Psycho-behavioural Influences on Vaccine Success: Towards a Brief, Non-pharmacological Primary Care Intervention

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By

Kieran Ayling BSc. (Hons), MSc.

Division of Primary Care

School of Medicine

Abstract

Vaccines against infectious diseases are less effective in older adults than in younger adults. This is of significant clinical importance as older adults are also the most vulnerable to contracting, and suffering the most severe consequences of, infectious diseases. Prior research demonstrates that behavioural and psychological factors can modulate the immune system and, in turn, influence how well vaccines work. However, there is a relative paucity of work focusing on older adults. Further, studies have tended to consider only one behavioural or psychological factor at a time - meaning it is unclear which factor, or combination of factors, should be the target of interventions to enhance vaccine effectiveness. This thesis presents three distinct, yet inter-related, pieces of original research which sought to further our understanding of the behavioural and psychological influences on vaccinations and inform the future development of interventions to enhance vaccine effectiveness.

First, current approaches to measuring the immune response to vaccination (e.g., ELISAs) are limited in that they require large volumes of sera, antigen, and other consumables. This makes them expensive and time consuming, which limits their utility for larger studies. Thus, the first phase of the research involved establishing a novel high-throughput multiplex antigen microarray assay for quantifying influenza-specific IgG levels in sera. This involved adapting an existing microarray assay and validating it in a series of laboratory experiments. The microarray assay demonstrated acceptable intra- and inter-assay reliability and correlated well with ELISA (H1N1: rho=.534, p<.01; H3N2: rho=.802, p<.00001; B: rho=.454, p<.01). Crucially, the protocols developed could be adapted to suit a variety of research purposes in the future.

Second, a prospective longitudinal observational cohort study was conducted to investigate the influence of modifiable psychological and behavioural factors on short and long-term antibody responses to influenza vaccination in older adults (n=138). Diary methods, pedometers, and anthropometric measurements were used to assess nutrition, sleep, physical activity, affect and perceived stress repeatedly over the 2 weeks prior to, and 4 weeks following influenza vaccination. Greater positive affect across the measurement period was found in multivariate regression models to be a significant independent predictor of both short- (β=.189, p=.036) and long-term H1N1 antibody responses following vaccination (β=.296, p=.003): with greater positive affect predicting a more robust antibody response. However, positive affect on the day of vaccination was found be even more salient, explaining greater variance than positive affect over the longer period (H1N1 short term model: R^2=.077 vs .058; long term model: R^2=.136 vs .125). Greater variability in perceived stress over the measurement period predicted poorer long-term antibody responses to both H1N1 (β=-.268, p<.05) and B strains (β=-.255, p<.05), even after taking overall stress exposure into account.

Third, in view of the observed relationship between positive affect and immune responses to vaccination, and in particular the role of positive affect on the day of vaccination, a systematic scoping review was conducted to examine the effects of brief positive mood interventions on immunity. A moderate-sized (31 studies, 38 interventions), but relatively low quality, literature was identified. Few studies included older adults and none examined the effects on *in-vivo* immune challenges such as vaccination. While there was considerable heterogeneity in the form of interventions that elicited mood improvements, the clear majority of studies reported

enhanced immunological outcomes (87.1%). However, many interventions were unsuitable in their current form for implementation within the current already resource-stretched UK health system.

Together, the work presented in this thesis points to the potential utility of brief, positive affect enhancing interventions as a way of enhancing immune responses to vaccination in older adults. Further methodologically rigorous research is needed to systematically develop and evaluate positive affect interventions that are both acceptable for older adults and feasible for implementation in the NHS.

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Chapter 1: Infectious Disease in the Elderly

Chapter Synopsis

The aim of the following chapter is to introduce the reader to the central issue addressed throughout this thesis: vaccination. This is the primary therapeutic method of preventing infectious disease, but is known to be considerably less effective in older adults than other groups. This is of significant clinical importance as in industrialised countries older adults are also the most vulnerable to contracting, and suffering the most severe consequences of, infectious diseases. The chapter begins by outlining the disproportionate burden of infectious diseases on the elderly in industrialised countries with the concept of immunosenesence, the natural declining of immune function with age, introduced as a key explanatory factor. The following sections consider the immunological mechanisms underlying successful vaccination and how immunosenesence can undermine these mechanisms. Finally, attention is turned to the need for novel approaches to enhance vaccination outcomes in the elderly and the growing interest in the immune modulating effects of psychological and behavioural factors, which is considered more fully in chapters 2 & 3.

1.1 *The Burden of Infectious Disease*

Infectious diseases account for approximately one quarter of all deaths worldwide (Lozano et al., 2012) as well as vast morbidity and economic burden (Fonkwo, 2008). Common infectious diseases include [estimated annual deaths in thousands shown in square brackets]: Tuberculosis [2661], HIV/AIDS [1196], Malaria [1170], Influenza [508], Hepatitis (all forms) [308], E-coli [212], Typhoid [190], Measles [125], Shigellosis [123],

Whooping cough [81], Tetanus [61], Cholera [58] and Rabies [26] (Lozano et al., 2012). While the largest effects of infectious disease are undoubtedly felt in poorer, developing countries (Bygbjerg, 2012), wealthier countries still face huge healthcare challenges associated with infectious diseases. For example, in the United States (US) and the United Kingdom (UK), influenza & pneumonia together rank among the 10 leading causes of death (Heron, 2013; Office for National Statistics, 2013) and total annual deaths from all infectious diseases are estimated to be over 170,000 and 35,000 in these countries respectively (Health Protection Agency, 2005; National Intelligence Council, 2000).

In industrialised countries, older adults[1] bear the greatest burden of infectious disease with the highest hospitalisation (K. Christensen et al., 2009) and mortality rates (Pitman et al., 2003) attributable to infectious causes. Approximately 80-90% of all influenza-related deaths occur in those aged over 65 (Feng et al., 2012; Lui & Kendal, 1987; W. Thompson, Shay, & Weintraub, 2003) and there is evidence that the overall number and percentage of hospitalisations attributed to infectious diseases in the elderly is on the increase (Curns, Holman, Sejvar, Owings, & Schonberger, 2005; Saliba, Fediai, Edelstein, Markel, & Raz, 2013).

This state of affairs is particularly concerning considering the current worldwide trend towards an ageing population. The proportion of people across the globe aged over 60 years is expected to double from 11% in 2000 to 22% by 2050 (World Health Organization, 2012). In the UK alone, the 10 million people aged over 65 years in 2010 is estimated to rise to 19 million

[1] In this thesis, the terms 'older adults' and 'elderly' interchangeably to denote those aged ≥65 years, unless explicitly stated otherwise.

by 2050 (Cracknell, 2010). Ultimately, this population shift will lead to more individual and societal costs resulting from infectious diseases, making research into the causes and prevention of infectious disease in the elderly salient and timely.

1.2 *Immunosenesence*

Instrumental in the disproportionate burden of infectious diseases in the elderly is a natural decline of immunological competence that occurs with age, known as immunosenesence (Goronzy & Weyand, 2013). Immunosenesence is a well-observed phenomenon and is a term used to denote the collective wide-ranging decrease in the functional capacities of both the innate and adaptive arms of the immune system that occur with age, though the most pronounced decline is seen in adaptive immunity (i.e., B- and T-cells and their products; Simpson & Spielmann, 2013). These changes collectively have been shown to increase older adults' susceptibility to infection (for detailed accounts see Duggal & Lord, 2013; Ginaldi, Loreto, Corsi, Modesti, & De Martinis, 2001; Müller & Pawelec, 2013). To provide an illustrative example: the thymus is known to decrease in size with age (thymic involution), which leads to reduced production of new T-cells. This results in reduced T-cell receptor diversity, an increased memory to naive T-cell ratio, and lower overall numbers of naive T-cells; making the elderly more susceptible to infections from novel antigens (Fagnoni et al., 2000; Kohler et al., 2005; Naylor et al., 2005).

Given the high rates of mortality and hospitalisation from infectious causes in the elderly, preventative measures to reduce infectious disease incidence are frequently targeted at those aged over 65 years. Foremost amongst these measures are vaccinations, of which many are given as a priority to

older adults in industrialised countries (e.g., influenza, varicella zoster, pneumococcal). However, as will be explored in the following sections, immunosenesence also impacts negatively on the protection provided by vaccines.

1.3 *Combating Infectious Disease: Vaccination*

Vaccination is considered one of the greatest health care advances ever documented (Global Public Health Achievements Team CDC, 2011) saving an estimated six million deaths worldwide annually (Ehreth, 2003). For infectious diseases that are unlikely to have been naturally encountered before vaccination (e.g., rubella in UK children), the central immunological aim of vaccination is to induce a primary immune response, which leads to the production of long-lived memory cells. These memory cells circulate throughout the body and if the pathogen is encountered again via natural exposure, a rapid and pronounced protective response from the immune system can occur. In more commonly-circulating infections that are often encountered through natural exposure, such as influenza, vaccines can also provide benefits through inducing a secondary, rather than primary response.[2] This leads to a relatively short-lived but robust increase in protective antibodies circulating in the body, during which time the individual is considerably less susceptible to infection if exposed to the pathogen. This secondary response is typified in the case of the annual influenza vaccination, which is typically administered in the UK between September and January to provide increased protection in the months where influenza

[2] As the names suggest, a primary response refers to the first-time exposure to a pathogen or its associated antigens. A secondary response occurs on subsequent exposures and is immunologically distinct, characterised by faster production of the protective IgG antibody serotype.

viruses commonly circulate amongst the population (Department of Health, 2013a).

1.3.1 The Immune System

Before describing the immunological processes underlying vaccination, it is first necessary to provide here a brief overview of the immune system (readers are directed to Parham, 2009; Todd & Spickett, 2011 for more comprehensive texts on this topic). The immune system refers to the network of organs, cells, tissues and molecules that function to protect an organism from foreign substances that can potentially cause disease, known as pathogens (e.g., bacteria, viruses, parasites and fungi). It does this by identifying, repelling and/or destroying potential pathogens that come into contact with the organism through a variety of mechanisms. As such, the immune system can be considered a homeostatic system, in that it acts to keep the internal environment of the organism stable (Husband, 1995).

In humans, the immune system can be broadly conceptualised as two arms that work synergistically: innate and adaptive immunity. Innate immunity, as the name suggests, is present from birth and includes physical barriers as well as specialist cells and proteins. Physical barriers, such as skin, hair and mucosal surfaces, provide a first line of defence preventing potential pathogens from entering the blood stream or tissues. While physical barriers prevent most pathogens from entering the body, some inevitably enter the tissues and blood stream (e.g., through damaged skin). At this point, internal cells and proteins (including macrophages, natural killer cells, and complement proteins) which circulate around, or reside within, the tissues of the body provide 'surveillance' and are rapidly able to identify and destroy most pathogens. To be more specific, these components destroy pathogens

using secreted chemicals that cause cell lysis (breaking down the cell membrane) or through phagocytosis (pathogen ingestion and breakdown). While rapid, innate immune responses are not always successful in completely clearing pathogens, as many have evolved a variety of mechanisms to evade recognition or destruction (Ploegh, 1998).

Adaptive immune responses take much longer than innate responses (days to weeks), but result in the production and proliferation of highly specific cells and proteins that are extremely effective at neutralising, clearing, and destroying pathogens that have evaded destruction by innate responses. Crucially, adaptive immune responses also lead to the development of long lived 'memory cells' that circulate in the body long after a pathogen has been encountered and subsequently destroyed. This means that should the pathogen be encountered again, the response to the pathogen is much faster, vigorous, and more effective.

The following section provides a basic overview of the immune response following vaccination, which primarily relies on adaptive immune responses – although innate immune components are vital to the process. It should, however, be noted that there is some variation between the immune responses to different vaccines. Therefore, the description that follows represents a somewhat simplified account of the immunological response to vaccinations.

1.3.2 Immunological Response to Vaccination

Vaccination allows for individuals to be exposed deliberately to specific pathogens (i.e., disease causing viruses or bacteria), or their associated antigens, to acquire a degree of protection against future exposure in a

manner in which disease should not result (Roitt & Rabson, 2000). On a mechanistic level, vaccination exploits a fundamental feature of the adaptive immune system known as immunological memory. Upon primary exposure to a potentially harmful foreign substance (known as a pathogen) a cascade of immune processes results in the development of cells specific to molecules present on the pathogen's surface (known as antigens). These can be cells that combat the pathogen directly (such as B-cells and their products) or long-lived memory cells, which remain in the body in case of future exposure. If the pathogen and/or its associated antigens are encountered again, these circulating memory cells allow the immune system to mount a larger and more rapid targeted response, allowing for more effective destruction or clearing of the pathogen.

To achieve this, vaccines often contain inactivated or attenuated live organisms (i.e., organisms unable to replicate), or parts of these organisms, which induce a profound immune response without exposing the individual to the dangers associated with natural exposure (Department of Health, 2013a). These pathogen-specific components are mixed with preserving chemicals and in some cases chemical adjuvants that enhance the immune response to the vaccine in a non-specific manner.

Typically, although not exclusively, vaccines are administered via intra-muscular injection where innate immune components residing within the tissues, including complement proteins and dendritic cells, recognise the presence of pathogen-associated molecular patterns (PAMPs) on the vaccines surface. PAMPs are evolutionarily conserved groupings of molecules common to many foreign substances, but not the body's own cells, and are recognised by pathogen recognition receptors (including toll-like receptors) on the surface of many immune cells. This recognition of the pathogen

stimulates complement proteins to opsonize, or tag, the pathogen (aiding phagocytosis, or ingestion, of the pathogen) as well as inducing the production of cytokines (chemical messengers that can affect other cells) and chemokines (chemical attractants) (Siegrist, 2012). The released cytokines and chemokines in turn play a crucial role in attracting more immune cells to the area.

Antigen presenting cells (APCs; which include dendritic cells, macrophages and Langerghans' cells) present at, or attracted to, the site of vaccination, engulf the vaccine components and break them down into small fragments, or peptides, that are then 'presented' on the APCs surface via major histocompatibility complex (MHC) molecules. These APCs as well as non-processed antigens migrate to the local secondary lymph organ, typically a lymph node, via the afferent lymphatic vessels where the non-processed antigens are recognised by naive B lymphocytes (B-cells) and the APCs present antigen to T lymphocytes (T-Cells) with receptors that are capable of specific binding to the antigen. B- and T-cells are present in huge numbers in lymph organs, each with receptors capable of binding only to specific antigens. The large number of cells with variable receptors ensures that essentially all potential foreign antigens will be recognised by one or more of these cells. Upon recognition of the antigen, T- and B-cells proliferate and differentiate into many daughter cells with identical receptors capable of recognising the antigen in question, which then migrate to the site of injection. More specifically, B-cells differentiate into antibody-secreting plasma cells or long lived B- memory cells.

While all these pathogen-specific cells are important for combating future natural exposures, the major immunological correlates of vaccine-induced protection are considered to be plasma cell secreted antibodies (Siegrist,

2012). Therefore, vaccines are primarily developed to induce large numbers of these protective antibodies against the pathogen in question. Structurally, antibodies (also known as immunoglobulins) are 'Y-shaped' molecules, made up of two 'light' and two 'heavy' amino-acid chains with two identical, highly specific antigen-binding sites (see Figure 1-1). Their role in the immune system is to combat extracellular pathogens by (1) binding directing to the pathogen, neutralising its ability to invade cells or replicate and (2) opsonizing (chemically tagging) the pathogen and thus promoting phagocytosis of the pathogen by macrophages and a clearance of the infection (Siegrist, 2012).

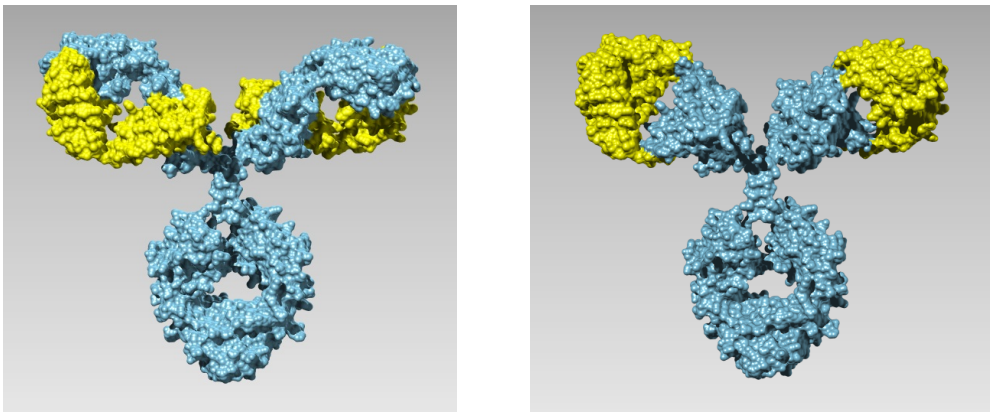


Figure 1-1: The structure of an antibody
On the left image, the heavy chain is shown in blue the light chain in yellow. On the right image antigen-binding receptors are shown in yellow. Images used with permisson from Dr Paddy Tighe.

There are five major classes of antibody in mammals (IgA, IgD, IgE, IgG, IgM), which can be distinguished by their differences in the amino acid sequences in the heavy chains. IgG can be further divided into four sub-classes, IgG1, IgG2, IgG3 and IgG4 and IgA into two subclasses IgA1 and IgA2. The primary antibody focus of vaccine research is typically total IgG, which represents the prime mechanism for antibody-mediated protection from infectious diseases in humans and makes up approximately 70-75% of the antibody pool found in serum. IgA and IgM responses also play

prominent roles in vaccine-induced immunity (IgA is important for pathogen clearance on mucosal surfaces and IgM is the first antibody produced after exposure). IgD and IgE collectively make up less than 1% of the serum antibody pool and therefore have received minimal attention in terms of their roles in vaccine-induced protection.

1.3.3 The Limits of Vaccination

While vaccines are developed to induce pronounced antibody responses in recipients, no current vaccines protect all recipients from contracting the disease. Fundamentally, as described above, the success of a vaccine depends on multiple inter-dependent immunological components responding appropriately to each other and the antigens contained within the vaccine. As such, any deficiencies in the number or function of any immune component involved in the vaccine response can lead to sub-optimal outcomes. While the immune system has evolved to contain considerable redundancy - and therefore minor impairment to one or more immune component does not result in catastrophic failure to address antigenic challenge - ultimately, those with greater immune impairment are less likely to achieve an optimal response to vaccination. As described in section 1.2, immunosenesence occurring with advancing age means that older adults are less likely to have optimally-functioning immune systems due to deficiencies in a wide range of immune components. This means that vaccines are least effective in those most vulnerable to infectious diseases.

In older adults, many vaccines are considerably less effective in terms of producing protective antibody responses than in younger adults (for a review see Chen, Kozlovsky, & Effros, 2009). For example, the annual trivalent influenza vaccination is estimated to be 17-53% effective in older adults,

compared to 70-90% in younger adults (Goodwin, Viboud, & Simonsen, 2006). Hepatitis B vaccine antibody responses reach established efficacy thresholds in less than 46% of older adults compared to 96% of younger adults (Denis et al., 1984) and pneumococcal vaccine outcomes are also comparatively low in the elderly (Koivula, Stén, Leinonen, & Mäkelä, 1997).

1.4 *Enhancing Vaccination: A Need for New Approaches*

Given the limited immunogenicity of vaccines in many older adults, some researchers have questioned the wisdom of public health vaccination programmes targeted specifically at this age group (most notably for influenza e.g., Osterholm, Kelley, & Sommer, 2012). Others have argued that although the protection provided to elderly recipients is reduced, vaccination remains relatively effective in reducing mortality and hospitalisation in this population. As such, the benefits of vaccination programmes outweigh the costs of delivery (Hannoun, Megas, & Piercy, 2004).

Though the validity and cost-effectiveness of vaccination programmes in the elderly is still debated, there have been multiple calls to research and develop new methods to improve vaccine efficacy among the elderly (e.g., Kelly & Valenciano, 2012; Lang et al., 2012; Lang, Govind, Mitchell, Siegrist, & Aspinall, 2011). While considerable efforts have been put into researching pharmacological methods such as chemical adjuvants, higher vaccine doses or booster vaccination, to date, these have demonstrated minimal benefit in addressing the sub-optimal outcomes associated with many vaccines in the elderly (Goronzy & Weyand, 2013). For example, the live-attenuated influenza vaccine, which compares favourably to inactivated vaccines in

children, is not licenced for use in older adults as it has repeatedly been found to be ineffective (Ambrose, Luke, & Coelingh, 2008).

An alternative avenue to enhancing vaccine responses in the elderly is to consider the individual differences that account for variations in older adults' vaccine responses. While many older adults do not develop adequate protection following certain vaccinations, many achieve pronounced antibody responses. This heterogeneity has led to a growing interest in modifiable factors that influence immune response to vaccines in older adults and more broadly the immune response to pathogenic challenge. Of particular focus in this regard, and in this thesis, is the potential role of behavioural and psychological factors in modulating vaccine outcomes. Such factors are of interest, in part, because of their high variability across populations and their potential amenability to change through psycho-behavioural interventions.

As will be explored in Chapters 2 and 3, a considerable body of literature now exists that behavioural and psychological factors can modulate immune function, including in older adults. It follows that altering these factors may provide a realistic alternative to pharmacological approaches to enhancing vaccine responses in the elderly. Chapter 2 briefly outlines the historical and theoretical antecedents of research into psychological and behavioural influences on vaccine outcomes, which is then described and critically evaluated in Chapter 3. As will be discussed, early psycho-behavioural interventions to enhance vaccine outcomes are promising, but limited. Further research is needed to understand and develop optimal psycho-behavioural interventions to address poor vaccine outcomes in older adults.

1.5 *Chapter Summary*

Infectious diseases are responsible for significant hospitalisation and mortality among older adults in industrialised countries. Declining immunological competence, known as immunosenesence, not only increases older adults' susceptibility to infections but also reduces the protection afforded by vaccination. Pharmacological methods of enhancing vaccine responses have had limited impact to date. Psycho-behavioural interventions could provide an alternative method for improving vaccine responses among the elderly. The evidence supporting this assertion is considered at length in Chapters 2 and 3.

Chapter 2: Historical and Theoretical Context of this Thesis

Chapter Synopsis

This chapter aims to situate this thesis in context by introducing the reader to key historical and theoretical antecedents to research into psychological and behavioural influences on vaccination responses. Specifically, attention is given to the biopsychosocial model and the concurrent identification of bi-directional connections between central nervous and immune systems, which provide the biological pathway by which psychological factors can influence immune function. Together, these developments contributed to the proliferation of psychoneuroimmunology (PNI) research, an area concerned with interactions between behavioural, neural, endocrine, and immune systems. This thesis is situated within this tradition, based on the premise that behaviour, psychology, and physiology interact to influence health outcomes. Within PNI, much research attention has focused on infectious diseases - primarily to understand psychological and behavioural factors that contribute to increased infection susceptibility. Vaccination has been widely studied in this context, not only because of its clinical importance, but also because the immune response following vaccination mirrors that following infectious disease exposure, but without the associated risks.

2.1 *Introduction*

The statement that behaviour can influence the physical body and health is unlikely to be challenged by any reader of this thesis. To give one of many everyday examples: when engaging in rigorous physical activity, a plethora of physiological changes occur in the body including increased respiration,

fat metabolism and heart rate (Burton, Stokes, & Hall, 2004). The mechanisms by which these changes result are primarily orchestrated by the central nervous system which, upon receiving electrochemical signals for increased energy demands in muscles, sends signals to other parts of the body to change activity to compensate (e.g., for the heart to beat faster). Many behaviours are recognised as strongly influencing the development and progression of multiple illnesses or conditions including obesity, type 2 diabetes, multiple cancers, dental disease and osteoporosis (Durstine, Gordon, Wang, & Luo, 2013; WHO / FAO Expert Consultation, 2003). Indeed, the well-established interdisciplinary field of behavioural medicine is primarily concerned with investigating behavioural influences on health. This is both a well-recognised and well-researched area (M. Conner & Norman, 2005; B. Gardner, 2014).

In contrast, the assertion that psychological factors, such as perceptions of stress and experienced emotions, can influence the physical body and health is perhaps less self-evident.[3] The idea behind this assertion, however, is by no means new. Writings interpreted as espousing a relationship between the mind and body can be traced back at least as far as Ancient Greece, as well as in some traditional Hindu medical practices (Solomon, 2002). While the majority of scientists now reject the notion of Cartesian dualism - that mind and body are separate entities - how subjective experiences of stress or emotion are physiologically manifested in the body, or how they might affect health, received little attention until the latter quarter of the 20th century. Prior to this, in scientific circles, health and illness were considered almost

[3] Although examples of physiological consequences of experienced emotions, such as the face flushing, increased heart rate and blood pressure that regularly accompany feelings of anger, will be familiar to most readers.

entirely in purely biomedical terms; that is, the focus was exclusively on the pathology, biochemistry or physiology of a given disease.

2.2 *The Biopsychosocial Model*

The biomedical approach was formally challenged by the work of George Engel who, in a seminal article published in *Science,* argued for a biopsychosocial model of understanding health and disease (Engel, 1977). According to Engel, the then dominant biomedical model was, by definition, reductionist. It did not account for important social, psychological, and behavioural aspects of illness and was therefore incomplete. Proposing an alternative, Engel drew heavily on a systems approach (Bertalanffy, 1950) which emphasises the need to understand large systems (e.g., organisms, organisations, ecosystems) as being made up of many inter-related hierarchical levels that all contribute to the 'health' of the system. Applying this to humans and disease, Engel highlighted that while important information was gained from understanding disease and health at molecular, biochemical, cell, and tissue levels; information derived from understanding patients as people who live within larger social systems was equally important in the understanding and treatment of illnesses. Patient history, social environment, personality, memories, experiences, and behaviours are therefore all potentially important additional sources of information in understanding a person's health, which interact with physiological aspects of disease pathology. Put another way, Engel advocated the understanding of health and disease as a combination of biological, psychological and social influences, hence the name biopsychosocial model.

An elegant illustration of the differences between a biomedical and biopsychosocial approach was provided by Rolf Adler (2009) with the aid of

the popular fictional character Miss Marple. In *Murder at the Gallop* (1963), a film adaption of one of Agatha Christie's novels, Miss Marple witnesses the death of a wealthy man (Mr Enderby) whose house she had been visiting, canvassing for charitable donations. Curious as to the cause of the sudden death, Miss Marple investigates around the house, finding mud clods containing shoe prints and a menacing cat in the vicinity of where Mr Enderby had died. Miss Marple grows suspicious and visits the local police inspector to report the man's death as a murder. Her hypothesis is inherently biopsychosocial: Mr. Enderby's existing poor cardiac condition (biological) was exacerbated by encountering a cat, to which he is known to have a pathological fear (psychological), which a nefarious visitor (social), had smuggled into his house. In contrast, the police inspector sticks to a rigidly biomedical view of the scenario, ruling the death of natural causes. The local doctor had confirmed heart failure and a history of heart problems (biological), which was deemed sufficient grounds to close the case (psychological and social factors were not considered). As might be expected, Miss Marple is ultimately proved correct in her suspicions, with a relation of Mr Enderby eventually confessing to planting the cat, with the aim of frightening him to death and inheriting his estate. While fictional, Adler's example alerts the reader to how solely biomedical explanations of health-related events cannot always be complete.

There is much empirical evidence to support the fundamental ideas formalised in the biopsychosocial model, namely that psychological and social factors influence health and disease. To give some examples from reviews: epidemiological studies have demonstrated that loneliness is as important a risk factor in premature mortality as is smoking (Holt-Lunstad, Smith, & Layton, 2010), more than 40 differences in immunological parameters have been reliably observed in those with a diagnosis of major

depressive disorder compared to those without (Zorrilla et al., 2001), and psychological stress has been implicated as an important predictive factor in the progression of cardiovascular disease, carotid artery disease, HIV and cancer (Chida & Vedhara, 2009; S. Cohen, Janicki-Deverts, & Miller, 2007; Steptoe & Kivimaki, 2012). However, while this evidence supports the basic tenants of the biopsychosocial model, some have strongly critiqued the biopsychosocial model for its lack of specificity and questioned whether it satisfies the criteria of a model, in the scientific sense of the word (Epstein & Borrell-Carrio, 2005; McLaren, 1998). For example, McLaren (1998) argues that scientific models are real world actualisations of scientific theories or ideas that must be able to test novel predictions and that the biopsychosocial 'model' in no-way meets this standard ("[it] does not reveal anything that would not be known (implicitly, if not explicitly) to any practitioner of reasonable sensitivity" (McLaren, 1998 p.91)). Further, others have argued that Engels 'model' is too vague and as such it invites multiple possible interpretations as to whether it should be understood as a model, a theory, a philosophy, a belief system, or a guide to clinical practice (Epstein & Borrell-Carrio, 2005).

While the biopsychosocial model may not satisfy the traditional criteria of scientific models, it is indisputable that the position advanced by Engel - that health is a complex interplay of biological, psychological, behavioural and social factors - has been, and indeed remains, hugely influential. In particular, the biopsychosocial model has inspired much research exploring how such factors are entwined (Adler, 2009; Alonso, 2004; Borrell-Carrio, Suchman, & Epstein, 2004; Dunkel Schetter, 2011; Novack et al., 2007). In this thesis, the biopsychosocial model is considered to be best understood as a conceptual framework which, at its core, emphasises a holistic approach

to understanding health and illness, focusing on both the person in a social context and disease pathology.

2.3 *Central Nervous and Immune System Integration*

Around the same time that Engel was arguing for a biopsychosocial approach in medicine, research conducted by Robert Ader and Nicholas Cohen was instrumental in launching a new field of enquiry investigating relationships between the central nervous system and the immune system. While conducting an experiment on the acquisition and extinction of conditioned taste aversion response in rats, Ader and Cohen noticed an unexpectedly high mortality rate in some conditions. Upon further observation, they discovered that mortality rates were related to the volume of saccharin (a harmless sweetener) rats received in extinction trials, to which they had a conditioned taste aversion response from prior pairings with the compound cyclophosphamide (a drug that causes nausea and has immunosuppressive qualities). They concluded that, in addition to conditioning a taste aversion response, the rats' immune systems were being classically conditioned to the immunosuppressive effects of cyclophosphamide (Robert Ader & Cohen, 1975). This finding was of great significance as historically the immune system had been considered to be an autonomous defence system, working independently from the central nervous system (CNS). However, Ader and Cohen's research provided some of the first evidence that the two systems might interact.[4] While initially met with scepticism, further research conducted throughout the 20th century firmly established an inextricable bi-

[4] Interestingly, similar effects had previously been demonstrated by Russian scientists Metal'nikov and Chorine (1926) many decades earlier, but had received minimal attention or interest (Robert Ader, 1995).

directional integration between the central nervous and immune systems (Robert Ader, 1995).

Multiple connections between these systems have now been identified and continue to be elucidated (for a review see Wrona, 2006). Primary efferent (CNS to immune) pathways of this relationship are neuroendocrine, via hypothalamic-pituitary-adrenal (HPA) axis released hormones, and neuronal, via direct innervation to primary and secondary lymphoid organs (Robert Ader, Cohen, & Felten, 1995; Eskandari & Sternberg, 2002; Marques-Deak, Cizza, & Sternberg, 2005; Wrona, 2006). To be more specific: regarding the neuroendocrine route, upon encountering stress (physical or psychological) the hypothalamus secretes corticotropin-releasing hormone (CRH) which, along with other secreted hormones, stimulates the release of adrenocorticotropin (ACTH) from the pituitary gland. In turn, ACTH stimulates the production and secretion of glucocorticoids from the adrenal glands, most notably cortisol in humans. Glucocorticoids regulate a variety of immune functions including cell trafficking, production, maturation and differentiation (Barnes & Adcock, 1993; Eskandari & Sternberg, 2002). These effects are exerted through specific hormone receptors present on many types of immune cells, including B-cells (the pre-cursors to antibodies), T-cells, macrophages, NK cells and granulocytes (Marques-Deak et al., 2005). Neuronal pathways are primarily conducted through sympathetic nerve fibres, which spread to immune organs including lymph nodes, bone marrow, spleen, and thymus (Robert Ader et al., 1995; D. Felten & Felten, 1988; S. Felten & Felten, 1991). These nerve fibres form close synapse-like junctions with immune cells, including lymphocytes and macrophages, which allows released neurotransmitters to affect immune cell function through receptors present on the cell surface (Eskandari & Sternberg, 2002).

Afferent pathways (immune to CNS) are currently less well delineated but are primarily thought to involve cytokines (Wrona, 2006). Cytokines released by immune cells can activate the HPA axis, most notably IL-1 for which receptors are expressed heavily in hippocampus and hypothalamus neurons (Besedovsky & Del Rey, 2007). Cytokines IL-2, IL-6, INF-γ and TNF-α also influence the HPA axis and in turn are influenced by HPA axis secreted glucocorticoids (E. Sternberg, 2001; Wrona, 2006). Perhaps the best demonstration of immune to CNS communication is the case of sickness behaviour. Sickness behaviour refers to a frequently observed suite of behavioural and cognitive changes that occur when a person encounters a pathogen and becomes infected. These can include lethargy, depression, a reduction in self-grooming behaviour and decreased appetite (Hart, 1987). These changes have been shown to be driven by pro-inflammatory cytokines that influence the brain on a cellular level during infection (Dantzer, 2004; Hart, 1987).

2.4 ***Psychoneuroimmunology***

Together, the establishment of CNS-immune integration and the increased awareness of psychological and social influences on health because of Engel's biopsychosocial model resulted in a proliferation of research. Specifically, considerable interest was sparked in whether behavioural and psychological factors might trigger CNS-immune pathways and thereby influence physiological aspects of health and immunity. This field of research is now referred to as psychoneuroimmunology (PNI), referring to the study of the (mal)adaptive interactions among behavioural, neural, endocrine, and immune systems (Robert Ader, 1995).

Since the term PNI was coined (Robert Ader, 1980), considerable evidence has accumulated that the pathways between the central nervous and immune systems can be triggered by external (stressors or social factors) or internal (psychological) factors. Much of this evidence concerns psychological stress, with experimental functional magnetic resonance imaging (fMRI) studies demonstrating that stressful experiences are physiologically manifested in the brain (Dedovic, D'Aguiar, & Pruessner, 2009), and that inducing stress results in modification of immune parameters (e.g., Dedovic, Renwick, Mahani, & Engert, 2005; Larson, Ader, & Moynihan, 2001). For example, Dedovic et al. (2005) demonstrated that participants completing the Montreal Imaging Stress Task - a timed mental arithmetic task designed to induce moderate stress – while undergoing fMRI showed increased activation in the angular gyrus region of the brain and increased salivary cortisol production. Similar research has been conducted on emotional experiences, such as happiness or anger, which have been shown to have distinct neural correlates (Phan, Wager, Taylor, & Liberzon, 2002; Vytal & Hamann, 2010) with experimental studies of induced positive and negative mood states finding causal relationships between mood changes and immunological parameters including production of salivary IgA, multiple cytokines (including IL-2, IL-3, TNF-α, and IL6), and natural killer cell activity (Pressman & Cohen, 2005).

This thesis is firmly grounded within this tradition of PNI research and is based on the premise that psychology, behaviour, and physiology interact to influence health outcomes. Specifically, this thesis is situated within a prominent strand of PNI research concerned with psychological or behavioural influences on human health conditions with immune aetiologies, with a view towards developing psycho-behavioural interventions that lead to improved health outcomes. This kind of research has been directed

towards many illnesses or conditions including cancer (Andersen et al., 2004), wound healing (Emery, Kiecolt-Glaser, Glaser, Malarkey, & Frid, 2005), and asthma (Castés et al., 1999).

Of particular relevance here, however, is a body of PNI research that has focused on infectious diseases (much of which is considered in more detail in Chapter 3). Early work in this area provided observational evidence that elevated stress and depressed mood were associated with greater incidence of self-reported upper-respiratory infections (e.g., Glaser et al., 1987; McClelland, Floor, Davidson, & Saron, 1980; Parens, McConville, & Kaplan, 1966; Sarason, Sarason, Potter, & Antoni, 1985). For example, Glaser et al. (1987) prospectively tracked 40 medical students during their first year in training and found greater incidence of self-reported symptoms of infectious illnesses during examination periods than non-exam periods. However, these studies were limited due to the reliance on self-reports of infection symptoms - as stressed and depressed individuals are known to show a cognitive bias towards increased symptom perception (S. Cohen, Doyle, & Skoner, 1995; Salovey & Birnbaum, 1989).

Better evidence came from studies that have examined associations between psychological and behavioural factors with serologically verified upper-respiratory infections, again showing, on balance, relationships between psychological stress/distress and infection susceptibility (e.g., Boyce et al., 1977; Graham, Douglas, & Ryan, 1986; Meyer & Haggerty, 1962). For example, Graham et al. (1986) prospectively studied the relationship between stress and incidence of upper-respiratory infections among 235 members of 94 families, finding that individuals classified as highly stressed had a greater number of verified illness episodes and self-reported symptom days. Additional research in this area focused on latent

viruses, which are a specific type of virus that can remain dormant in the body after initial exposure by incorporating their genetic material into the host thereby allowing it to be replicated. The 'reactivation' of these latent viruses, such as Herpes Simplex Viruses (HSV-1, HSV-2), have been found in numerous studies to be associated with stress exposure (e.g., Glaser et al., 1987; Kemeny, Cohen, Zegans, & Conant, 1989; Kiecolt-Glaser et al., 1988; Longo & Clum, 1989; VanderPlate, Aral, & Magder, 1988). While on balance this research points to the importance of psychological factors like stress in infectious disease incidence and severity, the above evidence is observational and therefore cannot establish causal relationships. Indeed, there are many potentially confounding factors (e.g., initial exposure, environmental conditions) that cannot be controlled in such observational designs.

Arguably the best evidence that psychological factors can trigger CNS-immune links and lead to changes in susceptibility to infectious diseases comes from highly controlled, live virus challenge studies. In such studies, participants are deliberately exposed to a standardised dose of an infectious agent (e.g., rhinovirus) and quarantined, with any resulting infections verified through nasal swabs or serum assays and severity measured via the extent of mucosal secretions. The primary advantage of this viral challenge paradigm is that the initial exposure to the infectious agent can be carefully controlled – something not possible in more naturalistic observational studies of infection. This can be a significant confound when unknown in other study designs, as the greater the initial exposure to viral particles, the more likely a resultant infection. Further, viral challenge studies allow many other potentially confounding variables (including environmental conditions, diet, sleep etc.) to be standardised, or accurately measured and controlled for. Ultimately, this gives researchers greater confidence that any

relationships found between psychological factors and infection susceptibility are causal, rather than simply correlational, in nature. A small number of large viral challenge studies have examined the relationship between behavioural and/or psychological factors in relation to infection susceptibility (e.g., Broadbent, Broadbent, Phillpotts, & Wallace, 1984; S. Cohen & Doyle, 2009; S. Cohen, Tyrrell, & Smith, 1991, 1993; Totman, Kiff, Reed, & Craig, 1980). Relevant findings from such studies are described in more detail in the following chapter, but here it is sufficient to note that these 'gold standard' studies have demonstrated that both psychological (higher levels of stress) and behavioural (too much or too little sleep) factors can increase susceptibility to infection at clinically meaningful level (S. Cohen & Doyle, 2009; S. Cohen et al., 1991).

While live virus challenge studies are highly rigorous, they cannot be used in many cases on ethical grounds. Many infectious agents cannot be used as they carry considerable risk of morbidity, and such studies cannot typically be conducted on older adults, due to the higher vulnerability to adverse events following infection. As a result of these limitations, researchers have frequently turned to vaccination models as an alternative (Kiecolt-Glaser, Glaser, Gravenstein, Malarkey, & Sheridan, 1996; Vedhara, Cox, et al., 1999). Vaccine administration causes an immune response, which, broadly speaking, mirrors natural infection exposure. In both vaccination and natural exposure, antigens enter the body and are recognised as foreign by the immune system, ultimately resulting in the formation of antigen-specific antibodies that can protect the host from infection. Vaccination is therefore a controlled and biologically relevant model in which the effects of psychological and behavioural factors on naturally acquired infection can be examined without the associated risks (Burns & Gallagher, 2010; Phillips, 2012). Beyond this, as discussed at length in the previous chapter,

vaccination responses are frequently sub-optimal - especially in older adults. This makes studying how behavioural or psychological factors might impact on vaccine responses clinically relevant and has contributed to the growth of research in this area. The remainder of this thesis is concerned with this type of research; specifically, how psychological and behavioural factors influence on vaccination responses.

2.5 *Chapter Summary*

Behaviour has historically been well accepted as influencing physical health outcomes. In contrast, the influence of psychological and social factors on physical health was largely ignored by scientists before the latter quarter of the 20th century. The biopsychosocial model proposed by George Engel (1977) and growing evidence of bi-directional communication between central nervous and immune systems has contributed to a proliferation of research into how psychological factors may influence immune function and disease. This thesis is situated within this research tradition and is specifically concerned with psychological and behavioural influences on vaccination, which have been widely studied as a model analogous to natural infection exposure, without the associated risks.

Chapter 3: Psycho-Behavioural Factors, Immunity, and Vaccination Responses

Chapter Synopsis

This chapter presents an overview of the current state of research concerning the influence of psychological and behavioural factors on human vaccination responses. Evidence for relationships between multiple behavioural (physical activity, nutrition, and sleep) and psychological (stress, negative affect, and positive affect) factors, immunity, and vaccination responses are critically reviewed, before several common methodological weaknesses and knowledge gaps where further research is needed are highlighted. Research that begins to address some of these knowledge gaps is presented in the remainder of this thesis.

3.1 *Chapter Scope*

The terms *'behaviour' and 'psychological factors'* are sufficiently broad that they encompass almost all human experience. Here, in accordance with an interest in developing psycho-behavioural interventions to improve vaccination responses, the discussion will be limited to factors that are (a) theoretically amenable to change through psycho-behavioural interventions, (b) proposed to be proximal behavioural or psychological determinants of immune response and (c) have been the subject of sustained research in the context of immunity and/or vaccination. To clarify, this means the following sections will exclude psychological or behavioural factors that are non-modifiable (e.g., having an existing diagnosis of a mental health

condition) and factors hypothesised to be more distal determinants of immune response (e.g., social support; Uchino, 2006).

Applying the above criteria, the factors considered in this chapter are: physical activity, nutrition, sleep, stress, and affect.[5] Even limiting the discussion in this way, the literature concerning these factors in relation to immunity and vaccination responses to date is vast. Each of these factors and their relationship with immune and vaccine outcomes could be, and in most cases have been, the subject of entire theses in their own right (e.g., Campbell, 2010; Hallam, 2013; Hash-Converse, 2008; Prather, 2010). To illustrate, a 2004 review identified over 300 articles examining the relationship between stress and immunity (Segerstrom & Miller, 2004) and in 2009, a review considering research on nutrition and vaccination responses alone identified over 130 articles (Savy et al., 2009). As such, detailing all this research is beyond the scope of this thesis. Therefore, the literature reviewed in this chapter should be considered representative, rather than exhaustive. In line with the subject of this thesis, particular attention is given to research that focused on the role of behavioural and psychological factors in antibody responses to vaccination among older adults.

3.2 ***Behavioural Factors, Immunity & Vaccine Response***

3.2.1 Exercise & Physical Activity

While physical activity refers to any movement produced by skeletal muscles that results in energy expenditure (Caspersen, Powell, & Christenson, 1985), research examining how physical activity influences immunity and/or vaccine responses has almost exclusively focused on exercise. Exercise is a

[5] The inexact distinctions drawn between psychological factors such as stress and affect are discussed in 3.3

sub-category of physical activity which is typically pre-planned and structured, with the objective of improvement or maintenance of physical fitness (Caspersen et al., 1985). Research in this area can be further separated into that which has investigated the effects of acute exercise (a single bout of exercise) or regular exercise (repeated exercise, typically over a period of weeks or months).

3.2.1.1 Acute Exercise

Considerable research has documented transient changes to immune dynamics during, and following, a single bout of moderate or strenuous exercise, and this has been reviewed extensively elsewhere (e.g., Nielsen, 2013; B. Pedersen & Hoffman-Goetz, 2000; Romeo, Wärnberg, Pozo, & Marcos, 2010; Simpson et al., 2012; Walsh, Gleeson, Pyne, et al., 2011; Walsh, Gleeson, Shephard, et al., 2011). To give just a few examples: levels of circulating cytokines IL-6 and TNF-α increase in marathon runners immediately post-race (Northoff & Berg, 1991; Ostrowski, Rohde, Zacho, Asp, & Pedersen, 1998). Natural killer (NK) cell activity is dramatically increased following moderate and intense bouts of treadmill running in non-athletes (Nieman, Miller, et al., 1993) but is supressed for several hours after if the exercise is prolonged (Gleeson & Bishop, 2005), and circulating T-cells, and to a lesser extent B-cells, increase during and immediately post-exercise (Walsh, Gleeson, Shephard, et al., 2011). Such exercise-induced immune alterations have been demonstrated in both young and older adults (Mazzeo et al., 1998; Simpson et al., 2008).

While transient immune alterations because of acute exercise are well established, the clinical effects of these accumulated changes depend on the length and intensity of the exercise in question. Immunological changes as

a result of prolonged, intense bouts of exercise (e.g., distance running) are typically associated with adverse immunological consequences, with greater susceptibility to upper respiratory tract infections and/or symptoms found in multiple observational and epidemiological studies of athletes undergoing intensive training (Davison, Kehaya, & Jones, 2016; Nieman, 2000). While some debate remains as to whether such associations are the result of actual infections or other exercise-induced inflammatory factors (Walsh, Gleeson, Shephard, et al., 2011), current evidence is at least consistent with the so-called 'open window' hypothesis (Nieman & Pedersen, 1999). This proposes that immunological changes as a result of intense and prolonged physical exertion can lead to higher than normal vulnerability to infection for between three and 72 hours post-exercise (Walsh, Gleeson, Shephard, et al., 2011).

In contrast, the changes to immune dynamics resulting from low-to-moderate length and/or intensity acute exercise may have beneficial consequences. Dhabhar (2002) has argued that the immunological activation that follows stress (physical or psychological) is part of an evolved 'fight-or-flight' response, preparing the organism to rapidly respond to dangerous pathogens should exposure occur (e.g., through a wound). This position is supported by multiple studies in animal models which have illustrated enhanced cellular and humoral immune responses to antigenic challenge immediately following mild physical stressors or exercise (see Campbell, 2010; Dhabhar, 2002 for reviews of these findings). Both prolonged and moderate bouts of acute exercise have been examined in relation to human vaccination responses, although the latter has received greater attention.

Evidence for Effects of Acute Exercise on Human Vaccination Responses

A recent systematic review on the effects of exercise on vaccination responses (Pascoe, Fiatarone Singh, & Edwards, 2014), identified only two studies investigating the effects of a single bout of prolonged exercise on human vaccination responses, both of which had observational designs. The first, notably on a very small sample of young adult marathon runners (n=4), found that marathon runners vaccinated against tetanus toxoid immediately after completing a marathon showed higher IgG responses at 2 weeks post-vaccination compared to non-runner controls (Eskola et al., 1978). A second larger, although still relatively small study, found no differences in 2-week post-vaccination antibody outcomes between young and middle-aged males (21-53 years) who completed a triathlon (n=22) prior to receiving diphtheria, tetanus toxoid and pneumococcal vaccinations, compared to resting athletes or sedentary controls (Bruunsgaard et al., 1997).

At this stage, there is simply too sparse and poor quality data to draw reasonable conclusions regarding the role of single bouts of prolonged and/or highly-intense exercise in relation to human vaccination responses. There have been no randomised controlled trials to date, and what observational evidence exists comes from small sample studies that do not adequately control the length and intensity of exercise performed by participants. The relevance of these findings to older adults is also questionable. No studies of prolonged exercise on vaccination responses have been conducted in older adults and, even if prolonged and/or high intensity exercise was found to reliably improve vaccination responses, very few older adults do, or can, perform this type of exercise. Indeed, older adults are far more likely, than younger adults, to have physical activity

levels below recommended guidelines (Townsend, Wickramasinghe, Williams, Bhatnagar, & Rayner, 2015).

Acute bouts of short-to-moderate length and/or intensity exercise are more feasible for most older adults, and have also been examined in the context of vaccination. The quality of evidence in this area is considerably stronger with multiple randomised controlled trials investigating the effects of brief or moderate exercise interventions undertaken immediately prior to, or following, vaccination. All but one of these trials come from a single research group, explicitly investigating the acute stress enhancement hypothesis (Campbell et al., 2010; Edwards et al., 2010, 2012, 2006; Edwards, Burns, Allen, et al., 2007; Long et al., 2012). This hypothesis, drawing heavily on the position of Dhabar (2002) described in the previous section, contends that short bouts of physical or psychological stress, typically operationalised as a brief bout of moderate intensity exercise, prime the immune system to respond to antigenic challenges such as vaccination (Edwards, Burns, Carroll, Drayson, & Ring, 2007).

Findings from these randomised controlled trials have been broadly supportive of the acute stress enhancement hypothesis. For example, young adults who completed either 45 minutes of cycling or a psychological stress task immediately prior to vaccination showed enhanced short- and long-term antibody responses to one of three influenza strains (females) and meningococcal A (males) following vaccination, compared to resting controls (Edwards et al., 2006, 2008). Further, women have shown enhanced antibody responses, compared to controls, to two of three influenza stains after completing 50 repetitions of bicep curl and lateral raise movements with a weight at 85% of their maximum one-repetition weight, at 6 hours prior to vaccination (Edwards, Burns, Allen, et al., 2007).

However, not all findings have been in line with the predictions of the acute stress enhancement hypothesis. For example, in the same trial where women had enhanced antibody responses following bicep curl exercises, exercising men showed reduced antibody responses compared to controls (Edwards, Burns, Allen, et al., 2007). Further, Campbell et al. (2010) found that participants who performed the 50 repetitions of bicep curl and lateral raise movements with a weight at 85% of their maximum one-repetition weight at either 48 hours, 6 hours or immediately prior to influenza vaccination showed no significant improvement in antibody responses compared to resting controls.

One explanation for this disparity in findings is that exercise may only enhance antibody responses when vaccine immunogenicity is low (i.e., when the vaccine is less successful in inducing an immune response). In the Edwards et al. (2007) study, males in the control group showed comparatively stronger antibody responses than women in the control group – suggesting there may have been more potential for exercise to enhance responses in females than males. In the Campbell et al. (2010) study, the control group showed strong antibody responses to all of the vaccine strains, meaning that 'ceiling effects' may have prevented any further enhancement because of exercise. Further support for this explanation comes from two trials that explicitly investigated this phenomenon by manipulating the vaccine dose received by participants. Edwards et al. (2010) gave participants a 50% dose of influenza vaccine after performing one of three intensities of exercise immediately prior to vaccination. Enhancing effects of exercise were observed in all exercise conditions over controls in the least immunogenic of the three vaccine strains, amongst men only in the next least immunogenic strain, but not in the most immunogenic strain.

Extending these findings, Edwards et al. (2012) compared young adults' antibody responses to either a full or 50% dose of pneumococcal vaccination, administered immediately after completing a 15 minute resistance exercise program or resting. Those performing exercise in the half-dose group showed greater average antibody levels over resting participants. For those receiving the full dose, there was no significant difference in antibody responses between exercisers and resting controls.

While the above evidence would suggest that an acute bout of moderate intensity exercise could potentially enhance weak responses to vaccination, such as those seen in the elderly, only one trial of this to date has included older adults as participants. Ranadive et al. (2014) compared antibody responses to influenza vaccination at 4 weeks post-vaccination between adults (55-75 years) who were randomised to perform 40 minutes of moderate intensity exercise (55-65% maximal aerobic capacity) or rest, immediately prior to vaccination. In men, no differences were found between conditions, but exercising women showed significantly greater antibody responses to one strain (H1N1) than resting controls.

