Cross-reactive immunity to SARS-CoV-2 and related coronaviruses

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"Nothing in life is to be feared,
only to be understood.
Now is the time to understand more,
so that we may fear less."

Marie Curie

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Chapters 3, 4, and 6

Sample collection and processing for the Family cohort were performed by Philip Goulder, Emily Adland, Anna Csala, Oliver Sampson, and Nicholas Lim. Peptide pools were provided by Anthony Brown. Stephanie Longet and Oliver Sampson assisted with the MSD immunoassays. Susanna Dunachie provided the data on adult immunity from the PITCH cohort. Wiebke Arlt at Imperial College London ran tandem mass spectrometry on steroid hormones in the adolescent cohort.

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List of Abbreviations

ACE2 . . . . . angiotensin converting enzyme 2

ADE . . . . . antibody-dependent enhancement

AID . . . . . activation-induced cytidine deaminase

AIM . . . . . activation-induced marker

APC . . . . . antigen-presenting cell

AMP . . . . . antimicrobial peptide

Bcl-2 . . . . . B-cell lymphoma 2

BCR . . . . . B-cell receptor

CCL . . . . . chemokine C-C motif ligand

CCR . . . . . chemokine C-C motif receptor

CD . . . . . cluster of differentiation

CD40L . . . . CD40 ligand

CD62L . . . . CD62 ligand

CDR3 . . . . . complementarity-determining region 3

CMV . . . . . cytomegalovirus

COVID-19 . . Coronavirus disease 19

Ct . . . . . cycle threshold

CTL . . . . . cytotoxic T-lymphocyte

CTLA-4 . . . . cytotoxic T-lymphocyte antigen 4

CTV . . . . . CellTrace Violet

CXCL . . . . . chemokine C-X-C motif ligand

CXCR . . . . . chemokine C-X-C motif receptor

DC . . . . . dendritic cell

DENV . . . . . Dengue virus

DMSO . . . . . dimethylsulphoxide

DMV . . . . . double-membrane vesicle

DNA . . . . . deoxyribonucleic acid

E . . . . . envelope

EBOV . . . . . Ebolavirus

EBV . . . . . Epstein-Barr virus

ELISA . . . . . enzyme-linked immunosorbent assay

ESN . . . . . exposed seronegative

EU . . . . . ELISA units

Fab . . . . . antigen binding fragment

Fc . . . . . crystallisable fragment

FCS . . . . . foetal calf serum

FOXP3 . . . . forkhead box P3

HA . . . . . haemagglutinin

HBV . . . . . Hepatitis B virus

HCoV . . . . . human coronavirus

HCV . . . . . hepatitis C virus

HCW . . . . . healthcare worker

HEV . . . . . hepatitis E virus

HIV . . . . . human immunodeficiency virus

HLA . . . . . human leukocyte antigen

HPV . . . . . human papillomavirus

IC50 . . . . . half-maximal inhibitory concentration

ICAM . . . . . intracellular adhesion molecule

ICS . . . . . intracellular cytokine staining

ICU . . . . . intensive care unit

IFN . . . . . interferon

Ig . . . . . immunoglobulin

IL . . . . . interleukin

IL2RG . . . . . IL-2 receptor gamma

ILC . . . . . innate lymphoid cell

JEV . . . . . Japanese encephalitis virus

kB . . . . . kilobase

kDa . . . . . kilodalton

LAIV . . . . . live-attenuated influenza vaccine

LCMV . . . . lymphocytic choriomeningitis virus

LFA-1 . . . . . lymphocyte function-associated antigen 1

LPS . . . . . lipopolysaccharide

LRR . . . . . leucine-rich-repeat

M . . . . . membrane

mAb . . . . . monoclonal antibody

MALT . . . . . mucosal-associated lymphoid tissue

MERS-CoV . Middle Eastern respiratory syndrome coronavirus

MHC . . . . . major histocompatibility complex

mRNA . . . . messenger RNA

MSD . . . . . Meso Scale Diagnostics

N . . . . . nucleocapsid

nAb . . . . . neutralising antibody

NFκB . . . . . nuclear factor kappa-light-chain-enhancer of activated B-cells

NK . . . . . natural killer

NLR . . . . . NOD-like receptor

NLRP3 . . . . NOD-containing, LRR-containing and pyrin-domain-containing protein 3

NOD . . . . . nucleotide oligomerisation domain

NSP . . . . . non-structural protein

NTD . . . . . N-terminal domain

OAS . . . . . original antigenic sin

ORF . . . . . open reading frame

PAMP . . . . . pathogen-associated molecular pattern

PBMC . . . . peripheral blood mononuclear cells

PBS . . . . . phosphate-buffered saline

PC . . . . . principal component

PCA . . . . . PC analysis

PCR . . . . . polymerase chain reaction

pDC . . . . . plasmacytoid dendritic cell

PHA . . . . . phytohaemagglutinin L

PITCH . . . . Protective Immunity from T-cells in Healthcare Workers

pp1a . . . . . polypeptide 1a

pp1ab . . . . . polypeptide 1ab

PRR . . . . . pattern recognition receptor

QIV . . . . . quadrivalent influenza vaccine

RAG . . . . . recombination activating gene

RBD . . . . . receptor binding domain

RIG-1 . . . . . retinoic acid-inducible gene 1

RLR . . . . . RIG-1-like receptor

RNA . . . . . ribonucleic acid

RSV . . . . . respiratory syncytial virus

RT . . . . . room temperature

RTC . . . . . replication-transcription complex

S . . . . . Spike

SAE . . . . . serious adverse event

SARS-CoV-1 . Severe acute respiratory syndrome coronavirus 1

SARS-CoV-2 . Severe acute respiratory syndrome coronavirus 2

SCF . . . . . stem cell factor

SD . . . . . standard deviation

SIV . . . . . simian immunodeficiency virus

SP . . . . . structural protein

TBEV . . . . . tick-borne encephalitis virus

TCR . . . . . T-cell receptor

$\mathbf{T_{FH}}$ . . . . . T-follicular helper

TGFβ . . . . . transforming growth factor beta

$\mathbf{T_H}$ . . . . . T-helper

TLR . . . . . Toll-like receptor

TMPRSS2 . . transmembrane serine protease S2

TNF . . . . . tumour necrosis factor

T_{REG} . . . . . Regulatory T-cell

UKHSA . . . . UK Health Security Agency

USN . . . . . unexposed seronegative

VOC . . . . . variant of concern

WNV . . . . . West Nile virus

WHO . . . . . World Health Organisation

XLA . . . . . X-linked agammaglobulinaemia

YFV . . . . . Yellow fever virus

ZIKV . . . . . Zika virus

1 Introduction

Contents

1.1 Introduction

The human immune system has evolved robust mechanisms for mediating protection against viral infection. Despite our comprehensive understanding of the complexities of human antiviral immunity, the continuous emergence of novel viral pathogens necessitates the constant investigation of human immune mechanisms in an ever-changing context of circulating pathogens. The coronavirus disease 19 (COVID-19) pandemic has provided an opportunity to study the role of antiviral immunity to a novel pathogen, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with unprecedented detail. Furthermore, the rapid deployment of vaccines against SARS-CoV-2 has offered an opportunity to understand vaccine-induced immunity

in what was initially a largely infection-naïve population.

Early in the pandemic, several researchers reported that pre-pandemic blood samples from SARS-CoV-2-unexposed individuals contained both T-cells and antibodies that were capable of recognising SARS-CoV-2.[1, 2] This was attributed to immune cross-reactivity between SARS-CoV-2 and its endemic relatives, the human coronaviruses (HCoVs) HCoV-HKU1, -OC43, -NL63 and -229E, as well as the more virulent severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) and Middle Eastern respiratory syndrome coronavirus (MERS-CoV) in the few individuals who had been exposed to these viruses.[3] However, the role of cross-reactive immunity in mediating protection against and resolution of SARS-CoV-2 infection is as yet unclear, and will be explored in this thesis.[4–8]

1.1.1 Canonical antiviral immunity

Innate recognition

Upon invading epithelial barriers and gaining entry to the interior of a host, viruses first trigger an inflammatory response through activation of innate immune components.[9] Primarily, virus-infected cells are recognised by phagocytic cells such as macrophages and granulocytes, which engulf infected cells and viruses by macropinocytosis, promoting the recognition of viral components by specific receptors. Recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on phagocytes, such as the recognition of nucleic acid derivatives by Toll-like receptors (TLRs), promotes the production of pro-inflammatory cytokines. These include interferons (IFNs) that are crucial for control of viral infection. For example, the recognition of single-stranded ribonucleic acid (RNA) by TLR7 in endosomes promotes the production of IFN: TLR signalling through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) induces the production of IFNα and IFNβ by most cell types. The detection of intracellular viral RNA by retinoic acid-inducible gene 1 (RIG-1) also contributes to the production of type 1 IFN. Plasmacytoid dendritic cells (pDCs) are key

producers of type 1 IFNs during viral infection, through activation of TLR7 and TLR9 by viral RNA and deoxyribonucleic acid (DNA). Secretion of cytokines by macrophages and dendritic cells (DCs) contribute to pathogen control by promoting fever, activating lymphocytes, increasing antibody production, and promoting access of effector cells such as neutrophils to sites of infection. In addition, natural killer (NK) cells and other types of innate lymphoid cell (ILC) are stimulated by cytokines during infection and generate immune mediators such as IFNα, as well as performing distinct cytotoxic functions.

Adaptive immunity

DCs represent the point of integration between the innate and adaptive immune systems by presenting antigen to lymphocytes for their activation. Adaptive immunity is mediated by the action of B- and T-cells, which, within an individual, consist of a remarkably diverse repertoire capable of specifically recognising many distinct pathogens. During lymphocyte development, V, D, and J gene segments undergo rearrangement through the action of recombination activating gene (RAG) to generate combinatorial diversity of gene segments. Diversity is further enhanced by somatic hypermutation by activation-induced cytidine deaminase (AID), as well as by junctional diversity at joint regions. These processes contribute a wide variety of T-cell receptors (TCRs) and B-cell receptors (BCRs), endowing lymphocytes with the ability to specifically recognise a wide variety of pathogens and promote effective viral control.[9]

T-cell development

The production of naïve T-cells occurs in central lymphoid tissues such as the bone marrow and thymus. T-cells originate from haematopoietic stem cells in the bone marrow, however, key stages of T-cell development occur in the thymus. T-cell precursors migrate from bone marrow to thymus, where Notch signalling induces T-cell fate in progenitor cells, leading to a period of proliferation. Gene rearrangement and changes in the expression of cell surface molecules such as CD3, CD4 and CD8 mark the stages of thymocyte development. Once the TCR

has been produced through gene rearrangement, T-cell development is reliant on interactions between peptide:major histocompatibility complex (MHC) molecules. They will undergo positive and negative selection to produce mature T-cells that interact weakly with self-antigens. Naïve T-cells will then circulate through the bloodstream and secondary lymphoid organs, such as the spleen, lymph nodes, and mucosal-associated lymphoid tissue (MALT). Secretion of chemokine C-C motif ligand- (CCL-) 19 by DCs attracts T-cells to lymph nodes through binding to chemokine C-C motif receptor- (CCR-) 7 on T-cells.

B-cell development

In contrast, B-cell development occurs continuously in the bone marrow. Stromal cells in contact with developing B-cells will direct development through the production of cytokines such as IL-7 and stem-cell factor. B-cells also originate from haematopoietic stem cells, which first undergo immunoglobulin (Ig) gene rearrangement of heavy chain and light chain segments, leading to their entry into the pre-B stage. Further gene rearrangement leads to IgM production on the cell surface, producing immature B-cells. Before migrating to the spleen, immature B-cells are assessed for autoreactivity, then will migrate to the spleen through recognition of DC-derived chemokine C-X-C motif ligand- (CXCL-) 13 by chemokine C-X-C motif receptor- (CXCR-) 5 on B-cells. At this stage, immature B-cells will differentiate into naıve, marginal zone, or follicular B-cells.[9, 10]

T-cell functions

T-cell activation occurs only when antigen-derived peptide fragments are displayed on the surface of infected cells in the context of the MHC family of proteins. Canonical models for T-cell activation require the integration of three distinct signals. The first signal for T-cell activation is the binding of the T-cell receptor (TCR) to the peptide:MHC complex on the antigen-presenting cell (APC). Co-receptors for this interaction are cluster of differentiation (CD) 4 and 8, which determine the specificity of the MHC interaction. CD4+ T-cells interact with MHC Class II molecules, which consist of two polypeptide chains and an open

peptide binding cleft that binds peptides of over 13 amino acids in length. MHC II molecules are expressed only on professional APCs such as DCs, macrophages, and B-cells. In contrast, CD8+ T-cells interact with the almost ubiquitously expressed MHC Class I, a polypeptide consisting of three subunits associated with β2-microglobulin. MHC I displays a more restricted binding cleft that associates with short peptides of 8-10 amino acids. The second signal for T-cell activation is the interaction of CD28, a member of the Ig superfamily, with B7.1 and B7.2 on the DC. To regulate T-cell activation, CD28 competes with another B7 receptor on T-cells, cytotoxic T-lymphocyte antigen 4 (CTLA-4). The inhibitory effects of CTLA-4 lie in its higher avidity for B7 compared to CD28, enabling competitive control of T-cell activation. The third and final signal for T-cell activation is the production of cytokines by the DC, such as interleukin (IL)-12 and type 1 IFN.[9, 11]

Once activated, T-cells undergo clonal expansion to produce progeny and begin secreting IL-2 to support their activation and differentiation. Upon differentiation into an effector cell, T-cells no longer require co-stimulation once they encounter their specific antigen. They stop recirculating through lymphoid tissues and instead migrate to sites of inflammation. DCs bind activated T-cells through interactions between lymphocyte function-associated antigen 1 (LFA-1) and CD2 with intracellular adhesion molecule (ICAM) 1 and 2 on the DC. Binding of a TCR to its cognate peptide:MHC complex promotes local effector function at sites of infection.[9]

CD4+ T-cell effector functions are determined by cytokine profiles during activation. For example, T-helper (T_H)1 cells produce IFNγ to activate macrophages, and this phenotype is driven by early IFNγ and IL-12 production by NK cells and other ILCs. T_H2 cells produce IL-5, IL-4, and IL-13 to recruit eosinophils, mast cells, and basophils, and resolve helminth infections. This is driven by the production of IL-4 by innate immune cells. T_H17 cells are driven by IL-6, IL-23, and transforming growth factor beta (TGFβ), and they secrete IL-17 to recruit neutrophils and IL-22

to produce antimicrobial peptides (AMPs). T-follicular helper (T_{FH}) cells migrate to B-cell follicles and promote the development of germinal centres, as well as providing B-cell help through the production of IFNγ or IL-4 to promote IgG or IgE class-switching, respectively. Regulatory T-cells (T_{REG}) suppress the activation of T-cells through constitutive expression of the IL-2 receptor CD25 and high expression of CTLA-4 and CD62 ligand (CD62L). Cytotoxic CD8+ T-cells mediate cell killing through the induction of apoptosis in target cells. Calcium-dependent release of cytotoxic granules containing granzyme, perforin, and granulysin by CD8+ T-cells induces apoptosis in target cells through the release of cytochrome C from mitochondria, activating caspases and leading to controlled cell death. This is particularly crucial during viral infection.[9]

B-cell functions

B-cells become activated through the interaction of CD40 with CD40 ligand (CD40L) on T-cells, which promotes B-cell survival through NFκB signalling. Furthermore, the production of IL-21 by T_{FH} cells activates B-cell proliferation, promoting differentiation into plasmablasts, germinal centre B-cells, plasma cells, and memory B cells. In contrast to the membrane-bound TCR, BCRs are secreted by plasma cells in the form of Ig. These antibody proteins consist of an antigen-binding variable region and an effector cell-binding constant region, consisting in total of two heavy and two light chains. The antigen binding regions (Fab) and the constant, crystallisable regions (Fc) are connected via disulphide bonds at the hinge region, which is flexible to allow binding to multiple antigens. In contrast to TCRs, which recognise linear peptides in the context of MHC molecules, antibodies bind to conformational epitopes with no requirement for an MHC association.

The constant region of an antibody confers specific function to the molecule – IgM and IgD are expressed on the surface of mature B-cells before class-switching occurs, whilst T-cell-mediated class-switching promotes the production of IgG, IgE, and IgA. Class-switching is regulated by the cytokine profile of T_{FH} cells, and

mediated through the interaction of CD40 and CD40L. Production of IL-21 and IFNγ promotes switching to IgG. This is the predominant antibody in blood and extracellular fluid, with roles in pathogen opsonisation and complement activation. IL-5 promotes the production of IgA, the major component of lamina propria secretions at the intestinal and respiratory tracts, whilst IL-4 promotes IgE switching beneath the skin and mucosa – this primarily functions to sensitise mast cells. In addition to class switching, germinal centre B-cells undergo somatic hypermutation at V-regions, resulting in the production of antibodies with greater affinities for antigen. This process is also reliant on CD40:CD40L interactions with T_{FH} cells. The adaptive immune response is therefore tightly integrated at the level of immune cell interactions, enabling specific and highly regulated responses to viral infection.[9]

B-cell memory

A key characteristic of adaptive immunity is the ability of lymphocytes to form memory cells that circulate in the body for long periods of time, even in the absence of their specific antigen, in case of re-infection with the same pathogen. Any circulating antibody still present in the blood upon secondary infection will be able to immediately bind to pathogens without the need for plasma cell generation and Ig production. If antibodies are insufficient to completely neutralise the pathogen, memory B-cells will bind antigens to initiate a secondary immune response. Memory B-cells, like naïve B-cells, circulate through secondary lymphoid tissues such as the spleen, lymph nodes, and Peyer's patches in the MALT. They arise from germinal centres as well as from plasma cells during primary infection, and often express class-switched isotype such as IgA or IgG. This is in contrast to naïve B-cells expressing IgM or IgD, or plasma cells that express low surface Ig. Upon secondary infection or exposure through vaccination, class-switched memory cells will produce class-switched antibody more rapidly than naïve B-cells that have not yet undergone class switching. Memory B-cells also express higher levels of MHCII molecules and B7.1 than naïve B-cells, enabling enhanced antigen presentation through interactions with CD28 on T_{FH} cells. As well as differentiating into plasma cells, memory B-cells

are also able to re-enter germinal centres to experience further affinity maturation and somatic hypermutation before becoming plasma cells. This process generates antibodies with continuously higher affinity and avidity for antigens.[9]

T-cell memory

Memory T-cells are also generated after primary infection. Memory T-cell survival is dependent upon IL-2, IL-7, and IL-15 signalling, both for CD4+ and CD8+ memory cells, and is less dependent on contact with self peptide:MHC complexes compared to naïve T-cells. A decrease in CD62L expression as well as increased CD44 expression results in memory T-cells migrating from blood to peripheral tissues rather than lymphoid tissues. Upon restimulation with antigen, memory T-cells rapidly produce cytokines including IL-2, TNF-α, and IFNγ.[9]

Memory T-cells can be classified into three major types – central memory, effector memory, and tissue-resident memory. Detailed immunophenotyping of memory T-cells through flow cytometry has enabled fine dissection of the cell surface markers and functional properties of these memory groups.[12] Central memory T-cells are marked by expression of CCR7, enabling trafficking through peripheral lymphoid tissues in a similar way to naïve T-cells. Central memory T-cells readily cross-link their TCRs, proliferate and express CD40L in response to antigen, though they are slow to develop effector functions following stimulation. Effector memory T-cells do not express CCR7 but instead express integrins to enable trafficking to inflamed tissues, as well as inflammatory chemokine receptors for quick maturation and early IL-4 and IL-5 expression. They circulate through the blood into peripheral nonlymphoid tissues, through the lymphatic system and into secondary lymphoid tissues. Finally, tissue-resident memory T-cells are retained at epithelial sites such as the dermis or lamina propria, through expression of chemokine receptors such as CXCR3 and CCR9 and induction of CD69. The three subsets of memory T-cells are themselves heterogeneous and fluid – loss of CCR7 expression by central memory T-cells enables differentiation into effector memory cells, for example.[9] Studies

of viral infection demonstrate that memory T-cells are crucial for viral clearance following secondary infection with a pathogen, and CD8+ memory T-cells have been demonstrated to survive for 75 years following primary exposure.[13]

1.1.2 SARS-CoV-2

SARS-CoV-2 was first identified as the causative agent of a severe pneumonia of previously unknown aetiology in December 2019 in Wuhan, China.[14] Following its rapid global spread, a pandemic was declared by the World Health Organisation (WHO) in March 2020, resulting in international lockdowns and severe global disruption. As of June 2023, over 600 million confirmed cases of SARS-CoV-2 infection have been recorded, leading to over six million deaths worldwide.[15] SARS-CoV-2 spread initially in Wuhan before transmitting throughout China, and finally, despite lockdown measures in China, ongoing international travel resulted in transmission on a global scale. Asymptomatic transmission combined with high transmissibility appeared to enable its global spread.[16]

SARS-CoV-2 is a member of the *Coronaviridae* family of positive sense, single-stranded RNA viruses, alongside SARS-CoV-1, MERS-CoV, and the endemic HCoVs HCoV-229E, HCoV-NL63, HCoV-HKU1, and HCoV-OC43. Previously, coronaviruses were believed to cause severe disease primarily in bird and non-human mammal populations, but, in recent years, their ability to transmit zoonotically and infect humans has been demonstrated by the emergence of SARS-CoV-1, MERS-CoV, and SARS-CoV-2.[17] Coronaviruses have some of the largest genomes of all RNA viruses: the 30 kilobase (kB) RNA genome of SARS-CoV-2 sits within the nucleocapsid (N) protein, surrounded by an envelope (E), membrane glycoprotein (M) and homotrimeric spike (S) proteins that mediate cell entry (Figure 1.1A). These four structural proteins (SPs) are critical for the assembly of intact virions. The SARS-CoV-2 S is highly similar to that of SARS-CoV-1, with an amino acid homology of 78%, and is composed of an N-terminal S1 region and C-terminal S2 region.[18] At the border of these subunits is a furin cleavage site that is essential

for viral entry. The S1 region contains the receptor-binding domain (RBD) that interacts with angiotensin converting enzyme 2 (ACE2) for host cell entry, whilst the S2 region mediates cleavage and membrane fusion (Figure 1.1B). By the binding of S to ACE2 on host cells, and interactions with transmembrane serine protease S2 (TMPRSS2), SARS-CoV-2 enters the cell through one of two pathways: endocytosis, or fusion directly with host cell membranes (Figure 1.2). Following uncoating, the positive-sense, single-stranded genome is directly translated. The SARS-CoV-2 genome encodes 16 non-structural proteins (NSPs), as well as 9 accessory proteins within 14 open reading frames (ORFs).[18] Proteins involved in RNA synthesis are encoded on 16 NSPs within ORF1a and ORF1b. This region accounts for around 70% of the viral genome, and the roles of the 16 NSPs are outlined in Table 1. Upon viral entry, ORF1a is translated to produce polypeptide 1a (pp1a), a 500 kilodalton (kDa) peptide that is cleaved into 11 NSPs. A frameshift upstream of the ORF1a stop codon enables continued translation of ORF1b, to produce polypeptide 1ab (pp1ab), a large protein that is cleaved into 15 NSPs by the proteolytic action of NSP3 and NSP5.[17] Viral replication occurs inside double-membrane vesicles (DMVs), after which nascent RNA is translated and mature virions assembled and released from cells (Figure 1.2).[19]

1.1.3 Human infection with SARS-CoV-2

In most cases, SARS-CoV-2 causes upper and lower respiratory tract infections that are mild in nature, and are associated with fever, cough, anosmia, and ageusia. An estimated 20-40% of cases are asymptomatic, whilst ∼15% of patients experience severe disease or death.[20, 21] Severe disease is associated with respiratory distress, systemic inflammation, cytokine storm, and widespread tissue damage.[21] The process of viral entry and replication makes SARS-CoV-2 vulnerable to detection by innate immune components, such as TLRs 1, 2, 4, 6 and 7, and enables production of type I IFN by NK cells and macrophages, as well as the production of tumour necrosis factor (TNF)α, IL-6, and IL-1.[21] Intracellularly, single-stranded RNA from SARS-CoV-2 can be recognised by RIG-1-like receptors (RLRs) and

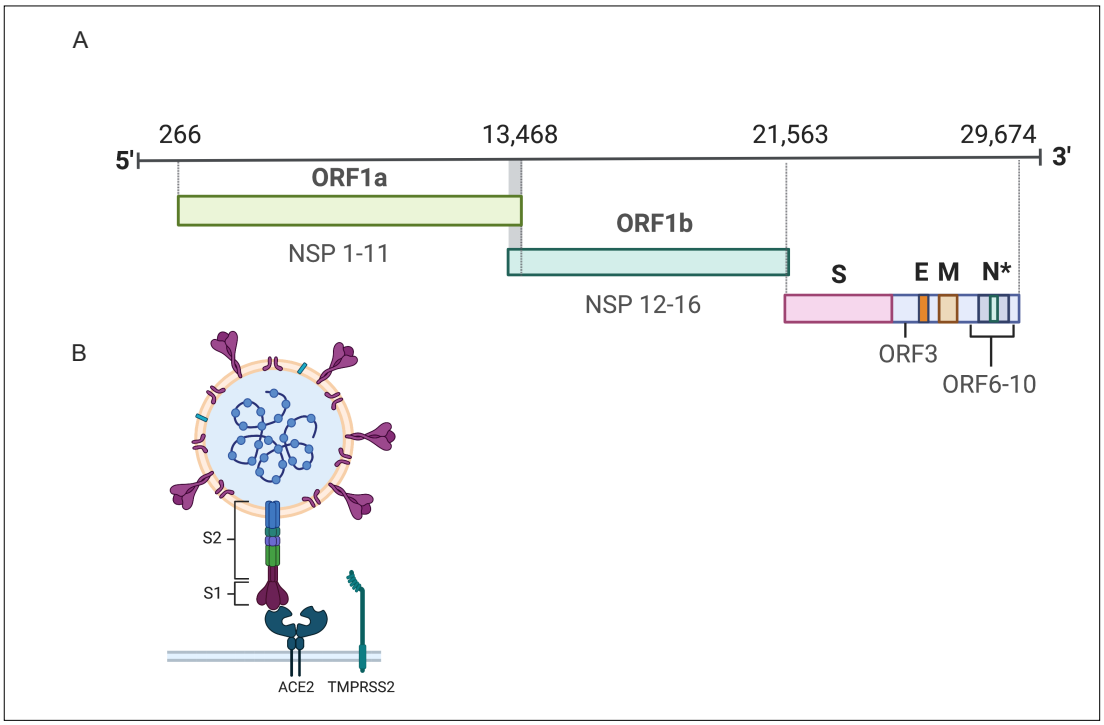


Figure 1.1: SARS-CoV-2 genome (A) and ACE2 binding (B)

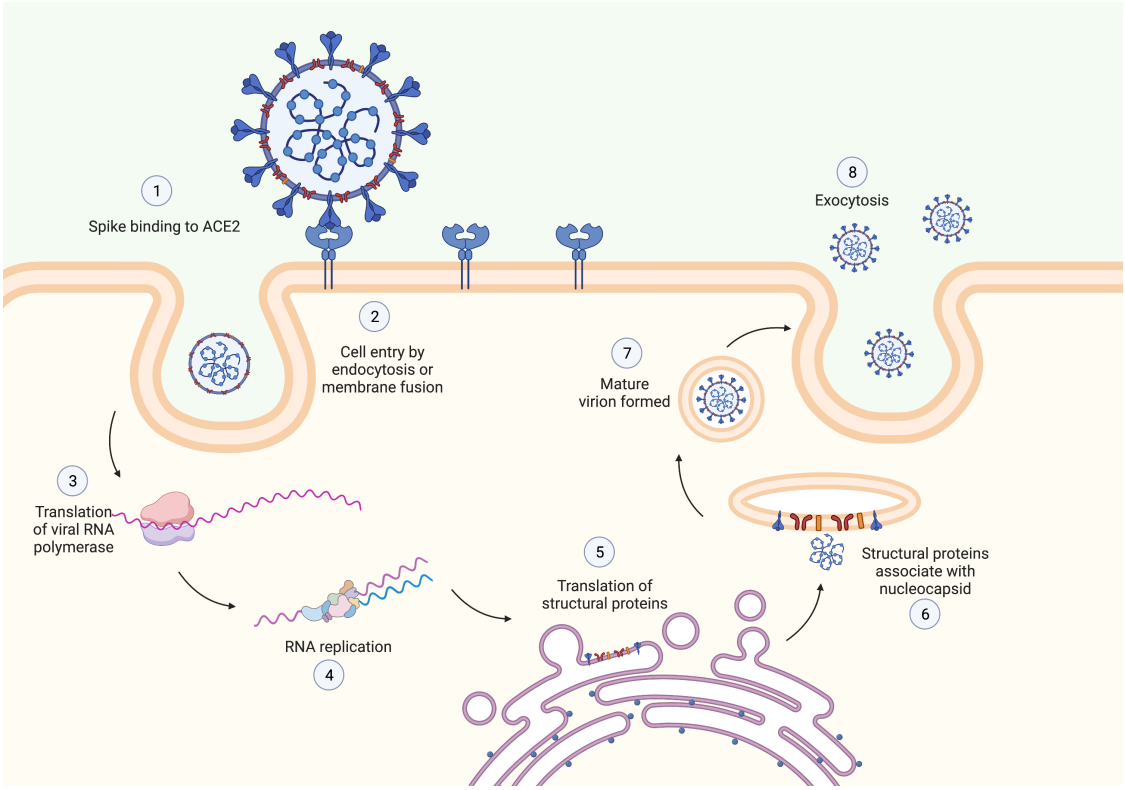


Figure 1.2: SARS-CoV-2 replication cycle

NSP	Function
NSP1	mRNA degradation, innate evasion
NSP2	Viral growth, RNA synthesis
NSP3	Protein scaffold, viral replication, protease activity
NSP4	Interaction with cell membrane
NSP5	Main protease
NSP6	Antiviral evasion
NSP7	Stabilisation of NSP8
NSP8	Catalysis of synthesis of RNA primers
NSP9	Nucleic acid binding
NSP10	Regulation of viral replicase
NSP11	Unknown
NSP12	RNA dependent RNA polymerase
NSP13	Helicase
NSP14	Exonuclease, reduction in incidence of mismatched nucleotides
NSP15	Endonuclease, degradation viral RNA
NSP16	Innate evasion

Table 1.1: SARS-CoV-2 NSP functions. Functions from Bai *et al.* [18]

nucleotide oligomerisation domain- (NOD-) like receptors (NLRs), facilitating further production of type I and type III IFN. Higher levels of pro-inflammatory cytokines such as IL-1β, IL-6, TNF, IL-12, IFNβ, IL-17, and IFNγ are associated with acute COVID-19. These cytokines are crucial for clearing infection yet can become dysregulated in severe disease and cause significant tissue damage.[21] Furthermore, SARS-CoV-2 is able to effectively evade innate immunity, such as by reducing levels of type I and III IFN in the blood through post-transcriptional modification of IFN transcripts.[22–24] In addition, interference with RLR and RIG-1 signalling pathways can further attenuate IFN and pro-inflammatory cytokine responses.[21, 25] Reduced IFN levels have been identified in patients with severe COVID-19, and it has been suggested that poor or delayed IFN responses can enable viral replication to gain a foothold and contribute to severe disease.[26, 27]

Mild disease and successful disease resolution are both associated with robust B- and T-cell-mediated immunity to SARS-CoV-2.[1, 28–32] S-specific CD4+ T-cells are identified in almost all COVID-19 convalescents, can be observed 2-4 days following symptom onset, and are associated with more mild disease.[2, 28,

30] T_{FH} cells are generated during acute infection and provide B-cell help, whilst T_H1 cells generate IFNγ, TNF, and IL-2.[28] T_H1 CD4+ T-cells are associated with viral control for many diseases, including COVID-19 and influenza, with T_H2 phenotypes often associated with severe disease.[33, 34] However, early concerns about T_H2 skewing and immunopathology were alleviated by the consistent T_H1 phenotype of specific CD4+ cells in COVID-19 patients.[2, 29, 30] The majority of CD4+ T-cells in COVID-19 convalescents display a central memory phenotype, with the development of polyfunctional stem cell-like memory T-cells that are durable for up to 10 months following infection.[35, 36] CD8+ T-cells are also correlated with milder disease in acute infection and can be detected early in disease course, although are less commonly identified compared to CD4+ cells. CD8+ cells in SARS-CoV-2 infection display classic cytotoxic functions, such as the expression of granzyme B and perforin.[29, 37] Severe COVID-19 is often associated with lymphocytopenia, characterised by loss of $\sim$80% of the detectable peripheral T-cell pool alongside CD8+ proliferation of the other 20%.[38]

Serostatus is used as the gold standard to assess prior SARS-CoV-2 infection due to the vast majority of infected individuals generating a robust IgA, IgG, and IgM response.[36, 39] Neutralising antibody (nAb) responses to SARS-CoV-2 primarily target the RBD region of S, and in some cases the N-terminal domain (NTD) as well as the S2 region, particularly the fusion peptide.[28] These antibodies exhibit low levels of somatic hypermutation and affinity maturation early in convalescence, with increasing breadth, affinity, and accumulation of somatic mutations in the 6 months after infection.[40] nAb titres in convalescence appear to correlate positively with disease severity and antigen load, which is also seen in SARS-CoV-1 and MERS-CoV.[41, 42] Enzyme-linked immunosorbent assay (ELISA) IgG titres and nAb titres peak between 3 and 4 weeks after symptom onset, and are stable for months following infection, particularly IgG.[43] Although SARS-CoV-2-specific antibodies appear less durable than T-cells, memory B-cells do persist once humoral immunity has waned.[44–46] Most memory B-cells are IgG, have undergone affinity

maturation, and are more RBD-focussed at later timepoints, reflecting germinal centre responses.[40]

A key challenge in maintaining robust immunity to SARS-CoV-2 is the evolution of immune escape mutations in SARS-CoV-2 that evade nAbs. Genomic surveillance of viral sequences has enabled detailed analyses of SARS-CoV-2 evolution.[47] Several SARS-CoV-2 variants of concern (VOCs) have been identified, with particular global interest focussed on those with apparent changes in transmissibility and disease severity such as B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.617.2 (Delta), and BA.1-5 (Omicron).[47] The E484K mutation was first identified following apparent reduced neutralising ability of some monoclonal antibodies (mAbs) to new lineages of SARS-CoV-2.[48] Additionally, the N439K mutation, which has arisen independently in multiple SARS-CoV-2 lineages, also confers evasion of antibody-mediated immunity.[49] More substantial immune escape has been described in the Omicron variant, which has accumulated more than 30 mutations in S and is associated with an over 20-fold decrease in neutralising activity.[50] However, T-cell responses in SARS-CoV-2 convalescents appear more resistant to escape mutations.[51, 52] This may result from the broader epitope repertoire of specific T-cells in COVID-19, as well as T-cell cross-reactivity, which will be explored later.[1]

1.1.4 1.4 Vaccine-induced immunity

Vaccination for viral disease is based upon the principle of immune memory, enabling more rapid and higher magnitude immune responses upon secondary exposure to a target pathogen. Classical vaccine platforms, such as inactivated or live attenuated virus vaccines, protein subunit vaccines, and virus-like particle vaccines, have been effectively employed to reduce the burden of diseases like polio, measles, mumps, and rubella, and human papillomavirus (HPV).[53] A good vaccine is safe, immunogenic, and results in a reduction in disease severity or, ideally, pathogen transmissibility.

In recent years, and accelerated by the COVID-19 pandemic, next-generation vaccine

platforms have been developed that do not rely on laboratory-grown virus and are therefore intrinsically more scalable. Nucleic acid-based vaccines and viral vector vaccines require only the viral sequence for development, and therefore booster doses can be updated as viral variants emerge. Messenger RNA (mRNA) vaccines contain synthetic mRNA encoding the viral antigen of interest, and incorporate modified nucleosides to prevent innate recognition until sufficient antigen translation can occur.[54] Viral vector vaccines include an attenuated recombinant virus containing a viral antigen of interest, resulting in antigen production within human cells and therefore the induction of robust cellular as well as humoral immunity.[53]

Vaccines against COVID-19 were developed at unprecedented speed and scale, with over 13 billion doses administered worldwide as of June 2023.[15] In the UK, COVID-19 vaccines approved for human use included the Oxford-AstraZeneca chimpanzee adenovirus-vectored vaccine ChAdOx1 nCoV-19 (AZ1222), the Pfizer-BioNTech mRNA vaccine (BNT162b2), and Moderna's mRNA vaccine (mRNA1273).[55, 56] Most SARS-CoV-2 vaccines target the S protein. Both mRNA and vectored vaccines promote the production of robust nAb responses to the Wuhan strain of SARS-CoV-2.[57–59] One dose of AZ1222 promotes the production of anti-S IgG and B-cell proliferation, as well as the induction of T_H1 and cytotoxic T-cells. Both humoral and cellular immunity is further promoted with a second dose, particularly after an extended time interval.[57, 60] Furthermore, mRNA vaccines generate IgG with increased breadth compared to natural infection.[61] Vaccine-induced T-cell immunity is largely conserved against VOCs, although humoral immunity is substantially affected.[51, 59, 62]

1.1.5 1.5 Age- and sex-related correlates of immunity

The impact of age on immunity is well appreciated; the decline in immune function with age that leads to increased morbidity and mortality following infection is collectively known as 'immunosenescence'.[63] Although complex, key age-related changes in immunity that characterise this senescence include:

1. Changes in the abundance of key cell subsets, such as a reduction in naïve T-cells and an increase in effector memory T-cells;

2. Promotion of a chronic low-grade inflammatory environment,[64] for example by increased production of IL-6, C-reactive protein, IL-1β, fibrinogen, and TNF;

3. Decline in cellular effector functions such as cytokine production by NK cells, macrophage phagocytosis, and expression of co-stimulatory molecules by DCs;

4. Reduction in B-cell functional responsiveness and diminished avidity of antibodies;

5. T-cell signalling defects; and

6. Dysregulation of fibrosis homeostasis, leading to increased collagen deposition, myofibroblast activation, and ultimately organ pathology.[65].

The hallmarks of immunosenescence outlined above have a detrimental impact on protective immunity and responses to vaccination in older individuals. Increased disease severity in older individuals has been described for infection with SARS-CoV-1, MERS-CoV and HCoVs,[66] influenza virus,[67] West Nile virus (WNV),[68] Chikungunya virus,[69] and respiratory syncytial virus (RSV),[63] among others. Furthermore, chronic infection with latent viruses such as human cytomegalovirus (CMV) can impair immunity in adulthood and old age: reduced T-cell diversity due to the proliferation of CMV-specific memory T-cells, as well as the secretion of pro-inflammatory cytokines, contribute to immunosenescence in older individuals. [63, 70] In addition, thymic involution - the reduction in thymic output with age - contributes to the lower T-cell diversity observed in older individuals.[71] High TCR diversity is crucial for generating robust and competitive T-cell responses to vaccination and infection, enabling the recognition of diverse antigens and reducing

the probability of immune escape.[72] Although difficult to measure accurately due to its vast repertoire, the paucity of rare sequences, and potential biases from PCR and sequencing methods, one study attempted to measure TCR diversity in 156 health donors aged 18-88 years using limiting dilution analysis and TCR cloning.[73] T-cell diversity was shown to remain high in healthy donors until 65 years of age, before declining dramatically. Another more recent study used high-throughput deep sequencing to characterise TCR diversity across the age course, and identified a linear decrease in diversity with age; although the oldest age group (average age 82 years) showed higher diversity than the second-oldest (average age 62 years), perhaps reflecting an association between TCR diversity and longevity in this cohort.[74]

It was therefore unsurprising that old age was considered the biggest risk factor for severe COVID-19 at the beginning of the pandemic. Early estimates of the SARS-CoV-2 case fatality rate were less than 1% overall, but exceeded 20% in over-80s.[75] Multiple mechanistic explanations for this trend have been offered. The involvement of the NOD-containing, leucine-rich-repeat- (LRR-) containing and pyrin-domain-containing protein 3 (NLRP3) inflammasome in severe COVID-19, and its overactivation in the elderly, provides a mechanism for the increased predisposition to 'cytokine storms' in older individuals.[76, 77] Detection of viral RNA by TLR7/8 and TLR3 stimulates the NLRP3 inflammasome, promoting production of IL-1β, IL-6, TNF-α, and type 1 IFN.[78] These play a significant role in pulmonary inflammatory diseases such as asthma, chronic obstructive pulmonary disease, and acute respiratory distress syndrome.[77] In COVID-19, NLRP3-mediated increases in pro-inflammatory cytokine expression in the elderly is associated with worse disease and death.[77] Age-related changes in adaptive immune components may also contribute to worse COVID-19 outcomes in older individuals. TCR diversity decreases modestly in the healthy elderly.[65, 79, 80] Furthermore, reduced TCR repertoire diversity was demonstrated in COVID-19 patients, and diversity further decreased with age.[81] Severe COVID-19 is also associated with

the production of autoantibodies targeting type 1 IFN, which increase in abundance with age.[26] A study of 987 patients with COVID-19 pneumonia identified anti-IFN autoantibodies capable of neutralising IFN activity in 101 individuals. Patients with autoantibodies were slightly older than the rest of the cohort, but autoantibodies were also present in individuals as young as 25.[26] Finally, other risk factors for severe COVID-19 such as cardiovascular disease, diabetes, and obesity are age-associated, further contributing to the increased risk of severe COVID-19 in the elderly.[76]

Responses to COVID-19 vaccines are also impaired in the elderly. A study of 66 individuals aged 22-95 who had been vaccinated a median of 44 days earlier with COVID-19 mRNA vaccines identified a negative correlation between age and nAb titre, as well as between age and S-specific memory B-cells. Furthermore, S-specific T-cell responses were significantly lower in older individuals.[63] Durability of vaccine-induced immunity is reduced in the elderly; waning of efficacy was found to be greater in over-65-year-olds than 40-64-year-olds vaccinated with either BNT162b2 or AZ1222 COVID-19 vaccines.[82]

In addition to understanding immune dynamics in older individuals, studies of SARS-CoV-2 infection in children enable the examination of paediatric immunity to a previously unencountered virus. Lower respiratory tract infections are the leading cause of death globally in children under the age of five,[83] particularly in low- and middle-income countries. Generally, young children and the elderly are the most at risk for severe disease following respiratory infection.[84] However, children experience low rates of COVID-19 mortality and severe disease. Similar patterns were observed following infection with SARS-CoV-1 and MERS-CoV,[85–87] a trend for which multiple explanations have been offered. Initially, it was suggested that children were more resistant to becoming infected with SARS-CoV-2, perhaps due to enhanced mucosal immunity during initial stages of infection.[88] This theory was supported by an early study of infection risk in the community which identified

a lower risk of infection in preschool-age children compared to adults,[89] as well as the finding that expression of ACE2 in children is reduced compared to adults.[90] However, this hypothesis was rejected after studies identified SARS-CoV-2-specific antibodies in a significant proportion of the paediatric population, suggesting widespread asymptomatic infection.[84] In addition, higher nasopharyngeal viral loads have been identified in hospitalised children and teenagers compared to hospitalised adults.[90]

Enhanced innate immune defences in children alongside increased numbers of naïve lymphocytes may offer children improved protection against infection and disease.[76, 91] Furthermore, children are less likely to mount 'storms' of pro-inflammatory cytokines, which have been strongly linked to severe disease and mortality in COVID-19.[92] However, children do on occasion mount cytokine storms following infection with influenza and RSV.[93, 94] Another potential explanation for the reduced severity of paediatric COVID-19 is the effect of concurrent infections with other pathogens; children have greater nasopharyngeal colonisation by bacteria and viruses and have a differential diversity of gastrointestinal microbes compared to adults.[76] More frequent infections with other pathogens may generate so-called 'trained immunity', leading to epigenetic changes in immune mediator genes that promote viral clearance.[76] Children also display enhanced levels of natural antibodies – antibodies produced in the absence of a pathogen or immunisation, generated by reactivity with self-antigens.[95] These are generally IgM class, broadly reactive and able to contain infection rapidly whilst memory B cells work to produce high-affinity class-switched antibodies.[96] Despite this, a study of hospitalised children and adults with severe COVID-19 identified increased T-cell and nAb responses in adults, suggesting that weak adaptive immunity is not the cause of the increased mortality from COVID-19 in adults.[88]

As well as age, the role of sex in the immune response to SARS-CoV-2 is of particular interest. Sex differences in susceptibility to illness are widespread: females make up

80% of autoimmune disease cases, increased viral loads in males with hepatitis C virus (HCV) and human immunodeficiency virus (HIV) have been described, and there is an increased prevalence of hepatitis A and tuberculosis in males.[97, 98] Females generally clear pathogens more quickly, with stronger immune responses that are also associated with increased autoimmunity and inflammation.[98] Immune sex differences are particularly evident following vaccination, both in childhood and into adulthood. Immune responses to influenza, yellow fever virus (YFV), measles, mumps, rabies, and smallpox vaccines, among others, are consistently higher in females.[99] Across the age course, female responses to influenza vaccination are higher, and half-doses of trivalent influenza vaccines in females have been demonstrated to generate antibody responses higher than those of males receiving full doses.[100] However, adverse events following vaccination are also more frequent in females.[101]

Sex differences are found in both innate and adaptive components of the human immune system. TLR7 is encoded on the X chromosome and able to escape X-inactivation in females, leading to increased expression in females and greater production of IFNα by pDCs.[98, 102] However, increased activation of TLR9 in males promotes greater production of IL-10 compared to females, and this correlates with the concentration of androgens such as testosterone, supporting a hormonal basis for this difference.[98] TLR4 expression on macrophages is increased in males, and lipopolysaccharide (LPS) stimulation promotes greater production of pro-inflammatory cytokines in males – this is also androgen-dependent.[98, 101] Immune cell frequencies and functionality differ between males and females – males have increased frequencies of NK cells, but reduced phagocytic activity of neutrophils and macrophages, and reduced efficacy of antigen presentation by APCs such as DCs.[98] Females have greater CD4+ T-cell numbers, whilst males display greater CD8+ numbers. However, following stimulation, both CD4+ and CD8+ T-cells are more highly activated in females.[98, 102] Differences in cytokine profile can also be observed – males preferentially produce IL-17 upon stimulation of naïve CD4+

T-cells, whereas females produce more IFNγ. Females generate higher Ig responses than males and have greater numbers of B-cells.[98]

As mentioned, mediators of these immune sex differences include location of genes on sex chromosomes, the effects of sex hormones, environmental factors such as nutrition and the microbiome, and the effects of reproductive status. Genes located on the X-chromosome include TLR7/8, cytokine receptors such as IL-2 receptor γ (IL2RG), and transcription factors including forkhead box P3 (FOXP3).[98] Furthermore, the Y chromosome encodes several immune regulatory genes.[103] Oestradiol has been shown to promote increased neutrophil frequencies in the blood, NK cell cytotoxicity, and pro-inflammatory cytokine production, as well as T_H2 immunity.[98] Testosterone can be immunosuppressive and can reduce NK cell activity, TLR4 expression on macrophages, and TNF production, but increase IL-10 and TGFβ production.[98, 104]

Male sex is a risk factor for death from COVID-19, despite similar rates of SARS-CoV-2 infection between males and females.[103] Early in the pandemic, male sex was strongly associated with an increased risk of COVID-19 fatality and 60% of global COVID-19 deaths were in men.[105, 106] This sex difference was particularly pronounced in males over 30 years of age.[103] A comprehensive longitudinal study of sex differences in antibody-, cytokine-, and cell-mediated-immunity in 98 SARS-CoV-2-infected individuals identified greater IFNα2 levels in females, but higher levels of IL-8 and IL-18 in males. Furthermore, the authors identified lower T-cell counts in males over the disease course, and greater T-cell activation in females at baseline, particularly CD8+ T-cells.[97] Another study identified a protective role for oestrogens in inhibiting inflammation during SARS-CoV-2 infection, suggesting that the reduction in oestrogen level associated with menopause in older women could partially explain increased their increased mortality from COVID-19 compared to younger women.[107]

1.1.6 1.6 Immune cross-reactivity

Cross-recognition of related pathogens by various immune components, particularly T-cells, had been described extensively before the emergence of SARS-CoV-2.[8, 108, 109] Cross-reactivity of TCRs results from only a few residues in the peptide:MHC complex being necessary for TCR activation, and the ability of TCRs to tolerate some amino acid substitutions in their cognate peptides.[109] Additionally, an 'induced fit' model of TCR complementarity has been suggested, whereby TCRs undergo conformational adjustments in complementarity-determining region 3 (CDR3) upon contact with an MHC molecule, allowing a TCR to recognise peptides with greater promiscuity.[110] Enhanced recognition repertoires may confer an evolutionary advantage to host immunity in their ability to recognise a greater diversity of pathogens.

T-cell cross-reactivity has been described between variants of influenza,[111] between influenza and rotavirus or HCV,[112, 113] and between flaviviruses.[108, 114, 115] Cross-reactivity is particularly common in CD8+ memory T-cells, and varies according to human leukocyte antigen (HLA) type.[116] The expansion of cross-reactive memory cells following pathogen exposure is believed to confer an advantage over naïve T-cells, enabling changes in the hierarchy of the T-cell response due to the increased frequency and speed of memory responses. In some cases, cross-reactive immunity can provide protection against disease or even death.[109] However, heterologous immunity can also promote immunopathogenesis through so-called 'original antigenic sin' (OAS), which was first described in influenza.[117] Cross-reactivity between SARS-CoV-2 and SARS-CoV-1, MERS-CoV, and endemic HCoVs has been described since 2019, although the significance of these cross-reactive responses in disease course is disputed, and will be the focus of this thesis.[3, 5, 6, 8, 118]

1.2 Aims of this Thesis

Using peripheral blood samples collected from naturally infected individuals and their households, as well as vaccinated individuals, this thesis aims to:

1. Understand the humoral and cellular immune dynamics of SARS-CoV-2 infection within and between families (Chapter 3)

2. Explore the role of cross-reactive T-cells in family members highly exposed to, but seronegative for, SARS-CoV-2 (Chapter 4)

3. Investigate the relationship between humoral immunity to endemic HCoVs and COVID-19 severity (Chapter 5)

4. Examine the immune response to SARS-CoV-2 and influenza following vaccination in UK adolescents (Chapter 6)

2 Methods

Contents

2.1 Chapters 3, 4, 5, and 6

2.1.1 Collection of clinical samples

Family study cohort

Samples were collected from October 2020 to June 2021. Eligible participants were individuals aged six and above who had experienced symptoms of COVID-19, as well as their family members. Individuals were recruited by word of mouth and blood samples were taken at home or at the laboratory. Samples from Wales were collected by GP recruitment as part of the CROWN study, which also sampled SARS-CoV-2 convalescent individuals and their families. As this study was initiated

before widespread polymerase chain reaction (PCR) and lateral flow testing became available, confirmed SARS-CoV-2 infection was determined by anti-S IgG ELISA above a threshold of 10 ELISA units (EU). ELISAs were carried out by collaborators at the Oxford Vaccine Group. Written informed consent was obtained from patients; ethical approval was given by the Central University Research Ethics Committee (CUREC R71346/RE001). Information sheets given to participants are available in Appendix 1.

As this cohort was primarily recruited by word of mouth, this leaves the study vulnerable to biases in sampling. The cohort is predominantly white (92%) and largely made up of HCWs, students, and individuals working in professional services such as business and law. The socioeconomic status of the individuals in this cohort are likely similar, and this may have affected their behaviour during lockdown such as rule-following and ability to work from home. This might also have impacted the level of mixing between households - for example, children whose parents were key workers were likely more exposed to SARS-CoV-2 compared to children whose parents worked from home during lockdowns.

Intensive care unit (ICU) cohort

Serum samples were obtained from the Arden Biobank (ethics number 18/SC/0180) University Hospital Coventry and Warwickshire. Eligible samples were from unvaccinated individuals recruited before April 2021 who had been admitted to ICU with severe COVID-19, with a blood sample taken <14 days after admission.

These individuals were recruited opportunistically during their time in the ICU, and therefore the cohort may be biased and not fully representative of the general population. 69% of individuals were male, reflecting the increased severity of COVID-19 in males, and the mean age was 80 years. This cohort is therefore representative of individuals at high risk for severe COVID-19, making it a useful cohort for the study in Chapter 5.

Time course cohort

Samples were made up of two groups: serially sampled healthcare workers (HCWs) from the Oxford Radcliffe Biobank (ref:19/ SC/0173) and individuals from the COVID-LIV study at the University of Liverpool.[119] Eligible samples were from unvaccinated individuals who had PCR-confirmed SARS-CoV-2 infection and for whom a pre-infection sampled had been taken. Oxford samples were taken up to 209 days pre-infection and up to 36 days post-infection as confirmed by PCR testing. COVID-LIV samples were taken on the day of PCR positivity and an average of 14 days later.

These cohorts were chosen for the study in Chapter 5 due to the availability of samples pre- and post-infection. Although no information is known on the age and sex distribution of these cohorts, the first cohort is made up of HCWs, which biases it towards working-age individuals with high levels of exposure to SARS-CoV-2 through their occupation. The second cohort is made of index patients from the COVID-LIV family study, making it biased towards individuals of child-rearing age with high levels of exposure to SARS-CoV-2 through their children. The two cohorts are therefore similar in their biases, and made up a population at high risk of SARS-CoV-2 infection, which is why they were chosen for the study in Chapter 5.

PITCH cohort

32–52-year-old healthy HCWs were recruited as part of the Protective Immunity from T-cells in HCWs (PITCH) consortium of HCWs under the GI Biobank Study 16/YH/0247, approved by the research ethics committee (REC) at Yorkshire and The Humber - Sheffield Research Ethics Committee. PBMCs, plasma and serum were collected before the first dose, four weeks after the first dose, eight weeks after the first dose, and four weeks after the second dose.

Cohort	n	Female	Male	Ages	Seropositivity	Timepoints
Family	264	145	119	6 to 88	54%	Convalescent
ICU	104	32	72	26 to 87	100%	Acute
Timecourse	26	N/A	N/A	N/A	100%	Pre- and post-infection
PITCH	79	N/A	N/A	21 to 71	59%	Pre and post-vaccination
Adolescent	34	16	18	12 to 16	52% to 71%	Pre and post-vaccination

Table 2.1: Cohorts

Adolescent cohort

This longitudinal cohort study was conducted from November 2021 to February 2022. Eligible participants were healthy adolescents aged 12-16 who either had no history of SARS-CoV-2 infection or had experienced mild COVID-19 prior to enrolment. Eligible participants were identified via their participation in school-based vaccination events. Written informed consent was obtained from all patients and ethical approval was given by the Central University Research Ethics Committee (reference: CUREC R71346/RE001).

For the BNT162b2 vaccination (dose 1 [V1] and dose 2 [V2]), patients received 30μg of vaccine intramuscularly. Live-attenuated influenza vaccine (LAIV) was administered immediately after V1 only; patients received 0.1mL intranasally in both nostrils. Whole blood samples from all 34 individuals were taken immediately before V1 (sample pre-V1). Samples from all 34 individuals were taken a mean of 37 days after V1 (33-39) (sample post-V1), from 23 individuals 2 days before V2 (0-8) and 96 days after V1 (81-114) (sample pre-V2), and from 14 individuals 35

days after V2 (30-40) (sample post-V2). All whole blood samples were processed the same day as collection. All serum samples were tested for anti-S and anti-N IgG; individuals were classified as seropositive if their anti-N IgG titre was above the previously determined Meso Scale Diagnostics (MSD) immunoassay cut-off at any point in the study or if their anti-S IgG titre was above the cut-off pre-V1.[60]

Sample processing

Whole blood samples that had not already been processed (the family study cohort and the adolescent cohort) were transported from their collection site to an academic laboratory and processed the same day. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated as described elsewhere.[120] Briefly, PBMCs were isolated using Lymphoprep (1.077 g/mL, Stem Cell Technologies, Canada) through density gradient centrifugation. Plasma and PBMCs were collected and plasma was spun at 2000g for 10 minutes to remove platelets. PBMCs were washed twice with R10 (RPMI 1640 [Sigma, USA] containing 10% heat inactivated foetal calf serum [FCS], 2mM L-glutamine, and 1mM penicillin/streptomycin [Sigma, USA]). Cells were resuspended in R10 and counted using a Muse Cell Analyser (Luminex Corporation, USA). Plasma and PBMCs were frozen and stored at -80°C for later use. An estimated 10 million PBMCs were thawed in a 37°C water bath and diluted into 30mL of warm R10, then spun and resuspended in 2mL of R10 for counting.

2.1.2 Statistical analysis

All analyses were performed in GraphPad Prism 9.0. For pairwise comparisons, two-tailed Mann-Whitney tests were used for unpaired data and Wilcoxon signed-rank tests for paired data. For multiple comparisons, Kruskal-Wallis tests with Dunn's multiple comparisons test were used. Principal component (PC) analysis (PCA), linear regression, and logistic regression were also performed in Prism.

2.2 Chapters 3, 4, and 6

Proliferation assay

T-cell responses were assayed using a CellTrace Violet (CTV) Proliferation assay as described elsewhere.[120] The CTV assay was chosen for this study due to its ability to pick up low magnitude T-cell responses in both seropositive and seronegative individuals.[120]. Other benefits of the CTV assay include its ability to discriminate between CD4+ and CD8+ T-cell subsets, and its status as a flow cytometric assay enables qualitative visualisation of cells to assess cell viability, rounds of division, and response strength. However, it is important to note the potential drawbacks and biases of this assay. The long incubation time (7 days) compared to an activation-induced marker (AIM), intracellular cytokine staining (ICS) or ELISpot assay means that the CTV assay is vulnerable to stochastic results due to clonal expansion of small subsets of cells early on in the assay. This may lead to an exaggerated measurement of proliferation. One solution to this bias is to repeat assays to determine the coefficient of variation between batches. However, due to limited cell availability, this was not possible in the cohorts assessed in this thesis. Other biases of the CTV assay include a skew towards detecting CD4+ T-cells which are more proliferative than CD8+ T-cells, and towards central memory rather than effector memory. In contrast, the IFNγ ELISpot assay measures IFNγproducing cells such as both T_H1 CD4+ effector memory T-cells and CD8+ T-cells. ICS can also discriminate between CD4+ and CD8+ T-cells if desired, but is dependent upon active production of cytokines. AIM, in contrast, measures the expression of activation markers, and therefore is not reliant upon cytokine production, making it useful in studying certain cell subsets that are hard to detect using ICS such as T_{FH} cells and T_{REG} cells. However, due to cell availaibility in the studies outlined in this thesis, proliferation assays were the main T-cell assays performed due to their ability to pick up low-magnitude responses in seronegative individuals.

Cells were washed twice with phosphate buffered saline (PBS) and stained with CTV (Life Technologies, USA) at 2.5μM for 10 minutes at room temperature (RT).

