Investigating protective mechanisms of anti-sporozoite vaccines



Duncan Grant Bellamy

Exeter College

University of Oxford

A thesis submitted for the degree of
Doctor of Philosophy

Michaelmas Term 2023

Word count ~ 50,000 (excluding acknowledgements, contents, tables, figure legends and appendix)

1 Abstract

Malaria remains a leading cause of childhood mortality, thereby highlighting the need for an effective vaccine. R21 is a virus-like particle-based (VLP-based) vaccine displaying the immunodominant NANP repeat region of the circumsporozoite protein (CSP) of *Plasmodium falciparum*. R21 is biosimilar to the current leading malaria vaccine candidate, RTS,S, in that CSP epitopes are found on the VLP surface. However RTS,S has a higher ratio of unbound Hepatitis B surface antigen (HBsAg) than CSP.

R21/Matrix M (R21/MM) was initially manufactured in Oxford with a C-tag for purification, but is now manufactured at the Serum Institute India (SII) without a C-tag. The products made using the two manufacturing processes elicit comparable immunogenicity and efficacy among malaria naïve adults in the UK using comparable dose regimens.

Three doses of R21/MM produce comparable immunogenicity in adults in the UK, Kenya and Burkina Faso, all with different transmission settings. Anti-NANP antibodies were comparable to antibodies measured in protected malaria naïve adults immunised with RTS,S/AS01. A fractional third dose and delayed fractional third dose whereby a third dose of one-fifth did not improve ELISA-measured antibody titres or efficacy. However, a delayed third dose administered at 5 months increased antibody avidity. Among R21/MM immunised malaria naïve adults anti-NANP IgG titres greater than 1100EU and anti-C term IgG greater than 1100EU were associated with protection.

R21/MM was examined in infants in both Kenya (low relatively consistent transmission) and Burkina Faso (seasonal transmission). Infants in both countries, who were administered the same dose, produced comparable antibody titres. Infants immunised with the higher dose adjuvant (50µg MM) produced higher antibody titres and functional antibodies as compared to low dose adjuvant (25µg), irrespective of the dose of R21. Furthermore, three doses of R21/MM and a booster administered 12 months later produced efficacy that met WHO's desired vaccine efficacy of 75% by 2030.

Both RTS,S and R21 induce high anti-NANP IgG responses following a 3-dose primary series regimen and after a boost. Anti-NANP IgG durability measured twelve months after primary series and boost were improved in R21 immunised infants. In addition, HBsAg titres were 8-fold higher with RTS,S. Increased CSP antibody durability and high cytophilic subclass antibody responses may contribute to the effectiveness of the R21 vaccine.

High protection and immunogenicity to CSP antigens induced by R21 along with large-scale manufacturing and reduced cost from the SII will support R21 for the Phase III study and aid WHO pre-qualification approval following the recent positive recommendation. Additional effective vaccines to accompany RTS,S will help alleviate supply help contribute towards increasing demand and, ultimately, reduce morbidity and mortality from malaria amongst the most vulnerable populations.

2 Acknowledgements

Firstly, I would like to thank and express my appreciation to my supervisors Professor Katie Ewer and Professor Adrian Hill for supervising and supporting me throughout my DPhil. I am grateful to them for giving me an opportunity to contribute towards a ground-breaking vaccine that has the potential to save many lives. It has been a privilege and honour to have worked with them both and to learn from two leading individuals in the field of malaria and vaccinology.

I would like to thank Professor Teresa Lambe who not only has supported me throughout my DPhil, she has always had her door open to me if I ever needed help or advice. In addition, I would like to thank Professor Alex Spencer who not only supported and provided excellent scientific advice but was a very supportive and helpful colleague to have worked alongside. I would also like to pay a special mention to Richard Morter, Andres Noe, Mustapha Bittaye, Sarah Silk and Robert Quinlan for their guidance and friendship during my DPhil and my time in Oxford.

My work presented in this thesis would not have been possible without the hard work and dedication of the clinical trial teams in the UK, Kenya and Burkina Faso. With special mention Kenyan research team Mainga Hamaluba (PI) Philip Bejon, Melissa Kapulu. Burkinabe clinicians: Dr. Hamtandi Magloire Natama and Dr. Athanse Some and lastly the UK clinicians to Dr. Mehreen Datoo, Dr Navin Venkatraman, Dr. Dan Silman and Dr. Meera Madhavan.

I would like to thank the excellent lab team and part of the Ewer group working on pre-erythrocytic malaria: Lisa Stockdale, Rebecca Makinson, Jeremy Aboagye, Sam Provstgaard-Morays, Helen Sanders, Danielle Woods, Katerina Rapi and Amy Flaxman. I have also had the privilege of working with Federica Cappuccini, Reshma Kailath, Sean Elias, Ekta Mukhopadhyay and Andres Noe and I am grateful for their invaluable assistance with the first-year immunology dataset for VAC076. I would

like to thank Adam Truby and Martha Ulasewski for their invaluable work in the insectary providing high-quality mosquitos to dissect to support our functional assays. Lastly, I would like to acknowledge the lead statistician – Nicola Williams for her work on the VAC076 efficacy analysis and in addition who assisted in the multivariate analysis presented in Chapter 2 and 3 of this thesis.

A special thank you to Lisa Stockdale for her amazing friendship, support and guidance while working in Oxford. I would like to thank Caroline Bundi who started her PhD in Kenya alongside me, who I have enjoyed working with and our academic discussions. I would also like to thank Ousmane Traore for his incredible work supporting the VAC076 programme and laboratory work in Burkina Faso and for his professionalism, support, and friendship throughout my work at the Jenner.

Finally, I would like to thank my many friends who have supported me throughout my time in Oxford. To, my parents, Janet, and Martin along with my sister Robyn for their consistent support throughout my life. Lastly to my amazing partner Fala for her love and patience in supporting my career and for always being there for me.

3 Statement of Authorship

I, Duncan Bellamy, certify that all work presented in this thesis has been produced, analysed, and written by Duncan Bellamy. The design of humoral assays and analysing the data, troubleshooting, statistical analysis, and graphing of the data was done by Duncan Bellamy. VAC053, VAC060 and VAC065 ELISAs were carried out by Duncan Bellamy with the assistance of Lisa Stockdale. Assistance with ELISA assays for the VAC076 Year 1 immunology data from Federica Cappuccini, Reshma Kailath, Sean Elias, Ekta Mukhopadhyay and Andres Noe. VAC076 Year 2, and VAC073 Year 2 ELISA assays were assisted by Jeremy Aboagye, Sam Provstgaard-Morys and Danielle Woods. Meso-scale discovery (MSD) data analysis was performed by Lisa Stockdale and Sam Provstgaard-Morays. C term avidity ELISA's were assisted by Sam Provstgaard-Morays. ISI assays were conducted by Duncan Bellamy with extensive help and assistance from Lisa Stockdale and additional help from Carol Bundi. ELISpot assays were carried out by Duncan Bellamy and the clinical trial immunology team. All other assays were conducted by Duncan Bellamy. Clinical trials were designed by Professors Adrian Hill and Katie Ewer and the clinical trials clinicians. Efficacy analysis for VAC076 was performed by Nicola Williams who is the lead statistician on that study. In addition, Nicola Williams performed the multivariate analysis presented in chapters 2 and 3 and confirmed my post-hoc sample size power calculations. Clinical trials regulatory and ethics applications, and clinical care were conducted by the clinical trials team, clinicians, and research nurses.

4 Papers from this thesis

1. **VAC053 and VAC060**
    a. Both Phase I studies submitted together in July 2023 and have been accepted into Lancet Microbe – awaiting publication.
2. **VAC065**
    a. Submitting in August 2023 and been accepted into Lancet Microbe in parallel with VAC053/60 paper – awaiting publication.
3. **VAC072**
    a. Draft manuscript in Progress
    b. Exploratory immunology manuscript and identifying associations with protection being drafted by Duncan Bellamy with the aim of submitting to Nature Communications in December 2023.
4. **VAC073**
    a. Clinical manuscript with anti-NANP ELISA immunogenicity submitted to Wellcome open in July 2023 and accepted in October 2023 – Sang *et al.*, 2023 [1]
    b. Exploratory immunology manuscript in progress by Caroline Bundi and Duncan Bellamy with the aim of being submitted to Frontiers of Immunology in November 2023
5. **VAC076**
    a. Year 1 efficacy and immunogenicity published – Datoo *et al.,* 2021 [2]
    b. Year 2 efficacy and immunogenicity published – Datoo *et al.,* 2022 [3]
    c. Year 3&4 efficacy and immunogenicity manuscript in progress by Oxford aiming to be published in Lancet ID by December 2024
    d. Exploratory immunology manuscript and identifying associations with protection being drafted by Duncan Bellamy with the aim of submitting to Nature Communications in December 2023
    e. R21 impact and cost-effectiveness analysis. Manuscript written by Azra Ghani's group at Imperial College and submitted to Lancet ID November 2023
6. **RTS,S Phase 3 study**
    a. Immunogenicity manuscript from data generated at the Jenner Institute being drafted by Duncan Bellamy and Dr. Lisa Stockdale with input from Prof. Brian Greenwood – manuscript draft aiming to be ready January 2024
7. **Methodology papers**

a. Anti-NANP IgG standardised ELISA validation paper
b. Inhibition of Sporozoite invasion assay methodology paper

5 Table of Contents

1 Abstract ii

2 Acknowledgements iv

3 Statement of Authorship vi

4 Papers from this thesis vii

5 Table of Contents ix

6 Figures and Tables xv

6.1 List of Figures xv

6.2 List of Tables xvii

7 Abbreviations xviii

8 Introduction 1

8.1 Vaccinology 1

8.2 Immunology 2

8.2.1 Innate immunity 3
8.2.2 Adaptive immunity 8
8.2.3 Cellular immune responses 9
8.2.4 T cells 9
8.2.5 TCR 10
8.2.6 Th subsets 12
8.2.7 B cells 16
8.2.8 BCR 17
8.2.9 B cell antibody production 18
8.2.10 Antibody subclass and isotypes 19

8.3 Malaria 22

8.3.1 Types of malaria 22
8.3.2 Global impact 22
8.3.3 Sub-Saharan Africa 23
8.3.4 Malaria lifecycle 24
8.3.5 *Anopheles* mosquito 28

8.4 Malaria symptoms, diagnosis and treatment 28

8.4.1 Symptoms 28
8.4.2 Diagnosis 29
8.4.3 Treatment 31

8.5 Pathogenesis 31

8.5.1 Uncomplicated malaria 31
8.5.2 Severe malaria 32
8.5.3 Asymptomatic infection 32

8.6 Immunity to malaria 33

8.6.1 The Innate response to malaria 33
8.6.2 Naturally acquired immunity 33
8.6.3 Adaptive immunity to pre-erythrocytic malaria 35

8.6.4 Adaptive immunity to liver stage malaria infection 36
8.6.5 Adaptive immunity to blood stage malaria 37
8.6.6 Cytokine production 38

8.7 Acquired immune mechanism dysfunction 38
8.7.1 Malaria co-infection with HIV 38
8.7.2 Atypical memory B cells 39
8.7.3 Dendritic cells 39
8.7.4 Tregs 40
8.7.5 Hemozoin 40
8.7.6 Sickle cell anaemia 41

8.9 Vaccines against malaria 42
8.9.1 Malaria development goals 42
8.9.2 Current malarial vaccines 42
8.9.3 WHO policy approvals 44
8.9.4 Pre-erythrocytic malaria vaccines 44
8.9.5 Erythrocytic vaccines 51
8.9.6 Placental vaccines 52
8.9.7 Transmission blocking vaccines 52
8.9.8 Adjuvants 53
8.9.9 Monoclonals 54

8.10 Correlates of protection 55
8.10.1 Bradford Hill criteria to identify causation 55
8.10.2 CoP terminology 56
8.10.3 CoP with malaria 58

8.11 Seasonal transmission of malaria. 60
8.11.1 Seasonal Malaria Chemoprevention 60
8.11.2 Dry season vs all-year-round transmission. 61
8.11.3 Rebound (resurgent) and delayed malaria. 62
8.11.4 CHMI models 63

9 Thesis aims 65

10 Methods and Trial Designs 66

10.1 Materials 66

10.2 Immunological Methods 66

10.3 Vaccine Trials assessed in this thesis 66
10.3.1 Trial locations 67
10.3.2 Clinical trial regulatory approvals 69
10.3.3 R21 vaccine formulations 69
10.3.4 Trial Designs and Immunology bleed schedules 70

10.4 Analyses and Statistics 81
10.4.1 Analyses 81
10.4.2 Sample sizes 82
10.4.3 Power calculations 82
10.4.4 Statistical analyses 82
10.4.5 Multivariate analyses 83

11 Chapter 1 - Characterising immunogenicity of R21 and adjuvant Matrix M across Phase I/II trials in different malaria settings 84

11.1 Chapter 1 - Introduction 84

11.2 Chapter 1 - Methods 86

11.2.1 Trial designs 86
11.2.2 Study Details 86
11.2.3 Sample sizes 86
11.2.4 Immunology and time points examined 86

11.3 Chapter 1 - Objectives 88

11.4 Chapter 1 - Results 89
11.4.1 Humoral responses to dose regimens of R21/MM in different age groups and different malaria settings 89
11.4.2 Comparing anti-NANP IgG responses across different trials 102
11.4.3 R21/MM induces low T cell response in UK adults 104
11.4.4 R21/ MM induce functional antibodies to block sporozoite invasion *in vitro* 108

11.5 Chapter 1 - Discussion 120
11.5.1 Overview 120
11.5.2 Assay development 120
11.5.3 Requiring an Adjuvant 120
11.5.4 Dose 121
11.5.5 Malaria endemic regions 121
11.5.6 Fractional and delayed regimens 122
11.5.7 Two weeks post second dose 123
11.5.8 Age 124
11.5.9 % ISI 124
11.5.10 Cellular Immune Responses 126

11.6 Chapter 1 - Conclusion 127

12 Chapter 2 - Examining the effects of different dose regimens of R21/MM on efficacy in malaria naïve adults 128

12.1 Chapter 2 - Introduction 128

12.2 Chapter 2 - Methods 130
12.2.1 R21 Vaccines (CBF and SII) 130
12.2.2 Matrix M 130
12.2.3 Trial assessed 130
12.2.4 CHMI 130
12.2.5 VE 130
12.2.6 Sample size 131
12.2.7 Post-hoc power calculations 131
12.2.8 Sample analysis and immunology assays 131
12.2.9 Post-hoc sample analysis to determine antibody threshold associated with protection 132
12.2.10 Multivariate analysis 132

12.3 Chapter 2 - Objectives 133

12.4 Chapter 2 - Results 134
12.4.1 Manufacturing R21 in the UK (CBF) vs India (SII) 134
12.4.2 VAC065 Efficacy 135
12.4.3 VAC065 parasite quantification 7.5 days post-CHMI 137
12.4.4 VAC072 efficacy 139
12.4.5 Anti-NANP IgG greater than 1100EU/ml are associated with protection 141
12.4.6 Does R21/MM induce anti-C term antibodies which are associated with protection? 145
12.4.7 MSD anti-IgG measured by novel multiplex platform 149
12.4.8 HBsAg IgG antibodies measured by MSD induced from R21 155
12.4.9 Avidity to NANP and C term IgG 156
12.4.10 Subclass and Isotype antibody responses in UK adults 161
12.4.11 VAC065 Subclass and Isotype do not associate with protection in UK adults 166
12.4.12 VAC072 subclass and isotype – IgG1, IgG3 and IgG4 associate with protection 168

12.4.13 ISI examining functional antibodies 170
12.4.14 Multivariate model to assess associations with protection 173

12.5 Chapter 2 - Discussion 176
12.5.1 Manufacturing 176
12.5.2 VE CBF vs SII 176
12.5.3 Infectivity controls failing to be diagnosed 177
12.5.4 Post-hoc associations with protection 177
12.5.5 Fractionated third dose 178
12.5.6 Delayed fractional third dose 179
12.5.7 Two doses vs three doses 181
12.5.8 Delay to parasitaemia 182
12.5.9 Viral vectors 182
12.5.10 ISI 183
12.5.11 Hepatitis B Surface antigen 183
12.5.12 Multivariate analysis 184
12.5.13 Limitations 184

12.6 Chapter 2 - Conclusion 186

13 Chapter 3 - Protection infants in Burkina Faso administered with R21/MM within highly seasonal malaria endemic settings 187

13.1 Chapter 3 - Introduction 187

13.2 Chapter 3 - Methods 188
13.2.1 Aim 188
13.2.2 VAC076 Trial design 189
13.2.3 VAC076 Immunology endpoint 189
13.2.4 VAC076 immunology cohort 189
13.2.5 Sample size 190
13.2.6 Post-hoc power calculation of VAC076 immunology cohort 191
13.2.7 Surveillance and case detection 191
13.2.8 Efficacy analysis 191
13.2.9 Immunogenicity assays and time points 191
13.2.10 Multivariate analysis 192

13.3 Chapter 3 - Objectives 194

13.4 Chapter 3 - Results 195
13.4.1 *Pf* cases indicating Burkina Faso is a highly seasonal malaria transmission area 195
13.4.2 Durable efficacy observed over 3 year period in Burkinabe infants 197
13.4.3 Characterising total IgG responses to NANP 198
13.4.4 Characterising total IgG responses to C term 208
13.4.5 IgG responses measured by new multiplex assay using MSD 211
13.4.6 Anti-HBsAg correlate with other NANP, C term and R21 IgG as measured by MSD 215
13.4.7 Protection associated with anti-NANP and anti-R21 IgG levels as measured by MSD 216
13.4.8 Anti-IgG avidity 218
13.4.9 Subclass and isotype antibody responses in Burkinabe infants 223
13.4.10 Functional antibodies unable to differentiate between protected and non-protected infants 227
13.4.11 Multivariate analysis 228
13.4.12 Alternate factors influencing immunogenicity and protection 231
13.4.13 Age does not impact antibody response after immunisation with R21/MM 232

13.5 Chapter 3 - Discussion 235
13.5.1 Burkina seasonality 235
13.5.2 Proof of concept of CSP-based vaccines in seasonal settings 235
13.5.3 Efficacy 235
13.5.4 NANP vs C term 236

13.5.5 Avidity 237
13.5.6 Durability 237
13.5.7 Subclass and Isotype 238
13.5.8 MSD 239
13.5.9 Time of day 239
13.5.10 Age and gender 239
13.5.11 ISI 240
13.5.12 Multivariate Immune combinations 240
13.5.13 Limitations 241

13.6 Chapter 3 - Conclusion 242

14 Chapter 4 - Immunology on two leading PEM vaccine candidates – R21/MM and RTS,S/AS01$_E$ 243

14.1 Chapter 4 - Introduction 243

14.2 Chapter 4 - Methods 245
14.2.1 Trial assessed 245
14.2.2 RTS,S immunology cohort 246
14.2.3 Primary immunological outcome 246
14.2.4 Immunology methods 246

14.3 Chapter 4 - Objectives 247

14.4 Chapter 4 - Results 248
14.4.1 Anti-NANP IgG responses in infants immunised with R21/MM or RTS,S/AS01$_E$ 248
14.4.2 Durability of R21 and RTS,S antibodies 12 months after primary series and boost 252
14.4.3 R21 and RTS,S have declining antibody titres post-boost as compared to primary series 253
14.4.4 Adjuvants AS01 and Matrix M produce comparable antibody titres with the same immunogen. 255
14.4.5 RTS,S and R21 induce high anti-HBsAg titres after 3 doses compared to baseline 256
14.4.6 Functional Antibodies induced from R21 and RTS,S 256

14.5 Chapter 4 - Discussion 259
14.5.1 Seasonal vaccination 259
14.5.2 ELISA 260
14.5.3 Antibody durability 260
14.5.4 Perennial transmission 261
14.5.5 AS01 vs Matrix M 261
14.5.6 HBsAg 262
14.5.7 HBsAg responses differing from published work 262
14.5.8 % ISI 263
14.5.9 Comparing VE 263
14.5.10 Limitations 263

14.6 Chapter 4 - Conclusion 265

15 Overview, Future Directions and Conclusions 266

15.1 Overview 266

15.2 Summary of findings 267
15.2.1 Characterising dose responses to R21/MM in different 267
15.2.2 R21 performance in different malaria endemic areas and age groups 269
15.2.3 Identifying immune mechanisms of protection 270
15.2.4 Examining immunogenicity of R21 and RTS,S 273

15.3 Future implications 274
15.3.1 WHO approval 274
15.3.2 Distribution 275

15.3.3 Multi-stage vaccines ... 275
15.3.4 Assay validation ... 276
15.3.5 Limitations ... 276

16 Final remarks ... 278

17 Appendix ... 279

17.1 Materials and reagents ... 279
17.1.1 Reagents ... 279
17.1.2 Solutions ... 281

17.2 Equipment ... 282

17.3 Investigational products ... 282
17.3.1 RTS,S ... 282
17.3.2 R21 ... 283
17.3.3 Matrix M ... 284
17.3.4 AS01 ... 284
17.3.5 Viral vectors – ChAd63 Me-TRAP ... 284
17.3.6 Viral vectors – MVA-ME-TRAP ... 285

17.4 Clinical Methods ... 286
17.4.1 Vaccination ... 286
17.4.2 CHMI ... 286
17.4.3 Blood collection ... 288

17.5 Immunological Methods ... 289
17.5.1 PBMCs & plasma separation ... 289
17.5.2 Serum separation ... 289
17.5.3 Counting PBMCs ... 290
17.5.4 Peptides ... 291
17.5.5 IFNγ ELISpot ... 293
17.5.6 Freezing PBMCs ... 294
17.5.7 Thawing PBMC ... 295
17.5.8 Culturing Huh7 cells ... 295
17.5.9 ISI assay ... 296
17.5.10 Total IgG ELISA's ... 297
17.5.11 Avidity ... 299
17.5.12 Isotype and subclass ... 300
17.5.13 Total IgG measured by MSD ... 300
17.5.14 Efficacy analyses ... 302

17.6 Peptide sequences ... 303
17.6.1 CSP peptide sequence and pools ... 303
17.6.2 TRAP peptide sequence and pools ... 304
17.6.3 ME peptide sequence ... 306

17.7 Gating strategies ... 307

18 References ... 308

6 Figures and Tables

6.1 List of Figures

Figure 1 - Innate vs Adaptive Immune response to repeated infections 3
Figure 2 - Complement cascade. 6
Figure 3 - T cell development in the thymus 11
Figure 4 - Th subsets 14
Figure 5 - B cell development 16
Figure 6 - Antibody structure with BCR. 19
Figure 7 – Estimated number of malaria deaths per country in 2021. 23
Figure 8 – Prevalence of total Pf cases and deaths in Africa 24
Figure 9 – Pf Malaria Life Cycle 25
Figure 10 - Innate immune responses to Pf 33
Figure 11 – Development of immunity with increasing age. 34
Figure 12 – Adaptive immune responses to Pf. 37
Figure 13 – Sickle cell disease and its protective functions against malaria. 41
Figure 14 – Individual vaccines targeting specific Pf lifecycle. 43
Figure 15 - Schematic of the Pf CSP and the important antigens 45
Figure 16 - R21 VLP design compared to RTS,S with respect to the CSP 48
Figure 17 - Seasonal distribution of rainfall 60
Figure 18 – Seasonal malaria in Mali 62
Figure 19 - Phase I/II trial sites of R21 and Matrix M examined in this thesis 67
Figure 20 - VAC053 Immunology bleed schedule 70
Figure 21 – VAC060 Immunology bleed schedule 71
Figure 22 – VAC065 Immunology bleed schedule 72
Figure 23 - VAC072 Immunology Bleed Schedule 75
Figure 24 – VAC073 bleed schedule 77
Figure 25 – VAC076 Trial Design 79
Figure 26 – VAC053 anti-NANP IgG in UK adults 89
Figure 27 – VAC060 Anti-NANP IgG in Burkinabe Adults 91
Figure 28 – VAC073 Anti-NANP IgG from Kenyan adults, young children, and infants 92
Figure 29 - Effect of a booster dose of R21/MM in Kenyan adults, Young Children, and Infants 94
Figure 30 - VAC073 antibody durability 96
Figure 31 – VAC065 anti-NANP IgG among different dose regimens of $R21_{CBF}$/MM. 97
Figure 32 – VAC072 anti-NANP titres after different dose regimens of $R21_{SII}$/MM 99
Figure 33 - VAC072 anti-NANP IgG after a booster administered 8 months 100
Figure 34 - VAC076 anti-NANP IgG in 450 Burkinabe infants after Immunisation of R21/MM. 101
Figure 35 – Trial comparison of anti-NANP IgG comparing adults in the UK, Burkina Faso and Kenya all with the same dose of R21/MM (10, 10, 10). 102
Figure 36 – Trial comparison of anti-NANP IgG in Infants in Kenya and Burkina Faso with identical doses of R21/MM. 103
Figure 37 – VAC053 T cell ELISpot response measured UK adults. 105
Figure 38 – VAC065 T cell ELISpot response measured UK adults. 106
Figure 39 - VAC072 ELISPOT T cell responses in UK adults. 107
Figure 40 - VAC053 / 60 - Inhibition of sporozoite invasion. 109
Figure 41 - VAC065 - Inhibition of sporozoite Invasion 111
Figure 42 - VAC072 - Inhibition of sporozoite invasion 112
Figure 43 - VAC073 Inhibition of sporozoite invasion. 114
Figure 44 - VAC076 inhibition of sporozoite invasion. 116

Figure 45 – Inhibition of Sporozoite Invasion across trials. 118
Figure 46 – Anti-NANP titres among adults in VAC065 ($R21_{CBF}$) vs VAC072 ($R21_{SII}$) 134
Figure 47 – VAC065 Parasite quantification by qPCR 138
Figure 48 – VAC065 anti-NANP IgG associations with protection 142
Figure 49 - VAC072 NANP IgG associates with protection 144
Figure 50 - VAC065 C term IgG and associations with protection. 146
Figure 51 - VAC072 C term IgG associations with protection. 148
Figure 52 – VAC065 MSD total IgG to NANP, C term, R21 and HBsAg. 150
Figure 53 - VAC072 MSD total IgG to NANP, C term, R21 and HBsAg. 152
Figure 54 - MSD total IgG induced from $R21_{CBF}$ and $R21_{SII}$ stratified by protection. 153
Figure 55 - MSD assay correlations – VAC065 and VAC072. 154
Figure 56 - Anti-HBsAg IgG measured by MSD for VAC065 and VAC072. 155
Figure 57 - VAC065 avidity - NANP and C term associations with protection. 157
Figure 58 - VAC072 Avidity - NANP and C term associations with protection. 159
Figure 59 - VAC065 antibody subclass and isotype responses. 162
Figure 60 - VAC072 antibody subclass and isotype responses. 164
Figure 61 – VAC065 antibody subclass and Isotype associations with protection. 166
Figure 62 - VAC072 antibody and subclass associations with protection. 168
Figure 63 - VAC065 ISI stratified by protection. 170
Figure 64 - VAC072 ISI stratified by protection. 172
Figure 65 - Transmission of Pf in Burkina Faso 195
Figure 66 – Anti-NANP IgG over 3 years and 1 month post third boost in the 4th year 199
Figure 67 - NANP IgG Durability over 3 years 200
Figure 68 - VAC076 anti-NANP IgG CoP 12 & 24 months. 203
Figure 69 – Protection stratified using published antibody EU thresholds. 204
Figure 70 – NANP IgG in third year stratified by protection. 205
Figure 71 – Decreasing anti-NANP IgG effect on multiple episodes. 206
Figure 72 - Anti C term IgG after primary series and subsequent boosters. 208
Figure 73 - Anti-C term IgG CoP at 12 and 24 months. 210
Figure 74 - MSD total IgG - NANP, C term, HBsAg and Full Length R21. 211
Figure 75 - MSD Antigen IgG Correlations. NANP vs C term vs R21. 213
Figure 76 - HBsAg IgG in Burkinabe infants measured by MSD. 214
Figure 77 - HBsAg Correlations with NANP, C term and R21 IgG. 215
Figure 78 - MSD IgG stratified by protection 12 and 24 months. 216
Figure 79 - Correlating IgG from ELISA with MSD - NANP vs C term. 217
Figure 80 - NANP avidity with Groups 1 and 2 merged. 218
Figure 81 – VAC076 Avidity (IC50) to NANP and C term IgG. 219
Figure 82 - NANP avidity stratified by protection. 221
Figure 83 - C term avidity stratified by protection. 222
Figure 84 - Subclass and isotype antibody responses at D84. 223
Figure 85 - Subclass and isotype antibodies stratified by protection. 224
Figure 86 - Isotype and Subclass antibodies with merged groups stratified by protection. 226
Figure 87 – Inhibition of sporozoite invasion and stratified by protection 227
Figure 88 - Time of vaccination (morning vs afternoon). 231
Figure 89 – Anti-NANP IgG stratified by age at third vaccination (5-12 months vs 12-17 months). 232
Figure 90 - Antibody response separated by gender. 234
Figure 91 – VAC076 anti-NANP IgG time course for infants immunised with R21/MM 248
Figure 92 – MAL099 anti-NANP IgG time course (3 years) RTS,S SMC trial. 249
Figure 93 – Peak anti-NANP IgG responses in infants immunised with RTS,S. 250
Figure 94 – Anti-NANP IgG induced by R21 vs RTS,S. 251
Figure 95 - Antibody durability from R21 and RTS,S after primary series and first boost. 252

Figure 96 – Assessing the ability of R21 and RTS,S booster doses compared to primary series. 253
Figure 97 - Matrix M and AS01 anti-NANP IgG. 255
Figure 98 - HBsAg responses between R21 and RTS,S. 256
Figure 99 - RTS,S inhibition of sporozoite invasion. 257
Figure 100 - % ISI induced from RTS,S and R21 in Burkinabe infants. 258
Figure 101 - ISI gating strategy. 307

6.2 List of Tables

Table 1 - Key features of innate and adaptive immune responses 2
Table 2 - Antibody Isotypes 21
Table 3 - Formulation of RTS,S and AS adjuvants. 46
Table 4 – Key trial developments and publications of RTS,S 47
Table 5 - Bradford Hill Criteria for assessing causation 56
Table 6 - CoP terminology 56
Table 7 - Summary of Clinical Trials Used in this thesis. 68
Table 8 - Clinical Trial Regulatory Approvals 69
Table 9 – VAC053 Trial Design 70
Table 10 – VAC060 Trial Design 71
Table 11 - VAC065 Trial Design 72
Table 12 - VAC072 Trial design 74
Table 13 - VAC073 Trial Design 76
Table 14 – VAC076 Trial Design 78
Table 15 - MAL099 - RTS,S + SMC Trial Design 80
Table 16 - MAL099 - Immunology sample numbers 81
Table 17 – VAC065 efficacy 136
Table 18 – VAC072 efficacy 140
Table 19 - Univariate and logistic regression and AUC analysis on UK adult immunology 174
Table 20 - VAC076 immunology cohort sample numbers 190
Table 21 - VAC076 Efficacy Results over 3 years 197
Table 22 - Univariate logistic regression and AUC analysis results. D84, 1B+30 and 2B+30 229
Table 23 - MAL099 RTS,S Immunology cohort 246
Table 24 - Commercially Available Reagents used in this thesis. 279
Table 25 - Commercially available lab equipment used in this thesis. 282
Table 26 - CSP ELISpot Plate layout (4 separate cell samples per plate) 292
Table 27 - ME-TRAP ELISpot Plate Layout (2 separate cell samples per plate) 292
Table 28 – ISI plate layout 297
Table 29 - Standardised ELISA antigen descriptions 298
Table 30 - Total IgG Avidity plate layout 299
Table 31 - Isotype and Subclass antibody assay plate layout 300
Table 32 - MSD plate layout 301
Table 33 - MSD LLoD values 302
Table 34 - CSP peptide sequences and ELISPOT peptide pool configurations 303
Table 35 - TRAP Peptide sequences and ELISPOT peptide pools 304
Table 36 - ME peptide sequences 306

7 Abbreviations

°C	-	Degree Celsius
% ISI	-	Percent Inhibition of Sporozoite invasion
1B	-	First Boost
2B	-	Second Boost
3B	-	Third Boost
95% CI	-	95% Confidence Intervals
ACT	-	Artemisinin Combination Therapy
Ad	-	Adenovirus
AMA	-	Apical Membrane Antigen
APC	-	Antigen-Presenting Cell
AS	-	Adjuvant System
ASC	-	Antibody Secreting Cell
AUC	-	Area Under the Curve
AUROC	-	Area under the receiver operating characteristic
B-H	-	Benjamini-Hochberg
BCR	-	B cell receptor
BF	-	Burkina Faso
BBIL	-	Bharat Biotech International Ltd
C-1	-	Challenge - 1day (day before CHMI)
CBF	-	University of Oxford Clinical Biomanufacturing Facility
CCVTM	-	Centre for Clinical Vaccinology and Tropical Medicine
CD	-	Cluster of Differentiation
CDC	-	Centre for Disease Control
ChAd	-	Chimpanzee Adenovirus
CHI	-	Controlled human infection
CHMI	-	Controlled Human Malaria Infection
CI	-	Chief Investigator
CI	-	Confidence Interval
CM	-	Central memory
CMV	-	Cytomegalovirus
CoP	-	Correlate of Protection
COVAX	-	COVID-19 Vaccines Global Access
COVID-19	-	Coronavirus Disease 2019
CRP	-	C Reactive Protein
CSA	-	Chondroitin sulphate A
CSP	-	Circumsporozoite Protein
CT	-	Cell Traversal
CTL	-	Cytotoxic T Lymphocyte
D	-	Day
DAMP	-	Danger Associated Molecular Protein
DAPI	-	4',6-diamidino-2-phenylindole
DC	-	Dendritic cell

DNA	-	Deoxyribonucleic acid
dPBS	-	Dulbecco's Phosphate Buffer Saline
DPhil	-	Doctor of Philosophy
DPT	-	Diphtheria, Pertussis and Tetanus
DTP	-	Diphtheria, Tetanus and Pertussis
EBV	-	Epstein Barr Virus
ELISA	-	Enzyme-Linked Immunosorbent Assay
ELISpot	-	Enzyme-Linked Immunosorbent Spot
E_M	-	Effector Memory
EMA	-	European Medial Agency
EPI	-	Expanded Programme on Immunisation
EU	-	European Union
EU	-	ELISA unit
Fab	-	Fragment antigen binding
FC	-	Fold Change
FCS	-	Foetal Calf Serum
FFS	-	Forward Scatter
Fig	-	Figure
G	-	Group
GAVI	-	Global Alliance for Vaccines and Immunisation
GC	-	Germinal Centre
GCLP	-	Good Clinical Laboratory Practice
GCP	-	Good Clinical Practice
GFP	-	Green Fluorescent Protein
GMP	-	Good Manufacturing Practice
GMT	-	Geometric Mean Titre
GP	-	Glycoprotein
GSK	-	GlaxoSmithKline
GVAP	-	Global Vaccine Action Plan
HBsAg	-	Hepatitis B Surface Antigen
HBV	-	Hepatitis B virus
HCV	-	Hepatitis C virus
HCW	-	Healthcare worker
HI	-	Heat-inactivated
HIV	-	Human Immunodeficiency Virus
HLA	-	Human Leucocyte Antigen
HR	-	Hazzard ratio
HSC	-	Haematopoietic stem cell
HSP	-	Heat Shock Protein
HSV	-	Human Herpes Virus
HZ	-	Haemozoin
IAVI-HIL	-	International AIDS Vaccine Initiative - Human Immunology Laboratory
ICS	-	Intracellular Cytokine Staining
IFN	-	Interferon
Ig	-	Immunoglobulin

IL	-	Interleukin
IM	-	Intramuscular
IPTp	-	Intermittent Preventive Treatment of Malaria in Pregnancy
IQR	-	Inter-Quartile Range
iRBC	-	infected red blood cell
IRS	-	Indoor residual spraying
ISI	-	Inhibition of Sporozoite Invasion
ITN	-	Insecticide Treated Nets
IU/ml	-	International units per millilitre
IV	-	Intravenous
KEMRI	-	Kenyan Medical Research Institute
Kg	-	Kilogram
LBP	-	LPS binding protein
LLoD	-	Lower Limit of detection
LLPC	-	Long-Lived Plasma Cell
LSA-1	-	Liver Stage Antigen 1
LSAP-2	-	Liver Stage Associated Protein 2
LSHTM	-	London School of Hygiene and Tropical Medicine
MAC	-	Membrane Attack Complex
mCoP	-	mechanistic correlate of protection
ME	-	Multiple Epitope String
ME-TRAP	-	Multiple Epitope – Thrombospondin Adhesion particle
MHC	-	Major Histocompatibility Complex
MHRA	-	Medicines and Healthcare products Regulatory Agency
mIU/ml	-	Micro International units per millilitre
Ml	-	Millilitre
MM	-	Matrix M™
MMR	-	Measles, Mumps and Rubella
MSD	-	Meso-Scale Discovery
MSP	-	Merozoite Surface protein
MVA	-	Modified Vaccinia Ankara Virus
MVIP	-	Malaria Vaccine Implementation Programme
N	-	Number
NAI	-	Naturally Acquired Immunity
NaSCN	-	Sodium Thiocyanate
nCoP	-	non-mechanistic correlate of protection
NHS	-	National Health Service
NIH	-	National Institute of Health
NK	-	Natural Killer
NO	-	Nitric Oxide
NP	-	Nucleoprotein
OD	-	Optical Density
P/a	-	per annum
PAMPs	-	Pathogen-Associated Recognition Receptor
PBMC	-	Peripheral Blood Mononuclear Cell

PBS	-	Phosphate buffered saline
PCR	-	Polymerase Chain Reaction
PD1	-	Programmed Cell Death Protein 1
PEM	-	Pre-Erythrocytic Malaria
Pf	-	*Plasmodium falciparum*
PFA	-	Paraformaldehyde
*Pf*EMP-1	-	*Plasmodium falciparum* Erythrocyte Membrane Protein 1
*Pf*SPZ	-	Cryopreserved *Plasmodium falciparum* Sporozoite Vaccine
PI	-	Principal Investigator
PID	-	Participant Identification
Pk	-	*Plasmodium knowlsei*
PMR	-	Parasite multiplication rate
pNPP	-	Para-nitrophenylphosphate
Po	-	*Plasmodium ovale*
PRR	-	Pathogen Recognition Receptors
PS	-	Primary Series
Pv	-	*Plasmodium vivax*
QC	-	Quality Control
qPCR	-	quantitative Polymerase Chain Reaction
R21	-	R21 manufactured in SII, India
R21c	-	R21 manufactured in CBF, Oxford
RAS	-	Radiation Attenuated Sporozoites
RBS	-	Red Blood Cell
RDT	-	Rapid Diagnostic Test
Re-CHMI	-	Repeat Controlled Human Malaria Infection
RH5	-	Reticulocyte-binding Homologue 5
RNA	-	Ribonucleic acid
ROS	-	Reactive Oxygen Species
RR	-	Relative Risk
RSV	-	Respiratory syncytial virus
RT	-	Room temperature
SA	-	South Africa
SD	-	Standard Deviation
SFC	-	Spot Forming Cell
SG	-	Salivary Gland
SII	-	Serum Institute of India
SMC	-	Seasonal Malaria Chemoprophylaxis
SoP	-	Surrogate of Protection
SOP	-	Standard Operating Procedure
SP+AQ	-	Sulfadoxine-Pyrimethamine plus Amodiaquine
Spz	-	Sporozoites
SSC	-	Side Scatter
STD	-	Sexually Transmitted Diseases
std	-	Standards
TB	-	Tuberculosis

T_{CM}	-	Central Memory T Cell
TCR	-	T Cell Receptor
T_{EM}	-	Effector Memory T Cell
TfH	-	T Follicular Helper Cell
TM	-	Trade Mark
TNF	-	Tumour necrosis factor
TP	-	Time Point
Treg	-	Regulatory T Cell
TRM	-	Resident Memory T Cell
UK	-	United Kingdom
UNICEF	-	United Nations International Children's Emergency Fund
USA	-	United States of America
VAC	-	Vaccine Trial prefix
VE	-	Vaccine Efficacy
VII	-	Vaccine-Induced Immunity
Vit	-	Vitamin
VLP	-	Virus-Like Particle
VP	-	Viral particles
VZV	-	Varicella zoster
WHO	-	World Health Organisation Programme
WRAIR	-	Walter Reed Army Institute of Research
WSV	-	Whole Sporozoite Vaccines
Yrs	-	Years
µg	-	Microgramme(s)
µl	-	Microlitre

8 Introduction

8.1 Vaccinology

Vaccines have transformed how we live by improving public health, reducing childhood mortality and extending life expectancy, and livestock vaccines have increased the worldwide distribution of food [4]. Countries which have high vaccination rates have seen diseases causing high childhood morbidity and mortality such as measles, pertussis and polio reduced or even eliminated, with clusters of outbreaks limited to communities with low vaccine coverage or vaccine scepticism [5, 6].

Efforts to control infectious diseases have been attempted over centuries and most notably recorded by the English aristocrat Lady Mary Wortley Montague in 1720 against smallpox [7]. Montague promoted the technique of 'variolation' whereby infectious material from smallpox lesions or scabs was scratched into the skin of a non-infected person. While variolation did induce protection in some, it also led to some infection or death [8].

In 1798 Edward Jenner observed that milkmaids infected with cowpox did not contract smallpox. He would go on to scrape samples from lesions on maids infected with cowpox and transfer this to the skin of an 8-year-old boy which resulted in his protection from smallpox [7]. This method of vaccination would revolutionise our understanding of disease prevention. Smallpox, which swept through much of the world in the 17th, 18th and 19th centuries and accounted for 29% of all deaths in children in the UK [9], was declared eradicated by the WHO in 1980 [10, 11].

Vaccines utilise the ability of the immune system to respond and remember foreign pathogens, antigens, and proteins. A vaccine is therefore a biological product used to induce an immune response that results in increased protection from infection or disease after exposure to that pathogen [12].

Understanding the target disease and the immune mechanisms involved is essential in designing a successful vaccine [13]. In addition, understanding the characteristics of the intended age group i.e., immune maturation in infants and immuno-senescence in older adults, factors into the success of a vaccine [14-16]. Understanding these mechanisms will enable scientists to improve vaccines against immune evasive diseases such as HIV, hard-to-reach diseases such as TB [17], diseases that can lead to outbreaks such as Ebola [18] and diseases with complex lifecycles such as malaria [19].

8.2 Immunology

The immune system is a mechanism which allows an organism to distinguish between 'self' and 'nonself' [20] and generate relevant protective immune responses. The human immune system comprises two parts: the innate (referred to as non-specific) and adaptive (referred to as acquired or specific) immune responses. For defence effector mechanisms, both the innate and adaptive immune responses work together and involve either direct cell to cell contact or the release of cytokines and chemokines to eliminate or supress a pathogen [21]. Key Features of each branch of the immune system are summarised in Table 1. The timescale of how the adaptive immune response responds to the second encounter with a known pathogen can be viewed in Figure 1.

Table 1 - Key features of innate and adaptive immune responses

Table adapted from Marshal et al., 2019 [21]

Innate Immune Responses	Adaptive Immunity
Germline encoded receptors which target pathogen molecular patterns	Clonally variable receptors generated somatically by rearranging gene elements
Immunisation does not lead to immune training	Requires B cell and T cell activation
Limited memory	Induces immunological memory
Includes physical barriers to pathogens	Involves antibody production and cytotoxic T cells

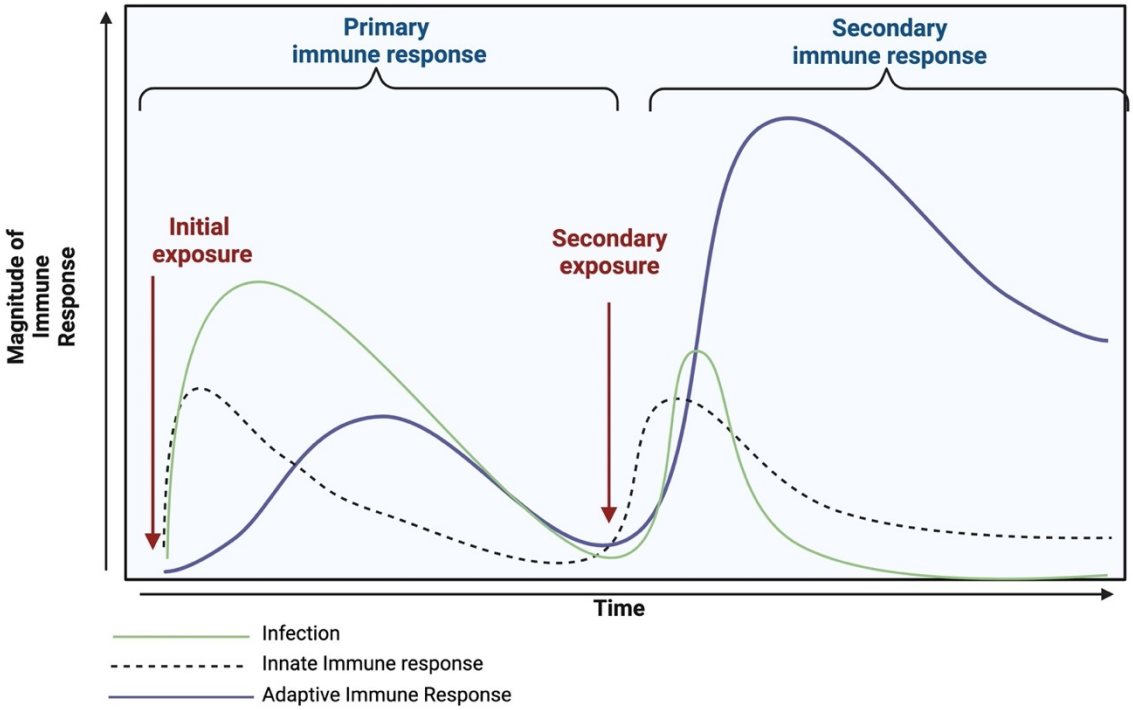


Figure 1 - Innate vs Adaptive Immune response to repeated infections

Figure made by D.G Bellamy and designed in Biorender

8.2.1 Innate immunity

The innate immune response is the first to respond to the detection of a foreign pathogen or microbe. Innate cells clear initial infection, recruiting other innate cells and activating the adaptive immune response. The innate immune system comprises four main components: anatomic (skin and mucous membrane), physiological (temperature, pH and chemical mediators), endocytic/ phagocytic functions (ability to ingest foreign particles) and inflammatory [22]. The innate immune response involves both a cellular and soluble protein component. Innate cellular components include basophils, eosinophils, natural killer (NK) and dendritic cells, the most common being macrophages and neutrophils [23]. Soluble protein components include the complement system, C reactive protein (CRP), antimicrobial peptides (AMP) and LPS binding protein (LBP) [24].

8.2.1.1 Recognising infection

An important part of the innate response is the rapid recruitment of immune cells to the site of infection. Pathogen recognition receptors (PRRs) are sensors on innate immune cells which identify pathogen-associated molecular patterns (PAMPs) on the surface of pathogens [24]. PAMPs are conserved structures in which an innate cell can recognise and respond rapidly to infection [25]. Other sensors include Toll-like receptors (TLRs) and C-type Lectin Receptors (CLRs) on the surface of innate cells allowing for a cascade of effector immune response to occur. PAMPs act as a ligand or activation for the TLR and other PRRs [26].

8.2.1.2 TLRs

TLRs are comprised of protein products from ten functional genes in humans (TLR 1-10) and eleven (TLR1–TLR9, TLR11–TLR13) in mice. In humans, TLR1, 2, 4, 5, 6, 10 are expressed on the cell surface and therefore detect extracellular components. TLR, 3, 7, 8 and 9 are expressed on the intracellular endosomal compartment [27]. Intracellular TLRs recognise nucleic acids from intracellular bacteria, viruses, and parasites. However, ligand binding by TLRs can be promiscuous [28].

Once a TLR recognises a PAMP, it activates intracellular signalling pathways causing gene transcription and subsequent protein expression such as an increase in the release of proinflammatory cytokines such as interferon (IFN), Interleukin-6 (IL-6), Interleukin 1B (IL-1B), Interleukin 12p70 (IL-12p70) and Tumour necrosis factor alpha (TNFα) [29]. Cytokines recruit and innate phagocytes and augment components of the adaptive response such as T cells which further augments the microbicidal activities of phagocytes. Activated macrophages, recruit other phagocytes such as neutrophils by chemotaxis or will engulf and digest a foreign pathogen when in contact.

TLRs can also recognise endogenous danger signals such as damage-associated molecular patterns (DAMPs). DAMPs activate TLR and release proinflammatory cytokines (e.g. Pro-IL-1B to active IL-1B)

and heat shock proteins being released from damaged cells such as after a sunburn to increase wound healing, prevent further infection and alleviate scarring [30]. Other sensory pathways include NOD-like receptors, Rig-II-like receptors, CLRs and scavenger receptors.

8.2.1.3 Innate effector mechanisms

Innate immune responses will either clear the pathogen or control the infection until an effective adaptive immune response is developed. Circulating phagocytes identify pathogens through cell surface receptors (described above) and release proinflammatory cytokine markers which recruit both a humoral innate response and a cellular innate response.

8.2.1.4 Humoral innate immunity

The complement system is a biological cascade of thirty plasma and membrane-associated serum proteins that function to identify and opsonise (coat) biological pathogens and is one of the major effector mechanisms of the innate immune system. The name is derived from the ability to 'complement' the antimicrobial antibodies in serum and plasma [31]. The complement system is activated in three pathways: classical, lectin and alternative [32, 33]. The classical pathway works with adaptive immune responses (usually IgG1 and IgM) whereas the lectin and alternative pathways are independent of the adaptive immune system. All three pathways opsonise pathogen cell surfaces and create membrane penetrating pores known as the membrane attack complex (MAC) [31]. The MAC causes cytolysis of the microbe or cell death of infected cells through neutrophil and macrophage recruitment.

Figure 2 shows a visual representation of the three arms of the complement system.

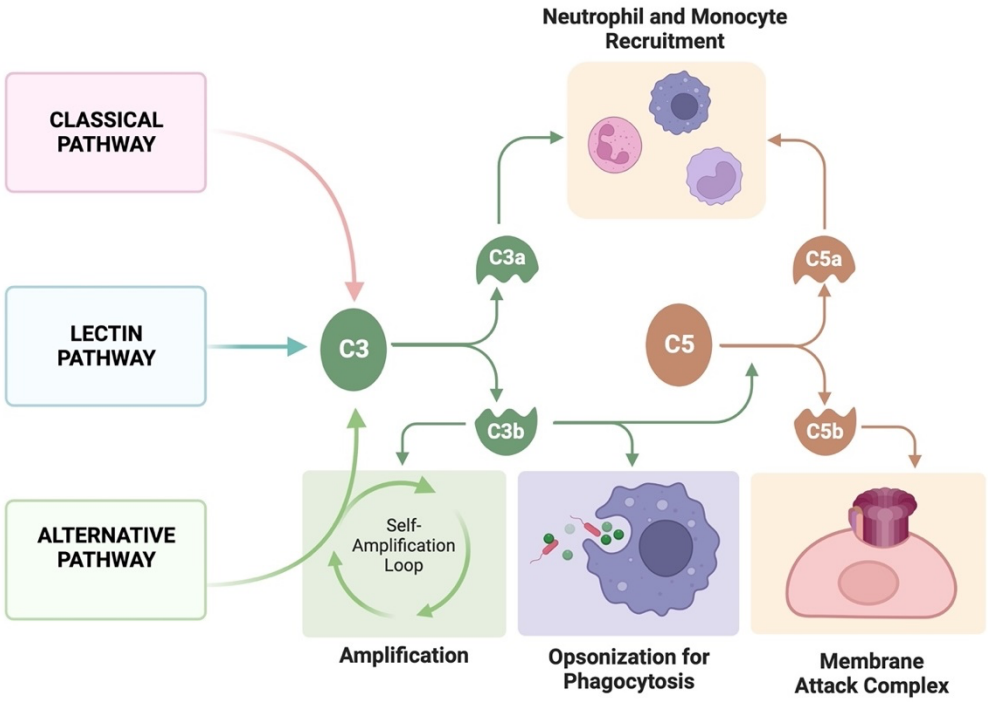


Figure 2 - Complement cascade.

Figure made by D.G Bellamy and designed in Biorender

8.2.1.5 Cellular innate responses

Phagocytes such as macrophages, monocytes, and neutrophils are activated through PRR activation or opsonised microbe surfaces due to complement. Activated macrophages will produce cytokines and chemokines to attract local inflammatory cells and leukocytes to the site of infection. Local inflammation can also activate the complement system. Phagocytes engulf (phagocytose) and clear microorganisms, foreign particles, and cell debris from circulation. Neutrophils are the most abundant of the innate immune system, first to respond and are short lived (six hours in circulation) [34]. Individuals with low neutrophil counts are vulnerable to bacterial and fungal infections [35]. Macrophages and monocytes are subsequently the predominant phagocytes several days after infection [36, 37].

Macrophages and monocytes kill through the release of microbicidal molecules such as reactive oxygen species (ROS) and nitric oxide (NO) which are toxic to microbes [38]. These molecules are

released extracellularly to kill extracellular pathogens and then engulf the killed microbe to clear it from circulation. Microbial molecules are also released intracellularly after a microbe has been engulfed and is contained within a phagosome which then fuses with lysosomes to form a phagolysosome which will kill the microbe with ROS or NO [35].

Another major innate cellular immune cell is the NK cell which accounts for 5-20% of mononuclear cells in the periphery. NK cells are termed by their spontaneous ability to kill tumour cells, however, are identified as subpopulations of lymphocytes but do not express B or T cell receptors and therefore do not possess memory. NK cells are regulated by a balance of inhibitory and activating receptors. NK cells recognise and selectively kill unhealthy, infected or malignant (tumour) cells through the recognition of MHC-1 expressed in healthy cells [39].

Activated NK cells as with lymphocytes, contain cytotoxic granules (perforin & granzymes) that can be released from plasma membranes using contact-dependant-killing resulting in the apoptosis of infected cells. Activated NK cells produce large quantities of IFNγ which mediates both macrophages' and monocytes' phagocytic functions as well as T cell activation. Certain viruses such as HIV, lead to reduced MHC class 1 expression thereby reducing viral presentation to NK and T cells. In addition, patients with NK cell deficiencies display increased susceptibility to Herpes Simplex virus (HSV), Epstein Barr virus (EBV), Cytomegalovirus (CMV) and Varicella Zoster virus [VZV] (a lineage of Herpes infections). Decreased NK cell frequencies have shown associations with autoimmune diseases such as type 1 diabetes and multiple sclerosis (MS) [40].

8.2.1.6 Role with adaptive immune system

The remaining role of the innate immune system is to alert and aid the adaptive immune system. Activated innate cells release cytokines and chemokines which activate and recruit leukocytes (white blood cells) to sites of inflammation and infection. Antigen-presenting cells (APCs) and mainly

dendritic cells (DCs) also play a key role in both innate and adaptive responses [41] and are efficient stimulators of T and B cells. DCs process and capture antigens in the periphery, releasing proinflammatory cytokines for recruitment of innate cells and then migrating to lymphoid organs to promote acquired immune response [42].

8.2.1.7 Major histocompatibility complex (MHC)

APCs express a group of proteins on their surface called the major histocompatibility complex (MHC) which help the immune system recognise foreign pathogens. It is a dense genomic region that includes the largest number of single nucleotide polymorphisms (SNPs) making it highly variable. In humans, the MHC is referred to as human leucocyte antigen (HLA) and is mainly associated with the adaptive immune response [43]. MHC and HLA are often used interchangeably, however in this thesis MHC will refer to the broad genomic region and HLA will refer to specific genes and protein products involved [44].

MHCs are split into two classes, class I and class II. MHC class I is subdivided into HLA-A, B, and C which are found on all healthy nucleated cells. The role of MHC class I is to present intracellular peptides from infected or healthy cells to cytotoxic T cells (CD8) and is often referred to as the endogenous pathway [45]. MHC class II is subdivided into HLA DP, DQ and DR and are only found on certain immune cells such as macrophages, APCs (mainly DCs) and B cells helper T cells (CD4) [46]. The role of MHC class II on macrophages and monocytes is to present extracellular proteins that have been phagocytosed, broken down by phagolysosomes resulting in the epitope peptide fragments being displayed and loaded onto MHC class II molecules on the cell surface [46].

8.2.2 Adaptive immunity

Adaptive immunity is initiated when the innate system fails to eliminate a pathogen and an infection has been established or induced by vaccination. The innate system recognises a wide variety of

pathogens, however is limited in the range of molecular patterns on pathogens it detects [47]. The innate system recognition receptors mentioned above (8.2.1.1) are all fully encoded in a cell's functional form due to the germline genome. However, pathogens have large structural antigenic variations and the ability to mutate and avoid host immunity has led to the formation of an adaptive immune response [48]. Adaptive immune cells have receptors that are tailored and selected through somatic recombination of a variety of gene segments. After a pathogen is encountered, immune cells are able to retain these new receptors and persist over lifetime, allowing for immunological memory and to rapidly respond if re-infection occurs [49].

8.2.3 Cellular immune responses

The acquired immune system is subdivided into T and B cell subsets. T lymphocytes which mature in the thymus deliver an effector cellular response [50]. B lymphocytes develop in the bone marrow and deliver a humoral-mediated immunity by the release of antibodies into circulation [51]. Lymphocytes can recognise, adapt, and respond to a wide host of antigens through specific receptors (BCR and TCR) which are explained below. Lymphocytes are also highly mobile after developing in primary lymphoid organs (thymus and bone marrow) after which they migrate to secondary lymph organs, lymph nodes and spleen, which serve to capture foreign antigens circulating in the blood with the aid of APCs. Thereafter, they migrate to areas of inflammation and infection to exert effector functions [49].

8.2.4 T cells

T cells recognise a diverse array of pathogens, allergens and tumours and can coordinate multiple roles in the adaptive immune response that can maintain immunological memory and self-tolerance. T cells are characterised by two main subsets: cytotoxic T lymphocyte (CTL) CD8+ and Helper T (Th) CD4+ which detect foreign antigens on MHC class I [47]. Before gaining immunological memory, T cells are referred to as naïve which have the capacity to respond to new microbes and pathogens [52]. For a T cell to contribute towards adaptive immunity, it needs to encounter an antigen, induced to

proliferate and differentiate into cells that assist with the removal of the pathogen thus creating memory [53] During childhood, long-term reserves of memory T cells are established due to constant exposure to novel antigens which plateaus in adulthood [54].

8.2.5 TCR

T cell development (thymopoiesis) is initiated after lymphocytes from bone marrow (BM) progenitors migrate to the thymus [55]. In the thymus, BM progenitors lacking CD4+ and CD8+ coreceptors are influenced by transcription factors causing T Cell receptor (TCR) rearrangement to generate CD4+CD8+ double positive (DP) thymocytes [56]. DP thymocytes undergo selection giving rise to single positive (SP) CD4+ or CD8+ thymocytes that will leave the thymus and into the periphery [56, 57] - Figure 3.

8.2.5.1 T Cell Development

DP thymocytes which develop a TCR with high affinity for self-peptide MHC complexes will be negatively selected for apoptosis (negative selection) whereas those with low affinity to TCR to self will develop into mature naïve T cells (positive selection) [58]. Naïve T cells leave the thymus and into the periphery expressing CD45RA+ and CCR7+ phenotypes [56] - Figure 3. Further differentiation of T cells involves antigen-independent processes in which a series of coordinated genomic rearrangements create functional genes of the TCR [49].

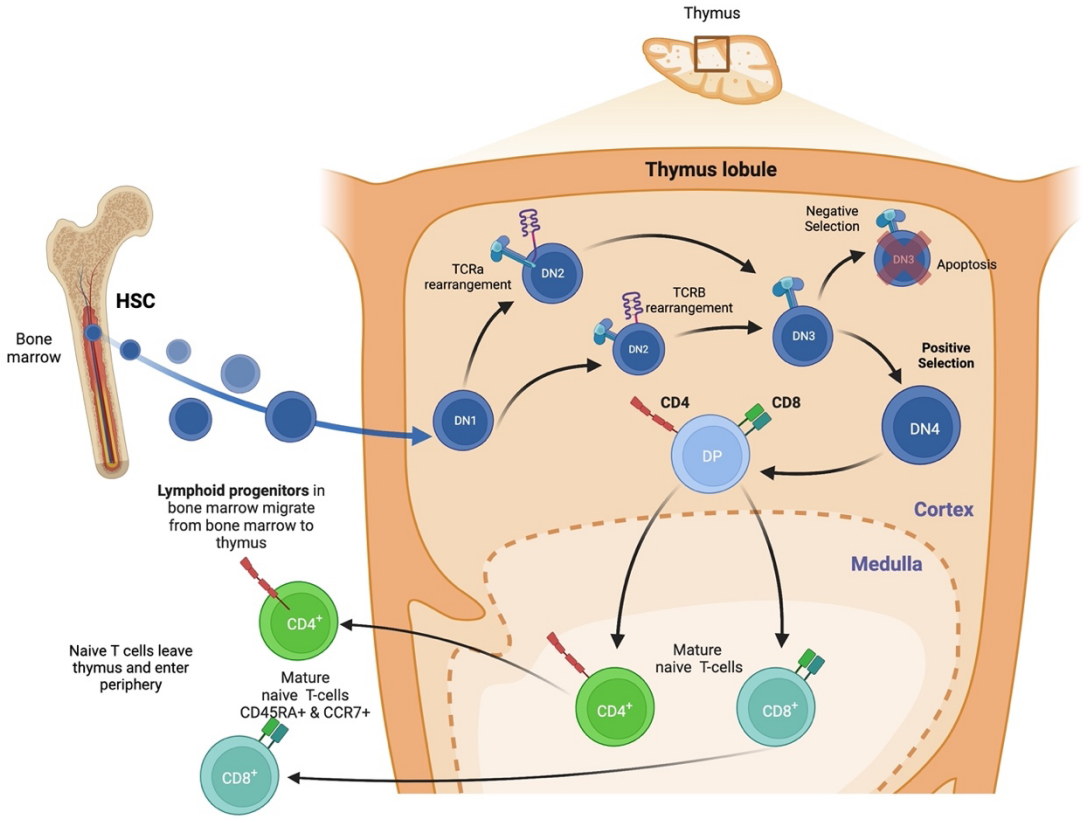


Figure 3 - T cell development in the thymus

Involving positive and negative selection of thymocytes to give rise to single CD4+ and single CD8+ naïve T cells. DN=double negative, and DP=double positive.

Figure is adapted from Chopp et al., 2023 [59] and designed by D.G Bellamy in Biorender

T cells recognise pathogens through antigen presentation on the MHC via the TCR. In their lymphocyte germline configuration, the TCR loci contain arrays of V (variable), D (diversity) and J (joining) segments and V(D)J segments [49]. The TCR is a heterodimer which consists of two TCR chains: α and β chains (majority of mature T cells) or γ and δ chains. Both heterodimers (αβ T cells and γδ T cells) form multiprotein complexes with cluster differentiation 3 (CD3 δ, γ, ε, and ζ) chains [58]. All TCR chains contain a variable immunoglobulin-like domain (VD) and a constant immunoglobulin-like domain (CD) which interact with CD3 chains [58]. The VD is assembled through the facilitation of recombinase (RAG1 and RAG2) which form gene segments for antigen recognition. These gene segments in the variable domain form the basis of large TCR diversity through somatic recombination. A diagram of the TCR can be viewed in Figure 6.

8.2.5.2 T cell activation

TCR activation is mediated through the exposure of chemokines and cytokines. APCs play a major role in T cell activation and migration to sites of inflammation. APCs can load antigenic peptides onto either MHC class I or II to allow interaction between both CD4+ and CD8+ cells. The CD4+ forms an immunological synapse between itself and APC releasing adhesion molecules called integrins [60]. Within the APC / T cell synapse, the first activation signal is the TCR-MHC complex causes intracellular signalling pathways which initiate T cell activation. A second co-stimulatory activation signal forms between the CD28 co-receptor on the T cell and CD80 co-receptor on the APC. The third signal is mediated by cytokines in the APC and will determine what subset the CD4 T cell will develop, Th1, Th2, Th17, TfH and Treg [61]. Figure 4, indicates the various Th subsets and their associated cytokines and transcription factors.

8.2.6 Th subsets

Of the Th subsets mentioned above, Th1 is predominantly a proinflammatory mediator through the release of cytokines: IFNγ and TNFα which will heavily influence CD8+ CTLs to tackle intracellular pathogens and assist the production of complement-fixing antibodies [62]. Th2 molecules respond to extracellular pathogens and therefore mainly produce IL4, IL5, IL9 and IL13, thereby initiating B cell activation and antibody production [52].

The Th17 subset of CD4 T cells is predominantly characterised by the release of IL17 and IL22 [63]. Studies have shown that Th17 cells have roles in inflammatory and haematopoietic effects on structural tissue such as epithelial, endothelium and fibroblasts due to the high expression of the IL-17 receptor on these tissues [62]. Therefore, Th17 can be a mediator in several autoimmune disorders, including MS and arthritis.

Regulatory T cells (Tregs) are important for immune peripheral tolerance, down-modulate the amplitude of an immune response [56] and prevent autoimmune and inflammatory disorders from occurring. Tregs are defined by the expression of the transcription factor FoxP3 and the IL2 receptor alpha (CD25) and primarily mediate Th cell activity [64]. The overall mechanisms by which Tregs modulate the immune system are not well understood, but immunosuppressive cytokines IL-10, TGF-β and IL-5 are secreted upon Treg activation. Immunosuppressive cytokines reduce Th1/Th2 activation and differentiation resulting in reduced secretion of proinflammatory cytokines IFNγ, IL-2 and TNFα. In addition, Tregs can downregulate MHC and co-stimulatory molecules on APCs, resulting in an overall suppression of the T cell response [62].

T follicular helper (TfH) cells are derived from the Th CD4+ lineage and play a role in B cell development, enhancing germinal centre activity, affinity maturation and therefore ultimately assist with the production of high affinity antibodies and long-term memory B cells [65]. This will be further explained in 8.2.10.1 below.

A diagram of Th subset differentiation and the cytokines involved is shown in Figure 4.

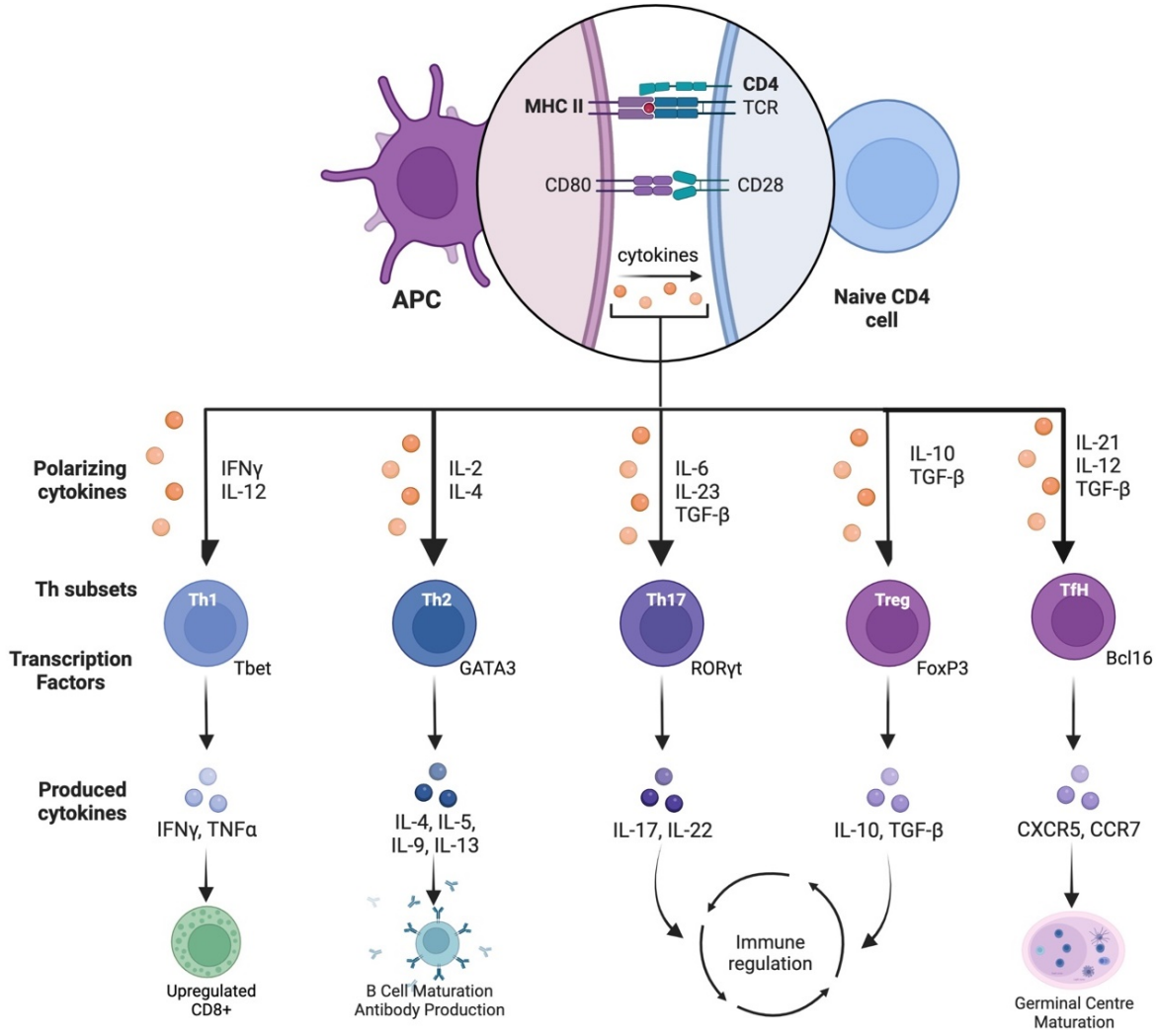


Figure 4 - Th subsets

From left to right, Th subsets along with associated transcription factors and cytokines.

Figure adapted from Crotty et al., 2015 [65] and designed by D.G. Bellamy in Biorender

8.2.6.1 T cell memory formation

CD8+ activation is initiated upon the recognition of MHC molecules by the TLR on APCs in secondary lymphoid organs. The CD8+ forms an immunological synapse between itself and the APC releasing adhesion molecules called integrins [60]. T cell memory (T_M) is based on four factors, effector function, location within the body, surface receptor expression and trafficking properties [66]. Once a CD8+ is activated it undergoes clonal expansion in which T_M, which has pathogen-specific memory, differentiates into T effector memory cells (T_{EMs}) and goes on to clear infection. Second is contraction,

in which the remaining T_{EMs}, after pathogen clearance, will die by apoptosis. Third, is the memory phase in which primed T cells will persist as long-term T_M cells that will reactivate upon re-infection [67]. Re-infection will result in the release of inflammatory cytokine IL2 which will drive the production of T_{EM}. If IL2 is absent or reduced, T_{EM} will either undergo apoptosis or develop into central memory T cells (T_{CM}) [68].

8.2.6.2 T cell effector mechanisms

Upon re-infection, activated CD8+s are recruited to the site of infection through the release of inflammatory cytokines, mainly IFNγ. Recognition of foreign cytosolic peptides on an infected cell is through the host MHC class I by the TLR. The cytotoxic killing is dependent on CD8+ contact with the infected cell through contact-dependant mechanisms. Within minutes, the CD8+ activates apoptotic cell death by mobilisation of cytotoxic granules perforin, and granzyme B to the immunological synapse of the target cell plasma membrane. The T cell releases perforin into the synapse, resulting in the formation of a pore in the target cell membrane allowing granzyme to enter. Granzyme will degrade cellular proteins followed by a Fas-ligand which causes the cell to undergo Fas-ligand apoptosis [49]

Another key role for CTLs is cytokine production notably interferon γ (IFNγ). IFNγ upregulates components of the MHCI and MHCII antigen presentation pathway, increasing the quantity and diversity of peptides presented for CD8+ and CD4+ T cell recognition respectively [69].

8.2.7 B cells

B cells are responsible for the short and long-term generation of humoral antibody responses. Unlike T cells, B cells respond directly to pathogens without the need for APCs through unique antibodies expressed on the cell surface [22] and under specific circumstances, B cells act as APCs. The principal function of B cells is the production of antibodies. In addition, B cells have antibody-independent processes such as antigen presentation, modulate T cell differentiation and production of both regulatory and proinflammatory cytokines [52].

8.2.7.1 B cell development

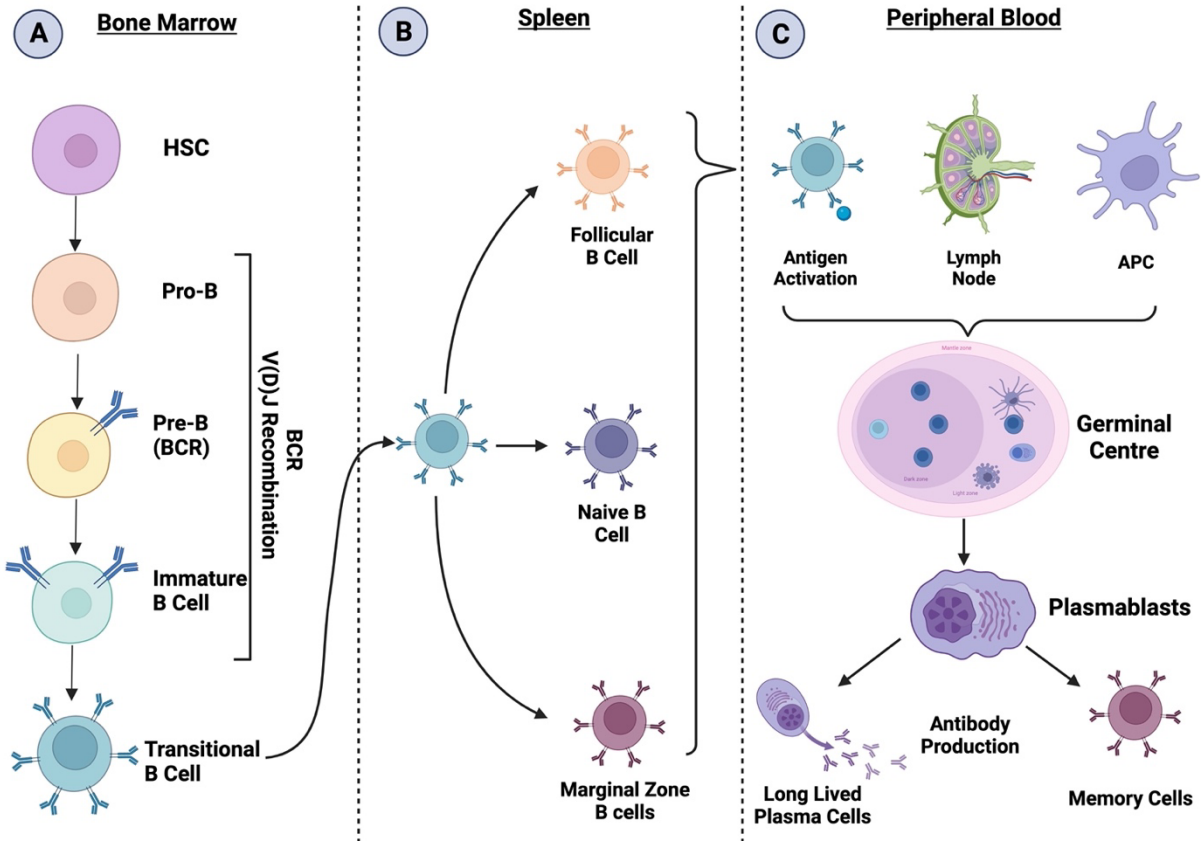


Figure 5 - B cell development

A) *B cell formation in bone marrow haematopoietic stem cells.* ***B)*** *B cell migration to the spleen for maturation.* ***C)*** *B cell activation from the presence of an antigen leads to memory B cells, LLPCs and antibody production.*

Figure adapted from Carsetti et al., 2022 [70] and Schulthei et al., 2022 [71] and designed by D.G Bellamy in Biorender.

B cells arise from HSCs in BM with a specific gene recombinant locus which results in the unique formation of the B cell receptor (BCR) [72]. The development of naïve B cells is shown in Figure 5 which, with the aid of IL-7 initiates an HSC to transform into a common lymphoid progenitor. A common lymphoid progenitor not only gives rise to naïve B cells but also to NK cells, some plasmacytoid DC and T cells [73]. B cell differentiation from T cell lineages is due to the absence or suppression of protein Notch-1 signalling [74].

B cells that have left the BM migrate to the spleen for further maturation and ultimately form naïve B cells that are ready to become antigen-specific upon the detection of an infection. B cell activation through the aid of Th2 cells expression GATA3 transcription factor (TF) and release IL-4, IL5, and IL13 assist B cell maturation and antibody production [65]. TH17 cells produce IL17 to assist in inflammation immune regulation and certain parasitic infections [63]. See Figure 5 for other transcription factors and cytokine productions from Th subsets.

Activated B cells can also enter a lymphoid follicle known as a germinal centre (GC). The GC facilitates more class switching and somatic hypermutations causing high affinity antibody responses to be produced. After an infection has been cleared, germinal centres transition into memory B cells and long-lived plasma cells (LLPCs) [75] - Figure 5. These LLPCs and memory B cells are crucial for vaccine success and long-lived protection [76].

8.2.8 BCR

During differentiation of B cells results in the formation of the BCR and genetic recombination occurs in genes that make membrane-bound immunoglobulin (mIgG). BCR immunoglobulin gene segments variable (V), diversity (D), and joining (J) are arranged into a heavy chain (H) locus and V and J gene segments are rearranged in the light chain (L) locus [77]. The BCR is composed of two Ig L chains and two Ig H chains which form two antigen binding sites (Figure 6) that are specific to each B cell clone

and able to recognise more than $5x10^{13}$ different antigens [78]. B cells also undergo somatic hypermutation where BCR generates diversity through gene sequence mutations allowing the BCR to adapt to new infections, producing increased affinity for the pathogen and ultimately better memory B and plasma cells [79].

8.2.9 B cell antibody production

B cells produce antibodies that are secreted as cell surface receptors and secreted molecules. The first antibodies that a newly formed B cell produces are not secreted but lie on the plasma membrane where they act as cell surface receptors. When a BCR recognises and binds to an antigen, the antigen is engulfed and expressed on MHC-II which is needed for recognition of a CD4+ T cell to complete BCR activation [80]. An activated BCR will receive further signalling from CD4+ T cells forming germinal centres in lymphoid organs transitioning it into a mature plasma cell (effector B cells) which are able to produce antibodies (see Figure 5). Each effector B cell produces a single species of antibody each with a unique antigen binding site. An activated B cell will produce antibodies continuously until an infection is cleared or the B cell dies [81].

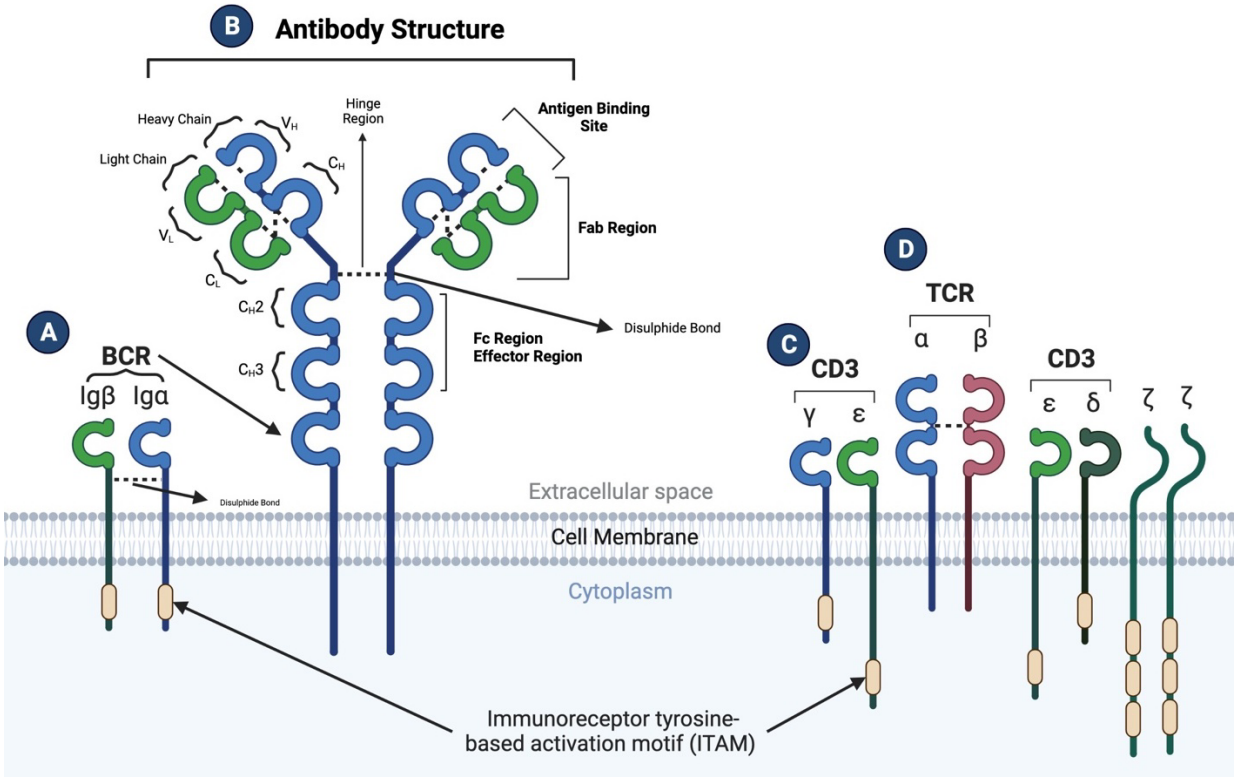


Figure 6 - Antibody structure with BCR.

A) *BCR.* ***B)*** *Antibody structure, consisting of two light chains and two heavy chains making the Fab region linked to the Fc region by disulphide bonds.* ***C)*** *CD3 receptor.* ***D)*** *TCR*

Figure adapted from Janeway Immunology, 2001 [82] and Hoffman et al., 2016 [83] and designed by D. G. Bellamy in Biorender.

8.2.10 Antibody subclass and isotypes

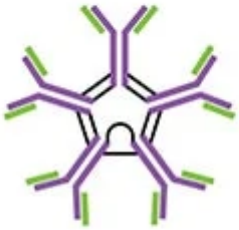
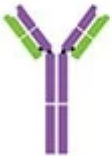
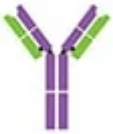
Antibodies also known as immunoglobulins (Ig) are small soluble Y-shaped proteins which bind to antigens with high affinity and assist with protein degradation of pathogens and microbes in various ways [83]. Antibodies that are secreted from a B cell have a fragment antigen binding (Fab) region containing two antigen-binding sites and a fragment crystallisable (Fc) region involved in effector functions through binding of Fc receptors - Figure 6. Antibodies bind to their target antigens and ligands, preventing interactions with the host receptors thereby neutralising the function of that ligand. An antibody that has attached to a particular antigen can cause infectious proteins to cross-link which makes it easier for monocytes and macrophages to locate and phagocytose [84].

B cells produce different isotypes of antibodies through a process called ‘class switching’ [85]. Naïve B cells express cell surface antibodies IgM and IgD, both of which are normally released during the initial stages of infection [81]. However, if a B cell is stimulated primarily through stimulations of CD4+ Th cell, the B cell can then change the isotype it produces. Class switching will occur depending on the type of immune response needed from different pathogens and the location of infection - Table 2. Upon activation, IgM and IgG B cells class switch to IgG, IgA and IgE. Class switching occurs by rearranging the heavy chains within the Fab region. Humans have five different heavy chains: IgA, IgD, IgE, IgG and IgM and their functions can be seen in Table 2.

IgG is the most abundant isotype found in humans and is further subdivided into four subclasses: IgG1-4 [86] named in the order of decreasing abundance [87]. IgG subclasses are ~90% identical on an amino acid level but each has a unique antigen-binding function, unique complement cascade activation [88], unique antibody half-life, unique triggering of effector cells for enhancing the phagocytic activity by opsonisation (8.2.1.4) and placental transport [87]. IgG1 is the most abundant subclass and low levels of IgG1 can result in lower total IgG levels (hypogammaglobulinemia) associated with repeat infections [89]. IgG2 primarily responds to bacterial infections. IgG3 have a major role in inducing effector functions and inflammatory responses. IgG4 respond to allergens and is often formed after repeated or prolonged exposure to an antigen in a non-infectious setting [87]. Generally, IgG1 and IgG3 are referred to as cytophilic due to their protective properties and high affinity to many Fc receptors. IgG2 and IgG4 are generally non-cytophilic with reduced protective properties with poor Fc receptor engagement [90].

Table 2 - Antibody Isotypes

Table taken from Aryal, 2022 [91]

The Five Immunoglobulin (Ig) Classes					
	IgM pentamer	IgG monomer	Secretory IgA dimer	IgE monomer	IgD monomer
					
Heavy chains	μ	γ	α	ε	δ
Number of antigen binding sites	10	2	4	2	2
Molecular weight (Daltons)	900,000	150,000	385,000	200,000	180,000
Percentage of total antibody in serum	6%	80%	13%	0.002%	1%
Crosses placenta	no	yes	no	no	no
Fixes complement	yes	yes	no	no	no
Fc binds to		phagocytes		mast cells and basophils	
Function	Main antibody of primary responses, best at fixing complement; the monomer form of IgM serves as the B cell receptor	Main blood antibody of secondary responses, neutralizes toxins, opsonization	Secreted into mucus, tears, saliva, colostrum	Antibody of allergy and antiparasitic activity	B cell receptor

8.2.10.1 Improved antibody production

Class switching and somatic hypermutation of the BCR are two of the many ways the adaptive system has antigenic diversity to create antigenic memory. This process takes time and co-stimulation from other cells such as Th subset differentiation. Once an infection has cleared, many of the T and B cells will apoptose and a subset will remain as memory. Upon re-infection and recognition of a foreign pathogen, memory cells can activate quickly having optimised the immune process including BCR and

TCR somatic hypermutation and B cell class switching. As a result, re-infection of pathogens is less severe and forms the premise for vaccination strategies - Figure 1.

8.3 Malaria

8.3.1 Types of malaria

Malaria is a vector-borne tropical disease caused by different species of the apicomplexan parasite *Plasmodium sp* and is spread by the bite of an infected female *Anopheles* mosquito [92]. Malaria can be found in 84 different countries [93] and there are more than 120 different species of *Plasmodium sp*, infecting mammals, birds and reptiles of which five are known to infect humans [94]. These species are *P. falciparum (Pf)*, *P. vivax (Pv)*, *P. malariae (Pm)*, *P. knowlesi (Pk) and P. ovale (Po)*. *Pf* and *Pv* result in most infections worldwide and both, particularly the former, can result in severe disease and death. Recent evidence shows that *Po* has split into two separate species: *P. ovale curtisi* and *P. ovale wallikeri*. These are understudied but severity of illness is like that of uncomplicated *Pv* [94].

This thesis will focus on vaccine trials and immunology related to disease reduction and elimination efforts against *Pf.*

8.3.2 Global impact

Great progress has been made in reducing malaria deaths globally with a decrease of approximately 897,000 deaths in 2000 reduced to approximately 568,000 in 2019 [95]. This 40% reduction in deaths can largely be attributed to intervention strategies such as insecticide treated nets (ITNs), insecticide residual spraying, seasonal malaria chemoprophylaxis (SMC) and advancement in treatment. The number of reported cases globally has remained consistent with 245 million cases in 2000 and 247million in 2021 [95]. However, the number of malaria deaths increased to 619,000 at the end of

2021, mainly because of a reduction in malaria in intervention services following the global Covid-19 pandemic [96].

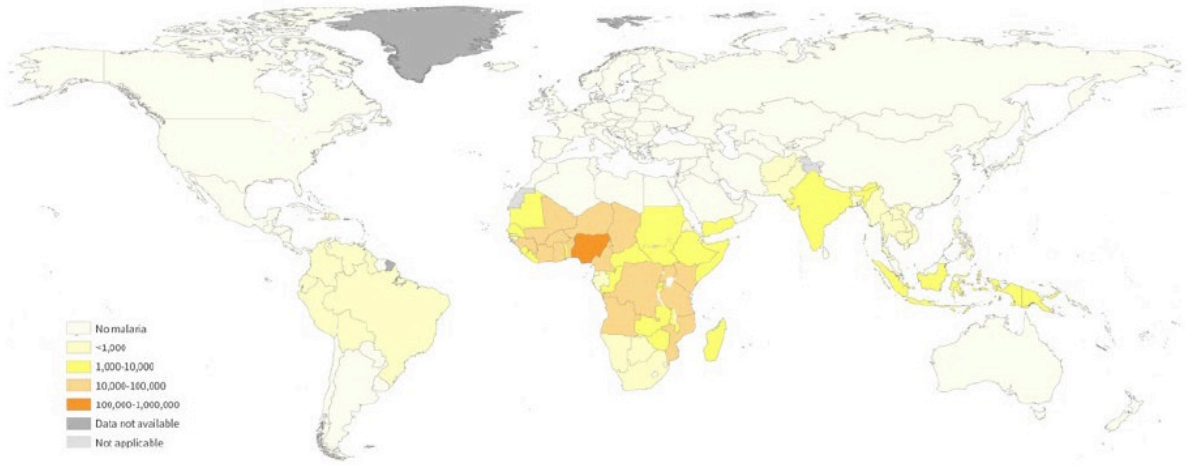


Figure 7 – Estimated number of malaria deaths per country in 2021.

Figure from WHO – World Health Statistics, 2022 [97]

8.3.3 Sub-Saharan Africa

Approximately 90% of all malaria cases occur in sub-Saharan Africa (Figure 7) with children under the age of five carrying the highest disease burden [94]. Currently, 40 countries with past transmission are reported as malaria-free by the WHO, the latest being China in 2021 [98]. Nonetheless, Sub-Saharan African countries continue to have optimal environmental conditions for the breeding of the female *Anopheles*' vector as well as wide-scale poverty [99]. Increase in temperature and rainfall in countries around the equator see the highest percentages of *Pf* transmission in four countries: Nigeria, Democratic Republic of Congo (DRC), Uganda and Mozambique, accounting for approximately half of the world's malaria cases Figure 8A and deaths [95]- Figure 8B. Furthermore, the effect of climate change has been estimated to place an additional 75.9 million people at risk of malaria by 2080 in central and Southern Africa [100]. Understanding mechanisms by which parasites survive in dry seasons is essential to elimination campaigns [101] – seasonal malaria will be covered in 8.11.

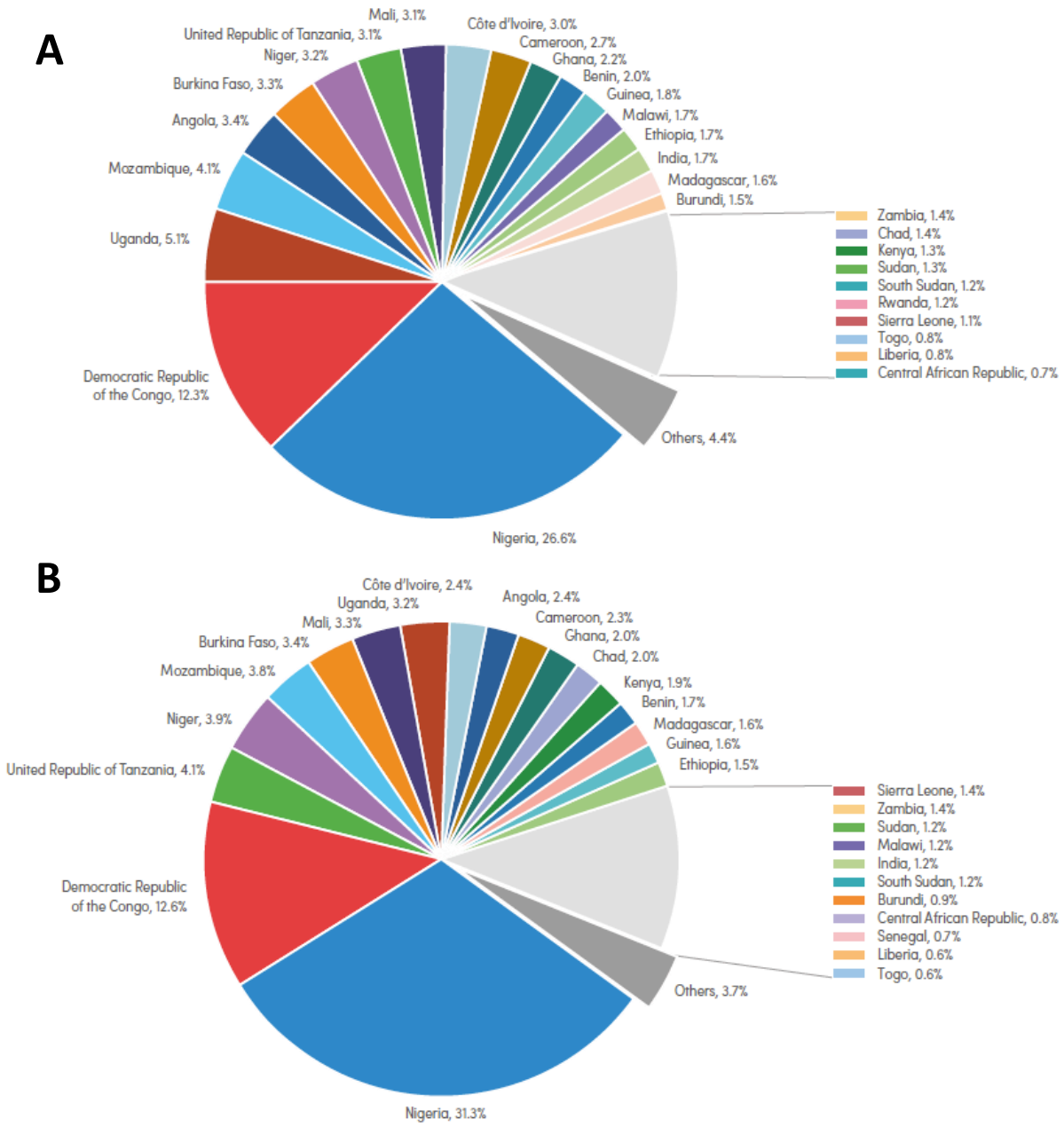


Figure 8 – Prevalence of total Pf cases and deaths in Africa

A) *Percentage distribution of Pf malaria cases in Africa.* ***B)*** *Percentage distribution of Pf malaria deaths in Africa*

Figure taken from World Malaria Report – 2022 [95]

8.3.4 Malaria lifecycle

Plasmodium species are global pathogens that predominantly occur in tropical areas where mosquitos are likely to breed successfully. All *Plasmodium* species have similar lifecycles and alternate between *Anopheles* mosquitos and vertebrate hosts that require unique parasite transformations (zoite forms)

to invade different cell types during the Plasmodium lifecycle [102]. The lifecycle can be subdivided into four sections: A) pre-erythrocytic stage/liver stage (8.3.4.1), the erythrocytic stage/blood stage (8.3.4.2), the sexual stage (8.3.4.3) and the mosquito stage. A schematic of the *Pf* lifecycle is presented in Figure 9 [103-105].

This thesis will predominantly focus on vaccine targets in the pre-erythrocytic stage.

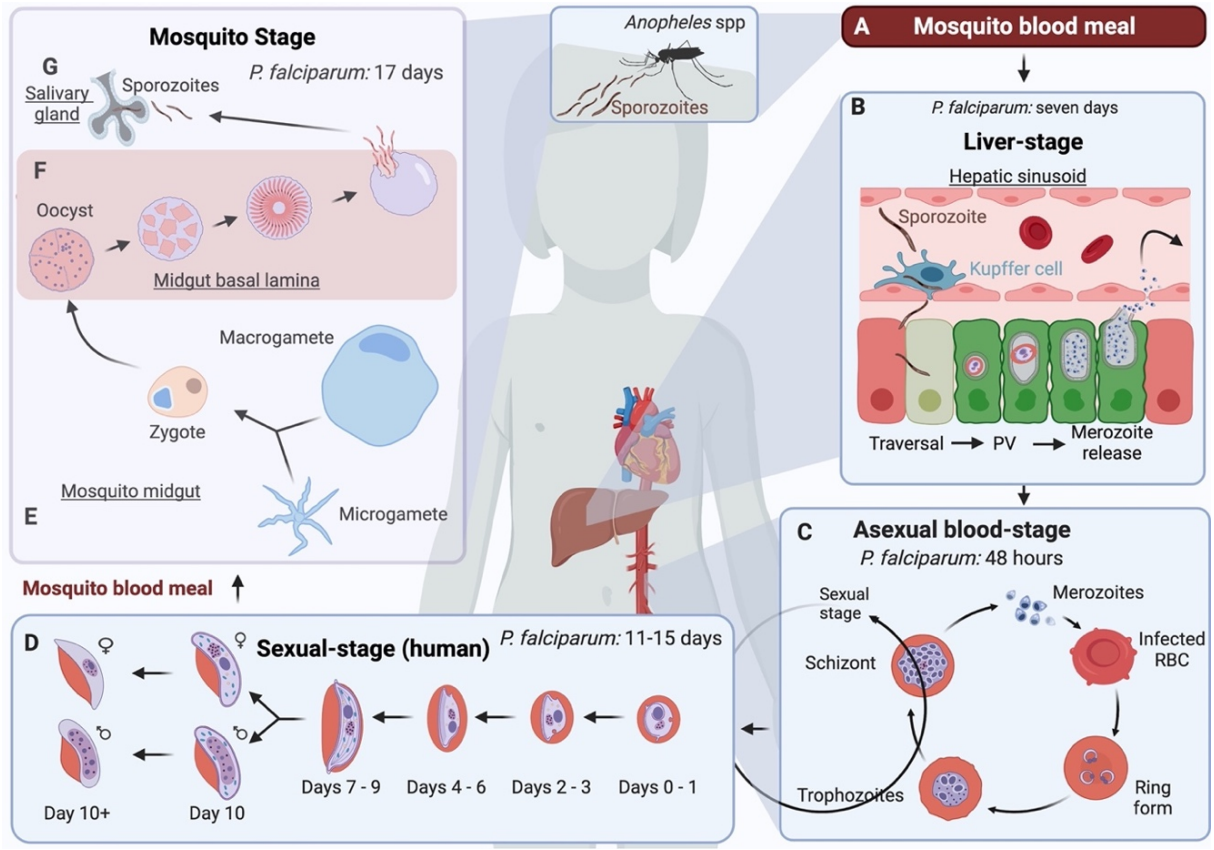


Figure 9 – Pf Malaria Life Cycle

(A) *Malaria Infection commences with the injection of SPZs through a bite from an infected female Anopheles mosquito into the dermis of a human host during a mosquito blood meal.* ***(B)*** *Sporozoites migrate through the dermis and into the vasculature to be transported to the liver. There they exit hepatic sinusoids, traverse through Kupffer cells until a suitable hepatocyte is located. In a hepatocyte they form a parasitophorous vacuole (PV), undergo schizogony and develop into merozoites where they will multiply and after approximately 7 days, thousands of merozoites are released into vasculature.* ***(C)*** *Merozoites in the bloodstream where they target and invade red blood cells (RBCs) and begin the asexual (schizogony) reproduction.* ***(D)*** *A proportion of merozoites differentiate from an asexual reproductive cycle to a sexual cycle (gametocytogenesis) to produce male and female gametocytes. Gametocytes migrate to the BM where they require a 10-15 day maturation period. Mature gametocytes then concentrate in skin capillaries to be taken up by the next mosquito meal.* ***(E)***

In the mosquito midgut, male and female gametocytes form micro and macrogametocyte fuse (mating) forming a zygote that migrates through the epithelial wall of the mosquito midgut forming an oocyst. ***(F)*** *The oocyst begins asexual sporogonic stage of reproduction.* ***(G)*** *The Oocyst ruptures releasing sporozoites into hemocoel and migrate to the salivary glands where the Pf lifecycle will continue after the next mosquito blood meal.*

Figure adapted from Cowmen et al., 2015 [102] and designed by D. G. Bellamy in Biorender.

8.3.4.1 Pre-erythrocytic stage

Upon biting a human, a mosquito injects an anaesthetic along with several thousand sporozoites (SPZs) into the dermis of the skin. Sporozoites propel themselves through the dermis by gliding motility invading the vasculature where they are transported to the liver, however the exact mechanism of how they reach the liver is unclear [106]. Sporozoites can remain in the dermis for up to six hours [107]. Those remaining in the skin will either drain into the lymphatics [102] or be destroyed by host immune responses in the skin. Animal models have shown the increasing role dermal immunity plays in malaria infection [108]. Once in the vasculature, sporozoite traversal involves crossing the hepatic sinusoidal barrier comprising of endothelial cells and macrophage like Kupfer cells [104]. Sporozoites have been reported to invade multiple hepatocytes before arresting in one and continuing the next stage of development within PVs. Sporozoites within the PV develop into a merozoite where they undergo multiple rounds of asexual reproduction before being released into the vasculature as merozoites in clusters of merosomes [102, 109, 110].

Several proteins are involved in cell traversal and each presents as a potential vaccination target. A key surface protein on the sporozoite surface is called CSP [103]. CSP is the most abundant protein on the parasite's surface and is essential for membrane structure, motility and invasion [102, 110]. Immunity to the sporozoite is viewed as a key vaccine target that can prevent downstream stages of infection which result in clinical malaria and onward transmission.

8.3.4.2 Erythrocytic stage – asexual stage

After seven days, merozoites enter vasculature circulation where they begin to invade RBC in a fast, dynamic three-step process that is complete within two minutes [111]. The first step 'pre-invasion' is the initial interaction of the merozoite with the erythrocyte whereby the merozoite reorientates itself for the next step, 'invasion', to occur [112]. Invasion causes the merozoite to seal itself in the PV which follows echinocytosis causing the parasite to shrink [109]. Once erythrocyte infection has been established, the merozoite will replicate (schizogony) over a 48-hour period resulting in the rupture and destruction of the erythrocyte releasing further merozoites into the circulation for the cycle to continue [113]. This cycle of invasion, schizogony and erythrocyte rupture every 48 hours results in the clinical presentation of cyclical fevers and anaemia associated with malaria [114].

8.3.4.3 Sexual stage

The transmission of malaria from humans to mosquitos and back to humans is dependent on the production of male and female gametocytes. During rounds of blood-stage schizogony, a small proportion of merozoites undergo a developmental switch that results in the initiation of sexual development of the gametocytes. The exact molecular mechanism of the developmental switch remains unclear, however, environmental factors can increase the production of gametocytes (gametogenesis) such as high parasitaemia or exposure to anti-malarial drugs [115]. Gametocytes migrate to the BM where they require an 11–15-day maturation before they are infectious to mosquitos. *Pf* has the longest gametocyte maturation of any *Plasmodium* species [116]. Migrating to the BM allows gametocytes to avoid humoral and cellular immune response as well as parasitic clearance from the spleen [117].

8.3.4.4 The mosquito stage

Mature male and female gametocytes are taken up in anopheline blood meal whereas, in the mosquito midgut, proteases form eight male microgametes and one female macrogamete which will

fuse to form a zygote [118]. The zygote transforms into a motile ookinete which then proceeds to cross the midgut epithelial wall of the mosquito and forms an oocyst. The oocyst allows the asexual production of several thousand sporozoites which are released in the haemolymph and migrate to the salivary glands where they will remain until the next mosquito blood meal [104].

8.3.5 *Anopheles* mosquito

Anopheles gambiae can have sporozoite yields from natural infection from 125 to 79,875 sporozoites [119], infected mosquitos grown *in vitro* have an average of 70,000 spz per mosquito [120]. *Anopheles gambiae* is the most common vector for *Pf* in sub-Saharan Africa [121].

8.4 Malaria symptoms, diagnosis and treatment

8.4.1 Symptoms

Naïve individuals infected with *Pf* will develop symptoms of malaria [102] and clinical symptoms develop once the parasite progresses into the erythrocytic stage (8.3.4.2) of the lifecycle. The pre-erythrocytic stage (8.3.4.1) is commonly referred to as clinically silent [94]. The clinical presentation of malaria can be separated into two disease presentations: uncomplicated and severe. Symptoms of *Pf* malaria will typically appear 10-14 days after infection with malaria naïve individuals typically presenting with 100 parasites per µl [122].

8.4.1.1 Uncomplicated malaria

Uncomplicated malaria produces non-specific symptoms which can include fever, chills, rigours, headache, cough, body ache, nausea, fatigue and diarrhoea therefore a clinical diagnosis based on symptoms is unreliable and can be improved with an accurate travel history within a malaria endemic country [123]. The most common (and a classic) symptom is the presentation of a cyclical fever that

occurs every 48 hours and increases in severity with each cycle [124]. If malaria is suspected, the most appropriate course of action is laboratory testing and prompt treatment [94].

8.4.1.2 Severe malaria

Severe malaria is complex, can infect many different tissues and primarily brings about physical damage through low oxygen supply [123]. The most common clinical manifestations of severe malaria are cerebral malaria, acute lung injury leading to respiratory distress syndrome, pulmonary oedema and acute kidney injury [125]. Severe anaemia, hypoglycaemia and concomitant sepsis is mostly presented in children under the age of five [94]. Malaria induced anaemia arises from the increased loss of red blood cells either by asexual reproduction of merozoites, immune mediated mechanisms or by dyserythropoiesis due to hemozoin affecting BM production of RBCs [126].

8.4.1.3 Placental malaria

Placental malaria is caused by *Pf*-infected red blood cells (iRBC) accumulating in placental capillaries and placental intervillous space [127]. This is due to the parasite ligand *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP-1) expressing a variant receptor called VAR2CSA that has an active Duffy-binding like protein which enables cytoadherence to the chondroitin sulphate A (CSA) in the placental intervillous space [128]. This leads to accumulation of maternal monocytes and macrophages causing increased inflammatory response. Placental malaria can restrict foetal growth causing low birth weight, congenital malaria, preterm birth and still birth [129].

8.4.2 Diagnosis

8.4.2.1 Microscopy

Diagnosing malaria typically involves the detection of parasites in the blood by microscopy on blood films that have been stained with Giemsa stain [130]. Microscopy involves the examination of slides to calculate parasite density and species of plasmodia and is the gold standard for diagnosis in the

field as it is inexpensive and can be used effectively in rural and remote locations [131]. However, microscopy has a sensitivity of 50-500 parasites per µl and is therefore relatively insensitive which may lead to misdiagnosis or delays in treatment. Furthermore, microscopy is subjective based on the skill of the microscopist leading to under or over-diagnosis resulting in the excessive use of anti-malarial treatment which can lead to an increase in drug resistance [131].

8.4.2.2 Rapid diagnostic tests

Rapid diagnostic tests (RDTs) have become more frequently used in clinical settings. Currently, there are over 200 brands of RDTs in use all of which involve immunochromatography of monoclonal antibodies (mAb) to detect blood stage antigens [132]. The most common antigen utilised in RDTs in Africa is Plasmodium histidine-rich protein 2 (*Pf*HRP-2) which is highly *Pf*-specific [133]. RDTs are inexpensive and easier to use than microscopy, however, sensitivity decreases with low parasitaemia (<100 parasites/µL) and is not always species-specific [132]. Furthermore, RDTs often report false positives as antigens such as *Pf*HRP-2 can persist in the blood for several days after an infection has cleared [134].

