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Plasmodium falciparum immunodominant IgG epitopes in subclinical malaria

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Incomplete non-sterile immunity to malaria is attained in endemic regions after recurrent infections by a large percentage of the adult population, who carry the malaria parasite asymptomatically. Although blood-stage *Plasmodium falciparum* rapidly elicits IgG responses, the target antigens of partially protective and non-protective IgG antibodies as well as the basis for the acquisition of these antibodies remain largely unknown. We performed IgG-immunomics to screen for *P. falciparum* antigens and to identify epitopes associated with exposure and clinical disease. Sera from malaria cases identified five prevalent antigens recognized by all analyzed patients' IgGs. Epitope mapping of them, using adult and children sera samples from an endemic malaria region in Ghana segregated into patients with positive or negative subclinical detection of *P. falciparum*, revealed binding specificity for two 20-mer immunodominant antigenic regions within the START-related lipid transfer protein and the protein disulfide isomerase PDI8. These 20-mer epitopes challenged with sera samples from children under 5 years old displayed specific IgG binding in those with detectable parasitemia, even at subclinical level. These results suggest that humoral response against START and PDI8 antigens may be triggered at submicroscopic parasitemia levels in children and may eventually be used to differentially diagnose subclinical malaria in children.

Endemic *Plasmodium falciparum* malaria causes million clinical cases and hundreds of thousand deaths worldwide¹, although the global disease toll probably exceed these numbers^{2,3}. Most deaths occur in sub-Saharan Africa (90%) and in children under 5 years old (70%)¹. In non-endemic areas, malaria could also be a public health threat due to international tourism and migration to and from endemic areas, which has led to the occasional re-emergence of this parasitic disease as imported infection⁴.

In-housing vector control, elementary clinical diagnosis and therapeutic drugs are the only available management strategy for both malaria prevention and treatment in endemic regions. As a necessary step towards malaria elimination neither vaccine nor diagnostics of subclinical reservoirs are widely available. After repeated exposure to the parasite, those who survive malaria early in life eventually acquire resistance to the disease⁵, but the mechanisms that underlie this immunity remain poorly understood⁶. Landmark studies in the 1960s showed that purified IgG from malaria-immune adults transferred to acute-malaria infected children leads to reduction of both fever and parasitemia⁷, thus indicating that antibodies against *P. falciparum* proteins play a critical role in controlling the blood stage of the infection. However, it is still unknown which of the 5,300 proteins presently annotated in the *P. falciparum* genome (PlasmoDB, www.plasmodb.org) elicit the production of protective antibodies⁸. In addition to allelic variations of MHC molecules, the degree of antigen exposure, antigen abundance and host

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immunodominance impede that all possible antigens could be recognized by natural immune responses⁹. With regard to host immune detection of the *P. falciparum* parasite, pioneer studies showed that a large number of antigens are recognizable and dispersed amongst a large fraction of the proteome^{10,11}. Among these, specific epitopes that do induce a response upon immunization with whole native antigens are defined as dominant¹². Although most of the putatively exposed parts of the Ag surface could be subject of recognition by antibodies, the selection mechanism by which only certain antigen regions become B-cell epitopes is not fully understood¹³. The accurate identification of B-cell epitopes constitutes a basis for development of antibody therapeutics¹⁴, peptide-based vaccines^{14–16}, and immunodiagnostic tool¹⁷.

Previous studies in areas endemic for malaria have been carried out to identify a correlation between malaria immunity and *P. falciparum*-specific antibodies. These studies determined antibody reactivity against either a few selected proteins available after cloning¹⁸ or a wider selection of the *P. falciparum* proteome (up to 23%) by means of microarray technology¹¹. More recently, protein microarray has been shown to be an useful tool to correlate plasmodial antigens with protective immunity^{19,20} and to discover new immunogenic epitopes from known vaccine candidates and hypothetical proteins²¹. Scanty information is available about the actual immunodominant B-cell epitopes from the identified antigens. Based on the spatial structure, epitopes can be categorized as a continuous (linear or sequential) and discontinuous (nonlinear or conformational); in the latter case amino acid residues are in close contact due to the three-dimensional conformation^{16,22}. The amount of amino acid sequence in native proteins required for the correct folding of a discontinuous epitope of B cells is always greater than 20 amino acids and can reach up to 400 amino acids. Using a less stringent definition for continuity, it has been proposed that the majority of discontinuous epitopes (over 70%) are composed of 1–5 linear segments of lengths of 1–6 amino acids²³. Currently, the Immune Epitope Database (http://www.iedb.org/home_v3.php) contains above 700 epitopes from 63 antigens of *P. falciparum*. Most of them have been identified in commonly identified antigens, i.e. MSP proteins^{24–26}, circumsporozoite protein (CSP)^{27,28} or ring-infected erythrocyte surface protein (RESA)²⁹ among others. Notwithstanding all these data, correlation between IgG response for the outlined *P. falciparum* proteins and malaria immunity have not been firmly established, suggesting that antibodies against these proteins might not play a role in protective immunity or, more likely, that IgG against single-parasite proteins are insufficient to confer protection. In addition, the identification of B-cell epitopes can also be used in the development of diagnostic tests by immunodetection approaches³⁰. This diversity of Plasmodium antigens found in different scenarios highlights the interest of identifying other repertoires of antigens and epitopes that are expressed during Plasmodium infection by different strategies. Thus, identifying more *P. falciparum* antigens can be instrumental to find links between disease state and its progression as well as in advancing in the design of effective diagnosis systems and vaccines.

In an effort to expand the search of *P. falciparum* antigens and immunodominant epitopes, we first analyzed immunoproteomic profiles shown in sera from patients with imported malaria. These patients living in a non-endemic region were not periodically re-infected after other continuous *P. falciparum* infections, but occasionally when they visit transiently their home country of endemic malaria. Thus, their humoral immune status may hypothetically reflect a selection for pre-immunogenic anti-*P. falciparum* IgG antibodies, eventually directed against epitopes that suppressed acute symptoms. The identification of IgG-reactive antigens in sera from imported malaria patients with a single recent restricted infection but with different levels of parasitemia would therefore be particularly informative, as this could allow for the identification of immunoreactive antigens that favour subclinical malaria and therefore may be relevant for both immunisation and diagnosis. In this work, the humoral response in imported malaria patients was analyzed for the identification of potential highly immunoreactive antigens and mapping of immunodominant epitopes using peptide microarrays. Subsequently, the specific IgG response to a selection of these epitopes in adult and child sera from a malaria endemic Ghanaian population was quantified, validating the antigens found and suggesting a possible correlation with the stage of development of the infection.

