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Supplemental information

Plasmodium vivax malaria serological exposure markers: Assessing the degree and implications of cross-reactivity with *P. knowlesi*

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Supplementary Information

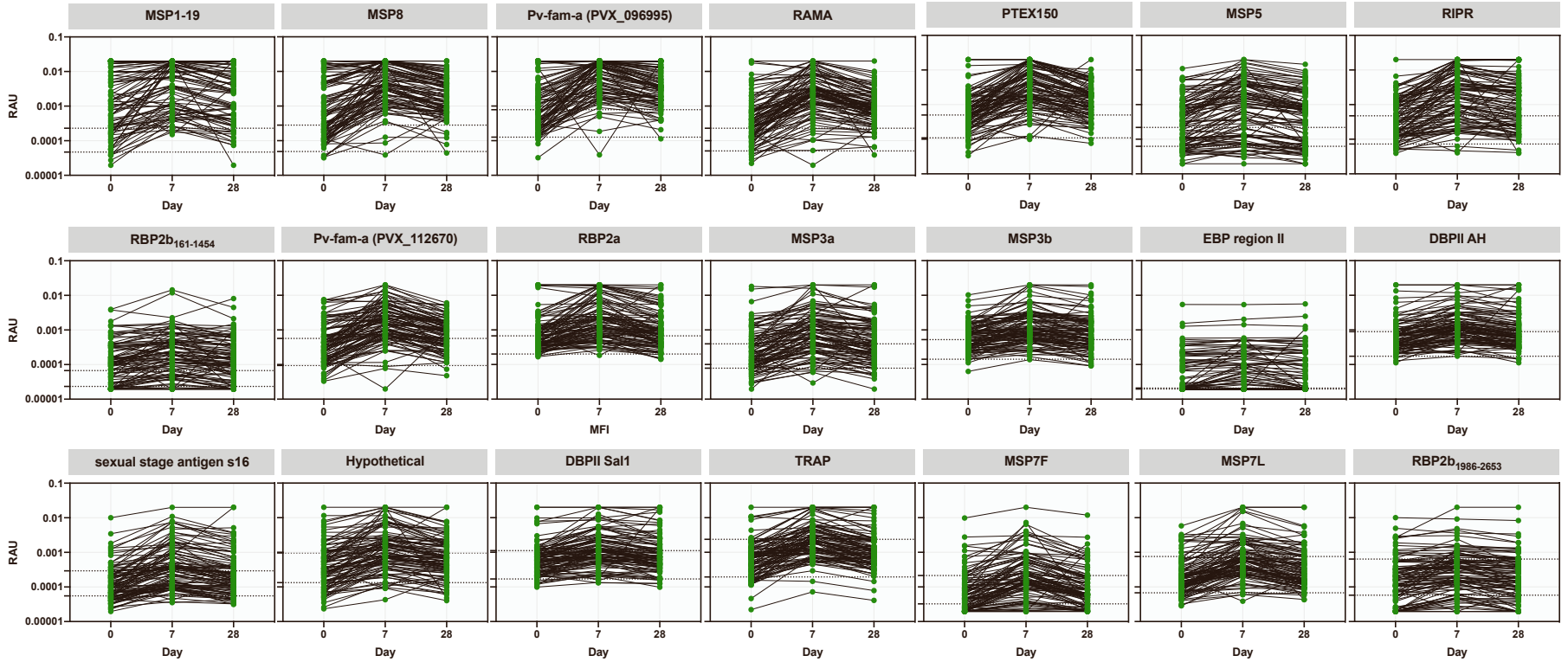

Figure S1: IgG antibody levels against 21 *P. vivax* proteins in 98 individuals with matched data over time. IgG levels were measured against the 21 *P. vivax* proteins using a multiplexed antibody assay. Samples were obtained and run at the time of *P. knowlesi* infection (day 0), and days 7 and 28 following enrolment (ACTKNOW cohort). Results are expressed as the relative antibody units (RAU). All samples were run in singlicate. The raw data were converted to RAU based on the protein-specific standard curve using a 5-paramater logistic function. Proteins are ordered by highest level of median IgG at day 7 compared to the seropositivity cut-off. Dashed lines indicate the malaria-naïve negative control samples (n=369, MSP3b n=213): lower = average of the negative control samples; upper = seropositivity cut-off (average plus 2x standard deviation). Related to Figure 1.

