Major Article

Induction and kinetics of complement-fixing antibodies against *Plasmodium vivax* MSP3α and relationship with IgG subclasses and IgM

Running title: Complement-fixing antibodies against *P. vivax*

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Summary: Complement-fixing antibodies targeting *P. vivax* PvMSP3α are prevalent in both children and adults with infection, with both IgG and IgM mediating complement fixation. Magnitudes of complement-fixing antibodies are influenced by antigenic region.

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Footnote Page

Conflicting of interest

All authors report no conflicts of interests. The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

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Meetings presented

Results from this study were previously presented in part at **1)** 1st World Malaria Congress, July 2018, Melbourne, VIC, Australia **2)** Lorne Infection & Immunity Conference, February 2019, Lorne, VIC, Australia **3)** 7th International Conference on *Plasmodium vivax* Research (ICPvR), June 2019, Paris, France.

Abstract

Background: Complement-fixing antibodies are important mediators of protection against *Plasmodium falciparum* malaria. However, complement-fixing antibodies remain uncharacterised for *P. vivax* malaria. *Plasmodium vivax* merozoite Surface Protein-3α (PvMSP3α) is a target of acquired immunity and a potential vaccine candidate.

Methods: Plasma from children and adults with *P. vivax* malaria in Sabah, Malaysia, were collected during acute infection, 7 and 28 days following drug treatment. Complement-fixing antibodies and IgM and IgG, targeting three distinctive regions of PvMSP3α were measured by ELISA.

Results: Seroprevalence of complement-fixing antibodies was highest against PvMSP3α Central region (77.6%). IgG1, IgG3, and IgM were significantly correlated with C1q-fixation and both purified IgG and IgM were capable of mediating C1q-fixation to PvMSP3α. Complement-fixing antibody levels were similar between age groups, but IgM was predominant in children and IgG3 more prevalent in adults. Functional antibodies increased following acute infection to 7 days after treatment, however rapidly waned by day 28.

Conclusion: Our study demonstrates PvMSP3α antibodies acquired during *P. vivax* infection can mediate complement-fixation and shows the important influence of age in shaping these specific antibody responses. Further studies are warranted to understand the role of these functional antibodies in protective immunity against *P. vivax* malaria.

Introduction

Plasmodium vivax malaria is a major contributor to global malaria burden, and remains a challenge for elimination and eradication efforts [1]. Morbidity from malarial infections arises from blood-stage replication, and its control is critical for the prevention of clinical disease [2]. Reduction of blood-stage infection also limits gametocyte formation, thus reducing transmission [3, 4]. The first step in each replication cycle of blood-stage malaria is merozoite invasion of red blood cells (RBCs). Blood exposure of merozoites to immune mediators and chemical inhibitors make this parasite stage a key therapeutic target [2, 5].

Antibodies are key mediators of malarial immunity. Functional antibodies can fix complement at the merozoite to prevent RBC invasion, and this immune mechanism is associated with protection from *P. falciparum* malaria [6-9]. Additionally, complement-fixing antibodies have been associated with better artemisinin treatment-efficacy [10]. Multiple antigen targets of complement-fixing antibodies that mediate protection have been identified in *P. falciparum* [8], and both IgG and IgM antibodies mediate complement fixation to the parasite [6, 9]. However, complement-fixing antibodies were more strongly associated with protection than

total IgG or IgM responses [9], suggesting it is the quality and not only quantity of the antibody response that is important in protection. A recent study provided initial evidence for the acquisition of complement-fixing antibodies against *P. vivax* merozoites [11]. However, no other studies have investigated complement-fixing antibodies targeting *P. vivax*, and the impact of protein regions and age on complement-fixing antibodies, kinetics of acquisition and decay, and the specific antibody isotypes and subclasses associated with functional complement-fixing antibodies are unknown.

PvMSP3α is a member of the PvMSP3 family characterised by an alanine-rich central domain containing a series of heptad repeats that are predicted to form coiled-coil structures involved in protein-protein interactions [12]. The carboxy terminal (C-terminal) and hydrophilic amino (N-terminal) domains of PvMSP3α are relatively conserved, while the Central domains (Block I and II sequences) are highly polymorphic [13]. In malaria endemic regions, naturally occurring antibodies to PvMSP3α (C-terminal and Central region) have been associated with protection from *P. vivax* malaria in different populations [14, 15]. Antibodies that target PvMSP3α are predominately cytophilic IgG1 and IgG3 [14]. These IgG subclasses have strong complement fixation potential [2], suggesting that complement-fixing antibodies may play an important role in immunity targeting PvMSP3. To date, no studies have investigated IgM reactivity against PvMSP3α, while recent work has shown that IgM mediates complement fixation and prevention of clinical *P. falciparum* [9]. Whether IgM mediates a similar functional complement mechanism in *P. vivax* malaria remains to be determined.

In this study, we investigated the induction of antibody-mediated complement fixation following acute *P. vivax* malaria episode from a low malaria endemic setting in Sabah, Malaysia. In order to increase our knowledge on the factors influencing development of functional complement fixing antibodies targeting *P. vivax* malaria, we investigated the

influence of age, antigenic region, and antibody composition of responses on antibody responses in children and adults with *P. vivax* malaria.

Methods

Detailed methods in Supplementary Data.

Ethics statement:

Written and informed consent was obtained from all study participants, with consent obtained from parents or guardians in the case of children. Studies were approved by the Ethics Committee of Menzies School of Health Research and the Medical Research and Ethics Committee, Ministry of Health Malaysia.

Study cohort

Plasma samples were obtained from patients with PCR-confirmed *P. vivax* malaria with fever, who were seeking treatment and subsequently enrolled on diagnosis in malaria cohort studies in Sabah, Malaysia [16, 17], an region of low *P. vivax* endemicity [18]. Patients were treated using hospital guidelines. Uninfected control plasma were obtained from visitors or patient's relatives with no history of fever in the last 48 hours, who were blood film negative by microscopy and confirmed negative for *Plasmodium* infection by PCR. Samples were grouped as children (≤15 years) and adults (>15 years).

Recombinant antigens

Proteins representing different regions of PvMSP3α were used; N-terminal (nucleotides 73-309), Central region (Block I-II) (nucleotides 316-2058), and C-terminal (nucleotide 2059-

2523). Proteins were PCR amplified from *P. vivax* (Belem strain), and expressed in a vector containing a C-terminal 6x His Tag [14].

ELISAs

ELISAs were performed as described [6, 8]. Plates were coated with PvMSP3α antigens (0.5 µg/ml), blocked and incubated with plasma (1/250). Total IgG was quantified with horseradish peroxidase (HRP)-conjugated sheep anti-human IgG (1/2000) (Life Technologies). For IgM and IgG subclasses, monoclonal mouse anti-human IgM and IgG antibodies (Life Technologies) and goat polyclonal anti-mouse IgG-HRP (Merck Millipore) were added (1/2000). For C1q fixation, recombinant C1q (10 µg/ml, Quidel) was added after human plasma, and C1q was detected with 1/2000 rabbit anti-C1q antibodies (Dako) and 1/4000 goat anti-rabbit HRP (Bio-Rad).

Malaria unexposed controls were obtained from Australian donors. Positive responses were defined as absorbance greater than the average of 7 malaria unexposed controls plus 3 standard deviations.

Antibody purification

Plasma samples from *P. vivax*-infected patients (n = 5) and malaria unexposed (n = 15) were pooled and immunoglobulins precipitated using saturated ammonium sulfate, and then dialysed in PBS. Purification of IgG and IgM was performed using NAb™ Protein G Spin Column (Thermo Scientific) as per manufacturer's instructions. IgM fraction refers to non-IgG fraction from the column. Both IgG and IgM fractions were dialysed overnight in PBS and concentrated using 10 kDa spin columns (Amicon, Merck Millipore).

Statistical analysis

All analyses were performed in STATA (ver15.0) and GraphPad Prism (ver7.03). Differences in antibody magnitudes between age groups were compared using Mann-Whitney non-parametric test. For comparison between antigen regions and follow-up time points, Friedman and Wilcoxon-matched pair test was used. Chi-square test was used to compare seroprevalence between antigen regions.

Results

Complement-fixing antibodies are prevalent in adults and children with acute *P. vivax* malaria

Antibodies against PvMSP3α were assessed in 21 uninfected subjects and 52 patients with *P. vivax* malaria (24 children and 28 adults, **Table 1**). Median parasitemia was similar between adults and children (p=0.449).

We first assessed antibody-mediated complement fixation targeting three regions of PvMSP3α in children and adults presenting with acute *P. vivax* malaria, and adults who were PCR negative for *P. vivax* infection [19] (**Table 1**). Uninfected adults in this cohort may have been previously exposed to malaria. We quantified antibody fixation of C1q, the first sera component of the antibody-dependent classical activation pathway (we have previously demonstrated that C1q fixation correlates with complement activation and formation of the membrane attack complex [8, 9]). Seroprevalence of C1q-fixing antibodies varied across antigen regions, with highest overall prevalence against Central region (children = 82.6%, adults = 73.1%), followed by C-terminal (children = 39.1%, adults = 57.7%) and N-terminal (children = 17.4%, adults = 19.2%) (**Figure 1A & Supplementary Figure 1A**). There was no difference in seroprevalence of C1q fixation between infected children and adults (**Figure 1A**). When compared with uninfected individuals, *P. vivax* infected individuals had higher

prevalence of C1q-fixing antibodies regardless of age, except for antibody responses in children against the C-terminal domain (**Figure 1A**). Overall, the majority of infected individuals (82.6% children and 80.8% adults) had C1q-fixing antibodies recognising at least one of the PvMSP3α region, compared with only 52.4% of uninfected individuals (**Figure 1B**). Similarly, the magnitude of C1q-fixing antibodies was not different between the two infected age groups, with both being higher than in uninfected individuals (**Figure 1C**). Uninfected individuals had higher magnitudes of C1q fixing antibodies than malaria unexposed individuals to the Central region (**Supplementary Figure 1B**). The magnitude of C1q-fixing antibodies were similar across PvMSP3α regions, except that responses for the Central region were significantly higher than for the C-terminus (**Supplementary Figure 1B**).