8.4.2.3 Quantitative polymerase chain reaction

Another diagnostic method is quantitative polymerase chain reaction (qPCR) which uses nucleic acid amplification and is the most sensitive tool for detecting parasitaemia (2-5 parasites/µl) [131]. The method applies primers to the 18S rRNA genes which through 35-40 cycles of heating and cooling amplifies malaria-specific DNA [135]. Parasite density is measured using a fluorescent probe against a known standard curve [136]. However, qPCR is expensive and requires specialised equipment making it unsuitable for rural and remote settings.

8.4.3 Treatment

Treatment guidelines for malaria are set by the WHO, last published in November 2022 [137]. Before prescribing anti-malarial medication, the species of plasmodia must be considered as well as the risk of possible drug resistance. Drug resistance to *Pf* anti-malarial, chloroquine, is widely reported [94] therefore artemisinin combination therapies (ACTs) are recommended for the treatment of uncomplicated malaria in children and adults. Artemisinin is an unusual compound derived from the leaves of the plant *Artemesia annua* and the derivatives of the artemisinin dihydroartemisinic acid (DHA), artesunate and artemether form the backbone of current treatments for malaria [138] and artemisinin combinations can include artemether-lumefantrine (AL) and artesunate-amodiaquine (AS+AQ) [137]. Individuals with severe malaria are administered with artesunate intramuscularly (IM) or intravenously (IV) followed by oral ACT [139, 140]. Oral ACT or IM/IV artesunate clears parasite infection leading to reports of sterile efficacy. However, artemisinin resistance has been reported, particularly in southeast Asia [141-143]. Accordingly, artemisinin in different combinations needs to continually be used and deployed to avoid further resistant strains of malaria [144, 145]. Drug resistance further promotes the need for new intervention strategies such as vaccines and new treatments.

8.5 Pathogenesis

8.5.1 Uncomplicated malaria

Non-specific symptoms of uncomplicated *Pf* are caused by the rupturing of RBC releasing erythrocyte and parasite debris (hemozoin) and glycophosphatidylinositol, together referred to as ‘malaria toxins’ [146, 147]. The release of malaria toxins results in the activation of peripheral blood mononuclear cells (PBMCs) which release an array of inflammatory and anti-inflammatory cytokines [123]. These inflammatory responses result in non-specific symptoms associated with uncomplicated malaria [148].

8.5.2 Severe malaria

Organ damage due to lack of oxygen is a result of iRBCs clogging and blocking small capillaries in the body caused by the parasites' ability to sequester and cytoadhere iRBCs to the endothelial cell linings of capillaries and venules of various tissues. This is the major pathologic mechanism in cerebral and placental malaria [149, 150]. Malaria parasites cause iRBCs to sequester in capillaries to avoid parasite clearance by the removal of damaged RBCs by the spleen [151].

Uninfected RBCs are biconcave, in contrast, iRBCs with later asexual stage parasites are spherical and swollen due to the large volume of merozoites within the cell [152]. Without the ability of the parasite to cytoadhere to capillaries, many iRBCs would be removed by the spleen [117]. Parasite count is often underestimated when assessed by microscopy as large portions of the parasite biomass sequester in the endothelial linings of blood vessels and organs. Assessing parasite density is far more accurate by PCR or histidine-rich protein (HCP) [153]. Parasite adherence is known to be associated only with *Pf* evidence of why *Pf* has remained the most common *Plasmodium* parasite in humans [154].

Oxygen restriction due to the obstruction of blood flow in capillaries from iRBC cytoadherence is caused by the release of *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) which binds ICAM-1 and CD36 expressed on the endothelial lining in capillaries lining of organs [151, 155].

8.5.3 Asymptomatic infection

Individuals, in particular adults living in malaria-endemic areas, who have naturally acquired immunity (NAI) present with much greater parasite densities before symptoms develop. Furthermore, many individuals in endemic regions are asymptomatic and are important drivers of malaria transmission [156].

8.6 Immunity to malaria

8.6.1 The Innate response to malaria

Innate immune responses have been outlined in (8.2.1). The various roles that innate immune mechanisms play in response to different *Pf* infections are summarised in Figure 10.

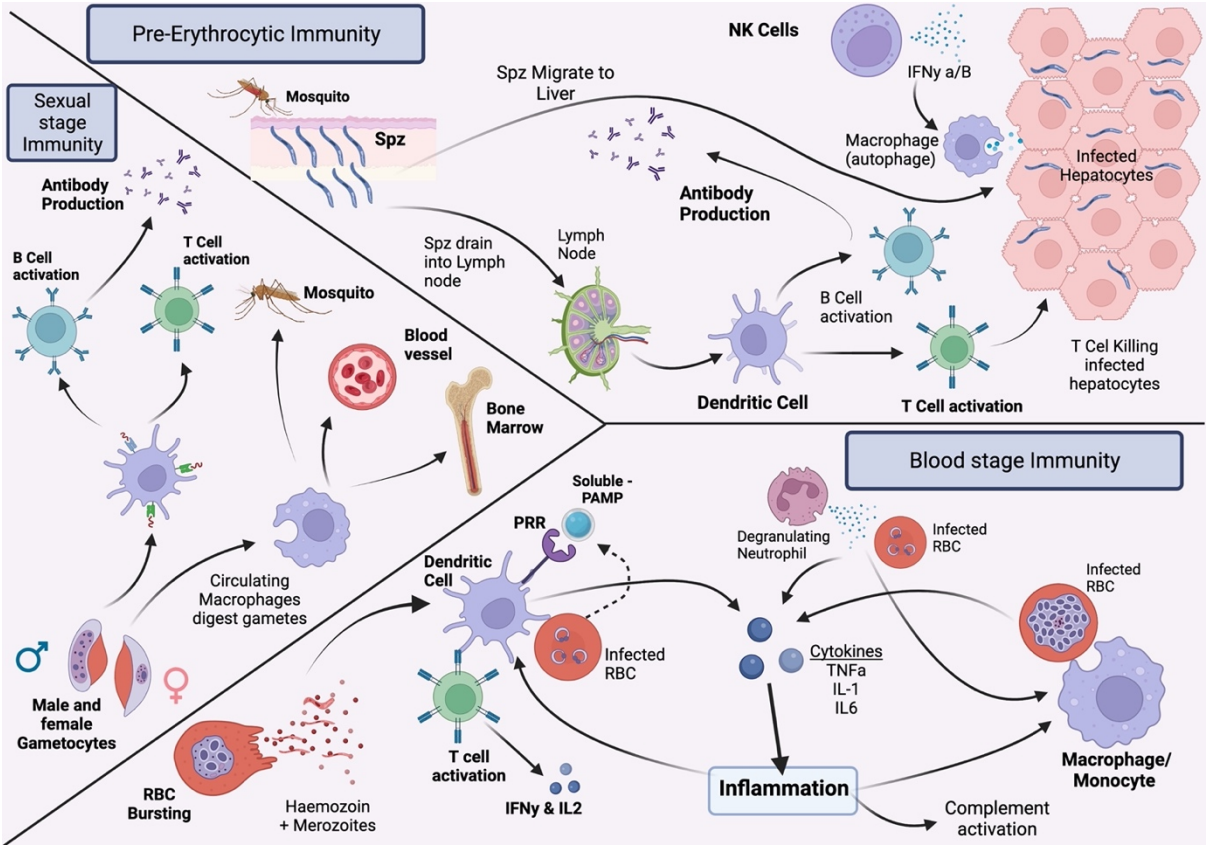


Figure 10 - Innate immune responses to Pf

Innate immune responses to pre-erythrocytic, blood stage and sexual stage Pf lifecycles and some of the key interactions with the adaptive immune response.

Figure by D. G Bellamy and designed in Biorender

8.6.2 Naturally acquired immunity

NAI refers to whether the infection induces protection. NAI can provide valuable insight into the type of immune responses required in order for a vaccine to induce the same level of protection [103]. Protection from natural infection is predominantly targeted to the blood stage lifecycle of malaria.

NAI takes many years to develop in populations with stable endemic transmission and is often associated with adults who present with low parasitaemia and who are asymptomatic of *Pf* [157]. This is further substantiated in that increasing risk and death is highest among young children declining rapidly with repeated exposure (see Figure 11). As NAI mainly induces protection to the blood stage of the malarial lifecycle, repeated infection reduces morbidity and mortality with increasing age. However, the risk of infection continues [102]. NAI is often misinterpreted as it does not provide protection against infection from the sporozoite, but rather protection against clinical disease from blood stage infection. Adaptive immune responses to pre-erythrocytic and blood stage malaria lifecycles can be seen in Figure 12.

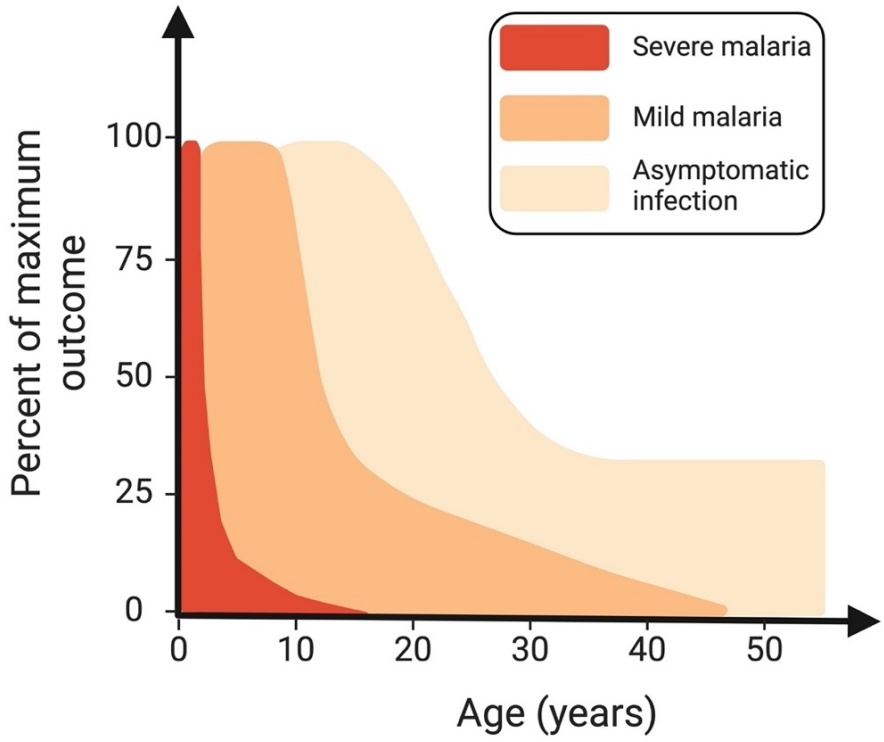


Figure 11 – Development of immunity with increasing age.

Diagram to illustrate the development of malaria immunity to different manifestations of malaria infection in malaria-endemic populations.

Figure adapted from Cowman et al., 2016 [102] and Langhorne et al., 2007 [158] and designed by D. G. Bellamy in Biorender

8.6.3 Adaptive immunity to pre-erythrocytic malaria

NAI to the pre-erythrocytic stage is mainly found in small numbers of adults living in areas of high malaria exposure [159]. This is mainly due to the complexity of the *Pf* lifecycle and the speed at which Spz invade hepatocytes providing limited-time antigen presentation to the immune system. Creating an effective immune response that targets the sporozoite is an effective prospect for vaccine design. Therefore, a vaccine able to induce durable memory B cell producing high-affinity antibodies and a robust Th2 and TfH T cell response is a promising mechanism to prevent and neutralise Spz before invading hepatocytes thereby preventing clinical malaria and onward transmission occurring.

In the 1970s, the initial vaccine design recreated CSP-specific responses in protected persons who were immunised with irradiated sporozoites [160, 161] mainly inducing antibodies targeting the conserved repeat region NANP on the sporozoite surface protein CSP [162]. Protection against sporozoites is mostly mediated by antibody Fc-dependant effector functions [163] of IgG antibodies. IgG can be subdivided into cytophilic IgG1 and IgG3 which are widely found to be associated with protection and non-cytophilic subclasses IgG2 and IgG4 [90, 164]. Antibodies (anti-CSP IgG) can bind to sporozoites and cross-link Fcγ receptors (FcγRs) on the surface of neutrophils and macrophages which will initiate phagocytosis and subsequently engage FcγRIIa and FcγRIIIa/b [34]. Fcγ receptors also mediate NK cells through cross-linking of bound IgG promoting antibody-dependent cellular cytotoxicity [165]. Further, antibodies IgG and IgM activate complement which can opsonise sporozoite activity, and inhibit motility and sporozoite invasion, ultimately leading to parasite death [166, 167]. Anti-CSP IgA has also been induced in children immunised with RTS,S, however, the role of IgA in protection remains unclear [168]. Quantifying antibody inhibitory activity is difficult to reliably perform and produce at scale in clinical trial samples [19].

8.6.4 Adaptive immunity to liver stage malaria infection

Cellular immunity for sporozoite-based vaccines is less understood when compared with humoral responses and involves the induction of CD4+ and CD8+ T cells [103]. Due to the complex nature of the *Pf* lifecycle, cellular effector mechanisms do not target the Spz but target infected hepatocytes, therefore, generating antigen-specific cytotoxic CD8+ T cells is critical to target infected hepatocytes [19]. Secondly, inducing CD4+ cells will aid and prolong immunity through a variety of mechanism by increasing survival and proliferation of CD8+ cells or direct effector mechanism through cytokine production [169].

CD8+ T cells mediated through the release of IFNγ have been shown *in vitro* to cluster around and eliminate infected hepatocytes [170] and there are IFNγ-independent mechanisms [171]. CD8+ memory is initiated from APCs and DCs that present antigens after uptake in secondary lymph nodes and organs such as the spleen. The magnitude of the CD8 response is mediated through $CD4^+$ T cells or γδ T cells during the initial stages of immunisation. Circulating antigen-specific CD8+ cells can migrate to the liver, usually due to re-infection or inflammation, becoming tissue-resident memory cells (TRM cells) [172]. TRMs migrate around hepatocytes through sinusoids in an LFA-1 dependent manner until they locate infected hepatocytes [173]. It is difficult to quantify the importance of CD8+ cells in pre-erythrocytic malaria mainly due to the cells being located in the periphery. However, studies have shown that *Pf*-specific TRMs in the liver contribute towards protective immunity against malaria [174, 175]. A strong cellular correlate of protection (CoP) targeting infected hepatocytes was the frequency of the Vγ9 Vδ2 subset of γδ T cells induced from the *Pf*SPZ vaccine [176]. γδ T cells, which recognise phosphoantigens produced by the *Plasmodium* species are important sources of interferon-γ (IFN-γ), which plays a role in protection [177]. High parasite specific $CD4^+$ and $CD8^+$ T cells expressing cytotoxic markers CD107a and granzyme B, respectively, are associated with protection [19].

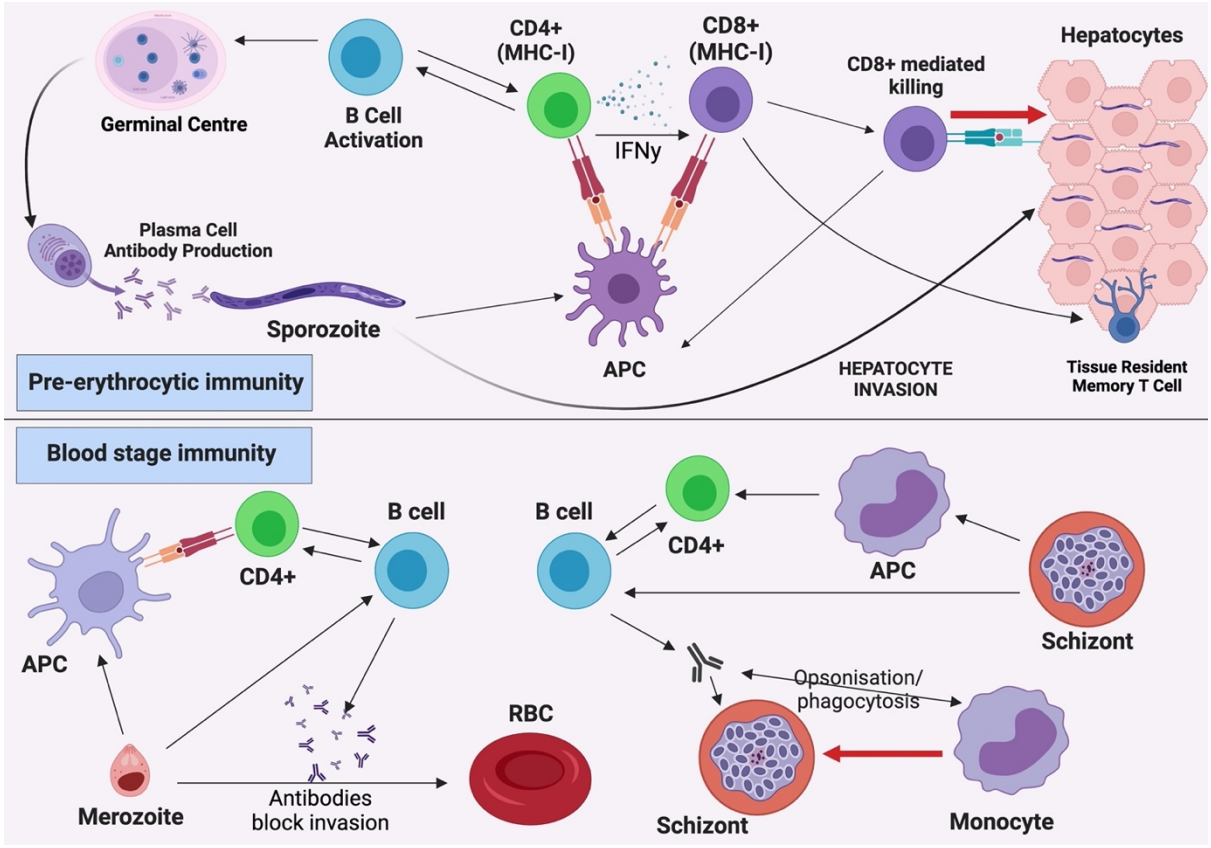


Figure 12 – Adaptive immune responses to Pf.

Adaptive immune responses to pre-erythrocytic and blood stage Pf Malaria Lifecycles.

Figure designed by D.G. Bellamy in Biorender

8.6.5 Adaptive immunity to blood stage malaria

The NAI to the blood stage malaria lifecycle is predominantly induced by antibodies to blood stage antigens that exist on the merozoite surface which facilitate the merozoite adhesion and invasion of RBC [154], as well as antigens that assist in avoiding parasite clearance by the spleen [110]. Many merozoite surface antigens are highly variable [178] and pose challenges in designing a blood stage malaria vaccine [179]. Therefore, NAI induces protection against blood stage antigens which are highly polymorphic [180].

8.6.6 Cytokine production

As mentioned, symptoms from clinical malaria are a result of the release of malaria toxins causing an increase in inflammatory and anti-inflammatory cytokines from PBMCs [123, 158]. There is a strong association with severe malaria and high levels of inflammatory cytokines tumour necrosis factor alpha (TNFα) [181, 182]. In addition, elevated inflammatory cytokines interleukin 1B (IL-1B), IL-6, IL-10 and IFNγ are associated with severe malaria [183]. IFNγ has long been considered as having protective properties. However, individuals who have increased disease severity have greater IFNγ responses [184]. Overproduction of IFNγ is well known in the development of severe malaria and confirmed with IFNγ receptor knockout mouse models that do not develop severe malaria [185].

8.7 Acquired immune mechanism dysfunction

8.7.1 Malaria co-infection with HIV

Sub-Saharan Africa is heavily affected by HIV/AIDS, accounting for 67% of global HIV cases and 88% of global HIV cases in children [186]. HIV depletes Th (CD4+) lymphocyte thereby increasing the risk of opportunistic secondary infections often leading to death [187, 188] and being infected with malaria while HIV positive can cause increased parasite densities leading to increased chances of severe malaria, especially in children [94]. Individuals who are HIV positive (HIV+) thus experience severe malaria and multiple episodes [189]. Pregnant women infected with HIV as well as placental malaria have reduced transfer of maternal antibodies (IgG) via umbilical cord and breastmilk leading to increased risk of malaria in infants and lower birth weight [190].

With antiretroviral therapy (ART) treatment, individuals can live normal lives with almost undetectable viral loads [191]. While it is important to administer vaccines to immunocompromised individuals to boost their immunity, vaccines should not negatively affect HIV disease progression, or interfere with ART medication or other immune responses during routine vaccination [192]. Generally,

most vaccines being administered to people who are HIV+ have been shown to be safe and effective although have lower vaccine immune responses compared to people who are HIV- [193].

8.7.2 Atypical memory B cells

Individuals living in areas with high malaria transmission present with atypical memory B cells (aMBCs) that resemble B cells found in individuals with constant viral infections [194]. Atypical memory B cells can be defined as lacking the classic memory marker CD27 [195]. Malaria is unusual in comparison with other infectious diseases in that acquisition of NAI is slow and can take many years of repeated infection to build. Malaria infection can drive aMBCs formation which will drive phenotypic changes resulting in an infection tolerance profile prolonging infection [196]. AMBCs present with diminished BCR and inhibitory receptor signalling resulting in reduced B cell activation and proliferation, reduced cytokine production and reduced antibody secretion [194].

In addition, constant malaria exposure can cause T cell (T_H1 type) activation of TfH cells [197, 198] leading to T cell exhaustion which results in reduced recruitment of other innate immune mechanisms. Atypical memory B cell and T cell exhaustion can lead to increased morbidity and promote asymptomatic infections, thereby increasing transmission as well as poorer immune responses after vaccination [177, 199-201]. However, it remains unclear whether reduced immune responses found in increasing malaria exposure are caused by aMBCs and exhausted lymphocytes or are caused by other factors such malnutrition and other coinfections [202]

8.7.3 Dendritic cells

Dendritic cells play a crucial role in communication between innate and adaptive immune responses [203] therefore are important for early vaccine responses and the development of acquired immunity. Pathogens including *Pf* have evolved immune evasion mechanisms specifically targeting DCs [204]. Constant exposure can result in reduced DC numbers and expression of antigen presentation markers,

as well as altered responsiveness to TLR4 stimulation leading to immunosuppression [205]. Furthermore, decreased DC numbers and phenotypic changes were observed 14 days after treatment suggesting that even with treatment *Pf* can result in prolonged DC impairment leading to vulnerability to future infections [206, 207] as a result of repeated exposure.

8.7.4 Tregs

Regulatory T cells (Tregs) are upregulated during infection, although increased Treg frequencies are not associated with protection. Furthermore, there is a trend for reduced symptomatic malaria among children with lower Treg frequencies [208] yet their exact protective mechanisms remain unknown including their role in parasite growth rate, in particular anti-parasite immune mechanisms.

8.7.5 Hemozoin

Haemoglobin is the principal component of RBCs accounting for ~95% of the cytosol [209]. Hemozoin (HZ) is a biocrystal that is synthesised by *Pf* to avoid the toxicity of free haem that is produced after Haemoglobin degradation during the invasion of an RBC [210]. Chloroquine, a once widely used anti-malarial, inhibited HZ formation indicating that the sequestration of haem into hemozoin as essential for *Pf* survival and therefore an attractive target for new drug therapies [211].

Phagocytes and in particular macrophages ingest iRBCs most commonly with trophozoites and schizonts. HZ is released into circulation during RBC lysis and is further taken up by phagocytes [212]. Phagocytes release proinflammatory cytokines IL-1β and TNFα. In addition, HZ can activate further immune responses through binding to the TLR9 and are associated with protective immunity to malaria [213] and as a possible adjuvant for malaria vaccines [214].

HZ can also impair immunity by downregulating DC function by the accumulation of pigment on the DC surface leading to reduced B and T cell responses [215]. Therefore, HZ is linked with malaria

immune tolerance of DC activity leading to smaller clusters of T Cells, cytokine production and migration of B cell follicles than would have been seen during the initial period of blood stage infection [215, 216]. HZ can also affect BM having long downstream consequences on both immunity and bone structure.

8.7.6 Sickle cell anaemia

Sickle cell disease (SCD) has long been shown to provide increased protection from *Pf*. HbAS is a result of a single point mutation on the beta-globin gene [217]. SCD causes red cells to sickle and occlude in smaller blood vessels causing vaso-occlusion resulting in shortness of breath, anaemia and increased risk of serious infections [218]. SCD protects from *Pf* in several ways [219] which are summarised in Figure 13.

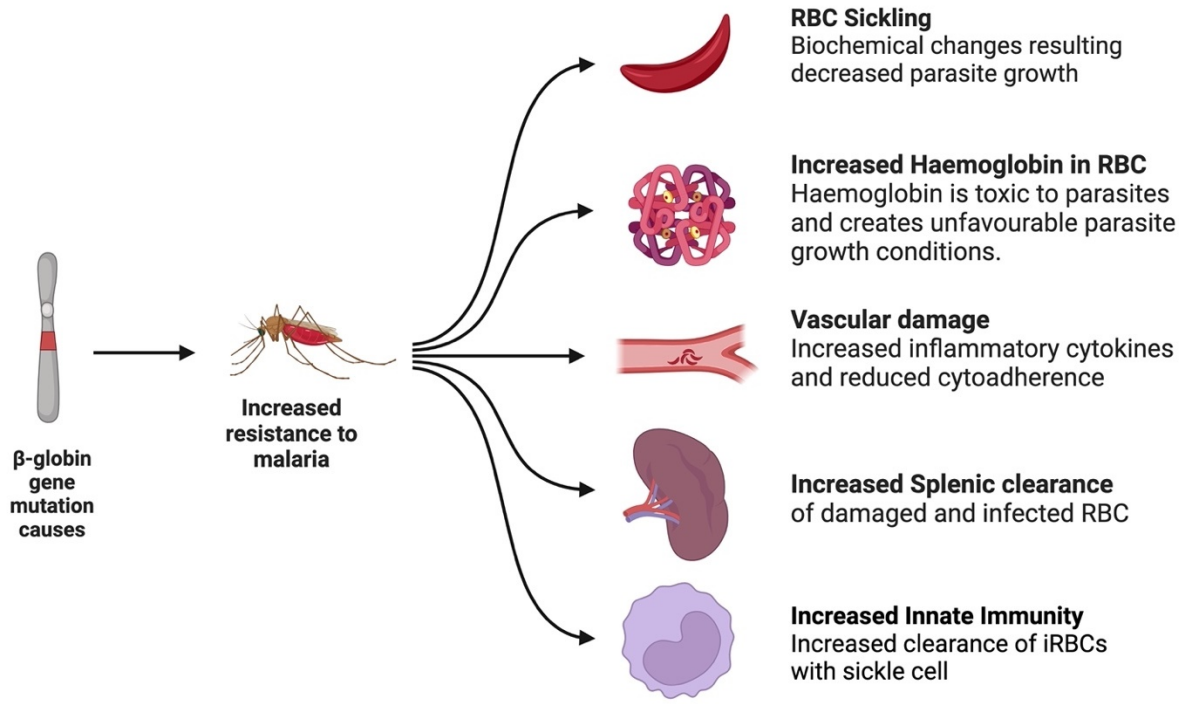


Figure 13 – Sickle cell disease and its protective functions against malaria.

Figure designed by D. G. Bellamy in Biorender.

8.9 Vaccines against malaria

Progress in reducing malaria morbidity and mortality has stalled despite interventions such as IRS, ITNs, SMC and treatment [95]. An efficacious vaccine has long been thought of as the next intervention to reduce the malarial burden and contribute towards malaria eradication [220]. At present and over the last 5-10 years, there have been 153 trials involving 89 malaria vaccine candidates in clinical development of which 82 target *Pf*, with the remaining 7 targeting *Pv* [221]. After decades of development, only one vaccine candidate has completed Phase 3 studies, RTS,S/AS01$_E$, with R21/MM currently in the second year of a Phase 3 trial.

8.9.1 Malaria development goals

In 2006, the malaria vaccine funders group came together to form the Malaria Vaccine technology roadmap to help coordinate a global strategy to reduce morbity and mortality caused by malaria. This included having a first-generation vaccine reaching a VE of 50% against severe malaria and death by 2015 which is sustained for a year [222]. In 2013, in response to changes in malaria transmission and epidemiology with an increase in clinical malaria seen in older children, the Malaria Vaccine Technology Roadmap was updated by the WHO to promote a proof of concept or second generation vaccine with efficacy greater than or equal to 75% [223].

8.9.2 Current malarial vaccines

All the current vaccines target one or two specific proteins or antigens within the malaria lifecycles (Figure 14). It is widely reported that in order to achieve malaria elimination and possibly eradication, multiple vaccines that target multiple lifecycle stages will be needed [224]. However, a multi-stage vaccine if found, would likely have significant manufacturing costs which may impact delivery of the vaccine to poorer countries in most need [225]. Furthermore, an effective vaccine would need to involve multiple aspects of the immune system. These include high B cell responses to produce quality humoral responses for circulating sporozoites, merozoites and gametocytes. In addition, CD8+ killer T

cells to target infected hepatocytes and possibly erythrocytes [226, 227] and CD4 responses for identification and recruitment of further immune responses. Designing a vaccine requires formulating a hypothesis regarding the type of adaptive immune response that will be required to mediate protection [103].

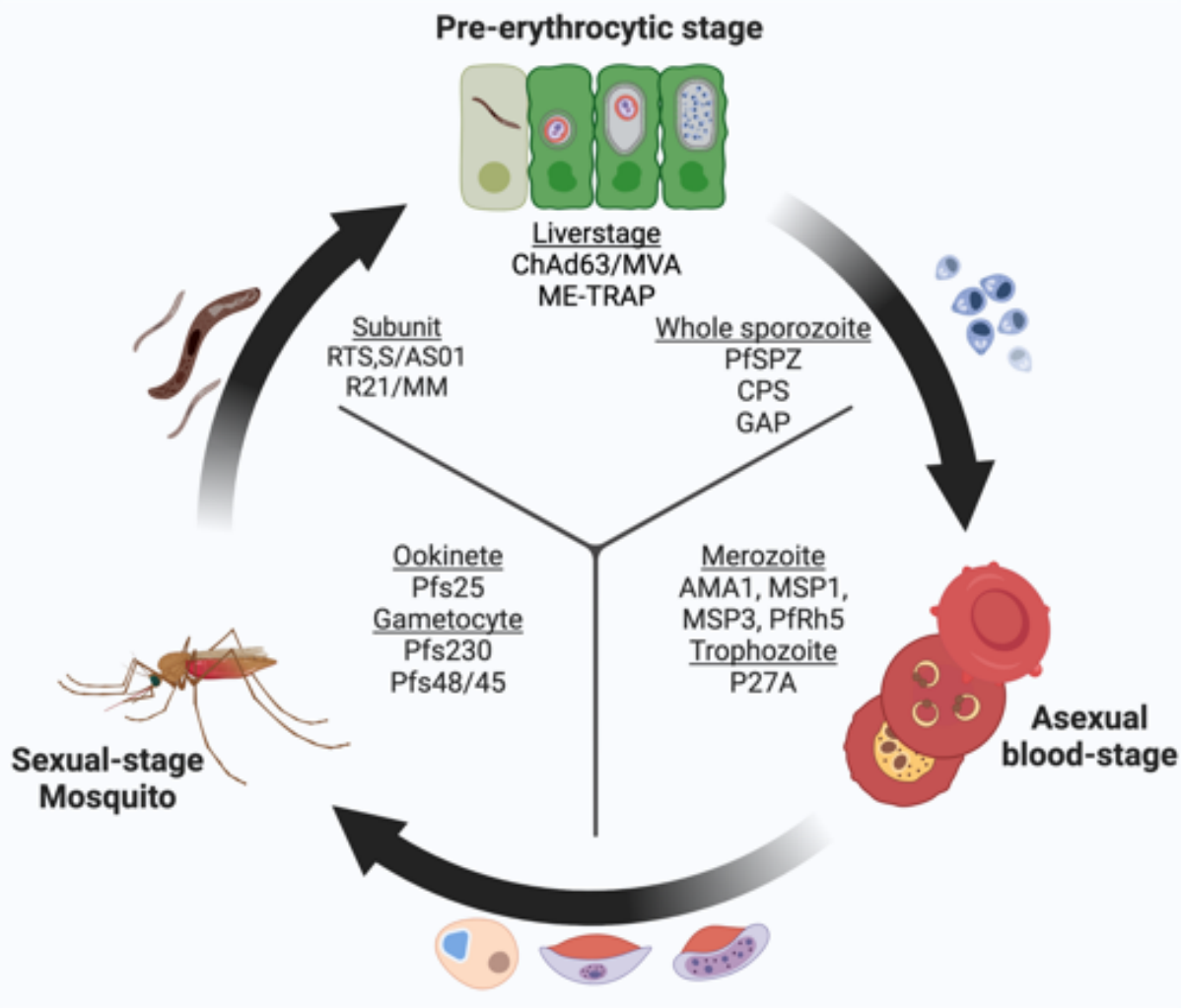


Figure 14 – Individual vaccines targeting specific Pf lifecycle.

Figure redrawn and adapted from Duffy et al., 2020 [224] and designed by D. G Bellamy in Biorender.

NAI (8.6.2) mainly involves protection from clinical disease and increases in immunity to the blood stage lifecycle of infection [228] which takes many years to develop involving constant reinfections resulting in asymptomatic infection but with high circulating parasitaemia [229]. Therefore, an effective vaccine will need to induce greater and more effective immune responses compared to NAI.

8.9.3 WHO policy approvals

RTS,S/AS01$_E$ received a positive scientific opinion from the European Medical Agency in 2015 [230], a positive policy recommendation from the WHO in 2021 [231] and pre-qualification in 2022 [232]. With pre-qualification, the GAVI alliance, an organisation established to fund vaccine supply to the world's poorest countries, has allocated US$ 155 million to support the introduction, procurement and delivery of RTS,S/AS01$_E$ to GAVI-eligible countries in sub-Saharan Africa [233]. The first 4 million doses to be used in a programmatic deployment are scheduled for distribution in 2023. In 2023, regulatory authorities in Burkina Faso, Mali and Nigeria licensed R21/MM after being granted an export licence from India [234]. Soon after the WHO provided a positive policy recommendation for the use of R21/MM in African infants below the age of 2, joining RTS,S/AS01$_E$ as the only malarial vaccines to receive this review from the WHO [235].

8.9.4 Pre-erythrocytic malaria vaccines

Pre-erythrocytic malaria (PEM) vaccines create an immune response preventing the malaria parasite from progressing to the blood stage lifecycle of infection. By preventing blood stage infection clinical malaria and onward transmission can be prevented.

PEM vaccines either target sporozoites or infected hepatocytes. Sporozoites are the first stage of the lifecycle that can be targeted by vaccination. Before reaching the liver, sporozoites are briefly in the skin and subsequently in the vasculature and therefore a target for humoral immunity [236]. The major surface protein on the surface of the sporozoite is the CSP which plays an important role in motility, attachment, and entry into hepatocytes [237]. *Pf*CSP is a 397 amino acid protein composed of a central repeat region of NANP and NVDP flanked by conserved N and C Terminal domains [238]. A full diagram of the CSP can be viewed in Figure 15. However, as mentioned (8.3.4.1), the sporozoite has a small window (~1 hour) for an effective immune response before hepatocyte invasion [107].

Therefore, extremely high anti-CSP antibodies are needed to prevent parasite invasion. Increased anti-NANP and anti-C term IgG are associated with increased levels of protection [19, 239].

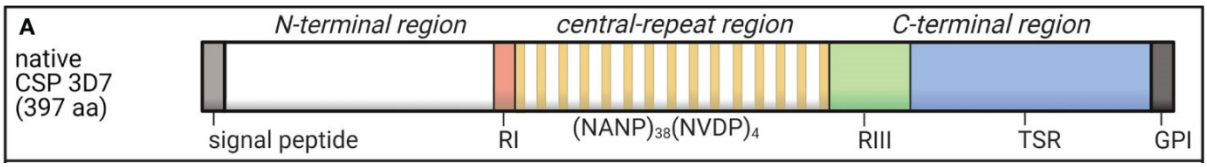


Figure 15 - Schematic of the Pf CSP and the important antigens

The native Pf CSP (3D7) comprising of the N Terminal region containing region 1 (RI), the central repeat region comprising various numbers of NANP repeats and some NVDP repeats, and the C terminal region including region 3 (RIII) and the thrombospondin type 1 repeat (TSR) domain.

This figure has been adapted from Kurtovic et al., 2021 [167].

8.9.4.1 Subunit sporozoite vaccines

Subunit vaccines consist of small amounts of virus like particles (VLP) or peptides or (glyco-) proteins that can produce an immune response to a particular pathogen. Subunit vaccines have been used in malaria vaccine development for decades. One of their drawbacks, however, is a lack of durable immune responses. They, therefore, often require multiple doses and adjuvants to reach desired levels of immunity and booster doses as well as be administered [240].

8.9.4.2 Virus like particles

VLP's are virus derived structures and are synthesised through self-assembly of recombinant viral capsid proteins. They mimic the size and form of a virus however lack any genetic replication material [241]. Structural proteins from several virsuses such human immunodeficiency virus (HIV), adeno-associated virus, Hepatitis B virus (HBV), Hepatitis C virus (HCV) have been used to produce VLPs. They are repetitive surface patterns, and have the capacity for induction of strong humoral and cellular responses [242]. They can also be loaded with immune modulators such as innate immune stimuli to promote immune responses.

VLPs contain an internal cavity and therefore can be used a delivery vehicles for the delivery of biological material making it useful application for vaccinology [241]. VLPs when administered as vaccine are taken up through phagocytosis or internalized into APCs, digested in the phaglyosome. Then loaded on to the MHC-II for Th cell activation through the TCR resulting in further signalling costimulatory receptors and cytokines [243]. Lastly, entrapment of the antigen of interest within the particle and with the addition of an adjuvant can potentially cross-present antigen, creat a depot effect increasing availability of antigen, thus supporting the generation of CD8+ T cell responses [240].

8.9.4.3 RTS,S

RTS,S is a VLP subunit vaccine developed at GSK in 1987 that targets the CSP [244]. Using knowledge gained during the development of the hepatitis B vaccine (EnergixB™vaccine) [245], GSK used the Hepatitis B Surface antigen (HBsAg) as a carrier matrix for the CSP central repeat region (18 copies of NANP) and added C term which contains T and B cell epitopes [246]. RTS,S has a 1:4 ratio of *Pf*CSP fused with HBsAg to unfused HBsAg and is administered with GSK AS (adjuvant system) adjuvant, either AS01 or AS02 with different doses identified for adults or infants - Table 3 [245].

Table 3 - Formulation of RTS,S and AS adjuvants.

**oil-in-water emulsion-based adjuvant systems. **Liposome-based adjuvant system*

Table adapted from Cohen et al., 2010 [245]

Formulation	RTS,S (µg)	MPL (µg)	QS21 (µg)	Final Volume (ml)
RTS,S/AS02A*	50	50	50	0.5
RTS,S/AS02D*	25	25	25	0.5
RTS,S/AS01B**	50	50	50	0.5
RTS,S/AS02$_E$**	25	25	25	0.5

In Phase III studies RTS,S/AS01$_E$ reported a vaccine efficacy (VE) of 56% [95% CI 50.6-60.4] against clinical malaria and 47.3% [95% CI 22.4-64.2] against severe malaria 12 months after the primary series in infants 5-17 months of age [247], reaching the 2006 Malaria Vaccine Technology Roadmap of a VE greater than 50% against severe malaria [222]. With the addition of a booster dose administered at

18 months, VE dropped to 36.3% [95% CI 31.8-40.5] against clinical malaria and 29% [95% CI 3.5-47·2] against severe malaria after four years [248]. Key RTS,S developments since 1987 can be viewed in Table 4.

Table 4 – Key trial developments and publications of RTS,S

Year	Details	Publication
1974	Proof of concept of CSP vaccine against malaria	[160, 161]
1984	GSK and WRAIR Collaboration to pursue vaccine against *Pf*	[249, 250]
1987	Preclinical studies show RTS,S produce antibodies that can inhibit spz invasion of hepatoma cells *in vitro*	[249, 251, 252]
1995	First Clinical Trial of RTS,S in humans	[253]
1997	First RTS,S Challenge Trial	[254, 255]
1998	First use of RTS,S in Adults (Gambia) in living in Malaria endemic area	[256]
2001	Efficacy of RTS,S / AS02 in adults in the Gambia	[257]
2001	Assessing 1, 2, or doses regimens of RTS,S in CHMI but administered in the delayed third dose	[258]
2004	RTS,S first safety and immunogenicity study in Gambian children	[259]
2004	RTS,S efficacious in young children in Mozambique	[260, 261]
2007	Phase 2 efficacy of Malaria endemic infants	[262-264]
2007	AS01 vs AS02 Adjuvant trial	[265]
2008	RTS,S/AS01E efficacy study in infants 5-17months Kenya	[266]
2011	RTS,S interference trial with EPI programme	[267]
2011	Phase III RTS,S/AS01E (Year 1) efficacy in children aged 5-17months	[247]
2012	Phase III RTS,S/AS01E (Year 1) efficacy in infants 6-12 weeks	[268]
2013	Efficacy of RTS,S in different malaria transmission settings	[269]
2014	Phase III RTS,S/AS01E efficacy at 18 months	[270]
2015	Phase III RTS,S/AS01E efficacy over 4 years	[248]
2015	Phase III RTS,S/AS01E Immunogenicity	[271]
2015	EMA Positive Scientific Opinion	[230]
2016	WHO Pilot Implementation Study	[272]
2019	Detailed safety analysis from the Phase 3 – RTS,S	[273]
2021	RTS,S with SMC	[274, 275]
2021	WHO – Positive vaccine recommendation	[231]
2022	Results from the pilot Implementation trial	[276]
2022	WHO – RTS,S Pre-qualification	[232]

8.9.4.4 R21

R21, developed at the University of Oxford, has a similar construct to RTS,S whereby the central repeat region NANP and C-terminus of the CSP are fused to the HBsAg. RTS,S has a four-fold excess of HBsAg unbound to the CSP whereas R21 does not [277, 278] - Figure 16. This causes a greater density of CSP

to be presented on the R21 surface thus potentially enhancing the immune response to CSP. N Terminal region is not included in R21 or RTS,S but studies have shown it to be involved in hepatocyte attachment and invasion by sporozoites [279]. Current Phase II studies show that R21/MM induces a point estimate VE 77% [95% CI 67-84] in Burkinabe infants after 12 months and 81% [95% CI 66-87] 12 months after a booster [2, 3]. A Phase III study assessing efficacy of R21 showed VE was 75% [95% CI 71-79] at the seasonal sites and 68% [95% CI 61-74] at the standard sites against clinical malaria after 12 months [280].

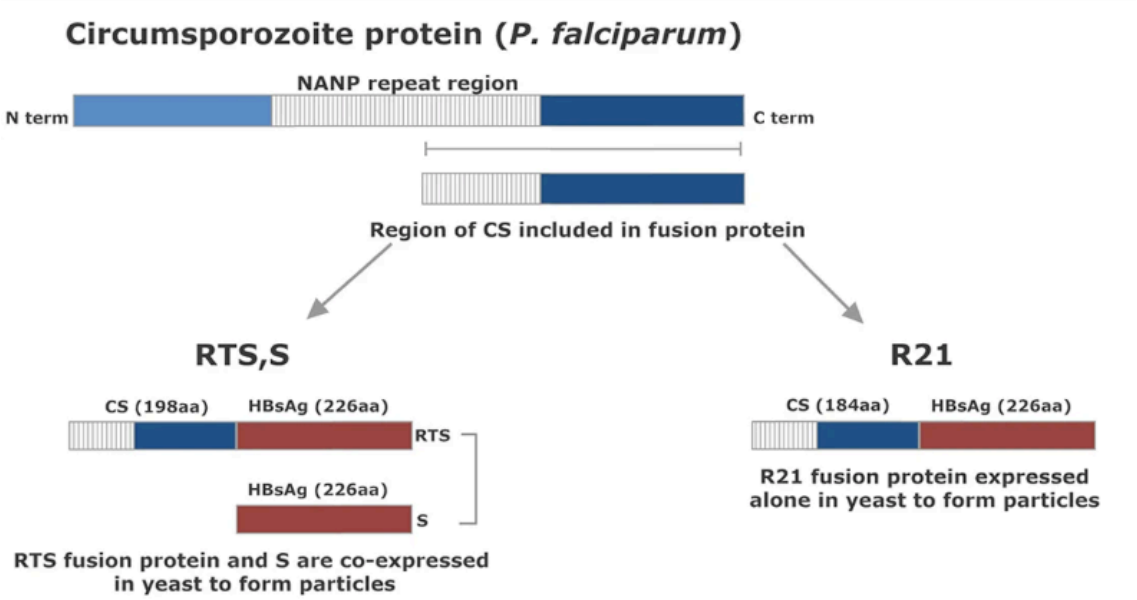


Figure 16 - R21 VLP design compared to RTS,S with respect to the CSP

Both R21 and RTS,S consist of C Terminal portion of CSP from Pf NF54 strain. Both are fused to the N-terminus of HBsAg however RTS,S has extra unbound HBsAg. R21 has 19 NANP repeats and RTS,S has 18.

Diagram taken from Collins et al., 2017 [278].

8.9.4.5 Whole Sporozoite vaccines

Whole sporozoite vaccines (WSV) have been in development since 1970 and are one of the first vaccines to show short-term high efficacy (~90%) against homologous CHMI against *Pf* malaria [281]. WSV involves injecting viable sporozoites that have undergone radiation so that they can infect hepatocytes but will self-arrest in the liver therefore preventing onward transmission [282] known as radiation-attenuated sporozoites (RAS). RAS has advantages in providing immunity to multiple

antigens and can replicate natural pre-erythrocytic infection. Furthermore, RAS are said to provide not only strong humoral responses [283] but potent T cell responses which are needed to locate infected hepatocytes [284, 285].

A variety of WSV have been developed through the RAS concept [284]. Currently, three WSVs have shown efficacy in clinical trials [19]: the first, RAS (*Pf*SPZ vaccine) developed by Sanaria which involves different methods to radiate Spz allowed to invade hepatocytes but self-attenuating at different stages [286]. *Pf*SPZ provided up to 80% protection when administered to malaria naïve adults under homologous CHMI but was less effective in field studies [176, 283, 285] with no efficacy in young children. The second involves using anti-malarial drugs to achieve chemo attenuation of hepatocyte infection known as *Pf*SPZ-C vac for chemoprophylaxis vaccination. *Pf*SPZ-C vac induced high efficacy that was durable for up to two years [287, 288]. The last is to genetically attenuate parasites (*Pf*SPZ-GAP) by deletion of genes to complete liver stage development [289]. The advantage of *Pf*SPZ-C vac and GAP is that the parasite self-arrests at a later stage allowing for longer antigen presentation to the immune system and number of doses and concentration.

RAS can mediate high protection against the pre-erythrocytic stage compared with natural exposure likely due to the multitude of sporozoites injected through vaccination across multiple doses [103] compared with several hundred administered with a single mosquito bite [290, 291]. The practical challenges of high manufacture cost, essential intravenous dosing, the need for pre-clearance of parasites in the field for good efficacy, lack of efficacy in young children and thermolability of sporozoites, requiring liquid nitrogen storage, remain problematic.

8.9.4.6 Viral vectored vaccines

Viral vectors have shown great potential to be used as a malaria vaccine because of their ability to induce both humoral and cellular immune responses [292-294]. The chimpanzee adenovirus 63

(ChAd63) has been widely assessed in humans in a variety of different infectious diseases and is safe and highly immunogenic [295-297]. ChAd63 holds advantages over other human adenovirus vectors such as adhu5 by reduced anti-vector immunity [298]. Modified Vaccinia Ankara (MVA) virus has also been shown to be safe and immunogenic. MVA induces protective mechanisms by interacting with APCs migrating to lymph nodes where antigen of interest is presented to CD4+ and CD8+ T cells [299].

Administering a combination of ChAd63 with MVA in a prime-boost regimen has shown to be a relatively potent combination [300]. Viral vectors expressing thrombospondin adhesion protein (TRAP) along with a string of T cell malaria epitopes (MEs) have shown protective efficacy against *Pf* [301] with 20-25% VE. Thrombospondin-related adhesion protein (TRAP) is presented on the surface of sporozoites and infected hepatocytes and therefore will induce humoral responses to prevent sporozoite invasion of hepatocytes and cellular responses to locate and kill infected hepatocytes. A viral vector approach has reported a modest efficacy of 45% in delay to patency in malaria naïve adults [302] using a new prime-target immunisation approach, and field efficacy in a small Kenyan trial in adults, however no efficacy in malaria endemic adults in Senegal [303].

8.9.4.7 mRNA malarial vaccines

The recent success of mRNA vaccines against SARS-COV-2 has sparked new interest in RNA and DNA vaccine platforms. Flexibility in antigen design and subsequently rapid development and saleability make them attractive technology for malaria vaccine development [304]. Developments such as lipid-based formulations allow RNA to enter cell membranes necessary to producing proteins of interest and induce broad humoral and cellular immune responses. Furthermore, the development of self-amplifying RNA has shown significantly greater immune responses in comparison with mRNA formulas allowing for the possibility of reduced dosing schedule or the need for boosters [305]. MRNA do have a big limitation as seen in with COVID-19 vaccines is their storage requirements to be -80°C adding

complexity of delivering a vaccine to remote regions [306]. At present only one mRNA based vaccine BioNTech company targeting the *Pf*CSP is in Phase I clinical trials (NCT05581641) [307].

8.9.5 Erythrocytic vaccines

Blood-stage malaria is an attractive vaccine target due to the lifecycle causing disease and commonly death and immunity is therefore aimed at preventing merozoite invasion and thereby reducing clinical malaria and onward transmission. The concept of blood-stage malaria immunity has been known for several decades due to the passive transfer of purified IgG from African adults to African children can clear parasitaemia [308]. While the objective seems simple, several challenges have arisen for anti-merozoite vaccines: the brief time between erythrocyte bursting and re-invasion of hepatocytes, high degree of merozoite protein polymorphism, reduced invasion pathways and large concentrations of parasites [224].

Blood-stage candidates merozoite surface protein (MSP), a highly abundant surface protein and apical membrane antigen 1 (AMA1) involved in merozoite invasion of erythrocytes, have dominated blood stage malaria vaccinology [224]. The aim was to induce high antibody titres against these antigens that would inhibit merozoite invasion and induce antibody-dependent cellular inhibition [309]. Unfortunately both CHMI and field trials assessing these two candidates elicited no convincing efficacy [310] most likely due to high antigenic diversity [311].

New blood stage research thereby targeted invasion pathways rather than surface proteins which were deemed less polymorphic. One such complex is the remarkably conserved *P. falciparum* reticulocyte-binding protein homologue 5 (*Pf*RH5) which is essential for RBC alignment and invasion [312]. *Pf*RH5 showed neutralising antibodies in mouse studies and protection in macaque studies [313, 314]. Three doses of *Pf*RH5.1/AS01b administered in a delayed fractional dose regimen when compared to 3 monthly intervals led to reduction in parasite growth rate [315, 316].

8.9.6 Placental vaccines

Placental malaria vaccines target those antigens which enable the parasite to sequester in the capillaries of the placenta. Top vaccination targets for expressing chondroitin-A (CSA)-binding protein [224] and *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) [317]. NAI to CSA-binding parasites in the placenta have been associated with increased protection [318]. Furthermore, anti-*Pf*EMP1 antibodies can block parasite binding to CSA. A study in 2019 indicated vaccine PAMVAC expressing both *PF*EMP1 and CSA was immunogenic with functional antibodies and no safety concerns among participants within the study [319].

8.9.7 Transmission blocking vaccines

Transmission-blocking vaccines (TBV) create immune responses that target the sexual stage of the malarial life cycle. Antibodies targeting gametes or zygotes will kill/inhibit parasites before a mosquito meal therefore interrupting transmission through the vector [320]. The leading candidates *Pf*s230 and *Pf*s48/45 which are gamete surface proteins and *Pf*s25 and *Pf*s28 which are grouped as zygote surface proteins [321]. Many of these antigens are cysteine-rich or contain epidermal growth factors (EGF)-like domains making them difficult to produce a properly folded protein, leading to scaling up related issues should the antigens reach Phase III testing [224]. Standard or direct membrane feeding assays (SMFAs or DMFAs) to assess functional activity have been the gold standard to assess TBV activity and involve mosquitos feeding on gametocyte-infected blood and dissection approximately once per week for detection of oocysts in mosquito midgut [322].

*Pf*s25 has been the most closely studied TBV with its stable good manufacturing product (GMP) protein and high antibody titres with higher serum-blocking transmission activity compared to other antigens [321, 323]. *Pf*s230 induces antibodies with functional activity against *Pf* in the presence of

complement [324]. All TBV candidates mentioned above require an adjuvant to boost humoral and cellular responses for which nanoparticle adjuvants are being designed.

Trials to assess the safety and immunogenicity of TBV are easy to perform. Further, antibodies can be assessed for functional activity *in vitro*, although designing efficacy trials in malaria-endemic areas remains a challenge. Efficacy trials for TBV will require a cluster randomised designs to measure herd immunity that reduces human and mosquito rates of infection [325]. Such cluster randomised trials will need to be large at a community or regional level, will be complex and costly to perform and difficult to analyse [326].

8.9.8 Adjuvants

Adjuvants are commonly used in vaccination practices as they increase efficacy by acting as carriers, depots and stimulators of the immune system [327]. Due to increased immune responses, adjuvants can reduce the antigen dose used in a vaccine as well as the number of doses needed. Adjuvants can also improve the stability of the antigen thereby extending the vaccine's half-life and, ultimately, providing longer-lasting protection and immune response [328].

There are two types of adjuvants based on their mechanism of action. First, immunostimulatory adjuvants (e.g. mineral salts and polysaccharides) directly activate and recruit immune cells or innate responses to the site of vaccination. Second, are delivery adjuvants (emulsion and liposomes) which act through effective antigen presentation to the immune system [329]. Delivery adjuvants can activate PRR pathways involving PAMPs and TLR causing a downstream immune recruitment through cytokine and chemokine release.

One of the major drawbacks to vaccinating against malaria is the lack of long-lasting/ durable immune responses [240, 242] and therefore, many anti-malarial vaccines incorporate the use of adjuvants.

RTS,S administered with a single adjuvant - alum or monophosphoryl lipid (MPL) or oil-in-water emulsion did not result in protection even though a “strong” antibody response was reported [255]. However, administering RTS,S with a novel AS which used a combination of MPL and the saponin QS21 with in an oil/water emulsion induced humoral and cellular responses and improved efficacy [239, 330]. Saponin is likely the key contributor towards efficacy due to its ability to initiate a balanced Th1/ Th2 immune response [331]. Therefore, most vaccines targeting malaria are administered with a combination of immunostimulants (adjuvants) [242].

8.9.9 Monoclonals

Monoclonal antibodies (mAbs) have increasingly been funded in prophylactic malaria trials. A mAb is defined as a single antibody (Ab) that has been cloned from a single B cell and which will bind to a specific epitope on a pathogen [332]. mAbs used in clinical trials have an excellent safety profile since they are naturally occurring within the body and they are highly specific and can deplete or block receptor functions [333] with no off-target effects [334]. Unlike vaccines, they do not rely on the immune system for production and therefore should have reduced variability in different populations but may also be less effective with increased Fc receptor variability in different populations. Furthermore, mAbs may have a reduced half-life and will need constant boosters to maintain mAb concentrations with no natural immune memory. Lastly, mAbs are expensive to produce and may not be viable for lower-income countries as widely distributed therapeutic or prophylactic medication [333].

Nonetheless, monoclonal CIS43LS administered to malaria naïve participants resulted in protection nine months after receiving the prophylactic monoclonal [335]. The mAB targets the NPDP tetrapeptide on the CSP [336, 337] and consistent protection holds exciting opportunities for travellers as an alternative to prophylactic anti-malarial medication which report low compliance to dosing schedules and side effects [338] and for military personnel sent to malaria-endemic areas [339].

Monoclonals also offer exciting opportunities in seasonal transmission settings where protection is needed in a few months of the year [337].

8.10 Correlates of protection

A correlate of protection (CoP) is an immune function that statistically correlates with and may be biologically responsible for vaccine-induced efficacy [340]. A CoP can assist in selecting vaccine candidates for clinical trials, informing trial design and implementation, facilitating interpretation of trial data and aiding vaccine licensure [341]. In addition, CoP may be a useful tool to reduce the need for large efficacy trials which are expensive and may not be feasible or ethical [342] such as in difficult or dangerous locations [343]. Lastly, they can be used to test the consistency of vaccine production over time and their performance in a population [344].

8.10.1 Bradford Hill criteria to identify causation

Before one can identify a CoP, it is important to define differences between association, correlation and causation. Associations should not be confused with causation, if *X* results in *Y*, then the two are associated however it may not be causal and the association may be a result of: chance (random error), bias (systematic error), confounding, reverse causality and true causality. Associations show one variable provides information about another and a correlation provides information whether two variables display an increasing or decreasing trend [345].

The Bradford Hill Criteria are a set of nine principles that are widely used to help determine whether epidemiological or immunological associations are causal [346]. These are: strength of association, consistency, specificity, temporality, biological gradient, plausibility, coherence, experiment, and analogy [347, 348] and are summarised in Table 5 below.

Table 5 - Bradford Hill Criteria for assessing causation

Table adapted from Hill et al., 1965 **[348]**.

Term	Definition
Strength	A weak association does not mean that there is not a causal effect, though the stronger the association, the more likely that it is causal.
Consistency	Consistent or reproducible findings observed by different persons in different places with different samples strengthen the likelihood of an effect.
Specificity	The more specific an association between a factor and an effect is, the bigger the probability of a causal relationship.
Temporality	The effect must occur after the cause.
Biological gradient	Greater exposure should generally lead to a greater incidence of the effect.
Plausibility	A plausible mechanism between cause and effect is helpful.
Coherence	Coherence between epidemiological and laboratory findings increases the likelihood of an effect.
Experiment	Occasionally it is possible to appeal to experimental evidence.
Analogy	The use of analogies or similarities between the observed association and any other associations.

8.10.2 CoP terminology

Large body of literature with many authors having different definitions. This thesis will follow terms published in Plotkin *et al.*, 2012 [342] who collaborated with authors in the field to establish consistent terminology and nomenclature. They established a statistical relationship between an immune marker and protection but does not necessarily imply causality. They established two additional to describe a CoP based on their causality: a CoP, a mechanistic correlate of protection (mCoP) and non-mechanistic correlate of protection (nCoP). These terms are summarised in Table 6.

Table 6 - CoP terminology

Table 6 has been adapted from Plotkin., 2010 [344] and Plotkin et al., 2012 [342].

Term		Definition
Correlate (CoP)		An immune response that is statistically associated with protection.
	mCoP	a marker that is mechanistically and causally responsible for protection.
	nCoP	a marker that is not mechanistically causal of protection.

CoP is a protective immune response, however that immune response may only be accompanied (associated) with protection but is not causally responsible for it - nCoP. Conversely if the immune response is causally responsible for protection - mCoP. Identifying an mCoP is preferable over an nCoP, however nCoP can be valuable as well [342].

An example of these two types of CoP can be seen with immune responses to the meningococcal vaccine measured by bactericidal antibodies (functional assay measuring lysis of the bacterial cells by antibody and complement) or by ELISA. Bactericidal antibodies are shown to be responsible for efficacy [349] as infants with the lowest bactericidal antibodies have the increased incidence of disease [350]. Whereas antibodies measured by ELISA are induced by the vaccine and associated with protection but not causal. Therefore bactericidal antibodies are a CoP and an mCoP and ELISA antibodies are CoP but nCoP [342].

Vaccine-induced immune responses are often multifaceted. A single immune component is unlikely to be the whole source of protection but rather a combination of a variety of responses [351]. Therefor there may be more than one CoP for a particular disease and may be referred to as 'cocorrelates' [343, 352]. A CoP can differ depending on the endpoint used, such as protection from infection, from disease, from severe disease or mortality [351]. An example, a vaccine against smallpox elicits protection by antibodies but protection from disseminated disease is mediated by $CD4^+$ and $CD8^+$ T cells [353]. The precision of the CoP must be precise which is examined using confidence intervals (CIs). Wide CIs will lead to imprecise interpretation of data [354].

8.10.2.1 Sample size

Sample sizes are a critical for clinical trials assessing efficacy, however many VE studies are not powered to assess immune correlates [355]. Depending on the hypothesis, the sample size should not be too small and on the contrary not be too big. Too small may prevent findings being extrapolated

or assuming as true a false premise. Too big may amplify the detection of differences which may emphasise statistical differences that are not clinically relevant to the trial [356]. Therefore, when designing a study, sample size calculations are important for any findings found and in the absence of this calculation, data should be examined with caution.

8.10.3 CoP with malaria

At present, there is still no malaria vaccine which produces immune responses that consistently and robustly predict protection among different populations and age groups [162]. This can result from different populations responding differently because of genetic, age, or environmental factors [354]. With PEM vaccines, some indications suggest that increased antibody titres targeting the CSP are associated with increased protection [271, 357]. In addition, other humoral immunological markers, such as avidity especially towards the C-Terminus region of the CSP protein [358, 359] and subclass IgG1 and IgG3, are increased in protected individuals [360]. Lastly, increased NK cell activity [361] elevated CD4 T cells secreting IL-2 and IFNγ [362] after administration of RTS,S shown to CoP. Simply quantifying individual immune variables does not appear to provide a consistent correlation or association with protection across various studies, therefore new methods to assess CoP need to be identified.

A method to improve the identification of CoP in vaccine studies which incorporates individual (univariate variables) immune responses and assesses whether different combinations of univariate variables better correlate with protection is a multivariate analysis [363]. Vaccines such as RTS,S have reported a broad range of efficacies in different malaria settings, therefore multivariate analyses help to extract and identify immune markers of protection across studies [364]. In malaria vaccine studies, multivariate models have shown immune combinations such as increased IgG avidity to CSP and C term antigens that correlate to antibody effector functions (Fab and Fc). Furthermore, multivariate models discriminated monocyte phagocytosis, NK cell and DC activation to be associated with

protection [365]. Another study found CoP combinations involving antibody subclass (IgG1 and IgG3), and fine specificity and avidity (total serum antibody dissociation phase area under the curve for NANP6, CSP, NPNA3, and N-interface binding using multivariate models [366]. Therefore, a multivariate model can be useful in identifying protective mechanisms which showed no association with protection when assessed individually or when more than one variable is independently associated with protection.

Another approach is 'systems biology' which identifies changes in molecular immune signatures (such as changes in gene expression) immediately after vaccination that correlate with and can later be used predict protective adaptive immune responses [367, 368]. Suscovich *et al.*, 2020 [369] reported that a qualitative functional humoral feature Fc gamma receptor 3A (FCGR3A) which involves antibody mediated phagocytosis and engagement predicted protection across multiple RTS,S/AS01 studies. Enhancing Fc-dependant antibody mediated effector functions (FcγRIIa, FcγRIIIa, or complement) is likely to be a worthwhile strategy for future RTS,S and other malaria vaccine studies [370].

IgG1 and IgG3 are cytophilic antibodies that assist with complement fixation, opsonic phagocytosis, and high affinity Fc receptors [371, 372]. The high production cytophilic antibodies in infants provides a possible explanation for the high protection found. IgG2 has mixed reports of being associated with malaria protection [373]. IgG2 and IgG4 are generally non-cytophilic, with a low affinity for complement and have poor engagement with Fc receptors [87].

Furthermore, CHMI models offer an ideal opportunity to investigate CoP in humans as well as assess immunogenicity and dosing in early Phase 1 trials [374]. However, CHMI models that report high efficacy results, often fail to replicate results in the field likely due to heterogeneity in parasite strains [375]. Therefore, univariate association with protection in CHMI studies is not enough to avoid future efficacy or field studies. Further details on CHMI models can be viewed in 8.11.4.

8.11 Seasonal transmission of malaria.

In 2012, it was estimated that 39 million children under the age of 5 were living in areas of seasonal rainfall and therefore suitable for SMC to be administered providing a high level of protection [376, 377]. However, in areas with constant rainfall or even two rainy seasons in a year, modelling four doses of SMC was not found to be as effective [378].

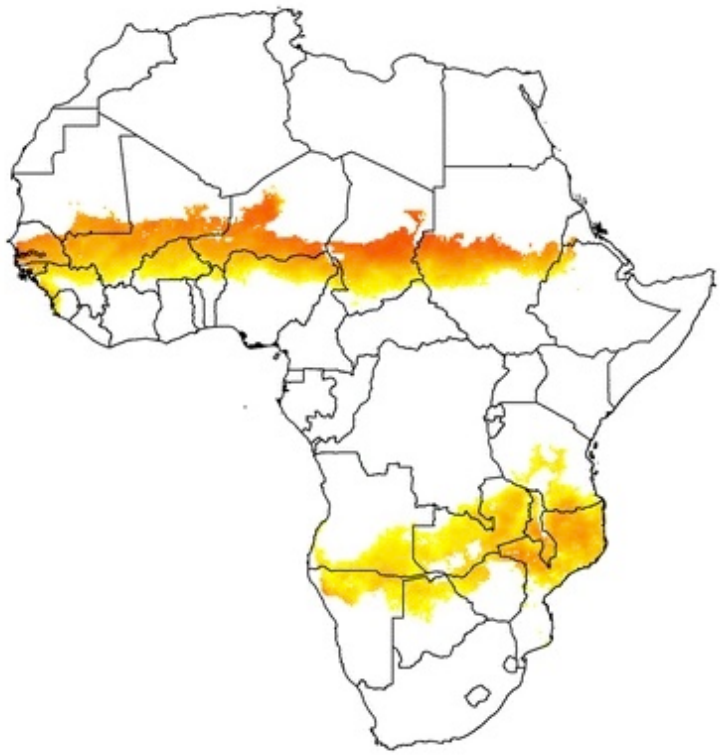


Figure 17 - Seasonal distribution of rainfall.

Areas in yellow indicate rainfall lasting ~3 months per year which are therefore ideal for seasonal SMC vaccination regimens

Figure taken from Greenwood et al., 2018 [379].

8.11.1 Seasonal Malaria Chemoprevention

The WHO has recommended the use of SMC for certain age groups since 2012 and there is growing evidence that it is highly effective [95, 380]. SMC involves administering 4 doses of sulfadoxine-pyrimethamine plus amodiaquine at monthly intervals [380] starting at the beginning of the rainy

season or malaria transmission season [381]. WHO has recommended SMC in areas in sub-Saharan Africa with periodic rainfall highlighted in yellow in Figure 17.

The downsides to SMC are logistical issues such as the very short half-life of anti-malarial drugs, completeness of effective delivery in rural areas and that a minimum of four doses of SMC requires administration monthly (as recommended by the WHO) [381], meaning travel challenges for rural families [379]. In addition, while there is no evidence to date that SMC has shown resistance [380], mass drug administration does risk potential resistance which occurred with artemisinin and piperaquine [141, 142, 382]. There is also a growing problem that by restricting mass deployment of SMC to children under the age of five, there is a risk of severe malaria in older children who no longer qualify [379, 383]. A study in Senegal indicates that continuing SMC prophylaxis in children up to age ten can be beneficial and results in a herd effect in both adults and children not on medication at all [384].

8.11.2 Dry season vs all-year-round transmission.

Another question relating to highly seasonal malaria areas relates to how the parasite persists in the dry season when all potential mosquito breeding grounds run dry. Almost no cases were recorded in malaria-endemic areas between January and July (Figure 18) in Malian children and adults in 2017 and 2018 [385]. Malaria parasites survive the dry season by undergoing transcriptional changes and have less cytoadherence [386]. This results in decreased immune evasion techniques, increased red blood cell clearance by the host spleen and a persistent low level of infection in adults [385, 387]. By undergoing transcriptional and phenotypic changes, the parasite can survive 6-9 months of the dry season [388], however, low levels of parasitaemia commonly associated in asymptomatic infections does not result increased blood stage immunity [389].

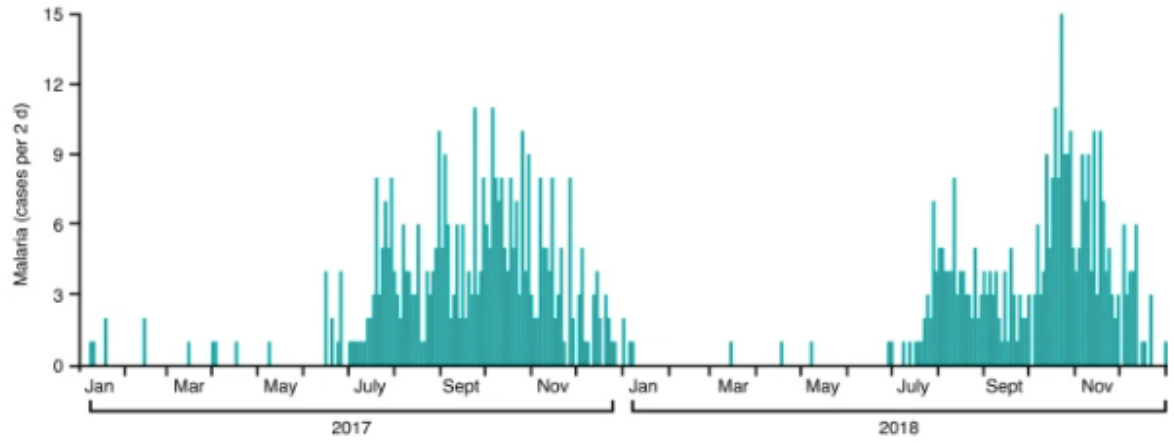


Figure 18 – Seasonal malaria in Mali

Number of malaria cases in Mali between 2017 and 2018. The X-axis indicates months of the year over 2017 and 2018. Y-axis represents the frequency of clinical malaria in a cohort of ~600 individuals aged 3 months to 45 years measured every 2 days over a 2-year period

Figure taken from Adrage et al., 2020 [385]

8.11.3 Rebound (resurgent) and delayed malaria

Individuals living in malaria-endemic regions will acquire some NAI to malaria over time through repeated infection. Introducing malaria interventions can impact the level of NAI in a population and therefore it is important to understand how the removal of interventions can impact a population. Two main epidemiological scenarios can occur following the removal of an intervention: Rebound (resurgent) and delayed malaria.

Rebound (resurgent) malaria is when an effective intervention or interventions are introduced into a community which already has some NAI and results in a marked reduction in malaria incidence. This then reverts to the previous level in a short period after the interventions are withdrawn. Therefore, malaria incidence is said to 'rebound' to its previous level because the population loses its NAI during the intervention period.

Delayed malaria describes a situation when young children or infants who have not acquired NAI are provided with an intervention which is then withdrawn, for example, SMC is given from 3 months to five years of age and then withdrawn [390]. This is often followed by an increase in malaria which would have occurred earlier in life had the children not received the intervention. Therefore, cases needed to induce effective NAI are 'delayed' often resulting in worse outcomes than if the intervention was not introduced in the first place [383]. Delayed malaria can also negatively impact VE over time when NAI in the control group overtakes protective immunity in the vaccine group thus creating negative VE [391]. This suggests that other control measures need to be maintained in the post-intervention period, particularly in areas with high transmission.

8.11.4 CHMI models

The application of controlled human infection (CHI) models whereby deliberately infecting a healthy person with the pathogen of interest is a promising approach for the acceleration of a vaccine [392]. CHI allows for testing vaccine effectiveness for diseases with low transmission or where there are sporadic outbreaks. A CHI must resemble the disease, participants need to be uniform, a control group must be used and a sufficient sample size to test the hypothesis is required [393].

For malaria, controlled human malaria infection (CHMI) models can be undertaken involving the inoculation of sporozoites (Spz) via mosquito bite or by direct venous injection (DVI) of Spz or administering *Pf*-infected blood [375]. CHMI facilitates testing VE without large and expensive clinical trials in malaria-endemic areas. CHMI is only possible due to extensive knowledge of parasite biology as well as having established and effective treatment [394]. The most common strains used in CHMI are the *Pf* NF54 (West African origin), 3D7 (clonal line from NF54, 7G8 - Brazilian isolate), HMP01 (isolate from Ghana) and NF135.C10 (Cambodian origin) [395]. CHMI can also be used to assess and model NAI in individuals living in different malaria transmission areas [396, 397]. However, CHMI is not perfect as it involves infection through only one *Pf* strain whereas there are antigenically diverse

heterologous parasites in the field [398]. In addition, DVI does not accurately reflect natural infection (NF), as the dermis of the skin is critical for the establishment of infection and induction of the host immune response [108, 374].

9 Thesis aims

- To characterise immune responses of different dose regimens of R21 and Matrix M in adults, young children, and infants in different malaria transmission regions.
- To report the effectiveness and/or necessity of a booster dose of R21 and Matrix M to maintain immunogenicity and efficacy in adults, young children, and infants in different malaria transmission regions.
- To identify immune responses that are associated with protection in malaria naïve adults.
- To identify immune responses that are associated with protection in infants and young children in highly seasonal malaria transmission areas.
- Characterise and identify immune responses after administration of R21 and RTS,S in children aged 5–17-months in Burkina Faso.

10 Methods and Trial Designs

10.1 Materials

All materials and reagents used in immunology assays can be found in the Appendix.

10.2 Immunological Methods

All clinical and immunological methods used in this thesis can be viewed in the Appendix.

10.3 Vaccine Trials assessed in this thesis

- **VAC053**: a Phase I clinical trial to assess safety and immunogenicity in different dose regimens of $R21_{CBF}$ with or without adjuvant Matrix M in UK adults – see Table 9 for trial design.
- **VAC060**: a Phase Ib single-blinded clinical trial to assess safety and immunogenicity after immunisation of $R21_{CBF}$ and MM in Burkina Faso – see Table 10 for trial design.
- **VAC065**: a Phase I/IIa first human challenge clinical trial to assess safety, immunogenicity and efficacy of participants immunised with different dose regimens of $R21_{CBF}$ and MM in healthy adults in the UK - see Table 11 for trial design.
- **VAC072**: a second Phase I/IIa sporozoite challenge clinical trial to further assess safety, immunogenicity and efficacy using different dose regiments of adjuvanted $R21_{SII}$ and MM in healthy adults in the UK – see Table 12 for trial design.
- **VAC073**: a Phase I, age de-escalation dose escalation clinical trial assessing the safety and immunogenicity of $R21_{SII}$ and MM in adults, young children, and infants in Kenya – see Table 13 for trial design.
- **VAC076**: a Phase IIb double-blinded randomised control clinical trial assessing the safety, immunogenicity, and efficacy of $R21_{SII}$ and MM in 5–17-month-old infants in Nanoro, Burkina Faso – see Table 14 for trial design.
- **MAL099**: a Phase III trial of seasonal vaccination with the RTS,S/$AS01_E$ vaccine, SMC and the combination of vaccination and chemoprevention – see Table 15 for trial design.

10.3.1 Trial locations

Data presented in this thesis is obtained from trials conducted in three locations: the UK, Kenya, and Burkina Faso - Figure 19. The sites examine the effect of R21/MM in different age groups and different malaria transmission areas.

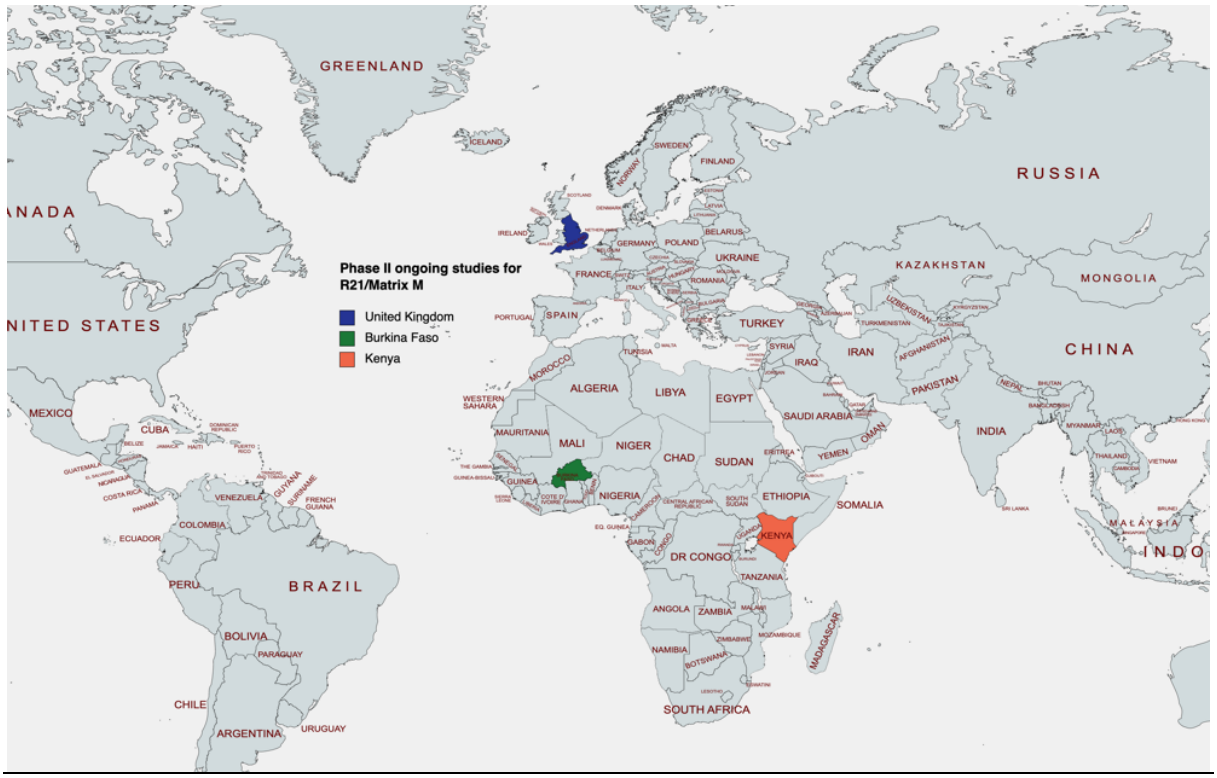


Figure 19 - Phase I/II trial sites of R21 and Matrix M examined in this thesis.

Blue: United Kingdom – VAC053 (adults), VAC065 (adults) and VAC072 (adults). Green: Burkina Faso – VAC060 (adults) and VAC076 (infants). Red: Kenya – VAC073 (adults, young children, and infants)

VAC053, VAC065 and VAC072 were trialled in adults in the UK. The UK is classified as a malaria naïve population with no natural occurrence of malaria transmission, with the last recorded transmissible case of malaria in 1921 [399]. VAC073, was trialled in Kenya in the coastal region of Kilifi County. Kilifi is characterised by a moderate to high year-round transmission of *Pf* with an estimated entomological inoculation rate of 22 – 53 infective bites per person per year [400, 401]. VAC060 was trialled in Banfora - Burkina Faso along with VAC076 trialled in Nanoro - Burkina Faso. In both Burkina Faso sites, *Pf* is endemic throughout the year however there is a peak of cases in the rainy season (June to November) [402, 403]. A summary of all trials can be viewed in Table 7.

Table 7 - Summary of Clinical Trials Used in this thesis.

Trial Code	Country	Vaccine	Phase	Challenge?	Age (years)	Route	Number of volunteers	Objectives & EPs	Publications/ Timeline
VAC053	UK	R21, administered with & without Matrix-M1	I	N	Adults (18-50)	IM	34	Safety and Immunogenicity	Accepted in Lancet Microbe - [404]
VAC056	UK	R21 and AS01B	I	N	Adults 18-50	IM	20	Safety and immunogenicity	Manuscript in Progress
VAC060	Burkina Faso	R21 and Matrix M	I	N	Adults 18-45	IM	16	Safety and Immunogenicity	Accepted in Lancet Microbe - [404]
VAC065	UK	R21 and Matrix M & combo R21, ChAd63/MVA-ME-TRAP	IIa	Y	Adults 18-45	IM	65	Sporozoite Challenge - Safety, immunogenicity, and efficacy	Accepted to Lancet Microbe
VAC072	UK	R21 & Matrix M	IIa	Y	Adults 18-45	IM	72	Sporozoite Challenge - Safety, immunogenicity, and efficacy	Manuscript in Progress
VAC073	Kenya	R21 and Matrix M	Ib	N	Adults (18-45), children(1-5yrs), infants (5-12months)	IM	90	Dose escalation, age de-escalation,	Published in Welcome open - [1]
VAC076	Burkina Faso	R21 MM	IIb	Field Efficacy Trial	Infants (5-17months)	IM	450	Safety, immunogenicity, and field efficacy	Year 1 – [2] Year 2 – [3] Year 3/4 – Manuscript in Progress
MAL099	Burkina Faso and Mali	RTS,S/AS01$_E$	IIIb	Field Efficacy Trial	Infants	IM	6000	Safety, immunogenicity, and field efficacy	Year 1-3 - [275] Year 4-5 - [274]

10.3.2 Clinical trial regulatory approvals

All studies included in this thesis were conducted in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guidelines, Good Clinical Practice (GCP), and the Declaration of Helsinki (2008). UK studies were also performed in accordance with the UK Genetically Modified Organisms Regulations (2000). Written informed consent was provided by all volunteers or a responsible parent. Each study was approved by the appropriate regulatory authorities and registered with ClinicalTrials.gov. Individual approval numbers and ethics reviews can be viewed in Table 8.

Table 8 - Clinical Trial Regulatory Approvals

N/A – not available

Trial Code	Trial Location	UK Ethical committee	Local regulatory board (where trial is outside of the UK)	NCT number
VAC053	UK	REC - 15/SC/0386	N/A	NCT02572388
VAC056	UK	REC - 15/LO/1353	N/A	NCT02600975
VAC060	Burkina Faso	OXTREC 36-15	Agence Nationale De Regulation Pharmaceutique - CERS 2014-10-118	NCT02925403
VAC065	UK	REC - 16/SC/0261	N/A	NCT02905019
VAC072	UK	REC - 18/SC/0679	N/A	NCT03970993
VAC073	Kenya	OXTREC 6-18	Pharmacy and poisons board (PBB) - PPB/ECCT/18/12/01/2019(046)	NCT03580824
VAC076	Burkina Faso	OXTREC 19-19	Agence Nationale De Regulation Pharmaceutique - N2019 237/MG/SG/ANRP/H1H1	NCT03896724
MAL099	Burkina Faso & Mali	N/A	N/A	NCT04319380

10.3.3 R21 vaccine formulations

Two different formulations of R21 were examined. Full details of each R21 formulation are detailed in the Appendix (0 - Investigational products).

- $R21_{CBF}$ – manufactured in the Clinical Biomanufacturing Facility (CBF) in Oxford.
- $R21_{SII}$ – manufactured in SII

10.3.4 Trial Designs and Immunology bleed schedules

10.3.4.1 VAC053 – UK adults Phase I

Table 9 – VAC053 Trial Design

UK Adults – 18-45 years of age

Week	0	4	8
Group 1 (n=11) Adults	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM
Group 2 (n=4) Adults	50µgR21	50µgR21	50µgR21
Group 3 (n=10) Adults	50µgR21 / 50µgMM	50µgR21 / 50µgMM	50µgR21 / 50µgMM
Group 4 (n=6) Adults	2µgR21 / 50µgMM	2µgR21 / 50µgMM	2µgR21 / 50µgMM

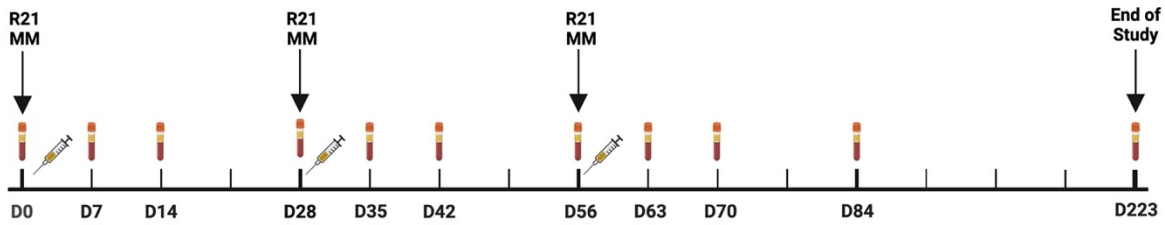


Figure 20 - VAC053 Immunology bleed schedule

Syringes represent time points where immunisations were administered. Vials represent time points where Plasma, Serum and PBMCs were taken for Immunogenicity assessment.

Figure made by D.G. Bellamy and designed in Biorender.

In VAC053, 31 participants were recruited into four groups - Table 9. Group 1 (G1) received three doses of 10µg $R21_{CBF}$ and 50µg MM administered one month apart (Figure 18). G2 received three doses of 50µg $R21_{CBF}$ with no adjuvant administered one month apart. G3 received three doses of 50µg $R21_{CBF}$ and 50µg MM administered one month apart. G4 received three doses of 2µg $R21_{CBF}$ and 50µg MM administered one month apart. Participants were followed up one month post post-third dose (D84) and six months post post-third dose (D236). Full bleed immunology schedules can be viewed in Figure 20.

10.3.4.2 VAC060 – Burkinabe adults

Table 10 – VAC060 Trial Design

Adults – 18-45 years of age

Week	0	4	8
Group 1 (n=11) Adults	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM
Group 2 (n=5) Adults	Controls Saline Placebo	Controls Saline Placebo	Controls Saline Placebo

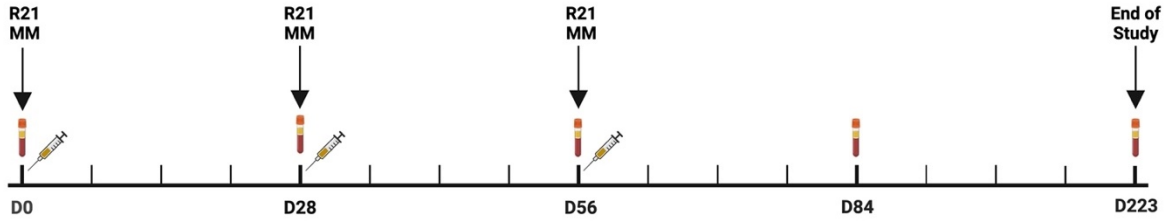


Figure 21 – VAC060 Immunology bleed schedule

Syringes represent time points where immunisations were administered. Vials represent time points where Plasma, Serum and PBMCs were taken for Immunogenicity assessment.

Figure made by D.G. Bellamy and designed in Biorender.

In VAC060, 15 adult participants were recruited and assigned into two groups - Table 10. 11. Participants in G1 received three doses of 10µg $R21_{CBF}$ and 50µg MM administered one month apart with the second group (G2) acting as the control receiving three doses of a saline placebo. Plasma, serum and PBMC were taken every 28 days during the first two vaccinations and taken every 7 days up until a month after the third dose, full bleed immunology schedules can be viewed in Figure 21.

10.3.4.3 VAC065 – UK adults

Table 11 - VAC065 Trial Design

Adults – 18-45 years of age. CHMI – controlled human malaria infection.

Group 1, *2*, *3* & 4a - *CHMI-A.* Group 5, 6, 7, 4b and G1rc (Re-challenge) – CHMI B

Week	0	1	4	8	9	12	45	78
Group 1 (n=13) Adults	10μgR21 / 50μgMM		10μgR21 / 50μgMM	10μgR21 / 50μgMM		CHMI - A	Repeat CHMI - B	
Group 2 (n=12) Adults	50μgR21 / 50μgMM		50μgR21 / 50μgMM	10μgR21 / 50μgMM		CHMI - A		
Group 3 (n=12) Adults	10μgR21 / 50μgMM	ChAd63 ME-TRAP	10μgR21 / 50μgMM	10μgR21 / 50μgMM	MVA ME-TRAP	CHMI - A		
Group 4a (n=6) Adults	Controls					CHMI - A		
Group 4b (n=6) Adults	Controls					CHMI - B		
Group 5 (n=8) Adults	10μgR21 / 50μgMM		10μgR21 / 50μgMM	2μgR21 / 50μgMM		CHMI - B		
Group 6 (n=2) Adults	10μgR21 / 50μgMM		10μgR21 / 50μgMM	10μgR21 / 50μgMM				CHMI -B
Group 7 (n=7) Adults			10μgR21 / 50μgMM	10μgR21 / 50μgMM		CHMI - B		

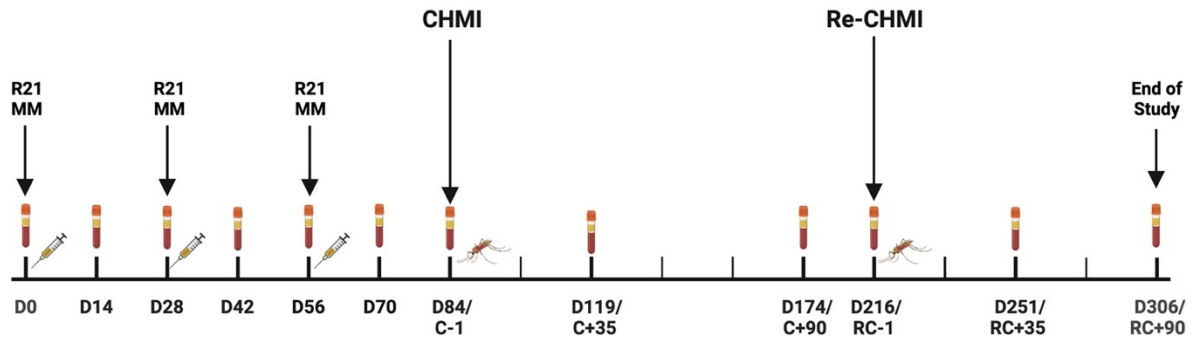


Figure 22 – VAC065 Immunology bleed schedule

Syringes represent time points where immunisations were administered. Vials represent time points where Plasma, Serum and PBMCs were taken for Immunogenicity assessment. Mosquitos indicate the first and second CHMI.

Figure made by D.G. Bellamy and designed in Biorender.

In VAC065, 65 UK adults were recruited in the $R21_{CBF}$ CHMI challenge study – Table 11, and participants were split across two CHMI's – challenge A and challenge B. Challenge A: 42 adult participants were divided into four groups. G1 received 10μg $R21_{CBF}$/50μg MM and G2 received 50μg $R21_{CBF}$/50μg MM,

both administered one month apart. G3 received the same dosing schedule as G1 (10µg $R21_{CBF}$/50µg MM), however, one week after the first dose viral vector, ChAd63 encoding ME-TRAP was administered and one week after the second dose of R21/MM viral vector MVA encoding ME-TRAP was administered. G4a acted as challenge control during CHMI-A. All vaccinated participants were challenged by CHMI one month post-third vaccination of R21/MM. Full immunology bleed schedules can be viewed in Figure 22.

Challenge B: a further 23 adults were recruited to assess safety, immunogenicity, and efficacy on further dose regimens of R21/MM. Participants in G5 received two doses of 10µg R21 and 50µg MM with the third dose being reduced by a fifth to 2µg $R21_{CBF}$ and 50µg MM to assess the effect of a fractional third dose. G6 included two participants from G1 in VAC053 who received a 10µg dose of R21/MM but were recruited to be challenged in VAC065, therefore, are classified as delayed challenges. Finally, 7 participants were recruited into G7 to assess whether immunogenicity and efficacy could be achieved or maintained with two doses of 10µg $R21_{CBF}$/50µg MM administered one month apart and challenged one month after the second dose. 6 unvaccinated participants were recruited to act as controls for challenge B. Lastly, 4 participants who were sterilely protected in G1 of challenge A were invited to be re-challenged to assess the long-term immunogenicity and efficacy of R21/MM.

10.3.4.4 VAC072 – UK adults CHMI

Table 12 - VAC072 Trial design

Adults – 18-45 years of age. CHMI – controlled human malaria infection.

Groups 1 & 5 were not challenged. Group 2 & 3 - challenge A. Boosted participants in groups 2 & 3 and vaccinated participants in group 4 – challenge B.

Week	**0** **Dose 1**	**4** **Dose 2**	**8** **Dose 3**			
Group 1a (n=10) Adults	10µg R21 / 50µg MM	10µg R21 / 50µg MM	10µg R21 / 50µg MM			
Week	**0** **Dose 1**	**4** **Dose 2**	**24** **Dose 3**	**28**	***56-80*** ***Boost 1***	**60-84**
Group 2 (n=12) Adults	10µg R21 / 50µg MM	10µg R21 / 50µg MM	10µg R21 / 50µg MM	CHMI - A	10µg R21 / 50µg MM	Repeat CHMI* - B
Week	**0** **Dose 1**	**4** **Dose 2**	**8** **Dose 3**	**12**	***40-64*** ***Boost 1***	**44-68**
Group 3 (n=16) Adults	10µg R21 / 50µg MM	10µg R21 / 50µg MM	10µg R21 / 50µg MM	CHMI - A	10µg R21 / 50µg MM	Repeat CHMI* - B
Week				**0**		**0**
Group 6 (n=6)				CHMI -A		
Group 7 (n=6)						CHMI - B
Week	**0** **Dose 1**	**4** **Dose 2**	**24** **Dose 3**			***28***
Group 4a (n=10)	50µg R21 / 50µg MM	50µg R21 / 50µg MM	10µg R21 / 50µg MM			*CHMI -B*
Week	**0** **Dose 1**	**4** **Dose 2**	**24** **Dose 3**			***28***
Group 5 (n=7)	10µg R21 / 50µg MM	10µg R21 / 50µg MM	2µg R21 / 50µg MM			

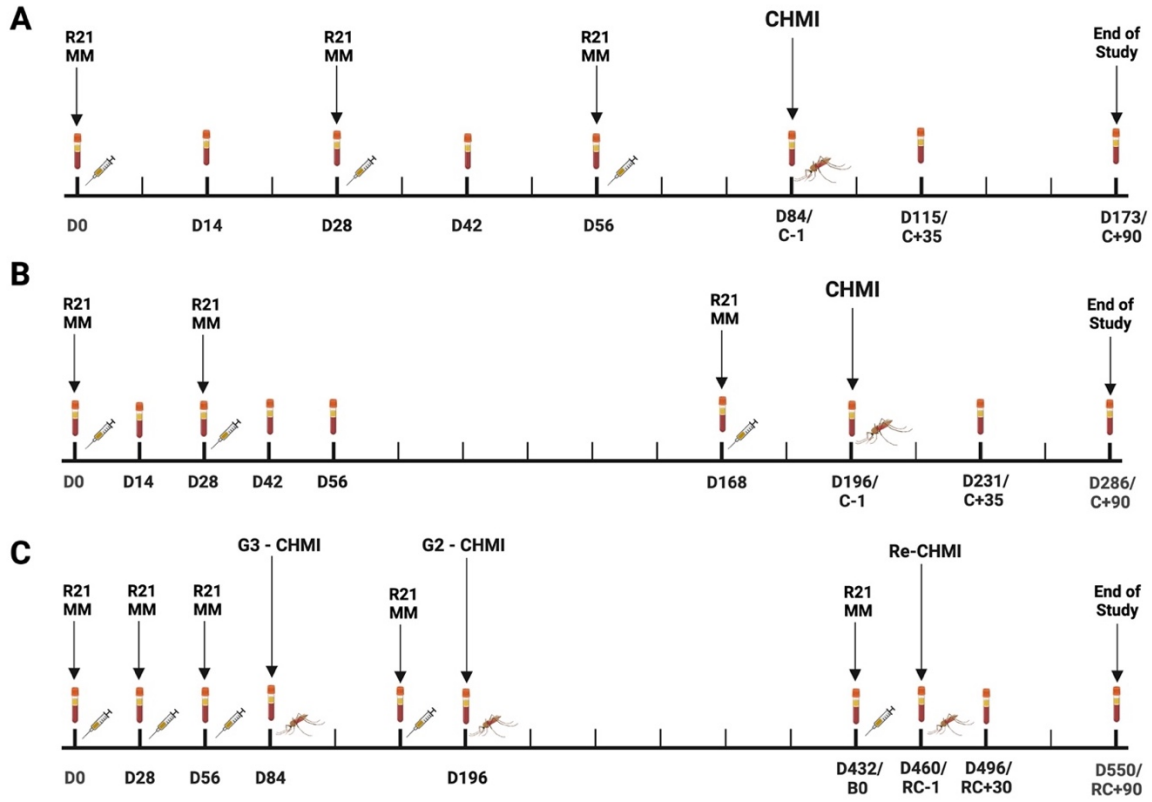


Figure 23 - VAC072 Immunology Bleed Schedule

A) *Immunology Bleed Schedule for participants in G1 and G3 who received the standard dose regimen.* ***B)*** *Immunology Bleed Schedule for participants in G2, G4 and G5 who received the delayed and the delayed fractional third dose regimen.* ***C)*** *Immunology Bleed schedule for participants in G2 and G3 who consented to receive a boost and undergo Re-CHMI.*

Syringes represent time points where immunisations were administered. Vials represent time points where Plasma, Serum and PBMCs were taken for Immunogenicity assessment. Mosquitos indicate the first and second CHMI.

Figure made by D.G. Bellamy and designed in Biorender.

In VAC072, the aim was to assess the effects of the standard three-dose regimen of 10µg $R21_{SII}$ with adjuvant Matrix M administered one month apart with a delayed third dose and a fractionating third dose. 67 participants were recruited and assigned to one of seven groups and split across two challenges labelled Challenge A and Challenge B - Table 12.

G1 and G3 received the standard 10µg $R21_{SII}$/50µg MM administered one month apart and G3 was challenged by CHMI one month post-third dose. G2 received two doses of R21/MM one month apart

with the third dose delayed by five months then challenged one month later. G2 and G3 were challenged in January 2020 (challenge A) - Figure 23A/B.

Additionally, G5 received a smaller delayed fractional third dose given at six months (10, 10, 2µg $R21_{SII}$) and G4 with higher delayed fractional third dose (50, 50, 10µg $R21_{SII}$) were investigated. Three participants from G2 and six from G3 from challenge A were recruited back, boosted with 10µg $R21_{SII}$ /50µg MM and re-challenged (challenge B) in September 2020 - Figure 23C.

10.3.4.5 VAC073 – Kenyan adults, young children and infants

Table 13 - VAC073 Trial Design

Adults - >18+ years of age. Young children – 1-5 years of age. Infants – 5-17 months of age. Length of Trial two years. All participants are to receive 4 doses of R21/MM.

Primary Series – 3 doses of R21/MM.

Boost – a single dose of R21/MM administered approximately 1 year after dose 3 of the primary series.

Week	0 (Year 1) Primary Series Dose 1	4 (Year 1) Primary Series Dose 2	8 (Year 1) Primary Series Dose 3	60 (Year 2) Boost
Group 1A (n=5) Adults	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM
Group 1B (n=15) Adults	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM
Group 2A (n=3) Young children	5µgR21 / 25µgMM	5µgR21 / 25µgMM	5µgR21 / 25µgMM	5µgR21 / 25µgMM
Group 2B (n=17) Young children	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM
Group 3A (n=3) Infants	5µgR21 / 25µgMM	5µgR21 / 25µgMM	5µgR21 / 25µgMM	5µgR21 / 25µgMM
Group 3C (n=15) Infants	5µgR21 / 25µgMM	5µgR21 / 25µgMM	5µgR21 / 25µgMM	5µgR21 / 25µgMM
Group 3B (n=3) Infants	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM
Group 3D (n=15) Infants	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM	10µgR21 / 50µgMM
Group 3E (n=15) Infants	5µgR21 / 50µgMM	5µgR21 / 50µgMM	5µgR21 / 50µgMM	5µgR21 / 50µgMM

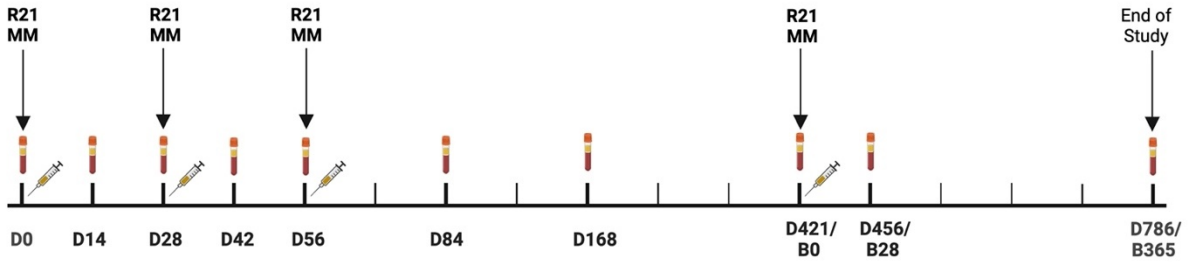


Figure 24 – VAC073 bleed schedule

Syringes represent time points where immunisations were administered. Vials represent time points where plasma, serum and PBMCs were taken for immunogenicity assessment.

Figure made by D.G. Bellamy and designed in Biorender.

In VAC073, each age category was assigned a specific group - Table 13. Adults were the first to be vaccinated with 10µg $R21_{SII}$/50µg MM. After finding no safety concerns in adults, three children in G2 were vaccinated with a half dose of 5µg $R21_{SII}$/25µg MM (G2a). Identifying no safety concerns, a further seventeen young children were vaccinated with a full dose of 10µg $R21_{SII}$/50µg MM (G2b). Finally, three infants were vaccinated with a half dose of 5µg $R21_{SII}$/25µg MM (G3a/c) followed by a further three infants with a full dose of 10µg $R21_{SII}$/50µg MM (G3b/d). A further three subsets had mixed R21/MM dose regimens of 5µg $R21_{SII}$/50µg MM (G3e). After identifying no safety concerns, a further fifteen infants were immunised in each G3 subset resulting in a total of fifty infants in VAC073. Full immunology bleed schedules can be viewed in Figure 24.

10.3.4.6 VAC076 – Burkinabe infants

Table 14 – VAC076 Trial Design

Year 1 (Primary Series) – Group 1 – 5µg R21 / 25µgMM. Group 2 – 5µg R21 50µg MM. G3 – Control vaccine (Rabivax).

Year 2 (First Booster) – Group 1 – 5µg R21 / 25µgMM. Group 2 – 5µg R21 50µg MM. G3 – Control vaccine (Rabivax).

Year 3 (Second Booster) – G1a & 2a – 5µg R21 / 50µg MM. G1b & 2b – Control vaccine (Rabivax). G3 – Control vaccine (Rabivax).

Year 4 (Third Booster) – G1aa & 2aa – 5µg R21 / 50µg MM. G1ab & 2ab – Control vaccine (Rabivax). G1bb & 2bb – Control vaccine (Rabivax). G3 – Control vaccine (Rabivax).

<table>
<tr><th>Week</th><th>0
(Year 1)
Primary Series</th><th>4
(Year 1)
Primary Series</th><th>8
(Year 1)
Primary Series</th><th>60
(Year 2)
First Booster</th><th>112
(Year 3)
Second Booster</th><th>164
(Year 4)
Third Booster</th></tr>
<tr><td rowspan="3">Group 1 (n=150)</td><td rowspan="3">5µgR21 /
25µgMM
G1</td><td rowspan="3">5µgR21 /
25µgMM
G1</td><td rowspan="3">5µgR21 /
25µgMM
G1</td><td rowspan="3">5µgR21 /
25µgMM
G1</td><td rowspan="2">5µgR21 /
50µgMM
G1a</td><td>5µgR21 /
50µgMM
G1aa</td></tr>
<tr><td>Control Vaccine
G1ab</td></tr>
<tr><td>Control Vaccine
G1b</td><td>Control Vaccine
G1b</td></tr>
<tr><td rowspan="3">Group 2 (n=150)</td><td rowspan="3">5µgR21 /
50µgMM
G2</td><td rowspan="3">5µgR21 /
50µgMM
G2</td><td rowspan="3">5µgR21 /
50µgMM
G2</td><td rowspan="3">5µgR21 /
50µgMM
G2</td><td rowspan="2">5µgR21 /
50µgMM
G2a</td><td>5µgR21 /
50µgMM
G2aa</td></tr>
<tr><td>Control Vaccine
G2ab</td></tr>
<tr><td>Control Vaccine
G2b</td><td>Control Vaccine
G2bb</td></tr>
<tr><td>Group 3 (n=150)</td><td>Control Vaccine</td><td>Control Vaccine</td><td>Control Vaccine</td><td>Control Vaccine</td><td>Control Vaccine</td><td>Control Vaccine</td></tr>
</table>

VAC076 was initially designed to assess safety, immunogenicity, and efficacy over a two-year period, with a primary series of three doses of $R21_{SII}$ and Matrix M and a booster dose administered one year later. After initial results revealed a high level of efficacy after the primary series and even higher efficacy after the booster, the trial was extended for a further two years to assess the effectiveness of yearly boosters up to year 4.

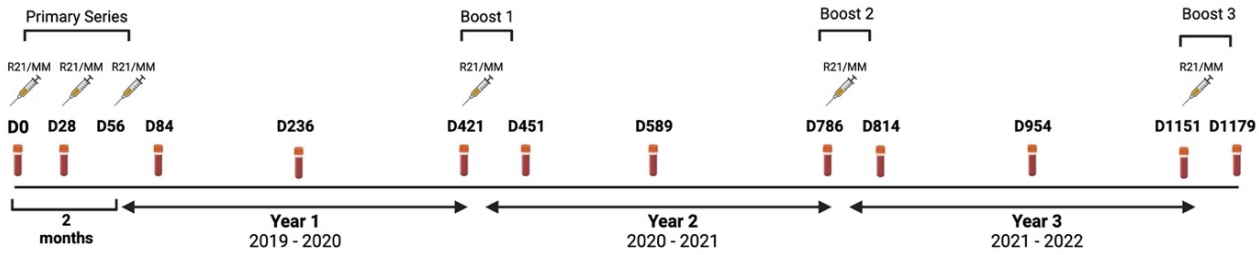


Figure 25 – VAC076 Trial Design

Trial Design covers all time points presented in this chapter from baseline at D0 to 1-month post-third boost at D1179/. Syringes represent immunisation time points. Blood tubes represent immunology bleeds. Sigmoidal curves are a representation of malaria transmission for each year of the trial with high transmission season occurring between August and December.

Figure designed by D.G Bellamy in Biorender.

Trial design with vaccination and immunology bleed schedules can be viewed in Table 14 and Figure 25. Blood that was taken for immunology in the first year (2019) was taken at D0, D28 and D84 during the primary series. Thereafter, blood was taken six (D236) and twelve (D421) months post post-third dose.

After the first year, a booster dose of R21/MM was administered at D421 (B0) and an immunology bleed was taken one month post-boost (1B+30 / D456). Follow-up immunology bleeds were taken at six- and twelve months post-boost. A second and third boost was administered in years 3 and 4, following the same immunology bleed schedules as in the year after the first boost.

10.3.4.7 MAL099 trial design – RTS,S + SMC Trial Design

Table 15 - MAL099 - RTS,S + SMC Trial Design

*SMC. *SMC or placebo will be given at monthly intervals on four occasions during the malaria transmission season.*

Table adapted from Chandramohan et al., 2020 [405].

Year	**Group 1** RTS,S/AS01$_E$ alone	**Group 2** SMC alone	**Group 3** RTS,S/AS01$_E$ + SMC
Year 1 - 2017			
April – June – July	RTS,S/AS01$_E$ x3	Rabies x3	RTS,S/AS01$_E$ x3
July – August – September - October	SMC* Placebo x4	SMC* x4	SMC* x4
Year 2 - 2018			
June	RTS,S/AS01$_E$ x1	Rabies x1	RTS,S/AS01$_E$ x1
July – August – September - October	SMC* Placebo x4	SMC* x4	SMC* x4
Year 3 - 2019			
June	RTS,S/AS01$_E$ x1	Rabies x1	RTS,S/AS01$_E$ x1
July – August – September - October	SMC* Placebo x4	SMC* x4	SMC* x4

MAL099 is a phase IIIb trial which enrolled 5920 infants aged 5-17 months across Mali and Burkina Faso [405]. Infants were randomised into three groups, the first group (G1) received three priming doses of RTS,S/AS01$_E$ and then received four placebo doses. G2 (control group) received three doses of rabies vaccine followed by four cycles of SMC (SP + SQ). G3 (combined group), received three primary doses in 2017 of RTS,S/AS01$_E$ one month apart followed by four cycles of SMC (SP + SQ) Table 15. Infants in each group were boosted at yearly intervals according to their assigned group.