Results

Immunoproteomics of *P. falciparum* blood stage antigens. The sera of 19 imported malaria patients showed *P. falciparum* infection through parasitological and serological analyses. Five of these patients showed clinical symptoms and were classified as imported clinical malaria (ICM) with high parasitemia. The remaining 14 patients were identified during routine medical checks and were classified as imported subclinical malaria (ISC), since no clinical symptoms of malaria were observed in addition to a submicroscopic parasitemia. Four additional sera from individuals who had never been in malaria-endemic regions were used as control. Mean values of specific anti-*P. falciparum* IgG antibodies quantified by indirect ELISA did not show significant differences between both patient groups, although very large intragroup variability was observed in the ISC group (Figure S1). No specific antibodies against *P. falciparum* were detected in unexposed controls (not shown).

To further identify differential IgG reactive antigens, detailed immunoproteomics was performed. Thus, the total *P. falciparum* intraerythrocytic proteome obtained in 2D-PAGE from a clinical isolate, originally causing subclinical malaria³¹, showed 862 ± 14 spots within the range at 3–11 isoelectric point and 3–260 kDa molecular weight (Figure S2). The corresponding 2D immunoblots using sera from imported malaria cases revealed different immunoreactivity patterns for each group of patients (Figure S2). Immunoblots obtained with ICM group sera showed a higher number of immunoreactive spots than ISC group sera, suggesting that parasitemia correlates with obtaining a diverse and strong humoral immune response. In addition, ISC sera showed a predominantly acidic protein pattern, in contrast to immunoblots obtained from ICM sera that showed a wide variety of both acid and basic antigens.

The quantification of signals from all samples allowed the selection of 83 immunoreactive protein spots that were present in at least 30% of the complete set of Westerns blots developed with the imported malaria patients' sera ($n = 19$). These spots were excised from the reference 2D gel and subjected to MALDI-TOF/TOF analysis

and subsequent search in *P. falciparum* databases for protein identification. Although nearly 60% of the removed spots could not be identified (<90 score), the remaining 34 spots (Figure S2) were successfully assigned to 17 proteins, as shown in Table 1, and classified into the main functional classes of the *P. falciparum* proteome³², resulting in seven of protein fate (protein degradation), five of metabolism, four of cell surface or organelles and only one of transport. Although some differences were observed for antigen reactivity, none of the identified proteins appeared to be significantly associated with clinical or subclinical stages. However, with the expectation of characterizing potential differences in epitope recognition, five antigens were selected based on relative immunodominance (four of which showed a positive identification of >50% by patient sera) and computational analysis of antigen function and recognition by T and B cells^{33,34}: i) Functions that are likely key for survival according to gene ontology terms of the proteins; ii) Secreted or bound to cell membranes; iii) Global antigenicity above 1.01 as determined by the method of Kolaskar & Tongaonkar³⁵; and iv) Immunogenicity considered as potential for recruiting antigen specific Th cells and determined as density of peptides predicted to bind HLA-DR molecules using RANKPEP³⁶.

These analyses resulted in the following five proteins: Putative protein kinase (Q8I2Q0), Heat shock protein 70 (HSP70-2), Heat shock protein 60 (HSP60) and Star-related lipid transfer protein (START), and the Protein disulfide isomerase 8 (PDI8). Among the five selected proteins, only HSP60 and PDI8 had been reported as antigenic in previous studies^{11,37}.

Mapping linear IgG epitopes with sera from an endemic malaria region. If any of the antigens detected in imported malaria cases are relevant to the maintenance of acquired malaria immunity, sera from asymptomatic individuals in endemic areas can be expected to contain specific antibodies against epitopes in these proteins. To analyze this possibility, random sampling of sera from patients visiting Breman-Asikuma Hospital (Ghana) for reasons other than malaria symptomatology was undertaken. Blood samples were quantified for *P. falciparum* parasitemia and IgG content, and subsequently HLA genotyped (see Table S4). The sera were classified into two groups: adults with subclinical malaria (ASC) with low parasitemia level (<0.01%); and adults with no parasitemia (ANP), and thus no having malaria. Both groups were further subdivided by their specific anti-*P. falciparum* IgG contents into high (>60 µg mL⁻¹) or low (<60 µg mL⁻¹) IgG. A list of the 38 samples used is shown in Table S2. Most samples used for further analysis belonged to the most frequent HLA-DRB1 genotype (Table S4).

Immunoreactive epitopes in the five selected antigenic proteins were mapped by high density peptide microarrays designed to contain 15-mer amino acid sequence overlapped by the following 14 residues in the next 15-mer peptide to cover the whole sequence of the selected antigens. The reactivity of IgG antibodies present in 38 serum samples from the malaria endemic region was individually tested in the peptide microarrays. The IgG antigenicity profile of the five antigens along their sequence by these sera is shown in Fig. 1. It is noteworthy that all five antigens showed a wide distribution of regions of IgG recognition along the protein sequences. The plots shown similar recognition patterns in both ASC and ANP samples. Although significant differences were found in the ratio between specific fraction of anti *P. falciparum* IgG and total IgG between the two studied groups (see footnote b to Table S2), the overall signal in sera with either high or low IgG levels were no significantly different (see legend to Fig. 1). To identify peptides that produce consistent reactivity among serum samples, a signal cut was established in the third quartile of the intensities recorded in the microarray within each serum group. Above this signal-to-noise ratio threshold, regions of high reactivity are distinguished even in samples with low IgG levels. Figure 2 shows the frequency of serum samples emitting a fluorescent signal above the threshold in both ASC and ANP groups at all 15-mer peptide positions in the five antigens. According to the aggregate pattern of the individual sera, the peptide reactivity along the sequences showed similar patterns with the ASC and ANP sera, which consequently did not allow the identification of any specific epitopes of the subclinical state. The greatest difference was observed in the 107–120 region of the Q8I2Q0 protein which showed >85% positive with ASC sera and <50% positive with ANP sera.

Validation of highly immunoreactive epitopes. To validate immunoreactive epitopes from the antigens, 20-mer length peptides were selected for ELISA immunodetection with sera from the malaria endemic area. The peptide sequences were chosen among those that fulfill the following three criteria from the data obtained in the peptide arrays: i) high reactivity with high IgG concentration sera from both ASC and ANP groups; ii) high recognition rate, namely including at least one 15-mer peptide showing signals above de threshold value in, at least, 75% of the high IgG sera challenged with than peptide, and iii) a theoretically calculated acidic or basic isoelectric point to anticipate their solubility. Moreover, the 107–120 region of antigen Q8I2Q0 which showed the largest differences in antibody reactivity in ASC and ANP sera was also included for validation. The sequence of the chosen peptides and their location in the antigens are shown in Table 2.