Antibody composition during infection is age dependent

Both IgG and IgM antibodies have C1q-fixing capacity against *P. falciparum* antigens [6, 9]. To investigate the relative importance of IgG and IgM antibody isotypes in mediating complement fixation on PvMSP3α, we first quantified magnitudes of total IgG (pan-anti-IgG detecting all subclasses) and IgM in *P. vivax* infected patients and uninfected individuals. During *P. vivax* infection, both IgG and IgM seroprevalence was high across all PvMSP3α regions (children; IgG = 52-61%, IgM = 96-100%, adults; IgG = 65-69%, IgM = 58-77%) (**Figure 2A & Supplementary Figure 2A**). There was no difference in IgG seroprevalence between infected children and adults, but children had significantly higher IgM seroprevalence (**Figure 2A**). IgG seroprevalence was significantly lower in uninfected individuals compared to infected individuals. There was no overall difference in seroprevalence of IgG and IgM in infected individuals across the PvMSP3α regions (**Supplementary Figure 2A**). A large proportion of children and adults had IgG and IgM recognition to at least a single PvMSP3α region (74-81% for IgG and 81-96% for IgM) (**Figure 2B**). The breadth of IgM responses in children was higher than breadth of IgG, with 95.7% recognising all 3 PvMSP3α regions as

opposed to 34.8% for IgG (χ^2 test, p<0.001). In uninfected individuals, IgM recognition to at least a single PvMSP3α region was also significantly higher than IgG (χ^2 test, p=0.013).

Similarly, the magnitude of IgG in infected children and adults was comparable, while the magnitude of IgM was significantly higher in children than adults against all PvMSP3α regions (**Figure 2C**). Uninfected individuals had lower IgG and IgM compared to infected individuals, except for IgM to the N-terminal region which was comparable (**Figure 2C**). However, uninfected individuals had higher magnitudes of IgG antibodies (to central and C-terminal) and higher magnitudes of IgM antibodies to all regions compared to unexposed controls, suggesting these individuals had prior malaria exposure and some levels of maintenance of acquired antibodies (**Supplementary Figure 2B**). Overall, the magnitude of both IgG and IgM were highest against C-terminal regions (**Supplementary Figure 2B**).

We next quantified the composition of the IgG subclass responses. Consistent with prior studies [14], IgG1 and IgG3 were the dominant antibodies to PvMSP3α (**Figure 3**) while IgG2 and IgG4 responses were below detection limits and therefore were not analysed further (data not shown). IgG1 and IgG3 seroprevalence were similar between infected children and adults across all regions of PvMSP3α (**Figure 3A**). Compared to uninfected individuals, seroprevalence in the infected individuals was significantly higher across all PvMSP3α regions, except for IgG3 seroprevalence against the N-terminal region (**Figure 3A**). Comparisons between regions show that IgG1 seroprevalence was highest against the N-terminal, followed by C-terminal and Central regions (**Supplementary Figure 3A**). For IgG3, seroprevalence was highest against the N-terminal, followed by the Central and C-terminal regions (**Supplementary Figure 3A**). The majority of infected children and adults had IgG1 and IgG3 recognition to PvMSP3α antigens, with 91-96% and 96-100% recognising at least a single PvMSP3α region, respectively (**Figure 3B**). Antibody recognition to at least a single PvMSP3α

region in uninfected individuals was moderate for IgG1 (52.4%) and high for IgG3 (95.2%) (**Figure 3B**).

The magnitude of IgG1 and IgG3 responses in infected children and adults was similar, except for significantly higher IgG3 responses against the Central region in adults (**Figure 3C**). IgG1 and IgG3 responses were significantly higher in infected individuals compared to uninfected individuals across all regions (**Figure 3C**). Compared to unexposed individuals, uninfected Malaysia individuals had higher magnitudes of IgG3 but not IgG1 to all protein regions (**Supplementary Figure 3B**). There was no difference in the magnitude of IgG1 between PvMSP3α regions (**Supplementary Figure 3B**). For IgG3, the magnitude was lowest against the N-terminal domain, with similar responses between the Central region and the C-terminal domain (**Supplementary Figure 3B**).

Both IgG and IgM mediate complement fixation to PvMSP3

To identify specific antibodies that may mediate complement fixation on PvMSP3α, we assessed correlations between C1q fixation and antibody responses. Total IgG was positively associated with C1q fixation in infected children and adults; the correlations were significant across all PvMSP3α regions in children while only the C-terminus was significantly correlated in adults (**Figure 4A**). IgG1 responses in children were significantly correlated with C1q fixation against the C-terminal region while elevated levels of IgG3 were associated with all antigenic regions of PvMSP3α. In comparison, significant correlations with C1q fixation were only observed for IgG1 against the Central region and IgG3 against the C-terminus in infected adults (**Figure 4A**). There was no significant correlation observed between IgG antibodies and C1q fixation in uninfected individuals. IgM was also associated with C1q fixation in children, but not in infected adults (**Figure 4A**). In uninfected adults, IgM response against the C-terminal region had significant correlation with C1q fixation. Overall, IgG1 and IgG3

responses in infected adults, and IgG1, IgG3, and IgM responses in infected children, were correlated with C1q fixation (**Supplementary Figure 4)**.

Our results suggest that both IgG and IgM antibodies contribute to complement fixation to PvMSP3α. To test this hypothesis, IgG and IgM were purified from a pool of *P. vivax*-infected (n = 5) and malaria unexposed individuals (n = 15). The purity of IgG and IgM fractions were confirmed via ELISA (**Figure 4B)**. Both IgG and IgM from *P. vivax* infected donors effectively fixation C1q to PvMSP3α-Central, compared to no C1q fixation in malaria unexposed donors (**Figure 4C)**.

Complement-fixing antibodies to PvMSP3α increase after *P. vivax* infection and then decay rapidly

To investigate the acquisition and decay of complement-fixing antibodies after *P. vivax* infection, we compared antibody levels in Sabah individuals during their infections with levels at 7 and 28 days after drug treatment and parasite clearance. C1q-fixing antibodies were significantly elevated at day 7 following treatment (**Figure 5**). Antibody decayed and returned to levels seen at acute infection by day 28 after treatment (**Figure 5**). Patterns of antibody kinetics for IgG1, IgG3, and IgM were similar as those of C1q-fixing antibodies (**Figure 5**), although levels of IgG1 were generally low. There was no marked difference in kinetics between infected children and adults (**Supplementary Figure 5 & 6**). Antibody levels at acute infection (day 0) were not associated with parasitemia (**Supplementary Figure 7A**). However, IgG (to N-terminal and Central region) antibodies at convalescences (day 28) were negatively associated with parasitemia during acute infection (**Supplementary Figure 7B**).

Discussion

Complement-fixing antibodies have recently been identified as important mediators of immunity against *P. falciparum* malaria [6, 8, 20], however, our understanding of the targets and acquisition of complement-fixing antibodies against *P. vivax* is limited. We investigated the acquisition of complement-fixing antibodies targeting three regions of a major *P. vivax* merozoite surface protein, PvMSP3α. Our data show that during infection, complement-fixing antibodies are prevalent in both children and adults, and the magnitude of complement-fixing antibodies differ between antigenic regions of PvMSP3α. The composition of antibody responses was age-dependent; IgM was more prominent in children, whereas IgG responses dominated in adults. Further, we established that both IgG and IgM mediated complement fixation to PvMSP3α, suggesting that both isotypes have important roles in functional immunity against *P. vivax* malaria.

We demonstrate that complement-fixing antibodies are highly prevalent in Sabah patients, especially to the variable Central region. However, the overall prevalence of complement-fixing antibodies is lower than total IgG and IgM antibodies. The Central region consists of two blocks of heptad repeats that have 18 out of 25 B-cell epitopes of PvMSP3α, thus providing the most recognition sites for antibody binding on the protein [14], a factor that may increase the induction of complement-fixing antibodies against this region. Differences in the antibody composition targeting each region of PvMSP3α may also influence the magnitude of complement-fixing antibodies. IgG responses against PvMSP3α were mainly IgG1 and IgG3 subclasses, consistent with previous findings [14]. However, the overall balance of IgG1 versus IgG3 was region-specific, with IgG1 highest against the conserved N-terminal region, and IgG3 highest towards the Central region; IgG3 has higher capacity to bind complement than IgG1 [21]. Consistent with these findings, previous *P. falciparum* studies show that antigenic regions with polymorphic sequences (such as MSP1 Block 2) tend to induce IgG3 whereas those with conserved regions (such as MSP1-19) tend to induce IgG1 responses [22, 23]. Other

studies of *P. falciparum* suggest that protein structure can also influence the nature of IgG subclasses responses [24]. Several other factors may also influence the ability of antibodies to mediate complement fixation including specific epitope of antibody binding [25], epitope distance from cell surfaces [26], levels of non-functional interfering antibodies that block complement fixation [27] and glycosylation of binding antibodies [28]. While more studies are required to elucidate how each of these factors influences complement fixation to *Plasmodium* antigens, together they may explain why overall levels of complement fixing antibodies are lower than the prevalence of total IgG and IgM antibodies.