Cold FCS was added to quench the reaction. Cells were plated at 250,000 cells per well in a 96-well round-bottom plate. Peptide pools covering SARS-CoV-2 S1, S2, M and N, NSP 1+2, NSP3A, NSP3B, NSP3C, NSP4, NSP5+6, NSP7-11, NSP12A, NSP12B, NSP13, NSP14, NSP15+16, HCoV-OC43 S, and HCoV-HKU1 S were added to stimulate cells at a final concentration of 1μg/mL. Media containing 0.1% dimethylsulphoxide (DMSO) (Sigma, USA) was used as a negative control. Phytohaemagglutinin L (PHA) (Sigma, USA) was used as a positive control at a final concentration of 2μg/mL. Plates were incubated at 37°C, 5% CO_2, and 95% humidity for 7 days, with a hemimedia change at day 4. On day 7, cells were washed in PBS and stained with fluorochrome-conjugated antibodies for CD4, CD8, and CD3 (BioLegend, USA) in PBS. LIVE/DEAD Fixable Aqua was used as a viability marker (Thermo Fisher, USA). Cells were fixed in 4% paraformaldehyde (Sigma, USA) for ten minutes at 4°C and washed in PBS before storing at 4°C in the dark. Samples were acquired on a MACSquant X (Miltenyi, Germany) and analysed in FlowJo (Gating shown in Chapter 3). Samples were run within three days of staining to ensure staining efficiency.

2.3 Chapters 5 and 6

ELISAs

IgG responses to influenza A/Victoria (H1N1), B/Washington (Victoria), A/Cambodia (H3N2), and B/Phuket (Yamagata) haemagglutinin (HA)(A/Victoria/2570/2019 [H1N1]pdm09-like virus [NCBI Accession Number: EPI1799581], amino acids 1-528 and C-terminal His-tag; Cambodia/e0826360/2020 [H3N2]-like virus [NCBI Accession Number: EPI1799580], amino acids 46-469 and C-terminal His-tag; B/Washington/02/2019 [B/Victoria lineage]-like virus [NCBI Accession Number: EPI1846769], amino acids 31-469 and C-terminal His-tag, B/Phuket/3073/2013 [B/Yamagata Lineage]-Like virus] [NCBI Accession Number: EPI1799823], amino acids 44-466 and C-terminal His-tag; The Native Antigen Company, UK), HCoV-OC43 and HCoV-HKU1 S and N (HCoV-OC43 N: amino acids 1-447 [HCoV-OC43/Seattle/USA/SC0841/2019]; HCoV-OC43 S: amino acids 1-753 [NCBI YP-

009555241.1; sequence strain: ATCC VR-759], HCoV-HKU1 N: 441 amino acids [NCBI: ABG77571.1], HKU1 S: amino acids 1-752 [NCBI: AZS52618.1; sequence strain: SC2521]) (The Native Antigen Company, UK), and SARS-CoV-2 S2 (Sino Biological, China) antigens were measured using indirect ELISA. Antigens of interest were diluted to 1μg/mL in PBS and used to coat 535 Nunc-Immuno 96-well plates (Thermo Fisher Scientific, USA) overnight at 4°C. Plates were washed three times in 0.1% PBS-Tween, before blocking with Casein-PBS Buffer for one hour at RT. Plasma was diluted (1:500 for S, 1:400 for N, 1:200 for HA) in Casein-PBS Buffer and added to plates in duplicate. A ten-point standard curve of pooled highly reactive sera beginning at 1:25 was prepared in duplicate and added to plates. Casein-PBS Buffer was used as a negative control. Plates were incubated for two hours at RT and washed six times in 0.1% PBS-Tween. Secondary antibody (goat anti-human IgG conjugated to alkaline phosphatase) (Sigma, USA) was diluted 1:1000 in Casein-PBS Buffer and added to plates. Plates were incubated for one hour at RT before washing six times in 0.1% PBS-Tween. 4-nitrophenyl phosphate in diethanolamine buffer (Pierce, UK) was added as a substrate and plates were incubated for 15 minutes. 405nm absorbance was read using an ELx800 microplate reader (Cole Parmer, UK).

MSD serological assay

IgG responses to SARS-CoV-2 S, N, and RBD as well as the S proteins of HCoV-OC43, HCoV-NL63, HCoV-229E, HCoV-HKU1, SARS-CoV-1, and MERS-CoV were measured using an MSD v-plex immunoassay 'Coronavirus panel 3' (MSD, USA) according to the manufacturer's protocol. Plates were incubated in Blocker A solution for 30 minutes at RT with shaking at 700 revolutions per minute (rpm). Plasma or serum was diluted at 1:1000 and 1:10000 in Diluent 100, and a seven-point standard curve of reference standard beginning at 1:10 was prepared in duplicate. Three internal controls and an in-house control of COVID-19 convalescent serum were also used, with Diluent 100 used as a blank. Plates were washed three times with MSD Wash Buffer and samples and standards added to the plate before incubation at RT for two hours with shaking at 700rpm. Plates were washed three

times with MSD Wash Buffer and detection antibody solution was added. Plates were incubated for one hour at RT with shaking at 700rpm. Plates were washed three times with MSD Wash Buffer. Neat MSD Gold Read Buffer was added, and plates were read immediately on a MESO QuickPlex SQ 120 (MSD, USA). Data was analysed using MSD Discovery Workbench software. Thresholds for seropositivity were taken from analyses of pre-pandemic sera, as published elsewhere,[121] and defined as 1160 AU/mL for SARS-CoV-2 S, 1169 for RBD, and 3874 for N.

2.4 Chapter 3

Enzyme-linked immunospot assay (ELISpot)

Multiscreen-I 96-well ELISpot plates (Millipore, USA) were coated with 50μl of 10μg/mL human IFNγ 1-D1K capture antibody (Mabtech, Sweden) and incubated at 4°C overnight. Coating solution was removed, and plates were washed twice with sterile PBS. Plates were blocked with 100μl R10 for 30 minutes at RT. Blocking solution was removed and 50μl of cell suspension at 4 million cells/mL in R10 was added to wells in triplicate. 50μl of prepared peptide stocks at 4 µg/mL were added to wells, as well as PHA as a positive control and R10 as a negative control. Plates were incubated for 16-18 hours at 37°C, 5% CO_2, and 95% humidity. Cells were removed and plates were washed six times with 100μl 0.1% PBS-Tween before addition of 50μl 1μg/mL anti-human 7-B6-1-Biotin detector antibody (Mabtech, Sweden). Plates were incubated for two to four hours at RT. Detector antibody was removed, plates were washed six times with PBS-Tween before addition of 50μl 1μg/mL anti-Biotin alkaline phosphatase antibody (Mabtech, Sweden), and were incubated for one to two hours at RT. Plates were washed six times with PBS-Tween. BCIP/NBT solution was filtered and 50μl added to each well for five to seven minutes, before washing plates thoroughly with tap water. Plates were air dried for two days before reading on an ELISpot reader (CTL Immunospot, USA). Spot-forming cells per million (SFC /10^6) were calculated by subtracting negative control well values from test wells and multiplying by five. Assays were repeated if background wells exceeded 20 SFU/10^6cells, or if positive control wells

contained less than 100 SFU/10^6. Values were calculated by taking the mean of triplicate wells. If any one triplicate value differed from the other two values by more than 10, it was excluded from the mean calculation. If all three values differed from each other by more than 10, the sample was re-run.

2.5 Chapter 5

Neutralisation assay

nAb titres were calculated using a SARS-CoV-2 lentivirus-based pseudovirus assay displaying a codon-optimized SARS-CoV-2 S protein (National Centre for Biotechnology Information (NCBI) reference sequence: YP_009724390.1) as described elsewhere.[122] Briefly, HEK293 T/17 cells were cultured in complete medium (Dulbecco's Modified Eagle Medium [DMEM] supplemented with 10% FCS, 1% penicillin/streptomycin, and 1% L-glutamine) and incubated at 37°C and 5% CO_2. Pseudotyped viruses were produced by transfecting HEK293T cells with 1μg codon optimized S, 1μg of gag/pol, and 1.5μg of a luciferase reporter in a plasmid-OptiMem solution.

For the microneutralization assay, a transfection mix was prepared using 2500ng of ACE2, 250ng of TMPRSS2, 1mL of OptiMem, and 9μl of Fugene (Promega, USA) as part of a plasmid-media mix. 10mL of cells were transfected with the plasmid-media mix 24 hours before the assay. 5μl of serum and 95μl of complete media was added to columns one and seven in a 96 well white opaque culture plate. 50μl of complete media was added to columns two to six and eight to 12, and serum was serially diluted across the plate in a 1:2 dilution. 50μl of pseudotyped virus was added to wells, mixed, and incubated for two hours at 37°C. Each plate contained six positive controls wells (no serum) and six negative control wells (no pseudovirus). 10^4 plasmid transfected HEK293 T/17 cells were added to each well and incubated for 48 hours at 37°C/5% CO_2. Supernatant was removed using vacuum filtration. Bright Glo (Promega, USA) was diluted 1:1 with sterile PBS. 50μl of the Bright Glo-PBS mixture was added to each well and allowed to lyse for five minutes after

which luciferase activity was measured using a ProMega GloMax (USA). Data was analyzed using Microsoft Excel and GraphPad Prism.

2.6 Chapter 6

MSD ACE2 inhibition assay

nAb titres targeting SARS-CoV-2 were quantified using MSD ACE2 inhibition assay 'Panel 27'(analytes: SARS-CoV-2 S, SARS-CoV-2 S [B.1.351], SARS-CoV-2 S [B.1.617.2; AY.4], SARS-CoV-2 S [BA.2], SARS-CoV-2 S [BA.2.12.1], SARS-CoV-2 S [BA.2+L452M], SARS-CoV-2 S [BA.2+L452R], SARS-CoV-2 S [BA.3], SARS-CoV-2 S [BA.4], SARS-CoV-2 S [BA.5]), according to the manufacturer's instructions. Plates were incubated in Blocker A solution for 30 minutes at RT with shaking at 700rpm. Serum was diluted at 1:10 and 1:100, and a seven-point standard curve of Calibration reagent was prepared with four-fold serial dilutions. Plates were washed three times with MSD Wash Buffer and samples and calibrator were added to the plate. Plates were incubated at RT for one hour with shaking at 700rpm. ACE2 protein was added to the plate and incubated at RT for one hour with shaking at 700rpm. Plates were washed three times with MSD Wash Buffer and MSD Gold Read Buffer was added. Plates were read immediately on a MESO QuickPlex SQ 120 (MSD, USA). Data was analysed using MSD Discovery Workbench software.

3

Immune responses to SARS-CoV-2 infection in families

Contents

3.1 Abstract

Family studies of viral infection provide an opportunity to assess virus-specific immunity in seropositive and seronegative individuals, as well as to examine the dynamics of viral infection within families. Transmission of SARS-CoV-2 between family members represented a major route for viral spread during the early stages of the pandemic, due to the nature of SARS-CoV-2 transmission through close contacts. This chapter analyses humoral and cellular immunity in 81 families from Oxford, London, and Cardiff where at least one family member experienced symptoms of COVID-19 early in the pandemic before widespread infection and vaccination had occurred. Multiple perspectives are explored, including risk factors for infection, the concordance between humoral and cellular immunity, common patterns of seropositivity within families, and the roles of age and sex in immune response. Overall, the chapter describes marked heterogeneity between families and between individuals, with robust cellular and humoral immunity into convalescence. Correlates of T-cell response magnitude include being older and being male, and worse symptoms are associated with stronger cellular immunity. Furthermore, T-cell responses in seronegative individuals are widespread, particularly in adults and in individuals exposed to SARS-CoV-2 through an infected family member. This chapter provides valuable insights into the transmission dynamics of and immune responses to SARS-CoV-2 infection within families, in a unique cohort of immunologically-naïve individuals of diverse ages.

3.2 Introduction

Aim: To understand the humoral and cellular immune dynamics of SARS-CoV-2 infection within and between families.

Following the emergence of SARS-CoV-2, a race to understand correlates of protection against infection, disease severity, and disease susceptibility began. Central to this understanding was characterising immune responses to SARS-CoV-2 following transmission through different routes. HCWs represented a highly infected population that was intensely studied early in the pandemic. However, as the UK entered a series of successive lockdowns, studies of family and household transmission of SARS-CoV-2 enabled the assessment of host immunity in family groups where transmission was highly likely between close family contacts. Family studies are a useful tool in assessing immune dynamics, with opportunities to serially sample hosts before and after infection, analyse the temporal characteristics of disease, and assess the effects of exposure versus infection on immune response. Furthermore, the close relationships between family members, in terms of intensity and frequency of close contact, provide further clarity that is absent in broader population-based transmission studies.

Intrafamilial viral spread has been well documented for viruses with remarkably different virological characteristics including members of Baltimore classification groups IV (HCV, Hepatitis E Virus [HEV], MERS-CoV, Enteroviruses), V (Ebolavirus [EBOV]), VI (HIV), and VII (Hepatitis B Virus [HBV]).[123–126] These studies have highlighted familial infection with pathogens such as HCV and enteroviruses as a significant risk factor for the acquisition of viral infection in family members. For epidemic viruses such as EBOV, non-compliance to public health measures in small numbers of families contribute to ongoing viral transmission. Spread of blood-borne diseases such as HCV and HIV between sexual partners represent a significant route of viral transmission within families.[127, 128] Intrafamilial spread of respiratory viruses such as MERS-CoV has also been reported,[129] although

transmission is limited.[130]

SARS-CoV-2 is understood to be transmitted both through respiratory droplets (>5 to 10 μm) and aerosols ($\leq$5 μm).[131] Close contact with an infected person is considered a major risk factor for becoming infected,[132], however, SARS-CoV-2 has been identified in aerosols over six feet from infected patients.[131] Alternative modes of SARS-CoV-2 transmission - such as through fomites (infected surfaces) - have also been suggested, making SARS-CoV-2 transmission a controversial topic. In the early stages of the pandemic, a significant degree of our understanding of SARS-CoV-2 transmission risk arose from small case studies, many of which involved intrafamilial transmission.[133–136] One case study from China published in April 2020 identified intrafamilial SARS-CoV-2 transmission in a family of three – a 35-year-old man who was symptomatic and PCR positive, a 33-year-old woman, and a three-year-old boy; the latter two remained asymptomatic but were PCR+.[133] The authors were unable to conclude the index case in this instance and hypothesized on the potential role of asymptomatic transmission. In February and March 2020, the Chicago Department of Public Health investigated two SARS-CoV-2 outbreaks associated with nonhousehold, community events held by families (one birthday party and one funeral).[134] These two outbreaks led to three deaths. This study provided evidence as to the temporal dynamics of infection risk and the role of large indoor events in disease spread. Another study of 15 individuals from Shandong Province, China in January-February 2020 comprising three families identified three asymptomatic cases, one paucisymptomatic, and four symptomatic; viral sequencing revealed high degrees of relatedness between the viruses (>99.99%).[136] This further reinforced the idea of asymptomatic transmission between family members as critical in SARS-CoV-2 spread. A larger study focussing on paediatric immunity recruited 74 children with confirmed SARS-CoV-2 infection who had been exposed via a family member and demonstrated that 23% (17) were asymptomatic.[137] However, these early studies did not measure the immune response of infected or exposed individuals in family groups, perhaps owing to the limited accessibility of serological

tests and SARS-CoV-2 specific reagents early in the pandemic, or the difficulty of taking blood samples in the context of social distancing regulations.

Later, longitudinal immunological studies of large cohorts consisting of multiple households queried the effects of sequential exposures to SARS-CoV-2 on population immunity.[137–139] From July 2020 to August 2021, a study of both urban and rural household cohorts in South Africa (n=1200, 222 households) used PCR testing, blood sampling and symptom monitoring for 13 months to measure serological and virological characteristics of households at a time before widespread vaccination. Prevalence of different VOCs, serological status, kinetics of viral shedding, and risk factors associated with infection were all assessed in this study. Most infections were asymptomatic, but these asymptomatic individuals were still able to transmit SARS-CoV-2 within their households. In addition, prior SARS-CoV-2 infection generated cross-reactive immunity to VOCs that conferred protection from reinfection.[137] Between March and September 2020 in Australia, a cohort of 92 individuals from 26 households enabled the study of infection and transmission dynamics through PCR testing and mucosal and systemic antibody quantification. The authors identified a secondary attack rate of 36% using PCR positivity, which increased to 76% with antibody data. In 'high transmission' families, in which everyone tested positive, plasma antibody responses to SARS-CoV-2 were elevated compared to 'low transmission' families. The study also identified unique functional signatures of salivary antibody responses in adults versus children.[138] Finally, the OpenSAFELY study examined the risk of SARS-CoV-2 infection in families with and without children in England, finding a small increased risk of infection in families with 12-18-year-olds compared to those with younger or no children, but no increased risk of death.[140]

As well as investigating transmission dynamics, a benefit of family studies of SARS-CoV-2 infection is the opportunity to interrogate age- and sex-related determinants of immunity. The role of age in disease severity and immune response is well

characterised: enhanced low-grade inflammation, thymic ageing, and reduced cellular functionality in older individuals associated with 'immunosenescence' promotes worse disease outcomes in the elderly.[78] This raises the question of whether a poorer adaptive immune response is associated with older age following infection with SARS-CoV-2. Children experience low rates of COVID-19 mortality, perhaps owing to their increased numbers of naïve T-cells, high degree of exposure to related respiratory viruses, and reduced inflammatory phenotypes.[76] Furthermore, sex-specific differences in immune response owing to the location of immune genes on sex chromosomes, the immunomodulatory effects of sex hormones, and differential cytokine profiles in males and females generally promote greater adaptive immune responses and stronger autoimmune phenotypes in females.[98] This pattern raised the possibility that weaker adaptive immune responses may contribute to the worse disease outcomes seen in males with COVID-19.[103] Studying SARS-CoV-2 infections in families enables the exploration of these patterns of immunity in males and females of diverse ages with differing levels of exposure to SARS-CoV-2.

Here, a large dataset of SARS-CoV-2-infected and -exposed families was generated through recruitment of families in Oxford, London, and Cardiff during the winter of 2020 and the spring of 2021. As well as assessing transmission dynamics of SARS-CoV-2, one aim of this study was to characterise immune dynamics of SARS-CoV-2 infection in family members, with a focus on differences in age and sex. Common family archetypes were identified, and immune dynamics of SARS-CoV-2 infection were compared within and between these types. Sex- and age-related differences were assessed within family groups as well as across the whole dataset, and immune correlates of disease severity were studied in asymptomatic and symptomatic individuals. This study is therefore one of the largest of its kind, and unique in assessing immune dynamics of infection and exposure in immunologically naïve individuals.

3.3 Results

3.3.1 Cohort

Families were eligible for this cohort study if at least one family member had experienced symptoms of COVID-19 between October 2020 and June 2021. Over a period of nine months of recruitment, a total of 264 individuals belonging to 81 families were recruited (Figure 3.1A). 55% of individuals were female, and the median age was 40, ranging from six to 88 years of age (Figure 3.1C). The cohort was predominantly white (92%). 54% of individuals were seropositive for SARS-CoV-2 anti-S IgG at the time of enrolment and 94% (n = 248) were unvaccinated; vaccinated individuals were excluded from later analysis. In most families (74%), the index patient recruited to the study was the mother, and in 51% of families, the index patient was an HCW. From each study participant, whole-blood EDTA samples were taken, and PBMCs and serum were isolated and cryopreserved (Figure 3.1B).

Among 127 patients that self-reported symptoms, sampling occurred a mean of 228 days (7.6 months) after self-reported symptom onset; however, the lack of widespread PCR and lateral flow testing at this point in the pandemic made determining exact dates of infection difficult. Symptoms were self-reported at the time of sampling and individuals were classified as symptomatic if they were seropositive and had one or more of the following symptoms: cough, fever, anosmia, gastrointestinal symptoms, sore throat, fatigue, myalgia, or a runny nose. Individuals were classified as asymptomatic if they were seropositive but did not report any of the above symptoms. 127 individuals reported symptoms; 91 of these were seropositive for SARS-CoV-2 S. 136 individuals did not report symptoms; 44 of these were seropositive for SARS-CoV-2 S. One patient did not provide symptom information and was excluded from analyses of symptoms.

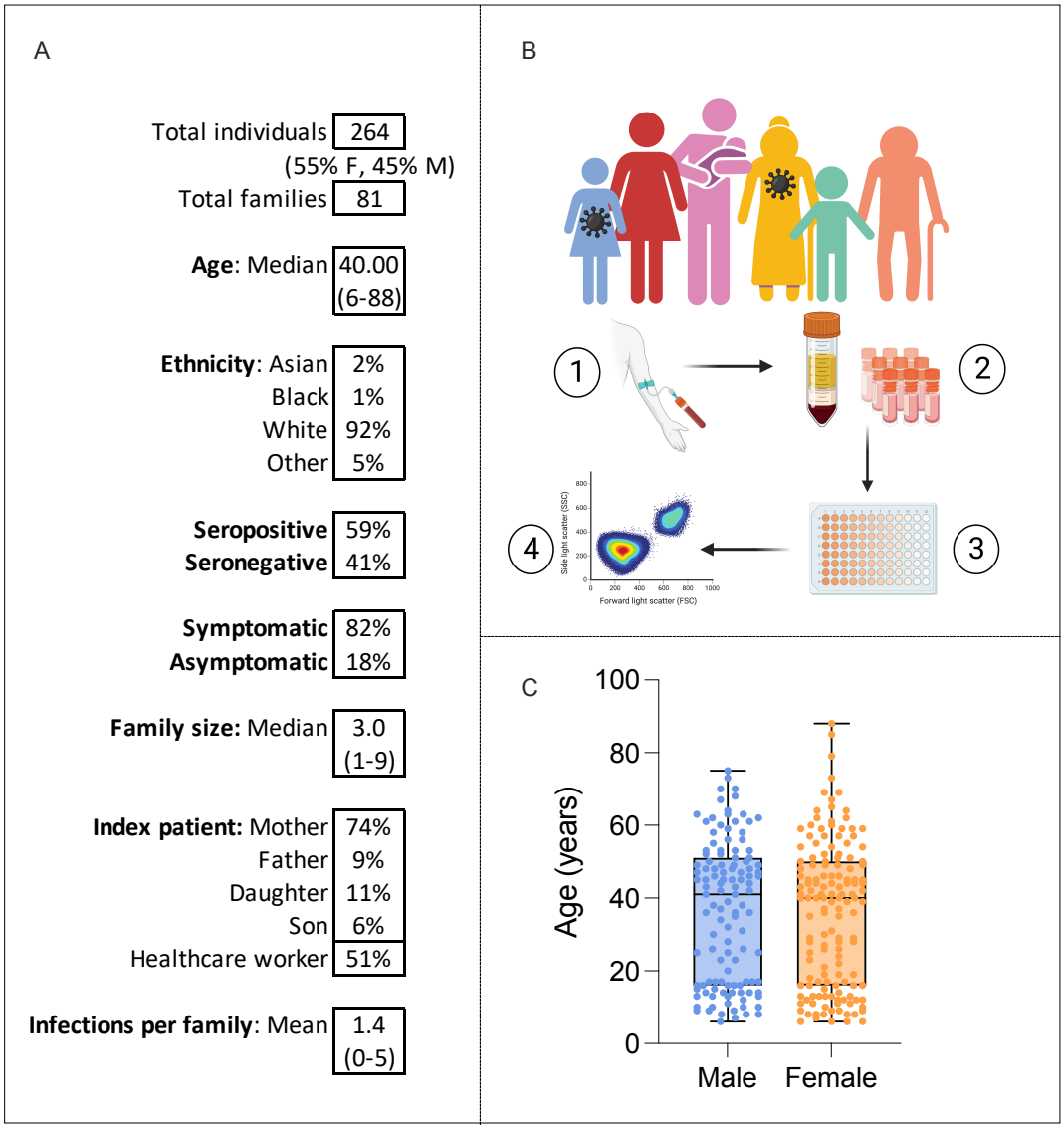


Figure 3.1: The Family Study Cohort. Characteristics of the cohort (A). Graphical representation of the study: sampling of infected individuals and their family members, PBMC and serum isolation, assays and analysis (B). Age distribution of the cohort by sex (C).

3.3.2 Validation of a T-cell proliferation assay

To assay T-cell responses to SARS-CoV-2 antigens in seropositive and seronegative individuals, a CTV flow cytometry-based assay and an IFNγ ELISpot assay were used. The CTV assay was chosen in an attempt to quantify low-magnitude responses in seronegative individuals, as demonstrated earlier,[120] and the ELISpot assay was chosen as a standard and widely employed assay for T-cell responses. The CTV assay measures T cell proliferation while the ELISpot measures the release of IFNγ by stimulated T cells. As mentioned in Chapter 2, the CTV and ELISpot assays capture different parameters from different cell populations are therefore are biased in different ways. In measuring proliferation, the CTV assay is useful in picking up highly proliferating cells such as CD4+ central memory T-cells. Its long incubation time makes it amenable to identifying low magnitude responses, and its semi-quantitative readout enables visualisation of the cell population and analysis of other parameters such as CD4+ vs CD8+ ratio, cell viability, and rounds of division. In contrast, the ELISpot measures only those cells that are actively secreting IFNγ. It is therefore less prone to false positive responses by early clonal proliferation of a few highly proliferating cells, but is also less sensitive to low-magnitude responses. Both assays involved stimulating PBMCs with peptide pools spanning the SARS-CoV-2 genome (Figure 3.2A). The CTV assay included seven days of stimulation with a hemimedia change on day four, followed by staining with antibodies conjugated with fluorophores against CD3, CD4, and CD8, as well as a viability stain (Figure 3.2B). The ELISpot assay, in contrast, involved cell stimulation for 16-18 hours and utilised immunosorbent plates coated with an anti-IFNγ antibody, an anti-IFNγ biotinylated secondary antibody, and an anti-biotin alkaline phosphatase tertiary antibody that was detected using BCIP/NBT. This resulted in the development of visible spots that were counted using a CTL ImmunoSpot analyser (Figure 3.2C). Figure 3.2DE displays the output from both the CTV and ELISpot assays for randomly sampled individuals assayed for S1 and S2-specific responses (n=22 for ELISpot, n=27 for CTV including the 22 used for ELISpot). Both assays were run using the same vial of PBMCs for each

	CTV CD4+	CTV CD8+	ELISpot
S1 seropositive	9/11	4/11	1/10
S2 seropositive	7/11	2/11	1/10
S1 seronegative	2/16	1/16	3/12
S2 seronegative	5/16	0/16	1/12

Table 3.1: Number of positive T-cell responses by CTV proliferation assay and ELISpot. Red text refers to ELISA-seropositive individuals, grey text refers to ELISA-seronegative individuals.

patient. The output from the CTV proliferation assay was the number of CTV_{LOW} cells as a percentage of the total CD4+ population or the total CD8+ population (Figure 3.2D). The cutoff for a positive response was 1% proliferation as determined previously.[120] In contrast, the ELISpot assay quantified peptide-specific cells as the number of spot-forming cells per million PBMCs (SFU /10^6). The cutoff for a positive ELISpot response was the mean of negative controls + three standard deviations (39 SFU /10^6). Overall, the proliferation assay appeared more sensitive to low magnitude responses (Table 3.1).

To compare the output of the proliferation assay to the ELISpot assay, the correlations between T-cell responses to the S1 pool from the proliferation assay and from the ELISpot assay were calculated for CD4+ and CD8+ cells for a subset of participants (Figure 3.2F). There was a weakly positive correlation for both (S1: r=0.38, p=0.01; S2: r=0.4, p=0.009, Spearman rank test). This weakness of this correlation is unsurprising, as the proliferation assay and ELISpot assays measure different populations of memory T-cells. Using both assays on the remainder of the cohort to measure both proliferating and IFNγ-secreting T-cells would have been insightful. However, due to cell availability, only the CTV assay was run on the remainder of samples. This was chosen due to its higher sensitivity and its ability to discriminate between CD4+ and CD8+ responses.

For the remainder of the proliferation assays, samples were run using PHA as a positive control and DMSO as a negative control. When the negative control

wells exceeded 1% proliferation, assays were repeated. When the positive control wells did not exceed 10% proliferation, assays were repeated.

3.3.3 Humoral and cellular immune responses in seronegative and seropositive individuals

Serological testing was performed by the Oxford Vaccine Group using an anti-S IgG ELISA; the cutoff for seropositivity was 10 EU. To validate the serological testing by ELISA assay, the MSD v-plex immunoassay was used to quantify total IgG targeting SARS-CoV-2 S, RBD, N, as well as responses to SARS-CoV-1, MERS-CoV, and endemic HCoVs. This assay had been used previously to detect SARS-CoV-2 specific IgG in vaccinated HCWs.[60, 121] The threshold for S, RBD and N positivity were set at 1160, 1169, and 3874 AU/ml, respectively, as previously determined through analysis of 103 pre-pandemic sera + three SD.[60] All individuals negative on anti-S IgG ELISA were negative on anti-S IgG MSD assay. One individual negative by anti-S ELISA was positive for anti-RBD and anti-N IgG by MSD (Figure 3.3A). However, ∼50% of ELISA-seropositive individuals were below the MSD threshold for IgG targeting N, suggesting some discordancy in IgG targeting different antigens. IgG targeting all three SARS-CoV-2 antigens (S, RBD and N) were significantly higher in ELISA-seropositive individuals (all $p<0.0001$; Mann-Whitney tests). nAb half maximal inhibitory concentration (IC50) determined by pseudovirus neutralisation assay was also significantly elevated in ELISA-seropositive individuals, as expected ($p<0.0001$, Mann-Whitney test).

To explore the dynamics of T-cell immunity in individuals seropositive and seronegative for SARS-CoV-2, T-cell proliferation to four pools comprising three structural antigens (S1, S2, M, and N) as well as pools spanning the majority of the genome (ORF3, ORF8, NSP1+2, NSP3A, NSP3B, NSP3C, NSP4, NSP5+6, NSP7-11, NSP12A, NSP12B, NSP13, NSP14, NSP15+16) was measured in seropositive and seronegative family members. CD4+ T-cell proliferation to structural pools was significantly higher in seropositive individuals compared to seronegative individuals

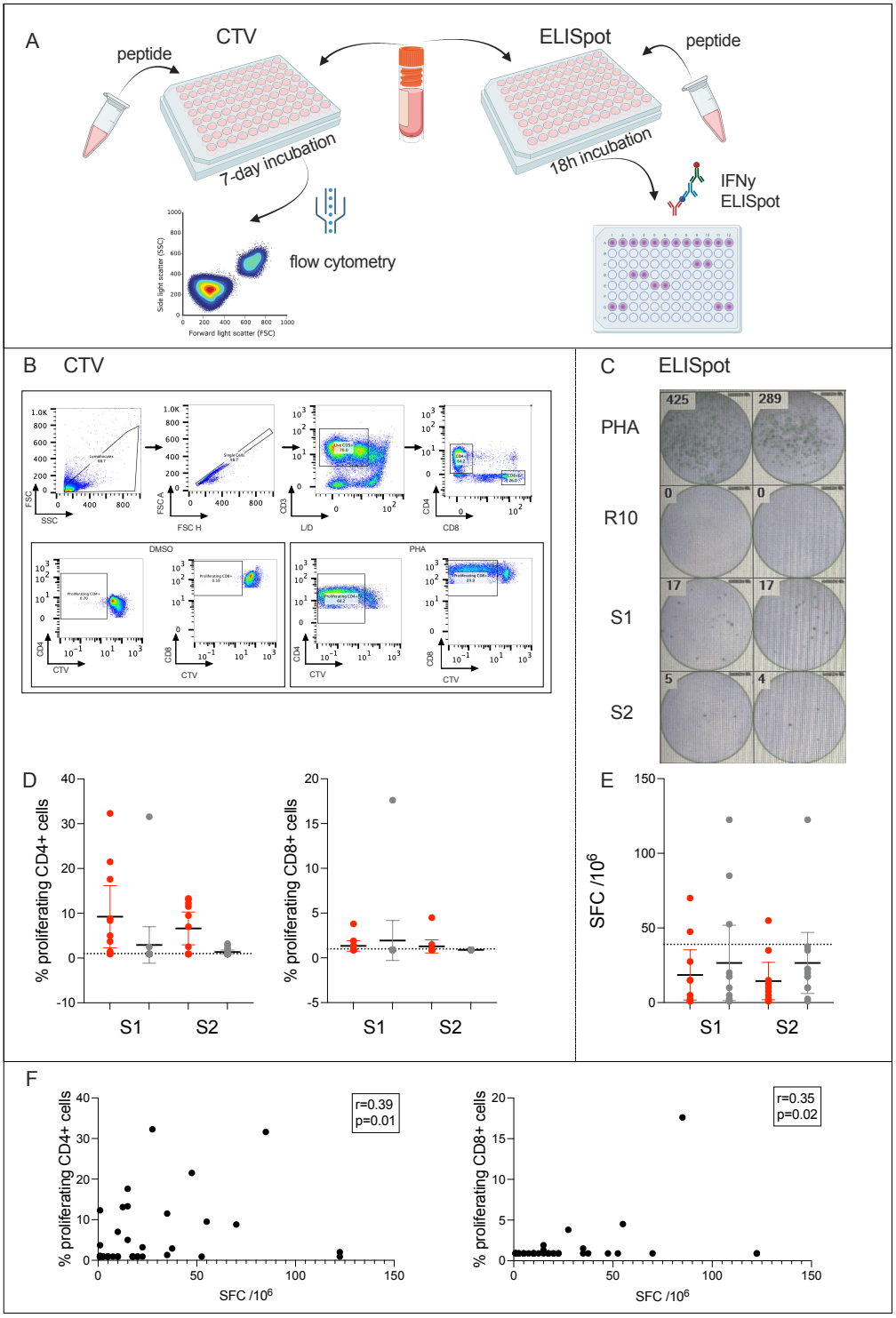


Figure 3.2: Validation of a T-cell proliferation assay. Comparison of proliferation and ELISpot assays (A). Gating strategy for proliferation assay (B). Example positive control (PHA), negative control (R10) and test wells (S1, S2) for ELISpot assay (C). Example CD4+ and CD8+ responses to S1 and S2 by proliferation assay. Seropositive individuals are shown in red, seronegative individuals in grey (D). Example responses to S1 and S2 pools by ELISpot assay (E). Correlation between ELISpot data and CD4+ or CD8+ proliferation assay data for S1 in both seronegative and seropositive individuals. p- and r- values from Spearman rank tests. Proliferation values below 1% were given nominal values of 0.9.

(all p<0.0001, Mann-Whitney tests) (Figure 3.3B), although a substantial number of seronegative individuals generated CD4+ responses, particularly to S1 and S2. There was a trend towards greater CD4+ T-cell proliferation in seropositive individuals for NSP pools compared to seronegative individuals, which reached significance for ORF3, ORF8 and NSP7-11 (ORF3: p=0.03; ORF8: p=0.02; NSP7-11: p=0.05; Mann-Whitney tests) (Figure 3.3C). These trends were mirrored in CD8+ T-cell responses (Figure 3.3DE); particularly elevated CD8+ T-cell responses in seropositive individuals included S1, S2, M, and N (all p<0.0001), ORF3 (, p=0.01), NSP1+2 (p=0.003), NSP3B (p=0.0002), NSP13 (p=0.01) and NSP14 (p=0.03) (Mann-Whitney tests), although taking into account multiple comparisons, some of these p-values may reflect statistical artefacts.

3.3.4 Transmission dynamics within families

One benefit of family studies of SARS-CoV-2 infection is the opportunity to examine risks of infection susceptibility and protection. Heterogeneity in susceptibility to COVID-19 was and still remains a challenge to understand. Mathematical analysis of the correlates of infection risk in this cohort provide some further clarity on determinants of infection susceptibility. At first glance, older female index patients appeared overrepresented in this cohort (74% of index patients were mothers). A chi-squared test of independence was performed to analyse the relationship between sex and infection risk. The relationship between these variables was not significant, X^2 (1, N = 232) = 8.9, p = 0.1. To further examine whether females were at increased risk of infection in this cohort, considering the potential effect of age, multiple logistic regression was carried out (Table 3.2). This included an interaction term between age and sex, in order to test whether the the effect of sex on infection risk was the same regardless of age. The fitted regression model was:

Infected? = Intercept + Age + Sex[Female] + (Age:Sex[Female])

There was no increased infection risk associated with age (p=0.16) or female

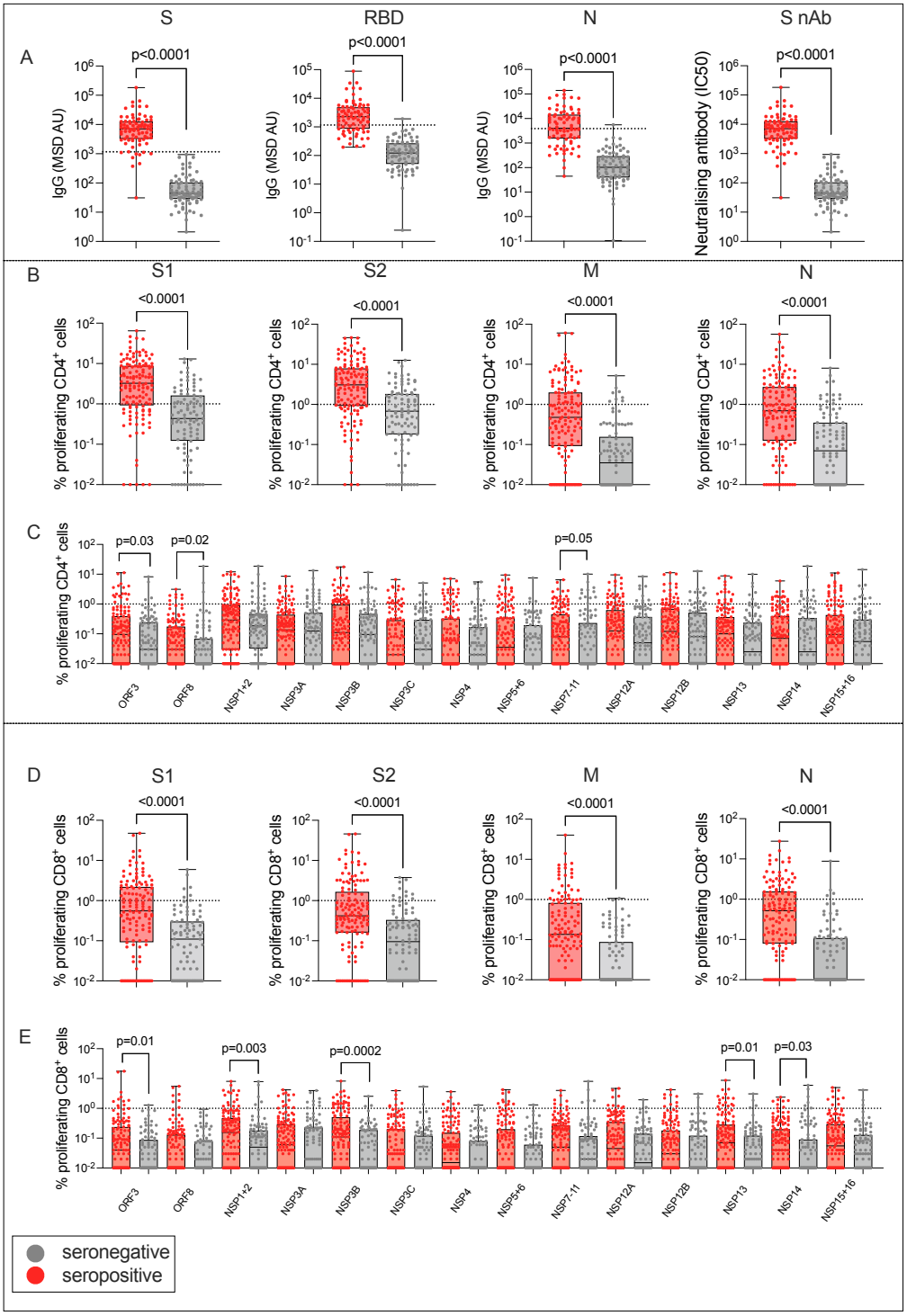


Figure 3.3: Humoral and cellular immune responses in seronegative and seropositive individuals. Total IgG responses to SARS-CoV-2 S, RBD, and N, in ELISA-seropositive (red) and seronegative (grey) individuals, as measured by MSD v-plex immunoassay, and nAb responses to SARS-CoV-2 as measured by pseudoneutralisation assay (A). CD4+ T-cell responses to SARS-CoV-2 S1, S2, M, and N as measured by proliferation assay (B). CD4+ T-cell responses to non-structural peptide pools (C). CD8+ T-cell responses to S1, S2, M, and N as measured by proliferation assay (D). CD8+ T-cell responses to non-structural peptide pools (E). p-values refer to Mann-Whitney tests. Dotted lines refer to cutoffs as determined previously.[60, 120] Proliferation values below 1% were given nominal values of 0.9%.

Parameter estimates	Variable	Odds ratio	Standard error	95% CI	|Z|	p-value
$\beta 0$	Intercept	1.68	0.42	-0.30 to 1.4	1.23	0.22
$\beta 1$	Age	0.98	0.01	-0.04 to 0.006	1.40	0.16
$\beta 2$	Sex[Female]	0.54	0.56	-1.7 to 0.5	1.10	0.27
$\beta 3$	Age : Sex[Female]	1.03	0.015	0.003 to 0.06	2.11	0.034

Table 3.2: Multiple logistic regression of the impact of age and sex on infection risk.

sex (p=0.27) alone. However, there was a significant interaction between age and sex (p=0.03), indicating that the impact of sex on infection risk varied by age.

To further understand how sex and age interact to impact infection risk in this cohort, two simple logistic regression models were carried out for males and females separately. Looking at the effect of age on infection risk in females only, the following model was created:
Infected? = -0.12 + 0.02*(Age)

The log odds of becoming infected were significantly associated with increased age (p=0.04) in females. For every one year increase in age, the log odds of infection increased by 2%. Looking at the effect of age on infection risk in males only, the following model was created:
Infected? = 0.49 – 0.01*(Age)

The log odds of becoming infected were not significantly associated with age (p=0.27) in males. Together, these models indicate that older females were more likely to be infected in this cohort, reflecting the bias towards infected mothers likely as a result of recruitment methods.

3.3.5 Family types

To examine the immune dynamics of infection within and between families, families with similar patterns of seropositivity were identified and grouped into seven family types (Figure 3.4). These included: all seropositive (18 families) (Figure 3.4A), all

seronegative (six families) (Figure 3.4B), seropositive father/male (three families) (Figure 3.4C), seropositive mother/female (17 families) (Figure 3.4D), seropositive children only (five families) (Figure 3.4E), serodiscordant parents with some seropositive children (six families) (Figure 3.4F) and seropositive seroconcordant parents with some seropositive children (seven families) (Figure 3.4G). Interestingly, the rarest groups, therefore, were seropositive children only and seropositive adult males only. All seropositive seroconcordant parents had at least one seropositive child. Overall, the families represented a diverse group, indicating that intrafamilial transmission can occur through multiple routes. The cohort was overwhelmingly made up of biological families living together. However, one non-family household was included (Family 2), which consisted of four cohabitating students. Although these individuals were not related, they were included in Figures 1.4 and 1.7 as they were one of only two households where the only seropositive individual was an adult male. However, they were excluded from larger analyses of parent-child dynamics and intra-couple dynamics.

Having identified families with common patterns of infection, immune dynamics within groups were analysed. Firstly, a representative case study from Group A was assessed in detail. A fully seropositive family (Family 8) consisting of a 39-year-old mother (orange circle), a 41-year-old father (purple square), two daughters (pink and yellow circles) and two sons (blue and green squares) are shown in Figure 3.5A. All individuals made IgG responses to SARS-CoV-2 S; the response of greatest magnitude was the father (1011 EU), and the response of lowest magnitude was the mother (18 EU). The greatest nAb response (IC50 = 74) and S1-specific CD4+ T-cell response (14% proliferation) also belonged the father. All individuals made CD4+ T-cell responses to either S1 or S2 pools; CD8+ responses were weaker.

In order to compare immune responses between all mothers, fathers, daughters and sons from the 'all seropositive' group, and assess whether any age- or sex-related differences in immunity were present in families where all individuals became

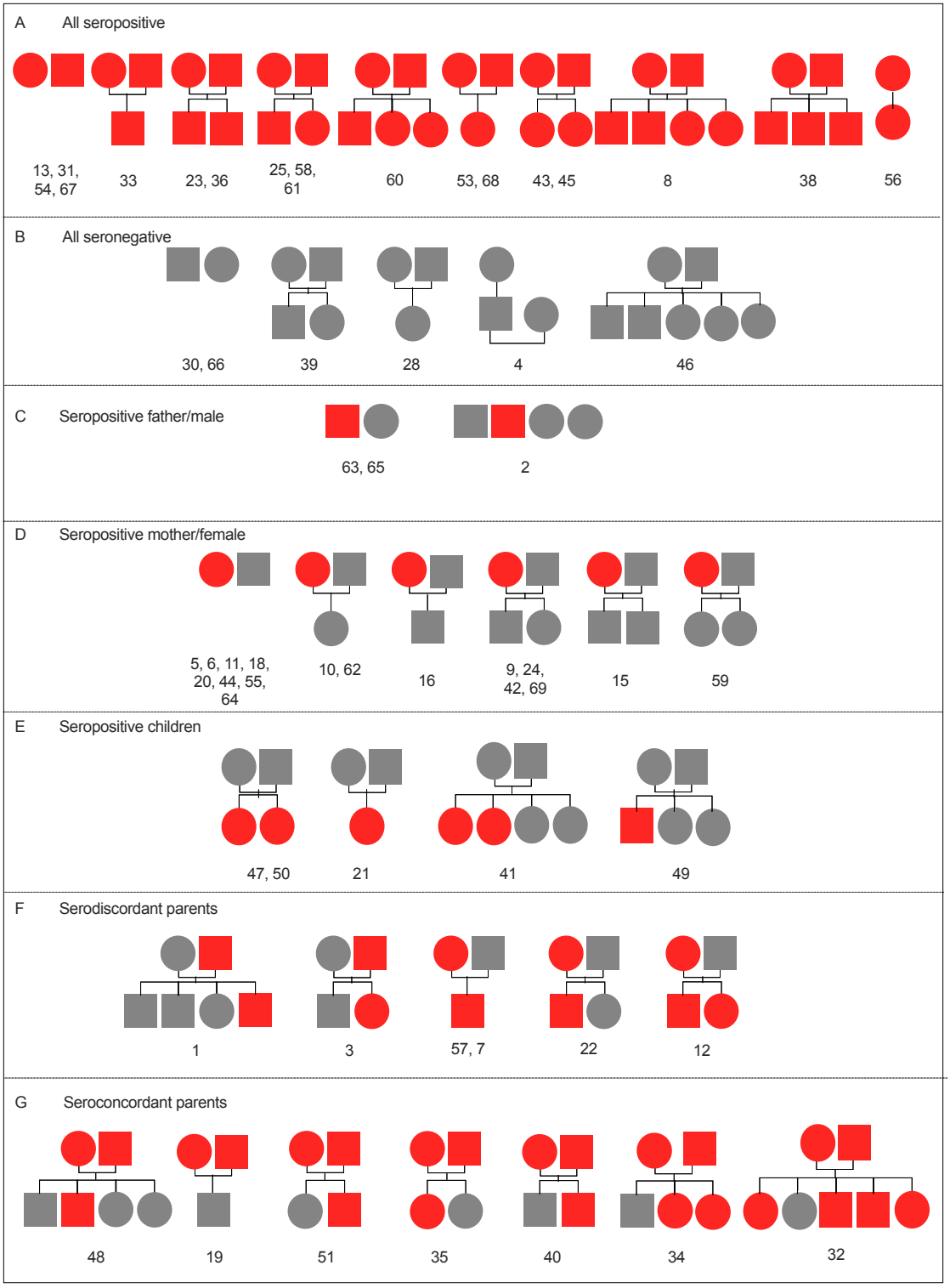


Figure 3.4: Family types. Example families where all individuals became infected (A). Families where all remained seronegative (B). Families where only the father or an adult male were infected (C). Families where only the mother was infected (D). Families where only children were infected (E). Families where one parent and a child/children were infected (F). Families where both parents and a child/children were infected (G). Squares are male, circles are female. Seropositive individuals are shown in red, seronegative in grey.

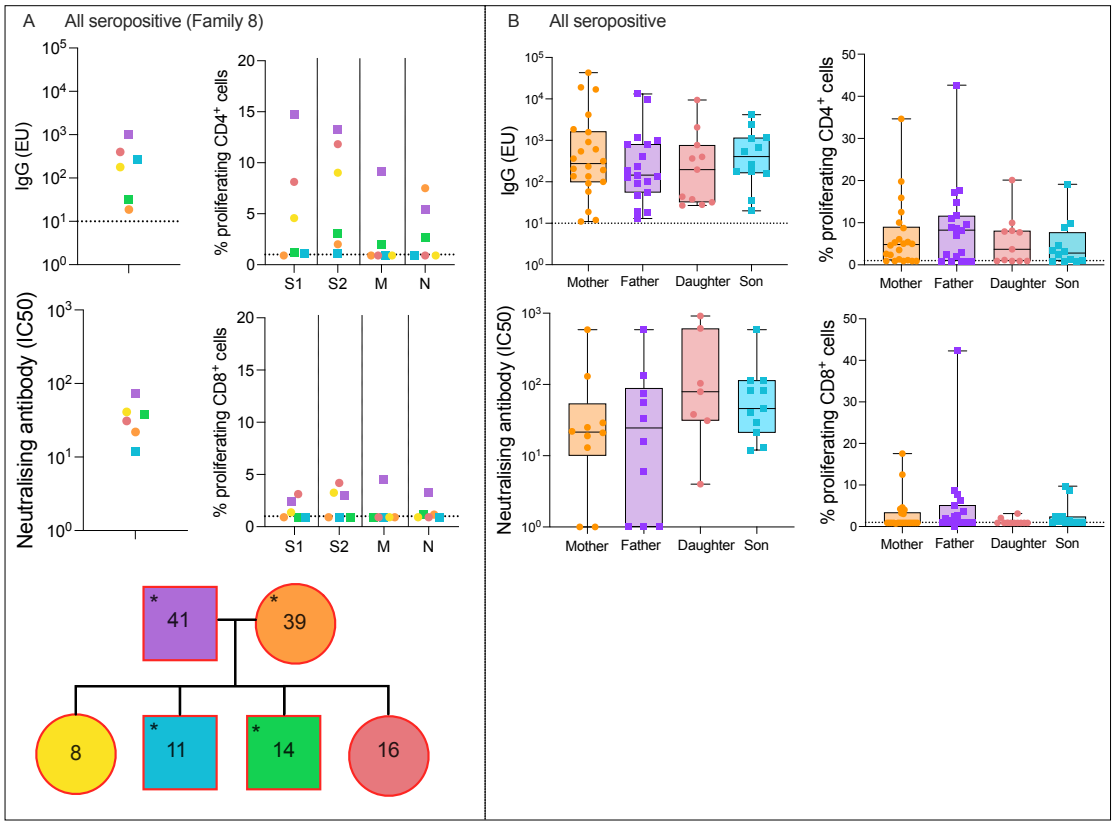


Figure 3.5: Family Type A. Anti-S IgG, nAb responses, CD4+ T-cell responses, and CD8+ responses in a seropositive family group consisting of mother, father, two sons and two daughters (A). IgG, nAb, and T-cell responses in all individuals from the 'All seropositive' family type (B). Males are squares, females are circles. Asterisks mark symptomatic individuals. Red outlines indicate seropositive individuals. Proliferation values below 1% were given nominal values of 0.9.

infected, IgG, nAb titre, and S1-specific CD4+ and CD8+ T-cell responses were compare between mothers (orange circles), fathers (purple squares), daughters (pink circles) and sons (blue squares) across all of the 'all seropositive' group (Figure 3.5B). Notably, there was no difference in IgG, nAb response, or CD4+ S1-specific T-cell response between any of the family members in this group.

Next, analysis was performed of families where no individuals became infected, to assess whether any pre-existing SARS-CoV-2-specific immunity was present in seronegative individuals. Although these individuals were seronegative, in order to be recruited onto the study, at least one individual self-reported symptoms of COVID-19. A representative fully seronegative family (Family 46) consisting of

a mother, father, three daughters, and two sons generated no IgG or nAbs to SARS-CoV-2, as defined (Figure 3.6A). T-cell responses were absent in most family members, although the 22-year-old daughter generated a weak CD4+ response to S2 (1.3% proliferation), and the 15-year-old daughter generated a weak CD4+ response to N (1.2% proliferation).

To determine if any form of SARS-CoV-2-specific immunity was present in all members of the 'all seronegative' group, and to uncover any age- and sex-specific trends, IgG, nAbs, and T-cell responses were compared between mothers, fathers, daughters and sons in the 'all seronegative' group (Figure 3.6B). By definition, no humoral immunity was observed. Of note, some mothers and fathers generated CD4+ T-cell responses to S1. However, no children generated a CD4+ or CD8+ response to S1, raising the question of whether pre-existing immunity is found at higher levels in older individuals.

Although some families were either fully seropositive or fully seronegative, the majority of families were of mixed serostatus. This is consistent with findings in the literature that demonstrate heterogeneity in infection susceptibility between family members.[138] Three case studies of such families are shown in Figure 3.7. Families where the only seropositive individual was an adult male were rare, perhaps as mothers were the most common index patient (74% of index patients) and fathers only represented 9% of index patients. One 'family', a household of four individuals aged 23-28, was examined closely, particularly as the seronegative 25-year-old male reported anosmia, cough and gastrointestinal symptoms when his seropositive 23-year-old male household member was also symptomatic. Interestingly, despite remaining seronegative and nAb negative, the 25-year-old symptomatic male and the other two seronegative females all generated CD4+ responses, in particular to S2 (Figure 3.7A). The seropositive male generated a strong IgG, nAb, CD4+ and CD8+ T-cell response, as expected.

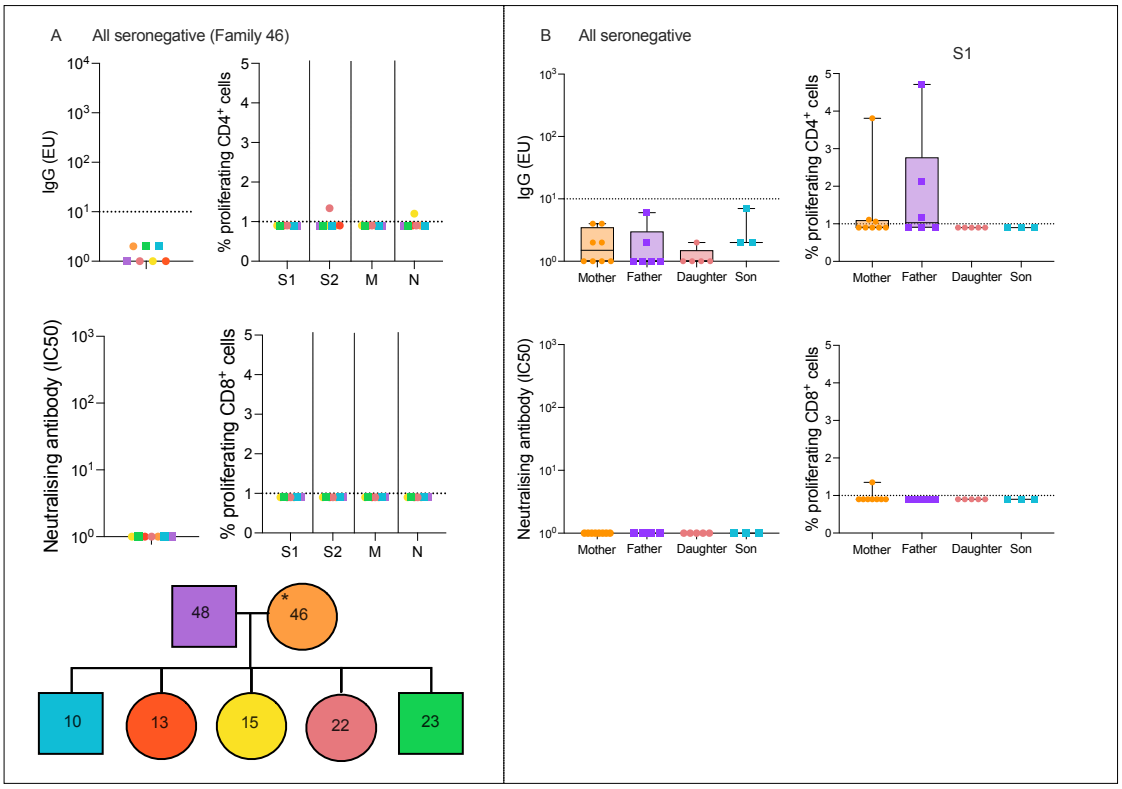


Figure 3.6: Family Type B. Anti-S IgG, nAb responses, CD4+ T-cell responses and CD8+ responses in a seronegative family group consisting of mother, father, two sons, and three daughters (A). IgG, nAb, and T-cell responses in all individuals from the 'All seronegative' family type (B). Males are squares, females are circles. Asterisks mark symptomatic individuals. Proliferation values below 1% were given nominal values of 0.9.

Families where the only seropositive individual was an adult female were common. Family 9 consisted of a 59-year-old mother, a 68-year-old father, a 30-year-old son and a 19-year-old daughter (Figure 3.7B). The mother generated strong IgG (308 EU) and nAb (IC50 63) responses to SARS-CoV-2. Interestingly, the seronegative father, who also reported symptoms, generated a moderate nAb response (IC50 12), and a moderate CD4+ response to S1 (1.3%). In contrast to the 'all seronegative' families where T-cell immunity was only present in parents, the seronegative son also generated a measurable CD4+ response to S1 (1.8%), and the daughter generated a CD4+ response to N (1.4%) despite no humoral immunity or cellular immunity to any other antigen. Only the seropositive mother had measurable CD8+ T-cells.

