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A Dual, Systematic Approach to Malaria Diagnostic Biomarker Discovery

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Background. The emergence and spread of *Plasmodium falciparum* parasites that lack HRP2/3 proteins and the resulting decreased utility of HRP2-based malaria rapid diagnostic tests (RDTs) prompted the World Health Organization and other global health stakeholders to prioritize the discovery of novel diagnostic biomarkers for malaria.

Methods. To address this pressing need, we adopted a dual, systematic approach by conducting a systematic review of the literature for publications on diagnostic biomarkers for uncomplicated malaria and a systematic in silico analysis of *P. falciparum* proteomics data for *Plasmodium* proteins with favorable diagnostic features.

Results. Our complementary analyses led us to 2 novel malaria diagnostic biomarkers compatible for use in an RDT format: glyceraldehyde 3-phosphate dehydrogenase and dihydrofolate reductase-thymidylate synthase.

Conclusions. Overall, our results pave the way for the development of next-generation malaria RDTs based on new antigens by identifying 2 lead candidates with favorable diagnostic features and partially de-risked product development prospects.

Keywords. malaria; diagnostics; biomarker; GAPDH; DHFR-TS.

For most of the 20th century, microscopy, at best, but often clinical diagnosis alone without parasitological confirmation remained the sole methods for diagnosing malaria. This inertia in the development of novel malaria diagnostics finally ended in 1991 with the characterization of histidine-rich protein 2 (HRP2) as a diagnostic biomarker for malaria [1]. The global diagnostics market has since become flooded with a variety of biomarker-based rapid diagnostic tests (RDTs).

The World Health Organization (WHO) Prequalification of In Vitro Diagnostics (IVDs) Program assesses the safety and performance of malaria diagnostics to determine their suitability for use in resource-limited settings. Malaria RDTs currently on the WHO list of prequalified IVDs rely on the detection of HRP2 and lactate dehydrogenase (LDH) [2]. The only US Food and Drug Administration–approved RDT for malaria (BinaxNOW Malaria) detects a pan-malarial antigen, aldolase, in addition to HRP2 [3]. HRP2 is a heat-stable, *Plasmodium falciparum*-specific malarial protein excreted in high abundance by the parasite throughout different stages of its life cycle in human blood [4]. *Plasmodium* LDH (pLDH) is an essential enzyme highly conserved among human-infecting *Plasmodium* species [4]. While species-specific detection of *P. falciparum* can be achieved using either pLDH- or HRP2-based RDTs, the latter are preferred for their lower limit of detection (LoD) and greater heat stability [5, 6]. The only quality-assured RDTs available for the detection of non-*falciparum* species target pLDH, either in a pan or species-specific manner. However, the limited analytical sensitivity of pLDH-based products restrains their efficient detection [4, 6–9]. Similarly, RDTs based on aldolase show highly variable clinical performance in detecting *Plasmodium* parasites in malaria-endemic settings [10, 11].

The widespread use of HRP2-based tests has also revealed their shortcomings [4, 12]. HRP2 contains multiple tandemly repeating short amino acid sequences that are recognized by monoclonal antibodies used in HRP2-based RDTs. The presence of such repeats helps improve the clinical sensitivity of the tests but possibly contributes to global variability in performance due to the high variation in these sequences [12–15]. Most importantly, HRP2 is not essential for *P. falciparum* growth, as shown by laboratory-based culture experiments [12, 16–20]. The first report of *P. falciparum* clinical isolates with *hrp2* deletions from the Amazon region in 2010 was, therefore, not surprising but rather troubling due to its potential impact on the utility of HRP2-based tests

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for case management [21]. The gradual spread of *hrp2*-deleted mutants in South America, Asia, and Africa has called into question our almost exclusive reliance on HRP2-based tests for *P. falciparum* detection [22–27]. These tests could potentially be substituted by pLDH-based tests but at the cost of lower sensitivity because efforts to match the LoD of pLDH-based tests with that of HRP2-based RDTs have fallen short to date.

Therefore, innovative malaria RDTs that can provide similar or improved levels of performance to those currently used are now a key focus of any road maps to malaria control and elimination [5, 28, 29]. Identification of novel diagnostic biomarkers for malaria is a sensible approach to lend impetus to the ongoing innovation efforts.

To identify novel biomarkers for malaria diagnosis, we adopted a 2-pronged, complementary approach: a systematic review of published evidence on nontraditional malaria biomarkers as well as an interrogation of *Plasmodium* proteomic databases to identify potential *P. falciparum* antigens that may constitute suitable diagnostic targets. Here, we present the findings of this comprehensive dual approach and suggest research and development starting points that could rapidly lead to innovation in the field of malaria RDTs.

METHODS

Systematic Review of Malaria Diagnostic Biomarkers

Systematic Review Protocol

A systematic review protocol was developed prior to searching databases and is registered in PROSPERO (PROSPERO 2019 CRD42019126038).

Searched Databases

A systematic approach, based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines, was used to search the following databases: Medline (PubMed), Web of Science, EMBASE, the Cochrane Central Register of Controlled Trials, and Latin America and Caribbean Health Sciences Literature. The search terms used are shown in Supplementary Table 1. These search terms were adapted as necessary for the other databases. Searches were carried out during February 2019. See Supplementary Methods for further details.

In Silico Analysis of *P. falciparum* Proteomics Data

PlasmoDB Data Access and Data Reduction

Proteomics databases were reviewed to identify potential antigen candidates for malaria diagnosis. Data for *P. falciparum* were downloaded from PlasmoDB [30] using an "Organism" search for *P. falciparum*. Records lacking a gene name were omitted. Data were also obtained for 4 additional species (*Plasmodium vivax*, *Plasmodium knowlesi*, *Plasmodium malariae*, and *Plasmodium ovale*), and comparisons were made to identify common gene names. See Supplementary Methods for further details.