During *P. vivax* malaria, there was no difference in the seroprevalence or magnitude of complement-fixing antibodies between infected children and adults. However, the overall composition of the antibody response was age dependent, with children mounting higher IgM while adults had higher IgG3. Both IgG and IgM were correlated with complement-fixing antibodies, and both purified IgG and IgM from *P. vivax* infected patients had the capacity to mediate complement fixation to PvMSP3α. The role of IgM in complement fixation against *P. vivax* is consistent with previous findings in *P. falciparum* malaria [8, 9, 20, 29], emphasising the importance for further studies of IgM to understand protective immunity against *Plasmodium* spp infecting humans.

The age-specific differences in antibody isotype levels could be attributed to different *P. vivax* infection histories. IgM, which is higher in children, is thought to be a rapidly induced response that dominates primary infection [30]. Currently, *P. vivax* transmission in Sabah is undergoing major reductions from the previously higher transmission intensity [31]. As such, children enrolled in our study may not have been previously exposed to *P. vivax*, while adults are more likely to have had prior infection(s) [32]. Thus, the adult-specific dominance of IgG3 may be due to the induction of antibodies from pre-existing B-cell memory. This is consistent with a PvMSP1 study in Brazil, where individuals with first malaria episodes mounted higher IgM

responses, while IgG responses were higher in previously exposed individuals [33]. Despite the IgM dominance in children, seroprevalence of IgM was also high in uninfected adults from malaria endemic Sabah, and the magnitude of *Pv*MSP3α IgM antibodies was significantly higher than detected in malaria unexposed donors. While the malaria history, and recency of infection of uninfected adults tested here is unknown, these data suggest that IgM responses to PvMSP3α are relatively long lived. Recent studies of antibody responses to *P. falciparum* merozoites in multiple populations have found that IgM is a prominent component of the response even in children and adults with high life-time exposure. Further, rather than rapidly decaying post-infection, IgM showed similar kinetics to IgG [9].

Following malaria treatment, complement-fixing, IgG and IgM antibodies to PvMSP3α increased rapidly within 7 days but declined to levels seen at acute presentation at day 28. Similar findings in *P. falciparum* also show that anti-merozoite antibodies peaked 7 days after malaria and quickly declined within weeks of infection [34]. Additionally, in other *P. vivax* antigens, antibodies against PvMSP1 also appear to be short-lived, with the majority of individuals becoming seronegative two months after anti-malarial treatment [33]. However, other studies have reported that IgG responses to some antigens are much better maintained [35-41]. Within our population, levels of IgG antibodies at day 28 after infection were negatively associated with parasitemia at acute infection. This finding is consistent with mouse models of malaria where high parasitemia has been associated with negative consequences for long lived and mature antibodies responses [42]. This observation in human infection with *P. vivax* warrants further investigation, however the lack of long-term follow-up on our patients precludes the study of the maintenance of antibody responses that may be generated from long-lived cells, and in memory phase immunity.

In *P. falciparum* infections, complement fixation of antibodies targeting merozoites and sporozoites are better correlates of protection than total antibody levels, highlighting its

significance in malaria immunity [8, 9, 20]. Due to the nature of our study, we were not able to define the association of complement fixation with protection from infection or disease. However, we show that PvMSP3α is a target of complement-fixing antibodies, and as such, future studies investigating the association between complement-fixing antibodies targeting PvMSP3α and other merozoite antigens and protection from *P. vivax* infection are warranted. Furthermore, evidence of cross-reactivity between other *P. vivax* and *P. knowlesi* antigens has been reported previously in Sabah [43], and we cannot exclude a contribution of PvMSP3α antibody responses from prior infection with *P. knowlesi*.

In conclusion, our study demonstrates that antibody-mediated complement fixation against PvMSP3α antigen is prevalent in individuals with *P. vivax* infection living in Sabah, Malaysia, and the prevalence and magnitudes of complement-fixing antibody responses are dependent on antigenic region. The composition of the antibody response during infection is age dependent, with IgM predominant in children and IgG3 the dominant in adults. However, IgG1, IgG3 and IgM antibodies targeting PvMSP3α all correlated with complement fixation, and both IgG and IgM mediated complement fixation to PvMSP3α. These findings are significant for understanding immunity to *P. vivax* malaria and for the potential development of vaccines against *P. vivax*. Indeed, if complement-fixing antibodies targeting *P. vivax* antigens are shown to be highly protective against *P. vivax* malaria, vaccines that induce high levels of functional complement-fixing antibodies may lead to increased protection and vaccine efficacy.

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553

554 **Tables**

555 ***Table 1. Demographic parameters of Sabah study samples***

	Uninfected (n = 21)	***P. vivax* Malaria Patients**	
		Children (n = 24)	**Adults (n = 28)**
Male (%)	12 (57%)	14 (58%)	24 (86%)
Median age (Range)	39 (18-67)	10 (5-15)	34 (16-57)
Parasitemia (iRBC/µl) Median (IQR)	0	3,518 (1,490 – 9,610)	2,470 (2,470 – 7,600)

556 Note: Parasitemia was measured during acute presentation of malaria by blood smear.
557 Uninfected samples are Sabah adults with parasite negative by PCR
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Figure Legends

Figure 1. Seroprevalence and magnitude of C1q-fixing antibodies targeting PvMSP3α in children and adults with* P. vivax *malaria and uninfected adults. ***(A)*** *Seroprevalence of C1q-fixing antibodies against different regions of PvMSP3α. Positive threshold for seroprevalence was calculated as above the mean plus three standard deviations of absorbance detected in malaria naïve Australian donors. Chi-square test is indicated for comparisons between groups; children, adults, and uninfected individuals.* ***(B*** *Cumulative C1q-fixing antibody responses targeting different regions of PvMSP3α. Data represent percentage of infected individuals who are positive for 0-3 proteins tested.* ***(C)*** *Magnitudes of C1q-fixing antibody responses against different regions of PvMSP3α. Mann-Whitney test is indicated for comparisons between groups; children, adults, and uninfected individuals. For boxplot, lower and upper hinges represent first and third quartiles, and whisker lines correspond to highest and lowest values no further than 1.5 interquartile range from hinges. Data beyond the whisker lines are treated as outliers. Median line is indicated across the box.*

Figure 2 Seroprevalence and magnitude of IgG and IgM antibodies to PvMSP3α in children and adults with* P. vivax *malaria and uninfected adults. ***(A)*** *Seroprevalence of IgG and IgM antibodies against different regions of PvMSP3α. Positive threshold for seroprevalence was calculated as above the mean plus three standard deviations of absorbance detected in malaria naïve Australian donors. Chi-square test is indicated for comparisons between groups; children, adults, and uninfected individuals.* ***(B)*** *Cumulative IgG and IgM antibody responses targeting different regions of PvMSP3α. Data represent percentage of infected individuals who are positive for 0-3 protein regions tested.* ***(C)*** *Magnitudes of IgG and IgM antibody responses against different regions of PvMSP3α. Mann-Whitney test is indicated for comparisons*

588 *between groups; children, adults, and uninfected individuals. For boxplot, lower and upper*
589 *hinges represent first and third quartiles, and whisker lines correspond to highest and lowest*
590 *values no further than 1.5 interquartile range from hinges. Data beyond the whisker lines are*
591 *treated as outliers. Median line is indicated across the box.*

592

593 ***Figure 3. Seroprevalence and magnitude of IgG1 and IgG3 antibodies to PvMSP3α in***
594 ***children and adults with* P. vivax *malaria and uninfected adults.*** ***(A)*** *Seroprevalence of IgG1*
595 *and IgG3 antibodies against different regions of PvMSP3α. Positive threshold for*
596 *seroprevalence was calculated as above the mean plus three standard deviations of absorbance*
597 *detected in malaria naïve Australian donors. Chi-square test is indicated for comparisons*
598 *between groups; children, adults, and uninfected individuals.* ***(B)*** *Cumulative IgG1 and IgG3*
599 *antibody responses targeting different regions of PvMSP3α. Data represent percentage of*
600 *infected individuals who are positive for 0-3 proteins tested.* ***(C)*** *Magnitudes of IgG1 and IgG3*
601 *antibody responses against different regions of PvMSP3α. Mann-Whitney test is indicated for*
602 *comparisons between groups; children, adults, and uninfected individuals. For boxplot, lower*
603 *and upper hinges represent first and third quartiles, and whisker lines correspond to highest*
604 *and lowest values no further than 1.5 interquartile range from hinges. Data beyond the whisker*
605 *lines are treated as outliers. Median line is indicated across the box.*

606

607 ***Figure 4. Functional C1q-fixing capacity of antibody isotypes.*** ***(A)*** *Correlations matrixes*
608 *between IgG1, IgG3, IgM, and C1q-fixing antibodies to PvMSP3α in children, adults, and*
609 *uninfected individuals. Spearman's correlation coefficient is indicated. Coloured boxes*
610 *indicate statistical significance, $P < 0.05$.* ***(B)*** *Total IgG and IgM absorbance readings against*
611 *PvMSP3α Central region from purified IgG and IgM fractions in malaria infected plasma*

pools. ***(C)*** *C1q fixation absorbance readings against PvMSP3α Central region from purified IgG and IgM fractions from malaria infected (n=5) and malaria unexposed (n=14) plasma pools.*