Table 16 - MAL099 - Immunology sample numbers

Number of infant samples taken from each site for immunology assessment. Primary Series indicates 3 doses of RTS,S AS01E or rabies administered in April, May and June in 2017. The first boost indicates a single dose of RTS,S/AS01$_E$ or rabies vaccine administered in June 2018. The second boost indicates a single dose of RTS,S/AS01$_E$ or rabies vaccine administered in June 2019.

Year	Burkina Faso	Mali	Combined
2017			
Pre-primary series	104	114	213
Post-primary series	104	114	213
2018			
Pre-first boost	132	147	279
Post-first boost	132	147	279
2019			
Pre-second boost	135	156	291
Post-second boost	135	156	291

Blood samples for immunology and serological assays were collected before and one month after the three priming doses for 213 infants in 2017, 279 infants before and one month after the first booster dose in 2018 and 291 infants before and one month after the second booster dose in 2019 - Table 16.

To avoid repeated venous blood sampling from the same child over a period of five years, a cohort of different infants was randomly selected each year for serology. Each cohort included approximately equal numbers who had received RTS,S/AS01$_E$ with or without SMC (groups G1 and G3) and a small number (10-20%) of controls who received a rabies vaccine with SMC (G2).

10.4 Analyses and Statistics

10.4.1 Analyses

Analytical plan and methodology are detailed in individual chapter methods.

10.4.2 Sample sizes

This sample size should allow an initial estimation to be made of the frequency and magnitude of outcome measures, rather than obtaining statistical significance for differences between groups. Group sizes reflect practical limitations on volunteer recruitment, ethical considerations limiting the number of volunteers that should receive a vaccine regimen without prior evidence of efficacy, and the desire to describe the efficacy within the study.

10.4.3 Power calculations

Studies presented in this thesis were not statistically powered to identify immunological correlates of protection. Sample sizes specific for efficacy are described in the corresponding chapter. Post-hoc power calculations were performed using a two-sample t-test to assess the power of current sample sizes and what sample size would be needed to provide power to identify associations between protected and unprotected individuals.

10.4.4 Statistical analyses

Data was tested for normal distribution by the D'Agostino-Pearson omnibus normality test. If the normality test passed, a parametric test was used and if the normality test failed, a non-parametric test was used. Medians were presented along with interquartile ranges (IQR) and geometric means were presented with 95% CIs. Most data was analysed using non-parametric methods due to the small sample sizes in the studies assessed.

A T-test or Mann-Whitney analysis was used to compare the differences arising between the two groups. A Kruskal-Wallis with Dunn's multiple comparisons post-test was used to compare responses across multiple groups at a given time point. Wilcoxon matched-pairs analysis was used to compare responses at two time points within a group. A Friedman-matched test with Dunn's multiple

comparisons was used to compare responses at three or more time points within a group. Spearman's rank was calculated for correlations. $P < 0.05$ was considered significant and all *P* values are two-tailed. Linear regression with line of best fit and 95% CIs (95% CI) was added to correlation plots. Analyses were performed in GraphPad Prism, version 10.0.

10.4.5 Multivariate analyses

In these analyses, protection will be the outcome variable and the log of the immune response as the independent variable. A univariate logistic regression analysis was performed to assess the association between immune response and protection. Results will be summarised as a list of all immune response variables together with their estimated odds ratios, 95% CI, and two-sided P-values. P-values are then corrected for multiple comparisons by estimating the false discovery rate using the Benjamini and Hochberg (B-H) method [406, 407]). q values (B-H) less than 0.20 will be considered as evidence of a significant association.

Secondly, the area under the receiver operating characteristic (AUROC) curve will be calculated for each variable. Immune variables with an AUROC between 0.80 and 0.90 will be considered good discriminators of high and low-risk infants while responses with AUROC greater than 0.90 will be considered excellent discriminators. An AUROC of 0.5 means it is no better than chance and an AUROC of 1.0 is a perfect discriminator. This would imply that the immune responses are higher in all protected participants than they are in all non-protected participants.

Thereafter, significant variables are added to a multivariate (forward stepwise logistic regression) model. The forward stepwise regression uses a sequence of steps to enter or leave a regression model individually based on inclusion or exclusion criteria. The significance level for removal (exclusion) from the model was set at 0.3 and the significance level for addition (inclusion) to the model was 0.25.

Chapter 1

11 Chapter 1 - Characterising immunogenicity of R21 and adjuvant Matrix M across Phase I/II trials in different malaria settings

11.1 Chapter 1 - Introduction

Malaria burden from *Plasmodium Falciparum* (*Pf*) has changed significantly over the past twenty years. Young children under the age of five have an increased risk of severe malaria and death and are therefore a target age group for a safe and efficacious malaria vaccine [408]. While children are the target population for malaria vaccine trials, a novel biological product must first be evaluated in adults who are able to give informed consent, show effectiveness and provide detailed safety reports before being evaluated in infants. Thus, age de-escalation dose escalation studies were conducted first [409].

Adults who live in high transmission areas have varying degrees of NAI [157]. Repeated malarial infections can have a profound impact on the immune system resulting in decreased memory B cell [195, 410] and TfH [198] function which impede the quality of antibody response [198]. Understanding pre-exposure will be valuable to further understanding of vaccine responses. Furthermore, individuals who present with blood-stage infection can have reduced vaccine response [411, 412]. Ensuring vaccinated participants are not infected will be essential for improved vaccine responses.

ISI assays aim to recreate malarial infection *in vitro* through transgenic *Pb* parasites expressing a copy of the antigen of interest from *Pf* [413] and assessing the functional ability of antibodies induced from vaccination to ISI. Other qualitative assays in use include cell traversal (CT) assays. Sporozoites traverse through several hepatocytes before committing to host cell invasion [414]. CT primes the parasite for

invasion, shown by knockout of traversal genes results in no blood stage infection. Secondly, CT releases hepatocyte growth factor making neighbouring hepatocytes susceptible to invasion [415]. CT is measured by the uptake of fluorescence dextran by traversed cells [416] or the release of calcein green (intracellular dye) by wounded cells [417, 418] and quantified by flow cytometry. Mouse studies using live imaging can visualise CT through the uptake of propidium iodide [419]. Antibodies targeting CSP can prevent invasion of CT making it a promising protein to target.

This chapter will prioritise antibody responses to NANP viewed as the immunodominant epitope of the CSP and is the primary immunological endpoint for R21 trials. Different dose concentrations and timing of dose regimens will be compared to assess peak antibody titres, durability, or the duration at which antibody titres can last before the need to administer a booster dose. Chapters 2 and 3 will examine broader exploratory immunology responses to other epitopes of the R21 vaccine.

11.2 Chapter 1 - Methods

11.2.1 Trial designs

Trials presented in this chapter and the trial designs can be found in the main methodology (10.3):

- VAC053 - Table 9 – VAC053 Trial Design
- VAC060 - Table 10 – VAC060 Trial Design
- VAC065 - Table 11 - VAC065 Trial Design
- VAC072 - Table 12 - VAC072 Trial design
- VAC073 - Table 13 - VAC073 Trial Design
- VAC076 - Table 14 – VAC076 Trial Design

11.2.2 Study Details

Full details of these studies may be found in the clinical trial reports listed in Table 7. All vaccinations were administered intramuscularly into the deltoid muscle of the arm. All humoral immunogenicity data was performed using plasma and cellular data from fresh PBMCs.

11.2.3 Sample sizes

Group sizes reflect practical limitations on volunteer recruitment and ethical considerations limiting the number of volunteers who should receive a vaccine regimen without prior evidence of efficacy.

11.2.4 Immunology and time points examined

The primary immunogenicity endpoint for R21 trials is total IgG antibody responses to the NANP repeat region of the CSP. In this chapter, immunogenicity across six Phase I/II trials, and IgG-specific antibodies towards NANP are presented at all timepoints. Indvidual antibody responses at D84 (1month post third dose – peak antibody response) from all groups, will be examined. A finding made in Rampling *et al*., 2018 [420] which saw antibody responses among RTS,S immunised adults peak at

D42 (2 weeks post second dose). Therefor D42 was also examined in this chapter with comparisons made to D84.

T cell responses measured by ELISPot to assess interferon-gamma (IFNγ) secretion after stimulation with CSP. ELISpot responses are only reported for UK trials on fresh PBMC, due to the assay only being validated for use in Oxford. Lastly, ISI assay to assess antibody function and the ability of vaccine-induced immunity to prevent sporozoite invasion *in vitro* is presented across all trials. Full details of each of these immunological methods are described in the appendix (17.5).

11.3 Chapter 1 - Objectives

- To assess total IgG responses to NANP from different dose regimens of R21 and adjuvant Matrix M in different age categories and different malaria settings.
- To assess cellular responses to CSP by IFNγ ELISpot among UK adults.
- To assess the functional ability of antibodies induced from immunisation of R21 and Matrix M to block sporozoite invasion *in vitro* from different age categories and different malaria settings.

11.4 Chapter 1 - Results

11.4.1 Humoral responses to dose regimens of R21/MM in different age groups and different malaria settings

11.4.1.1 VAC053 – R21/MM induces anti-NANP IgG titre

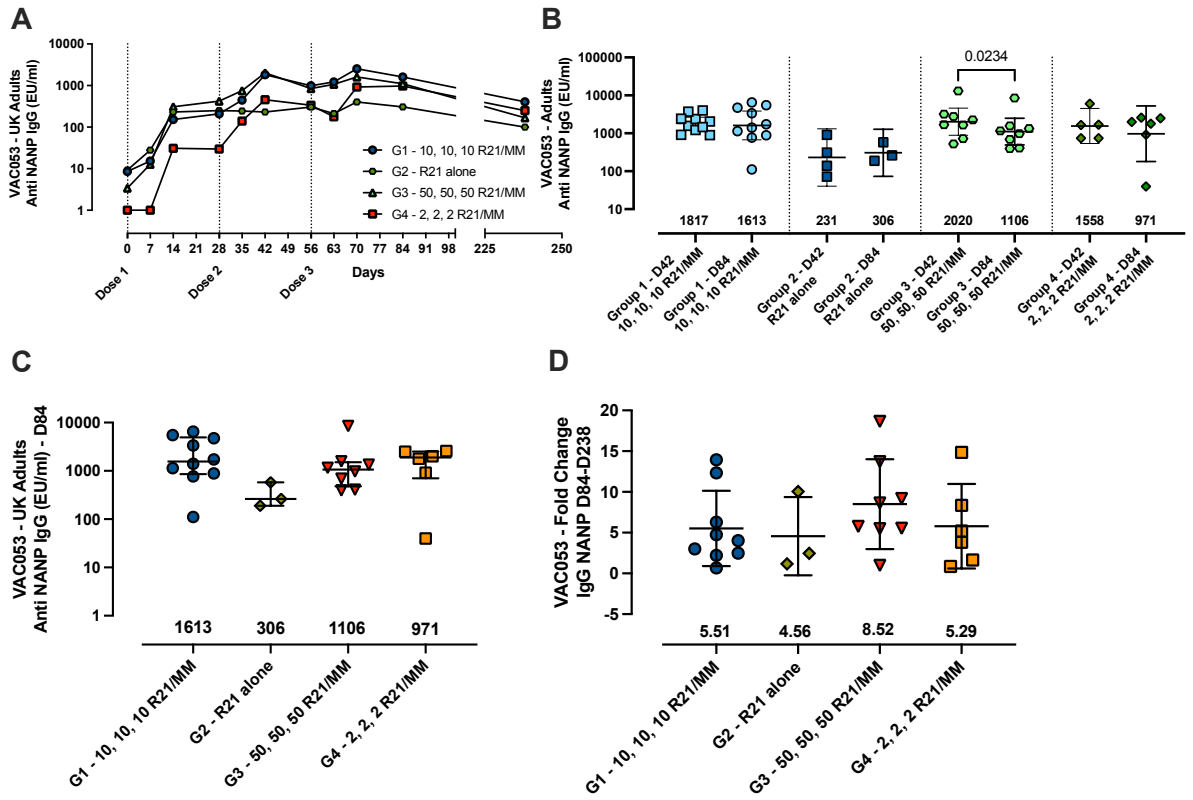


Figure 26 – VAC053 anti-NANP IgG in UK adults

A) *Median anti-NANP time courses. R21/MM vaccinations are marked with dotted lines with the dose number appearing below the x-axis.* ***B)*** *Comparing anti-NANP IgG 2 weeks post second dose with 1 month post third dose for different dose regimens. Geometric means with 95% CI. Mann-Whitney P=0.02.* ***C)*** *Peak anti-NANP IgG titres 1-month post third vaccination – D84. Geometric means with 95% CI.* ***D)*** *Antibody durability measured by fold change between D84 (1-month post third dose) and D238 (6 months post third dose)*

Values above the x-axis indicate geometric means.

In VAC053, 14/28 UK adults recruited had a small amount of detectable anti-NANP IgG (antibody titres) measured at baseline. The 14 participants with pre-existing antibody responses had a GMT

15.6EU [95% CI 8.77 – 27.8]. After immunisation, UK adults produced high anti-NANP IgG titres from varied doses of R21 with or without adjuvant Matrix M - Figure 26A. Antibody titres across all groups followed similar time course antibody kinetics whereby they increased after the first two doses of R21, peaked at D42 (2 weeks post the second dose), and dropped slightly at D56 before the third vaccination - Figure 26A. A similar finding made in Rampling *et al*., 2018 [420]

After receiving the third dose, participants' antibody titres at D84 returned to levels seen at D42 with only G3 (50, 50, 50µg) having a significantly lower titre at D84 (GMTs 1106EU [95% CI 490-2494]) compared with D42 (GMT 2020EU [95% CI 887-4597]) - Figure 26B.

No significant difference in antibody titres across G1 (10, 10, 10µg), G3 and G4 (2, 2, 2µg) who received R21 and MM was seen at D84 - Figure 26C. Group 2 (10, 10, 10µg but no MM) was removed from comparing antibody titres at D84 due to low sample numbers (n=3). The durability of antibody responses in VAC053 was measured by calculating the fold change in antibody titres between D84 and D238.

All participants showed a significant reduction in antibody titres between D84 and D238 (data not shown), however, G3 (50, 50, 50µg) had the largest reduction in antibody titres with a mean fold change of 6.59 [95% CI 3.9 – 13.1] - Figure 26D.

11.4.1.2 VAC060 – R21/MM induce anti-NANP IgG in Burkinabe adults.

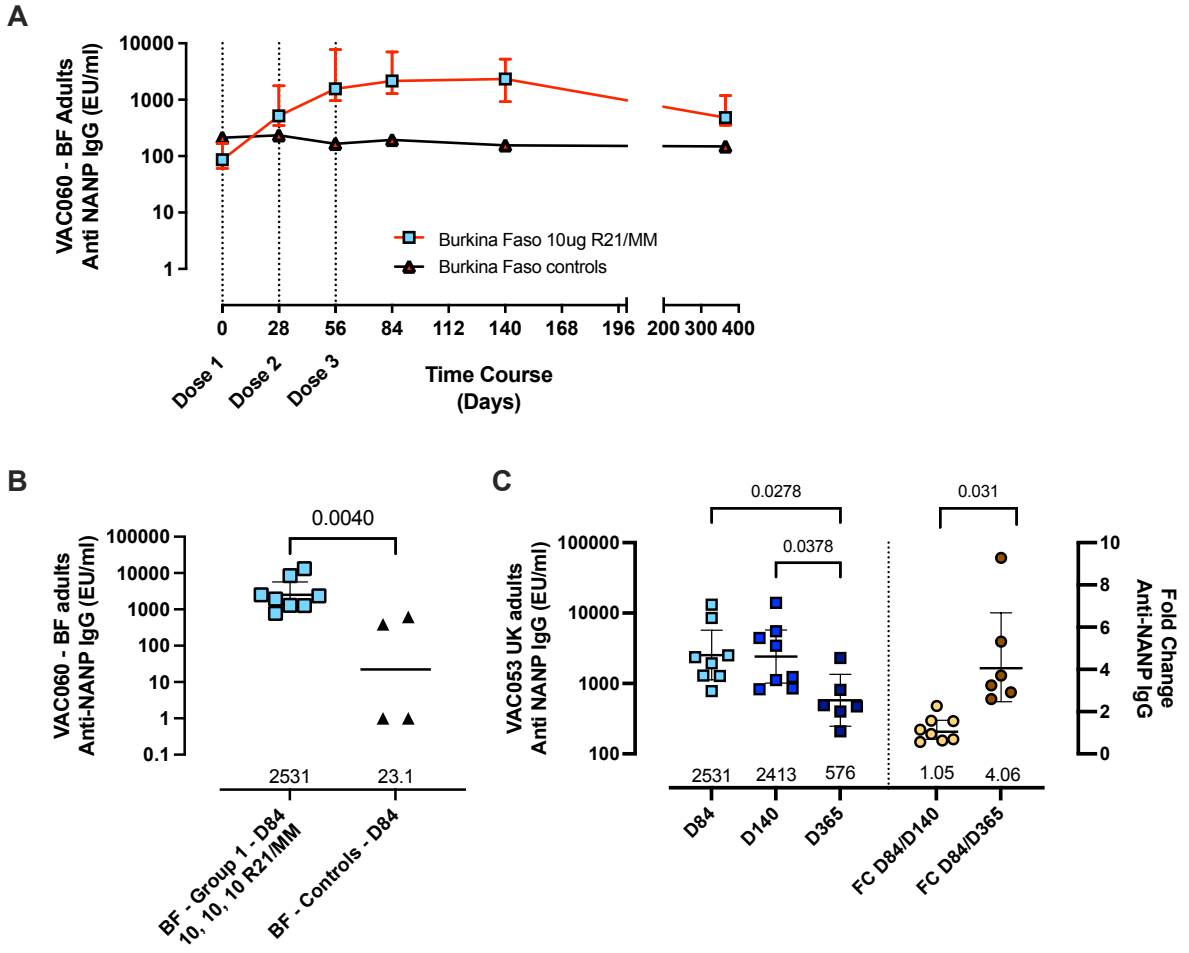


Figure 27 – VAC060 Anti-NANP IgG in Burkinabe Adults

A) *Median and IQR anti-NANP IgG Time Course for adults either immunised with 10µg R21 / 50µg MM or controls. IQR only shown for Burkina Faso controls not shown due to the small sample size (n=4).* ***B)*** *Geometric mean anti-NANP IgG titres 1 month post third dose for both vaccinated and controls. Error bars indicate 95% CI and numbers on the x-axis are geometric means values.* ***C -Left)*** *geometric mean anti-NANP IgG at D84, D140 and D365.* ***C-right)*** *Fold change between D84/D140 and fold change between D84/D365.*

Error bars indicate 95% CI and numbers on the x-axis are geometric mean values.

In VAC060, 9/12 adults recruited had detectable anti-NANP IgG titres at baseline with a GMT of 155EU [95% CI 81.2 – 296] - Figure 27A. Consistent anti-NANP IgG was observed in controls over the course of the trial period (1 year), consistent with adults living in a malaria endemic area. Vaccinated adults in G1 (10, 10, 10µg) peaked at D84 (one month post third dose) – GMT 2551EU [95% CI 1125 – 5693] and significantly higher than controls – GMT 23.1EU [95% CI 0.07 – 6600] - Figure 27B. Among

vaccinated, antibody titres were well maintained at D140 (two months post third dose) – GMT 2431EU [95% CI 1013 – 5738] with no fold reduction – 1.05 Figure 27C. Antibody levels waned over the next eight months with a 4.06 fold reduction measured at D365 with a GMT 576EU [95% CI 246 – 1345] - Figure 27C.

11.4.1.3 VAC073 – R21/MM induces anti-NANP IgG in Kenyan adults, young children and infants

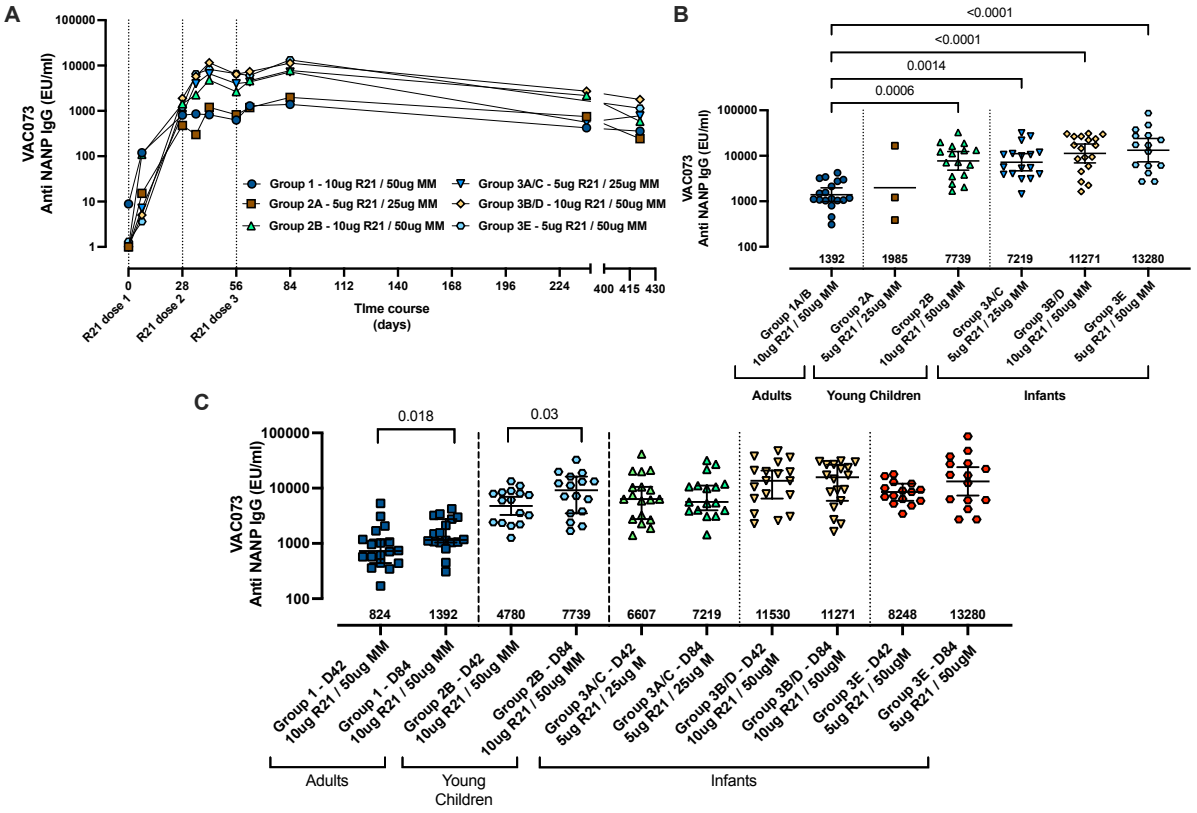


Figure 28 – VAC073 Anti-NANP IgG from Kenyan adults, young children, and infants

A) *Median with IQR anti-NANP IgG Time Course for adults, young children and infants with different dose regimens of R21/MM. The dotted lines represent each dose number of R21/MM for each group. Time course presents anti-NANP titres up to 1 year post third dose.* ***B)*** *Geometric mean anti-NANP titres at D84 (1-month post third dose for each group. Values above the x-axis are geometric mean titres. P-values represent significance. Kruskal-Wallis with Dunn's multiple comparison correction.* ***C)*** *Geometric means of anti-NANP titres comparing D42 with D84. Values above the x-axis represent geometric means for each group. P-values represented significance calculated. Mann-Whitney with a Bonferroni correction for multiple comparisons.*

In VAC073, 9/18 Kenyan adults had detectable anti-NANP IgG baseline (D0) (GMT 77.3EU [95% CI 50.8 – 118]). Whereas no detectable anti-NANP IgG was found at baseline among young children and

infants. All groups in all age categories follow similar antibody kinetics after each dose of R21/MM whereby antibody levels increase after the first two doses and tend to plateau at D42 (two weeks post second dose) - Figure 28A.

As seen with previous trials for R21 [421] and RTS,S [420, 422], antibody titres peaked at D84 for all dose groups. Immunised young children in G2b (10µg R21/50µg MM) have a 5.5-fold significantly higher antibody titre (GMT 7739EU [95% CI 4819 – 12428]) at D84 compared to adults in G1 (10µg R21/50µg MM) (GMT 1392EU [95% CI 984 – 1969]), receiving the same dose - Figure 28B. In addition, Infants in G3B/D (10µg R21/50µg MM) have an 8-fold higher antibody titre compared to adults GMT 11271EU [95% CI 6995-18160]) - Figure 28B all receiving the same dose regimen.

Infants in G3A/C (5µg R21/25µg MM) receiving the lower dose of immunogen - R21 and adjuvant MM have comparable titres to young children in G2B who received double the dose of R21/MM (10µg R21 / 50µg MM) at D84, however not significant - Figure 28B. Infants in G3E (5µg R21/50µg MM) who have a low dose of R21 and high dose of adjuvant have the highest antibody titres at D84 (GMT 13280EU [95% CI 7366 – 23942]) - Figure 28B. All groups, irrespective of their anti-NANP IgG titres at one month post-third vaccination (D84) dropped by 3-fold at six months post-third vaccination (D226) - Figure 28A.

Data from UK adults in VAC053 (11.4.1.1) indicate comparable antibody titres at D42 (two weeks post second dose) with D84 (one month post third dose) - Figure 28B. In contrast, adults in Kenya reported a significantly (P=0.018) higher antibody titre at D84 (GMT 1392EU [95% CI 984 – 1969]) compared with D42 (GMT 824EU [95% CI 543 – 1250]) - Figure 28C. Young children in Kenya follow a similar trend with Kenyan adults and have a significantly (P=0.02) higher antibody titre at D84 (GMT 7739EU [95% CI 4819 – 12428]) compared with D42 (GMT 4780EU [95% CI 3274 – 6980]). Conversely, infants in all three-dose groups had no significant difference in antibody titre between D42 and D84 - Figure 28C.

11.4.1.4 VAC073 – A booster dose at 12 months returns anti-NANP IgG to peak levels observed after primary series in Kenyan Infants

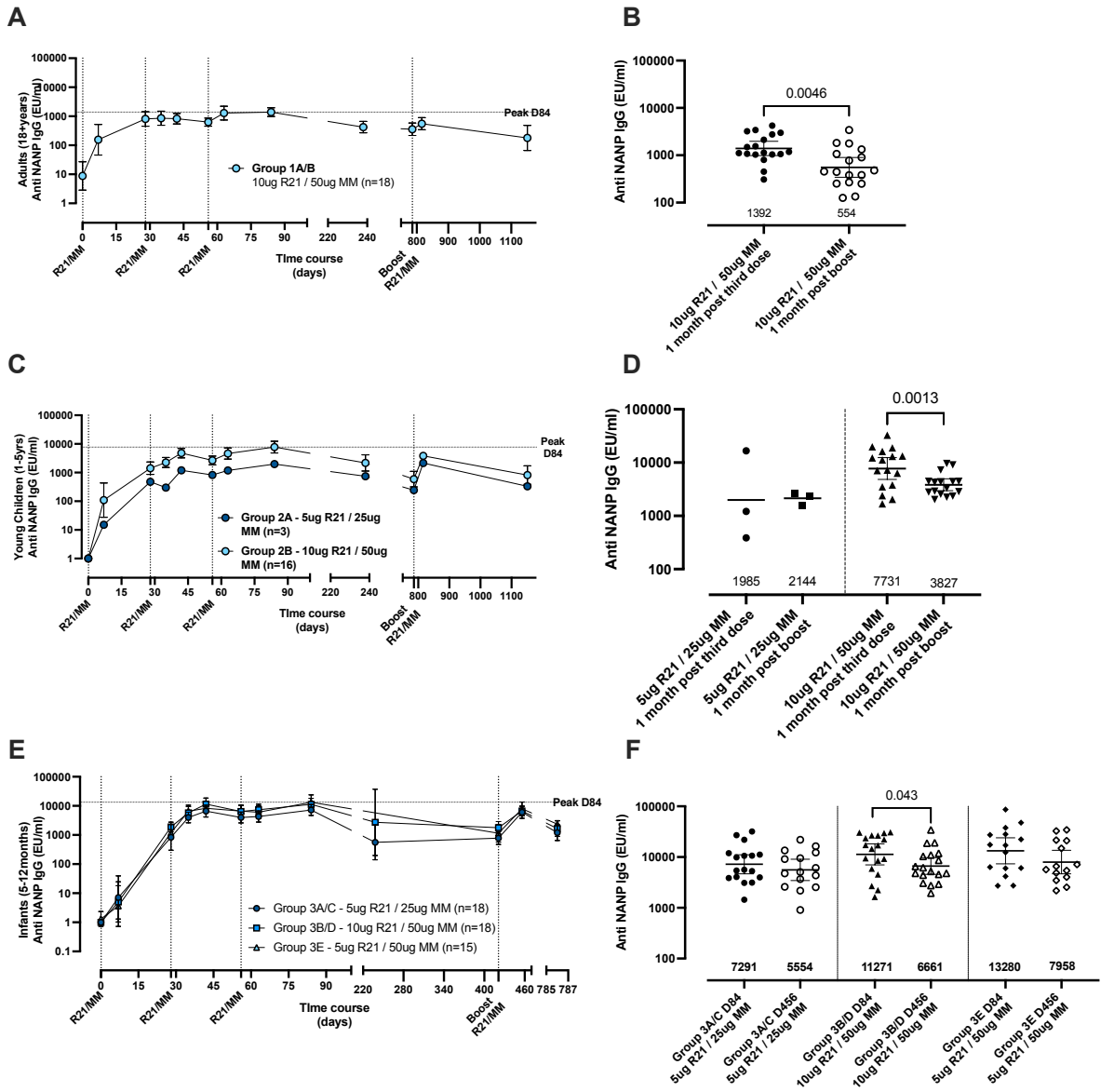


Figure 29 - Effect of a booster dose of R21/MM in Kenyan adults, Young Children, and Infants

A,C,E. *Median Time courses of anti-NANP IgG for adults, young children and infants. Vertical dotted lines represent each dose of R21/MM at D0, D28 and D84 with booster dose administered at D421.* ***B)*** *Geometric mean anti-NANP IgG 1-month post third dose and 1-month post boost. Wilcoxon matched-pairs signed rank test P=0.0046. GMT is displayed above the x-axis for each time point.* ***D)*** *Geometric mean anti-NANP IgG 1-month post third dose and 1-month post boost. Wilcoxon matched-pairs signed rank test P=0.0013. GMT is displayed above the x-axis for each time point.* ***F)*** *Geometric mean anti-NANP IgG 1-month post third dose and 1-month post boost. Wilcoxon matched-pairs signed rank test P=0.043. GMT is displayed above the x-axis for each time point.*

In VAC073, Kenyan adults in G1 showed a significant 4-fold reduction in anti-NANP IgG between D84 (one month post third dose) - GMT 1392 EU [95% CI 984- 1969] and D421 (one year post third dose) - GMT 356EU [95% CI 218 -583] - Figure 29A. A booster dose given to adults at D421 returns anti-NANP IgG titres to less than half at D456 (one-month post boost) with a GMT 554EU [95% CI 343 – 897] of what was seen at D84 - Figure 29B.

Young children in G2B (10µg R21/50µg MM) have a significant 13-fold reduction in anti-NANP IgG between D84 - GMT 7739EU [95% CI 4819 – 12428] and D421 – GMT 588EU [95% CI 316 – 1096] – Figure 29C. Similar to adults, a booster dose of (10µg R21/50µg MM) given at D421 returns antibody levels to a GMT 3827EU [95% CI 2960 – 4948] at D456, approximately a 2-fold decrease in antibody titres - Figure 29D.

Infants in G3A/C (5µgR21/25µg MM) have a significant 9-fold reduction in antibody titres between D84 – GMT 7219EU [95% CI 4684 – 11126] and D421 – GMT 778EU [95% CI 474 – 1277] - Figure 29E. With the addition of a booster administered at one year, antibody titres returned to levels seen after the primary series at D456 with no significant difference - Figure 29F. Infants in G3B/D (10µgR21/50µg MM) had a 10-fold reduction in total IgG titres after a year at D421 – GMT 1780EU [95% CI 1093 – 2897] - Figure 29E. For G3B/D, antibody titres measured at D458 – 6661EU [95% CI 4558 – 9874] failed to reach levels that were seen at D84, a significant reduction - Figure 29F. Infants in G3E (5µgR21/50µg MM) had an 11-fold reduction in antibody titres one year after primary series D421 – 1144EU [95% CI 570 – 2295] compared with D84 - Figure 29E. Immunogenicity measured at D458 – 7958EU [95% CI 4662 – 13584] saw antibody titres return 1.6 times the levels seen at peak - Figure 29F.

11.4.1.5 VAC073 - Boosting improves durability in younger age categories compared to adults in Kenya

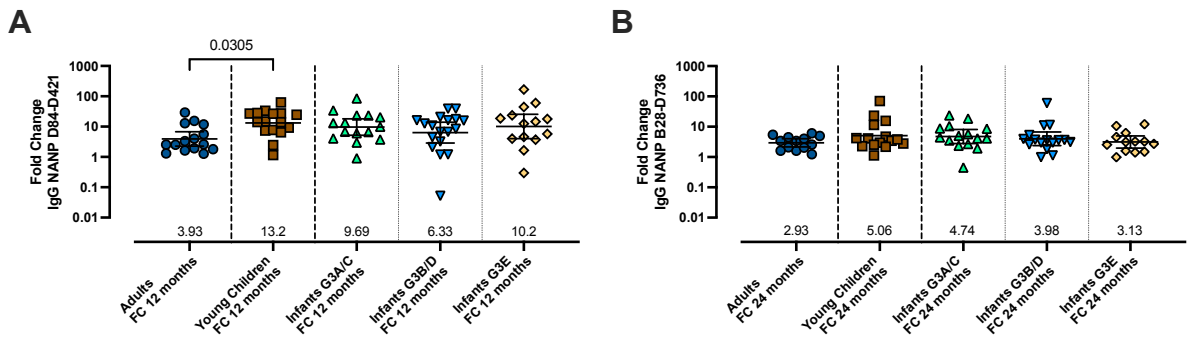


Figure 30 - VAC073 antibody durability.

A) *Antibody durability among all groups in the first year of trial, measured by the fold change between D84 (1 month post third dose) and D421 (1 year post third dose).* ***B)*** *Antibody durability among all groups in the second year of trial, measured by fold change between D456 (1 month post boost) and D721 (1 year post first boost).*

Values above the x-axis indicate geometric means.

In VAC073, all groups saw a decline in antibody levels in the twelve months after the primary series - Figure 28A and twelve months after boost - Figure 29A/C/E. Fold changes at 12 months after the primary series and booster dose were calculated to compare the effect of age and dose on the durability of the response.

In the first year of the trial, adults had significantly lower antibody levels at D84, as compared to young children and infants – Figure 28B. However, Kenyan adults had the smallest reduction/fold change – 3.93 in antibody titres measured between D84 and D421 compared with young children – 13.2 and infants – 8.74 (average fold change of all infant groups) - Figure 30A. These data indicate that R21/MM results in better antibody durability in adults compared with young children and infants in the first year after vaccination however age and immune maturation may be a confounding factor.

The effect of a booster administered at D421 (one year after the primary series) and measured at D458 results in antibody titres increasing but not reaching levels seen at D84 – Figure 29B/D/E. While antibody titres after the boost are not as high, all age groups showed an improvement in the reduction

of titres measured at D721 - Figure 29A/C/E. Adults have the lowest fold change – 2.93 when comparing D458 with D721. All young children and infants have a large ~2-fold reduction in antibody titre between years 1 and 2 - Figure 30B, indicating that boosting improves antibody durability.

11.4.1.6 VAC065 – R21/MM induces high anti-NANP IgG in UK adults 1-month post third dose

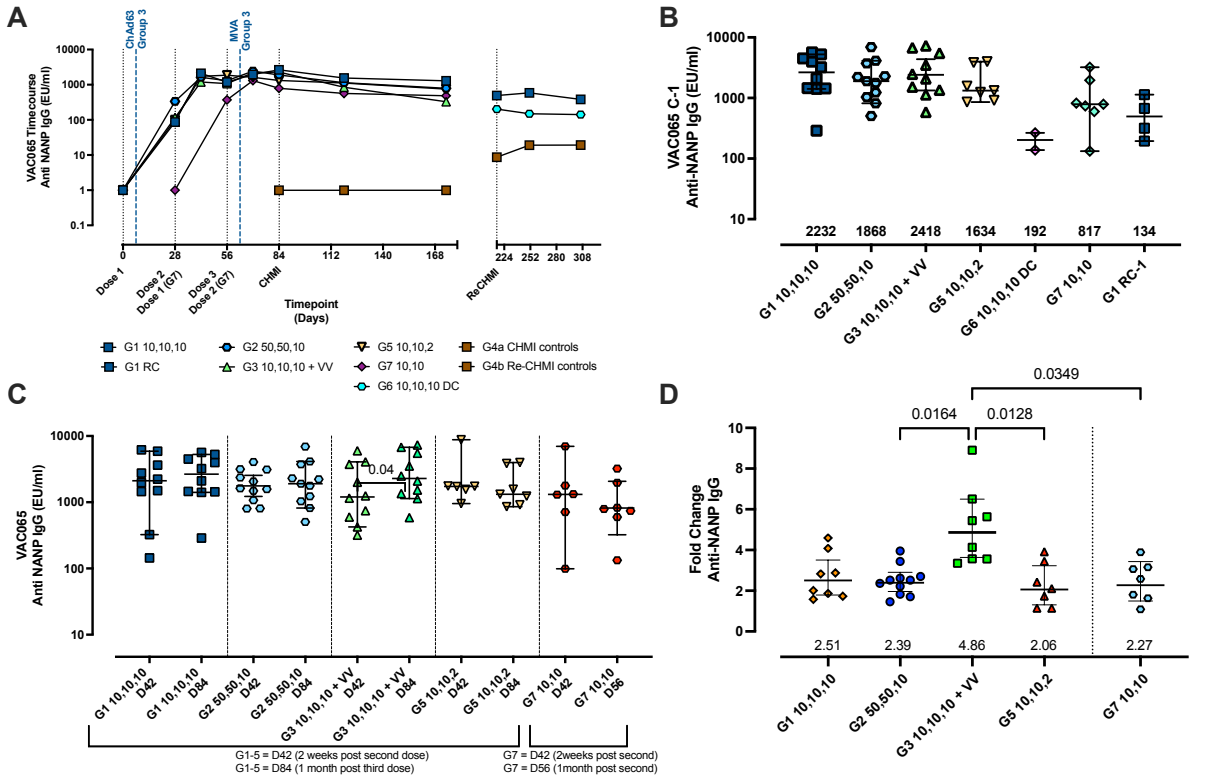


Figure 31 – VAC065 anti-NANP IgG among different dose regimens of R21$_{CBF}$/MM.

A) *Median anti-NANP time courses. R21/MM vaccinations are marked with dotted lines with dose numbers for different groups appearing below the x-axis. Viral vectored vaccinations for group 3 are marked with purple dashed lines with vector names appearing above the Figure. CHMI and Re-CHMI are represented by black dotted lines and labelled below the x-axis.* ***B)*** *Geometric means with 95% CI of anti-NANP titres measured at 1 month post final dose for each group. Geometric means values are displayed above the x-axis.* ***C)*** *Geometric means with 95% CI of anti-NANP IgG comparing D42 and D84 for groups 1, 2, 3, 5 and D42 with D56 for group 7. Wilcoxon matched-pairs signed rank test P=0.04.* ***D)*** *Geometric means with 95% CI of fold change calculated between D84 (1 month post third dose) and D174 (4 months post third dose). Kruskal-Wallis corrected with Dunn's multiple comparisons.*

In VAC065, all UK adults apart from one recruited into this CHMI study were negative for anti-NANP specific IgG measured at baseline (data not shown). All groups, irrespective of dosage, followed similar

antibody kinetics, wherein antibody titres increased after the first two doses, peaked at D42, dropped at D56 and with the addition of a booster dose titres peaked at D84 - Figure 31A.

Adults in G1 (10μgR21/50μgMM) and G3 (10μgR21/50μgMM & viral vectors – ME-TRAP) exhibited the highest antibody titres at D84 with G1 having a GMT - 2232EU [95% CI 1170 – 4260] and G3 having a GMT – 2418EU [95% CI 1333 – 4386]. The lowest antibody titres were seen in G7 receiving two doses of 10μg R21/50μg MM measured one month post second dose (D56) - 817EU [95% CI 323 – 2068] - Figure 31B.

All groups had comparable anti-NANP IgG between D42 and D84 with no significant differences except G3 (10μgR21/50μg/MM & viral vectors – ME-TRAP) which at D84 had a GMT 2418EU [95% CI 917 – 4481] had significantly higher titres than at D42 GMT 1318EU [95% CI 642 – 2707] - Figure 31C.

To investigate whether a third dose is needed to maintain durable immunogenicity, a two-dose regimen of 10μg R21/50μg MM was added in G7. Immunogenicity measured at D84 (one month post third dose) and D174 (four months after the final dose) had a significant reduction in antibody titres across all groups, data not presented. All groups had a 2-2.5 fold reduction in antibody titres at four months post final dose with the largest reduction in antibody titres occurring in G3 (10μgR21/50μg/MM & viral vectors – ME-TRAP) which had a 4.86 fold reduction in antibody titres - Figure 31D. G3 had a significantly larger reduction than G2, G5 and G7 Figure 31D.

11.4.1.7 VAC072 – A delayed third dose of either 10µg or 2µg R21 with MM improves anti-NANP IgG compared to the standard 3-dose regimen

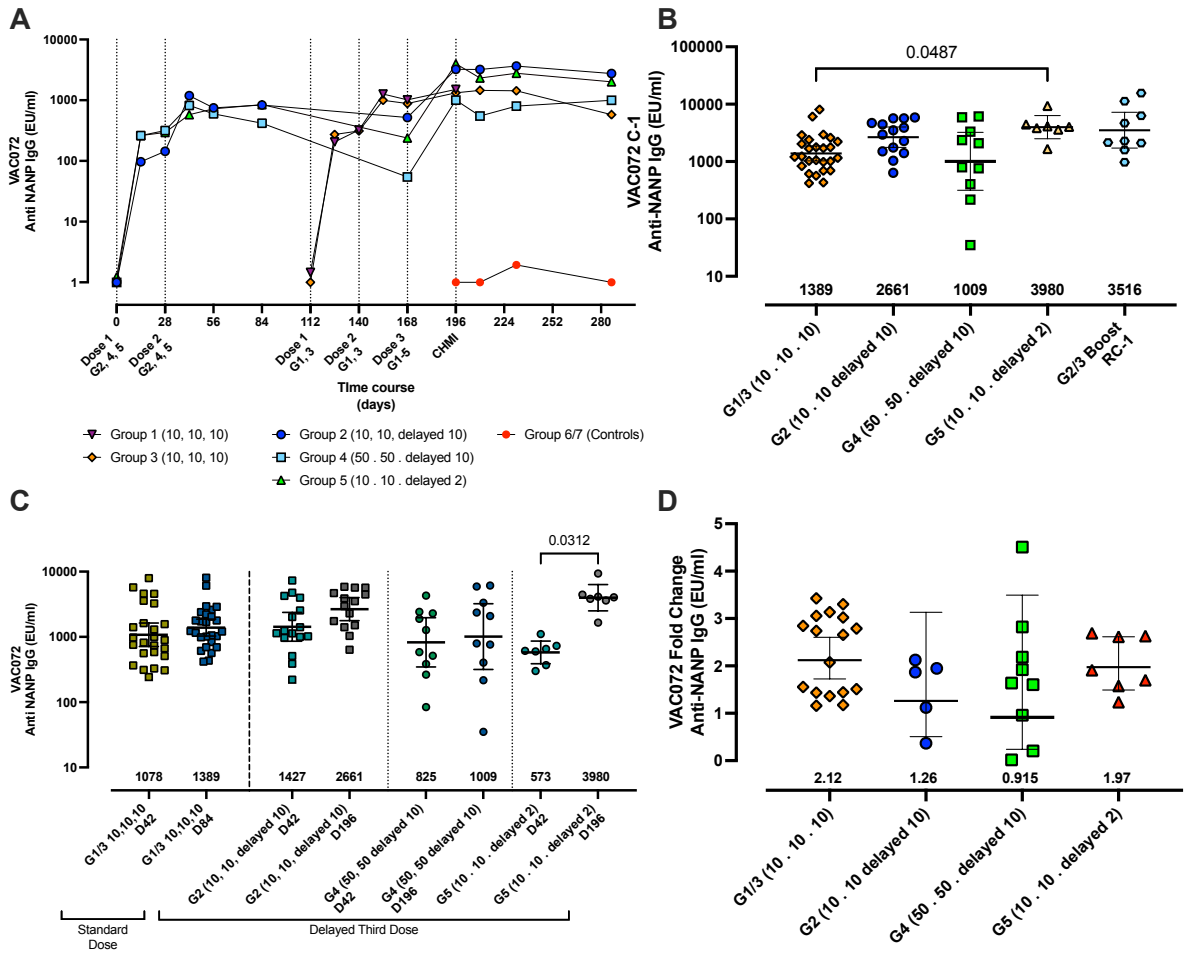


Figure 32 – VAC072 anti-NANP titres after different dose regimens of R21$_{SII}$/MM

A) Median Anti-NANP IgG time course. R21/MM vaccinations are marked with dotted lines with dose numbers for different groups appearing below the x-axis. Error bars not shown. ***B)*** *Geometric means with 95% CI of peak anti-NANP IgG titres at 1-month post third dose for all groups 1-5- and 1-month post boost. Kruskal-Wallis with Dunn's multiple comparison test P=0.0487.* ***C)*** *Geometric means with 95% CI of anti-NANP IgG time course for participants at D42 compared with D84.* ***D)*** *Fold change with 95% CI of anti-NANP IgG 1-month post-third dose, and 4 months post-third dose.*

In VAC072, only 2/65 UK adults recruited to the second CMHI had detectable anti-NANP IgG at baseline - Figure 32A. Both standard dose regimens G1 and G3 (10, 10, 10µg), delayed dose regimen G2 (10, 10 delayed 10µg) and delayed fractional dose regimens G4 (50, 50 delayed 10µg) and G5 (10, 10 delayed 2µg) followed similar antibody kinetics after the first two doses of R21/MM and peaked/plateaued at D42 - Figure 32A. For G2, G4 and G5, who received delayed third dose regimens,

antibody titres declined between D42 and 5 months (D168). However, with the addition of a third dose, antibody titres increased - Figure 32A.

UK adults in G2 (10, 10 delayed 10µg) and G5 (10, 10 delayed 2µg) had the highest anti-NANP titres one month post-third dose with G5 having significantly higher titres when compared with G1/3 standard dose regimen (10, 10, 10 µg) - Figure 32B. G4 with the highest delayed fractional dose regimen of 50, 50 delayed 10µg had the lowest antibody titres one month post third dose – 1009EU [95% CI 316 – 3221] - Figure 32B.

After two doses at D42, NANP-specific titres were comparable to those seen at one month post-third dose except in G5 which had a significant 6.9-fold increase in responses from 573 to 3980EU [95% CI 2500 – 6337] - Figure 32C. G5 (10, 10 delayed 2µg) had the lowest anti-NANP IgG titres two weeks after two doses when compared with G1/3 (10, 10, 10µg) and G2 all received the same first two doses in the first month, however, no significant difference was observed - Figure 32C.

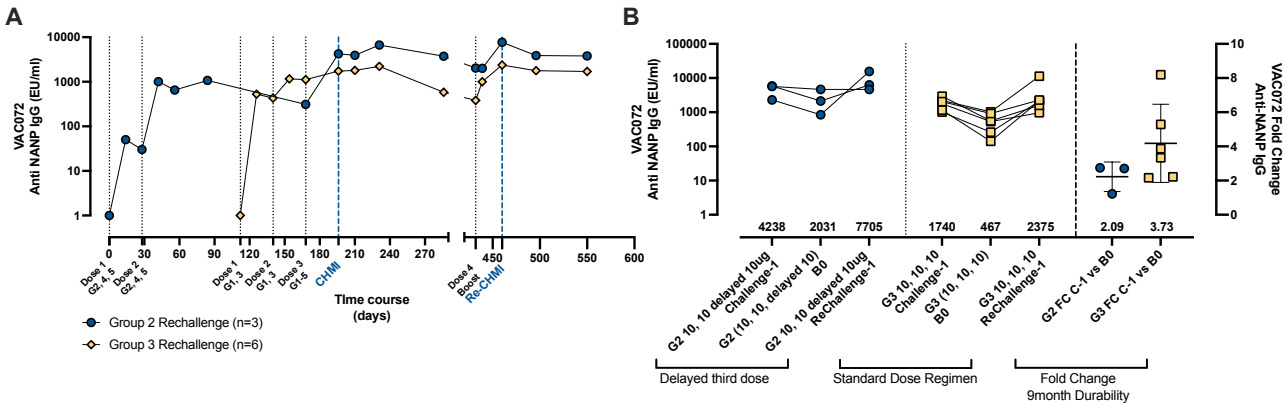


Figure 33 - VAC072 anti-NANP IgG after a booster administered 8 months

A) *Median time course (error bars show IQR). Vertical black dotted lines show R21/MM Immunisations for each group. Vertical blue dashed lines show CHMI and Re-CHMI.* ***B-left)*** *Individual anti-NANP IgG responses at D84 (G3 1 month post third dose) and D196 (G2 1 month post third dose), B0 (9 months after 3rd vaccination and day of boost) and RC-1 (30 days post boost measure against left y-axis.* ***B-right)*** *Fold change with 95% CI between C-1 and B0 to represent a reduction in IgG titre measured on the right y-axis.*

Re-challenged participants from G2 (n=3) saw anti-NANP IgG levels drop by 2-fold in eight months after the first challenge and returned to levels seen one month after the third dose - Figure 33A. Re-challenged participants in G3 saw anti-NANP IgG reduce 7.8-fold eight months after the first challenge and return to levels seen one month after the third dose - Figure 33B.

11.4.1.8 VAC076 – R21/MM induces high anti-NANP IgG in Burkinabe infants

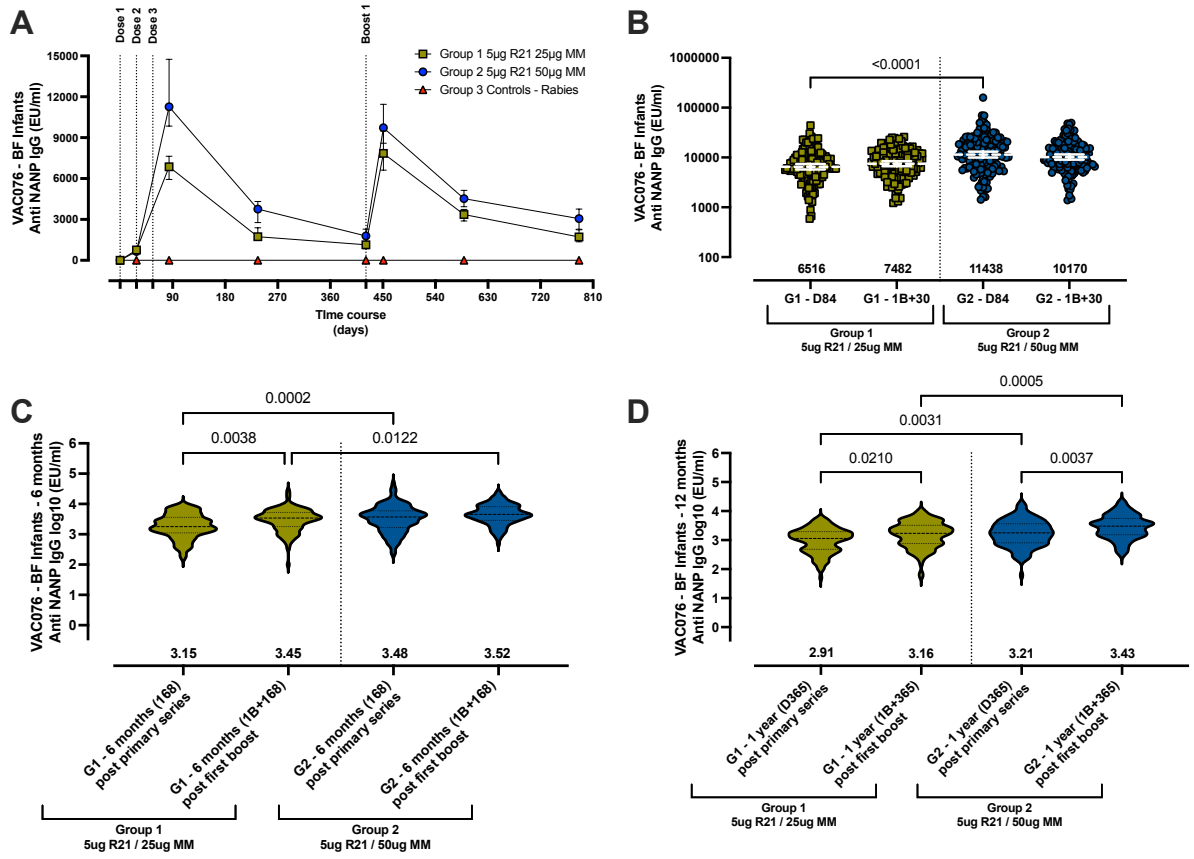


Figure 34 - VAC076 anti-NANP IgG in 450 Burkinabe infants after Immunisation of R21/MM.

A) *Median (error bars indicate 95% CI) over the duration of 2 years. The primary series of 3 vaccinations 1 month apart in the year of follow-up after boost. G1 are depicted by green squares and, G2 are blue circles. Vertical black lines represent immunisations of R21/MM. Horizontal black dotted lines indicated threshold protection titre reported in Datoo et al., 2022 [3].* ***B)*** *Geometric means with (error bars indicating 95% CI) of individual responses at peak time points for G1 and G2. D84 – 1-month post-primary series, 1B+30 – 1-month post-first boost. Kruskal-Wallis corrected with Dunn's multiple comparisons.* ***C)*** *Violin plots (log10) of anti-NANP IgG at 6 months post primary series and 6 months post first boost. G1 P=0.0009 & G2 P=0.0271 Mann-Whitney.* ***D)*** *Violin plots (log10) of anti-NANP IgG at 12 months post primary series and 12 months post first boost. G1 P=0.0008 & G2 P<0.0006 Mann-Whitney.*

Error bars in violin plots indicate interquartile ranges. Values above the x-axis indicate geometric means.

Three doses of R21/MM provided high levels of immunogenicity one-month post-third vaccination for both doses of adjuvant 25µg (G1) vs 50µg (G2) MM - Figure 34A, but declined by 3-fold in six months after the third vaccination and a further 2.4-fold reduction between 6 months and a year after the primary series of vaccination - Figure 34A. At peak, the highest anti-NANP IgG titres were observed in G2 – 11438EU [95% CI 9985-13102] which had significantly higher titres than G1 - Figure 34B. A booster dose administered at one year (D365) and measured one month after the boost increased anti-NANP titres to levels seen at the peak, with no significant difference between one-month post-boost and one-month post-primary series for each group - Figure 34B.

Boosting significantly improved durability as measured by antibody titres at six and twelve months after a boost compared to antibody titres six and twelve months post primary series - Figure 34C/D.

11.4.2 Comparing anti-NANP IgG responses across different trials

11.4.2.1 R21/MM produces comparable anti-NANP IgG antibodies in adults in the UK, Kenya and Burkina Faso

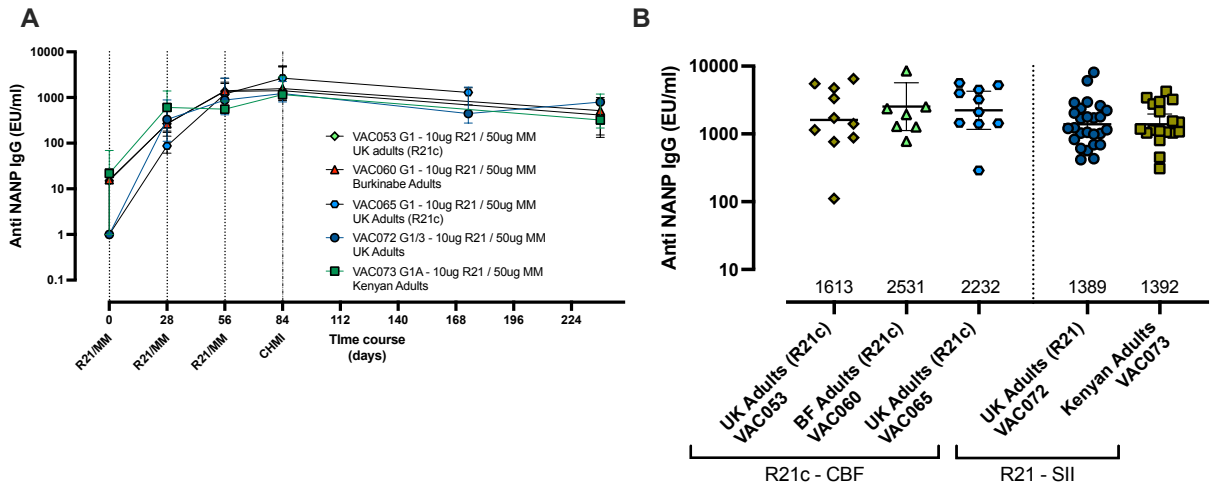


Figure 35 – Trial comparison of anti-NANP IgG comparing adults in the UK, Burkina Faso and Kenya all with the same dose of R21/MM (10, 10, 10).

A) *Median anti-NANP time course (error bars indicate IQR) followed up to six months post primary series of vaccination. Vertical black dotted lines indicated immunisation of R21/MM and vertical black dashed line indicate CHMI for UK adults (VAC065/72).* ***B)*** *Geometric mean anti-NANP IgG individual titres measured at D84 (error bars represent 95% CI). Values are presented directly above the x-axis.*

All adults in the UK (VAC053, VAC065 and VAC072), Kenya (VAC073) and Burkina Faso (VAC060) followed similar antibody kinetics over a six-month timescale with immunisation of 10µg R21/50µg MM - Figure 35A. No significant difference in antibody titres was found between adults when measured at D84 (one-month post-third vaccination) across the trials - Figure 35B. In addition, R21 manufactured in SII without the C-tag required for vaccine purification provides comparable anti-NANP IgG with $R21_{CBF}$ manufactured in CBF amongst UK adults immunised with the same dose regimen of 10µg R21/50µg MM (VAC065 vs VAC072) - Figure 35B. Efficacy and exploratory immunology on $R21_{CBF}$ vs $R21_{SII}$ in UK adults is explored in Chapter 2.

11.4.2.2 R21/MM produces comparable anti-IgG antibodies in infants in Kenya and Burkina Faso

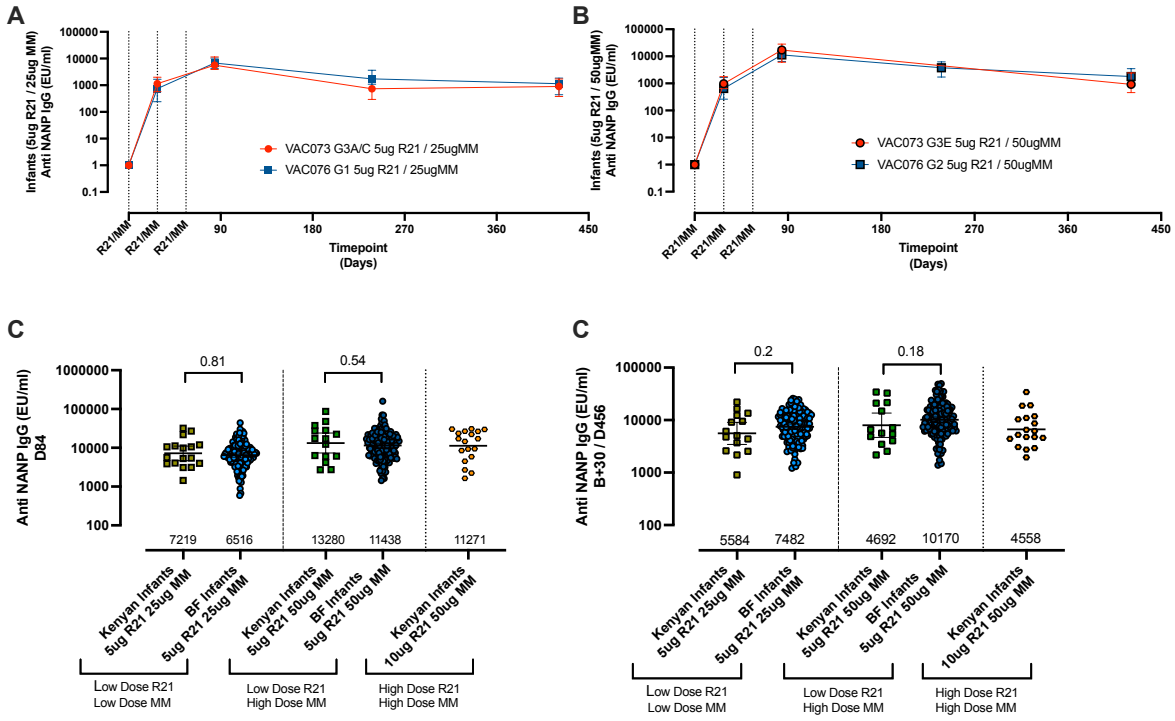


Figure 36 – Trial comparison of anti-NANP IgG in Infants in Kenya and Burkina Faso with identical doses of R21/MM.

A) *Median anti-NANP time courses of infants immunised with 5µg R21 and 25µg MM (error bars indicate IQR).* ***B)*** *Median time course for infants immunised with 5µg R21 and 50µg MM (error bars indicate IQR).* ***C)*** *Individual points comparing peak anti-NANP titres at D84 for all groups in Kenya and Burkina Faso (error bars are geometric means with (95% CI). Low dose R21 & low dose MM, P=0.81 Mann-Whitney. Low dose R21 & high dose MM, P=0.54 Mann-Whitney.* ***D)*** *Individual points comparing peak anti-NANP titres at D84 for all groups in Kenya and Burkina Faso (error bars are geometric means with (95% CI).*

As with adults, infants in Kenya (VAC073) and Burkina Faso (VAC076) immunised with the same dose regimen were examined for comparison. Infants in Kenya and Burkina Faso followed similar antibody kinetics to the primary series and after boost, where the time courses in the two years these infants were examined overlap - Figure 36A/B. Infants in both studies had similar antibody titres at one month post-third dose with no significant differences arising between infants of the same dose regimen - Figure 36C. Conversely, infants in Kenya had a 2-fold lower titre one-month post boost than Burkinabe infants, however this was not statistically significant - Figure 36D.

11.4.3 R21/MM induces low T cell response in UK adults

The T cell response to R21/MM was measured by interferon γ (IFNγ) enzyme-linked immunosorbent spot (ELISpot) assay after stimulation of CSP. See the appendix for the CSP peptide sequence (17.6.1) and CSP plate layout (Table 34). T cell response was assessed in UK trials VAC053, VAC065 and VAC072 due to the assays requiring stimulation on fresh PBMCs and the assay is only validated when performed in Jenner Laboratories in Oxford.

11.4.3.1 VAC053 – T cell response measured by IFNγ secretion in UK adults

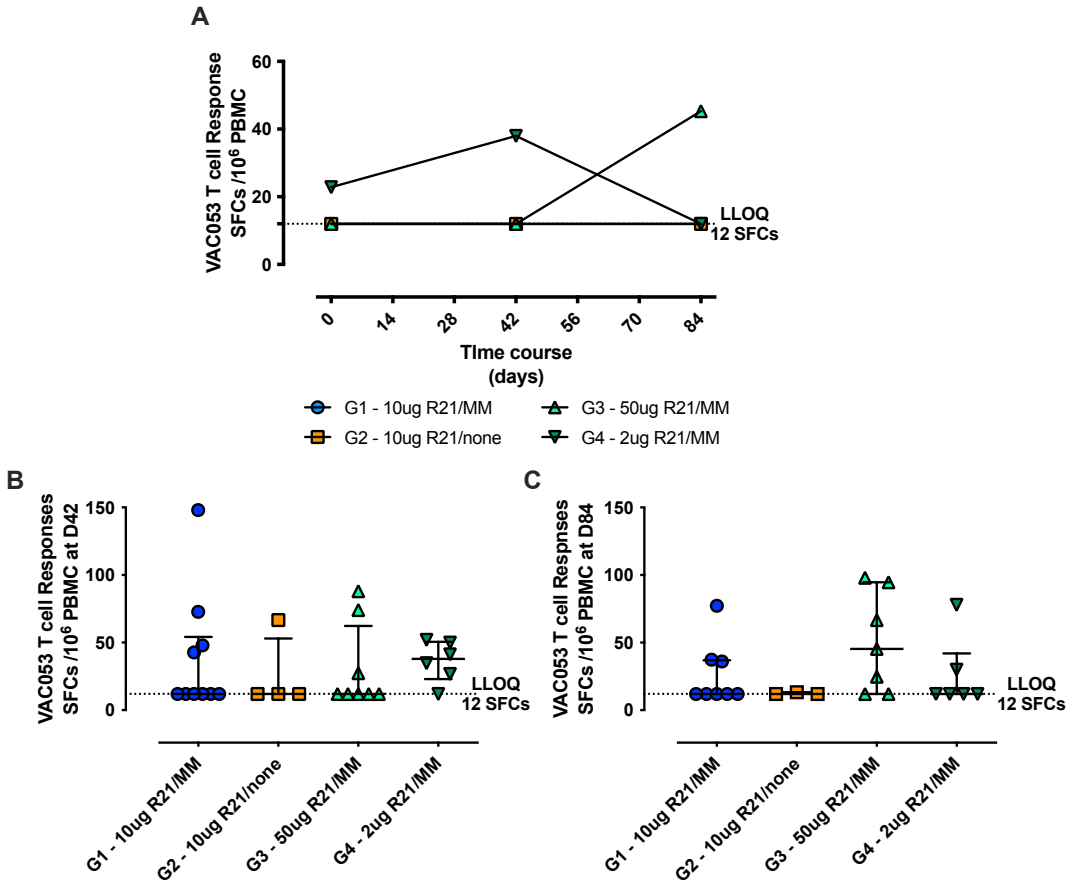


Figure 37 – VAC053 T cell ELISpot response measured UK adults.

A) *Median IFNγ ELISpot time courses – summed pools of CSP peptides measure at D0 (baseline), D42 (2 weeks post second dose) and D84 (1-month post third dose). Dose groups are viewed in the key on the right side of the graph.* ***B)*** *Individual responses comparing IFNγ production at D42.* ***C)*** *Individual responses comparing IFNγ production at D84. Error bars indicate 95% CI. The horizontal dashed line represents LLoQ = 12 SFC per million PBMC.*

In VAC053, CSP-specific T cell response induced in UK adults after immunisation of varied dose regimens of R21/MM were measured at D0 (baseline), D42 (2 weeks post second dose) and D84 (one month post third dose). At baseline, 8/30 participants had detectable IFNγ production after being stimulated with CSP. After vaccination, responses were weak in comparison with baseline responses in all groups – Figure 37A and most participants had no change in response throughout the duration of the trial. Participants who had a T cell response, peaked at D42 – Figure 37B and were maintained at D84 – Figure 37C with significant differences between the time points. At D84, G4 (50, 50, 50µg)

the high dose group had the highest T cell response, however not significant when compared with other dose regimens.

11.4.3.2 VAC065 – T cell response measured by IFNγ production in UK adults before and after CHMI

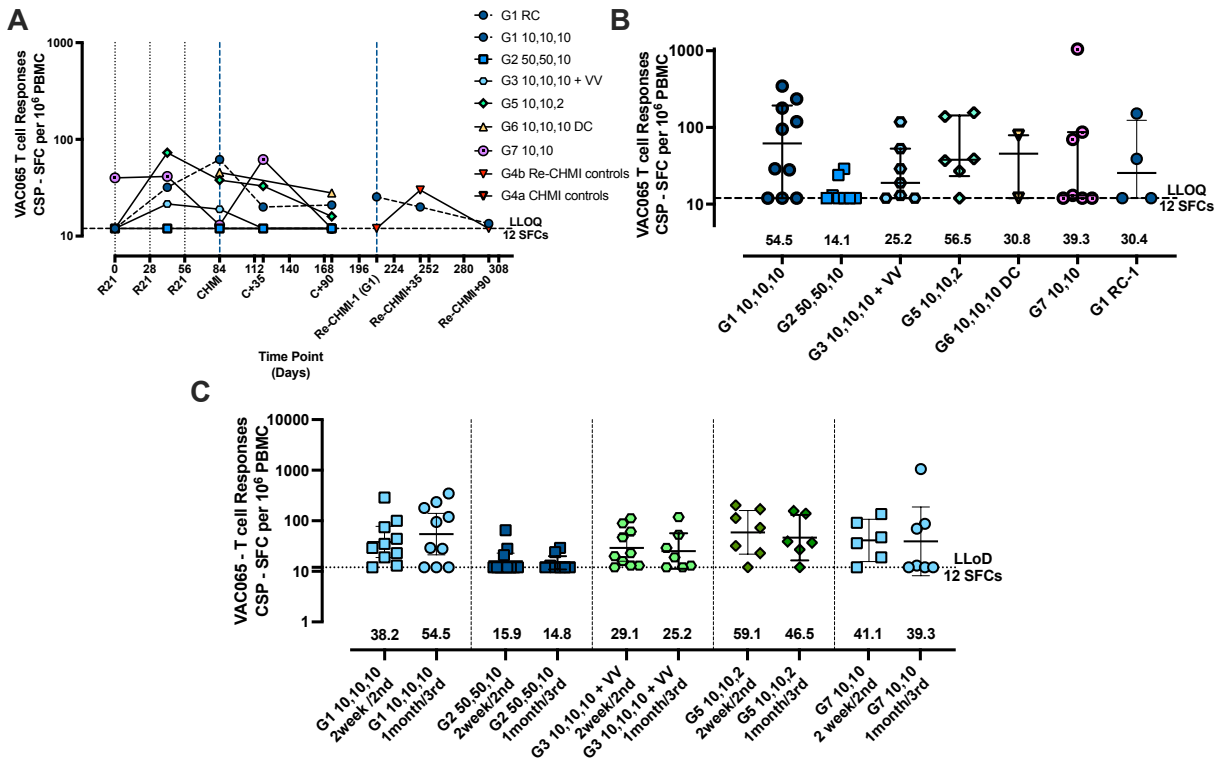


Figure 38 – VAC065 T cell ELISpot response measured UK adults.

A) *Median IFNγ ELISpot time courses – summed pools of CSP peptides measure at D0 (baseline), D42 (2 weeks post second dose), D84 (1-month post third dose), D119 (35 days post-CHMI) and D174 (90 days post-CHMI). Dose groups are viewed in the key on the right side of the graph. Vertical black dotted lines represent R21/MM vaccination and vertical blue dashed lines represent CHMI and Re-CHMI.* ***B)*** *Peak (1 month post third dose) geometric mean CSP specific IFNγ ELISpot. Values above the x-axis are geometric means for each group.* ***C)*** *Geometric means comparing D42 (2 weeks post second dose) with D84/D196 (1-month post third dose). Values presented above the x-axis are the geometric means for each group.*

In Phase II CHMI – VAC065 as with the Phase I trial in UK adults – VAC053, R21/MM elicited little or no T cell response when stimulated with CSP peptides. The T cell response across all groups did not follow consistent kinetics as seen with antibody responses after immunisation of R21 and MM. T cell response measured after stimulation of ME-TRAP saw an increase in response after each viral vector

dose of ChAd63 and MVA ME-TRAP with detectable responses measured at D168 approximately three months after immunisation with MVA ME-TRAP – Figure 38C.

At D84, the timepoint where the highest antibody production was found, minimal T cell responses were reported with no significant differences observed between any groups – Figure 38B. Most participants in all groups had no response and therefore the lower limit of detection (LLoD) was reported (CSP – 12sfc per million PBMC) – Figure 38B. At D84, G1 (10, 10, 10µg) showed a trend for higher T cell responses after stimulation with CSP peptide when compared with other groups, however not significant. G3 (10, ChAd63, 10, 10µg, MVA) recorded lower ELISpot responses in comparison. No difference was observed between two weeks after the second dose and one month after post third dose for any group - Figure 38C.

11.4.3.3 VAC072 – A delayed third dose of R21/MM improves T cell responses measured by IFNγ production in UK adults

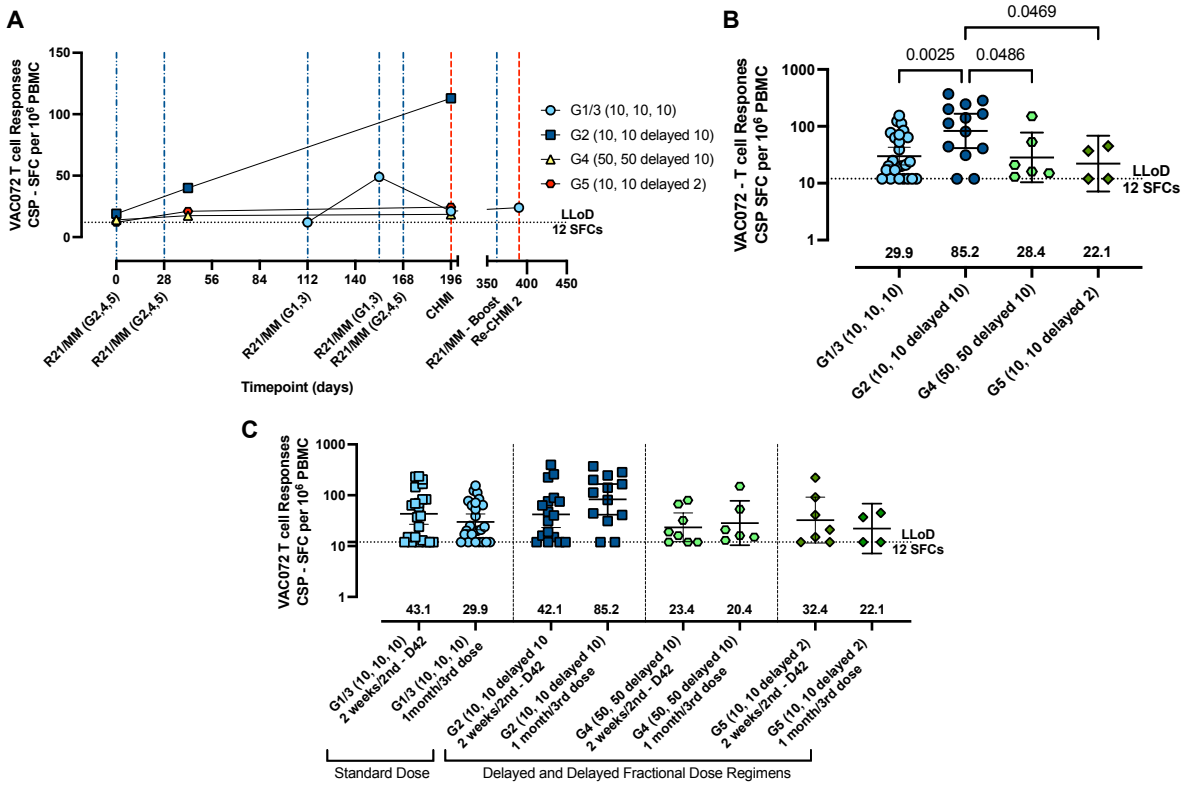


Figure 39 - VAC072 ELISPOT T cell responses in UK adults.

A) *Median IFNγ ELISpot time courses – summed pools of CSP peptides. Dose groups are viewed in the key on the right side of the graph. The time course is staggered to accurately reflect the linear scale of standard dose regimens to delayed dose regimens. Vertical blue dotted lines represent R21/MM vaccination and vertical black dashed lines represent CHMI and Re-CHMI.* ***B)*** *Peak (1 month post third dose) geometric mean CSP specific IFNγ ELISpot. Values above the x-axis are geometric means for each group. P-values 0.0025, 0.0486 and 0.0469 – Kruskal-Wallis corrected for Dunn's multiple comparisons.* ***C)*** *Geometric means comparing D42 (2 weeks post second dose) with D84/D196 (1-month post third dose).*

Values presented above the x-axis are the geometric means for each group.

In the second UK challenge – VAC072 study, CSP-specific T cell responses were measured at D0, D42 (two weeks post second dose) and D84/D196 (one month post third dose). Additional responses for participants who consented to be boosted and re-challenged were measured at B+30 (one-month post boost) – Figure 39A.

The highest responses in VAC072 were measured in G2 (10, 10, delayed 10µg) – 85.2SFC [41.4-167] at D196 (1-month post third dose). In addition, G2 had significantly higher responses compared to G1/3 (10, 10, 10µg) and G4 (50, 50 delayed 10µg) – Figure 39B. Lowest responses were observed in G5 (10, 10 delayed 2µg). There were no differences in T cell responses between D42 and D84 for any dose regimen in this trial. However, the delayed dose regimen in G2 (10, 10 delayed 10µg) showed a trend for a higher ELISpot response one-month post-third dose In comparison to the other dose regimens - Figure 39C.

11.4.4 R21/ MM induce functional antibodies to block sporozoite invasion *in vitro*

ISI is a functional assay used to test whether immunity elicited by vaccination with R21/MM can block the invasion of transgenic *Pb* sporozoites expressing a copy of the *Pf*CSP antigen of interest *in vitro*. ISI was tested on a selected number of samples from trials listed in this chapter. ISI in-human hepatocytic cell line (Huh7 cells) was blocked using serum to compare with previous ISI assays performed at the Jenner Institute. In VAC073 and VAC076, ISI was performed using plasma as no serum samples were collected in these trials.

11.4.4.1 VAC053 – R21/MM induces antibodies in UK adults that block sporozoite invasion *in vitro*.

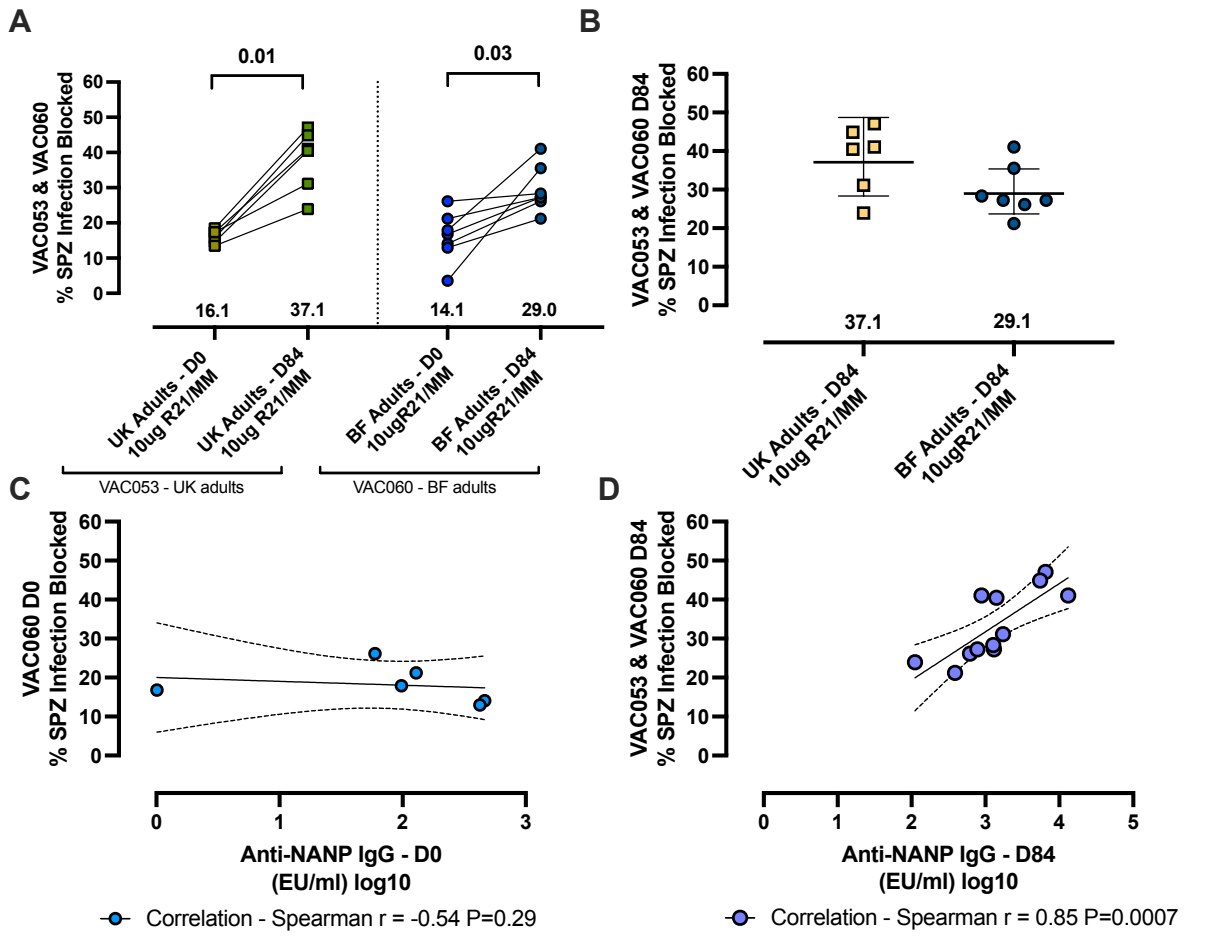


Figure 40 - VAC053 / 60 - Inhibition of sporozoite invasion.

A) *Percentage of inhibition that is increased between baseline (D0) and 1-month post-third dose (D84) for both VAC053 (left) and VAC060 (right). Samples at each timepoint are paired and matched – Wilcoxon Matched-Pairs signed rank test.* ***B)*** *Percentage of sporozoite invasion into human hepatocytes that was blocked by serum at 1-month post-third dose for UK adults (VAC053) and Burkinabe adults (VAC060). Mann-Whitney.* ***C)*** *Relationship between baseline (D0) anti-NANP IgG titres and percentage sporozoite inhibition amongst Burkinabe adults who presented with anti-NANP IgG titres at baseline. Spearman rank correlation with line of best fit calculated with linear regression with 95% CI.* ***D)*** *Relationship between 1-month post-third dose (D84) anti-NANP IgG titres and per cent sporozoite inhibition amongst UK adults and Burkinabe adults who presented with anti-NANP IgG titres at baseline. Spearman rank correlation with line of best fit calculated with linear regression with 95% CI.*

Geometric means with error bars indicating 95% CI. Values presented above the x-axis indicate geometric mean values.

Six UK adults in VAC053 G1 and six Burkinabe adults in VAC060 G1 immunised with 10µg $R21_{CBF}$ / 50µg MM were selected to assess antibody inhibition sporozoite invasion *in vitro*. Serum from VAC053 and VAC060 were selected at 1-month post-third dose (D84) and paired with matched baseline (D0). Limited numbers of mosquitos resulted in assessing only six participants from each trial.

Serum from UK adults at baseline in VAC053 blocked 16.1% of sporozoite invasion compared to the positive control, despite being negative for anti-NANP IgG. Burkinabe adults in VAC060 at baseline were able to block 14.1% of sporozoites invading hepatocytes compared to positive control - Figure 40A. There was no statistical difference in % ISI between UK and Burkinabe adults despite Burkinabe adults having pre-existing NANP IgG antibodies (155EU/ml) - Figure 27.

All adults had significant (P=0.01 and P=0.03) increases in % ISI between baseline samples and samples taken 1 month post third dose - Figure 40A. At 1 month post-third dose, no difference was observed in % ISI between UK adults (37.1% inhibition) and Burkinabe adults (29.0% inhibition) - Figure 40B.

Anti-NANP IgG titres at baseline for Burkinabe adults were correlated with % ISI which found a no association between antibody titres and ISI although the trend was negative (Spearman r = -0.54, P=0.29) - Figure 40C. At 1 month post-third dose, antibody titres and % ISI from both UK and Burkinabe adults were comparable and therefore % ISI data was merged to assess association. After three doses of R21/MM, an increase in antibody titres at D84 highly correlated with an increase in % ISI (Spearman r = 0.85, P=0.0007) - Figure 40D.

11.4.4.2 VAC065 – R21/MM induces antibodies in UK adults that inhibit sporozoite invasion *in vitro*

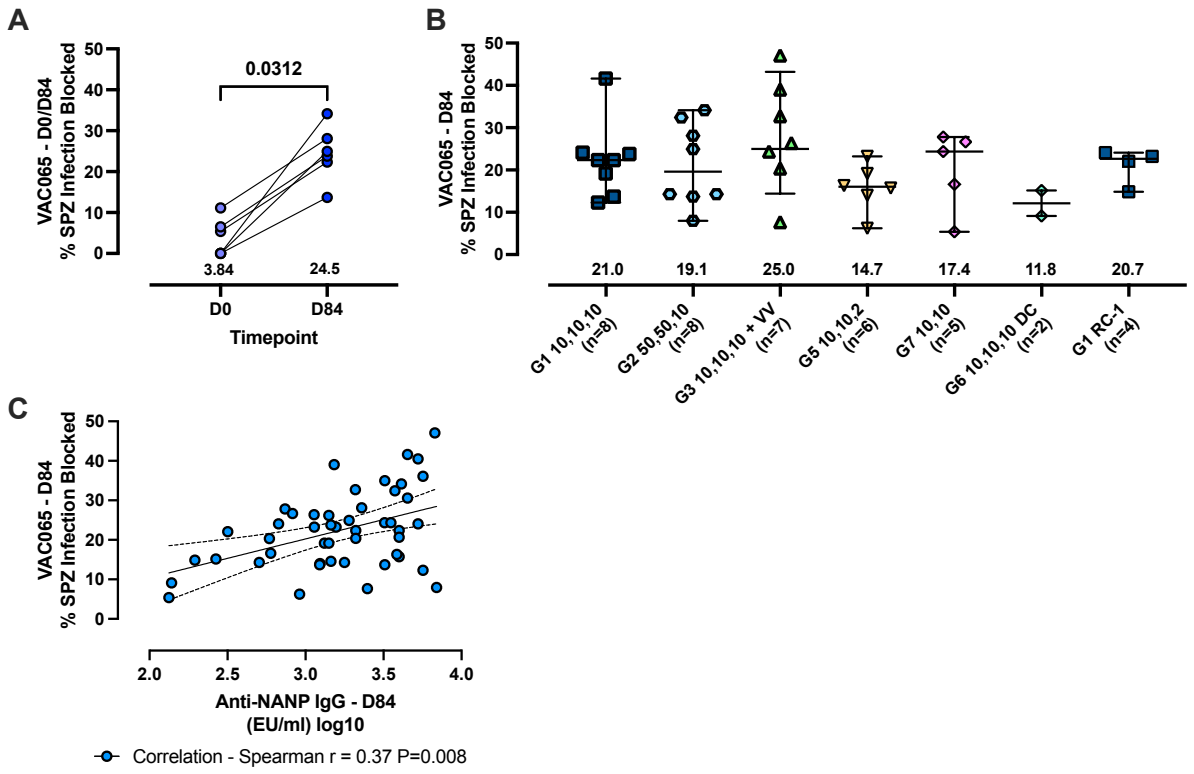


Figure 41 - VAC065 - Inhibition of sporozoite Invasion

A) *Percentage of inhibition that is increased between baseline (D0) and 1-month post-third dose (D84). Samples at each time point are paired and matched.* ***B)*** *Percentage of sporozoite invasion into human hepatocytes that was blocked by serum at 1-month post-third dose for UK adults. Comparison of vaccinated groups G1, G2, G3, G5, G7, G6 and Re-CHMI. No significant difference was calculated using Kruskal-Wallis corrected with Dunn's multiple comparisons.* ***C)*** *Relationship between C-1 NANP IgG titres and sporozoite blocking ability in individuals that received three full doses of R21/MM. Spearman rank correlation. The line of best fit was calculated via linear regression with 95% CI.*

Geometric means with error bars indicating 95% CI. Values presented above the x-axis indicate geomeans.

In VAC065, 40 adults immunised with varied doses of R21/MM across seven groups (G1, G2, G3, G5, G6, G7 and G1 RC-1) were selected to assess the functional ability of antibodies to inhibit sporozoite invasion *in vitro*. In addition, six samples at baseline were assessed as a negative control for the potential inhibitory mediators despite being negative for anti-NANP IgG. Adults had a significant increase (P=0.031) in the percentage inhibition of Spz from 3.5% at baseline (D0) to 24.5% at 1-month post-third dose (D84) - Figure 41A.

When comparing samples from 1-month post-third dose in all groups in VAC065, G1 (10, 10, 10µg R21) and G3 (10, 10, 10µg R21 with viral vectors) - both of which had the same dose regimen of R21/MM had the higher inhibition of Spz of 21.0% and 25.0% respectively, however not significant - Figure 41B. G6 had the lowest % ISI with 11.6% which corresponds to them having the lowest anti-NANP IgG titre. Anti-NANP IgG titres at 1-month post-third dose positively correlated (Spearman r = 0.37, P=0.008) with % ISI - Figure 41C.

11.4.4.3 VAC072 – Delayed third dose does not improve functional antibodies to block invasion

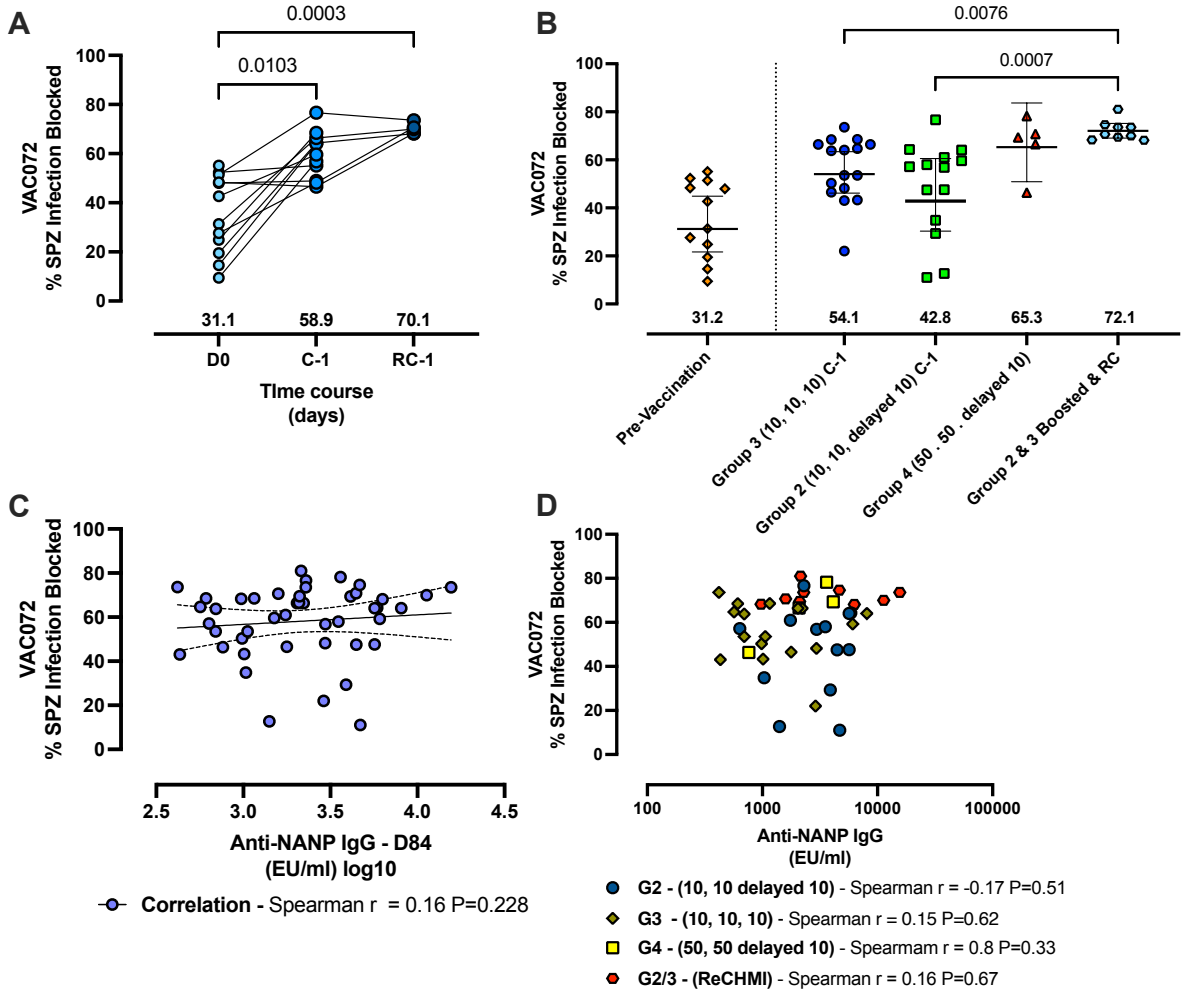


Figure 42 - VAC072 - Inhibition of sporozoite invasion

A) *Percentage of sporozoite inhibition that is increased between D0, C-1 and RC-1. Samples at each time point are paired and matched.* ***B)*** *Percentage of sporozoite invasion into human hepatocytes that was blocked by serum at 1-month post-third dose for UK adults. Comparison of vaccinated groups G2,*

G3, G4 and Re-CHMI and baseline ISI (pre-vaccinate). P=0.0027 - Kruskal-Wallis corrected with Dunn's multiple comparisons. ***C)*** *Relationship between C-1 NANP IgG titres and % ISI in individuals that received three full doses of R21/MM. Spearman r correlation. Line of best fit calculated with linear regression with 95% CI.* ***D)*** *Relationship between C-1 NANP IgG titres and sporozoite blocking ability in individuals separated by group. Spearman r correlation.*

All participants in VAC072 that underwent CHMI were tested for sporozoite inhibition with samples taken at one month post-third dose. Ten participants who consented to be re-challenged (Re-CHMI) were also tested for ISI and baseline samples were selected to form a time course for ISI. A relatively higher amount of sporozoite inhibition was reported among baseline samples with a mean of 30% Spz inhibition indicating potential non-specific factors contributing to inhibition such as cross-reacting antibodies or other molecular factors that could contribute to inhibition in naïve samples although this has not been fully investigated – Figure 42A. In a paired comparison between samples at baseline and at one month post-third dose, 10/12 samples from adults showed increased % ISI - Figure 42A.

Of adults who received 3 doses, G3 (10, 10, 10µg) reported a higher percentage of sporozoite inhibition - 54.1% compared with G2 (10, 10 delayed 10µg) - 42.8%. The highest % ISI was found in adults who received the higher delayed fractional dose in G4 (50, 50, delayed 10µg) - 65.3% sporozoite infection blocked. Participants who were boosted and re-challenged (receiving 4 doses) had the highest % ISI – 72.1% - Figure 42B. No correlation was identified between anti-NANP IgG titres and % ISI with all groups in VAC072 (Spearman r = 0.16, P=0.228) - Figure 42C.

11.4.4.4 VAC073 ISI – Increased % ISI was associated with increased anti-NANP IgG in Kenyan young children and infants, but not adults

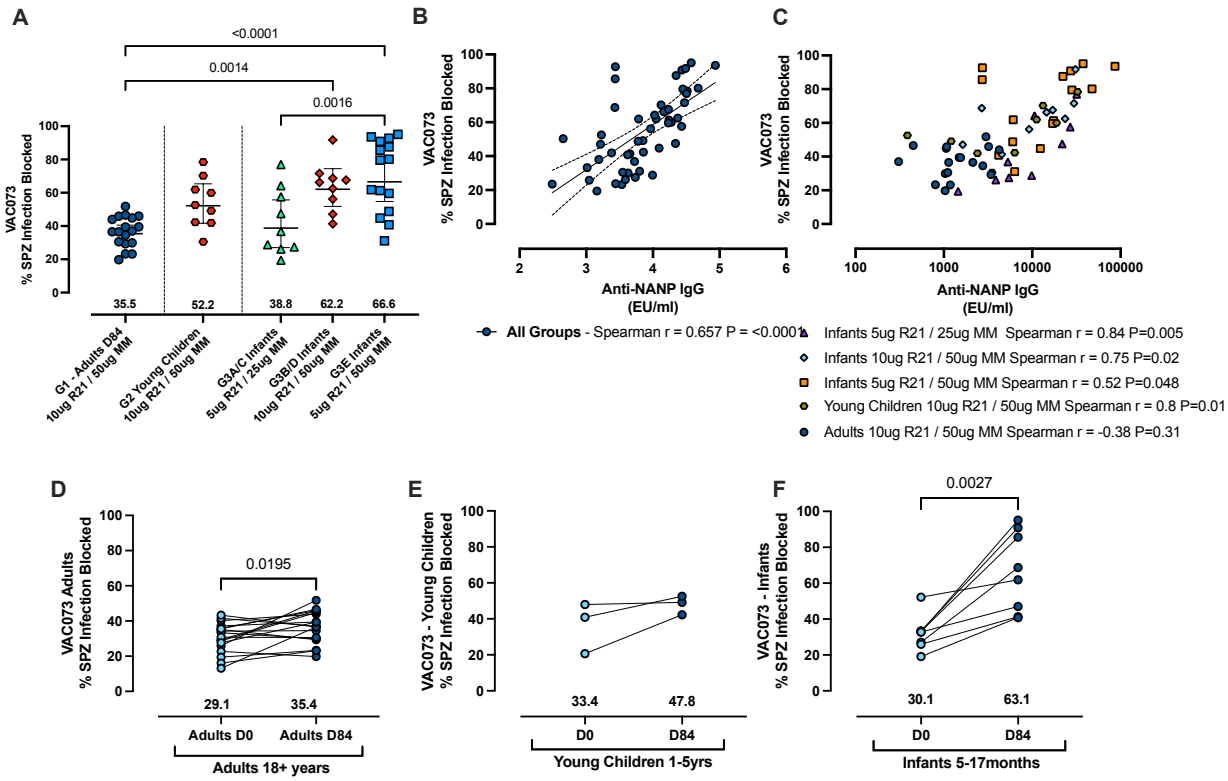


Figure 43 - VAC073 Inhibition of sporozoite invasion.

A) *Percentage of sporozoite invasion into human hepatocytes that was blocked by plasma at D84 (1-month post third dose) for Kenyan adults, young children, and infants. Error bars are the geometric mean of percentages with 95% CI.* ***B)*** *Relationship between ISI and anti-NANP IgG antibody titres for all groups and ages combined Spearman r=0.657 P=<0.0001.* ***C)*** *Relationship between ISI and anti-NANP IgG antibody titres separated by age and group. Correlations calculated using Spearman r.* ***D)*** *Percent increase of ISI among adults pre and post-vaccination. P=0.0195 Mann-Whitney.* ***E)*** *Percent increase of ISI among young children pre and post-vaccination.* ***F)*** *Percent increase of ISI among infants pre and post-vaccination. P=0.0027, Mann-Whitney.*

Values presented above the x-axis are geometric means of ISI percentages.

Plasma from Kenyan adults (G1) immunised with 10µg R21 and 50µg MM showed low ISI in comparison with young children and infants - Figure 43A. A 35.5% reduction in sporozoite inhibition among adults was found in comparison with positive controls. A 52.2% reduction in sporozoite invasion among Kenyan young children in G2 (10µg R21/50µg MM) was found in comparison with positive controls. However, no significant difference when compared with adults - Figure 43A.

Kenyan infants in G3A/C (5µg R21/25µg MM) demonstrated the lowest reduction in sporozoite invasion (38.8%) compared with infants in G3B/D (10µg R21/50µg MM) who reported a 62.2% reduction. Infants in G3E (5µg R21/50µg MM) who received low dose R21, and high dose MM reported the highest percentage reduction of 66.6% in sporozoite invasion with a significantly higher reduction between Kenyan adults and Kenyan infants who received the low dose of R21 and MM (5µg R21/50µg MM) - Figure 43A.

Comparing anti-NANP titres with % ISI for all participants and groups from samples taken one-month post-third dose, a positive correlation (Spearman r = 0.657, P<0.0001) can be observed - Figure 43B. Further stratifying correlations of anti-NANP IgG and sporozoite inhibition by each age group, a significant positive correlation can be observed for all infant groups and young children - Figure 43C. A negative correlation (Spearman r = -0.38, P=0.31) was found between adults – Figure 43C.

In our study, 9/18 Kenyan adults had detectable anti-NANP IgG titres at baseline - Figure 28B. Due to the limited number of mosquitos, a subgroup of participants from each group was selected to run an ISI. Eight adults were selected at D84 and paired with the baseline samples, ensuring each sample had a range of different antibody titres at both D0 and D84 to evaluate whether pre-existing immunity would affect sporozoite inhibition. No young children and infants had detectable AB titres at baseline, therefore only three baseline (D0) samples and nine, one-month post-third doses were selected for young children. Two infants from each group were selected at baseline. Nine D84 samples from infants who received 5µg R21/25µg MM, 9 from 10µg R21/50µg MM and all 15 infants who received 5µg R21/50µg MM were tested for ISI.

All Kenyan adults had significant increases in the percentage reduction of sporozoite invasion compared to baseline 29.% to 35.5% (P=0.01) - Figure 43D. All young Kenyan children recorded an

increase in percentage reduction of sporozoite invasion compared to baseline 33.4% to 47.8% (Sample size too small to show significance) - Figure 43E. All young children who were tested at baseline showed an increase in infection blocked in samples taken one-month post-third dose 30.1% to 63.1% (P=0.0027) - Figure 43F.

11.4.4.5 VAC076 ISI – Infants in Burkina Faso immunised with R21 produce functional antibodies to block sporozoite invasion

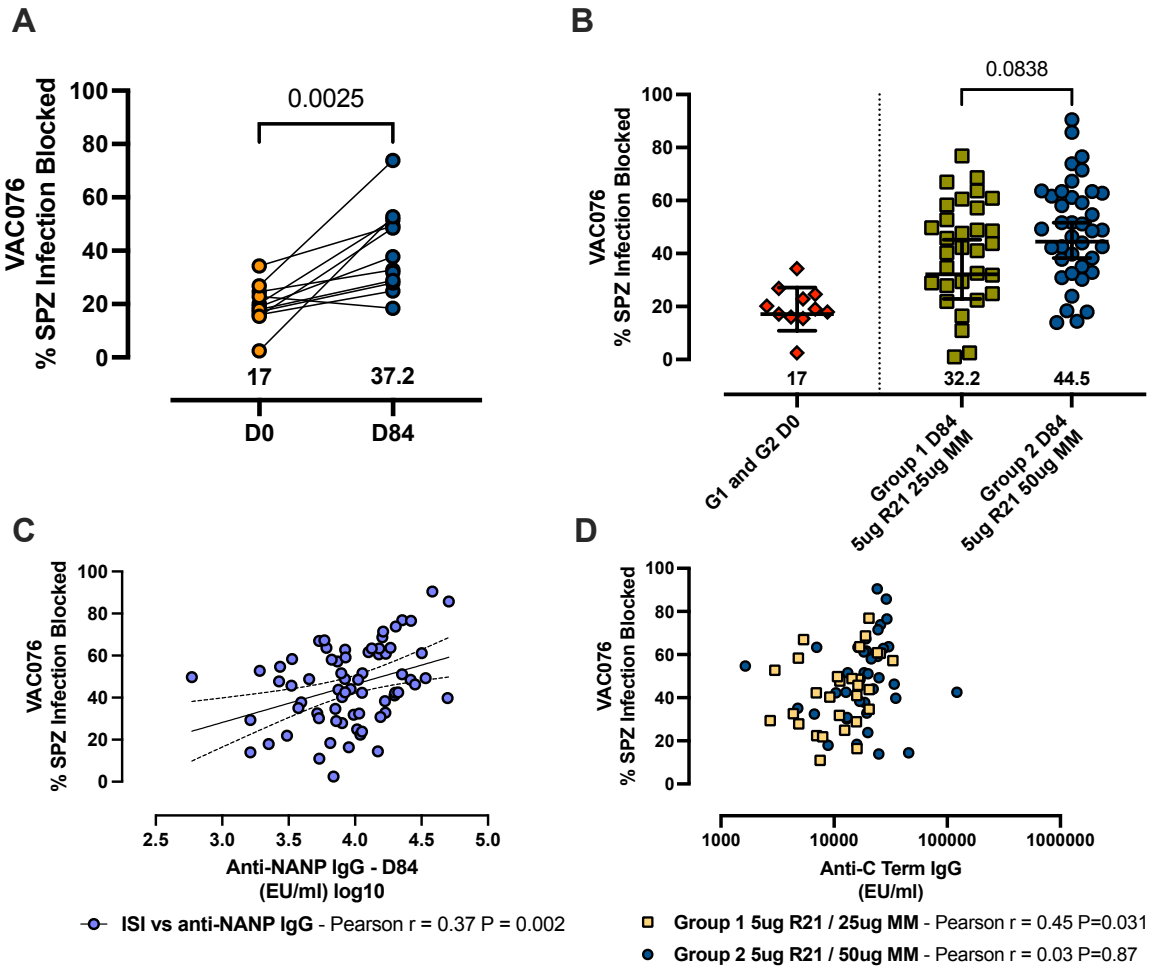


Figure 44 - VAC076 inhibition of sporozoite invasion.

A) *Percentage increase of ISI among adults pre and post-vaccination from both vaccinated groups in Burkinabe infants. P=0.0025, Mann-Whitney.* ***B)*** *Percentage of sporozoite inhibition into human hepatocytes that was blocked by plasma at D84 separated by group. P=0.0838, Mann-Whitney. C) Relationship between ISI and anti-NANP IgG antibody titres for both groups 1 and 2 combined. Correlation calculated by Spearman r=0.37 P=0.002. The line of best fit is calculated through a linear regression with 95% CI.* ***D)*** *Relationship between ISI and anti-NANP IgG antibody titre separated by group. Correlation calculated by Spearman r.*

In VAC076, 72 plasma samples (details of the VAC076 immunology cohort can be viewed in - Table 20 in Chapter 3) taken from G1 and G2 at D84 (one-month post-third dose) from infants in VAC076 were tested for vaccine-induced immunity to block sporozoite invasion *in vitro*. 31 samples from G1 (5µg R21/25µgMM) and 39 (G2 – 5µg R21/50µg MM) were selected from the immunology cohort. Of the 72, 12 samples were run from baseline and paired – Figure 44A. Due to the limited number of mosquitos, no other time points were evaluated.

In our study, 11/12 infants had a significant increase in percentage reduction of sporozoite invasion between baseline (D0) - 17% and one-month post-third dose (D84) - 37.2% - Figure 44A. Infants in G2 who received the higher dose adjuvant group had a higher percentage reduction of sporozoite invasion – 44.5% than G1 – 32.2%, however, this increase was not significant - Figure 44B.

A significant positive correlation (Spearman r = 0.37, P=0.002) was observed between anti-NANP titres and % ISI with G1 and G2 merged - Figure 44C. When responses were stratified by group, G2 (Spearman r = 0.45, P=0.004) had a stronger and more significant correlation compared with G1 (Spearman r = 0.29, P=0.13) – Figure 44D.