RESULTS

Malaria Biomarkers Systematic Review

The initial search identified 3914 publications (Figure 1). Following the sequential screening of titles, abstracts, and full texts by 2 independent reviewers, 88 publications reporting on 98 unique biomarkers or biomarker signatures were included in the review.

The 98 biomarker candidates were classified into 4 major biomarker categories: host origin (N = 55), parasite origin (N = 39), mechanical (N = 3), or proxy (N = 1; Figure 2). Cytokines, chemokines, and other proteins comprised most biomarkers of host origin. All but 5 biomarkers of parasite origin were proteins.

To comprehensively examine the status of the malaria biomarker pipeline, we adopted an existing framework to validate biomarkers under development targeted for specific use cases [31]. The framework allows an assessment of the level of evidence regarding the diagnostic value of a given candidate vs its deployability at different levels of a health system, given the method used to detect the candidate of interest in its current state. This revealed that most diagnostic biomarker candidates for malaria are at an early stage of development (Figure 2). Few candidates were found to be usable at the lower levels of a health system at their current stage of development. For instance, topoisomerase I activity was a valuable measure of malaria infection in saliva samples when tested in a lateral flow assay (LFA); however, it is still at an early stage of development, requiring validation of its diagnostic value [32]. Similarly, TPx-1, a well-conserved enzyme from the antioxidant family, was identified as a potential diagnostic biomarker for malaria when measured in culture isolates using a proof-of-concept LFA [33].

Ninety-three of the 98 candidates identified in the review were reported in just 1 publication (Supplementary Figure 1). C-reactive protein was the most studied biomarker, with 10 publications. Additionally, most biomarkers targeted *P. falciparum* single infection or coinfection with other species (Supplementary Figure 2).

According to the summarized results for study quality and risk of bias based on a revised version of QUADAS (Quality Assessment of Diagnostic Accuracy Studies)-2, the majority of 26 validation studies showed a high risk of bias in terms of study design, reference standard chosen, and recruitment timing (Supplementary Figure 3).

We focused on parasite proteins for further analysis, as they are the easiest to detect in terms of reagent development, assay format, infrastructure requirement, and level of training entailed [34]. Thirty publications reported

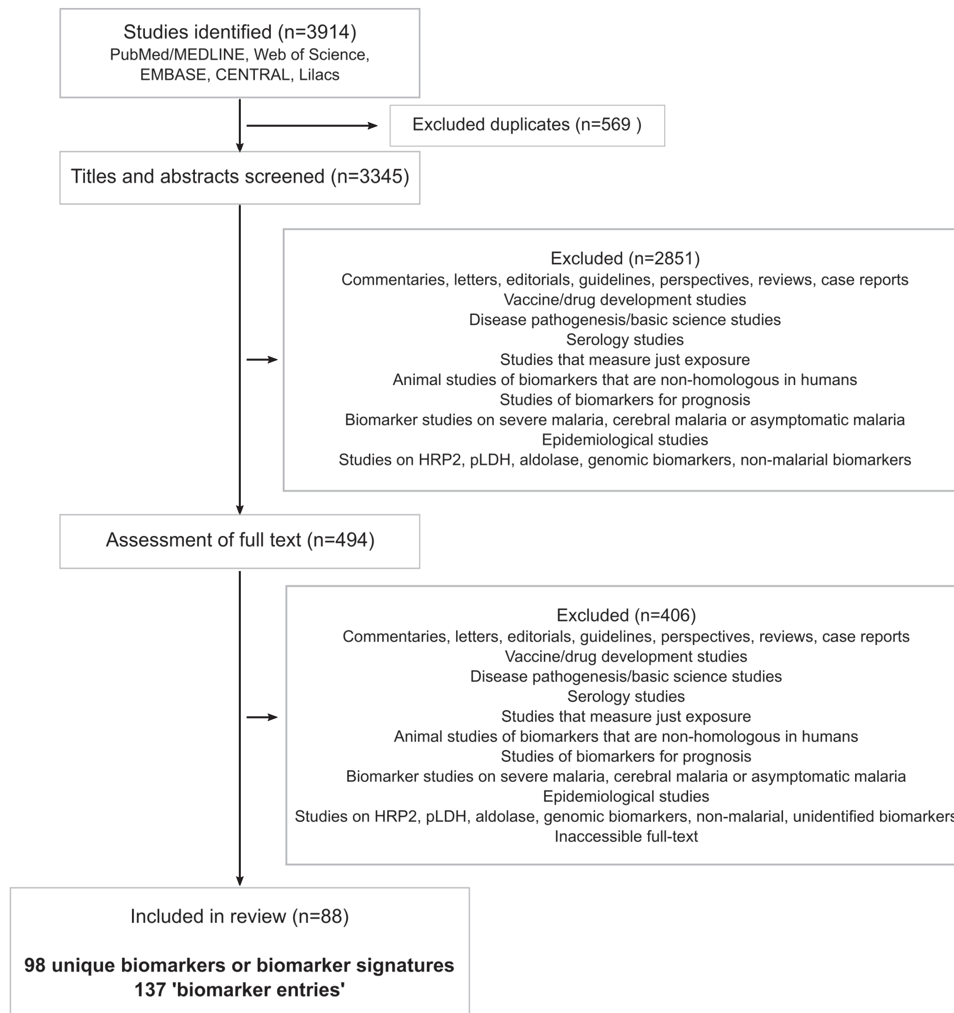


Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow diagram. Abbreviations: CENTRAL, Cochrane Central Register of Controlled Trials; LILAC, Latin America and Caribbean Health Sciences Literature; pLDH, *Plasmodium* lactate dehydrogenase.