Figure 5. Antibody kinetics profile across 28 days follow up against PvMSP3α. *Magnitudes of C1q-fixing antibodies, IgG1, IgG3, and IgM against different regions of PvMSP3α were compared between Day 0 and Day 7 and 28 follow-ups. Wilcoxon signed-ranked test is indicated. Grey dots and lines represent individual antibody magnitude over time. Median line is indicated.*

Figure 1

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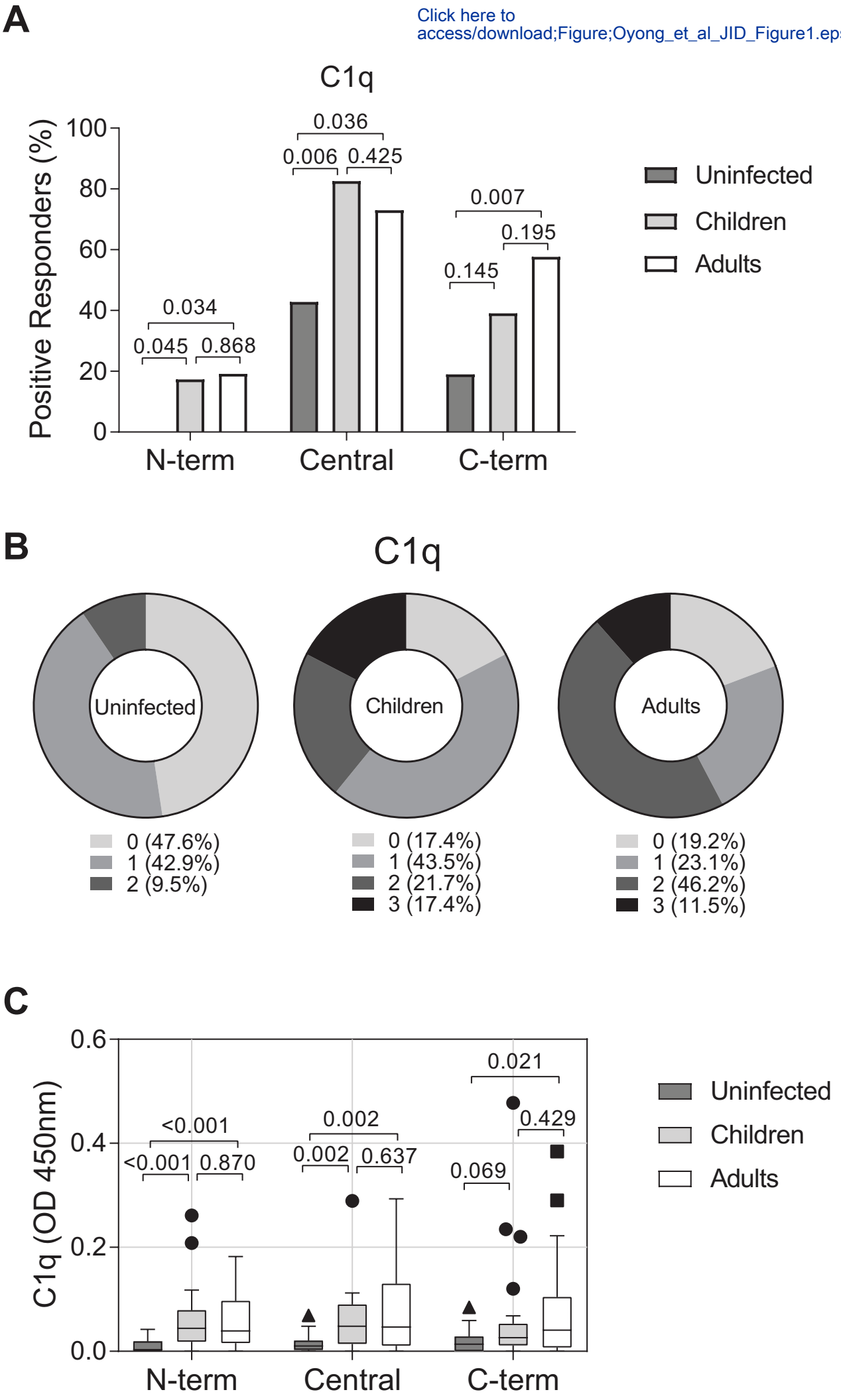

Figure 2

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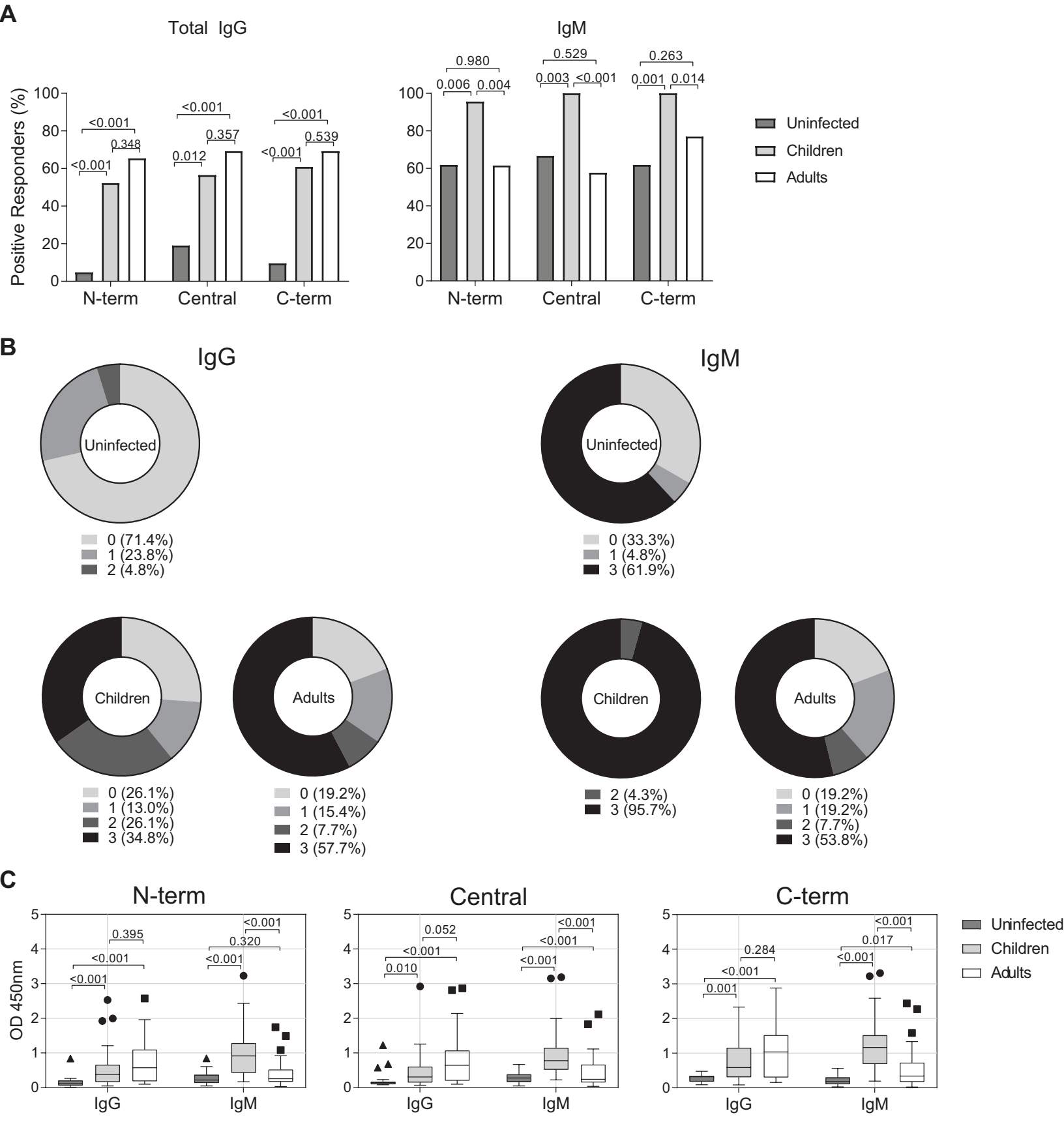