11.4.4.6 Different malaria transmission areas do not affect ISI in adults or infants

Levels of sporozoite inhibition from individuals immunised with various dose regimens of R21 / MM were compared using samples taken at baseline (D0) and 1 month post-third dose (D84 or D196) amongst adults in the UK (VAC053, VAC065 and VAC072) adults in Burkina Faso (VAC060) and adults in Kenya (VAC073). Furthermore, the degree of sporozoite inhibition was assessed between baseline and one-month post-third dose samples from infants in Kenya (VAC073) and Infants in Burkina Faso (VAC076).

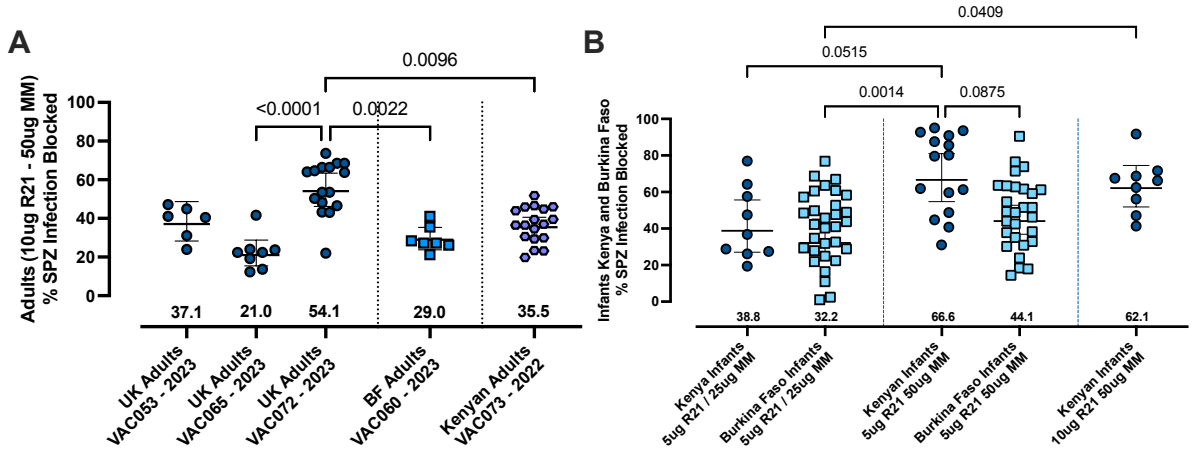


Figure 45 – Inhibition of Sporozoite Invasion across trials.

A) *Percentage of sporozoite inhibition into human hepatocytes that was blocked by plasma at D84 in adults across different trials and malaria endemic areas all of whom received 3 doses of 10µg R21 / 50µg MM administered 1 month apart. UK adults- VAC053, VAC065 and VAC072. Kenyan adults – VAC073 and Burkinabe adults – VAC060. Statistical analysis was calculated with Kruskal-Wallis and corrected with Dunn's multiple comparisons.* ***B)*** *Percentage of sporozoite inhibition into human hepatocytes that was blocked by plasma at D84 in infants in Kenya and Burkina Faso. Statistical analysis was calculated with Kruskal-Wallis and corrected with Dunn's multiple comparisons.*

Values presented above the x-axis indicate medians with IQR

Adults in the UK, Kenya and Burkina Faso exhibited a high degree variation of inhibition of sporozoite invasion from different trials in different malaria endemic areas who received the same dose regimen (10µg R21/50µg MM) - Figure 45A. Adults in the UK who received 10µg R21 manufactured in the SII (VAC072) had significantly higher % ISI (54.1%) than adults who received R21c VAC053 – 37.1% and VAC065 (21.0%) manufactured in the CBF. UK adults in VAC072 had a % ISI compared with Kenyan adults VAC073 – 35.5% and higher than Burkinabe adults VAC060 – 29.0% - Figure 45A.

Infants in Burkina Faso and Kenya who received 5µg R21/25µg MM had comparable percentage inhibitions of sporozoite invasion of 38.8% and 32.2%, respectively - Figure 45A. Infants in Burkina Faso who received 5µg R21/50µg MM had less reduction in sporozoite invasion, 44.5% than Kenyan infants 66.6% although not significant - Figure 45. Furthermore, Kenyan infants who received 5µg

R21/50µg MM had a significantly higher percentage reduction of sporozoite invasion than both Kenyan and Burkinabe infants receiving the lower dose adjuvant - Figure 45B.

11.5 Chapter 1 - Discussion

11.5.1 Overview

In this chapter, serology, T cell responses and functional antibody responses across the first six trials assessing different dose regimens of R21 in different age groups across different malaria transmission settings were assessed. Here follows an immunological overview of the first 6 clinical trials which led to the dose selection for Phase 3 evaluation.

11.5.2 Assay development

R21 with adjuvant Matrix M targets CSP on the sporozoite and is therefore primarily an antibody-mediated vaccine. R21/MM aims to induce high circulating antibodies targeting CSP on the sporozoite preventing it from invading hepatocytes and onward malarial infection. Therefore, measuring antibodies and specifically IgG responses to immunodominant repeat region NANP [423] is the primary immunological endpoint for R21 clinical trials. Prior to this DPhil project, the anti-NANP ELISA assay and the ISI assay had not been in use since December 2017 and therefore various optimisation steps were undertaken to ensure that the assay was comparable and had improved efficiency. Optimisation steps have not been presented due to word limitations but are planned to be included in an ELISA validation and ISI methodology paper. All data presented were performed after October 2020 allowing for highly reproducible results.

11.5.3 Requiring an Adjuvant

In the first-in-human trial assessing R21/MM (VAC053), safety data was favourable and immunogenic [404] and used to inform dose selection decisions in subsequent studies. VAC053 indicated that R21 administered with adjuvant MM had significantly higher antibody responses compared with groups receiving R21 alone. The R21 alone group had a small sample size (n=4) but responses were wide

enough to make adequate conclusions on the necessity of an adjuvant and are in line with reports from another subunit vaccine (RTS,S), [242] which uses AS01 to improve immune responses [240].

Among infants in Burkina Faso and Kenya the higher dose of Matrix M (50µg), irrespective of the dose of R21, produces significantly higher antibody titres than the lower dose of Matrix M (25µg). Further adding to the value of using an adjuvant with R21. The higher dose of Matrix M was subsequently selected for the Phase III trial of R21 currently underway in four countries in sub-Saharan Africa [221, 424].

11.5.4 Dose

The low dose R21 group (2, 2, 2µg) had lower antibody titres than the intermediate R21 dose group (10, 10, 10µg) and the high R21 dose group (50, 50, 50µg). The intermediate dose administered one month apart (referred to as the 'standard dose regimen') induced the highest antibody titres to the immunodominant repeat region NANP in UK adults (1106EU) and even higher antibody titres Burkinabe adults (2531EU). Antibody titres reported in Phase I (VAC053) have previously been associated with protection in UK adults immunised with RTS,S/AS01b [420, 422, 425] when assessed using the Jenner standardised NANP ELISA. Achieving equivalent antibody titres with a lower dose is encouraging as it allows for vaccine dose sparing, lower costs and potentially increased safety [426]. While sample sizes were small, it prompted further efficacy studies by CHMI which are evaluated in chapter 2.

11.5.5 Malaria endemic regions

This chapter examines humoral antibody responses across individuals living in different malaria-endemic areas. The standard dose regimen produced comparable antibody titres across UK adults (VAC053, VAC065 and VAC072), Kenyan adults (VAC073) and Burkinabe adults (VAC060). In addition, comparable immunogenicity was observed in Kenyan and Burkinabe infants. Malaria exposure can

reduce immune responses [90, 164, 427] such as B cell exhaustion or atypical memory B cells, however, this is mainly seen in adults. While sample numbers are small (n=66 from three different malaria endemic regions) this is a very positive result and can generate confidence in countries seeking to use R21/MM irrespective of whether a clinical trial has been conducted in their country.

11.5.6 Fractional and delayed regimens

Subunit vaccines such as R21 and RTS,S are commonly administered as 3 doses administered 1 month apart. There is evidence that altering the timing and concentration between doses can improve immunogenicity and efficacy including RTS,S [428-430], COVID-19 vaccines [431], and in other malaria vaccine programmes [432]. A reduced third dose (one-fifth) has been shown to improve somatic hypermutation and increase avidity ultimately improving antibody quality. Furthermore, a reduced dose can result in increased competition between B and T cells leading to greater affinity maturation. Delaying the third dose can have similar effects on B and T cell competition as well as increased priming of GCs leading to a greater differentiation of plasma cells and ultimately a quality antibody response. Reduced third doses have logistical advantages in dose sparing and increased timing between doses allows flexibility for remote communities whereby it may be difficult to adhere to a strict vaccine schedule.

Administration of the fractional third dose of R21 in VAC065 without the delay did not improve the antibody response, however a delayed fractional third dose in VAC072 significantly improved antibody responses in malaria naïve adults. However, participants who received the higher delayed fractional third dose (50, 50 delayed 10µg R21) had lower responses compared with G2 and G5 in VAC072, indicating that a higher dose can be detrimental and reduced antibody production from incorrectly primed B cells.

11.5.7 Two weeks post second dose

Antibody responses across all age groups and dose regimens appeared to peak at two weeks following the second dose of R21/MM (D42). After this, antibody titres appeared to decline slightly but with the addition of a third dose at 2 months (D56), antibody responses returned to levels seen at D42 and maintained until at least 3 months post-third dose. A hypothesis remains as to whether two doses of R21 are sufficient to induce high antibody titres but that three doses improve overall antibody durability. Whether two doses versus three doses effects efficacy is discussed further in Chapter 2.

UK adults in VAC065 who received two doses (10, 10µg $R21_{CBF}$) had lower peak antibody titres to three-dose groups, although this was not statistically significant. Two doses had a comparable reduction in antibody titre when looking at the fold reduction between D84 and D174 (4 months). A two-dose regimen of R21 inducing durable antibody titres is promising and may still provide some protection in individuals who fail to adhere to a three-dose vaccine schedule, however low sample sizes (n=7) limit the power of this analysis.

In VAC065, all groups (apart from G3 who received two additional viral vectored doses) had an approximately two-fold decrease in antibody responses to NANP between one month and four months post-third dose. This may be due to interference of viral vectors expressing ME-TRAP creating immune competition of cellular immune responses. In Rampling *et al.,* 2018 [420], it was suggested that MVA (ME-TRAP) enhanced the T cell response of RTS,S however this finding was not found with the same MVA and R21 in VAC065.

Burkinabe adults (VAC060) had improved antibody durability four months post-third vaccination compared to UK adults. This is possibly due to natural infection acting as a booster for low levels of

underlying immunity within a transmission site as well as low consistent antibody titres among not vaccinated controls.

11.5.8 Age

Kenyan infants and young children have significantly higher antibody titres after the primary series and boost when compared with adults. However, while antibody titres were higher in younger age groups, this was followed by a much greater antibody decline compared to adults indicating immune maturation [433].

In the Phase IIb efficacy trial (VAC076) in Burkina Faso, R21/MM had a good safety profile and was immunogenic after 3 dose primary series and while antibody titres did decline, the addition of a booster saw antibody titres return to levels seen at the peak. Furthermore, the addition of a booster improved antibody durability at 6 and 12 months compared to the same time points after the primary series. Adding to a delay can improve GC and antibody production. This is examined further in Chapter 3.

11.5.9 % ISI

During my research, I optimised and ran a functional assay known as ISI which aims to recreate a *Pf* infection *in vitro.* The ISI assay aims to assess whether antibodies produced from R21/MM have functional properties that can inhibit transgenic parasite-invading hepatocytes [434, 435]. Bowyer *et al.*, 2018 [425] showed that protected individuals immunised with various dose regimens of RTS,S/AS01 had significantly higher sporozoite inhibition compared to non-protected UK adults after CHMI. Furthermore, anti-NANP IgG correlated with % ISI in that study promoted that increased anti-NANP IgG is associated with increased protection.

All participants from all age groups immunised with R21 had increased ISI when measured at 1 month post-third dose compared to baseline. Indicating R21/MM produces antibodies that target sporozoites preventing onward malarial infection. All positive correlation was observed between % ISI and anti-NANP titre adding evidence that increased antibody response may result in increased protection.

All adults immunised with the standard dose regimen inhibit between 20-40% of Spz invasion from serum that has been diluted to 1%. Infants who received the higher dose of MM had increased ISI over the lower dose adjuvant. Infants and young children have significantly higher antibody titres and % ISI compared with adults irrespective of the dose. There was high assay variability between trials as seen in VAC072 with the standard dose regimen having significantly higher ISI compared to other trials. ISI data from VAC065 and VAC072 will be discussed further in Chapter 2 and ISI data from VAC076 will be addressed in Chapter 3 which assesses antibody data with protection.

Analysing trials separately, adults in VAC065 found no difference between the standard dose regimen and the fractional third dose. This corresponded with anti-NANP titre which also found no difference between groups. In VAC072 a delayed third dose group had lower ISI despite having increased antibody titres over the standard dose regimen however not significant. Further, adults who received the higher delayed fractional third dose (G5) group in VAC072 had the highest ISI when they had the lowest anti-NANP titre. While sample numbers are small, this reverse relationship between antibody titre and ISI is different to the rest of the data presented. Lastly, adults who were boosted at nine months after the first challenge, and subsequently re-challenged, showed significantly higher ISI compared to adults in VAC065 who were re-challenged but not boosted. Indicating that the delay between primary series boost is necessary and provides highly functional antibodies from antigen priming of germinal centres from the delay.

Infants in Kenya and Burkina Faso who received the same dose regimen had comparable % ISI. As seen with antibody data, the higher dose of adjuvant, irrespective of the dose of R21 administered, produced significantly higher ISI among infants. Results provide further confidence in R21 dose selection for the Phase III trial. Both Kenyan and Burkinabe infants were negative at baseline for NANP-specific antibodies therefore baseline correlations could not be performed. Kenyan and Burkinabe adults who did have detectable antibody titres had negative correlations with % ISI indicating that NAI does not inhibit sporozoite invasion and potentially there are other factors in serum which may be contributing to inhibition measured at baseline.

11.5.10 Cellular Immune Responses

The role of cellular-mediated immunity in infants, the target population, is difficult to study due to challenges in obtaining PBMCs in remote areas and requiring trained medical professionals to take blood from infants [163]. Secondly, cell-mediated immunity in infants may be less important with regard to CSP-based vaccines and more important in the liver stage in which T cells and TRMs function [172]. In addition, cellular-mediated immunity is mainly involved in the blood stage of malaria infection [103]. T cell responses by IFNγ ELISpot were examined after immunising with three doses of R21 and MM. R21 does not induce high IFNγ production amongst malaria naïve adults in the UK which corresponds with similar responses seen in malaria naïve adults immunised with RTS,S [330]. Furthermore, CSP is not a good target for a T cell response, which has been seen in studies using viral vectors encoding CSP which induced increased T cell immune responses but little VE.

11.6 Chapter 1 - Conclusion

In this chapter, it can be concluded that various dose regimens of R21 presented are immunogenic. As with other VLP vaccines, data shows that administering R21 with an adjuvant Matrix M significantly improves antibody titres compared to R21 alone in UK adults. Antibody titres remain consistent among participants who live in different malaria-endemic areas. Booster doses help return antibody titres to peak levels seen after the primary series as well as improving the overall durability of responses when measured at 6 and 12 months after vaccination. R21/MM induces functional antibodies that can block sporozoite invasion *in vitro* however it is clear that R21/MM induces very little T cell immune response. Accordingly, the results provide a promising basis for further research in the Phase III trials.

Chapter 2

12 Chapter 2 - Examining the effects of different dose regimens of R21/MM on efficacy in malaria naïve adults

12.1 Chapter 2 - Introduction

Chapter 1 reported immunogenicity involving antibody responses, in which T cell responses and the functional ability of antibodies to inhibit sporozoite invasion induced by varying dose regimens of R21 and Matrix M immunisation are presented. However, to truly show VE, one needs to prove that a vaccine elicits protection [343, 436]. Understanding the disease lifecycle, mode of transmission, and immune mechanisms involved are key to designing an effective vaccine [437].

VE is a measure of how well a vaccine prevents an outcome of interest (usually disease) compared to a control group (placebo) [438]. Once a trial is complete, the number of outcome variables in each group is compared and the relative risk (RR) is calculated to determine how well the vaccine lowers the risk of developing the outcome variable. A VE of 90% does not mean that 10% will fall ill but rather 90% fewer people will contract the disease if they become exposed. Distinguishing VE, effectiveness and efficiency are important terms to understand. Efficacy is a measure of vaccine capacity to perform in ideal conditions of a clinical trial. Effectiveness is how well a vaccine performs in the real world, outside of a clinical trial and efficiency is the production/distribution term used when assessing the outcome relative to the amount of resources used.

Initial safety and immunogenicity of three doses of RTS,S administered 1 month apart resulted in high immunogenicity to CSP, although participants presented with high reactogenicity after the third dose [253] resulting in delayed fractional third dose 7 months. This alleviated safety concerns and improved immunogenicity and protection [254, 255]. This led to the hypothesis that altering the immunisation

regimen can improve immunogenicity and efficacy. In 2016, a delayed fractional third dose of RTS,S administered at 7 months among 30 participants led to improved VE (86.7% [95% CI 66.8–94.6]), and increased antibody titre and avidity over the standard dose regimen (VE 62.5% [95% CI 29.4–80.1]) [439]. In 2018, a fractional third dose with no delay (2 months) found comparable high VE (88.9% [95% CI 43.3–98.4] to VE of the standard dose regimen (75% [95% CI 31.5–93.1]) and no change in immunogenicity [420]. Leading to the prospect of vaccine dose-sparing.

Examining novel malarial vaccine candidates has been accelerated through CHMI models. CHMIs provide critical information on pathogenesis, inform strain selection for vaccines, explore cross-protective immunity, identify potential CoP induced by vaccination, guide decisions on appropriate trial endpoints, and help to evaluate VE [393]. Details of CHMI models can be viewed in the introduction (8.11.4). In this chapter, malaria naïve adults underwent CHMI and Re-CHMI approximately 1 month post final dose which is when immune response are seen to have peaked.

In this chapter, immunogenicity will be assessed with R21 manufactured at two different sites and using different manufacturing methods. Secondly, immunogenicity induced from varied dose regimens of R21 and Matrix M impacts protection and whether any immunological associations with protection can be identified in malaria naïve adults after a CHMI.

12.2 Chapter 2 - Methods

12.2.1 R21 Vaccines (CBF and SII)

Details of both $R21_{CBF}$ and $R21_{SII}$ are described in the appendix methods (17.3.2).

12.2.2 Matrix M

Full details on the manufacturing of Matrix M can be viewed in the appendix methods (17.3.3).

12.2.3 Trial assessed

This chapter will focus on two Phase 2 trials (VAC065 and VAC072) which assessed efficacy after CHMI in malaria naïve adults (ages 18-45 years)in the UK after various dose regimens of R21 and adjuvant Matrix M. Full details of trial designs and immunology bleed schedules have been outlined in Methods (10.3.4).

- Table 11 - VAC065 Trial Design
- Table 12 - VAC072 Trial design

12.2.4 CHMI

Full details of the CHMI procedure are described in the Appendix (17.4.2).

12.2.5 VE

VE was calculated as 1 – estimated RR where relative risk (RR) is calculated by MV/MC where MV is the number of individuals diagnosed with malaria in the vaccinated group and MC is the number of individuals diagnosed with malaria in the control group (controls from both challenges were combined for analysis). Incidence rates were calculated as the number of subjects with malaria divided by the number of subjects at risk. The 95% confidence intervals (95% CIs) for VE were calculated with asymptotic estimates of the variances of the RR (precision-based estimates) without adjustment for

multiple comparisons. Differences in time to parasitemia were calculated by log-rank (Mantel-Cox) analysis of Kaplan Meier curves (not presented) by comparing time to PCR positivity in vaccinated groups with unvaccinated controls from each arm in both VAC065 and VAC072. A value of $p < 0.05$ was considered significant.

12.2.6 Sample size

VAC065 and VAC072 were the first efficacy studies involving $R21_{CBF}$ and $R21_{SII}$ with MM. As such, it was unable to be powered to detect efficacy between groups due to ethical considerations limiting the number of volunteers that should receive a vaccine regimen without prior evidence of efficacy. In addition, sample sizes were hindered due to practical limitations on volunteer recruitment and funding to compensate volunteers. Therefore, both studies are not able to detect statistical differences in efficacy between groups. However, a one-tailed Fisher exact test was used to assess individual groups to controls to unvaccinated controls. Further details on sample sizes are outline in Introduction (8.10.2.1).

12.2.7 Post-hoc power calculations

Using a two-sample t-test allowing for unequal variance it was shown that across VAC065 and VAC072 a 26% power was detected to observe differences in immunological associations between protected and non-protected participants.

12.2.8 Sample analysis and immunology assays

Vaccine studies in this chapter will focus on immunogenicity at one time point on the day before CHMI (C-1) or re-challenge (RC-1) which is viewed as the peak immune response after immunisation with R21/MM. In addition to immunogenicity presented in Chapter 1, further immunology assays investigate IgG-specific responses to the C term. Avidity to both NANP and C term peptides will be described. Subclass and Isotype antibodies are measured and described. IgG to NANP, C term, HBsAg

and full-length R21 protein measured by multiplex assay MSD will be measured. Finally, inhibition of ISI assays to functional antibodies is induced in volunteers immunised with R21/MM. Immune responses measured will be stratified by protection status to assess any associations with protection.

12.2.9 Post-hoc sample analysis to determine antibody threshold associated with protection

Quantifying anti-NANP IgG responses induced from R21 was the primary immunological endpoint written into the analytical plan. After completing the anti-NANP ELISA assays I undertook a post-hoc analysis of antibody titre to identify an antibody titre that could act as a statistical threshold that could be associated with increased protection among malaria naïve adults. This post-hoc exploratory analysis began with anti-IgG data from VAC072. All antibody data from all groups was merged and the responses were stratified based on the protection status after CHMI. Upon viewing the data, a cluster of unprotected participants was viewed who had an ELISA titre <1100EU/ml. Using this value, antibody data of protected and unprotected was further stratified by 1100EU/ml and a Fisher's exact statistical test was used to test the significance of 1100EU/ml being associated with increased protection. This same approach was used for VAC065 anti-NANP IgG data. In addition, the post-hoc analysis was conducted on anti-C term IgG data from VAC065 and VAC072.

12.2.10 Multivariate analysis

Details outlining the statistical methods for the multivariate analysis can be viewed in the methods and trial design section (10.4.5).

12.3 Chapter 2 - Objectives

- Assess whether manufacturing R21 in CBF or SII impacts efficacy and immunogenicity.
- Characterise immune responses measured at 1-month post third dose or 1-month post boost after being immunised with different dose regimens R21/MM.
- Determine whether altering dose regimens and timing between doses of R21/MM impacts efficacy and immunogenicity.
- Identify any immunological associations with protection found amongst UK adults who undergo CHMI after being immunised with R21/MM.
- Assess whether functional antibodies measured by ISI are associated with protection among malaria naïve UK adults.

12.4 Chapter 2 - Results

12.4.1 Manufacturing R21 in the UK (CBF) vs India (SII)

12.4.1.1 R21 manufactured in SII produces immunogenicity comparable to R21 manufactured in the UK among UK adults

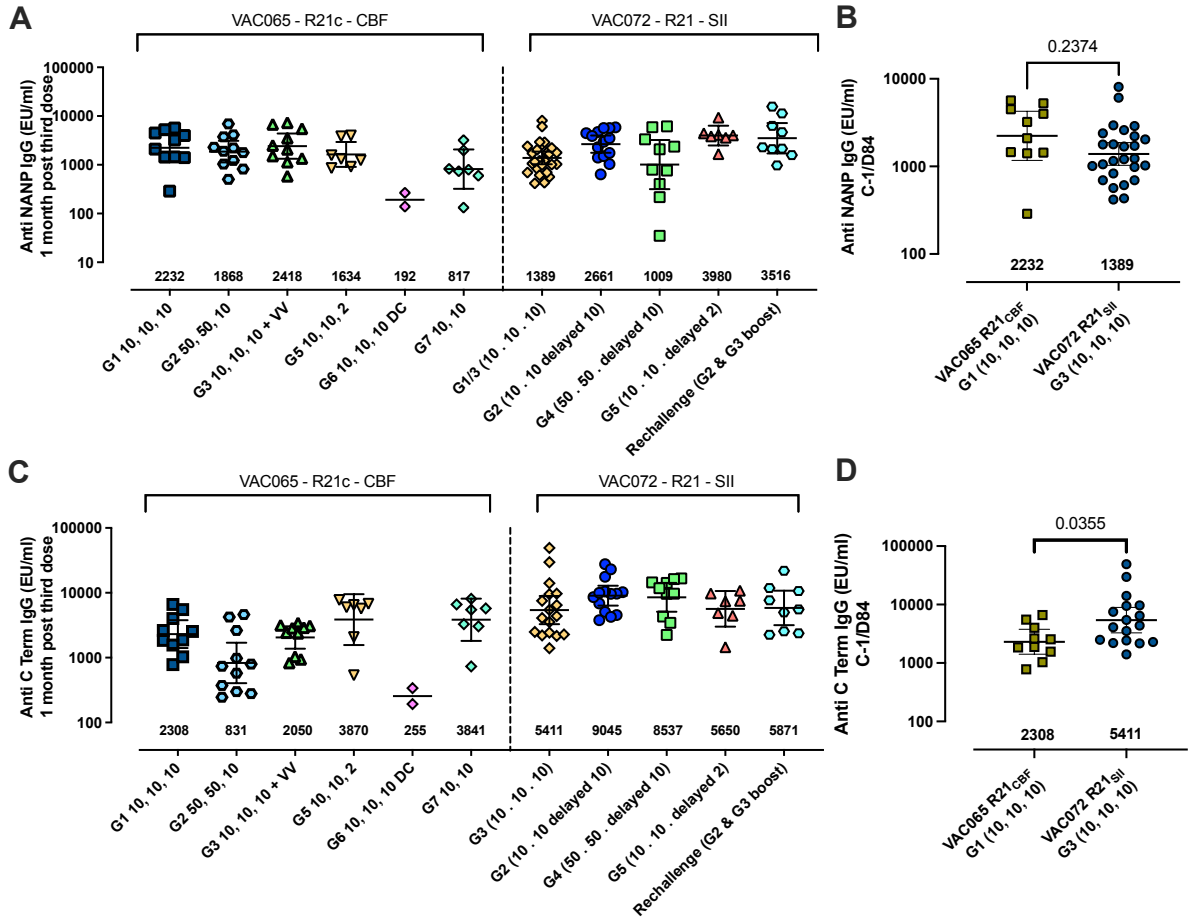


Figure 46 – Anti-NANP titres among adults in VAC065 ($R21_{CBF}$) vs VAC072 ($R21_{SII}$)

A) *Geometric mean anti-NANP IgG titres at 1-month post-third dose from both VAC065 ($R21_{CBF}$) and VAC072 ($R21_{SII}$), vertical dashed indicates the separation of both trials.* ***B)*** *Direct comparison of geometric means of ant-NANP IgG from identical dose regimens (10, 10, 10) from both VAC065 ($R21c_{BF}$) and VAC072 ($R21_{SII}$).* ***C)*** *Geometric means anti-C term IgG from both VAC065 ($R21c_{BF}$) and VAC072 ($R21_{SII}$), vertical dashes indicate separation of both trials.* ***D)*** *Direct comparison of geometric means of ant-NANP IgG from identical dose regimens (10, 10, 10) from both VAC065 ($R21c_{BF}$) and VAC072 ($R21_{SII}$)*

Values are geometric means with error bars to indicate 95% CI. Values presented above the x-axis indicate geometric specific per group. P-values in Figures B and D indicate significance according to Mann-Whitney. (DC - delayed challenge)

Both $R21_{CBF}$ manufactured at the CBF and $R21_{SII}$ manufactured in SII induce a range of anti-NANP IgG titres - (Figure 46A) and anti-C term IgG titres – (Figure 46C) from different dose regimens of R21/MM.

To assess whether different manufacturing processes (12.2.1) impact immunogenicity, the same dose regimens of both $R21_{CBF}$ and $R21_{SII}$ (10µg R21/50µg MM) administered one month apart were directly compared from both trials. Anti-NANP IgG measured from G1 in VAC065 ($R21_{CBF}$) induced higher GMTs than G1/3 in VAC072 ($R21_{SII}$) – (2232EU [95% CI 1170 – 4260]) versus (1389EU [95% CI 1031 – 4260]) respectively, although this was not found to be statistically significant (P=0.24). Thus, $R21_{CBF}$ and $R21_{SII}$ produce comparable anti-NANP IgG titres - Figure 46B. Conversely, significantly higher anti-C term IgG were found in VAC072 ($R21_{SII}$) – (5411EU [95% CI 3276 – 8939]) compared with VAC065 ($R21_{CBF}$) – (2308EU [95% CI 1409 – 3779])- Figure 46D. Anti-C term IgG titres will be repeated to confirm the result.

Anti-NANP and anti-C term IgG titres are not directly comparable due to different coating concentrations of peptide and development times. Furthermore, this assay is novel to this thesis and was developed in 2021, after NANP IgG titres were assessed.

12.4.2 VAC065 Efficacy

VAC065, was split into challenge A and challenge B: Challenge A assessed efficacy among participants in G1-3 and G4a, whereas challenge B assessed efficacy among G5-7, G4b and sterilely protected participants from G1 in challenge A who consented to be boosted and re-challenged.

Table 17 – VAC065 efficacy

The table indicates group, challenge A or B, dose of $R21_{CBF}$ and regimen, number per group, number of sterile protected, and percentage protection per group compared with 12 infectivity controls who did not receive any vaccines prior to challenge. Of the 12 infectivity controls, 2 did not meet diagnostic criteria for malaria infection. RC; re-challenge, CHMI.

**Log-rank (Mantel-Cox) efficacy compared Groups 1,2 and 3 with n=6 unvaccinated controls from challenge A and Groups 5, 6 and 7 with n=6 unvaccinated controls from challenge B, using days to parasitaemia by blood film for diagnosis.*

*** VE = 1-RR (One-tailed Fishers exact test) with 95%CI*

Group	Challenge	Regime (dose $R21_{CBF}$ µg)	Number Recruited	N sterilely protected	Protection %	P-value*	Vaccine Efficacy, (%) **	95% CI	P-value
1	A	10, 10, 10	11	9/11	82	0.0009	80	60 - 94	0.0028
1	B	10, 10, 10 RC	5	3/5	60	0.184	72	-12 - 93	0.117
2	A	50, 50, 10	11	7/11	64	0.0043	63	14 - 86	0.029
3	A	10, ChAd63, 10, 10, MVA ME-TRAP	9	6/9	67	0.0057	69	17 - 90	0.029
4a*	A	Infectivity Controls	6	1/6	n/a	n/a	n/a	n/a	n/a
5	B	10, 10, 2	7	5/7	71	0.027	77	21 - 94	0.029
6	B	10, 10, 10 (delayed CHMI)	2	0/2	0	0.89	n/a	n/a	n/a
7	B	10, 10	7	4/7	57	0.079	65	-8 - 89	0.947
4b*	B	Infectivity Controls	6	1/6	n/a	n/a	n/a	n/a	n/a

Participants in VAC065 challenge A, 5/6 unvaccinated infectivity control participants (group 4a) developed malaria with a median time to diagnosis by blood film of 11.25 days (IQR 11-12, SD 0.45). Among vaccinated participants, 82% (9/11) of G1 (10, 10, 10µg $R21_{CBF}$), 64% (7/11) of G2 (50, 50, 10µg $R21_{CBF}$) and 67% (6/9) of G3 remained undiagnosed with *Pf* 21 days post-CHMI. When all vaccine groups from challenge A (Groups 1, 2 and 3) are combined, we see a significant delay in parasitaemia compared with challenge A controls (per-protocol log-rank (Mantel-Cox) analysis at all tested

thresholds using Kaplan Meier curves (not presented). Group 1 reported the highest VE of 80% [95% CI; 60-90] - Table 17.

In challenge B, 5/6 unvaccinated infectivity control participants (group 4b) with a with a median time to diagnosis of 13.0 days (IQR 12-14.5, SD 1.05). 71% (5/7) of G5 (10, 10, 2µg $R21_{CBF}$), 0% (0/2) of G6 (delayed challenge) and 57% (4/7) of G7 (10, 10µg $R21_{CBF}$) were protected and undiagnosed with *Pf* 21 days post-CHMI – Table 17. When all vaccine groups from challenge B (Groups 5, 6 and 7) are combined, we see a significant delay to parasitaemia except for G6 compared with challenge B controls (per-protocol log-rank (Mantel-Cox) analysis). Five protected subjects from group 1 agreed to be re-challenged (at challenge B) with 60% (3/5) remaining protected at 7.5 months after vaccination by log-rank (Mantel-Cox test) analysis compared with unvaccinated controls Table 17. Due to small sample sizes, the CI for the estimates of VE is wide Table 17, resulting in insufficient statistical power to provide meaningful conclusions of group-to-group comparisons.

This study (VAC065) saw the first cases (one in each challenge) of an unvaccinated infectivity volunteer failing to develop clinical malaria in a CHMI study conducted at the University of Oxford. Similar instances have been reported in other studies [258]. Controls failing to be infected poses questions to the validity of the challenge, estimates of VE using RR consider non-infected controls. Further discussion on the outcome of this challenge are discussed in the discussion.

12.4.3 VAC065 parasite quantification 7.5 days post-CHMI

It is worth assessing whether anti-sporozoite vaccines can delay blood-stage malaria infection when comparing vaccinated individuals with infectivity controls. As such, G3 were administered ChAd63 and MVA encoding ME-TRAP to promote cellular CTL immunity to liver-stage infection involving hepatocytes which could prevent further protection or delay blood-stage infection further. Here

parasite density is only presented for challenge A, as it was viewed as a 'hotter' challenge meaning that unprotected vaccinees and controls were diagnosed with malaria significantly earlier after CHMI.

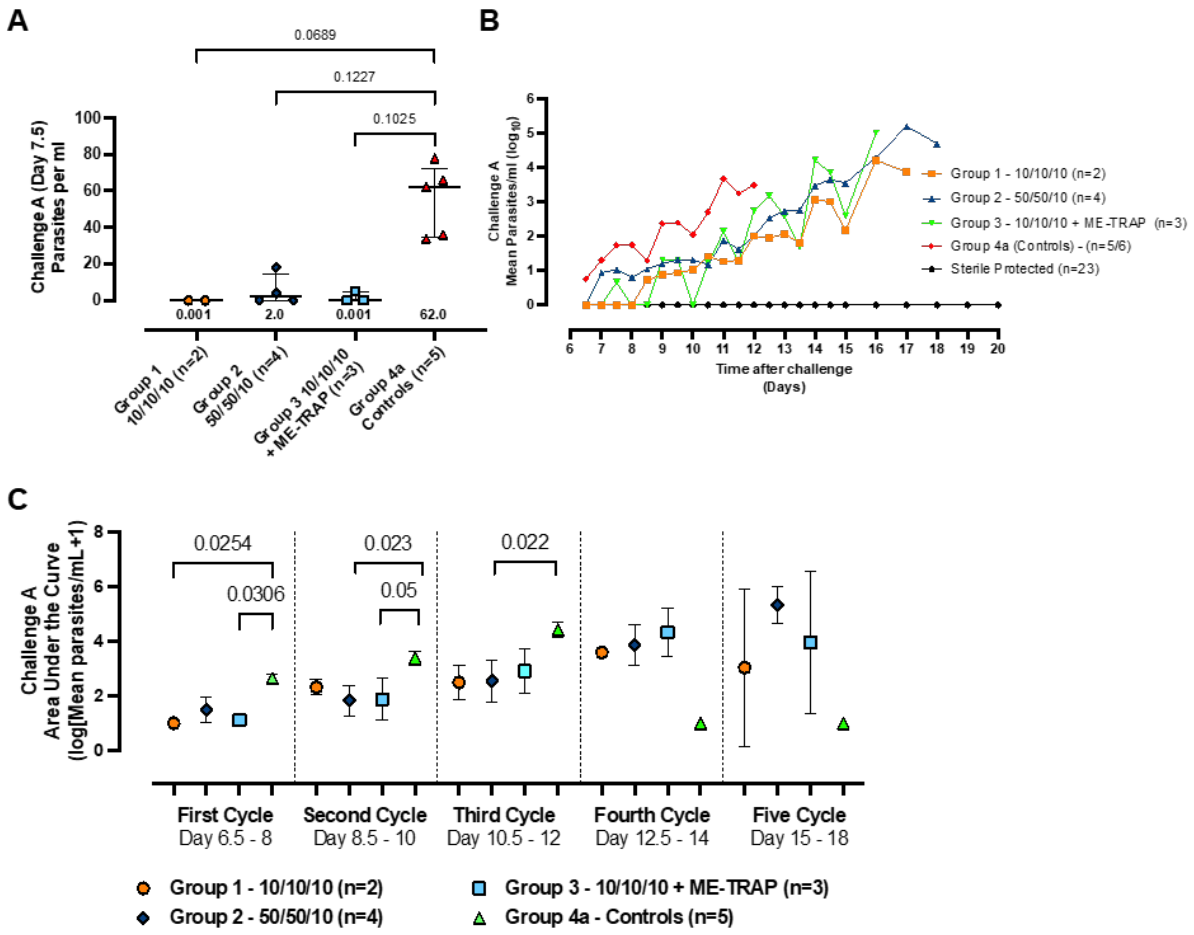


Figure 47 – VAC065 Parasite quantification by qPCR

Parasite quantification as measured by qPCR 6.5 days after CHMI. **A)** *Parasite burden measured by qPCR at 7.5 after CHMI. Medians with error bars indicating IQR. Kruskal-Wallis and corrected with Dunn's multiple comparisons test. Values presented above the x-axis indicate medians.* **B)** *Comparison of mean parasite density, between grouped vaccinees and control volunteers in challenge A.* **.** *Fractions represent the number of infected volunteers included in Figure.* **C)** *Area under the curve (AUC) analysis for parasite densities comparing controls to vaccinated groups in 5 replication cycles after CHMI, day 6.5-8 (the first cycle after hepatocyte release), days 8.5–10 (the second cycle), and days 10.5–12 (the third cycle), day 12.5-14 (the fourth cycle) and day 15-18 (the fifth cycle). Means of log [parasite density + 1] were compared for each vaccine group to those of controls, using the Kruskal-Wallis test within each cycle.*

At day 7.5 post-CHMI, controls had higher parasitaemia (52.3 p/ml [95% CI 32.7 – 83.5]) compared to unprotected vaccinees who all, apart from 2, were negative for malaria parasites at day 7.5 post-CHMI – Figure 47A. Parasite density was measured by qPCR every 12 hours starting 6.5 days post-CHMI. The

48-hour blood stage lifecycle can be seen in Figure 47B whereby parasite density increases exponentially every 48 hours and decreases within each 48-hour cycle. During the first (day 6.5 - 8), second (day 8.5 – 10) and third (day 10.5 – 12) blood stage replication cycles had reduced parasite densities in vaccinated groups compared with unvaccinated controls but ultimately led to a diagnosis - Figure 47B.

To show whether vaccinating with different dose regimens of R21/MM impacted the delay to diagnosis, an exploratory analysis of parasite densities measuring area under the curve (AUC) was conducted. The addition of ChAd63 and MVA encoding ME-TRAP to the 3 R21/MM vaccinations has no additional reduction in parasite density at the first second or third replication cycle compared with R21/MM alone - Figure 47C.

12.4.4 VAC072 efficacy

VAC072 was split into challenge A and challenge B: Challenge A assessed efficacy among participants in G2 and G3. Challenge B assessed efficacy among 4 participants in G4 along with sterilely protected participants protected in challenge A who consented to be boosted and re-challenged. Group 4 had reduced sample numbers due to caution among participants in travelling during the COVID-19 pandemic.

Table 18 – VAC072 efficacy

The table indicates group, challenge A or B, dose of $R21_{SII}$ and regimen, number per group, number of sterile protected, and percentage protection per group compared with 12 infectivity controls who did not receive any vaccines prior to challenge. Of the 12 infectivity controls, 2 did not meet diagnostic criteria for malaria infection. RC; re-challenge, CHMI, n/a = not applicable and nc = not challenged.

**Log-rank (Mantel-Cox) efficacy compared Groups 2 and 3 with n=6 unvaccinated controls (Group 6) from challenge A and Groups 4, 2 RC and 3 RC with n=6 unvaccinated controls (Group 7) from challenge B, using days to parasitaemia by blood film for diagnosis.*

*** VE = 1-RR (One-tailed Fisher's exact test) with 95% CI*

RC; re-challenge, CHMI, n/a = not applicable and nc = not challenged.

Group	Challenge	Regimen (dose of R21 µg)	No. Recruited	No. Sterilely protected	Protection %	P-value*	Vaccine Efficacy, (%) **	95% CI	P-value
1	nc	10, 10, 10	10	nc	nc	nc	nc	nc	nc
2	A	10, 10, delayed 10	12	9/12	75	<0.0001	77	57 - 91	0.003
3	A	10, 10, 10	18	11/16	69	<0.0001	66	35 - 85	0.012
6	A	Infectivity Controls	6	0/6	n/a	n/a	n/a	n/a	n/a
4	B	50, 50, 10	11	2/4	50	0.14	77	-0.09 - 94	0.14
5	n/a	10, 10, 2	8	nc	nc	nc	nc	nc	nc
2/3 RC^	B	Boost 10	9	8/9	89	0.004	91	58 – 98	0.0004
7	B	Infectivity Controls	6	1/6	n/a	n/a	n/a	n/a	n/a

In challenge A, 6/6 unvaccinated participants assigned to G6 who acted as infectivity controls underwent CHMI, all were diagnosed for malaria. In challenge B, 5/6 unvaccinated controls assigned to G7, were diagnosed with malaria post-CHMI. Since not all infectivity controls in G7 were infected, controls in challenge A and challenge B were not merged as in VAC065 mentioned above.

In G3 (10, 10, 10µg), 68% (11/16) participants were sterilely protected providing VE of 66% (95% CI 35-85%) when compared to infectivity controls. In G2 (10, 10, delayed 10µg), 75% (9/12) participants were sterilely protected with a VE of 77% (95% CI 57-91%) when compared to infectivity controls. Both

G3 and G2, had significant protection as calculated by 1-RR and delayed to parasitaemia compared to infectivity controls calculated by log-rank (Mantel-Cox) – Table 18.

In G4 (50, 50, delayed 10μg), 2/4 participants were sterilely protected with a VE of 77% (95% CI -0.09-94%) when compared to infectivity controls however large confidence intervals do need to be considered. Participants in G2/G3 who were boosted 10μg R21 50μg MM eight months after the first challenge and re-challenged one month later, 8/9 were sterilely protected with a VE of 91% (95% CI 58-98%) compared to infectivity controls and a significant delay to parasitaemia in challenge B – Table 18.

As with VAC065, due to small sample sizes, the CI for the estimates of VE are wide (Table 18), resulting in insufficient statistical power to provide meaningful conclusions of group-to-group comparisons. Furthermore, another unvaccinated infectivity control failing to be infectivity poses questions on CHMI process which is discussed further in the discussion.

12.4.5 Anti-NANP IgG greater than 1100EU/ml are associated with protection

Participants in VAC065 ($R21_{CBF}$) and VAC072 ($R21_{SII}$), immunised with $R21_{CBF}$ and $R21_{SII}$ respectively produced high anti-NANP IgG titres one month post-third dose (D84) – Figure 46. A post-hoc analytical approach outlined in chapter methods (12.2.9) was used to try and identify any potential antibody threshold titres for both anti-NANP and anti-C term IgG that could be used to stratify protected and unprotected participants in an attempt to observe an association with protection.

12.4.5.1 VAC065 – High anti-NANP IgG antibodies induced from R21$_{CBF}$/MM.

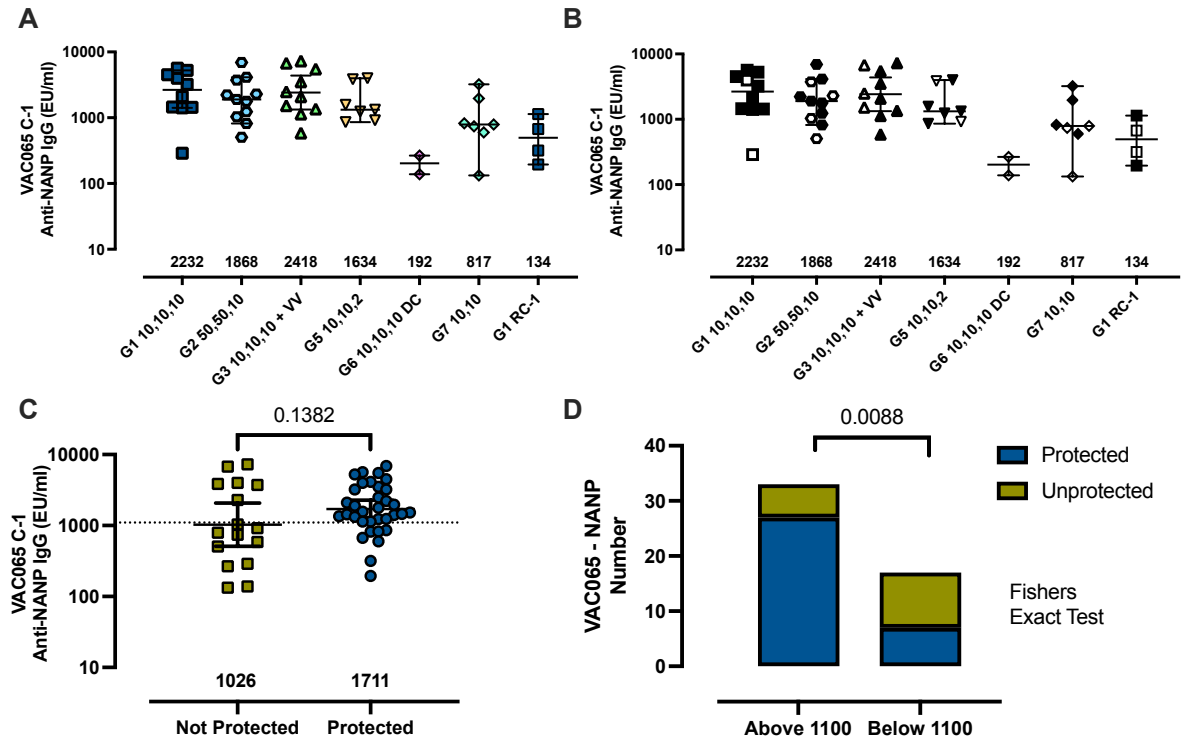


Figure 48 – VAC065 anti-NANP IgG associations with protection

Geometric mean anti-NANP IgG among all groups in VAC065. ***B)*** *Geometric mean anti-NANP IgG among all groups in VAC065 with protected participants identified. Closed shapes indicate sterilely protected participants and open shapes indicate infected participants.* ***C)*** *Geometric mean anti-NANP IgG separated by protected and not protected. P=0.138 Mann-Whitney. The horizontal dotted line Y=1000 represents the threshold of protection* ***D)*** *Participants grouped by an anti-NANP IgG titre above or below 1100EU. P=0.008 Fisher's exact test.*

Geometric means with error bars indicate 95% CI. Values presented above the x-axis indicate geometric mean values. VV = viral vectors, DC = delayed challenge and RC-1 = re-challenge minus 1

All participants in VAC065 underwent CHMI. Participants in G1 and G3 (10, 10, 10µg R21$_{CBF}$) presented with the highest anti-NANP IgG titres at D84, with G1 having a GMT – 2232EU [95% CI 1170 – 4260] and G3 having a GMT of 2418EU [95% CI 1333 – 4386], respectively. There was no indication that the addition of viral vectors affects anti-NANP titres. Participants in G2 and G5 who received a fractionated third dose presented with lower anti-NANP IgG at D84 than the standard dose regimen with G2 having a GMT of 1868EU [95% CI 1119 – 3118] and G5 having a GMT of 1634 [95% CI 910 – 2933]. G7 participants receiving two doses (10, 10µg R21c) presented with the lowest anti-NANP titres (GMT 817EU [95% CI 323 – 2068]) – Figure 48A.

Protected and unprotected participants across all groups of VAC065 had a range of antibody titres – Figure 48B. To assess whether a threshold of anti-NANP IgG titre is associated with protection, all participant responses at D84 were stratified according to CHMI outcome – Figure 48C. Protected participants in VAC065 had a higher anti-NANP IgG (GMT 1711EU [95% CI 1287 – 2273]) compared with unprotected (GMT 1026EU [95% CI 509 – 2067]), although this was not statistically significant.

A cluster of unprotected participants was observed to have an anti-NANP IgG titre of <1100EU. Protected and unprotected participants were further stratified by anti-NANP IgG titre of above and below 1100EU – Figure 48D. Protected participants who had an anti-NANP IgG titre greater than 1100EU were significantly more likely to be protected measured by Fisher's exact test (p=0.0088).

12.4.5.2 VAC072 - A delayed and delayed fractional third dose improves anti-NANP IgG and 1100EU/ml continues to associate with increased protection.

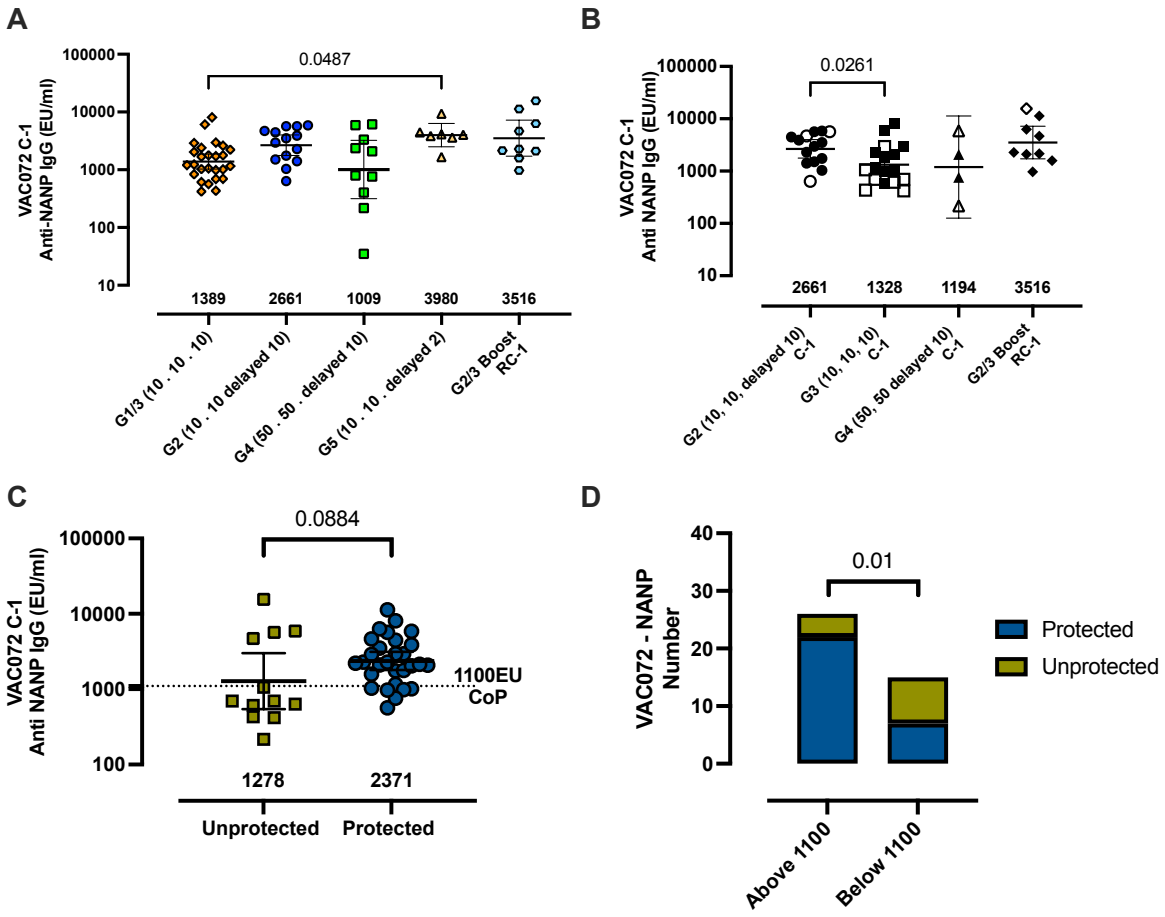


Figure 49 - VAC072 NANP IgG associates with protection

A) *Geometric mean anti-NANP IgG among all groups in VAC072.* ***B)*** *Geometric mean anti-NANP IgG among all groups in VAC072 with protected participants identified. Closed shapes indicate sterilely protected participants and open shapes indicate infected participants.* ***C)*** *Geometric mean anti-NANP IgG separated by protected and not protected. P=0.088 Mann-Whitney. Horizontal dotted line Y=1100 represents the threshold of protection* ***D)*** *Participants grouped by an anti-NANP IgG titre above or below 1100EU. P=0.01 Fisher's exact test.*

Geometric means with error bars indicate 95% CI. Values presented above the x-axis indicate geometric mean values. C-1 = Challenge minus 1 day, and RC-1 = re-challenge minus 1 day

In VAC072 R21/MM induces high anti-NANP titres in UK adults. Full anti-NANP IgG titres are presented in Chapter 1 - Figure 32. Here, the focus is on peak antibody responses measured one day prior to CHMI.

In VAC072 - Figure 49A presents the anti-NANP IgG of all participants one-month post-third dose. Figure 49B presents the anti-NANP IgG of participants who underwent CHMI. In G2 (10, 10 delayed 10μg R21), 12/16 participants recruited underwent CHMI. In G3 (10, 10, 10μg R21), 16/18 underwent CHMI. In G4, (50, 50, delayed 10μg R21), 4/11 participants underwent CHMI. Of the 10 participants who received a fourth booster dose, 9 underwent Re-CHMI.

To evaluate immune associations with protection in UK adults after varied doses of R21/MM, G2 and G3 were focussed due to larger sample sizes. G2 had higher efficacy comparable to G3 – Table 18 and had yet higher anti-NANP IgG – Figure 49A. However, protected, and unprotected participants had mixed responses, with some participants with the highest anti-NANP IgG not protected - Figure 49. In G2, 2/3 of unprotected participants had anti-NANP IgG greater than 4500EU - Figure 49B.

Anti-NANP IgG were merged across all participants who underwent CHMI and stratified by protected and unprotected. Protected participants in VAC072 had a higher GMT (2371EU [95% CI 1793 – 3005]) compared to unprotected (GMT 1278EU [95% CI 544 – 3005]), however this was not significant - Figure 49C. A cluster (8/12) of unprotected participants was identified to have an ELISA unit less than 1100, as observed in VAC065 - Figure 48C. Grouping protected and unprotected participants by an anti-NANP IgG titre above or below 1100EU indicated a significant difference with a contingency P=0.015 by Fisher's exact test - Figure 49D.

12.4.6 Does R21/MM induce anti-C term antibodies which are associated with protection?

Unlike with NANP IgG, full anti-C term IgG time courses across every time point were not assayed due to time limitations. Only peak response one month post-third dose for both VAC065 and VAC072 were assessed. Baseline samples were assumed negative due to negative anti-NANP IgG titres reported in

Chapter 1. Furthermore, anti-C term IgG measured at baseline in infants in Burkina Faso were negative when anti-NANP IgG samples were negative. A post-hoc analytical approach outlined in chapter methods (12.2.9) was used to try and identify any potential antibody threshold titres for anti-C term IgG that could be used to stratify protected and unprotected participants to observe an association with protection.

12.4.6.1 VAC065 – High anti-C term IgG induced from R21$_{CBF}$/MM are associated with increased protection

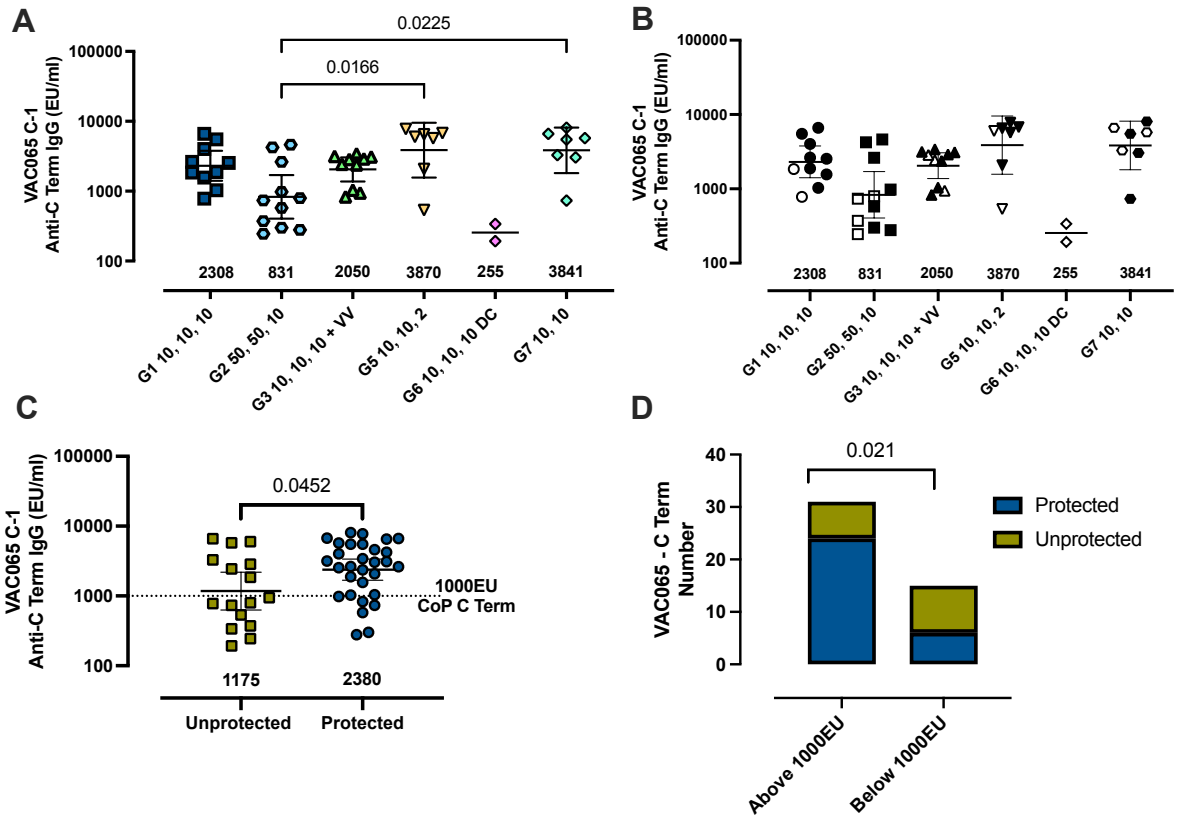


Figure 50 - VAC065 C term IgG and associations with protection.

A) *Geometric mean anti-C term IgG among all groups in VAC065. P-values calculated by Kruskal-Wallis were corrected with Dunn's multiple comparison.* ***B)*** *Geometric mean anti-C term IgG among all groups in VAC065 with protected participants identified. Closed shapes indicate sterilely protected participants and open shapes indicate infected participants.* ***C)*** *Geometric mean anti-C term IgG separated by protected and not protected. P=0.045 Mann-Whitney. The horizontal dotted line Y=1000 represent the threshold of protection.* ***D)*** *Participants grouped by an anti-C term IgG titre above or below 1000EU. P=0.021 Fisher's exact test.*

Geometric means with error bars indicate 95% CI. Values presented above the x-axis indicate geometric mean values.

$R21_{CBF}$ / MM elicited high anti-C term titres across all groups in VAC065. The highest anti-C term IgG was observed in G5 (10, 10, 2µg $R21_{CBF}$) GMT 3870EU [95% CI 1570 – 9542] and G7 (10, 10) (GMT 3841EU [95% CI 1811 – 8145]) - Figure 50A. Both G5 (10, 10, 2µg $R21_{CBF}$) and G7 (10, 10µg $R21_{CBF}$) had significantly higher anti-C term IgG titres compared to G2 (50, 50, 10µg $R21_{CBF}$) (GMT 831EU [95% CI 405 – 1704]). G1 (10, 10, 10µg $R21_{CBF}$) and G3 (10, VV, 10, VV, 10µg $R21_{CBF}$) which received the same dose of $R21_{CBF}$ had comparable anti-C term IgG titres with similar 95% CI - Figure 50A.

Protected participants represented by closed symbols and unprotected by open symbols in Figure 50B have a range of responses similar to those observed with anti-NANP IgG titres in Figure 48B and Figure 49B with no clear distinction of anti-C term IgG titres between protected and unprotected per group. To assess whether there is a threshold of total anti-C term antibodies that are associated with protection, all participants' anti-C term IgG titres were merged and separated by protection - Figure 50C. Protected participants have a significantly (P=0.045) higher anti-C term IgG (GMT 2380EU [95% CI 1677 – 3376]) compared with unprotected (GMT 1175EU [95% CI 630 – 2193]).

As seen with NANP in Figure 48 and Figure 49, a cluster of unprotected participants had an anti-C term IgG titre below 1000EU and a cluster of protection above 1000EU - Figure 50C. Further stratifying anti-C term titres above and below an anti-C term IgG titre of 1000EU, protected participants have a significantly (P=0.021) higher association with protection with a titre >1000EU - Figure 50D.

12.4.6.2 VAC072 – A delayed third dose improves anti-C term IgG, yet is not associated with protection

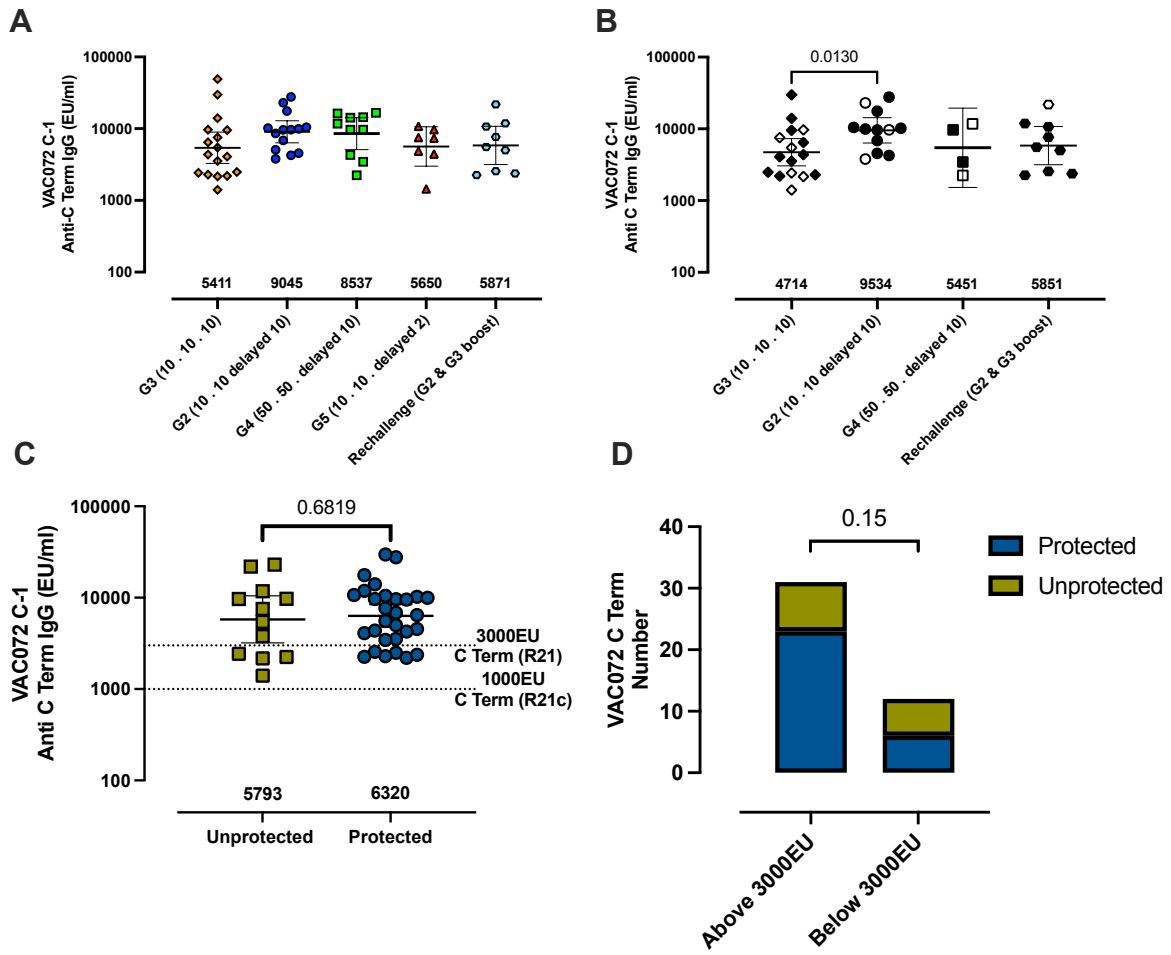


Figure 51 - VAC072 C term IgG associations with protection.

A) *Geometric mean anti-C term IgG among all groups in VAC072.* ***B)*** *Geometric mean anti-C term IgG among all groups in VAC072 with protected participants identified. Closed shapes indicate sterilely protected participants and open shapes indicate infected participants.* ***C)*** *Geometric mean anti-C term IgG separated by protected and not protected. P=0.045 Mann-Whitney. The horizontal dotted line Y=1000 represents the threshold of protection observed in VAC072 Figure 50C and Y=3000 observed in VAC072.* ***D)*** *Participants grouped by an anti-C term IgG titre above or below 3000EU. P=0.15 Fisher's exact test.*

Geometric means with error bars indicate 95% CI. Values presented above the x-axis indicate geometric mean values.

As reported in Figure 46D, anti-C term IgG titres were significantly higher in VAC072 ($R21_{SII}$) as compared to VAC065 ($R21_{CBF}$) and, therefore, anti-C term IgG should be repeated on UK adults to confirm the reported increase in significance.

In VAC072, R21 $_{SII}$ elicits strong detectable anti-C term IgG responses across all groups with no significant differences found between groups - Figure 51A. Unlike with anti-NANP titres - Figure 49A in G4 (50, 50, delayed 10µg R21$_{SII}$) elicited relatively high anti-C term IgG responses (GMTs 8537EU [95% CI 5116 – 142995]) in comparison with G3 (10, 10, 10µg R21 $_{SII}$) and G5 (10, 10, delayed 2µg R21 $_{SII}$)- Figure 51A.

Among participants who were challenged, G2 (10, 10, delayed 10µg R21$_{SII}$) presented with significantly (P=0.013) higher anti-C term IgG GMTs (9534EU [95% CI 6359 – 12865]) compared with G3 GMTs (4714EU [95% CI 3046 – 7294]) - Figure 51B. There was no clear distinction of anti-C term antibodies between protected and unprotected per group – Figure 10B.

To assess whether there is a threshold of total anti-C term antibodies that are associated with protection in VAC072 (R21$_{SII}$), all participants with anti-C term IgG titres were merged and separated by protection. Due to the significant increase in anti-C term IgG titres measured in VAC072 compared with VAC065, the threshold of 1000EU (Figure 50C/D) could not be used. No cluster of participants could be observed apart from four unprotected participants who had an anti-C term IgG <3000EU - Figure 51C. Further stratifying anti-C term IgG titres by above or below 3000EU, no significant association with protection was found - Figure 51D.

12.4.7 MSD anti-IgG measured by novel multiplex platform

The MSD platform was selected as the assay technology platform for immunology in the Phase III trial of R21/MM (VAC078). Extensive assay validation of a custom multiplex serology kit measuring responses to R21 was performed prior to use on clinical trial samples. Selected samples from VAC065 and VAC072 were run at baseline (D0), two weeks post second dose (D42), one month post-third dose (D84 or D196) and ninety days post-challenge (C+90). In this thesis, only one-month post-third dose data is presented.

12.4.7.1 VAC065 MSD – Anti-NANP, anti-C term, anti-R21 and anti-HBsAg IgG measured by MSD

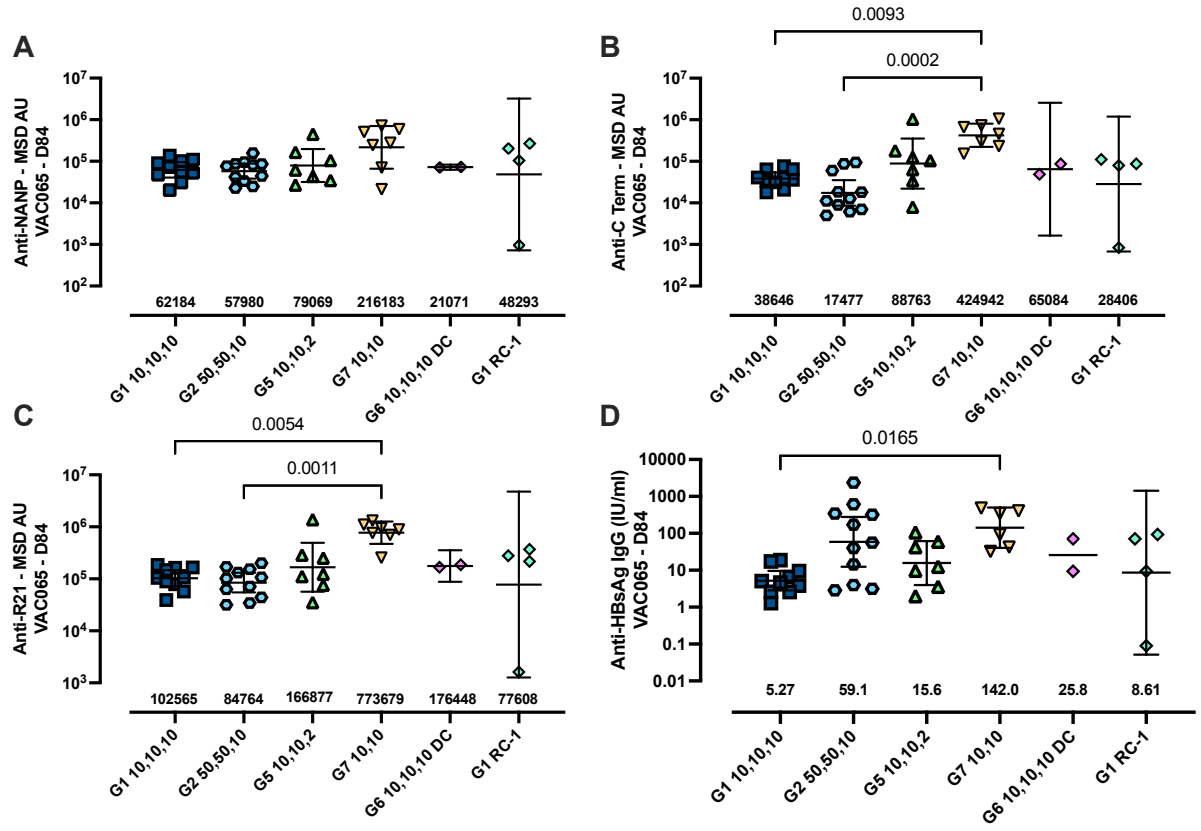


Figure 52 – VAC065 MSD total IgG to NANP, C term, R21 and HBsAg.

A) *Anti – NANP (AU) MSD IgG.* ***B)*** *Anti-C term (AU) MSD IgG.* ***C)*** *Anti-R21 MSD IgG.* ***D)*** *Anti-HBsAg MSD IgG (IU/ml). The horizontal dotted line represents the WHO threshold of protection of 0.01 IU/ml.*

Figures show GMTs with 95% CI for G1 and G2 at specific time points. P-values compare time points within each group – Kruskal-Wallis corrected with Dunn's multiple comparisons.

For VAC065 ($R21C_{BF}$), all adults had a large increase in anti-NANP, anti-C term, anti-R21 and anti-HBsAg IgG between baseline (D0) and 1 month after 2-3 doses of $R21_{CBF}$ / MM (baseline data not presented). All UK adults were seronegative for anti-NANP, anti-C term IgG and anti-R21 MSD IgG.

For anti-NANP total IgG, all groups presented with high anti-NANP total IgG titres across all groups with no significant differences observed. The highest titres were found in the two-dose regimen (10, 10µg $R21_{CBF}$) of G7 – GMTs 216183AU [95% CI 66036 – 707721] and the lowest response observed in

the higher fractional dose regimen (50, 50, 10µg $R21_{CBF}$) in G2 (GMTs 57980AU [95% CI 38304 – 87764]) – Figure 52A.

High levels of anti-C term IgG were detected in all groups after vaccination. As with NANP, G7 (10,10µg $R21_{CBF}$) had the highest anti-C term IgG with GMTs 424942AU [95% CI 223402 – 808300]. G7 had significantly higher anti-C term IgG compared with G1 (10, 10, 10µg $R21_{CBF}$) (GMTs 38646AU [95% CI 27501 – 54309]) and G2 (50, 50, 10µg $R21_{CBF}$) (GMTs 17477AU [95% CI 8596 – 35532]) - Figure 52B.

Anti-R21 total IgG titres had similar trends to those observed with anti-NANP and anti-C term MSD IgG titres whereby all participants had significant increases in anti-R21 IgG titres after immunisation with R21/MM. G7 (10, 10µg $R21_{CBF}$) produced significantly higher anti-R21 MSD IgG titres GMTs (773679AU [95% CI 472403 – 1267094]) than G1 (10, 10, 10µg $R21_{CBF}$) (GMTs 102565AU [95% CI 71525 – 147075]) and G2 (50, 50, 10µg $R21_{CBF}$) (GMTs 84764AU [95% CI 54903 – 130865]) - Figure 52C.

Immunising with varied doses of R21/MM saw anti-HBsAg IgG measured by MSD increase significantly between baseline and 1 month post-third dose. G7 (10, 10µg R21c) produced the highest response (142AU [95% CI 40.3 – 503] compared to all other groups – Figure 52D.

12.4.7.2 VAC072 MSD – Anti-NANP, anti-C term, anti-R21 and anti-HBsAg IgG measured by MSD

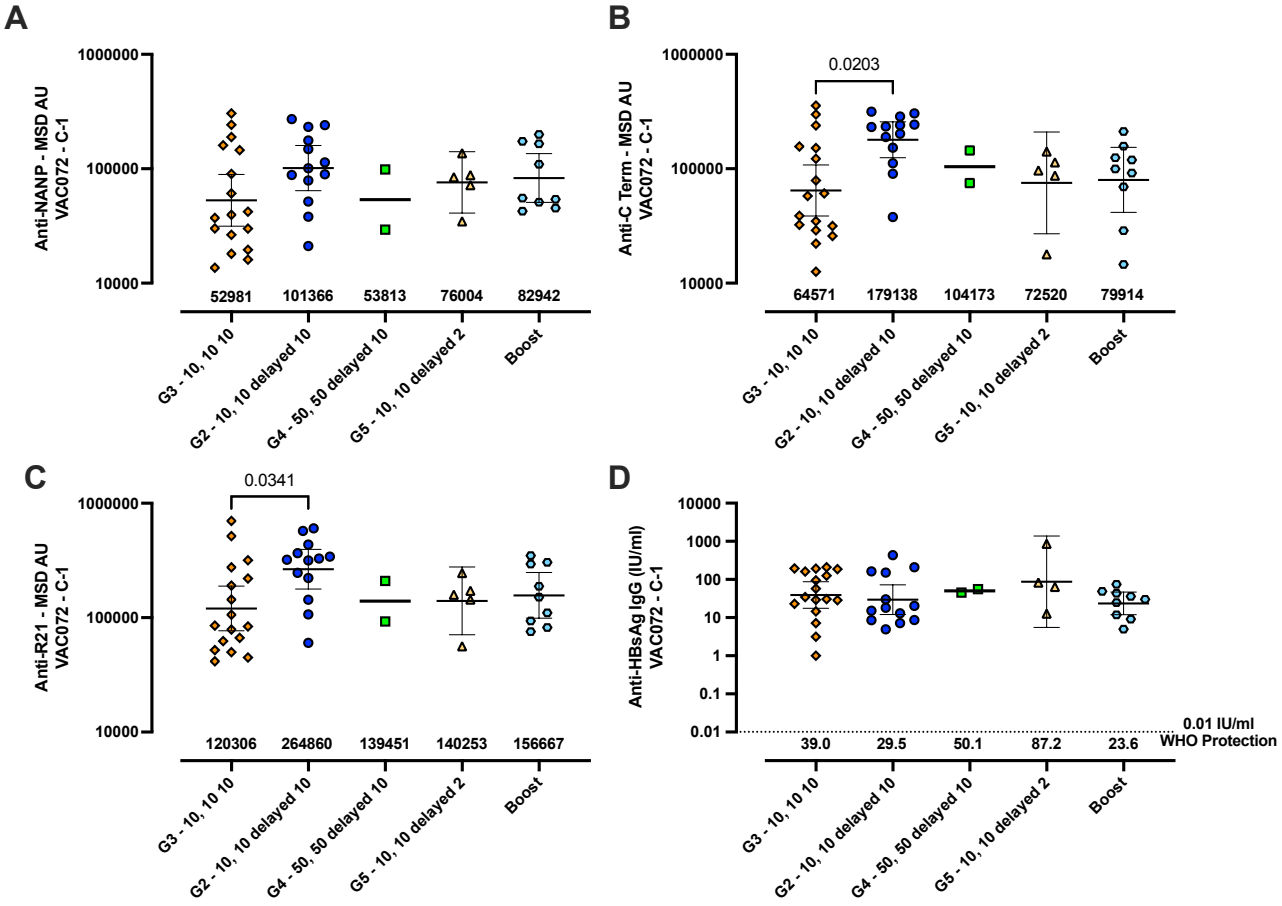


Figure 53 - VAC072 MSD total IgG to NANP, C term, R21 and HBsAg.

A) *Anti – NANP MSD IgG.* ***B)*** *Anti-C term MSD IgG.* ***C)*** *Anti-HBsAg MSD IgG (IU/ml). The horizontal dotted line is the WHO threshold of protection 0.01 IU/ml.* ***D)*** *Anti-R21 MSD IgG.*

Figures show GMTs with 95% CI for G1 and G2 at specific time points. P-values compare time points within each group – Kruskal-Wallis corrected with Dunn's multiple comparisons.

MSD was used to assess anti-NANP, anti-C term, anti-R21 and anti-HBsAg MSD IgG titres for VAC072 ($R21_{SII}$) and to compare with VAC065 ($R21_{CBF}$) - Figure 53. Across all antigens, G2 (10, 10, delayed 10µg $R21_{SII}$) had higher IgG responses compared to all other groups apart from anti-HBsAg MSD IgG. Furthermore, G2 had significantly higher anti-C term MSD IgG and anti-R21 IgG than G3 (10, 10, 10µg $R21_{SII}$).

12.4.7.3 Increased IgG antibodies measured by MSD are associated with protection

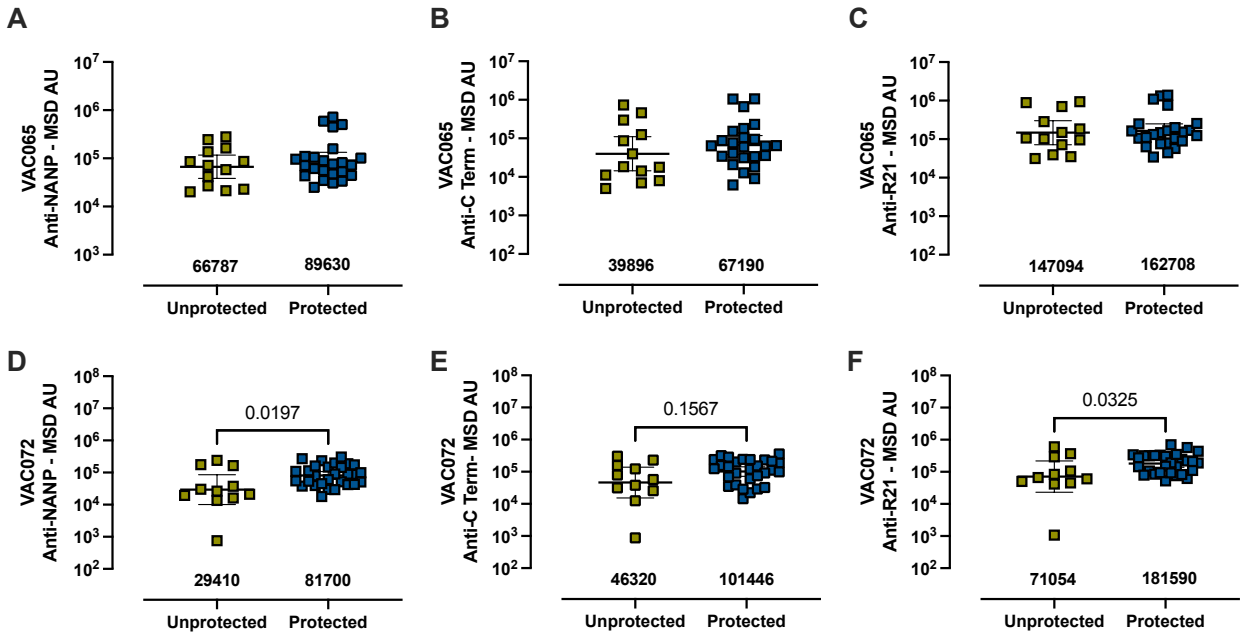


Figure 54 - MSD total IgG induced from $R21_{CBF}$ and $R21_{SII}$ stratified by protection.

A) *Anti-NANP MSD IgG for VAC065 ($R21_{CBF}$) measured at 1-month post-third dose stratified protection.*
B) *Anti-C term MSD IgG for VAC065 ($R21_{CBF}$) measured at 1-month post-third dose stratified protection.*
C) *Anti-R21 MSD IgG for VAC065 ($R21_{CBF}$) measured at 1-month post-third dose stratified protection.*
D) *Anti-NANP MSD IgG for VAC072 ($R21_{SII}$) measured at 1-month post-third dose stratified protection.*
E) *Anti-C term MSD IgG for VAC072 ($R21_{SII}$) measured at 1-month post-third dose stratified protection.*
F) *Anti-R21 MSD IgG for VAC072 ($R21_{SII}$) measured at 1-month post-third dose stratified protection.*

All figures present GMT with error bars indicating 95% CI antibody titres measured by MSD. P-values, as calculated by Mann-Whitney, are presented between protected and unprotected subjects for each group.

IgG responses to NANP, C term and R21, as measured by the MSD multiplex platform, were stratified by protection to assess for immune associations with protection. For both VAC065 and VAC072, IgG responses for each antigen were merged and then subsequently separated by protection status after CHMI. A general observation can be made that protected and unprotected responses in VAC065 show similar antibody results with adults in VAC065.

For VAC065, no difference in antibody response against NANP, C term and R21 was detected between protected and unprotected participants Figure 54. A cluster of unprotected volunteers have anti-

NANP titres below 66000AU which could determine a threshold of protection for adults in VAC065 - Figure 54A however, this has not yet been evaluated.

In VAC072, protected participants had significantly higher anti-NANP MSD IgG GMTs (81700AU [95% CI 61717 – 108154]) than unprotected participants (GMTs 29410AU [95% CI 10100 – 85638]) - Figure 54D. Protected participants had a much higher anti-C term MSD IgG titre however this was not significant - Figure 54E. Protected participants had significantly higher anti-R21 MSD IgG GMTs (181590AU [95% CI 139356 – 236624]) than unprotected participants GMTs (71054AU [95% CI 23243 – 217211]) - Figure 54F.

Anti-HBsAg IgG titres were stratified with protection but no association was found – data not presented.

12.4.7.4 Antibodies induced by $R21_{CBF}$ and $R21_{SII}$ measured by MSD are correlated

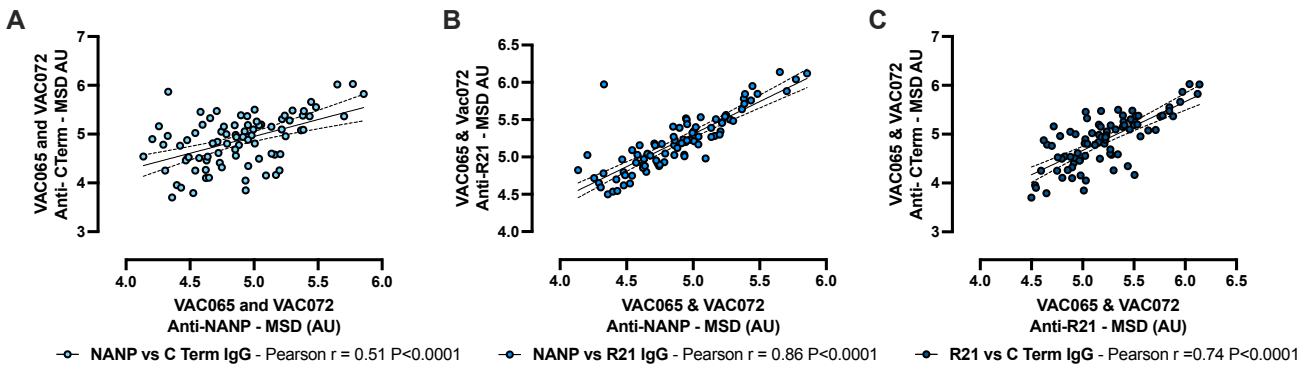


Figure 55 - MSD assay correlations – VAC065 and VAC072.

A) *Correlation between anti-NANP IgG and anti-C term IgG.* ***B)*** *Correlation between anti-NANP IgG and anti-R21 IgG* ***C)*** *Correlation between anti-R21 IgG and anti-C term IgG.*

The time point presented is 1-month post-third dose. Correlations calculated by Pearson r. Line of best fit – Linear Regression model with 95% CI.

Antibodies specific to NANP, C term and R21 measured by MSD multiplex platform from UK adults immunised with R21 highly correlate with each other. Anti-NANP and anti-C term IgG have a significant positive correlation (Pearson r – 0.51 P<0.0001) - Figure 55A. Anti-NANP with anti-R21 also have a

significant positive correlation (Pearson r = 0.86 P<0.0001) - Figure 55B. Finally, the anti-C term and anti-R21 IgG also reveal a significant positive correlation (Pearson r = 0.74 P<0.0001) - Figure 55C.

12.4.8 HBsAg IgG antibodies measured by MSD induced from R21

12.4.8.1 $R21_{CBF}$ significantly increase HBsAg antibodies in UK adults

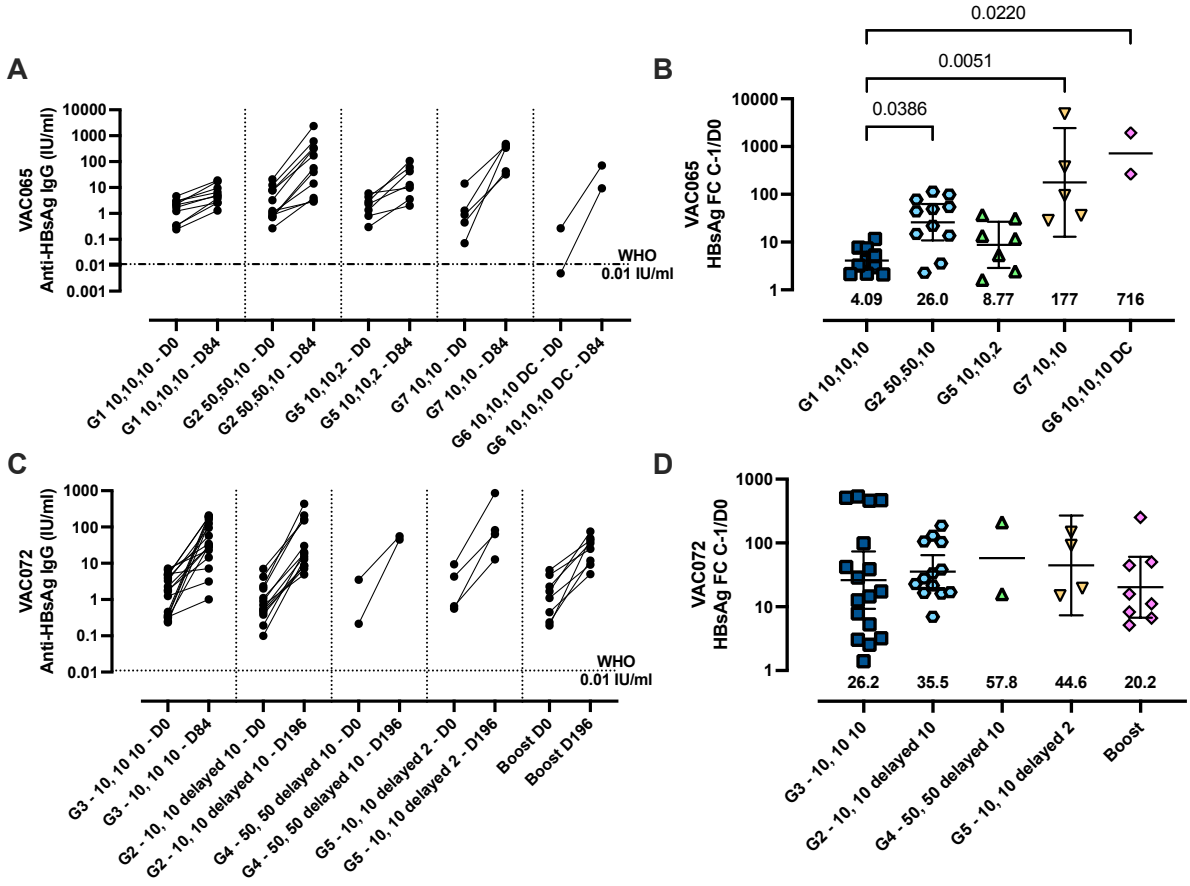


Figure 56 - Anti-HBsAg IgG measured by MSD for VAC065 and VAC072.

A) *Anti-HBsAg MSD IgG for VAC065 ($R21_{CBF}$) connecting baseline with 1-month post third dose. The horizontal dotted dashed line indicates the WHO protection serology threshold of 0.01IU/ml.* ***B)*** *Fold change representing fold increase in HBsAg between baseline and 1-month post third dose for VAC065. P-values calculated by Kruskal-Wallis corrected with Dunn's multiple comparisons. Geometric means with error bars indicating 95% CI.*

C) *Anti-HBsAg MSD IgG for VAC072 ($R21_{SII}$) connecting baseline with 1 month post third dose. The horizontal dotted dashed line indicates the WHO protection serology threshold of 0.01IU/ml.* ***D)*** *Fold change representing fold increase in HBsAg between baseline and 1-month post-third dose for VAC072. Geometric means with error bars indicating 95% CI.*

Values above the x-axis represent geometric mean values.

Measuring HBsAg responses at baseline, all participants in VAC065 ($R21_{CBF}$) and VAC072 ($R21_{SII}$) were seropositive for HBsAg IgG greater than 0.03 IU/ml [440] and all participants apart from one had a HBsAg titre greater than the WHO protective threshold of 0.01 IU/ml [441, 442] - Figure 56A/C.

All participants were seropositive at baseline and, to evaluate how R21/MM affects anti-HBsAg IgG titres, the fold-change (FC) was calculated between D0 and D84. All participants in both VAC065 ($R21_{CBF}$) and VAC072 ($R21_{SII}$) had a positive fold increase in anti-HBsAg IgG titres measured by MSD - Figure 56B/D. For VAC065, the largest FC was observed in the two-dose group of G7 (10, 10µg $R21_{CBF}$) with an FC of GMT 177 and with the lowest FC observed in G1 receiving the standard dose regimen (10, 10, 10µg R21c) with a FC of 4.09 - Figure 56B. In VAC072, the largest FC in anti-HBsAg IgG titre was observed in G4 (50, 50, delayed 10µg $R21_{SII}$) with an FC of 57.8. As with VAC065 the standard dose regimen G3 (10, 10, 10µg $R21_{SII}$) in VAC072 presented with the lowest FC in anti-HBsAg titre with a FC of 26.2 - Figure 56D.

12.4.9 Avidity to NANP and C term IgG

Avidity was measured by sodium thiocyanate displacement of IgG. Full methods of both NANP and C terms can be viewed in appendix methods (17.5.11). Avidity to NANP was assessed at the Jenner Institute approximately four years before the start of this DPhil project. Therefore, an assay comparability to previous avidity data was undertaken before data was generated for this thesis. The standardised ELISA to C term peptide is novel to this thesis.

Results from avidity assays to NANP and C term peptides are not directly comparable as each assay uses a different coating concentration on the ELISA plate. However, avidity is calculated by normalising antibody titres by altering the dilutions to ensure all samples develop uniformly to an OD_{405}=1.

Normalising antibody concentrations creates the same quantity of antibodies in each well. Therefore, a reasonable association between the two assays can be made.

12.4.9.1 VAC065 Avidity –Three doses of R21CBF do not increase avidity, irrespective of dose

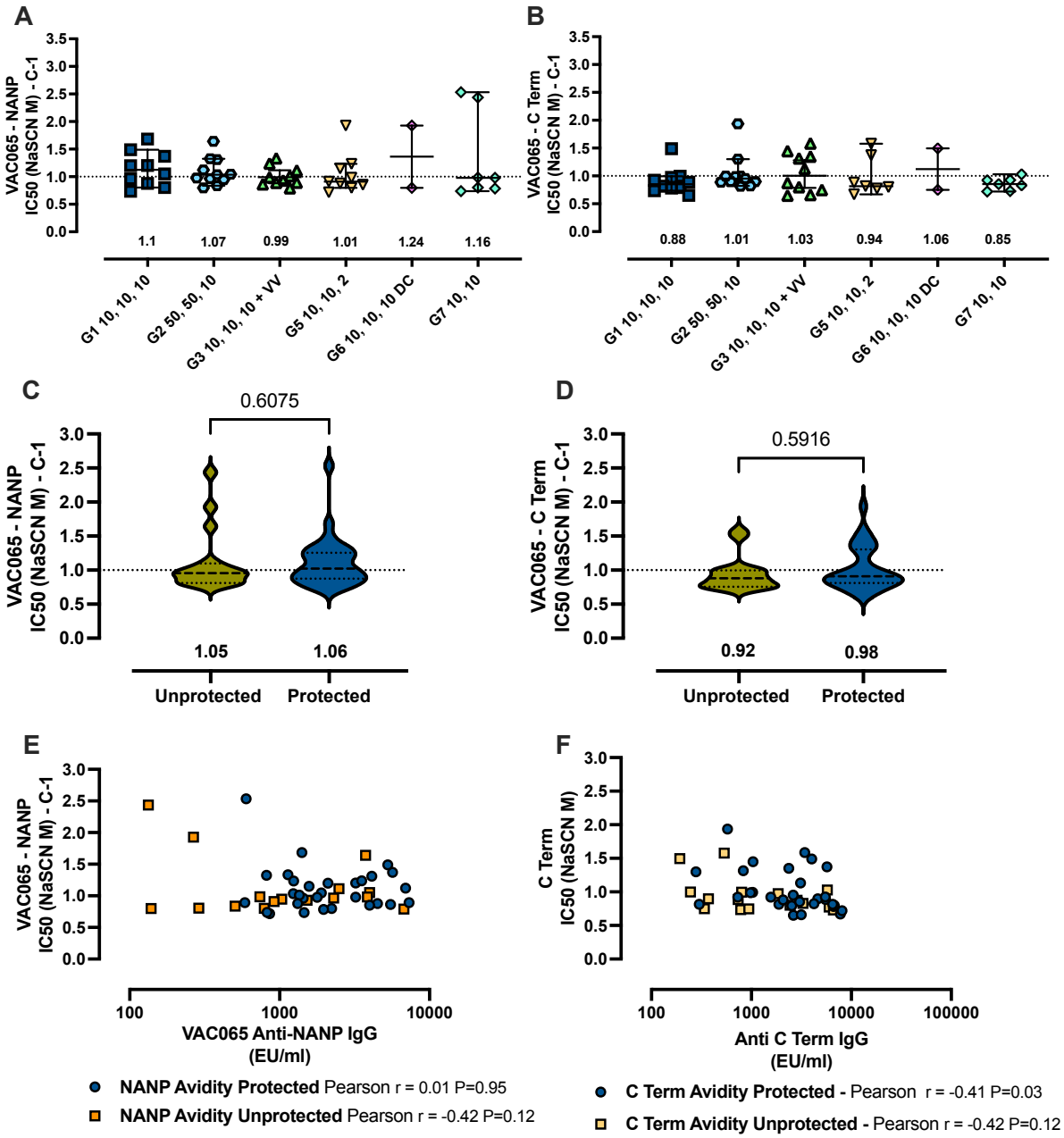


Figure 57 - VAC065 avidity - NANP and C term associations with protection.

A) *Avidity to anti-NANP IgG with 1-month post-third dose for VAC065 (R21c). GMTs with error bars indicating 95% CI. Values above the x-axis indicate GMT values.* ***B)*** *Avidity to anti-C term IgG with 1-month post-third dose for VAC065 (R21c). GMTs with error bars indicating 95% CI. Values above the x-axis indicate GMT values.* ***C)*** *Violin plot representing avidity to anti-NANP IgG stratified by protection. Lines indicate quartiles. Values above the x-axis indicate GMT values* ***D)*** *Violin plot representing avidity*

to anti-C term IgG stratified by protection. Lines indicate quartiles and values above the x-axis indicate GMT values. ***E)*** *Association between anti-NANP IgG titres and avidity. Correlation calculated by Pearson r.* ***F)*** *Association between anti-NANP IgG titres and avidity. Correlation calculated by Pearson r.*

Across all groups in VAC065, avidity to NANP was consistent, with an average IC50 of 1.1 with no significant differences observed – Figure 57A. A similar observation was made with avidity to the C term, with no significant differences observed - Figure 57B. Accordingly, groups were merged for both NANP and C terms and then stratified into protected and unprotected. No significant difference was observed between protected and unprotected for both NANP - Figure 57C and C term - Figure 57D.

Avidity was correlated with total IgG antibody titre for the NANP and C term-specific antibodies to assess if an association could be made between protected and unprotected participants. For NANP, there was no association between avidity and NANP IgG titre (Pearson r = 0.01, P=0.95), thus no increase or decrease in antibody titres correlated with avidity. For unprotected participants, a negative correlation was observed (Pearson r = -0.42, P=0.12), although this was not statistically significant - Figure 57E. Avidity correlations with C term-specific IgG indicated a significant negative correlation with protected participants (Pearson r = -0.41 P=0.03) as well as a negative correlation with unprotected participants (Pearson r = -0.42 P=0.12), although this was statistically insignificant - Figure 57F.

12.4.9.2 VAC072 Avidity – a delayed and delayed fractional third dose improves avidity to NANP and C term in UK adults

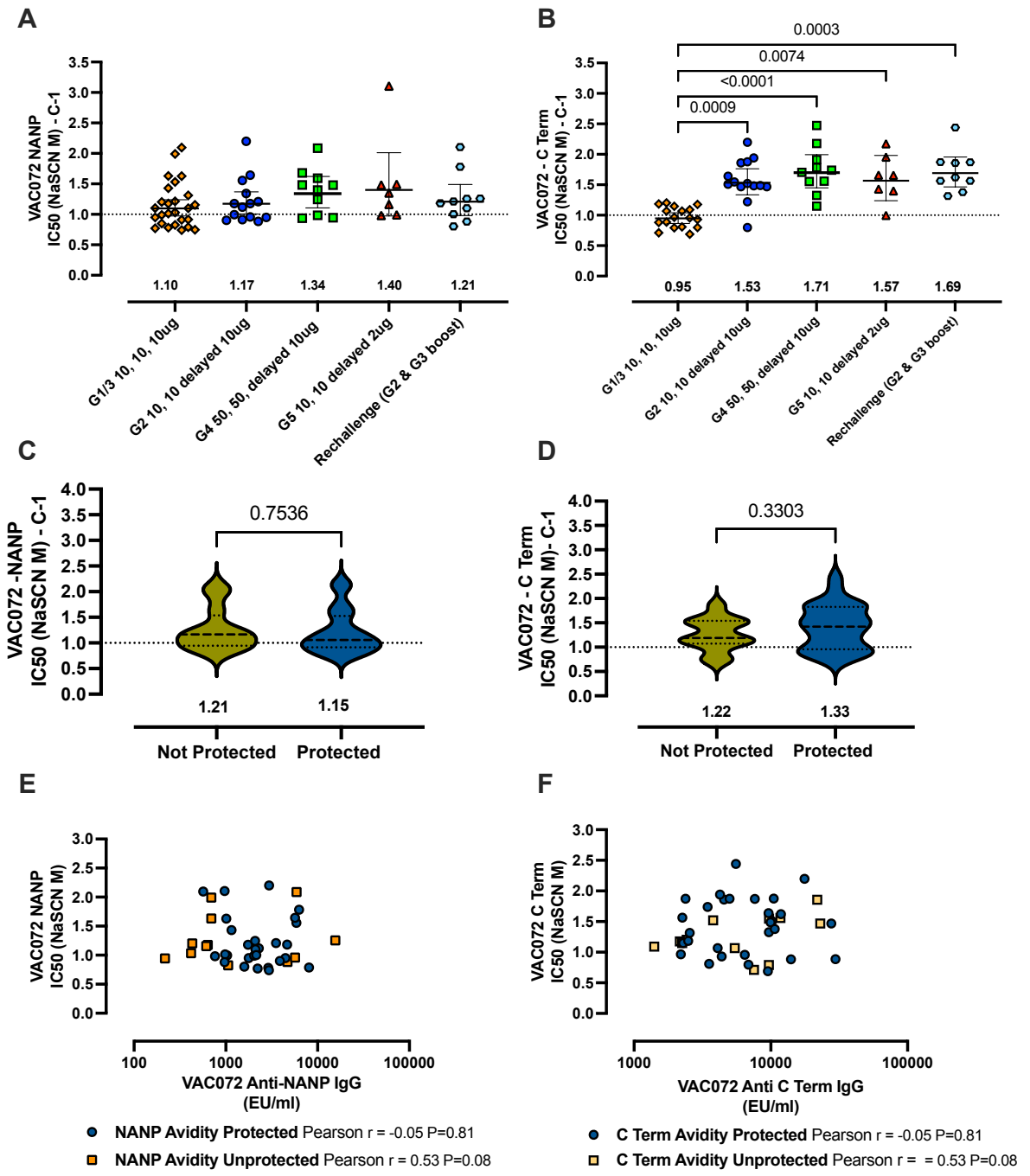


Figure 58 - VAC072 Avidity - NANP and C term associations with protection.

A) *Avidity to anti-NANP IgG with 1-month post-third dose for VAC072 (R21). GMTs with error bars indicating 95% CI. Values presented above the x-axis indicate GMT values.* ***B)*** *Avidity to anti-C term IgG with 1-month post third dose for VAC072 (R21). GMTs with error bars indicating 95% CI. Values presented above the x-axis indicate GMT values.* ***C)*** *Violin plot representing avidity to anti-NANP IgG stratified by protection. Lines indicate quartiles. Values above the x-axis indicate GMT values* ***D)*** *Violin plot representing avidity to anti-C term IgG stratified by protection. Lines indicate quartiles and values*

above the x-axis indicate GMT values. ***E)*** *Association between anti-NANP IgG titres and avidity. Correlation calculated by Pearson r.* ***F)*** *Association between anti-NANP IgG titres and avidity. Correlation calculated by Pearson r.*

Avidity was also measured against NANP and C term-specific IgG from samples in VAC072 at the one-month post-third dose time point across all groups and one-month post boost, from participants who consented to a boost.

Avidity in the standard dose regimen in G1/3 (10, 10, 10µg R21) resulted in a GMT IC50 of 1.1 [95% CI 0.97 – 1.24] - Figure 58A, which is comparable to avidity seen in the standard dose regimen in VAC065 G1 - Figure 57A. The delayed dose G2 (10, 10, delayed 10µg R21) resulted in a small increase in avidity to NANP IgG (1.17 [95% CI 1.01 – 1.37]) and was not significant compared to G1 - Figure 58A. Avidity to NANP IgG in delayed fractional dose G4 (50, 50, delayed 10µg R21) resulted in a greater increase in IC50 (1.34 [95% CI 1.11 – 1.62]) and delayed fractional dose G5 (10, 10, delayed 2µg R21) further increased IC50 (1.40 [95% CI 0.96 – 2.01]). However, no dosing regimen was significantly different when compared to G1 - Figure 58A.

The standard dose regimen resulted in comparable C term specific IgG between VAC065 - Figure 57B and VAC072 - Figure 58B with an IC50 of 0.95 [95% CI 0.86 – 1.04]. However, all delayed and delayed fractional third dose groups had significantly higher avidity to C term-specific IgG when compared to the standard dose regimen - Figure 58B. Further, the participants who received a booster also had a significant increase in avidity to the standard dose regimen.

The increase in avidity to NANP-specific IgG and a significant increase in avidity C term IgG prompted a potential association with protection in avidity for delayed and delayed fractional dose groups. To assess if a threshold of avidity to both NANP and C term-specific IgG correlates with protection, groups were merged for both NANP and C terms, and then stratified into protected and unprotected. For NANP, protected participants had a decreased avidity to NANP with an IC50 1.15 [95% CI 1.02 – 1.31]

compared to unprotected with an IC50 1.21 [95% CI 0.99 – 1.46], however not statistically significant - Figure 58C. For C term, protected participants had a higher avidity IC50 1.33 [95% CI 1.17 – 1.52] than unprotected IC50 1.22 [95% CI 1.02 – 1.46], however not statistically significant - Figure 58D.

As with VAC065, avidity was correlated with total IgG antibody titre for the NANP and C term-specific antibodies to assess if an association could be made between protected and unprotected participants. No correlation between avidity and NANP-specific IgG between protected participants (Pearson r = -0.05, P=0.81) was found and a positive correlation (Pearson r = 0.53, P=0.08) was observed in unprotected participants but not significant. A significant negative correlation was observed between avidity and C term-specific IgG in protected participants (Pearson r = -0.41, P=0.03) and a negative correlation in unprotected participants (Pearson r = -0.42, P=0.12) was observed although not significant - Figure 58F.

12.4.10 Subclass and Isotype antibody responses in UK adults

Subclass (IgG1-4) and Isotype antibodies (IgA and IgM) were assessed to NANP portions of R21 vaccine with samples taken one month post-third dose for VAC065 and VAC072. Subclass antibody IgG4 is not presented in VAC065 dataset due to irregular plate variability - Figure 59. Subclass and Isotype antibodies to C term and full-length R21 protein are assessed and reported. However, due to time limitations, these samples were not assayed.

Subclass and Isotype assays presented in this thesis are comparable as they were performed using plates coated with the same NANP peptide concentration, subclass and isotype secondary antibodies are added at the same concentration, and plates are developed so that internal controls ensure the plates are comparable across experiments and results are reported as ODs.