on 30 individual parasite proteins and 3 biomarker signatures comprising multiple parasite proteins [28, 29, 32, 33, 35–40, 41–60]. Detecting multiple targets in an RDT format is complex; therefore, we excluded biomarker signatures from further analysis and focused on individual proteins, evaluating their potential as diagnostic biomarkers for malaria (Table 1). Eleven candidates targeted *P. falciparum*, 10 *P. vivax*, 2 *P. knowlesi*, and 2 pan. Phosphoethanolamine N-methyltransferase (PMT) was evaluated for its ability to indicate *P. falciparum*, *P. vivax*, and *P. knowlesi* [37]. Aldolase (FBPA) made it onto the list of parasite proteins since the article we included in our review identified a *P. vivax*-specific version of this biomarker [47]. Nonetheless, most of these reports are early-stage discovery studies; clinical diagnostic performance data are limited (Supplementary Figure 4). Overall, the reported diagnostic performance of 5 candidates (CelTOS, FBPA, HSPATR, MSP-1, and MSP-3) showed high specificity but varying sensitivity [35, 41, 43, 44, 47, 48, 59].

Our review of malaria biomarkers summarized available evidence on novel biomarker candidates for malaria diagnosis but also revealed that few parasite proteins reported were selected as a result of an unbiased approach. Exceptions were mature erythrocyte surface antigen, glutamate-rich protein, and the *P. vivax*-specific proteins PVX_110940, PVX_083555, PVX_090265, PVX_003545, and PVX_094303, identified using high-throughput analysis techniques, for example, mass spectrometry [36, 39, 42, 61]. These studies failed to describe the intrinsic diagnostic features of the identified candidates. Therefore, we performed an in silico analysis of available *P. falciparum* proteomics data to evaluate the diagnostic value of candidates identified in the review and to identify additional candidates of interest.

IN SILICO ANALYSIS OF *P. FALCIPARUM* PROTEOMICS DATA

An "Organism" search of PlasmoDB for *P. falciparum* yielded 89 841 genes across 16 strains; 64 457 genes with no protein

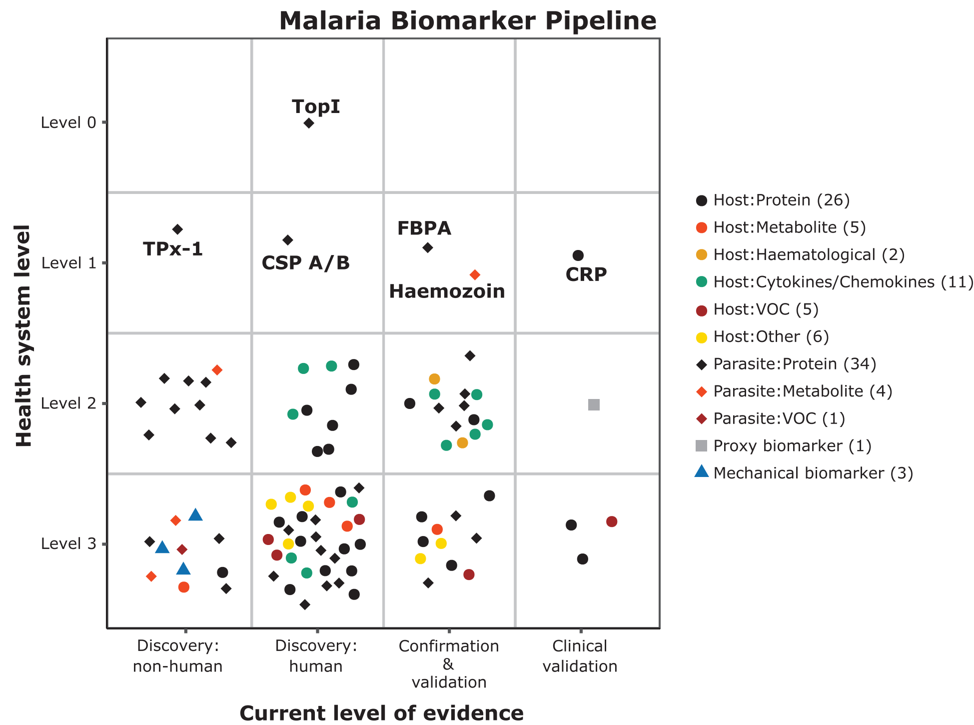


Figure 2. Malaria biomarker development pipeline. Level 0, community (prerequisite: noninvasive tests, no blood draw, eg, urine rapid diagnostic tests [RDTs], saliva RDTs); level 1, health clinic/post (prerequisite: blood draw but with easy-to-use/basic tests that can be done in health centers, eg, finger-prick blood–based RDT, microscopy); level 2, peripheral laboratory (prerequisite: blood draw with/without the use of plasma and advanced equipment/complex tests, eg, enzyme-linked immunosorbent assay, Western blot); level 3, centralized reference laboratory/hospital (prerequisite: highly advanced equipment/complex tests, eg, mass spectrometry, cytometry, suspension array). Abbreviations: CRP, C-reactive protein; CSP, circumsporozoite protein; FBPA, aldolase; TopI, topoisomerase I; Tpx-1, thioredoxin peroxidase 1; VOC, volatile organic compounds.

sequence or no gene name but a protein sequence were excluded from further analysis as they were likely inferred from hypothetical genes. This dataset was further reduced by limiting the search to *P. falciparum* 3D7 (Pf 3D7), yielding 2380 genes. A filtering cascade was established to identify *P. falciparum* proteins associated with favorable diagnostic features (Figure 3). In the absence of data on the subcellular localization of the protein products of these genes in the parasite, the data were further filtered to select those whose human orthologue, when available, has a nonnuclear localization in the cell; this resulted in a list of 30 proteins, 29 excluding pLDH.