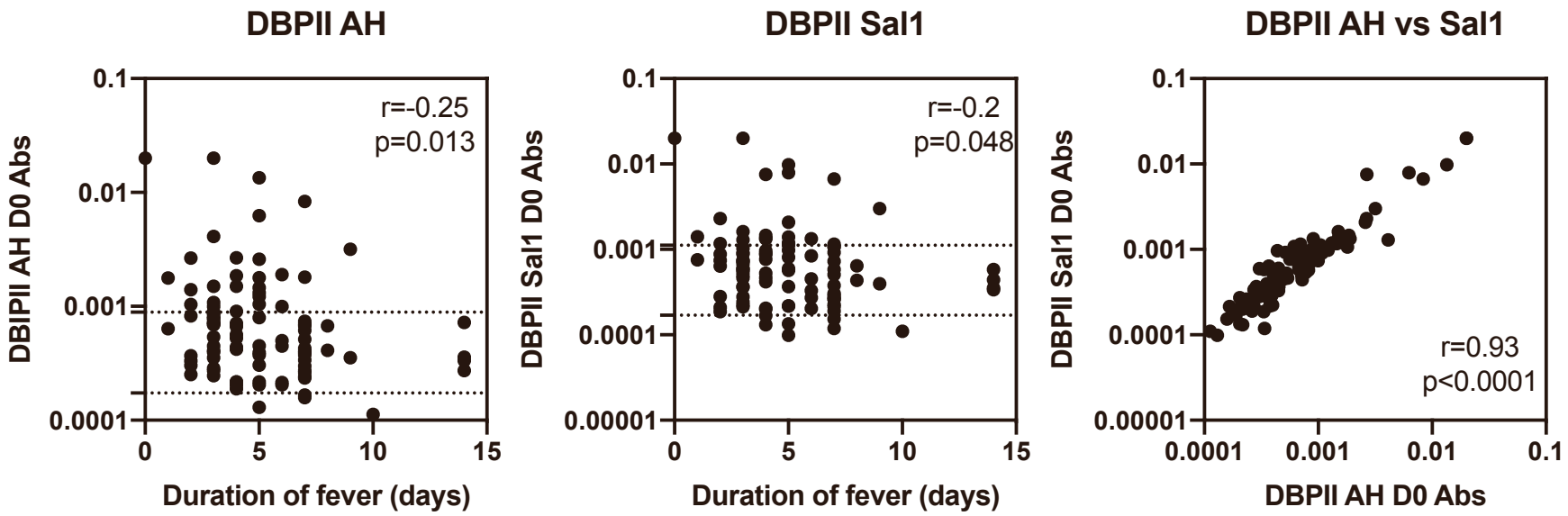

Figure S2: Correlation between acquisition of IgG antibodies at Day 0 to *P. vivax* DBPII constructs and duration of fever of *P. knowlesi* patients. IgG levels were measured against the 21 *P. vivax* proteins using a multiplexed antibody assay. Samples were obtained and run at the time of *P. knowlesi* infection (day 0), and days 7 and 28 following enrolment (ACTKNOW cohort). Results are expressed as the relative antibody units (RAU). All samples were run in singlicate. The raw data were converted to RAU based on the protein-specific standard curve using a 5-paramater logistic function. Here, IgG antibody data at day 0 were correlated with the duration of fever (days) reported by the *P. knowlesi* patients. Data are shown for the only two proteins with a statistically significant correlation, DBPII AH and Sal1. Dashed lines indicate the malaria-naïve negative control samples (n=369): lower = average of the negative control samples; upper = seropositivity cut-off (average plus 2x standard deviation). The third plot shows the correlation between antibodies between both DBPII constructs. Spearman correlation co-efficients are shown. Related to Table 4.