12.4.10.1 VAC065 – Antibody isotype IgA, IgM and subclass IgG1-3 responses to NANP

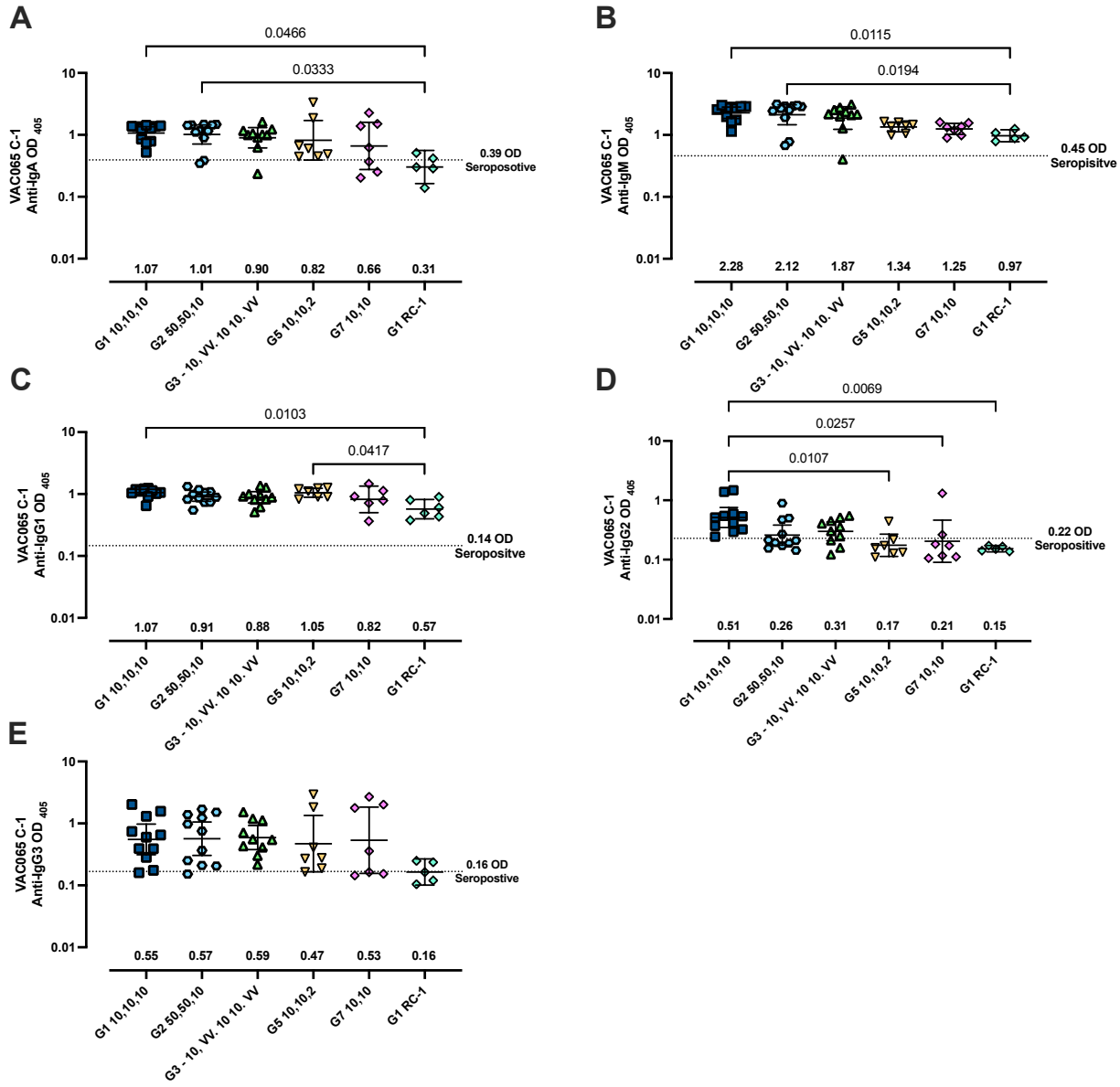


Figure 59 - VAC065 antibody subclass and isotype responses.

A) *OD_{405} of anti-NANP IgA measured 1-month post third dose for VAC065 - $R21_{CBF}$* ***B)*** *OD_{405} of anti-NANP IgM measured 1-month post-third dose for VAC065 – $R21_{CBF}$.* ***C)*** *OD_{405} of anti-NANP IgG1 measured 1-month post-third dose for VAC065 - R21c.* ***D)*** *OD_{405} of anti-NANP IgG2 measured 1-month post third dose for VAC065 – $R21_{CBF}$.* ***E)*** *OD_{405} of anti-NANP IgG3 measured 1-month post third dose for VAC065 - $R21_{CBF}$.*

Error bars indicate a geometric mean with a 95% CI. Values presented above the x-axis are GMTs. P-values calculated by Kruskal-Wallis were corrected with Dunn's multiple comparisons.

For VAC065 ($R21_{CBF}$), detectable subclass and isotype responses were observed across all assays. Seropositive values for subclass and isotype samples are represented by horizontal dotted lines in Figure 59.

For isotype IgA responses in VAC065, the standard dose regimen G1 (10, 10, 10µg $R21_{CBF}$) resulted in the highest IgA OD_{405} with GMT 1.07 OD_{405} [95% CI 0.05 – 1.35] – Figure 59A, followed by G2 (50, 50, 10µg $R21_{CBF}$) group with a GMT 1.01 OD_{405} [95% CI 0.711 – 1.45]. Both G1 and G2 had significantly higher ODs than participants who were re-challenged and who had GMTs 0.303 OD_{405} [95% CI 0.16 – 0.56]. Similar observations were made for IgM with G1 and G2 reporting the highest ODs in all the groups assessed with significantly higher OD_{405s} responses compared to re-challenged participants - Figure 59B.

Subclass responses in VAC065 were varied with the larger responses occurring in IgG1 - Figure 59C and IgG3 - Figure 59E. For IgG1, the standard dose regimen of G1 (10, 10, 10µg $R21_{CBF}$) reported the highest response with an OD_{405} of 1.07 [95% CI 0.93 – 1.19] followed by G5 (10, 10, 2µg R21c) with an OD_{405} of 1.05 [95% CI 0.89 – 1.24] - Figure 59C. Both G1 and G3 had significantly higher OD_{405} than the re-challenged participants.

Reponses in Ig2 were comparable with ODs from subclass antibodies IgG1 and IgG3 with most participants having ODs to IgG2 below the seropositive threshold of 0.22 OD_{405} – Figure 59D. G1 (10, 10, 10µg R21c). Participants who received the standard dose regimen presented with the highest IgG2 OD_{405} with a GMT 0.513 [95% CI 0.35 – 0.76] with significantly higher ODs compared with G5 (10, 10, 2µg $R21_{CBF}$), G7 (10, 10µg $R21_{CBF}$) and re-challenged participants – Figure 59D.

12.4.10.2 VAC072 - Antibody Isotype IgA & IgM and subclass IgG1-4 responses to NANP

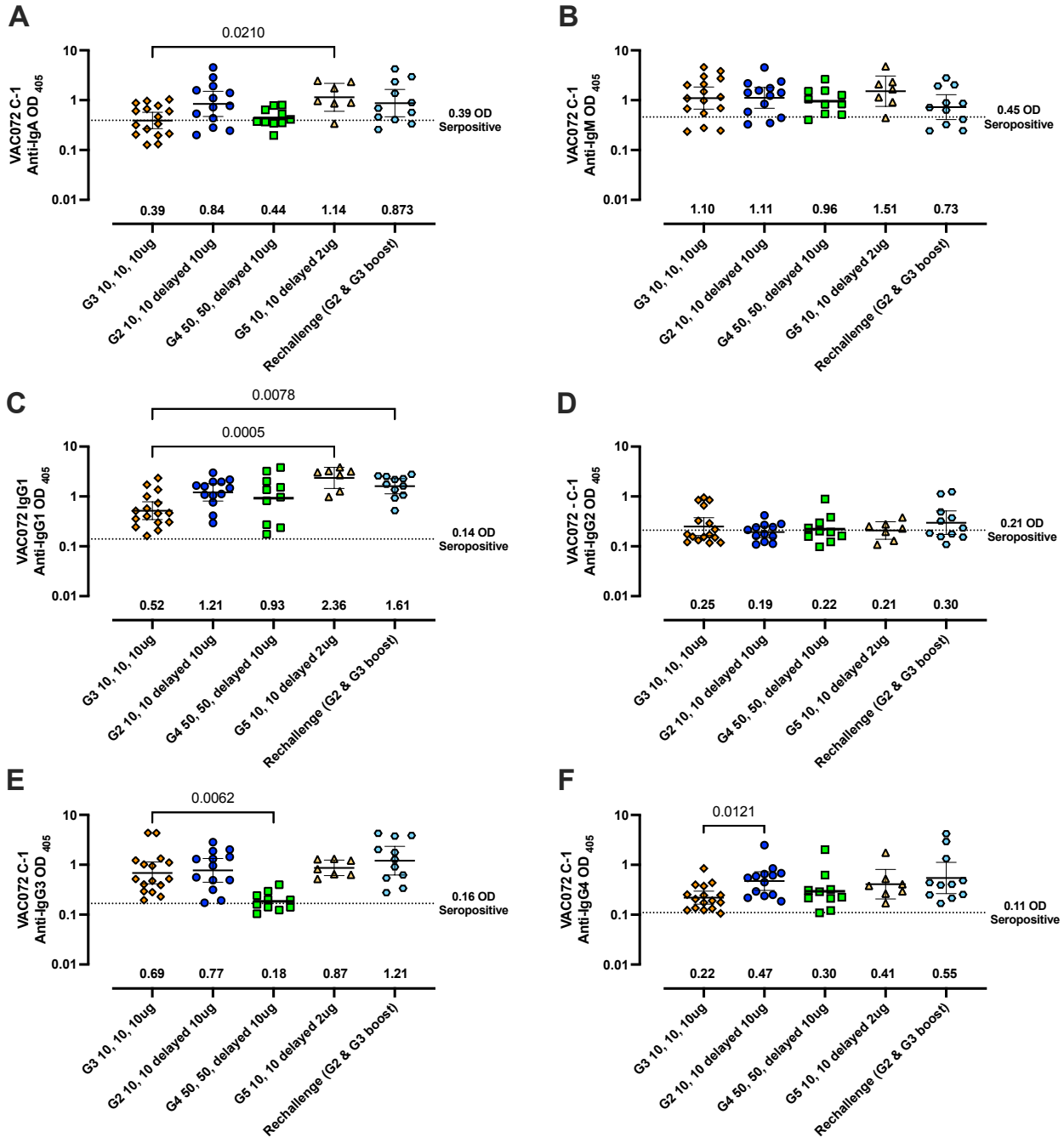


Figure 60 - VAC072 antibody subclass and isotype responses.

A) *OD_{405} of anti-NANP IgA measured 1-month post third dose for VAC072 - $R21_{SII}$* ***B)*** *OD_{405} of anti-NANP IgM measured 1-month post third dose for VAC072 – $R21_{SII}$.* ***C)*** *OD_{405} of anti-NANP IgG1 measured 1-month post third dose for VAC072 - R21.* ***D)*** *OD_{405} of anti-NANP IgG2 measured 1-month post third dose for VAC072 – $R21_{SII}$.* ***E)*** *OD_{405} of anti-NANP IgG3 measured 1-month post third dose for VAC072 - $R21_{SII}$.* ***F)*** *OD_{405} of anti-NANP IgG4 measured 1-month post third dose for VAC072.*

Error bars indicate geometric means with a 95% CI. Values presented above the x-axis are GMTs. P-values calculated by Kruskal-Wallis were corrected with Dunn's multiple comparisons.

For VAC072 ($R21_{SII}$), detectable ODs for all antibody subclass and isotypes could be detected above the seropositivity threshold reported and represented by horizontal dotted lines in each Figure - Figure 60.

For isotype responses with IgA and IgM in VAC072 ($R21_{SII}$), ODs for IgA and IgM among participants who received the standard dose regimen from VAC065 - Figure 59A/B, and VAC072 - Figure 60A/B, have significant differences in ODs. However, when assay comparability was undertaken to cross-reference assay performance, no significant difference was found aside from IgG2.

For isotype IgA, G5 (10, 10, delayed 2µg) reported the highest OD_{405} GMT 1.14 [95% CI 0.6 – 2.18] and significantly higher compared to G3 (10, 10, 10µg) with an OD_{405} GMT 0.391 [95% CI 0.23 – 0.57]. G2 (10, 10, delayed 10µg) reported the second highest IgA response with a GMT 0.84 [95% CI 0.47 – 1.51] - Figure 60A. For Isotype IgM, a similar response to what was seen in IgA was observed, in that the G5 (10, 10, delayed 2µg) and G2 (10, 10, delayed 10µg) dose group had the highest subclass and isotype OD_{405}. However, no significant difference was observed - Figure 60B.

In VAC072, subclass antibodies IgG1-4, as with VAC065 (Figure 59), the highest subclass responses were observed with IgG1 and IgG3 - Figure 60C/E. For IgG1, delayed and delayed fractional dose groups had ODs greater than ODs reported from the standard dose regimen G1 (10, 10, 10µg) with significantly higher responses in G5 (10, 10 delayed 10µg) and boosted groups - Figure 60C. For IgG3, the highest delayed fractional dose G4 (50, 50 delayed 10µg) had the lowest OD_{405} reported with a GMT 0.19 [95% CI 0.18 – 0.25] and significantly lower compared to G3 with a GMT of 0.69 [95% CI 0.42 – 1.13] - Figure 60D.

Responses to IgG2 in VAC072, the majority of participants presented with ODs below the seropositivity threshold with no significant differences between any of the groups - Figure 60D. Subclass antibody

IgG4 measurements were low in comparison to IgG1 and IgG3, but unlike IgG2, the majority of participants were seropositive for IgG4. G2 (10, 10, delayed 10µg) dose group reported the highest IgG4 with a OD_{405} GMT of 0.47 [95% CI 0.31 – 0.73] and significantly higher IgG4 OD_{405} compared to G3 (10, 10, 10µg) with a GMT 0.22 [95% CI 0.16 – 0.3] - Figure 60F.

12.4.11 VAC065 Subclass and Isotype do not associate with protection in UK adults

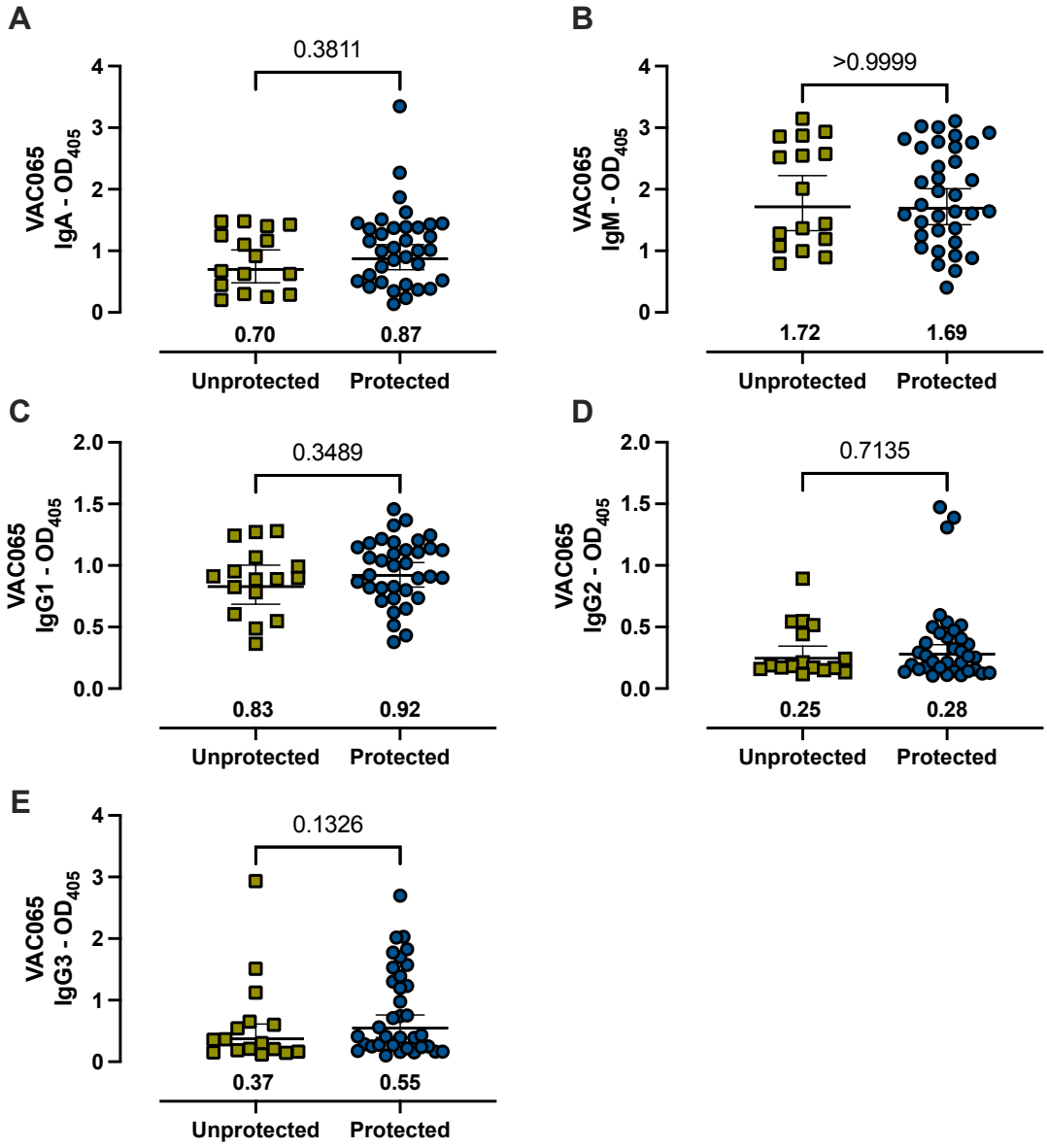


Figure 61 – VAC065 antibody subclass and Isotype associations with protection.

A) *OD_{405} anti-NANP IgA measured at D84 stratified by protection in VAC065 – $R21_{CBFI}$.* ***B)*** *OD_{405} anti-NANP IgM measured at D84 stratified by protection in VAC065 – $R21_{CBF}$. C) OD_{405} anti-NANP IgG1 measured at D84 stratified by protection in VAC065 – $R21_{CBF}$.* ***D)*** *OD_{405} anti-NANP IgG2 measured at*

*D84 stratified by protection in VAC065 – $R21_{CBF}$. **E)** OD_{405} anti-NANP IgG3 measured at D84 stratified by protection in VAC065 – $R21_{CBF}$.*

Error bars indicate Geometric Means with 95% CI. Values above the x-axis are GMTs. P-values were calculated by Mann-Whitney.

In VAC065 ($R21_{CBF}$) antibody subclass and isotype were assessed further to identify a potential CoP. OD_{405} for all antibody subclasses and isotypes OD_{405} were merged and then stratified based on the degree of protection afforded. No significant difference could be observed for any antibody subclass and isotype antibody measured in VAC065 - Figure 61A/B/C/D/E.

12.4.12 VAC072 subclass and isotype – IgG1, IgG3 and IgG4 associate with protection

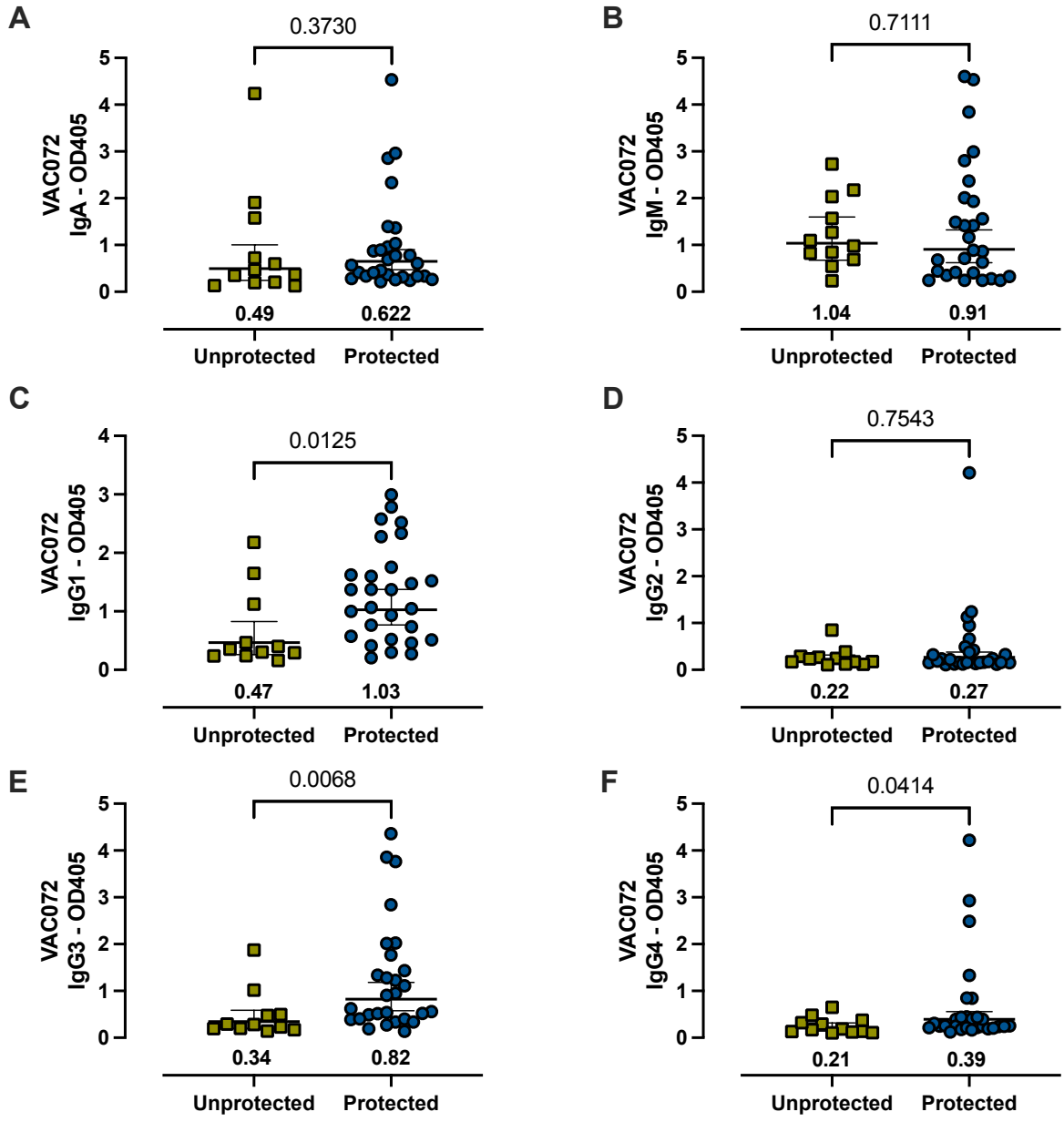


Figure 62 - VAC072 antibody and subclass associations with protection.

A) *OD_{405} Anti-NANP IgA measured 1-month post-third dose stratified by protection in VAC072 – $R21_{SII}$.* ***B)*** *OD_{405} anti-NANP IgM measured 1-month post-third dose stratified by protection in VAC072 – $R21_{SII}$.* ***C)*** *OD_{405} anti-NANP IgG1 measured 1-month post-third dose stratified by protection in VAC072 – $R21_{SII}$.* ***D)*** *OD_{405} anti-NANP IgG2 measured 1-month post-third dose stratified by protection in VAC072 – $R21_{SII}$.* ***E)*** *OD_{405} anti-NANP IgG3 measured 1-month post-third dose stratified by protection in VAC072 – $R21_{SII}$.* ***F)*** *OD_{405} anti-NANP IgG4 measured 1-month post-third dose stratified by protection in VAC072 – $R21_{SII}$.*

Error bars indicate Geometric Means with 95% CI. Values presented above the x-axis are GMTs. P-values were calculated by Mann-Whitney.

As with VAC065, in VAC072 antibody subclass and isotype were assessed for associations with protection. OD_{405} were merged across all groups for each antibody subclass and isotype response and stratified for protection - Figure 62.

For antibody isotypes IgA and IgM, no significant difference in GMT between protected and unprotected was found - Figure 62A/B. For antibody subclass assayed for VAC072, significant differences between protected and unprotected were found for IgG1, IgG3 and IgG4- Figure 62. Antibody subclass IgG2 indicated no significant difference between protected and unprotected participants - Figure 62D.

For subclass IgG1, protected participants had a significantly higher (P=0.013) (GMT 1.03 OD_{405} [95% CI 0.77 – 1.37]) than unprotected participants (GMT 0.47 OD_{405} [95% CI 0.26 – 0.83]) - Figure 62C. For subclass IgG3, protected participants had a significantly higher (P=0.0068) (GMT 0.83 OD_{405} [95% CI 0.58 – 1.18]) than unprotected participants (GMT 0.34 OD_{405} [95% CI 0.20 – 0.59]) - Figure 62E. For antibody subclass IgG4, protected participants had a significantly higher (P=0.041) (GMT 0.39 OD [95% CI 0.276 – 0.56]) than unprotected GMT participants (0.21 OD_{405} [95% CI 0.144 – 0.32]) - Figure 62F.

12.4.13 ISI examining functional antibodies

12.4.13.1 Antibodies induced by $R21_{CBF}$/MM in VAC065 can inhibit sporozoite invasion

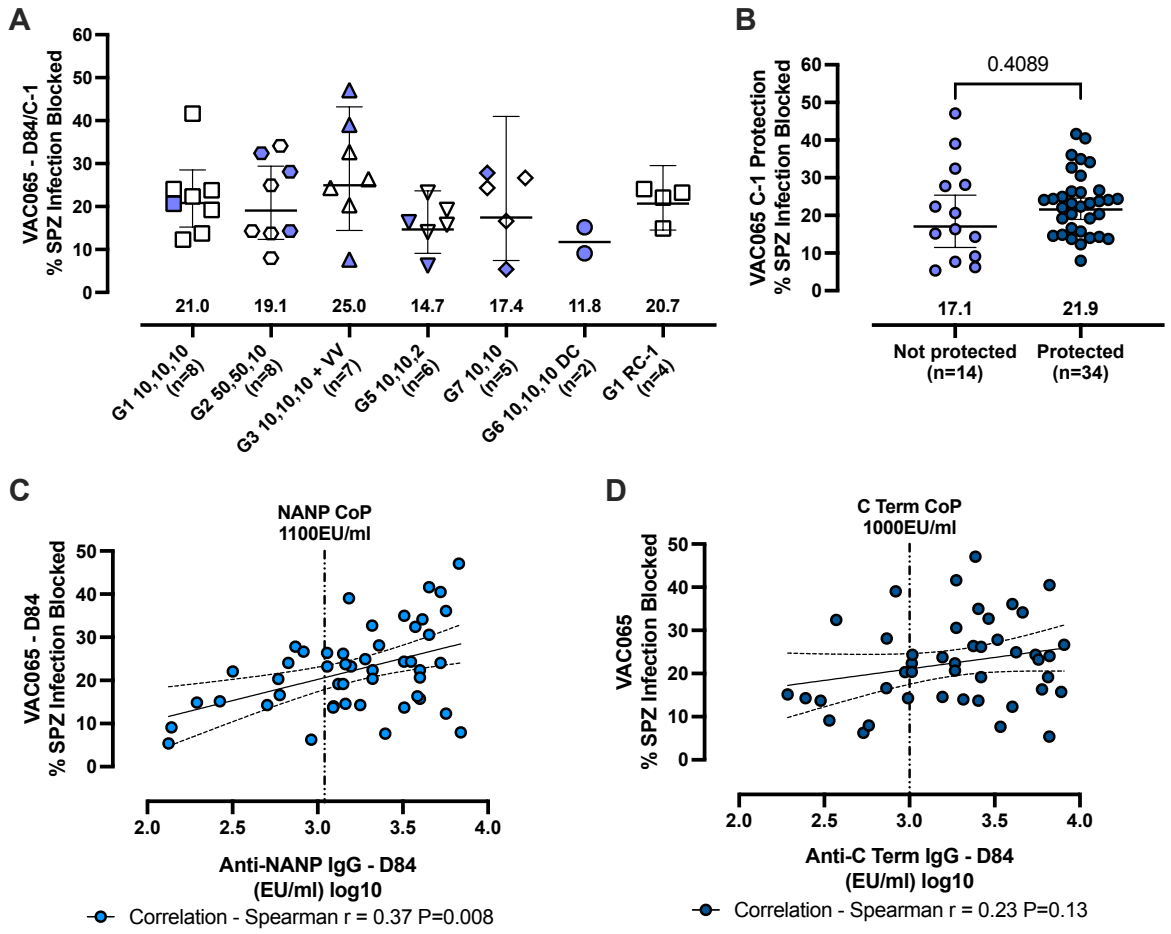


Figure 63 - VAC065 ISI stratified by protection.

A) *Percent sporozoite infection blocked in different groups among challenged participants in VAC072.* ***B)*** *Percent sporozoite infection blocked among protected and unprotected participants in VAC072. P=0.44 Mann-Whitney.* ***C)*** *Relationship between C-1 NANP IgG titres and sporozoite blocking ability in individuals that received three full doses of R21/MM. Spearman rank correlation. Vertical dashed line (x=3.04) 1100EUml CoP)* ***D)*** *Relationship between C-1 C term IgG titres and sporozoite blocking ability in individuals that received three full doses of R21/MM. Spearman rank correlation. Vertical dashed line (x=3.04) 1100EUml CoP)*

Geometric means with error bars to indicate a 95% CI. Values presented above the x-axis indicate geometric mean values.

Due to the limited number of mosquitos available for dissection, the total number of samples from challenged participants had to be reduced. Samples in each group were randomly selected ensuring an even distribution of antibody titres. Due to the high number of protected participants in VAC065,

unprotected participants in each group were prioritised to facilitate potential associations with protection analysis.

In Chapter 1 (11.4.4), a significant increase in % ISI from baseline to vaccinated samples was reported. The highest percentage of inhibition was found in participants who received (10, 10, and 10μg R21) in G1 and G3. At 1-month post third dose (D84), Figure 63A represents the identical % ISI in Figure 41A, although protected and unprotected are highlighted by open and closed symbols and as with antibody responses, and the % ISI was evenly distributed among protected and unprotected within each group - Figure 63A.

As no significant difference was found, % ISI from each group was merged and then stratified by protection status. Protected adults in VAC065 had a higher % ISI (21.9%) compared with unprotected adults (17.1%) however no significant difference was found - Figure 63B indicating that ISI is an ineffective immunological marker to predict protection.

A significant positive correlation (Spearman r=0.37, P=0.008) was found between anti-NANP IgG titres and % ISI for adults in VAC065 - Figure 63C. Anti-C term IgG titres also positively correlated (Spearman r=0.23 P=0.13) with % ISI. This association was however not statistically significant - Figure 63D.

12.4.13.2 VAC072 ISI – delayed and delayed fractional dose groups improve ISI but do associate with protection

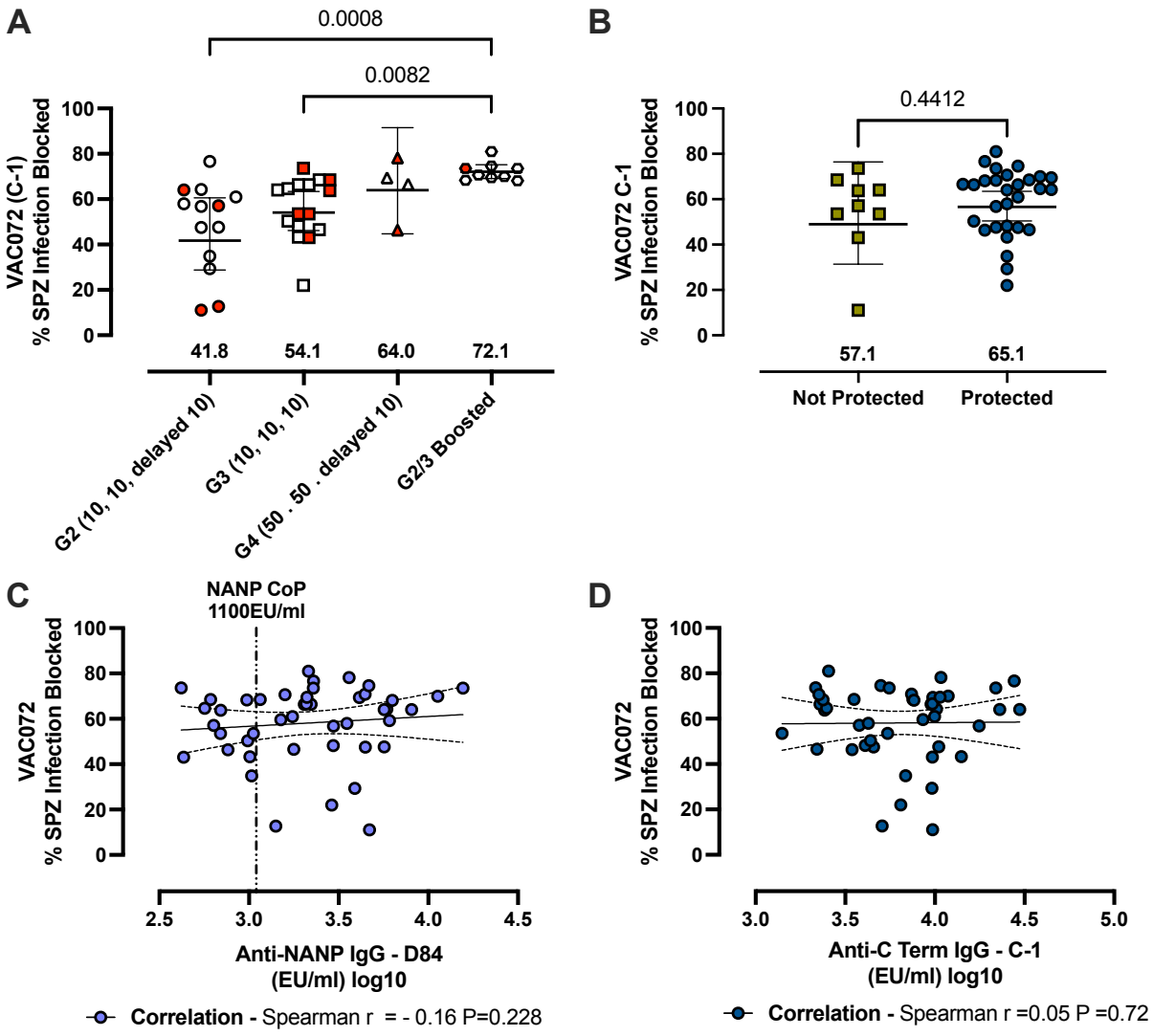


Figure 64 - VAC072 ISI stratified by protection.

A) *Percent sporozoite infection blocked in different groups among challenged participants in VAC072.* ***B)*** *Percent sporozoite infection blocked among protected and unprotected participants in VAC072. P=0.44 Mann-Whitney.* ***C)*** *Relationship between C-1 NANP IgG titres and sporozoite blocking ability in participants that received three full doses of R21/MM. Spearman rank correlation. Vertical dashed line (x=3.04) 1100EUml CoP)* ***D)*** *Relationship between C-1 C term IgG titres and sporozoite blocking ability in participants that received three full doses of R21/MM. Spearman rank correlation.*

Geometric means with error bars to indicate a 95% CI. Values presented above the x-axis indicate geometric mean values.

Due to the limited numbers of mosquitos and yield of sporozoites per mosquito obtained, only samples from participants who underwent CHMI were tested in VAC072. In Chapter 1, a significant increase in % ISI from baseline to vaccinated samples was reported. The highest percentage inhibition was found in participants who were boosted and re-challenged (72.1%) and the lowest inhibition of sporozoites was observed in G2 (10, 10, delayed 10µg). In Figure 64A, samples from protected and unprotected are highlighted by open and closed symbols and as with antibody responses, % ISI was evenly distributed among protected and unprotected within each group.

To assess if a certain % ISI correlated with protection, % ISI across all groups assayed were merged and subsequently separated by protected and unprotected UK adults - Figure 64B. Protected participants had a higher percentage ISI – 64.3 % compared to unprotected participants with a median percentage ISI of 57.1% with no significant difference between the two groups. Both anti-NANP and anti-C term IgG titres showed no correlation with % ISI titres.

12.4.14 Multivariate model to assess associations with protection

All immune responses from participants who underwent CHMI in both VAC065 and VAC072 irrespective of group were merged and a univariate logistic regression analysis was performed to assess the association between immune response and protection. In addition, AUROC analysis was performed to help discriminate immune variables which are associated with protection. Immunogenicity data from participants who were re-challenged were excluded as values will not be independent and will likely correlate with each other. Full methods for the multivariate analysis can be found in methods (10.4.5).

Table 19 - Univariate and logistic regression and AUC analysis on UK adult immunology

Immunological data measured at C-1 across both VAC065 and VAC072 was merged and assessed for association with efficacy post-CHMI. The outcome variable is protection following challenge. All immune response variables have been log-transformed except for % ISI and NANP IgM.

**Estimated odds ratio from a logistic regression model where protection is the outcome variable and the log of the immune response is the independent variable (except ISI and NANP IgM)*

^ Benjamini-Hochberg adjusted P-value with a false discovery rate of 0.2 (variable to be included in the multivariate model if B-H adjusted P-value ≤0.2)

AUROC – area under the receiving operating characteristic (variable to be included in the multivariate model if AUROC≥0.8)

Variable	Immune Timepoint	N	Outcome Variable	OR*	95% CI	P-value	B-H adjusted P value ^	AUROC
% ISI	C-1	65	C+21	1.01	0.98 to 1.04	0.509	0.626	0.564
Avidity C term	C-1	78	C+21	1.87	0.39 to 8.93	0.432	0.576	0.540
C term IgG ELISA	C-1	78	C+21	1.55	1.01 to 2.39	0.047	0.154	0.629
IFNγ CSP ELISpot	C-1	71	C+21	1.44	0.90 to 2.29	0.129	0.229	0.627
C term IgG MSD	C-1	67	C+21	1.41	0.92 to 2.15	0.110	0.229	0.617
HBsAg IgG MSD	C-1	66	C+21	0.86	0.64 to 1.15	0.308	0.448	0.412
IgG NANP MSD	C-1	67	C+21	1.87	1.01 to 3.48	0.048	0.154	0.651
IgG R21 MSD	C-1	67	C+21	1.49	0.84 to 2.63	0.173	0.277	0.623
Avidity NANP	C-1	78	C+21	0.84	0.18 to 3.89	0.826	0.826	0.511
NANP IgA	C-1	76	C+21	1.74	0.92 to 3.32	0.091	0.229	0.589
NANP IgG1	C-1	76	C+21	2.48	1.08 to 5.67	0.031	0.154	0.646
NANP IgG2	C-1	76	C+21	1.22	0.61 to 2.42	0.577	0.626	0.504
NANP IgG3	C-1	76	C+21	1.92	1.08 to 3.41	0.027	0.154	0.658
NANP IgG4	C-1	45	C+21	2.56	0.79 to 8.28	0.117	0.229	0.670
NANP IgM	C-1	76	C+21	1.14	0.71 to 1.85	0.587	0.626	0.535
IgG NANP ELISA	C-1	78	C+21	2.37	1.34 to 4.18	0.003	0.048	0.690

Five variables (C term IgG ELISA, IgG NANP MSD, NANP IgG1, NANP IgG3 and IgG NANP ELISA) highlighted in yellow in Table 19 had an association with protection (B-H adjusted P-value of ≤0.2). No variable had an AUROC≥0.8.

We then attempted to construct a multivariate (forward stepwise logistic regression) model. After inclusion in the model of anti-NANP IgG (as measured by the standardised NANP ELISA), no further

variable fulfilled the inclusion criterion, indicating that no additional variable was significantly predictive of protection, independent of anti-NANP IgG.

12.5 Chapter 2 - Discussion

12.5.1 Manufacturing

Data presented in Chapter 1 indicated that $R21_{CBF}$ manufactured at the CBF has induced high antibody titres that are comparable to antibody titres from protected participants immunised with RTS,S/AS01B [420, 422, 425] when using the Jenner standardised NANP ELISA. In 2017 [443], Oxford entered into an arrangement with the SII and Novavax to manufacture R21/MM at scale with plans to manufacture 200 million doses annually by 2023 [444]. SII optimised the manufacturing of R21/MM removing the C-tag for vaccine purification [445], resulting in a novel vaccine. Thus, VAC072 was the first in-human study to test R21 manufactured in SII for both safety, immunological and efficacy comparisons. In this chapter, I examined potential immunological differences between $R21_{CBF}$ and $R21_{SII}$. In addition, changes in dose and timing of dose regimens can impact immunogenicity and efficacy after CHMI and possible immune mechanisms mediating protection in malaria naïve adults.

12.5.2 VE CBF vs SII

Antibody responses induced from standard adult dose regimen (10, 10, 10µg $R21_{SII}$) were comparable to the standard dose $R21_{CBF}$ (VAC065 G1 – 10, 10, 10µg $R21_{CBF}$) manufactured in CBF. Furthermore $R21_{SII}$ and $R21_{CBF}$ induced comparable point estimates of VE ($R21_{CBF}$ - 80% [95% CI 60-94] and R21SII – 66% [95% CI 35-85]) with overlapping 95% CI in malaria naïve adults in the UK. However, low sample sizes in these groups do not allow power for group-to-group comparisons. It is not uncommon to observe variations in VE results when comparable dose regimens with other anti-sporozoite vaccines [420, 422, 439]. Possible reasons are due to volunteer immune heterogeneity, vaccine-induced and non-specific immunity, and quality of CHMI or number of infectious bites. However, CIs overlap providing confidence in vaccine performance.

Comparable anti-NANP IgG were found between manufacturing sites for $R21_{CBF}$ and $R21_{SII}$ however statistically higher anti-C term titres were found with $R21_{SII}$ compared with $R21_{CBF}$. This may be an anomaly caused by the initial assay setup considering anti-NANP IgG responses were comparable and anti-C term IgG titres measured by MSD were comparable. Given more time, anti-C term IgG titres would be repeated for VAC072 to confirm the significant difference between $R21_{SII}$ and $R21_{CBF}$.

12.5.3 Infectivity controls failing to be diagnosed

One concern in both VAC065 and VAC072 was the first cases of an unvaccinated control volunteer failing to develop clinical malaria in a CHMI study conducted at the Jenner Institute, in collaboration with Imperial. Similar instances have been reported in several other CHMI trials undertaken at other centres [258] and are likely a result of non-specific immunity or variations in mosquito-administered sporozoite dose. Cross-reactive immunity is an unlikely reason as all controls had low anti-NANP titres as presented in Chapter 1. Furthermore, the mean time to diagnosis in the control groups is otherwise within the expected range (~10-14 days). Regardless, these occurrences pose no significant limitation on the principal aim of each study to provide a measure of whether R21/MM can elicit protection and estimates of VE using RR take non-infected controls into account.

12.5.4 Post-hoc associations with protection

Both $R21_{SII}$ and $R21_{CBF}$ produce high anti-NANP and anti-C term IgG which are associated with increased protection after CHMI. However, many participants across both trials had a wide range of antibody titres with overlapping CIs between protected and non-protected participants. NANP and C term antibodies remain critical for protection against *Pf* [446] and the positive antibody associations found in this chapter could be described as a SoP whereby an immunological parameter correlates with reduced risk [447], whereby anti-CSP antibodies may be able to predict protection in a defined population [271]. Further details can be viewed in Table 6.

Anti-NANP>1100EU and C term>1000EU thresholds could be valuable going forward, creating a benchmark for future R21 trials to achieve. However, the large overlap in antibody titres between protected and non-protected participants implies additional immune mechanisms contribute to protection induced by these vaccines. Furthermore, using a post-hoc approach in visually selecting a titre to associate with protection may be statistically flawed and result in an increased likelihood of finding significance. In addition, the low sample numbers across both trials and the large ratio of protected to non-protected participants across each group provide low power. Larger efficacy studies may be needed to find additional immune correlates. Being able to achieve a significant CoP would be invaluable in designing new trials without field efficacy or CHMI which is expensive and increases risk for participants challenged with *Pf* [448] as well as the gold standard for vaccines to ascertain [449]. Antibody thresholds achieved are a step forward in potentially immunising adults or using R21/MM as a traveller's vaccine [450].

12.5.5 Fractionated third dose

Our group previously reported among malaria naïve adults in the UK who received RTS,S/AS01b, that a reduced third dose resulted in improved VE (89% [95% CI 43.3–98.4]) compared with the same dose given at all three visits (75% [95% CI 31.5–93.1]) although no differences in antibody responses were found [420]. In VAC065, comparable VE from two fractional third dose regimens G2 (50, 50, 10µg $R21_{CBF}$) – 63% [95% CI 14-86] and G5 (10, 10, 2µg $R21_{CBF}$) - 77% [95% CI 21-94] was found compared to the standard dose regimen. Comparable anti-NANP IgG titres were seen in both fractional third dose groups but the higher fractional dose group had significantly lower anti-C term responses to the lower fractional dose group. This C term disparity in the fractional dose group was also found using the MSD anti-C term data. Reasons for this remain to be disentangled.

A lower dose regimen and even a fractional third dose producing comparable immunogenicity and efficacy have significant logistical advantages for vaccine dose sparing and ease of adherence to strict

dosing schedules. A reduced third dose can lead to a better quality of antibody response by increasing GC and affinity maturation from increased antigen competition from T and B cells [451]. To assess possible improvement of antibody quality from fractional doses I investigated antibody avidity by sodium thiocyanate displacement and found no differences in avidity to both NANP and C term-specific binding of IgG in any group in VAC065. Furthermore, no difference in avidity was found between protected and non-protected participants. Avidity to C term peptide has been reported as a potential immune marker associated with protection for RTS,S/AS01$_E$ by Dabonao *et al.,* 2019 [358] however this finding has yet to be repeated.

R21$_{CBF}$ was able to seroconvert most adults in VAC065 with subclass (IgG1 and IgG3) and isotype (IgA and IgM) antibodies greater than the LLoD. The highest responses were found in those adults immunised with the standard dose regimen, and a significant IgG2 response for which most of the dose regimens were seronegative. IgG2 in standard dose regimen was a concern being associated with malaria risk [90]. Nevertheless, the ratio between cytophilic subclass IgG1/3 to non-cytophilic subclasses IgG2/4 found in VAC065 was positive and has been linked with increased protection [164, 452]. However, no subclass or isotype associations were found between protected and unprotected in this dataset.

12.5.6 Delayed fractional third dose

VAC072 explored whether delaying the third dose and a delayed fractional third dose improved immunogenicity and efficacy as observed in other studies [258, 431, 453]. A delayed third dose of R21/MM at 5 months improved anti-NANP IgG and anti-C term titres when compared to the standard dose regimen of R21$_{SII}$ which is consistent with delayed third dose RTS,S in malaria naïve adults immunised with RTS,S [258]. However, a delayed third dose of RTS,S resulted in reduced efficacy and immunogenicity compared to 3 dose standard regimen in African children [267].

A delayed fractional third dose of R21/MM was also investigated in VAC072 following promising data presented by Regules *et al.,* 2017 [439], which saw improved efficacy and immunogenicity. Conclusions on whether efficacy for the delayed fractional dose groups remain to be investigated as only 4/9 participants in G4 (50, 50, delayed 10 $R21_{SII}$) were challenged and no participants in G5 (10, 10, delayed 2µg $R21_{SII}$) underwent CHMI. The lower delayed fractional dose G5 induced significantly higher anti-NANP antibodies that were above the threshold of protection of 1100EU and anti-C term IgG that was comparable to G2 (10, 10, delayed 10µg $R21_{SII}$) and G3(10, 10, 10µg $R21_{SII}$) both of which provided high levels of protection. G4 (50, 50, delayed 10µg $R21_{SII}$) had wide a range of anti-NANP IgG amongst protected and non-protected participants, alluding that a larger dose of R21 can be detrimental and produce low anti-NANP IgG titres. While a delayed fractional dose improves immunogenicity in malaria naïve adults, large field efficacy studies indicate that a delayed fractional third dose of RTS,S/$AS01_E$ did not improve immunogenicity and efficacy in infants and young children in malaria-endemic regions [267, 454]. It is yet unclear what immune mechanisms might be influencing the combination of a delay and fractional third dose, and studies conducted thus far have been unable to disentangle the two aspects.

A delayed fractional third dose of R21/MM improved the avidity of both NANP IgG and substantially increased avidity C term specific IgG compared to the standard dose regimen, implying that increased timing between doses and dose variation will allow greater GC priming following an encounter with an antigen [455] improve functional B cells and ultimately antibody quality assessed by avidity [430]. As with VAC065, avidity was not associated with protection in VAC072, indicating that IgG avidity is an inaccurate immune marker associated with protection against malaria, although useful to describe changes in antibody quality. This corresponds with avidity data that a fractional third dose of RTS,S improves avidity and is not a good immunological marker for protection [420]. However, avidity measured in RTS,S administered with a delayed fractional dosed samples measured by biolayer interferometry (BLI) and binding antibody multiplex assay (BAMA) found that IgG magnitude, as well

as avidity, were associated with protection [366]. This is likely because of the sensitivity of the assay being able to accurately determine when an antibody attaches to an antigen (on rate) and when it detaches (off rate) known as the area under the dissociation Phase binding response curve (AUC_{diss}). Remodelling RTS,S data is significantly improved when incorporating avidity with antibody titre, suggesting increased titre along with increased avidity in volunteers is associated with increased protection [456]. However, in African field trials in infants, avidity was not associated with protection [457, 458].

In VAC072, delayed fractional dose G2 (10, 10, delayed 10µg R21) induced higher cytophilic antibodies IgG1/3 compared to the standard dose regimen. But the higher delayed fractional dose G4 (50, 50, delayed 10µg R21) had the lowest subclass responses which is to be expected, following low total IgG responses. QC of IgG4 saw experiment failures on VAC065 samples but increased IgG4 in delayed and delayed fractional dose regimens of VAC072 corresponds to IgG4 responses induced from delayed fractional dose of RTS,S [429]. Both $R21_{SII}$ and $R21_{CBF}$ induced strong levels of isotype antibodies IgA and IgM and their roles in protection are not yet clearly defined [163]. Protected participants had increasing levels of IgM which is associated to be increased levels in controls from NAI [459].

12.5.7 Two doses vs three doses

A two-dose vaccine schedule has merited exploration as this may be sufficient to protect people living in malaria-endemic regions who have been primed through natural exposure. In addition, showing efficacy from 2 doses can promote some protection for individuals who do not adhere to a three-dose schedule. Two doses of $R21_{CBF}$ elicited a VE of 65% [95% CI -8 to 89%] however low sample numbers and high confidence intervals make it difficult to make any meaningful conclusions aside from protection can be provided using two doses. No significant difference in antibody response was found between two and three doses of R21 and two doses induced functional antibodies which inhibited Spz invasion of hepatocytes *in vitro*. This two-dose regimen may merit further development, possibly with a delayed third (booster) dose at a later visit. However, data from RTS,S has shown a three-dose

regimen superior to a two-dose regimen in field studies [460]. Taken together, these data support the further evaluation of two doses as having some useful efficacy in the CHMI model.

12.5.8 Delay to parasitaemia

$R21_{CBF}$ produced a delay to blood-stage parasitaemia at day 7.5, however, this was only seen in challenge A of VAC065. Indicating R21/MM provided partial protection as measured by few parasites leaving the liver 7 days post-CHMI compared to controls. All participants who presented with blood stage parasitaemia regardless of the delay were diagnosed with *Pf* by day 15. Few anti-sporozoite vaccines have replicated this finding, although White *et al.*, 2013 [362] reported a similar finding with a 96.1% reduction in liver to blood stage inoculum because of immunising with RTS,S. This result has been observed in other RTS,S studies in the field where delayed blood stage antigens were reduced in infants immunised with RTS,S promoting protection from clinical malaria [461] which persists for many years [462]. This finding in challenge A of VAC065 is unique amongst CHMIs performed in malaria naïve adults with anti-sporozoite vaccines, but caution in interpretation is required due to the small sample size and is likely challenge dependant and specific to the infectivity of sporozoites in mosquitos.

12.5.9 Viral vectors

RTS,S administered with viral vectors that target CSP does not result in better efficacy or antibody responses, however, a notable improvement with a CD4 and CD8 cellular response compared with RTS,S on its own [463]. The addition of MVA and ChAd63 encoding ME-TRAP along with three doses of R21/MM did not improve efficacy compared standard dose regimen in contrast to what was seen with a similar regimen involving RTS,S administered concomitantly ChAd63 and MVA ME-TRAP [420]. No interference with anti-NANP or anti-C term IgG responses was found in comparison to the standard dose regimen. In addition, R21 with additional viral vectors did not reduce blood stage parasitaemia at day 7.5 compared to the R21 alone groups. Adding a viral vector that encodes another malaria

antigen of a different malaria life cycle presents a promising hypothesis and may benefit a multi-stage malaria vaccine [227, 464].

12.5.10 ISI

The ISI assay is a useful tool to assess the functional ability of antibodies from anti-sporozoite vaccines such as R21/MM to block infection *in vitro* [413]. In malaria naïve adults in VAC065, anti-C term IgG titres followed a similar trend to anti-NANP with a positive correlation, however, there was no such association for both VAC065 and VAC072. This finding differs from previous data from UK adults vaccinated with RTS,S/AS01B where a significant difference in % ISI between protected and unprotected was observed [425]. While both assays utilised similar SOPs, key differences in procedure were applied. Bowyer *et al.*, 2018 [425] infected with 10,000spz per well whereas, in this thesis, 15,000spz were used to infect cells. The increases in sporozoite infection concentration were due to low infectivity in early experiments. The second is that serum samples were diluted to 10% for RTS,S/AS01B samples whereas serum was diluted to 1%. This was done because I found that the ISI assay is very sensitive to antibody concentration, as shown by the positive correlation between antibody titre and % ISI. Therefore, changes to the number of Spz infected and serum dilutions could impact associations with protection [413]. Further assay optimisations need to be made which are discussed in the main discussion of this thesis.

12.5.11 Hepatitis B Surface antigen

All UK adults in VAC065 and VAC072 were seropositive (anti-HBsAg >0.003IU/ml [440]) at baseline. Irrespective of the dose and dose regimen, all UK adults had increased anti-HBsAg after two or three doses of R21/MM in VAC065 and VAC072 that were higher than the WHO threshold of protection (anti-HBsAg > 0.01 IU/ml) [441, 442]. Most surprising was that the highest anti-HBsAg titres were found in the two-dose regimen in VAC065. A similar finding was made in RTS,S where two doses of RTS,S produced comparable HBsAg responses compared to three doses of RTS,S [368]. Additional time

points should be assessed in three-dose groups and compared to two doses of R21/MM on anti-HBsAg titres.

12.5.12 Multivariate analysis

NANP has long been identified as the immunodominant epitope with anti-CSP vaccines [271]. This is consistent with data found in this chapter where after univariate logistic regression models to identify associations with protection found total anti-NANP IgG, anti-NANP IgG1 and anti-NANP IgG3 consistent with what was found in other univariate models [366]. Furthermore, a significantly associated variable in a multivariate model found that total anti-NANP is the variable most associated with protection in R21 immunised malaria naïve adults. Post-hoc power calculations found the dataset was significantly underpowered (26%). To gain additional power (>90%) to achieve additional associations with protection, one would need at least 221 protected adults and 111 unprotected participants to achieve significance.

12.5.13 Limitations

Limitations of this study include the small sample size, and that only 10 out of 12 participants in the infectivity control group developed parasitemia. CHMI studies are, by design, small proof of concept studies to accelerate the down-selection of promising vaccines by generating an early indication of efficacy. Sample size was restricted by cost, supply of GMP-grade mosquitos for CHMI, and clinical trial site capacity to undertake the intensive follow-up required after the challenge agent was administered. Recruitment to CHMI studies can be challenging due to the stringent safety requirements, inflexible timelines due to the need to challenge all participants simultaneously and time requirements for participants to attend clinic visits. Lack of infection of control volunteers in malaria CHMI studies can also impact VE. Five infectious bites are normally 100% infective in most cases with unvaccinated controls and this is likely a chance finding. CHMI performed in this chapter

used a homologous parasite strain for CHMI and therefore efficacy found may not be replicable in field studies.

Lastly, a limitation in this chapter is the lack of cellular data on B and TfH cells and their relation to antibodies induced from R21. It is known that delaying the third dose and a delayed fractional third dose can improve the GC function of B cells producing a better quality of antibodies [430]. A delayed third dose and delayed fractional third dose increase antigen avidity by competitive antigen binding in GCs which leads to the expansion and survival of B cells [439]. Going forward, B cell and TfH phenotyping will be undertaken in samples from UK adults who were administered different dose regimens of R21/MM.

12.6 Chapter 2 - Conclusion

It is shown that R21 manufactured in CBF and SII show comparable immunogenicity and efficacy. Delayed and delayed fractional dosing improves antibody titre and promotes avidity to the C term portion of the vaccine. Significant associations between anti-NANP (1100EU/ml) and anti-C term titre (1000EU/ml) IgG titre were associated with protection. Recreating *Pf* infection *in vitro* using the ISI assay has shown no association with protection *in vitro* and is therefore not a good surrogate marker for predicting protection from R21/MM samples. However, the assay does correspond to dosage regimens which will induce the highest antibody response which corresponds with increased antibody titre results in a greater protection. R21 induces strong HBsAg responses that appear not to interfere with other CSP antigen responses. R21/MM induces strong cytophilic subclass antibodies which are associated with protection in other studies. Delayed and delayed fractional dose regimens offer interesting insights into boosting immunity and efficacy, however will be difficult to deploy at scale and likely only be beneficial for adults.

Chapter 3

13 Chapter 3 - Protection infants in Burkina Faso administered with R21/MM within highly seasonal malaria endemic settings

13.1 Chapter 3 - Introduction

Malaria has a significant impact on communities in Burkina Faso [465] and in 2021, had one of the highest burdens of malaria cases and deaths per thousand in the world, with approximately 3.3% of cases and 3.4% of deaths [95]. Young children under the age of 5 experience the highest risk of severe malaria and death, with even higher rates in one-year-olds [408] thus the highest disease burden falls in the youngest of the population [335]. Despite promising vaccine results from vaccine programmes and the WHO positively recommending the implementation of the RTS,S vaccine [231], vaccine scepticism, arising due to a lack of education, misinformation and conspiracy theories, could impact future vaccine roll-outs [466].

Burkina Faso is an area renowned for having a highly seasonal malaria transmission due to its rainfall being significantly concentrated to 4-6 months of the year (August – December) [402, 467]. It is, therefore, ideally suited for the implementation of SMC [376]. Current malaria vaccines which have reached Phase III have shown a high VE in the first few months after immunisation, although protection declines rapidly thereafter [248, 257, 468]. This sharp decline in protection is associated with a sharp decline in antibody titres [271] and has led to the premise of seasonal vaccine regimens, immunising individuals before the malaria season to induce the highest level of immunity during the highest levels of transmission [379].

Antibodies are generally the primary immunological function that induces protection in clinically available vaccines [344] and with malaria, antibodies are critical for protection against sporozoite infection. A variety of immunogenicity data is available from numerous trials involving malarial anti-sporozoite vaccines, however, precise levels of antibody concentrations are inconsistently associated across trials and the cellular methods upon which antibody production changes because of vaccination remain enigmatic [369]. In malaria vaccine development is understanding protective mechanisms induced by NAI and vaccine-induced immunity (VII) [469]. Establishing an immunological CoP can aid the rational design of novel vaccine strategies, thereby accelerating vaccine development and potentially removing the need for large, expensive efficacy trials [162].

In this chapter, multiple humoral immune responses induced from an R21/MM trial in infants in Nanoro, Burkina Faso are explored. Aiming to identify the optimal immunogenic dose regimen that will inform vaccine trial design for the Phase III assessment of R21/MM. In addition, whether any of the univariate immunological parameters are associated with protection and whether any of the variables qualify to be included in a multivariate model are assessed.

13.2 Chapter 3 - Methods

13.2.1 Aim

The aim was to assess the efficacy of R21/MM administered in seasonal vaccine regimens, whereby vaccinations and subsequent boosters would occur in monthly intervals so that the final vaccination happens preferably near the beginning of the malaria season, which typically runs from June to November.

13.2.2 VAC076 Trial design

VAC076 trial design and bleed schedule can be found in methods (10.3.4.6). In Chapter 1, immunogenicity was presented from the first two years (11.4.1.8), here immunogenicity has been assessed for a further two years examining the effects of a second and third booster.

13.2.3 VAC076 Immunology endpoint

As per the lab analysis plan, the primary immunological endpoint for VAC076 is anti-NANP IgG responses assessed by the standardised Jenner ELISA. Therefore every plasma sample taken from every timepoint was assessed had NANP IgG quantified. Due to the large sample size of the VAC076 trial (n=450) and time limitations, all other exploratory immunology assays presented in this chapter, a subset of samples was selected (n=72), which will be referred to as 'VAC076 immunology cohort' – Table 20.

13.2.4 VAC076 immunology cohort

To create the immunology cohort, with the immunology team still blinded, samples were ranked by order of magnitude of their anti-NANP IgG response based on their response at one month post-third dose – D84. The PID with the highest and lowest anti-NANP IgG titre was selected. Thereafter, 70 additional PIDS were randomly selected into the immunology cohort until n=72. In the final year of this DPhil (February 2022), the study was unblinded and information on the immunology cohort may be viewed in Table 20. Sample size and power calculations can be found in 13.2.5.

Table 20 - VAC076 immunology cohort sample numbers

Year 1 *(Primary Series) - Group 1 – 5µg R21 / 25µgMM. Group 2 – 5µg R21 50µg MM.*
Year 2 *(First Booster) - Group 1 – 5µg R21 / 25µgMM. Group 2 – 5µg R21 50µg MM.*
Year 3 *(Second Booster) - G1a & 2a – 5µg R21 / 50µg MM. G1b & 2b - Control vaccine (Rabivax).*
Year 4 *(Third Booster) - G1aa & 2aa – 5µg R21 / 50µg MM. G1ab & 2ab - Control vaccine (Rabivax). G1bb & 2bb - Control vaccine (Rabivax).*

	0 (Year 1) Primary Series	4 (Year 1) Primary Series	8 (Year 1) Primary Series	60 (Year 2) First Booster	112 (Year 3) Second Booster	164 (Year 4) Third Booster
G1aa	5µgR21 / 25µgMM n=32	5µgR21 / 25µgMM n=32	5µgR21 / 25µgMM n=32	5µgR21 / 25µgMM n=32	5µgR21 / 50µgMM n=16	5µgR21 / 50µgMM n=6
G1ab						Control Vaccine n=9
G1bb					Control Vaccine n=11	Control Vaccine n=10
G2aa	5µgR21 / 50µgMM n=40	5µgR21 / 50µgMM n=40	5µgR21 / 50µgMM n=40	5µgR21 / 50µgMM n=40	5µgR21 / 50µgMM n=24	5µgR21 / 50µgMM n=11
G2ab						Control Vaccine n=12
G2bb					Control Vaccine n=10	Control Vaccine n=10

13.2.5 Sample size

The primary endpoint is the time to the first episode of malaria meeting the primary case definition of clinical malaria episode, over a period of 6 months. The study is powered to provide an initial point estimate of the efficacy of the malaria vaccine if the VE over 6 months is greater than 50%.

Sample sizes were not powered to assess immunological associations with protection. The statistical power of these comparisons is constrained by the number of participants available and by whether a plasma sample was taken due to difficulty in bleeding infants.

13.2.6 Post-hoc power calculation of VAC076 immunology cohort

The VAC076 immunology cohort listed in (13.2.4) before I was unblinded by group and efficacy. A post-hoc power calculation using a two-sample t-test showed that the VAC076 immunology cohort has 83% power to detect the difference observed between the protected and non-protected infants.

13.2.7 Surveillance and case detection

Cases were counted from 14 days after the third dose in the primary series and 14 days following the subsequent booster doses. In VAC076, the primary efficacy endpoint used a mix of both active and passive case detection of clinical malaria.

13.2.8 Efficacy analysis

Efficacy analyses were performed by the lead statistician from PHE – Nicola Williams. Cox regression models were used to analyse the time to first episodes of clinical malaria from fourteen days following the third dose in the primary series and fourteen days following subsequent boosters. The Cox proportional hazards model was used to calculate the HR and VE was calculated as 1 minus the HR. Cases were counted and reported at six and twelve months post primary series and six- and twelve months post boosters. The primary analysis for efficacy was prespecified as being between group 1 (G1) and G3, G2 and G3, and G1 and G2 combined versus G3.

13.2.9 Immunogenicity assays and time points

In this chapter, the anti-NANP IgG ELISA being the primary immunological endpoint, all time points will be measured and assessed for associations with protection. Immunological associations with efficacy (clinical malaria) were made with anti-NANP IgG at twelve months post-third dose in the primary series (12 months), twelve months post-first boost (24 months) and 12 months post-second

boost (36 months). Furthermore, anti-NANP IgG will also be stratified by the number of malaria episodes.

Several exploratory immunology assays involving the VAC076 immunology cohort (13.2.3) are presented. These include IgG-specific responses to the C term. Avidity to both NANP and C term peptides will be assessed. Subclass and Isotype antibodies against the NANP peptide will be assessed. IgG to NANP, C term, HBsAg and full-length R21 protein measured by multiplex assay developed by MSD. ISI at D84 and a select number of baseline samples were assessed for antibody functionality. All immunology assays in this chapter using the VAC076 immunology cohort are correlated and stratified by efficacy to determine whether a potential immunological association with protection can be identified. Immunological associations against multiple episodes will are not assessed due to small event numbers. Details of immunogenicity methods used in this chapter can be found in the appendix methods section (17.5). Peptide and protein sequences can be viewed in appendix (17.6)

Antibody half-life was calculated using a linear exponential regression model. Anti-NANP IgG titres peak at 1-month post third dose (D84) of the primary series and 1-month post first boost (1B+30). Thereafter antibody titres wane over time and thus half-life was calculated using a one-phase decay function 6 and 12 months after peak. A limitation to the model is the use of only 3 timepoints, making the model unstable and therefore interpreted with caution.

13.2.10 Multivariate analysis

For each assay, a series of univariate logistic regression analyses were performed to assess the association between D84, 1B+30, and 2B+30 immune response and risk of malaria infection (meeting primary case definition) in the twelve months following fourteen days post-vaccination and in the three and twelve months following fourteen days post subsequent boosters. In this analyses, the occurrence of malaria infection was the outcome variable and the log of the immune response at each

time point was the independent variable. These models assume all immune responses are independent of each other therefore each immunogenicity association is separated by time points to avoid highly correlated variables.

Full methods of the univariate logistic regression model and AUROC which are used to assess significant association with efficacy and inclusion in a multivariate stepwise regression model can be viewed in methods - 10.4.5.

13.3 Chapter 3 - Objectives

- Assess efficacy in infants immunised with R21/MM over 3 years.
- Assess the effectiveness of booster doses administered at yearly intervals to maintain protection.
- Assess how different doses of Matrix M impact immunogenicity.
- Assess changes in immunogenicity after three doses of R21 and booster doses administered at yearly intervals.
- Assess if univariate immune response can associate with protection at selected time points.
- Assess if combinations of immune responses correlate with protection (multivariate analysis).

13.4 Chapter 3 - Results

13.4.1 *Pf* cases indicating Burkina Faso is a highly seasonal malaria transmission area

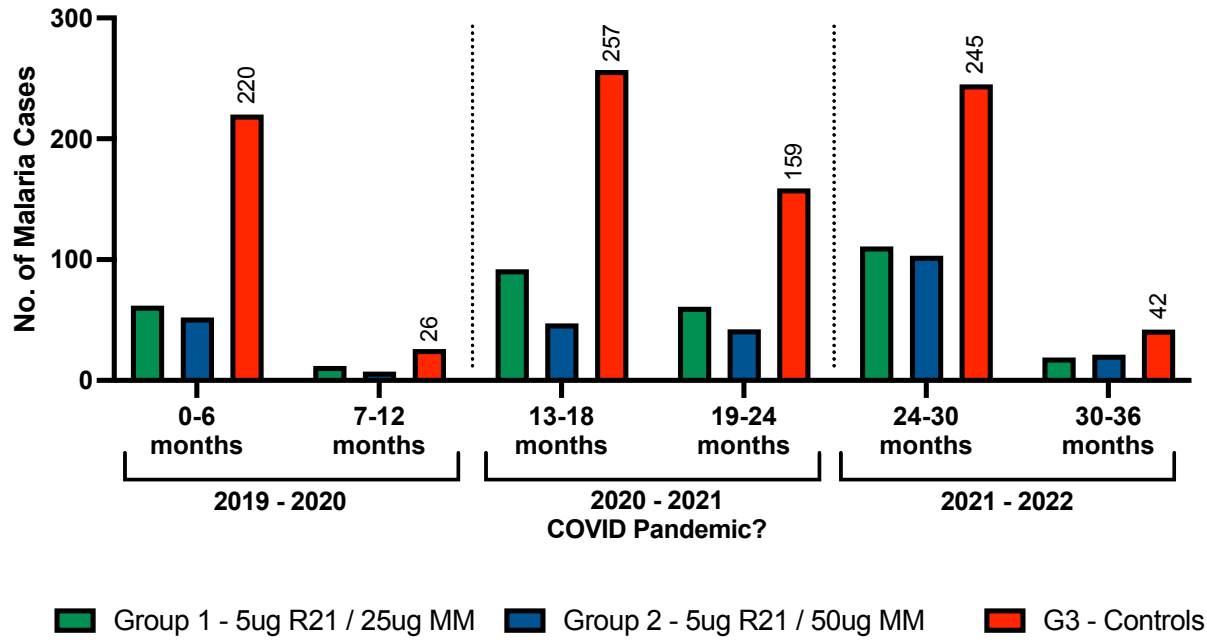


Figure 65 - Transmission of Pf in Burkina Faso

Bar chart summing the total number of Pf cases recorded per group irrespective of whether they receive 1 or multiple cases as per primary case definition of Pf. Chart is stratified per group in 6- 6-month intervals measured 2 weeks after the primary series of vaccinations for the annual 2019-2020 period, 2 weeks after the first booster in 2020-2021, and weeks after the second booster in 2021-2022 over 3 years of the trial. In 2021-2022, G1a and G1b were merged along with G2a and G2b.

Values above the red bar chart list the total number of cases in the control group. CIs are not presented due to exact accumulated sum of recorded Pf cases.

Burkina Faso has a highly seasonal malaria transmission pattern [403, 470], which coincides with the rainy or wetter season around the months of June-December [402]. The number of cases per infant per group irrespective of whether they experience one or multiple cases was summed at six-month intervals over the three years in which the trial was conducted (2019 – 2020, 2020 – 2021 & 2021 – 2022) and increased during the second half of the year, Figure 65. In 2019 – 2020 when vaccinations commenced in the second half of the year (rainy, wetter season), 220 cases were recorded in this period (0-6 months) in the G3 control group compared to 26 cases in the first six months (7-12

months). In the second year (2020 – 2021), a higher number of cases were recorded in all groups in the rainy/ wetter season (13-18 months) with 257 cases recorded in the control G3 when compared to the first six months of the 2019 – 2020 year. In the third year, cases remained high (24-30 months) in the rainy/ wetter season consistent with transmission trends seen in the first two years - Figure 65. At 18-24 months, however, a significantly higher number of cases were recorded in the dry season compared to the first and third years with 159 cases recorded in the control (G3). This remains an outlier as cases dropped significantly in the 30-36 months of the third year - Figure 65.

13.4.2 Durable efficacy observed over 3 year period in Burkinabe infants

Table 21 - VAC076 Efficacy Results over 3 years

Efficacy includes time to the first episode of clinical malaria as per the primary case definition measured 14 days to 12 months after the third vaccination in year 1, 14 days to 12 months after the first boost in year 2 and 14 days to 12 months after the second boost in year 3.

Cox proportional hazards model with 95% CI, adjusted for sex, age category (5–9 months, 10–12 months, and >12 months) and adequate insecticide-treated net use. All P-values comparing vaccination groups to the control group for efficacy were less than 0·0001.

Year 1 – (2019 to 2020). Year 2 – (2020 to 2021). Year 3 – (2021 – 2022).

Group	Subgroup	Year 1 2019-2020		Year 2 2020-2021		Year 3 2021-2022	
		Time to first Pf Episode	Time to first Pf Episode 95% CI	Time to first Pf Episode	Time to first Pf Episode 95% CI	Time to first Pf Episode	Time to first Pf Episode 95% CI
Group 1 (n=150)	1 (n=146)	74%	63-82				
	1 (n=132)			71%	54-81		
	1a (n=80)					67%	58-77
	1b (n=39)					59%	47-75
Group 2 (n=150)	2 (n=146)	77%	67-84				
	2 (n=136)			80%	66-87		
	2a (n=83)					60%	45-78
	2b (n=40)					64%	44-79

Efficacy in Burkinabe infants has been assessed over a 3-year period with the first two years published [2, 3]. In this chapter, VE will be presented as the time to the first clinical episode according to the primary case definition using Cox regression models (13.2.5). Table 21 presents VE percentages for groups G1 and G2 between 14 days and 12 months after vaccination over a three-year period.

In the first year, G1 infants who received the low dose adjuvant (5µg R21/25µg MM) had an unadjusted VE of 71% [95% CI 63–82] at the time to the first episode when compared to the unvaccinated controls in G3. Infants in G2 who received the high dose of adjuvant (5µg R21/50µg MM) had an unadjusted VE of 77% [95% CI 67–84] when compared to the unvaccinated controls in G3. No significant difference in disease incidence was found between G1 and G2.

In the second year, VE measured at twelve months after boost, G1 reported an unadjusted VE of 71% (95% CI 64–81), while G2 reported an unadjusted VE of 80% [95% CI 66–87] when compared to the unvaccinated controls in G3. There was a significant difference in efficacy between G1 and G2 (HR 0·68 [95% CI 0.48–0.98]; p=0.038).

In the third year, infants in G1a who received a booster dose of the higher dose adjuvant (5µg R21/50µg MM) had an unadjusted VE of 67% [95% CI 58–77] when compared to unvaccinated controls (G3). Infants in G1b who did not receive a booster reported an unadjusted VE of 60% [95% CI 47–75]. Infants in G2a who received the boost had an unadjusted VE of 60% [95% CI 45–78] compared with 64% [95% CI 44–79] in G2b who did not - Table 21.

13.4.3 Characterising total IgG responses to NANP

In this chapter, anti-NANP IgG was assessed from plasma samples taken from baseline until 1 month post-third boost of the fourth year.

13.4.3.1 Total IgG to NANP increases after immunisation with R21/MM

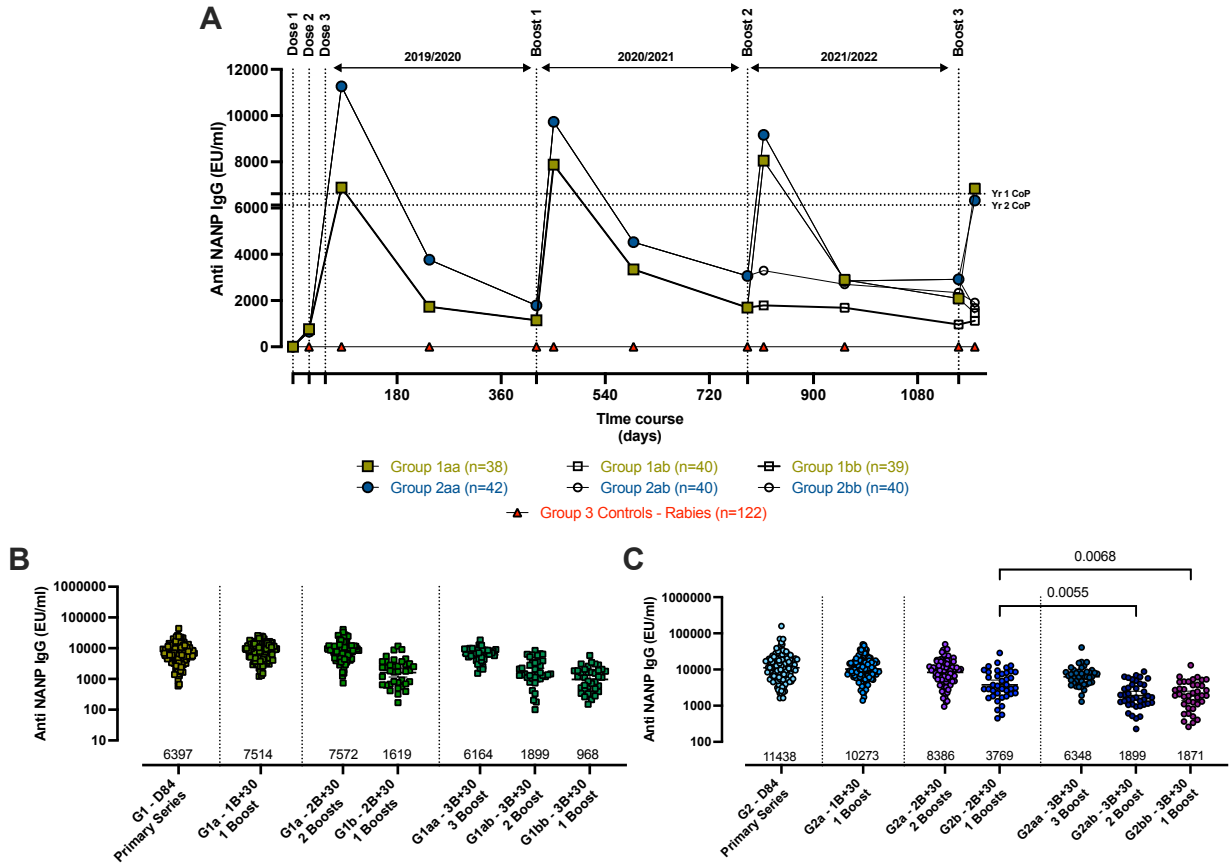


Figure 66 – Anti-NANP IgG over 3 years and 1 month post third boost in the 4th year.

A) *Median time courses (error bars indicate IQR) over the duration of 4 years. The primary series of 3 vaccinations 1 month apart in the first year followed up 3 booster doses given at yearly intervals. G1 are depicted by green squares and, G2 are blue circles. Vertical black lines represent immunisations of R21/ MM. Horizontal black dotted lines indicated threshold protection titre reported in Datoo et al., 2022 [3].* ***B)*** *Geometric means of individual responses at peak time points for G1. Kruskal-Wallis corrected with Dunn's multiple comparison* ***C)*** *Geometric means of individual responses at peak time points for G2. Kruskal-Wallis corrected with Dunn's multiple comparison.*

GMTs with error bars indicate 95% CI. Values above the x-axis indicate GMTs.

In Chapter 1, anti-NANP IgG from the first two years was presented - Figure 34. Here time course figures have been extended to include post-second and third boost data from re-randomised infants to investigate the effects of booster doses of R21/MM to maintain immunogenicity. Infants who received multiple booster doses followed similar antibody kinetics over the three years of the trial - Figure 66. Antibody titres declined one year after the primary series and boost but then significantly

increased one-month post boost for infants in both G1 and G2 - Figure 66A. Infants who were not boosted saw their anti-NANP IgG plateau at 1500 – 3000EU - Figure 66A.

Infants in G1a and G2a who received the same second booster dose (5µg R21/50µg MM) in the 2021/2022 segment of the trial had a significant increase in antibody titres compared to infants in G1b and G2b who did not receive a boost - Figure 66C/D. Similar responses can be seen in the fourth year 2022-2023 segment one month after a third boost whereby infants who received the third booster had a large increase in anti-NANP IgG but not significant compared to infants in G1ab/ G1bb, and G2ab/ G2bb.

13.4.3.2 Boosting improves antibody durability compared to the primary series

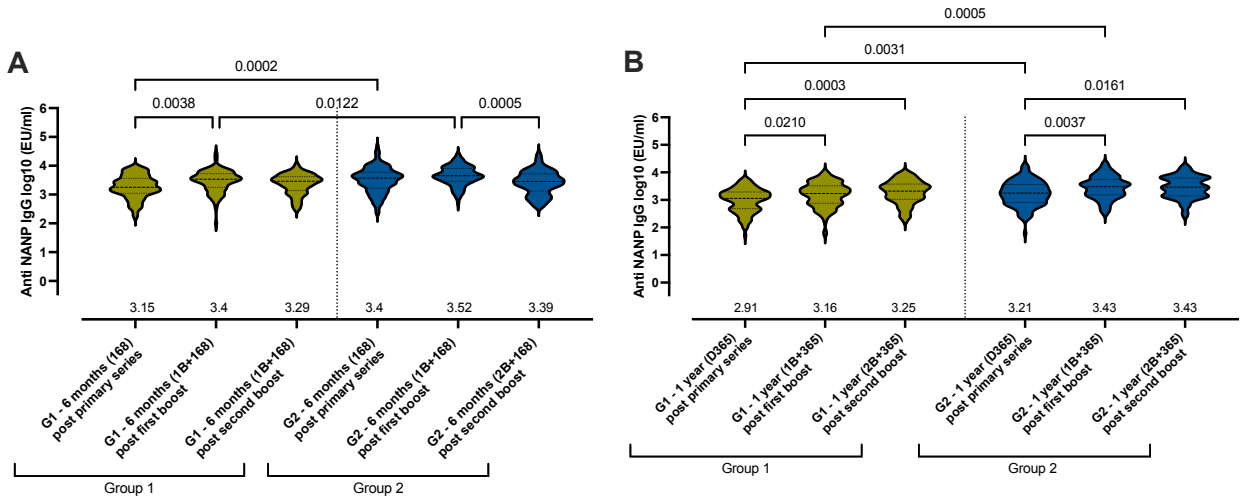


Figure 67 - NANP IgG Durability over 3 years

A) *Violin plots comparing log10 anti-NANP IgG 6 months post primary series (D236), 6 months post first boost (1B+168) and 6 months post second boost (2B+168).* ***B)*** *Violin plots comparing log10 anti-NANP IgG 12 months post primary series (D421), 12 months post first boost (1B+365) and 12 months post second boost (2B+365). Non-boosted infants in G1b & G2b removed.*

Violin plots dotted and dashed lines represent median and quartiles. P-Values, Kruskal-Wallis corrected for Dunn's multiple comparisons.

Antibody durability was assessed by comparing six and twelve months post primary series with six and twelve months after boost 1 and boost 2 covering the first three years of the VAC076 (13.2.1), with

age as a possible confounder. At six months, G1 had significantly higher titres anti-NANP IgG post-first boost (log10 3.45 [95% CI 3.38 – 3.52]) compared with six months post primary series (log10 3.24 [95% CI 3.17 – 3.32]) but no significant difference was observed at six months after the second boost - Figure 67A. At twelve months, infants in G1 had significantly higher anti-NANP IgG titres post-first boost (log 10 3.16 [95% CI 3.07 – 3.24]) and post second boost (log10 3.25 [95% CI 31.16 – 3.35]) when compared to twelve months post primary series log10 2.95 [95% CI 2.87 – 3.04] - Figure 67B.

Infants in G2 had significantly higher anti-NANP titres at six months post primary series (log10 3.48 [95% CI 3.40 – 3.56]) compared with G1. A trend in infants in G2 indicated a higher titre at six months post boost (log10 3.64 [95% CI 3.58 – 3.70]) compared to six months post primary series and significantly higher than six months post second boost (log10 3.39 [95% CI 3.3 – 3.48]) - Figure 67A.

At twelve months, infants in G2 had significantly higher anti-NANP titres post-primary series (log10 3.20 [95% CI 3.12 – 3.28]) and post-first boost (log10 3.43 [95% CI 3.36 – 3.50]) when compared with G1 at those time points. Following the trend from G1, infants in G2 had significantly higher titres twelve months post first and second boost than compared with the primary series - Figure 67B.

13.4.3.3 Boosting with R21/MM improves antibody half-life

Table 22 - VAC076 antibody half-life for group 2

Antibody half-life was calculated through a non-linear exponential regression model to calculate the one-phase decay across 3 time points.

Year 1 – 1 month post third dose (D84), 6 months post third dose (D236) and 12 months post third dose (D421).

Year 2 – 1 month post first Boost (1B+30), 6 months post first boost (1B+168) and 12 months post third dose (B365).

Year	Half-Life (days)	95% Confidence Interval
Year 1 2019 - 2020	120.63	88.58 – 186.21
Year 2 2020 - 2021	185.69	148.29 – 240.31

Antibody half-lives were calculated for G2 (5µg R21/50µg MM) using a non-linear exponential regression model across 3 time points post-primary series (D84, D236 and D421) and 3 time points post-first boost (1B+30, 1B168 and B365). Antibody half-life in the first year was calculated to be 120.63 days [95% CI 88.58-186.21], which improved after a boost to 180.69 days [95% CI 148.29–240.31]. The CIs are large due to the instability of the model as half-life was assessed over only 3 time points and the inclusion of more time points would have improved certainty.

13.4.3.4 Protected infants have significantly higher anti-NANP IgG

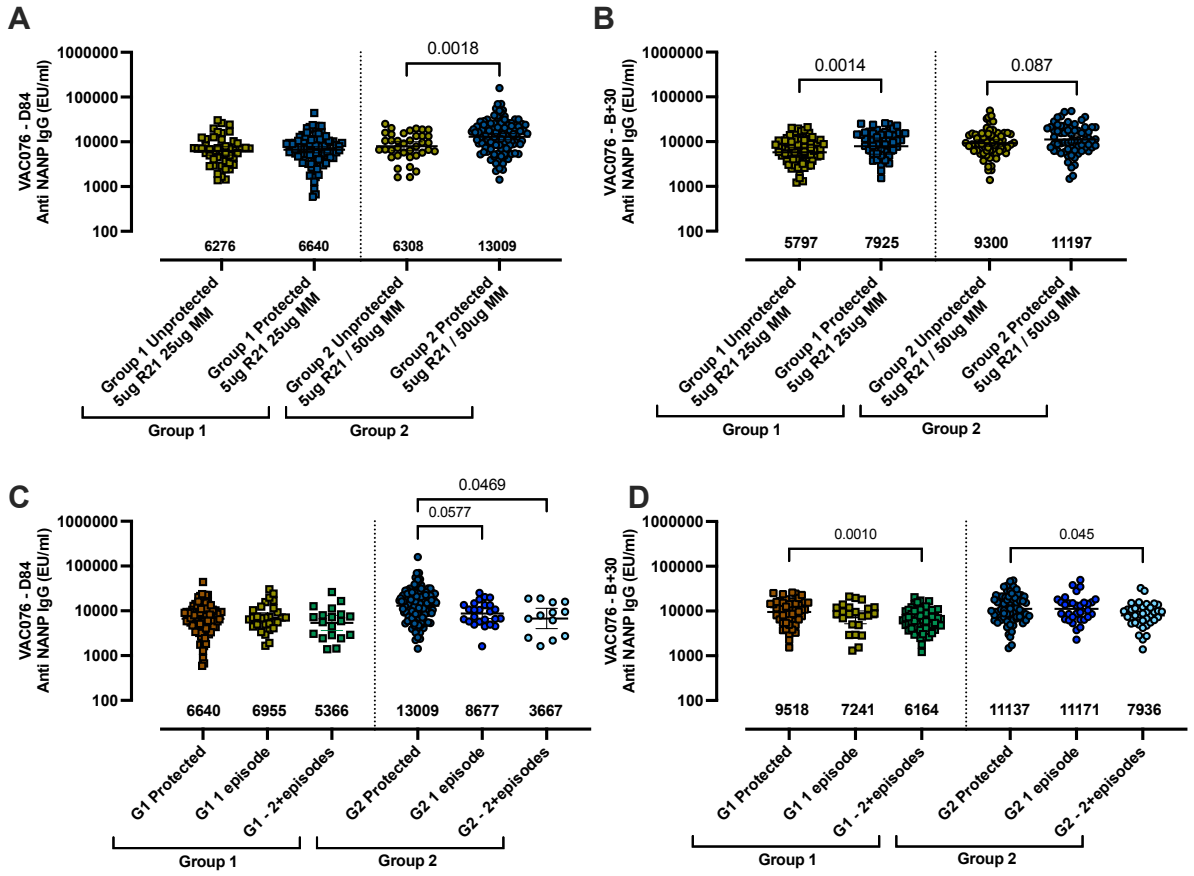


Figure 68 - VAC076 anti-NANP IgG CoP 12 & 24 months.

A) *Anti-NANP IgG measured at D84 stratified by efficacy at 12 months.* ***B)*** *Anti-NANP IgG measured at 1B+30 stratified by efficacy at 24 months.* ***C)*** *Anti-NANP IgG measured at D84 stratified by multiple episodes (protected, 1 episode and 2 or more episodes) at 12 months.* ***D)*** *Anti-NANP IgG measured at 1B+30 stratified by multiple episodes (protected, 1 episode and 2 or more episodes) at 24 months.*

Geometric means with error bars indicating 95% CI. Values above the x-axis indicate geometric means. P-values were calculated by Kruskal-Wallis with Dunn's correction for multiple comparisons.

When stratifying infants by protection by the primary case definition at twelve months, protected participants in G2 had significantly higher anti-NANP IgG titres at D84 one month post third dose – (GMTs 13009EU [95% CI 11092 – 15258]) compared with unprotected – (GMTs 7960EU [95% CI 6308 – 10043]) - Figure 68A. Groups were not merged as G2 had significantly higher antibody titres than G1 - Figure 34. No difference was observed between protected and unprotected in G1 - Figure 68A.

A multiple-episode analysis was investigated. No significant difference in the first twelve months was found in G1 between protected and multiple episodes - Figure 68C. In G2, a significant difference (P=0.47) was observed between protected participants and participants with two or more episodes, and a trend for a higher anti-NANP IgG between protected and those with one episode was observed - Figure 68C.

In the second year, immunogenicity measured at one month post-first boost (1B+30) was stratified by protection observed at twelve months after the first boost. Both protected participants in G1 and G2 had significantly higher anti-NANP IgG than unprotected participants - Figure 68B. A similar association can be observed when comparing multiple episodes. Protected infants in G1 and G2 had significantly higher anti-NANP IgG than infants who had two or more episodes - Figure 68D.

13.4.3.5 Threshold of protection in Year 1 and Year 2

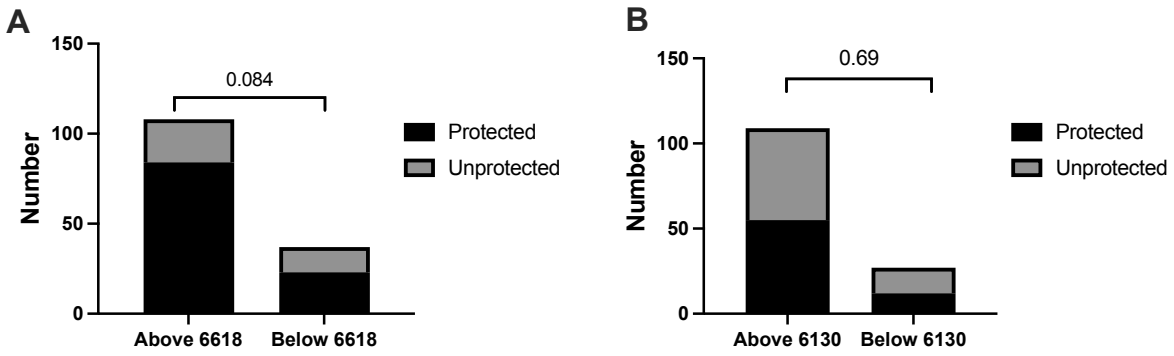


Figure 69 – Protection stratified using published antibody EU thresholds.

A) *Antibody threshold of <6618EU> and stratified by protection at 12 months - P. Fischer's exact test.*
B) *Antibody threshold of <6130EU> and stratified by protection at 24 months - P. Fischer's exact test.*

Both antibody thresholds of <6618EU> and <6130EU> were identified by lead statistician Nicola Williams in Datoo et al. 2022 [3]

Datoo *et al.,* 2022 [3], NANP antibodies were significantly higher in G2 among participants that remained malaria-free during both first years of follow-up (mean difference 0·21 [95% CI 0·10–0·33]; p=0·0004) and second year of follow-up (mean difference 0·12 [0·01–0·22]; p=0·032). The paper

reported two thresholds of protection using reverse cumulative distribution curves from data generated in G2. In year one, VE was 77% and found that infants who had antibody titres greater than 6618EU [95% CI 5565–8397] were associated with a 77% reduction in malaria. In year two, VE for G2 was 80% and therefore in the second year following the first boost, infants who had an antibody titre greater than 6130EU [95% CI 5347–7179] had an 80% reduction in clinical malaria.

Using this threshold, participants in G2 were stratified by protection and antibody level in both year 1 6618EU or year 2 6130EU - Figure 69A and B respectively, and Fischer's exact test was performed on cumulative values. No association or significant difference could be detected using this method.

13.4.3.6 A second boost of R21/MM in year 3 results in increased protection in infants

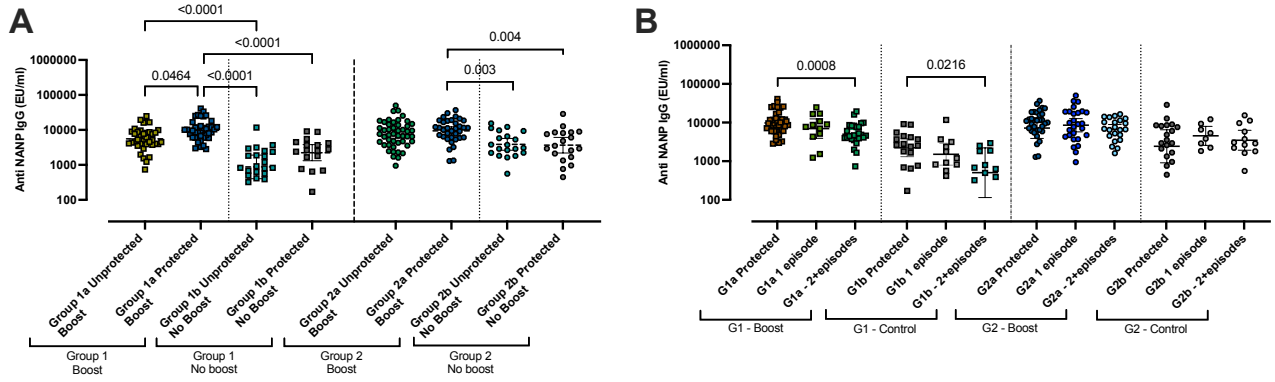


Figure 70 – NANP IgG in third year stratified by protection.

A) *Ant-NANP IgG measured at D84 stratified by efficacy at 36 months.* ***B)*** *Anti-IgG measured at D84 stratified by multiple episodes (protected, 1 episode and 2 or more episodes) at 36 months.*

GMTs with 95% CI. Kruskal-Wallis and corrected for multiple comparisons.

In the third year, participants in G1a who received a boost had significantly higher anti-NANP IgG titres (GMTs 9786EU [95% CI 7960 – 12029]) than unprotected who were boosted (GMTs 5554EU [95% CI 4314 – 7150]). Conversely, no significant difference in anti-NANP IgG titres was found between unprotected in G1a and protected in G1b (GMTs 2233EU [95% CI 1315 – 3790]) but were significantly higher than unprotected in G1b (GMTs 874EU [95% CI 402 – 1900]) – Figure 70A. When stratifying by

multiple episodes, protected participants in both boosted G1a and non-boosted G1b had significantly higher titres than those with multiple episodes - Figure 70B.

For participants in G2a who received a boost, no significant difference in anti-NANP IgG was observed between protected (GMTs 9433EU [95% CI 7209 – 12344]) and unprotected (GMTs 7509EU [95% CI 5815 – 9697]). Protected participants in G2a had significantly higher titres than protected and unprotected participants in G2b who were not boosted. Conversely, unprotected participants in G2a had comparable titres to those who were not boosted - Figure 70a. When assessing multiple episodes, there was a trend for both boosted G2a and non-boosted G2b, for protected to have higher titres than participants with one or two or more episodes, although no statistically significant difference was found – Figure 70B.

13.4.3.7 Decreasing anti-NANP IgG has little impact on more malaria episodes

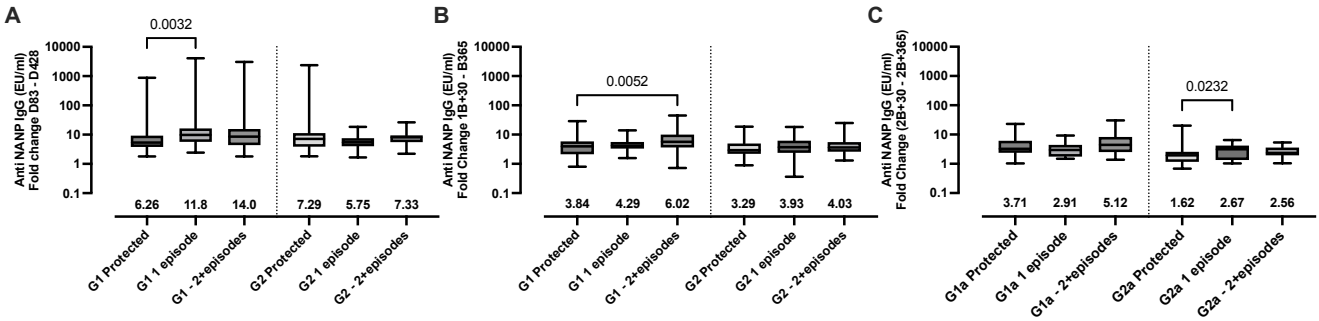


Figure 71 – Decreasing anti-NANP IgG effect on multiple episodes.

A) *Year 1 - FC of anti-NANP titre between 1-month post-primary series and 1-year post-primary series (D84 and D428).* ***B)*** *Year 2 – FC of anti-NANP IgG between 1-month post-first boost and 1 year post-first boost (1B+30 and 1B+365).* ***C)*** *Year 3 - FC of anti-NANP IgG between 1-month post-second boost and 1-year post-second boost (2B+30 and 2B+365). P- values Kruskal-Wallis test correct for multiple comparisons.*

Values presented above the x-axis are GMTs of FC calculations.

Figure 71 examines whether infants who have a larger decline in antibody titres result in more malaria episodes. Antibody FC was calculated between 1 month and 12 (D84/D421) months post the third dose of the primary series - Figure 71A. In addition, the same FC calculation was applied to the first

booster (1B+30/1B+365) - Figure 71B and second booster doses (2B+30/2B+365) - Figure 71C. Each FC was then stratified by the number of malaria episodes within a twelve-month segment between vaccinations. There was an overall trend across both groups across all three years to have a higher FC amongst participants with a higher number of episodes. Protected infants in G1 had a lower FC across all three years compared to G2 which is likely due to having lower peak titres after each vaccination. However, there are many confounding factors that are not adjusted for which can create 'noise' in the data such as infants location to a water source thereby increasing the concentration of mosquitos and therefor potential increased infection and vice versa.

13.4.4 Characterising total IgG responses to C term

13.4.4.1 R21/MM increases anti-C term IgG responses in Burkinabe infants

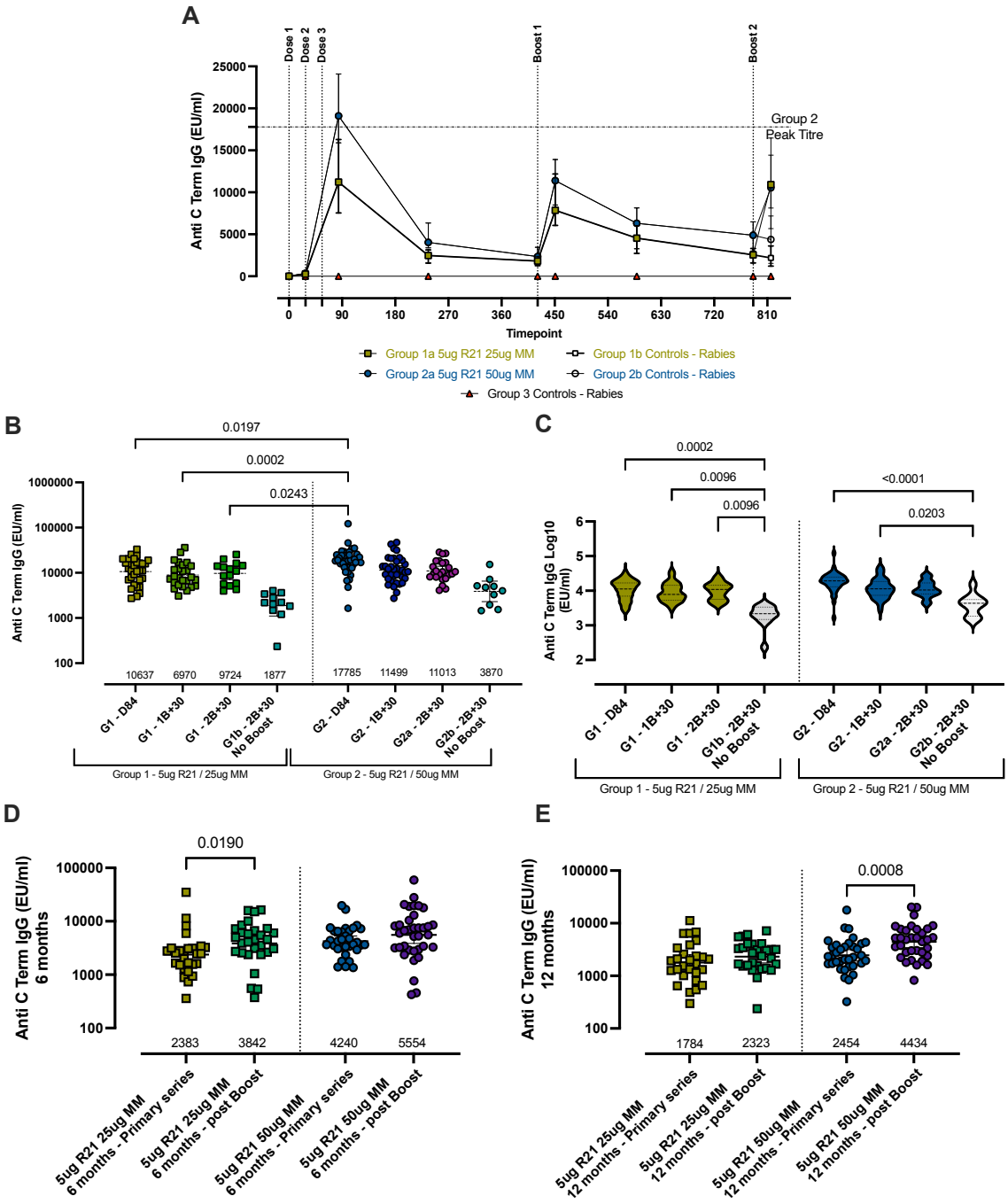


Figure 72 - Anti C term IgG after primary series and subsequent boosters.

A) *Median anti-C term IgG time course (error bars indicate IQR).* ***B)*** *Anti-C term IgG (GMTs with 95% CI) at peak responses after primary series and post boost time points. P-values comparing all time points - Kruskal-Wallis corrected with Dunn's multiple comparisons.* ***C)*** *Log10 violin plot anti-C term*

IgG at peak responses after primary series and post boost time points. P-values G1 and G2 matched paired analysis – Friedman test corrected for multiple comparisons. ***D)*** *Anti-C term IgG at 6 months post PS and PB (GMT 95% CI). Wilcoxon matched-pairs signed rank test.* ***E)*** *Anti-C term IgG at 12 months post-PS and PB (GMT 95% CI). Wilcoxon matched-pairs signed rank test.*

Values presented above the x-axis indicate GMT values.

Total IgG responses to the C term peptide were measured on the VAC076 immunology cohort (13.2.3). Due to time limitations, samples six months and twelve months post-second boost as well as one month post-third boost have not yet been assessed.

All infants were negative for anti-C term IgG at baseline. The time course - Figure 72A - follows similar antibody kinetics observed in the anti-NANP IgG time course - Figure 66A whereby anti-C term IgG increased after the primary series and peaked one-month post-third dose – D84. After D84, anti-C term IgG declined in the year after the third dose until a booster dose was given and anti-C term IgG increased, whereupon the trend is repeated.

At D84 - Figure 72B, G2 had significantly higher antibody titres (GMT 17,786EU [95% CI 14317 – 22091]) when compared to G1 – (GMT 10,637EU [95% CI 8332 – 13579]). When comparing the primary series with the peak antibody titres post first and second boost, antibody titres declined, however, no significant difference was found for either G1 or G2 - Figure 72B. Infants in G1b and G2b who did not receive a second boost had a significant decrease in anti-C term IgG compared to the primary series and first boost time points - Figure 72C.

As with peak responses, G2 had significantly higher responses at six months and twelve months compared with G1. Durability was directly measured to assess six and twelve months post primary series with six- and twelve months post-first boost. When comparing durability between doses, at six months both G1 and G2 had higher responses post boost however G1 had significantly higher anti-C term IgG post-boost (GMT 3842EU [95% CI 2681 – 5519]) than the primary series (GMT 2383EU [95%

CI 1669 – 3403]) - Figure 72D. At twelve months, both G1 and G2 had higher anti-C term IgG post boost than the primary series, however G2 had significantly higher IgG post boost (GMT 4434EU [95% CI 3434 -5725]) than the primary series (GMT 2454EU [95% CI 1909 – 3156]) - Figure 72E.

13.4.4.2 Anti-C term IgG is associated with protection in G2 only

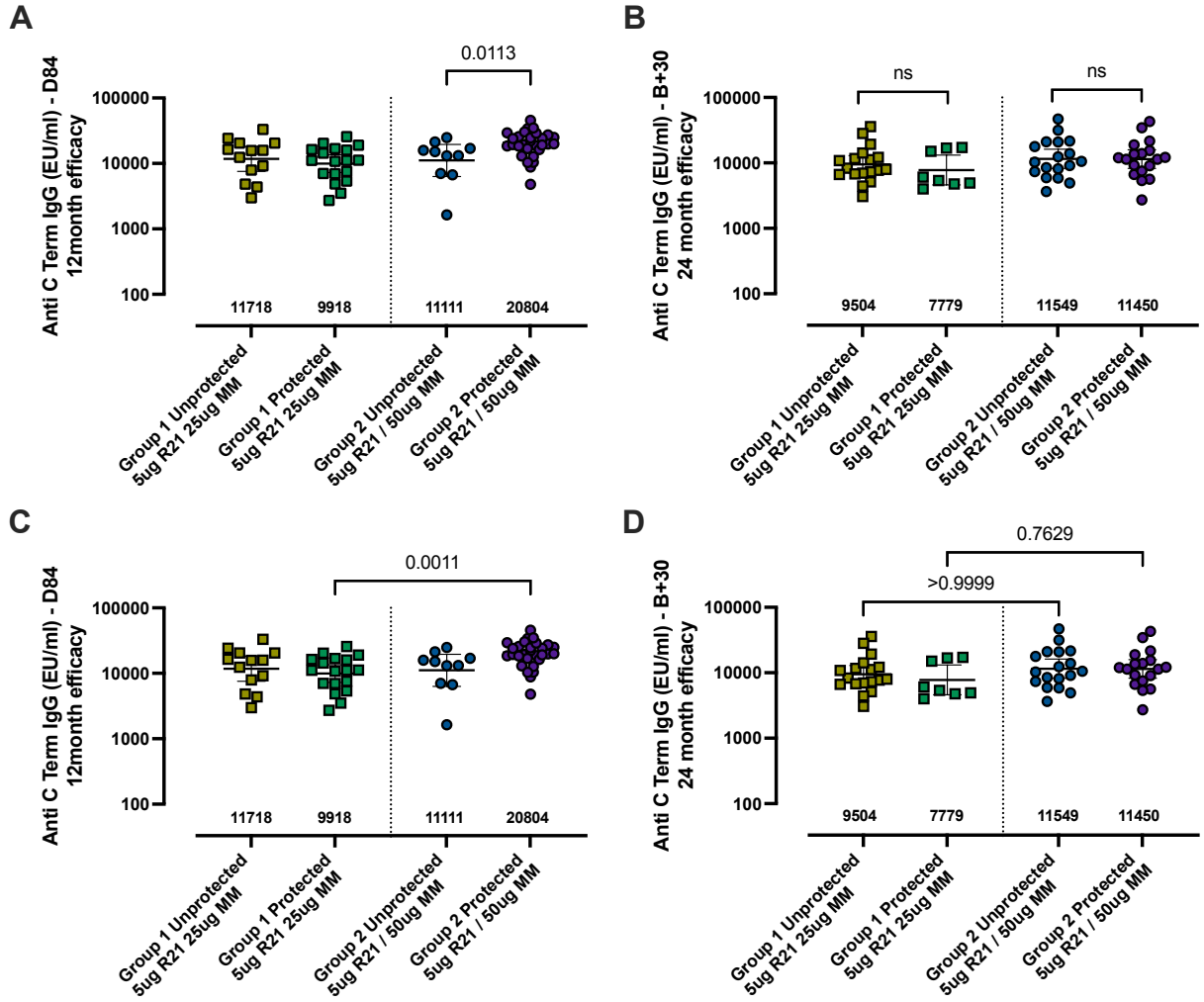


Figure 73 - Anti-C term IgG CoP at 12 and 24 months.