It is noteworthy that multiple studies cited in this section, and indeed throughout this chapter, show evidence of gender differences. However, the directionality of these differences is not consistent, with some studies showing greater effects among females, others among males, and a large number not reporting any such differences. Understanding these discrepancies is challenging because of multiple competing influences of gender in these studies. On the one hand, there are sex-specific immunological and endocrine differences between men and women (Klein, Marriott, & Fish, 2014). Females are typically found to show greater immune responses to antigenic challenges, including vaccination, than males

(Giefing-Kroll et al., 2015). For psychoneuroimmunological research, this might suggest that some effects of psychological and behavioural factors may be less evident in females due to ceiling effects. On the other hand, there is evidence that immune responses to psychological and behavioural factors such as stress and exercise differs between males and females (Pullinen, Mero, Huttunen, Pakarinen, & Komi, 2002; Stupka et al., 2000). For example, males have a greater inflammatory response to exercise than females (Stupka et al., 2000) increasing the trafficking of immune cells important in the adaptive immunity cascade. Beyond this, there is the possibility that some of these findings are 'chance' data artefacts, with gender-differences often only explored (and indeed reported) if significant and where main effects are not evident. Further research is needed, but for now it is important that researchers in this area consider, and if necessary control for, any gender effects in their studies.

On balance, there is currently modest evidence that brief bouts of moderate duration and/or intensity exercise immediately prior to vaccination can enhance antibody responses to vaccination in humans. Enhanced antibody responses as a result of exercise may only be evident in cases where responses to vaccination are weak. This potentially bodes well for the potential of this form of exercise to act as a psycho-behavioural vaccine adjuvant in older adults (Chen et al., 2009). However, the relative paucity of research conducted on older adults in this area needs to be addressed.

3.2.1.2 Regular Exercise

Regular performance of physical activity/exercise has been widely linked to beneficial health effects, including reduced incidence of many chronic diseases with immunological aetiology (Durstine et al., 2013; Warburton,

Nicol, & Bredin, 2006). The impact of regular exercise on immune dynamics and function has received substantial attention, with multiple observational and cross-sectional studies identifying differences in the immune profiles of regularly exercising individuals compared to sedate individuals, particularly amongst older adults (Simpson & Guy, 2010; Simpson & Spielmann, 2013). T-cell proliferation in vitro is greater in older women with good physical conditioning than sedentary counterparts (Nieman, Henson, et al., 1993) as well as in older male recreational runners compared to non-runners (Shinkai et al., 1995). Leukocyte telomere lengths, which are proxy indicator of morbidity and mortality in humans (Simpson & Spielmann, 2013), are shorter in less physically active older adults (Cherkas et al., 2008; Ludlow et al., 2009) and IL-2 production is greater in elderly habitual exercisers than sedate elderly (Ogawa, Oka, Yamakawa, & Higuchi, 2003). These accumulated changes to the immune environment provide a pathway by which regular exercise can influence both disease progression and response to antigenic challenge. In the case of infectious diseases, accumulated immunological changes because of regular exercise appear to be clinically meaningful. A recent epidemiological cohort study of over 114,000 responders to a Canadian population health survey found that moderate and highly physically active responders were significantly less likely to have received an influenza-coded physician or hospital visit than those who were classified as sedate. Importantly, this finding was found to be primarily driven by older adults (Siu, Campitelli, & Kwong, 2012).

Evidence for Effects of Regular Exercise on Human Vaccination Responses

Multiple cross-sectional observational studies have examined relationships between regular physical activity performance and vaccine responses (Keylock et al., 2007; Kohut, Cooper, Nickolaus, Russell, & Cunnick, 2002;

Schuler et al., 1999; T. P. Smith, Kennedy, & Fleshner, 2004). One study, found no effect of self-reported exercise on influenza vaccination responses in college students (Schuler et al., 1999) but the remainder have all focused on older adults and shown some association between those self-reporting greater physical activity levels and enhanced antibody responses to vaccination (Keylock et al., 2007; Kohut et al., 2002; Schuler, Leblanc, & Marzilli, 2003; T. P. Smith et al., 2004). Among these studies in older adults, influenza vaccination has been the most frequently studied, with active older adults found to have enhanced antibody responses (to some but not all strains) compared to sedate older adults in three studies (Keylock et al., 2007; Kohut et al., 2002; Schuler et al., 2003). Antibody responses following vaccination with Keyhole Limpet Hemocyanin has also been found to be enhanced in active older adults compared to sedentary older adults (T. P. Smith et al., 2004).

While the above studies point to a beneficial effect of regular exercise on vaccination responses among older adults, it is not possible to disentangle whether this effect is due to the performance of exercise *per se,* or simply greater physical fitness. Physical fitness has been found to influence vaccination responses; for example, Keylock et al. (2007) found antibody responses to influenza vaccination in older adults to be higher in those who demonstrated greater physical fitness in a VO_{2max} treadmill test. While physical fitness and exercise performance are inextricably linked, these observational studies are limited in that as exercise performance was pre-existing and not manipulated they cannot establish a causal relationship between exercise and vaccine responses. Nor can they establish whether engaging in physical activity would enhance vaccine responses in those who are typically sedate.

Better evidence for the effects of regular exercise on vaccine responses comes from randomised controlled trials of regular exercise interventions. In the first of such studies, Kohut et al. (2004) randomised a small group (n=27) of older adults (≥64 years), who previously did not engage in regular physical activity, into a 10-month exercise condition that was completed prior to vaccination or a control comparison condition in which participants retained their previous activity level. Following influenza vaccination, older adults in the exercise condition had significantly greater mean fold-increases in antibodies to two out of three strains contained in the vaccine at both 4 weeks and 3 months post-vaccination compared to those in the control condition. In a larger (n=144), but similar, study conducted by Woods et al. (2009), older adults assigned to a 10-month cardiovascular training programme were more likely to have protective levels of antibodies at 24 weeks after influenza vaccination (administered 4 months into the exercise intervention) in 2 out of 3 strains than older adults assigned to a flexibility and balance programme of the same length; although no differences were found at 3 or 6 weeks post-vaccination. A subset of older adults in this study were also vaccinated with Keyhole Limpet Hemocyanin 8 months into the intervention and had IgG1 and IgM responses at 2, 3 and 6 weeks post-vaccination that were significantly higher in those in the cardiovascular training condition compared to those in the flexibility and balance condition (R. Grant et al., 2008). Interestingly, intervention-induced improvement in physical fitness as measured by change in VO_{2peak} was not related to antibody responses, suggesting it may be the exercise – rather than just improved physical fitness – that was driving the enhanced vaccination responses.

While the majority of randomised controlled trials of regular physical activity have shown beneficial effects on antibody responses to vaccination (R. Grant et al., 2008; Kohut et al., 2004; Woods et al., 2009; Yang et al., 2007), two

recent trials have found no evidence for such effects (Hayney et al., 2014; Long et al., 2013). However, it is worth noting that in these two later studies, exercise interventions were comparatively short in length (3-4 months, compared to 9+ months in most other studies), perhaps indicating that a minimum period of regular exercise engagement may be necessary to obtain benefits in terms of antibody responses to vaccination. It is also worthy of note that there seems to be little consistency in the timing of exercise in relation to vaccination across studies. Some, have vaccinated participants once a prolonged exercise intervention is complete (e.g., Kohut et al., 2004), whereas others have provided vaccination at various lengths into the intervention (e.g., R. Grant et al., 2008; Woods et al., 2009). Whether there are any benefits from continuing exercise for a period post-vaccination (at which point the vaccine components are interacting with the immune system) is yet to be established.

In sum, there is reasonably strong evidence that the long-term performance of regular exercise can enhance antibody responses to vaccination, particularly in older adults. Observational evidence, while limited, suggests that older adults who regularly perform exercise have greater antibody responses following vaccination than those who do not. Further, randomised controlled trial evidence shows that the antibody responses following vaccination of previously sedate older adults can be improved by regular exercise. While this evidence is convincing, by definition, interventions of this type cannot be brief and must begin a significant time before a target vaccination. Regular exercise has widespread health benefits and many public health initiatives have targeted population increases in exercise performance (Department of Health, 2011, 2014). However, the evidence that these interventions can produce sustained behaviour change is limited (Hillsdon, Foster, Crombie, & Naidoo, 2005). These factors together

potentially limit the practicality of using regular exercise interventions as a method of improving vaccine responses in older adults.

Surprisingly, there is a lack of research examining how physical activity performed in day-to-day living influences vaccine outcomes in older adults. This is despite evidence that greater free-living physical activity in older adults is associated with increased secretion of IgA in saliva, a proxy measure of mucosal immune function (Shimizu et al., 2007). For many older adults, free-living activity – including housework, shopping etc. – accounts for the majority of all physical activity performed and there is a need for research examining whether performance of this kind of physical activity is also related to antibody responses following vaccination.

3.2.2 Nutrition

There is a wealth of evidence linking nutrition to the dynamics and functional status of the immune system, which has been documented extensively in multiple reviews (Calder, 2013; Calder & Jackson, 2000; R. Gross & Newberne, 1980; Maijó, Clements, Ivory, Nicoletti, & Carding, 2014; Pae, Meydani, & Wu, 2012). The immune system is constantly active and requires a continual production of new cells; a process which is heightened during pathogenic challenge (Calder, 2013). The production of these cells requires an adequate source of various proteins and micronutrients (Calder, 1995; Lesourd, 2006). If the body does not have adequate sources of nutrients required through diet or bodily stores, then immune cell production can be reduced and/or poorer in functional capacity (Calder, 2013). Protein-energy malnutrition as well as deficiencies in a number of individual micronutrients (primarily vitamins A, D, Iron, and Zinc) have been associated with sub-optimal immune component levels and function across all age groups (Calder

& Jackson, 2000; R. Gross & Newberne, 1980). A selection of these effects are presented in Table 3.1.

Table 3.1: Nutritional deficiencies and immunity adapted from Savy et al. (2009)

Nutritional Deficiency	**Associated Immunological Response**
Protein-Energy	Decrease of innate immunity (physical barriers, complement, natural killer (NK) cell activity) Thymic atrophy Decrease of T lymphocyte number and function Decrease in Th1-type cytokines
Vitamin A	Impact on innate immunity (integrity of mucosal epithelia, monocytes, macrophages, and NK numbers and activity) Decrease of T lymphocyte number (especially CD-4) Decrease of B lymphocyte numbers
Iron	Decrease of innate immunity (neutrophils, NK, macrophage activity) Decrease of T lymphocyte number
Zinc	Decrease of innate immunity (macrophages, NK activity) Decrease of T lymphocyte number and function Th1/Th2 balance shifts to Th2 Decrease of B lymphocyte number and function (from animal studies)
Vitamin D	Decrease of innate immunity (macrophage activity) Decrease of T lymphocyte number and function Th1/Th2 balance shifts to Th1

In higher income countries, nutritional deficiencies are particularly prevalent among the elderly. Up to half of community-living 75-year-olds have decreased intakes in at least one micronutrient, compared to recommended guideline levels (Lesourd, Mazari, & Ferry, 1998). Such nutritional deficiencies are associated with greater susceptibility to infectious disease (Schaible & Kaufmann, 2007). For example, nutritional deficiencies have been identified as a major risk factor for hospital acquired infections (S. M. Schneider et al., 2004). Recent reviews examining the role of nutrition in immunity (Lesourd, 2006; Maijó et al., 2014; Pae et al., 2012) have concluded that nutritional deficiencies likely play a role in immunosenesence,

with optimal nutrition helping to maintain immune function for longer in the elderly.

Evidence for Effects of Nutrition on Human Vaccination Responses[6]

A substantial amount of research has been conducted looking at the value of the co-administration of nutritional supplements (e.g., Vitamin A) alongside vaccination schedules for infants in low-income countries, where nutritional deficiencies are highly prevalent. This co-administration of supplements with vaccination is recognised as beneficial in 'at-risk' areas by the World Health Organization (2011) not as a method of improving vaccination responses *per se*, but rather as a pragmatic opportunity to reduce common nutritional deficiencies which exacerbate morbidity and mortality resulting from infectious diseases in young children. This proximity of nutritional supplementation to vaccination has provided many opportunities for researchers to investigate the effects of nutritional supplements on vaccine responses in malnourished children (e.g., Ahmed, Arifuzzaman, Lebens, Qadri, & Lundgren, 2009; Albert et al., 2003; Bahl et al., 1999; Benn et al., 1997; Bhaskaram & Rao, 1997; Cherian, Varkki,

[6] Readers familiar with this literature will note the omission of research authored by Ranjit Kumar Chandra from this section. This is a deliberate omission due to questions that have been raised regarding the legitimacy of this research. In 2005, the journal *Nutrition* retracted an article written by Chandra (2001) after serious concerns were raised about the veracity of the research (Meguid, 2005). The *British Medical Journal* had raised serious questions into the articles credibility with the journal's statistical reviewer concluding the article showed "all the hallmarks of being entirely invented" (White, 2004 p.67). Multiple critiques were, and have since, been written about the article and another earlier article authored by Chandra (1992) was brought under heavy critique. Further, concerns over conflicts of interest have been raised regarding undisclosed financial interests relating to patented vitamin supplements (Roberts, 2007; S. Sternberg & Roberts, 2006). These specific papers focused on the role of vitamin supplements in improving memory. No other papers of Chandra's have since been retracted. However, many have argued his entire body of work should be viewed as, at the very least, highly suspicious (Roberts, 2007; S. Sternberg & Roberts, 2006). While no position is taken in this thesis as to the truth or falsehood of these accusations, the decision has been made to not include this work in this review of the literature.

Raghupathy, Ratnam, & Chandra, 2003; Semba et al., 1995). Here, due to our primary focus on enhancing vaccine responses in UK older adults, this literature will not be described. Interested readers are directed to a comprehensive review of this literature by Savy and colleagues (2009) who concluded that malnutrition has little or no effect on vaccine responses in malnourished children and that evidence for adjunctive effects of supplementation at vaccination was weak. However, importantly they noted that "the paucity, poor quality, and heterogeneity of data make it difficult to draw firm conclusions" (Savy et al., 2009, page 2154S) calling for further, more methodologically rigorous research.

This heterogeneity of research and poor methodological quality described by Savy et al. (2009) is also highly evident across research examining the effects of nutritional factors on older adult vaccine responses. Cross-sectional observational evidence for nutritional effects on vaccination is plentiful, yet inconsistent. Typically, these types of studies have assessed multiple markers of nutrition in serum at the time of vaccination and examined their association with antibody responses at 2-6 weeks post-vaccination (e.g., Fülöp et al., 1999; E. Gardner et al., 2000; Hara, Tanaka, & Hirota, 2005; Sundaram et al., 2013; Sundaram, Meydani, Vandermause, Shay, & Coleman, 2014). Findings from these studies have been contradictory. For example, Fülöp and colleagues (1999) found pre-vaccination serum levels of iron and total protein to be significantly higher among institutionalised elderly patients who demonstrated a four-fold increase in antibody titre to at least one influenza vaccination strain at 4 weeks post-vaccination, than those who did not. No associations were found for any of the many other nutritional factors measured including Vitamin E. In contrast, Hara et al. (2005) found that serum levels of Vitamin E in older adults were associated with improved antibody responses to influenza

vaccination. A similar pattern is true for research on Zinc, with serum levels shown to be associated with antibody response to pneumococcal vaccination (Hamza et al., 2012) but not with antibody response to influenza vaccination (Sundaram et al., 2014).

Overall, no consistent patterns emerge from the cross-sectional observational literature concerning nutritional influences on antibody responses to vaccination. While the causes of such disparity in findings are unclear, it is important to note that such studies are methodologically limited. As most biochemical markers of nutrition can be assessed in serum with relative ease, most cross-sectional studies of this type compare antibody responses against a large number of nutritional factors, thereby increasing the risk of false-positive associations. Further, all of the above studies rely on measuring nutrition via single time-point measures in serum. Such measures have been noted to be unreliable due to their high within-person variability across time (Shvetsov et al., 2009). As such, it is important that future observational research in this area should include more comprehensive assessments of diet, collected over a longer period.

Higher quality evidence comes from several randomised controlled trials of long-term nutritional supplementation, as an intervention to improve vaccine responses, many of which have been conducted in older adult populations. Multi-nutritional supplements have received significant attention presumably based on the rationale that while nutritional deficiencies are common in the elderly, the specific deficiencies differ between individuals and can be costly to identify. Long-term supplementation of diets with multi-nutritional formulas made up of many essential micro- and or macro-nutrients may therefore eliminate, or reduce, any possible nutritional deficiencies in a given population. Five trials of multi-

nutritional supplementation have been conducted in older adults populations (Allsup et al., 2004; Bunout et al., 2004; Langkamp-Henken et al., 2006, 2004; Wouters-Wesseling et al., 2002), with three finding some evidence of enhanced antibody effects of multi-nutritional supplementation on response to influenza vaccination (Langkamp-Henken et al., 2006, 2004; Wouters-Wesseling et al., 2002). However, all of these positive trials had significant methodological limitations. Wouters-Wesseling et al. (2002) found that older adults who consumed a multi-nutritional drink for 6 months prior to, and 1 month following, influenza vaccination had greater mean fold increases in antibody to one influenza strain; however, the sample size for this trial was extremely small (n=19). Two larger studies in institutionalised elderly participants by Langkamp-Henken and colleagues (2006, 2004) found similar enhanced responses to some influenza strains among older adults who consumed a daily multi-nutritional drink compared to those who consumed a commercially available control drink with considerably less nutritional value. However, both of these trials suffered considerable non-compliance (>40%) – making the validity of these findings questionable.

Two trials found no benefits of multi-nutritional supplementation for either influenza or pneumococcal vaccination. Allsup et al. (2004) reported that care home residents taking a multi-micronutrient supplement for 4 weeks before and after vaccination were no more likely to demonstrate a 4-fold increase in antibody titre to any influenza vaccine strains at 4 weeks post-vaccination compared to those given placebo, despite significant increases in serum concentrations of many of the micronutrients in the supplemented group suggesting good adherence. In the only trial in free-living older adults, Bunout et al. (2004) found that a daily multi-nutritional supplement for 6 months (4 months prior to vaccination and 2 months following) did not enhance antibody responses to influenza and pneumococcal vaccinations in

Chilean elderly compared to those who maintained their usual diet at 6 months post-vaccination.

A handful of specific micro-nutrient supplements have also been examined individually or in small combinations as a method of enhancing vaccination response in older adults. Zinc has been included in three trials, with two finding evidence of enhancing effects on influenza (Girodon et al., 1999) and tetanus vaccination (Duchateau, Delepesse, Vrijens, & Collet, 1981) respectively, and one showing no effect on influenza vaccination (Provinciali et al., 1998). One study reported that high levels of vitamin E supplementation (200mg/day) for 4 months can enhance antibody responses to Hepatitis B and Tetanus vaccines, but not diphtheria vaccination compared to placebo (Meydani et al., 1997) However, Girodon et al. (Girodon et al., 1999) found no enhancing effects of vitamin E supplementation on influenza vaccination responses when given in lower, more typical doses (15mg/day).

Probiotics (live bacteria and yeasts that are promoted as having benefits to health) have also received some preliminary attention as an intervention to improve older adults vaccination responses. Boge et al. (2009) reported two trials (pilot and confirmatory) that indicated that nursing home residents who consumed a fermented dairy drink containing the probiotic strain *lactobacillus casei* for 4 weeks prior to, and 9 weeks following, influenza vaccination showed greater antibody responses to some vaccine strains at up to 5 months post-vaccination compared to placebo controls. However, it is noteworthy that these trials were sponsored by the drinks manufacturer and that a very similar, but larger, non-industry trial of the same probiotic strain found no evidence of enhanced influenza vaccination responses

consuming the probiotic drink compared to placebo controls (Puyenbroeck et al., 2012).

Finally, one trial has investigated whether more naturalistic dietary changes, in this case increasing fruit and vegetable consumption, can enhance vaccine responses in older adults (Gibson & Edgar, 2012). Participants who were asked to eat ≥5 portions of fruit and/or vegetables per day for 12 weeks prior to, and 4 weeks following, pneumococcal and tetanus vaccines demonstrated greater IgG antibody responses to pneumococcal, but not tetanus, vaccination at 4 weeks post-vaccination than controls who continued their normal diet of ≤ 2 portions of fruit or vegetables per day.

In sum, there is weak-to-moderate evidence that nutritional factors can influence responses to vaccination. The evidence that nutrition can influence immune dynamics is considerable, with nutritional deficiencies preventing optimal immune function and increasing susceptibility to disease. However, vaccine specific studies are less convincing and are frequently of poor methodological quality. Findings from cross-sectional vaccination studies are inconsistent; but there is, on balance, suggestive evidence from randomised controlled trials that correcting nutritional deficiencies in the elderly may have beneficial effects on responses to vaccination. However, the exact nutritional factors that are most influential are not currently established. Little is known regarding the timing of adequate nutrition in relation to vaccine responses, with most intervention studies beginning supplementation at differing lengths of time prior to vaccination and continuing until post-vaccination follow-up.

3.2.3 Sleep

It is estimated that humans spend up to a third of their lives asleep (Committee on Sleep Medicine and Research, 2006), yet relatively little is known about the exact functions of sleep and its related physiological effects (Imeri & Opp, 2009). Recent societal changes (e.g., changes to working patterns, increased 24-hour access to media) have led to a growing interest in how sleep might influence physiological systems – in part, fuelled by a concern that the population is becoming chronically sleep deprived with potentially detrimental effects to health. Over the last 25 years, there has been a population-wide decline in sleep duration (Jean-Louis, Kripke, Ancoli-Israel, Klauber, & Sepulveda, 2000) with the proportion of adults sleeping less than 6 hours per night increasing rapidly (Centers for Disease Control and Prevention, 2008). Older adults are the most likely to be sleep deprived, sleeping less than younger adults (Klerman & Dijk, 2008), frequently reporting persistent sleep complaints (Foley et al., 1995) and declining sleep quality with age (Neubauer, 1999). These age-related declines in sleep duration and quality mirror the decline in immune function observed with age (see section 1.2) – leading some to hypothesise that it is this sleep disruption that leads to immunosenesence (e.g., Hampson, Rossi, Arora, Lord, & Taheri, 2013).

While a causal relationship between sleep and age-related immunological decline is far from established, there is considerable consensus that sleep and immune function are closely entwined (Bryant, Trinder, & Curtis, 2004; Hampson et al., 2013; Kiecolt-Glaser & Glaser, 1988; Penedo & Dahn, 2005). Indeed, in a review of the evidence linking sleep and immune function, Bryant et al. (2004) went as far as to conclude that "sleep should be considered a vital part of the immune system" (p.465). Evidence for

sleep-immune links is substantial. Many immune components (including cytokines, monocytes and dendritic cells) show systematic fluctuations over a 24-hour period, known as circadian rhythms, that reach their peak, or nadir, during sleep - suggesting some relationship between immune components and the sleep-wake cycle (Lange, Dimitrov, & Born, 2010). Further, human and animal evidence demonstrates that sleep patterns are altered by infection (Norman et al., 1992; Toth & Krueger, 1988; Toth, Rehg, & Webster, 1995), with sleep duration typically found to be extended during symptomatic phases of infection (Bryant et al., 2004). This extended sleep is hypothesised to be immunologically restorative (Imeri & Opp, 2009; Moldofsky, 1994; Opp & Imeri, 1999) with evidence from studies in rabbits demonstrating that longer sleep duration during infection is associated with improved survival chances (Toth, Tolley, & Krueger, 1993).

Of particular importance in the relationship between sleep and the immune system is a specific sleep stage known as slow wave sleep (SWS). Sleep is commonly categorised into 5 stages (see Figure 3.1), distinguished by characteristic changes in brain activity when measured by electroencephalogram (EEG). SWS is typified by high amplitude EEG waves and is accompanied by decreased heart rate, blood pressure, body temperature and breathing rate (Somers, Dyken, Mark, & Abboud, 1993) as well as a range of endocrine changes including the increased release of growth hormone and prolactin and the inhibition of cortisol secretion (Lange et al., 2010). SWS is often found to be extended during both human and animal infections (Bryant et al., 2004) with pro-inflammatory cytokines (such as IL-1β and TNF-α) thought to play a crucial role in a feed-forward loop between the immune system during infection and sleep – such that SWS is increased during an ongoing immune response (Imeri & Opp, 2009; Toth, 1995). The importance of pro-inflammatory cytokines in this process

is further evidenced by studies in animals which have shown that administering TNF-α and IL-1β increases SWS in animals (Dickstein, Moldofsky, Lue, & Hay, 1999; Shoham, Davenne, Cady, Dinarello, & Krueger, 1987) whereas blocking the actions of these cytokines reduces SWS duration (Opp & Krueger, 1994; S. Takahashi, Kapas, Fang, & Krueger, 1995). Notably, SWS duration is reduced in older, compared to younger, human adults (Vgontzas et al., 2003) rapidly declining from midlife onwards (Van Cauter, Leproult, & Plat, 2000) – further supporting the hypothesis that sleep may be crucial in the maintenance of optimal immunological functioning with advancing age.

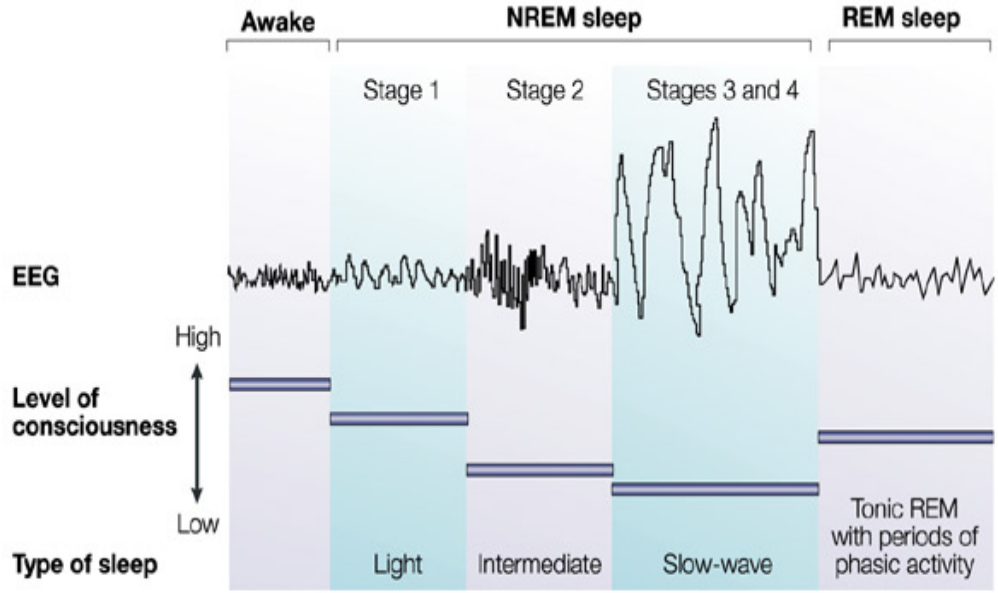


Figure 3.1: Stages of sleep
Adapted from Bryant et al. (2004)

While many of the immunoregulatory mechanisms responsible for the relationship between sleep and immune function remain to be elucidated, there is convincing evidence that this relationship is responsible for clinically meaningful effects on health outcomes in humans. Multiple large prospective epidemiological cohort studies have demonstrated predictive relationships between sleep duration and all-cause mortality, with too much, or too little, sleep associated with increased mortality (Cappuccio, D'Elia, Strazzullo, & Miller, 2010). Infectious disease susceptibility also appears to be influenced meaningfully by sleep. Healthy adults that self-report shorter sleep duration (<7 hours) or poorer sleep efficiency are more likely to develop infection following a standardised controlled viral exposure, compared to those who sleep longer (≥ 8 hour) or had good sleep efficiency (S. Cohen & Doyle, 2009).

Evidence for Effects of Sleep on Human Vaccination Responses

In comparison to the other behaviours considered in this chapter, relatively little research has examined the relationship between sleep and antibody responses to vaccination. The majority of existing studies have concerned the effects of acutely-induced sleep deprivation in small experimental trials. These studies, typically conducted within the confines of a sleep laboratory, involve the comparison of vaccine responses between participants who have their sleep restricted, or prevented, for a given period to those who are allowed to keep their usual sleep/wake cycle. Sleep deprivation is consistently shown in these studies to negatively impact on antibody production following vaccination (Benedict, Brytting, Broman, Markström, & Schiöth, 2012; Lange, 2003; Lange, Dimitrov, Bollinger, Diekelmann, & Born, 2011; Spiegel, Sheridan, & Van Cauter, 2002), although the persistence of this effect has differed. For example, Benedict and colleagues (2012) found that adults kept awake for 24 hours immediately after vaccination against H1N1 (Swine Flu) produced fewer antigen-specific antibodies at 5 days, but not 10 days, post-vaccination compared to those who kept their normal sleep/wake cycle. Similar short-term effects have been reported in relation to the trivalent influenza vaccination, with young adults who had sleep restricted to 4 hours for 6 nights following vaccination (and then extended sleep during the following 7 nights) showing lower antibody levels at 10 days post-vaccination than normally sleeping controls, but no difference at 4 weeks post-vaccination (Spiegel et al., 2002). In contrast, others have described more persistent effects of acute sleep deprivation. Lange (2003) found reduced antibody responses at 28 days post-vaccination in adults who did not sleep for 36 hours following Hepatitis A vaccination and in a further study (Lange et al., 2011) found evidence of decreased IgG1 responses to both Hepatitis A and B vaccinations at up to a

year post-vaccination in participants who did not sleep the night after each part of the triple vaccination schedule. These differing findings most likely relate to the extent of sleep deprivation induced, with the studies with longer deprivation associated with more persistent negative impacts on vaccination responses (e.g., Lange, 2003). However, the differences in the type of vaccine studied, immunological assays used, and whether the sleep deprivation was induced on one occasion or consecutive days makes direct comparisons between these studies challenging.

While these acutely induced sleep deprivation studies provide useful insights into how sleep might impact on vaccination, they have exclusively focused on small samples (n<40) of young healthy adults in laboratory settings, limiting their relevance to community dwelling older adults, or those with chronic ill-health or disease. Further, the acute sleep deprivation established in these studies (over days immediately following vaccination) is qualitatively different to the type of chronic sleep deprivation that is common in older adults (established over months or years prior to vaccination) making it unclear how findings might relate to real-world sleep deprivation (Hampson et al., 2013).

Very few studies have examined the role of sleep deprivation on vaccination responses in more naturalistic scenarios. One small study (n=31) found no difference in antibody response to influenza vaccination between those with obstructive sleep apnoea (which can disturb sleep) compared to healthy controls (Dopp et al., 2007). However, findings from this study should be treated with caution, as the participants with sleep apnoea demonstrated minimal sleep disturbance across the study. Stronger evidence comes from one comparatively large observational study of middle-aged adults (n=125; 40-60 years) in their homes (Prather et al., 2012). In this study, sleep

duration was measured using a combination of self-report diaries and objective actigraphy measures on the days surrounding a 3-part Hepatitis B vaccination schedule. Participants who were found to have shorter sleep duration post-vaccination were less likely to have protective levels of Hepatitis B antibodies at 6 months and lower antibody responses to the second and third vaccination doses (secondary responses).

In total, there is strong evidence of links between sleep and immune function and moderate evidence that sleep deprivation over short or long periods can negatively influence antibody responses following vaccination. That being said, this area would benefit from more research attention. In particular, there is a lack of research investigating the role of sleep on vaccination outcomes in older adults, which is surprising considering this age group are most likely to be sleep deprived (Foley et al., 1995; Klerman & Dijk, 2008). Further, the lack of studies conducted in naturalistic environments remains to be addressed.

3.3 *Psychological Factors, Immunity & Vaccine Response*

While the behaviours considered above are easy distinguished (i.e., sleeping is self-evidently different to exercise), clearly demarcating psychological factors such as stress, distress, affect, emotion or mood is more challenging. There are considerable conceptual overlaps between these terms, and while each has specific meanings within the psychological literature, in reality, they are generally applied interchangeably in healthcare research (Mayne, 1999; Pressman & Cohen, 2005; Reber & Reber, 2001).

Here, to provide some structure for the reader, the imperfect distinction has been made to separate the literature concerning psychological influences on

immunity and antibody responses to vaccination into three broad categories. First, research concerning psychological stress is considered. Stress has been the most widely investigated psychological influence on immunity and arguably could be considered the cornerstone of PNI research. Second, attention will be turned to other emotional experiences that are typically considered to be negative (for which we use the nomenclature: negative affect) – including research that has measured overall negative affective states (i.e., how a person feels at a single time-point), traits (a person's typical affective disposition), and more discrete emotions. Finally, research concerning positive emotional experiences (positive affect), immunity, and antibody responses to vaccination are reviewed.

3.3.1 Psychological Stress

While there is no universally agreed upon definition of stress, typically researchers broadly conceptualise stress as a suite of cognitive, behavioural, and physiological responses to stimuli (stressors) perceived as threatening a person's well-being or goals (Lazarus & Folkman, 1984; McEwen, 2010). Stress, in this context, is most frequently measured via self-report, either to establish the number of stressors encountered over a given period – be that daily hassles or major life events (e.g., divorce) - or to establish the extent to which responders have felt 'stressed'. The latter method is arguably superior in that it allows for individual differences in how a people perceive the same stressor (e.g., encountering a tiger may be less stress-inducing for a zookeeper than an academic). However, both methods are widely used in the study of stress and immunity.

The relationship between stress and the immune system has been the most widely investigated of all the factors considered in this chapter, with multiple

reviews and commentaries of this literature consistently concluding that there exist relationships between stress and immune responses (Glaser, 2005; Glaser & Kiecolt-Glaser, 2005; Gouin, Hantsoo, & Kiecolt-Glaser, 2008; Herbert & Cohen, 1993b; Segerstrom & Miller, 2004). The most notable of these reviews was conducted by Segerstrom & Miller (2004) who compiled a seminal and comprehensive meta-analytic review of over 300 articles examining the effects of psychological stress on the immune system. They concluded that there was convincing evidence that stress is associated with immune responses, with effects dependent on the stressor duration. In response to short (<100 minutes), experimentally induced stress, meta-analyses revealed highly significant changes to many immune component distributions or function including those of T-Lymphocytes, NK Cells and the cytokine IL-6. These short lived changes are hypothesised to be beneficial and adaptive, in that immune components are redistributed to where they will be most effective should pathogens enter the body (Dhabhar & McEwen, 1997). Longer, but still time limited, stressors (e.g., exams) were found to be most associated with changes in cytokine production (IL-1, IL-2, IF-γ) and other markers of cellular immunity suppression such as increased antibody production to latent viruses and NK cell cytotoxicity. Chronic stressors (e.g., caregiving for someone with dementia) had the most pernicious impact on immunity, being associated with multiple functional dysregulations of both cellular and humoral immunity.

That stressors of differing durations may influence the immune system in different ways is captured theoretically in the Allostatic Load Model (McEwen, 1998; McEwen & Stellar, 1993). This model posits that physiological adaptation to stress (also known as allostasis) is essential to maintaining homeostasis, but in scenarios where such stress-induced changes are overly frequent or prolonged (allostatic load) this causes 'wear and tear' on the

body leaving it more vulnerable to disease. Further support for this model comes from multiple viral challenge studies which have found that among healthy adults volunteers, greater perceptions of stress and stressor exposure are associated with greater susceptibility to, and severity of, objectively measured common colds viruses (S. Cohen, Doyle, & Skoner, 1999; S. Cohen et al., 1998, 1991, 1993), with one study demonstrating that only chronically stressful events lasting at least one month in duration were associated with greater susceptibility to infections (S. Cohen et al., 1998).

Evidence for Effects of Psychological Stress on Human Vaccination Responses

Multiple reviews have specifically examined the evidence that psychological stress can influence antibody response following vaccination in humans (Burns, Carroll, Ring, & Drayson, 2003; S. Cohen, Miller, & Rabin, 2001; A. Pedersen, Zachariae, & Bovbjerg, 2009; Powell, Allen, Hurnagle, Sheridan, & Baily, 2012) consistently concluding there is strong evidence that a high level of sustained psychological stress is associated with poorer antibody responses. The majority of this evidence is observational in design and has explored associations between self-reports of experienced stressors or perceived stress measured at a single time-point and antibody response at 2-6 weeks post-vaccination. Hepatitis B vaccination has been the most commonly studied in young adults, presumably because of the availability of medical students who routinely receive this vaccination (Burns et al., 2003). This vaccine is typically administered in three doses, with participants usually screened to ensure they have no Hepatitis B antibodies prior to the first dose. This means researchers are theoretically able to examine either

primary or secondary response to the Hepatitis B vaccination depending when blood sampling occurs.

Only one observational study to date has looked at primary response to Hepatitis B vaccine (Glaser et al., 1992), finding that medical students who had 'seroconverted' - a term not defined in the paper but presumably referring to having any identifiable antibody response to the vaccine – at one month post-vaccination had lower levels of perceived stress than those that did not. Secondary responses to Hepatitis B have been examined more frequently, although findings are mixed. Two studies have found a relationship between stress and secondary antibody response to Hepatitis B, although in opposite directions. Jabaaij et al. (1993) vaccinated 95 students with a low dose of the Hepatitis B vaccine course and found that at 7 months after the first dose (1 month after completion of the vaccination schedule), those with higher scores on a composite stress index (comprising life events and psychological symptoms scales) at the time of the second dose had lower antibody responses than those with low stress. Stress index scores at the time of third dose (6 months after first dose) were not related to antibody response, suggesting the importance of stress during the earlier stages of the immune response. In contrast, Petry et al. (1991) found that higher perceived stress over the six months following the first vaccine dose was associated with higher peak antibody response following the third dose of the vaccine series. Two similarly designed studies found no evidence of a relationship between stress and secondary antibody responses to Hepatitis B in medical students (Jabaaij et al., 1996; Marsland, Cohen, Rabin, & Manuck, 2001) although the heterogeneity of stress measures used, and the times relative to vaccination at which they were completed – makes synthesising these findings difficult.

The largest Hepatitis B and stress study to date (n=260) examined cross-sectional observational associations between exposure to stressful life events having protective antibody titres against Hepatitis B in medical students (Burns, Carroll, Ring, Harrison, & Drayson, 2002). For participants that were vaccinated against Hepatitis B over a year prior to the study, those with higher exposure to stressful life events in the previous 12 months were less likely to have adequate antibody levels (defined as >100mIU/ml) than those with lower exposure. This association was not observed among participants who were vaccinated less than 12 months prior to the study, of whom the clear majority had adequate Hepatitis B antibody levels. While the cross-sectional design and reliance of participants being able to report retrospectively over a 12-month period on stressful life events are limitations of this study, it does provide preliminary evidence that stress may be of importance in the long-term maintenance of vaccine responses. Considerable further research is needed to verify these associations. Notably, no studies to date have looked at the relationship between stress and Hepatitis B vaccination in older adults.

Influenza vaccination has also received significant research attention in context of the potential influence of stress, both in young and older adults. A 2009 review and meta-analysis of these studies, found a small-to-moderate sized significant negative relationship (r=-.18) between stress and antibody response, with the strongest relationship (r=-.25) evident in studies conducted in older adults (A. Pedersen et al., 2009). Among older adults, a frequently used paradigm has been to compare the antibody response to influenza vaccination among a cohort of participants considered to be chronically stressed (e.g., spousal caregivers) to age-matched non-chronically stressed control group. For example, Vedhara et al. (Vedhara, Cox, et al., 1999) found that only 16% of older adults caring for a spouse

with dementia achieved a four-fold increase in antibodies to at least one vaccine strain following influenza vaccination compared to 39% of non-carer older adults. Congruent findings have also been reported in two other similar studies of older adult caregivers (Glaser, Kiecolt-Glaser, Malarkey, & Sheridan, 1998; Kiecolt-Glaser et al., 1996). In contrast, one recent study of elderly Hong Kong Chinese caregivers found lower cell-mediated responses, but not antibody responses, following influenza vaccination compared to non-carers (Wong et al., 2013). This differing result may be explained by the seemingly high levels of pre-vaccination antibodies observed among both caregivers and controls prior to vaccination, as well as differences in statistical analyses, specifically how baseline antibody levels where accounted for. In the earlier studies, participants were considered 'responders' if they demonstrated a four-fold increase in antibody levels from baseline. However, a limitation of this approach is that vaccine-induced antibody changes are strongly influenced by pre-vaccination antibody levels, with those with low pre-vaccination antibodies more likely to exhibit greater fold-increases (Sasaki et al., 2008). The study by Wong et al. (2013) may have found differing results as they controlled for this by adjusting fold-increases for baseline antibody levels, although it is unclear from the published article how this adjustment was achieved.

A small number of intervention trials have explicitly examined whether stress reduction interventions can enhance antibody responses to vaccination, finding mixed results (Loft et al., 2012; Moynihan et al., 2013; Vedhara et al., 2003). One trial has been conducted in younger adult medical students preparing for examinations, finding no effect of a 45-minute weekly massage, during the period prior to vaccination, on responses at 2- and 6-weeks following Hepatitis B vaccination (administered post-intervention) compared to controls. However, notably self-report measures of perceived

stress did not differ between groups, suggesting that the weekly massage intervention was not sufficient to reduce the stress caused by the examinations. Two stress-reduction intervention trials have been conducted in older adults, both finding significant effect – but in opposite directions. Vedhara et al. (2003) conducted a non-randomised pragmatic trial of an 8-week group stress-management intervention prior to vaccination, finding that 50% of elderly spousal carers of dementia patients who received the intervention produced a four-fold increase in antibody titre following influenza vaccination compared to 7% of carers who did not receive the intervention and 29% of non-carer controls. In contrast, a more recent and relatively large (n=201) randomised controlled trial of a group based mindfulness-based stress reduction intervention, delivered prior to vaccination, in older adults found *poorer* antibody responses at 24 weeks post-vaccination to Keyhole Limpet Hemocyanin at various doses in those receiving the intervention to those in a waitlist control (Moynihan et al., 2013). There are many potential explanations for this disparity in findings. For example, it is noteworthy that the reliability of findings in the study conducted by Moynihan and colleagues (2013) are limited by the fact that between group differences in baseline KLH antibodies were observed (to which it had been assumed participants would be negative) potentially confounding results. Further, differences could relate to disparities in the intervention design, vaccine choice, or the fact that the trial conducted by Vedhara and colleagues (2003) focussed on older adult carers (who demonstrated high levels of pre-intervention stress) whereas the study conducted by Moynihan and colleagues (2013) recruited 'typical' older adults (who demonstrated relatively low-levels of pre-intervention stress). Considerable further research is needed to demonstrate whether, and in whom, stress-reduction interventions can improve vaccination outcomes.

In sum, there is strong evidence that psychological stress can influence the immune system, with moderate-to-strong evidence that chronic stress can impair antibody responses following vaccination. These effects appear to be most evident in older adults. While some interventions aiming to reduce stress have been trialled as a method of enhancing vaccine responses, results are mixed and complicated by numerous methodological disparities, indicating the need for additional research. Also, no intervention trial to date has focused on the impact of psychological stress reduction in the period immediately following vaccination (when the vaccine components are interacting with the immune system) – an area in need of further investigation.

3.3.2 Affect

It is worth addressing at this point the relationship between positive and negative affect, and how they are conceptualised in this thesis. While lay descriptions of emotions classify someone as being in either a good or bad mood - researchers interested in affect most frequently conceptualise positive and negative affect as being separate dimensions of emotional experience as opposed to opposite ends of a single continuum (e.g., Remington, Fabrigar, & Visser, 2000; Watson, Clark, & Tellegen, 1988). Put another way, positive affect is typically not seen as just the absence of negative affect, indeed researchers have noted that strong experiences of positive and negative can co-occur (Hershfield, Scheibe, Sims, & Carstensen, 2013). Here, findings relating to positive and negative affect are presented separately, to highlight this distinction. A further distinction to note is between state and trait measures of affect. Conceptually, state measures capture momentary, or short-term affective states that are often related to the situational or environmental circumstances experienced by the

respondent. Trait measures, in contrast, seek to capture a more enduring characteristic of an individual as having a more positive or negative affective disposition. Most of studies reviewed in the following sections capture state measures of affect, however studies that have explicitly measured trait affect are highlighted.

3.3.2.1 Negative Affect

Alongside research on stress, within PNI there has been a closely-related tradition of research investigating negative emotional experiences on immunity. Multiple studies have demonstrated that inducing negative emotion states (e.g., by watching a sad video) are accompanied by changes to immune dynamics (e.g., Knapp et al., 1992; Labott, Ahleman, Wolever, & Martin, 1990) and that those with a diagnosis of depression - a condition characterised by prolonged high levels of negative affect - show altered immunological profiles to non-depressed individuals (Zorrilla et al., 2001).

At a clinical level, higher levels of negative affect are often hypothesised to lead to poorer immune functioning, with multiple observational and experimental studies finding relationships consistent with this (for reviews on this literature see Herbert & Cohen, 1993a; Kiecolt-Glaser, McGuire, Robles, & Glaser, 2002; Koh, 1998). To give just a few examples here: higher state levels of negative affect have been found to increase susceptibility to cold viruses in viral challenge studies (S. Cohen et al., 1995, 1993). Greater levels of anxiety and depression following the death of a loved one has been associated with reduced NK cell function at up to 6 months post-bereavement (Gerra et al., 2003); and in two innovative daily-diary studies, lower salivary secretions of IgA to an orally administered antigen (rabbit albumin) were observed on days where participants self-

reported higher levels of negative affect (Stone, Cox, Valdimarsdottir, Jandorf, & Neale, 1987; Stone et al., 1994).

However, while much evidence supports the position that negative affect can adversely influence immune dynamics and function – the characterisation of negative affect as immunosuppressive is overly simplistic. In a review of negative affect on health outcomes, Mayne (1999) argued that brief bursts of negative affect can actually enhance immunological outcomes, in a similar manner to that observed in acute bursts of exercise or stress, as long as this is not prolonged. This is because emotion can cause activation of the sympathetic branch of the autonomic nervous system (Hughes, Uhlmann, & Pennebaker, 1994), which leads to the increased release of immune cells from lymphatic organs into circulation, as well as enhancing the function of some immune cells (Elenkov, Wilder, Chrousos, & Vizi, 2000). There is some evidence to support this position; for example, in a study of 18 HIV-positive men who were asked to imagine reliving both positive and negative emotional experiences, those who showed greatest sympathetic activation during the task showed a marked increase in NK cell function (O'Leary, Temoshok, Sweet, & Jenkins, 1989). While research into any potentially beneficial immunological effects of experiencing negative affect remains relatively sparse, the possibility accords well with the research examined above on both stress and exercise, which has shown that the severity and duration of either stressors or exercise exert differing effects on immune function (e.g., Segerstrom & Miller, 2004).

Evidence for Effects of Negative Affect on Human Vaccination Responses

Multiple observational studies have investigated the relationship between negative affect (in its different conceptions) and antibody responses following vaccination (e.g., Afsar, Elsurer, Eyileten, Yilmaz, & Caglar, 2009;

Kiecolt-Glaser et al., 1996; Li et al., 2007; Marsland et al., 2001; Moynihan et al., 2004; Pressman et al., 2005). A slight majority of these studies support an inverse relationship between negative affect and antibody response to vaccination (Afsar et al., 2009; Li et al., 2007; Marsland et al., 2001; Pressman et al., 2005). Higher trait levels of negative affect, as measured by a combination of validated self-report measures, has been found to be associated with lower antibody response to a Hepatitis B vaccination course in young undergraduate students (Marsland et al., 2001). Among spousal and offspring caregivers for older adults with Alzheimer's disease, higher depressive symptoms have been found to be associated with lower antibody titres following tetanus vaccination (Li et al., 2007) and dialysis patients achieving 'protective' levels of antibodies following Hepatitis B vaccination (as determined by a pre-established cut-off) scored lower on measures of depressive symptoms than those who did not (Afsar et al., 2009). However, two studies in older adults have found no relationship between either general negative affect (Moynihan et al., 2004) or depressive symptoms (Kiecolt-Glaser et al., 1996) and antibody response to influenza vaccination. Further, one study in young adults found higher levels of depression to predict enhanced antibody responses to Hepatitis B vaccination at 3 months following the vaccine schedule (Petry et al., 1991). The reasons for this lack of consistency could be explained by many factors, including differences in the psychological measures and immunological assays used, the varying degree or duration of negative affect between samples or time post-vaccination at which antibody response was assessed. However, it is also important to note that the majority of these studies are all limited by the fact that negative affect was measured via self-report at a single time point. This is problematic because self-reports of affect are known to fluctuate over time (Eid & Diener, 1999) – and even measures that purport to capture affect over a period of time are likely influenced by recall

biases and the affective state of the responder at the time of completion (Kahneman & Krueger, 2006; Shiffman et al., 2008).

Studies by Pressman et al. (2005) and Rosenkranz et al. (2003) in this area have used more sophisticated measures of negative affect, both finding a relationship between negative affect and vaccination response. Pressman et al. (2005) measured loneliness among college freshman via 4-times daily self-report measures over a 13 day period, encompassing the period both immediately prior to and after influenza vaccination. Aggregating these scores, they found that greater loneliness was associated with poorer antibody response to one of three stains included in the influenza vaccine at both 1 and 4 months post-vaccination. In a particularly novel experimental study, Rosenkranz et al. (2003) induced positive and negative affect states in late, middle aged adults (57-60 years) by asking them to write about their best or worst 'time or experience in life' for 5 minutes prior to receiving influenza vaccination. Using electroencephalography (EEG), they found that participants who showed greater activation in the right-prefrontal region of the brain – an area previously established as playing a role in affective processing (Davidson, Jackson, & Kalin, 2000) – at baseline and as a result of negative affect induction had weaker antibody responses to influenza vaccination at 6 months post-vaccination.

Very few intervention trials have explicitly sought to improve responses to vaccination by reducing negative affect. However, a small literature on written emotional disclosure is relevant here. Written emotional disclosure interventions involve asking participants to write, in private, about a personal negative emotional experience on 3 to 4 occasions over a brief period (typically within 2 weeks). The assumption behind this method is that non-disclosure of negative emotional experiences is effortful – and acts as a

low level stressor that, if disclosed, can reduce stress and/or negative affect potentially having immunological and wider health benefits (Smyth, Pennebaker, & Arigo, 2012). Two small randomised controlled trials (n<50) of written emotional disclosure interventions on vaccine responses have been conducted to date (Petrie, Moss-Morris, Grey, & Shaw, 2004; Stetler, Chen, & Miller, 2006) finding differing effects. Petrie et al. (2004) randomised medical students to write either about a personally traumatic experience or their recent and upcoming schedules on four consecutive days. After this intervention, participants were given the first of a 3-course Hepatitis B vaccination. Students in the emotional disclosure condition demonstrated higher antibody responses to Hepatitis B vaccination at both 4 months and 6 months after the vaccine schedule began. Interestingly, self-report measures indicated students in the emotional disclosure condition experienced significantly greater sadness, guilt and 'heart pounding' following writing sessions raising the possibility that an increase in negative affect and its associated physiological activation may account for the enhanced vaccine responses. In contrast, Stetler and colleagues (2006) found that black adults who wrote about personal experiences of racial discrimination had reduced antibody responses to influenza vaccination to two of three viral strains at 1 and 3 months post-vaccination, compared to control participants who wrote about their daily schedules. These divergent findings may relate to the type of experience being disclosed in each study. In the Petrie et al. (2004) study, participants were disclosing details of a distinct traumatic event that occurred in the past and is was therefore time-limited and unlikely to re-occur. In contrast, in the study by Stetler et al. (2006) participants disclosed experiences of racial discrimination – a continuing stressor that may influence participants future experiences as well as past ones. It is possible that emotional disclosure interventions are

less successful in situations where the experienced disclosed are perceived to be likely to re-occur.

In sum, there is moderate evidence that greater negative affect can influence responses to vaccination. Negative affect has been associated with changes to immune dynamics and susceptibility to infectious diseases in both observational and experimental studies. Observational studies of the relationship between negative affect on vaccine responses are mixed, but on balance suggest that greater negative affect (especially when longitudinally measured) is associated with reduced antibody responses following vaccination. Intervention trials on written emotional disclosure are inconclusive and contradictory, however other potential negative affect altering interventions, and their timing in relation to vaccination, remain to be investigated. Research in this area would be strengthened by less reliance on single time-point measures of negative affect and addressing the relative paucity of studies conducted exclusively in older adult populations.