Five families had seropositive children and seronegative parents. One of these, family

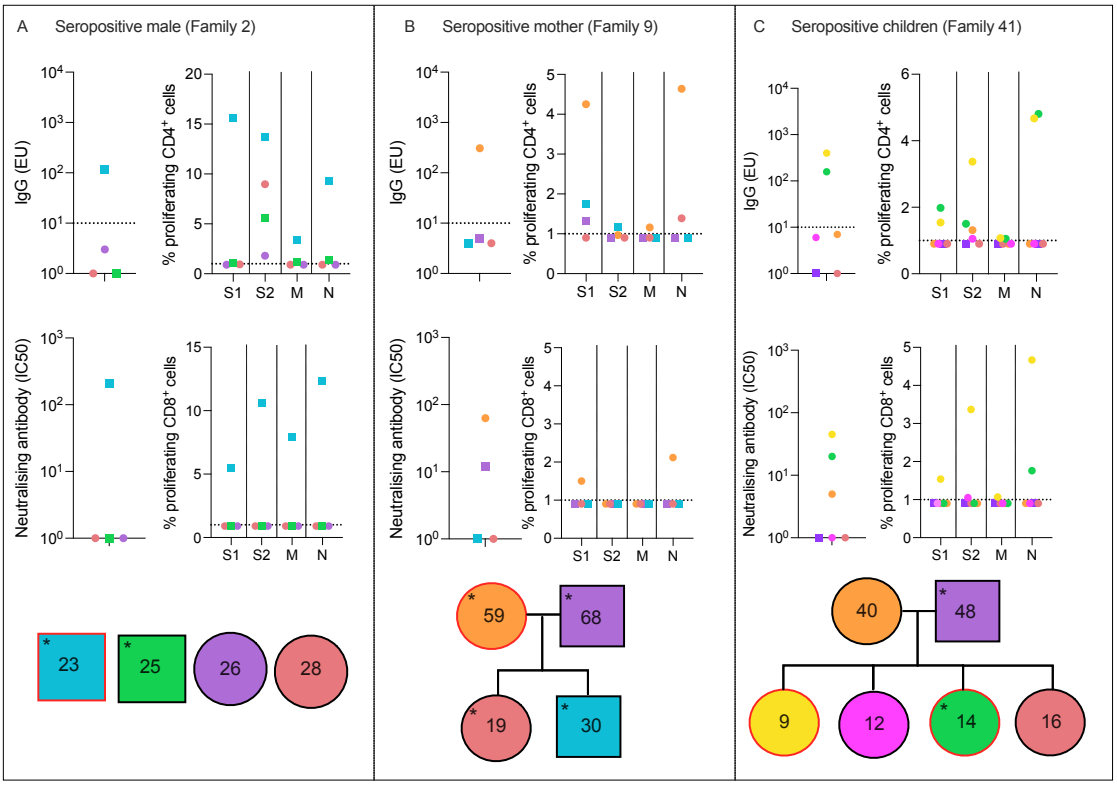


Figure 3.7: Family Types C, D and E. Anti-S IgG, nAb responses, CD4+ T-cell responses, and CD8+ responses in a household group consisting of a seropositive male, a seronegative male and two seronegative females (A). IgG, nAb and T-cell responses in a household group consisting of a seropositive mother, and seronegative father, daughter, and son (B). IgG, nAb, and T-cell responses in a household group consisting of seronegative parents, two seropositive daughters, and two seronegative daughters (C). Seropositive family members are outlined in red. Males are squares, females are circles. Asterisks mark symptomatic individuals. Proliferation values below 1% were given nominal values of 0.9.

41, was made up of a 40-year-old mother, 48-year-old father, and four daughters aged 16, 14, 12, and 9 (Figure 3.7C). Two daughters were seropositive (14 and 9) and two were seronegative (16 and 12). The 14- and 9-year-old seropositive daughters generated nAb responses (IC50 20 and 45, respectively), and the seronegative mother generated a small nAb response (IC50 5). Both seropositive daughters generated CD4+ responses to S1, S2, M, and N, and the mother generated a response to S2 (1.5%). CD8+ responses were highest in the 9-year-old daughter but also measurable in the other seropositive 14-year-old daughter. The seronegative daughters did not generate any measurable immunity other than small CD4+ T-cell responses to S2.

The final two family types were 'serodiscordant parents' and 'seroconcordant parents'. These were mixed serostatus families where either one (serodiscordant) or both (seroconcordant) parents were seropositive, and at least one child was also seropositive. Family 1, a 'serodiscordant parents' family, consisted of a 51-year-old father, a 44-year-old mother, three sons aged 13, 16, and 17, and an 8-year-old daughter (Figure 3.8A). Only the father and 13-year-old son were seropositive and generated IgG and nAb responses. However, CD4+ responses to S1 were observed in the father, seropositive 13-year-old son, seronegative 17-year-old son, and mother, and the seronegative 16-year-old son generated a response to N. Only the father generated CD8+ responses to S1, S2, M, and N.

Family 32 represented a 'seroconcordant parents' family, where both parents became infected. This family consisted of a 38-year-old father, a 35-year-old mother, three daughters aged 13, 11, and six, and two sons aged 14 and eight. All were seropositive except the 11-year-old daughter (Figure 3.8B). nAb responses were varied but were highest in the 14-year-old son. Surprisingly, only the mother generated measurable CD4+ and CD8+ T-cell responses.

3.3.6 Immune responses in serodiscordant and seroconcordant couples

To assess correlates of immunity in households where both parents or just one parent were seropositive, IgG (Figure 3.9A) and CD4+ T-cell responses to S1 (Figure 3.9B) were compared between the following individuals: seropositive and seronegative parents and their seropositive and seronegative children, and seropositive seroconcordant parents and their seropositive and seronegative children. Due to the potential role of a robust immune response in reducing peak viral load and preventing onwards transmission of SARS-CoV-2, it was hypothesised that a stronger immune response would be seen in seropositive partners of seronegative individuals compared to seroconcordant parents where both became infected. Though there was a trend of greater IgG and CD4+ responses in seropositive serodiscordant parents

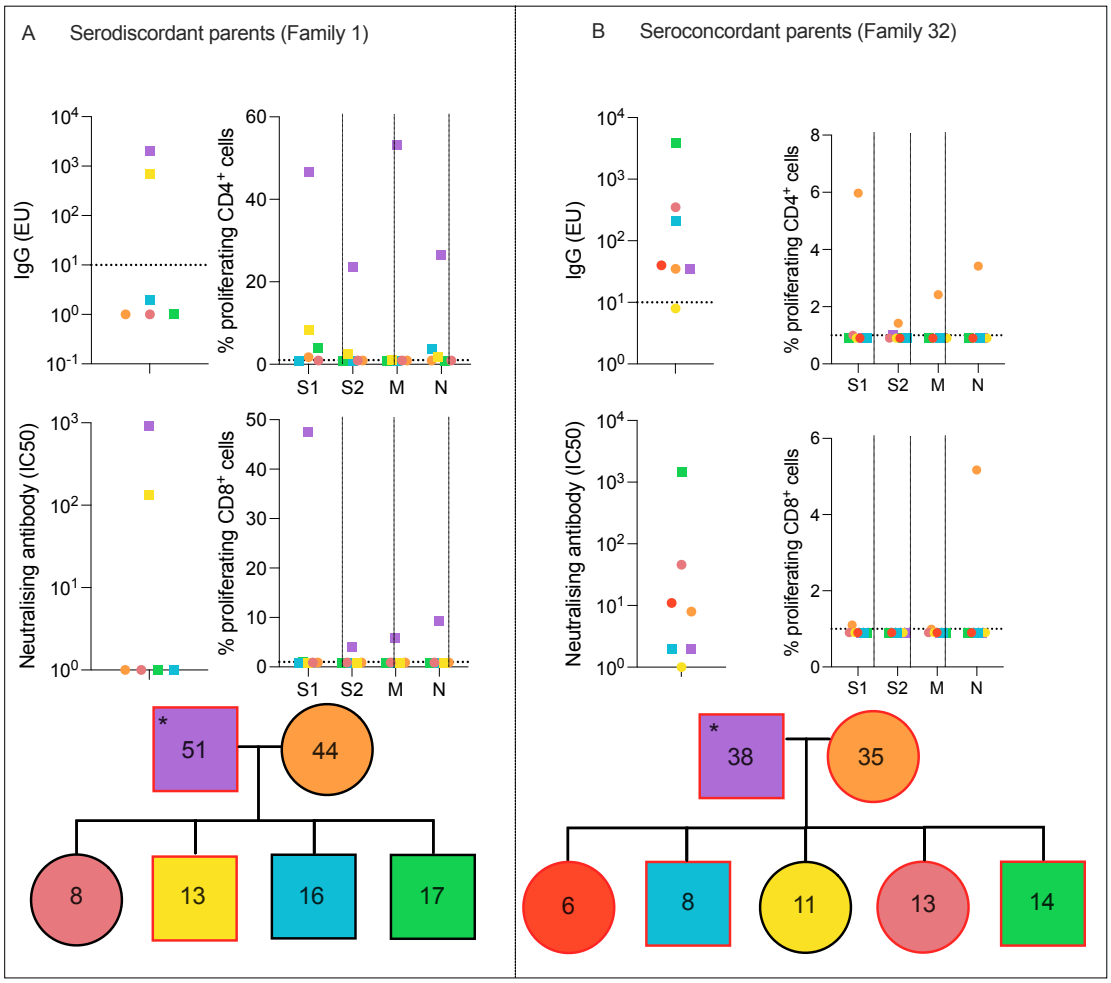


Figure 3.8: Family Types F and G. Serodiscordant parent and seroconcordant parent families. Anti-S IgG, nAb responses, CD4+ T-cell responses, and CD8+ responses in a family group consisting of a seropositive father, seronegative mother, two seronegative sons, one seronegative daughter, and a seropositive son (A). IgG, nAb, and T-cell responses in a family group consisting of seropositive parents, two seropositive sons, two seropositive daughters, and a seronegative daughter (B). Males are squares, females are circles. Seropositive family members are outlined in red. Asterisks mark symptomatic individuals. Proliferation values below 1% were given nominal values of 0.9.

compared to seropositive seroconcordant parents, this was not significant (IgG: $p>0.99$; CD4+: $p=0.25$, Kruskal-Wallis tests with Dunn's multiple comparisons). It was also hypothesised that immune responses in children of seroconcordant parents would be greater than children of serodiscordant parents, owing to a larger number of family members being infected and therefore perhaps a greater intensity of viral exposure. However, there was no significant difference in IgG or CD4+ T-cell response between seronegative children of serodiscordant parents and

seronegative children of seroconcordant parents (both p>0.99, Kruskal-Wallis test with Dunn's multiple comparisons) and no significant difference in IgG response between seropositive children of serodiscordant parents and seropositive children of seroconcordant parents (IgG: p>0.99, Kruskal-Wallis test with Dunn's multiple comparisons). However, S1-specific CD4+ responses were higher in seropositive children of serodiscordant parents compared to seropositive children of seropositive parents (p=0.029, Kruskal-Wallis test with Dunn's multiple comparisons).

To further explore whether the immune response of an index patient was associated with the infection status of their partner, and to determine if stronger immunity in an index patient might reduce their partner's likelihood of becoming infected, IgG response, nAb titre, and CD4+ response targeting S1, S2, M, and N in seropositive individuals from serodiscordant couples and seroconcordant couples were compared (Figure 3.10). There was no significant difference between serodiscordant index patients and seroconcordant index patients for any of the cellular and humoral immune components studied. However, there was a trend towards greater nAb responses (Figure 3.10B), and greater T-cell immunity to S1, S2, and N (Figure 3.10CDF) in serodiscordant index patients (i.e individuals who failed to pass on their infection to their partner).

3.3.7 Age and sex differences in immune response

As females have been shown to generate greater antibody-mediated immunity than males following viral infection,[98] and males experience worse outcomes following SARS-CoV-2 infection,[103] we sought to compare immune responses to SARS-CoV-2 between males and females in this cohort (Figure 3.11). Unexpectedly, there was no significant difference in humoral or cellular immunity in seropositive males versus females, either for total anti-S IgG (Figure 3.11A), nAb IC50 (Figure 3.11B), or CD4+ T-cell responses to the four structural pools (Figure 3.11C-F)

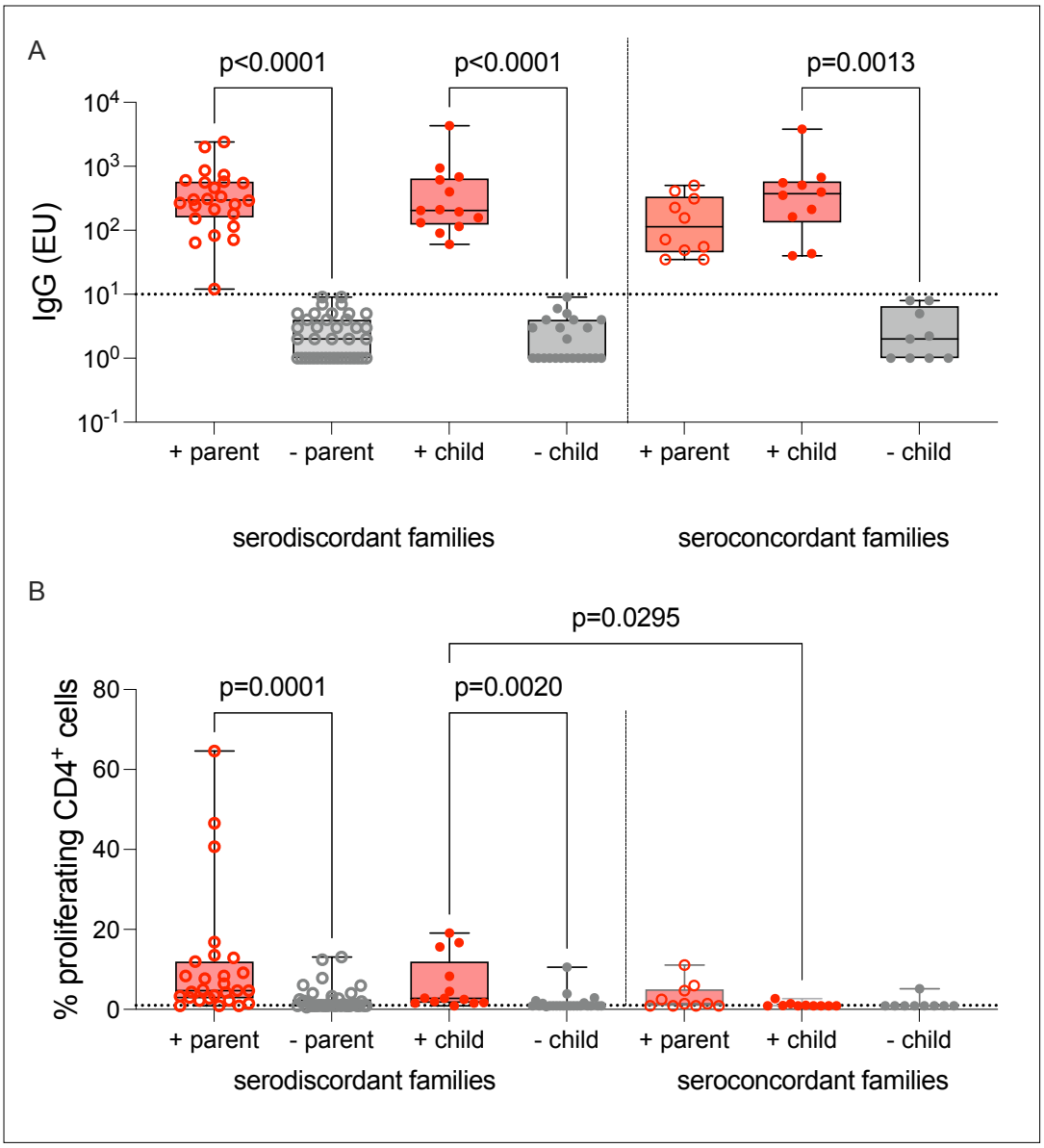


Figure 3.9: Serodiscordant and seroconcordant families. IgG (A) and S1-specific CD4+ T-cells (B) in seropositive partners of seronegative individuals, seronegative partners of seropositive individuals, seropositive children of serodiscordant parents, seronegative children of serodiscordant parents, seropositive partners of seropositive individuals, seropositive children of seroconcordant parents, and seronegative children of seroconcordant parents. Seropositive individuals are red, seronegative individuals are grey. Parents are unfilled circles, children are filled circles. p-values refer to Kruskal-Wallis tests with Dunn's multiple comparisons. Proliferation values below 1% were given nominal values of 0.9.

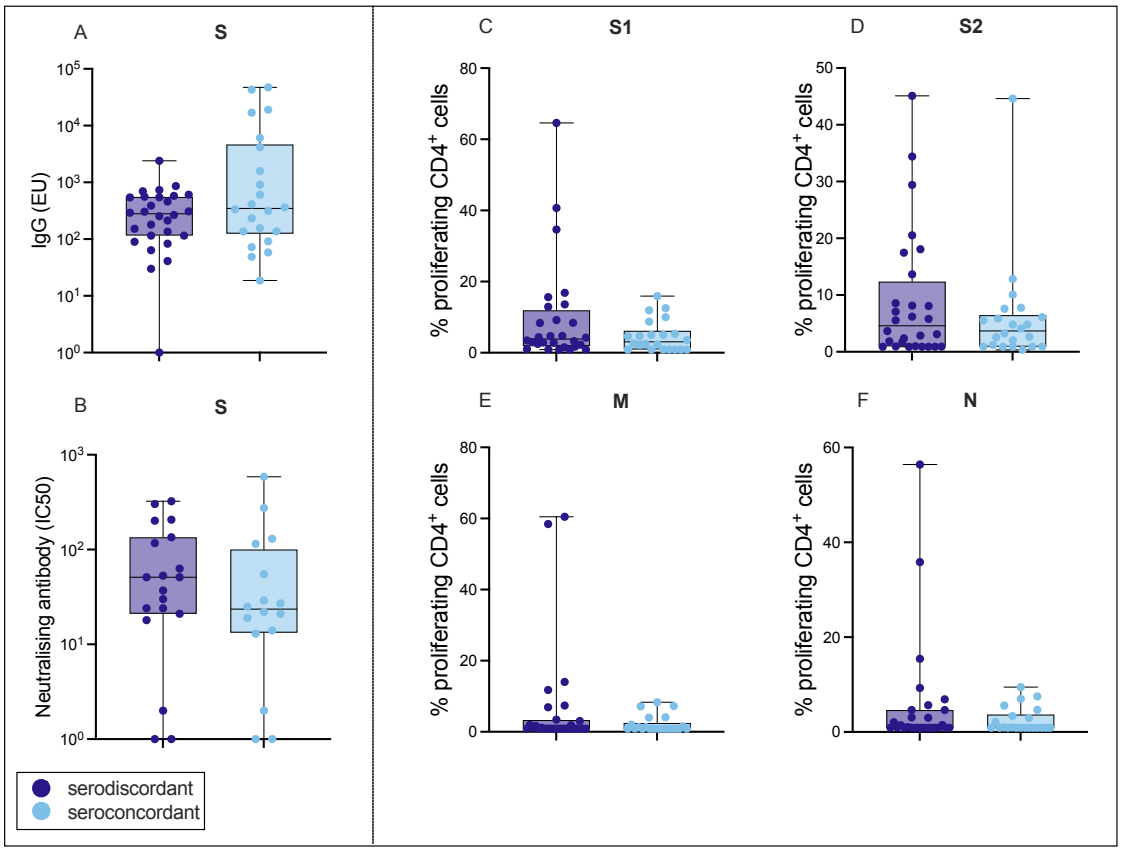


Figure 3.10: Serodiscordant and seroconcordant couples.. Anti-S IgG (A), nAb responses (B), and CD4+ T-cell responses to S1 (C), S2 (D), M (E), and N (F) in seropositive individuals from serodiscordant couples (navy) and seroconcordant couples (light blue). Values below 1% proliferation were given nominal values of 0.9.

(Mann-Whitney tests).

This cohort was unique in comprising individuals of diverse ages, from six to 88 years old. It was therefore possible to explore the impact of age on immunity following infection with SARS-CoV-2. It was hypothesised that older individuals would generate weaker immune responses than children, due to the reduction in adaptive immune functionality associated with immunosenescence.[77] Correlations between age and both humoral and cellular responses in seropositive individuals were calculated using Spearman's rank tests (Figure 3.12). There was a weak positive correlation between age and S1-specific CD4+ response that approached significance (r=0.17, p=0.06) and a weak positive correlation between age and M-specific CD4+ response (r=0.19, p=0.04) (Spearman's rank tests).

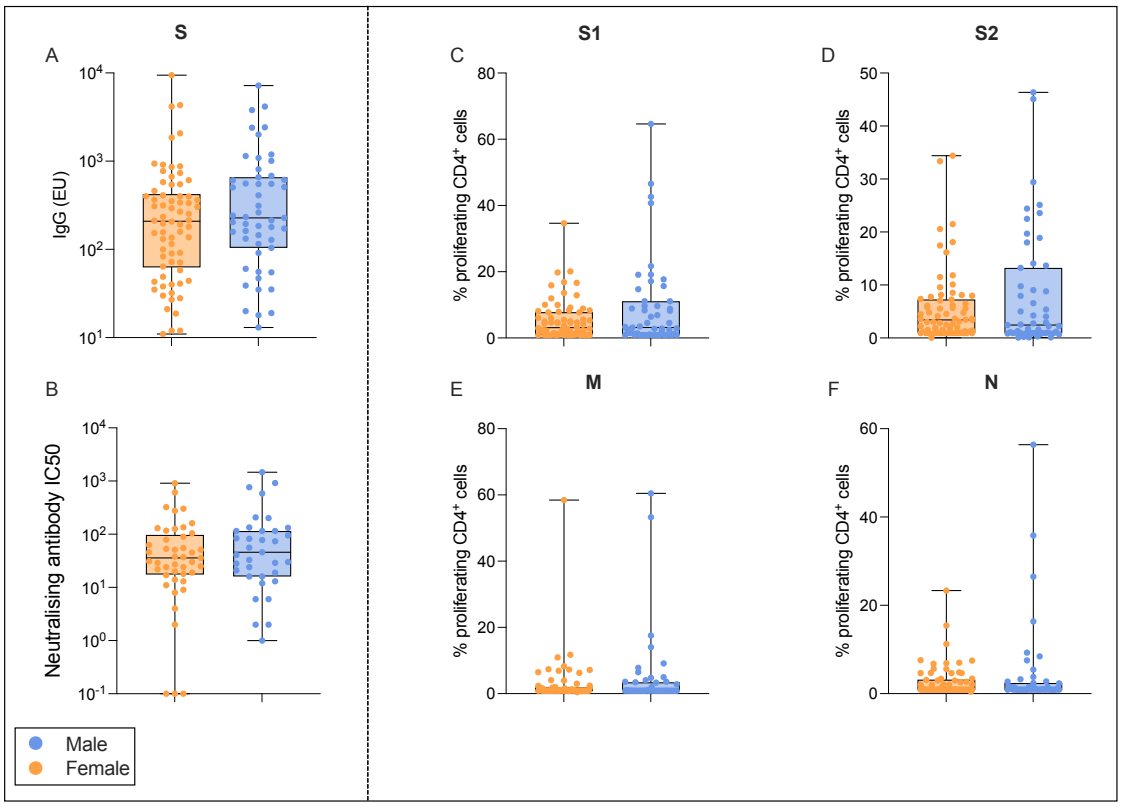


Figure 3.11: Humoral and cellular immunity by sex. Anti-S IgG (A), nAb response (B), and CD4+ T-cell response to S1 (C), S2 (D), M (E), and N (F) in females (orange) and males (blue). Proliferation values below 1% were given nominal values of 0.9.

To confirm these results, and to analyse more closely if age and sex had an impact on immunity, two multivariable linear regressions were run. Model one calculated the effects of age, sex, and days since symptom onset on total IgG response in seropositive individuals. The fitted regression model was:

IgG = 2744-15.78*(Age) -195.2*(Sex[Male]) – 5.808*(Days since symptoms)

The overall regression was statistically significant ($R^2 = 0.22$, $F(3, 35) = 3.29$, p=0.03). It was found that neither age ($\beta = -15.8, p = 0.26$), nor male sex ($\beta = -195.2, p = 0.67$) significantly predicted IgG response. However, days since symptoms ($\beta = -5.81, p = 0.017$), was negatively associated with IgG response, indicating some waning of humoral immunity over time (Table 3.4).

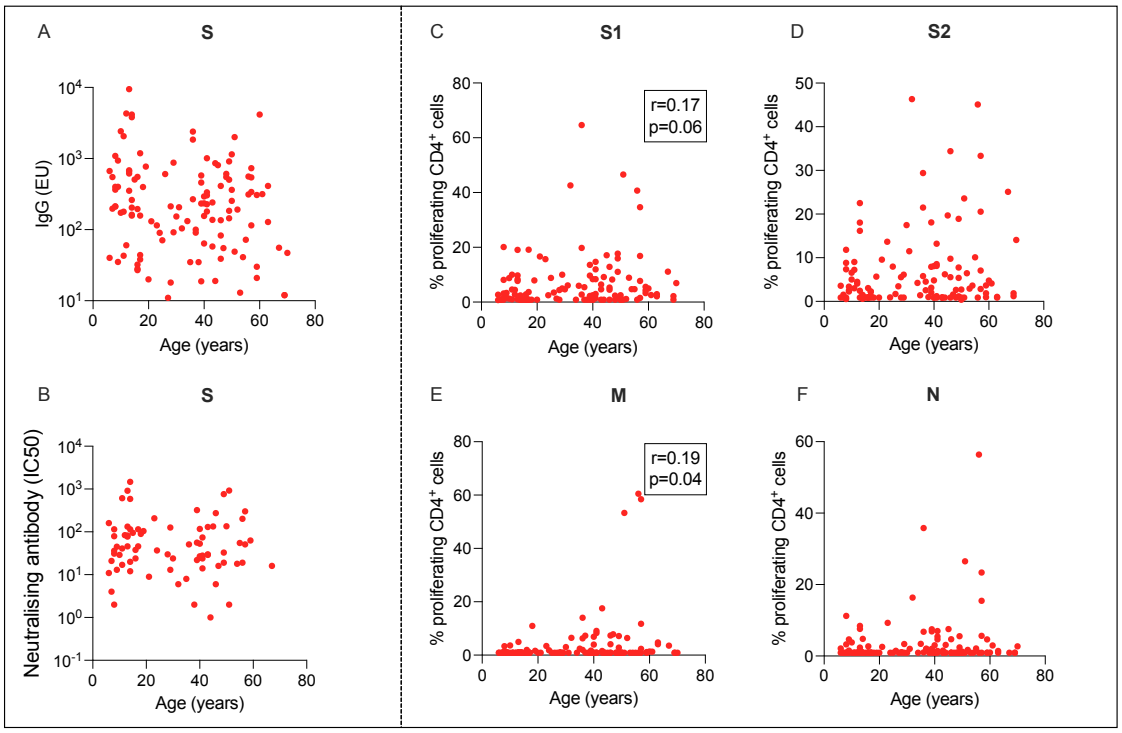


Figure 3.12: Humoral and cellular immunity by age. Correlation between age and anti-S IgG (A), nAb response (B), and CD4+ T-cell response to S1 (C), S2 (D), M (E), and N (F) in seropositive individuals. Proliferation values below 1% were given nominal values of 0.9.

Parameter estimates	Variable	Estimate	Standard error	95% CI	|t|	p-value
$\beta 0$	Intercept	2744	726.4	1270 to 4219	3.78	0.0006
$\beta 1$	Age	-15.8	13.91	-44.02 to 12.47	1.13	0.26
$\beta 2$	Sex[Male]	-195.2	460.2	-1129 to 739.1	0.42	0.67
$\beta 3$	Symptoms	-5.81	2.317	-10.51 to -1.105	2.51	0.017

Table 3.3: Multiple linear regression of the impact of age, sex and days since symptoms on total anti-S IgG response in seropositive individuals.

Model two calculated the effects of age, sex and days since symptom onset on S1-specific CD4+ T-cell responses in seropositive individuals. The fitted regression model was:

CD4+ response = 4.82+ 0.23*(Age) + 8.57*(Sex[Male]) – 0.037*(Days since symptoms)

Parameter estimates	Variable	Estimate	Standard error	95% CI	|t|	p-value
$\beta 0$	Intercept	4.82	4.37	-4.05 to 13.7	1.10	0.28
$\beta 1$	Age	0.23	0.084	0.064 to 0.40	2.80	0.0084
$\beta 2$	Sex[Male]	8.57	2.77	2.95 to 14.2	3.10	0.0038
$\beta 3$	Symptoms	-0.037	0.014	-0.065 to -0.0087	2.66	0.012

Table 3.4: Multiple linear regression of the impact of age, sex and days since symptoms on total anti-S CD4+ T-cell response in seropositive individuals.

The overall regression was statistically significant ($R^2 = 0.36$, $F(3, 35) = 6.69$, p=0.001). It was found that age ($\beta = 0.23, p = 0.0084$), male sex ($\beta = 8.57, p = 0.0038$), and days since symptoms ($\beta = -0.037, p = 0.012$) significantly associated with CD4+ response (Table 3.5). This suggested that older males generated stronger CD4+ T-cell responses to S1, with some waning in T-cell immunity with time since symptoms.

Observation of 'all seronegative' families suggested that pre-existing T-cell immunity was more prevalent in older individuals. To test this hypothesis, the correlation between age and T-cell responses were assessed in seronegative individuals (Figure 3.13). Notably, proliferating CD4+ T-cells targeting S1 were correlated with age in seronegative individuals (r=0.28, p=0.006, Spearman's rank test).

3.3.8 The relationship between symptoms and immunity

To explore the relationship between symptoms and immune response, humoral and cellular immunity was compared between asymptomatic and symptomatic individuals (Figure 3.14). There was no significance difference in total anti-S IgG or nAb titre between asymptomatic and symptomatic individuals (p=0.23, p=0.37, respectively, Mann-Whitney tests). However, symptomatic individuals generated significantly higher proliferative CD4+ T-cell responses to three of four structural

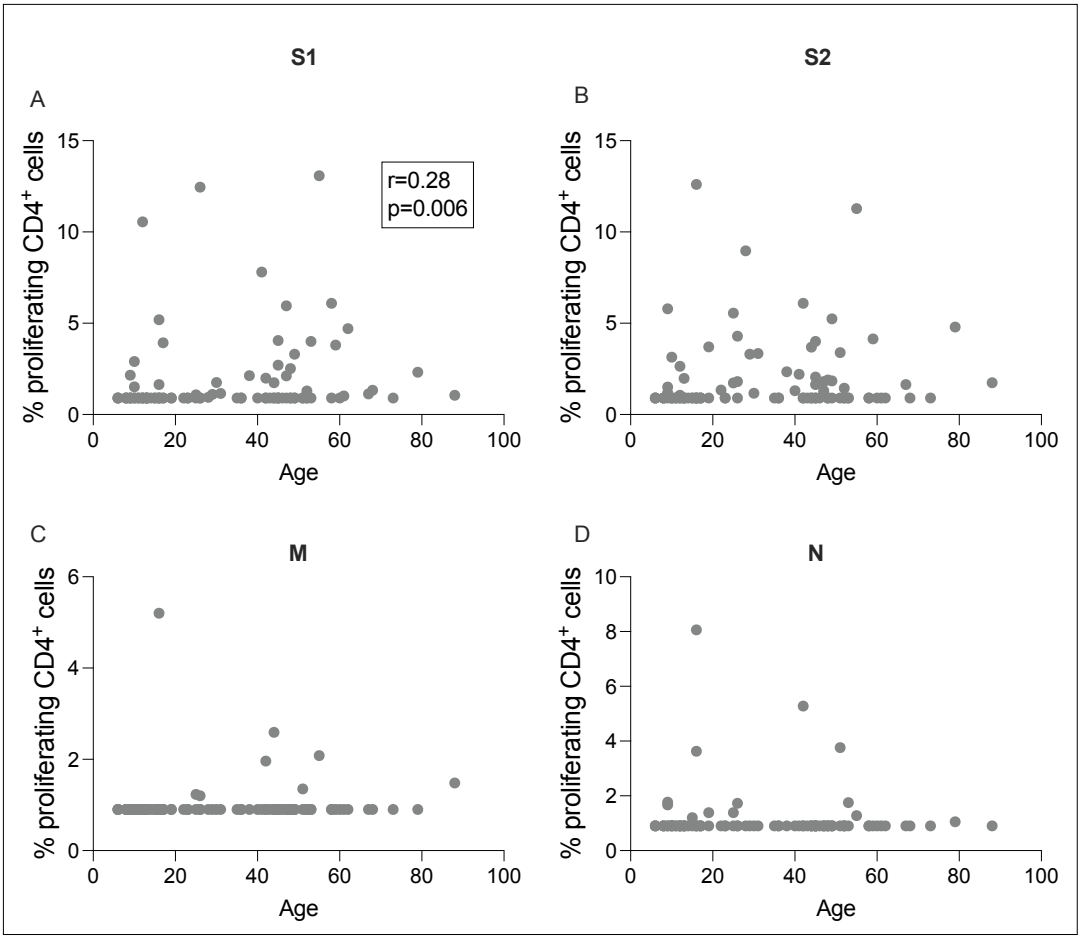


Figure 3.13: Cellular immunity in seronegative adults and children. Correlation between CD4+ T-cell responses to S1, S2, M, and N and age in seronegative individuals. Values below 1% were given nominal values of 0.9. p- and r- values refer to Spearman's rank correlation values.

pools (S1: p=0.002; S2: p=0.14, M: p=0.004; N: p=0.009, Mann-Whitney tests). To further assess the relationship between immunity and symptoms in a way that incorporated disease severity rather than just a binary value, individuals were assigned a symptom score based on the number of symptoms self-reported. There was a significant positive correlation between symptom score and all parameters measured(IgG: r=0.23, p=0.008; S1: r=0.32, p=0.0002; S2: r=0.23, p=0.0008; M: r=0.28, p=0.0002; N: r=0.3, p=0.0007; nAbs: r=0.27, p=0.002, Spearman's rank tests). Overall, these data demonstrate heightened immune responses in symptomatic individuals and in individuals that reported a greater number of symptoms.

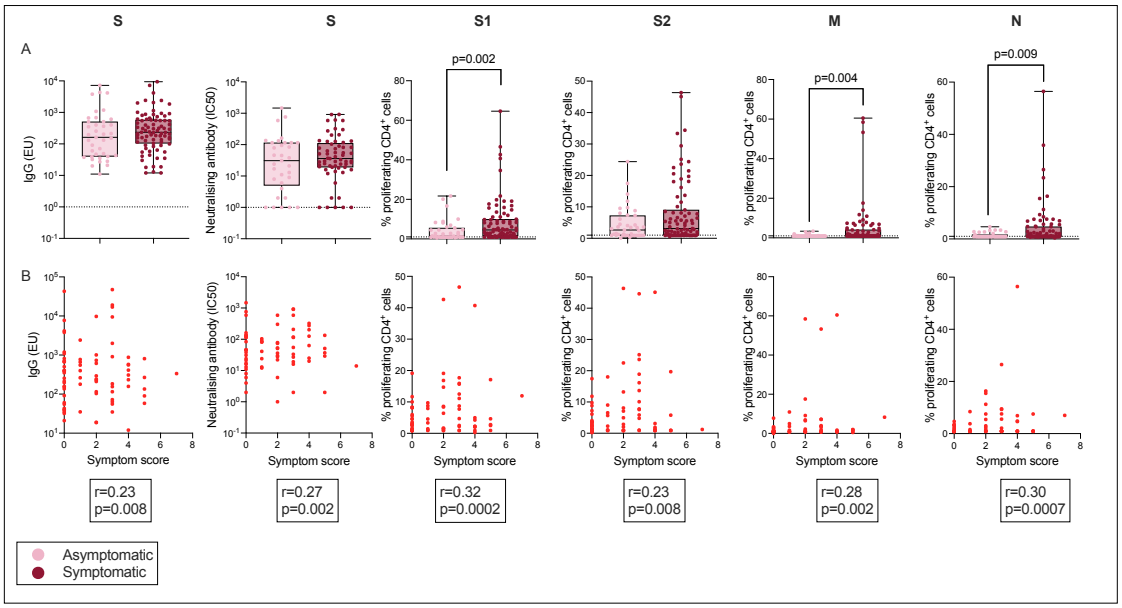


Figure 3.14: The relationship between symptoms and immunity. Anti-S IgG, nAb response, and CD4+ T-cell response to S1, S2, M, and N in asymptomatic (pink) and symptomatic (burgundy) seropositive individuals (A). The correlation between symptom score and anti-S IgG, nAb response, and CD4+ response to S1, S2, M and N in symptomatic seropositive individuals. Pairwise p-values from Mann-Whitney tests, r- and p-values from Spearman's rank tests.

The samples used in this study were taken a mean of 228 days (7.6 months) into convalescence. To assess the stability of cellular and humoral responses in seropositive individuals during convalescence, the correlations between days since symptom onset and IgG, nAb titre, and CD4+ T-cells targeting S1, S2, M, and N were calculated for seropositive individuals for whom temporal data was available (n=43) (Figure 3.15). There was a negative correlation between days since symptom onset and IgG (r=-0.36, p=0.02), nAb titre (r=-0.43, p=0.007), and CD4+ T-cells targeting S1 (r=-0.32, p=0.05), S2 (r=-0.38, p=0.02), and N (r=-0.46, p=0.004), but not M (r=-0.1, p=0.5) (Spearman's rank tests).

The cohort was broadly divided into individuals sampled 200-400 days post-symptoms (n=34) and individuals sampled 0-100 days post-symptoms (n=9). When divided into two subgroups, and correlations re-calculated for each subgroup, the correlation was lost, except for the 200-400 group for N (r=-0.40, p=0.03, Spearman

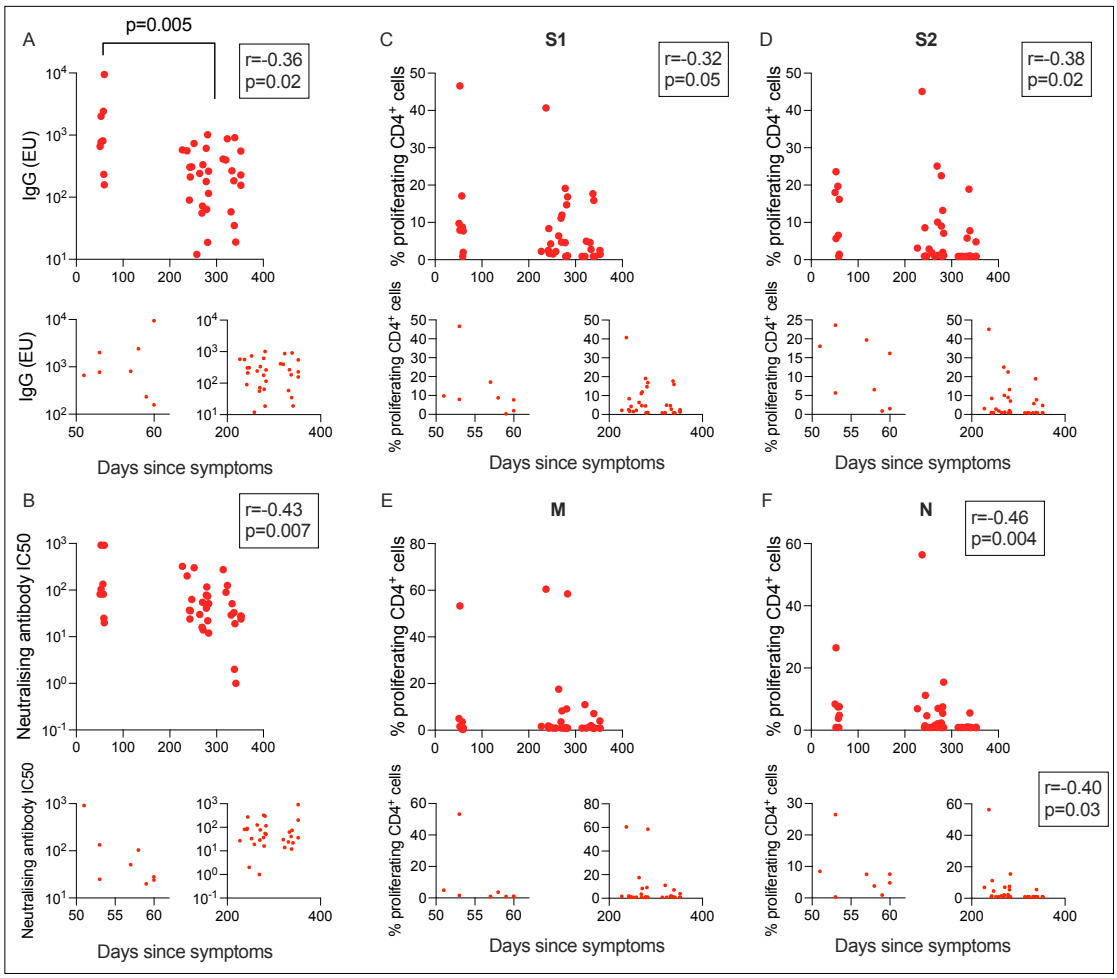


Figure 3.15: Durability of humoral and cellular immune responses. Correlation between the days since symptom onset and anti-S IgG (A), nAb response (B), and CD4+ T-cell response to S1 (C), S2 (D), M (E), and N (F) in seropositive individuals. r- and p-values from Spearman's rank tests for correlations, Mann-Whitney test for the pairwise comparison in A. Proliferation values below 1% were given nominal values of 0.9.

rank test). When comparing the two groups directly, median anti-S IgG response in the 0-100 group was significantly higher than the 200-400 group (790 vs 242, p=0.005, Mann-Whitney test), whilst T-cell and nAb responses were not significantly different between subgroups.

3.4 Discussion

Studies of viral disease within families provide an opportunity to assess correlates of infection susceptibility, disease severity, and immune response in individuals of differing age and sex. For SARS-CoV-2, to which exposure often occurred within households during nationwide lockdowns, this family study enabled a detailed assessment of transmission and immune dynamics to a novel viral pathogen at a time when exposure was highly likely. Here, we assessed humoral and cellular immunity in individuals infected by or exposed to SARS-CoV-2 through intra-household transmission, as well as in families with no documented SARS-CoV-2 infection.

CTV proliferation assays have been used to assess specific T-cell responses to viral pathogens, including SARS-CoV-2, [120, 141] ZIKV,[142] and Epstein-Barr virus (EBV),[143] as well as in oncological studies.[144, 145] Here, CTV proliferation assay was shown to be highly sensitive and T-cell data was well correlated with results from the standard ELISpot assay. Any variability between assays may result from the different parameters that the assays quantify: the proliferation assay assesses memory T-cell responses following a 7-day stimulation, whereas the ELISpot may be more biased towards IFNγ-secreting effector T-cell responses. Furthermore, the proliferation assay enables a finer dissection of CD4+ versus CD8+ responses, which the ELISpot assay does not. In a previous study, the CTV proliferation assay was shown to reliably pick up cross-reactive T-cell responses to S1 and S2 in seronegative individuals, whilst IFNγ ELISpot did not,[120] suggesting that ELISpot assays are perhaps more suited to identifying higher magnitude responses in PCR-confirmed seropositive individuals. However, SARS-CoV-2 T-cell responses in seronegative individuals have been detected by ELISpot previously.[7, 146] Here, it was demonstrated that the CTV assay was more sensitive to T-cell responses in both seronegative and seropositive individuals, which is why the assay was used to quantify cellular immunity in the remainder of the cohort.

Other assays to quantify virus-specific T-cells include the activation-induced marker (AIM) assay and intracellular cytokine staining (ICS) assay. Both have been used to identify cross-reactive T-cell responses in SARS-CoV-2 seronegative individuals.[1, 2] AIM is a cytokine-independent assay that uses flow cytometry to quantify expression of T-cell activation markers including CD69, CD40L, OX40, CD25, or CD137.[147, 148] ICS can be used in parallel to further elucidate the functionality and cytokine profile of specific CD4+ and CD8+ T-cells.[149] Although the employment of multiple T-cell assays was limited by cell availability in this study, it is important to note the different quantities they measure. The use of the CTV proliferation assay may bias the data described herein by picking up low-magnitude memory responses with a different functionality compared to those assayed by ELISpot, AIM, or ICS. However, for a cohort with mixed SARS-CoV-2 infection history, the proliferation assay was beneficial for identifying small responses in seronegative individuals, and therefore was well suited to this study.

CD4+ T-cell responses were identified in most seropositive individuals as well as some seronegative individuals in this cohort. In seropositive individuals, responses primarily targeted S1 and S2 regions of S, with some individuals generating responses to M and N as well as several NSPs including ORF3, ORF8, and NSP7-11. Seronegative individuals also targeted these NSPs, although most of the cellular responses in seronegative individuals targeted S1 and S2. Few seronegative individuals generated T-cell responses to M and N - this is in accordance with previous findings from Ogbe *et al.* (2021).[120] These results are also in accordance with Mateus *et al.* (2020),[150] which reported S as the primary target of T-cell responses in uninfected individuals (54% of the total positive T-cell response). 11% of the response targeted the RBD region of S, whilst 44% targeted non-RBD. From the data described herein, responses to S1 and S2 were comparable in seronegative individuals, although in seronegative individuals, the median response to S2 was higher than to S1, whilst in seropositive individuals, the median response to S1 was higher than to S2.

Over half of the cohort was seropositive, suggesting that transmission between family members was consistently occurring, or family members were being concurrently infected by an outside source. In general, the former explanation is more parsimonious due to the restrictions on social mixing over the course of this study. Most ELISA-seropositive individuals were also seropositive for S by MSD, although nine individuals fell slightly below this threshold, and one individual was far below (30 AU/ml). Generally, ELISA and MSD serostatuses were concordant.

A key consideration is the fact that an average of 7.6 months passed between infection and sampling in seropositive individuals. This opens up a possibility of infections within a household occurring at different time points over this period, which cannot be ruled out in this cohort. Additionally, multiple seropositive individuals within a household may be more indicative of riskier behaviour, leading to multiple infections, rather than transmission between family members. This is particularly important to consider in a family study where parental behaviour likely has a strong impact on the behaviour of children in the household.

Responses to SARS-CoV-2 N were varied in magnitude in S-seropositive individuals, suggesting that that not all S-seropositive individuals make a robust response to other SARS-CoV-2 antigens such as N. Moderate correlations between anti-S and anti-N IgG have been demonstrated elsewhere.[40] However, anti-N IgG has also been suggested to decline more rapidly than anti-S IgG, which may explain the lack of N-specific antibody in some seropositive individuals in this cohort.[36] nAb responses were assessed using a pseudovirus neutralisation assay.[122] The significantly elevated nAb response in seropositive individuals demonstrates that total IgG and nAb responses are well associated following natural infection, as shown previously,[40] even many months into convalescence. nAb titres following infection have been shown to be durable and detectable after eight months, as supported

here, a mean of seven months post-infection.[151] Correlates of protection for SARS-CoV-2 are still uncertain, and it is unclear whether total IgG or nAb response is a better marker for protective immunity. However, the data described herein suggests that at least for natural infection, the two measures are well associated and are both durable into convalescence.

The breadth of responses, both humoral and cellular, to SARS-CoV-2 SPs and NSPs demonstrates just how varied the immune signature following SARS-CoV-2 infection is compared to vaccination with S-targeting viral vectored or mRNA vaccines.[60, 152] S-targeting vaccines appear highly effective at preventing severe disease,[153] but are rarely sterilising.[154] The diverse immune responses generated following infection with SARS-CoV-2 raises the question of whether vaccines targeting multiple antigens might further improve vaccine efficacy. Vaccines targeting N administered alongside S-targeting vaccines have been demonstrated to enhance efficacy and promote immunity in distal sites such as the brain.[155, 156] A multivalent adenoviral-vectored intranasal vaccine expressing the SARS-CoV-2 S, N, and RNA-dependent RNA polymerase proteins induced local and systemic Ig and cellular immunity.[157] Furthermore, several SARS-CoV-2 NSPs that are translated early in the viral replication cycle are targets of T-cell responses in this cohort, including the replication-transcription complex (RTC) composed of NSP7, NSP8, NSP12, and NSP13.[7] Inclusion of early-translated proteins such as the RTC into SARS-CoV-2 vaccines may promote sterilising immunity by enabling rapid viral control early in disease course.

Assessing immunity through the lens of family groups enabled some exploration of correlates of transmission and infection susceptibility. The most common family type was a seropositive mother with a seronegative family. Although chi-squared analysis did not report a difference in the proportion of infected males and females, logistic regression of infection risk by age and sex demonstrated that seropositive older females were overrepresented in this cohort. As families were recruited by

word-of-mouth or through the enrolment of index patients from studies of HCWs, it is likely that the dataset is biased towards mothers and female HCWs through this mode of recruitment. An alternative explanation is that older females are more susceptible to SARS-CoV-2 infection. Other studies of intrafamilial SARS-CoV-2 transmission and infection identified similar rates of male to female and female to male transmission between serodiscordant couples,[158] although a study of SARS-CoV-1 in mice identified greater susceptibility to infection in males.[159] Sex-based differences in TMPRSS2 expression through androgen-mediated regulation have been suggested,[103] which provides a mechanism for this difference, but it is unclear if this would have a distinct biological effect on infection susceptibility. A more parsimonious explanation of the bias towards infected older females in this cohort is by recruiting female HCWs as index patients. Infection risk was also positively associated with an increased proportion of families being infected. This is intuitive, as a higher proportion of individuals shedding infectious virus likely increases risk of transmission. Previously, household exposure intensity as measured by the combined viral shedding intensity of seropositive family members was associated with an increased risk of infection in seronegative individuals, particularly in early phases of infection when individuals are most infectious.[137]

Case studies of individual families provided the opportunity to assess immunity within families in more detail. In families where all individuals were seropositive for SARS-CoV-2, humoral and cellular responses were varied and did not appear to associate with age or sex. Previous studies have identified lower respiratory cycle threshold (Ct) values and higher plasma IgG in 'high transmission' families where all individuals become infected compared to 'low transmission' families, here described as serodiscordant.[138] Nasopharyngeal sampling of this cohort would have enabled this analysis and further provided an opportunity to assess what makes these 'all seropositive' families all become infected, and whether this derives from viral-associated factors such as viral load. In families where all members were seronegative for SARS-CoV-2, cellular immunity was observed, but only in parents.

In contrast, in mixed serostatus families where parents were either seroconcordant or serodiscordant, cellular immunity was present in children of serodiscordant parents that remained seronegative. This raised the possibility of a role for exposure to SARS-CoV-2 in promoting cellular immunity even in the absence of seroconversion, which will be discussed in more detail in Chapter 4.

Comparing immunity between family groups demonstrated a trend towards elevated nAb and CD4+ T-cell responses in seropositive individuals from serodiscordant couples compared to those in seroconcordant couples. It could be speculated that stronger immune responses in an index patient may protect against onward transmission of virus to a partner; however, samples were obtained into convalescence, and therefore assessing immune dynamics early in infection course is difficult. Furthermore, this trend was not significant. Rates of intrahousehold transmission of SARS-CoV-2 appears to reduce following vaccination of one family member,[160, 161] supporting the idea that improved immunity in one individual can reduce onwards transmission to close contacts. Enhanced transmission of SARS-CoV-2 by so-called 'super spreaders' has been associated with higher viral load and a higher number of close contacts,[162] although this has not been associated with a suboptimal immune response in the 'super spreader'. Further research into the relationship between immune response and SARS-CoV-2 transmission is warranted.

One hypothesis generated at the beginning of this study was that females would generate stronger immune responses, as is consistent for infections with other viruses, and that this could partially explain the increased mortality in males infected with SARS-CoV-2.[103, 106] However, males are more at risk for adverse events following vaccination with SARS-CoV-2 mRNA vaccines,[163] which is in contrast to established trends that rates of adverse events following vaccination are higher in females.[101] Analysis of family groups demonstrated that, unlike trends for other viral infections,[98] fathers often generated the strongest T-cell, IgG, and nAb response within their family. Furthermore, although an overall

difference in immune response was not observed between the sexes by Mann-Whitney test, male sex was associated with increased T-cell immunity by multiple linear regression. In contrast, a previous study of sex differences in SARS-CoV-2-specific immunity identified higher T-cell activation in females during acute disease, as well as a correlation between worse disease outcome and poor T-cell response in males.[97] The discordance between the results described herein and published findings may result from sampling in convalescence and the fact that individuals in this cohort only experienced mild disease. Furthermore, the CTV assay measures total proliferation, whereas Takahashi *et al.* (2020) phenotyped expression of HLA-DR and CD38 instead to identify higher T-cell activation in females.[97] Further immunophenotyping of sex differences in cohorts of differing disease severity utilising multiple techniques are required to make firm conclusions on the role of sex in COVID-19.

Another hypothesis generated before this study was undertaken was that immunity would be weaker in older individuals, due to the reduced cellular functionality associated with ageing and 'immunosenescence'. Pre-existing cellular immunity in seronegative individuals was significantly higher in individuals 18 and over, compared to under-18s, and although there was no correlation between age and either humoral or cellular immunity to SARS-CoV-2 in seropositive individuals, age was positively associated with T-cell response by multiple linear regression. A study of children and adults with severe COVID-19 also identified higher cellular and humoral immunity in adults.[164] Another study identified higher levels of anti-N IgG in adults compared to children, as well as greater nAb responses in adults.[165] Humoral immunity in this cohort was not associated with age by multiple linear regression. However, it is possible that crude measures of immunity such as total IgG, total nAbs, and T-cell proliferation do not adequately capture the nuances of age-related changes in immunity such as cytokine profiles, cell effector functionality, and abundance of different cellular subsets.

Studying correlates of disease severity was limited in this dataset due to all seropositive individuals experiencing either asymptomatic or mild disease. However, self-reported information regarding the number and types of symptoms experienced during putative COVID-19 illness was taken at the time of sampling, and therefore broad trends were able to be studied in this cohort. Greater cellular but not humoral immunity was identified in symptomatic individuals and in individuals with higher numbers of symptoms (increased symptom score). An enhanced T-cell response targeting S1, but not S2, in symptomatic individuals suggests that T-cell immunity to S2 may be associated with asymptomatic infection. This finding was reported in a recent study by Augusto *et al.*(2023), where individuals with the HLA allele HLA-B*15:01 were found to generate T-cell immunity to a SARS-CoV-2 epitope located in the S2 region, which was associated with a 4- to 8-fold higher chance of asymptomatic infection.[166] Elevated cellular immunity has been identified in individuals with more severe COVID-19,[30] a trend that appears to hold true even in mild and asymptomatic disease in this cohort. Whether this is a consequence of a suboptimal humoral response for which cellular immunity compensates, or, perhaps more parsimoniously, associated with higher viral load that promotes increased cellular immunity whilst also worsening symptoms, is unclear and warrants further investigation. Higher viral load is associated with increased mortality, although not worse symptoms in mild disease.[167, 168] Nasopharyngeal sampling of this cohort during acute infection would have enabled such analyses to be run, but as these individuals were sampled in convalescence, this was not possible.

As samples were taken a mean of seven months post-SARS-CoV-2 infection, it was possible to broadly assess the durability of cellular and humoral immunity following infection in previously naïve hosts. Immune responses in seropositive individuals were varied but appeared stable at a mean of seven months into convalescence, particularly to S1 and S2. The magnitude of IgG, nAb, and T-cell responses were negatively correlated with the number of days since symptoms in seropositive

individuals, and this was confirmed by multiple linear regression for IgG and T-cell responses. It was observed that the cohort could be divided into individuals sampled 200-400 days since symptoms, and individuals sampled 0-100 days since symptoms. This was a consequence of the timing of national lockdowns and participant availability. When split into two groups, there was no longer a negative correlation between days since symptoms and immune response for each group. However, total IgG responses were significantly higher in the 0-100 group than the 200-400 group, indicating some waning of antibody responses over the course of several months.

Other estimates of durability of SARS-CoV-2-specific immune responses following natural infection show stable nAb responses and T-cell immunity at six to seven months post-infection, and stable anti-RBD IgG after one to two months followed by a decline at six to seven months.[169] Another study demonstrated a rapid reduction in anti-RBD IgG levels in 34 individuals over the course of 120 days post-infection.[170] In contrast, vaccination with mRNA vaccines appears to generate higher IgG responses that are more durable,[171] particularly in individuals previously infected with SARS-CoV-2 before vaccination.[172] Seasonal vaccination and reinfection of individuals now makes long-term durability of responses less critical for maintaining protection against infection and severe disease; nonetheless, understanding the durability of SARS-CoV-2-specific immunity is useful in planning immunisation regimes going forward. Furthermore, it is important to consider the implications of waning IgG and T-cell responses on cross-sectional studies of immunity such as this one. Longitudinal and repeated sampling of individuals enables the incorporation of temporal data into assessments of immune durability. However, in this study where samples were taken at only one timepoint, response waning can only be assessed retrospectively based on self-reported symptom onset. Here, 'days post infection' was incorporated into linear models of response magnitude, assuming a linear decay in response. Rate of decay may differ for humoral and cellular immune responses, and may decay nonlinearly for one or both arms of

the immune system, and this will impact the interpretability of results. For both IgG and T-cell responses in this cohort, days since symptoms was significantly associated with response magnitude. However, T-cell responses appeared more stable by correlation analysis, and this has been reported elsewhere.[173–175] This raises the possibility that long-term T-cell memory may offer enhanced protection against SARS-CoV-2 reinfection compared to humoral immunity. However, the role of cellular immunity in protection is less well understood. T-cells specific for influenza are able to restrict viral replication in the upper respiratory tract.[176] It could be speculated that SARS-CoV-2-specific T-cells also share this ability to prevent COVID-19 in isolation, as has been suggested previously.[7, 177]

Assessing immunity within and between family groups had several benefits. Households represent a well-controlled environment where exposure to SARS-CoV-2 via an infected family member is highly likely, and therefore examinations of transmission risk, susceptibility to infection, and the role of exposure intensity are all possible. This is also true for studies of HCWs where occupational exposure to SARS-CoV-2 is again highly likely. However, family studies also have the extra benefit of the genetic relatedness between family members. Shared HLA types, although not assessed in this study, enable analyses of the genetic components of immunity and disease susceptibility. Furthermore, genetic relatedness could act to standardise immune responses within families, reducing the confounding effects of genetic background that are present in cohort studies of unrelated individuals (although this could also be seen as a limitation, if findings are not generalisable to other genetic demographics or HLA types). The composition of a family is often somewhat equally divided between males and females, such as between parents or sex-discordant siblings. This provides opportunities to assess sex-related differences in immunity within families or in the cohort as a whole. In a similar vein, the age discordancy between grandparents, parents, and children enables assessment of immunity across the life course, in contrast to the solely working age individuals recruited onto studies of HCWs. Finally, all individuals within a family can largely be expected to become

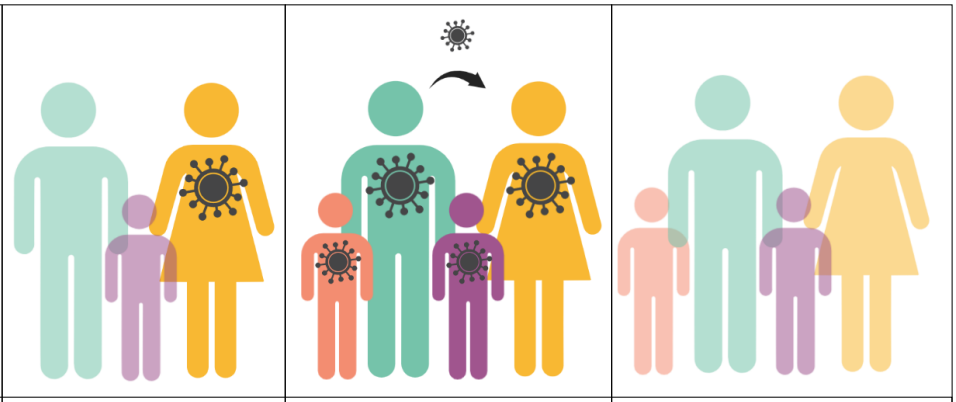
infected at the same point in time, and so comparisons of immune response within families is protected from the unwanted effects of waning of responses. Overall, family studies provide a microcosmic environment particularly suited to case studies of immune response in individuals of different life stages.

