Malaria Parasite Survival Depends on Conserved Binding Peptides' Critical Biological Functions

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Dedicated to the memory of Professors Henry G. Kunkel and Bruce Merrifield (Rockefeller University) and Peter Perlmann (Stockholm University) who introduced us to the fascinating worlds of immunology, peptide chemistry and malaria, respectively.

Abstract
Biochemical, structural and single amino acid level analysis of 49 *Plasmodium falciparum* protein regions (13 sporozoite and 36 merozoite proteins) has highlighted the functional role of each conserved high activity binding peptide (cHABP) in cell host-microbe interaction, involving biological functions such as gliding motility, traversal activity, binding invasion, reproduction, nutrient ion transport and the development of severe malaria. Each protein's key function in the malaria parasite's asexual lifecycle (pre-erythrocyte and erythro-cyte) is described in terms of cHABPs; their sequences were located in elegant work published by other groups regarding critical binding regions implicated in malarial parasite invasion. Such cHABPs represent the starting point for developing a logical and rational methodology for selecting an appropriate mixture of modified cHABPs to be used in a completely effective, synthetic antimalarial vaccine. Such methodology could be used for developing vaccines against diseases scourging humanity.

Introduction
One of the most relevant conserved functions for successful cell host-microbe interaction and parasite survival is binding to host cell molecules to mediate parasite invasion and multiplication.

Transcriptome analysis of *P. falciparum* has shown that ~50 of the ~5,600 proteins are directly involved in merozoite (Mrz) invasion of red blood cells (RBC) (Bozdech et al., 2003) and ~30 are involved in sporozoite (Spz) invasion of liver cells (Kaiser et al., 2004; Lasonder et al., 2008); however, only those undoubtedly shown to be on Spz and Mrz surface and/or directly mediating host-cell microbe functional interactions their relevant cHABPs will be analysed here. These represent the Achilles' heel of the *P. falciparum* malaria parasite (Patarroyo et al., 2015a).

A very robust, sensitive, specific synthetic peptide methodology, involving the *Plasmodium falciparum* parasite (infecting ~200 million people and killing ~584,000 of them annually) (World Health Orgaanization, 2014) as our leading, model disease for vaccine development has been thoroughly used for identifying ~300 cHABPs in this parasite's most relevant molecules for Spz binding to and invasion of liver cells (Garcia et al., 2006) and Mrz binding to and invasion of erythrocytes, and binding to endothelial cells (Rodriguez et al., 2008).

cHABPs become excellent candidate components for a minimal subunit based, multi-epitope, multistage, chemically-synthesised antimalarial vaccine when properly modified (mHABPs) (Patarroyo et al., 2011; Patarroyo et al., 2005; Patarroyo and Patarroyo, 2008), since blocking or destroying their biological functions may represent one of the most effective methods for impeding functions or killing the parasite

The aforementioned cHABPs are shown here at single amino acid and/or atomic level (when their 3Dstructure is available), representing the first attempt at comprehensively describing cell-host-microbe interactions at the deepest level, particularly regarding the *P. falciparum* parasite. Exquisite, relevant, biological functions have not yet been determined for a few of these cHABPs; however, it is hoped that they will be so in the near future, similar to what occurred during the last 25 years after the first cHABPs were identified (Calvo et al., 1991) based on the recognition that some SPf66 peptides (first chemically-synthesised, anti-malarial vaccine developed by us 28 years ago) strongly and specifically bound to RBCs (Calvo et al., 1991; Patarroyo et al., 1988; Patarroyo et al., 1987).

Our institute has led research into two different, complementary directions aimed at developing a logical and rational methodology for a minimal subunit-based, multi-epitope, multistage, fully and completely protective anti-malaria vaccine and defining physicochemical and immunological principles for vaccine development: a functional biological approach (here deeply analysed) for identifying important regions of the most relevant molecules involved in *P. falciparum* malaria invasion and infection and their biological functions and a simultaneous immunochemical-immunogenetic approach to render these cHABPs into highly immunogenic, protection-inducing components (beyond the scope of this manuscript, but deeply analysed and reviewed in Patarroyo et al., 2015b; Patarroyo et al., 2011).

The functional biological approach

Differently to a purely immunological approach based on large sero-epidemiological information suggesting that the most significant and relevant molecules for vaccine development were highly antigenic or immunogenic ones and highly variable (thousands of genetic variants being present in the *P. falciparum* genome) as a mechanism for escaping immune pressure, we suggested 25 years ago that the most relevant fragments or amino acid sequences to be included in a vaccine should be those directly involved in biological functions like invasion, infection and some other critical biological functions and that a deep analysis (at the atomic level if possible) of this very complex parasite should be performed during this parasite's different functionally invasive stages. We predicted that specific receptor-ligand interactions could lead to a deep understanding of this parasite's biology and the pertinent physicochemical rules and that such understanding could lead to a logical and rational methodology for vaccine development, the *raison d'être* of this manuscript.

Conserved binding sequences or cHABPs have been confirmed after a deep analysis of all amino acids sequences from the proteins described here which have been derived from different *P. falciparum* strains and isolates deposited in the National Center for Biotechnology Information (NCBI), cHABPs (by definition) only being those not showing any amino acid sequence variation in all strains or the few displaying one variation 1 or 2 residues downstream the N-terminus or upstream the C-terminus. The rationale being that since these cHABPs are 20 mer long they can be shortened or extended 1 or 2 residues to exclude variable residues for mHABP design without dramatically modifying or changing these peptides' 3D structure.

The sporozoites' journey to the liver

Spz-derived cHABPs perform different biological functions

Gliding motility and Spz displacement

Once under the skin (where they can stay for ~60 minutes), the 100-1,000 Spz (Figure 1A) inoculated during an *Anopheles* mosquito bite begin their journey (Vaughan et al., 2008). They move at ~2-4μm/second (Amino et al., 2008) (Figure 1B) with characteristic slip-stick displacement movements (gliding motility) modulated by the turnover of discrete adhesion sites (Munter et al., 2009). Such movement is mediated by a set of proteins, such as thrombospondin-related anonymous protein (TRAP) (Sultan et al., 1997) and TRAP-like protein (TLP) (Moreira et al., 2008), secreted by the micronemes at the Spz apical pole (Figures 1A and 1C) and translocated to the membrane, together with the membrane coat multifunctional circumsporozoite protein 1 (CSP-1) (Figure 1D and Figure 2A), prior to hepatocyte invasion.

Note: From here on Figure 2 shows all molecules' PlasmoDB code numbers, molecular weight, relative size and cHABP location and Table 1 shows cHABP amino acid sequences, with their initial and last amino acid numbers. Only critical residues whose biological functions have been clearly determined (in bold) will be mentioned in the text (location number as superscript to the left).

TRAP is a 63kDa type I microneme protein which is essential for Spz gliding motility conserved in all *Plasmodium* species (Sultan et al., 1997); it has an acidic C-terminal cytoplasmic tail, a transmembrane region and four extracellular domains: a proline-rich region, a hyper-variable region, a thrombospondin-type-related region 1 (TSR) and a ~200 amino acid-long von Willebrand factor A-like (vWA) domain (Figure 1D and 2A) (Rogers et al., 1992).

cHABP 3271 is contained in vWA domain (involved in cell-cell, cell-matrix, matrix-matrix interactions); such domain includes a metal-ion dependent adhesion site (MIDAS) where cHABP 3271 ^{162}D, ^{167}S, ^{170}D ^{171}S residues (Table 1) display typical geometric and coordinated symmetry to bind one Mg^{++} atom (Pihlajamaa et al., 2013).

It has been shown that the vWA domain is involved in *Pf*TRAP dimerisation for attachment to stromal surfaces and fast gliding motility (Pihlajamaa et al., 2013); hybrid cHABP 3277/79 covering the ^{205}C-C^{212} loop is located in this domain. cHABP 3277 ^{197}AFNR^{200} establishes H-bonds with cHABP 3279 ^{201}FLV^{203} sequence to form a niche where an unrecognised receptor binds (Figure 1D). cHABP 3287, including ^{250}(WSPCSV)^{255} motif in the TSR-1 region, and 3289, completely including ^{254}(SVTCGK)^{259} in the TSR-2 region (Song et al., 2012), contain a β-ribbon region (Table 1 and Figure 1D) connecting vWA and TSR domains to allow TRAP to become elongated and straightened to resist the tensile force exerted by receptor-bound TRAP and the intracytoplasmic actino-myosin motor machinery interaction (Song et al., 2012).

cHABP 3289 ^{259}K, ^{262}R, ^{264}R and ^{265}K residues and side-chain^{247}W and ^{250}W residues in cHABP 3287 in the 2 antiparallel (A and B) and ripped β-sheet form a continuous, positively-charged surface where ligands like heparin and heparin sulphate bind (Figure 1D). X-ray crystallography has shown a fucose residue interacting with ^{261}T in the β-turn formed by ^{258}(GKGT)^{261}connecting the A and B strands and present in cHABP 3289 (Tucker, 2004), suggesting this carbohydrate is a liver ligand in a still unrecognised receptor for this cHABP.

TRAP cHABP 3347, completely included in the 34 mer-long aldolase binding peptide connecting TRAP with the actin-myosin motor machinery propelling Spz gliding motility (Buscaglia et al., 2003), is located 20 residues downstream this protein's canonical cleavage site from Spz surface by a rhomboid protease (Ejigiri et al., 2012) recognised as being essential for Spz motility and infectivity. The only TRAP cHABP for which no function has yet been assigned is 3243 which binds with high affinity to hepatocytes and may be involved in host cell entry.