We identified a human orthologue for 24 candidates, with percent identity between *P. falciparum* and human orthologues ranging from 24% to 72%. Sixteen proteins were expressed during both the asexual stage and the sexual stage of the *P. falciparum* life cycle; 13 were specific to the asexual stage, opening the possibility for stage-specific markers. Single-nucleotide polymorphisms (SNPs), which are indicative of genetic diversity, in the 29 candidates ranged from 1 to 140, with most genes (21 of 29) showing less than 22.5 (the median number of SNPs across 2380 Pf 3D7 genes). We also identified potential immunogenic epitopes in 8 of the proteins. Through reference to the iSP-RAAC web server, 4 of the proteins were characterized as secretory (PF3D7_1029600, PF3D7_1110700, PF3D7_1401800, and PF3D7_1211700) [62].

We evaluated the diagnostic utility of the 26 biomarkers identified in the systematic review with respect to the filtering cascade (Figure 3; Supplementary File 2). Only 2 candidates, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and dihydrofolate reductase-thymidylate synthase (DHFR-TS), were found to meet all of the criteria established. In addition, PMT met all but 1: expression by all human-infecting *Plasmodium* species. A *P. malariae* orthologue appears to be lacking in PlasmoDB, even though a putative orthologue is predicted to exist [37]. The studies describing these 3 candidates were classified as early (nonhuman) discovery studies with a level 2 deployability based on the enzyme-linked immunosorbent assay (ELISA) used for detection [28, 37, 51]. Conversely, merozoite surface protein 1 and rhoptry protein 3 possessed all of the favorable diagnostic features but did not reach the ring-stage abundance threshold established. Thioredoxin peroxidase 1 (Tpx-1 or Trx-Px1) failed to meet the established criteria, but other enzymes of the redox network, thioredoxin 1 and glutaredoxin 1, ranked among the candidates identified via our in silico analysis

Table 1. Individual Parasite Proteins Identified in the Systematic Review

Short Name	Full Name	Study	Detection Method	Sample Type	Target *Plasmodium* spp.
AMA-1	Apical membrane antigen 1	Haghi 2012	ELISA	Blood	Pv
CelTOS	Cell traversal protein for ookinetes and sporozoites	Shamriz 2018	ELISA	Blood	Pf
CSP A/B	Circumsporozoite protein (variant VK210, A/B types)	Nam 2014	RDT	Blood	Pv
CSP variant VK247	Circumsporozoite protein (variant VK247)	Kim 2011	WB	Blood	Pf, Pv
DHFR-TS	Bifunctional dihydrofolate reductase-thymidylate synthase	Kattenberg 2012	ELISA	Blood	Pf, Pv
FBPA	Fructose-bisphosphate aldolase	Dzakah 2013	Immunoassay	Blood	Pv
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Krause 2017	ELISA	Blood	Pf
GDH	NADP-specific glutamate dehydrogenase	Ling 1986	Immunoassay, WB	Blood	Pf
GLURP	Glutamate-rich protein	Kattenberg 2012; Zerpa 2006	ELISA, WB	Blood	Pf
HDP	Heme detoxification protein	Kattenberg 2012	ELISA	Blood	Pf, Pv
Hsp70	Heat shock protein 70	Guirgis 2011	Immunoassay	Blood	Pf
IDEh	Insulin-degrading enzyme homolog	Mu 2017	ELISA, quantitative real-time polymerase chain reaction;	Blood	Pf
MESA	Mature parasite-infected erythrocyte surface antigen	Zainudin 2015	MS	Blood	Pf
MSP-1	Merozoite surface protein 1	Militao 1993	WB	Urine	Pv
$MSP\text{-}1_{19}$	Merozoite surface protein 1 (19 kDa fragment)	Sonaimuthu 2015; Lau 2014	WB	Blood	Pk; Pf, Po, Pv, Pk
$MSP\text{-}1_{33}$	Merozoite surface protein 1 (33 kDa fragment)	Cheong 2013	ELISA, WB	Blood	Pan
MSP-2	Merozoite surface protein 2	Khosravi 2013	WB	Blood	Pf
MSP-3	Merozoite surface protein 3	Silva 2016	ELISA, WB	Blood	Pk
Pcalp	Calpain	Choi 2009	WB, confocal microscopy	Blood	Pf
PMT	Phosphoethanolamine N-methyltransferase	Krause 2018	ELISA	Blood	Pf, Pv, Pk
PVX-003545	*Plasmodium*-exported protein, unknown function	Venkatesh 2018	MS	Blood	Pv
PVX-083555	Hypothetical protein	Venkatesh 2018	MS	Blood	Pv
PVX-090265	Tryptophan-rich antigen (Pv-fam-a)	Venkatesh 2018	MS	Blood	Pv
PVX-094303	Pvstp1, putative	Venkatesh 2018	MS	Blood	Pv
PVX-101520	Pv-fam-d protein	Venkatesh 2018	MS	Blood	Pv
PVX-110940	Hypothetical protein, conserved	Gualdron-lopez 2018	MS, WB	Blood	Pv
Rhop-3	High-molecular-weight rhoptry protein 3	Saleh 2012	Immunoassay	Buffer	Pf
SPATR	Secreted protein with altered thrombospondin repeat domain	Palaeya 2013	ELISA, WB	Blood	Pk
TopI	Topoisomerase I	Hede 2018	Rolling circle enhanced enzyme activity detection	Saliva	Pan
TPx-1	Thioredoxin peroxidase 1	Hakimi 2015	Immunochromatography, WB	Blood	Pf

Abbreviations: ELISA, enzyme-linked immunosorbent assay; MS, mass spectrometry; Pf, *Plasmodium falciparum*; Pk, *Plasmodium knowlesi*; Pm, *Plasmodium malariae*; Po, *Plasmodium ovale*; Pv, *Plasmodium vivax*; RDT, rapid diagnostic test; WB, Western blot.