However, family and household studies face associated limitations. The mode of recruitment for this study was primarily word-of-mouth, through individuals who were aware of the study or who had been recruited for similar studies such as the CROWN study of HCWs. This led to biases in the cohort that likely impacted the dataset. The cohort was predominantly white, with a bias towards female HCW index patients. The unstructured nature of recruitment meant that individuals were recruited on an ad-hoc basis and did not require a positive PCR or rapid test for recruitment. Family studies are partially paediatric in nature and acquiring sufficient blood samples from healthy children has its own ethical limitations. In addition, the degree of close contact between family members likely varies significantly between families – the relationship between a parent and a young child likely involves more contact than between two teenage siblings, for example. Assessing the degree of 'closeness' between family members is largely impossible yet could impact the findings of family studies. Furthermore, extrapolating from small case studies of individual families is difficult, and was challenging for this cohort where several different 'family types' were identified. Maintaining the dimensionality of the original data whilst making meaningful comparisons across large groups was a particularly difficult task in this study.

The lack of baseline samples in SARS-CoV-2 seropositive individuals made accurate estimates of correlates of protection against infection difficult. Assaying pre-existing immunity in individuals who later did or did not become infected would have strengthened the study and enabled further dissection of correlates of protection and susceptibility. One possible solution to this limitation would have been follow-up of seronegative individuals in case of later infection. However, many individuals in

the UK population were most likely vaccinated before they became infected, which would have prevented accurate estimates of infection-induced immunity. Finally, the spread of time post-infection in this cohort (mean 228 days, range 51-352 days) reduces confidence in the dataset, as it is difficult to discern where responses have waned. Incorporating days since symptoms into the multiple linear regressions carried out in this study was one solution to this issue. Sampling earlier on in convalescence or at more consistent timepoints between individuals would have been preferred but was difficult in the context of lockdowns, social distancing and a lack of available PCR and rapid testing to confirm when infection took place.

Overall, the humoral and cellular immune dynamics of SARS-CoV-2 infection within and between families appears diverse and varied, with some common patterns emerging including: robust and stable cellular and humoral immunity into convalescence, cross-reactive T-cell immunity in seronegative individuals (particularly adults), some evidence for greater T-cell immunity in older individuals and males, and an association between cellular immunity and symptom severity. A summary of findings is provided in Figure 3.16. These results enhance our understanding of mild SARS-CoV-2 infection in immunologically naïve hosts, providing new insights into how robust and long-lasting immunity to a novel pathogen develops in the family context.



	Mixed serostatus families	Seropositive families	Seronegative families
Transmission	Older women most frequently seropositive	Abundant transmission between family members	-
Humoral immunity	Robust IgG and nAbs in seropositive members	Robust IgG and nAbs, some waning with time	No cross-reactive IgG or nAbs
Cellular immunity	Cellular immunity to S1 and S2 in seronegative adults and children	Greater cellular immunity in older males	Cellular immunity to S1 and S2 in adults
Other trends	Children of seroconcordant parents often seropositive	Cellular immunity higher in symptomatic individuals	-

Figure 3.16: Dynamics of SARS-CoV-2-specific immunity in families.

4

T-cell responses in exposed seronegative individuals

Contents

4.1 Abstract

Pathogen-specific T-cell immunity has been described in individuals highly exposed to, but seronegative for, diverse viruses including HIV and HCV. Recently, this phenomenon has been extended to include SARS-CoV-2. Individuals exposed to SARS-CoV-2 through their occupation as HCWs or through close contact with an infected family member have been reported to generate specific T-cells in the absence of detectable antibody. In this chapter, we demonstrate SARS-CoV-2-specific T-cell immunity in seronegative family members highly exposed to SARS-CoV-2 through intrafamilial exposure. We show that the magnitude of this response is associated with the number of seropositive individuals in a family, with a greater number of seropositive family members leading to stronger T-cell immunity in seronegative individuals. The source of these seronegative T-cell responses to SARS-CoV-2 has been suggested as cross-reactive immunity to endemic HCoVs that is expanded upon SARS-CoV-2 exposure. However, in this chapter, no association between HCoV-specific immunity and seronegative T-cell immunity to SARS-CoV-2 is identified, suggesting that *de novo* T-cell immunity may be generated in seronegative SARS-CoV-2 exposed individuals.

4.2 Introduction

Aim: To explore the role of cross-reactive T-cells in individuals highly exposed to, but seronegative for, SARS-CoV-2.

Canonical immunity to SARS-CoV-2 is well understood despite the pathogen's recent emergence. Delayed innate immune activation resulting from viral evasion of IFN responses enables infection to occur, and this delay in initial defences likely promotes asymptomatic transmission of SARS-CoV-2.[28] Impaired IFN responses and enhanced pro-inflammatory cytokine responses in the absence of IFN have been associated with worse disease outcomes,[178] and inborn errors in IFN-mediated immunity are associated with severe COVID-19.[26] Additionally, both humoral and cellular mechanisms are implicated in viral control,[1] and stronger T-cell immunity is associated with milder disease.[28] Weak or delayed adaptive responses can lead to severe or fatal COVID-19, with immunopathology and cytokine hyperactivation characteristic of end-stage disease.[28] CD4+ and CD8+ T-cell responses are generated after most cases of COVID-19, and CD4+ T-cells can be detected early in infection, up to 2 days post symptom onset.[29] CD4+ responses are higher in magnitude than CD8+ responses,[1, 29] and are associated with improved viral control in mice.[28] Superior T-cell functionality is also associated with asymptomatic compared to symptomatic COVID-19.[179] In contrast, neutralising antibody responses do not correlate with disease severity during acute illness,[29] highlighting the importance of T-cell immunity.

Further testament to the role of T-cell immunity in COVID-19 is the ability of immunocompromised individuals with B-cell deficiencies to clear SARS-CoV-2 infection. Buckland *et al.* (2020) described a patient with X-linked agammaglobulinaemia (XLA) who generated a robust T-cell response and, despite persistent lung inflammation, was able to clear the virus with the aid of a course of remdesivir.[180] Two further XLA patients who were admitted to hospital with pneumonia and B-cell lymphopaenia, but normal T-cell numbers, were also able to clear infection

when treated with intravenous Ig replacement therapy (the standard treatment for XLA).[181] Whilst the increased severity of COVID-19 in XLA patients indicates that antibodies and B-cells are important in preventing severe disease, they are not essential in viral clearance; T-cells may contribute to preventing mortality in these instances. In addition, a study of COVID-19 patients with haematological cancers identified worse outcomes compared to patients with solid cancers, although improved CD8+ T-cell responses were associated with survival in haematological patients with B-cell deficiencies.[182]

As T-cells appear to contribute to resolving disease in the absence of B-cells, this raises the question of whether T-cells can prevent infection. Exposure to viral pathogens does not guarantee infection; the clearest examples of this phenomenon are in the failure of test subjects in human challenge studies to consistently become infected.[183–185] Variation in host susceptibility has been linked to host genetics, inoculum viral load, and prior exposure to related pathogens.[184–188] Among those individuals who are exposed but fail to become infected, a small but well-documented population generate pathogen-specific T-cell responses in the absence of viraemia or antibodies, so-called 'exposed seronegatives' (ESNs).[7, 189] The earliest reports of this phenomenon occurred in the late 1980s concerning apparent HIV resistance in at-risk individuals.[128, 190–192] In the 30 years since initial reports in HIV, the phenomenon has been appreciated to occur following exposure to HCV, HBV, Herpes Simplex virus 2 (HSV-2),[189, 193–196] and recently SARS-CoV-2.[7, 197]

HCWs represent a population where ongoing SARS-CoV-2 exposure in clinical settings enables the study of ESNs. A study of SARS-CoV-2-exposed HCWs from Swadling *et al.* (2022) found elevated T-cell immunity in seronegative SARS-CoV-2-exposed HCWs compared to unexposed controls.[7] Similarly, da Silva Antunes *et al.* (2021) recruited 26 PCR+ HCWs, 32 seronegative HCWs at high risk of exposure, and 33 community controls, and demonstrated that ESNs displayed

higher T-cell responses to SARS-CoV-2 than unexposed individuals.[198] Finally, Ogbe *et al.* (2020) identified SARS-CoV-2-specific T-cell responses in exposed HCWs: three of 10 generated IFNγ responses by ELISpot to S, M, and N, whilst all generated responses to M and N by proliferation assay.[120] T-cell responses in ESNs were of greater magnitude than unexposed controls for CD4+ but not CD8+ cells, although both CD4+ and CD8+ cells targeted a greater number of antigens in ESNs compared to unexposed controls.[120]

As well as HCWs, studies of family members and close contacts of SARS-CoV-2-infected individuals have provided an opportunity to assess the role of viral exposure on immunity in seronegative individuals. Wang *et al.* (2021) assessed cellular immunity in 90 seropositive individuals and 69 seronegative contacts who had been within 1.5m of a SARS-CoV-2-infected patient for over one hour or in the same household for over 24 hours.[199] The authors identified higher CD4+ and CD8+ T-cell activation in seronegative family members of infected individuals compared to unexposed controls, as measured by IFNγ production following stimulation with S, M, N, and E peptides.[199]

A potential source of memory T-cells in SARS-CoV-2 ESN individuals is from cross-reactive immunity specific for endemic HCoVs. SARS-CoV-2 is closely related to the endemic HCoVs HCoV-OC43, HCoV-HKU1, HCoV-NL63, and HCoV-229E, to which most people have been exposed through 'common cold' infections. These viruses are seasonal, causing mostly mild disease, and in 2019 made up 10—20% of viral respiratory infections in Scotland.[3] SARS-CoV-2 and endemic HCoVs have sequence homology, reflecting their shared ancestry. This is evidenced by their high sequence identity (number of matching base pairs between sequences) and sequence similarity (resemblance between sequences), particularly for non-structural regions of the genome (Figure 4.1).[7, 200] The high DNA sequence identity between SARS-CoV-2 and endemic HCoVs, particularly the betacoronaviruses HCoV-OC43 (69%) and HCoV-HKU1 (68%),[3] make cross-reactivity unsurprising, particularly

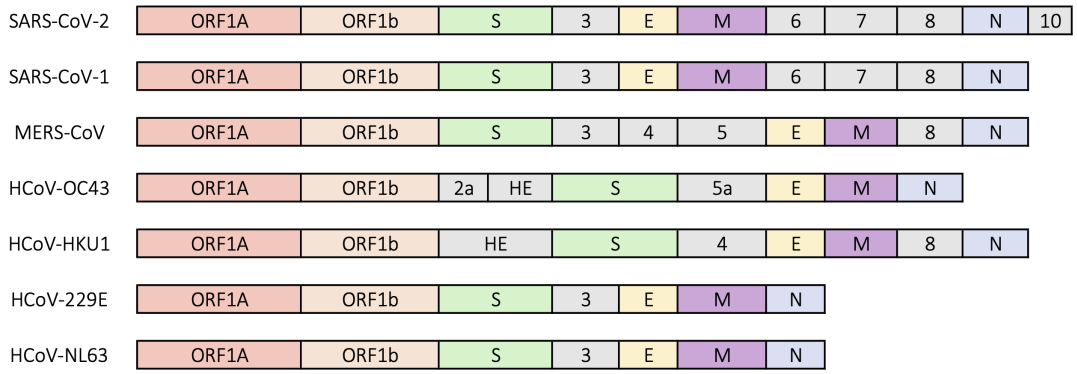


Figure 4.1: Genome sequence comparison of coronaviruses. Adapted from Kim *et al.* (2023).[202]

for T-cell immunity which tends to be less susceptible to the effects of small amino acid changes,[201] such as those that define SARS-CoV-2 VOCs.[51] T-cells capable of recognising SARS-CoV-2 in seronegative individuals were described early the pandemic.[1, 2, 158] One study estimated that 40-60% of unexposed individuals generated SARS-CoV-2-specific T-cell responses, particularly to non-S epitopes.[1] Epitope mapping through the use of short-term T-cell lines specific for SARS-CoV-2 epitopes demonstrated high reactivity with HCoV epitopes, and epitopes with the highest homology between SARS-CoV-2 and HCoVs were more likely to be cross-reactive.[2] HCWs display higher levels of HCoV-specific T-cells than community controls, which may contribute to the abundance of T-cell immune responses amongst seronegative HCWs.[198]

These findings raise the question of whether pre-existing cellular immunity provides protection against infection with SARS-CoV-2 – a question that has yet to be conclusively answered. Protection against infection mediated by pre-existing cross-reactive immunity has been described between strains of influenza,[203, 204] and between flaviviruses.[108, 205] Patients hospitalised with COVID-19 display lower frequencies of cross-reactive CD4+ T-cells than those with mild disease,[206] and CD8+ responses in individuals with severe disease target less conserved epitopes than those with mild disease.[207] Furthermore, in a study of 52 close contacts of

SARS-CoV-2-infected individuals, cross-reactive T-cell responses were identified at higher levels in individuals who resisted infection versus individuals who later became infected,[5] supporting a model whereby cross-reactive T-cells are protective against infection.

Analysis of the antigens targeted by this elevated cross-reactive immunity in ESNs is informative in understanding their source and their role in protection. The regions of SARS-CoV-2 with highest homology to HCoVs include N and the S2 domain of S.[3, 208] Cross-reactive T-cell responses in SARS-CoV-2-seronegative individuals preferentially target S2 rather than the RBD.[2] In addition, T-cells targeting the RTC of SARS-CoV-2 have been described in ESNs.[7] The RTC is comprised of the RNA polymerase NSP12, co-factors NSP7 and NSP8, and the helicase NSP13.[209] Its expression early in the SARS-CoV-2 replication cycle makes the RTC a likely target for rapidly induced T-cell responses. Furthermore, the RTC is highly conserved between SARS-CoV-2 and HCoVs.[210] In Swadling *et al.* (2022), fivefold-higher RTC-specific T-cell responses were identified in ESNs compared to unexposed controls; in addition, cellular immunity in ESNs preferentially targeted the RTC over SPs compared to seropositive individuals.[7] This data supports a model whereby rapid memory T-cell responses targeting early translated, highly conserved NSPs may prevent SARS-CoV-2 infection from gaining a foothold.

In Chapter 3, cellular immunity was identified in seronegative individuals – particularly in adults and individuals who had a seropositive family member. However, it was unclear if these cellular responses originated from cross-reactive immunity, because of intrafamilial exposure to SARS-CoV-2, or both. Here, using family serostatus as a marker for whether exposure to SARS-CoV-2 has occurred, we test the hypothesis that SARS-CoV-2 exposure generates elevated T-cell immunity that is higher than expected from mere cross-reactivity. Furthermore, we assess whether these responses appear to originate from cross-reactive immune responses to endemic HCoVs. Unlike previous studies, T-cell responses are assessed across

the entirety of the SARS-CoV-2 genome. Furthermore, we were able to quantify the intensity of SARS-CoV-2 exposure by considering the number of seropositive individuals within a family. Together, this data aims to replicate previous findings on ESN immunity in a new cohort, whilst also providing new insights into the source and quality of this immunity.

4.3 Results

4.3.1 Elevated T-cell immunity in ESN individuals

In Chapter 3, T-cell responses specific for SARS-CoV-2 were identified in seronegative individuals. However, in individuals who did not have a seropositive family member, these T-cell responses were only observed in adults. In contrast, seronegative individuals who had been exposed to SARS-CoV-2 via an infected family member generated T-cell responses to SARS-CoV-2, both in parents and children. This observation raised the possibility that exposure to SARS-CoV-2 can promote the development of cellular immune responses in individuals who otherwise do not display this immunity.

To test the hypothesis that exposure to SARS-CoV-2 through an infected family member generates cellular immunity in a seronegative context, we first compared T-cell responses between individuals seropositive for SARS-CoV-2, individuals seronegative for SARS-CoV-2 who had at least one seropositive family member (ESN), and individuals seronegative for SARS-CoV-2 who had no seropositive family members (unexposed seronegative, USN) (Figure 4.2).

As expected, seropositive individuals generated significantly greater CD4+ responses than ESNs (all $p<0.0001$, Mann-Whitney tests), and significantly greater CD8+ responses (S1, M, and N: $p<0.0001$; S2: $p=0.002$; Mann-Whitney tests). Of note, ESNs generated significantly greater CD4+ T-cell responses to S1 ($p=0.05$, Mann-Whitney test) compared to USNs, and this trend held for S2, M, and N, although they were not significant (S2: $p=0.6$, M: $p=0.5$, N: $p=0.16$, Mann-Whitney tests). There was also a trend of greater CD8+ responses to S1 in ESNs compared to USNs, although again this was not significant ($p=0.49$, Mann-Whitney test). To assess whether cellular immunity was also more widespread as well as higher in magnitude in ESNs compared to USNs, the percentage of individuals with cellular responses above the 1% cutoff for each group was assessed. There was a higher proportion

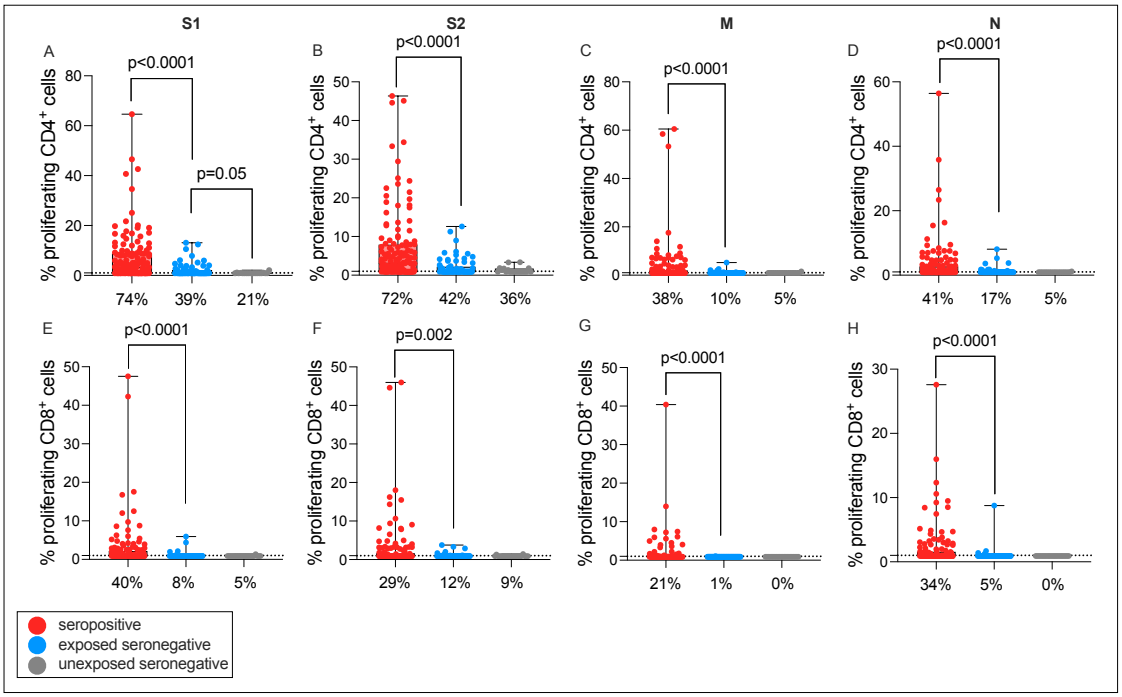


Figure 4.2: Elevated T-cell immunity in ESN individuals. CD4+ T-cell responses targeting S1 (A), S2 (B), M (C), and N (D) in seropositive (red), ESN (blue), and USN (grey) individuals. CD8+ T-cell responses targeting S1 (E), S2 (F), M (G), and N (H) in seropositive, ESN, and USN individuals. p-values refer to Mann-Whitney test values. Values below 1% proliferation were given nominal values of 0.9. Percentages refer to the percentage of responses above 1% proliferation.

of positive responses to all antigens in ESNs compared to USNs, however, this difference was not significant by Fisher's exact test (CD4: S1 p=0.14, S2 p=0.8; M p>0.99, N p=0.29, CD8: all p>0.99).

It has been demonstrated that a target of T-cell responses in ESNs is the RTC, an early-translated protein that is highly conserved between SARS-CoV-2 and endemic HCoVs. To test the hypothesis that ESN individuals generate enhanced responses to the RTC (NSP7, NSP8, NSP12, NSP13), both CD4+ and CD8+ T-cell responses to peptide pools consisting of overlapping peptides spanning the rest of the SARS-CoV-2 genome (ORF3, ORF8, NSP1+2, NSP3A, NSP3B, NSP3C, NSP4, NSP5+6, NSP7-11, NSP12A, NSP12B, NSP13, NSP14, and NSP15+16) were compared between seropositive, ESN, and USN individuals (Figure 4.3). In addition, responses to NSP7-11, NSP12A, NSP12B and NSP13 were summed together to

assess responses to the RTC. One ESN individual, a 16-year-old male with two seropositive parents, made very high (>10%) CD4+ responses to ORF8, NSP1+2, NSP3A, NSP3B, NSP12B, NSP14 and NSP15+16. Similarly to structural antigens, responses were generally greater in seropositive individuals than ESNs, reaching significance for CD8+ responses to NSP3B (p=0.019, Kruskal-Wallis test with Dunn's multiple comparisons). There was no significant difference in responses to individual RTC pools between ESN and USN individuals, and no obvious trend of greater responses in ESNs. However, when summed, responses to the RTC trended higher in ESNs compared to USNs. This supports a model whereby early T-cell responses to the RTC are induced following exposure to SARS-CoV-2. However, this difference was not significant by Mann-Whitney test (p=0.5). As well as response magnitude, the percentage of positive responses (>1%) was calculated for seropositive individuals, ESNs, and USNs, for individual RTC peptides as well as the summed RTC plot. Fisher's exact test was used to assess if there were significantly more positive T-cell responses in ESNs compared to USNs for the summed RTC plot. This was not significant (p=0.8).

4.3.2 Stronger T-cell responses are associated with greater SARS-CoV-2 exposure intensity

It was hypothesised that a greater number of seropositive individuals in a household would be associated with stronger T-cell immunity in ESNs, due to higher levels of exposure to SARS-CoV-2. To test this hypothesis, CD4+ and CD8+ T-cell responses were compared between seropositive and seronegative individuals with different numbers of infected family members (Figure 4.4). T-cell responses in seropositive individuals with different numbers of family members were significantly different (p=0.01, Kruskal-Wallis test) with a trend of increased T-cell immunity in seropositive individuals with fewer infected family members, although no pairwise comparison was significant by Dunn's multiple comparison test (Figure 4.4AB).

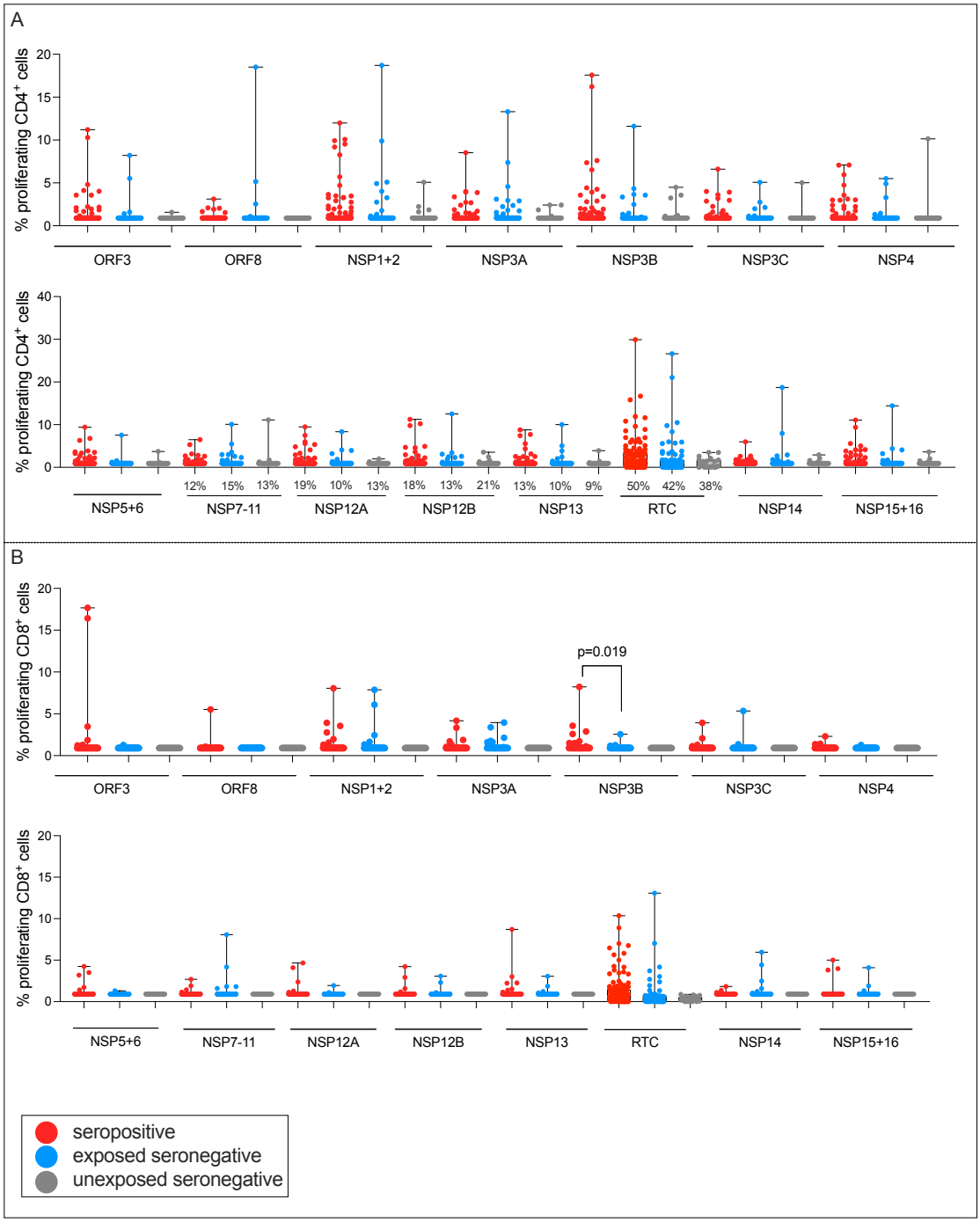


Figure 4.3: T-cell responses to NSPs. CD4+ T-cell responses targeting SARS-CoV-2 NSPs in seropositive (red), ESN (blue), and USN (grey) individuals (A). CD8+ T-cell responses targeting SARS-CoV-2 NSPs in seropositive, ESN, and USN individuals (B). p-values refer to Kruskal-Wallis test values with Dunn's multiple comparisons. Values below 1% were given nominal values of 0.9. Percentages refer to the percentage of responses above 1% proliferation.

CD4+ responses in seronegative individuals with two infected family members were significantly higher than in seronegative individuals with no infected family members (USNs) (p=0.0006, Kruskal-Wallis test with Dunn's multiple comparisons) (Figure 4.4C). There was no significant difference in T-cell response between individuals with no and one infected family member (p=0.5, Mann-Whitney test). Interestingly, seronegative individuals with two infected family members made significantly stronger T-cell responses than seronegative individuals with one infected family member (p=0.0001, Kruskal-Wallis test with Dunn's multiple comparisons), indicating that greater exposure intensity is associated with a stronger cellular immune response, and providing a direct link between viral exposure and cellular immunity in seronegative individuals (Figure 4.4C). To test whether additional infected family members affected the prevalence of positive T-cell responses as well as magnitude, the percentage of positive responses above 1% proliferation were compared between seronegative family members in families with 0, 1, 2, and >2 seropositive family members. The distribution of positive responses was significantly different between groups (Fisher's exact test, p=0.0001).

4.3.3 T-cell immunity and age

In Chapter 3, it appeared that cross-reactive immunity was found at higher levels in seronegative adults than seronegative children, and T-cell immunity was positively correlated with age in seronegative individuals. To test the hypothesis that SARS-CoV-2 exposure generates T-cell immunity in children as well as adults, Spearman rank correlations were calculated between age in years and S1- or S2-specific CD4+ T-cell response for ESNs, USNs and seropositive individuals (Figure 4.5). S1-specific T-cells were correlated weakly with age in both USNs and ESNs, although the correlation was weaker in ESNs (USN: r=0.42, p=0.05; ESN: r=0.28, p=0.02, Spearman rank test). In contrast to USNs, several ESNs under the age of 20 generated S1- and S2-specific immunity.

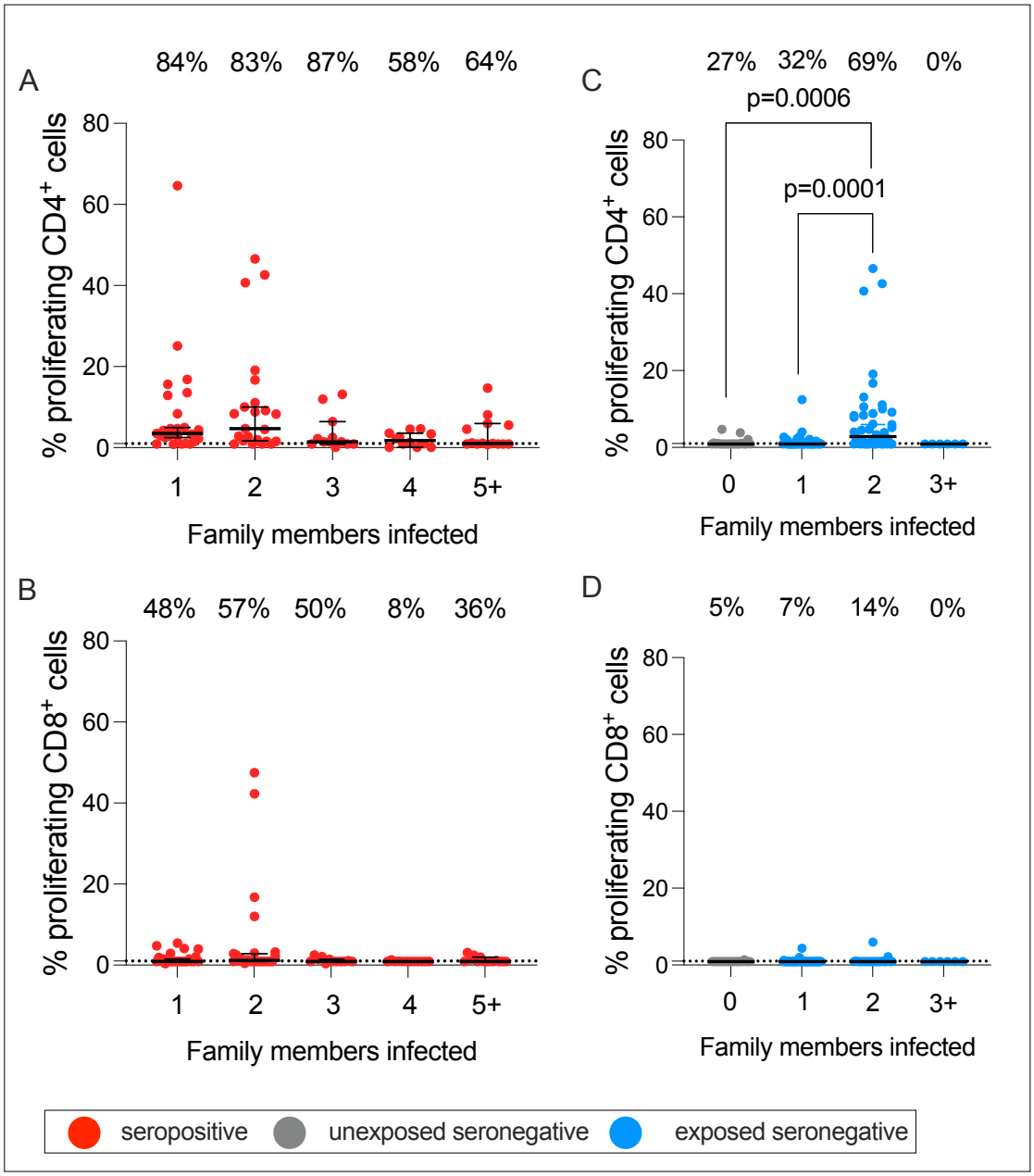


Figure 4.4: S1-specific T-cell responses by number of family members infected. CD4+ (A) and CD8+ (B) T-cell responses in seropositive (red) individuals with 1-5+ family members infected. CD4+ (C) and CD8+ (D) T-cell responses in USN (grey) and ESN (blue) individuals with 0-5+ family members infected. p-values refer to Kruskal-Wallis test values with Dunn's multiple comparisons. Values below 1% were given nominal values of 0.9. Percentages refer to the percentage of responses above 1%.

4.3.4 Humoral immune responses in ESNs

To assess whether there was any small increase in humoral immune responses in seronegative individuals exposed to SARS-CoV-2, total IgG targeting SARS-CoV-2 S RBD, S2, and N, and nAb titres, were compared between seropositive, ESN, and USN individuals (Figure 4.6). IgG targeting S, RBD, and N were quantified using the MSD v-plex assay as described in Chapter 3. IgG targeting S2 was quantified using an anti-S2 IgG ELISA. By definition, responses to S, and unsurprisingly to RBD, S2, and N were significantly higher in seropositive individuals compared to ESN individuals (S: $p<0.0001$; RBD: $p<0.0001$; S2: $p=0.003$; N: $p<0.0001$; Mann-Whitney tests). nAb responses, quantified by pseudovirus neutralisation assay, were also significantly higher in seropositive individuals compared to ESNs ($p<0.0001$, Mann-Whitney test). Whilst responses to S2 were higher in ESNs compared to USNs ($p=0.003$, Mann-Whitney test) and IgG responses to total S were not significantly different between ESNs and USNs, unexpectedly, responses to RBD were higher in USNs than ESNs (173 vs 116, $p=0.03$, Mann-Whitney test) Taking into account multiple comparisons as well as the small magnitude of these responses, the difference in RBD IgG may reflect an artefact.

4.3.5 T-cell responses in ESNs are not associated with enhanced HCoV-specific immunity

To further evaluate the nature of the T-cell response in ESNs, and to assess whether T-cell responses in ESNs target more structural antigens than USNs, the ratio between T-cells targeting SARS-CoV-2 SPs to T-cells targeting NSPs was calculated for seropositive individuals, ESNs, and USNs, for both CD4+ and CD8+ T-cells (Figure 4.7A). Seropositive individuals had significantly higher SP:NSP ratios than

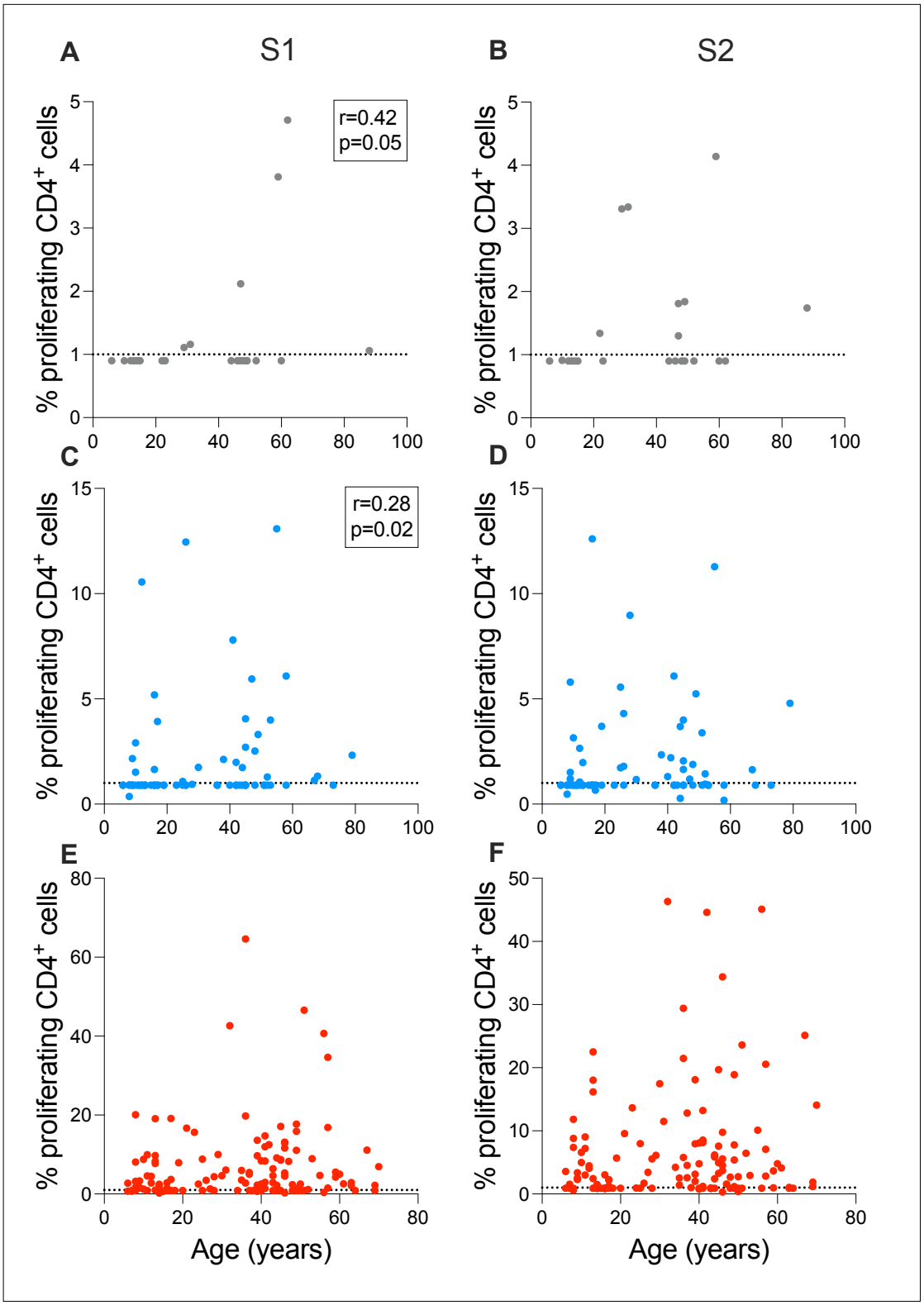


Figure 4.5: S1- and S2-specific immunity by age. The correlation between CD4+ T-cells targeting S1 (A) and S2 (B) and age in USNs (grey), ESNs (blue), and seropositive individuals (red). p- and r- values refer to Spearman rank correlation values. Prolifeation values below 1% were given nominal values of 0.9.

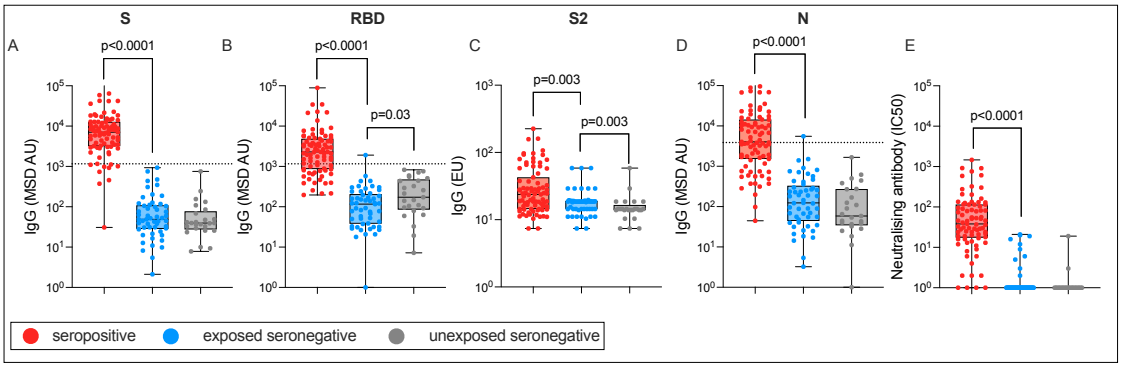


Figure 4.6: Humoral immune responses in ESNs. IgG targeting S (A), RBD (B), S2 (C) and N (D), and nAb titres (E) in seropositive (red), ESN (blue), and USN (grey) individuals. p-values refer to Mann-Whitney test values.

ESNs for both CD4+ and CD8+ T-cells (both p<0.0001) (Mann-Whitney tests). There was no significant different in SP:NSP ratio between ESNs and USNs.

A potential source of elevated T-cell responses in ESNs is cross-reactive HCoV-specific memory T-cells that are expanded upon SARS-CoV-2 exposure.[7] To assess the levels of circulating HCoV-specific T-cells in these individuals, CTV proliferation assays were carried out on a subset of individuals (n=35) using pools of peptides spanning the S2 region of S from HCoV-OC43 and HCoV-HKU1 (Figure 4.7BC). Responses were very low magnitude in all groups, and there was no significant difference in T-cell responses to HCoV-OC43 or HCoV-HKU1 between seropositive individuals, ESNs, or USNs, although there was a trend of greater CD4+ responses in seropositive individuals.

To test the hypothesis that elevated responses to structural antigens in ESNs was due to expansion of pre-existing, cross-reactive T-cells targeting conserved regions of SARS-CoV-2 S, T-cell responses to a pool of 63 peptides from the S region of the proteome highly conserved between SARS-CoV-2 and endemic HCoVs were assayed using the CTV proliferation assay, as defined by Mateus *et al.*[2] Furthermore, to distinguish between the expansion of total S responses versus expansion of conserved responses specifically, the ratio between responses to conserved peptides and total S1 response was calculated and compared between

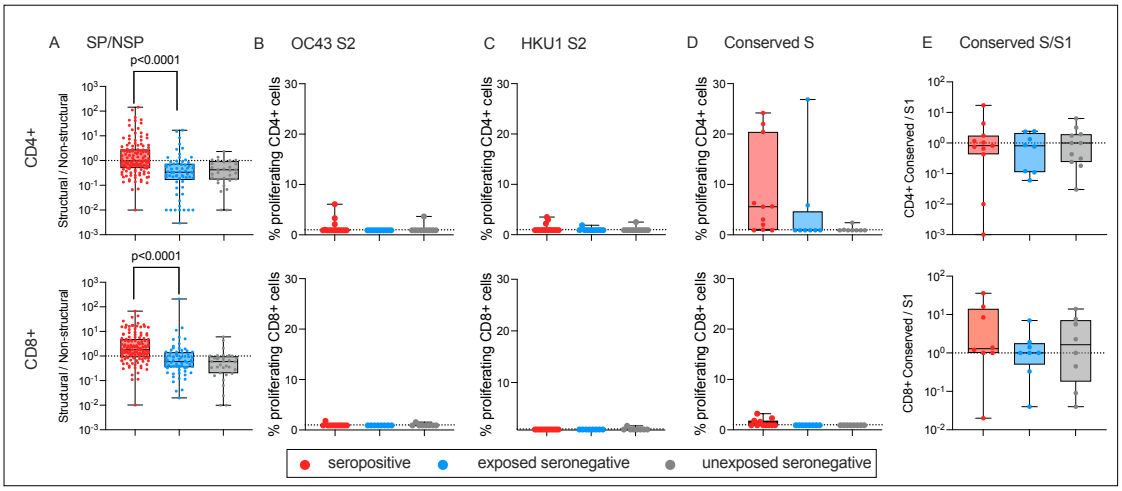


Figure 4.7: T-cell responses in ESNs are not associated with enhanced HCoV-specific immunity. Ratio of CD4+ and CD8+ SP to NSP responses (A), T-cells targeting HCoV-OC43 S2 (B), T-cells targeting HCoV-HKU1 S2 (C), T-cells targeting a pool of 63 conserved peptides (D), and ratio of response to the conserved pool to total S1 response (E) in seropositive (red), ESN (blue), and USN (grey) individuals. p-values refer to Mann-Whitney test values.

groups. There was no significant difference in the magnitude of responses to the conserved pool between seropositive individuals, ESNs and USNs (Figure 4.7D), although there was a trend towards stronger responses in seropositive individuals. Furthermore, there was no significant difference in conserved S:S1 ratio between seropositive individuals, ESNs and USNs (Figure 4.7E). This does not support a model of expanded cross-reactive S-specific immunity in ESNs, instead suggesting that *de novo* responses may also play a part.

4.3.6 PCA reveals cellular immune signatures of exposure and infection

Finally, to assess the quality of the T-cell response in seropositive, ESN, and USN individuals, PCA was employed. The following variables were included: CD4+ and CD8+ responses to S1, S2, M, N, ORF3, ORF8, NSP1+2, NSP3A, NSP3B, NSP3C, NSP4, NSP5+6, NSP7-11, NSP12A, NSP12B, NSP13, NSP14, and NSP15+16. The first two PCs with the highest eigenvalues were selected for further analysis

(PC1=8.3, PC2=4.5). PC1 explained 23.1% of the variance in the data, and PC2 explained 12.5% (Figure 4.8A). All variables were positively correlated with PC1; those that were the most positively correlated were generally structural responses to S1, S2, M, and N, as well as NSP1+2 (Figure 4.8BC). T-cell responses to NSPs, particularly NSP7-16, appeared positively correlated with PC2, whilst T-cell responses to SPs as well as ORF3 were negatively correlated with PC2. (Figure 4.8BC).

Data points were coloured according to their status as seropositive, ESN, or USN. Broadly, the three groups did not cluster discretely. However, some trends were visible. There was a large spread in the seropositive group along both the PC1 axis and the PC2 axis. USNs tended to cluster more in PC1<0 whilst ESNs and particularly seropositives were also scattered into PC1>0. As PC1 correlated positively with responses to SPs, this likely reflects the more structurally focussed T-cell response in ESNs and seropositive individuals compared to USNs.

Along PC2, ESNs tended to cluster mostly at PC2≥0. Interestingly, PC2 is positively correlated with NSPs in the RTC such as NSP7-11, NSP12A, NSP12B and NSP13 as well as other NSPs such as NSP3A and NSP14. The majority of seropositives were found at PC2<0 – as PC2 was negatively correlated with SPs S1, S2, M, and N, this further supports a more structurally-focussed response in seropositives. In summary, PCA identified a high degree of overlap between the three groups, with some trends: more structurally-focussed responses in seropositive individuals, a greater spread of data along PC1 in seropositive individuals likely reflecting high magnitude structural responses in some individuals, more NSP-focussed responses in ESNs, particularly those more 3' in the genome from NSP7 onwards, and tightly clustered USNs generally negatively correlated with PC1 and therefore associated with more non-structural responses.

As ESNs were not clustered discretely away from seropositive individuals and

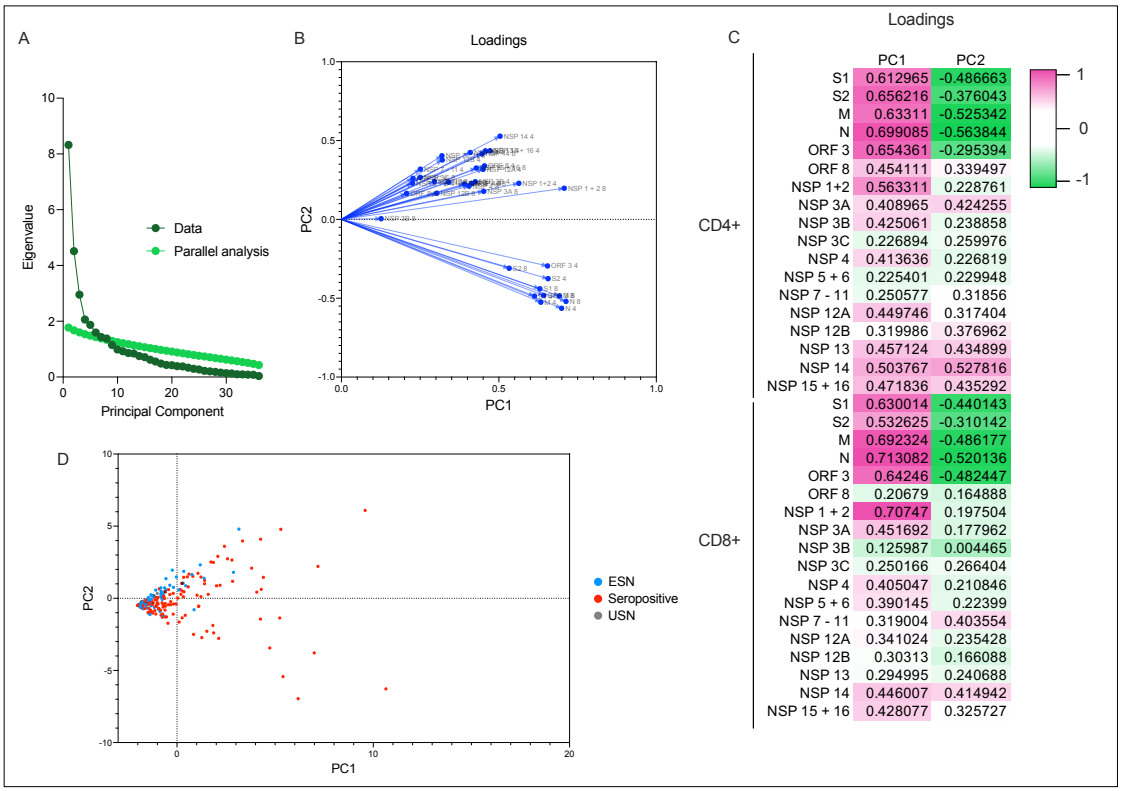


Figure 4.8: PC analysis of T-cell responses in seropositive, ESN and USN individuals. Eigenvalues for PCs as calculated by data (dark green) and by parallel analysis (light green) (A). Loadings for PC1 and PC2 (B). Loadings for PC1 and PC2 by antigen. Coloured by magnitude where green=low and pink=high within each PC (C). PC scores coloured by group in ESN (blue), seropositive (red) and USN (grey) individuals.

USNs, this suggests that ESNs may reflect points upon a spectrum of infection, from low-level exposure to overt infection. In this model, T-cells are induced at lower levels of viral exposure than required for antibody production, and individuals tend towards more structurally-focussed T-cell immunity as their antibody levels increase. To test this hypothesis, the correlation between SP:NSP ratio and total anti-S IgG was calculated for seropositive individuals. Notably, there was a weak positive correlation between IgG and SP:NSP ratio in seropositive individuals (r=0.32, p=0.0005). This supports the theory that infection is a spectrum, from T-cell responses to NSPs with a low contribution from IgG to a more structurally-focussed T-cell response associated with higher antibody involvement.

4.4 Discussion

The family study described in Chapter 3 provided an opportunity to assess SARS-CoV-2-specific cellular immunity in seronegative individuals where confirmed exposure has occurred. As such, the aim of this chapter was to identify the role of cross-reactive T-cells in ESNs. Here, it was demonstrated that elevated T-cell immunity is in fact present in ESNs, in accordance with previous studies,[7, 120, 199] although whether this immunity is wholly cross-reactive in nature remains unclear.

In this cohort, elevated CD4+ T-cell responses in ESNs targeted S1 and S2 regions of S, with a trend towards greater responses in M and N. This is in accordance with previous findings that demonstrate enhanced CD4+ S-, M-, and N-specific immunity in ESN HCWs by proliferation assay.[120] The response in ESNs is overwhelmingly CD4+, which may reflect helper T-cell activation early after exposure and before widespread cellular infection and cytotoxic T-cell activation. A trend towards elevated T-cell immunity to non-S regions of the SARS-CoV-2 proteome has also been identified.[198] In the data described herein, there is a trend towards stronger T-cell immunity in ESNs for some NSPs such as ORF3, ORF8, NSP3A, NSP3C, and others, compared to USNs.

T-cells targeting the RTC were shown to be elevated in ESN HCWs.[7] The role of early-translated proteins in ESNs is not unique to SARS-CoV-2: a study of six ESN sexual partners of HSV-2-infected individuals identified T cell responses skewed towards peptides expressed early in the virus replication cycle. In contrast, HSV-2 seropositive individuals more frequently generated responses to SPs present in virions.[189] In this study, T-cell responses targeting individual RTC peptide pools were not significantly elevated in ESNs compared to USNs, in contrast to previous findings.[7] However, there was a trend towards stronger T-cell responses to the sum of RTC pools in ESNs compared to USNs, supporting a model whereby RTC-specific T-cell immunity is generated following exposure to SARS-CoV-2 in

seronegative individuals.

Although both CD4+ and CD8+ responses were significantly more structurally focussed in seropositive individuals, there was no difference in SP:NSP ratio between ESNs and USNs in this cohort. This suggested that ESNs generate a T-cell response more reminiscent of cross-reactivity to NSPs in USNs than of infection in seropositive individuals. The ratio of structural to non-structural response has been studied previously and provides some insight into the origins of responses in SARS-CoV-2 ESNs. Studies of cross-reactivity in flavivirus infection, between Dengue virus (DENV) and ZIKV for example, demonstrate a skew towards T-cells targeting NSPs during ZIKV infection in individuals previously exposed to DENV due to the high homology between flavivirus NSPs.[211] For SARS-CoV-2, where the most conserved regions of the genome are also the NSPs,[7, 200] we would also expect to see a more non-structurally focussed response in ESNs compared to USNs, but this is not the case: the overall SP:NSP ratio does not differ significantly.

PCA provides further insight into the quality of T-cell responses in seropositive individuals, ESNs, and USNs. T-cell responses in ESNs were more positively correlated with PC2 than USNs, and as PC2 is positively associated with responses to NSPs such as NSP7-11, NSP12B, NSP13, and NSP14, this hints at some enhanced responses to the RTC in this cohort. Seropositive individuals tended to be more negatively correlated with PC2 than ESNs – PC2 is negatively associated with structural antigens, further suggesting that seropositive individuals generate more structural responses than ESNs. Together, this suggests that ESNs are generating stronger T-cell responses to NSPs than seropositive individuals, with some evidence of increased responses to the RTC compared to USNs. However, these differences are by no means obvious, and the significant overlap between the three groups by PC analysis suggests a spectrum of of T-cell responses in individuals of different infection status.

It is also important to consider the potential variability in results across studies owing to the use of different techniques. In this study, CTV assays were employed measuring cellular proliferation to peptides spanning the whole SARS-CoV-2 genome. The increased RTC-specific responses in Swadling *et al.* (2022), in contrast, were identified through IFNγ ELISpot.[7] Assaying IFNγ responses versus cellular proliferation may lead to discrepancies between studies. For example, a study of 52 household contacts of SARS-CoV-2-infected individuals identified higher frequencies of IL-2-, but not IFNγ-, secreting T-cells in ESNs who resisted infection compared to exposed individuals that later became infected.[5] Furthermore, the use of an IFNγ-release assay to assess T-cell responses in ESN household members did not identify significantly increased T-cell responses to SARS-CoV-2 antigens.[212] In Chapter 3, the CTV assay was shown to be more sensitive to responses in both SARS-CoV-2 seropositive and seronegative individuals compared to ELISpot.

The role of exposure intensity in promoting T-cell responses to SARS-CoV-2 in ESNs has not been studied. Here, we describe increased T-cell responses in seronegative individuals with two seropositive family members compared to those with only one seropositive family member, indicating that enhanced exposure intensity is associated with stronger cellular immunity. This may be due to an increased viral dose to which the ESN is exposed. The role of dose in ESNs has been studied for HCV and simian immunodeficiency virus (SIV) in non-human primates. Low doses of HCV are associated with T-cell immunity in the absence of seroconversion, whilst higher doses are associated with infection and seroconversion: a study of two chimpanzees described transient T-cell responses when chimpanzees were exposed to increasing doses of HCV at six month intervals.[213] 12 months later, both chimpanzees were exposed to a tenfold greater dose of the virus and became infected. The chimpanzee with consistently stronger T-cell responses cleared infection while the other developed chronic disease. Furthermore, macaques exposed to infectious doses of SIV seroconverted but generated weak cellular responses, whilst those exposed to sub-infectious doses generated cellular responses only.[214] These findings

suggest that dose may factor into which arm of adaptive immunity dominates upon viral exposure. Similar challenge studies in primates or humans exposed to different doses of SARS-CoV-2 would be necessary to make conclusions about the role of dose in SARS-CoV-2 ESNs. However, the findings described herein suggest that increased dose may promote enhanced cellular immunity in ESNs, whilst perhaps pushing individuals towards a dose threshold which surpassing leads to infection and seroconversion.

An alternative explanation is that an increased number of seropositive individuals within a family increases the duration, rather than the dose, of viral exposure in ESNs. Most mild COVID-19 symptoms such as anosmia can last between 2 to 15 days,[168, 215] whilst the mean duration of viral detection by nucleic acid testing was calculated as 12.1 days in the upper respiratory tract, and 24.1 days in the lower respiratory tract.[216] Multiple seropositive individuals within a family, if one is infected by the other, can effectively double the duration of exposure to other seronegative family members. This may have a significant impact on the magnitude or durability of cellular immunity in ESNs. An increased duration of HCV exposure is associated with stronger T-cell responses in ESN injection drug users and is also associated with an increased durability of response.[217] Furthermore, although unlikely as samples were taken early in the pandemic, it is also possible that different family members became infected at different times. This could lead to a prime-boost effect in ESNs, where individuals are exposed twice and therefore cellular immunity is enhanced. Particularly as individuals in this study were sampled in convalescence, multiple exposures to SARS-CoV-2 in ESN family members may also result in more durable immune responses that were more easily picked up in this study.

The data described herein also raises the question of how to define infection versus exposure. Here, infection was defined by seropositivity, and serological testing is often the gold standard for assessing infection history for SARS-CoV-2. However, the presence of SARS-CoV-2-reactive T-cells in ESNs makes hard delineation between

infected and uninfected individuals difficult. Furthermore, it is also possible that these ESN individuals did generate antibody response, but these have waned since infection. More detailed immunophenotyping and transcriptomic analysis can aid in understanding whether ESNs are simply exposed or if they are becoming infected and experiencing active viral replication. A study of SARS-CoV-2 ESN HCWs identified that the T-cell activation markers HLA-DR and CD38 were upregulated in PCR+ HCWs, but not in ESNs.[198] The authors concluded that ESNs had therefore not been infected, and that cellular responses were due to exposure rather than infection and waning antibody. In contrast, Swadling *et al.* (2022) found a significant correlation between the magnitude of the T-cell response in ESNs and transcript levels of IF127, an IFN-inducible marker of early infection.[7] It was concluded that transient infection had occurred but was aborted by early T-cell responses. However, ESNs with elevated IFI17 transcripts only represented 25% of total ESNs, and mean IFI17 expression was lower in ESNs than seropositive HCWs. ESNs may therefore represent not one discrete group, but a spectrum of immune engagement, from subclinical exposure to transient infection.

Another element to consider is the stability of this cellular immunity and its role in protection. Some evidence suggests that T-cell durability following SARS-CoV-2 infection is higher than antibody durability.[28, 169, 173, 175] If this is the case, then T-cell immunity may represent a more appropriate correlate of protection if SARS-CoV-2-specific antibodies have waned or were never generated in the first place. The T-cell responses observed in these ESN individuals derive from samples collected a mean of 7.6 months into convalescence, suggesting that cellular immunity is stable for many months even in the absence of seroconversion. A study of T-cell immunity in seronegative HCWs exposed to HCV-contaminated needles identified CD4+ responses in four of 10 individuals within eight weeks of injury. However, these responses were absent after 2.5 years.[218] Furthermore, another study of cellular immunity in HCWs exposed to HCV via needlestick, cut, or mucosal exposure identified HCV-specific proliferation and IFNγ production in 48% (n=30)

and 42% (n=26) of individuals, respectively. These responses were transient and returned to baseline within months, unlike canonical long-lived memory responses to HCV.[219] Follow-up of individuals exposed to SARS-CoV-2 would be essential to determine the durability of cellular immunity in the absence of seroconversion, however, frequent reinfection and re-exposure as well as the effects of vaccination make this difficult.