These eight synthesized peptides were tested for IgG reactivity using serum samples from the endemic malaria region. Figure 3 shows the amount of specific IgG for each 20-mer peptide detected in 8 ASC sera, 8 ANP sera and 4 control sera of individuals never exposed to the parasite. The 16 serum samples from the endemic area were randomly selected to include 4 of each of the four serum groups (ASC-high IgG; ASC-low IgG; ANP-high IgG; ANP-low IgG), hence most possible situations found in a malaria-endemic area were represented. Although the signals recorded for the ANP group appear to be consistently higher than those found in the ASC group, no statistically significant differences could be determined for any of the peptides ($P < 0.05$). Analysis of the immunoreactivity signal of the 20-mer epitopes between ASC or ANP and control sera samples, showed that all epitopes except HSP70-2 (2.2) displayed significant differences among them, although only START (1.1 and 1.3) and PDI8 (5.1 and 5.3) epitopes showed median values of IgG antibodies in serum above 1.5 ng/mL (see Fig. 3). These results suggest that both START and PDI8 contain immunodominant epitopes

Spot n.	Protein	Gene name	Accession	Score	ISC%	ICM%	Totaln/19	Localization/ Function	Functional category	Gene Expression (All Stages)**	Protein detection in erythrocytic stages ***
1,2,3,4	Putative protein kinase		Q8I2Q0*	485	86	80	16	Unknown/putative protein kinase	Protein fate	Sexual & Asexual TR (very low)	MR, TR, GM
13,14,15,16	Heat shock protein 70	HSP70-2	Q8I2X4*	1220	71	100	15	Cytoplasm/Stress response protein binding	Protein fate	Sexual & Asexual TR	ALL
17,18,19	Heat shock protein hsp70 homologue	HSP70-3	Q9GUX1	1110	71	60	13	Cytoplasm/Stress response protein binding	Protein fate	Sexual (mostly) Early-Mid TR	ALL (TR predominant)
7,8,9,10,11,12	Heat shock protein 60	HSP60	Q8IJN9*	1310	64	80	13	Cytoplasm/Stress response protein binding	Protein fate	Sexual & Asexual Late RG-Mid TR (low)	ALL (TR predominant)
20,21	Heat shock protein 70	HSP70	Q8IB24	819	57	60	11	Cytoplasm/Stress response protein binding	Protein fate	Sexual & Asexual RG-TR	ALL (TR predominant)
5,6	Star-related lipid transfer protein	START	Q8I298*	509	50	80	11	Integral membrane/Phospholipid transfer	Transport	Asexual ALL (low)	MR, GM
27	Plasmeprin I	PMI	Q7KQM4	881	50	40	9	Vacuole/Aspartic endopeptidase	Metabolism	Asexual (low)	MR, GM, TR (TR predominant)
28	Merozoite surface protein 9	MSP9	C5HEE4	435	43	80	10	Membrane/erythrocyte invasion	Surface or organelles	Asexual (predominant) Late TR-Early SC (high)	MR, TR
29	Merozoite surface protein 1	MSP1	A5A7C2	889	50	40	9	Membrane/erythrocyte invasion	Surface or organelles	Asexual Mid TR-Late SC (very high)	ALL (MR predominant)
33	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	BCKDH-E2	O97227	707	43	60	9	Mitochondrion/acetyltransferase activity	Metabolism	Sexual & Asexual Early TR-Early SC	MR, GM, TR (GM predominant)
30	Ferredoxin reductase-like protein		Q8IBP8	279	36	60	8	Oxidoreductase	Metabolism	Sexual (low) & Asexual TR (low)	SC, GM (Predominant GM)
23,24,25	Protein disulfide isomerase	PDI8	C0H4Y6*	1300	43	20	7	Endoplasmic reticulum/protein folding	Protein fate	Sexual & Asexual TR	ALL
31	Dihydrolipoyl dehydrogenase	LPD1	Q8I5A0	1040	43	40	8	Cytoplasm/Cell redox homeostasis	Metabolism	Sexual & Asexual TR	ALL (TR & GM predominant)
32	V-Type proton ATPase subunit B		Q6ZMA8	1090	43	20	7	Vacuole/ATP hydrolysis coupled proton transport	Transport	Sexual & Asexual Early TR-Early SC	ALL (MR, TR & GM predominant)
26	Plasmeprin II	PMII	P46925	623	36	40	7	Vacuole/Aspartic endopeptidase	Metabolism	Sexual & Asexual TR (low)	MR, TR, GM (TR predominant)
22	Heat shock protein 90	HSP90	Q8IC05	637	36	40	7	Cytoplasm/Stress response protein binding	Protein fate	Sexual & Asexual RG, TR	ALL (TR predominant)
34	Rhoptry-associated protein 2	RAP2	Q8I484	93	29	40	6	Rhoptry/cell invasion	Surface or organelles	Asexual (only) Early-mid SC	MR, TR, GM (MR predominant)

Table 1. Immunoproteomics-based identification of *P. falciparum* proteins that showed IgG reactivity with serum samples from malaria patients. Gene name, Accession No., and identification score is provided. Columns ISC and ICM indicates the percentage of individual Western blots where the protein spot showed up using patient sera from each group, ISC and ICM. Under column “Total” is given the total number of different patient sera used in Western blots where the protein spot showed up. A total number of 19 different Western blots were run including both groups. Information on each antigen includes its functional category and the developmental stages where its gene expression and protein have been detected from previous studies^{32,50}. An asterisk under “Accession” indicate those proteins selected for epitope mapping. Two asterisks under “Gene expression” compile data on stage expression from the Malaria Cell Atlas⁵⁰ as follows: Ring (RG), Trophozoite (TR), Schizont (SC), All Asexual Stages (ALL). Three asterisks under “Protein detection in erythrocytic stages” compile data on stage expression from³² as follows: Ring (RG), Trophozoite (TR), Schizont (SC), Merozoite (MR), Gametocyte (GM), All Erythrocytic Stages (ALL).

that elicit high amounts of specific IgG in the sera of populations exposed to malaria. The constant immunoreactivity observed in sera from the ANP group that has not detectable parasites suggests a long persistence of this IgG after elimination of the infection.

