Developing a novel vaccine for the prevention of severe disease caused by respiratory syncytial virus

Christopher A Green

The Queen's College, University of Oxford

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Supervisors:

Professors Andrew J Pollard and Paul Klenerman

Abstract

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Christopher A. Green The Queen's College, University of Oxford
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Respiratory syncytial virus, or RSV, is a major global pathogen responsible for severe respiratory disease in infants, immune-compromised adults and the elderly. Infants suffer the greatest burden of disease, with up to 1 in 6 paediatric beds required each winter for cases of bronchiolitis (up to 80% caused by RSV) and 2-6% of these admissions require paediatric intensive care support. In resource poor areas of the world RSV is second only to malaria in all-cause infant mortality between the first month and first year of life. Re-infection occurs throughout life and severe immune compromise re-establishes a risk of severe disease and death, with outbreak mortality as high as 80% in haematopoietic stem cell transplant patients. Immune senescence and co-morbidities results in a 10% mortality from RSV in the elderly where estimates of hospital disease burden and death from RSV appear comparable to seasonal influenza. Despite over 50-years of clinical trials there remains no effective treatment for RSV infection and no vaccine to prevent severe disease.

The work presented in this thesis uses a logical path towards the evaluation of a novel biological platform applied as an RSV vaccine candidate for the first time. Beginning with defining the epidemiological burden of disease, time-trend analyses from multiple hospital admission datasets reported a progressive rise in emergency hospital admission rates for infants with bronchiolitis in England, and this has been sustained year-on-year over the last decade and associated with a wide variability in admission rates across regions. The next step was to further define the problem of immunity that protects from severe disease but one that fails to protect from re-infection throughout life despite repeated seasonal exposure. The first of two observational studies showed the quantity of infant RSV G-antibody and IFNγ-producing T-cells lags behind the development of RSV F-specific antibody development seen in children. The second observational study used paediatric healthcare workers and adults of presumed lesser RSV exposure, and observed the failure to maintain total and RSV F-protein specific memory IgA B-cells in peripheral circulation after the winter months of RSV transmission. The remainder of this thesis concentrates on the design, setup, execution and analysis of a phase I (first-in-man) clinical trial of two novel genetic RSV vaccine candidates in healthy younger and older adults. Chimpanzee adenovirus (PanAd3) and modified vaccinia virus Ankara (MVA) were used as genetically-modified viral vectors for the delivery of RSV antigen. The vaccines were safe, well tolerated, and proved capable of inducing desirable humoral and cellular immunity to RSV despite robust pre-existing natural immunity and PanAd3 (vector) neutralising antibody. Final explorative analysis describes the early host genetic response to vaccination with correlation to important clinical and immunological responses measured at later time points. Viral-vectored vaccines for RSV have now entered clinical stage evaluation and these data represent a new and promising approach towards meeting this global need.

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It is with great gratitude that Professor Andrew Pollard gave me the opportunity to work at the Oxford Vaccine Group and later enrol as his DPhil student through which he has supported me throughout. I have learned so much about clinical trials, RSV and myself that I fear I am now indebted to Andrew in ways I might never be able to repay. My co-supervisor, Professor Paul Klenerman, has been a parallel source of calm, guidance and support throughout. In no small measure I am grateful for the friendship, guidance and simply good times with Charles Sande, Thomas Darton, Amber Thompson, Cathy de Lara, Slywester Pawluk and Christoph Blohmke in particular. Amber, Charles and Cathy somehow never tired of teaching me how to perform proper scientific experiments when it was clear I had little natural aptitude for them. Indeed there is a large family that at Oxford, one that is too large to mention individually, to whom I am forever grateful for the friendship, guidance and support I have received during my studies. The research presented here would not have been possible without them, the engagement of collaborators in Italy who trusted us with the next phase of their hard work, and the many members of the public that were so willing to play their part in supporting this work and who entrusted their wellbeing with us to translate these vaccines into man for the first time.

This DPhil and the work it represents over the last four years comes from a lifelong endurance of unfaltering love and support from my parents Bob and Arely, grandmother Doña Marina, and the love and support of Rebecca with whom I look forward to starting the next (married) chapter of our lives together. The success of this research is as much their success as it is mine, and it is with sadness that Fidel, Robert and Clara were unable to witness what would have been their success too. Finally, I wish to thank Professor Susanne Cholerton, Dr Shirley Varnish, Dr John Frampton and James Heath who were instrumental at critical times of my life and who helped me to be where I am today.

Publications arising from this thesis

The following contributions to the published literature were born as a result of my work presented in this thesis. Where results and sections of prose are identical in print and in this thesis, the work represents my own contribution to the publication and not that of co-authors.

Published papers

CA Green, E Scarselli, CJ Sande, AJ Thompson, CM de Lara, K Taylor, K Haworth, M Del Sorbo, B Angus, L Siani, S Di Marco, C Traboni, A Folgori, S Colloca, S Capone, A Vitelli, R Cortese, P Klenerman, A Nicosia, AJ Pollard.

Chimpanzee adenovirus and MVA-vectored respiratory syncytial virus vaccine is safe and immunogenic in adults

Science Translational Medicine 2015 Aug 12;7(300):300ra126.[1]

Abstract

Respiratory syncytial virus (RSV) causes respiratory infection in annual epidemics, with infants and the elderly at particular risk of developing severe disease and death. However, despite its importance, no vaccine exists. The chimpanzee adenovirus, PanAd3-RSV, and modified vaccinia virus Ankara, MVA-RSV, are replication defective viral vectors encoding the RSV proteins F, N and M2-1 for the induction of humoral and cellular responses. We performed an open-label, dose-escalation, phase 1 clinical trial in 42 healthy adults in which four different combinations of prime/boost vaccinations were investigated for safety and immunogenicity, including both intra-muscular and intra-nasal administration of the adenoviral vectored vaccine. The vaccines were safe and well tolerated, with the most common reported adverse events being mild injection site reactions. No vaccine-related serious adverse events occurred. RSV neutralising antibody titres rose in response to intramuscular (IM) prime with PanAd3-RSV, and after IM boost for individuals primed by the intra-nasal (IN) route. Circulating anti-F IgG and IgA antibody secreting cells (ASCs) were observed after IM prime and IM boost. RSV-specific T-cell responses were increased after IM PanAd3-RSV prime and were most efficiently boosted by IM MVA-RSV. IFNγ secretion after boost was from both CD4+ and CD8+ T-cells, without detectable Th2 cytokines that have been previously associated with immune pathogenesis following exposure to RSV after formalin inactivated RSV vaccine. In conclusion, PanAd3-RSV and MVA-RSV are safe and immunogenic in healthy adults. These vaccine candidates warrant further clinical evaluation of efficacy to assess their potential to reduce the burden of RSV disease.

Trial registration number: NCT01805921.

CA Green, D Yeates, A Goldacre, C Sande, RC Parslow, P McShane, AJ Pollard, M Goldacre.

Admission to hospital for bronchiolitis in England: trends over five decades, geographical variation and association with perinatal characteristics and subsequent asthma.

Archives of Disease in Childhood 2016 Feb; 101(2):140-6. [2]

Abstract

Background: Admission of infants to hospital with bronchiolitis consumes considerable healthcare resources each winter. We report an analysis of hospital admissions in England over five decades.

Methods: Data were analysed from the Hospital In-Patient Enquiry (HIPE, 1968-1985), Hospital Episode Statistics (HES, 1989-2011), Oxford Record Linkage Study (ORLS, 1963-2011) and Paediatric Intensive Care Audit Network (PICANet, 2003-2012). Cases were identified using International Classification of Diseases (ICD) codes in discharge records. Bronchiolitis was given a separate code in ICD9 (used in England from 1979). Geographical variation was analysed using Local Authority area boundaries. Maternal and perinatal risk factors associated with bronchiolitis and subsequent admissions for asthma were analysed using record-linkage.

Results: All-England HIPE and HES data recorded 468 138 episodes of admission for bronchiolitis in infants aged <1 year between 1979 and 2011. In 2011 the estimated annual hospital admission rate was 46.1 (95% CI 45.6 to 46.6) per 1000 infants aged <1 year. Between 2004 and 2011 the rates rose by an average of 1.8% per year in the all-England HES data, whereas admission rates to paediatric intensive care changed little (1.3 to 1.6 per 1000 infants aged <1 year). A fivefold geographical variation in hospital admission rates was observed. Young maternal age, low social class, low birth weight and maternal smoking were among factors associated with an increased risk of hospital admission with bronchiolitis.

Conclusions: Hospital admissions for infants with bronchiolitis have increased substantially in recent years. However, cases requiring intensive care have changed little since 2004.

CA Green, E Scarselli, M Voysey, S Capone, A Vitelli, A Nicosia, R Cortese, AJ Thompson, CS Sande, C de Lara, P Klenerman, AJ Pollard.

Safety and immunogenicity of novel respiratory syncytial virus (RSV) vaccines based on the RSV viral proteins F, N and M2-1 encoded by simian adenovirus (PanAd3-RSV) and MVA (MVA-RSV); protocol for an open-label, dose-escalation, single-centre, phase 1 clinical trial in healthy adults.

BMJ Open 2015 Oct 28;5(10):e008748. [3]

Abstract

Introduction: Respiratory syncytial virus (RSV) infection causes respiratory disease throughout life, with infants and the elderly at risk of severe disease and death. RSV001 is a phase 1 (first-in-man), open-label, dose-escalation, clinical trial of novel genetic viral-vectored vaccine candidates PanAd3-RSV and modified vaccinia virus Ankara (MVA)-RSV. The objective of RSV001 is to characterise the (primary objective) safety and (secondary objective) immunogenicity of these vaccines in healthy younger and older adults.

Methods and analyses: Heterologous and homologous 'prime'/boost combinations of PanAd3-RSV and single-dose MVA-RSV are evaluated in healthy adults. 40 healthy adults aged 18-50 years test one of four combinations of intramuscular (IM) or intranasal (IN) PanAd3-RSV prime and IM PanAd3 or IM MVA-RSV boost vaccination, starting at a low dose for safety. The following year an additional 30 healthy adults aged 60-75 years test either a single dose of IM MVA-RSV, one of three combinations of IN or IM PanAd3-RSV prime and PanAd3-RSV or MVA-RSV boost vaccination used in younger volunteers, and a non-vaccinated control group. Study participants are self-selected volunteers who satisfy the eligibility criteria and are assigned to study groups by sequential allocation. Safety assessment includes the daily recording of solicited and unsolicited adverse events for 1 week after vaccination, as well as visit (nursing) observations and safety bloods obtained at all scheduled attendances. Laboratory measures of RSV-specific humoral and cellular immune responses after vaccination will address the secondary end points. All study procedures are performed at the Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), Oxford, UK.

Ethics and dissemination: RSV001 has clinical trial authorisation from the Medicines and Healthcare Products Regulatory Agency (MHRA) and ethics approval from NRES Berkshire (reference 13/SC/0023). All study procedures adhere to International Conference on Harmonisation (ICH) Good Clinical Practice guidelines. The results of the trial are to be published in peer-reviewed journals, conferences and academic forums.

Trial registration number: NCT01805921.

SB Drysdale, CJ Sande, **CA Green**, AJ Pollard.

RSV vaccine use – the missing data

Expert Review of Vaccines 2016;15(2):149-52.[4]

Abstract

Respiratory syncytial virus (RSV) infection is the most important cause of hospitalization in infants and is one of the leading global causes of infant mortality and as such its prevention through vaccination is a public health priority. While essential for the successful implementation of vaccine programs, there remains a paucity of data on the epidemiology of the virus in different settings and age groups and limited knowledge about virus transmission and the health-care costs of the disease. Such data are now needed to populate health economic models and to inform optimal approaches to disease control through vaccination.

SB Drysdale, **CA Green**, CJ Sande.

Best practice in the prevention and management of paediatric respiratory syncytial virus infection

Therapeutic Advances in Infectious Diseases 2016 Apr;3(2):63-71.[5]

Abstract

Respiratory syncytial virus (RSV) infection is ubiquitous with almost all infants having been infected by 2 years of age and lifelong repeated infections common. It is the second largest cause of mortality, after malaria, in infants outside the neonatal period and causes up to 200,000 deaths per year worldwide. RSV results in clinical syndromes that include upper respiratory tract infections, otitis media, bronchiolitis (up to 80% of cases) and lower respiratory tract disease including pneumonia and exacerbations of asthma or viral-induced wheeze. For the purposes of this review we will focus on RSV bronchiolitis in infants in whom the greatest disease burden lies. For infants requiring hospital admission, the identification of the causative respiratory virus is used to direct cohorting or isolation and infection control procedures to minimize nosocomial transmission. Nosocomial RSV infections are associated with poorer clinical outcomes, including increased mortality, the need for mechanical ventilation and longer length of hospital stay. Numerous clinical guidelines for the management of infants with bronchiolitis have been published, although none are specific for RSV bronchiolitis. Ribavirin is the only licensed drug for the specific treatment of RSV infection but due to drug toxicity and minimal clinical benefit it has not been recommended for routine clinical use. There is currently no licensed vaccine to prevent RSV infection but passive immunoprophylaxis using a monoclonal antibody, palivizumab, reduces the risk of hospitalization due to RSV infection by 39-78% in various high-risk infants predisposed to developing severe RSV disease. The current management of RSV bronchiolitis is purely supportive, with feeding support and oxygen supplementation until the infant immune system mounts a response capable of controlling the disease. The development of a successful treatment or prophylactic agent has the potential to revolutionize the care and outcome for severe RSV infections in the world's most vulnerable infants.

Articles in preparation

Green *et al*. **Transcriptional changes following chimpanzee adenovirus- and MVA viral-vectored vaccines for RSV and correlates with vaccine responses in healthy adults.**

Green *et al*. **Healthy adults develop temporary B-cell IgA memory to respiratory syncytial virus in winter months.**

Green *et al*. **Novel respiratory syncytial virus vaccines based on chimpanzee adenovirus and MVA is safe and expands humoral and cellular immunity in healthy adults aged 60-75 years of age.**

Green *et al*. **An analysis of volunteer recruitment, exclusions and adherence to protocol for a phase 1 vaccine trial in younger and older healthy adults.**

Statement of responsibility

This thesis is based on the results from one epidemiological study, two observational studies and a phase I (first-in-man) clinical vaccine trial. The work presented in this thesis is my own work, although this could not have been achieved without the considerable cooperative effort of many people and through partnerships with industry and the public. Where content of this thesis is not my own it has been reproduced with permission and appropriately accredited within the text. In particular I wish to acknowledge and thank Amber Thompson, Charles Sande, Cathy de Lara, Hannah Gerretsen and Tamsin Cargill who setup, optimised and validated many of the immunological assays in their previous work and in advance of the RSV research projects.

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Dr Christopher A Green
25th February 2017

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Lead physician	**Christopher Green**
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Ethics submission	Michael Goldacre, Roger Parslow
Data collection	Michael Goldacre, Roger Parslow, David Yeates, Phillip McShane, Allie Goldacre
Data analysis	**Christopher Green**, Michael Goldacre
Data interpretation	**Christopher Green**
Manuscript preparation	**Christopher Green**

RSV Observational study in infants and children

Study design	Andrew Pollard, Paul Klenerman, **Christopher Green**, ReiThera Srl
Lead physician	**Christopher Green**
Lead nurse	Sharon Westcar
Project manager	Rebecca Beckley
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Monitoring	Clinical trials research group (CTRG), University of Oxford
Protocol	**Christopher Green**
Informed consent form	**Christopher Green**
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Ethics submission	**Christopher Green**
Local R&D submission	Rebecca Beckley
Recruitment	Sharon Westcar, Louise Willis, Roser Farras-Araya, Emma Plested, Tessa John
Sample collection	Sharon Westcar, Louise Willis, Roser Farras-Araya
Sample processing	Harri Hughes, Amber Thompson, Laura Silva Reyes, Charles Sande, Cathy de Lara, **Christopher Green**
Database setup	Sylwester Pawluk, **Christopher Green**
Serum ELISA assay	ReiThera Srl
Serum PRNA assay	Laura Silva Reyes, Charles Sande
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RSV Observational study in paediatric healthcare workers

Study design	Andrew Pollard, Paul Klenerman, **Christopher Green**, ReiThera Srl
Lead physician	**Christopher Green**
Lead nurse	Sarah Kelly
Project manager	Katherine Gantlett
Sponsor	University of Oxford
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Monitoring	Clinical trials research group (CTRG), University of Oxford
Protocol	**Christopher Green**
Informed consent form	**Christopher Green**
Study information booklet	**Christopher Green**
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Local R&D submission	Katherine Gantlett
Recruitment	**Christopher Green**, Sarah Kelly, Emma Plested, Sharon Westcar, Tessa John, Katherine Haworth, Anna Peters
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Sample collection	**Christopher Green**, Katherine Haworth, Anna Peters, Sarah Kelly, Sharon Westcar
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Serum ELISA assay	ReiThera Srl,
Serum FACS assay	ReiThera Srl
Memory B-cell ELISpot assay	Amber Thompson, **Christopher Green**
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Data interpretation	**Christopher Green**
Manuscript preparation	**Christopher Green**

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Senior nurse	Hannah Robinson
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Informed consent form	**Christopher Green**
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A	A549	Human alveolar cancer cell line
	AE	Adverse event
	AEC	3-amino-9-ethylcarbazole
	AID	Autoimmun Diagnostika
	ALT	Alanine transaminase
	ALP	Alkaline phosphatase
	ANCOVA	Analysis of co-variance
	ANOVA	Analysis of variance
	AP	Alkaline phosphatase
	APC	Antigen presenting cell
	aPTT	Activated partial thromboplastin time
	ASC	Antibody secreting cell
B	BAL	Broncho-alveolar lavage
	BCIP	5-bromo-4-chloro-3-indolyphosphate p-toluidine salt
	BD	Beckton Dickinson
	BEI	Biodefense and Emerging Infections
	BMI	Body mass index
	BSA	Bovine serum albumin
	BTM	Blood transcription module
C	CA	Croup associated virus
	CBA	Cytometric bead array
	CC	Cysteine-cysteine
	CCA	Chimpanzee coryza agent
	CCL	CC chemokine ligand
	CCVTM	Centre for Clinical Vaccinology & Tropical Medicine
	CD	Cluster of differentiation
	CF	Complement fixation
	CI	Confidence interval
	CMV	Cytomegalovirus
	ConA	Concanavalin A
	CONSORT	Consolidated standards of reporting trials
	COPD	Chronic obstructive pulmonary disease
	CPE	Cytopathic effect
	CpG	Cysteine-phosphate-guanine
	CRN	Clinical research network
	CSM	Centre for statistics in medicine
	CSP	Clinical study plan
	CSPD	Chemiluminescent substrate
	CTL	Cytotoxic T-lymphocyte
	CTRG	Clinical Trials Research Governance
	CXC	Cysteine-(other amino acid)-cysteine
	CXCR	Cysteine-(other amino acid)-cysteine receptor

D	DAIDS	Division of AIDS
	DNA	Deoxyribonucleic acid
	DMSO	Dimethyl sulphoxide
	DNA	Deoxyribonucleic acid
	dPBS	Dulbecco's PBS
	DSMC	Data safety monitoring committee
E	EBV	Epstein-Barr virus
	EC	Effective concentration
	ECG	Electrocardiogram
	ECT	Electroconvulsive therapy
	EDTA	Ethylenediaminetetraacetic acid
	ELISA	Enzyme-linked immunosorbent assay
	ELISpot	Enzyme-linked immunospot
	EudraCT	European clinical trials database
F	F	Fusion protein
	FACS	Fluorescence-activated cell sorting
	FCS	Foetal calf serum
	FDA	Federal Drug Administration
	FDR	False discovery rate
	FEC	Flu/EBV/CMV
	FI-RSV	Formalin-inactivated RSV
	FITC	Fluorescein isothiocyanate
G	G	Attachment glycoprotein
	GCP	Good Clinical Practice
	GFP	Gylcosaminoglycan sulphate
	GLP	Good Laboratory Practice
	GMO	Genetically modified organism
	GMP	Good Manufacturing Practice
	GMT	Geometric mean titre
	GP	General practioner
	GSEA	Gene set enrichment analysis
	GSK	GlaxoSmithKline
	GSMC	Genetic modification safety committee
H	HAE	Human airway epithelial
	HBD	Heparin binding domain
	HBsAg	Hepatitis B virus surface antigen
	HBV	Hepatitis B virus
	HCV	Hepatitis C virus
	HEK	Human embryonic kidney
	HEp-2	Human epithelial cells type 2
	HES	Hospital Episode Statistics
	HI	Haemagglutination inhibition
	HIPE	Hospital In-Patient Enquiry
	HIV	Human immunodeficiency virus
	HLA	Human leucocyte antigen
	HOCM	Hereditary obstructive cardiomyopathy
	HRP	Horse radish peroxidase
	HSA	Human serum albumin
	HSCT	Human stem cell transplant

I	ICD	International classification of diseases
	ICH	International conference on harmonisation
	ICOS	Inducible co-stimulator
	ICS	Intra-cellular staining
	IFA	Immunoflorescence assay
	IFNγ	Interferon-gamma
	IgA	Immunoglobulin A
	IgG	Immunoglobulin G
	IgM	Immunoglobulin M
	IL	Interleukin
	IM	Intra-muscular
	IMD	Index of multiple deprivation
	IN	Intra-nasal
	IQR	Interquartile range
	ITT	Intention-to-treat
	IU	International units
	IVIG	Intravenous immunoglobulin
J	JAK	Janus-activated kinase
K	KEMRI	Kenya Medical Research Institute
L	L	Large protein
	LAIV	Live-attenuated influenza
	LLD	Lower limit of detection
	LPS	Lipopolysaccharide
	LRTI	Lower respiratory tract infection
M	M	Matrix protein
	MD-2	Lymphocyte antigen 96
	MEDI	MedImmune
	MEM	Minimum essential media
	MHC	Major histocompatibility complex
	MHRA	Medicines & healthcare products regulatory agency
	MVA	Modified vaccinia virus Ankara
N	N	Nucleocapsid protein
	NBBS	New born bovine serum
	NBT	Nitro-blue tetrazolium chloride substrate
	NCT	National clinical trial
	NES	Normalised enrichment score
	NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
	NHAIS	National Health Applications and Infrastructure Services
	NHS	National Health Service
	NHSBT	National Health Service blood transfusion service
	NIHR	National Institute for Health Research
	NLR	Nucleotide-binding oligomerisation domain-like receptor
	NRES	National Research Ethics Service
	NS	Non-structural protein
O	OD	Optical density
	ODN	Oligodeoxynucleotides
	ONS	Office for National Statistics
	ORLS	Oxford Record Linkage Study
	OUHL	Oxford University Hospitals laboratory
	OVC	Oxford Vaccine Centre
	OVG	Oxford Vaccine Group
	OVGL	Oxford Vaccine Group laboratory

P	P	Phosphorylation protein
	PAMPS	Pathogen associated molecular patterns
	PanAd3	*Pan paniscus* adenovirus 3
	PATH	Program for appropriate technology in health
	PBMC	Peripheral blood mononuclear cell
	PBS	Phosphate buffered saline
	PCR	Polymerase chain reaction
	PD1	Programmed cell death protein 1
	PFP	Purified F-protein
	PHA	Phytohemagglutinin
	PHE	Public Health England
	PICU	Paediatric intensive care unit
	PMA	Phorbol 12-myrstate 13-acetate
	PP	Per-protocol
	PRNA	Plaque-reduction neutralisation assay
	PRNT	Plaque-reduction neutralisation titre
	PRR	Pathogen recognition receptors
	PVF	Polyvinylidene flouride
	PVM	Pneumonia virus of mice
	PWM	Poke weed mitogen
R	RANTES	Regulation on activation, normal T-cell expressed and secreted
	RIG	Retinoic acid inducible gene
	RNA	Ribonucleic acid
	RSV	Respiratory syncytial virus
	RSV001	Phase RSV vaccine trial code
	RSVA	Respiratory syncytial virus subtype A
	RSVB	Respiratory syncytial virus subtype B
S	SAC	*Staphylococcus aureus* Cowan's strain
	SAE	Serious adverse event
	SAP	Statistical analysis plan
	SAR	Serious adverse reaction
	SARS	Severe acute respiratory syndrome
	SD	Standard deviation
	SEAP	Secreted alkaline phosphatase
	SH	Small hydrophobic protein
	SIB	Study information booklet
	SOP	Standard operating procedure
	SP-A	Surfactant protein A
	ssRNA	Single-stranded ribonucleic acid
	STAT	Signal transducer and activator of transcription
T	Tfh	T-follicular helper cell
	TIV	Trivalent influenza vaccine
	TLR	Toll-like receptor
	TMB	Tetramethylbenzidine
	TNFα	Tumour necrosis factor α
	TOPS	The over-volunteering prevention system
U	UK	United Kingdom
	URTI	Upper respiratory tract infection
	US	United States

Chapter 1: Respiratory syncytial virus

1.1 The discovery of RSV

"The first change seen in infected cultures was the formation of a syncytial area with loss of cell boundaries, rapidly followed by a breaking away from the surrounding cell sheet. Numerous small vacuoles made their appearance within the cytoplasm of cells, and together with the increased granularity which accompanied the loss of discernable cell structure produced a picture best described as "sponge-like". Within 24 to 28 hours of the first change 80 to 90 per cent of the epithelial sheet was affected and off the glass, while the remaining normal appearing epithelium persisted in a "Swiss cheese" like pattern. A similar sequence of events followed inoculation of human amnion cultures, which in a simultaneous test proved to be 10 times more sensitive than monkey kidney cultures in the detection of minimal quantities of the virus."

Figure 1-1. The first description of the cytopathogenic effect of "croup-associated' virus from throat swab fluids taken from two infants diagnosed with croup in November 1955, later to become known as respiratory syncytial virus (RSV).
Primary insolation was in monkey epithelial cells and cytopathogenic changes were not observed until the 10th and 15th day after inoculation for each sample. Mumps produced early cytopathogenic changes similar to croup-associated (CA) virus but without the progression to form small vacuoles. This description is copied from the paper by Chanock et al. and formed the basis for early discovery of RSV infection.[6]

In October 1955 a colony of 20 healthy chimpanzees at the Walter Reed Army Institute of Research developed an acute respiratory illness. A novel cytopathogenic agent was recovered from nasal swabs of these animals and in 1956 Morris *et al*. named the new pathogen "chimpanzee coryza agent" (CCA)[7]. Koch's postulates for causality was fulfilled when other chimpanzees were experimentally inoculated intra-nasally with tissue culture of the new agent and coryzal symptoms ensued 3-days later. The same year, in November 1955 in Cincinnati, Chanock *et al*. recovered and characterised a new cytopathogenic myxovirus from 2/12 infants (aged 11 and 4 months) suffering from croup which he called "croup-associated virus" (CA virus)[6]. These "Greer" and "Lorentz" isolates were identical in cytopathogenic effect, incubation period and

antigenic character as CCA. The sponge-like cytopathogenic changes were unlike any other known viral cytopathogenic effect and these infants had developed significant increases in haemoagglutination-inhibition (HI), complement fixation (CF) and neutralising antibody during convalescence. Sera from a further 3 infants with similar disease, and from whom no virus had been isolated, also recorded a rise in one or more of these serological measures of exposure. Antibody measures from infants aged 2-34 months (a separate group of infants who did not have signs of respiratory infection), adults aged 21-30 years, chimpanzees and Cynomolgus monkeys showed HI titres of 1:10 or more in 25% of infants, 90% of adults and were absent in non-human primates until after challenge with CA virus. Here was the first cumulative evidence that CA virus was a common human pathogen and that primary infection occurred early in life. After submission of this seminal paper, Chanock received a further 10 strains isolated from 15 infants with croup during the winter of 1955/56 from Toronto, and he reported identical tissue culture and antigenic properties to the "Greer" strain of CA virus in support of his findings.

The following year, in 1957, the similarity between CCA and CA viruses became increasingly apparent. Chanock and others reported the recovery of CA virus from an infant with croup and another infant with pneumonia, with retrospective serological evidence of infection from a larger number of infants from whom no sample had been obtained[8,9]. The continued recovery or virus from infants with respiratory infection and serological responses from outpatient infants with respiratory infection supported an implied causality between CCA and respiratory infections in infants. Hospitalised infants without respiratory infection also demonstrated a high rate of serological conversion and a further study using samples collected prospectively from inpatients and outpatients in the winter of 1958/59 provided further evidence of this new agent causing respiratory disease in infants[10].

In 1960 Beem *et al*. published their findings from samples collected between December 1958 and June 1959, when 291 patients had been divided into four groups designated inpatient/outpatient plus with/without respiratory disease[10]. Two agents were isolated from throat swabs in the first month of the study and were characterised by the unusual formation of syncytia and giant cells in human epithelial (or HEp-2) cells. They called these agents "Randall virus", named for reasons unknown and not used elsewhere, and over the next 5-months another 48 similar agents were recovered and isolated from nose and throat samples from 41 patients (27 inpatients, 14 outpatients and none from infants without respiratory disease).

Most infants recorded a notable rise in complement fixation and neutralisation titres in convalescent sera and the results from complement fixation tests from paired sera of 5 patients from whom Randall virus was isolated showed antigenic similarity between Randall virus and two strains of chimpanzee coryza agent (CCA). Overall a third of hospitalised respiratory diseases in infants could be associated with Randall virus infection, compared with 16% of outpatient cases. Furthermore, the clinical disease phenotype associated with Randall virus now included any acute respiratory illness, bronchiolitis (including fatal bronchiolitis) and pneumonia but not croup or asthma. 70% of Randall virus infections occurred between December and February though it remained unknown if the disease was endemic or occurred in sporadic community epidemics. The term "respiratory syncytial virus" (or RSV) appears from 1960 when isolated from a case of pneumonia and thereafter this virus has retained this single identity[11].

The method of direct inoculation from throat swab specimens to HEp-2 tissue culture without freeze/thawing, a technique used by Beem and Hamre, improved the rate of viral isolation. More publications emerged showing the association between RSV isolation and clinical respiratory disease in infants and serological evidence of an even greater disease burden. Bronchiolitis, an inflammation of the small airways of infants, in the first 6-months of life was now recognised at the most common manifestation of severe disease caused by RSV infection[12]. In 1961 evidence from the west coast of the United States and Australia indicated RSV disease was a worldwide problem[13-15]. More importantly the causal relationship between RSV and respiratory tract disease, and especially severe disease in infants, was now accepted (although the detection of RSV antibody in cattle in 1968 caused some concern[16]). Adams *et al* described the clinical phenotype of the disease in more detail as well noting nosocomial transmission to healthcare workers and the deaths of two infants, both of which had medical conditions we now recognise as pre-dispositions to severe RSV disease (Down's syndrome and congenital heart disease)[13]. Autopsy findings on the latter fatality and cases in other hospitals described a "necrotising bronchiolitis with peribronchial infiltration of mononuclear cells". In 1961, using a wild-type isolate of RSV obtained in 1957 from an infant with pneumonia, Kravetz *et al* successfully challenged 41 male prisoners, aged 21 to 35 years, who had been pre-selected for low serum antibody titres to RSV[17]. 20 developed clinical signs of infection and the virus was recovered from 13/21 'volunteers' who did not become unwell. 75% of those who developed clinical infection developed a four-fold rise in complement fixing or neutralising antibody titre,

compared with 2/13 who did not develop disease but from whom the virus could be recovered after challenge. The RSV challenge strain was recovered in rhesus monkey kidney tissue culture, though unfortunately the challenge inoculum for 27/41 men had been contaminated with simian kidney vacuolating virus (SV_{40}) that caused subclinical infection in 14/27 volunteers. Further challenge experiments were performed by Fridewald *et al*[18] and by the end of the 1960s this work, and the work of others, demonstrated that immunity to RSV following natural infection was incomplete and that re-infection was possible[19]. The oldest cases of fatal RSV-bronchiolitis, confirmed using antigen staining, date back as far as 1931 from autopsy specimens from the Vanderbilt Medical School[20,21].

As early as the 1960's RSV was established as a major human pathogen and work immediately began to develop a vaccine to protect infants against developing severe infection. 60-years after the discovery of RSV there is a new appreciation of the worldwide burden of disease and despite great advances in the understanding of the genetics, structure and biology of the virus, RSV continues to evade the development of effective treatments and a safe and effective vaccine.

1.2 Basic virology

Human RSV is a single, linear, negative-stranded, enveloped, non-segmented, RNA virus 15.2 kilobases in length of the order *Mononegavirales*, family *Paramyxoviridae*, sub-family *Paramyxoviriniae*, genus *Pneumovirus*. The genus also includes bovine RSV (BRSV) and the closest relative to human RSV, and within the same sub-family, is human metapneumovirus (hMPV) which was first described in 2001 (genus *Metapneumovirus*)[22]. There is only a single RSV serotype with differences in the variable regions of one of the surface expressed proteins (the G attachment protein) used to define two antigenic subgroups by monoclonal antibody binding. These subtypes are termed RSVA and RSVB (although earlier studies believed there to be more subtypes[23]) and subtypes and different genotypes can circulate simultaneously. The most common ancestor for RSVA and RSVB is likely to have been in the early 1940s and between 1938-55 respectively[24,25].

The RSV genome comprises of 10 genes that encode 11 proteins, as illustrated in Figure 1-2 and Figure 1-3. The overlapping open reading frames in the M2 gene result in two matrix proteins, M2-1 and M2-

2. The viral envelope contains three surface proteins designated the F (fusion) glycoprotein, attachment (G) glycoprotein and the small hydrophobic protein (SH) protein. The F glycoprotein mediates the fusion of viral and host cell membranes and the entry of viral RNA into the target cell. Ultimately this results in the characteristic cytopathic effect of syncytia formation that bears the virus name. The G glycoprotein acts as an attachment protein for free virus to bind onto the target cell surface and is not essential for *in vitro* infection. The SH protein contains a porin-like structure that is not essential for *in vitro* infection and whose function remains unknown. Within the virus particle are six internal proteins concerned with the nucleocapsid construction and RNA replication; the L (large) protein which is an RNA dependent RNA polymerase, N (nucleocapsid) protein required for protection of the viral RNA and the transition from transcription to replication, P (phosphylation) protein as a polymerase co-factor, M (Matrix) protein for viral assembly and M2 (matrix M2-1 and M2-2) proteins for transcription anti-termination factor and regulation of transcription respectively. Two non-structural proteins, called NS1 and NS2, complete the RSV complement of gene products and are concerned with inhibition of the host cell response to infection.

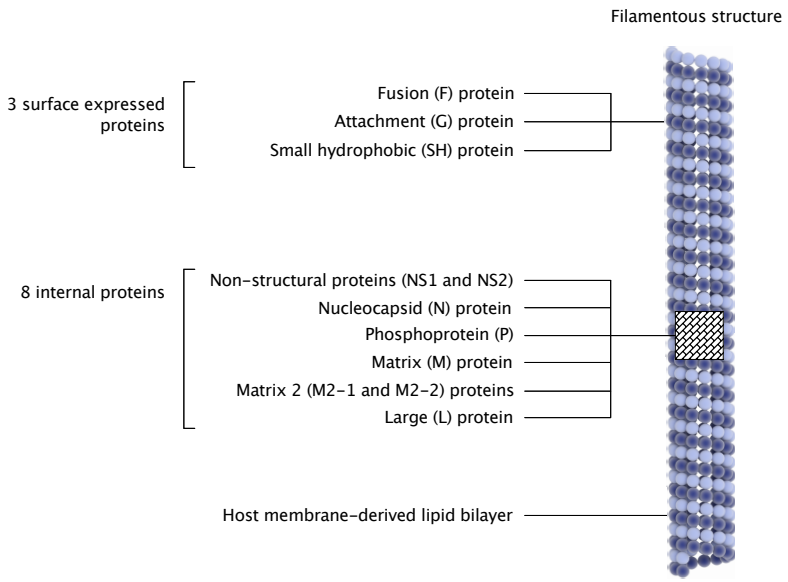


Figure 1-2. Schematic representation of the RSV particle and RSV proteins.
The RSV virus is made up of 11 proteins; 3 surface expressed proteins and 8 internal proteins.

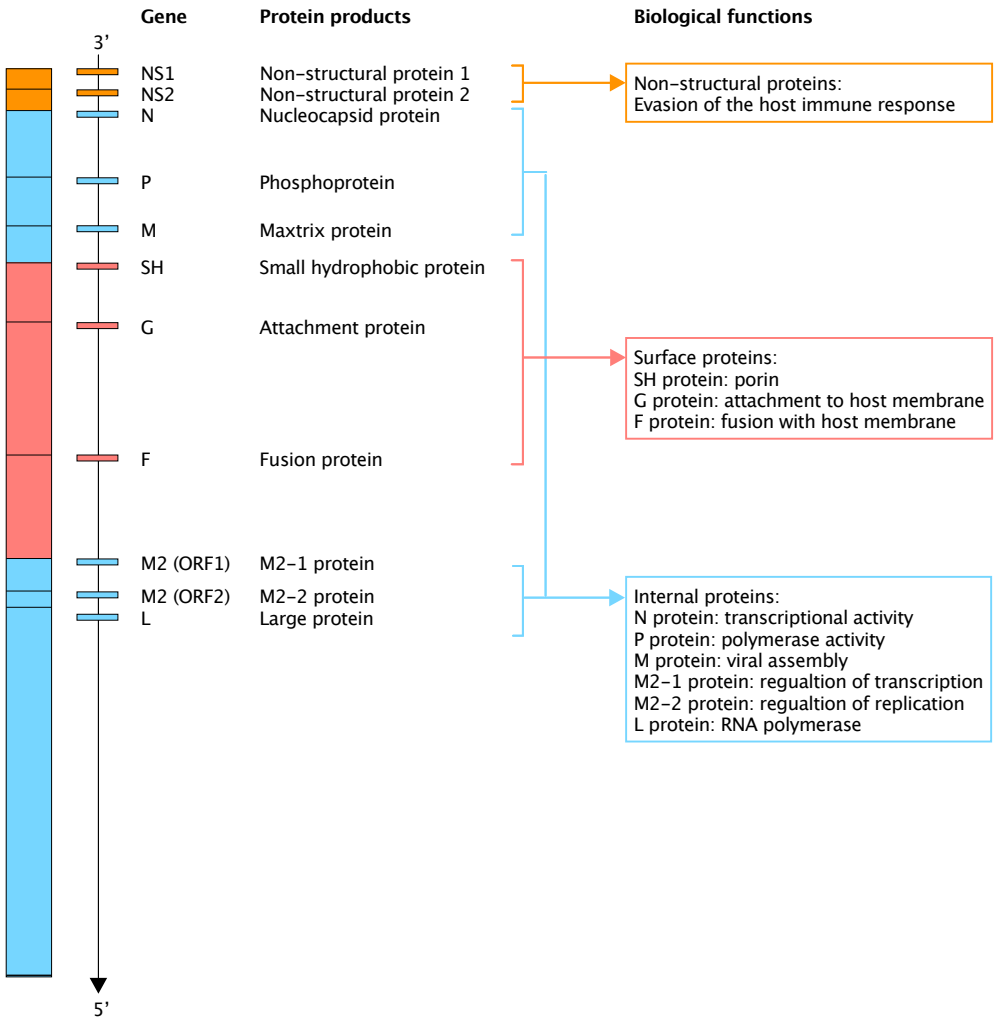


Figure 1-3. Schematic representation of the human RSV genome.
The 10 genes are ordered from 3' to 5' as top to bottom. The size of each block is proportional to the size of each gene (total 581.8-634.3 kDa; the SH gene ranges between 7.5-60 kDa and represented here as 40kDa). The 11 protein products are annotated next to each gene; the M2 gene contains two open-reading frames (ORFs) that generate two protein products (M2-1 and M2-2). Non-structural, surface expressed and internal structural proteins are colour coded and a summary of the biological function of each gene product is annotated.

RSV is restricted to infecting humans. The closest animal relatives to human RSV are bovine RSV, which shares 70% amino acid sequence homology, and pneumonia virus of mice (PVM). As such the selective pressure for RSV in the absence of a vaccine or effective antivirals comes from the immunity of its only host. The G-glycoprotein in particular has undergone temporal genetic evolution that may have been driven by immune selection selective pressure, although these changes do not appear to be associated with the loss of G-specific antibody neutralisation[24-26].

1.3 Clinical disease

Disease is a product of viral infection and the host immune response to clear the infection. Clinical disease from RSV ranges from asymptomatic infection through to fatal disease. The majority of RSV infections are mild and involve the upper respiratory tract to cause local symptoms of rhinorrhoea, nasal congestion, coryza, malaise and cough. RSV can also be responsible for cases of otitis media in infants. Severe RSV disease is more commonly recognised as disease of the lower respiratory tract (viral replication below the level of the trachea) with compromise of respiratory function (and/or feeding in the case of infants). Severe RSV-bronchiolitis can involve extra-pulmonary sites, with direct and indirect evidence of involvement with the myocardium[27-30], liver[31] and central nervous system[32,33] that causes periods of apnoea[34,35] and seizures[36].

Respiratory viral RNA can be recovered from droplets (particle size >μ5m) or aerosols (particle size <μ5m) from the cough and secretions of infected people, though it is likely that RSV is transmitted by droplet spread only[37,38]. RSV can also survive on the skin, fomites and contact surfaces for several hours as an alternative source of infection[39,40]. Surveillance data indicates children of school age are a common source of RSV spread within households and the community[41,42]. In temperate climates RSV transmission peaks during the colder (winter) months concurrent to several other seasonal respiratory viruses such as influenza, para-influenza, human metapneumovirus, rhinovirus and coronaviruses and co-infections are not uncommon. In the UK the onset and duration of the RSV transmission season, as measured by reported cases of infection from infants, has been consisted over many years[43]. Less temperate climates record an incidence of RSV infection that is more constant throughout the year.

1.3.1 Pathophysiology

RSV infection begins with the attachment of free virus onto the surface of the host cell. The host cell receptor(s) remain to be identified but nucleolin and fractalkine have been advanced as a likely candidate receptors[44,45]. The main RSV targets are airway epithelial cells, though mononuclear cells in peripheral circulation have also shown to be infected experimentally and following natural infection[46,47]. The process of attachment is mediated by the surface expressed G (attachment) glycoprotein of RSV. Once attached, another surface expressed glycoprotein, the F (fusion) glycoprotein, changes from a relatively

unstable pre-fusion (pre-F) configuration into a more stable post-fusion (post-F) confirmation, and in doing so releases energy that merges the virus and host cell membranes. This allows entry of the viral ssRNA into the host cell cytoplasm and from here RNA replication can begin, starting with the construction of a positive-sense RNA template for new viral progeny. The M2 gene products, M2-1 and M2-2, regulate the replication of RSV with M2-1 mediating the transcription of viral mRNA and M2-2 controlling the transition from viral transcription to replication[48-50]. The nucleocapsid forms viral particles of helical symmetry assembled into filamentous conformations up to 100-350nm in length, with lipid envelopes derived from the host cell and viral particles are typically assembled in cholesterol rich domains of the infected host cell[51,52]. Complete viral replication in the upper airways presumably confers an advantage in onward droplet transmission and evasion of host systemic immunity.

1.3.2 Disease in infants and children

Infants were immediately recognised as the main population susceptible to developing clinical disease simultaneous to the discovery of the virus in chimpanzees[6,9]. The peak age of disease is two months and infection after 6-months of age is associated with lower rates of severe disease and hospitalisation, although reinfection occurs throughout life and is even possible within the first year of life[53,54]. Between 30-70% of infants have their first RSV infection in the first year of life[42,55-58] and although most infections are relatively mild and can be managed at home, approximately 2-3% of primary infections will require admission to hospital (when feeding and/or breathing becomes compromised)[59,60] and 2-6% of these admissions need management on paediatric intensive care units (PICUs)[60-63]. Infants with bronchiolitis account for up to 18% of winter paediatric admissions and infection by RSV is responsible for up to 80% of cases with co-infection with other respiratory viruses not uncommon[63-65]. Prematurity, low birth-weight, male sex, broncho-pulmonary dysplasia, congenital heart disease, immunodeficiency, cerebral palsy and Down's syndrome are recognised risk factors for severe RSV-bronchiolitis,[53,59,66-68] but 50-80% of emergency admissions occur in otherwise healthy infants born at term[53,67]. Twin studies have estimated the heritable contribution to susceptibility to severe RSV disease between 16-22%[69] and single nucleotide polymorphisms (SNPs) and genetic polymorphisms in immune genes indicate that susceptibility to severe disease is partially governed at a genetic level[70-80].

RSV-bronchiolitis therefore represents the single greatest burden to paediatric hospital resources each year. RSV morbidity alone is estimated to cost between 13.5% and 21.6% of the $2.25 billion spent each year on infants and between 9.7% and 15.5% of the $3.73 billion spent each year on children under 5-years hospitalised with lower respiratory tract disease in the US[81]. Worldwide, RSV disease in children under the age of 5-years account for an estimated 33.8 million lower respiratory tract infections, 3.4 million hospitalisations and up to 200,000 deaths annually[82]. Access to mechanical ventilation on PICUs has maintained a low mortality in industrialised nations (0-1.5% in otherwise healthy infants with RSV)[83] and RSV-associated deaths are almost exclusive to resource poor areas of the world where RSV is second only to malaria in all-cause infant mortality between one and 12-months of age[82,84].

After the first year of life the risk of developing severe disease and hospitalisation dramatically declines. However, bronchiolitis in the first year of life is associated with sequelae of functional airway impairment, wheeze and possibly asthma in later years[85-88]. The declining rates of wheeze following the use of palivizumab (Synagis®, MedImmune), an F-specific humanised mouse monoclonal antibody, has implied a causal relationship between RSV infection in infancy and childhood wheeze; infants who received palivizumab recorded a reduction in wheeze at 3- and 5-years and more recent randomised controlled trial data have shown a reduction of 61% in the total number of wheezing days in the first year of life following palivizumab RSV prophylaxis[89-93].

There have been frustratingly few successes in developing treatments and other prophylactic measures for RSV infection in infants. Randomised, double-blind, placebo-controlled trials of aerosolised ribavirin in infants in the 1980's demonstrated a reduction in the severity of symptoms and ribavirin is a Federal Drug Administration (FDA) approved treatment for infants with severe RSV disease, but concerns about drug delivery and toxicity have impacted on its use[94,95]. Cysteinyl leucotrienes (monteleukast) and corticosteroids have failed to show an improvement on the clinical course of disease[96,97]. Care for infants with RSV infection therefore remains supportive until the infant immune system is capable of controlling disease (oxygen, feeding support, bronchodilators and mechanical ventilation if required). With the aim of delaying susceptibility to RSV to an older age with an associated lesser risk of developing severe disease, human immunoglobulin of high neutralising antibody titres (RSV-IVIG, or Respigam™, MedImmune) was licenced and given to infants from 1996. This was later superseded by palivizumab in 1998 after clinical trials

showed this could reduce hospital admissions in high-risk infants by 45-55%[90,98-100]. Paviluzimab remains the only cost-effective licenced product for prophylaxis against severe RSV disease. Incomplete protection and the logistics of administering five injections over the winter period have restricted its impact in reducing the incidence of severe disease in this sub-population of hospital admissions, and pavilizumab has been prohibitively expensive for resource poor healthcare economies. A modified version of palivizumab, motavizumab (MedImmune), with improved antibody affinity has shown superior *in vitro* viral neutralisation but was associated with a minor increased incidence of hyper-sensitivity reactions and drug-specific antibodies in human trials and, as a result, further clinical development has been suspended.

Research in neonates presents ethical and practical challenges to explain the propensity for RSV to cause severe disease in infants. Disease is multi-factorial and the lack of a clear and comprehensive understanding of these factors has been, and remains, a serious obstacle in developing therapies and vaccines. In infants the virus needs to travel a relatively short distance to the reach the bronchioles and oxygenating respiratory apparatus, and mucin plugs are more likely to completely plug or occlude smaller airways. Even if the airway is not occluded, Poiseuille's law of flow dynamics would indicate that (with all other variables being equal) the laminar flow through respiratory airways is very sensitive to the radius of the lumen (flow rate is proportionate to the radius to the power of 4). In the small bronchioles of the infant lung this would mean any minor loss in airway radius (from mucin or inflammation, for example) carries a dramatic effect on the airflow needed for gas exchange. Another anatomical considerations are that RSV infection does not need to reach oxygenating tissues of the lower respiratory tract to compromise respiratory function, babies have a reduced physiological reserve that may be due to the relatively high volume of anatomical dead space, and the ribs are positioned horizontally with a floppy rib cage. Infants are obligate nasal breathers meaning local replicating virus causing nasal congestion alone can be sufficient to disrupt breathing and feeding.

The severity of clinical disease in infants can be correlated with the burden of virus in blood in several (but not all) studies[73,101-103]. Data from natural infection in the first 2-years of life have associated viral load with hospital admissions, length of hospital stay, rates of respiratory failure and the need for ventilation[104]. Bronchoavleolar lavage samples from the upper and lower respiratory tracts of intubated infants have been characterised as having large numbers of neutrophils (up to 85% of the total cell count)

and relatively low quantities of CD4+, CD8+ and CD19+ cells (0.1-9% of the total cell count)[105-109]. Furthermore, the changes in cellularity and pro-inflammatory cytokines TNFα and IL-6 in BAL specimens over time on ventilation appear to be different between term and premature infants[110,111]. The peak emergence of neutrophils in blood correlates with clinical disease severity and RSV viral load (albeit with a 48-72 hour lag) and precedes CD8+ T-cells that peak 9-12 days after the onset of symptoms and during convalescence[109,112]. It remains unclear what role these neutrophils perform in severe disease, and whether this can signify secondary bacterial infection or a facet of immune dysregulation. Quantities of chemokines, such as CXC and to a lesser extent CC chemokines, recovered from BAL specimens of intubated infants appeared to correlative with disease severity[113].

Histological examination of historical lung tissue samples obtained from the pre-intensive care and pre-antiviral era can give a rare insight into natural severe RSV pathology[21]. Three female infants and children[a] who died of 'atypical pneumonia' between 1931 and 1949 and who were RSV antigen positive on autopsy specimens demonstrated an assortment of pulmonary pathology. These included airway obstruction (with inflammatory debris, oedema and mucin), external airway compression (from hyperplastic lymphoid follicles), viral infection of the small airways and bronchiolar epithelium, type I and II pneumocytes and an infiltration of CD68+ alveolar macrophages and monocytes and double negative CD3+ T-cells, CD8+ T-cells and neutrophils. The sample from 1931 remains the oldest confirmed case of fatal RSV infection. Similar pathologies were observed from a 15-month old child who was ambulatory and 'well' with RSV infection diagnosed 36-hours earlier (concurrent with complex congenital heart disease) but who was tragically killed in a road traffic accident in 1999, and therefore did not die from RSV infection. Histology recorded bronchiolar-associated lymphoid tissue rich in CD20+, CD8+ and CD3+ cells as before. However, more recent autopsy research into the natural pathophysiology of fatal RSV-bronchiolitis has given a different perspective[114]. Here, the lung histology from 9 infants (median age of 3-months, 7 cases with pre-disposing co-morbidities for severe RSV disease[b]) also recorded extensive RSV antigen in exfoliated bronchiolar epithelial cells but with a poverty of key host cellular immune responses (such as CD4+ and CD8+ T-cells, CD56+ natural killer cells and granzyme) and an abundance of CD16+ cells (macrophages and

[a] One infant aged one month with congenital syphilis and 2-days of symptoms, one child aged 3-years with
[b] Two cases with Down syndrome and five cases with congenital heart disease

neutrophils) and caspase 3 and Fas (on epithelial cells and markers of apoptosis). These observations, which were also observed in cases of fatal influenza, imply a fatal absence of a protective Th1 lymphocyte adaptive immunity in the presence of lower respiratory tract infection. The innate and adaptive immune response to infection may therefore contribute to disease in infants.

1.3.3 Disease in adults

Healthy adults do not develop severe life-threatening disease. The population aged 18 to 65-years can expect a 9-11% annual risk of developing mild, self-limiting, upper respiratory tract infection and outbreak infection rates can reach up to 17%[115-117]. In one sentinel prospective study of nearly 3000 healthy working adults, aged 18-60 years, the RSV infection rate was 7%[116]. From these, 84% of infections were symptomatic and 26% included symptoms of lower respiratory tract disease (tracheobronchitis, bronchitis or wheezing), with a mean duration of illness lasting 9.5 days (longer than influenza) and 38% requiring time off work (a lower proportion compared with influenza). A 'bad cold' represents a significant burden to society with medical attendances and time off from work, and healthy adults make up the stratum of society nursing infants or caring for the elderly, and immunity in healthy adults represents a significant obstacle for RSV transmission and a selective pressure for the virus. Viral shedding typically ranges from 4 to 7-days and viral load and nasal pro-inflammatory cytokines appear to drive the manifestations of RSV disease in experimental human challenge models[118,119].

Severe immune suppression re-establishes the risk of developing severe disease and death from RSV infection in adults. This was recognised in the context of haematological malignancies and immune-suppressive chemotherapy from the 1980s and 1990s when 50% of viral respiratory infections were due to RSV with associated high rates of intubation and mortality estimates of 50-55%[120-122]. RSV infection is most serious in allogeneic and autologous haematopoietic stem cell transplant (HSCT) patients during the period of pre-engraftment or within one-month post-engraftment, with mortality ranging from 20-80%[120,123-127]. An important factor for the restriction of disease progression from the upper to lower respiratory tract (from retrospective multivariate modelling) is an absolute lymphocyte count in excess of 1000 cells/mm^3 at the time of symptom onset. Counts less than 1000 cells/mm^3, smoking and whole-body irradiation were associated with disease progression, and no association was found with serum anti-RSV antibody, viral

subtypes or use of corticosteroids[128]. Early recognition and treatment with antiviral drugs and IVIG may reduce the likelihood of progressing to lower respiratory tract disease and death[129-131] but approximately 25-35% of post-HSCT patients with upper RSV infection progress to lower respiratory tract disease[128,132,133]. Although palivizumab was safe and well tolerated in phase I trials in HSCT patients with maintenance of palivizumab levels for 21-days post-infusion, subsequent retrospective analyses on the use of palivizumab failed to show any protection against developing severe lower respiratory tract disease or mortality[134-137]. The use of aerosolised ribavirin and intravenous immunoglobulin (IVIG) has been expensive, difficult to administer, confounded by toxicity and is unsupported in data from controlled clinical trials although surveys of treatment options in transplant centres has reported a wide range of oral/inhaled ribavirin and IVIG use[138,139].

Pathogenic and iatrogenic immune suppression confer an increased risk of infection and severe RSV disease, but so does attending hospital. Molecular sequencing of RSV isolates from patients undergoing HSCT therapy as outpatient care revealed 50-90% of RSV strains were identical and representative of clonal outbreaks of disease[140-142]. The importance of infection control measures in preventing nosocomial RSV outbreaks in HSCT patients is clear, with HSCT RSV-pneumonia outbreak mortality as high as nearly 80%[143]. RSV remains the greatest nosocomial infection risk to HSCT patients and also accounts for a significant on-going morbidity and mortality on HSCT survivors of acute infection. One respective study of over 100 patients receiving their first allogeneic transplant recorded 38% of the 132 confirmed respiratory tract infections within 100-days post HSCT was associated with a significant decline in airflow by one-year post transplantation; the odds of developing this late complication were significantly increased by parainfluenza virus and RSV (odds ratio 17.9 and 3.6 respectively)[144].

1.3.4 Disease in the elderly

Senescence of the immune system and comorbid conditions place the elderly at risk of developing severe RSV disease and death. Recent estimates of the hospital burden and mortality from RSV in the elderly vary but may be comparable to seasonal influenza[145-150]. RSV was rapidly established as a major paediatric pathogen and, possibly because of presumed serological protection from severe disease throughout adulthood, it was not until the 1970s and outbreaks of severe disease in long-term elderly

healthcare facilities that attention focussed on this population as being at risk of developing severe disease; here 20/50 residents developed acute respiratory disease (4 died)[151] in one care facility and 40/101 (8 died) in another[152]. Work from Scandinavia as early as 1967 might have given an earlier indication of RSV disease in the elderly with an estimated incidence of 7% each winter[153]. Infection rates for the elderly living in the community and care homes range from 5% to 10% each year, which is broadly comparable to the infection rate observed in younger adults[154-156]. Further analysis showed the attack rate increases with age (6.1% in adults aged 65-69 years, 7.1% aged 70-74 years, 8.9% aged 75-79 years and 8.5% aged 80 or more years). In total 8/41 (19.5%) RSV infections required hospitalisation[157,158]. UK surveillance data from primary care sources over four successive winters registered a 2-3 week lag between the onset of confirmed cases of influenza and RSV infection in infants and children, and acute respiratory disease and deaths in the elderly[159]. Disease modelling using time-series linear regression analyses from UK databases (collected between 1995 and 2009) estimated nearly half a million GP attendances, 18'000 hospitalisations and 8482 deaths were attributed to RSV per season in all adults aged 18 and over and the estimated rates for adults aged over 65 years were twice as high for GP attendances and death and four-times greater for hospitalisation[160]. RSV testing is commonly practiced for infants but not the elderly, which has limited disease burden estimates in this population.

Where younger adult and elderly populations differ is with the frequency of progressing towards developing severe disease with respiratory failure (8-13%) and death (2-5%)[157]. Co-morbid factors drive rates of hospitalisation and death from RSV in the elderly; both RSV and influenza A result in similar lengths of stay, rates of intensive care (15% and 12% respectively) and mortality (8% and 7% respectively). RSV infection is responsible for a significant proportion of hospitalisations for pneumonia (10.6%), chronic obstructive pulmonary disease (11.4%), congestive cardiac failure (5.4%) and asthma (7.2%)[c 147]. Elderly RSV-related hospitalisation rates of up to 10% have been reported consistently, with nearly 90% of RSV-infected hospitalised patients having one or more underlying medical conditions with 30-day and 60-day mortality rates of 9.1% and 11.9% respectively[145,154,161]. Not surprisingly older age is associated with greater mortality in both HSCT and solid organ transplant recipients[162].

[c] N=1388 patients, prospectively collected data on hospitalisation in patients over 65-years over four winters in the US

RSV is a major global pathogen and the aging population common to many parts of the globe indicate that RSV infection in older adults will exert an increasing burden to healthcare resources in the future. By 2050 an estimated 2 billion of the human population will be 65-years or older and represent 25% of the population in developed healthcare economies[163]. Targeting the development of therapeutic and vaccination measures to prevent severe RSV disease in the elderly is therefore of major public health interest.

1.4 Immunity to RSV

The issue of immunity to RSV is extremely complex, poorly understood and remains the subject of intense research in developing successful strategies to mitigate the burden of disease in infants, immune-compromised adults and the elderly. Immunity to RSV is incomplete, difficult to sustain and may even contribute to disease.

1.4.1 Innate immunity

Protection from invading pathogens involves a complex dynamic between early innate immune recognition and signalling systems that are used to engage a more effective, pathogen and organ-specific adaptive humoral and cellular immune response. All the while this process requires tight regulation to minimise injury to the host and to generate lasting immunity to re-exposure. The anti-viral innate immune response relies upon an array of dendritic cells embedded at mucosal and other sites throughout the body that use soluble and cellular receptors to recognise evolutionary conserved foreign structural components, commonly referred to as pathogen-associated molecular patterns (PAMPs), which trigger a cascade of signalling infection through mediators such as interferons. Dendritic cells are antigen-presenting cells (APCs) and provide a critical connection between the innate and adaptive immune response. The receptors on these sentinel cells, commonly referred to as pattern recognition receptors (PRRs), are described as coming from three classes that include Toll-like receptors (or TLRs, of which there are 11 known at present), retinoic acid inducible gene (RIG)-like receptors (or RLRs, such as RIG-I and MDA5) and nucleotide-binding oligomerization domain-like receptors (or NLRs, such as Nod1, Nod2 and NALP3). These receptors recognise

various PAMPs such as viral nucleic acid and foreign surface glycoproteins that results in the activation of transcription factors, cell signalling molecules and cell migration to lymph nodes.

The net result is the production of pathogen-specific antibody, activation of naïve T-cells and the direction of an effector T-cell response. TLRs, RLRs and NLRs have all been implicated in the innate anti-viral response to RSV infection (TLRs summarised in Table 1-1)[164,165]. The point of entry for RSV is the respiratory epithelium where dendritic cells of the lung are populated by two major subsets, namely the conventional or myeloid dendritic cells (CD11b+ cells) and plasmacystoid (CD103+) dendritic cells located at mucosal sites. Mouse models of RSV infection have indicated that several dendritic cell populations are involved with MHC class I and II antigen presentation and the lymph node activation for T-cell effector responses (although it is worth noting that mice are profoundly different from humans and only semi-permissive hosts of RSV infection)[166].

The activation of innate immunity has been studied in clinical disease. Peripheral monocytes from infants with severe RSV-bronchiolitis showed increased TLR4 expression which resolved during convalescence, TLR4 expression was greater in BAL neutrophils (as opposed to peripheral blood, and no differences were seen for TLRs 2, 7, 8 and 9) and genetic polymorphisms in the TLR4 gene have been associated with an increased risk of severe disease, although this remains controversial[107,167-172]. Cytokine markers of Th2 immune responses have also been implicated in severe disease[173,174].

Toll-like receptor	Ligand(s)	Summary evidence of interaction with RSV
TLR2	Glycolipids, lipopeptides, lipoproteins, lipoteichoic acid, heat shock proteins	TLR2 is required for the promotion of TNFα, IL-6, CCL2 and CCL5 in knockout mice models[175] A549 cells respond to RSV infection by up-regulation of TLR2[176]
TLR3	dsRNA	Deregulation of mucus production, IL-13 and IL-5 production and accumulation of eosinophils in the airways in TLR3 knockout mice models[177] A549 cells respond to RSV infection by up-regulation of TLR3 for interferon signalling[178,179] RSV challenge in mice results in an up-regulation of TLR3 expression in the lung[180] TLR3 functions in chemokine production in HEK cell lines[181]
TLR4	LPS, heparin sulphate fragments, heat shock proteins	RSV F-protein induction of IL-6 in monocytic cells (expressing TLR and CD14)[182], and later challenged[183] Interaction required between RSV, TLR4 and the co-receptor MD-2[184] CD14 and MD-2 TLR4 co-receptor antagonism in normal human bronchial epithelial cells impairs the production of IL-6 and IL-8 and protects from syncytia formation after RSV inoculation[185] TLR4-deficient mice have impaired natural killer, CD14+ trafficking, IL-12 expression and viral clearance after RSV challenge[186] Alveolar macrophages use TLR4-dependent pathways to signal RSV infection[187] RSV infection induces expression and membrane localisation in A549 cells[188]
TLR6	Diacyl lipopeptides	Knock out mice fail to mount pro-inflammatory responses to RSV challenge[175]
TLR7	ssRNA	Induction of interferon in human plasmacytoid dendritic cells [189] Peripheral eosinophils expressing TLR7 were associated with improved viral clearance in the lungs of infected mice[190]
TLR9	CpG-rich DNA domains	Induction of interferon in human plasmacytoid dendritic cells [189]

Table 1-1. Summary of the experimental evidence of the role of Toll-like receptors (TLRs) in the recognition of pathogen-associated molecular patterns (PAMPs) and the activation of innate immunity to RSV infection. *TLRs 2, 4 and 6 are cell surface expressed whereas TLRs 3, 7 and 9 are within dendritic cells. The list of ligands is not exhaustive. dsRNA, double-stranded ribonucleic acid. LPS, lipopolysaccharide. ssRNA, single-stranded ribonucleic acid. Cp-G oligodeoxynucleotide. TNFα, Tumour necrosis α. IL-6, interleukin-6. CCL2, chemokine (C-C) motif) ligand 2 (also referred to as chemoattractant protein 1). CCL5, chemokine (C-C) motif) ligand 5 (also referred to as RANTES or regulation on activation, normal T cell expressed and secreted). A549 cells, human alveolar cancer cell line. HEK cell, human embryonic kidney cell line.*

1.4.2 *Targets of adaptive immunity*

The adaptive immune response is pathogen-specific and responsible for immune memory and protection from re-infection. Upon exposure to RSV an effector response is mounted through the production of antibody for the neutralisation and/or opsonisation of free virus and B- and T-cell populations support an effective antibody response and clear infected cells. The adaptive immunity derived from repeated seasonal exposure in infancy and throughout life confers protection from severe disease but not re-infection. Critically the immune correlates of protection from infection and severe disease from RSV infection remain unknown.

The surface expressed F- and G-proteins are the only viral antigens that induce neutralising antibodies and protection from disease[191]. The nucleotide and sequence homology of the G gene between subtypes A and B is 67% and 37% respectively[192-194], meaning the protection afforded by G-specific antibody is restricted to previous subtype exposure. The RSV fusion (F) protein is a more reliable target of protective immunity being a highly conserved protein between different strains of RSV (nucleotide and sequence homology of 79% and 89% respectively[195]) and is essential for viral replication. An elegant demonstration that antibodies directed towards the F protein alone can protect from lower respiratory tract RSV disease in infants comes from the use of the F-specific monoclonal antibody palivizumab[90]. Palivizumab targets an epitope exhibited on both pre-F and post-F protein conformations designated antigenic site II (or site A)[196,197] and antigenic site IV (or site C) is another epitope present in both the pre- and post-F conformational protein structures and is the target of protective monoclonal antibodies MAb19 and 101F used in animal studies[196,198-200]. Recent advances in the field of structural biology has characterised an epitope unique to the pre-F trimer (designated antigenic site zero, or ø). Antibodies that bind to antigenic site ø, such as 5C4, Am22 and D25, are 10- to 100-fold more potent at neutralising virus than palivizumab antibodies in mouse, macaque and rabbit models[201-204]. A major research objective is now to stabile pre-F epitopes in vaccine formulations for use in man. The advent of HLA tetramers has allowed for exploration of the MHC class I restricted RSV-specific CD8+ effector T-cell response to infection and epitopes have been identified on the F, N, M, M2 and NS2 proteins of the virus[205-209].

1.4.3 *Adaptive immunity in infants and children*

Infant life begins with an immature immune system restricted in its capacity to produce signalling cytokines, reduced numbers of dendritic cells, unbalanced T-cell responses and delayed B-cell production of antibody which is often poorly sustained and of relatively low affinity (reviewed here[210]). Infants inherit a starting titre, or fixed-dose, of maternal antibody that rapidly wanes in the few weeks of life. The gift of passive immunity from the mother contains high-affinity neutralising antibody to the RSV F- and G-glycoproteins and are delivered through active placental transfer (IgG) and colostrum (IgG and IgA). These antibodies can protect from infection, lower-respiratory tract disease and hospitalisation but the estimated maternal antibody half-life is between 26 and 79-days, meaning this period of protection is short[211-218]. Once maternal antibody has fully waned the infant is especially vulnerable to developing disease. Furthermore the presence of maternal antibody may inhibit the development of infant humoral and cellular immunity following natural exposure[219,220]. Infant immunity develops slowly with repeated exposure and only 50% of infants will have detectable RSV antibody by 11-months of age and primary infection induces a neutralising antibody response that wanes within 3-months[57,221,222]. Several studies have described a negative correlation between serum neutralising antibody titres and the risk of re-infection in infants and children[55,214,218,223,224].

The relative balance between T-cells and the production of Th1-associated cytokines (such as IFNγ and IL-2), Th2-associated cytokines (such as IL-4, IL-5 and IL-13) and Th17-associated cytokines (such as IL-6, IL-17A, IL-1β and IL-23) can be an important determinant in clearing or contributing to disease in infants. Several, but not all, observational studies in infants with severe disease have associated a dominance of Th1 responses with viral clearance and a skew towards Th2 and Th17 responses with disease severity and wheeze later in life[173,225-234]. The innate and cellular immune response can therefore support recovery or exacerbate the clinical disease, and determinants of these factors are unknown. T-cells also play an important role after the peak of disease severity. Healthy infants typically stop shedding virus within 21-days of infection, but infants with underlying disorders of T-cell immunity can continue to shed virus for several months after infection further supporting a cellular role in controlling and clearing infection[29,235].

1.4.4 Adaptive immunity in adults

Unlike infants, healthy adults can be experimentally challenged with RSV for a better characterisation of immunity to infection and severe disease. Early RSV challenge experiments from the 1970's gave first indication that mucosal antibody plays an important role in protection from infection, which has been observed in later RSV challenge models and observational studies[236-238]. Multivariate analysis from observational studies described low nasal IgA RSV-specific antibody titres as an independent risk factor for RSV infection, and that severity of disease was partially abrogated with higher serum RSV-neutralising antibody in adults and the elderly[239,240]. Experimental human challenge models and observational studies have also reported a positive correlation with serum neutralisation antibody titres and protection from infection[223,241,242]. One proposal has been that measures of serum and mucosal immunity can be independent correlates of protection from infection and disease[238]. Interestingly the F- and G-specific serum neutralising antibody response to natural infection cannot be sustained for long and afforded little protection from re-infection even in healthy young adults. In one study, 15 naturally RSVA infected adults were nasally challenged with the same strain 2, 4, 8, 14, 20 and 26-weeks later. Re-infection was possible for 50% and 67% of volunteers at 2 and 8-months after natural infection respectively and serum neutralising antibody (not nasal IgA) could be correlated with protection from re-infection, and even 1 in 4 could be re-infected as early as 2-months after natural infection[223]. This might explain some of the wide variation seen in baseline serum neutralising antibody titres and their fluctuation; paired sera over two consecutive years detected a >2-fold rise in 3% and a >2-fold titre drop in antibody titre in 1.8-4.8% of healthy adult volunteers in another observational study[243].

Protection from severe disease in adults is associated with a strong Th1 immune response and lower levels of innate cytokines that were commonly reported from infants with severe disease[244,245]. The effector T-cell response in blood and the lungs differ in activation and temporal kinetics after infection, and resident memory lung CD8+ T-cells (CD69+CD103+) correlate with a reduction in symptoms after challenge and remain active for weeks after resolution of the clinical symptoms[246]. Furthermore, depletion of cellular and not serum neutralising antibody was the predictor of disease progression in HSCT patients[128].

1.4.5 *Adaptive immunity in the elderly*

Increasing age after 65-years is associated with a progressive functional loss in innate and adaptive immunity, more commonly referred to as immunosenescence. This is partly responsible for age-related higher incidences of malignancy, autoimmune disorders and susceptibility to developing severe disease with respiratory viral infections such as influenza and RSV. In particular, aging is associated with changes in T-cell immunity (such as a contraction in the diversity of naïve T-cells, an expansion of CD8+ memory T-cells and altered T-cell signalling) as a result of thymic involution and waning marrow lymphopoiesis.

From the perspective of RSV-immune responses with advancing age, comparative analysis of RSV-specific humoral immunity between younger and older subjects have found little/no measureable quantitative or qualitative deficit with antibody responses, mucosal immune responses or memory B-cell responses and increasing age[247,248]. Here, Falsey *et al* measured the background total and functional antibody titres to RSV in blood and noted comparable titres between age groups and even a greater fold increase in antibody after infection in elderly subjects compared with younger adults (79% vs. 64% ≥ 4-fold rise)[247]. Higher baseline and inducible antibody titres in the frail elderly did not confer greater viral neutralisation, as measured by the micro-neutralisation assay, suggesting an accumulation of non-protective antibody and immune dysregulation with age. High RSV-specific neutralising antibody titres from natural exposure persist into later life but relatively lower titres are associated with the development of severe disease in the elderly[240,242,248,249]. The rise in neutralising antibody seen after infection in the elderly, and higher titres of antibody in the elderly who attend day care (with presumed higher social contact and exposure)[247], could mean the wide range of neutralising antibody titres in this population is, in part, a reflection of the time elapsed since last exposure instead of a manifestation of aging. There have been no serial studies of neutralising antibody titres after infection to measure the rate of neutralising antibody decay, or experimental challenge models in elderly humans, but approximately 15% of PCR-confirmed RSV infections are not associated with changes in serum or nasal RSV-specific antibody from a cohort of elderly COPD patients, 73% of whom were symptomatic of infection[250].

Compared with healthy younger adults the resting RSV-specific CD4+ and CD8+ memory T-cell population in peripheral circulation from elderly adults contracts with increasing age and is associated with an expansion of suppressive regulatory T-cells and Th1 to Th2 switch of functional phenotype[208,248,251]. Age-

related changes in central and effector memory T-cell and F-specific memory B-cell populations were not observed[248,251]. These age-related defects in cellular immunity are likely contributory factors towards severe disease and mortality in the elderly.

1.4.6 RSV escape and evasion of the host immune response

The fact that RSV can, with little antigenic variation, cause recurrent infection throughout life demonstrates an effective armoury of immune suppression and evasion. The non-structural proteins NS1 and NS2, the G (attachment) protein and the small hydrophobic protein are the principal mediators of this potent evasion of the host innate and adaptive immune response (see summary in Table 1-2).

NS 1 and NS2 are the first two genes from the 3' promoter-proximal end of the genome and are unique to the paramyxovirus family. Recombinant wild-type RSV deleted of the NS1 and NS2 genes (rA2ΔNS1 and rA2ΔNS2) are significantly attenuated in human cell cultures[252-254], mice[254,255], cotton rats[252], calves (with bovine RSV)[256], African green monkeys[257] and chimpanzees[258,259]. The accessory protein products NS1 and NS2 contain 139 and 124 amino acids respectively and show little sequence homology with each other. They do not form part of the virion structure and are not packaged in mature progeny virions. Instead they appear in large quantities early after host cell invasion to inhibit and subvert innate and adaptive immune responses[260]. NS1 and NS2 specifically target both the inhibition of type I and type III interferon synthesis (IFNα, β and γ) and the response to interferon signalling from other cells through the interferon α/β receptor (and therefore the infected cells resistance to infection). NS1 and NS2 can operate independently and co-operatively with NS1 described as more dominant in man, although NS2 dominance is observed in mice and bovine models[252,261,262]. NS1 effects in man alone may involve up to 221 host proteins[263-265]. The NS1 and NS2 target interferon resistance to viral infection, the suppression of Th1 and Th17 cellular immune responses and the suppression of dendritic cell maturation. Furthermore, NS1 appears to promote cell growth and extend the life and infectivity of an infected cell by inhibition of early apoptosis (seen also in interferon α/β receptor negative Vero cell lines) and the promotion of transforming-growth β (TGFβ)-mediated arrest of infected A549 (human lung adenocarcinoma cells) in G_0/G_1 phase[252,260,266,267].

The transmembrane attachment (G) glycoprotein was first described in immortalised HeLa cells in 1977 and was described as having attachment properties 10-years later[268,269]. The mechanism of attachment

remains unclear but host receptor candidates include heparin sulphate, CX3CR1, surfactant protein A (SP-A) and Annexin A. The centrally conserved 13-amino acid region of the G protein contains both a heparin-binding domain (HBD) and a region of sequence homology with the CX3C chemokine fractalkine (amino acid positions 182-186)[270,271]. Peptide blockage of the HBD can abrogate attachment in immortalised Hep-2 cells but apical surfaces of ciliated human airway epithelial (HAE) cells that lack heparin sulphate remain readily infected[254,270,272]. Here, attachment uses mimicry of the CXC3 motif to bind with the CX3CR1 receptor on the apical surfaces of HAE and establish infection[44]. The G-protein contribution to immune evasion comes from a soluble truncated form (lacking the N-terminal 65-amino acids) that can act as decoy antigen for neutralising antibody, two mucin-rich variable regions that flank the centrally conserved region, and interaction through the CX3CR1 receptor to modulate (and possibly promote) the host inflammatory response and pulmonary inflammation[35,273-275]. Furthermore T-cell epitope mapping has implied CD8+ cells fail to recognise G protein with only CD4+ cells mapped onto epitopes of the centrally conserved region[276].

The contribution from the small hydrophobic (SH) porin-like protein is less well characterised. The SH protein contains 64 or 65 amino-acid residues (RSVA and RSVB respectively) and unlike the non-structural proteins there are similar counterparts for the SH protein in simian paramyxovirus virus 5 and mumps[277,278]. SH deleted mutant virus (ΔSH) failed to replicate effectively in the upper-respiratory tract (but replicated in the lower-respiratory tract) of mice, but the reverse was observed with ΔSH virus in calves[259,279]. In the context of para-influenza virus, SH protein has been shown to impede TNFα-mediated apoptosis[280].

Finally, palivizumab and natural antibody pressure can result in single amino acid substitutions and other escape mutants resistant to palivizumab neutralisation, with no loss of biological fitness in competition assays, and a similar process might occur for naturally produced antibody[281-285].

RSV proteins	Effects on host innate immune responses	Effects on host adaptive immune responses
NS1 and NS2	[A549 cells] ΔNS1 and ΔNS2 virus induced high levels of IFNα and IFNβ[264] [A549 cells] ΔNS1 failed to inhibit IRF3 gene transcription and IFNβ synthesis[286] NS1 and NS2 can degrade STAT2[287] [HEp-2 cells and A549 cells] NS1 and NS2 can degrade STAT2 [A549 cells] NS1 (and less efficiently NS2) can decrease levels of TRAF3 and NS1 (not NS2) can decrease levels of IKKε and disrupt interferon-inducing signals[288] [A459 cells] NS2 can inhibit RIG-I and early IRF3 activation[289] [HAE cells] NS2 can interfere with JAK/STAT signalling downstream of the interferon α/β receptor, preventing the activation of interferon-stimulated genes[290] [Cotton rats] siNS1 significantly reduced post-challenge RSV ling titres and induction of hyperplasia and eosinophils[291] [Mice] Intra-nasal treatment siNS1 nanoparticles recorded lower lung RSV titres and lung inflammation following challenge[254] [Vero and A549 cells] rA2ΔNS2 reduced the activation of NF-kB in IFN α/β receptor deplete Vero cells and rA2ΔNS1 and rA2ΔNS2 simulated lower levels of RANTES, IL-8, and TNFα[262] [Bovine RSV and Vero cells] Recombinant rabies virus expressing NS1 and NS2 abrogated interferon-mediated suppression of viral replication[256]	[Human dendritic cells] rA2ΔNS1 and rA2ΔNS2 enhances the expression of maturation markers in infected cells[265] [Human dendritic cells] Infection with rA2ΔNS1, but not rA2ΔNS2, showed increased responses for CD8+ CD103+ (mucosal epithelial cell homing) and Th17 cells with reduced CD4+ IL-4 producing cells (associated with Th2 vaccine immunopathology)[292] Recombinant wild type RSV A2 virus deleted of the NS2 gene (rA2ΔNS2) was significantly attenuated in chimpanzee challenge studies[259] [Human dendritic cells] siNS1 produced elevated type I interferon and induced the differentiated of naïve CD4+ T-cells to T-helper type 1 (Th1 cells) [254] [Mice] rA2ΔNS1 and rA2ΔNS2 reduced and expanded Foxp3+ regulatory (Treg) cell populations[255]
G	Centrally conserved CX3C-frackalkine-like region that inhibits transcription nuclear factor (NF)-κ B G-protein can interact with host genetic transcription and control of the cell cycle and cytokine production in A459 cells[293] Acts on lectin of dendritic cells for ERK1/2-phosphorylation mediated maturation inhibition[294]	Produces a soluble truncated form as a decoy antigen for neutralising antibody (sG)[273,275] Up to 80% of G-protein is secreted as a truncated soluble decoy protein and sG is released shortly before the release of mature viral progeny[274]
SH	Inhibition of TNFα-mediated apoptosis and propagation of infected cell survival in mutated para-influenza virus to express RSV SH protein (rPIV5ΔSH-RSV SH)[280]	

Table 1-2. Summary of experimental evidence for the putative mechanisms of RSV effects host innate and adaptive immune responses to infection.

The evidence presented in this table are not exhaustive. A549, human alveolar carcinoma cells. Vero cells, kidney epithelial cells from African green monkeys. Hep-2 cells, human epithelial cells. IRF3, interferon-regulatory factor 3. RIG-I, retinoic acid- inducible gene I (RIG-I). JAK, Janus-activated kinase (JAK). STAT, signal transducer and activator of transcription (STAT). siNS1, short interfering RNA against the NS1 gene. rA2 Δ NS1 and rA2 Δ NS2, recombinant wild type RSV A2 virus deleted of the NS1 and NS2 genes. NF-kB, nuclear factor kappa-light-chain-enhancer of activated B-cells. HAE, human alveolar cells. RANTES, regulation on activation, normal T-cell expressed and secreted.

1.5 50-years of RSV vaccine development

This year, 2016, will mark 60-years since the discovery of RSV and half a century of clinical vaccine research. The current field has nanoparticle vaccines in early phase III development, subunit vaccines in phase II development, and a plethora of other vaccine designs at phase I evaluation. A snapshot of the current field of RSV vaccine development is provided by PATH and is shown in Figure 1-4. Despite these many clinical trials and testing of different vaccine platforms (see Table 1-3) that have included whole-cell, live-attenuated, sub-unit and nanoparticle vaccine formulations, there remains no licenced, safe and effective vaccine to mitigate the burden of RSV disease.

The disastrous formalin-inactivated RSV (FI-RSV) vaccine trial in the in 1960s, an incomplete understanding of immune correlates of protection and the greatest burden of disease from infants in the first months of life have made RSV vaccine development especially challenging. This section will outline some of the critical lessons learned from the history of RSV vaccine successes and failures in humans.

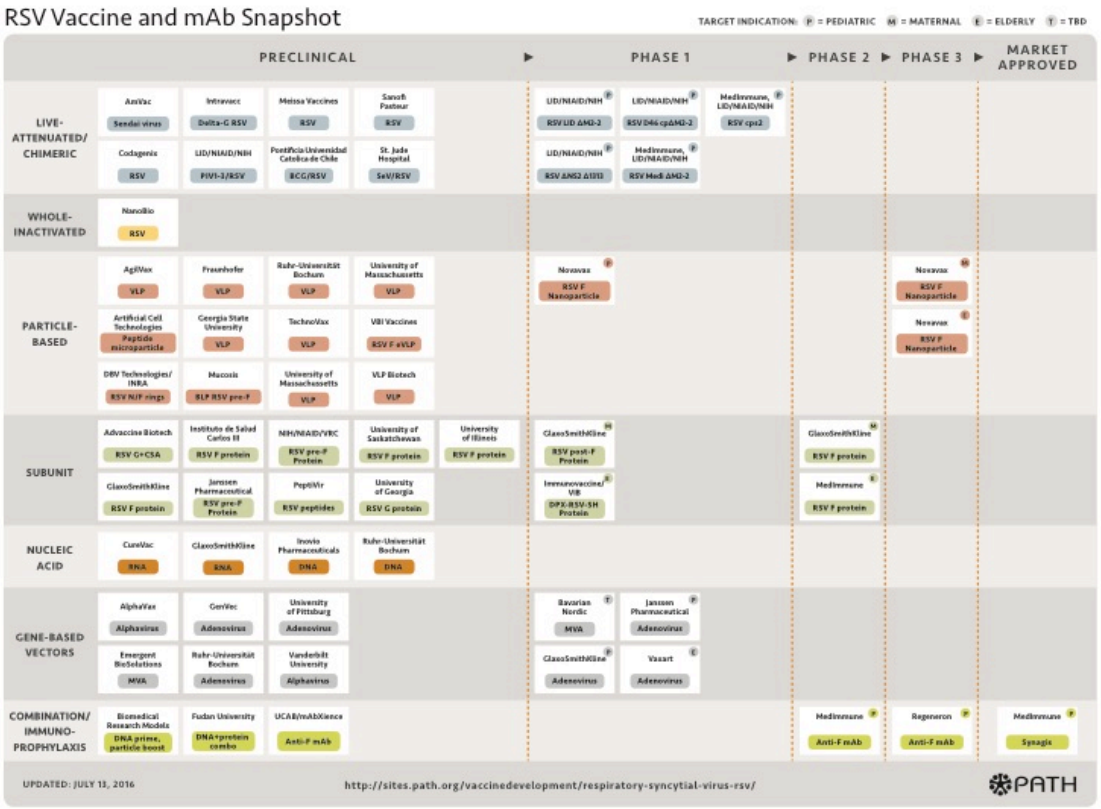


Figure 1-4. Schematic snapshot of the current field of RSV vaccine development.
The figure is produced by the Program for Appropriate Technology in Health (PATH) and reproduced with permission[295]. Last accessed July 2016.

Whole-virus vaccine	Live-attenuated vaccines	Subunit vaccines	Peptide vaccines	Nanoparticle vaccine
FI-RSV[296-299]	*cp*RSV[18,300] RSV *ts*-1[301-303] RSV *ts*-2[304] RSV *ts*-1 A, B C[241,305] *cpts*248/955[306] *cpts*530/1009[306] *cp*-52B[307] *cpts*248/404[308,309] rA2cp248/404ΔSH[310] rA2cp248/404/1030ΔSH[310] rA2cpΔNS2[311] rA2cp248/404/1030ΔNS2[311] rA2cp530/1009ΔNS2[311] MEDI-559[312] MEDI-534[313-315]	PFP1[316-321] PFP2[216,309,322-325] PFP3[326] F, G, M subunit[327,328]	BBG2Na[329,330]	F-nanoparticle[331,332]

Table 1-3. RSV-vaccine formulations evaluated in man.
Summary of the RSV vaccine candidates that have been evaluated in humans, and does not contain reference to vaccine candidates in preclinical evaluation. PFP, purified F-protein. MEDI, MedImmune.

1.5.1 Inactivated whole-virus vaccines; the disastrous Lot 100 vaccine

Successes with formalin-inactivation and vaccines for polio, measles, influenza and para-influenza encouraged a similar approach for the first RSV vaccine. RSV had been discovered only 10-years earlier and with limited preclinical immunogenicity data from hamsters the formalin-inactivated RSV vaccine (FI-RSV) was given to infants in the 1960's. The "Lot 100" FI-RSV product was made by Pfizer using the Bernett stain of RSV which was propagated in human embryonic kidney cells (x3), then vervet monkey kidney cells (x10), formalin-inactivated (0.4% formaldehyde for 72-hours), concentrated by ultracentrifugation and adsorbed to 4mg/mL aluminium hydroxide. FI-RSV was given by 1-3 IM injections to infants and children across four centres in the US over the winter of 1965/66. Control groups of children received either no vaccine or formalin-inactivated para-influenza vaccine. The trial was a disaster that resulted in the death of two infants following natural RSV exposure and a 16-fold rise in hospitalisation rate in other vaccine recipients[296-299]. Rates of RSV-related disease were the same in controls and formalin-inactivated para-influenza vaccine recipients (approximately 5%). It was clear that FI-RSV primed the infant immune system towards enhanced disease following natural infection, and this enhanced respiratory disease (or ERD) had a predilection for infants under 6-months of age. FI-RSV induced an antibody response after vaccination (43% showed a four-fold rise in serum neutralising antibody and 93% showed a four-fold rise in complement fixation) but these antibodies failed to recognise, neutralise and protect from natural RSV. Lung histology from the two infant fatalities confirmed the presence of high titres of RSV in the lower respiratory tract and further implicated ERD as a vaccine-primed Th2-associated immunopathology with "peribronchiolar moncytic infiltration with some excess of eosinophils". Re-examination of the autopsy material later revised this description, with immunopathology seen in the bronchi (a dense infiltrate made up of 50% neutrophils, 30% macrophages and 20% lymphocytes), bronchial epithelium (10% neutrophils, 10% macrophages and 80% lymphocytes), complement activation and eosinophils making up only 1-2% of the total infiltrate[333]. It has since been speculated that the original interpretation of eosinophilic pathology may have arisen due to *Klebsiella* and *E. coli,* which was cultured from the first and second autopsies respectively, being interpreted as super-infection that was unrelated to the underlying pathology (and cause of neutrophils) at a time when neutrophilic pneumonia had not (yet) been recognised as a manifestation of RSV disease[333,334]. The fact that ERD had a predilection for RSV-naïve infants and that it did not recur on

subsequent RSV seasons suggested that immunity derived from natural RSV exposure was protective for FI-RSV ERD.

The natural recourse was to reproduce and then explore the mechanisms behind FI-RSV ERD in animal models, which could then be applied to screen future RSV vaccine candidates. In 1971 the semi-permissive susceptibility of the *Sigmodon hispidus* cotton rat to human RSV was described and in 1986 the cotton rat model successfully reproduced some of the histological pathology seen in infants after FI-RSV vaccination[335,336]. Over the many decades that have followed several other animal models have been developed (including mice, African green monkeys, calves and lambs) and not withstanding the limitations of each model the mechanisms behind FI-RSV ERD remain unclear and the subject of active enquiry. Animal challenge experiments and post-mortem lung histology from the infant fatalities have implicated ERD as a vaccine-primed Th2-associated immunopathology following exposure to natural RSV infection which continues to cast a long shadow over subsequent vaccine design and development. Future RSV vaccine candidates would require careful pre-clinical evaluation in animal challenge experiments,and, provided no FI-RSV associated immunopathology was observed, a cautious progression from phase 1 trials in healthy adults through to series of age de-escalation trials towards infants that includes the monitoring of infants over the next RSV transmission season.

1.5.2 Live-attenuated virus vaccines

Clinical evaluation of live-attenuated RSV vaccine candidates also began in the 1960's and have been the only RSV vaccine design to be given safely to RSV-naïve infants[337]. Live-attenuated RSV vaccines candidates have been delivered intra-nasally with the intended benefits of promoting mucosal immunity while avoiding the need to overcome maternal antibody inhibition. The first two types of live-attenuated RSV vaccine were generated in the 1960's by repeated passage under cold temperature (*cp*) or by chemical mutagenesis to generate temperature sensitive (*ts*) virus. Tested in healthy prisoners as early as 1968 it was intended that the vaccines would be restricted to replication in the cooler upper respiratory tract and unable to replicate in the lower respiratory tract with the potential to cause harm. Live-attenuated vaccines were developed further using combinations of cold passage and chemical mutagenesis, and from the 1990's by genetic engineering and the deletion of select amino acids or whole genes/open reading frames. More

recently chimeric viruses with para-influenza 3 (MEDI-534) have been evaluated in man. However, despite the number of different types live-attenuated RSV vaccine candidates being safely tested none have been successful to warrant licensure. Instead they have been troubled by nasal congestion (young infants are obligate nasal breathers), genetic instability, vaccine virus transmission, and limited immunogenicity[308,310,312,313,338]. The balance between vaccine virus attenuation required for safety, and vaccine virus virulence needed to stimulate and an effective and durable immune response for future protection, has proved very difficult to achieve.

1.5.3 Sub-unit, peptide, nanoparticles and viral-like particle virus vaccines

Purified RSV F-protein (PFP) isolated from infected Vero cell lines have been tested in healthy adults, the elderly, pregnant mothers and sero-positive infants. Although the data from these trials reported a good safety profile, concerns about vaccine primed immunopathology to natural exposure in RSV-naïve infants following animal challenge experiments using subunit vaccine, combined with inadequate immunogenicity in the elderly, has focussed RSV subunit vaccine development as maternal vaccines[322,323,332,334,339]. One subunit vaccine, purified fusion protein 2 (PFP-2), has been given by IM injection to 35-mothers in the third trimester of pregnancy. No adverse events from mother or babies were observed and infants of vaccinated mothers recorded a 4-fold induction in maternal antibody (although the effect on neutralising antibody titres were disappointing)[216]. Subunit vaccines expressing F, G and M proteins have been safe and immunogenic in adult and elderly populations. However pulmonary immunopathology similar to FI-RSV ERD was observed to other RSV G glycoprotein subunit candidate vaccines in the mouse challenge model[339]. Although the mechanisms of disease are different for FI-RSV and G glycoprotein vaccine ERD, the mouse model was characterised by the involvement of CD4+ cells (with a loss of CD8+ cell inhibition) and the production of IL-4, IL-5 and IL-13 cytokines[339-342]. Preclinical concerns also contributed to the termination of a G vaccine candidate, BBG2Na. BBG2Na was G protein peptide (amino acids 130-230) fused to the albumin-binding domain of Streptococcus G protein as a carrier molecule. This vaccine candidate appeared overall to be safe and immunogenic in healthy adults and the elderly, though some volunteers reported adverse events, including rash and nephritis reactions, but Th2 immunopathology in the rhesus macaque challenge model meant this vaccine candidate would not be developed for use in an infant

population[329,330,343]. Finally, in the last 3-years clinical data from phase I and phase II clinical trials of healthy adults has shown novel insect-cell derived RSV F-nanoparticle vaccines are safe and powerful inducers of RSV-neutralising antibody, and are now in development as maternal vaccine entering phase III clinical trials[331,332].

1.5.4 Requirements for developing a safe and effective RSV vaccine

The high rate of emergency admissions, lack of universal and cost-effective preventative measures and the magnitude of seasonal disease incidence maintain RSV as a major priority for vaccine development[344]. The obstacles facing RSV vaccine development are hard to understate. In short, an effective RSV vaccine is required to better natural infection that sets an extremely high bar for any vaccine. Immune correlates of protection from infection and severe disease are likely to be different and remain unknown. The immune response from infants would benefit from the parallel induction of neutralising antibody and Th1-balanced immunity at an effective strength before the peak onset of disease aged 2-months and in the presence of maternal antibody. Immune supressed adults and the elderly would require restoration of the cellular immune response in the presence of pathological/iatrogenic immune suppression and immune senescence respectively. This all sits on the background of FI-RSV and subunit vaccine immunopathology in semi-permissive animal challenge models, barring the use of whole-cell or subunit vaccines in RSV-naïve infants. A new and different biological platform, genetically modified virus used as vectors for the expression of vaccine antigen, has become the next recourse in the pursuit of a safe and effective RSV vaccine that could be used in infants and other target populations in need of vaccination.

1.6 Aim and objectives of this thesis

This thesis aims to address the burden of severe RSV disease through the careful development of a safe and effective vaccine. The objectives of this thesis are:

1. To revise the estimated burden of emergency hospital admissions for infants with bronchiolitis in England and analyse temporal trends in hospital admission rates over several decades.
2. To design, setup, execute and analyse observational studies to characterise humoral and cellular RSV-specific immune responses in paediatric healthcare workers, children and infants.
3. To design, setup and execute an open-label, dose-escalation, single-site, phase I (first-in-man), vaccine trial of two novel viral-vectored vaccines for RSV (PanAd3-RSV and MVA-RSV) in healthy adults aged 18-50 years and 60-75 years.
4. To analyse the safety and tolerability of the experimental viral-vectored vaccines PanAd3-RSV and MVA-RSV in healthy adults aged 18-50 years and 60-75 years.
5. To analyse the induction of humoral and cellular responses to the experimental viral-vectored vaccines PanAd3-RSV and MVA-RSV in healthy adults aged 18-50 years and 60-75 years.
6. To describe the host transcriptional response to the experimental viral-vectored vaccines PanAd3-RSV and MVA-RSV in healthy adults aged 18-50 years.

The overview of this thesis has been to address each objective in a separate chapter. This way I hope to begin with new epidemiological data analyses that underpin the case for developing an RSV vaccine for use in the United Kingdom (Chapter 3). The next chapter seeks to characterise the immune response to natural RSV exposure in paediatric healthcare workers and infants, and explore where vaccination may learn lessons from the robust immunity from repeated seasonal exposure (Chapter 4). These lead into the design, setup and execution of a phase 1 (first-in-man) vaccine trial of two viral-vectored experimental vaccine candidates for RSV (designated RSV001). I then analysed the performance of the RSV001 trial (Chapter 5), the primary endpoint of safety and tolerability (Chapter 6) and secondary endpoints of immunogenicity (Chapter 7). Finally I explored early host genetic responses to vaccination and where these responses could be correlated with clinical and immunological responses at later time points in an exploratory analysis (Chapter 8). The final chapter is a discussion of this work and thoughts on future direction (Chapter 9).

Chapter 2: General laboratory materials and methods

This chapter details the reagents and assays used to define and quantify RSV-specific immune responses to natural exposure and vaccination in later chapters. The optimisation and validation of these assays was performed ahead of their use on clinical samples, and the data for these setup experiments are not presented in this thesis.

2.1 Buffers

The chemical solutions used across Oxford Vaccine Group laboratory (OVGL) assays for the measurement of RSV-specific immune responses are detailed in the table below.

Buffer	Preparation	Storage
10x Phosphate buffered saline solution (PBS)	800mLs of dH_2O was mixed with 80 g/L sodium chloride, 20.6 g/L sodium phosphate dibasic heptahydrate, 3.14 g/L potassium phosphate monobasic and 1.6 g/L potassium chloride and titrated to a pH between 7.2 and 7.4 using 10M sodium hydroxide (NaOH, Sigma-Aldrich) and 50% hydrochloric acid (HCl, Sigma-Aldrich) as appropriate. The final volume was made up to 1 L with dH_2O.	Room temperature for no more than 6 months
1x Phosphate buffered saline solution (PBS)	100mLs of 10x PBS was added to 900mLs of dH_2O and pH corrected to between 7.2 and 7.4 as described above.	Room temperature for no more than 6 months
PBS with 0.25% Tween solution	2.5mLs Tween detergent (VWR International, BDH 663684B) was added to 1 L of 1xPBS	Room temperature for no more than 6 months
PBS with 0.05% Tween solution	0.5 mL of Tween detergent (VWR International, BDH 663684B) was added to 1L of 1xPBS	Room temperature for no more than 6 months
1% milk in PBS solution	100mLs of 1xPBS solution was added to 1g of skimmed milk powder (Fluka 70166) and mixed gently	4°C for no more than one week
4% milk in PBS solution	100mLs of 1xPBS solution was added to 4g of skimmed milk powder (Fluka 70166) and mixed gently	4°C for no more than one week
5% milk in PBS solution	100mLs of 1xPBS solution was added to 5g of skimmed milk powder (Fluka 70166) and mixed gently	4°C for no more than one week
PBS-EDTA-0.5%NBBS solution	Dissolve 5 PBS tablets and 0.744g EDTA powder in 800mLs sdH_2O, made up to a volume of 1L with dH_2O and pH corrected to 7.2 using 10M NaOH/50%HCl. Under sterile conditions then add 5mLs NBBS-HI.	4°C for no more than six months
R0 media	500mLs RPMI media with phenol red indicator and 25mM hepes modification (Sigma-Aldrich R-5886) was mixed with 5mL L-glutamine at 2mM (Sigma-Aldrich G-7513) and 5mLs penicillin-streptomycin solution (containing 50U/mL and 0.05g/mL of each respectively, Sigma-Aldrich P-4458)	4°C for no more than one month
R10 media	50mLs of heat-inactivated new born bovine serum (NBBS, Sigma-Aldrich) was added to 450mLs R0 media	4°C for no more than one week
R10+ media	500mLs of R10 media with 5mLs of 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Gibco 15630)	4°C for no more than one month
M0 media	5mLs of penicillin-streptomycin (Sigma-Aldrich, P4458-100ML) added to 500mLs of Eagle Minimum Essential Media (MEM, Sigma-Aldrich, M4655-500L)	4°C for no more than one month
M10 media	5mLs of penicillin-streptomycin (Sigma-Aldrich, P4458-100ML), 50mLs of foetal bovine serum added to 450 mLs of Eagle Minimum Essential Media (MEM, Sigma-Aldrich, M4655-500L)	4°C for no more than one month
Thawing media	1L RPMI-1640 with 5mLs L-glutamine at 2mM (containing 50U/mL, Sigma-Aldrich G-7513) and 5mLs penicillin-streptomycin solution (containing 0.05g/mL, Sigma-Aldrich P-4458) – 9mLs plus 1mL CTL serum free wash buffer [details] with 100 microL DNAase (Sigma-Aldrich)	4°C for no more than one month
0.5% Bovine serum albumin solution in PBS	100mLs 1xPBS to 0.5g BSA powder (Sigma-Aldrich, A7030). BSA was batch tested and approved for use on clinical trial samples in accordance with local SOPs.	4°C for no more than one month

Table 2-1. Preparation and storage of chemical solutions for OVGL immunological assays for the measurement of RSV-specific immune responses.
The preparation of each solution was guided by local OVGL standard operating procedures (SOPs).

2.2 Sample collection and processing

The collection, transportation, processing and storage of all samples in all studies adhered to OVGL and OUHL standard operating procedures (SOPs), good clinical practice (GCP) and good laboratory practice (GLP) standards.

2.2.1 PBMC collection, separation and storage

Blood samples were collected in 50mL Falcon tubes (BD) with added preservative free heparin for injection (1000 Units/mL, Pharmacy, DAI 043); 15µLs heparin per 1mL whole blood collected for assays that used peripheral blood mononuclear cells (PBMCs). PBMCs were isolated within 6-hours of sample collection from a 1:1:1 mix of heparinised blood with R0 by density centrifugation (1000g for 20-minutes) through Lymphoprep™ (Alere). PBMCs were harvested into 50mLs Falcon tubes (BD), washed with 45mLs R0 media and centrifuged again (800g, 10-minutes). The supernatant was discarded and the pellet re-suspended in 1mL R10 media per 10mLs of whole blood. A 1mL aliquot of cell suspension was immediately prepared for each fresh B- or T-cell enzyme-linked immunospot (ELISpot) assay, as described later. The mean live PBMC concentration of the remaining suspension was estimated using a haemocytometer and 10µLs of a 1:1:1 mix of cell suspension, 1xPBS and trypan blue (trypan blue stain, WVR International 700003416). The suspension of PBMCs was then centrifuged a final time (500g for 10-minutes), the supernatant discarded and the pellet reconstituted to a PBMC concentration of $10x10^6$/mL with Recovery™ Cell Freezing Medium (10% dimethyl sulfoxide, or DMSO, and calf serum, Invitrogen). 1mL aliquots were stored at -80°C overnight and then cryopreserved for later analysis.

2.2.2 Serum collection, preparation and storage

Blood was collected in serum separation tubes (BD) and allowed to clot at room temperature for a minimum of 30-minutes. The tubes were then centrifuged for 10-minutes at 3000g. Sera supernatant was aspirated and aliquoted (2mLs sera per vial) and cryopreserved at -80°C for later analysis.

2.2.3 *Nasal sample collection, preparation and storage for the detection and quantification of RSV-specific immune responses to vaccination*

Nasal samples were collected by mid-turbinate flocked swab (Copan Diagnostics Inc) in 1mL M4RT media (Remel). One swab was inserted into both nostrils and rotated three times. The swab was immersed in the M4RT media for a minimum of 30-minutes at room temperature before being discarded. A protease inhibitor (Roche, 4693159001) was added at a 1 in 40 M4RT media volume dilution. Samples were equally divided into two vials and cryopreserved at -80°C for later analysis.

2.2.4 *Nasal sample collection, preparation and storage for the detection of viral infections*

Nasal samples were collected by mid-turbinate swab in Copan Universal Transport Medium kit (UTM-RT mini, Copan Diagnostics Inc) according to the manufacturers instructions. One swab was inserted into both nostrils and rotated three times. Samples were stored at room temperature and transported on the same day as collection to OUHL for polymerase chain reaction (PCR) analysis.

2.2.5 *Nasal sample collection, preparation and storage for the detection of PanAd3-RSV vaccine virus shedding*

Nasal samples were collected by mid-turbinate flocked swab (Copan Diagnostics Inc) in 3mLs M4RT media (Remel). One swab was inserted into both nostrils and rotated three times. Samples were cryopreserved at -80°C and transported to WuXi AppTec (USA) for analysis.

2.2.6 *Functional genomics samples*

Tempus RNA™ (Applied Biosystems) tubes with 2-3mLs of whole blood were stored at room temperature for 2-hours and cryopreserved within 24-hours of sample collection and stored at -80°C for later analysis.

2.3 OUHL safety and diagnostics assays

Blood and nasal samples collected to determine volunteer eligibility and monitor safety were transported to the Oxford University Hospitals NHS Trust Laboratory (OUHL) at the John Radcliffe Hospital for reporting by an accredited National Health Service (NHS) laboratory via the CaseNotes (version 5) hospital intranet system.

2.3.1 Routine haematology and biochemistry, and screening for IgA deficiency and blood borne viruses

Local OUHL protocols were applied for the collection of bloods samples for haematology (in ethylenediaminetetraactetic acid, EDTA tubes), biochemistry (in lithium tubes) and quantification of IgA antibody, the detection of anti-HIV (human immunodeficiency virus) and anti-HCV (hepatitis C) antibody, and the detection of hepatitis B virus surface antigen (HbsAg) (in serum separation tubes).

2.3.2 Viral PCR detection from nasal samples during an influenza-like illness

Viral diagnostics were performed by PCR for RSV, influenza A, parainfluenza 1/2/3, rhinovirus, coronaviruses, adenovirus, metapenumovirus, enterovirus, parechovirus, bocavirus, and *Mycoplasma pneumoniae*. The PCR panel was developed, modified according to prevalent viral strains and validated by internal and external quality control standards for use on clinical trial and NHS patient samples by local SOPs.

2.4 OVGL immunological assays

2.4.1 Enzyme linked immunosorbent assay (ELISA) for the detection and quantification of F-specific IgG and IgA antibody in serum and nasal samples

Nunc Maxisorp 96-well plates were coated with RSV recombinant F protein antigen (Sino Biological Inc) diluted in PBS at a concentration of 5mg/mL for the serum ELISA and 20mg/mL for the nasal ELISA. The plates were sealed and stored at 4°C for no more than 72-hours before use. On the day of use, the plates were blocked with 200µL/well 5% milk-PBS solution blocking buffer for 1-hour (5% CO_2, 37°C, 95% humidity). 50µL/well of a 1:50 dilution of sera and undiluted nasal antibody (primary antibody) was added in duplicate and an R10 negative control and positive control samples (pooled donated samples) was included on each plate. Plates were then incubated for 1-hour (5% CO_2, 37°C, 95% humidity). The plates were then washed (200µLs/well PBS, three times) before 100µL/well of 1:10000 dilution of anti-human IgG and IgA conjugated to horseradish peroxidase (HRP) in 5% milk-PBS solution was added (secondary antibody). The plates were then incubated for 1-hour (5% CO_2, 37°C, 95% humidity). Plates were washed (200µLs/well PBS, three times) before 100µL/well tetramethylbenzidine substrate (TMB, Sigma-Aldrich) was added for 5-minutes under light-protected conditions (tertiary antibody). The reaction was terminated at 5-minutes by the addition of 50µL/well of 2M H_2SO_4. Each plate was read at an absorbance of 450nm using a Biotek Elx808 microplate reader. The optimal coating concentrations and antibody dilutions were determined by checkerboard assay in accordance with local SOPs.

2.4.2 Plaque reduction neutralisation assay (PRNA) for the quantification of serum RSV-neutralising antibody

An RSV-A2 strain was kindly donated from stocks at the KEMRI Wellcome Trust research facility in Kilifi, Kenya, courtesy of Charles Sande and James Nokes. The local biological safety officer approved the use of live RSV virus, a category II micobiological hazard, after formal risk assessment (reference OVGL062-F1). Virus and human samples were handled in class II microbiological safety cabinets, in accordance with local SOPs.

50 plaque-forming units (pfu) of RSV A2 virus in 50µLs was mixed with 50µLs of heat-inactivated sera from volunteers, in a serum dilution series from 1:20 to 1:10'240 in M0 media. Sera was heat-inactivated by 56°C water bath for 30-minutes. RSV A2 virus mixed with volunteer sera was incubated for 1-hour (5%

CO_2, 37°C, 95% humidity) before 50μLs/well added to a confluent monolayer of HEp-2 cells. The Hep-2 cells, donated from the Sir William Dunn School of Pathology at the University of Oxford, had been seeded onto 96-well plates (flat bottomed clear tissue culture plates, BD Biosciences, 353072) at $3x10^4$ cells/well earlier, and each plate was removed of M10 media and washed twice with 200μLs/well PBS before the addition of virus and sera. A total of 6-cycles of 30-minutes incubation (5% CO_2, 37°C, 95% humidity) followed by 10-minutes of platform rotation (angle 30° and 40 revolutions/minute) were completed before allowing the Hep-2 cells/RSV A2/volunteer heat-inactivated sera to incubate a further 60-hours (5% CO_2, 37°C, 95% humidity). Each sample was tested in duplicate. Controls included A2 virus with sera that had not been heat-inactivated, media with heat-inactivated sera (no RSV A2 virus), and human anti-RSV reference serum standards (Biodefense and Emerging Infections, BEI, Resources Repository NR-4020). Infant sero-negative samples were not available to calibrate the assay, which was done by titration of the BEI control standard serum instead.

After 60-hours incubation the plates were fixed with cold acetone/methanol (80:20%, v/v, 100μLs/well), washed with PBS (200μLs/well, twice) and air-dried before storage at 4°C. Plaque staining used primary mouse anti-RSV antibody (AbD serotec 7950-1705, diluted 1:500 PBS dilution, 100μLs/well) for 60-minutes incubation at room temperature, followed by washing (200μLs/well PBS, three times) and addition of goat anti-mouse horse radish peroxidase (HRP) conjugated secondary antibody (AbD serotec STAR120P, 1:1000 PBS dilution, 100μLs/well) for 60-minutes incubation at room temperature. The plates were then washed (200μLs/well PBS, three times) before immunostaining with 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich) for 30-minutes at room temperature and final PBS wash. Plates were air-dried and stored at room temperature. Plaques were enumerated using an Autoimmun Diagnostika (AID version 5.0) reader. The serum RSV-neutralising antibody titre was defined as the sera dilution at which 50% of plaques survived (ND_{50}), and calculated using the Spearman-Karber method[345]. The lower limit of detection was a titre of 20; titres below 20 were assigned an arbitrary value of half the lower limit of detection (LLD) in accordance with local standard practice.

2.4.3 *Ex vivo enzyme-linked immunospot (ELISpot) assay for the detection and quantification of IgG and IgA antibody secreting cells (ASCs) in peripheral blood*

MultiScreen$_{HTS}$ HA 96-well plates (Millipore MSHAN4510) were coated with RSV F protein antigen (5μg/mL) (Sino Biological Inc), human serum albumin (10μg /mL) (HSA, Sigma-Aldrich), tetanus toxoid protein (5μg/mL) (Statens Serum Institut), and polyvalent goat anti-human immunoglobulin (10μg/mL) (Caltag). Plates were sealed and stored at 4°C overnight and for no more than 72-hours before use. On the day of use, the plates were washed (200μLs/well PBS, four times) and blocked with 1% milk-PBS solution for 45-minutes in an incubator (5% CO_2, 37°C, 95% humidity). The 1% milk-PBS block was then replaced with 100μLs/well R10.

Freshly separated PBMCs suspended in R10, as described in section 2.2.1, were added at three concentrations; $2x10^6$, $1x10^6$ and $5x10^5$ cells/mL to F, HSA and Tet wells, and at dilutions of $2x10^5$, $2x10^4$ and $2x10^3$ cells/mL to goat anti-human immunoglobulin control wells. Each cell concentration was tested in triplicate. Plates were incubated overnight (5% CO_2, 37°C, 95% humidity) before the cells were discarded and the plates were washed (200μLs/well PBS, four times, followed by 200μLs/well PBS with 0.05% Tween, four times) in preparation for development. Secondary antibodies were diluted in 4% milk-PBS solution and added to each well (1 in 2000 dilution for IgG and 1 in 1000 dilution for IgA, 100μLs/well), with anti-human IgG-fluorescein isothiocyanate (FITC, Sigma-Aldrich F1641-.5ML) and anti-human IgA-biotin (AbD Serotec 205008), and incubated a minimum of 4-hours (5% CO_2, 37°C, 95% humidity). The plates were washed (200μLs/well PBS, four times, followed by 200μLs/well PBS with 0.05% Tween, four times) and tertiary antibody in 4% milk-PBS solution was added to each well (1 in 1000 dilution, 100μLs/well), using anti–FITC–alkaline phosphatase (AP, Sigma-Aldrich A1812-.2ML) and streptavidin–horseradish peroxidase (AbD Serotec STAR5B), for 30-minutes at room temperature. After washes (200μLs/well PBS with 0.05% Tween, four times), the AEC substrate (Sigma-Aldrich AEC101-1KT, made up according to manufacturers instructions, 100μLs/well) was added for 30-minutes at room temperature. Plates were washed once with water before adding the Vector Blue alkaline phosphatase substrate kit (Vector Laboratories SK-5300, made up according to manufacturers instructions, 100μLs/well) for 10-minutes at room temperature. The plates were washed once with water and allowed to air-dry at room temperature.

IgG and IgA spots were enumerated using an AID ELISpot reader (spot intensity range 5-225, size 30-5000 and gradient 1-90) using the optimal cell concentration for counting as determined by the operator. Plates were rejected (failed) in the absence of any spots in human immunoglobulin controls, or if the wells were too discoloured for counting. All AID-counted plates were manually reviewed for artefacts. Responses were measured as antigen-specific spots per million PBMCs with HSA background subtracted. One spot per million PBMCs was set as the lower limit of detection, and negative and zero values after HSA background subtraction were reverted to half the lower limit of detection in accordance with standard practice (a value of 0.5 spots per million PBMCs).

2.4.4 Memory B-cell ELISpot for the detection and quantification of IgG and IgA immune responses

PMBCs frozen in Recovery™ Cell Freezing Medium (10% DMSO, and calf serum, Invitrogen) at $10x10^6$cells/mL were thawed rapidly using a water bath and with thawing medium added slowly. Polyclonal stimulants cysteine-phosphate-guanine-ODN 2006 (CpG, human TLR-9 stimulatory oligonucleotide, Bioscience UK tlrl-2006-5, final concentration 1.7μg/ml), pokeweed mitogen (PWM, Sigma-Aldrich L-9379, final concentration 83.33ng/ml) and *Staphylococcus aureus* Cowans strain 1 (SAC, VWR International 507861-50, final dilution 1 in 50'000) were combined as an antigen mix. 100μLs of PBMC suspension (total $2x10^5$ cells) was added to 100μLs CpG/PWM/SAC antigen mix in each well of a 96-well plate[d] and incubated for 6-days (5% CO_2, 37°C, 95% humidity). PBMCs were harvested from the culture plate to a universal centrifuge tube, topped up with wash buffer (PBS-EDTA-NBBS) and centrifuged (1000g for 10-minutes). Supernatant was removed, and cells re-suspended in wash buffer, and spun again (250g for 10 minutes); this wash step was repeated once more before cells were re-suspended in 1mL R10. PBMCs were counted using the method described in section 2.2.1, and a dual-colour IgG and IgA ELISpot assay was then performed as described earlier.

[d] Or as many wells as allowed given the recovered cell count

2.4.5 *Ex-vivo ELISpot assay for the detection and quantification of interferon-gamma (IFNγ)-producing T-cells in peripheral blood*

96-well Multiscreen polyvinylidene fluoride membrane plates (PVF, Millipore MAIPS4510) were coated overnight with mouse anti-human IFNγ clone 1-D1K (MAbTec 3420-3) in Dulbecco's PBS (dPBS, Gibco 14190) at a dilution of 1 in 200 (100µLs/well). The plates were sealed and stored at 4°C overnight and for no more than 72-hours before use.

Coated plates were washed (250µLs/well sterile dPBS, four times) and blocked with R10+ (200µLs/well) for a minimum of 2-hours (5% CO_2, 37°C, 95% humidity). The 1mL aliquot of freshly separated PBMCs suspended in R10, as described in section 2.2.1, were counted using the methods described in section 2.2.1, and the required R10+ volume was added to give a final concentration of $4x10^6$ cells/mL.

RSV peptide pools consisted mainly of 15-mer sequences with 11 amino acid overlaps that covered the sequence of RSV proteins F, N and M2-1 (JPT Peptide Technologies). The 269 peptides were dissolved in 100% DMSO (Sigma-Aldrich D2650) and arranged into four separate pools; Fa (64 peptides representing the N-terminal half for the F protein), Fb (64 peptides representing the C-terminal half for the F protein, N (95 peptides) and M (46 peptides). The RSV peptides were donated from ReiThera Srl. The concentration of each pool was adjusted to 0.3µg/mL single peptide. Control wells included DMSO background (DMSO, cells, no peptides), R10+ media (no cells, no peptides), cytomegalovirus cell lysate, mixed human leucocyte antigen (HLA) class I-restricted peptides from influenza, Epstein-Barr virus and CMV (FEC) and concanavalin A (ConA, Sigma-Aldrich). Peptides were diluted in R10+ and filtered through 0.2µm before use.

PBMCs were added to each well (200'000 PBMCs/well in 50µL) in triplicate with peptides (50µLs/well), as appropriate, and incubated overnight (5% CO_2, 37°C, 95% humidity). Plates were washed (250µLs/well, 1xPBS with 0.05% Tween, seven times) and mouse anti-human IFNγ clone 7-B6-1 biotinylated antibody (MAbTec 3240-6) added (50µLs/well of 1 in 2000 dilution in 1xPBS with 0.5% BSA, filtered through 0.2µm before use) and allowed to incubate at room temperature for 2-4 hours. The plates were washed (250µLs/well, 1xPBS with 0.05% Tween, four times) before adding anti-biotin alkaline phosphatase conjugate (50µLs/well of 1 in 750 dilution in 1xPBS with 0.5% BSA, Vector Laboratories SP3020) and incubated at room temperature for 2-hours. The plates were washed (250µLs/well, 1xPBS with 0.05% Tween, four times) and 50µLs of 0.2µm filtered 5-bromo-4-chloro-3'-indolyphosphate p-toluidine

salt/nitro-blue tetrazolium chloride substrate was added to each well (BCIP/NBT 1-Step solution, Pierce). The plates were incubated at room temperature for 5-15 minutes, or until dark pots appeared in the ConA wells, before the reaction was terminated with three final dH_2O plate washes and allowed to air-dry. Supernatants were retained for use in the CBA assay (described later).

Spots were enumerated with an AID ELISpot reader (spot intensity 20, minimum size 30 and gradient 3 settings) and AID counts were manually checked for artefacts. The mean plus 4 standard deviations of the DMSO background from all samples was used to establish a threshold for rejection from analysis. This resulted in samples with DMSO counts ≥55 spots per million PBMCs being excluded from analysis. Samples were also rejected if 5 or more spots were detected in the R10+ media well, and samples were failed if no spots were observed in any of the CMV, FEC or ConA wells. Calculation of the triplicate variance, described elsewhere [346], was performed for DMSO and RSV-peptide pools and a threshold variance of 10 was applied for exclusion from analysis. The minimum spot count was one spot in only one well of the three replicates of peptide pool wells that each contained 200'000 PBMCs (equivalent to 1.6 spots per million PBMCs). The lower limit of detection for the assay was then approximated to 1 spot per million PBMCs, and zero values were reverted to half the lower limit of detection in accordance with standard practice (assigned value of 0.5 spots per million PBMCs). The results were reported for each peptide pool without DMSO background subtraction, and the overall response was defined as the sum of each peptide pool response minus 4xDMSO background.

2.4.6 ELISpot assay for the detection and quantification of IFNγ-producing T-cells in peripheral blood from frozen PBMCs

PMBCs frozen at $10x10^6$/mL Recovery™ Cell Freezing Medium (10% DMSO, and calf serum, Invitrogen) were thawed as described in section 2.4.4 and rested overnight in thawing medium (5% CO_2, 37°C, 95% humidity). The IFNγ ELISpot assay was then performed as described in the last section.

2.4.7 ELISpot assay for the detection and quantification of IL-13, IL-17 and IL-4 in peripheral blood from frozen PBMCs

96-well Multiscreen PVF plates (Millipore MAIPS4510) were coated overnight with IL-13 antibody (MAbTec IL13-I), IL-17A (MAbTec MT44.6) and IL-4 (MAbTec IL4-I). The plates were sealed and stored at 4°C

overnight and for no more than 72-hours before use. PMBCs frozen at $10x10^6$/mL in Recovery™ Cell Freezing Medium (10% DMSO, and calf serum, Invitrogen) were thawed as described in section 2.4.4 and rested overnight in thawing medium (5% CO_2, 37°C, 95% humidity). The ELISpot assay was then performed as described earlier. Biotinylated secondary antibodies for IL-13, IL-17 and IL-4 were obtained from MAbTec (IL13-3, MT504 and IL4-II respectively).

2.4.8 *Intra-cellular staining (ICS) for the detection and quantification of CD4+ and CD8+ production of IFNγ, IL-2, TNFα and IL-5 in peripheral blood*

PMBCs frozen at $10x10^6$/mL in Recovery™ Cell Freezing Medium (10% DMSO, and calf serum, Invitrogen) were thawed as described in section 2.4.4, centrifuged (1500rpm for 10-minutes) and the cells re-suspended in 4mLs R10 and incubated overnight (5% CO_2, 37°C, 95% humidity). The next day, the cell suspension was centrifuged (1500rpm for 5-minutes) and the pellet re-suspended in 1mL R10 for counting using the method described earlier. After counting, additional R10 media was added to achieve a final concentration of $1x10^7$ cells/mL, and $1x10^6$ cells in 100µLs was added to each well of a 96-well plate (96-well U-bottomed plate, Thermo-Fisher). DMSO, Fa, Fb, N, M and FEC peptides were added at the same concentration for the IFNγ ELISpot assay described earlier (2µL/well for DMSO, Fa, Fb, N and M peptides; 1.3µL/well FEC peptides). Phorbol 12-myrstate 13-acetate/ionomycin (PMA/ionomycin, Sigma-Aldrich P8139 and I3909) and phytohemagglutinin (PHA, Sigma-Aldrich L2646) were used as polyclonal stimulant controls. Each well had 1µL of brefeldin A added (1 in 100 dilution of 5mg/mLs stock in R10) and the plate was incubated overnight (5% CO_2, 37°C, 95% humidity). The plates were then washed (1xPBS, 100µLs/well) and centrifuged (1500rpm for 5-minutes).

Fluorochrome-conjugated monoclonal surface staining antibodies (as a mix diluted in PBS, 50µLs/well) were added and included; 1 in 300 dilution of live/dead or L/D-APC (allophycocyanin)-Cy7 (Life Technologies Ltd), 1 in 50 dilution of CD3-efluor450 (Affymetrix, eBioscience Inc), 1 in 100 dilution of CD4-APC (BioLegend) and 1 in 100 dilution of CD8-VioGreen (Miltenyi Biotec). Fluorochrome-conjugated monoclonal surface staining antibodies were given 20-30 minutes protected from light and at room temperature before the plates were washed (1xPBS) and centrifuged (1500rpm for 5-minutes). Next, 1% FACS Fix (1mL formaldehyde mixed with 36mLs PBS) was added to each well for 20-minutes (100µLs/well) protected from light before another wash (1xPerm buffer, eBioscience Inc) and centrifugation (1500rpm for

5-minutes). 200μLs/well Perm buffer (eBioscience Inc) was added for 20-minutes protected from light and plates were then centrifuged (1500rpm for 5-minutes). Intra-cellular antibodies were diluted in Perm buffer and added for 25-minutes, protected from light and at room temperature, as a single mix (50sμLs/well) that included; 1 in 50 dilution of IFNγ-FITC, 1 in 50 dilution of IL-2 PerCPCy5.5 (peridinin chlorophyll protein), 1 in 50 dilution of TNFα-PE (phycoerythrin) and 1 in 25 dilution of IL-5-PE. Another 150μLs/well Perm buffer was added and the plates centrifuged (1500rpm for 5-minutes), 200μLs/well PBS added and centrifuged again (1500rpm for 5-minutes). Each well was finally re-suspended in 200μLs/well PBS.

Samples were analysed by fluorescence-activated cell sorting (FACS) using a MACSQuant flow cytometer (Miltenyi Biotec) and FlowJo software (version X0.7 for Mac). Samples were rejected if no responses were observed in either the PMA/ionomycin or PHA controls. ICS-measured responses to Fa, Fb, N and M peptides were reported as DMSO background subtracted with negative values reverted to zero.