Spz cHABPs as mediators of cell traversal activity

A sporozoite leaves the skin by gliding in a random, freely cork-screw-like movement to find a small blood vessel to

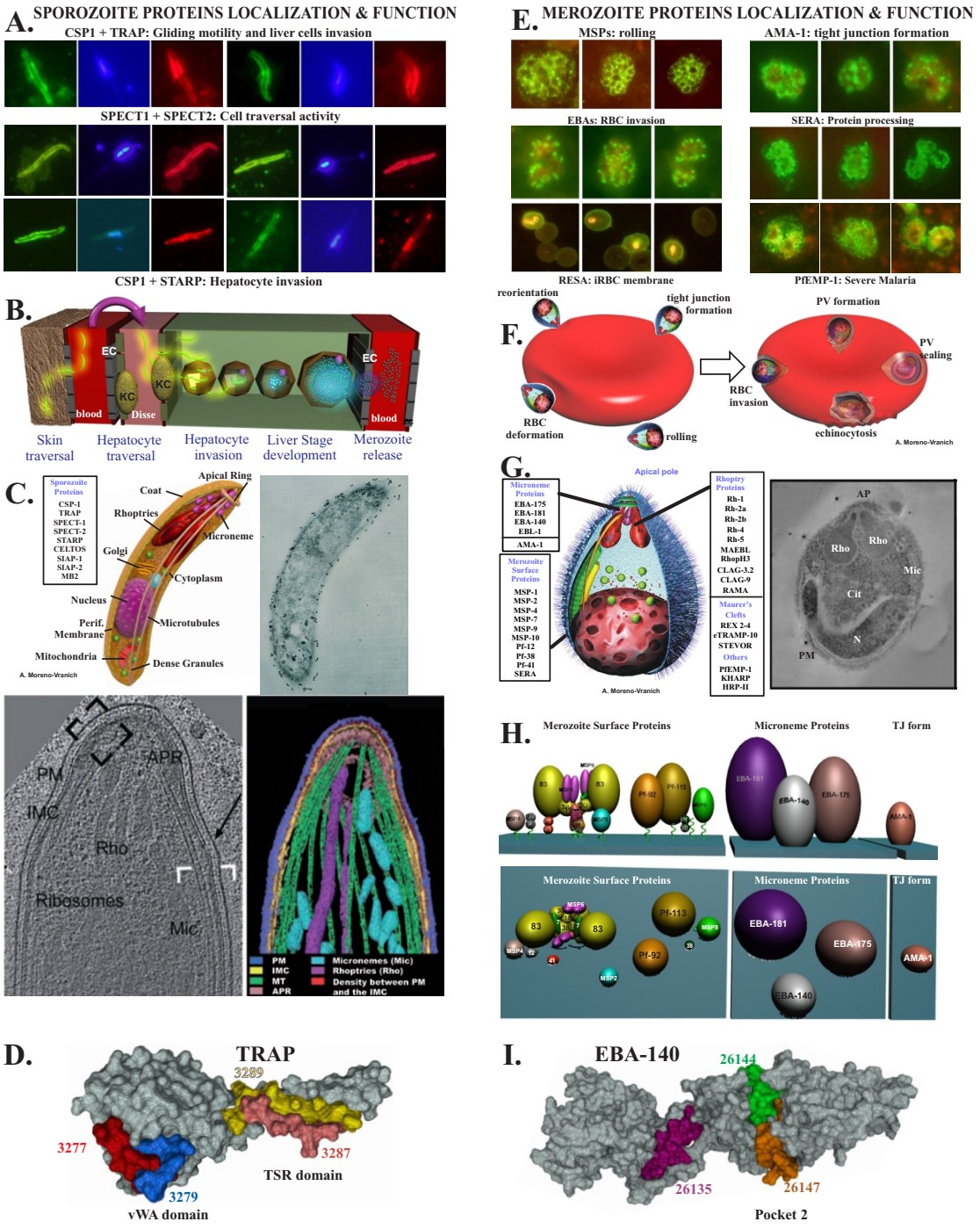

Figure 1. From parasites to atoms

Column 1. *Plasmodium falciparum* (A) sporozoite protein location is shown, as detected by double immunofluorescence antibody test (IFA) staining with Aotus monkey sera immunised Spz protein-derived mHABPs. The top line shows CSP1 on the membrane (green) and TRAP micronemes (red) involved in gliding motility and cell invasion; the middle line shows SPECT 1 (green) and SPECT 2 (red) involved in cell traversal; the bottom line shows CSP1 on the membrane (green) and intracytoplasmic STARP (red). (B) The sporozoite's journey (fluorescent larvae-like structures: KC (Kupffer cells), EC (endothelial cells) and activities after passing the skin to the liver, adapted from (Vaughan et al., 2008). (C) *P. falciparum* sporozoite's structural features. Top: Spz anatomy showing its invasion machinery proteins and essential organelles adapted from (Kudryashev et al., 2010) and immune electron microscopy showing anti-CSP antibody reactivity with Spz membrane (black dots on the membrane), adapted from (Kudryashev et al., 2010). Bottom: Electron microscopy of Spz apex positioning and subpellicular network. PM; peripheral membrane, DGP, dense granules; Mic, micronemes; Rho, rhoptry; Mt, microtubules; Ct, cytostome; ApPR, apical, pole ring; N, nucleus; Mit, mitochondria and Ap, apicoplast. Spz apical end, displayed as a projection through a tomogram (left) and volume rendered (right). (D) TRAP 3D structure (PDB: 4F1J) and thrombospondin repeat (TSR) type 1 domain, von Willebrand factor A and cHABP location. **Column 2.** *P. falciparum* (E) merozoite protein location is shown, as determined by IFA and functions: rolling on the membrane by MSPs, TJ formation mediated by AMA-1-and apical rhoptries proteins, RBC deformation and invasion by microneme EBAs, protein processing by intracytoplasmic SERA-5, iRBC membrane expression by RESA and severe malaria (SM) and echinocytosis by PfEMP-1. (F) Steps (clockwise) involved in RBC invasion by merozoites. (G) Protein location in organelles recognised by EM. (H) Representation of Mrz protein location and interactions according to approximate molecular weight (I) EBA-140 3D structure (PDB 4GF2) and cHABP location.

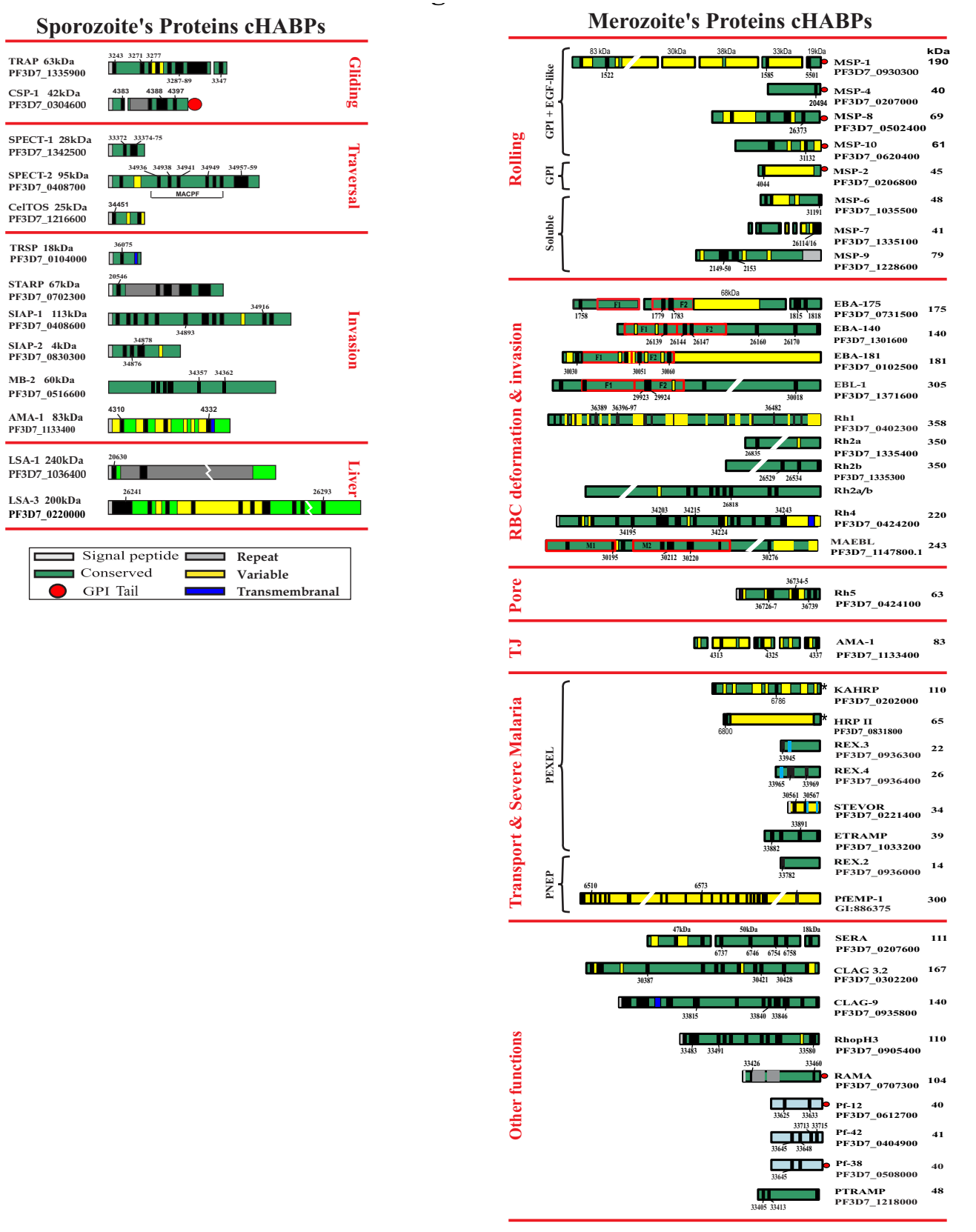

Figure 2. A. Schematic representation of the most important *Plasmodium falciparum* Spz proteins and the location of their functional cHABPs (black). **B**. Schematic representation of the most important *Plasmodium falciparum* Mrz proteins and the location of their functional cHABPs (black).The molecular mass and sequence accession codes are shown for each molecule. The bar length represents approximate molecular weight. The colour code is described in the convention summary at the bottom of this Figure. *Abbreviations:* GPI, glycosylphosphatidylinositol anchor; EGF, epidermal growth factor-like structures; HMIC, high molecular weight complex; PEXEL, plasmodium export element; Gly, glycophorin A, B or C.

Table 1. Conserved high activity binding peptides (cHABPs) perform critical biological functions in *P. falciparum*.

The sequence is shown for each cHABP and associated with the relevant functions which they perform in malarial parasite invasion and development. Each critical residue in each cHABP is shown in bold and the physicochemical constants (dissociation constant, Kd and number of receptor sites per cell, NRSC) regarding interactions between cHABPs and host cells are shown in columns. An additional bar has been included to show cHABPs which are common to Rh2a and Rh2b proteins, called Rh2a/b. ND = not determined, NS = non-saturated, EGF=epidermal growth factor-like, HMIC=high molecular weight complex, PEXEL=plasmodium export element, Gly=glycophorin A, B or C. PSAC=*Plasmodium* surface anion channel.