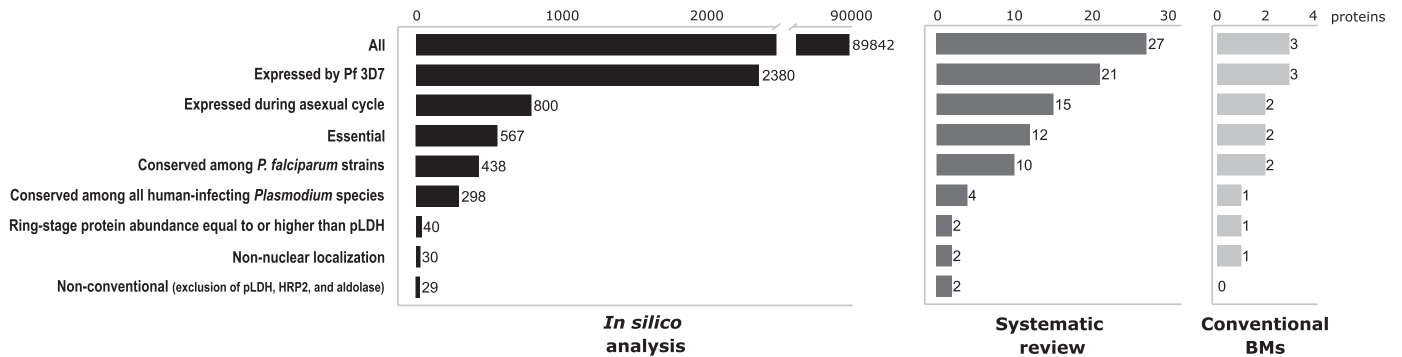


Figure 3. Filtering cascade adopted to identify *Plasmodium falciparum* proteins with favorable diagnostic features. Abbreviations: BM, biomarker; pLDH, *Plasmodium* lactate dehydrogenase.

(Table 2), suggesting that proteins involved in oxidative stress responses could be propitious targets for malaria diagnostic biomarker development [63].

We also investigated whether the conventional malaria diagnostic biomarkers, HRP2, pLDH, and aldolase, harbor favorable diagnostic features and found that only pLDH possessed all selected features (Figure 3; Supplementary File 2). HRP2 was filtered out because of its nonessential and *P. falciparum*-specific nature. Aldolase, on the other hand, showed lower expression levels than pLDH in ring-stage parasites.

MALARIA DIAGNOSTIC BIOMARKER LEADS: GAPDH AND DHFR-TS

Our dual, systematic approach singled out GAPDH and DHFR-TS as the 2 biomarker candidates for malaria diagnosis with not only favorable diagnostic features but also actual evidence of their diagnostic value (Table 3). Table 3 compares GAPDH and DHFR-TS with the commonly used malarial antigens pLDH and HRP-2.

GAPDH is a highly conserved, essential glycolytic enzyme responsible for oxidative phosphorylation of glyceraldehyde-3-phosphate in cells (Supplementary Figure 5A) [64]. Antibody-based evidence confirmed that *P. falciparum*-specific and pan epitopes in GAPDH are detectable, albeit only in culture isolates [28]. Our in silico analysis of the GAPDH protein sequence to identify immunogenic B-cell epitopes pointed to 1 probable C-terminal epitope, with low prediction scores (Figure 4A). The number of nonsynonymous SNPs identified in GAPDH by the in silico analysis was found to be low, in line with its essential cellular function (Table 3). The low genetic diversity of GAPDH is likely to obviate the risk of variable test performances due to sequence variability of the target marker.

DHFR-TS is a bifunctional enzyme with roles in the folate pathway and pyrimidine and DNA synthesis that is expressed in all human-infecting *Plasmodium* species (Supplementary Figure 5B [65]. Pan-specific monoclonal antibodies developed against DHFR-TS were shown to detect *P. falciparum* and *P. vivax* isolates in an ELISA [51]. Two immunogenic B-cell epitopes with low prediction scores but surface exposure were identified by our in silico analysis (Figure 4B; 4C). The number of nonsynonymous SNPs in DHFR-TS was low, in accordance with published results (Table 3; [66, 67]). Additionally, we investigated the number of repeats in GAPDH and DHFR-TS and found that amino acid repeats are not common in either protein (Figure 4D).

DISCUSSION

We sought novel malaria biomarkers for use in malaria RDTs that can be deployed in malaria-endemic areas with widespread *hrp2/hrp3* deletions. We adopted a dual, systematic approach and identified 2 candidates, GAPDH and DHFR-TS, that featured in both approaches, indicating that not only do they have favorable diagnostic properties in silico but also experimental evidence warranting their anticipated diagnostic value. Intriguingly, GAPDH has been proposed as a biomarker for various conditions, from infections to cancer, for diagnostic and prognostic purposes [70–75]; however, to date, it has not been used for clinical decision-making. To the best of our knowledge, the only follow-up study on the diagnostic potential of DHFR-TS for malaria failed to confirm the findings in the study included in this review when the reagents to recognize DHFR-TS were tested using clinical isolates, likely due to the low affinity of the antibodies obtained through classic animal immunization procedures (Foundation for Innovative New Diagnostics, unpublished data). Therefore, an immediate next step would be the development of high-affinity and highly specific reagents that target these selected biomarkers and the subsequent evaluation of these reagents using geographically diverse clinical isolates of different *Plasmodium* species to assess the use-case-relevant utility of these candidates. Moreover, antimalarial antifolates that, in the past, have been commonly used have led to resistance mutations in the DHFR domain of DHFR-TS [76]. It is, therefore, critical to enable an impact assessment on the detection of malarial parasites using DHFR-TS–based RDTs by the circulation of the *dhfr-ts* mutations.