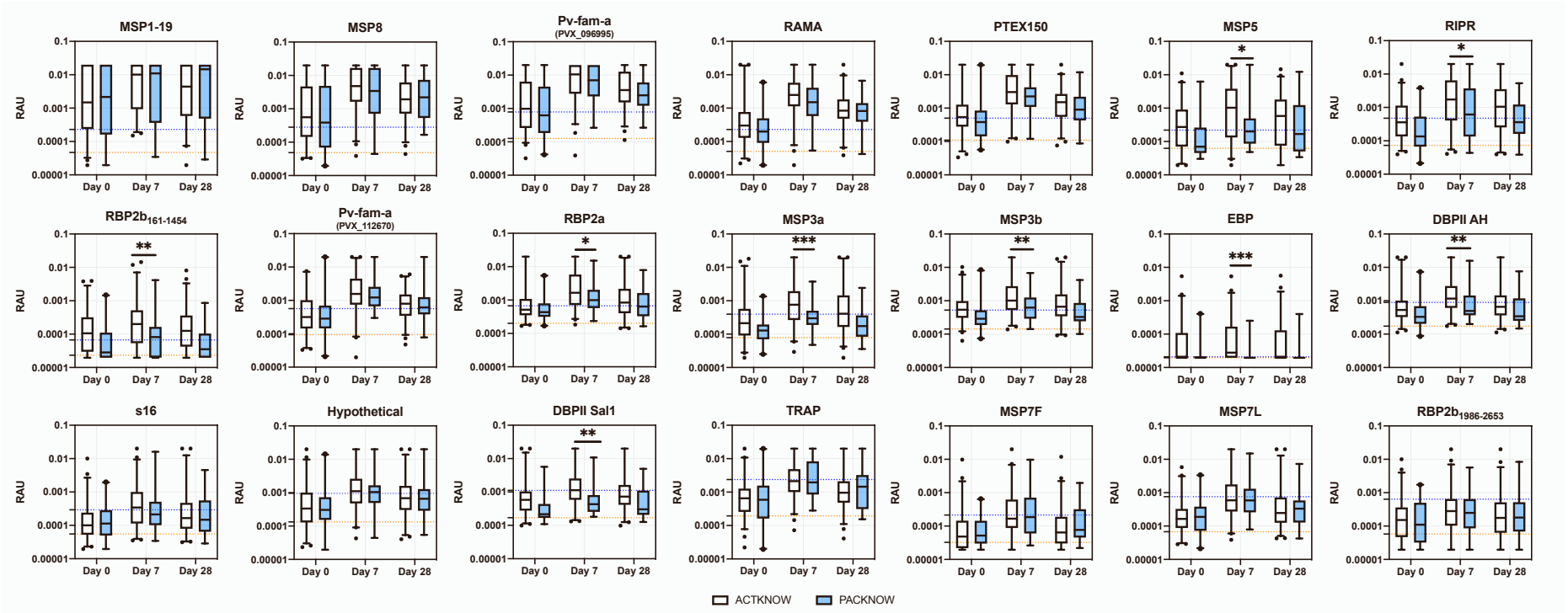

Figure S3: IgG antibody levels against 21 *P. vivax* proteins in *P. knowlesi* patients split via clinical trial. IgG levels were measured against the 21 *P. vivax* proteins using a multiplexed antibody assay. Samples were obtained and run at the time of *P. knowlesi* infection (day 0), and days 7 and 28 following enrolment (ACTKNOW and PACKNOW cohorts). Further data timepoints were available for PACKNOW but are not shown. Results are expressed as the relative antibody units (RAU). All samples were run in singlicate. The raw data were converted to RAU based on the protein-specific standard curve using a 5-paramater logistic function. Proteins are ordered by highest level of median IgG at day 7 compared to the seropositivity cut-off. Dashed lines indicate the malaria-naïve negative control samples (n=369, MSP3b n=213): lower = average of the negative control samples; upper = seropositivity cut-off (average plus 2x standard deviation). Data are split via clinical trial; the ACTKNOW cohort (n=99) and the PACKNOW cohort (n=41 at day 0). Statistical difference in antibody levels between clinical trial groups was only assessed at the peak timepoint of day 7, using Mann-Whitney U tests. 9 of 21 *P. vivax* proteins induced significantly higher levels in ACTKNOW vs PACKNOW: RBP2b$_{161-1454}$ (p=0.0019), MSP3b (p=0.0095), DBPII AH (p=0.0064), RIPR (p=0.013), MSP3a (p=0.0002), MSP5 (p=0.021), RBP2a (p=0.046), DBPII Sal1 (p=0.0049) and EBP (p=0.0007). Related to Figures 1 and 2.