2.4.9 Cytometric bead array (CBA) for the detection and quantification of Th1/Th2/Th17 cytokines in peripheral blood

BD™ Cytometric Bead Arrays (CBA) have been used to characterise vaccine immunogenicity[347,348] and a human Th1/Th2/Th17 cytokine kit was applied to the supernatants of the *ex vivo* IFNγ ELISpot assay according to the manufacturers instructions. 35μLs of the pooled triplicate supernatants of DMSO, Fa, Fb, N and M wells were mixed with 5μLs aliquots of cytokine capture beads and 35μLs of PE-conjugated antibody as detection reagent. Capture beads included human IL-2, IL-4, IL-6, IL-10, TNFα, IFNγ and IL-17A. Samples were given 3-hours protected from light and at room temperature before 800μLs wash buffer was added and the sample was centrifuged (200g for 5-minutes). The supernatant was discarded and the capture beads were re-suspended in 200μLs wash buffer. Cytokine detection used an LSRII FACS machine (BD), BD FACSDiva software (version 6.0 for Windows) and FlowJo software (version X0.7 for Mac). Cytokine quantities were measured from the kit control beads and responses to Fa, Fb, N and M were reported as DMSO background subtracted with resulting negative values reverted to zero.

2.4.10 FACS-based assay for the detection and quantification of T follicular helper cells, IgA memory B-cells and plasmablasts in peripheral blood

PMBCs frozen at $10x10^6$/mL in Recovery™ Cell Freezing Medium (10% DMSO, and calf serum, Invitrogen) and were thawed and added to 1mL of thawing medium (9mLs thawing medium with 100µLs DNAase added). The samples were centrifuged (1600rpm for 6-minutes), the supernatant discarded and the pellet was re-suspended in 1mL R10 for counting using the same method described earlier. After centrifugation (1600rpm for 6-minutes) the PBMCs were re-suspended in R10 at a concentration of $5x10^6$ cells/mL. 800'000 PBMCs in 160µLs were added to each well of a 96-well U-bottom plate (Thermo-Fisher), centrifuged (1600rpm for 6-minutes) and then a 50µLs/well mix of fluorochrome-conjugated monoclonal surface staining antibodies in PBS was added for 30-minutes at 4°C protected from light. Staining antibodies for T-follicular helper cells included CXCR3-PerCPCy5.5 (BD), CXCR5-AF647 (BD), PD-1-PE (BD), ICOS-FITC (Miltenyi), CD4-Pacific Blue (Miltenyi), CD45RA-VioGreen (Miltenyi) and NIR live/dead stain (Invitrogen). Staining antibodies for B-cell responses included CD38-PerCPCy5.5 (BD), IgA-FITC (Miltenyi), IgD-PE (BD), CD27-PECy7 (eBioscience Inc), CD19-Pacific Blue (BD), CD20-Pacific Orange (Invitrogen) and NIR live/dead stain (Invitrogen). Plates were then centrifuged (1600rpm for 6-minutes), the supernatants discarded, and PBMCs were re-suspended in 200µLs/well PBS. Samples were analysed by FACS using a MACSQuant flow cytometer (Miltenyi Biotec) and FlowJo software (version X0.7 for Mac).

2.5 Immunological assays conducted in external laboratories

2.5.1 Serum anti-F and anti-G IgG and IgA enzyme-linked immunosorbent assay (ELISA) for the RSV Observational studies

This ELISA assay was performed by ReiThera Srl has been summarised as follows. Nunc 96-well plates (Nunclon) were coated at 4°C overnight with 0.5µg/100µLs recombinant F protein (SinoBiologicals) in 0.05M $NaHCO_3$ buffer. Data are expressed as endpoint titres calculated from 1-hour absorbance readings, defined as the highest dilution of sample giving an OD_{405} reading greater than three standard deviations above the mean of naïve samples. The lower limit of detection was a titre of 150; titres below this threshold were assigned a value half the lower limit of detection in accordance with standard local practice.

2.5.2 *FACS-based assay for the quantification of serum RSV-neutralising antibody for the RSV Observational studies*

This assay was performed by ReiThera Srl and has been summarised as follows. Serum RSV-neutralizing antibody titres were measured by a FACS-based assay that relies upon recombinant RSV expressing glycosaminoglycan sulphate (RSV-GFP) and has been described in more detail elsewhere[349,350]. RSV-GFP was generously gifted from Mark Peeples, Nationwide Children's Hospital, Columbus, Ohio. Briefly, HEp-2 cells were plated onto 96-well plates ($5x10^4$ cells/100µLs/well) and incubated for 2-hours (5% CO_2, 37°C, 95% humidity). Heat-inactivated sera, serially diluted in DMEM 7% FCS, were incubated with RSV-GFP (0.1 MOI) for 1-hour (5% CO_2, 37°C, 95% humidity) and then 100µLs/well added to the HEp-2 cell layer and incubated for 20-22 hours (5% CO_2, 37°C, 95% humidity). Palivizumab F-specific monoclonal antibody (Synagis® 100mg, Abbott Cat. 131-026-19) was used as positive control. HEp-2 were detached by trypsin, collected in PBS containing 1% foetal bovine serum and 1% formaldehyde and fluorescence was read using a FACS Canto (BD) and FACS DiVa Software (BD). The percentage of fluorescent cells for each dilution was calculated with respect to the negative control (100% of infection). The EC_{50} was calculated by curve fitting and non-linear regression in GraphPad Prism (GraphPad Software Inc, USA).

2.5.3 *PanAd3-RSV vaccine virus shedding for the RSV001 clinical trial*

This assay was performed by WuXi App Tec Inc and is summarised as follows (modified from Green et al [1]). Nasal samples were inoculated onto monolayers of human embryonic kidney (HEK) 293 and A549 cells to detect both replication-competent and replication-incompetent adenovirus by the presence of virus-induced cytopathic effects (CPEs) using a 14-day *in vitro* assay, and immunofluorescence detection (IFA) as a confirmatory assay. Briefly, each nasal sample was thawed to 35-39°C, vortexed and filtered through 0.8nm and 0.2mm filters. The filtrate was inoculated onto 24-well plates and 35-mm dishes of HEK-293 and A549 cells, and was incubated for 50-70 minutes (5% CO_2, 37°C, 95% humidity). The inoculum was removed, the monolayers were washed with PBS, and fresh culture medium was added. Dish cultures were visually monitored for CPEs for 14 days. On day 3 or 4, 24-well plates were processed for IFA using a monoclonal antibody against hexon adenovirus. The nasal swab samples were considered positive if positive immunofluorescence and/or CPEs consistent with viral infection were observed in the sample inoculated HEK-293 or A549 cultures.

2.5.4 *Anti-PanAd3 (vector) neutralising antibody assay for the RSV001 clinical trial*

This assay was performed by ReiThera Srl and has been summarised as follows (modified from Green et al [1]). PanAd3 neutralising antibody titres were quantified using a secreted alkaline phosphatase (SEAP) assay [351]. Briefly, human embryonic kidney 293 (HEK-293) cells were seeded at a concentration of $3.5x10^4$ cells/well in a 96-well plate for 48-hours. SEAP-expressing PanAd3 was pre-incubated for 1-hour at 37°C alone or with serial dilutions (1:18, 1:72, 1:288, 1:1152, and 1:4608) of heat-inactivated serum from trial volunteers and added to the 95-100% confluent HEK-293 cells for 1-hour at 37°C. The supernatant was then removed and replaced with 10% foetal calf serum (FCS) in Dulbecco's modified Eagle's medium. SEAP activity in the supernatant was measured after 22-26 hours using the chemiluminescent substrate (CSPD, Phospha-Light kit, Tropix) according to the manufacturer's instructions. Light signal output, expressed as relative light units, was measured 45-minutes after the addition of the CSPD substrate using a luminometer (EnVision 2102 Multilabel Reader, PerkinElmer). The neutralization titre was defined as the reciprocal of sera dilution required to inhibit SEAP expression by 50%, compared with the SEAP expression of virus infection alone.

Chapter 3: The burden of emergency hospital admissions for bronchiolitis

3.1 Introduction

There is evidence of a rise in admission rates for bronchiolitis occurring over time in the US and Canada during the 1990s that has been maintained to this decade[352-354]. This chapter reports a retrospective analysis of hospital admissions in infants with bronchiolitis in England over the last five decades. The main objective was to describe a time-trend analysis of admissions to hospitals and to paediatric intensive care units (PICUs) in England. Additional objectives were to map geographical and socioeconomic variation in hospital admission rates. The results of this chapter have been published[2]. Sections copied from this publication and reproduced here represent my own work with acknowledgment to my co-authors and their essential contribution[e].

[e] In the published manuscript there are additional analyses that used mother-to-child linked data from a specialist maternal dataset (collected as part of the ORLS) that explored maternal and perinatal factors that contribute towards the likelihood of emergency hospital admission for bronchiolitis, and the risk of admission for wheeze in later years. This work was not my own, although the interpretation was, and is therefore not included in this chapter.

3.2 Methods

3.2.1 HIPE, HES and ORLS

Discharge codes from any diagnostic position were analysed from hospital admission datasets that are routinely collected in England including the Hospital In-Patient Enquiry (HIPE), Hospital Episode Statistics (HES) and Oxford Record Linkage Study (ORLS). Hospital admissions for bronchiolitis were identified using International Classification of Diseases (ICD) codes J21.0, J21.1, J21.8 and J21.9 in the 10th revision of the ICD (1999 to 2011) and 466.1 in the 9th revision of the ICD (1979-1998). A case was included if any code had been recorded at any diagnostic position for a patient aged less than one year. In the early years of the ORLS (1963-1978) and HIPE (1968-1978), covered by ICD7 and ICD8, cases were identified from codes 500 (ICD7 until 1965; bronchitis) and 466 (ICD8, 1965-1978; acute bronchitis and bronchiolitis), in infants aged less than one year, and therefore cover acute bronchitis and bronchiolitis without distinguishing them for most of the period before 1979.

National Health Service (NHS) admission statistics from 1968-1985 were collected on a one-in-ten basis for the whole of England in the HIPE and were scaled up (by multiplying by 10) to represent all admissions. Admissions from 1989, including day case admissions, were recorded in HES data. Linked HES data from 1999 permitted an analysis of successive admissions for individuals within the population. The current population in England is estimated at 53 million. The ORLS is essentially a fully linked regional subset of HES, collected independently of HIPE and HES, without sampling, from 1963-1998; and compiled as a subset from national HES for the same region since then. ORLS covers the period between 1985 and 1989, when no HIPE or HES data were collected.

In the trend analyses, hospital admission rates are presented as episode-based and person-based admission rates of cases per 1000 population, for both sexes combined, aged under one year expressed as an annual rate with 95% confidence intervals (CI). The population denominator for each year was obtained from mid-year population estimates from the Office for National Statistics (ONS). With the episode-based admission rate, a person admitted several times for the condition is counted as many times as he/she has admissions. The person-based admission rate counts individuals once, and only once, at the first admission per year in which they were admitted. In the geographical analyses, place of residence was determined from

the HES record and population denominators for the local areas were obtained from ONS. Person-based rates are given and anyone admitted more than once is counted just once. The boundaries of the Local Government Areas of England were used to map geographical and socioeconomic variations in the recorded rates of admissions. The Index of Multiple Deprivation (IMD 2004) score was used as a measure of socioeconomic status for each local area.

3.2.2 PICANet

The Paediatric Intensive Care Audit Network (PICANet) dataset is a national audit that has collected data on admissions to all paediatric intensive care units (PICUs) across England since 2002. The primary diagnosis for admission used Read codes X100C, XSDOK, H061, H0615, H061z, X100D, Xa0BK, H0612 and XaPOd to identify admissions for bronchiolitis, deaths and length of stay between 2004 and 2012. The PICANet population denominator for each year was estimated from ONS data, and was 729,674 infants aged less than 12-months in 2012.

3.2.3 Statistics

For the trends and mapping statistical analyses were performed in Statistical Analysis System (SAS Institute). For the analyses of PICU admissions the analyses were performed using GraphPad Prism for Mac version 6.0 (GraphPad Software, USA).

3.2.4 Regulatory approvals and funding

Ethical approval for research using the ORLS, HIPE and HES datasets was granted from the Central and South Bristol Research Ethics (reference 04/Q20006/176). The construction of these datasets was funded by the National Institute for Health Research (reference RNC/035/02). Collection of anonymised data used to generate the PICANet dataset was granted from the Patient Information Advisory Group (now the NHS Health Research Authority Confidentiality Advisory Group) and ethical approval granted by the Trent Medical Research Ethics Committee (reference 05/MRE04/17+5).

3.3 Results

3.3.1 Hospital admission rates for bronchiolitis over time

All-England HIPE and HES data recorded 468,138 episodes of admission of infants aged less than one year between 1979 and 2011, accounting for 92.6% of all bronchiolitis admissions in children of all ages in the same period. HES data between 1999 and 2011 recorded 299,397 episodes and 259,044 infants aged less than one year admitted for bronchiolitis. The mean ratio of episodes to people was 1.16. The annual average episode-based admission rate reached 46.1 (95% CI 45.6 to 46.6) in 2011, up from 6.6 (95% CI 6.0 to 7.2) in 1979 when bronchiolitis discharge codes came into use (a near 7-fold rise in 32-years), Figure 3-1. The annual average person-based admission rate reached 37.2 (95% CI 36.7 to 37.7) in 2011, from 31.2 (95% CI 30.8 to 31.7) per 1000 population aged less than one year in 1999.

The ORLS recorded a total of 25,852 episodes and 23,319 infants aged less than one year admitted for bronchiolitis in this sub-population of England between 1979 and 2011. The mean ratio of bronchiolitis episodes to number of infants was lower at 1.11, and 98.2% of all bronchiolitis admissions in children were aged less than 12-months. Per 1000 infants aged less than one year, the annual average episode-based admission rate reached 40.4 (95% CI 38.5 to 42.3) in 2011, from 4.5 (95% CI 3.7 to 5.3) in 1979 (a near 9-fold rise in 32-years), Figure 3-1. The annual average person-based admission rate reached 33.1 (95% CI 31.3 to 34.8) in 2011, from 4.1 (95% CI 3.4 to 4.9) in 1979 (an 8-fold rise in 32-years). Between 2004 and 2011, when data on admissions to paediatric intensive care were recorded, the HES and ORLS datasets recorded an episode-based average annual increment in hospitalisation rates of 1.8% and 1.6% respectively. A small, relatively fixed, difference between the episode-based and person-based rates of admission emerged from 1995 onwards. The pre-1979 rates, when the codes included bronchitis as well as bronchiolitis in the infants, were lower than those for bronchiolitis alone after 1979.

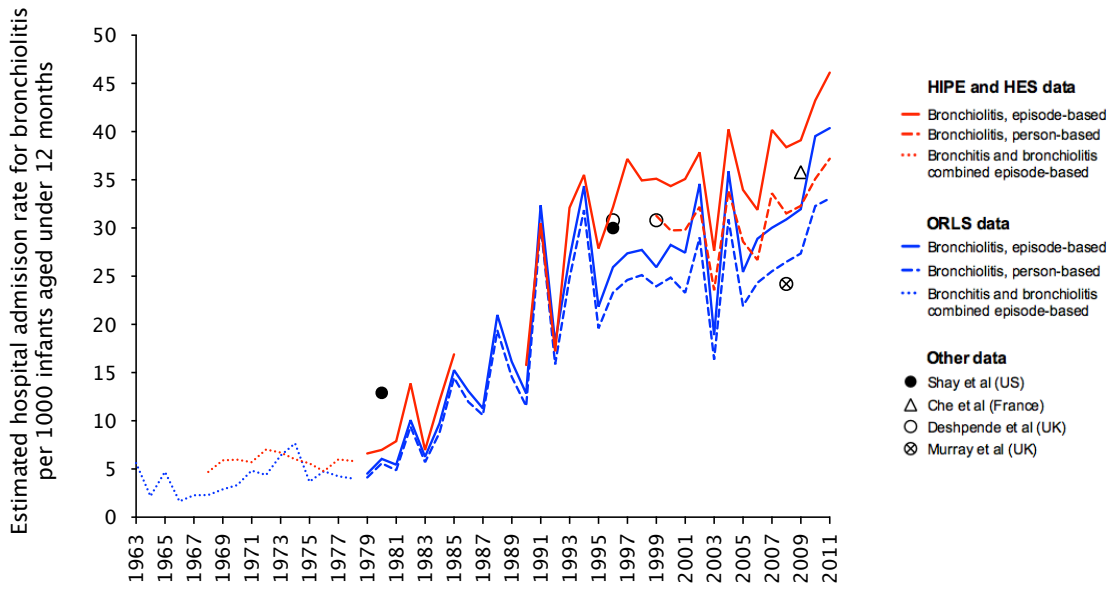


Figure 3-1. Point estimates for the annual average episode and person-based hospital admission rate for bronchiolitis per 1000 infants aged less than 12 months from 1979-2011, episode-based combined acute bronchitis and bronchiolitis rates from 1965-1979, and bronchitis before 1965.
Person-based hospital admission rates for males were consistently higher than females (see supplementary material).

3.3.2 Bronchiolitis admissions to PICU over time

A total of 102,126 infants aged less than 5-years of age were admitted to PICUs between 2004 and 2012. Of the 64,066 infants aged less than 12 months, 8172 were diagnosed with bronchiolitis. Infants aged less than 12-months accounted for 93% of all bronchiolitis admissions to PICU, and represented an average 11.8% (95% CI 10.5 to 13.1) of all the PICU admissions for each year, typically between October and March with little year-to-year variation. The estimated PICU admission rate ranged between 1.33 and 1.6 per 1000 infants aged less than one year between 2004 and 2012, with year-to-year variability showing no consistent trend towards increasing admissions (Figure 3-2). The most frequent age of admission was one month and overall 5083/8172 (62.2%) of infants were aged less than 2-months. By 2012 the mean age was significantly lower than it was in 2008, though the mean age initially rose between 2004 and 2006. For infants aged between 1 and 5 years the estimated rate rose from 0.01 to 0.03 admissions per 1000 infants. The annual average length of stay for all ages combined ranged from 5.4 to 6.7 days (mean 6.1 days). 158 bronchiolitis admissions resulted in death, representing a PICU case-fatality of 1.75% in infants aged less than 12 months and 4.4% in infants aged 12 months and older.

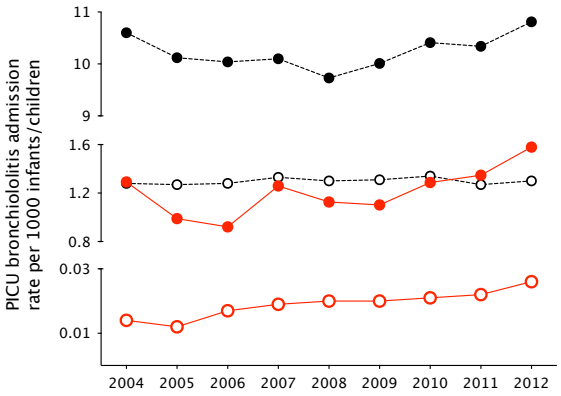


Figure 3-2. PICU rates of admission per 100 infants aged under months and children aged 1-5 years, for all causes and bronchiolitis.
Data from between 2004 and 2012, expressed as the rate for 1000 infants/children of the population.

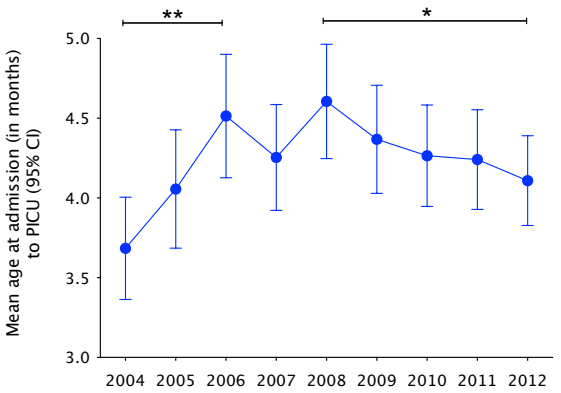


Figure 3-3. Mean age of admission to PICU for bronchiolitis.
*Data from between 2004 and 2012, mean age with 95% confidence intervals. * and ** denotes p<0.05 and <0.005 respectively.*

3.3.3 *Variation in hospital admission rates for bronchiolitis across England*

The mean person-based rate of admissions between 1999 and 2011 was 32.4 per 1000 infants aged less than one year (95% CI 32.2-32.5). The person-based rate of admission across the 352 Local Government Areas ranged 5.3-fold, from 12.2 (95% CI 10.2-14.5) to 65.0 (95% CI 61.8-68.3), with rates of 20.0 and 44.5 at the 10th and 90th percentiles respectively. A positive correlation (r^2= 0.24), with high admission rates for bronchiolitis in areas with high socio-economic deprivation, was found between local person-based admission rates and IMD 2004 score.

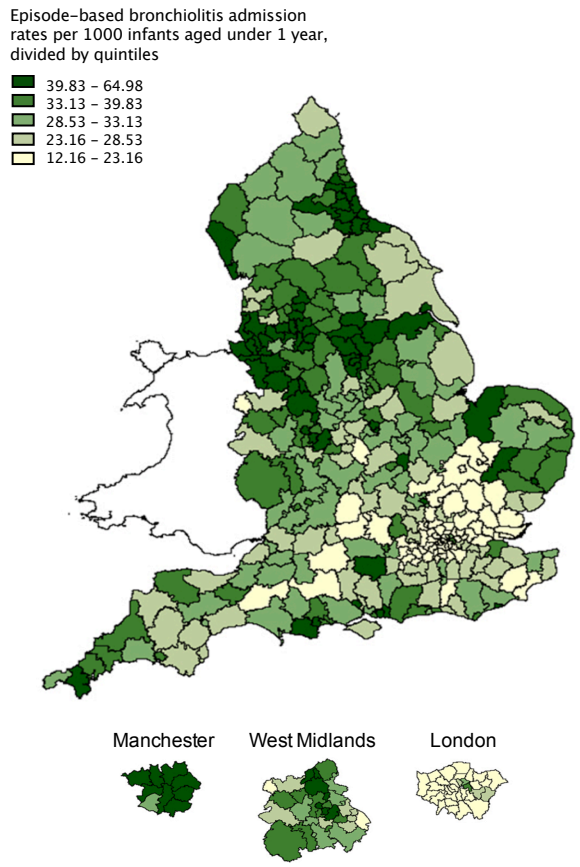


Figure 3-4. National variation in the person-based bronchiolitis hospital admission rates, 1999 to 2011.

Bronchiolitis admission rate per 1000 infants aged less than one year, derived from HES data for both sexes and mapped using the boundaries of each Local Government Area. This figure was generated by the Unit of Health-care Epidemiology, University of Oxford, and is reproduced with permission.

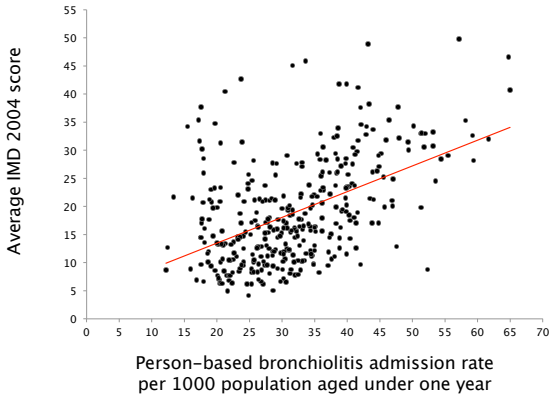


Figure 3-5. Index of Multiple Deprivation (IMD) score plotted against the person-based bronchiolitis admission rate per 1000 infants aged less than one year for each Local Government Area.

1999 to 2011, males and females combined. Each dot represents one local health authority. There was a positive correlation between the measure of socioeconomic deprivation and hospital admission rates (r^2=0.24). This figure was generated by the Unit of Health-care Epidemiology, University of Oxford, and is reproduced with permission.

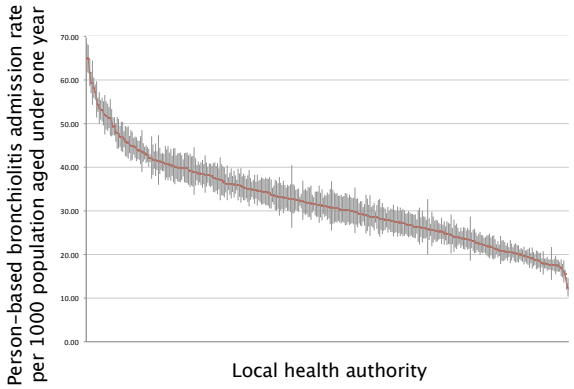


Figure 3-6. Person-based bronchiolitis admission rate per 1000 infants aged less than one year for each Local Government Area.

1999 to 2011, males and females combined. Each red dot is the point estimate for a Local Government Area and the vertical bars are the 95% confidence intervals on the point estimates. This figure was generated by the Unit of Health-care Epidemiology, University of Oxford, and is reproduced with permission.

3.4 Discussion

3.4.1 Summary of main findings

The combined analysis of several independent hospital admission datasets recorded a substantial rise in emergency hospitalization rates for bronchiolitis in recent decades. In recent years a year-on-year rise of up to 1.8% was observed, although PICU admission rates changed little during the same period. These data, together with the wide variation in hospital admission rates across England, indicate that changes in the threshold used to decide admission and a lack of standardised practice were major drivers behind this consuming trend in paediatric hospital resources each winter.

3.4.2 Hospital and PICU admissions

Data on hospital admission trends in the US, measured per 1000 infants aged less than 12 months, showed a 2.4-fold rise from 12.9 in 1980 to 31.2 in 1996[352]. In Canada, between 1981 and 1999, the bronchiolitis admission rate per 1000 infants aged 0-6 months rose 3.5-fold (estimated from 12 to 42) and 2.1-fold in infants aged 6-12 months (estimated from 11 to 23 per 1000 infants of the same age range)[353]. UK data from a single region between 1996 and 1999 recorded an average rate of 30.8[60] and the last national estimate from an industrialised nation was single-season data from France, which estimated 35.8 admissions per 1000 infants aged less than 12 months in 2009.[355] Although PICU admissions for bronchiolitis have steadily increased as a proportion of all PICU admissions since 2002[356] these data suggest that there has not been a substantial increase in disease severity over time as a driver for increasing rates of hospital admission.

The candidate drivers behind changes in recorded hospital admission rates include the increasing prevalence of maternal and perinatal factors that predispose to severe disease, social factors such as parents' expectations, and changes to the way care is provided and used. Improvements in neonatal care (the estimated prevalence of premature infants is 7.5%) has led to an increase in the population at enhanced risk: hospital admission rates are 24.2 per 1000 for all infants under one year of age, and 47.3 per 1000 amongst premature infants under one year of age[53,357,358]. These co-morbidities were not measured in this analysis that sought the single discharge code for bronchiolitis admissions. In 2003, 7-9% of hospital bronchiolitis admissions were re-admissions[60,359] and more recent data estimated 21% of infants had more

than one bronchiolitis admission during the first year of life[53]. It is also noteworthy that overall admission rates in England for children under 15 years of age with lower respiratory tract infections have risen 40% in the last decade and there have also been large increases in paediatric admissions for upper respiratory tract infections, urinary infections and gastroenteritis[360]. The striking increases in hospital admission rates for bronchiolitis should be considered in this context with the likelihood that important factors behind the increases in paediatric admissions, generally, represent a lowering of clinical thresholds for referral and admission.

Furthermore major changes in the organisation of healthcare across England in the last two decades may have contributed to changing admission rates. A major change has been decreased access to medical assessment in the community by general practitioners (GPs) as a result of both a reduction in out-of-hours-care by GPs under a new contract since 2004[361] and the introduction of a non-medical protocol-based telephone triage system (NHS Direct) in 1999[362]. These changes tend to direct parents to hospital care, where availability of new technology over this period, including oximeters,[363] and a drive to reduce emergency department assessment times may have inadvertently encouraged clinicians to lower the threshold for admission in order to meet waiting time targets and as a result of clinical protocols. Only 25% of the geographic variation in hospital admission rates could be attributed to socioeconomic deprivation, which is consistent with two publications of HES data which reported a 15-fold variation in the rates of hospital admission for bronchiolitis and a 6-fold variation in the mean length of stay across local areas in England between 2007 and 2010[53,364]. The finding of geographical variation, as well as the finding discussed above of increasing admission rates, indicate that there is clinical uncertainty, probably combined with organisational factors, about when to admit a sick child.

3.4.3 General limitations

While the length of the time-period of data collection was a considerable strength there may have been some variation over time in the quality of diagnostic coding which may have reduced the accuracy of the analysis. These levels of independence increase confidence in the time trend analyses. The incidence rates reported from the HES and ORLS datasets, as shown in Figure 3-1, showed the estimated rates were consistent across studies both in the UK and elsewhere, and suggested that the large increase in admissions

are real even if affected to some extent by coding variation. There has been only one all-England estimate of burden of disease, which recorded an admission rate of 24.2 per 1000 infants aged less than 12 months[53] using discharge codes in the primary diagnostic position only, which may explain the lower estimated admission rate for 2007/08. Although the overall trend is clear, significant year-to-year variations in the hospitalisation rate could not be explained. The year-to-year rate in the 16-year US and 3-year UK hospital data are not published, but Canadian admission rates varied from year-to-year between 1981 and 1999[353]. This variation appeared to have been less than in these data reported in this analysis, although the direction and magnitude of the overall rising trend was comparable. Other factors such as changes in the prevalence of premature births and other conditions that predispose to developing severe disease, and ethnicity, may also have been important factors that influenced changes in admission rates that could not be addressed in these datasets[365].

3.4.4 Conclusions

Hospital admissions for bronchiolitis in England are increasing and exceed recent estimates from similar industrialized nations. PICU admission rates have changed little over the same period and indicate a need to examine the threshold used for hospital admission and explore the medical and social factors behind the rising hospital admission trend. The burden of RSV disease presented here provides a strong rationale for development of strategies for prevention through vaccination.

Chapter 4: The immune response to natural RSV exposure in healthcare workers and infants

4.1 Introduction

The development of RSV immunity in infants and maintenance of RSV immunity in adult healthcare providers are important factors relevant to the problem of nosocomial disease and in characterising two populations at polar ends of the risk of developing severe disease. Defining the RSV-specific humoral and cellular immunity from natural exposure in paediatric healthcare workers, infants and children can be used to develop the case for vaccination in these populations as well learn what the immune profile of healthy adults is following what should be significant repetitive priming and maintenance from natural exposure.

This chapter details the setup, execution and analysis of two non-interventional observational studies performed independent of each other. The objectives of each study were the same; namely to profile RSV-specific humoral and cellular immune responses derived from natural exposure. The first study was conducted in paediatric healthcare workers and the second study was in infants and children. The specific objectives were to quantify RSV-specific immune responses in blood that included the quantification of F-specific and G-specific IgG and IgA antibody, titres of serum RSV-neutralising antibody, the quantification of F-specific IgG and IgA B-cell memory cells and the quantity of interferon-gamma (IFNγ)-producing and Th2-producing T-cells in peripheral circulation.

4.2 Methods

4.2.1 Study setup and design of the paediatric healthcare workers study

The paediatric healthcare workers study was a non-interventional longitudinal observational study performed in Oxford, United Kingdom, in 2012. Volunteers were self-selected males and females aged 18-60 years working as either clinical paediatric NHS staff or members of the public responding to advertisements inviting them to register an expression of interest in taking part. A total of 20 nurses and doctors caring for RSV-infected infants on acute medical paediatric wards and the paediatric intensive care unit at the Children's Hospital, Oxford University Hospitals NHS Trust, were recruited as a cohort of presumed healthy RSV-exposed healthcare workers. Another 10 adults, presumed to be healthy and who were not health-care, child-care or parents of young children, were recruited as a control group of presumed lesser RSV-exposure for comparative analysis. The full eligibility criteria are detailed in Table 4-1. Public Health England (PHE) surveillance of laboratory confirmed RSV-infections in infants was used to determine the end of the 2011/12 season when each volunteer donated the first of two 50mLs blood samples[43]. A second blood sample was obtained 4-6 months later and volunteers were compensated £10 for each donation. Blood samples were tested for RSV-specific immune responses, as described in section 4.2.5 using the methods described earlier.

Inclusion criteria – all must be satisfied
1. Volunteer must be willing and able to consent to take part in the study
2. At the time of enrolment; aged 18-60 years inclusive
3. A Be able and willing to comply with study requirements
4. [Paediatric healthcare workers group only] Working on a paediatric ward with acute medical paediatric admissions during the RSV season
Exclusion criteria – none can be satisfied
1. History of any immunodeficiency or immunological disorder that could affect the acquisition of RSV responses
2. Use of immunosuppressive medications such as steroids
3. [Non-healthcare control group only] Working on an NHS ward or in close contact with populations at higher risk of RSV transmission (e.g. nursery workers, care home workers, parents of children under the age of 5 years)

Table 4-1. Eligibility criteria for the paediatric healthcare workers study.

4.2.2 Study setup and design of the infant and children study

The infant and children study was an observational study performed in Oxford, United Kingdom, over 2013 and 2014. Volunteers were infants aged 2-4 months (n=5, born after the last winter), infants aged 6-12 months (n=20, with a single season of RSV exposure) and children aged 3-6 years (n=10, with multiple seasons of RSV exposure). The eligibility criteria are set out in Table 4-2. Parents were approached when their infant/child required a blood sample or peripheral cannula insertion for another clinical indication at the Children's Hospital, University Hospitals Oxford NHS Trust. If written informed consent had been obtained, an additional 5mLs (infants age 2-4 months) or 6mLs (infants aged 6-12 months and children aged 3-6 years) was obtained for research purposes. Samples were collected outside the 2013/14 RSV transmission season, as determined by PHE data described earlier[43].

Inclusion criteria – all must be satisfied
1. Parent or guardian must be willing and able to consent to take part in the study
2. Age suitable for the study (2-4.9 months, 6-12.9 months or 3-6.9 years)
3. Delivery at 36 weeks gestation or later
4. Having a blood sample or insertion of cannula for clinical reasons
Exclusion criteria – none can be satisfied
1. Any impaired function of the immune system, by medication or pathological process, which could augment or impair the immune response to RSV
2. Any acute or chronic infection
3. Any chronic illness which, in the opinion of the investigator, may lead to incorrect or inaccurate immunology data

Table 4-2. Eligibility criteria for the infant and children study.

4.2.3 Statistics

No sample size calculations were performed to power the studies and the applied statistics were descriptive in nature. Comparative statistics represent post-hoc analyses and the tests applied to analyse the data are annotated with the results. A p-value <0.05 was considered statistically significant. Graphical presentations of the data and statistics were performed using GraphPad Prism for Mac version 6.0 (GraphPad Software, USA).

4.2.4 *Regulatory approvals, funding, study sites and monitoring*

Ethical approval and amendments for the paediatric healthcare workers study and the infants and children study were granted by NRES Berkshire (reference 12/SC/0023) and NRES Central Bristol (reference 13/SW/0130), and each study was registered with clinicaltrials.gov (reference NCT 01563692 and 01640652 respectively). Both studies were sponsored by the University of Oxford, funded by ReiThera Srl and monitored by the Clinical Trials Research Governance (CTRG) office, University of Oxford. Study procedures were conducted at the Children's Hospital, Oxford University Hospitals NHS Trust, and at the Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), Oxford. Written informed consent was obtained from all volunteers, parents or legal guardians before study any procedures were performed. Procedures were performed in accordance with ICH Good Clinical Practice (GCP) and local standard operating procedures (SOPs).

4.2.5 *Measures of RSV-specific humoral and cellular immune responses*

RSV-specific immune measures from blood obtained from both the paediatric healthcare worker study and infant and children study are summarised below. Immunological assays for the detection and quantification of RSV-specific humoral and cellular immune responses were performed on samples from every time point, using the methods described earlier. Immune measures included;

- Serum anti-F and anti-G IgG antibody quantity measured by enzyme-linked immunosorbent assay (ELISA)
- Serum antibody-mediated RSV-neutralisation by plaque-reduction neutralisation assay (PRNA, infant and children study) and fluorescence-activated cell sorting (FACS, paediatric healthcare workers study)
- Quantification of anti-F IgG and anti-F IgA memory B-cell responses measured by enzyme-linked immunospot assay (ELISpot) from frozen peripheral blood mononuclear cells (PBMCs)
- Quantification of IFNγ producing T-cells measured by ELISpot from frozen PBMCs
- Quantification of IL-4, IL-13 and IL-17 producing T-cells measured by ELISpot from frozen PBMCs (subset of volunteers only)

4.3 Recruitment, study population and sample collection

4.3.1 Paediatric healthcare workers study

A total of 34 expressions of interest were received and 30 volunteers were enrolled, as planned. A volunteer was considered enrolled at the time of donation of the first blood sample (termed visit 1, or V1). All volunteers donated two 50mLs blood samples (at V1 and V2) and completed the study according to protocol. A Consolidated Standards of Reporting Trials (CONSORT) map is provided in Figure 4-1 and the characteristics of the enrolled study population summarised in Table 4-3. Overall, the cohort of paediatric healthcare workers was generally younger and higher proportion was female. The first blood samples at V1 were collected over a 35-day period between 01/03/2012 and 05/04/2012, and the second blood samples at V2 were collected over a 45-day period between 09/07/2012 and 23/08/2012. The median interval between V1 and V2 was 124-days (range 112-174 days) and 144-days (range 125-147 days) for paediatric healthcare workers and non- healthcare workers respectively. There were no adverse events from any volunteer during the study.

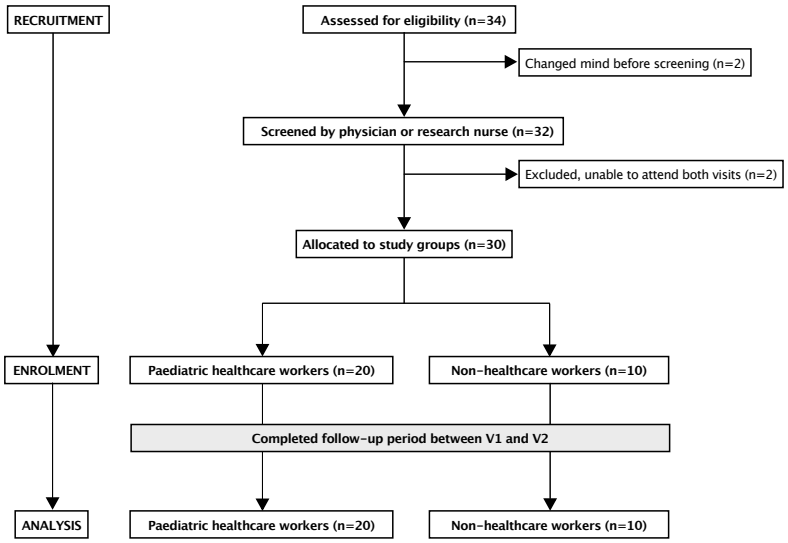


Figure 4-1. CONSORT map of recruitment, enrolment and completion of the paediatric healthcare workers study.

	Paediatric healthcare workers	Non-healthcare workers
N volunteers	20	10
Median age, years (range)	27.9 (20-52)	34.7 (24-58)
Male, n/N (%)	2/20 (10)	4/10 (40)

Table 4-3. The baseline physical characteristics of volunteers enrolled into the paediatric healthcare workers study. *The ethnicity of volunteers was not recorded.*

4.3.2 *Infant and children study*

A total of 66 expressions of interest were received and 35 infants and children were enrolled, as planned. A volunteer was considered enrolled on the first attempted blood donation. A CONSORT map and the characteristics of the enrolled study population are summarised below. There were no adverse events during the study. The non-continuous age distribution across both studies, as shown in supplementary material, meant age group was used as a binary variable (as defined by the study groups) in later comparative post-hoc analyses.

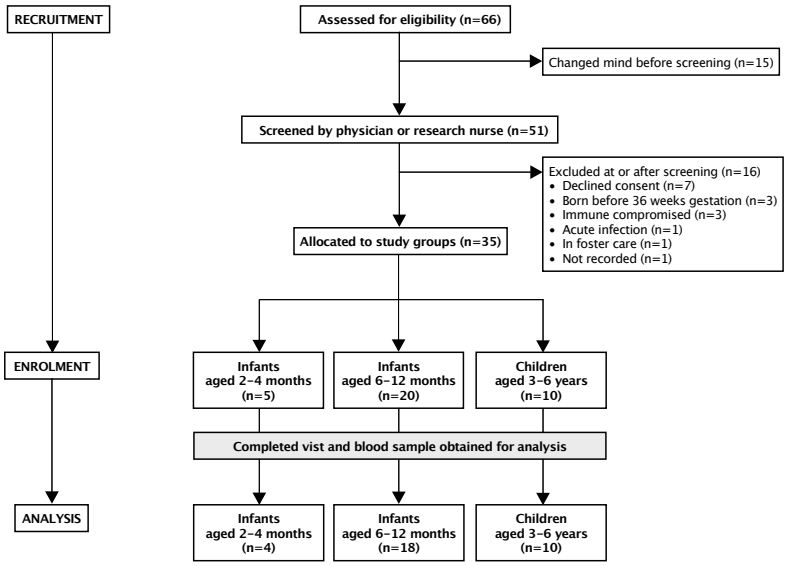


Figure 4-2. CONSORT map of recruitment, enrolment and completion of the infant and children study.
No blood was obtained from one infant aged 2-4 months and 2/20 infants aged 6-12 months. Only a small volume of blood (<1mL) was obtained from an additional 3 infants; one infant aged 2-4 months and two infants aged 6-12 months.

	Infants aged 2-4 months	**Infants aged 6-12 months**	**Children aged 3-6 years**
N volunteers	5	20	10
Median age (range)	13 weeks (11-18 weeks)	9 months (6-12 months)	4.8 years (3.8-5.6 years)
Ethnicity, n/N	4/5 W European 1/5 not recorded	15/20 W European 2/20 Central Asia 1/20 SE Asia 1/20 W Caribbean 1/20 not recorded	8/10 W European 1/10 W Asian 1/10 W African

Table 4-4. The baseline physical and demographic characteristics of volunteers of the infant and children study.
The sex of volunteers was not recorded.

4.4 Analysis of humoral immune responses to RSV after natural exposure

4.4.1 Serum IgG antibody response to RSV F and G protein exposure

Maternally derived anti-F IgG antibody was detectable in all 3/3 infants aged 2-4 months (geometric mean titre, GMT, 2814). The titres in infants aged 6-12 months were significantly lower (p=0.028, two-tailed Mann-Whitney test) with 4/17 (24%) failing to register a titre above the lower limit of detection for the assay (GMT 319, 95%CI 151-677). From the children aged 3-6 years, F-specific antibody titres were indistinguishable from adults in the paediatric healthcare workers study (GMT 12798, 95%CI 6679-24526, p=0.901, one-way Kruskal-Wallis ANOVA). The titres obtained from the paediatric healthcare study found no statistically significant differences in GMTs across study groups and time points (p=0.936, one-way Kruskal-Wallis ANOVA). For adult paediatric healthcare workers at the end of the RSV season the anti-F IgG GMT was 15181 (95% CI 11325-20349).

In contrast, maternal G-specific antibody was detectable from 1/3 infants to RSV subtype A and all 3/3 infants to RSV subtype B. In infants aged 6-12 months, G-specific antibody was detectable in 3/17 (18%) and 6/17 (35%) of volunteers for subtypes A and B respectively. Although antibody titres had risen significantly in children aged 3-6 years for both RSV subtype A (GMT 382, 95%CI 197-740) and subtype B (GMT 561, 95%CI 297-1063), these antibody titres remained significantly lower than adult samples from the paediatric healthcare workers study (p=0.004 and p=0.027, two-tailed Wilcoxon test for RSV subtypes A and B respectively). Adults failed to show any significant differences in subtype A or B anti-G antibody titres across paediatric healthcare workers and control groups and across time points (p=0.955 and p=0.925, one-way Kruskal-Wallis ANOVA, subtypes A and B respectively). For adult paediatric healthcare workers at the end of the RSV season the anti-G IgG GMTs were 1296 (95%CI 845-1986) and 1708 (95%CI 1009-2891) for subtypes A and B respectively. Serum end-point titres of F-specific antibody correlated with end-point titres of subtype A G-specific antibody in paediatric healthcare workers at both time points, and with non-healthcare workers at the end of the RSV season at V1. For subtype B, the only significant correlation with F-specific antibody titres was from paediatric healthcare workers in the summer months at V2 (Figure 4-4). Analysis of the subtype A and B responses to G-protein found statistically significant correlations in paediatric healthcare workers at both time points (Figure 4-4).

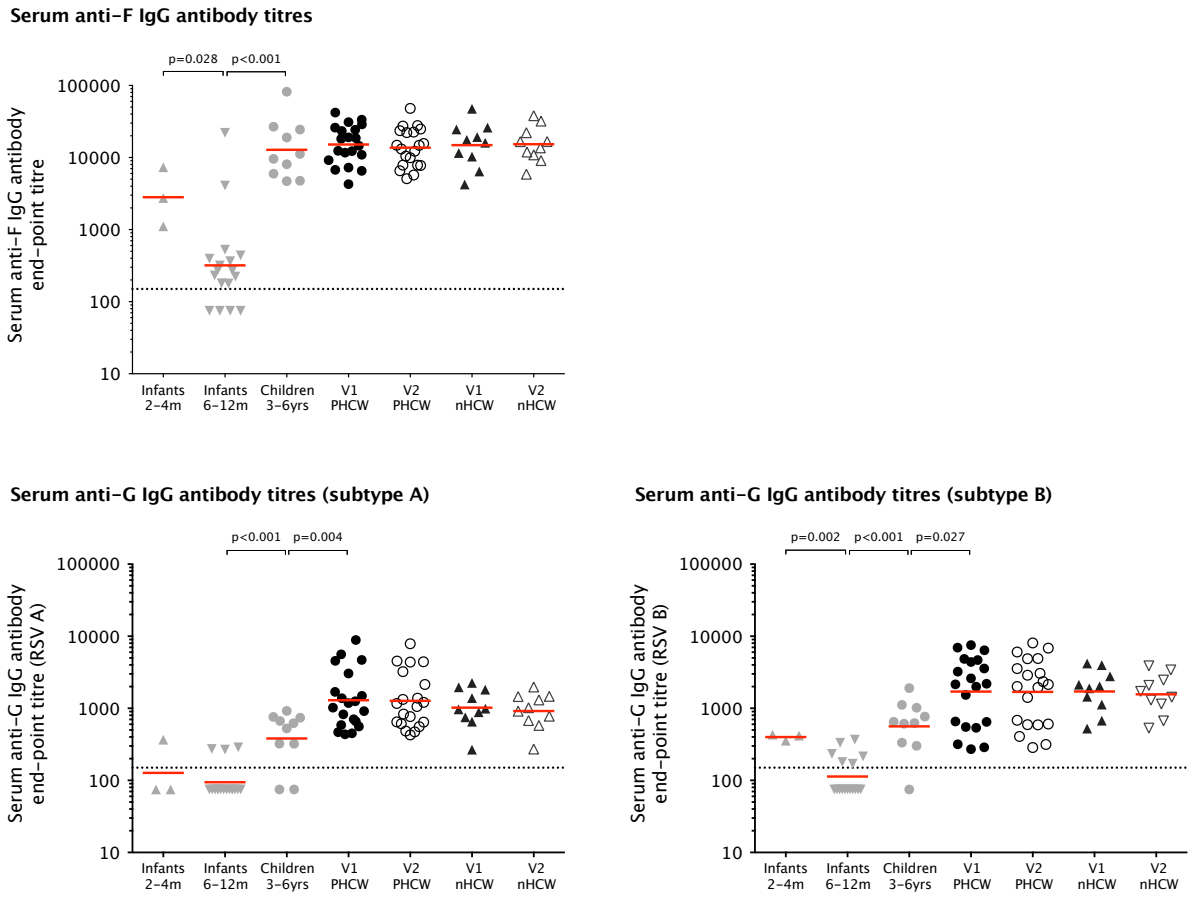


Figure 4-3. End-point titres for serum anti-F and anti-G IgG antibody (RSV subtypes A and B).
ELISA data. The red lines denote the geometric mean titre. The lower limit of detection for the assay is marked by the dotted horizontal line. Annotated p-values were generated from two-tailed Wilcoxon tests.

	Geometric mean antibody titre (with 95% CI)		
	Anti-F	**Anti-G (subtype A)**	**Anti-G (subtype B)**
Infants aged 2-4 months	2814 (-)	127 (-)	400 (-)
Infants aged 6-12 months	319 (151-677)	94 (73-123)	113 (83-154)
Children aged 3-6 years	12798 (6679-24526)	382 (197-740)	561 (297-1063)
Paediatric healthcare workers (V1)	15181 (11325-20349)	1296 (845-1986)	1708 (1009-2891)
Paediatric healthcare workers (V2)	13716 (10297-18271)	1268 (838-1919)	1692 (1025-2792)
Non-healthcare workers (V1)	14866 (8968-24644)	1020 (649-1603)	1711 (1044-2806)
Non-healthcare workers (V2)	15291 (10187-22952)	917 (608-1384)	1566 (986-2486)

Table 4-5. Geometric mean titres for serum IgG antibody directed against the F and G proteins of RSV. *ELISA data. Confidence intervals were not calculated for infants aged 2-4 months (n=3).*

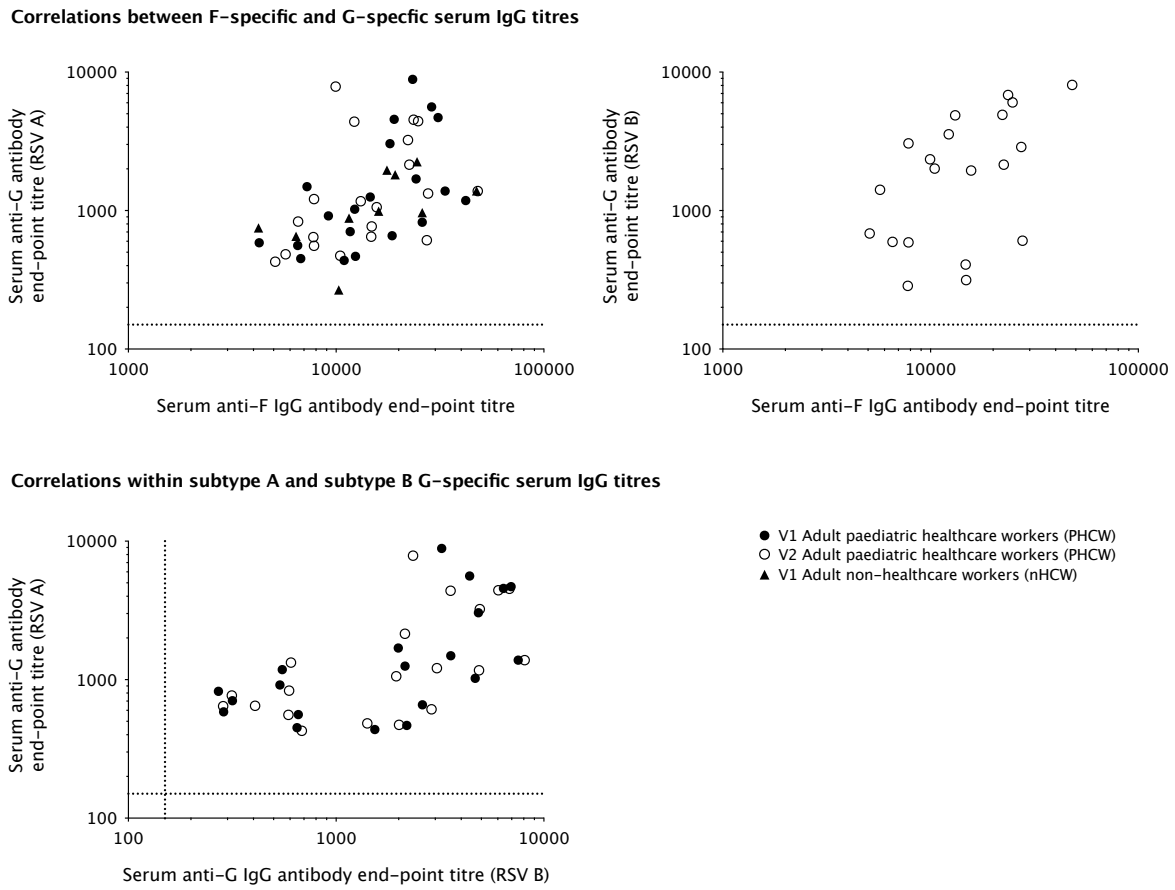


Figure 4-4. Correlation analyses between F- and G-specific serum IgG antibody titres.
*ELISA data. Correlation analyses used a two-tailed Spearman's test. The lower limit of detection of each assay is marked by the dotted lines (**Top left**) Statistical significance was observed with F- and G (subtype A)-specific titres from paediatric healthcare workers at the end of the RSV season (V1, p=0.004, r=0.62) and 4-6 months later (V2, p=0.020, r=0.52), and non-healthcare workers at the end of the RSV season (V1, p=0.027, r=0.71). (**Top right**) Statistical significance was observed with anti-F and anti-G (subtype B) antibody titres from paediatric healthcare workers from blood obtained in the summer months (V2, p=0.024, r=0.50). (**Bottom left**). Between subtypes A and B of the anti-G IgG titres, statistical significance was observed with paediatric healthcare workers at the end of the RSV season (V1, p=0.003, r=0.63) and 4-6 months later (V2, p=0.003, r=0.64). Non-healthcare workers recorded a borderline significant association (p=0.05, r=0.64, data not shown).*

4.4.2 Serum RSV-neutralising antibody response to natural RSV exposure

Serum RSV-neutralising antibody titres were measured at every time point by plaque-reduction neutralisation assay (PRNA) for the infant and children observational study and by fluorescence-activated cell sorting (FACS) for the paediatric healthcare workers study. The results are summarised in Figure 4-5. Serum RSV-neutralising antibody was detected in 2/3 infants aged 2-4 months and 6/17 (35%) of infants aged 6-12 months. Antibody titres had significantly risen and were detectable in all 10/10 children aged 3-6 years (GMT 1063, 95%CI 526-2148). In adults and the study of paediatric healthcare workers there were recordable titres from all volunteers and no statistically significant differences in GMTs of serum RSV-neutralising antibody titres were observed across adult study groups and time points ($p=0.96$, one-way Kruskal-Wallis ANOVA).

Further exploration sought to identify correlations between the F-specific and G-specific serum antibody titres measured by ELISA and the functional measure of antibody mediated viral neutralisation by PRNA/FACS assay. The only statistically significant correlation found was in in children aged 3-6 years and with F-specific antibody titres ($p<0.001$, $r=0.90$, two-tailed Spearman's correlation) (Figure 4-6). All other study groups and time points failed to record a statistically significant correlation, except for a borderline correlation with subtype A G-specific antibody titres in paediatric healthcare workers at V2 ($p=0.044$, $r=0.45$, see supplementary material).

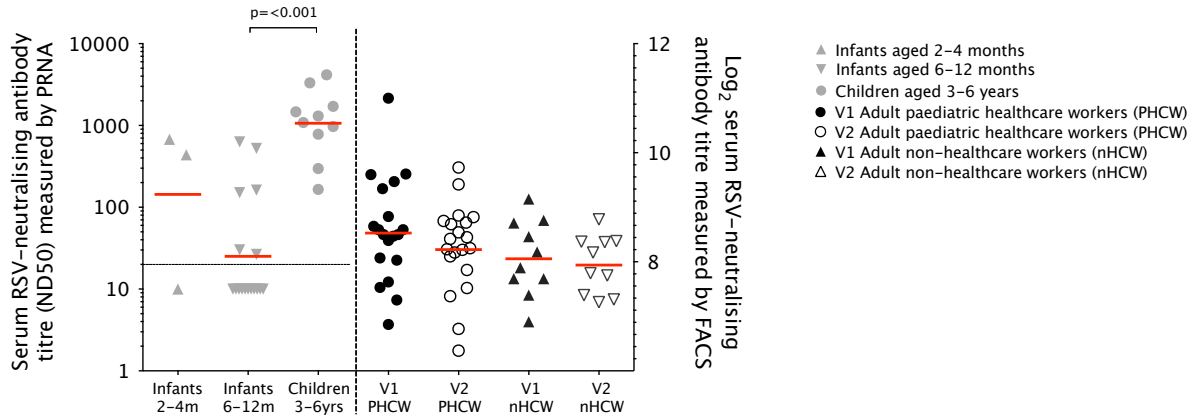


Figure 4-5. Serum RSV-neutralising antibody titres.
The red lines denote the geometric mean titre. Titres were measured using a plaque-reduction neutralisation assay (PRNA) for infants and children, and by fluorescence-activated cell sorting (FACS) for the paediatric healthcare workers study. The lower limit of detection for the PRNA assay is 20 (dotted line). The only statistically significant difference was between infants aged 6-12 months and children aged 3-6 years (p<0.001, two-tailed Wilcoxon test).

	Geometric mean antibody titre (with 95% CI)
Infants aged 2-4 months (PRNA assay)	144 (-)
Infants aged 6-12 months (PRNA assay)	25 (12-54)
Children aged 3-6 years (PRNA assay)	1063 (526-2148)
Paediatric healthcare workers (V1) (FACS assay)	8.5 (8.1-9.0)
Paediatric healthcare workers (V2) (FACS assay)	8.2 (7.8-8.6)
Non-healthcare workers (V1) (FACS assay)	8.1 (7.6-8.6)
Non-healthcare workers (V2) (FACS assay)	7.9 (7.6-8.3)

Table 4-6. Geometric mean titres of serum RSV neutralising antibody.
Confidence intervals were not calculated for infants aged 2-4 months (n=3).

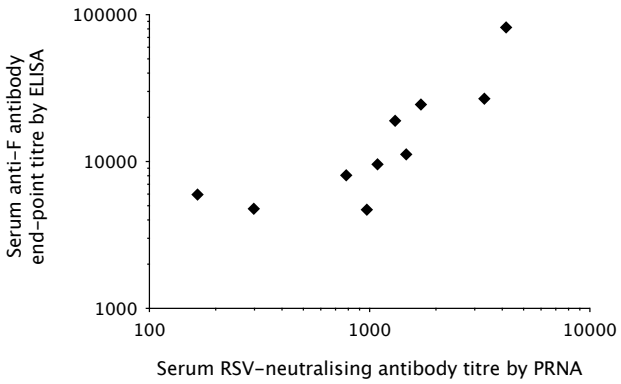


Figure 4-6. Correlation between serum F-specific IgG antibody titres and serum RSV-neutralising antibody titres.
Data from children aged 3-6 years only. A statistically significant correlation was observed (p=0.001, r=0.90, two-tailed Spearman's test).

4.5 Analysis of the cellular immune responses to RSV after natural exposure

4.5.1 IgG and IgA memory B-cell responses to natural RSV exposure

The magnitude of memory IgG B-cell responses to natural exposure was statistically indistinguishable across study groups and time points in the paediatric healthcare workers study. With the infants and children study both total and F-specific IgG B-cell memory responses shown no differences across study groups (p=0.930 and p=0.275 respectively, one-way Kruskal-Wallis ANOVA not inclusive of infants aged 2-4 months where n=1)(Figure 4-7). There were no statistically significant correlations between F-specific IgG memory B-cell responses and the F-specific IgG end-point antibody titres reported in the last section (data not shown).

IgA memory B-cell responses were different. F-specific IgA B-cell memory was undetectable in blood from all infant and children samples. In paediatric healthcare workers there were F-specific IgA memory B-cell responses detectable in 9/18 (50%) samples at the end of the RSV season, and 4-6 months later this proportion had declined to 5/20 (25%) and were of a lower magnitude of response measured earlier (p=0.179 and p=0.027, Fisher's exact test and two-tailed Wilcoxon test respectively). For non-healthcare workers, only 2/8 (25%) volunteers at the end of the RSV season and 0/9 volunteers 4-6 months later had any measurable F-specific IgA-memory B-cell response in peripheral circulation (p>0.05, Fisher's exact test and two-tailed Wilcoxon test). Total IgA memory B-cells, representative of the full repertoire of IgA specificities, were detectable in some but not all infant volunteers. In paediatric healthcare workers all 18/18 samples recorded a measure total-IgA memory B-cell response at the end of the winter viral transmission period compared with only 11/20 (55%) of samples obtained in the summer months 4-6 months later (p=0.001, Fisher's exact test)(Figure 4-7). Furthermore there was a clear binary distribution to the data between detectable and non-detectable responses, combined with a notable drop in the magnitude of response from volunteers with continued detectable total IgA memory responses at V2 (p<0.001, two-tailed Wilcoxon test). Compared with paediatric healthcare workers the non-healthcare worker cohort recorded comparable total-IgA memory B-cells responses at equivalent time points. At the end of the RSV season 8/8 non-healthcare worker samples recorded a total-IgA response compared with 7/9 at the later time point (p=0.471, Fisher's exact test), but measured responses were lower (p=0.012, two-tailed Wilcoxon test).

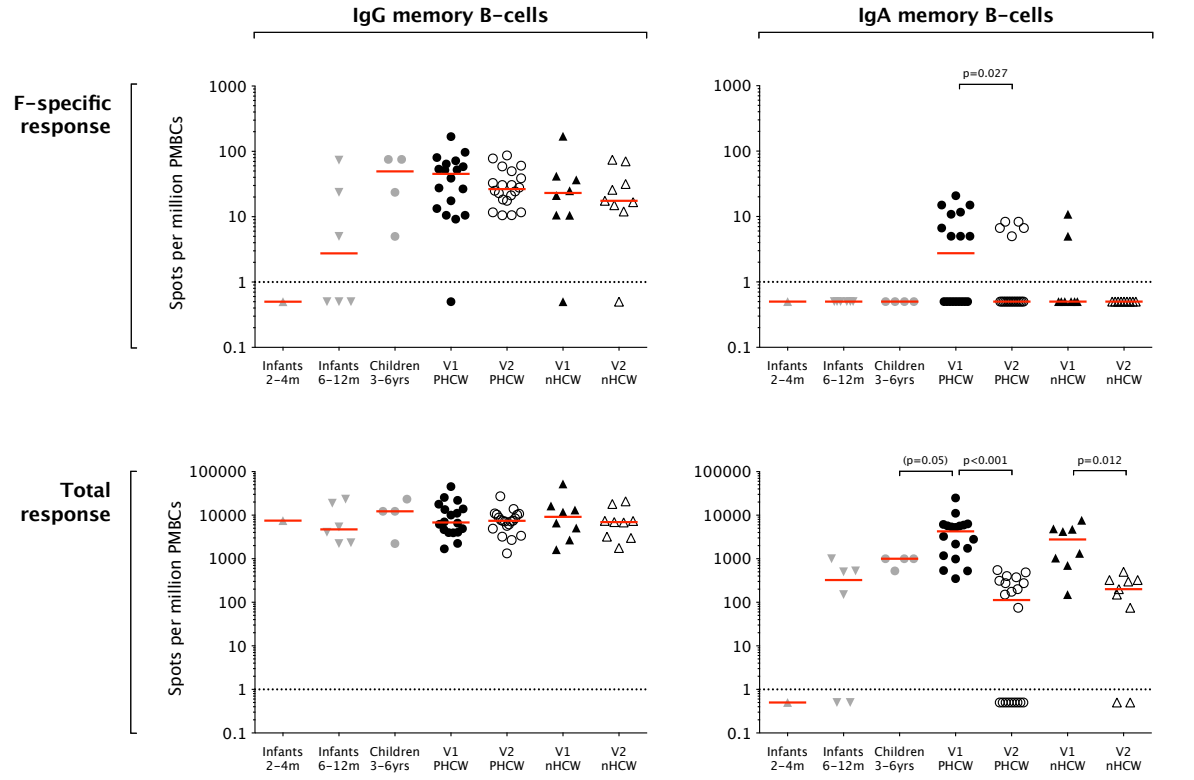


Figure 4-7. Total and F-specific IgG and IgA memory B-cell results. *ELISpot data. The red lines are the median and the lower limit of detection for the assay is marked by the dotted horizontal line (1 spot per million PBMCs). The top pair of figures show the RSV F-protein specific memory B-cell response for IgG and IgA respectively. The bottom pair of figures show the anti-Ig (or total) IgG and IgA memory B-cell responses respectively.*

	Geometric mean spots per million PBMCs (95% CI)			
	F-specific IgG	**Total IgG**	**F-specific IgA**	**Total IgA**
Infants aged 2-4 months	0.5 (-)	7500 (-)	0.5 (-)	0.5 (-)
Infants aged 6-12 months	3.2 (-13 – 48)	6063 (-377 – 19119)	0.5 (0.5 – 0.5)	46.3 (-46 – 772)
Children aged 3-6 years	28.6 (-13 – 102)	9399 (-1224-26274)	0.5 (0.5 – 0.5)	851.2 (503 – 1259)
Paediatric healthcare workers (V1)	28.7 (27 – 68)	7910 (5939 – 16751)	2.2 (2 – 9)	2912 (2148 – 7833)
Paediatric healthcare workers (V2)	27.4 (23 – 44)	6834 (5680-10779)	0.9 (0.7 – 4)	15.6 (77 – 251)
Non-healthcare workers (V1)	17.2 (-6 – 85)	8123 (-69 – 27398)	0.9 (-0.7 – 6)	1794 (862 – 5305)
Non-healthcare workers (V2)	16.9 (9 – 49)	6329 (3257-13466)	0.5 (0.5 – 0.5)	59.2 (79 – 338)

Table 4-7. Geometric mean memory B-cell responses to RSV F-protein and total (spots per million PBMCS with 95% confidence intervals).
Confidence intervals were not calculated for infants aged 2-4 months (n=3).

4.5.2 T-cell responses to natural RSV exposure

The total T-cell IFNγ response to RSV and responses to the separate peptide pools of Fa, Fb, M and N are shown in Figure 4-8. PBMCs from 2/3 infants aged 2-4 months and 10/20 (50%) infants aged 6-12 months failed to record any detectable IFNγ responses, with low-magnitude responses recorded from those infants that did compared with children aged 3-6 years who recorded a detectable response in all but one sample ($p<0.001$, two-tailed Mann-Whitney test). Children aged 3-6 years recorded a magnitude of response comparable to adults from the paediatric healthcare workers study ($p=0.283$, two-tailed Mann-Whitney test). All adult samples, except one, recorded IFNγ responses to RSV exposure. There were no differences in the mean response across study groups and time points ($p=0.281$, one-way Kruskal-Wallis ANOVA). Spearman's correlation analyses between the total IFNγ response with RSV-specific antibody (F and G endpoint titres) and F-specific memory B-cells failed to register any statistical significance, except for an inverse relationship with IgG F-specific antibody titres in children aged 3-6 years ($p=0.008$, $r=-0.83$, Spearman's correlation)(Figure 4-9).

Analysis of the peptide pool specific IFNγ responses found no detectable response above DMSO background to any peptide pool before aged 3-6 years. In this age group there were only four recorded responses above background. From adult samples the DMSO background cut off of 35 spots per million PBMCs commonly defined the median value for each peptide pool response. The breadth of peptide pool responses were not different between paediatric healthcare workers and non-healthcare workers, or within each group and between time points ($p>0.05$, one-way Kruskal-Wallis ANOVA).

PBMCs selected at random from the infant and children study, and a subset of adult PBMCs from paediatric healthcare workers from the end of the 2013/14 RSV season (V1, n=10), were used to explore Th2 immune responses to natural RSV exposure by IL4, IL-13 and IL-17 ELISpot assay. Low-level responses were detectable in some volunteers and not others, and without discernable patterns in relation to different study populations (see Figure 4-10).

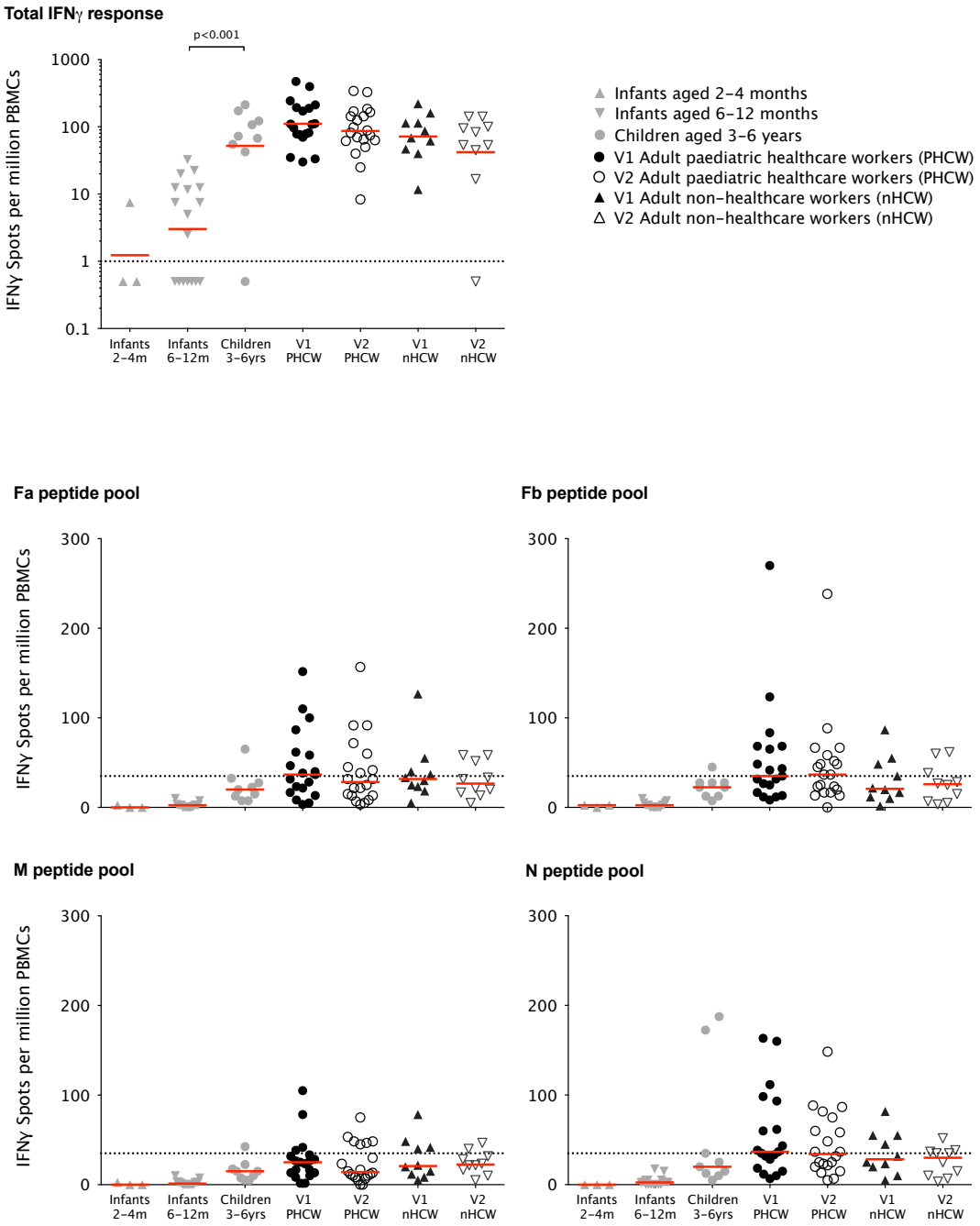


Figure 4-8. T-cell IFNγ responses to separate RSV peptide pools from natural exposure.
*ELISpot data. (**Top figure**) Total IFNγ response to natural exposure. The red line is the geometric mean value. The dotted line is the lower limit of detection (1 spot per million PBMCs). The total IFNγ response was calculated as the sum of the 4 separate peptide pools with 4xDMSO background subtraction. (**Bottom panel of 4 figures**) The peptide pool specific IFNγ response to natural exposure. The red line is the median and the dotted line is the mean + 3 standard deviations of the DMSO background (35 spots per million PBMCs).*

	Geometric mean spots per million PBMCs (95% CI)
Infants aged 2-4 months	1.2 (-)
Infants aged 6-12 months	3.0 (1.3 – 7.1)
Children aged 3-6 years	52.0 (12.8 – 210.4)
Paediatric healthcare workers (V1)	111.0 (76.5 – 161.1)
Paediatric healthcare workers (V2)	86.8 (58.0 – 129.8)
Non-healthcare workers (V1)	71.7 (39.6 – 129.9)
Non-healthcare workers (V2)	42.0 (12.6 – 139.7)

Table 4-8. Geometric mean IFNγ spots per million PBMCs.
Confidence intervals were not calculated for infants aged 2-4 months (n=3).

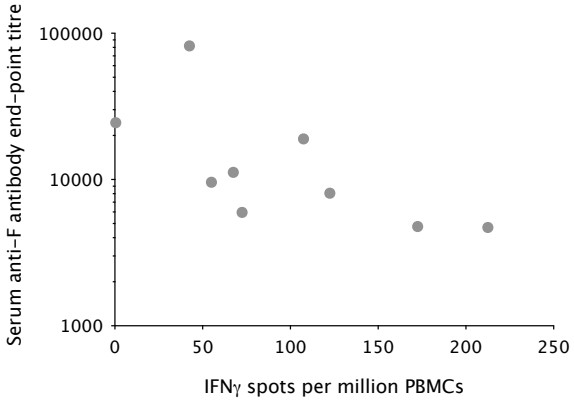


Figure 4-9. Correlation analysis between total IFNγ spots per million PBMCs and end-point titres for serum F-specific antibody in children aged 3-6 years.
A Spearman's correlation analysis found this relationship to have statistical significance (p=0.008, r=-0.83).

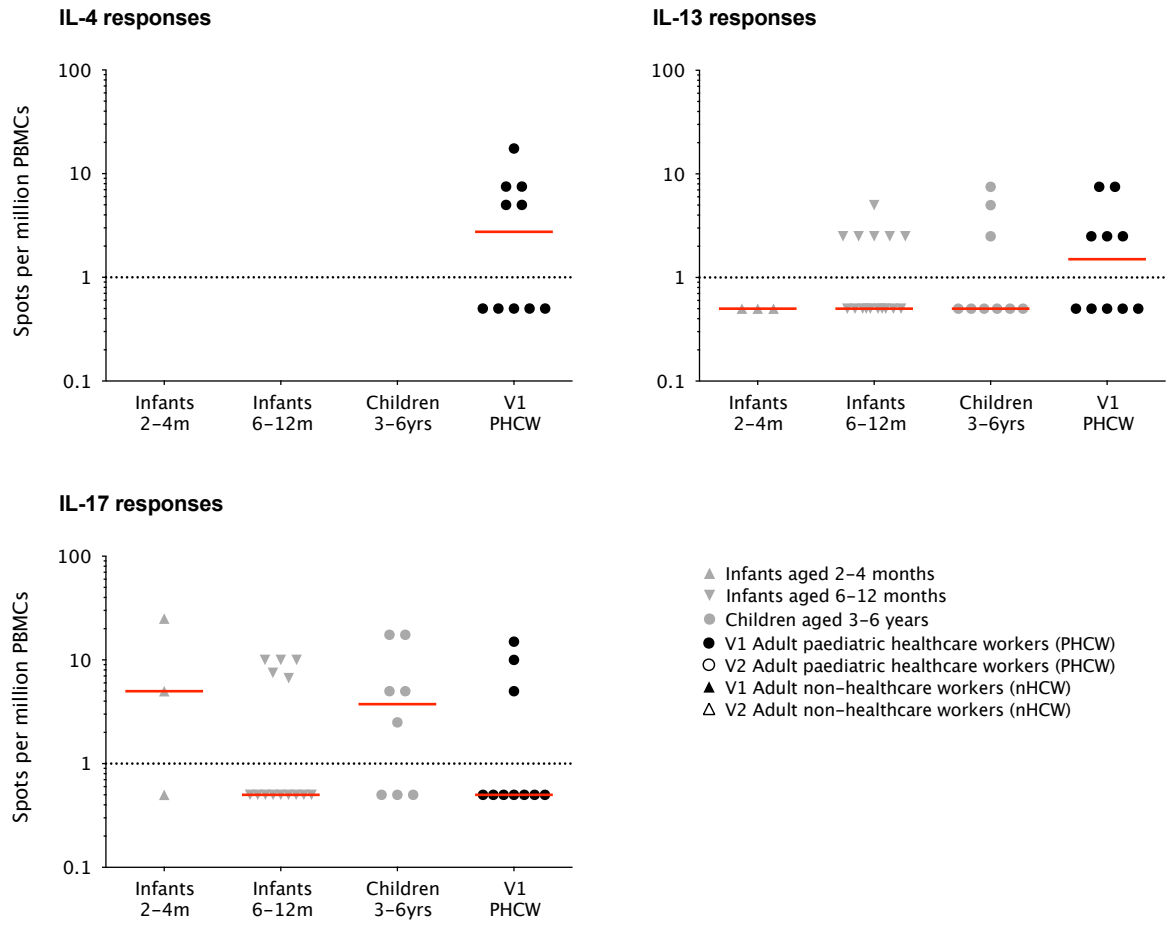


Figure 4-10. IL-4, IL-13 and IL-17 responses to natural RSV exposure.
ELISpot data. The red lines denote the median. The dotted line represents the lower limit of detection for the assay (1 spot per million PBMCs; values below this were reverted to 0.5) The summed IL-4, IL-13 and IL-17 responses are the summed response to the separate RSV peptide pools Fa, Fb, M and N with 4xDMSO background subtraction.

4.6 Discussion

4.6.1 Summary of main findings

Paediatric healthcare workers recorded robust immunity to RSV. Titres of antibody and measures of cellular immune responses were indistinguishable from non-healthcare or non-child minding adults of presumed lesser exposure. Both adult populations recorded defective maintenance of total and F-specific IgA memory B-cells in summer months. From the children and infant study there were notable differences in the development of G-antibody and T-cell immune responses with cumulative natural exposure.

4.6.2 The humoral and cellular immune response to natural RSV exposure

Antibodies play a significant role in protection from infection and disease. The partial protection afforded by maternal antibody and palivizumab, an F-specific humanised mouse monoclonal antibody, gives a clear indication that antibodies alone can protect infants from lower respiratory tract disease[90]. In developing natural immunity, which includes cellular immunity, this takes time and repeated exposure. As described earlier, 50% of infants will have developed detectable antibody by 11-months of age and primary infection induces a neutralising antibody response that wanes within 3-months, which was comparable with the data reported here[57,221,222]. By the age of 3-6 years, when the risk of severe disease has declined significantly, F-specific antibody titres matched those recorded in adults although G-specific antibody titres lagged behind. Adults, irrespective of occupation and presumed exposure or time of sampling, exhibited stable antibody levels but with significant within group and time point variation.

The most striking and unexpected observation was the apparent defect in maintaining F-specific IgA memory B-cells in peripheral circulation. This may represent an Achilles heel related to re-infection in healthy adults. Interestingly IgA memory B-cells were absent from healthy volunteers before and after RSV challenge experiments and this was interpreted as a global inability of RSV to induce IgA memory B-cell immunity[237]. The data presented here demonstrate IgA memory B-cells are inducible in some but not all individuals, presumed in relation to recent or repeated exposure, and that these responses in particular are difficult to maintain. Nasal IgA can be used as a correlate of protection from infection in healthy adults but the relationship between IgA memory B-cells and mucosal IgA is not clear. Equally unclear is the mechanism for a deficiency in the maintenance of RSV-specific IgA B-cell memory. Natural and experimentally controlled RSV infection is capable of inducing antigen-specific IgA antibody secreting cells (ASCs) indicating there is no defect in this early B-cell IgA ASC response, which is likely to be derived from memory B-cells[237]. Influenza and cholera are capable of inducing durable IgA memory B-cell populations. Although RSV has several potent mechanisms for subverting the host adaptive immune response the depletion of total IgA memory B-cells in summer months is indicative of a mechanism not wholly and uniquely attributable to RSV, and the persistence of total and RSV-specific IgG memory B-cells is indicative of a process not common to the maintenance of memory B-cells of both antibody classes[237,366,367]. B-cell differentiation, class switching, antibody maturation and the selection of memory B-cells cells are

supported by CD4+ T-follicular helper (Tfh) cells in germinal centres (reviewed here[368]) and this may be the point at which the maintenance of IgA memory B-cell immunity to RSV and other pathogens is imperfect. An alternative consideration is whether IgA memory B-cells are typically resident in pulmonary lymphoid organs, and the detection of these in peripheral circulation is an effect of 'spill-over' in periods of high demand and the possibility that their absence in peripheral circulation in summer months may not be indicative of a susceptibility to RSV re-infection.

In considering the maturation of infant immunity further, T-cell immunity is difficult to maintain in early years and only 80% of infants develop RSV-specific CD8+ T-cell responses after the first year of exposure, which is largely consistent with the data reported here[369,370]. Although not measured in these studies, it is likely that other T-cell populations could follow a similar and slow kinetic in developing into mature function. Given the role of CD4+ T-follicular helper (Tfh) cells in supporting B-cell maturation, class switching and antibody protection in germinal centres it seems intuitive that any further exploration of the mechanisms behind the development of RSV-specific IgA memory B-cells and immunity in infants and healthy adults should include a wider consideration of cell populations than were measured here.

4.6.3 General limitations

Greater RSV exposure in paediatric healthcare workers was an assumed qualitative variable used here to define the difference between the small cohort of paediatric healthcare workers and the even smaller cohort of non-paediatric healthcare workers used for comparative analysis. While it was reasonable to assume that NHS staff working on acute paediatric medical wards and PICU would have invariably come into contact with sick infants shedding virus it remains unknown if, at a biological level, this was considerably different to the exposure needed to boost or maintain RSV immunity in the general population. It may be that only a little RSV exposure is needed to maintain this immunity. The small group sizes were evidently prone to misrepresentation of the wider population by chance occurrence. Furthermore, RSV exposure is not a binary variable or event, and it is possible that frequent contact with the virus can give rise to a different kinetic of immune response, or that some individuals were last exposed closer to the time of sampling than others. Given the point of contact with RSV is the respiratory mucosa then responses in blood cannot be representative of total immunity to RSV. Nasal sampling was not

performed in these studies and, as described in earlier sections, mucosal immunity is a major determinant of susceptibility to infection and onward transmission.

4.6.4 Conclusions

Infant RSV-specific G-protein specific antibody and IFNγ producing T-cells develop slowly with cumulative exposure to RSV. Adults maintain robust titres of RSV-neutralising antibody and IFNγ producing T-cells in peripheral circulation, but total and F-specific IgA memory B-cells were poorly maintained over the summer months and offers an interesting question of whether IgA memory B-cells can be related to susceptibility to reinfection. The restoration of IgA memory B-cell cells in circulation might therefore become a target for vaccine immunogenicity.

Chapter 5: Clinical trial RSV001 – setup, trial design and analysis of recruitment, study population & trial execution

5.1 Introduction

Whole-virus, live-attenuated, subunit and nanoparticle RSV vaccines candidates have, to date, failed to reach the standard required for licensure. Novel recombinant viral-vectored vaccines, never before applied to the problem of RSV, have an advantage over other subunit vaccine in the induction of T-cell immunity in addition to antibody responses. Heterologous and homologous prime/boost combinations of genetically modified, replication-defective viral-vectored vaccines have been safe and powerfully immunogenic in man when used as vaccines for malaria[371-379], HIV[380-383], tuberculosis[384-388], hepatitis C[389,390], influenza[391,392], Ebola[393-398], dengue[399,400], Japanese encephalitis and cancer vaccines, and several have been tested safely in infants. This chapter, and the remaining chapters of this thesis, is concerned with translational research and a phase I, first-in-man, clinical evaluation of two novel viral-vectored vaccines engineered to deliver RSV antigen in healthy adult volunteers. The objectives of this chapter are to describe the setup and design of the RSV001 clinical trial, followed by reporting a retrospective analysis of trial recruitment and execution. The clinical trial protocol has been published in full elsewhere[3f].

[f] Sections of this chapter copied from the clinical trial protocol and the published manuscript represent my own work.

5.2 The experimental vaccines PanAd3-RSV and MVA-RSV

The vaccines, designated PanAd3-RSV and MVA-RSV, were conceived, constructed and evaluated in preclinical models by ReiThera Srl, Italy, in collaboration with Dr Geraldine Taylor at the Pirbright Institute, United Kingdom. In summary, an artificial, consensus-based, RSV antigen was generated and packaged as a single codon-optimised DNA fragment for insertion and the expression of RSV F (F0ΔTM), N and M2-1 proteins by replication defective adenovirus, PanAd3, and modified vaccinia virus Ankara, MVA. Upon transfection of a mammalian cell, a Foot and Mouth disease virus 2A region self-cleavage site releases a truncated version of the F protein (F0ΔTM, deleted of the trans-membrane portion) as soluble antigen in the cell supernatant. The N and M2-1 proteins are retained within the host mammalian cell for MHC co-expression. A schematic illustration is provided in Figure 5-1. Clinical grade PanAd3-RSV and MVA-RSV were manufactured under Good Manufacturing Practice (GMP) conditions by Advent (Italy) and Impfstoffwerk Dessau-Tornau (Germany) respectively.

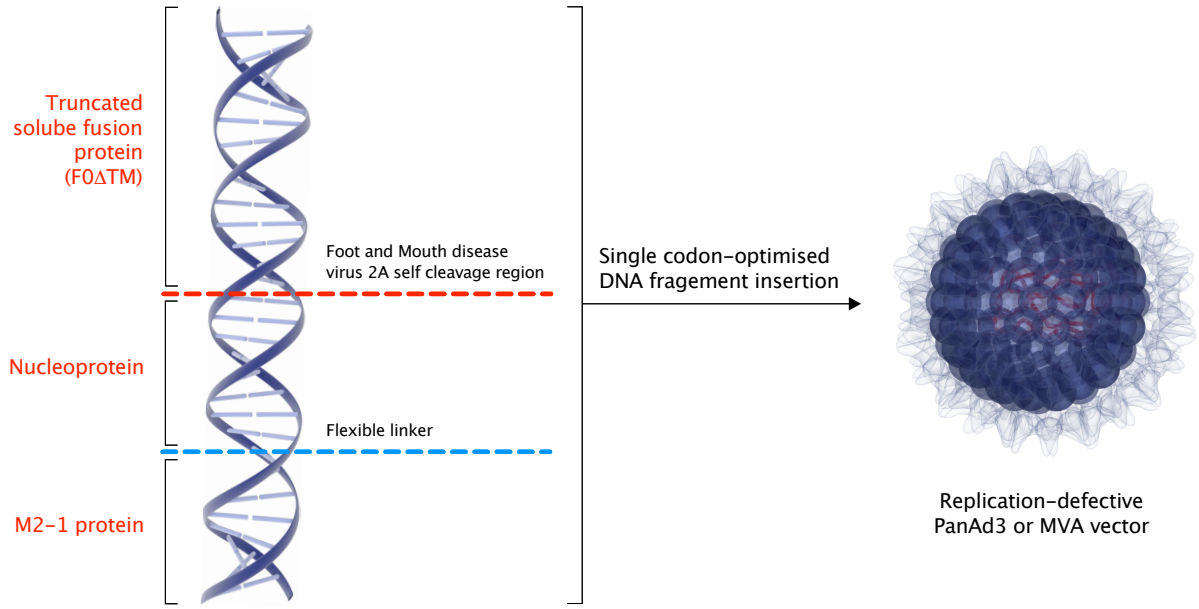


Figure 5-1. Schematic illustration of the PanAd3-RSV and MVA-RSV genetically modified organisms.
A single codon-optimised DNA fragment was used to encode the RSV F, N and M2-1 proteins (red). The same DNA fragment was inserted into the replication-defective PanAd3 and modified vaccinia virus Ankara (MVA) viral-vectors. After transfection into the mammalian host cell, a cleavage region from Foot and Mouth disease virus 2A releases a soluble truncated form of the RSV F protein (F0ΔTM, devoid of the trans-membrane region of the protein). The N and M2-1 proteins are retained within the mammalian host cell.

PanAd3 is a hexon group C chimpanzee derived adenovirus (ChAd) originally isolated from faecal samples of the bonobo *Pan paniscus*. Deletion of the E1 and E4 loci renders the chimpanzee adenovirus replication-defective. The PanAd3 vector was selected for its robust *in vitro* immunogenicity and low rates of sero-prevalence of neutralising antibody in the human population (estimated 3%)[380,401]. PanAd3 had never been tested in humans before RSV001, and was therefore a first-in-man vector as well as a first-in-man vaccine. PanAd3 shares significant sequence homology with other hexon group C adenoviruses such as human Ad5, human Ad6 and chimpanzee adenovirus 3 (ChAd3) that have returned favourable safety and desirable immunological potency in humans[389,401]. Modified vaccinia virus Ankara, or MVA, is a poxvirus strain obtained originally from Ankara, Turkey, and passaged between donkeys and calves before serial attenuation by >500 passages through chick embryo fibroblasts (CEFs) in Germany. MVA was administered to >120'000 people, including in high-risk populations such as the young and old, in the final stages of the smallpox eradication campaign without significant side effects. The loss of approximately 12% of the genome included many genes that could interfere with the host immune response[402-405]. This has resulted in MVA becoming restricted to avian cells and cannot successfully replicate in mammalian cells, and yet remains a potent inducer of type I interferons. MVA has been used safely in several vaccine formulations in man that include malaria[406], HIV[380,381], tuberculosis, hepatitis C, influenza, Ebola and in cancer vaccine trials for prostate, colorectal, breast and melanoma (reviewed here[407]).

Preclinical evaluation of PanAd3-RSV and MVA-RSV included homologous and heterologous combinations, including the intra-nasal route, in BALB/c mice, cotton rats, non-human primates and young calves. The results are published elsewhere, as was as the use of PanAd3 as an influenza vaccine vector in mice[408-410]. In summary a single dose of intra-nasal (IN) or intra-muscular (IM) vaccine fully protected the lower respiratory tract from viral replication after challenge with human RSV in BALB/c mice and cotton rats, with the IN route also capable of inducing sterilising immunity in the upper respiratory tract. The calf model, where calves were separated from the mother's colostrum and only source of maternal antibody, was used as a translational model for the development of a vaccine for use with sero-negative infants. All regimens fully protected the lower respiratory tract from bovine RSV infection in the calf and heterologous combinations resulted in sterilizing immunity in both upper and lower respiratory tracts. Importantly the

immunogenicity and protective efficacy of PanAd3-RSV and MVA-RSV was not associated with evidence of immunopathology associated with ERD after vaccination and subsequent challenge.

5.3 Clinical trial setup, design and objectives

The next stage was to translate these finding into man. RSV001 was an open label, dose-escalation, single-site, phase 1 (first-in-man) clinical trial of the experimental vaccines PanAd3-RSV and MVA-RSV in healthy adult volunteers. In 2013, a total of 42 healthy adults aged 18-50 years tested four prime/boost combinations of PanAd3-RSV and MVA-RSV. The four prime/boost combinations were selected from the preclinical experiments in rodents and calves[408,409]. As described in earlier chapters, adults have robust RSV-specific immune responses derived from repeated natural exposure and the term 'prime' used here has been inherited from viral-vectored vaccine trials in antigen naïve populations. Here, the term 'prime' is used to denote the first dose of vaccine and 'boost' as the second dose of vaccine.

Based upon favourable safety and immunogenicity data from the planned interim data analysis, in 2014 the trial was expanded to include an additional 30 healthy older adults aged 60-75 years and tested MVA-RSV as a single-dose of vaccine, three prime/boost combinations selected and tested in younger adults and now included a non-vaccinated control group.

The trial objectives were to characterise the safety and tolerability (primary objective) and immunogenicity (secondary objective) of PanAd3-RSV and MVA-RSV in healthy adults aged 18-50 and 60-75 years. The primary and secondary endpoints are listed in Table 5-1.

		Endpoint measures
Primary objective: vaccine safety and tolerability	Solicited and unsolicited symptoms recorded daily for one week after each vaccine dose	Oral temperature Solicited symptoms; headache, nausea and/or vomiting, malaise, myalgia and arthralgia Local injection site adverse events; pain and/or tenderness, erythema, induration and swelling Local nasal site adverse events; pain and/or tenderness, irritation and discharge Any other event not listed above (unsolicited symptoms)
	Nursing observations obtained at all visit attendances	Resting heart rate, resting respiratory rate, systolic and diastolic blood pressure and oral temperature
	Blood obtained at scheduled visit attendances for the routine safety monitoring of haematology and biochemistry	Haematology: haemoglobin, total white cell count, platelet count, haematocrit, red cell count, mean cell volume, mean haemoglobin, mean haemoglobin concentration, neutrophil count, lymphocyte count, monocyte count, eosinophil count and basophil count Biochemistry: sodium, potassium, urea, amylase, C-reactive protein, creatinine, bilirubin, alanine transaminase, alkaline phosphatase and albumin
Secondary objective: vaccine immunogenicity	Humoral immune response to vaccination	Serum anti-F IgG and IgA antibody titre by ELISA Serum antibody-mediated RSV neutralisation by PRNA assay Serum anti-PanAd3 neutralising antibody titre by SEAP assay
	Cellular immune response to vaccination	Quantification of circulating anti-F IgG and IgA antibody secreting cells by dual-colour ELISpot assay Quantification of circulating interferon-gamma (IFNγ) producing T-cells by ELISpot assay CD4+ and CD8+ subset analysis of the immune response to vaccination by ICS/FACS assay
	Exploratory immunology of the immune response to vaccination	Nasal and salivary anti-F IgG and IgA antibody titre by ELISA Cytokine response to vaccination by CBA assay Gene expression after vaccination analysis by DNA microarray

Table 5-1. The primary and secondary endpoints of the RSV001 clinical trial.
Enzyme-linked immunosorbent assay (ELISA). Enzyme-linked immunospot assay (ELISpot). Plaque-reduction neutralisation assay (PRNA). Secreted alkaline phosphatase assay (SEAP). Intra-cellular staining (ICS). Fluorescence-activated cell sorting (FACS). Cytometric bead array (CBA). Deoxyribonucleic acid (DNA).

5.3.1 Study groups, vaccine dose and dose-escalation

RSV001 consisted of a total of 9 study groups, each defined by different prime/boost combinations in younger and older healthy adult volunteers (see Table 5-3 and shown schematically in Figure 5-2). In all heterologous prime/boost combinations, the prime was PanAd3-RSV administered by intra-muscular (IM) injection or intra-nasal (IN) spray. The boost vaccine was by IM injection only, using either PanAd3-RSV or MVA-RSV. Homologous 'double prime' IM PanAd3-RSV was tested in both younger and older adults, and single dose IM MVA-RSV was tested in older adults only. The prime/boost combinations used in 2014 in younger adults were selected from preclinical data[408,409]. Based upon the interim analysis of safety and immunogenicity data in younger adults, the trial expansion in 2014 with older adults selected to test IM MVA-RSV alone, three prime/boost combinations trialled in younger adults and a non-vaccinated control group.

Within each study group of younger adults (study groups 1-4), the first two individuals tested a low-dose of vaccine before remaining volunteers received normal-dose (or target-dose) vaccine. The vaccine doses are set out in Table 5-2. Halting rules for dose-escalation included the occurrence of any serious adverse reaction (SAR) or if two or more subjects experienced a severe adverse event (grade 3) that was clinically significant with a reasonable possibility of being related to the vaccine. Formal approval from the independent data safety monitoring committee (DSMC) was required before normal-dose prime and normal-dose boost vaccinations (Figure 5-3). All vaccines given to older adults were at normal-dose.

	Low-dose vaccine	Normal-dose vaccine
IM or IN PanAd3-RSV	5×10^9 vp	5×10^{10} vp
IM MVA-RSV	1×10^7 pfu	1×10^8 pfu

Table 5-2. Low-dose and normal-dose of IM/IN PanAd3-RSV and IM MVA-RSV.
Doses were prepared by dilution to the desired volume with sterile 0.9% saline solution. Vp, viral particles. Pfu, plaque forming units.

	N volunteers	Prime vaccine	Boost vaccine	Prime/boost interval
2013/14 - Healthy adults aged 18-50 years:				
Group 1	10	IM PanAd3-RSV	IM MVA-RSV	8 weeks
Group 2	10	IM PanAd3-RSV	IM PanAd3-RSV	4 weeks
Group 3	10	IN PanAd3-RSV	IM MVA-RSV	8 weeks
Group 4	10	IN PanAd3-RSV	IM PanAd3-RSV	8 weeks
2014/15 - Healthy adults aged 60-75 years:				
Group 5	6	None	None	Not applicable
Group 6	6	IM MVA-RSV	None	Not applicable
Group 7	6	IM PanAd3-RSV	IM PanAd3-RSV	4 weeks
Group 8	6	IN PanAd3-RSV	IM MVA-RSV	8 weeks
Group 9	6	IM PanAd3-RSV	IM MVA-RSV	8 weeks

Table 5-3. The 9 study groups in RSV001.
PanAd3-RSV prime was administered by intra-muscular (IM) injection or intra-nasal (IN) spray. All boost vaccines were given by IM injection. The first two volunteers in study groups 1-4 received a low-dose of vaccine; all other volunteers received the normal-dose (or target-dose) of vaccine. The interval between prime and boost vaccine was 8 weeks for all study groups scheduled to use two doses of vaccine, except for study groups 2 and 7 who received the homologous 'double prime' IM PanAd3-RSV with a 4-week interval between doses.

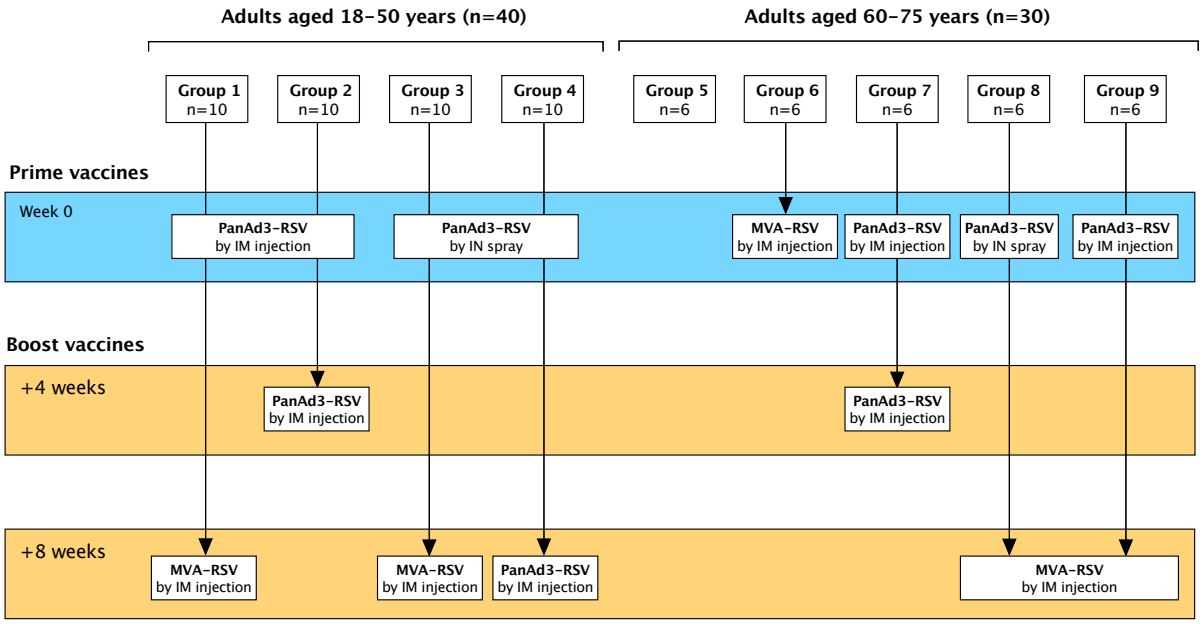


Figure 5-2. Schematic of the RSV001 vaccination schedules that defined the 9 study groups.
Study group 5 was a non-vaccinated control arm.

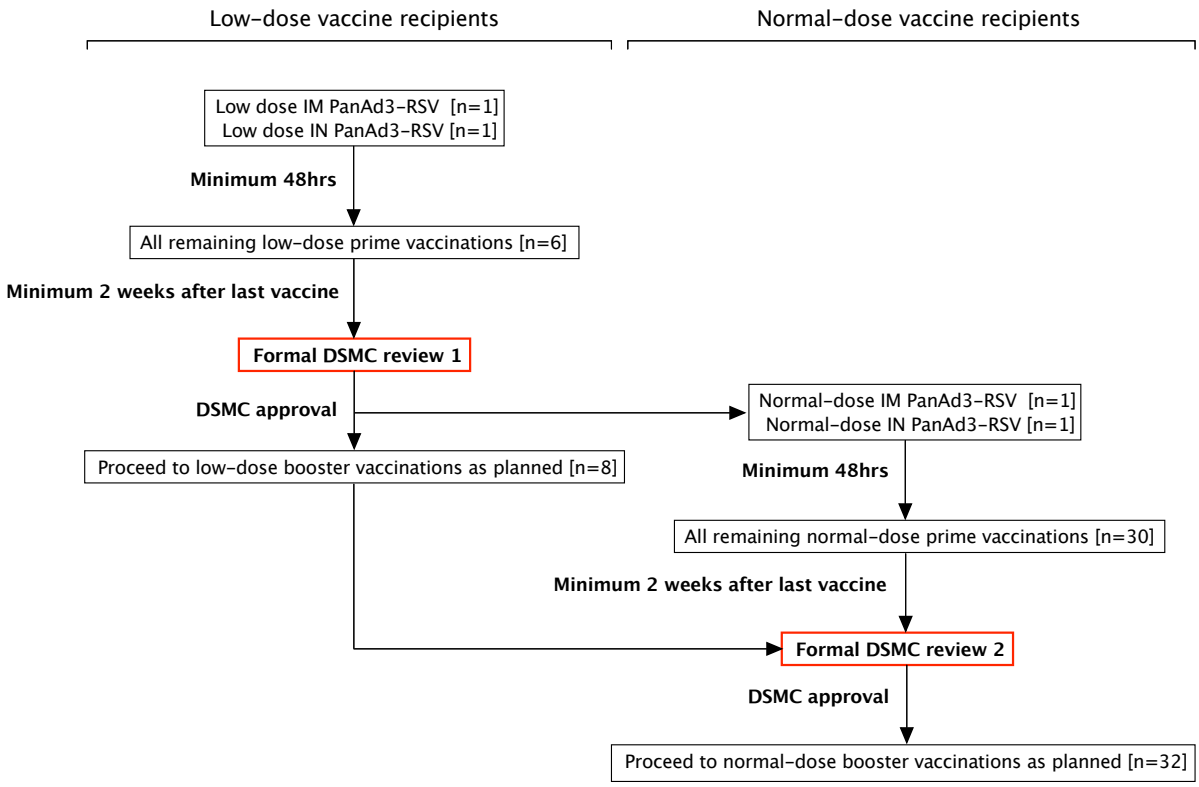


Figure 5-3. Schematic for dose and group size escalation for adults aged 18-50 years.
At each dose, prime vaccination with IM and IN PanAd3-RV was performed in one individual and safety assessed for a minimum of 48hrs before the remaining prime vaccinations were administered. Formal data safety monitoring committee (DSMC) approval was required before administration of the low-dose booster and high-dose prime vaccinations, and again for high-dose boost vaccinations. For older adults aged 60-75 years there were no dose or group size escalation rules and no scheduled formal reviews for the DSMC.

5.3.2 Volunteer eligibility

Age-appropriate healthy volunteers who satisfied the eligibility criteria set out in Table 5-5, and who were willing and able to take part and had given written informed consent we eligible for the trial.

Inclusion criteria – all must be satisfied
1. Volunteer must be willing and able to consent to take part in the clinical trial
2. At the time of enrolment; aged 18-50 years inclusive for study groups 1-4, or aged 60-75 years inclusive for study group 5-9
3. In good health as determine by medical history, physical examination and in the judgement of the study investigators
4. Willing to use effective contraception (if sexually active); Females: Oral contraceptive pill, contraceptive implant or barrier methods from one month prior and for the duration of the study Males: Barrier contraceptive from V1 until 3 months after the last dose of vaccine
5. Willing to allow his/her General Practitioner and/or Consultant, if appropriate, to be notified of participation in the study
6. Confirmation from the General Practitioner that they are aware of the inclusion and exclusion criteria and are satisfied from their knowledge of the volunteer that they are suitable to enrol
7. Willing to provide their National Insurance/Passport number for the purpose of TOPS registration
Exclusion criteria – none can be satisfied
1. History of any significant organ/system disease that interfere with trial conduct or completion. These include any history of significant disease in the following; • Cardiovascular disease including congenital heart disease, previous myocardial infarction, valvular heart surgery (or history of rheumatic fever), previous bacterial endocarditis, history of cardiac surgery (including pacemaker insertion), personal or family history of cardiomyopathy or sudden death • Respiratory disease such as asthma (excluding childhood asthma) and chronic obstructive pulmonary disease • Endocrine disorders such as diabetes mellitus and Addison's disease • Significant renal or bladder disease, including a history of renal calculi • Biliary tract disease • Gastro-intestinal disease such as inflammatory bowel disease, abdominal surgery within the last two years, coeliac disease and liver disease • Neurological disease such as seizures and myasthaenia gravis • Metabolic disease such as glucose-6-phosphate dehydrogenase deficiency • Psychiatric illness requiring hospitalisation or depression whose severity is deemed clinically significant • Non-benign cancer, including squamous cell carcinoma, basal cell carcinoma of the skin and cervical carcinoma in situ • Clinically significant contact dermatitis
2. Have any known or suspected impairment or alteration of immune function, resulting from, for example: congenital or acquired immune deficiency, human immunodeficiency virus or symptoms/signs of an HIV-associated condition, autoimmune disease, receipt of immunosuppressive therapy such as chemotherapy or radiation therapy in the preceding 12 months or long-term corticosteroid therapy, receipt of immunoglobulin or any blood product transfusion within 3 months of the study start
3. A vaccination history indicative of; planning to receive another vaccine (other than the study vaccine) within 4 weeks of vaccination, a history of anaphylaxis reaction to a vaccine, history of allergic disease or reactions likely to be exacerbated by any component of the vaccine (e.g. Kathon), previously having received a recombinant simian or human adenoviral vaccine, previously having received a recombinant MVA vaccine
4. Detection of any of the following at screening; IgA deficiency, anti-HIV antibody, hepatitis B surface antigen, anti-HCV antibody, any other significant abnormalities at the discretion of the study investigator
5. Known or suspected drug and/or alcohol misuse (alcohol misuse defined as an intake > 42 units/week).
6. Nasal septal pathology including; congenital deformities such as an abnormal septum or polyps, previous cauterisation, rhinoplasty or surgery of any kind, recurrent epistaxis
7. Scheduled procedures requiring general anaesthesia during the study
8. Participation in another study involving an investigational product in the past 12 weeks, or are planning to do so during the study
9. Inability, in the opinion of the investigator, to comply with study requirements
10. Female participants who are pregnant, lactating or planning pregnancy during the course of the study
11. Has donated blood within 4 months before starting the trial, or is intending to donate blood during the trial and up to 12 weeks after completing the trial
12. Any other significant disease or disorder that, in the opinion of the investigator, may put the participant at risk because of participation in the study, influence the result of the study or impair the participant's ability to take part on the trial

Table 5-4. Summary of the inclusion and exclusion criteria for RSV001 volunteers.

5.3.3 *Data safety monitoring committee and safety oversight*

A data safety monitoring committee (DSMC) was convened to provide advice and independent oversight of the continuous safety monitoring performed by the trial physician and members of the study team. The DSMC assessed weekly safety data and formal review was required by the DSMC to give approval for dose escalation and group size expansion, as shown in Figure 5-3. The DSMC acted in accord with a trial specific, pre-specified, DSMC charter written in accordance with DAMOCLES guidance[411] and agreed upon before the trial started.

5.3.4 *Regulatory approvals and trial registration*

The RSV001 clinical trial was conducted in accordance with the UK Medicines for Human Use (Clinical Trials) Regulations 2004, and subsequent amendment[412,413]. RSV001 was granted the following regulatory approvals before study procedures commenced;

- Clinical Trials Authorisation from the UK Medicines and Healthcare Products Regulatory Agency (MHRA reference 35082/0003/001-0001)
- University of Oxford Genetic Modification Safety Committee (GSMC reference GM462.11.64)
- Research Ethics Committee (NRES Berkshire reference 13/SC/0023)
- Local site and NHS R&D approvals

RSV001 was registered with clinicaltrials.gov (reference NCT01805921) and the European Medicines Agency's European Clinical Trials Database (EudraCT; reference 2011-003589-34). The trial was portfolio adopted by the National Institute for Health Clinical Research Network.

5.3.5 Setting and timing of the clinical trial

RSV001 was performed at the Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), Churchill Hospital, Oxford, United Kingdom. The trial workload was pre-planned in respect of every visit for every volunteer to ensure both a manageable clinical and laboratory workload, and for all vaccinations and sample collection up to 28-days after vaccination to occur in summer months, when there was minimal risk of natural RSV exposure and the potential for natural induction of RSV-specific immune responses and false interpretation of vaccine immunogenicity. Public Health England (PHE) surveillance data on laboratory confirmed RSV infections reported in all ages between the 1999/2000 and 2012/13 seasons were used to anticipate the beginning and peak RSV transmission periods in 2013/14 and 2014/15[43]. The date of each scheduled attendance was recorded and analysed in respect of the presumed RSV transmission season in each year.

5.3.6 Funding, sponsorship and monitoring

RSV001 was funded and sponsored by ReiThera Srl (formerly Okairos Srl), which was acquired by GlaxoSmithKline Biologicals SA during the trial. NIHR Oxford Biomedical Research and the Wellcome Trust provided salary support for members of the study team. Clinical Trials Research Governance (CTRG), University of Oxford, monitored RSV001 according to ICH Good Clinical Practice (GCP).

5.4 Study procedures

All study procedures adhered to the International Conference on Harmonisation Good Clinical Practice 1996 guidelines (ICH GCP) and principles of the Declaration of Helsinki[414,415].

5.4.1 Recruitment

A range of methods was used to engage the public and solicit an expression of interest from members of the public who considered themselves eligible to take part (see Table 5-5). The relative use of each method was based on experience of recruiting to clinical trials in earlier years.

For the analysis of recruitment, data were collected on where and when recruitment material was distributed. How potential volunteers heard of the trial invitation was recorded at the time of responding to an expression of interest or at physician screening. 'No interest', 'return to sender' and the absence of a response from mailed addresses were collectively interpreted as not having had an interest in taking part. The time taken from physician screening to enrolment was recorded which included the time taken for GPs to complete a short questionnaire from community medical records, and changes in haemoglobin between screening an enrolment were used to measure sub-clinical changes in haemoglobin concentrations before the phlebotomy demands of the trial commenced.

Methods used to invite expressions of interest in taking part in the clinical trial
Poster advertising throughout local hospitals and doctor's surgeries, tertiary education institutions and other public places with the permission of the owner/ proprietor.
Direct mail-out of the study information sheet to adults whose names and addresses were obtained via the Electoral register. Those people had indicated that they did not wish to receive postal mail–shots had their names removed prior to the Investigators being given the names and addresses. The company providing this service is registered under the Data Protection Act 1998.
Email campaign via representatives of local tertiary education establishments and local employers.
Clinical trials database contact; the Oxford Vaccine Centre (OVC) database for healthy volunteers. A direct email and link to members of the public who registered an interest in potentially volunteering for clinical trials conducted by OVC. This secure database was maintained by OVC and members of the public registered here had given consent to have their details recorded and be contacted expressly for this purpose of being notified when a trial opens for recruitment. It was understand this is was not a commitment to volunteering for any trial they are contacted about.
Media advertising by local media, newspaper and website advertisement placed in locations relevant for the target age group with brief details of the study and contact details for further information.
Website advertising on the study group website; www.ovg.ox.ac.uk.
Exhibitions using stalls or stands at exhibitions and/or fairs, such as University Fresher's Fairs.
Open Exeter; potential study participants were identified via National Health Applications and Infrastructure Services (NHAIS) who hold the central NHS patient database (Open Exeter).

Table 5-5. Summary of the methods used to invite expressions of interest in volunteering for the clinical trial. *Open Exeter was not available in 2013 and the recruitment of younger adults.*

5.4.2 Assessment of volunteer eligibility and volunteer consent

Assessment of eligibility (screening) began from receipt of an expression of interest from a potential volunteer. A schematic to show the steps taken to identify appropriate volunteers is shown in Figure 5-4. Initial volunteer contact was by telephone and lasted 10-20 minutes. This was performed by clinical and non-clinical GCP trained staff and relied on information freely provided from potential volunteers as no consent was yet obtained. The purpose was to ensure the content of the study information booklet (SIB) was understood and to answer any immediate questions from potential volunteers. An invitation for physician screening was made provided the candidate remained interested in taking part and had not volunteered any information that would result in exclusion from the study.

Physician screening would involve a detailed explanation of the rationale of the study, the study requirements, potential risks of taking part and provided a further opportunity to ask questions. The process lasted 90-120 minutes for each potential volunteer. Potential volunteers were required to have read and understood the SIB in advance of physician screening and the structure and content of the SIB was used to structure the face-to-face interview. Particular attention was drawn to the first-in-man nature of the vaccines and PanAd3 vector, the limitations of pre-clinical models, and the use of inferred expectations of safety and immunogenicity from viral-vectored vaccines given to humans in the context of HIV, malaria, smallpox, tuberculosis and hepatitis C. It was made clear that taking part in RSV001 cannot be assumed to have any benefit to volunteers with respect to protection from RSV infection or severe RSV disease, and that volunteers were free to withdraw from the study at any time, for any reason, with no obligation to give the reason for withdrawal. Each participant was allowed time to consider all the information received, and given the opportunity to question the researcher, their GP or other independent parties such as INVOLVE [416] to help decide whether to participate in the study. If the potential volunteer wished to proceed, each personally signed and dated the latest approved version of the informed consent form that was countersigned by the physician who obtained consent. Provided written informed consent was obtained, formal assessment of eligibility could then be performed (see Table 5-6). The reasons for exclusion at all stages of the eligibility assessment were recorded for later analysis.

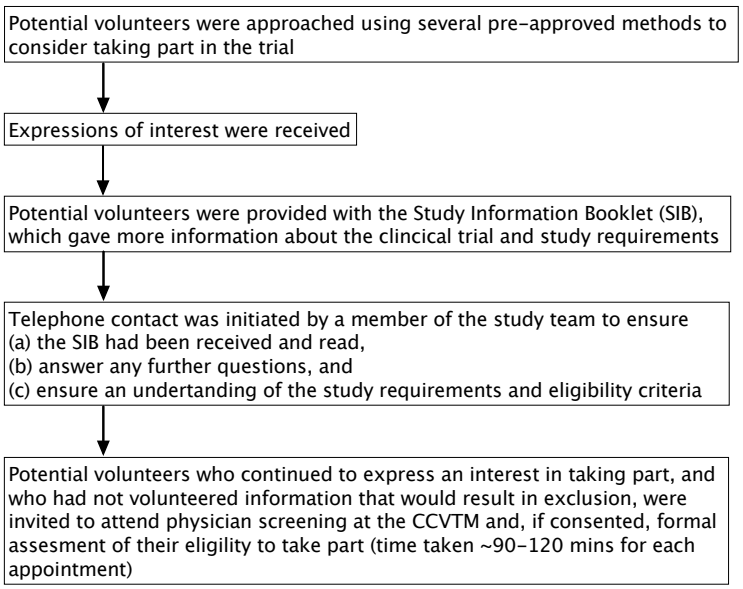


Figure 5-4. Schematic to show the process used to recruit eligible volunteers for the clinical trial. *Potential volunteers were self-selected members of the public responding to advertisements inviting them to consider volunteering for the trial. The flow diagram in shows the method applied to efficiently direct screening resources towards volunteers eligible to participate.*

Investigations in the assessment of study eligibility
1. Recording of volunteer demographics; age, sex, body mass index, ethnicity, occupation
2. Assessment of current and past medical history
3. Physical examination; cardiovascular system, respiratory system, abdomen and neurology
4. 12-lead electrocardiogram
5. Blood sampling; routine haematology and biochemistry, detection of blood born viruses (anti-HIV antibody, hepatitis B surface antigen, anti-hepatitis C virus antibody)
6. Urine sampling; microscopic haematuria, pyuria or proteinuria. Urinary human chorionic gonadotrophin pregnancy testing for all females irrespective of age
7. Registration with The Over-volunteering Prevention System (TOPS) database
8. General Practioner assessment of study eligibility, based upon a copy of the eligibility criteria and their knowledge and records of the potential volunteer.

Table 5-6. Summary of investigations used to determine the suitability of potential volunteers consented to enter RSV001. *Formal assessment of study eligibility was performed after written informed consent was obtained. The TOPS database was setup for phase I trials to guard against the potential for harm arising from excessive volunteering for clinical trials*[417].

5.4.3 Study group allocation

The schedule of attendances for each study group was set in advance and allowed for matching of volunteer availability to specific study groups. Eligible volunteers were assigned to study groups by sequential allocation based upon their availability for scheduled visits and not by randomisation or choice.

5.4.4 Enrolment

A volunteer was considered enrolled at the point of receiving the prime vaccine. For Group 5 volunteers, who did not receive any vaccine, enrolment occurred on sample collection at the first visit (V1). Replacements were permitted if a volunteer was withdrawn after enrolment. In both years the rate of enrolment was predetermined for safety oversight and so as not to overwhelm resources as the trial expanded. The time taken from physician screening to enrolment was recorded for reporting.

5.5 Vaccination procedures

The experimental vaccines were administered according to OVG SOPs and a trial-specific clinical study plan (CSP). PanAd3-RSV and MVA-RSV were used in accordance with GMO (Contained Use) Regulations 2000[418].

5.5.1 PanAd3-RSV and MVA-RSV by intra-muscular injection

0.5mls of vaccine product was injected into the non-dominant deltoid muscle using a 23-gauge 25mm needle and syringe. The 'Z' technique was used to minimise any leakage from the needle track and the site of inoculation was covered with a dressing after immunization. This absorbed any GMO virus that may have leaked through the needle track and the dressing was removed after 30 minutes and disposed as GMO waste by autoclaving in accordance with relevant SOPs.

5.5.2 PanAd3-RSV by intra-nasal spray

0.15mls of PanAd3-RSV was sprayed into nostril in the sitting position using a syringe attached to an LMA MAD Nasal™ needle-free delivery system (LMA, San Diego). Each volunteer remained in the clinic room for 30-minutes after dosing to contain any vaccine expelled by sneezing. The LMA MAD Nasal™ needle-free delivery system was disposed as GMO waste by autoclaving in accordance with the relevant SOPs.

5.5.3 Temporary exclusion criteria for vaccination

Prime or boost vaccination was deferred if any of the following temporary exclusion criteria applied;

- Presence of a low-grade febrile illness (oral temperature ≥37.5°C)
- Any recent moderate or severe illness irrespective of temperature (such as diarrhoea or a mild upper respiratory infection)
- A laboratory adverse event that required time and/or investigation to resolve or stabilise prior to another dose of vaccine being administered
- Receipt of a live vaccine within 4 weeks or killed vaccine within 7-days prior to vaccination

5.6 Data collection

The schedule of research site attendances, study procedures and data collection for both primary and secondary endpoint measures are set out in Table 5-7. The number of scheduled attendances for each volunteer depended upon the study group;

- Volunteers in study groups 1, 3 and 4 were invited to attend a total of 12 visits over 34 weeks
- Volunteers in study group 2 were invited to attend a total of 11 visits over 30 weeks.
- Volunteers in study groups 5, 8 and 9 were invited to attend 13 visits over 52 weeks
- Volunteers in study groups 6 and 7 were invited to attend 12 visits over 52 weeks

Volunteers who reported an influenza-like illness at any time during the study were invited to attend additional 'unscheduled visit(s)' for the detection of RSV or other viral infection by PCR on nasal swabs. A maximum of four unscheduled visits per volunteer were permitted. Nasal samples were tested for the presence of influenza A/B, rhinovirus, coronaviruses, parainfluenza 1/2/3, adenovirus, human metapneumovirus, RSV, enterovirus, parechovirus, bocavirus and *Mycoplasma pneumoniae*.

Visit number	Days after vaccination (±window, days)	Vaccine continued eligibility	Solicited adverse events	Unsolicited adverse events	Visit observations	Nasal sample for RSV detection	Nasal sample for PanAd3-RSV detection*	Nasal sample for anti-F antibody	Salivary sample for anti-F antibody	Safety bloods (mLs)	Serum anti-PanAd3 antibody (blood, mLs)	Serum anti-F antibody (blood, mLs)	B-cell responses (blood, mLs)	T-cell responses (blood, mLs)	RNA for gene expression analysis*** (blood, mLs)
V1 (P)	n/a	◆	◆	◆	◆			◆	◆	5 5	5 5	10 7	30 25	60 25	3 3
V2	3 ±1		◆	◆	◆		◆			5 5					3 3
V3	7 ±2		◆	◆	◆					5 5			30 25		3 3
V4	14 ±3			◆	◆			◆	◆	5 5		10 7		60 50	
V5	28 ±4			◆	◆			◆	◆	5 5		10 7		60 50	3 3
V6 (B)	n/a	◆	◆	◆	◆			◆	◆	5 5	5 5	10 7	30 25	60 25	3 3
V7	3 ±1		◆	◆	◆					5 5					3 3
V8	7 ±2		◆	◆	◆					5 5			30 25	60 25	3 3
V9	14 ±3			◆	◆			◆	◆	5 5		10 7		60 50	
V10	28 ±4			◆	◆			◆	◆	5 5	5** 5**	10 7	30 25	60 25	3 3
V11	70 ±7			◆	◆			◆	◆	5 5		10 7	30 25	80 25	
V12	182 ±12			◆	◆					5 5		10 7		60 50	
V13 ****	332 ±28			◆	◆					5 5		n/a 7	n/a 25	n/a 25	
UN	Any			◆	◆	◆						10 7			

Table 5-7. Full table of procedures and samples collected for all volunteers in RSV001.
*Each volunteer also attended a screening visit (V0, not shown) before enrolment into the study and received prime vaccine (P) at V1 and boost vaccine (B) at V6. The prime/boost interval was group dependent. Study groups 2 and 7 did not have a V5 and group 6 did not have a V7. Vaccine continued eligibility tests included an interim medical history (see temporary exclusion criteria) and urine pregnancy testing. * Nasal samples for PanAd3-RSV shedding was performed only in volunteers who received IN PanAd3-RSV prime (groups 3, 4 and 8). ** Serum anti-PanAd3 antibody samples at V10 were obtained from volunteers who received IM PanAd3-RSV vaccine at prime and boost (groups 2 and 7). *** No RNA samples were taken from the single-dose MVA group (group 6) from V6 onwards. ****There was no V13 time-point for adults aged 18-50 years (study groups 1-4). For each blood sample, the top number was the volume obtained from adults aged 18-50 years (study groups 1-4); the bottom number was the volume obtained from adults aged 60-75 years (study groups 5-9). In addition to scheduled attendances, each volunteer was allowed a maximum of 4 unscheduled visits (UN1, UN2, UN3 and UN4) for reasons of safety or influenza-like illnesses and the detection of RSV infection. Not applicable, n/a. ◆ denotes collection of these data at these visits.*

5.6.1 Clinical safety data collection

The clinical data collected for the assessment of safety and tolerability of each vaccine used three principal methods;

1. Symptoms within one week of vaccination were entered directly by the volunteers themselves each day after vaccination onto a University of Oxford server hosted, password-protected, purpose built, electronic diary (eDiary version 1.0) using encrypted transfer. Volunteers were provided with an oral thermometer (Omron Eco Temp basic thermometer) and transparent measuring tape for injection site measurements, with written instructions on how to recorded these data.
2. Nurse recorded visit observations were entered directly onto OpenClinica™ Community Edition (version 3.1.3 – beta), hosted on a University of Oxford server with password-protection and encrypted transfer.
3. Blood results for haematology and biochemistry were reported through the Oxford University Hospitals NHS Trust CaseNotes system (version 5), hosted on OUHL servers with password protection and encrypted transfer.