3.3.2.2 Positive Affect

Since its inception as a scientific field of enquiry, PNI research has primarily focused on those psychological experiences that are broadly viewed, albeit somewhat simplistically, as negative (e.g., stress, depression). However, while such research remains considerably more abundant, more recently there has been growing interest in how more positive psychological experiences might influence health and immune outcomes (Marsland, Pressman, & Cohen, 2007; Pressman & Cohen, 2005). This increasing attention on so called 'positive psychology' has resulted in an accumulation of evidence that both trait positive affect and experiences of more discrete emotions (e.g., happiness, joy) are associated with beneficial health

outcomes, including those which may have immunological aetiologies (Barak, 2006; Chesney et al., 2005; Consedine & Moskowitz, 2007; Dockray & Steptoe, 2010; Pressman & Cohen, 2005; Steptoe, Dockray, & Wardle, 2009).

One common method of investigating how positive affect might influence immunity has been through experimental mood induction studies. In these laboratory based studies, immune components are measured prior to and immediately following a manipulation designed to improve mood. Such manipulations have included: watching a humorous film (Dillon, Minchoff, & Baker, 1985), listening to music (Hucklebridge et al., 2000), reflecting on pleasant experiences (Knapp et al., 1992), and hypnosis (Zachariae et al., 1991). Multiple immune components have been measured in these studies, although the most researched is salivary IgA secretions, which have consistently been shown to increase following positive affect inducement (Dillon et al., 1985; Harrison et al., 2000; Hucklebridge et al., 2000; Labott et al., 1990; McCraty, Atkinson, Rein, & Watkins, 1996; Njus, Nitschke, & Bryant, 1996; Watanuki & Kim, 2005). Other immune components have been less comprehensively investigated, but some studies have demonstrated increased numbers of circulating cytokines IL-2 and IL-3, B-cells, T-Cells and percentage of NK cells as a result of positive affect induction (Berk, Felten, Tan, Bittman, & Westengard, 2001; Futterman, Kemeny, Shapiro, & Fahey, 1994; Mittwoch-Jaffe, Shalit, Srendi, & Yehuda, 1995). It is noteworthy that two of these studies have provided evidence that positive and negative mood manipulations may not differentially influence immune responses – with similar increases in salivary IgA observed immediately after both positive and negative mood is induced (Hucklebridge et al., 2000; Njus et al., 1996). This indicates that it may be the physiological arousal of emotional experiences, rather than the positive-

negative valence of the emotion that stimulates immunological changes, at least in the short-term.

While experimental studies such as those described above provide evidence that transient experiences of positive affect have immunological consequences, they have all been conducted on young healthy adults, making it difficult to interpret whether the findings are generalisable to older adults. Further, they provide little insight into how real-world fluctuating experiences of positive affect over time might influence the immune system and its ability to deal with antigenic challenge.

More naturalistic observational studies have drawn upon daily diaries or cross-sectional trait measures of positive affect to understand how average experiences of positive affect over time might influence immunity. For example, in two daily diary studies conducted by Stone and colleagues (1987, 1994), secretory IgA levels in response to an oral antigen were higher on days where participants reported high states of positive affect. In contrast, a similar diary study conducted by Evans et al. (1993) found no effect of daily positive affect on secretory IgA. However, this study included a sample of only 12 students – potentially rendering it inadequately powered to find effects.

Only two observational studies of positive mood and immunity have included older adults (Lutgendorf et al., 2001; Ryff, Singer, & Dienberg Love, 2004). Lutgendorf et al. (2001) examined immune responses among a relatively small sample of 30 older adults who were moving to assisted living and 28 non-moving controls. It was found that higher levels of positive affect (vigour), measured at 3 time points, was associated with greater NK cell cytotoxicity and that greater positive affect at 2-weeks post-move was

associated with lower antibody titers to the latent Epstein-barr virus. No relationship was observed between positive affect and IL-6. This lack of association between IL-6 and positive affect was replicated in a sample of 135 older women by Ryff et al. (Ryff et al., 2004) who measured positive affect repeatedly over four days.

Taken together, the research described above demonstrates a relationship between positive affect and immune dynamics/function. Such a relationship would seem to be clinically meaningful with two viral challenge studies finding that those with more positive emotional styles (greater positive affect over a period of time) have a reduced risk of developing serologically verified infection after standardised viral challenge (S. Cohen, Alper, Doyle, Treanor, & Turner, 2006; S. Cohen, Doyle, Turner, Alper, & Skoner, 2003).

Evidence for Effects of Positive Affect on Human Vaccination Responses

Despite convincing evidence that positive affect can influence immune dynamics, in possibly clinically meaningful ways, very few studies to date have examined positive affect on responses to vaccination. Only one observational study has measured both positive affect and vaccine-specific antibody responses, finding that graduate students who reported higher trait levels of positive affect had greater antibody responses to Hepatitis B vaccination at 5 months post-vaccination (Marsland, Cohen, Rabin, & Manuck, 2006). No independent effect of negative affect on antibody response was found in this study, with moderation analyses indicating that physical activity played a protective role for those participants with low positive affect. A further study has examined *ex-vivo* cytokine response to influenza vaccination in older adults, by stimulating peripheral blood mononuclear cells with the vaccine – with greater trait optimism associated

with increased IL-2, but not INF-γ or IL-10, production (Costanzo et al., 2004).

While multiple trials of psychosocial interventions aimed at improving immune responses to vaccination have focussed on reducing 'negative' emotional experiences (see 3.3.1 and 3.3.2.1), the related role of increasing positive affect is rarely considered. However, one would expect that in addition to reducing negative affect or stress, interventions such as mindfulness training or stress management might have the related consequence of increasing positive affect, as has been evidenced previously (Nyklíček & Kuijpers, 2008; Orzech, Shapiro, Brown, & McKay, 2009). Unfortunately, most trials in this area have not investigated this possibility, making it impossible to untangle whether between group improvements in vaccine response are driven by changes to negative affect, positive affect, or both. There is however, some preliminary evidence that intervention induced changes in positive affect may influence vaccine responses. In a randomised controlled trial, Davidson et al. (2003) found that young adult participants who underwent an 8-week mindfulness training program, delivered prior to vaccination, showed greater left-sided anterior brain activation (previously associated with experiencing positive affect) in response to mood inductions and had greater response to influenza vaccination at 4 weeks post-vaccination compared to wait-list controls. Importantly, the change from pre- to post-intervention in brain-activation also predicted the magnitude of antibody response to the vaccination.

In sum, there is currently too little evidence to be confident that positive affect influences responses to vaccination, although existing evidence is consistent with this hypothesis. Indeed, the considerable experimental evidence suggesting that transient experiences of positive affect can

influence immune dynamics makes further investigation warranted. Future research in this area should strive to include multiple measurements of positive affect over time, as well as negative affect – to begin to untangle the importance of emotion valence (i.e., whether the relative attractiveness/aversiveness of emotions result in differential impacts on immunity). Further, including measurement periods both pre- and post-vaccination will allow researchers to better understand if positive affect is more influential at certain times in relation to vaccination (e.g., pre- or post-vaccination).

3.4 ***Summation of Evidence***

Taken together, there is strong evidence, both observational and experimental, that each of the behavioural and psychological factors considered in this chapter influence the dynamics and/or function of the immune system. They, in turn, have also been shown (to a greater or lesser extent) to impact on the body's ability to mount an effective antibody response to vaccination. That being said, it is clear that in many areas there is a need for considerably more, methodologically rigorous, research. While some attempts have been made to modulate immune response to vaccination via psycho-behavioural interventions, their success is best described as promising, but limited.

While the disparate nature of the literatures considered make any grand synthesis of findings challenging, a few common themes do emerge. For example, influences of behavioural and psychological factors on antibody responses to vaccination appear to be most evident where recipients have sub-optimal immune function (e.g., older adults) or in cases where overall vaccine immunogenicity is low. Reduced, or null, effects in younger adults

or highly immunogenic vaccines are likely due to ceiling effects, with immune responses being so robust that behavioural or psychological influences are unlikely to be observed. This finding points to the importance of appropriately targeting any psycho-behavioural intervention to improve vaccination responses at individuals most likely to benefit or in cases where vaccine efficacy is known to be poor.

Further, intensity and duration are important determinants in how a behavioural or psychological factor influences the immune system and thus antibody responses following vaccination. Short or moderate physiological activation (such as that which accompanies acute exercise performance, acute bouts of stress or affect) has been demonstrated to have enhancing effects on immune function and vaccine responses. In contrast, prolonged or high intensity physiological activation (such as that which accompanies long-term stress, marathon running) can result in immunological wear-and-tear, leading to a pernicious decline of immunological competence and impairing vaccine responses. Future interventions may find benefits by combining these effects: aiming to induce short-term changes in psychological or behavioural factors close to the point of vaccination, while simultaneously seeking to reduce chronic negative influences that cause immunological dysfunction.

3.5 ***Common Methodological Limitations & Areas in Need of Further Research***

3.5.1 Over-reliance on Single Time-point, Retrospective Measures

In measuring each of the behavioural and psychological factors considered above, the vast majority of studies sought to retrospectively ask participants to self-report their performance of a behaviour, or psychological experience

over a given period (typically weeks or months), using standardised and validated questionnaire measures. The assumption underpinning this methodology is that participants can accurately recall their experiences and articulate these in their questionnaire responses. However, this assumption of unbiased memory recall has been critiqued extensively (e.g., Bradburn, Rips, & Shevell, 1987; N. Schwartz & Sudman, 1995), most notably because such responses are open to systematic cognitive biases. Such biases result from the fact that in retrieving autobiographical memories respondents often rely on a variety of cognitive heuristics, or shortcuts, to estimate – rather than logically aggregate and recall – their experiences (Shiffman et al., 2008). Many such heuristics have been described (see Tversky & Kahneman, 1974), including a tendancy for individuals to recall experiences based on its most salient or recent aspects. For example, Redelmeier and Kahneman (Redelmeier & Kahneman, 1996) found patients' retrospective memories of pain during colonoscopy and lithotripsy procedures were predicted by peak and late-procedure pain reported during the procedures, but not overall levels of reported pain or length of procedure. Further, recall has also been demonstrated to be influenced by current affective state, with participants in a negative mood more likely to recall negative information than positive, and *vice versa* (Teasdale & Russell, 1983). Studies that employ more sophisticated and repeated measurements of behavioural and psychological influences on vaccination response are therefore needed.

3.5.2 Heterogeneity in Operationalising Antibody Outcomes Following Vaccination

In examining the influence of behavioural or psychological factors on antibody outcomes following vaccination, the studies reviewed above have operationalised antibody outcomes in multiple and disparate ways. For

example, some classified participants as having successfully achieved, or not achieved, a level of serum antigen-specific antibodies deemed to be clinically protective using pre-defined cut-offs (e.g., Woods et al., 2009). In contrast, others have focussed more specifically on change in antibody levels from pre- to post-vaccination, either by classifying participants as having an increase above or below a cut-off (Vedhara, Cox, et al., 1999) or using a continuous measure of change from baseline (e.g., Yang et al., 2007). Beyond this, there have been some additional statistical differences in the approaches taken, with some studies controlling for baseline antibody levels in situations where participants have detectable pre-vaccine antibodies (e.g., Wong et al., 2013) and others not (e.g., Kiecolt-Glaser et al., 1996).

The approach taken in operationalising antibody outcomes following vaccination is of critical importance and can potentially influence the conclusions drawn regarding how behavioural or psychological factors might be implicated in vaccine responses. For example, an individual who demonstrates a relatively large change in antibody level following vaccination could be classified as a 'successful case' using one approach, but as an 'unsuccessful case' in another if the post-vaccination antibody levels are still below the pre-defined threshold. Conversely, another individual with high pre-vaccination antibody levels might demonstrate little increase in antibody levels following vaccination (due to ceiling effects) and therefore be identified as an 'unsuccessful case' in some approaches, whereas others approaches, such as those focussing on absolute levels of antibody at a given time post-vaccination, result in this individual being seen as a 'successful case'. Further, with different studies taking different approaches to operationalising antibody outcomes, this makes comparisons across studies challenging especially for those seeking to synthesis findings using meta-analyses.

While no simple fix for the existing heterogeneity exists, those conducting further studies in this area should be mindful of the wide variety of approaches used to operationalise antibody responses to vaccination. Ideally, all studies investigating how behavioural or psychological factors influence antibody responses to vaccination would adopt the same operationalised definitions, but reaching such a consensus is challenging. Indeed, while some approaches are more conceptually and statistically robust than others (an issue that will be discussed in chapter 4), all are defendable. For now, researchers in this area should report, as a minimum, results for how behavioural and psychological factors influence on both absolute post-vaccination antibody levels (as a surrogate measure of protection) and a measure of change from pre- to post-vaccination.

3.5.3 The Potential Role of Intra-Individual Variability

In the broader literature of psychological predictors of health, it is almost ubiquitous for researchers to focus on exploring whether individuals' mean levels of particular factors (e.g., average stress level) are determinants of any given health outcome. However, there is now growing recognition that other characteristics of psychological factors may have additional predictive value – such as the stability of these factors over time. This measure of stability, or lack thereof, is typically termed *intra-individual variability* and is receiving greater prominence amongst studies that incorporate multiple repeated measures. For example, greater intra-individual variability in various psychological constructs have been associated with the frequency of health complaints (Watson, 1988), physical and psychological health status (Chan, Zhang, Fung, & Hagger, 2016; Gruber, Kogan, Quoidbach, & Mauss, 2013; Hardy & Segerstrom, 2016), and even mortality rates in older adults (Eizenman, Nesselroade, Featherman, & Rowe, 1997). As highlighted in

3.5.1, existing literature concerning psycho-behavioural influences on vaccine responses has been limited by an over-reliance of single-time point measures. Therefore, as a result, no study to date has examined whether intra-individual variability in psychological factors may be associated with vaccine outcomes. While entirely speculative, there is some biological plausibility for such a relationship. As described throughout section 3.3, inducing positive or negative psychological states has been shown to result in immunological changes (Berk et al., 2001; Dillon et al., 1985; Hucklebridge et al., 2000). Frequent oscillation between more positive and more negative emotional states could potentially therefore result in the kind of chronic activation associated with long-term stress which is associated with considerable immune dysregulation (Segerstrom & Miller, 2004). The incorporation of momentary, repeated measures of psychological factors across time into future studies would allow this possibility to be explored.

3.5.4 Identifying the Active Ingredients of Effective Interventions

At present, no psycho-behavioural intervention to improve vaccine efficacy is delivered widely within any major healthcare system or is recommended by healthcare bodies. Long-term interventions, such as regular exercise interventions, require significant resources and are therefore perhaps of limited practical benefit for widespread implementation. To achieve wider acceptance and recommendation, psycho-behavioural interventions to improve vaccine efficacy need to be established as practical, effective and economical. Such an intervention would ideally be brief, low-cost, and potentially self-deliverable.

To develop such interventions that improve vaccine responses, it is important to establish the essential features of an effective intervention. Currently it is unknown which psycho-behavioural factor, or combination of

factors, are associated with optimal vaccine responses and are therefore ideal targets for interventions. The clear majority of research described above has focused on the role of a *single* psychological or behavioural factor. This is likely due to differences in researcher's domains and expertise, where sports scientists have traditionally been interested in the role of exercise on immunity, nutritionists on nutritional factors and immunity, and health psychologists on psychological factors and immunity. Where multiple psychological or behavioural factors are measured, they are typically only considered as 'controlling factors' for analyses rather than as a primary explanatory predictor. Overall, there is a dearth of vaccine research in PNI that has compared *multiple* psycho-behavioural factors in conjunction comparing their relative influence on immunity. As a result, there is currently very little evidence to support the targeting of one behavioural or psychological factor over another in developing novel interventions to enhance vaccine effectiveness. While the evidence described in this chapter points to the importance of multiple behavioural and psychological influences on immunity and vaccine responses, it is unknown whether these effects are additive or not. This deficiency in the literature requires considerable attention.

A further requirement is to establish the most appropriate timing for psycho-behavioural interventions (i.e., when, in relation to vaccine administration, do psycho-behavioural factors have the greatest influence?). To date, intervention studies in this area have differed with regards their timing in relation to vaccination, some have occurred entirely before vaccination, some immediately post-vaccination and others have spanned both periods. In general, such intervention studies have not provided a rationale for the particular timings of the studied intervention and there is a paucity of evidence to support any specific time period being more influential than

others. However, on an immunological level one could speculate that there may be critical points at which the dynamics of the immune system most influence antibody generation. For example, immediately following vaccination where the components of the vaccine interact with the immune system. While plausible, research examining whether there exist critical periods at which behavioural and psychological factors can most influence vaccine response is greatly lacking. Longitudinal observational studies may be useful in this regard, where the influence of behavioural and psychological factors on vaccine response can be examined across all potentially critical time periods.

3.6 *Chapter Summary*

This chapter has critically reviewed the literature concerning behavioural and psychological influences on immunity and vaccine responses. While there is currently reasonably strong evidence that behavioural and psychological factors can influence immune function and, as a result, responses to vaccination, there is a need for additional, more methodologically rigorous research. Specifically, this chapter has highlighted the need for further research in older adult populations that explore multiple psychological and behavioural factors and their influence on vaccine outcomes. Further, this chapter has emphasised concerns regarding the over-reliance on single time-point, retrospective measures of psychological and behavioural factors. The remainder of this thesis presents novel research that begins to address these areas, increasing the evidence base for developing novel and effective psycho-behavioural interventions to improve vaccination responses.

Chapter 4: The BeHIVE-65 Study – Design and Methods

Chapter Synopsis

This chapter outlines the development and implementation of a prospective cohort observational study designed to investigate the influence of modifiable psychological and behavioural factors on influenza vaccination responses in older adults. Initial considerations that shaped the study design are described, as well as changes resulting from the input of a patient and public involvement group. In the study that was ultimately conducted, measures of implicit and explicit positive and negative affect, perceived stress, nutrition, physical activity, and sleep were collected using diary measures and pedometers for a period of 6 weeks (2 weeks prior to, and 4 weeks following, influenza vaccination). Short- and long-term antibody responses to the vaccine were measured by a novel antigen microarray assay at 4 and 16 weeks post-vaccination respectively, the development and validation of which is described in Chapter 5.

4.1 *Background*

As described in the previous chapter, interventions targeting psychological and behavioural factors to improve vaccine responses in older adults have shown some promise. Developing more effective interventions requires targeted research that seeks to identify the critical components or, to borrow a term from pharmacology, 'active ingredients' that can make such interventions effective. As a starting point, this requires knowledge of which psychological and behavioural factors exert the greatest influence on vaccination outcomes; as well as if these influences are additive or not.

While much research has investigated individual psychological or behavioural influences on vaccine outcomes, to date, there has been little research, and therefore understanding, of the respective contribution of different psychological and behavioural influences on vaccination responses. Additionally, an over-reliance on single time-point, retrospective measures of both behavioural and psychological factors in previous observational studies means that little is known about at which time-point(s) psychological and behavioural factors exert the greatest influence on vaccination responses. This information is crucial for targeting psycho-behavioural interventions at an optimal time-point to maximise efficacy.

To begin to address these issues, a prospective cohort observational study in older adults (named BeHIVE-65 – BeHaviours that Influence Vaccine Efficacy in the over 65's) was planned. The primary aim of this study was to identify the influence of physical activity, nutrition, sleep, stress and affect on short- (4 weeks post-vaccination) and long-term (16 weeks post-vaccination) antibody responses to influenza vaccination among the elderly. Secondary aims included identifying time-points (pre-, post-vaccination, day of vaccination) at which psycho-behavioural factors were most predictive of antibody responses and to explore the role of intra-individual variability in psychological factors on vaccine responses.

4.2 ***Initial Considerations***

4.2.1 Study Design

Two types of study design were initially considered: experimental and observational. Experimental designs, particularly in the form of randomised controlled trials, are typically considered to provide a higher standard of evidence than observational designs (Barton, 2000). This is because

experimental designs allow causal inferences to be made about how a factor of interest influences a particular outcome variable. Experimental designs, by definition, require that the researcher control or manipulate a factor of interest (e.g., by assigning participants to conditions) while holding other influencing factors stable, to measure their effects on an outcome. This gives the researcher a degree of certainty that it was the control or manipulation of this factor that caused any changes in the outcome. While this is self-evidently desirable, in cases where there are multiple variables of interest, or the time period during which factors must be controlled is prolonged, experimental designs can become both unfeasible and/or unethical.

Observational designs, in contrast, are highly suited to exploring the influence of multiple factors on a given outcome. Such designs do not require researchers to manipulate or control these factors, just measure them. Associations, but not causal relationships, between explanatory factors and outcomes can then be identified. Observational designs are arguably more ecologically valid than experimental designs, in that there is no control or manipulation of variables on the part of the researcher; meaning behavioural or psychological factors can be measured as they might naturally occur in the study population. The most substantial limitation of observational designs is that they are potentially open to systematic biases resulting from unmeasured factors, known as confounding variables.

On balance, an observational design was chosen as the most suitable to meet the primary aims of this study. As many psychological and behavioural factors were of interest, ethical (unreasonable participant burden and the potential for distress) and practical considerations (costs, feasibility) prohibited an experimental design. Specifically, a prospective, cohort observational study was chosen as the most appropriate design to address

aims outlined above. This design involves the recruitment of a cohort of patients, prior to receipt of a given intervention (in this case vaccination), who are then followed over time to assess outcomes. This design was considered to provide the strongest observational evidence that any given factor (e.g., physical activity) influences a given outcome (e.g., antibody response following vaccination).

4.2.2 Vaccination Choice

A variety of vaccines have been used in research investigating behavioural and psychological influences on vaccination responses including: Hepatitis A (Lange, 2003), Hepatitis B (Prather et al., 2012), Keyhole Limpet Hemocyanin (R. Grant et al., 2008) and Pneumococcus (Glaser, Sheridan, Malarkey, MacCallam, & Kiecolt-Glaser, 2000). Studies in older adult populations have frequently opted to investigate influenza vaccination responses (e.g., Kiecolt-Glaser, Glaser, Gravenstein, Malarkey, & Sheridan, 1996; Moynihan et al., 2004; Segerstrom, Hardy, Evans, & Greenberg, 2012). The reasons for this are relatively straightforward: first, the annual seasonal influenza vaccine is the most common vaccine received by older adults, with over 7 million administered annually in the UK alone (Public Health England, 2015). In the UK, it is recommended that all those over 65, for whom it is not contraindicated, should be vaccinated for influenza annually (Department of Health, 2013a). Second, the administration of influenza vaccination raises few ethical concerns and greater practicality because the vaccine frequently forms part of standard care. Participants are therefore not subjected to an additional medical procedure and studies can be designed to 'piggyback' on an existing vaccination schedule. The major limitation of using influenza vaccination in studies such as this is that older adults have typically had prior exposure to influenza virus antigens, either

through previous vaccinations or natural exposure. This means that prior to the vaccination being researched, most participants have some existing antibodies against the antigens present in the vaccine. Immune responses to the vaccination therefore typically represent secondary immune responses, the robustness of which are influenced by existing levels of antibodies (Goronzy et al., 2001; Sasaki et al., 2008). That being said, investigating secondary immune responses may be particularly salient in older adult populations as they are more likely to have encountered a wider range of antigens previously than younger counterparts.

Alternative vaccinations that avoid this limitation are those against diseases that are rarely encountered in Western countries such as yellow fever, or specialist vaccines against an antigen that would not be naturally encountered such as keyhole limpet hemocyanin (KLH). These vaccines tend to produce primary immune responses in participants – removing some of the variability brought about by baseline levels of antibodies. However, using such vaccinations in research presents significant challenges, primarily in terms of getting ethical approval and recruiting participants willing to be subject to an arguably unneeded medical procedure.

On balance it was decided that the present study would focus around influenza vaccination. This decision was primarily driven by the need to recruit a substantial sample of between 100 and 200 patients, the cost of less common vaccinations (e.g,. KLH, yellow fever) was prohibitive, and as the overall aim of the wider programme of research was targeted at developing interventions to enhance vaccination responses in older adults, it made sense to use the most commonly received vaccination for this population.

4.2.3 Study Length

A key consideration in determining the length of the observation period (during which psychological and behavioural data would be collected) was the time course of IgG antibody generation in the human body. Prior research indicated that peak IgG antibody levels are reached at approximately 28 days post-vaccination (P. Gross et al., 1996), therefore it was considered essential for the post-vaccination observation period to last until this point. As it was hypothesised that psychological and behavioural factors in the weeks prior to vaccination may also influence vaccination responses it was initially proposed that an additional pre-vaccination observation period of the 4 weeks would be included, making the total observational period 56 days (8 weeks). However, after consultation regarding the study with a PPI group (see 4.3), the pre-vaccination observation period was reduced to 2 weeks, making the overall observational period 42 days (6 weeks).

Serum samples were scheduled to assess both short- and long-term antibody responses to vaccination. As is standard across the majority of studies in this area (Edwards et al., 2008; Segerstrom, Schipper, & Greenberg, 2008; Vedhara, Cox, et al., 1999), short-term antibody responses were measured in serum samples taken at 4 weeks post-vaccination and compared to baseline samples. There are no previously established standards for assessing long-term antibody responses, with previous research measuring long-term responses anywhere between 3 and 24 months post-vaccination. In the UK, influenza vaccination schedules typically start towards the end of September with the majority of patients being vaccinated in October or November. For pragmatic reasons, long-term antibody responses were measured in serum samples taken at 16 weeks

post-vaccination so that participants would not be required to attend clinics around the Christmas period. This follow-up length is also of clinical relevance because it covers the minimum time period for most patients between vaccination and when influenza viruses circulate most frequently in the UK population (January-March; Public Health England, 2014).

4.2.4 Using Diary Methods

Observational research investigating the relationships between psychological or behavioural factors in relation to vaccine responses has frequently relied on single time-point retrospective questionnaires (e.g., Afsar et al., 2009; Marsland et al., 2001, 2006). These measures require that participants respond to a set of questions regarding their behaviour or psychological state over a relatively lengthy period of time (often weeks, months or years). As discussed in the previous chapter (section 3.5.1), while widely used, sole reliance on single time-point retrospective questionnaires is limited in that (i) they rely on the respondent's ability to accurately and unbiasedly recall their experience over a prolonged period of time and (ii) they cannot capture the inherent variability of people's behaviour or psychological states (Bolger, Davis, & Rafaeli, 2003; T. Conner & Barrett, 2012).

An alternative, and increasingly applied method of capturing psychological and behavioural phenomena is to take repeated measurements of a person's momentary experience, which requires that they regularly recall their experience or behaviour over much briefer windows of time (e.g., in the present moment or over hours or days). This use of multiple, repeated measurements has been variously labelled as experience sampling (T. Christensen, Barrett, Bliss-moreau, Lebo, & Kaschub, 2003), ecological

momentary assessment (Smyth & Stone, 2003) or, as will be used in this thesis, diary methods (Bolger et al., 2003). Diary methods remove (in the case of momentary measures) or greatly reduce (in the case of brief time-span retrospective measures) recall biases that are inherent in typical retrospective measures (Bradburn et al., 1987). Further, diary methods allow researchers to capture the inherent day-to-day variability in behaviour and psychological states (Bolger et al., 2003). For this study, incorporating diary methods was especially desirable in light of evidence that psychological factors measured by diary methods better predict observed immunological functioning and health outcomes than single time-point retrospective or trait measures (for a review of this evidence see T. Conner & Barrett, 2012). For example, in a viral challenge study, Cohen et al. (2003) found that aggregated daily measures of affect better predicted susceptibility to cold viruses than trait affect measures. Similar enhanced predictive ability of repeated measures over trait or retrospective measures have been demonstrated for the relationship between stress and atherosclerosis risk (Bhattacharyya, Whitehead, Rakhit, & Steptoe, 2008) as well as affect and cortisol secretion (Steptoe, Leigh Gibson, Hamer, & Wardle, 2007). Drawing on this literature, the BeHIVE-65 study was designed to incorporate diary methods where possible. Participants' momentary psychological experience and recent behaviour was measured on three days per week during the 2 weeks prior to and 4 weeks following influenza vaccination.

4.2.5 Capturing Affect: Both Explicit and Implicit

In measuring affect (feelings and emotions), researchers typically ask participants to explicitly self-report on their emotional experiences; often in relation to rating scales (e.g., The Positive and Negative Affect Schedule; Watson, Clark, & Tellegen, 1988). These measures have face validity and

are underpinned by the understanding that participants can introspectively assess their own emotional state(s) and accurately report these back to the researcher. However, despite this approach appearing eminently reasonable, there is increasing acknowledgement among researchers interested in emotion that these measures may only capture a portion of affective cognition. Indeed, it has been argued that a (possibly large) proportion of emotional processing occurs outside of conscious awareness (Brosschot, 2010; Gyurak, Gross, & Etkin, 2011; Koole, Webb, & Sheeran, 2015; Quirin, Kazén, & Kuhl, 2009; Robinson & Clore, 2002) and is therefore not amenable to typical explicit self-report. For example, Gyurak et al. (2011) has proposed a dual process model of emotion regulation in which emotions occur and are regulated in two parallel processes, explicit processing – which demands conscious effort and monitoring - and implicit processing – which occur automatically in response to external stimuli but are not monitored and occur without insight or awareness. Asking participants to articulate their emotional state therefore only taps the explicit form of emotion processing, leaving implicit emotional experiences unmeasured (Quirin, Kazén, & Kuhl, 2009).

While less abundant than explicit measures, measures of implicit affect have been developed and validated (e.g., Gemar, Segal, Sagrati, & Kennedy, 2001; Quirin, Kazén, & Kuhl, 2009). For psychoneuroimmunology studies, capturing implicit affect may be particularly salient in light of evidence that implicit positive and negative affect measures can predict cortisol regulation where explicit measures do not (Mossink, Verkuil, Burger, Tollenaar, & Brosschot, 2015; Quirin, Kazén, Rohrmann, & Kuhl, 2009). For example, Mossink et al. (2015) measured salivary cortisol, implicit and explicit affect in young adults at multiple time points over a 24-hour period, finding that implicit positive and negative affect (but not explicit affect) were inversely

related to cortisol levels. Cortisol is a steroid hormone that is known to have strong influence on many immune cells and their function in humans (Jefferies, 1991). Therefore, given this importance of cortisol in altering immune function and the potential that implicit but not explicit measures of affect might influence cortisol levels, it was deemed prudent that the present study should include measures of both *explicit* and *implicit* affect.

4.2.6 Participant Burden

A number of ethical issues were considered in the study development process. Of particular note was the balance between evidence quality and potential participant burden. In terms of evidence quality, multiple daily measures of psychological factors, using full-length scales with excellent psychometric properties, would be preferable. However, the burden of this over a relatively prolonged period would be substantial. While those choosing to participate in the study would be volunteers who had been fully informed of the study requirements - it was considered essential that the number of measures and questionnaire items where kept to a minimum, while maintaining the scientific integrity of the study. Bearing these factors in mind, it was proposed that measures would be collected on 3 days each week during the observational period, with shortened versions of scales used where available. Further to this, it was decided that participants would have the option of completing measures via paper diaries, online forms or telephone interviews, thus giving participants the opportunity to opt for a method to minimise burden on their lives.

4.3 ***Patient & Public Involvement***

To aid the design and implementation of the observational study, a patient and public involvement (PPI) group was convened. In the UK, PPI is

considered integral to the process of healthcare research (Mockford, Staniszewska, Griffiths, & Herron-marx, 2012) and successive National Health Service constitution documents have supported the need for greater public involvement in healthcare services and research (Department of Health, 2010, 2013b). This increased involvement aims to shift research from being "to", "about" or "for" members of the public to being "with" or "by" them (INVOLVE, 2015). For many funding bodies, PPI is now a mandatory aspect of all healthcare research funding applications (Maguire, Jones, Williams-Yesson, & Stevens, 2011). Beyond this clear mandate for PPI in health research, PPI provides numerous potential benefits in the development and implementation of healthcare research including: clarifying research questions and objectives, improving research designs resulting in reduced attrition, improving readability of participant documentation, addition of outcomes important for patients, and greater dissemination of research findings (Boote, Telford, & Cooper, 2002; Entwistle, Renfrew, Yearley, Forrester, & Lamont, 1998).

For this study, eight older adults (aged 65-85 years) who were eligible for the 2014/15 influenza vaccine, spoke fluent English and had no significant cognitive impairment, were recruited from a local GP community practice in Nottingham. The group participated in a semi-structured focus group concerning the design, development and outputs of the study, as well as reviewing an initial draft of participant materials. Participants were provided with a written (Appendix A) and verbal description of the proposed study and asked to reflect and comment upon whether they would consider participating in the study, its challenges and strengths. Further specific questions where then posed to the group concerning: suitability of potential measures (e.g., "to measure physical activity levels, is the use of pedometers acceptable?"), appropriate levels of inconvenience payments

(e.g., "how should participants be compensated for their time and efforts – are proposed payments adequate?") and how to best contact potential participants (e.g., "how could we best contact those who might wish to participate in this research?, Is the proposed method of sending an information letter to all suitable participants acceptable?").

In summary, there was broad agreement from PPI participants that this kind of research was beneficial and useful for older adults. Specific study issues deemed to be of priority to the PPI group included: reducing participant burden where feasible, maintaining regular contact throughout the study with participants to make them feel "part of something bigger", and ensuring simplicity and clarity throughout participant documentation. With regard to more specific questions, most measures where viewed favourably (e.g., pedometers), although the group encouraged reducing 'mood' questionnaire lengths where possible. Further, contacting potential participants via GP letter was deemed the only suitable approach.

As a result of this input from the PPI group a number of changes to the originally proposed study design were made. These included: shortening of the study length from 8 to 6 weeks (reducing pre-vaccination observation from 4 to 2 weeks), increasing the frequency of reminders and general 'keep-in-touch' contact throughout the study, and reducing the number and length of measures included during the diary period. The group also suggested minor clarity amendments to original versions of the participant documentation, helped identify the most appropriate level of inconvenience payments (£10 per practice visit), and gave crucial practical guidance on how to best interact with participants during the study to maintain engagement (e.g., explaining while not patronising, being respectful and interested).

4.4 *Final Study Design & Overview*

Following PPI input, the final iteration of the study adopted a prospective cohort observational design. The impact of behavioural and psychological factors (Explicit and Implicit Affect, Physical Activity, Stress, Nutrition, and Sleep) on short and long-term influenza vaccine responses in older adults were assessed via diary measures with antibody levels measured at baseline to determine pre-vaccination antibody levels; 4 weeks post-vaccination to determine the short-term antibody responses to vaccination and 16 weeks post-vaccination to determine the long-term antibody responses to vaccination. The primary outcome measure was baseline-adjusted IgG antibody levels (see 4.9.2) against each of the three virus strains contained within the 2014/15 trivalent influenza vaccination, as measured by antigen microarray.

Participants attended community practices at baseline (T0), 2 weeks (T1), 6 weeks (T2) and 18 weeks (T3). Blood samples were obtained at baseline (T0), 6 weeks (T2) and 18 weeks (T3), with one standard dose of the 2014/15 influenza vaccination administered at 2 weeks (T1). Between baseline (T0) and 6 weeks (T2), participants also wore a pedometer and completed written booklets including food diaries and brief psychological measures (see 4.8) on 3 consecutive, but randomly selected (computer generated), days each week (see Figure 4-1). This 6 week period is hereafter refered to as the 'diary period'.

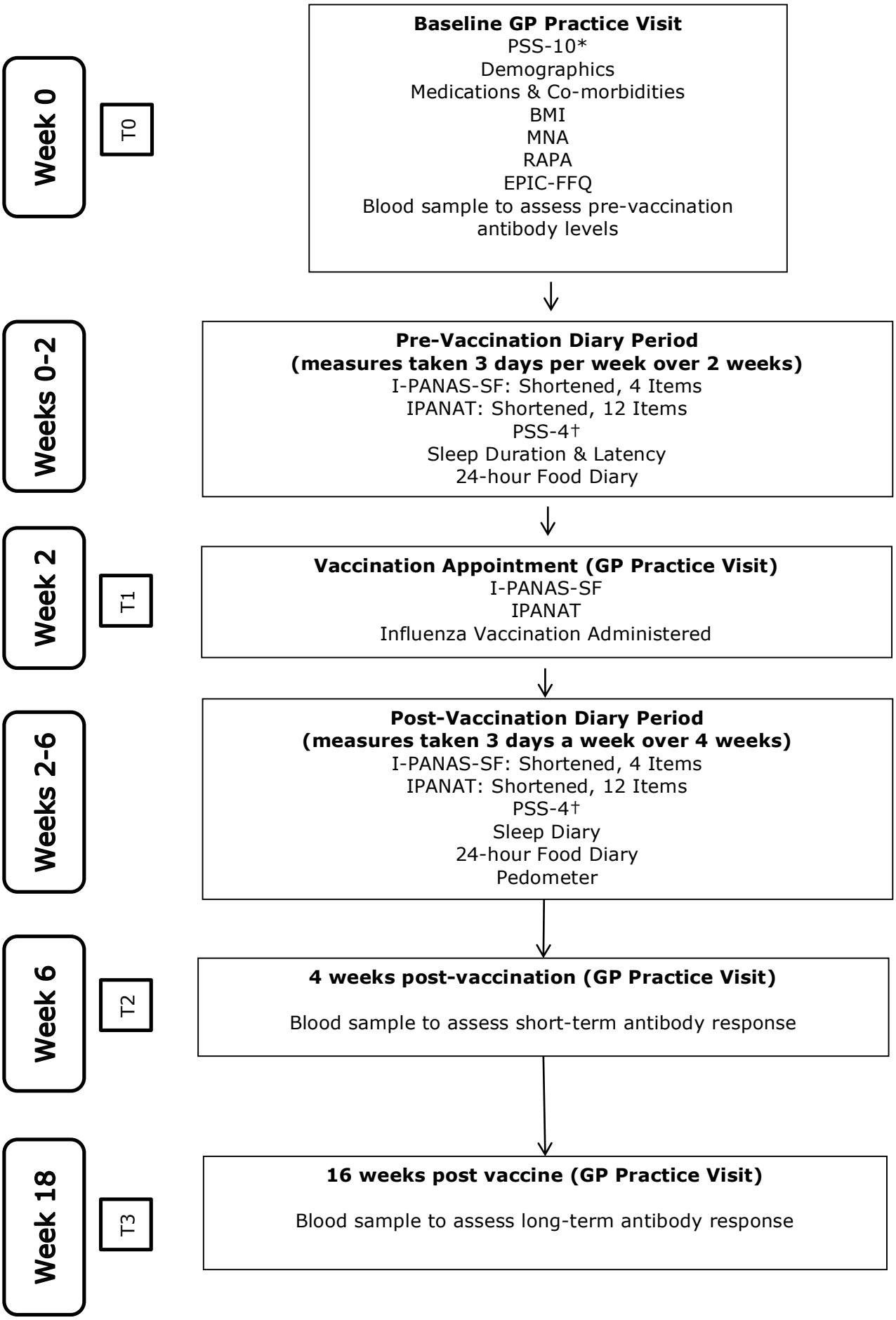


Figure 4-1: Participant measures during the BeHIVE-65 study

I-PANAS-SF = International Positive and Negative Affect Schedule Short Form; IPANAT = Implicit Positive and Negative Affect Test; PSS-10 = Perceived Stress Scale 10 Item Version; BMI = Body Mass Index; MNA = Mini Nutritional Assessment; RAPA = Rapid Assessment of Physical Activity; EPIC-FFQ = EPIC-Norfolk Food Frequency Questionnaire; PSS-4 Perceived Stress Scale 4 Item Version. (*) = Instructions require responses over "the past month"; (†) Instructions require responses over "the past few days".

4.5 ***Recruitment Strategy***

4.5.1 Inclusion and Exclusion Criteria

As responses to influenza vaccination are influenced by previous influenza vaccine history and exposure (Goronzy et al., 2001; Sasaki et al., 2008), recruitment was limited to those aged between 65 years and 85 years (inclusive) who had been vaccinated the previous year (2013/14). Two alternative methods of controlling for this prior exposure were rejected: (1) recruiting only participants who had never been vaccinated and (2) recruiting participants regardless of vaccine history and controlling for previous vaccination in analytic models. The former of these was rejected because this would likely represent a very select portion of the older adult population, thereby reducing generalisability of findings. After the age of 65, all adults are offered the annual influenza vaccination and uptake rates are typically over 70% year-on-year (Public Health England, 2015). Further, even the minority of individuals that are not vaccinated against influenza are likely to have encountered influenza viruses through natural exposure and as a result have influenza-specific antibodies prior to vaccination. The latter option of controlling for previous vaccination history statistically was rejected because of concerns that long-term vaccination history may not be well recorded, and that given the number of predictor variables of interest adding additional controlling variables to statistical model would be undesirable and could potentially obscure findings. The chosen option of only recruiting those who had been vaccinated in the previous year (2013/14) is not without its limitations – in that it is only partially accounts for previous exposures – but did ensure that all participants had each at least received one prior exposure to influenza viruses, of exactly the same strains and dosage.

By chance, the viral strains included in the 2013/14 and 2014/15 northern hemisphere influenza vaccinations were the same (A/California/7/2009 (H1N1) pdm09; A/Texas/50/2012 (H3N2); B/Massachusetts/2/2012), as such all participants had previously been exposed to the strains contained within the vaccine. This has only occurred twice in the last 20 years and only became known after study ethical approval was given and planning complete. As will become apparent when results of the study are presented in Chapters 6 & 7, this repeated vaccination has serious implications, because in receiving the exact same vaccination approximately 12 months apart, there was an increased likelihood of ceiling effects - in that individuals with high levels of antibodies may generate fewer new antibodies as existing levels are already sufficient to provide protection. Further, there is greater potential for *negative interference* (D. Smith, Forrest, Ackley, & Perelson, 1999) where antibodies generated from the earlier vaccine respond and clear some of the second vaccine antigens before they are able to induce an immune response, thereby dampening response to the second vaccination.

To maximise representativeness and generalisability of the sample, other exclusion criteria were kept to a minimum with only those deemed by their health care provider to be too physically frail to participate, diagnosed with a cognitive condition (e.g., dementia) that would make participation difficult, to have insufficient command of the English language or where influenza vaccination was contraindicated, excluded. It is worth addressing here that some studies investigating influences on vaccine outcomes exclude 'unhealthy' older adults on the rationale that medical conditions, medications or frailty might confound immunological findings (e.g., de Bruijn et al., 1997; Edwards et al., 2008; Glaser, Robles, Sheridan, Malarkey, & Kiecolt-Glaser, 2003). The most rigorous method for achieving this is applying the SENIEUR protocol – a stringent set of criteria based on clinical, laboratory and

pharmacological data - which aims to exclude potential participants with any sign of disease or non-standard immune functioning (Ligthart et al., 1984). While noble in its intention, a significant limitation of applying the SENIUR protocol is that only an estimated 15-20% of older adults meet the criteria (Penedo & Dahn, 2005). As such, adults meeting the SENIUR protocol are not typical of older adults, making it unclear how applicable findings are to older adults in general. A simpler, frequently employed, solution is to exclude only those participants with a diagnosed disease or medication that is known to influence immune function (e.g., excluding those with a cancer diagnosis or taking immunosuppressive drugs such as glucocorticoids). However, this strategy is also problematic in that (a) it still limits generalisability, although to a lesser extent than the SENIUR protocol, and (b) it does not necessarily remove all influences of disease and medication on findings. This is because the immune consequences of most diseases and medications are poorly understood (Castle, 2000), therefore participants with many diseases or medications are not excluded, despite the likelihood they influence immunological outcomes. As the ultimate goal of this research was to inform the development of psycho-behavioural interventions to improve vaccine responses across the older adult population, it was important that participants in this study represented, as much as was possible, a typical older adult sample. Therefore, we did not exclude 'unhealthy' older adults from participating in this study.

4.5.2 Sample Size

No prior study has simultaneously investigated role of diet, physical activity, sleep, stress and affect on immune response following vaccination. To inform the sample size for this study indicative power calculations were performed on the basis of a previous observational study conducted by Vedhara et al.

(Vedhara, Cox, et al., 1999), which examined the effects of stress on influenza vaccination in the elderly. This study found 16% of elderly carers who reported high stress achieved a four-fold increase in antibody levels to one or more vaccine strain, compared with 39% of age-matched non-carers reporting low stress. This corresponds to a small-to medium effect size of r=.25 or f^2 =.07. As it was not possible *a priori* to predict the number of variables that would be included in final regression models, a series of calculations were performed for different scenarios using G*Power version 3.1.9.2 (Faul, Erdfelder, Lang, & Buchner, 2013) using the effect size from Vedhara et al. (Vedhara, Cox, et al., 1999). It was calculated that, for individual regression models, a sample of 121 would give 80% power at 5% significance to detect effects in the antibody response to influenza vaccination. For multiple regression models containing four to eight predictors, sample sizes of 181, 194, 206, 217 and 236 respectively would be needed to detect effects at the same level. Further consideration of sample size is required for intensive diary-based designs, as this frequently requires a trade-off between higher-level (participants) and lower-level (responses during the observational period) units (Scherbaum & Ferreter, 2009).

Balancing the aims of the study, expenses, feasibility, the performed calculations and additional expert advice from the project statistician, recruitment aims were set at 200 participants. This, allowing for some participant attrition, would provide sufficient power for all individual regression models and multiple regression models that included up to four predictors, as it was assumed not all predictors would be included in the final models.

4.6 ***Participants***

In total, 2000 invitations to join the study were sent to eligible older adults by four medium-sized primary care community practices in Nottingham between August and October 2014 on the assumption of a 10% uptake rate. From these, 209 expressions of interest were received (10.45%) and of those 161 eligible older adults were successfully scheduled to attend an enrolment session at their local community practice. Following enrolment sessions, 138 older adults consented to the study (6.9% uptake rate). The characteristics of these participants are presented as part of the results in Chapter 6. The flow of participants through the study and reasons of loss to follow-up are shown in Figure 4.2. Attrition across the study was low with 136 older adults returning for the 6-week appointment (1.4% attrition; 2 Males) and 122 for the final 18-week appointment (11.6% attrition; 10 Males, 8 Females). There were no significant differences in age, gender, body mass index, baseline scores of positive affect, negative affect, perceived stress and pre-vaccination antibody levels between completers and non-completers (Table 4.1).

Table 4.1: Differences between completers and non-completers (Mean (Standard Deviation) unless otherwise stated)

	Completers (max. n=122)	Non-Completers (max. n=16)	Statistical Test	*df*	*p*-value
Age	72.36 (8.68)	72.22 (4.51)	t = .06	134	.95
Gender (females)*	53 (44.2)	8 (44.4)	χ^2 = .00	1	.98
Body mass index (kg/m^2)	28.05 (5.86)	26.97 (5.29)	t = .73	135	.47
Baseline positive affect	15.96 (4.37)	17.63 (3.90)	t = -1.45	131	.15
Baseline negative affect	6.78 (2.66)	6.50 (2.07)	t = .40	130	.69
Baseline perceived stress	11.04 (6.28)	10.53 (7.19)	t = .31	131	.76
Baseline antibody H1N1 (µg/ml)	1.94 (1.94)	1.84 (1.41)	t = .21	135	.83
Baseline antibody H3N2 (µg/ml)	7.11 (6.90)	5.87 (6.26)	t = .70	135	.48
Baseline antibody B (µg/ml)	5.31 (4.77)	4.27 (3.80)	t = .86	135	.39

* Frequency (%)

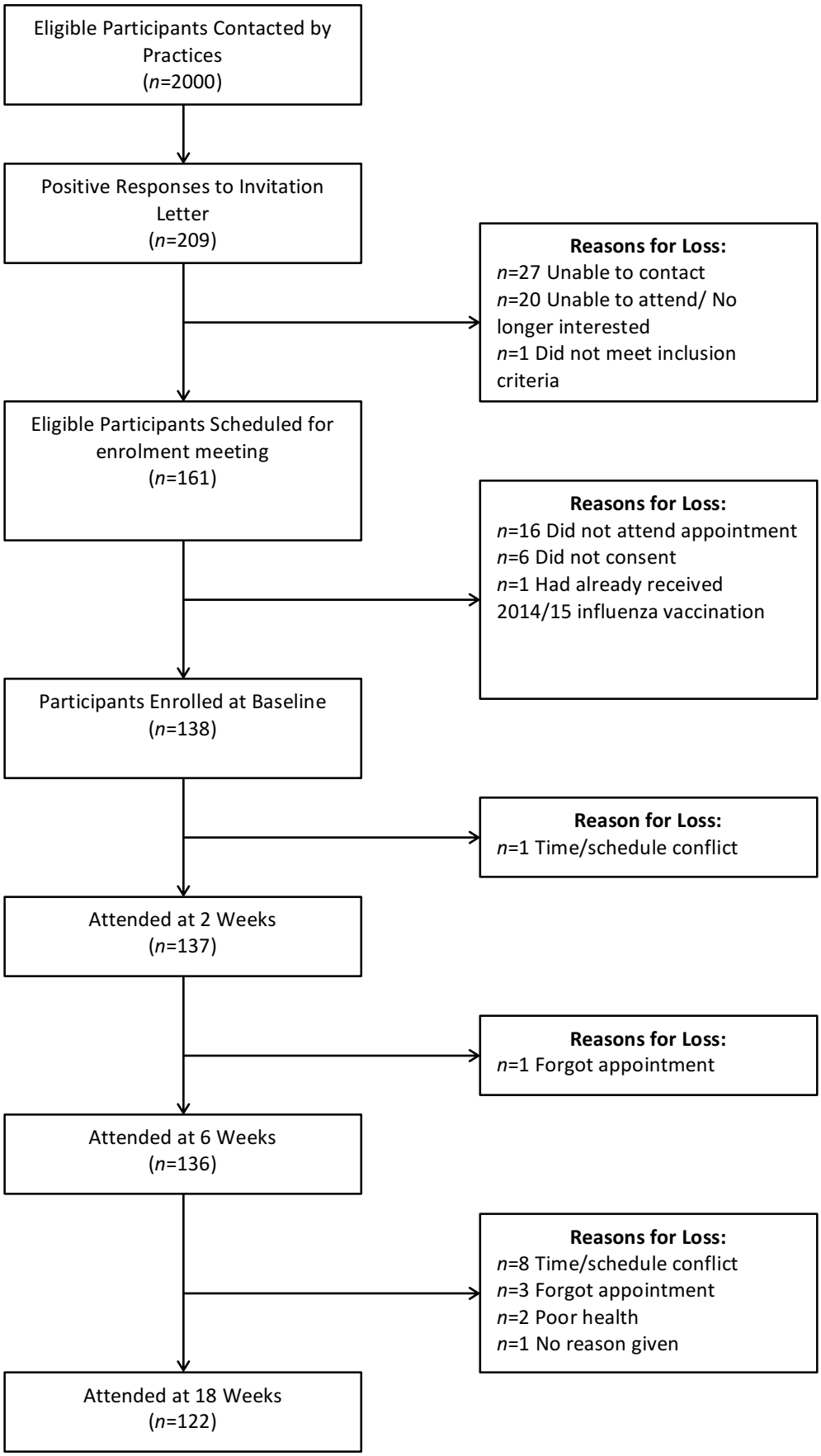


Figure 4.2: Flow of participants through observational study

4.7 ***Procedure***

Ethical approval and research governance approval was obtained prior to study commencement (REC: 14/EM/0201) (Appendix B). The study was also portfolio adopted by the Clinical Research Network (UKCRN Ref: 16840). Eligible individuals received a letter inviting them to participate in the study from their primary care community practice, containing summary information and researcher contact details (Appendix C). Interested individuals contacted researchers via telephone or by returning a reply slip provided with a freepost envelope. Potential participants were then individually contacted and invited to attend a session at their primary care community practice. At this session, all potential participants were informed about the requirements of the study, provided with an information sheet and given the chance to discuss and ask questions about the study. For those happy to participate, written informed consent was obtained.