Whether T-cell responses in ESNs are protective is unclear. Kundu *et al.* (2022) identified elevated T-cell immunity in ESNs compared to seronegative individuals who later became infected, suggesting seronegative cellular immunity can offer some protection.[5] However, in a model whereby cellular immunity in ESNs is protective, one might expect that the magnitude of the cellular response in ESNs would be greater than in seropositive individuals, to compensate for the lack of humoral immunity. In Wang *et al.* (2021), the magnitude of the SARS-CoV-2 specific CD4+ T-cell response was twice as high in infected individuals compared to ESNs, and in this data, CD4+ T-cell responses are stronger in seropositive individuals compared to ESNs.[199] This casts doubt on whether ESN T-cell immunity is of a sufficient magnitude to be protective. However, it is uncertain whether a high magnitude T-cell response is a correlate for protection against SARS-CoV-2 infection. A study of IFNγ-producing T-cells from vaccinated individuals who later either became infected or remained uninfected identified enhanced protection (5.4% risk) in individuals with high magnitude T-cell immunity compared to those with low-magnitude T-cell responses (43.2% risk). Despite this difference, however, several individuals with low-magnitude T-cell responses remained protected against infection. The authors also identified no correlation between IgG targeting S, RBD or N and risk of infection, highlighting the importance of cellular immunity in protection.[220] Another study of vaccinated individuals identified a correlation between higher magnitude antibody and T-cell responses, as well as higher memory T-cell diversity and cross-reactivity, with protection against Omicron infection.[221]

Some evidence for T-cell-mediated protection is provided by studies of influenza virus. During influenza virus infection, cytotoxic T-cells target conserved NSPs whilst antibodies target the divergent neuraminidase and HA proteins and are thus strain-specific. In 1983, McMichael and colleagues demonstrated that individuals with cross-reactive T-cells targeting influenza A were able to clear heterotypic infection in the absence of subtype-specific antibody.[222] Later studies showed that cross-reactive CD4+ and CD8+ T-cells are associated with milder disease in influenza-infected individuals lacking cross-reactive antibody.[223, 224] However, whether these findings are applicable to SARS-CoV-2 is unclear. Challenge studies of SARS-CoV-2 infection in ESN individuals may shed further light on this topic. Another possibility is that antigen is being cleared solely by the action of the innate immune system, with T-cell production as an epiphenomenon due to the presence of viral antigen, but not responsible for viral clearance.

To prevent infection before seroconversion, a rapid cellular response appears critical. Chandran *et al.* (2021) assayed weekly nasopharyngeal swabs and blood samples from HCWs and demonstrated that SARS-CoV-2-specific T-cell proliferation can occur early in infection, even preceding PCR positivity.[225] One explanation for these rapid responses is pre-existing, cross-reactive T-cell memory specific for endemic HCoVs. However, although T-cell responses to HCoV-OC43 appeared elevated in seropositive individuals, there was no significant difference between seropositive individuals (n=15), ESNs (n=10), and USNs (n=10) assayed here. This, combined with the lack of an increased response to conserved peptides in ESNs compared to USNs, raises the question of whether ESNs are expanding their cross-reactive T-cell responses or generating *de novo* responses to SARS-CoV-2-specific peptides. However, the median S2-specific response in ESNs is greater than the median S1-specific response, in contrast to seropositive individuals where the S1-specific response is greater. Furthermore, it is also possible that ESNs are generating both a cross-reactive memory response and a *de novo* response to non-conserved peptides. Evidence from HCV exposure suggests that HCV ESNs generate

a memory-like response, due to the peaking of T-cell responses four to six weeks after exposure, rather than seven to 14 weeks as is typical of canonical seropositive HCV infection.[226] It is unclear whether cross-reactive T-cells contribute to immunity in HCV-exposed individuals. Some cross-reactivity between HCV and influenza A has been described, with HCV-seronegative individuals generating T-cell responses to a cross-reactive HCV epitope.[113] However, human viruses with homology to other viruses for which the ESN phenomenon has been described such as HIV, HBV or HSV-2 have not been reported, and are unlikely to be the driver of the ESN phenomenon for all viruses.

Rather than the expansion of pre-existing, cross-reactive T-cells specific for endemic HCoVs, an alternative explanation for the rapidly induced T-cell immunity in ESNs is rapid *de novo* immunity arising from high precursor frequency naive T-cells, as explored by Milighetti *et al.* (2023).[227] In this study, the authors describe expanded populations of naive T-cells specific for SARS-CoV-2 and non-cross-reactive with endemic coronaviruses that are detectable early in infection or even before infection, paralleling the innate immune response. The authors also demonstrate a similar phenomenon in LCMV infection, suggesting that high-frequency naive T-cells may be a general feature of early responses to human pathogens. These findings may be applicable to this chapter, providing an alternative explanation for the rapid and low-magnitude T-cell immunity observed in ESNs that does not originate from cross-reactivity with endemic coronaviruses.

A model for the dynamics of adaptive immunity in infection versus exposure is shown in Figure 4.9. In canonical SARS-CoV-2 infection (Figure 4.9A), T-cells and antibodies reduce viral load and contribute to disease resolution. In ESNs (Figure 4.9B), T-cells proliferate alone and at lower levels than canonical infection, though higher levels than expected from cross-reactivity in unexposed individuals. Viral load never reaches detectable levels and clinical disease does not occur. This may result from early proliferation of cross-reactive T-cells but could also be due to

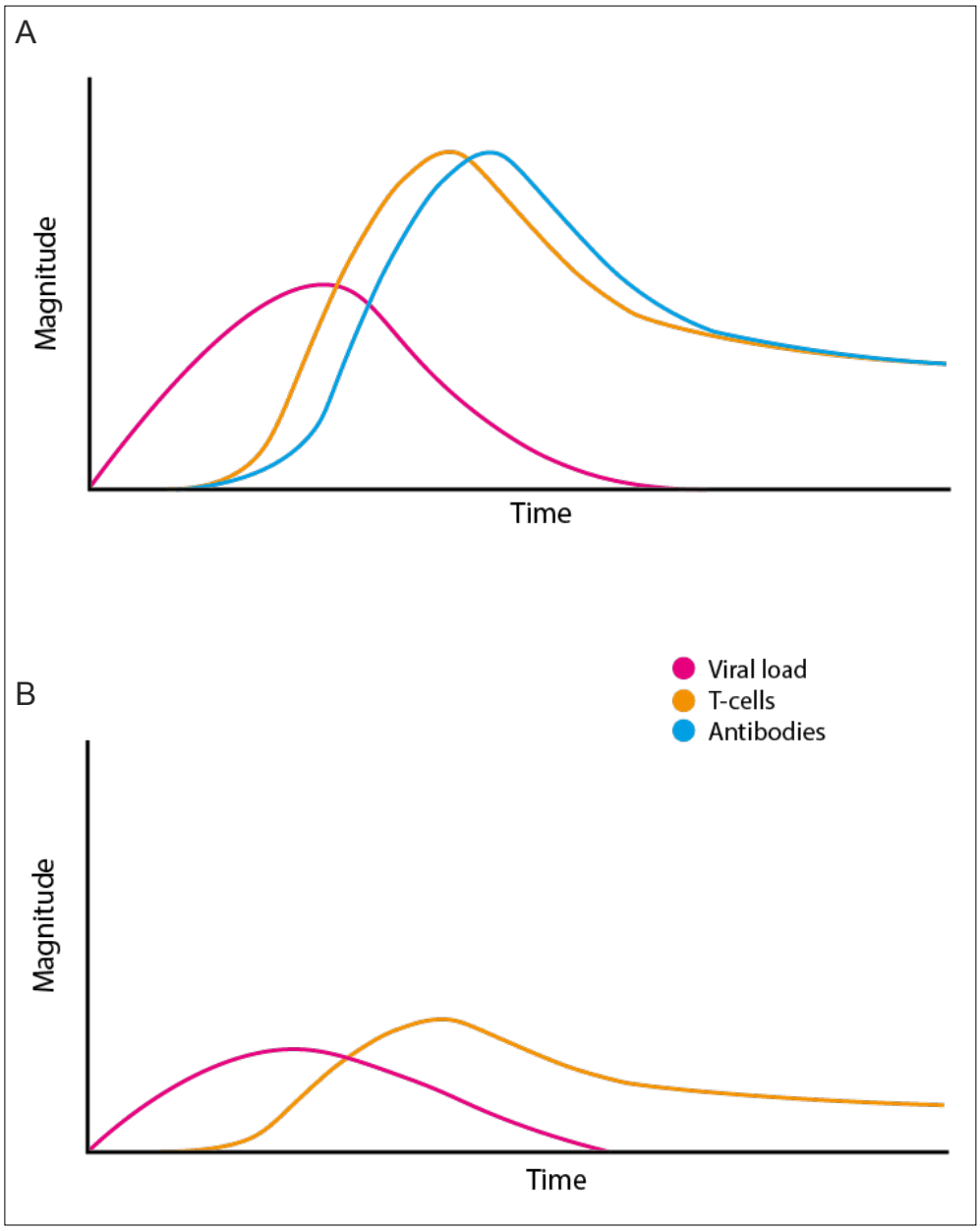


Figure 4.9: Immune dynamics in ESNs. Adaptive immunity in seropositive individuals (A) and SARS-CoV-2 ESNs (B).

abortion of infection by rapidly-induced *de novo* T-cells targeting non-conserved SARS-CoV-2 epitopes. As demonstrated by PCA, rather than forming a discrete group, ESNs represent points along a spectrum of immune engagement influenced by viral and host factors (Figure 4.10). Discrepancies between this study and other studies likely reflect individuals at different points along this spectrum, dependent upon viral dose, existing cross-reactive immunity, and duration of exposure.

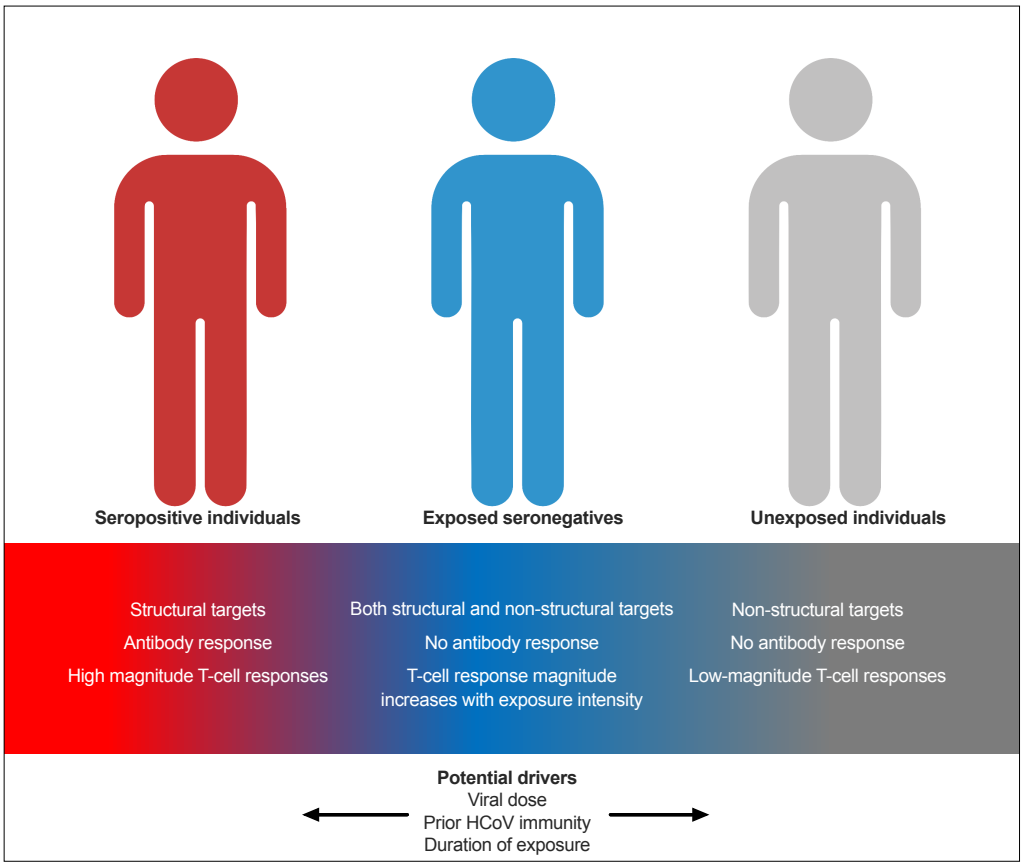


Figure 4.10: A model for immunity in seropositive individuals, ESNs and USNs. Individuals represent points along a spectrum from USN to seropositive, modulated by viral dose, prior immunity to endemic HCoVs, and duration of exposure.

The aim of this chapter was to explore the role of cross-reactive T-cells in individuals highly exposed to, but seronegative for, SARS-CoV-2. Here, elevated T-cell immunity was identified in SARS-CoV-2-exposed family members that remained seronegative. These T-cells targeted structural antigens but were less structurally focussed than seropositive individuals. It was demonstrated that the magnitude of this response was associated with the number of individuals infected within a family, highlighting a role for exposure intensity, either through increased duration or dose of SARS-CoV-2 exposure. Finally, cross-reactive T-cells were implicated in this immunity through enhanced S2-specific responses, but analysis of HCoV-specific immunity and T-cell responses to peptides conserved between SARS-CoV-2 and HCoVs suggest that *de novo* T-cell responses likely also play a role. Overall, this

data has implications for estimates of SARS-CoV-2 correlates of protection and highlights the fact that using seropositivity as the sole marker of prior SARS-CoV-2 infection and protection may be overly simplistic.

5

Cross-reactive antibodies and COVID-19 severity

Contents

5.1 Abstract

The role of cross-reactive antibodies in modulating COVID-19 severity has been widely disputed. Antibodies targeting common cold coronaviruses are widespread by the time an individual reaches adolescence. Serum samples from SARS-CoV-2-infected or vaccinated individuals contain antibodies that recognise endemic HCoVs, and pre-pandemic samples contain antibodies that recognise SARS-CoV-2,[1, 2], although these are rare in the general population.[228] A key question that arose early in the pandemic, and has yet to be conclusively answered, is whether this cross-reactivity offers protection against SARS-CoV-2 infection and severe disease, or whether it can promote worse disease through original antigenic sin (OAS) or antibody-dependent enhancement (ADE). In this chapter, we identify an association between prior exposure to endemic betacoronaviruses and worse disease outcomes in two separate cohorts of individuals with mild and severe COVID-19. In addition, we identify age-associated differences in HCoV exposure that may contribute to worse disease. Unlike the potentially protective role of T-cells as explored in Chapter 4, this chapter identifies a contrasting role for cross-reactive humoral immunity in promoting worse disease during SARS-CoV-2 infection and attempts to offer a mechanistic explanation for how this might occur.

5.2 Introduction

Aim: Investigate the relationship between humoral immunity to endemic HCoVs and COVID-19 severity.

Antibodies and T-cells sample antigen through different mechanisms, resulting in markedly different functional properties. Both antibodies and T-cells react with target antigens through protein-protein interactions determined by the secondary structure of both the target antigen and the immune component. T-cells recognise antigen as MHC-peptide complexes, which enables them to sample the intracellular space and, as a result, they can and must recognise a larger number of epitopes than are available for antibody recognition.[201] In addition, T-cells do not undergo affinity maturation and express a fixed constant region, in contrast to B-cells. Rather than generating highly specific T-cells for each of the estimated 10^{17} 14-mer peptides that the MHC Class II could theoretically present, the recognition process for T-cells allows for cross-reactivity between closely related antigens.[201, 229] T-cells recognise linear epitopes, and therefore may be less sensitive to changes in the peptide primary sequence. In contrast, antibodies recognise conformational rather than linear epitopes, and thus have a lower capacity for cross-reactivity due to the more significant effects of point mutations on antigen secondary structure.[201] Despite this constraint, cross-reactive antibodies have been identified in the context of many viral diseases.[230–235] This may result from conformational flexibility of antibodies, particularly at glycine or aromatic residues.[236]

Perhaps the most well described instances of serological cross-reactivity arise from studies of flaviviruses. Flaviviruses are positive-sense, single-stranded, enveloped RNA viruses, 40 of which are disease-causing in humans.[237] These include ZIKV, DENV, YFV, WNV, tick-borne encephalitis virus (TBEV), and Japanese encephalitis virus (JEV). Flaviviruses are arthropod-borne viruses (arboviruses) and their host vector is a strong determinant of genetic relatedness: phylogenetically, they cluster by vector, either mosquitoes (DENV, YFV, WNV, JEV) or ticks (TBEV,

Kyasanur forest disease virus, Powassan viruses).[237] Their infection can cause a wide range of symptoms in humans, from self-limiting fever to haemorrhagic fever and encephalitis, and they represent a major public health concern - particularly in tropical and subtropical regions.[238] High degrees of homology between flaviviruses enable cross-recognition of viral proteins by antibodies and T-cells alike.[237] The main antigenic target during acute infection is the E surface glycoprotein, the primary surface molecule on the flavivirus virion.[232, 233] Between 15%-84% of antibodies specific for non-DENV flaviviruses (YFV, ZIKV, JEV, and TBEV) cross-react with DENV, and cross-reactivity is elevated in individuals previously infected with antigenically similar species.[238] Mice with pre-existing immunity to JEV develop faster nAb responses to DENV than JEV-naïve animals through recall of memory responses.[237]

In addition to flaviviruses, cross-reactivity between strains of influenza virus (also known as 'heterosubtypic immunity') is well established and believed to be somewhat protective.[204, 222] Influenza strains are classified by their neuraminidase and HA proteins, and circulating strains vary each year through both antigenic drift driven by selection of nAb escape mutations, and antigenic shift due to novel strains emerging into naïve populations.[204] 18 HA subtypes have been described, broadly divided into group 1 and group 2 molecules.[239] Protective immunity against influenza virus infection is mediated by anti-HA antibodies targeting immunodominant epitopes in the HA head region, although these are predominantly strain specific.[239] Therefore, antigenic drift leads to annual outbreaks as viruses escape existing protective immunity.[240] However, *in vitro* and *in vivo* cross-reactivity between strains has been recognised since the mid-1960s, when infection with one influenza A virus was found to be protective against heterosubtypic challenge in mice.[241] Later, vaccination of mouse mothers with inactivated influenza A virus during pregnancy was found to protect pups against heterosubtypic challenge.[242] Convalescent blood samples from individuals infected with the pandemic H1N1 influenza strain in 2009 contained plasmablasts cross-reactive to three strains included in recent seasonal

influenza vaccines.[235] Finally, mAbs that target the head region of multiple subtypes of HA have been described.[239]

A key question when assessing cross-reactive immunity to SARS-CoV-2 is whether it can confer protection against infection. Cross-reactive antibodies to flaviviruses have been shown to be protective – antibodies induced by immunisation against JEV were protective against WNV in hamsters, and serum from DENV patients neutralised ZIKV.[232, 243] mAbs derived from memory B-cells of humans recently vaccinated against influenza A were shown to react broadly to different influenza subtypes including H1, H2, H5, H6, H8, and H9, and the most potent antibody identified conferred protection against lethal H5N1 and H1N1 challenge in mice.[244] Another study identified a mAb able to inhibit H1, H2, H3, H5, H9, and H13 influenza A subtypes.[245] In addition, reduced disease susceptibility to the 2009 H1N1 pandemic in the elderly was associated with pre-existing immunity to childhood strains of influenza.[246] Furthermore, a recent study identified two broadly neutralising mAbs: one targeting both respiratory syncytial virus and human metapneumovirus, and the other targeting both human parainfluenza virus types one and three.[247]

Despite the protective effects of cross-reactive antibodies, there is also evidence that they can enhance pathogenesis for certain viral diseases. Initial reports by Halstead *et al.* (1977) that serum from DENV convalescent individuals promoted enhancement of *in vitro* DENV infection in cell culture models provided evidence that primary infection with one virus could enhance disease upon secondary infection.[248] Primary DENV infection is generally mild and acute and promotes production of nAbs.[249] However, it is now appreciated that low titres of heterotypic anti-DENV antibodies do not neutralise other strains of DENV but instead enhance disease. DENV replicates in phagocytes, and the binding of low titre, non-neutralising anti-DENV antibodies promotes recognition of intact virions by Fcγ receptors, enhancing viral entry into phagocytic cells.[250] This then allows the virus to exist in a reservoir of infected phagocytes, spread through the body systemically, and promote more

severe disease.[251] This process is known as ADE. In particular, production of highly cross-reactive antibodies specific for the DENV precursor-membrane protein of the viral surface is associated with ADE; incomplete maturation of this protein during virion assembly results in variable amounts on the viral surface, making it a poor target for humoral responses.[252] Furthermore, prior exposure to ZIKV has been implicated as a risk factor for DENV ADE, and vice versa, due to the presence of cross-reactive ZIKV-specific antibodies that are poor neutralisers of DENV but bind sufficiently to promote phagocytic uptake.[251] A study of DENV pathogenesis in mice identified worse disease severity following DENV challenge in pups of ZIKV-immune mothers compared to naïve mothers.[253] In addition, serum against DENV was shown to be highly cross-reactive with ZIKV and drove ADE of ZIKV infection *in vitro* in a myeloid cell line through poorly neutralising binding dynamics.[252]

As well as ADE, pre-existing cross-reactive antibodies can mediate worse disease through OAS. The term OAS was coined by Thomas Francis with reference to influenza strains: exposure to a new strain of influenza virus boosted pre-existing memory responses to a previously encountered strain, with little or no development of novel responses to neoepitopes.[254, 255] The worse disease outcomes associated with OAS may result from poorly neutralising responses, delayed development of *de novo* responses, and competition between memory and naïve B-cells.[255] Several studies have demonstrated that OAS can be observed following successive influenza infections: antibody titres of highest magnitude are often specific for strains encountered in childhood,[246] and a cross-sectional study of humoral immunity to influenza in China identified higher nAb titres to strains that circulated in the first decade of an individual's life.[256] However, whether this enhances disease severity in later infections is less well defined. OAS likely also plays a role in flaviviral ADE. Increased disease severity following secondary infection with a different subtype is characteristic of DENV infection.[233] Midgley *et al.* (2011) demonstrated boosting of low-level DENV cross-reactive immune responses following heterotypic infection

with another DENV subtype; these cross-reactive antibodies had a higher avidity for primary serotype antigens than for the secondary serotype.[257] A study of vaccination against human CMV in CMV seropositive individuals identified boosting of pre-existing antibody responses, but no *de novo* responses to novel epitopes, indicating a substantial impact of prior immunity on the antibody response following vaccination.[258]

Although antibody-mediated OAS is the focus of this chapter, T-cell mediated OAS has also been demonstrated. OAS mediated by CTLs has been described in lymphocytic choriomeningitis virus (LCMV) in mice, where exposure to an initial LCMV strain and subsequent exposure to variants generated a CTL response specific for the original epitope rather than subsequent variant epitopes.[259] In addition, studies of DENV infection have described CD8+ T-cell responses in children acutely infected with DENV that were of low affinity for the infecting virus and were specific for other, previously encountered strains of DENV. These CD8+ T-cells were highly activated and apoptotic.[260]

The role of cross-reactive antibodies in COVID-19 severity is controversial. There is substantial evidence that pre-pandemic serum samples contain HCoV-specific antibodies that can cross-recognise SARS-CoV-2, particularly those targeting the S2 region of S.[3, 228, 261] In addition, pre-existing HCoV-specific memory B-cells expand in early SARS-CoV-2 infection.[46] However, their role in modulating COVID-19 outcomes is contested. Levels of HCoV-specific antibodies are boosted following infection, but in some studies, baseline levels do not associate with susceptibility to infection,[262] whilst other studies show an adverse impact of pre-existing cross-reactive antibodies on disease outcome.[263–265] One study of COVID-19 patients in an ICU identified an association between antibodies targeting HCoV-OC43 and death from COVID-19.[6] In addition, this study showed that although antibody responses to the HCoV-OC43 N protein were not boosted following SARS-CoV-2 infection, they were elevated in individuals who died of

COVID-19. Conversely, a study of 48 SARS-CoV-2-unexposed individuals described pre-existing antibodies capable of neutralising SARS-CoV-2 in vitro,[228] while another study identified an association between anti-HCoV-NL63 antibody and enhanced neutralisation of SARS-CoV-2 in COVID-19 convalescent plasma.[266]

Cross-reactive humoral immunity to SARS-CoV-2 and related coronaviruses is evident in the literature, but the role of this immunity in disease severity is unclear. To investigate the role of cross-reactive antibodies in modulating COVID-19 severity, humoral responses to multiple structural HCoV antigens were assessed in the family cohort, as well as an ICU cohort and a cohort of HCWs sampled before and after SARS-CoV-2 infection. In this chapter, evidence for an association between cross-reactive HCoV-specific IgG and worse disease outcomes both in mild and severe disease is presented, with implications for pan-coronavirus vaccines and the emergence of future human coronaviruses.

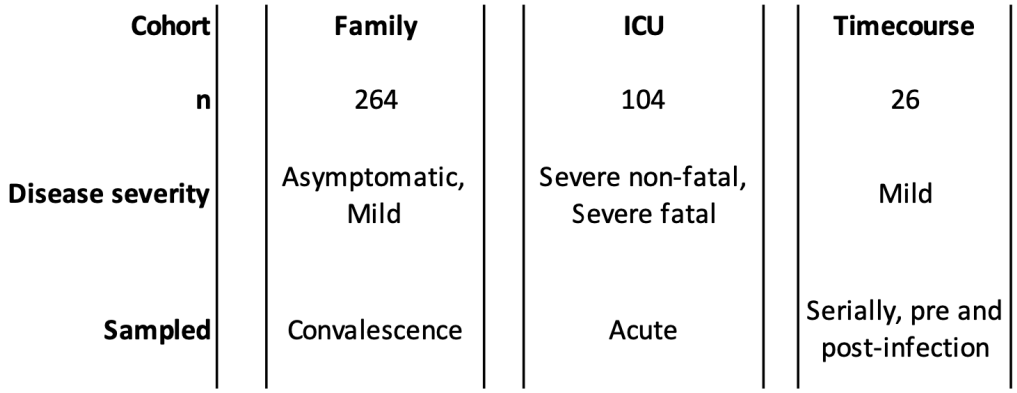


Figure 5.1: Cohorts. The three cohorts studied in this chapter: the family cohort, the ICU cohort, and the timecourse cohort.

5.3 Results

5.3.1 Cohorts

In order to understand the role of pre-existing cross-reactive antibodies in modulating disease severity following SARS-CoV-2 infection, three cohorts of differing COVID-19 disease severity and sampling date relative to infection were assayed for their humoral responses to HCoV antigens: the family cohort as outlined in Chapter 3 sampled during the convalescent phase of infection, a cohort of ICU patients sampled during the acute phase of infection, and a cohort of individuals sampled serially pre- and post-infection (Figure 5.1). Further characteristics of the family study cohort are detailed in Chapter 3.

The ICU cohort consisted of 104 individuals who were hospitalised with severe COVID-19; samples were taken during acute disease. Individuals were categorised based on whether they died of COVID-19 (fatal group, n=58) or survived (non-fatal group, n=46). The mean age was 80 years and ranged from 26 to 87. 69% of individuals were male (72/104). The median number of days from ICU admission to death in the fatal group was 13 (1 to 41), and the median number of days from ICU admission to sampling in all individuals was 3 (-3 to 15).

The timecourse cohort was made up of two groups: serially sampled HCWs from the Oxford Radcliffe Biobank, and individuals sampled pre- and post-SARS-CoV-2 infection from the COVID-LIV study at the University of Liverpool.[119] Both Oxford and COVID-LIV cohorts were made up of individuals with mild disease. Samples were taken up to 209 days pre-infection and up to 36 days after PCR-confirmed SARS-CoV-2 infection.

5.3.2 IgG responses to HCoVs are boosted following SARS-CoV-2 infection

To assess the levels of IgG targeting coronaviruses in SARS-CoV-2 S ELISA seronegative and seropositive individuals with mild disease, an MSD immunoassay (Panel 3) was run on serum from the family cohort (Figure 5.2). IgG targeting the S proteins of SARS-CoV-1, MERS-CoV, HCoV-OC43, HCoV-HKU1, HCoV-NL63, and HCoV-229E was quantified and compared between seronegative and seropositive individuals. IgG responses to betacoronaviruses were significantly elevated in SARS-CoV-2 seropositive individuals (SARS-CoV-1: $p<0.0001$, MERS-CoV: $p<0.0001$, HCoV-OC43: $p=0.001$, HCoV-HKU1: $p=0.02$, Mann-Whitney tests). Levels of IgG targeting HCoV-NL63 and HCoV-229E were not significantly different between SARS-CoV-2 seronegative and seropositive individuals. Interestingly, the magnitude of IgG boosting appeared to correlate with genetic relatedness to SARS-CoV-2, with boosting to SARS-CoV-1 being the highest and to the alphacoronaviruses HCoV-NL63 and HCoV-229E being the lowest.

5.3.3 IgG responses to HCoVs correlate with age in SARS-CoV-2 seropositive individuals

Older age is a primary risk factor for severe COVID-19, and in Chapter 3, age was positively correlated with T-cell responses to SARS-CoV-2 in seronegative individuals.[76] To understand the relationship between age and IgG responses to SARS-CoV-2 and related coronaviruses, the correlation between age and IgG targeting SARS-CoV-2 S and RBD, SARS-CoV-1 S, MERS-CoV S, HCoV-OC43 S,

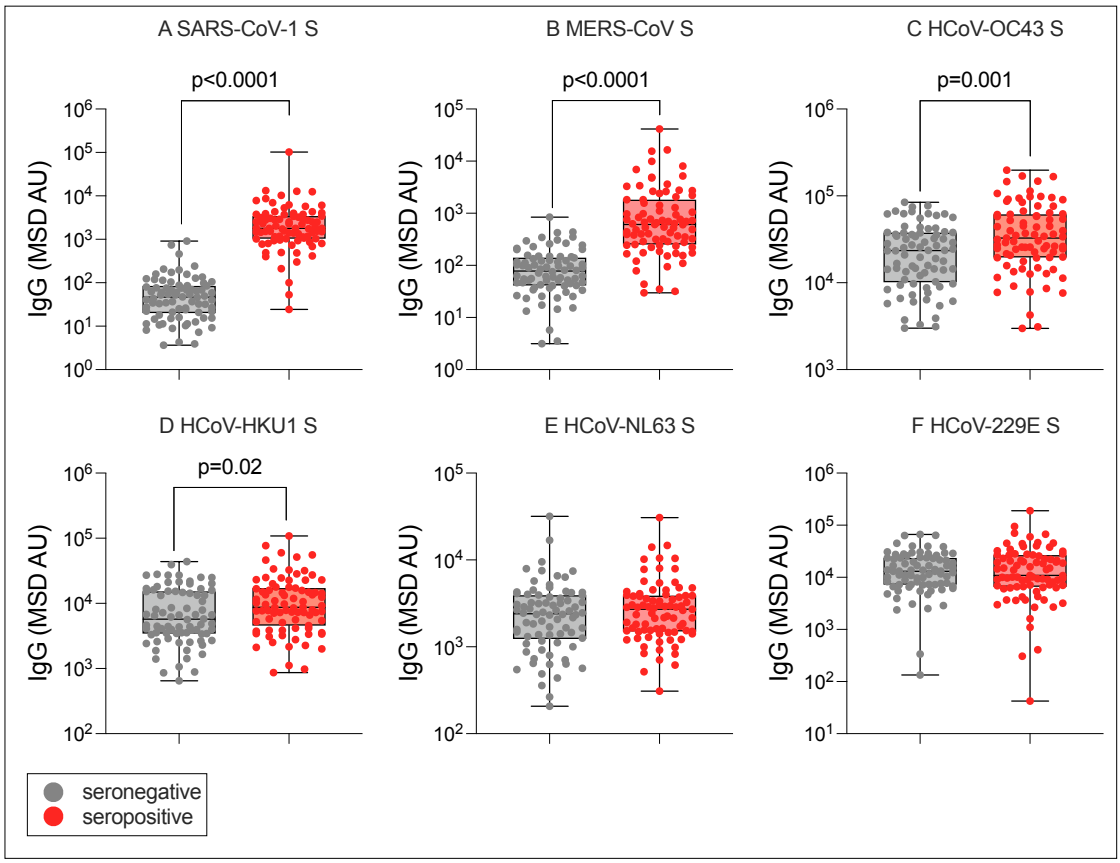


Figure 5.2: IgG responses to HCoVs are boosted following SARS-CoV-2 infection. Total IgG targeting SARS-CoV-1 S (A), MERS-CoV S (B), HCoV-OC43 S (C), HCoV-HKU1 S (D), HCoV-NL63 S (E), and HCoV-229E S (F) in SARS-CoV-2 S seronegative (grey) and seropositive (red) individuals from the family cohort. p-values refer to Mann-Whitney test values.

HCoV-HKU1 S, HCoV-NL63 S, and HCoV-229E S was calculated. As detailed in Chapter 3, there was no correlation between age and antibody responses to SARS-CoV-2 antigens in seropositive individuals. In addition, there was no correlation between age and IgG targeting SARS-CoV-1 or MERS-CoV S. However, age was positively correlated with IgG targeting HCoV-OC43 (r=0.37, p=0.0006), HCoV-HKU1 (r=0.53, p<0.0001), HCoV-NL63 (r=0.36, p=0.0008) and HCoV-229E (r=0.58, p<0.0001) in SARS-CoV-2 seropositive individuals only (Spearman rank tests) (Figure 5.3). Only IgG targeting HCoV-HKU-1 was correlated with age in seronegative individuals (r=0.33, p=0.003, Spearman rank test).

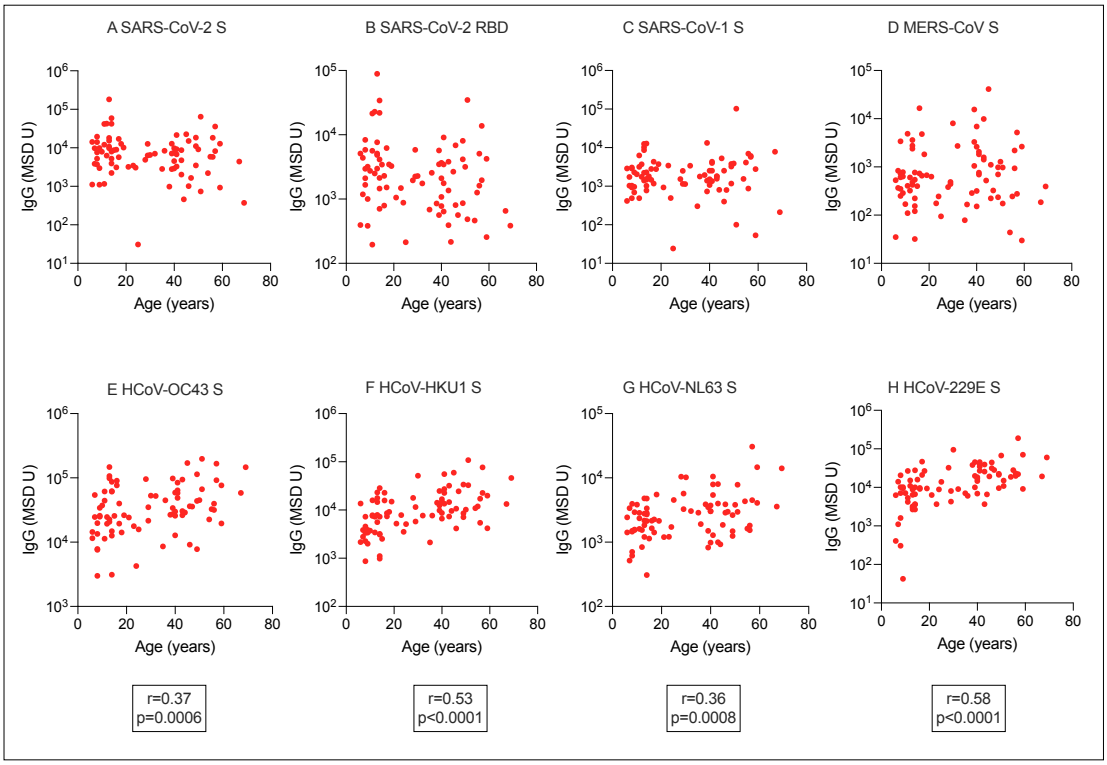


Figure 5.3: IgG responses to HCoVs correlate with age in SARS-CoV-2 seropositive individuals. The correlation between age and IgG targeting SARS-CoV-2 S (A), SARS-CoV-2 RBD (B), SARS-CoV-1 S (C), MERS-CoV S (D), HCoV-OC43 S (E), HCoV-HKU1 S (F), HCoV-NL63 S (G), and HCoV-229E S (H) in SARS-CoV-2 S seropositive individuals from the family cohort. p- and r-values refer to Spearman rank tests.

5.3.4 IgG responses to conserved regions of S are elevated in symptomatic individuals

To explore the relationship between cross-reactive IgG and disease severity, IgG targeting coronaviruses was compared between seropositive asymptomatic individuals and seropositive individuals who self-reported at least one symptom of COVID-19 from the family cohort. As described in Chapter 3, IgG responses to SARS-CoV-2 S were not significantly elevated in symptomatic individuals compared to asymptomatic individuals. Furthermore, IgG responses to the RBD region of S were also not significantly different between asymptomatic and symptomatic individuals (Figure 5.4A). However, IgG responses to the S2 region of S, as measured by an S2 ELISA, were elevated in symptomatic individuals (p=0.01, Mann-Whitney test) (Figure 5.4B). Furthermore, IgG targeting other coronaviruses were elevated

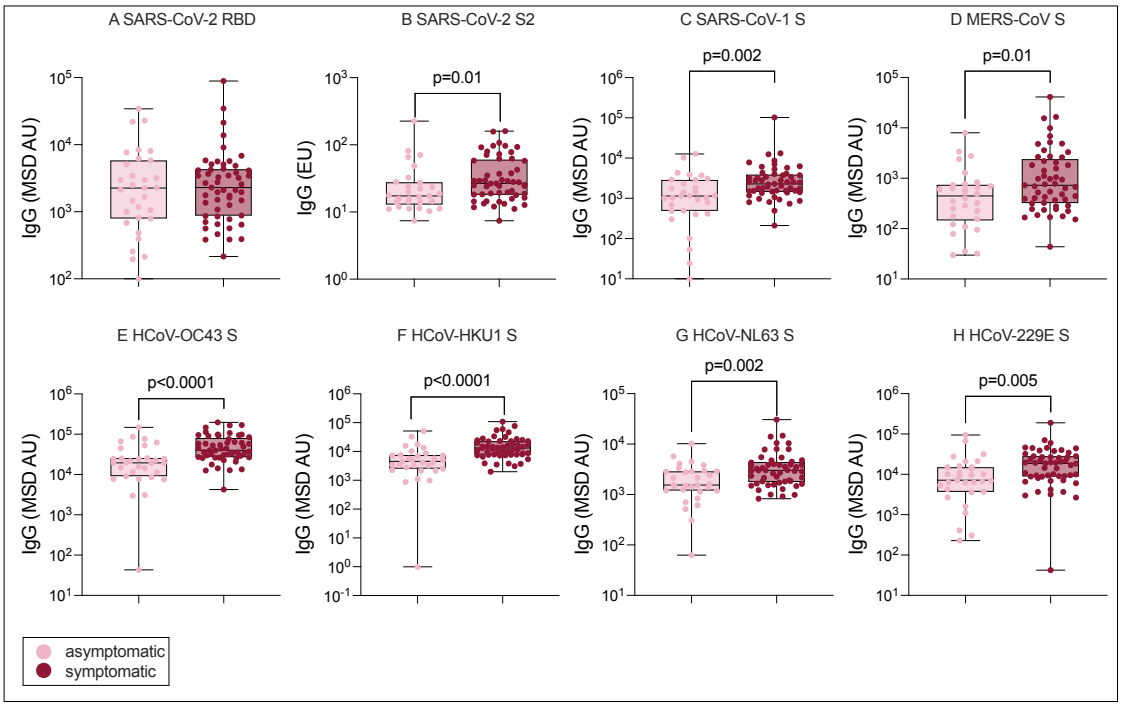


Figure 5.4: IgG responses to conserved regions of S are elevated in symptomatic individuals. IgG targeting SARS-CoV-2 RBD (A), SARS-CoV-2 S2 (B), SARS-CoV-1 S (C), MERS-CoV S (D), HCoV-OC43 S (E), HCoV-HKU1 S (F), HCoV-NL63 S (G), and HCoV-229E S (H) in SARS-CoV-2 S seropositive asymptomatic (pink) and symptomatic (maroon) individuals. p-values refer to Mann-Whitney test values.

in symptomatic compared to asymptomatic seropositive individuals (SARS-CoV-1 S: p=0.002, MERS-CoV S: p=0.01, HCoV-OC43 S: p<0.0001, HCoV-HKU1 S: p<0.0001, HCoV-NL63 S: p=0.002, HCoV-229E S: p=0.005, Mann-Whitney tests) (Figure 5.4C-H). The lack of difference in total S or RBD response, but the difference in S2 and HCoV-specific antibody responses suggests that IgG responses to conserved regions of SARS-CoV-2 S are associated with worse symptoms in mild disease.

5.3.5 No association between HCoV-specific IgG and anti-SARS-CoV-2 nAb titres

To assess whether increased cross-reactive IgG responses were associated with weak neutralising activity against SARS-CoV-2, correlations between nAb titres and IgG targeting SARS-CoV-2 RBD, SARS-CoV-2 S2, SARS-CoV-1 S, MERS-CoV, HCoV-OC43, HCoV-HKU1, HCoV-NL63, and HCoV-229E were calculated (Figure 5.5). As expected, there was a positive correlation between SARS-CoV-2 nAb titres

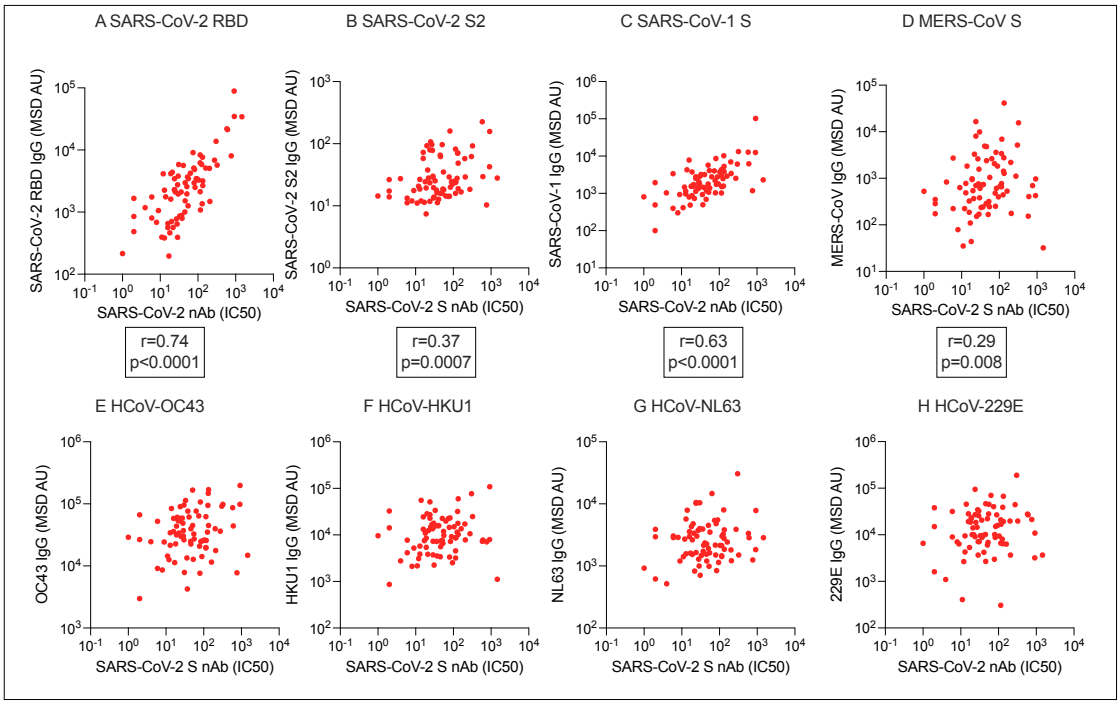


Figure 5.5: No association between HCoV-specific IgG and anti-SARS-CoV-2 nAb titres. Correlations between SARS-CoV-2 neutralisation and IgG targeting SARS-CoV-2 RBD (A), SARS-CoV-2 S2 (B), SARS-CoV-1 S (C), MERS-CoV S (D), HCoV-OC43 S (E), HCoV-HKU1 S (F), HCoV-NL63 S (G), and HCoV-229E S (H) in SARS-CoV-2 S seropositive individuals from the family cohort. p- and r-values refer to Spearman rank test values.

and IgG targeting SARS-CoV-2 RBD (r=0.74, p<0.0001) SARS-CoV-2 S2 (r=0.37, p=0.007), SARS-CoV-1 S (r=0.63, p<0.0001) and MERS-CoV S (r=0.29, p=0.008) (Spearman rank tests). However, there was no correlation between anti-HCoV IgG and SARS-CoV-2 neutralisation.

5.3.6 IgG responses in severe COVID-19

In order to compare immune responses following mild disease with immune responses during severe COVID-19, IgG responses in seropositive individuals from the family cohort were compared to IgG responses in individuals who had been admitted to the ICU with COVID-19. As individuals from the ICU cohort were sampled during acute disease, whilst the family cohort was sampled in convalescence, direct comparisons between these two cohorts are confounded by sampling date and potential waning of responses. As expected, individuals in the ICU cohort generated significantly

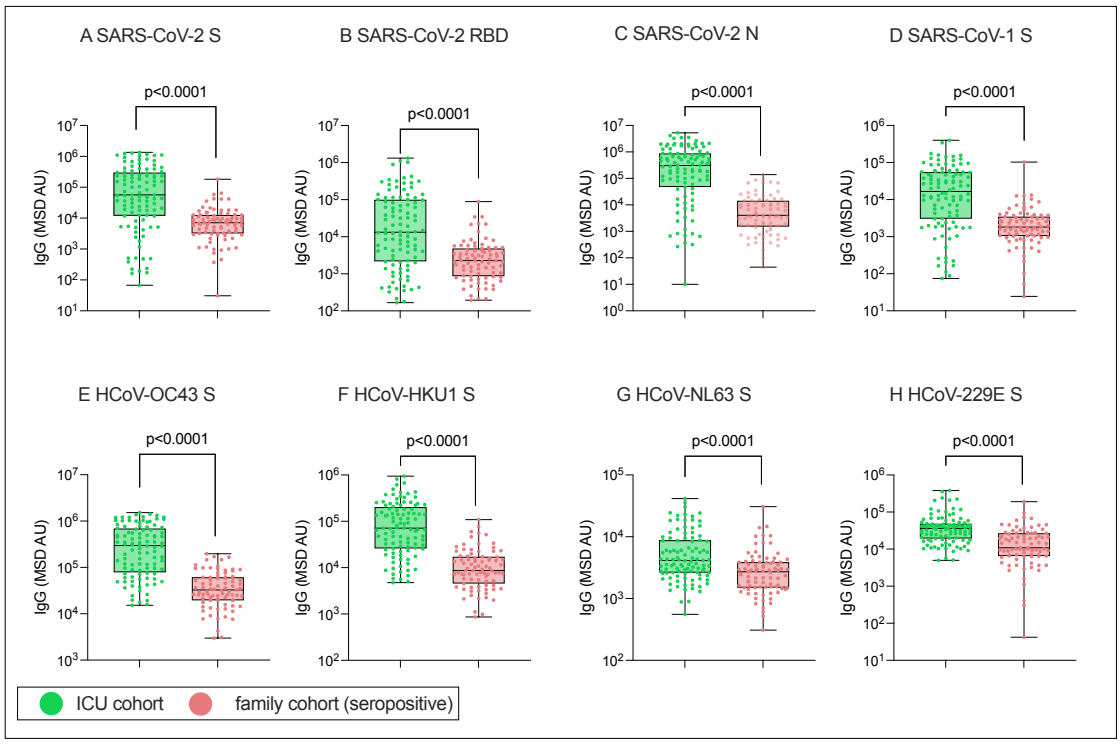


Figure 5.6: IgG responses in mild versus severe disease. IgG targeting SARS-CoV-2 S (A), SARS-CoV-2 RBD (B), SARS-CoV-2 N (C), SARS-CoV-1 S (D), HCoV-OC43 S (E), HCoV-HKU1 S (F), HCoV-NL63 S (G) and HCoV-229E S (H) in the ICU cohort (green) and SARS-CoV-2 seropositive individuals from the family cohort (pink). p-values refer to Mann-Whitney test values.

higher IgG responses to all antigens tested ($p<0.0001$ for all, Mann-Whitney tests) (Figure 5.6).

To explore the effects of age and sex in the ICU cohort, and to determine if any confounding factors may influence interpretation of results, correlations between age and IgG response to coronaviruses were calculated in this cohort (Figure 5.7). Surprisingly, there was no correlation between age and IgG response to most coronavirus antigens, although there a weak negative correlation between age and IgG targeting HCoV-NL63 S ($r=-0.2$, $p=0.04$, Spearman rank test) (Figure 5.7G). In addition, there was no significant difference in IgG response to SARS-CoV-2 antigens between males and females, although males generated stronger IgG responses to HCoV-229E S ($p=0.03$, Mann-Whitney test). In the non-fatal group, 65% of individuals were male, whereas in the fatal group, 72% were male. However,

there was no significant overrepresentation of males in the fatal group (p=0.5, Fisher's exact test).

The association between age and risk of death was measured using binary logistic regression. The fitted regression model was:

Fatal outcome? = Intercept + Age

The odds of death from COVID-19 increased by 6.7% (95% CI [1.03 to 1.11]) for every one-year increase in age (p=0.0007).

5.3.7 No difference in IgG responses to HCoVs between fatal and non-fatal COVID-19

Responses to conserved regions of S were associated with symptomatic COVID-19 in the family cohort. In order to validate this finding in a separate cohort of increased disease severity, IgG responses to SARS-CoV-RBD, SARS-CoV-2 NTD, SARS-CoV-2 N, SARS-CoV-1 S, HCoV-OC43 S, HCoV-HKU1 S, HCoV-NL63, and HCoV-229E were compared between non-fatal and fatal groups (Figure 5.8). In contrast to the family cohort, there was no significant difference in IgG titre between the two groups. However, there was a trend towards greater IgG in the non-fatal group, perhaps reflecting impaired humoral immunity in the fatal group.

5.3.8 IgG responses to HCoV-HKU1 and HCoV-OC43 N are not boosted following SARS-CoV-2 infection

The finding that symptomatic individuals display elevated levels of cross-reactive IgG targeting conserved regions of S supports the hypothesis that cross-reactive IgG may worsen COVID-19 disease course through OAS. To determine whether cross-reactive antibodies are associated with worse outcomes irrespective of the impacts of boosting during infection, responses to HCoV-HKU1 and HCoV-OC43 S and N were assayed pre- and post-SARS-CoV-2 infection. The N protein is conserved and highly cross-reactive.[267] However, it was recently demonstrated

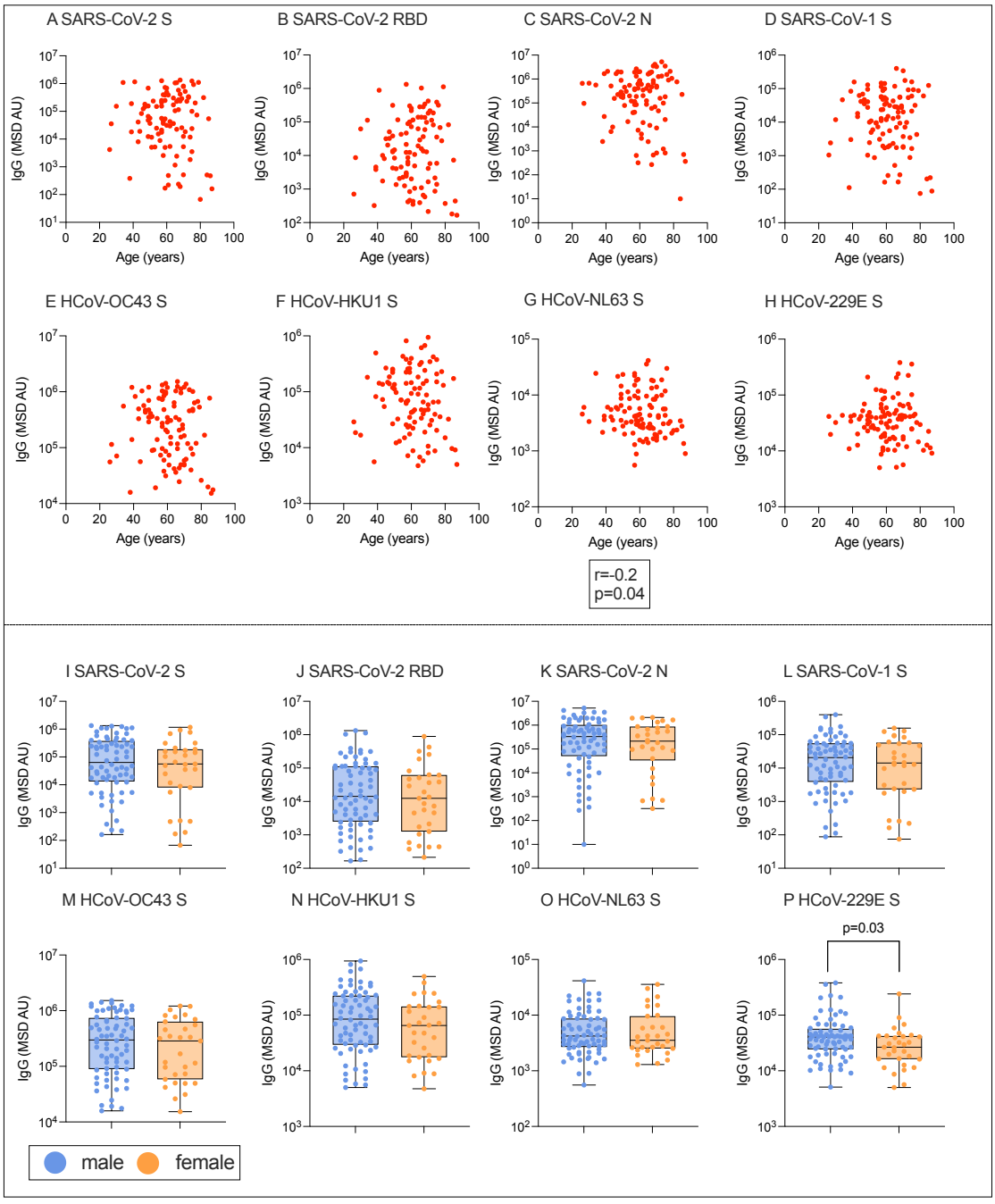


Figure 5.7: Age- and sex-specific immunity in severe COVID-19. Correlations between age and IgG targeting SARS-CoV-2 S (A), SARS-CoV-2 RBD (B), SARS-CoV-2 N (C), SARS-CoV-1 S (D), HCoV-OC43 S (E), HCoV-HKU1 S (F), HCoV-NL63 S (G), and HCoV-229E S (H) in individuals from the ICU cohort. IgG targeting SARS-CoV-2 S (I), SARS-CoV-2 RBD (J), SARS-CoV-2 N (K), SARS-CoV-1 S (L), HCoV-OC43 S (M), HCoV-HKU1 S (N), HCoV-NL63 S (O), and HCoV-229E S (P) in males (blue) and females (orange) in the ICU cohort. p-values represent Mann-Whitney test values for pairwise comparisons and p- and r-values represent Spearman rank test values for correlations.

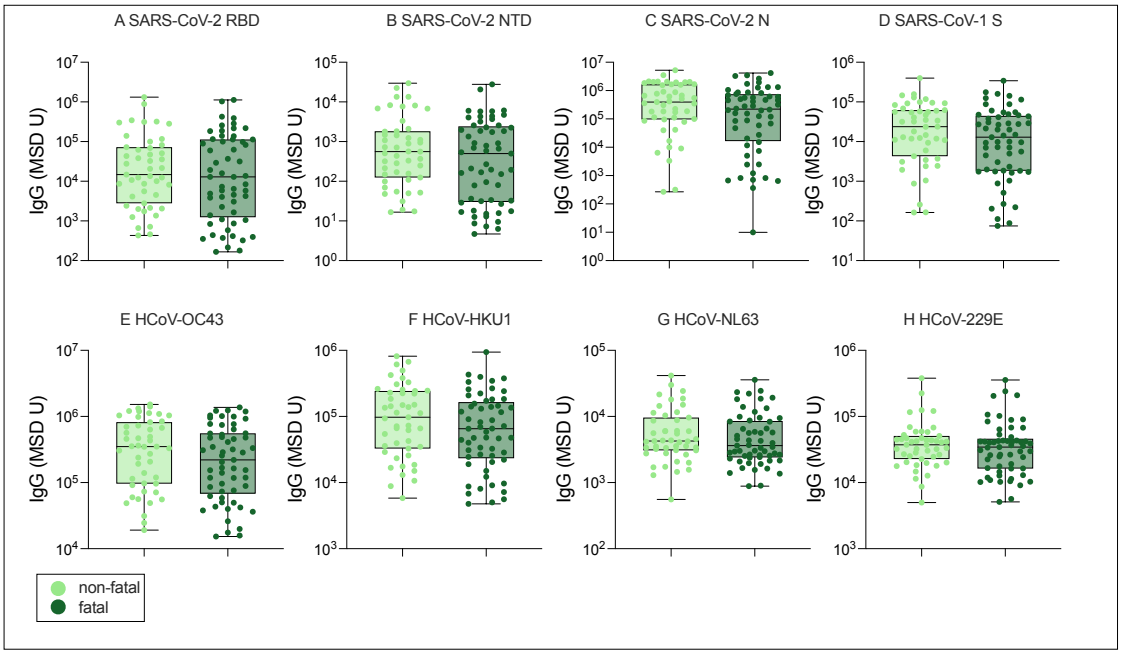


Figure 5.8: No difference in IgG responses to HCoVs between fatal and non-fatal COVID-19. IgG targeting SARS-CoV-2 RBD (A), SARS-CoV-2 NTD (B), SARS-CoV-2 N (C), SARS-CoV-1 S (D), HCoV-OC43 (E), HCoV-HKU1 (F), HCoV-NL63 (G), and HCoV-229E (H) in individuals admitted to ICU who survived (light green) and who died of COVID-19 (dark green).

that responses to HCoV-OC43 N are not boosted following SARS-CoV-2 infection.[6] This suggests anti-HCoV-OC43 N IgG may be used as a marker of prior exposure to HCoV-OC43 in the absence of baseline samples. As expected, responses to both HCoV-OC43 and HCoV-HKU1 S were significantly elevated following SARS-CoV-2 infection ($p<0.0001$ for both, Wilcoxon tests of timepoints immediately pre- and post-infection) (Figure 5.9AB). In contrast, responses to HCoV-OC43 and HCoV-HKU1 N did not increase significantly following infection and instead remained stable through time (HKU1: $p=0.16$, OC43: $p=0.98$) (Figure 5.9CD). This indicates that IgG targeting the N protein of endemic betacoronaviruses can be used as a marker of prior HCoV immunity that is not impacted by SARS-CoV-2 infection.