The data from each of these reporting systems was collated onto a single purpose built Microsoft Excel workbook (Excel for Mac 2011 version 14.5.4) for on-demand safety assessment and weekly reporting of all available data to the DSMC and Sponsor.

5.6.2 Immunogenicity data collection

The collection and storage of nasal, salivary and blood samples for the analysis of immune responses to vaccination were detailed earlier. Samples were collected according to the schedule detailed in Table 5-7.

5.6.3 BioBank

DNA and/or serum were, with specific separate volunteer consent, transferred to the Oxford Vaccine Group BioBank for future sero-epidemiological and other vaccine related research (ethics reference LREC 10/H0504/25).

5.7 Volunteer reimbursement

Study volunteers were compensated for their time (£20/visit), travel costs (£15/visit) and the inconvenience of procedures (£10/blood or nasal sample). The tariffs were generated using guidance from NHS and Royal College of Physicians London publications[419-421] and were approved by NRES Berkshire ethics committee. Four extra visits (designated unscheduled visits) were included in the total remuneration for each volunteer regardless of whether they were used to remove any incentive to report an influenza-like illness during the study. Volunteers in study groups 2, 6 and 7 were compensated the same as all other participants despite having one less scheduled visit. Remuneration was on a *pro rata* basis if a volunteer failed to complete all scheduled visits and/or study requirements. The maximum compensation for volunteers in study groups 1-4 and study groups 5-9 was £845 and £920 respectively.

5.8 Statistics and data analysis

Analyses were intended to be descriptive in nature; the purpose of the study was to characterise the safety and immunogenicity of PanAd3-RSV and MVA-RSV in healthy adults. There were no pre-specified hypotheses on which to power the study and no formal sample size calculations were performed. The sample sizes were considered standard to assess phase 1 stage product safety and tolerability.

All clinical data were collated and interpreted un-blinded to study group. Laboratory samples were analysed blinded to study group, and were un-blinded after completion of the assay. There were no imputations for missing data and analyses were performed on data locked after monitoring and quality assurance assessment. Trial reporting adhered to a trial-specific statistical analysis plan (SAP), written before any analyses commenced by the Centre for Statistics in Medicine (CSM), University of Oxford, and the lead trial physician. In accordance with the SAP and clinical trial protocol, descriptive interim study reports for study groups 1-4 and 5-9 were generated to examine safety and select immunogenicity data one month after the last dose of vaccine in each age group.

Where comparative statistics have been applied these represent post-hoc analyses in excess of the objectives specified in the pre-specified SAP. A p-value less than 0.05 was considered statistically significant

and tests that were applied to the data are annotated in the text. Normality of data were assessed by histogram plot or by formal testing (using the D'Augustino-Pearson omnibus normality test and Shapiro-Wilk test) to guide appropriate formal statistical test selection.

5.8.1 Study populations for analysis

Adverse events and immune responses occur transiently and within predictable windows after vaccination. Therefore, timely attendance to scheduled appointments represented an important factor in accurately addressing the safety and immunogenicity of the vaccines. Protocol-defined windows around scheduled visits, specified in Table 5-7, were used to identify a per-protocol (PP) study population separate from the intention-to-treat (ITT) population (all volunteers, all data).

5.8.2 Software

Graphs, descriptive statistics and comparative analyses for this chapter were generated using GraphPad Prism for Mac versions 6.0 and 7.0 (GraphPad Software, USA). Logistic regression analysis was performed using STATA version 11.2 for Windows (StataCorp, USA).

5.9 Analysis of recruitment methods

Initial application for clinical trial authorisation was submitted to the MHRA in December 2012. Final regulatory approvals (NHS site approvals) were granted by April 2013 (see Table 5-8). Recruitment commenced the following week and within the 60-day period expected of the NIHR.

Regulatory body	Approval date
Medicines and Healthcare Products Regulatory Agency (MHRA)	07/01/2013
National Research Ethics Service (NRES Berkshire)	08/03/2013
University of Oxford Genetic Modification Safety Committee (GSMC)	21/01/2013
Local site NHS R&D approvals (Oxford University Hospitals NHS Trust)	23/04/2013

Table 5-8. Pre-trial regulatory approvals.
Each application was accepted as valid on the initial submission and was granted approval without the requirement for significant modification or amendment.

5.9.1 How volunteers heard about the study

A total of 36'297 posted study information leaflets, several posters (number unrecorded), three exhibitions and advertising through the OVG website and OVC database of healthy volunteers were used in the spring and early summer of 2013 and 2014 to enrol 72 eligible volunteers into the trial (Table 5-9). Mailed recruitment material and the study website were the most common sources that lead to registering an interest in taking part in the trial. No record was made in 2013 of how potential volunteers had heard about the study before physician screening. The proportion of potential volunteers engaged by mailed material was similar at physician screening and at enrolment in both years. For younger adults in 2013, mailed material accounted for 44/101 (44%) of potential volunteers at physician screening and 18/42 (43%) at enrolment. For older adults recruited in 2014, this proportion was greater with 63/79 (80%) at physician screening and 24/30 (80%) at enrolment. At the time of initial telephone contact, mailed material accounted for 746/840 (89%) of expressions of interest in older adults (Figure 5-5).

Recruitment method	2013: Adults 18-50yrs (target n=40)	2014: Adults 60-75yrs (target n=30)
Mailing the study information leaflet via electoral roll	Total of 20'934 letters to OX3 and OX4 postcode prefix areas	Total of 10'073 letters to 20 postcode prefix areas
Mailing the study information leaflet via Open Exeter	n/a	Total of 5'290 letters to 2 postcode prefix areas
OVC database of potentially healthy adult volunteers	April and July 2013 electronic newsletters	July 2014 electronic newsletter
Presentations at exhibitions and meetings	Oxford Brookes University Fresher's Fair, University of Oxford Fresher's Fair	University of the Third Age meeting
Poster advertising	Yes	Yes
OVG website advertising	Yes	Yes
Email list distribution of information booklet	Yes	No

Table 5-9. The relative use of recruitment materials and methods for RSV001 in 2013 and 2014.
Open Exeter was not available (n/a) in 2013 for the recruitment of younger adults. No records were made of the number of website hits and email recipients of recruitment material. Oxford Vaccine Centre (OVC). Oxford Vaccine Group (OVG).

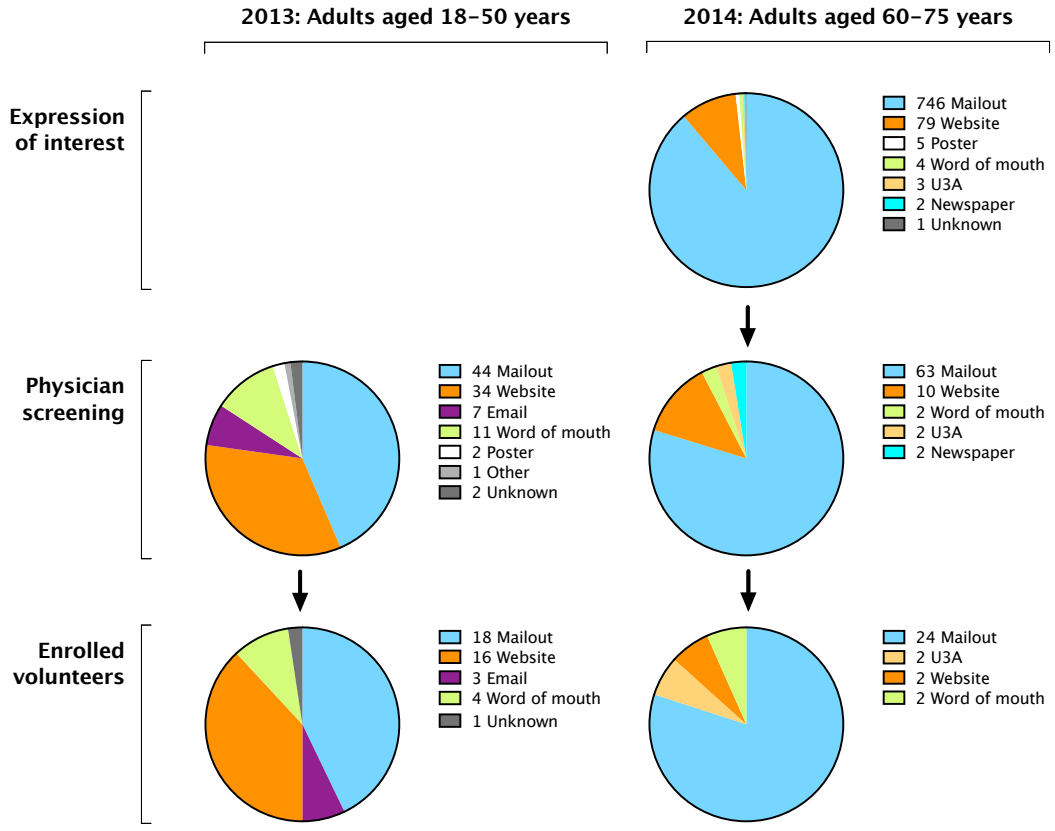


Figure 5-5. Pie-chart proportionate representation of how volunteers heard about the RSV001 clinical trial. *At the point of telephone contact (**top row**), physician screening (**middle row**) and at enrolment (**bottom row**). Overall the majority of expressions of interest were received from the mailing out of recruitment material and the study (OVG) website. No record was retained of how younger adults heard about the study until physician screening. University of the Third Age, U3A.*

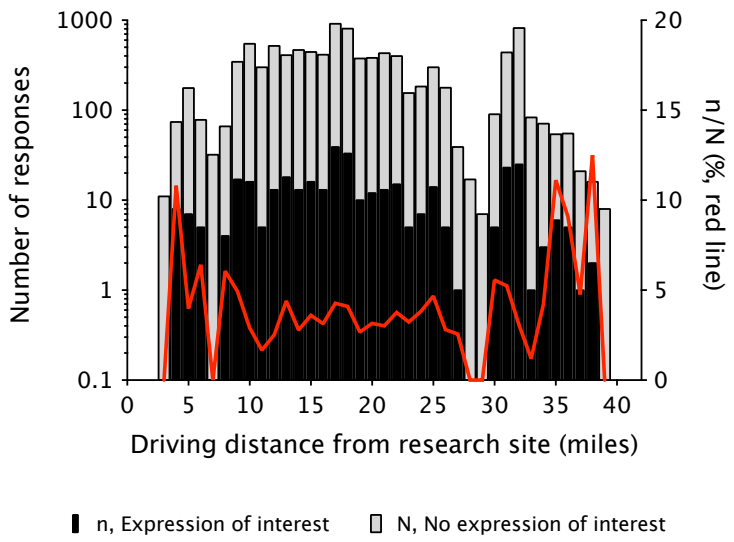
5.9.2 *Analysis of mailed recruitment material and recruitment to the trial*

In 2013, targeting younger adults in two postcodes prefix areas close to the CCVTM research site, a ratio of 523 study information leaflets was mailed for every volunteer of the target population of 40 adults. This ratio was marginally lower in 2014, with 512 study information leaflets per required volunteer aged 60-75 years, mailed across 20 postcodes prefix areas up to 31 miles from the research site. In 2013, the expression of interest rate from younger adults was low, estimated 1.2%, compared with 4.5% from older adults in 2014 (see Table 5-10). The estimated total cost of consumables for recruitment by mail was £23'282.10 in 2013 and £11'129.47 in 2014. This represented a cost of £1293.45 and £463.73 per younger and older enrolled study volunteer respectively who reported hearing about the study by mail. The total consumable cost for the 30/72 (42%) enrolled volunteers recruited in both years by other means was not recorded, but was considerably less expensive.

The time taken to receive expressions of interest to mailed recruitment material, and the distance from each expression of interest from the research site, was used to further explore responses to posted recruitment material in older adults and the electoral roll. 10'073 names and addresses were acquired for recruitment from the electoral roll in 2014 using the postcode prefix for area selection. Full postcode records for 9720 (96%) of these were available for analysis. 7535/9720 were unique postcodes and implied nearly a quarter (23%) of names identified from electoral roll resided in the same household as another member of the public contacted by mail. Interested members of the public responded quickly. The median interval from posting recruitment material to receiving an expression of interest in 2014 was 8 days (interquartile range 6-15 days) for adults aged 60-75 years, with little variation between postcodes prefix areas (Figure 5-6). The full postcode was available for 686/740 (92%) expressions of interest, irrespective of how volunteers had heard of the trial, and logistic regression analysis. In this analysis there was no effect of increasing distance from the research site on the odds of receiving an expression of interest, with some older adult volunteers willing to travel a 75-mile round trip for up to 15 appointments over 12-months to take part in the trial.

Postcode prefix area used to acquire names and addresses	Distance from postcode prefix to the research site (miles)	Number of expressions of interest received/number of letters sent (%)	Median number of days after posting (inter-quartile range) [number recorded]
Adults aged 18-50 years (recruitment 2013):			
OX4 (via electoral roll register)	2.9	Not recorded by postcode	Not recorded
OX5 (via electoral roll register)	10.5	Not recorded by postcode	Not recorded
All postcodes (adults aged 18-50yrs)	**-**	**256/20934 (1.2%)**	**-**
Adults aged 60-75 years (recruitment 2014):			
OX3 (via Open Exeter)	4.0	175/3157 (5.5)	8 (6 – 15) [174]
OX5 (via Open Exeter)	10.5	133/2133 (6.2)	8 (7 – 15) [131]
OX9 (via electoral roll register)	12.9	22/429 (5.1)	7 (6.5 – 17) [22]
OX10 (via electoral roll register)	14.2	9/725 (1.2)	7 (5.5 – 10) [9]
OX11 (via electoral roll register)	18.6	28/832 (3.4)	8.5 (6 – 14) [28]
OX12 (via electoral roll register)	21.5	26/820 (3.2)	8 (7 – 11) [26]
OX13 (via electoral roll register)	12.7	26/418 (6.2)	7.5 (4.8 – 12) [26]
OX14 (via electoral roll register)	12.1	40/1293 (3.1)	9 (7 – 16) [40]
OX15 (via electoral roll register)	36.0	21/587 (3.6)	7 (6 – 15) [21]
OX16 (via electoral roll register)	30.8	52/1336 (3.9)	12 (8.3 – 18) [52]
OX17 (via electoral roll register)	29.4	35/624 (5.6)	8 (5 – 19) [35]
OX18 (via electoral roll register)	21.0	23/681 (3.4)	11 (10 – 17) [23]
OX20 (via electoral roll register)	12.7	5/199 (2.5)	10 (7 – 13) [5]
OX25 (via electoral roll register)	17.4	8/242 (3.3)	10 (6 – 14) [8]
OX26 (via electoral roll register)	15.6	32/743 (4.3)	7 (6 – 10) [32]
OX27 (via electoral roll register)	18.8	4/160 (2.5)	8.5 (8 – 9) [4]
OX33 (via electoral roll register)	5.7	11/259 (4.2)	8 (6 – 23) [11]
OX39 (via electoral roll register)	17.2	9/263 (3.4)	6 (4.5 – 26) [9]
OX44 (via electoral roll register)	6.5	21/321 (6.5)	8 (6 – 15) [21]
OX49 (via electoral roll register)	16.8	6/141 (4.3)	11 (8 – 16) [6]
All postcodes (adults aged 60-75yrs)	**-**	**686/15363 (4.5%)**	**8 (6 – 15) [687]**

Table 5-10. Results for the response to mailed recruitment material posted to different postcode prefix areas in 2013 and 2014.
Open Exeter was used for two postcodes prefix areas in 2014, and all other mailed material used the electoral roll for names and addresses



Distance from research site (miles)	Expressions of interest rate (95% CI)
0 – 4	0.057 (0.03-0.09)
5 – 9	0.039 (0.03- 0.05)
10 – 14	0.030 (0.02-0.04)
15 – 19	0.037 (0.03-0.04)
20 – 24	0.037 (0.01-0.03)
25 – 29	0.030 (0.01-0.05)
30 – 34	0.040 (0.03-0.05)
35 – 38	0.080 (0.03-0.13)

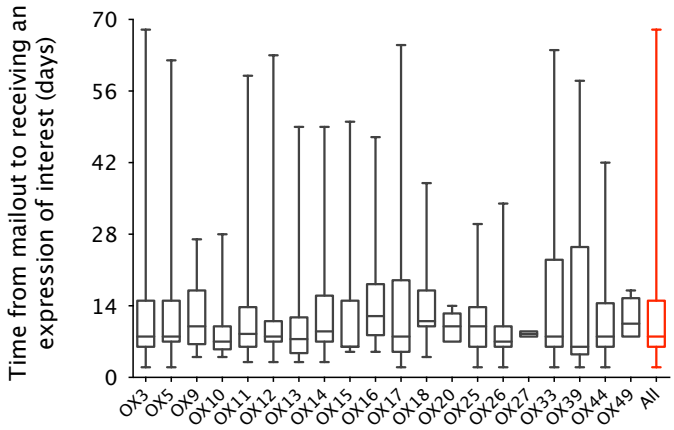


Figure 5-6. The effect of distance from the research site and the response to posted recruitment material. *Data from the recruitment of older adults aged 60-75 years via the electoral roll and mailed material in 2014 only. (**Top left and table**) The distance from the research site appeared to have no significant effect on the rate of favourable expressions of interest. The red line denotes the expression rate. Data table is courtesy of Charles Sande and is reproduced with permission. (**Bottom figure**) Box and whiskers plot of the time taken to receive an expression of interest from each post code prefix area mailed to. Whiskers represent the full range, the box the interquartile range, and the bar is the median. There was no significant variation across postcode prefix areas (p=0.210, Kruskal-Wallis ANOVA).*

5.10 Analysis of eligibility assessment and reasons for exclusion

5.10.1 Physician screening

The rate of physician screening for both years of recruitment are shown below. The greater response rate to recruitment material in older adults supported an accelerated screening rate in 2014, compared with younger adults in 2013. Physician screening stopped when the accrual of fully assessed and eligible volunteers reached the target number with a 10-20% excess to allow for the replacement of withdrawals after enrolment in later weeks. Recruitment formally closed after enrolment of the last volunteer and a total of 17-weeks of physician screening was performed in 2013 for younger adults, compared with only 6-weeks in 2014 for older adults. Dose and groups size escalation rules applied to younger adults in 2013, described earlier, meant the interval from physician screening to enrolment was longer in 2013 for several volunteers who needed to wait to enter the trial. In 2013, the median time from screening to enrolment was 32-days (IQR 19-54 days) and in 2014 it was 26-days (IQR 21-34 days). GPs returned a prompt response in support of eligibility assessment, with a median time of 10-days and 49/71 (69%) of GP paperwork being completed within 7 calendar days of the physician screening appointment.

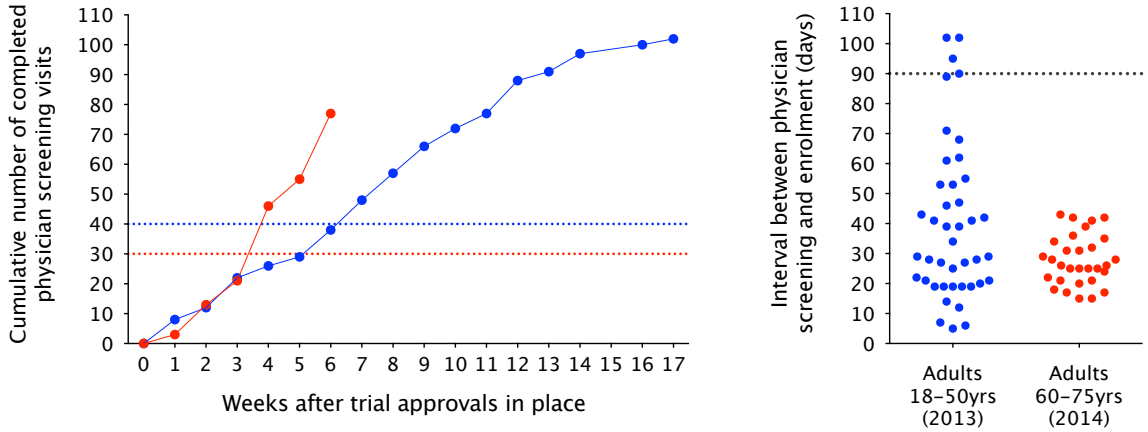


Figure 5-7. The cumulative number of physician screening assessments and time taken from screening to enrolment in younger adults in 2013 and older adults in 2014.
*(**Left figure**) The cumulative rate of physician screening. The blue and red dotted lines represent the target number for enrolment in younger and older adults respectively. (**Right figure**) Scatter plot illustration of the interval period between physician screening and enrolment into the trial. The dotted line denotes the protocol defined time cut of for needing repeated blood measures of eligibility before enrolment.*

5.10.2 Exclusions before physician screening

A total of 1598 expressions of interest were contacted by phone and 180 were invited for physician screening to ultimately enrol 72 suitable volunteers into the RSV001 trial. In both years a proportion of potential volunteers declined to participate in the trial at initial contact; 118/374 (32%) of younger adults in 2013 and 79/1224 (6%) of older adults in 2014. A further 155/374 (41%) of younger adults in 2013 and 640/1224 (52%) of older adults in 2014 were identified as ineligible at the point of initial telephone contact or shortly after using information freely given before consent. As a result, in 2013 for adults aged 18-50 years, only about a third of all initial expressions of interest for the study were invited for physician screening. In 2014, for adults aged 60-75 years, this proportion was smaller (6.6%). The high response rate to recruitment material in 2014 contributed to a large excess of responses (approximately 50%) needing to be excluded because recruitment completed within 6-weeks. Health concerns made up another third of the reasons for exclusion in this older population (see Figure 5-8).

5.10.3 Exclusions after physician screening

Initial telephone contact, which was dependent on information freely volunteered, resulted in physician screening populations that that were similar in respect of the proportion of subsequent exclusions in both years. Information volunteered or obtained from physician screening resulted in approximately two thirds of volunteers being determined as ineligible for the study. Health concerns were the main reason for exclusion; 32/59 (54%) and 22/49 (45%) in younger and older adults respectively. Medically important information detected at physician screening was communicated to the potential volunteers GP, with permission, for further management. The details of medical exclusions are reported later. Potentially preventable physician screening appointments, based on the content of the study information booklet and in the opinion of the investigators expectations of general public understanding of their own current/past medical history, numbered 11/59 (17%) and 9/49 (18%) of exclusions after physician screening respectively for younger (in 2013) and older adult (in 2014) recruitment respectively (see Figure 5-8).

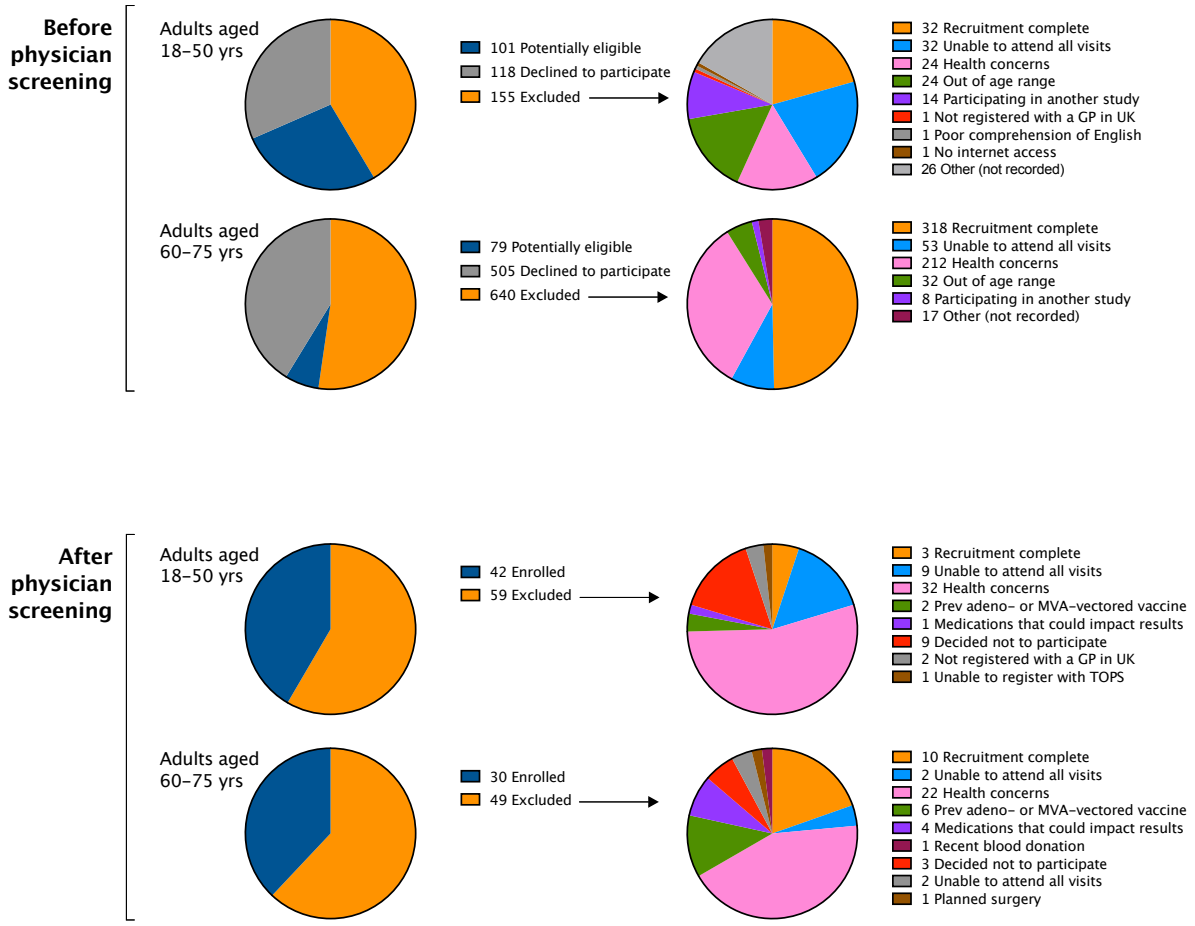


Figure 5-8. Pie chart frequency plots detailing the number and proportions of exclusions by category during the screening process for eligibility assessment.
(***Top charts***) *Exclusions before physician screening used information freely offered by potential volunteers at the point of initial telephone contact with the study team, or potential volunteers declining to participate after further consideration of the information provided, or recruitment was complete.* (***Bottom charts***) *Exclusions at or after physician screening were most commonly related to the medical health history of potential volunteers.*

5.10.4 Haemoglobin changes between physician screening and enrolment

Of potential clinical significance, given the planned volume of blood sampling, was the detection of 5 females in the younger cohort whose haemoglobin levels at enrolment were below the minimum threshold applied for blood donation by NHS Blood Transfusion (NHSBT) service. Although NHSBT guidelines were not used in the formal assessment of volunteer eligibility, they had been used to justify total blood volumes to volunteers in application for regulatory approval. All haemoglobin measures at screening were above this threshold. No significant changes in haemoglobin levels were observed in the period between physician screening and enrolment, although haemoglobin levels in males aged 60-75 years were significantly lower (statistically) at enrolment compared with the younger male cohort (Figure 5-9). There were no significant correlations between the changes in haemoglobin levels from physician screening to enrolment with the interval between the two points of sampling (data not shown).

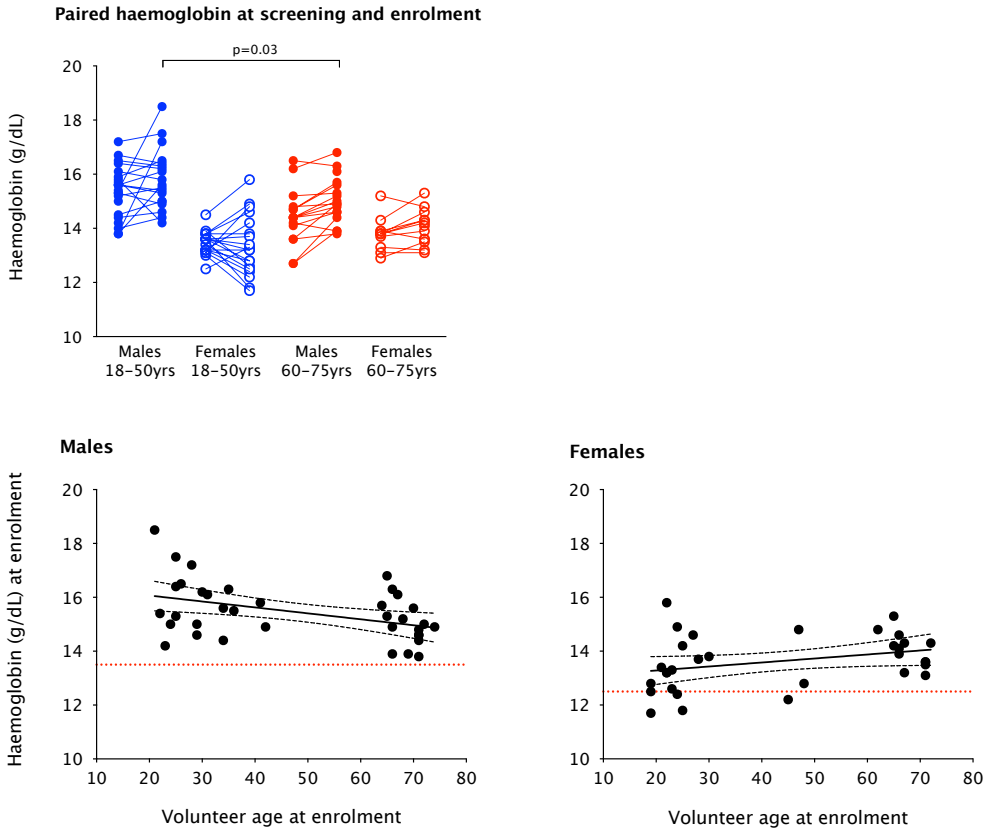


Figure 5-9. Haemoglobin values obtained at physician screening and enrolment.
(**Top**) *Paired haemoglobin measures from physician screening (left) and at the point of enrolment (right). The only statistically significant difference was between younger and older males (p=0.03, two-tailed unpaired t-test).* (**Bottom**) *Liner regression analysis of haemoglobin at enrolment with age, with 95% confidence intervals (dotted lines). A significant negative trend for males was observed that was not apparent in females (two-tailed Pearson correlations; males r^2=0.18, p=0.009 and females r^2=0.10, p=0.073). The red line denotes the threshold from NHS Blood Transfusion guidelines when blood donation would be deferred.*

5.11 Description of the enrolled study population and CONSORT reporting

The baseline physical and demographic characteristics of the enrolled study population are summarised in Figure 5-10 and Table 5-11. There were no statistically significant differences in volunteer age (within study groups 1-4 and 5-9), body mass index, smoking and alcohol consumption (one-way ANOVA, $p>0.05$ for each measure). Overall 40/72 (56%) volunteers were male and the ethnic mix reflected the local population and was dominated by white Caucasians.

Although RSV001 was not a randomised controlled trial the Consolidated Standards of Reporting Trials (CONSORT) guidelines were adopted[422,423]. The flow diagrams detail the medical reasons for exclusion after physician screening and show the retention of volunteers for later analysis of the trial objectives (Figure 5-11 and Figure 5-12). Two volunteers elected to withdraw from the trial after receiving the prime vaccine and were replaced per protocol, meaning a total of 72 healthy adults were enrolled into the clinical trial. The reasons for withdrawal were unrelated to the vaccine and could not have been anticipated at physician screening; one withdrawal was due to personal circumstances and the other after a change of employment. One volunteer from group 5 was withdrawn following a diagnosis of terminal oesophageal cancer and was not replaced due to the late stage of the withdrawal and only the final visit pending.

	Healthy adults aged 18-50 years				Healthy adults aged 60-75 years				
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**	**Group 7**	**Group 8**	**Group 9**
Number of volunteers:									
N volunteers	11	10	10	11	6	6	6	6	6
Sex:									
Male (%)	5 (45)	5 (50)	6 (60)	7 (64)	3 (50)	4 (68)	3 (50)	3 (50)	4 (68)
Age (years):									
Median age (range)	24.0 (19-42)	27.5 (22-48)	28.0 (19-41)	27.0 (19-48)	68.0 (62-71)	66.5 (66-71)	68.0 (64-74)	71.0 (65-72)	67.5 (65-72)
Body mass index (BMI, kg/m^2):									
Median BMI (range)	21.6 (19-32)	23.0 (22-37)	26.1 (18-29)	24.0 (20-32)	27.5 (24-34)	27.6 (23-31)	25.9 (24-38)	25.5 (21-31)	27.2 (22-33)
Alcohol consumption (units/week):									
Median/week (range)	4.0 (0-22)	5.0 (0-20)	8.5 (0-14)	4.0 (0-20)	7.0 (4-18)	13.0 (0-20)	1.8 (0-20)	7.0 (0-10)	1.5 (0-30)
Current smoking (cigarettes/week):									
Median/week (range)	0 (0-5)	0 (0-2)	0 (0-5)	0 (0-2)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-6)
Employment status, n (%)									
Full time	4 (36)	3 (30)	7 (70)	8 (73)	3 (50)	1 (17)	3 (50)	0 (0)	3 (50)
Part time	0 (0)	1 (10)	0 (0)	1 (9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Student	7 (64)	5 (50)	3 (30)	2 (18)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Unemployed	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Retired	0 (0)	0 (0)	0 (0)	0 (0)	3 (50)	4 (67)	3 (50)	6 (100)	3 (50)
Self-reported ethnicity, n (%):									
White British	8 (73)	6 (60)	7 (70)	7 (64)	6 (100)	6 (100)	6 (100)	6 (100)	5 (83)
White other	0 (0)	1 (10)	2 (20)	2 (18)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
White Irish	0 (0)	1 (10)	0 (0)	1 (9)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)
Mixed	1 (9)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pakistani	1 (9)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Indian	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Black other	0 (0)	0 (0)	0 (0)	1 (9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Chinese	1 (9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 5-11. The baseline physical and social characteristics of volunteers enrolled into the RSV001 clinical trial.

Younger adults aged 18-50 years (study groups 1-4) were recruited and enrolled in 2013, and older adults aged 60-75 years (study groups 5-9) were recruited and enrolled in 2014.

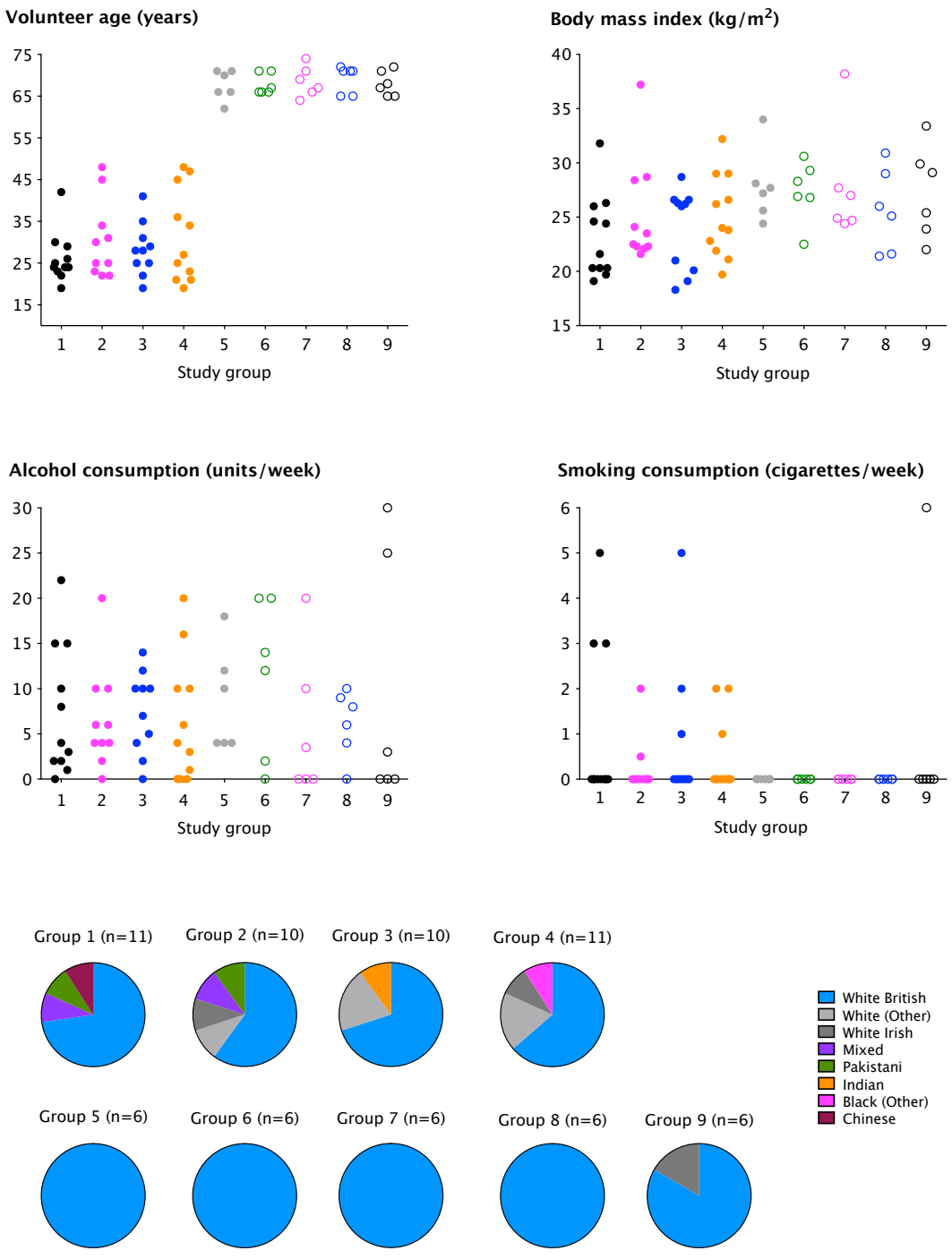


Figure 5-10. The baseline physical and demographic characteristics of volunteers enrolled into the RSV001 clinical trial.
*Illustrated by study group. (**Top left**) The volunteer age at enrolment. (**Top tight**) The recorded body mass index (BMI) of each volunteer at enrolment. (**Middle left and right**) The declared alcohol and cigarette weekly consumption of enrolled volunteers respectively. (**Bottom figure**) The self-reported ethnicity by frequency pie-chart for each study group.*

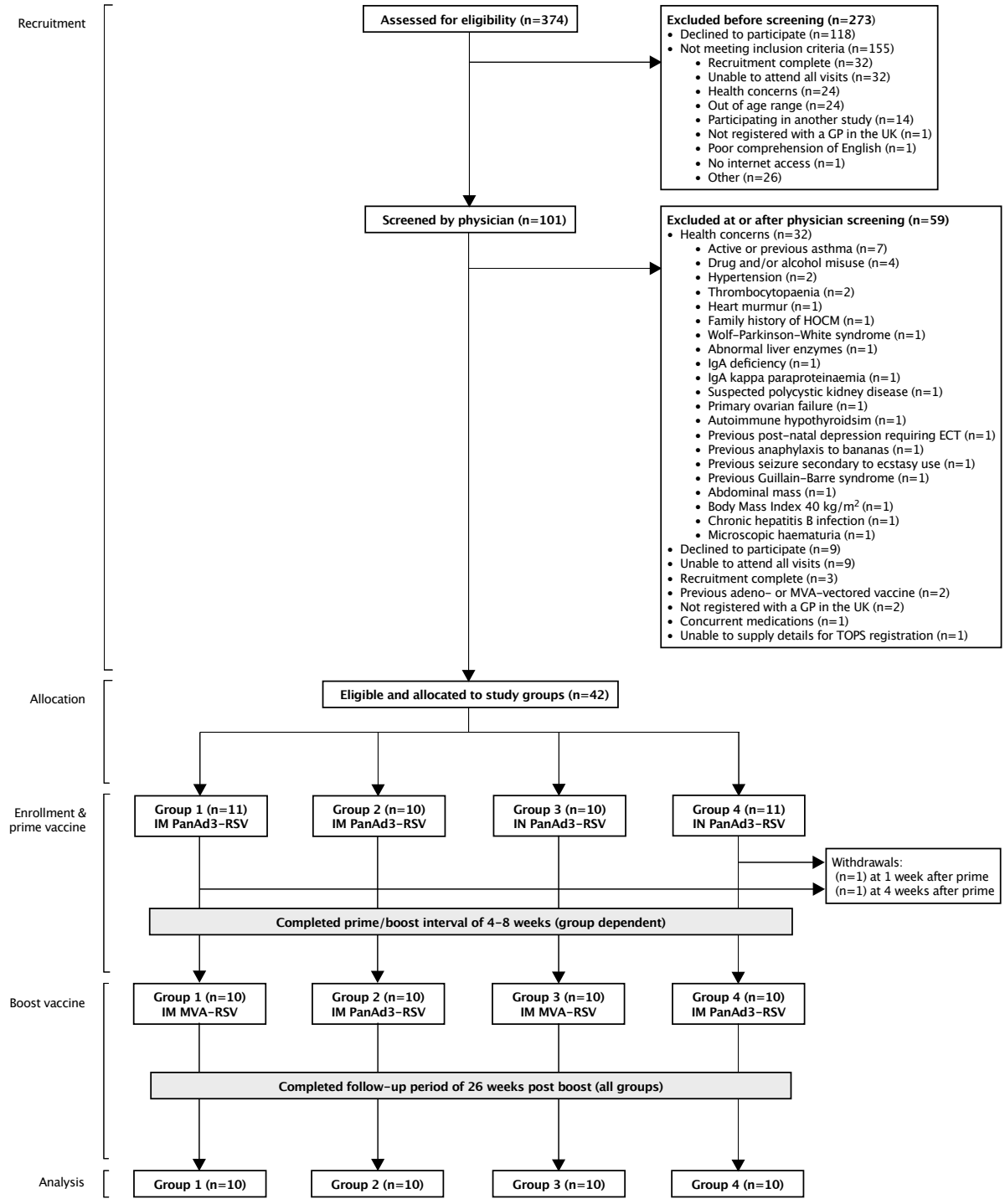


Figure 5-11. CONSORT flow diagram for the recruitment and completion of the RSV001 clinical trial in healthy adults aged 18-50 years in 2013/14.

The two withdrawals were not related to the vaccine, and withdrawn volunteers were replaced. The medical reasons for exclusion are detailed here. Hypertrophic obstructive cardiomyopathy (HOCM), electro-convulsive therapy (ECT), The Over-volunteering Prevention System (TOPS).

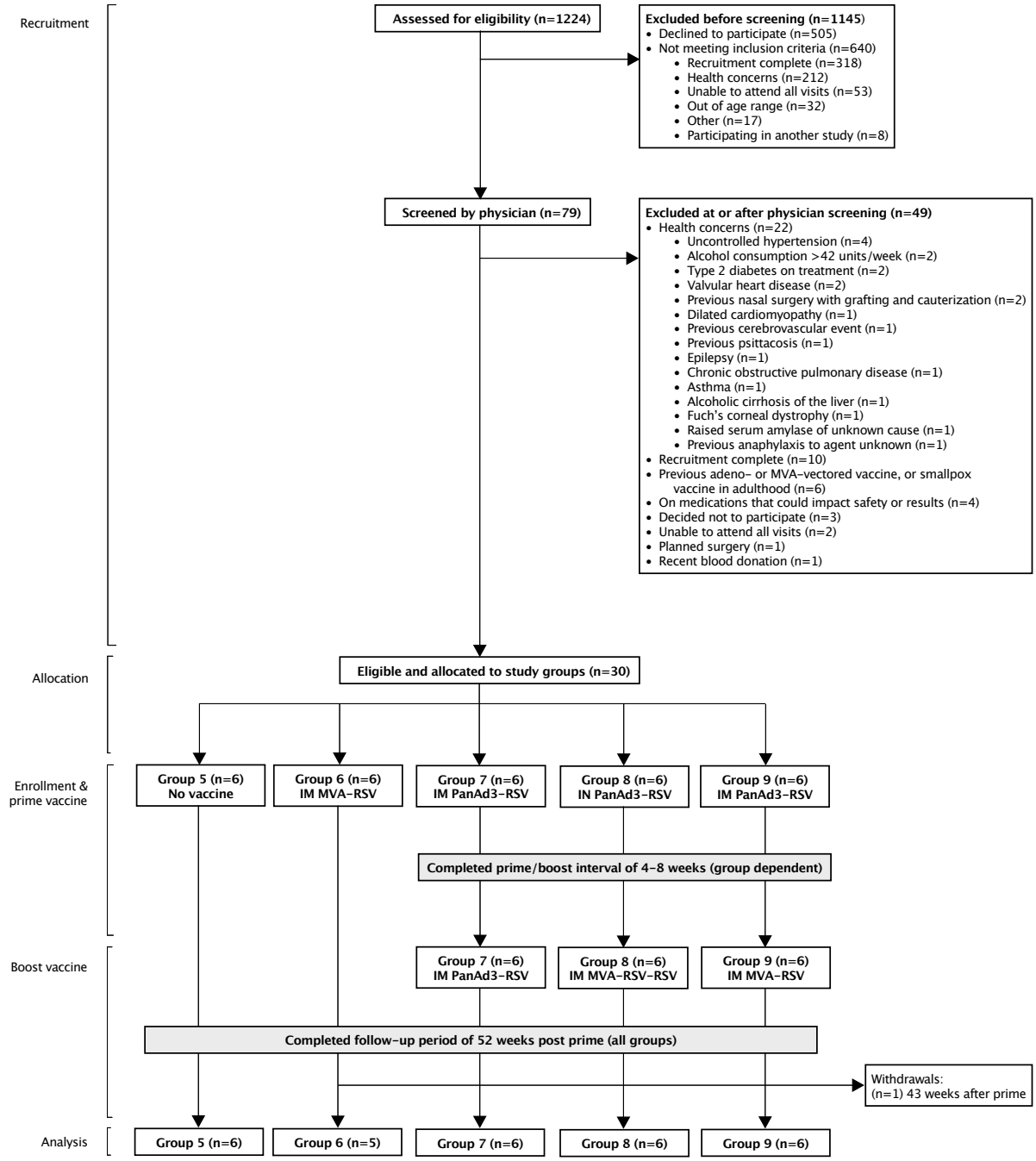


Figure 5-12. CONSORT flow diagram for the recruitment and completion of the RSV001 clinical trial in healthy adults aged 60-75 years in 2014/15.

One volunteer from group 6 was withdrawn after being diagnosed with terminal oesophageal cancer before the final visit.

5.12 Analysis of trial execution

5.12.1 *Vaccination and sample collection in relation to periods of background RSV transmission*

Public Health England (PHE) reported surveillance of confirmed RSV infections in all ages between 1999/2000 and 2012/13 seasons report a consistent pattern of winter epidemics of disease[43]. Each year confirmed RSV-infections appeared to rise from calendar week 40, peak between weeks 45 and 52, and then decline to baseline by week 14 the following year. All prime vaccines were administered in summer months as planned. All boost vaccines, with the exception of the two replacements for withdrawn volunteers in 2013, were vaccinated with more than 4 weeks remaining before the likely peak period of background RSV transmission (Figure 5-13).

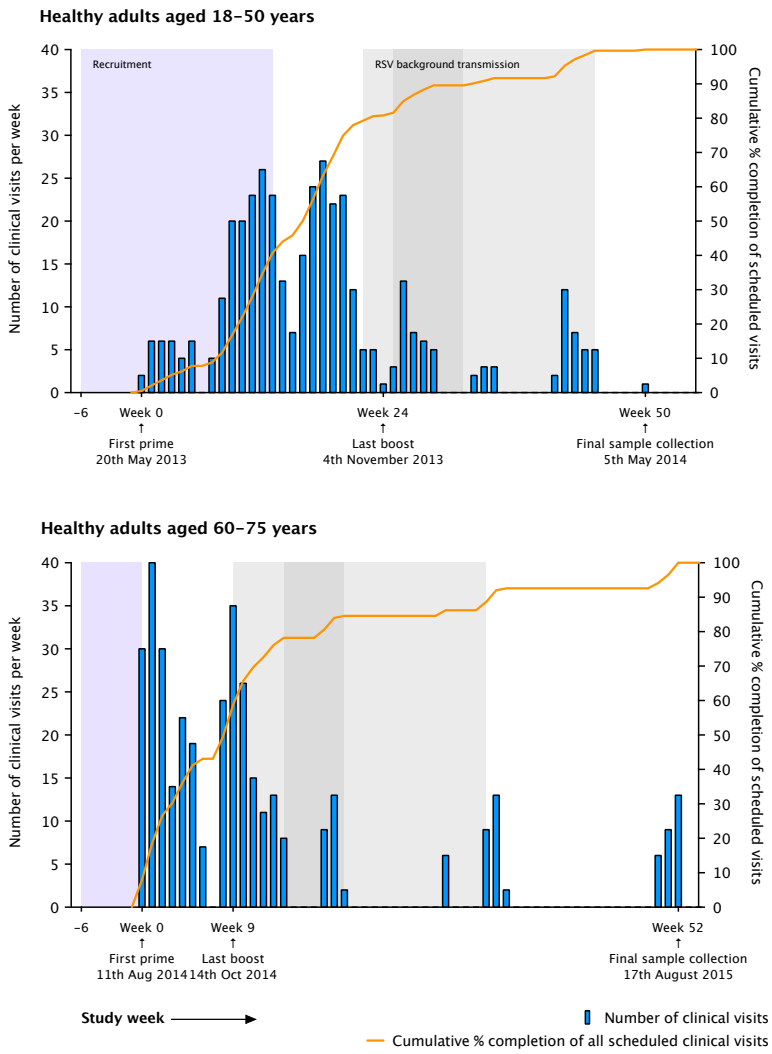


Figure 5-13. Data collection in relation to the RSV transmission periods.
*Each column represents one week of the clinical trial. The blue columns denote the number of scheduled visits performed in each study week, with the orange line showing the cumulative completion of clinical visits. The purple area shows the recruitment period. The light grey boxes illustrate the likely RSV transmission period based on Public Health England data, with the dark grey box denoting the peak of period of RSV cases reported to PHE. (**Top figure**) Data from 42 healthy adults aged 18-50 years in 2013. Dose and group size escalation rules meant the clinical and laboratory workload was scheduled to expand rapidly after initial safety assessments. (**Bottom figure**) Data from 2014 and 30 older adults aged 60-75 years, without the need for dose or group size escalation rules.*

5.12.2 ITT and PP populations for analysis

RSV001 completed the final visit of the last volunteer in August 2015. After withdrawals only 3/851 (0.4%) scheduled visits were unattended. A total of 834/851 (98%) of all scheduled visits were attended within the pre-specified windows after vaccination and satisfied the per-protocol population definition (shown in Figure 5-14 and Table 5-12). As a result, all analyses of safety and immunogenicity reported in this thesis used the ITT population (all volunteers, all data) without any separate analysis using the per-protocol population only.

Analysis of protocol deviations in respect of the date of scheduled attendances found most instances were due to volunteer availability and 14/17 (82%) were from the cohort of younger adults. The protocol breaches in older adults were all from non-vaccinated controls (study group 5). From younger adults and study groups 1-4 there were 3 significant deviations from the planned prime/boost interval. One volunteer from study group 1 received a live vaccine (Yellow Fever) for leisure travel within one-month of the planned boost vaccine (a temporary exclusion criteria for the boost vaccine) and was boosted on her return to the UK 48-days later than planned. The other two volunteer prime/boost deviations were due to volunteer availability (volunteer from group 2, 6-days later than planned) and scheduling error (volunteer from group 4, 14-days earlier than planned). Subsequent post-boost attendances adhered to the planned sampling schedule.

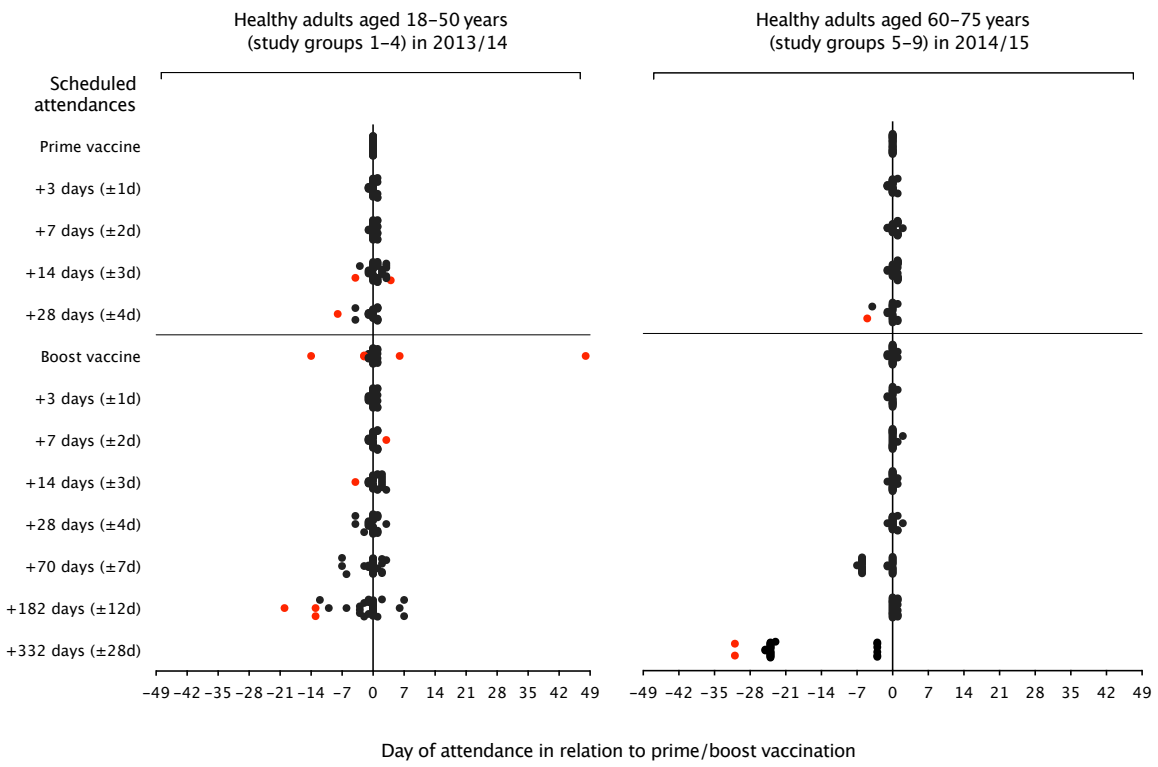


Figure 5-14. Schematic representation of the ITT and PP populations from RSV001.
Each dot represents one attended scheduled visit from one volunteer. Black dots denote those visits that occurred within the protocol-defined window for each visit after vaccination. The red dots were protocol deviations when the visit was conducted outside of the protocol-defined window.

	Study groups of adults aged 18-50 years				Study groups of adults aged 60-75 years					Total
	1	2	3	4	5	6	7	8	9	
Prime	11/11	10/10	10/10	11/11	6/6	6/6	6/6	6/6	6/6	72/72
+3d	11/11	10/10	10/10	10/10	6/6	6/6	6/6	6/6	5/5	70/70
+7d	11/11	10/10	10/10	11/11	6/6	6/6	6/6	6/6	6/6	72/72
+14d	10/11	10/10	9/10	10/10	6/6	6/6	6/6	6/6	6/6	69/71
+28d	11/11	-	9/9	9/10	5/6	6/6	6/6	6/6	6/6	53/54
Boost	10/10	6/10	10/10	8/10	6/6	6/6	6/6	6/6	6/6	64/70
+3d	10/10	10/10	10/10	10/10	6/6	-	6/6	6/6	6/6	64/64
+7d	9/10	10/10	10/10	10/10	6/6	6/6	6/6	6/6	6/6	69/70
+14d	8/9	10/10	10/10	10/10	6/6	6/6	6/6	6/6	6/6	68/69
+28d	10/10	10/10	10/10	10/10	6/6	6/6	6/6	6/6	6/6	70/70
+70d	10/10	10/10	10/10	10/10	6/6	6/6	6/6	6/6	6/6	70/70
+182d	9/10	10/10	9/10	9/10	6/6	6/6	6/6	6/6	6/6	67/70
+332d	-	-	-	-	4/6	5/5	6/6	6/6	6/6	27/29
Total	120/124	106/110	117/119	118/122	75/78	71/71	72/72	78/78	77/77	834/851

Table 5-12. The proportion of per-protocol attendances of scheduled visits from RSV001.
n (nominator) = number of volunteers attended visits per protocol (per protocol population, PP). N (denominator) = total number of volunteer attended visits (intention-to-treat population, ITT).

5.13 Discussion

5.13.1 Summary of main findings

RSV001 was the first viral-vectored vaccine trial for RSV and used a first-in-man adenovirus vector. After meeting all regulatory requirements the trial recruited and was conducted according to protocol and vaccinated outside of the RSV transmission season. The resulting data integrity supported meaningful analysis of the safety and immunogenicity of these vaccines.

The results have provided a detailed characterisation of the critical recruitment period required to initiate and deliver a phase I vaccine trial. In both years, members of the public were required to consider the risks of early vaccine development, similar trial commitments and other factors that can influence whether to pursue an interest in volunteering. This supports drawing some comparative observations between younger and older adults. When trial recruitment was analysed it revealed a real willingness from the older adult population to take part in clinical trials. There are, to my knowledge, no similar published data looking at recruitment in healthy younger and older adults/elderly to the same vaccine trial.

5.13.2 Recruitment

Public engagement, recruitment and selection of suitable study volunteers represents a critical and often defining period in the successful and timely delivery of clinical trials. Cited and modifiable obstacles to trial delivery have included delays in opening trial sites[424], the lack of timely volunteer accrual[425-428] and high rates of exclusion[429]. In addition, an estimated 2% of potential volunteers to healthy adult trials received a new medical diagnosis that resulted in their exclusion from the research, which was comparable to the data analysis presented here[430]. The attrition rate from the number of expressions of interest in both years was remarkable and, in part, justified the large-scale use of unsolicited mailed material. Telephone contact using GCP trained non-clinical staff was able to greatly reduce inappropriate physician screening appointments and resources. The time taken to enter the trial after physician screening was variable, but there were no significant changes in haemoglobin as a function of the time from screening to enrolment that would give concern about the large blood volume taken subsequently. An age-related decline in haemoglobin has been described before, with some studies indicating a higher differential drop in males[431,432].

The elderly and especially elderly with co-morbid conditions are generally under-represented in clinical trials of dugs and vaccines, even when intended for use in this population. Here the observation has been that older adults are especially interested to be involved in clinical trials, with response rates and the speed of responses that significantly exceeded younger adults with the methods used in this trial. Adults aged 60-75 years were almost four-times more likely to make contact with a view to taking part in the trial in response to unsolicited posted recruitment material. Analysis of elderly recruitment to pneumococcal vaccine trial in the Netherlands recorded an 18% inclusion rate from positive responses, with a negative correlation with increasing distance from the research site and age[433]. Interest from the healthy elderly for RSV001 was undiminished with increasing distance from the research site and it was remarkable that enrolled volunteers were prepared to travel 70-miles (round trip) for up to 17 visits over a one-year period. Although several papers have cited difficulties in recruiting the elderly with medical conditions of interest into clinical trials, there are comparably few publications on the recruitment of healthy elderly to early stage vaccine trials to compare with these observations[433,434]. Recruitment to phase 1 vaccine trials remains frequently under analysed or under reported, even in the exceptional context of having required accelerated trials for pandemic influenza in 2009[435]. To fully address the problems of trial delivery and recruitment common to so many clinical trial units, careful examination and analysis of these data in other trials is needed to better engage and select suitable volunteers for clinical research.

5.13.3 Trial execution

The design of the first phase I viral-vectored vaccine trial for RSV and small group sizes mandated careful consideration of the timing of the trial to occur outside the periods of high background RSV transmission, the need to minimise withdrawals and strict adherence to the schedule for sample collection after vaccination. Given the small study group sizes, any missing or mistimed data could have had a disproportionate effect on the validity of the data, and significant care was taken to select volunteers who could complete all scheduled visits on time. Prior planning of these dates allowed potential volunteers to confirm their availability for all appointments and protect study requirements from competing demands. This helped to achieve the high levels of data integrity at appropriate times after vaccination and supported confidence in the interpretation of the results. The two withdrawals could not have been anticipated at

physician screening and their replacements were vaccinated before the onset of the RSV season. Overall 98% of scheduled attendances were performed within the protocol-defined window after vaccination that allowed the use of the ITT population data to interpret the safety and immunogenicity of the experimental vaccines.

5.13.4 General limitations

This chapter describes the setup, design and execution of the RSV001 clinical trial. The conduct and oversight of all clinical trials of investigational medicinal products, and especially phase I trials, are controlled by legislation and independent monitoring. The design of RSV001 therefore conformed to regulatory requirements and the standards required of phase I vaccine trials. These included small group sizes that are not uncommon to phase I trials, and the inevitable inherent risk of confounding chance observations that would not be representative of the wider population or the non-occurrence of serious but uncommon adverse events. The prime/boost combinations in younger adults were determined from the pre-clinical rodent and calf challenge experiments of PanAd3-RSV and MVA-RSV by the trial Sponsor[408,410]. A non-vaccinated control arm was not incorporated until the trial was expanded to include a cohort of older adults.

This trial expansion allowed for an opportunistic comparison of recruitment between younger and older members of the public in considering the same vaccines, risks and benefits of taking part. It was not the purpose of RSV001 or was it incorporated into the design of the trial to undertake this analysis, which proved to be insightful and addresses some of the major obstacles in performing clinical trials. The response rates and costs, for both age groups, in respect of postal recruitment may have been be underestimated; an expression of interest was solicited by return post of a reply slip, telephone/email contact or via the OVG website and several website responses were from volunteers living in postcode areas who recently received recruitment material by mail. Volunteers were not asked who and how they had been directed to the website. These cost estimates did not include the time taken by administrative and clinical staff to manage the responses to recruitment material, and the intense 6-week period of recruitment in 2014 would not necessarily make for a cheaper (or more efficient) model of recruitment if inadequately resourced. Understanding the magnitude and consistent kinetics of the response rate from target populations to

recruitment material can be used to improve the use of this approach in future trials. The relatively inexpensive recruitment methods that lead to enrolment from volunteers who heard by other means should be investigated and exploited further. Other research units have reported successes in using social media to engage the interest of younger adults[436,437] and an exploration of these methods, or the effect of sex, ethnicity, education and employment status on volunteering for clinical trials was not investigated here.

5.13.5 Conclusions

The RSV001 clinical trial was executed with close adherence to the protocol and resulted in the timely collection of data to address the primary and secondary endpoints of the trial. Post-hoc exploration of the factors that can influence the recruitment and selection of suitable volunteers may allow research sites to optimise public engagement and improve recruitment to vaccine trials in other clinical trial units.

Chapter 6: Analysis of safety and the clinical response to vaccination

6.1 Introduction

As reported in Section 5.2, hexon group C human and chimpanzee adenovirus and MVA-vectored vaccines have been used safely across multiple antigenic inserts and has included safe use in infants. Pre-clinical safety evaluation of the genetically modified viral-vectored vaccines PanAd3-RSV and MVA-RSV has supported the development of these vaccines for use in man, albeit with clear limitations in the translation of the results from animals. This chapter describes the results and analysis of the primary objective and endpoints of the trial, namely the safety and tolerability of PanAd3-RSV and MVA-RSV and their first use in man. The results from younger adults (study groups 1-4) have been published and this chapter explores these findings in more detail and includes the data from older adults (study groups 5-9)[1g].

[g] Short sections of this chapter copied from the first publication of results from the RSV001 trial represent my own work.

6.2 Safety data integrity

A total of 32297/33013 (97.8%) of expected safety data points were available for analysis (Table 6-1). The minor losses of safety blood data were due to haemolysed samples on arrival to the OUHL lab or OUHL laboratory error. There was a disproportionate loss of blood safety data for group 4 due to laboratory error on a specific week of the trial. This included four pre-prime haematology and two pre-boost haematology and biochemistry blood samples. For these volunteers the last recorded values (screening bloods in the case of pre-prime losses) were used as surrogate baseline measures for monitoring changes in haemoglobin during the trial (the change from baseline was used to grade adverse event severity for haemoglobin). These surrogate values were removed from the post-hoc analyses and the volunteer data was excluded. All missing data from solicited symptoms were omitted temperature recordings.

	Solicited symptoms	Visit observations	Haematology and biochemistry bloods	Sub-total
All groups	8985/8995 (99.9)	4293/4330 (99.1)	19019/19688 (96.6)	**32297/33013 (97.8)**
Healthy adults aged 18-50 years, n/N (%):				
Group 1	1468/1470 (99.9)	620/625 (99.2)	2774/2875 (96.5)	4862/4970 (97.8)
Group 2	1396/1400 (99.7)	598/600 (99.7)	2483/2530 (98.1)	4477/4530 (98.8)
Group 3	1330/1330 (100)	585/600 (97.5)	2639/2760 (95.6)	4554/4690 (97.1)
Group 4	1393/1393 (100)	610/615 (99.2)	2507/2829 (88.6)	4510/4837 (93.2)
Healthy adults aged 60-75 years, n/N (%):				
Group 5	502/504 (99.6)	390/390 (100)	1742/1794 (97.1)	2634/2688 (98)
Group 6	420/420 (100)	360/360 (100)	1655/1656 (99.9)	2435/2436 (100)
Group 7	840/840 (100)	360/360 (100)	1656/1656 (100)	2856/2856 (100)
Group 8	797/798 (99.9)	390/390 (100)	1794/1794 (100)	2981/2982 (100)
Group 9	839/840 (99.9)	380/390 (97.4)	1769/1794 (98.6)	2988/3024 (98.8)

Table 6-1. RSV001 safety data integrity.
Each study group is presented overall and sub-divided by the three primary sources of safety data. n is the number of data points collected for analysis, and N is the number of safety data points expected after adjustment for withdrawals.

6.3 Serious adverse events (SAEs)

There were two severe adverse events in younger adults and one from the older adult cohort. None were related to the vaccine and each SAE is summarised in the table below.

SAE event number	Temporal relationship to vaccination	Description of the event	Outcome
1	16-weeks after high-dose IN PanAd3-RSV prime and 8-weeks after high dose IM MVA-RSV boost. [Group 3 volunteer]	Admitted to hospital for serologically confirmed scrub typhus infection on return from travelling through Myanmar.	Resolved.
2	22-weeks after low-dose IN PanAd3-RSV prime, 16-weeks after a Yellow Fever travel vaccination, and 7-weeks after low-dose IM PanAd3-RSV boost. [Group 4 volunteer]	A recurrence of transient hypnopompic visual hallucinations with flaccid paralysis that occurred at a frequency of 3-4 times per week. After consultation with the DSMC, the frequency and severity of her symptoms warranted designation as an SAE. She recalled milder and shorter episodes (once per week for 2-3 weeks) of these symptoms had occurred 6-months before enrolment, and these were not declared at physician screening or known to her GP. This discounted causality from the vaccine.	She was referred to a sleep specialist by her GP, and her symptoms improved and resolved spontaneously after 7 weeks intermittent duration. The specialist referral was cancelled.
3	43-weeks after high-dose IM MVA-RSV (single dose). [Group 6 volunteer]	Developed a short history of dysphagia in early 2015 and he was diagnosed with terminal oesophageal carcinoma complicated by multiple pulmonary emboli.	Died.

Table 6-2. Summary descriptions of the 3 severe adverse events (SAEs) from the RSV001 trial.
None were considered related to the study vaccines.

6.4 Dose and group size escalation

There were no severe adverse reactions or clinically significant severe (grade 3) adverse events from low-dose vaccine recipients to trigger an immediate halt to the planned dose or group size escalation. Two formal reviews of the DSMC were conducted as planned, one before first low-dose boost and one before first high-dose boost, and DSMC approval was granted for dose escalation and group size expansion to proceed according to protocol.

6.5 Volunteer reported symptoms after vaccination

Volunteers reported the severity score for each solicited symptom every day after vaccination for one week. Systemic solicited symptoms included headache, nausea and/or vomiting, myalgia, malaise, arthralgia and oral temperature. Local solicited symptoms following an intra-muscular vaccine included pain and/or tenderness at the site of injection and the maximal diameter reaction for redness, induration and swelling. Local solicited symptoms following PanAd3-RSV prime by intra-nasal spray included nasal pain/discomfort, nasal irritation and nasal discharge. Volunteers used the severity criteria defined in the supplementary material to grade the severity of solicited symptoms as none (grade 0), mild (grade 1), moderate (grade 2) or severe (grade 3). A total of 8985/8995 (99.9%) of expected solicited symptoms after vaccination were recorded and available for analysis.

6.5.1 Symptoms reported before vaccination

There were no solicited symptoms recorded before prime or boost vaccination from any volunteer, except for one elderly non-vaccinated volunteer in group 5 who recently joined a gym and attended one class and subsequently reported mild (grade 1) muscular aches for the duration of the boost reporting period.

6.5.2 *Solicited symptoms after vaccination*

Overall the prime and boost vaccines were safe and well tolerated. Solicited adverse events were generally mild, resolved within the one-week period of observation after vaccination, and commonly recorded as local injection site events. Table 6-3 and Table 6-4 details the counts and proportions of volunteers who experienced an adverse event by study group after prime and boost vaccination respectively, and the data are summarised as the maximum severity adverse event during the reporting period for each group in Figure 6-1 and Figure 6-2. A narrative description of each solicited symptom is provided in Table 6-5. The proportion of reported severity grades recorded for each solicited symptom each day after vaccination, as an illustration of the temporal nature of adverse events, is presented in supplementary material.

After prime there was one severe headache reported two days after IM PanAd3-RSV and one severe fever (temperature 39.9°C) 3-days after IN PanAd3-RSV. There were two recordings of fever from this volunteer, reported 2-days and 3-days after IN PanAd3-RSV. The severe febrile episode was concurrent with moderate severity headache, nausea, malaise and coryzal symptoms that developed in the short period after vaccination, and rhinovirus was detected on a nasal sample obtained 3-days post vaccination. This volunteer withdrew from the trial at the end of the 7-day reporting period for reasons unrelated to the vaccine and by which time her symptoms had resolved in full. All seven severe adverse events reported after boost vaccination were following IM MVA-RSV and included malaise (n=2), myalgia (n=2), arthralgia (n=1) and injection site pain (n=2).

All solicited adverse events after vaccination that extended beyond the one-week period of mandatory volunteer data collection were followed up, where possible, until complete resolution of the event. A total of 42 unresolved solicited symptoms, from 28/66 (42%) vaccinated volunteers, unresolved on day 7 after vaccination represented 3.5% of the data collected on the final day of reporting. Although all solicited adverse events were known to resolve in full, the total duration of these events was recorded in only 13/42 (31%) cases. From these, 12/13 had resolved within 9 days of vaccination and one volunteer experienced a total of 15 days injection site tenderness after IM MVA-RSV boost.

	Adults aged 18-50 years				Adults aged 60-75 years				
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**	**Group 7**	**Group 8**	**Group 9**
Prime vaccine	IM PanAd3-RSV	IM PanAd3-RSV	IN PanAd3-RSV	IN PanAd3-RSV	No vaccine	IM MVA-RSV	IM PanAd3-RSV	IN PanAd3-RSV	IM PanAd3-RSV
N volunteers	11	10	10	11	6	6	6	6	6
Any systemic adverse event after vaccination, n/N (%):									
Headache	**7/11 (64)**	**7/10 (70)**	**4/10 (40)**	**6/11 (55)**	0/6 (0)	**2/6 (33)**	0/6 (0)	**2/6 (33)**	**2/6 (33)**
Low dose	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-
High dose	6/9 (66.7)	6/8 (75.0)	3/8 (37.5)	5/9 (55.6)	0/6 (0)	2/6 (33.3)	0/6 (0)	2/6 (33.3)-	2/6 (33.3)
Nausea	1/11 (9.1)	**2/10 (20)**	**1/10 (10)**	**2/11 (18)**	0/6 (0)	0/6 (0)	0/6 (0)	**1/6 (17)**	0/6 (0)
Low dose	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	1/9 (11.1)	2/8 (25.0)	1/8 (12.5)	2/9 (22.2)	0/6 (0)	0/6 (0)	0/6 (0)	1/6 (16.7)	0/6 (0)
Malaise	**4/11 (36)**	**4/10 (40)**	**2/10 (20)**	**3/11 (27)**	0/6 (0)	**3/6 (50)**	0/6 (0)	**1/6 (17)**	**1/6 (17)**
Low dose	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	4/9 (44.4)	4/8 (50.0)	2/8 (25.0)	3/9 (33.3)	0/6 (0)	3/6 (50.0)	0/6 (0)	1/6 (16.7)	1/6 (16.7)
Myalgia	**6/11 (55)**	**5/10 (50)**	**1/10 (10)**	1/11 (9.1)	**1/6 (17)**	**2/6 (33)**	0/6 (0)	**2/6 (33)**	**1/6 (17)**
Low dose	1/2 (50.0)	1/2 (50.0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	5/9 (55.6)	4/8 (50.0)	1/8 (12.5)	1/9 (11.1)	1/6 (16.7)	2/6 (33.3)	0/6 (0)	2/6 (33.3)	1/6 (16.7)
Arthralgia	**3/11 (27)**	**2/10 (20)**	**1/10 (10)**	0/11 (0)	0/6 (0)	**2/6 (33)**	0/6 (0)	**2/6 (33)**	0/6 (0)
Low dose	1/2 (50.0)	0/2 (0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	2/9 (22.2)	2/8 (25.0)	1/8 (12.5)	0/9 (0)	0/6 (0)	2/6 (33.3)	0/6 (0)	2/6 (33.3)	0/6 (0)
Fever	0/11 (0)	0/10 (0)	0/10 (0)	1/11 (9.1)	0/6 (0)	**2/6 (33)**	0/6 (0)	0/6 (0)	0/6 (0)
Low dose	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	0/9 (0)	0/9 (0)	0/9 (0)	1/9 (11.1)	0/6 (0)	2/6 (33.3)	0/6 (0)	0/6 (0)	0/6 (0)
Any local adverse event after intra-muscular (IM) vaccination, n/N (%):									
Pain	**6/11 (55)**	**7/10 (70)**	n/a	n/a	n/a	**6/6 (100)**	0/6 (0)	n/a	**1/6 (17)**
Low dose	1/2 (50.0)	2/2 (100)	-	-	-	-	-	-	-
High dose	5/9 (55.6)	5/8 (62.5)	-	-	-	6/6 (100.0)	0/6 (0)	-	1/6 (16.7)
Induration	0/11 (0)	**4/10 (40)**	n/a	n/a	n/a	**2/6 (33)**	0/6 (0)	n/a	**1/6 (17)**
Low dose	0/2 (0)	1/2 (50.0)	-	-	-	-	-	-	-
High dose	0/9 (0)	3/8 (37.5)	-	-	-	2/3 (33.3)	0/6 (0)	-	1/6 (16.7)
Redness	**3/11 (27)**	**5/10 (50)**	n/a	n/a	n/a	**1/6 (17)**	0/6 (0)	n/a	**1/6 (17)**
Low dose	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-	-	-
High dose	2/9 (22.2)	4/8 (50.0)	-	-	-	1/6 (16.7)	0/6 (0))	-	1/6 (16.7)
Swelling	**7/11 (64)**	**8/10 (80)**	n/a	n/a	n/a	**2/6 (33)**	0/6 (0)	n/a	**1/6 (17)**
Low dose	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-	-	-
High dose	5/9 (55.6)	7/8 (87.5)	-	-	-	2/6 (33.3)	0/6 (0)	-	1/6 (16.7)
Any local adverse event after intra-nasal (IN) vaccination, n/N (%):									
Pain	n/a	n/a	0/10 (0.0)	1/11 (9.1)	n/a	n/a	n/a	0/6 (0)	n/a
Low dose	-	-	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	-	-	0/8 (0)	1/9 (11.1)	-	-	-	0/6 (0)	-
Discharge	n/a	n/a	**1/10 (10)**	1/11 (9.1)	n/a	n/a	n/a	**3/6 (50)**	n/a
Low dose	-	-	1/2 (50.0)	0/2 (0)	-	-	-	-	-
High dose	-	-	0/8 (0)	1/9 (11.1)	-	-	-	3/6 (50.0)	-
Irritation	n/a	n/a	**1/10 (10)**	**2/11 (18)**	n/a	n/a	n/a	**2/6 (33)**	n/a
Low dose	-	-	1/2 (50.0)	0/2 (0)	-	-	-	-	-
High dose	-	-	0/8 (0)	2/9 (22.2)	-	-	-	2/6 (33.3)	-

Table 6-3. Counts and proportions of any adverse event reported after prime vaccination by dose and study group within 7-days of vaccination.

Counts and proportions for the occurrence of any solicited symptom (grade 1-3) in each study group within one week of vaccination. The data are presented as the number of volunteers who reported an adverse event (n) and the number of volunteers in the study group (N). The frequencies of mild (grade 1), moderate (grade 2) and severe (grade 3) adverse events for each study group and each day after vaccination are presented in supplementary results. n/a (not applicable). Adverse events that occurred in ≥10% of volunteers are shown in bold.

	Adults aged 18-50 years				Adults aged 60-75 years				
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**	**Group 7**	**Group 8**	**Group 9**
Boost vaccine	IM MVA-RSV	IM PanAd3-RSV	IM MVA-RSV	IM PanAd3-RSV	No vaccine	No vaccine	IM PanAd3-RSV	IM MVA-RSV	IM MVA-RSV
N volunteers	10	10	10	10	6	-	6	6	6
Any systemic adverse event after vaccination, n/N (%):									
Headache	**7/10 (70)**	**4/10 (40)**	**5/10 (50)**	**3/10 (30)**	**1/6 (17)**	n/a	0/6 (0)	**5/6 (83)**	**3/6 (50)**
Low dose	1/2 (50.0)	0/2 (0)	0/2 (0)	1/2 (50.0)	-	-	-	-	-
High dose	6/8 (75.0)	4/8 (50.0)	5/8 (62.5)	2/8 (25.0)	1/6 (16.7)	-	0/6 (0)	5/6 (83.3)	3/6 (50.0)
Nausea	**2/10 (20)**	**1/10 (10)**	**3/10 (30)**	0/10 (0)	0/6 (0)	n/a	0/6 (0)	0/6 (0)	**1/6 (17)**
Low dose	1/2 (50.0)	1/2 (50.0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	1/8 (12.5)	0/8 (0)	3/8 (37.5)	0/8 (0)	0/6 (0)	-	0/6 (0)	0/6 (0)	1/6 (16.7)
Malaise	**6/10 (60)**	**4/10 (40)**	**8/10 (80)**	0/10 (0)	0/6 (0)	n/a	**2/6 (33)**	**5/6 (83)**	**2/6 (33)**
Low dose	0/2 (0)	0/2 (0)	1/2 (50.0)	0/2 (0)	-	-	-	-	-
High dose	6/8 (75.0)	4/8 (50)	7/8 (87.5)	0/8 (0)	0/6 (0)	-	2/6 (33.3)	5/6 (83.3)	2/6 (33.3)
Myalgia	**7/10 (700)**	**6/10 (60)**	**8/10 (80)**	**2/10 (20)**	**1/6 (17)**	n/a	0/6 (0)	**2/6 (33)**	**2/6 (33)**
Low dose	1/2 (50.0)	0/2 (0)	1/2 (50.0)	0/2 (0)	-	-	-	-	-
High dose	6/8 (75.0)	6/8 (75.0)	7/8 (87.5)	2/8 (25.0)	1/6 (16.7)	-	0/6 (0)	2/6 (33.3)	2/6 (33.3)
Arthralgia	**6/10 (60)**	**2/10 (20)**	**5/10 (50)**	**1/10 (10)**	0/6 (0)	n/a	**1/6 (17)**	**1/6 (17)**	**2/6 (33)**
Low dose	1/2 (50.0)	0/2 (0)	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-
High dose	5/8 (62.5)	2/8 (25.0)	4/8 (50.0)	0/8 (0)	0/6 (0)	-	1/6 (16.7)	1/6 (16.7)	2/6 (33.3)
Fever	0/10 (0)	0/10 (0)	0/10 (0)	**1/10 (10)**	0/6 (0)	n/a	0/6 (0)	0/6 (0)	0/6 (0)
Low dose	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	0/8 (0)	0/8 (0)	0/8 (0)	1/8 (12.5)	0/6 (0)	-	0/6 (0)	0/6 (0)	0/6 (0)
Any local adverse event after intra-muscular (IM) vaccination, n/N (%):									
Pain	**10/10 (100)**	**8/10 (80)**	**8/10 (80)**	**7/10 (70)**	n/a	n/a	0/6 (0)	**6/6 (100)**	**4/6 (67)**
Low dose	2/2 (100)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-
High dose	8/8 (100)	7/8 (87.5)	7/8 (87.5)	6/8 (75.0)	-	-	0/6 (0)	6/6 (100)	4/6 (66.7)
Induration	**2/10 (20)**	**2/10 (20)**	**3/10 (30)**	0/10 (0)	n/a	n/a	0/6 (0)	**2/6 (33)**	**2/6 (33)**
Low dose	0/2 (0)	0/2 (0)	1/2 (50.0)	0/2 (0)	-	-	-	-	-
High dose	2/8 (25.0)	2/8 (25.0)	2/8 (25.0)	0/8 (0)	-	-	0/6 (0)	2/6 (33.3)	2/6 (33.3)
Redness	**6/10 (60)**	**2/10 (20)**	**5/10 (50)**	**2/10 (20)**	n/a	n/a	0/6 (0)	**4/6 (67)**	**1/6 (17)**
Low dose	2/2 (100)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-
High dose	4/8 (50.0)	1/8 (12.5)	4/8 (50.0)	1/8 (12.5)	-	-	0/6 (0)	4/6 (66.7)	1/6 (16.7)
Swelling	**10/10 (100)**	**6/10 (60)**	**4/10 (40)**	**3/10 (30)**	n/a	n/a	**1/6 (17)**	**2/6 (33)**	**1/6 (17)**
Low dose	2/2 (100)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-
High dose	8/8 (100)	5/8 (62.5)	3/8 (37.5)	2/8 (25.0)	-	-	1/6 (16.7)	2/6 (33.3)	1/6 (16.7)

Table 6-4. Counts and proportions of any adverse event reported after boost vaccination by dose and study group within 7-days of vaccination.

Counts and proportions for the occurrence of any solicited symptom (grade 1-3) in each study group within one week of vaccination. The data are presented as the number of volunteers who reported an adverse event (n) and the number of volunteers in the study group (N). The frequencies of mild (grade 1), moderate (grade 2) and severe (grade 3) adverse events for each study group and each day after vaccination are presented in supplementary results. n/a (not applicable). Adverse events that occurred in ≥10% of volunteers are shown in bold.

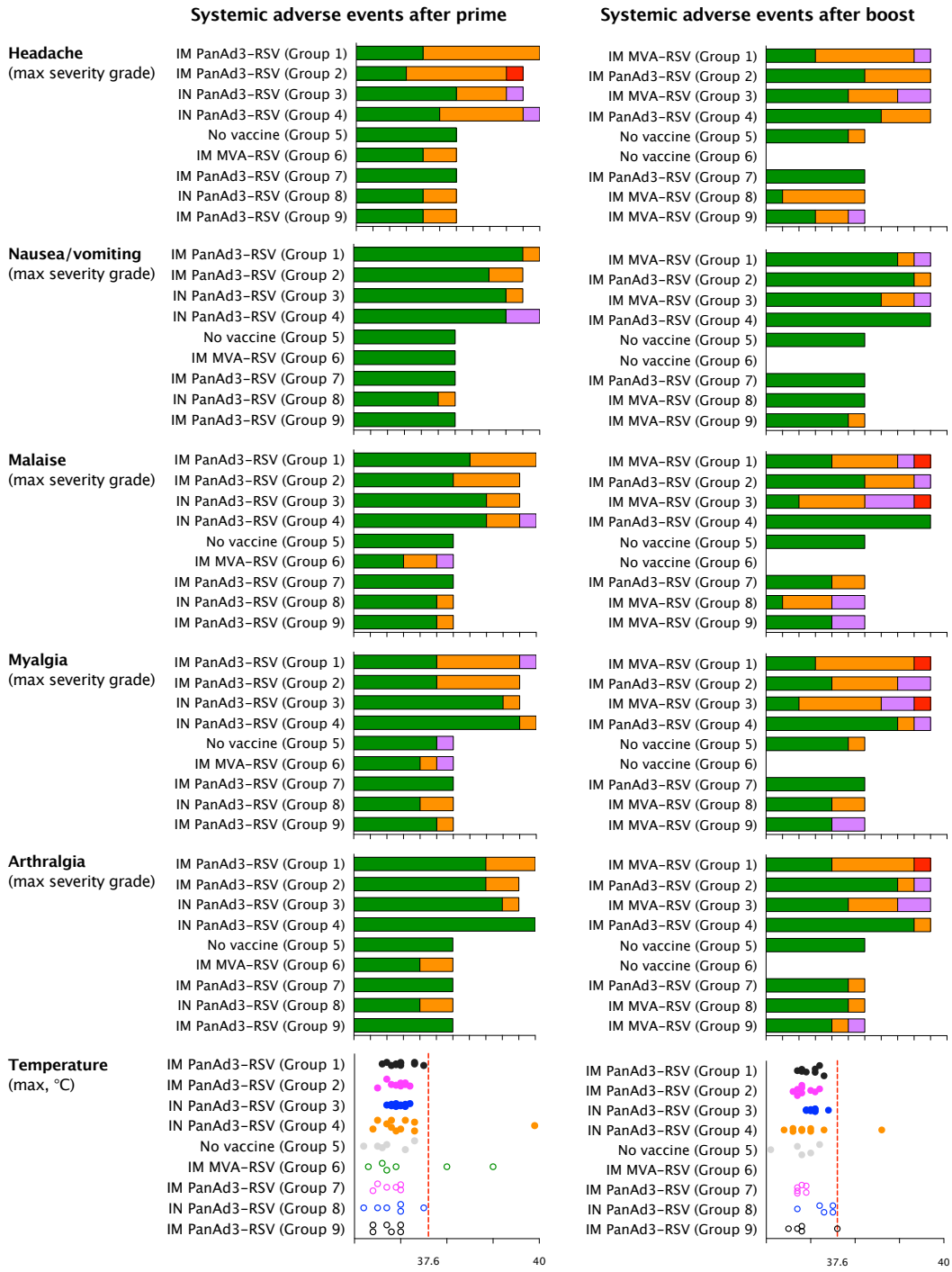


Figure 6-1. The maximum severity systemic solicited adverse event reported within one week of prime or boost vaccination.
Each volunteer maintained a daily record of severity grades from none (grade 0, green bars), mild (grade 1, yellow bars), moderate (grade 2, purple bars) and severe (grade 3, red bars). The number of volunteers is across the x-axis. The dotted red line for temperature, induration and redness are the grade 1 threshold.

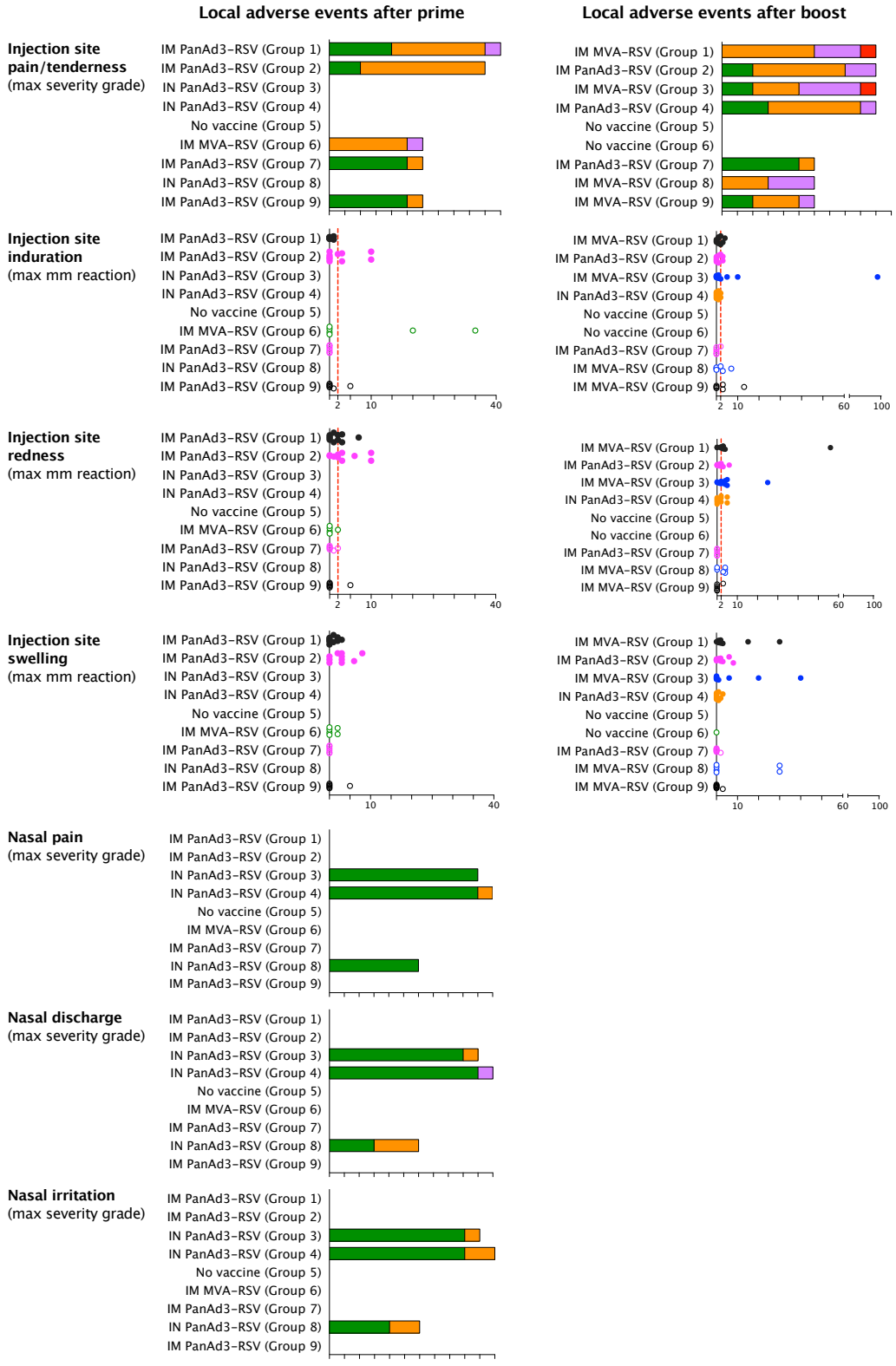


Figure 6-2. The maximum severity local solicited adverse event reported within one week of prime or boost vaccination.
Each volunteer maintained a daily record of severity grades from none (grade 0, green bars), mild (grade 1, yellow bars), moderate (grade 2, purple bars) and severe (grade 3, red bars). The number of volunteers is across the x-axis. The dotted red line for temperature, induration and redness are the grade 1 threshold.

	Description
Headache	Headache was commonly reported in all study groups after prime and boost. After IM PanAd3-RSV prime the peak frequency for headache reporting was 24hrs after vaccination, with no discernable day of peak frequency after IN PanAd3-RSV or IM MVA-RSV prime. After boost, the frequency of headache reporting was approximately twice as common following IM MVA-RSV relative to PanAd3-RSV, and the peak frequency of headache was 24hrs after boost vaccination.
Nausea and/or vomiting	There were no reports of vomiting. Nausea within the week following IN PanAd3-RSV prime was reported in ≥10% of volunteers, with too few occurrences to discern any day of peak frequency after vaccination. After boost, nausea was common between 24-48hrs following IM MVA-RSV in younger adults.
Malaise	Malaise was reported in ≥10% of volunteers from all study groups after prime and boost vaccination. After IM PanAd3-RSV prime the peak frequency was 24hrs after vaccination, and 24hrs after vaccination was the only day of malaise reported in older adults who received IM MVA-RSV prime. There was no discernable day of peak frequency for malaise in IN PanAd3-RSV prime volunteers. Despite the few numbers of volunteers who received low-dose vaccine, an indication of a dose effect was observed in the frequency reporting malaise after vaccination. Malaise was unreported in all low-dose volunteers, but was reported in 8/17 (47%) of high-dose IM and 5/21 (24%) of high-dose IN PanAd3-RSV 'primed' younger adults respectively. After boost, malaise was reported at approximately three times greater frequency following IM MVA-RSV relative to IM PanAd3-RSV, the peak frequency being 24hrs after vaccination. As observed following the prime vaccine, the 30-80% frequency of reporting of malaise after vaccination was from high-dose vaccine recipients only, with the exception of one low dose IM MVA-RSV volunteer.
Myalgia	Myalgia was a common occurrence (≥10% of volunteers in a study group) following any prime vaccine given by injection (IM PanAd3-RSV and IM MVA-RSV), the peak frequency being 24hrs after vaccination after IM PanAd3-RSV and with no discernable day of peak frequency after IN PanAd3-RSV prime. After boost, myalgia was reported twice as common following IM MVA-RSV relative to IM PanAd3-RSV. The peak frequency was 24hrs after boost vaccination.
Arthralgia	Arthralgia was infrequently reported after prime, but common 24hrs after IM MVA-RSV boost.
Fever	In addition to the severe fever following IM PanAd3-RSV prime described earlier, in older adults there were an additional two reports of fever from different volunteers in the group that received IM MVA-RSV prime. After boost, there was one recorded fever to IM PanAd3-RSV and one recorded fever to IM MVA-RSV.
Injection site pain and/or tenderness	Pain and/or tenderness at the site of vaccine site injection were a common experience in all study groups. IM PanAd3-RSV administered at prime caused pain in most volunteers, with a peak frequency of pain 24-48hrs after vaccination. IM MVA-RSV prime in older adults caused pain in all volunteers between 24-48hrs after vaccination. After boost, IM PanAd3-RSV caused a frequency and severity of pain comparative to the post-prime reporting. IM MVA-RSV boost caused mild to moderate pain in nearly all volunteers, with a greater proportion graded as moderate severity and of longer duration compared with IM PanAd3-RSV.
Injection site induration, redness and swelling	Induration, redness and swelling at the site of injection was infrequent and with too few occurrences to discern any day of peak frequency after prime or boost.
Nasal site pain, discharge and irritation	The intra-nasal route was well tolerated and adverse events were infrequently reported.

Table 6-5. Description of the solicited systemic and local adverse events after vaccination.
Data from prime and boost vaccination without distinction between low-dose and high-dose vaccine or prime/boost combination.

6.5.3 *Post-hoc analysis of solicited symptoms after vaccination*

Post-hoc analyses used volunteers data pooled by vaccine vector and route (instead of study group) and the calculated symptom scores for the 7-day reporting period after vaccination. The symptom score was the sum of severity grades reported for each of the seven days, sum oral temperatures and sum of local reaction measures recorded. There were no imputations for missing data and an incomplete 7-day record resulted in the exclusion of that volunteer's symptom score from post-hoc analysis.

There were no missing data from any solicited symptoms except missing temperature data for three volunteers after prime (one after IM and two after IN PanAd3-RSV prime) and five volunteers after boost (two after IM PanAd3-RSV, two after IM MVA-RSV boost and one non-vaccinated control) that resulted in these volunteers being excluded from analysis. The counts and proportions of adverse events and symptom scores for each solicited symptom after prime and boost vaccination are presented in Table 6-6 and Table 6-7 respectively.

Analysis of the data related to prime vaccine found the only statistically significant between-vaccine difference was in the mean severity scores reported for pain and/or tenderness at the site of vaccine injection. IM MVA-RSV prime caused pain in all 6 recipients with a mean symptom score of 3.67, compared with a mean score of 1.24 for the 32 volunteers who received IM PanAd3-RSV (p=0.001, two-tailed Mann-Whitney test). Differences in the mean symptom scores for other solicited systemic and local adverse events failed to reach statistical significance (Table 6-6). After boost, statistically significant differences in the mean severity scores were observed for headache, malaise, arthralgia, injection site pain, injection site induration and injection site swelling (Table 6-7). In all instances IM MVA-RSV was responsible for greater severity of reactions compared with IM PanAd3-RSV. Nausea, myalgia, oral temperatures and injection site swelling were reported at a greater frequency after IM MVA-RSV compared with IM PanAd3-RSV, but failed to reach statistical significance.

A two-tailed Spearman's test was used to explore correlations between volunteer age and symptom scores. Oral temperature declined with increasing age in both the IM and IN PanAd3-RSV prime groups (p=0.004 and p=0.027 respectively, Pearson's correlation). The summed temperatures were applied as the dependent variable in an analysis of co-variance (ANCOVA) model and volunteer age (p=0.013) and the baseline temperature recorded at the clinical visit before vaccination (p=0.001) had statistically

significant effects on the summed temperature 7-days after vaccination. A moderate correlation between age and baseline oral temperature was observed (r=-0.58, p=0.001, Pearson's correlation, see supplementary material), meaning older adults tended to record lower temperatures before vaccination with a possible impact on subsequent measurements after vaccination. A statistically significant, but weak, negative correlation with older age was detected for injection site pain scores following IM PanAd3-RSV prime and IM MVA-RSV boost, oral temperature after IM MVA-RSV boost and a trend for myalgia after IM MVA-RSV boost (see supplementary material).

		IM PanAd3-RSV	IN PanAd3-RSV	IM MVA-RSV	No vaccine	p-value
Study group combination [number of volunteers]:		1, 2, 7 and 9 [33]	3, 4 and 8 [27]	6 [6]	5 [6]	-
Systemic adverse events after vaccination:						
Headache	Any AE, n/N (%)	**16/33 (48.5)**	**12/27 (44.4)**	**2/6 (33.3)**	0/6 (0)	-
	Mean severity score	1.12	0.96	0.33	0	0.149 [a]
Nausea	Any AE, n/N (%)	3/33 (9.1)	**4/27 (14.8)**	0/6 (0)	0/6 (0)	-
	Mean severity score	0.09	0.37	0	0	0.466 [a]
Malaise	Any AE, n/N (%)	**9/33 (27.3)**	**6/27 (22.2)**	**3/6 (50.0)**	0/6 (0)	-
	Mean severity score	0.58	0.48	0.67	0	0.312 [a]
Myalgia	Any AE, n/N (%)	**12/32 (36.4)**	2/27 (7.4)	**2/6 (33.3)**	**1/6 (16.7)**	-
	Mean severity score	0.73	0.44	0.67	1.67	0.329 [a]
Arthralgia	Any AE, n/N (%)	**5/33 (15.2)**	1/27 (3.7)	**2/6 (33.3)**	0/6 (0)	-
	Mean severity score	0.15	0.33	0.33	0	0.417 [a]
Fever	Any AE, n/N (%)	0/33 (0)	1/27 (3.7)	**2/6 (33.3)**	0/6 (0)	-
	Mean sum of measures (°C)	255.4	254.6	255.3	254.1	0.225 [b]
Local adverse events after intra-muscular (IM) vaccination:						
Pain	Any AE, n/N (%)	**16/33 (48.5)**	n/a	**6/6 (100.0)**	n/a	-
	Mean severity score	1.24	-	3.67	-	**0.001** [a]
Induration	Any AE, n/N (%)	**5/33(15.2)**	n/a	**2/6 (33.3)**	n/a	-
	Mean sum of measures (mm)	2.27	-	27.83	-	0.635 [a]
Redness	Any AE, n/N (%)	**9/33 (27.3)**	n/a	**1/6 (33.3)**	n/a	-
	Mean sum of measures (mm)	4.58	-	24.33	-	0.664 [a]
Swelling	Any AE, n/N (%)	**16/33 (48.5)**	n/a	**2/6 (33.3)**	n/a	-
	Mean sum of measures (mm)	3.15	-	1.8	-	0.762 [a]
Local adverse events after intra-nasal (IN) vaccination:						
Pain	Any AE, n/N (%)	n/a	1/27 (3.7)	n/a	n/a	-
	Mean severity score	-	0.04	-	-	-
Discharge	Any AE, n/N (%)	n/a	**5/27 (18.5)**	n/a	n/a	-
	Mean severity score	-	0.59	-	-	-
Irritation	Any AE, n/N (%)	n/a	**5/27 (18.5)**	n/a	n/a	-
	Mean severity score	-	0.33	-	-	-

Table 6-6. Post-hoc analysis of adverse events reported after prime vaccination.
Volunteers are grouped by vaccine and not study group. The number of volunteers who reported an adverse event of any severity grade (n), and the number of volunteers who received the same vaccine (N) is shown for IM PanAd3-RSV, IN PanAd3-RSV, IM MVA-RSV prime and no vaccine. Between groups statistical tests used Kruskall-Wallis [a] and ANOVA [b]. Adverse events that occurred in ≥10% of volunteers and statistically significant results are shown in bold.

		IM MVA-RSV	IM PanAd3-RSV	No vaccine	p-value [a]
Study group combination [number of volunteers]:		1, 3, 8 and 9 [32]	2, 4 and 7 [26]	5 [6]	-
Systemic adverse events after vaccination:					
Headache	Any AE, n/N (%)	**20/32 (62.5)**	**8/26 (30.8)**	**1/6 (16.7)**	-
	Mean severity score	1.19	0.62	0.17	**0.013**[a]
	Pairwise comparisons [c]	**IM MVA-RSV vs IM PanAd3-RSV (p=0.017)** IM MVA-RSV vs no vaccine (p=0.05) IM PanAd3-RSV vs no vaccine (p=0.655)			
Nausea	Any AE, n/N (%)	**6/32 (18.8)**	1/26 (3.8)	0/6 (0)	-
	Mean severity score	0.44	0.08	0	0.113 [a]
Malaise	Any AE, n/N (%)	**21/32 (65.6)**	**6/26 (23.1)**	0/6 (0)	-
	Mean severity score	1.75	0.54	0	**<0.001** [a]
	Pairwise comparisons [c]	**IM MVA-RSV vs IM PanAd3-RSV (p=0.002)** **IM MVA-RSV vs no vaccine (p=0.008)** IM PanAd3-RSV vs no vaccine (p=0.408)			
Myalgia	Any AE, n/N (%)	**19/32 (59.4)**	**8/26 (30.8)**	**1/6 (16.7)**	-
	Mean severity score	1.7	1.12	1.17	0.094 [a]
Arthralgia	Any AE, n/N (%)	**14/32 (43.8)**	**4/26 (15.4)**	0/6 (0)	-
	Mean severity score	1.0	0.35	0	**0.014** [a]
	Pairwise comparisons [c]	**IM MVA-RSV vs IM PanAd3-RSV (p=0.02)** IM MVA-RSV vs no vaccine (p=0.051) IM PanAd3-RSV vs no vaccine (p=0.313)			
Fever	Any AE, n/N (%)	1/32 (3.1)	1/26 (3.8)	0/6 (0)	-
	Mean sum of measures (°C)	256.06	255.44	254.48	0.131 [b]
Local adverse events after intra-muscular (IM) vaccination:					
Pain	Any AE, n/N (%)	**28/32 (87.5)**	**15/26 (57.7)**	n/a	-
	Mean severity score	4.09 (4)	1.5 (2)	-	**<0.001** [a]
Induration	Any AE, n/N (%)	**9/32 (28.1)**	2/26 (7.7)	n/a	-
	Mean sum of measures (mm)	10.16 (10)	1.69 (3)	-	**0.047** [a]
Redness	Any AE, n/N (%)	**16/32 (50.0)**	**4/26 (15.4)**	n/a	-
	Mean sum of measures (mm)	11.13 (12)	3.42 (5)	-	**0.033** [a]
Swelling	Any AE, n/N (%)	**17/32 (53.1)**	**9/26 (34.6)**	n/a	-
	Mean sum of measures (mm)	19.75 (9)	3.54 (4)	-	0.429 [a]

Table 6-7. Post-hoc analysis of adverse events reported after boost vaccination.
Volunteers are grouped by vaccine and not study group. The number of volunteers who reported an adverse event of any severity grade (n), and the number of volunteers who received the same vaccine (N) is shown for IM PanAd3-RSV, IM MVA-RSV boost and no vaccine. Between groups statistical tests used Kruskall-Wallis [a] and ANOVA [b]. Pairwise comparisons for significant between group effects used Wilcoxon ranked sum tests with Bonferroni correction [c]. Adverse events that occurred in ≥10% of volunteers and statistically significant results are shown in bold.

6.5.4 *Unsolicited symptoms after vaccination*

Volunteers were encouraged to report any additional symptoms within the one-week reporting period after vaccination, irrespective of any perceived relationship to the vaccine, as unsolicited events graded for severity using the criteria defined in supplementary material. A total of 51 unsolicited adverse events were recorded and are summarised in Table 6-8 and Figure 6-3. Of note was that 5/21 younger adults who received IN PanAd3-RSV prime in 2013 reported a mild sore throat reaction within one week of vaccination. These were self-limiting, clinically insignificant and lasted 24-48hrs. With the agreement of the DSMC these reactions were considered related to IN PanAd3-RSV. No sore throat reactions were reported to any other vaccine except one after IM MVA-RSV in older adults the following year. The frequencies of unsolicited respiratory symptoms were higher after prime vaccines compared with boost, and unsolicited gastrointestinal symptoms were reported at a greater frequency following IN PanAd3-RSV prime.

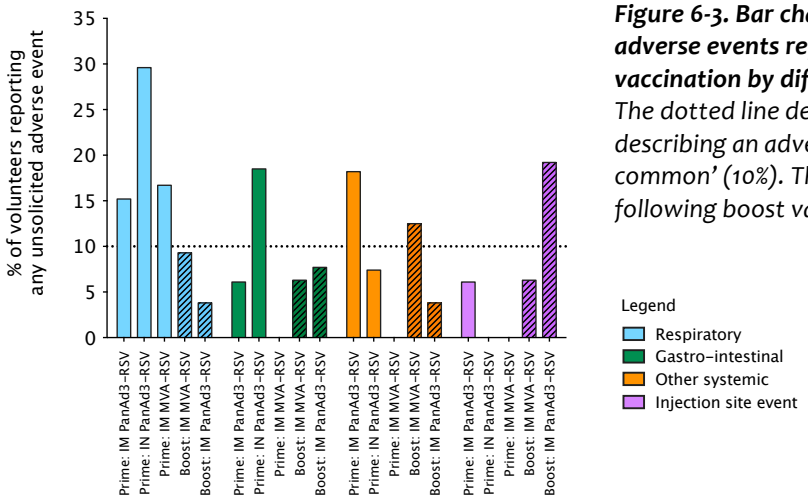


Figure 6-3. Bar chart of the frequency of unsolicited adverse events reported within one week of vaccination by different prime and boost vaccine. *The dotted line denotes the MHRA threshold for describing an adverse event frequency as 'very common' (10%). The hashed columns denote events following boost vaccines.*

	Adverse events after prime			Adverse events after boost	
	IM PanAd3-RSV	**IN PanAd3-RSV**	**IM MVA-RSV**	**IM MVA-RSV**	**IM PanAd3-RSV**
Study group combination [N total]:	1, 2, 7 and 9 [33]	3, 4 and 8 [27]	6 [6]	1, 3, 8 and 9 [32]	2, 4 and 7 [26]
Unsolicited respiratory symptoms, n/N (%):					
Runny nose	1/33 (3.0)	**3/27 (11.1)**	0/6 (0)	2/32 (6.3)	0/26 (0)
Sore throat	0/33 (0)	**5/27 (18.5)**	**1/6 (16.7)**	0/32 (0)	1/26 (3.8)
Cough	**4/33 (12.1)**	0/27 (0)	0/6 (0)	0/32 (0)	0/26 (0)
Breathlessness	0/33 (0)	0/27 (0)	0/6 (0)	1/32 (3.1)	0/26 (0)
Any unsolicited respiratory symptom	**5/33 (15.2)**	**8/27 (29.6)**	**1/6 (16.7)**	3/32 (9.3)	1/26 (3.8)
Unsolicited gastro-intestinal symptoms, n/N (%):					
Mouth ulcers	0/33 (0)	0/27 (0)	0/6 (0)	0/32 (0)	1/26 (3.8)
Taste alteration	0/33 (0)	1/27 (3.7)	0/6 (0)	0/32 (0)	0/26 (0)
Abdominal pain and/or discomfort	0/33 (0)	**3/27 (11.1)**	0/6 (0)	2/32 (6.3)	1/26 (3.8)
Diarrhoea	2/33 (6.1)	1/27 (3.7)	0/6 (0)	0/32 (0)	0/26 (0)
Any unsolicited gastro-intestinal symptom	2/33 (6.1)	**5/27 (18.5)**	0/6 (0)	2/32 (6.3)	2/26 (7.7)
Unsolicited systemic symptoms, n/N (%):					
Perspiration	1/33 (3.0)	0/27 (0)	0/6 (0)	0/32 (0)	0/26 (0)
Excessive tiredness	3/33 (9.1)	1/27 (3.7)	0/6 (0)	1/32 (3.1)	1/26 (3.8)
Disturbed sleep	1/33 (3.0)	0/27 (0)	0/6 (0)	0/32 (0)	0/26 (0)
Shivering	0/33 (0)	0/27 (0)	0/6 (0)	3/32 (9.4)	0/26 (0)
Cold-like symptoms	1/33 (3.0)	1/27 (3.7)	0/6 (0)	0/32 (0)	0/26 (0)
Any unsolicited systemic symptom	**6/33 (18.2)**	2/27 (7.4)	0/6 (0)	**4/32 (12.5)**	1/26 (3.8)
Unsolicited events at the site of vaccine injection, n/N (%):					
Ipsilateral hand pain	1/33 (3.0)	n/a	0/6 (0)	0/32 (0)	0/26 (0)
Ipsilateral shoulder pain	0/33 (0)	n/a	0/6 (0)	1/32 (3.1)	0/26 (0)
Bilateral shoulder pain	0/33 (0)	n/a	0/6 (0)	0/32 (0)	1/26 (3.8)
Ipsilateral swelling under arm	1/33 (3.0)	n/a	0/6 (0)	0/32 (0)	0/26 (0)
Itching at the injection site	0/33 (0)	n/a	0/6 (0)	1/32 (3.1)	0/26 (0)
Blueish spot developed at the injection site	0/33 (0)	n/a	0/6 (0)	0/32 (0)	1/26 (3.8)
Bruising	0/33 (0)	n/a	0/6 (0)	0/32 (0)	**3/26 (11.5)**
Any unsolicited injection site event	2/33 (6.1)	n/a	0/6 (0)	2/32 (6.3)	**5/26 (19.2)**

Table 6-8. Summary of unsolicited adverse events within one-week of vaccination.
Unsolicited adverse events by counts and proportions for each vaccine. There were no unsolicited adverse events in the 6 non-vaccinated older adult controls. An adverse event frequency ≥10% is shown in bold. n/a, not applicable.

6.6 Vaccine virus shedding after IN PanAd3-RSV prime

No shedding of the GMO PanAd3-RSV vaccine virus was detected on nasal swabs taken 3-days after IN PanAd3-RSV prime from any of the 27 volunteers in study groups 3, 4 and 8.

6.7 Influenza-like illnesses and unscheduled attendances during the trial

Volunteers were encouraged to report the onset of any influenza-like illness at any time during the trial. An influenza-like illness was described to volunteers as the onset of common cold symptoms, malaise, rhinorrhoea/congestion or any other symptom that they perceived to be the onset of a viral-like illness irrespective of severity. Up to four unscheduled visits per volunteer were allowed for nasal sampling and respiratory virus PCR diagnostics while symptomatic. Unscheduled visits were also permitted for repeated safety measures, if required.

In 2013/14, for adults aged 18-50 years a total of 27 unscheduled visits were performed for an influenza-like illness during the trial for 21/42 (50%) of vaccinated volunteers. 11/27 (41%) of visits were required within 5-weeks of receiving either the prime or boost vaccine (see Figure 6-4) with 9/11 (82%) tested positive for rhinovirus (the other two samples failing to detect any virus). There was no increased risk attributed to a particular vaccine or route of immunisation (3 following IN PanAd3-RSV prime, 2 following IM PanAd3-RSV prime, 3 following IM MVA-RSV boost and one following IM PanAd3-RSV boost). No influenza-like illnesses due to another respiratory virus occurred until mid-November when a plethora of respiratory viral pathogens were detected across all study groups for the remainder of the trial in younger adults. Only two further rhinovirus infections were detected in the winter months, and a total of four volunteers tested positive for RSV (three samples concurrent with other respiratory viruses) from 10-weeks after boost. An additional five unscheduled visits were performed for different volunteers for reasons of safety; two SAE reviews, two repeat measures of haematology/biochemistry blood results to correct laboratory error and one volunteer for additional testing. This last volunteer was noted to have a small drop in haemoglobin concurrent with a mild rise in bilirubin and, on the recommendation of the DSMC, attended for assessment of conjugated/unconjugated bilirubin and a direct Coombs test that showed no evidence for haemolysis. His haemoglobin and bilirubin returned to normal on later measures.

In 2014/15 and for adults aged 60-75 years there were a total of 24 unscheduled visits. Five were from non-vaccinated controls, and the remaining 19 episodes were from 15/24 (63%) vaccinated volunteers. Looking at the same 5-week period after vaccination, 4/24 (17%) of vaccinated volunteers reported an influenza-like illness and one tested positive for rhinovirus (two samples failed to detect any virus and one

sample was compromised by laboratory error). Overall 15/24 (63%) of nasal samples failed to detect any respiratory virus, four tested positive for rhinovirus (two from the same volunteer 6-months apart), three were compromised by laboratory error and one tested positive for influenza A (non-vaccinated control, but was flu vaccinated by his GP) and one positive for RSV that occurred 11-weeks after boost. No unscheduled visits were required for safety in older adults. A schematic for the occurrences of unscheduled visits in relation to vaccination and the time of year is shown in Figure 6-4.

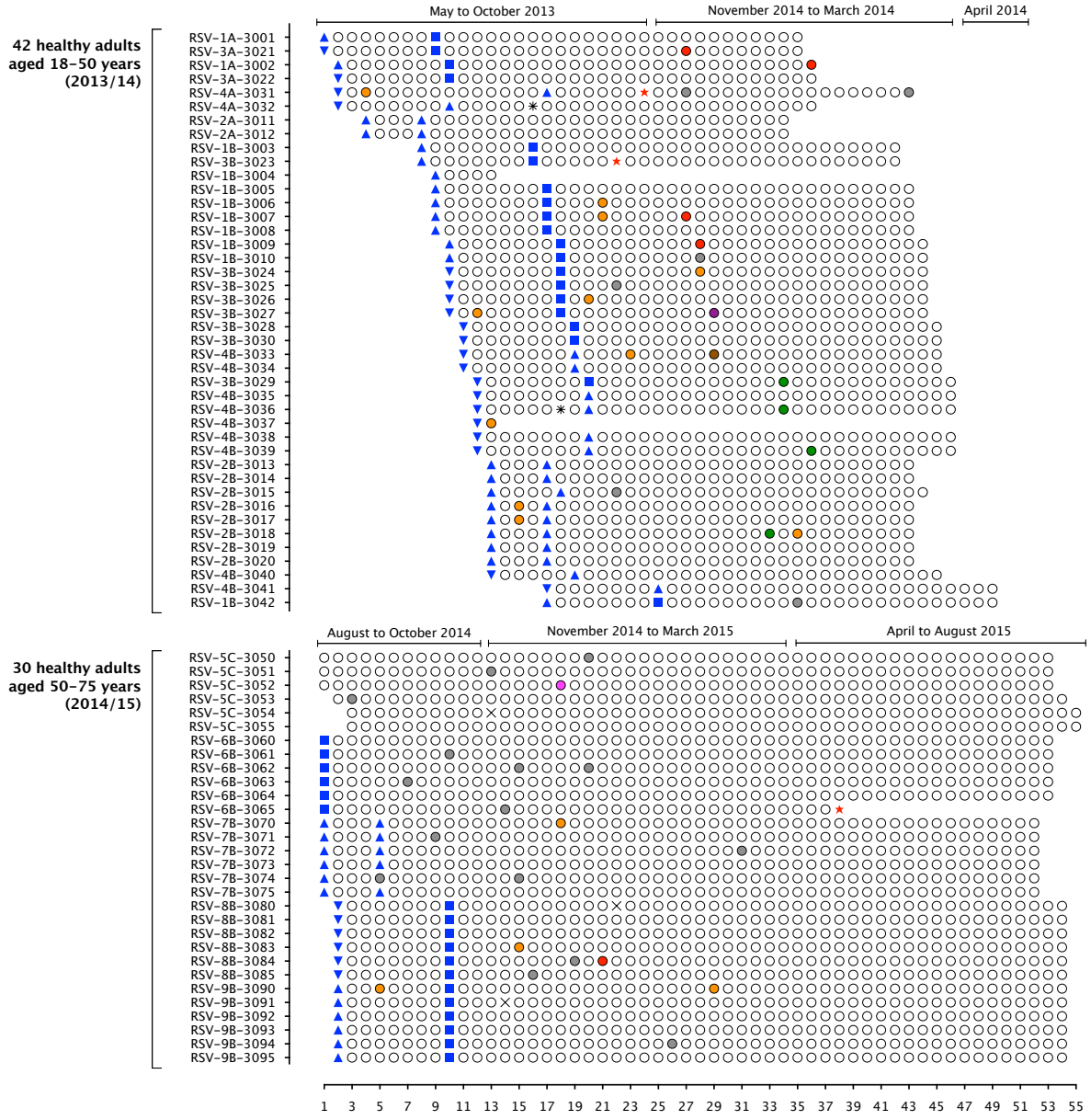


Figure 6-4. Schematic for unscheduled visits for safety and influenza-like illnesses during the RSV001 trial.

Each row represents an enrolled volunteer and each column one week relative to the first vaccination for younger and older adults respectively. Volunteer identifications are noted as RSV- (for RSV001) study group (1-9, A for-low dose, B for high-dose and C for non-vaccinated controls)- unique number. For example, volunteer RSV-4B-3401 received high-dose IN PanAd3-RSV prime and high-dose IM PanAd3-RSV boost.

6.8 Visit observations

Oral temperature, resting respiratory rate, resting heart rate and blood pressure measurements were obtained from volunteers at every clinic attendance. Adverse event severity grades were applied to these measures according to the criteria defined in supplementary material. Overall 4293/4330 (99.1%) of the expected data for visit observations were available for analysis. There were no adverse events recorded in low-dose recipients (data not shown) and the analysis was performed on all volunteers without distinction between low-dose and normal-dose vaccine (study groups 1-4; study groups 6-9 received high-dose only).

6.8.1 Baseline visit observations

The differences in baseline measures before prime and boost vaccination were not clinically significant and in cases where the baseline observations satisfied grade 1 or 2 adverse event severity criteria a repeated measure taken later during the visit was normal (data not shown). Summary statistics of the baseline and later time point measures for visit observation data are shown in supplementary material.

6.8.2 Visit observations after vaccination

PanAd3-RSV and MVA-RSV did not appear to cause any clinically significant deviations in measures of physiological function after vaccination. Severe adverse events were not considered related to vaccination. The results for visit observations made at clinic attendances before and after vaccination are shown in the supplementary figure of Appendix B.

Severe (grade 3) bradycardia was recorded from two volunteers who each recorded bradycardia prior to enrolment and had been deemed suitable to enrol. The first volunteer was an athletically fit 23 year old volunteer allocated to group 2 with sinus bradycardia on his screening ECG. The second volunteer was aged 65 years who recorded sinus bradycardia with isolated T-wave inversion in lead III on his screening ECG and had a history of asymptomatic coronary vessel disease. Private healthcare screening angiography had been performed when he retired early, 3-years before the clinical trial, and discovered a 70% stenosis of the left anterior descending coronary artery. He underwent went a successful elective stent insertion and had never developed symptoms of coronary vessel disease and received 5mg bisoprolol daily. After IM MVA-RSV his resting heart rate appeared to drop further and satisfy grade 3 severity criteria (from 56 to 44

beats/minute) and a repeat ECG was performed which confirmed his ECG findings from screening without any new abnormalities. In December 2014 (approximately 3 months after boost vaccine) he felt lightheaded on standing and was referred by his GP for specialist opinion and was diagnosed with paroxysmal atrial fibrillation. A transthoracic echocardiogram and myocardial perfusion scan were normal and he commenced warfarin therapy pending elective cardioversion. Neither volunteer with severe bradycardia was deemed to be caused or exacerbated by vaccination and no other adverse events for heart rate in other volunteers were deemed clinically significant.

One volunteer from group 6 recorded severe hypertension (systolic blood pressure >156 mmHg) at the pre-boost measure (although no boost vaccine was given for this group) and was noted to have had several elevated readings after prime. With his consent he was referred to his GP and commended anti-hypertensive therapy (amlodipine 5mg once daily). Another volunteer from group 9 recorded severe hypertension before prime and again before boost that persisted on the subsequent two recordings. His screening blood pressures were normal and with his consent he was referred to his GP for further investigation and management. The only severe systolic hypotension (76 mmHg) was recorded 14-days after IM MVA-RSV from one volunteer allocated to group 9, who was asymptomatic and the pressure recovered at the next visit. Two volunteers recorded severe diastolic hypertension (>100 mmHg); one was an isolated recording from the same volunteer with systolic hypertension before boost and the other was from a volunteer in group 3 after IM MVA-RSV boost at two time-points.

There were no recorded fevers (oral temperature ≥37.6°C) from any volunteer at any visit attendance during the trial; the only fevers after vaccination that were recorded were from volunteers at home as part of the solicited symptoms described in the last section. The results for respiratory rate measures recorded several measures that satisfied adverse event criteria, but many resolved on repeated measures at the same visit (data not shown) and none were considered clinically significant or showed any consistency within or between study groups.

6.8.3 Post-hoc analysis of visit observations safety data

No between groups post-hoc comparative analyses were performed on visit observations safety data due to the low frequency and inconsistency of adverse events after vaccination.

6.9 Safety monitoring of routine haematology and biochemistry

Routine haematology (haemoglobin, total white cells, platelets, haematocrit, red cell count, mean cell volume, neutrophils, lymphocytes, monocytes, eosinophils and basophils) and routine biochemistry (sodium, potassium, urea, creatinine, bilirubin, alanine transaminase, alkaline phosphatase, albumin, amylase and C-reactive protein) were measured from volunteers at every scheduled attendance. Adverse event severity criteria were modified Federal Drug Administration (FDA) and Division of AIDS (DAIDS) criteria defined in supplementary material. Several haematological measures (namely haematocrit, mean cell volume, lymphocytes, monocytes, eosinophil and basophil counts) did not have adverse event severity criteria assigned to them and were interpreted using the normal physiological ranges defined by the Oxford University Hospitals NHS Trust. Four pre-prime and two pre-boost haematology samples were not available due to the sample clotting or laboratory error. These samples included one volunteer from group 1, another from group 3 and two from group 4 before prime, and two from group 4 before boost. The most recent blood sample was used a surrogate for monitoring changes after vaccination during the trial, with DSMC approval.

A total of 19019/19688 (96.6%) of expected haematology and biochemistry blood results were collected and available for analysis, and the data were analysed without distinction between low-dose and normal-dose (study groups 1-4; study groups 6-9 received normal-dose only).

6.9.1 Baseline routine haematology and biochemistry measures

The pre-vaccination haematological and biochemical measures from volunteers in each study group were screened for significant variation by ANOVA and no statistically significant differences were observed, with the exception of monocyte counts before boost ($p=0.02$, one-way ANOVA). The results are summarised in supplementary material.