Figure 3

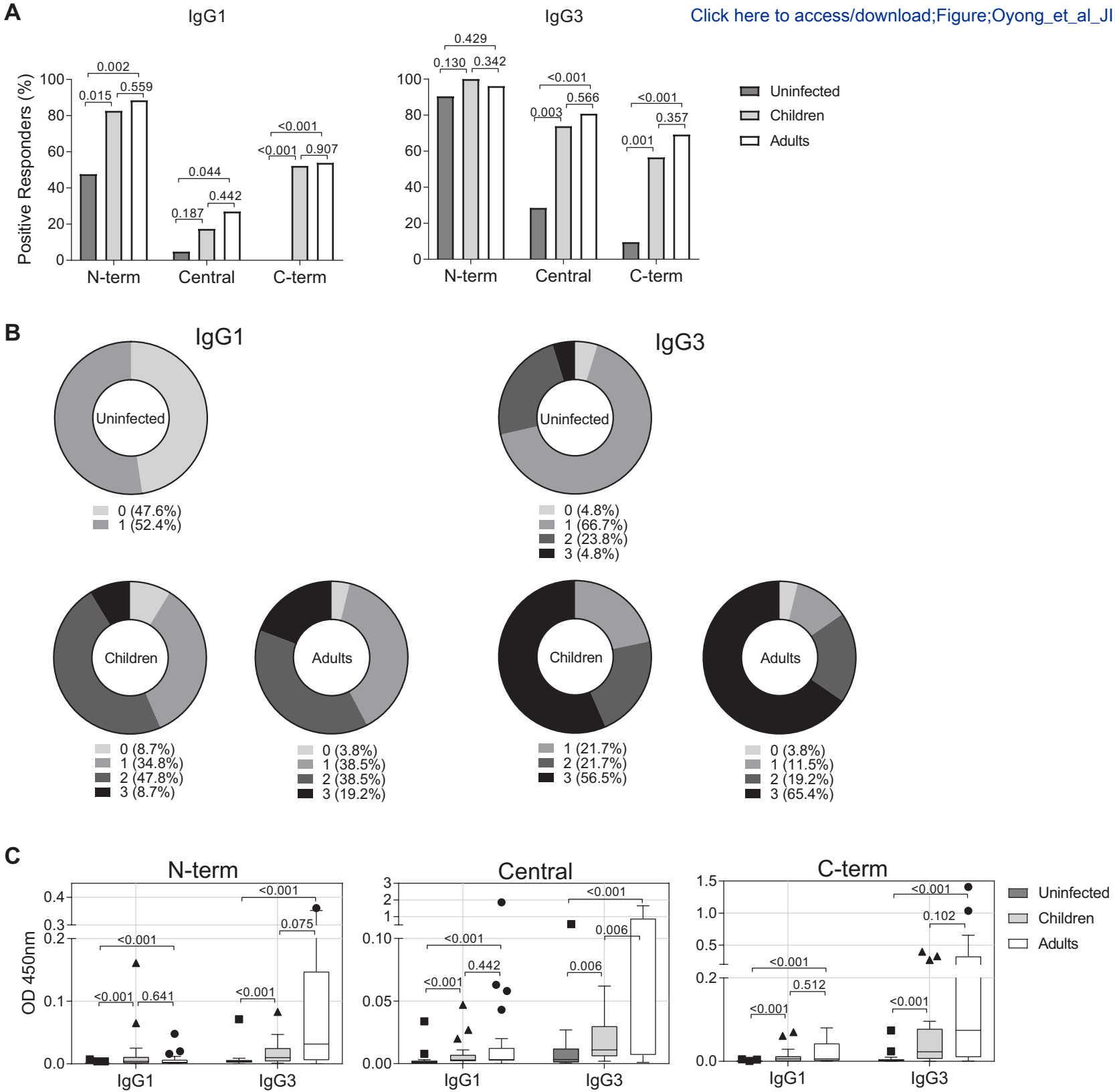

Figure 4

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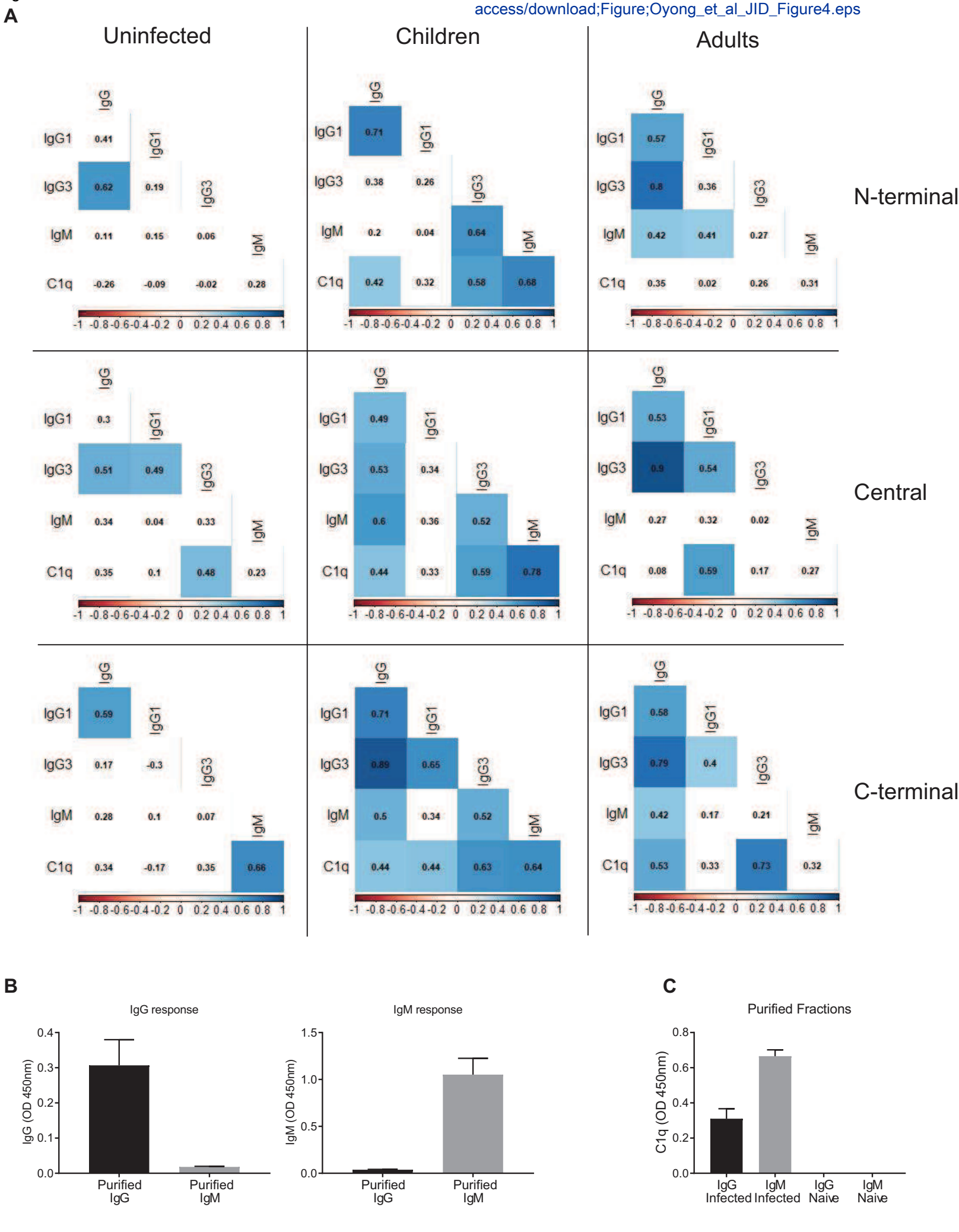

Figure 5

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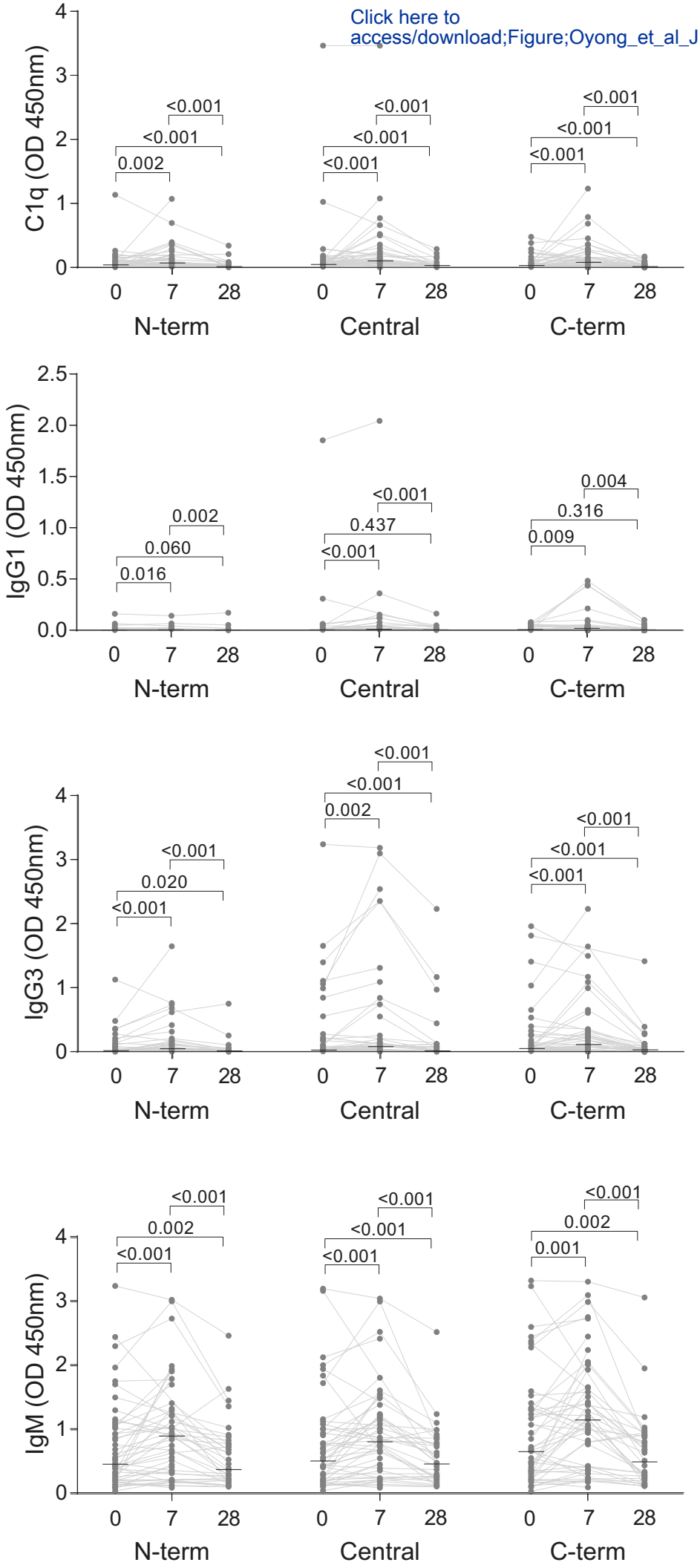