Once recruited, participants completed baseline questionnaire packs (details provided in 4.8.1), had physiological measurements taken (weight, height, waist-circumference, mid-arm circumference, and calf circumference), had a pre-vaccination blood sample (8ml) taken by a practice nurse or phlebotomist, and received instructions on how and when to wear their pedometer. Participants then began a 6-week diary period during which they completed questionnaire measures on 3 consecutive, but randomly selected[7] days each week (see 4.8.2). Participants also wore a pedometer for the same three days each week and noted down readings in provided booklets.

[7] Randomisation of the first response day each week occurred using a randomization computer algorithm implemented on Microsoft Excel software.

After 2 weeks, participants (n=137) attended their local primary care community practice where they completed additional questionnaire measures (see 4.8.3) and received a standard dose of the 2014/15 seasonal influenza vaccine. At 6 (n=135) and 18 (n=122) weeks post-baseline, participants again attended their local primary care community practice to provide follow-up blood samples. Participants received inconvenience payments for their time and travel expenses of up to a total of £30 (£10 per non-standard visit to their community practice).

4.8 ***Measures***

To assess the internal reliability of scales, Cronbach's alpha (α) was calculated for all scales, with the exception of 2-item scales (explicit positive and negative affect measures over the diary period) for which Spearman-Brown statistics were calculated in accordance with recommendations (Eisinga, Grotenhuis, & Pelzer, 2013). Demographics (age, gender, ethnicity, highest level of education, household income, occupation, marital status, living status, smoking status) and number of current prescribed medications and co-morbidities were obtained through self-report items at baseline. Body mass index (BMI) was calculated (kg/m^2) from measurements on a portable height measure and a set of calibrated mechanical scales.

4.8.1 Behavioural and Psychological Measures Collected at Baseline (2 weeks pre-vaccination)

4.8.1.1 Habitual Purposive Exercise (Appendix D-1)

Typical levels of purposive exercise were measured at baseline using the nine-item Rapid Assessment of Physical Activity (RAPA; Topolski & LoGerfo,

2006). Participants are asked whether statements regarding the level and nature of exercise they usually do (e.g., "I rarely or never do any physical activities") accurately describes them, with 'yes' or 'no' response options to nine questions. The RAPA comprises two sub-scales with the first 7 items assessing cardiovascular activity and the final 2 items assessing whether respondents regularly engage in strength ("I do activities to increase muscle strength, such as lifting weights or calisthenics, once a week or more") and flexibility training ("I do activities to improve flexibility, such as stretching or yoga, once a week or more"). Once scored, the 7-item cardiovascular scale can be used to categorise participants as sedentary, underactive, regular underactive (light activities), regular underactive, and regular active based on the highest level of regular physical activity reported. RAPA has been validated in older adults and compares favourably to other established self-report measures of exercise in terms of sensitivity, specificity and predictive value (Topolski & LoGerfo, 2006).

4.8.1.2 Habitual Dietary Intake (Appendix D-2)

Habitual dietary intake was assessed at baseline via the EPIC-Norfolk Food Frequency Questionnaire (FFQ; European Prospective Investigation of Cancer Team, 2013). The FFQ requires participants to indicate their level of consumption over the previous year for 130 different foods listed with common portion sizes with nine response options for each ranging from "never or less than once/month" to "6 times a day". Participants are asked to respond to additional questions to further classify listed food items including details on cooking methods, visible fat on meat and brands of breakfast cereal. Responses to the FFQ are entered into dietary analysis software to estimate habitual intake of a range of nutrients. The FFQ

correlates acceptably with weighed records and various urinary and serum biomarkers (Bingham et al., 1997; McKeown et al., 2001).

4.8.1.3 Nutritional Status (Appendix D-3)

Nutritional status was assessed in community practice visits at baseline via the Mini Nutritional Assessment (MNA; Vellas, Guigoz, Garry, & Nourhashemi, 1999). The MNA involves a combination of anthropometric measurements (weight, height, demi-span, waist, mid-arm and calf circumferences) and self-report dietary questions (e.g. "How many full meals do you eat daily?") to assess the likelihood of malnourishment. Overall, the MNA includes 18 items and is scored by summing item totals with a maximum possible score of 30. Scores greater than or equal to 24 indicate adequate nutrition, scores between 17 and 23.5 indicate at risk of malnutrition, and scores below 17 indicates protein-calorie malnutrition. This scoring has been found to have high sensitivity, specificity and predictive value in prior validation studies (Vellas et al., 1999).

4.8.1.4 Perceived Stress (Appendix D-4)

Perceived stress refers to the extent an individual perceives that life events exceed their ability to cope (S. Cohen, Kamarck, & Mermelstein, 1983; S. Cohen & Williamson, 1988). In measuring perceived stress, the researcher is not purely measuring stressor exposure, but also taking account an individual's appraisal (perception) of events (Lazarus & Folkman, 1984).

Perceived stress over the previous month was measured at baseline using the 10-item version (PSS-10) of the perceived stress scale (S. Cohen et al., 1983; S. Cohen & Williamson, 1988). Participants are required to indicate how often, over a given time period, they have experienced negative

thoughts and feelings because of overloading, unpredictable or uncontrollable situations (e.g., "In the last month, how often have you felt that you were unable to control the important things in your life?") on a 5-point scale ranging from 0 (never) to 4 (very often). This scale has been widely used within the psychological stress literature, including in older adults (e.g. Rueggeberg, Wrosch, & Miller, 2012).

While a longer version of the perceived stress scale exists (PSS-14), the PSS-10 is the preferred long-form version of the scale due to its superior psychometric qualities (S. Cohen & Williamson, 1988) including excellent internal reliability (α =.78 to .91; S. Cohen & Janicki-Deverts, 2012). In the present study, the PSS-10 was found to have excellent internal reliability statistics (α=.82)

4.8.2 Behavioural and Psychological Measures Collected During the Diary Period

4.8.2.1 Daily Physical Activity

Physical activity levels were estimated using step counts via Yamax SW-200 pedometers. Pedometers are widely used as a proxy measure of physical activity and correlate acceptably with observed behaviour (Crouter, Schneider, Karabulut, & Bassett, 2003). The Yamax SW-200 has been widely used and validated in previous studies, correlating highly with gold-standard actigraph measures (e.g. de Blok et al., 2006; Motl, McAuley, Snook, & Scott, 2006; P. Schneider, Crouter, & Bassett, 2004). Participants were asked to note pedometer readings within the diary booklets at the end of each response day (3 days/week).

4.8.2.2 Dietary Intake (Appendix D-5)

Dietary intake throughout the six-week diary period was assessed via the EPIC-Norfolk food diary (McKeown et al., 2001). Participants were required to keep a record of food and drink intake, along with quantities, brands and ingredients (if homemade) during each 24-hour response day (3 days/week). Participants were given a detailed instruction booklet containing photographs to help estimate portion size and illustrative examples of completed diaries. The EPIC-Norfolk diary (seven-day version) has been shown to correlate well with multiple urine and blood biomarkers of nutrition (McKeown et al., 2001).

Food diary data was entered into specialist dietary software DietPlan6 (Forestfield Software Ltd, 2015). Where any food or drink item was noted but no quantity given, the item was entered according to average portion sizes provided by DietPlan6. If no average portion size was available in DietPlan6, data provided by the Food Standards Agency (Mills, Crawley, & Patel, 2002) were used. In rare cases where no average portion size was available through either method – estimates were gathererd drawing on supermarket size information and portion sizes stored on the online database MyFitnessPal (MyFitnessPal Inc., 2015). In such cases, the agreed 'average portion' was stored in a database that could be referred to if the item appeared again.

4.8.2.3 Sleep (Appendix D-6)

Sleep duration (total time spent asleep) and efficiency (proportion of time in bed spent asleep) were measured on each response day during the diary period using three adapted items from the Pittsburgh Sleep Quality Index (PSQI; Buysse, Reynolds, Monk, Berman, & Kupfer, 1989). Participants were

asked to provide details each morning of what time they went to bed the previous night, what time they got up and total sleep duration (accounting for disturbances). This brief measure of self-report sleep demonstrates face validity and mirrors assessment of sleep duration and efficiency in other widely-used sleep diary instruments (e.g., The Pittsburgh Sleep Diary; Monk et al., 1994).

4.8.2.4 Explicit Affect (Appendix D-7)

Explicit affect refers to a generalised account of an individual's emotional state that is consciously experienced and can be articulated if required. It is contrasted with implicit affect (4.8.2.5), which are emotional experiences that may not be so easily articulated and may occur without insight or awareness (see 4.2.5).

Positive and negative explicit affect was measured using the International Positive and Negative Affect Schedule Short Form (I-PANAS-SF; E. Thompson, 2007). The I-PANAS-SF is a shortened, cross-culturally validated version of the widely used Positive and Negative Affect Schedule (PANAS; Watson et al., 1988). The 10-item scale is split into two subscales, positive affect (PA) and negative affect (NA). PA and NA sub-scales have shown minimal correlation, demonstrating 'quasi-independence' in line with theories that PA and NA are orthogonal dimensions of affect as opposed to ends of a continuum (E. Thompson, 2007). In completing the scale, participants rated the extent to which they feel (or felt) in relation to five positive (e.g., active) and five negative emotion adjectives (e.g., nervous) on a five-point Likert-type scale from 1 (very slightly or not at all) to 5 (extremely). Scores are summed for PA and NA subscales, with higher scores denoting greater PA and NA respectively.

While the I-PANAS-SF was developed as a measure of trait affect, the original full-length PANAS has been validated both to measure trait, and state affect over multiple retrospective time periods (e.g., over the last few weeks, months) or momentary experience (Watson et al., 1988). Momentary measures of affect are considered more reliable because they are subject to less memory bias and have been found to correlate more strongly with immune function than retrospective accounts of affect (T. Conner & Barrett, 2012). Therefore, momentary experience instructions from the original PANAS measures were used in conjunction with I-PANAS-SF items ("indicate the extent to which you feel this way right now, that is, at the present moment").

For each response day during the diary period, participants completed a shortened version of the scale comprising four randomly selected items (two positive, two negative). Randomisation was computer generated and occurred in cycles without replacement, so that all items appeared at equal frequencies throughout the diary period. Participants all completed the same items at the same time points. Full versions of the I-PANAS-SF have previously (E. Thompson, 2007) demonstrated high internal reliability (α = .75 to .82 for PA and .74 to .76 for NA). For the present study, internal reliability was found to be excellent for shortened explicit positive affect measures (Spearman-Brown=.83) but poor for negative affect (Spearman-Brown =.43) meaning results relating to negative affect during the diary period should be treated with appropriate caution.

4.8.2.5 Implicit Affect (Appendix D-8)

Implicit affect measures aim to tap into automatic emotional processes and states that are not consciously monitored (see 4.2.5). In this study, Implicit affect was measured using the Implicit Positive and Negative Affect Test (IPANAT; Quirin, Kazén, & Kuhl, 2009). Participants rated the extent to which nonsense words (e.g., VIKES, SUKOV) express three positive and three negative mood words (e.g., happy, tense) on a 4-point scale ranging from 1 (doesn't fit at all) to 4 (fits very well). In total, IPANAT includes six nonsense words that are judged in relation to three positive and three negative mood words (36 word pairs in total). For each response day during the diary period, participants completed a shortened version of the scale comprising two nonsense items (randomly selected without replacement, as above), resulting in 12 word pairs in total. Participants all completed the same items at the same time points. The IPANAT has been previously shown to have excellent internal reliability (α = .82 to .89 for both PA and NA Items) and the measure is also sensitive to changes resulting from presentation of affective stimuli (Quirin, Kazén, & Kuhl, 2009). Internal reliability statistics for this study showed acceptable levels of internal reliability for both implicit positive (α=.88) and implicit negative subscales (α=.77).

4.8.2.6 Perceived Stress (Appendix D-9)

For each response day during the diary period a shortened, 4 item version of the perceived stress scale (PSS-4) was used (S. Cohen & Williamson, 1988). The PSS-4 has demonstrated only a moderate loss of reliability (α = .60 to .72; S. Cohen et al., 1983; S. Cohen & Williamson, 1988) when compared to the PSS-10 and is recommended for research where brevity is essential (S. Cohen & Williamson, 1988). When completing the PSS-4

participants were instructed to answer in relation to their experiences "in the last few days". In the present study, internal reliability for the PSS-4 was found to be acceptable (α=.74).

4.8.3 Behavioural and Psychological Measures Collected on the Day of Vaccination

4.8.3.1 Explicit Affect (Appendix D-10)

On the day of vaccination, participants completed full (10-item) versions of the I-PANAS-SF (as described above in 4.8.2.4). Internal reliability of the I-PANAS-SF on the day of vaccination was found to be excellent for the positive affect subscale (α=.89) and acceptable for the negative affect subscale (α=.64).

4.8.3.2 Implicit Affect (Appendix D-11)

Participants also completed full versions (36 items) of the IPANAT (as described above in 4.8.2.5). Internal reliability of the I-PANAS-SF on the day of vaccination was found to be excellent for both the positive (α=.95) and negative affect (α=.92) subscales.

4.9 ***Measuring Antibody Responses***

4.9.1 Serum Sampling

Venous blood samples (8 ml) were obtained at baseline, four weeks post-vaccination, and 16 weeks post-vaccination. Samples were collected via venepuncture by trained phlebotomists using BD Vacutainer® tubes containing clot activator and gel for separating serum. After clotting at room temperature, samples were centrifuged at 2000*g* for 10 minutes after which

sera were separated and aliquoted into multiple Eppendorf tubes. Samples were stored at -80°C until analysis.

4.9.2 IgG Response to Vaccination

Influenza IgG antibodies for each of the three strains contained in the vaccine (H1N1 A/California/7/2009, H3N2 A/Texas/50/2012, B/Massachusetts/2/2012) were measured via antigen microarray. This novel method was optimised and validated for this study and is documented in chapter 5.

As discussed in the previous chapter (see 3.5.2), multiple different approaches to operationalising antibody outcomes following influenza vaccination have been employed. One major conceptual divide in these approaches relates to whether researchers focus on (1) whether vaccine recipients achieve a degree of clinical protection post-vaccination (often according to some pre-defined threshold) or (2) whether focus is on the increase in antibodies that exclusively result from vaccination (i.e., change from pre- to post-vaccination). The former (clinical protection) has particular relevance for health care professionals interested in whether a vaccine will protect recipients against a disease, but is complicated by the fact that for many vaccines (e.g., influenza) participants often have pre-existing levels of antibodies against influenza prior to vaccination. On a practical level this means that some participants with high baseline levels of antibodies may already meet pre-defined thresholds for clinical protection – yet still be included in analyses demonstrating that the vaccine is effective at inducing protection. The latter method (change exclusively because of vaccination, taking baseline levels into account) is arguably a 'purer' measure of vaccine

response but has comparatively limited clinical utility – as even a large antibody increase may not necessarily be sufficient to provide protection.

Even once the researcher has decided whether they are interested in either clinical protection, change exclusively as a result of vaccination, or indeed both – there have been multiple methods of implementing these approaches. A brief evaluation of the most commonly employed methods is provided below.

4.9.2.1 Approaches to Operationalising 'Clinical Protection'

4.9.2.1.1 Binary Approach: Classifying Individuals as 'Protected' vs. 'Not-protected'

Many studies have sought to classify vaccine recipients based on whether they achieve some *a priori* criterion for clinical protection. For example, in studies where influenza-specific antibodies are measured via hemagglutination inhibition assay (HIA), a threshold of antibody titre > 40 is frequently used to denote clinical protection (e.g., Sundaram et al., 2013; Talbot et al., 2012). This threshold was classically established in an early viral challenge study by Hobson et al. (1972) which demonstrated that having a pre-challenge titre of 40 gave participants a 50% probability of becoming infected after being exposed to a standardised dose assumed to be similar to that typically encountered in natural exposure. More recently, higher thresholds have been used (e.g., HIA titre > 160; Hayney et al., 2014) on the basis that this represents a higher percentage probability of protection if exposed to influenza via natural exposure (Coudeville et al., 2010; de Jong et al., 2003). However, there are significant limitations of this method most notably that the dichotomising of data results in an overly simplistic view of protection. Unlike in viral challenge studies, real world

exposure to influenza viruses can vary substantially across individuals (Hobson et al., 1972), therefore any given level of serum antibodies does not guarantee protection. Grouping individuals with antibody levels well above the threshold with those who just reach it also loses potentially useful information. Further, many assays do not have established cut-offs for protection making this approach unsuitable for many studies.

4.9.2.1.2 Continuous Approach: More Abundant Antibody Equals Greater Protection

An alternative approach has been to consider the quantity of post-vaccination vaccine-specific antibody as a continuous proxy measure of protection. Naturalistic and viral challenge studies have shown that vaccine-specific antibody quantity is a good, although not perfect, correlate of clinical protection with more antibodies corresponding to a greater degree of protection, up to a ceiling point where only minimal benefit is gained from having more antibodies (Coudeville et al., 2010). This simple approach is highly defensible and does not suffer from the same loss of information as the binary approach presented above.

4.9.2.2 Approaches to Operationalising Response to Vaccination

4.9.2.2.1 Binary Approach: Classifying Individuals as Responders or Non-responders

In measuring change in antibody levels as a result of vaccination, a large number of studies have opted to classify vaccine recipients as 'responders' or 'non-responders' according to whether the change from pre- to post-vaccination meets a pre-defined threshold – most commonly a four-fold increase (e.g., Kiecolt-Glaser et al., 1996; Phillips, Burns, Carroll, Ring, & Drayson, 2005; Phillips, Carroll, Burns, & Drayson, 2009; Vedhara, Fox, & Wang, 1999). The four-fold increase threshold has its origins in studies of infection, in which a four-fold increase in antibody is seen as indicative of recent infection (Stanley, 2002). While this method has been widely used, in addition to the loss of data subtlety resulting from dichotomising the data, it is inherently arbitrary. Further, this approach does not take into consideration how differing pre-vaccination levels may influence responses. Notably, there is evidence that greater fold-increases are associated with lower baseline antibody levels following vaccination (Sasaki et al. 2008).

4.9.2.2.2 Continuous Approach: Absolute or Proportional Change

An alternative method employed has been to operationalise vaccine response as the change in antibody levels (considered as continuous data) from pre- to post-vaccination either in absolute or proportional terms (e.g., Yang et al., 2007). This method addresses the issues of data lost from classifying participants as 'responders' or 'non-responders' but like the above approach does not account for the fact that low pre-vaccination levels are associated with greater proportional increases.

4.9.2.2.3 Continuous Approach: Baseline-adjusted Post-Vaccination Levels Via Linear Regression

One of the more rigorous approaches to operationalising vaccine response aims to control for the effect of pre-vaccination antibody levels by calculating a baseline-adjusted post-vaccination antibody level (e.g., Segerstrom et al., 2012). This method was developed by Beyer et al. (2004) to address some of the limitations of other approaches outlined above. Specifically, Beyer et al. compared multiple methods of defining vaccine-response across 16 previous influenza vaccine studies (including a total of 1176 participants) demonstrating that in many circumstances traditional methods of operationalising antibody responses (such as those described above) did not truly account for pre-vaccine antibody levels and could seriously over- or underestimate vaccine effectiveness.

To address this, Beyer et al. proposed the following two-step method of adjustment. First, a linear regression is performed with log transformed post-vaccination antibody as the dependent variable and log transformed pre-vaccination antibody as a predictor. The regression slope (unstandardised B) is then used to calculate a baseline-adjusted of post-vaccination antibody using the formula below:

Baseline-adjusted Post-Vaccination antibody level = Post-Vaccination antibody level – (Regression Slope x Pre-Vaccination antibody level)

This correction method deals with the issues of baseline antibody influence and as a continuous approach does not result in data loss. When applied to the same 16 studies, Beyer et al. concluded this method represented a more accurate measure of antibody responses for influenza vaccination.

4.9.2.3 Approach Taken in the Present Study

Given the conceptual differences in approaches that seek to operationalise (i) clinical protection and (ii) vaccine-induced antibody response, a measure of each was examined in this study. Specifically, as a surrogate measure of clinical protection, absolute IgG levels (see 4.9.2.1.2) were used as outcome measures (hereafter described as non-adjusted antibody levels). As a measure of vaccine-induced antibody response, the more robust baseline-adjusted post-vaccination IgG levels method (see 4.9.2.2.3) was used (hereafter described as baseline-adjusted antibody levels). The primary outcome measure was baseline-adjusted antibody levels, as this a comparatively 'purer' measure of vaccine-induced response.

4.10 ***Data Entry***

All participant completed booklets, except for food diaries, were entered into custom-built Microsoft Access databases. Data entry accuracy was checked using a continuous sampling procedure (King & Lashley, 2000). To begin with, a visual check was completed between the original documents and computer database records for the first 10 booklets. Given that booklets used for this study were large (number of items > 200); an acceptable error rate was considered to be anything less than 2%. If none of the first 10 booklets exceeded this threshold, then the checks continued at a rate of 1 in every 10 booklets. If at any point data entry error rates were found to be above 2% for one booklet, the process repeated in an iterative manner with the next 10 booklets examined for errors before returning to 1 in every 10. An advantage of a continuous sampling procedure such as this in large studies is that if data entry error rates are high then nearly all records are checked. However, if data entry error rates are acceptably low then the process is substantially quicker than double data entry without

compromising accuracy (King & Lashley, 2000). For food diaries, the same continuous sampling procedure was followed with each original diary entry checked against computer database records.

4.11 *Chapter Summary*

This chapter has described the development of longitudinal observational cohort study designed to examine relationships between multiple behavioural and psychological factors with influenza vaccination outcomes in older adults. The initial design was refined with input from a PPI group and adopted a primarily diary-based methodology where key predictor variables were psycho-behavioural factors measured over the period 2 weeks pre-vaccination to, and 4 weeks following influenza vaccination. Outcome measures included vaccine-specific IgG antibody levels (baseline-adjusted and non-adjusted) at 4 and 16 weeks post-vaccination. To assess IgG levels in serum, a novel assay methodology was optimised and validated which is described in detail in the following chapter.

Chapter 5: Optimisation and Validation of a Microarray Assay to Assess Antibody Response Following Vaccination[8]

Chapter Synopsis

This chapter presents a series of laboratory experiments conducted to optimise and validate the use of a novel microarray assay to assess antibody response to the 2014/15 influenza vaccination. First, existing assays that have previously been used to assess antibody response following influenza vaccination are described, with limitations of these methods highlighted. Second, an alternative and potentially superior method of antigen microarray is introduced and described conceptually. Finally, a series of optimisation and validation experiments are presented that established the suitability of this method for use in the BeHIVE-65 study.

5.1 *Introduction*

Previous studies described in this thesis that examined influenza vaccination responses measured antibody levels in serum samples using two specific immunological assays: the hemagglutination inhibition (HAI) assay (e.g., Benedict et al., 2012; Kohut et al., 2005; Sundaram et al., 2014; Woods et al., 2009) and/or the enzyme-linked immunosorbent assay (ELISA) (e.g., Hallam, 2013; Vedhara, Fox, et al., 1999; Vedhara et al., 2002; Whitham & Blannin, 2003). While widely employed, both of these methods have

[8] Adapted sections from this chapter have been published in Ayling, K., Bowden, T., Tighe, P., Todd, I., Dilnot, E. M., Negm, O. H., Fairclough, L., & Vedhara, K. (2016). The application of protein microarray assays in psychoneuroimmunology. *Brain Behavior and Immunity*. Published online ahead of print. doi:10.1016/j.bbi.2016.09.013

significant limitations that reduce their utility (e.g., throughput, sample volume) and reliability (range, sensitivity), especially for studies where a large number of samples are in need of analysis (see 5.2 for a discussion of these issues). Because of this, a novel assay was developed for analysing sera in the BeHIVE-65 study taking advantage of recent technological advances that allow for the miniaturisation of immunological assays (so called microarrays) that can detect multiple antibody types in small quantities of sera. This microarray technique has previously been used successfully to detect antibodies against other clinically relevant antigens such as *Clostridium difficile* (Negm et al., 2015) but has never previously been used to assess responses to influenza vaccination.

5.2 ***Existing Methods for Measuring Influenza-specific Antibodies***

The following sections describe and evaluate the two most commonly used immunological assays for the detection of influenza-specific antibodies highlighting their strengths and limitations.

5.2.1 Hemagglutination Inhibition Assay (HAI)

The HAI assay relies on characteristic property of red blood cells to form a lattice-like suspension structure in the presence of the influenza virus (J. Pedersen, 2014). This process, known as hemagglutination, occurs because receptors on the surface of red blood cells bind to hemagglutinin glycoproteins that are present on the surface of the influenza virus. Importantly, if present in sufficient quantities, antibodies specific to the hemagglutinin on the influenza viruses (e.g., those produced following vaccination) can inhibit or prevent this hemagglutination, because they bind to the same glycoproteins on the viral surface, preventing them from interacting with the red blood cells.

Practically, these characteristics of red blood cells, influenza virus, and hemagglutination inhibiting antibodies are manipulated in the HAI assay which is illustrated in Figure 5.1. Serum samples are serially diluted to increasing levels (e.g., 1:4, 1:8, 1:16, 1:32 etc.) and then incubated in a microtiter plate with a standardised quantity of influenza viral antigen. Red blood cells are then added to the plate and if antibodies are present in sufficient quantities this will inhibit the hemagglutination process, with red blood cells settling in a small pellet at the bottom of the well. Antibody titres are then read as the reciprocal of the highest dilution at which hemagglutination is inhibited (i.e., if no pellet of red blood cells is observed at the serum dilution of 1:32, the antibody titre is 32).

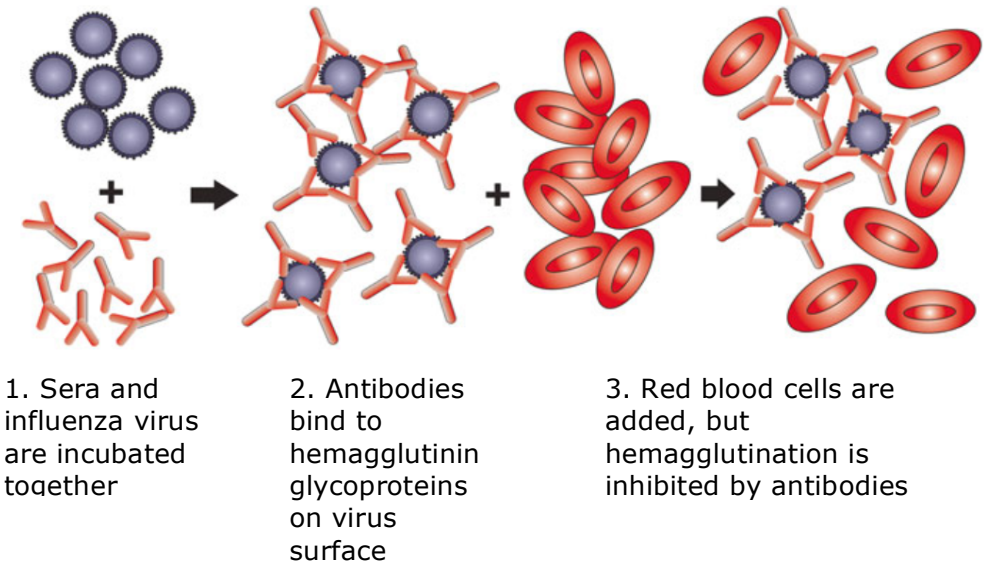


Figure 5.1: The hemagglutination inhibition assay
Image adapted from (J. Pedersen, 2014)

The main advantages of the HAI assay are that it is relatively low cost, simple to perform, and does not require much specialist equipment. Further, the HAI assay has been examined in multiple viral challenge and epidemiological studies, meaning that estimated thresholds for clinical protection have been established (Coudeville et al., 2010; de Jong et al.,

2003; Hobson et al., 1972). However, the HAI assay has many limitations. First, results of the assay are read visually by the researcher, potentially adding a degree of interpretation and potential biases, especially when the researcher is not blind to the research questions. Second, while simple to perform, the HAI assay process is fairly labour intensive, especially if multiple replicates are desired to improve reliability. A standard 96-well microtiter plate can only hold 7 sera diluted to 12 concentrations and 1 negative control. Therefore, if 100 sera require analysis for antibody titres for each of three influenza vaccine strains, each with 3 replicates, this would require at least 129 plates to be processed, as well as considerable volumes of sera, antigens and red blood cells. Finally, the most limiting feature of the HAI assay is that due to its requirement for titration (the increasing dilution of sera), samples can only be designated one of up to 12 possible discrete values (i.e., 4, 8, 16 etc.). This means significant variation is lost and actual antibody levels cannot be known with a high degree of specificity.

5.2.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA is an extremely common laboratory method used to detect a substance (typically an antigen or antibody) in a liquid sample. Many variations of ELISA exist (e.g., direct and competitive ELISA), but of relevance here is a variation known as a sandwich ELISA (so called because the detected substance is 'sandwiched' between two other substances) that can be used to detect antigen-specific antibodies in a given sample. In this context, ELISA is used to assess levels of influenza vaccine-specific IgG antibody levels and involves the coating of wells on reactive microtiter plates with the antigens present in the vaccine, followed by incubation with the human serum samples. Antibodies specific to the antigens then bind to the antigen on the coated wells, with other non-binding sera contents washed

away. A detection antibody is then added, in this case an antibody to detect Human IgG, which binds to the antibodies from the serum sample. Finally, an enzymatic substrate solution is added that reacts with the detection antibody resulting in a measurable colour change that can be read by an optical scanner, with increasing optical absorbance indicating higher quantities of antibody (Figure 5-2).

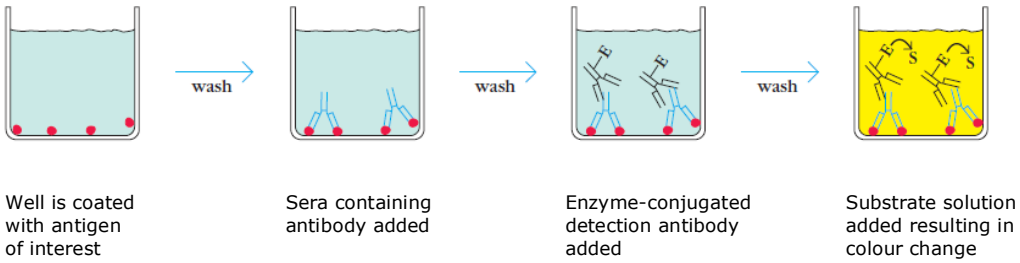


Figure 5-2: Schematic diagram of an indirect sandwich ELISA.
Adapted from Owen, Punt & Stanford (2013).

Alongside the wells containing serum samples, a calibration curve (also known as a standard curve) is also included on the plate made up of a serially diluted sample with a known concentration. In the above example this would be a commercially purchased Human IgG 'standard'. The optical absorbance of the known sample at different dilutions can then be plotted graphically, against which unknown samples can be compared to accurately quantify the level of antibody in all the other samples. This process is known as interpolation.

ELISA offers multiple benefits compared to the HAI assay, in that it produces a continuous measure of antibody levels (as opposed to limited discrete values) and can be easily adapted to measure any antibody isotype (e.g., IgA, IgM). Further, in ELISA absorbance is measured objectively by an optical scanner and fewer consumables are required per sample compared to the HAI assay, as titration is not performed. Like the HAI assay, ELISAs

are relatively simple to perform, low cost and the only specialist equipment required is the optical scanner that is common to most laboratories.

There are however, again, limitations of ELISA. To begin, ELISA has a fairly small dynamic range (values at which there is a linear relationship between absorbance and antibody quantity) meaning that serum samples often require considerable dilution for the majority to fall within this range (e.g., 1:8000 or greater). Even with such dilution, samples with particularly high or low antibody levels may fall outside this dynamic range and therefore require re-analysis at a different dilution, potentially exaggerating differences (Leng et al., 2008). Further, while ELISA requires less consumables than the HAI assay – reagent, sera, and labour requirements are still relatively high as only one antibody type can be measured per well. This limits the suitability of ELISA for where a large number of samples require processing (e.g., the 400+ samples collected during the BeHIVE-65 Study).

5.3 *Antigen Microarray Assay*

To address the limitations of both HAI assays and ELISA, a novel microarray assay (specifically an antigen microarray assay) was optimised and validated to assess influenza-specific antibodies in human sera (described conceptually below in section 5.3.1). While a relatively recent methodological and technological development (Chang, 1983), microarray assays have been widely used in genetic research, most frequently to examine the expression of multiple gene sequences by simultaneously by 'printing' DNA or RNA in minute quantities that are bound on a reactive glass slide (e.g., Jiang et al., 2004; Shena, Shalon, Davis, & Brown, 1995). More recently, this technology has been adapted to allow antibodies against

specific antigens to be measured in multiple, miniaturised assays (so called antigen microarrays) of which thousands can theoretically be performed on a single glass slide (Leng et al., 2008; Negm et al., 2015; Selvarajah et al., 2014).

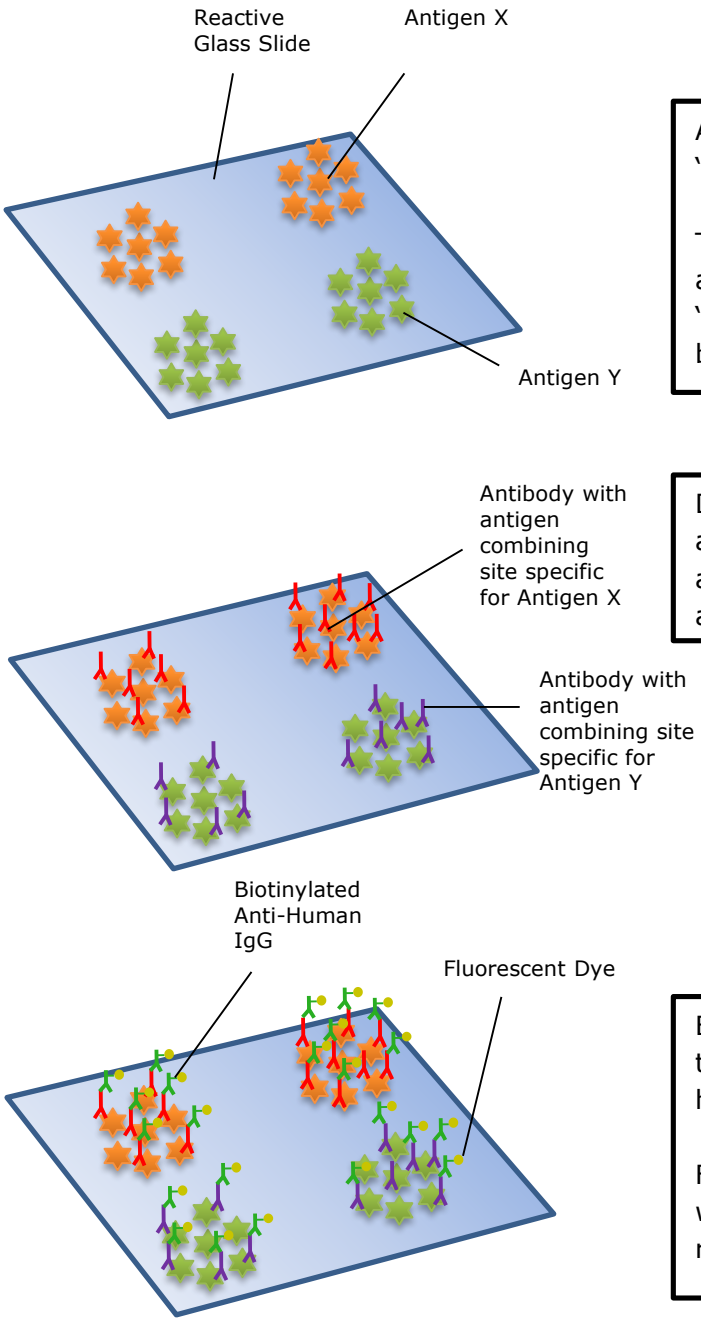
One of the most significant advantages of antigen microarray assays are that they are miniaturised and multiplex, meaning that many types of antibodies can be detected within a single processing of a sample (Negm et al., 2015). This vastly reduces the quantities of sera, antigen, reagents, and time required perform the assay (Leng et al., 2008). Large parts of the process are automated and resultant fluorescence signals are read through laser scanning techniques, which produce a highly specific continuous outcome with a large dynamic range. Further, antigen microarrays can be made increasingly robust as a large number of replicates can be performed simultaneously and they can be adapted to include multiple internal quality control measures (Negm et al., 2015). The most limiting factor of this assay is that it requires expensive, specialist equipment. However, such equipment is becoming increasingly common in modern laboratories due to their many potential applications and was available for use in the BeHIVE-65 study.

5.3.1 Conceptual Overview of Antigen Microarray

Antigen microarrays are a type of protein microarray in which tiny spots of antigen (<200 microns in diameter) are 'printed' onto a reactive surface (in this case a treated glass slide) to detect antibodies specific to the antigen in a given sample. Theoretically, the antigen microarray assays used in this thesis are, in essence, miniaturised versions of the ELISA assay. However, unlike ELISA methods, the miniaturised nature of antigen microarray assays mean that many replicates can be performed simultaneously, for multiple

antigens, with small quantities of sample and high throughput. The generalised process of antigen microarray is described below and illustrated in Figure 5-3.

First, the antigens of interest are bound in small 'spots' onto a reactive surface before the remainder of the surface is blocked to prevent further binding. Diluted serum samples are then added to the surface, at which point antibodies in the sample with specificity to epitopes on the antigen surface bind to the antigen. After unbound serum is washed away, a secondary antibody that binds specifically to human IgG antibodies (known as anti-human IgG) is added. This secondary antibody is 'labelled' with biotin, a small molecule that can be readily recognised by the protein streptavadin. Finally, a fluorescent dye (cyanine5) which is conjugated to streptavidin is added. This, in turn, can be detected by laser scanning. The fluorescence of a given sample (measured in arbitrary fluorescence units) is proportional to the number of antibodies in the serum sample that bound to the printed antigen, with greater fluorescence indicating more antibodies. As with ELISA, a calibration curve of Human IgG standard is also printed and probed, against which fluorescence values of all samples can be quantified through interpolation.



Antigens of interest are printed in 'spots' onto a reactive glass slide.

The remaining surface to which antigens are not bound, are then 'blocked' preventing further binding.

Diluted Human serum is then added, at which point any antibodies specific to the printed antigens bind.

Biotinylated Anti-Human IgG is then added, which binds to the human IgG antibodies.

Finally, a fluorescent dye is added, which binds to biotin and can be read by laser scanning.

Figure 5-3: Schematic diagram of antigen microarray

5.4 *Optimisation of the Antigen Microarray Assay*

A series of experiments were performed to optimise and validate the microarray assay used in this study; these are detailed below. Some experiments were conducted concurrently, but key features are presented separately for clarity. In all experiments 'antigens' refer to those contained in the three strains included within the 2014/15 influenza northern hemisphere vaccine (**H1N1**: *NYMC X-179A A/California/7/2009*; **H3N2**: *NYMC X-223 A/Texas/50/2012*; **B**: *NYMC BX-51B B/Massachusetts/2/2012*; National Institute of Biological Standards and Control). Antigens and IgG calibration curves were printed on coated glass slides in a 16 block array format using a Biorobotics MicroGridII arrayer (Microgrid 610, Digilab, Malborough, MA, USA) and left on the arrayer overnight for at least 12 hours before probing. Slides were then loaded into 16-well slide holders with hydrophobic barriers to separate blocks on the slide surface (thereafter described as wells). Antigens and antibodies that were printed on the slides were diluted in a printing buffer of PBS-Trehalose-Tween. Unless stated otherwise, all washes and incubations were carried out at room temperature with gentle agitation on a microplate shaker. Prior to scanning, slides were dried by centrifugation at 1200*g* for up to 10 minutes. Slides were scanned using a GenePix 4200AL scanner at a wavelength of 635nm, and 100% power. Photomultiplier gain (PMT) was set for each set of slides to ensure the highest concentration was not above the maximum level of quantification (i.e., not saturated). Resultant TIFF images were processed for fluorescence data via Axon Genepix Pro-6 Microarray Image Analysis software (Molecular Services Inc.).

5.4.1 Experiment 1: Slide Surface Coatings

In antigen microarray assays, antigens are fixed to a solid surface (in this case glass slides) by a reactive coating. A wide variety of coatings are available and pre-coated slides can be purchased commercially or produced in-house (Angenendt, 2005). Each coating has different functional properties, therefore it is essential to ensure the coating used is appropriate for a given assay (Angenendt & Glökler, 2004). Of interest here are Aminosilane and Aldehyde coatings which are known to bind human IgG (Bacarese-Hamilton, Mezzasoma, Ardizzoni, Bistoni, & Crisanti, 2004; Tang, Mei, Zhang, Zhu, & Lu, 2002). In previous internal research, commercially-coated Aminosilane slides and in-house coated Aldehyde slides have been successfully used for a variety of microarray assays. Commercially-produced slides are more expensive, making in-house produced Aldehyde slides are more economical for large sample sizes. However, of central importance for this thesis is reliability for the specific assay to be performed (identifying human IgG antibodies to influenza antigens). In this experiment, the ability to reliably bind known quantities of human IgG in decreasing concentrations (a so-called standard curve or calibration curve) was compared between commercially bought pre-coated Aminosilane slides were and 'in-house' produced Aldehyde coated slides.

Human IgG (R&D solutions) was initially diluted to 50µg/ml in printing buffer before eight, two-fold serial dilutions into printing buffer were performed (range 50µg/ml - 0.195µg/ml). These were printed on glass slides in multiple replicates (triplicate for Aminosilane slides, quadruplicate for Aldehyde slides). Wells were blocked and washed in accordance with internally developed protocols for each slide coating (see below). While minor differences exist between these protocols, these differences would not

influence the essential elements of this experiment. Aminosilane wells were blocked in 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 hour followed by five cycles of 3 minutes in 150µl 0.1% Tween-20 solution in PBS per well. Aldehyde wells were blocked in 3% BSA in PBS for 1 hour followed by a 10-minute wash in 150µl 0.1% Tween-20 solution in PBS per well. Human serum (randomly selected baseline and 4 weeks post-vaccination samples from one participant) were then diluted at 1:1000 and 1:2000 (in PBS for Aldehyde wells, in antibody diluent (Dako) for Aminosilane wells) with 100µl of added to separate wells for 1 hour. Wells were then aspirated and washed (as above) followed by 100µl of biotinylated Anti-human IgG at 1:20,000 in 5% BSA in PBS (Aminosilane) or 3% BSA in PBS (Aldehyde) for 1 hour. Wells were then washed again, with followed by 100µl of streptavidin cyanin 5 added to each well. Wells were then washed (as above), before being rinsed in PBS, then distilled water in turn. Slides were then dried and scanned.

Mean fluorescence values of standard curve replicates (one line per well) for each slide type are in Figure 5.4. As is clear, standard curves on the Aminosilane slides were more reliable and were in line with expected results. Average inter-well coefficients of variation (CV%) was acceptable for Aminosilane slides (<20%) for all Human IgG concentrations, with the exception of the highest dilutions (those <0.8µg/ml). In-house produced Aldehyde slides performed less well, with many concentrations failing to reliably bind and therefore produce a signal in all wells, all inter-block CV% were above acceptable limits (>30%). From these results it was decided that commercially produced Aminosilane slides would be used for all experiments in this thesis going forward.

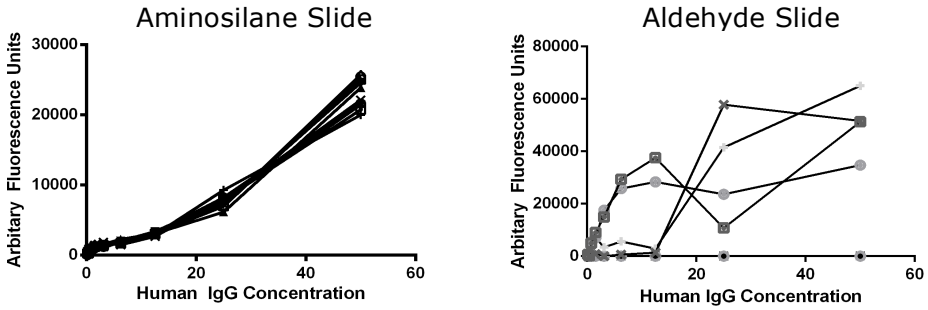


Figure 5.4: Fluorescence signals for human IgG standard curve printed on Aminosilane (Left) and Aldehyde (Right) slides

5.4.2 Experiment 2: Positive and Negative Control Test

To ensure the microarray assay could identify influenza vaccine antibodies, a control test was performed using positive sheep antiserum at multiple dilutions and PBS negative control samples. Antiserum, by definition, contains many antibodies against the antigens in question; whereas PBS contains none. For the assay to be working as required it would be expected that after slides were probed, strong signals would be found from positive controls (with signals reducing as antiserum was diluted) with very low signals above background for negative controls.

Antigens (including 5 additional closely related influenza antigen strains) were initially diluted to 100μgHA/ml in printing buffer and added to a 384-well plate (Genetix). Sheep IgG (R&D Systems) was also diluted to 1mg/ml and serially diluted in two-fold dilutions nine times (1mg/ml to 1.95μg/ml) into printing buffer and added to the plate. All dilutions were printed in triplicate onto an Aminosilane coated glass slide. Wells (each an identical array of antigens) were blocked with 3% BSA in PBS for 1 hour with gentle agitation, before being aspirated and rinsed 3 times with 150μl of 0.1% Tween-20 solution in PBS, with the final administered wash solution left in the wells for 10 minutes before aspiration. Next, antisera (A/California/7/2009 antiserum, A/Texas/50/2012 antiserum,

B/Massachusetts/02/2012-like antiserum; National Institute of Biological Standards and Control) was diluted to 1:5,000; 1:10,000; 1:20,000 and 1:40,000 in PBS with 100µl of each added to one of the 16 wells for 1 hour alongside 4 negative (PBS only) controls. Wells were then aspirated and washed again as described above before 100µl biotinylated anti-sheep IgG (R&D Systems) diluted at 1:750 in 3% BSA in PBS was added 1 hour. Wells were then aspirated and washed again, before a final 15-minute incubation under aluminium foil with 100µl Streptavidin-Cyanin5 dye (ebioscience) diluted to 1:3000 in PBS. Wells were then aspirated and washed for a final time before the slide was removed from holders and rinsed in PBS, then distilled water in turn. The slide was then dried and scanned as described above.

Examples of a magnified positive and negative control scans are shown in Figure 5-5. Brighter spots indicate stronger signals, and therefore greater number of detected antibodies. The assay performed as expected; strong signals were detected in blocks treated with antisera (left image), with the strength of signals reducing as antisera was diluted (Figure 5.6). Signals from PBS negative controls were not distinguishable from background signals (right image). The calibration curve shows strong signals regardless of the sample processed as would be expected.

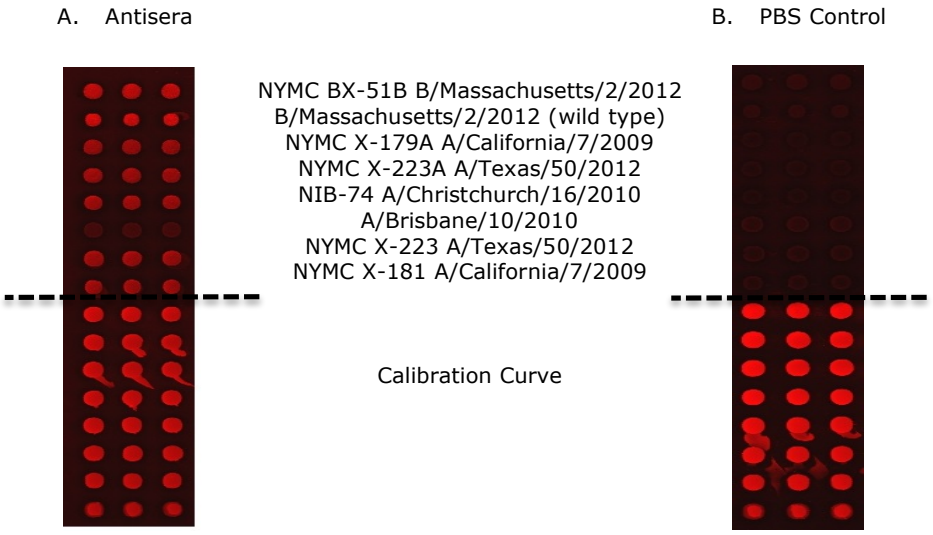


Figure 5-5: Scanned sections of positive control (B/Massachusetts Antiserum; left) and negative control (PBS; right) blocks. Above the dotted lines are signals for each antigen considered here plus five closely related influenza antigens, below is the Sheep IgG Calibration Curve which would be expected to have strong signals regardless of the sample being processed.

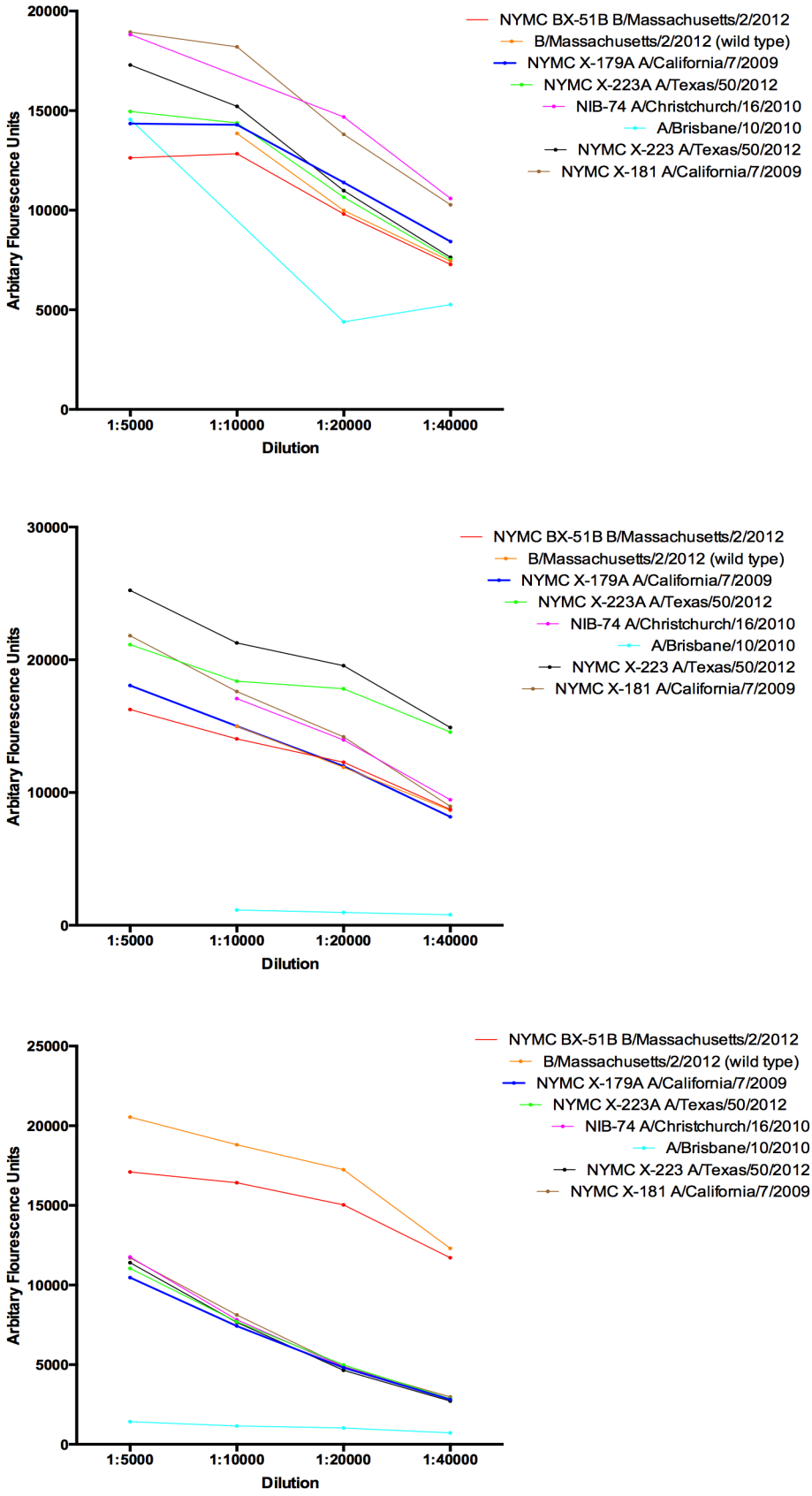


Figure 5.6: Fluorescence signals of antigens following probing with increasing dilutions of antiserum. A/California/7/2009 antiserum (top), A/Texas/50/2012 antiserum (middle) and B/Massachusetts/02/2012-like antiserum (bottom). Each line represents one antigen.