6.9.2 Haematological changes after vaccination

The change in haemoglobin from baseline was used for grading the severity of haemoglobin adverse events after vaccination. A drop in haemoglobin after vaccination was common and a total of 9/72 (13%) volunteers recorded a severe (grade 3) drop of 2-5 g/dL during the trial; one after IM PanAd3-RSV

prime, 3 after IN PanAd3-RSV prime, 3 after IM MVA-RSV boost and 2 after IM PanAd3-RSV boost (see Figure 6-5). One grade 4 severity event was recorded 7-days after IM MVA-RSV boost from a volunteer allocated to group 3. For this individual the haemoglobin drop of 6 g/dL (from 15.5 to 9.5 g/dL) was concurrent with a drop in the total white cell count (from 6.04 to 3.5 $x10^9$/L) and platelets (from 257 to 36 $x10^9$/L). The haematological measures returned to within the normal range at the next sample taken 14-days after vaccination. Clinical anaemia (haemoglobin <12 g/dL) developed in only a few individuals and as transient occurrences (see supplementary material). Furthermore, where IM/IN PanAd3-RSV at prime or boost (or no vaccine) was associated with a drop in haemoglobin in some individuals and not in others, the data from 3-days and 7-days recorded a drop in haemoglobin in 37/38 volunteers after a dose of IM MVA-RSV vaccine (Figure 6-5).

In total there were 5 occurrences of recorded clinical thrombocytopaenia (platelet count <140 $x10^9$/L) with two episodes qualifying as severe (<100 $x10^9$/L); one volunteer as described earlier and another volunteer from group 4 recorded an isolated drop (from 234 to 98 $x10^9$/L) 28-days after IN PanAd3-RSV prime. A mild transient drop in the total white cell count to below 3.5 $x10^9$/L was common and changes in neutrophil counts reflected this pattern. Neutrophil counts <2 $x10^9$/L (the threshold for definition as an adverse event) were most common 3-days after vaccination and frequent following IM MVA-RSV. One volunteer from group 1 developed severe neutropaenia (neutrophil count <1 $x10^9$/L) after IM PanAd3-RSV prime and again after IM MVA-RSV boost (see supplementary material). A transient rise in monocyte count was observed for several volunteers after IM MVA-RSV. The results for all haematological parameters and summary statistics are available in supplementary material.

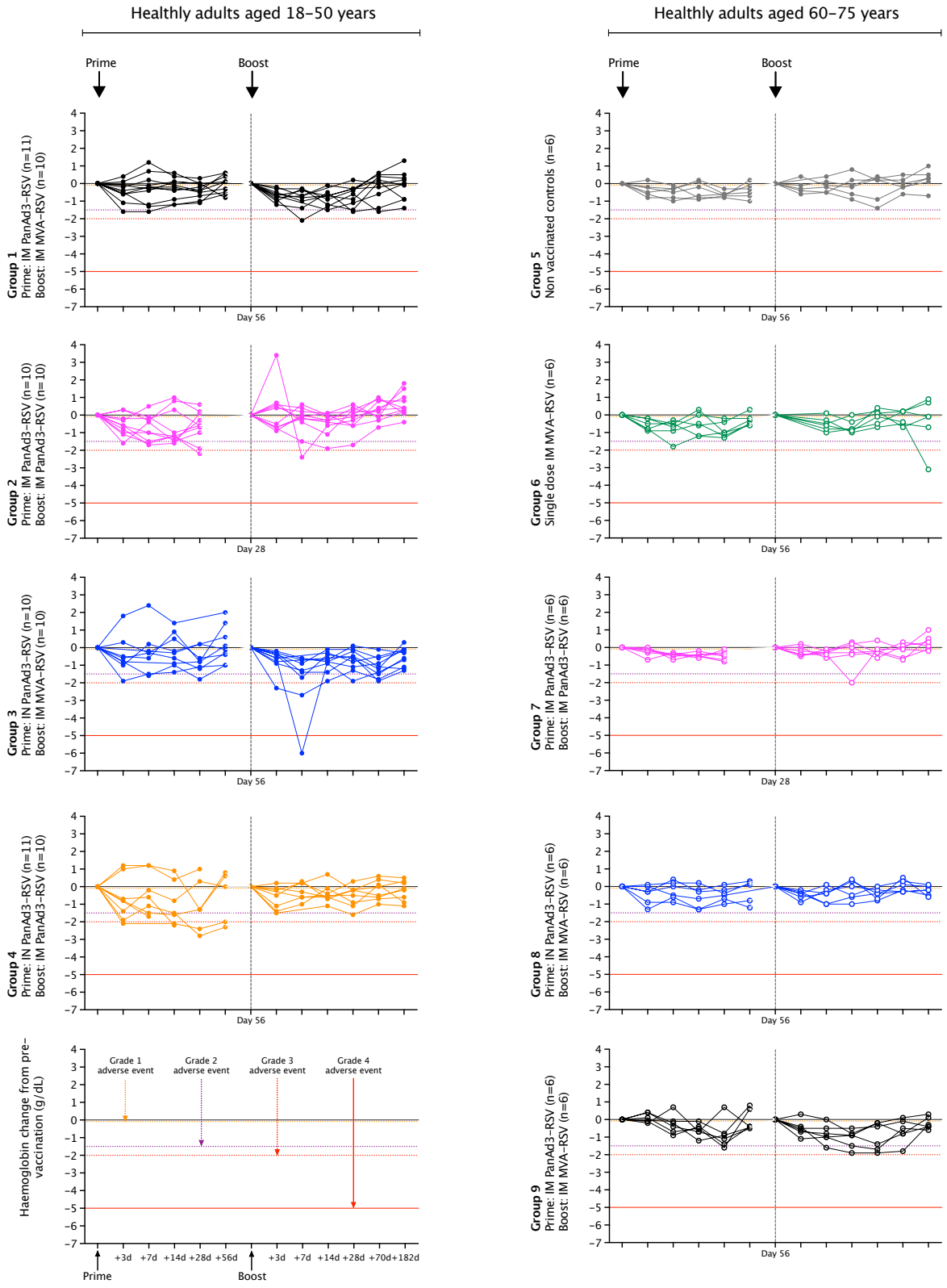


Figure 6-5. Haemoglobin changes after vaccination (g/dL).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6 that did not receive boost vaccine.

6.9.3 *Post-hoc analysis of changes in haemoglobin after vaccination*

The post-hoc analysis pooled volunteers by vaccine route and vector (and not study group). This explorative analysis concerned the observed changes in haemoglobin, white cells, neutrophils and platelets that had potential for clinical and statistical significance. Four pre-prime and two pre-boost haematology samples were not available due to the sample clotting or laboratory error (one from group 1, one from group 3 and two from group 4 before prime, and two from group 4 before boost) and these volunteers were removed from post-hoc haematology analyses.

Figure 6-6 illustrates the change in haemoglobin using the modified volunteer grouping. After prime there were no statistically different differences in mean haemoglobin changes between IM PanAd3-RSV, IN PanAd3-RSV and IM MVA-RSV at any time point after vaccination (p=0.482, one-way between groups ANOVA all time points and vaccine groups). In contrast, haemoglobin changes after boost were statistically significant across vaccine groups and time points (p<0.001, one-way between groups ANOVA all time points and vaccine groups) and this was explored by paired t-tests between vaccine groups. The results showed the magnitude of drop in haemoglobin was significantly greater after IM MVA-RSV compared with IM PanAd3-RSV at all time points up to and including 70-days after boost vaccination (Figure 6-6). At this late time-point a total of 5/32 (16%) of IM MVA-RSV boosted volunteers continued to record a drop from pre-boost baseline in excess of 1.5 g/dL (a grade 2 severity adverse event). Only the differences in mean haemoglobin drop at 7-days post-boost remained statistically significant after Bonferroni correction for multiple testing (IM MVA-RSV vs IM PanAd3-RSV boost, mean difference -0.73 g/dL, 95% CI -1.42 to -0.05). The mean drop in haemoglobin 7-days after IM MVA-RSV was -1.05 g/dL (95%CI -1.46 to -0.63, derived from 30 volunteers) compared with -0.32 g/dL after IM PanAd3-RSV boost (95%CI -0.60 to -0.04, derived from 23 volunteers), with statistical significance (difference of median 0.6, p=0.0001, two-tailed Mann-Whitney test). This haemoglobin drop 7-days after boost correlated significantly with changes in haematocrit (p<0.001 and p=0.006 for IM MVA-RSV and PanAd3-RSV respectively, two-tailed Spearman's correlation) and changes in neutrophil counts with IM MVA-RSV (p=0.001, two-tailed Spearman's correlation). There were no correlations with changes in bilirubin (p=0.434 and p=0.494 for IM MVA-RSV IM PanAd3-RSV respectively, two-tailed Spearman's correlation) or changes in

platelet count (p=0.508 and p=0.399 for IM MVA-RSV IM PanAd3-RSV respectively, two-tailed Spearman's correlation).

It was also noted that within the pooled group of volunteers who received IM MVA-RSV boost there was a progressive haemoglobin drop between 3-days and 7-days after vaccination that was statistically significant (median of differences -0.35 g/dL, p=0.007, two-tailed Wilcoxon test). The pre-boost sample blood volume was 70 and 113 mLs (in older and younger adults respectively) and only an additional 8 mLs of blood was obtained 3-days after vaccination before the next haemoglobin measure taken 7-days after vaccination (see Figure 6-6).

An analysis of covariance (ANCOVA) model was used to explore, and then adjusted for, the potential for other factors to influence the change in haemoglobin 3-days and 7-days after boost vaccination. The vector and pre-boost haemoglobin measures were found to have statistically significant effects on the magnitude of haemoglobin loss 3-days after boost vaccination (p=0.05 and p=0.008 respectively) without interaction between these factors (p=0.289). No statistical significance was found for volunteer age (p=0.289), sex (p=0.448), vaccine dose (p=0.989), prime vaccine (p=0.167) and the cumulative blood loss from sampling since starting the trial (p=0.207) and these factors were subsequently removed from the ANCOVA model. The estimated marginal means derived from the ANCOVA analysis (the mean change in haemoglobin adjusted for pre-boost haemoglobin as the only covariate) found the mean haemoglobin differences 3-days after vaccination were no longer statistically different between IM PanAd3-RSV and IM MVA-RSV. For the haemoglobin changes 7-days after vaccination the vector and pre-boost haemoglobin measures continued to exert statistically significant effects on the dependent variable (the change in haemoglobin) in the model (p=0.01 and p=0.049 respectively) without interaction between these factors (p=0.688). As before, no statistically significant effect was found for volunteer age (p=0.097), sex (p=0.704), vaccine dose (p=0.687), prime vaccine (p=0.648) and cumulative blood sample volume since screening (p=0.185). Pairwise comparisons between vaccine groups using the estimated marginal means found the greater drop in haemoglobin 7-days after IM MVA-RSV continued to reach statistical significance when compared with IM PanAd3-RSV after adjustment for the effect of pre-boost haemoglobin (Table 6-9).

		IM MVA-RSV boost	IM PanAd3-RSV boost	No vaccine
3 days after boost vaccination	Number of volunteers	32	22	5
	Estimated marginal mean for haemoglobin change (95% CI)	-0.578 (-0.814 to -0.343)	-0.188 (-0.472 to 0.096)	-0.191 (-0.783 to 0.402)
	Pairwise comparisons between vaccine groups using the estimated marginal means with Bonferroni correction	IM MVA-RSV and IM PanAd3-RSV: p=0.119 IM MVA-RSV and no vaccine: p= 0.683 IM PanAd3-RSV and no vaccine: p=1.0		
7 days after boost vaccination	Number of volunteers	30	23	6
	Estimated marginal mean for haemoglobin change (95% CI)	-1.02 (-1.348 to -0.700)	-0.346 (-0.716 to 0.024)	-0.153 (-0.875 to 0.570)
	Pairwise comparisons between vaccine groups using the estimated marginal means with Bonferroni correction	**IM MVA-RSV and IM PanAd3-RSV: p=0.024** IM MVA-RSV and no vaccine: p= 0.095 IM PanAd3-RSV and no vaccine: p=1.0		

Table 6-9. ANCOVA estimated marginal means for the change in haemoglobin 3-days and 7-days after boost vaccination.

The estimated marginal mean represents the likely mean change in haemoglobin after vaccination corrected for the effect of pre-boost haemoglobin measures. Statistically significant results are shown in bold.

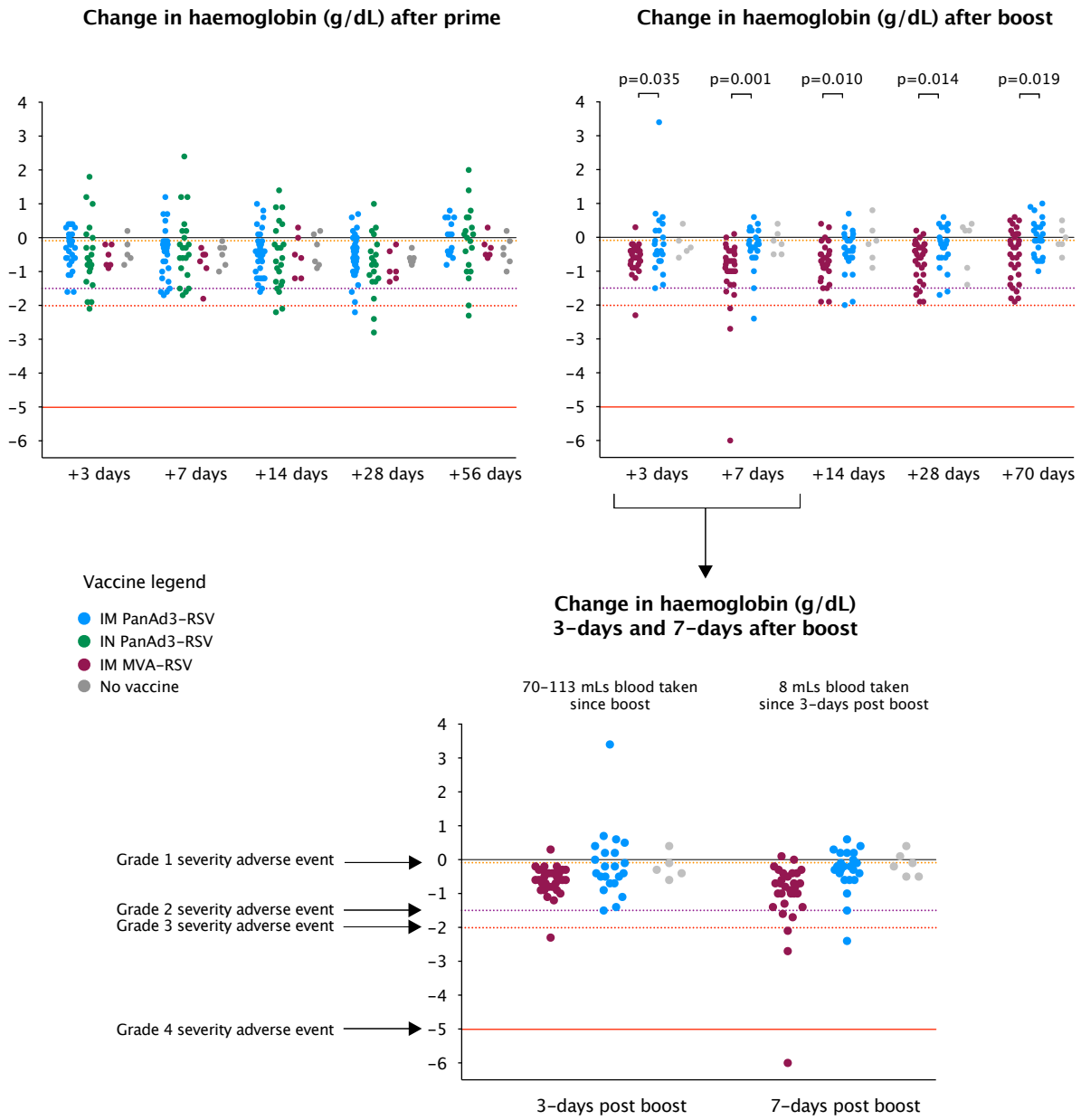


Figure 6-6. Changes in haemoglobin (g/dL) after vaccination by vaccine route and vector.
*The colour coded adverse event thresholds are the same as Figure 6-5. Volunteers were pooled by vaccine as follows; IM PanAd3-RSV (groups 1,2,7 and 9 at prime and groups 2, 4 and 7 at boost), IN PanAd3-RSV (groups 3, 4 and 8 at prime), IM MVA-RSV (group 6 at prime, groups 1, 3, 8 and 9 at boost) and no vaccine (group 5 prime and boost, group 6 after boost only). (**Top left**) A between groups one-way ANOVA found no statistically significant differences in the mean haemoglobin change after prime across vaccine groups and time points (p=0.482). (**Top right**) The p-values after boost compare responses to IM MVA-RSV and IM PanAd3-RSV at each time point using a two-tailed Mann-Whitney test without correction for multiple testing (only 7-day post boost differences remained statistically significant after Bonferroni correction for multiple tests). Differences in the mean haemoglobin change between IM PanAd3-RSV and non-vaccinated controls were not statistically significant at any time point after boost vaccination. (**Bottom figure**) Expansion of the 3-days and 7-days post boost haemoglobin drop, with reference to the observation that haemoglobin continued to drop in volunteers who received IM MVA-RSV boost despite the minimal blood sample volumes taken between 3- and 7-days and in contrast to IM PanAd3-RSV and non-vaccinated volunteers.*

6.9.4 Post-hoc analysis of changes in other haematological measures vaccination

Changes from baseline total white cell counts after vaccination reached borderline significance after prime (p=0.052, one-way ANOVA of all vaccine groups and post-prime time points) and between vaccine group pairwise testing revealed the only statistically significant differences after Bonferroni correction for multiple tests was at 3-days after vaccination for the route used to primed volunteers with PanAd3-RSV (mean difference -1.43 $x10^9$/L, 95%CI -2.76 to -0.09). Here the drop in total white cell count was significantly greater in volunteers who received IM PanAd3-RSV (mean change -0.72 $x10^9$/L, 95%CI -1.10 to -0.33) compared with the same vaccine by IN spray (mean change -0.14 $x10^9$/L, 95%CI -2.21 to 2.50) (p=0.001, two-tailed unpaired t-test). Total white cell counts recovered by 7-days after vaccination onwards. The changes in total white cell count from pre-boost measures were also statistically significant following vaccination (p=0.012, one-way ANOVA of all vaccine groups and post-boost time points). Between vaccine group pairwise testing found the mean drop in total white cell count was significantly greater in volunteers who received IM MVA-RSV compared with IM PanAd3-RSV boost (p=0.012, two-tailed unpaired t-test), but no pairwise tests remained significant after Bonferroni correction for multiple testing. As described before, an ANCOVA model was applied to the 3-days post-prime time point and found the difference in changes of total white cell counts 3-days after IM and IN PanAd3-RSV prime, when adjusted for the pre-vaccination total white cell count, remained statistically significant (p=0.021 with Bonferroni correction). The ANCOVA adjustment for pre-boost total white cell count resulted in the loss of statistical significance in total white cell count changes between IM MVA-RSV and IM PanAd3-RSV 3-days after boost (p=0.126 with Bonferroni correction). There were no significant correlations between the changes in haemoglobin and total white cell count 3-days after boost for IM MVA-RSV (p=0.955), IM PanAd3-RSV (p=0.694) and non-vaccinated controls (p=0.747) by two-tailed Spearman's test.

The changes in neutrophil count, which form the main component of the total white cell count, correlated with changes in total white cell count 3-days after prime (p<0.001 for IM and IN PanAd3-RSV, p=0.01 for IM MVA-RSV and p=0.05 for non-vaccinated controls, Spearman's correlation) and boost (p<0.001 for IM MVA-RSV and IM PanAd3-RSV, p=0.104 for non-vaccinated controls, Spearman's correlation). ANOVA of the changes in neutrophil counts after prime did not reach statistical significance (p=0.149, one-way ANOVA of all vaccine groups and post-prime time points) but pairwise comparisons

corrected for multiple testing showed that, at 3-days after prime, the fall in neutrophils was marginally greater for IM compared with IN PanAd3-RSV (mean difference -1.27 $x10^9$/L, p=0.003, two-tailed Mann-Whitney test). Investigated by ANCOVA with adjustment for the neutrophil count before vaccination, statistical significance remained for the greater magnitude of neutrophil drop when PanAd3-RSV was given by IM injection compared with IN spray at prime (p=0.03 with Bonferroni correction). The ANOVA analysis of changes in neutrophil counts after boost vaccination was significant (p=0.015, one-way ANOVA for all vaccine groups and post-boost time points). The mean drop in neutrophils was greater for IM MVA-RSV compared with IM PanAd3-RSV (difference in median 0.42, p=0.037, two-tailed Mann-Whitney test) 3-days after vaccination, although this significance was lost if corrected for multiple testing. Both IM MVA-RSV and IM PanAd3-RSV boost recorded a significant drop in neutrophils compared with the non-vaccinated group of older adults (p<0.001 and p=0.008 respectively, two-tailed Mann-Whitney test). ANCOVA analysis, with correction for the effect of pre-boost neutrophil count, found the differences in mean between IM MVA-RSV and IM PanAd3-RSV were no longer statistically significant (p=0.201 with Bonferroni correction), but the neutrophil depletion after both boost vaccines remained statistically significant compared with non-vaccinated older adults (p=0.001 and p=0.021 respectively with Bonferroni correction).

There were no discernable patterns in platelet count changes at vaccine group level after prime (p=0.325, one-way ANOVA of all vaccine groups and all time points). Significant differences were observed following boost (p=0.008, one-way ANOVA of all vaccine groups and all time points) but multiple pairwise testing failed to detect any statistically significant differences after Bonferroni correction. What appeared to be a modest excess in platelet fall 3-days after IM MVA-RSV (mean -22.0, 95%CI -35.0 to -9.1) relative to IM PanAd3-RSV (mean -10.1, 95%CI -24.1 to 3.8) was not statistically significant (p=0.215, two-tailed unpaired t-test). No ANCOVA analysis with correction for baseline platelet counts was performed. For lymphocyte counts 3-days after boost with IM PanAd3-RSV there was minor increase in lymphocytes relative to a minor drop with IM MVA-RSV (mean change from baseline 0.21 vs -0.14, p= 0.017, two-tailed unpaired t-test). A transient rise in monocyte count was observed for several volunteers after IM MVA-RSV. Analysed by vaccine group the mean rise of monocytes of 0.15 (95%CI 0.07-0.22) 3-days after IM MVA-RSV was statistically significant when compared the mean rise of 0.04 (95%CI -0.01- 0.09) after IM MVA-RSV boost (p=0.027, two-tailed unpaired t-test).

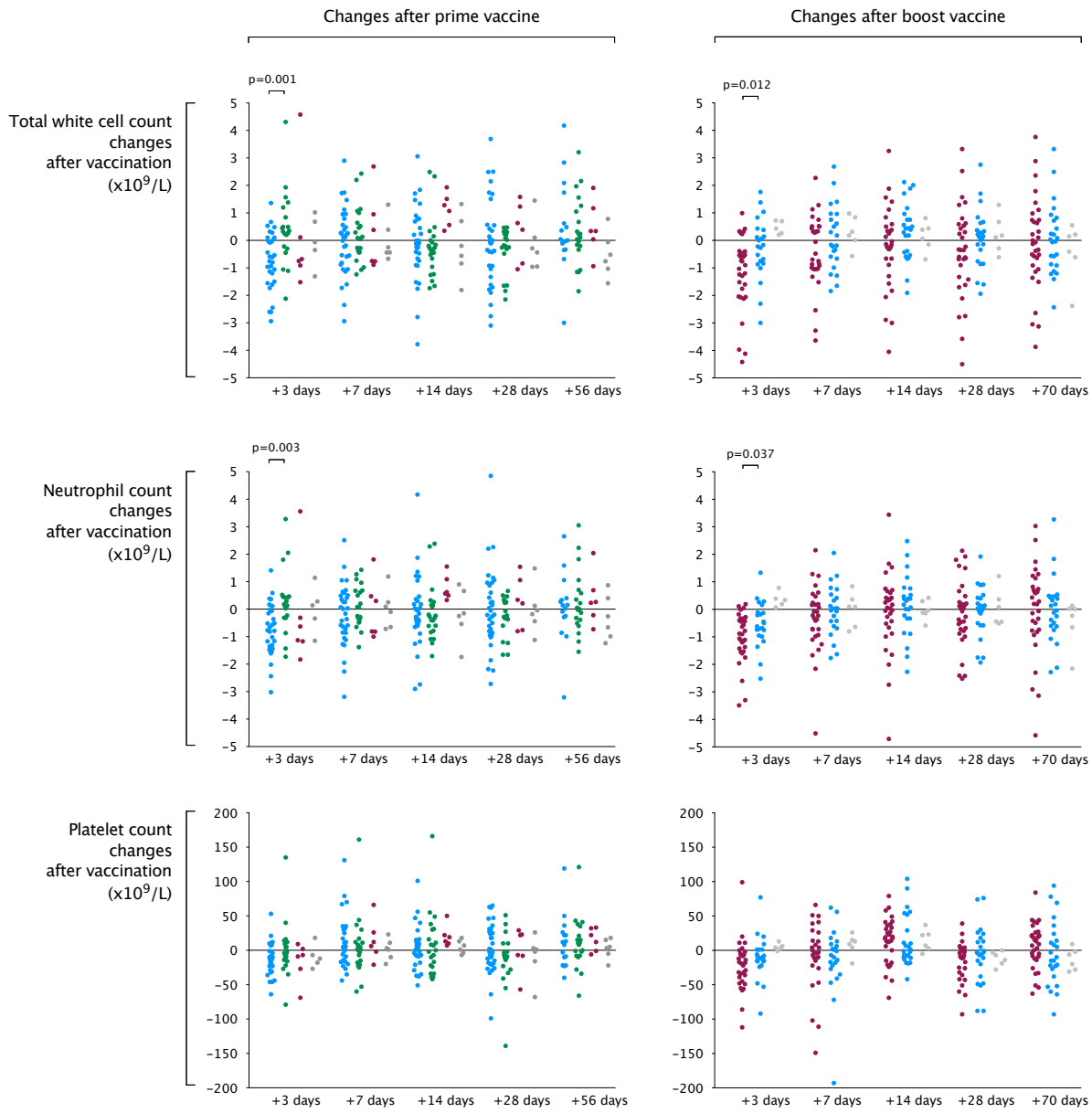


Figure 6-7. Changes in total white cell, neutrophil and platelet counts (x10⁹/L) after vaccination by vaccine route and vector.
Volunteers were pooled by vaccine as follows; IM PanAd3-RSV (groups 1,2,7 and 9 at prime and groups 2, 4 and 7 at boost), IN PanAd3-RSV (groups 3, 4 and 8 at prime), IM MVA-RSV (group 6 at prime, groups 1, 3, 8 and 9 at boost) and no vaccine (group 5 prime and boost, group 6 after boost only). The p-values after prime compare the IM and IN route of PanAd3-RSV prime. The p-values after boost compare IM MVA-RSV to IM PanAd3-RSV. Only significant results are shown and determined by a two-tailed Mann-Whitney test, without correction for multiple testing.

6.9.5 *Biochemical changes after vaccination*

Routine biochemistry blood samples were obtained from volunteers at every scheduled attendance and measured serum sodium, potassium, urea, creatinine, bilirubin, alanine transaminase, alkaline phosphatase, albumin, amylase and C-reactive protein. Adverse event severity criteria were modified Federal Drug Administration (FDA) and Division of AIDS (DAIDS) criteria defined in supplementary material. The results for all biochemical parameters and summary statistics are available in supplementary material (Appendix B). A change in the OUHL laboratory assay between 70-days and 182-days after boost in the older adult cohort meant the readings were much lower at the later time point measures.

In the opinion of the trial safety team and independent DSMC there were no biochemical safety concerns in relation to vaccination. This was despite the narrow severity grading criteria that resulted in several severe adverse events being recorded. For example, a difference in potassium of 0.5 mmol/L was the difference between no adverse event (grade 0) and a severe adverse event (grade 3) (see supplementary material). Biochemical adverse events were transient and no study or vaccine specific group effects were observed.

In summary of the renal function of volunteers, there was one grade 3 severe recording of hypernatraemia recorded at baseline for a volunteer in group 1 (sodium 149 mmol/L) and one grade 3 severe hyponatraemia (sodium 126 mmol/L) for a volunteer before boost in group 4, and both returned to within normal range at the next measure taken 3 days after vaccination. Several adverse events were recorded for sodium levels and occurred transiently in most study groups without any discernable pattern of change in relation to vaccination (see supplementary material). For potassium, several severity grades of hypokalaemia were recorded in all study groups. Grade 3 and 4 severity measures of potassium were recorded in >10% of younger adults in study groups 1-4, <10% of volunteers in older adult groups 5 and 6, and no volunteers in study groups 7-9. A total of 7 adverse event-defining rises in serum urea were recorded from 5 volunteers (4 mild, 3 moderate, none severe) and resolved. No adverse events recorded for creatinine measures after vaccination.

Liver function tests were generally unaltered after vaccination. Gilbert's syndrome had been diagnosed before enrolment, by their GP, in 3 volunteers and are the outliers seen in study groups 4, 6 and 8 in (see supplementary material). There was one grade 3 severe hyperbilirubinaemia recorded 126 days after IM MVA-RSV prime in group 6 and another 70 days after IM MVA-RSV boost in group 9, for reasons

unknown and not considered related to the vaccine. Changes in bilirubin did not bear any resemblance to the changes in haemoglobin, as described earlier. Mild (grade 1) severity elevation to ALT was present before prime in 2/5 volunteers who recorded a mild elevation to ALT during the trial and vaccination appeared to have no effect on the recorded ALT measures. No adverse events were recorded for alkaline phosphatase and only one recorded adverse event was recorded for albumin (25 g/L 28-days after IN PanAd3-RSV), which recovered and was unlikely to have any relationship to vaccination.

Finally, a total of 4 volunteers recorded episodes of amylase elevation; one moderate (grade 2) and three mild (grade 1) without any clear relationship to vaccination (although one developed 14-days after IM PanAd3-RSV prime in older adults, group 9). A short spike in C-reactive protein was observed 3-days after an injectable vaccine in most volunteers, with a greater magnitude following IM MVA-RSV.

6.9.6 Post-hoc analysis of biochemical changes after vaccination

No post-hoc analyses of the biochemical data were performed as the overall profile appeared that changes in these parameters (with the exception of the C-reactive protein) were not attributable to any vaccine after prime or boost.

6.10 Discussion

6.10.1 Summary of main findings

Development of an effective RSV and safe vaccine remains a high public health priority for infants, the elderly and immune compromised adults. This chapter details the first human data on the safety of viral-vectored vaccines expressing RSV proteins in healthy adults. Overall the vaccines were safe and well tolerated, with the most common adverse events having been mild/moderate local reactions to the site of vaccine injection. The reported rates of local and systemic adverse events after PanAd3-RSV and MVA-RSV vaccination were consistent with reported rates from similar vaccines and the transient nature and severity of these adverse events did not give cause for concern about developing these vaccines towards infants and the elderly with co-morbid conditions. Several other clinical trials of heterologous adenovirus/MVA vaccination have also recorded higher rates and severity of adverse events to the MVA vector, and volunteers were not warned of this to avoid any reporting bias[389]. There was no increased reporting of solicited symptom scores in the 'double-prime' adenovirus-vector study groups to suggest receipt of the adenovirus-vectored vaccine at prime precipitated an increase of reactogenicity at boost with the same vaccine by the same route. The observation of subclinical MVA-associated transient drops in haemoglobin shortly after vaccination has not been described in the published literature before, and was not a safety concern in this study population.

6.10.2 Mucosal immunisation

Intra-nasal live-attenuated RSV vaccines have been reported to cause nasal congestion in infants (see section on live-attenuated vaccines, page 64). It is unclear if these symptoms were a manifestation of local replication of attenuated vaccine virus, or whether the mucosal immune response was a contributory factor. Irrespective of the mechanism, infants are obligate nasal breathers and therefore any local adverse events to intra-nasal immunisation are likely to constitute an absolute bar for further development towards an infant population. Healthy adults failed to report nasal congestion in relation to live-attenuated RSV vaccine and close observation for local adverse events to intra-nasal routes of immunisation are mandatory through age de-escalation trials intended for use as vaccine in infants. Intra-nasal PanAd3-RSV was generally very well tolerated but caused a proportion of younger adult volunteers to develop a mild, self-

limiting sore throat related to vaccination. The occurrence of a sore throat was an unsolicited adverse event that did not require reporting and therefore the true incidence may have been higher. The reasons for this unexpected adverse event are unknown and may have been a non-specific effect of the mucosal immune response, but importantly no vaccine virus shedding was detected from any volunteer and only one older adult reported a sore throat reaction after IM MVA-RSV.

6.10.3 Rhinovirus infection after RSV vaccination

RSV infection has not been associated with an increased risk of another respiratory viral infection in the weeks after exposure. The high rates of symptomatic rhinovirus infection in younger adults in 2013 showed no predilection for any particular route or vector and rhinovirus infection after vaccination was not observed the following year and older adults. Furthermore it was likely that there was a reporting bias towards the immediate post vaccination period, when there were more frequent scheduled attendances required for sampling the immune response to vaccination, which contributed to the detection rate of rhinovirus and influenza-like illnesses after vaccination. Although 20-40% of bronchiolitis cases are attributable to respiratory viruses other than RSV, such as rhinovirus or co-infections with RSV, the lack of rhinovirus infection in the subsequent year suggested a seasonal exposure to rhinovirus in the summer of 2013 and not a vaccine-associated risk. Respiratory sampling of symptomatic upper respiratory tract infections of vaccine recipients will be required in future trials to evaluate this further.

6.10.4 Haematological changes after vaccination

In-depth characterisation of clinical and sub-clinical changes in haematological parameters following adenovirus and MVA-vectored vaccination is surprisingly absent from the published literature. Recent adenovirus-vectored vaccine trials with Ebola antigen reported the occurrence of transient neutropaenia and lymphopaenia in 5-20% and 11-50% of healthy adults respectively, although this was often observed in placebo study groups[394-396,438]. Asymptomatic prolongation of the activated partial thromoplastin time (or aPTT) was reported in a total of four volunteers that was further investigated and was attributed to the induction of anti-phospholipid antibody and *in vitro* prolongation of the aPTT, and not indicative of *in vivo* vaccine-induced coagulopathy[393,394,438]. The rates of neutropaenia attributed with PanAd3-RSV would be consistent with the rates published from recent adenovirus vaccines for Ebola. Data

on neutropaenia and other haematological changes after adenovirus vaccination to other infectious diseases are lacking in the literature, and for Ebola the rates of haemoglobin change after adenovirus-vectored vaccination were rarely reported and of no clinical significance[438].

Despite the extensive use of MVA in multiple vaccines formulations I could find no detailed description of changes in haematological parameters after vaccination. This may be because adverse haematological events were frequently below the clinical threshold for reporting according to Federal Drug Administration (FDA) or Division of AIDS (DAIDS) criteria (see supplementary material) or, in the opinion of the investigators, not clinically significant or important enough to report within the constraints of reporting clinical adverse events and immunogenicity data. Indeed the first paper that described the safety and immunogenicity of PanAd3-RSV and MVA-RSV in man, written by myself and others, drew little attention to these observations of an MVA-associated excess of haemoglobin depletion relative to an adenovirus vector expressing the same vaccine antigen[1]. The post-hoc use of an ANCOVA model to identify and then statistically correct for other factors that contributed to the magnitude of haemoglobin change appeared to confirm an MVA-specific effect on vaccinated volunteers. The absence of concurrent changes in platelets and bilirubin has offered some reassurance that this was not representative of haemolysis or coagulation, but samples were not obtained for direct or indirect measures of clotting activity. The estimated mean haemoglobin loss between 0.7 and 1.4 g/dL 7-days after IM MVA-RSV was not clinically hazardous for otherwise healthy adults but confirmation and investigation of these observations is warranted in the consideration of developing MVA for use in infants and the elderly with co-morbidities and lower baseline haemoglobin measures[431].

6.10.5 General limitations

The use of a small number of carefully pre-selected healthy adults is a requirement for phase I first-in-man clinical trials. This introduced an inevitable inherent risk of confounding chance observations and limitations on extrapolating these data to a wider population. A larger population of healthy adults and other populations (adults with co-morbidities or infants, for example) may reveal adverse safety signals that failed to manifest in early clinical evaluation. Nonetheless these data are useful in the development of vaccine for use in target populations and informing the design of future trials. A comprehensive

interpretation of these early clinical data would have benefited from a non-vaccinated control group, and this was only introduced when the trial was extended to include a cohort of adults aged 60-75 years.

PanAd3-RSV and MVA-RSV were tested in an open-label study design, which might have led to adverse event reporting bias by volunteers. Furthermore, the nasal sampling from volunteers with an influenza-like illness was not intended to infer anything regarding potential protective efficacy of these vaccines, but was used to support the distinction of whether laboratory measures of RSV-specific immune responses reflected a response to vaccine or natural boost. The estimated annual rate of infection is 9-11% and typically over the winter months, but asymptomatic infection is not uncommon and would not have been captured using this method of surveillance[115,116]. To explore protective efficacy would require investigation on a much larger scale with a control group, or could be more efficiently investigated by deliberate infection in a controlled human challenge model.

6.10.6 Conclusions

PanAd3-RSV and MVA-RSV were safe and well tolerated in healthy adult and healthy elderly volunteers. No vaccine-related serious adverse events or medically significant vaccine-related adverse events were recorded that would bar the development of these vaccine towards populations in need of a safe and effective RSV vaccine.

Chapter 7: Analysis of the immune response to vaccination

7.1 Introduction

As reported in Section 5.2, adenovirus and MVA viral-vectored vaccines used in homologous and heterologous prime/boost combination have registered potent immune responses to several vaccine targets. Although immune correlates of protection from RSV remain unknown, the preclinical results of PanAd3-RSV and MVA-RSV reported sterilising immunity, broad induction of antibody and cell-mediated immunity, and no evidence of ERD immunopathology under experimental challenge conditions. The aim of this chapter is to provide the results and analysis of the secondary objectives and endpoints of the clinical trial, namely the immune responses to vaccination and post hoc explorative analyses of these responses. The results from younger adults (study groups 1-4) have been published and this chapter explores these findings in more detail, with many additional experiments, and includes the data from older adults (study groups 5-9)[1h].

[h] Short sections of this chapter copied from the first publication of results from the RSV001 trial represent my own work.

7.2 Nasal RSV F protein specific IgG and IgA antibody

Nasal samples were obtained from volunteers before each dose of vaccine and again 14-days and 28-days later (plus an additional sample 70-days post boost). The levels of anti-F IgG and IgA were determined using an ELISA assay as described earlier. Nasal samples from younger adults (study groups 1-4) at vaccination and two weeks later were available for analysis (n=42 volunteers at prime) and data were analysed without distinction for low-dose and target-dose vaccine recipients.

7.2.1 Analysis of baseline anti-F IgG and IgA nasal antibody

The pre-prime measures of anti-F IgG and IgA antibodies were representative of the baseline mucosal immune response to RSV derived from repeated natural seasonal exposure. The results shown in the figure below showed that immune responses were detectable in all volunteers without statistically significant differences between study groups before vaccination.

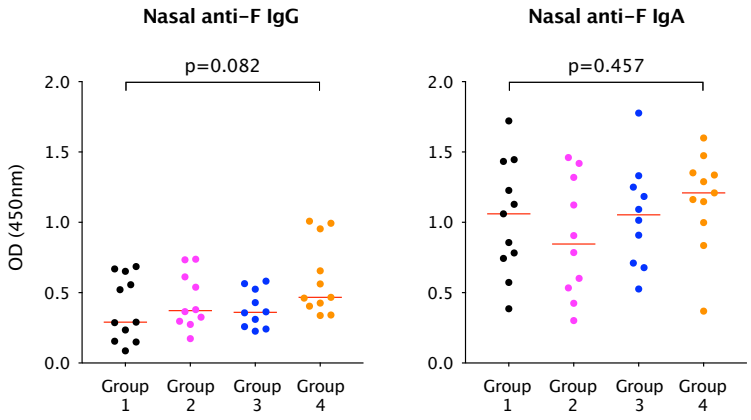


Figure 7-1. Nasal F-specific IgG and IgA antibody from baseline natural exposure.
*ELISA data from adults aged 18-50 years only (study groups 1-4). The red bar denotes the mean optical density (OD) measure for each study group. (**Left**) Anti-F IgG antibody and (**right**) anti-F IgA antibody from nasal samples collected at baseline. The p-values represent the one-way ANOVA analysis between study groups.*

7.2.2 The F-protein specific antibody response to vaccination from nasal samples

The results shown in Figure 7-2 indicated no discernable or consistent change in nasal antibody after vaccination. The IN route of PanAd3-RSV did not appear to induce changes in antibody two-weeks after prime, and IM PanAd3-RSV induced a significant IgG rise in volunteers from group 2 (and not group 1).

Boost vaccine also failed to record significant changes in antibody, and a drop in IgA was observed from volunteers of group 3 (IN PanAd3-RSV prime/IM MVA-RSV boost).

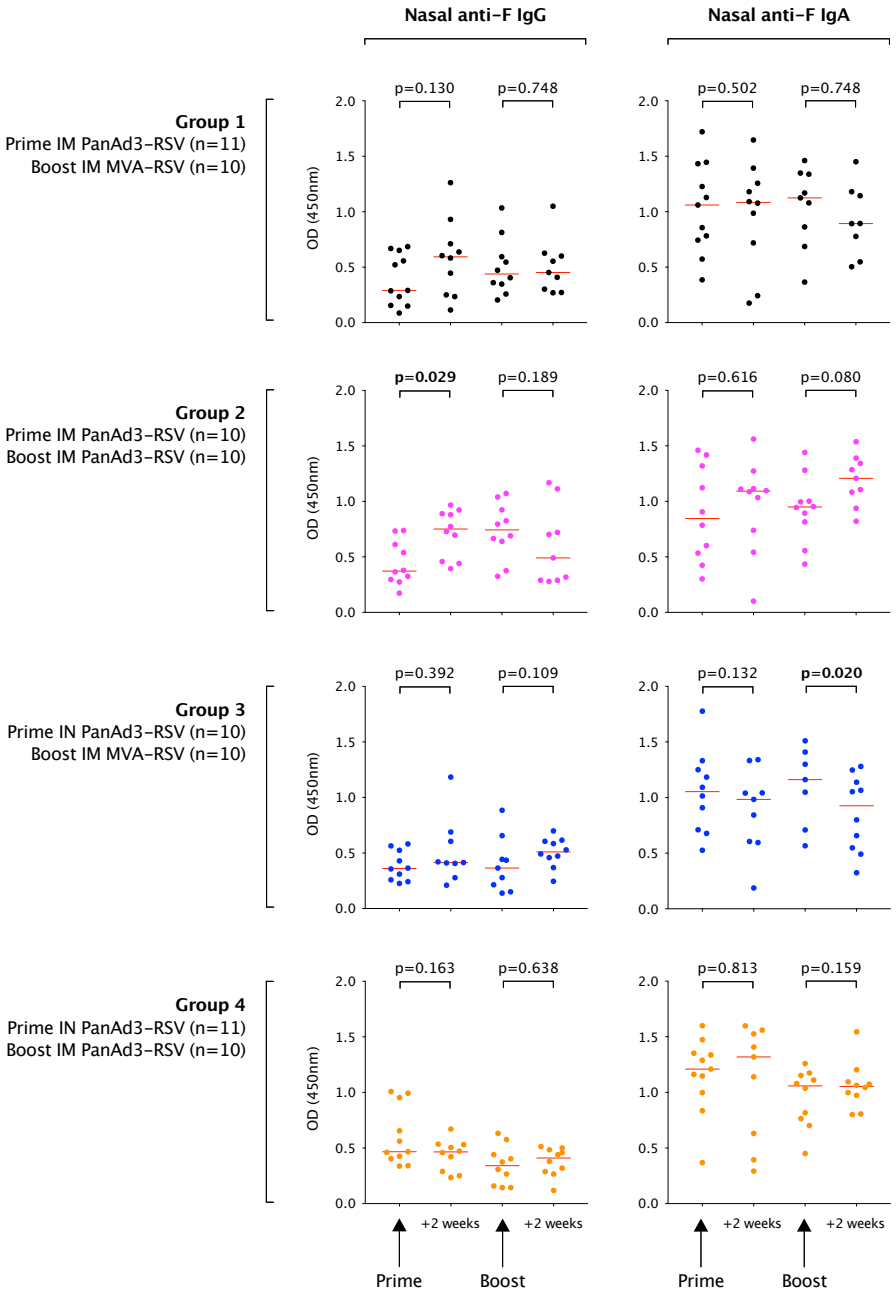


Figure 7-2. Nasal F-specific IgG and IgA antibody response to vaccination.
*ELISA data from adults aged 18-50 years only (study groups 1-4) without distinction for low-dose and target-dose vaccine recipients. The red bar denotes the mean optical density (OD) measure for each study group. (**Left column**) Anti-F IgG antibody and (**right column**) anti-F IgA antibody from nasal samples collected at baseline, two weeks after prime, at boost and two weeks after boost. A two-way paired t-test was performed within each group to determine statistical significance in changes of antibody response after each dose of vaccine. The only comparisons to reach statistical significance (shown in bold) was an increase in anti-F IgG antibody 2-weeks after IM PanAd3-RSV prime in group 2 and a decrease in anti-F IgA antibody 2-weeks after IM MVA-RSV boost in group 3 (primed with IN PanAd3-RSV). No corrections were made for multiple testing.*

7.3 Serum anti-PanAd3 (vector) neutralising antibody

Serum obtained before prime and before boost was used for *in vitro* quantification of anti-PanAd3 (vector) neutralising antibody titres using the secreted alkaline phosphatase (SEAP) assay described earlier. Only samples from younger adults who completed the trial were available for analysis (n=40 volunteers) and antibody titres were measured before prime and boost vaccine doses only.

7.3.1 Analysis of the baseline serum PanAd3 vector neutralising antibody titres

The median baseline antibody titre was 120 (IQR 9-254; GMT 87, 95% CI 50-152) and 15/40 (38%) of volunteers recorded a titre >200. There was no correlation between baseline antibody titre and volunteer age (Figure 7-3). Volunteers were not screened for anti-PanAd3 neutralising antibody titres before group allocation. The proportion of volunteers with titres >200 and overall antibody titres in volunteers allocated to receive IM PanAd3-RSV prime was lower than volunteers allocated to receive the prime by intra-nasal spray (difference in median 165.5, p=0.043, two-tailed Mann-Whitney test).

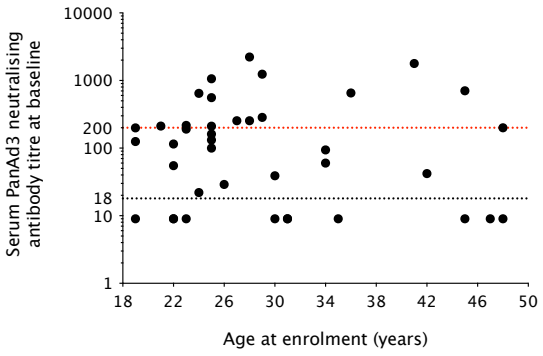


Figure 7-3. Titres of baseline serum anti-PanAd3 (vector) neutralising antibody from natural exposure correlated with volunteer age at enrolment.
Data for adults aged 18-50 years only. Each dot represents the measured titre for one volunteer at baseline correlated with volunteer age. The red dotted line marks a titre of 200, and the black dotted line was the lower limit of detection for the assay (a titre of 18). There was no correlation between log_{10} transformed antibody titres and volunteer age (r=-0.18, p=0.604, two-tailed Pearson's correlation).

7.3.2 *The serum PanAd3 vector neutralising antibody response to IM/IN PanAd3-RSV prime*

The intra-muscular route of PanAd3-RSV prime induced a significant rise in serum vector neutralising antibody titres when intra-nasal vaccination did not (Figure 7-4). Log_{10} transformed serum anti-PanAd3 antibody titres increased an average 1.62 fold over baseline for IM primed volunteers (95% CI 1.3 to 1.9), compared with an average 1.07 fold change over baseline after IN PanAd3-RSV (95% CI 1.0 to 1.1). No dose effect was observed and there was no significant difference in the mean fold-change of antibody 4-weeks and 8-weeks after IM prime in groups 2 and group 1 respectively. The magnitude of PanAd3 antibody induction did not correlate with volunteer age and a trend was observed for greater induction of vector neutralising antibody responses with lower baseline titres. At the time of boost, median titres after IM and IN PanAd3-RSV prime were 565 (IQR 159-1510; GMT 344, 95% CI 148-802) and 247 (IQR 58-746; GMT 193, 95% CI 86-433) respectively. The differences were not statistically significant (difference in median 318, p=0.242, two-tailed Mann-Whitney test). The proportion of volunteers with titres >200 increased to 75% following IM PanAd3-RSV, which made the overall proportions of volunteers with titres >200 comparable in all study groups before boost.

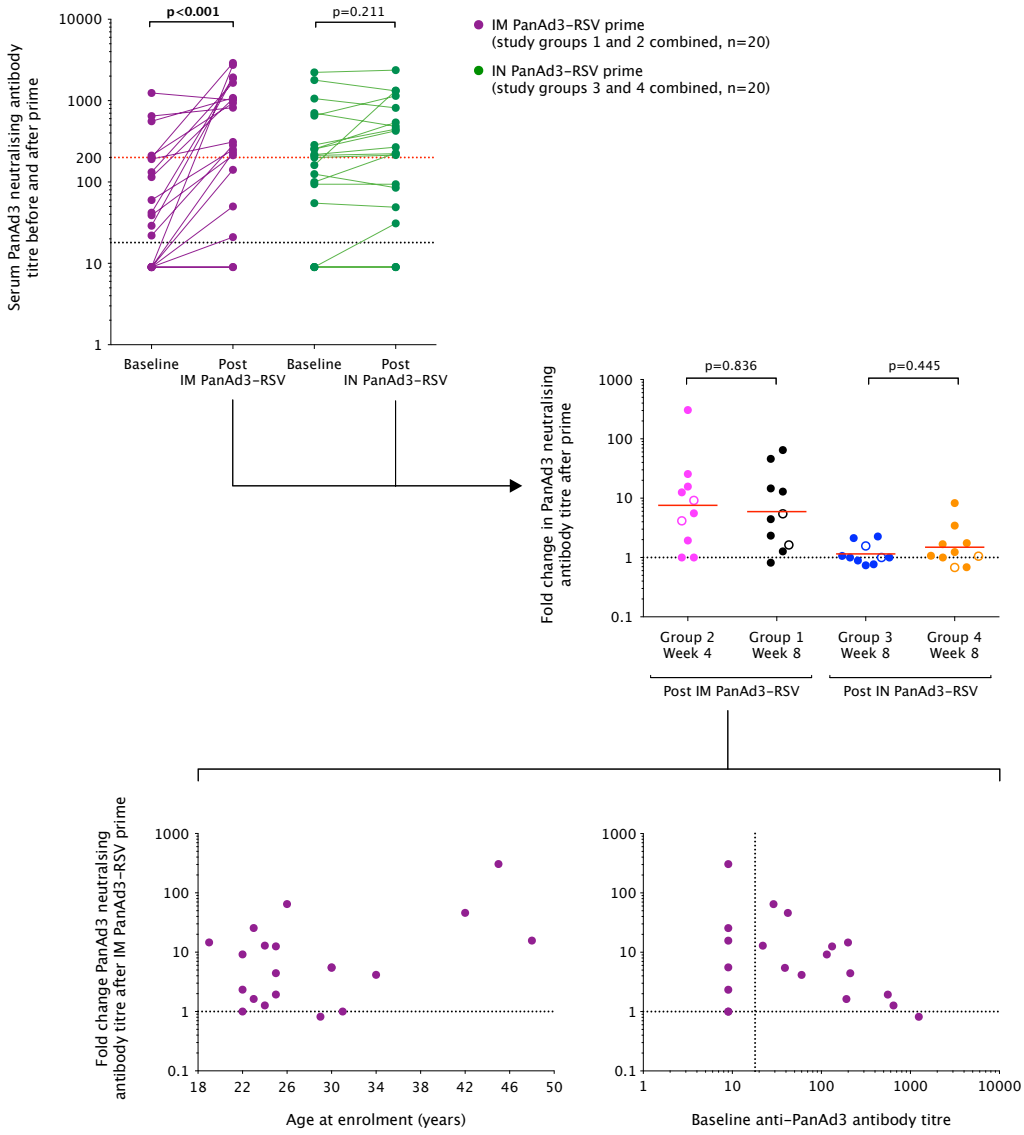


Figure 7-4. Serum anti-PanAd3 (vector) neutralising antibody response to IM and IN PanAd3-RSV prime vaccination.

*Serum PanAd3 vector neutralising antibody titres measured by SEAP assay. Data for adults aged 18-50 years before prime and before boost vaccine. (**Top left figure**) The absolute antibody titres from volunteers pooled by the route of prime. Each linked dot represents the titre for one volunteer at baseline and after prime vaccination (measured before boost 4- or 8-weeks later). The red line marks a titre of 200 and the black line shows the lower limit of detection for the assay (a titre of 18). Baseline measures were statistically significant between IM/IN groups (p=0.043, two-tailed Mann-Whitney test). After IM prime antibody titres increased significantly (p<0.001, two-tailed Wilcoxon test). After IN prime there were no significant changes in antibody titres measured 8-weeks later (p=0.211, two-tailed Wilcoxon test). Pre-boost titres were not statistically different between IM and IN primed volunteers (p=0.242, two-tailed Mann-Whitney test). (**Middle right figure**) The fold-change in neutralising antibody from baseline by study group. The red bar denotes the mean geometric fold-change and empty circles are volunteers who received a low-dose of vaccine. The fold-change in antibody titres were not significant 4- and 8-weeks after IM prime between study groups 1 and 2 and between groups 3 and 4 measured 8-weeks after IN prime (p=0.836 and p=0.445 respectively, two-tailed Mann-Whitney test). (**Bottom left figure**) The fold-change in antibody after IM prime did not correlate with volunteer age (r=0.235, p=0.3172, two-tailed Spearman's correlation). (**Bottom right figure**) The vertical dotted line denotes the lower limit of detection for the assay (a titre of 18). The fold-change in antibody after IM prime observed a negative trend with baseline antibody titres that did not reach statistical significance (log_{10}-transformed data, r^2=0.14, p=0.110, two-tailed Pearson's correlation).*

7.4 Serum RSV F protein specific IgG and IgA antibody

Serum samples for the detection of F-specific IgG and IgA antibody were obtained from all volunteers before each dose of vaccine and again 14-days and 28-days later. Antibody quantitation was performed using an ELISA assay described earlier. Serology from younger adults (study groups 1-4) were available for analysis (n=42 volunteers at prime). Data were analysed without distinction for low-dose and target-dose vaccine recipients.

7.4.1 Analysis of baseline serum anti-F IgG and IgA antibody

As expected, volunteers recorded RSV F-protein specific antibody before vaccination as a result of repeated seasonal exposure to the virus. Although the variability of IgA antibody was greater than IgG there were no statistically significant differences in the mean antibody measures between study groups (p>0.05, one-way ANOVA).

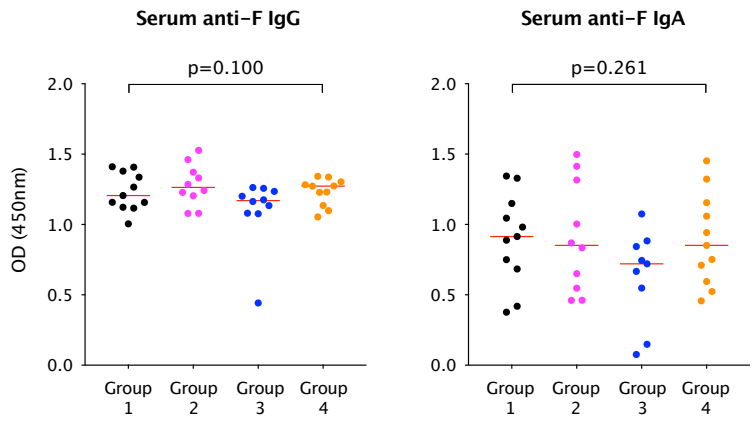


Figure 7-5. Serum F-specific IgG and IgA antibody from baseline natural exposure.
*ELISA data from adults aged 18-50 years only (study groups 1-4). The red bar denotes the mean optical density (OD) measure for each study group. (**Left**) Anti-F IgG antibody and (**right**) anti-F IgA antibody from serum samples collected at baseline. The p-values represent the one-way ANOVA analysis between study groups.*

7.4.2 *The F-protein specific antibody response to vaccination from serum*

The results are shown in Figure 7-6. From the analysis of the anti-F IgG antibody response to vaccination there was a modest but significant rise from volunteers in group 1 measured two-weeks after IM PanAd3-RSV prime (p=0.020, two-tailed paired t-test). There was no similar observation from volunteers in study group 2 who received the prime by the same route, or from volunteers in groups 3 and 4 who received IN PanAd3-RSV prime. Following boost there was only one significant change, with volunteers in group 4 recording higher antibody measures two-weeks after IM PanAd3-RSV boost (p=0.005, two-way paired t-test).

The results from F-specific IgA antibody analysis were more interesting and appeared to indicate a pattern of antibody dependency on the first dose of IM vaccine. Volunteers from both study groups to receive IM PanAd3-RSV prime (groups 1 and 2) recorded a significant rise in serum anti-F IgA two-weeks after prime (p=0.008 and p=0.028 respectively, two-way paired t-tests). For these study groups there were no further significant changes in IgA antibody after boost, irrespective of the boost vector. This pattern was reversed for study groups 3 and 4 who received IN PanAd3-RSV prime. Here there were no significant changes in serum IgA after intra-nasal prime in either group 3 or 4, but a statistically significant rise was detected two-weeks after the boost (and first IM) vaccine (p=0.001 and p=0.007 for groups 3 and 4 respectively, two-tailed paired t-tests). The choice of boost vector appeared irrelevant for this observation of IgA increasing after boost. A further and more detailed characterisation of the serum antibody response to vaccination was to measure antibody–mediated RSV-neutralisation by plaque-reduction neutralisation assay, as detailed in the next section.

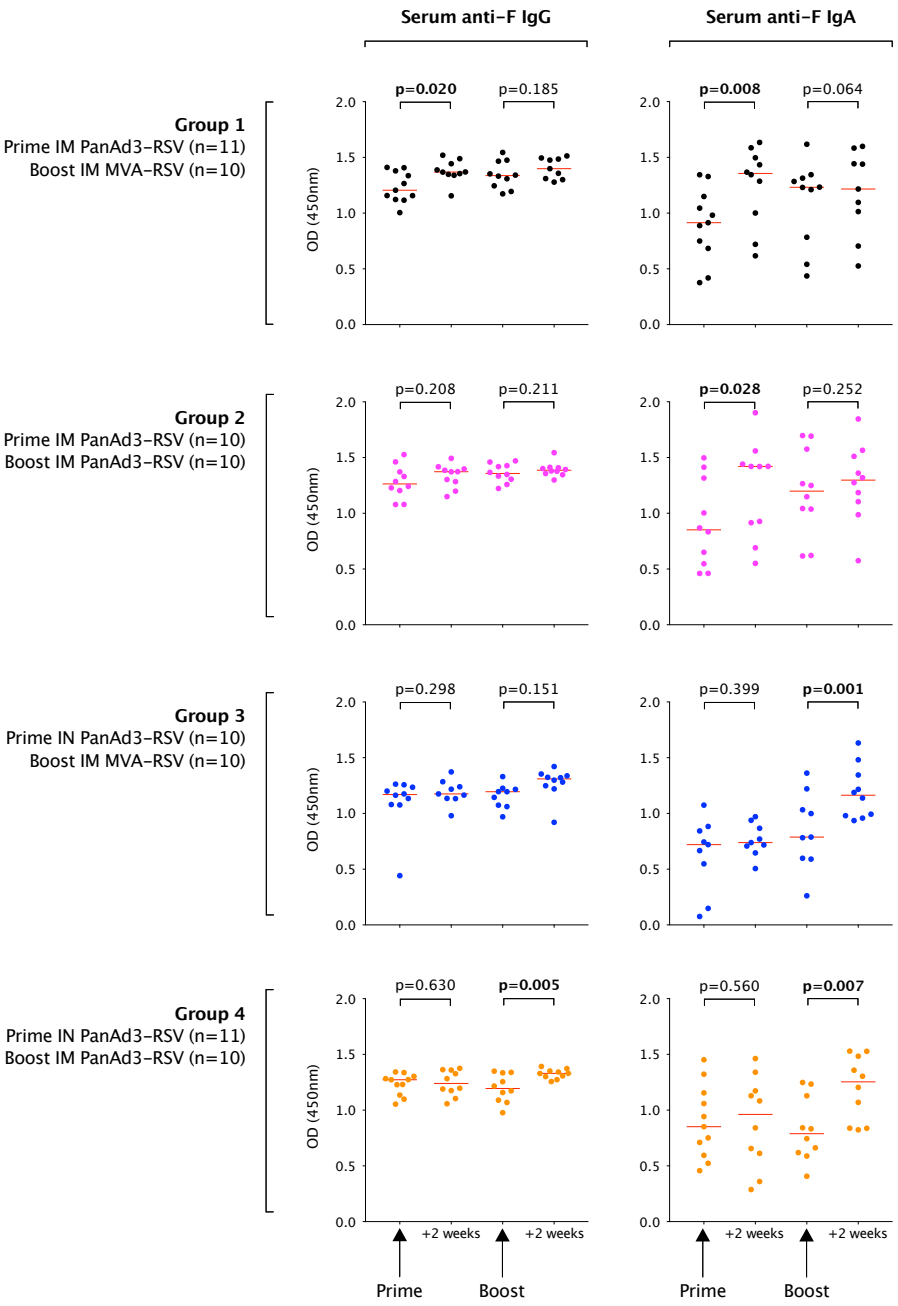


Figure 7-6. Serum F-specific IgG and IgA antibody from baseline natural exposure.
*ELISA data from adults aged 18-50 years only (study groups 1-4) without distinction for low-dose and target-dose vaccine recipients. The red bar denotes the mean optical density (OD) measure for each study group. (**Left column**) Anti-F IgG antibody and (**right column**) anti-F IgA antibody from serum samples collected at baseline, two weeks after prime, at boost and two weeks after boost. A two-way paired t-test was performed within each group to determine statistical significance in changes of antibody response after each dose of vaccine. The statistical comparisons that reached significance are shown in bold).*

7.5 Serum RSV-neutralising antibody

A plaque-reduction neutralisation assay was used for the *in vitro* measurement of serum RSV-neutralising antibody responses to vaccination. The assay was described earlier. Sera were obtained before prime and then 2- and 4-weeks after prime, and before boost and then 2-, 4-, 10- and 26-weeks after boost. Only samples from younger adults were available for analysis and samples from one volunteer was not available (total for analysis n=39 volunteers aged 18-50 years). In total, 12/60 (20%) of plates failed by either positive or negative control criteria. Frozen sera were used to repeat the failed plates and generate a complete data set from the 39 volunteers.

7.5.1 Analysis of the baseline serum RSV-neutralising antibody titres

The baseline antibody titres are indicative of the background humoral immunity to RSV derived from repeated natural exposure. There were no statistically significant differences in baseline RSV-neutralising antibody titres between study groups and the geometric mean titre (GMT) from all volunteers combined was 2788 (95% CI 2336 to 3323). No significant correlations between serum RSV-neutralising antibody titres and volunteer age or vector neutralising antibody titres were observed (Figure 7-7).

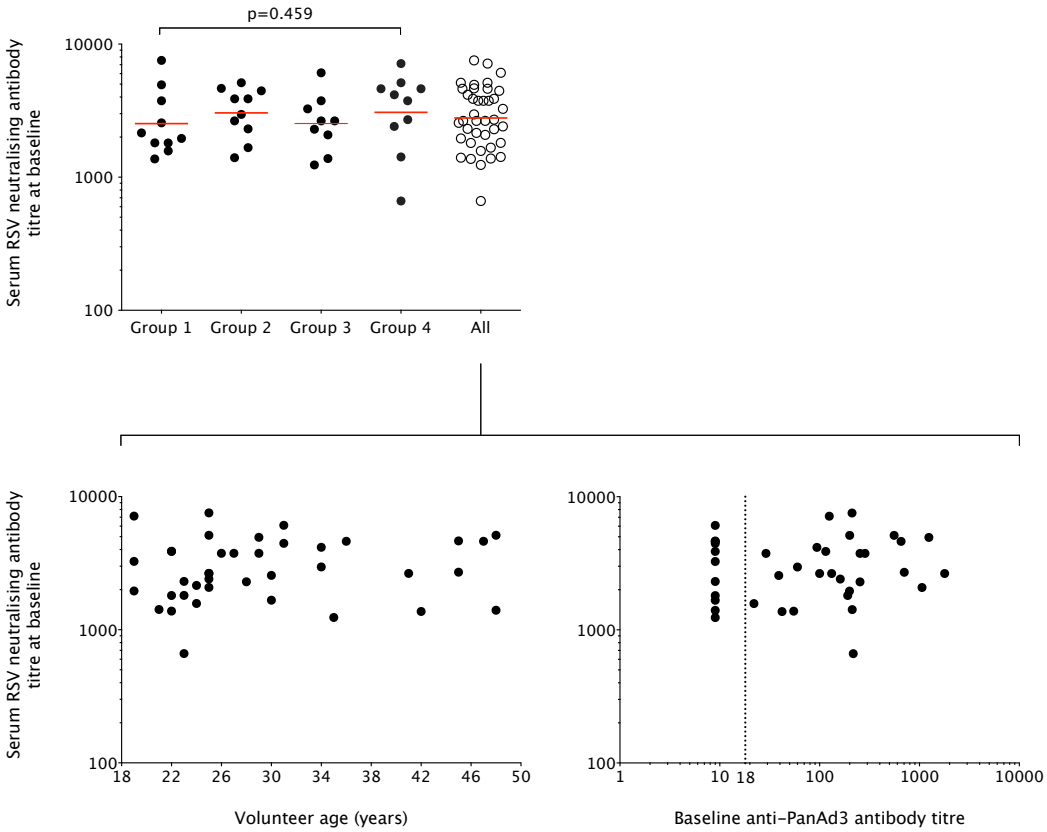


Figure 7-7. Baseline serum RSV-neutralising antibody titres from natural exposure.
*Plaque-reduction neutralisation data (PRNT) from adults aged 18-50 years only (study groups 1-4). (**Top figure**) The red lines denote the geometric mean titre (GMT) for each study group and for all volunteers combined before prime vaccination. There were no statistically significant differences in antibody titres across study groups at baseline (p=0.459, two-tailed Kruskall-Wallis ANOVA). (**Bottom left figure**) Baseline titres with volunteer age found no statistical significance (r=0.20, p=0.223, two-tailed Spearman's correlation). (Bottom right figure) Baseline antibody titres correlated with baseline PanAd3 (vector) neutralising antibody titres. The vertical dotted line denotes the lower limit of detection of serum anti-PanAd3 (vector) neutralising antibody (a titre of 18). There was no significant correlation (r=0.12, p=0.459, two-tailed Spearman's correlation).*

7.5.2 The serum RSV-neutralising antibody response to vaccination

IM PanAd3-RSV induced a significant rise in serum RSV-neutralising antibody titre by 4-weeks after vaccination. In contrast serum antibody titres after IN PanAd3-RSV were unchanged from baseline. After IM MVA-RSV and IM PanAd3-RSV boost there were no further rises in antibody titres from those volunteers primed with IM PanAd3-RSV (study groups 1 and 2 respectively). However, volunteers primed with the intra-nasal route mounted a detectable rise in serum antibody titres 4-weeks after IM MVA-RSV or IM PanAd3-RSV boost (study groups 3 and 4 respectively). Serum antibody titres inexorably waned from the peak responses 4-weeks post IM PanAd3-RSV prime (in study groups 1 and 2) and 4-weeks post IM MVA-RSV or IM PanAd3-RSV boost (in groups 3 and 4) to baseline antibody measures. There were no differences in serum RSV-neutralising antibody titres of low-dose and high-dose vaccine recipients, and further analyses were performed on all volunteers without distinction for dose.

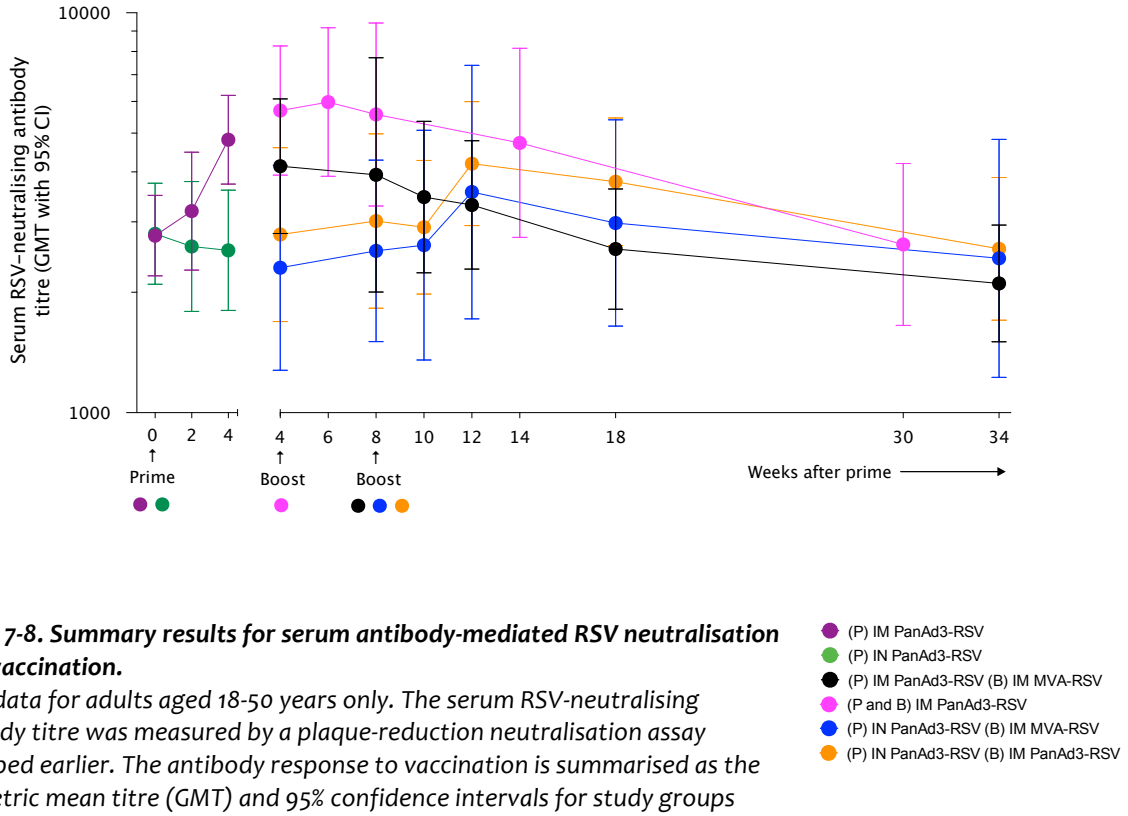


Figure 7-8. Summary results for serum antibody-mediated RSV neutralisation after vaccination.
PRNT data for adults aged 18-50 years only. The serum RSV-neutralising antibody titre was measured by a plaque-reduction neutralisation assay described earlier. The antibody response to vaccination is summarised as the geometric mean titre (GMT) and 95% confidence intervals for study groups combined after prime (P) vaccine and separated by study groups after week 4 when the first boost (B) vaccine was administered to group 2. The lower limit of detection for the assay was a titre of 20.

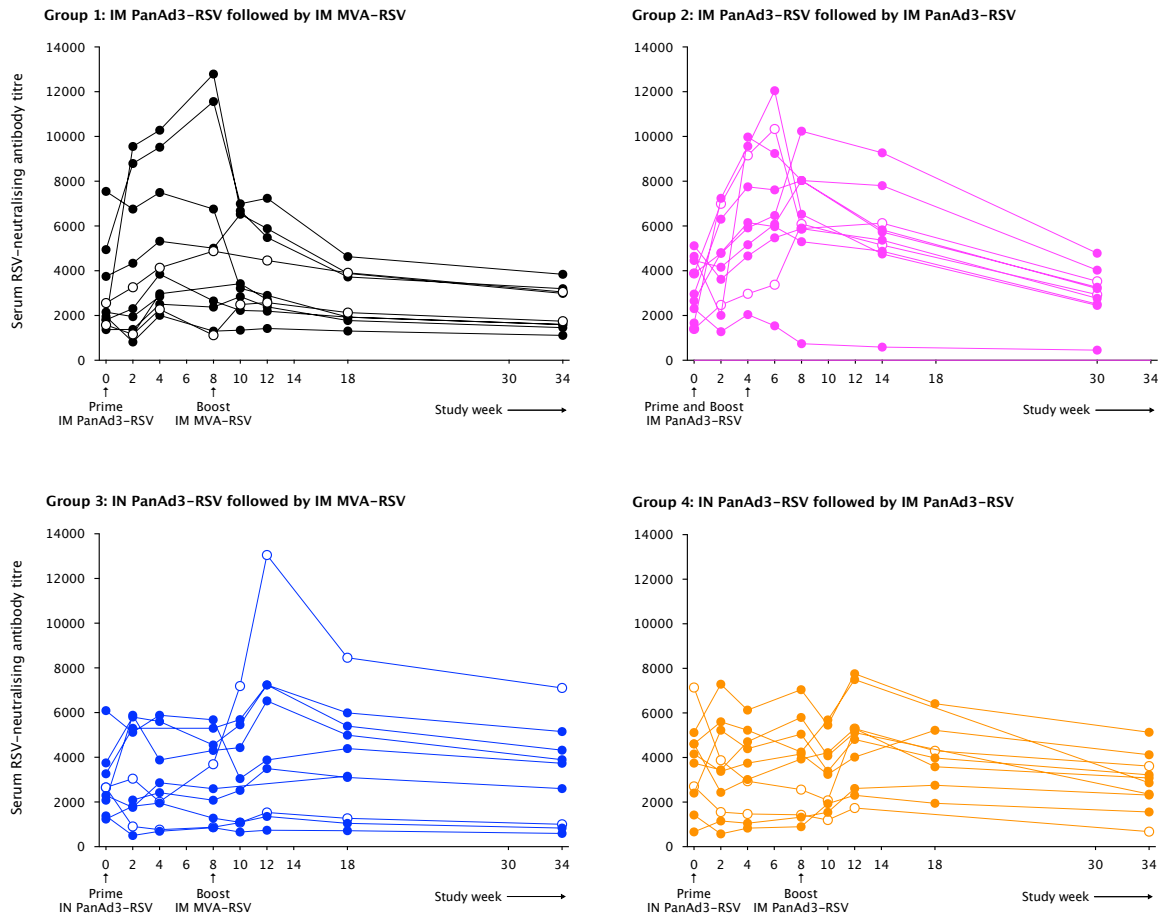


Figure 7-9. Serum RSV-neutralising antibody titres by dose and study group after prime/boost vaccination. *PRNT data for adults aged 18-50 years only. Each connected dot represents one volunteer. Empty circles are low-dose vaccine recipients and there were no discernable dose effects in the measured antibody response to vaccination.*

	Group 1	Group 2	Group 3	Group 4	ANOVA
Prime vaccine [N volunteers]:	IM PanAd3-RSV [10]	IM PanAd3-RSV [10]	IN PanAd3-RSV [10]	IN PanAd3-RSV [10]	
Boost vaccine [N volunteers]:	IM MVA-RSV [10]	IM PanAd3-RSV [10]	IM MVA-RSV [10]	IM PanAd3-RSV [10]	
Geometric mean serum RSV-neutralising antibody titres before and after prime vaccination (95% CI):					
Pre-prime	2528 (1706-3744)	3040 (2213-4177)	2531 (1736-3688)	3070 (1855-5079)	0.452
28-days post vaccination	4135 (2807-6090)	5697 (3927-8266)	2303 (1277-4155)	2789 (1690-4602)	**0.015**
Geometric mean serum RSV-neutralising antibody titres before and after boost vaccination (95% CI):					
Pre-boost	3933 (2004-7716)	5697 (3927-8266)	2540 (1506-4281)	3015 (1824-4984)	0.057
28-days post vaccination	3308 (2286-4786)	5568 (3287-9432)	3562 (1718-7388)	4196 (2936-5998)	0.161

Table 7-1. Summary descriptive statistics of the serum RSV-neutralising antibody response to vaccination. *PRNT data for adults aged 18-50 years only. Between groups analysis used a one-way ANOVA for significance testing; the only time point to reach statistical significance was 28-days post prime.*

7.5.3 *Exploration of the serum RSV-neutralising antibody response to IM PanAd3-RSV prime vaccination*

Younger adult volunteers were combined by the route of prime for post-hoc analysis of the serum RSV-neutralising antibody responses 4-weeks post vaccination. The antibody geometric mean ratio (GMR) following the IM route of vaccination was 1.8 (95% CI 1.4-2.7) and attained significantly greater titres compared with the IN route of prime, which had no effect on serum antibody titres (GMR 0.92, 95% CI 0.7-1.2).

Correlation analyses were used to explore potential effects of baseline and other antibody factors on the heterogeneity of responses to IM PanAd3-RSV prime (see Figure 7-10). The peak RSV-neutralising antibody response was 28-days post-prime and was expressed as the fold-change from baseline. The results showed that baseline RSV-neutralising antibody titres from natural exposure had no effect on the magnitude of the prime response. Furthermore the pre-prime antibody titres directed against the PanAd3 vector did not appear to impair the magnitude of the RSV-neutralising antibody response. The plaque-reduction neutralising antibody assay employs a plethora of RSV-specific antibodies of varying epitope specificity (including G-specific IgG and IgA) and the final post-hoc analysis sought to correlate ELISA data and F-specific serum IgG and IgA after vaccination to infer the contribution of these antibodies in functional viral neutralisation as measured in the plaque-reduction neutralisation assay. As described earlier, there was minimal anti-F IgG antibody change 14-days after IM PanAd3-RSV prime and unsurprisingly the serum F-specific IgG antibody response (expressed as a fold-change in OD measure from baseline) bore no relationship to the overall PRNT. Changes in F-specific serum IgA however, measured 14-days post prime, recorded a significant positive correlation with the induction of overall serum RSV-neutralising antibody titre measured 28-days post prime ($p=0.004$, two-tailed Pearson's correlation).

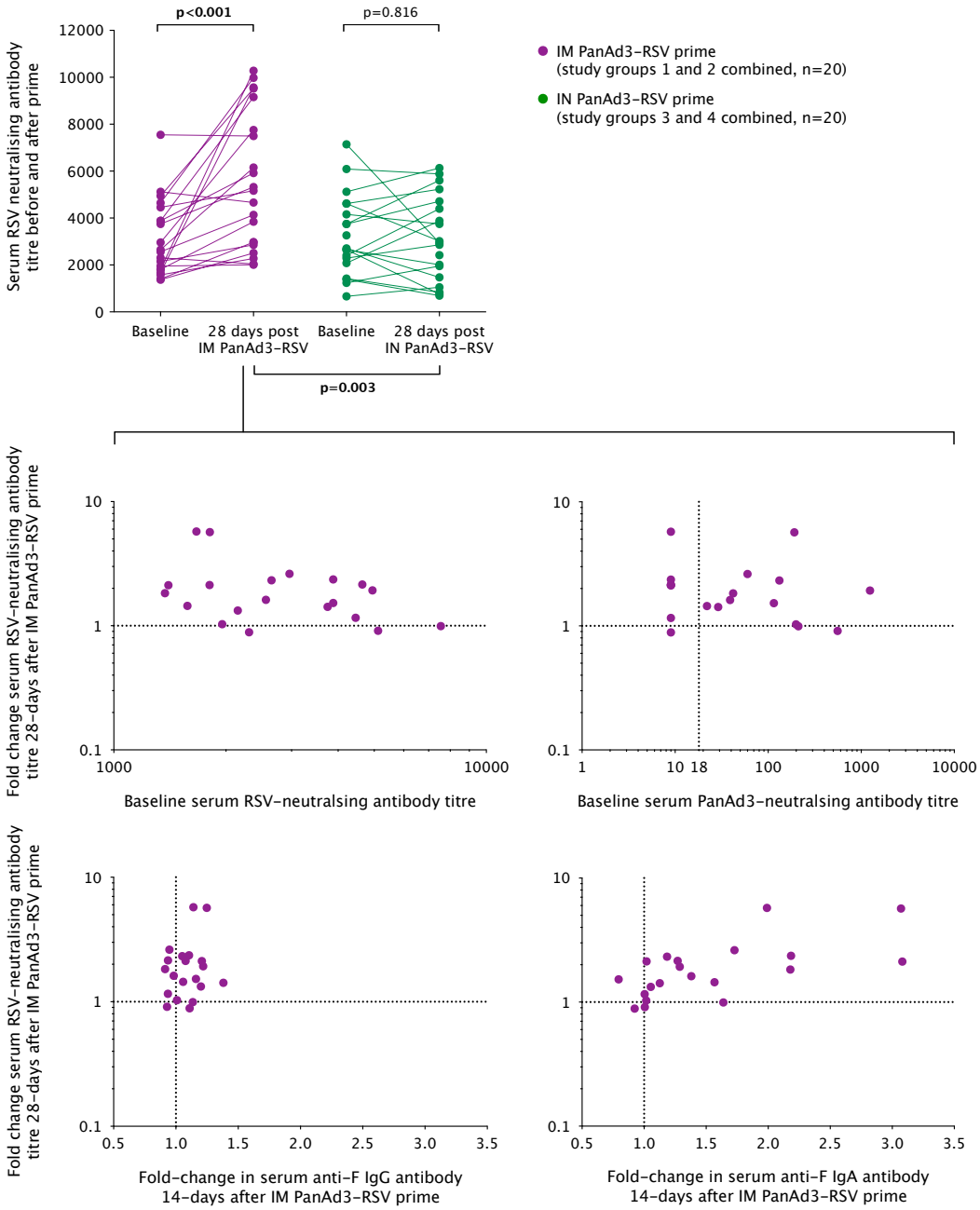


Figure 7-10. Serum RSV-neutralising antibody response 4-weeks after IM or IN PanAd3-RSV prime.
*PRNT data for adults aged 18-50 years only. Groups 1 and 2 were combined as IM PanAd3-RSV prime and groups 3 and 4 were combined as IN PanAd3-RSV prime. (**Top figure**) IM PanAd3-RSV induced a significant increase in antibody titres when IN PanAd3-RSV did not (p<0.001 and p=0.816 respectively, two-tailed paired t-test). The resulting antibody titres 4-weeks after prime were significantly greater from primed with IM PanAd3-RSV (p=0.003, two-tailed unpaired t-test). (**Middle left and middle right figures**) For IM PanAd3-RSV responses there were no statistically significant correlations with the fold-induction of antibody and the baseline serum RSV (r=-0.36, p=0.121, two-tailed Pearson's correlation) or PanAd3 (vector) neutralising antibody titres measured by SEAP assay (r=-0.17, p=0.475, two-tailed Spearman's correlation). The vertical dotted line in the bottom right figure denotes the lower limit of detection for serum vector neutralising antibody (a titre of 18). (**Bottom left and bottom right figures**) The fold-change in serum RSV-neutralising antibody titre measured 28-days after IM PanAd3-RSV prime did not correlate with the fold-change in serum anti-F IgG measured 14-days after prime by ELISA assay (r=0.24, p=0.308, two-tailed Pearson's correlation) but was significantly correlated with the serum anti-F IgA measured 14-days after prime by ELISA assay (r=0.62, p=0.004, two-tailed Pearson's correlation).*

7.5.4 *Exploration of the serum RSV-neutralising antibody response to IM MVA-RSV and IM PanAd3-RSV boost*

The data presented earlier indicated there was little/no measurable rise in serum RSV-neutralising antibody in response to boost vaccination from volunteers primed by IM PanAd3-RSV (groups 1 and 2). Conversely a measureable serum antibody increase was observed 4-weeks after boost from volunteers primed with IN PanAd3-RSV (study groups 3 and 4), which was a similar pattern observed from the ELISA data. Summary analyses of the serum RSV-neutralising antibody responses to boost are shown in Figure 7-11. Increases in serum RSV-neutralising antibody that reached statistical significance were from volunteers in group 4 ($p=0.035$, two-tailed paired t-test). A correlation analysis between the fold-changes in serum RSV-neutralising antibody and fold changes in anti-F IgG and IgA measured by ELISA 14-days post boost, as applied earlier to the post-prime response, failed to record any correlation with F-specific IgA as it had done with IM PanAd3-RSV prime.

Further exploration sought to characterise the effects of pre-boost serum RSV-neutralising antibody titres on the subsequent boost response in all study groups. The absence of detectable rises in neutralising antibody after boost from IM PanAd3-RSV primed volunteers was found to include a negative correlation with antibody titres before vaccination. This pattern was also observed from volunteers in group 4 who were primed intra-nasally (Figure 7-12). Finally, correlation analyses were applied to test whether pre-boost titres of serum PanAd3 (vector) neutralising antibody, which was a heterogeneous antibody population derived from natural adenoviral exposure and post PanAd3-RSV prime, had an appreciable association with the magnitude of serum RSV-neutralising antibody response to PanAd3-vectored boost (study groups 2 and 4). No such association could be demonstrated (Figure 7-12).

Antibody titres 30 and 34 weeks after boost were statistically indistinguishable from each study group baseline antibody titres. There was no rise in serum RSV-neutralising antibody titres in the three volunteers with confirmed RSV infection detected before the final time point measure (data not shown).

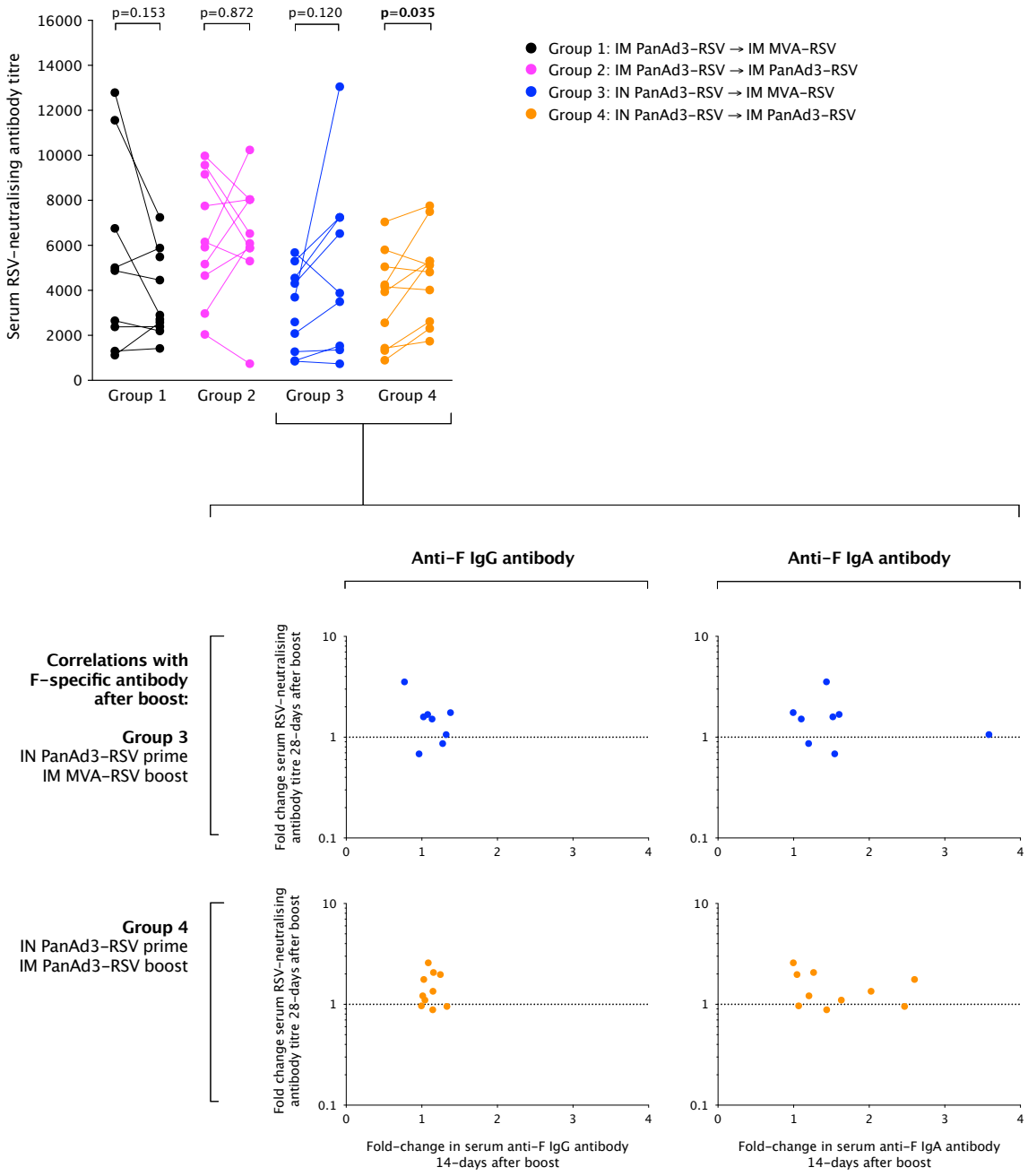


Figure 7-11. Serum RSV-neutralising antibody titres before and 28-days after IM MVA-RSV or IM PanAd3-RSV boost.

*Data for adults aged 18-50 years only. (**Top figure**) The absolute serum RSV-neutralising antibody titres measured by plaque-reduction neutralisation assay before and 28-days after boost. Each paired points represents the titre from one volunteer. Pairwise comparisons were two-tailed paired t-tests; the only statistically significant difference was the mean rise in titres for volunteers in study group 4. (**Bottom panel of four figures**) Correlation analyses between the fold-changes in serum RSV-neutralising antibody titre 28-days after boost and the F-specific IgG and IgA antibody measured by ELISA assay 14-days after boost. No correlations were statistically significant (clockwise, p=0.138, p=0.610, p=0.963 and p=0.084 by two-tailed Pearson's correlation).*

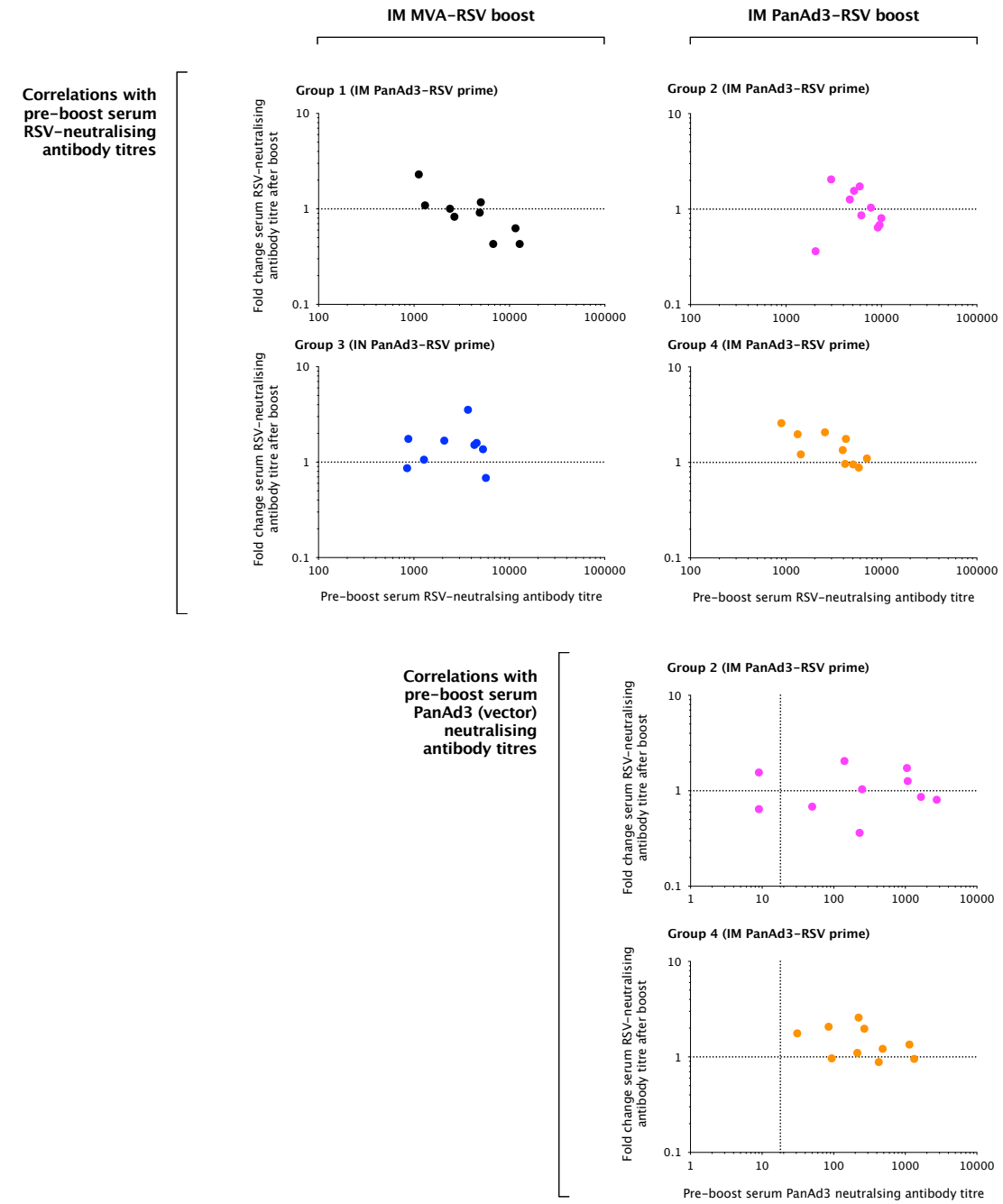


Figure 7-12. Correlations between the fold-change in serum RSV-neutralising antibody titres 4-weeks after boost with pre-boost serum RSV-neutralising and PanAd3-neutraising antibody titres.
*Data for adults aged 18-50 years only. Two-tailed Pearson's correlation analyses were applied to the log_{10}-transformed data for each study group. The horizontal dotted lines denote a fold-change of 1 for serum RSV-neutralising antibody 4-weeks after boost. (**Top panel of four figures**) Correlations between the fold change of serum RSV neutralising antibody titre 4-weeks following boost vaccination and pre-boost RSV serum neutralising antibody titres. Volunteers from group 1 were boosted with IM MVA-RSV (r^2=0.66, p=0.008); group 2 were boosted with IM PanAd3-RSV (r^2<0.001, p=0.978); group 3 were boosted with IM MVA-RSV (r^2=0.01, p=0.787) and group 4 were boosted with IM PanAd3-RSV (r^2=0.53, p=0.017). (**Bottom panel of two figures**). Correlations between the fold change of serum RSV-neutralising antibody titre 4-weeks following boost vaccination and pre-boost serum PanAd3 (vector) neutralising antibody titres. The vertical dotted lines denote the lower limit of detection for the vector neutralising antibody assay (a titre of 18). Volunteers from study groups 2 and 4 were boosted with IM PanAd3-RSV. There was no association for group 2 (r^2<0.01, p=0.870) or group 4 (r^2=0.16, p=0.250).*

7.6 Anti-F IgG and IgA antibody secreting cells (ASC)

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the methods described earlier. Fresh PBMCs were used to detect and quantify the F-specific IgG and IgA antibody-secreting cell (ASC) at baseline and one week after prime and one week after boost vaccination. The *ex-vivo* dual-colour ELISpot assay was used to generate data from all nine study groups (n=72 volunteers).

The ASC response to vaccination in younger adults (study groups 1-4) was measured in 2013 and a total of 14/50 (26%) of plates for anti-F IgG and 10/50 (20%) of plates for anti-F IgA ASC responses were compromised by error. This resulted in 34/124 (27%) of samples for anti-F IgG and 29/124 (23%) of samples for anti-F IgA ASC analysis being unavailable for analysis. The loss of each sample underwent a root cause analysis to optimise sample processing and minimise the future loss of samples as the trial progressed, and the results of these investigations are provided in supplementary material. The trial intensity was the main reason for error, and due to the trial design the loss of data impacted on specific groups disproportionately. The ASC response to vaccination in older adults (study groups 5-9) was measured in 2014 and only 1/29 (3%) of anti-F IgG and 1/29 (3%) anti-F IgA plates were compromised by laboratory error. This resulted in the loss of 2/90 (2%) IgG and IgA ASC samples (both from group 6).

7.6.1 Analysis of baseline anti-F IgG and IgA ASCs in peripheral circulation

Anti-F ASCs were essentially absent or undetectable from peripheral circulation before vaccination, with only a few individuals who registered low frequencies of ASCs (see Figure 7-13). The analysis of the ASC response to vaccination was therefore performed without further reference to any baseline ASC responses.

7.6.2 The anti-F IgG and IgA ASC response to vaccination

In the cleanest demonstration of vaccine immunogenicity, antigen specific ASCs that were not detectable in peripheral blood from natural exposure appeared 7-days after the first dose of vaccine given by IM injection; IM PanAd3-RSV or IM MVA-RSV at prime and IM PanAd3-RSV or IM MVA-RSV at boost in volunteers primed with intra-nasal PanAd3-RSV.

In 2013 and the trial of younger adults, 19/19 and 18/20 volunteers had a detectable anti-F IgG and IgA ASC response respectively after IM PanAd3-RSV (study groups 1 and 2 combined). The anti-F IgG median was 91.7 spots per million PBMCs (IQR 36.7 to 226.7) and anti-F IgA median was 31.4 spots per million PBMCs (IQR 13.4 to 123.3). In contrast, IN PanAd3-RSV prime induced a detectable ASC response in blood at the same time point in lower frequency and magnitude; 7/14 and 7/13 volunteers for anti-F IgG and IgA respectively. Following IN PanAd3-RSV the anti-F IgG median response was 4.4 spots per million PBMCs (IQR 0.5 to 16.8) and anti-F IgA median response was 5.0 spots per million PBMCs (IQR 0.5 to 13.3). There appeared to be no differences in the magnitude of the immune response with the dose of vaccine used in younger adults (data not shown). In older adults in 2014, all 12/12 and 10/12 volunteers had measurable F-specific IgG and IgA responses 7-days after IM PanAd3-RSV prime, compared with 3/6 for both IgG and IgA ASCs after IN priming. For older adults the anti-F IgG median response was also 91.7 spots per million PBMCs (IQR 49.6 to 183.3) and anti-F IgA median response was 25.8 spots per million PBMCs (IQR 6.2 to 45.0) after IM PanAd3-RSV prime. The IN route induced a median response of 4.4 IgG spots per million PBMCs (IQR 0.5 to 11.7) and 4.4 IgA spots per million PBMCs (IQR 0.5 to 11.3). Group 6, single dose IM MVA-RSV prime in older adults, recorded IgG and IgA responses in 3/4 volunteers (IgG median 170.0 spots per million PBMCs, IQR 40.4 to 315.0; IgA median 50.0, IQR 3.7to 106.7).

The F-specific ASC response to boost vaccination appeared dependent on whether this vaccine was the first IM dose. IM PanAd3-RSV primed volunteers from study groups 1, 2, 7 and 9 did not mount a notable response to IM MVA-RSV or IM PanAd3-RSV boost. In contrast, volunteers from study groups 3, 4 and 8 made a comparable response seen to IM PanAd3-RSV prime, irrespective of the boost vector (see Figure 7-13 and Table 7-2).

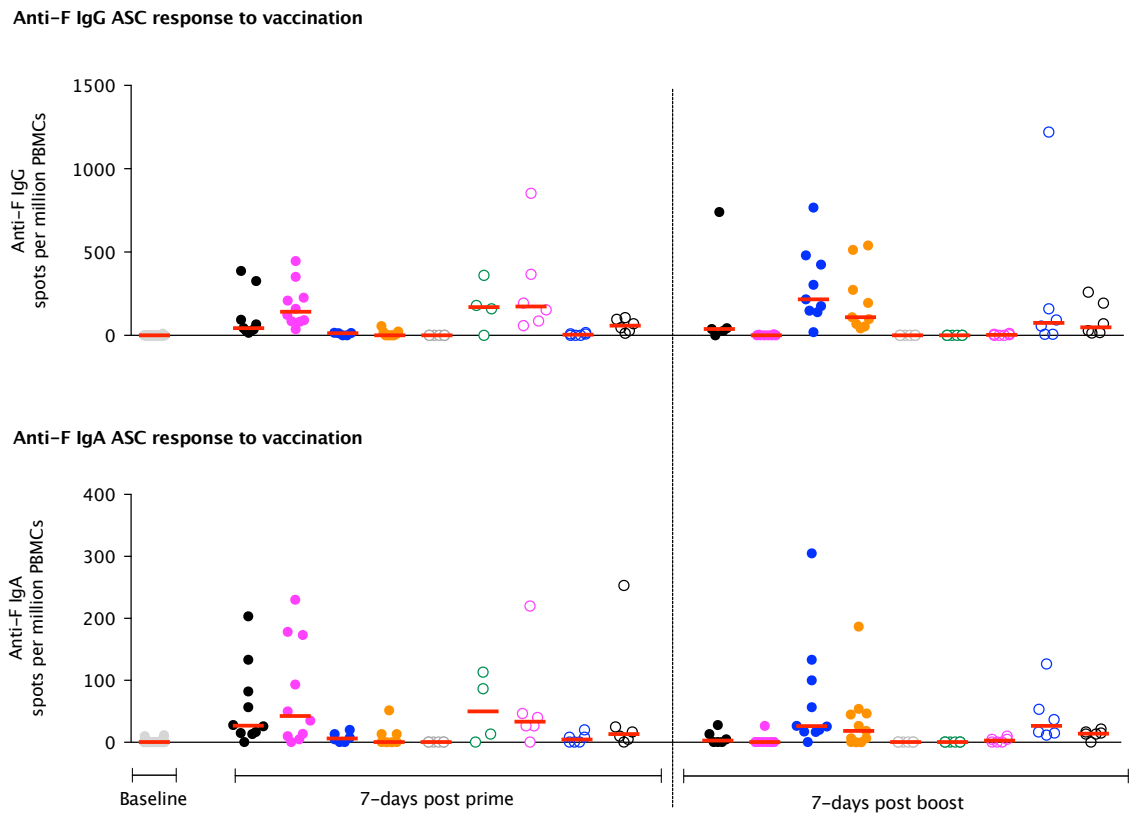


Figure 7-13. Summary results for anti-F IgG and IgA ASC response to vaccination.
*Scatter plot of the anti-F IgG (**top**) and anti-F IgA (**bottom**) count of spots per million PBMCs measured at baseline and one week after prime and one week after boost by study group. The red bar indicates the median. The colour coding matches prime/boost combinations in younger and older adults. Note that groups 5 and 6 did not receive a boost vaccine. The lower limit of detection for the assay was 1 spot per million PBMCs.*

	Adults aged 18-50 years				Adults aged 60-75 years					ANOVA
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**	**Group 7**	**Group 8**	**Group 9**	
Prime [N]	IM PanAd3-RSV [11]	IM PanAd3-RSV [10]	IN PanAd3-RSV [10]	IN PanAd3-RSV [11]	None	IM MVA-RSV [6]	IM PanAd3-RSV [6]	IN PanAd3-RSV [6]	IM PanAd3-RSV [6]	
Boost [N]	IM MVA-RSV [10]	IM PanAd3-RSV [10]	IM MVA-RSV [10]	IM PanAd3-RSV [10]	None	None	IM PanAd3-RSV [6]	IM MVA-RSV [6]	IM MVA-RSV [6]	
Median anti-F IgG ASC spots per million PBMCs after vaccination [N samples] (inter-quartile range):										
Prime vaccine	43.9 [9] (28-210)	141.7 [10] (84-258)	13.3 [5] (0.5-15)	0.5 [9] (0.5-22)	0.5 [6] (0.5-0.5)	170.0 [4] (40-315)	173.3 [6] (80-488)	4.4 [6] (0.5-12)	58.3 [6] (25-99)	p<0.001
Boost vaccine	38.3 [5] (14-392)	0.5 [8] (0-4)	216.7 [9] (144-453)	108.9 [9] (61-393)	0.5 [6] (0.5-0.5)	0.5 [6] (0.5-0.5)	2.8 [6] (0.5-7.9)	75.0 [6] (7-425)	49.1 [6] (16-210)	p<0.001
Median anti-F IgA ASC spots per million PBMCs after vaccination [N samples] (inter-quartile range):										
Prime vaccine	26.8 [10] (15-95)	42.5 [10] (8-175)	6.3 [6] (0.5-15)	0.5 [7] (0.5-13)	0.5 [6] (0.5-0.5)	50.0 [4] (4-107)	33.3 [6] (20-90)	4.4 [6] (0.5-11)	13.3 [6] (4-82)	p<0.001
Boost vaccine	2.8 [6] (0.5-17)	0.5 [9] (0.5-0.5)	26.1 [10] (17-108)	18.3 [10] (0.5-47)	0.5 [6] (0.5-0.5)	0.5 [6] (0.5-0.5)	2.8 [6] (0.5-6)	26.7 [6] (14-72)	14.2 [6] (10-18)	p<0.001

Table 7-2. Median and interquartile ranges of the anti-F IgG and IgA ASC responses 7-days after vaccination.
A one-way Kruskall Wallis ANOVA was used to test differences in the median responses between study groups.

7.6.3 Exploration of the anti-F IgG and IgA ASC response to IM PanAd3-RSV prime vaccination

Post-hoc analysis of the ASC response to prime concerned IM PanAd3-RSV vaccinated volunteers only. Volunteers from study groups 1 and 2 were combined to represent a larger cohort of younger adults aged 18-50 years (n=21 volunteers, ELISpot assay performed in 2013, no distinction between low-dose and normal-dose). Volunteers from study groups 7 and 9 were combined to represent a larger cohort of older adults aged 18-50 years (n=12 volunteers, ELISpot assay performed in 2014). The F-specific IgG and IgA ASC responses from IN PanAd3-RSV primed volunteers were low and infrequent, and there were too few older volunteers in study group 6 (single dose IM MVA-RSV) to support meaningful further exploration from these volunteers. Data were analysed without any correction for baseline ASC responses, as these were infrequently detected and mostly negligible. Following IM PanAd3-RSV prime the magnitude of the F-specific IgG ASC response offered no correlation to the magnitude of the IgA response (p=0.608 and p=0.438 for younger and older adults respectively, p=0.364 for all volunteers combined, two-tailed Spearman's correlation). F-specific IgG and IgA ASC responses were therefore analysed separately to other immune responses to vaccination (Figure 7-14).

Volunteer age at enrolment did not appear to have an impact upon the magnitude of F-specific ASC responses to IM PanAd3-RSV prime. Age was analysed as a categorical variable (younger and older adults) and as a continuous variable, and found no statistically significant relationships (Figure 7-15, top panel).

Exploration of the ASC response in relation to serum neutralising antibody titres against RSV and the PanAd3 vector were possible for younger adults only (n=21 volunteers). Here, the baseline serum RSV-neutralising antibody titres had no correlation with the subsequent IgG or IgA ASC response to prime. The F-specific IgG ASC response 7-days after prime had a weak positive correlation with the fold change in serum RSV-neutralising antibody titres measured 28-days after vaccination (p=0.021, two-tailed Spearman's correlation) and there was not a statistically significant correlation with the F-specific IgA ASC response (Figure 7-15, middle panel). Finally, baseline titres of serum anti-PanAd3 (vector) neutralising antibody titres were measured against the F-specific IgG and IgA ASC response to IM PanAd3-RSV prime in younger adults. Here a weak but statistically significant negative correlation with the subsequent anti-F IgA ASC response was observed (p=0.040, two-tailed Pearson's correlation with log10 transformed vector

neutralising antibody titres). No similar correlation to F-specific IgG ASC responses was observed (Figure 7-15, bottom panel).

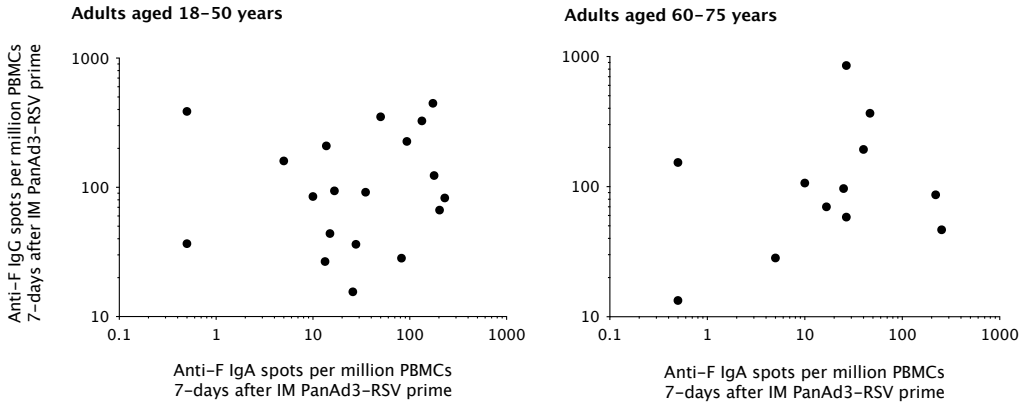


Figure 7-14. Correlation analysis between the anti-F IgG and IgA ASC response 7-days after IM PanAd3-RSV. *Ex-vivo ELISpot data. There were no correlations in the response from younger adults and older adults (p=0.608 and p=0.438 respectively, and p=0.364 for all volunteers combined, by two-tailed Spearman's correlation).*

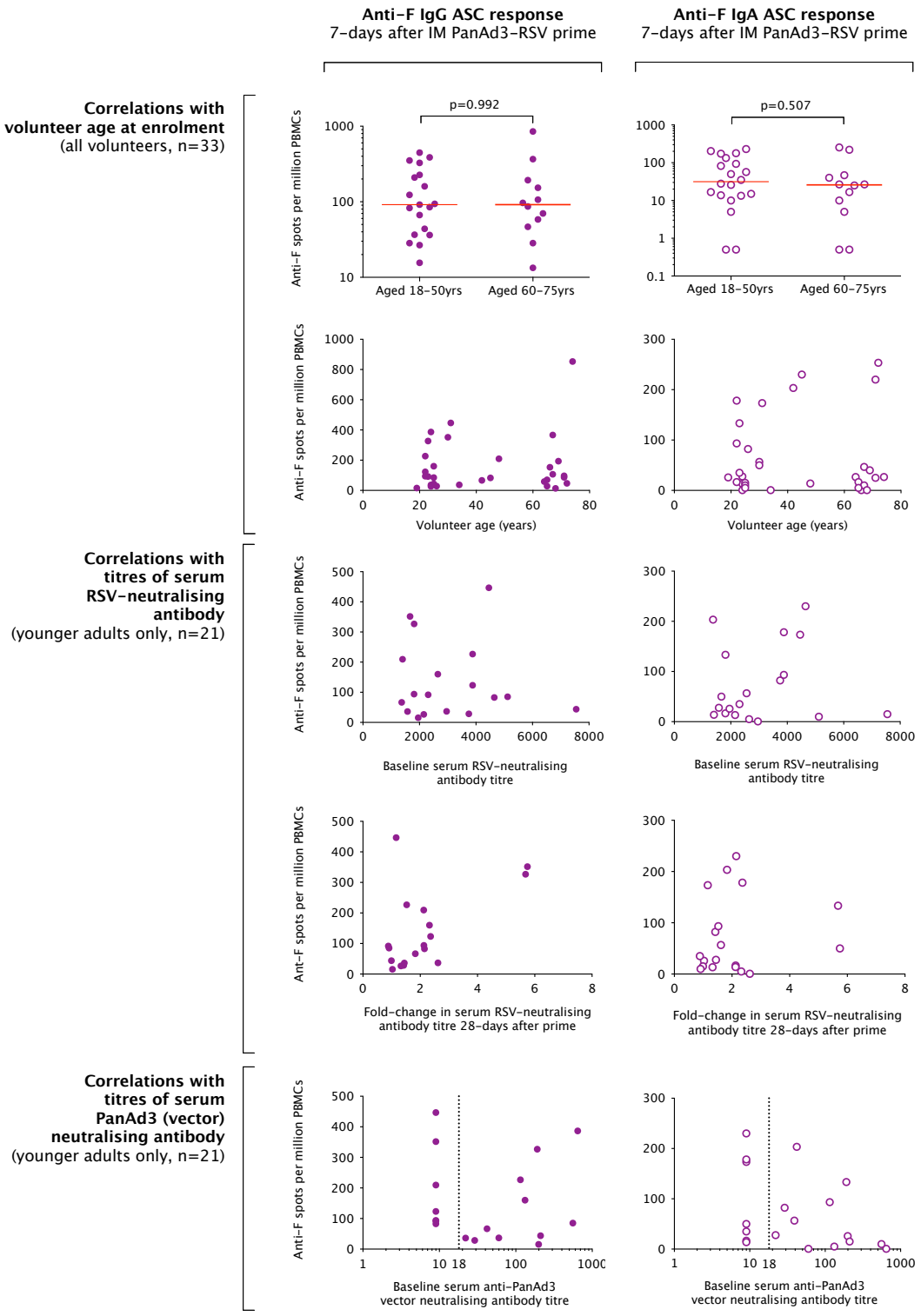


Figure 7-15. Post-hoc analyses of the anti-F IgG and IgA ASC response to IM PanAd3-RSV prime vaccine.
*The F-specific antibody secreting cell (ASC) response was measured by dual-colour ex-vivo ELISpot 7-days after IM PanAd3-RSV prime. (**Top panel**) The red bar indicates the median. No correlations with volunteer age were observed (p=0.608 and p=0.438 respectively, and p=0.364 for all volunteers combined, two-tailed Spearman's correlation). (**Middle panel**) There were no correlations with baseline titres of serum RSV-neutralising antibody (two-tailed Spearman's correlation; IgG r=0.00, p=0.99; IgA r=0.04, p=0.878). Greater anti-F IgG ASC responses were positively correlated with the fold change in serum RSV neutralising antibody titres 4-weeks after vaccination (r^2=0.29, p=0.021, two-tailed Pearson's correlation) but not with the IgA ASC response to vaccination (r=0.15, p=0.538, two-tailed Pearson's correlation). (**Bottom panel**) The dotted lines mark the*

lower limit of detection for the assay (a titre of 18). There was no correlation with IgG ASC responses (r^2=0.01, p=0.735) and titres of vector neutralising antibody but a weak statistically significant correlation was observed with IgA ASC responses (using log_{10} transformed antibody titres, r^2=-0.23, p=0.040, two-tailed Pearson's correlation).

7.6.4 Exploration of the anti-F IgG and IgA ASC response to IM MVA-RSV and IM PanAd3-RSV boost vaccination

Volunteers primed with IM PanAd3-RSV made little or no detectable response in peripheral blood 7-days after boost with either IM MVA-RSV or PanAd3-RSV. The anti-F IgG and IgA ASCs detectable in peripheral circulation 7-days after boost were from volunteers primed by intra-nasal PanAd3-RSV vaccination (volunteers from groups 3, 4 and 8; total n=26 volunteers) and were subjected to post hoc exploration.

There were no statistically significant differences in the magnitude of the F-specific IgG and IgA ASC response with the MVA or PanAd3 vector in younger adults (p=0.380 and p=0.243 for IgG and IgA respectively, two-tailed Mann-Whitney tests)(Figure 7-16). In addition, and unlike the observations made after IM PanAd3-RSV prime, a positive correlation between anti-F IgG and IgA ASC responses was observed following both IM MVA-RSV and IM PanAd3-RSV boost vaccination in younger adults (Figure 7-17).

Heterologous IN PanAd3-RSV prime followed by IM MVA-RSV boost was performed in younger and older adults (study groups 3 and 8 respectively); although these assays were performed in different years the volunteer age at enrolment appeared to have no correlation to the ASC response to boost (Figure 7-16). Correlation analyses between ASC responses to boost with serum RSV- and PanAd3-neutralising antibody titres were possible for younger adults only (study groups 3 and 4). For both MVA and PanAd3-vectored booster vaccines the titres of pre-boost serum RSV-neutralising antibody did not have a statistically significant impact on the subsequent F-specific IgG and IgA ASC responses. There were statistically significant positive correlations with the anti-F IgG ASC (but not anti-F IgA ASC) responses 7-days after both IM MVA-RSV and IM PanAd3-RSV boost and the fold change in serum RSV-neutralising antibody measured 28-days after vaccination (p=0.022 for both IM MVA-RSV and IM PanAd3-RSV boost vaccines, two-tailed Spearman's correlation). Finally, no significant interference from pre-boost serum anti-PanAd3 vector neutralising antibody could be demonstrated with the IgG and IgA ASC response to IM PanAd3-RSV boost vaccine from volunteers in study group 4 (Figure 7-18).

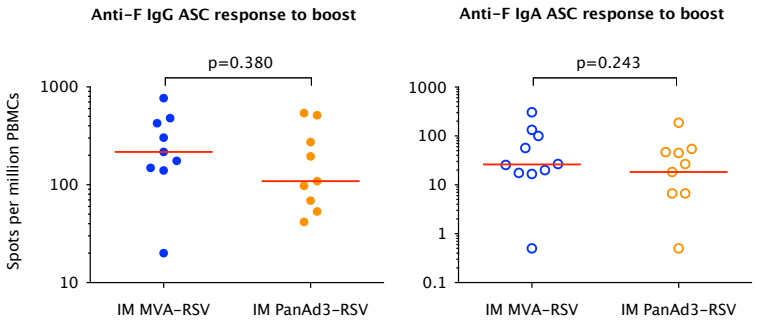


Figure 7-16. The F-specific IgG and IgA ASC response to boost vaccination from IN PanAd3-RSV primed volunteers.
Data for younger adults only. The antibody secreting cell (ASC) response was measured by ex vivo dual-colour ELISpot 7-days after IM MVA-RSV boost (blue dots, volunteers from group 3, n=10) and IM PanAd3-RSV boost (yellow dots, volunteers from group4, n=10 volunteers). Comparative statistical tests were two-way Mann-Whitney t-tests.

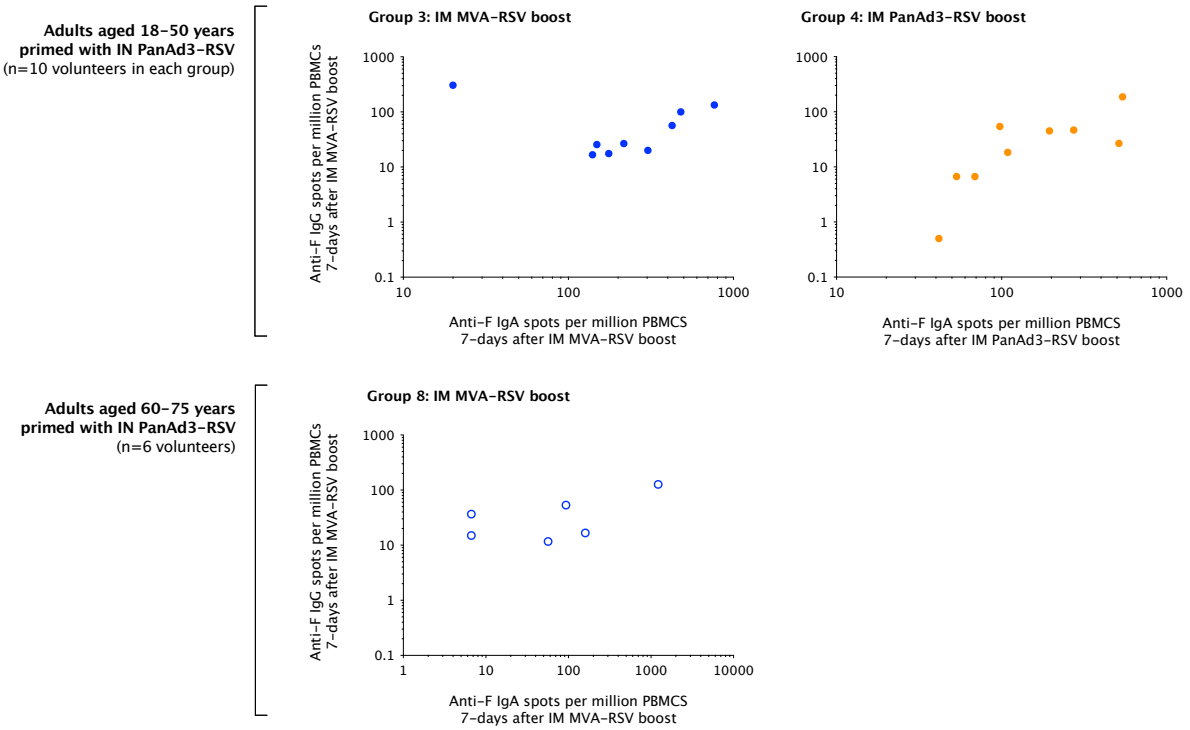


Figure 7-17. Correlation between anti-F IgG and IgA ASC responses 7-days after IM MVA-RSV or IM PanAd3-RSV boost in IN PanAd3-RSV primed volunteers.
The antibody secreting cell (ASC) response was measured by ex vivo dual-colour ELISpot 7-days after boost. The only correlation to reach statistical significance was in from volunteers in study group 4 (r=0.78, p=0.018, two-tailed Spearman's correlation). There was no statistical significance for responses from volunteers in study group 3 (r=0.31, p=0.410, two-tailed Spearman's correlation) due to one volunteer who did mount a measurable IgA ASC response. The 6 older volunteers from study group 8 did not show a significant correlation (r=0.55, p=0.271, two-tailed Spearman's correlation) and group 9 (r=0.32, p=0.5556, two-tailed Spearman's correlation).

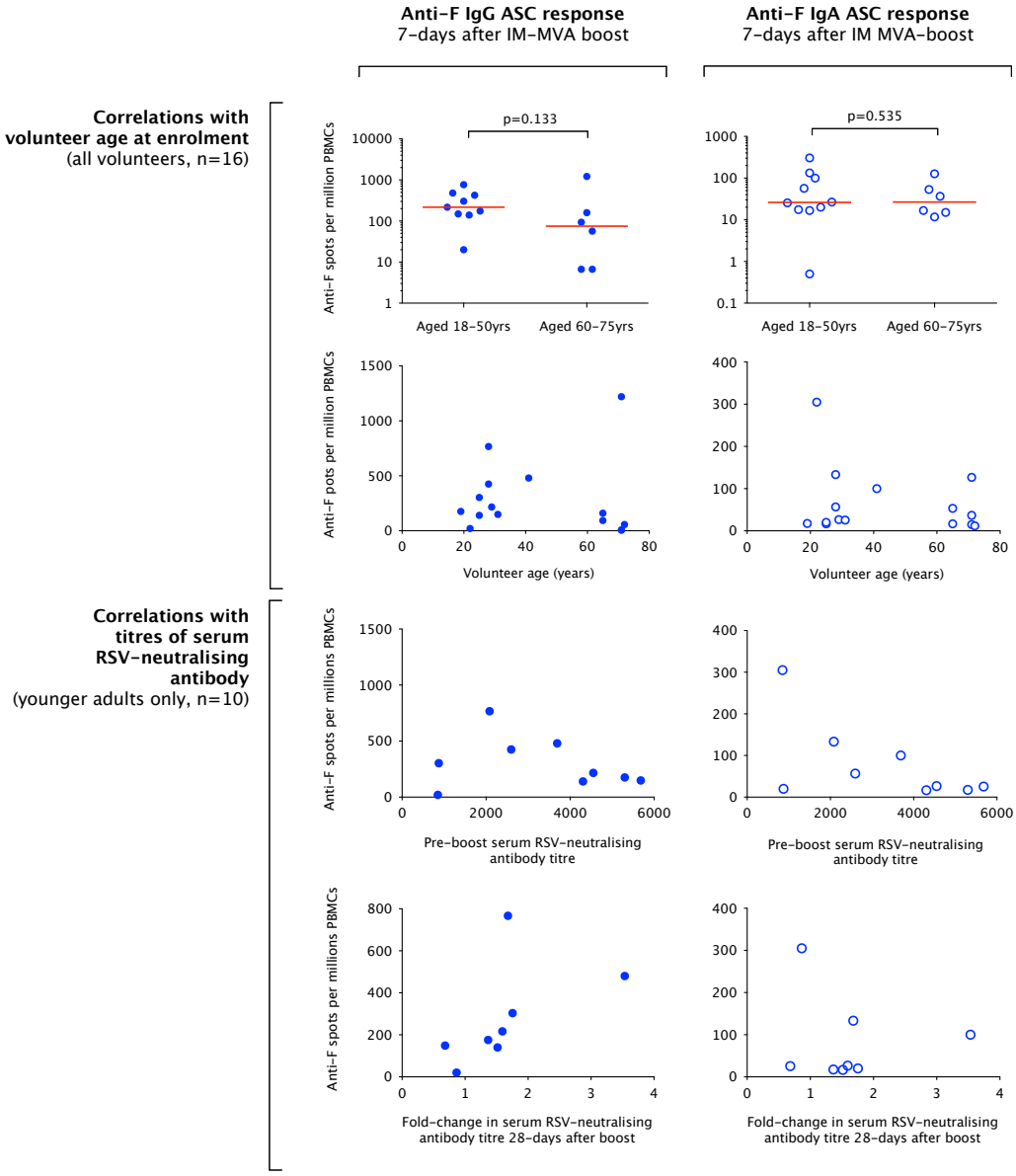


Figure 7-18. Post-hoc analyses of the anti-F IgG and IgA ASC response to IM MVA-RSV boost vaccine in volunteers primed with IN PanAd3-RSV.