Supplementary Experimental Procedure

ELISA based complement fixation and antibody isotypes assays

ELISAs were carried out as described previously [6, 8]. Ninety-six well flat bottom Maxisorp® plates (Nunc) were coated overnight at 4°C with recombinant PvMSP3α proteins at 0.5 µg/ml. Washing steps were done three times with PBS-tween (0.5% w/v). Plates were blocked with 1% casein for 2 hours at 37°C, followed by plasma samples incubation at 1/250 in 0.1% casein for 2 hours at room temperature (RT). Levels of total IgG was quantified by adding horseradish peroxidase (HRP)-conjugated sheep anti-human IgG at 1/2000 in 0.1% casein for 1 hour at RT (Life Technologies). For quantification of IgM and IgG subclasses, monoclonal mouse anti-human IgM and IgG antibodies (Life Technologies) and goat polyclonal anti-mouse IgG HRP (Merck Millipore) were added, each diluted at 1/2000 in 0.1% casein and incubated for 1 hour at RT. For detection and quantification of C1q fixation, recombinant C1q at 10 µg/ml (Quidel) in 0.1% casein was added after human plasma step and incubated for 30 minutes at RT. C1q fixation was detected with rabbit anti-C1q antibodies at 1/2000 (Dako) and goat anti-rabbit HRP (Bio-Rad) at 1/4000, each incubated for 1 hour at RT. Antibodies and C1q binding were detected using Tetramethylbenzidine (TMB) (Sigma) substrate, with reactions being stopped after 30 minutes to 1 hour by adding 1M HCl. Absorbance was then measured using spectrophotometer at 450 nm.

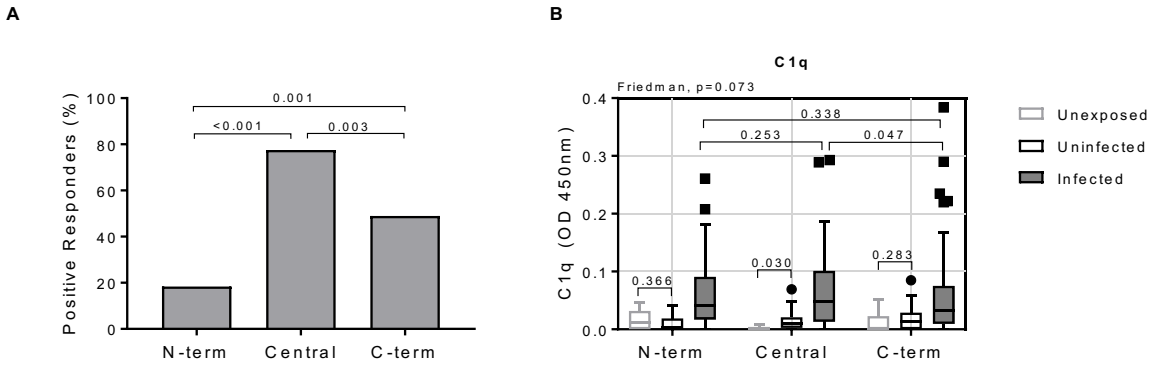
Antibody purification

Plasma samples were pooled from 1) *P. vivax*-infected patients with high IgG and IgM responses to PvMSP3α Central region (n = 5, Absorbance @450 nm >1) and 2) malaria naïve controls (n = 15). Crude immunoglobulins were obtained using saturated ammonium sulfate precipitation method. All steps were performed on ice. Pooled plasma samples were first centrifuged at high speed for 5 minutes to remove insoluble precipitates. Saturated ammonium

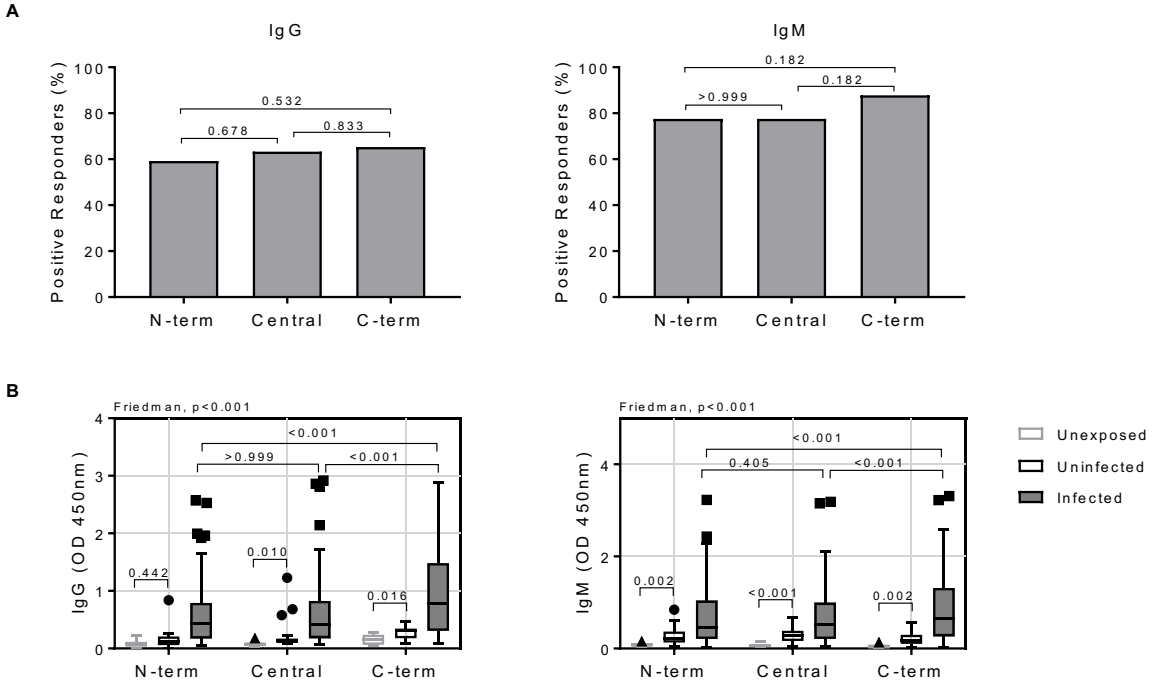
sulfate was added dropwise in increasing concentration of 0-50% to the pooled plasma with continuous agitation. Mixture was then incubated for 30 minutes with occasional gentle swirl to allow immunoglobulin precipitation. To obtain immunoglobulin pellet, samples were centrifuged at maximum speed for 10 minutes and supernatant was discarded. Pellet was then washed once with 50% ammonium sulfate, centrifuged, and any remaining supernatant was carefully removed. Immunoglobulin pellet was resuspended and dialysed overnight in PBS with dialysis tubes (Sigma).

Purification of IgG and IgM from crude immunoglobulins was performed using NAb™ Protein G Spin Column (Thermo Scientific) as per manufacturer's instructions. Where indicated, IgM fraction refers to non-IgG fraction purified from the Protein G column. IgM fraction was re-loaded once into the column to further purify from IgG antibody. Both IgG and IgM fractions were then dialysed overnight in PBS and concentrated using 10 kDa spin columns (Amicon, Merck Millipore). Subsequent ELISA procedures to determine IgG/IgM purity and quantify C1q fixation were as above.