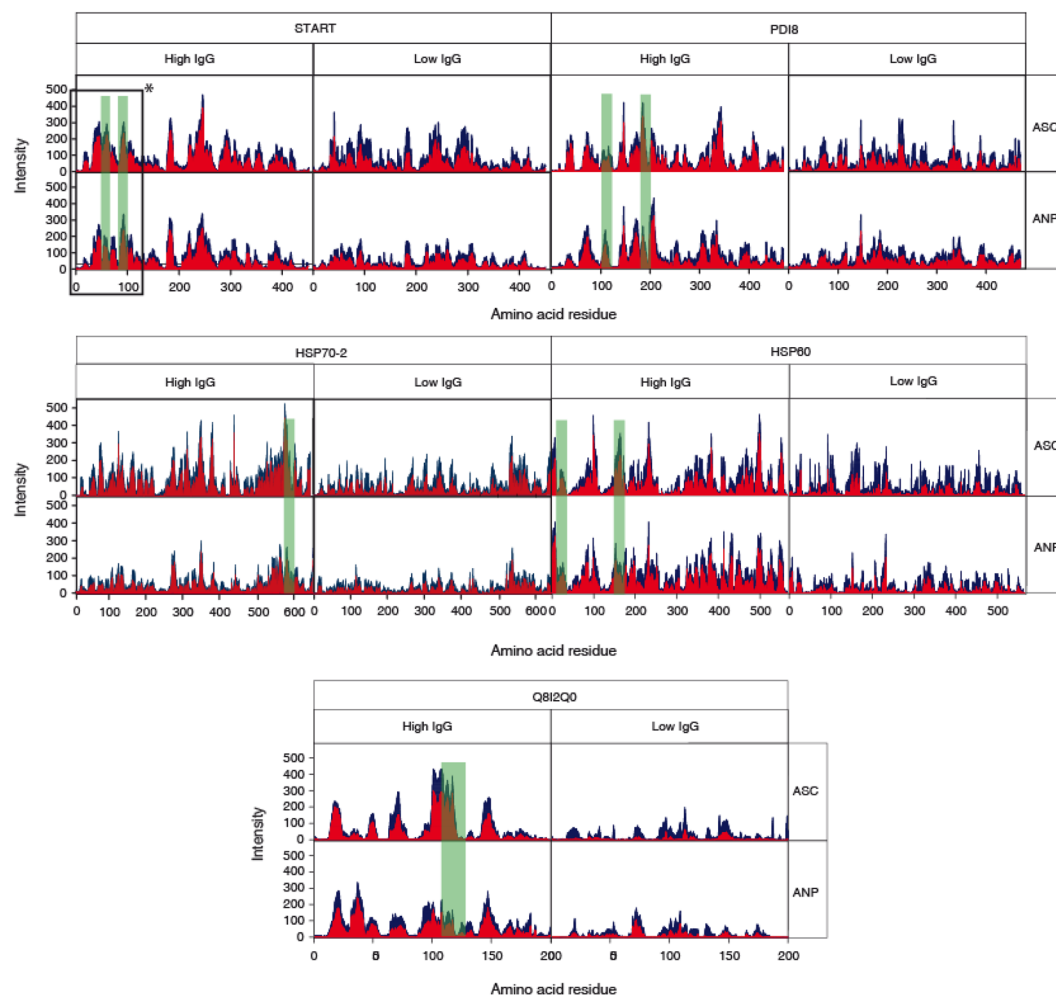


Figure 1. Epitope mapping in the five *P. falciparum* antigens. Data was recorded from 15-mer microarrays of overlapping peptides covering the whole protein sequence. Data shows average fluorescence m (blue) and standard error (s) subtracted average fluorescence $m-s$ (red) along the sequence of the indicated antigens. Data is presented in a set of 4 plots to distinguish incubation with sera from ASC or ANP patients and for high IgG ($>60 \mu\text{g mL}^{-1}$) and low IgG sera. n values are as follow: 10 for ASC/High IgG; 8 for ANP/Low IgG; 11 for ASC/Low IgG; 9 for ANP/High IgG. The positions of the selected 20-mer peptides are shown as overlay green bars. Ratios of cumulative signal intensities to Pf-specific IgG were not significantly different between High and Low IgG groups: average values $7.6 \times 10^{-3} \pm 0.01$ and $5.6 \times 10^{-3} \pm 0.01$, respectively.

Since the persistence of anti-START and anti-PDI8 IgG in the adult population of the malaria-endemic region may be a consequence of repeated exposure to the parasite, it was conceivable that a less exposed population group would show a different pattern. To test this hypothesis, serum samples from children younger than 5 years old in the same endemic area were analyzed to detect specific IgG against peptides 1.1, 1.3, 5.1 and 5.3 that were the most immunoreactive of START and PDI-8 in adults. In this trial, the sera of children with symptoms of malaria or asymptomatic, corresponding to those showing parasites by microscopic screening (parasitemia $>0.1\%$, clinical malaria: CCM), those for whom *P. falciparum* was detected only by PCR of its DNA but not by microscopy (subclinical malaria: CSC), and those who did not show the presence of the parasite by either method (CNP). The samples from the CCM, CSC and CNP groups were defined and split upon analysis since were obtained from children enrolled from clinic visits for any medical reason, and not necessarily malaria suspicion. The results, shown in Fig. 4, confirm the immunoreactivity of both antigens in sera collected from children, but unlike adults, the signal obtained in cases of subclinical malaria was significantly higher than the signal in uninfected cases ($P < 0.05$).

Discussion

Despite the variety of *P. falciparum* antigenic proteins identified by different approaches^{10,11,37–40}, only a few have been shown to be; albeit partially, relevant to malaria immunity¹⁰ or for detection purposes^{41,42}. Previously, the humoral response to *P. falciparum* in patients with imported malaria was used to identify immunogenic proteins by western blot 1D analysis.³⁷ Here, we have used a 2D immunoproteomics approach using imported malaria patient sera to increase search resolution, mapped the position of potential epitopes in a selection of

