobin (g/dL) concentration with *P. falciparum* proteotypic peptides (PTPs) abundance. PTPs abundance was measured as ratio of light peptide peak area (endogenous) to heavy labeled peptide peak area (spiked). Pearson's correlation analysis was used to measure the association and significance of the MS measurements with parasite density and hemoglobin concentration. A p-value < 0.05 was considered statistically significant.

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P. falciparum proteins in mild or convalescent cases. The lower sensitivity of the Q-TOF measurement in combination with a "diluting" effect associated with sample pooling may partially explain this discrepancy.

Severe anemia is an important clinical feature of falciparum malaria.[24] Indeed, *P. falciparum* parasitemia progresses through sequential cycles of erythrocytic invasion and destruction, which results in lower blood hemoglobin concentration and eventual compromise of oxygen delivery to peripheral tissues. pLDH and pFBPA proteins were higher in severe than mild malaria cases and showed a good correlation with parasite density. Interestingly, SRM measurements showed a remarkable linearity with hemoglobin concentration, indicating that a higher concentration of parasite proteins was found in those cases with the highest level of erythrocyte lysis (lower hemoglobin concentration). It is beyond the scope of this study to prove any pathogenic associations between the proteins identified and the development of anemia. However, the correlation of parasite proteins with severity of disease may reflect frequent (or faster) cycles of parasite multiplication and erythrocyte destruction. More importantly, these clinical correlations provide strong evidence to support the value of SRM assays for quantification purposes in clinical samples.

The development of new enzyme immunoassays (EIA) to validate candidate biomarkers in clinical samples requires a considerable investment of time and resources and depends on the immunogenicity of the candidate protein. Targeted proteomics has developed as a suitable alternative methodology to immunoassays for protein quantification.[25−27] Although independent biochemical confirmation of candidate biomarkers is the method of choice for validation, the possibility of measuring proteins in clinical samples independently of antibody availability is particularly attractive for pathogen-oriented research.

■ CONCLUSIONS

The development of faster and more sensitive mass spectrometers and better methods to efficiently reduce sample complexity will allow a higher coverage of the dynamic range and facilitate the analysis of the deep proteome.[28,29] With this methodology we have identified and validated a novel biomarker pHPRT to diagnose acute *P. falciparum* infection and provided further evidence to support the potential role of SRM assays for pathogen-oriented proteomic research. We anticipate that faster and more sensitive platforms will maximize the promising clinical impact of this methodology. The use of pHPRT to diagnose acute malaria should be validated prospectively.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1: Absolute quantification and linear range of the *P. falciparum* protein pHPRT based on VLVPNGVIK purified peptide. Table S1: Optimized SRM method for quantification *P. falciparum* proteins. Table S2: *P. falciparum* protein/peptides identified in shotgun proteomic experiments. Table S3: *P. falciparum* proteins identified in plasma samples from children with severe malaria with only 1 or more peptides but with shared sequence with human proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ccasals@well.ox.ac.uk. Phone: +0044(0)1865 287784. Fax: +0044 (0)1865 287787.

Author Contributions

▽These authors contributed equally to the manuscript.

Notes

The authors declare the following competing financial interest(s): The University of Oxford (through ISIS Ltd) has filed a patent on pHPRT to diagnose malaria..

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