Table 2. Candidate Biomarkers Identified by In Silico Analysis of *Plasmodium falciparum* Proteomics Data

Gene ID	Short Name	Full Name	Relative Abundance	Percent Identity With Human Orthologues	Availability of Crystal Structure	Expression During Sexual Cycle	Number of Single-Nucleotide Polymorphisms	Immunogenic Epitope Identified	Reported in Systematic Review
PF3D7_1123200	LRR11	Leucine-rich repeat protein	1.09	24	No	No	39	Yes	No
PF3D7_1211700	MCM5	DNA replication licensing factor MCM5, putative	1.08	49	No	Yes	1	No	No
PF3D7_1110700	ALP1	Actin-like protein, putative	1.06	43	Yes	Yes	10	No	No
PF3D7_239200	ApiAP2	AP2 domain transcription factor, putative	1.06	N/A	No	No	140	Yes	No
PF3D7_0501800	CAF1	Chromatin assembly factor 1 subunit A	1.05	N/A	No	No	112	Yes	No
PF3D7_1244600	ARFGAP	ADP-ribosylation factor GTPase-activating protein	1.04	30	Yes	No	6	Yes	No
PF3D7_0317200	CRK4	cdc2-related protein kinase 4	1.04	24	Yes	No	113	Yes	No
PF3D7_0306300	GRX1	Glutaredoxin 1	1.03	49	Yes	Yes	5	No	No
PF3D7_1029600	ADA	Adenosine deaminase	1.03	39	Yes	No	12	No	No
PF3D7_1401800	CK	Choline kinase	1.03	33	Yes	No	6	No	No
PF3D7_0606900	GLP2	Glutaredoxin-like protein	1.03	29	No	No	6	No	No
PF3D7_1012400	HGPRT	Hypoxanthine-guanine phosphoribosyl transferase	1.03	55	Yes	No	5	No	No
PF3D7_1414400	PP1	Serine/threonine protein phosphatase PP1	1.01	72	No	Yes	3	No	No
PF3D7_0417200	**DHFR-TS**	**Bifunctional dihydrofolate reductase-thymidylate synthase**	**1.01**	**31**	**Yes**	**No**	**12**	**No**	**Yes**
PF3D7_1457200	TRX1	Thioredoxin 1	1.01	51	Yes	No	4	No	No
PF3D7_0312400	GSK3	Glycogen synthase kinase 3	1.01	52	Yes	No	17	Yes	No
PF3D7_0512700	OPRT	Orotate phosphoribosyl transferase	1.01	31	Yes	No	27	No	No
PF3D7_0316500	NUF2	Kinetochore protein NUF2, putative	1.01	N/A	No	No	7	No	No
PF3D7_1449500	ApiAP2	AP2 domain transcription factor AP2-O5, putative	1.01	N/A	No	No	62	Yes	No
PF3D7_1403900	CPPED1	Serine/threonine protein phosphatase CPPED1, putative	1.00	40	No	Yes	3	No	No
PF3D7_1037700	ERH	Enhancer of rudimentary homolog, putative	1.00	42	No	Yes	1	No	No
PF3D7_1462800	**GAPDH**	**Glyceraldehyde-3-phosphate dehydrogenase**	**1.00**	**68**	**Yes**	**Yes**	**4**	**No**	**Yes**
PF3D7_1204300	EIF5A	Eukaryotic translation initiation factor 5A	1.00	57	No	Yes	1	No	No
PF3D7_1126000	ThrRS	Threonine-tRNA ligase	1.00	37	No	Yes	76	Yes	No
PF3D7_1015900	ENO	Enolase	1.00	71	No	Yes	9	No	No
PF3D7_0214000	CCT8	T-complex protein 1 subunit theta	1.00	45	No	Yes	4	No	No
PF3D7_1324900	**LDH**	**L-lactate dehydrogenase**	**N/A**	**42**	**Yes**	**Yes**	**2**	**No**	**N/A**
PF3D7_0922500	PGK	Phosphoglycerate kinase	1.00	66	Yes	Yes	6	No	No
PF3D7_0922200	SAMS	S-adenosylmethionine synthetase	1.00	61	No	No	7	No	No
PF3D7_0810800	PPPK-DHPS	Hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase	1.00	N/A	Yes	Yes	23	No	No

In addition to the traditional biomarker lactate dehydrogenase, biomarkers identified in the systematic review are highlighted in bold.

Abbreviation: N/A, not available.

Table 3. Characteristics of Glyceraldehyde 3-Phosphate Dehydrogenase and Dihydrofolate Reductase-Thymidylate Synthase in Comparison With *Plasmodium* Lactate Dehydrogenase and HRP-2