Sporozoite's cHABPs

	PEPTIDE	SEQUENCE	K_d (nM)	NRSC (x10^5)	Function
CSP 1	4383	^{68}NSRSLGENDDGNNEDNEKLR^{87}	80	120	PEXEL, CSP Trans.
	4388	^{278}GNGQGHNMPNDPNRNVDENA^{297}	80	140	cB act. HSPG bind.
	4397	^{323}IQNSLSTEWSPCSVTCGNGI^{342}	ND	ND	RII$^+$ HFobic core
TRAP	3243	^{25}YLVNGRDVQNNIVDE^{35}	290	145	Entry
	3271	^{161}TDGIPDSYQDSLKES^{175}	290	15	MIDAS Mg^{2+}
	3277/9	^{192}QGINVAFNRYLVGCHPSDGKC^{212}	240	80	HSPG
	3287	^{241}TASCGVWDEWSPYSV^{255}	28	14	Fucose HSPG extensible
	3289	^{251}SPCSVTCGKGTRSRK^{265}	180	85	Heparin
	3347	^{541}YAGEPAPFVEPLGEE^{555}	541	40	Aldolase
SPECT 2	34936	^{281}LSKEGIANDLSTLQPVNGWIY^{301}	ND	ND	
	34938	^{321}YTKSLSAEAKVSGSYWGIAS^{340}	770	19	Ca^{++} binding
	34941	^{381}WDKTTAYKNAVNELPAVFTG^{400}	ND	ND	KN sulphate
	34946	^{481}SAGGSTDVNSSNSSANDEQS^{500}	ND	ND	
	34949	^{541}KLTPISDSFDSDDLKESYDK^{560}	750	68	
	34951	^{581}DKDIIKILTNADTVTKNSAP^{600}	750	68	KN sulphate
	34957	^{701}SSSGRINSAEYVYSTPCIPG^{720}	ND	ND	Condroitins bind.
	34958	^{721}MKSCSLNMNNDNQKSYIYVL^{740}	460	34	
	34959	^{741}CVDTTIWSGVNNLSLVALDG^{760}	600	30	Cholesterol bind.
SPECT 1	33372	^{81}ASLEEVSDHVVQNISKYSLT^{100}	850	84	Cavity
	33375	^{143}TDLILKKLKKLENVNKLIK^{161}	750	241	Cavity
STARP	20546	^{41}VIKHNRFLSEYQSNFLGGGY^{60}	70±5	90	PEXEL
CELTOS	34451	^{21}NVLCFRGNNGHNSSSSLYNG^{40}	500	7200	
TRSP	36075	^{41}SDVRYNKSFINNRLLNEHAH^{60}	NS	ND	PEXEL
SIAP 1	34893	^{421}KVQGLSYLLRRKNGTKHPVY^{440}	ND	ND	KN sulphate
	34916	^{881}SSKPANEYHDGETIVSLSFN^{900}	460	17.3	
SIAP 2	36876	^{12}KKEMNNKLEQQTNQNNNTIH^{140}	ND	ND	
	36878	^{161}LKNQDSSNSLISTNSNTMDE^{180}	ND	ND	KN sulphate
MB 2	34357	^{1001}INHGKTSLFDYICKTNEQKK^{1020}	ND	ND	GTP binding
	34362	^{1101}KVDIPNVDVDRIINDLLYHD^{1120}Y	ND	ND	
LSA 1	20630	^{21}INGKIIKNSEKDEIIKSNLR^{40}Y	450	700	LS develop. KN sulphate
LSA 3	26241	^{81}KKLNKLFNRSLGESQVNGEL^{100}Y	880	900	PEXEL
	26293	^{889}FKSESDVITVEEIKDEPVQK^{908}Y	NS	ND	

Merozoite's cHABPs I

	PEPTIDE	SEQUENCE	K_d (nM)	NRSC (x10^5)	Function
MSP 1	1522	^{217}QIPYNLKIRANELDVLKKLV^{236}	150	6.3	Gly A
	1585	^{1343}GEVLYLKPLAGVYRSLKKQLE^{1363}	180	10.6	Heparin K5-NSOS-H
	5501	^{1605}MLNISQHQCVKKQCPQNSY^{1622}	230	11.8	EGF
MSP 4	20494	^{221}EYVGNRRVKCKCKEGYKLEG^{240}	470	24	EGF
MSP 8	26373	^{498}CPLNSNCYVIDDEETCRCLP^{517}	450	200	EGF
MSP 10	31132	^{422}KCGPNSRCYIVEKDKEQCRC^{441}	**600**	17.6	EGF
MSP 6	31191	^{352}EIDSTINNLVQEMIHLFSNN^{371}	150	128	HMIC Tetramer
MSP 7	26114	^{301}KDKEYHEQFKNYIYGVYSYA^{320}	NS	ND	HMIC
	26116	^{332}KPEEEYKKFLEYSFNLLNTM^{351}	450	85.3	
MSP 9	2149	^{141}KKHLIYKNKSYNPLLLSCVK^{160}	73	9.0	Hemolysin
	2150	^{161}KMNMLKENVDYIQKNQNLFK^{180}	80	7.6	HMIC
	2153	^{221}QKYQEVNDEDDVNDEEDTNP^{240}	ND	ND	
MSP 2	4044	^{2}KNESKYSNTFINNAYNMSIR^{21}	140	12	Lipids bind.
EBA 175	1758	^{80}KSYGTPDNIDKNMSLINKHN^{90}	112	15	
	1779	^{356}NIDRIYDKNLLMIKEHILAI^{375}	175	14.6	R II-F1
	1783	^{436}HRNKKNDKLYRDEWWKVIKK^{455}	139	7.5	Glycan5, dimer. R II-F2
	1815	^{1247}YTNQNINISQERDLQKHGFH^{1266}	106	8.0	RV micron. traf.
	1818	^{1247}NNNFNNIPSRYNLYDKKLDL^{1328}	146	9.4	RVI micron. traf.
EBA 140	26139	^{441}DIASQINVNDLRGFGCNYKS^{460}			
	26144	^{541}DLADIIKGSDIIKDYYGKKM^{560}	350	3.8	Acetamide GlyC in F2
	26147	^{601}LKNKETCKDYDKFQKIPQFL^{620}	500	6.8	
	26160	^{861}GHSESSLNRTTNAQDIKIGR^{880}	590	1.1	
	26170	^{1061}CNNEYSMEYCTYSDERNSSP^{1080}	600	3.1	
EBA 181	30030	^{81}KKVKIISRPVENTLHRYPVS^{100}	394	2.1	
	30051	^{501}LLWKKHGTILDNQNACKYIN^{520}	595	1.5	
	30060	^{681}KDRKLFSLAKDKNVTTFLKE^{700}	178	0.6	PEXEL
EBL 1	29923	^{620}CNAILGSYADIGDIVRGLDV^{639}	415	10.5	Gly B
	29924	^{640}WRDINTNKLSEKFQKIFMGG^{659}	450	7	Gly B
	30018	^{2501}LEDIINLSKKKKKSINDTSFY^{2520}	245	8	

Merozoite's cHABPs II

	PEPTIDE	SEQUENCE	K_d (nM)	NRSC (x10^5)	Function
Rh1	36389	^{601}IYIQPILNNLHTLKQVQNNK^{620}	ND	ND	
	36396	^{741}KYILKQKDIELTQHVYTDEK^{760}	ND	ND	
	36397	^{761}INDYLEEIKNEQNKIDKTID^{780}	ND	ND	Ca^{++} signal
	36482	^{2462}IKEQKNIYTQKEIDISKIIK^{2481}	ND	ND	
Rh2a/b	26818	^{2421}NITNLLGRINTFIKELDKYQ^{2440}	300	97	
Rh2a	26835	^{2761}LEREKQEQLQKEEELKRQEQ^{2780}	250	5.3	
Rh2b	26529	^{3021}SDIHMDSVDIHDSIDTDENA^{3040}	200	10	
	26534	^{3121}ITEKLVDIYPSTYRTLDEPM^{3140}	300	40	PEXEL
Rh4	34195	^{461}CTNIKKYTDDICLSIKPKAL^{480}	870	180	CR1 binding
	34203	^{621}QIYKNELKDRIKETQTKINL^{640}	590	90	CR1 binding
	34215	^{861}LFDYTHLWDNAQFTRTKENI^{880}	1200	150	Trypsin, chymotrypsin ultra-sens.
	34224	^{1041}YDKLNEHVINNLYTKSKDSL^{1060}	480	60	Trypsin, chymotrypsin ultra-sens.
	34243	^{1421}LNTILHRNEQTKNATRSYNM^{1440}	850	90	Trypsin, chymotrypsin ultra-sens.
Rh5	36727	^{20}GKYIAVDAFIKKINETYDKV^{220}	1300	65	Aotus RBC
	36735	^{361}DEYIHKLILSVKSKNLNKDL^{380}	ND	ND	Basigin
	36739	^{441}KIKLNIWRTFQKDELLKRIL^{460}	ND	ND	Basigin
MAEBL	30195	^{401}TGSCYFLKKKPTCVLKKENH^{420}	220	16.4	
	30212	^{741}KSKIFSNRFTMKEYDPKTRL^{760}	300	18	
	30220	^{901}KSKVFGTFDQKTGKCKSLMD^{920}	380	8	
	30276	^{2021}EMDLNYDKIYTLAMINNEET^{2040}	ND	ND	Salivary Spz
AMA 1	4310	^{74}QHAYPIDHEGAEPAPQEQNL^{93}	670	270	Prodomain
	4313	^{134}DAEVAGTQYRLPSGKCPVFG^{153}	120	11	
	4325	^{374}MIKSAFLPTGAFKADRYKSH^{393}	100	5.1	
	4332	^{514}AEVTSNNEVVVKEEYKDEYA^{533}	890	665	ROM 1 site
	4337	^{603}WGEEKRASHTTPVLMEKPYY^{622}	ND	ND	Ⓟ +Aldolase
Pf. EMP 1	6510	^{128}GACAPYRRLHVCDQNLEQIE^{147}	119	37	A blood group
	6573	^{1257}TDISHDGACMPPRRQKLCLY^{1275}	ND	ND	Motif
KHARP	6786	^{378}KSKKHKDHDGEKKKSKKHKD^{397}	190	1	PEXEL
HRP II	6800	^{24}NNSAFNNNLCSKNAKGLNLN^{43}	200	6	2xPEXEL
REX 2	33782	^{21}DTLGSSNFSPLKPCEGLECL^{40}	NS	ND	PNEP
REX 3	33945	^{1}MQTRKYNKMLSKVETKQFIY^{20}	440	84.3	
	33947	^{41}EGSSFRQLSEPVVEEQDLKK^{60}	ND	ND	PEXEL
REX 4	33965	^{61}REININKNIPSYPVKFSKLE^{80}	500	48.2	10x PEXEL
STEVOR	30561	^{41}MKSRRLAEIQLPKCPHYNND^{60}	110	7.7	PEXEL
	30567	^{161}ASCCKVHDNYLDNLKKGCFG^{180}	90	4.0	Gly C
ETRAMP 10.2	33882	^{41}EQDLQQKKNRKRNLILYSLG^{60}	900	466	PNEP
	33891	^{221}TPTESSHGISDGKKDTSTND^{240}	NS	ND	
SERA	6737	^{409}YDNILVKMFKTNENNDKSELI^{428}	500	120	
	6746	^{589}DQGNCDTSWIFASKYHLETI^{608}	150	30	Enzyme
	6754	^{749}YKKVQNLCGDDTADHAVNIVG^{768}	1100	100	Enzyme
	6758	^{829}KTTKKESKIYDYYLKASPEF^{48}	ND	ND	Enzyme block
CLAG 3.2	30387	^{340}VYYSEKKRRKTYLKVDRSST^{359}	107	2.2	
	30421	^{1020}NSLLPPYAKKPITQLKYGKT^{1039}	255	1.8	PSAC
	30428	^{1160}LDAYKSFPGGFGPAIKEQTQ^{1179}	416	3.4	
CLAG 9	33815	^{501}EKCYIRNRKNRSFSHRIASI^{520}	600	1.400	
	33840	^{1001}MYDKLTNVFFPMNIKKPTIQ^{1020}	300	480	
	33846	^{1121}GGNMLYRNILYFPNHLPEEL^{1140}	NS	ND	
RhopH3	33483	^{81}KFRVPKHLKDKNIHNFTPTL^{100}	465	168	HMIC
	33491	^{623}PKEFELIKSRMIHPNIVDRI^{642}	540	176	
	33570	^{643}LKGIDNLMKSTRYDKMRTMY^{662}	680	38	
RAMA	33426	^{79}NINILSSVHRKGRILYDSF^{97}	400	180	PEXEL
	33460	^{765}HKKREKSISPHSYQKVSTKVQ^{785}	400	34	H-ATPase
PI 12	33631	^{221}IGFKCPSNYSVEPHDCFVSA^{240}	ND	ND	
	33633	^{261}MDHYNNTFYSRLPSLISDNW^{280}	1000	23.7	
PI 38	33645	^{141}VLRIHISNGVLRKIPGCDFNY^{160}	550	24,6	
PI 42	33713	^{261}AGKVNNKVCKIQGKPGELVG^{280}	350	24	
	33715	^{301}LHKNKVTDLKTLIPGYASYT^{320}	550	40.2	
PTRAMP	33405	^{21}YISSNDLTSTNLKVRNNWEH^{40}	170	17	
	33413	^{177}LEGPIQFSLGKSSGAFRIN^{195}	200	17	