A) *Anti-C term IgG measured at D84 stratified by efficacy at 12 months.* ***B)*** *Anti-C term IgG measured at 1B+30 stratified by efficacy at 24 months.* ***C)*** *Anti-C term IgG measured at D84 stratified by multiple episodes (protected, 1 episode and 2 or more episodes) at 12 months.* ***D)*** *Anti-C term IgG measured at 1B+30 stratified by multiple episodes (protected, 1 episode and 2 or more episodes) at 24 months.*

GMTs with 95% CI. Values presented above the x-axis indicate GMT values.

When assessed for associations with protection anti-C term IgG, protected participants in G2 at twelve months had significantly higher anti-C term IgG – (GMT 20,804EU [95% CI 16833 – 25712]) at D84 than unprotected – (GMT 11,111EU [95% CI 6304 – 19582]) - Figure 73A. In addition, protected participants in G2 had significantly higher anti-C term IgG than protected participants in G1 (GMT 9918EU [95% CI 7254 – 13561]) - Figure 73C.

At twenty-four months, there was no significant difference between protected and unprotected participants for G1 and G2 when looking at antibodies measured one-month post-first boost. Secondly, no difference was found when comparing protected participants in G2 with protected or unprotected participants in G1 - Figure 73D.

13.4.5 IgG responses measured by new multiplex assay using MSD

MSD, a multiplex assay (see Appendix – 17.5.13) was implemented to be a higher throughput assay for future R21 trials. MSD produces results for all components of the R21 vaccine, NANP, C term and HBsAg, as well as responses to a full length of the R21 protein.

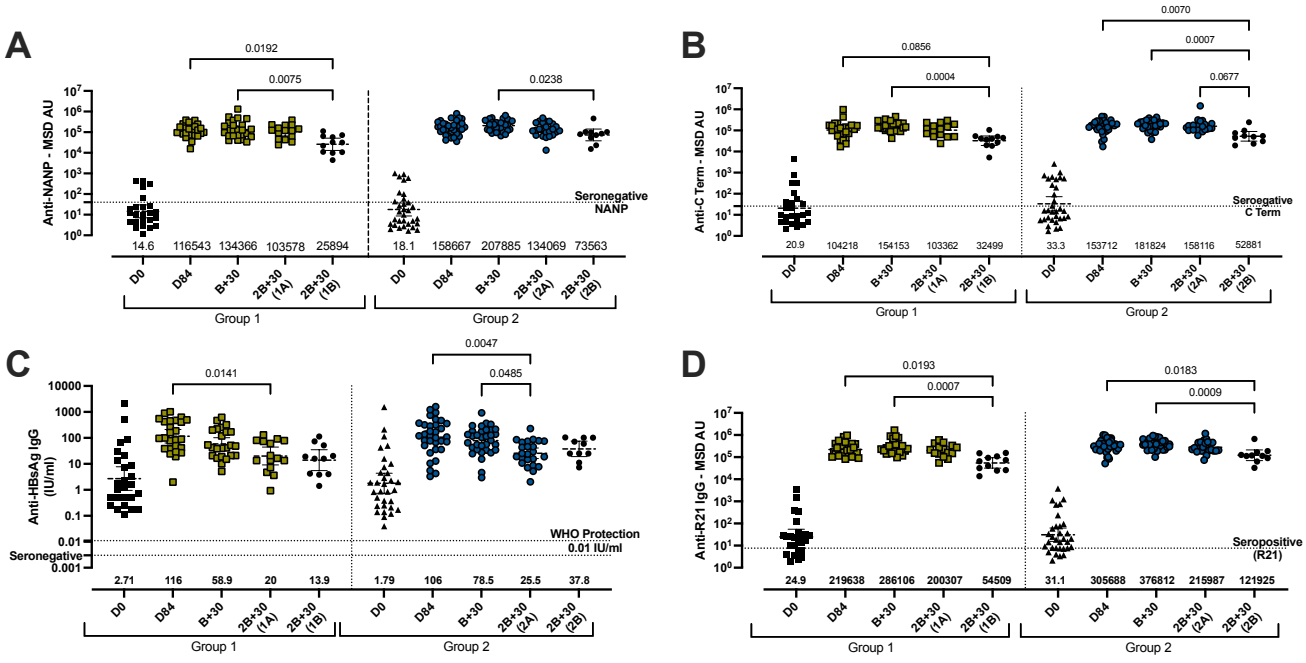


Figure 74 - MSD total IgG - NANP, C term, HBsAg and Full Length R21.

A) *Anti – NANP MSD IgG.* ***B)*** *Anti-C term MSD IgG.* ***C)*** *Anti-HBsAg MSD IgG (IU/ml). The horizontal dotted line is the WHO threshold of protection 0.01 IU/ml.* ***D)*** *Anti-R21 MSD IgG.*

Figures show GMTs with 95% CI for G1 and G2 at specific time points. Values above the x-axis indicate GMT values. P-values compare time points within each group – Kruskal-Wallis corrected with Dunn's multiple comparisons. Horizontal dashed lines represent seropositive cut-offs calculated in Appendix - 17.5.13.1.

MSD is a recent collaboration for the R21 programme and therefore only selected time points were selected for this thesis and was one of the final assays completed. MSD assays were run on the VAC076 cohort (13.2.3) and samples at baseline (D0), one-month post-primary series (D84), one-month post-first boost (1B+30), and one-month post second boost were assessed (2B+30).

As samples had detectable limit and range to calculate IgG values for all antigens - lower limits of detection for MSD can be viewed in Table 33. Samples were classified as seronegative for antigen-specific IgG from malaria naïve baseline samples from VAC065 and VAC072, averaged and seronegative values calculated as two standard deviations - Table 33.

At baseline, 19/72 infants were seronegative for anti-NANP MSD IgG (<40.29AU) - Figure 74A with a geometric mean titre of 16.4AU [95% CI 10.6 – 25.3]. 26/72 infants were seronegative for anti-C term MSD IgG (<25.58AU) - Figure 74B with GMT titres of 24.4AU [95% CI 15.2 – 31.9]. All infants were seropositive anti-HBsAg MSD IgG (<0.003 IU/ml) at baseline - Figure 74C with a geometric mean titre of 1.56 IU/ml [95% CI 0.884 – 2.74]. 17/72 infants were seronegative anti-R21 MSD IgG (<7.638) at baseline Figure 74D with a GMT titre of 31.9AU [95% CI 19.2 – 53.0].

For anti-NANP IgG (MSD), G2 had significantly higher antibody titres at all time points assessed than G1 (data not shown). At one-month post first boost (1B+30), both G1 (GMT 134,366AU [95% CI 90808 – 198600]) and G2 (GMT 207,885AU [95% CI 166649 – 259326]) presented with higher titres compared to the primary series - Figure 74A. While not significantly different, it contrasts with what was measured on anti-NANP IgG measured by ELISA - Figure 66A. Infants in G1b who did not receive the

second boost had significantly lower anti-NANP IgG (MSD) than D84 and 1B+30 but not 2B+30. Infants in G2b, only had significantly lower antibody titres than 1B+30.

Anti-C term IgG (MSD), followed similar trends to anti-NANP IgG (MSD), whereby the highest antibody levels were recorded in the one-month post-first boost for both G1 and G2 - Figure 74B. Infants in G2b who did not receive the second boost had significantly lower antibody levels compared to all other time points assayed. Similar observations were found for anti-R21 MSD IgG - Figure 74D.

13.4.5.1 Correlation with antibody titres to R21

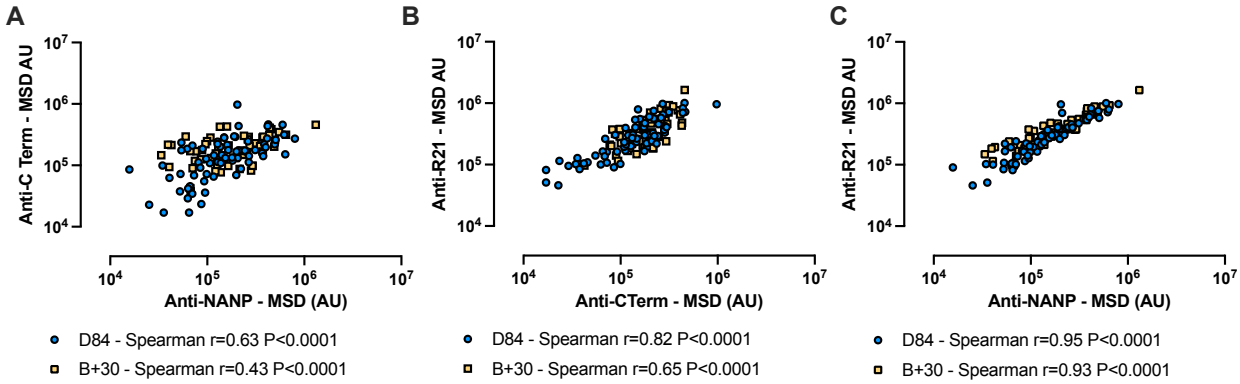


Figure 75 - MSD Antigen IgG Correlations. NANP vs C term vs R21.

A) *Correlating anti-NANP MSD IgG with anti-C term MSD IgG.* ***B)*** *Correlating anti-C term MSD IgG with anti-R21 MSD IgG.* ***C)*** *Correlating anti-NANP MSD IgG with anti-R21 MSD IgG.*

Correlations comparing samples measured at D84 and B+30 with Spearman rank with significant P-values.

Anti-NANP, anti-C term and anti-R21 MSD IgG positively correlate with each other with significantly positive correlation co-efficient - Figure 75A/B/C. Data indicates NANP and C Term do not interfere with antibody responses nor to the full length R21 protein. MSD and single antigen ELISA assay responses are corelated below. And anti-HBsAg IgG was correlated with NANP, C Term and full length R21 protein below.

13.4.5.2 R21/MM significantly increases anti-HBsAg antibody responses in Burkinabe infants

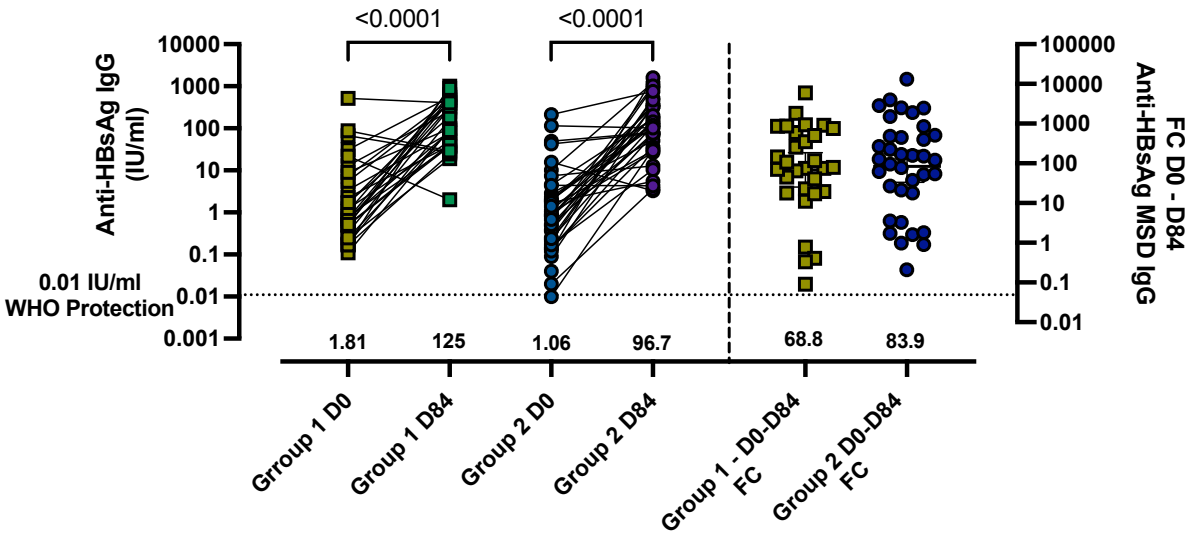


Figure 76 - HBsAg IgG in Burkinabe infants measured by MSD.

Left of the Figure*, comparing baseline D0 with D84 anti-HBsAg MSD IgG (IU/ml) for G1 and G2. P<0.0001 Mann-Whitney. The horizontal dotted line at Y=0.001 represents the WHO threshold of protection.* ***Right of the Figure****, FC between anti-HBsAg IgG between D0 and D84 for G1 and G2.*

Values above the X-axis indicate geometric mean values.

All infants were seropositive at baseline for anti-HBsAg MSD IgG with G1 reporting a GMT titre of 1.81IU/ml [95% CI 0.823 – 3.99] - Figure 74C and Figure 76 and G2 reporting a GMT titre of 1.06 IU/ml [95% CI 0.499 – 2.24]. Seropositivity is described in Jan *et al.,* 2020 [440] as having an antibody level greater than 0.003 IU/ml. All infants at baseline had anti-HBsAg IgG above the WHO protection threshold of 0.01IU/ml [441].

After three doses of R21, anti-HBsAg MSD IgG measured in infants in G1 (GMT 125 IU/ml [95% CI 72.9 – 214]) was higher when compared with infants in G2 (GMT - 96.7 IU/ml [95% CI 55 – 171]) although not significant. G1 had a fold increase of 68.8 in HBsAg MSD IgG D84 and G2 (5µg R21 / 25µg MM) had an increase of 83.9 - Figure 76.

13.4.6 Anti-HBsAg correlate with other NANP, C term and R21 IgG as measured by MSD

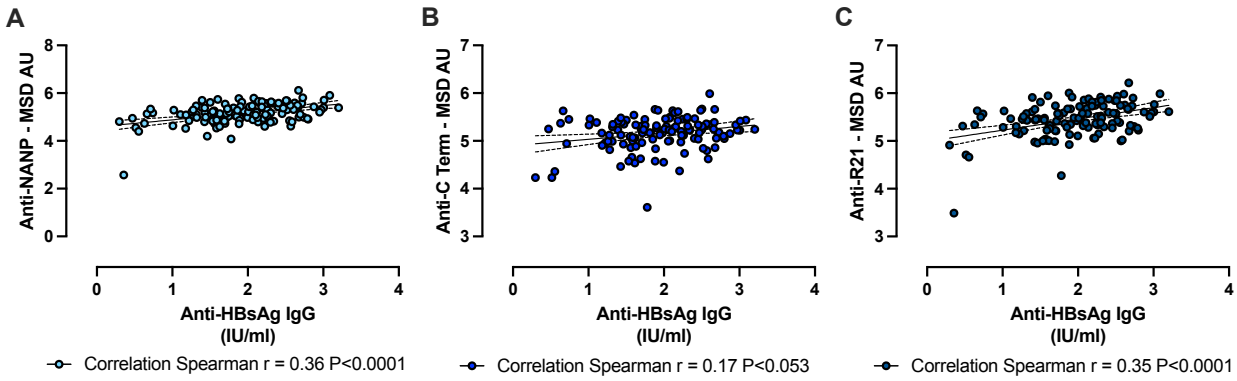


Figure 77 - HBsAg Correlations with NANP, C term and R21 IgG.

A) *Correlating anti-HBsAg MSD IgG with anti-NANP MSD IgG.* ***B)*** *Correlating anti-HBsAg MSD IgG with anti-C term MSD IgG.* ***C)*** *Correlating anti-HBsAg MSD IgG with anti-R21 MSD IgG.*

Correlations calculated by Spearman r with P-values. The line of best fit calculated by linear regression with 95% CI

Anti-HBsAg MSD IgG positively correlates with anti-NANP MSD IgG (Spearman r = 0.36, P<0.0001) - Figure 77A and with anti-R21 MSD IgG (Spearman r = 0.35, P<0.0001) Figure 77C. However, the anti-C term MSD IgG does not correlate with HBsAg IgG (Spearman r = 0.17, P=0.053) - Figure 77B. Data indicates HBsAg antibody titres has no impact or interreference on responses to other peptides from R21 vaccine.

13.4.7 Protection associated with anti-NANP and anti-R21 IgG levels as measured by MSD

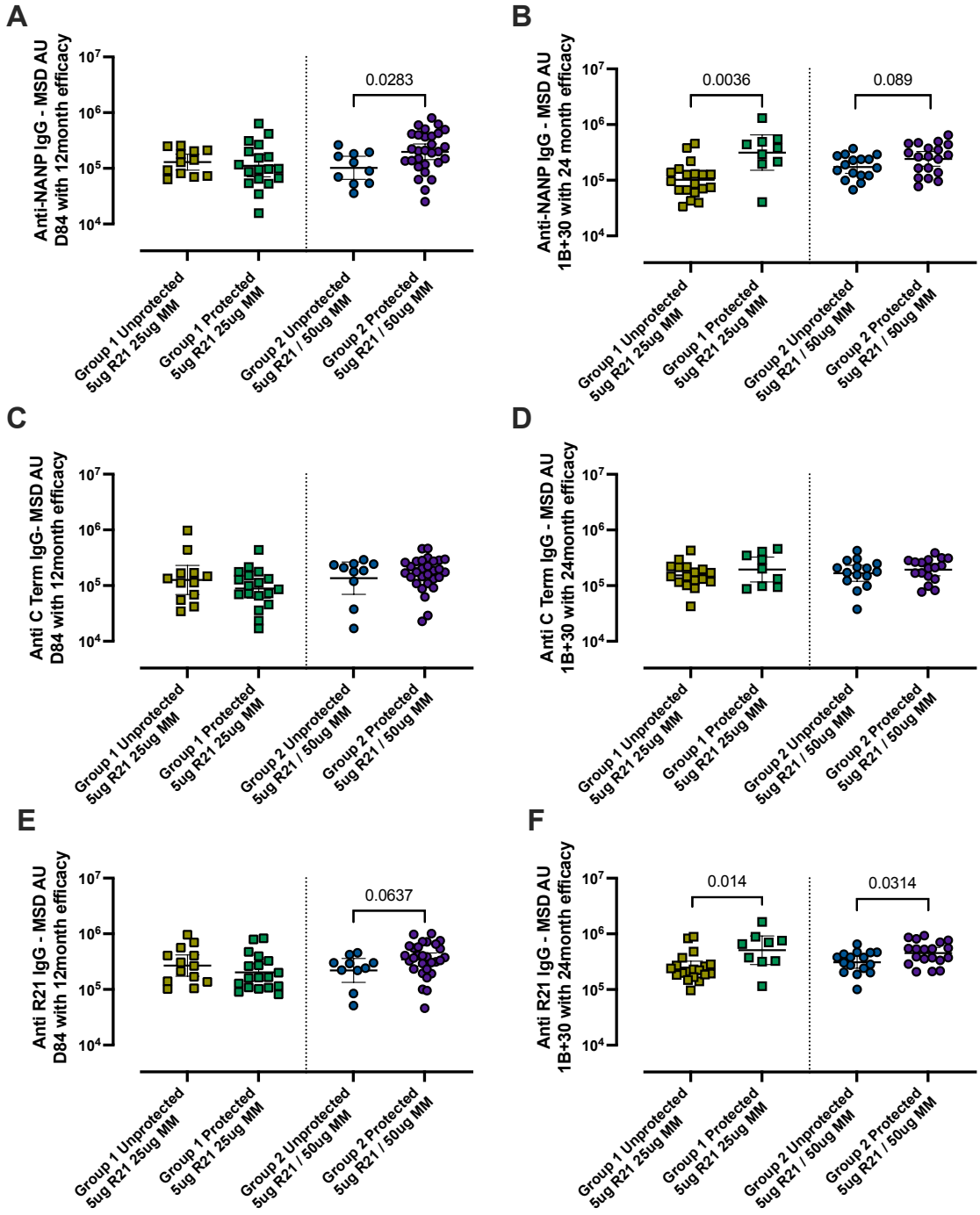


Figure 78 - MSD IgG stratified by protection 12 and 24 months.

A) *Anti-NANP MSD IgG measured at D84 stratified by efficacy at 12 months.* ***B)*** *Anti-NANP MSD IgG measured at 1B+30 stratified by efficacy at 24 months.* ***C)*** *Anti-C term MSD IgG measured at D84 stratified by efficacy at 12 months.* ***D)*** *Anti-C term MSD IgG measured at 1B+30 stratified by efficacy*

at 24 months. ***E)*** *Anti-R21 MSD IgG measured at D84 stratified by efficacy at 12 months.* ***F)*** *Anti-R21 MSD IgG measured at 1B+30 stratified by efficacy at 24 months.*

All figures present GMT with 95% CI antibody titres as measured by MSD. P-values calculated by Mann-Whitney between protected and unprotected for each group.

Anti-NANP IgG (MSD), protected infants in G2 at D84 had significantly higher antibody titres (197889AU [95% CI 143300 – 273274]) compared to unprotected (102247AU [95% CI 63328 – 165084]) but not significant when compared with G1 - Figure 78A. At one-month post-first boost 1B+30, both protected infants in G2 and G1 had significantly higher titres than unprotected infants – Figure 78B.

No association with protection was observed for anti-C term IgG (MSD) in either G1 or G2 for both D84 and 1B+30 time points - Figure 78C/D. With anti-R21 IgG (MSD), protected infants at G2 had significantly higher antibody titres (345272AU [95% CI 263172 – 452983]) compared with unprotected (219570AU [95CI% 133915 – 360013]). There was no difference between protected in G2 with infants in G1 - Figure 78E. As with NANP IgG (MSD), both protected and unprotected in G1 and G2 at 1B+30 had significantly higher titres than unprotected - Figure 78F. Lastly, no association with protection was found with anti-HBsAg IgG titres – data not shown.

13.4.7.1 Correlation of MSD with single antigen ELISA

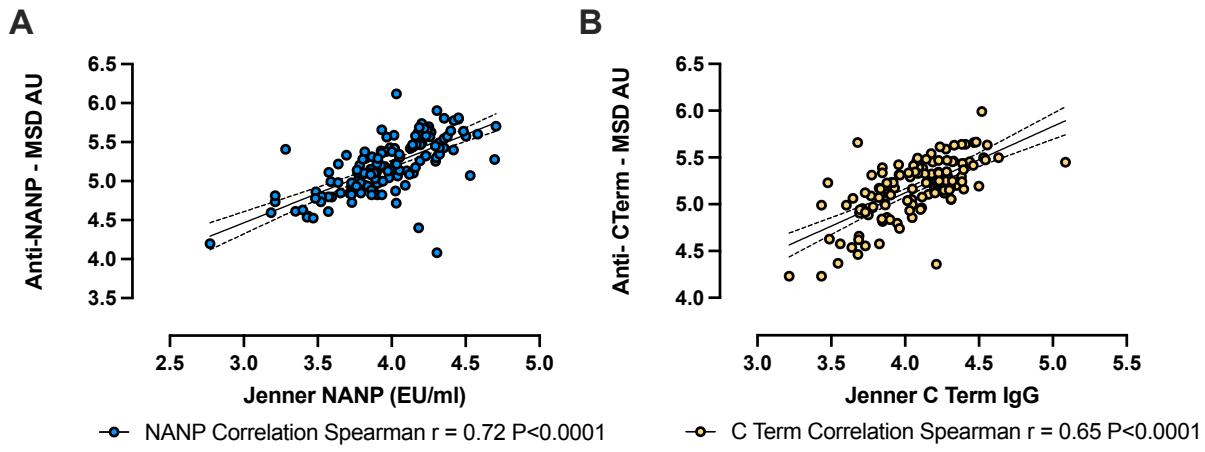


Figure 79 - Correlating IgG from ELISA with MSD - NANP vs C term.

A) *Correlating anti-NANP IgG with anti-NANP MSD IgG at D84 and 1B+30.* ***B)*** *Correlating anti-C term IgG with anti-C term MSD IgG at D84 and 1B+30.*

Correlations calculated with Spearman rank and linear regression with 95% CI represent the line of best fit.

Both NANP and C term-specific IgG measured by ELISA and MSD positively correlate. Figure 79A/B indicates that both assays produce reproducible data to measure IgG induced in infants immunised with R21.

13.4.8 Anti-IgG avidity

Avidity at baseline was not assessed as all infants were seronegative at baseline for anti-NANP IgG and for anti-C term IgG. Using the VAC076 immunology cohort (13.2.3), avidity against NANP and C term was assessed at key time points D84, 1B+30 and 2B+30. In addition, avidity against NANP was assessed at 1-month post first dose (D28) of the primary series but not for C term due to time limitations. Assays are not directly comparable due to different coating concentrations. However, antibodies are normalised to have an OD of one, permitting reasonable observation of comparability between assays.

13.4.8.1 Avidity to NANP increases after vaccination

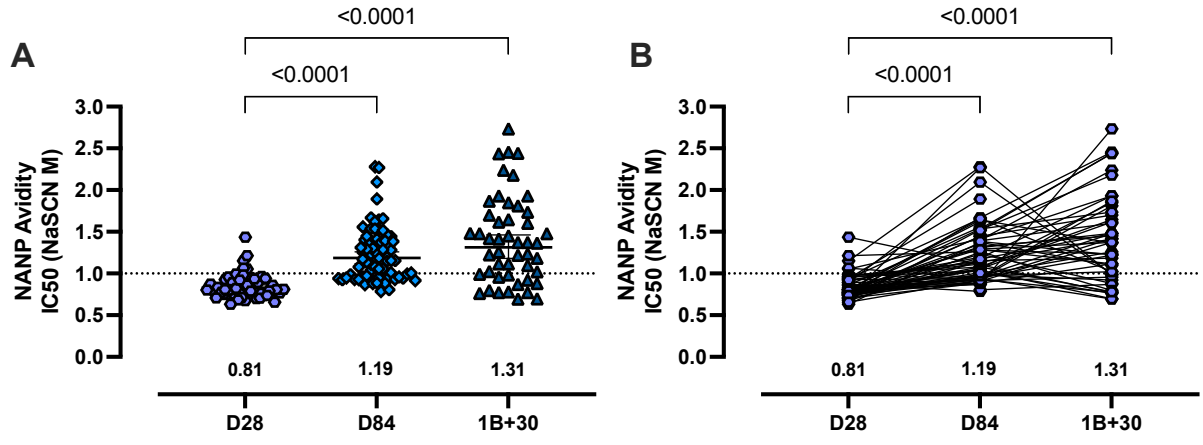


Figure 80 - NANP avidity with Groups 1 and 2 merged.

A) *Scatterplot representing GMY with 95% CI of IC50 of anti-NANP IgG P-values Kruskal-Wallis corrected with Dunn's multiple comparisons.* ***B)*** *Scatter plot with connecting participant IDs.*

Time points presented – 1 month post first dose (D28), 1 month post third dose (D84) and 1-month post first boost (1B+30). The horizontal dotted line Y=1 is a point of reference as a visual aid. Numbers above the x-axis are GMTs.

Avidity measured by sodium thiocyanate displacement of IgG to NANP peptides increased significantly between D28 (IC50 0.81 [95% CI 0.794 – 0.863]) and D84 (IC50 1.19 [95% CI 1.12 – 1.26]) - Figure 80A. When pairing time points together, most participants increased in avidity from D28 to D84 to one month post-boost – Figure 80B.

13.4.8.2 Avidity to NANP and C term increases significantly after first and second boost

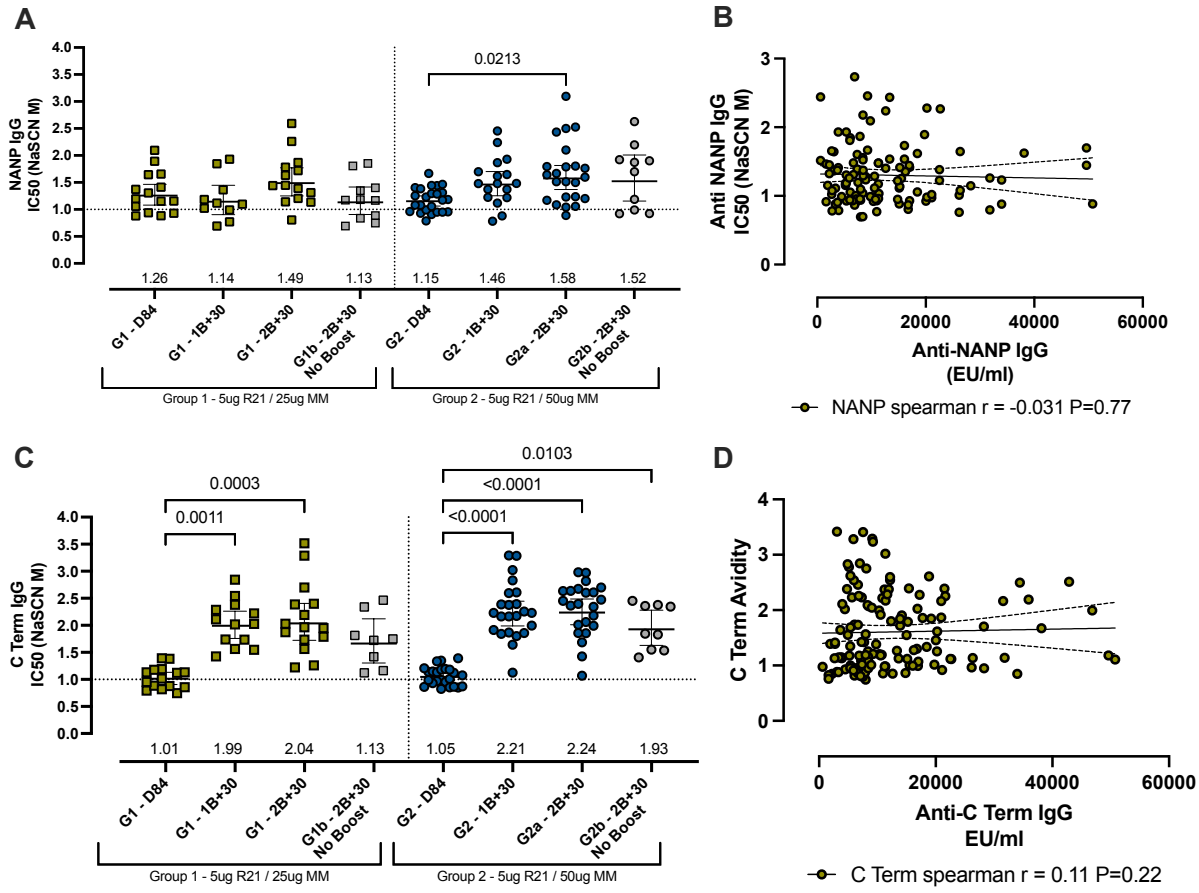


Figure 81 – VAC076 Avidity (IC50) to NANP and C term IgG.

A) *Avidity to anti-NANP IgG with D84, 1B+30 and 2B+30 time points. GMTs with 95% CI. The P-values are from a Kruskal-Wallis corrected with Dunn's multiple comparisons.* ***B)*** *Correlating (Spearman r correlation) anti-NANP IgG with NANP avidity. The line of best fit was calculated with linear regression with 95% CI.* ***C)*** *Avidity to anti-C term IgG with D84, 1B+30 and 2B+30 time points. GMTs with 95% CI. P-values are a Kruskal-Wallis corrected with Dunn's multiple comparisons.* ***D)*** *Correlating (Spearman r*

correlating anti-C term IgG with C term avidity. The line of best fit was calculated with linear regression with 95% CI.

Figures present GMTs with 95% CI. Values above the x-axis indicate GMTs.

Avidity to both NANP and C term were assayed using the VAC076 cohort (13.2.3) at time points D84, 1B+30 and 2B+30. At D84, no significant difference was observed between G1 and G2 for both NANP and C terms - Figure 81A/C. Avidity to NANP was shown to increase one-month post boost and significantly (P=0.02) one-month post-second boost with the larger increase observed in G2 - Figure 81A. Infants in G2b who did not receive the second boost maintained antibody avidity comparable to those who received the first and second boosts. Infants in G2b had a numerically higher GMT avidity of IC50 1.52 [95% CI 1.16 – 2.01] at 2B+30 compared with an IC50 1.15 [95% CI 1.06 – 1.25] at D84, although not significant - Figure 81A.

IgG avidity to C Term appeared to show larger increases post-first boost (1B+30) and second boost (2B+30) in comparison with IgG avidity to NANP, with significant differences observed for both G1 and G2 when compared to D84. The highest avidity was measured in G2 with an IC50 2.21 [95% CI 1.99 – 2.44] at 1B+30 and an IC50 2.24 [95% CI 2.01 – 2.49] and 2B+30 in comparison with D84 - Figure 81C. Infants in G2b who did not receive a second boost had significantly higher avidity at 2B+30 IC50 1.93 [95% CI 1.63 – 2.28] compared to D84 with no overlapping 95% confidence intervals - Figure 81C.

When comparing whether avidity correlates with anti-NANP and anti-C term IgG, no positive or negative correlation was observed with antibodies measured at D84 and B+30 - Figure 81B/D. 2B+30 was not added to the correlation as a third of participants at this timepoint did not receive a boost.

13.4.8.3 Avidity to NANP does not impact protection in Burkinabe infants

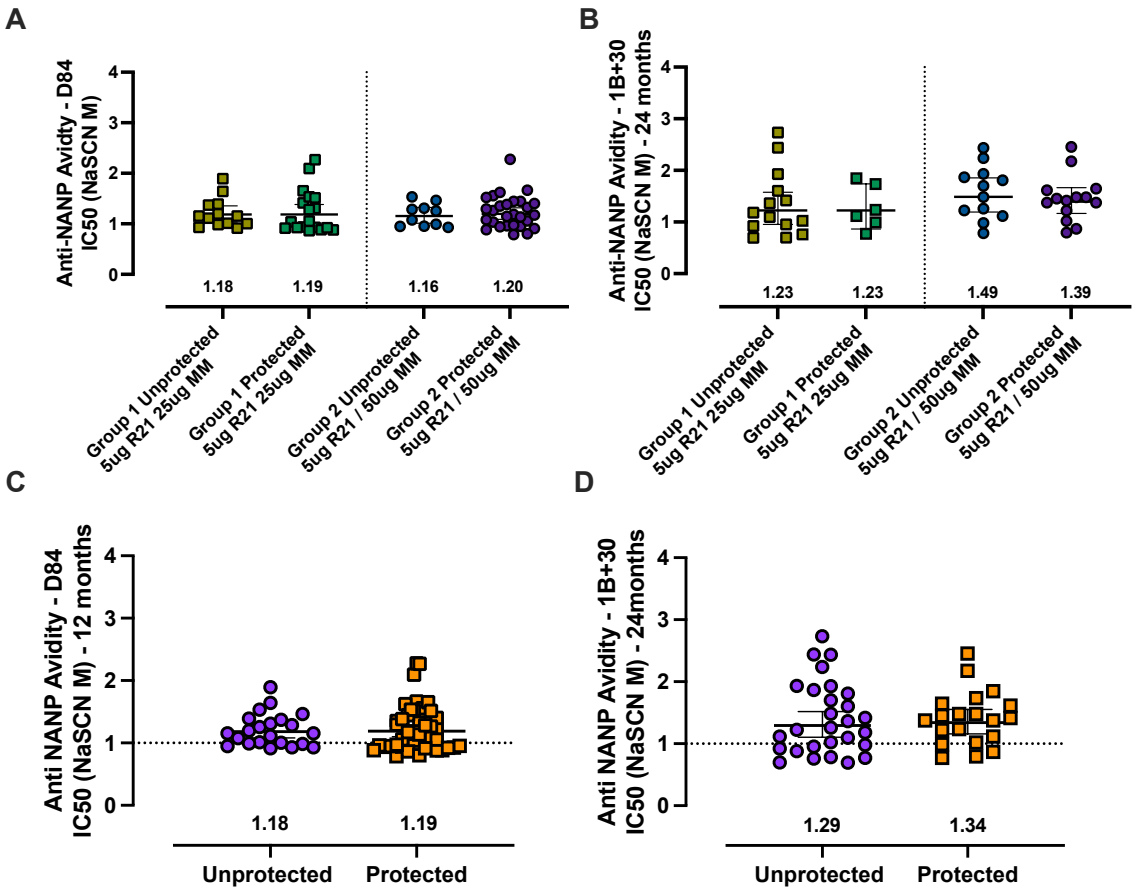


Figure 82 - NANP avidity stratified by protection.

A) *Anti-NANP avidity IgG measured at D84 stratified by efficacy at 12 months.* ***B)*** *Anti-NANP avidity IgG measured at 1B+30 stratified by efficacy at 24 months.* ***C)*** *Anti-NANP avidity IgG measured at D84, protected participants and unprotected infants in G1 & G2 merged at 12-month efficacy.* ***D)*** *Anti-NANP avidity IgG measured at 1B+30, protected participants, and unprotected infants in G1 & G2 merged at 24-month efficacy.*

Figures present GMTs with 95% CI. Values above the x-axis indicate GMTs.

The avidity of NANP-specific IgG was assessed for potential associations with protection. Avidity measured in infants at D84 and 1B+30 and stratified by protection at twelve and twenty-four months respectively months, saw no immunological associations - Figure 82.

13.4.8.4 Avidity to C term IgG does not impact protection

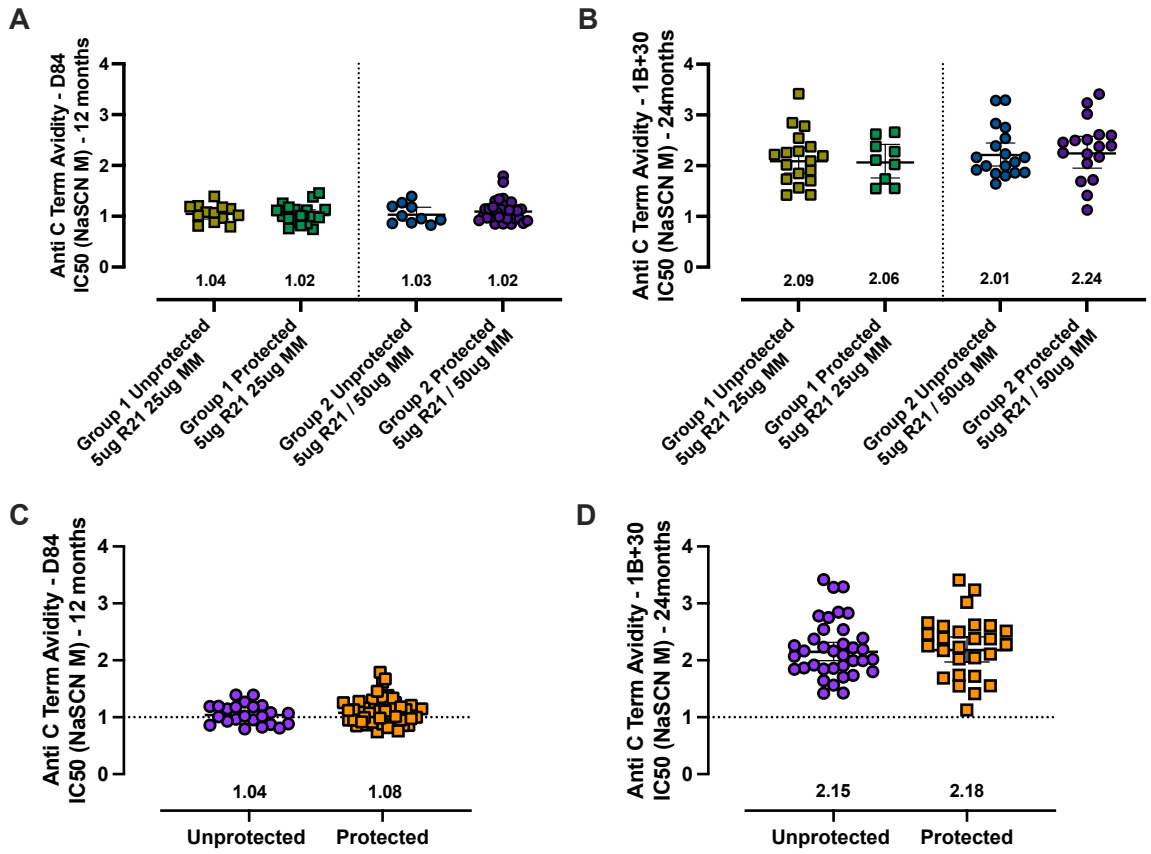


Figure 83 - C term avidity stratified by protection.

A) *Anti-C term avidity IgG measured at D84 stratified by efficacy at 12 months.* ***B)*** *Anti-C term avidity IgG measured at 1B+30 stratified by efficacy at 24 months.* ***C)*** *Anti-C term avidity IgG measured at D84, protected participants and unprotected infants in G1 & G2 merged at 12-month efficacy.* ***D)*** *Anti-C term avidity IgG measured at 1B+30, protected participants, and unprotected infants in G1 & G2 merged at 24-month efficacy.*

Figures present GMTs with 95% CI. Values above the x-axis indicate GMTs.

In terms of avidity to anti-C term IgG, no differences could be found between protected and unprotected at both D84 and 1B+30 immunological time points - Figure 83A/B. Similar to NANP, avidity at key time points for both G1 and G2 were comparable permitting the data to be merged. With avidity merged at D84 and 1B+30 and stratified again by protection, no differences could be found - Figure 83C/D.

13.4.9 Subclass and isotype antibody responses in Burkinabe infants

Isotype antibodies IgA and IgM along with IgG subclass antibodies IgG1, IgG2, IgG3 and IgG4 were assayed at D84 using samples selected from the VAC076 cohort (13.2.3).

13.4.9.1 Subclass and Isotype responses in Burkinabe infants

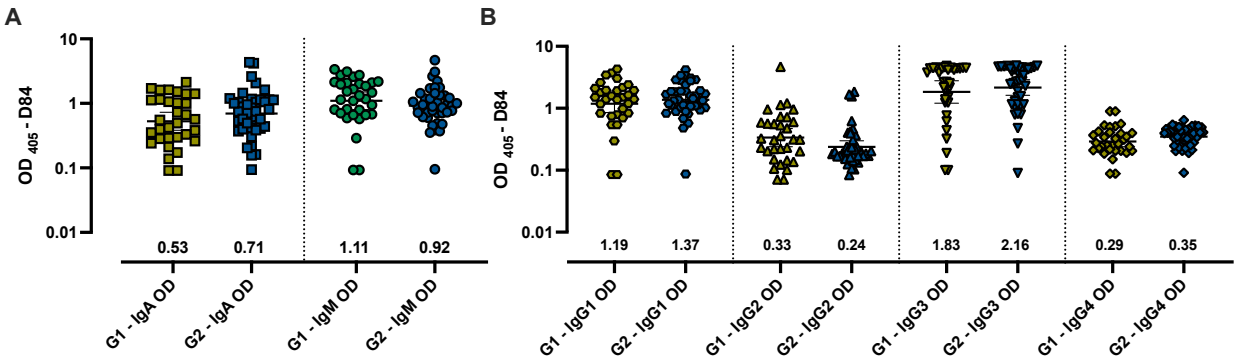


Figure 84 - Subclass and isotype antibody responses at D84.

A) *OD_{405} of anti-NANP IgA and anti-NANP IgG comparing G1 vs G2 at D84.* ***B)*** *OD_{405} of anti-NANP IgG1, anti-NANP IgG2, anti-NANP IgG3 and anti-NANP IgG4 comparing G1 vs G2 at D84.*

Geometric Means with Error bars indicate 95% CI. Values above the x-axis are GMT values. No statistically significant comparisons.

G2 had comparable isotype responses when compared with G1 with no significant differences between groups for both anti-NANP IgA and anti-NANP IgM – Figure 84A. Subclass antibodies anti-NANP IgG1 and IgG3 reported higher optical density readings compared to anti-NANP IgG2 and IgG4 - Figure 84B. No significant differences could be found between groups for subclass antibodies IgG1-4, unlike with total IgG against NANP which saw infants in G2 have significantly higher antibody titres at D84 compared with G1 - Figure 66D.

A portion of IgG3 readings resulted in an OD_{405} in the range of 4.1 – 4.4. This range is the upper limit of what can be read using an alkaline phosphatase-developing solution. A higher dilution was applied, however numerous samples had an OD_{405} less than 0.1nm and therefore were not able to be

accurately recorded. However, due to results being reported in OD_{405}, the same dilution was used across samples for comparability.

13.4.9.2 No isotype or subclass was associated with protection in Burkinabe infants

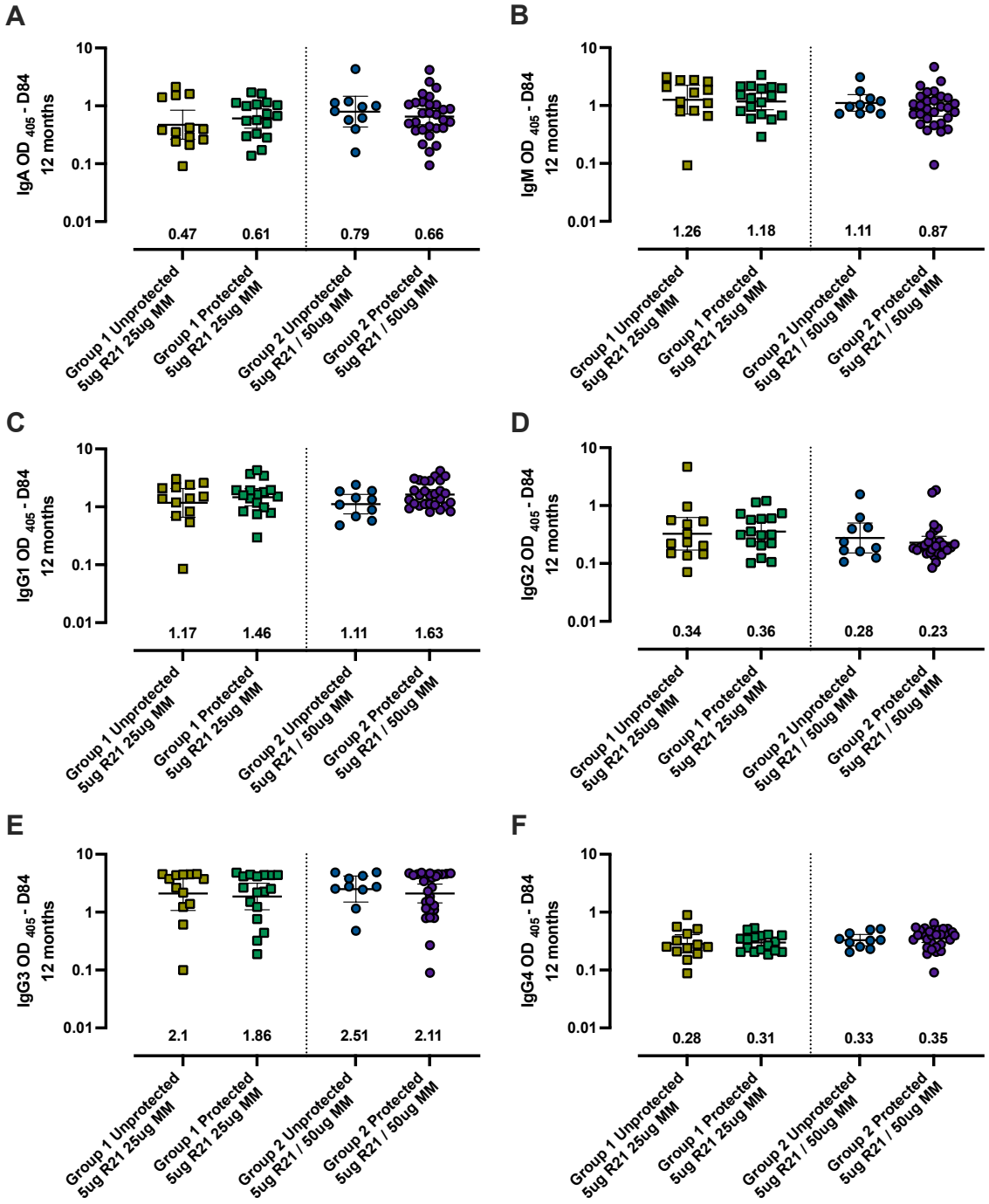


Figure 85 - Subclass and isotype antibodies stratified by protection.

A) *Anti-NANP IgA measured at D84 stratified by efficacy at 12 months separated by G1 & G2.* ***B)*** *Anti-NANP IgM measured at D84 stratified by efficacy at 12 months separated by G1 & G2.* ***C)*** *Anti-NANP*

IgG1 measured at D84 stratified by efficacy at 12 months separated by G1 & G2. ***D)*** *Anti-NANP IgG2 measured at D84 stratified by efficacy at 12 months separated by G1 & G2.* ***E)*** *Anti-NANP IgG3 measured at D84 stratified by efficacy at 12 months separated by G1 & G2.* ***F)*** *Anti-NANP IgG4 measured at D84 stratified by efficacy at 12 months separated by G1 & G2.*

Error bars indicate Geometric Means with 95% CI. Values above the x-axis are GMTs. No statistically significant comparisons were found.

In stratifying by protection against subclass and isotype antibodies, no significant differences could be found between protected and unprotected participants from samples taken at D84 and stratified by efficacy at twelve months - Figure 85A-F. With no significant differences observed between groups, subclass, and isotype ODs G1 and G2 were merged and stratified by efficacy at twelve months to identify a threshold of protection. Merged groups showed no difference in subclass anti-NANP titre between protected and unprotected at D84 - Figure 86 A-F.

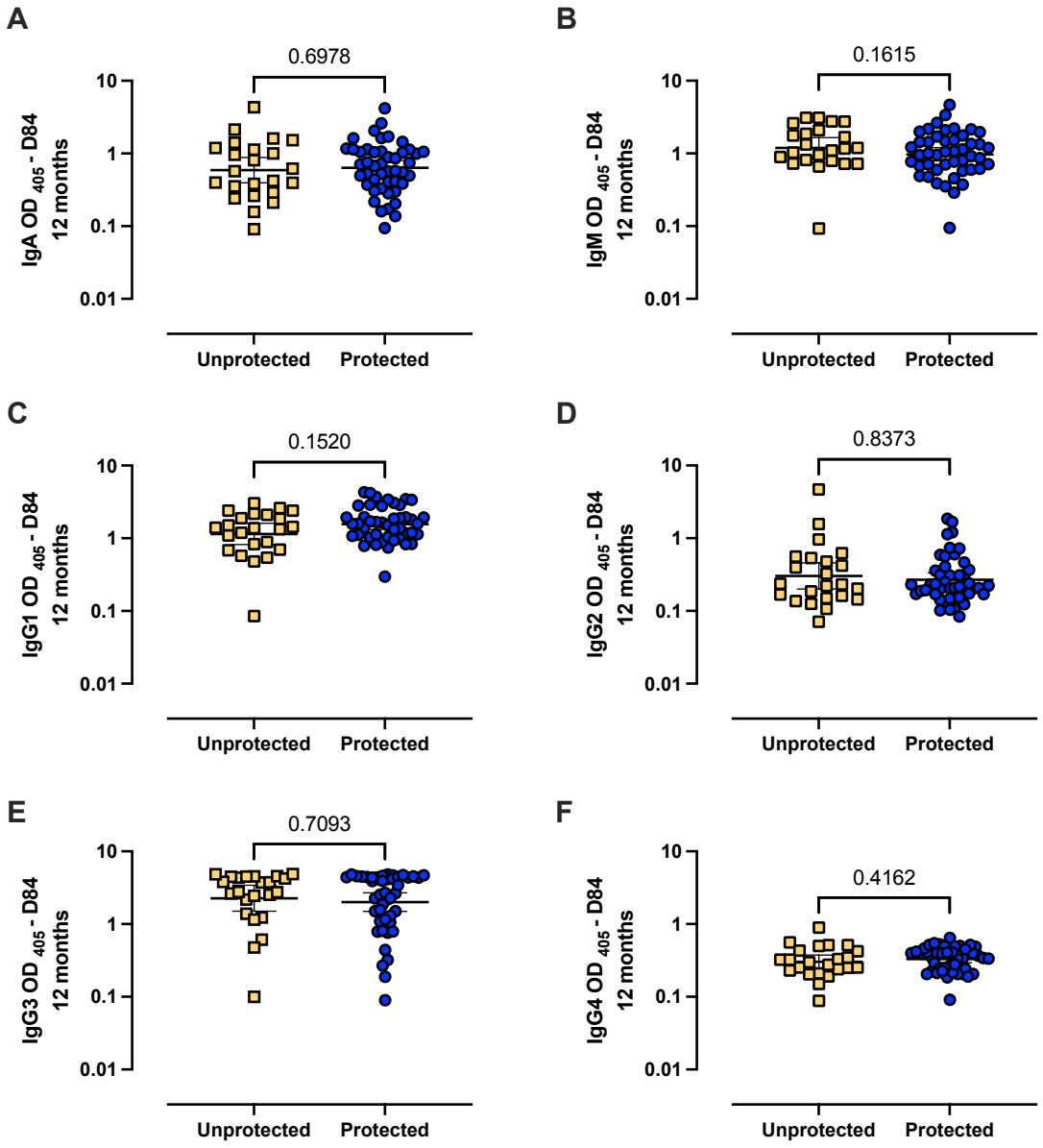


Figure 86 - Isotype and Subclass antibodies with merged groups stratified by protection.

A) *Anti-NANP IgA measured at D84 stratified by efficacy at 12 months.* ***B)*** *Anti-NANP IgM measured at D84 stratified by efficacy at 12 months.* ***C)*** *Anti-NANP IgG1 measured at D84 stratified by efficacy at 12 months.* ***D)*** *Anti-NANP IgG2 measured at D84 stratified by efficacy at 12 months.* ***E)*** *Anti-NANP IgG3 measured at D84 stratified by efficacy at 12 months.* ***F)*** *Anti-NANP IgG4 measured at D84 stratified by efficacy at 12 months.*

Error bars indicate Geometric Means with 95% CI. Values above the x-axis are GMTs. No statistically significant comparisons were found.

13.4.10 Functional antibodies unable to differentiate between protected and non-protected infants

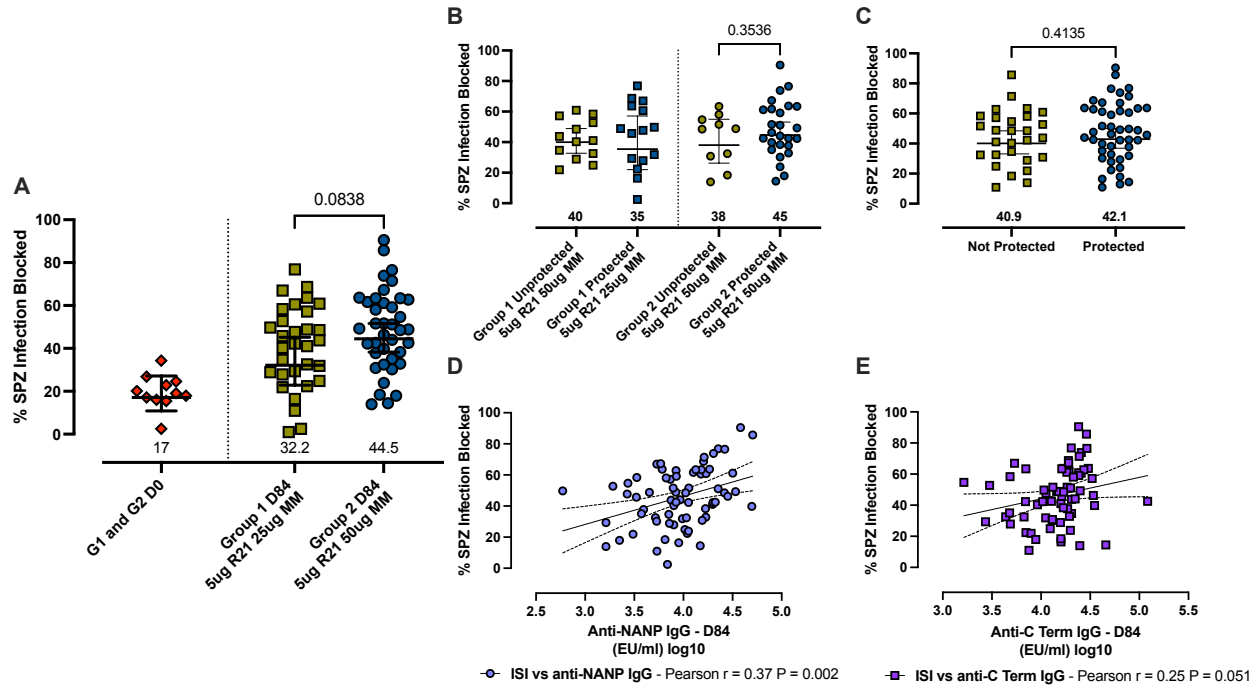


Figure 87 – Inhibition of sporozoite invasion and stratified by protection

A) *Percent increase of ISI among Burkinabe infants pre and post-vaccination from both vaccinated groups in Burkinabe infants. P=0.0025, Mann-Whitney.* ***B)*** *Percentage ISI measured at D84 stratified by efficacy at 12 months separated by G1 & G2.* ***C)*** *Percent ISI measured at D84, protected participants and unprotected infants in G1 & G2 merged at 12-month efficacy.* ***D)*** *Correlation between percent ISI and anti-NANP IgG at D84.* ***E)*** *Correlation between percent ISI and anti-NANP IgG at 1B+30.*

Values above the x-axis indicate GMT percentages and 95% CI. Correlation calculated with Pearson r. line of best of fit calculated by linear regression with 95% CI.

VAC076 ISI data is presented in Chapter 1 compared similar dose regimens from a Phase I immunogenicity study in Kenya - Figure 45. In this chapter, ISI data presented in Chapter 1 will be assessed to determine if the assay can be associated with protection in infants. As with other exploratory immunology assays presented in this chapter, the VAC076 cohort (13.2.3) was assessed for ISI. A subset of baseline samples was run as negative controls and D84 was used to test for ISI - Figure 87A.

No difference in % ISI was apparent when comparing protected and unprotected individuals in either G1 or G2 at 12 months. Protected participants in G2 appeared to have a higher percentage of sporozoite invasion blocked – 45% compared with 38% in unprotected participants, however not statistically significant (P=0.35) – Figure 87B. Protected infants in G1 had a large spread in terms of the percentage of Spz invasion blocked with a mean percentage that was lower than unprotected infants, however not statistically significant - Figure 87B.

With no significant differences observed between groups - Figure 87A/B, the percentage of sporozoite invasion from both groups was merged and stratified by efficacy. Merged groups indicated no significant difference between protected and unprotected - Figure 87C.

Anti-NANP IgG antibodies positively correlate with ISI (Pearson r = 0.37 P = 0.002) - Figure 87D. Anti-C term IgG antibodies also positively correlate with ISI (Pearson r = 0.25 P = 0.051), however not as positive as anti-NANP IgG and not significant - Figure 87E.

13.4.11 Multivariate analysis

Eighteen immunology variables were measured at D84 and assessed for significant association with efficacy at 12 months, no variables had a B-H adjusted P-value of ≤0.2 and no variable had an AUROC ≥0.8 – Table 4. Of the 9 immunology variables measured at 1B+30 and assessed at year 2 efficacy - Table 22 – six variables highlighted in orange have an adjusted (B-H) P-value of ≤0.2. These variables are IgG C Term, IgG NANP, MSD NANP, MSD C term, MSD HBsAg and MSD R21, however, none of these variables has an AUROC≥0.8. Of the nine immunology variables measured at 2B+30 and assessed at year 3 efficacy - Table 22, five variables highlighted in blue have an adjusted (B-H) P-value of ≤0.2. These variables are the IgG C Term, IgG NANP, MSD NANP, MSD C term, and MSD R21. However, none of these variables has an AUROC≥0.8.

Table 22 - Univariate logistic regression and AUC analysis results. D84, 1B+30 and 2B+30

**Estimated odds ratio from a logistic regression model where the occurrence of malaria infection is the outcome variable and the log of the immune response is the independent variable.*

^ Benjamini-Hochberg adjusted P-value with a false discovery rate of 0.2 (variable to be included in the multivariate model if B-H adjusted P-value ≤0.2).

AUROC – area under the receiving operating characteristic (variable to be included in the multivariate model if AUROC ≥ 0.8).

Variable	Timepoint	N	Outcome Variable	OR*	95% CI	P-value	B-H adjusted P value^	AUROC
Avidity C term	D84	72	12 months	0.35	0.02 to 5.38	0.451	0.823	0.472
IgG C Term	D84	71	12 months	0.53	0.25 to 1.09	0.084	0.51	0.375
IAVI ELISA C term IgG	D84	66	12 months	0.94	0.50 to 1.74	0.836	0.861	0.462
IAVI ELISA NANP	D84	66	12 months	0.67	0.36 to 1.26	0.218	0.785	0.401
ISI (% infection blocked)	D84	70	12 months	0.99	0.96 to 1.01	0.316	0.801	0.423
Avidity NANP	D84	70	12 months	0.73	0.09 to 5.67	0.761	0.856	0.502
IgG NANP	D84	72	12 months	0.59	0.32 to 1.08	0.085	0.51	0.347
IgG R21	D84	57	12 months	0.46	0.22 to 0.97	0.042	0.51	0.345
MSD – NANP IgG	D84	71	12 months	0.76	0.43 to 1.35	0.356	0.801	0.416
MSD - C term IgG	D84	71	12 months	1.14	0.65 to 2.01	0.64	0.823	0.523
MSD – HBsAg IgG	D84	71	12 months	0.92	0.67 to 1.27	0.616	0.823	0.476
MSD - R21 IgG	D84	71	12 months	0.88	0.47 to 1.65	0.695	0.834	0.458
IgA - NANP	D84	70	12 months	0.95	0.53 to 1.71	0.861	0.861	0.481
IgM- NANP	D84	70	12 months	1.52	0.72 to 3.22	0.271	0.801	0.604
IgG1 - NANP	D84	70	12 months	0.64	0.32 to 1.28	0.205	0.785	0.411
IgG2 – NANP	D84	70	12 months	1.18	0.64 to 2.18	0.592	0.823	0.51
IgG3 – NANP	D84	70	12 months	1.2	0.70 to 2.04	0.507	0.823	0.538
IgG4 - NANP	D84	70	12 months	0.69	0.22 to 2.16	0.529	0.823	0.45
Avidity C term	1B+30	63	24 months	1.12	0.43 to 2.95	0.812	0.888	0.498
IgG C Term	1B+30	62	24 months	0.52	0.23 to 1.18	0.117	0.176	0.38
Avidity NANP	1B+30	48	24 months	0.89	0.19 to 4.25	0.888	0.888	0.486
IgG NANP	1B+30	66	24 months	0.21	0.08 to 0.58	0.003	0.009	0.264
IgG R21	1B+30	61	24 months	0.68	0.32 to 1.44	0.31	0.399	0.452
MSD – NANP IgG	1B+30	64	24 months	0.18	0.07 to 0.47	<0.0001	0.0005	0.194
MSD - C term IgG	1B+30	59	24 months	0.3	0.11 to 0.86	0.025	0.056	0.332
MSD – HBsAg IgG	1B+30	64	24 months	0.67	0.44 to 1.01	0.057	0.103	0.369
MSD - R21 IgG	1B+30	64	24 months	0.09	0.03 to 0.34	<0.0001	0.0005	0.458

Avidity C term	2B+30	55	36 months	0.38	0.05 to 2.75	0.337	0.387	0.391
IgG C Term	2B+30	60	36 months	0.59	0.33 to 1.05	0.071	0.128	0.381
Avidity NANP	2B+30	58	36 months	0.48	0.10 to 2.21	0.344	0.387	0.432
IgG NANP	2B+30	61	36 months	0.92	0.71 to 1.18	0.496	0.496	0.306
IgG R21	2B+30	46	36 months	0.26	0.10 to 0.67	0.005	0.025	0.237
MSD – NANP IgG	2B+30	61	36 months	0.44	0.24 to 0.82	0.01	0.025	0.267
MSD - C-Terminus IgG	2B+30	60	36 months	0.41	0.20 to 0.82	0.011	0.025	0.315
MSD – HBsAg IgG	2B+30	61	36 months	0.78	0.52 to 1.17	0.231	0.347	0.428
MSD - R21 IgG	2B+30	61	36 months	0.36	0.18 to 0.75	0.006	0.025	0.258

13.4.11.1 Stepwise regression Model

The six immunology variables measured at 1B+30 (highlighted in orange in Table 22) were assessed were included in a forward stepwise logistic regression model. In addition, the five immunology variables measured at the 2B+30 timepoint (highlighted in blue in Table 22) were included in a separate forward stepwise logistic regression model. After this process, the only variable remaining in the model for both time points was IgG NANP. Concluding that anti-NANP IgG is the variable most associated with protection in Burkinabe infants

A limitation is the observed immunogenicity at peak immunogenicity time points (1-month post-primary and 1-month post-boosts were assessed). Potentially using immunogenicity at 6 months post primary series or boosts would help pick apart the analyses. A post-hoc power calculation found that the immunology cohort was 83% powered to detect associations with protection, making this dataset sufficiently powered.

13.4.12 Alternate factors influencing immunogenicity and protection

13.4.12.1 Vaccinating in the morning or afternoon does not influence immunogenicity

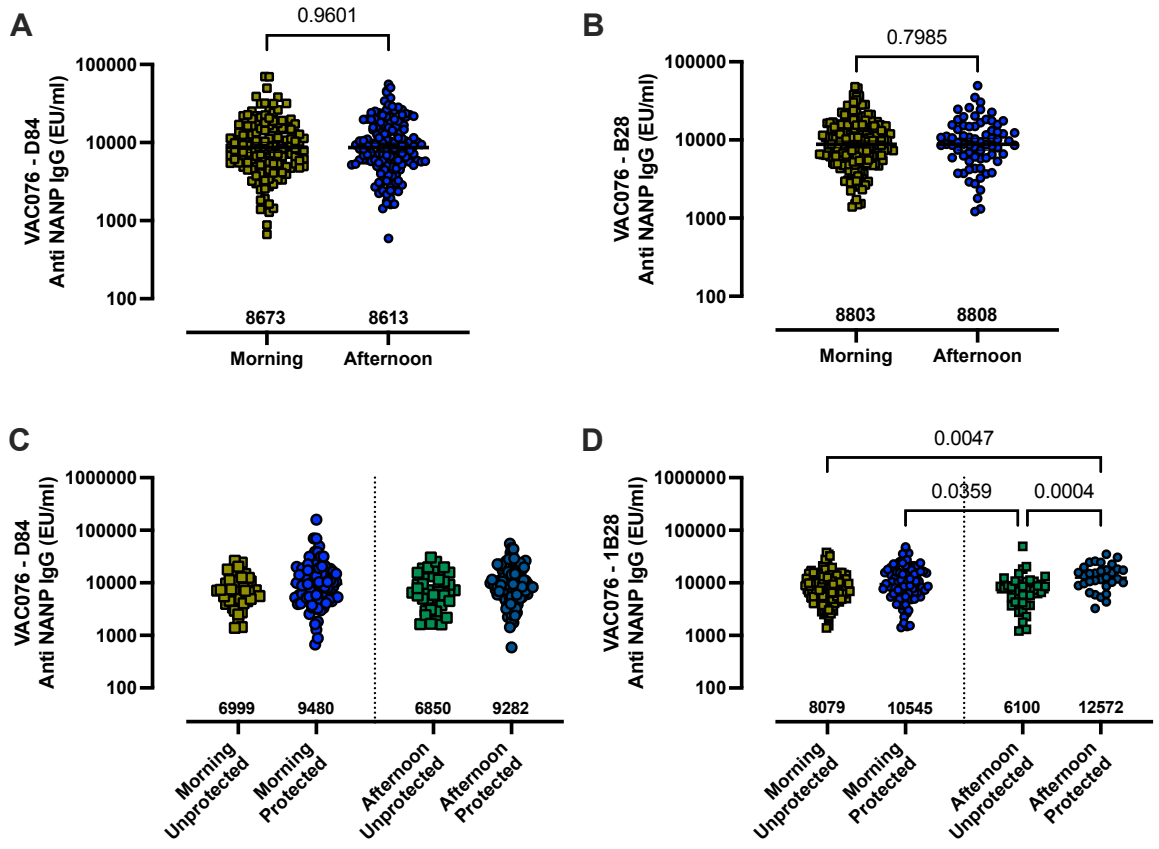


Figure 88 - Time of vaccination (morning vs afternoon).

A) *Anti-NANP IgG at D84 by morning (3rd dose of primary series < 12:00 pm) and afternoon (3rd dose of primary series >12:00 pm). Geometric means with error bars indicating 95% CI. Mann-Whitney.* ***B)*** *Anti-NANP IgG at 1B+30 by morning (first boost < 12:00 pm) and afternoon (first boost >12:00 pm). Geometric means with error bars indicating 95% CI. Mann-Whitney.* ***C)*** *Anti-NANP IgG at D84 by morning and afternoon stratified by protection at 12 months. Geometric means with error bars indicating 95% CI. Kruskal-Wallis was corrected by Dunn's multiple comparisons.* ***D)*** *Anti-NANP IgG at 1B+30 by morning and afternoon stratified by protection at 12 months. Geometric means with error bars indicating 95% CI. Kruskal-Wallis corrected by Dunn's multiple comparison*

Data represents geometric mean with error bars indicating 95% CI. Values above the x-axis are GMTs values.

Recent literature has suggested that the time of vaccination can impact immune responses [471]. Accordingly, anti-NANP IgG titres measured at one-month post-primary series (D84) were stratified by whether the infants received their third vaccination of the primary series before or after 12 pm. In

addition, anti-NANP IgG values measured one month post-boost (1B+30) were also stratified by whether the booster dose was administered before or after 12 pm.

An even distribution of participants was immunised in the morning (n=138) or afternoon (n=151) at 3rd vaccination of the primary series, however no significant differences (P=0.96) were found at D84 - Figure 88A. At boost, more than double the number of infants received their boost in the morning (n=195) compared to the afternoon (n=72) but no differences in anti-NANP IgG at one-month post boost were found - Figure 88B.

The time of vaccination was further stratified by protection at D84. Both morning and afternoon immunised infants who were protected had higher anti-NANP IgG titres at D84, although no statistically significant differences were found - Figure 88C. One month post-boost, protected infants immunised in the afternoon had significantly higher titres than unprotected infants with protected infants immunised in the afternoon presenting with the highest anti-NANP IgG GMTs 12572EU [95% CI 10236 – 15442] - Figure 88D.

13.4.13 Age does not impact antibody response after immunisation with R21/MM

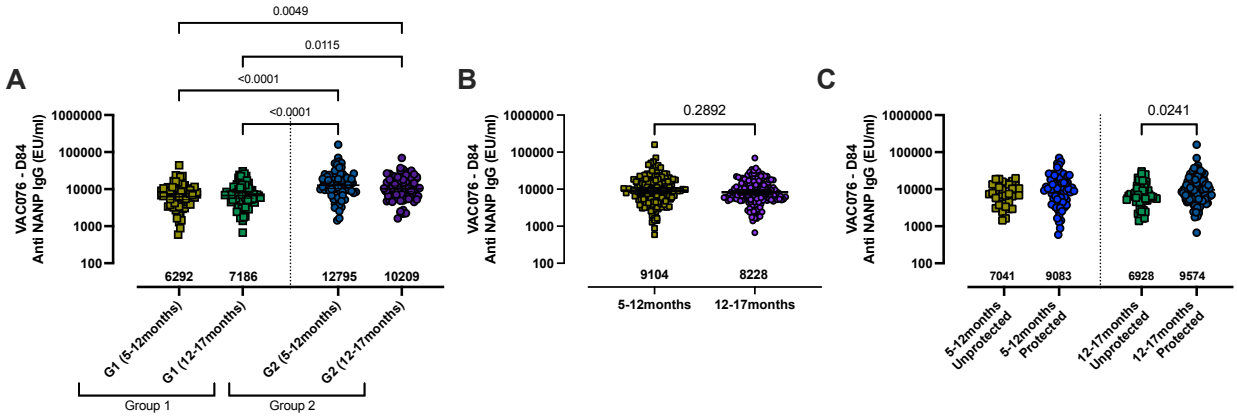


Figure 89 – Anti-NANP IgG stratified by age at third vaccination (5-12 months vs 12-17 months).

A) *Group 1 (5µg R21 / 25µg MM) & Group 2 (5µg R21 / 50µg MM). Geometric means with error bars indicating 95% CI. Kruskal-Wallis with Dunn's correction for multiple comparisons.* ***B)*** *Groups merged*

and stratified by age. Geometric means with error bars indicating 95% CI. Mann-Whitney. ***C)*** *Groups merged, separated by age, and stratified by protection. Geometric means with error bars indicating 95% CI. Mann-Whitney.*

Data represents geometric mean with error bars indicating 95% CI. Values above the x-axis are GMT values.

Infants between the ages of 5 and 17 months were recruited into VAC076. To assess whether age affects immunogenicity outcomes and potentially affects protection, anti-NANP IgG responses were stratified by age of infants between the ages of 5-12 months and 12-17 months. Infants in G2, irrespective of their age at the time of third vaccination had significantly higher anti-NANP IgG than infants in G1 - Figure 89A.

As no significant differences were observed between groups, G1 and G2 were merged and subsequently stratified by age. Infants aged 5-12 months had anti-NANP IgG (GMT 9104EU [95% CI 7826 – 10592]) compared to infants 12-17 months (GMT 8228EU [95% CI 7255 – 9332]) with no significant difference calculated (P=0.29) - Figure 89B. When further stratifying by protected or unprotected, both infants 5-12 months and 12-17 months who were protected had higher anti-NANP IgG than unprotected - Figure 89C. Protected infants aged 12-17 months had significantly higher anti-NANP IgG (P=0.02) with (GMTs 9574EU [8196 – 11185]) compared with unprotected (GMTs 6928EU [95% CI 5637 – 8516]).

13.4.13.1 Gender does not impact antibody response after immunisation

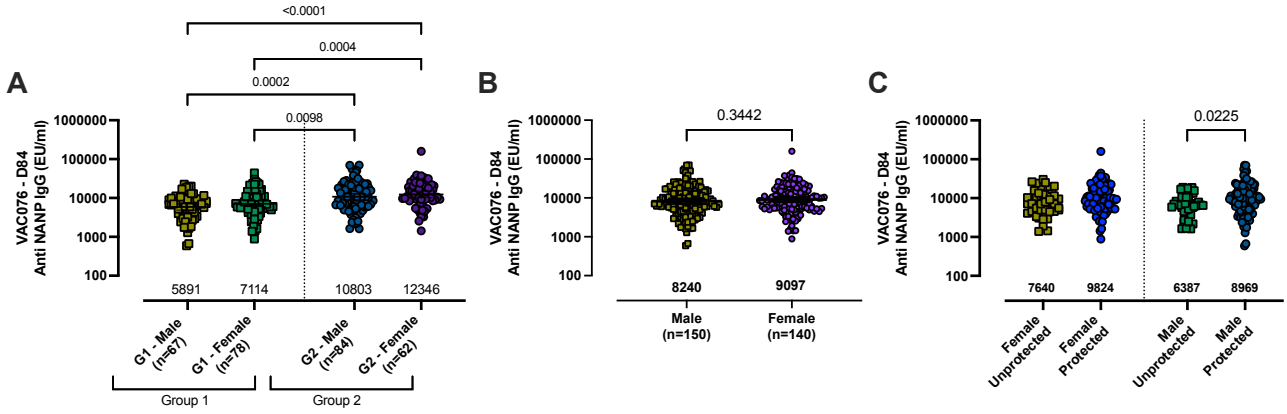


Figure 90 - Antibody response separated by gender.

A) *Anti-NANP IgG Group 1 (5µg R21 / 25µg MM) & Group 2 (5µg R21 / 50µg MM) separated by gender. Geometric means with error bars indicating 95% CI. Kruskal-Wallis with Dunn's correction for multiple comparisons.* ***B)*** *Groups merged and stratified by gender. Geometric means with error bars indicating 95% CI. Mann-Whitney.* ***C)*** *Groups merged, separated by gender, and stratified by protection. Geometric means with error bars indicating 95% CI. Mann-Whitney.*

Data represents geometric mean with error bars indicating 95% CI. Values above the x-axis are GMT values.

Males and females in G1 and G2 had no significant differences in anti-NANP IgG antibodies. However, both males and females in G2 had significantly higher antibody titres than males and females in G1 - Figure 90A. As no significant differences were found between males and females within each group, anti-NANP IgG titres were merged and stratified by gender. Females had a small increase in anti-NANP IgG (GMTs 9097EU [95% CI 7942 – 10421]) compared with males with (GMTs 8240EU [955CI 7160 – 9482]) however no significant difference was found - Figure 90B. Gender did not impact protection, although both protected males and protected females had higher anti-NANP IgG responses over unprotected males and females, with protected males having significantly higher responses than unprotected males (P=0.02) - Figure 90C.

13.5 Chapter 3 - Discussion

13.5.1 Burkina seasonality

The number of cases amongst the control groups in clinical trials confirms that Burkina Faso has highly seasonal malaria transmission. The high proportion of cases observed in the control groups in the first six months of each year of the clinical trials confirms the advantages of a seasonal immunisation regimen to ensure the greatest immune response during peak transmission [402].

13.5.2 Proof of concept of CSP-based vaccines in seasonal settings

A five-year study of RTS,S, in combination with SMC has shown high VE [274, 405] in Mali and Burkina Faso of 71.7% [95% CI 63.8 to 77.8] and dropping over five years to 57.1% [95% CI 53.3-61.7] [472]. This provides proof of concept that protection can be maintained when an anti-sporozoite vaccine is administered seasonally and with yearly boosters. A cost-effectiveness analysis has shown seasonal administration of RTS,S in combination with SMC reduces a further 42,000– 67,000 cases of malaria per 100,000 children under the age of five [473]. R21 is now into the fourth year of trial following a seasonal vaccination regimen, with certain groups receiving as many as six doses of R21/MM and should expect a similar number of cases averted. A similar or improved cost-effectiveness analysis could be seen among R21 immunised infants with increased manufacturing from SII and reduced cost per dose while administered at $1/5^{th}$ the dose of RTS,S [280].

13.5.3 Efficacy

During the first year, high VE was reported in both low dose G1 (5µg R21/25µgMM – 71% [95% CI 63-82]) and high dose G2 (5µg R21/50µg MM – 77% [95% CI 67-84]) adjuvant groups [2]. Efficacy in the high-dose adjuvant group met the goal of VE greater than 75%, listed in the 2013 Malaria Vaccine Technology Roadmap [223]. In the second year, VE in G1 (71% [95% CI 54-81%]) remained high and G2 (81% [95% CI 66-87]) increased and 12 months after the first boost [3]. Both groups saw antibody

titres increase after a boost to levels seen 1 month after the primary series with significantly higher titres in the high-dose adjuvant (G2) group compared to the low-dose (G1) group. Higher efficacy along with significantly higher titres in the high-dose adjuvant group provides evidence for further evaluation in Phase III trials.

The necessity of a second booster dose was assessed in year 3 whereby infants were randomised to receive the control vaccine. At thirty-six months, VE in the boosted group (G2a – 60% [95% CI 45-78]) was comparable to the non-boosted control group (G2b - 64% [95% CI 44-79]) with overlapping confidence intervals. While sample sizes are even smaller this is a positive result and indicates the use of a single booster being required. Immunogenicity in infants in non-boosted groups saw antibody titres plateau around 3000EU/ml and protection maintained with anti-NANP titres significantly lower than the threshold of 6618EU reported in Datoo *et al.,* 2022 [3]. Further evaluation of the antibody associations with protection will be assessed in Phase 3 with much larger sample sizes, but indications from non-boosted groups show protection can be achieved with lower antibody titres.

13.5.4 NANP vs C term

Antibody responses to both NANP and C term in Burkinabe infants immunised seasonally with R21 followed similar kinetics to what was seen in RTS,S with peak responses found 1-month post-vaccination and waned over the course of the following year [474]. Stratifying by number of episodes there is a trend in infants who have higher antibody responses to have fewer episodes of malaria. However, although increased antibody responses are associated with protection, many infants with the highest antibody titres report multiple episodes. Further supporting antibody quantity alone is not the only mediator in protection and additional immune mechanisms are involved. In this chapter, strong anti-NANP and C term IgG titres across various time points are reported with both in-house single antigen ELISA and by MSD. However, only anti-C term IgG titres in G2 were associated with protection when assessed at 1 month post-third dose of the primary series.

13.5.5 Avidity

Antibody 'quality' [456, 475] can be assessed by avidity which quantifies antibody strength of binding and therefore an indicator of improved quality [457]. Avidity can be described as the strength at which an antibody binds to an antigen and should be distinguished from affinity which is the ability of an antibody to bind to antigen [476, 477]. Avidity generally increases over time following the encounter with the antigen and therefore avidity can be described as the result of successful priming and therefore strong memory responses [455]. Avidity in R21/MM has been shown to significantly increase after boosting, likely as a result of a strong memory response from GC and B cell maturation [455]. This strong memory response was found in non-boosted groups, yet maintained high levels of avidity. Boosting or the delay between doses has been reported in other studies [455] to promote avidity, but a striking observation made in this chapter was the significant increase in avidity to C term after the first and second boost when compared to the primary series. Data from an RTS,S/AS01$_E$ study in African children and infants show that IgG avidity to C Term is important for protection against malaria induced by RTS,S [478] however not seen in R21 immunised infants. IgG avidity to NANP did increase after the boost, however only after the second boost was a significant P-value identified. Future cellular experiments by flow cytometry will be planned to assess changes in TfH and B cell phenotypic changes after boosting compared to the primary series.

13.5.6 Durability

Boosting with R21/MM in Burkinabe infants improves durability whereby anti-NANP IgG titres are significantly higher at six and 12 months after the first and second compared to the primary series. Indicating that the delay between boosts allows for improved antibody production. Peak antibody titres measured 1 month post-immunisation are reduced after each boost, and the rate of antibody decline is improved. This may be a result of a better quality of antibody production rather than quantity however age may play a confounding factor with the reduction in peak titre observed after

the boost. Furthermore, infants who had a smaller rate of antibody decline had fewer episodes of malaria and increased protection [103, 479].

Boosting also improved antibody half-life compared to the half-life after the primary series, which may explain efficacy being maintained in the second year. Whereas short and long-lived half-lives reported by White *et al.,* 2015 after perennial immunisation [271] of RTS,S/AS01$_E$ decreased however in that Phase 3 study from 2011 to 2015 study booster doses were administered at 18 months compared to 12 months in VAC076 of R21.

13.5.7 Subclass and Isotype

Strong IgG1 and IgG3 responses to NANP were found in Burkinabe infants at D84 with no differences between G1 and G2. This is unsurprising considering IgG1/3 is the most abundant subclass IgG in circulation [199] and corresponds to RTS,S-induced subclass isotypes [164, 452]. However, no subclass antibody was found to associate with protection. Whereas with IgG subclass induced by RTS,S assessed found IgG1 to be associated with protected infants in Ghana and Mozambique [90]. Increased sample sizes across more time points may allow for a greater power to detect associations or rather a more sensitive assay to detect antibody on/off rates which detect lower reported responses [480, 481].

Isotypes IgA and IgM against NANP were found in VAC076 Burkinabe infants immunised with R21, but no difference between G1 and G2 was found or between protected and unprotected. RTS,S improves IgG and IgM [370] and while IgA is mainly involved with mucosal pathogens, Tan *et al.,* 2021 [482] show a potential role of IgA in using a sporozoite challenge murine model using IgA monoclonal antibodies that target the sporozoite. IgM has also been shown to play a role in infection and to persist in naturally immune subjects without constant re-infection [483]. Further, IgM has been shown to inhibit the invasion of hepatocytes *in vitro* [484].

13.5.8 MSD

IgG responses quantified by the novel multiplex assay from MSD found comparable responses measured by in-house ELISAs for NANP and C term and similar trends when stratifying by protection. All infants had comparable anti-HBsAg IgG titres at baseline above the WHO protection threshold of 0.01 IU/ml [441]. This is unsurprising considering infants at five months would have had three doses of the hepatitis B vaccine as part of the EPI programme in BF [485]. What is striking is that three doses of R21 significantly increase anti-HBsAg responses compared to HBsAg titres at baseline allowing R21 to be classed as a strong hepatitis B vaccine.

13.5.9 Time of day

Considerable data indicates that the immune system changes over the course of a day, often referred to as the immune circadian clock [471, 486]. Circadian clocks refer to endogenous physiological processes that change approximately every 24 hours [487]. A BCG study reported that vaccinating in the morning resulted in a higher Th1 cytokine response compared to those immunised afternoon [488]. Additionally, a seasonal influenza vaccine trial reported significantly higher antibody responses in the morning compared to the afternoon [489]. As such, immunogenicity was stratified by time of day to determine if this was a potential problem in infants immunised with R21 [490]. As such no association in anti-NANP IgG titres was found when stratifying by morning (8 am – 12 pm) or afternoon (12 pm – 5 pm). Potentially further stratifying time points may indicate difference, although statistical power would be lost. Additionally, the time-of-day stratification was only assessed on the third vaccination of the primary series.

13.5.10 Age and gender

No association with age or gender was found between anti-NANP IgG titres one month after the third vaccination. However, gender was only assessed in the first year of study, and gender differences post-

first boost and second boost have yet to be assessed. GSK was required to further evaluate RTS,S due to a safety signal with increased female mortality and an increase in cases of meningitis [273, 491] in the RTS,S group as compared to the control group, although this was later disproved. In addition, age and gender have found immunological differences post-pubertal females reported significantly increased immune responses after Sanaria's PfSPZ vaccine although VE was comparable with males [492].

13.5.11 ISI

In Chapter 1 (11.4.4.5), high anti-NANP IgG titres result in increased ISI and a higher % ISI was found with G2 although not significant. In this chapter Burkinabe infant samples positively correlate with NANP and C term IgG titres. This has been observed throughout this thesis that both NANP and C term antibodies are associated with an increase in % ISI. However, no association with protection in % ISI could be found. Future improvements to the ISI assay are discussed in the main discussion (14.6).

13.5.12 Multivariate Immune combinations

Datoo *et al.,* 2022 [3] reported a threshold of protection of anti-NANP IgG titres of 6618EU after the primary series and 6130EU/ml after the boost respectively. Aside from increased antibody titre measure by single antigen ELISA's and MSD no other immunological variable presented significantly associated with protection. Univariate logistic regression analyses confirmed associations of protection. However, it was hypothesised potential combinations of immune responses may correlate with protection using a multivariate analysis.

Ultimately after the potential inclusion of immune variables in a multivariate analysis, IgG NANP remains the most significant variable associated with protection in infants in Burkina Faso. This concords with the existing literature which posits that NANP IgG is the dominant antigen for eliciting protection. Larger immunology sample sizes may indicate that more variables may potentially qualify

for future multivariate models however post-hoc power calculations show the VAC076 immunology cohort was sufficiently powered. Ubillos *et al.,* 2018 [164] ran a similar multivariate model on 195 African infant samples across two trial sites, and found similar results with IgG1 and IgG3 to both NANP and C terms were associated with protection.

13.5.13 Limitations

Limitations of this study include the small sample size of the immunology cohort (n=72). Post-hoc power calculations indicated an increase of n=50 could provide more statistical power to see a significant immune association with protection. The immunology cohort did not include any of the control group, due to the assay specificity to detect low levels of immune response. However, the MSD assay was validated to detect low immune responses and therefore running more controls on the MSD assay would have been helpful to compare immune responses with protection status as well as run a mixed effects model across multiple time points. In addition, the overall sample size in the trial (n=450) could restrict the identification of less common adverse events and reduce the statistical power to identify a potential CoP.

Another limitation is the lack of cellular data in response to R21/MM. Durable immune responses and increased avidity after the boost suggest an improved cellular response. Assessing changes in B cell and TfH phenotypes before and after boost would be invaluable to improving the understanding of R21's influence on long-lived memory responses and ultimately how protection is mediated. Immunogenicity and efficacy of the R21/MM vaccine in VAC076 were conducted in one area of high seasonal malaria transmission. Investigations of VE in areas with different transmission patterns will be required if R21/MM is to be administered more widely.

13.6 Chapter 3 - Conclusion

It is shown that in VAC076, the high-dose adjuvant group induces higher efficacy/protection and significantly higher immune responses post-vaccination compared to the lower adjuvant group. Protected infants have significantly higher antibody titres reduced number of malaria episodes to have lower antibody levels. A booster dose maintains efficacy and improves antibody durability and avidity. There is an indication that only one boost may be needed as comparable efficacy was found between infants who received a second boost compared with infants who received the first boost. However, increased follow-up of these infants needs to be undertaken to assess for delayed malaria. Further questions remain as to what antibody quantity driving protection potential other immune combinations are involved. Data presented in this chapter provides confidence for 5µg R21/50µg MM to be taken forward for Phase III evaluation.

Chapter 4

14 Chapter 4 - Immunology on two leading PEM vaccine candidates – R21/MM and RTS,S/AS01$_E$

14.1 Chapter 4 - Introduction

At present, there are over 82 vaccine-candidate clinical trials targeting the *Pf* lifecycles [221]. RTS,S, first developed in 1987 [493, 494] has seen approximately US$750 million invested in development [495] with more than 830,000 doses administered across a variety of age groups [496]. RTS,S is currently the most advanced vaccine in development with Phase III trial results in 2015 reporting VE in infants (5-17months) after a perennial regimen of 55.8% (95% CI 50.6-60.4) after twelve months [247], dropping to 45% (95% CI 42.3-50.0) at eighteen months prompting the need for a booster dose [270]. VE at forty-eight months, after a booster dose at eighteen months was 36.3% (95% CI 31.8-40.5) [248].

It is estimated that a broad distribution of RTS,S among the ten countries with the highest burden could prevent 5.3 million cases and 24,000 deaths in young children each year [497]. A WHO endorsement is a significant step forward in fostering vaccine confidence and promoting vaccine uptake in vaccine-hesitant individuals. Yet, even with an approved vaccine and an endorsement of $155.7 million from GAVI to produce, procure and deliver malaria vaccines from 2022-2025 [498], most of the 40 million children at risk [498], many will not receive any vaccine due to manufacturing supply issues. GSK has pledged to deliver 18 million doses by 2025, the maximum that the manufacturing facility in Wavre, Belgium can produce [499]. In 2021, GSK signed a product transfer with Bharat Biotech (BBIL) of India to increase antigen production of RTS,S. GSK will retain the production of adjuvant (AS01E) and its supply to BBIL [500].

Vaccine trial designs were altered to potentially improve the efficacy of anti-sporozoite vaccines to involve seasonal vaccine administration with the addition of SMC [501], creating maximum immunity and protection during periods where infections are highest [379]. SMC was implemented in Burkina Faso in 2017 and allows every child between the ages of 3-59 months to receive SMC drugs at monthly intervals during the peak malaria transmission season. Each course of SMC is known as a cycle and is claimed to protect for up to 12 months [502].

RTS,S administered with SMC in a seasonal regimen reported VE of 71.7% (95% CI 63.8- 77.8) and VE after 12 months with the addition of a single booster plus SMC dropped to 63.2% (95% CI 56.8-68.6) [275]. Modelling studies have shown that administering RTS,S along with SMC in seasonal transmission settings can avert an additional 14,000–47,000 cases per 100,000 children under the age of five over fifteen years [473]. These studies included a booster dose at twelve-month intervals to maintain protection. SMC is only administered up to the age of five as per WHO guidelines [137]. This can lead to reduced NAI immunity in children causing increased risk of severe malaria [503]. Reduced number of natural infections due to interventions such a vaccination and SMC leads to reduced NAI leading to an epidemiological effect know as delayed malaria [462].

The current WHO recommendation is a three-dose regimen with a booster administered at twelve months [137]. Approximately 40 million children are born each year in malaria-endemic regions. Therefore, approximately 160 million doses will be required to vaccinate children under the age of two with many more doses required if a vaccine is to be successful in malaria elimination programmes. GSK's pledge of 18 million doses thus falls short of the desired demand.

Primary immunogenicity assays for all RTS,S trials are performed at the Centre for Vaccinology at Ghent University in Belgium (CEVAC). There they report CSP-based IgG responses to the R32LR protein which comprises two NVDP oligopeptides and thirty NANP repeats linked to the dipeptide Leu-Arg

(NVDP[NANP]$_{152}$LR) [504]. Further RTS,S immunology assays are reported from the IAVI-HIL lab at Imperial College assessing IgG responses to both NANP and C term peptides as well as avidity to NANP and C term [358].

SII together with Novavax who manufacture Matrix M adjuvant have reported capabilities of producing 200 million doses per year [499]. R21/MM has shown high VE against clinical malaria of 77% (95% CI 67-84) and 80% (95% CI 66-87) in the first two years of a Phase II trial in Burkina Faso [2, 3] with similar VE being reported in preliminary data from the Phase III trial currently underway [505]. Due to pre-qualification already in place for RTS,S, it is hoped that R21 should receive a quick approval process, considering both vaccines are similar in design [163] and the high safety and immunogenicity data released by Novavax on the use of Matrix M in multiple Phase III trials [506, 507].

In this chapter, immunogenicity induced from R21/MM and with RTS,S/AS01$_E$ the same assays will be presented among Burkinabe infants aged 5-36 months. Both studies were conducted in Burkina Faso and therefore evidence similar levels of malaria transmission. In addition, both studies followed a seasonal vaccine regimen however VAC076 had varied levels of SMC with ~93% of infants at least receiving 1 cycle of SMC and ~50% receiving 4 cycles as per the WHO guidelines [390].

14.2 Chapter 4 - Methods

14.2.1 Trial assessed

In this chapter, data from (R21/MM) and (RTS,S/AS01$_E$) with or without SMC among infants in Burkina Faso will be compared. Both trials were undertaken in the same age group (infants aged 5-17 months) which has the highest disease burden [335] and in the same malaria endemic area (Burkina Faso). Both trials included a seasonal vaccination regimen of three doses administered one month apart before the rainy season (August to December) [403] which sees a seasonal surge in malaria cases

[402]. Both trials included multiple boosters administered at yearly intervals. Trial Designs and sample numbers can be found in methods (10.3.4.6 & 10.3.4.7).

14.2.2 RTS,S immunology cohort

Table 23 - MAL099 RTS,S Immunology cohort

Group 1 – 3 doses of RTS,S/AS01$_E$ only. G3 – 3 doses of RTS,S/AS01$_E$ followed by 4 doses of SMC.

Group	No. of infants pre - vaccination	No. of infants post - vaccination
2017		
Group 1 (RTS,S only)	4	15
Group 1 (RTS,S + SMC)	4	13

After the primary immunological assay was completed, several samples that had been administered RTS,S were assessed to evaluate the functional ability of antibodies to inhibit ISI of hepatocytes. At the time of experimental design, the NANP IgG data had yet to be unblinded, therefore samples were randomly selected for ISI according to the anti-NANP IgG response at D84. IgG titres at D84 were ranked from highest to lowest and samples were selected for a range of different responses. Due to assay limitations, a maximum of 50 samples can be run at any given time. Further, if sporozoite yields are lower than expected after dissection, then the sample sizes had to change to meet the infection criteria.

14.2.3 Primary immunological outcome

All serological samples available in these studies were tested using standard NANP ELISA. All other exploratory immunology assays for R21 samples were undertaken only on the VAC076 immunological cohort (Table 20) and the MAL099 immunology cohort for RTS,S samples (Table 23).

14.2.4 Immunology methods

The full dataset for anti-NANP IgG in Burkinabe infants who took part in VAC076 is presented in Chapter 3. Immunological assay methods can be viewed in the appendix (17.5).

14.3 Chapter 4 - Objectives

- Report antibody responses for R21/MM and RTS,S/AS01$_E$ using the Jenner NANP standardised ELISA.
- Evaluate antibody responses after booster doses of R21/MM and RTS,S/AS01$_E$ administered at yearly intervals.
- Evaluate the durability of antibody responses induced by R21/MM and RTS,S/AS01$_E$.
- Evaluate the performance of Matrix M vs AS01 using the same immunogen (R21).
- Evaluate the HBsAg responses induced from R21/MM and RTS,S/AS01$_E$ at one month post-third dose using the MSD multiplex platform.
- Evaluate functional antibodies induced from R21/MM and RTS,S/AS01$_E$ to inhibit sporozoite invasion of hepatocytes *in vitro*.

14.4 Chapter 4 - Results

14.4.1 Anti-NANP IgG responses in infants immunised with R21/MM or RTS,S/AS01$_E$

Samples from the Phase IIIb RTS,S SMC trial in Mali and Burkina Faso were transferred to the Jenner Institute of Immunology requested by the LSHTM. Due to time constraints, only the Burkina Faso cohort immunology cohort was assessed. The Mali cohort has now subsequently been run.

14.4.1.1 Anti-NANP IgG responses induced from R21/MM

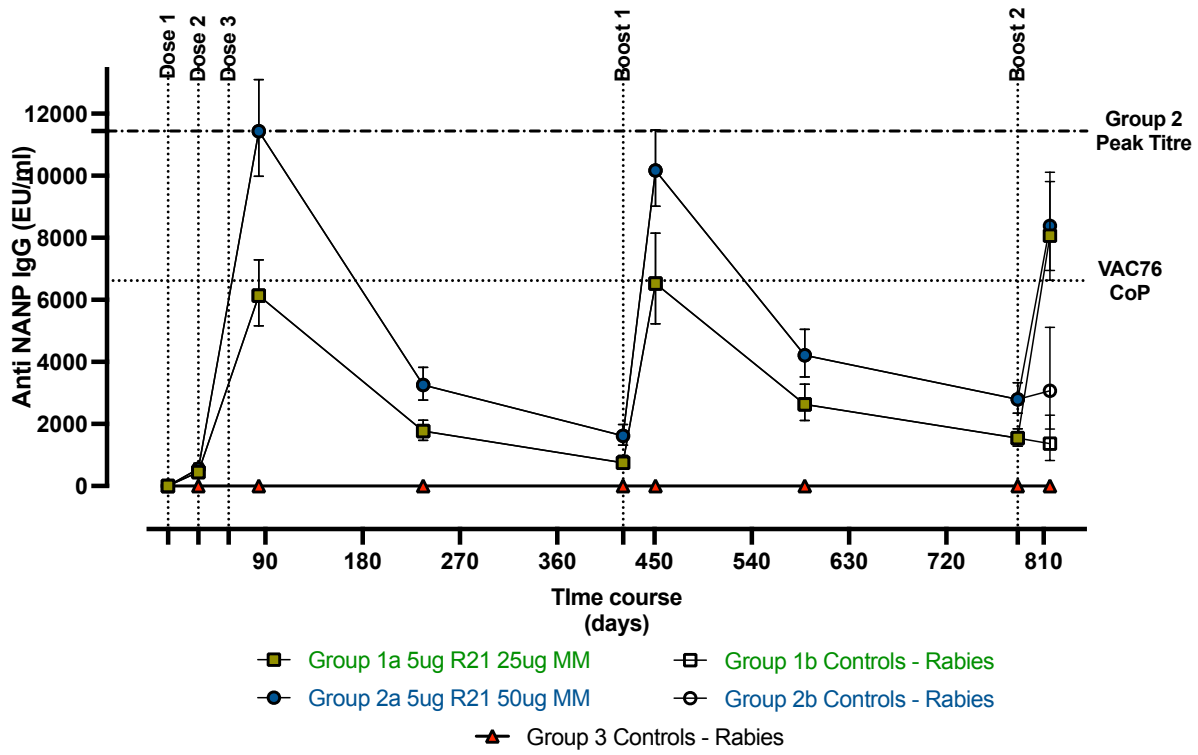


Figure 91 – VAC076 anti-NANP IgG time course for infants immunised with R21/MM.

Median time courses (error bars indicate IQR) over the duration of 4 years. The primary series of 3 vaccinations 1 month apart in the first year followed up by 3 booster doses given at yearly intervals. G1 are depicted by green squares and, G2 are blue circles. Vertical black lines represent immunisations of R21/ MM. The horizontal black dotted line (y=6618) indicated the threshold protection titre reported in Datoo et al., 2022 [3]. Horizontal dashed line (y=11438) peak G2 IgG titre at D84.

Infants immunised with R21/MM show antibody titres that increase after the primary series and peak at one month post-third dose with significantly higher antibody titres found in G2 (5µg R21/50µg MM). Thereafter, antibody titres significantly decline six months and twelve months after the primary series.

However, with the addition of a booster dose administered twelve months after the primary series, anti-NANP IgG responses return to levels seen at D84 - Figure 91. This cycle is repeated in subsequent boosts, examined in Chapter 3 (13.4.3).

14.4.1.2 Anti-NANP IgG responses induced from RTS,S/AS01$_E$

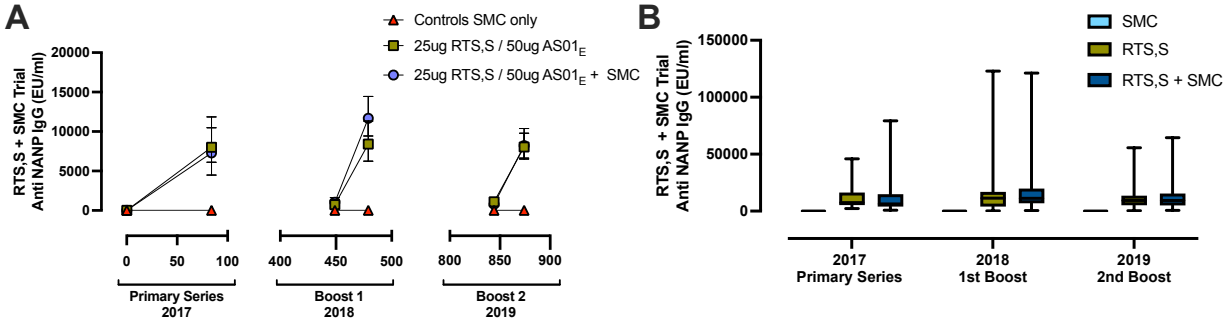


Figure 92 – MAL099 anti-NANP IgG time course (3 years) RTS,S SMC trial.

A) *Anti-NANP IgG titres pre and post-primary series (2017), pre and post-first boost (2018) and pre and post-second boost (2019). Medians with 95% CI.* ***B)*** *Box and whisker plot to indicate anti-NANP IgG peak responses in the first year (2017), second year (2018) and third year (2019) intervals.*

In the Phase IIIb RTS,S SMC trial, 104 infants were recruited in Burkina Faso in 2017 to explore serology responses with samples taken at baseline and one-month post-third dose (D84). In year two, a new cohort of infants was selected to have serology measured before and one-month post boost one. The same recruitment plan took place after boost two [275, 405]. Accordingly, infants cannot be directly compared between primary series and subsequent boosts for RTS,S samples.

Anti-NANP IgG time course over three years among infants immunised with RTS,S - Figure 92 follows very similar antibody kinetics with infants immunised with R21/MM in Burkina Faso – Figure 91A. Antibody titres increase after three doses in the primary series, peaking at one-month post third dose and followed by a large decline in antibody titres in the twelve months after the primary series Figure 92B in both studies.

14.4.1.3 SMC does not interfere with antibody titres induced from RTS,S/AS01$_E$

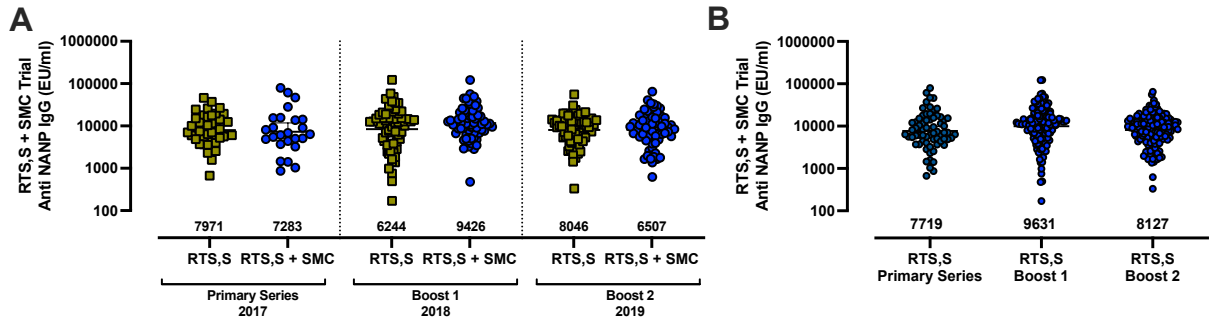


Figure 93 – Peak anti-NANP IgG responses in infants immunised with RTS,S.

A) *Anti-NANP IgG between G1 (RTS,S only) and G3 (RTS,S + SMC) pre and post-primary series (2017), pre and post-first boost (2018) and pre and post-second boost (2019).* ***B)*** *Anti-NANP IgG with G1 and G3 merged at 1-month post-primary series, 1-month post-first boost, and 1-month post second boost.*

Geometric means with error bars indicating 95% CI. Values above the x-axis indicated geometric mean values.

Antibody responses 1 month after primary series between infants in G1 (RTS,S only) were compared with infants in G3 (RTS,S + SMC) to assess whether SMC interferes with anti-NANP responses. Infants in G1 had a comparable anti-NANP IgG titre (7971EU [95% CI 6098 - 10472]) when compared with G3 (7238EU [95% CI 4481 – 11838]) – Figure 93A.

An observation in VAC076 (Chapter 3) is that anti-NANP IgG titres significantly increase one month after the first and second boost. However, the boost responses do not reach levels seen after the primary series. Only immunogenicity assessments are being made with no indication that SMC interferes with anti-NANP titres between time points, therefore data from G1 and G3 who received RTS,S were merged. If immunogenicity was stratified by protection, G1 and G3 would be separated. Antibody responses in infants from both vaccinated groups in the primary series peaked at D84 (7719EU [95% CI 6069 - 9820]) and antibody responses after the first boost (9931EU [95% CI 8277 - 11914]) were comparable to levels seen at the primary series. Furthermore, after the second boost, anti-NANP IgG titres (8127EU [95% CI 7002 - 9432]) remained consistent with titres after the primary series and second boost with no statistical significance – Figure 93B.