Organism	*Plasmodium falciparum* 3D7				*Plasmodium vivax* P01		
Gene ID	PF3D7_1462800	PF3D7_0417200	PF3D7_1324900	PF3D7_0831800	PVP01_1244000	PVP01_0526600	PVP01_1229700
Gene name	GAPDH	DHFR-TS	LDH	HRP2	GAPDH	DHFR-TS	LDH
Product description	Glyceraldehyde-3-phosphatedehydrogenase	Bifunctionaldihydrofolatereductase-thymidylate synthase	L-lactate dehydrogenase	Histidine-rich protein II	Glyceraldehyde-3-phosphate dehydrogenase, putative	Bifunctional dihydrofolate reductase-thymidylate synthase, putative	L-lactate dehydrogenase
Coding sequence length	1014	1827	951	918	1014	1875	951
Protein length	337	608	316	305	337	624	316
Molecular weight (Da)	36 635	71 736	34 107	32 408	36 690	71 031	34 222
Isoelectric point	7.77	7.22	7.63	7.02	7.83	7.42	7.50
Essentiality	Nonmutable	Nonmutable	Nonmutable	Mutable	N/A	N/A	N/A
Noncoding SNPs	16	1	0	11	43	0	0
Nonsynonymous SNPs	3	11	2	132	2	55	2
SNPs with stop codons	1	1	0	0	0	2	0
Synonymous SNPs	4	2	0	115	28	67	37
Total SNPs	24	15	2	258	73	124	39
Number of transmembrane domains	0	0	1	0	0	0	1
Average expression at ring stage	4.52	4.58	4.52	N/A	N/A	N/A	N/A
Average expression at trophozoite stage	3.81	3.81	3.91	N/A	N/A	N/A	N/A
Average expression at schizont stage	3.70	3.58	3.58	N/A	N/A	N/A	N/A
Percent identity with *Plasmodium falciparum*	N/A	N/A	N/A	N/A	87.5	73.7	90.5
Percent identity with *Plasmodium vivax*	87.5	73.7	90.5	[No gene]	N/A	N/A	N/A
Percent identity with *Plasmodium knowlesi*	86.9	73.0	90.2	[No gene]	94.4	89.0	97.2
Percent identity with *Plasmodium malariae*	87.2	77.8	92.7	[No gene]	90.2	78.5	90.5
Percent identity with *Plasmodium ovale*	89.3	72.5	92.7	[No gene]	93.5	73.9	89.2
Human orthologue gene name	GAPDH	TYMS	LDHC	[No gene]	GAPDH	TYMS	LDHC
Percent identity with human orthologue	68.3	31.4	41.6	N/A	67.5	32.2	40.7

Abbreviations: DHFR-TS, dihydrofolate reductase-thymidylate synthase; GADPH, glyceraldehyde 3-phosphate dehydrogenase; N/A, not available; SNP, single-nucleotide polymorphism; TYMS, thymidylate synthetase.

(A) > GAPDH

Pf-3D7 1 MAVTKLGINGFGRIGRLVFRAAFGRKDIEVVAINDPFMDLNHLCYLLKYDSVHGQFPCEVTHADGFLLIGEKKVSVFAEKDPSQIPWGKCQVDVVCESTG 100

Pf-3D7 101 VFLTKELASSHLKGGAKKVIMSAPPKDDTPIYVMGINHHQYDTKQLIVSNASCTTNCLAPLAKVINDRFGIVEGLMTTVHASTANQLVVDGPSKGGKDWR 200

Pf-3D7 201 AGRCALSNIIPASTGAAKAVGKVLPELNGKLTGVAFRVPIGTVSVVDLVCRLQKPAKYEEVALEIKKAAEGPLKGILGYTEDEVVSQDFVHDNRSSIFDM 300

Pf-3D7 301 KAGLALNDNFFKLVSWYDNEWGYSNRVLDLAVHITNN 337

* BepiPRED:0.54, IUPRED:0.25, ANCHOR:0.29

> DHFR-TS

Pf-3D7 1 MMEQVCDVFDIYAICACCKVESKNEGKKNEVFNNYTFRGLGNKGVLPWKCNSLDMKYFCAVTTYVNESKYEKLKYKRCKYLNKETVDNVNDMPNSKKLQN 100

Pf-3D7 101 VVVMGRTSWESIPKKFKPLSNRINVILSRTLKKEDFDEDVYIINKVEDLIVLLGKLNYYKCFIIGGSVVYQEFLEKKLIKKIYFTRINSTYECDVFFPEI 200

Pf-3D7 201 NENEYQIISVSDVYTSNNTTLDFIIYKKTNNKMLNEQNCIKGEEKNNDMPLKNDDKDTCHMKKLTEFYKNVDKYKINYENDDDDEEEDDFVYFNFNKEKE 300

Pf-3D7 301 EKNKNSIHPNDFQIYNSLKYKYHPEYQYLNIIYDIMMNGNKQSDRTGVGVLSKFGYIMKFDLSQYFPLLTTKKLFLRGIIEELLWFIRGETNGNTLLNKN 400

Pf-3D7 401 VRIWEANGTREFLDNRKLFHREVNDLGPIYGFQWRHFGAEYTNMYDNYENKGVDQLKNIINLIKNDPTSRRILLCAWNVKDLDQMALPPCHILCQFYVFD 500

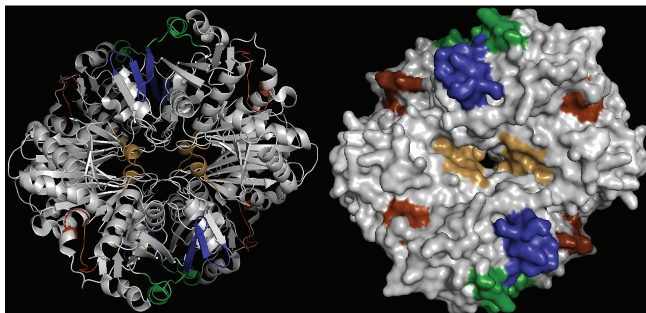
Pf-3D7 501 GKLSCIMYQRSCDLGLGVPFNIASYSIFTHMIAQVCNLQPAQFIHVLGNAHVYNNHIDSLKIQLNRIPYPFPTLKLNPDIKNIEDFTISDFTIQNYVHHE 600

Pf-3D7 601 KISMDMAA 608

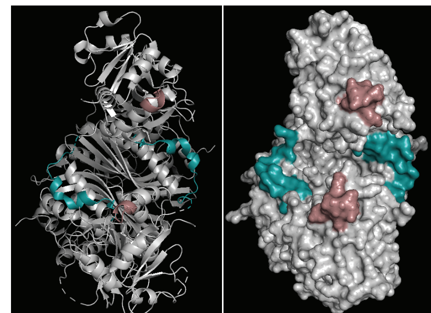
** BepiPRED:0.61, IUPRED:0.19, ANCHOR:0.28

*** BepiPRED:0.65, IUPRED:0.31, ANCHOR:0.36

(B)