traverse endothelial cells twice, entering it to navigate in the blood stream and leaving it when arriving at the liver (Amino et al., 2008). It stops in the Disse space and the liver sinusoidal cell layer to start searching for a hepatocyte to infect (Figure 1B). The liver sinusoids are a unique vascular system having a fenestrated endothelium where an extracellular highly-rich heparin sulphate proteoglycan (HSPG) matrix protrudes, separating endothelial cells from hepatocytes where Kupffer (phagocytic) cells are also present to destroy all potentially dangerous particles (Figure 1B).

Cell trespassing (or cell traversal activity) (Mota et al., 2002) is mediated by a set of proteins, the most relevant being sporozoite protein essential for cell traversal 1 and 2 (SPECT 1 and 2) (Ishino et al., 2004) and cell traversal protein for ookinetes and sporozoites (CelTOS) (Kariu et al., 2006) and thrombospondin related sporozoite protein (TRSP) (Labaied et al., 2007) (Figures 1C and 2A).

SPECT-2 (*Plasmodium* perforin-like protein 1 (PPL-1)), a Spz micronemal 95kDa protein translocated to Spz membrane during infection (Kaiser et al., 2004) (Figure 1A), contains a membrane attack complex/perforin-related (MACPF) domain. When SPECT 1 and 2 are genetically knocked out (KO), such Spz can glide but cannot traverse through host cells *in vivo* or *in vitro* (Ishino et al., 2004).

The SPECT-2/MACPF domain is highly homologous to complement system proteins C6 to C9, especially C8α, having a similar function regarding pore formation and permeabilisation of host cell membranes, very similar to many microbial pore-forming toxins (PFT) and cholesterol-dependent cytolysins (CDC) secreted by Gram-positive bacteria (Rosado et al., 2008).

The C8α MACPF domain (recombinant fragment 103-462 residues) 3D structure determined by X-ray crystallography shows centrally-kinked, ~90° four-stranded, β-sheets surrounded by α-helices and β-strands forming the D1 and D3 structural segments, similar to the intermedilysin (ILY) CDC proteins which also contain D2 and D4 segments. D4 mediates membrane binding in CDCs and D2 links D1 and D3 for pore formation (Hadders et al., 2007); (Giddings et al., 2004).

cHABP 34936 contains ^{301}Y which creates a hydrophobic niche in this MACPF structure, establishing an H-bond and π resonant structure with cHABP 34938 ^{335}Y where host cell membrane phosphocholine (PC) binds to mediate cell traversal activity (De Colibus et al., 2012). SPECT 2 cHABP 34941 contains the KN sequence also present in cHABP 34951 suggested as the binding motif for heparin-sulphate (HS) moieties (Polekhina et al., 2005).

MACPF C2 region 34949 peptide contains the ^{547}DX^{549}FXX^{552}D^{553}D motif which, together with ^{487}D in cHABP 34946, has the coordinated sequence and orientation for Ca^{++} binding (in the **c**alcium **b**inding **r**egion **1** or CBR1) after which a strong interaction with host membrane begins (Law et al., 2010).

Outside the MACPF domain, cHABP 34957 binds chondroitin sulphate (CS) in a niche created with 34958. cHABP 34959 contains the ^{744}TT/I/W^{747} motif which specifically binds cholesterol in ILY (CDC) microbial perforins.

Recent interest has been shown in SPECT-1; its 3D structure has shown (Hamaoka and Ghosh, 2014) that host cell cholesterol and/or heparin-like and/or dematan-like oligosaccharides having high or low sulfate content can fit in the ~750 Å^3 cavity entirely containing cHABP 33372 (^{81}A to ^{97}Y) and ^{156}N and ^{161}K in cHABP 33375. SPECT 1 cHABP 33372 also contains ^{98}S, ^{99}F/L and ^{100}T/S forming a juxtaposing deep pocket where a still unrecognised receptor binds (Hamaoka and Ghosh, 2014).

CelTOS, a Spz protein, is present in different host-invasive stages, playing a critical role in breaking through cell barriers. Targeted disruption of the *CelTOS* gene reduces parasite infectivity 200-fold in the mosquito host and Spz infectivity in the liver, abolishing Spz cell-passage ability (Kariu et al., 2006). Two HABPs have been identified: highly conserved 34451 and highly variable, antigenic and immunogenic HABP 34458 (therefore not included in this manuscript) (Curtidor et al., 2012).

MB2 is a 185 kDa protein expressed in Spz, liver stage (LS), blood-stage (BS) parasites and gametocytes. This protein has an amino-terminal basic, a central acidic and a carboxyl-terminal domain, the latter having great similarity with the GTP-binding domain where peptide 34357 containing XX^{1004}GTK^{1006} and 34362 containing ^{1101}KDV^{1103}coordinate and bind GTP from prokaryotic translation initiation factor 2 (Nguyen et al., 2001) (Arévalo-Pinzón and Curtidor, unpublished results).

Sporozoite invasion-associated protein-1 and -2 (SIAP-1/S5 and SIAP-2) (113kDa and 45kDa respectively) have similar location on Spz membrane to that of CSP-1 and participate in cell traversal and hepatocyte invasion. Differential proteomic analysis has shown that these proteins' expression is increased 10X and 4.4X, respectively, when Spz incubation temperature rises from 24°C to 37°C, similar to what occurs when Spz are transmitted from a mosquito's salivary glands to human skin during the mosquito bite (Siau et al., 2008).

SIAP-1 cHABPs 34893 and 34916 bind specifically to HeLa and hepatocyte cells, the former containing KN sulphate-binding motifs while SIAP-2 cHABPs 36876 and 36878 only bind HepG2 cells (Arevalo-Pinzon et al., 2011); the latter also has the KN binding motif. Antibodies against SIAP-2 have significantly decreased cell traversal percentage in dose-dependent inhibition of invasion (Siau et al., 2008). *Anopheles* mosquitoes infected with *siap-1*(−) parasites cannot transmit malaria to susceptible rodents, despite the normal formation of Spz in their midgut (Engelmann et al., 2009).

Multi-functional CSP-1

Last but not least, due to its tremendous impact in the malaria vaccine development process the CSP-1

multifunctional protein which accounts for 5-15% of total Spz [^{35}S] methionine incorporation, densely coating Spz surface (Figure 1C) displays a common characteristic molecular structure in all *Plasmodium* parasites, having a variable length and composition central tandem repeat region (CRR) consisting of a highly antigenic and immunogenic major tetrapeptide (NANP) repeated 30-40 times intercalated 4 times with a minor repeat (NVDP) sequence (Dame et al., 1984). CRR (originally suggested as the most relevant epitope for antimalarial vaccine development, though discarded after numerous human trials) has recently been found to be critical for Spz formation and maturation during sporogony in oocyst development inside the mosquito's midgut (Ferguson et al., 2014).

The CRR is flanked by two relatively conserved regions (RI and RII) (Dame et al., 1984). RI has two RxLxE *Plasmodium* export element (PEXEL) motifs, one completely included in the N-terminus of cHABP 4383 involved in CSP entry to hepatocyte cytoplasm to promote parasite development in the liver (Singh et al., 2007). 4383 also contains the target amino acid sequence for protective antibody induction in its C-terminal portion (^{81}E to ^{87}R) (Espinosa et al., 2015). cHABP 4383 is located 5 residues upstream the ^{101}(KKLKQP)^{106} motif used by CSP-1 to bind to glucosamine glycan (GAG) and heparan sulphate (HS) moieties present on hepatocyte membrane (Rathore et al., 2002). This KKLKQP sequence also becomes the CSP-1 cleavage site once Spz contacts the highly sulphated proteoglycans on hepatocyte membrane (but not dermal cells), releasing a ~10kDa N-terminal fragment covering, protecting and masking mature CSP-1 protein and its adhesive cell domain in mosquito salivary gland Spz to be exposed to the hepatocyte cell adhesive domain in a vertebrate host (Coppi et al., 2011). KKLKQ has very recently been suggested as an equally efficient non-canonical PEXEL motif (Schulze et al., 2015).

cHABP 4388, completely containing an amino acid sequence linking CRR to RII, is located 15 residues upstream the high content HSPG binding region on hepatocyte membrane to which Spz bind and halt their motion to start invasion and reproduction inside liver cells (Frevert et al., 1993).

3D structure has shown that RII in the II^{+} region completely contains cHABP 4397, immediately followed by VRVRKRKNV (nuclear localisation signal, NLS), to enter the hepatocyte nucleus (Singh et al., 2007). cHABP 4397 is topologically located in strand 1 where ^{331}W interacts with neighbouring non-binding ^{345}R, generating a π-cation interaction where hepatocyte HSPG can bind in a hydrophobic groove where ^{327}L forms part of the wall. This region structurally and functionally resembles TRAP TSR region, having two antiparallel β-strands and defined β-turns stabilised by ^{334}C and ^{338}C, rather than the ripped β-strand in TRAP having an α1 helix in CSP-1 (Tossavainen et al., 2006).

TRSP is a 18kDa (163 aa long) protein located in Spz rhoptries, containing a characteristic signal sequence (SS) and a C-terminal hydrophobic region and a TSR domain in its N-terminal region, playing a relevant role in hepatocyte entry (Kaiser et al., 2004). cHABP 36075 is located 3 residues upstream this protein's RII^{+} region and has a PEXEL motif (Curtidor et al., 2012).