*The F-specific antibody secreting cell (ASC) response was measured by dual-colour ex-vivo ELISpot 7-days after IM MVA-RSV boost. (**Top panel**) The red bar indicates the median. No correlations with volunteer age were observed (IgG p=0.351, IgA p=0.324, two-tailed Spearman's correlation). (**Bottom panel**) The pre-boost titres of RSV-neutralising antibody did not correlate significantly with the subsequent F-specific IgG and IgA ASC response to IM MVA-RSV boost (p=0.540 and p=0.084 respectively, two-tailed Pearson's correlation). The fold-changes in serum RSV-neutralising antibody titre reached statistical significance with the anti-F IgG ASC response 7-days after boost (r=0.81, p=0.022, two-tailed Spearman's correlation) but not with the anti-F IgA ASC response (p=0.840, two-tailed Spearman's correlation).*

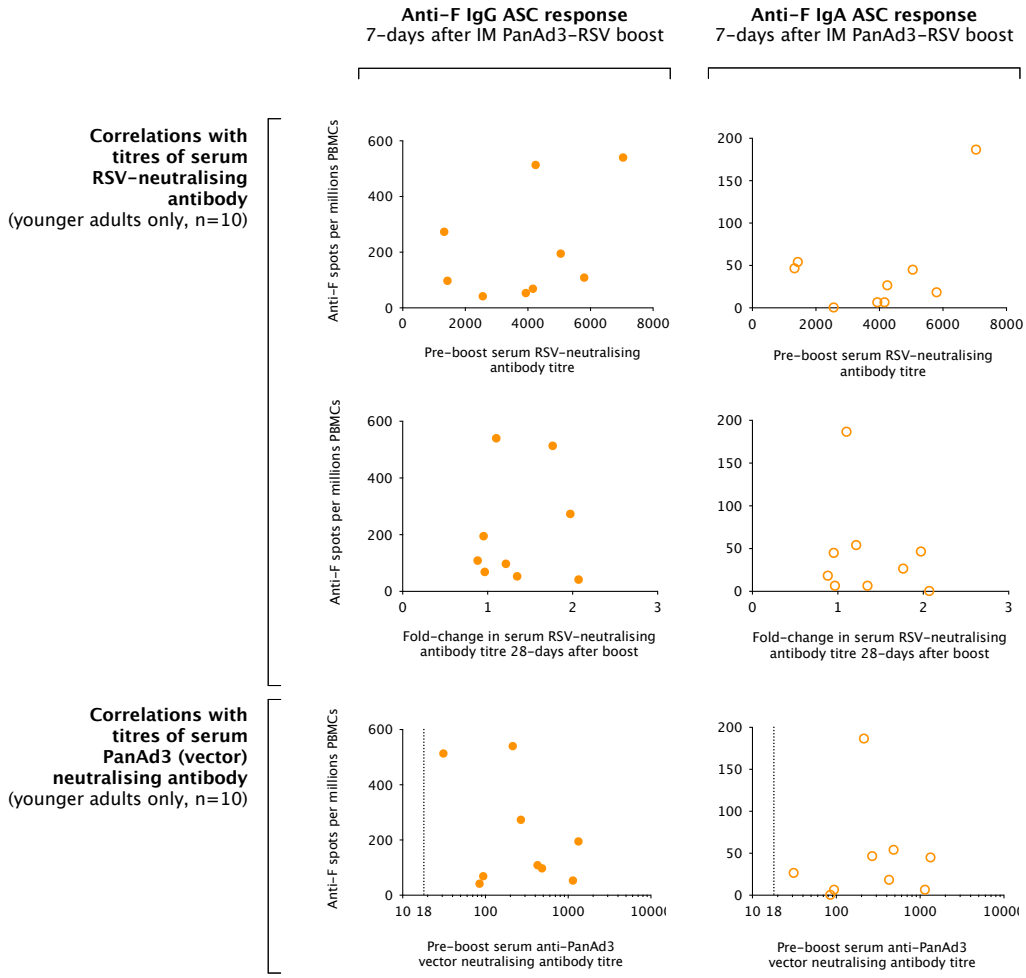


Figure 7-19. Post-hoc analyses of the anti-F IgG and IgA ASC response to IM PanAd3-RSV boost vaccine in volunteers primed with IN PanAd3-RSV.

*The F-specific antibody secreting cell (ASC) response was measured by dual-colour ex-vivo ELISpot 7-days after IM PanAd3-RSV boost. Only younger adults received this combination of prime/boost regime. (**Top panel**) Pre-boost titres of RSV-neutralising antibody did not correlate significantly with the subsequent F-specific IgG and IgA ASC response to IM PanAd3-RSV boost (p=0.269 and p=0.204 respectively, two-tailed Pearson's correlation). The fold-changes in serum RSV-neutralising antibody titre reached statistical significance with the anti-F IgG ASC response 7-days after boost (r=0.81, p=0.022, two-tailed Spearman's correlation) but not with the anti-F IgA ASC response (p=0.840, two-tailed Spearman's correlation). (**Bottom panel**) There were no significant correlations with anti-F IgG ASC (r=-0.12, p=0.776, two-tailed Spearman's correlation) or anti-F IgA ASC (r=0.24, p=0.528, two-tailed Spearman's correlation) responses and serum vector neutralising antibody titres at the time of boost vaccination.*

7.7 T-cell interferon-gamma (IFNγ)

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the methods described earlier. An *ex vivo* ELISpot assay used fresh PBMCs to detect and quantify the peripheral T-cell interferon-gamma (IFNγ) responses to vaccination. The assay was described earlier. PBMCs were collected at baseline, 14-days after prime, before boost and 7-days after boost. IFNγ ELISpot data was available for analysis from all nine study groups (n=72 volunteers).

In 2013 the trial measured the IFNγ response to vaccination in younger adults (study groups 1-4). Overall 13/68 (19%) of plates failed by either the positive or negative control criteria, resulting in the loss of 30/163 (18%) of samples. Trial intensity was the main cause for loss of data and the root cause analysis summary is provided in supplementary material. In 2014 and measuring the IFNγ response to vaccination in older adults, from 40 plates and 120 samples only one sample failed by background DMSO criteria as a result of presumed pipette error (all other DMSO wells on the plate passed). After exclusions for plate failures, a triplicate variance > 10 were detected for 5 triplicates in 2013 and 4 triplicates in 2014 (total 9/1260 triplicates, 0.7%) and were excluded from analysis (see supplementary material).

7.7.1 Analysis of baseline RSV-specific T-cell IFNγ responses

The baseline IFNγ immune responses represent the immunity derived from cumulative natural exposure. The baseline IFNγ measures were significant greater in the 42 younger adults measured in 2013 compared with the 30 older adults measured in 2014 ($p<0.001$, two-tailed Mann-Whitney test). Within younger and older adults there were no statistically significant differences in the median values for each study groups ($p=0.835$ and $p=0.165$ respectively, one-way Kruskall-Wallis ANOVA) (Figure 7-20). No correlation was detected between naturally acquired baseline IFNγ measures and serum RSV-neutralising antibody titres in younger adults (Figure 7-21).

The total IFNγ response represents the summed T-cell response to four separate peptide pools (designated Fa, Fb, M and N, see supplementary methods). A strong positive correlation existed between the different peptide pools ($p<0.001$, two-tailed Spearman's correlation, data not shown). The baseline peptide-pool specific IFNγ responses within each study group were analysed and the median responses to

each pool were not significantly different, with the exception of volunteers allocated to study group 3 (see Figure 7-22). A strong correlation between each peptide pool was observed (data not shown, p<0.001, two-tailed Spearman's correlation) and the decline in the total IFNγ response in older adults was explored at peptide-pool level to determine if there were any disproportionate, age-related, peptide pool specific changes. Using age as a continuous variable recorded a statistically significant negative correlation with the Fa, Fb and N peptide pool IFNγ responses, but not with the M peptide pool (Figure 7-23).

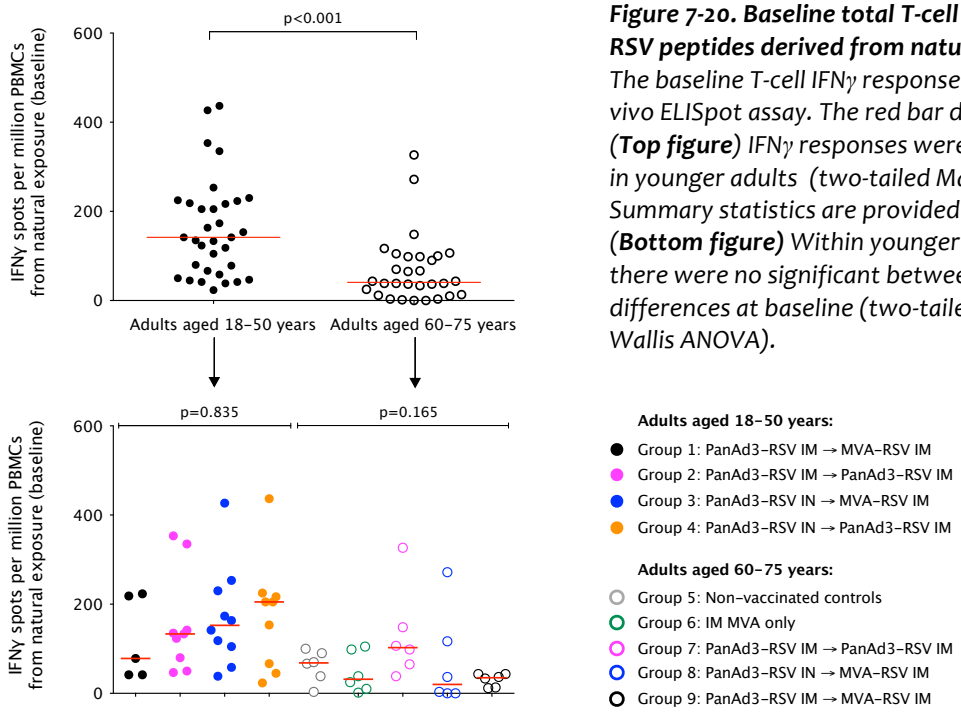


Figure 7-20. Baseline total T-cell IFNγ response to RSV peptides derived from natural exposure.
*The baseline T-cell IFNγ response was measured by ex vivo ELISpot assay. The red bar denotes the median. (**Top figure**) IFNγ responses were significantly greater in younger adults (two-tailed Mann-Whitney test). Summary statistics are provided in Table 7-3. (**Bottom figure**) Within younger and older adults there were no significant between study-group differences at baseline (two-tailed, one-way Kruskall-Wallis ANOVA).*

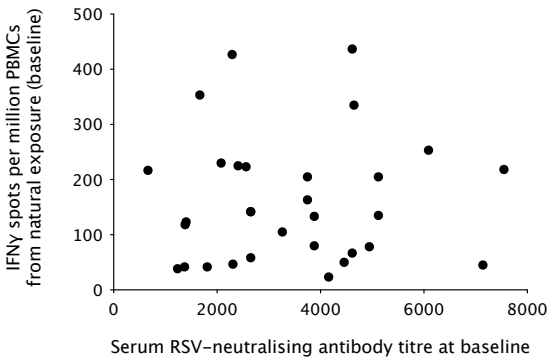


Figure 7-21. Correlation between the total IFNγ response to RSV peptides and serum RSV-neutralising antibody titres from natural exposure.
Data from younger adults only. There was no correlation between these measures at baseline (r=0.10, p=0.606, two-tailed Spearman's correlation).

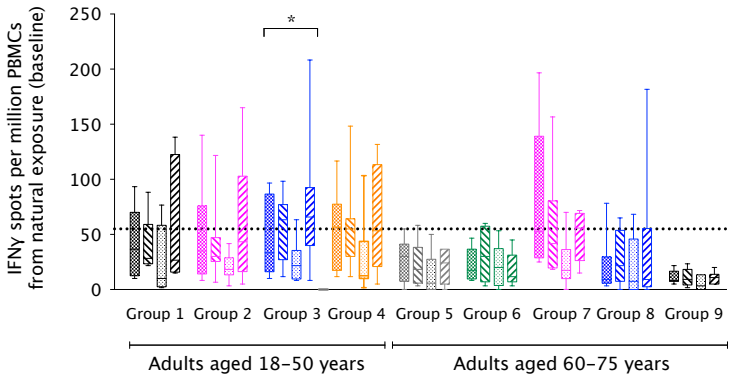


Figure 7-22. Baseline responses to each RSV peptide pool by study group.
*The IFNγ response to Fa, Fb, M and N peptide pools by box and whiskers plot by each study group at baseline. The dotted line marks the mean and 4 standard deviations of the DMSO background (55 spots per million PBMCs). *The variation in peptide pool responses in group 3 were statistically significant (p=0.042, one-way Kruskall-Wallis ANOVA).*



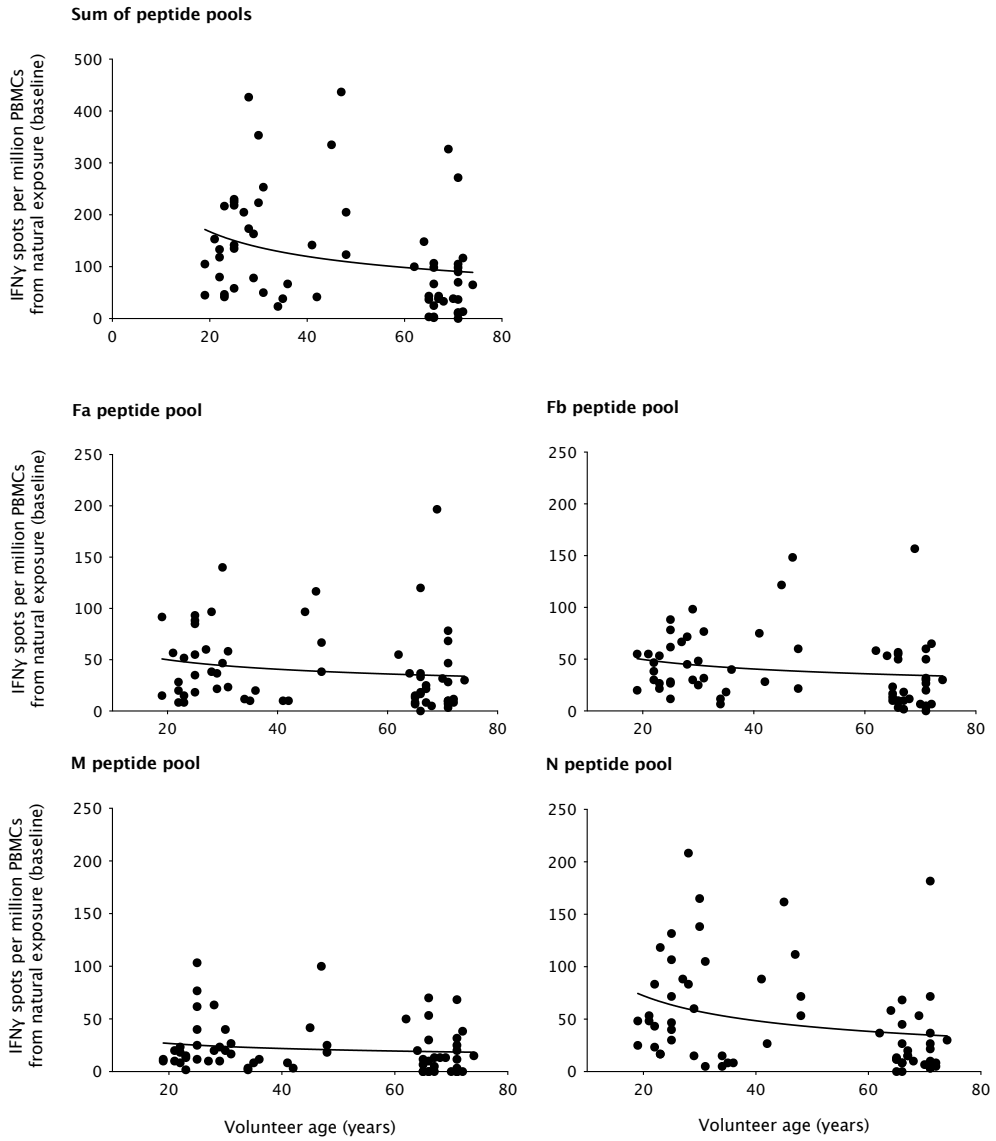


Figure 7-23. Correlation analyses between the baseline RSV-peptide specific IFNγ responses from natural exposure and volunteer age at enrolment.

*ELISpot data from younger and older adults (assays performed in 2013 and 2014 respectively). Each dot represents a single volunteer and the line is the non-linear best-fit description of the relationship with age. Correlation analyses used a two-tailed Spearman's test. (**Top figure**) The overall baseline IFNγ T-cell response to natural exposure, expressed as the sum of peptide pool responses with 4xDMSO background subtracted. A statistically significant decline in IFNγ responses with increasing age was observed (r=-0.40, p=0.001). (**Bottom panel of four figures**). The IFNγ response to peptide pools Fa, Fb, M and N not adjusted for DMSO background. A statistically significant decline in IFNγ responses with increasing age was observed for Fa (r=-0.27, p=0.032), Fb (r=-0.31, p=0.013) and N (r=-0.41, p=0.001) peptide pools. There was no statistical significance for the M peptide pool (r=-0.20, p=0.110).*

7.7.2 The T-cell IFNγ response to vaccination

IM PanAd3-RSV and IM MVA-RSV induced significant IFNγ T-cell responses in peripheral blood after vaccination. The intra-nasal route for PanAd3-RSV did not provoke detectable changes from baseline natural exposure 14-days after prime. The total IFNγ measures from each time point are presented in Figure 7-24. Peptide pool specific IFNγ responses remained balanced across each peptide pool (representing the three vaccine antigens) without dominance from one peptide pool relative to another (Figure 7-25 and Figure 7-27). There were no differences in the magnitude of the IFNγ response to low-dose and high-dose vaccine in younger adults (data not shown), and further analyses were performed without distinction for dose.

The expansion of IFNγ producing T-cells in peripheral circulation 14-days following prime was statistically significant for IM PanAd3-RSV (younger and older adults) and for IM MVA (older adults only). The peptide pool specific results, shown in Figure 7-28, showned each peptide pool contributed to the response to IM PanAd3-RSV or IM MVA-RSV prime with several peptide pool responses reaching statistical significance in the small study groups. By counts and proportions measures, 10/10 younger adults and 11/12 older adults primed with IM PanAd3-RSV recorded an increase in the summed IFNγ response. For IN PanAd3-RSV the proportions were lower; 9/17 younger adults and 4/6 older adults recorded an increase. IM MVA-RSV prime induced an IFNγ increase in 6/6 older adult volunteers and reached a magnitude of response comparable to IM PanAd3-RSV prime in older adults.

IFNγ responses measured before boost vaccine (4-weeks or 8-weeks after prime, depending on study group) had returned to baseline for most volunteers. Where the magnitude of the IFNγ response to prime was contingent on the IM route of vaccination, the greatest IFNγ responses 7-days following IM boost were vector associated. The greatest IFNγ responses were to IM MVA-RSV, exceeding the magnitude of response to prime and were irrespective of whether the prime had used the IM or IN route. All 15/15 younger adults and 9/9 older adults boosted with IM MVA-RSV recorded a rise in IFNγ responses 7-days after boost. A lower proportion of volunteers recorded a rise in IFNγ responses after IM PanAd3-RSV boost; 1/2 younger and 0/5 older from homologous IM PanAd3-RSV (groups 2 and 7) and 6/8 younger adults primed with IN PanAd3-RSV (group 4).

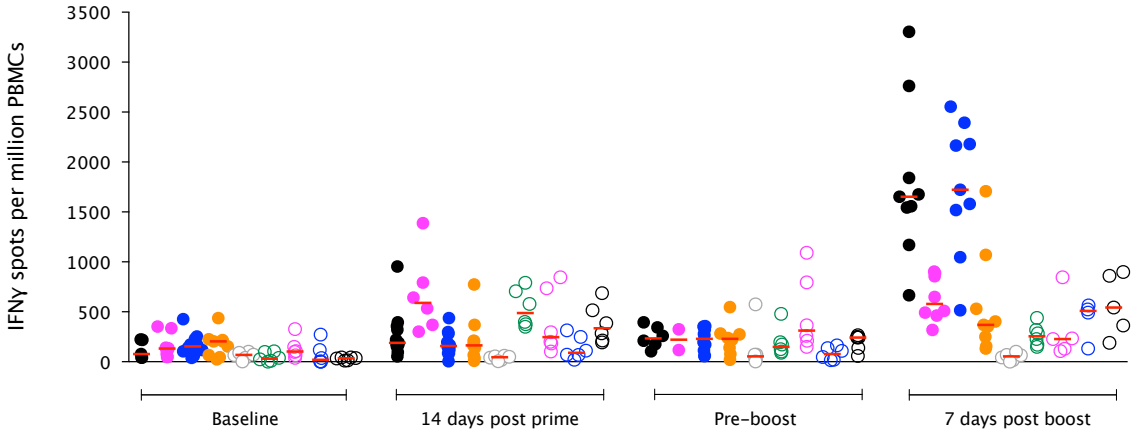


Figure 7-24. Summary results for the total T-cell IFNγ response to vaccination measured by ex vivo ELISpot.
Scatter plot of the summed IFNγ response measured by ELISpot, presented by study group at baseline, 14-days after prime, before boost and 7-days after boost. The red bar denotes the median. The colour coding matches prime/boost combinations in younger and older adults. Note that groups 5 and 6 did not receive a boost vaccine.

Adults aged 18–50 years:
- Group 1: PanAd3–RSV IM → MVA–RSV IM
- Group 2: PanAd3–RSV IM → PanAd3–RSV IM
- Group 3: PanAd3–RSV IN → MVA–RSV IM
- Group 4: PanAd3–RSV IN → PanAd3–RSV IM

Adults aged 60–75 years:
- Group 5: Non–vaccinated controls
- Group 6: IM MVA only
- Group 7: PanAd3–RSV IM → PanAd3–RSV IM
- Group 8: PanAd3–RSV IN → MVA–RSV IM
- Group 9: PanAd3–RSV IM → MVA–RSV IM

	Adults aged 18-50 years				Adults aged 60-75 years					p-value
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**	**Group 7**	**Group 8**	**Group 9**	
Prime vaccine	IM PanAd3-RSV	IM PanAd3-RSV	IN PanAd3-RSV	IN PanAd3-RSV	No vaccine	IM MVA-RSV	IM PanAd3-RSV	IN PanAd3-RSV	IM PanAd3-RSV	
Boost vaccine	IM MVA-RSV	IM PanAd3-RSV	IM MVA-RSV	IM PanAd3-RSV	No vaccine	No vaccine	IM PanAd3-RSV	IM MVA-RSV	IM MVA-RSV	
Median total responses (interquartile range):										
Before prime	78.3 (42-221)	133.3 (47-238)	152.5 (93-236)	205.0 (56-221)	68.3 (30-93)	31.7 (8-100)	102.5 (58-193)	20.0 (0-155)	35.0 (13-43)	p=0.003
14-days post prime	190.0 (152-357)	589.2 (353-942)	157.5 (86-286)	165.0 (52-290)	45.8 (15-55)	488.3 (370-728)	247.5 (163-764)	90.8 (56-265)	335.8 (208-558)	p<0.001
Before boost	235.0 (159-358)	220.8 (117-325)	229.2 (101-330)	230.0 (94-281)	54.2 (20-198)	148.3 (99-268)	312.5 (195-869)	78.3 (19-143)	243.3 (118-253)	p=0.04
7-days post boost	1653.0 (1358-2302)	580.0 (470-880)	1723.0 (1283-2287)	370.0 (203-800)	54.2 (11-76)	255.8 (165-349)	226.7 (118-541)	508.3 (223-555)	543.3 (277-880)	p<0.001

Table 7-3. Summary statistics of the total IFN γ responses to vaccination.
A one-way Kruskall-Wallis between groups ANOVA analysis was used to generate a p-value for the differences in median responses at each time point across study groups.

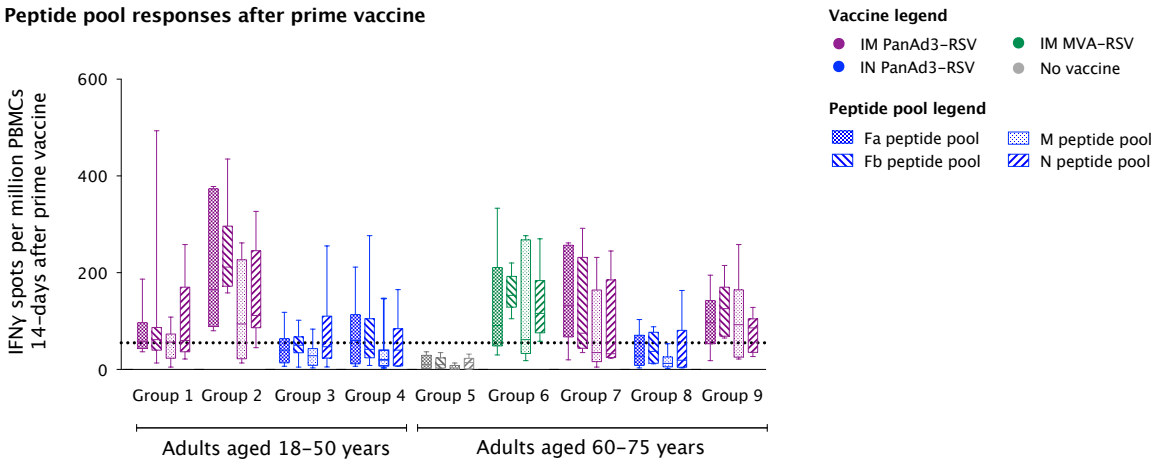


Figure 7-25. Responses to each RSV peptide pool to prime vaccination by study group.
Ex-vivo ELISpot data. The IFNγ response to Fa, Fb, M and N peptide pools by box and whiskers plot by each study group 14-days after prime vaccination. The dotted line marks the mean and 4 standard deviations of the DMSO background. The variations in median between RSV peptide pools within each study group were not statistically significant (p>0.05, one-way Kruskall-Wallis ANOVA).

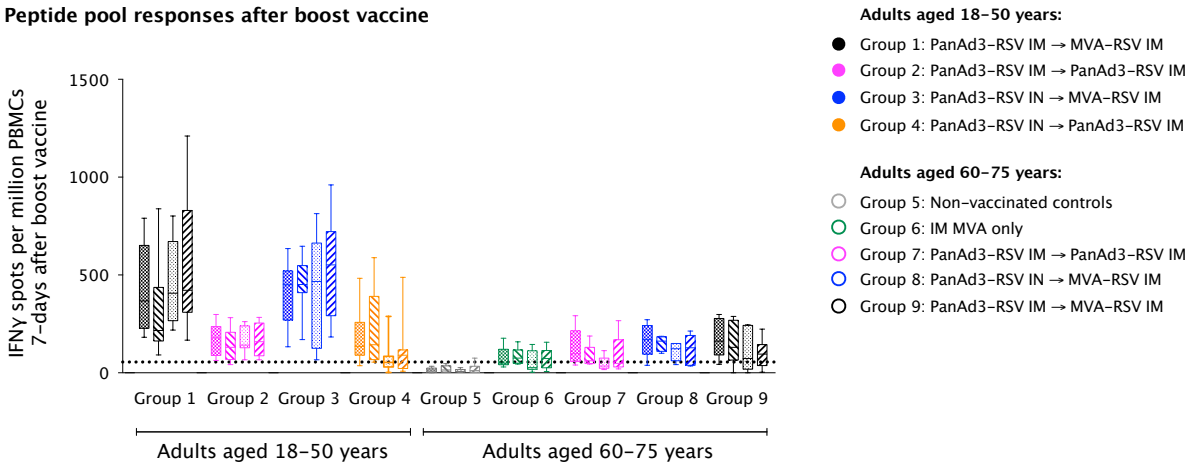


Figure 7-26. Responses to each RSV peptide pool to boost vaccination by study group.
Ex-vivo ELISpot data. The IFNγ response to Fa, Fb, M and N peptide pools by box and whiskers plot by each study group 7-days after boost vaccination. The dotted line marks the mean and 4 standard deviations of the DMSO background. The variations in median between RSV peptide pools in each study group were not statistically significant (p>0.05, one-way Kruskall-Wallis ANOVA). The peptide pool legend is the same as Figure 7-25.

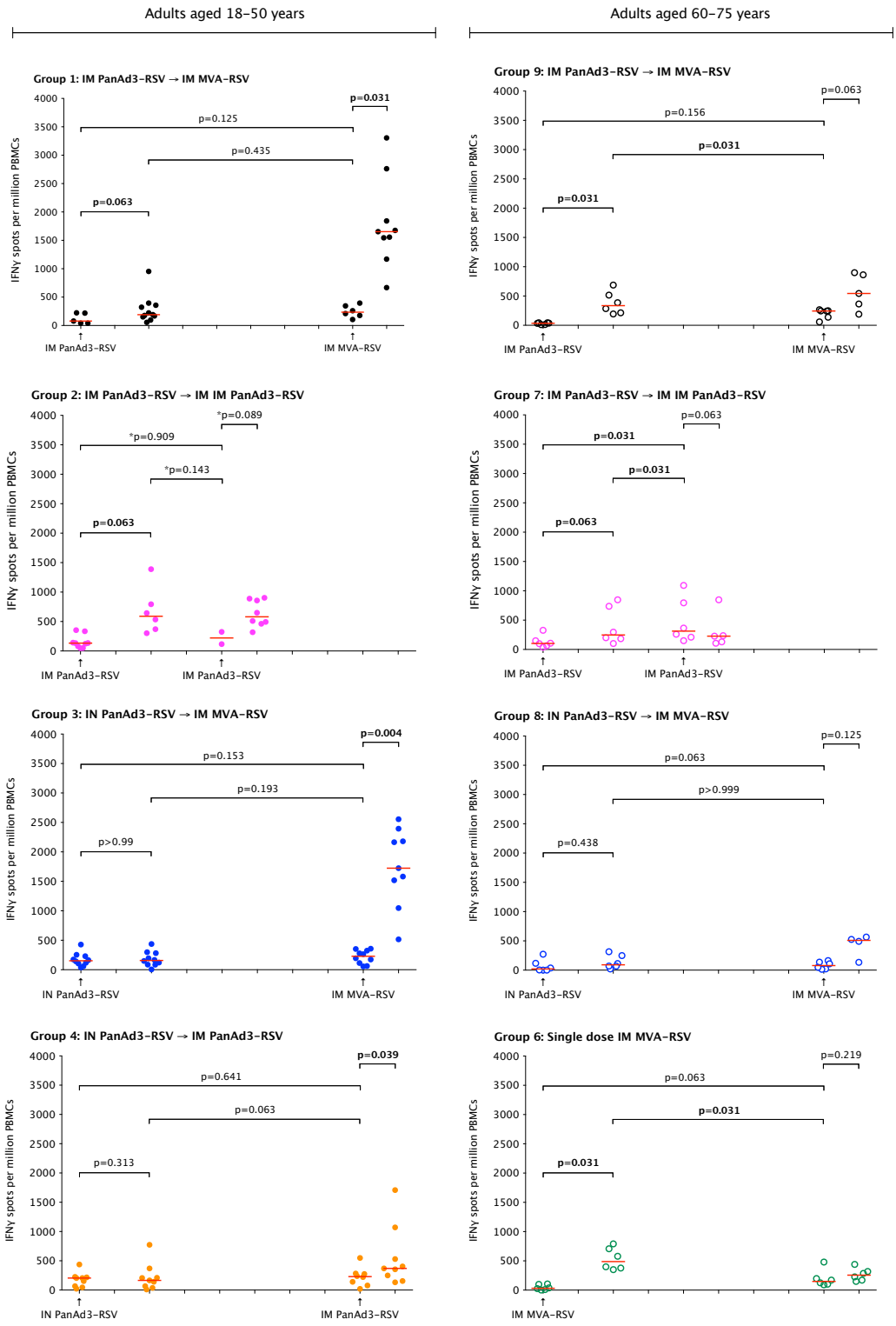


Figure 7-27. T-cell IFNγ response to vaccination measured by ex vivo ELISpot for each study group presented by timeline.

*Summed IFNγ response only. The x-axis shows the number of weeks after prime. The study groups are graphically paired for prime/boost combinations in younger and older adults, except for group 4 and group 6. The results for group 5 non-vaccinated controls are not shown (there were no differences across all time points for group 5, p=0.501, one-way ANOVA). Each pairwise comparison is a two-way Wilcoxon test, except those marked is an asterix in groups 2 where there were too few pre-boost samples (*two-tailed Mann-Whitney test applied). Statistically significant results are shown in bold.*

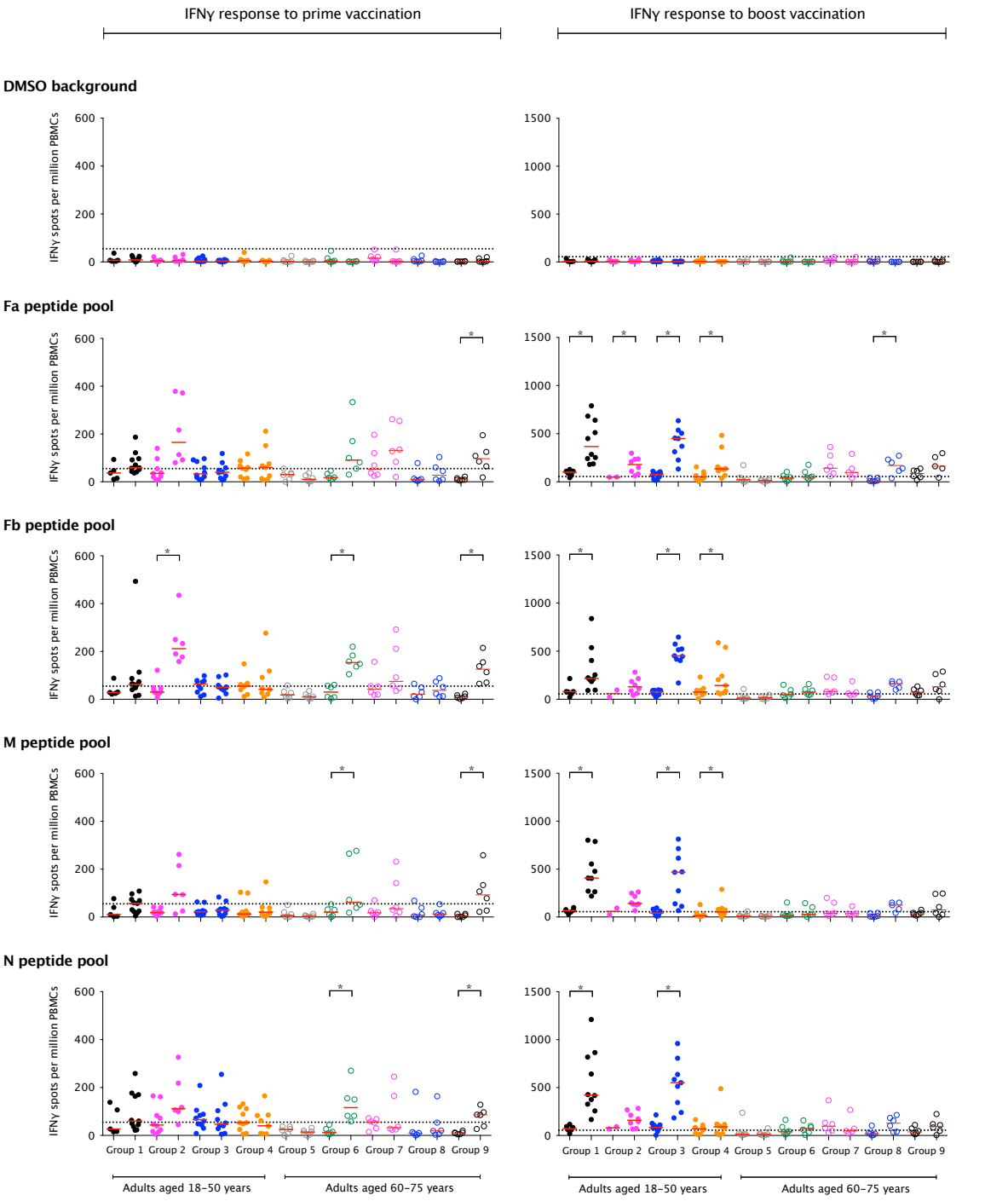


Figure 7-28 DMSO background and RSV peptide pool specific IFNγ responses to vaccination for each study group after prime and boost.
*Scatter plot of the IFNγ response paired before (**left panel**) and after vaccination (**right panel**). The red bar denotes the median and dotted line the mean DMSO +4 standard deviations background. The colour coding matches prime/boost study group vaccine combinations in younger and older adults. Pairwise tests were two-tailed Wilcoxon tests, except for group 2 due to insufficient pre-boost samples (two-tailed Mann-Whitney tests applied). Statistically significant differences are marked with an asterix (p-value <0.05).*

Adults aged 18–50 years:
- Group 1: PanAd3-RSV IM → MVA-RSV IM
- Group 2: PanAd3-RSV IM → PanAd3-RSV IM
- Group 3: PanAd3-RSV IN → MVA-RSV IM
- Group 4: PanAd3-RSV IN → PanAd3-RSV IM

Adults aged 60–75 years:
- Group 5: Non-vaccinated controls
- Group 6: IM MVA only
- Group 7: PanAd3-RSV IM → PanAd3-RSV IM
- Group 8: PanAd3-RSV IN → MVA-RSV IM
- Group 9: PanAd3-RSV IM → MVA-RSV IM

7.7.3 Exploration of the T-cell IFNγ response to IM PanAd3-RSV prime

As described earlier, an IFNγ response from circulating T-cells was detectable from volunteers primed by intra-muscular vaccination only. The post hoc exploration of the IFNγ prime response used volunteers primed with IM PanAd3-RSV (n=33 volunteers from study groups 1, 2, 7 and 9). The small number of older adults from group 6 (n=6 volunteers) did not support further analysis.

The post hoc analysis used the fold change in IFNγ spots per million PBMCs from baseline as an adjustment for variations in the baseline and background immune responses from natural exposure. Correlation analyses explored the potential for statistical relationships with volunteer age and other immune responses to vaccination.

Volunteer age was not statistically correlated with the fold-changes in IFNγ after prime, although a positive trend towards a significant correlation with increasing age was observed. Of note was that baseline measures of IFNγ from natural exposure and baseline titres of serum PanAd3 vector neutralising antibody recorded a statistically significant negative correlation with the fold-change in IFNγ after vaccination ($p<0.001$ and $p=0.003$ respectively, two-tailed Spearman's correlation). There were no statistically significant correlations with the serum RSV-neutralising antibody titres (baseline or the fold-change of antibody 28-days after prime) or with the F-specific IgG and IgA responses to vaccination (Figure 7-29).

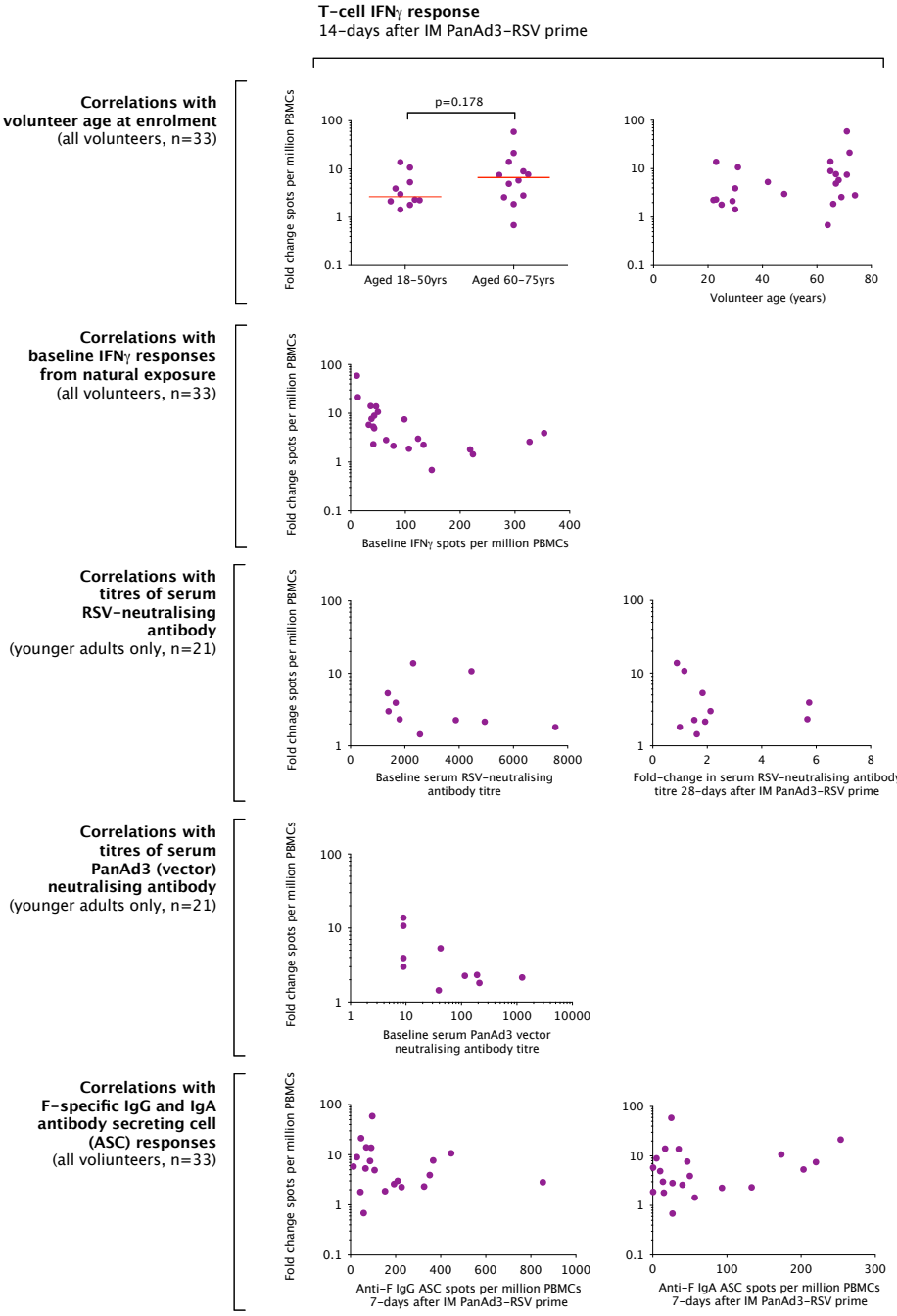


Figure 7-29. Post-hoc analyses of the T-cell IFNγ response to IM PanAd3-RSV prime vaccine.
*The T-cell IFNγ response to IM PanAd3-RSV prime was measured by ex-vivo ELISpot 14-days after vaccination and expressed as the log_{10} fold-change from baseline. (**Top row**) The red bar indicates the median. No statistically significant differences were observed between younger and older adults (left, p=0.177, two-tailed Mann-Whitney test) although a trend was observed for greater IFNγ fold-changes with increasing age (right, r=0.40, p=0.068, two-tailed Spearman's correlation). (**Second row**) A significant negative correlation was observed with the baseline IFNγ responses from natural exposure (r=-0.73, p<0.001, two-tailed Spearman's correlation). (**Middle row**) The baseline and 28-days post-prime fold change in serum RSV-neutralising antibody titres bore no statistical correlation with the fold-change induction of IFNγ after IM PanAd3-RSV prime. (**Fourth row**) The baseline titres of serum PanAd3 (vector) neutralising antibody had a significant negative association with the fold-changes of IFNγ after IM PanAd3-RSV prime (r=-0.67, p=0.030, two-tailed Spearman's correlation). (**Bottom row**) There were no statistically significant correlations for either the anti-F IgG ASC (left) or anti-F IgA (right) response to IM PanAd3-RSV prime (p=0.519 and p=0.618 respectively, two-tailed Spearman's correlation).*

7.7.4 Exploration of the T-cell IFNγ response to IM MVA-RSV and IM PanAd3-RSV boost

The MVA vector conferred the greatest magnitude of IFNγ responses 7-days after boost vaccination irrespective of the route of PanAd3-RSV prime. Further post hoc exploratory analysis concerned the IFNγ response to IM MVA-RSV boost vaccine only (n=32 volunteers from study groups 1, 3, 8 and 9).

As before, correlation analyses were applied to the IFNγ fold change 7-days after IM MVA-boost boost and investigated the potential for immune response variability due to volunteer age and pre-boost immune responses derived from natural exposure and prime vaccination. Age analysed as a binary or continuous variable correlated negatively with the magnitude of the IFNγ response to vaccination (p=0.029, two-tailed Mann Whitney test, see Figure 7-30), and no statistically significant relationship was observed with pre-boost measures of IFNγ, serum RSV- or PanAd3-neutralising antibody titres.

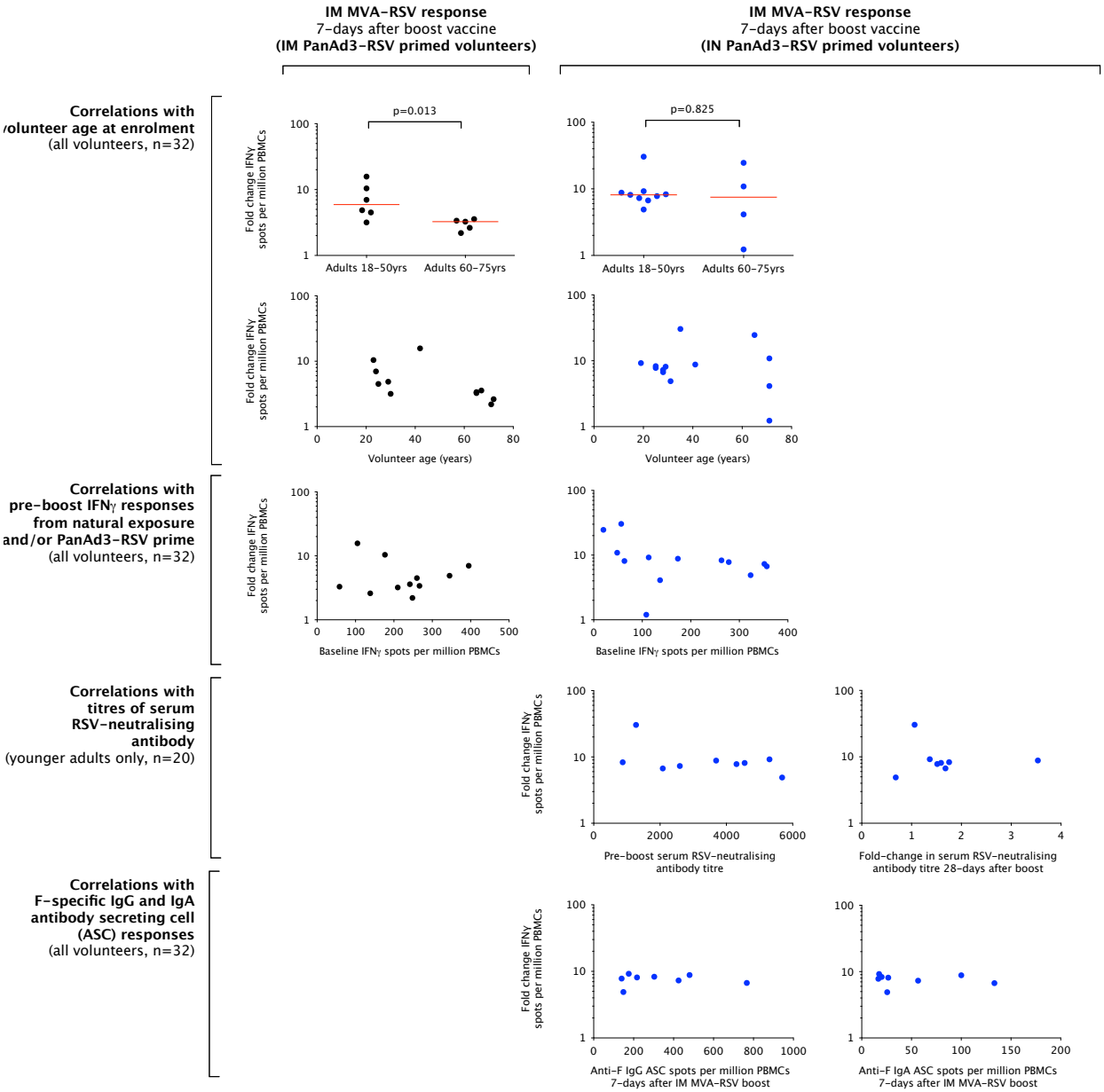


Figure 7-30. Post-hoc analyses of the T-cell IFNγ response to IM MVA-RSV boost vaccine from both IM and IN PaAd3-RSV primed volunteers.

*The T-cell IFNγ response to IM MVA-RSV boost was measured by ex-vivo ELISpot 7-days after boost and is represented as the log_{10} fold-change from pre=boost measures. (**Top row**) The red bar indicates the median. For younger adult volunteers primed with IM PanAd3-RSV (study group 1) the fold-changes in IFNγ producing T-cells was significantly greater than the old adults who received the same prime/boost combination (study group 9)(p=0.013, two-tailed Mann-Whitney test). There were no statistically significant differences between IFNγ responses to IM MVA-RSV boost from younger (study group 3) and older adult (study group 8) volunteers (p=0.825, two-tailed Mann-Whitney test). (**Second row**) The fold changes in IFNγ responses were negatively correlated with volunteer age in IM primed and not IN PAnAd3-RSV primed volunteers (p=0.013 and p=0.780 respectively, two-tailed Spearman's correlation). (**Middle row**) No statistically significant correlations with baseline IFNγ responses from natural exposure and/or prime were observed from IM PanAd3-RSV primed volunteers, but a significant negative correlation was observed with volunteers primed intra-nasally (p=0.714 and p=0.036 respectively, two-tailed Spearman's correlation). (**Fourth row**). The IFNγ response to IM MVA-RSV boost was measured against serum RSV-neutralising antibody responses from IN PanAd3-RSV primed volunteers only, as antibody responses from IM primed volunteers were not notable (data available from younger adults only). There were no statistically significant correlations with baseline or 28-day post-boost*

*titres of serum RSV-neutralising antibody (p=0.204 and 0.882 respectively, two-tailed Spearman's correlation). (**Bottom row**) The IFNγ response to IM MVA-RSV boost was measured against F-specific ASC responses from IN PanAd3-RSV primed volunteers only, as little/no ASC from IM primed volunteers had been observed. There were no correlations with anti-F IgG and IgA ASC responses (p=0.977 and p=0.501 respectively, two-tailed Spearman's correlation).*

7.8 Intra-cellular staining and FACS analysis of immune responses to boost vaccination

Next the phenotype of vaccine induced CD4+ and CD8+ T-cells was explored by intracellular cytokine staining (ICS) and fluorescence-activated cell sorting (FACS) analysis on frozen PBMCs stimulated with the same peptide pools used in the IFNγ ELISpot assay. The ICS assay, which was described in more detail earlier, included the measurement of IFNγ, TNFα, IL-2 and IL-5 at baseline and 7-days after boost vaccination. Only samples from younger adult volunteers were available for analysis without distinction for vaccine dose (total n=40 volunteers). The gating strategy used to quantify immune responses from ICS FACS data is shown in Figure 7-31.

Low levels of CD8+ and CD4+ T cell IFNγ responses were recorded at baseline and no between-groups analyses of the baseline were performed. There was a modest but consistent increase in IFNγ producing CD4+ T cells from each study group, of a magnitude consistent with the *ex vivo* ELISpot data, in response to boost vaccination with responses to all peptide pools. CD8+ T cell IFNγ responses were not altered by boost vaccination (Figure 7-32). T-cell production of TNFα was not altered by vaccination across CD4+ and CD8+ populations (Figure 7-33). More significantly was the absence of detectable Th2 immune responses (IL-2 and IL-5 producing T-cells) 7-days after IM MVA-RSV and IM PanAd3-RSV boost vaccination (Figure 7-34 and Figure 7-35).

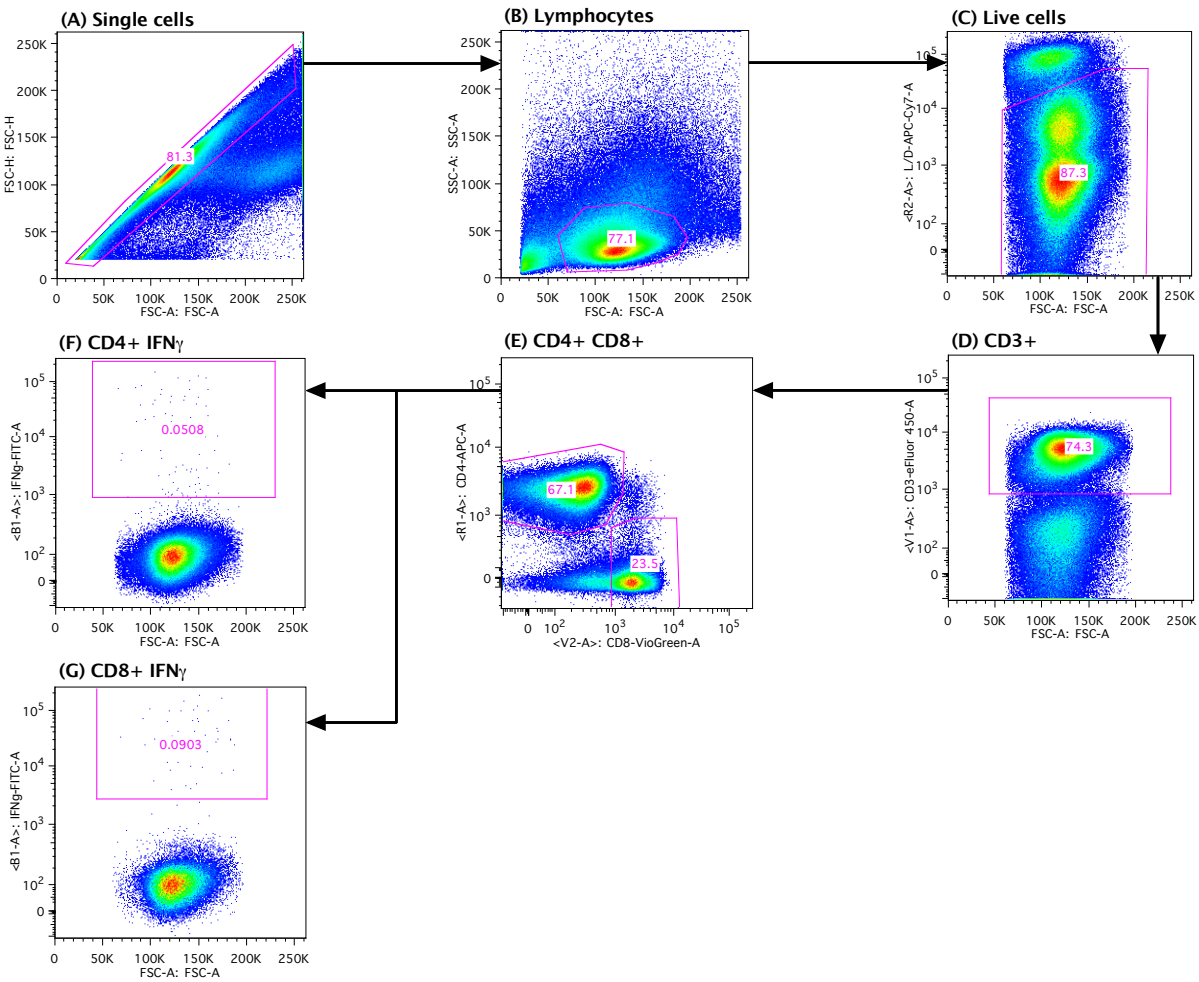


Figure 7-31. The gating strategy used for the quantification of IFNγ, TNFα, IL-2 and IL-5 responses after IM MVA-RSV and IM PanAd3-RSV boost vaccination by ICS FACS analysis.
The example used here is the IFNγ response from one volunteer to the N peptide pool measured 7-days after high-dose IM MVA-RSV vaccination. PBMCs were gated from (A) single cells, (B) lymphocytes, (C) live cells, (D) CD3+ cells and then (F) CD4+ IFNγ and (G) CD8+ IFNγ cells. Screenshots are courtesy of Catherine de Lara.

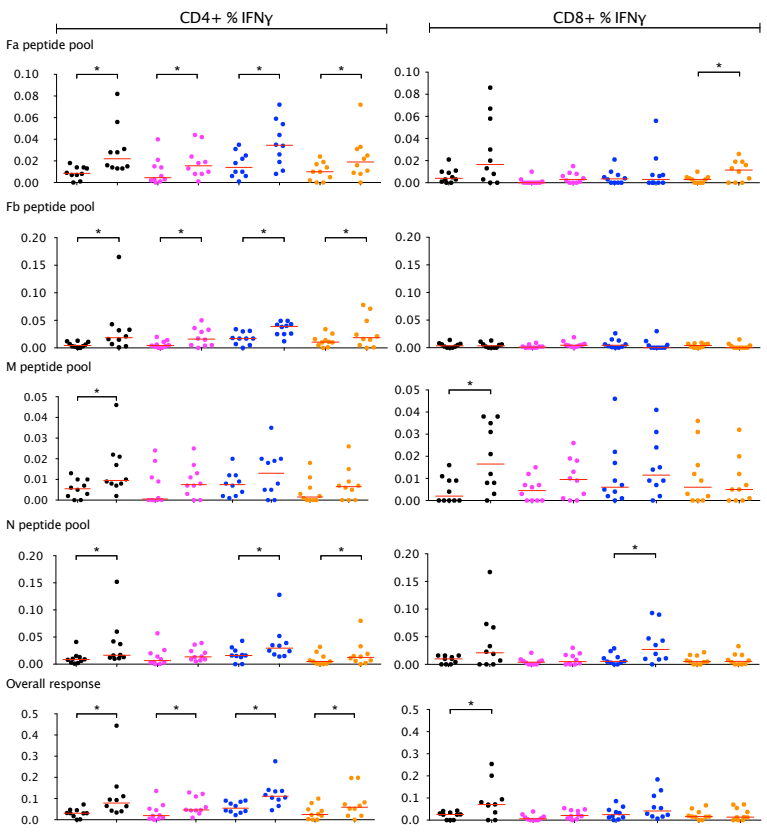


Figure 7-32. CD4+ and CD8+ IFNγ response to boost vaccination by ICS FACS analysis.
Data from younger adults only. Frozen PMBCs were stimulated with the same RSV peptide pools as the IFNγ ELISpot. Peptide pool specific responses are DMSO background subtracted (negative values reverted to zero), and the overall response is the sum of peptide responses. For each study group the left scatter plot are the baseline responses from natural exposure, and the right scatter plot is the response measured 7-days post boost vaccination. The red line is the median. Pairwise comparisons used a two-tailed Wilcoxon test, with significant differences marked with an asterix (p<0.05).

- Group 1: PanAd3-RSV IM → MVA-RSV IM
- Group 2: PanAd3-RSV IM → PanAd3-RSV IM
- Group 3: PanAd3-RSV IN → MVA-RSV IM
- Group 4: PanAd3-RSV IN → PanAd3-RSV IM

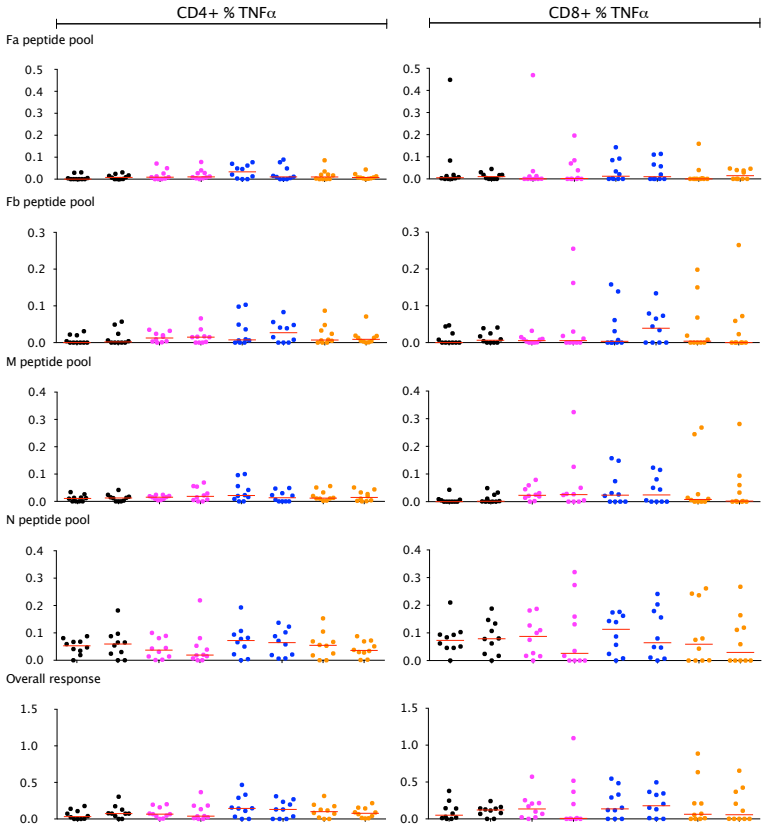


Figure 7-33. CD4+ and CD8+ TNFα response to boost vaccination by ICS FACS analysis.
Data from younger adults only. Frozen PMBCs were stimulated with the same RSV peptide pools as the IFNγ ELISpot. Peptide pool specific responses are DMSO background subtracted (negative values reverted to zero), and the overall response is the sum of peptide responses. For each study group the left scatter plot are the baseline responses from natural exposure, and the right scatter plot is the response measured 7-days post boost vaccination. The red line is the median. Pairwise comparisons used a two-tailed Wilcoxon test, with significant differences marked with an asterix (p<0.05).

- Group 1: PanAd3-RSV IM → MVA-RSV IM
- Group 2: PanAd3-RSV IM → PanAd3-RSV IM
- Group 3: PanAd3-RSV IN → MVA-RSV IM
- Group 4: PanAd3-RSV IN → PanAd3-RSV IM

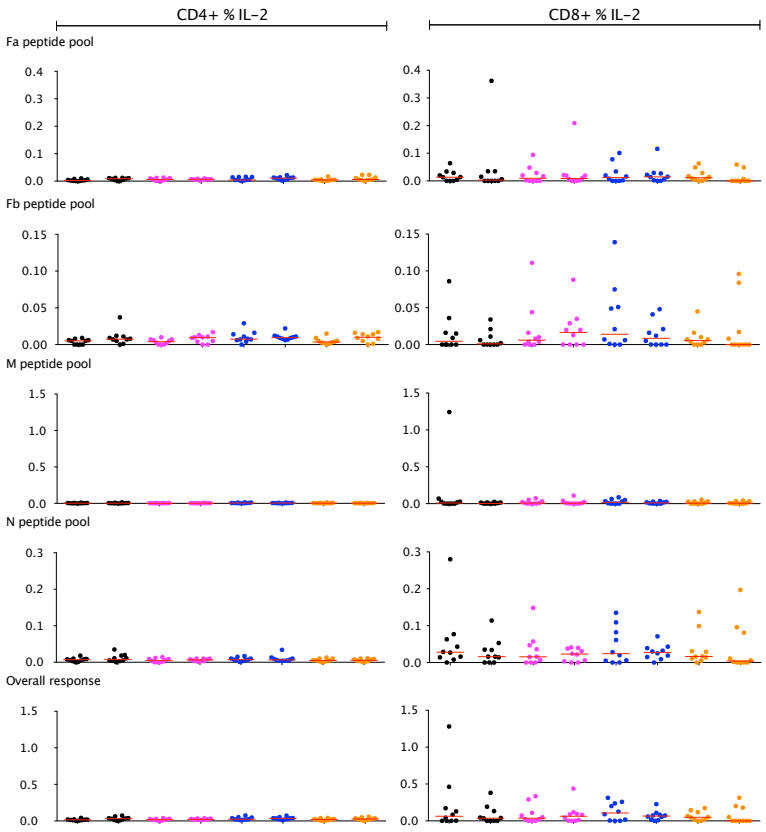


Figure 7-34. CD4+ and CD8+ IL2 response to boost vaccination by ICS FACS analysis.

Data from younger adults only. Frozen PMBCs were stimulated with the same RSV peptide pools as the IFNγ ELISpot. Peptide pool specific responses are DMSO background subtracted (negative values reverted to zero), and the overall response is the sum of peptide responses. For each study group the left scatter plot are the baseline responses from natural exposure, and the right scatter plot is the response measured 7-days post boost vaccination. The red line is the median. Pairwise comparisons used a two-tailed Wilcoxon test, with significant differences marked with an asterix ($p<0.05$).

- Group 1: PanAd3-RSV IM → MVA-RSV IM
- Group 2: PanAd3-RSV IM → PanAd3-RSV IM
- Group 3: PanAd3-RSV IN → MVA-RSV IM
- Group 4: PanAd3-RSV IN → PanAd3-RSV IM

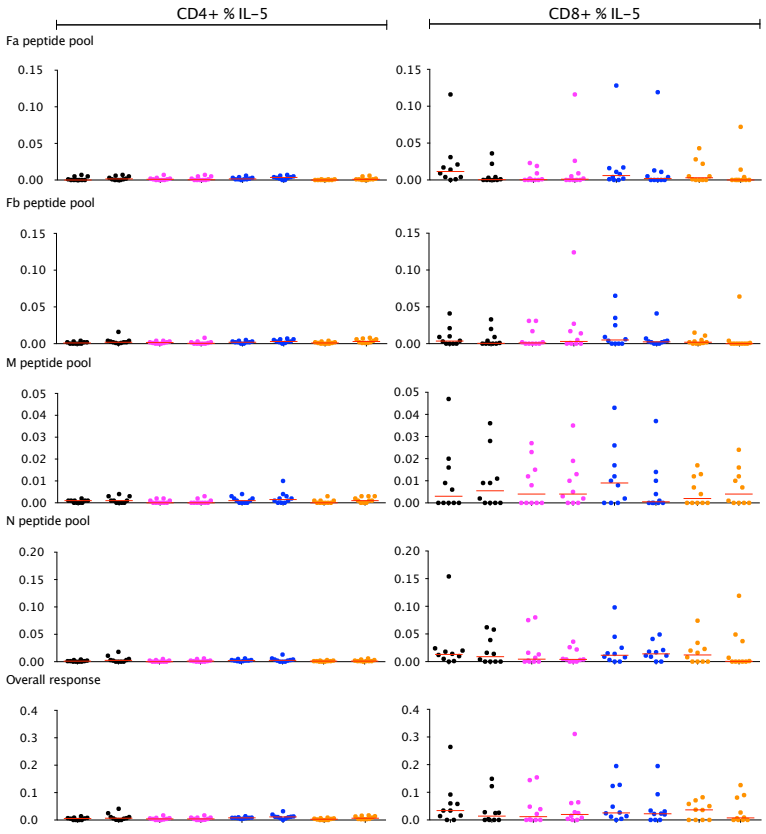


Figure 7-35. CD4+ and CD8+ IL5 response to boost vaccination by ICS FACS analysis.

Data from younger adults only. Frozen PMBCs were stimulated with the same RSV peptide pools as the IFNγ ELISpot. Peptide pool specific responses are DMSO background subtracted (negative values reverted to zero), and the overall response is the sum of peptide responses. For each study group the left scatter plot are the baseline responses from natural exposure, and the right scatter plot is the response measured 7-days post boost vaccination. The red line is the median. Pairwise comparisons used a two-tailed Wilcoxon test, with significant differences marked with an asterix ($p<0.05$).

- Group 1: PanAd3-RSV IM → MVA-RSV IM
- Group 2: PanAd3-RSV IM → PanAd3-RSV IM
- Group 3: PanAd3-RSV IN → MVA-RSV IM
- Group 4: PanAd3-RSV IN → PanAd3-RSV IM

7.9 Cytokine response to boost vaccination

To further explore the phenotype of vaccine-induced immune responses the production of cytokines was measured using supernatants of the *ex vivo* IFNγ ELISpot and a modified BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 kit. Three normal-dose vaccine recipients from each study group were selected as high-, mid- and low- responders according to the *ex vivo* IFNγ ELISpot result 7-days after boost vaccination (total n=11 volunteers, samples from one volunteer in group 3 were not available). After DMSO background subtraction there was no detectable IFNγ (presumably captured in the IFNγ ELISpot), IL-2, IL-4, IL-10 and only low-level baseline TNFα responses from one volunteer only (data not shown). IL-6 and IL-17 production was detected mostly in response to N peptide pool stimulation, but there was no consistency in changes across baseline or after vaccination.

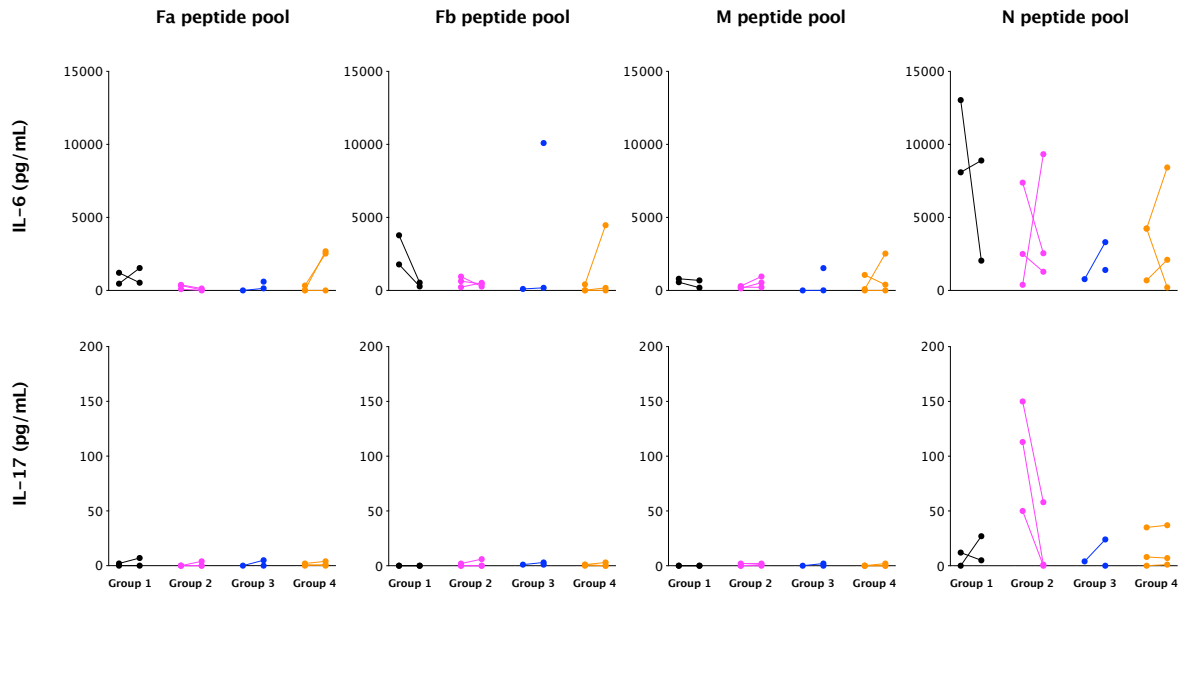


Figure 7-36. IL-6 and I-L17 responses to boost vaccination measured by cytometric bead array.
Data for adults aged 18-50 years only. IL-6 and IL-17 were measured from the supernatant of the ex vivo IFNγ ELISpot in three volunteers of each study group at baseline and 7-days post IM MVA-RSV and IM PanAd3-RSV boost vaccination. After DMSO background subtraction there were no consistent responses for IL-6 and IL-17, and no IFNγ, IL-2, IL-4, IL-10 or TNFα responses above background were recorded at baseline or after vaccination (data not shown).

7.10 Peripheral T-follicular helper (Tfh) cells measured by FACS

The T follicular helper (Tfh) cell response to vaccination was explored by intracellular staining (ICS) and fluorescence-activated cell sorting (FACS) assay[i]. The assay was described in more detail in earlier sections. Un-stimulated frozen PBMCs from peripheral blood of volunteers who received IM PanAd3-RSV prime vaccine (study groups 1, 2, 7 and 9) were analysed for Tfh cell frequencies and expression of PD1 and ICOS activation markers before vaccination and one-week post prime/boost (n=26 volunteers). Volunteer age was applied as a categorical variable (younger/older adults) based upon the distribution shown in the figure below.

Tfh cells are essential in the development of B-cell effector and memory responses within lymphoid follicles and can be characterised by the surface expression of CXCR5 (CD185), the receptor for CXCL13. Tfh cells (CD4+ CXCR5+ CD45-) were subdivided into Th1 (CXCR3+, CCR6-), Th2 (CXCR3- CCR6-) and Th17 (CXCR3- CCR6+) subsets[439]. Finally, within each subset the co-expression of markers PD1 and ICOS were used to characterise the activated cell phenotype. The FACS gating strategy is shown by example in Figure 7-38. The Tfh response to vaccination (overall, by subset and by activation markers) is expressed as the percentage of the parent population from the gating strategy applied.

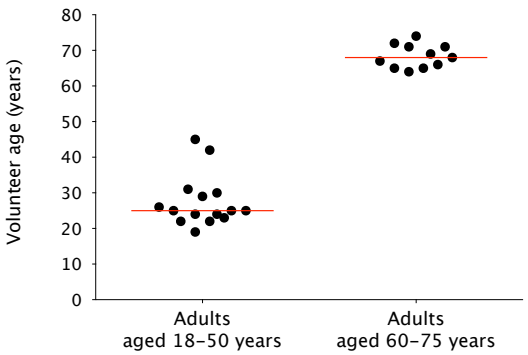


Figure 7-37. The age distribution of IM PanAd3-RSV primed volunteers selected for T-follicular helper cell ICS/FACS analysis.
Adults aged 18-50 years; n=15, median age 25 years (IQR 19-30), 9/16 (56%) male. Adults aged 60-75 years; n= 11, median age 68 years (IQR 64-71), 7/11 (64%) male.

[i] The term T follicular helper (Tfh) cells strictly applies to T-cells resident in follicles. Here the term is used to denote T-cells in peripheral circulation with Tfh-like markers, as measured by FACS.

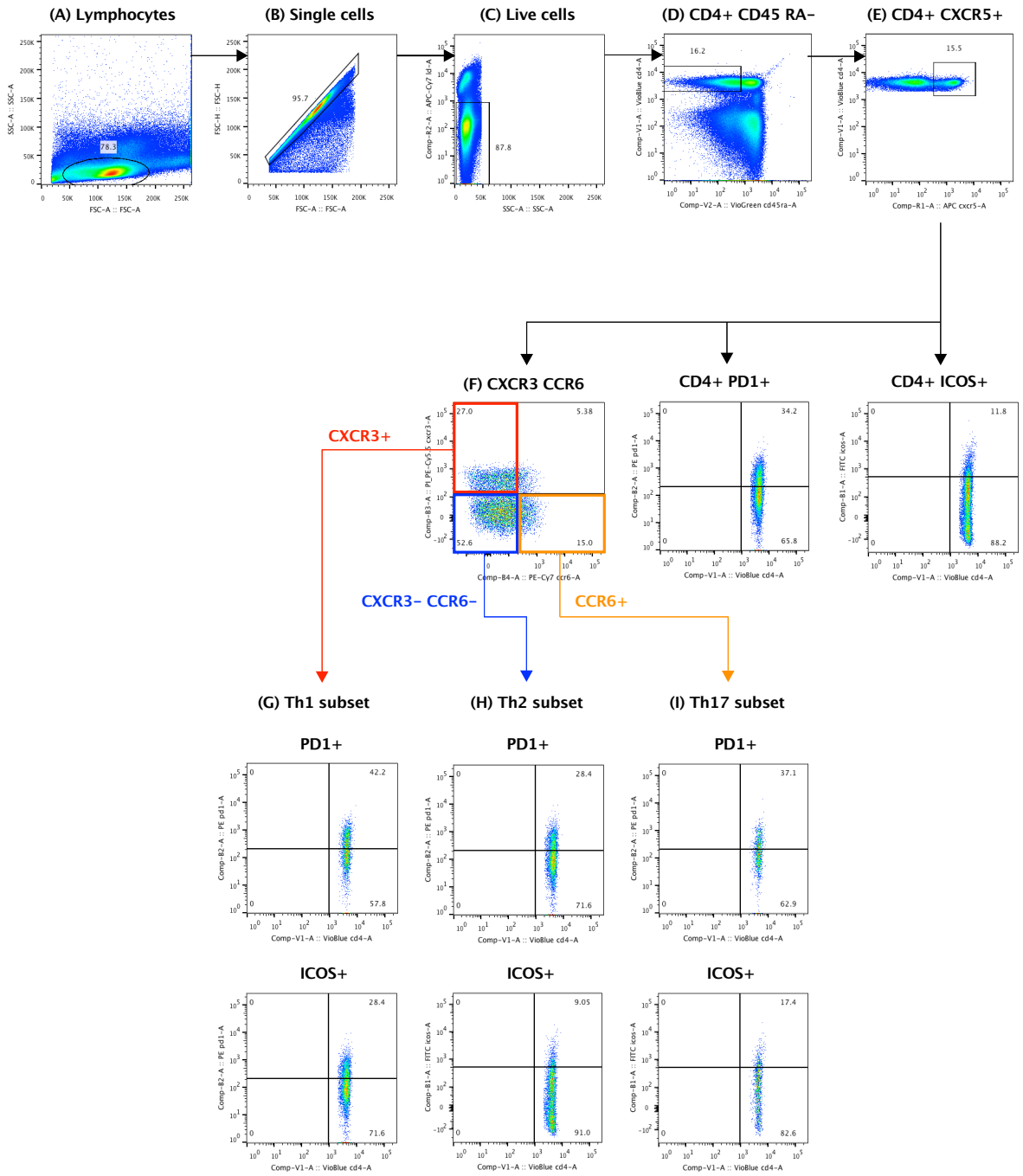


Figure 7-38. The gating strategy for the quantification of Tfh cell immune responses after vaccination. *The example used here is from a volunteer 7-days after high-dose IM PanAd3-RSV prime. PBMCs were un-stimulated and gated from (A) lymphocytes, (B) single cells, (C) live cells, (D) CD4+ CD45RA- memory T-cells, (E) CD4+ T-cells with CXCR5 expression, (F) lymphocytes gated on CCR6 into (G) Th1 subset (CD4+ CD45RA- CXCR5+ CXCR3+), (H) Th2 subset (CD4+ CD45RA- CXCR5+ CXCR- CCR6-) and (I) Th17 subset (CD4+ CD45RA- CXCR5+ CCR6+). CXCR5+ Th1/Th2/Th17 subsets were further gated on PD1+ and ICOS+. Screenshots are courtesy of Tamsin Cargill.*

7.10.1 Analysis of the baseline T-follicular helper cell (Tfh) expression in peripheral blood

The total Tfh cell populations at baseline derived from natural exposure were not statistically different between younger and older adults. This was also observed for each of the Th1/Th2/Th17 subsets. Statistically significant baseline differences were observed with the expression of PD1 and ICOS activation markers. In all CXCR5+ cell populations (total, Th1, Th2 and Th17) the percentage of cells from the parent population with ICOS expression was significantly lower in older adults. The proportion of cells with PD1 expression was generally lower in older adults too, with the difference within the CXCR5+ Th2 subset reaching statistical significance (borderline significance for CXCR5+ Th17 subset). Baseline Tfh cell frequencies, subsets and expression markers bore no relationship to the baseline serum RSV-neutralising antibody titres in younger adults (see supplementary material).

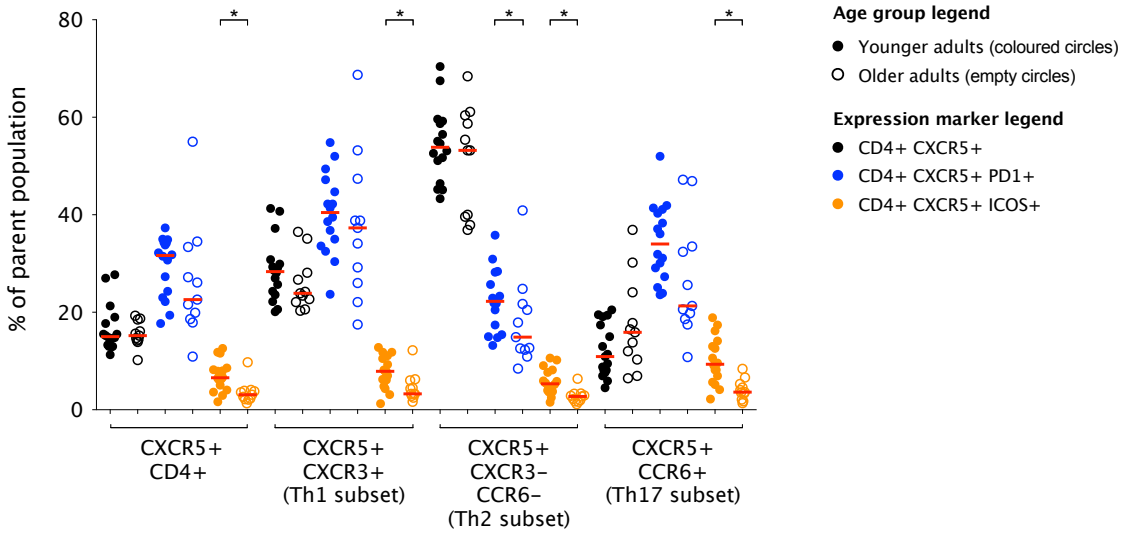


Figure 7-39. T-follicular helper cell profile in peripheral blood from younger and older adults at baseline. *Data from IM PanAd3-RSV primed volunteers only. Scatter plot of %CD4+ CXCR5+ and Th1/Th2/Th17 subsets of un-stimulated PBMCs before vaccination. Red bars indicate the median. Pairwise comparisons are two-tailed Mann-Whitney tests, significant differences are shown by asterix ($p<0.05$).*

7.10.2 The T-follicular helper cell (Tfh) response to IM PanAd3-RSV prime

IM PanAd3-RSV prime was associated with paradoxical changes in peripheral Tfh cell populations of younger and older adults 7-days after vaccination. In adults aged 18-50 years there was a small but statistically significant rise in the total circulating Tfh cell population 7-days after prime. Within the total Tfh cell population, the proportion of the Th1 and Th17 subsets remained unchanged but the proportion of Th2 Tfh cells decreased significantly. In contrast, for adults aged 60-75 years the total circulating Tfh cell population significantly decreased 7-days after prime. Within the total Tfh cell population the proportion of the Th1 and Th2 subsets remained unchanged but the proportion of Th17 Tfh cells decreased significantly. The expression of PD1 and ICOS were analysed as surrogates of Tfh cell activation. In both younger and older adults PD1 expression on the total Tfh population did no change significantly after prime. In younger adults PD1 expression did not change after prime in the Th1 and Th2 subsets, but fell in the Th17 subset. In older adults PD1 expression did not change after prime in the Th2 or Th17 subsets, but increased in the Th1 subset. Despite PD1 expression remaining consistent, ICOS expression on total Tfh cells significantly decreased after prime in younger adults and this was consistent in the Th1 (trend only), Th2 and Th17 subsets. In contrast ICOS expression in older adults did not record any statistically significant differences between baseline and 7-days post-vaccination. ICOS expression remained significantly lower at boost for all subsets in younger adults and was lower at boost for the Tfh and Th2 and Th17 subsets of older adults.

Analysis of PBMCs obtained 7-days post IM MVA-RSV boost recorded a small but statistically significant rise in the Th1 subset of younger adults and Th2 subset of older adults, and a fall in the Th17 subset of younger adults. PD1 expression increased in the Th17 subset of younger and older adults only (trend in older adults only). ICOS expression increased significantly in total Tfh, Th1 and Th2 but not Th17 subsets. Finally, analysis of the Tfh response to IM PanAd3-RSV boost recorded significant rises in Tfh (younger adults), Th1 subsets (older adults) and a fall in the Th17 subset (younger adults). PD1 expression was unchanged in Tfh and Th1 subsets, depressed in the Th2 subset (trend in older adults only) and depressed in the Th17 subset of younger adults. ICOS was unchanged across all subsets in both younger and older adults.

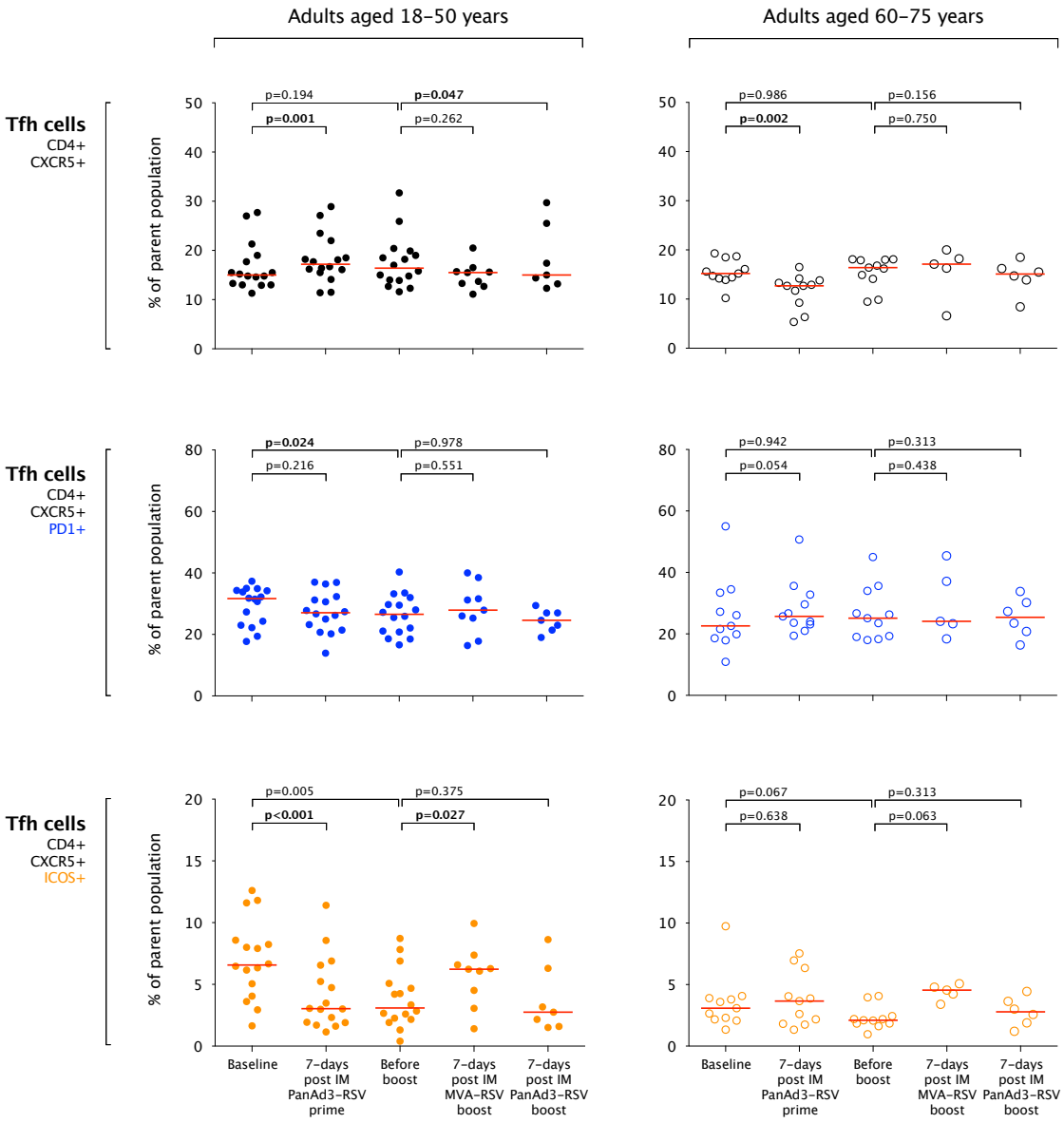


Figure 7-40. The T-follicular helper cell (CD4+ CXCR5+ CD45-) cell frequency response to vaccination.
*FACS data from un-stimulated PBMCs from IM PanAd3-RSV primed volunteers (study groups 1, 2, 7 and 9). All cell frequencies are presented as the % of parent population with red lines used to mark the median value. Time points included were baseline, 7-days after IM PanAd3-RSV prime, before boost (4- weeks post prime for groups 2 and 7; 8-weeks post prime for groups 1 and 9) and 7-days after boost for IM MVA-RSV and IM PanAd3-RSV respectively. Comparative statistics were two-way paired t-tests without correction for multiple testing. (**Top row**) The overall T-follicular helper (Tfh) cell populations in younger and older adults and (**middle row**) with PD1 co-expression and (**bottom row**) ICOS co-expression.*

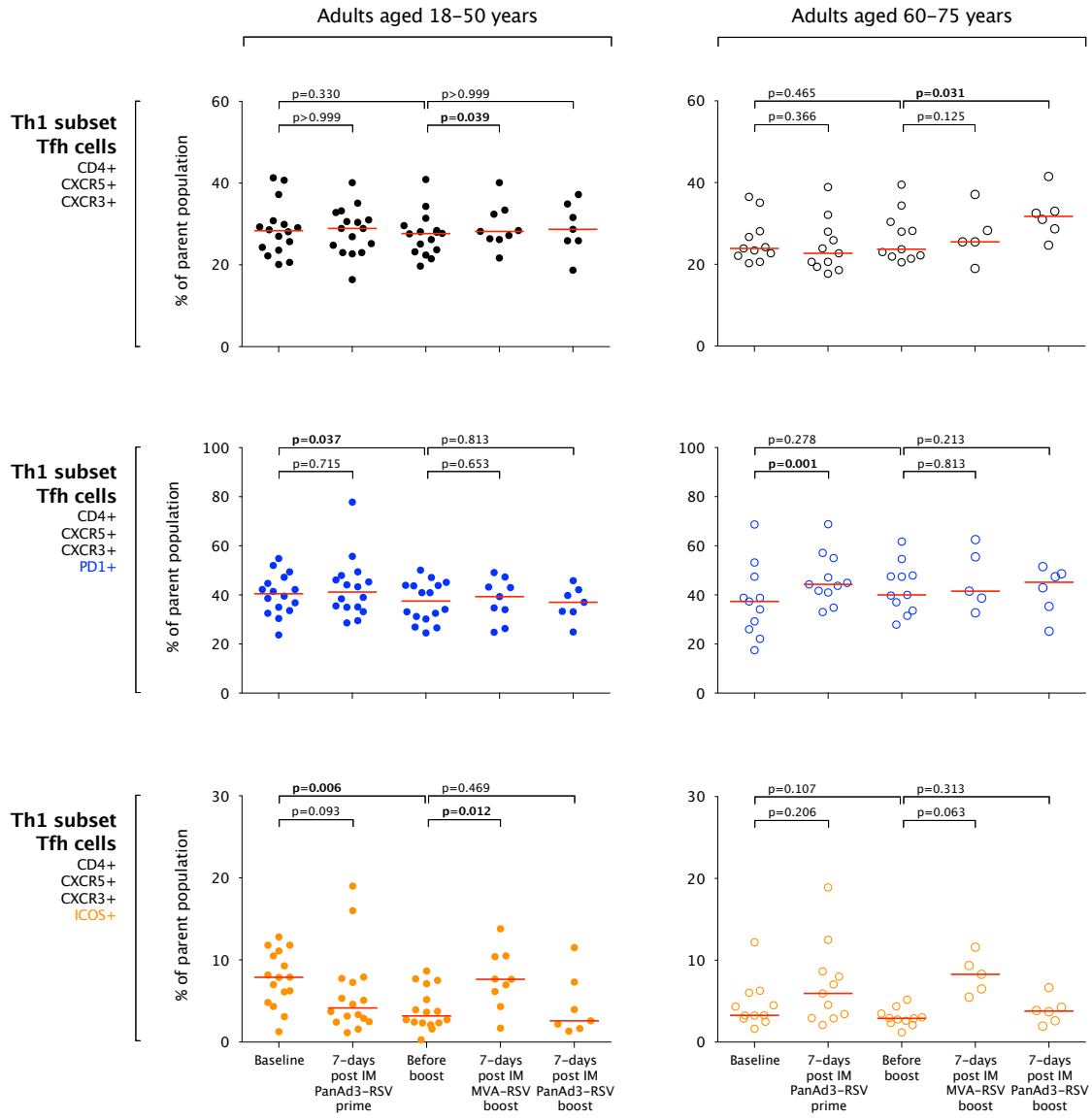


Figure 7-41. The Th1 subset of T-follicular helper cell (CD4+ CXCR5+ CD45- CXCR3+) cell frequency response to vaccination.

*FACS data from un-stimulated PBMCs from IM PanAd3-RSV primed volunteers (study groups 1, 2, 7 and 9). All cell frequencies are presented as the % of parent population with red lines used to mark the median value. Time points included were baseline, 7-days after IM PanAd3-RSV prime, before boost (4- weeks post prime for groups 2 and 7; 8-weeks post prime for groups 1 and 9) and 7-days after boost for IM MVA-RSV and IM PanAd3-RSV respectively. Comparative statistics were two-way paired t-tests without correction for multiple testing. (**Top row**) The overall T-follicular helper (Tfh) cell populations in younger and older adults and (**middle row**) with PD1 co-expression and (**bottom row**) ICOS co-expression.*

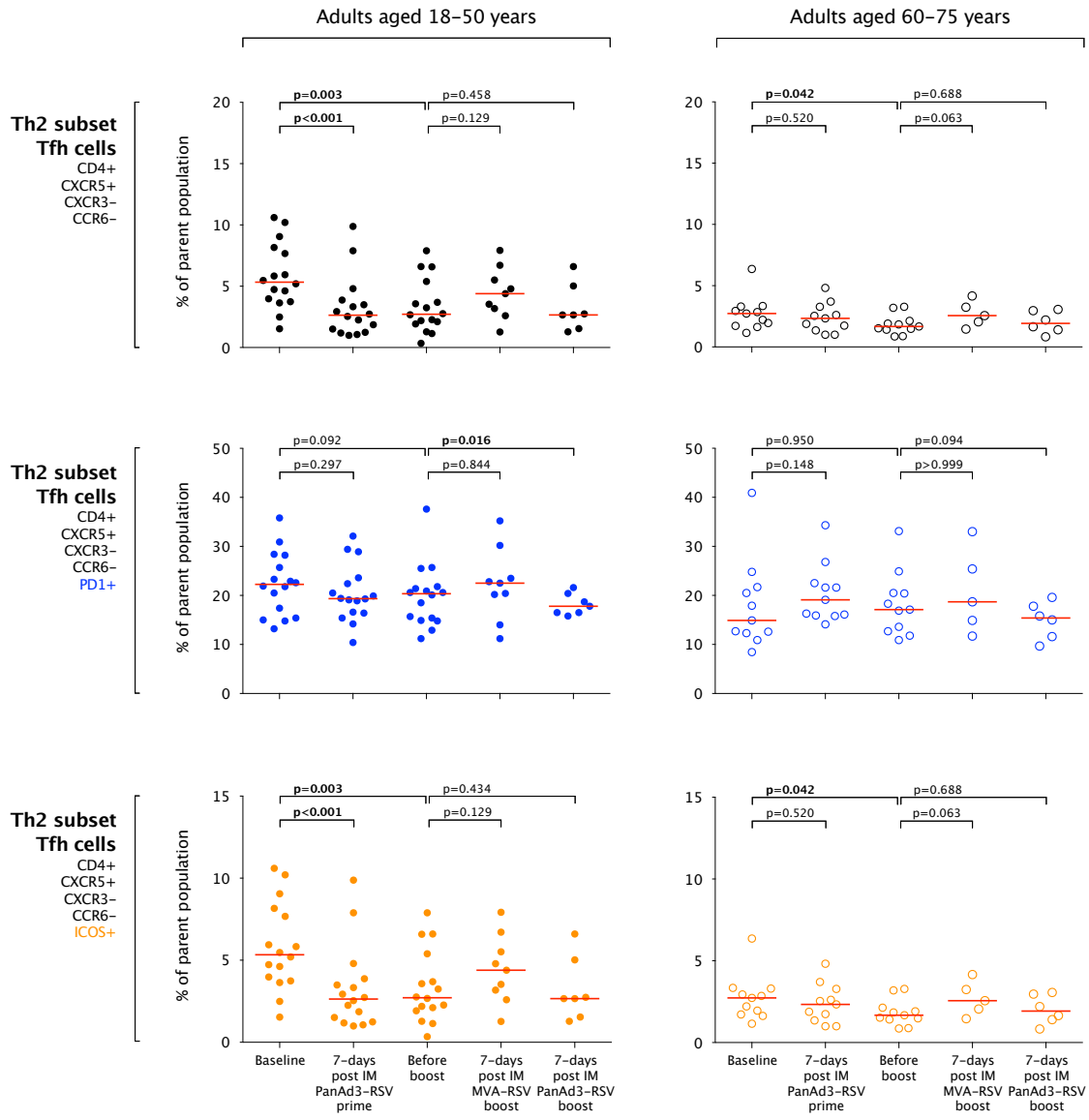


Figure 7-42. The Th2 subset of T-follicular helper cell (CD4+ CXCR5+ CD45- CXCR3- CCR6-) cell frequency response to vaccination.

*FACS data from un-stimulated PBMCs from IM PanAd3-RSV primed volunteers (study groups 1, 2, 7 and 9). All cell frequencies are presented as the % of parent population with red lines used to mark the median value. Time points included were baseline, 7-days after IM PanAd3-RSV prime, before boost (4- weeks post prime for groups 2 and 7; 8-weeks post prime for groups 1 and 9) and 7-days after boost for IM MVA-RSV and IM PanAd3-RSV respectively. Comparative statistics were two-way paired t-tests without correction for multiple testing. (**Top row**) The overall T-follicular helper (Tfh) cell populations in younger and older adults and (**middle row**) with PD1 co-expression and (**bottom row**) ICOS co-expression.*

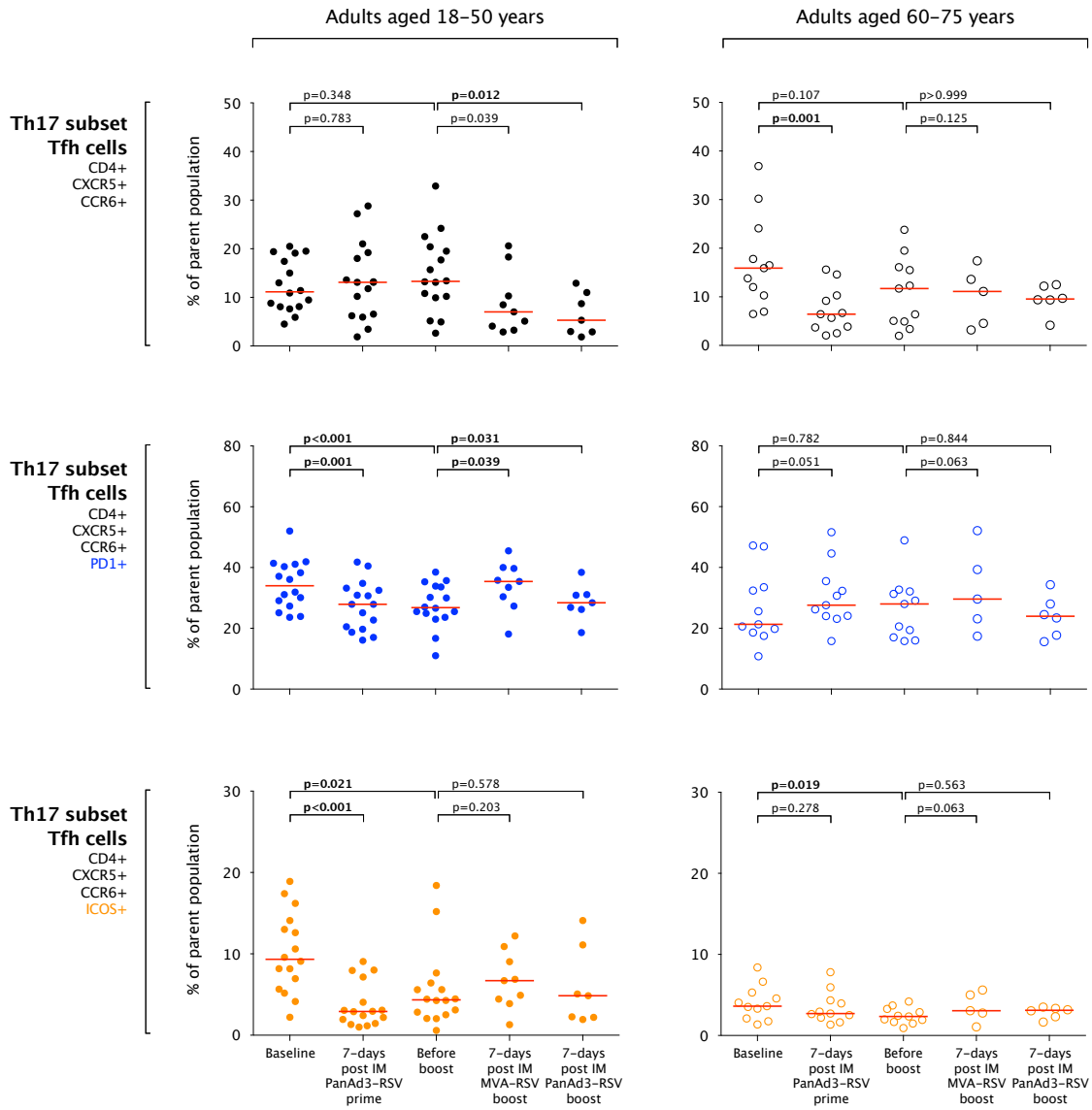


Figure 7-43. The Th17 subset of T-follicular helper cell (CD4+ CXCR5+ CD45- CCR6+) cell frequency response to vaccination.

*FACS data from un-stimulated PBMCs from IM PanAd3-RSV primed volunteers (study groups 1, 2, 7 and 9). All cell frequencies are presented as the % of parent population with red lines used to mark the median value. Time points included were baseline, 7-days after IM PanAd3-RSV prime, before boost (4- weeks post prime for groups 2 and 7; 8-weeks post prime for groups 1 and 9) and 7-days after boost for IM MVA-RSV and IM PanAd3-RSV respectively. Comparative statistics were two-way paired t-tests without correction for multiple testing. (**Top row**) The overall T-follicular helper (Tfh) cell populations in younger and older adults and (**middle row**) with PD1 co-expression and (**bottom row**) ICOS co-expression.*

7.10.3 Exploration of the T-follicular (Tfh) cell response to IM PanAd3-RSV prime vaccination

Further post-hoc exploratory analyses sought to characterise any relationships between Tfh cells and humoral immune responses to vaccination. Volunteers primed with IM PanAd3-RSV made antibody responses that were not reproduced after boost, as described in earlier sections, and so this analysis focussed on data from 7-days post IM PanAd3-RSV prime response only.

Baseline measures of Tfh cells and the Th1/Th2/Th17 subsets were correlated with RSV-specific B-cell responses as measured by the serum RSV-neutralising antibody titre 28-days post prime (expressed as the fold-change from baseline) and the induction of F-specific IgG and IgA antibody-secreting cells (ASCs) measured 7-days post prime (absolute number of spots per million PBMCs). The results are shown in the following figures. The only statistically significant correlation was from younger adults and the Th2 subset negatively associated with the fold-change in serum RSV-neutralising antibody 28-days later (Figure 7-44). There were no significant correlations with anti-F IgG and IgA specific ASC responses 7-days after prime (Figure 7-45).

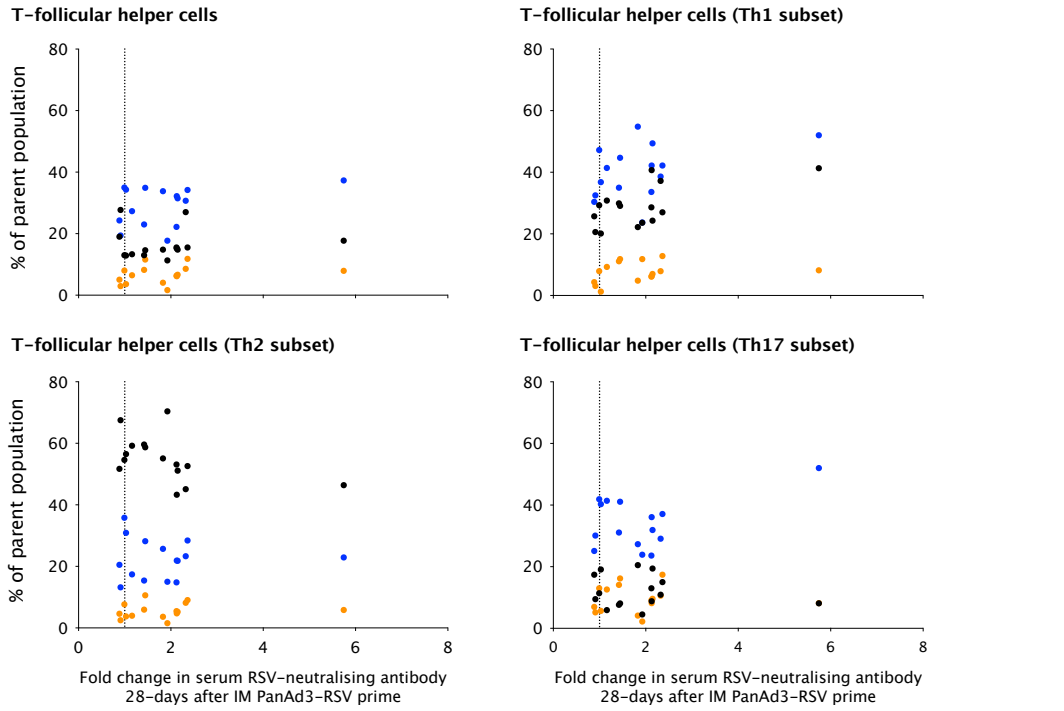


Figure 7-44. Correlations between baseline Tfh cells and the fold change in serum RSV-neutralising antibody titre 28-days after IM PanAd3-RSV prime.

Data from IM PanAd3-RSV primed younger adults only. T-follicular helper cells were measured by ICS/FACS assay. Serum RSV-neutralising antibody titres were measured by plaque-reduction neutralisation assay. All correlations were not statistically significant, except for the Th1 subset (black dots, r=0.62, p=0.0137, two-tailed Pearson's correlation). There were no significant correlations with the absolute antibody titre measured 28-days after vaccination (data not shown).

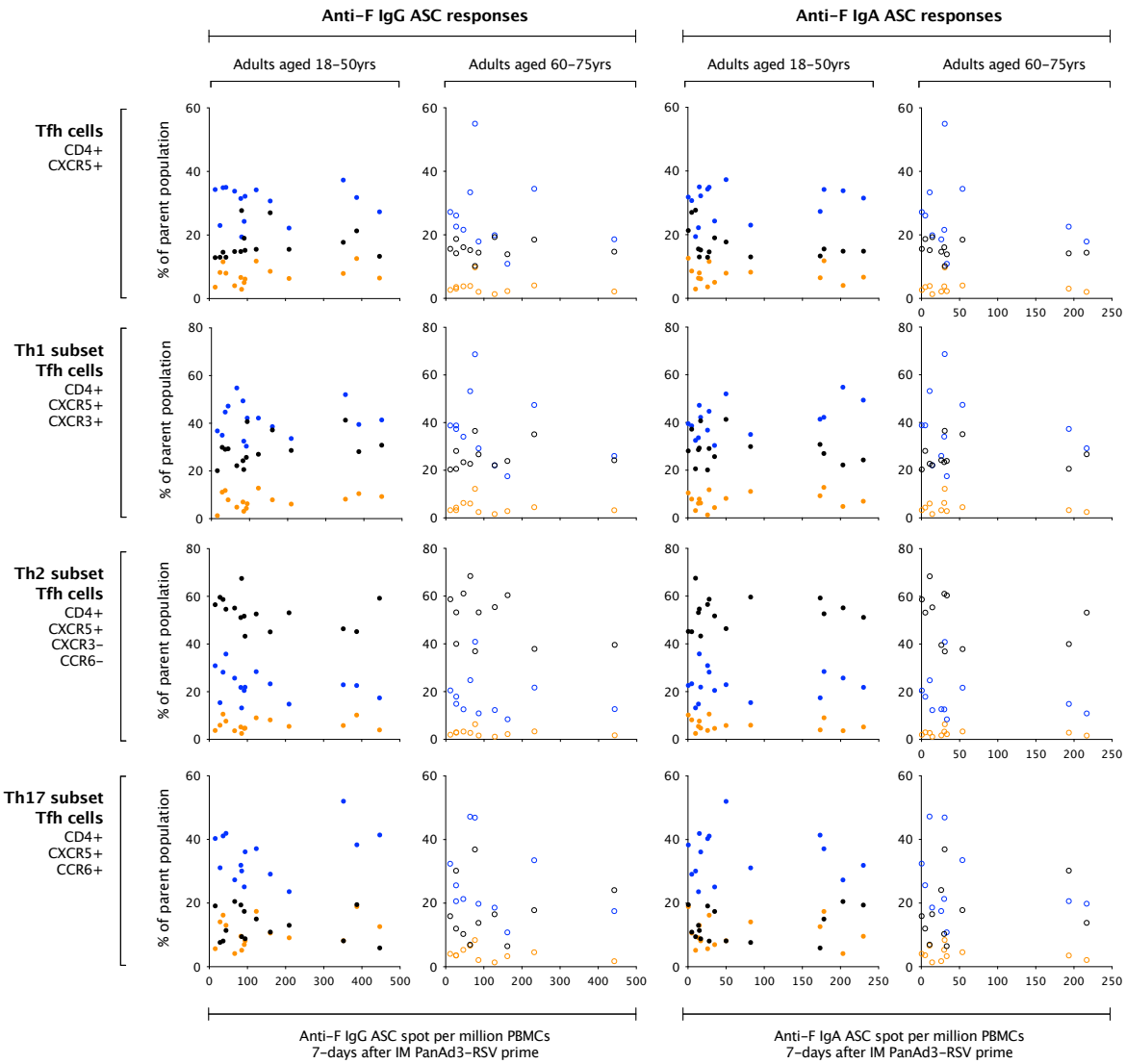


Figure 7-45. Correlations between baseline Tfh cells and the F-specific IgG and IgA antibody secreting cell (ASC) response 7-days after IM PanAd3-RSV prime.

Data from IM PanAd3-RSV primed younger and older adults. T-follicular helper cells were measured by ICS/FACS assay. ASC responses were measured by dual-colour ELISpot assay. All correlation analyses were not statistically significant (two-tailed Pearson's correlation).

T-follicular (Tfh) cells:
- CD4+ CXCR5+
- CD4+ CXCR5+ PD1+
- CD4+ CXCR5+ ICOS+

Tfh cells (Th1 subset):
- CD4+ CXCR5+ CXCR3+
- CD4+ CXCR5+ CXCR3+ PD1+
- CD4+ CXCR5+ CXCR3+ ICOS+

Tfh cells (Th2 subset):
- CD4+ CXCR5+ CXCR3- CCR6-
- CD4+ CXCR5+ CXCR3- CCR6- PD1+
- CD4+ CXCR5+ CXCR3- CCR6- ICOS+

Tfh cells (Th17 subset):
- CD4+ CXCR5+ CCR6+
- CD4+ CXCR5+ CCR6+ PD1+
- CD4+ CXCR5+ CCR6+ ICOS+

7.11 B-cell responses measured by FACS

The same PBMCs thawed for the investigation of T-follicular cell responses to vaccination were used to analyse the specific issue of IgA B memory cell responses that appeared short after natural exposure in paediatric healthcare workers and absent after experimental challenge[237]. Un-stimulated PBMCs were analysed by FACS using the methods described earlier from 26 younger and older IM PanAd3-RSV primed adults before and one week after each normal-dose vaccine (see Figure 7-37). The gating strategy is shown by example below.

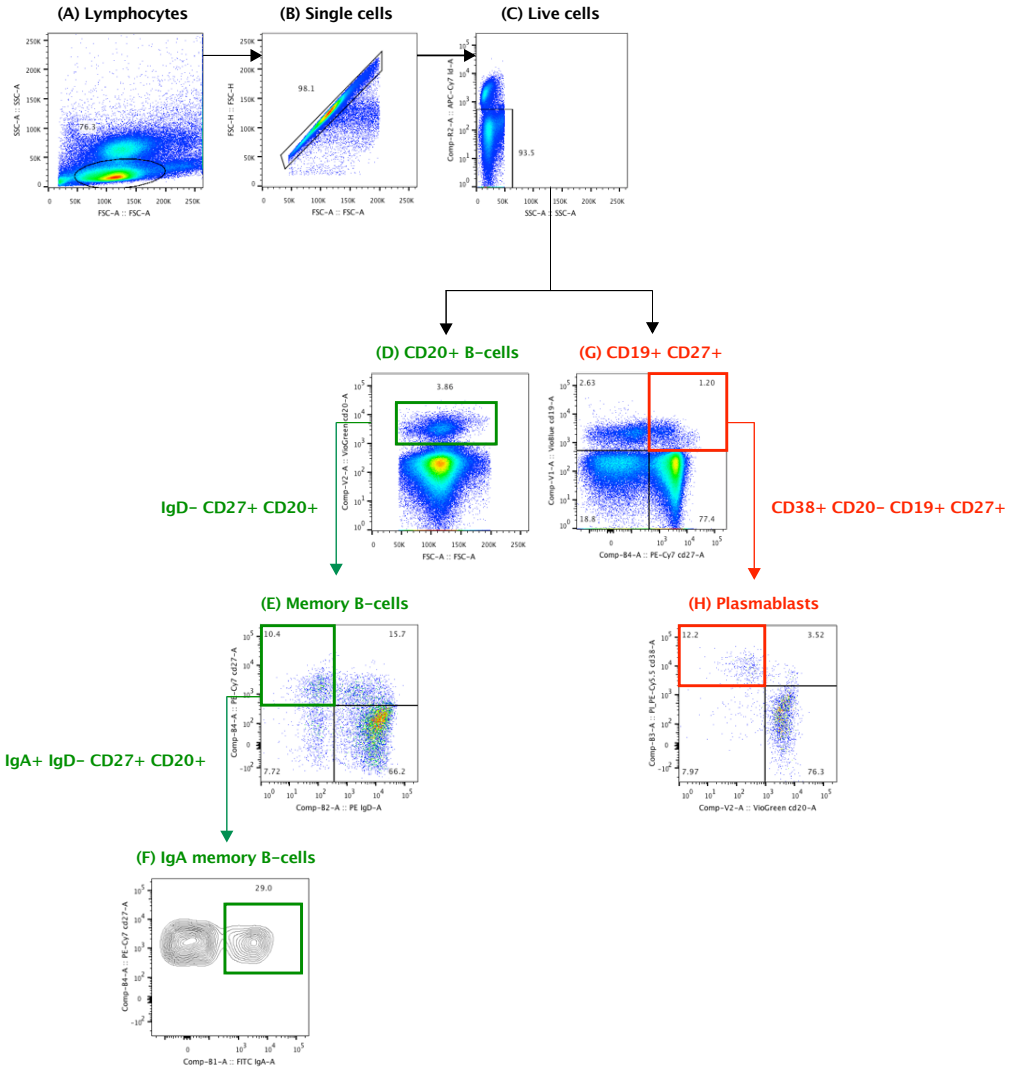


Figure 7-46. The gating strategy for the quantification of B-cell immune responses after vaccination. *The example used here is from a volunteer 7-days after high-dose IM PanAd3-RSV prime. PBMCs were un-stimulated and gated from (A) lymphocytes, (B) single cells and (C) live cells. For IgA memory, (D) CD20+ B-cells, (E) IgD- CD27+ CD20+ memory B-cells and (F) IgA+ IgD- CD27+ CD20+ memory B-cells. For plasmablasts from (C), (G) CD19+ CD27+ and then (H) CD38+ CD20- CD19+ CD27+ plasmablasts. Screenshots are courtesy of Tamsin Cargill.*

7.11.1 *Baseline observations*

There were no differences in the frequencies of circulating memory B-cells, IgA memory B-cells and plasmablasts (measured as a proportion of the parent population) between younger and older adults before prime vaccination (Figure 7-47).

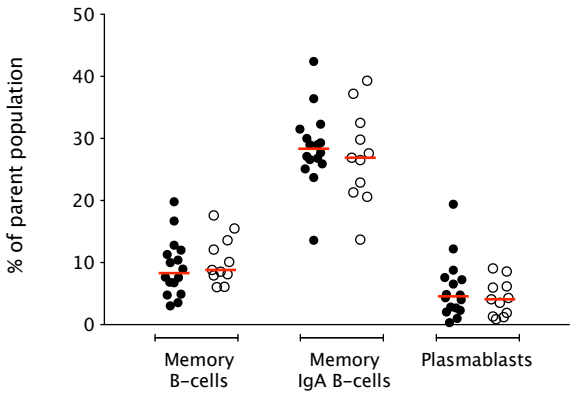


Figure 7-47. Memory B-cell, IgA memory B-cell and plasmablast cell frequencies by FACS analysis at baseline.
The red bar denotes the median. There were no significant differences at baseline between younger and older adults (as determined by two-way unpaired t-test).

7.11.2 *Results of the B-cell plasmablast and IgA memory responses to vaccination*

The results shown in Figure 7-48 indicated that vaccination had no effect on un-stimulated B-cell frequencies in circulation. A repeated-measures one-way ANOVA was used to detected statistically significant differences in the measured outcomes at each time point. For the memory B-cells there was a modest but statistically significant increase after prime in older adults and with plasmablasts there was a significant expansion after prime in both younger and older adults. Plasmablasts expanded again after IM MVA-RSV boost in younger adults and a similar (non-significant) trend was seen in the older cohort. The only result of statistical significance following IM PanAd3-RSV boost was with IgA memory B-cells in younger adults. None of these statistically significant pairwise comparisons survived Bonferroni correction for multiple testing.

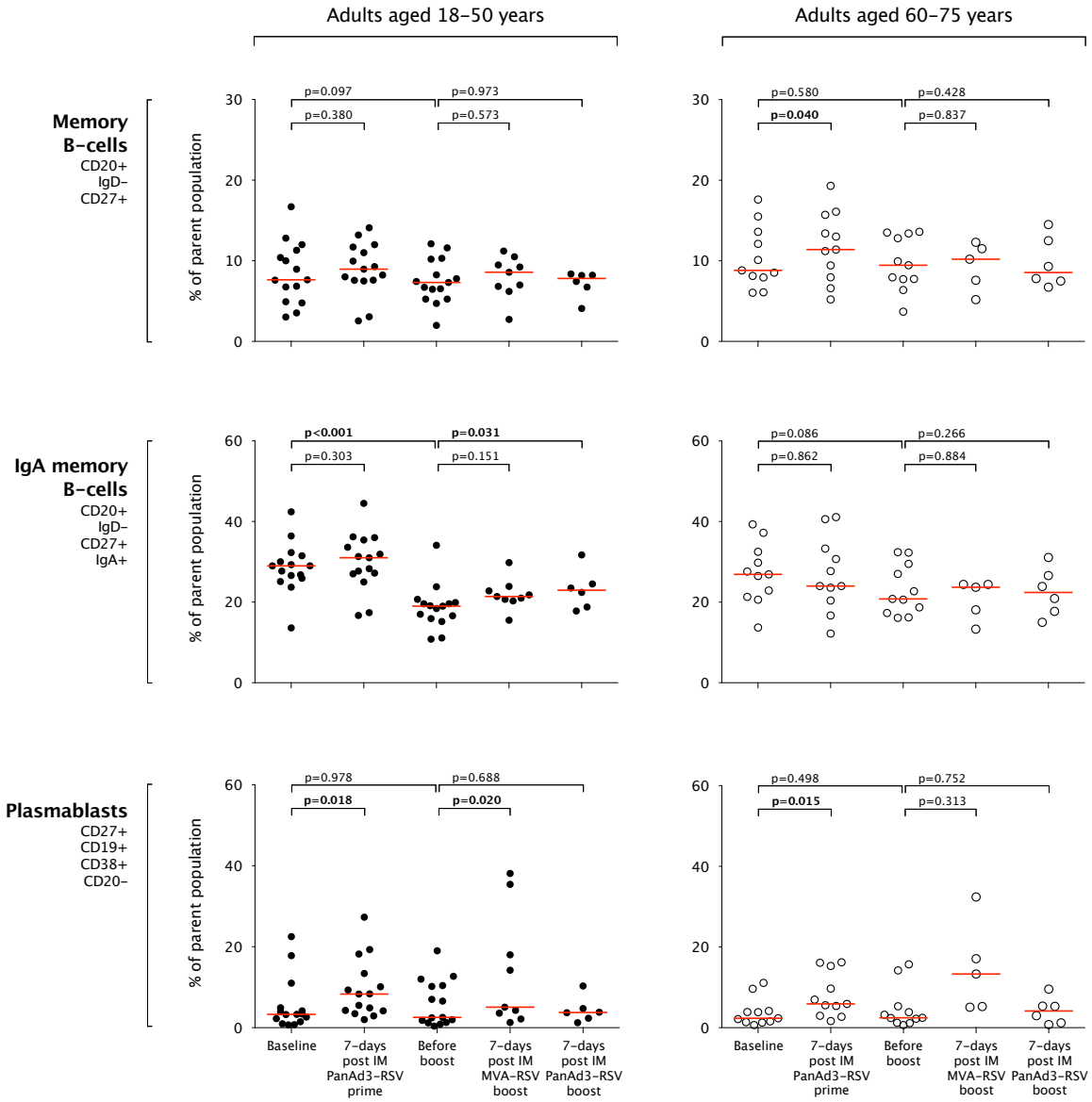


Figure 7-48. Changes in frequencies of memory B-cells, IgA memory B-cells and plasmablasts after vaccination.