14.4.1.4 Both R21 and RTS,S induce high anti-NANP titres after primary series and subsequent booster doses

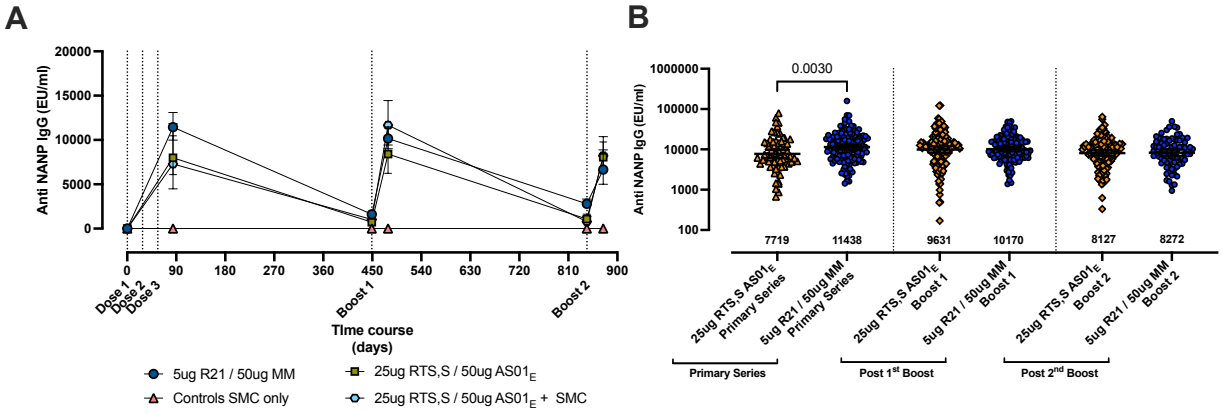


Figure 94 – Anti-NANP IgG induced by R21 vs RTS,S.

A) *Median anti-NANP IgG time courses for R21 and RTS,S overlapped pre and post-primary series, pre and post-first boost and pre and post-second boost. Vertical dotted lines indicate immunisation with either R21/MM or RTS,S/AS01$_E$.* ***B)*** *Geometric mean anti-NANP IgG comparing 25µg RTS,S and AS01E (G1 & G3 merged) 5µg R21 & 50µg MM at 1-month post-primary series, 1-month post first boost and 1-month post second boost. P=0.003 Mann-Whitney with Bonferroni's correction for multiple comparisons.*

Geometric means with error bars indicating 95% CI. Values above the x-axis indicated geometric mean values.

Anti-NANP IgG time courses from infants immunised with both R21/MM and RTS,S infants in Burkina Faso were merged and overlapped – Figure 94. Due to both VAC076 and MAL099 incorporate similar trial designs in that the primary series includes three doses of each vaccine administered one month apart over the summer months prior to the malaria season starting in August [467]. Anti-NANP IgG time courses for both R21/MM and RTS,S follow similar kinetics when the same time points are overlapped - Figure 94A.

High anti-NANP titres are produced from R21 and RTS,S, with R21/MM (5µg R21/50µgMM) producing 11,438EU [95% CI 9985–13102]) and infants immunised with RTS,S/AS01$_E$ (25µg RTS,S/50µg AS01E) producing 7719EU [95% CI 6069 - 9820]. However, the sample sizes with RTS,S (n=67) and R21/MM (n=147) do differ.

One month post boost, responses produced by RTS,S (9931EU [95% CI 8277-11914]) and R21/MM (10170EU [95% CI 9016-11472] are comparable with narrow overlapping confidence intervals. Responses in the second boost show similar trends to the first boost with RTS,S/ASO1$_E$ (8127EU [95% CI 7002 – 9432]) and R21/MM (8277EU [95% CI 6887 – 9949]) - Figure 94B.

14.4.2 Durability of R21 and RTS,S antibodies 12 months after primary series and boost

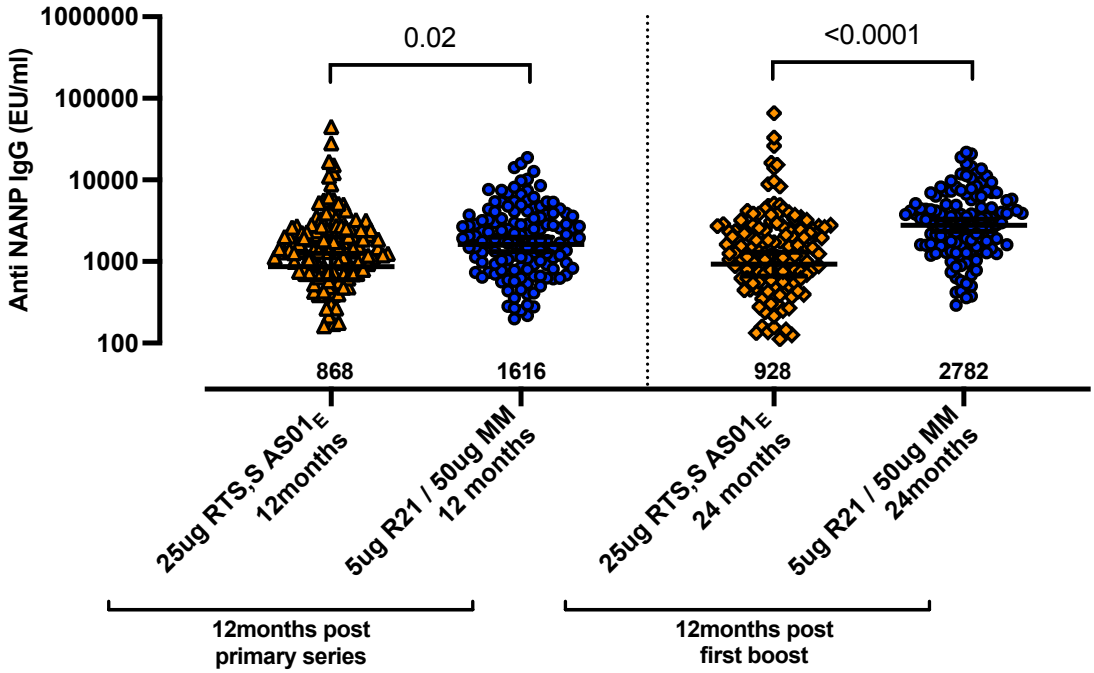


Figure 95 - Antibody durability from R21 and RTS,S after primary series and first boost.

Anti-NANP IgG induced from R21 and RTS,S at 12-months post-primary series and 12-months post-boost. P=0.02 and P<0.0001 Mann-Whitney with Bonferroni's correction for multiple comparisons.

Geometric means with error bars indicating 95% CI. Values presented above x-axis indicated geometric mean values.

Assessing the durability of antibody titres is important to understanding the effectiveness of the vaccine. Anti-NANP IgG titres peaked at D84 for RTS,S and R21 - Figure 93A. A significant (P=0.02) decline in anti-NANP IgG titres was observed twelve months after the primary series in the RTS,S (874EU [95% CI 606 – 1262]) cohort relative to R21 (1616EU [95% CI [1320 – 1979]).

The durability of anti-NANP IgG responses twelve months post boost of RTS,S and R21 were measured. Both RTS,S and R21, antibody titres peaked at approximately 10200EU one month post first boost - Figure 94B. Twelve months post first boost saw anti-NANP IgG titres decline 10-fold for RTS,S (680EU [95% CI 680 -1347]) whereas anti-NANP IgG for R21 (2782EU [95% CI 2339 – 3309]) declined 4.5-fold - Figure 95B.

14.4.3 R21 and RTS,S have declining antibody titres post-boost as compared to primary series

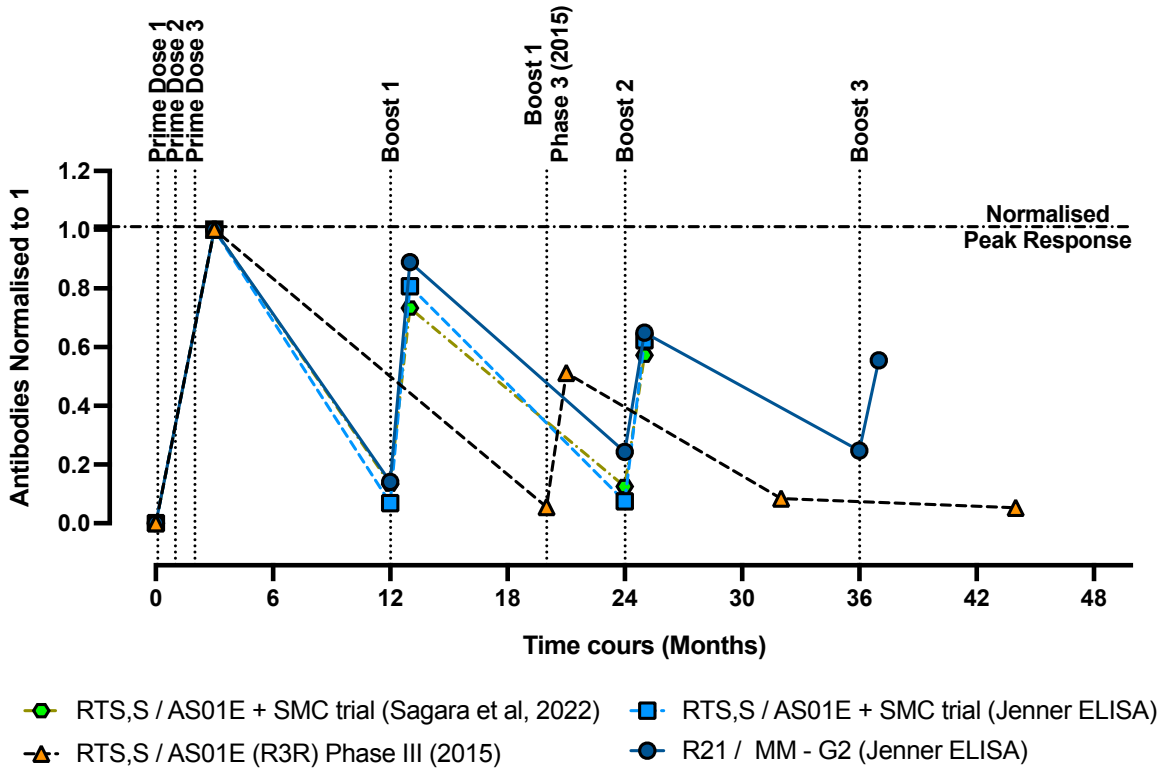


Figure 96 – Assessing the ability of R21 and RTS,S booster doses compared to primary series.

Immunogenicity at 1 month post third dose (D84) from RTS,S Phase 3 trial in 2011-2015 [271], 1 month post third dose from RTS,S SMC trial in 2017 – 2021 [474] was normalised to a value of 1. Immunogenicity measured using the Jenner Institute standardised NANP ELISA measuring anti-NANP IgG in VAC076 [2] and RTS,S SMC samples [275] at 1 month post third dose was normalised to a value of 1. Lines depict whether a boost was able to return antibody titres to levels seen at peak.

In order to compare the effect of booster doses of R21 and RTS,S, antibody titres retrieved from the publications of the RTS,S Phase III trial conducted in 2011 to 2015 [248, 271] and antibody titres

retrieved from publications of the RTS,S SMC trial [474] were compared to anti-NANP titres from R21/MM and RTS,S titres using the Jenner Institute standardised NANP ELISA. Anti-NANP titres at one month post-third dose from each database were normalised to a value of 1 and responses after booster doses were plotted to assess whether booster responses from each trial could return titres to levels seen at the peak - depicted in Figure 96 - by the value of 1.

In the RTS,S Phase III (2011-2015) trial [271], a booster was administered at eighteen months whereas in the RTS,S SMC trial (MAL099) and R21/MM (VAC076) a booster was administered at twelve months post primary series. In the RTS,S Phase III trial, antibody responses only returned to 51% of that seen at peak after boost at eighteen months. In the RTS,S SMC trial anti-NANP IgG titres returned to 73% of that seen at peak after the first boost at twelve months - data from Sagara *et al.,* 2022 [474]. Applying data from the Jenner Institute standardised NANP ELISA on the RTS,S SMC samples, anti-NANP IgG titres returned to 80% of those observed at the peak. Finally, anti-NANP IgG titres from a boost of R21/MM in VAC076 saw titres return to 90% of that seen at the peak - Figure 96.

Anti-NANP IgG data from the second boost of RTS,S showed that antibody levels only returned to 61% of the levels seen at the peak. A second boost of R21/MM produced anti-NANP IgG titres of 66% when compared to the peak of the primary series. Anti-NANP IgG titres after a third boost of R21/MM in Burkinabe infants were measured and yielded anti-NANP IgG titres only 55% of those of the peak in the primary series - Figure 96.

14.4.4 Adjuvants AS01 and Matrix M produce comparable antibody titres with the same immunogen.

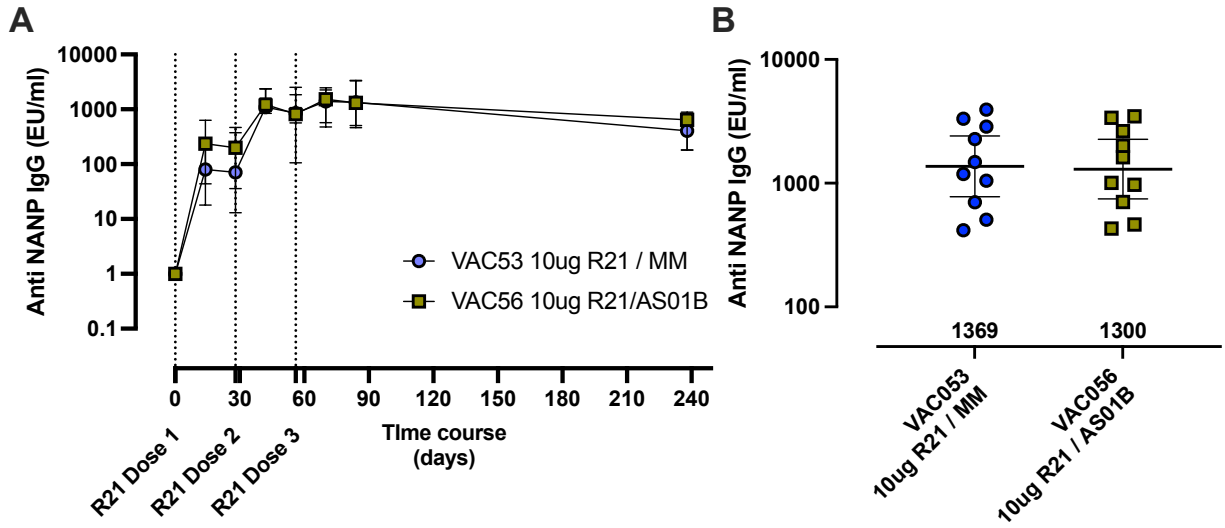


Figure 97 - Matrix M and AS01 anti-NANP IgG.

A) *Median (error bars indicate IQRI) anti-NANP IgG time courses after 3 doses of 10µg R21 with either 50µg MM (VAC053) or 50µg AS01B (VAC056) in malaria naïve adults in the UK.* ***B)*** *Geometric means of anti-NANP IgG (error bars indicate 95% CI) between 10µg R21 with 50µg MM and 50µg AS01b at 1 month post third dose. No significant difference as tested by Mann-Whitney.*

Values presented above the x-axis indicate a 95% CI.

VAC053 and VAC056 trialled malaria naïve adults in the UK immunised with R21 and with adjuvant MM and adjuvant AS01b, respectively. Trial designs have previously been outlined in Chapter 1. Comparing anti-NANP IgG responses in adults immunised with 10µg R21 with either 50µg MM or 50µg AS01b have comparable overlapping time courses - Figure 97A. Comparing peak responses at one month third dose showed comparable peak responses between 10µg R21 with 50µg MM (1369EU [95% CI 777 – 2415]) and 50µg AS01B (1300EU [95% CI 746 – 2266]) with nearly identical confidence intervals - Figure 97B.

14.4.5 RTS,S and R21 induce high anti-HBsAg titres after 3 doses compared to baseline

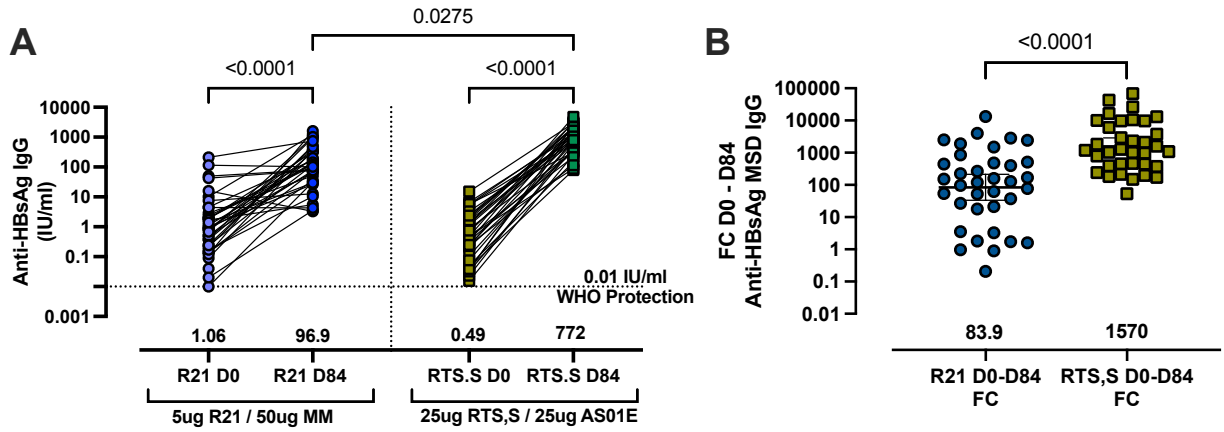


Figure 98 - HBsAg responses between R21 and RTS,S.

A) *Anti-HBsAg IgG at baseline (D0) and 1-month post-third dose (D84) for R21/MM (left) and RTS,S/AS01$_E$ (Right). P-values are presented for Kruskal-Wallis corrected with Dunn's multiple comparisons. The horizontal dotted line (Y=0.01) represents the WHO threshold of protection = 0.01IU/ml [442].* ***B)*** *FC of HBsAg responses between 1-month post third dose (D84) and baseline (D0) for R21/MM (left) and RTS,S/AS01$_E$ (Right). P<0.0001 Mann-Whitney.*

Data is geometric means with error bars indicating 95% CI. Values presented above the x-axis are geometric mean values.

Anti-HBsAg IgG titres for both R21/MM and RTS,S/AS01$_E$ samples were measured by MSD multiplex serology plates at baseline (D0) and one month post-third dose (D84). At baseline, all infants for both R21/MM and RTS,S/AS01$_E$ had anti-HBsAg titres above the WHO protection threshold of 0.01IU/ml [442] - Figure 98A. All Burkinabe infants across both trials had a significant increase in HBsAg IgG titres at one month post-third dose. Furthermore, infants who received RTS,S had an anti-HBsAg titre of 772IU/ml [95% CI 534 – 1115] and infants who received R21/MM 96.9IU/ ml [95% CI 55.0 – 171] - Figure 98A. Infants who received RTS,S had a high fold increase (baseline to 1 month post third dose) in HBsAg IgG titres of 1570 and infants receiving R21/MM had a fold increase of 83.9 - Figure 98B.

14.4.6 Functional Antibodies induced from R21 and RTS,S

Plasma from infants in VAC076 was tested for functional antibodies induced from R21/MM. ISI data presented in Figure 44 has been evaluated in Chapter 1 (11.4.4.5).

14.4.6.1 RTS,S induces functional antibodies that can block sporozoite invasion

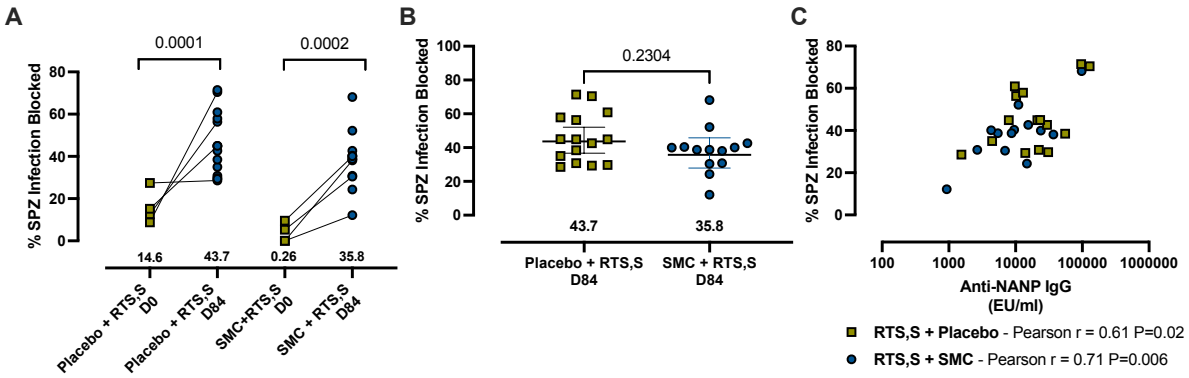


Figure 99 - RTS,S inhibition of sporozoite invasion.

A) *Percent increase of ISI among Burkinabe infants pre and post-vaccination from both vaccinated groups of RTS,S only and RTS,S + SMC in Burkinabe Infants. P=0.0001 & P=0.0002 Mann-Whitney with Bonferroni's correction for multiple comparisons.* ***B)*** *Percent increase of ISI among Burkinabe infants between 1 month post third dose between RTS,S vaccinated groups. P=0.23 – Mann-Whitney. Lines represent GMT with error bars indicating 95% CI.* ***C)*** *Correlation between per cent ISI and anti-NANP IgG at D84. Correlation calculated with Pearson r.*

Values presented above the x-axis indicate geometric means.

RTS,S SMC immunised infants were assessed for functional antibodies induced using the ISI assay. After unblinding, four of the baseline samples and fifteen of the D84 samples were part of G1 (RTS,S only). The remaining four baseline samples and thirteen D84 samples were part of G3 (RTS,S + SMC) - Figure 100.

Infants in G1 (RTS,S only) had a 14.6% mean ISI at baseline and all infants in G1 had an increase in ISI at D84 to a mean of 43.7% - Figure 99A. Infants in G3 (RTS,S + SMC) had a mean baseline ISI of 0.26% and all infants in G3 exhibited an increase in ISI at D84 to 35.8% - Figure 99A. No significant difference (P=0.2) between RTS,S groups was observed - Figure 99B. Both RTS,S groups' ISI percentages were correlated with anti-NANP IgG titres. G1 (RTS,S only) had a significant positive correlation (Pearson r = 0.61, P=0.02) between ISI and anti-NANP IgG titres. G3 (RTS,S + SMC) also positively correlated

(Pearson r = 0.71, P=0.006) between ISI and anti-NANP IgG Figure 99C, suggesting that an increase in anti-NANP IgG is highly associated with increased % ISI.

14.4.6.2 R21 and RTS,S induce functional antibodies measured by ISI

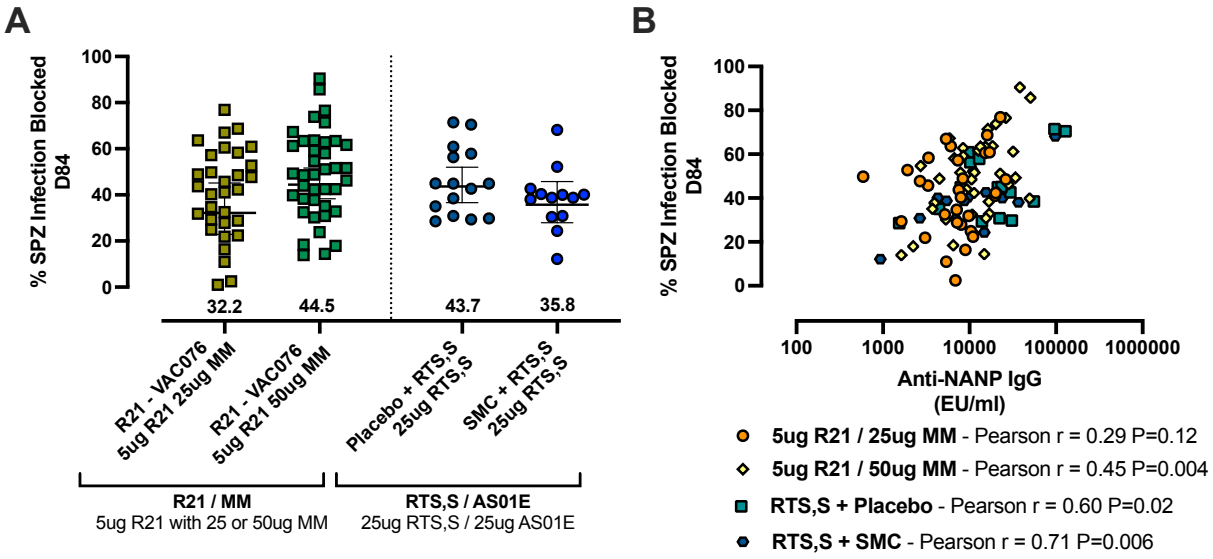


Figure 100 - % ISI induced from RTS,S and R21 in Burkinabe infants.

A) *Percent increase of ISI among Burkinabe infants at 1-month post third dose between RTS,S and R21 vaccinated groups. Geometric means with error bars indicating 95% CI.* ***B)*** *Correlation between percent ISI and anti-NANP IgG at D84. Correlation calculated with Pearson r.*

Values presented above the x-axis indicate geometric means values.

ISI data generated from samples immunised with R21/MM and RTS,S/AS01$_E$ were evaluated in two experiments approximately nine months apart. As mentioned in Chapter 1, assay variability is a problematic factor.

Both R21/MM and RTS,S/AS01E induced similar levels of ISI in Burkinabe infants with R21/MM (5µg R21/50µg MM) inhibiting 44.5% (95% CI 38.3 – 51.6) of sporozoite invasion and RTS,S (25µg RTS,S / 25µg AS01$_E$) with 43.7% (95% CI 36.7 – 52.0) – Figure 100A. Both R21/MM and RTS,S/AS01$_E$ % ISI percentages positively correlated with anti-NANP IgG - Figure 100B.

14.5 Chapter 4 - Discussion

14.5.1 Seasonal vaccination

Many sub-Saharan countries have a highly seasonal malaria transmission, with the main burden of malaria occurring within the three to four months of the rainy season and the period immediately afterwards. SMC was developed to take account of the epidemiology of malaria in these areas and has proved very successful, providing a high level of protection. A study presented here (MAL099) assessed whether the protective efficacy of SMC could be enhanced by combining SMC with seasonal vaccination with the RTS,S/AS01$_{E.}$ During the initial three years of the trial, combining the RTS,S/AS01$_{E}$ malaria vaccine with SMC gave 50-70% enhanced VE over either intervention given alone for a range of trial endpoints including asymptomatic malaria infection, clinical attacks of malaria, severe malaria requiring hospital admission and deaths from malaria. Chapter 3 of this thesis reported high VE percentages from R21/MM with VE CIs that overlap with the Phase 3 (MAL099) study of RTS,S. However, comparing VE from two different studies should be avoided due to many confounding variables and can erode public confidence in vaccines.

This chapter aims to present immunological differences found from R21 and RTS,S vaccinated plasma samples both of which used a seasonal immunisation regimen with multiple boosters administered in yearly intervals. The purpose of this chapter is not aimed at proving one vaccine is better than the other but rather to further our knowledge of the immune responses induced from both R21 and RTS,S under similar dose regimens and when administered in a seasonal vaccine setting. An increased immune knowledge will help guide future vaccine programmes and potentially improve second or third-generation vaccine programmes. Immunological differences presented do not provide any real evidence for differences in protection but both show R21 and RTS,S are immunogenic, and published papers provide details of safety and efficacy.

14.5.2 ELISA

Immunogenicity on R21 and RTS,S was reported using the standardised Jenner NANP ELISA providing antibody data to the 6 NANP repeats (NANP6) of the CSP. R21/MM contains 19 NANP repeats [277, 278], RTS,S contains 18 NANP repeats [246, 306] and the polymorphic CSP contains 18-38 NANP repeats [508]. Using NANP6 peptide allows for accurate detection of NANP IgG responses induced from R21 and RTS,S vaccines. The Jenner NANP ELISA assay is robust and provides accurate anti-NANP IgG data from several anti-CSP based vaccines such as RTS,S/AS01b trials [420, 422, 425], viral vectors ChAd63, and MVA encoding full-length CSP [302], and R21/MM trials [2, 3, 404, 509].

Using the Jenner standardised NANP ELISA, anti-NANP responses from R21/MM and RTS,S in Burkinabe infants measured one month after the first and second boost are almost identical with overlapping CIs. The initial peak response was higher for R21 after the primary series compared with RTS,S but the addition of a booster dose of both vaccines saw antibody titres returned to levels seen at peak. High antibody titres are found to be associated with increased protection for both vaccines. In addition, a booster dose at 12 months returns antibody titres to peak levels seen in the previous year. Furthermore, the timing of when the booster dose is administered has an important role. A booster dose of RTS,S at 12 months had improved immunogenicity (Figure 96) and efficacy compared to boosting at 18months [248]. Which has now been used by the WHO in their policy recommendation for dosing schedules of both R21 and RTS,S [235].

14.5.3 Antibody durability

In VAC076, VE in the second year appeared to improve from the first year (77% [95% CI 67-84]) to 81% [95% CI 66-87] [2, 3]. Chapter 3 suggests an increased VE because of improved durability of antibody titres 12 months after the boost compared to 12 months post primary series. The RTS,S/SMC trial

reported a slight reduction in VE from the first year (71.7% [95% CI 63.8-77.8]) to the second year (63.2% [95% CI 56.8-68.6] although CIs overlap.

Here we see higher antibody durability among R21 vaccinated infants than RTS,S vaccinated infants. Although VE efficacy in VAC076 was assessed from Phase 2 (n=450) and the RTS,S/SMC was a Phase 3 (n=6861) with increased statistical power. In addition, immunology blood samples were taken from each infant at key time points therefore a paired analysis could be performed. In the RTS,S SMC trial, infants were re-randomised to have immunology bloods taken each year therefore samples taken in year two could not be directly compared to year one however an association can be made. Anti-NANP responses were significantly higher in infants immunised with R21 at twelve months post primary series and post boost when compared to RTS,S. This improved durability will be investigated further to evaluate its role in sustained protection and help reduce epidemiological consequences such as delayed malaria from occurring.

14.5.4 Perennial transmission

In areas with more perennial transmission, VE was lower within Phase III studies of RTS,S than seasonal administration. More recently VE of R21 (68% [95% CI 61-74]) in perennial sites [280] appear to be higher than perennial sites of RTS,S. However, Phase 3 testing after a single booster is yet to be reported but will likely produce comparable efficacy considering reboosted vaccine responses.

14.5.5 AS01 vs Matrix M

Both AS01 and Matrix M (MM) incorporate liposome formulations from purified saponin [242]. The saponin in Matrix M is formulated in cholesterol and phospholipid making a nanoparticle [510] whereas AS01 is formulated with MPL [242]. It is useful to assess whether differences in immunogenicity to the CSP are attributed vaccine or the adjuvant. To assess immunogenic differences between adjuvants, R21 administered with AS01 adjuvant was compared to R21 administered with

MM from VAC053. Data suggests that using the same immunogen (10µg R21), adjuvants produced comparable immunogenicity. However, this study was limited by small sample sizes (n=10) and greater sample sizes and tresting in infants living in malaria endemic areas will be needed to confirm this result. Therefore, any differences in immunogenicity could be attributed to different CSP to HBsAg ratios however as indicated in chapter 3, HBsAg does not interfere with CSP responses. Lastly, RTS,S has five times the dose of immunogen (5µg R21 vs 25µg RTS,S) but half the dose of adjuvant (50µg MM vs 25µg $AS01_E$) when administered to infants [245]. Further comparability studies of doses and adjuvant are needed.

14.5.6 HBsAg

HBsAg responses from infants immunised with RTS,S and infants with R21 were directly compared using the novel MSD multiplex assay at the Jenner Institute. Anti-HBsAg titres amongst RTS,S immunised infants were significantly higher than anti-HBsAg from R21/MM immunised infants. This finding can be attributed to the four-fold higher ratio of unbound HBsAg and the higher dose used as mentioned above. However, there is no direct evidence to indicate whether increased or decreased HBsAg responses impact immunogenicity or efficacy against malaria.

14.5.7 HBsAg responses differing from published work

The MSD multiplex assay reports anti-HBsAg titres in international units per microlitre (IU/ml), therefore units reported should be comparable to publications using the same scale. In this chapter (Figure 98) anti-HBsAg titres for RTS,S samples from MAL099 have a GMT 772IU/ml. However, Valea *et al.,* 2018 [511] reported anti-HBsAg titres in infants across multiple RTS,S/$AS01_E$ Phase II trials in Africa of 6412.7mIU/ml at one-month post-third dose (D84) which is equivalent to 6.4127IU/ml. In addition, Macete *et al.,* 2006 [512] reported RTS,S/AS02A anti-HBsAg IgG titres among children aged 1-5 years as 10386.8mIU/ml, equivalent to 10.386IU/ml. Conversion of units should equate to similar titres among infants immunised with RTS,S irrespective of the assay used. Furthermore, Valea *et al.,*

2020 [513] reported that anti-HBsAg measured at one-month post boost increased by 70-fold as compared to the one-month post-primary series. Therefore HBsAg IgG data produced using MSD cannot be made to other publications [511, 513, 514] but HBsAg responses between R21 and RTS,S using the platform show increased responses from RTS,S.

14.5.8 % ISI

Using the ISI assay, we show that both R21 and RTS,S induce functional antibodies that can block sporozoite invasions *in vitro*. However as mentioned in the previous chapter, due to high inter-assay variation samples run in different experiments cannot be accurately compared, despite appearing comparable in the functional data presented here. If more time was available, samples would be assessed be assessed in the same experiment for functional antibodies to block sporozoite invasion.

14.5.9 Comparing VE

Directly comparing vaccine efficacies can have negative implications on vaccine demand irrespective of the positive public health impact they produce. In COVID-19, it was incorrectly reported that the Oxford/AstraZeneca (ChAdOx1-nCov-19) vaccine was only 8% effective in people over the age of 65, leading to a widespread erosion in confidence in the vaccine, resulting in individuals opting to wait until another vaccine was available [515]. At a time when governments were desperately trying to protect their populations and the vaccine supply was limited, this was a major setback. This cannot be repeated with RTS,S or R21 when vaccine supply is limited and demand is high at a time when morbidity and mortality are increasing again.

14.5.10 Limitations

Limitations in this chapter are the small sample sizes and the limited number of assays that were able to be assessed. While SMC was shown to have no interference in immunogenicity, the amount of SMC administered to each infant differs between MAL099 (4 rounds) and VAC076 (1-4 rounds) making

comparisons on immunogenicity and potential associations with protection difficult to do. Further observations on immunological differences cannot be determined whether it is vaccine design (increased unbound HBsAg), dose (5µg vs 25µg) or adjuvant (AS01 or MM). Lastly, clinical efficacy data was not available to be able to stratify immune respnses by protectoion induced by RTS,S to assess any associations.

14.6 Chapter 4 - Conclusion

Data presented in this chapter provides evidence that three doses of R21 and RTS,S vaccine induce high antibody responses which peak one month after the third dose and with the addition of a booster dose twelve months see antibody titres return to levels seen at peak. R21 has observed high antibody durability at 12 months after the first boost compared to primary series providing possible explanations for sustained VE. R21 VLP has a smaller ratio of CSP to HBsAg resulting in a reduced anti-HBsAg response than RTS,S however there is no evidence to suggest this impacts VE. Both vaccines elicit high HBsAg response above a protective antibody threshold set by the WHO, meaning both vaccines can be marketed as a dual vaccine for both malaria and hepatitis. RTS,S pre-qualification from the WHO is a major step forward in combatting malaria. R21 now given a positive recommendation from the WHO has shown durable antibody titres and can be manufactured at scale with reported reduced cost that would meet world demand [516], making R21 a promising new vaccine that could join RTS,S in pre-qualified vaccines to combat *Pf* malaria.

15 Overview, Future Directions and Conclusions

15.1 Overview

A vaccine targeting *Pf* remains a significant prospect in reducing malaria morbidity and mortality [220]. Global mortality from malaria has seen a 60% reduction in deaths since 2000 [517] however the global burden of malaria has not changed, with 247 million cases worldwide reported in 2021, the same number as in the year 2000 [93]. Further, the reduction in the number of *Pf* deaths has stalled at ~619,000 despite significant investment in malaria intervention methods ($ 2.7bn in 2017 alone) [518]. In the last 20 years, US$30 billion has been invested in various malaria intervention measures such as the distribution of 2.5 billion ITNs, 3.5 billion ACTs and 3 billion RDTs [519], thus, new interventions such as vaccines against multiple malarial life cycles must be developed to reduce overall morbidity and mortality [520].

This thesis aimed to investigate the immune responses induced from different dose regimens of the novel malaria vaccine R21 and adjuvant Matrix M among different age groups and malaria endemic backgrounds. This project provides a detailed account of the first 6 Phase I and II clinical trials on the development of R21, and how the immune responses induced contributed towards the optimal dose regimen chosen for Phase III assessment in African infants, who carry the highest disease burden of malaria [335]. The immunology presented tries to disentangle what immune responses are most likely causing protection in our vaccinated participants and whether these responses correspond with literature from other anti-sporozoite vaccines.

All immunology data reported in this thesis were carried out after extensive assay optimisation to improve performance, efficiency, and reproducibility. Optimisation is not presented or discussed due to word limit constraints. The key findings from this project are discussed below.

15.2 Summary of findings

15.2.1 Characterising dose responses to R21/MM in different

The first trials R21 were conducted in malaria naïve adults in the UK (VAC053) and Burkinabe adults indicated that R21 was immunogenic and produced antibody titres similar to those which provide protection in other anti-CSP vaccines (RTS,S/AS01B) [420, 422]. Additionally, antibodies taken from participants saw a functional ability to block sporozoite invasion assessed by ISI. Furthermore, data showed the necessity of an adjuvant to be to be used to promote immune responses consistent with data from other subunit vaccines [404]. Later investigations of adjuvants showed no difference in antibody titres from adults immunised with MM and AS01.

A three-dose regimen of 10µg R21 administered 1 month apart (standard dose regimen) was then assessed to see whether it could provide protection in CHMI. The standard dose regimen provided high-level efficacy (VAC065 G1 – 80% [95% CI 60-94] and appeared superior to a two-dose regimen (VAC065 G7 – 65% [95% CI -8-89]) although small sample sizes and large confidence intervals provide insufficient statistical power to make any meaningful conclusions. A fractional (one-fifth) third dose did not improve immunogenicity or efficacy compared to the standard dose regimen in contrast to other vaccines.

In 2019, a deal was made with SII to continue producing R21 at scale for future Phase II/III trials. New manufacturing techniques led to $R21_{SII}$ being produced without a C-tag and ultimately new vaccine. The standard dose regimen of R21SII produced comparable VE (VAC072 G3 – 66% [95% CI 35-85]) to $R21_{CBF}$ and anti-NANP IgG titres. Anti-C term titres were significantly higher with $R21_{SII}$, however, this was concluded as false and uniquely due to comparable NANP and C term titres measured by the new multi-plex assay from MSD.

A delayed third dose (VAC072 G2 – 10, 10 delayed 10µg R21) delivered at five months improved immunogenicity however VE had overlapping CIs as compared to adult standard regimen. A delayed fractional third dose (one-fifth of 2µg R21) significantly improved immunogenicity compared to the standard and delayed dose regimen however these groups were not challenged due to logistical issues associated with the COVID-19 pandemic. Many vaccines, and not just those for malaria, have shown exciting data with delayed and delayed fractional third dose however, using these regimens in African infants with RTS,S has failed to improve efficacy over the standard three-dose regimen. Administering a delayed fractional third dose of RTS,S in infants in an African field study resulted in reduced efficacy and increased severe malaria compared to the standard three-dose regimen [454]. This has led to the speculation that delayed fractional doses may work best in adults who have more mature immune systems to effectively promote GC activity [521].

R21 was next evaluated in malaria-endemic infants and Kenya following an age de-escalation/dose escalation in adults and young children. Infants, immunised with the high dose matrix M (5µg R21 – 50µg MM) induced the highest immune responses irrespective of the dose of R21. Providing further conclusions on the effectiveness of Matrix M.

A field efficacy trial involving three doses of R21 (5µg) with either 25µg or 50µg MM (VAC076 G1 and G2) was evaluated in infants living in a highly seasonal endemic area in Burkina Faso. Three doses of R21/MM administered seasonally induced high levels of protection in both groups ((G1 - 74% [95% CI 63-82]) and (G2 - 77% [95% CI 67-84])) respectively. These high efficacy results meet the WHO malaria roadmaps set out in 2006 (VE>50%) and 2012 roadmap (VE>75%) [223]. As with Kenyan infants, the higher dose (50µg) MM in Burkinabe infants had significantly higher immune responses compared with the low dose group most notably total IgG to both NANP and C term, as well as increased in IgG1 and IgG3. Corresponding with protective immune responses found in other literature sources.

Antibody levels did wane twelve months after the primary series in infants. However, a booster dose administered at twelve months saw antibody titres rebound to levels seen at the peak in all infants and led to sustained high VE 81% [95% CI 66-87] at 24 months in Burkinabe infants. Malaria naïve adults who also received a boost and underwent Re-CHMI, found antibody titres significantly increase resulting in 8/9 boosted adults to be protected and very high functional antibodies at preventing sporozoite invasion. Suggesting a booster dose is highly effective in sustaining immune responses and protection against *Pf*.

VAC076 was extended to assess whether further booster doses were needed. Vaccinated groups were re-randomised in the third year to receive a booster (G2a) or placebo (G2b) at 36 months high VE ((G2a - 60% [95% CI 45-78]) and (G2b - 64% [95% CI 44-79]). Similar VE and overlapping CIs suggest only a single booster may be needed to provide protection. However, group sample sizes have been reduced further providing hesitancy on the validity of results. Nevertheless, an exciting result prompting further assessment in Phase III. Non-boosted groups show no indication of increased malarial episodes or of severe malaria indicative of sustained immunity preventing delayed or rebound malaria. The trial protocol will ensure all infants in VAC076 maintain access and use of other malaria interventions such as SMC and ITNs and be monitored for surges in delayed or rebound malaria.

15.2.2 R21 performance in different malaria endemic areas and age groups

This thesis aimed to examine whether R21 performed differently in individuals living at different levels of transmission. Often individuals living in sub-Saharan Africa have reported reduced immune responses to vaccines compared with western regions. Often a vaccine looks promising in initial trials but when taken into an African setting has reported worse responses. This can be attributed to greater exposure to a wider range of pathogens such as CMV [522], HIV [187, 188], Helminth infections [523], Anaemia [524] and malnourishment [525] which are known to result in reduced immune response to

vaccination. Constant malarial re-infection can lead to atypical memory B cells as well as B and T cell exhaustion [202] resulting in less effective downstream immune responses. Individuals who are infected at the time of vaccination can negatively influence immune responses from PEM vaccines [526]. Quantifying blood stage immunity as a marker of exposure and correlating it with immunogenicity induced from R21 would be useful to understand the effect of pre-exposure on R21 despite comparable immune responses from different sites.

R21 was examined in the UK, Kenya, and Burkina Faso and produced comparable immune responses when given the standard adult dose regimen of R21. Furthermore, infants in Kenya or Burkina Faso found comparable immune responses from comparable dose regimens. Comparable immune responses across wide age ranges of participants and different levels of malaria exposure is an encouraging result which will assist regulators in approving the use of a vaccine in different countries and avoid the need for individual trials across different transmission settings to assess vaccine effectiveness. However, sample sizes are small with many potential confounding variables.

Antibody titres reported between adults and infants were significantly different. Adults who are found to be sterilely protected have antibody titres approximately 7-10 fold less than infants. It is widely considered that an increase in antibody titres is associated with protection [236], one cannot ignore the difference in antibody concentration therefore other immunological mechanisms are driving protection. Infants have immature immune systems compared with adults [14] who produce a greater immune quality over quantity and may be important [456], therefore the amount of immune maturation is a factor when designing vaccine dosage [527].

15.2.3 Identifying immune mechanisms of protection

Antibody durability contributing to duration of protection is a key determinant in vaccine design. Both R21 and RTS,S induce high antibody responses that decline quickly leading to seasonal vaccine

regimens along with SMC [528-530]. A booster dose returns high antibody titres but antibodies do continue to wane. Both Burkinabe and Kenyan infants immunised with R21 showed improved durability six- and twelve months post-first boost compared with the primary series. Furthermore, infants with increased durability saw fewer malaria episodes indicating durability is associated with increased protection.

A booster dose improves GC activity over the primary series leading to improved LLPCs and B cell production leading to higher affinity antibodies [479]. This improved durability resulted in a substantial increase in antibody half-life in Burkinabe infants. In addition, adults who received a boost showed a significantly higher immunogenicity and improved efficacy than malaria naïve adults who did not receive a boost Re-CHMI. A booster dose also saw significant increases in antibody avidity compared to the primary series in both adults and infants. In addition, a delayed and delayed fractional third dose found increases in IgG avidity particularly to the C term sequence of R21 and improved functional activity of ISI *in vitro*. Suggesting that the delay and increased timing of doses results in better germinal centres priming/activation and increased engagement of memory B cells and LLPCs [103] and ultimately promoting an improved antibody quality over quantity [236]. Significant increases in C term avidity over NANP are interesting and not seen in other CSP-based vaccines. C term avidity has been shown to correlate with protection in certain studies [358] further promoting the C term involvement in mediating protection.

However, no association with avidity and protection was found indicating avidity induced by R21 measured by sodium thiocyanate a poor immunological assay to associate with protection despite being observed in protection in studies involving RTS,S [358, 365]. Although the sensitivity of the assays used in those studies was likely much greater and therefore better to find associations with protection.

It can be concluded that all samples from participants immunised with varying doses of R21 and Matrix M have antibodies that can block the invasion of human hepatocytes. All participants had a % ISI increase between baseline and post-vaccination. The ISI assay supports dose regimens that induce the highest antibody titre however the assay is unable to predict protection which has been seen in other studies using the ISI assay at the Jenner Institute, possibly due to the assay being influenced by antibody concentration irrespective of other factors such as avidity or antibody subclass or isotype. Antibody titres are likely saturating each well, preventing invasion irrespective of the quality of antibody response. The assay requires serum to be diluted to 1%, lower than other studies - 10% [425] and 20% [413] using invasion assays. A possible solution would be to normalise the antibody concentration based on antibody titres using a similar method to how samples for avidity are calculated to ensure the same antibody concentration in each well resulting in samples with higher antibody titres that do not saturate the well. Further evaluation to improve the assay may be conducted in the future.

For CSP-based vaccines, no clear immunological parameter consistently correlates with protection. However, there is a trend that larger antibody titres to various CSP proteins are associated with protection [531]. This trend is reported in this chapter in that both malaria naïve adults and Burkinabe infants who are protected have statistically greater anti-NANP and anti-C term IgG titres compared with non-protected.

In malaria naïve adults, an anti-NANP IgG titre of 1100EU and anti-C term IgG titre of 1000EU significantly associated with protection was found. This association can be characterised as an nCoP found in malaria naïve adults immunised and is a working baseline for future R21 or CSP-based vaccines to achieve. However, this is not a definitive mCoP, as several adults with some of the highest antibody titres were not protected, suggesting these values only give a likelihood of protection. Secondly, the analysis is very rudimentary and done using a post-hoc approach of arbitrarily selecting

a titre that found a significant association. Nevertheless, protection observed in all UK adults suggests a step forward towards using R21 as a travellers' vaccine [450].

A threshold of protection was found using anti-NANP IgG responses in Burkinabe infants in VAC076. In the first year, it was found that 77% of infants who had anti-NANP IgG titres greater than 6618EU and in the second year 81% of infants who had anti-NANP greater than 6130EU were significantly associated with protection [3]. Overall, infants with higher antibody titres had fewer episodes of clinical malaria. Lastly, infants who had a greater decline of antibody responses after vaccination were not shown to have an increased risk of clinical malaria or multiple episodes. This questions whether the antibody threshold reported by Datoo *et al.*, 2022 [3] may be too high to accurately associate with protection. This is supported further in that efficacy was maintained in the third year in infants who did not receive a boost and antibody titres were significantly lower than boosted, yet those infants had similar levels of protection to infants who were boosted. This Indicates that long-lived antibodies induced in the primary series and boost may be enough to sustain protection.

A multivariate analysis undertaken in this thesis confirmed that anti-NANP IgG responses remained the most important marker to be associated with protection. Confirm NANP as the immunodominant epitope. Subclass IgG1 and IgG3 antibodies were significantly increased in protected participants in both malaria naïve adults in the UK and Burkinabe infants, however did not meet the inclusion criteria to be included in the multivariate model. This finding follows similar patterns to protective antibodies found after immunising with RTS,S/AS01 [90, 164, 452]. The UK adult data was severely underpowered due to the large ratio of protected to unprotected participants. A commensurate increase in sample size, especially in the unprotected group, may allow for further associations to be made.

15.2.4 Examining immunogenicity of R21 and RTS,S

R21 and RTS,S while biosimilar, differ in small ways such as the number of NANP repeats, the 4-fold excess of unbound HBsAg and the adjuvant used. Comparable efficacy is found between the two

vaccines after 1 year when administered seasonally with SMC suggesting comparable performance as indicated by similarly high peak titres. In the second year, efficacy from R21 appears to improve which can be attributed to improved durability 12 months after the boost over the primary series. This was not seen with RTS,S however this is insufficient substantive evidence to support the notion that increased durability corresponds with increased protection however one can conclude that R21 induces very durable antibody titres. Finally, anti-HBsAg responses were significantly greater in RTS,S vaccinated samples compared to R21, likely due to increased dose (25µg RTS,S vs 5µg R21) or the 4-fold excess unbound HBsAg. However, there is no evidence to indicate HBsAg interfere with CSP responses. But both vaccines can be labelled as successful hepatitis B vaccines which induce antibody titres well over the WHO-required protective threshold.

While making such comparisons is valuable to our understanding of immune differences produced, it could produce problematic concerns of vaccine favouritism or individual selection of what vaccine to get. A problem when vaccine supply is limited. This was seen during the COVID-19 pandemic when a German newspaper incorrectly reported that the Oxford/AstraZeneca vaccine had decreased immunity in older adults. This led to vaccine hesitancy and dose wastage due to many people preferring to wait until their preferred vaccine was available. This must not happen for malaria vaccines, both RTS,S and R21 have shown protective VE and any comparisons made must be done carefully when demand is high.

15.3 Future implications

15.3.1 WHO approval

At present, only the RTS,S/AS01E vaccine has been given a positive scientific opinion from the EMA 2016 [230], a positive recommendation in 2021 [231] and pre-qualification status from WHO in 2022 [232]. Recently R21/MM has been given a positive recommendation by the WHO [235]. A positive recommendation is given after an extensive review of safety, immunogenicity and efficacy data giving

individual countries confidence to purchase vaccines. Pre-qualification occurs after a second review of data by the WHO and allows funding from organisations such as GAVI or UNICEF to be made available for less economically developed nations to purchase vaccines [532].

15.3.2 Distribution

Manufacturing limitations result RTS,S supply falling short of world demand. A second vaccine R21 received a positive recommendation after comparable safety and efficacy with RTS,S reported that increased manufacturing supply can help to close the gap in terms of world supply and demand. It is expected that R21/MM will be a third of the cost of RTS,S [516] and seven times the cost of PfSPZ (without cold chain requirements) [533]. With the collaboration of SII, who have committed to producing 100 million doses per year [534] of R21/MM, with capabilities of increasing if needed. Lastly, R21/MM is being supplied as a single vial formulation restricting the need for the vaccine to be mixed on site reducing any potential mistakes as well reducing storage capacity with two vial formulations.

RTS,S is expected to be rolled out in early 2024 and R21 by the middle of 2024 [535]. Secondly, policy for both R21 and RTS,S follows the same dose regimen, making it very easy for countries to adopt the two vaccines into their vaccination schedule if supply is an external circumstance that influences vaccine production from a single site.

15.3.3 Multi-stage vaccines

For malaria elimination to effectively occur, we will likely need a malaria vaccine that targets all stages of the malaria lifecycle. Blood stage and TBVs still have yet to provide sterile protection, nevertheless identifying methods to combine them should be planned with an optimal delivery strategy for each vaccine to ensure vaccine performance. A combination vaccine against Plasmodium could be effective and provide possible additive or synergistic effects on VE [536]. However current malaria combination

regimens in humans have not been effective [537]. Vaccines need not interfere with each other from antigen competition [538]. Interference trials of co-administered vaccines need to be assessed such as same or different sites (e.g. opposite arms), mixed or given staggered at different time points. Yet, combination regimens are possible as vaccines such as the measles, mumps and rubella (MMR) vaccine and the diphtheria, pertussis and tetanus [DPT] vaccine, as well as many others in the EPI schedule [539].

RTS,S and R21 are set to be administered with countries' EPI vaccine infrastructure. This is needed to ensure vaccination uptake and coverage is high as well as having monitoring programmes and broad surveillance systems for case detection in place [530]. RTS,S has extensive safety data and with EPI vaccines such as yellow fever, measles, mumps and rubella need to be performed [540] therefore R21 will need to be monitored closely due to the much smaller interreference data.

15.3.4 Assay validation

The standardised NANP ELISA was the primary immunological endpoint for the six R21 studies presented in this thesis. This assay is now being replaced by the multiplex assay from MSD. During the initial months of my DPhil, I undertook extensive assay optimisation and validation however due to word limitations, I have not presented but aim to publish. This will be valuable for the validity of the data that is already published [2, 3] and the data in this thesis. The experiments undertaken in this thesis fulfil the ICH criteria of precision, linearity, stability and robustness that are adopted by the regulatory authorities for assay validation [541]. Having a validated standard assay is a key goal of the vaccine technology roadmap of 2006 [542].

15.3.5 Limitations

The high efficacy produced in the first two years of the Phase II trial in Burkina Faso is as a result seasonal vaccination regimen. This has been criticised as unrepresentative of vaccine performance in

countries with year-round transmission. From a logistical standpoint, it may be impractical to attempt to vaccinate all infants under the age of two with three doses in communities that have been identified where seasonal vaccination will be effective [377]. Phase III results of R21 show comparable efficacy between seasonal and perennial vaccination sites [280], aiding the WHO's positive recommendation to align R21/MM with existing infrastructure EPI programmes [543] as is used with RTS,S. However, a limitation of the R21 Phase III is that it does not provide VE among high perennial malaria locations and only low perennial transmission settings and high seasonal settings (Phase 3). This was an important result found in the Phase III RTS,S trial where high perennial transmission saw reduced point VE estimates [248].

16 Final remarks

I believe the work presented in this thesis adds an important body of knowledge to the malaria and vaccine field. For future vaccines against malaria and other infectious diseases to be developed, a greater understanding of the protective immune responses will be needed. A variety of immunology experiments are planned (listed above) to try further disentangling and identify potential immune correlates and associations of protection,

The global response to the coronavirus pandemic has highlighted how investment and collaboration among research organisations, industry, regulatory authorities, governments, and other stakeholders can greatly accelerate vaccine development and implementation [163]. Malaria eradication will take a combined effort and while it is extremely positive that an RTS,S/AS01 malaria vaccine (Mosquirix) has been pre-qualified by the WHO. Now with R21/MM receiving a positive recommendation from the WHO will be a major addition to help improve the supply and reduce Africa's vaccine demand.

I feel immensely proud that a large portion of the data produced went towards licensure of vaccines in Ghana, Nigeria and Burkina Faso and the recent positive recommendation given by the WHO.

17 Appendix

17.1 Materials and reagents

17.1.1 Reagents

Table 24 below lists commercially available reagents and materials used in this thesis. Antibody concentrations used in flow cytometry are provided in the appendix along with their conjugated fluorochromes.

Table 24 - Commercially Available Reagents used in this thesis.

Reagent/ Material	Supplier	Catalogue Number
150ml Beakers		
15mL falcons	Sarstedt	554502
175cm2 cell culture flask	Corning	431086
25cm2 cell culture flask	Corning	430639
4-nitro-phenyl-phosphate	Sigma-Aldrich	N2765-100TAB
50mL falcons	Starstedt	547254
5mL polypropylene FACS tubes	BD Biosciences	352063
5mL polystyrene FACS tubes with cell strainer cap	BD Biosciences	352235
75cm2 cell culture flask	Corning	430641
96-well cell culture plate	Corning	3595
96-well U-bottom plates	BD Biosciences	353077
96-well V-bottom plates	Thermo Fisher Scientific	611V96
Aluminium Foil	Thermo Fisher Scientific	AKL-300-040J
Anti-C term mAB Control	MVI-PATH	000646
Anti-human IgG-AlexaFluor488	Invitrogen	A11013
Anti-human IgG-Alkaline Phosphatase (AP)	Sigma-Aldrich	A3187
Anti-human IgM-AlexaFluor594	Invitrogen	A21216
Anti-NANP mAB Control	MVI-PATH	000647
AQUA Live/Dead stain	Invitrogen	L34957
ARC beads	Invitrogen	A10346
BCIP/NBT development buffer	Europa Bioproducts	MO711A
Benzonase	Millipore	70664
Blocker Casein in PBS	Thermo Fisher Scientific	37528
Bovine serum albumin (BSA)	Sigma-Aldrich	A7906-100G
Brefeldin-A	Invitrogen	00-4506-51
Brilliant stain buffer	BD Biosciences	563794

Carbonate bicarbonate tablets	Sigma-Aldrich	C3041-100CAP
Casyton	OLS OMNI Life Science	5651808
Chambered microscope slides	Thermo Scientific	C24779
CoolCell™ FTS30	Corning™	432006
CoolCell™ LX	Corning™	432001
Cryovials	Star lab	E3110-6112
DAPI stain	Sigma-Aldrich	D9542-5MG
DEA buffer	Thermo Fisher Scientific	34064
Dimethyl-sulfoxide (DMSO)	Sigma-Aldrich	D2650
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific	12491015
Dulbecco's PBS (DPBS)	Sigma-Aldrich	D8537-500ML
Foetal Calf Serum (FCS)	Gibco	10500-064
Goat anti-human IgA-AP	Sigma-Aldrich	A9669-1ML
Goat anti-human IgG-HRP	ADI	10120
Goat anti-human IgM biotin	Sigma-Aldrich	B1265-2ML
IFNγ SA-ALP antibody kits	Mabtech	3420-25A
L-Glutamine	Sigma-Aldrich	G7513-100ML
Leucosep tubes	Thermo Fisher Scientific	15805547
Lymphoprep	STEMCELL Technologies	07851
Mouse anti-human IgG1 Fc biotin	Invitrogen	MH1515
Mouse anti-human IgG2 biotin	Invitrogen	05-3540
Mouse anti-human IgG3 biotin	Invitrogen	MH1031
Mouse anti-human IgG4 Fc biotin	Invitrogen	MH1542
Multiscreen IP ELISpot plates	Millipore	MAIPS4510
NaSCN	Sigma	251410
Nunc-Immuno MaxiSorp plates	Thermo Fisher Scientific	442404
One-Comp beads	eBioscience	01-1111-41
Paraformaldehyde (PFA)	Alfa Aesar	43368
Penicillin-Streptomycin	Sigma-Aldrich	P0781-100ML
PHA	InvivoGen	inh-phap
Phosphate buffered saline (PBS) powder	Sigma-Aldrich	P2194
PBS tablets	Sigma-Aldrich	P4417-100TAB
PBS Solution	Thermo Fisher Scientific	70011044
Rabbit anti-human IgG-HRP conjugate	DAKO	P0214
Reagent Reservoirs (Costar 4870)	Thermo Fisher Scientific	PMP-331-010C
Red blood cell lysis solution	Qiagen	158904
RPMI-1640 medium	Sigma-Aldrich	R0883-500ML
Sodium azide (NaN3)	Sigma-Aldrich	S2002
Sodium thiocyanate	Sigma-Aldrich	251410-500G
Staphylococcal enterotoxin B (SEB)	Sigma-Aldrich	S4881
Stop solution	ADI	80100
Sureblue TMB 1-Component Peroxidase Substrate	KPL	52-00-02

TMB ELISA substrate	ABCAM	171522
TMB Stop Solution	KPL	50-85-06
TMB substrate	ADI	80091
Trypan blue	Gibco	15250061
Trypsin	Gibco	12604-013
Tween-20	Sigma-Aldrich	P7949-500ML
Countess-Cell Counter Slides	Thermo Fisher Scientific	**C10312**
Dilution Plates	Greiner	780215
96 well plates for aliquots	VWR	732-2620
Titre Tops	Thermo Fisher Scientific	Z688630-1PAK
-80C Temperature logger	VWR	620-2442
Leucosep tubes	Greiner	G163290
SULFO-TAG™ Anti-human IgG] Antibody	MSD	3D3cc

17.1.2 Solutions

R0 Medium – RPMI (sigma), 1% sterile Penicillin-Streptomycin and 1% L-Glutamine (sigma).

R10 Medium - RPMI (sigma), 1% sterile Penicillin-Streptomycin and 1% L-Glutamine (sigma) and 10% heat-inactivated, sterile filtered FCS. FCS was previously tested for low reactivity and contamination (LabTech International).

D10 – Dulbecco's Modified Eagle's Medium (DMEM) - high glucose (Sigma-Aldrich, D5796), 1% sterile Penicillin-Streptomycin and 1% L-Glutamine (sigma) and 10% heat-inactivated, sterile filtered FCS. FCS was previously tested for low reactivity and contamination (LabTech International).

FACS Buffer - PBS containing 0.1% BSA and 0.01% sodium azide.

Coating Buffer - Carbonate bicarbonate tablets (Sigma, C3041) dissolved in PBS (15mM Na2CO3, 35mM NaHCO3, pH 9.3). Autoclaved for sterility.

PBS (PBS) - PBS (Sigma, P2194)

PBS/Tween (PBS/T) - PBS (Sigma, P2194) with 0.5% Tween-20.

IAVI ELISA assay buffer – PBS, FCS.

ISI Buffer 1 – Sterile PBS with 1% FCS.

ISI Buffer 2 – Sterile PBS with 10% FCS.

MSD GOLD™ Read Buffer B

17.2 Equipment

Table 25 - Commercially available lab equipment used in this thesis.

Equipment Name	Manufacturer	Model	Product Code
Cell counting Light Microscope	Leica	DMI3000-B	n/a
Centrifuge	Beckman Coulter	Allegra X12R	392302
Cell Counter - Countess	Thermo Fisher Scientific	Countess 3 Automated Cell Counter	AMQAX2000
Dissection Microscope			
ELISA Reader New	Biotek	ELx808	25-315S
ELISA Reader Old	Biotek	EL800	n/a
ELISPOT Reader	AID	AID iSpot	ELR08IFL1510229
Flow Cytometer	BD Biosciences	LSR-II	BF-LSRII
Flow Cytometer	BD Biosciences	Fortessa X-20	n/a
Fumigation Hood	ScanLaf	Mars	CF127
Incubator	Avantor	CB 170	390-1616
MSD Reader	MSD	MESO Quick Plex SQ120	n/a
Multi-(8) Channel Electronic Pipette (50 - 1000ul)	Satorious	The 8-channel Picus	735391
Pipette Boy	Intergra	PIPETBOY acu 2	155015
Plate Washer	Biotek	50 TS Washer	50TS12
QIA symphony SP system	Qiagen	QIAsymphony SP system	n/a
qPCR	Applied Biosystems	Step One Plus qPCR machine system	4376598
Single Channel Electronic Pipette (5 - 120µl)	Satorious	Picus 5 – 120µl	735041
Single Channel Electronic Pipette (50 - 1000µl)	Satorious	Picus 50 – 1000ul	735081
Cell Counter – Cacy Counter	OMNI Life Science	CASY 150µm	5651736

17.3 Investigational products

17.3.1 RTS,S

RTS,S/AS01B was developed and manufactured by GSK Vaccines. RTS,S consists of two proteins, RTS and S, expressed in *Saccharomyces cerevisiae*. RTS is a polypeptide formed from a portion of the CSP antigen from the *P. falciparum* strain NF54 fused to the amino-terminal end of the hepatitis B virus

surface antigen (HBsAg). The other polypeptide is the HBsAg expressed alone. RTS and S spontaneously assemble into mixed polymeric particulate structures, forming hepatitis B viral-like particles. A ratio of RTS to S of 1:4 was required for particles to form.

17.3.2 R21

R21, a novel malaria vaccine candidate, has been developed by the Jenner Institute at the University of Oxford [278]. R21 has a similar construct to RTS,S in that both are VLP vaccines where the central repeat region NANP and C-terminus of the CSP are fused to the HBsAg. RTS,S has a four-fold excess of HBsAg unbound to the CSP whereas R21 does not.

17.3.2.1 R21 – Manufactured at CBF

R21 is produced by combining recombinant HBsAg expressing the central (NANP – 19copies) repeat and the C-Terminus of the CSP. The R21 DNA sequence was inserted in *Pichia pastoris* yeast strain which spontaneously assembles the VLP – R21 [278, 421]. R21c was manufactured and vialed under GMP conditions at the CBF University of Oxford. R21c, ('c' being the identifier) the C-tag comprising the four amino acid EPEA sequence used as a short extension of R21 to facilitate the purification process using a camelid antibody to EPEA.

17.3.2.2 R21 – Manufactured at SII

Improvements in manufacturing at the SII have resulted in the manufacture of R21 without the 'c' identifying tag (C-tag) [544]. R21 was produced at scale at SII using a novel recombinant high-expressing yeast strain *Hansenula polymorpha from* ARTES Biotechnology GmbH. The final manufacturing process was optimised by taking multiple experimental and engineering batches of a main seed fermenter (100 L) which increases the biomass of the R21 protein. R21 is then labelled, packaged, and stored at 2-8°C until distribution.

17.3.3 Matrix M

Matrix M (MM) is a 40nm-sized complex containing the adjuvant-active saponin *Quillaja 284aponaria*, containing phospholipid and cholesterol. Quillaja saponins are triterpene glycoside substances derived from the bark of the *Quillaja Saponaria* tree [545]. Saponins are surface-active compounds with a variety of applications including in agriculture, feed, food and beverage, mining, and veterinary vaccines, and over the last twenty years have been increasingly investigated in human vaccine clinical trials. Matrix M is being developed by Novavax as an adjuvant for their new influenza vaccine adjuvanted with Matrix M[546]. Novavax are seeking licensure successfully in many countries of their COVID-19 vaccine adjuvanted with Matrix M creating a large safety profile of the use of Matrix M adjuvant [547].

17.3.4 AS01

Adjuvant System – 1 (AS01) contains two immunostimulants formulated with liposomes: monophosphoryl lipid A (MPL, 50µg, produced by GSK) and *Quillaja saponaria* Molina, fraction 21 (QS-21, 50µg licensed by GSK from Antigenics Inc). AS01 can be administered in two different doses whether administered to adults or infants respectively – AS01B (50µg) or $AS01_E$ (25µg) [245].

17.3.5 Viral vectors – ChAd63 Me-TRAP

Chimpanzee adenovirus strain 63 (ChAd63) was isolated from a captivity-born chimpanzee at the New Iberia research facility (NIRC, University of Louisiana at Lafayette, New Iberia, LA) and has been in development since 1999. Deletion of the E1 region within the viral genome rendered the virus replication-deficient [297] and subsequent deletions/modifications to E3 and E4 regions to optimise growth rate and yield [548]. ChAd63-ME-TRAP was manufactured using HEK293 cells under GMP conditions by the CBF.

The viral vector encodes for the insert ME-TRAP, which is a multiple epitope string (ME) fused to the full sequence of the *P. falciparum* T9/96 pre-erythrocytic antigen TRAP. ME consists of CTL epitopes from a variety of potentially protective antigens and is included to ensure an immune response to the vaccine in most vaccinated individuals. These epitopes include 14 CD8+ T cell epitopes and three CD4+ epitopes from tetanus toxoid, Bacille Calmette-Guérin (BCG) and *P. falciparum* CSP, (PfCSP), two B cell epitopes, (NANP) from *Pf*CSP and the adhesion motif of TRAP (TRAP-AM).

17.3.6 Viral vectors – MVA-ME-TRAP

MVA is a highly attenuated, replication-deficient strain of the vaccinia virus. This was produced by over 500 passages through chicken embryo fibroblast (CEF) cells. The viral vector expresses the same ME-TRAP construct as described above for ChAd63 ME-TRAP. Clinical grade MVA-ME-TRAP was produced under GMP conditions by Impfstoffwerk Dessau-Tornau GmbH, Rosslau, Germany.

17.4 Clinical Methods

17.4.1 Vaccination

All adult and young children participants in the studies discussed in this thesis received vaccinations via intramuscular (IM) injection into the deltoid muscle of the arm, preferably the non-dominant arm. Infant and young children participants under the age of 2 years received IM vaccinations in the anterolateral thigh. All vaccinations were administered by trained medical representatives at each trial site.

17.4.2 CHMI

CHMI whereby deliberately infecting healthy adults with malaria from infected mosquitos grown in a lab was first used in 1971 [549]. The CHMI procedure is now well-established and has been pioneered by the University of Maryland (UMB) and Walter Reid Army Institute (WRAIR) to evaluate malaria intervention methods [550, 551]. The first documented CHMI was recorded by Chulay *et al.*, 1986 [552]. However, differences between natural experimental infections made it difficult to reliably interpret the results of CHMI [550]. The lack of genetic diversity of malaria strains used in CHMI which generally only use 1 strain during infection has been reported to show differences with field efficacy results [553]. Another general difference is the parasite load during infection, which instantly delivers five infectious bites during CHMI, which may increase the parasite burden during CHMI and may not naturally replicate natural infection [554].

CHMI studies utilised by the Jenner Institute were designed in accordance with research conducted by the World Health Organisation Initiative for Vaccine Research (WHO-IVR), PATH Malaria Vaccine Initiative (MVI), European Vaccine Initiative (EVI) and United States Agency for International Development (USAID) who in 2011 produced a comprehensive guide “Standardisation of Design and Conduct of *P. falciparum* Sporozoite Challenge Trials” to conduct CHMI studies [555].

Mosquitos (*Anopheles stephensei*) were infected at the Walter Reid Army Institute, Maryland by allowing mosquitos to feed on a membrane feeder containing parasite material (fully sensitive 3D7 *Plasmodium falciparum*) plus fresh O+ red cells and A+ human serum. By-products used for mosquito infection were screened by WRAIR for *Trypanosoma cruzi* (Tc), CMV, HIV 1 and 2, HBV, HCV, HTLV, West Nile Virus, Zika Virus, Babesia and syphilis in accordance with Red Cross guidelines [556]. Mosquitos are infected with the chloroquine-resistant Nf54 strain or the 3D7 clone of *Pf*. Infected mosquitos are then transferred from WRAIR to the Sir Alexander Fleming Building, Imperial College London where CHMI takes place at Imperial Colleges' CL3 Insectary.

During CHMI, each volunteer has a net-covered paper cup containing five infectious mosquitos placed on their forearm allowing the mosquitos to bite and take up a blood meal. Infection is confirmed when five mosquitos with blood in the mosquito abdomen/midgut are dissected and sporozoite load is evaluated (graded 0 to +4; a gland rating of +2 or more, representing 10 or more observed sporozoites, qualifies as infectious). The bite-challenge procedure continues until the subject has been bitten by five infectious mosquitos. Five infectious bites ensure a high probability that all volunteers develop patent parasitaemia.

After seven days post-CHMI, participants attend clinic visits at the CCVTM, Oxford at approximately 12-hour intervals for detection of blood stage *Pf*. Diagnosis of clinical malaria was based on development of clinical symptoms (ref intro) and detection of blood-stage parasitaemia by microscopy of a thick blood smear and/or qPCR. Volunteers were treated as soon as participants reached the primary diagnostic endpoint specific to each trial and VE was calculated by time to diagnosis in comparison with time to diagnosis of unvaccinated controls.

17.4.3 Blood collection

Volunteers were enrolled onto each trial after being screened by physicians. On the day of the appointment set out by the trial design, blood was drawn from volunteers and collected in a variety of different vacutainers depending on the purpose. Lithium heparin vacutainers for PBMC and Plasma collection, plain (untreated) vacutainers for serum collection, EDTA vacutainers for qPCR analysis after CHMI and Paxgenes for RNA preservation. All blood samples were processed in under four hours from blood drawn to ensure PBMC viability and quality.

17.5 Immunological Methods

17.5.1 PBMCs & plasma separation

All cellular work was conducted under sterile conditions in a level 2 fumigation hood. Different bleed volumes were taken for different trials and time points, therefore approximately 15-30ml of heparinised whole blood was poured into one or two Leucocep tubes prefilled with 15ml of Lymphoprep suspended below the porous frit disc.

PBMCs were separated by centrifuging each Leucocep tube at 1000g for 13 mins at room temperature with no break. Using a Pasteur pipette, approximately 2-4mls of plasma was aliquoted into 2ml or 5ml labelled cryovials (trial laboratory analytical plan dependant) and frozen down for serological analysis.

The remaining plasma and PBMC layers were poured into a sterile 50ml falcon tube and topped up to 40ml with warm R0 and centrifuged at 1800rpm (764g) for 5mins at RT. After this first wash, the supernatant was poured off, the cell pellets were resuspended and combined into a single tube in in 30mls of R0 and centrifuged again at 1800rpm (764g) for 5 mins at RT. After the second wash, if clear red cell contamination was visible, RBCs were lysed for ~5mins with Red Cell Lysis Buffer. Thereafter, 25ml of R0 was added and centrifuged again at 1800rpm (764g) for 5mins at RT. Finally, cells were resuspended in 10ml of R10 and rested for a minimum of 30 mins in 37°C and C02 incubator before counting.

17.5.2 Serum separation

Plain tubes (heparinised tubes) were centrifuged at 1800rpm (Beckman Coulter Allegra X-12R Centrifuge) or 764g for 5 mins and transferred to labelled cryovials for further frozen at -80°C for further serological analysis.

17.5.3 Counting PBMCs

17.5.3.1 Cacy counter

For all clinical trial samples, cells were counted using an automated cell counter - CASY counter (Roche Applied Science) set up to count human PBMC. Two independent counts were measured and the average of the two counts was then multiplied by the volume of media cells that were suspended. Cells were then resuspended to a concentration of $5x10^6$ PBMCs per ml or an alternate concentration for assays being conducted.

17.5.3.2 Using haemocytometer

For exploratory cellular assays involving smaller concentrations of cells, cells were counted manually using a light microscope and haemocytometer. Approximately 50µl of cell mixture were pipetted into a single well of a 96-well U-bottom plate (or an alternate vessel). Next, 50µl of trypan blue was added to well-containing cells in the U-bottom plate. Both cells and trypan blue were gently mixed using a pipette and 10µl was transferred to a single section of a haemocytometer.

Cells-stained purple indicated dead cells and non-stained cells indicated live cells. Using 10x magnification, live cells were counted across 16 squares (ignoring cells overlapping with inner square borders). If the number of live cells was too concentrated or diluted to accurately count and adjust the trypan blue-to-cell suspension ratio. Total cell count was calculated by the number of cells in 16 squares multiplied by 10^{16} multiplied dilution of cells suspension to trypan blue multiplied by the volume of cell suspension (see formula below). Cells were then resuspended to a concentration of $5x10^6$ PBMCs per ml or an alternate concentration for assays being conducted.

$$\text{Total cell count} = (\text{count in 16 squares}) \times (10^{16}) \times (\text{dilution}) \times (\text{volume})$$

17.5.4 Peptides

Peptides were ordered and custom synthesised to a purity of 70%. Peptides were individually dissolved in sterile DMSO to a stock concentration of between 50 to 200mg per ml and pooled at working concentrations for cellular assays. Final peptide concentrations of 5µg per ml (CSP) and 20µg/ml (ME-TRAP) according to SOPs for pooling peptides. Peptide Sequences and individual peptide pools can be found in the appendix in 17.6.

17.5.4.1 CSP

CSP peptides used in PBMC stimulated assays to span the length of the antigen that comprises 15mer peptides which are overlapped by 11 amino acids (aa) from the 3D7 strain. Peptides were split across 3 peptide pools (17.6) to a concentration of 5µg per ml. Pools were selected in order to compare immunogenicity from GSK induced by RTS,S vaccines. The CSP ELISpot plate layout can be viewed in Table 26.

Table 26 - CSP ELISpot Plate layout (4 separate cell samples per plate)

Medium = R10. CSP, 1, 2 and 3 = CSP peptide pools (see supplementary). CTL = Cytotoxic T lymphocyte stimulant comprising of peptides from Flu, EBV and CMV (FEC), PHA (lectin from Phaseolus vulgaris) / SEB (staphylococcus enterotoxin B) = positive control

	Volunteer 1			Volunteer 2			Volunteer 3			Volunteer 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium
B	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium
C	CSP 1	CSP 1	CSP 1	CSP 1	CSP 1	CSP 1	CSP 1	CSP 1	CSP 1	CSP 1	CSP 1	CSP 1
D	CSP 2	CSP 2	CSP 2	CSP 2	CSP 2	CSP 2	CSP 2	CSP 2	CSP 2	CSP 2	CSP 2	CSP 2
E	CSP 3	CSP 3	CSP 3	CSP 3	CSP 3	CSP 3	CSP 3	CSP 3	CSP 3	CSP 3	CSP 3	CSP 3
F	CSP Megapool	CSP Megapool	CSP Megapool	CSP Megapool	CSP Megapool	CSP Megapool	CSP Megapool	CSP Megapool	CSP Megapool	CSP Megapool	CSP Megapool	CSP Megapool
G	CTL	CTL	CTL	CTL	CTL	CTL	CTL	CTL	CTL	CTL	CTL	CTL
H	PHA/SEB	PHA/SEB	PHA/SEB	PHA/SEB	PHA/SEB	PHA/SEB	PHA/SEB	PHA/SEB	PHA/SEB	PHA/SEB	PHA/SEB	PHA/SEB

Table 27 - ME-TRAP ELISpot Plate Layout (2 separate cell samples per plate)

Medium = R10. TT 1-57 are from the PF - TRAP T9/96 strain. TD 1-51 are from the PF - TRAP 3D7 strain. ME = Multiple Epitope string [557]. PHA (lectin from Phaseolus vulgaris) / SEB (staphylococcus enterotoxin B) = positive control

	Volunteer 1						Volunteer 2					
	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	Medium	Medium	TD 1-10	TD 1-10	TD 1-10	Medium	Medium	Medium	TD 1-10	TD 1-10	TD 1-10
B	TT 1-20	TT 1-20	TT 1-20	TD 11-20	TD 11-20	TD 11-20	TT 1-20	TT 1-20	TT 1-20	TD 11-20	TD 11-20	TD 11-20
C	TT 11-20	TT 11-20	TT 11-20	TD 21-30	TD 21-30	TD 21-30	TT 11-20	TT 11-20	TT 11-20	TD 21-30	TD 21-30	TD 21-30
D	TT 21-30	TT 21-30	TT 21-30	TD 31-40	TD 31-40	TD 31-40	TT 21-30	TT 21-30	TT 21-30	TD 31-40	TD 31-40	TD 31-40
E	TT 31-40	TT 31-40	TT 31-40	TD-41-51	TD-41-51	TD-41-51	TT 31-40	TT 31-40	TT 31-40	TD-41-51	TD-41-51	TD-41-51
F	TT 41-51	TT 41-51	TT 41-51	TT Megapool	TT Megapool	TT Megapool	TT 41-51	TT 41-51	TT 41-51	TT Megapool	TT Megapool	TT Megapool
G	TT 52-57	TT 52-57	TT 52-57	TD Megapool	TD Megapool	TD Megapool	TT 52-57	TT 52-57	TT 52-57	TD Megapool	TD Megapool	TD Megapool
H	ME	ME	ME	PHA/SEB	PHA/SEB	PHA/SEB	ME	ME	ME	PHA/SEB	PHA/SEB	PHA/SEB

17.5.5 IFNγ ELISpot

Ex vivo ELISpots performed in this thesis were performed under sterile conditions in a fumigation cat 2 hood using Multiscreen IP ELISpot plates, human IFNγ/SA-ALP antibody kits and BCIP NBT-plus chromogenic substrate. All ELISpots were performed using fresh PBMC.

The coating solution was prepared by adding 10µl catcher anti-IFNγ catcher antibody (1-D1K) per ml of coating buffer creating a final concentration of 10µg/ml. 50µl of coating solution was added per well and left at RT for 3-8 hours or at 4°C for 8-72 hours before use. After coating, plates were washed 3 times with 100µl sterile PBS and then blocked with 100µl of R10 at RT for 1-8 hours or at 4°C for 72 hours. Pre-prepared ELISpot pooled peptide plates containing either ME-TRAP (20µg/ml) and at CSP (5µg/ml) were thawed and 50µl from each pool was added per well in triplicate matching peptide layout seen in Table 26 (CSP) and Table 27 (ME-TRAP) respectively. In addition, 50µl of R10 containing 250,000 PBMCs were added to all wells containing peptides, resulting in a final concentration of peptides as 10µg/ml and 2.5µg/ml accordingly. A mixture of staphylococcal enterotoxin B (SEB, 0.02µg/mL) and phytohemagglutinin-L (PHA, 10µg/mL) was used as a positive control in all ELISpot assays. Wells with R10 only and no peptide were included in all assays as a negative control. For CSP ELISpots, a CTL pool (10µg/mL final concentration), also referred to as FEC, was included as an additional positive control. CTL is a well-defined pool of 23 CD8+ epitopes from influenza A, EBV and CMV [558] - Table 26.

After an 18-20 hour (overnight) incubation at 37°C and 5% CO2, cells were discarded and plates were subsequently washed 6 times with PBS/T (non-sterile). After washing, the detector antibody (biotinylated anti-IFNγ) was prepared at 1µg/ml in PBS, 50µl per well was added and incubated for 2-4 hours at RT. Plates were washed again 6 times with PBS/T and 50µl/ well of 1:1000 with SA-ALP for 1-2hours at RT. Plates were washed again and 50µl of BCIP/ NBT-plus (ELISpot development buffer)

was added for 3-5 mins until spots were clearly visible. Thereafter, BCIP/ NBT-plus was washed off thoroughly with tap water and plates were left to dry for a minimum of 24 hours in darkness before counting.

After drying, the spots in each well were counted using an automated plate counter (AID Diagnostika GmbH, algorithm C). All plates were counted using identical settings and any non-biological artefacts were removed. Total spot-forming cells for ME-TRAP and CSP ELISpot were calculated by taking the average of each triplicate within each peptide pool and multiplying by four to produce a count per million PBMC (SFC/10^6 PBMC). Next, the average of spots in the unstimulated (negative control) wells was subtracted from each peptide pool. Finally, counts across each peptide pool were summed for each strain of TRAP antigen (TD or TT) and CSP. Responses greater than the median + 2 SDs of the negative control wells and greater than the LLoD were considered positive. The LLoD for this assay was defined as the lowest number of spots that could accurately be identified from the unstimulated control well and calculated as the median + 2 SDs of all unstimulated control wells run for each study. The LLoD for studies presented in this thesis was 28 SFC/10^6 PBMC for the ME-TRAP ELISpot and 12 SFC/10^6 PBMC for the CSP ELISpot. All responses below the LLoD were adjusted to the LLoD.

17.5.6 Freezing PBMCs

Any PBMCs remaining after completing assays that require fresh PBMC were frozen down at 5-10x10^6 cells per ml per vial. Cells were centrifuged at 1800rpm (764g) for 5 minutes and resuspended with 0.5ml of FCS for 30 minutes at 4°C. An equal volume of cold 20% DMSO in FCS was added and gently mixed with a Strippette and then transferred to labelled cryovials and frozen for 24 hours at -80°C in a CoolCell. After 24-72 hours at -80°C, PBMCs were transferred to liquid nitrogen.

17.5.7 Thawing PBMC

Each cryopreserved PBMC vial was thawed by suspension in a 37-degree water bath until approximately two-thirds thawed and then transferred to a labelled 15ml Falcon Tube containing R10 ensuring a final volume of 10ml R10. Cells were centrifuged at 1800rpm (764g) for 5 minutes, supernatant was poured off and resuspended in 2-5ml of warm R10 depending on the number of cells in suspension – 2ml of R10 for every $10x10^6$ PBMC. Benzonase was added to prevent cellular clumping (1µl Benzonase per $10x10^6$ PBMC). Cells were rested in 37°C, 5% CO2 incubator for a minimum of three hours. After resting, cells were centrifuged at 1800rpm (764g) for 5 minutes to remove any Benzonase and subsequently resuspended in R10 for counting.

17.5.8 Culturing Huh7 cells

Long-term cell cultures performed during this project involved a human hepatoma cell line – Huh7. Huh7 cells were thawed using the procedure mentioned above (17.5.7), and cells were transferred to sterile T75 or T175 flasks and placed in a sterile 37°C, 5% CO2 incubator. The Huh7 cell line has a doubling time of approximately 24 hours. Flasks were cultured (split or discarded) every 3-4 days or when Huh7 confluency reached 80—90% when observed through a 5x magnification light microscope.

Cultured Huh7 cells identified to be split had D10 media removed using a sterile Strippette. Cells were washed twice with 15-30ml (T75 or T175 flask respectively) of sterile PBS. Thereafter, 4-7mls of trypsin was added to each flask until a thin layer of trypsin was evenly distributed across the surface. Each flask containing 4-7ml of trypsin was placed in a 37°C, 5% CO2 incubator for 5 minutes. After 5 minutes, the flask was gently tapped to dislodge Huh 7 cells from the surface of the flask causing a milky appearance within the D10 media. Warm sterile D10 was immediately added (26ml for the T75 flask or 33ml for the T175 flask) to neutralise trypsin for a final volume of 40ml. Cells were transferred to a labelled 50ml falcon tube and centrifuged at 1800rpm (764g) for 5 minutes. Thereafter, the

supernatant was discarded and Huh 7 cells and cells were resuspended in 10ml of D10. Cells were split by transferring 1-5ml to a new sterile flask, topped up to 30 or 60ml of D10 and placed in a 37°C, 5% CO2 incubator to maintain ongoing cell culture.

17.5.9 ISI assay

The inhibition of sporozoite invasion assay performed has been modified from previously described protocols [413, 425]. Updated procedures include an increase in sporozoite infection concentration from 10000spz/well to 15000 spz per well, changes in culture medium from R10 to D10, and the method of removing cells by the addition of trypsin to Huh7 cells on day of cellular acquisition was changed.

The ISI assay is performed over a 3-day period. A human hepatocytic cell line (Huh7) was cultured (17.5.8) approximately 2 weeks prior to commencement of the ISI assay to ensure sufficient cell numbers and no cellular continuation. Cultured Huh7 cells were counted, concentration adjusted and added to a 96-well plate at 30,000 cells/well and left to adhere in a 37°C, 5% CO2 incubator overnight. Viable GFP-labelled *P. berghei* (*Pb*) sporozoites expressing *Pf* CSP at the *P. berghei* CSP locus (*P. berghei* PfCSP@CSP) were obtained by dissecting the salivary glands of infected *Anopheles stephensi* (*As*) mosquitos. Dissected salivary glands were pooled in a homogeniser in DMEM media and left on ice until ready to count. Upon counting, salivary glands were homogenised and sporozoites were counted using a 40x magnification microscope and diluted to a concentration of 150,000 spz/ml in DMEM medium.

Culture medium from Huh7 cells was aspirated, serum/plasma diluted 1:50 in D10 and 100µl added into each well (final serum/plasma concentration 1% in D10 per well) along with 100µl of sporozoite dilution (15,000spz/ml). Samples taken 1 month post-third dose along with a subset of baseline samples were tested in duplicate and arranged in a layout described in Table 28. Along with diluted

plasma/serum samples, 4 positive controls (Spz 1-4, containing Huh7 cells and sporozoites but no plasma/serum) and 6 negative controls (Cells only, Huh7 only wells) were added.

Table 28 – ISI plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	Blank	SPZ - 1 Only	SPZ - 2 Only	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Cells Only
C	Blank	SPZ - 3 Only	SPZ - 4 Only	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Cells Only
D	Blank	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Cells Only
E	Blank	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Cells Only
F	Blank	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	Cells Only
G	Blank	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	Cells Only
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

After incubating for 20-26 hours (overnight) in 37°C, 5% CO2 incubator, D10 media was aspirated and plates were washed twice with 100µl sterile dPBS. Thereafter, 50µl was added to each well and to increase trypsin activity on cells, the solution was gently mixed ~10-15 times using a manual multi-channel pipette. After 10mins, trypsin was neutralised using ISI buffer 1 (dPBS with 10% FCS). Cells were transferred to a 96-well V-bottom plate and centrifuged at 1800rpm (764g) for 5 minutes. Cells were resuspended in 80µl of ISI buffer 2 (dPBS with 1% FCS) and immediately acquired using a BD LSRII and FACSDiva v6.2. A DAPI stain (1:1000 dilution) was added to each sample just before acquisition. Data was analysed in FlowJo software v10.6 (TreeStar Inc., Ashland, Oregon) using the gating strategy in Figure A8.

17.5.10 Total IgG ELISA's

Standardised ELISA methods in this thesis were used to measure total IgG-specific antibodies against the antigens expressed from the R21 vaccine of the *Pf* CSP. All standardised ELISA methods followed the same standard operating procedure (SOP) using plasma, however utilising different coating concentrations - Table 29.

Table 29 - Standardised ELISA antigen descriptions

Antigen		Concentration (µg/ml)	Sequence	Manufacturer
NANP(6)	Peptide	0.2	NANPNANPNANPNANPNANPNANP	Thinkpeptides, ProImmune, Oxford, UK
C term	Peptide	1.5	epsdkhikeylnkiqnslstewspcsvtcgngiqvrikpgsankpkdeldyandiekkickmekcs	Thinkpeptides, ProImmune, Oxford, UK
R21 protein	Protein	0.2	MDPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANP NANPNANPNANPNANPNANPNA NPNANPNKNNQGNGQGHNMPNDPNRNVDENANANSAVKNNNNEEPSD KHIKEYLNKIQNSLSTEWSPCSVTCGNGIQVRIKPGSANKPKDELDYANDIEKKICKMEKCSSVPV TNMENITSGFLGPLLVLQAGFFLLTRILTIPQSLDSWWTSLNFLGGSPVCLGQNSQSPTSNHSP TSCPPICPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYQGMLPVC PLIPGSTTTNTGPCKTCTTPAQGNSMFPSCCCTKPTDGNCTCIPIPSSW AFAKYLWEWASVRFSWLSLLVPFVQWFVGLSPTVWLSA IWMMWYWGPSLYSIVSPFIPLLPIFFC LWVYI	SIIPL

Plates were coated with synthetically produced peptide (NANP and C term) or full-length R21 protein in carbonate bicarbonate (coating buffer) solution overnight at 4°C. After blocking, 100µl Blocker™ Casein was added and incubated for 1–1.5 hours at RT. Next, 50µl of diluted plasma samples were added for a further 2 hours at RT. A minimum dilution for samples from UK trials was performed at 1:100, however for samples obtained in malaria-endemic regions a minimum dilution of 1:500 was used to reduce non-specific binding or background in the assay. Standard curve and internal control were made from sera containing high amounts of NANP6C specific antibodies induced by RTS,S vaccination from a clinical trial previously run at the Jenner Institute (VAC055). The standard curve was added in a two-fold serial dilution to produce ten standard points and assigned arbitrary EUs. Secondary goat anti-human IgG (γ-chain)-alk phos was added at 1:1000 and incubated further for 1 hour at RT. Finally, plates were washed with PBS/T and developer solution consisting of Dilute 5X Diethanolamine substrate buffer (Pierce, 34064) to 1X in dH_2O and 20mg 4-Nitrophenyl Phosphate Tablets and 100µl added to each well in the plate. An ELx808 microplate reader was used to provide optical density measurement of the plates at 405nm (OD_{405}). Plates were developed until the internal control reached an OD_{405} of 2 (1.7–2.2). Standard Curve pass/ fail criteria were examined on a 4-parameter hyperbolic curve generated in Gen5 v3.09, BioTek. A seropositivity cut-off OD405 of 0.2

using the minimum dilution mentioned above was used and given a numerical value of 1EU. The seropositivity threshold was conducted by a previous staff in the Jenner before this DPhil project.

17.5.11 Avidity

IgG antibody avidity against NANP and C term peptides was assessed by sodium thiocyanate (NaSCN)-displacement ELISA. Avidity assays were conducted using Total IgG ELISA SoP (17.5.10) except that plasma was diluted to a level calculated to reach an OD_{405} of 1.0 (using total IgG EU results initially measured for each sample) and 50µl was added in 16 wells of 96 well ELISA plate (avidity plate layout - Table 30) and incubated for 2 hours at RT. Thereafter, a chaotropic agent (NaSCN) was added in duplicate at increasing concentrations down the plate and incubated for 15 mins at RT before washing with PBS/T and then incubated with secondary antibody as per the total IgG method (17.5.10). Plates were developed so that the top row with no NaSCN added developed to an OD405 (0.8-1.2). The concentration of NaSCN required to reduce the OD405 to 50% of that in wells where no NaSCN (IC50) was used as a measure of avidity.

Table 30 - Total IgG Avidity plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1 (PBS)	Sample 1 (PBS)	Sample 2 (PBS)	Sample 2 (PBS)	Sample 3 (PBS)	Sample 3 (PBS)	Sample 4 (PBS)	Sample 4 (PBS)	Sample 5 (PBS)	Sample 5 (PBS)	Sample 6 (PBS)	Sample 6 (PBS)
B	Sample 1 (1M)	Sample 1 (1M)	Sample 2 (1M)	Sample 2 (1M)	Sample 3 (1M)	Sample 3 (1M)	Sample 4 (1M)	Sample 4 (1M)	Sample 5 (1M)	Sample 5 (1M)	Sample 6 (1M)	Sample 6 (1M)
C	Sample 1 (2M)	Sample 1 (2M)	Sample 2 (2M)	Sample 2 (2M)	Sample 3 (2M)	Sample 3 (2M)	Sample 4 (2M)	Sample 4 (2M)	Sample 5 (2M)	Sample 5 (2M)	Sample 6 (2M)	Sample 6 (2M)
D	Sample 1 (3M)	Sample 1 (3M)	Sample 2 (3M)	Sample 2 (3M)	Sample 3 (3M)	Sample 3 (3M)	Sample 4 (3M)	Sample 4 (3M)	Sample 5 (3M)	Sample 5 (3M)	Sample 6 (3M)	Sample 6 (3M)
E	Sample 1 (4M)	Sample 1 (4M)	Sample 2 (4M)	Sample 2 (4M)	Sample 3 (4M)	Sample 3 (4M)	Sample 4 (4M)	Sample 4 (4M)	Sample 5 (4M)	Sample 5 (4M)	Sample 6 (4M)	Sample 6 (4M)
F	Sample 1 (5M)	Sample 1 (5M)	Sample 2 (5M)	Sample 2 (5M)	Sample 3 (5M)	Sample 3 (5M)	Sample 4 (5M)	Sample 4 (5M)	Sample 5 (5M)	Sample 5 (5M)	Sample 6 (5M)	Sample 6 (5M)
G	Sample 1 (6M)	Sample 1 (6M)	Sample 2 (6M)	Sample 2 (6M)	Sample 3 (6M)	Sample 3 (6M)	Sample 4 (6M)	Sample 4 (6M)	Sample 5 (6M)	Sample 5 (6M)	Sample 6 (6M)	Sample 6 (6M)
H	Sample 1 (7M)	Sample 1 (7M)	Sample 2 (7M)	Sample 2 (7M)	Sample 3 (7M)	Sample 3 (7M)	Sample 4 (7M)	Sample 4 (7M)	Sample 5 (7M)	Sample 5 (7M)	Sample 6 (7M)	Sample 6 (7M)

17.5.12 Isotype and subclass

Isotype and subclass antibody (IgA, IgM and IgG1-4) responses were assessed against the NANP peptide. An individual ELISA plate was prepared for each isotype and subclass antibody using the method mentioned in (17.5.10). However, all plasma samples were diluted to 1:100 and added in duplicate (Isotype and subclass plate layout - Table 31) for 2 hours at RT. Plates were washed with PBS/T and one of six secondary antibodies against IgA, IgM, IgG1, IgG2, IgG3 and IgG4, was diluted at 1:1000 and 50µl was added to each plate in casein. Plates were washed again with PBS/T and 50µl of Extravadin-AP diluted 1:1000 in casein was added and incubated for 30 mins at RT. Plates were washed a final time before developing as for the standardised Total IgG ELISA method above. Blank wells and internal development controls were included on each plate. Raw OD_{405} values were recorded and a seropositivity threshold for each sample was calculated using malaria naïve samples using mean + 3 SD of baseline sample OD_{405}.

Table 31 - Isotype and Subclass antibody assay plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
B	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
C	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
D	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
E	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36
F	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36
G	IgG - 1	IgG - 2	IgG - 3	IgG - 4	Kenyan Pos	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43
H	IgG - 1	IgG - 2	IgG - 3	IgG - 4	Kenyan Pos	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43

17.5.13 Total IgG measured by MSD

The MSD multiplex assay is a novel assay developed for measuring the concentrations of multiple antigen targets induced from the R21 vaccine within a single experiment. Each assay plate is pre-coated with 4 different antigens (NANP, C term, HBsAg and R21 (full length)) arranged in independent spots on the base of each well.

Prior to the experiment, each MSD plate was brought to RT and all samples, standards, and regents prepared. Standards were prepared using the same serum pool used for the total standardised ELISA (17.5.10) starting with a 1:1000 dilution for standard 1 (std1) and using a serial fold dilution of 1:4 for the next seven standards (std 2-8). Samples were diluted 1:1000 for baseline samples and 1:100,000 for post-vaccination samples. Assay controls were prepared and diluted at 1:40,000 for High Qc control, 1:10,640,000 for medium QC control and 1:240,000 for low QC control. See Table 32 for the MSD plate layout. A Detection Antibody SULFO-TAG Anti-human IgG antibody was diluted to a final concentration of 1 µg/mL in casein.

Table 32 - MSD plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	High QC control	Mid QC control	Low QC control	Blank	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
B	High QC control	Mid QC control	Low QC control	Blank	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
C	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
D	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
E	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
F	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
G	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36
H	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36

Once all reagents were prepared, plates with 150µl of casein, were sealed with an adhesive plate sealer and placed on a shaker (500rpm) for 30 mins at RT. Plates were then washed with MSD wash buffer and 50µl of reference standard, controls and diluted samples were added to each well (See Table 32), sealed and placed on a shaker (500rpm) for 1 hour. Plates were again washed and 50µL/well of detection antibody solution was added to the plate, sealed and placed on a shaker (500rpm) for a further 1 hour. Plates were washed with MSD and 150 µL/well of MSD GOLD Read Buffer B to the

plate and read using the MSD plate reader within 15mins. Antibody concentrations are calculated and reported using MSD Methodical Mind Software.

17.5.13.1 MSD Seronegative values

Table 33 - MSD LLoD values

Values were calculated from the mean response from samples taken at baseline from malaria naïve adults in the UK.

	MSD - R21	MSD - HBsAg	MSD - NANP	MSD – C Terminus
LLoD - MSD	0.0021	0.000011	0.0036	0.0032
LLoD Jenner 1-St Dev	3.8192	n/a	20.1417	12.7913
LLoD Jenner 2-St Dev	7.6385	n/a	40.2834	25.5826

17.5.14 Efficacy analyses

Cox regression models were used to analyse the time to first episodes of clinical malaria fourteen days following the third dose in the primary series and fourteen days following subsequent boosters. Cox's proportional hazards model was used to calculate the HR and VE was calculated as 1 minus the hazard ratio (HR). Cases were counted and reported at six and twelve months post primary series and six and twelve months post boosters. The primary analysis for efficacy was prespecified as being between groups 1 and 3, groups 2 and 3, and groups 1 and 2 combined versus 3.

17.6 Peptide sequences

17.6.1 CSP peptide sequence and pools

Table 34 - CSP peptide sequences and ELISPOT peptide pool configurations

Pool	Region	Peptide number	Amino acid sequence
1	**NANP and Conserved Region**	1	MMAP DPNANPNANPN
		2	NANP NANPNANPNAN
		3	DPNA NPNANPNKNNQ
		4	NPNA NPNKNNQGNGQ
		5	NPNK NNQGNGQGHNM
		6	NNQG NGQGHNMPNDP
		7	NGQG HNMPNDPNRNV
		8	HNMP NDPNRNVDENA
		9	NDPN RNVDENANANS
		10	RNVD ENANANSAVKN
		11	ENAN ANSAVKNNNNE
2	**TH2R region**	12	ANSA VKNNNNEEPSD
		13	VKNN NNEEPSDKHIK
		14	NNEE PSDKHIKEYLN
		15	PSDK HIKEYLNKIQN
		16	HIKE YLNKIQNSLST
		17	YLNK IQNSLSTEWSP
		18	IQNS LSTEWSPCSVT
		19	LSTE WSPCSVTCGNG
3	**TH3R/CS. T3T region**	20	WSPC SVTCGNGIQVR
		21	SVTC GNGIQVRIKPG
		22	GNGI QVRIKPGSANK
		23	QVRI KPGSANKPKDE
		24	KPGS ANKPKDELDYA
		25	ANKP KDELDYANDIE
		26	KDEL DYANDIEKKIC
		27	DYAN DIEKKICKMEK
		28	DIEK KICKMEKCSSV
		29	KICK MEKCSSVFNVV
		30	MEKC SSVFNVVNSSI
		31	KCSS VFNVVNSSIGL

17.6.2 TRAP peptide sequence and pools

Table 35 - TRAP Peptide sequences and ELISPOT peptide pools

Peptide showing overlapping conserved sequences between the T9/96 and 3D7 strains. 10T and 37T do not exist due to an insertion in the 3D7 sequence causing a shift in the T9/96 sequence to aid alignment.

Pool	Peptide Name	Peptide sequence T9/96	T996 Pools	Peptide sequence 3D7 (if different from T9/96)	3D7 Pools
1	TRAP-1	MNHLGNVKYLVIVFLIFFDL	1		1
	TRAP-2	VIVFLIFFDLFLVNGRDVQN			
	TRAP-3	FLVNGRDVQNNIVDEIKYSE		FLVNGRDVQNNIVDEIKYRE	
	TRAP-4	NIVDEIKYSEEVCNDQVDLY		NIVDEIKYREEVCNDEVDLY	
	TRAP-5	EVCNDQVDLYLLMCSGSIR		EVCNDEVDLYLLMCSGSIR	
	TRAP-6	LLMCSGSIRRHNWVNHAVP			
	TRAP-7	RHNWVNHAVPLAMKLIQQLN			
	TRAP-8	LAMKLIQQLNLNDNAIHLYV		LAMKLIQQLNLNDNAIHLYA	
	TRAP-9	LNDNAIHLYVNVFSNNAKEI		LNDNAIHLYASVFSNNAREI	
	TRAP-10	LNDNAIHLYVNVFSNNAKEI		SVFSNNAREIIRLHSDASKN	
2	TRAP-11	IRLHSDASKNKEKALIIIRS	2	IRLHSDASKNKEKALIIIKS	2
	TRAP-12	KEKALIIIRSLLSTNLPYGR		KEKALIIIKSLLSTNLPYGK	
	TRAP-13	LLSTNLPYGRTNLTDALLQV		LLSTNLPYGKTNLTDALLQV	
	TRAP-14	TNLTDALLQVRKHLNDRINR			
	TRAP-15	RKHLNDRINRENANQLVVIL			
	TRAP-16	ENANQLVVILTDGIPDSIQD			
	TRAP-17	TDGIPDSIQDSLKESRKLSD			
	TRAP-18	SLKESRKLSDRGVKIAVFGI			
	TRAP-19	RGVKIAVFGIGQGINVAFNR			
	TRAP-20	GQGINVAFNRFLVGCHPSDG			
3	TRAP-21	FLVGCHPSDGKCNLYADSAW	3		3
	TRAP-22	KCNLYADSAWENVKNVIGPF			
	TRAP-23	ENVKNVIGPFMKAVCVEVEK			
	TRAP-24	MKAVCVEVEKTASCGVWDEW			
	TRAP-25	TASCGVWDEWSPCSVTCGKG			
	TRAP-26	SPCSVTCGKGTRSRKREILH			
	TRAP-27	TRSRKREILHEGCTSEIQEQ		TRSRKREILHEGCTSELQEQ	
	TRAP-28	EGCTSEIQEQCEEERCPPKW		EGCTSELQEQCEEERCLPKR	
	TRAP-29	CEEERCPPKWEPLDVPDEPE		CEEERCLPKREPLDVPDEPE	
	TRAP-30	EPLDVPDEPEDDQPRPRGDN			
4	TRAP-31	DDQPRPRGDNSSVQKPEENI	4	DDQPRPRGDNFAVEKPNENI	4

	TRAP-32	SSVQKPEENIIDNNPQEPSP		FAVEKPNENIIDNNPQEPSP	
	TRAP-33	IDNNPQEPSPNPEEGKDENP		IDNNPQEPSPNPEEGKGENP	
	TRAP-34	NPEEGKDENPNGFDLDENPE		NPEEGKGENPNGFDLDENPE	
	TRAP-35	NGFDLDENPENPPNPDIPEQ		NGFDLDENPENPPNPPNPPN	
	TRAP-36	NPPNPDIPEQKPNIPEDSEK		NPPNPPNPPNPPNPPNPPNP	
	TRAP-37	NONE		PPNPPNPPNPDIPEQKPNIP	
	TRAP-38	DIPEQKPNIPEDSEKEVPSD		DIPEQKPNIPEDSEKEVPSD	
	TRAP-39	EDSEKEVPSDVPKNPEDDRE			
	TRAP-40	VPKNPEDDREENFDIPKKPE			
5	TRAP-41	ENFDIPKKPENKHDNQNNLP	5		5
	TRAP-42	NKHDNQNNLPNDKSDRNIPY		NKHDNQNNLPNDKSDRYIPY	
	TRAP-43	NDKSDRNIPYSPLPPKVLDN		NDKSDRYIPYSPLAPKVLDN	
	TRAP-44	SPLPPKVLDNERKQSDPQSQ		SPLAPKVLDNERKQSDPQSQ	
	TRAP-45	ERKQSDPQSQDNNGNRHVPN			
	TRAP-46	DNNGNRHVPNSEDRETRPHG			
	TRAP-47	SEDRETRPHGRNNENRSYNR			
	TRAP-48	RNNENRSYNRKYNDTPKHPE			
	TRAP-49	KYNDTPKHPEREEHEKPDNN			
	TRAP-50	REEHEKPDNNKKKGESDNKY			
6	TRAP-51	KKKGESDNKYKIAGGIAGGL	6		6
	TRAP-52	KIAGGIAGGLALLACAGLAY			
	TRAP-53	ALLACAGLAYKFVVPGAATP			
	TRAP-54	KFVVPGAATPYAGEPAPFDE			
	TRAP-55	YAGEPAPFDETLGEEDKDLD			
	TRAP-56	TLGEEDKDLDEPEQFRLPEE			
	TRAP-57	EPEQFRLPEENEWN			

17.6.3 ME peptide sequence

Table 36 - ME peptide sequences

The epitope string was codon optimised for mammalian translation. The table has been adapted from McConkey et al., 2003 [557].