5.4.3 Experiment 3: Optimising Serum and Antigen Concentrations

Ensuring signals fall within the microarray assays sensitivity range is a balance of serum sample and antigen concentrations. If either of these elements are too concentrated, then signals can fall above the detection of the scanner or the calibration standard curve to which signals are compared. If any are too diluted, then signals may be below detection or be highly variable. This experiment was designed to identify optimal dilutions of sample and antigen concentrations for identifying pre- and post-vaccination influenza specific antibody levels in human serum samples.

Antigens were initially diluted to 50µg/ml in printing buffer before three, two-fold serial dilutions into printing were performed (range 50µg/ml – 6.25µg/ml) in a 384 well plate. Human IgG was initially diluted to 50µg/ml in printing buffer and added to the plate before a further nine, two-fold serial dilutions into printing buffer were performed (range 50µg/ml - 0.098µg/ml). Dilutions were printed overnight on Aminosilane coated glass slides in each block in triplicate. The following morning, wells were blocked in 5% BSA in PBS for 1 hour followed by aspiration and five wash cycles of 3 minutes with 150µl 0.1% Tween-20 solution in PBS per well. Four Human serum samples (n=4; randomly selected baseline and 4 weeks post-vaccination samples from two participants) were then diluted at 1:1,000, 1:2,000, 1:4,000, 1:8,000, 1:16,000 and, 1:32,000 in antibody diluent with 100µl added to separate wells each for 1 hour. Wells were then aspirated and washed (as above) followed by 100µl of biotinylated Anti-human IgG at 1:20,000 in 5% BSA in PBS for 1 hour. Wells were aspirated then washed again, followed by a 15-minute incubation with 100µl of streptavidin cyanin 5 added at a concentration 1:1,000 in PBS. Wells were then aspirated and washed (as

above), before being rinsed in PBS then distilled water, in turn. Slides were then dried and scanned.

Fluorescence signals (AFU) for the Human IgG Calibration curves in all wells are shown in Figure 5-7. Median fluorescence signals for the calibration curve ranged from 24,444 AFU for Human IgG at 50ug/ml to 282 AFU for 0.098µg/ml.

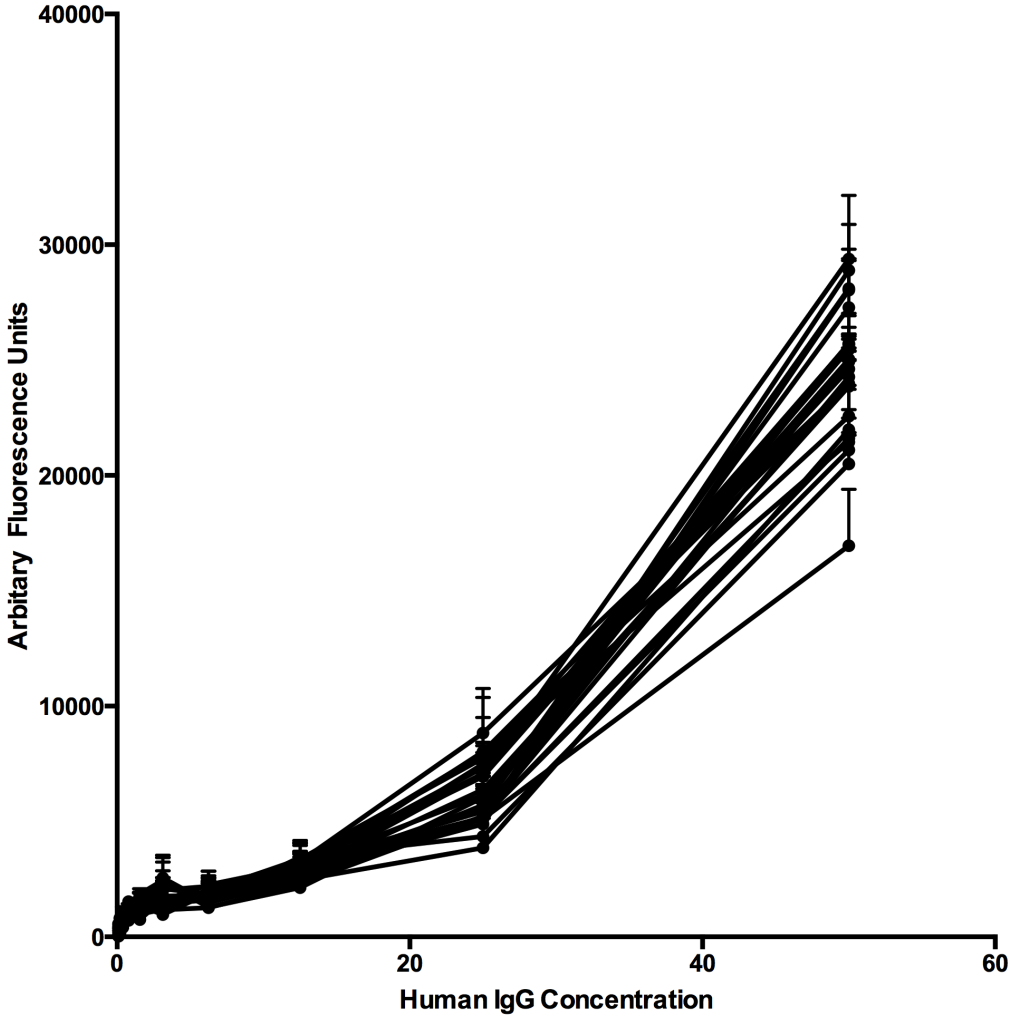


Figure 5-7: Fluorescence signals for human IgG standard curves for optimising serum and antigen concentrations

As signals from serum samples would be interpolated against this calibration curve, only those combinations of diluted serum and antigen concentrations

that produced signals within the range on the most linear portion of the curve (assessed visually as being between 25µg/ml and 6.25ug/ml; 6,094 – 1,753 AFU) were deemed acceptable.

Table 5.1, Table 5.2, and Table 5.3 show the median fluorescence signals for each combination of serum and antigen combinations across samples for H1N1, H3N2 and B antigens respectively.

Multiple combinations of serum dilution and antigen concentrations fell within the most linear region of the human IgG calibration curve for each antigen, but only the combination of serum at 1:8000 and antigen concentration at 50µg/ml fell within this range for all three antigens. Therefore, this combination was used in all microarray assays in this thesis going forward.

Table 5.1: Median fluorescence signals (AFU) for H1N1 antigen concentration and serum dilution combinations

	Serum Dilution (1:X)					
	1000	2000	4000	8000	16000	32000
50µg/ml	9472.33	6009.67*	3403.33*	**2172.33***	1234.50	698.83
25µg/ml	3444.67*	2479.17*	1358.00	864.17	491.33	309.00
12.5µg/ml	3502.83*	2053.67*	1245.83	647.67	481.33	322.67
6.25µg/ml	2189.50*	1407.50	863.17	569.67	412.67	196.33

* indicates falls within most linear portion of the calibration curve

Table 5.2: Median fluorescence signals (AFU) for H3N2 antigen concentration and serum dilution combinations

	Serum Dilution (1:X)					
	1000	2000	4000	8000	16000	32000
50µg/ml	20208.67	14322.67	9383.67	**5371.00***	3300.67*	1883.67*
25µg/ml	16885.17	12571.33	7958.17	4135.50*	2442.33*	1376.83
12.5µg/ml	13824.83	9167.83	5685.00*	3324.67*	1781.33*	1054.50
6.25µg/ml	12127.50	7664.67	4909.17*	2776.83*	1642.17	839.00

* indicates falls within most linear portion of the calibration curve

Table 5.3: Median fluorescence signals (AFU) for B antigen concentration and serum dilution combinations

	Serum Dilution (1:X)					
	1000	2000	4000	8000	16000	32000
50µg/ml	23880.67	15192.67	10585.33	**6078.67***	3282.50*	1836.17*
25µg/ml	18769.00	11303.17	7489.67	4299.17*	2303.00*	1202.83
12.5µg/ml	13448.67	9003.66	5567.83*	3288.83*	1674.00	937.66
6.25µg/ml	6965.00	3601.33*	2394.33*	1771.17*	963.67	642.16

* indicates falls within most linear portion of the calibration curve

5.5 ***Final Protocol***

Incorporating the findings of the above experiments, the following protocol was used for analysing all samples from the BeHIVE-65 Study.

Antigens for the 3 strains contained within the 2014/15 influenza northern hemisphere vaccine (NYMC X-179A A/California/7/2009; NYMC X-223 A/Texas/50/2012; NYMC BX-51B B/Massachusetts/2/2012; National Institute of Biological Standards and Control) were diluted to 50µg/ml in printing buffer (PBS Trehalose Tween) and added to a 384-well plate (Genetix). Alongside this, 18 two-fold serial human IgG dilutions (range 50µg/ml - 0.2ng/ml) were added to the plate to form a calibration curve. Antigens and Human IgG dilutions were spotted (8 and 4 replicates respectively) on Aminosilane coated glass slides (Schott) in a 17 x 16 array format using a Biorobotics MicroGridII arrayer (Microgrid 610, Digilab, Malborough, MA, USA).

Spotted slides were loaded into 16-well slide holders with hydrophobic barriers to separate wells. Wells were blocked for 1 hour with 100µl of 5% BSA in PBS. Wells were then aspirated and washed for five cycles of 3 minutes with 150µl 0.1% Tween-20 solution in PBS. Sera were diluted at 1:8,000 in a two-step process with the final dilution being made into antibody diluent (Dako). 100µl of diluted sera was added to each well for 1 hour. Wells were then aspirated and washed again as described above before 100µl biotinylated anti-human IgG (Vector Labs) diluted at 1:20,000 in 5% BSA in PBS was added for 1 hour. Wells were then aspirated and washed again, before a final incubation of 100µl Streptavidin Cyanin 5 dye (ebioscience) diluted to 1:1000 in antibody diluent for 15 minutes (covered in foil). Wells were then aspirated and washed (as above), before a final wash of 3 minutes with PBS only. All washes and incubations were carried out at room temperature with gentle agitation on a microplate shaker.

Slides were then removed from holders and rinsed with distilled water before being dried by centrifugation at 1200*g* for up to 10 minutes. Slides were

scanned using a GenePix 4200AL scanner excited at a wavelength of 635nm, and 100% power. Photomultiplier gain (PMT) was set for each set of slides to ensure the highest concentration from the Human IgG curve was not above the level of detection (typically PMT = 400). Produced TIFF images were processed for fluorescence data via Axon Genepix Pro-6 Microarray Image Analysis software (Molecular Services Inc.).

It is common practice for microarray data to be systematically pre-processed and filtered in line with pre-defined criteria to remove the influence of aberrant spots that can occur in printing or probing stages. This is a particular advantage of microarray methods and can dramatically improves the reliability of the assay (Tighe, Ryder, Todd, & Fairclough, 2015). For this assay, aberrant spots were defined as those with circularity values <50, diameter <70 microns, and with less than 50% percent of spot pixels at least 2 standard deviations above the local background, and were removed prior to analysis. The outcome variable of interest was spot median fluorescence minus the local background (F635 Median – B635). Interpolation of arbitrary fluorescence units was performed against the human IgG calibration curve in Graphpad Prism (Graphpad Software Inc., 2013) using a point-to-point spline fit, as no mathematical curve fit (e.g., Linear, Sigmoidal, second-order polynomial) reliably matched observed data points. Mean interpolated values where carried forward for use in further analyses.

5.6 ***Reproducibility Tests***

To assess the reproducibility of microarray assay, 30 randomly selected human serum samples (including baseline, 4 weeks and 16 weeks post-vaccination samples) were processed three times, each on separate days.

Slides were printed and probed in accordance with the protocol described above. Coefficients of variation (CV) were calculated for all antigens to assess both intra-assay reliability (referring to variability in signals between replicates within a block) and inter-assay reliability (variability in interpolated values obtained for each sample across the three times processed).

Intra-assay reliability was found to be excellent, with median CV's calculated at 3.96% for H1N1, 4.66% for H3N2 and 5.40% for B. Median CV's for all human IgG dilutions in the calibration curve were less than 7%. Inter-assay reliability was calculated at 27.53% for H1N1, 24.56% for H3N2, and 27.51% for B. These inter-assay CV's are slightly elevated (ideal <20%) but this is most likely a reflection of the low absolute values obtained. Coefficients of variation are highly sensitive to variation where absolute values are low. This is because the coefficient is calculated proportionally so an absolute difference of, for example, 1µg/ml results in a much higher CV where the mean value is low (e.g., mean of 5µg/ml would give CV of 20%) compared to when the mean value is high (e.g., mean of 50ug/ml would give CV of 2%). In this assay, mean interpolated levels were relatively low (mean <10µg/ml) meaning that small absolute differences would give large CVs. Median absolute differences (between highest and lowest interpolated values obtained for each sample) were small at only 0.92µg/ml for H1N1, 2.72µg/ml for H3N2, and 2.53µg/ml for B and median standard deviations were 0.47 for H1N1, 1.40 for H3N2 and 1.36 for B, suggesting acceptable inter-assay reliability of the antigen microarray.

5.7 *Validation of the Antigen Microarray Assay against ELISA*

To validate the microarray assay against a more established assay, a random selection of samples (n=40) were analysed by both microarray and ELISA with results compared. The ELISA protocol is described below.

Wells of reactive ELISA plates (Fisher Scientific) were coated, in triplicate, with 50ul of antigens (NYMC X-179A A/California/7/2009; NYMC X-223 A/Texas/50/2012; NYMC BX-51B B/Massachusetts/2/2012) diluted to 1µgHA/ml in carbonate-bicarbonate buffer (Sigma-Aldrich), alongside a calibration human IgG standard curve (25µg/ml to 0.098µg/ml) and refrigerated at 4°C overnight. The following morning, wells were aspirated and washed 3 times with 0.05% Tween-20 in PBS using an automated plate washer (BioTek) and blocked for 1 hour with 100µl of 3% BSA. Plates were then aspirated and washed again (as above) before 50µl of serum diluted at 1:8,000 in PBS was added and incubated for 1 hour. After washing, 50µl of biotinylated anti-human IgG diluted at 1:20,000 in PBS was added 1 hour. Plates were then washed again, before 50µl of Streptavidin-HRP (R&D systems) at 1:40 in PBS was added to each well and incubated for 15 minutes. After a final wash, 50µl of Tetramethylbenzidine substrate solution (Sigma-Aldrich) was added to each well for 10 minutes, before the reaction was stopped with 50µl of 1 normal solution of H_2SO_4 (sulphuric acid). Plates were then scanned at 450nm on a FLUOstar Omega plate reader (BMG Labtech). Like microarray data processing, final antibody levels were obtained by interpolating luminescence signals against the human IgG calibration curve included on the plate on which each sample was processed.

As some strains were not normally distributed (assessed visually via histograms and via significant Kolmogorov-Smirnov tests), the comparison

of microarray and ELISA performance was made using the non-parametric Spearman's rank correlation coefficient (rho). Correlation between antibody levels obtained using microarray and ELISA were moderately strong-to-strong and highly significant, indicating parity in findings between the assays (H1N1: rho(28)= .534, p<.01; H3N2: rho(37) = .802, p<.00001; B: rho(37) = .454, p<.01). This is shown graphically in 'before-and-after' plots for H1N1 (Figure 5.8), H3N2 (Figure 5.9) and B (Figure 5.10) antigens respectively.

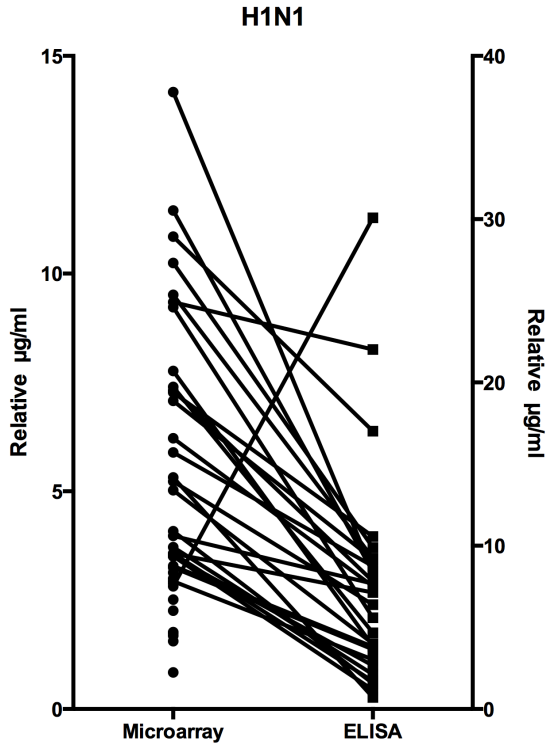


Figure 5.8: Before and after plot of relative H1N1 antibody levels assessed via microarray and ELISA

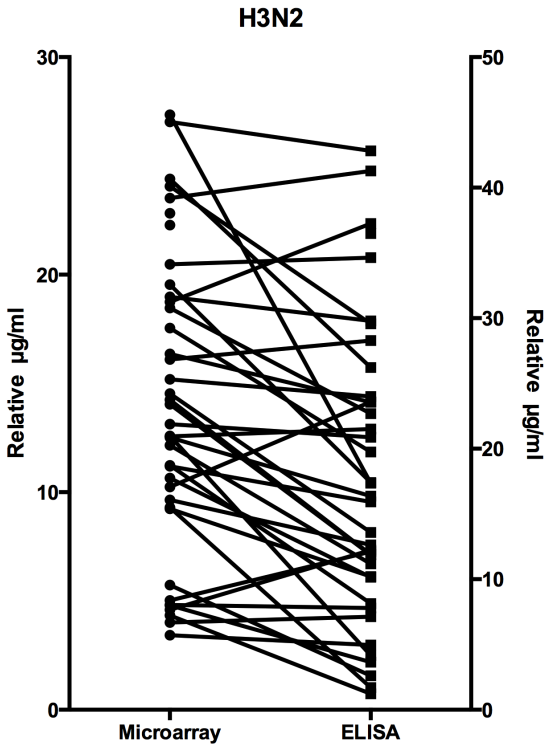


Figure 5.9: Before and after plot of relative H3N2 antibody levels assessed via microarray and ELISA

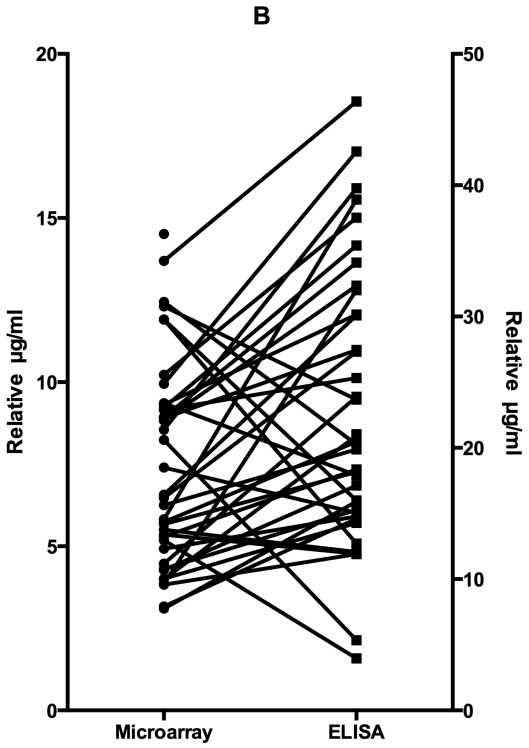


Figure 5.10: Before and after plot of relative B antibody levels assessed via microarray and ELISA

5.8 *Chapter Summary*

HAI assays and ELISA have been frequently used to measure influenza-specific antibodies in human sera to quantify vaccination responses. However, both assays have significant limitations in terms of reliability and/or utility that make them undesirable for use in large studies such as the BeHIVE-65 study described in Chapter 4. Recently developed antigen microarrays address many of these issues and therefore a novel microarray assay was optimised and validated to measure antibody response to the 2014/15 influenza vaccination for use in the BeHIVE-65 study. A series of experiments identified optimal parameters of the microarray assay and validation studies demonstrated acceptable reliability and equivalence in findings compared to ELISA.

Chapter 6: BeHIVE-65 Study Results I: Psycho-Behavioural Influences on Short-Term Influenza Vaccination Responses

Chapter Synopsis

This chapter presents results from the first phase of the BeHIVE-65 study, up to and including 4 weeks post-vaccination. A number of specific issues relating to the treatment of data are first discussed including the testing of statistical assumptions, dealing with missing data, analysing longitudinal diary data, and the multiple comparisons problem. Then, descriptive statistics for the participant sample are presented before results relating to each of the following aims are presented and discussed in turn:

- To explore whether long-term modifiable and non-modifiable participant characteristics predict short-term antibody responses following influenza vaccination in older adults.
- To explore whether behavioural and psychological factors in the period immediately prior to, on the day of, and following vaccination predict short-term antibody responses following influenza vaccination in older adults.
- To examine intra-individual variability in psychological factors among older adults and its influence on short-term antibody responses following influenza vaccination.

Finally, the chapter ends with a brief discussion of above findings and their implications, while addressing the potential impact of the previous year's influenza vaccine on the present study.

6.1 ***Background***

Considerable research, outlined at length in Chapter 3, has provided support for the hypothesis that behavioural and psychological factors can influence immune responses following vaccination. However, this thesis has highlighted a paucity of research investigating multiple behavioural and psychological influences in the same study. This means that little is known about the relative independent contribution of each factor and whether interventions targeting more than one behavioural or psychological factor may have greater impact than those that target single factors. Further, previous chapters have highlighted methodological limitations of the current literature, with an over reliance on single time-point retrospective measures and failure to consider both pre- and post-vaccination influences. The BeHIVE-65 study (BeHaviours that Influence Vaccine Efficacy in the over 65's) was designed as a starting point to address some of these issues. The development and design of the BeHIVE-65 study was described in Chapter 4. This chapter presents results from the first phase of this study, up to and including 4 weeks post-vaccination - a time point that is traditionally thought to represent the 'peak' of vaccine-induced antibody production (P. Gross et al., 1996). Figure 6.1 presents an overview of the BeHIVE-65 study timeline. This figure will be repeated throughout this and the following chapter, with time points under consideration highlighted in red.

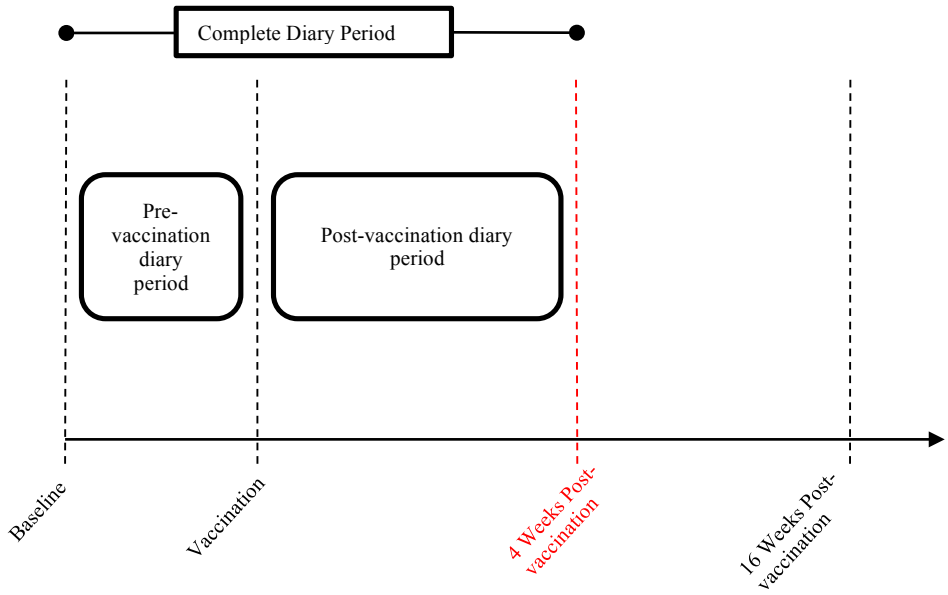


Figure 6.1: Summary diagram of BeHIVE-65 study – highlighting focus of Chapter 6

6.2 *Treatment of Data*

6.2.1 Testing Assumptions

Normality of distribution was assessed via the calculation of skewness statistics for all variables and the visual inspection of histograms prior to performing parametric analyses. Where evidence of non-normality was suggestive in histograms, skewness statistics outside of the range of -2 to +2 were considered sufficiently skewed to require transformation (George & Mallery, 2010). Antibody levels at all time points were found to be significantly positively skewed and were therefore log (base 2) transformed. Re-analysis of histograms and skewness statistics indicated these transformations successfully produced an acceptably normal distribution. Explicit negative affect scores were also found to be significantly positively skewed, however attempts at transformation (log, square root, and inverse) were unsuccessful at sufficiently improving the normality of the distribution. Therefore, in subsequent analyses the original scores were used, with non-

parametric tests employed where possible, although findings related to this variable should be treated with appropriate caution.

Univariate outliers were also assessed via visual inspection of histograms, and removed – if necessary – prior to analysis. Homogeneity of variance was assessed for 3 or more group comparisons (e.g., ANOVAs) using Levene's test, with significant values ($p<.05$) indicating unequal variances. If heterogeneity of variance was indicated, test statistics are reported where equal variances were not assumed.

For multiple regression models, multicollinearity was assessed by bivariate Pearson's correlations, variance inflation factors and tolerance statistics. Correlations larger than .8, variance inflation factors above 10 and tolerance statistics below .1 were used as thresholds for unacceptable levels of multicollinearity. Normality of residuals was assessed visually using residual plots. Multivariate outliers were identified by examining Mahalanobis distances, with any cases with a distance beyond the critical χ^2 value at $p<.001$ with degrees of freedom equal to the number of predictors in the model removed from the analysis (Tabachnick & Fidell, 2007). All analyses were performed using SPSS software version 21 (IBM Corp., 2012) or Mplus software version 7.4 (Muthén & Muthén, 2015).

6.2.2 Missing Data

Diary studies, due to requiring participants to respond to multiple items on multiple occasions, often inevitably result in missing data. There are no accepted guidelines for the level of missing data that is acceptable and, while there are numerous options for dealing with missing data, there is no universal consensus on procedures that should be followed (Tabachnick &

Fidell, 2007). Where data are shown to be missing completely at random, or at random, a common procedure is to impute values where data are missing. Several imputation methods have been proposed, the simplest and most widely used being to replace missing values with the participant's or sample's mean, median or mode. Increasingly complex, but also arguably increasingly robust, methods include applying an expectation maximization algorithm or performing multiple imputation (Scheffer, 2002). However, these more complex imputations provide computational challenges with large data sets (e.g., non-convergence, need for additional processing power) which can limit their practicality in intensive repeated measures designs. For this study, the nature of missing data was assessed using Little's MCAR test. This showed no significant deviation from randomness (χ^2 = 11155.95, df = 13394, p=1.00) indicating data were suitable for imputation. For calculating scores of multi-item psychological and behavioural measures at all time points, where at least 50% of the scales items were present, missing items were imputed using the participant's mean for that scale. Where less than 50% were present, no imputation was performed and no scale score was calculated for that time point. Missing data levels for all multi-item psychological and behavioural measures at all time points were relatively low (mean 14.1%), with imputation decreasing this only marginally to 12.1% (see Appendix E). During the diary period, missing single items (e.g., iron intake, sleep latency) were imputed using the participants mean from the two other response days for that week. If missing, baseline assessed nutritional status and habitual activity performance measures were replaced with the sample mode. No imputation was performed for anthropomorphic measures, demographics or antibody outcomes. Sensitivity analyses were also performed with non-imputed datasets, finding no meaningful differences that would alter interpretations

between analyses presented in the remainder of this thesis, performed on imputed datasets, compared with non-imputed datasets.

6.2.3 High Levels of Missing Data for Implicit Affect Measures

In inspecting missing data prevalence, it was observed that there was a concerning level of missing data for measures of implicit positive and negative affect (approximately 40-50%), as measured by the implicit positive and negative affect test (IPANAT), throughout the study. All other measures demonstrated minimal and expected levels of missing data (<15%). Informal feedback provided by participants throughout the study indicated several issues with the IPANAT. First, participants reported a lack of comprehension in what the questions were seeking to measure. While this is an intentional feature of the IPANAT, in that it seeks to not explicitly ask about emotional states, this lack of meaning was viewed negatively by many participants in this study who expressed concerns about "wasting their time" and the questions "being nonsense". So as not to compromise the collection of other measures, where participants expressed concern regarding the IPANAT, they were reminded that they did not have to respond to every question if they did not wish to, which may have further contributed to the levels of missing data for this measure. Second, others reported a lack of clarity in how to answer the questions, or indicated they were just randomly choosing responses. Based on this feedback, and the considerable levels of missing data, it was decided that the validity of the IPANAT in this study was irreparably compromised and was therefore not considered in further analyses described in this thesis.

6.2.4 Treatment of Repeated Measures Data

The most appropriate strategies for analysing intensive longitudinal data, such as that derived from diary studies, have been the subject of significant debate and commentary (Bolger & Laurenceau, 2013; J. Schwartz & Stone, 1998, 2007; Shiffman et al., 2008). The most basic, and commonly used strategy, for such data is to first perform simple mean aggregation - in which average scores over time are calculated for a given period (J. Schwartz & Stone, 1998). This condenses the data in a manner that easily then allows traditional statistical tests (e.g., multiple regressions) to be performed. Beyond ease of use, the rationale of this approach is that the pooling of repeated measures obtained throughout the study potentially provides a more reliable average than could be potentially ascertained on a single measurement occasion. However, the resultant loss of data is a limitation of this approach, which ultimately restricts the questions that can be asked of the data. Further, simple mean aggregation ignores any temporal relationships between repeated measures, such as autocorrelation. Autocorrelation refers to a non-independence of repeated measurements, often observed in diary studies where measures taken closer together in time are more similar to those taken further apart (Bolger & Laurenceau, 2013). Failing to account for such autocorrelation, especially when measurement intervals are unequal, can result in inaccurate estimates of average levels, ultimately leading to inference errors from subsequent statistical tests.

More recent commentaries have highlighted the benefits of multi-level modelling for analysing diary data (Bolger & Laurenceau, 2013). This method incorporates the natural nested structure of longitudinal data (repeated measurements are nested within individuals) and retains all the

information allowing researchers to answer questions regarding temporal relationships between factors measured at multiple time points. However, despite these attractive features, traditional multi-level models do not allow for the consideration of bottom up effects with a single measurement outcome (e.g., do scores on repeated perceived stress questionnaires, predict antibody levels at 4 weeks post-vaccination?). Therefore, this approach was unsuitable for the primary aims of this research.

An alternative approach to the analysis of diary data involves latent growth modelling (LGM), which is frequently used as part of a structural equation modelling framework (S. Gross, Meier, & Semmer, 2012). LGM allows repeated measures data to be modelled on an individual level (i.e., a regression line is fitted to best fit measurement observations over time). The properties of that model (e.g., the starting point, average level, or rate of change) can then be used as new variables to predict a given outcome. Additional attractive features of a LGM approach are that it is highly flexible (for example properties of the model can be fixed or allowed to vary freely depending on research requirements), it can examine bottom up effects, it can handle unequal measurement intervals, and is relatively insensitive to missing data (i.e., it does not require all measurement occasions to have data). Also, crucially, LGM incorporates temporal information regarding the measures in the modelling, meaning that unequal distribution of measurement occasions and any autocorrelation can be easily incorporated into each individual's model.

Given the benefits of LGM approaches described above, a hybrid LGM-mean aggregation approach was adopted for addressing research questions relating to the repeated diary measures that were collected. Mplus software was used to perform LGM in which linear regression lines were fitted to each

individual's scores over time on all variables measured during the diary period (e.g., stress, positive affect etc.), such that it minimised any residuals (errors) between observed and predicted values. As change over time was not of interest for these analyses, or evident from visual graph inspection for most behavioural and psychological variables, slopes of the regression lines for each individual were fixed to 0, so that the intercept represented the average level for that variable over the examined period. For each variable of interest, autocorrelation was assessed by examining the correlation between adjacent residuals and adjacent-plus-one residuals and were retained in the model if significant (p<.05). LGM derived intercepts for each individual were then exported into SPSS for use as predictor variables in univariate and multivariate analyses. Conceptually, these LGM intercepts can be understood as a participant's mean level on that variable, after accounting for the relative timings of the measures and any autocorrelation.

6.2.5 Antibody Outcome Measures

As discussed at length in Chapter 4, in this study antibody responses to vaccination were operationalised in two ways: one where baseline (pre-vaccination) antibody levels were accounted for (a measure of vaccine-induced response) and one where absolute antibody levels were considered (a surrogate marker for clinical protection). However, it is important to emphasise that, conceptually, these outcomes are closely related - with those showing greater vaccine-induced responses more likely to have higher absolute antibody levels post-vaccination. In the present study, correlations between these outcomes at both 4 and 16 weeks post-vaccination were high (r>.7 for all strains). Therefore, to avoid unnecessary duplication of similar results, the analyses presented in this, and the following chapter, relate to the primary outcome baseline-adjusted antibody levels, with non-adjusted

antibody level results only described briefly where differences were observed. Detailed results for non-adjusted absolute antibody levels can be seen in Appendix F.

In the following analyses, antibody responses to each antigen strain within the influenza vaccine (H1N1, H3N2 and B) are considered separately, as is common among previous studies in this field (e.g., Boge et al., 2009; Woods et al., 2009). Some researchers have opted to combine responses to different strains using some form of composite measure (e.g., Segerstrom et al., 2012). However, this approach was rejected here on the grounds that it assumes that responses to different strains (as well as the influence that psycho-behavioural factors will have on them) are sufficiently similar. Research outlined in Chapter 3, challenged this assumption - with effects of psycho-behavioural factors on antibody responses differing in strength across strains (e.g., Edwards et al., 2010, 2012; Woods et al., 2009) with the relative immunogenicity of each strain noted as a critical influence on previous findings.

6.2.6 The Multiple Comparisons Problem

Prior to presenting the analysis of the BeHIVE-65 data, it is important to briefly address the issue of the multiple comparisons problem. The multiple comparisons problem refers to the increased probability of making a type-1 error (false-positive) when many comparisons are made within a data set (McDonald, 2014). Classically, within the sciences, a critical p-value of .05 is used to denote statistical significance which, put another way, means that if the null hypothesis was true there is only a 5% chance you would get the observed result. Logically, it follows that if one was to do 100 statistical tests in which the null hypothesis was true, approximately 5 would be significant

at the 0.05 level by chance alone (yet all would represent false-positives). The implication is that studies in which many statistical comparisons are made – are likely to find spurious significant findings by chance alone. This is relevant here because, due to the focus on multiple psychological and behavioural influences on antibody outcomes (at two time points and for three antibody strains), the analysis of the BeHIVE-65 data will involve multiple comparisons.

The multiple comparisons problem is often addressed by the 'correction' of p-values, making accepted levels of significance more stringent based on the number of comparisons being made. The most common of these corrections is the Bonferroni correction, in which the originally accepted p-value of .05 is divided by the number of statistical tests performed. Thus, if 50 comparisons are made, a p-value of <.001 would be required for one or more of those tests to be considered significant. While this approach has some appeal and is relatively simple to apply, it has been widely criticised for being too conservative (e.g., Perneger, 1998) and results in the requirements of substantially larger, and often impractical, sample sizes to ensure studies are sufficiently powered to find effects (Witte, Elston, & Cardon, 2000).

While other, less conservative corrections have been proposed a more general critique of 'correcting' for multiple comparisons in this manner is that it does not truly resolve the issue of spurious effects. To illustrate, consider two independent researchers interested in predictors of outcome Z:

> Researcher One measures 25 variables (A-Y) and finds a single interesting association between variable A and outcome Z with a p-

value of .04. Correcting for multiple comparisons, Researcher One concludes this association does not meet their newly defined level of significance and accepts the null hypothesis that there is no association between variable A and outcome Z.

Researcher Two on the other hand, unaware of Researcher one's research, measures just one of those predictor variables (A) recording identical results to Researcher one. A p-value of .04 is observed, and Researcher Two concludes this association is of statistical significance and rejects the null hypothesis.

In both cases the pertinent data (variable A and its association with outcome Z) are identical, yet different conclusions are drawn. Further, in neither case is it clear that the relationship is, or is not, spurious. To be more confident in this finding, the same relationship would need to be observed in a different sample, or in another study. Further, while type-1 errors may be reduced, type-2 errors are necessarily increased (Rothman, 1990).

Based on the above, in the analysis that follows – no corrections have been made for multiple comparisons. While it is important to acknowledge this as a potential limitation, the stance taken in this thesis is that expressed by Rothman (1990) that missing possibly important effects is worse than reporting effects that turn out to be data artefacts.

6.3 ***Descriptive Statistics***

6.3.1 Participant Demographics

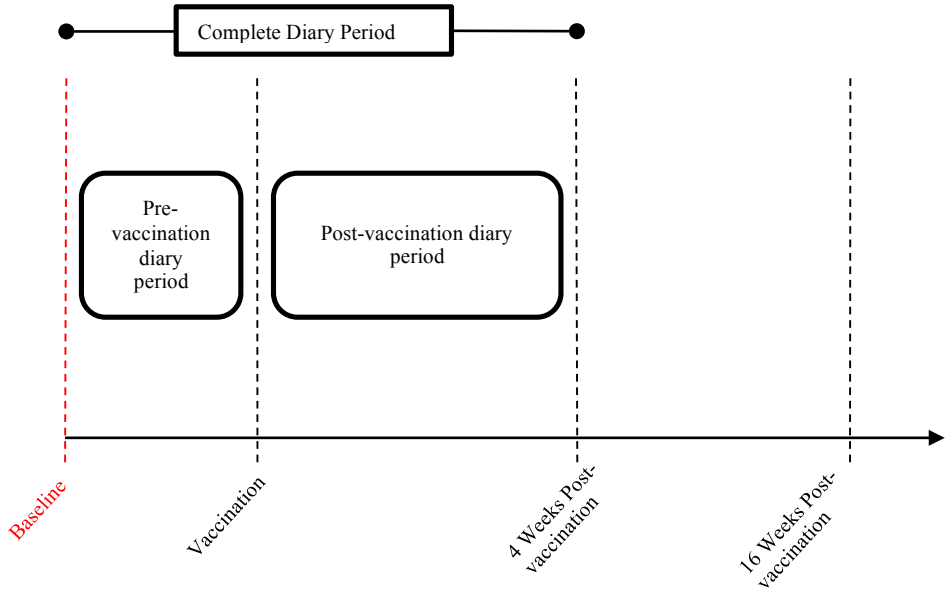


Figure 6.2: Highlighting baseline measures in BeHIVE-65 study diagram

Demographic and medical information of the 138 older adult participants is presented in Table 6.1. Just over half of participants were male (55.1%); they were predominantly white (97.8%), married (64.5%), with a school level of education (63.8%) and aged just under 73 years (M= 72.82, SD = 5.43). The mean number of self-reported illnesses and current medication prescriptions were 2.07 and 3.57 respectively, although there was considerable variation across the sample. The average body mass index (BMI) was 27.9, which is moderately overweight.

Table 6.1: Participant demographics and medical characteristics at baseline

		Mean (SD), Max-Min
Age in Years		72.82 (5.43), 65.2 – 85.0
		Frequency (%)
Gender	Male	76 (55.1)
	Female	61 (44.2)
	Did Not Respond	1 (0.7)
Ethnicity	White	135 (97.8)
	Black	1 (0.7)
	Asian	0 (0)
	Mixed	1 (0.7)
	Other	0 (0)
	Did Not Respond	1 (0.7)
Marital Status	Married	89 (64.5)
	Single, never married	7 (5.1)
	Separated/Divorced	14 (10.1)
	Widowed	26 (18.8)
	Co-habiting	1 (0.7)
	Did Not Respond	1 (0.7)
Highest Level of Education	School	88 (63.8)
	University (Undergraduate)	10 (7.2)
	University (Postgraduate)	23 (16.7)
	Other	13 (9.4)
	Did Not Respond	4 (2.9)
Current Smoker		10 (7.2)
		Mean (SD), Max-Min
# Medical Conditions (n=119)		2.07 (1.45), 0-6
# Prescribed Medications (n=116)		3.57 (2.79), 0-16
BMI (n=137)		27.9 (5.79), 17.07-58.00

SD= Standard Deviation; BMI=Body Mass Index

6.3.2 Baseline Habitual and Long-term Behavioural and Psychological Characteristics

Table 6.2 presents an overview of behavioural and psychological characteristics measured at baseline. Few participants reported being habitually sedentary (2.2%) with the majority being classified as regular underactive (52.9%) or regular active (33.3%). The majority of participants did not regularly complete any strength or flexibility training (63.8%). In terms of dietary intake, most participants met nutritional recommendations (Committee on Medical Aspects of Food Policy, 1991) for protein, iron, zinc, vitamin C, and vitamin E, but not for calories, selenium, vitamin A or vitamin D. The overall nutritional status of participants, as measured by the mini nutritional assessment (Vellas et al., 1999), was typically adequate (86.2%, scores ≥ 24) with a minority being classified at risk of malnutrition (10.1%, scores between 17 and 23.5). Average perceived stress scores were 10.98, slightly lower than previously reported normative data (Mean= 12.0) for over 65's (S. Cohen & Williamson, 1988).

Table 6.2: Participant habitual and long-term characteristics

		Frequency (%)
Habitual Physical Activity Performance – Aerobic Subscale (RAPA)	Sedentary	3 (2.2)
	Underactive	3 (2.2)
	Regular Underactive (Light Activities)	13 (9.4)
	Regular Underactive	73 (52.9)
	Regular Active	46 (33.3)
	Missing	0 (0)
Habitual Physical Activity Performance – Strength and Flexibility Subscale (RAPA)	No Strength or Flexibility	88 (63.8)
	Strength Only	1 (0.7)
	Flexibility Only	32 (23.2)
	Strength and Flexibility	17 (12.3)
	Missing	0 (0)
Habitual Dietary Intake (EPIC-FFQ)	Met Calories EAR (n=137)	40 (29.2)
	Met Protein RNI (n=137)	131 (95.6)
	Met Iron RNI (n=138)	116 (84.1)
	Met Zinc RNI (n=137)	83 (60.6)
	Met Selenium RNI (n=137)	61 (44.5)
	Met Vitamin A RNI (n=137)	47 (34.3)
	Met Vitamin C RNI (n=138)	134 (97.1)
	Met Vitamin D RNI (n=138)	4 (2.9)
	Met Vitamin E RNI (n=137)	120 (87.6)
Nutritional Status (MNA)	Adequate Nutrition	119 (86.2)
	At Risk of Malnutrition	14 (10.1)
	Malnourished	0 (0)
	Missing	5 (3.6)
		Mean (SD)
Perceived Stress over the past month (PSS-10)	(n=133)	10.98 (6.38)

RNI= Recommended Nutrient Intake; EAR= Estimated Average Requirement; SD= Standard Deviation; RAPA= Rapid Assessment of Physical Activity; EPIC-FFQ= The European Prospective Investigation into Cancer and Nutrition – Norfolk Food Frequency Questionnaire; MNA= Mini Nutritional Assessment; PSS-10= Perceived Stress Scale 10 item version

6.3.3 Antibody Responses to Vaccination

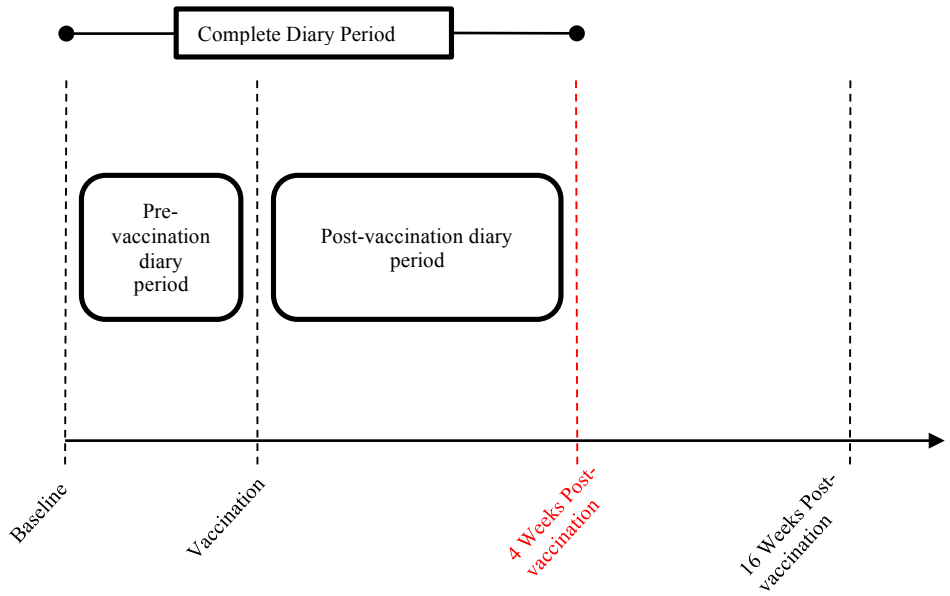


Figure 6.3: Highlighting short-term antibody outcome in BeHIVE-65 study diagram

Table 6.3 presents mean interpolated IgG antibody levels for each antigen contained in the 2014/15 northern hemisphere influenza vaccine at baseline and 4 weeks following vaccination. As expected, prior to vaccination, participants had existing antibodies to these strains, having been vaccinated against them in the previous year. Paired samples t-tests were performed to assess change in $\log_2$ transformed antibody levels from baseline to 4 weeks post-vaccination. These demonstrated there was a significant increase in serum antibody levels in all three strains from baseline to 4 weeks post-vaccination. H1N1 antibody levels demonstrated smaller increases between baseline and 4 weeks' post-vaccination and had lower absolute levels at both time points comparted to H3N2 and B, suggesting this was the least immunogenic strain.

Table 6.3: Interpolated IgG antibody levels at baseline and 4 weeks post-vaccination

	Baseline	4 Weeks Post-Vaccination	t-statistic+	p-value
H1N1	1.93 (1.88)	2.21 (2.19)	-2.11	.036*
H3N2	6.96 (6.81)	8.45 (6.91)	-2.87	.005**
B	5.18 (4.66)	6.13 (4.89)	-3.27	.001***

+Based on $\log_2$ transformed values
*p<.05, **p<.01, ***p<.001

6.3.4 Behavioural and Psychological Characteristics During the Diary Period

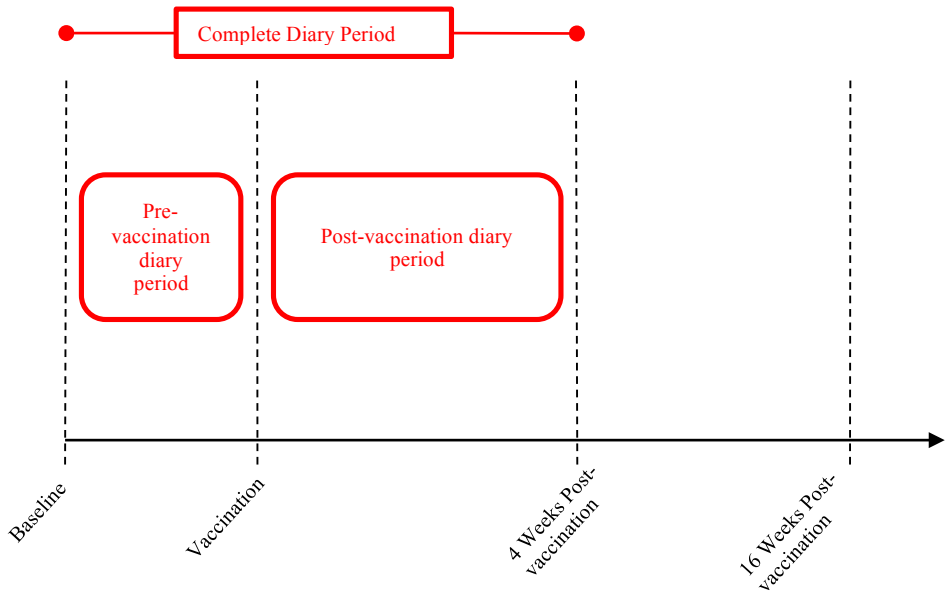


Figure 6.4: Highlighting pre-vaccination, post-vaccination, and complete diary periods in BeHIVE-65 study diagram

Table 6.4 presents descriptive statistics relating to behavioural and psychological factors measured during the pre-vaccination, post-vaccination, and complete diary periods. Group averages were similar across all periods. Across the complete diary period participants reported moderate levels of explicit positive affect as well as low levels of explicit negative affect and perceived stress. The average number of steps per day across the complete diary period was 5969, which falls within the range of previously described normative data for US older adults (Tudor-Locke et al., 2011), but

would be classified as 'low active' per Tudor-Locke et al.'s (2008) physical activity hierarchy. Participants reported an average sleep duration of a little over 7 hours a night (M=7:05) which is very similar to normative sleep data (M=7:08) previously described in a large sample of French older adults (Ohayon & Vecchierini, 2005). Mean sleep efficacy (% of time spent sleeping while in bed) was around 83% indicating that on average participants spent 17% of their time in bed not sleeping. In terms of dietary intakes, results were broadly similar to that found by examining habitual dietary intake, with the exception of selenium, vitamin A and vitamin E for which the number of participants typically meeting recommended intake levels over the diary period was considerably less than was reported for habitual intake. Based on food diary entries, the majority of participants met nutritional reference intakes for protein, iron, zinc, vitamin C, and vitamin E, but were below recommendations for calories, selenium, vitamin A, and vitamin D.

Table 6.4: Participant behavioural and psychological characteristics during the pre-vaccination, post-vaccination, and complete diary periods

	Pre	**Post**	**Complete**
	Mean (SD) unless otherwise stated		
Explicit Positive Affect	6.24 (1.62)	6.52 (1.63)	6.45 (1.56)
Explicit Negative Affect	2.40 (0.72)	2.34 (0.59)	2.35 (0.54)
Perceived Stress	3.39 (2.41)	3.27 (2.42)	3.29 (2.34)
Steps/d	6097 (3295)	5877 (2986)	5969 (2910)
Sleep Duration hours/d	6:59 (0:59)	7:06 (0:57)	7:05 (0:54)
Sleep Efficiency (%)	81.15 (12.89)	83.26 (13.84)	82.84 (13.01)
Calories/d	1877 (460)	1881 (414)	1888 (398)
Met Calories EAR n (%)	36 (25.9)	38 (31.4)	42 (34.7)
Protein (g)/d	74.30 (16.02)	76.00 (15.93)	75.61 (14.71)
Met Protein RNI n (%)	124 (96.1)	117 (96.7)	118 (97.5)
Iron (mg)/d	11.23 (3.47)	11.41 (3.79)	11.38 (3.46)
Met Iron RNI n (%)	97 (74.6)	94 (77)	97 (79.5)
Zinc (mg)/d	8.96 (2.40)	9.43 (2.74)	9.29 (2.36)
Met Zinc RNI n (%)	75 (58.1)	81 (66.9)	77 (63.6)
Selenium (μg)/d	41.90 (15.47)	43.27 (12.02)	42.97 (11.28)
Met Selenium RNI n (%)	7 (5.4)	4 (3.3)	5 (4.1)
Vitamin A (μg)/d	687.31 (1044.14)	583.86 (767.77)	625.70 (660.87)
Met Vitamin A RNI n (%)	28 (21.7)	25 (20.7)	32 (26.4)
Vitamin C (mg)/d	106.66 (98.95)	108.09 (98.95)	107.79 (118.50)
Met Vitamin C RNI n (%)	112 (86.2)	103 (84.4)	109 (89.3)
Vitamin D (μg)/d	3.37 (2.49)	3.60 (2.62)	3.55 (2.28)
Met Vitamin D RNI n (%)	3 (2.3)	3 (2.5)	2 (1.6)
Vitamin E (mg)/d	7.75 (3.82)	7.84 (3.74)	7.91 (3.53)
Met Vitamin E RNI n (%)	65 (50.4)	70 (57.9)	71 (58.7)

RNI= Recommended Nutrient Intake; EAR= Estimated Average Requirement; /d= per day; SD= Standard Deviation.