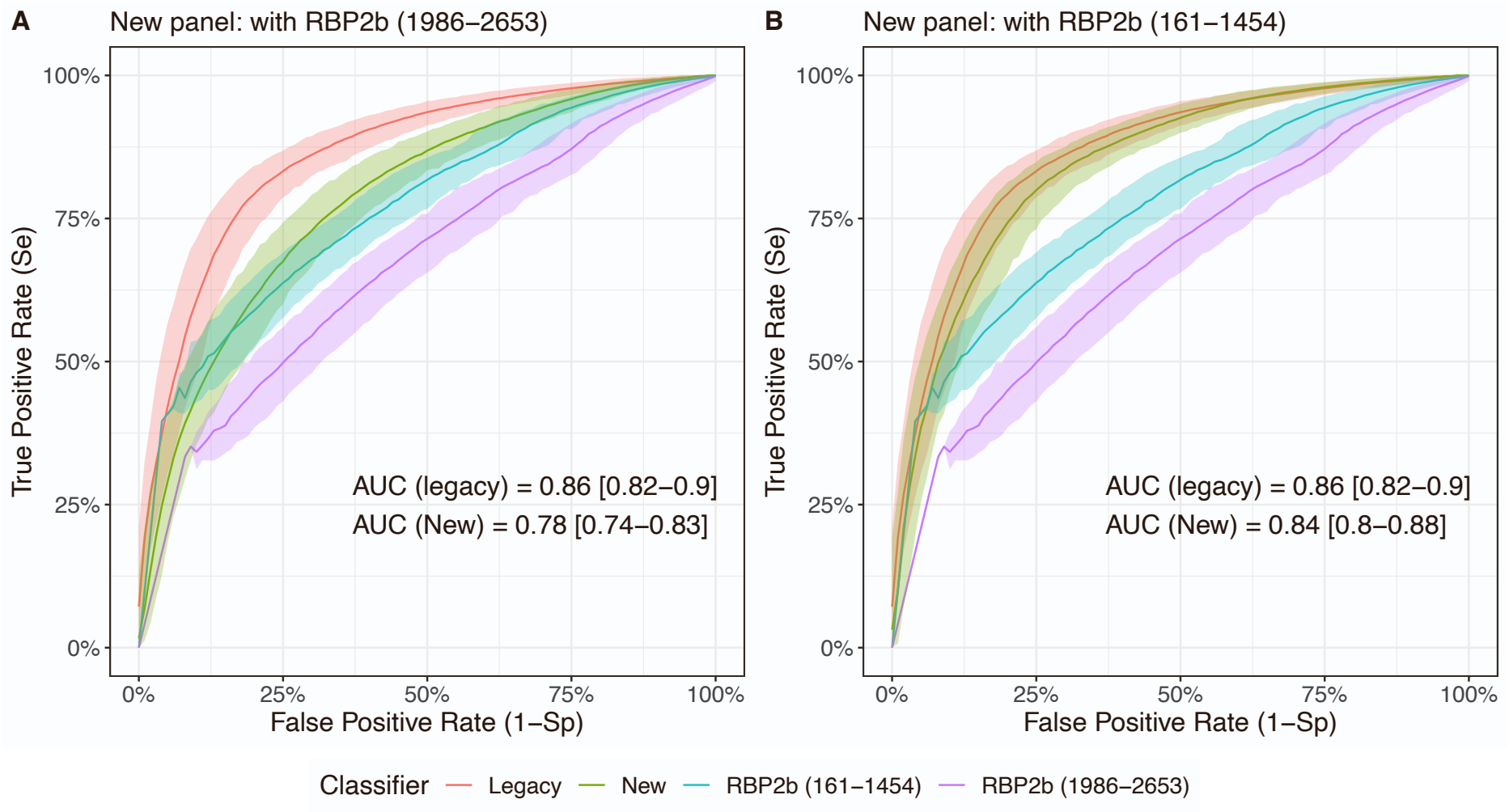

Figure S4: Classification of recent *P. vivax* infections using an adjusted *P. vivax* antigen panel. Receiver operator characteristic curve for classifying individuals with PCR-detected *P. vivax* infections within the prior 9 months, using a random forest classification algorithm with antibody responses to a modified set of 8 *P. vivax* antigens (green, "new") compared to the original 8 (red, "legacy"). In panel A) $RBP2b_{1986-2653}$, MSP7L, MSP7F, TRAP, DBPII Sal1, hypothetical (PVX_097715), s16 and EBP, and B) $RBP2b_{161-1454}$, MSP7L, MSP7F, TRAP, DBPII Sal1, hypothetical (PVX_097715), s16 and EBP. Both panels A and B also include classification using either of the $RBP2b_{1986-2653}$ (purple) or $RBP2b_{161-1454}$ (blue) responses alone. Antibody data was from Thailand (n=829), Brazil (n=928), the Solomon Islands (n=754) and malaria-naïve negative controls (n=370). All samples were run in singlicate. Related to Table 3 and STAR Methods.

Table S1: Proteins used in this study. Related to STAR Methods, Figures 1, 2, 3, Tables 1 and 4.

Protein	PlasmoDB code/GenBank ID	Full Annotation	Region, amino acids (size)	Expression system	µg protein coupled to $2.5x10^6$ beads
$RBP2b_{161-1454}$	PVX_094255	Reticulocyte binding protein 2b (n-terminal fragment)	161-1454 (1294)	*E. coli,* WEHI	0.6
MSP1-19	PVX_099980	Merozoite surface protein 1 - 19	1622-1729 (108)	WGCF, CellFree Sciences	4
$RBP2b_{1986-2653}$	PVX_094255	Reticulocyte binding protein 2b (c-terminal fragment)	1986-2653 (667)	WGCF, CellFree Sciences	8