5.3.9 IgG responses to HKU1 and OC43 N are elevated in worse COVID-19 outcomes

In order to test the hypothesis that prior immunity to endemic HCoVs is associated with worse COVID-19 outcomes in mild disease, IgG targeting HCoV-OC43 and

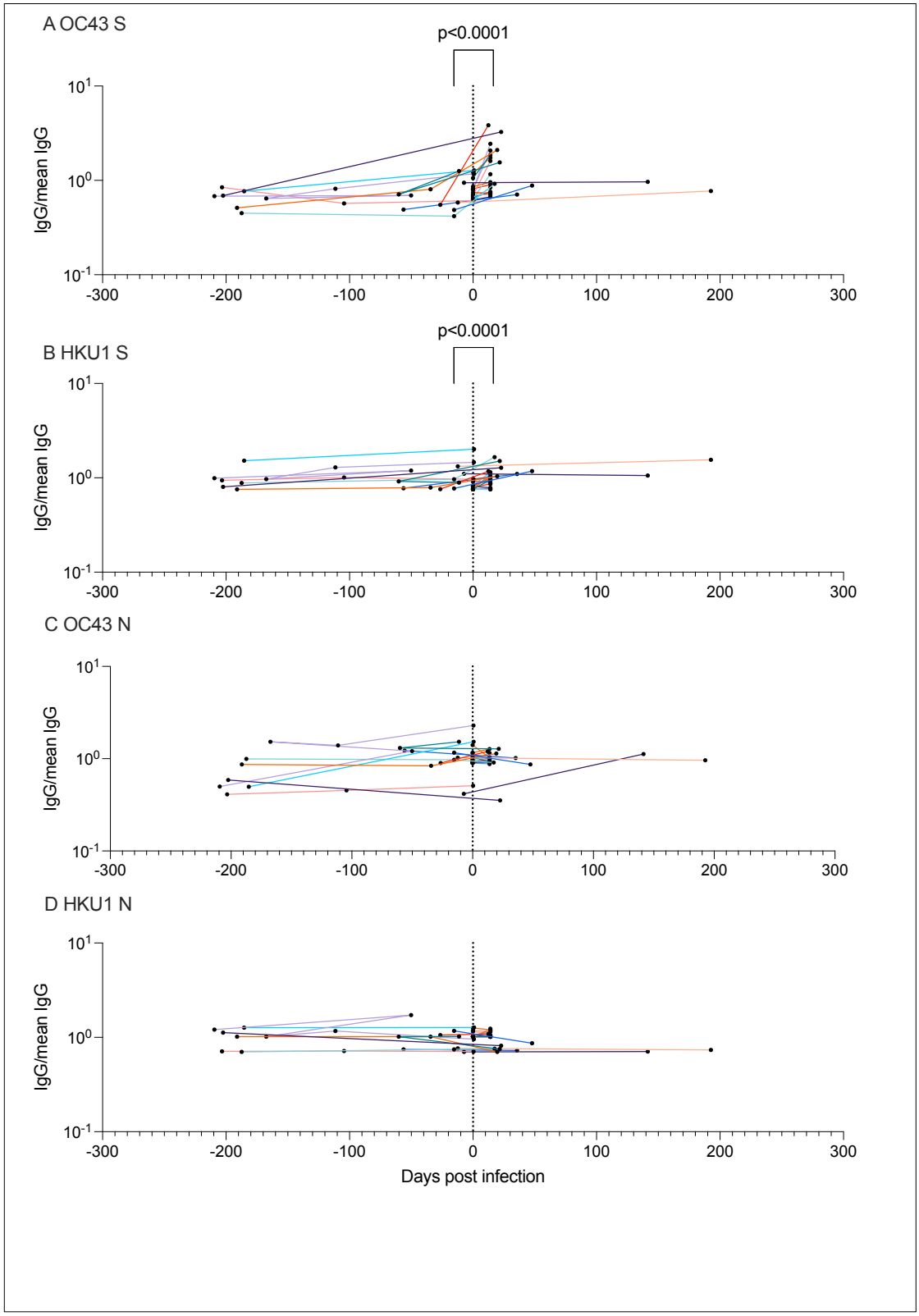


Figure 5.9: IgG responses to HCoV-HKU1 and HCoV-OC43 N are not boosted following SARS-CoV-2 infection. IgG targeting HCoV-OC43 S (A), HCoV-HKU1 S (B), HCoV-OC43 N (C), and HCoV-HKU1 N (D) in individuals serially sampled before and after SARS-CoV-2 infection. Data was standardised by dividing by the mean response for each plate to account for batch effects between plates. p-values refer to Wilcoxon test values from pairwise comparisons between data points immediately before and after infection.

HCoV-HKU1 N was compared between asymptomatic and symptomatic individuals from the family study cohort (Figure 5.10AB). Anti-N IgG was elevated in symptomatic individuals for both HCoV-OC43 and HCoV-HKU1; this increase was significant for HCoV-OC43 (p=0.003, Mann-Whitney test) but not HCoV-HKU1 (p=0.34, Mann-Whitney test). Anti-N IgG was also compared between non-fatal and fatal ICU groups for both HCoV-OC43 and HCoV-HKU1 (Figure 5.10CD). Individuals who died from COVID-19 made stronger responses to N (HCoV-OC43: p=0.03, HCoV-HKU1: p=0.01, Mann-Whitney tests). This data supports the hypothesis that prior exposure to endemic betacoronaviruses is associated with worse COVID-19 outcomes in both mild and severe disease.

Finally, to assess whether elevated IgG responses to HCoV-OC43 and HCoV-HKU1 N were associated with older age, correlations were calculated between HCoV-OC43 and HCoV-HKU1 N IgG and age for the family and ICU cohorts. For the family cohort, there was a weak positive correlation between age and IgG targeting HCoV-OC43 N (r=0.38, p=0.0001) and HCoV-HKU1 N (r=0.21, p=0.007) (Spearman's rank tests). For the ICU cohort, there was no correlation between age and IgG targeting HCoV-OC43 N (r=0.06, p=0.4) or HCoV-HKU1 N (r=0.18, p=0.07). In the family cohort, symptomatic individuals were significantly older than asymptomatic individuals (p=0.0006, Mann-Whitney test) and in the ICU cohort, individuals who died were significantly older than individuals who survived (p=0.0001, Mann-Whitney test). This indicates that the increased cross-reactive immunity in individuals with worse outcomes is age-associated in the family study cohort but not the ICU cohort.

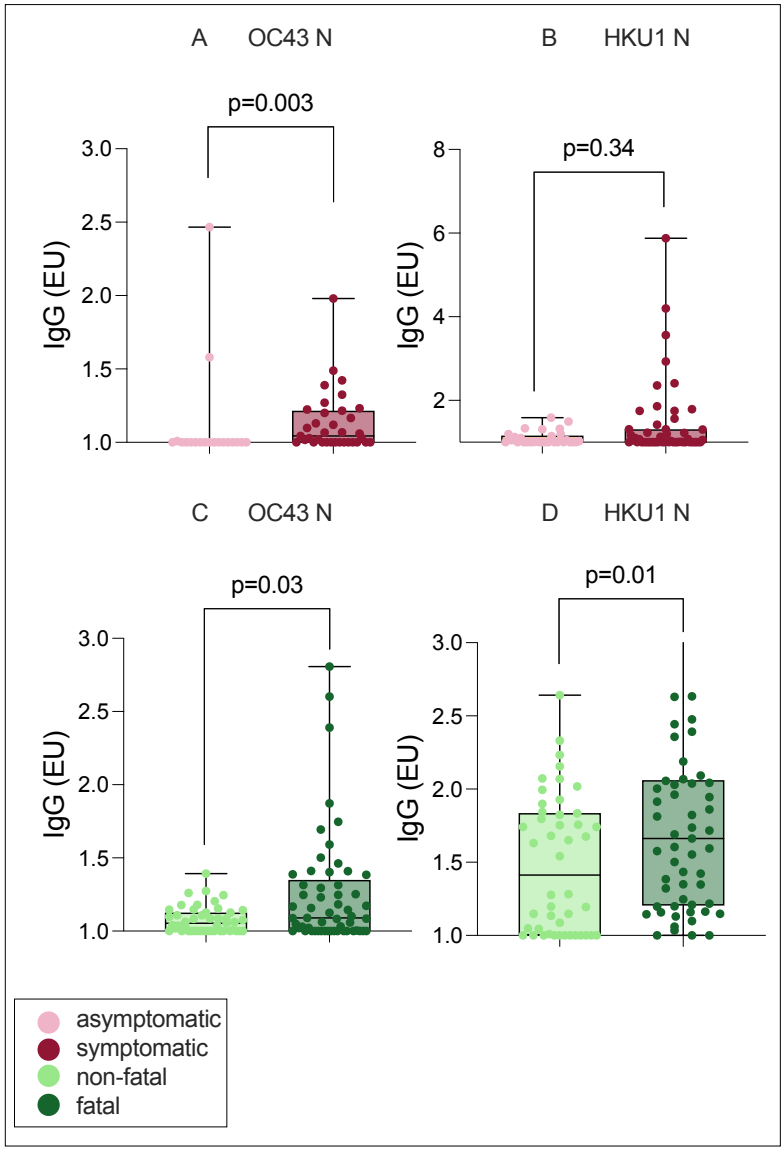


Figure 5.10: IgG responses to HKU1 and OC43 N are elevated in worse COVID-19 outcomes. IgG targeting HCoV-OC43 N (A)and HCoV-HKU1 N (B) in asymptomatic (pink) and symptomatic (maroon) individuals from the family cohort. IgG targeting HCoV-OC43 N (C) and HCoV-HKU1 N (D) in individuals from the ICU cohort who survived (light green) or died from COVID-19 (dark green). p-values refer to Mann-Whitney test values.

5.4 Discussion

OAS is an immunological concept believed to worsen disease severity through the expansion of pre-existing cross-reactive immunity. This is accepted to occur during flavivirus infection, whereas the role of cross-reactive IgG in modulating COVID-19 outcomes is contested. Here, cross-reactive IgG targeting the N antigen of endemic betacoronaviruses is associated with worse disease in two cohorts of differing disease severity.

Responses to conserved regions of SARS-CoV-2 have previously been shown to correlate with more severe outcomes.[6, 264, 265] Analysis of antibody responses in an ICU cohort found elevated responses to HCoV-OC43 S in ICU patients compared to individuals with mild or asymptomatic disease.[6] Furthermore, the authors identified an association between IgG targeting HCoV-OC43 N and fatal outcomes in the ICU cohort, as well as between fatal outcomes and IgG targeting the S2 region of S.[6] In a study of SARS-CoV-2 convalescent donors of differing disease severity, elevated S-specific IgG titres targeting SARS-CoV-1 and HCoV-OC43, but not HCoV-NL63 or HCoV-229E, were associated with higher disease severity scores.[265] The study also identified higher levels of anti-RBD IgG in patients with mild compared to severe disease, indicating that an antibody response to novel epitopes in less conserved regions of S may be protective.[265] Furthermore, boosting of S2-specific antibodies targeting HCoV-OC43 was elevated in critically ill individuals in another study.[264] An additional study identified boosting of cross-reactive IgG with poor SARS-CoV-2 neutralising capacity in COVID-19 patients with severe disease.[4] Evidence of OAS during SARS-CoV-1 infection has also been described – individuals with prior HCoV-OC43 or HCoV-229E infection who became infected by SARS-CoV-1 demonstrated boosted HCoV antibody responses but no increase in SARS-CoV-1-specific antibody.[268]

A requirement for OAS to occur is cross-reactivity of immune responses, leading to boosting of pre-existing immune memory following infection with a heterologous

virus. Antibodies that cross-react between endemic HCoVs and SARS-CoV-2 have been widely described, owing to the high homology between SARS-CoV-2 and HCoV-OC43 (69%), HCoV-HKU1 (68%), HCoV-NL63, and HCoV-229E (both 65%).[1–3] In particular, the highest areas of homology in the SARS-CoV-2 genome are the S2 domain of S, N, and several NSPs.[3] Here, we show that total anti-S IgG responses to SARS-CoV-1, MERS-CoV, and endemic betacoronaviruses HCoV-OC43 and HCoV-HKU1 are boosted in SARS-CoV-2 seropositive individuals and remain elevated months after infection. This is in accordance with published literature stating that, as well as boosting pre-existing T-cell immunity, infection with SARS-CoV-2 promotes the production of cross-reactive IgG. In a previous study, two- to four-fold increases in the level of HCoV-specific IgG, and 11- to 123-fold increases in SARS-CoV-1- and MERS-CoV-specific IgG, respectively, were observed in SARS-CoV-2 convalescents compared to pre-pandemic samples, preferentially targeting the S2 domain of S.[269] In another study, up to 50% of COVID-19 convalescents generated IgG responses to HCoV-OC43 epitopes that were not present in pre-pandemic controls.[270]

The lack of boosting of IgG targeting alphacoronaviruses HCoV-NL63 and HCoV-229E is unexpected in this study due to their high homology. In Grobben *et al.* (2021), IgG targeting HCoV-229E and HCoV-NL63 S proteins was significantly elevated in COVID-19 convalescents compared to pre-pandemic controls as measured by a Luminex assay.[269] However, in McNaughton *et al.* (2022), there was no difference in HCoV-229E or HCoV-NL63-specific IgG between uninfected individuals, SARS-CoV-2-infected individuals, asymptomatic individuals, or patients admitted to ICU, as determined by the same MSD v-plex assay used in this chapter.[6] In Grobben *et al.* (2021), patients were recruited four to six weeks post symptom onset, whereas in McNaughton *et al.* (2022), patients with mild disease were recruited during routine blood donation, and ICU patients were recruited during acute disease. The lack of HCoV-229E or HCoV-NL63 boosting described herein may be the result of waning of antibody responses in convalescence, or due to recent circulation of

these HCoVs in seronegative individuals that led to already elevated IgG titres in SARS-CoV-2 seronegative individuals.

Another requirement to demonstrate that OAS may be occurring is a suboptimal immune response following secondary infection, associated with worse disease outcomes. Here, there was an association between IgG responses to S2, but not RBD, and symptomatic disease, indicating that preferential targeting of conserved S epitopes is associated with worse outcomes following SARS-CoV-2 infection. Furthermore, IgG targeting the S protein of HCoVs was elevated in symptomatic individuals in the family cohort, further supporting this hypothesis. Alternatively, it could be concluded that this difference between asymptomatic and symptomatic individuals is due to overall greater humoral responses during symptomatic disease, due to enhanced inflammation and immune activation. However, the lack of a significant difference between IgG targeting SARS-CoV-2 RBD or total S between asymptomatic and symptomatic individuals, as well as the lack of a difference in nAb titre between asymptomatic and symptomatic individuals shown in Chapter 3, makes this conclusion less likely.

In the ICU cohort described herein, we identified no difference in the magnitude of IgG targeting the S protein of endemic HCoVs or SARS-CoV-2 between fatal and non-fatal groups, in accordance with McNaughton *et al.* (2022) and Wratil *et al.* (2021).[6, 264] This may be due to the hyperinflammation and immunopathology characteristic of severe COVID-19.[271, 272] Additionally, the impact of cross-reactive IgG may be relevant early on in disease course, but by the time patients are experiencing severe disease in ICU, nuances in the levels of pre-existing immunity to cross-reactive antigens such as S may become impossible to decipher.

For the reason outlined above, in the absence of baseline samples, it is critical to have a marker of prior immunity that is not impacted by the immune hyperactivation observed during severe COVID-19. The possibility of using HCoV-OC43 N as this

marker was highlighted by McNaughton *et al.* (2022).[6] Here, neither IgG responses to HCoV-OC43 nor HCoV-HKU1 N increased following SARS-CoV-2 infection in two cohorts of serially sampled individuals, supporting the use of both as markers of prior immunity to endemic HCoVs. N is a helical protein that binds the RNA genome of SARS-CoV-2 to form the ribonucleoprotein complex. This maintains the conformation of the RNA for transcription and replication.[135] N is involved in regulation of transcription, the cell cycle, apoptosis, and structural dynamics, and is expressed early in the viral replication cycle.[273] Cross-reactivity between HCoV-OC43 N and SARS-CoV-1 N has been reported to the extent that false-positive results in a SARS-CoV-1 ELISA were identified.[274] It is therefore unclear why IgG targeting HCoV-HKU1 and HCoV-OC43 N is not boosted significantly following SARS-CoV-2 infection. This could be due to N not being expressed on the external virion surface and therefore only being visible to the cellular immune system after cross-presentation by APCs. Additionally, mAbs targeting SARS-CoV-1 N that lack cross-reactivity with HCoV-OC43 and HCoV-229E have been described.[273]

Elevated IgG responses to HCoV-HKU1 and HCoV-OC43 N were identified in individuals with worse disease outcomes in both cohorts assayed here, supporting a model whereby prior, recent exposure to endemic betacoronaviruses are associated with worse COVID-19 disease, irrespective of the SARS-CoV-2-specific immune response. Higher IgG responses to HCoV-OC43 N have been identified in ICU patients with fatal COVID-19 outcomes.[6] In contrast, another study sampling individuals of varying disease severity during acute COVID-19 identified individuals without anti-HCoV-OC43 N antibodies as having an elevated risk of critical disease.[275] An additional study identified an association between anti-HCoV-229E and anti-HCoV-NL63 N IgG and worse outcomes,[154] whilst a study of infection susceptibility in HCWs identified a lower risk of infection in individuals with high anti-HCoV-OC43 N IgG.[276] The discordancy in findings may result from studies at different stage of disease: pre-infection studies of susceptibility and risk, studies of acute infection, and studies of convalescent individuals as described here.

Furthermore, the findings from these studies are likely confounded by factors such as age and pre-existing health conditions that also contribute to worse COVID-19 outcomes.

A confounder for this analysis is the effect of age on disease severity and exposure to endemic betacoronaviruses. In a study of HCoV seroprevalence among individuals with respiratory illness in Scotland, prevalence of anti-HCoV IgG was highest in children and the elderly.[277] Another studied identified highest HCoV infection frequency in children under five compared to 6-11, 12-17, 18-49, or over 50 age groups.[278] However, in both studies, infection with HCoV-OC43 was highest in children, whereas infection with HCoV-229E was highest in the elderly.[277, 278] In addition, one study identified elevated levels of cross-reactive SARS-CoV-2 S IgG in pre-pandemic samples of children compared to adults.[228] Here, individuals with worse outcomes were significantly older in both cohorts, and in the family cohort only, HCoV-HKU1 and HCoV-OC43 N IgG was correlated with age. However, in the ICU cohort, there was no correlation between HCoV N IgG and age. This suggests that the elevated levels of cross-reactive antibodies in individuals with fatal outcomes is likely not a result of the confounding effects of age in the ICU cohort. However, in the family cohort where a greater range of ages were sampled (family cohort: 6 to 88 years, standard deviation: 19.4; ICU cohort: 26 to 87 years, standard deviation: 10.5), older individuals were more likely to be symptomatic and also to have elevated cross-reactive immunity. One interpretation is that higher cross-reactivity leading to OAS may promote worse outcomes in older individuals. Another interpretation is that older individuals have higher levels of cross-reactive memory B-cells and are also predisposed to more severe disease for other reasons.

The findings from this chapter support a potential model for OAS in SARS-CoV-2 infection. The mechanism of this OAS is unclear, but one hypothesis can be put forward: in individuals with pre-existing cross-reactive immunity to endemic HCoVs, infection with SARS-CoV-2 activates B-cell memory responses, leading

to the rapid production of cross-reactive antibodies targeting conserved epitopes such as those in the S2 region. These may be poorly neutralising and unable to efficiently control viral replication, enabling disease to occur. Eventually, *de novo* antibody responses targeting novel epitopes in the RBD are generated and control infection, but these appear later when infection has already gained a foothold (Figure 5.11A). In contrast, individuals who have not been recently exposed to endemic HCoVs generate a *de novo* response to SARS-CoV-2 upon exposure, which is more neutralising and capable of controlling replication sooner. This leads to asymptomatic or less severe disease (Figure 5.11B). The temporal element of this model explains why IgG targeting SARS-CoV-2 RBD is not significantly different between asymptomatic and symptomatic individuals: both groups generate anti-RBD IgG eventually, but the delay in this response mediated by expansion of B-cell memory in individuals with pre-existing immunity enable worse disease to occur. The timing of immune control of SARS-CoV-2 appears to be critical to the manifestations of COVID-19.[279] Further studies of the temporal dynamics of different B-cell subsets are required to test this hypothetical mechanism.

When making comparisons between adaptive immune responses and disease severity, it is important to consider the limitations of only assessing immunity at one timepoint, as was done for the family study cohort and the ICU cohort. Having only a snapshot of adaptive immunity, rather than assessing dynamics longitudinally throughout infection, enables only a limited understanding of how severity and adaptive immunity interact. It has been widely reported that SARS-CoV-2 antibodies are induced within 4-5 days after symptom onset, and that they are often higher in individuals with more severe disease. In addition, early detection of SARS-CoV-2-specific T-cells is associated with milder disease and rapid viral clearance.[179] However, in the family and ICU cohorts, it is difficult to establish causation between adaptive immunity and disease severity in the absence of longitudinal kinetic studies. For example, the elevated HCoV-specific IgG in more severe disease shown in this chapter may be the result of individuals who

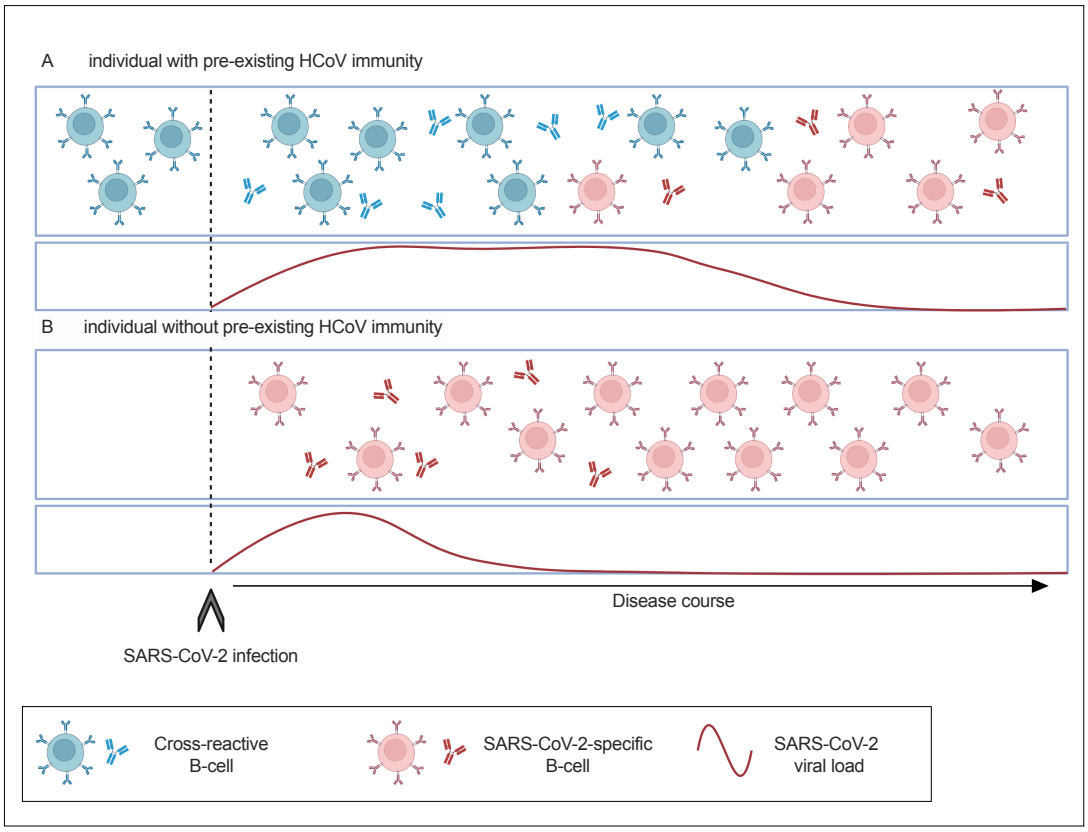


Figure 5.11: A model for antibody dynamics of OAS in COVID-19. Individuals with pre-existing cross-reactive immunity to endemic HCoVs generate a memory B-cell response upon SARS-CoV-2 infection, with *de novo* responses appearing later (A). Individuals lacking cross-reactive immunity generate a *de novo* response to SARS-CoV-2 more rapidly, enabling viral clearance sooner (B).

make poor early immune responses to both HCoVs and SARS-CoV-2, leading to increased susceptibility to infection, more severe disease, and higher levels of immunopathology later in infection. This might then result in prolonged levels of antigen exposure and ultimately higher levels of specific antibody later in disease course. This provides an alternative explanation for the data described in this chapter.

A key next question in understanding cross-reactive immunity to SARS-CoV-2 and HCoVs is the impact of cross-reactivity on future infections with SARS-CoV-2 VOCs as well as booster vaccinations. Sequential SARS-CoV-2 infections have been studied in both unvaccinated and vaccinated cohorts.[137, 280] Protection

against reinfection with the Omicron variant in household members in South Africa was conferred by initial infection with Beta or Delta, and where reinfections did occur, viral shedding was shorter in duration and of lower magnitude.[137] However, one study by Quandt *et al.* (2022) assessed B-cell memory in individuals infected with Omicron BA.1 after two or three doses of the Pfizer-BioNTech (BNT162b2) COVID-19 vaccine. Omicron infection led to expansion of B-cells specific for S and RBD from the Wuhan strain, Alpha, Delta, and Omicron BA.1, but not Omicron BA.4 or BA.5. The B-cell response was primarily a memory response with expansion of B-cells specific for conserved epitopes shared among VOCs.[281] Similarly, a study of Omicron BA.1 breakthrough infection in double- or triple-vaccinated individuals showed that infection enhanced neutralisation responses to both Omicron and Wuhan strains. Furthermore, most antibodies had higher affinities for the Wuhan RBD than the Omicron BA.1 RBD.[282] Vaccination with the ancestral SARS-CoV-2 strain followed by infection with Omicron B.1.1.529 generated enhanced immune responses to the ancestral strain but not to Omicron, suggesting OAS mediated by prior exposure to SARS-CoV-2 VOCs.[283]

Another, more optimistic implication of cross-reactive humoral immunity is the potential to develop broadly reactive vaccines against pathogens where antigenic diversity is a barrier to eradication. Studies identifying cross-reactive antibodies that recognise not only all influenza A viruses but also influenza B viruses make this appear possible for influenza – indeed, the development of a universal influenza vaccine remains a global goal.[235, 284, 285] Seasonal vaccines for influenza target the head region of HA, though these vaccines are strain-specific. In contrast, the stem region of the HA molecule is highly conserved and therefore a target for broadly neutralising cross-reactive immunity. Immunisation with an avian H5N1 virus vaccine induces broadly cross-reactive HA stem-specific antibodies,[239] and several novel vaccine technologies are currently being employed to promote the production of cross-reactive antibodies targeting multiple influenza strains.[286] Production of vaccines that elicit broadly reactive antibodies against EBOV species

is also a goal following the isolation of cross-reactive antibodies from survivors of filoviral infections.[287] In addition, universal vaccines against flaviviruses and pan-DENV vaccines are in development.[288] However, efforts to develop such broadly protective vaccines are hamstrung by the complexities of heterosubtypic immunity: in particular, the adverse effects of prior exposure to related viruses such as ADE and OAS.[251, 289] An example of this is Dengvaxia, a vaccine manufactured by Sanofi Pasteur against DENV that failed its primary efficacy endpoint in a paediatric Phase II trial in Thailand. Although effective in DENV-seropositive individuals, the vaccine appeared not to reduce rates of infection and to even enhance disease in DENV-seronegative children.[290] Several explanations for this result have been offered, including a suboptimal cellular immune response, or the mimicking of a primary infection leading to ADE,[291] which has been demonstrated in mouse models.[292]

Whether a universal coronavirus vaccine is possible is as yet unknown. Several pan-coronavirus vaccines are in development, such as 'variant-proof' SARS-CoV-2 vaccines, pan-sarbecovirus vaccines, and pan-betacoronavirus vaccines, with some early evidence of immunogenicity.[293] Studies of vaccination against multiple VOCs of SARS-CoV-2 can offer some insight into whether multivalent vaccination can protect against multiple SARS-CoV-2 VOCs or indeed multiple different coronaviruses. Administration of bivalent booster vaccines appear to increase neutralising responses to ancestral strains to a greater extent than novel VOCs.[294] Single-cell transcriptomics of vaccine-induced immunity provided evidence that a single dose of BNT162b2 activated a weakly neutralising memory response targeting the more conserved S2 region of S, whereas a second dose of vaccine promoted neutralising responses to S1 and RBD.[295] However, OAS in the context of vaccination does not appear to increase mortality from COVID-19,[296] and therefore its clinical importance is yet unclear.

The aim of this chapter was to investigate the relationship between humoral

immunity to endemic HCoVs and COVID-19 disease severity. The data presented herein demonstrates an association between cross-reactive antibody responses to endemic betacoronaviruses and worse disease severity in two clinically different cohorts. This may interact with age, as older age is a risk factor for worse COVID-19 outcomes. Demonstrating an association between disease severity and prior immunity to endemic HCoVs is made possible through the use of the N antigen which is not boosted by SARS-CoV-2 infection. Future research will be necessary to test the hypothetical mechanism of this association, and to examine the clinical consequences of OAS in the context of seasonal vaccination against and reinfection with SARS-CoV-2.

6

Immune responses to SARS-CoV-2 vaccination in adolescents

Contents

6.1 Abstract

Sex- and age-specific immune differences have a wide impact on outcomes from infections and immunisations. Typically, adult females make stronger immune responses and have better disease outcomes but suffer more adverse events following vaccination and are more prone to autoimmune disease. To better understand the mechanisms underlying these differences in vaccine responses, we studied immune responses to two doses of BNT162b2 in an adolescent cohort (n=34, ages 12-16). At the same time, we evaluated immune responses to a co-administered influenza vaccine, which has been shown to induce stronger immune responses in females. As previously demonstrated, total IgG responses to SARS-CoV-2 S antigens were significantly higher among vaccinated adolescents compared to adults (32-52 years of age) who received BNT162b2. However, unexpectedly, antibody responses to BNT162b2 and the influenza vaccine were not higher among female adolescents compared to males; among infection-naïve adolescents, antibody responses to BNT162b2 were higher in males than females. No sex difference was identified in vaccinated adults. These unexpected findings may result from the introduction of novel mRNA vaccination platforms, generating patterns of immunity divergent from established trends.

6.2 Introduction

Aim: Examine the immune response to SARS-CoV-2 and influenza following vaccination in UK adolescents.

The race to rapidly develop, trial, and manufacture effective vaccines against COVID-19 has been facilitated by novel, scalable vaccine platforms, widespread transmission of SARS-CoV-2 enabling early estimates of efficacy, and a dedicated global effort. Numerous COVID-19 vaccines have now been developed and licensed, both targeting the original Wuhan strain of SARS-CoV-2 as well as monovalent and bivalent vaccines targeting novel VOCs. In the UK, over 151 million vaccine doses have been administered.[56] COVID-19 vaccines approved for administration in the UK include the Moderna (mRNA-1273) mRNA vaccine, the Novavax (NVX-COV2373) recombinant vaccine, the Sanofi GlaxoSmithKline (VidPrevtyn Beta) recombinant vaccine, the Oxford-AstraZeneca ChAdOx1 (AZD1222) viral-vectored vaccine, the Janssen (Ad26.COV2-S) viral-vectored vaccine, and the Pfizer-BioNTech Comirnaty (BNT162b2) mRNA vaccine.[297]

As of April 2023, in Europe, the most highly administered vaccines were BNT162b2 (∼665 million doses, 73%), mRNA-1273 (∼150 million doses, 17%), and ChAdOx1-S (∼67 million doses, 7%) of a total of ∼905 million doses.[298] mRNA-1273 is a lipid nanoparticle-formulated RNA vaccine containing a prefusion stabilised full-length SARS-CoV-2 S protein. The flexibility and scalability of mRNA vaccine platforms enabled rapid design and production of mRNA-1273 in early 2020. Early Phase III trials of mRNA-1273 efficacy reported low reactogenicity and no vaccine-enhanced disease with some common adverse events including fatigue and headache. Overall efficacy in this trial was calculated as 94.1% for prevention of symptomatic disease and 100% for prevention of severe disease or death.[299] A study of 3,732 adolescents (12-17 years) who received mRNA-1273 demonstrated noninferior nAb responses compared to young adults (18-25 years) and an estimated efficacy of 93.3% two weeks after the second dose.[300] mRNA-1273 efficacy after the second dose in

children 6-11-years-old was estimated at 88%.[301]

AZD1222, manufactured by AstraZeneca and the University of Oxford, is a viral vectored vaccine employing a replication-deficient chimpanzee adenovirus vector expressing full length SARS-CoV-2 S.[302] Viral vectored vaccines are strong inducers of cellular as well as humoral immunity, making them a promising early candidate for preventing COVID-19, a disease for which the development of T-cell immunity following infection is widespread and believed to be protective.[1, 302] Phase I/II trial data displayed strong T_H1 type immunity and IgG production after one dose of AZD1222.[302] Later phase III trials identified an overall vaccine efficacy of 74% with low reactogenicity.[303] Following a concerning safety signal associated with venous thrombosis and thrombocytopaenia following AZD1222 vaccination in some individuals, AZD1222 is no longer offered in the UK.[304, 305] However, the pricing of AZD1222 at cost resulted in a step forward for vaccine equity that enabled this vaccine to save more lives from COVID-19 than any other vaccine in its first year of circulation.[306]

Finally, BNT162b2 is another lipid nanoparticle-formulated nucleoside-modified RNA vaccine encoding the full-length S protein of SARS-CoV-2.[307] BNT162b2 contains two proline mutations to force it into the prefusion conformation of S, which more closely resembles intact SARS-CoV-2.[308] Phase III trials of BNT162b2 efficacy identified 95% protection against disease in individuals over 16 years of age, with similar safety profiles to other licensed vaccines.[307, 309] Cumulative COVID-19 case incidence diverged 12 days after the first dose, and efficacy between the two doses was 52%, later increasing to 91% in the first week after the second dose and 95% at least seven days after the second dose.[307] In children 5-11 years old, two 10µg doses of BNT162b2 administered 21 days apart generated no serious adverse events, with similar nAb titres in 5-11-year-olds compared to 16-25-year-olds (ratio: 1.04, 95% CI: 0.93-1.18).[310] Estimated efficacy against COVID-19 7 days after the second dose was 90.7%.[310] Another study of BNT162b2 immunogenicity and

efficacy in 12-15-year-olds was undertaken by Frenck *et al.* (2021).[311] 2,260 SARS-CoV-2-infection-naïve adolescents received two doses of either 30µg BNT162b2 or placebo (saline) at a 21-day-interval. Reactogenicity was mostly mild to moderate including headache, fatigue and fever, and there were no vaccine-related serious adverse events reported. Efficacy against COVID-19 was 100%.[311]

Vaccination in UK children and adolescents began in late 2021. BNT162b2 was authorised for 12-15-year olds in June 2021 in the UK as a 30µg one-dose regimen by the Medicines and Healthcare Products Regulatory Agency.[56] This was extended to a two-dose regimen in early 2022. By July 2022, 62.4% of 12-15-year-olds and 80.5% of 16-17-year-olds had received one or more doses, and 45.3% and 69.8%, respectively, had received two or more doses.[312] The rate of SARS-CoV-2 infections was significantly lower in schools where first-dose uptake exceeded 60% compared to schools where 0-20% of pupils were vaccinated.[312]

In the UK, the first dose of BNT162b2 was often administered in adolescents alongside the intranasal seasonal live-attenuated influenza vaccine (LAIV) Fluenz Tetra, manufactured by AstraZeneca. This vaccine is believed to induce broader influenza-specific immunity than inactivated virus vaccines, and has been demonstrated to generate stronger protection against infection in children compared to the trivalent inactivated influenza vaccine,[313] with an estimated 83% efficacy against disease in six-month-olds to seven-year-olds.[314, 315] During the 2019-2020 influenza season in Europe, when influenza A (H1N1)pdm09, A(H3N2), and B/Victoria (Washington) were co-circulating, the LAIV had an efficacy of 68% against infection with any influenza strain in children aged two to six years old.[316] In the 2021-2022 influenza season, the following lineages were contained in the LAIV: A/Victoria/2570/2019 (H1N1)pdm09-like virus, A/Cambodia/e0826360/2020 (H3N2)-like virus, B/Washington/02/2019 (B/Victoria lineage)-like virus, and a B/Phuket/3073/2013-like virus.[317] Influenza A (H3N2) dominated in the 2021-2022 season in the UK, with some B/Victoria and A(H1N1)pdm09 in 2022.[318]

Studying the immunogenicity of BNT162b2 and the LAIV in adolescents was of particular interest due to the substantial effects of ageing on immune responses to infection and vaccination. Vaccine efficacy is often lower in the elderly – influenza vaccination, for example, reduces in efficacy from 70-90% in adults (17-59 years) to 17-53% in the elderly (58-104 years), and induces a lower magnitude antibody response in the elderly.[319] Responses to HBV and pneuomococcal vaccines are also reduced in the elderly, and antibody durability decreases with age.[320] These patterns are likely a result of the sequelae of immune age-related changes collectively known as immunosenescence: fewer naïve lymphocytes, impaired innate immune responses, and increased numbers of dysfunctional memory cells due to chronic stimulation with antigens such as CMV.[321] Infants also generate weak immune responses to vaccination;[320, 322] antibody responses to measles vaccination were stronger in children over 15 months old than children six months old,[323] although seroconversion rate following LAIV administration was higher in five-to-eight-year-olds than nine-to-17-year-olds or 18-49-year-olds (68%, 64% and 47%, respectively).[324] These findings suggest that immune responses may be strongest in childhood compared to early infancy, adulthood and old age.

Older age is also a primary risk factor for severe COVID-19, perhaps due to the persistent inflammation and cellular dysfunction characteristic of immunosenescence.[325] The majority of young people experience mild COVID-19; severe disease and multisystem inflammatory syndrome only occurs in a minority of paediatric patients.[326] Adolescents and children display rapid and adaptable immune responses that may contribute to improved resolution of infections, such as abundant IgM memory B-cells, broad and rapidly produced natural antibodies, and lower inflammatory cytokine responses.[76, 96] Differential COVID-19 outcomes between adults and children may also be influenced by pre-existing immune responses to endemic coronaviruses that might circulate at higher levels in children.[76] COVID-19 vaccine efficacy is also reduced in the elderly.[327, 328] In addition, adolescents

between 12 and 15 years of age generate 1.76-fold higher IgG responses to BNT162b2 than 16-25-year olds, indicating either potential age-related changes in immune response even during adolescence, or an increase in cross-reactivity with endemic coronaviruses that enhances vaccine responsiveness and declines with age.[311] Finally, older individuals are more likely to have immunodeficiencies or chronic diseases which increase their risk of severe COVID-19.

In addition to age, understanding the role of sex in immune response is crucial for developing more effective vaccines. Adult females often generate stronger immune responses following viral infection than males.[101] In addition, females generate greater antibody responses to vaccines,[98] and are more at risk for serious adverse events (SAEs) after vaccination.[98, 329] In one study, adult females given half-dose influenza vaccine made marginally stronger antibody responses compared to age-matched males who received full-dose vaccine.[100] The prevalence of IgG specific for measles, mumps, and rubella following vaccination was higher in females in one cross-sectional study of vaccinated adolescents.[330] Furthermore, adult females consistently generate stronger responses to Hepatitis A vaccination compared to males.[331] Local and systemic reactions to influenza vaccination are also more commonly reported in females.[332] Greater vaccine-induced immune responses in females could potentially facilitate reduced dosing regimens for females, which may minimise incidence of SAEs, improve vaccine uptake, and improve vaccine supply.

Adult males are at increased risk of death from COVID-19 than females.[103] This may result from differential regulation of ACE2 expression by sex hormones, increased innate sensing of SARS-CoV-2 in females due to enhanced TLR7 expression owing to its location on the X chromosome, or the stronger adaptive immune responses mounted by females.[103] There has been no reported sex difference in humoral or cellular immune responses to COVID-19 vaccines.[60, 121, 333] However, adolescent males experience more vaccine-induced myocarditis after BNT162b2, suggesting that immune responses to mRNA vaccines may be differentially influenced

by sex.[163, 334] Additionally, a meta-analysis of vaccine efficacy by sex identified higher efficacy of COVID-19 vaccines in males compared to females.[335] Adolescents undergoing puberty face significant changes in levels of sex hormones such as testosterone and oestrogen, which are known to modulate immunity to SARS-CoV-2 and influenza.[104, 107]

To explore sex- and age-specific differences in humoral and cellular immunity to BNT162b2, we studied adolescent and adult cohorts in the UK who received this vaccine. Data collected from adolescents in this study was compared to the PITCH cohort of vaccinated HCWs, who received two doses of BNT162b2 and also represented a mixture of previously-infected and infection-naïve individuals.[121] We explored age-specific effects on immunity within the adolescent cohort as well as between adolescents and adults. Furthermore, we examined whether sex-specific immune effects were evident. As not all adolescents also received the LAIV, we were also able to assess whether co-administration of LAIV appeared to influence the magnitude of response to BNT162b2. Finally, many studies of adolescent responses to BNT162b2 have used prior SARS-CoV-2 infection as an exclusion criterion.[311, 336] Here, we enrolled both SARS-CoV-2 infection-naïve and previously-infected adolescents to understand the role of prior or ongoing infection in vaccine response.

6.3 Results

6.3.1 Cohort and study design

In November and December of 2021, 34 adolescents aged between 12 and 16 were recruited onto the study through their enrolment at schools in Oxford, UK (Figure 6.1A). All 34 individuals received the first dose of BNT162b2; 26 (76%) also received the LAIV on the same day as the first dose of BNT162b2. 47% of individuals (n =16) were female, and the median age was 14.1 years (12.2-16) (Figure 6.1B). 33 individuals were Caucasian, one individual was Asian. No individuals were on any regular medication.

For the BNT162b2 vaccination (dose 1 [V1] and dose 2 [V2]), patients received 30μg of vaccine intramuscularly. LAIV was administered immediately after V1 only; patients received 0.1mL intranasally in both nostrils. Whole blood samples from all 34 individuals were taken immediately before V1 (sample pre-V1). Samples from all 34 individuals were taken a mean of 37 days after V1 (33-39) (sample post-V1), from 23 individuals 2 days before V2 (0-8) and 96 days after V1 (81-114) (sample pre-V2), and from 14 individuals 35 days after V2 (30-40) (sample post-V2). All whole blood samples were processed the same day as collection as described in the methods. All serum samples were tested for anti-S and anti-N IgG; individuals were classified as seropositive if their anti-N IgG titre was above the previously determined MSD immunoassay cut-off at any point in the study or if their anti-S IgG titre was above the cut-off pre-V1.[60]

The adult cohort to which adolescent data was compared was the PITCH cohort of vaccinated HCWs.[60, 121] This cohort consisted of 589 adults aged 32-52 who had received two doses of BNT162b2 28 days apart. Here, MSD IgG data from 170 adults and MSD nAb data from 10 adults was used to compare to data from adolescents.

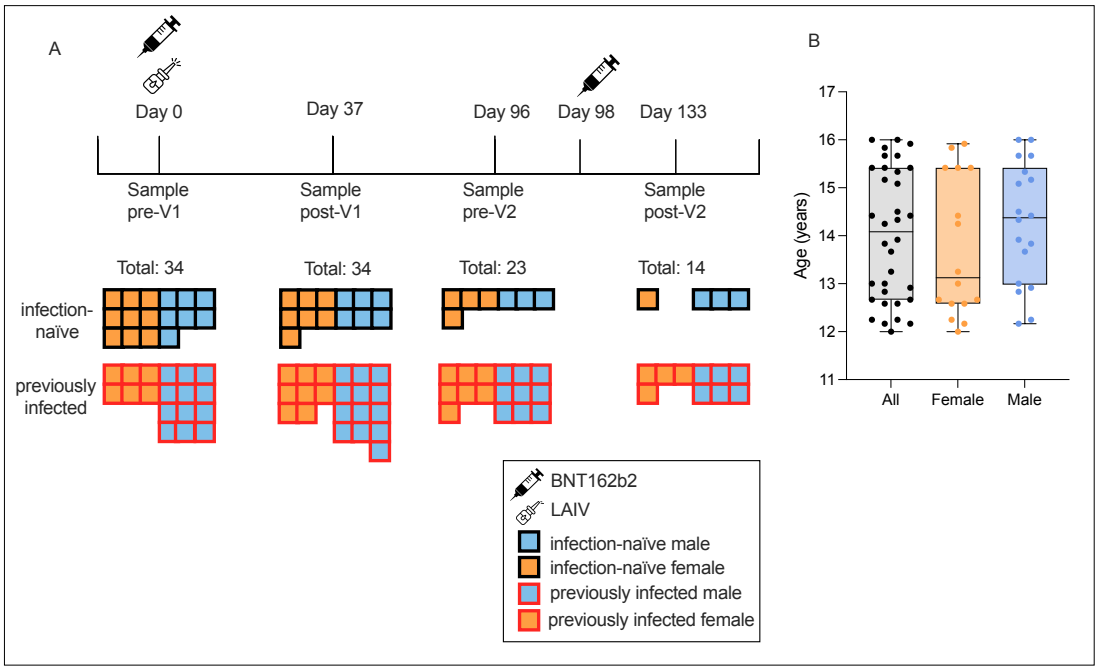


Figure 6.1: Adolescent cohort. Timeline of dosing and number of individuals at each timepoint (A). Distribution of ages by sex (B).

6.3.2 Humoral immune responses to BNT162b2 vaccination

To evaluate the immunogenicity of the BNT162b2 vaccine among adolescents, humoral responses to SARS-CoV-2 were first characterised using MSD-platform immunoassays to measure quantitatively the total IgG responses to SARS-CoV-2 S, RBD, and N (Figure 6.2A). Both infection-naïve and previously-infected adolescents made significantly greater IgG responses to S post-V1 than pre-V1 ($p=0.0005$ and $p<0.0001$, respectively, Wilcoxon signed-rank test) and greater anti-RBD IgG responses ($p=0.0005$ and $p<0.0001$, respectively, Wilcoxon signed-rank test) (Figure 6.2A). Anti-S and RBD IgG responses increased post-V2 in all groups, but only anti-RBD IgG increased significantly and only in previously-infected individuals ($p=0.008$, Wilcoxon signed-rank test). Notably, two doses of BNT162b2 in infection-naïve individuals gave similar levels of IgG to one dose of vaccine in previously-infected individuals.

Since nAbs as well as total IgG are reported to be a correlate of protective immunity against symptomatic COVID-19,[337] a surrogate of nAb activity was assessed using

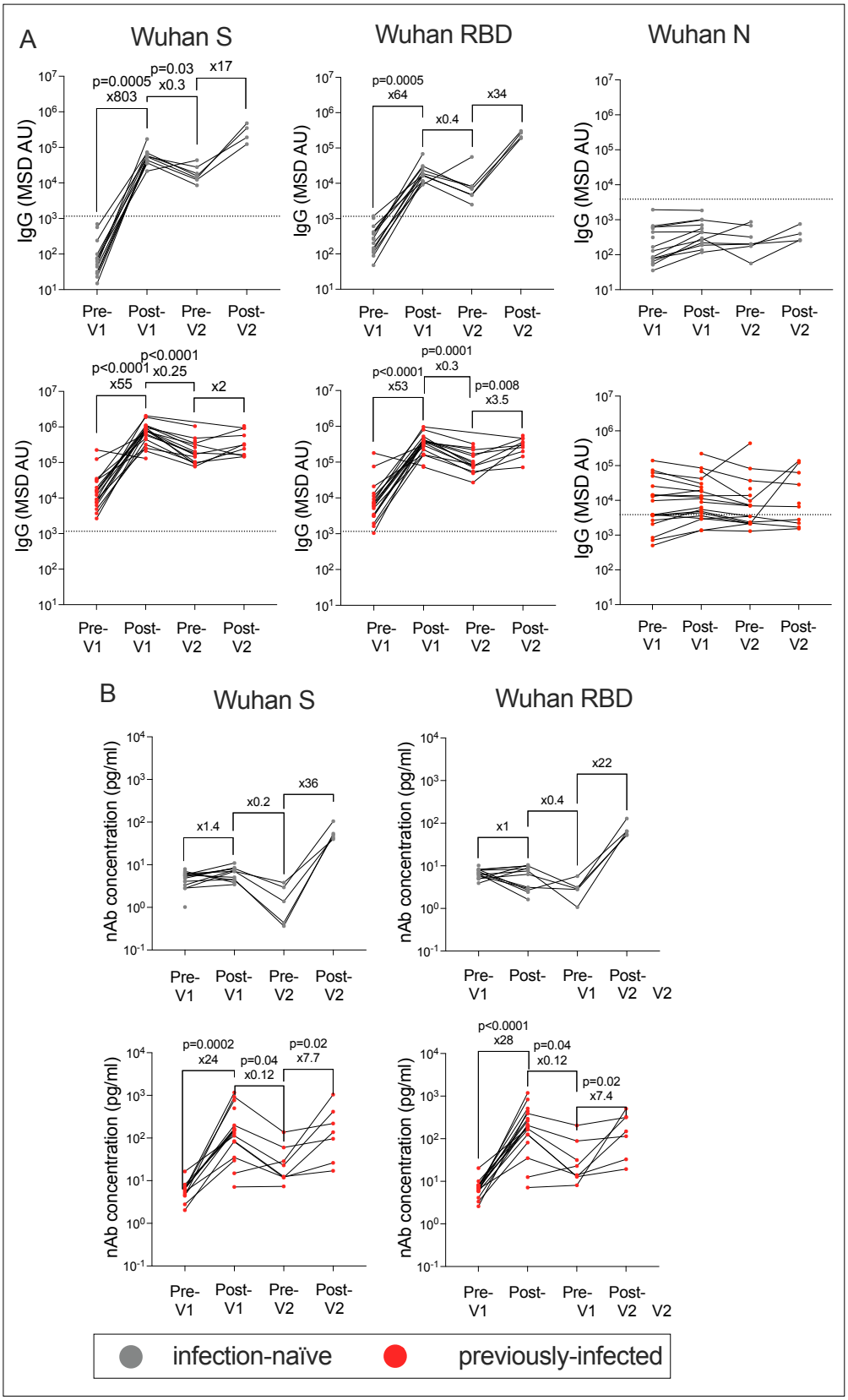


Figure 6.2: Humoral responses to SARS-CoV-2 antigens. IgG targeting SARS-CoV-2 S, RBD, and N in infection-naïve (grey) and previously-infected (red) individuals (A). nAb responses to S and RBD in infection-naïve and previously-infected individuals (B). p-values refer to Wilcoxon signed-rank test values. Dotted lines show IgG cutoffs for seropositivity. [60]

the MSD-platform ACE2 inhibition assay,[338] which is well correlated with live virus neutralisation assays.[59, 60, 121] In contrast to IgG responses, only previously-infected individuals generated increased nAb responses to both S and RBD following the first dose of vaccine ($p=0.0002$ and $p<0.0001$, respectively, Wilcoxon signed-rank test) (Figure 6.2B), and fold change in nAb response to S and RBD was higher in previously-infected individuals post-V1 compared to infection-naïve individuals ($p<0.0001$ and $p=0.0002$, respectively, Mann-Whitney tests) (Figure 6.3). After two doses of BNT162b2, infection-naïve individuals reached similar nAb titres to previously-infected individuals after one dose, supporting the idea that two doses of vaccine are required for a robust neutralising response in infection-naïve individuals.

To determine how breadth of nAb response to SARS-CoV-2 variants is impacted by vaccination and prior infection, the MSD-platform ACE2 inhibition assay was carried out against the common variants of SARS-CoV-2 in both infection-naïve and previously-infected individuals (Figure 6.4). Notably, previously-infected individuals made broad nAb responses against all studied variants following the first dose ($p\leq0.0001$ for all,Wilcoxon signed-rank test), whereas high-titre nAb responses against these variants were only observed following the second dose in infection-naïve individuals.

To assess the impact of BNT162b2 vaccination on IgG responses to related coronaviruses such as SARS-CoV-1, MERS-CoV, and endemic HCoVs, total IgG targeting the S protein of these viruses were quantified using the MSD v-plex immunoassay (Figure 6.5). Post-V1, IgG targeting betacoronaviruses was significantly elevated in both infection-naïve individuals (SARS-CoV-1: $p<0.0001$, MERS-CoV: $p<0.0001$, HCoV-HKU1: $p=0.0185$, HCoV-OC43: $p=0.01$, Wilcoxon signed-rank tests) and previously-infected individuals (SARS-CoV-1: $p<0.0001$, MERS-CoV: $p<0.0001$, HCoV-HKU1: $p=0.0006$, HCoV-OC43: $p=0.0062$, Wilcoxon signed-rank tests). There was no significant impact of vaccination on IgG targeting HCoV-NL63 or HCoV-229E in either infection-naïve or previously-infected individuals. There was

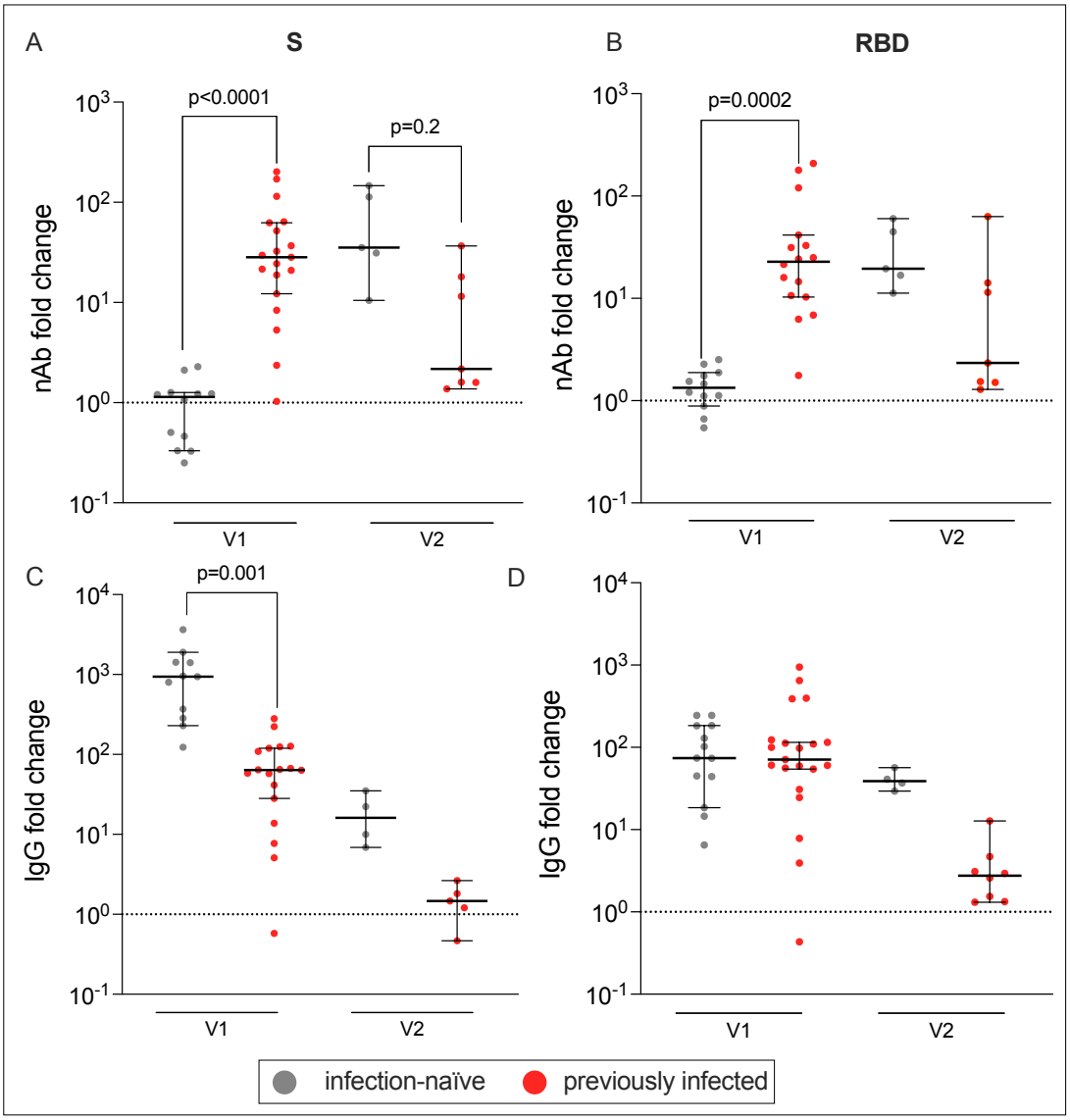


Figure 6.3: Fold change in nAb and IgG response. Fold change in nAbs targeting S (A) and RBD (B), and fold change in IgG targeting S (C) and RBD (D), in infection-naïve (grey) and previously-infected (red) individuals post-V1 and post-V2. Dotted lines show a fold change of 1. p-values refer to Mann-Whitney test values.

no significant impact of a second dose of BNT162b2 on cross-reactive IgG to any coronavirus, although IgG responses to SARS-CoV-1 S waned significantly between post-V1 and pre-V2 timepoints in previously-infected adolescents (p=0.04, Wilcoxon signed-rank test).