No.	Peptide Name	Sequence	Antigen Source	Epitope Type	Length
1	st8	MINAYLDKL	STARP	CD8	9
2	ls50	ISKYEDEI	LSA1	CD8	8
3	pb9	SYIPSAEKI	PbCS	CD8	9
4	ls8	KPNDKSLY	LSA1	CD8	8
5	cp26	KPKDELDY	CSP	CD8	8
6	ls6	KPIVQYDNF	LSA1	CD8	9
7	tr42/43	ASKNKEKALII	TRAP	CD8	11
8	tr39	GIAGGLALL	TRAP	CD8	9
9	cp6	MNPNDPNRNV	CSP	CD8	10
10	tr26	HLGNVKYLV	TRAP	CD8	9
11	ls53	KSLYDEHI	LSA1	CD8	8
12	tr29	LLMDCSGSI	TRAP	CD8	9
13	csp	DPNANPNVDPNANPNV	CSP	CD4	16
14	38H BCG	QVHFQPLPPAVVKL	BCG	CD4	14
15	FTTp	QFIKANSKFIGITE	TT	CD4	14
16	cp39	YLNKIQNSL	CSP	CD8	9
17	la72	MEKLKELEK	LSA3	CD8	9
18	ex23	ATSVLAGL	Exp1	CD8	8
19	nanp	NANPNANPNANPNANP	CSP	B cell	16
20	trap-AM	DEWSPCSVTCGKGTRSRKRE	TRAP	Heparin Binding Motif	20

17.7 Gating strategies

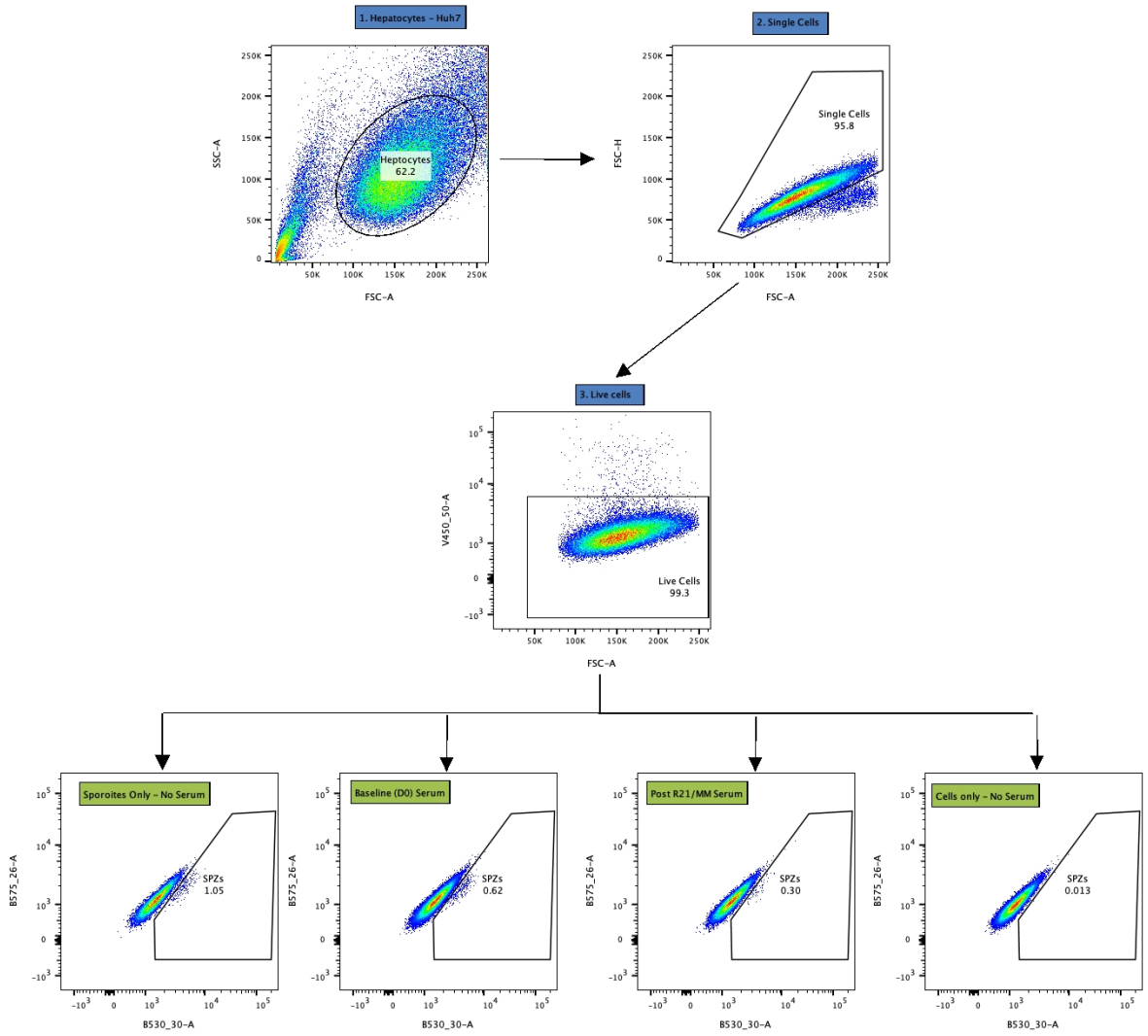


Figure 101 - ISI gating strategy.

18 References

1. Sang, S., et al., *Safety and immunogenicity of varied doses of R21/Matrix-M™ vaccine at three years follow-up: A phase 1b age de-escalation, dose-escalation trial in adults, children, and infants in Kilifi-Kenya.* Wellcome Open Research, 2023. **8**: p. 1-13.
2. Datoo, M.S., et al., *Efficacy of a low-dose candidate malaria vaccine, R21 in adjuvant Matrix-M, with seasonal administration to children in Burkina Faso: a randomised controlled trial.* Lancet, 2021. **397**(10287): p. 1809-1818.
3. Datoo, M.S., et al., *Efficacy and immunogenicity of R21/Matrix-M vaccine against clinical malaria after 2 years' follow-up in children in Burkina Faso: a phase 1/2b randomised controlled trial.* Lancet Infect Dis, 2022. **22**(12): p. 1728-1736.
4. Bonanni, P., *Demographic impact of vaccination: a review.* Vaccine, 1999. **17 Suppl 3**: p. S120-5.
5. WHO, *Global Vaccine Action Plan Monitoring, Evaluation & Accountability: Secretariat Annual Report 2020.* Immunization, Vaccines and Biologicals, 2020(ISBN: 978-92-4-001432-9): p. 1 - 24.
6. WHO, *Global Vaccine Action Plan 2011 - 2013.* Immunization, Vaccines and Biologicals, 2013(ISBN: 9789241504980): p. 1 - 77.
7. Riedel, S., *Edward Jenner and the history of smallpox and vaccination.* Proc (Bayl Univ Med Cent), 2005. **18**(1): p. 21-5.
8. Pollard, A.J. and E.M. Bijker, *Publisher Correction: A guide to vaccinology: from basic principles to new developments.* Nat Rev Immunol, 2021. **21**(2): p. 129.
9. Davenport, R.J., M. Satchell, and L.M.W. Shaw-Taylor, *The geography of smallpox in England before vaccination: A conundrum resolved.* Soc Sci Med, 2018. **206**: p. 75-85.
10. Fenner, F., *A successful eradication campaign. Global eradication of smallpox.* Rev Infect Dis, 1982. **4**(5): p. 916-30.
11. Strassburg, M.A., *The global eradication of smallpox.* Am J Infect Control, 1982. **10**(2): p. 53-9.
12. Pollard, A.J. and E.M. Bijker, *A guide to vaccinology: from basic principles to new developments.* Nat Rev Immunol, 2021. **21**(2): p. 83-100.
13. Rappuoli, R., S. Black, and P.H. Lambert, *Vaccine discovery and translation of new vaccine technology.* Lancet, 2011. **378**(9788): p. 360-8.
14. Vasto, S., M. Malavolta, and G. Pawelec, *Age and immunity.* Immun Ageing, 2006. **3**: p. 2.
15. Pawelec, G., *Age and immunity: What is "immunosenescence"?* Exp Gerontol, 2018. **105**: p. 4-9.
16. Weng, N.P. and G. Pawelec, *Research on immunity and ageing comes of age.* Immun Ageing, 2019. **16**: p. 8.
17. Hatherill, M., R.G. White, and T.R. Hawn, *Clinical Development of New TB Vaccines: Recent Advances and Next Steps.* Front Microbiol, 2019. **10**: p. 3154.
18. Matz, K.M., A. Marzi, and H. Feldmann, *Ebola vaccine trials: progress in vaccine safety and immunogenicity.* Expert Rev Vaccines, 2019. **18**(12): p. 1229-1242.
19. Beeson, J.G., et al., *Challenges and strategies for developing efficacious and long-lasting malaria vaccines.* Sci Transl Med, 2019. **11**(474).
20. McComb, S., et al., *Introduction to the Immune System.* Methods Mol Biol, 2019. **2024**: p. 1-24.
21. Robert Rich, D.C., *The human immune response.* In Clinical Immunology, 2019. **Elsevier**(E11): p. 3 - 11.
22. Marshall, J.S., et al., *An introduction to immunology and immunopathology.* Allergy Asthma Clin Immunol, 2018. **14**(Suppl 2): p. 49.
23. Prame Kumar, K., A.J. Nicholls, and C.H.Y. Wong, *Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease.* Cell Tissue Res, 2018. **371**(3): p. 551-565.
24. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.
25. Jang, J.H., et al., *An Overview of Pathogen Recognition Receptors for Innate Immunity in Dental Pulp.* Mediators Inflamm, 2015. **2015**: p. 794143.

26. Wicherska-Pawlowska, K., T. Wrobel, and J. Rybka, *Toll-Like Receptors (TLRs), NOD-Like Receptors (NLRs), and RIG-I-Like Receptors (RLRs) in Innate Immunity. TLRs, NLRs, and RLRs Ligands as Immunotherapeutic Agents for Hematopoietic Diseases.* Int J Mol Sci, 2021. **22**(24).
27. Kawasaki, T. and T. Kawai, *Toll-like receptor signaling pathways.* Front Immunol, 2014. **5**: p. 461.
28. Wicherska-Pawlowska, K., et al., *Infection and expression of Toll-like receptors in lymphoid malignancy patients after autologous stem cell transplantation.* Cent Eur J Immunol, 2021. **46**(4): p. 463-469.
29. Salwa Refat El-Zayat, H.S.a.F.A.M., *Toll-like receptors activation, signaling, and targeting: an overview.* Bulletin of the National Research Centre, 2019. **43**: p. 1-12.
30. D'Arpa, P. and K.P. Leung, *Toll-Like Receptor Signaling in Burn Wound Healing and Scarring.* Adv Wound Care (New Rochelle), 2017. **6**(10): p. 330-343.
31. Dunkelberger, J.R. and W.C. Song, *Complement and its role in innate and adaptive immune responses.* Cell Res, 2010. **20**(1): p. 34-50.
32. Walport, M.J., *Complement. Second of two parts.* N Engl J Med, 2001. **344**(15): p. 1140-4.
33. Walport, M.J., *Complement. First of two parts.* N Engl J Med, 2001. **344**(14): p. 1058-66.
34. Feng, G., et al., *Mechanisms and targets of Fcgamma-receptor mediated immunity to malaria sporozoites.* Nat Commun, 2021. **12**(1): p. 1742.
35. Douglas R. McDonald, O.L., *Innate Immunity.* Clinical Immunology, 2019. **Principals and Practice**: p. 39-53.
36. Netea, M.G., et al., *Innate immune memory: a paradigm shift in understanding host defense.* Nat Immunol, 2015. **16**(7): p. 675-9.
37. Quintin, J., et al., *Innate immune memory: towards a better understanding of host defense mechanisms.* Curr Opin Immunol, 2014. **29**: p. 1-7.
38. Collin, M. and V. Bigley, *Monocyte, Macrophage, and Dendritic Cell Development: the Human Perspective.* Microbiol Spectr, 2016. **4**(5).
39. Abel, A.M., et al., *Natural Killer Cells: Development, Maturation, and Clinical Utilization.* Front Immunol, 2018. **9**: p. 1869.
40. Orange, J.S., *Unraveling human natural killer cell deficiency.* J Clin Invest, 2012. **122**(3): p. 798-801.
41. Mellman, I., *Dendritic cells: master regulators of the immune response.* Cancer Immunol Res, 2013. **1**(3): p. 145-9.
42. Banchereau, J., *Dendritic cells: therapeutic potentials.* Transfus Sci, 1997. **18**(2): p. 313-26.
43. Neefjes, J., et al., *Towards a systems understanding of MHC class I and MHC class II antigen presentation.* Nat Rev Immunol, 2011. **11**(12): p. 823-36.
44. Wieczorek, M., et al., *Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation.* Front Immunol, 2017. **8**: p. 292.
45. Blum, J.S., P.A. Wearsch, and P. Cresswell, *Pathways of antigen processing.* Annu Rev Immunol, 2013. **31**: p. 443-73.
46. Roche, P.A. and K. Furuta, *The ins and outs of MHC class II-mediated antigen processing and presentation.* Nat Rev Immunol, 2015. **15**(4): p. 203-16.
47. Warrington, R., et al., *An introduction to immunology and immunopathology.* Allergy Asthma Clin Immunol, 2011. **7 Suppl 1**(Suppl 1): p. S1.
48. Cooper, M.D. and M.N. Alder, *The evolution of adaptive immune systems.* Cell, 2006. **124**(4): p. 815-22.
49. Bonilla, F.A. and H.C. Oettgen, *Adaptive immunity.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S33-40.
50. Alcover, A., B. Alarcon, and V. Di Bartolo, *Cell Biology of T Cell Receptor Expression and Regulation.* Annu Rev Immunol, 2018. **36**: p. 103-125.
51. Nothelfer, K., P.J. Sansonetti, and A. Phalipon, *Pathogen manipulation of B cells: the best defence is a good offence.* Nat Rev Microbiol, 2015. **13**(3): p. 173-84.
52. Booth, J.S. and F.R. Toapanta, *B and T Cell Immunity in Tissues and Across the Ages.* Vaccines (Basel), 2021. **9**(1).
53. Janeway CA Jr, T.P., Walport M, *The Immune System in Health and Disease*. Immunobiology. Vol. 5th eddition. 2001, New York: Garland Science.

54. Saule, P., et al., *Accumulation of memory T cells from childhood to old age: central and effector memory cells in CD4(+) versus effector memory and terminally differentiated memory cells in CD8(+) compartment.* Mech Ageing Dev, 2006. **127**(3): p. 274-81.
55. Hedrick, S.M., *Thymus lineage commitment: a single switch.* Immunity, 2008. **28**(3): p. 297-9.
56. Kumar, B.V., T.J. Connors, and D.L. Farber, *Human T Cell Development, Localization, and Function throughout Life.* Immunity, 2018. **48**(2): p. 202-213.
57. Jenkinson, E.J., et al., *The thymus and T-cell commitment: the right niche for Notch?* Nat Rev Immunol, 2006. **6**(7): p. 551-5.
58. Shah, K., et al., *T cell receptor (TCR) signaling in health and disease.* Signal Transduct Target Ther, 2021. **6**(1): p. 412.
59. Chopp, L., et al., *From thymus to tissues and tumors: A review of T-cell biology.* J Allergy Clin Immunol, 2023. **151**(1): p. 81-97.
60. Dustin, M.L., *The cellular context of T cell signaling.* Immunity, 2009. **30**(4): p. 482-92.
61. Kurup, S.P., N.S. Butler, and J.T. Harty, *T cell-mediated immunity to malaria.* Nat Rev Immunol, 2019. **19**(7): p. 457-471.
62. Broere, F. and W. van Eden, *T cell subsets and T cell-mediated immunity.* Nijkamp and Parnham's principles of immunopharmacology, 2019: p. 23-35.
63. Tesmer, L.A., et al., *Th17 cells in human disease.* Immunol Rev, 2008. **223**: p. 87-113.
64. Kondelkova, K., et al., *Regulatory T cells (TREG) and their roles in immune system with respect to immunopathological disorders.* Acta Medica (Hradec Kralove), 2010. **53**(2): p. 73-7.
65. Crotty, S., *T follicular helper cell differentiation, function, and roles in disease.* Immunity, 2014. **41**(4): p. 529-42.
66. Gattinoni, L., et al., *A human memory T cell subset with stem cell-like properties.* Nat Med, 2011. **17**(10): p. 1290-7.
67. Gourley, T.S., et al., *Generation and maintenance of immunological memory.* Semin Immunol, 2004. **16**(5): p. 323-33.
68. Mitchell, D.M., E.V. Ravkov, and M.A. Williams, *Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8+ effector and memory T cells.* J Immunol, 2010. **184**(12): p. 6719-30.
69. Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. **75**(2): p. 163-89.
70. Carsetti, R., et al., *Comprehensive phenotyping of human peripheral blood B lymphocytes in healthy conditions.* Cytometry A, 2022. **101**(2): p. 131-139.
71. Schultheiss, C., et al., *B cells in autoimmune hepatitis: bystanders or central players?* Semin Immunopathol, 2022. **44**(4): p. 411-427.
72. Tonegawa, S., *Somatic generation of immune diversity.* Biosci Rep, 1988. **8**(1): p. 3-26.
73. Nagasawa, T., *Microenvironmental niches in the bone marrow required for B-cell development.* Nat Rev Immunol, 2006. **6**(2): p. 107-16.
74. Cruickshank, M.N. and D. Ulgiati, *The role of notch signaling in the development of a normal B-cell repertoire.* Immunol Cell Biol, 2010. **88**(2): p. 117-24.
75. Robinson, M.J., et al., *Long-lived plasma cells accumulate in the bone marrow at a constant rate from early in an immune response.* Sci Immunol, 2022. **7**(76): p. eabm8389.
76. Chackerian, B. and D.S. Peabody, *Factors That Govern the Induction of Long-Lived Antibody Responses.* Viruses, 2020. **12**(1).
77. Pieper, K., B. Grimbacher, and H. Eibel, *B-cell biology and development.* J Allergy Clin Immunol, 2013. **131**(4): p. 959-71.
78. Herzog, S., M. Reth, and H. Jumaa, *Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling.* Nat Rev Immunol, 2009. **9**(3): p. 195-205.
79. Papavasiliou, F.N. and D.G. Schatz, *Somatic hypermutation of immunoglobulin genes: merging mechanisms for genetic diversity.* Cell, 2002. **109 Suppl**: p. S35-44.
80. LeBien, T.W. and T.F. Tedder, *B lymphocytes: how they develop and function.* Blood, 2008. **112**(5): p. 1570-80.
81. Alberts B, J.A., Lewis J, *Molecular Biology of the Cell*. 4th eddition ed. B Cells and Antibodies. 2002, New York: Garland Science.

82. Janeway Jr, C.A., Travers, P., Walport, M., & Shlomchik, M. J, *The structure of a typical antibody molecule.* The Immune System in Health and Disease, 2001.
83. Hoffman, W., F.G. Lakkis, and G. Chalasani, *B Cells, Antibodies, and More.* Clin J Am Soc Nephrol, 2016. **11**(1): p. 137-54.
84. Charles A Janeway, J., Paul Travers, Mark Walport, and Mark J Shlomchik, *Antigen receptor structure and signaling pathways.* Immunology, 2001. **The Immune System in Health and Disease**.
85. Stavnezer, J., J.E. Guikema, and C.E. Schrader, *Mechanism and regulation of class switch recombination.* Annu Rev Immunol, 2008. **26**: p. 261-92.
86. Chiu, M.L., et al., *Antibody Structure and Function: The Basis for Engineering Therapeutics.* Antibodies (Basel), 2019. **8**(4).
87. Vidarsson, G., G. Dekkers, and T. Rispens, *IgG subclasses and allotypes: from structure to effector functions.* Front Immunol, 2014. **5**: p. 520.
88. Lu, L.L., et al., *Beyond binding: antibody effector functions in infectious diseases.* Nat Rev Immunol, 2018. **18**(1): p. 46-61.
89. Jefferis, R. and D.S. Kumararatne, *Selective IgG subclass deficiency: quantification and clinical relevance.* Clin Exp Immunol, 1990. **81**(3): p. 357-67.
90. Dobano, C., et al., *Differential Patterns of IgG Subclass Responses to Plasmodium falciparum Antigens in Relation to Malaria Protection and RTS,S Vaccination.* Front Immunol, 2019. **10**: p. 439.
91. Aryal, S. *Antibody- Structure, Classes and Functions*. 2022 01/06/2023]; Available from: https://microbiologyinfo.com/antibody-structure-classes-and-functions/.
92. Sato, S., *Plasmodium-a brief introduction to the parasites causing human malaria and their basic biology.* J Physiol Anthropol, 2021. **40**(1): p. 1.
93. WHO, *World Malaria Report.* 2022. **Geneva**.
94. Ashley, E.A., A. Pyae Phyo, and C.J. Woodrow, *Malaria.* Lancet, 2018. **391**(10130): p. 1608-1621.
95. WHO, *World Malaria Report 2022.* 2022.
96. Weiss, D.J., et al., *Indirect effects of the COVID-19 pandemic on malaria intervention coverage, morbidity, and mortality in Africa: a geospatial modelling analysis.* Lancet Infect Dis, 2021. **21**(1): p. 59-69.
97. WHO, *World Health Statistics.* GHO - THE GLOBAL HEALTH OBSERVATORY, 2022(ISBN 978-92-4-005115-7): p. 135.
98. WHO, *From 30 million cases to zero: China is certified malaria-free by WHO.* News Release, 2021.
99. Badmos, A.O., et al., *What sub-Saharan African countries can learn from malaria elimination in China.* Trop Med Health, 2021. **49**(1): p. 86.
100. Ryan, S.J., C.A. Lippi, and F. Zermoglio, *Shifting transmission risk for malaria in Africa with climate change: a framework for planning and intervention.* Malar J, 2020. **19**(1): p. 170.
101. Snow, R.W., et al., *The prevalence of Plasmodium falciparum in sub-Saharan Africa since 1900.* Nature, 2017. **550**(7677): p. 515-518.
102. Cowman, A.F., et al., *Malaria: Biology and Disease.* Cell, 2016. **167**(3): p. 610-624.
103. Cockburn, I.A. and R.A. Seder, *Malaria prevention: from immunological concepts to effective vaccines and protective antibodies.* Nat Immunol, 2018. **19**(11): p. 1199-1211.
104. Venugopal, K., et al., *Plasmodium asexual growth and sexual development in the haematopoietic niche of the host.* Nat Rev Microbiol, 2020. **18**(3): p. 177-189.
105. Lee, A.H., L.S. Symington, and D.A. Fidock, *DNA repair mechanisms and their biological roles in the malaria parasite Plasmodium falciparum.* Microbiol Mol Biol Rev, 2014. **78**(3): p. 469-86.
106. Tavares, J., et al., *Role of host cell traversal by the malaria sporozoite during liver infection.* J Exp Med, 2013. **210**(5): p. 905-15.
107. Yamauchi, L.M., et al., *Plasmodium sporozoites trickle out of the injection site.* Cell Microbiol, 2007. **9**(5): p. 1215-22.
108. Aliprandini, E., et al., *Cytotoxic anti-circumsporozoite antibodies target malaria sporozoites in the host skin.* Nat Microbiol, 2018. **3**(11): p. 1224-1233.

109. Bullen, H.E., et al., *The Plasmodium falciparum parasitophorous vacuole protein P113 interacts with the parasite protein export machinery and maintains normal vacuole architecture.* Mol Microbiol, 2022. **117**(5): p. 1245-1262.
110. Chan, J.A., et al., *Targets of antibodies against Plasmodium falciparum-infected erythrocytes in malaria immunity.* J Clin Invest, 2012. **122**(9): p. 3227-38.
111. Wilson, D.W., et al., *Macrolides rapidly inhibit red blood cell invasion by the human malaria parasite, Plasmodium falciparum.* BMC Biol, 2015. **13**: p. 52.
112. Riglar, D.T., et al., *Super-resolution dissection of coordinated events during malaria parasite invasion of the human erythrocyte.* Cell Host Microbe, 2011. **9**(1): p. 9-20.
113. Das, S., et al., *Processing of Plasmodium falciparum Merozoite Surface Protein MSP1 Activates a Spectrin-Binding Function Enabling Parasite Egress from RBCs.* Cell Host Microbe, 2015. **18**(4): p. 433-44.
114. Salinas, N.D., W.K. Tang, and N.H. Tolia, *Blood-Stage Malaria Parasite Antigens: Structure, Function, and Vaccine Potential.* J Mol Biol, 2019. **431**(21): p. 4259-4280.
115. Mantel, P.Y., et al., *Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system.* Cell Host Microbe, 2013. **13**(5): p. 521-534.
116. Hawking, F., M.E. Wilson, and K. Gammage, *Evidence for cyclic development and short-lived maturity in the gametocytes of Plasmodium falciparum.* Trans R Soc Trop Med Hyg, 1971. **65**(5): p. 549-59.
117. Joice, R., et al., *Plasmodium falciparum transmission stages accumulate in the human bone marrow.* Sci Transl Med, 2014. **6**(244): p. 244re5.
118. Sologub, L., et al., *Malaria proteases mediate inside-out egress of gametocytes from red blood cells following parasite transmission to the mosquito.* Cell Microbiol, 2011. **13**(6): p. 897-912.
119. Kabiru, E.W., et al., *Sporozoite loads of naturally infected Anopheles in Kilifi District, Kenya.* J Am Mosq Control Assoc, 1997. **13**(3): p. 259-62.
120. Eappen, A.G., et al., *In vitro production of infectious Plasmodium falciparum sporozoites.* Nature, 2022.
121. Coetzee, M., M. Craig, and D. le Sueur, *Distribution of African malaria mosquitoes belonging to the Anopheles gambiae complex.* Parasitol Today, 2000. **16**(2): p. 74-7.
122. Bousema, T. and C. Drakeley, *Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination.* Clin Microbiol Rev, 2011. **24**(2): p. 377-410.
123. Mawson, A.R., *The pathogenesis of malaria: a new perspective.* Pathog Glob Health, 2013. **107**(3): p. 122-9.
124. Bartoloni, A. and L. Zammarchi, *Clinical aspects of uncomplicated and severe malaria.* Mediterr J Hematol Infect Dis, 2012. **4**(1): p. e2012026.
125. Marsh, K., et al., *The pathogenesis of severe malaria in African children.* Ann Trop Med Parasitol, 1996. **90**(4): p. 395-402.
126. Awandare, G.A., et al., *Mechanisms of erythropoiesis inhibition by malarial pigment and malaria-induced proinflammatory mediators in an in vitro model.* Am J Hematol, 2011. **86**(2): p. 155-62.
127. Zakama, A.K., N. Ozarslan, and S.L. Gaw, *Placental Malaria.* Curr Trop Med Rep, 2020. **7**(4): p. 162-171.
128. Omer, S., et al., *Impact of placental malaria on maternal, placental and fetal cord responses and its role in pregnancy outcomes in women from Blue Nile State, Sudan.* Malar J, 2021. **20**(1): p. 35.
129. Lawford, H.L.S., et al., *Associations between malaria in pregnancy and neonatal neurological outcomes.* Int J Infect Dis, 2021. **112**: p. 144-151.
130. Fleischer, B., *Editorial: 100 years ago: Giemsa's solution for staining of plasmodia.* Trop Med Int Health, 2004. **9**(7): p. 755-6.
131. Berzosa, P., et al., *Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea.* Malar J, 2018. **17**(1): p. 333.

132. Kavanaugh, M.J., S.E. Azzam, and D.M. Rockabrand, *Malaria Rapid Diagnostic Tests: Literary Review and Recommendation for a Quality Assurance, Quality Control Algorithm.* Diagnostics (Basel), 2021. **11**(5).
133. Ugah, U.I., et al., *Evaluation of the utility value of three diagnostic methods in the detection of malaria parasites in endemic area.* Malar J, 2017. **16**(1): p. 189.
134. Humar, A., et al., *Parasight F test compared with the polymerase chain reaction and microscopy for the diagnosis of Plasmodium falciparum malaria in travelers.* Am J Trop Med Hyg, 1997. **56**(1): p. 44-8.
135. Sazed, S.A., M.G. Kibria, and M.S. Alam, *An Optimized Real-Time qPCR Method for the Effective Detection of Human Malaria Infections.* Diagnostics (Basel), 2021. **11**(5).
136. Foquet, L., et al., *Molecular detection and quantification of Plasmodium falciparum-infected human hepatocytes in chimeric immune-deficient mice.* Malar J, 2013. **12**: p. 430.
137. WHO, *WHO Guidelines for Malaria (25 November 2022).* World, 2022. **Manual and Guidleine**.
138. Hanboonkunupakarn, B. and N.J. White, *Advances and roadblocks in the treatment of malaria.* Br J Clin Pharmacol, 2022. **88**(2): p. 374-382.
139. Ashley, E.A. and A.P. Phyo, *Drugs in Development for Malaria.* Drugs, 2018. **78**(9): p. 861-879.
140. Ashley, E.A. and J.R. Poespoprodjo, *Treatment and prevention of malaria in children.* Lancet Child Adolesc Health, 2020. **4**(10): p. 775-789.
141. Zhu, L., et al., *Artemisinin resistance in the malaria parasite, Plasmodium falciparum, originates from its initial transcriptional response.* Commun Biol, 2022. **5**(1): p. 274.
142. Ashley, E.A., et al., *Spread of artemisinin resistance in Plasmodium falciparum malaria.* N Engl J Med, 2014. **371**(5): p. 411-23.
143. Intharabut, B., et al., *Artemisinin Resistance and Stage Dependency of Parasite Clearance in Falciparum Malaria.* J Infect Dis, 2019. **219**(9): p. 1483-1489.
144. van der Pluijm, R.W., et al., *Triple Artemisinin-Based Combination Therapies for Malaria - A New Paradigm?* Trends Parasitol, 2021. **37**(1): p. 15-24.
145. van der Pluijm, R.W., et al., *Triple artemisinin-based combination therapies versus artemisinin-based combination therapies for uncomplicated Plasmodium falciparum malaria: a multicentre, open-label, randomised clinical trial.* Lancet, 2020. **395**(10233): p. 1345-1360.
146. Clark, I.A. and W.B. Cowden, *The pathophysiology of falciparum malaria.* Pharmacol Ther, 2003. **99**(2): p. 221-60.
147. Schofield, L. and F. Hackett, *Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites.* J Exp Med, 1993. **177**(1): p. 145-53.
148. Kwiatkowski, D., et al., *Tumour necrosis factor production in Falciparum malaria and its association with schizont rupture.* Clin Exp Immunol, 1989. **77**(3): p. 361-6.
149. Seydel, K.B., et al., *Brain swelling and death in children with cerebral malaria.* N Engl J Med, 2015. **372**(12): p. 1126-37.
150. Ayres Pereira, M., et al., *Placental Sequestration of Plasmodium falciparum Malaria Parasites Is Mediated by the Interaction Between VAR2CSA and Chondroitin Sulfate A on Syndecan-1.* PLoS Pathog, 2016. **12**(8): p. e1005831.
151. Moxon, C.A., et al., *New Insights into Malaria Pathogenesis.* Annu Rev Pathol, 2020. **15**: p. 315-343.
152. Cranston, H.A., et al., *Plasmodium falciparum maturation abolishes physiologic red cell deformability.* Science, 1984. **223**(4634): p. 400-3.
153. Hendriksen, I.C., et al., *Defining falciparum-malaria-attributable severe febrile illness in moderate-to-high transmission settings on the basis of plasma PfHRP2 concentration.* J Infect Dis, 2013. **207**(2): p. 351-61.
154. Haldar, K., et al., *Malaria: mechanisms of erythrocytic infection and pathological correlates of severe disease.* Annu Rev Pathol, 2007. **2**: p. 217-49.
155. Franke-Fayard, B., et al., *Sequestration and tissue accumulation of human malaria parasites: can we learn anything from rodent models of malaria?* PLoS Pathog, 2010. **6**(9): p. e1001032.
156. Andolina, C., et al., *Sources of persistent malaria transmission in a setting with effective malaria control in eastern Uganda: a longitudinal, observational cohort study.* Lancet Infect Dis, 2021. **21**(11): p. 1568-1578.

157. Griffin, J.T., et al., *Gradual acquisition of immunity to severe malaria with increasing exposure.* Proc Biol Sci, 2015. **282**(1801): p. 20142657.
158. Langhorne, J., et al., *Immunity to malaria: more questions than answers.* Nat Immunol, 2008. **9**(7): p. 725-32.
159. Offeddu, V., et al., *Naturally acquired immune responses against Plasmodium falciparum sporozoites and liver infection.* Int J Parasitol, 2012. **42**(6): p. 535-48.
160. Clyde, D.F., *Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites.* Am J Trop Med Hyg, 1975. **24**(3): p. 397-401.
161. Clyde, D.F., et al., *Immunization of man against sporozite-induced falciparum malaria.* Am J Med Sci, 1973. **266**(3): p. 169-77.
162. Stanisic, D.I. and M.B.B. McCall, *Correlates of malaria vaccine efficacy.* Expert Rev Vaccines, 2021. **20**(2): p. 143-161.
163. Beeson, J.G., et al., *The RTS,S malaria vaccine: Current impact and foundation for the future.* Sci Transl Med, 2022. **14**(671): p. eabo6646.
164. Ubillos, I., et al., *Baseline exposure, antibody subclass, and hepatitis B response differentially affect malaria protective immunity following RTS,S/AS01E vaccination in African children.* BMC Med, 2018. **16**(1): p. 197.
165. Schwenk, R., et al., *Opsonization by antigen-specific antibodies as a mechanism of protective immunity induced by Plasmodium falciparum circumsporozoite protein-based vaccine.* Parasite Immunol, 2003. **25**(1): p. 17-25.
166. Kurtovic, L., et al., *Human antibodies activate complement against Plasmodium falciparum sporozoites, and are associated with protection against malaria in children.* BMC Med, 2018. **16**(1): p. 61.
167. Kurtovic, L., et al., *Antibody Targets and Properties for Complement-Fixation Against the Circumsporozoite Protein in Malaria Immunity.* Front Immunol, 2021. **12**: p. 775659.
168. Suau, R., et al., *RTS,S/AS01(E) malaria vaccine induces IgA responses against CSP and vaccine-unrelated antigens in African children in the phase 3 trial.* Vaccine, 2021. **39**(4): p. 687-698.
169. Carvalho, L.H., et al., *IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages.* Nat Med, 2002. **8**(2): p. 166-70.
170. Schofield, L., et al., *Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites.* Nature, 1987. **330**(6149): p. 664-6.
171. Oliveira, G.A., et al., *Class II-restricted protective immunity induced by malaria sporozoites.* Infect Immun, 2008. **76**(3): p. 1200-6.
172. Noe, A., et al., *Deep Immune Phenotyping and Single-Cell Transcriptomics Allow Identification of Circulating TRM-Like Cells Which Correlate With Liver-Stage Immunity and Vaccine-Induced Protection From Malaria.* Front Immunol, 2022. **13**: p. 795463.
173. McNamara, H.A., et al., *Up-regulation of LFA-1 allows liver-resident memory T cells to patrol and remain in the hepatic sinusoids.* Sci Immunol, 2017. **2**(9).
174. Fernandez-Ruiz, D., et al., *Harnessing liver-resident memory T cells for protection against malaria.* Expert Rev Vaccines, 2021. **20**(2): p. 127-141.
175. Fernandez-Ruiz, D., et al., *Liver-Resident Memory CD8(+) T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection.* Immunity, 2019. **51**(4): p. 780.
176. Seder, R.A., et al., *Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine.* Science, 2013. **341**(6152): p. 1359-65.
177. Moncunill, G., et al., *Antigen-stimulated PBMC transcriptional protective signatures for malaria immunization.* Sci Transl Med, 2020. **12**(543).
178. Masserey, T., M.A. Penny, and T.E. Lee, *Patient variability in the blood-stage dynamics of Plasmodium falciparum captured by clustering historical data.* Malar J, 2022. **21**(1): p. 300.
179. Salamanca, D.R., et al., *Plasmodium falciparum Blood Stage Antimalarial Vaccines: An Analysis of Ongoing Clinical Trials and New Perspectives Related to Synthetic Vaccines.* Front Microbiol, 2019. **10**: p. 2712.
180. Fowkes, F.J., et al., *The relationship between anti-merozoite antibodies and incidence of Plasmodium falciparum malaria: A systematic review and meta-analysis.* PLoS Med, 2010. **7**(1): p. e1000218.

181. Kwiatkowski, D., *Tumour necrosis factor, fever and fatality in falciparum malaria.* Immunol Lett, 1990. **25**(1-3): p. 213-6.
182. Kwiatkowski, D., et al., *TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated Plasmodium falciparum malaria.* Lancet, 1990. **336**(8725): p. 1201-4.
183. Lyke, K.E., et al., *Serum levels of the proinflammatory cytokines interleukin-1 beta (IL-1beta), IL-6, IL-8, IL-10, tumor necrosis factor alpha, and IL-12(p70) in Malian children with severe Plasmodium falciparum malaria and matched uncomplicated malaria or healthy controls.* Infect Immun, 2004. **72**(10): p. 5630-7.
184. Riley, E.M., et al., *Immune response to soluble exoantigens of Plasmodium falciparum may contribute to both pathogenesis and protection in clinical malaria: evidence from a longitudinal, prospective study of semi-immune African children.* Eur J Immunol, 1991. **21**(4): p. 1019-25.
185. Amani, V., et al., *Involvement of IFN-gamma receptor-medicated signaling in pathology and anti-malarial immunity induced by Plasmodium berghei infection.* Eur J Immunol, 2000. **30**(6): p. 1646-55.
186. Eriksson, P., et al., *Vaccine vial monitor availability and use in low- and middle-income countries: A systematic review.* Vaccine, 2017. **35**(17): p. 2155-2161.
187. Hochman, S. and K. Kim, *The Impact of HIV Coinfection on Cerebral Malaria Pathogenesis.* J Neuroparasitology, 2012. **3**.
188. Hochman, S. and K. Kim, *The Impact of HIV and Malaria Coinfection: What Is Known and Suggested Venues for Further Study.* Interdiscip Perspect Infect Dis, 2009. **2009**: p. 617954.
189. Ejigu, S., D. Haile, and Y. Solomon, *Effect of malaria and HIV/AIDS co-infection on red blood cell indices and its relation with the CD4 level of patients on HAART in Bench Sheko Zone, Southwest Ethiopia.* PLoS One, 2022. **17**(3): p. e0263865.
190. Alonso, S., et al., *HIV infection and placental malaria reduce maternal transfer of multiple antimalarial antibodies in Mozambican women.* J Infect, 2021. **82**(4): p. 45-57.
191. Kemnic, T.R. and P.G. Gulick, *HIV Antiretroviral Therapy*, in *StatPearls*. 2023: Treasure Island (FL).
192. Beeson, J.G. and F.J.I. Fowkes, *A malaria vaccine in children with HIV.* Lancet Infect Dis, 2016. **16**(10): p. 1087-1089.
193. William J Moss, J.C., Neal A Halsey, *Immunization of children at risk of infection with human immunodeficiency virus.* Bulletin World Health Organisation, 2003. **81**: p. 61 - 70.
194. Portugal, S., et al., *Malaria-associated atypical memory B cells exhibit markedly reduced B cell receptor signaling and effector function.* Elife, 2015. **4**.
195. Weiss, G.E., et al., *Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area.* J Immunol, 2009. **183**(3): p. 2176-82.
196. Ubillos, I., et al., *Chronic Exposure to Malaria Is Associated with Inhibitory and Activation Markers on Atypical Memory B Cells and Marginal Zone-Like B Cells.* Front Immunol, 2017. **8**: p. 966.
197. Obeng-Adjei, N., et al., *Malaria-induced interferon-gamma drives the expansion of Tbethi atypical memory B cells.* PLoS Pathog, 2017. **13**(9): p. e1006576.
198. Obeng-Adjei, N., et al., *Circulating Th1-Cell-type Tfh Cells that Exhibit Impaired B Cell Help Are Preferentially Activated during Acute Malaria in Children.* Cell Rep, 2015. **13**(2): p. 425-39.
199. Aguilar, R., et al., *Changing plasma cytokine, chemokine and growth factor profiles upon differing malaria transmission intensities.* Malar J, 2019. **18**(1): p. 406.
200. Moncunill, G., et al., *Transcriptional correlates of malaria in RTS,S/AS01-vaccinated African children: a matched case-control study.* Elife, 2022. **11**.
201. Moncunill, G., et al., *RTS,S/AS01E Malaria Vaccine Induces Memory and Polyfunctional T Cell Responses in a Pediatric African Phase III Trial.* Front Immunol, 2017. **8**: p. 1008.
202. Illingworth, J., et al., *Chronic exposure to Plasmodium falciparum is associated with phenotypic evidence of B and T cell exhaustion.* J Immunol, 2013. **190**(3): p. 1038-47.
203. Orsini, G., et al., *Enumeration of human peripheral blood dendritic cells throughout the life.* Int Immunol, 2012. **24**(6): p. 347-56.
204. Rogers, N.J., et al., *A model for sequestration of the transmission stages of Plasmodium falciparum: adhesion of gametocyte-infected erythrocytes to human bone marrow cells.* Infect Immun, 2000. **68**(6): p. 3455-62.

205. Yap, X.Z., et al., *Dendritic Cell Responses and Function in Malaria.* Front Immunol, 2019. **10**: p. 357.
206. Urban, B.C., et al., *The frequency of BDCA3-positive dendritic cells is increased in the peripheral circulation of Kenyan children with severe malaria.* Infect Immun, 2006. **74**(12): p. 6700-6.
207. Urban, B.C., et al., *Peripheral blood dendritic cells in children with acute Plasmodium falciparum malaria.* Blood, 2001. **98**(9): p. 2859-61.
208. Boyle, M.J., et al., *Decline of FoxP3+ Regulatory CD4 T Cells in Peripheral Blood of Children Heavily Exposed to Malaria.* PLoS Pathog, 2015. **11**(7): p. e1005041.
209. Francis, S.E., D.J. Sullivan, Jr., and D.E. Goldberg, *Hemoglobin metabolism in the malaria parasite Plasmodium falciparum.* Annu Rev Microbiol, 1997. **51**: p. 97-123.
210. Coronado, L.M., C.T. Nadovich, and C. Spadafora, *Malarial hemozoin: from target to tool.* Biochim Biophys Acta, 2014. **1840**(6): p. 2032-41.
211. Garcia-Bustos, J.F. and F.J. Gamo, *Antimalarial drug resistance and early drug discovery.* Curr Pharm Des, 2013. **19**(2): p. 270-81.
212. Schwarzer, E., et al., *Hemozoin and the human monocyte--a brief review of their interactions.* Parassitologia, 2008. **50**(1-2): p. 143-5.
213. Gowda, N.M., X. Wu, and D.C. Gowda, *TLR9 and MyD88 are crucial for the development of protective immunity to malaria.* J Immunol, 2012. **188**(10): p. 5073-85.
214. Coban, C., et al., *The malarial metabolite hemozoin and its potential use as a vaccine adjuvant.* Allergol Int, 2010. **59**(2): p. 115-24.
215. Millington, O.R., et al., *Malaria impairs T cell clustering and immune priming despite normal signal 1 from dendritic cells.* PLoS Pathog, 2007. **3**(10): p. 1380-7.
216. Zinselmeyer, B.H., et al., *In situ characterization of CD4+ T cell behavior in mucosal and systemic lymphoid tissues during the induction of oral priming and tolerance.* J Exp Med, 2005. **201**(11): p. 1815-23.
217. Ingram, V.M., *Abnormal human haemoglobins.* Proc R Soc Med, 1958. **51**(8): p. 645-6.
218. Ilesanmi, O.O., *Pathological basis of symptoms and crises in sickle cell disorder: implications for counseling and psychotherapy.* Hematol Rep, 2010. **2**(1): p. e2.
219. Gong, L., et al., *Biochemical and immunological mechanisms by which sickle cell trait protects against malaria.* Malar J, 2013. **12**: p. 317.
220. Penny, M.A., et al., *Future use-cases of vaccines in malaria control and elimination.* Parasite Epidemiol Control, 2020. **10**: p. e00145.
221. WHO, *WHO review of malaria vaccine clinical development.* Observatories, 2022. **Global Observatory of Health Research and SDevelopment**.
222. WHO, *Malaria Vaccine Technology Roadmap.* Technical Document, 2013.
223. Moorthy, V.S., R.D. Newman, and J.M. Okwo-Bele, *Malaria vaccine technology roadmap.* Lancet, 2013. **382**(9906): p. 1700-1.
224. Duffy, P.E., Patrick Gorres, *Malaria vaccines since 2000: progress, priorities, products.* npj Vaccines, 2020. **5**(48).
225. Hill, A.V., *Vaccines against malaria.* Philos Trans R Soc Lond B Biol Sci, 2011. **366**(1579): p. 2806-14.
226. Ewer, K.J., et al., *Protective CD8+ T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation.* Nat Commun, 2013. **4**: p. 2836.
227. Ewer, K.J., et al., *Progress with viral vectored malaria vaccines: A multi-stage approach involving "unnatural immunity".* Vaccine, 2015. **33**(52): p. 7444-51.
228. Gonzales, S.J., et al., *Naturally Acquired Humoral Immunity Against Plasmodium falciparum Malaria.* Front Immunol, 2020. **11**: p. 594653.
229. Lindblade, K.A., et al., *The silent threat: asymptomatic parasitemia and malaria transmission.* Expert Rev Anti Infect Ther, 2013. **11**(6): p. 623-39.
230. EMA, *Mosquirix: Opinion on medicine for use outside EU.* 2015.
231. WHO, *WHO recommends groundbreaking malaria vaccine for children at risk.* 2021.
232. WHO, *Mosquirix - Prequalification.* Vaccines (Basel), 2022.
233. WHO, *Over 1 million African children protected by first malaria vaccine.* WHO News, 2021.

234. PATH, M. *PATH welcomes WHO recommendation of a second malaria vaccine for young children*. Malaria Vaccines & Biologics 2023 10th November 2023]; Available from: https://www.malariavaccine.org/news-events/news/path-welcomes-who-recommendation-second-malaria-vaccine-young-children#:~:text=R21%20has%20been%20granted%20an,Faso%2C%20Ghana%2C%20and%20Nigeria.&text=RTS%2CS%20was%20developed%20by,partnership%20with%20PATH%20since%202001.
235. WHO. *WHO recommends R21/Matrix-M vaccine for malaria prevention in updated advice on immunization*
. WHO News 2023 2nd October 2023 6th October 2023]; Available from: https://www.who.int/news/item/02-10-2023-who-recommends-r21-matrix-m-vaccine-for-malaria-prevention-in-updated-advice-on-immunization.
236. Wahl, I. and H. Wardemann, *How to induce protective humoral immunity against Plasmodium falciparum circumsporozoite protein.* J Exp Med, 2022. **219**(2).
237. Coppi, A., et al., *The malaria circumsporozoite protein has two functional domains, each with distinct roles as sporozoites journey from mosquito to mammalian host.* J Exp Med, 2011. **208**(2): p. 341-56.
238. Daubenberger, C.A. and G. Moncunill, *Next-generation malaria subunit vaccines to reduce disease burden in African children.* Lancet Infect Dis, 2022. **22**(12): p. 1655-1656.
239. Bonam, S.R., et al., *Plasmodium falciparum Malaria Vaccines and Vaccine Adjuvants.* Vaccines (Basel), 2021. **9**(10).
240. Pirahmadi, S., et al., *How can we develop an effective subunit vaccine to achieve successful malaria eradication?* Microb Pathog, 2021. **160**: p. 105203.
241. Nooraei, S., et al., *Virus-like particles: preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers.* J Nanobiotechnology, 2021. **19**(1): p. 59.
242. Pirahmadi, S., et al., *A review of combination adjuvants for malaria vaccines: a promising approach for vaccine development.* Int J Parasitol, 2021. **51**(9): p. 699-717.
243. Donaldson, B., et al., *Virus-like particle vaccines: immunology and formulation for clinical translation.* Expert Rev Vaccines, 2018. **17**(9): p. 833-849.
244. Ballou, W.R., et al., *The development of molecular vaccines against malaria sporozoites.* Adv Exp Med Biol, 1989. **251**: p. 295-313.
245. Cohen, J., et al., *From the circumsporozoite protein to the RTS, S/AS candidate vaccine.* Hum Vaccin, 2010. **6**(1): p. 90-6.
246. Laurens, M.B., *RTS,S/AS01 vaccine (Mosquirix): an overview.* Hum Vaccin Immunother, 2020. **16**(3): p. 480-489.
247. Rts, S.C.T.P., et al., *First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children.* N Engl J Med, 2011. **365**(20): p. 1863-75.
248. Rts, S.C.T.P., *Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial.* Lancet, 2015. **386**(9988): p. 31-45.
249. Ballou, W.R., et al., *Immunogenicity of synthetic peptides from circumsporozoite protein of Plasmodium falciparum.* Science, 1985. **228**(4702): p. 996-9.
250. Young, J.F., et al., *Expression of Plasmodium falciparum circumsporozoite proteins in Escherichia coli for potential use in a human malaria vaccine.* Science, 1985. **228**(4702): p. 958-62.
251. Ballou, W.R., et al., *Safety and efficacy of a recombinant DNA Plasmodium falciparum sporozoite vaccine.* Lancet, 1987. **1**(8545): p. 1277-81.
252. Egan, J.E., et al., *Efficacy of murine malaria sporozoite vaccines: implications for human vaccine development.* Science, 1987. **236**(4800): p. 453-6.
253. Gordon, D.M., et al., *Safety, immunogenicity, and efficacy of a recombinantly produced Plasmodium falciparum circumsporozoite protein-hepatitis B surface antigen subunit vaccine.* J Infect Dis, 1995. **171**(6): p. 1576-85.
254. Stoute, J.A., et al., *Long-term efficacy and immune responses following immunization with the RTS,S malaria vaccine.* J Infect Dis, 1998. **178**(4): p. 1139-44.

255. Stoute, J.A., et al., *A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group.* N Engl J Med, 1997. **336**(2): p. 86-91.
256. Doherty, J.F., et al., *A phase I safety and immunogenicity trial with the candidate malaria vaccine RTS,S/SBAS2 in semi-immune adults in The Gambia.* Am J Trop Med Hyg, 1999. **61**(6): p. 865-8.
257. Bojang, K.A., et al., *Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial.* Lancet, 2001. **358**(9297): p. 1927-34.
258. Kester, K.E., et al., *Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental Plasmodium falciparum malaria.* J Infect Dis, 2001. **183**(4): p. 640-7.
259. Bojang, K.A., et al., *Safety and immunogenicty of RTS,S/AS02A candidate malaria vaccine in Gambian children.* Vaccine, 2005. **23**(32): p. 4148-57.
260. Alonso, P.L., et al., *Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial.* Lancet, 2005. **366**(9502): p. 2012-8.
261. Alonso, P.L., et al., *Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial.* Lancet, 2004. **364**(9443): p. 1411-20.
262. Aponte, J.J., et al., *Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial.* Lancet, 2007. **370**(9598): p. 1543-51.
263. Sacarlal, J., et al., *Long-term safety and efficacy of the RTS,S/AS02A malaria vaccine in Mozambican children.* J Infect Dis, 2009. **200**(3): p. 329-36.
264. Sacarlal, J., et al., *Safety of the RTS,S/AS02A malaria vaccine in Mozambican children during a Phase IIb trial.* Vaccine, 2008. **26**(2): p. 174-84.
265. Kester, K.E., et al., *A phase I/IIa safety, immunogenicity, and efficacy bridging randomized study of a two-dose regimen of liquid and lyophilized formulations of the candidate malaria vaccine RTS,S/AS02A in malaria-naive adults.* Vaccine, 2007. **25**(29): p. 5359-66.
266. Bejon, P., et al., *Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age.* N Engl J Med, 2008. **359**(24): p. 2521-32.
267. Asante, K.P., et al., *Safety and efficacy of the RTS,S/AS01E candidate malaria vaccine given with expanded-programme-on-immunisation vaccines: 19 month follow-up of a randomised, open-label, phase 2 trial.* Lancet Infect Dis, 2011. **11**(10): p. 741-9.
268. Rts, S.C.T.P., et al., *A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants.* N Engl J Med, 2012. **367**(24): p. 2284-95.
269. Bejon, P., et al., *Efficacy of RTS,S malaria vaccines: individual-participant pooled analysis of phase 2 data.* Lancet Infect Dis, 2013. **13**(4): p. 319-27.
270. Rts, S.C.T.P., *Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites.* PLoS Med, 2014. **11**(7): p. e1001685.
271. White, M.T., et al., *Immunogenicity of the RTS,S/AS01 malaria vaccine and implications for duration of vaccine efficacy: secondary analysis of data from a phase 3 randomised controlled trial.* Lancet Infect Dis, 2015. **15**(12): p. 1450-8.
272. WHO, *Malaria Vaccines: WHO Position Paper - January 2016.* Weekly Epidemiological Record, 2016. **4**(91): p. 33-52.
273. Guerra Mendoza, Y., et al., *Safety profile of the RTS,S/AS01 malaria vaccine in infants and children: additional data from a phase III randomized controlled trial in sub-Saharan Africa.* Hum Vaccin Immunother, 2019. **15**(10): p. 2386-2398.
274. Cairns, M., et al., *The duration of protection against clinical malaria provided by the combination of seasonal RTS,S/AS01(E) vaccination and seasonal malaria chemoprevention versus either intervention given alone.* BMC Med, 2022. **20**(1): p. 352.
275. Chandramohan, D., et al., *Seasonal Malaria Vaccination with or without Seasonal Malaria Chemoprevention.* N Engl J Med, 2021. **385**(11): p. 1005-1017.

276. Praet, N., et al., *Assessing the safety, impact and effectiveness of RTS,S/AS01(E) malaria vaccine following its introduction in three sub-Saharan African countries: methodological approaches and study set-up.* Malar J, 2022. **21**(1): p. 132.
277. Collins, K.A., et al., *Ultra-low dose immunization and multi-component vaccination strategies enhance protection against malaria in mice.* Sci Rep, 2021. **11**(1): p. 10792.
278. Collins, K.A., et al., *Enhancing protective immunity to malaria with a highly immunogenic virus-like particle vaccine.* Sci Rep, 2017. **7**: p. 46621.
279. Rathore, D., et al., *Binding and invasion of liver cells by Plasmodium falciparum sporozoites. Essential involvement of the amino terminus of circumsporozoite protein.* J Biol Chem, 2002. **277**(9): p. 7092-8.
280. Datoo, M., *A Phase III Randomised Controlled Trial Evaluating the Malaria Vaccine Candidate R21/Matrix-M™ in African Children.* The Lancet Preprint, 2023.
281. Hoffman, S.L., et al., *Progress toward malaria preerythrocytic vaccines.* Science, 1991. **252**(5005): p. 520-1.
282. Marques-da-Silva, C., K. Peissig, and S.P. Kurup, *Pre-Erythrocytic Vaccines against Malaria.* Vaccines (Basel), 2020. **8**(3).
283. Epstein, J.E., et al., *Protection against Plasmodium falciparum malaria by PfSPZ Vaccine.* JCI Insight, 2017. **2**(1): p. e89154.
284. Luke, T.C. and S.L. Hoffman, *Rationale and plans for developing a non-replicating, metabolically active, radiation-attenuated Plasmodium falciparum sporozoite vaccine.* J Exp Biol, 2003. **206**(Pt 21): p. 3803-8.
285. Lyke, K.E., et al., *Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection.* Proc Natl Acad Sci U S A, 2017. **114**(10): p. 2711-2716.
286. Itsara, L.S., et al., *The Development of Whole Sporozoite Vaccines for Plasmodium falciparum Malaria.* Front Immunol, 2018. **9**: p. 2748.
287. Roestenberg, M., et al., *Protection against a malaria challenge by sporozoite inoculation.* N Engl J Med, 2009. **361**(5): p. 468-77.
288. Roestenberg, M., et al., *Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study.* Lancet, 2011. **377**(9779): p. 1770-6.
289. Mueller, A.K., et al., *Genetically modified Plasmodium parasites as a protective experimental malaria vaccine.* Nature, 2005. **433**(7022): p. 164-7.
290. Hafalla, J.C., et al., *Short-term antigen presentation and single clonal burst limit the magnitude of the CD8(+) T cell responses to malaria liver stages.* Proc Natl Acad Sci U S A, 2002. **99**(18): p. 11819-24.
291. Muller, K., et al., *Low immunogenicity of malaria pre-erythrocytic stages can be overcome by vaccination.* EMBO Mol Med, 2021. **13**(4): p. e13390.
292. Bliss, C.M., et al., *Assessment of novel vaccination regimens using viral vectored liver stage malaria vaccines encoding ME-TRAP.* Sci Rep, 2018. **8**(1): p. 3390.
293. Bliss, C.M., et al., *Viral Vector Malaria Vaccines Induce High-Level T Cell and Antibody Responses in West African Children and Infants.* Mol Ther, 2017. **25**(2): p. 547-559.
294. Ewer, K.J., et al., *Viral vectors as vaccine platforms: from immunogenicity to impact.* Curr Opin Immunol, 2016. **41**: p. 47-54.
295. Elias, S.C., et al., *Analysis of human B-cell responses following ChAd63-MVA MSP1 and AMA1 immunization and controlled malaria infection.* Immunology, 2014. **141**(4): p. 628-44.
296. Ewer, K., et al., *A Monovalent Chimpanzee Adenovirus Ebola Vaccine Boosted with MVA.* N Engl J Med, 2016. **374**(17): p. 1635-46.
297. O'Hara, G.A., et al., *Clinical assessment of a recombinant simian adenovirus ChAd63: a potent new vaccine vector.* J Infect Dis, 2012. **205**(5): p. 772-81.
298. Colloca, S., et al., *Vaccine vectors derived from a large collection of simian adenoviruses induce potent cellular immunity across multiple species.* Sci Transl Med, 2012. **4**(115): p. 115ra2.
299. Norbury, C.C., et al., *Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells in vivo.* Nat Immunol, 2002. **3**(3): p. 265-71.

300. Hill, A.V., et al., *Prime-boost vectored malaria vaccines: progress and prospects.* Hum Vaccin, 2010. **6**(1): p. 78-83.
301. Schneider, J., et al., *Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara.* Nat Med, 1998. **4**(4): p. 397-402.
302. Hodgson, S.H., et al., *Evaluation of the efficacy of ChAd63-MVA vectored vaccines expressing circumsporozoite protein and ME-TRAP against controlled human malaria infection in malaria-naive individuals.* J Infect Dis, 2015. **211**(7): p. 1076-86.
303. Mensah, V.A., et al., *Safety, Immunogenicity and Efficacy of Prime-Boost Vaccination with ChAd63 and MVA Encoding ME-TRAP against Plasmodium falciparum Infection in Adults in Senegal.* PLoS One, 2016. **11**(12): p. e0167951.
304. Tsoumani, M.E., C. Voyiatzaki, and A. Efstathiou, *Malaria Vaccines: From the Past towards the mRNA Vaccine Era.* Vaccines (Basel), 2023. **11**(9).
305. Fotoran, W.L., et al., *Establishment of an Antiplasmodial Vaccine Based on PfRH5-Encoding RNA Replicons Stabilized by Cationic Liposomes.* Pharmaceutics, 2023. **15**(4).
306. Laurens, M.B., *Novel malaria vaccines.* Hum Vaccin Immunother, 2021. **17**(11): p. 4549-4552.
307. Cliicaltrials.gov. *Safety and Immune Responses After Vaccination With an Investigational RNA-based Vaccine Against Malaria*. 2023 8th October 2023]; Available from: https://classic.clinicaltrials.gov/ct2/show/NCT05581641?term=BNT165-01&draw=2&rank=1.
308. Cohen, S., G.I. Mc, and S. Carrington, *Gamma-globulin and acquired immunity to human malaria.* Nature, 1961. **192**: p. 733-7.
309. Sirima, S.B., S. Cousens, and P. Druilhe, *Protection against malaria by MSP3 candidate vaccine.* N Engl J Med, 2011. **365**(11): p. 1062-4.
310. Ogutu, B.R., et al., *Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya.* PLoS One, 2009. **4**(3): p. e4708.
311. Thera, M.A., et al., *A field trial to assess a blood-stage malaria vaccine.* N Engl J Med, 2011. **365**(11): p. 1004-13.
312. Baum, J., et al., *Reticulocyte-binding protein homologue 5 - an essential adhesin involved in invasion of human erythrocytes by Plasmodium falciparum.* Int J Parasitol, 2009. **39**(3): p. 371-80.
313. Douglas, A.D., et al., *A PfRH5-based vaccine is efficacious against heterologous strain blood-stage Plasmodium falciparum infection in aotus monkeys.* Cell Host Microbe, 2015. **17**(1): p. 130-9.
314. Douglas, A.D., et al., *The blood-stage malaria antigen PfRH5 is susceptible to vaccine-inducible cross-strain neutralizing antibody.* Nat Commun, 2011. **2**: p. 601.
315. Minassian, A.M., et al., *Reduced blood-stage malaria growth and immune correlates in humans following RH5 vaccination.* Med (N Y), 2021. **2**(6): p. 701-719 e19.
316. Nielsen, C.M., et al., *Delayed boosting improves human antigen-specific Ig and B cell responses to the RH5.1/AS01B malaria vaccine.* JCI Insight, 2023. **8**(2).
317. Beeson, J.G., et al., *Parasite adhesion and immune evasion in placental malaria.* Trends Parasitol, 2001. **17**(7): p. 331-7.
318. Fried, M., et al., *Maternal antibodies block malaria.* Nature, 1998. **395**(6705): p. 851-2.
319. Mordmuller, B., et al., *First-in-human, Randomized, Double-blind Clinical Trial of Differentially Adjuvanted PAMVAC, A Vaccine Candidate to Prevent Pregnancy-associated Malaria.* Clin Infect Dis, 2019. **69**(9): p. 1509-1516.
320. Carter, R. and D.H. Chen, *Malaria transmission blocked by immunisation with gametes of the malaria parasite.* Nature, 1976. **263**(5572): p. 57-60.
321. Kapulu, M.C., et al., *Comparative assessment of transmission-blocking vaccine candidates against Plasmodium falciparum.* Sci Rep, 2015. **5**: p. 11193.
322. Miura, K., et al., *Qualification of standard membrane-feeding assay with Plasmodium falciparum malaria and potential improvements for future assays.* PLoS One, 2013. **8**(3): p. e57909.
323. Menon, V., et al., *Assessment of Antibodies Induced by Multivalent Transmission-Blocking Malaria Vaccines.* Front Immunol, 2017. **8**: p. 1998.

324. Read, D., et al., *Transmission-blocking antibodies against multiple, non-variant target epitopes of the Plasmodium falciparum gamete surface antigen Pfs230 are all complement-fixing.* Parasite Immunol, 1994. **16**(10): p. 511-9.
325. Duffy, P.E., *Transmission-Blocking Vaccines: Harnessing Herd Immunity for Malaria Elimination.* Expert Rev Vaccines, 2021. **20**(2): p. 185-198.
326. Delrieu, I., et al., *Design of a Phase III cluster randomized trial to assess the efficacy and safety of a malaria transmission blocking vaccine.* Vaccine, 2015. **33**(13): p. 1518-26.
327. Facciola, A., et al., *An Overview of Vaccine Adjuvants: Current Evidence and Future Perspectives.* Vaccines (Basel), 2022. **10**(5).
328. Apostolico Jde, S., et al., *Adjuvants: Classification, Modus Operandi, and Licensing.* J Immunol Res, 2016. **2016**: p. 1459394.
329. Aguilar, J.C. and E.G. Rodriguez, *Vaccine adjuvants revisited.* Vaccine, 2007. **25**(19): p. 3752-62.
330. Kester, K.E., et al., *Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection.* J Infect Dis, 2009. **200**(3): p. 337-46.
331. Wang, P., *Natural and Synthetic Saponins as Vaccine Adjuvants.* Vaccines (Basel), 2021. **9**(3).
332. Liu, J.K., *The history of monoclonal antibody development - Progress, remaining challenges and future innovations.* Ann Med Surg (Lond), 2014. **3**(4): p. 113-6.
333. Aleshnick, M., et al., *Monoclonal antibodies for malaria prevention.* Mol Ther, 2022. **30**(5): p. 1810-1821.
334. Hansel, T.T., et al., *The safety and side effects of monoclonal antibodies.* Nat Rev Drug Discov, 2010. **9**(4): p. 325-38.
335. Kisalu, N.K., et al., *Author Correction: A human monoclonal antibody prevents malaria infection by targeting a new site of vulnerability on the parasite.* Nat Med, 2019. **25**(1): p. 188-189.
336. Gaudinski, M.R., et al., *A Monoclonal Antibody for Malaria Prevention.* N Engl J Med, 2021. **385**(9): p. 803-814.
337. Kayentao, K., et al., *Safety and Efficacy of a Monoclonal Antibody against Malaria in Mali.* N Engl J Med, 2022. **387**(20): p. 1833-1842.
338. Kuna, A. and M. Gajewski, *Malaria vaccine for travellers - where are we now?* Int Marit Health, 2019. **70**(1): p. 65-67.
339. Teneza-Mora, N., J. Lumsden, and E. Villasante, *A malaria vaccine for travelers and military personnel: Requirements and top candidates.* Vaccine, 2015. **33**(52): p. 7551-8.
340. Plotkin, S.A., *Recent updates on correlates of vaccine-induced protection.* Front Immunol, 2022. **13**: p. 1081107.
341. Kulkarni, P.S., et al., *Establishing Correlates of Protection for Vaccine Development: Considerations for the Respiratory Syncytial Virus Vaccine Field.* Viral Immunol, 2018. **31**(2): p. 195-203.
342. Plotkin, S.A. and P.B. Gilbert, *Nomenclature for immune correlates of protection after vaccination.* Clin Infect Dis, 2012. **54**(11): p. 1615-7.
343. Plotkin, S.A., *Vaccines: correlates of vaccine-induced immunity.* Clin Infect Dis, 2008. **47**(3): p. 401-9.
344. Plotkin, S.A., *Correlates of protection induced by vaccination.* Clin Vaccine Immunol, 2010. **17**(7): p. 1055-65.
345. Altman, N. and M. Krzywinski, *Association, correlation and causation.* Nat Methods, 2015. **12**(10): p. 899-900.
346. Fedak, K.M., et al., *Applying the Bradford Hill criteria in the 21st century: how data integration has changed causal inference in molecular epidemiology.* Emerg Themes Epidemiol, 2015. **12**: p. 14.
347. Hill, A.B., *The environment and disease: association or causation? 1965.* J R Soc Med, 2015. **108**(1): p. 32-7.
348. Hill, A.B., *The Environment and Disease: Association or Causation?* Proc R Soc Med, 1965. **58**(5): p. 295-300.
349. Borrow, R., P. Balmer, and E. Miller, *Meningococcal surrogates of protection--serum bactericidal antibody activity.* Vaccine, 2005. **23**(17-18): p. 2222-7.

350. Frasch, C.E., R. Borrow, and J. Donnelly, *Bactericidal antibody is the immunologic surrogate of protection against meningococcal disease.* Vaccine, 2009. **27 Suppl 2**: p. B112-6.
351. Krammer, F., *A correlate of protection for SARS-CoV-2 vaccines is urgently needed.* Nat Med, 2021. **27**(7): p. 1147-1148.
352. Holmgren, J., et al., *Correlates of protection for enteric vaccines.* Vaccine, 2017. **35**(26): p. 3355-3363.
353. Slifka, M.K., *Immunological memory to viral infection.* Curr Opin Immunol, 2004. **16**(4): p. 443-50.
354. Plotkin, S.A., *Increasing Complexity of Vaccine Development.* J Infect Dis, 2015. **212 Suppl 1**: p. S12-6.
355. Gilbert, P.B., H.E. Janes, and Y. Huang, *Power/sample size calculations for assessing correlates of risk in clinical efficacy trials.* Stat Med, 2016. **35**(21): p. 3745-59.
356. Faber, J. and L.M. Fonseca, *How sample size influences research outcomes.* Dental Press J Orthod, 2014. **19**(4): p. 27-9.
357. White, M.T., et al., *A combined analysis of immunogenicity, antibody kinetics and vaccine efficacy from phase 2 trials of the RTS,S malaria vaccine.* BMC Med, 2014. **12**: p. 117.
358. Dobano, C., et al., *Concentration and avidity of antibodies to different circumsporozoite epitopes correlate with RTS,S/AS01E malaria vaccine efficacy.* Nat Commun, 2019. **10**(1): p. 2174.
359. Chaudhury, S., et al., *Breadth of humoral immune responses to the C-terminus of the circumsporozoite protein is associated with protective efficacy induced by the RTS,S malaria vaccine.* Vaccine, 2021. **39**(6): p. 968-975.
360. Sanchez, L., et al., *Antibody responses to the RTS,S/AS01(E) vaccine and Plasmodium falciparum antigens after a booster dose within the phase 3 trial in Mozambique.* NPJ Vaccines, 2020. **5**: p. 46.
361. Horowitz, A., et al., *Antigen-specific IL-2 secretion correlates with NK cell responses after immunization of Tanzanian children with the RTS,S/AS01 malaria vaccine.* J Immunol, 2012. **188**(10): p. 5054-62.
362. White, M.T., et al., *The relationship between RTS,S vaccine-induced antibodies, CD4(+) T cell responses and protection against Plasmodium falciparum infection.* PLoS One, 2013. **8**(4): p. e61395.
363. Haynes, B.F., et al., *Immune-correlates analysis of an HIV-1 vaccine efficacy trial.* N Engl J Med, 2012. **366**(14): p. 1275-86.
364. Duffy, P.E., *Making a good malaria vaccine better.* Trends Parasitol, 2022. **38**(1): p. 9-10.
365. Das, J., et al., *Delayed fractional dosing with RTS,S/AS01 improves humoral immunity to malaria via a balance of polyfunctional NANP6- and Pf16-specific antibodies.* Med (N Y), 2021. **2**(11): p. 1269-1286 e9.
366. Seaton, K.E., et al., *Subclass and avidity of circumsporozoite protein specific antibodies associate with protection status against malaria infection.* NPJ Vaccines, 2021. **6**(1): p. 110.
367. Pulendran, B., *Systems vaccinology: probing humanity's diverse immune systems with vaccines.* Proc Natl Acad Sci U S A, 2014. **111**(34): p. 12300-6.
368. Kazmin, D., et al., *Systems analysis of protective immune responses to RTS,S malaria vaccination in humans.* Proc Natl Acad Sci U S A, 2017. **114**(9): p. 2425-2430.
369. Suscovich, T.J., et al., *Mapping functional humoral correlates of protection against malaria challenge following RTS,S/AS01 vaccination.* Sci Transl Med, 2020. **12**(553).
370. Kurtovic, L., et al., *Multifunctional Antibodies Are Induced by the RTS,S Malaria Vaccine and Associated With Protection in a Phase 1/2a Trial.* J Infect Dis, 2021. **224**(7): p. 1128-1138.
371. Dekkers, G., et al., *Affinity of human IgG subclasses to mouse Fc gamma receptors.* MAbs, 2017. **9**(5): p. 767-773.
372. Osier, F.H., et al., *Opsonic phagocytosis of Plasmodium falciparum merozoites: mechanism in human immunity and a correlate of protection against malaria.* BMC Med, 2014. **12**: p. 108.
373. Oeuvray, C., et al., *Cytophilic immunoglobulin responses to Plasmodium falciparum glutamate-rich protein are correlated with protection against clinical malaria in Dielmo, Senegal.* Infect Immun, 2000. **68**(5): p. 2617-20.
374. Kibwana, E., M. Kapulu, and P. Bejon, *Controlled Human Malaria Infection Studies in Africa-Past, Present, and Future.* Curr Top Microbiol Immunol, 2022.

375. Stanisic, D.I., J.S. McCarthy, and M.F. Good, *Controlled Human Malaria Infection: Applications, Advances, and Challenges.* Infect Immun, 2018. **86**(1).
376. Cairns, M., et al., *Estimating the potential public health impact of seasonal malaria chemoprevention in African children.* Nat Commun, 2012. **3**: p. 881.
377. Cairns, M.E., et al., *Seasonality in malaria transmission: implications for case-management with long-acting artemisinin combination therapy in sub-Saharan Africa.* Malar J, 2015. **14**: p. 321.
378. Roca-Feltrer, A., et al., *A simple method for defining malaria seasonality.* Malar J, 2009. **8**: p. 276.
379. Greenwood, B., et al., *Seasonal vaccination against malaria: a potential use for an imperfect malaria vaccine.* Malar J, 2017. **16**(1): p. 182.
380. Ashley, E.A. and A. Yeka, *Seasonal malaria chemoprevention: closing the know-do gap.* Lancet, 2020. **396**(10265): p. 1778-1779.
381. WHO, *WHO policy recommendation: seasonal malaria chemoprevention (SMC) for plasmodium falciparum malaria control in highly seasonal transmission areas of the Sahel sub-region in Africa.* 2012: p. 1-4.
382. Suresh, N. and K. Haldar, *Mechanisms of artemisinin resistance in Plasmodium falciparum malaria.* Curr Opin Pharmacol, 2018. **42**: p. 46-54.
383. Greenwood, B., et al., *Resurgent and delayed malaria.* Malar J, 2022. **21**(1): p. 77.
384. Cisse, B., et al., *Effectiveness of Seasonal Malaria Chemoprevention in Children under Ten Years of Age in Senegal: A Stepped-Wedge Cluster-Randomised Trial.* PLoS Med, 2016. **13**(11): p. e1002175.
385. Andrade, C.M., et al., *Increased circulation time of Plasmodium falciparum underlies persistent asymptomatic infection in the dry season.* Nat Med, 2020. **26**(12): p. 1929-1940.
386. Lee, W.C., B. Russell, and L. Renia, *Sticking for a Cause: The Falciparum Malaria Parasites Cytoadherence Paradigm.* Front Immunol, 2019. **10**: p. 1444.
387. Bejon, P., *Malaria parasites hide in plain sight in the dry season.* Nat Med, 2020. **26**(12): p. 1816-1818.
388. Ouedraogo, A.L., et al., *Dynamics of the Human Infectious Reservoir for Malaria Determined by Mosquito Feeding Assays and Ultrasensitive Malaria Diagnosis in Burkina Faso.* J Infect Dis, 2016. **213**(1): p. 90-9.
389. Portugal, S., et al., *Treatment of Chronic Asymptomatic Plasmodium falciparum Infection Does Not Increase the Risk of Clinical Malaria Upon Reinfection.* Clin Infect Dis, 2017. **64**(5): p. 645-653.
390. WHO, *Seasonal malaria chemoprevention with sulfadoxine–pyrimethamine plus amodiaquine in children: a field guide.* WHO Publications, 2023.
391. Bell, G.J., et al., *Malaria Transmission Intensity Likely Modifies RTS, S/AS01 Efficacy Due to a Rebound Effect in Ghana, Malawi, and Gabon.* J Infect Dis, 2022. **226**(9): p. 1646-1656.
392. Spring, M., M. Polhemus, and C. Ockenhouse, *Controlled human malaria infection.* J Infect Dis, 2014. **209 Suppl 2**: p. S40-5.
393. Choy, R.K.M., et al., *Controlled Human Infection Models To Accelerate Vaccine Development.* Clin Microbiol Rev, 2022. **35**(3): p. e0000821.
394. Chavatte, J.M. and G. Snounou, *Controlled human malaria infection-Maker and breaker of dogma.* PLoS Med, 2021. **18**(4): p. e1003591.
395. Moser, K.A., et al., *Strains used in whole organism Plasmodium falciparum vaccine trials differ in genome structure, sequence, and immunogenic potential.* Genome Med, 2020. **12**(1): p. 6.
396. Kapulu, M.C., et al., *Controlled human malaria infection (CHMI) outcomes in Kenyan adults is associated with prior history of malaria exposure and anti-schizont antibody response.* BMC Infect Dis, 2022. **22**(1): p. 86.
397. Stanisic, D.I., et al., *Vaccination with chemically attenuated Plasmodium falciparum asexual blood-stage parasites induces parasite-specific cellular immune responses in malaria-naive volunteers: a pilot study.* BMC Med, 2018. **16**(1): p. 184.
398. Goh, Y.S., D. McGuire, and L. Renia, *Vaccination With Sporozoites: Models and Correlates of Protection.* Front Immunol, 2019. **10**: p. 1227.
399. Walker, M.D., *The last British malaria outbreak.* Br J Gen Pract, 2020. **70**(693): p. 182-183.
400. Health, K.M.o., *Kenya Malaria Indicator Survey 2020.* Government Document, 2020.
401. WHO, *Malaria 2021 Country Profile: Kenya.* 2021.

402. Natama, H.M., et al., *Malaria incidence and prevalence during the first year of life in Nanoro, Burkina Faso: a birth-cohort study.* Malar J, 2018. **17**(1): p. 163.
403. WHO, *Malaria 2021 Country Profile: Burkina Faso.* 2021.
404. Navin Venkatraman, A.B.T., Georgina Bowyer, Jonathan Powlson, Katharine A. Collins, Sam Coulibaly, Mehreen Datoo, Daniel Silman, Alphonse Ouedraogo, Issa Nébié, Egeruan Imoukhuede, Florian Brod, Pedro Folegatti, Emma Dickinson, Sophie Jamieson, Edith C. Bougouma, Daniel Wright, Duncan Bellamy, Amidou Diarra, Carly M. Bliss, Richard Morter, Greg Glenn, Louis F. Fries, Jenny Reimer, Karin Lovgren-Bengtsson, Megan Baker, Ian Poulton, Sarah Moyle, Eleanor Berrie, Nicola Green, Ekta Mukhopadhyay, Nicola Viebig, Brian Angus, Alison Lawrie, Rachel Roberts, Sarah C. Gilbert, David J.M. Lewis, Sodiomon B. Sirima, Katie J. Ewer, Adrian V. S. Hill, *Phase I assessments of first-in-human administration of a novel malaria anti-sporozoite vaccine candidate, R21 in matrix-M adjuvant, in UK and Burkinabe volunteers.* MedRxiv, 2019.
405. Chandramohan, D., et al., *Seasonal malaria vaccination: protocol of a phase 3 trial of seasonal vaccination with the RTS,S/AS01(E) vaccine, seasonal malaria chemoprevention and the combination of vaccination and chemoprevention.* BMJ Open, 2020. **10**(9): p. e035433.
406. Benjamini, Y. and R. Cohen, *Weighted false discovery rate controlling procedures for clinical trials.* Biostatistics, 2017. **18**(1): p. 91-104.
407. Benjamini, Y., et al., *Controlling the false discovery rate in behavior genetics research.* Behav Brain Res, 2001. **125**(1-2): p. 279-84.
408. Doolan, D.L., C. Dobano, and J.K. Baird, *Acquired immunity to malaria.* Clin Microbiol Rev, 2009. **22**(1): p. 13-36, Table of Contents.
409. Harbin, A., N. Laventhal, and M. Navin, *Ethics of age de-escalation in pediatric vaccine trials: Attending to the case of COVID-19.* Vaccine, 2023. **41**(9): p. 1584-1588.
410. Weiss, G.E., et al., *A positive correlation between atypical memory B cells and Plasmodium falciparum transmission intensity in cross-sectional studies in Peru and Mali.* PLoS One, 2011. **6**(1): p. e15983.
411. Greenwood, B.M., et al., *Immunosuppression in children with malaria.* Lancet, 1972. **1**(7743): p. 169-72.
412. Williamson, W.A. and B.M. Greenwood, *Impairment of the immune response to vaccination after acute malaria.* Lancet, 1978. **1**(8078): p. 1328-9.
413. Rodriguez-Galan, A., et al., *An in vitro assay to measure antibody-mediated inhibition of P. berghei sporozoite invasion against P. falciparum antigens.* Sci Rep, 2017. **7**(1): p. 17011.
414. Loubens, M., et al., *Plasmodium sporozoites on the move: Switching from cell traversal to productive invasion of hepatocytes.* Mol Microbiol, 2021. **115**(5): p. 870-881.
415. Yang, A.S.P., et al., *Cell Traversal Activity Is Important for Plasmodium falciparum Liver Infection in Humanized Mice.* Cell Rep, 2017. **18**(13): p. 3105-3116.
416. Sinnis, P., et al., *Quantification of sporozoite invasion, migration, and development by microscopy and flow cytometry.* Methods Mol Biol, 2013. **923**: p. 385-400.
417. Sinnis, P. and A. Coppi, *A long and winding road: the Plasmodium sporozoite's journey in the mammalian host.* Parasitol Int, 2007. **56**(3): p. 171-8.
418. Sinnis, P., et al., *Mosquito heparan sulfate and its potential role in malaria infection and transmission.* J Biol Chem, 2007. **282**(35): p. 25376-84.
419. Formaglio, P., et al., *Loss of host cell plasma membrane integrity following cell traversal by Plasmodium sporozoites in the skin.* Parasitol Int, 2014. **63**(1): p. 237-44.
420. Rampling, T., et al., *Safety and efficacy of novel malaria vaccine regimens of RTS,S/AS01B alone, or with concomitant ChAd63-MVA-vectored vaccines expressing ME-TRAP.* NPJ Vaccines, 2018. **3**: p. 49.
421. Venkatraman, N., et al., *Safety and Immunogenicity of a Heterologous Prime-Boost Ebola Virus Vaccine Regimen in Healthy Adults in the United Kingdom and Senegal.* J Infect Dis, 2019. **219**(8): p. 1187-1197.
422. Rampling, T., et al., *Safety and High Level Efficacy of the Combination Malaria Vaccine Regimen of RTS,S/AS01B With Chimpanzee Adenovirus 63 and Modified Vaccinia Ankara Vectored Vaccines Expressing ME-TRAP.* J Infect Dis, 2016. **214**(5): p. 772-81.

423. Pholcharee, T., et al., *Structural and biophysical correlation of anti-NANP antibodies with in vivo protection against P. falciparum.* Nat Commun, 2021. **12**(1): p. 1063.
424. Hein, I., *Malaria Vaccine Candidate 'Impressive' in Phase III Test.* Med Page Today, 2022.
425. Bowyer, G., et al., *CXCR3(+) T Follicular Helper Cells Induced by Co-Administration of RTS,S/AS01B and Viral-Vectored Vaccines Are Associated With Reduced Immunogenicity and Efficacy Against Malaria.* Front Immunol, 2018. **9**: p. 1660.
426. Roozen, G.V.T., A.H.E. Roukens, and M. Roestenberg, *COVID-19 vaccine dose sparing: strategies to improve vaccine equity and pandemic preparedness.* Lancet Glob Health, 2022. **10**(4): p. e570-e573.
427. Kurtovic, L., et al., *Induction and decay of functional complement-fixing antibodies by the RTS,S malaria vaccine in children, and a negative impact of malaria exposure.* BMC Med, 2019. **17**(1): p. 45.
428. Chaudhury, S., et al., *The biological function of antibodies induced by the RTS,S/AS01 malaria vaccine candidate is determined by their fine specificity.* Malar J, 2016. **15**: p. 301.
429. Chaudhury, S., et al., *Delayed fractional dose regimen of the RTS,S/AS01 malaria vaccine candidate enhances an IgG4 response that inhibits serum opsonophagocytosis.* Sci Rep, 2017. **7**(1): p. 7998.
430. Pallikkuth, S., et al., *A delayed fractionated dose RTS,S AS01 vaccine regimen mediates protection via improved T follicular helper and B cell responses.* Elife, 2020. **9**.
431. Martinez, D.R. and E.E. Ooi, *A potential silver lining of delaying the second dose.* Nat Immunol, 2022. **23**(3): p. 349-351.
432. Hou, M.M., et al., *Impact of a blood-stage vaccine on Plasmodium vivax malaria.* medRxiv, 2022.
433. Simon, A.K., G.A. Hollander, and A. McMichael, *Evolution of the immune system in humans from infancy to old age.* Proc Biol Sci, 2015. **282**(1821): p. 20143085.
434. Mendes, A.M., et al., *Pre-clinical evaluation of a P. berghei-based whole-sporozoite malaria vaccine candidate.* NPJ Vaccines, 2018. **3**: p. 54.
435. Prudencio, M., M.M. Mota, and A.M. Mendes, *A toolbox to study liver stage malaria.* Trends Parasitol, 2011. **27**(12): p. 565-74.
436. Plotkin, S.A., *Immunologic correlates of protection induced by vaccination.* Pediatr Infect Dis J, 2001. **20**(1): p. 63-75.
437. De Gregorio, E. and R. Rappuoli, *Vaccines for the future: learning from human immunology.* Microb Biotechnol, 2012. **5**(2): p. 149-55.
438. WHO. *Vaccine efficacy, effectiveness and protection*. Feature Stories 2021 9th October 2024]; Available from: https://www.who.int/news-room/feature-stories/detail/vaccine-efficacy-effectiveness-and-protection.
439. Regules, J.A., et al., *Fractional Third and Fourth Dose of RTS,S/AS01 Malaria Candidate Vaccine: A Phase 2a Controlled Human Malaria Parasite Infection and Immunogenicity Study.* J Infect Dis, 2016. **214**(5): p. 762-71.
440. Jan, C.F., et al., *Doses of hepatitis B revaccination needed for the seronegative youths to be seropositive to antibody against hepatitis B surface antigen.* Fam Pract, 2020. **37**(1): p. 30-35.
441. Ocan, M., et al., *Antibody levels and protection after Hepatitis B vaccine in adult vaccinated healthcare workers in northern Uganda.* PLoS One, 2022. **17**(1): p. e0262126.
442. WHO, *The Immunological Basis for Immunization Series.* Immunisations, Vaccines and Biologicals, 2012. **Module 22: Hepatitis B**: p. 12.
443. Novavax, *Novavax and Serum Institute of India to Develop and Commercialize Malaria Vaccine Candidate.* 2020.
444. News, O., *Malaria vaccine becomes first to achieve WHO-specified 75% efficacy goal.* Oxford News and Events, 2021.
445. Jin, J., et al., *Accelerating the clinical development of protein-based vaccines for malaria by efficient purification using a four amino acid C-terminal 'C-tag'.* Int J Parasitol, 2017. **47**(7): p. 435-446.
446. Feng, G., et al., *Induction, decay, and determinants of functional antibodies following vaccination with the RTS,S malaria vaccine in young children.* BMC Med, 2022. **20**(1): p. 289.

447. Qin, L., et al., *A framework for assessing immunological correlates of protection in vaccine trials.* J Infect Dis, 2007. **196**(9): p. 1304-12.
448. Matuschewski, K. and S. Borrmann, *Controlled Human Malaria Infection (CHMI) Studies: Over 100 Years of Experience with Parasite Injections.* Methods Mol Biol, 2019. **2013**: p. 91-101.
449. Moormann, A.M. and V.A. Stewart, *The hunt for protective correlates of immunity to Plasmodium falciparum malaria.* BMC Med, 2014. **12**: p. 134.
450. Thompson, R.F., D.M. Bass, and S.L. Hoffman, *Travel vaccines.* Infect Dis Clin North Am, 1999. **13**(1): p. 149-67, vii.
451. Batista, F.D. and M.S. Neuberger, *Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate.* Immunity, 1998. **8**(6): p. 751-9.
452. Ubillos, I., et al., *Optimization of incubation conditions of Plasmodium falciparum antibody multiplex assays to measure IgG, IgG(1-4), IgM and IgE using standard and customized reference pools for sero-epidemiological and vaccine studies.* Malar J, 2018. **17**(1): p. 219.
453. Silva, P.J.S., et al., *Optimized delay of the second COVID-19 vaccine dose reduces ICU admissions.* Proc Natl Acad Sci U S A, 2021. **118**(35).
454. Samuels, A.M., et al., *Efficacy of RTS,S/AS01(E) malaria vaccine administered according to different full, fractional, and delayed third or early fourth dose regimens in children aged 5-17 months in Ghana and Kenya: an open-label, phase 2b, randomised controlled trial.* Lancet Infect Dis, 2022. **22**(9): p. 1329-1342.
455. Goldblatt, D., A.R. Vaz, and E. Miller, *Antibody avidity as a surrogate marker of successful priming by Haemophilus influenzae type b conjugate vaccines following infant immunization.* J Infect Dis, 1998. **177**(4): p. 1112-5.
456. Thompson, H.A., et al., *Modelling the roles of antibody titre and avidity in protection from Plasmodium falciparum malaria infection following RTS,S/AS01 vaccination.* Vaccine, 2020. **38**(47): p. 7498-7507.
457. Olotu, A., et al., *Avidity of anti-circumsporozoite antibodies following vaccination with RTS,S/AS01E in young children.* PLoS One, 2014. **9**(12): p. e115126.
458. Ajua, A., et al., *The effect of immunization schedule with the malaria vaccine candidate RTS,S/AS01E on protective efficacy and anti-circumsporozoite protein antibody avidity in African infants.* Malar J, 2015. **14**: p. 72.
459. Mugo, R.M., et al., *Seven-year kinetics of RTS, S/AS01-induced anti-CSP antibodies in young Kenyan children.* Malar J, 2021. **20**(1): p. 452.
460. Owusu-Agyei, S., et al., *Randomized controlled trial of RTS,S/AS02D and RTS,S/AS01E malaria candidate vaccines given according to different schedules in Ghanaian children.* PLoS One, 2009. **4**(10): p. e7302.
461. Bejon, P., et al., *Effect of the pre-erythrocytic candidate malaria vaccine RTS,S/AS01E on blood stage immunity in young children.* J Infect Dis, 2011. **204**(1): p. 9-18.
462. Ndungu, F.M., et al., *A seven-year study on the effect of the pre-erythrocytic malaria vaccine candidate RTS,S/AS01 (E) on blood stage immunity in young Kenyan children.* Wellcome Open Res, 2019. **4**: p. 42.
463. Ockenhouse, C.F., et al., *Ad35.CS.01-RTS,S/AS01 Heterologous Prime Boost Vaccine Efficacy against Sporozoite Challenge in Healthy Malaria-Naive Adults.* PLoS One, 2015. **10**(7): p. e0131571.
464. Yusuf, Y., et al., *A Viral-Vectored Multi-Stage Malaria Vaccine Regimen With Protective and Transmission-Blocking Efficacies.* Front Immunol, 2019. **10**: p. 2412.
465. Sachs, J. and P. Malaney, *The economic and social burden of malaria.* Nature, 2002. **415**(6872): p. 680-5.
466. Aremu, T.O., et al., *Assessing Public Awareness of the Malaria Vaccine in Sub-Saharan Africa.* Trop Med Infect Dis, 2022. **7**(9).
467. Natama, H.M., et al., *Modulation of innate immune responses at birth by prenatal malaria exposure and association with malaria risk during the first year of life.* BMC Med, 2018. **16**(1): p. 198.
468. Olotu, A., et al., *Seven-Year Efficacy of RTS,S/AS01 Malaria Vaccine among Young African Children.* N Engl J Med, 2016. **374**(26): p. 2519-29.

469. McCall, M.B.B., P.G. Kremsner, and B. Mordmuller, *Correlating efficacy and immunogenicity in malaria vaccine trials.* Semin Immunol, 2018. **39**: p. 52-64.
470. WHO, *Burkina Faso Malaria Surveilannce.* 2017.
471. Wang, C., et al., *The circadian immune system.* Sci Immunol, 2022. **7**(72): p. eabm2465.
472. Dicko, A., et al., *Seasonal vaccination with RTS,S/AS01(E) vaccine with or without seasonal malaria chemoprevention in children up to the age of 5 years in Burkina Faso and Mali: a double-blind, randomised, controlled, phase 3 trial.* Lancet Infect Dis, 2023.
473. Thompson, H.A., et al., *Seasonal use case for the RTS,S/AS01 malaria vaccine: a mathematical modelling study.* Lancet Glob Health, 2022. **10**(12): p. e1782-e1792.
474. Sagara, I., et al., *The Anti-Circumsporozoite Antibody Response of Children to Seasonal Vaccination With the RTS,S/AS01E Malaria Vaccine.* Clin Infect Dis, 2022. **75**(4): p. 613-622.
475. Lambert, P.H., M. Liu, and C.A. Siegrist, *Can successful vaccines teach us how to induce efficient protective immune responses?* Nat Med, 2005. **11**(4 Suppl): p. S54-62.
476. Rudnick, S.I. and G.P. Adams, *Affinity and avidity in antibody-based tumor targeting.* Cancer Biother Radiopharm, 2009. **24**(2): p. 155-61.
477. Rudnick, S.I., et al., *Influence of affinity and antigen internalization on the uptake and penetration of Anti-HER2 antibodies in solid tumors.* Cancer Res, 2011. **71**(6): p. 2250-9.
478. Dobano, C., et al., *RTS,S/AS01E immunization increases antibody responses to vaccine-unrelated Plasmodium falciparum antigens associated with protection against clinical malaria in African children: a case-control study.* BMC Med, 2019. **17**(1): p. 157.
479. Nutt, S.L., et al., *The generation of antibody-secreting plasma cells.* Nat Rev Immunol, 2015. **15**(3): p. 160-71.
480. Dennison, S.M., et al., *Qualified Biolayer Interferometry Avidity Measurements Distinguish the Heterogeneity of Antibody Interactions with Plasmodium falciparum Circumsporozoite Protein Antigens.* J Immunol, 2018. **201**(4): p. 1315-1326.
481. Tomaras, G.D., et al., *Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia.* J Virol, 2008. **82**(24): p. 12449-63.
482. Tan, J., et al., *Functional human IgA targets a conserved site on malaria sporozoites.* Sci Transl Med, 2021. **13**(599).
483. Boyle, M.J., et al., *IgM in human immunity to Plasmodium falciparum malaria.* Sci Adv, 2019. **5**(9): p. eaax4489.
484. Zenklusen, I., et al., *Immunization of Malaria-Preexposed Volunteers With PfSPZ Vaccine Elicits Long-Lived IgM Invasion-Inhibitory and Complement-Fixing Antibodies.* J Infect Dis, 2018. **217**(10): p. 1569-1578.
485. WHO, *Vaccination schedule for Burkina Faso.* 2022.
486. Zhang, X., et al., *Effects of time of day and taxi route complexity on navigation errors: An experimental study.* Accid Anal Prev, 2019. **125**: p. 14-19.
487. Labrecque, N. and N. Cermakian, *Circadian Clocks in the Immune System.* J Biol Rhythms, 2015. **30**(4): p. 277-90.
488. de Bree, L.C.J., et al., *Circadian rhythm influences induction of trained immunity by BCG vaccination.* J Clin Invest, 2020. **130**(10): p. 5603-5617.
489. Long, J.E., et al., *Morning vaccination enhances antibody response over afternoon vaccination: A cluster-randomised trial.* Vaccine, 2016. **34**(24): p. 2679-85.
490. Fernandes, G., et al., *Circadian rhythmic plaque-forming cell response of spleens from mice immunized with SRBC.* J Immunol, 1976. **117**(3): p. 962-6.
491. Gessner, B.D., et al., *Could the RTS,S/AS01 meningitis safety signal really be a protective effect of rabies vaccine?* Vaccine, 2017. **35**(5): p. 716-721.
492. Kc, N., et al., *Increased levels of anti-PfCSP antibodies in post-pubertal females versus males immunized with PfSPZ Vaccine does not translate into increased protective efficacy.* Front Immunol, 2022. **13**: p. 1006716.
493. Young, J.F., W.R. Ballou, and W.T. Hockmeyer, *Developing a human malaria sporozoite vaccine.* Microb Pathog, 1987. **2**(4): p. 237-40.

494. Ballou, W.R. and C.P. Cahill, *Two decades of commitment to malaria vaccine development: GlaxoSmithKline Biologicals.* Am J Trop Med Hyg, 2007. **77**(6 Suppl): p. 289-95.
495. Maxmen, A., *Scientists hail historic malaria vaccine approval - but point to challenges ahead.* Nature, 2021.
496. Furrer, E., *Will RTS,S/AS01 be the first malaria vaccine recommended as part of routine immunization to reduce illness and death in African children?* American Society of Tropical Medicine and Hygiene, 2021. **Annual Meeting**(Symposium 76).
497. Hogan, A.B., P. Winskill, and A.C. Ghani, *Estimated impact of RTS,S/AS01 malaria vaccine allocation strategies in sub-Saharan Africa: A modelling study.* PLoS Med, 2020. **17**(11): p. e1003377.
498. WHO, *WHO welcomes historic decision by Gavi to fund the first malaria vaccine.* 2021. **News Release**.
499. Willyard, C., *The slow roll-out of the world's first malaria vaccine.* Nature, 2022. **612**(7941): p. S48-S49.
500. GSK. *GSK, PATH, and Bharat Biotech sign product transfer agreement to help ensure long-term supply of RTS,S/AS01E malaria vaccine*. 2021 9th October 2023]; Available from: https://www.gsk.com/en-gb/media/press-releases/gsk-path-and-bharat-biotech-sign-product-transfer-agreement-to-help-ensure-long-term-supply-of-rts-sas01e-malaria-vaccine/.
501. Greenwood, B., et al., *Combining malaria vaccination with chemoprevention: a promising new approach to malaria control.* Malar J, 2021. **20**(1): p. 361.
502. Zongo, I., et al., *Randomized Noninferiority Trial of Dihydroartemisinin-Piperaquine Compared with Sulfadoxine-Pyrimethamine plus Amodiaquine for Seasonal Malaria Chemoprevention in Burkina Faso.* Antimicrob Agents Chemother, 2015. **59**(8): p. 4387-96.
503. Tinto, H., et al., *Long-term incidence of severe malaria following RTS,S/AS01 vaccination in children and infants in Africa: an open-label 3-year extension study of a phase 3 randomised controlled trial.* Lancet Infect Dis, 2019. **19**(8): p. 821-832.
504. Clement, F., et al., *Validation of an enzyme-linked immunosorbent assay for the quantification of human IgG directed against the repeat region of the circumsporozoite protein of the parasite Plasmodium falciparum.* Malar J, 2012. **11**: p. 384.
505. Hill, A., *The R21/Matrix-M malaria vaccine: Results from a phase III trial and future plans.* American Society of Tropical Medicine & Hygiene, 2022. **Trop Med**(LB-5492).
506. Heath, P.T., et al., *Safety and Efficacy of NVX-CoV2373 Covid-19 Vaccine.* N Engl J Med, 2021. **385**(13): p. 1172-1183.
507. Shinde, V., et al., *Comparison of the safety and immunogenicity of a novel Matrix-M-adjuvanted nanoparticle influenza vaccine with a quadrivalent seasonal influenza vaccine in older adults: a phase 3 randomised controlled trial.* Lancet Infect Dis, 2022. **22**(1): p. 73-84.
508. Kurtovic, L., M.J. Boyle, and J.G. Beeson, *Epitope masking may limit antibody boosting to malaria vaccines.* Immunol Cell Biol, 2021. **99**(2): p. 126-129.
509. Attenello, F.J., et al., *Suboccipital decompression for Chiari malformation-associated scoliosis: risk factors and time course of deformity progression.* J Neurosurg Pediatr, 2008. **1**(6): p. 456-60.
510. Magnusson, S.E., et al., *Matrix-M adjuvant enhances immunogenicity of both protein- and modified vaccinia virus Ankara-based influenza vaccines in mice.* Immunol Res, 2018. **66**(2): p. 224-233.
511. Valea, I., et al., *Immune response to the hepatitis B antigen in the RTS,S/AS01 malaria vaccine, and co-administration with pneumococcal conjugate and rotavirus vaccines in African children: A randomized controlled trial.* Hum Vaccin Immunother, 2018. **14**(6): p. 1489-1500.
512. Macete, E., et al., *Safety and immunogenicity of the RTS,S/AS02A candidate malaria vaccine in children aged 1-4 in Mozambique.* Trop Med Int Health, 2007. **12**(1): p. 37-46.
513. Valea, I., et al., *Long-term immunogenicity and immune memory response to the hepatitis B antigen in the RTS,S/AS01(E) malaria vaccine in African children: a randomized trial.* Hum Vaccin Immunother, 2020. **16**(6): p. 1464-1470.
514. Macete, E.V., et al., *Evaluation of two formulations of adjuvanted RTS, S malaria vaccine in children aged 3 to 5 years living in a malaria-endemic region of Mozambique: a Phase I/IIb randomized double-blind bridging trial.* Trials, 2007. **8**: p. 11.

515. Boytchev, H., *Why did a German newspaper insist the Oxford AstraZeneca vaccine was inefficacious for older people—without evidence?* BMJ, 2021. **372**(Medicine and the Media).
516. Ledford, H., *Malaria vaccine booster prolongs protection.* Nature, 2022.
517. WHO, *World Malaria Report.* 2019.
518. Dhiman, S., *Correction to: Are malaria elimination efforts on right track? An analysis of gains achieved and challenges ahead.* Infect Dis Poverty, 2019. **8**(1): p. 19.
519. White, N.J., et al., *Have we really failed to roll back malaria?* Lancet, 2022. **399**(10327): p. 799-800.
520. Draper, S.J., et al., *Malaria Vaccines: Recent Advances and New Horizons.* Cell Host Microbe, 2018. **24**(1): p. 43-56.
521. Giefing-Kroll, C., et al., *How sex and age affect immune responses, susceptibility to infections, and response to vaccination.* Aging Cell, 2015. **14**(3): p. 309-21.
522. Bowyer, G., et al., *Reduced Ebola vaccine responses in CMV+ young adults is associated with expansion of CD57+KLRG1+ T cells.* J Exp Med, 2020. **217**(7).
523. Nouatin, O., et al., *Exploratory analysis of the effect of helminth infection on the immunogenicity and efficacy of the asexual blood-stage malaria vaccine candidate GMZ2.* PLoS Negl Trop Dis, 2021. **15**(6): p. e0009361.
524. Hill, D.L., et al., *Immune system development varies according to age, location, and anemia in African children.* Sci Transl Med, 2020. **12**(529).
525. Kollmann, T.R., et al., *Protecting the Newborn and Young Infant from Infectious Diseases: Lessons from Immune Ontogeny.* Immunity, 2017. **46**(3): p. 350-363.
526. Chora, A.F., M.M. Mota, and M. Prudencio, *The reciprocal influence of the liver and blood stages of the malaria parasite's life cycle.* Int J Parasitol, 2022. **52**(11): p. 711-715.
527. Adkins, B., C. Leclerc, and S. Marshall-Clarke, *Neonatal adaptive immunity comes of age.* Nat Rev Immunol, 2004. **4**(7): p. 553-64.
528. JL, N.D., et al., *Correction: Safety of Seasonal Malaria Chemoprevention (SMC) with Sulfadoxine-Pyrimethamine plus Amodiaquine when Delivered to Children under 10 Years of Age by District Health Services in Senegal: Results from a Stepped-Wedge Cluster Randomized Trial.* PLoS One, 2016. **11**(12): p. e0168421.
529. Baird, J.K., et al., *Seasonal malaria attack rates in infants and young children in northern Ghana.* Am J Trop Med Hyg, 2002. **66**(3): p. 280-6.
530. Silal, S.P., *Seasonal targeting of the RTS,S/AS01 malaria vaccine: a complementary tool but sustained funding is required.* Lancet Glob Health, 2022. **10**(12): p. e1693-e1694.
531. Chatterjee, D. and I.A. Cockburn, *The challenges of a circumsporozoite protein-based malaria vaccine.* Expert Rev Vaccines, 2021. **20**(2): p. 113-125.
532. Zarocostas, J., *Gavi unveils malaria vaccine plans.* Lancet, 2023. **401**(10387): p. 1485.
533. Garcia, C.R., et al., *Comparative cost models of a liquid nitrogen vapor phase (LNVP) cold chain-distributed cryopreserved malaria vaccine vs. a conventional vaccine.* Vaccine, 2013. **31**(2): p. 380-6.
534. Naddaf, M., *Second malaria vaccine to win global approval is cheaper and easier to make.* Nature, 2023.
535. Mahase, E., *WHO recommends second vaccine for malaria prevention in children.* BMJ, 2023. **383**: p. p2291.
536. White, M.T. and D.L. Smith, *Synergism from combinations of infection-blocking malaria vaccines.* Malar J, 2013. **12**: p. 280.
537. Bauza, K., et al., *Tailoring a Combination Preerythrocytic Malaria Vaccine.* Infect Immun, 2015. **84**(3): p. 622-34.
538. Insel, R.A., *Potential alterations in immunogenicity by combining or simultaneously administering vaccine components.* Ann N Y Acad Sci, 1995. **754**: p. 35-47.
539. Jatana, S.K. and M. Nair, *Combination Vaccines.* Med J Armed Forces India, 2007. **63**(2): p. 167-71.
540. Asante, K.P., et al., *Immunogenicity and safety of the RTS,S/AS01 malaria vaccine co-administered with measles, rubella and yellow fever vaccines in Ghanaian children: A phase IIIb,*

multi-center, non-inferiority, randomized, open, controlled trial. Vaccine, 2020. **38**(18): p. 3411-3421.
541. EMA. *NOTE FOR GUIDANCE ON VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY* 1995; Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002662.pdf.
542. consortium, O., et al., *Towards validated assays for key immunological outcomes in malaria vaccine development.* Vaccine, 2011. **29**(17): p. 3093-5.
543. WHO, *Essential Programme on Immunisation.* 2022.
544. Mukhopadhyay, E., et al., *Production of a high purity, C-tagged hepatitis B surface antigen fusion protein VLP vaccine for malaria expressed in Pichia pastoris under cGMP conditions.* Biotechnol Bioeng, 2022. **119**(10): p. 2784-2793.
545. Bengtsson, K.L., et al., *Matrix-M adjuvant: enhancing immune responses by 'setting the stage' for the antigen.* Expert Rev Vaccines, 2013. **12**(8): p. 821-3.
546. Toback, S., et al., *Safety, immunogenicity, and efficacy of a COVID-19 vaccine (NVX-CoV2373) co-administered with seasonal influenza vaccines: an exploratory substudy of a randomised, observer-blinded, placebo-controlled, phase 3 trial.* Lancet Respir Med, 2022. **10**(2): p. 167-179.
547. Heath, P.T., et al., *Safety and Efficacy of the NVX-CoV2373 COVID-19 Vaccine at Completion of the Placebo-Controlled Phase of a Randomized Controlled Trial.* Clin Infect Dis, 2022.
548. Dicks, M.D., et al., *A novel chimpanzee adenovirus vector with low human seroprevalence: improved systems for vector derivation and comparative immunogenicity.* PLoS One, 2012. **7**(7): p. e40385.
549. Clyde, D.F., et al., *Specificity of protection of man immunized against sporozoite-induced falciparum malaria.* Am J Med Sci, 1973. **266**(6): p. 398-403.
550. Sauerwein, R.W., M. Roestenberg, and V.S. Moorthy, *Experimental human challenge infections can accelerate clinical malaria vaccine development.* Nat Rev Immunol, 2011. **11**(1): p. 57-64.
551. Friedman-Klabanoff, D.J., et al., *The Controlled Human Malaria Infection Experience at the University of Maryland.* Am J Trop Med Hyg, 2019. **100**(3): p. 556-565.
552. Chulay, J.D., et al., *Malaria transmitted to humans by mosquitoes infected from cultured Plasmodium falciparum.* Am J Trop Med Hyg, 1986. **35**(1): p. 66-8.
553. Takala, S.L. and C.V. Plowe, *Genetic diversity and malaria vaccine design, testing and efficacy: preventing and overcoming 'vaccine resistant malaria'.* Parasite Immunol, 2009. **31**(9): p. 560-73.
554. Genton, B., et al., *Randomized double-blind controlled Phase I/IIa trial to assess the efficacy of malaria vaccine PfCS102 to protect against challenge with P. falciparum.* Vaccine, 2010. **28**(40): p. 6573-80.
555. Laurens, M.B., et al., *A consultation on the optimization of controlled human malaria infection by mosquito bite for evaluation of candidate malaria vaccines.* Vaccine, 2012. **30**(36): p. 5302-4.
556. *What Happens to Donated Blood.* American Red Cross, 2023.
557. McConkey, S.J., et al., *Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans.* Nat Med, 2003. **9**(6): p. 729-35.
558. Harris, S.A., et al., *Process of assay selection and optimization for the study of case and control samples from a phase IIb efficacy trial of a candidate tuberculosis vaccine, MVA85A.* Clin Vaccine Immunol, 2014. **21**(7): p. 1005-11.