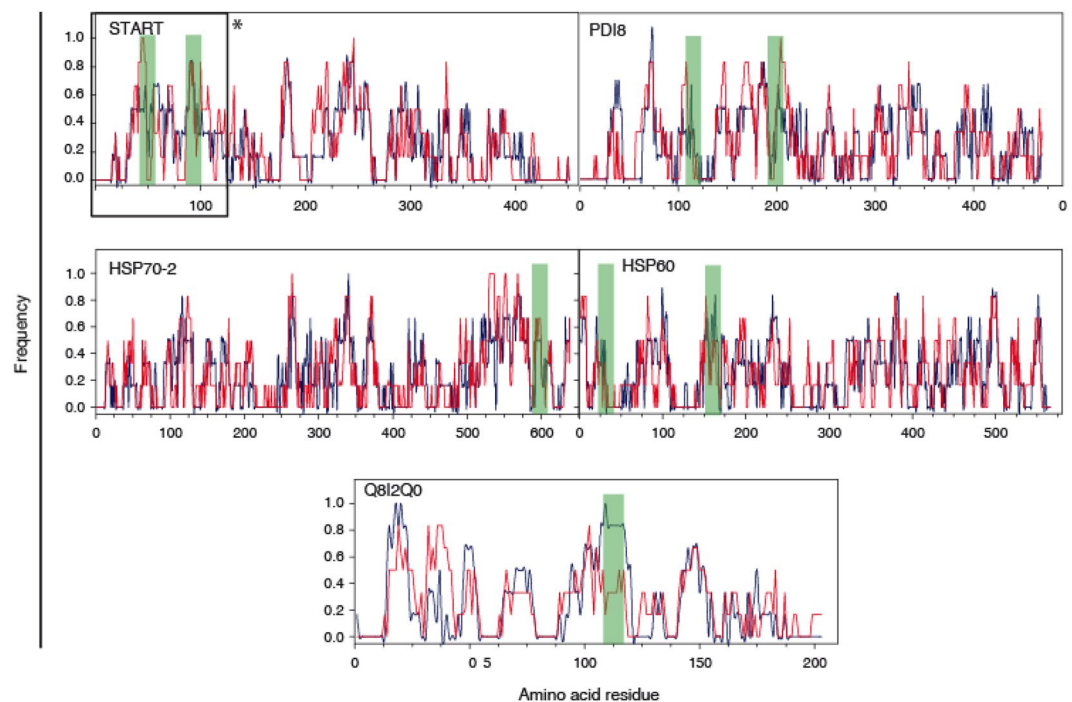


Figure 2. Comparison of IgG reactivity frequency between serum from adult patients with (blue: ASC) and without (red: ANP) parasitemia from Bremo-Asikuma. X-axis shows amino acid sequence length of each antigen. Y-axis shows fluorescence signals above a cutoff established as the third quartile of the recorded data. Data from high IgG sera are shown. The positions of the selected 20-mer peptides are shown as overlay green bars.

Protein	Peptide Name	Sequence	Amino acid position
START	MP_1.1	NVYKNKIKSRILKENKEESL	43–57
	MP_1.3	SCNYSTPSSVFNNNDKENTAS	86–100
HSP70-2	MP_2.2	VKDAEDWLNNNSNADSEALK	594–613
Putative protein kinase Uniprot Q8I2Q0	MP_3.3	NKPLDENTTTVEEFNASIYL	113–132
HSP 60	MP_4.1	NIQKRNIKDIRFGSDARTA	25–44
	MP_4.3	KGVEKVLEYLNSIKKDVTTT	151–170
PDI8	MP_5.1	YPTLILFNKKNKINYGGGRT	106–125
	MP_5.3	NREIAKYFVKKNDKHNKLF	183–202

Table 2. Peptides selected for IgG immunoreactivity analysis with patients sera from Bremo-Asikuma (adults and children).

five immunodominant antigens, and validated their immunogenicity using serum samples from individuals in a malaria-endemic region. Although imported malaria samples used for immunoproteomics were classified into two groups based on different clinical stage, a clear association between individual antigens and disease stage could not be determined. In fact, no significant differences in total serum anti-*P. falciparum* IgG levels were found between the two groups, as high antibody levels are associated not to protection but to higher exposure to *P. falciparum*⁴³.

Eight of the 17 antigens identified by immunoproteomics had not been previously reported, including experimental approaches and computational prediction of the entire proteome¹⁰. It is noteworthy that different populations and experimental approaches may yield the identification of different sets of antigens, potentially associated to immune response in endemic areas. Residents in a endemic area of Kenya monitored for reinfection after administration of antimalarial drugs mostly showed in their sera the presence of antibodies against widely known antigens as the liver-stage antigen (LSA), merozoite surface proteins (MSP) or Pf Erythrocyte membrane protein PfEMP1, among others¹⁹. In other study using only alpha helical coiled-coil protein motifs, differences were observed in the reactivity of sera antigens between different ethnicity and age²⁰. This supports the notion that broad and diverse experimental approaches are necessary to obtain a more complete range of *P. falciparum* immunogenic proteins. Chaperones, within the fate category including the heat shock proteins HSP70, HSP70-2, HSP70-3 and HSP60, were the most frequently recognized by sera IgGs. In the metabolic category,

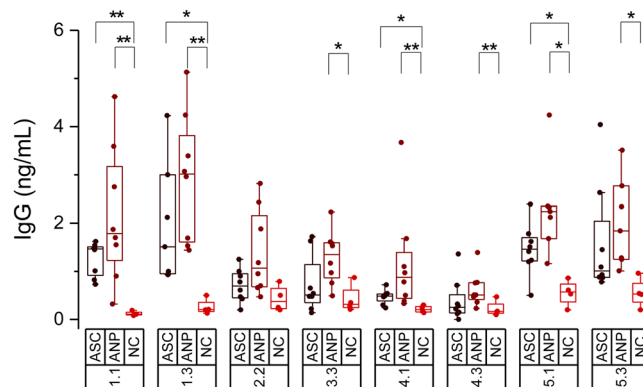


Figure 3. IgG reactivity of adult serum samples from the endemic malaria region with eight selected 20-mer epitopes. Data shows specific IgG concentration present in sera against 20-mer peptides sequences of START (1.1, 1.3); HSP70-2 (2.2); putative protein kinase Uniprot Q8I2Q0 (3.3); HSP60 (4.1, 4.3); and PDI8 (5.1, 5.3). Three different sera groups were used: asymptomatic subclinical (ASC, $n = 8$), undetected malaria parasite (ANP, $n = 8$) and no malaria exposed controls (NC, $n = 4$). The 16 sera used from endemic malaria region were selected from the patients indicated with an asterisk in Table S2. Mean values are given by horizontal lines; boxes depict 25/75 percentiles and whiskers cover data excluding outliers at 1.5 coefficient. Mann-Whitney nonparametric tests were run: * $P < 0.05$; ** $P < 0.01$.