Disappointing results have been obtained in countless human trials using large recombinant, DNA or vector-based vaccines including *P. falciparum* Spz proteins such as CSP-1. This would suggest the need to include some others functionally-relevant epitopes in such vaccine, like cHABPs candidates derived from CSP-1, TRAP, SPECT 1, 2, CelTOS, MB2, TRSP, SIAP-1 and 2 to be used as components of a minimal subunit-based, multi-epitope, multistage, chemically-synthesised antimalarial vaccine (Curtidor et al., 2011; Garcia et al., 2006; Patarroyo et al., 2011).

Liver stage cHABPs

Spz dramatically change their morphology after invading hepatic cells (Hegge et al., 2010; Kappe et al., 2003); the liver stage (LS) reproduction cycle begins, implicating several molecules. The most studied from the immunological point of view have been liver stage antigens 1 and 3 (LSA 1, 3), STARP and SPATR (Daubersies et al., 2000; Fidock et al., 1994b; Fidock et al., 1997; Kurtis et al., 1999). Interestingly, all these LS proteins have degenerated highly antigenic and immunogenic tandem repeat regions, varying in length and copy number, mostly deposited as flocculent material inside infected hepatocytes and considered decoy fragments to distract the immune response (Fidock et al., 1994b; Fidock et al., 1997).

Only cHABP 20630 has been found in the 240 kDa LSA-1 protein in non-repeat region A (NR-A); it has high binding affinity to both hepatocytes and RBC and has the sulphate binding KN motif, lacking any other known biological function (Curtidor et al., 2011).

The very relevant, highly immunogenic 200 kDa LSA-3, now in human trials, expressed on Spz and the periphery of maturing hepatic Mrz, has one NR-A, followed by repeat I region, along with R2, an NR-B, short R3 and a C-terminal NR-C (Daubersies et al., 2000). cHABP 26241 has a PEXEL motif in the NR-A, suggesting that this protein could be transported thorough the membranes to infected liver cell surface, whilst cHABP 26293 in NR-B has been assigned no recognised biological function to date (Curtidor et al., 2011).

Little is known about the biological function of 78 kDa **s**porozoite **t**hreonine/**a**sparagine **r**ich **p**rotein (STARP) but the fact that it is a Spz membrane protein (identified by electron microscopy and immunofluorescence in the early ring stages of erythrocyte development (Fidock et al., 1994a)) confers appropriate support for its inclusion as a vaccine component. This is further supported by the fact that cHABP 20546 has a PEXEL motif, suggesting that it is transported to hepatocyte and RBC membranes and large-scale serological analysis in African hyper-endemic areas has placed STARP as the second most relevant molecule

in sterile protective immunity induction prior to the high malaria transmission season (Fidock et al., 1997).

Most Spz proteins and their corresponding cHABPs which are relevant in the cell host-microbial interactions described here have been recognised by total and putative proteomics of *P. falciparum* salivary gland Spz (Lindner et al., 2013).

The asexual blood stage

New strategies for new targets: RBC

Spz have incredibly fast proliferation and differentiation speed where infected hepatocytes produce 30,000 new descendants in one week which can change their morphology into round, pear-shaped structures, named Mrz, having completely different biochemical and functional characteristics to enable them to invade their new target: the RBC (Figure 1E, G). The very elegant work by Alan Cowman (Weiss et al., 2015) and some other groups during the last few years has shown the coordinated sequence of events in Mrz invasion of RBC through live cell imaging filming and super resolution (Riglar et al., 2011).

Mrz cHABPs involved in initial contact with RBC

Initial contact involves weak receptor-ligand interaction during Mrz rolling on RBC surface, inducing slight erythrocyte deformation mediated by heparin-like receptors (Boyle et al., 2010) and MSP proteins like MSP1, -2, -4, -8, -10 anchored to the RBC membrane via a glycosyl-phosphate-inositol (GPI) tail (Figure 1F) (Sanders et al., 2005; Sanders et al., 2006) which are non-covalently associated with other peripheral membrane proteins like MSP-6, -7 and 9, forming high molecular invasion complexes (HMIC) (Figure 1H) (Boyle et al., 2010).

The above is followed by dramatic RBC deformation, depending on actin-myosin motor activation mediated by strong receptor-ligand interactions, involving microneme stored and surface transport erythrocyte binding antigens (EBA 175, 140, 181, and EBL) and rhoptry stored and surface discharged reticulocyte binding-like proteins (Rh1, Rh2a, 2b, Rh4) (Figure 1G). Pore formation then involves Rh5-basigin interaction, followed by tight junction (TJ) formation mediated by the AMA1-RON2 complex which facilitates invasion of RBC. Transient echinocytosis formation of infected red blood cells (iRBC) lasts 5-10 minutes, probably caused by RBC dehydration and recovery to their normal shape when Mrz become rings to start the reproduction cycle (Paul et al., 2015; Weiss et al., 2015). All these proteins' cHABP fundamental functions are analysed below (Figure 1F).

MSP-1 is the most abundant 200 kDa protein expressed on Mrz surface (Gilson et al., 2006). It forms an HMIC with the MSP6 and MSP7 in the endoplasmic reticulum (Figure 1H), mediating weak receptor-ligand interactions during parasite rolling (Kauth et al., 2006). MSP1 undergoes primary proteolytic cleavage on the Mrz surface giving rise to N-terminal 83 kDa, internal 30 kDa and 38 kDa and C-terminal 42 kDa fragments, followed by a second (calcium enzymatic dependent) cleavage of $MSP1_{42}$ fragment into $MSP1_{33}$ and $MSP1_{19}$ segments (Blackman and Holder, 1992). The second cleavage releases the HMIC to the milieu (Figure 2B), with only the C-terminal GPI-anchored $MSP1_{19}$ fragment remaining anchored to the parasite membrane to enter RBC. $MSP1_{19}$ has two EGF-like domains shown to be involved in parasitophorous vacuole (PV) development and sealing, where it remains until the end of the intracellular cycle (Dluzewski et al., 2008).

Note: All cHABPs amino acid sequences are shown in Table 1.

Analysis of the MSP1 amino acid sequence and critical binding residues has revealed that all our cHABPs have been deeply involved in these very relevant biological functions (Urquiza et al., 1996). cHABP 1522 is present in the $MSP1_{83}$ fragment which interacts with the glycophorin A protein fragment (residues 31 to 72) (Baldwin et al., 2014); cHABP 1585, located five residues downstream the primary cleavage of MSP1 (generating the $MSP1_{42}$ fragment), interacts with K5-NSOS-H heparin moieties (Boyle et al., 2010). cHABP 5501 is located at the beginning of the first EGF-like domain, containing at its N-terminus the MSP1 secondary cleavage site that yields the MSP1 19 kDa. It interacts with the band 3 (5ABC) sequence (residues 726-761) (Li et al., 2004).

Parasite rolling and subsequent weak RBC deformation mediated by GPI-anchored proteins and the EGF domain containing cHABPs

Other MSPs have been putatively involved in morphologically defined rolling and RBC surface deformation (originally weak, later becoming very strong) (Figures 1F and H), i.e. MSP4, MSP8, MSP10 anchored to Mrz membrane via a GPI tail. Strikingly, together with other detergent-resistant membrane (DRM) proteins, Pf12 and Pf38 are GPI anchored, this being a very common characteristic of Mrz-derived proteins involved in RBC attachment (Figure 2B) (Sanders et al., 2005). This anchor is very rare in Spz-derived proteins or Mrz proteins involved in some other biological functions (Figure 2B).

The 272 amino acid-long 40 kDa MSP-4 has only one cHABP (20494) located at the C-terminal end within the EGF-like domain (Rodriguez et al., 2008); 70 kDa MSP8 also contains an EGF-like domain including cHABP 26373 while MSP-10 cHABP 31132 is contained in a 61 kDa fragment which is further processed into a 36 kDa (Rodriguez et al., 2008). These 3 cHABPs, together with MSP-1 cHABP 5501, bind to RBC band 3 protein fragment 5ABC (residues 726 to 761), thereby mediating initial stages during invasion. It has been suggested that such redundant sequences are used to escape immune pressure by switching just their C-C residue location, due to amino acid sequence similarity in their EGF domains (Puentes et al., 2003).

The 48 kDa soluble glutamate-rich MSP6 protein is cleaved at ^{161}S in HMIC formation to yield an $MSP6_{36}$ fragment having great similarity with another member of the family, the MSP-3 protein. $MSP6_{36}$ forms a tetramer molecule in a tail-tail configuration mediated by cHABP 31191 which is

non-covalently bound to the $MSP1_{38}$ fragment (Trucco et al., 2001).

351 amino acid-long 41 kDa MSP7 is processed by removing the SS preceding a 38 kDa polypeptide processed further on at residues ^{176}Q and ^{177}S to generate 17 kDa N-terminal and 22 kDa C-terminal fragments. The latter is further processed to yield a 20.7 kDa fragment and sequentially another 19 kDa one, the latter binding to the $MSP1_{83}$ and $MSP1_{38}$ fragments to form the Mrz-derived HMIC (Pachebat et al., 2007). MSP-7 cHABPs 26114 and 26116 binding becomes totally abolished by RBC trypsin and chymotrypsin treatment, suggesting that these cHABPs could form a link between RBC and HMIC (Garcia et al., 2007).

Soluble 85 kDa MSP9 or acid basic repeat antigen (ABRA) participates in HMIC formation. cHABPs 2149, 2150 and 2153 are present in ABRA, 2149 having very high homology with a human cytosolic phospholipase A_2 active site (Table 1, highlighted in bold), so much so that this cHABP has dose-dependent haemolytic activity at low concentrations (50μM), while cHABPs 2150 and 2153 in the MSP9Δ1a (residues 77-241) recombinant fragment also bind to RBC band 3.0 in the 5ABC peptide (Li et al., 2004; Rodriguez et al., 2008)

MSP-2, an abundant, intrinsically disordered membrane coat protein is anchored to Mrz surface via a GPI tail (Figure 2B). It has two allele forms (3D7 and FC27) having numerous variations, displaying N- and C-terminal, highly-conserved regions flanking a hypervariable and unordered central region, tending to self-aggregate and form microfibrils (Adda et al., 2009). The only cHABP (4044) located in this protein's N-terminus has been found to have an amphipathic structure (Edmunson's wheel) between residues ^{10}T to ^{22}R with amino acids ^{12}I, ^{16}Y and ^{20}I strongly and specifically interacting with dodecyl phosphocholine (DPC) and phosphatidyl inositol (PI) moieties on RBC membrane (MacRaild et al., 2012; Zhang et al., 2008), stressing this cHABP's very relevant role in aiding parasite invasion. MSP2, together with MSP4 (another GPI anchored protein), are the only complete, unprocessed MSPs carried inside the RBC (Boyle et al., 2014).

cHABPs in strong RBC deformation promoting invasion

It has been suggested that low potassium (K^+) level in blood triggers Mrz Ca^{++} release thereby activating microneme EBL (EBA-175, 140, 181, EBL-1) and rhoptry stored *Pf*Rh (Rh1, Rh2a, Rh2b, Rh4, Rh5) release (Singh et al., 2010); these proteins cooperate alternatively in a strong receptor-ligand type interaction to cope with RBC receptor genetic variability and immune pressure (Persson et al., 2008). These molecules have similar complementary and exquisitely redundant functions regulated by epigenetic factors (Cortes et al., 2007).