EBP	KMZ83376.1	Erythrocyte binding protein	109-432 (324)	*E. coli,* Institut Pasteur	0.5
s16	PVX_000930	Sexual stage antigen S16	31-end (110)	WGCF, CellFree Sciences	2
RIPR	PVX_095055	Rh5 interacting protein	552-1075 (524)	*E. coli,* WEHI	2
MSP3a	PVX_097720	Merozoite surface protein 3	25-end (828)	WGCF, CellFree Sciences	2
Hypothetical	PVX_097715	Hypothetical protein	20-end (431)	WGCF, CellFree Sciences	3
DBPII AH	AAY34130.1	Duffy binding protein region II (strain AH)	1-237 (237)	*E. coli,* Institut Pasteur	1.4
MSP8	PVX_097625	Merozoite surface protein 8	24-463 (440)	WGCF, CellFree Sciences	1.4
Pv-fam-a	PVX_112670	Pv-fam-a	34-end (302)	WGCF, CellFree Sciences	2.26
RAMA	PVX_087885	Rhoptry associated membrane antigen	462-730 (269)	WGCF, CellFree Sciences	1.2
Pv-fam-a	PVX_096995	Pv-fam-a	61-end (420)	WGCF, CellFree Sciences	3
MSP3b	PVX_097680	Merozoite surface protein 3	21-end (996)	WGCF, CellFree Sciences	0.8
MSP7L	PVX_082700	Merozoite surface protein 7	23-end (397)	WGCF, CellFree Sciences	1.5
MSP5	PVX_003770	Merozoite surface protein 5	23-365 (343)	WGCF, CellFree Sciences	0.2
MSP7F	PVX_082670	Merozoite surface protein 7	24-end (388)	WGCF, CellFree Sciences	1
TRAP	PVX_082735	Thrombospondin related adhesion protein (also known as SSP2)	26-493 (468)	WGCF, CellFree Sciences	2
DBPII Sal1	PVX_110810	Duffy binding protein region II (strain Sal1)	193-521 (329)	*E. coli,* Institut Pasteur	1.5
PTEX150	PVX_084720	translocon component PTEX150, putative	24-908 (885)	WGCF, CellFree Sciences	1
RBP2a	PVX_121920	Reticulocyte binding protein 2a	160-1135 (976)	*E. coli,* WEHI	1.5