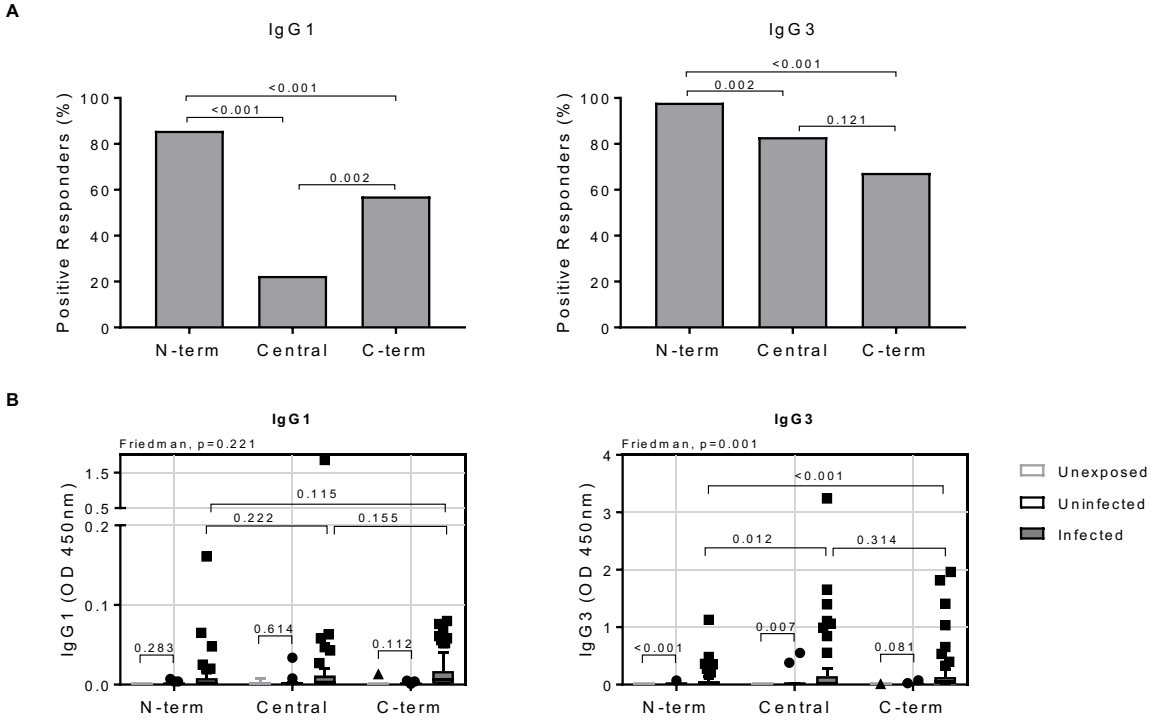
Supplementary Figures



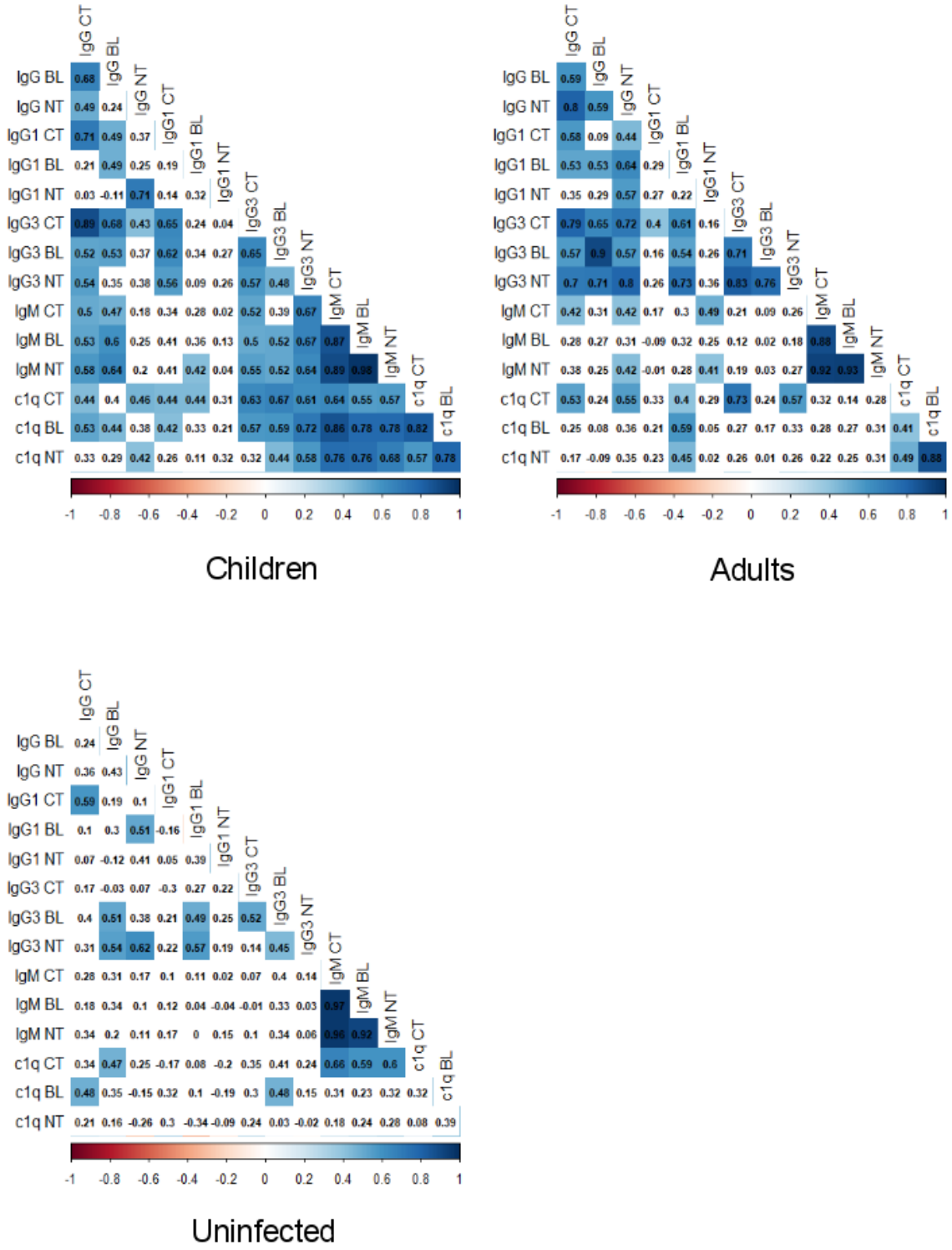
Supplementary Figure 1. Seroprevalence and magnitude of C1q-fixing antibodies against different PvMSP3α regions. ***(A)*** *Seroprevalence of C1q-fixing antibodies against different regions of PvMSP3α. Positive threshold for seroprevalence was calculated as above the mean plus three standard deviations of absorbance detected in malaria naïve Australian donors. Chi-square test is indicated for comparison between antigen regions* ***(B)*** *Magnitude of C1q-fixing antibodies against different regions of PvMSP3α. Friedman test and Wilcoxon-matched pair test is indicated for comparison between protein regions. For boxplot, lower and upper hinges represent first and third quartiles, and whisker lines correspond to highest and lowest values no further than 1.5 interquartile range from the hinges. Data beyond the whisker lines are treated as outliers. Median line is indicated across the box.*



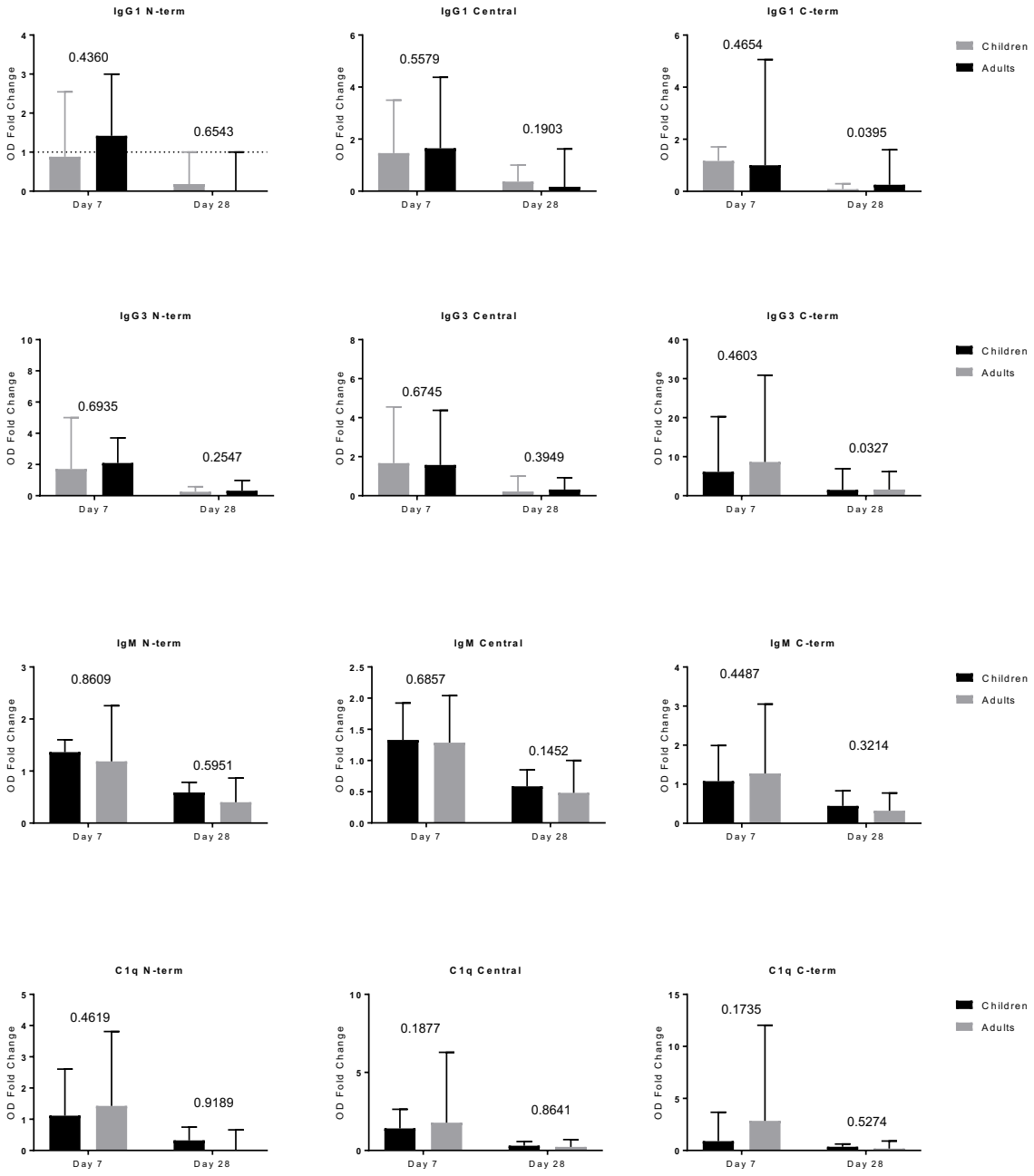
Supplementary Figure 2. Seroprevalence and magnitude of IgG and IgM antibodies against different PvMSP3α regions. ***(A)*** *Seroprevalence of IgG and IgM antibodies against different regions of PvMSP3α. Chi-square test is indicated for comparison between antigen regions. Positive threshold for seroprevalence was calculated as above the mean plus three standard deviations of absorbance detected in malaria naïve Australian donors* ***(B)*** *Magnitude of IgG and IgM antibodies against different regions of PvMSP3α. Friedman test and Wilcoxon-matched pair test is indicated for comparison between protein regions. For boxplot, lower and upper hinges represent first and third quartiles, and whisker lines correspond to highest and lowest values no further than 1.5 interquartile range from the hinges. Data beyond the whisker lines are treated as outliers. Median line is indicated across the box.*