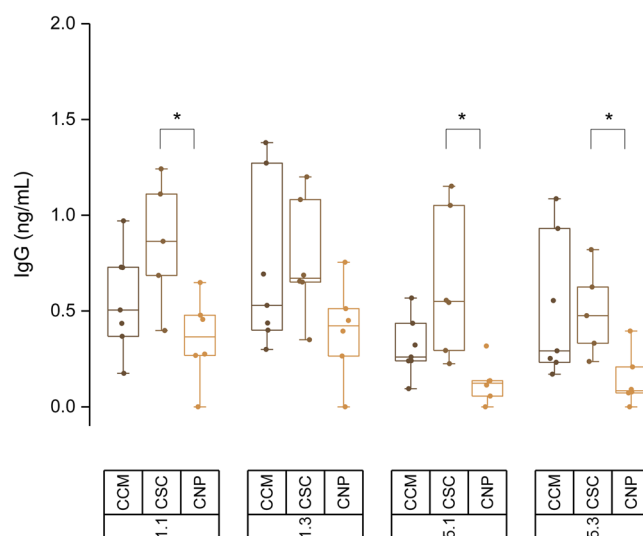


Figure 4. IgG reactivity of children serum samples from the endemic malaria region with four selected 20-mer epitopes. Data shows specific IgG concentration present in sera against 20-mer peptides sequences of START (1.1, 1.3) and PDI8 (5.1, 5.3). Three different sera groups were used: clinical malaria samples (CCM, parasitemia $> 0.1\%$); asymptomatic subclinical (CSC), and undetected malaria parasite (CNP). Mean values are given by horizontal lines; boxes depict 25/75 percentiles and whiskers cover data excluding outliers at 1.5 coefficient. Mann-Whitney nonparametric tests were run: * $P < 0.05$.

IgGs anti-Plasmeplins I and II, (proteases involved in host hemoglobin digestion) were similarly found in sera from both imported malaria groups. Plasmeplins I has been detected in intraerythrocytic young stages whereas Plasmeplins II expression has been associated with mature stages⁴⁴. A similar distribution was observed in the generation of antibodies against dihydrolipoamide dehydrogenase and dihydrolipoamide acyltransferase, both of them components of the multienzyme pyruvate dehydrogenase complex. Two further metabolism-related proteins from the parasite, ferredoxin reductase-like protein, involved in fatty acid metabolism, and vacuolar ATP synthase subunit b, likely involved in acidification of the digestive vacuole⁴⁵, raised antibodies against them in sera from both groups. However, while antibodies against ferredoxin-reductase-like protein were found mainly in patients with clinical symptoms, the vacuolar ATP synthase subunit b was more antigenic in those individuals without clinical signs of malaria. The most frequently detected antigen was initially identified as a conserved protein of unknown function Uniprot Q8I2Q0, corresponding to gene *PF3D7_0925900* in the genome of *P. falciparum* 3D7. Further analysis by sequence comparison (not shown) revealed that this sequence may correspond to an uncomplete gene duplication of a putative protein kinase Uniprot Q8I2P9, corresponding to gene

PFIT_PFI1270W, located 20 kpb downstream *PF3D7_0925900*. Within the cell surface class, three immunogenic proteins which assist in the process of erythrocyte invasion⁴⁶ were identified. Two of them, MSP-1 and MSP-9, are located at the merozoite surface⁴⁷ and RAP2, associated with the low-molecular-weight rhoptry complex in the rhoptry organelles⁴⁸. Finally, a new antigenic protein, integral membrane STAR-related lipid transport START, raised IgG antibodies in about 55% of the samples from all three groups. Although the humoral response against some of these proteins has previously been reported in other studies using samples from malaria-endemic regions^{11,38–40}, antigens such as putative protein kinase, HSP70-2, V-Type proton ATPase subunit B, START, LPD1, PDI8, ferredoxin reductase-like protein and plasmepsins I and II, may be indicative of differences in the specific IgG range shown in individuals with imported malaria caused only by an isolated infection in a partially protected individual. It is therefore conceivable that antibodies against several of these housekeeping proteins are merely markers of exposure rather than part of acquired protective immunity, as previously reported for other malaria antigens⁴⁹.

To investigate epitope immunoreactivity in the identified antigens, five of them were selected for mapping of their epitopes by humoral response with sera from an endemic malaria population. Putative protein kinase Q8I2Q0, HSP70-2, HSP60 and START were the most frequently detected antigens by ISC sera, suggesting that these proteins may induce a strong humoral response that lasts after parasite clearance. These five selected proteins are not only present in the asexual stage of merozoite³² guaranteeing potential immunogenic contact by the host during the intraerythrocytic cycle (see Table 1), but have also recently been guaranteed gene expression throughout the asexual and sexual stages of the *Plasmodium* cycle at discrete levels⁵⁰, thus implying non-saturating exposure to the immune system. START antigen was also detected even more frequently than other well-known antigenic membrane proteins, such as MSPs. The protein disulfide isomerase PDI8 was also included in the epitope mapping as it was also detected with high frequency and its function associated with multiple proteins during the parasite cycle suggests that it may be widely exposed to the host immune system. Complete mapping of potential linear epitopes in these proteins using overlapping 15-mer peptide microarrays revealed high IgG reactivity sequences homogeneously distributed throughout the sequence in all antigens analyzed. In this sense, it has been previously shown that known plasmodial antigens such as MSP-1, GLURP, LSA1 and SEA1 analyzed by high-density peptide mapping, also display a wide collection of highly immunoreactive epitopes not revealed by other techniques²¹. As the collection of sera used for antigen screening was confined to asymptomatic adults, potential epitopes were expected to represent sequences associated with acquired immunity and/or subclinical infections. Subsequent ELISA analysis of a selection of 20-mer peptides showing both a high IgG recognition signal and a high binding frequency in endemic malaria samples allowed quantification of specific IgG concentrations in sera. Since no statistically significant differences in recognition in microarray patterns or in the IgG binding levels to the 20-mer peptides were observed between ASC endemic region samples with subclinical infection and non-infected ANP controls, we can conclude that the host IgG collection directed against these epitopes represents a subpopulation of long-term IgG antibodies present in individuals after repeated exposure to the parasite.

The persistence of anti-malarial IgG response may be due to a long-lasting B-cell memory or a high number of long-lasting plasma cells (LLPC)⁵¹. On the other hand, the longevity of the antibody response observed in the adult population of malaria-endemic regions has been explained as a result of a progressive increase in LLPC over the course of recurrent reinfections by highly antigenic variable parasites⁵². The titration of specific IgG bound to different epitopes of the five selected antigens revealed that only START and PDI8 epitopes yielded high IgG absolute titers and significantly higher IgG signal above background in sera from this malaria-endemic region. Humoral response against START and PDI8 in uninfected individuals from endemic areas may be caused by repeated exposure to the parasite and increased production of specific IgG against these two proteins by LLPC.