EBA 175 is the dominant ligand in the EBL family, having very similar 3D structural and functional characteristics. It is synthesised as a 175kDa type 1 transmembrane protein, consisting of an SS followed by region II (RII) subdivided into two tandem Duffy binding-like (DBL) cysteine-rich related regions (F1 and F2) and regions III-IV linking RII to RV and RVI, a small cysteine-rich region followed by a transmembrane region and a small cytoplasmic tail (Sim et al., 1990) (Table 1).

3D structural analysis of an RII recombinant fragment containing F1 (residues 8-282) and F2 (residues 297-603) regions (Tolia et al., 2005) has located cHABP 1783 in F2, where ^{436}H allowed dimerisation with a contralateral ^{435}V in an inverted EBA-175 molecule, forming a "hand-shake-like" structure. Residues ^{439}K and ^{442}D bound glycophorin A glycan 5 Neu5Ac1 via H-bonds. cHABP 1783 ^{450}W has also induced fold stabilisation at this site in the same RII and contained critical residue ^{446}R establishing a salt bridge with ^{30}D, thereby dramatically reducing RBC binding when ^{446}R is mutated (Rodriguez et al., 2008; Tolia et al., 2005).

cHABP 1779 is in close proximity to glycan 5 and 6 binding sites in F1 but does not interact directly with them (Salinas et al., 2014; Wanaguru et al., 2013a); antibodies against 1779 could therefore have exerted such influence by steric-hindrance. It has been found recently that EBA-175 high activity binding to RBC has depended on both the DBL domains (F1 and F2) where 1779 and 1783 are located, such binding being inhibited and neutralised by antibodies but not galactose (Salinas and Tolia, 2014).

Region V cHABP 1815 contains a conserved sequence present in all EBL molecules where critical binding residues ^{1258}R and ^{1265}F link cHABP 1815 with 1818 in N-terminal region VI for mediating this protein's correct microneme trafficking (Sakura et al., 2013). Furthermore, EBA-175 cHABP 1818 has been located close to the cleavage site by rhomboid 4 protease, releasing EBA 175 to the milieu during invasion to allow PV formation. The complete recombinant EBA-175 ectodomain (PfEBA-175FL) binds RBC in a trypsin- and neuraminidase-sensitive manner, having greater affinity than the isolated RII fragment, thereby confirming the role of cHABPs located outside RII in RBC invasion (Rodriguez et al., 2008; Wanaguru et al., 2013a).

EBA-140 has a similar structure to EBA-175; however, the EBA-140 receptor-binding region contains two conserved Duffy binding-like (DBL) domains. Monomer EBA-140 has two glycan binding sites in the DBL, Pocket 1 (P1) and Pocket 2 (P2), contrasting with six glycan binding sites per dimer in PfEBA-175, suggesting that EBL proteins can create multiple binding pockets to accommodate different receptors (Lin et al., 2012; Malpede et al., 2013). EBA-140 ^{541}D^{550}D^{551}I in cHABP 26144 and ^{618}Q in cHABP 26147 are P2 components (Figure 1I), where cHABP 26144 ^{556}Y establishes H-bonds with the glycophorin C glycan 2 sialic acid acetamide group (Malpede et al., 2013; Rodriguez et al., 2008). Mutations in ^{556}Y have resulted in a 35.2% decrease in RBC binding ability while ^{457}N in HABP 26139 P2 interacts with carboxyl group GlyC. Our corresponding peptides had very low binding capacity in EBA-140 P1 3D structure and critical ^{182}Q was followed by variable ^{185}I, thereby altering RBC binding ability, which is why they were not considered in our studies (Malpede et al., 2013). Apart

from sialic acid-dependent EBA-140 interaction with sialoproteins, it has also been found that this protein also strongly interacts with glycophorin C backbone residues; however, such specific receptor sites have not yet been described, even though cHABPs 26160 and 26170 located in this protein's region III and V could perform this function, since their binding to chymotrypsin-treated RBC became reduced by >80% (Lin et al., 2012; Malpede et al., 2013; Rodriguez et al., 2008).

EBA-181 binds to a putative W receptor suggested to be a band 4.1 10 kDa fragment which is susceptible to neuraminidase and chymotrypsin treatment (Lanzillotti and Coetzer, 2006). cHABPs 30030 (located in this protein's binding domain) and 30051 are very susceptible to neuraminidase treatment (≥75% binding reduction), the latter being extremely susceptible to chymotrypsin treatment (≥92% binding reduction), completely fulfilling the enzymatic profile established for this molecule. cHABP 30060 has a non-canonical PEXEL motif ^{683}RK^{685}LF^{687}S, suggesting this protein's transport through membranes (Rodriguez et al., 2008).

It has been reported that the erythrocyte-binding-like 1 (EBL-1) protein's D2 domain or F2 region binds to a receptor in glycophorin B which is resistant to trypsin but sensitive to chymotrypsin and neuraminidase (Mayer et al., 2009). The core binding site is contained within the 69 amino acid region, named F2i (residues ^{601}C to ^{669}V), where cHABPs 29923 and 29924 are completely located (Li et al., 2012; Rodriguez et al., 2008). The cHABP 30018 receptor is extremely susceptible to trypsin (65% binding reduction) and chymotrypsin (95% reduction) and is located 10 residues upstream rhomboid 4 cleavage site and the Cys-rich region; another binding site could thus exist in a different region, as in the other EBL proteins (Table 1).

The MAEBL 243 kDa hybrid protein expressed in Mrz and Spz rhoptries, having a similar structure to other EBLs, has a carboxyl terminal (R6) cysteine-rich domain, duplicated AMA-1-like domains (D1 and D2) instead of a DBL domain in R2 (Ghai et al., 2002). AMA-1-like domains have cell binding abilities where cHABPs 30212 and 30220 have been found in the M2 domain, the main RBC binding domain (Ghai et al., 2002; Rodriguez et al., 2008). cHABP 30195 in M1 is very susceptible to chymotrypsin. A MAEBL isoform containing the C-terminal transmembrane domain (ORF1) is essential for Spz invasion of the *Anopheles* mosquito's salivary glands and cHABP 30276 (residues 2021-2040) is located in this region containing critical residues ^{2021}E, ^{2030}Y, ^{2038}E and ^{2039}E (Rodriguez et al., 2008; Saenz et al., 2008).

The role of the PfRh family in RBC invasion

This family of rhoptry proteins, having high homology with *P. vivax* reticulocyte binding proteins (Rh or RBL), includes Rh1, 2a, 2b and 4, having very high molecular weights (~350kDa) (Figure 2B and Table 1), except for the recently described Rh5 (63kDa). PfRhs are recognised by their interactions with receptors having variable susceptibility to enzymes, specifically neuraminidase where the Rh1 receptor is sialic acid dependent (SAD) and Rh2b and Rh4 are sialic acid independent (SAI) (DeSimone et al., 2009; Stubbs et al., 2005).

Sialic acid dependence for triggering functional Ca^{++}

The 358 kDa Rh1 SAD protein, processed into a 240 kDa N-terminal and 120 kDa C terminal fragments before Mrz release, contains 8 cHABPs whose interaction with neuraminidase-treated RBC becomes completely abolished (Arevalo-Pinzon et al., 2013). cHABP 36389, which is extremely susceptible to RBC trypsin treatment, is located in RII-3, thereby blocking RBC invasion (Gao et al., 2008). Intermediate binding (~1.5% specific binding) HABPs 36396 and 36397 contain the ^{757}TDEKINDYLEE^{767} sequence triggering the calcium (Ca^{++}) signal during RBC invasion (Arevalo-Pinzon et al., 2013; Gao et al., 2013). cHABP 36482 has been found in the ~10kDa portion, remaining attached to the parasite when the 120kDa fragment in the C-terminus has been cleaved, the 10kDa part being carried into ring stage RBC (Triglia et al., 2009).

Sialic acid independence

PfRh4 is a 220 kDa protein which releases a ~160 kDa fragment when undergoing proteolytic processing (Triglia et al., 2009). The Rh4 region which binds to a receptor on RBC consists of a 30 kDa fragment (^{328}N to ^{588}D) where cHABP 34195 is found (Garcia et al., 2010; Gaur et al., 2007). PfRh4 erythrocyte-binding ability has been shown to be SAI and trypsin and chymotrypsin sensitive, thereby agreeing with RBC complement receptor 1 (CR1) binding to the most membrane-distal of the 30 complement control proteins (CCP) domain, where CCP1 residues ^{7}H, ^{9}L, ^{18}N and ^{20}F form the PfRh4-binding site (Park et al., 2014). cHABPs 34195, 34215, 34224 and 34243 binding has been seen to be extremely sensitive to treatment with trypsin and chymotrypsin (Garcia et al., 2010).

Rh2a, Rh2b cooperate with Rh4 for efficient SAI invasion

P. falciparum strains express different proteins due to the complexity of the host cell-microbe interaction, depending on the RBC receptor's genetic makeup.

Rh2a (~360kDa) and Rh2b have great sequence similarity, where Rh2a is cleaved at its N-terminus to release a 90 kDa fragment and another 270 kDa one, this being further processed into a 130 kDa portion and a 140 kDa fragment which is then transported to the TJ where it has been suggested that it plays a role in helping Mrz enter RBC (Gunalan et al., 2011). Enzymatic treatment has rendered the N-terminus fragment susceptible to neuraminidase, while 270 kDa and 140 kDa are extremely susceptible to chymotrypsin. cHABP 26835 is exclusive to Rh2a while 26529 and 26534 are exclusive to C-terminus region Rh2b where 26534 containing the ^{3134}RT^{3136}LD^{3138}E PEXEL motif is located 50 residues upstream the cleavage site by a rhomboid protease, while cHABP 26818 is common to both Rh2a and Rh2b (Table 1). The critical RBC binding residues for all of them have been identified by glycine analogue scanning (Rodriguez et al., 2008). Rh2b antibodies do not inhibit Mrz invasion when EBA-181 is

absent, suggesting that these two proteins cooperate during invasion (Lopaticki et al., 2011)

cHABPs involved in pore formation

The soluble ~63kDa Rh5 protein is an atypical integrant of the Rh family which interacts with two proteins located in the micronemes: *P. falciparum* Rh5 interacting protein (PfRipr) and GPI-anchored cysteine-rich protective antigen (CyRPA) (Reddy et al., 2015). This complex mediates pore formation through Rh5 binding to basigin and RBC from different species (Wanaguru et al., 2013b). A 45 kDa fragment observed in parasite lysate specifically interacts with CD147 (basigin) a receptor protein expressed on RBC membrane and other cells (Crosnier et al., 2011). The contact residues between basigin and Rh5 are concentrated between residues 197 to 448 where cHABP 36727 is located, containing ^{207}D which, together with ^{362}E/D located in peptide 36735, contact the basigin C-terminal domain. Furthermore, cHABP 36727 is located one residue downstream of those contacting the basigin amino terminal extreme (Wright et al., 2014). Point mutations at Rh5 cHABP 36727 residue I204K have been involved in binding activity and specie-specific invasion of *Aotus* monkey RBC (Arevalo-Pinzon et al., 2012). cHABP 36739 (unpublished results) is located in the Rh5 C-terminal fragment (residues ^{447}WRT^{449}) and interacts with basigin (Arevalo-Pinzon et al., 2012).