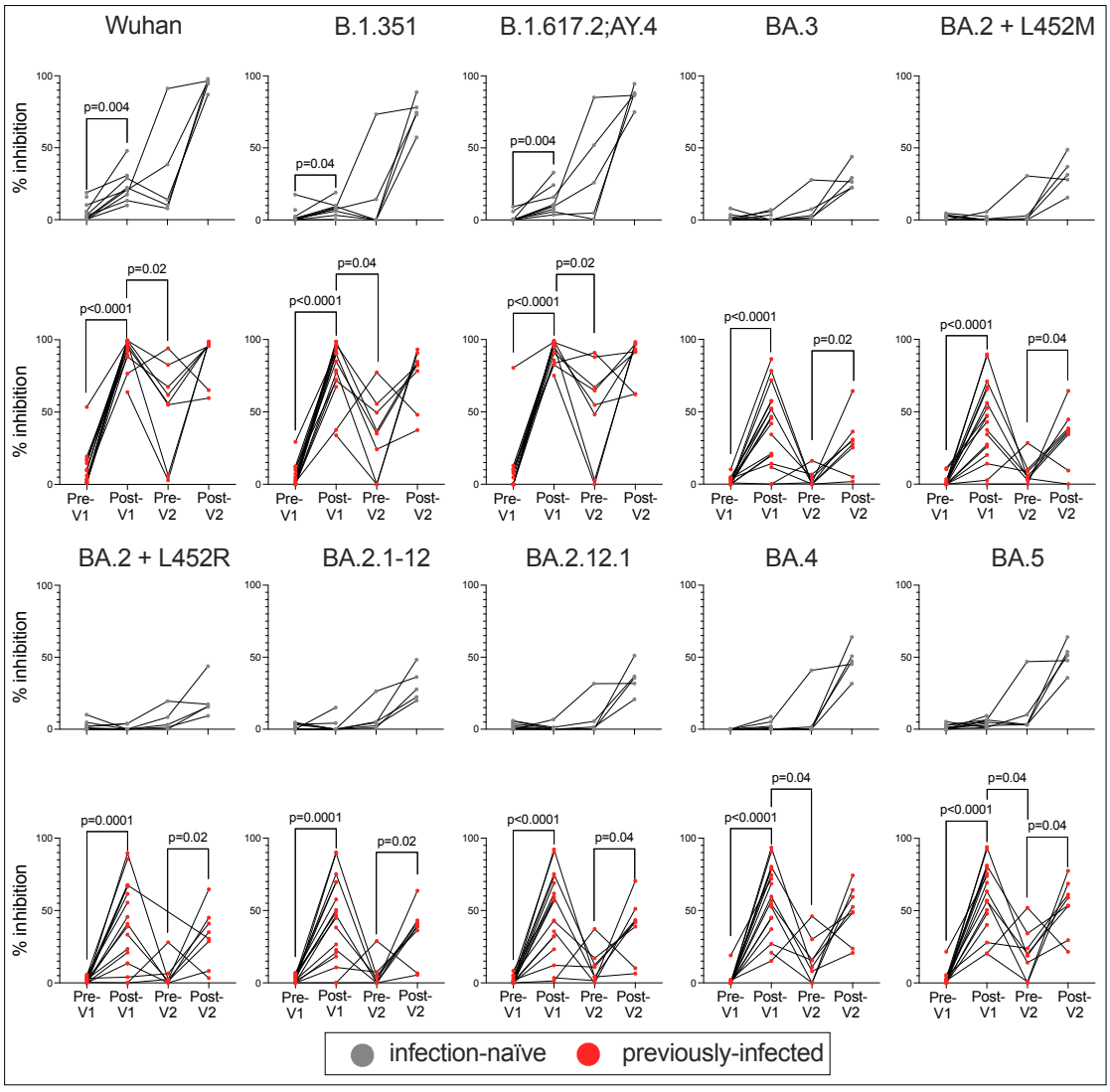


Figure 6.4: Neutralising responses to SARS-CoV-2 VOCs. % inhibition of ACE2-S binding for common VOCs: Ancestral Wuhan lineage, B.1.351, B.1.617.2;AY.4, BA.3, BA.2 + L452M, BA.2 + L452R, BA2.1-12, BA.4, and BA.5 in infection-naïve (grey) and previously-infected (red) individuals. p-values refer to Wilcoxon test values.

6.3.3 Cellular immune responses to BNT162b2 vaccination

Next, cellular immune responses in adolescents following first and second doses of BNT162b2 were assessed. Proliferation assays with pools of SARS-CoV-2 peptides spanning the S1 and S2 regions of S, as well as M and N, were performed at pre-V1, post-V1, pre-V2 and post-V2 timepoints (Figure 6.6). In contrast to humoral responses to BNT162b2, SARS-CoV-2-specific CD4+ T-cell proliferative responses were similar in infection-naïve compared to previously-infected individuals after

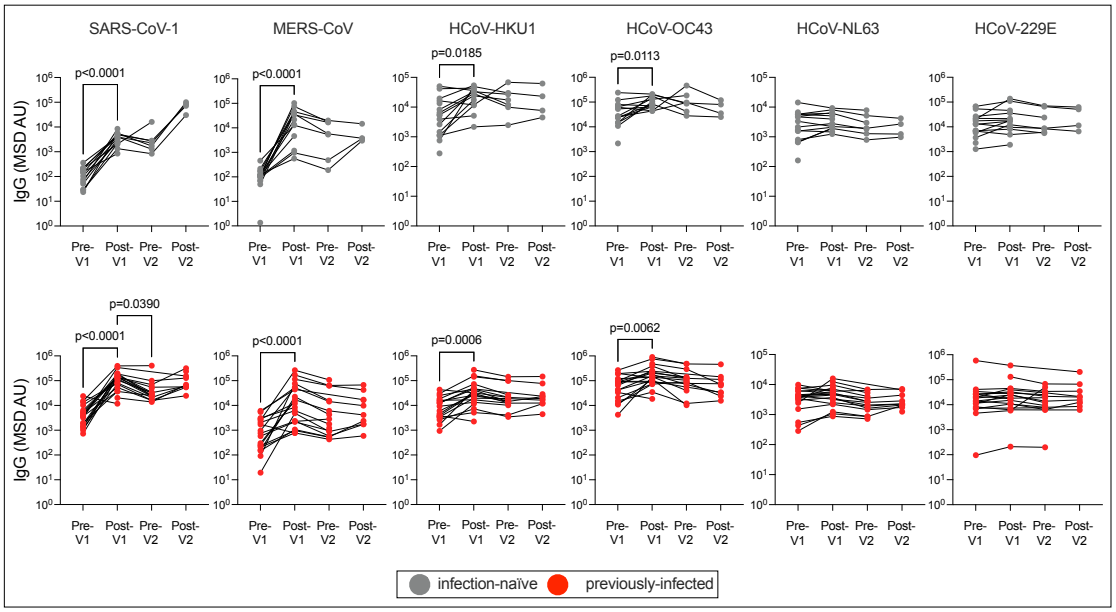


Figure 6.5: Humoral responses to related coronaviruses. IgG targeting SARS-CoV-1, MERS-CoV, HCoV-HKU1, HCoV-OC43, HCoV-NL63 and HCoV-229E in infection-naïve (grey) and previously-infected (red) individuals. p-values refer to Wilcoxon signed-rank test values.

a single dose of vaccine. In infection-naïve individuals, CD4+ responses to S1 (p=0.02) and to S2 (p=0.03) increased significantly post-V1 (Wilcoxon signed-rank tests). The T-cell response in previously-infected individuals increased following vaccination, but not significantly, suggesting stable cellular immunity over time.

To assess the impact of BNT162b2 vaccination on cross-reactive T-cell responses to endemic HCoVs, stimulation of PBMCs with peptides spanning the S2 region of HCoV-OC43 and HCoV-HKU1 S was undertaken using the CTV assay. There was no significant difference in T-cell response to any antigen between pre-V1, post-V1, pre-V2, and post-V2 timepoints. Interestingly, responses to both HCoVs appeared highest at pre-V2 timepoints in previously-infected individuals only (Figure 6.7).

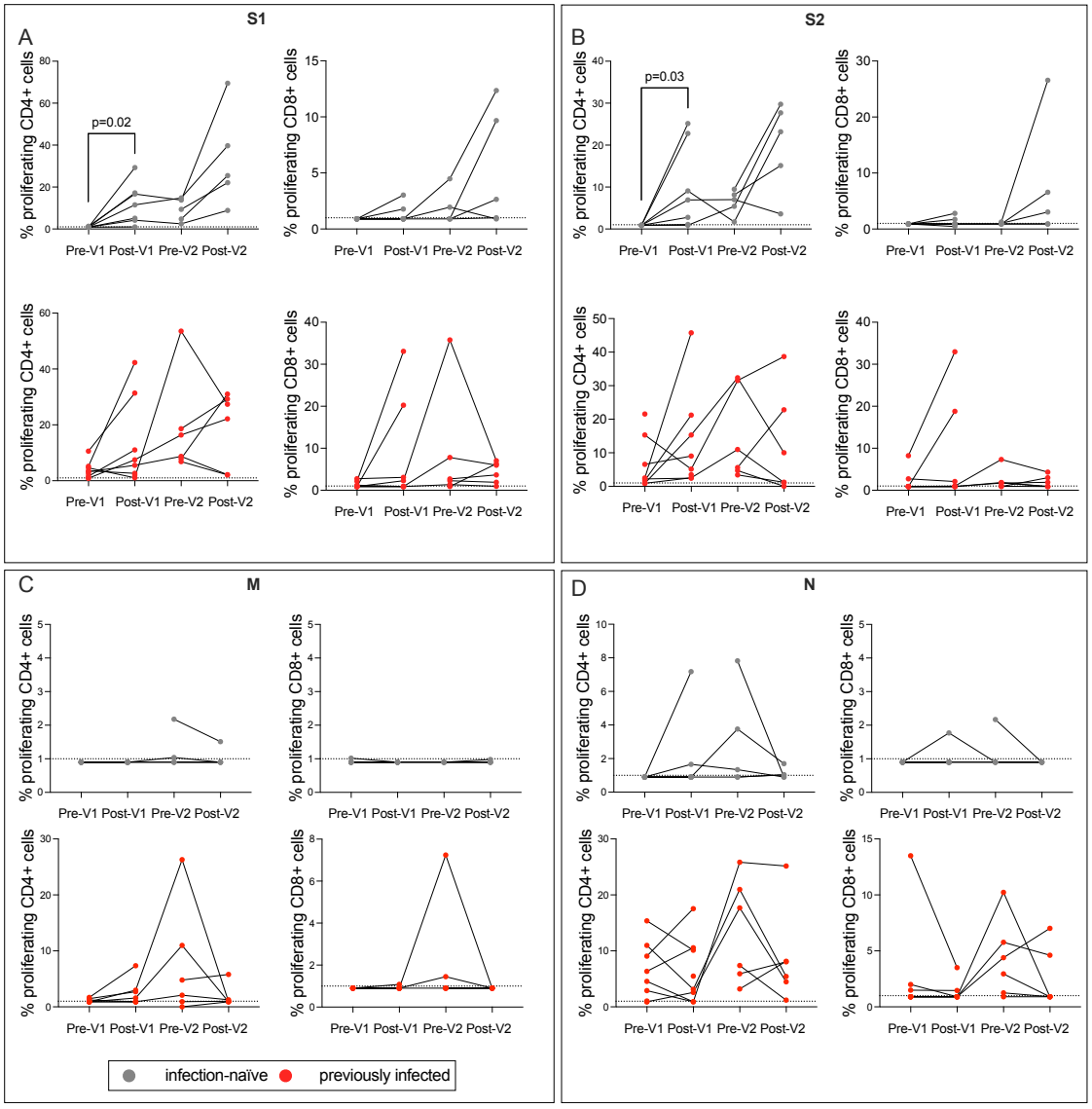


Figure 6.6: Cellular responses to BNT162b2 vaccination. % Proliferating CD4+ and CD8+ T-cells targeting S1 (A), S2 (B), M (C), and N (D) in infection-naïve (grey) and previously-infected (red) individuals. p-values refer to Wilcoxon signed-rank test values. Values below 1% were given nominal values of 0.9. Dotted lines refer to the cutoff for positivity of 1% proliferation.[120]

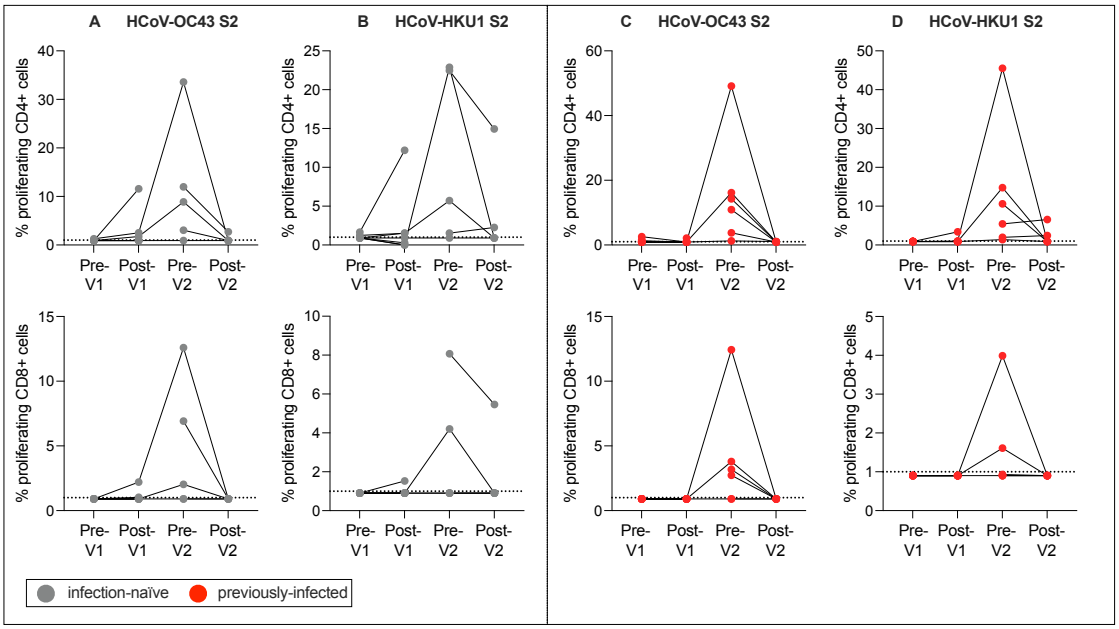


Figure 6.7: Cellular responses to endemic HCoVs. % Proliferating CD4+ and CD8+ T-cells targeting HCoV-OC43 S2 (A) and HCoV-HKU1 S2 (B) in infection-naïve individuals (grey). % proliferating CD4+ and CD8+ T-cells targeting HCoV-OC43 S2 (C) and HCoV-HKU1 S2 (D) in previously-infected individuals (red). Values below 1% were given nominal values of 0.9. Dotted lines refer to the 1% cutoff for positivity.[120]

6.3.4 Higher magnitude antibody responses to BNT162b2 in adolescents versus adults

The role of age in immune response to vaccination was of particular interest in this study. To determine whether the responses observed in adolescents to the BNT162b2 vaccine were stronger than those observed in adults, as previously shown,[311] the adolescent data was compared to humoral responses in adults from the PITCH cohort 28 days after the first dose of BNT162b2 (Figure 6.8).[60, 121] PITCH is a consortium of universities and UK Health Security Agency (UKHSA) with the aim of characterising infection-acquired and vaccine-induced immunity to SARS-CoV-2 in HCWs. Here, as reported for adolescents receiving two vaccines,[311, 336] post-V1, infection-naïve adolescents generated higher magnitude anti-S IgG responses than infection-naïve adults ($p=0.03$, Mann-Whitney test) and previously-infected adolescents generated greater anti-S IgG responses than previously-infected adults ($p<0.0001$, Mann-Whitney test) (Figure 6.8A). Post-V1 nAb responses did not differ significantly between adolescents and adults (Figure 6.8B).

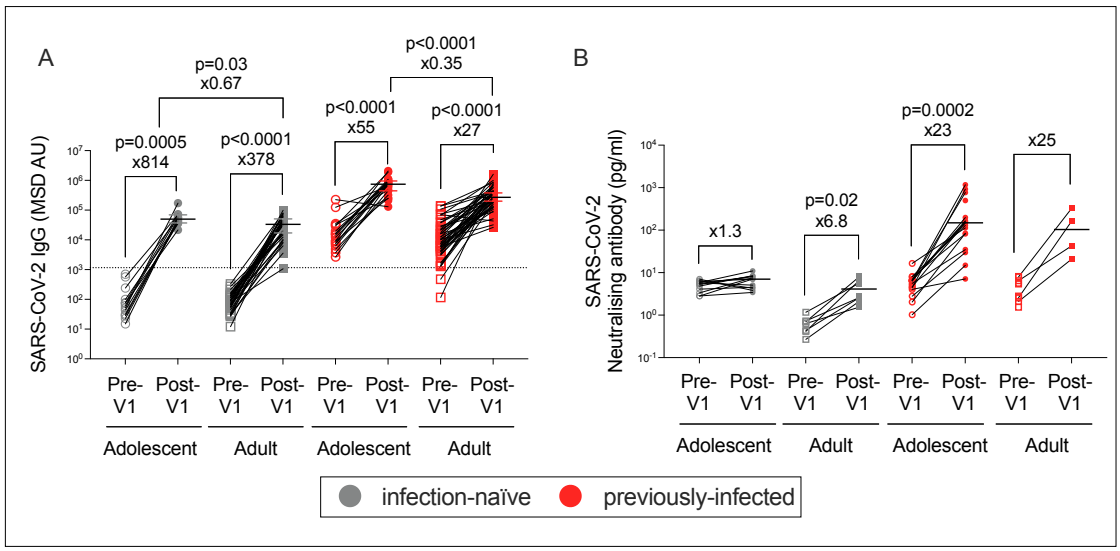


Figure 6.8: Humoral responses to BNT162b2 in adults and adolescents. IgG (A) and nAb responses (B) pre- and post-V1 in adolescents (circles) and adults (squares), either previously-infected (red) or infection-naïve (grey). p-values from paired comparisons are Wilcoxon test values, p-values from unpaired comparisons are Mann-Whitney test values. The dotted line refers to the cutoff for seropositivity of 1160 AU.[60]

To investigate why infection-naïve adolescents do not generate significantly increased nAb titres post-V1, despite a strong total IgG response, it was hypothesised that cross-reactive antibody responses to endemic HCoVs might be present at higher levels in infection-naïve adolescents, thereby interfering with the generation of novel SARS-CoV-2-specific responses to BNT162b2, as has been suggested previously.[6, 8] As displayed in Chapter 5, humoral responses to HCoVs have been associated with worse COVID-19 outcomes, through the inhibition of novel responses to SARS-CoV-2 as a result of immune imprinting or OAS.[6]

Our data supports the hypothesis that cross-reactive antibody responses to HCoVs are associated with weaker vaccine-induced neutralising responses: in this study, the ratio of IgG targeting betacoronaviruses HCoV-HKU1 and HCoV-OC43 S to IgG targeting SARS-CoV-2 S was significantly higher in infection-naïve adolescents versus infection-naïve adults (HKU1: $p<0.0001$; OC43: $p<0.0001$, Kruskal-Wallis test with Dunn's multiple comparisons) and versus previously-infected adolescents

(HKU1: $p<0.0001$; OC43: $p<0.0001$, Kruskal-Wallis test with Dunn's multiple comparisons) post-V1 (Fig. 6.9AB). Furthermore, the ratio of IgG targeting HCoV-HKU1 and HCoV-OC43 S to IgG targeting SARS-CoV-2 S was negatively correlated with nAb response (HKU1: $r=-0.75$, $p<0.0001$; OC43: $r=-0.84$, $p<0.0001$) in all adolescents, though this significance was lost when adolescents were divided into infection-naïve and previously-infected (Figure 6.9CD).

To test the hypothesis that a first exposure to SARS-CoV-2 generates primarily a cross-reactive recall response targeting S2 epitopes, and secondary exposure generates more of a SARS-CoV-2-specific response to RBD, the ratio of IgG targeting SARS-CoV-2 RBD to SARS-CoV-2 S was calculated for infection-naïve and previously-infected adolescents post-V1 and post-V2 (Figure 6.9E). Of note, previously-infected adolescents generated more of an RBD-focussed IgG response than infection-naïve adolescents ($p=0.0145$, Kruskal-Wallis test with Dunn's multiple comparisons) post-V1. In addition, post-V2, infection-naïve adolescents generated more of an RBD-focussed IgG response than post-V1 ($p=0.0004$, Kruskal-Wallis test with Dunn's multiple comparisons). This suggests that exposure to SARS-CoV-2 through infection or vaccination pushes the IgG response away from conserved regions of S. Furthermore, the RBD:S ratio in infection-naïve adolescents post-V2 was higher than in previously-infected adolescents post-V1, albeit not significantly. This suggests that vaccination may be more effective than natural infection at generating RBD-specific responses.

6.3.5 Humoral responses to LAIV vaccination

As well as the immune response to BNT162b2, co-administration of the LAIV enabled the characterisation of immunity against influenza following vaccination. To determine the effect of the LAIV on lineage-specific anti-HA IgG titres, ELISAs were performed on pre- and post-LAIV samples for the 26 individuals who received

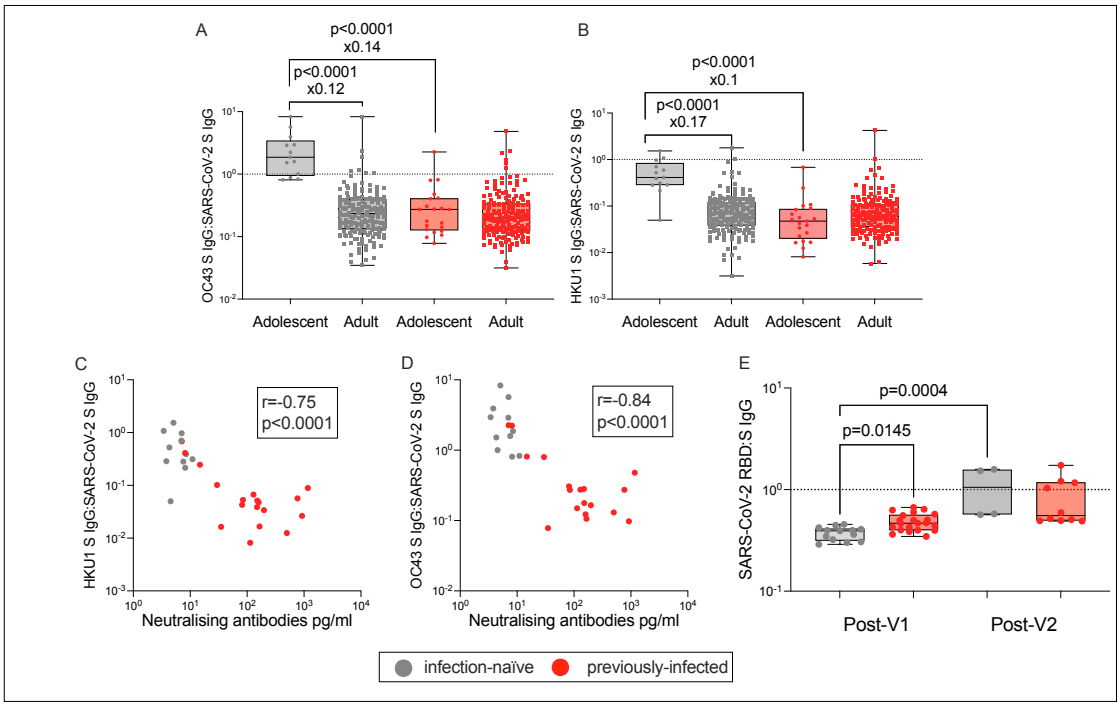


Figure 6.9: Evidence of immune imprinting in infection-naïve adolescent IgG responses. Ratio of IgG targeting HCoV-OC43 S (A) or HCoV-HKU1 S (B) to SARS-CoV-2 S in infection-naïve (grey) and previously-infected (red) adults (squares) and adolescents (circles). Correlation between nAb response and HCoV-HKU1 (C) or HCoV-OC43 (D) to SARS-CoV-2 IgG ratio post-V1. Ratio between SARS-CoV-2 RBD to total S response post-V1 and post-V2 in adolescents (E). p-values for pairwise comparisons refer to Kruskal-Wallis tests with Dunn's multiple comparisons. r- and p-values for correlation analysis refer to Spearman rank test values. Dotted lines show a ratio of 1:1.

the LAIV (Figure 6.10). As expected, IgG titres were significantly higher post-LAIV for A/Cambodia (H3N2), A/Victoria (H1N1), and B/Phuket (Yamagata) ($p<0.0001$, $p=0.0002$, $p<0.0001$, respectively, Wilcoxon signed-rank tests) (Figure 6.10A). Post-LAIV anti-HA IgG responses towards the B/Washington (Victoria) lineage were not significantly increased compared to pre-LAIV. A possible explanation lies in the observation that responses to B/Washington (Victoria) were strongly correlated with age for both pre- and post-vaccine timepoints ($r=0.61$, $p=0.0001$; $r=0.57$, $p=0.0008$, respectively, Spearman rank test) (Figure 6.10BC). By contrast, there was no correlation between age and post-LAIV IgG for A/Cambodia (H3N2) or B/Phuket (Yamagata), and for A/Victoria (H1N1) pre-LAIV IgG levels only were weakly correlated with age ($r=0.39$, $p=0.02$, Spearman rank test).

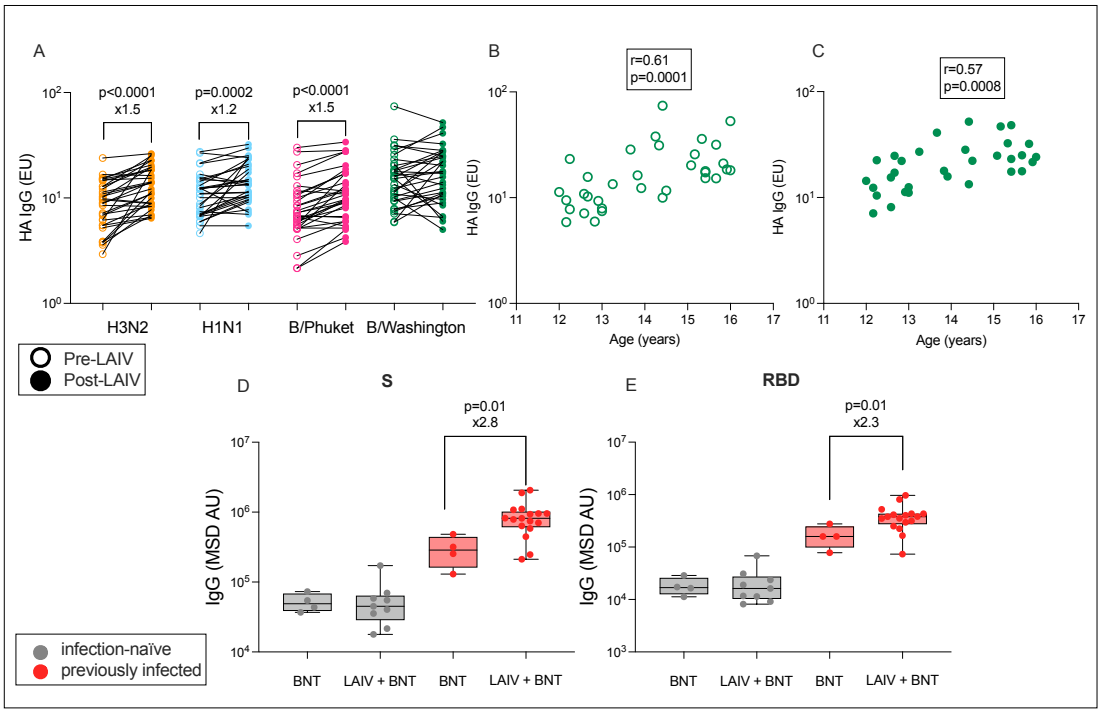


Figure 6.10: Humoral responses to LAIV vaccination. IgG targeting influenza HA pre- and post-LAIV administration in adolescents (A). Correlation between age and IgG targeting B/Washington lineage pre-LAIV (B) and post-LAIV (C). IgG targeting SARS-CoV-2 S (D) and RBD (E) in infection-naïve (grey) and previously-infected (red) adolescents who received only BNT162b2 (BNT) or both BNT162b2 and the LAIV (LAIV + BNT).

Unexpectedly, and in contrast to previous studies,[339] adolescents previously-infected with SARS-CoV-2 who received both BNT162b2 and the LAIV generated significantly higher post-V1 IgG targeting both S and RBD compared to adolescents previously-infected with SARS-CoV-2 who received BNT162b2 alone (both p=0.01, Mann-Whitney tests), although this analysis involved very small numbers of individuals (Figure 6.10DE).

6.3.6 Sex differences in response to vaccination

Females typically make stronger IgG responses than males following vaccination,[98, 102, 104, 329] including to influenza vaccines.[100, 104] Surprisingly, therefore, here, infection-naïve males generated significantly higher post-V1 IgG targeting

both SARS-CoV-2 S and RBD than females (p=0.008, p=0.02, respectively, Mann-Whitney tests) (Figure 6.11A). There was no significant difference in IgG response between the sexes in previously-infected individuals (Figure 6.11B). Furthermore, there was a trend towards a stronger RBD and S nAb response post-V1 in infection-naïve males compared to infection-naïve females, although this was not significant (p=0.07 and p=0.15, respectively, Mann-Whitney tests) (Figure 6.11C). There was no significant different in baseline IgG responses between males and females, and there was no sex difference in the humoral response to BNT162b2 in adults.[60]

Stronger responses to influenza vaccination in females have been described to the extent that a half-dose of influenza vaccine generated higher IgG responses in females than a full dose in males.[100] Surprisingly, here, we did not find a sex difference in IgG response to the four influenza lineages studied (Figure 6.11E).

A potential mediator of immune sex differences is the effect of sex hormones. Males start puberty on average 2 years later than females. To ensure males in this cohort had entered puberty, steroid hormones including testosterone, dihydrotestosterone (DHT) and progesterone were measured by Tandem mass spectrometry. All but the two youngest males (12 years 2 months and 12 years 10 months) demonstrated pubertal androgen levels. Testosterone correlated with age in males only (r=0.47, p=0.05).

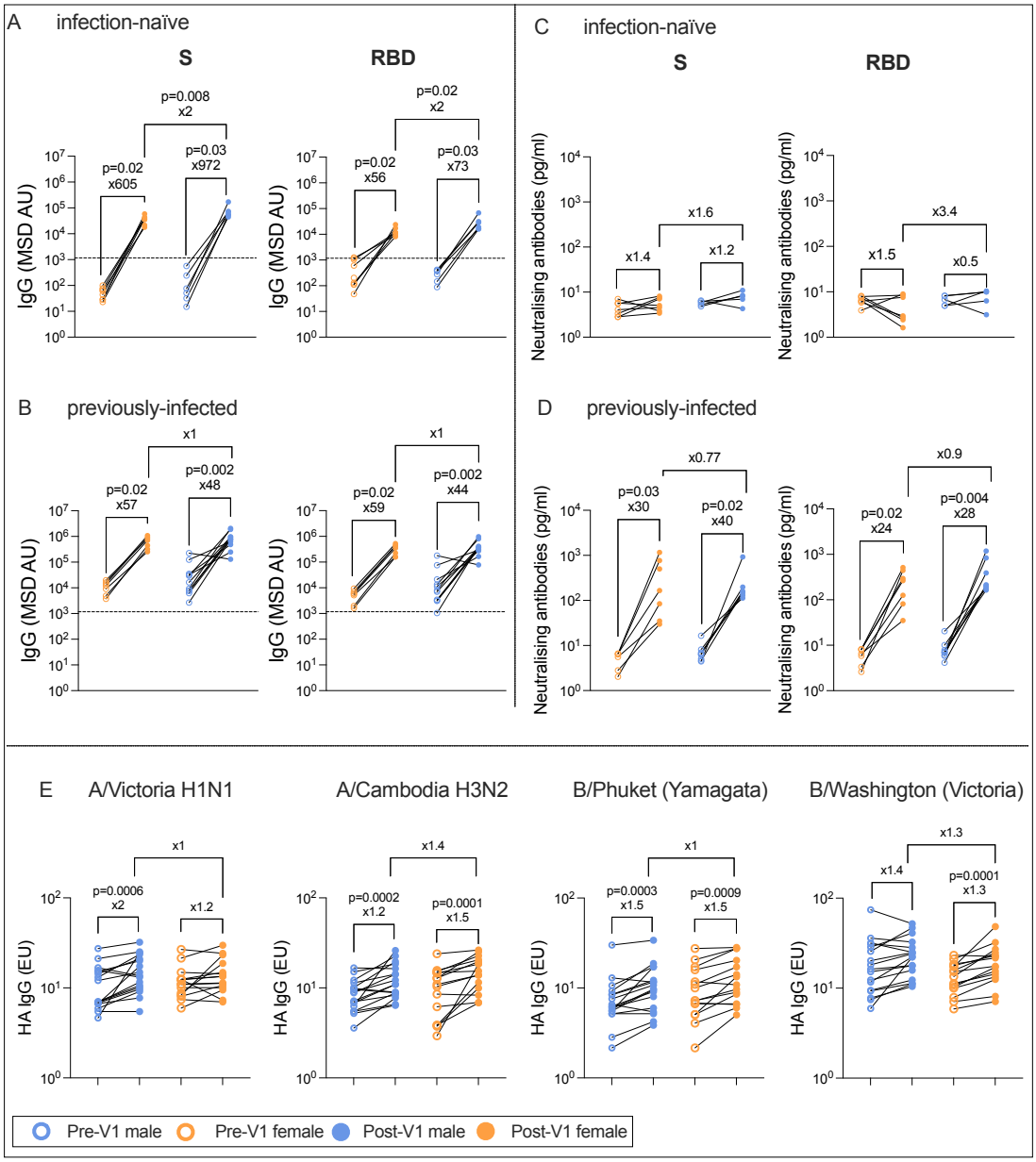


Figure 6.11: Sex differences in response to vaccination. IgG targeting SARS-CoV-2 S and RBD pre- and post-V1 in infection-naïve (A) and previously-infected (B) females (orange) and males (blue). nAb responses to S and RBD in infection-naïve (C) and previously-infected (D) individuals. IgG targeting the four influenza lineages pre- and post-LAIV in males (blue) and females (orange) (E). Dotted lines in A and B refer to seropositivity cutoffs.[60]

6.4 Discussion

Understanding the quantitative markers of vaccine immunogenicity, as well as confounding patient demographic factors, will help better define correlates of protection against SARS-CoV-2 and improve interpretability of future vaccine trials. The data presented in this chapter demonstrates robust SARS-CoV-2-specific IgG production in adolescents following vaccination with BNT162b2. Frenck *et al.* (2021) identified noninferior nAb production in 12-15-year-olds compared to 16-25-year-olds receiving two doses BNT162b2.[311] In addition, a study of adults and adolescents receiving two doses of either BNT162b2 or CoronaVac demonstrated 100% S-RBD IgG seropositivity following one dose of BNT162b2 in adolescents.[336]

Two doses of BNT162b2 is known to elicit robust T_H1 T-cell responses in adults, with widespread IFNγ production.[121, 340] S-specific T-cell responses following vaccination with BNT162b2 were generated post-V2, but not post-V1, in another cohort of infection-naïve adolescents.[336] This is in contrast with the data described herein, where one dose of BNT162b2 is sufficient to induce a significant increase in S-specific CD4+ T-cells in infection-naïve adolescents. The lack of a significant reduction in T-cell response across timepoints suggests that these responses are stable over several months, as reported elsewhere,[341, 342] and as evidenced in Chapter 3 following natural infection. The longevity of these responses is uncertain due to the lack of an extended follow up in this cohort but should be the focus of future studies.

The data described herein also suggests that a nAb response is prompted in infection-naïve adolescents after two doses but previously-infected adolescents only require one dose for a robust neutralising response. Due to the discrepancy between IgG and nAb response in infection-naïve adolescents, these data support the use of nAb titre as well as total IgG when assessing vaccine immunogenicity.[343, 344] Other studies have established that BNT162b2 and the CoronaVac inactivated virus vaccine elicit robust nAb responses post-V2 in infection-naïve adolescents.[311, 336] Strong neutralising responses have also been described in adolescents receiving

other COVID-19 vaccines, such as CoronaVac, where 100% of adolescents generated nAbs following two doses of vaccine.[344]

This data demonstrates that a similar SARS-CoV-2-specific IgG response is elicited in adolescents after natural infection and one vaccine dose compared to two vaccine doses only. Previous studies in adults have evaluated COVID-19 vaccine-induced versus infection-induced immunity: Payne *et al.* (2021) identified significantly higher IgG and T-cell responses in previously-infected adults four weeks after the second dose of BNT162b2 compared to infection-naïve individuals. In addition, previously-infected adults generated a similar anti-S IgG response following one dose compared to infection-naïve adults after two doses, and a stronger cellular immune response.[60] A similar study of infection-naïve and previously-infected HCWs receiving two doses of BNT162b2 identified a similar T-cell response in previously-infected individuals following one dose compared to two doses in infection-naïve individuals; the study also reported higher anti-S IgG in previously-infected HCWs after one dose than infection-naïve HCWs after two doses.[121] Studies of BNT162b2 immunogenicity in adolescents have so far excluded previously-infected individuals, and therefore this study is one of the first to assess the impact of prior SARS-CoV-2 infection on BNT162b2 response in adolescents.[311, 336]

As SARS-CoV-2 evolves and the global population faces infection with and vaccination against novel VOCs, understanding how prior immunity through natural infection or vaccination is able to protect against VOCs is essential. The data presented in this chapter demonstrates strong neutralising activity against many SARS-CoV-2 VOCs, including several Omicron sublineages, in previously-infected adolescents after one dose of BNT162b2. Infection-naïve adolescents do generate neutralising responses to SARS-CoV-2 VOCs after two doses, indicating that vaccination against the Wuhan strain of SARS-CoV-2 does generate cross-reactive humoral immunity that is capable of neutralising novel VOCs. However, responses

to all assayed VOCs after two doses were inferior to the nAb responses in previously-infected adolescents after one dose, highlighting the superiority of natural infection in this context. Furthermore, it is likely that many of these previously-infected adolescents were initially infected with Delta and Omicron lineages, as sampling took place in the winter of 2021-2022. This may explain why neutralisation of variants was superior in previously-infected adolescents. Studies of immunity to SARS-CoV-2 VOCs following BNT162b2 vaccination have demonstrated cross-reactivity with VOCs, though neutralisation of the Beta variant was reduced compared to the ancestral lineage in one study of adults after two doses of BNT162b2.[121] However, immune escape by Omicron sublineages appeared to reduce after a third dose of BNT162b2 in adults.[281] It is unlikely that adolescents in the UK will receive additional doses of COVID-19 vaccine at this time, though future research on the impact of sequential SARS-CoV-2 infection by different VOCs will shed further light on the extent of cross-variant immunity in adolescents.

Another goal of this study was to identify age-related differences in response to vaccination. Here, both infection-naïve and previously-infected adolescents generated stronger IgG responses than adults. BNT162b2 has been shown to promote greater IgG production in younger individuals.[311, 336] In Frenck *et al.* (2021), 190 12-15-year-old adolescents who received two doses of BNT162b2 generated nAb titres 1.76x higher than 170 16-25-year-olds.[311] Similarly, in Rosa Duque *et al.* (2022), two doses of BNT162b2 generated stronger SARS-CoV-2 nAb titres and promoted higher anti-S IgG avidity in adolescents compared to adults; in addition, two doses of CoronaVac also generated stronger anti-S IgG, anti-N IgG, and nAbs in adolescents compared to adults.[336] Furthermore, a study of mRNA-1273 in a cohort of 12-17-year-olds identified noninferior nAb titres in adolescents compared to 18-25-year-olds.[300]

Elevated vaccine responses in adolescents may result from higher or more recent exposure to endemic HCoVs compared to adults, promoting a stronger IgG response

to conserved SARS-CoV-2 antigens. In Chapter 4, stronger cross-reactive T-cell immunity was identified in adults compared to under-18s, and in Chapter 5, age was positively correlated with IgG targeting endemic HCoVs in SARS-CoV-2 seropositive individuals. However, pre-pandemic samples of adults and children have demonstrated higher pre-existing SARS-CoV-2 S-specific IgG in children, although higher class-switched IgG specific for conserved regions of S2 and N have been identified in adults.[3] To reconcile these findings, it could be theorised that SARS-CoV-2 infection generates higher levels of cross-reactive IgG in adults due to activation of accumulated memory B-cells over an individual's lifetime. Children, conversely, may contain higher levels of baseline circulating antibodies specific for HCoVs to which they are routinely exposed, albeit with less accumulated memory, which are cross-reactive for SARS-CoV-2 and therefore manifest as stronger SARS-CoV-2-specific IgG responses in adolescents post-vaccination.

This hypothesis may also explain the poorer neutralising responses in infection-naïve adolescents compared to previously-infected adolescents, as well as the higher HCoV:SARS-CoV-2 IgG ratio and lower RBD:S IgG ratio in infection-naïve adolescents. Despite the strong IgG response in infection-naïve adolescents post-V1, this appears to largely consist of cross-reactive IgG and is poorly neutralising, targeting more conserved regions of S than previously-infected adolescents. Weaker immune responses associated with pre-existing cross-reactive immunity is a feature of OAS as described in Chapter 5, and this may explain the patterns described herein. Once adolescents have been exposed to SARS-CoV-2, either through infection as seen in previously-infected adolescents post-V1 or through vaccination as seen in both groups post-V2, their IgG response is less prone to the effects of OAS and is more RBD-focussed, with a smaller contribution from cross-reactive IgG and a higher neutralising capacity. This is also supported by the strong negative correlation between HCoV:SARS-CoV-2 IgG ratio and nAb response.

Notably, in this cohort, increased post-V1 anti-S and anti-RBD IgG responses in

infection-naïve males compared to infection-naïve females were observed, in contrast to expectations based on other vaccines such as inactivated influenza vaccines.[98, 100] Immune responses to many adult and childhood vaccines, as well as responses to natural infection with viral pathogens, are consistently higher in females and associated with increased inflammation and autoimmunity as well as CD4+-skewed T-cell responses and greater B cell activation and IgG production.[98, 102, 104, 329] Female IgG responses to influenza vaccines have been shown to reach twice the magnitude of male IgG responses, and females also report more frequent SAEs to viral vaccines.[104, 329] Mechanisms of stronger adaptive immunity to vaccination in females include higher pro-inflammatory responses, CD4+ T-cell counts and antibody responses.[329] Oestrogen appears to stimulate IgG and IgM production, as well as B-cell class-switching and TNFα production by macrophages.[345] It is therefore unclear why infection-naïve males generated stronger IgG targeting SARS-CoV-2 S and RBD in this cohort. Another trend to which COVID-19 mRNA vaccines appear to be an exception is the higher rates of SAEs in females. Following mRNA vaccination, vaccine-induced myocarditis is more frequent in young males.[163, 334] This is believed to by mediated by testosterone; a study of coxsackievirus-induced myocarditis in mice identified worse disease in males, and mice who received exogenous testosterone and progesterone displayed more localisation of virus to the heart than mice receiving oestrogen.[346] The same mechanism that promotes a higher incidence of myocarditis in males receiving COVID-19 mRNA vaccines may also be responsible for the higher antibody responses identified here in infection-naïve males. However, these findings have not been reported elsewhere, and the trend is not visible in previously-infected males, for reasons which are as yet unclear. Further research into innate and adaptive mechanisms of immunity in males and females following mRNA vaccination are required to interpret these findings. No significant sex difference in anti-HA IgG titres following the LAIV was identified in this cohort. This is surprising in the context of established literature,[98, 329] but may be obscured by the very small increase in anti-HA IgG post-V1 in this cohort, the effect of a live-attenuated rather than inactivated influenza vaccine, the use

of different serological assays, or the result of co-administration with BNT162b2.[347]

Finally, the study identified boosting of IgG responses to A/Cambodia (H3N2), B/Phuket (Yamagata), and A/Victoria (H1N1) following LAIV administration, but no boosting of IgG targeting B/Washington (Victoria). This, combined with the correlation between age and IgG targeting B/Washington (Victoria), highlights the possibility that natural exposure to B/Washington (Victoria) is so frequent in this cohort that vaccination against this strain of influenza does not add significantly to the natural immunity that is accumulated over adolescence. Pre-existing immunity to influenza has been widely described, from prior infection and vaccination, in support of this finding.[348, 349]

A potential confounder for this study is that adolescents of this age group are likely at different stages of puberty and therefore have diverse levels of testosterone, oestrogen, and progesterone. Furthermore, males experience puberty at older ages compared to females, and therefore the sex difference identified herein may result from the confounding effects of puberty. If many male adolescents had not gone through puberty at the time of sampling, the increased humoral responses to vaccination in males may result from an absence of the immunosuppressive effects of androgens. Steroid hormones were measured in this cohort, and it was identified that all but two males had pubertal levels of testosterone. This promotes confidence in the results of comparisons between sexes, as the majority of males had undergone puberty at time of sampling.

The data presented herein indicates that co-administration of BNT162b2 with the LAIV results in enhanced IgG responses to SARS-CoV-2 antigens. This is in contrast with findings for NVX-COV2373 in adults, where co-administration with inactivated quadrivalent influenza vaccines (QIV) reduced SARS-CoV-2-specific IgG titres (NVX-CoV2373/QIV: 31,236.1 vs NVX-CoV2373: 44,678.3).[339] However, studies of co-administration of COVID-19 mRNA vaccines with QIV in adults

have reported no reduction in antibody response compared to administration of mRNA vaccines alone.[309, 350] Izikson *et al.* (2022) studied adults aged 65 and older who had previously received two doses of mRNA-1273. Participants either received a QIV and third dose of mRNA-1273, QIV alone, or a third dose of mRNA-1273 alone. nAbs targeting HA were similar between QIV/mRNA-1273 and QIV alone groups, and nAbs targeting SARS-CoV-2 were also similar between QIV/mRNA-1273 and mRNA-1273 alone groups.[350] Lazarus *et al.* (2021), as part of the UK ComFluCOV trial, studied adults who had previously received one dose of either BNT162b2 or AZD1222; participants received a second dose of either vaccine as well as a placebo or an influenza vaccine (six cohorts in total: AZD1222/cellular quadrivalent, BNT162b2/cellular quadrivalent, AZD1222/MF59C adjuvanted trivalent, BNT162b2/MF59C adjuvanted trivalent, AZD1222/recombinant quadrivalent, BNT162b2/recombinant quadrivalent). Anti-S IgG 21 days after vaccination was similar between co-administered cohorts and placebo cohorts. However, titres of anti-HA nAbs targeting A/H1N1 and B lineages were higher in the BNT162b2/recombinant quadrivalent cohort compared to placebo/recombinant quadrivalent. The authors speculated that this may have resulted from the RNA in BNT162b2 acting as an adjuvant, although it was unclear why this result was only identified for the recombinant quadrivalent influenza vaccine and not cellular or adjuvanted trivalent vaccines.[309] In this chapter, it is possible that the live attenuated viruses in the LAIV may have also adjuvanted antibody responses in the BNT162b2/LAIV adolescents. This could enhance innate immune activation, particularly in the nasal mucosa, leading to greater SARS-CoV-2-specific local T-helper cell activation. However, it is unclear why this would only impact previously-infected individuals, and further studies of innate response following COVID-19 and influenza vaccination would shed further light on this result.

This study has some limitations. The small numbers of adolescents assayed in this cohort make broad conclusions difficult, particularly when making comparisons

between small sub-groups such as co-administered LAIV/BNT162b2 and BNT162b2-alone individuals. No mucosal samples were taken and so mucosal immunity is not assessed in this cohort. Neutralisation responses to SARS-CoV-2 were estimated using the MSD-ACE2 inhibition assay. This has shown to correlate with live virus assays,[59, 60, 121] but live virus neutralisation is likely a more accurate measure of nAbs. In addition, no neutralisation assays were carried out for influenza lineages, which would have shed further light on the functionality of humoral immunity to influenza. Furthermore, the lack of an extended follow-up in this study makes assessments of immune durability impossible but should be the focus of future studies. Finally, the adolescents recruited to this study were aged between 12 and 16, an age group likely going through puberty and therefore changes in sex hormones, which may influence the results in this study. A more detailed analysis of levels of circulating sex hormones would have enabled closer dissection of the impacts of sex hormones on immune response in this cohort, though this is out of the scope of this thesis.

The aim of this chapter was to examine the immune response to SARS-CoV-2 and influenza following vaccination in UK adolescents. Taken together, these data paint a complex picture of vaccine-induced immunity in adolescents, with a potential role for immune sex and age differences in determining antibody responses to vaccination. These findings have important implications for paediatric vaccination regimes, such as the potential benefit of co-administration with influenza vaccines, and the necessity to consider sex and age when studying vaccine-induced immunity.

7
General Discussion

When SARS-CoV-2 emerged in the winter of 2019, a key question that arose was why some individuals experience severe or even fatal COVID-19, whilst others are largely spared from severe illness or, indeed, symptoms at all. Three years later, reasons for the heterogeneity of susceptibility to COVID-19 in human populations remain unclear. However, progress can be made in understanding this heterogeneity through close examination of immune responses in individuals from different demographics and with different disease severities. In addition, analysis of vaccine-induced immunity sheds further light on what constitutes a good immune response to SARS-CoV-2, and what factors affect the strength of this response.

In this thesis, adaptive immunity to SARS-CoV-2 was assessed through several lenses that enable the dissection of these contributory factors: primarily, the role of cross-reactive immunity in COVID-19, but also the influence of age, sex, prior infection, intrafamilial transmission of SARS-CoV-2, and vaccination with mRNA vaccines, as summarised in Figure 7.1. In Chapter 3, immunity was described in the context of family groups. This enabled the assessment of age- and sex-specific trends in immunity, as well as the impact of intrafamilial exposure to SARS-CoV-2 on infection likelihood. Key takeaways from this chapter included: robust humoral responses in infected families, abundant transmission between

family members, some indication of stronger cellular immunity in older males and symptomatic individuals, and an apparent lack of cross-reactive immunity in unexposed children. Finally, strong cross-reactive cellular immune responses in exposed individuals of different ages raised the question of whether exposure to SARS-CoV-2 could promote expansion of pre-existing cellular immunity. This was examined in Chapter 4, where elevated T-cell immunity targeting the S1 region of SARS-CoV-2 S was identified in seronegative individuals exposed to SARS-CoV-2 compared to unexposed individuals. This immunity was stronger in individuals with more than one infected family member, but responses didn't appear to originate solely from endemic HCoVs; immunity to conserved epitopes was similar between seropositive individuals, ESNs, and USNs. Although it has been suggested elsewhere that pre-existing cellular immunity to HCoVs might protect against SARS-CoV-2 infection,[7] cross-reactive antibody responses have been described as promoting worse COVID-19 outcomes in some cases.[6] In Chapter 5, this phenomenon was explored using HCoV-OC43 and HCoV-HKU1 N as markers for prior immunity to HCoVs. It was demonstrated that prior exposure to endemic betacoronaviruses was associated with worse disease outcomes in two cohorts of mild and severe COVID-19. Although the mechanism for this 'OAS' remains unclear, one interpretation was offered that suggested a delay in SARS-CoV-2-specific antibody responses in individuals with prior HCoV-specific immunity, due to an initial expansion of weakly neutralising memory B-cell responses, leading to worse disease outcomes. Finally, Chapter 6 assessed adaptive immunity to SARS-CoV-2 through the lens of vaccination in adolescents and identified the following trends: better neutralising responses and less cross-reactive humoral responses in previously-infected adolescents compared to infection-naïve adolescents, stronger humoral immunity in adolescents compared to adults, and elevated IgG responses in infection-naïve males compared to females - this diverges from established trends of vaccine-induced immunity in males and females.

The emergence of SARS-CoV-2 offered an unprecedented opportunity to study

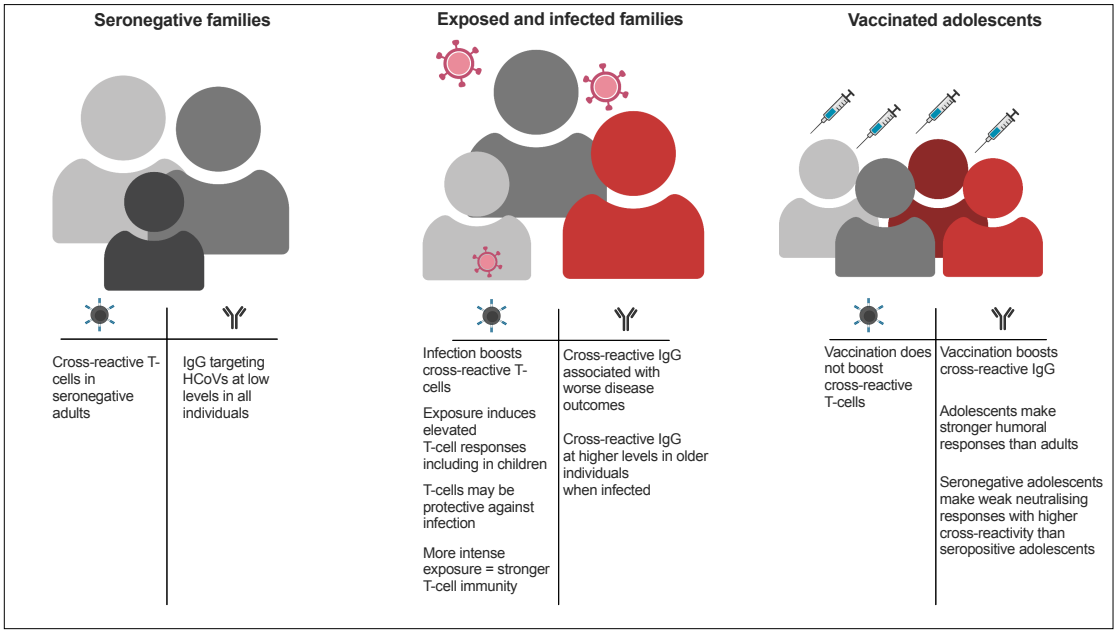


Figure 7.1: Summary of findings. Trends in cross-reactive antibody- and T-cell mediated immunity in seronegative families, seropositive and mixed serostatus families, and vaccinated adolescents.

immunity to a novel pathogen in an immunologically naïve population. This enabled robust assessments of immune responses pre- and post-infection and facilitated comparisons of response between individuals that were all experiencing primary infection. Furthermore, this allowed for a comparison between primary infection and prime vaccination in order to better understand the similarities and differences in vaccine-induced immunity and immunity induced by natural infection. Overall, primary infection appeared to induce both IgG and nAb responses as well as cellular immunity, whilst prime vaccination in immunologically naïve individuals only generated IgG with a weak nAb response. Furthermore, natural infection appeared to induce broad responses to SARS-CoV-2 VOCs, whilst vaccination in the absence of prior immunity was ancestral strain-specific in most individuals. Additionally, we were also able to compare heterologous immunity following infection and vaccination to two doses of vaccine alone – this was similar, albeit IgG responses appeared slightly higher in heterologous individuals who had experienced prior

infection.

Another unique aspect of the COVID-19 pandemic was the prevalence of pre-existing prior immunity to closely related coronaviruses in the global population, owing to endemic circulation of 'common cold' HCoVs. This offered an opportunity to study not only the relevance of prior immunity for COVID-19 severity, but also the impact of SARS-CoV-2 infection on the levels of HCoV-specific immunity. From the data outlined in this thesis, as well as evidence from existing literature,[5, 7] opposing roles for pre-existing cellular and humoral immunity in modulating COVID-19 disease course can be postulated. T-cells specific for SARS-CoV-2 S are expanded upon exposure to SARS-CoV-2 in seronegative individuals; the most parsimonious explanation for the source of these responses is boosting of pre-existing cellular immunity specific for endemic HCoVs. In this thesis, however, pre-existing responses appear not to be the sole source of T-cell immunity in ESNs; indeed, responses to the less conserved S1 region of S are elevated in ESNs, and responses to conserved peptides appear not to differ between ESNs and USNs. Nonetheless, T-cell immunity to HCoVs likely contributes to responses in ESNs, and a role for pre-existing T-cells in protection against infection has been well demonstrated.[5, 7, 351] In contrast, cross-reactive antibodies specific for endemic betacoronaviruses are associated with worse disease in this thesis, both in mild and severe cohorts. Altogether, this suggests opposing roles for cross-reactive cellular and humoral immunity in COVID-19 severity. The causes of this contrast are unclear but may result from the differing functionality of T- and B-cell-mediated immunity. Cross-reactive antibodies likely face reduced neutralising function from binding weakly to highly conserved regions of SARS-CoV-2 S, and if a large proportion of the B-cell pool targets poorly neutralising epitopes, the overall humoral response will be suboptimal. In contrast, T-cells are more able to recognise any region of SARS-CoV-2 S – conserved or non-conserved – and still function as efficient killers or helpers. This conjecture allows for cross-reactive T-cells to still function effectively despite recognising conserved regions, whilst cross-reactive antibodies would experience a

decline in function. The complexity of the immune system coupled with individual heterogeneity in prior exposure and immune function makes these nuances difficult to pull apart, as evidenced in the abundant contradictory literature on the subject.[3]

Despite the concerted global effort to understand immunity to SARS-CoV-2, and the unprecedented progress made on the topic in the last three years, ongoing endemic circulation of SARS-CoV-2 as well as seasonal vaccination in at-risk populations means future research must retain momentum to keep up with the evolving features of population immunity. Research should continue to query the effects of prior vaccination against ancestral SARS-CoV-2 strains on infection with novel VOCs, and vice versa. Cross-reactivity between VOCs is varied but generally high, and therefore OAS or the lack thereof should be a focus of studies of sequential infection with VOCs. Finally, emergence of novel coronaviruses from animal reservoirs remains a global threat. Most of the world's population now has some level of immunity against betacoronaviruses – whether this offers protection against future emerging coronaviruses is as yet unknown.

Overall, this thesis explores cross-reactive immunity to SARS-CoV-2 through multiple perspectives: intrafamilial transmission and exposure, T-cell immunity in seronegative individuals, cross-reactive antibodies in COVID-19 severity, and vaccination with BNT162b2 in adolescents. The data paints a complex picture of the adaptive immune system following viral infection and vaccination, but it also provides some clarity as to the mechanisms behind heterogeneous susceptibility to COVID-19. Furthermore, this thesis calls for a cautious optimism that cross-reactive immunity, although not always beneficial in COVID-19, is able to continuously evolve following infection and vaccination, tending asymptotically towards protection in the face of ever-changing viral threats.

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8 Appendix

8.1 Consent forms

Study title: SARS-CoV-2 Immunity in Healthcare Worker Family/Household Members

INFORMATION SHEET FOR ADULT STUDY PARTICIPANTS

Ethics Approval Reference: R71346/RE001

Philip Goulder philip.goulder@paediatrics.ox.ac.uk
Peter Medawar Building for Pathogen Research, South Parks Rd, Oxford OX1 3SY

We would like to invite you to be part of this study being undertaken at The University of Oxford and Cardiff University investigating immune responses to coronavirus SARS-CoV-2 (the virus causing COVID-19 or "COVID"). We very much hope you would like to take part, but before you decide, it is important that you understand why the study is being done and what it will involve.

What are we trying to find out?

Outcome from exposure to SARS-CoV-2, the virus that causes COVID-19, is extremely variable. Young people – children and adolescents – appear to become infected less easily by the virus, and even if they do become infected, they suffer less disease than older people. Also, it is clear that, despite exposure to other family or household members who have been infected by SARS-CoV-2, some family/household members appear to remain uninfected. The reasons for these differences in outcome from exposure to SARS-CoV-2 are unknown, and this is what this study aims to find out. Understanding what immune responses may prevent infection or may reduce illness in someone who becomes infected will help to develop treatments to reduce the impact of the SARS-CoV-2 epidemic.

More information about the project can be obtained by contacting the research team lead (contact details below).