To further explore this possibility, serum samples from children under 5 years old from the same endemic area were analyzed. It is now accepted that resistance to severe malaria is rapidly attained within the first five years of life⁵³, while immunity to non-severe malaria is slowly acquired over years of repeated re-infection, often leading to a subclinical course of the disease that can only be detected by PCR amplification⁵⁴. Remarkably, only *P. falciparum*-infected children displayed anti-START or anti-PDI8 IgGs, indicating that the presence of these antibodies in adults may be associated with the development of acquired immunity against non-severe malaria.

Taken together, these results suggest that *P. falciparum* antigens involved in the maintenance of acquired immunity show a wide range of B-cell epitopes of which at least the START and PDI8 epitopes lead to the production of a long-lasting IgG response that is maintained after parasite clearance, which may be indicative of a relevant role of these antigens in naturally acquired immunity. Nevertheless, we cannot discard that the CNP population has been previously infected with malaria. Thus, the low START and PDI8 antibody levels in this group suggest that the IgG response is not long-lived initially. It seems more likely that only after repeated infection(s) do these antibody responses persist. In addition, the presence of anti-START and/or anti-PDI8 IgG in children with subclinical malaria would also be useful as a marker for such undetected reservoir infection. Thus, these epitopes could be used as a rapid method of ELISA immunodetection at large-scale in elimination programs instead of PCR diagnosis.

Methods

Sample collection and population. Four sets of human sera were used in the present study. The first set (n = 19) was obtained from adult sub-Saharan African patients resident in Spain with imported malaria detected after a recent trip from Africa (Table S1). The second set (n = 38) was obtained from adult donors (17–40 years old, excluding pregnant women) living at Breman-Asikuma (Ghana) attending to a local hospital; Our Lady of Grace Hospital, but showing no symptoms of malaria or other endemic infections (tuberculosis, hepatitis B, hepatitis C, HTLV, dengue and HIV) at the time of collection (Table S2). The third set (n = 20) was collected

at the same location from children aged 6 weeks to 5 years old, including patients showing malaria symptoms (Table S3).

A fourth set of 4 European volunteers never exposed to malaria was used as a control.

Ethics Statement. This study was approved by both, the Ethical Review Board at Research Institute Hospital 12 de Octubre, Madrid (Spain) and the Ethical and Protocol Review Committee from University of Ghana's College of Health Sciences (Ghana). The study was conducted according to the guidelines laid down by the Declaration of Helsinki, with written informed consent obtained for each adult participant, or in children a parent or guardian of child participant provided written informed consent on their behalf. The samples were specifically obtained for this study including complementary analysis of co-infections and polymorphisms in connection to malaria immunity that are not yet published.

Protein extracts from *in vitro* cultures of *Plasmodium falciparum*. Clinical *P. falciparum* isolate UCM7³¹ from West Africa was cultured to high parasitemia (>50%) following the previously described method⁵⁵. Briefly, the culture media consisted of standard RPMI 1640 (Sigma-Aldrich) supplemented with 0.5% Albumax I (Gibco), 100 μ M Hypoxanthine (Sigma-Aldrich), 25 mM 101 HEPES (Sigma-Aldrich), 12.5 μ g/mL Gentamicine (Sigma-Aldrich) and 25 mM NaHCO₃ (Sigma-Aldrich). Culture at 37 °C with 5% CO₂ started by mixing a cryopreserved stock with fresh erythrocytes to achieve 1% hematocrit. Growth was monitored by microscopy in thin blood smears and media changes and synchronization steps following defined criteria⁵⁵ according to parasite stage and parasitemia.

Protein extracts from *P. falciparum* isolate UCM7 were obtained from three 150 ml culture flasks of infected red blood cells (iRBC) synchronized to mature forms (schizonts) at high parasitemia⁵⁵. Cultures were harvested and erythrocytes resuspended 1:1 in PBS containing 0.1% saponin to gently vortex during 15 s for lysing RBC. Released parasites were centrifuged at 800 $\times$ g during 5 min and the pellet was 3x washed in cold PBS to subsequently centrifuged at 15,000 $\times$ g, 10 min. Pellets were solubilized in 50 mM Tris-HCl pH8 containing 50 mM NaCl, 3% CHAPS, 0.5% MEGA 10 and protease inhibitor cocktail from Roche. Resuspension was gently mixed at 4 °C during 15 min followed by 4x freeze-thaw cycles. Cold (4 °C) centrifugation at 15,000 $\times$ g during 10 min resulted in a supernatant considered the parasite extract. Protein concentration was quantified using Bradford based *Protein Assay* from Bio-Rad and total protein lysates stored at -20 °C until use.

Total and *P. falciparum* specific immunoglobulin-G quantification. Immunoglobulin-G (IgG) in sera samples was quantified using indirect IgG ELISA detection kit (Bethyl Laboratories) following the manufacturer's instructions. For total IgG quantification, microtiter plates were coated with 1 μ g/well of anti-human IgG (Bethyl Laboratories) in 100 μ l carbonate/bicarbonate buffered solution (Sigma C3041) for 1 hour at room temperature; while specific anti-*P. falciparum* IgG were quantified using 0.5 μ g/well of parasite protein extract (see above) in the same buffer as coating antigen overnight at 4 °C. From this step onwards, all wells followed same protocol. Plates were blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline solution and diluted human serum was incubated for 1 h at room temperature, using 1:50,000 dilution for total IgG and 1:5,000 dilution for specific IgG quantification. IgG binding was detected with goat anti-human IgG conjugated with horseradish peroxidase (HRP) at 1:75,000 dilution. The enzymatic reaction was developed using 3,3',5,5' tetramethyl benzidine (TBM) as enzyme substrate and stopped by the addition of 100 μ l of 4 N H₂SO₄. Absorbance readings were obtained at 652 nm in a Varian Cary 50 Biospectrophotometer. Serum samples from non-exposed European individuals were used to establish the negative cut-off of the ELISA (mean OD+2 S.D.). Human reference serum from Bethyl Laboratories was used to generate a logistic four-parameter sigmoidal standard curve.