Tight junction formation

Electron microscopy has shown that Mrz reorient their apical tip after initial rolling to face erythrocyte surface to form an electron-dense structure moving rapidly inside RBC (i.e. TJ formation), partly mediated by microneme apical membrane antigen 1 (AMA-1) (Mitchell et al., 2004) (Figure 1E and F) and the rhoptry neck protein 2 (RON2) (Collins and Blackman, 2011; Giovannini et al., 2011).

The bulk of 83 kDa precursor AMA1 is formed by the ectodomain, divided into a pro-domain and three structural domains (I, II, III) defined by a pattern of 16 conserved cysteines contributing 8 disulphide bonds (Hodder et al., 1996) and a transmembrane helix followed by a cytoplasmic C-terminal tail (Figure 2 and Table 1). Domains I and II are similar and belong to the plasminogen apple nematode (PAN) super-family, forming a protein fold having a long hydrophobic trough surrounded by major polymorphic sites in domain I and dimorphic residues in domains II and III involved in receptor binding (Bai et al., 2005).

Domain I ^{134}D and ^{143}R cHABP 4313 establishes two H-bonds with cHABP 4325 ^{390}Y^{391}K (domain II) to form a trough or channel where a still unrecognised receptor binds (Patarroyo et al., 2011). cHABP 4337 contains the complete intracytoplasmic domain (^{603}W to ^{622}Y), including^{610}S and ^{613}T phosphorylation sites which are critical in AMA1 invasion, and ^{622}Y the aldolase binding residue (Leykauf et al., 2010). AMA-1 is also involved in Spz invasion of hepatocytes and it has been shown that cHABP 4310 forms a niche stabilised by H-bonds to bind to HepG2 cells (Patarroyo et al., 2011; Schussek et al., 2013); it is topologically very close to cHABP 4332 in domain III (one in front of the other). cHABP 4332 is cleaved between ^{517}T^{518}S while two residues downstream (^{94}F^{95}S) cHABP 4310 is cleaved, these being the only two fragments remaining as stubs entering the RBC. Only the cytoplasmic, transmembrane region and an adjacent 29 residue membrane fragment can be detected in ring-stage parasites (Howell et al., 2001). cHABP 4332 in the *P. yoelii* orthologous system containing region was able to induce sterilising protective immunity against Spz challenge (Schussek et al., 2013).

The role of iRBC surface cHABPs in severe malaria

It has been postulated that *P. falciparum* parasites have developed a series of highly polymorphic molecules and clonal antigenic variation on iRBC membrane for binding different cell types to escape spleen surveillance and clearance. iRBC accumulation in different organs is a key factor in this disease's pathogenesis due to the microvascular obstruction or inflammation induced by it. Virulence-associated proteins would include *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP-1), the subtelomeric variable open reading frame family (STEVOR), early transcribed membrane protein (ETRAMP), ring exported (REX) protein, surface-associated interspersed gene family (SURFIN) (collectively known as variable surface antigens: VSA). Some, like PfEMP-1, are assembled and anchored to RBC cytoskeleton molecules spectrin and actin by molecule like knob-associated histidine-rich protein (KAHRP) which, together with VSA, forms a RBC membrane rigid structure (i.e. knobs), inducing reduced membrane deformability (Spillman et al., 2015).

*Pf*EMP-1 is a 200-350 kDa protein which is encoded by the differentially expressed 59 *var* multigene family (including 56 *var* genes and 3 small *var* genes) where each parasite produces a single *Pf*EMP-1 variant per iRBC (Zhang et al., 2014). This protein mediates adhesion to host cells and molecules like uninfected RBC, ABO blood groups, vascular endothelial, syncytiotrophoblast placental cells, ICAM, CD31 and CD36 molecules (Salanti et al., 2004; Smith et al., 2013; Vigan-Womas et al., 2012).

*Pf*EMP-1 has an extracellular region consisting of 2 to 9 domains which are extremely variable regarding amino acid sequence, composition and length (Figure 2B and Table 1). These domains include an N-terminal segment (NTS), a Duffy binding-like (DBL) 1α domain, a cysteine inter-domain region (CIDR) α1 (all forming the head structure) and DBL2X, C2, DBL3X-DBL4ε to 7ε domains followed by a transmembrane region (TM) and an intracytoplasmic acidic terminal segment (ATS) inserted into iRBC membrane (Smith et al., 2013).

The thousands of PfEMP-1 sequences have revealed this molecule's tremendous variability, having very few and very short conserved sequences. Our approach for identifying cHABPs, working with the Dd2 var 1 clone able to bind C32 cells and RBC, has revealed just two HABP pairs where DBL 1α cHABP 6510 establishes an H-bond between ^{139}C and ^{168}E from HABP 6512 binding to A1 blood group α-1,3 linked N-acetyl galactosamine (Patarroyo et al., 2014).

DBL3X cHABP 6573 ^{1268}R establishes an H-bond with HABP 6583 ^{1456}E and HABP 6583 ^{1257}W and ^{1261}E with HABP 6584 ^{1511}Y and ^{1518}E, present in the same region, creating a niche to accommodate chondroitin sulphate proteoglycans (CSPG). Strikingly, cHABP 6510 and 6573 have the conserved GACXPXRRXXLC motif, being one of the few conserved sequences having a characterised biological function (Patarroyo et al., 2014). The strategy for tackling this molecule's tremendous amino acid sequence polymorphism will involve a completely different methodology, based on in-depth 3D structural knowledge working with restricted configuration peptides (Calvo et al., 2003), pseudopeptides or mimotopes (Lozano et al., 2013).

The cytoadherence-linked asexual gene (*clag9*), encoding at least 9 exons, belongs to the high molecular weight RhopH complex (containing clag/RhopH1, RhopH2 and RhopH3) expressed in blood stages (Trenholme et al., 2000) (Figure 2B and Table 1). CLAG-9 is implicated in cytoadherence, binding to CD36 (the most widespread receptor on endothelial cells) and involved in trafficking of EMP-1 or initial remodelling of host red blood cells so that these proteins can be trafficked to the appropriate location (Gupta et al., 2015; Trenholme et al., 2000). CLAG-9 has cHABPs 33815, 33840 and 33846 where enzymatic treatment of RBC with trypsin and chymotrypsin has significantly reduced cHABP specific binding, suggesting that the cHABP receptor on RBC membrane has a protein composition (Pinzon et al., 2010).

KAHRP, one of the classical members of the transportome concept, is a 80-100 kDa molecule; it consists of an N-terminal histidine-rich domain (region I, residues 41-300), a central lysine-rich domain (region II, residues 301-480) and a C-terminal decapeptide repeat domain (region III, residues 481-660). The classical PEXEL motif ^{54}RT^{56}LA^{58}Q is present in region I, while cHABP 6786 (residues 381-400) is located in region II.

HRPII is 100 kDa, is stored in Maurer's clefts and is assembled on iRBC membrane where cHABP 6800 (residues 24-43) is located two residues before a canonical PEXEL motif (^{45}**R**L^{47}**L**H^{49}**E**) (Lopez-Estrano et al., 2003), suggesting this cHABP's exposure on iRBC membrane could be an important target for vaccine development.

The STEVOR protein family encodes ~30 *stevor* genes organised similarly to *rifin* genes where exon I encodes SS and exon II encodes a family of 34 kDa integral proteins having two transmembrane domains flanking a hypervariable region located in the apical end of the Mrz and transported to iRBC membrane (Blythe et al., 2008). STEVOR transcription peaks at 22-32h in late trophozoites and early schizonts and has been unambiguously demonstrated to be inserted into iRBC membrane (Niang et al., 2009). cHABP 30561 (residues 41-60) has a PEXEL motif in ^{44}RR^{46}LA^{48}E; it is highly susceptible to erythrocyte binding after trypsin treatment of RBC and cHABP 30567 (residues 161-180), which is extremely sensitive to neuraminidase and trypsin treatment, is located in the N-terminal portion close to the transmembrane region and binds glycophorin C to mediate Mrz invasion of RBC and rosseting by iRBC (Bachmann et al., 2015; Garcia et al., 2005; Niang et al., 2014; Sanyal et al., 2012).

Ring exported (REX) proteins are another VSA protein family, consisting of a family of 4 transcripts (REX1, 2, 3 and 4) in all stages of intra-erythrocyte development, mainly expressed ~20 hours (early rings) after RBC invasion (Hawthorne et al., 2004; Spielmann et al., 2006). REX4 (a 26kDa protein), containing cHABP 33965 (residues 61-80), is highly susceptible to chymotrypsin and trypsin treatment of RBC and displays a PEXEL motif 10 residues upstream the cHABP. REX3 has the classical PEXEL motif ^{45}RN^{47}LS^{49}E 25 residues downstream cHABP 33945. PEXEL negative exported protein (PNEP) REX 2 displays cHABP 33782 which is extremely susceptible to neuraminidase treatment of RBC (100% reduction in binding) (Garcia et al., 2009a).

PEXEL motifs (RxLxE/D/Q) are cleaved by an endoplasmic reticulum (ER) resident plasmepsin V after conserved L. They are further acetylated to allow these proteins' maturation and solubility and transport from the parasite's PV and parasitophorous vacuole membrane (PVM) to host cells which, together with proteins lacking the PEXEL motif called PEXEL negative exported proteins (PNEP), are transported to the Maurer's clefts and peripheral membrane (Goldberg, 2012; Gruring et al., 2012).

Likewise, among the 13 family members of early transcribed membrane proteins (eTRAMP), eTRAMP 10.2 displays cHABPs 33882 (residues 41-60) containing a PEXEL motif in residues ^{52}RN^{54}LI^{56}L and 33891 which is extremely susceptible to neuraminidase treatment of RBC (0% binding) and trypsin (9% binding activity) (Garcia et al., 2009a).

These cHABPs also represent excellent targets for *P. falciparum* blood stage vaccine development, being so relevant in protein transport and expressed on iRBC during early *P. falciparum* parasite development stages.