(C)



(D)

	Longest 1-mer Repeat	Longest 2-mer Repeat	Longest 3-mer Repeat	Longest 4-mer Repeat	Longest 5-mer Repeat	Most Frequent 1-mer	Most Frequent 2-mer	Most Frequent 3-mer	Most Frequent 4-mer	Most Frequent 5-mer
PfGAPDH	1:AA	0:[NA]	0:[NA]	0:[NA]	0:[NA]	35:V	5:LA	2:FGR	1:DPSQ	1:DTKQL
PvGAPDH	1:AA	0:[NA]	0:[NA]	0:[NA]	0:[NA]	34:V	5:VV	2:KYD	1:YDNE	1:KVLPE
PfDHFR-TS	3:DD	1:GVGV	0:[NA]	0:[NA]	0:[NA]	63:N	11:KN	4:KKL	2:LKYK	1:MYDNY
PvDHFR-TS	4:LL	2:LLL	0:[NA]	0:[NA]	0:[NA]	51:L	9:GG	3:GGD	3:GGDN	2:GGDNT
PfLDH	2:AA	0:[NA]	0:[NA]	0:[NA]	0:[NA]	32:V	7:IV	2:NNK	2:LDTS	1:LKRYI
PvLDH	2:AA	1:PKPK	0:[NA]	0:[NA]	0:[NA]	35:V	6:GG	3:IGG	2:LDTS	1:LKRYI
PfHRP2	2:AA	1:LNLN	2:HHAHHA	0:[NA]	0:[NA]	110:A	51:AH	50:AHH	46:AHHA	27:DAHHA

Figure 4. *A*, GAPDH and DHFR-TS amino acid sequences with peptide epitopes. BepiPred, IUPRED, and ANCHOR scores for the immunogenic epitopes predicted in this study are shown below the sequences. The scores are not available for the epitopes identified in a previous study [28]. *B*, Location of the epitopes in the GAPDH tetramer [68] and *(C)* in DHFR-TS [69]. *D*, Contiguous repeated subsequences within protein sequences. Abbreviations: DHFR-TS, dihydrofolate reductase-thymidylate synthase; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

A third potential biomarker of value is PMT, an essential protein involved in *Plasmodium* lipid biosynthesis [77, 78]. Both GAPDH and PMT were identified as biomarker candidates for malaria in a prior in silico analysis [28, 37]. In our analysis, we applied essentiality, conservation across *Plasmodium* strains and species, and high expression during the ring stage of the parasite life cycle as additional filtering criteria to minimize the risk of selecting a target that cannot be readily detected in parasitized human blood samples because of its deletion, high diversity, and/or low abundance. Another in silico analysis of malaria biomarkers identified 8 candidates that are highly expressed by asexual stage parasites, essential and conserved across different parasite strains [38]. However, none of these 8 candidates were present in our final list of 30 proteins. Four of them (PF3D7_1250100, PF3D7_0500800, PF3D7_1016300, and PF3D7_0220000) were found to be dispensable for parasite growth in our dataset. To assess the dispensability of *Plasmodium* genes, we sourced data from a study in which the mutability of the parasite genes was assessed by saturation mutagenesis [79], whereas the

previous study used data from an earlier large-scale gene-knockout study with a focus on proteins exported into red blood cells [80]. Three of the candidates, PF3D7_1118300, PF3D7_1110400, and PF3D7_1120000, had no gene name and were excluded. Nonetheless, we found that even though 2 of those, PF3D7_1110400 and PF3D7_1120000, met our essentiality and expression during asexual cycle criteria, they had lower abundance in the ring stage than pLDH (data not shown). Finally, PF3D7_0401800 was also found to be less abundant than pLDH at the ring stage.

Efforts to improve the performance of currently available pLDH-based RDTs to match that of HRP2-based tests have recently intensified; however, they have yet to yield a product for use in malaria-endemic settings where *hrp2/hrp3* deletions exist. The question remains, therefore, whether improved pLDH-based tests will be sufficient to close the diagnostic gap created by the decreased utility of HRP2-based tests. Regardless, relying solely on pLDH for the diagnosis of hundreds of millions of suspected malaria cases annually may increase the risk of driving mutations, abolishing the epitopes targeted in pLDH-based RDTs due to strong selective pressure, as is predicted to be the case for HRP2 [81, 82]. Functional diagnostic-resistant pLDH variants may emerge, in a similar manner to what has been occurring for highly conserved essential enzymes in response to drug pressure [83, 84].

There were some limitations to this study. First, it was not possible to perform a meta-analysis of the studies identified in our review due to heterogeneity between the studies and the small number of studies per biomarker; this made direct comparisons difficult. Second, the review excluded biomarkers for severe malaria. However, this is a less important limitation since early and accurate detection of uncomplicated malaria is likely to reduce severe malaria cases. Finally, information stored in public databases is subject to errors that may have occurred during data entry, archiving processes, and changes as new information becomes available. Errors may also occur during data processing and analysis. Manual steps were taken to verify data content with original, noted sources (when available) to ensure data transformations maintained the data integrity.

In this study, we champion the development of novel malaria RDTs based on unconventional antigens by identifying promising candidates and highlighting 2 antigens for which not only favorable in silico but also in vitro evidence exists and that could represent ideal starting points for a rapid and partially de-risked research and development effort toward effective malaria RDTs based on new antigens.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. S. Y. and X. C. D. designed the study. S. Y., E. D. A. O., and X. C. D. developed the systematic review protocol. E. D. A. O. and A. F. screened the titles and extracted the data. S. Y. validated the data and performed the data analysis. R. K. D. retrieved the proteomics data and conducted the in silico analysis. S. Y. drafted the manuscript. E. D. A. O., A. F., R. K. D., and X. C. D. provided critical editing and review.

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