Table 6.5 presents bivariate correlations between behavioural and psychological factors measured over the complete diary period. Many significant correlations were as expected, for example all nutritional components were significantly positively correlated with each other. Positive and negative explicit affect scores showed a small but significant correlation, which is in line with previous findings (Watson et al., 1988). Perceived stress measures were strongly positively associated with explicit negative affect and strongly negatively associated with explicit positive affect measures. Greater physical activity levels were associated with greater calorie intake, as well as greater intake of protein, iron, vitamin C and vitamin E. Sleep duration and efficiency were very strongly positively correlated (r=.738). There were some less predictable associations including between greater calories and vitamin A intake with greater sleep efficiency; and lower vitamin C and protein intakes with explicit negative affect and perceived stress respectively. However, these associations were of comparatively lower magnitude.

Table 6.5: Correlations (Pearson's unless otherwise specified) between psychological and behavioural factors measured over the complete diary period

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Explicit Positive Affect	_													
2. Explicit Negative Affect^{+}	**-.183***	_												
3. Perceived Stress	**-.570*** **	**.592*** **	_											
4. Steps/d	.067	-.048	.025	_										
5. Sleep Duration	.021	-.063	-.123	.025	_									
6. Sleep Efficiency	-.005	.025	-.066	.122	**.738*** **	_								
7. Calories	-.002	-.075	-.082	**.211***	.030	**.258** **	_							
8. Protein	.109	-.025	**-.193***	**.231***	.013	.187*	**.762*** **	_						
9. Iron	.035	-.085	-.038	**.307** **	-.003	.151	**.685*** **	**.602*** **	_					
10. Zinc	.148	-.027	-.122	.146	.044	.163	**.605*** **	**.740*** **	**.617*** **	_				
11. Selenium	.053	.007	-.106	.176	-.009	.138	**.480*** **	**.618*** **	**.428*** **	**.457** **	_			
12. Vitamin A^{+}	.153	-.136	-.144	.177	.090	**.181***	**.517*** **	**.441*** **	**.395*** **	**.456*** **	**.351*** **	_		
13. Vitamin C^{+}	.068	**-.210***	-.068	**.183***	-.062	.034	**.394*** **	**.301** **	**.400*** **	**.272** **	**.299** **	**.267** **	_	
14. Vitamin D^{+}	.108	-.114	-.113	.171	.074	.080	**.394*** **	**.470*** **	**.425*** **	**.397*** **	**.474*** **	**.322*** **	**.441*** **	_
15. Vitamin E	.033	-.095	.002	**.207***	-.127	.136	**.651*** **	**.424*** **	**.511*** **	**.376*** **	**.418*** **	**.491*** **	**.533*** **	**.507*** **

*p<.05, **p<.01, ***p<.001
$^{+}$non-parametric correlation (Spearman's rho)

6.3.5 Psychological Factors Measured on the Day of Vaccination

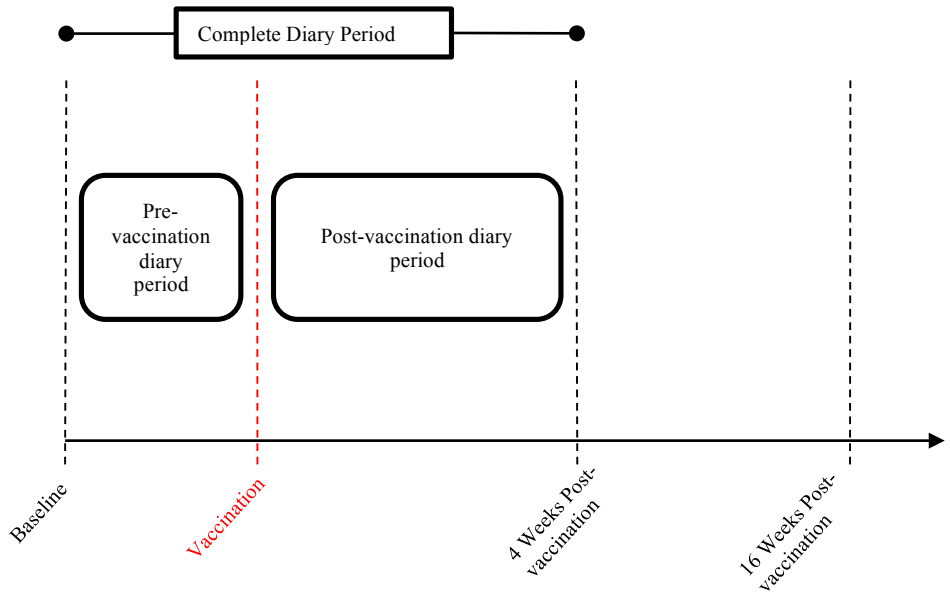


Figure 6.5: Highlighting day of vaccination measures in BeHIVE-65 study diagram

Explicit positive and negative affect were measured on the day of vaccination using full versions of the I-PANAS-SF (E. Thompson, 2007). Average explicit positive affect levels were moderately high (M= 17.58, SD= 4.24; range 5-25) and were in line with previously described cross-cultural levels (Mean range 17.38-20.53; E. Thompson, 2007). In contrast, explicit negative affect levels were comparatively low (M = 6.12, SD= 1.90; range 5-14) and lower than previously described cross-cultural levels (Mean range 10.76-14.25). Table 6.6 presents bivariate correlations between explicit positive and negative affect measures taken on the day of vaccination and across the complete diary period. As expected measures taken on the day of vaccination strongly correlated with average levels across the complete diary period.

Table 6.6: Correlations (Pearson's unless otherwise specified) between psychological factors measured on the day of vaccination and across the complete diary period

	1	2	3	4
1. Explicit Positive Affect on Day of Vaccination	_			
2. Explicit Negative Affect on Day of Vaccination[+]	-.174	_		
3. Explicit Positive Affect across complete diary period	**.803***	-.175	_	
4. Explicit Negative Affect across complete diary period[+]	-.119	**.639***	**-.183***	_

*p<.05, **p<.01, ***p<.001
[+]non-parametric correlation (Spearman's rho)

6.4 *Results*

Aim 1: To explore whether long-term modifiable and non-modifiable participant characteristics predict short-term antibody responses following influenza vaccination in older adults.

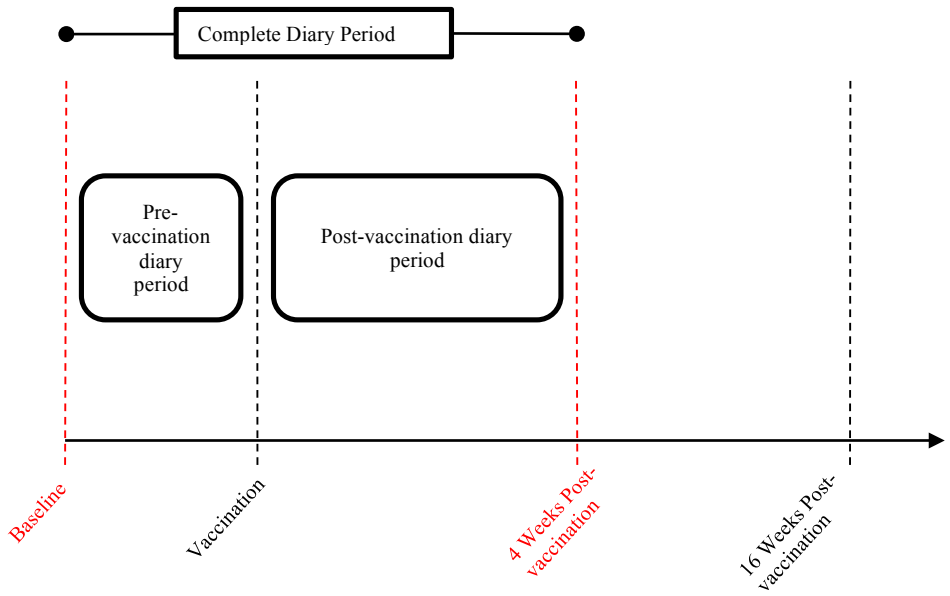


Figure 6.6: Highlighting time points under consideration in aim 1 on BeHIVE-65 study diagram

6.4.1 Research Question 1.1

Are demographic (age, gender) or clinical factors (number of self-reported illnesses, number of self-reported medications prescribed) associated with short-term influenza vaccination responses in older adults?

6.4.1.1 Hypotheses

As advancing age and greater ill-health have previously been found to be associated with poorer antibody responses following immunological challenge (P. Gross, Quinnan, Weksler, Setia, & Douglas, 1989; Grubeck-Loebenstein, Della Bella, & Iorio, 2009), it was hypothesised that greater age, number of reported illnesses, and number of prescription medications would be associated with lower baseline-adjusted antibody levels at 4 weeks post-vaccination. No directional hypotheses were developed for gender because of mixed findings in previous research.

6.4.1.2 Analyses

Pearson's and point-biserial correlations were performed to examine relationships between demographic and clinical factors with baseline-adjusted antibody levels at 4 weeks post-vaccination for each of the three antigens contained within the 2014/15 influenza vaccine. A *p*-value of less than .05 was considered statistically significant, with sizes of any significant correlations interpreted in accordance with Cohen (J. Cohen, 1992).

While correlation coefficients were in the expected direction, there were no statistically significant associations between age, gender, number of illnesses or number of medications with baseline-adjusted antibody levels for any strain at 4 weeks post-vaccination.

Table 6.7: Correlations (Pearson's unless otherwise indicated) between demographic and clinical factors with baseline-adjusted antibody levels at 4 weeks post-vaccination

	H1N1	H3N2	B
Age	-.143	-.124	-.066
Gender[+]	.029	.092	.151
Illnesses	-.151	-.039	-.117
Medication	-.062	-.033	-.040

*p<.05, **p<.01, ***p<.001
[+] Point-biserial correlation, positive values indicate females have greater antibody levels

6.4.1.3 Summary of Results & Comparisons to Previous Literature

In contrast to the hypotheses, there was no evidence that within this sample of older adults: age, gender or clinical well-being (as indicated by self-reported measures of diagnosed illnesses and prescribed medication) meaningfully influenced short-term influenza vaccination antibody outcomes. Given the relatively narrow age range (65-85 years) of this study, it is perhaps understandable that previously reported age-related differences – which typically relate to differences between younger (<65 years) and older adults (>65 years) – were not replicated in this study. The finding that clinical characteristics such as diagnosed illnesses and prescribed medication did not significantly influence vaccine responses is less expected given previous findings that greater ill-health is related to poorer vaccine responses (P. Gross et al., 1989). One explanation for this is that due to the self-selecting nature of our ambulatory participant sample there was likely fewer individuals with significant ill-health (median number of conditions = 2, range 0-6) than have been present in other studies (e.g., those which recruit institutionalised elderly). Further, it may be that measures of diagnosed illnesses and prescribed medication are not a good proxy for ill-health; indeed, individuals who have a diagnosed condition that is well managed with medication may be healthier than those with undiagnosed conditions or not taking medications.

6.4.2 Research Question 1.2

Do long-term modifiable participant characteristics (habitual aerobic activity, habitual strength and flexibility activity, stress over the previous month, habitual diet, nutritional status, and BMI) predict short-term influenza vaccination responses in older adults?

6.4.2.1 Hypotheses

Previous research (discussed at length in Chapter 3) has indicated that regular exercise, lower stress, adequately nutritious diets, and healthy BMI's are associated with more optimal immune function. Therefore, it was hypothesised that greater levels of habitual physical activity and lower levels of stress over the previous month would be associated with higher baseline-adjusted antibody levels at 4 weeks post-vaccination. With regards nutritional status, it was hypothesised that those who were malnourished, or at risk of malnutrition would have lower baseline-adjusted antibody levels at 4 weeks post-vaccination than those with adequate nutrition. Similarly, it was predicted that those who reported habitually not meeting recommended intake levels for individual dietary components would have lower baseline-adjusted antibody levels at 4 weeks post-vaccination compared to those who met recommended intake levels for those dietary components. Finally, it was hypothesised that those who had a healthy BMI (18-25 kg/m^2) would have higher baseline-adjusted antibody levels at 4 weeks post-vaccination than those who were under or overweight.

6.4.2.2 Analyses

To address this research question, a two-stage analysis approach was followed. In the first, exploratory stage, potentially meaningful relationships between long-term modifiable participant characteristics and baseline-

adjusted antibody levels were identified. This was achieved using Pearson's, point-biserial and biserial correlations (for continuous, naturally-occurring binary, and imposed binary variables respectively) with a *p*-value of <.1 considered a potentially meaningful relationship worthy of further investigation. Dietary factors were dichotomised based on meeting recommended intake levels prior to analysis. For many dietary components, there was little evidence of heterogeneity - as nearly all participants (range 84-97% of sample) met recommended intakes for protein, iron, vitamin C and vitamin E and did not meet recommended intakes for vitamin D. In contrast, more sample variability was observed in habitual intakes levels for calories, zinc, selenium and vitamin A with between 29-61% of participants meeting recommended intake levels. Therefore, only these dietary factors were considered in these analyses. For exercise variables, which were coded as multiple groups, one-way ANOVAs were performed, with post-hoc Tukey tests employed to ascertain whether between-group differences were significant. If post-hoc Tukey tests revealed any significant contrasts, these were further examined in isolation using biserial correlations to assess the significance and strength of the relationship using the same criteria as above.

Participant characteristics identified in the above exploratory analyses as having potentially meaningful relationships with antibody outcomes were then entered into the second stage, a series of hierarchical multiple regressions to see if these characteristics added significant and independent beyond age and gender - which were retained in all models due to the strength of previous findings that indicate these may influence immune responses following vaccination (Boraschi & Italiani, 2014; Müller & Pawelec, 2013). If, in the exploratory analyses, multiple participant characteristics were identified as possible predictors, they were entered one at a time into

the model, starting with the predictor showing the greatest correlation statistic and descending, until all predictors were entered into the model.

Stage 1: Exploratory Analyses

Summaries of exploratory analyses are presented in Table 6.8 (correlations) and Table 6.9 (ANOVAs). Habitual intake for selenium was associated with significantly lower baseline-adjusted antibody levels at 4 weeks post-vaccination for all three strains, with a small-to-moderate effect size. For the H1N1 strain, there was also the indication of meaningful relationships with habitual intake for vitamin A (negative) and BMI (positive), although these did not meet traditional criteria of statistical significance ($p<.05$). For analyses relating to non-adjusted antibody levels at 4 weeks post-vaccination (Appendix E - Table F.2) the only differences in findings related to Vitamin A, which was not identified as having a potentially meaningfully relationship with the H1N1, but a potentially meaningful negative association with the H3N2 strain (r_b=-.185, p<.1).

Table 6.8: Correlations (Pearson's unless otherwise indicated) between long-term modifiable participant characteristics with baseline-adjusted antibody levels at 4 weeks post-vaccination

	H1N1	H3N2	B
Perceived Stress	-.020	-.056	.022
BMI[≠]	**.212[t]**	.138	.107
Calories[+]	-.069	-.122	.011
Zinc[+]	-.133	-.069	.038
Selenium[+]	**-.248***	**-.366*****	**-.229***
Vitamin A[+]	**-.192[t]**	-.150	-.041
Nutritional Status[~]	-.065	.096	.022

[t] $p<.1$, *$p<.05$, **$p<.01$, ***$p<.001$
[≠] Biserial correlation. Positive values indicate those with a healthy BMI have greater antibody levels
[+] Biserial correlation. Positive values indicate those with intakes meeting recommended levels have greater antibody levels
[~] Biserial correlation. Positive values indicate those with adequate nutrition have greater antibody levels

Table 6.9: One-way ANOVAs for categorical long-term modifiable participant characteristics on baseline-adjusted antibody levels at 4 weeks post-vaccination

	H1N1			H3N2			B		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Aerobic	4,130	0.737	.568	4,130	0.298	.879	4,130	0.881	.477
Strength and Flexibility	3,131	0.023	.995	3,131	0.902	.442	3,131	0.440	.725

[t]*p*<.1, **p*<.05, ***p*<.01, ****p*<.001[9]

Stage 2: Predicting antibody outcomes

H1N1 Strain

Table 6.10 presents a hierarchical multiple regression analyses to predict H1N1 baseline-adjusted antibody levels at 4 weeks post-vaccination. In step one, age and gender were entered into the model explaining 2.3% of the variance in H1N1 antibody responses from baseline at 4 weeks post-vaccination, although the model was not significant. In step two, habitual intake for selenium RNI was added into the model, with the revised model becoming significant, explaining an additional 4.3% of the variance. This was a significant improvement to the model's predictive ability. Habitually meeting selenium RNI significantly predicted H1N1 baseline-adjusted antibody levels at 4 weeks post-vaccination independently of age and gender, such that those habitually met selenium RNI had lower baseline-adjusted antibody levels than those who did not at 4 weeks post-vaccination. In step 3, having a healthy BMI was added into the model, explaining an additional 2.8% of the variance, which fell just short of a statistically significant improvement to the model (p=.051). In step 4, habitually meeting vitamin A RNI was added into the model, explaining an additional 0.3% of the variance, which was not a significant improvement to the model.

[9] p<.1 is highlighted as this is the threshold for being entered into the multivariate models in stage 2. This applies to all subsequent exploratory analysis tables.

Table 6.10: Hierarchical multiple regression analysis for long-term modifiable participant characteristics predicting H1N1 baseline-adjusted antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.958	0.890	
Age	-0.021	0.012	-.147
Gender	0.041	0.132	.027
R^2 =.023, *F*(2,130)=1.514, *p*=.224			
Step 2			
(Constant)	2.045	0.874	
Age	-0.020	0.012	-.144
Gender	0.088	0.131	.058
Selenium	-0.320	0.131	**-.209***
R^2=.066, ΔR^2=.043, *F*(3,129)=3.019, *p*=.032*, Δ*F*(1,129)=5.915, *p*=.016*			
Step 3			
(Constant)	2.086	0.865	
Age	-0.022	0.012	-.156
Gender	0.048	0.131	.031
Selenium	-0.318	0.130	**-.208**
BMI	0.267	0.135	.168
R^2=.093, ΔR^2=.028, *F*(4,128)=3.288, *p*=.013, Δ*F*(1,128)=3.893, *p*=.051			
Step 4			
(Constant)	1.986	0.879	
Age	-0.020	0.012	-.144
Gender	0.054	0.132	.036
Selenium	-0.294	0.135	-.192
BMI	0.259	0.136	.164
Vitamin A	-0.097	0.142	-.061
R^2=.096, ΔR^2=.003, *F*(5,127)=2.712, *p*=.023*, Δ*F*(1,127)=0.464, *p*=.497			

*p<.05, **p<.01, ***p<.001

H3N2 Strain

Table 6.11 presents a hierarchical multiple regression analyses to predict H3N2 baseline-adjusted antibody levels at 4 weeks post-vaccination. In step one, age and gender were entered into the model explaining 2.6% of variance, although the model was not significant. In step two, habitually meeting selenium RNI was added into the model, with the revised model becoming significant explaining an additional 9.3% of the variance. This was a significant improvement to the model's predictive ability. Habitually meeting selenium RNI significantly predicted H3N2 baseline-adjusted antibody levels at 4 weeks post-vaccination independently above age and gender, such that those habitually met selenium RNI had lower H3N2

baseline-adjusted antibody levels at 4 weeks post-vaccination than those that did not.

Table 6.11: Hierarchical multiple regression analysis for long-term modifiable participant characteristics predicting H3N2 baseline-adjusted antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*B*
(Constant)	3.549	1.173	
Age	-0.024	0.016	-.131
Gender	0.177	0.174	.088
R^2 =.026, *F*(2,131)=1.719, *p*=.183			
Step 2			
(Constant)	3.747	1.121	
Age	-0.024	0.015	-.128
Gender	0.264	0.168	.131
Selenium	-0.623	0.168	-**.308*****
R^2=.119, ΔR^2=.093, *F*(3,130)=5.845, *p*=.001*, Δ*F*(1,130)=13.761, *p*<.001***			

*p<.05, **p<.01, ***p<.001

B Strain

For the B strain (Table 6.12), in step one, age and gender were entered into the model explaining 2.7% of the variance in baseline-adjusted antibody levels at 4 weeks post-vaccination, although the model was not significant. In step two, habitual selenium intake was added into the model, explaining an additional 4.2% of the variance. This was a significant improvement to the model's predictive ability and the model became significant. Habitual selenium intake predicted B antibody responses significantly above the effects of age and gender, such that those who met recommended intake levels for selenium had lower responses. For equivalent non-adjusted antibody analyses, selenium was not found to be a significant independent predictor (Appendix E - Table F.6).

Table 6.12: Hierarchical multiple regression analysis for long-term modifiable participant characteristics predicting B baseline-adjusted antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.312	1.009	
Age	-0.010	0.014	-.064
Gender	0.258	0.150	.149
R^2 =.027, *F*(2,131)=1.805, *p*=.169			
Step 2			
(Constant)	2.426	0.992	
Age	-0.010	0.014	-.062
Gender	0.308	0.148	**.177***
Selenium	-0.359	0.149	**-.206***
R^2=.069, ΔR^2=.042, *F*(3,130)=3.101, *p*=.026*, Δ*F*(1,130)=5.832, *p*=.017*			

*p<.05, **p<.01, ***p<.001

6.4.2.3 Summary of Results & Comparisons to Previous Literature

Across all strains, habitually meeting selenium intake was associated with lower antibody responses at 4 weeks post-vaccination. This finding was highly surprising, especially given the established importance of selenium in immune cell development and function, which is perceived as particularly important in older adults (Hoffmann & Berry, 2008). In the context of vaccines, selenium has received little attention. However, one previous study in older adults did find that, paired with zinc, 6 months of selenium supplementation enhanced influenza vaccine outcomes (Girodon et al., 1999). Given current understandings of selenium's role in immune function, a healthy degree of scepticism is warranted for the finding in the present study. There were substantial differences between levels of selenium intake indicated by habitual measures and diary measures. The above results reflect habitual measures - where participants are asked to recall the typical frequency at which they ate a variety of foods over the previous 12 months. Findings from this measure indicated that 44.5% of participants met recommended levels of selenium intake. However, food diary measures – which only require participants to report on each day's food and drink intake (either continuously throughout the day or at bedtime) and thus is generally considered more accurate – indicated that only one participant met selenium

intake during the time around vaccination. This disparity was not evident for most other dietary components and could potentially reflect seasonal variations in diet. Given the lack of biological plausibility and disparity between habitual and food diary selenium intake levels it is possible this observed effect is spurious and would need to be replicated in future studies before acceptance.

No other component of habitual dietary intake, habitual physical activity performance, BMI, or perceived stress over the past month were independent predictors of peak post-vaccination antibody levels. While previous findings in relation to dietary factors and BMI influencing vaccine outcomes have been equivocal, the null findings related to habitual physical activity performance and perceived stress were less expected. Considering first habitual physical activity, there has been a considerable number of prior studies demonstrating a relationship between being a regular exerciser and having larger antibody responses following vaccination (Keylock et al., 2007; Kohut et al., 2002; Schuler et al., 2003; T. P. Smith et al., 2004). While no such relationship was observed in this study, this may be explained by the fact that there were relatively few participants in this sample who were not habitually participating in physical activity. In the present sample, only six participants reported being habitually sedate (n=3) or underactive (n=3), therefore the reliability of comparisons made between sedentary and regularly active older adults is questionable.

The finding that perceived stress over the past month was not associated with peak post-vaccination antibody levels is also not consistent with the conclusions of a recent meta-analysis examining the relationship between stress and antibody responses to influenza vaccination which found a small-to-moderate sized significant negative relationship (r=-.18) between stress

and antibody response, with the strongest relationship (r=-.25) evident in studies conducted in older adults (A. Pedersen et al., 2009). However, upon closer examination of the studies included in that review – this pooled-effect is primarily driven by a considerable number of studies which compared outcomes between a very high stress group (e.g., primary carer for a spouse with Alzheimer's) and a more typical sample. Only one previous study explored the relationship between stress and antibody responses following influenza vaccination in a general population sample of older adults, also found no evidence of an association (Moynihan et al., 2004). The present study accords with this finding, indicating that in older adults, stressful experiences must be of a sufficiently large magnitude to meaningfully influence vaccine outcomes.

Aim 2: To explore whether behavioural and psychological factors in the period immediately prior to, on the day of, and following vaccination predict short-term antibody responses following influenza vaccination in older adults.

6.4.3 Research Question 2.1

Which behavioural (physical activity, diet, sleep duration, and sleep efficiency) or psychological (explicit positive affect, explicit negative affect, and perceived stress) factors as measured during the complete diary period predict short-term influenza vaccination antibody responses in older adults?

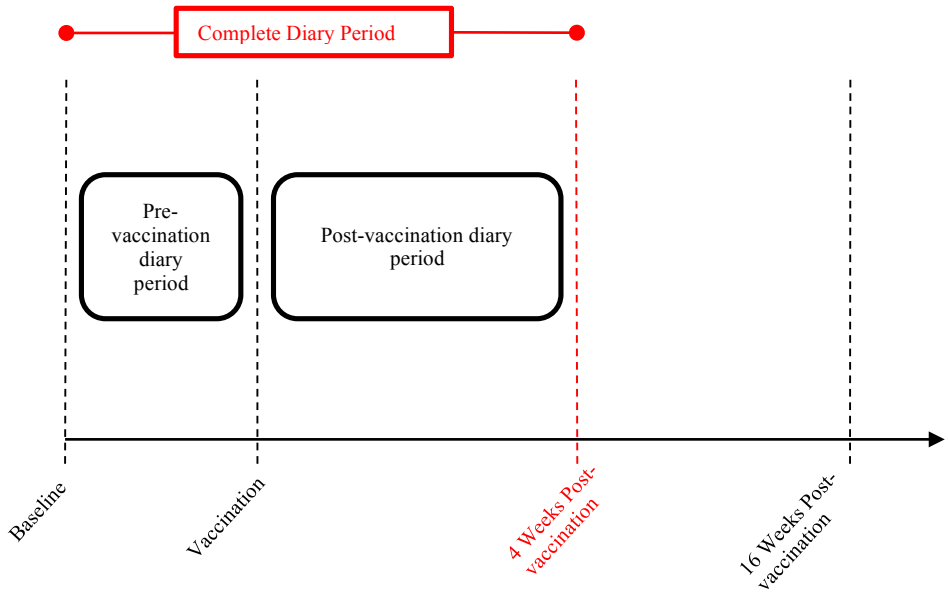


Figure 6.7: Highlighting time points under consideration for research question 2.1 on BeHIVE-65 study diagram

6.4.3.1 Hypotheses

In line with the research outlined in Chapter 3 that illustrated the potential influence of behavioural and psychological influences on immunity and vaccination responses, it was hypothesised that greater levels of physical activity, sleep duration, sleep efficiency, and explicit positive affect across the diary period, would be associated with higher baseline-adjusted antibody levels at 4 weeks post-vaccination. In contrast, it was hypothesised that greater levels of explicit negative affect and perceived stress would be associated with lower baseline-adjusted antibody levels at 4 weeks post-vaccination. For dietary factors it was hypothesised that those who did not meet recommended intake levels during this period would have lower baseline-adjusted absolute antibody levels at 4 weeks post-vaccination compared to those who met recommended intake levels.

6.4.3.2 Analyses

To address this question the two stage approach (exploratory analyses followed by multiple regressions) as described above 6.4.2.2 was taken using LGM derived intercept variables for the whole diary period (as described in 6.2.4) as predictors. As noted previously, there was minimal sample heterogeneity for many dietary components - as nearly all participants (range 81-99% of sample) met recommended intakes for protein, iron, and vitamin C and did not meet recommended intakes for selenium, vitamin A and vitamin D (range 1-6.2%). In contrast, more sample variability was observed in habitual intakes levels for calories, zinc, and vitamin E with between 27-68% of participants meeting recommended intake levels. Therefore, only these dietary factors were considered in the following analyses.

Stage 1: Exploratory Analyses

Correlations between the psycho-behavioural factors measured over the complete diary period and baseline-adjusted antibody levels at 4 weeks post-vaccination are shown in Table 6.13. A significant, small-to-moderate sized, correlation was observed between H1N1 baseline-adjusted antibody levels at 4 weeks post-vaccination and explicit positive affect, such that those with higher levels of positive affect had greater antibody levels. There was also suggestive evidence of a relationship between meeting recommended intake levels for vitamin E and greater H1N1 response. No significant correlations were observed between the psycho-behavioural factors and H3N2 or B baseline-adjusted antibody levels at 4 weeks post-vaccination. When analyses were repeated using non-adjusted antibody levels (Appendix E - Table F.7) a similar pattern of results were found, although there was no evidence of a potential relationship between vitamin

E intake and H1N1 levels and suggestive evidence that perceived stress was negatively associated with H1N1 levels (r=-.164, p<.1).

Table 6.13: Correlations (Pearson's unless otherwise indicated) between psychological and behavioural factors over the complete diary period with baseline-adjusted antibody levels at 4 weeks post-vaccination

	H1N1	H3N2	B
Explicit Positive Affect	**.198***	.085	.085
Explicit Negative Affect[+]	-.011	-.064	-.008
Perceived Stress	-.100	-.056	-.018
Physical Activity	.081	.095	.006
Sleep Duration	-.132	-.025	-.099
Sleep Efficiency	-.060	-.048	-.085
Calories[~]	.154	.127	.181
Zinc[~]	.042	-.027	.056
Vitamin E[~]	**.198[t]**	.024	.147

[t] $p<.1$, * $p<.05$, ** $p<.01$, *** $p<.001$
[+] Non-parametric correlation (Spearman's rho)
[~] Biserial correlation. Positive correlations indicate those with intakes meeting recommended levels have greater antibody levels

Stage 2: Predicting antibody outcomes

H1N1 Strain

A hierarchical multiple linear regression analysis was conducted to explore whether explicit positive affect and vitamin E intake predicted H1N1 baseline-adjusted antibody levels at 4 weeks post-vaccination independently of age and gender (see Table 6.14). In step one, age and gender were entered into the model explaining 2.4% of the variance in H1N1 baseline-adjusted antibody levels at 4 weeks post-vaccination, although the model was not significant. In step two, explicit positive affect was added into the model, and accounted for a further 3.5% of the variance – significantly improving the model, with greater explicit positive affect being associated with higher H1N1 baseline-adjusted antibody levels. In step 3, vitamin E levels were added and accounted for a further 2.4% of the variance. This was not a significant improvement to the model. For the equivalent analyses on H1N1 non-adjusted antibody levels at 4 weeks post-vaccination (Appendix E - Table F.8), perceived stress was not an independent predictor

above age, gender and explicit positive affect – nor did it improve on the model containing age, gender and explicit positive affect alone.

Table 6.14: Hierarchical multiple regression analyses for psychological and behavioural factors over the complete diary period predicting H1N1 baseline-adjusted antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.745	0.085	
Age	-0.018	0.012	-.133
Gender	0.104	0.130	.071
R^2 =.024, *F*(2,123)=1.480, *p*=.232			
Step 2			
(Constant)	1.117	0.921	
Age	-0.017	0.012	-.126
Gender	0.064	0.129	.044
Explicit Positive Affect	0.091	0.043	**.189***
R^2=.058, ΔR^2=.035, *F*(3,122)=2.514, *p*=.062, Δ*F*(1,122)=4.499, *p*=.036*			
Step 3			
(Constant)	0.843	0.926	
Age	-0.014	0.012	-.106
Gender	-0.002	0.133	-.002
Explicit Positive Affect	0.086	0.043	**.179***
Vitamin E	0.241	0.135	.164
R^2=.082, ΔR^2=.024, *F*(4,121)=2.716, *p*=.033*, Δ*F*(1,121)=3.187, *p*=.077			

*p<.05, **p<.01, ***p<.001

H3N2 Strain

No predictors were carried through from the exploratory analysis stage.

B Strain

No predictors were carried through from the exploratory analysis stage.

6.4.3.3 Summary of Results & Comparisons to Previous Literature

Greater explicit positive affect was found to be the only psycho-behavioural factor measured during the complete diary period that significantly predicted 4-week post-vaccination antibody levels following influenza vaccination in older adults above age and gender. However, this effect was only evident for the H1N1 strain of the vaccine. As noted in 6.2.5, the H1N1 strain was the least immunogenic strain of the vaccine with serum antibody levels for

this strain lower than H3N2 and B strains at all time points. Previous studies have noted that psycho-behavioural influences on influenza vaccination are often only observed in strains that are comparatively poor at inducing an immune response, or have been given in lower doses (Edwards et al., 2010, 2012, 2006; Edwards, Burns, Allen, et al., 2007) – presumably because of ceiling effects with strongly immunogenic strains unlikely to be further influenced by psycho-behavioural factors. The relationship between positive affect and H1N1 antibody responses observed in the present study is consistent with the findings of Marsland et al. (2006) who found higher levels of trait positive affect were associated with greater responses to hepatitis B vaccination. While the strength of this relationship was only small-to-moderate, it is noteworthy that even small effects can be clinically meaningful in large public health initiatives, such as vaccination. Indeed, many common medications demonstrate similar sized effects on health outcomes, including statins for the prevention of major cardiovascular events, and aspirins for prevention of vascular disease (Leucht, Helfer, Gartlehner, & Davis, 2015).

No significant relationships were observed between the other psycho-behavioural factors measured across the diary period and short-term antibody levels, despite previous studies finding such relationships. One plausible explanation for this disparity is that the high pre-vaccination antibody levels observed in this study, because of receiving the exact same vaccine 12 months previously, introduced significant ceiling effects. Thus, there was likely limited potential for strong psycho-behavioural influences on antibody responses to be observed. This issue is discussed at length in section 6.5.2.

6.4.4 Research Question 2.2

Are behavioural or psychological influences on short-term influenza vaccination responses greater during the pre- or post-vaccination period, as compared to the complete diary period?

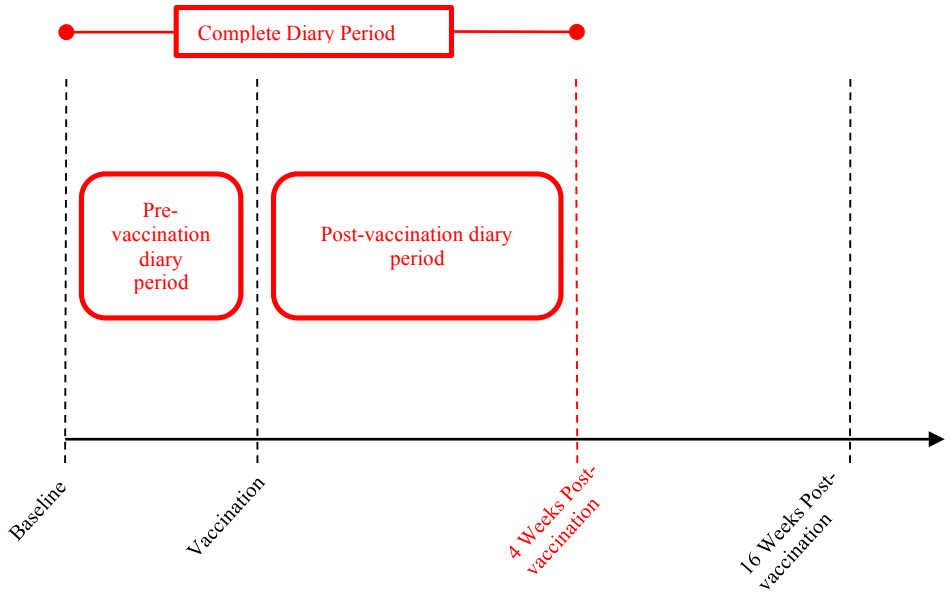


Figure 6.8: Highlighting time points under consideration for research question 2.2 on BeHIVE-65 study diagram

6.4.4.1 Hypotheses

As highlighted in Chapter 3, there has been a paucity of research investigating at what time points, relative to vaccination, behavioural and psychological factors have the most influence on antibody responses to vaccination. Despite this, it was hypothesised that behavioural and psychological factors during the post-vaccination period would be more predictive of baseline-adjusted antibody levels at 4 weeks post-vaccination than those factors measured pre-vaccination. This is because it is during this period that the vaccine is interacting with the immune system, therefore psycho-behavioural influences on immune dynamics may have greater consequences.

6.4.4.2 Analyses

To address this question, LGMs (as described in 6.2.4) for each behavioural and psychological factor were calculated separately using only diary responses at time points prior to vaccination and post-vaccination respectively. Correlations were then calculated between intercepts derived from these models and baseline-adjusted antibody levels at 4 weeks post-vaccination. Effect sizes were then compared across time points with factors showing significant, or close to significant ($p<.1$), correlations being entered into multiple regression models with age and gender (as previously described) with the relative predictive strengths of the models (r^2) and their included variables compared.

Stage 1: Exploratory Analyses

Correlations between behavioural and psychological factors measured over the pre- and post-vaccination diary periods with baseline-adjusted antibody responses at 4 weeks post-vaccination are presented in Table 6.15, alongside analogous correlations for the complete diary period. Considering first the pre-vaccination period, there was suggestive evidence of a relationship between baseline-adjusted H1N1 antibody levels at 4 weeks post-vaccination and explicit positive affect, such that those with higher levels of positive affect during the pre-vaccination period had greater H1N1 antibody levels. However, this relationship was weaker than the correlation observed over the complete diary period. During the post-vaccination period, a positive correlation was observed between explicit positive affect, meeting recommended intake levels for vitamin E during this period and baseline-adjusted H1N1 levels at 4 weeks post-vaccination, such that those with greater levels of explicit positive affect or who on average met recommended vitamin E intake across the post-vaccination period had higher H1N1 antibody baseline-adjusted antibody levels at 4 weeks post-

vaccination. These correlations were larger than during the pre-vaccination or complete diary periods. For the equivalent analyses on non-adjusted antibody levels at 4 weeks post-vaccination (Appendix E - Table F.9), a similar pattern of results were observed. However, there was also suggestive evidence of a relationship between explicit positive affect over the pre-vaccination period with B antibody levels (r=.151, $p<.1$) and no evidence of a relationship between meeting recommended intake levels for vitamin E during the post-vaccination period and H1N1 antibody levels.

Table 6.15: Correlations (Pearson's unless indicated otherwise) between psycho-behavioural factors as measured during the pre-vaccination, post-vaccination, and complete diary period with baseline-adjusted antibody levels at 4 weeks post-vaccination

	H1N1			H3N2			B		
	Pre	Post	Complete	Pre	Post	Complete	Pre	Post	Complete
Explicit Positive Affect	**.158**[t]	**.214***	**.211***	.077	.081	.122	.115	.068	**.151**[t]
Explicit Negative Affect[+]	-.039	-.002	-.030	-.024	-.052	.005	-.014	.021	-.014
Perceived Stress	-.047	-.050	-.119	-.041	.007	-.085	.002	.045	-.028
Physical Activity	.077	.074	.082	.123	.079	.127	-.017	.004	-.019
Sleep Duration	-.118	-.138	-.080	-.045	-.024	-.039	-.063	-.133	-.039
Sleep Efficiency	-.053	-.063	-.084	-.073	-.041	-.113	-.070	-.117	.005
Calories[~]	.094	.005	.123	.159	-.125	.129	.172	-.025	.116
Zinc[~]	.031	.082	-.013	.027	.022	.015	.102	.121	.072
Vitamin E[~]	.107	**.196**[t]	.077	-.019	-.020	-.073	.050	.140	.003

[t]$p<.1$, *$p<.05$, **$p<.01$, ***$p<.001$

[+] Non-parametric correlation (Spearman's rho)

[~] Biserial correlation. Positive correlations indicate those with intakes meeting recommended levels have greater antibody levels

Pre= Pre-vaccination period (from 2 weeks prior to vaccination to point of vaccination); Post= Post-vaccination period (from point of vaccination to 4 weeks after vaccination); Complete= Complete diary period (from 2 weeks prior to vaccination to 4 weeks after vaccination).

Stage 2: Predicting antibody outcomes

H1N1 Strain (Pre-Vaccination Period)

Multiple regression analyses concerning only the pre-vaccination period demonstrated that explicit positive affect did not independently predict baseline-adjusted H1N1 antibody levels at 4 weeks post-vaccination, above age and gender (Table 6.16). This is in contrast to measures of explicit positive affect over the complete diary period which significantly independently predicted baseline-adjusted H1N1 antibody levels at 4 weeks post-vaccination above the effects of age and gender. However, for the same analyses on non-adjusted H1N1 antibody levels (Appendix E -Table F.10), explicit positive affect was a significant independent predictor above age and gender explaining an additional 4.3% of variance in H1N1 antibody levels. However, the proportion of variance explained by this final model including only pre-vaccination measures of explicit positive affect (6.6%) was lower than explained by the same model including measures of explicit positive affect over the complete diary period (9.2%).

Table 6.16: Hierarchical multiple regression analysis for pre-vaccination measurements of psycho-behavioural factors predicting baseline-adjusted H1N1 antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	β
(Constant)	1.633	0.881	
Age	-0.016	0.012	-.122
Gender	0.080	0.129	.056
R^2 =.018, F(2,122) = 1.128, p=.327			
Step 2			
(Constant)	1.128	0.923	
Age	-0.016	0.012	-.116
Gender	0.043	0.130	.030
Explicit Positive Affect	0.075	0.044	.154
R^2=.041, ΔR^2=.023, F(3,121)=1.726, p=.165, ΔF(1,121)=2.888, p=.092			

*p<.05, **p<.01, ***p<.001

H3N2 Strain (Pre-Vaccination Period)

No variables were carried through from the exploratory analysis stage.

B Strain (Pre-Vaccination Period)

For baseline-adjusted B antibody levels no variables were carried through from the exploratory analysis stage. For non-adjusted B antibody levels at 4 weeks post-vaccination hierarchical multiple regression analyses showed explicit positive affect over the pre-vaccination period was not a significant independent predictor beyond age and gender (Appendix E - Table F.11).

H1N1 Strain (Post-Vaccination Period)

During the post-vaccination period, multiple regression analyses indicated that explicit positive affect independently predicted baseline-adjusted H1N1 antibody levels at 4 weeks post-vaccination, above age and gender (explaining 4.4% of additional variance) although meeting vitamin E RNI did not significantly add further to that model (Table 6.17). The model including explicit positive affect over the post-vaccination period, age and gender explained 7.2% of the variance in baseline-adjusted H1N1 antibody levels at 4 weeks post-vaccination. This was marginally higher than the percentage of variance explained by explicit positive affect over the complete diary period, age and gender (5.8%), indicating explicit positive affect over the post-vaccination period was a marginally better predictor of baseline-adjusted H1N1 antibody levels than explicit positive affect as measured over the complete diary period, or the pre-vaccination period.

Table 6.17: Hierarchical multiple regression analysis for post-vaccination measurements of psycho-behavioural factors predicting baseline-adjusted H1N1 antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.849	0.931	
Age	-0.019	0.013	-0.140
Gender	0.123	0.136	0.083
R^2 =.027, *F*(2,116)=1.589, *p*=.208			
Step 2			
(Constant)	1.162	0.960	
Age	-0.019	0.012	-.140
Gender	0.096	0.134	.065
Explicit Positive Affect	0.107	0.046	**.210***
R^2=.071, ΔR^2=.044, *F*(3,115)=2.914, *p*=.037*, Δ*F*(1,115)=5.441, *p*=.021*			
Step 3			
(Constant)	0.864	0.967	
Age	-0.017	0.012	-.119
Gender	0.029	0.138	.020
Explicit Positive Affect	0.104	0.045	**.204***
Vitamin E	0.214	0.141	.161
R^2=.094, ΔR^2=.023, *F*(4,114)=2.957, *p*=.023*, Δ*F*(1,114)=2.940, *p*=.089			

*p<.05, **p<.01, ***p<.001

H3N2 Strain (Post-Vaccination Period)

No variables were carried through from the exploratory analysis stage.

B Strain (Post-Vaccination Period)

No variables were carried through from the exploratory analysis stage.

6.4.4.3 Summary of Results & Comparisons to Previous Literature

The above results suggest that, on balance, there are minimal differences in associations between psycho-behavioural factors with short-term antibody responses following influenza vaccination when measured pre-vaccination, post-vaccination or across both time periods. Examination of correlations in Table 6.15 shows only minor differences in relationships for measures collected during the post-vaccination period compared to the pre-vaccination period, with differences were small and not always consistent in direction. Post-vaccination measures of explicit positive affect were more predictive of

peak H1N1 antibody levels than pre-vaccination measures, although, again, the differences were marginal.

While no previous studies have explicitly addressed this issue of timing, the finding that explicit positive affect may be slightly more influential during the post-vaccination period is in line with our hypothesis. One can speculate that if positive affect has an impact on the number, function, or quality of immune cells then this would be most likely to be relevant in the post-vaccination period, as this is the time when the vaccine components are interacting with the immune system. As discussed in 1.3.2, a plethora of immune components at are involved in triggering the adaptive immune response to vaccination that ultimately leads to the formation of protective IgG antibodies. This interaction between vaccine components and immune system occurs over a period post-vaccination (days to weeks) with processes including: the recognition of vaccine components as 'foreign' to the host; phagocytosis of antigens; transport of vaccine antigens through the lymphatic system to lymph organs; and the presentation to, and proliferation of, T-cells and B-cells with matching antigen binding sites. Each of these processes rely on a supply of adequately functioning immune cells and appropriate endocrine environments. Thus, it is during this period any beneficial immune alterations resulting from increased positive affect is most likely to influence vaccination responses.

However, that is not to say that psycho-behavioural factors in the short and long-term pre-vaccination period are not influential – as these factors may contribute the initial immune environment into which the vaccine is administered. With regards informing future interventions, the above findings indicate that, while differences may be marginal, in deciding whether to target a positive affect intervention to improve vaccine outcomes

in the weeks either prior to, or following vaccination, the latter may be marginally preferable.

6.4.5 Research Question 2.3

Does explicit positive affect and negative affect on the day of vaccination predict short-term antibody responses to influenza vaccination in older adults?

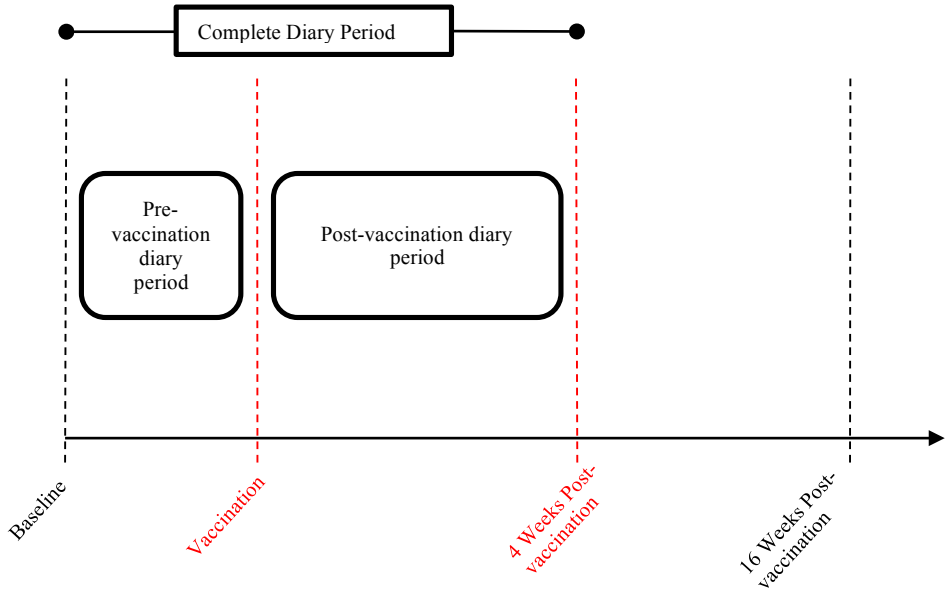


Figure 6.9: Highlighting time points under consideration for research question 2.3 on BeHIVE-65 study diagram

6.4.5.1 Hypotheses

Previous research has indicated that the modulation of psychological factors on the day of vaccination can influence immune responses (Edwards, Burns, Allen, et al., 2007). In line with this, it was hypothesised that greater explicit positive affect and lower negative affect on the day of vaccination would be associated with baseline-adjusted antibody levels at 4 weeks post-vaccination. Other psycho-behavioural factors were not examined as they were not measured on the day of vaccination.

6.4.5.2 Analyses

Stage 1: Exploratory Analyses

Following the two-stage analysis approach of above sections, correlations between measures of explicit positive (Pearson's) and negative affect (Spearman's rho) at the point of vaccination and baseline-adjusted antibody levels at 4 weeks post-vaccination are shown in Table 6.18. Higher levels of explicit positive affect on the day of vaccination was associated with greater baseline-adjusted antibody levels for all three vaccine strains at 4 weeks post-vaccination, although for the H3N2 strain this did not meet traditional criteria for significance ($p<.05$). Explicit negative affect was not significantly associated with antibody from baseline at 4 weeks post-vaccination for any strain. For non-adjusted antibody levels, the same pattern of results was observed (Appendix E - Table F.13) with the exception that the relationship between explicit positive affect on the day of vaccination and H3N2 antibody levels at 4 weeks post-vaccination was statistically significant according to conventional criteria ($r=.19$, $p<.05$).

Table 6.18: Correlations (Pearson's unless otherwise indicated) between measures of affect on the day of vaccination and baseline-adjusted antibody levels at 4 weeks post-vaccination

	H1N1	H3N2	B
Explicit Positive Affect	**.258****	**.158^{t}**	**.187***
Explicit Negative Affect^{+}	-.108	-.081	-.112

$^{t}p<.1$, $*p<.05$, $**p<.01$, $***p<.001$
$^{+}$indicates non-parametric correlation calculated (Spearman's rho)

Stage 2: Predicting antibody outcomes

H1N1 Strain

For H1N1, hierarchical multiple regression showed that explicit positive affect on the day of vaccination significantly predicted baseline-adjusted antibody levels at 4 weeks post-vaccination above the effects of age and gender, adding 5.6% of variance explained with the combined model collectively explaining 7.7% of the variance in H1N1 antibody levels (see

Table 6.19). This was a better predictive model of baseline-adjusted H1N1 antibody levels at 4 weeks post-vaccination than those including aggregated explicit positive affect measures over the complete diary period (5.8%), pre- (4.2%), or post-vaccination periods (7.1%).

Table 6.19: Hierarchical multiple regression analysis for measures of affect on the day of vaccination predicting baseline-adjusted H1N1 antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.691	0.931	
Age	-0.017	0.013	-.123
Gender	0.106	0.134	.072
R^2 =.020, *F*(2,119)=1.245, *p*=.292			
Step 2			
(Constant)	0.681	0.983	
Age	-0.013	0.012	-.093
Gender	0.055	0.132	.037
Explicit Positive Affect (Day of Vaccination)	0.042	0.016	**.241****
R^2=.077, ΔR^2=.056, *F*(3,118)=3.267, *p*=.024*, Δ*F*(1,118)=7.181, *p*=.008**			

*p<.05, **p<.01, ***p<.001

H3N2 Strain

For the H3N2 strain, hierarchical multiple regression analysis showed explicit positive affect on the day of vaccination was not a significant independent predictor of baseline-adjusted antibody levels above age and gender (see Table 6.20).