Some other critical functions associated with cHABPs

P. falciparum proteins simultaneously display functions different to receptor-ligand interactions, such as serine repeat antigen (SERA) 5 (one of the 9 members of the SERA family). This 114 kDa protein is processed during Mrz release into a 47 kDa N-terminal, a 56 kDa inner region having serine-like protease activity region and a 18 kDa C-terminal portion. cHABP 6737 is located in the 56 kDa inner region 18 residues downstream (i.e. the subtilisin-1 (SUB1) cleavage site), while cHABPs 6746 and 6754 are more centrally located (Rodriguez et al., 2008). The 3D structure of a recombinant fragment has revealed a non-canonical serine protease active site (Hodder et al., 2009), stabilised by 2 H-bonds between cHABP 6746 ^{588}S and 6754 ^{755}H^{756}A, suggestive of this papain-like cysteine protease's active site. Replacing S^{596}C in the recombinant protein led to such modification inducing clear cysteine protease enzymatic activity (Stallmach et al., 2015). Further cleavage of the 56 kDa located towards the C-terminus liberates a 6 kDa fragment where our 6758 cHABP is completely included. The last C-terminal residues

of this cHABP inhibit this protein's enzymatic activity in an allosteric-like interaction; molecular docking studies have suggested this peptide binds to the SERA-5 active site (Kanodia et al., 2014).

The CLAG 3.2 protein

Plasmodium surface anion channel (PSAC) linked to *P. falciparum* CLAG 3.2 mediates iRBC nutrition, contributing towards ion and nutrient entry. It has been clearly demonstrated that a couple of amphipathic sequences (Edmunson's wheels) in one of this 142 kDa protein's transmembrane domains (adjacent to extracellular motifs) are involved in malaria parasite nutrient channel formation (Nguitragool et al., 2014). These sequences, traversing the RBC membrane, have been located in the C-terminal area, distal to the hypervariable region limits spanning ^{1100}S to ^{1120}Y and ^{1200}F to ^{1220}Y. cHABP 30428 (^{1160}L to ^{1180}Q) has been located in between these two regions in the CLAG 3.2 surface exposed region. cHABP 30421 binds with very high capacity to both C32 and RBC (Rodriguez et al., 2008).

The RAMA protein

This late ring, early trophozoite and immature schizont expressed rhoptry associated membrane antigen (RAMA), synthesised as a 170 kDa precursor, is cleaved to produce a 60kDa C-terminal fragment anchored to the membrane by a GPI tail via a 25 mer hydrophobic sequence (Topolska et al., 2004). It contains cHABP 33460, located in the histidine ATPase region. RAMA has a PEXEL motif in cHABP 34426 (^{91}RI^{93}LY^{95}D) before the first acidic domain, suggesting that this protein is transported through membranes to iRBC surface via this sequence (Pinzon et al., 2008a).

The RhopH3 protein

It has been very recently shown that the 110 kDa RhopH3 protein encoded by seven exons is involved in HMIC formation. This rhoptry's molecule, appearing 30 hours after invasion, binds to RBC band 3 in the 5ABC region through a C-terminal portion (residues 734-865) containing cHABPs 33580 and 33581 (extremely susceptible to trypsin treatment) and to the $MSP1_{19}$ fragment anchored by a GPI tail to the RBC membrane, suggesting that these cHABPs are involved in parasite rolling and initial steps of RBC invasion (Baldwin et al., 2014; Pinzon et al., 2008b; Ranjan et al., 2011).

Pf12, Pf41 and Pf38

The multistage Cys6 family contains some GPI anchored proteins; 6 notably conserved Cys residues form similar domains. Pf12 cHABP 33633 establishes 6 H-bonds between ^{271}RLP^{273} residues with intermediate conserved binding HABP 33631 ^{218}ND^{219} (Arredondo et al., 2012) to generate a niche where an extremely sensitive neuraminidase, trypsin and chymotrypsin receptor binds (Garcia et al., 2009b). Likewise, the Pf38 GPI anchored DRM protein contains cHABP 33645 having the same extreme enzymatic susceptibility. Pf41 without a GPI tail establishes 3 H-bonds between cHABPs 33713 and 33715 and GPI anchored Pf12, having antiparallel orientation to be presented to the host cell (Tonkin et al., 2013). There is a controversy about these proteins' role in RBC invasion.

PTRAMP

Plasmodium thrombospondin-related apical merozoite protein (PTRAMP) is located in the micronemes and subsequently becomes relocated to Mrz surface. This protein contains a TSR domain within its ectodomain and has a cytoplasmic domain which has been shown to weakly interact with aldolase (Thompson et al., 2004). As attempts to delete it have failed, it appears to have an essential and conserved biological function (Thompson et al., 2004). cHABP 33405, located in the PTRAMP amino-terminal region, contained a PEXEL-like sequence in its C-terminal portion whilst HABP 33413 was located in the protein's central region just before the TSR domain (Calderon et al., 2008).

Conclusions

Thorough amino acid level analysis of 49 (13 Spz- and 36 Mrz-derived) of the most important proteins involved in *P. falciparum* infection during the last 25 years has demonstrated that many cHABPs perform very relevant biological functions, such as Spz gliding motility, cell traversal activity, invasion of hepatocytes and reproduction in the liver cells (Ferguson et al., 2014; Ishino et al., 2004; Kariu et al., 2006; Song et al., 2012; Sultan et al., 1997). Other functions are directly mediated by Mrz in RBC invasion as elegantly documented by (Weiss et al., 2015) where the sequence of events like Mrz rolling on RBC surface, strong erythrocyte deformation and invasion, pore and TJ formation, expression on iRBC, ion and nutrient transport are associated with these cHABPs.

This manuscript has provided, for the first time, a deep molecular analysis (at the atomic level when possible) of these cHABPs' fundamental biological functions in host-cell microbe interactions and parasite survival, making them excellent targets for multi-epitope, multistage, minimal subunit based, chemically synthesised vaccine development and some of them even for drug design.

Further work with new methodologies, like the very recently described RBC protein binding Mrz ligands such as basigin allowing recognition of Rh5 as a key molecule in Mrz invasion (Crosnier et al., 2011; Wright et al., 2014) and complement-regulatory protein CD55 (a receptor for bacterial and viral pathogens (Coyne and Bergelson, 2006; O'Brien et al., 2008) whose absence did not enable parasite invasion when EBA-140, EBA-175, EBA-181, Rh1, Rh2a or Rh2b were deleted (Egan et al., 2015), blood group A binding RIFINs proteins, deeply involved in severe malaria (Goel et al., 2015), has suggested that some cHABPs present in proteins for which direct functional activity has not yet been assigned could be directly involved in invasion and other pathogenic mechanisms regarding this very complex parasite, thereby strongly supporting our functional-biological methodology as a way of identifying potential vaccine or drugs targets. Furthermore, these molecules interact in a very synchronic orchestrated way (LaCount et al., 2005; Weiss et al., 2015; Wuchty, 2007), suggesting that their blocking by immune

reactions or drugs could impede the cascade of events leading to host cell invasion; however, future analysis needs further work.

This approach, the first of this kind, has been further supported by the disappointing results which have recently been reported regarding the recombinant RTS,S/ASO1 malaria vaccine candidate tested on 15,459 children and infants who were followed-up for ~4 years (Rts, 2015) yielding 28.3% to 36.3% protective efficacy against clinical malaria and only 17.3% to 10.3% protection against severe malaria defining a malaria case as any individual with fever >37.5°C and more than 5000 parasites/μL (1 infected RBC x 1000 RBC), clearly showing that this is not the appropriate approach for vaccine development. Only one of our CSP-1 functionally-relevant cHABPs (4397, shadowed in Table 1) is present in this vaccine candidate's amino acid sequence, without any further modification, stressing once again the tremendous complexity of the parasite's lifecycle and the impossibility of killing it with a single "magic bullet". Such failure and many more frustrating malaria vaccine human trials involving thousands of people reinforce the importance of the basic work developed by many laboratories throughout the world for decades now, trying to understand this tricky parasite's biology at its deepest level to ensure a logical and rational methodology for effective vaccine development.

Based on large sero-epidemiological results obtained with complex microarray technologies, some malaria experts involved in vaccine development have recently suggested that mixtures of some of the most relevant proteins (whether complete or recombinant fragments) could be the answer to developing a fully complete vaccine against this deadly disease (Boes et al., 2015; Osier et al., 2014). Nevertheless, it is clear that all the molecules required from this complex parasite with tremendous genetic polymorphism in the same molecule, with multiple invasion mechanism, as shown here, with redundant protein systems to evade immune pressure, plus human genetic variability, might complicate such approach.

Therefore, an appropriate mixture of all, or most, of the aforementioned very short HABPs (20 mer long), when properly modified (mHABPs), should lead to a multi-epitope, multistage, minimal subunit-based, fully protective, complete, definitive synthetic vaccine against malaria opening the gate for the development of new vaccines against scourging diseases for humankind, malaria being one of them, as we have been systematically suggesting for more than 30 years now.

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Conflict of interest disclosure
The authors declare no competing financial interest.

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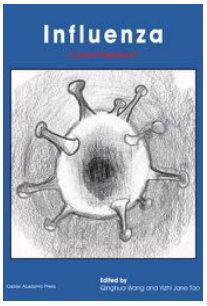

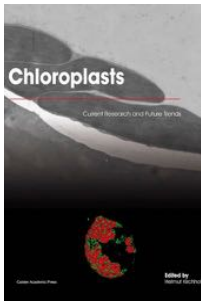

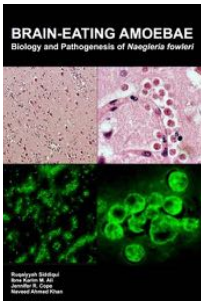

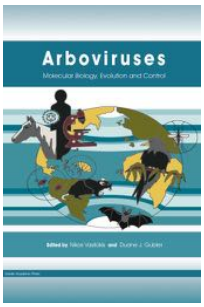

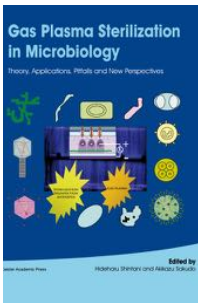

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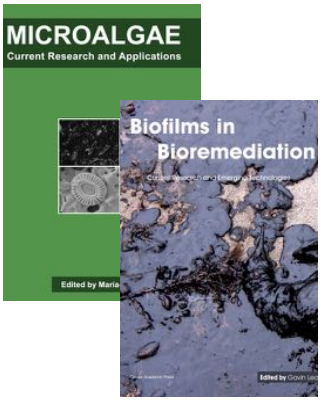

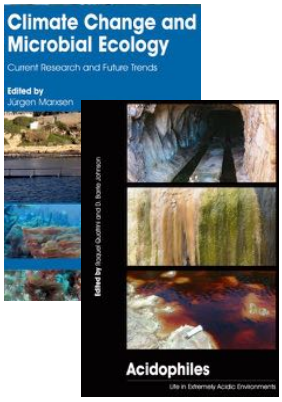

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