Table S2: Association between peak antibody levels at day 7 with parasitaemia and gender. Sample size = 134. Regression analyses were performed univariably. Antigens are ordered by the fold change in the peak antibody level at day 7 compared to the seropositivity cut-off based on the negative control samples (=Fold Δ IgG). Fold change data shown is from the ACTKNOW cohort. CI = confidence interval. Related to Table 4.

		Parasitaemia		**Gender**	
Protein	**Fold Δ IgG**	*Coefficient (95% CI)*	*p value*	*Coefficient (95% CI)*	*p value*
MSP1-19	43.98	-2.22e-6 (-8.69e-6-4.25e-6)	0.498	0.051 (-0.23-0.33)	0.724
MSP8	17.07	-3.31e-7 (-3.9e-6 - 3.24e-6)	0.855	0.17 (-0.06-0.42)	0.146
Pv-fam-a (PVX_096995)	13.39	-5.35e-7 (-4.02e-6-2.94e-6)	0.761	0.12 (-0.07-0.32)	0.207
RAMA	10.86	-1.41e-6 (-4.02e-6-1.2e-6)	0.287	0.23 (-0.015-0.47)	0.065
PTEX150	6.08	3.64e-7 (-2.59e-6-3.32e-6)	0.808	0.11 (-0.13-0.34)	0.376
MSP5	4.70	-1.92e-6 (-5.28e-6-1.43e-6)	0.258	0.34 (0.049-0.64)	0.023
RIPR	3.66	-2.8e-6 (-7.78e-6-2.18e-6)	0.267	0.25 (-0.015-0.51)	0.065
RBP2b$_{161-1454}$	3.00	1.59e-6 (-3.74e-6 - 6.95e-6)	0.557	0.073 (-0.18-0.32)	0.56
Pv-fam-a (PVX_112670)	2.76	9.23e-7 (-1.62e-6-3.46e-6)	0.473	-0.036 (-.24-0.17)	0.724
RBP2a	2.45	-2.78e-6 (-5.99e-6-4.35e-7)	0.09	-0.02 (-2.2-0.18)	0.842
MSP3b	1.93	7.7e-7 (-1.16e-6 - 2.7e-6)	0.432	0.26 (0.053-0.46)	0.014
MSP3a	1.92	-7.73e-7 (-3.5e-6-1.95e-6)	0.575	0.28 (0.047-0.52)	0.019
EBP	1.33	-2.02e-6 (-4.83e-6-7.94e-7)	0.158	0.057 (-0.16-0.27)	0.603
DBPII AH	1.31	-3.6e-9 (-5.52e-6 - -6.03e-7)	0.015	0.178 (0.005-0.35)	0.044
S16	1.18	8.4e-7 (-3.57e-6-5.25e-6)	0.707	0.23 (-0.035-0.49)	0.089
Hypothetical	1.16	-7.69e-7 (-4.23e-6-2.69e-6)	0.661	0.18 (-0.067-0.430	0.152
DBPII Sal1	1.02	-2.13e-6 (-5.29e-6-1.03e-6)	0.185	0.17 (-0.022-0.36)	0.082
TRAP	0.90	3.31e-7 (-3.95e-6 - 4.61e-6)	0.878	0.11 (-0.096-0.31)	0.297
MSP7L	0.78	-8.79e-7 (-3.09e-6-1.34e-6)	0.434	0.16 (-0.077-0.40)	0.185
MSP7F	0.78	4.55e-6 (1.29e-6-7.82e-6)	0.007	0.16 (-0.093-0.41)	0.217
RBP2b$_{1986-2653}$	0.44	2.67e-6 (-1.06e-6 - 6.4e-6)	0.159	0.20 (-0.055-0.45)	0.125