Table 6.20: Hierarchical multiple regression analysis for positive affect on the day of vaccination predicting baseline-adjusted H3N2 antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	3.776	1.279	
Age	-0.028	0.017	-.143
Gender	0.267	0.184	.131
R^2 =.038, *F*(2,119)=2.368, *p*=.098			
Step 2			
(Constant)	3.052	1.380	
Age	-0.025	0.018	-.128
Gender	0.231	0.185	.113
Explicit Positive Affect (Day of Vaccination)	0.030	0.022	.125
R^2=.053, ΔR^2=.015, *F*(3,118)=2.215, *p*=.090, Δ*F*(1,118)=1.874, *p*=.174			

*p<.05, **p<.01, ***p<.001

B Strain

For the B strain, hierarchical multiple regression analysis showed explicit positive affect on the day of vaccination was not a significant predictor of baseline-adjusted antibody levels above age and gender (see Table 6.21). However, for non-adjusted B antibody levels, explicit positive affect on the day of vaccination was an independent predictor above age and gender, explaining 3.1% of additional variance. The final model (also including age and gender) explained 5.4% of the variance in B antibody levels at 4 weeks post-vaccination (Appendix E - Table F.16).

Table 6.21: Hierarchical multiple regression analysis for positive affect on the day of vaccination predicting baseline-adjusted B antibody levels from baseline at 4 weeks post-vaccination

Step 1	B	SE B	β
(Constant)	2.183	1.109	
Age	-0.009	0.015	-.052
Gender	0.280	0.159	.159
R^2 =.028, F(2,119)=1.728, p=.182			
Step 2			
(Constant)	1.367	1.190	
Age	-0.005	0.015	-.032
Gender	0.238	0.159	.135
Explicit Positive Affect (Day of Vaccination)	0.034	0.019	.163
R^2=.054, ΔR^2=.030, F(3,118)=2.241, p=.087, ΔF(1,118)=3.203, p=.076			

*p<.05, **p<.01, ***p<.001

6.4.5.3 Summary of Results & Comparisons to Previous Literature

Explicit positive affect at the time of vaccination was found to be a significant predictor of short-term baseline-adjusted H1N1 antibody levels over and above the effects of age and gender. While this was not the case for H3N2 and B strains, correlations indicated some positive association between explicit positive affect at the time of vaccination and short-term post-vaccination antibody levels. For non-adjusted B antibody levels, explicit positive affect on the day of vaccination also was found to be an important predictor. Such relationships were not observed for explicit positive affect

over the complete diary period. Further, the proportion of variance explained for H1N1 4 weeks post-vaccination antibody levels was marginally larger than predicted by explicit positive affect over the diary period. Together, this provides some evidence suggestive of the fact that the time of vaccination is particularly salient for the influence of positive affect on vaccine outcomes.

While this finding is novel is relation to positive affect, it is congruent with the more established literature relating to exercise and stress, in which it has been observed that interventions immediately prior to vaccination can enhance vaccination responses (Dhabhar & Viswanathan, 2005; Edwards & Campbell, 2011; Edwards et al., 2006; Edwards, Burns, Allen, et al., 2007). The implications of this finding are that explicit positive affect interventions, delivered on the day of vaccination, may be the most appropriate target for a psycho-behavioural intervention to enhance influenza vaccination responses in older adults.

Explicit negative affect, on the day of vaccination, was not associated with 4 week post-vaccination antibody outcomes, further adding to the literature demonstrating positive and negative affect are distinct emotional dimensions (Watson et al., 1988) which may have differential effects on health and immunity. However, a degree of caution is warranted here, as an alternative explanation of the null findings relating to negative affect are that scores were comparatively homogeneous across the sample (compared to positive affect). Most participants expressed very low average levels of negative affect, with mean levels well below that of previously described levels among the general population (see 6.3.5) – thereby limiting the potential for any relationship to be observed.

Aim 3: To examine intra-individual variability in psychological factors among older adults and its influence on short-term antibody responses following influenza vaccination.

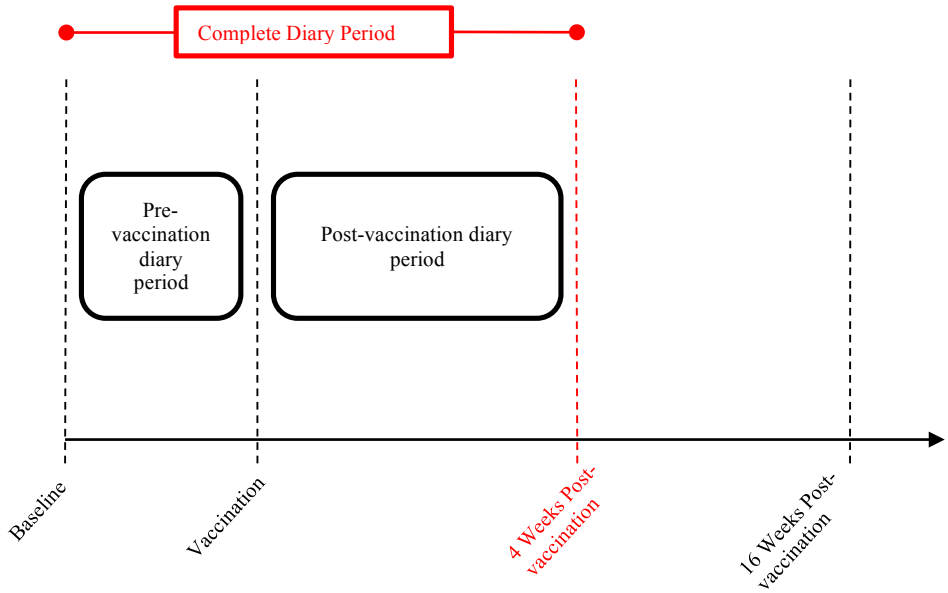


Figure 6.10: Highlighting time points under consideration for aim 3 on BeHIVE-65 study diagram

6.4.6 Research Question 3.1

How variable are psychological factors (explicit positive affect, explicit negative affect, and perceived stress) in older adults, and is variability distinct from mean levels of those factors?

6.4.6.1 Hypotheses

Intra-individual variability in psychological states have previously been found to be sufficiently distinct from mean levels to be considered a unique 'trait-like' aspect of psychological experience (Eid & Diener, 1999). It was hypothesised that intra-individual variability levels in psychological states would be associated with a participants' mean level for that psychological

factor, such that those with higher levels of negative affect and perceived stress, and lower levels of positive affect would show greatest variability. However, it was also hypothesised that mean levels in these factors would only explain a small proportion of this variability.

6.4.6.2 Analyses

Intra-individual variability was assessed by calculating the mean squared successive difference (MSSD) for each variable across the entire diary period (Jahng, Wood, & Trull, 2008; Von Neumann, 1941). MSSD, conceptually, is an average of the differences between values at adjacent measurement points. This approach is a widely used and accepted measure of intra-individual variability (Gruber et al., 2013; Human et al., 2015), having the advantage of being considerably less sensitive to changes over time than other common measures such as individual standard deviation (Wang, Hamaker, & Bergeman, 2012). In line with previous research (Koval et al., 2013) and to aid interpretation, a square-root of MSSD was calculated (rMSSD) to make the value analogous to a standard deviation and on the same scale as the original measurements. rMSSD scores were found to be significantly positively skewed and were therefore log (base 2) transformed. Re-analysis of histograms indicated these transformations successfully produced an acceptably normal distribution. Simple linear regression modelling was then performed to examine the proportion of intra-individual variation for each psychological factor explained by mean levels in that factor (operationalised as LGM intercept values for the entire diary period).

rMSSD scores for each psychological factor is shown in Table 6.22. Average variability was smaller for negative affect, than positive affect or perceived stress. Regression analyses between intra-individual variability and average

levels for explicit positive affect, explicit negative affect and perceived stress are presented in Table 6.23, Table 6.24, and Table 6.25 respectively. As predicted, lower average levels of explicit positive affect were associated with greater variability in explicit positive affect, although mean explicit positive affect levels only explained 7.7% of the variance in rMSSD scores. For explicit negative affect, greater negative affect was significantly associated with greater variability in explicit negative affect over the complete diary period, but again mean levels only explained a relatively small percentage of the variation (5.2%). Against predictions, variability in perceived stress was not significantly associated with average levels of stress.

Table 6.22: Root mean successive difference scores (rMSSD) for psychological factors over the complete diary period

	Mean (SD)
Explicit Positive Affect	1.54 (0.60)
Explicit Negative Affect	0.61 (0.62)
Perceived Stress	1.46 (0.86)

Table 6.23: Linear regression analysis predicting intra-individual variability in positive affect over the complete diary period

	B	SE B	*β*
(Constant)	1.249	0.233	
Mean Explicit Positive Affect	-.111	0.035	**-.278****
R^2 =.077, *F*(1,119)=9.995, *p*=.002**			

*p<.05, **p<.01, ***p<.001

Table 6.24: Linear regression analysis predicting intra-individual variability in negative affect over the complete diary period

	B	SE B	*β*
(Constant)	-0.078	0.243	
Mean Explicit Negative Affect	0.261	0.102	**.229***
R^2 =.052, *F*(1,119)=6.590, *p*=.011*			

*p<.05, **p<.01, ***p<.001

Table 6.25: Linear regression analysis predicting intra-individual variability in perceived stress over the complete diary period

	B	SE B	*β*
(Constant)	0.335	0.155	
Mean Perceived Stress	0.036	0.038	.091
R^2 =.008, *F*(1,109)=0.915, *p*=.341			

*p<.05, **p<.01, ***p<.001

6.4.6.3 Summary of Results & Comparisons to Previous Literature

Intra-individual variability in negative affect over the study was found to be lower in the present sample of older adults than for positive affect or perceived stress. This differs from previous findings that have shown similar levels of variability across positive and negative emotion experiences (Eid & Diener, 1999). However, this finding may in part be explained by issues with the explicit negative affect measure, was showed comparatively low internal reliability, or because the participants in the present study reported very low levels negative affect in general across the study.

The above results also demonstrate that intra-individual psychological variability is largely distinct from mean levels in psychological states over time. Less than 10% of intra-individual variability in positive affect, negative affect, and perceived stress was explained by mean levels in those factors. This level of distinctness is concordant with previous findings (Eid & Diener, 1999), highlighting that intra-individual variability in psychological factors over time should be considered as a separate aspect of emotional experience. While further data is needed on this issue, it has previously been argued that intra-individual variability in affective states are sufficiently stable to be considered a trait (Eid & Diener, 1999; Eid & Langeheine, 2003). This is closely related to research on trait emotional reactivity - for example Larsen & Ketelaar (1991) demonstrated strong between-person differences in the intensity of reactions to positive and negative stimuli. Together, this prior research and the findings of the present study point to the importance of measuring (and assessing the influence of) variability in psychological states across time.

6.4.7 Research Question 3.2

Does intra-individual variability in psychological factors predict short-term antibody responses to influenza vaccination in older adults?

6.4.7.1 Hypotheses

Based on recent evidence (discussed in 3.5.3) that greater intra-individual variability in psychological factors are associated with poorer physical health (Chan et al., 2016; Hardy & Segerstrom, 2016), it was hypothesised that greater intra-individual variability in all of the psychological factors would be associated with lower baseline-adjusted antibody levels at 4 weeks post-vaccination.

6.4.7.2 Analyses

Bivariate Correlations for intra-individual variability and baseline-adjusted antibody levels at 4 weeks post-vaccination are presented in Table 6.26. There were no statistically significant associations between intra-individual variability in explicit positive affect, explicit negative affect, and perceived stress with antibody responses from baseline for any strain at 4 weeks post-vaccination.

Table 6.26: Correlations (Pearson's unless indicated otherwise) between intra-individual variability and baseline-adjusted antibody levels at 4 weeks post-vaccination

	H1N1	H3N2	B
Explicit Positive Affect	.072	.062	.101
Explicit Negative Affect	-.042	.041	.045
Perceived Stress	.088	.004	.066

*p<.05, **p<.01, ***p<.001

6.4.7.3 Summary of Results & Comparisons to Previous Literature

Against predictions, intra-individual variability in psychological factors was not associated with short-term antibody outcomes in older adults following

influenza vaccination. No previous research has examined this question, however associations between greater variability in psychological factors and poorer physical health have been previously reported (Chan et al., 2016; Hardy & Segerstrom, 2016). Further research will be needed to ascertain whether these null findings are due to a true lack of association between intra-individual variability in psychological factors and short-term antibody responses - or are explained by characteristics of the present sample. For example, the level of intra-individual variability in psychological factors observed in this study appear small, at an average difference between measurement occasions of 0.5-1.5 units on each scale (see Table 6.22). While there are no normative values against which we compare this level, it may be that the nature of the present sample (e.g., being comparatively healthy, active, and showing low levels of negative affect) resulted showed lower levels of variability than would be found in the wider population, thereby limiting the possibility that significant effects would be found.

6.5 ***Discussion***

This chapter has presented results from the first phase of the BeHIVE-65 study, which adopted a prospective longitudinal observational design. The overall aim of the BeHIVE-65 study was to examine short- and long-term psycho-behavioural influences on antibody responses to influenza vaccination in older adults, with a view to informing the development of novel interventions to improve the effectiveness of vaccinations in older adults. This chapter focussed on psycho-behavioural influences on short-term antibody responses at 4 weeks post-vaccination, a time point commonly adopted in vaccine studies due to its association with peak IgG antibody generation (P. Gross et al., 1996). While findings have been discussed individually and compared to previous literature throughout this

chapter, this short discussion section provides a brief re-cap of the key findings and addresses some general implications of the findings. Further, the potential influence of the identical vaccination preparation in the 2013/14 and 2014/15 influenza vaccinations on the findings and the representativeness of the sample are discussed. A more comprehensive discussion of the BeHIVE-65 studies contribution to the literature, strengths and weaknesses appears in Chapter 7.

6.5.1 Re-cap of Key Findings & Implications for Novel Interventions

The first aim addressed in this chapter was to examine whether long-term modifiable and non-modifiable factors were predictors of short-term influenza vaccination antibody outcomes in older adults. Long-term factors examined included demographic (age, gender), clinical (BMI, medical conditions, medication), behavioural (habitual diet, habitual physical activity), and psychological factors (stress). In contrast to previous research, most these factors did not appear to be related to short-term antibody outcomes following influenza vaccination. Only having a habitual diet meeting recommended levels of selenium significantly predicted antibody outcomes, which in all strains was found to negatively predict antibody responses and overall antibody levels at 4 weeks. This finding was highly counterintuitive, given the previously well-established role of selenium in maintaining optimal immune function (Hoffmann & Berry, 2008). It was argued above that this finding may be a spurious artefact of the data, given the lack of congruence between this habitual measure of selenium (which required participants to report on typical consumption) and diary measures (which required participants to report on that day's intake). An implication of these findings for developing novel interventions to improve vaccine effectiveness in older adults is that there is no reason, based on this

research, to specifically target sub-populations of older adults because of demographic, clinical or long-term lifestyle factors.

The second aim examined in this chapter concerned the influence of physical activity, diet, sleep, affect, and stress in the six-week period around vaccination on short-term influenza vaccination responses in older adults. Here, while most factors did not show significant relationships with vaccine outcomes, explicit positive affect was found to be a significant independent predictor of H1N1 antibody responses, the weakest immunogenic strain of the vaccine. This accords with previous studies that have noted psycho-behavioural influences on vaccine responses are only observed in strains that are comparatively weak (Edwards et al., 2012). The relationship between 4-week H1N1 antibody responses and explicit positive affect was marginally greater when considering explicit positive affect measured during the post-vaccination period compared to measures taken during the pre-vaccination period or the entire diary period, but stronger still when considering just the measure of explicit positive affect on the day of vaccination. This suggests the importance of the immediate immune environment at the point of vaccination and how levels of explicit positive affect at this time may be particularly influential on vaccine outcomes.

Positive affect may influence vaccine outcomes through multiple pathways, both direct and indirect (Dockray & Steptoe, 2010). Indirectly, positive affect may enhance vaccination responses by influencing the performance of health behaviours or engagement, which in turn improve general health and the individual's ability to respond appropriately to vaccine antigens. Certainly, there is prior evidence that positive affect is associated with healthier lifestyles (N. Grant, Wardle, & Steptoe, 2009), and that better health status is associated with improved responses to vaccination (P. Gross

et al., 1989). However, in the present study no association between health status (as measured by number of diagnosed illnesses and prescribed medications) and short-term antibody responses were observed. Nor was positive affect significantly correlated with the behaviours measured (sleep, nutrition, physical activity). That being said, it is not possible to preclude the possibility that unmeasured health behaviours may have been influenced by positive affect and in turn influences vaccination responses.

While indirect influences of positive affect may partially underlie the effects observed, more direct pathways between positive affect and the immune system may provide a better explanation for the fact that positive affect on the day of vaccination was the strongest predictor of antibody responses. Multiple direct biological pathways between sub-cortical regions of the brain responsible for affective processing and the immune system were discussed in 2.3 – including the direct innervation of lymphatic organs extending from the central nervous system (D. Felten & Felten, 1988; S. Felten & Felten, 1991) and that receptors found on many immune cells respond to HPA-axis released hormones secreted in response to psychological experiences (Marques-Deak et al., 2005). To give a few more specific examples here, a variety of mood induction and ambulatory measurement studies have demonstrated associations between positive affective states and: CNS parasympathetic activation (Brosschot & Thayer, 2003; James, Yee, Harshfield, Blank, & Pickering, 1986); lower cortisol secretions (Hoppmann & Klumb, 2006; Jacobs et al., 2007); increased cellular immune competence (Lutgendorf et al., 2001), greater NK cytotoxicity (Valdimarsdottir & Bovbjerg, 1997), lower levels of c-reactive protein (Steptoe, O'Donnell, Badrick, Kumari, & Marmot, 2008), and secretory IgA (Stone et al., 1987). Together, these biological correlates of positive affect provide some evidence of mechanisms by which immune function, and therefore antibody

responses to vaccination, might be influenced. However, additional research to further delineate these pathways remains important.

In terms of developing novel interventions, the findings of the present study suggest a brief, positive affect intervention delivered very close to the time of vaccination could be effective. While very few previous studies have examined the relationship between positive affect and vaccine responses, those that have all show a relationship that accords with the findings of this study (Marsland et al., 2006; Rosenkranz et al., 2003). These findings point to the need for further research in this relatively under-researched area.

The third, and final, aim examined in this chapter concerned whether intra-individual variability in psychological factors was associated with short-term influenza vaccination antibody outcomes in older adults. Intra-individual variability in psychological factors was shown to represent a distinct trait to mean levels in those factors but were not significantly associated with short-term antibody outcomes. As this study is the first to address this question, this finding cannot be compared to previous analogous work.

6.5.2 The Effects of the 2013/14 Influenza Vaccination on Findings

A key discrepancy between the findings presented above and the previous literature that was reviewed in Chapter 3 is that so few of the measured factors were found to be related to short-term antibody outcomes. Each of the psychological and behavioural factors measured during the diary period had previously been shown to be related to immunity and/or vaccination responses in a relatively large number of studies – with a key rationale for the present study being to examine these factors concurrently to see which of these factors most strongly predicted antibody responses. However,

instead, we found that for short-term antibody responses – only explicit positive affect significantly predicted antibody levels in the hypothesised direction, with other psycho-behavioural factors showing minimal associations.

The explanation for this discrepancy likely relates to the previous year's vaccination introducing a ceiling effect in vaccination responses. To be more specific, as noted in chapter 4, the 2014/15 northern hemisphere influenza vaccine contained antigens for the exact same 3 influenza strains as the 2013/14 vaccine. This is not typical and was not known at the inception of study planning as annual influenza vaccine components are not agreed upon until around 6 months prior to administration. Thus, due to our pre-determined inclusion criteria that all participants in this study had received the influenza vaccination a year previously – all participants had significant baseline antibody levels to the 2014/15 strains even prior to vaccination. Vaccine-induced antibody increases have regularly been found to be lower in those with higher pre-vaccination levels. Conceptually, this is because there exists an 'antibody ceiling' at which the body will no longer increase serum antibody levels in response to further challenge. Evidence for such a ceiling has been observed for repeated influenza vaccination with those with the highest levels of antibody failing to further increase levels post-vaccination (Beyer et al., 1996; Sasaki et al., 2008; D. Smith et al., 1999). Further, high baseline antibody levels introduce the possibility of negative interference – a phenomenon where pre-existing antibodies bind to vaccine antigens clearing them from the body – thus dampening the induced antibody response (D. Smith et al., 1999).

A possible implication this repeated vaccination scenario is that the BeHIVE-65 study presents an almost worst-case scenario to observe the true extent

of the effects of psycho-behavioural influences on influenza vaccination responses. Yet, despite this, significant relationships (particularly in relation to explicit positive affect) were evident. It is reasonable to argue that the influence of explicit positive affect would likely have been considerably greater in a 'more typical' influenza vaccination year – where at least some of the vaccine strains are different. Further research is warranted to explore this possibility in more detail.

6.5.3 Representativeness of the Sample

A further overarching consideration in how generalisable the findings presented above are concerns how representative the participants in the BeHIVE-65 study were of the older adult population. Due to the voluntary nature of participation in health research, recruiting truly representative samples is a common problem across research areas especially in older adults (Carter, Elward, Malmgren, Martin, & Larson, 1991; Leinonen, Koponen, & Hartikainen, 2015). It is at best challenging, and at worst an unrealistic goal. In the present study, for many demographic characteristics (including age, gender, marital status, and education) the sample recruited showed considerable diversity. However, the sample was almost exclusively Caucasian, which may limit the generalisability of the findings to other ethnic groups. This is an important limitation to acknowledge, because previous research has demonstrated race/ethnicity based differences in immune responses to some vaccinations (Haralambieva et al., 2013). Representative recruitment of individuals from minority ethnic groups is a common challenge for healthcare researchers (Yancey, Ortega, & Kumanyika, 2006), and while many strategies to improve this situation has been suggested (Waheed, Woodham, Hughes-Morley, Allen, & Bower, 2015) there is little evidence that these are effective (UyBico, Pavel, & Gross, 2007). That being

said, no specific measures were put in place in the present study to address the possibility of under-representation of particular demographic or social groups – which, in hindsight, may have been advisable.

Examination of behavioural characteristics over the diary period would suggest the present sample was representative in this regard where comparisons were possible - falling within established normative ranges for physical activity performance and sleep. However, for psychological functioning, the sample diverged from previously-described normative values, notably reporting low levels of perceived stress and negative affect. The cause of this may be the intensive nature of the present study, with those who are more stressed or exhibiting low mood less likely to commit their time to participating in this research. While speculative, it has been discussed throughout this chapter how these low levels of 'negative' psychological experiences in the present sample may explain some divergence in findings from previous studies relating to these variables.

6.6 *Chapter Summary*

This chapter has presented findings from the first phase of a prospective longitudinal observational study exploring the influence of multiple psycho-behavioural factors on influenza vaccination responses in older adults. It was found that explicit positive affect in the two weeks prior to, and four weeks following vaccination was associated with short-term H1N1 antibody responses. This was not true for any other demographic or clinical factor measured – indicating the potential importance of explicit positive affect in altering immune dynamics and influencing vaccination responses. There was also suggestive evidence that the influence of explicit positive affect was greatest during the post-vaccination period and, intriguingly, on the day of

vaccination. It was also observed that having a long-term diet sufficient in selenium was associated with poorer antibody responses across vaccine strains – although the validity of this finding was questioned given discrepancies in selenium intake estimates between food frequency and daily diary measures. In contrast to previous literature, many psychological and behavioural factors were not found to be associated with vaccination responses in older adults, although this may be partially explained by the unusual scenario in which identical influenza vaccines were received by participants 12 months apart in this study.

Chapter 7: BeHIVE-65 Study Results II: Psycho-Behavioural Influences on Long-term Influenza Vaccination Responses

Chapter Synopsis

This chapter presents results from the long-term follow-up phase of the BeHIVE-65 study, at 16 weeks post-vaccination. Specifically, this chapter aims to address the following aims:

- To explore whether long-term modifiable and non-modifiable participant characteristics predict long-term antibody responses following influenza vaccination in older adults.
- To explore whether behavioural and psychological factors in the period immediately prior to, on the day of, and following vaccination predict long-term antibody responses following influenza vaccination in older adults.
- To examine whether intra-individual variability in psychological factors among older adults predict long-term antibody responses following influenza vaccination.

The chapter concludes with a discussion of the contribution of the BeHIVE-65 study to the existing literature concerning psycho-behavioural influences on immunity and vaccination responses and the implications for developing novel interventions. Strengths and limitations of the BeHIVE-65 study are also explored.

7.1 *Background*

Long-term maintenance of vaccine-induced antibodies levels is critical for protection against influenza, as clinical infection cases are most prevalent a number of months following when vaccinations are routinely administered

(Public Health England, 2014). Despite this, relatively few studies have examined the relationship between psycho-behavioural factors and long-term antibody responses, more frequently focussing on 'peak' antibody responses at 4 weeks post-vaccination (P. Gross et al., 1996). In this chapter, using the same analytic techniques described in the previous chapter, the influence of multiple psychological and behavioural factors on longer-term antibody responses (16 weeks post-vaccination) are explored. This follow-up period is clinically significant, as it represents the most likely minimum time-period to bridge the gap between influenza vaccination administration (typically October) and peak circulation of influenza viruses among the population (typically January-March).

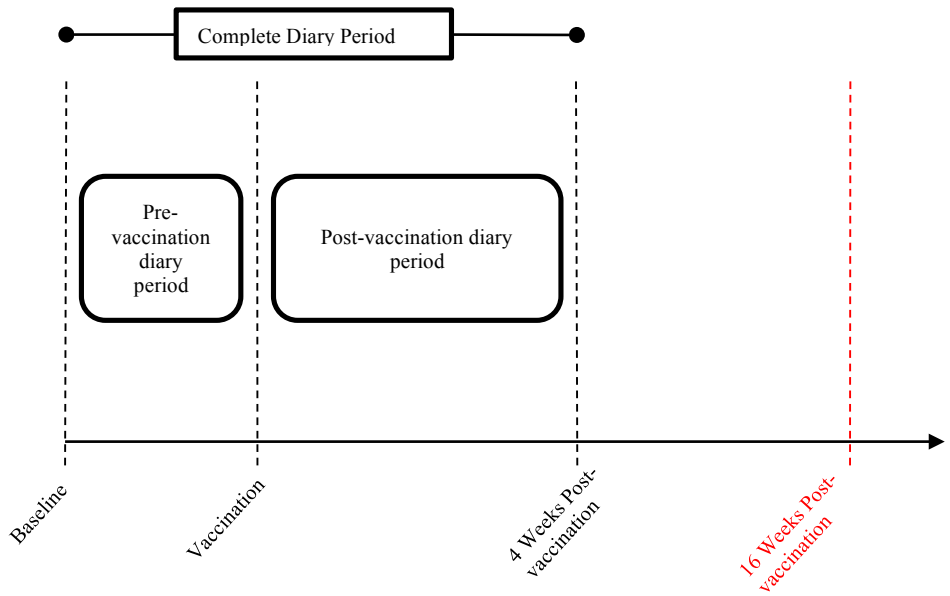


Figure 7.1: Summary diagram of BeHIVE-65 study – highlighting focus of Chapter 7

7.2 ***Results***

7.2.1 Antibody Responses to Vaccination at 16 Weeks

Table 7.1 presents mean IgG antibody levels for all antigens at baseline and 16 weeks post-vaccination. IgG antibody levels were significantly greater in all strains at 16 weeks post-vaccination than at baseline. As at 4 weeks, the lowest average absolute level and change from baseline was seen in the H1N1 strain, indicating this was the least immunogenic strain of the vaccine. It was expected that antibody levels would slightly decline between 4 and 16 weeks post-vaccination – as 4 weeks post-vaccination has previously been reported as the 'peak' time point for IgG (P. Gross et al., 1996). However, in the present study a minor increase in mean antibody levels across strains was observed between 4 and 16 weeks post-vaccination. Paired sample t-tests on log transformed antibody levels indicated no group level significant differences between antibody levels at 4 and 16 weeks post-vaccination in all strains (H1N1: $t(119)=-.969$, $p=.335$; H3N2: $t(119)= -1.731$, $p=.086$; B: $t(119)=-.287$, $p=.775$).

Table 7.1: Interpolated IgG antibody levels at baseline and 16 weeks post-vaccination

	Baseline	16 Weeks Post-Vaccination	t-statistic[+]	p-value
H1N1	1.93 (1.88)	2.37 (2.39)	-2.97	.004**
H3N2	6.96 (6.81)	9.49 (7.52)	-4.71	<.001***
B	5.18 (4.66)	6.11 (6.46)	-3.45	.001***

+Based on $\log_2$ transformed values
*p<.05, **p<.01, ***p<.001

Aim 4: To explore whether long-term modifiable and non-modifiable participant characteristics predict long-term antibody responses following influenza vaccination in older adults.

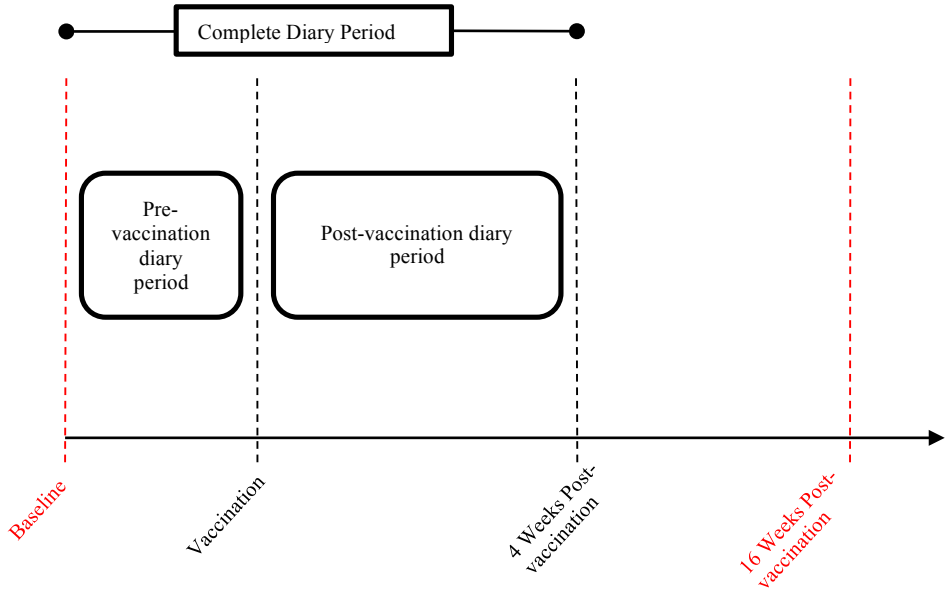


Figure 7.2: Highlighting time points under consideration for aim 4 on BeHIVE-65 study diagram

7.2.2 Research Question 4.1

Are demographic (age, gender) or clinical factors (number of self-reported illnesses, number of self-reported medications prescribed) associated with long-term influenza vaccination antibody responses in older adults?

7.2.2.1 Hypotheses

It was hypothesised that greater age, number of reported illnesses, and number of prescription medications would be associated with lower baseline-adjusted antibody levels at 16 weeks post-vaccination. No directional hypotheses were developed for gender because of mixed findings in previous research.

7.2.2.2 Analyses

Pearson's and point-biserial correlations examining the relationship between demographic and clinical factors with baseline-adjusted antibody levels at 16 weeks post-vaccination are shown in Table 7.2. Number of illnesses was found to significantly negatively correlate with baseline-adjusted H1N1 levels at 16 weeks post-vaccination. However, this finding was not replicated when examining non-adjusted antibody levels at 16 weeks (Appendix E - Table F.18). There were no significant relationships between age, gender, or number of medications with antibody levels for any strain at 16 weeks post-vaccination.

Table 7.2: Correlations (Pearson's unless otherwise stated) between demographic and clinical factors with baseline-adjusted antibody levels at 16 weeks post-vaccination

	H1N1	H3N2	B
Age	-.138	-.013	-.058
Gender[+]	.047	.140	.002
Illnesses	**-.197***	-.042	-.008
Medication	-.094	-.117	-.021

*p<.05, **p<.01, ***p<.001
[+] Point-biserial correlation. Positive correlation indicates females have greater antibody levels

7.2.2.3 Summary of Results & Comparisons to Previous Literature

As with 4-week post-vaccination antibody levels, there was no evidence within this sample of older adults that age, gender or numbers of prescribed medications were significantly related to long-term influenza vaccination antibody outcomes. There was some evidence, that a greater number of illnesses was associated with lower long-term H1N1 responses to vaccination which is in-line with predictions, given that those with greater ill-health have previously been found to have poorer responses to vaccination (P. Gross et al., 1989). Given this finding, number of illnesses (in addition to age and gender) were included in all subsequent models predicting baseline-adjusted H1N1 antibody levels at 16 weeks.

7.2.3 Research Question 4.2

Do long-term modifiable participant characteristics (habitual aerobic activity, habitual strength and flexibility activity, stress over the previous month, habitual diet, nutritional status, and BMI) predict long-term influenza antibody responses to vaccination in older adults?

7.2.3.1 Hypotheses

It was hypothesised that greater levels of habitual physical activity and lower levels of stress over the previous month would be associated with higher baseline-adjusted antibody levels at 16 weeks post-vaccination. Those who were malnourished or at risk of malnutrition in terms of nutritional status, were under or overweight in terms of BMI, or habitually reported not meeting recommended intake levels for individual dietary components (Calories, Zinc, Selenium, Vitamin A) would have lower baseline-adjusted antibody levels at 16 weeks post-vaccination compared to their counterparts.

7.2.3.2 Analyses

As described at length in the previous chapter, a two-stage analysis approach was followed to first identify relationships of interest (retaining any variable demonstrating a relationship significant at the $p<.1$ level), before using these to predict antibody outcomes after accounting for age, gender, and, in the case of baseline-adjusted H1N1 levels, number of illnesses.

Stage 1: Exploratory Analyses

Pearson's and biserial correlations (for continuous and imposed binary variables respectively) and one-way ANOVAs examining the relationships between long-term modifiable participant characteristics and baseline-

adjusted antibody levels at 16 weeks post-vaccination are presented in Table 7.3 and Table 7.4. In terms of correlational analyses, a significant relationship was observed between nutritional status and baseline-adjusted H1N1 levels at 16 weeks post-vaccination (and to a less significant extent H3N2 levels), such that those who were classified as having an adequate nutritional status had lower antibody levels than those at risk of malnutrition. These findings were not replicated when examining non-adjusted antibody levels (H1N1: r_b=-.092, p>.1; H3N2: r_b=-.052, p>.1; Appendix E - Table F.20). Possible relationships were also observed between BMI with H1N1 and H3N2 baseline-adjusted antibody levels at 16 weeks post-vaccination such that those with a healthy BMI had lower antibody levels than those who were under-or-overweight. A similar pattern of findings was true for non-adjusted antibody levels, with the relationship reaching traditional significance levels for H3N2 antibody levels (r_b=-.253, p<.05) and showing a possible association with B strain antibody levels (r_b=-.200, p<.1; Appendix E - Table F.20). The only additional non-adjusted antibody level relationship with long-term modifiable participant characteristics related to selenium and H1N1 levels, in which a trend-level negative relationship was observed (r_b=-.190, p<.1). ANOVAs revealed significant between group differences in baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination and strength and flexibility training performance. Post-hoc Tukey tests revealed those who completed flexibility training (such as stretching or yoga, at least once a week) had lower H3N2 antibody levels (p=.04) than those who did not. Engaging in flexibility training was therefore retained as a binary predictor for H3N2 antibody levels in the next stage of analysis.

Table 7.3: Correlations (Pearson's unless indicated otherwise) between long-term modifiable participant characteristics with baseline-adjusted antibody levels at 16 weeks post-vaccination

	H1N1	H3N2	B
Perceived Stress	-.096	-.130	-.070
BMI[‡]	**-.183[t]**	**-.204[t]**	-.098
Calories[+]	.044	-.061	-.080
Zinc[+]	.008	.084	-.019
Selenium[+]	-.147	-.174	-.176
Vitamin A[+]	-.170	-.048	-.160
Nutritional Status[~]	**-.349***	**-.242[t]**	-.195

[t]$p<.1$, *$p<.05$, **$p<.01$, ***$p<.001$
[‡]Biserial Correlation. Positive correlations indicate those with a healthy BMI have greater antibody levels
[+]Biserial Correlation. Positive correlations indicate those with intakes meeting recommended levels have greater antibody levels
[~]Biserial Correlation. Positive correlations indicate those with adequate nutrition have greater antibody levels

Table 7.4: One-way ANOVAs for categorical long-term modifiable participant characteristics on baseline-adjusted antibody levels at 16 weeks post-vaccination

	H1N1			H3N2			B		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Aerobic	4,115	1.444	.224	4,115	0.418	.795	4,115	1.197	.316
Strength and Flexibility	2,117	1.259	.288	**2,117**	**3.441**	**.035***	2,117	1.578	.211

[t]$p<.1$, *$p<.05$, **$p<.01$, ***$p<.001$

Stage 2: Predicting Antibody Outcomes

H1N1 Strain

Table 7.5 presents a hierarchical multiple regression analysis to predict baseline-adjusted H1N1 antibody levels at 16 weeks post-vaccination. In step one, age, gender, and number of illnesses were entered into the model explaining 4.1% of the variance in baseline-adjusted antibody levels at 16 weeks post-vaccination, although the model was not significant. In step 2, nutritional status was added to the model, with the model remaining non-significant explaining only 1.9% additional variance. In step 3, BMI was added to the model explaining 0.1% of additional variance, with the final model remaining non-significant.

Table 7.5: Hierarchical multiple regression analysis for long-term modifiable participant characteristics predicting baseline-adjusted H1N1 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	β
(Constant)	1.183	0.959	
Age	-0.008	0.013	-.060
Gender	-0.002	0.145	-.001
Illnesses	-0.096	0.050	-.190
R^2 =.041, *F*(3,99)=1.394, *p*=.249			
Step 2			
(Constant)	1.330	0.960	
Age	-0.009	0.013	-.072
Gender	-0.004	0.145	-.002
Illnesses	-0.095	0.049	-.190
Nutritional Status	-0.360	0.256	-.139
R^2=.060, ΔR^2=.019, *F*(4,98)=1.552, *p*=.193, Δ*F*(1,98)=1.984, *p*=.162			
Step 3			
(Constant)	1.180	1.071	
Age	-0.009	0.013	-.070
Gender	0.005	0.148	.003
Illnesses	-0.100	0.051	-.198
Nutritional Status	-0.352	0.258	-.135
BMI	0.005	0.016	.033
R^2=.061, ΔR^2=.001, *F*(5,97)=1.251, *p*=.292, Δ*F*(1,97)=0.103, *p*=.748			

*p<.05, **p<.01, ***p<.001
Note: 1 multivariate outlier was excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

H3N2 Strain

Table 7.6 presents a hierarchical multiple regression analysis to predict baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination. After age and gender were added in step 1, nutritional status was added in step 2, explaining only a further 1.2% of variance with the overall model remaining non-significant. In step 3, engaging in long-term flexibility training was added, explaining an additional 6.2% of variance in H3N2 antibody levels. This was a significant improvement to the model's predictive ability. Long-term flexibility training significantly predicted baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination independently above age, gender, and nutritional status, such that those who engaged in flexibility training had lower antibody levels than those who did not. Adding BMI (step 4) did not improve the model, indeed the model containing BMI was non-significant.

Table 7.6: Hierarchical multiple regression analysis for long-term modifiable participant characteristics predicting baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	β
(Constant)	1.530	1.181	
Age	0.001	0.016	.004
Gender	0.233	0.177	.129
R^2 =.017, F(2,102)=0.864, p=.425			
Step 2			
(Constant)	1.642	1.183	
Age	0.000	0.016	-.002
Gender	0.215	0.178	.119
Nutritional Status	-0.335	0.301	-.110
R^2=.029, ΔR^2=.012, F(3,101)=0.991, p=.400, ΔF(1,101)=1.242, p=.268			
Step 3			
(Constant)	1.976	1.158	
Age	-0.003	0.016	-.019
Gender	0.232	0.173	.128
Nutritional Status	-0.424	0.294	-.139
Flexibility vs None	-0.515	0.197	**-.251***
R^2=.091, ΔR^2=.062, F(4,100)=2.488, p=.048*, ΔF(1,100)=6.808, p=.010*			
Step 4			
(Constant)	1.951	1.334	
Age	-0.003	0.016	-.019
Gender	0.232	0.175	.129
Nutritional Status	-0.422	0.302	-.138
Flexibility vs None	-0.513	0.203	**-.251***
BMI	0.001	0.018	.004
R^2=.091, ΔR^2=.000, F(5,99)=1.971, p=.090, ΔF(1,99)=0.001, p=.970			

*p<.05, **p<.01, ***p<.001
Note: 1 multivariate outlier was excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

B Strain

For baseline-adjusted B antibody levels, no variables were retained from the exploratory analyses. For non-adjusted B antibody levels, only BMI was retained as a possible predictor from exploratory analyses. Hierarchical multiple regression analysis revealed BMI did not significantly predict B antibody levels at 16 weeks post-vaccination beyond age and gender (Appendix E - Table F.24).

7.2.3.3 Summary of Results & Comparisons to Previous Literature

Exploratory analyses revealed several surprising 'trend-level' associations between long-term modifiable patient characteristics and long-term influenza antibody outcomes. Specifically, the findings that having adequate nutritional status (as measured by the MNA), and having a healthy BMI were associated with poorer outcomes were against predictions and at odds with some previous findings (Hara et al., 2005; Talbot et al., 2012). However, for these variables relationships with antibody levels were not independently significant beyond age, gender, and number of illnesses suggesting they were not important predictors of post-vaccination antibody responses.

One surprising finding that remained significant was that those participants who reported regularly engaging in flexibility training had poorer H3N2 antibody responses and post-vaccination protection than those that did not. Woods et al. (2009) previously reported older adults who engaged in a regular 10-month long flexibility training program had poorer antibody responses to influenza protection at 24 weeks post-vaccination, compared to those who engaged in a cardiovascular training program. However, the authors concluded that this effect was driven by improvements within the cardiovascular training group rather than any detrimental effects of flexibility training. From a mechanistic perspective, it does not seem plausible that engaging in flexibility training could causally have detrimental effects on vaccination responses. A more likely explanation of the observed negative relationship between flexibility training and antibody responses may relate to the underlying cause behind why some older adults choose to regularly engage in flexibility training. Flexibility training has been shown to benefit range of motion, and may improve functional and daily living outcomes among the elderly (Stathokostas, Little, Vandervoort, & Paterson, 2012). Difficulties in these areas are associated with increased frailty (Al Snih et al.,

2009). It may therefore be that those actively seek to participate in flexibility training do so because they are already experiencing increased frailty or other ill-health symptoms – and that it is this that accounts for the relationship observed in the present study. Unfortunately, the present study did not include measures of frailty or activities of daily living limitations that can be used to directly test this hypothesis. Future research may benefit from greater consideration of these issues.

As with 4-week post-vaccination outcomes, against predictions we did not find an association between perceived stress over the previous month and 16-week post-vaccination antibody levels, although correlations were in the predicted directions. While previous studies have demonstrated associations between long-term perceived stress and influenza vaccination responses (A. Pedersen et al., 2009) these studies have typically been conducted in highly stressed populations (e.g., spousal carers). In the present study, the comparatively small and non-significant association observed may be explained by the low levels of long-term stress reported by participants in this study.

Aim 5: To explore whether behavioural and psychological factors in the period immediately prior to, on the day of, and following vaccination predict long-term antibody responses following influenza vaccination in older adults.

7.2.4 Research Question 5.1

Which behavioural (physical activity, diet, sleep duration, and sleep efficiency) or psychological (explicit positive affect, explicit negative affect, and perceived stress) factors as measured during the complete diary period predict influenza vaccination long-term antibody outcomes in older adults?

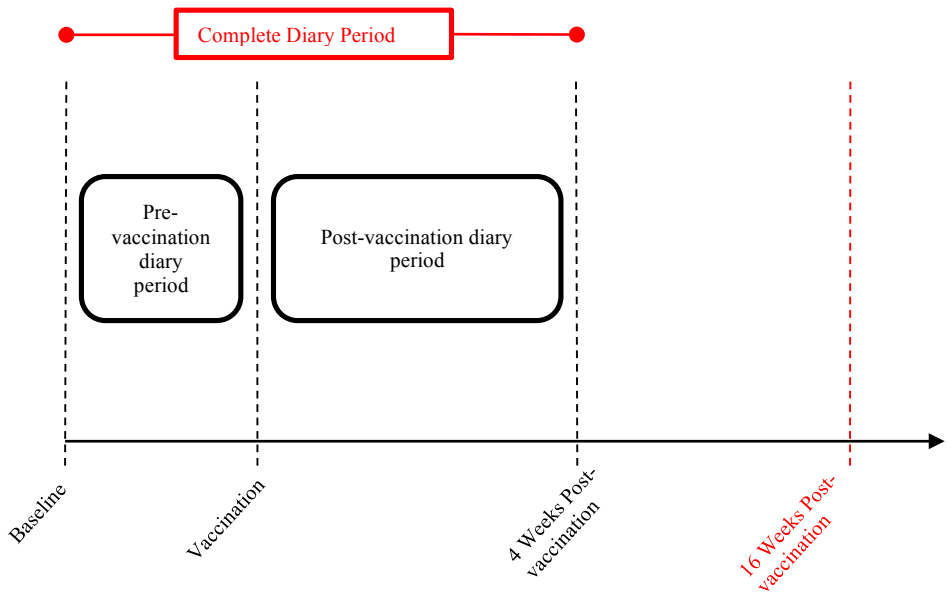


Figure 7.3: Highlighting time points under consideration for research question 5.1 on BeHIVE-65 study diagram

7.2.4.1 Hypotheses

As per 4-week post-vaccination outcomes, it was hypothesised that greater levels of physical activity, sleep duration, sleep efficiency, and explicit positive affect across the diary period, would be associated with higher baseline-adjusted antibody levels at 16 weeks post-vaccination. In contrast,

it was hypothesised that greater levels of explicit negative affect and perceived stress would be associated with lower baseline-adjusted antibody levels at 16 weeks post-vaccination. For dietary factors it was hypothesised that those who did not meet recommended intake levels during this period would have lower baseline-adjusted antibody levels at 16 weeks post-vaccination compared to those who met recommended intake levels.

7.2.4.2 Analyses

This research question was addressed using the same two-stage approach (exploratory correlation analyses followed by hierarchical multiple regressions) as previous analyses using LGM derived intercept variables for behavioural and psychological factors for the complete diary period as predictors (see 6.2.4).

Stage 1: Exploratory Analyses

Correlations between behavioural and psychological factors measured over the complete diary period with baseline-adjusted antibody levels at 16 weeks post-vaccination are shown in Table 7.7. For baseline-adjusted H1N1 antibody levels, a significant small-to-moderate sized correlation was observed with explicit positive affect such that those with higher levels of explicit positive affect had greater antibody levels. Possible associations (significant at the $p<.1$ level) with physical activity and perceived stress were also observed with greater physical activity and lower perceived stress associated with greater antibody levels. For non-adjusted H1N1 levels (Appendix E - Table F.25) the same pattern of associations was observed with the exception that at the $p<.1$ level, physical activity was not significant, but explicit negative affect was negatively associated with

antibody levels (*rho*=-.164, $p<.1$) and the association with perceived stress reached traditional criteria for statistical significance (r=-.224, $p<.05$).

For baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination, a significant negative relationship was observed with explicit negative affect. This finding was also true for non-adjusted H3N2 antibody levels, with perceived stress (r=-.199, $p<.05$) and meeting recommended vitamin E intake (r_b=-.306, $p<.01$) also showing significant associations (Appendix E - Table F.25).

No significant relationships between B strain antibody levels at 16 weeks post-vaccination and any psychological or behavioural factor measured during the complete diary period were observed.

Table 7.7: Correlations (Pearson's unless indicated otherwise) between baseline-adjusted antibody levels at 16 weeks post-vaccination and psycho-behavioural factors over the complete diary period

	H1N1	H3N2	B
Explicit Positive Affect	**.269****	.064	.115
Explicit Negative Affect^{+}	-.140	**-.243****	-.145
Perceived Stress	**-.157^{t}**	-.136	-.076
Physical Activity	**.160^{t}**	.038	.037
Sleep Duration	-.078	-.054	-.092
Sleep Efficiency	-.078	-.104	-.069
Calories$^{\sim}$	.078	.059	.142
Zinc$^{\sim}$	.109	-.039	.064
Vitamin E$^{\sim}$	.025	-.081	.087

$^{t}p<.1$, $*p<.05$, $**p<.01$, $***p<.001$

$^{+}$indicates non-parametric correlation calculated (Spearman's rho)

$^{\sim}$ Biserial correlation. Positive correlations indicate those with intakes meeting recommended levels have greater antibody levels.

Stage 2: Predicting antibody outcomes

H1N1 Strain

Table 7.8 presents a hierarchical multiple linear regression analyses that was conducted to explore whether explicit positive affect, physical activity, and perceived stress over the complete diary period predicted baseline-adjusted antibody levels at 16 weeks post-vaccination independently of age, gender, and number of illnesses. In step one, age, gender, and number of illnesses explained 4% of the variance in H1N1 antibody levels, although the model was not significant. In step two, explicit positive affect was added as a predictor, explaining an additional 8.5% of variance. This was a strong, significant improvement to the model's predictive ability. Greater explicit positive affect was associated with higher baseline-adjusted H1N1 antibody levels independent of other variables entered into the model. In step three, physical activity was added to the model, explaining an additional 2.2% of variance – although this was not a significant improvement nor an independent predictor. Finally, in step 4 perceived stress across the complete diary period was added to the model, again failing to improve the predictive ability of the model.

Table 7.8: Hierarchical multiple regression analysis for baseline-adjusted H1N1 antibody levels at 16 weeks post-vaccination and psycho-behavioural factors over the complete diary period

Step 1	B	SE B	*β*
(Constant)	1.213	1.028	
Age	-0.008	0.014	-.059
Gender	-0.018	0.152	-.012
Illnesses	-0.101	0.054	-.190
R^2 =.040, *F*(3,94)=1.292, *p*=.282			
Step 2			
(Constant)	0.217	1.041	
Age	-0.009	0.013	-.065
Gender	-0.042	0.146	-.028
Illnesses	-0.074	0.052	-.140
Explicit Positive Affect	0.155	0.052	**.296****
R^2=.125, ΔR^2=.085, *F*(4,93)=3.308, *p*=.014*, Δ*F*(1,93)=9.022, *p*=.003**			
Step 3			
(Constant)	-1.050	1.326	
Age	0.004	0.016	.027
Gender	-0.066	0.146	-.044
Illnesses	-0.066	0.052	-.124
Explicit Positive Affect	0.162	0.052	**.309****
Physical Activity	0.050	0.033	.175
R^2=.146, ΔR^2=.022, *F*(5,92)=3.150, *p*=.011*, Δ*F*(1,92)=2.331, *p*=.130			
Step 4			
(Constant)	-1.022	1.357	
Age	0.004	0.016	.028
Gender	-0.066	0.147	-.044
Illnesses	-0.065	0.053	-.122
Explicit Positive Affect	0.158	0.062	**.302***
Physical Activity	0.051	0.033	.177
Perceived Stress	-0.004	0.040	-.013
R^2=.146, ΔR^2=.000, *F*(6,91)=2.599, *p*=.023*, Δ*F*(1,91)=0.012, *p*=.912			

*p<.05, **p<.01, ***p<.001

Note: 1 multivariate outlier was excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

H3N2 Strain

Table 7.9 presents a hierarchical multiple linear regression analysis examining whether explicit negative affect independently predicts baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination. When explicit negative affect was added into the model at step two, it was found not to be a significant independent predictor explaining 2.7% of additional variance above age and gender, which was not a significant improvement to the model. The equivalent analysis for non-adjusted antibody levels, which also

included vitamin E intake and perceived stress, found the only significant independent predictor of H3N2 antibody levels at 16 weeks post-vaccination above age and gender was vitamin E intake – such that those who on average met their recommended intake levels for vitamin E had lower antibody levels (Appendix E - Table F.27).