Why have I been invited to take part?

We are inviting you to take part because you are an adult living in the same family/household as one of the healthcare workers enrolled onto our related research study of immune responses in healthcare workers who have tested positive for coronavirus SARS-CoV-2 infection. This means that you are more likely to have been exposed to SARS-CoV-2 infection. We are inviting approximately 200 adults – that is, the healthcare worker and their partner - in addition to 200 young people aged 8-18yrs, to take part in this study.

Do I have to take part?

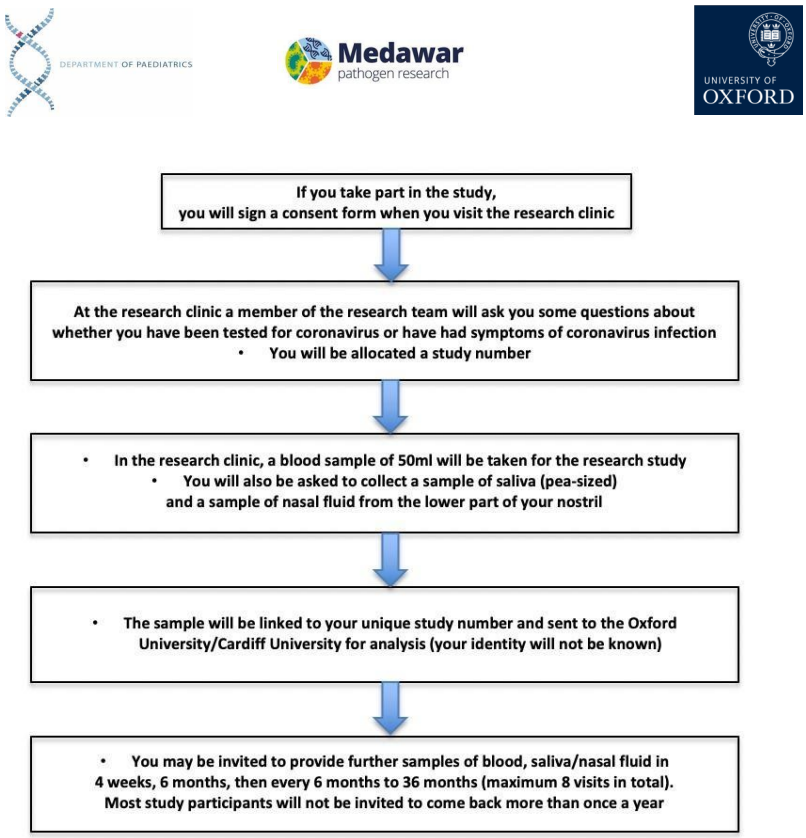
No. You can ask questions about the study before deciding whether or not to participate. If you do agree to participation, you may withdraw from the study at any time, without giving a reason and without penalty, by advising the researchers of this decision.

Figure 8.1: Appendix 1.1. Information sheet for Family cohort adult participants (Page 1/5).

What will happen I take part?

If you decide to take part in the study, then we will ask for your permission (via a consent form) to ask a few simple questions via an email questionnaire about previous tests or symptoms of coronavirus SARSCoV-2 infection. This questionnaire will take approximately 5 minutes to complete. General data protection regulation (GDPR) will be followed according to the University of Oxford and HealthFirst Consulting Ltd data protection policies. At the initial clinic visit, we will take a 50 ml blood sample, equivalent to three tablespoons. You will also be asked to collect a saliva sample and a nasal swab from the lower part of your nostril. Over the next 3 years following this initial visit you will be invited to provide further samples of blood, saliva and nasal swab at time intervals as described below and in the diagram. How often we invite you back for further visits depends on whether immune responses to SARS-CoV-2 are detectable in the samples taken at the initial visit. If we detect responses to SARS-CoV-2 at the initial visit we will invite you back in 4 weeks, and every 6 months. If no SARS-CoV-2 responses are detectable at the initial visit, we will invite you back once a year to see if responses have developed in that time. The following diagram summarises what will happen if you choose to take part in the study:

Figure 8.2: Appendix 1.2. Information sheet for Family cohort adult participants (Page 2/5).



The research study is planned to continue for up to 3 years and, if you are willing, you would also be involved in the research study for that time. The research team may then continue to study the samples you provided for a further 5 years beyond this 3 years – a total of 8 years from the start of the study. In most cases, following the initial visit, you would be asked to attend visits no more than once every 12 months. In a smaller subset of individuals, you would also be asked to attend for a repeat visit 4 weeks after the initial visit, and then to attend visits no more than once every 6 months. In this subset of people participating in the study, we will also request a saliva sample to be collected at 12 weeks following the initial visit. However, this will not require an additional clinic visit as you will be able to collect the saliva sample yourself at home. Research visits will take place at the University clinic at the Churchill Hospital in Oxford or a similar University (non-NHS) clinic in Oxford. In Cardiff, this will be at a non-NHS facility at an independent research clinic registered with Health Inspectorate Wales, HealthFirst Consulting Limited, or via a home visit.

We would expect each clinic visit would take less than 30 minutes for each person attending the clinic. If there were 3 family members attending together, the visit might be expected to take an hour in total for all 3 family members.

What are the advantages / disadvantages of taking part?

There are no direct benefits to you of taking part in the study. The benefits are indirect in contributing to knowledge about the immune responses that reduce SARS-CoV-2 infection or reduce illness

Figure 8.3: Appendix 1.3. Information sheet for Family cohort adult participants (Page 3/5).

following SARSCoV-2 infection. The disadvantages of taking part are the inconvenience of attending research clinics and the discomfort associated with giving blood samples, which may result in fainting, bruising or localised pain, although discomfort from having blood taken can be reduced by application of local anaesthetic gel 30-45 minutes prior to the blood sample being taken.

NB Please see the supplementary information sheet detailing COVID safety measures for this study.

What happens to the data provided?

The information you provide during the study is the **research data**. Any research data from which you can be identified (name, age, sex and contact details) are known as **personal data**. This includes more sensitive categories of personal data such as your racial/ethnic origin and data concerning your health (previous symptoms of SARS-CoV-2 infection or tests done that indicate previous SARS-CoV-2 infection).

Personal / sensitive data held on the paper questionnaire will be kept in a fireproof lockable cabinet in the research premises by the clinical research team. This document will link your name and contact details to the study ID number. Data captured electronically from the paper questionnaire will then be transferred by typing onto an electronic storage means, excluding your name and contact details, and stored on encrypted electronic storage devices. Each study participant will be provided with a unique study number, and the laboratory researchers who process/analyse your samples will only see this study ID number, therefore will not be able to identify you. In this way, your personal/sensitive data will only be known to the clinical research team who will meet you and collect the blood and other samples as described above. Your contact details will be destroyed 3 years after the start of the study. Personal data that is not linked to name and contact details that are stored on encrypted storage devices will all be destroyed 8 years after the start of the study. Responsible members of the University of Oxford and Health Inspectorate Wales may be given access to data for monitoring and/or audit of the research.
Other research data will be also destroyed within 8 years after the start of the study.

Will the research be published?

The research may be published in scientific journals and/or on websites. You and your child will not be identifiable from these publications/websites.

What will happen to any samples taken from me?

We will use your samples to study immune responses to SARS-CoV-2, the virus that causes COVID19. We will study the antibody responses in blood, saliva and in the nasal secretions. We will study the T-cell responses against the virus in the blood. T-cells are immune cells that can recognise and kill viruses. Finally, using the blood samples, we will study the immune activity that forms the 'first line of defence' against the virus – known as the innate immune response. The samples will be stored in the Peter Medawar Building for up to 8 years after the start of the study of the samples, in order that the samples may be used for future studies to further evaluate the immune responses to SARS-CoV-2. If you wish to know more on this, please discuss with a member of the research team (contact details below).

Who is conducting this research?

Figure 8.4: Appendix 1.4. Information sheet for Family cohort adult participants (Page 4/5).

The research project is organised by Professor Philip Goulder of the University of Oxford, who is a Professor of Immunology as well as being a paediatrician in the University Department of Paediatrics. The research is funded by the University Medical Sciences Division funding for COVID-19. This study has been reviewed by, and received ethics clearance through, the University of Oxford's Central University Research Ethics Committee, [CUREC reference number R71346/RE001].

What if there is a problem?

If you have a concern about any aspect of this study, please contact Professor Philip Goulder 01865-281884,Philip.goulder@paediatrics.ox.ac.uk or Dr Donal Skelly (01865 281884 donal.skelly@ndcn.ox.ac.uk) or in Wales, Dr Lucy C Jones (07792244418), lucy.jones14@wales.nhs.uk) and we will do our best to answer your query. We will acknowledge your concern within 10 working days and give you an indication of how it will be dealt with. If you remain unhappy or wish to make a formal complaint, please contact the Chair of the Medical Sciences Interdivisional Research Ethics Committee at the University of Oxford who will seek to resolve the matter as soon as possible: Email: ethics@medsci.ox.ac.uk; Address: Research Services, University of Oxford, Wellington Square, Oxford OX1 2JD

Data Protection

The University of Oxford is the data controller with respect to your personal data and, as such, will determine how your personal data are used in the study. The University will process your personal data for the purpose of the research outlined above. Research is a task that we perform in the public interest. Further information about your rights with respect to your personal data is available from https://compliance.web.ox.ac.uk/individual-rights.

What should I do next? Research Team contact details:

Please send an email to the study lead researchers Professor Philip Goulder at philip.goulder@paediatrics.ox.ac.uk or Dr Donal Skelly at donal.skelly@ndcn.ox.ac.uk or, in Wales, Dr Lucy C Jones at lucycjones@wales.nhs.uk, if you would like to take part in this study or if you wish to obtain further information about the study. Please remember that you may withdraw from the study at any time, without penalty and without giving a reason, by notifying the researcher.

Figure 8.5: Appendix 1.5. Information sheet for Family cohort adult participants (Page 5/5).

Study title: SARS-CoV-2 Immunity in Healthcare Worker Family/Household Members

INFORMATION SHEET FOR YOUNG PEOPLE AGED 8 TO 10

YEARS Ethics Approval Reference: R71346/RE001

Our names are Philip Goulder, Donal Skelly and Lucy Jones. Philip and Donal work at the University of Oxford in the Department of Paediatrics and Lucy works at Cardiff University, Wales. We are doing some research and would like you to join in.

Research is a way we try to find out the answers to questions. This research is about the virus called coronavirus that causes the disease known as COVID. We want to understand why some people get very unwell when they are infected with the virus whereas other people do not get ill at all. Children and young people usually do not get ill if they become infected with this virus, but older people can get very ill. This is why we want to study young people such as yourself.

You can talk to your family, friends, or the researchers if you want to before you agree to join in.

Why have I been asked?

We are asking you if you would like to take part because you are between 8 and 10 years old.

We are asking 200 children to help us.

Your parent/guardian has said it is OK for you to join in.

Do I have to join in?

No, you don't have to if you don't want to! You can ask questions before choosing whether you want to join in.

You can change your mind at any time by telling the researcher or your parent/guardian. You don't have to say why.

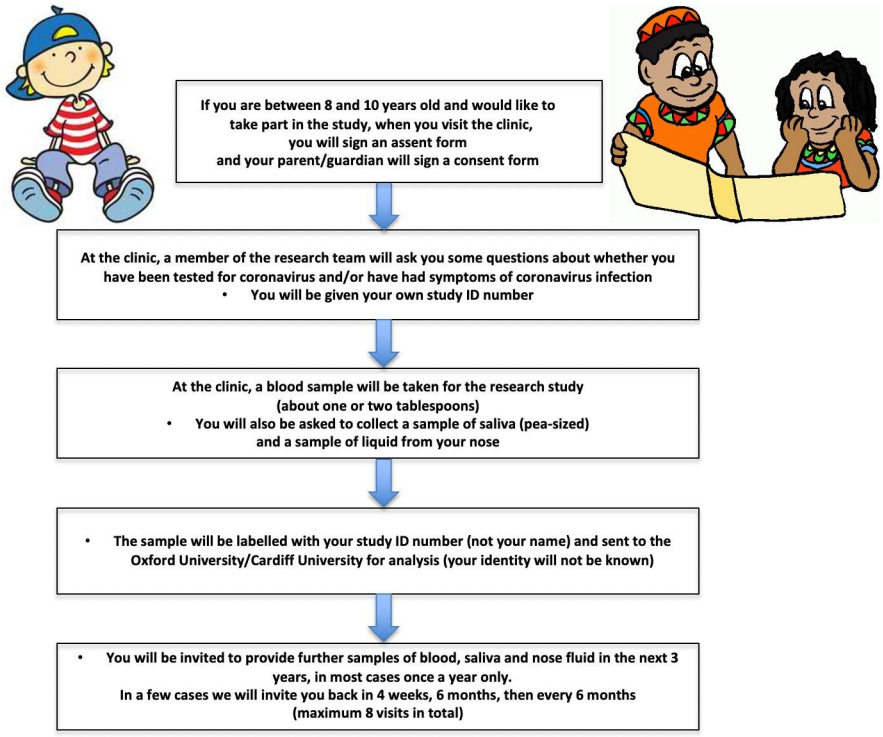
If you decide to stop, no one will be upset with you.

What will happen?

If you decide to take part in the study, we will ask your parent/guardian to bring you to our clinic. If you have any questions we will try to answer them. Then if you and your parent/guardian give us permission to go ahead with you as part of the study, we will first ask a few simple questions about previous tests or symptoms of coronavirus infection. This will take about 5 minutes. We will then take a blood sample from you. The amount of blood we will take is equivalent to one to two tablespoons. We will also collect a saliva sample and a nose swab from the lower part of your nostril. We will invite you to come back to the clinic to provide further samples of blood, saliva and nose swabs over the next 3 years after this first clinic visit. You won't need to miss any school to take part.

Figure 8.6: Appendix 2.1. Information sheet for Family cohort child participants (Page 1/3).

The following diagram summarises what will happen if you choose to take part in the study:



These research visits will take place at a clinic in Oxford or Cardiff. We expect each clinic visit to take less than 30 minutes for each person attending the clinic, or one hour in total for all family members.

Will anything about the research upset me?

No-one likes having blood tests. If you are worried about the blood tests you do not need to join in. However, if you do want to do this, your parent/guardian can put some numbing gel on your arm before you come into the clinic to reduce the pain of the blood test . The saliva test and the swab in your nose do not hurt.

Will joining in help me?

The study will not help you, but it might help other children in the future.

Figure 8.7: Appendix 2.2. Information sheet for Family cohort child participants (Page 2/3).

Will anyone else know I'm doing this?

The people in our research team will know you are taking part. No one else will know that you have helped us with this research - unless, of course, you tell them yourself!

What happens to what the researchers find out?

When we collect information from you, we will keep it in a safe place and only the people doing the research, or helping with the research, can look at it. We will use the information to publish the results in scientific publications (like newspapers) so that we can share what we have discovered from the study. But no-one will be able to identify you from anything that we publish.

Is this study OK to do? Before any research involving people happens it has to be checked by a group of people known as a Research Ethics Committee to make sure that it is fair. This study has been checked by the Ethics Committee at the University of Oxford.

What if there is a problem or something goes wrong? If you are not happy because of something that happened in the study, please talk to your parent/guardian who will let the researcher know.

Thank you for reading – please ask us any questions.

Figure 8.8: Appendix 2.3. Information sheet for Family cohort child participants (Page 3/3).

ID	Sequence
SARS-CoV-2 S1	
S_1	MFVFLVLLPLVSSQCVNL
S_2	PLVSSQCVNLTTRTQL
S_3	CVNLTTRTQLPPAYTNSF
S_4	QLPPAYTNSFTRGVYY
S_5	TNSFTRGVYYPDKVFR
S_6	GVYYPDKVFRSSVLHSTQ
S_7	FRSSVLHSTQDLFLPFF
S_8	STQDLFLPFFSNVTWF
S_9	LPFFSNVTWFHAIHV
S_10	NVTWFHAIHVSGTNGTKR
S_11	HVSGTNGTKRFDNPVLPF
S_12	KRFDNPVLPFNDGVYF
S_13	VLPFNDGVYFASTEKSNI
S_14	YFASTEKSNIIRGWIF
S_15	KSNIIRGWIFGTTLDSK
S_16	WIFGTTLDSKTQSLLIV
S_17	DSKTQSLLIVNNATNVVI
S_18	IVNNATNVVIKVCEFQF
S_19	VVIKVCEFQFCNDPFLGV
S_20	QFCNDPFLGVYYHKNNK
S_21	LGVYYHKNNKSWMESEFR
S_22	NKSWMESEFRVYSSANNC
S_23	FRVYSSANNCTFEYV
S_24	SANNCTFEYVSQPFLMDL
S_25	YVSQPFLMDLEGKQGNFK
S_26	DLEGKQGNFKNLREFVFK
S_27	FKNLREFVFKNIDGYFKI
S_28	FKNIDGYFKIYSKHTPI
S_29	FKIYSKHTPINLVRDL
S_30	HTPINLVRDLPQGFSAL
S_31	RDLPQGFSALEPLVDLPI
S_32	ALEPLVDLPIGINITRF
S_33	LPIGINITRFQTLLALHR
S_34	RFQTLLALHRSYLTPGDS
S_35	HRSYLTPGDSSSGWTAGA
S_36	DSSSGWTAGAAAYYVGYL
S_37	GAAAYYVGYLQPRTFLLK
S_38	YLQPRTFLLKYNENGTI
S_39	LLKYNENGTITDAVDCAL
S_40	TITDAVDCALDPLSETK
S_41	CALDPLSETKCTLKSFTV
S_42	TKCTLKSFTVEKGIY
S_43	KSFTVEKGIYQTSNFRV
S_44	GIYQTSNFRVQPTESIVR
S_45	RVQPTESIVRFPNITNL
S_46	IVRFPNITNLCPFGEVF
S_47	TNLCPFGEVFNATRFASV
S_48	VFNATRFASVYAWNRKRI
S_49	SVYAWNRKRISNCVADY
S_50	KRISNCVADYSVLYNSAS
S_51	DYSVLYNSASFSTFKCY
S_52	SASFSTFKCYGVSPTKL
S_53	KCYGVSPTKLNDLCFTNV
S_54	KLNDLCFTNVYADSFVIR
S_55	NVYADSFVIRGDEVRQI
S_56	VIRGDEVRQIAPGQTGKI
S_57	QIAPGQTGKIADYNYKL
S_58	GKIADYNYKLPDDFTGCV
S_59	KLPDDFTGCVIAWNSNNL
S_60	CVIAWNSNNLDSKVGGNY
S_61	NLDSKVGGNYNYLYRLFR
S_62	NYNYLYRLFRKSNLKPF
S_63	LFRKSNLKPFERDISTEI
S_64	PFERDISTEIYQAGSTPC
S_65	EIYQAGSTPCNGVEGF
S_66	STPCNGVEGFNCYFPL
S_67	VEGFNCYFPLQSYGF
S_68	CYFPLQSYGFQPTNGVGY
S_69	GFQPTNGVGYQPYRVVVL
S_70	GYQPYRVVVLSFELL
S_71	RVVVLSFELLHAPATV
S_72	FELLHAPATVCGPKK
S_73	APATVCGPKKSTNLVKNK
S_74	KKSTNLVKNKCVNFNF
S_75	VKNKCVNFNFNGLTGTGV
S_76	NFNGLTGTGVLTESNKKF
S_77	GVLTESNKKFLPFQQFGR
S_78	KFLPFQQFGRDIADTTDA
S_79	GRDIADTTDAVRDPQTL
S_80	TDAVRDPQTLEILDI
S_81	DPQTLEILDITPCSFGGV
S_82	DITPCSFGGVSVITPGTN
S_83	GVSVITPGTNTSNQVAVL
S_84	TNTSNQVAVLYQDVNCTE
S_85	VLYQDVNCTEVPVAI
S_86	VNCTEVPVAIHADQL
S_87	VPVAIHADQLTPTWRVY
S_88	DQLTPTWRVYSTGSNVF
S_89	RVYSTGSNVFQTRAGCLI
S_90	VFQTRAGCLIGAEHV
S_91	AGCLIGAEHVNNSYECDI
S_92	HVNNSYECDIPIGAGI
S_93	ECDIPIGAGICASYQTQT

Figure 8.9: Appendix 3.1. Peptides for T-cell proliferation and ELISpot assays.

SARS-CoV-2 S2	
S_94	GICASYQTQTNSPRRAR
S_95	TQTNSPRRARSVASQSII
S_96	ARSVASQSIIAYTMSL
S_97	QSIIAYTMSLGAENSVAY
S_98	SLGAENSVAYSNNSIAI
S_99	VAYSNNSIAIPTNFTISV
S_100	AIPTNFTISVTTEILPV
S_101	ISVTTEILPVSMTKTSV
S_102	LPVSMTKTSVDCTMYI
S_103	KTSVDCTMYICGDSTECS
S_104	YICGDSTECSNLLLQY
S_105	TECSNLLLQYGSFCTQL
S_106	LQYGSFCTQLNRALTGI
S_107	TQLNRALTGIAVEQDK
S_108	LTGIAVEQDKNTQEVF
S_109	EQDKNTQEVFAQVKQIYK
S_110	VFAQVKQIYKTPPIKDF
S_111	IYKTPPIKDFGGFNFSQI
S_112	DFGGFNFSQILPDPSK
S_113	FSQILPDPSKPSKRSFI
S_114	PSKPSKRSFIEDLLFNKV
S_115	FIEDLLFNKVTLADAGFI
S_116	KVTLADAGFIKQYGDCL
S_117	GFIKQYGDCLGDIAARDL
S_118	CLGDIAARDLICAQKF
S_119	ARDLICAQKFNGLTVL
S_120	AQKFNGLTVLPPLLTDEM
S_121	VLPPLLTDEMIAQYTSAL
S_122	EMIAQYTSALLAGTI
S_123	YTSALLAGTITSGWTF
S_124	AGTITSGWTFGAGAALQI
S_125	TFGAGAALQIPFAMQMAY
S_126	QIPFAMQMAYRFNGIGV
S_127	MAYRFNGIGVTQNVLY
S_128	GIGVTQNVLYENQKLI
S_129	NVLYENQKLIANQFNSAI
S_130	LIANQFNSAIGKIQDSL
S_131	SAIGKIQDSLSSTASAL
S_132	DSLSSTASALGKLQDVV
S_133	SALGKLQDVVNQNAQAL
S_134	DVVNQNAQALNTLVKQL
S_135	QALNTLVKQLSSNFGAI
S_136	KQLSSNFGAISSVLNDIL
S_137	AISSVLNDILSRLDKV
S_138	NDILSRLDKVEAEVQIDR
S_139	KVEAEVQIDRLITGRL
S_140	QIDRLITGRLQSLQTYV
S_141	GRLQSLQTYVTQQLIR
S_142	QTYVTQQLIRAAEIR
S_143	QQLIRAAEIRASANL
S_144	AAEIRASANLAATKM
S_145	ASANLAATKMSECVL
S_146	AATKMSECVLGQSKRVDF
S_147	VLGQSKRVDFCGKGYHLM
S_148	DFCGKGYHLMSFPQSAPH
S_149	LMSFPQSAPHGVVFLHV
S_150	APHGVVFLHVTYVPAQEK
S_151	HVTYVPAQEKNFTTAPAI
S_152	EKNFTTAPAICHDGKAHF
S_153	AICHDGKAHFPREGVFV
S_154	AHFPREGVFVSNGTHWFV
S_155	FVSNGTHWFVTQRNFY
S_156	HWFVTQRNFYEPQII
S_157	QRNFYEPQIITTDNTFV
S_158	QIITTDNTFVSGNCDVVI
S_159	FVSGNCDVVIGIVNNTVY
S_160	VIGIVNNTVYDPLQPEL
S_161	TVYDPLQPELDSFKEEL
S_162	PELDSFKEELDKYFK
S_163	FKEELDKYFKNHTSPDV
S_164	YFKNHTSPDVDLGDISGI
S_165	DVDLGDISGINASVVNI
S_166	SGINASVVNIQKEIDRL
S_167	VNIQKEIDRLNEVAKNL
S_168	DRLNEVAKNLNESLIDL
S_169	KNLNESLIDLQELGKY
S_170	LIDLQELGKYEQYIKWPW
S_171	KYEQYIKWPWYIWLGFI
S_172	WPWYIWLGFIAGLIAIVM
S_173	FIAGLIAIVMVTIMLCCM
S_174	VMVTIMLCCMTSCCSCLK
S_175	CMTSCCSCLKGCCSCGSC
S_176	LKGCCSCGSCCKFDEDDS
S_177	SCCKFDEDDSEPVLKGVK
S_178	FDEDDSEPVLKGVKLHYT
SARS-CoV-2 M	
M(ORF5)_1	MADSNGTITVEELKKLL
M(ORF5)_2	ITVEELKKLLEQWNLVI
M(ORF5)_3	KLLEQWNLVIGFLFLTWI
M(ORF5)_4	VIGFLFLTWICLLQFAY
M(ORF5)_5	TWICLLQFAYANRNRFLY
M(ORF5)_6	AYANRNRFLYIIKLIFLW
M(ORF5)_7	LYIIKLIFLWLLWPVTL
M(ORF5)_8	FLWLLWPVTLACFVLAAV
M(ORF5)_9	TLACFVLAAVYRINWI

Figure 8.10: Appendix 3.2. Peptides for T-cell proliferation and ELISpot assays.

M(ORF5)_10	LAAVYRINWITGGIAIAM
M(ORF5)_11	WITGGIAIAMACLVGLMW
M(ORF5)_12	AMACLVGLMWLSYFIASF
M(ORF5)_13	MWLSYFIASFRLFARTR
M(ORF5)_14	ASFRLFARTRSMWSF
M(ORF5)_15	FARTRSMWSFNPETNILL
M(ORF5)_16	SFNPETNILLNVPLHGTI
M(ORF5)_17	LLNVPLHGTILTRPLL
M(ORF5)_18	HGTILTRPLLESELVI
M(ORF5)_19	RPLLESELVIGAVILR
M(ORF5)_20	ELVIGAVILRGHLRI
M(ORF5)_21	AVILRGHLRIAGHHLGR
M(ORF5)_22	LRIAGHHLGRCDIKDLPK
M(ORF5)_23	GRCDIKDLPKEITVATSR
M(ORF5)_24	PKEITVATSRTLSYYKL
M(ORF5)_25	TSRTLSYYKLGASQRV
M(ORF5)_26	YYKLGASQRVAGDSGF
M(ORF5)_27	SQRVAGDSGFAAYSRYRI
M(ORF5)_28	GFAAYSRYRIGNYKL
M(ORF5)_29	SRYRIGNYKLNTDHSSSS
M(ORF5)_30	KLNTDHSSSSDNIALLV
M(ORF5)_31	KLNTDHSSSSDNIALLVQ

SARS-CoV-2 N

N(ORF9)_1	MSDNGPQNQRNAPRITF
N(ORF9)_2	NQRNAPRITFGGPSDSTG
N(ORF9)_3	TFGGPSDSTGSNQNGER
N(ORF9)_4	STGSNQNGERSGARSKQR
N(ORF9)_5	ERSGARSKQRRPQGL
N(ORF9)_6	RSKQRRPQGLPNNTASWF
N(ORF9)_7	GLPNNTASWFTALTQHGK
N(ORF9)_8	WFTALTQHGKEDLKFPR
N(ORF9)_9	HGKEDLKFPRGQGVPI
N(ORF9)_10	KFPRGQGVPINTNSSPDD
N(ORF9)_11	PINTNSSPDDQIGYYRR
N(ORF9)_12	PDDQIGYYRRATRRIR
N(ORF9)_13	YYRRATRRIRGGDGKMK
N(ORF9)_14	RIRGGDGKMKDLSPRWYF
N(ORF9)_15	MKDLSPRWYFYYLGTGPE
N(ORF9)_16	YFYYLGTGPEAGLPY
N(ORF9)_17	GTGPEAGLPYGANKDGII
N(ORF9)_18	PYGANKDGIIWVATEGAL
N(ORF9)_19	IIWVATEGALNTPKDHI
N(ORF9)_20	GALNTPKDHIGTRNPANN
N(ORF9)_21	HIGTRNPANNAAIVLQL
N(ORF9)_22	ANNAAIVLQLPQGTTLPK
N(ORF9)_23	QLPQGTTLPKGFYAEGSR
N(ORF9)_24	PKGFYAEGSRGGSQASSR
N(ORF9)_25	SRGGSQASSRSSSRSR
N(ORF9)_26	ASSRSSSRSRNSSRNSTP
N(ORF9)_27	SRNSSRNSTPGSSRGTSP
N(ORF9)_28	TPGSSRGTSPARMAGNGG
N(ORF9)_29	SPARMAGNGGDAALALLL
N(ORF9)_30	GGDAALALLLLDRLNQL
N(ORF9)_31	LLLLDRLNQLESKMSGK
N(ORF9)_32	NQLESKMSGKGQQQQGQT
N(ORF9)_33	GKGQQQQGQTVTKKSAAE
N(ORF9)_34	QTVTKKSAAEASKKPRQK
N(ORF9)_35	AEASKKPRQKRTATKAY
N(ORF9)_36	RQKRTATKAYNVTQAFGR
N(ORF9)_37	AYNVTQAFGRRGPEQTQG
N(ORF9)_38	GRRGPEQTQGNFGDQELI
N(ORF9)_39	QGNFGDQELIRQGTDYK
N(ORF9)_40	ELIRQGTDYKHWPQIAQF
N(ORF9)_41	YKHWPQIAQFAPSASAFF
N(ORF9)_42	QFAPSASAFFGMSRIGM
N(ORF9)_43	AFFGMSRIGMEVTPSGTW
N(ORF9)_44	GMEVTPSGTWLTYTGAIK
N(ORF9)_45	TWLTYTGAIKLDDKDPNF
N(ORF9)_46	IKLDDKDPNFKDQVILL
N(ORF9)_47	PNFKDQVILLNKHIDAYK
N(ORF9)_48	LLNKHIDAYKTFPPTEPK
N(ORF9)_49	YKTFPPTEPKKDKKKK
N(ORF9)_50	TEPKKDKKKKADETQAL
N(ORF9)_51	KKKADETQALPQRQKK
N(ORF9)_52	TQALPQRQKKQQTVTLL
N(ORF9)_53	QKKQQTVTLLPAADLDDF
N(ORF9)_54	LLPAADLDDFSKQLQQSM
N(ORF9)_55	DFSKQLQQSMSSADSTQA

HCoV-OC43 S

OC43_S_1	MFLILLISLPTAFAVI
OC43_S_2	ISLPTAFAVIGDLNCPL
OC43_S_3	AVIGDLNCPLDPRLKGSF
OC43_S_4	PLDPRLKGSFNNRDTGPP
OC43_S_5	SFNNRDTGPPSISTDTV
OC43_S_6	GPPSISTDTVDVTNGL
OC43_S_7	TDTVDVTNGLGTYYVLDR
OC43_S_8	GLGTYYVLDRVYLNTTLF
OC43_S_9	DRVYLNTTLFLNGYY
OC43_S_10	NTTLFLNGYYPTSGSTYR
OC43_S_11	YYPTSGSTYRNMALK
OC43_S_12	GSTYRNMALKGTDLLSTL
OC43_S_13	LKGTDLLSTLWFKPPFL
OC43_S_14	STLWFKPPFLSDFINGIF
OC43_S_15	FLSDFINGIFAKVKNTKV
OC43_S_16	IFAKVKNTKVFKDGVMY
OC43_S_17	TKVFKDGVMYSEFPAITI

Figure 8.11: Appendix 3.3. Peptides for T-cell proliferation and ELISpot assays.

OC43_S_18	MYSEFPAITIGSTFV	OC43_S_66	FGFIEDSVFVPQPTGVF
OC43_S_19	PAITIGSTFVNTSYSVVV	OC43_S_67	VFVPQPTGVFTNHSVVY
OC43_S_20	FVNTSYSVVVQPRTI	OC43_S_68	GVFTNHSVVYAQHCFK
OC43_S_21	YSVVVQPRTINSTQDGV	OC43_S_69	SVVYAQHCFKAPKNF
OC43_S_22	RTINSTQDGVNKLQGLL	OC43_S_70	QHCFKAPKNFCPCSSCPG
OC43_S_23	DGVNKLQGLLEVSVCQY	OC43_S_71	NFCPCSSCPGKNNGI
OC43_S_24	GLLEVSVCQYNMCEHPHT	OC43_S_72	SSCPGKNNGIGTCPAGTN
OC43_S_25	QYNMCEHPHTICHPNL	OC43_S_73	GIGTCPAGTNYLTCDNL
OC43_S_26	HPHTICHPNLGNHFKELW	OC43_S_74	GTNYLTCDNLCTLDPITF
OC43_S_27	NLGNHFKELWHLDTGVV	OC43_S_75	NLCTLDPITFKAPDTYK
OC43_S_28	ELWHLDTGVVSCLYKRNF	OC43_S_76	ITFKAPDTYKCPQTKSLV
OC43_S_29	VVSCLYKRNFTYDVNATY	OC43_S_77	YKCPQTKSLVGIGEHCSG
OC43_S_30	NFTYDVNATYLYFHFY	OC43_S_78	LVGIGEHCSGLAVKSDY
OC43_S_31	NATYLYFHFYQEGGTFY	OC43_S_79	CSGLAVKSDYCGNNSCTC
OC43_S_32	HFYQEGGTFYAYFTDTGF	OC43_S_80	DYCGNNSCTCQPQAFLGW
OC43_S_33	FYAYFTDTGFVTKFLFNV	OC43_S_81	TCQPQAFLGWSADSCL
OC43_S_34	GFVTKFLFNVYLGMAL	OC43_S_82	FLGWSADSCLQGDKCNIF
OC43_S_35	LFNVYLGMALSHYYVMPL	OC43_S_83	CLQGDKCNIFANFILHDV
OC43_S_36	ALSHYYVMPLTCISRRDI	OC43_S_84	IFANFILHDVNNGLTCST
OC43_S_37	PLTCISRRDIGFTLEYWV	OC43_S_85	DVNNGLTCSTDLQKANTE
OC43_S_38	DIGFTLEYWVTPLTPRQY	OC43_S_86	STDLQKANTEIELGVCV
OC43_S_39	WVTPLTPRQYLLAFNQDG	OC43_S_87	NTEIELGVCVNYDLYGI
OC43_S_40	QYLLAFNQDGIIFNAV	OC43_S_88	VCVNYDLYGISGQGIFV
OC43_S_41	NQDGIIFNAVDCMSDFM	OC43_S_89	YGISGQGIFVEVNATYY
OC43_S_42	NAVDCMSDFMSEIKCK	OC43_S_90	IFVEVNATYYNSWQNLLY
OC43_S_43	SDFMSEIKCKTQSIAPPT	OC43_S_91	YYNSWQNLLYDSNGNLY
OC43_S_44	CKTQSIAPPTGVYELNGY	OC43_S_92	LLYDSNGNLYGFRDYI
OC43_S_45	PTGVYELNGYTVQPIADV	OC43_S_93	GNLYGFRDYITNRTFMI
OC43_S_46	GYTVQPIADVYRRKPDL	OC43_S_94	DYITNRTFMIHSCYSGRV
OC43_S_47	ADVYRRKPDLPNCNIEAW	OC43_S_95	MIHSCYSGRVSAAYHANS
OC43_S_48	DLPNCNIEAWLNDKSV	OC43_S_96	RVSAAYHANSSEPALLFR
OC43_S_49	IEAWLNDKSVPSPLNWER	OC43_S_97	NSSEPALLFRNIKCNYVF
OC43_S_50	SVPSPLNWERKTFSNCNF	OC43_S_98	FRNIKCNYVFNNSLTRQL
OC43_S_51	ERKTFSNCNFNMSSLMSF	OC43_S_99	VFNNSLTRQLQPINYSF
OC43_S_52	NFNMSSLMSFIQADSF	OC43_S_100	RQLQPINYSFDSYLGCVV
OC43_S_53	LMSFIQADSFTCNNI	OC43_S_101	SFDSYLGCVVNAYNSTAI
OC43_S_54	QADSFTCNNIDAAKIYGM	OC43_S_102	VVNAYNSTAISVQTCDL
OC43_S_55	NIDAAKIYGMCFSSITI	OC43_S_103	TAISVQTCDLTVGSGYCV
OC43_S_56	YGMCFSSITIDKFAIPNR	OC43_S_104	DLTVGSGYCVDYSKNRR
OC43_S_57	TIDKFAIPNRRKVDLQL	OC43_S_105	YCVDYSKNRRSRRAI
OC43_S_58	PNRRKVDLQLGNLGYL	OC43_S_106	SKNRRSRRAITTGYRF
OC43_S_59	DLQLGNLGYLQSSNYRI	OC43_S_107	RRAITTGYRFTNFEPFTV
OC43_S_60	GYLQSSNYRIDTTATSCQ	OC43_S_108	RFTNFEPFTVNSVNDSL
OC43_S_61	RIDTTATSCQLYYNL	OC43_S_109	FTVNSVNDSLEPVGGLY
OC43_S_62	ATSCQLYYNLPAANVSV	OC43_S_110	DSLEPVGGLYEIQIPSEF
OC43_S_63	YNLPAANVSVSRFNPSTW	OC43_S_111	LYEIQIPSEFTIGNMEEF
OC43_S_64	SVSRFNPSTWNKRFGFI	OC43_S_112	EFTIGNMEEFIQTSSPKV
OC43_S_65	STWNKRFGFIEDSVFV	OC43_S_113	EFIQTSSPKVTIDCAAFV

Figure 8.12: Appendix 3.4. Peptides for T-cell proliferation and ELISpot assays.

OC43_S_114	KVTIDCAAFVCGDYAACK
OC43_S_115	FVCGDYAACKLQLVEY
OC43_S_116	AACKLQLVEYGSFCDNI
OC43_S_117	VEYGSFCDNINAILTEV
OC43_S_118	DNINAILTEVNELLDTTQ
OC43_S_119	EVNELLDTTQLQVANSLM
OC43_S_120	TQLQVANSLMNGVTLSTK
OC43_S_121	LMNGVTLSTKLKDGVNF
OC43_S_122	STKLKDGVNFNVDDINF
OC43_S_123	VNFNVDDINFSPVLGCL
OC43_S_124	INFSPVLGCLGSECSK
OC43_S_125	LGCLGSECSKASSRSAI
OC43_S_126	CSKASSRSAIEDLLFDKV
OC43_S_127	AIEDLLFDKVKLSDVGFV
OC43_S_128	KVKLSDVGFVEAYNNCTG
OC43_S_129	FVEAYNNCTGGAEIRDLI
OC43_S_130	TGGAEIRDLICVQSYKGI
OC43_S_131	LICVQSYKGIKVLPPLL
OC43_S_132	KGIKVLPPLLSENQISGY
OC43_S_133	LLSENQISGYTLAATSAS
OC43_S_134	GYTLAATSASLFPPW
OC43_S_135	ATSASLFPPWTAAAGVPF
OC43_S_136	PWTAAAGVPFYLNVQYRI
OC43_S_137	PFYLNVQYRINGLGVTM
OC43_S_138	YRINGLGVTMDVLSQNQK
OC43_S_139	TMDVLSQNQKLIANAF
OC43_S_140	QNQKLIANAFNNALHAI
OC43_S_141	NAFNNALHAIQQGFDATN
OC43_S_142	AIQQGFDATNSALVKI
OC43_S_143	DATNSALVKIQAVVNANA
OC43_S_144	KIQAVVNANAEALNNLL
OC43_S_145	ANAEALNNLLQQLSNRF
OC43_S_146	NLLQQLSNRFGAISASL
OC43_S_147	NRFGAISASLQEILSRL
OC43_S_148	ASLQEILSRLDALEAEAQ
OC43_S_149	RLDALEAEAQIDRLINGR
OC43_S_150	AQIDRLINGRLTALNAYV
OC43_S_151	GRLTALNAYVSQQLSDST
OC43_S_152	YVSQQLSDSTLVKFSAAQ
OC43_S_153	STLVKFSAAQAMEKV
OC43_S_154	FSAAQAMEKVNECVK
OC43_S_155	AMEKVNECVKSQSSRINF
OC43_S_156	VKSQSSRINFCGNGNHII
OC43_S_157	NFCGNGNHIISLVQNAPY
OC43_S_158	IISLVQNAPYGLYFIHF
OC43_S_159	APYGLYFIHFNYVPTKYV
OC43_S_160	HFNYVPTKYVTAKVSPGL
OC43_S_161	YVTAKVSPGLCIAGNRGI
OC43_S_162	GLCIAGNRGIAPKSGYFV
OC43_S_163	GIAPKSGYFVNVNNTWMY
OC43_S_164	FVNVNNTWMYTGSGYYY
OC43_S_165	WMYTGSGYYYPEPITENN
OC43_S_166	YYPEPITENNVVVMSTCA
OC43_S_167	NNVVVMSTCAVNYTKAPY
OC43_S_168	CAVNYTKAPYVMLNTSI
OC43_S_169	APYVMLNTSIPNLPDFK
OC43_S_170	TSIPNLPDFKEELDQWFK
OC43_S_171	FKEELDQWFKNQTSV
OC43_S_172	DQWFKNQTSVAPHLSLDY
OC43_S_173	SVAPHLSLDYINVTFLDL
OC43_S_174	DYINVTFLDLQVEMNRL
OC43_S_175	LDLQVEMNRLQEAIKVL
OC43_S_176	NRLQEAIKVLNHSYINLK
OC43_S_177	VLNHSYINLKDIGTYEYY
OC43_S_178	LKDIGTYEYYVKWPWYVW
OC43_S_179	YYVKWPWYVWLLICLAGV
OC43_S_180	VWLLICLAGVAMLVLLFF
OC43_S_181	GVAMLVLLFFICCCTGCG
OC43_S_182	FFICCCTGCGTSCFKK
OC43_S_183	TGCGTSCFKKCGGCCDDY
OC43_S_184	KKCGGCCDDYTGYQELVI
OC43_S_185	CDDYTGYQELVIKTSHDD
	HCoV-HKU1 S
HKU1_S_Cl2_1	MFLIIFILPTTLAVIGDF
HKU1_S_Cl2_2	PTTLAVIGDFNCTNSFI
HKU1_S_Cl2_3	GDFNCTNSFINDYNKTI
HKU1_S_Cl2_4	SFINDYNKTIPRISEDVV
HKU1_S_Cl2_5	TIPRISEDVVDVSLGL
HKU1_S_Cl2_6	EDVVDVSLGLGTYYVLNR
HKU1_S_Cl2_7	GLGTYYVLNRVYLNTTLL
HKU1_S_Cl2_8	NRVYLNTTLLFTGYFPK
HKU1_S_Cl2_9	TLLFTGYFPKSGANFRDL
HKU1_S_Cl2_10	PKSGANFRDLALKGSIYL
HKU1_S_Cl2_11	DLALKGSIYLSTLWYK
HKU1_S_Cl2_12	SIYLSTLWYKPPFLSDF
HKU1_S_Cl2_13	WYKPPFLSDFNNGIFSKV
HKU1_S_Cl2_14	DFNNGIFSKVKNTKLYV
HKU1_S_Cl2_15	SKVKNTKLYVNNTLYSEF
HKU1_S_Cl2_16	YVNNTLYSEFSTIVIGSV
HKU1_S_Cl2_17	EFSTIVIGSVFVNTSYTI
HKU1_S_Cl2_18	SVFVNTSYTIVVQPHNGI
HKU1_S_Cl2_19	TIVVQPHNGILEITACQY
HKU1_S_Cl2_20	GILEITACQYTMCEY
HKU1_S_Cl2_21	TACQYTMCEYPHTVCKSK
HKU1_S_Cl2_22	EYPHTVCKSKGSIRNESW
HKU1_S_Cl2_23	SKGSIRNESWHIDSSEPL

Figure 8.13: Appendix 3.5. Peptides for T-cell proliferation and ELISpot assays.

HKU1_S_Cl2_24	SWHIDSSEPLCLFKKNF
HKU1_S_Cl2_25	EPLCLFKKNFTYNVSADW
HKU1_S_Cl2_26	NFTYNVSADWLYFHFY
HKU1_S_Cl2_27	SADWLYFHFYQERGVFY
HKU1_S_Cl2_28	HFYQERGVFYAYYADVGM
HKU1_S_Cl2_29	FYAYYADVGMPTTFLFSL
HKU1_S_Cl2_30	GMPTTFLFSLYLGTIL
HKU1_S_Cl2_31	LFSLYLGTILSHYYVMPL
HKU1_S_Cl2_32	ILSHYYVMPLTCNAI
HKU1_S_Cl2_33	YVMPLTCNAISSNTDNET
HKU1_S_Cl2_34	AISSNTDNETLEYWVTPL
HKU1_S_Cl2_35	ETLEYWVTPLSRRQYLL
HKU1_S_Cl2_36	TPLSRRQYLLNFDEHGVI
HKU1_S_Cl2_37	LLNFDEHGVITNAVDCSS
HKU1_S_Cl2_38	VITNAVDCSSSFLSEI
HKU1_S_Cl2_39	DCSSSFLSEIQCKTQSF
HKU1_S_Cl2_40	SEIQCKTQSFAPNTGVY
HKU1_S_Cl2_41	QSFAPNTGVYDLSGFTVK
HKU1_S_Cl2_42	VYDLSGFTVKPVATVYRR
HKU1_S_Cl2_43	VKPVATVYRRIPNLPDCD
HKU1_S_Cl2_44	RRIPNLPDCDIDNWLNNV
HKU1_S_Cl2_45	CDIDNWLNNVSVPSPLNW
HKU1_S_Cl2_46	NVSVPSPLNWERRIF
HKU1_S_Cl2_47	SPLNWERRIFSNCNFNL
HKU1_S_Cl2_48	RIFSNCNFNLSTLLRLV
HKU1_S_Cl2_49	FNLSTLLRLVHVDSF
HKU1_S_Cl2_50	LLRLVHVDSFSCNNLDK
HKU1_S_Cl2_51	DSFSCNNLDKSKIFGSCF
HKU1_S_Cl2_52	DKSKIFGSCFNSITVDKF
HKU1_S_Cl2_53	CFNSITVDKFAIPNRRR
HKU1_S_Cl2_54	DKFAIPNRRRDDLQL
HKU1_S_Cl2_55	PNRRRDDLQLGSSGFL
HKU1_S_Cl2_56	DLQLGSSGFLQSSNYKI
HKU1_S_Cl2_57	GFLQSSNYKIDISSSSCQ
HKU1_S_Cl2_58	KIDISSSSCQLYYSLPLV
HKU1_S_Cl2_59	CQLYYSLPLVNVTINNF
HKU1_S_Cl2_60	PLVNVTINNFNPSSWNRR
HKU1_S_Cl2_61	NFNPSSWNRRYGFGSFNL
HKU1_S_Cl2_62	RRYGFGSFNLSSYDVVY
HKU1_S_Cl2_63	FNLSSYDVVYSDHCFSV
HKU1_S_Cl2_64	VVYSDHCFSVNSDFCPCA
HKU1_S_Cl2_65	SVNSDFCPCADPSVV
HKU1_S_Cl2_66	FCPCADPSVVNSCVKSK
HKU1_S_Cl2_67	SVVNSCVKSKPPSAI
HKU1_S_Cl2_68	CVKSKPPSAICPAGTKYR
HKU1_S_Cl2_69	AICPAGTKYRHCDLDTTL
HKU1_S_Cl2_70	YRHCDLDTTLYVKNWCR
HKU1_S_Cl2_71	TTLYVKNWCRCSCLPDPI
HKU1_S_Cl2_72	CRCSCLPDPISTYSPNTC
HKU1_S_Cl2_73	PISTYSPNTCPQKKVVV
HKU1_S_Cl2_74	NTCPQKKVVVGIGEHCPG
HKU1_S_Cl2_75	VVGIGEHCPGLGINEEK
HKU1_S_Cl2_76	CPGLGINEEKCGTQL
HKU1_S_Cl2_77	INEEKCGTQLNHSSCF
HKU1_S_Cl2_78	GTQLNHSSCFCSPDAFL
HKU1_S_Cl2_79	SCFCSPDAFLGWSFDSCI
HKU1_S_Cl2_80	FLGWSFDSCISNNRCNIF
HKU1_S_Cl2_81	CISNNRCNIFSNFIFNGI
HKU1_S_Cl2_82	IFSNFIFNGINSGTTCSN
HKU1_S_Cl2_83	GINSGTTCSNDLLYSNTE
HKU1_S_Cl2_84	SNDLLYSNTEISTGVCV
HKU1_S_Cl2_85	NTEISTGVCVNYDLYGI
HKU1_S_Cl2_86	VCVNYDLYGITGQGIFK
HKU1_S_Cl2_87	YGITGQGIFKEVSAAYY
HKU1_S_Cl2_88	IFKEVSAAYYNNWQNLLY
HKU1_S_Cl2_89	YYNNWQNLLYDSNGNII
HKU1_S_Cl2_90	LLYDSNGNIIGFKDFL
HKU1_S_Cl2_91	GNIIGFKDFLTNKTYTIL
HKU1_S_Cl2_92	FLTNKTYTILPCYSGRV
HKU1_S_Cl2_93	TILPCYSGRVSAAFY
HKU1_S_Cl2_94	YSGRVSAAFYQNSSSPAL
HKU1_S_Cl2_95	FYQNSSSPALLYRNLK
HKU1_S_Cl2_96	SPALLYRNLKCSYVLNNI
HKU1_S_Cl2_97	LKCSYVLNNISFISQPFY
HKU1_S_Cl2_98	NISFISQPFYFDSYLGCV
HKU1_S_Cl2_99	FYFDSYLGCVLNAVNL
HKU1_S_Cl2_100	YFDSYLGCVLNAVNLTSY
HKU1_S_Cl2_101	VLNAVNLTSYSVSSCDLR
HKU1_S_Cl2_102	SYSVSSCDLRMGSGFCI
HKU1_S_Cl2_103	DLRMGSGFCIDYALPSSR
HKU1_S_Cl2_104	CIDYALPSSRRKRRGI
HKU1_S_Cl2_105	PSSRRKRRGISSPYRFV
HKU1_S_Cl2_106	SSRRKRRGISSPYRFVTF
HKU1_S_Cl2_107	ISSPYRFVTFEPFNVSFV
HKU1_S_Cl2_108	TFEPFNVSFVNDSVETV
HKU1_S_Cl2_109	SFVNDSVETVGGLFEIQI
HKU1_S_Cl2_110	TVGGLFEIQIPTNFTI
HKU1_S_Cl2_111	EIQIPTNFTIAGHEEFI
HKU1_S_Cl2_112	FTIAGHEEFIQTSSPKV
HKU1_S_Cl2_113	EFIQTSSPKVTIDCSAFV
HKU1_S_Cl2_114	KVTIDCSAFVCSNYAACH
HKU1_S_Cl2_115	FVCSNYAACHDLLSEY
HKU1_S_Cl2_116	AACHDLLSEYGTFCDNI
HKU1_S_Cl2_117	SEYGTFCDNINSILNEV
HKU1_S_Cl2_118	DNINSILNEVNDLLDI
HKU1_S_Cl2_119	LNEVNDLLDITQLQV

Figure 8.14: Appendix 3.6. Peptides for T-cell proliferation and ELISpot assays.

HKU1_S_Cl2_120	DLLDITQLQVANALMQGV
HKU1_S_Cl2_121	QVANALMQGVTLSSNL
HKU1_S_Cl2_122	MQGVTLSSNLNTNLHSDV
HKU1_S_Cl2_123	NLNTNLHSDVDNIDFKSL
HKU1_S_Cl2_124	DVDNIDFKSLLGCLGSQC
HKU1_S_Cl2_125	SLLGCLGSQCGSSSRSLL
HKU1_S_Cl2_126	QCGSSSRSLLEDLLFNKV
HKU1_S_Cl2_127	LLEDLLFNKVKLSDVGFV
HKU1_S_Cl2_128	KVKLSDVGFVEAYNNCTG
HKU1_S_Cl2_129	FVEAYNNCTGGSEIRDLL
HKU1_S_Cl2_130	TGGSEIRDLLCVQSFNGI
HKU1_S_Cl2_131	LLCVQSFNGIKVLPPIL
HKU1_S_Cl2_132	NGIKVLPPILSETQISGY
HKU1_S_Cl2_133	ILSETQISGYTTAATV
HKU1_S_Cl2_134	ISGYTTAATVAAMFPPW
HKU1_S_Cl2_135	ATVAAMFPPWSAAAGVPF
HKU1_S_Cl2_136	PWSAAAGVPFSLNVQYRI
HKU1_S_Cl2_137	PFSLNVQYRINGLGVTM
HKU1_S_Cl2_138	YRINGLGVTMDVLNKNQK
HKU1_S_Cl2_139	TMDVLNKNQKLIANAFNK
HKU1_S_Cl2_140	QKLIANAFNKALLSI
HKU1_S_Cl2_141	NAFNKALLSIQNGFTATN
HKU1_S_Cl2_142	SIQNGFTATNSALAKI
HKU1_S_Cl2_143	TATNSALAKIQSVVNANA
HKU1_S_Cl2_144	KIQSVVNANAQALNSLL
HKU1_S_Cl2_145	ANAQALNSLLQQLFNKF
HKU1_S_Cl2_146	SLLQQLFNKFGAISSSL
HKU1_S_Cl2_147	NKFGAISSSLQEILSRL
HKU1_S_Cl2_148	SSLQEILSRLDNLEAQV
HKU1_S_Cl2_149	SRLDNLEAQVQIDRLI
HKU1_S_Cl2_150	EAQVQIDRLINGRLTAL
HKU1_S_Cl2_151	RLINGRLTALNAYVSQQL
HKU1_S_Cl2_152	ALNAYVSQQLSDITLIK
HKU1_S_Cl2_153	QQLSDITLIKAGASRAI
HKU1_S_Cl2_154	LIKAGASRAIEKVNECVK
HKU1_S_Cl2_155	AIEKVNECVKSQSPRINF
HKU1_S_Cl2_156	VKSQSPRINFCGNGNHIL
HKU1_S_Cl2_157	NFCGNGNHILSLVQNAPY
HKU1_S_Cl2_158	ILSLVQNAPYGLLFIHF
HKU1_S_Cl2_159	APYGLLFIHFSYKPTSFK
HKU1_S_Cl2_160	HFSYKPTSFKTVLVSPGL
HKU1_S_Cl2_161	FKTVLVSPGLCLSGDRGI
HKU1_S_Cl2_162	GLCLSGDRGIAPKQGYFI
HKU1_S_Cl2_163	GIAPKQGYFIKQNDSWMF
HKU1_S_Cl2_164	FIKQNDSWMFTGSSYYY
HKU1_S_Cl2_165	WMFTGSSYYYPEPISDK
HKU1_S_Cl2_166	YYYPEPISDKNVVFM
HKU1_S_Cl2_167	PISDKNVVFMNSCSVNF
HKU1_S_Cl2_168	VFMNSCSVNFTKAPFIYL
HKU1_S_Cl2_169	NFTKAPFIYLNNSIPNL
HKU1_S_Cl2_170	IYLNNSIPNLSDFEAEL
HKU1_S_Cl2_171	PNLSDFEAELSLWFK
HKU1_S_Cl2_172	FEAELSLWFKNHTSI
HKU1_S_Cl2_173	SLWFKNHTSIAPNLTF
HKU1_S_Cl2_174	HTSIAPNLTFNSHINATF
HKU1_S_Cl2_175	TFNSHINATFLDLYYEM
HKU1_S_Cl2_176	INATFLDLYYEMNVI
HKU1_S_Cl2_177	LDLYYEMNVIQESIKSL
HKU1_S_Cl2_178	NVIQESIKSLNSSFINLK
HKU1_S_Cl2_179	SLNSSFINLKEIGTYEMY
HKU1_S_Cl2_180	LKEIGTYEMYVKWPWYIW
HKU1_S_Cl2_181	MYVKWPWYIWLLIVILFI
HKU1_S_Cl2_182	IWLLIVILFIIFLMILFF
HKU1_S_Cl2_183	FIIFLMILFFICCCTGCG
HKU1_S_Cl2_184	FFICCCTGCGSACFSK
HKU1_S_Cl2_185	TGCGSACFSKCHNCCDEY
HKU1_S_Cl2_186	SKCHNCCDEYGGHNDFVI
HKU1_S_Cl2_187	CDEYGGHNDFVIKASHDD

Figure 8.15: Appendix 3.7. Peptides for T-cell proliferation and ELISpot assays.

Cross-reactive S peptides

MFVFLVLLPLVSSQCVNL
TNSFTRGVYYPDKVFR
GVYYPDKVFRSSVLHSTQ
DSKTQSLLIVNNATNVVI
IVNNATNVVIKVCEFQF
QFCNDPFLGVYYHKNNK
FRVYSSANNCTFEYV
SANNCTFEYVSQPFLMDL
FKNIDGYFKIYSKHTPI
HTPINLVRDLPQGFSAL
LPIGINITRFQTLLALHR
RFQTLLALHRSYLTPGDS
TKCTLKSFTVEKGIY
KSFTVEKGIYQTSNFRV
GIYQTSNFRVQPTESIVR
RVQPTESIVRFPNITNL
IVRFPNITNLCPFGEVF
TNLCPFGEVFNATRFASV
VFNATRFASVYAWNRKRI
QIAPGQTGKIADYNYKL
GKIADYNYKLPDDFTGCV
KLPDDFTGCVIAWNSNNL
NLDSKVGGNYNYLYRLFR
STPCNGVEGFNCYFPL
VKNKCVNFNFNGLTGTGV
HVNNSYECDIPIGAGI
ARSVASQSIIAYTMSL
QSIIAYTMSLGAENSVAY
SLGAENSVAYSNNSIAI
VAYSNNSIAIPTNFTISV
YICGDSTECSNLLLQY
TECSNLLLQYGSFCTQL
LQYGSFCTQLNRALTGI
TQLNRALTGIAVEQDK
EQDKNTQEVFAQVKQIYK
VFAQVKQIYKTPPIKDF
DFGGFNFSQILPDPSK
FSQILPDPSKPSKRSFI
PSKPSKRSFIEDLLFNKV
FIEDLLFNKVTLADAGFI
CLGDIAARDLICAQKF
ARDLICAQKFNGLTVL
VLPPLLTDEMIAQYTSAL
EMIAQYTSALLAGTI
AGTITSGWTFGAGAALQI

DVVNQNAQALNTLVKQL
KQLSSNFGAISSVLNDIL
NDILSRLDKVEAEVQIDR
KVEAEVQIDRLITGRL
LMSFPQSAPHGVVFLHV
APHGVVFLHVTYVPAQEK
EKNFTTAPAICHDGKAHF
AICHDGKAHFPREGVFV
AHFPREGVFVSNGTHWFV
HWFVTQRNFYEPQII
PELDSFKEELDKYFK
FKEELDKYFKNHTSPDV
DVDLGDISGINASVVNI
VNIQKEIDRLNEVAKNL
DRLNEVAKNLNESLIDL
KYEQYIKWPWYIWLGFI

Figure 8.16: Appendix 3.8. Cross-reactive peptides for T-cell assays.