*Data from IM PanAd3-RSV primed volunteers only. Cell frequencies are presented as the % of parent population with the red lines used to mark the median value. Data from younger and older adults side-by-side. Time points included were baseline, 7-days after IM PanAd3-RSV prime, before boost (4- weeks post prime for groups 2 and 7; 8-weeks post prime for groups 1 and 9) and 7-days after boost for IM MVA-RSV and IM PanAd3-RSV respectively. Comparative statistics were two-way Wilcoxon paired t-tests without correction for multiple testing. (**Top row**) Memory B-cells, (**middle row**) IgA memory B-cells and (**bottom row**) plasmablasts.*

7.11.3 Exploration of the B-memory and plasmablast response to IM PanAd3-RSV prime

This post-hoc analyses explored potential for any relationships between baseline, naturally derived, memory B-cells and plasmablasts with humoral immune responses to vaccination. As with the Tfh analyses, volunteers primed with IM PanAd3-RSV made minimal antibody responses to boost and this final analysis concentrated on data from 7-days post IM PanAd3-RSV prime only. Measures of memory B-cells, IgA memory B-cells and plasmablasts were correlated with RSV-specific B-cell responses as measured by the serum RSV-neutralising antibody titre 28-days post prime (expressed as the fold-change from baseline) and the induction of F-specific IgG and IgA antibody-secreting cells (ASCs) measured 7-days post prime (absolute number of spots per million PBMCs).

The only statistically significant correlation with the induction of serum RSV-neutralising antibody was with baseline IgA memory B-cells (r=0.56, p=0.031, two-way Pearson's correlation), with higher baseline measures associated with a greater induction of antibody. No neutralising antibody data were available from older adults for analysis (see Figure 7-49). The multitude of correlation analyses with anti-F IgG and IgA ASC responses after prime (shown in Figure 7-50) recovered a single observation of statistical significance. Here it was found that higher baseline measures of memory B-cells were associated with lower IgG ASC responses in older adults (r=-0.64, p=0.037, two-way Spearman's correlation). A similar trend was observed for IgA ASC responses in this age group but did not reach statistical significance.

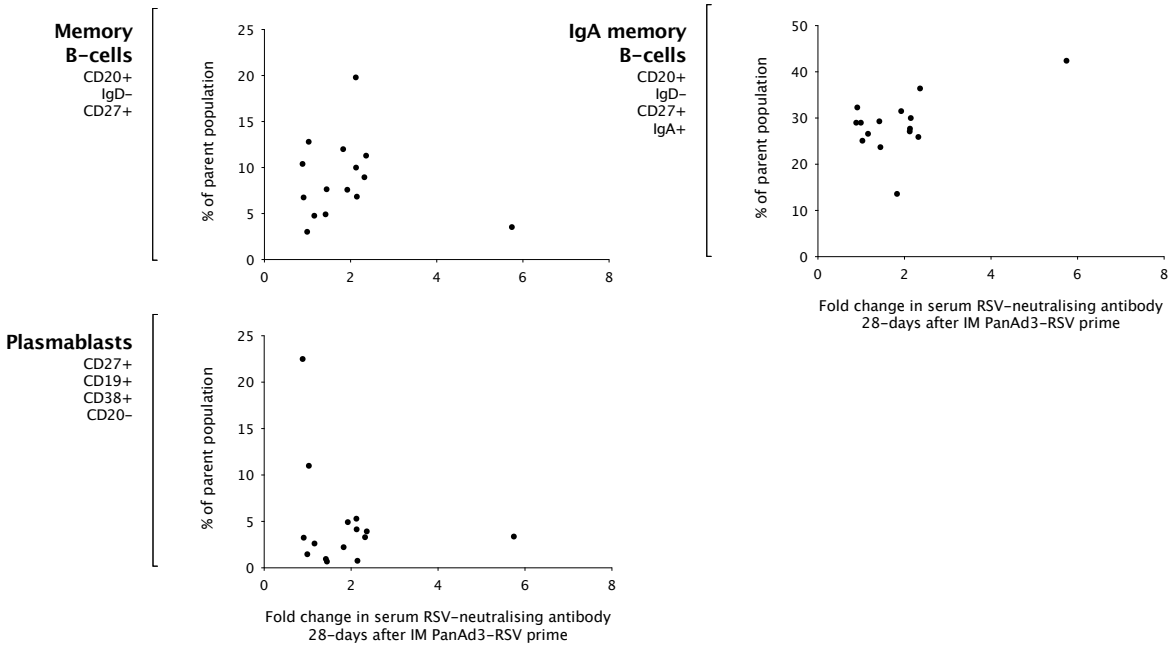


Figure 7-49. Correlations between naturally derived baseline memory B-cell, IgA memory B-cell and plasmablasts with the fold change in serum RSV-neutralising antibody titre 28-days after IM PanAd3-RSV prime.

*Data from younger adults only (n=15 paired samples). B-cell populations were measured by ICS/FACS assay. Serum RSV-neutralising antibody titres were measured by plaque-reduction neutralisation assay. The fold-change in serum RSV-neutralising antibody titre was correlated against the baseline memory B-cell population (**top left**), IgA memory B-cell population (**top right**) and plasmablast population (**bottom left**). The only statistically significant correlation was with baseline memory IgA B-cells (r=0.56, p=0.031, two-way Pearson's correlation).*

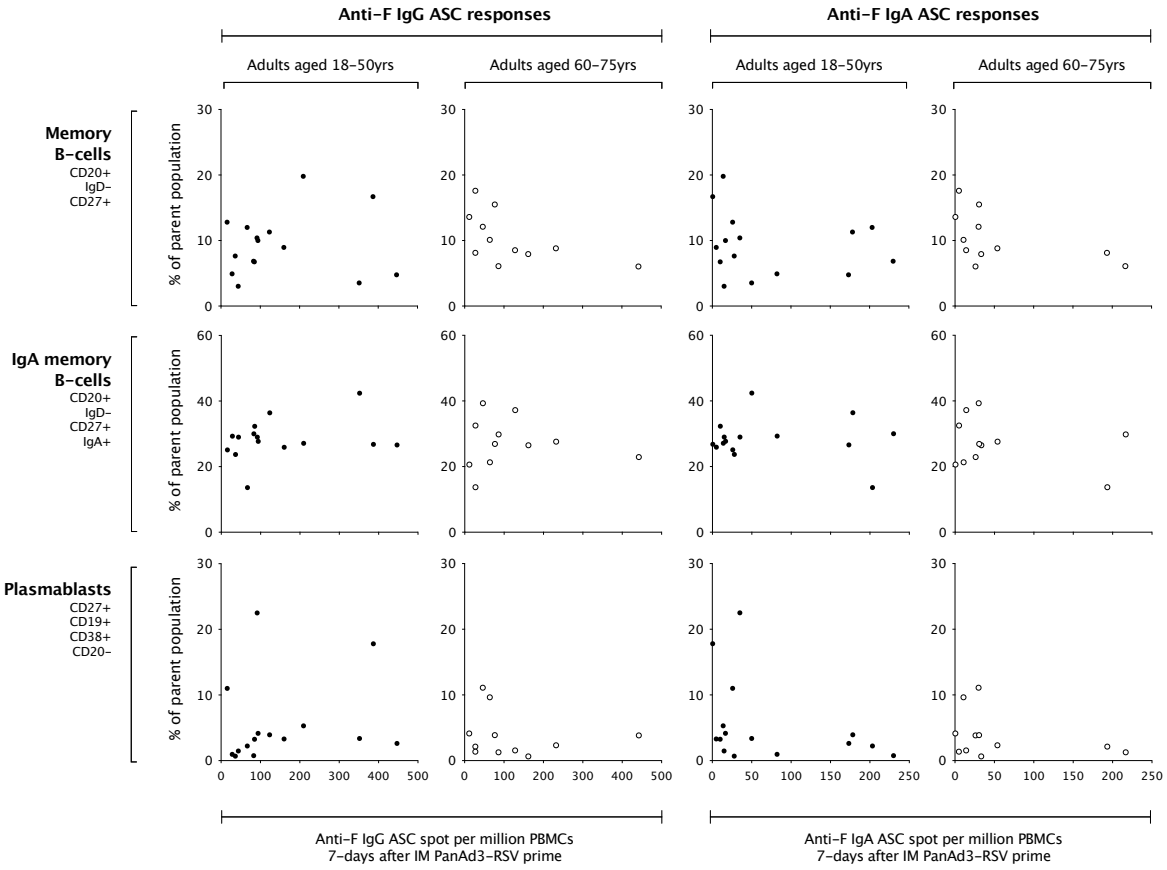


Figure 7-50. Correlations between naturally derived baseline memory B-cell, IgA memory B-cell and plasmablasts with the F-specific IgG and IgA antibody secreting cell (ASC) response 7-days after IM PanAd3-RSV prime.

*Data from younger and older adults (n=15 and n=11 paired samples respectively B-cell populations were measured by ICS/FACS assay and included the memory B-cell population (**top row**), IgA memory B-cell population (**middle row**) and plasmablasts (**bottom row**). ASC responses were measured by dual-colour ELISpot assay. The only statistically significant correlation was with baseline memory B-cells and anti-F IgG ASC responses in older adults (top row second from left; r=-0.64, p=0.037, two-way Spearman's correlation).*

7.12 Discussion

7.12.1 Summary of main findings

RSV disease is propagated within the host by free virus (targets of neutralising antibody) produced from infected cells (targets of cellular immunity). Safe and optimal vaccine-induced protection from severe disease might therefore require the parallel induction of desirable humoral and cellular RSV-specific immune responses. The results of this chapter showed that the viral-vectored vaccines PanAd3-RSV and MVA-RSV boosted RSV-specific humoral and cellular immunity in the presence of pre-existing, naturally acquired, serum RSV- and PanAd3-vector neutralising antibody and without Th2-associated cytokines that might give cause for concern for vaccine immunopathology. Following prime, vaccine immunogenicity measures recorded in blood were dependent upon the IM route. Following boost there was little/no further increase in measures of serum RSV-neutralising antibody and F-specific ASCs from IM primed volunteers, but there were measurable responses from volunteers primed by the IN route. Overall, in blood, the measures of vaccine immunogenicity were dependent and responsive to the first dose of IM vaccine. This pattern was different for the IFNγ−response to IM boost, where the frequency and magnitude of the response was greater for the MVA vector irrespective of the route used to prime.

7.12.2 Mucosal antibody response to vaccination

Mucosal immunity plays an important role in antibody-mediated protection and antigen recognition and processing. Licenced mucosal vaccines for influenza, typhoid and polio have used live-attenuated pathogens to elicit safe and protective immunity to disease and MVA viral vectored vaccines for tuberculosis administered by aerosol spray have been safely evaluated in phase 1 clinical trials[387]. RSV-specific mucosal IgA has been a correlate of protection from infection in experimental challenge conditions and observational studies in adult populations[236-240].

Although F-specific IgG and IgA antibody was detectable by ELISA in nasal samples, the intra-nasal route of PanAd3-RSV prime did not elicit a detectable rise in nasal antibody 2-weeks after vaccination. All other immunology endpoints indicated that intra-nasal prime did not induce detectable immune responses in peripheral blood for many younger adult volunteers. The different prime/boost vaccine regimens selected for use in the RSV001 trial were selected from pre-clinical animal challenge experiments in rodents and

sero-negative calves. Here the IN route resulted in improved protective efficacy in the upper respiratory tract despite having induced lower levels of systemic RSV-specific T-cells than IM PanAd3-RSV, and of note was that IN prime generated comparable levels of immunity in the lung[408,409]. The PanAd3 vector used as an IN influenza vaccine in mice induced greater IgG antibody responses in broncho-alveolar lavage samples and greater CD8+ IFNγ T-cell responses in the lungs compared with the same vaccine given by intramuscular injection, which generated greater responses in the spleen[410]. Therefore immune responses measured in blood are not representative of the overall immune response to vaccination or infection. Although live-attenuated RSV vaccine candidates in sero-negative infants have often failed to induce serum neutralising antibody titres, these infants were able to significantly restrict (by 100-fold) the replication of a 'second challenge' subsequent vaccines dose[310]. Selective accumulation of memory T-cells in the lung against respiratory pathogens like RSV has been described in humans and pulmonary T-cells may have contributed substantially to protection afforded by mucosal immunisation[246,440,441]. The only licensed intra-nasal vaccine, the live attenuated influenza vaccine (LAIV, Flumist®), protects despite its limited ability to induce humoral and cellular effector responses in blood compared with the injectable trivalent inactivated vaccine (TIV)[442-444]. Instead of generating plasmablasts, as was the response to trivalent inactivated influenza vaccine (TIV), LAIV elicited increases in naïve and transitional B-cells 30-days after vaccination, supporting evidence that influenza and RSV infection and vaccination though different routes engages a different blood kinetic and mechanism of action towards host protection[445-447]. In considering viral-vectored vaccines for possible intra-nasal use for infants, some of the major obstacles for live-attenuated vaccines have been local viral replication (and inflammation) and the partial reversion to wild-type and transmission of vaccine virus. PanAd3-RSV virus could not be detected in any of the 21 intra-nasally primed volunteers 3-days after vaccination by PCR, which was encouraging.

Intra-nasal PanAd3-RSV prime may have generated desirable immune responses that cannot be appreciated in the assays applied in this clinical trial. IN PanAd3-RSV did not induce vector-neutralising antibody (discussed in more detail later) and the mucosal route can bypass the negative effects of serum maternal antibody in infants[448]. The mucosal immune response to IN PanAd3-RSV in humans remains potentially valuable for protection from infection in the upper respiratory tract, and supported the use of an

IM boost vaccine, even in naturally primed volunteers, to induce RSV-specific immune responses in blood in supporting protection from severe disease in the lower respiratory tract.

7.12.3 Serum antibody and B-cell response to vaccination

Serum neutralising antibodies alone constitute a significant obstacle for RSV and the risk of developing severe disease[197,215,216237]. Passive immune prophylaxis with F-specific monoclonal antibody can reduce hospitalization for severe RSV-bronchiolitis in high-risk infants by 45-55% and has been a clear demonstration that serum F-specific antibodies alone can confer protection in the lower respiratory tract in infants[90,100]. In adults, high RSV-specific neutralising antibody titres persist into later life though, according to some reports, relatively lower titres are associated with the development of severe disease in the elderly[240,242,248,249]. Unfortunately, due to the timing of writing this thesis, the results for RSV serum-neutralising antibody in older adults were not available. In the younger adult cohort, IM administered PanAd3-RSV and MVA-RSV induced up to a two-fold rise in neutralising titre 28-days after vaccination despite a negative correlation with baseline natural immunity. The effect of pre-vaccination titres may account for the lack of antibody response to the second dose of IM vaccine after boost, and would be of relevance when considering maternal and elderly vaccines for RSV. A similar fold-change in neutralising antibody titres was observed following a promising RSV nanoparticle vaccine, now entering late stage clinical evaluation as maternal vaccine candidate, which also observed the negative effects of natural pre-existing antibody on vaccine-induced immunity to RSV that has been reported in other vaccine trials[332,449,450]. Further exploration by ELISA revealed modest increments in F-specific IgA antibody, and to a lesser extent IgG, characterised the serum response to IM PanAd3-RSV prime and IN PanAd3-RSV/IM MVA-RSV boost. The small changes in serum anti-F IgA antibody measured by ELISA could be correlated with the rise in serum RSV-neutralising antibody measured by PRNA after IM PanAd3-RSV prime (see figure page 209). Data from RSV infected adults showed no correlation between the magnitude of the RSV-specific ASC response and RSV serum-neutralising antibody titres, but a correlation was observed under human challenge conditions[237,447]. The IgA ELISA result could be interpreted as tentative indirect evidence that the first dose of IM vaccine (PanAd3-RSV or MVA-RSV) can repair a defect in IgA immunity implied from the absence of IgA memory B-cells described in Section 4.5.1 (page 106) and by others, although the connection

to B-cell memory is unknown and duration of this serum anti-F IgA response is likely to have been short-lived if it was truly related to titres of serum RSV-neutralising antibody that waned after vaccination[237]. Exploration of the B-memory IgA response to vaccination by ICS/FACS failed to show an expansion in this population of cells after vaccination in younger or older adults, and only the frequencies of memory B-cells could be correlated with the serum RSV-neutralising antibody response in younger adults (see figure page 256). An expansion of plasmablasts was observed by FACS analysis, which corroborated the observation of antibody-secreting cells in circulation by ELISpot at the same time. Vaccine induced F-specific antibodies might therefore fulfil functional and biologically relevant roles in protection against RSV in infants and the elderly, although it remains unknown whether the truncated soluble F-protein antigen exposed epitopes for pre-F or post-F (or both) for the induction of neutralising antibody and how this could impact upon protection from severe disease. The vaccine-induced serum RSV-neutralising antibody response waned from a few weeks after the first IM vaccine, a kinetic similar to natural disease, but the relevance of this for a paediatric sero-negative population cannot be anticipated as even a modest antibody-mediated reduction in viral load could have an disproportionate impact on the rates of severe disease and hospitalisation[104,451].

Serum antibody is derived from long-lived plasma cells that reside in the bone marrow, spleen and target organs such as the respiratory tract. Antibody-secreting cells (ASCs) are generated in secondary lymphoid organs and appear transiently in the peripheral circulation en route to their final destination. These cells appear for a short period between 4-10 days into the primary immune response, typically peaking at day 7 or 8[443,446,447,452-456]. A large proportion of these cells will be short-lived plasmablasts, programmed for antibody production and then apoptosis, while other cells receive the necessary co-stimulatory signals to become long-lived plasma cells that maintain immunity to re-infection. A clear signal of vaccine take after IM PanAd3-RSV, and later IM MVA-RSV, was observed in all vaccinated subjects by the detection of circulating anti-F IgG and IgA antibody secreting cells (ASCs) which were undetectable in peripheral blood at baseline. F-specific IgG ASC responses correlated with the serum RSV-neutralising antibody response (but not IgA ASCs, in contrast to the correlation with serum anti-F IgA ELISA), which was observed in healthy adults in RSV challenge conditions and after trivalent influenza vaccination[237,367,453]. The mean anti-F IgG ASC response 7 days post-IM PanAd3-RSV prime was 149 (±SD 136) and 173 (±SD 234) spots per million PBMCs in younger and older adults respectively. F-specific IgG and IgA ASC cell frequencies from

natural exposure (expressed as percentage of the total population by FACS analysis) have shown no differences between younger and older adults in other studies[248], and the rise in ASC responses after vaccination compares favourably with post RSV infection clinical data in elderly adults who, approximately 7-days into symptomatic infection, recorded a mean anti-F IgG ASC response of 200 spots per million PBMCs (±SD 256)[447]. The absence of detectable ASC responses 7-days after boost in IM primed volunteers, and not IN primed volunteers, may be an effect of vector neutralising antibody or reflect differences in the kinetics and magnitude of ASC responses after primary and secondary immunisations. In other vaccine trials the peak ASC response following a booster vaccine appeared around day 7 for rabies, capsular group C meningococcal, pneumococcal and oral cholera vaccines[457-460]. ASCs responses to the trivalent influenza (TIV) vaccine in children were reported to be lower compared with adults and where a second influenza dose is not indicated in healthy adults and the elderly it remains a requirement for naïve infants for an effective immune response, indicating that favourable and demonstrable immunogenicity after two intramuscular injections remains possible in sero-negative infants when it was not observed in an adult population[443].

The generation and maintenance of an effective antibody response benefits from the support and interaction of B-cells and specialist CD4+ T-follicular helper (Tfh) cells. Tfh cells support the growth, differentiation, class switching, affinity maturation and memory selection of B-cells in germinal centres (reviewed here[368]). Tfh cell frequencies can be correlated with the development of broadly neutralising serum-neutralising antibody to HIV and the expansion of Tfh cells in peripheral circulation following influenza vaccination correlated with an increase in activity of memory B-cells (not naïve B-cells) and influenza serum-neutralising antibody in young adults, which was lost in elderly populations[461-463]. Baseline measures of the total Tfh cells frequencies were lower in older adults and within each of the Th1/Th2/Th17 subsets the relative expression of the activation marker ICOS was lower in older adults. Although changes were observed in some cell phenotypes and not others following vaccination, no clear pattern was observed and no correlations could be drawn with the induction of a serum RSV-neutralising antibody response to vaccination in younger adults (and no analyses sought to correlate Tfh responses with the waning of antibody at later time points).

7.12.4 T-cell response to vaccination

RSV-specific T-cells play an essential role in controlling the severity of disease and clearing infection. A poor or absent Th1 response, T-cell deficiencies in infants and T-cell immune senescence in the elderly confer a substantial risk of developing severe disease[208,242,248,251]. In infants Th2-associated activity has been associated with severe disease and subsequent wheeze in later life and a Th2 immune-pathological reaction was implicated in the disastrous FI-RSV vaccine trial in the 1960s.

Earlier clinical trials of heterologous prime/boost combinations of viral-vectored vaccine candidates for malaria, HIV, tuberculosis and hepatitis C vaccines have consistently recorded a robust induction of antigen-specific T-cell immunity[401]. Consistent with the findings reported in these trials, IM PanAd3-RSV and IM MVA-RSV were capable of expanding the population of RSV-specific IFNγ producing T-cells in peripheral blood. We did not seek to identify *de novo* priming of naïve T cells but several volunteers who later developed clear responses after vaccination had failed to record T-cell reactivity to RSV peptide pools at baseline. IFNγ responses appeared independent of serum RSV-neutralising antibody and F-specific ASC responses. Instead baseline IFNγ responses and vector neutralising antibody titres were negatively associated with the magnitude of IFNγ producing T-cell expansion after vaccination. Phenotypic analysis showed baseline CD4+ responses were generally below 0.1%, which was within the range for healthy adults reported elsewhere (0.05 and 0.3%)[464], and baseline CD8+ T-cell responses were similar to those against HLA-B7 restricted epitopes to RSV nucleoprotein[206]. Analysis of the cell population frequencies one week after IM PanAd3-RSV or IM MVA-RSV boost showed a balanced expansion of CD4+ and CD8+ T-cells producing IFNγ. A similar RSV-specific Th1-biased CD4+ T-cell response was observed after immunization of PanAd3-RSV prime/MVA-RSV boost in relevant preclinical models such as non-human primates and sero-negative calves[408,409]. The identification of RSV epitopes for CD4+ and CD8+ cells remain an active area of investigation[209], but in the context of influenza a positive correlation was reported between the frequency of memory IFNγ CD4+ cells and protection from clinical symptoms[465].

It seems likely that an optimal vaccine for the elderly should re-establish T-cell effectiveness. Not withstanding the *ex-vivo* IFNγ ELISpot assay being performed in different years, IFNγ responses in older adults at baseline were substantially lower than the cohort of younger adults. The older volunteers however recorded comparable fold-changes in the expansion of IFNγ–producing T-cells after IM PanAd3 prime with

younger adults. For younger adults the greatest IFNγ activity was seen to the MVA vector irrespective of the vaccine route used for prime. MVA-vectored vaccines encoding influenza antigen were found to be safe and potent inducers of IFNγ responses in older adults, but age related decline in the magnitude of the T-cell response has been observed and was observed here too[391]. The reasons for this are unclear and are possibly a direct effect of an aging immune system that failed manifest in response to the adenovirus vector. In considering our cohort of older adults, most would have received smallpox vaccine in childhood[j] and we did not seek to measure immune responses to the MVA vector. Antibody and T-cell responses to MVA persist for decades after small pox vaccination[466-471]. Longitudinal studies on vaccinia-specific antibody persistence after smallpox vaccination, in the absence of any known natural boosting by endemic orthopoxviruses, estimated the antibody half-life was over 90-years. Cross-reactivity from prior vaccination up to 20-years earlier with smallpox vaccine in adults was associated with a small but statistically significant impairment in IFNγ responses to malaria antigen delivered by heterologous prime/boost with recombinant attenuated fowlpox virus FP9 and MVA viral vectors (although in children the first dose of FP9 did not appear to induce cross-reactive responses to the MVA given at the second dose)[375]. Several trials continue to use and report successes with repeated MVA, suggesting MVA-vector immunity may be a relatively ineffective obstacle for vaccination. This might deserve further investigation in future trials of elderly subjects.

The MVA-dependent boost in younger, and lesser extent older adults, indicated added immunogenicity from a boost vaccine following vaccine 'prime' or naturally primed healthy adults, which is of potential value when considering the development of prime/boost combinations for seropositive children and the elderly where impaired T-cell responses have been associated with severity of disease. Previous use of single dose MVA-vectored vaccines, or use of these vectors for priming, has not proved sufficiently immunogenic in formulations developed for malaria, tuberculosis or HCV because of the presumed need for effective priming by an alternative vaccine or natural exposure[371,386,389]. The data presented here, possibly from IN primed and MVA boosted volunteers in younger and certainly single-dose IM MVA-RSV in older adults[k], indicated that IFNγ T-cell responses to IM MVA-RSV could be generated

[j] This was not an exclusion criterion and would have resulted in the exclusion of almost all other adults had it been.
[k] Study groups 4 and 6 respectively

independent of vaccine priming in naturally exposed adult volunteers. This had been observed in naturally exposed MVA-vectored influenza vaccine trials in the elderly and recent data from phase I antigen-naïve Ebola vaccine trials recorded responses to the MVA-vector at prime which, when boosted with an adenovirus vector, superseded T-cell responses to the conventional adeno/MVA combination[391,396].

The favourable absence of ERD-associated cytokine responses to vaccination in the respective ICS and CBA assays in younger adults, together with pre-clinical RSV-challenge experiments in rodents and calves, offers some but only limited reassurance of their likely safety in an infant population. The human cytokine response following FI-RSV vaccination is unknown and instead it has been informed from the results of RSV exposure in FI-RSV vaccine animal challenge experiments. It is important to note that the assays presented here were performed after vaccination and not following natural RSV exposure and all volunteers had prior natural exposure to RSV that was a protective factor in older infants following FI-RSV[472]. The absence of detectable Th2 cytokine responses in a small number of volunteers after boost, while encouraging, does not discount the induction of undesirable vaccine immunogenicity in other populations after vaccination followed by natural infection. This will require continued evaluation in the elderly and throughout age de-escalating development of these vaccines in seropositive children and infants towards RSV-naïve infants.

7.12.5 Adenovirus vector immunity

Naturally acquired serum anti-adenovirus serotype 5 (Ad5) neutralising antibody titres >200 have been associated with an impairment of vaccine immunogenicity to Ad5-vectored HIV vaccine and increased rates of HIV-acquisition in circumcised men (although the association with HIV-acquisition waned over time)[473-475]. Serum vector-neutralising antibody to the hyper-variable regions of the adenovirus hexon protein can therefore interfere with vaccine immunogenicity[476,477]. Although anti-Ad5 antibody targets multiple hyper-variable regions, vector immunity could be subverted using a chimeric Ad5-vector containing the hyper-variable regions of another adenovirus serotype (Ad48) to successfully deliver HIV antigen and elicit a more robust HIV-specific immune response[476,478]. An alternative approach has been to select non-human vectors with little/no potential for serum neutralising antibody in the general population. Significant variation in the prevalence of neutralising antibody to different human and non-human

adenoviruses in different geographical locations has been observed[479,480]. Successful chimpanzee derived adenoviral vectors for use in humans were selected from a process of screening potential vectors by *in vitro* immunogenicity testing and estimates of low sero-prevalence of neutralising antibody[380,398,401]. The same strategy was applied for the selection of the PanAd3 vector and RSV001 was the first use of this vector in man.

Data for the PanAd3 vector neutralising antibody was available for younger adults only. Volunteers were excluded at screening if they had had, at any time, received another adenoviral or MVA-vectored vaccine, and yet the proportion of volunteers with pre-existing neutralising anti-PanAd3 titres >200 (38%) greatly exceeded the 3% expected from earlier estimates from US and European populations[380]. The source of these antibodies is unclear and the PanAd3 hexon protein results in its classification within subgroup C of human adenovirus and therefore the potential for cross-neutralising antibodies from other adenovirus exposure[380]. There was no baseline correlation with increasing age and IM PanAd3-RSV induced a significant rise in serum vector neutralising antibody. While our data do not identify a clear effect from anti-PanAd3 neutralising antibody on serum antibody and antibody-secreting cell responses to vaccination, there appeared to be a negative correlation with the expansion of IFNγ producing T-cells (see page 233). Mouse models anticipated that vector neutralising antibody would not interfere with T-cell responses to vaccination and anti-AdHu5 neutralising antibody had no effect on T-cell responses to AdHu5 vectored vaccines[384,481]. This could be a chance observation from small group sizes, but Ad5 specific adenovirus T-cell immunity impaired CD4+ and CD8+ HIV immunogenicity independent of serum vector neutralising antibody and the predictive value of *in vitro* neutralization assays on adenovirus-vectored vaccination has been a matter of debate[482,483]. The antibody immune responses from robustly pre-primed individuals and cumulative natural exposure to RSV produce highly functional antibodies that may have masked a loss of vaccine immunogenicity caused by vector neutralisation. The mechanisms of adenoviral vector immunity and potential for interference with vaccine responses is not fully understood and may not be readily measured using standard assays. The potential limiting effect of pre-existing and *de novo* vaccine-induced vector neutralising antibody and cellular immunity on the magnitude of immune responses to viral vectored vaccines remains an interesting question and relevant to the development of viral-vectored vaccines in the future.

7.12.6 General limitations

This phase I study had several limitations inherent in all early phase studies. The study was intended to give a description of the immunogenicity of these vaccines and the small group sizes risk confounding chance observations and mandate caution in the use of post-hoc comparative statistics that were applied to further interpret the results. There are no known immune correlates of protection for RSV disease and the selection of healthy adults aged 18-75 years enrolled into this trial are not representative of a population at risk of developing severe RSV disease. Robust pre-existing RSV immunity and the effect this has on immune responses to vaccination means there cannot be an expectation that the data provide inevitable insight into protective efficacy of these vaccines in infants and the elderly. This would require additional clinical trials.

The analysis of the F-specific IgG and IgA antibody-secreting cell (ASC) immune response and IFNγ T-cell response to vaccination used fresh PBMCs meaning laboratory errors that compromised the assay could not be repeated to generate a complete and comparable dataset. The setup and intense nature of the clinical trial meant errors on specific days resulted in nearly 20% of the data was lost in younger adults (in 2013) with a disproportionate effect on some study groups (see supplementary material). For the remaining data generated from these fresh cell assays it is worth noting the assays were performed almost one-year apart for younger and older adults respectively, due to the trial being extended, although assay procedures were unchanged and used the same batch of reagents with little operator change. There were no common controls that could be used to standardise responses in each year.

7.12.7 Conclusions

This chapter sought to characterise the immune response to vaccination. PanAd3-RSV and MVA-RSV induced serum RSV-neutralising antibody, ASCs and IFNγ producing T-cells. These desirable immune responses warrant their development towards populations in need of protection from severe disease caused by RSV.

Chapter 8: Analysis of the transcriptomic response to vaccination

8.1 Introduction

The final chapter of this thesis is an exploratory analysis of the host genetic response to vaccination. Measures of antibody, cytokines and cells are used to characterise clinical and immune responses to vaccination, but the machinery used to generate these complex proteins is initiated and regulated at a genetic level. In earlier chapters, concerning the clinical and immune responses to vaccination, several baseline factors could be identified that influenced the breath and magnitude of vaccine responses. Underpinning these would have been host genetic responses to vaccination, with genes expressed very early after vaccination, which could be critical determinants of the type, magnitude and variability in clinical and immunological responses to vaccination. The aim of this chapter was to describe and analyse the host transcriptomic response to vaccination through the identification and quantification of differentially expressed genes, and explore potential relationships between host gene expression and important clinical and immunological responses to vaccination described in earlier chapters.

8.2 Methods

RNA from younger adults was used for the analysis of the transcriptomic response to vaccination (study groups 1-4; n=42 volunteers at prime, n=40 at boost).

8.2.1 Sample collection, RNA extraction, hybridization and scanning

3mLs of whole blood was collected in Tempus RNA™ tubes (Applied Biosystems, UK) immediately before and 3, 7 and 28-days after prime and boost vaccination from each volunteer. RNA obtained 28-days after vaccination were not analysed based on the absence of detectable differentially expressed genes from this time point in other viral-vectored vaccine trials (unpublished data). Tempus RNA™ tubes were stored at room temperature for 1-2 hours to precipitate RNA and then frozen to -80°C for later analysis. A schematic illustration of the sample processing steps before data analysis is presented in Figure 8-1.

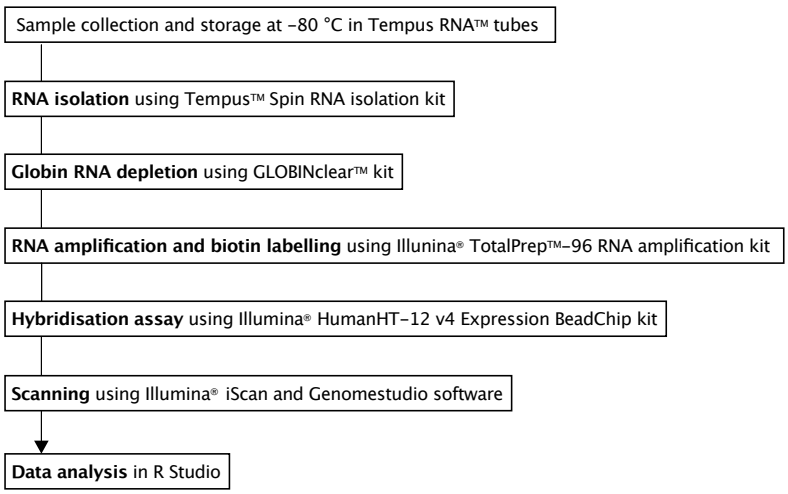


Figure 8-1. Flow diagram for RNA sample processing before analysis.
Each step was performed according to the manufacturer's instructions and was quality controlled according manufacturer's guidance and local SOPs.

Messenger RNA (mRNA) was extracted from total RNA in four batches using a Tempus™ Spin RNA Isolation Kit (Applied Biosystems, Life Technologies, California, USA) and included DNAse digestion for 15-minutes at room temperature. The next step was the depletion of α- and β-globin mRNA using magnetic bead separation and the GLOBINclear™ Kit (Ambion, Life Technologies). Quality and quantity of remaining mRNA concentration was measured using an Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer (Agilent Technologies). Samples with a mRNA concentration <150ng/uL were concentrated by vacuum

concentration. The mRNA was amplified and labelled using an Illumina® TotalPrep™-96 RNA Amplification Kit (Illumina Inc). Quality control checks were performed by the Jenner Institute. 750ng of amplified, biotin labelled, complimentary RNA (cRNA) was directly hybridised onto gene-specific probes using an Illumina® HumanHT-12 v4 Expression BeadChip Kit (Illumina Inc). Samples were randomised across beadchips to minimize clustering of volunteers or time-points after vaccination by batch effect. The fluorescent intensity values were scanned with an Illumina® iScan System and raw data were extracted and formatted using GenomeStudio software. These data were then imported into R for final quality checks and analysis of the transcriptomic response to vaccination.

8.2.2 Quality control of Illumina HT-12 v4 data

Quality control and microarray analyses used the bioconductor platform in R Studio (Mac version 3.1.3 - "Smooth Sidewalk"). Raw fluorescent Illumina probe intensities were read into R using the 'read.ilmn' function of the *limma* package[484]. Probe quality data was assessed using the 'arrayQualitymetrics' package[485]. As part of the 'neqc' function within the *limma* package on $\log_2$ transformed data, background correction was performed from negative control probes followed by quantile normalisation from both positive and negative control probe references. Probes were then filtered for detection above background (p-value < 0.05) in at least 10% of the samples, and probes without a gene symbol or map to hg19 build were excluded[486].

8.2.3 *Identification of differentially expressed probes after vaccination*

Linear regression with an empirical Bayes model was applied to the expression value of each probe after prime and boost with adjustment for volunteer age, sex, vaccine dose, day of sample collection and sample scan date using the 'lmfit' and 'eBayes' functions of the limma R package[487]. The duplicate correlation option was applied to account for the paired sampling and arrays were weighted by quality score [488,489]. A fold change ≥1.25 combined with a 5% false discovery rate (FDR) adjusted p-value <0.05 were used as threshold criteria for the identification of differentially expressed probes 3-days and 7-days after vaccination compared with the pre-vaccination baseline. Publicly available gene ontology databases were used to infer the biological function of differentially expressed genes that mapped onto the Illumina probe gene symbols.

8.2.4 *Gene set enrichment analysis*

Gene Set Enrichment Analysis (GSEA) is a method to interpret gene expression based on a ranked list of genes against a database of known gene sets (i.e. KEGG, REACTOME, Blood Transcriptional Modules or BTMs)[490]. Where genes were presented by more than one probe, the highest expressing probe was used as representative genes. Normalized genes and filtered differentially expressed genes were ranked by fold change and run against BTMs developed by Li *et al*[456] producing normalized enrichment scores (NES) and p-values using randomly generated gene sets in 1000 iterations using R. The same approach was performed on gene lists pre-ranked based on $\log_2$ fold-change per subject (single sample GSEA).

8.2.5 Statistical correlations with clinical and immunological responses to vaccination

Differentially expressed probes 3-days after vaccination were used as a subset list of probes for the analysis of transcriptomic correlates of vaccine responses measured at later time points. Using the list of differentially expressed probes after vaccination generated earlier, the log_2 fold change of each probe for each volunteer was correlated with individual clinical and immune responses to vaccination. Correlations were filtered for statistical significance (Pearson's p-value <0.05) with a correlation rho value >0.5 or <-0.5 to identify probes of interest. The phenotypes selected for investigation were derived from the results described in earlier chapters and are summarised below.

Phenotypic responses to vaccination selected for the exploration of correlates to early transcriptomic changes
The absolute change from pre-vaccination haemoglobin measured 7-days post IM PanAd3-RSV prime (groups 1 and 2 combined) and IM MVA-RSV boost vaccination (groups 1 and 3 separately)
The absolute change from pre-vaccination haemoglobin measured 7-days post IM PanAd3-RSV prime and IM MVA-RSV boost vaccination (all groups)
The log_2 fold-change from baseline in anti-PanAd3 (vector) neutralising antibody titre measured by SEAP assay 4- and 8- weeks after IM PanAd3-RSV prime vaccination (groups 1 and 2 combined)
The log_2 fold-change from pre-vaccination in serum RSV-neutralising antibody titre measured by PRNT 28-days post IM PanAd3-RSV prime (groups 1 and 2 combined) and IM MVA-RSV boost vaccination (groups 1 and 3 separately)
The log_2 transformed anti-F IgG antibody secreting cell (ASC) counts measured by *ex vivo* ELISpot 7-days after IM PanAd3-RSV prime (groups 1 and 2 combined) and IM MVA-RSV boost vaccination (groups 1 and 3 separately)
The log_2 fold-change from pre-vaccination in T-cell IFNγ counts measured by *ex vivo* ELISpot 14-days after IM PanAd3-RSV prime (groups 1 and 2 combined) and 7-days after IM MVA-RSV boost vaccination (groups 1 and 3 separately)

Table 8-1. The pre-selected clinical and immunological responses to vaccination for correlation analysis with differential gene expression 3-days after vaccination.
Correlations by Pearson's test with log_2 transformed data, where appropriate.

8.3 Data integrity and quality assurance

Quality control detected three samples with fewer gene expression signals than the other 241 samples (defined as recording fewer genes than the mean minus 3 standard deviations of all samples). Repeat mRNA amplification and hybridisation resulted in 2/3 samples becoming suitable for analysis. Overall 3/246 (1.2%) of expected samples were unavailable for analysis and all three were from high-dose group 4 volunteers in relation to IN PanAd3-RSV prime; two samples were not collected (one volunteer feinted during phlebotomy and another failed to attend the visit) and one baseline sample failed quality controls for reasons unknown and despite vacuum concentration (see supplementary material). Background correction and quantile normalisation are shown in supplementary material. After quality control analyses a total of 25'758 probes (corresponding to 16'731 individual genes) on 243 samples were available for analysis.

8.4 Differentially expressed probes after vaccination

8.4.1 Analysis of the baseline gene expression

At baseline there were no differentially expressed probes between study groups 1 and 2 allocated to receive IM PanAd3-RSV prime, and no differentially expressed probes were detected between study groups 3 and 4 allocated to receive IN PanAd3-RSV prime. One probe (gene symbol CLEC12A) was differentially expressed between groups 1 and 3, and two probes (gene symbols GSTT1 and TREML4) were differentially expressed between groups 2 and 4. Within each study group there were no differentially expressed probes before prime and before boost vaccination 4- or 8-weeks later. Prior to boost only one probe was differentially expressed between groups 1 and 2 (gene symbol C21orf81), and two probes were differentially expressed between groups 1 and 3 (gene symbols STOX1 and CLEC12A).

8.4.2 *Differentially expressed genes after vaccination*

Between none and 554 differentially expressed probes were detected after vaccination in different study groups and different time points, as illustrated in Figure 8-2. IM PanAd3-RSV primed volunteers (groups 1 and 2 combined) recorded a total of 305 differentially expressed probes 3-days after vaccination, which declined to only 7 probes by 7-days post-prime. The transcriptomic analysis of the same vaccine given by intra-nasal spray (IN PanAd3-RSV) found no differentially expressed probes at any time point measure (study groups 3 and 4, analysed combined and separately).

IM MVA-RSV boost was responsible for the greatest number of differentially expressed probes 3-days after boost, and the magnitude of this response was different between IM or IN PanAd3-RSV primed volunteers. In contrast, IM PanAd3-RSV boost failed to provoke measurable changes in gene expression (only 3 differentially expressed probes). Interestingly Group 4 volunteers who had failed to mount measurable transcriptional or immune responses to IN prime vaccination, implying a lack of vaccine stimulus or one measurable in blood at this time-point, did not make a transcriptional response similar to the first dose IM PanAd3-RSV at prime for study groups 1 and 2. For IN PanAd3-RSV primed volunteers the IM PanAd3-RSV boost resulted in only one differentially expressed probe compared with the 305 probes from groups 1 and 2 combined after their first dose of IM PanAd3-RSV vaccine.

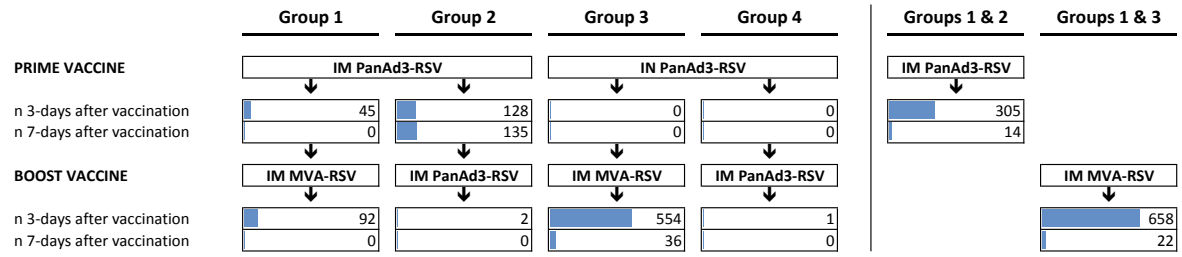


Figure 8-2. Illustration of the number of differentially expressed probes after vaccination by study group and route/vector combination.
The number (n) within each cell is the number of differentially expressed probes at each time point. The blue bars are used to illustrate the relative number of differentially expressed probes between groups and time points. The DNA microarray contained a total of 25'758 separate probes.

8.4.3 *Exploration of differentially expressed probes after IM PanAd3-RSV prime vaccination*

Compared with baseline a total of 305/25758 (1.2%) probes, corresponding to 265 genes and 45 splice variants, were differentially expressed 3-days following IM PanAd3-RSV prime (volunteers from study groups 1 and 2 combined). 216 probes (181 genes) were up-regulated and 89 probes (84 genes) were down-regulated at this time-point (Figure 8-3). Analysed by separate study group there were differences in the number of differentially expressed probes between volunteers in groups 1 and 2, with 45 differentially expressed probes (38 genes) from the 11 volunteers from group 1 and 135 differentially expressed probes (120 genes) from the 10 volunteers from group 2, although the differences between groups were not statistically significant (see supplementary material). Gene annotation indicated that differentially up-regulated probes were enriched for interferon signalling and innate anti-viral immune responses and down-regulated probes were enriched for the regulation of apoptosis and cell metabolism. The addition of baseline anti-PanAd3 (vector) neutralising antibody titre, which were different between study groups 1 and 2 volunteers, as a covariate into the *lmfit* linear regression model did not significantly alter the number of differentially expressed probes following IM PanAd3-RSV prime (data not shown).

By 7-days after vaccination only 14/25758 (0.05%) of probes (representing 8 genes) were differentially expressed from baseline following IM PanAd3-RSV (groups 1 and 2 combined). None of these had been differentially expressed 3-days after vaccination. 13/14 probes were up-regulated and gene symbol annotations indicated adaptive humoral immune responses such as antibody assembly and B-cell development. The biological function of the one down-regulated probe is unknown (gene symbol AK023464). Analysed by study group, no differentially expressed probes were detected 7-days after vaccination from group 1 volunteers and 128 differentially expressed probes (118 genes) were detected from group 2 volunteers. These included 12 probes (5 genes; PPAP2C, RRNAD1, TBL1X, CLDN18, RNF145 and SNRNP70) that were differentially expressed at both 3-days and 7-days after vaccination.

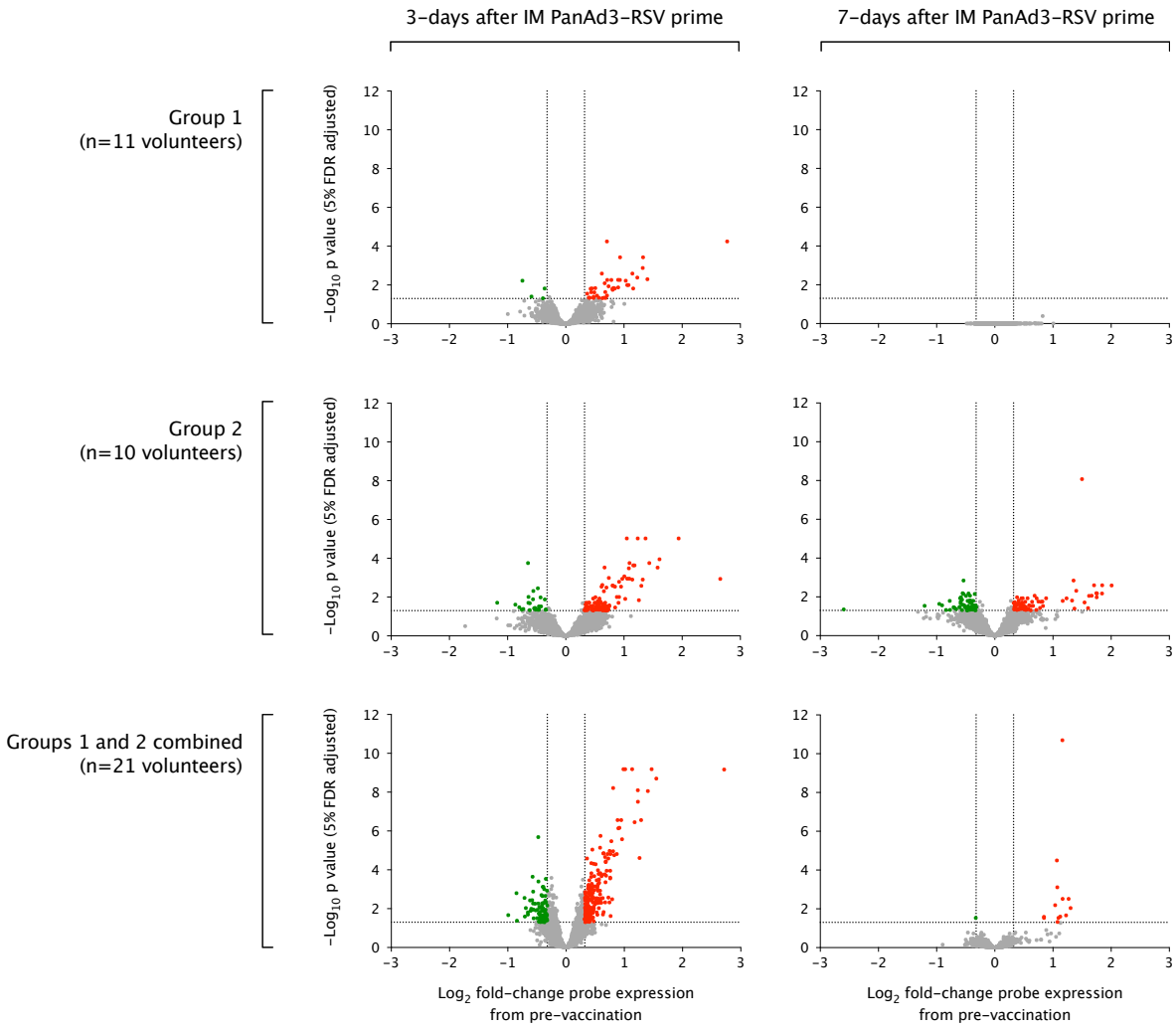


Figure 8-3. Volcano plots of the transcriptomic response 3-days and 7-days following IM PanAd3-RSV prime vaccination measured by DNA microarray.

The dotted lines denote the cut off for definition of being differentially expressed from baseline; log_2 fold change ≥1.25 or <-1.25 (vertical lines) and 5% FDR-adjusted p-value <0.05 (horizontal line). Each probe is represented by a single dot.

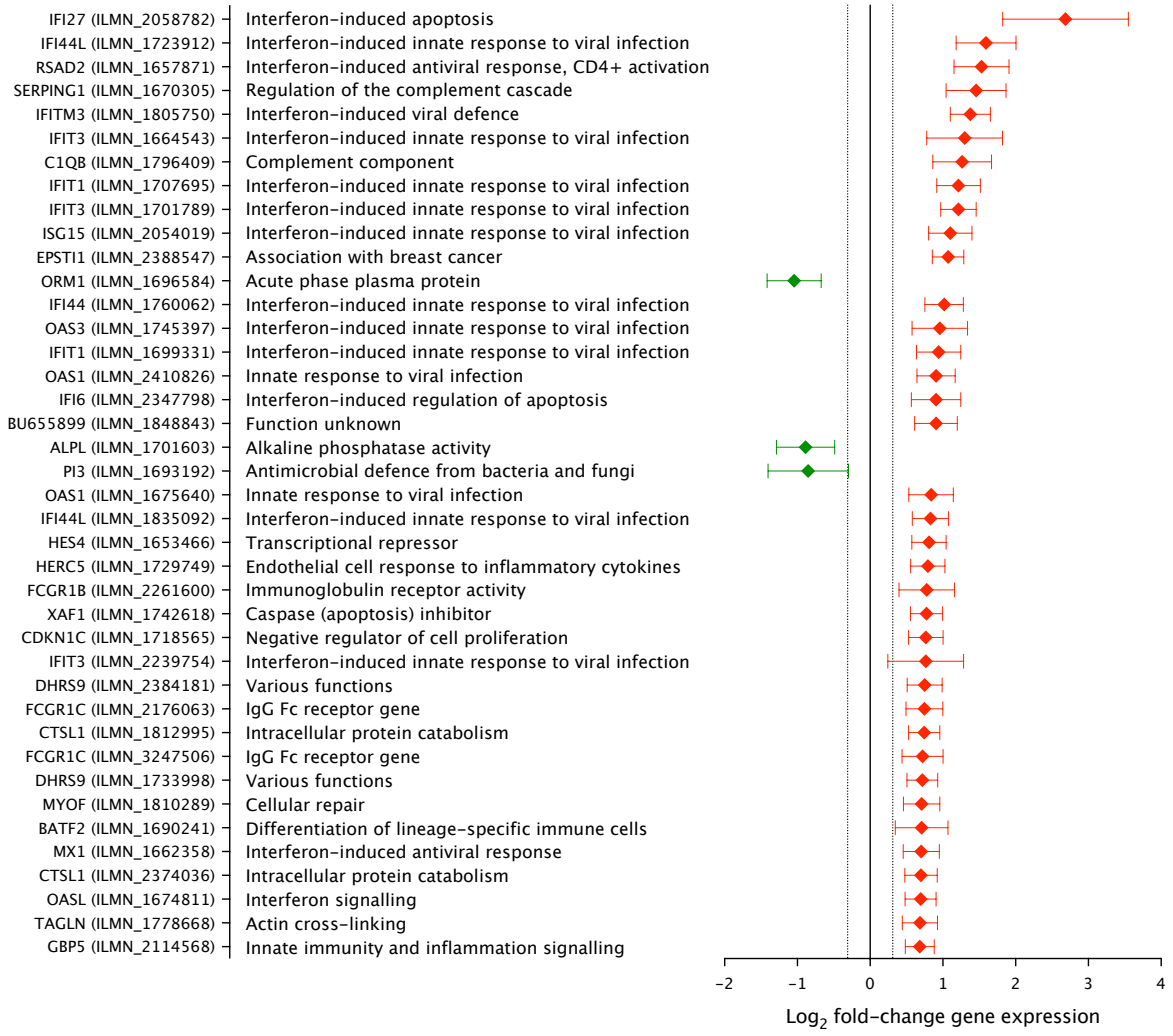


Figure 8-4. Illustration of the top-40 differentially expressed probes 3-days after IM PanAd3-RSV prime. *Volunteers from study groups 1 and 2 combined (n=21). Probes are ranked by log_2-fold change in expression from baseline, irrespective of direction, and the vertical dotted lines denote the threshold cut off for defining a probe as differentially expressed. The functional annotations for each gene symbol are summarised from publicly available databases. The diamond symbol denotes the mean fold-change in probe expression from baseline with the bars used to indicate the 95% confidence interval about the mean. Red, green and black colours are used to indicate up-regulated, likely differentially expressed and down-regulated probes respectively.*

8.4.4 Exploration of differentially expressed probes after IM MVA-RSV boost vaccination

A total of 658/25758 (2.6%) probes, corresponding to 540 genes and 118 splice variants, were differentially expressed from pre-boost expression in peripheral whole blood samples 3-days after IM MVA-RSV boost (volunteers from groups 1 and 3 combined). 409 of these (62.2%) were up-regulated with gene symbol annotations related to interferon signalling, innate immune responses, complement and apoptosis regulation. 20 of the top 25 differentially up-regulated probes (representing 14 separate genes) were shared with the top 25 differentially up-regulated probes 3 days after IM PanAd3-RSV prime. 249/658 (37.8%) of differentially expressed probes were down-regulated with gene symbol annotations implicating biological activity with molecular/intracellular signalling and inflammatory responses. 9 of the top 25 differentially down-regulated probes (representing 6 genes) had been down-regulated 3-days after IM PanAd3-RSV prime. By 7-days after IM MVA-RSV boost only 22/25758 (0.1%) probes were differentially expressed. 21/22 were up-regulated and included probes for IFI27, which was the only differentially expressed gene shared with the 3-day response and the probe that recorded the greatest fold-induction at both time points. One probe (gene symbol TRPC4AP) was down-regulated and has been functionally annotated with ion transportation and the cell cycle.

An added complexity was that the magnitude of the transcriptomic response to IM MVA-RSV boost appeared different depending upon the route of PanAd3-RSV prime. At 3-days after boost, volunteers primed with IN PanAd3-RSV (group 3) recorded 554 differentially expressed probes (462 genes) compared with 92 differentially expressed probes (77 genes) from volunteers primed by IM injection (group 1). The intercept of the Venn diagram in Figure 8-2 is used to illustrate shared probes that were differentially expressed between groups 3-days after IM MVA-RSV boost; for IM primed volunteers (group 1), 85/92 (92%) of differentially expressed probes were shared with volunteers IN primed volunteers (group 3), leaving a total of 469 probes 'uniquely' differentially expressed (as defined by the analysis thresholds) after IM MVA-RSV boost in volunteers primed by IN spray (Figure 8-7). The $\log_2$ fold change in expression of the 85 shared probes were tightly correlated between IM and IN primed volunteers ($p<0.001$, two-tailed Pearson's correlation, $r^2=0.96$, see supplementary material). By 7-days after IM MVA-RSV boost there were no differentially expressed probes detected from IM primed volunteers and only 36 probes (35 genes) were detected from IN primed volunteers.

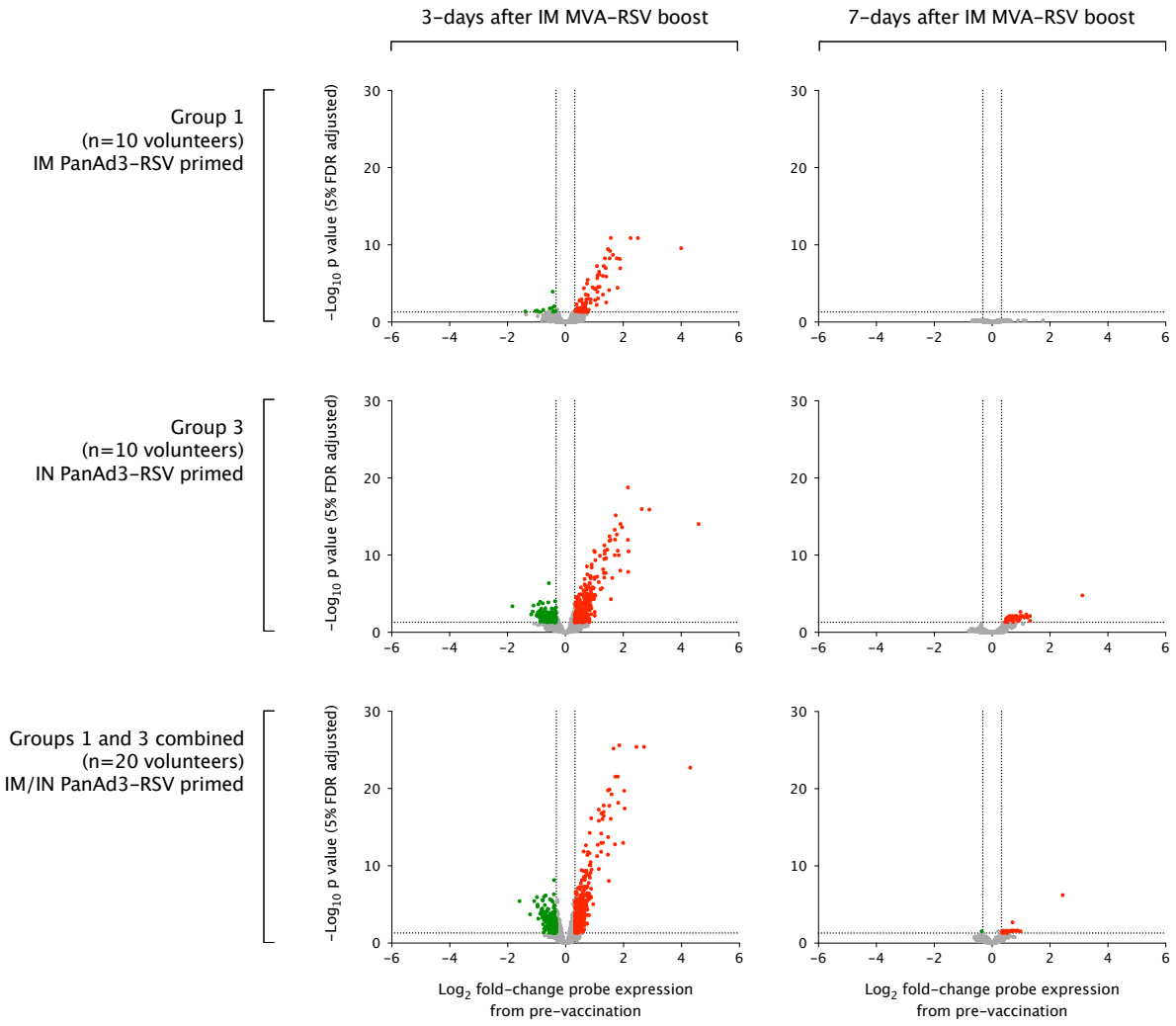


Figure 8-5. Volcano plots of the transcriptomic response 3-days and 7-days following IM MVA-RSV boost vaccination measured by microarray.

The dotted lines denote the cut off for definition of being differentially expressed from baseline; log_2 fold change ≥1.25 or <-1.25 (vertical lines) and 5% FDR-adjusted p-value <0.05 (horizontal line). Each probe is represented by a single dot.

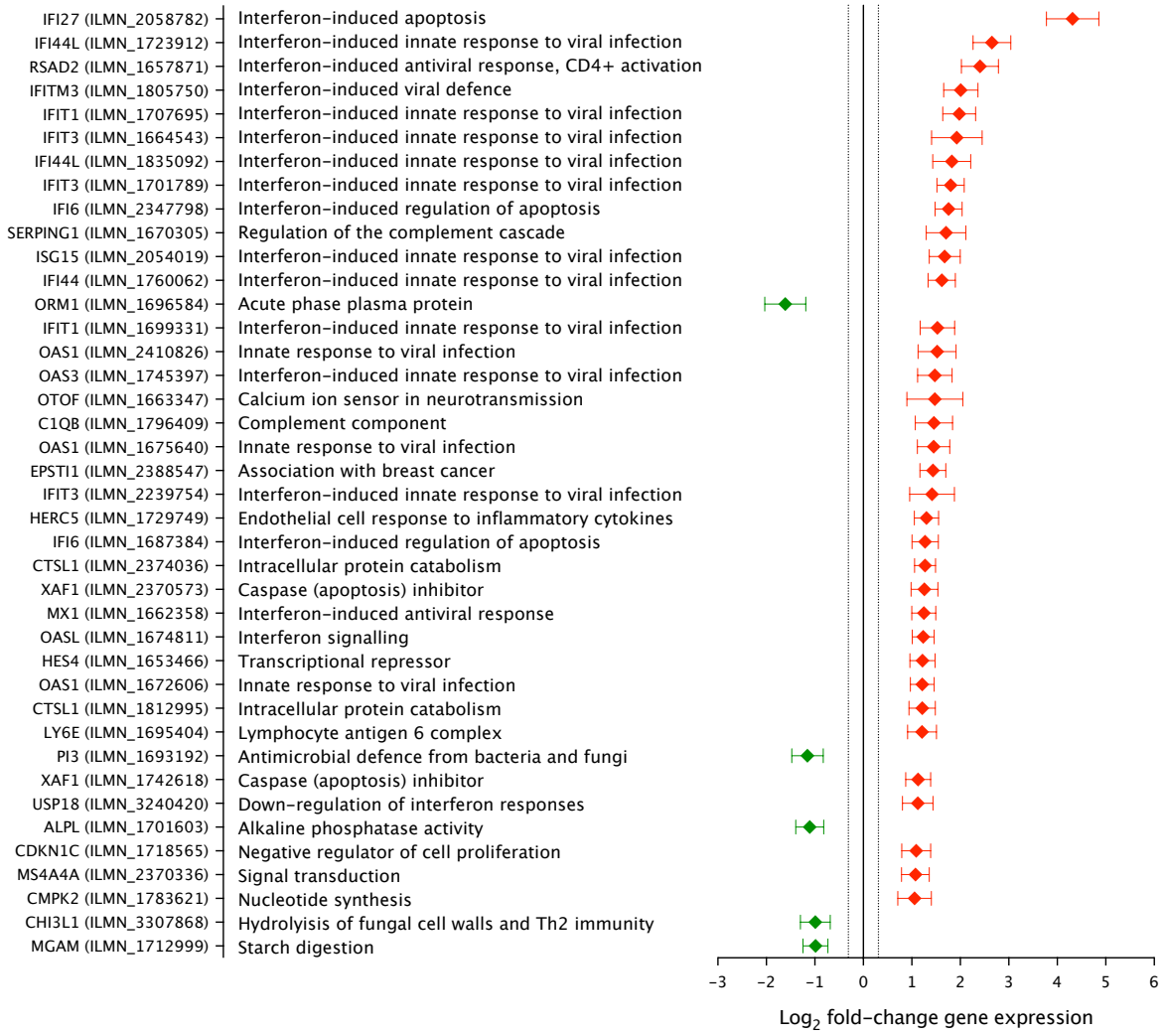


Figure 8-6. Illustration of the top-40 differentially expressed probes 3-days after IM MVA-RSV boost. *Volunteers from study groups 1 and 3 combined (n=20). Probes are ranked by log_2-fold change in expression from baseline, irrespective of direction, and the vertical dotted lines denote the threshold cut off for defining a probe as differentially expressed. The functional annotations for each gene symbol are summarised from publicly available databases. The diamond symbol denotes the mean fold-change in probe expression from baseline with the bars used to indicate the 95% confidence interval about the mean. Red and green are used to indicate up-regulated and down-regulated probes respectively.*

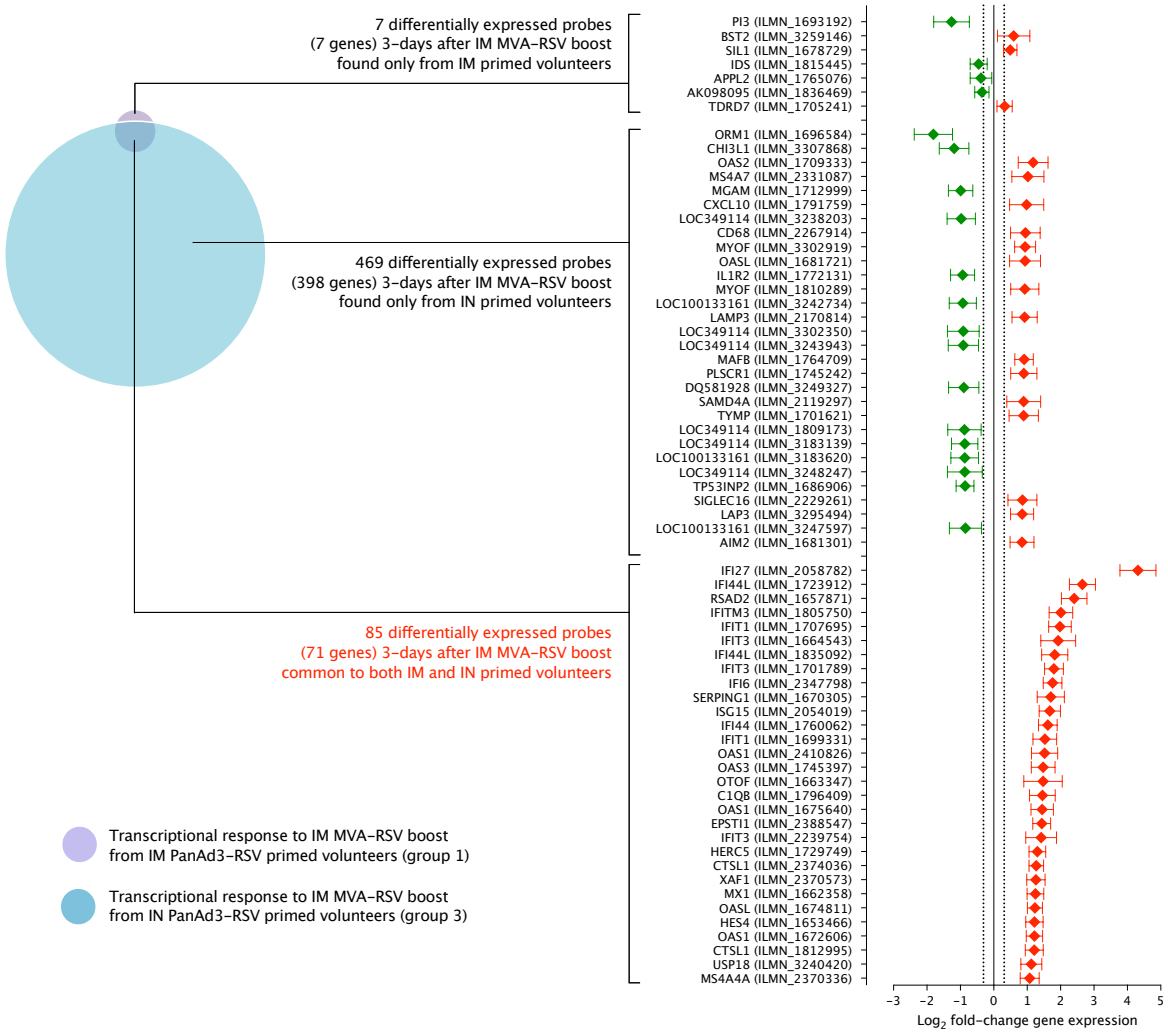


Figure 8-7. Venn diagram of the shared differentially expressed probes 3-days after IM MVA-RSV boost vaccination in volunteers who received IM or IN PanAd3-RSV prime.

The Venn diagram shows the relative number of differentially expressed probes 3-days after IM MVA-RSV for volunteers primed by IM PanAd3-RSV (group 1, n=10 after boost) and from volunteers primed by IN PanAd3-RSV (group 3, n=10 after boost). The sizes of the purple and blue bubbles are proportionate to the total number of differentially expressed probes 3-days after IM MVA-RSV boost. Within each plot the probes from group 1, group 3 and shared across groups are ranked by the mean log_2 fold-change in gene expression with the top 30 probes shown.

8.4.5 Exploration of differentially expressed probes after IM PanAd3-RSV boost vaccination

For IM PanAd3-RSV boost there were only four differentially expressed probes (gene symbols IFI27, CTSL1, RSAD2 and RRAS) up-regulated 3-days after vaccination (study groups 2 and 4 combined). Three of these four differentially expressed probes were detected at the equivalent time-point after IM MVA-RSV boost (gene symbols IFI27, CSTL1 and RSAD2). Analysed by study group, two probes were up-regulated in volunteers primed by IM PanAd3-RSV (group 2, gene symbols IFI27 and RRAS) and one probe down-regulated in volunteers primed by IN spray of vaccine (group 4, gene symbol PRSS1). By 7-days post IM PanAd3-RSV boost there were no differentially expressed probes (study groups 2 and 4 analysed combined and separately).

8.4.6 Exploration of differentially expressed probes common to each intra-muscular dose of viral-vectored vaccine

A total of 68 differentially expressed probes, corresponding to 57 genes, were commonly differentially expressed following IM PanAd3-RSV prime and IM MVA-boost 3-days after vaccination (Figure 8-8). 62 probes were up-regulated and 6 were down-regulated, and included all four differentially expressed probes after IM PanAd3-RSV boost. Furthermore, these 68 commonly differentiated expressed probes recorded the greatest fold-changes in expression after vaccination. Gene ontology, as described in public databases, indicated enrichment for interferon-induced apoptosis and activation of antiviral innate immunity. By 7-days after vaccination, none of the 68 "common core" of differentially expressed probes remained detectable after IM PanAd3-RSV (from 13 probes detected) and only probes for IFI27 remained differentially expressed after IM MVA-RSV boost (from 23 probes detected).

Gene symbol (Illumina probe ID)	Mean log_2 fold change gene expression	Annotation of biological gene function
IFI27 (ILMN_2058782)	4.0	Interferon-induced apoptosis
IFI44L (ILMN_1723912)	2.5	Interferon-induced innate response to viral infection
RSAD2 (ILMN_1657871)	2.3	Interferon-induced antiviral response, CD4+ activation
IFITM3 (ILMN_1805750)	1.9	Interferon-induced viral defence
IFIT1 (ILMN_1707695)	1.9	Interferon-induced innate response to viral infection
IFIT3 (ILMN_1664543)	1.8	Interferon-induced innate response to viral infection
IFIT3 (ILMN_1701789)	1.8	Interferon-induced innate response to viral infection
IFI6 (ILMN_2347798)	1.6	Interferon-induced regulation of apoptosis
IFI44 (ILMN_1760062)	1.6	Interferon-induced innate response to viral infection
IFI44L (ILMN_1835092)	1.5	Interferon-induced innate response to viral infection
ISG15 (ILMN_2054019)	1.5	Interferon-induced innate response to viral infection
SERPING1 (ILMN_1670305)	1.5	Regulation of the complement cascade
IFI6 (ILMN_1687384)	1.5	Interferon-induced regulation of apoptosis
IFIT1 (ILMN_1699331)	1.4	Interferon-induced innate response to viral infection
IFIT3 (ILMN_2239754)	1.4	Interferon-induced innate response to viral infection
OAS1 (ILMN_2410826)	1.4	Innate response to viral infection
EPSTI1 (ILMN_2388547)	1.4	Association with breast cancer
OAS1 (ILMN_1675640)	1.3	Innate response to viral infection
C1QB (ILMN_1796409)	1.3	Complement component
OAS3 (ILMN_1745397)	1.3	Interferon-induced innate response to viral infection
XAF1 (ILMN_2370573)	1.2	Caspase (apoptosis) inhibitor
HERC5 (ILMN_1729749)	1.2	Endothelial cell response to inflammatory cytokines
MX1 (ILMN_1662358)	1.2	Interferon-induced antiviral response
OTOF (ILMN_1663347)	1.1	Calcium ion sensor in neurotransmission
LY6E (ILMN_1695404)	1.1	Lymphocyte antigen 6 complex
OAS1 (ILMN_1672606)	1.1	Innate response to viral infection
HES4 (ILMN_1653466)	1.1	Transcriptional repressor
CTSL1 (ILMN_2374036)	1.1	Intracellular protein catabolism
XAF1 (ILMN_1742618)	1.1	Caspase (apoptosis) inhibitor
OAS1 (ILMN_1658247)	1.1	Innate response to viral infection

Table 8-2. Top-30 differentially expressed probes 3-days after each dose of IM viral-vectored vaccine.
The probes are ranked by mean fold-change from pre-vaccination baseline (mean across study groups). Biological gene functions were annotated using GeneCards (www.genecards.com).

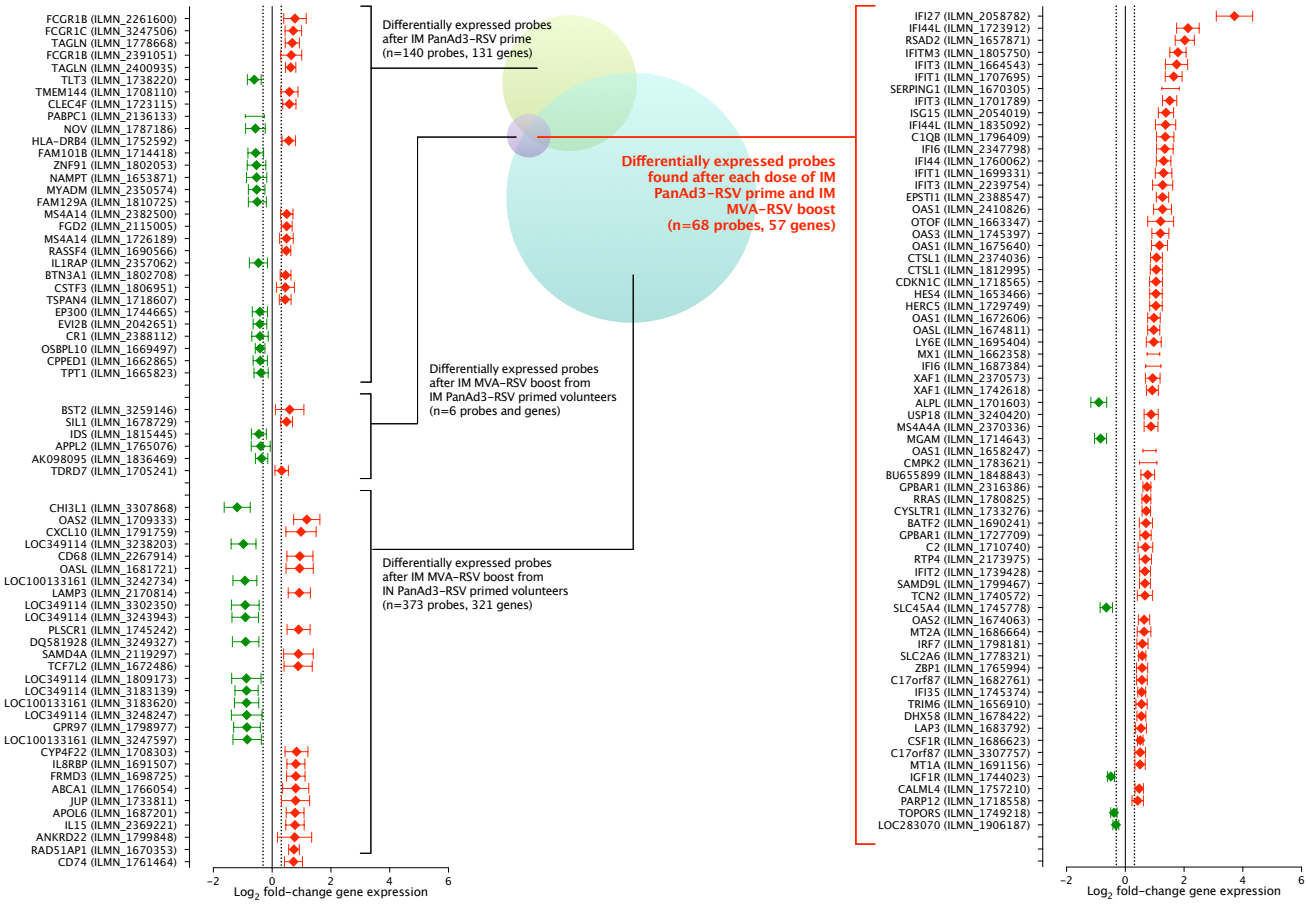


Figure 8-8. Differentially expressed probes 3-days after IM PanAd3-RSV and IM MVA-RSV vaccination.
The size of each bubble of the Venn diagram is representative of the total number of differentially expressed probes 3-days after IM PanAd3-RSV prime (green bubble, n=21 volunteers from groups 1 and 2 combined, 305 differentially expressed probes), IM MVA-RSV boost after IM prime (purple bubble, n=10 volunteers from group 1, 92 differentially expressed probes) and IM MVA-RSV boost after IN prime (blue bubble, n=10 volunteers from group 3, 554 differentially expressed probes). The top-15 probes are shown and ranked by the log_2 fold change from pre-vaccination expression (mean for all groups in the case of common probes).

8.5 Gene set enrichment analysis (GSEA)

Expression in certain BTMs characterised some differences in transcriptomic responses 3-days after IM or IN PanAd3-RSV prime (see Figure 8-9). These included M37.0 (Immune activation – generic cluster), M4.1 (Cell Cycle), M4.5 (mitotic cell cycle in stimulated CD4 T cells) and M76 (DNA repair). Single sample GSEA showed reasonable separation between the two groups with only 8/39 samples clustering in the opposite group (data not shown). Interestingly, although no significant perturbation on the gene level was observed following IN PanAd3-RSV prime, modular responses to IM MVA-RSV boost in IN primed individuals were markedly increased compared with those primed with IM PanAd3-RSV (group 3 and group 1 volunteers).

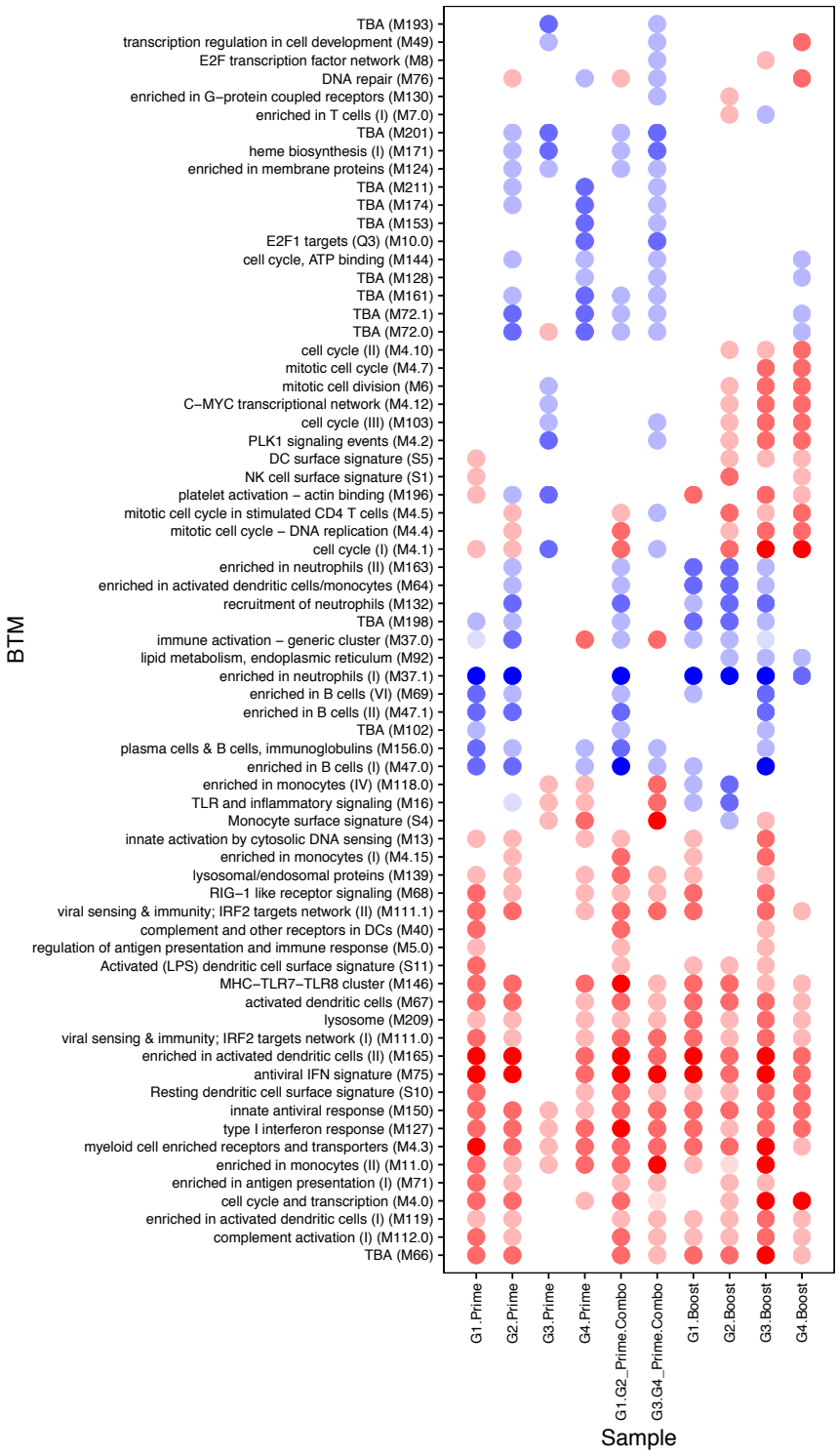


Figure 8-9. Gene set enrichment analysis of the transcriptomic response to vaccination.
Data for younger adults only (study groups 1-4). Blood transcription modules (BTMs) that were significantly enriched (adjusted p-value<0.05) in at least one group are plotted on a grid highlighting marked similarity between the global responses following IM/IN PanAd3-RSV prime. The GSEA analysis and this figure was performed exclusively by Dr Christoph Blohmke, and is reproduced with permission for a more in depth description of the transcriptomic response to vaccination. G1, group 1 (IM PanAd3-RSV prime/IM MVA-RSV boost). G2, group 2 (IM PanAd3-RSV prime/IM PanAd3-RSV boost). G3, group 3 (IN PanAd3-RSV prime/IM MVA-RSV boost). G4, group 4 (IN PanAd3-RSV/IM PanAd3-RSV boost).

8.6 Statistical correlations between differentially expressed probes and vaccine responses

The final stage of analysis was to explore the potential for statistical correlations between the observed transcriptional response 3-days following vaccination with pre-selected clinical and immunological responses to vaccination measured at later time points (as defined earlier) using Pearson's test. The results indicated that early transcriptional changes in approximately 15-30% of differentially expressed probes correlated with one or more of the pre-selected vaccine responses.

Genetic responses to vaccination with correlations to clinical/immunological vaccine responses following IM PanAd3-RSV prime vaccination are shown in Figure 8-10 (study groups 1 and 2 combined, n=21). In total, 66/305 (21.6%) of differentially expressed probes correlated significantly with one or more of the pre-specified clinical/immunological responses to vaccination measured at later time points (see Table 8-1). Gene probes for HERC5 (interferon-induced pro-inflammatory responses), MX1 (interferon-induced antiviral responses) and OAS3 (interferon-induced viral resistance) were found to correlate significantly with both the serum PanAd3 vector and RSV-neutralising antibody responses. From the probes with correlation to the anti-F IgG ASC response, gene probes for SLC37A2 (transporter activity) and SGK1 (cellular stress responses) were also correlated significantly to the serum RSV-neutralising antibody and T-cell IFNγ responses respectively.

Following IM MVA-RSV boost, a total of 26/92 (28.2%) and 103/554 (18.6%) of differentially expressed probes 3-days after vaccination could be correlated with one or more of the pre-specified clinical/immunological responses to vaccination measured at later time points in volunteers primed by IM and IN PanAd3-RSV respectively (Figure 8-11 and Figure 8-12 for study groups 1 and 3 respectively, n=10 for each group).

The transcriptomic IM MVA-RSV boost response from volunteers in group 1, primed with IM PanAd3-RSV, found 4/26 of the differentially expressed genes correlated with more than one of the pre-selected vaccine responses. These included HES4 (encoding a transcription factor) correlated with both the changes in haemoglobin and the anti-F IgG ASC responses, RRAS (cell signalling) correlated with both the changes in haemoglobin and the serum RSV-neutralising antibody responses and both CYSLTR1 (leukotriene receptor)

and TRIM6 (function unknown) that correlated with both the serum RSV-neutralising antibody and anti-F IgG ASC responses to boost vaccination.

The transcriptomic IM MVA-RSV boost response from volunteers in group 3, primed with IN PanAd3-RSV, found 7/103 of the correlating differentially expressed genes correlated with more than one of the pre-selected vaccine responses. From the 47 probes that correlated with observed changes in haemoglobin after vaccination, 2 probes (ANXA2, regulation of cell growth and EPSTI1, association with breast cancer) also correlated with the IFNγ response and 4 probes (BLVRA, biliverdin reductase; BST2, growth and development of B-cells; JUP, cytoskeleton protein and KIT, cell survival, proliferation and haematopoiesis) also correlated with the serum RSV-neutralising antibody response to vaccination. The final shared probe, PLA2G15 encoding proteins annotated to lysosome pathways, was shared between the serum RSV-neutralising antibody and anti-F IgG ASC responses to vaccination.

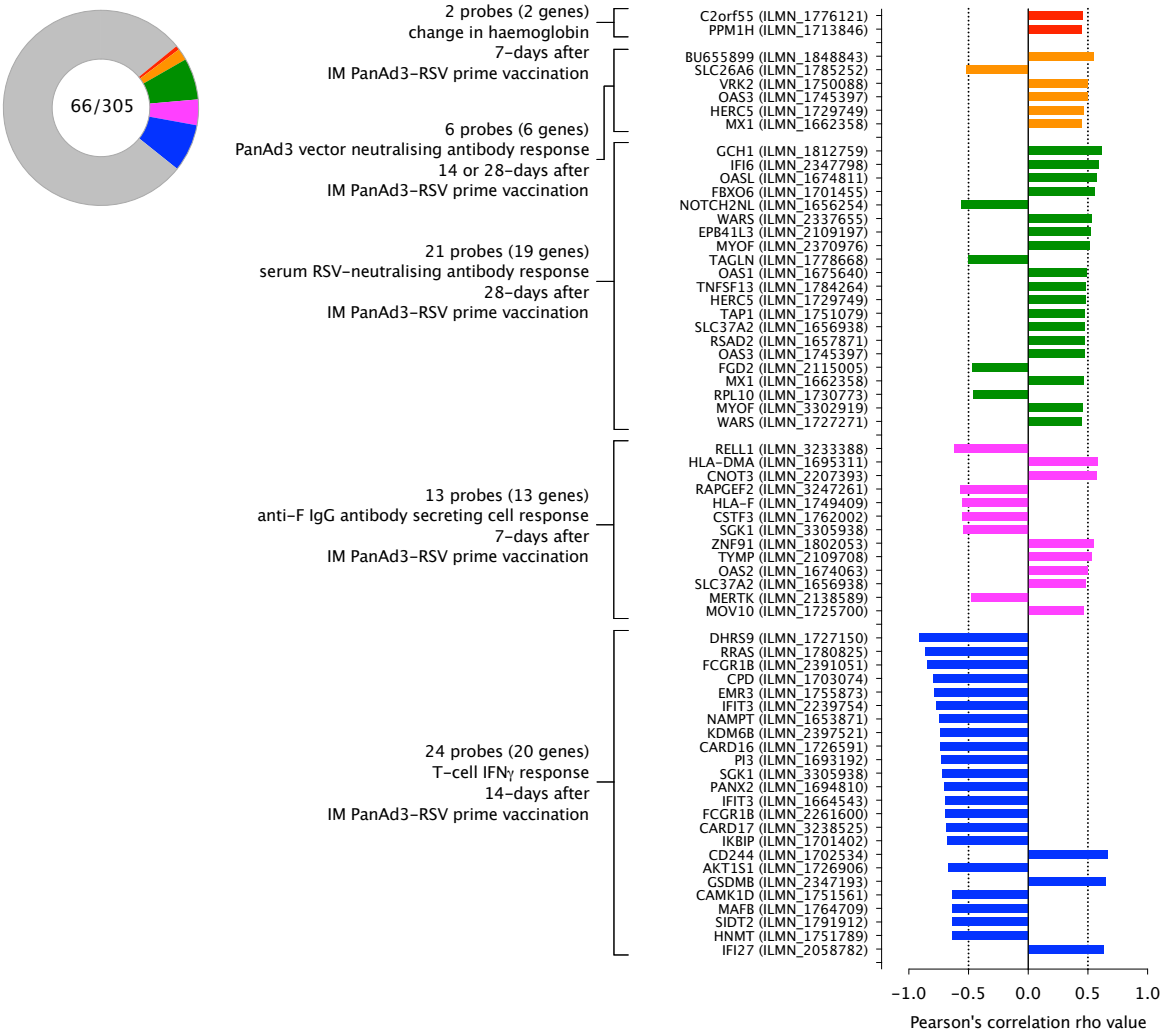


Figure 8-10. Differentially expressed probes 3-days after IM PanAd3-RSV prime vaccination with significant statistical correlation to clinical/immunological responses measured at later time points.
Data from volunteers in study groups 1 and 2 combined (n=21). The log_2 fold-changes in gene expression from 66/305 (21.6%) of differentially expressed probes 3-days after IM PanAd3-RSV prime significantly correlated with one or more clinical/immunological responses to vaccination measured at later time points (Pearson's p-value <0.05, probes ranked by correlation rho value irrespective of orientation). The pre-selected vaccine responses included the haemoglobin change 7-days after vaccination, log_2-transformed fold-change in serum anti-PanAd3 vector neutralising antibody 4- or 8-weeks after vaccination, log_2-transformed fold-change in serum RSV-neutralising antibody titre 28-days after vaccination, log_2-transformed anti-F IgG antibody secreting cell count 7-days after vaccination and the log_2-transformed fold-change in IFNγ count 14-days after prime.

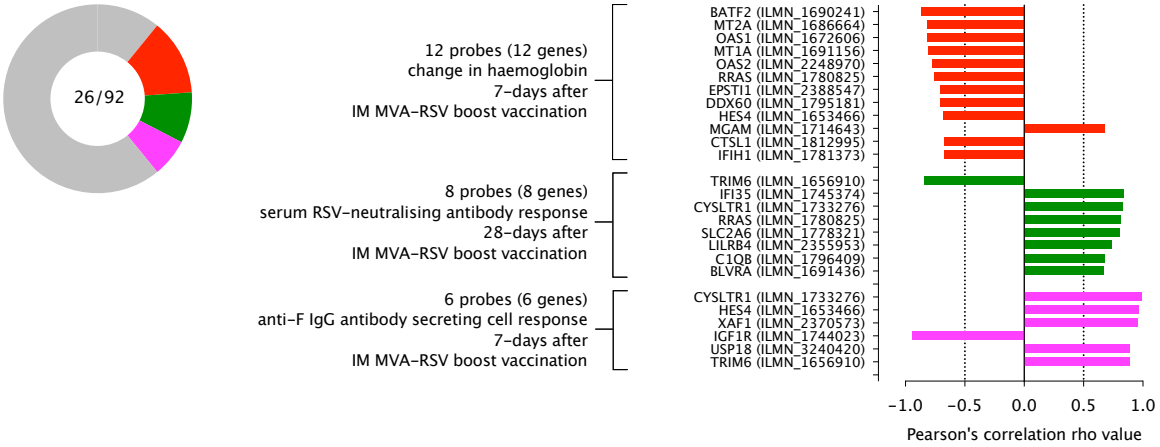


Figure 8-11. Differentially expressed probes 3-days after IM MVA-RSV boost vaccination with significant statistical correlation to clinical/immunological responses measured at later time points in volunteers primed with IM PanAd3-RSV.

Data from volunteers in study group 1 only (n=10). The log_2 fold-changes in gene expression from 26/92 (28.2%) of differentially expressed probes 3-days after IM MVA-RSV boost significantly correlated with one or more clinical/immunological responses to vaccination measured at later time points (Pearson's p-value <0.05, probes ranked by correlation rho value irrespective of orientation). The pre-selected vaccine responses included the haemoglobin change 7-days after vaccination, log_2-transformed fold-change in serum RSV-neutralising antibody titre 28-days after vaccination and the log_2-transformed anti-F IgG antibody secreting cell count 7-days after vaccination.

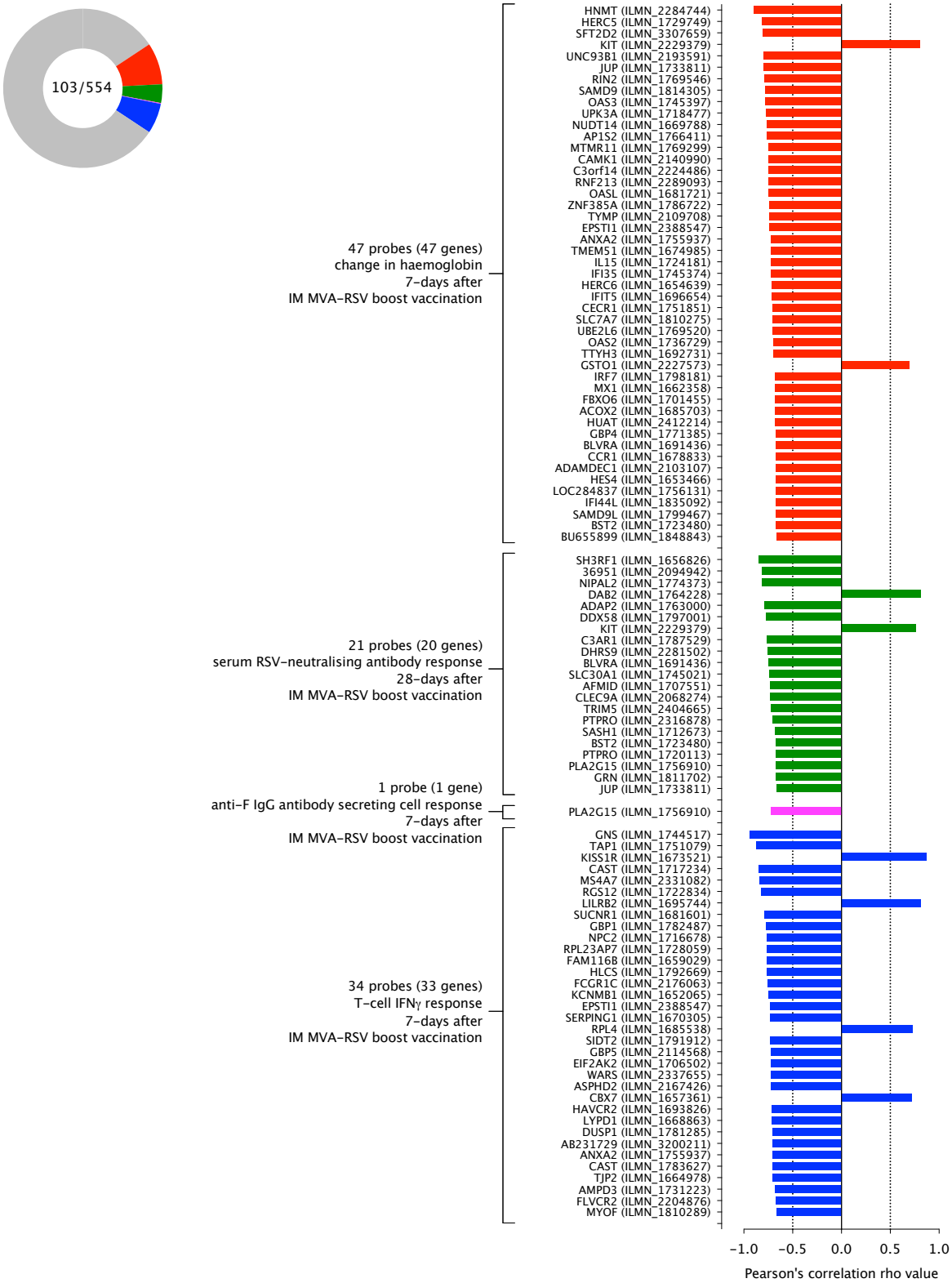


Figure 8-12. Differentially expressed probes 3-days after IM MVA-RSV boost vaccination with significant statistical correlation to clinical/immunological responses measured at later time points in volunteers primed with IN PanAd3-RSV.

Data from volunteers in study group 3 only (n=10). The log_2 fold-changes in gene expression from 103/554 (18.6%) of differentially expressed probes 3-days after IM MVA-RSV boost significantly correlated with one or more clinical/immunological responses to vaccination measured at later time points (Pearson's p-value <0.05, probes ranked by correlation rho value irrespective of orientation).

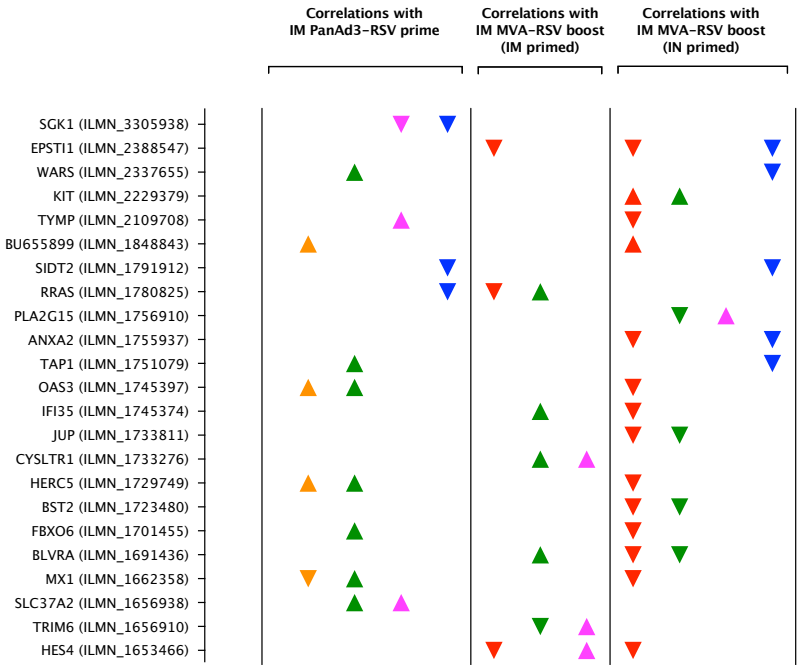


Figure 8-13. Illustration of differentially expressed probes 3-days after vaccination with significant statistical correlation to more than one clinical/immunological response measured at later time points.
A total of 23 differentially expressed probes could be correlated with more than one vaccine response within one vaccine dose or between prime/boost combinations of vaccines. Upward triangles denote positive correlations and downward triangles negative correlations with the gene symbols listed to the side (annotated with the Illumina probe ID in brackets). Colours denote the vaccine response as changes in haemoglobin (red), serum anti-PanAd3 (vector) neutralising antibody (gold), serum RSV-neutralising antibody (green), anti-F IgG antibody secreting cells (magenta) and T-cell IFNγ (blue).

8.6.1 Early gene expression correlates with changes in haemoglobin after vaccination

The log_2 fold-changes in gene expression from 2, 12 and 47 differentially expressed probes 3-days after vaccination could be correlated with the absolute changes in haemoglobin measured 7-days after IM PanAd3-RSV prime and IM MVA boost vaccination in IM and IN PanAd3-RSV primed volunteers respectively.

Of the 305 differentially expressed probes detected 3-days after IM PanAd3-RSV prime (volunteers in groups 1 and 2 combined, n=21), the log_2 fold changes in expression from only 2 probes (Corf55 and PPM1H) bore a statistically significant correlation to the minor absolute change in haemoglobin 7-days after prime (Figure 8-14).

Following IM MVA-RSV boost the transcriptional changes in 12/92 (13%) and 47/554 (8%) differentially expressed probes from IM primed (group 1) and IN primed (group 3) volunteers respectively correlated with the greater absolute changes in haemoglobin measured 7-days after boost (Figure 8-14). The majority of these probe expression changes were negatively correlated and 2 probes were shared in the changes with IM and IN primed volunteers (EPSTI1 and HES4)(Figure 8-13).

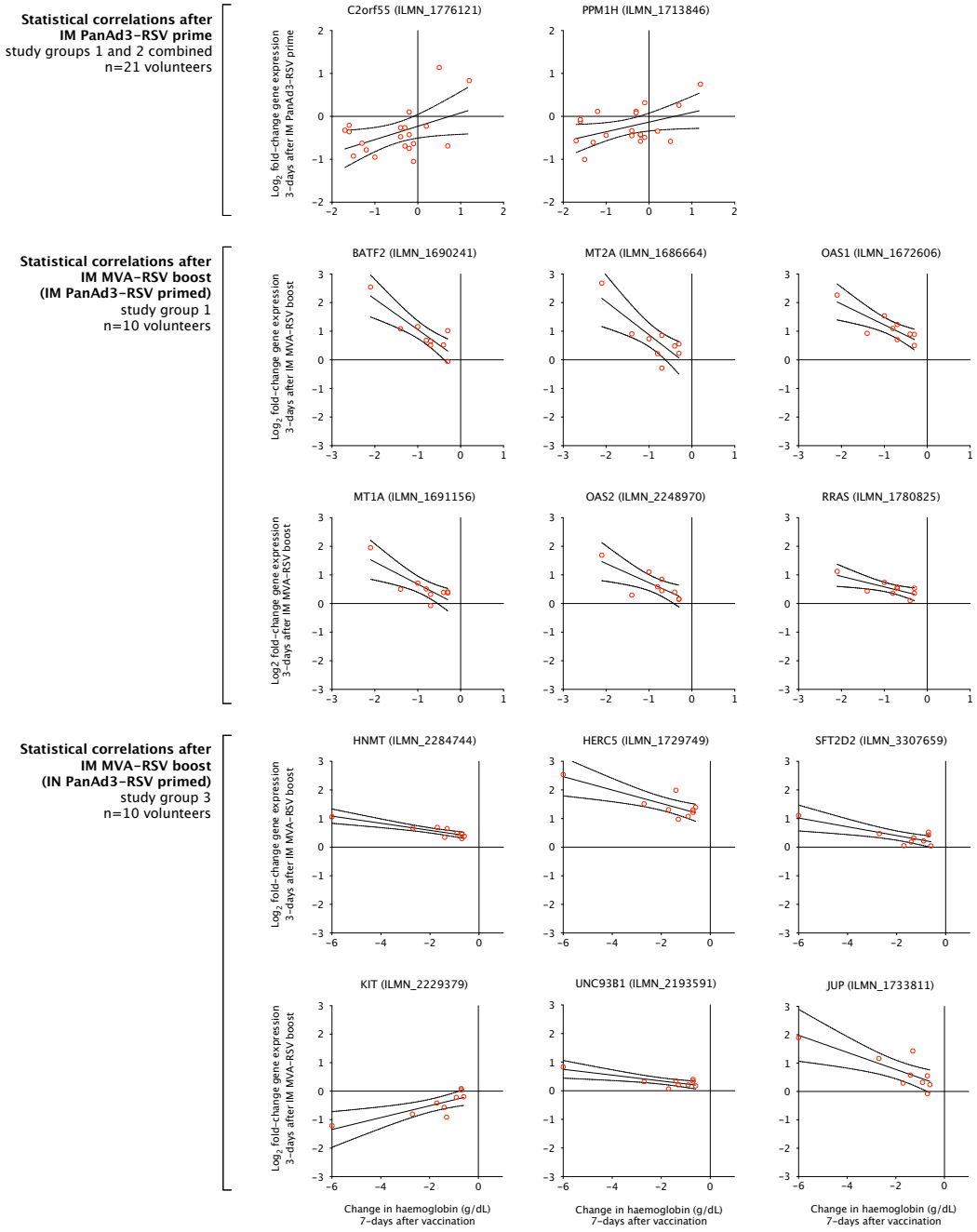


Figure 8-14. Statistically significant correlations between transcriptional changes 3-days after vaccination and changes in haemoglobin 7-days after vaccination.

The log_2-fold change in gene expression from each differentially expressed probe 3-days after vaccination was correlated by Pearson's correlation with the change in haemoglobin (g/dL) 7-days after vaccination. Statistically significant ($p<0.05$) top-ranked correlates (ranked by rho value) are shown in the above figure. The line is the linear regression line and dotted lines denote the 95% confidence intervals.

8.6.2 *Early gene expression correlates with the induction of serum anti-PanAd3 (vector) neutralising antibody after IM PanAd3-RSV prime*

The transcriptional response from a total of 6/305 (2%) differentially expressed probes (representing 6 genes) measured 3-days after IM PanAd3-RSV prime correlated significantly with the induction of serum vector neutralising antibody titres measured 4- or 8-weeks after vaccination (study groups 1 and 2 combined, n=21 volunteers). 3/6 probes also correlated with the fold-changes in serum RSV-neutralising antibody after IM PanAd3-RSV prime (gene symbols OAS3, HERC5 and MX1) and 4/6 probes correlated with the changes in haemoglobin after IM MVA-RSV boost from volunteers primed with IN (and not IM) PanAd3-RSV (gene symbols BU55899, OAS3, HERC5 and MX1) (see Figure 8-13).

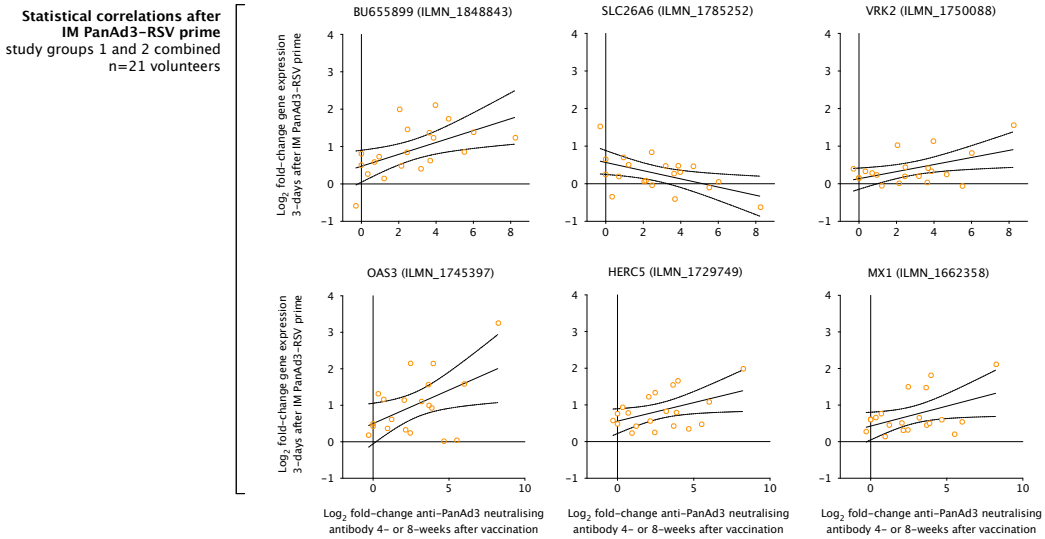


Figure 8-15. Statistically significant correlations between transcriptional changes 3-days after IM PanAd3-RSV prime and changes in serum titres of anti-PanAd3 (vector) neutralising antibody measured 4- and 8-weeks after vaccination.
The log_2-fold change in gene expression from each differentially expressed probe 3-days after vaccination was correlated by Pearson's correlation with the log_2 transformed fold-change in serum anti-PanAd3 (vector) neutralising antibody measured 28-days (group 2) and 56-days (group 1) after vaccination. Statistically significant ($p<0.05$) top-ranked correlates (ranked by rho value) are shown in the above figure. The line is the linear regression line and dotted lines denote the 95% confidence intervals. Serum vector neutralising antibody titres were not recorded after boost vaccination.

8.6.3 *Early gene expression correlates with the induction of serum RSV-neutralising antibody after vaccination*

After IM PanAd3-RSV prime the fold-changes in probe expression from 21/305 (6.8%) of all differentially expressed probes (representing 17 genes) correlated significantly with the fold-changes of RSV-neutralising antibody measured 28-days after vaccination. Analysis of the IM MVA-RSV boost revealed 8/92 (8.7%) and 21/554 (3.8%) differentially expressed probes could be correlated with the fold-change in serum RSV-neutralising antibody titre from IM and IN primed volunteers respectively (study groups 1 and 3). In general, the correlations were positive from IM primed volunteers (who made no discernable increment in titres following boost) and negative from IN primed volunteers (who did respond favourably to the boost) with only one gene symbol shared. This probe, gene symbol BLVRA, correlated positively with the IM MVA-RSV boost in IM primed (group 1) and negatively with the IM MVA-RSV boost in IN primed (group 3) responses (see Figure 8-13).

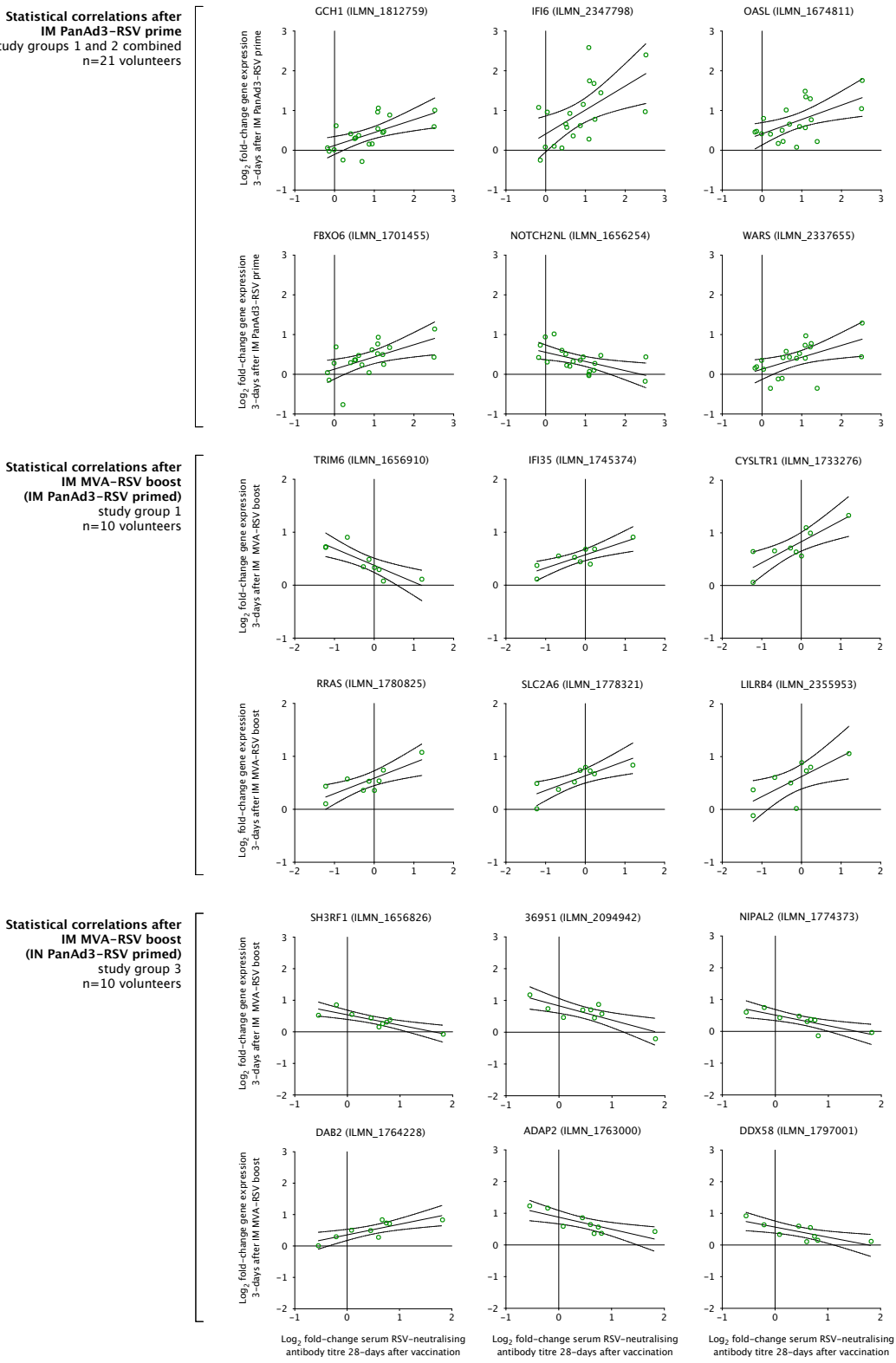


Figure 8-16. Statistically significant correlations between transcriptional changes 3-days after vaccination and changes serum RSV-neutralising antibody titres 28-days after vaccination.

The log_2-fold change in gene expression from each differentially expressed probe 3-days after vaccination was correlated by Pearson's correlation with the log_2 fold-change in serum RSV-neutralising antibody titre 28-days after vaccination. Statistically significant ($p<0.05$) top-ranked correlates (ranked by rho value) are shown in the above figure. The line is the linear regression line and dotted lines denote the 95% confidence intervals.

8.6.4 *Early gene expression correlates with the anti-F IgG antibody secreting cell (ASC) response to vaccination*

A total of 13/305 (4.2%) of differentially expressed genes after IM PanAd3-RSV prime correlated with the induction of F-specific IgG ASC in peripheral blood observed 7-days after vaccination. Gene symbol annotations identified four genes with probable immune function; three genes involved in antigen processing and MHC class I presentation (LILRB1, HLA-F and HLA-DMA) and the MX1 gene. Annotations for the remaining gene symbols did not describe immune-specific functions (cell structure, cell signalling and metabolic processes).

The 6 probes (6 genes) correlating with the IM MVA-RSV boost from volunteers primed with IM PanAd3-RSV could use results from only 4/10 individuals, due to laboratory failures described in earlier chapters. Only one probe correlated with transcriptomic changes after IM MVA-RSV in IN primed volunteers (gene symbol PLA2G15). Several probes that correlated with the anti-F IgG ASC response also correlated with the serum RSV-neutralising antibody response to vaccination; one probe after IM PanAd3-RSV prime (gene symbol SLC37A2), two probes after IM MVA-RSV in IM primed volunteers (gene symbols CYSLTR1 and TRIM6) and the PLA2G15 probe after IM MVA-RSV in IN primed volunteers (see Figure 8-13).

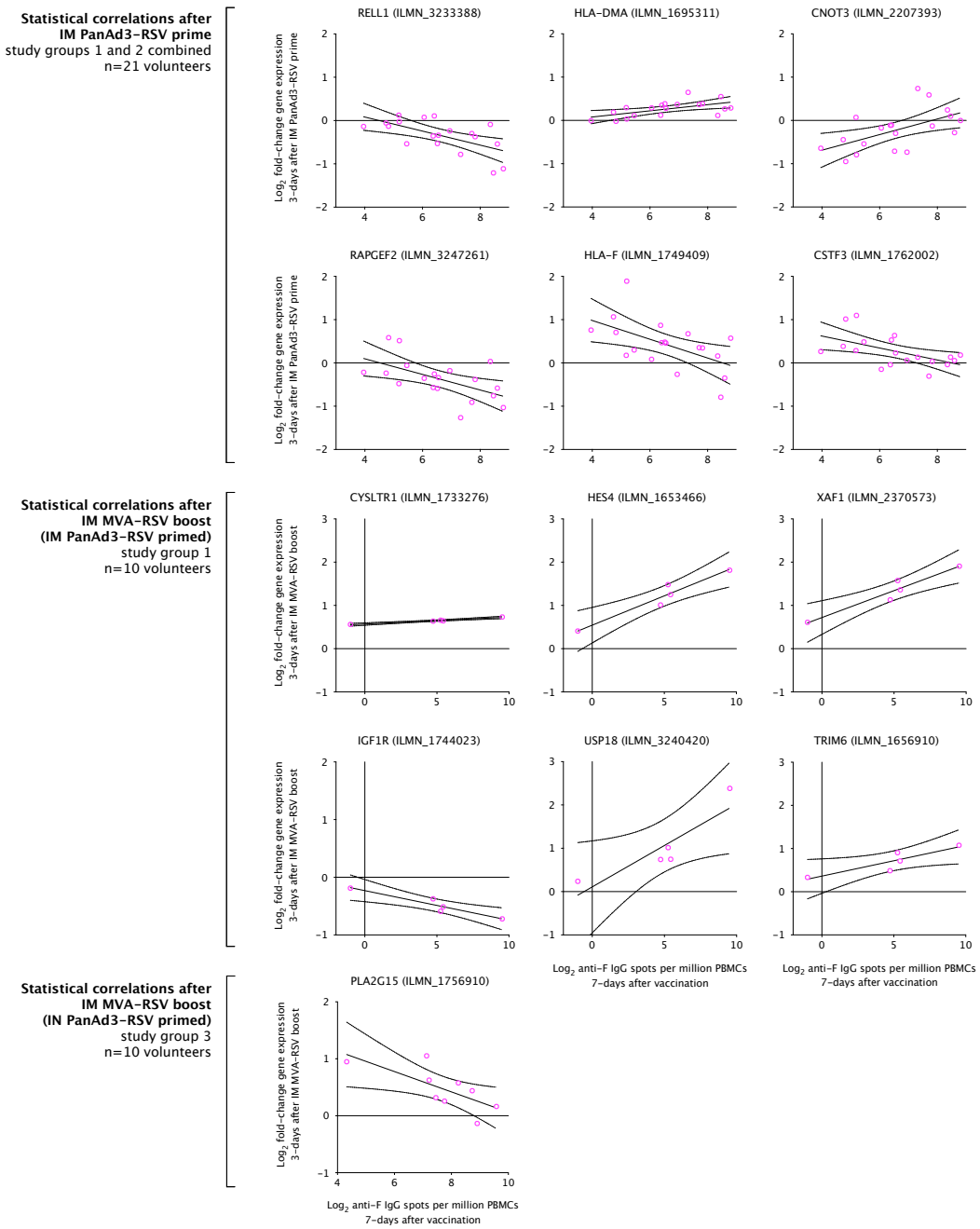


Figure 8-17. Statistically significant correlations between transcriptional changes 3-days after vaccination and F-specific IgG antibody secreting cell response 7-days after vaccination.
The log_2-fold change in gene expression from each differentially expressed probe 3-days after vaccination was correlated by Pearson's correlation with the log_2 anti-F IgG antibody secreting cell (ASC) spot per million PBMCs 7-days after vaccination. Statistically significant ($p<0.05$) top-ranked correlates (ranked by rho value) are shown in the above figure. The line is the linear regression line and dotted lines denote the 95% confidence intervals.

8.6.5 *Early gene expression correlates with the induction of IFNγ after vaccination*

The strongest statistical associations were observed for the IFNγ response to vaccination. The fold-changes from 24/305 (7.7%) differentially expressed probes (representing 20 genes) 3-days after IM PanAd3-RSV prime correlated significantly with the fold-changes in T-cell IFNγ measured 14-days after vaccination; 21/24 were negatively correlated and several gene symbols were annotated to have immune functions. The remaining gene symbols were annotated with structural proteins, cell metabolism and cellular stress responses.

The highest ranked correlation from all vaccine responses was with gene probe DHRS9 after IM PanAd3-RSV prime. Here, the correlation between with log_2-transformed fold-changes in DHRS9 expression and T-cell IFNγ recorded a p-value <0.001 and rho-value of -0.91 (Pearson's correlation). The resulting r^2 value of 0.83 meant that 83% of the variability in IFNγ induction measured 14-days after prime could be attributed, statistically, to the induction of this gene 11-days earlier.

Following IM MVA-RSV boost there were no significant correlations from IM PanAd3-RSV primed volunteers. For volunteers primed with IN PanAd3-RSV there were 34/554 (6.1%) differentially expressed probes (representing 33 genes) that correlated significantly with the IFNγ induction measured 7-days post-boost. As with the correlations after IM prime, most of the correlations after IM MVA-RSV boost were negative and the only shared probe from IM prime was SIDT2.

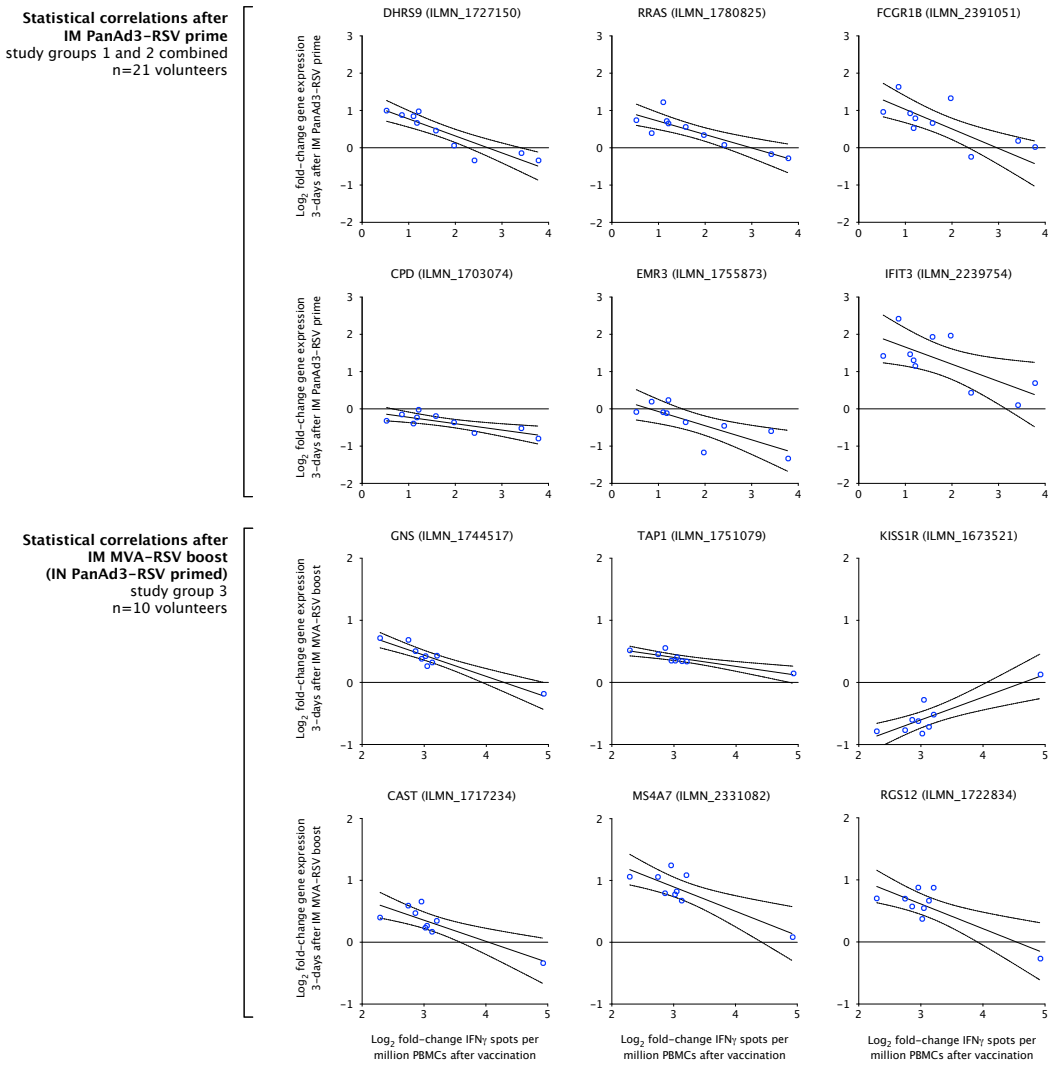


Figure 8-18. Statistically significant correlations between transcriptional changes 3-days after vaccination and the T-cell IFNγ response after vaccination.