Immunoproteomics for antigen identification. *P. falciparum* UCM7 extracts were run in 2D-PAGE (300 μ g for mass-spectrometry identification / 150 μ g for immunoblot) for subsequent Western blot analysis using sera from patients with imported malaria. Protein extract were cup loaded onto IPG strips (pH 3 – 11, 18 cm) previously hydrated overnight with a buffer containing 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1.2%, DeStreak reagent and 2% ampholites pH 3–11. For first-dimensional separation, IEF was performed using the IPGphor 3 IEF system (GE Healthcare) at 20 °C. Voltage was gradually ramped in a step-and-hold manner to 1,000 V in three steps: 1 h at 120 V, 1 h at 500 V, and 2 h at 500 – 1,000 V. Then, voltage increased to 4,000 V in linear gradient (1,000–4,000 V) over the next 9 hours. Run terminated after $\approx$ 70,000 V/h. The focused strips were equilibrated twice with gentle vertical shaking. The first equilibration during 12 min was performed with a solution containing 100 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 0.5% DTT. In the second equilibration solution, DTT was replaced with 4.5% iodoacetamide and incubation continued 5 min. SDS-PAGE using a Ettan-Daltsix unit at 2 W/gel for 30 min followed by 20 W/gel for 4 h was run on homogeneous 12.5% pre-casted gels.

To visualize and further excise and identify protein spots, the 2D-gels loaded with 300 μ g were fixed in 10% methanol/7% acetic acid for 30 min and overnight stained with Sypro Ruby (Sigma). Two 2D-gels for staining and protein-spot excising (300 μ g) and 20 replicated 2D-gels for Western-blot (150 μ g) were run in parallel, in group of 6, under identical voltage conditions to warrant replica. The 2D-gels loaded with 150 μ g were transferred to polyvinylidene difluoride (PVDF) membranes. Transfer efficiency was confirmed by: (i) gel staining with Sypro Tangerine before transfer; and (ii) membrane staining with Sypro Ruby after transfer. Stained gels or blots were scanned in a Typhoon 9400 variable mode imager (GE Healthcare). For immunodetection with individual patient serum, the transferred PVDF membranes were blocked for 2 hours in 10% non-fat skimmed milk (w/v) in PBS containing 0.05% Tween-20 followed by overnight incubation with patient serum at 1: 10,000 dilution. Then, goat anti-human IgG HRP linked (Amersham Bioscience) as secondary antibody was incubated for 1 h at room temperature at a 1:10,000 dilution. Antigen-antibody reaction was visualized using the SuperSignal chemiluminescent substrate (Pierce) and exposure to X-ray film. Human sera from Europeans never exposed to malaria

were used as negative controls. Gel and immunoblot images were analyzed using PDQuest software, version 8.0.1 (BioRad Laboratories Inc., Munich, Germany), and spots of interest were manually excised from the 2D gels, in-gel reduced, alkylated, and then digested with trypsin as described elsewhere⁵⁶. Excised spots were washed twice in water, shrunk for 15 min with 100% acetonitrile, and dried in a Savant SpeedVac for 30 min. The samples were then reduced using 10 mM DTT in 25 mM ammonium bicarbonate for 30 min at 56 °C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min in the dark. Finally, samples were digested with 12.5 ng/μL sequencing grade trypsin in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37 °C. After digestion, the supernatant was collected, and 1 μL was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Next, 0.4 μL of a 3 mg/mL of α-cyano-4-hydroxytranscinnamic acid matrix in 50% acetonitrile was added to the dried peptide spots and allowed, again, to air-dry at room temperature. MALDI time-of-flight (TOF) MS analyses were performed in a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Warrington, U.K.) operated in positive reflector mode, with an accelerating voltage of 20,000 V. Mass spectra were then acquired for peptide mass fingerprinting (PMF). Proteins were identified by comparing the trypsin-digested peptide masses with the data provided in two databases (NCBI residues and UniprotKB-SwissProt v.56.6) separately using MASCOT 1.9 (<http://www.matrixscience.com>) through the Global Protein Server v3.5 from Applied Biosystems. All of the identified proteins fulfilled the criterion of being significant ($p < 0.005$) according to probability based on the Mowse score⁵⁷. Statistical analyses were performed using the Statgraphics Plus 5.1 software package.

High-density Peptide Arrays. Sera samples from subjects from Brehman-Asikuma were tested for the recognition of peptides in arrays. They were synthesized on glass slides using photolithographic peptide array⁵⁸ using a layout with 12 identical sectors per slide. Each sector contained 6630 peptide fields. The surface coating on the slides was made by incubation of Nexterion E slides (Schott AG) with a 2% w/v linear copolymer of N,N'-dimethylacrylamide and aminoethyl methacrylate as described⁵⁹. Each sector of the arrays was incubated with a different serum diluted 1:200 in buffer (0.05 M Tris/acetate pH 8.0, 0.13 M NaCl, 0.1% v/v Tween20, 1 g/L BSA) for 2 hours in after blocking. Then, after washing in same buffer, the slides were incubated for 2 hours at room temperature with 1 μg/mL Cy3-conjugated goat anti-human IgG (H+L) (Sigma) and washed again. The slides were centrifuged in a slide spin-dryer and scanned at 1 μm resolution in an InnopSys 900 laser scanner using an excitation wavelength of 532 nm. The images were analyzed using the PepArray analysis program (Schaefer-N) and the fluorescent signal in each field was calculated as the weighted average of the pixel intensities in that field.

Peptide-based ELISA. Specific IgG binding in serum samples to selected peptides was tested by ELISA. Microtiter plates (Maxisorb, NUNC) were coated in duplicate with peptides by adding 100 μL of a 2.5 μg mL⁻¹ solution in 0.05 M Carbonate-Bicarbonate buffer, pH 9.6. After overnight incubation at 4 °C, plates were washed with 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0 buffer five times, 200 μL of blocking buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) were added and incubated at room temperature for 30 minutes. After one more wash, 100 μL of serum diluted 1:450 in wash buffer was added. After incubation at room temperature during 60 min, the supernatant was removed, the wells were washed once more and 100 μL of goat Anti-IgG-Fc HRP conjugated at 1 mg mL⁻¹ (Bethyl) were added at 1:15000 dilution. HRP activity was detected as indicated above. Mann-Whitney non parametric test was used for statistical analysis of IgG binding signal between groups.

Statistical analysis. Statistical analysis of quantitative variables was performed using either parametric Student's t-test (normal distribution) or Mann-Whitney non-parametric test (non-normally distribution), as required, to determine significant differences between groups, if any. All statistical analyses were carried out using OriginPro software (OriginLab Corporation Northampton MA, USA). A P value ≤ 0.05 was considered significant.

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Author contributions

J.M.B., A.P., and A.D. designed and conceptualized research; I.G.A., P.M.G., P.A., S.P.B., E.P.A., P.A.R., J.F., J.M.R. and A.D. performed research; J.M.B., A.P., A.D., I.G.A., and P.M.G. analyzed data; J.M.B., A.P., I.G.A., and P.M.G. supervised research and wrote the paper; and J.M.B. and A.P. acquired financial support for the project leading to this publication.

Competing interests

The authors declare no competing interests.

Additional information

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