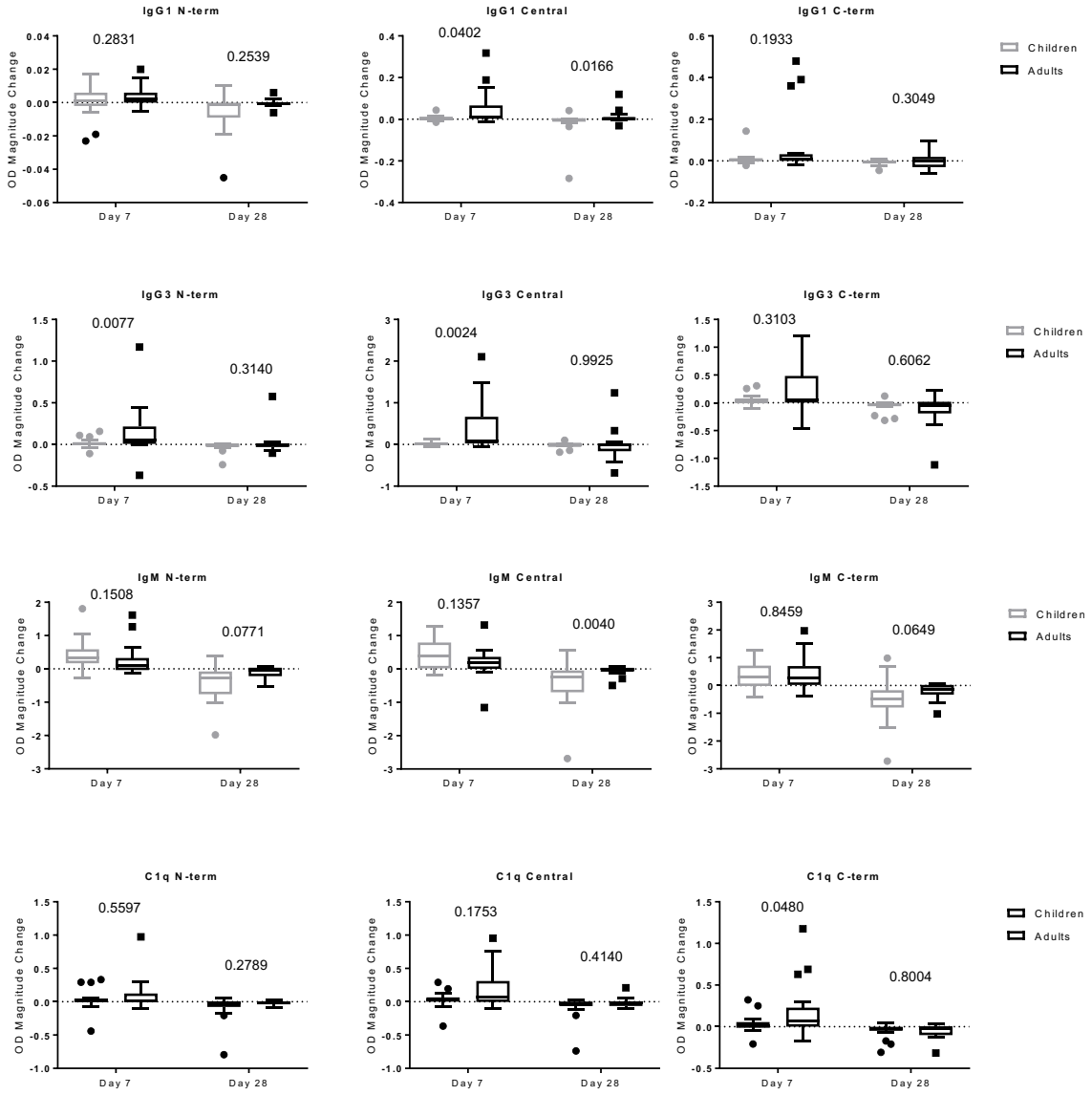
Supplementary Figure 3. Seroprevalence and magnitude of IgG1 and IgG3 antibodies against different PvMSP3α regions. ***(A)*** *Seroprevalence of IgG1 and IgG3 antibodies against different regions of PvMSP3α. Chi-square test is indicated for comparison between antigen regions* ***(B)*** *Magnitude of IgG1 and IgG3 antibodies against different regions of PvMSP3α. Friedman test and Wilcoxon-matched pair test is indicated for comparison between protein regions. For boxplot, lower and upper hinges represent first and third quartiles, and whisker lines correspond to highest and lowest values no further than 1.5 interquartile range from the hinges. Data beyond the whisker lines are treated as outliers. Median line is indicated across the box.*



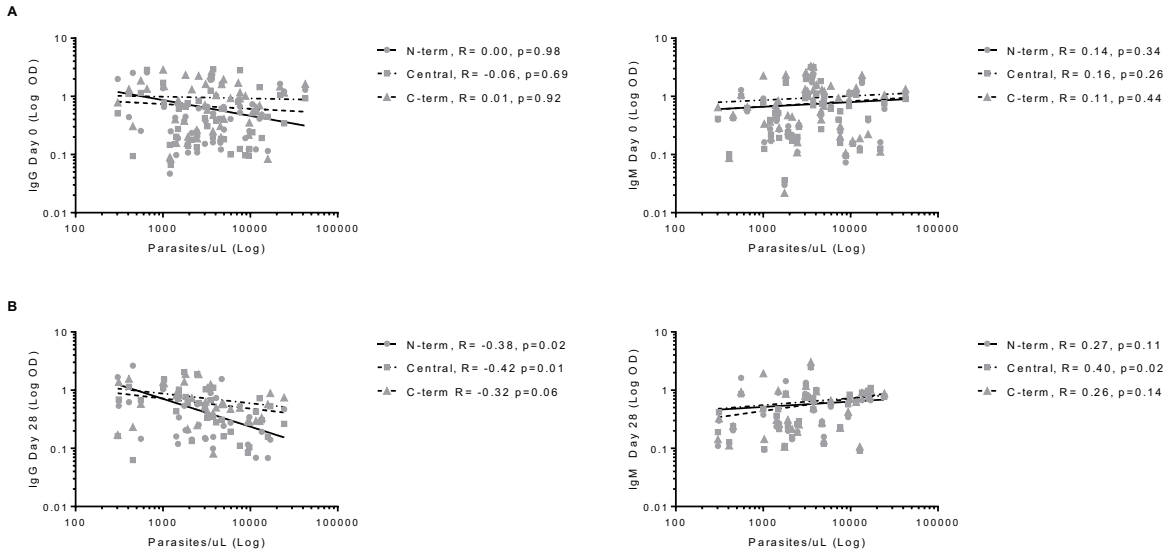
Supplementary Figure 4. Correlations matrixes between Total IgG, IgG1, IgG3, IgM, and C1q fixation against PvMSP3α antigens from different groups; children, adults, and uninfected individuals. *Spearman's correlation coefficient is indicated. Coloured boxes indicate statistical significance, $P < 0.05$.*



Supplementary Figure 5. Comparison of fold change to anti-PvMSP3α antibody response between children and adults over 28 days follow-up periods. *Mann-Whitney test is indicated for comparison between age groups. NOTE: Fold change is defined as Day 7 and Day 28 follow-up absorbance (OD 450nm) divided by Day 0. Bar graph indicates median and whisker lines correspond to interquartile range.*



Supplementary Figure 6. Comparison of magnitude change to anti-PvMSP3α antibody response between children and adults over 28 days follow-up periods. *Mann-Whitney test is indicated for comparison between age groups. NOTE: Magnitude change is defined as Day 7 and Day 28 follow-up absorbance (OD 450nm) subtracted with Day 0. Lower and upper hinges represent first and third quartiles, and whisker lines correspond to highest and lowest values no further than 1.5 interquartile range from the hinges. Data beyond the whisker lines are treated as outliers. Median line is indicated across the box.*



Supplementary Figure 7. Correlations between IgG and IgM antibody responses and parasite count in P. vivax-infected patients. (A) *At day 0 follow-up and* ***(B)*** *day 28 follow-up. Spearman's correlation test is indicated. Parasite count was determined by blood smear microscopy at enrolment.*