The log_2-fold change in gene expression from each differentially expressed probe 3-days after vaccination was correlated by Pearson's correlation with the log_2 fold-change T-cell IFNγ spots per million PBMCs measured 14-days after IM PanAd3-RSV prime and 7-days IM MVA-RSV boost vaccination. Statistically significant ($p<0.05$) top-ranked correlates (ranked by rho value) are shown in the above figure. The line is the linear regression line and dotted lines denote the 95% confidence intervals. There were no significant correlations with the IFNγ response following IM MVA-RSV boost from volunteers primed with IM PanAd3-RSV (study group 1).

8.7 Discussion

8.7.1 Summary of main findings

This chapter sought to characterise the host genetic response to vaccination. A total of 305 differentially expressed probes were detected 3-days following IM PanAd3-RSV prime. Consistent with observations of phenotypic responses to vaccination, the IM route of PanAd3-RSV prime induced transcriptional responses in blood when the IN prime route did not. Although there were no measurable transcriptomic responses following IN PanAd3-RSV prime, there was a surprising poverty of differentially expressed genes following the first IM dose of vaccine with IM PanAd3-RSV boost from the IN PanAd3-RSV primed volunteers (only four probes compared with the 305 detected following IM PanAd3-RSV prime). This implied there might have been responses to IN prime not detected by the microarray assay or at this time point. The GSEA used to explore this further supported the notion that there was a weak systemic transcriptomic response to IN prime, which may be responsible for the attenuated transcriptomic response to IM PanAd3-RSV boost following IN prime.

Analysis of the transcriptomic response to boost showed there was a greater number and magnitude of differential gene expression associated with the MVA vector. The IM prime was associated with relatively fewer differentially expressed probes after IM MVA-RSV boost compared with IN prime (92 vs 554 probes). The combination of responses to IM prime and boost resulted in the identification of 57 genes that were differentially expressed 3-days after each IM dose of viral-vectored vaccine. These genes also represented the greatest magnitude of gene expression changes, closely reproduced/correlated with the response seen to each vaccine, and supported the notion of being a core family of genes that defined common transcriptional responses to viral-vectored vaccines in man.

The final analysis in this chapter revealed that changes in approximately 20% of differentially expressed probes could be correlated with one or more clinical/immunological response to vaccination measured at later time points. Although this association is statistical and not biological, the strength of the association for some genes warrants further exploration as potential biomarkers for vaccine immunogenicity and in defining the innate transcriptomic pathways behind vaccine responses. For example, 80% of the variability in expansion of IFNγ-producing T-cells measured 14-days after IM PanAd3-RSV

(expressed as a fold-change from baseline) could be statistically attributed to changes in gene expression in two probes 3-days after vaccination.

8.7.2 Analysis of differentially expressed genes

Despite several viral-vectored vaccines being used in man there has been, to my knowledge, no published characterisation of the transcriptomic response to this vaccine type in man from whole blood samples. Other researchers have used pre-selected PBMCs to discern the cell specific transcriptomic signatures that correlate to immune responses to tuberculosis after BCG prime/MVA boost[491,492]. In the analysis from the RSV001 trial, approximately 22'000 genes were reduced to 305 genes of interest after IM PanAd3-RSV prime and 554 genes of interest after IN prime/IM MVA-RSV boost, having been defined as differentially expressed genes 3-days after vaccination. The relative excess of a greater number of differentially expressed genes associated with the MVA-vector was consistent with unpublished data from 24-hours after adenovirus/MVA vaccination for malaria. Furthermore, genes such as OAS1, OAS2, OAS3, MX1, IFI27, IFI44, IFI44L, IFIT1, IFIT2, IFIT3 have been reported as genetic signatures of the antiviral response to yellow fever and influenza vaccination[493,494].

Of particular interest from the list of differentially expressed genes was IFI27, which recorded the greatest fold-changes in induction after IM PanAd3-RSV and IM MVA-RSV 3-days and 7-days after vaccination. IFI27 expression has been identified as a biomarker for RSV infection with a high degree of conservation between RSV-specific signatures derived from PBMCs, airway epithelial cells and clinical disease severity scores[495,496]. IFI27, together with genes such as IFI44, OAS3, EPSTII and IFI44L, was significantly up-regulated in whole blood from infants admitted to hospital with severe RSV-bronchiolitis and indicates that several genetic responses to vaccination are also likely to span the same genes responding from mucosal infection and viraemia in natural infection[497]. Interferon-stimulated genes (including IFIT1, IFIT2 and IFIT3) have been induced after RSV infection in mice and A549 (human alveolar carcinoma cell line) models, and are targets of the interferon-suppressive NS1 RSV protein[498,499]. Whole blood gene expression from infants with RSV-bronchiolitis has also described activation of the innate immune response, especially interferon pathways, without being able to distinguish between mild or severe

disease[500]. Clinical data such as these can be used as evidence that PanAd3-RSV and MVA-RSV induced a genetic response biologically relevant and similar to the response observed following natural RSV infection.

Although IN PanAd3-RSV prime did not appear to elicit a transcriptomic response to vaccination in whole blood, the GSEA analysis and notable absence of transcriptomic responses to IM PanAd3-RSV boost in IN primed volunteers (as opposed to the response seen to IM PanAd3-RSV prime) indicated a reactivity to IN prime not captured by the methods used here. Similar to our observations but from influenza vaccines, the injectable inactivated influenza vaccine (TIV) induced the expression of interferon genes shortly after vaccination. In contrast, intra-nasal live-attenuated influenza vaccine (LAIV) induced interferon gene expression 7-days after vaccination and only in children less than 5-years old[445]. Therefore it cannot be discounted that mucosal responses to IN prime could have occurred with implications for the host response to boost vaccination.

8.7.3 Correlations with responses to vaccination

Systems biology approaches have computationally identified discrete genetic networks that determine immune responses to vaccination. The definition of these early transcriptional signatures has been further refined to include the characterisation of a single or a few key genes that predict antigen-specific antibody responses to polysaccharide and conjugate meningococcal and inactivated influenza vaccines, and antigen-specific antibody and CD8+ T-cell responses to live-attenuated yellow fever YF-17D vaccine[456,493,494,501]. Some gene signatures such as expression of TNFRS17 were shared across different vaccines in relation to antibody production, but for the most part these data have shown diversity in the use of genetic networks to generate immune responses to different vaccines.

At a far simpler level, the exploration of transcriptional correlates of vaccine responses to with PanAd3-RSV and MVA-RSV returned a surprisingly high rate of statistically singificant correlations. Statistical correlation cannot be assumed to have biological significance, but in the case of many probes the statistical relationships warrant further investigation as putative effectors and determinants of vaccine responses. As genes work within networks of genetic pathways and co-correlate in expression, it would be misleading to claim that any individual gene(s) identified here have sole statistical correlation to certain vaccine outcomes in a dependent manner. Instead they highlight where pathways may operate and serve as

targets for future exploration. The highest ranking correlating probe was for gene DHRS9 and the T-cell IFNγ response to IM PanAd3-RSV prime. The Pearson's r^2 value of 0.83 implied that 83% of the variability in IFNγ responses 14-days after prime (expressed as a fold-change from baseline) was attributable to gene expression changes in DHRS9 11-days earlier. The biological functions of DHRS9 are poorly characterised, having been described as a 'moonlighting' protein due to its polyfunctionality yet thought to be over-represented in the respiratory tract[486,502]. Data such as these therefore bring a new functional perspective unto genes that have yet to declare their full role in immune responses to infection or vaccination. Interestingly few probes correlated with the same phenotype response to prime and boost, suggesting that pathways could be selected by the route of prime and type of boost vector. These observations should be validated in another populations (the elderly samples, for example), functionally assessed with gene knockout models in PBMCs or animal models (as used elsewhere[493,494]), and from more in-depth statistical modelling of genetic networks.

8.7.4 General limitations

The collection of RNA from vaccinated volunteers and the analysis of the transcriptomic response to vaccination was an opportunistic project. The RSV001 clinical trial was not designed for the analysis of the host genetic responses to viral-vectored vaccines. There were only a small number of volunteers in this trial, with potential for confounding chance observations, but also the numbers were too few to support any direct comparison between groups or vaccines. Instead the analysis had to focus on within-group genetic responses compared with the individual baseline pre-vaccination expression values. Despite these limitations the analyses have been insightful and although a direct between-groups comparison was not possible, the use of an identical genetic insert in all vaccine doses allowed for some observations and commentary on the effects of route at prime (IM vs IN) and effects of vector at boost (MVA vs PanAd3). Other general limitations concern the nature of the DNA microarray and the statistics used to interrogate the data. Although all effort was made to correct for baseline heterogeneity through the *lmfit* model, it cannot be assumed that the variability and magnitude of differential gene expression was driven by vaccine alone. For example, more differentially expressed probes were detected from volunteers 3-days after IM PanAd3-RSV prime in study group 2 compared with volunteers from study group 1, when 6/10 group 2

volunteers were actually sampled 2-days after vaccination and all other volunteers were obtained 3-days after vaccination (this was permissible in the trial protocol). Adding the individual interval from vaccine to sampling, as well as other variables such as the baseline serum PanAd3 (vector) neutralising antibody titre which was also significantly different between study groups 1 and 2 volunteers, as covariates into the *lmfit* linear regression model did not significantly alter the number of differentially expressed probes following IM PanAd3-RSV prime (data not shown). Furthermore, recent research has shown that baseline gene expression from a small number of genes can be a powerful modulator of the risk of developing tuberculosis[503], and the effect of baseline gene expression on vaccine responses were not investigated in this study. Finally these data were generated using a DNA microarray and whole blood. The resulting picture of gene expression is therefore derived from a diversity of cell populations in circulation. What remains unclear is the extent to which the fold-change in gene expression in whole blood following vaccination can be influenced by relative changes in cell populations after vaccination. Changes in whole blood gene expression may represent, in part, an expansion or contraction of certain cell populations. From earlier observations neutrophils transiently dropped 3-days after vaccination, but there was no correction for this in the *lmfit* model. In other work the gene expression scores from infants infected with RSV failed to correlate the peripheral neutrophil count but did correlate with lymphocyte and monocyte cell frequencies[500]. Finally, there was no correction for multiple testing during when identifying statistical correlations between the magnitude of differential gene expression and the magnitude of pre-selected clinical/immunological responses to vaccination measured at later time points.

8.7.5 Conclusions

PanAd3-RSV and MVA-RSV viral-vectored vaccines induced differential gene expression in a "core" family of genes, with MVA inducing a greater magnitude of transcriptional changes relative to the adenovirus vector. Early transcriptional changes from several of these probes can be statistically correlated with clinical and antigen-specific immune responses. These data represent the first exploration of transcriptomic responses to viral-vectored vaccines in man with correlation to vaccine responses, and provided insight into specific genes and putative gene pathways as mechanisms of action for viral-vectored vaccination.

Chapter 9: Summary of the novel and important insights described in this thesis

9.1 Summary of the new and interesting findings resulting from this thesis

9.1.1 The epidemiology of disease

This work began with defining the problem with the intended purpose of testing a novel vaccine design for use in man. The results from HIPE, HES, ORLS and PICANet dataset analyses served to demonstrate an alarming and increasing burden on limited paediatric hospital resources from RSV infection each year. The magnitude and seasonal epidemic nature of the disease provides a commanding impetus to develop a vaccine as an international priority. Although this conclusion is easy to draw and is readily supported in the finding of others, this was the longest and most comprehensive analysis from England. Some subtle observations to concern us included the relatively static rates of bronchiolitis on PICUs in the context of rising emergency hospital admissions, which can be interpreted as there being significant social drivers behind this alarming trend and the wide range of admission rates across the country. This highlights the need to fully understand the burden of disease outside the hospital and identification of these social factors that could be addressed through vaccination. Although it is premature to make assertions on how effective an RSV vaccine would be in alleviating hospital resources it is not too early to develop treatment and referral protocols to standardise practice across the NHS, and to define the burden of disease further in the community where over 95% of cases of primary RSV infection in infants are managed.

9.1.2 *The strengths and weaknesses of natural exposure*

The requirement for an RSV vaccine to improve upon natural infection is an exceedingly high target for any vaccine. The observational studies in infants, children and paediatric healthcare workers identified some key observations in defining potential novel targets for vaccination. The first was the slow and incomplete development of immunity from the first few weeks of life through childhood (and has been reported before), with relevance to vaccine designs that target the parallel induction of antibody and cellular immunity (such as a viral-vectored vaccine). From the cohort of presumed high-exposure, highly maintained immune responses of paediatric healthcare workers there appeared to be little differences in the profile of RSV immunity with non-healthcare workers, but this included a deficiency in maintaining total and RSV-specific IgA memory B-cells in peripheral circulation. The implications of this remain unclear and worthy of pursuit as to whether this can be related to repeated infections throughout life and potentially a new target for vaccine immunogenicity.

9.1.3 *The RSV001 clinical trial*

It is difficult to articulate or understate the enormous amount of work and effort required to prepare, setup and then execute a phase 1 vaccine trial for RSV. After all documents were prepared and regulatory approval from the required agencies were in place, over 30'000 members of the public were contacted with 180 physician screened in order to enrol 72 suitable volunteers. Analysis of the recruitment showed a real willing from older adults to engage with clinical trials in particular. The tight management of the trial and resulting data integrity supported meaningful exploitation of the phase 1 trial data.

PanAd3-RSV and MVA-RSV were safe and well tolerated in this population, with reported adverse event frequencies comparable to other trials of adenovirus and MVA-vectored vaccine. A new observation came from the differential rate of haemoglobin drop associated with MVA-RSV that has not been reported in other MVA vectored vaccine trials. Although this was not clinically significant in the study population used here, confirmation of this finding and developing an understanding of this the mechanism behind this has already started with new animal (rabbit) experiments and enhanced surveillance in future phase 1 RSV viral vectored clinical trials (data unpublished).

Despite the limitations placed by pre-existing RSV immunity and higher than anticipated titres of PanAd3 vector neutralising antibody, IM PanAd3-RSV and IM MVA-RSV were capable of eliciting a robust induction of both humoral and cellular antigen-specific immunity. For serum RSV-neutralising antibody, F-specific antibody-secreting cells and IFNγ T-cell responses these were dependent on the first dose of IM vaccine. The boost vaccine was required for an immune response in volunteers primed by the IN route, and for T-cell IFNγ-producing cells the greatest responses after boost (an irrespective of the route of prime) were specific for the MVA vector. MVA alone, possibly supported by natural priming, was sufficient to generate immune responses in older adults who, as a cohort, recorded lower baseline and post-vaccination IFNγ responses. CD4+ and CD8+ responses appeared balanced after boost in younger adults without signals of Th2-assocaited cytokine activation.

More detailed exploratory analysis sought to provide the first description of the host genetic response to viral-vectored vaccines in man. From over 20'000 probes less than 600 were differentially expressed, and nearly 60 of these could be characterised as members of a 'core' set of genes responsive to each dose of vaccine. At a statistical level a handful of these differentially expressed genes could be correlated with important clinical and immune responses to vaccination measured at later time points. These data provide a new insight into the genetic pathways used by viral-vectored vaccines and provide direction on where genetic networks can be targeted for improved responses to vaccination.

9.2 Future directions

Based upon these data from RSV001[1], GlaxoSmithKline (GSK), who acquired PanAd3-RSV and MVA-RSV during the trial, have commission another phase 1 vaccine trial on a paediatric development pathway (EudraCT number 2014-005333-31). Chimpanzee-adenovirus 155 (ChAd155) has replaced PanAd3 as the adenovirus vector (for reasons of manufacturing productivity) and uses the same single codon-optimised DNA insert. ChAd155-RSV has been employed in a homologous double-prime dosing schedule by IM injection one-month apart and the results of this trial are expected within the next year.

The burden of clinical disease gives clear definition of target populations for vaccination and how different vaccine strategies could be needed to meet this aim. Infants are the population with the most to

gain from an effective vaccine. However, an effective RSV vaccine would be required to protect from the first weeks of life, meaning there would be insufficient time for more than one dose of vaccine and this single dose would need to succeed in the presence of maternal antibody and in the context of an immature immune system. This, combined with concerns about immunopathology from subunit vaccine in RSV-naïve infants, has focussed development on maternal vaccination. Alternative options would consider the value of intra-nasal immunisation, which does not appear to suffer from maternal antibody or induce vector immunity, or the immunisation of siblings who act as principal importers of the virus.

For maternal vaccination it remains unknown when would be the best time in pregnancy to vaccinate, and what order of immune response to vaccination in pregnancy would be required to confer meaningful protection in the infant. Active antibody transfer begins after 32 weeks gestation, but maternal immunisation with acellular pertussis vaccine in the second trimester has been associated with greater antigen-specific cord antibody titres compared with mothers vaccinated in the third trimester[504]. Although pregnant women are easily accessed as part of routine prenatal care, factors around the limitations of mother's ability to boost antibody have been only partly explored. Several studies have shown that maternal vaccination does not readily conform to predictable cord antibody levels; HIV, malaria and diseases of the placenta can reduce the capacity for active transportation of antibody and competition for placental Fc receptors from high maternal antibody can reduce the transportation of antigen-specific antibody[505-507]. Maternal vaccines for diseases such as influenza have been argued on the basis of protection for the mother from enhanced disease as well as protection for the new born but in developing an RSV vaccine for infants through maternal immunisation this argument cannot be applied from existing data[508]. It is reasonable to expect that the medical community would have detected an increased RSV severity of disease associated with pregnancy, and therefore even without the explicit observational data needed to confirm this assertion it seems unlikely that "redundant" immune responses induced in the mother will be an obstacle to introducing a maternal RSV vaccine program. The ethical principal of vaccinating one individual, who does not directly benefit from vaccination, in order to protect another individual cannot constitute an obstacle to the development and licensure of a maternal RSV vaccine. Pertussis vaccine is integrated into the maternal vaccination programme in the United Kingdom on this basis. For RSV the benefits of maternal vaccination would need to be powered from data in infants and the incidence of severe

disease. Maternal antibody alone has not been associated with enhanced RSV disease and despite the inexorable decline in maternal antibody, and whose kinetics may be altered by maternal vaccination, it is clear that even a small nudge towards an older age in disease susceptibility could translate into a large change in disease burden in hospital admissions and the later development of wheeze.

Another consideration would be targeted vaccine development as transmission blocking vaccines for sero-positive siblings. Intra-nasal live-attenuated influenza vaccine (LAIV) offers little benefit to the vaccinated child but confers a desirable reduction in influenza shedding and risk reduction to vulnerable members of the community such as the elderly. For the targeted protection of infants through school children immunisation, additional thought could be applied to the design of vaccines as barriers to transmission more than barriers to severe disease, as well as the recognition that young children (under 5-years) infected with RSV continue to exert a significant demand on healthcare services and society. While reducing the period of viral shedding would be desirable in reducing transmission to infants and the elderly, it remains to be seen how this could be optimally achieved when considering the options of intra-nasal immunisation, use of G-protein antigen, and delivery by subunit vaccines in a population that enjoys natural protection from ERD. In the pursuit of protecting infants in the first 6-months of life the focus so far has been, understandably, a race towards direct immunisation of the new-born. With an increasing recognition that this may not be achievable using live attenuated vaccines it is worth exploring alternative strategies to meet this aim.

The modelling and assumptions placed in existing epidemiological models of RSV vaccine impact remain underdeveloped and lacking in critical data. Understanding the full burden of disease, in infants and the elderly, is needed to inform decisions on the cost-effectiveness of implementing an RSV vaccine should one become available in the future. With increasing cumulative evidence for causality between RSV-bronchiolitis and wheeze in later years, trial data expected later this year may result in the notion that the first effective RSV vaccine for infants could also become the first vaccine for the prevention of wheeze or even asthma. It is also with considerable excitement that the future RSV vaccines will draw upon significant advances in understanding the structural biology of the pre-fusion F-trimer and the identification of powerful new antigenic sites for antibody-mediated viral neutralisation. This year a promising nanoparticle vaccine[331] entered phase III clinical trials as a maternal vaccine candidate for RSV, which marks only the

second vaccine design to reach this stage and major milestone. A final noteworthy development has been the creation of F-specific monoclonal antibody (motavizumab-YTE, MedImmune) with a higher affinity and 2 to 4-fold extension of half-life compared to palivizumab, giving hope for passive immune prophylaxis that spans the winter months with a single dose[509].

The challenges for vaccination of adults are going to be different. Adults of working age do not need an RSV vaccine unless undergoing severe immune suppression or are employed in the provision of care to vulnerable populations (hospital patients, care homes and the elderly). For the former the requirement may be in the restoration of cellular immunity as a priority. For the latter population the main objective will be the prevention of viral transmission and nosocomial/institutional outbreaks of disease. The low (5-10%) infection rate in naturally primed adults and the elderly is a major obstacle in evaluating new vaccines and the scale of clinical trials that would be needed to demonstrate protective efficacy. In these circumstances the use of a human challenge model for RSV in healthy adults has several advantages that give might a proof-of-concept indication of which vaccine or therapeutics have better potential and would be worthy of further investment. Already novel oral cytidine nucleoside analogues have shown promise in reducing RSV viral load and disease symptoms in proof-of-concept healthy adult challenge models with low pre-existing serum RSV-neutralising antibody titre[119]. Inhaled RSV-specific short interfering RNA (si-RNA) inhibitors are also entering critical stages of evaluation and new therapeutics will continue to be required to augment any vaccine prevention strategies[510,511]. However the results of phase III trials of the promising RSV-nanoparticle vaccine were very disappointing (article in press), and motavizumab and siRNA development has also been discontinued. The field for developing novel vaccine and therapeutic interventions for the protection from severe RSV disease has expanded dramatically in recent years and has never been so rich, and remains an exciting and unpredictable challenge for the future.

9.3 Final comment

The development of a safe and effective vaccine to prevent severe disease caused by RSV is a major global health priority. RSV vaccine development continues to suffer from an incomplete understanding of protective immunity and destructive immunopathology. Viral-vectored vaccines for RSV have now successfully transitioned from pre-clinical models into man. Encouragingly PanAd3-RSV and MVA-RSV safely expanded desirable RSV humoral and cellular immunity in healthy younger and older adults. This heralds a new and exciting dimension to the pursuit of developing an RSV vaccine that has eluded the medical and scientific community for over 60-years.

Appendix A: Supplementary methods

	Adverse event	Severity grade	Definition
Systemic adverse events	Oral temperature	0	< 37.6 °C
		1	37.6 – 38.0 °C
		2	38.1 – 39.0 °C
		3	> 39.0 °C
	Headache, nausea and/or vomiting, malaise, myalgia and arthralgia and any other systemic symptom	0	Absence or resolution of symptom
		1	Awareness of symptom but tolerated; transient or mild discomfort, little/no medical intervention
		2	Discomfort enough to cause limitation of usual activity; some medical intervention or therapy required
		3	Incapacitating, absent from work or bed rest required; hospitalisation possible
Local adverse events after IM injection	Tenderness/pain at the site of injection	0	None
		1	Painful to touch; easily tolerated
		2	Painful when the limb is moved; interferes with daily activity
		3	Severe pain at rest; prevents daily activity
	Redness and induration at the site of injection	0	0 – 2 mm maximal diameter reaction
		1	3 – 50 mm maximal diameter reaction
		2	51 – 100 mm maximal diameter reaction
		3	> 100 mm maximal diameter reaction
	Swelling at the site of injection	0	No visible reaction
		1	1 – 20 mm maximal diameter reaction
		2	21 – 50 mm maximal diameter reaction
		3	> 50 mm maximal diameter reaction
Local adverse events after IN spray	Nasal tenderness/pain	0	None
		1	Painful on touch only and minimal
		2	Painful without touch, moderate severity
		3	Painful without touch, severe, interferes with smell
	Nasal irritation	0	None
		1	Mild irritation only
		2	Moderate irritation, distracting
		3	Severe irritation, disturbs rest or interferes with normal activity
	Nasal discharge	0	None
		1	Mild discharge
		2	Moderate discharge, readily controlled
		3	Severe discharge, interferes with normal activity

Supplementary table 1. Adverse event grading criteria for solicited and unsolicited adverse events after vaccination.
Adverse events were graded as 0 (normal), 1 (mild), 2 (moderate) or 3 (severe) from the recorded outcome measures in this table.

	Adverse event	Severity grade	Definition
Systemic adverse events	Oral temperature	0	< 37.6 °C
		1	37.6 – 38.0 °C
		2	38.1 – 39.0 °C
		3	> 39.0 °C
	Headache, nausea and/or vomiting, malaise, myalgia and arthralgia and any other systemic symptom	0	Absence or resolution of symptom
		1	Awareness of symptom but tolerated; transient or mild discomfort, little/no medical intervention
		2	Discomfort enough to cause limitation of usual activity; some medical intervention or therapy required
		3	Incapacitating, absent from work or bed rest required; hospitalisation possible
Local adverse events after IM injection	Tenderness/pain at the site of injection	0	None
		1	Painful to touch; easily tolerated
		2	Painful when the limb is moved; interferes with daily activity
		3	Severe pain at rest; prevents daily activity
	Redness and induration at the site of injection	0	0 – 2 mm maximal diameter reaction
		1	3 – 50 mm maximal diameter reaction
		2	51 – 100 mm maximal diameter reaction
		3	> 100 mm maximal diameter reaction
	Swelling at the site of injection	0	No visible reaction
		1	1 – 20 mm maximal diameter reaction
		2	21 – 50 mm maximal diameter reaction
		3	> 50 mm maximal diameter reaction
Local adverse events after IN spray	Nasal tenderness/pain	0	None
		1	Painful on touch only and minimal
		2	Painful without touch, moderate severity
		3	Painful without touch, severe, interferes with smell
	Nasal irritation	0	None
		1	Mild irritation only
		2	Moderate irritation, distracting
		3	Severe irritation, disturbs rest or interferes with normal activity
	Nasal discharge	0	None
		1	Mild discharge
		2	Moderate discharge, readily controlled
		3	Severe discharge, interferes with normal activity

Supplementary table 2. Adverse event grading criteria for solicited and unsolicited adverse events after vaccination.
Adverse events were graded as 0 (normal), 1 (mild), 2 (moderate) or 3 (severe) from the recorded outcome measures in this table.

	Adverse event	Severity grade	Definition
Systemic adverse events	Oral temperature (°C)	0	< 37.6
		1	37.6 – 38.0
		2	38.1 – 39.0
		3	> 39.0
Heart rate (beats/minute)	Tachycardia	0	55 – 100
		1	101 – 115
		2	116 – 130
		3	> 130
	Bradycardia	0	55 – 100
		1	50 – 54
		2	45 – 49
		3	< 45
Blood pressure (mmHg)	Systolic hypotension	0	90 – 140
		1	85 – 89
		2	80 – 84
		3	< 80
	Systolic hypertension	0	90 – 140
		1	141 – 150
		2	151 – 155
		3	> 155
	Diastolic hypertension	0	< 91
		1	91 – 95
		2	96 – 100
		3	> 100
Respiratory rate (breaths/minute)	Tachyopnea	0	< 17
		1	17 – 20
		2	21 – 25
		3	> 25

Supplementary table 3. Adverse event grading criteria for visit observations collected at every visit. *Adverse events were graded as 0 (normal), 1 (mild), 2 (moderate) or 3 (severe) from the recorded outcome measures in this table.*

	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 (Emergency)
Haemoglobin change from baseline (g/dL)	< 1.5	1.5 – 2.0	2.1 – 5.0	> 5
Total white cell count (x10^9/L): elevated	10.8 – 15.0	15.1 – 20.0	20.1 – 25.0	>25.0
Total white cell count (x10^9/L): depressed	2.5 – 3.5	1.5 – 2.4	1.0 – 1.4	< 1.0
Neutrophil count (x10^9/L)	1.5 – 2.0	1.0 – 1.4	0.5 – 0.9	< 0.5
Platelet count (x10^9/L)	125 - 140	100 - 124	25 - 99	< 25
Sodium (mmol/L): hyponatraemia	132 - 134	130 - 131	125 - 129	< 125
Sodium (mmol/L): hypernatraemia	144 - 145	146 - 147	148 - 150	> 150
Potassium (mmol/L): hypokalaemia	3.5 – 3.6	3.3 – 3.4	3.1 – 3.2	< 3.1
Potassium (mmol/L): hyperkalaemia	5.1 – 5.2	5.3 – 5.4	5.5 – 5.6	> 5.6
Urea (mmol/L)	8.2 – 8.9	9.0 - 11	> 11	RRT
Creatinine (umol/L)	132 - 150	151 - 176	177 - 221	>221 or RRT
ALT (IU/L)	1.1 – 2.5 x ULN	2.6-5.0 x ULN	5.1 – 10 x ULN	> 10 x ULN
Bilirubin (umol/L): with increased ALT	1.1 – 1.25 x ULN	1.26-1.5 x ULN	1.51-1.75 x ULN	> 1.75 x ULN
Bilirubin (umol/L): with normal ALT/AST	1.1 – 1.5 x ULN	1.6 – 2.0 x ULN	2.0-3.0 x ULN	> 3.0 x ULN
Alkaline phosphatase (IU/L)	1.1 – 2.0 x ULN	2.1 – 3.0 x ULN	3.1 – 10 x ULN	> 10 x ULN
Amylase (IU/L)	1.1 – 1.5 x ULN	1.6 – 2.0 x ULN	2.1 – 5.0 x ULN	> 5.0 x ULN
Albumin (g/L): hypoalbuminaemia	28 - 31	25 - 27	< 25	n/a
C-reactive protein (mg/L)	11 - 30	31 - 100	101 - 200	> 200

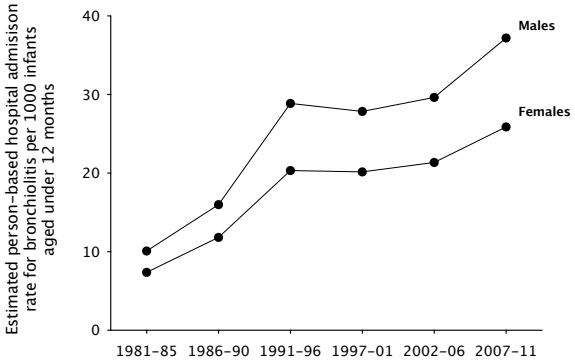
Supplementary table 4. Modified FDA and DAIDs criteria for adverse event grading criteria for haematology and biochemistry blood results measured at every scheduled visit.
Adverse events were graded as 0 (normal), 1 (mild), 2 (moderate) or 3 (severe) from the recorded outcome measures in this table. Renal replacement therapy (RRT), upper limit of normal (ULN), alanine transaminase (ALT), not applicable (n/a). The ULN for ALT, bilirubin, alkaline phosphatase and amylase were derived form the Oxford University Hospitals NHS Trust criteria, and were 45IU/ml, 17umol/L, 115IU/L and 125IU/L respectively.

Appendix B: Supplementary results

Supplementary results for the burden of emergency hospital admissions with bronchiolitis

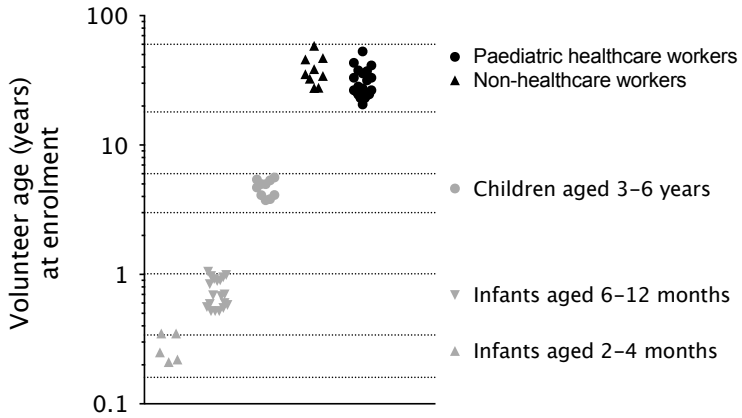
	Hospital In-Patient Enquiry (HIPE) and Hospital Episode Statistics (HES)		Oxford Record Linkage Study (ORLS)	
	Episode-based rate	Person-based rate	Episode-based rate	Person-based rate
1979	6.61 (5.95 - 7.27)	No data	4.52 (3.72 - 5.32)	4.12 (3.36 - 4.88)
1980	6.98 (6.31 - 7.65)	No data	6.04 (5.14 - 6.95)	5.58 (4.71 - 6.46)
1981	7.88 (7.18 - 8.59)	No data	5.44 (4.58 - 6.31)	4.87 (4.05 - 5.69)
1982	13.87 (12.92 - 14.83)	No data	10.07 (8.87 - 11.27)	9.40 (8.25 - 10.5)
1983	6.96 (6.29 - 7.63)	No data	6.26 (5.32 - 7.21)	5.70 (4.80 - 6.61)
1984	12.08 (11.19 - 12.96)	No data	9.73 (8.56 - 10.9)	8.77 (7.65 - 9.89)
1985	16.90 (15.86 - 17.94)	No data	15.25 (13.8 - 16.70)	14.4 (13.0 - 15.8)
1986	No data	No data	13.07 (11.75 - 14.40)	11.9 (10.6 - 13.2)
1987	No data	No data	11.28 (10.06 - 12.50)	10.5 (9.41 - 11.7)
1988	No data	No data	20.98 (19.36 - 22.61)	19.3 (17.8 - 20.9)
1989	No data	No data	16.15 (14.72 - 17.58)	14.5 (13.2 - 15.9)
1990	15.78 (15.47 - 16.09)	No data	12.83 (11.56 - 14.11)	11.5 (10.3 - 12.7)
1991	30.50 (30.08 - 30.93)	No data	32.36 (30.51 - 34.21)	30.3 (28.5 - 32.1)
1992	17.21 (16.89 - 17.53)	No data	18.10 (16.72 - 19.48)	15.8 (14.5 - 17.1)
1993	32.10 (31.65 - 32.54)	No data	26.88 (25.16 - 28.60)	24.8 (23.1 - 26.4)
1994	35.50 (35.03 - 35.96)	No data	34.34 (32.39 - 36.30)	31.8 (29.9 - 33.7)
1995	27.88 (27.46 - 28.30)	No data	21.84 (20.29 - 23.40)	19.5 (18.1 - 21.0)
1996	32.16 (31.71 - 32.61)	No data	25.93 (24.21 - 27.65)	23.2 (21.6 - 24.9)
1997	37.16 (36.68 - 37.64)	No data	27.37 (25.63 - 29.11)	24.6 (22.9 - 26.2)
1998	34.93 (34.46 - 35.41)	No data	27.73 (25.97 - 29.49)	25.1 (23.4 - 26.7)
1999	35.10 (34.63 - 35.58)	31.27 (30.82 - 31.72)	25.93 (24.22 - 27.63)	23.9 (22.3 - 25.5)
2000	34.35 (33.87 - 34.83)	29.75 (29.30 - 30.19)	28.25 (26.46 - 30.04)	24.8 (23.1 - 26.5)
2001	35.08 (34.59 - 35.58)	29.79 (29.34 - 30.24)	27.43 (25.65 - 29.22)	23.2 (21.6 - 24.9)
2002	37.84 (37.33 - 38.35)	32.19 (31.72 - 32.67)	34.58 (32.55 - 36.61)	29.0 (27.1 - 30.8)
2003	27.64 (27.21 - 28.07)	23.54 (23.14 - 23.94)	18.89 (17.42 - 20.36)	16.3 (14.9 - 17.7)
2004	40.22 (39.71 - 40.73)	33.86 (33.39 - 34.33)	35.88 (33.90 - 37.87)	30.8 (29.0 - 32.7)
2005	33.96 (33.49 - 34.42)	28.59 (28.17 - 29.02)	25.43 (23.77 - 27.10)	21.9 (20.3 - 23.4)
2006	31.88 (31.43 - 32.32)	26.69 (26.28 - 27.10)	28.89 (27.13 - 30.64)	24.3 (22.7 - 25.9)
2007	40.17 (39.68 - 40.66)	33.61 (33.16 - 34.06)	30.02 (28.28 - 31.77)	25.5 (23.9 - 27.1)
2008	38.38 (37.91 - 38.85)	31.52 (31.09 - 31.94)	30.88 (29.15 - 32.61)	26.4 (24.8 - 28.0)
2009	39.10 (38.63 - 39.58)	32.30 (31.86 - 32.73)	31.95 (30.19 - 33.72)	27.3 (25.7 - 28.9)
2010	43.23 (42.73 - 43.72)	35.10 (34.65 - 35.54)	39.52 (37.58 - 41.47)	32.2 (30.5 - 34.0)
2011	46.11 (45.60 - 46.62)	37.18 (36.73 - 37.64)	40.36 (38.42 - 42.31)	33.0 (31.3 - 34.8)

Supplementary table 5. Annual average episode- and person-based bronchiolitis admission rates per 1000 infants aged less than 12 months.
Data from HIPE, HES and ORLS datasets between 1979 and 2011 (95% confidence intervals). There are no all-England data for 1986-89 and HIPE and HES linked data was collected from 1999 onwards.

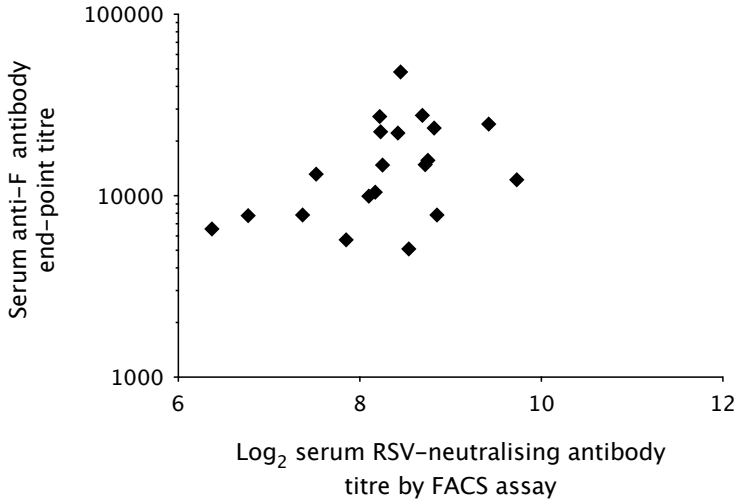


Supplementary figure 1. Male and female person-based bronchiolitis admission rates.
Infants aged less than 12 months.

Supplementary results for the analysis of immune responses to natural RSV exposure

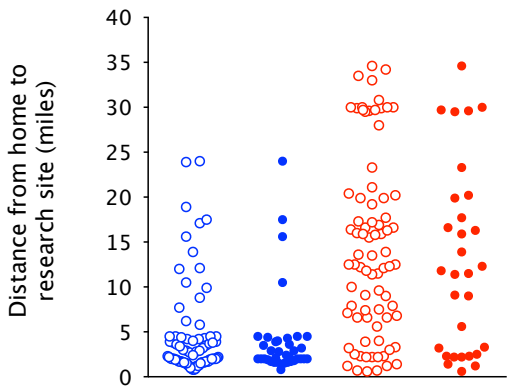


Supplementary figure 2. The age distribution of volunteers enrolled into the paediatric healthcare workers study and infant and children study.
Age was not treated as a continuous variable in the analysis of the immune response to natural RSV exposure.



Supplementary figure 3. Correlation between serum F-specific antibody titres and serum RSV-neutralising antibody titres in paediatric healthcare workers at V2.
A two-tailed Spearman's correlation test; p=0.044, r=0.45.

Supplementary results for the analysis of recruitment to the RSV001 clinical trial

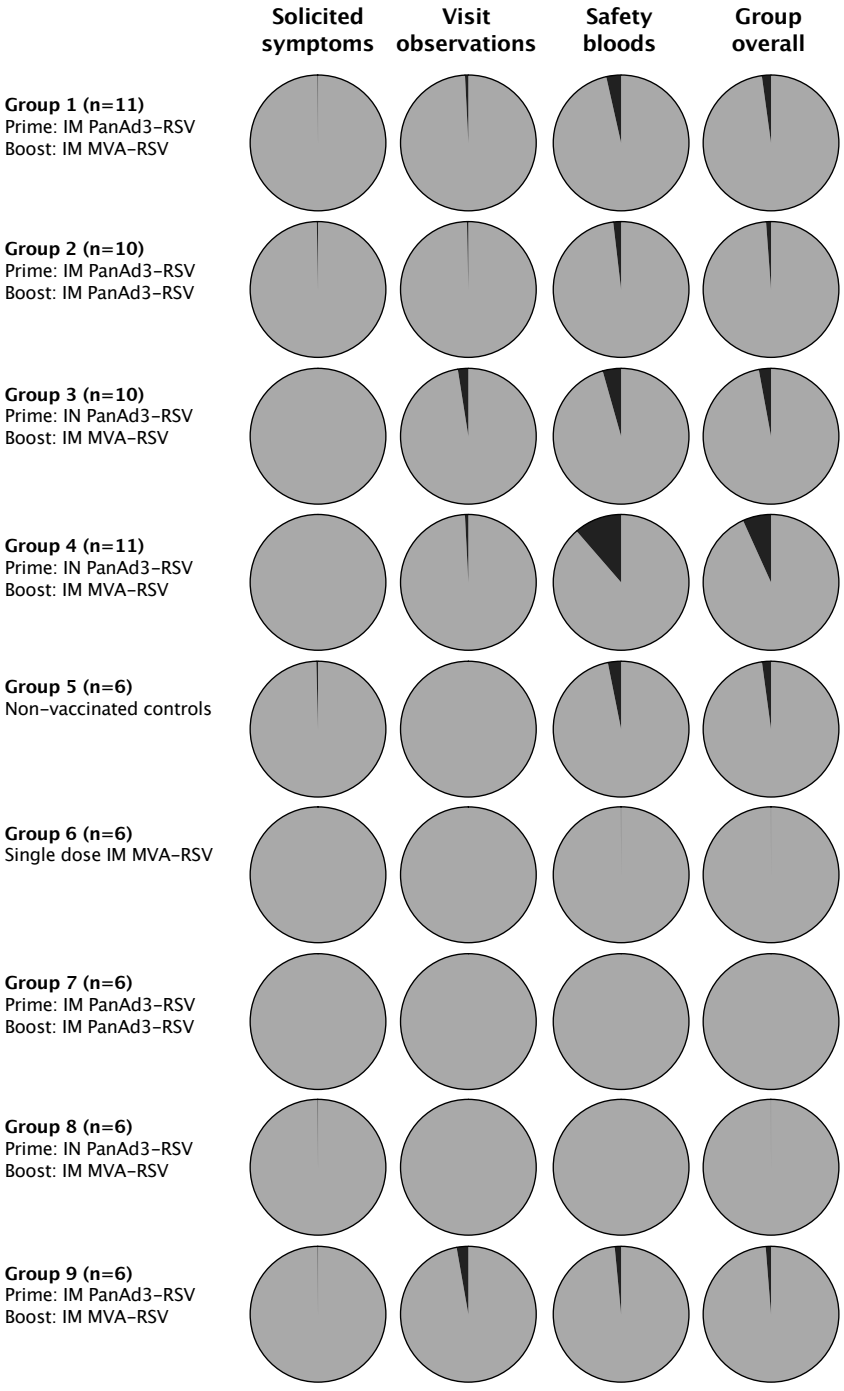


Supplementary figure 4. Scatter plot of the distance from the research site and homes of potential volunteers at screening and for volunteers enrolled into the trial.
The distance was calculated, in miles, using the postcode addresses and publicly available software.

Healthy adults aged 18–50 years:
- ○ Screened
- ● Enrolled

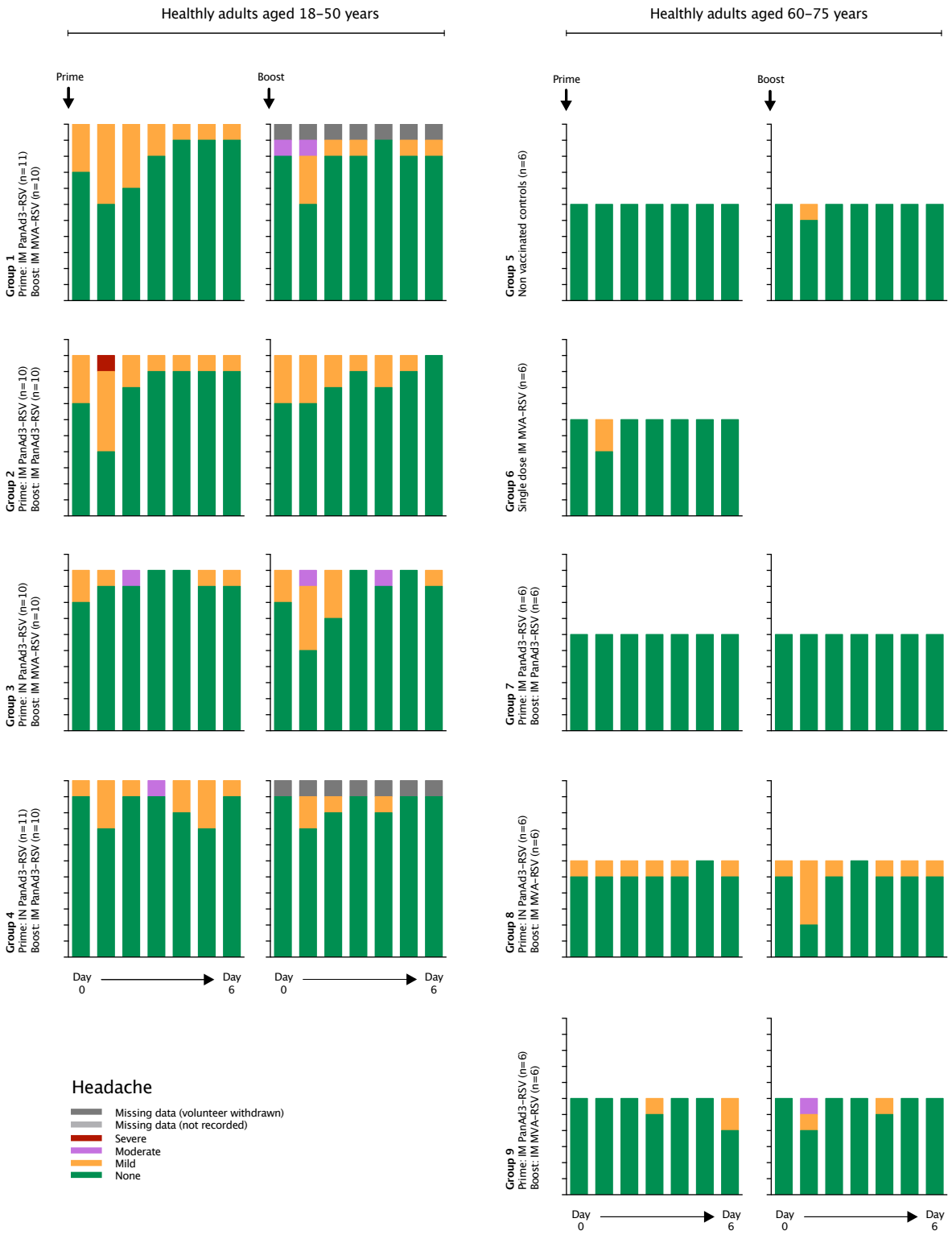
Healthy adults aged 60–75 years:
- ○ Screened
- ● Enrolled

Supplementary results for the analysis of the clinical response to PanAd3-RSV and MVA-RSV

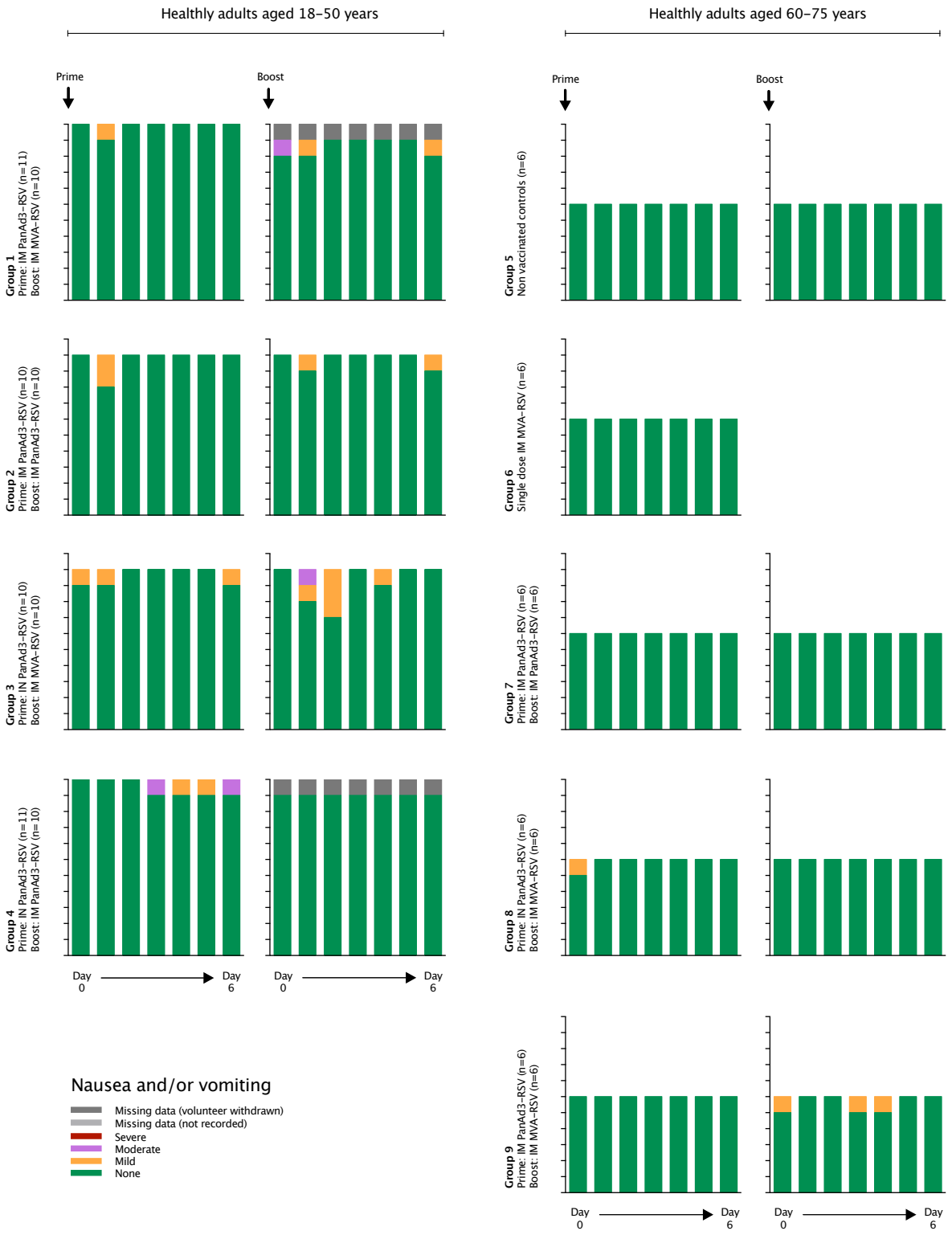


Supplementary figure 5. Frequency pie-chart representation of the safety data integrity RSV001.
Each study group is presented overall and sub-divided by the three sources of safety data in this pie chart frequency plot. Grey areas represented the proportion of data collected for analysis. Black areas represent proportion of missing data. There was a disproportionate loss of blood safety data for group 4 due to laboratory error on a specific week of the trial.

Solicited symptoms after vaccination

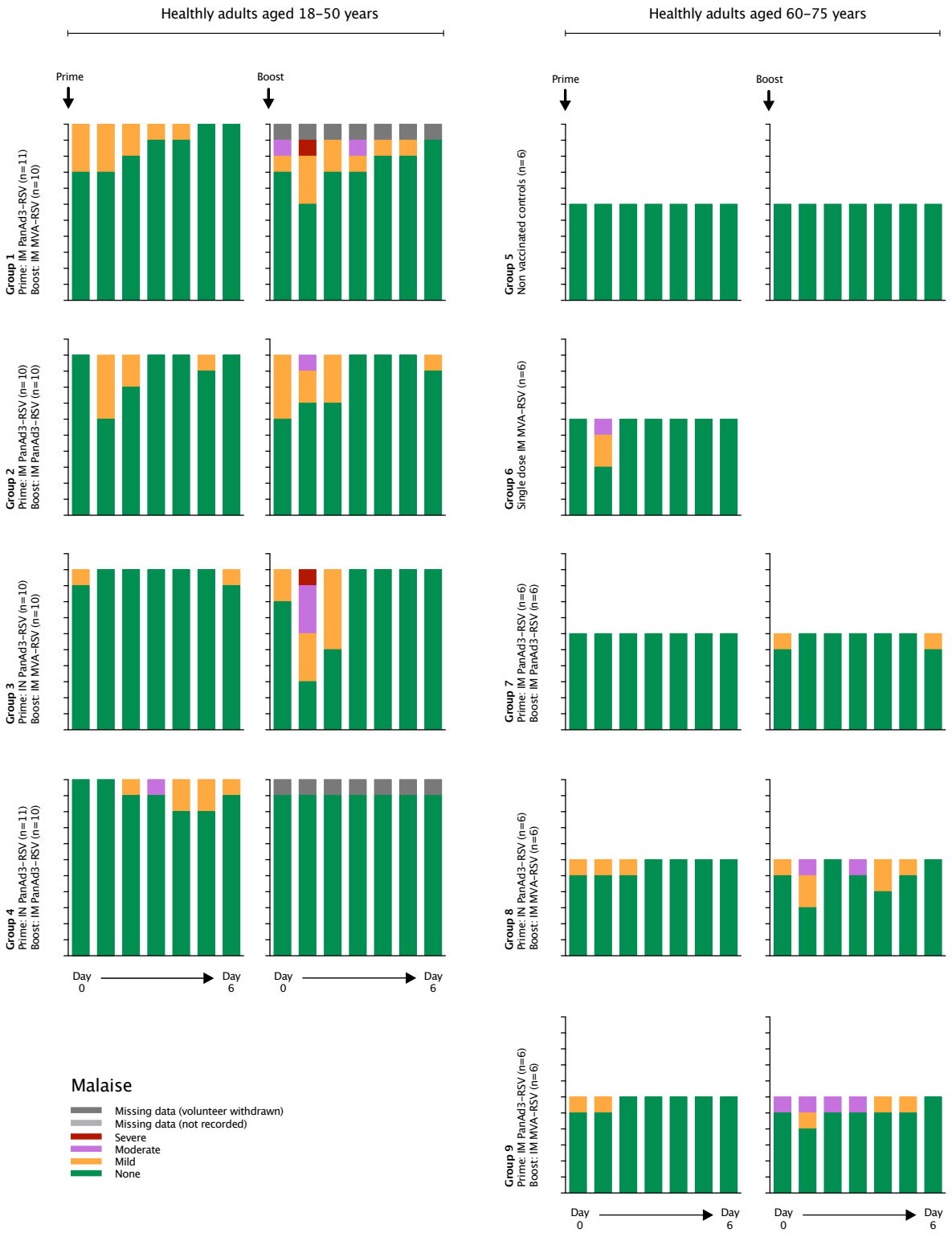


Supplementary figure 6. Solicited symptoms by severity grade for each day after vaccination; headache. *The number of volunteers is recorded on the y-axis, and each column represents one day after vaccination. Volunteers reported the worst severity grade every evening for one week after vaccination, with day 0 being the same day of vaccination.*

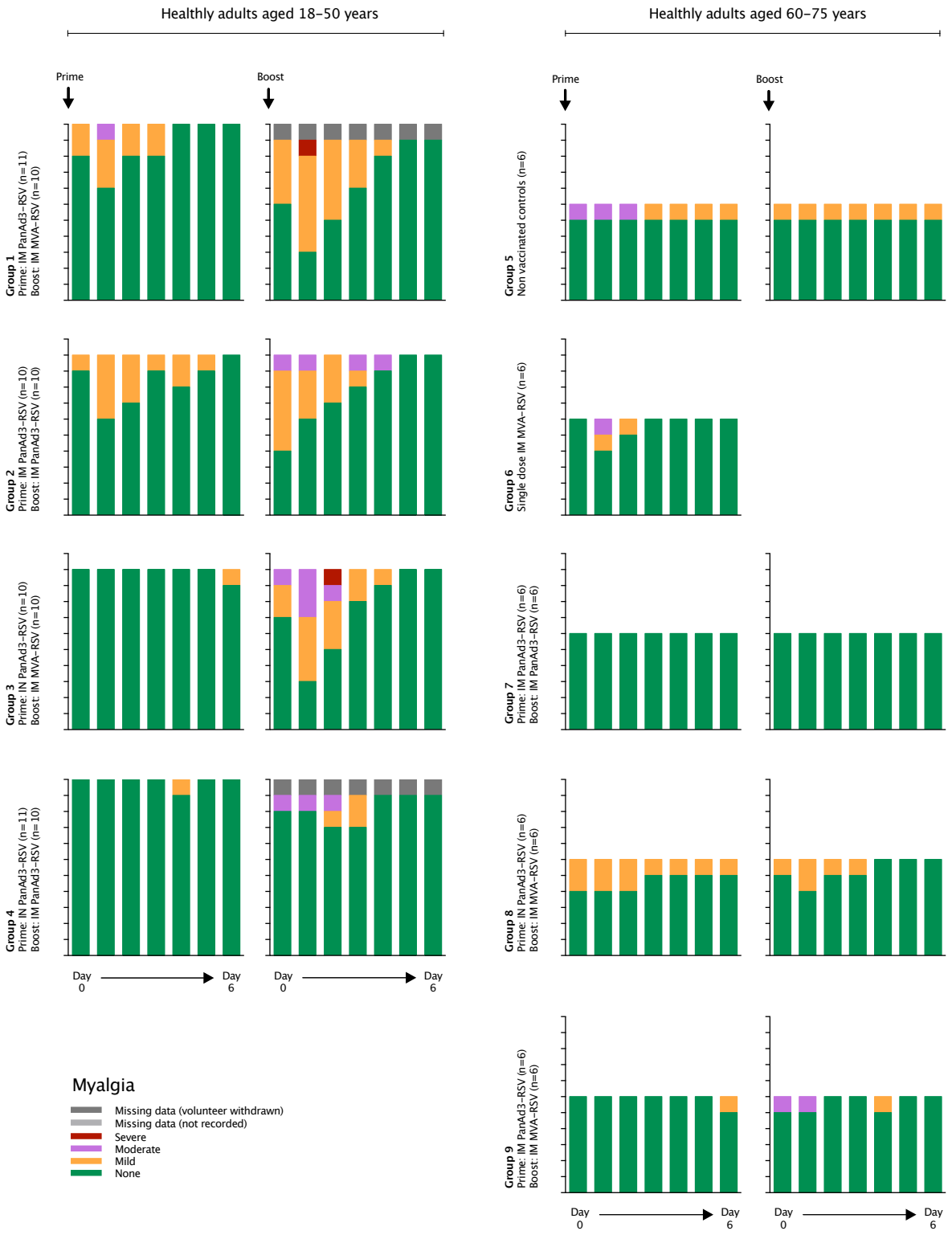


Supplementary figure 7. Solicited symptoms by severity grade for each day after vaccination; nausea and/or vomiting.

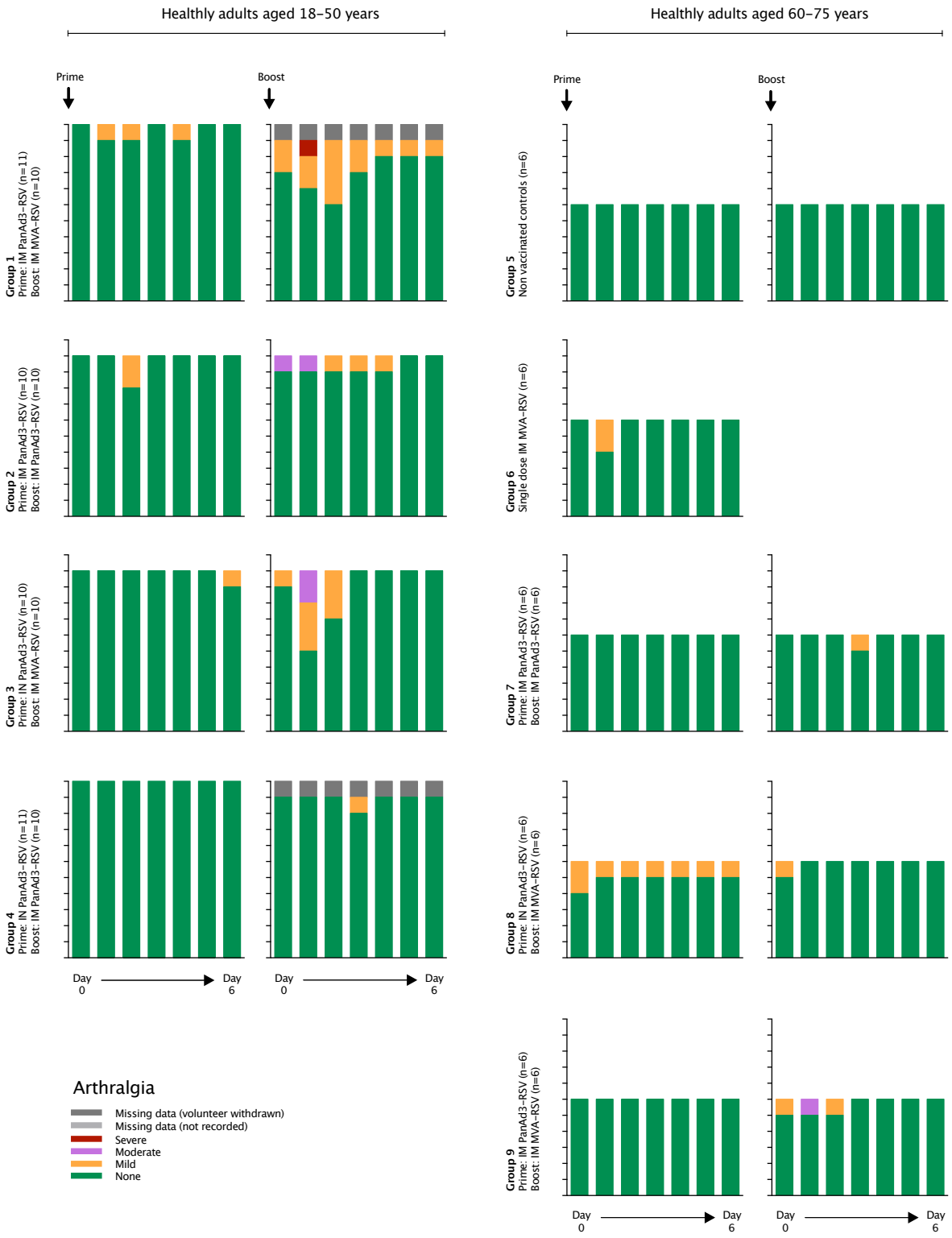
The number of volunteers is recorded on the y-axis, and each column represents one day after vaccination. Volunteers reported the worst severity grade every evening for one week after vaccination, with day 0 being the same day of vaccination.



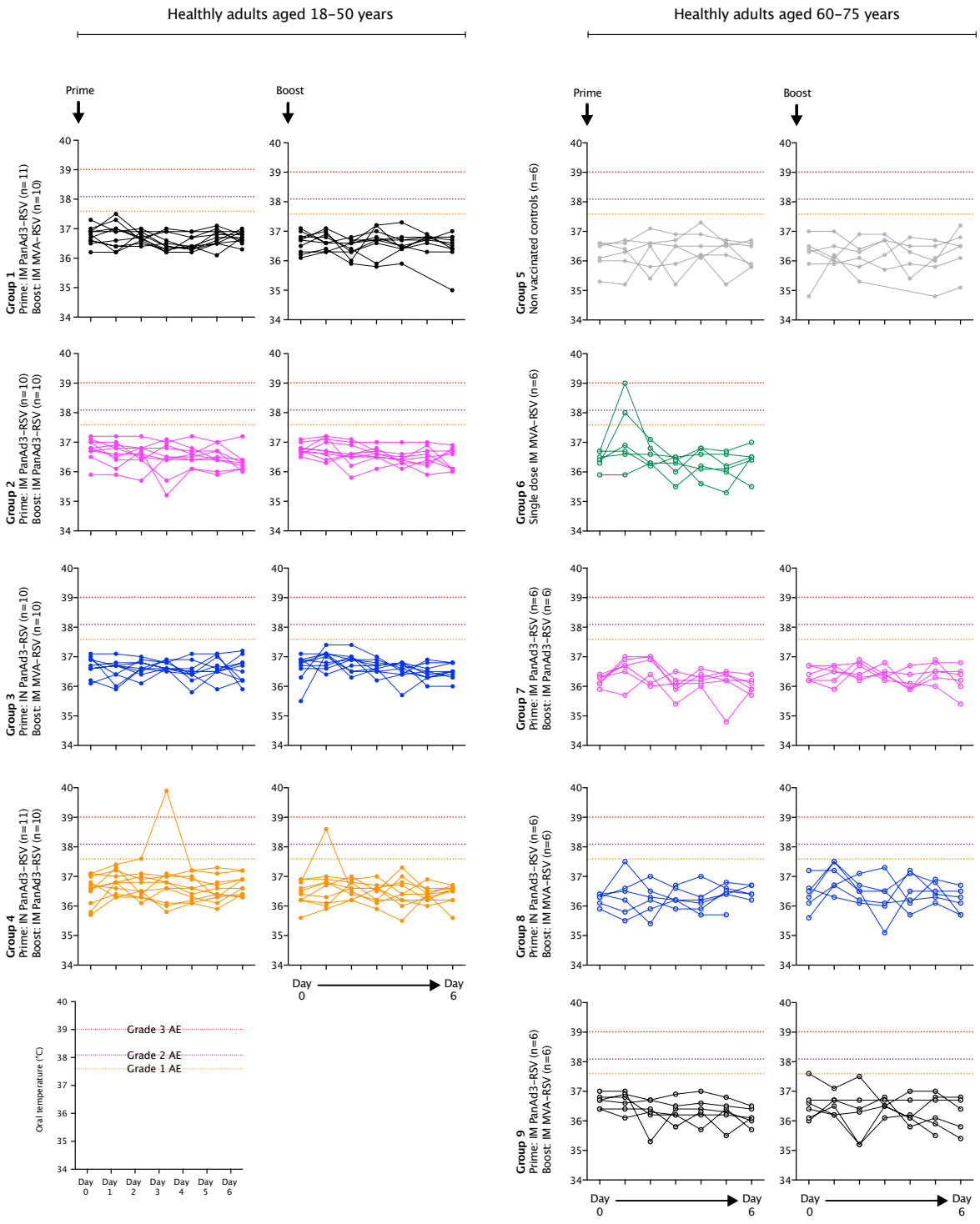
Supplementary figure 8. Solicited symptoms by severity grade for each day after vaccination; malaise.
The number of volunteers is recorded on the y-axis, and each column represents one day after vaccination. Volunteers reported the worst severity grade every evening for one week after vaccination, with day 0 being the same day of vaccination.



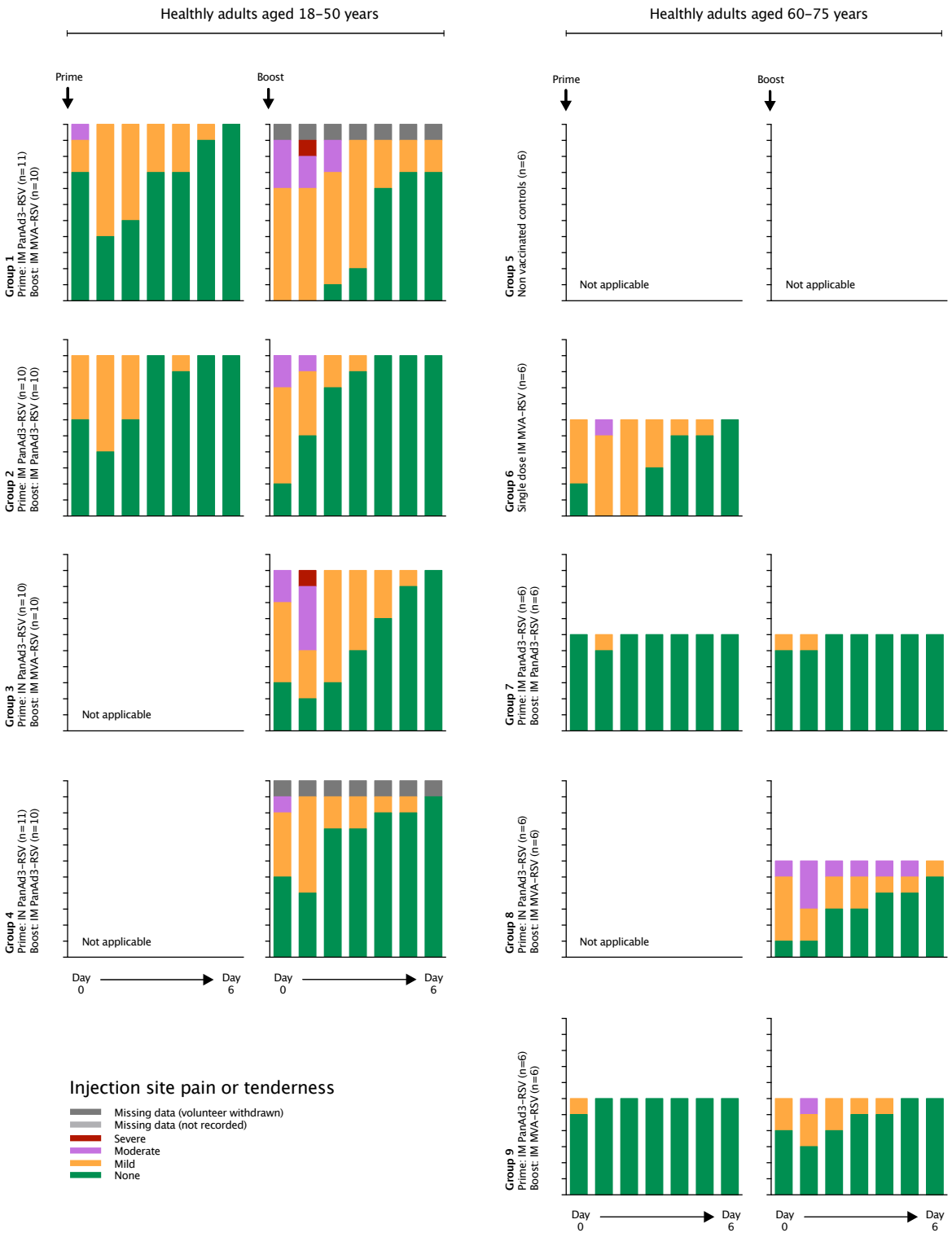
Supplementary figure 9. Solicited symptoms by severity grade for each day after vaccination; myalgia.
The number of volunteers is recorded on the y-axis, and each column represents one day after vaccination. Volunteers reported the worst severity grade every evening for one week after vaccination, with day 0 being the same day of vaccination.



Supplementary figure 10. Solicited symptoms by severity grade for each day after vaccination; arthralgia. *The number of volunteers is recorded on the y-axis, and each column represents one day after vaccination. Volunteers reported the worst severity grade every evening for one week after vaccination, with day 0 being the same day of vaccination.*

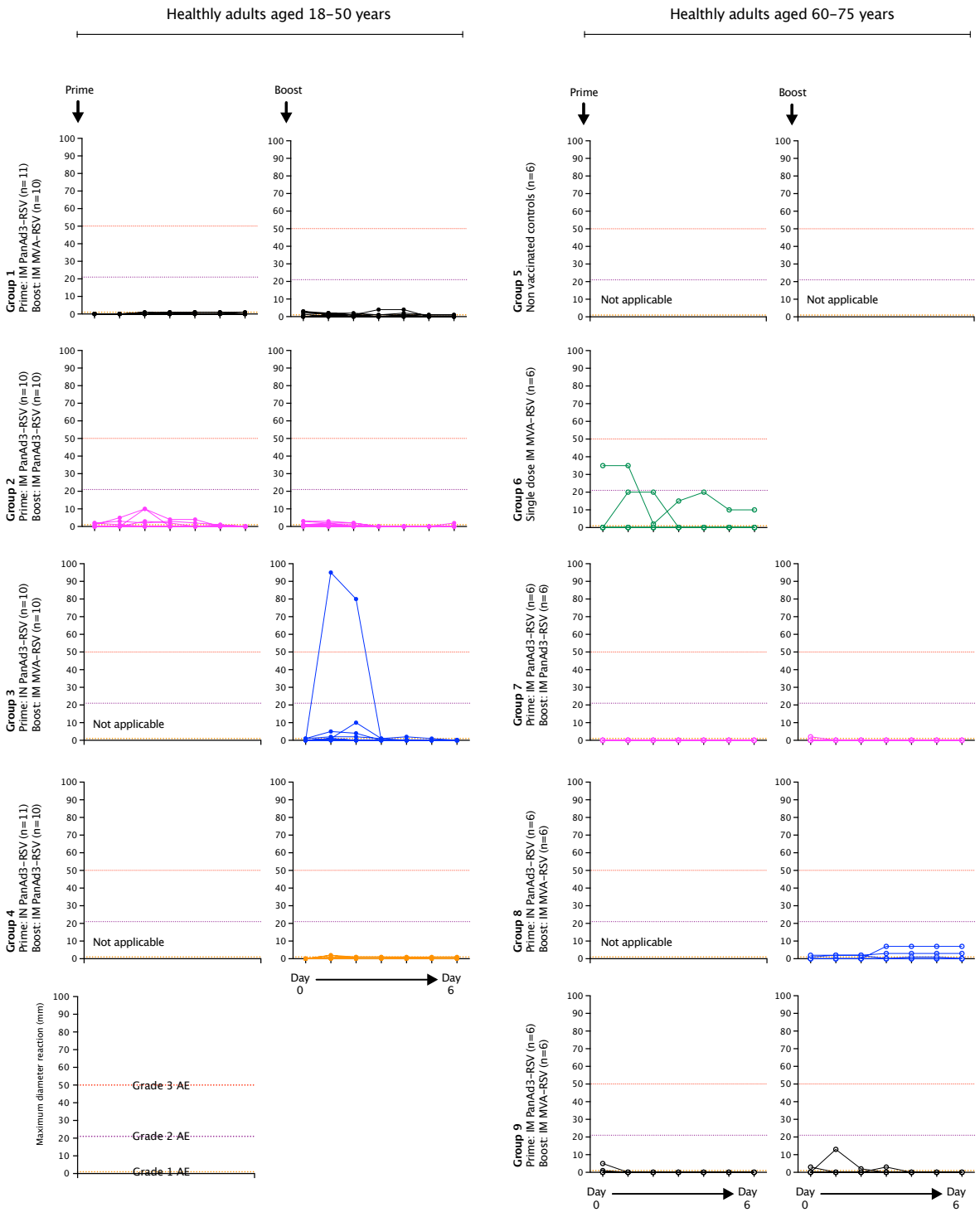


Supplementary figure 11. Solicited symptoms each day after vaccination; oral temperature (°C).
An individual line represents each volunteer. The dotted lines denote severity grade criteria for oral temperature recordings. The cases of fever in study group 4 were from different individuals.

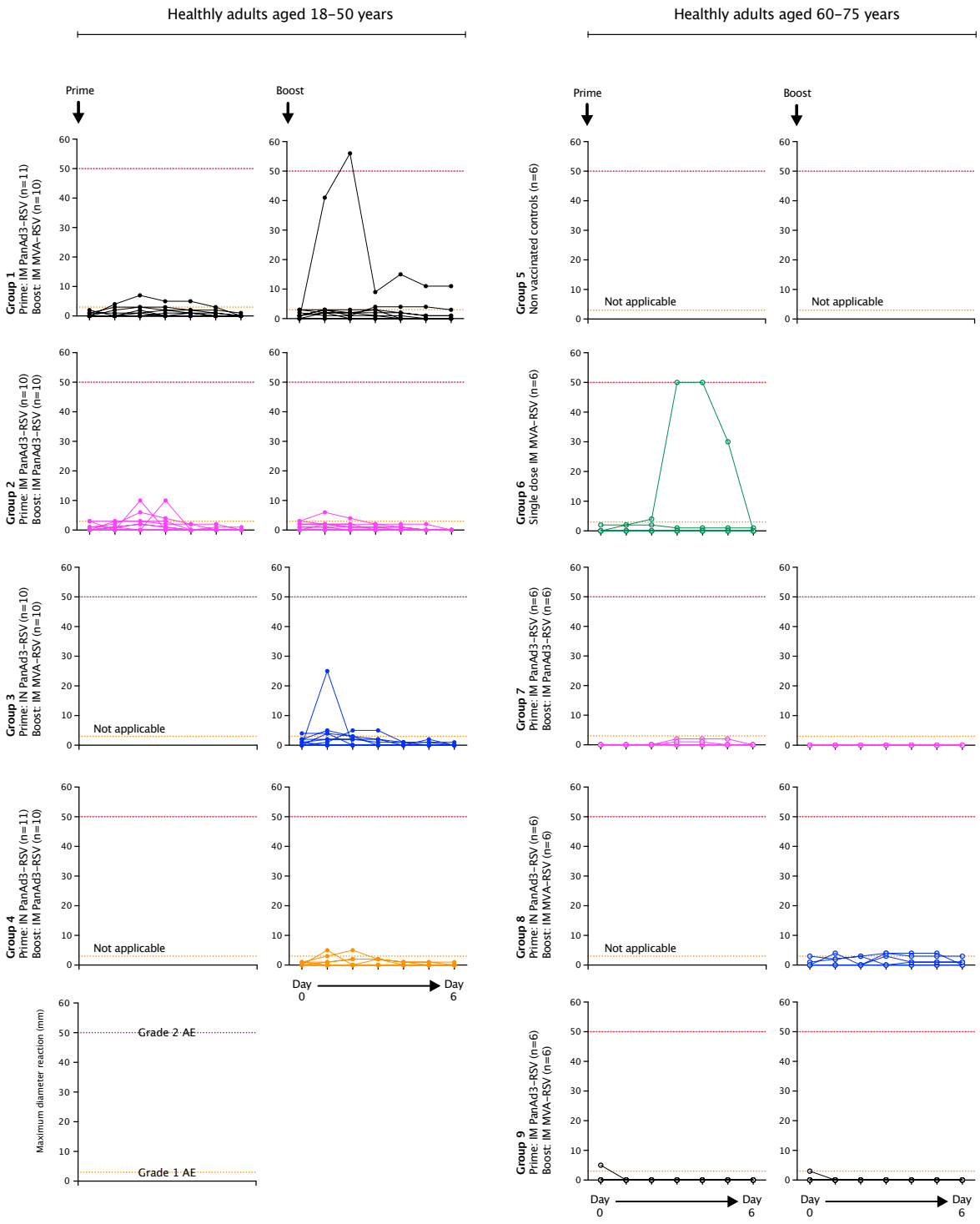


Supplementary figure 12. Solicited symptoms by severity grade for each day after vaccination; injection site pain or tenderness.

The number of volunteers is recorded on the y-axis, and each column represents one day after vaccination. Volunteers reported the worst severity grade every evening for one week after vaccination, with day 0 being the same day of vaccination.

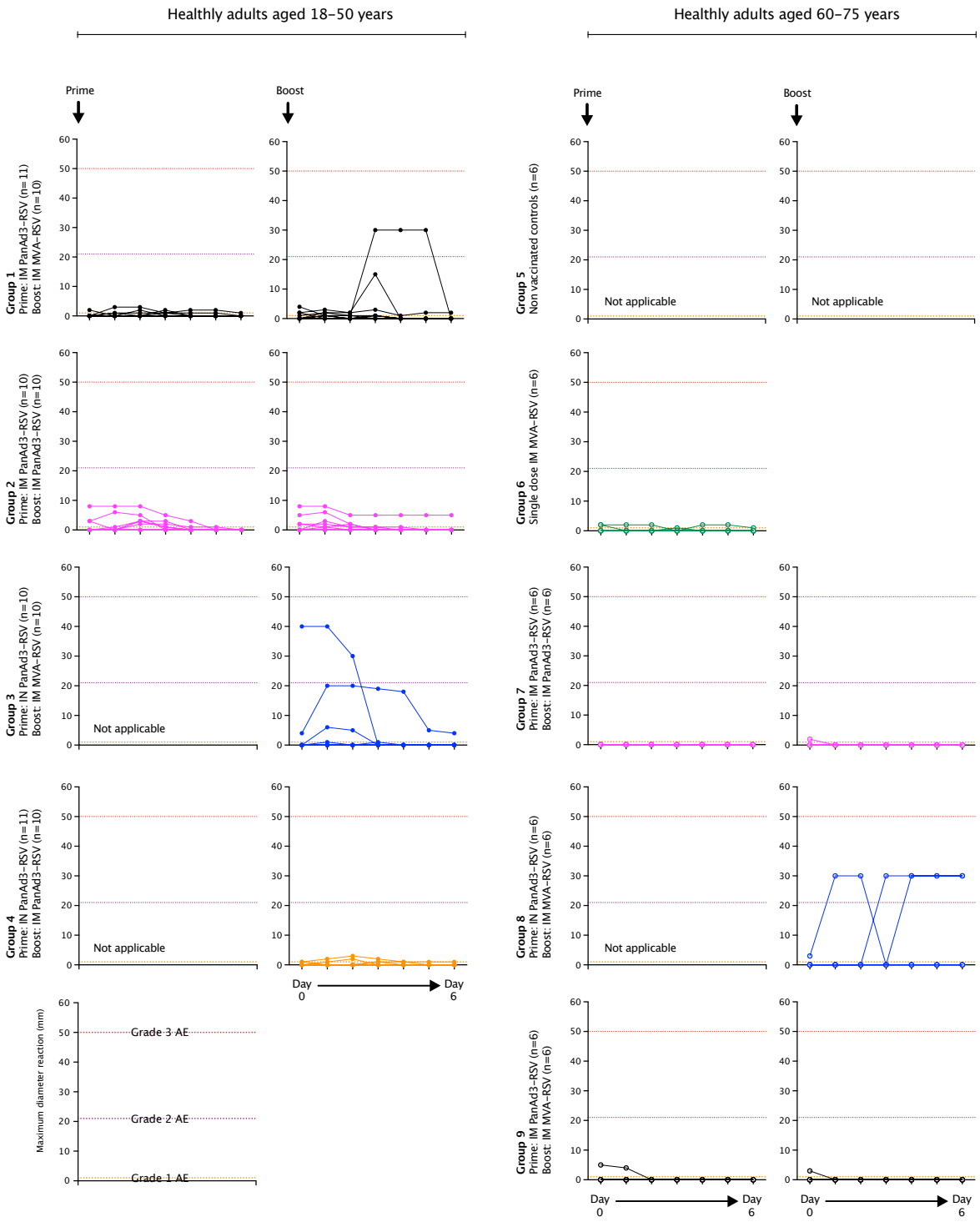


Supplementary figure 13. Solicited symptoms by severity grade for each day after vaccination; injection site induration (mm reaction).
Each line represents an individual volunteer.

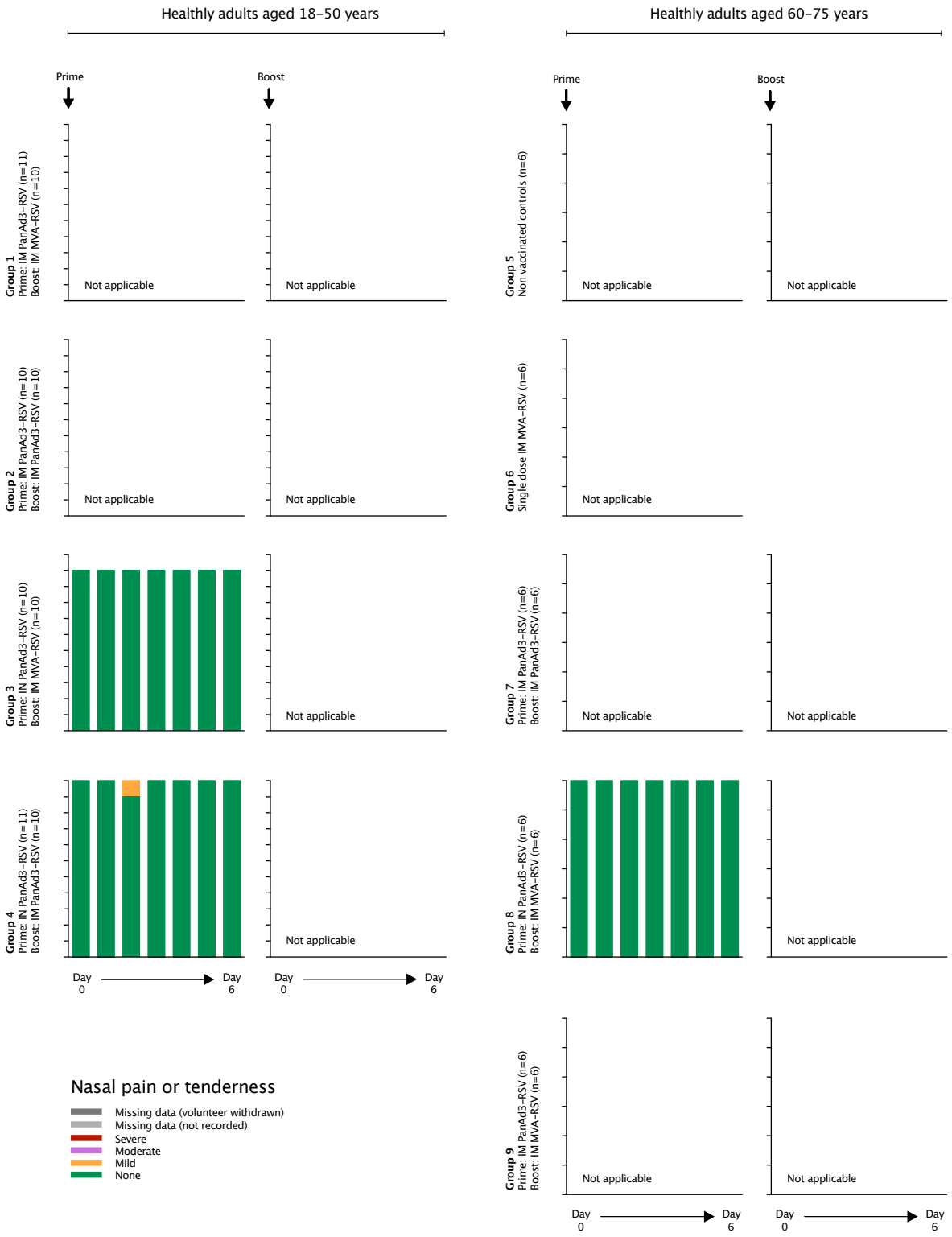


Supplementary figure 14. Solicited symptoms by severity grade for each day after vaccination; injection site redness (mm reaction).

Each line represents an individual volunteer.

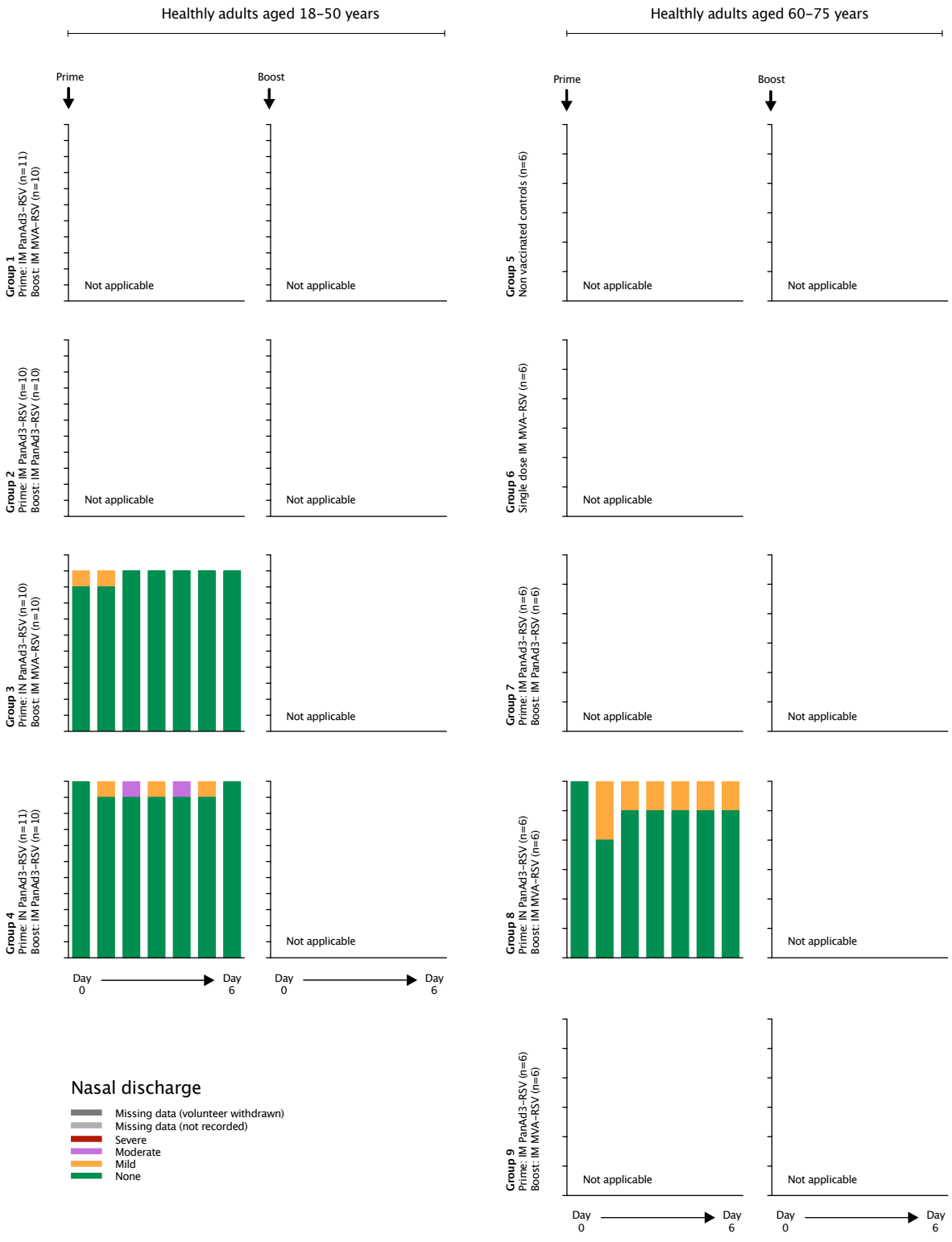


Supplementary figure 15. Solicited symptoms by severity grade for each day after vaccination; injection site swelling (mm reaction).
Each line represents an individual volunteer.



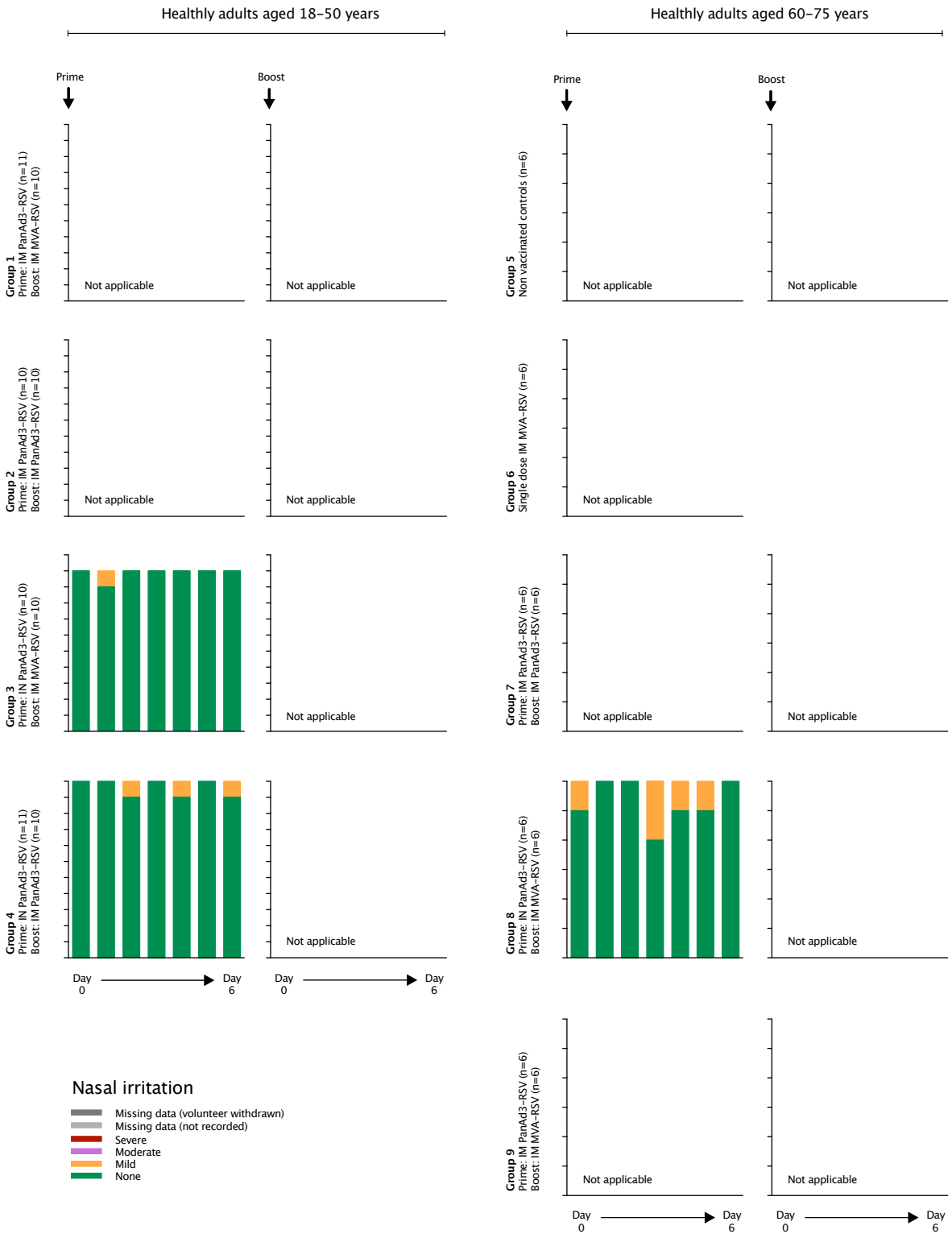
Supplementary figure 16. Solicited symptoms by severity grade for each day after vaccination; nasal pain or tenderness.

The number of volunteers is recorded on the y-axis, and each column represents one day after vaccination. Volunteers reported the worst severity grade every evening for one week after vaccination, with day 0 being the same day of vaccination.



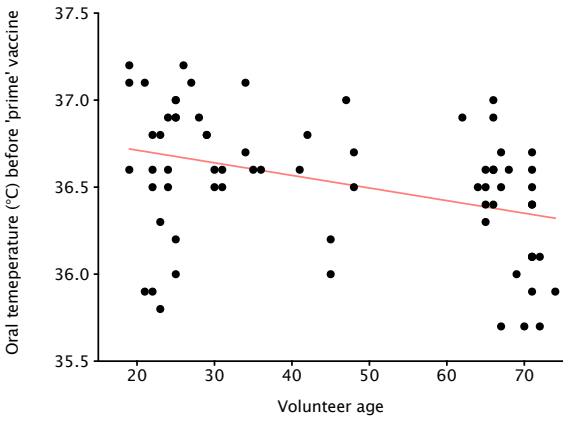
Supplementary figure 17. Solicited symptoms by severity grade for each day after vaccination; nasal discharge.

The number of volunteers is recorded on the y-axis, and each column represents one day after vaccination. Volunteers reported the worst severity grade every evening for one week after vaccination, with day 0 being the same day of vaccination.

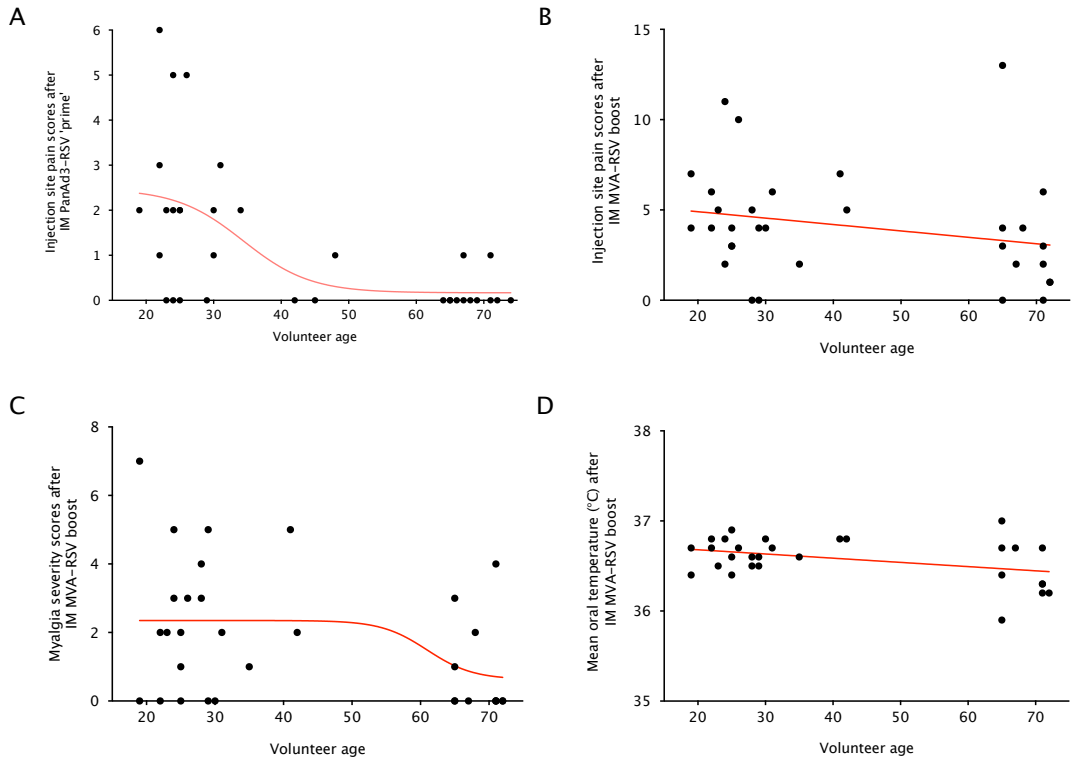


Supplementary figure 18. Solicited symptoms by severity grade for each day after vaccination; nasal irritation.

The number of volunteers is recorded on the y-axis, and each column represents one day after vaccination. Volunteers reported the worst severity grade every evening for one week after vaccination, with day 0 being the same day of vaccination.

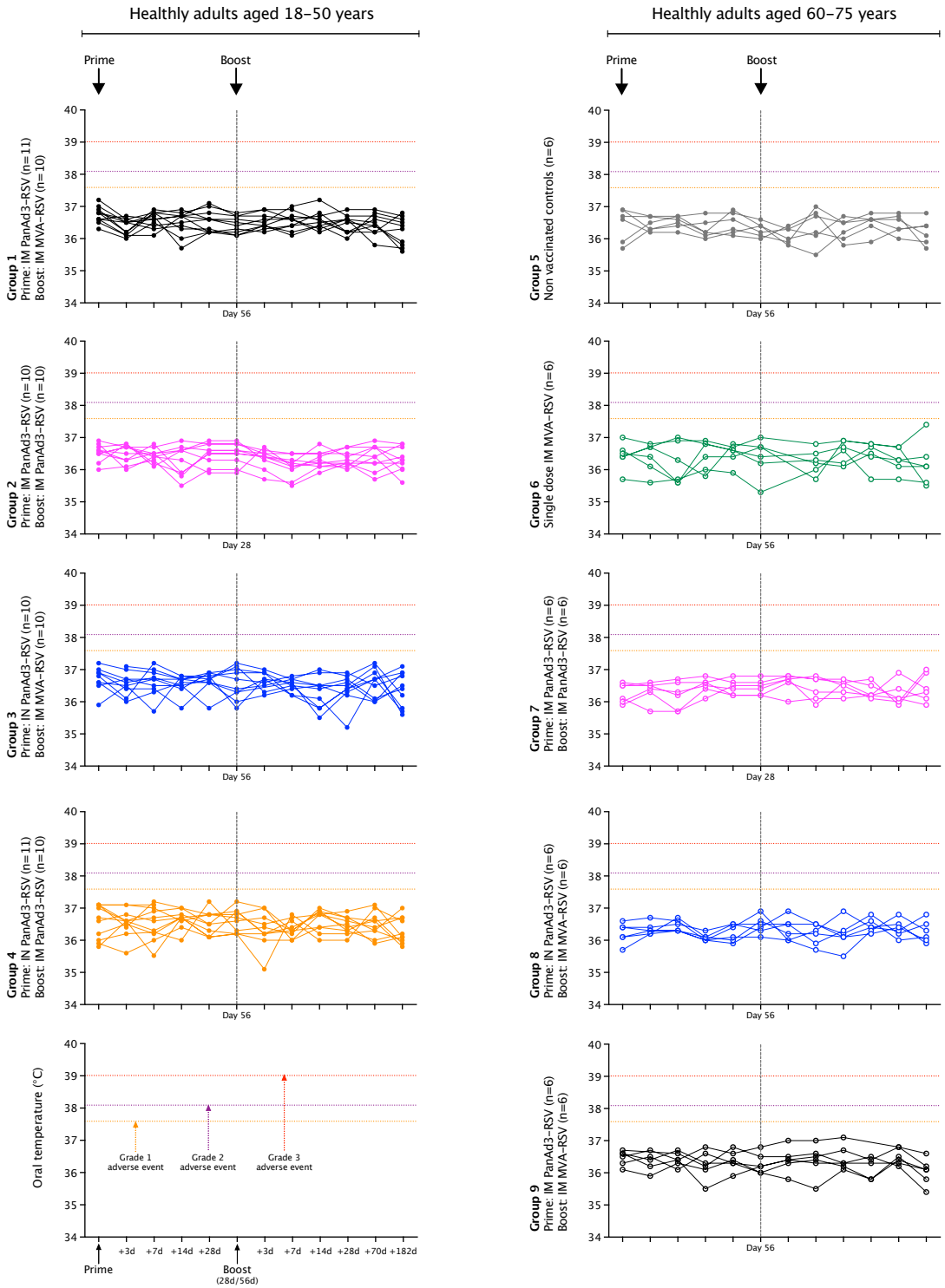


Supplementary figure 19. The relationship between oral temperature and volunteer age.
Oral temperature taken before prime vaccination declined with increasing age (r= 0.39, p =0.001, Pearson's correlation).

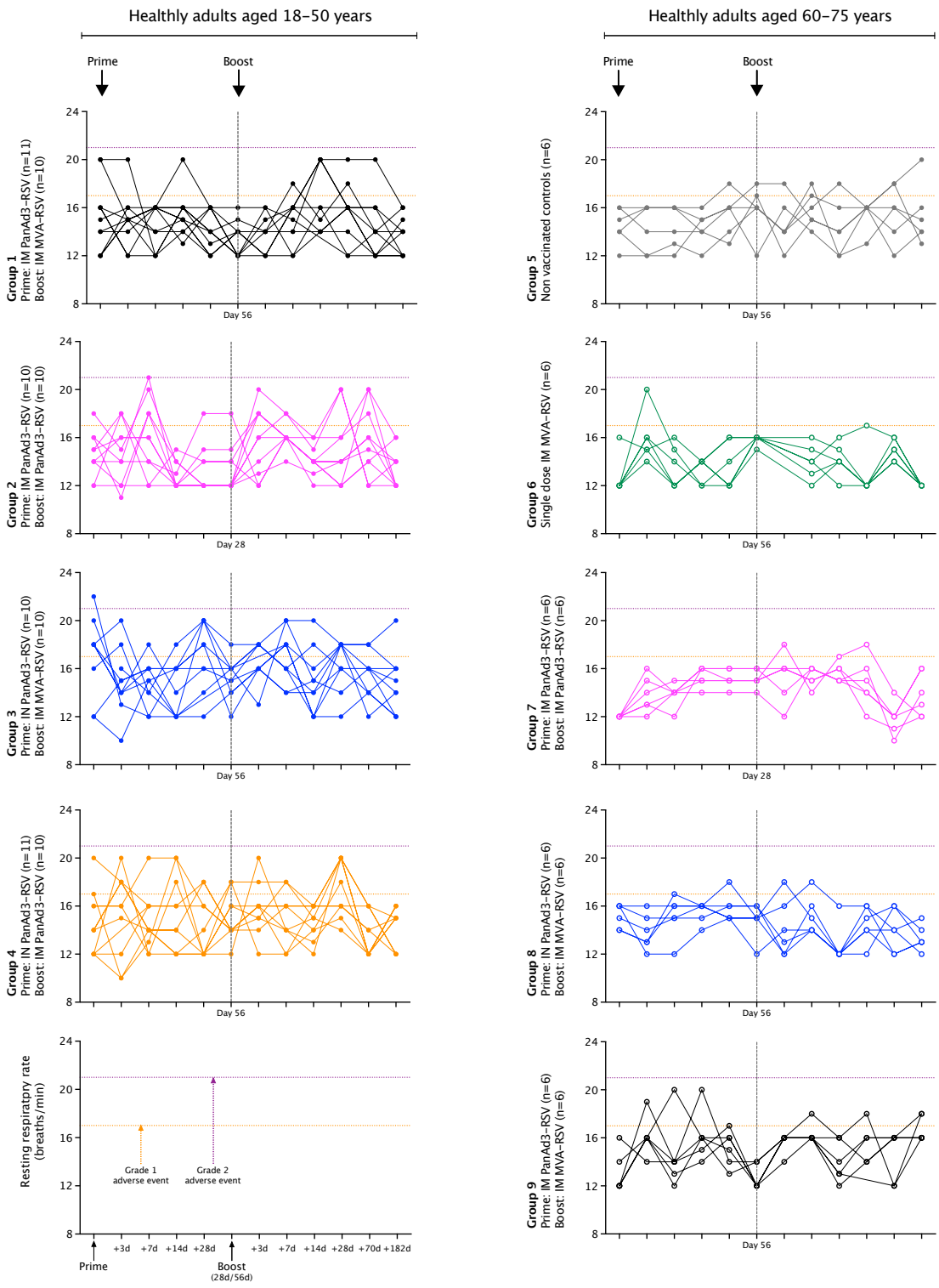


Supplementary figure 20. Post hoc analysis of solicited adverse events after vaccination; correlations with volunteer age and vaccine.
The red lines indicate the linear or non-linear line of best fit. (A) Volunteer age and injection site severity scores after IM PanAd3-RSV prime; r=-0.58, p=0.004, two-tailed Spearman's correlation. (B) Volunteer age and injection site severity scores after IM MVA-RSV boost; r=-0.37, p=0.037, two-tailed Spearman's correlation. (C) Volunteer age and myalgia severity scores after IM MVA-RSV boost; r=-0.34, p=0.055, two-tailed Spearman's correlation. (D) Volunteer age and the mean oral temperature after IM MVA-RSV boost; r=-0.38, p=0.003, two-tailed Pearson's correlation.

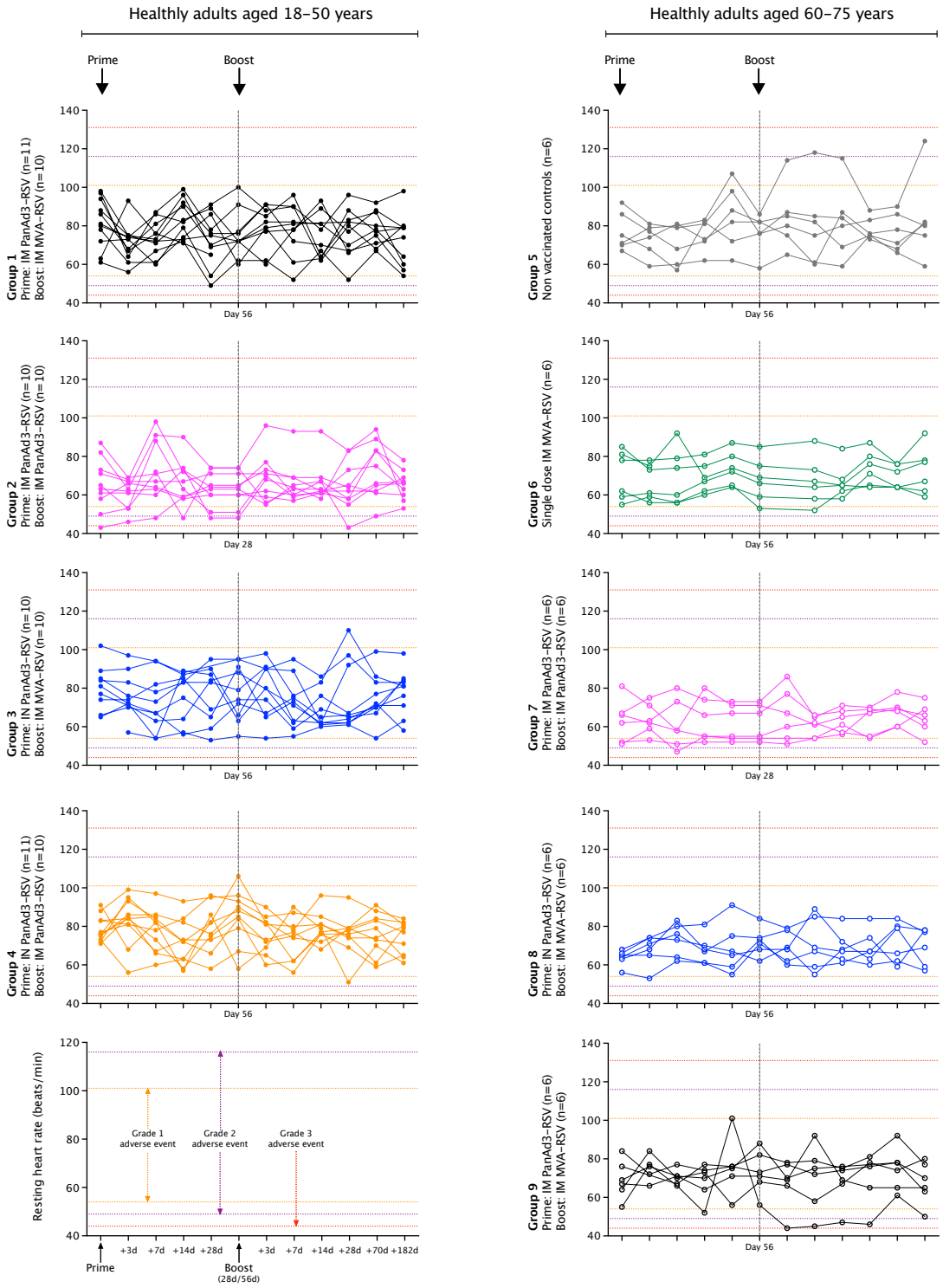
Visit observations



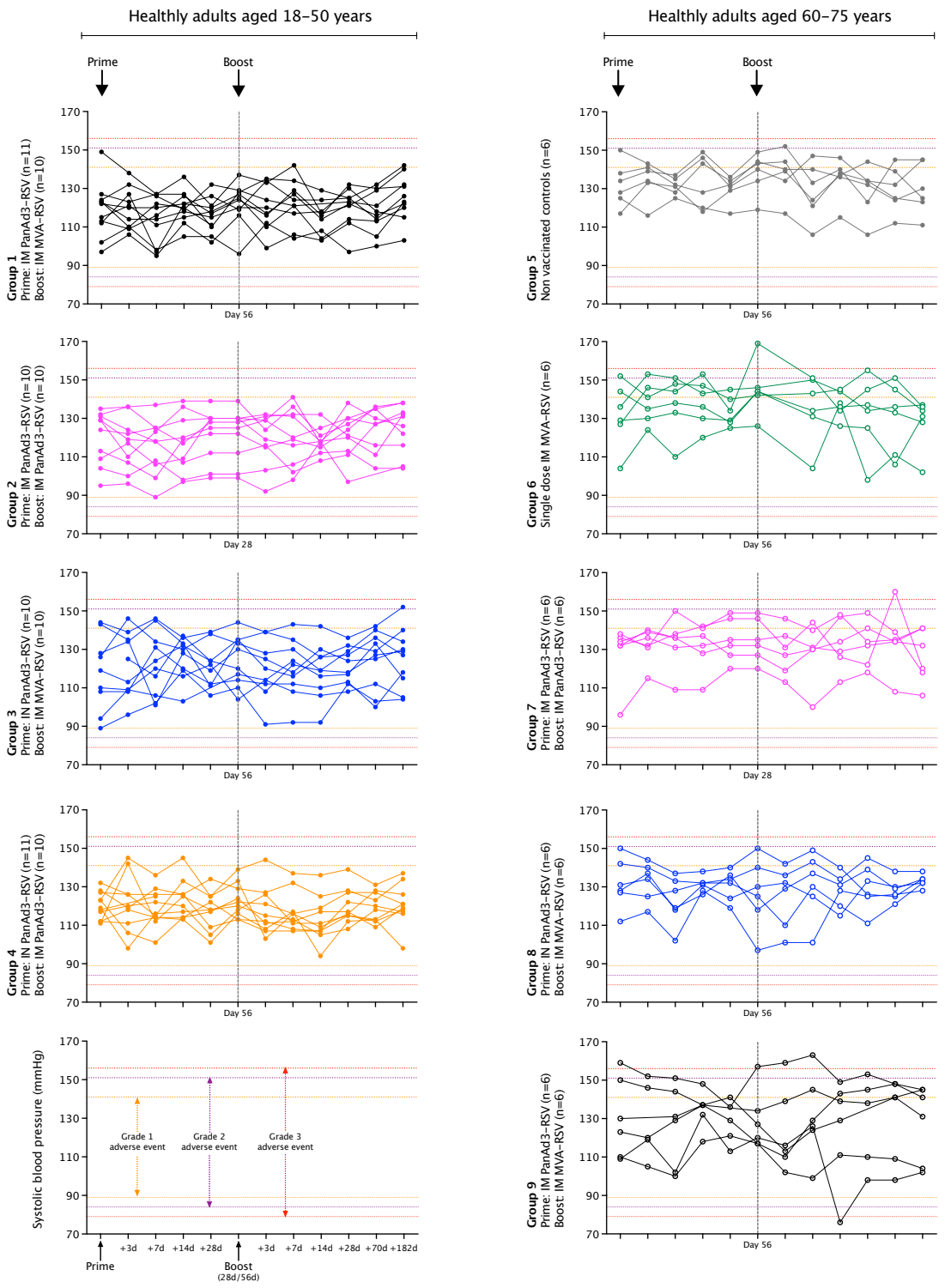
Supplementary figure 21. Visit observations from the RSV001 trial: oral temperature (°C).
Each line represents a single volunteer for each study group. The repeated measures ANOVA was significant for group 1 (p=0.003) due to a statistically significant drop in the recorded oral temperatures between prime and 3 days after prime (mean difference 0.4 °C, 95% CI 0.1-0.6, p=0.007 with Bonferonni correction). Pairwise testing revealed the significant within-group ANOVA for group 8 was not a difference from baseline but between 3 days and 14 days after prime. After boost, the significant within-group ANOVA from group 2 found no significant pairwise comparisons after correction for multiple tests.



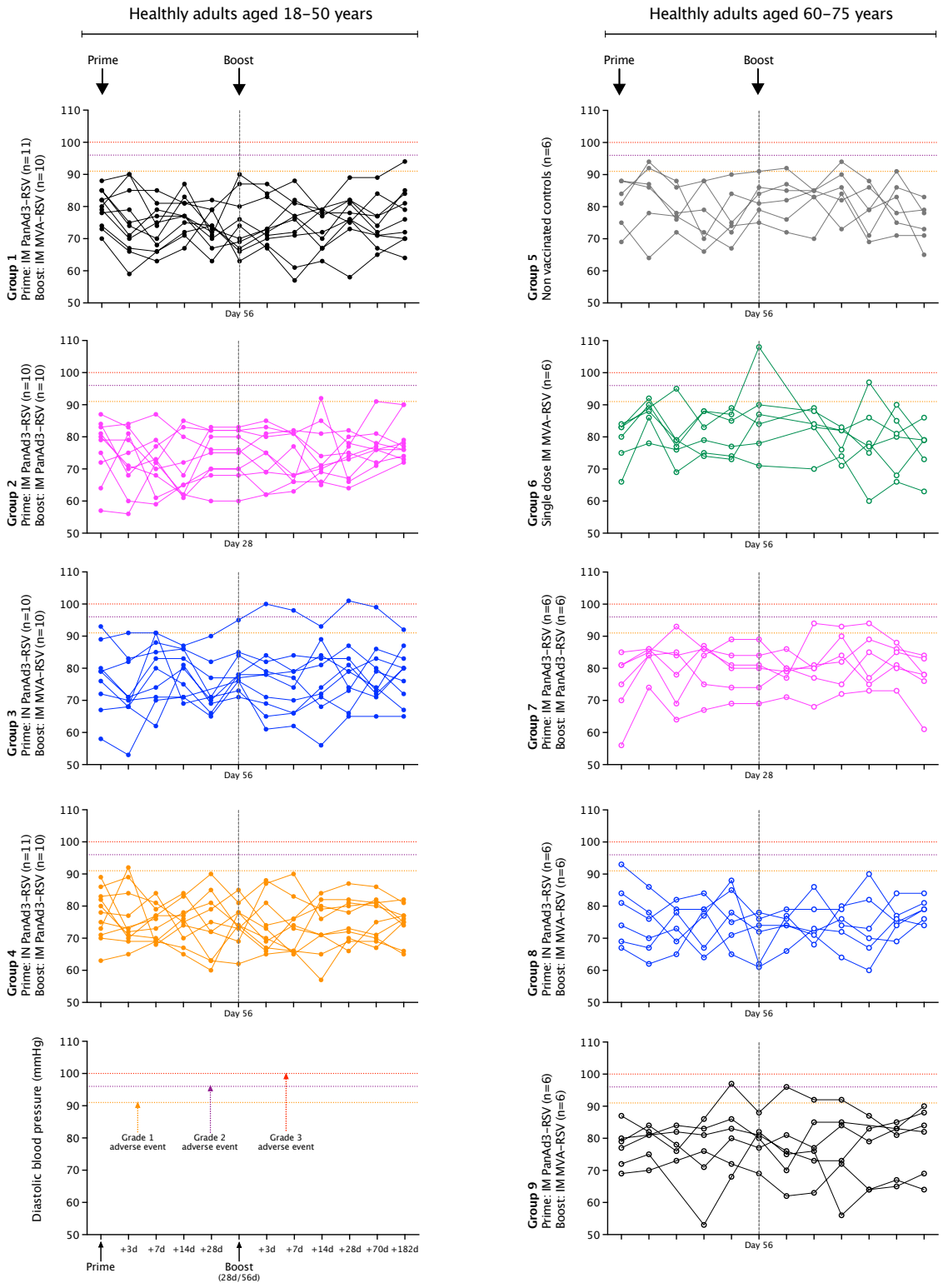
Supplementary figure 22. Visit observations from the RSV001 trial: resting respiratory rate (breaths/min). *Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6. ANOVA significance after prime vaccination for groups 2 and 6 found no statistically significant differences after correction for multiple testing. For group 7 the rate was higher 14-days and 18-days after prime compared with pre-vaccination (p=0.002 and p=0.001 respectively). For group 9 the significant ANOVA was due to differences between 7 days and 56 days after vaccination (p=0.022). After boost, ANOVA significance was found for study groups 1, 2, 4, 6, 7 and 9. From these, the only statistically significant change from pre-boost measure was for group 9 and a rise 7 days after boost (p<0.001).*



Supplementary figure 23. Visit observations from the RSV001 trial: resting heart rate (beats/min).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6. Grade 3 bradycardia was noted for one volunteer in group 2 and one volunteer in group 9. Within-groups ANOVA testing of changes after prime found statistical significance in groups 1, 4 and the non-vaccinated control group 5. For group 1 the statistically significant difference was the rise between 7- and 14-days after prime vaccination. There were no statistically significant differences between time-point measures in group 4 after correction for multiple testing. Analysis of the heart rate within groups after boost found no statistically significant changes by ANOVA.



Supplementary figure 24. Visit observations from the RSV001 trial: systolic blood pressure (mmHg). *Each line represents a single volunteer from each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6. Grade 3 hypertension was noted for one volunteer from group 6 and one volunteer from group 9. Within-groups ANOVA after prime was not significant for any study group. After boost, within-groups ANOVA were significant for study groups 1, 3 and 4. Pairwise comparisons were not significant after correction for multiple tests in groups 1 and 3, and for group 4 the mean systolic blood pressure was lower 14 days after IM PanAd3-RSV boost (p=0.014, two-tailed paired t-tests with Bonferroni correction).*



Supplementary figure 25. Visit observations from the RSV001 trial: diastolic blood pressure (mmHg). *Each line represents a single volunteer from each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6. Grade 3 diastolic hypertension was noted for one volunteer from group 3 and one volunteer from group 6. A within-group ANOVA significance from group 3 after prime was lost when pairwise comparisons were corrected for multiple testing. There were no significant within-group effects by ANOVA testing after boost vaccine.*

Temperature (degrees C)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	36.7 (36.6-36.8)	36.6 (36.5-36.7)	36.5 (36.3-36.7)	36.4 (36.2-36.6)	36.6 (36.4-36.8)	36.6 (36.4-36.8)
Group 2	36.5 (36.3-36.7)	36.4 (36.3-36.5)	36.3 (36-36.6)	36.5 (36.3-36.7)	36.1 (35.9-36.3)	36.3 (36.1-36.5)
Group 3	36.7 (36.5-36.9)	36.6 (36.3-36.9)	36.6 (36.4-36.8)	36.6 (36.3-36.9)	36.5 (36.4-36.6)	36.3 (36-36.6)
Group 4	36.6 (36.3-36.9)	36.5 (36.2-36.8)	36.7 (36.5-36.9)	36.6 (36.4-36.8)	36.4 (36.2-36.6)	36.6 (36.4-36.8)
Group 5	36.5 (36.1-36.9)	36.5 (36.3-36.7)	36.3 (36.1-36.5)	36.3 (36.1-36.5)	36.4 (36-36.8)	36.3 (36-36.6)
Group 6	36.4 (36.1-36.7)	36.2 (35.7-36.7)	36.5 (36.1-36.9)	36.4 (35.9-36.9)	36.3 (36-36.6)	36.6 (36.3-36.9)
Group 7	36.3 (36.1-36.5)	36.2 (35.9-36.5)	36.5 (36.3-36.7)	36.5 (36.3-36.7)	36.4 (36.1-36.7)	36.5 (36.3-36.7)
Group 8	36.2 (35.9-36.5)	36.5 (36.4-36.6)	36.1 (36-36.2)	36.5 (36.3-36.7)	36.2 (35.9-36.5)	36.2 (35.8-36.6)
Group 9	36.5 (36.3-36.7)	36.4 (36.2-36.6)	36.3 (35.9-36.7)	36.3 (36-36.6)	36.4 (36-36.8)	36.5 (36.2-36.8)

Supplementary table 6. Summary statistics for oral temperature after vaccination (°C) recorded at scheduled visits.
Mean and 95% confidence intervals.

Respiratory rate (breaths/min)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	15 (13-17)	15 (14-16)	15 (14-16)	14 (13-15)	15 (14-16)	17 (15-19)
Group 2	15 (14-16)	16 (14-18)	13 (12-14)	13 (12-14)	16 (15-17)	14 (13-15)
Group 3	17 (15-19)	15 (14-16)	14 (13-15)	15 (14-16)	17 (16-18)	15 (13-17)
Group 4	14 (12-16)	15 (14-16)	15 (13-17)	15 (14-16)	15 (14-16)	15 (14-16)
Group 5	15 (14-16)	15 (14-16)	14 (13-15)	16 (14-18)	16 (15-17)	14 (12-16)
Group 6	13 (12-14)	14 (13-15)	13 (12-14)	16 (16-16)	14 (13-15)	14 (13-15)
Group 7	12 (12-12)	14 (13-15)	15 (14-16)	15 (14-16)	15 (14-16)	16 (15-17)
Group 8	15 (14-16)	15 (14-16)	16 (15-17)	15 (14-16)	15 (14-16)	13 (12-14)
Group 9	13 (12-14)	15 (13-17)	16 (14-18)	13 (12-14)	16 (15-17)	14 (13-15)

Supplementary table 7. Summary statistics for resting respiratory rates (breaths/min) recorded at scheduled visits.
Mean and 95% confidence intervals.

Heart rate (beats/min)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	82 (75-89)	74 (69-79)	83 (77-89)	75 (68-82)	78 (70-86)	74 (67-81)
Group 2	65 (57-73)	72 (62-82)	66 (59-73)	63 (58-68)	66 (60-72)	66 (60-72)
Group 3	80 (73-87)	73 (64-82)	77 (69-85)	78 (69-87)	72 (64-80)	69 (63-75)
Group 4	79 (75-83)	78 (72-84)	72 (65-79)	85 (76-94)	74 (67-81)	79 (74-84)
Group 5	77 (69-85)	71 (62-80)	76 (70-82)	77 (69-85)	80 (63-97)	82 (67-97)
Group 6	70 (60-80)	70 (58-82)	69 (63-75)	68 (59-77)	67 (57-77)	67 (60-74)
Group 7	63 (54-72)	61 (51-71)	64 (55-73)	62 (54-70)	60 (56-64)	63 (58-68)
Group 8	64 (61-67)	73 (66-80)	68 (62-74)	72 (66-78)	71 (60-82)	69 (62-76)
Group 9	69 (61-77)	70 (67-73)	68 (61-75)	73 (64-82)	70 (57-83)	68 (59-77)

Supplementary table 8. Summary statistics for resting heart rates (beats/min) recorded at scheduled visits.
Mean and 95% confidence intervals.

Systolic blood pressure (mmHg)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	119 (111-127)	114 (107-121)	120 (115-125)	122 (115-129)	123 (116-130)	115 (110-120)
Group 2	120 (111-129)	115 (106-124)	117 (108-126)	122 (114-130)	120 (111-129)	118 (114-122)
Group 3	118 (106-130)	123 (113-133)	125 (118-132)	124 (116-132)	120 (111-129)	119 (110-128)
Group 4	120 (116-124)	119 (113-125)	123 (117-129)	123 (118-128)	117 (111-123)	113 (106-120)
Group 5	132 (123-141)	131 (127-135)	134 (123-145)	138 (130-146)	129 (117-141)	135 (127-143)
Group 6	132 (119-145)	137 (125-149)	138 (128-148)	145 (134-156)	136 (122-150)	137 (131-143)
Group 7	128 (115-141)	133 (122-144)	132 (122-142)	135 (126-144)	129 (117-141)	133 (122-144)
Group 8	132 (121-143)	123 (113-133)	131 (128-134)	127 (112-142)	131 (117-145)	128 (121-135)
Group 9	130 (113-147)	126 (109-143)	135 (127-143)	129 (117-141)	131 (114-148)	125 (103-147)

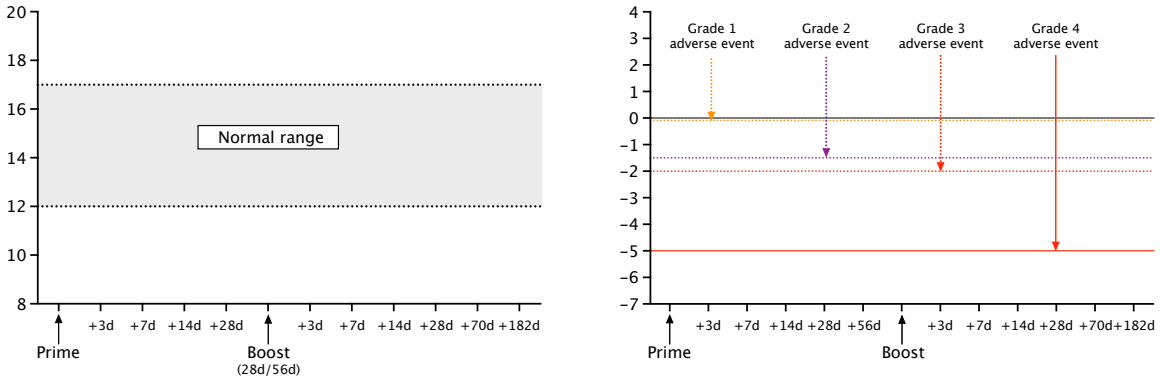
Supplementary table 9. Summary statistics for systolic blood pressure (mmHg) recorded at scheduled visits.
Mean and 95% confidence intervals.

Diastolic blood pressure (mmHg)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	80 (77-83)	73 (69-77)	77 (74-80)	74 (68-80)	74 (68-80)	72 (68-76)
Group 2	76 (70-82)	72 (67-77)	70 (64-76)	75 (70-80)	73 (68-78)	74 (69-79)
Group 3	77 (70-84)	80 (74-86)	79 (75-83)	79 (75-83)	76 (69-83)	77 (70-84)
Group 4	77 (72-82)	75 (72-78)	75 (71-79)	75 (71-79)	74 (69-79)	74 (69-79)
Group 5	81 (75-87)	80 (75-85)	77 (69-85)	83 (79-87)	82 (77-87)	85 (79-91)
Group 6	79 (73-85)	79 (72-86)	81 (76-86)	86 (76-96)	83 (78-88)	78 (74-82)
Group 7	75 (67-83)	79 (70-88)	81 (75-87)	80 (74-86)	80 (73-87)	83 (76-90)
Group 8	78 (70-86)	74 (69-79)	75 (69-81)	71 (65-77)	75 (70-80)	74 (69-79)
Group 9	77 (72-82)	74 (64-84)	75 (65-85)	80 (75-85)	78 (70-86)	77 (67-87)

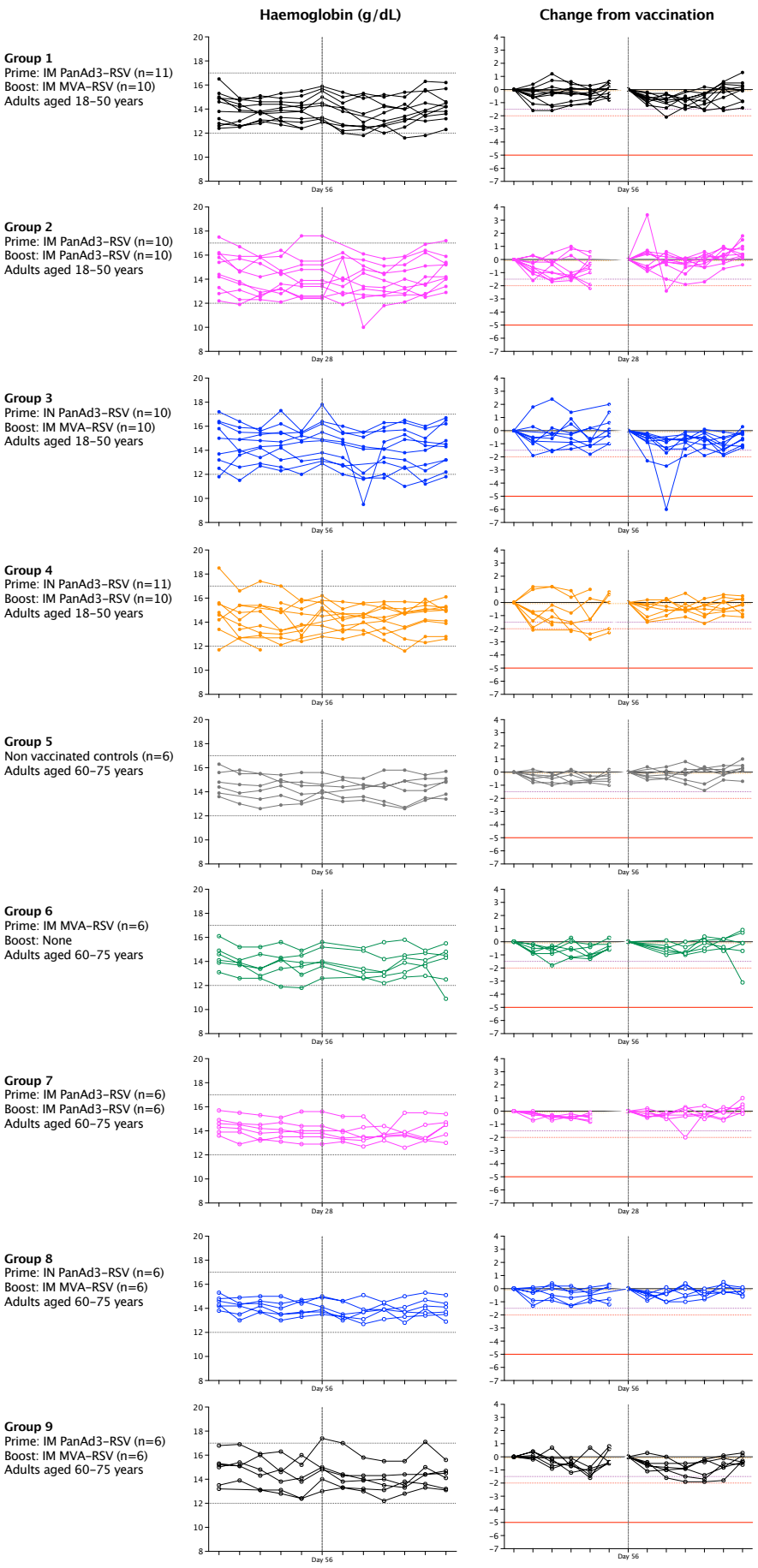
Supplementary table 10. Summary statistics for diastolic blood pressure (mmHg) recorded at scheduled visits.
Mean and 95% confidence intervals.

Safety monitoring of routine haematology and biochemistry

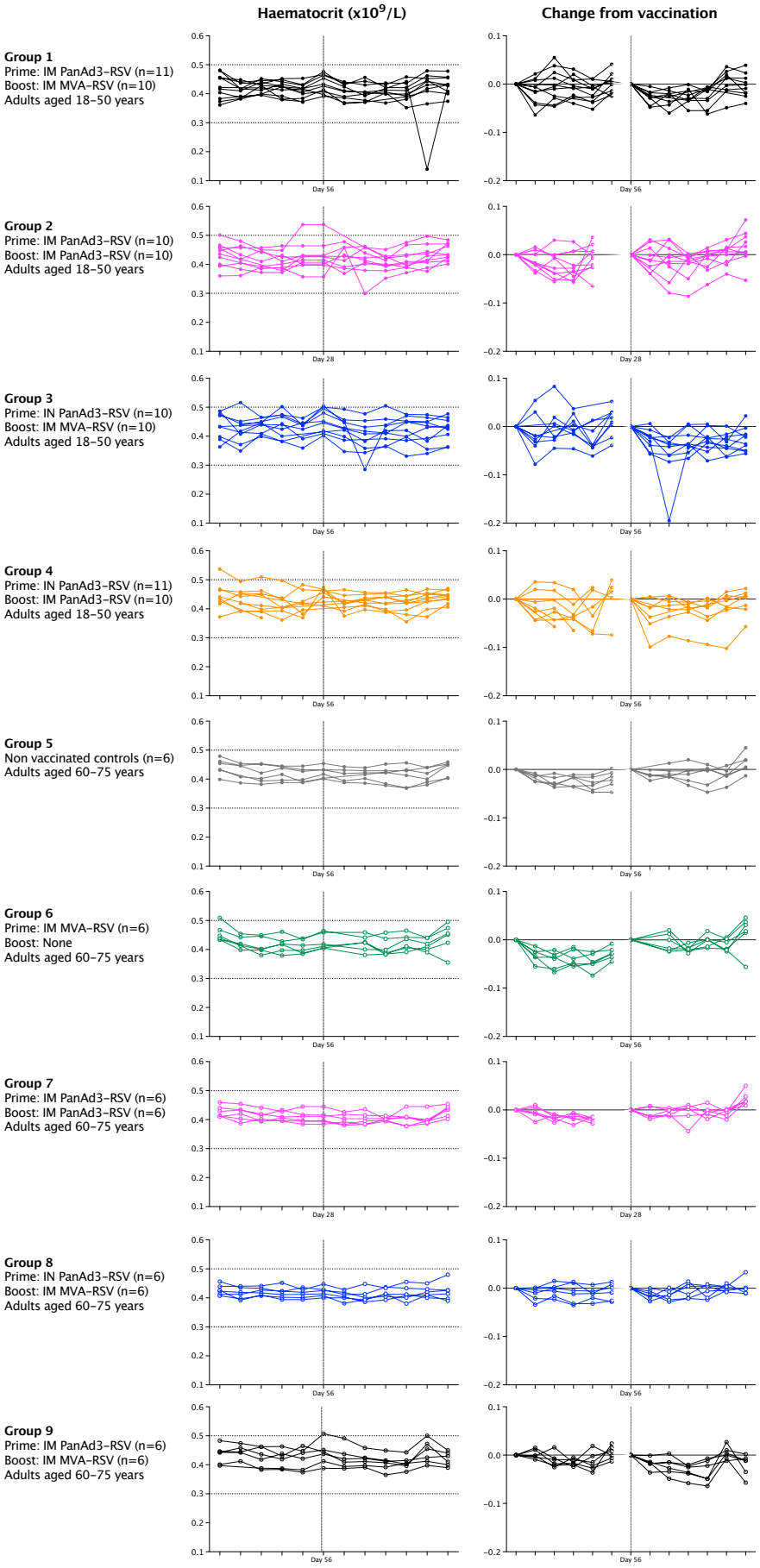
The figures within this section of supplementary results use a common legend, as shown below.



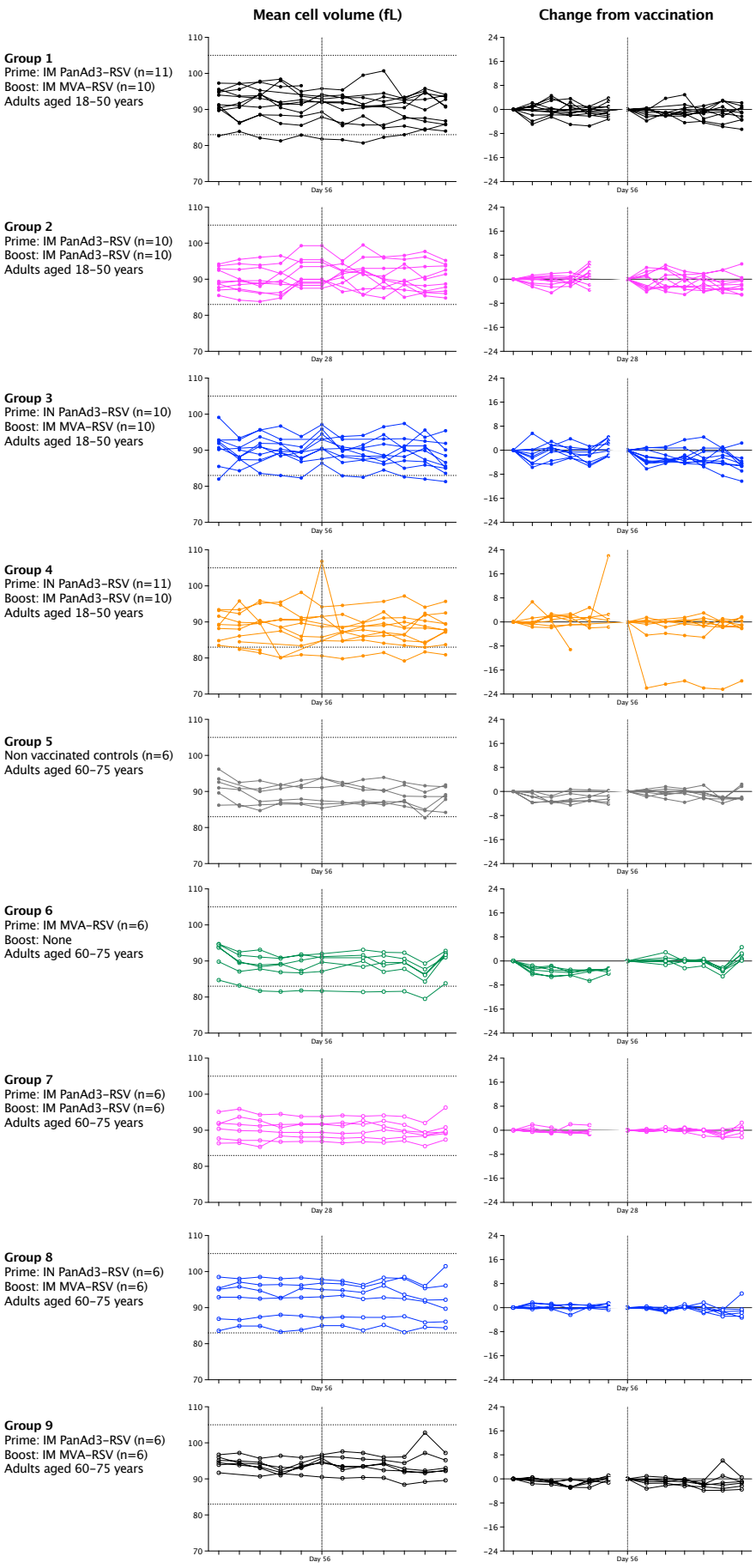
Supplementary figure 26. Example of the change in haemoglobin after vaccination.
This above figure is used to explain the additional markings on the figures contained within this section. Some safety bloods were interpreted using the normal physiological ranges from Oxford University Hospitals NHS Trust (left figure), while others used modified FDA and DAIDS criteria (right figure), as defined earlier. The data for the final visit in older adults, completed one year after boost, is not shown.



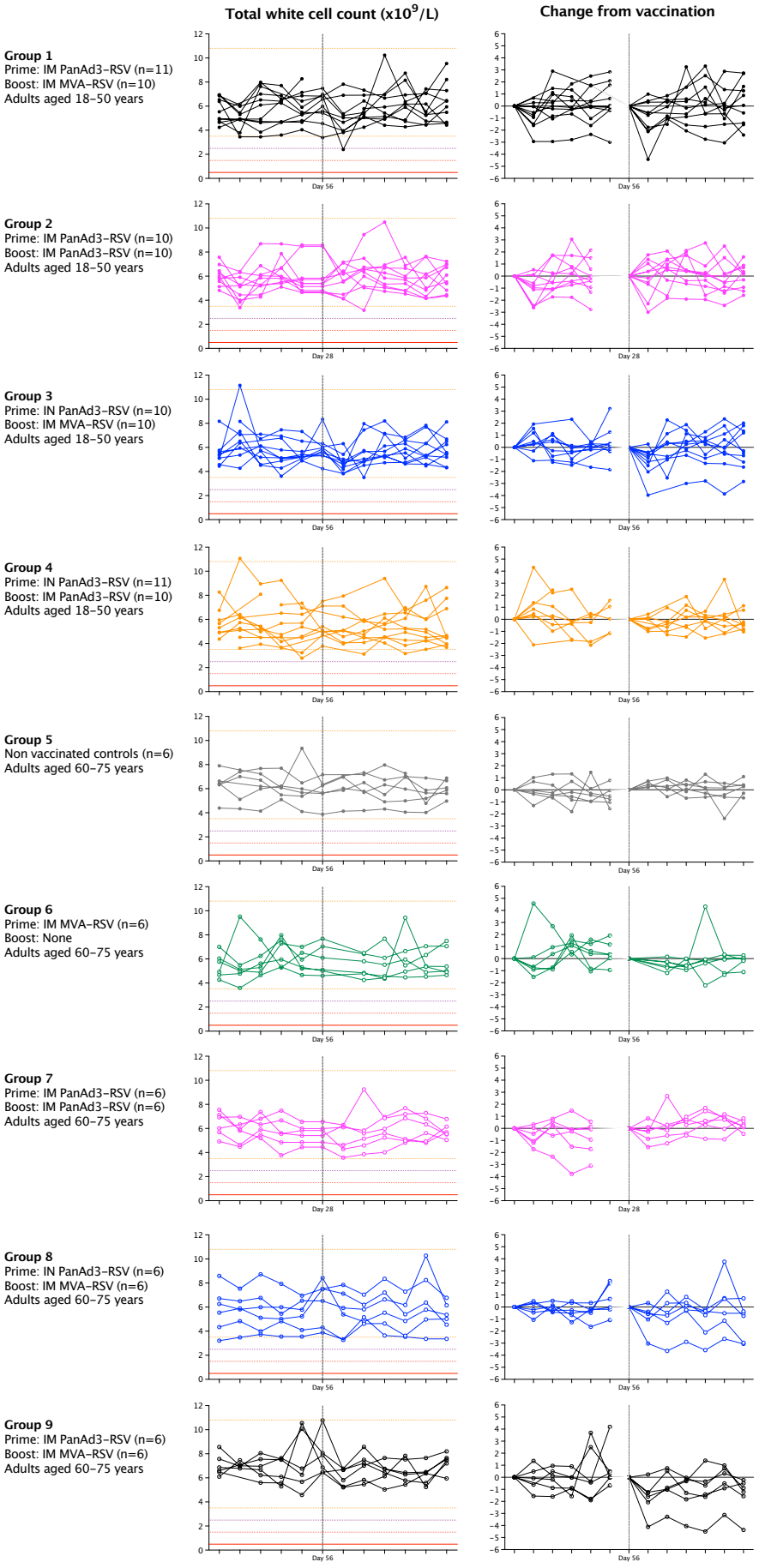
Supplementary figure 27. Safety bloods from the RSV001 trial: haemoglobin (g/dL).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



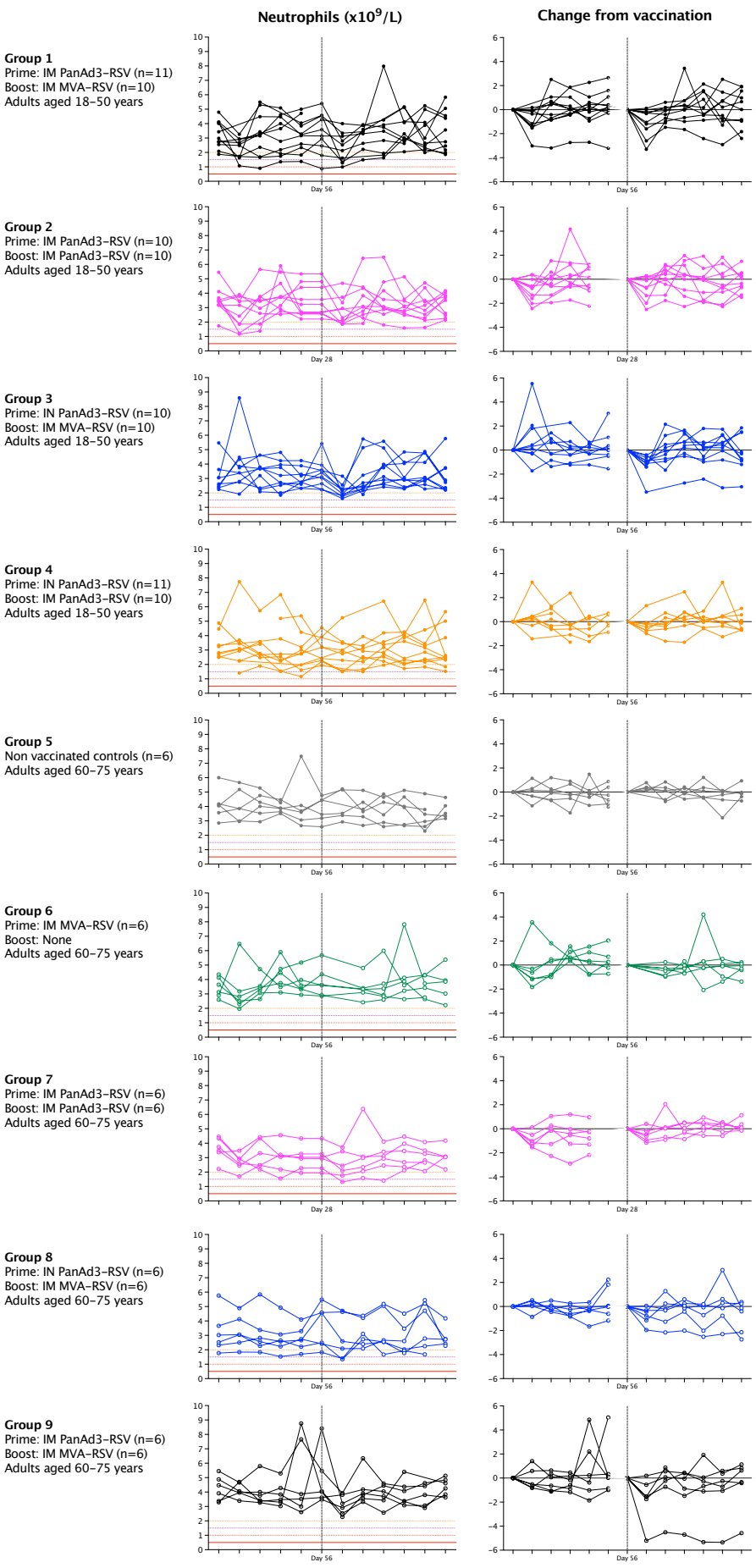
Supplementary figure 28. Safety bloods from the RSV001 trial: haematocrit (x10⁹/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



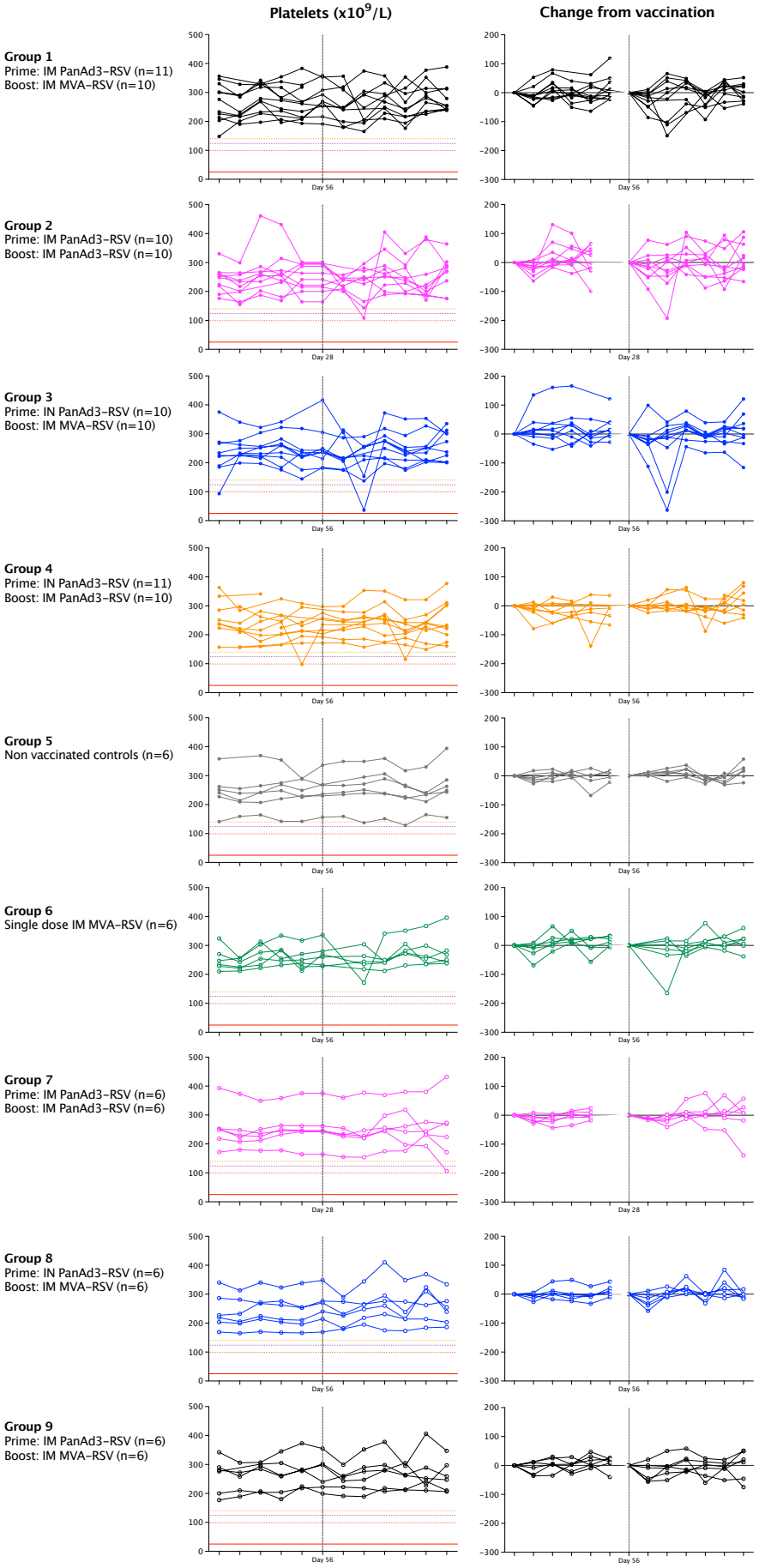
Supplementary figure 29. Safety bloods from the RSV001 trial: mean cell volume (fL). *Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.*



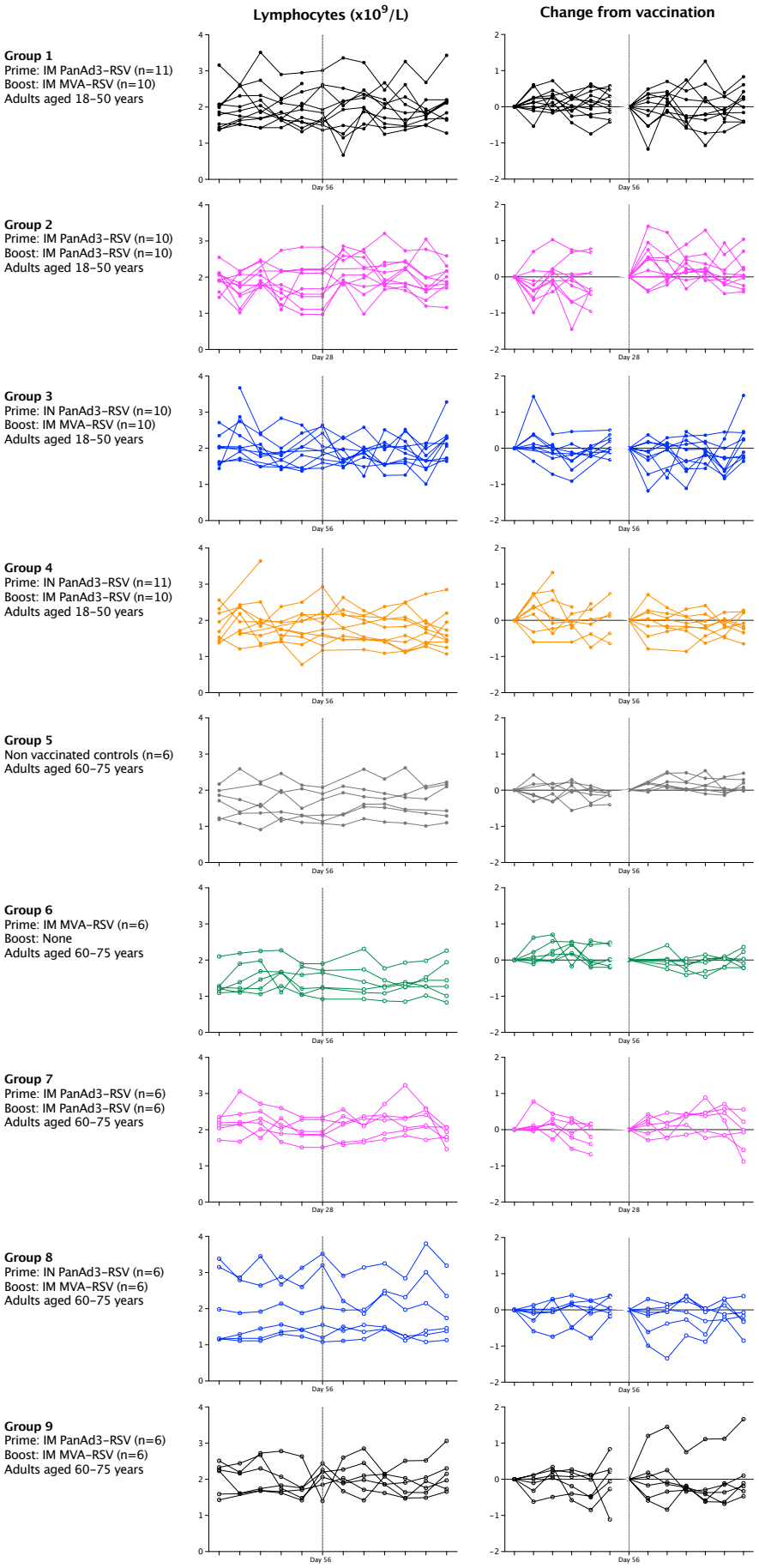
Supplementary figure 30. Safety bloods from the RSV001 trial: total white cell count (x10⁹/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



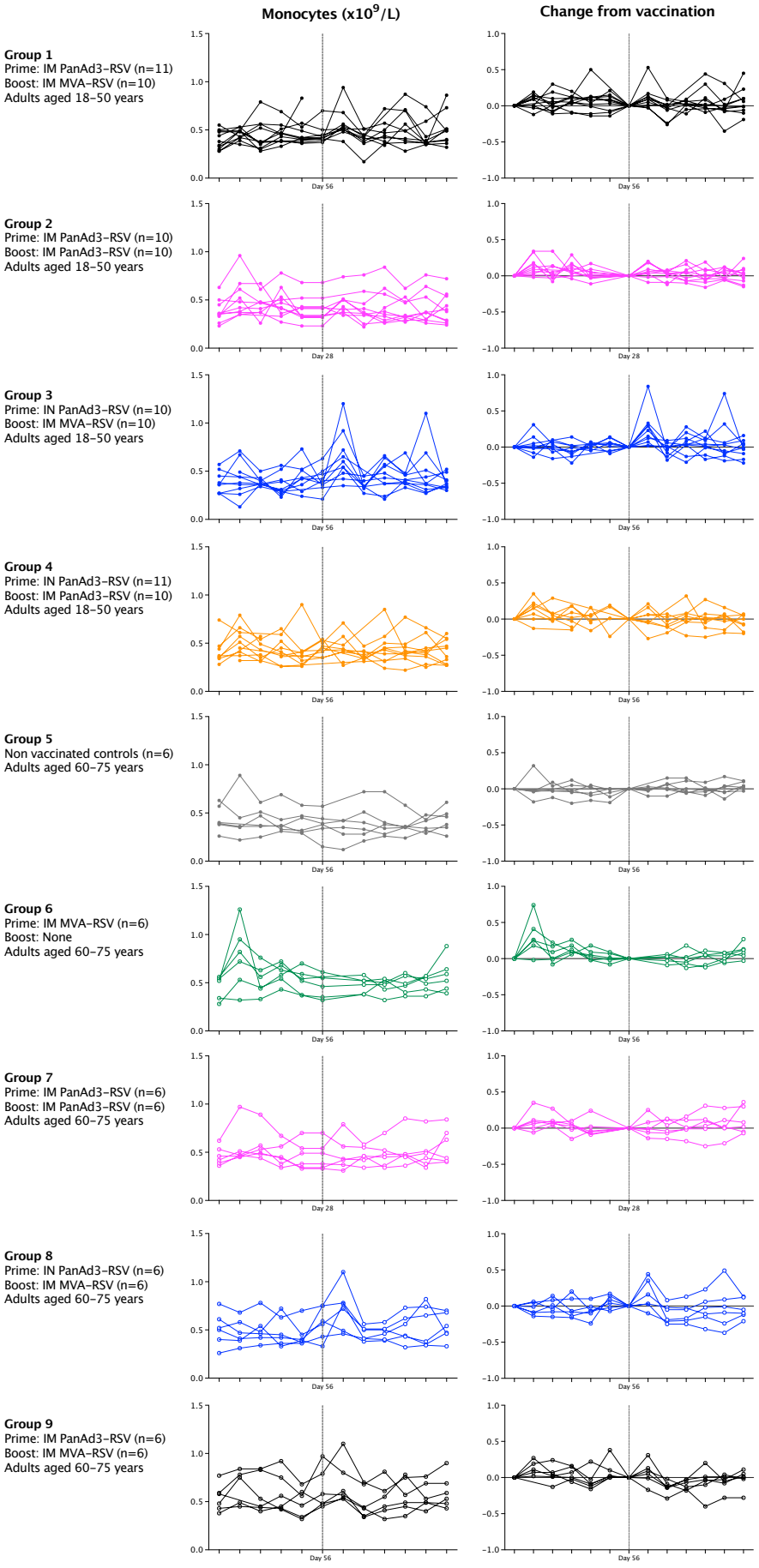
Supplementary figure 31. Safety bloods from the RSV001 trial: neutrophil count (x10⁹/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



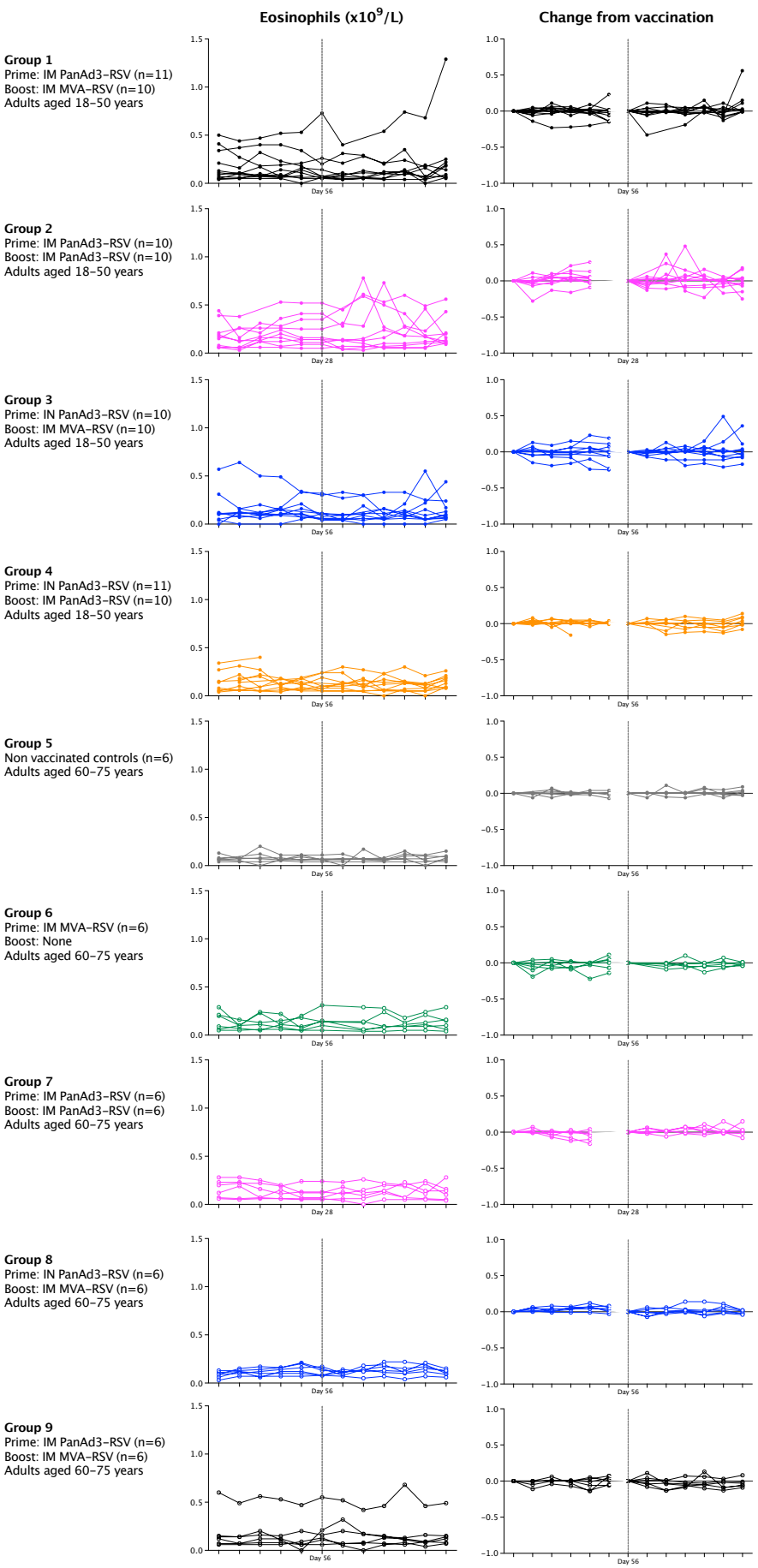
Supplementary figure 32. Safety bloods from the RSV001 trial: platelet count (x10⁹/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



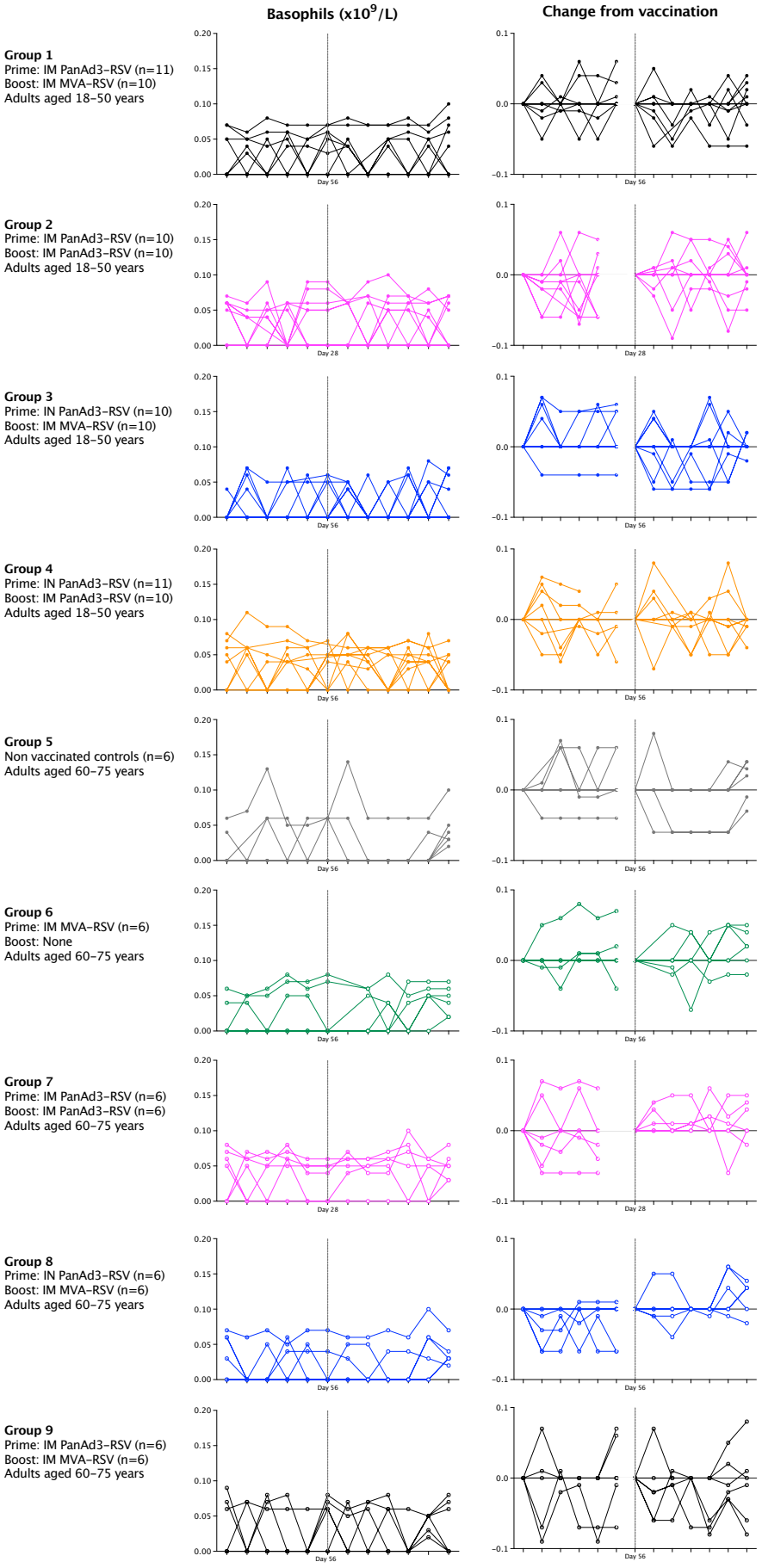
Supplementary figure 33. Safety bloods from the RSV001 trial: lymphocyte count (x10^9/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



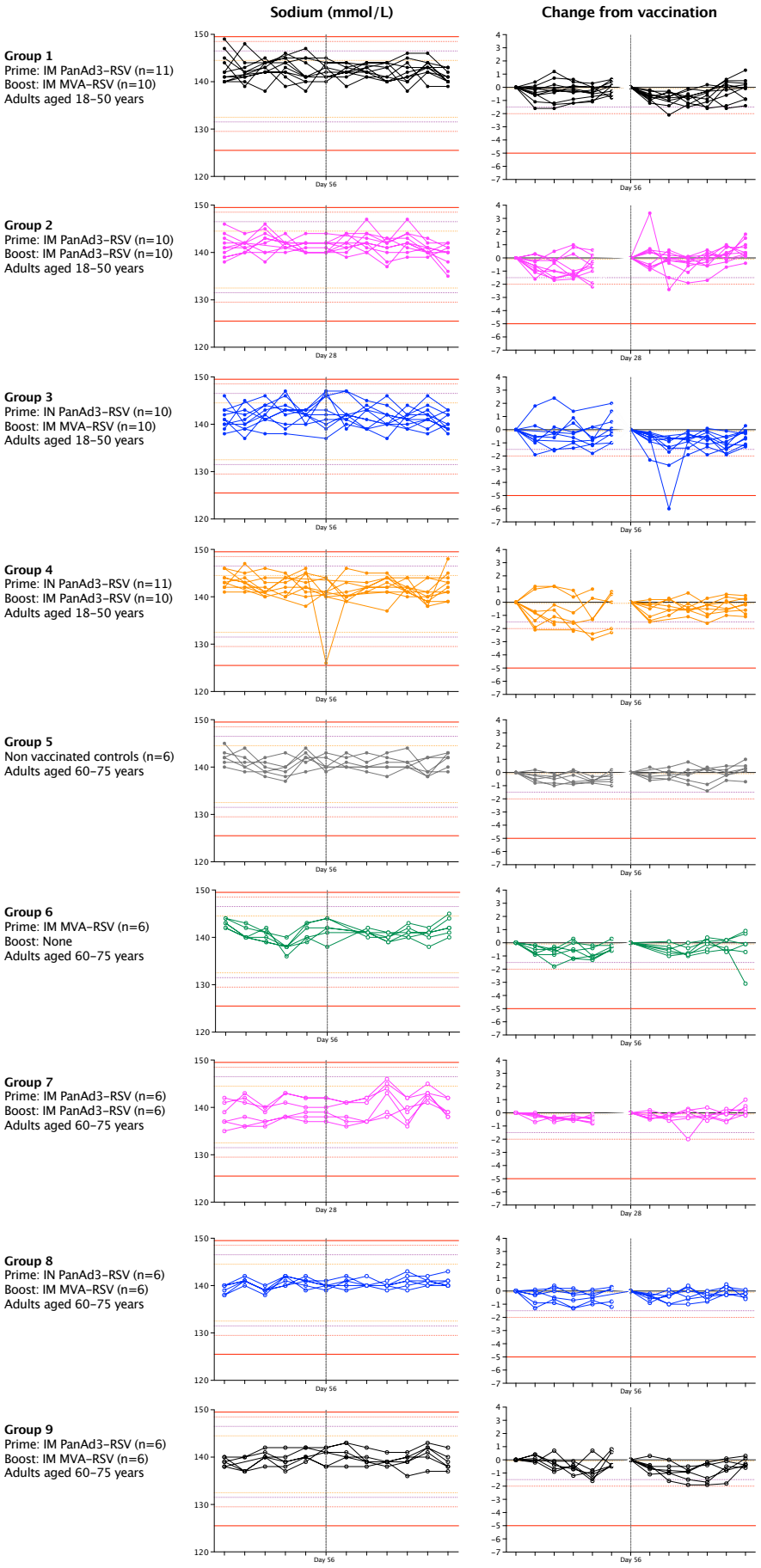
Supplementary figure 34. Safety bloods from the RSV001 trial: monocyte count (x10^9/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



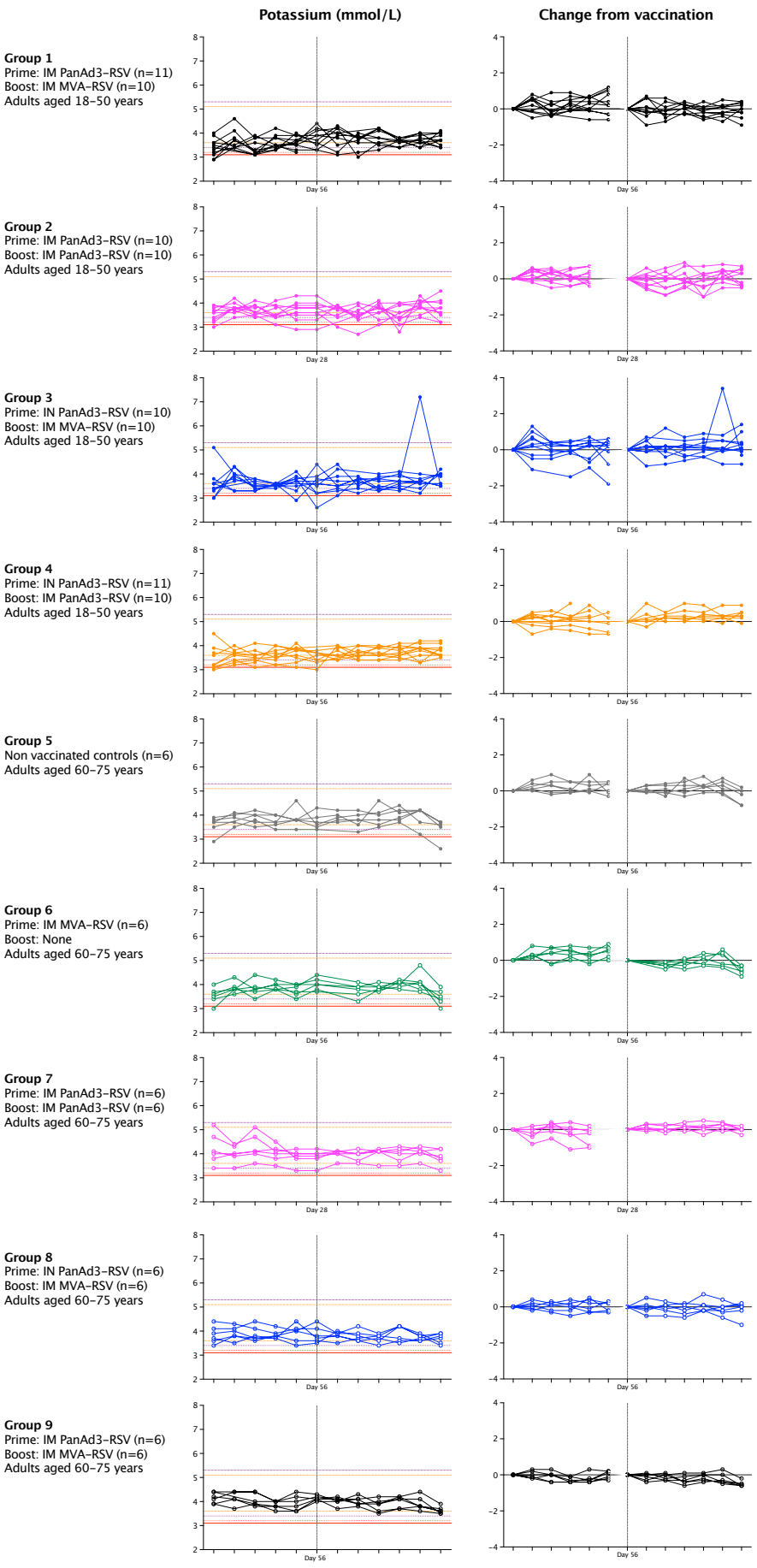
Supplementary figure 35. Safety bloods from the RSV001 trial: eosinophil count (x10^9/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



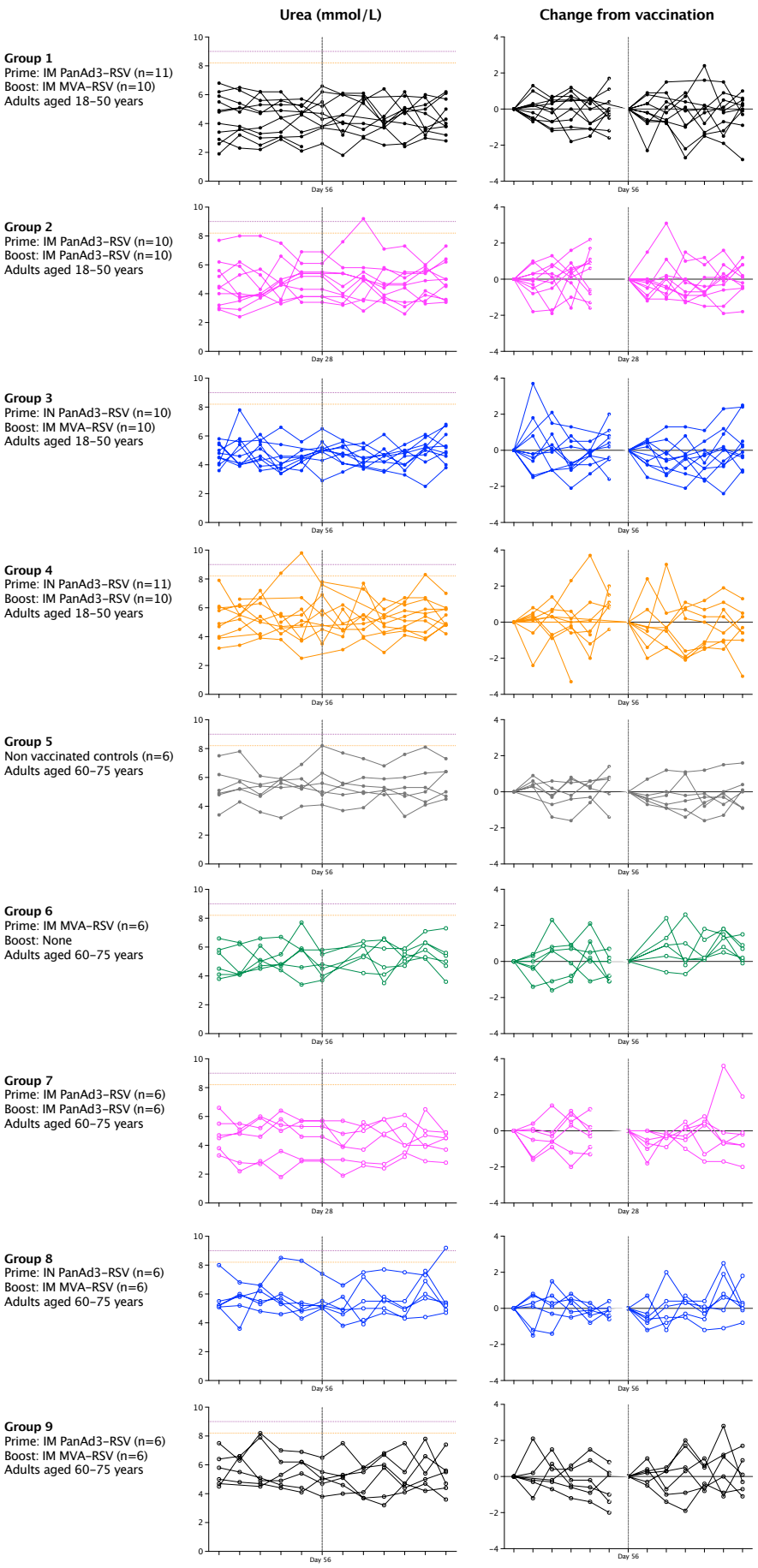
Supplementary figure 36. Safety bloods from the RSV001 trial: basophil count (x10⁹/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



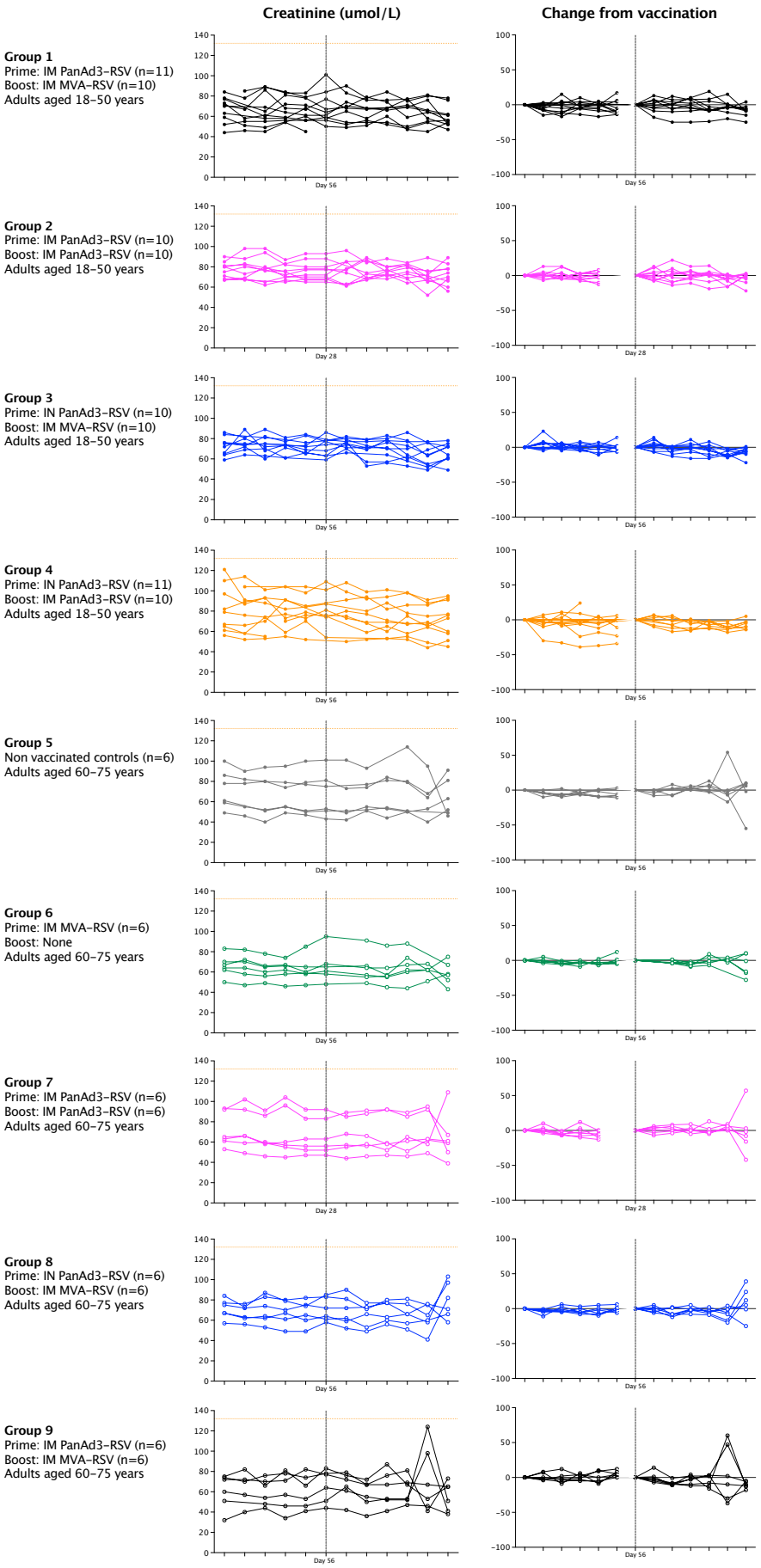
Supplementary figure 37. Safety bloods from the RSV001 trial: sodium (mmol/L). *Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.*



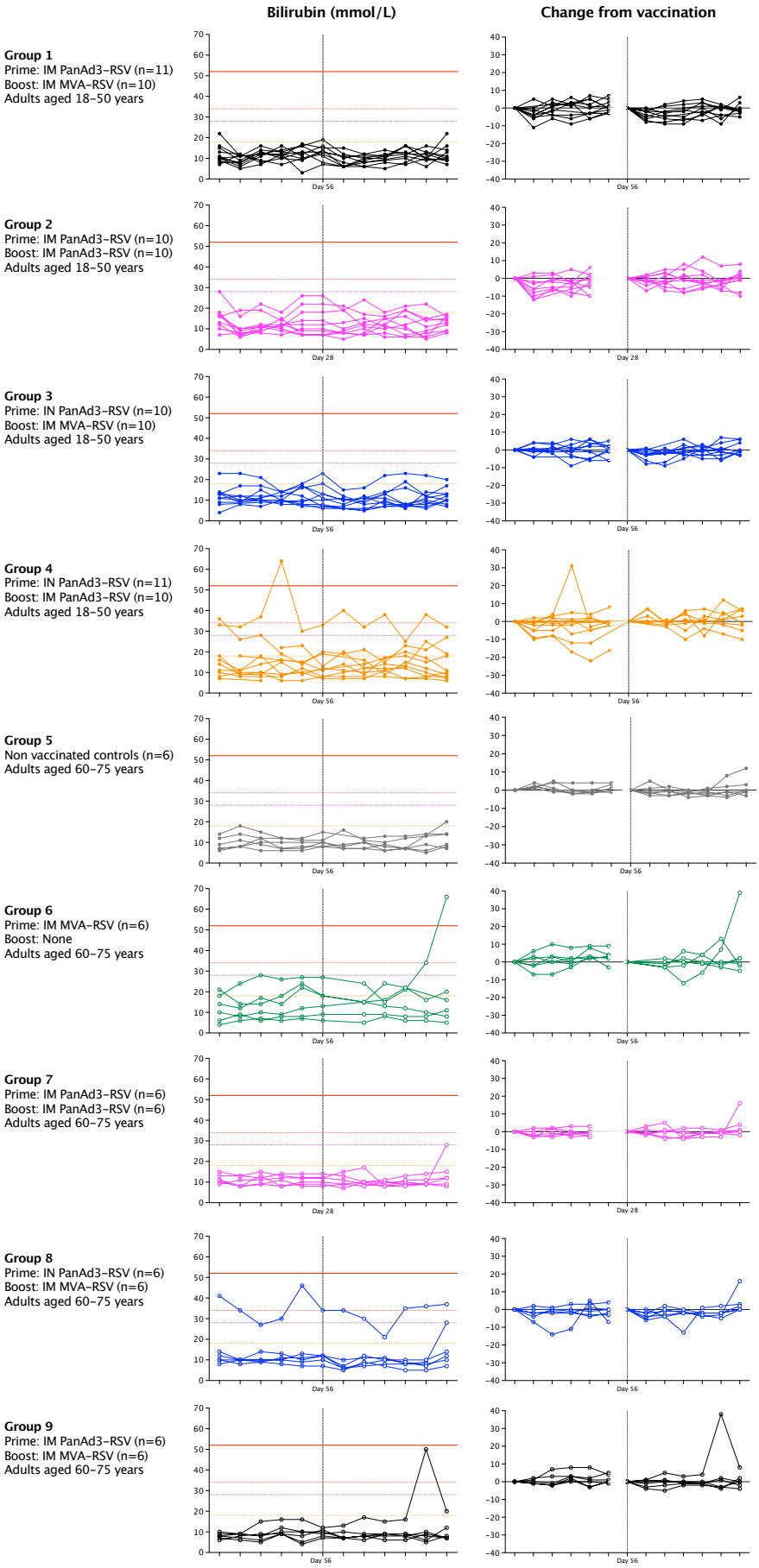
Supplementary figure 38. Safety bloods from the RSV001 trial: potassium (mmol/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



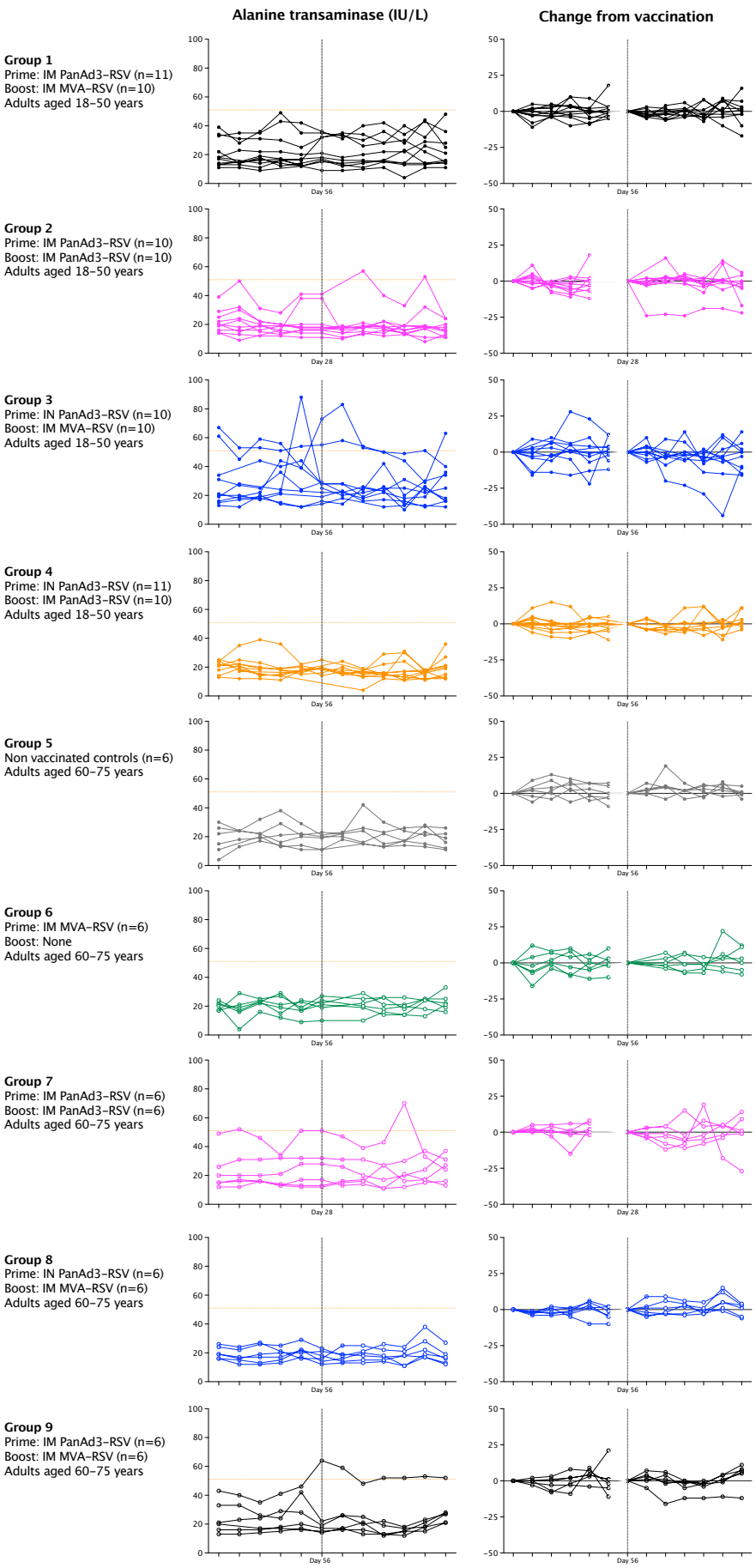
Supplementary figure 39. Safety bloods from the RSV001 trial: urea (mmol/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



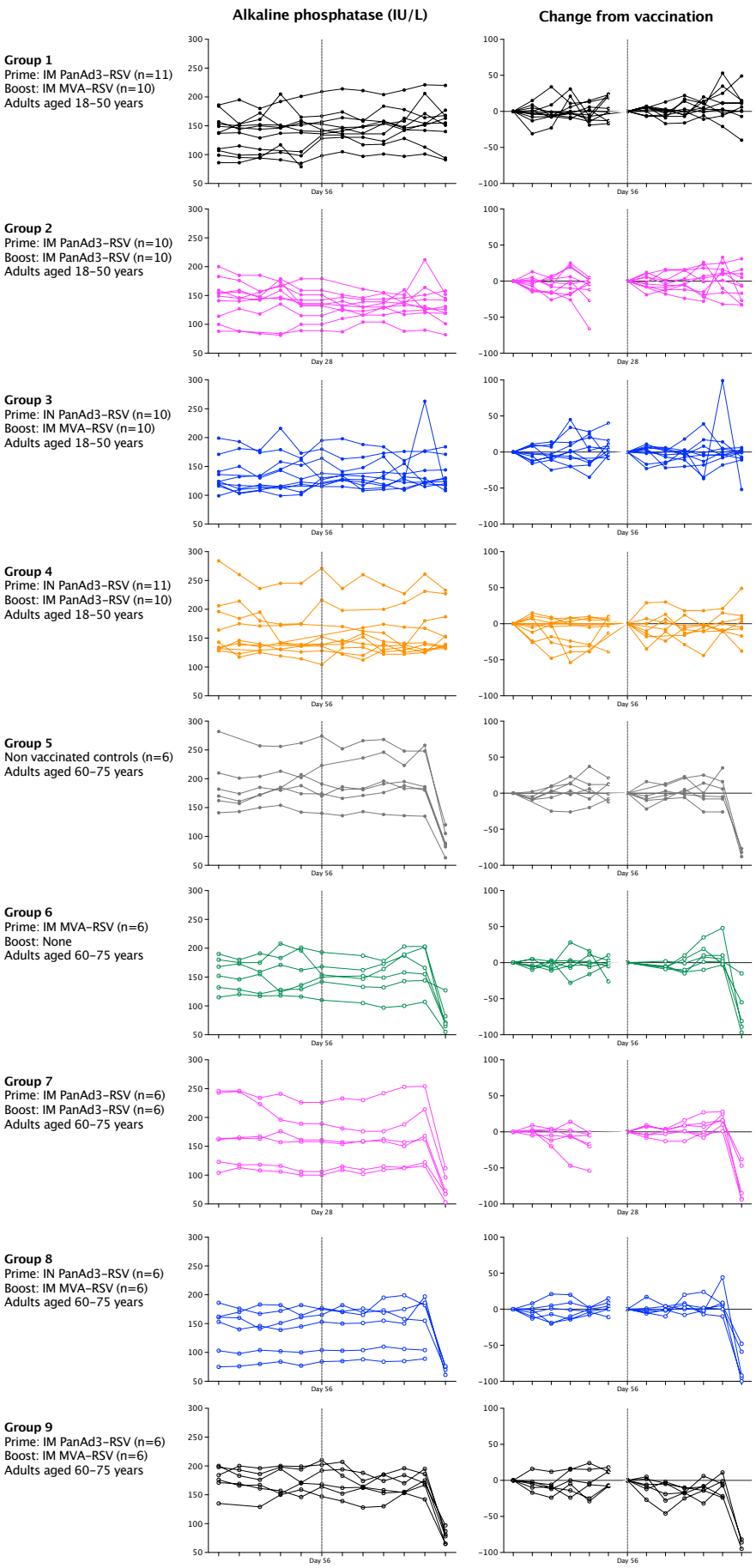
Supplementary figure 40. Safety bloods from the RSV001 trial: creatinine (umol/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



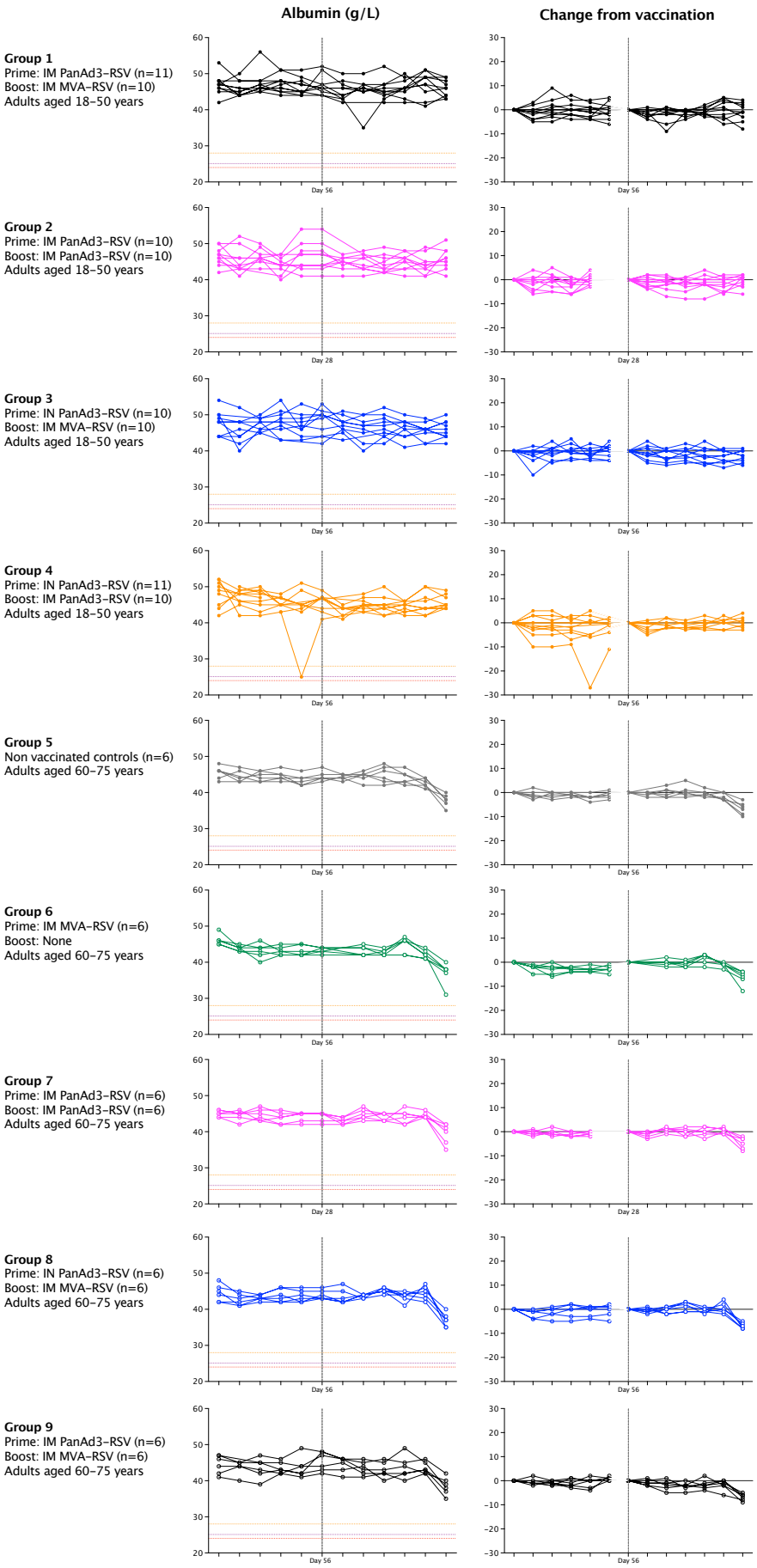
Supplementary figure 41. Safety bloods from the RSV001 trial: bilirubin (umol/L). *Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.*



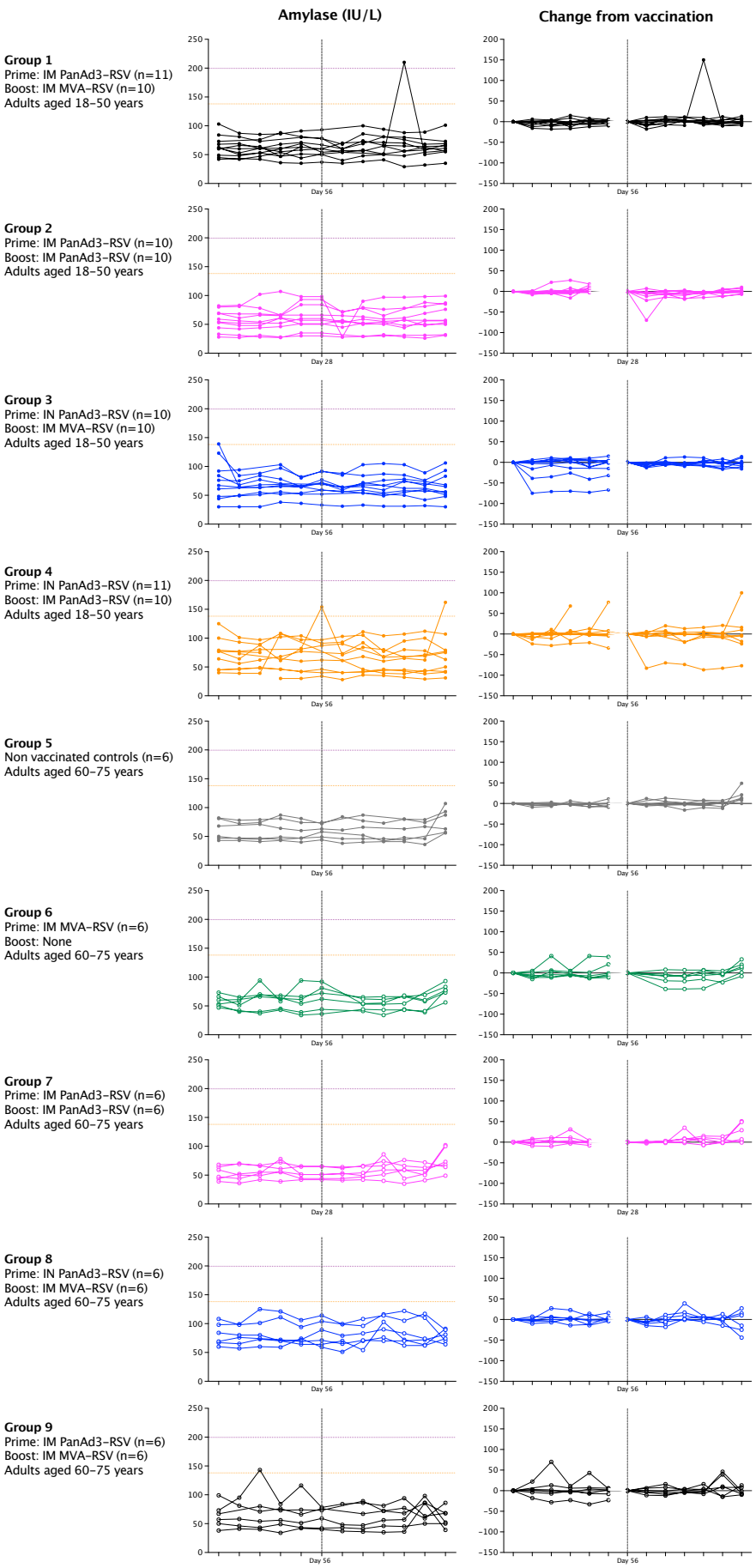
Supplementary figure 42. Safety bloods from the RSV001 trial: alanine transaminase (IU/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.



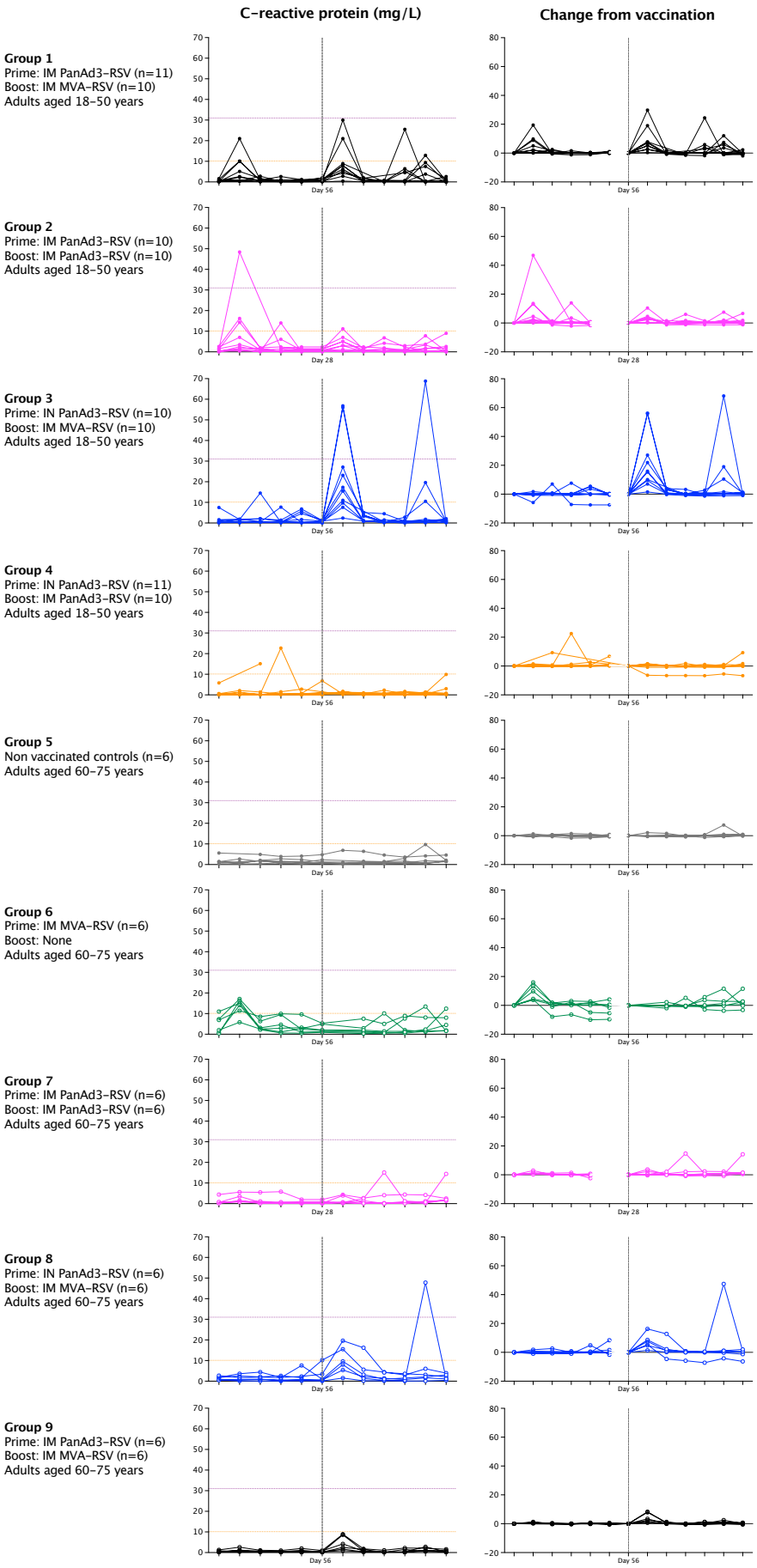
Supplementary figure 43. Safety bloods from the RSV001 trial: alkaline phosphatase (IU/L). *Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.*



Supplementary figure 44. Safety bloods from the RSV001 trial: albumin (g/L). *Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.*



Supplementary figure 45. Safety bloods from the RSV001 trial: amylase (IU/L). *Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.*



Supplementary figure 46. Safety bloods from the RSV001 trial: C-reactive protein (mg/L).
Each line represents a single volunteer for each study group. The vertical dotted line denotes the boost vaccine time-point, except for groups 5 and 6.

Haemoglobin (g/dL)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	14.1 (13.4-14.9)	13.8 (13.4-14.3)	13.9 (13.3-14.4)	14.3 (13.6-15.0)	13.4 (12.5-14.3)	13.6 (12.8-14.3)
Group 2	14.7 (13.7-15.8)	14.1 (13.1-15.1)	14.0 (13.2-14.9)	14.1 (13.1-15.1)	13.7 (12.6-14.9)	13.7 (12.9-14.4)
Group 3	14.6 (13.4-15.9)	14.3 (13.5-15.0)	14.5 (13.5-15.5)	14.8 (13.8-15.8)	13.2 (11.8-14.6)	14.0 (13.0-15.0)
Group 4	14.7 (13.4-16.1)	14.3 (13.0-15.5)	14.1 (13.0-15.1)	14.8 (14.0-15.6)	14.4 (13.8-14.9)	14.3 (13.6-14.9)
Group 5	14.7 (13.9-15.5)	14.2 (13.3-15.1)	14.3 (13.6-15.1)	14.3 (13.7-14.9)	14.2 (13.6-14.7)	14.2 (13.3-15.0)
Group 6	14.4 (13.6-15.2)	13.6 (12.8-14.4)	13.9 (12.9-14.8)	14.1 (13.2-15.0)	13.6 (12.7-14.5)	13.5 (12.5-14.4)
Group 7	14.5 (13.8-15.1)	14.0 (13.4-14.6)	14.0 (13.4-14.6)	14.0 (13.2-14.7)	13.7 (13.0-14.4)	13.6 (13.3-13.9)
Group 8	14.5 (14.0-14.9)	14.2 (13.8-14.6)	13.9 (13.3-14.4)	14.1 (13.6-14.6)	13.7 (13.0-14.3)	13.9 (13.5-14.3)
Group 9	14.8 (13.7-15.8)	14.5 (13.5-15.6)	14.2 (13.2-15.2)	14.8 (13.6-16.0)	14.0 (13.2-14.8)	13.7 (12.8-14.6)

Supplementary table 11. Summary statistics for haemoglobin measures after vaccination (g/dL).
Mean and 95% confidence intervals.

Total white cell count ($x10^9$/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	5.6 (5.0-6.3)	5.8 (4.9-6.8)	5.7 (4.9-6.5)	5.9 (5.1-6.7)	5.7 (5.1-6.3)	6.0 (4.9-7.2)
Group 2	6.0 (5.5-6.5)	5.8 (4.9-6.7)	6.3 (5.6-7.0)	5.8 (4.9-6.7)	6.2 (5.2-7.2)	6.3 (5.3-7.3)
Group 3	5.5 (4.7-6.2)	5.7 (5.1-6.3)	5.5 (4.7-6.2)	5.8 (5.2-6.5)	5.5 (4.5-6.4)	6.0 (5.3-6.7)
Group 4	5.7 (4.9-6.6)	5.8 (4.5-7.0)	5.4 (4.2-6.7)	5.4 (4.5-6.3)	4.8 (4.2-5.4)	5.5 (4.6-6.5)
Group 5	6.3 (5.4-7.2)	6.3 (5.3-7.3)	6.1 (5.3-6.8)	5.8 (4.9-6.7)	6.1 (5.1-7.0)	5.9 (4.8-7.0)
Group 6	5.4 (4.6-6.2)	5.7 (4.8-6.5)	6.5 (5.6-7.5)	5.9 (4.9-6.9)	5.4 (4.6-6.1)	5.4 (4.4-6.4)
Group 7	6.3 (5.5-7.1)	6.1 (5.5-6.8)	5.6 (4.6-6.7)	5.5 (4.8-6.1)	5.7 (4.2-7.2)	5.7 (4.8-6.6)
Group 8	5.7 (4.2-7.2)	5.7 (4.2-7.2)	5.4 (4.2-6.6)	6.3 (4.8-7.8)	5.5 (4.8-6.3)	6.0 (4.6-7.3)
Group 9	7.0 (6.3-7.7)	6.8 (6.1-7.5)	6.6 (5.7-7.4)	7.7 (6.4-9.0)	6.9 (6.0-7.8)	6.4 (5.7-7.1)

Supplementary table 12. Summary statistics for total white cell count measures after vaccination ($x10^9$/L).
Mean and 95% confidence intervals.

Platelet count ($x10^9$/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	266 (227-305)	283 (254-312)	272 (239-305)	276 (242-309)	253 (209-296)	274 (242-306)
Group 2	242 (215-269)	268 (216-319)	260 (215-306)	248 (218-277)	222 (183-261)	269 (228-310)
Group 3	229 (179-278)	250 (223-276)	254 (220-287)	250 (207-292)	201 (149-253)	268 (235-301)
Group 4	260 (215-305)	235 (193-276)	237 (207-268)	230 (200-260)	244 (207-280)	249 (213-284)
Group 5	246 (190-302)	248 (192-303)	251 (195-307)	249 (201-296)	257 (200-313)	263 (206-320)
Group 6	252 (219-284)	266 (236-296)	272 (243-301)	267 (236-298)	239 (203-274)	255 (219-290)
Group 7	256 (196-315)	241 (195-288)	254 (207-301)	255 (201-310)	241 (182-299)	265 (213-316)
Group 8	240 (191-290)	247 (200-294)	240 (195-285)	252 (204-301)	255 (214-296)	274 (211-337)
Group 9	260 (211-309)	266 (227-304)	259 (209-308)	269 (222-316)	262 (215-308)	276 (227-326)

Supplementary table 13. Summary statistics for platelet count measures after vaccination ($x10^9$/L).
Mean and 95% confidence intervals.

Neutrophil count ($x10^9$/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	3.2 (2.6-3.7)	3.1 (2.3-4.0)	3.1 (2.3-4.0)	3.3 (2.4-4.2)	3.1 (2.5-3.6)	3.5 (2.2-4.7)
Group 2	3.4 (2.9-4.0)	3.2 (2.3-4.0)	3.8 (3.1-4.5)	3.4 (2.7-4.0)	3.4 (2.6-4.2)	3.5 (2.7-4.3)
Group 3	3.0 (2.3-3.7)	3.2 (2.6-3.8)	3.1 (2.5-3.7)	3.3 (2.7-3.8)	3.1 (2.2-4.0)	3.6 (2.9-4.2)
Group 4	3.3 (2.6-3.9)	3.1 (2.3-3.9)	3.1 (2.0-4.3)	2.9 (2.3-3.5)	2.6 (2.1-3.1)	3.2 (2.5-4.0)
Group 5	4.1 (3.2-4.9)	4.1 (3.4-4.8)	3.9 (3.6-4.2)	3.8 (3.1-4.4)	3.8 (3.1-4.4)	3.7 (3.0-4.5)
Group 6	3.4 (2.9-4.0)	3.4 (2.8-4.0)	4.2 (3.4-5.0)	3.8 (2.9-4.6)	3.3 (2.7-4.0)	3.5 (2.5-4.5)
Group 7	3.5 (2.9-4.2)	3.2 (2.4-3.9)	2.9 (2.1-3.7)	2.9 (2.2-3.6)	3.0 (1.6-4.4)	2.9 (2.1-3.6)
Group 8	3.1 (2.0-4.3)	3.1 (1.9-4.2)	2.8 (1.9-3.7)	3.5 (2.3-4.7)	3.1 (2.3-3.9)	3.2 (2.0-4.4)
Group 9	4.2 (3.5-4.9)	3.9 (3.1-4.6)	3.8 (3.2-4.5)	4.8 (3.3-6.3)	4.1 (3.3-5.0)	3.7 (3.2-4.3)

Supplementary table 14. Summary statistics for neutrophil count measures after vaccination ($x10^9$/L).
Mean and 95% confidence intervals.

Sodium (mmol/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	142 (141-144)	142 (141-143)	143 (141-144)	142 (141-143)	142 (141-143)	142 (140-143)
Group 2	141 (139-142)	142 (140-144)	141 (141-142)	141 (140-142)	142 (141-143)	140 (139-142)
Group 3	141 (139-142)	142 (140-143)	142 (140-144)	142 (140-145)	141 (140-143)	141 (139-142)
Group 4	143 (142-144)	142 (140-143)	142 (141-143)	139 (135-143)	142 (141-143)	142 (140-143)
Group 5	142 (140-143)	139 (138-141)	139 (137-141)	140 (139-141)	140 (139-141)	140 (139-142)
Group 6	143 (142-143)	140 (139-141)	138 (136-139)	141 (140-143)	141 (140-141)	140 (139-140)
Group 7	138 (136-140)	138 (136-139)	140 (138-142)	139 (138-141)	139 (137-141)	142 (139-145)
Group 8	139 (138-139)	139 (138-139)	141 (140-141)	140 (139-140)	140 (139-140)	140 (139-140)
Group 9	139 (138-139)	140 (139-141)	139 (137-140)	140 (138-141)	139 (138-140)	139 (138-139)

Supplementary table 15. Summary statistics for sodium measures after vaccination (mmol/L).
Mean and 95% confidence intervals.

Potassium (mmol/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	3.4 (3.1-3.6)	3.4 (3.2-3.5)	3.6 (3.4-3.8)	3.7 (3.5-4.0)	3.6 (3.4-3.8)	3.8 (3.6-4.0)
Group 2	3.5 (3.3-3.7)	3.7 (3.5-3.8)	3.6 (3.4-3.7)	3.6 (3.4-3.9)	3.5 (3.2-3.7)	3.7 (3.5-3.8)
Group 3	3.5 (3.2-3.9)	3.5 (3.3-3.6)	3.5 (3.4-3.5)	3.5 (3.2-3.8)	3.6 (3.4-3.7)	3.6 (3.4-3.7)
Group 4	3.4 (3.1-3.8)	3.5 (3.3-3.7)	3.6 (3.4-3.7)	3.4 (3.2-3.6)	3.6 (3.5-3.8)	3.7 (3.5-3.8)
Group 5	3.5 (3.2-3.8)	3.8 (3.6-4.0)	3.7 (3.5-3.9)	3.7 (3.4-3.9)	3.7 (3.5-4.0)	3.9 (3.6-4.2)
Group 6	3.5 (3.2-3.7)	3.8 (3.5-4.0)	3.9 (3.8-4.0)	4.0 (3.8-4.2)	3.7 (3.5-3.9)	3.8 (3.7-3.9)
Group 7	4.2 (3.6-4.7)	4.2 (3.8-4.6)	4.0 (3.7-4.2)	3.8 (3.6-4.1)	3.9 (3.7-4.0)	4.0 (3.8-4.2)
Group 8	3.8 (3.5-4.1)	3.8 (3.6-4.1)	3.8 (3.7-3.9)	3.8 (3.5-4.1)	3.8 (3.6-3.9)	3.7 (3.5-3.8)
Group 9	4.1 (3.9-4.3)	4.0 (3.8-4.2)	3.8 (3.7-3.9)	4.1 (4.0-4.2)	4.0 (3.8-4.1)	3.8 (3.6-4.0)

Supplementary table 16. Summary statistics for potassium measures after vaccination (mmol/L).
Mean and 95% confidence intervals.

Urea (mmol/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	4.4 (3.4-5.3)	4.3 (3.4-5.1)	4.4 (3.7-5.2)	4.6 (3.8-5.4)	4.7 (3.8-5.5)	4.1 (3.4-4.8)
Group 2	4.6 (3.7-5.6)	4.7 (3.8-5.6)	4.8 (4.0-5.6)	4.9 (4.2-5.6)	5.0 (3.9-6.1)	4.7 (3.9-5.4)
Group 3	4.7 (4.2-5.1)	4.9 (4.3-5.4)	4.3 (3.7-4.9)	4.9 (4.3-5.5)	4.5 (4.1-4.9)	4.5 (4.0-5.0)
Group 4	5.1 (4.2-6.1)	5.3 (4.5-6.0)	5.1 (4.2-6.0)	5.8 (4.7-6.8)	5.3 (4.5-6.2)	4.9 (4.3-5.6)
Group 5	5.3 (4.2-6.4)	5.0 (4.3-5.7)	5.2 (4.4-6.1)	5.6 (4.5-6.8)	5.4 (4.4-6.3)	5.5 (4.9-6.0)
Group 6	5.0 (4.1-5.9)	5.3 (4.6-5.9)	5.1 (4.4-5.8)	4.7 (4.0-5.3)	5.5 (4.9-6.2)	5.2 (4.1-6.2)
Group 7	4.7 (3.7-5.6)	4.5 (3.3-5.7)	4.6 (3.3-6.0)	4.5 (3.5-5.5)	4.1 (3.1-5.2)	4.3 (3.1-5.5)
Group 8	5.6 (4.7-6.5)	5.8 (5.2-6.4)	5.9 (4.8-6.9)	5.5 (4.8-6.2)	5.5 (4.3-6.7)	5.7 (4.8-6.5)
Group 9	5.6 (4.7-6.5)	5.8 (4.5-7.2)	5.4 (4.5-6.2)	5.1 (4.3-5.8)	4.7 (3.9-5.5)	5.3 (4.1-6.6)

Supplementary table 17. Summary statistics for urea measures after vaccination (mmol/L).
Mean and 95% confidence intervals.

Creatinine (umol/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	67 (59-74)	66 (56-75)	67 (60-75)	67 (58-77)	65 (58-72)	66 (60-73)
Group 2	76 (71-81)	78 (71-86)	74 (70-79)	75 (70-81)	76 (71-82)	76 (72-79)
Group 3	72 (66-77)	73 (67-79)	72 (68-76)	72 (67-77)	69 (63-75)	71 (65-77)
Group 4	82 (67-96)	77 (66-89)	78 (67-88)	83 (72-95)	76 (66-87)	73 (63-84)
Group 5	72 (56-87)	66 (49-83)	67 (53-82)	67 (49-85)	67 (53-80)	63 (47-79)
Group 6	66 (57-74)	62 (54-70)	62 (54-69)	65 (53-78)	63 (51-75)	60 (49-71)
Group 7	71 (57-84)	66 (52-80)	69 (50-88)	65 (51-79)	67 (52-82)	66 (50-82)
Group 8	71 (63-78)	70 (59-81)	67 (58-76)	70 (61-79)	64 (55-73)	68 (60-77)
Group 9	60 (47-74)	59 (49-69)	61 (46-76)	66 (53-78)	57 (47-68)	62 (48-76)

Supplementary table 18. Summary statistics for creatinine measures after vaccination (umol/L).
Mean and 95% confidence intervals.

Bilirubin (mmol/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	11 (9-14)	11 (9-13)	11 (10-13)	12 (10-15)	9 (7-10)	10 (8-11)
Group 2	15 (11-18)	12 (9-15)	12 (10-13)	13 (9-17)	12 (9-15)	12 (9-14)
Group 3	11 (8-14)	12 (9-15)	11 (9-12)	11 (8-14)	9 (6-11)	11 (8-14)
Group 4	17 (10-23)	15 (8-22)	18 (7-30)	15 (9-21)	14 (9-19)	15 (9-20)
Group 5	9 (6-11)	10 (8-13)	9 (6-11)	10 (8-12)	9 (7-11)	8 (6-10)
Group 6	12 (6-17)	13 (7-20)	13 (7-19)	15 (9-21)	13 (8-19)	14 (9-18)
Group 7	11 (9-13)	11 (9-13)	11 (8-13)	10 (9-12)	10 (8-13)	9 (8-10)
Group 8	15 (5-25)	13 (7-18)	13 (7-20)	14 (6-22)	12 (5-19)	11 (7-15)
Group 9	8 (6-9)	8 (5-11)	10 (8-13)	9 (8-10)	9 (6-12)	9 (6-11)

Supplementary table 19. Summary statistics for bilirubin measures after vaccination (mmol/L).
Mean and 95% confidence intervals.

Alanine transaminase (IU/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	21 (15-26)	20 (15-26)	23 (15-31)	23 (17-29)	22 (15-29)	23 (16-30)
Group 2	21 (17-26)	19 (16-23)	17 (14-20)	20 (14-27)	21 (13-28)	19 (14-24)
Group 3	29 (17-41)	30 (19-41)	32 (22-41)	30 (19-42)	28 (19-38)	29 (20-37)
Group 4	20 (17-23)	19 (14-24)	18 (13-22)	18 (16-20)	15 (12-18)	16 (13-19)
Group 5	18 (10-25)	22 (17-26)	21 (13-29)	17 (13-21)	23 (14-31)	19 (13-24)
Group 6	20 (17-22)	22 (19-24)	20 (15-25)	20 (15-25)	20 (15-25)	20 (16-24)
Group 7	22 (11-33)	24 (14-33)	21 (13-28)	25 (13-37)	22 (14-30)	22 (12-32)
Group 8	20 (16-23)	19 (13-24)	18 (15-21)	17 (13-20)	18 (15-22)	18 (15-22)
Group 9	24 (15-33)	22 (15-28)	24 (16-31)	25 (9-40)	23 (13-33)	21 (9-34)

Supplementary table 20. Summary statistics for alanine transaminase measures after vaccination (IU/L).
Mean and 95% confidence intervals.

Alkaline phosphatase (IU/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	136 (117-156)	135 (116-153)	143 (121-165)	146 (128-164)	145 (124-166)	148 (127-169)
Group 2	144 (122-165)	142 (123-160)	143 (121-165)	133 (116-150)	130 (120-141)	133 (123-143)
Group 3	134 (116-153)	133 (115-150)	139 (117-162)	140 (122-158)	135 (118-153)	138 (121-155)
Group 4	168 (134-202)	159 (134-184)	155 (130-180)	160 (122-198)	156 (127-184)	157 (133-181)
Group 5	191 (151-231)	190 (160-219)	195 (167-223)	195 (157-233)	196 (160-233)	202 (164-240)
Group 6	156 (133-179)	153 (129-176)	155 (125-185)	152 (130-174)	147 (125-169)	148 (124-173)
Group 7	173 (125-221)	168 (127-210)	165 (124-205)	156 (118-195)	155 (118-193)	160 (121-199)
Group 8	140 (106-173)	136 (105-167)	138 (107-169)	143 (111-174)	142 (112-171)	147 (113-181)
Group 9	177 (158-196)	169 (150-187)	175 (155-195)	180 (160-200)	163 (147-179)	164 (147-181)

Supplementary table 21. Summary statistics for alkaline phosphatase measures after vaccination (IU/L).
Mean and 95% confidence intervals.

Albumin (g/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	47 (45-48)	47 (45-49)	47 (45-48)	46 (45-48)	45 (42-47)	45 (43-47)
Group 2	46 (44-48)	46 (45-47)	44 (42-45)	46 (43-48)	44 (43-46)	44 (42-45)
Group 3	47 (45-49)	47 (46-48)	47 (45-50)	47 (45-50)	46 (43-48)	46 (44-48)
Group 4	48 (45-50)	46 (44-48)	45 (44-46)	45 (43-47)	45 (44-46)	44 (43-46)
Group 5	45 (44-46)	44 (43-45)	44 (43-45)	44 (43-45)	44 (43-45)	45 (43-46)
Group 6	46 (44-47)	43 (41-44)	43 (42-44)	43 (42-43)	43 (42-44)	42 (42-43)
Group 7	45 (44-45)	44 (43-45)	43 (42-45)	44 (43-45)	44 (43-46)	44 (43-44)
Group 8	44 (42-46)	43 (42-43)	43 (42-45)	44 (42-45)	43 (43-44)	45 (44-45)
Group 9	44 (42-46)	43 (41-45)	43 (42-44)	45 (43-47)	43 (42-44)	43 (41-44)

Supplementary table 22. Summary statistics for albumin measures after vaccination (g/L).
Mean and 95% confidence intervals.

Amylase (IU/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	64 (54-74)	61 (53-69)	60 (49-70)	62 (52-73)	66 (54-79)	63 (52-74)
Group 2	57 (45-68)	57 (42-73)	57 (43-71)	62 (48-76)	58 (45-70)	56 (44-69)
Group 3	76 (55-97)	64 (52-76)	69 (57-81)	67 (56-78)	65 (52-78)	64 (49-78)
Group 4	72 (54-90)	69 (56-83)	71 (49-92)	76 (48-10)	69 (50-88)	62 (48-75)
Group 5	61 (47-75)	59 (45-73)	61 (46-76)	60 (50-69)	61 (46-76)	50 (35-65)
Group 6	58 (50-66)	62 (45-79)	56 (48-65)	64 (47-81)	53 (45-60)	52 (42-61)
Group 7	53 (44-62)	55 (47-63)	60 (49-71)	53 (45-60)	53 (45-61)	62 (49-75)
Group 8	81 (65-96)	85 (66-10)	83 (63-10)	83 (65-10)	80 (64-95)	94 (79-11)
Group 9	64 (47-80)	71 (41-10)	62 (46-77)	61 (47-75)	61 (42-79)	60 (46-74)

Supplementary table 23. Summary statistics for amylase measures after vaccination (IU/L).
Mean and 95% confidence intervals.

C-reactive protein (mg/L)	Prime			Boost		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Group 1	0.5 (0.2-0.8)	0.8 (0.4-1.3)	0.6 (0.1-1.0)	0.8 (0.4-1.2)	0.8 (0.4-1.3)	0.3 (0.2-0.5)
Group 2	1.1 (0.5-1.7)	1.0 (0.4-1.5)	2.7 (0.0-5.40)	0.8 (0.3-1.3)	0.8 (0.3-1.3)	1.6 (0.2-3.0)
Group 3	1.3 (0.0-2.7)	2.4 (-0.5-5.4)	1.3 (-0.1-2.7)	0.6 (0.3-0.9)	2.3 (1.2-3.4)	0.9 (0.1-1.8)
Group 4	0.9 (-0.3-2.1)	2.0 (-1.2-5.2)	3.3 (-2.1-8.7)	1.3 (-0.2-2.9)	0.3 (0.1-0.5)	0.5 (0.1-0.9)
Group 5	1.9 (0.4-3.4)	2.1 (0.9-3.3)	1.8 (0.8-2.9)	1.8 (0.5-3.1)	1.9 (0.1-3.7)	1.6 (0.4-2.8)
Group 6	4.7 (1.3-8.2)	4.2 (2.1-6.3)	4.8 (1.5-8.0)	2.7 (1.2-4.2)	2.5 (0.4-4.6)	3.1 (0.1-6.2)
Group 7	1.1 (-0.1-2.4)	1.4 (-0.2-3.0)	1.3 (-0.5-3.1)	0.7 (0.1-1.2)	1.2 (0.3-2.2)	3.3 (-1.5-8.1)
Group 8	1.4 (0.7-2.1)	1.7 (0.5-2.9)	1.2 (0.4-2.0)	2.6 (-0.5-5.7)	4.8 (0.0-9.5)	1.9 (0.4-3.4)
Group 9	0.5 (0.2-0.9)	0.6 (0.3-0.8)	0.4 (0.2-0.6)	0.5 (0.2-0.7)	0.9 (0.4-1.4)	0.4 (0.1-0.6)

Supplementary table 24. Summary statistics for C-reactive protein measures after vaccination (mg/L).
Mean and 95% confidence intervals.

Supplementary results for the immune response to vaccination

Performance of the assay and ASC data integrity; root cause analysis of failed samples

Plate failures: *ex-vivo* anti-F IgG ASC ELISpots

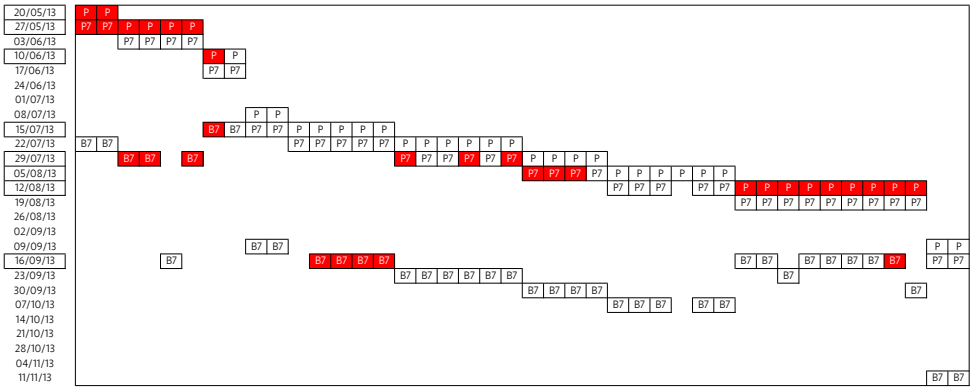


Plate number	N samples	Date	Failure reason	Comments
Plate 002	2	21/05/13	No IgG spots	IgG secondary antibody failure
Plate 004	4	28/05/13		
Plate 005	2			
Plate 013	1	11/06/13	Dark discoluration to all wells	No cause determined
Plate 029	1	16/07/13		
Plate 045	2	30/07/13		
Plate 047	4			
Plate 052	3	05/08/13	Laboratory error	Plates miscoded
Plate 061	4	13-14/08/2013	Contaminated	Speckling throughout the plate, presumed media contaminant
Plate 062	3			
Plate 065	3			
Plate 099	4	18/09/13	Not recorded	No cause determined
Plate 101	1			

Plate failures: *ex-vivo* anti-F IgA ASC ELISpots

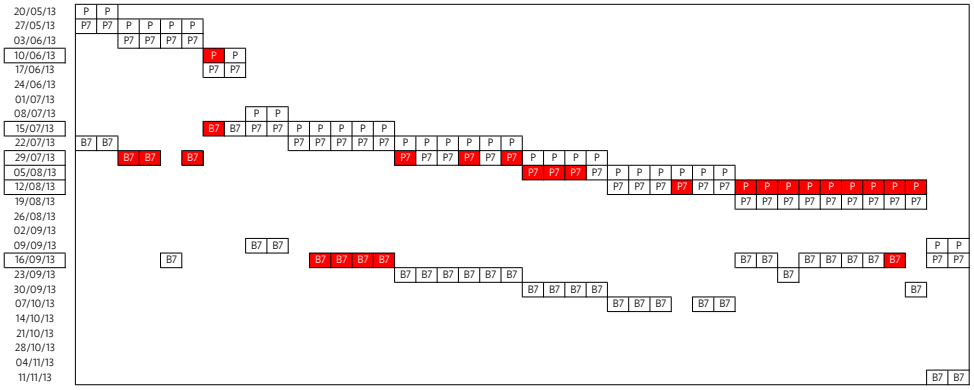


Plate number	Number of samples	Date	Failure reason	Comments
Plate 012	1	10/06/13	Dark discoluration to all wells	No cause determined
Plate 026	4	16/07/13		
Plate 045	2	30/07/13		
Plate 047	4			
Plate 052	3	05/08/13	Laboratory error	Plates miscoded
Plate 061	4	13 and 14/08/2013	Contaminated	Speckling throughout the plate, presumed media contaminant
Plate 062	3			
Plate 065	3			
Plate 099	4	18/09/13	Not recorded	No cause determined
Plate 101	1			

Supplementary figure 47. Ex-vivo IgG and IgA ASC ELISpot failures in 2013.
Each column represents one volunteer who was enrolled in 2013 (younger adults, n=42) by the week commencing in the left hand column. A boxed week identifies a week when one or more samples failed. P denotes the week of 'prime' vaccination, P7 and B7 were the visits conducted 7-days after 'prime' and boost respectively to measure IgG and IgA ASC responses to vaccination by dual-colour ex vivo ELISpot. Failed samples are shown in red, and a summary of the root cause analysis is in the table below. There was only one failed plate in 2014 from the trial in older adults (data not shown).

Performance of the ex vivo IFNg assay and ASC data integrity; root cause analysis

Plate failures: *ex-vivo* IFNgamma ELISpots

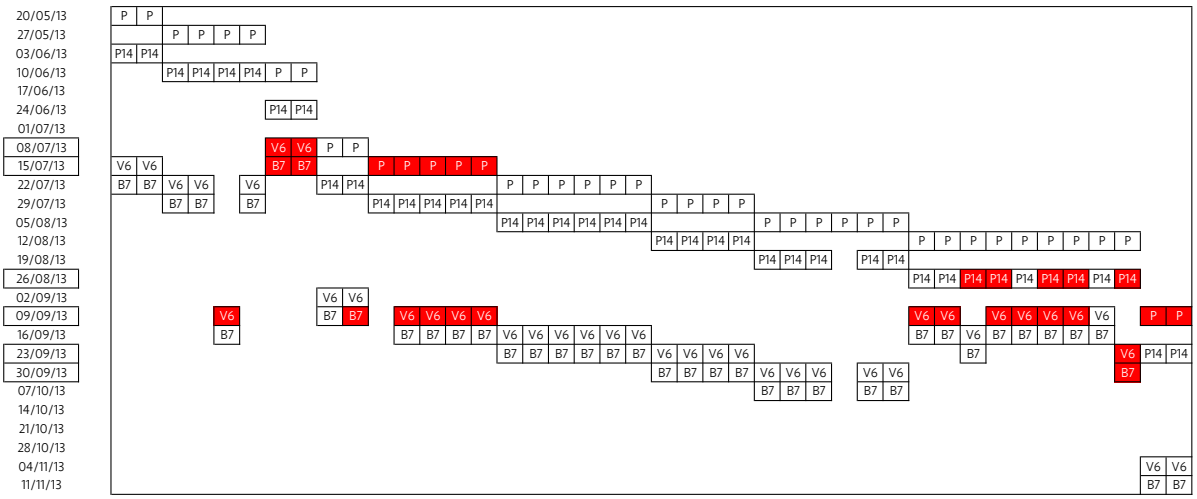
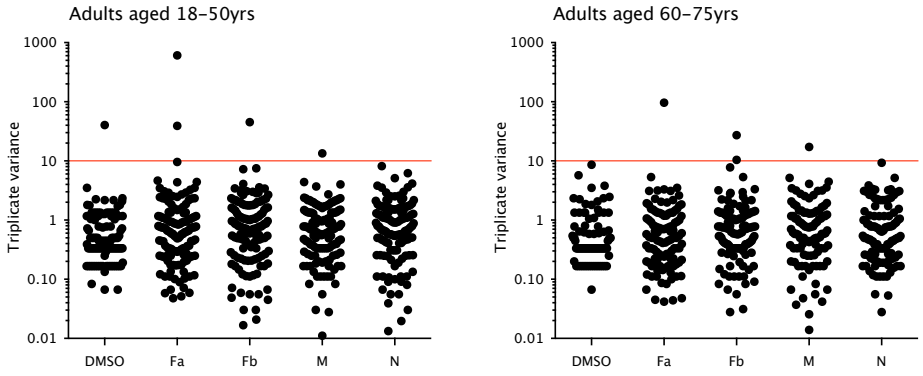


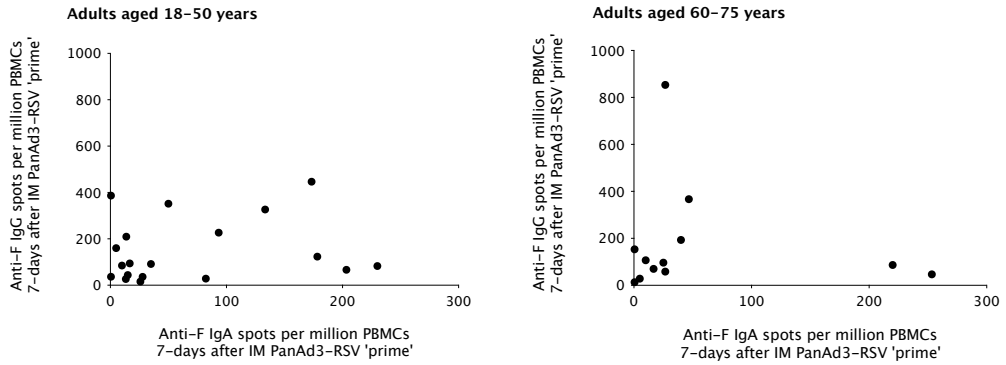
Plate number	N samples	Date	Failure reason	Comments
Plate 022	2	09/07/13	Positive control failure	Possible cell sedimentation or pipette error
Plate 025	3	16/07/13		
Plate 027	4			
Plate 074	2	28/08/13	Negative control failure	Presumed IFNgamma response to media
Plate 075	2			
Plate 076	1			
Plate 079	4	09/09/13		IFNg response to media (noted old preparation)
Plate 080	4			
Plate 082	1			
Plate 083	4			
Plate 084	1			
Plate 109	1	25/09/13		Presumed IFNgamma response to media
Plate 113	1	01/10/13		

Supplementary figure 48. Ex-vivo IFNγ ELISpot failures in 2013 by positive and negative control criteria.
Each column represents one volunteer who was enrolled in 2013 (younger adults, n=42) by the week commencing in the left hand column. A boxed week identifies a week when one or more samples failed. P denotes the week of 'prime' vaccination, P14 and B7 were the visits conducted 14-days after 'prime' and 7-days after boost respectively to measure the IFNγ response to vaccination by ex vivo ELISpot. Failed samples are shown in red, and a summary of the root cause analysis is in the table below. There was only one failed plate in 2014 from the trial in older adults (data not shown).

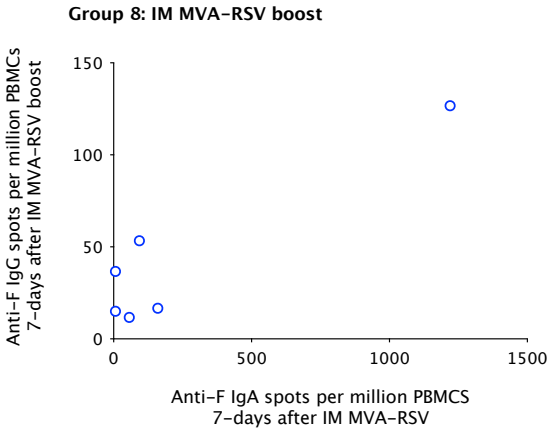


Supplementary figure 49. Ex vivo IFNγ ELISpot failures by triplicate variance criteria.
The triplicate variance for each peptide pool and DMSO background was calculated using the method by Moodie et al[346], and the result was excluded if the triplicate variance exceeded 10 (red line).

Supplementary correlation plots

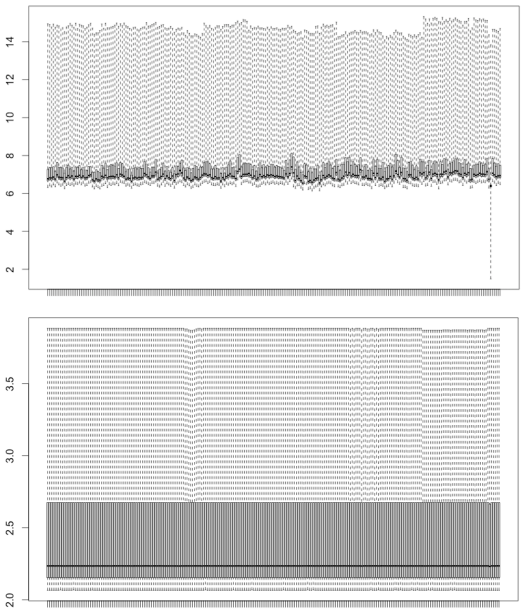


Supplementary figure 50. Correlation plot of anti-F IgG and IgA ASC responses to IM PanAd3-RSV prime vaccination in younger and older adults.

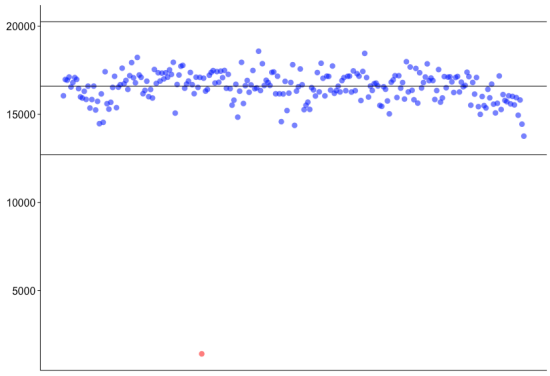


Supplementary figure 51. Correlation between the anti-F IgG and IgA ELISpot results for group 8 after IM PanAd3-RSV boost (older adults).
The number of volunteers was 6 and did not support correlation analysis; there did not appear to be any association in the anti-F IgG and IgA ASC responses to boost vaccination.

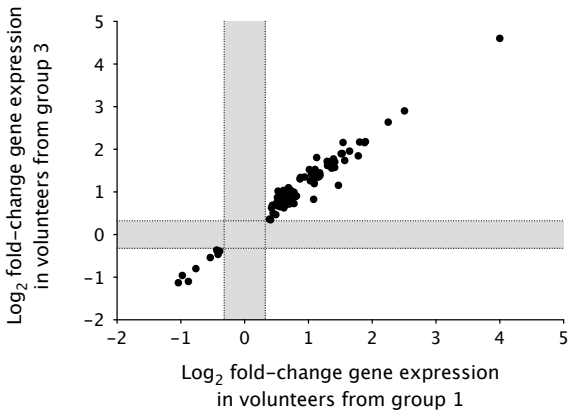
Supplementary results for the genetic response to vaccination



Supplementary figure 52. Box-plot figures before and after background correction and quantile normalisation.
The boxplot in each column represents one control gene probe by log2 florescent intensity. The box is the interquartile range, the bar is the median and the whiskers denote the range. The results are shown for before (top panel) and after normalisation (bottom) panel.

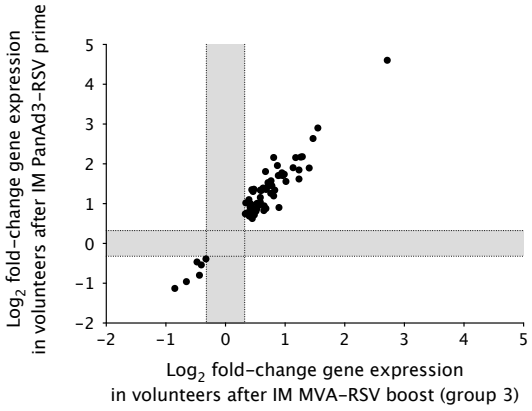


Supplementary figure 53. Outlier detection for one failed sample after repeat mRNA amplification and probe hybridization.
Each sample is represented by a single dot, and the number of genes detected is on the y-axis. The blue dots fall within the mean and 3 standard deviations of the number of genes detected (horizontal lines) and were accepted for analysis. The solitary red dot was a failed sample, for reasons unknown and despite repeat amplification and hydridization, with less than 2000 genes detected. This sample was a pre-vaccination time point, from a volunteer who then received high dose IN PanAd3-RSV 'prime'.



Supplementary figure 54. Correlation plot of the log_2 fold-change in gene expression 3-days after IM MVA-RSV boost for probes shared between IM and IN primed volunteers.

A total of 85 probes with a log_2 fold less than -1.25 or greater than 1.25 with 5% FDR-adjusted p-value <0.05 were differentially expressed in volunteers from both group 1 (IM PanAd3-RSV 'prime') and group 3 (IN PanAd3-RSV 'prime') 3-days after IM MVA-RSV boost vaccination. Differential probe expression was tightly correlated (p<0.001, two-tailed Pearson's correlation, r^2=0.96). They grey areas denote the threshold of exclusion for probes below the fold-change criteria.



Supplementary figure 55. Correlation plot of the log_2 fold-change in gene expression 3-days after IM MVA-RSV boost for probes shared between IM and IN primed volunteers.

A total of 85 probes with a log_2 fold less than -1.25 or greater than 1.25 with 5% FDR-adjusted p-value <0.05 were differentially expressed in volunteers from both group 1 (IM PanAd3-RSV 'prime') and group 3 (IN PanAd3-RSV 'prime') 3-days after IM MVA-RSV boost vaccination. Differential probe expression was tightly correlated (p<0.001, two-tailed Pearson's correlation, r^2=0.96). They grey areas denote the threshold of exclusion for probes below the fold-change criteria.

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