Table 7.9: Hierarchical multiple regression analysis for baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination and psycho-behavioural factors over the complete diary period

Step 1	B	SE B	*β*
(Constant)	1.955	1.171	
Age	-0.005	0.016	-.033
Gender	0.264	0.177	.144
R^2 =.022, *F*(2,105)=1.173, *p*=.313			
Step 2			
(Constant)	3.143	1.349	
Age	-0.007	0.016	-.044
Gender	0.257	0.175	.140
Explicit Negative Affect	-0.466	0.270	-.165
R^2=.049, ΔR^2=.027, *F*(3,104)=1.789, *p*=.154, Δ*F*(1,104)=2.978, *p*=.087			

*p<.05, **p<.01, ***p<.001
Note: 5 multivariate outliers were excluded from this analysis based on having a Mahalanobis distance above the critical χ^2 threshold.

B Strain

No variables were carried through from the exploratory analysis stage.

7.2.4.3 Summary of Results & Comparisons to Previous Literature

As with 4-week post-vaccination outcomes, explicit positive affect was found to be the only significant independent psycho-behavioural predictor of baseline-adjusted antibody levels (a measure of vaccine-induced response), but only in the least immunogenic strain (H1N1) of the vaccine. This relationship was also observed when examining non-adjusted antibodies (a proxy measure of post-vaccination protection). Notably, the relationship between explicit positive affect and baseline-adjusted H1N1 antibody levels was stronger for long-term than short-term antibody levels. This suggests that positive affect may play a role in the maintenance of the serum antibody

level – which is of importance given the aim of the seasonal influenza vaccination to boost protection during 'flu season' which typically occurs around 2-4 months after vaccines are most frequently administered. The effect size and proportion of variance in 16-week post-vaccination H1N1 antibody levels explained by explicit positive affect is not trivial, especially when considering the size of the population who receive annual influenza vaccinations. Indeed, the correlation effect size (r=.27 which corresponds to a Cohen's *d*=.56) observed was larger than many commonly prescribed medications such as statins for preventing major cardiovascular events (*d*= .15) and aspirin for preventing vascular disease (*d*=.12) (Leucht et al., 2015). Further, as outlined in 6.5.2, the present study is possibly a 'worse-case-scenario' to fully observe the effects of explicit positive affect on influenza vaccination response, given the high baseline antibody levels observed due to previous receipt of the same vaccine strains. In a more typical year, where at least some strains are changed from the previous year, the influence of explicit positive affect on antibody responses could be stronger. Indeed, this may explain why many previously observed relationships for other psychological and behavioural factors were not replicated in the present study.

In considering non-adjusted levels of H3N2 antibodies 16-weeks post-vaccination, it was surprising to observe lower antibody levels in those having adequate vitamin E intake over the complete diary period compared to those who did not. This finding was in the opposite direction to predictions and contrasts with a large body of evidence demonstrating the importance of vitamin E in retaining optimal immune function, particularly in the elderly (Meydani, Han, & Wu, 2005; Pae et al., 2012; Pekmezci, 2011). An explanation for this counter-intuitive finding may relate to how 'adequate vitamin E intake' was defined in the present study. Based on

recommendations set forward by the British Dietetic Association (Gandy, 2014), we used an intake of 8mg/day for men, and 6mg/day for women as a cut-off for achieving recommended intake. However, unlike most other nutritional factors examined in this thesis, it is ambiguous as to whether this is the most appropriate cut-off point. NHS guidelines recommend a lower cut-off of 4mg/day for men and 3mg/women (NHS Choices, 2015) whereas in the US, recommendations are considerably higher at 15mg/day (Otten, Hellwig, & Meyers, 2006). Further complicating this picture is that vitamin E uptake is known to be influenced by polyunsaturated fat intake (Harris & Embree, 1963), resulting in calls for vitamin E recommended intakes to be defined in relation to this. The present study used the higher UK recommendations but did not take account of polyunsaturated fat intake levels (as consensus guidelines as to how to achieve this are, as yet, not available). Post-hoc analyses showed that if either of these cut-offs had been used or vitamin E had been considered as a continuous predictor, no significant relationships between vitamin E intake and H3N2 antibody levels would have been found. Based on these factors, it is not reasonable to conclude that reducing vitamin E intake would improve antibody responses to vaccination – as the finding is likely an artefact of how vitamin E intake was defined in this study.

7.2.5 Research Question 5.2

Are behavioural or psychological influences greater during the pre- or post-vaccination period, as compared to the complete diary period?

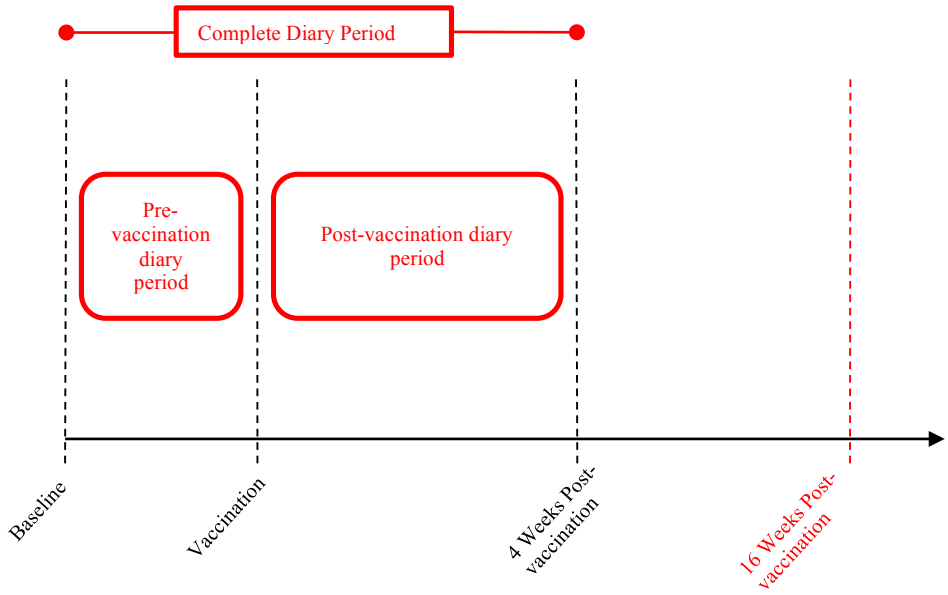


Figure 7.4: Highlighting time points under consideration for research question 5.2 on BeHIVE-65 study diagram

7.2.5.1 Hypotheses

It was hypothesised that behavioural and psychological factors during the post-vaccination period would be more predictive of baseline-adjusted antibody levels at 16 weeks post-vaccination than those factors measured pre-vaccination. This is because it is during this period that the vaccine is interacting with the immune system, therefore psycho-behavioural influences on immune dynamics may have greater consequences.

7.2.5.2 Analyses

To address this question, LGMs (as described in 6.2.4) for each behavioural and psychological factor were calculated separately using only diary responses at time points prior to vaccination and post-vaccination respectively. Correlations were then calculated between intercepts derived

from these models and baseline-adjusted antibody levels at 16 weeks post-vaccination. Effect sizes were then compared across time points with factors showing significant, or close to significant ($p<.1$), correlations being entered into multiple regression models with age and gender (as previously described) with the relative predictive strengths of the models (r^2) and their included variables compared.

Stage 1: Exploratory Analyses

Correlations between baseline-adjusted 16 week post-vaccination antibody responses with behavioural and psychological factors measured over the pre-vaccination, post-vaccination, and complete diary periods are presented in Table 7.10. Considering first pre-vaccination measures, significant relationships were observed between explicit positive affect with baseline-adjusted H1N1 levels (positive correlation) and explicit negative affect with baseline-adjusted H3N2 levels (negative correlation). Possible positive associations (trend-level) were also observed between pre-vaccination measures of physical activity with baseline-adjusted H1N1 levels and meeting recommended calorie intake with baseline-adjusted H3N2 & B levels.

For equivalent analyses on non-adjusted antibody levels (Table F.28), the same relationship was observed between pre-vaccination measures of explicit positive affect and H1N1 antibody levels and additionally at a trend level for B antibody levels ($r=.159$, $p<.1$). A negative relationship between explicit negative affect during the pre-vaccination period and non-adjusted H3N2 levels was only evident at the trend level ($rho=-.174$, $p<.1$). No relationships between non-adjusted antibody levels at 16 weeks were observed for physical activity or calories, but there was a significant negative

correlation between non-adjusted H1N1 levels and perceived stress (r=-.193, p<.05) and a trend level association for H3N2 levels (r=-.169, p<.1).

Considering post-vaccination measures, a significant positive relationship existed between explicit positive affect during the post-vaccination period and baseline-adjusted H1N1 levels at 16 weeks post-vaccination. This correlation was larger than during the pre-vaccination period or measures taken during the complete diary period as a whole. A significant negative correlation was also observed between explicit negative affect and baseline-adjusted H3N2 antibody levels, although this relationship was marginally weaker than during the pre-vaccination or complete diary periods. For non-adjusted antibody levels (Table F.29), the same pattern of results was observed. However, in addition, a significant negative relationship was observed between meeting recommended intake in vitamin E intake during the post-vaccination period and H3N2 antibody levels (r_b=-.329, p<.01) and a trend level association with H1N1 levels(r_b=-.190, p<.1). Further, trend level negative associations between explicit negative affect (r_b=-.178, p<.1) and perceived stress (r_b=-.179, p<.1) with non-adjusted H1N1 levels at 16 weeks were evident.

Table 7.10: Correlations (Pearson's unless indicated otherwise) between psycho-behavioural factors as measured during the pre-vaccination, post-vaccination, and complete diary period with baseline-adjusted antibody levels at 16 weeks post-vaccination

	H1N1			H3N2			B		
	Pre	Post	Complete	Pre	Post	Complete	Pre	Post	Complete
Explicit Positive Affect	**.256****	**.301****	**.269****	.073	.099	.064	.125	.139	.115
Explicit Negative Affect[+]	-.119	-.149	-.140	**-.229***	**-.202***	**-.243****	-.125	-.118	-.145
Perceived Stress	-.135	-.137	**-.157**[t]	-.127	-.060	-.136	-.052	-.046	-.076
Physical Activity	**.175**[t]	.140	**.160**[t]	-.030	.030	.038	-.062	.016	.037
Sleep Duration	-.015	-.103	-.078	-.030	-.068	-.054	-.062	-.081	-.092
Sleep Efficiency	-.003	-.103	-.078	-.059	-.068	-.104	-.031	-.081	-.069
Calories[~]	.144	-.033	.078	**.222**[t]	-.088	.059	**.232**[t]	.031	.142
Zinc[~]	.028	.055	.109	-.028	.083	-.039	.079	.062	.064
Vitamin E[~]	.093	-.073	.025	.014	-.124	-.081	.058	.039	.087

[t]$p<.1$, *$p<.05$, **$p<.01$, ***$p<.001$

[+] Non-parametric correlation (Spearman's rho)

[~] Biserial correlation. Positive correlations indicate those with intakes meeting recommended levels have greater antibody levels

Pre= Pre-vaccination period (from 2 weeks prior to vaccination to point of vaccination); Post= Post-vaccination period (from point of vaccination to 4 weeks after vaccination); Complete= Complete diary period (from 2 weeks prior to vaccination to 4 weeks after vaccination).

Stage 2: Predicting antibody outcomes

H1N1 Strain (Pre-Vaccination Period)

For baseline-adjusted H1N1 levels, explicit positive affect and physical activity performance over the pre-vaccination period were found to be significant independent predictors, above the effects of age, gender and number of diagnosed illnesses (Table 7.11). The final model explained 14% of the variance in 16 week post-vaccination baseline-adjusted H1N1 antibody levels, which was marginally lower than the proportion of variance explained by those same variables over the complete diary period (14.6%; Table 7.8). For equivalent analyses on non-adjusted antibody levels at 16 weeks post-vaccination, explicit positive affect was the only psycho-behavioural factor measured over the pre-vaccination period to be a significant independent predictor for H1N1 (Appendix E - Table F.30). The proportion of variance explained by the most parsimonious model (step 2) was marginally greater than the same model using measures during the complete diary period (12.2% vs 10.8%).

Table 7.11: Hierarchical multiple regression analysis for pre-vaccination measurements of psycho-behavioural factors predicting baseline-adjusted H1N1 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	0.602	1.015	
Age	0.000	0.014	.003
Gender	-0.077	0.151	-.052
Illnesses	-0.085	0.053	-.165
R^2 =.030, *F*(3,92)=0.948, *p*=.421			
Step 2			
(Constant)	-0.367	1.057	
Age	0.001	0.013	.007
Gender	-0.097	0.147	-.066
Illnesses	-0.053	0.053	-.104
Explicit Positive Affect	0.138	0.054	**.261***
R^2=.094, ΔR^2=.064, *F*(4,91)=2.367, *p*=.059, Δ*F*(1,91)=6.455, *p*=.013*			
Step 3			
(Constant)	-2.080	1.301	
Age	0.017	0.015	.128
Gender	-0.146	0.146	-.099
Illnesses	-0.029	0.053	-.057
Explicit Positive Affect	0.148	0.053	**.282****
Physical Activity	0.070	0.032	**.251***
R^2=.140, ΔR^2=.045, *F*(5,90)=2.922, *p*=.017*, Δ*F*(1,90)=4.752, *p*=.032*			

*p<.05, **p<.01, ***p<.001

H3N2 Strain (Pre-Vaccination Period)

For baseline-adjusted H3N2 antibody levels, explicit negative affect and calories over the pre-vaccination period were not found to be significant independent predictors above age and gender.

Table 7.12: Hierarchical multiple regression analysis for pre-vaccination measurements of psycho-behavioural factors predicting baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.428	1.202	
Age	0.002	0.016	.010
Gender	0.269	0.180	.145
R^2 =.021, *F*(2,103)=1.112, *p*=.333			
Step 2			
(Constant)	2.060	1.294	
Age	0.004	0.016	.021
Gender	0.230	0.182	.124
Explicit Negative Affect	-0.330	0.256	-.128
R^2=.037, ΔR^2=.016, *F*(3,102)=1.300, *p*=.278, Δ*F*(1,102)=1.662, *p*=.200			
Step 3			
(Constant)	1.838	1.310	
Age	0.005	0.016	.032
Gender	0.170	0.191	.092
Explicit Negative Affect	-0.302	0.257	-.117
Calories	0.243	0.228	.110
R^2=.048, ΔR^2=.011, *F*(4,101)=1.262, *p*=.290, Δ*F*(1,101)=1.140, *p*=..288			

*p<.05, **p<.01, ***p<.001
Note: 4 multivariate outliers were excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

B Strain (Pre-Vaccination Period)

For baseline-adjusted B antibody levels, calories over the pre-vaccination period was not found to be a significant independent predictor above age and gender.

Table 7.13: Hierarchical multiple regression analysis for pre-vaccination measurements of psycho-behavioural factors predicting baseline-adjusted B antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.621	1.078	
Age	-0.006	0.015	-.037
Gender	0.055	0.161	.032
R^2 =.002, *F*(2,110)=0.136, *p*=.873			
Step 2			
(Constant)	1.426	1.075	
Age	-0.004	0.015	-.023
Gender	-0.035	0.168	-.021
Calories	0.350	0.205	.170
R^2=.028, ΔR^2=.026, *F*(3,109)=1.065, *p*=.367, Δ*F*(1,109)=2.917, *p*=.091			

*p<.05, **p<.01, ***p<.001

H1N1 Strain (Post-Vaccination Period)

Table 7.14 presents a hierarchical multiple regression analyses for psycho-behavioural predictors of baseline-adjusted H1N1 levels at 16 weeks post-

vaccination. Explicit positive affect was found to independently predict baseline-adjusted H1N1 levels above the effects of age, gender and number of illnesses (9.1% additional variance explained). The final model explained 13.1% of variance, a marginally higher proportion than the same variables measured over the pre-vaccination (9.4%) or complete diary periods (12.5%).

Table 7.14: Hierarchical multiple regression analysis for post-vaccination measurements of psycho-behavioural factors predicting baseline-adjusted H1N1 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	β
(Constant)	1.212	1.033	
Age	-0.008	0.014	-.059
Gender	-0.014	0.153	-.009
Illnesses	-0.102	0.054	-.192
R^2 =.040, F(3,93)=1.298, p=.280			
Step 2			
(Constant)	0.195	1.041	
Age	-0.009	0.013	-.066
Gender	-0.029	0.147	-.019
Illnesses	-0.075	0.052	-.141
Explicit Positive Affect	0.158	0.051	**.306****
R^2=.131, ΔR^2=.091, F(4,92)=3.472, p=.011*, ΔF(1,92)=9.632, p=.003**			

*p<.05, **p<.01, ***p<.001

H3N2 Strain (Post-Vaccination Period)

Table 7.15 shows that post-vaccination measures of explicit negative affect did not significantly predict baseline-adjusted H3N2 antibody levels above age and gender. For equivalent analyses on non-adjusted antibody levels at 16 weeks post-vaccination (in which meeting recommended Vitamin E intake was first entered into the model), meeting recommended vitamin E intake over the post-vaccination period was a significant independent predictor of non-adjusted H3N2 responses above age and gender, such that those who met vitamin E intake had lower H3N2 levels. This model explained 11.8% of the variance in non-adjusted H3N2 antibody levels at 16 weeks post-vaccination. Adding explicit negative affect did not significantly improve this model (Appendix E - Table F.34).

Table 7.15: Hierarchical multiple regression analysis for post-vaccination measurements of psycho-behavioural factors predicting baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	β
(Constant)	1.901	1.193	
Age	-0.005	0.016	-.032
Gender	0.301	0.178	.166
R^2 =.028, F(2,102)=1.487, p=.231			
Step 2			
(Constant)	2.938	1.322	
Age	-0.006	0.016	-.035
Gender	0.300	0.176	.165
Explicit Negative Affect	-0.446	0.255	-.169
R^2=.057, ΔR^2=.029, F(3,101)=2.032, p=.114, ΔF(1,101)=3.062, p=.083			

*p<.05, **p<.01, ***p<.001
Note: 3 multivariate outliers were excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

B Strain (Post-Vaccination Period)

No variables were carried through from the exploratory analysis stage.

7.2.5.3 Summary of Results & Comparisons to Previous Literature

As with short-term antibody responses there was very little evidence for differential associations between psycho-behavioural factors measured pre-vaccination, post-vaccination, or across both time periods for long-term antibody responses. Notably, differences in correlations, in all cases, were marginal. No previous studies have explicitly addressed this issue of timing. Based on these, there is no clear blanket answer to the question of whether behavioural or psychological influences on long-term antibody responses following vaccination are greater in the pre- or post-vaccination period. The analyses conducted suggest that if seeking to improve long-term (as opposed to short-term) antibody responses using a psycho-behavioural intervention, there is little reason to favour pre- or post-vaccination based on possible improved efficacy.

7.2.6 Research Question 5.3

Does explicit positive affect and negative affect on the day of vaccination of predict long-term antibody levels at 16 weeks post-vaccination in older adults?

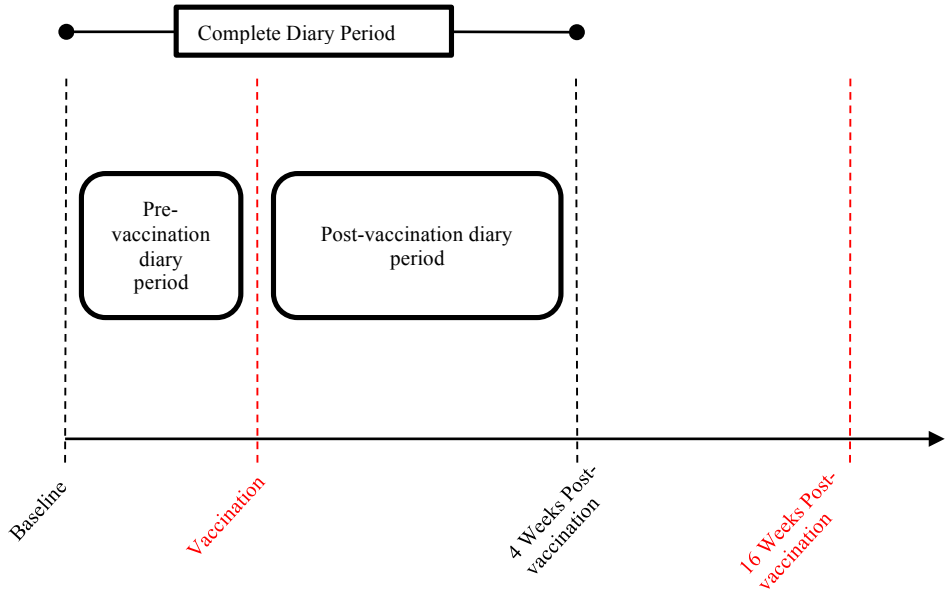


Figure 7.5: Highlighting time points under consideration for research question 5.3 on BeHIVE-65 study diagram

7.2.6.1 Hypotheses

Interventions manipulating psychological factors on the day of vaccination have previously been shown to influence immune responses (Edwards, Burns, Allen, et al., 2007). In line with this, it was hypothesised that greater explicit positive affect and lower negative affect on the day of vaccination would be associated with baseline-adjusted antibody levels at 16 weeks post-vaccination.

7.2.6.2 Analyses

Stage 1: Exploratory Analyses

Following the two-stage analysis approach of above sections, correlations between measures of explicit positive (Pearson's) and negative affect (Spearman's rho) at the point of vaccination and baseline-adjusted antibody levels at 16 weeks post-vaccination are shown in Table 7.16. Higher levels of explicit positive affect on the day of vaccination was strongly associated with greater baseline-adjusted H1N1 levels at 16 weeks post-vaccination with a moderate-to-strong effect size. In addition, explicit positive affect on the day of vaccination was associated at the trend level ($p<.1$) with greater B antibody responses. Explicit negative affect on the day of vaccination was negatively associated at the trend level ($p<.1$) with baseline-adjusted H3N2 levels at 16 weeks post-vaccination.

Table 7.16: Correlations (Pearson's unless indicated otherwise) between cross-sectional measures of affect with baseline-adjusted antibody levels at 16 weeks post-vaccination

	H1N1	H3N2	B
Explicit Positive Affect	**.350*** **	.096	**.187^{t}**
Explicit Negative Affect^{+}	-.102	**-.188^{t}**	-.097

$^{t}p<.1$, $*p<.05$, $**p<.01$, $***p<.001$
$^{+}$indicates non-parametric correlation calculated (Spearman's rho)

Stage 2: Predicting Antibody Outcomes

H1N1 Strain

Table 7.17 presents a hierarchical multiple regression analysis to examine whether explicit positive affect on the day of vaccination independently predicted baseline-adjusted H1N1 levels at 16 weeks post-vaccination above age, gender and number of illnesses. When explicit positive affect on the day of vaccination was added to the model (step 2) it was found to be a significant independent predictor above other variables in the model, explaining an additional 11.1% of variance in 16 week post-vaccination baseline-adjusted H1N1 antibody levels. The direction of the relationship indicates that those participants with greater explicit positive affect on the day of vaccination had greater H1N1 antibody responses at 16 weeks post-

vaccination. The final model explained 13.6% of variance, marginally higher than when explicit positive affect over the complete (12.5%), pre-vaccination (9.4%), post-vaccination (13.1%) periods were examined.

For equivalent analyses on non-adjusted antibody outcomes, both explicit positive affect and explicit negative affect on the day of vaccination were carried forward from stage 1 as possible predictors of non-adjusted H1N1 levels at 16 weeks post-vaccination. Explicit positive affect on the day of vaccination was found to be a strong predictor of non-adjusted H1N1 levels, explaining 13% of additional variance above age and gender (Appendix E - Table F.36). Adding explicit negative affect on the day of vaccination to the model did not significantly improve the model further.

Table 7.17: Hierarchical multiple regression analysis for psychological factors on the day of vaccination predicting baseline-adjusted H1N1 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	β
(Constant)	0.825	1.042	
Age	-0.003	0.014	-.022
Gender	-0.044	0.151	-.030
Illnesses	-0.081	0.054	-.152
R^2 =.024, F(3,93)=0.777, p=.510			
Step 2			
(Constant)	-0.900	1.106	
Age	0.003	0.014	.019
Gender	-0.105	0.144	-.071
Illnesses	-0.036	0.053	-.068
Explicit Positive Affect	0.070	0.020	**.348*** **
R^2=.136, ΔR^2=.111, F(4,92)=3.618, p=.009, ΔF(1,92)=11.867, p=.001*** **			

*p<.05, **p<.01, ***p<.001

H3N2 Strain

Table 7.18 presents hierarchical multiple regression analysis examining whether explicit negative affect on the day of vaccination independently predicted baseline-adjusted H3N2 levels at 16 weeks post-vaccination above age and gender. When added to the model, explicit negative affect on the day of vaccination was found to be a significant independent predictor, explaining an additional 3.8% of variance. However, the final model still had poor predictive ability, remaining non-significant.

Table 7.18: Hierarchical multiple regression analysis for psychological factors on the day of vaccination predicting baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.998	1.244	
Age	-0.007	0.017	-.038
Gender	0.301	0.182	.164
R^2 =.028, *F*(2,99)=1.434, *p*=.243			
Step 2			
(Constant)	2.950	1.315	
Age	-0.006	0.017	-.033
Gender	0.229	0.183	.124
Explicit Negative Affect	-0.173	0.086	**-.199***
R^2=.066, ΔR^2=.038, *F*(3,98)=2.322, *p*=.080, Δ*F*(1,98)=4.009, *p*=.048*			

*p<.05, **p<.01, ***p<.001
Note: 8 multivariate outliers were excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

B Strain

Table 7.19 presents hierarchical multiple regression analysis examining whether explicit positive affect on the day of vaccination independently predicted baseline-adjusted B levels at 16 weeks post-vaccination above age and gender. Explicit positive affect on the day of vaccination was found to just be above criteria for statistical significance (p=.054), although even with this included the predictive ability of the model was poor and non-significant.

Table 7.19: Hierarchical multiple regression analysis for psychological factors on the day of vaccination predicting baseline-adjusted B antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.396	1.112	
Age	-0.003	0.015	-.016
Gender	0.023	0.164	.014
R^2 =.000, *F*(2,106)=0.023, *p*=.977			
Step 2			
(Constant)	0.378	1.215	
Age	0.003	0.015	.016
Gender	-0.015	0.163	-.009
Explicit Positive Affect	-0.038	0.20	.191
R^2=.035, ΔR^2=.035, *F*(3,105)=1.281, *p*=.285, Δ*F*(1,105)=3.796, *p*=.054			

*p<.05, **p<.01, ***p<.001

7.2.6.3 Summary of Results & Comparisons to Previous Literature

On the day of vaccination, participants reporting greater explicit positive affect had greater long-term H1N1 antibody responses. Importantly, explicit positive affect on the day of vaccination appeared to be a better predictor of long-term H1N1 responses than explicit positive affect levels over the longer diary period. However, these factors are strongly correlated (r= .803) – with those having greater positive affect over the six-week diary period more likely to have greater positive affect on the day of vaccination. Therefore, the analyses presented should be considered as *preliminary* evidence supporting the hypothesis that positive affect on the day of vaccination influences vaccination responses. Long-term H3N2 responses also appear to be related to explicit negative affect on the day of vaccination, although the strength of this relationship was weak-to-moderate. Together, these findings further strengthen the argument presented in the previous chapter that the day of vaccination may be a particularly salient time at which affect can impact on vaccine outcomes. It follows that, interventions to increase positive affect delivered on the day of vaccination, may enhance older adults responses to the vaccination, thus conferring additional protection. This is discussed further in section 7.3.

The above findings are congruent with evidence reviewed in Chapter 3 that demonstrated psycho-behavioural interventions delivered close to vaccination (primarily in relation to exercise and stress) can impact on immunity and more specifically vaccination responses (Edwards et al., 2006; Edwards, Burns, Allen, et al., 2007). However, an alternative explanation for these results are that explicit affect was measured more accurately on the day of vaccination than over the diary period. On the day of vaccination, participants completed full (10 item) versions of the I-PANAS-SF, whereas throughout the diary period participants regularly completed randomly selected items from the scale (4 items per response day). Therefore, it may be that the shorter measure gave a less complete assessment of affect. However, the validity of this explanation is limited given that participants responded to different items from the I-PANAS-SF, repeatedly, on up to 18 occasions – arguably giving a much more accurate assessment of affect than a single time-point measure. As a result, it seems reasonable to conclude that the findings reflect a meaningful relationship between affect on the day of vaccination and long-term antibody responses.

Aim 6: To examine whether intra-individual variability in psychological factors among older adults predict long-term antibody responses following influenza vaccination

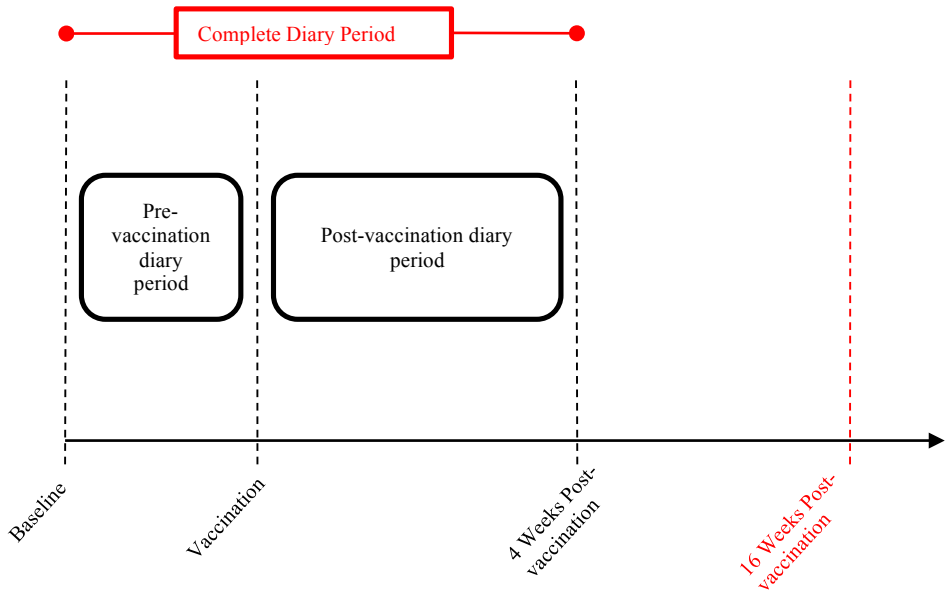


Figure 7.6: Highlighting time points under consideration for aim 6 on BeHIVE-65 study diagram

7.2.7 Research Question 6.1

Does intra-individual variability in psychological factors predict long-term antibody responses?

7.2.7.1 Hypotheses

In line with evidence demonstrating intra-individual variability in psychological factors is associated with poorer physical health (Chan et al., 2016; Hardy & Segerstrom, 2016), it was hypothesised that greater intra-individual variability in explicit positive affect, explicit negative affect, and perceived stress would be associated baseline-adjusted antibody levels at 16 weeks post-vaccination.

7.2.7.2 Analyses

Bivariate Correlations for intra-individual variability and baseline-adjusted antibody levels at 16 weeks post-vaccination are presented in Table 7.20. Significant correlations were observed for intra-individual variability in perceived stress and baseline-adjusted antibody levels at 16 weeks in all strains and for explicit positive affect variability and baseline-adjusted H1N1 levels. These relationships were of a weak-to-moderate negative magnitude, such that greater variability in perceived stress and explicit positive affect over the complete diary period were associated with lower antibody levels. For non-adjusted antibody levels, the same pattern of results was found for perceived stress, although the relationship with B-strain antibody levels at 16 weeks post-vaccination did not meet traditional statistical criteria. No relationships were found between intra-individual variability in explicit positive and negative affect in any strain (Table F.37).

Table 7.20: Correlations (Pearson's unless indicated otherwise) between intra-individual variability and baseline-adjusted antibody levels at 16 weeks post-vaccination

	H1N1	H3N2	B
Explicit Positive Affect	**-.206***	-.025	-.114
Explicit Negative Affect	.004	.050	.086
Perceived Stress	**-.273****	**-.214***	**-.258***

$^{t}p<.1$, $*p<.05$, $**p<.01$, $***p<.001$

H1N1 Strain

Table 7.21 presents a hierarchical multiple regression analysis exploring whether intra-individual variability in explicit positive affect and perceived stress predict baseline-adjusted H1N1 antibody levels at 16 weeks post-vaccination above previously identified non-modifiable predictors and average levels of those factors over the complete diary period. In step 2, intra-individual variability in perceived stress was added to the model explaining an additional 7.5% of variance above that explained by age,

gender, and number of illnesses. This was a statistically significant improvement to the model. Adding intra-individual variability in explicit positive affect over the complete diary period (Step 3) did not significantly improve the model. When average levels (LGM-intercepts) of perceived stress and explicit positive affect over the complete diary period were added (Step 4), intra-individual variability in perceived stress and average explicit positive affect over the complete diary period were found to be the only significant independent predictors of baseline-adjusted H1N1 antibody levels at 16 weeks post-vaccination.

Table 7.21: Hierarchical multiple regression analysis for intra-individual variability of psychological factors predicting baseline-adjusted H1N1 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.009	1.094	
Age	-0.005	0.015	-.041
Gender	0.012	0.165	.008
Illnesses	-0.093	0.057	-.174
R^2 =.032, *F*(3,85)=0.923, *p*=.433			
Step 2			
(Constant)	1.201	1.060	
Age	-0.007	0.014	-.049
Gender	-0.028	0.160	-.018
Illnesses	-0.094	0.055	-.176
IIV Perceived Stress	-0.244	0.092	**-.276****
R^2=.107, ΔR^2=.075, *F*(4,84)=2.515, *p*=.048*, Δ*F*(1,84)=7.091, *p*=.009**			
Step 3			
(Constant)	1.257	1.057	
Age	-0.006	0.014	-.040
Gender	-0.029	0.159	-.019
Illnesses	-0.107	0.056	-.200
IIV Perceived Stress	-0.225	0.093	**-.264***
IIV Explicit Positive Affect	-0.216	0.173	-.133
R^2=.123, ΔR^2=.016, *F*(5,83)=2.337, *p*=.049*, Δ*F*(1,83)=1.558, *p*=.215			
Step 4			
(Constant)	0.194	1.112	
Age	-0.010	0.014	-.070
Gender	-0.091	0.153	-.046
Illnesses	-0.090	0.055	-.168
IIV Perceived Stress	-0.226	0.089	**-.256***
IIV Explicit Positive Affect	-0.144	0.170	-.088
Perceived Stress (LGM-Intercept)	0.017	0.042	.049
Explicit Positive Affect (LGM-Intercept)	0.196	0.069	**.332****
R^2=.215, ΔR^2=.092, *F*(7,81)=3.168, *p*=.005, Δ*F*(2,81)=4.722, *p*=.011***			

**p*<.05, ***p*<.01, ****p*<.001

Note: 1 multivariate outlier was excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

H3N2 Strain

Table 7.22 presents a hierarchical multiple regression analysis examining whether intra-individual variability in perceived stress predicted baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination above age, gender, and average levels of perceived stress over the complete diary period. The analysis shows that when intra-individual variability in perceived stress is added to the model with age and gender alone, it is a significant independent predictor – explaining an additional 4% of variance in H3N2 antibody levels. However, the predictive ability of the model as a whole is poor and non-significant. When average levels of perceived stress are added neither variable is independently significant with the overall model remaining non-significant.

Table 7.22: Hierarchical multiple regression analysis for intra-individual variability of psychological factors predicting baseline-adjusted H3N2 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.334	1.227	
Age	0.002	0.017	.015
Gender	0.337	0.188	.181
R^2 =.033, *F*(2,95)=1.609, *p*=.206			
Step 2			
(Constant)	1.436	1.209	
Age	0.002	0.016	.015
Gender	0.305	0.186	.164
IIV Perceived Stress	-0.217	0.108	**-.200***
R^2=.073, ΔR^2=.040, *F*(3,94)=2.450, *p*=.068, Δ*F*(1,94)=4.031, *p*=.048*			
Step 3			
(Constant)	1.632	1.227	
Age	0.002	0.016	.011
Gender	0.296	0.186	.159
IIV Perceived Stress	-0.205	0.109	-.188
Perceived Stress (LGM-Intercept)	-0.041	0.043	-.095
R^2=.081, ΔR^2=.009, *F*(4,93)=2.059, *p*=.092, Δ*F*(1,93)=2.059, *p*=.347			

*p<.05, **p<.01, ***p<.001

B Strain

Table 7.23 presents the same analysis but for baseline-adjusted B antibody levels at 16 weeks post-vaccination. It shows that intra-individual variability

in perceived stress is a significant independent predictor, above and beyond age and gender, explaining 6.5% additional variance in B antibody levels at 16 weeks post-vaccination. When average perceived stress was added to the model, intra-individual variability in perceived stress remained independently significant. However, the model did not improve with average perceived stress added, and the final model remained non-significant indicating that it is a poor predictive model.

Table 7.23: Hierarchical multiple regression analysis for intra-individual variability of psychological factors predicting baseline-adjusted B antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.428	1.183	
Age	-0.003	0.016	-.021
Gender	0.077	0.181	.044
R^2 =.002, *F*(2,95)=0.117, *p*=.889			
Step 2			
(Constant)	1.553	1.151	
Age	-0.003	0.016	-.021
Gender	0.038	0.177	.022
IIV Perceived Stress	-0.264	0.103	**-.256***
R^2=.068, ΔR^2=.065, *F*(3,94)=2.274, *p*=.085, Δ*F*(1,94)=6.573, *p*=.012*			
Step 3			
(Constant)	1.570	1.174	
Age	-0.003	0.016	-.022
Gender	0.037	0.178	.021
IIV Perceived Stress	-0.263	0.104	**-.255***
Perceived Stress (LGM-Intercept)	-0.004	0.041	-.009
R^2=.068, ΔR^2=.000, *F*(4,93)=1.689, *p*=.159, Δ*F*(1,93)=0.007, *p*=.931			

*p<.05, **p<.01, ***p<.001

7.2.7.3 Summary of Results & Comparisons to Previous Literature

In contrast to findings for short-term antibody response, the above results demonstrate that greater variability in an individual's perception of stress over the period surrounding influenza vaccination was found to associated with poorer long-term antibody responses for all strains of the vaccine. This relationship was independent of overall perceptions of stress for H1N1 and B strains (the first and second least immunogenic strains of the vaccine),

but not the H3N2 strain (the most immunogenic strain). Variation in explicit positive and negative affect around vaccination were not found to be important determinants of long-term antibody responses. In the case of explicit negative affect, this may relate to the relatively low levels of variability seen across the sample. For explicit positive affect, as has been seen throughout this chapter, average levels around vaccination appear to be an important predictive factor in antibody responses to vaccination, but not variability over time. Together, these findings add further weight to calls emphasising the importance of studying individual-variability in psychological factors, as well as mean levels, to understand physical health (Chan et al., 2016).

Previous research has illustrated that greater intra-individual variability in psychological factors is associated with poorer physical health (Chan et al., 2016; Hardy & Segerstrom, 2016). The above findings are highly novel in that they show, for the first time, this relationship also extends to immunological function (as assessed by vaccination responses), providing a possible insight into the mechanisms behind how intra-individual variability might influence more broad physical health complaints. Exactly how greater variability in perceived stress impacts on the immune system remains to be elucidated, however one possible explanation is that high-levels of stress fluctuation acts much like chronic stress creating an over-activation of allostatic systems that ultimately leads to 'wear-and-tear' (McEwen, 1998). As discussed at length in section 3.3.1, chronic stress is strongly associated with marked deleterious effects on innate and adaptive immunity, morbidity, and mortality. Further research is needed to explore the extent to which variability in perceived stress is similarly problematic.

7.3 ***Discussion***

This chapter has presented results from the second phase of the BeHIVE-65 study, examining the influence of multiple psycho-behavioural influences on long-term antibody responses to influenza vaccination in older adults. Below is a brief re-cap of the key findings presented above and the implications for future research. Following this, the contribution of the BeHIVE-65 study to understanding psychological and behavioural influences on vaccination responses in older adults and limitations of the study are discussed.

7.3.1 Re-cap of Key Findings & Implications for Future Research

The first aim addressed in this chapter related to whether long-term modifiable and non-modifiable participant characteristics predicted long-term influenza vaccination antibody outcomes in older adults. In line with predictions, it was found that greater ill-health, as measured by number of self-reported illnesses, was associated with poorer long-term H1N1 responses to vaccination. This suggests that interventions to enhance antibody responses in older adult could be most beneficial for those exhibiting the greatest ill-health. In addition, there was also the unexpected finding that participants who habitually engaged in flexibility training (such as stretching or yoga, once a week or more) had poorer long-term H3N2 responses than those who did not. It was argued that this finding may be best explained by the underlying confounder of frailty – with frailer individuals more likely to be recommended flexibility training, but also have greater ill-health.

The second aim concerned the influence of behavioural and psychological factors in the period immediately prior to, following, and on the day of vaccination in predicting long-term antibody responses following influenza

vaccination in older adults. It was reliably found, across all time periods considered, that greater explicit positive affect was associated with greater long-term vaccine-induced increases in H1N1 antibodies and absolute antibody levels. Extending from the findings reported in Chapter 6, this means that those older adults with the greatest positive mood around influenza vaccination were most likely to respond robustly to the vaccination and have greater levels of protection in both the short- and long-term after vaccination. Collectively these findings indicate novel interventions targeting improvements in explicit positive affect could improve weak vaccine responses in older adults.

There was also evidence that explicit negative affect negatively correlated with long-term H3N2 antibody responses and absolute levels. While hierarchical multiple regression analyses found this effect may not be significant once age and gender were accounted for – these analyses must be treated with caution because of the significant skew in explicit negative affect score that can violate the assumptions of these parametric analyses. Given the robust findings for positive affect and inherent relationship with explicit negative affect, this further strengthens the case for explicit positive mood interventions to enhance vaccine responses in older adults – as such interventions may also decrease negative affect simultaneously.

With regards the time periods at which psycho-behavioural factors have the greatest influence on vaccine responses in older adults, there was little evidence to support differential strengths of influence on long-term antibody responses between the pre- and post-vaccination period. However, as with short-term antibody outcomes, there was good evidence that explicit positive affect on the day of vaccination was a better predictor of long-term H1N1 antibody responses than explicit positive affect over the complete

diary period, or the pre- and post-vaccination periods alone. This points to the possible utility of brief, positive mood enhancing interventions that could be delivered on the day of vaccination as a method of improving poor immune responses to vaccination in older adults. The following chapter presents a systematic scoping review of such interventions and their relationship to immunity, to aid in the development of a novel mood enhancing intervention for older adults to improve vaccination outcomes, suitable for use in or around primary care.

The final aim of this chapter was to examine whether intra-individual variability in psychological factors among older adults predict long-term antibody responses following influenza vaccination. In contrast to findings on short-term antibody responses where no such predictive relationships were observed, it was found that greater variability in the perception of stress over the complete diary period was found to be associated with poorer long-term antibody responses for all strains of the vaccine. These effects were independent of mean perceived stress levels for 2 out of 3 strains. This study is the first to explore intra-individual variability as a predictor of vaccine-responses – although it is receiving increased attention in relation to being a negative influence on health outcomes more generally (e.g., Chan et al., 2016; Hardy & Segerstrom, 2016). The findings presented here point to the possible benefit of interventions designed to reduce stress variability as a method of enhancing vaccine outcomes in older adults. However, to date, little research has examined how to reduce variability in psychological states. Mindfulness training may be one potential avenue of interest in this regard, as individuals found to have greater levels of dispositional mindfulness have been found to exhibit less variability in psychological factors and arousal (Rau, 2016). However, additional research into how intra-individual variability might impact on immunity, particularly in older

adults, is warranted before concluding that such interventions are a 'best-bet' for improving vaccine outcomes. More broadly these findings highlight the need researchers to provide greater prominence to intra-individual variability in psychological factors that can be explored in tandem with average levels.

7.3.2 Contributions & Limitations

The BeHIVE-65 study included many novel features that contribute to a greater understanding of how psychological and behavioural factors influence vaccination responses in older adults. First, this study is the first to explicitly examine and compare the contribution of affect, perceived stress, diet, physical activity and sleep to vaccination in a single study. This side-by-side comparison identified that, on balance, explicit positive affect was the most influential of these factors on influenza vaccination responses in older adults, and is therefore a good target for novel interventions.

Second, the BeHIVE-65 study is one of the first in this area to explicitly address the issue of when psychological and behavioural factors might have the greatest influence on vaccines, highlighting the day of vaccination as a particularly salient time-point at which psychological factors (particularly positive affect) may influence vaccination responses. As behavioural factors were not measured on the day of vaccination, it remains an open question if these too are particularly salient at this time. Importantly, this finding points to the potential benefit of brief interventions – which are more practical than many longer interventions that have previously been trialled in this area.

Third, the BeHIVE-65 study adopted a diary-based longitudinal design addressing a number of limitations of previous studies which over-relied on single time point retrospective measures of psychological and behavioural factors which are subject to recall biases. Here, the extensive use of repeated, momentary measures of psychological factors minimised such biases and allowed for the exploration of intra-individual variability, something not possible in most previous studies. Throughout the previous two Chapters, advantages of the repeated measurement approach has been evident. For example, differences were observed in habitual compared to daily diary measures of various nutritional intakes – indicating that accurately reporting such factors based on long-term recall is subject to significant error. Further, we found intriguing findings that variability in perceived stress, but not average levels in perceived stress, predicted long-term antibody outcomes. This issue was only examinable because of the repeated-measures design employed. It is hoped these findings will inspire greater interest and use of similar research designs and methodologies - reducing the over-representation of studies employing single time-point retrospective measures.

Finally, as described in Chapter 5, antibody outcomes were assessed in this study using a newly developed antigen microarray assay that boasts significant improvements over traditionally used assays in terms of reliability, reproducibility, speed, and reduced consumables costs. The use of this assay in the present study demonstrates its practicality for increasingly large studies in this area, where existing assays have often prevented such studies due to their high costs and labour requirements.

There are however, a number of limitations of the BeHIVE-65 study that are worthy of discussion. First and foremost amongst these is a limitation that

comes with any observational study: the inability to establish causality. While explicit positive affect was repeatedly found to be related to, and predict H1N1 antibody responses following influenza vaccination; on the strength of this study we cannot conclude that this relationship is not explained by an unmeasured confounding variable. That being said, previous experimental work has demonstrated immunological changes as a result of positive affect induction – suggesting a causal link (Hewson-Bower & Drummond, 1996; Hucklebridge et al., 2000; Knapp et al., 1992). However, such studies have not included vaccination as an outcome and therefore additional research, ideally in the form of a randomised controlled trial, would establish whether manipulating positive affect can improve vaccine responses.

A further limitation of note is that the sample size of the present study (n=138) was smaller than originally sought (n=200) possibly limiting its power to identify smaller effect sizes as significant. The primary reason for the lower than anticipated recruitment was an issue with invitations reaching eligible participants, as one practice incorrectly ran their searches and sent invites to participants who should have been excluded. By the time this issue was identified and new invitations sent out, most potential participants had already received the 2014/15 influenza vaccination, meaning very few participants were recruited from this relatively large site. Despite this issue, the BeHIVE-65 study is still one of the largest observational studies conducted in this area, with most previous studies (as described in Chapter 3) having less than 100 participants. Further, the low attrition rate achieved (<3% at 4 weeks post-vaccination) and relatively low missing data levels means a greater degree of confidence can be placed in the findings.

Another limitation of the present study is that it was unable to address whether implicit affect influenced vaccine responses, or whether this had differential effects compared to explicit affect. It was hypothesised in 4.2.5 that implicit affect may be particularly influential on vaccination responses based on previous research that demonstrated implicit measures of affect predicted cortisol regulation where explicit measures did not (Mossink et al., 2015; Quirin, Kazén, Rohrmann, et al., 2009). Unfortunately, the measure of implicit affect used in this study (IPANAT) was poorly received by the older adult participants in this study, with responses to the measure ranging from confusion to annoyance. Ultimately, this measure suffered from such substantial missing data that it could not be considered reliable. Previous researchers have not reported similar issues with using the IPANAT in longitudinal studies (e.g., Massey, 2015) although these were not conducted in older adults. Alternative methods of assessing implicit affect exist, including word-stem completion (Egloff, Weck, & Schmukle, 2008) and implicit association tasks (DeWall & Baumeister, 2007) which may be viewed more positively by this population.

A final important limitation of the BeHIVE-65 study is that it did not comprehensively explore interactions between the behavioural and psychological factors measured. Throughout this thesis these factors have been discussed separately, however there is no doubt that they are closely entwined. To give a few examples from prior literature: exercise can lead to a reduction in perceived stress and negative affect (Scully, Kremer, Meade, Graham, & Dudgeon, 1998); sleep problems are associated with lower levels of positive affect (Steptoe, O'Donnell, Marmot, & Wardle, 2008) and dietary changes, prolonged negative affect and sleep disturbance are all criteria for a diagnosis of depression (American Psychiatric Association, 2000). In the present study, multiple significant correlations between psychological and

behavioural measures were observed (see Table 6.5). Together, the behavioural and psychological factors considered in the BeHIVE-65 study are part of a complex and dynamic system that have the potential to impact on immune function and antibody responses to vaccination.

The reasons for not addressing interaction effects (including moderation and mediation) in this thesis is two-fold. First, there is little theoretical basis on which to select certain interactions for examination over others. The biopsychosocial model points to bi-directional interactions between these variables, meaning a huge number of possible interactions could be considered. Exploring all of these would have been impractical and vastly inflated the chances of type 1 errors. Second, to adequately examine interaction effects requires considerably larger sample sizes than needed for main effects. Here, given lower than anticipated recruitment (discussed above) the sample size of the present study was insufficient to justify the detailed exploration of interaction effects. Epidemiological research, based on a large UK-wide sample, would be better placed to explore these issues.

7.4 *Chapter Summary*

This chapter has presented findings from the follow-up phase of a prospective longitudinal observational study exploring the influence of multiple psycho-behavioural factors on influenza vaccination responses in older adults. It was found that explicit positive affect in the two weeks prior to, and four weeks following vaccination predicted long-term H1N1 antibody responses. There was also suggestive evidence that the influence of explicit positive affect was greatest during the post-vaccination period and, intriguingly, on the day of vaccination. Together, this points to brief, positive mood enhancing interventions delivered on the day of vaccination as the

most appropriate target for a psycho-behavioural intervention to enhance vaccine outcomes in older adults. In a highly novel finding, variability in perceived stress was also found to predict long-term antibody responses to two of three vaccine strains independent of mean perceived stress levels, highlighting the benefit of using longitudinal repeated measures designs and indicating need for additional research on this topic.

Chapter 8: Brief Positive Affect Interventions & Immunity: A Systematic Scoping Review

Chapter Synopsis

Building on findings from the longitudinal observational study, that positive affect on the day of vaccination predicted antibody responses to influenza vaccination in older adults - this chapter presents a systematic scoping review of brief mood enhancing interventions and their effects on immunity. This, in turn, will inform the development of novel positive affect interventions for older adults designed to enhance vaccination outcomes. Inclusion in the review was limited to studies reporting a single session intervention/manipulation demonstrating an improvement in mood and measuring some aspect of immunity. Twenty-eight articles reporting 38 mood-enhancing interventions were identified. While overall study quality was low, the vast majority (>80%) of interventions reported changes to some aspect of immunity following mood enhancement. The utility of different intervention strategies employed and their suitability for use in or around primary care are discussed.

8.1 *Background*

The longitudinal observational cohort study, described in the previous chapters, identified positive affect as the most suitable psycho-behavioural target for a non-pharmacological intervention to improve influenza vaccine responses in older adults. In the weakest immunogenic strain of the vaccine (H1N1), positive affect showed the strongest relationship with both short- and long-term antibody responses. Further, there was suggestive evidence that positive affect on the day of vaccination was more predictive of antibody

responses than positive affect over the 2 weeks prior to, and 4 weeks following vaccination. This, when considered in light of previous research findings that psycho-behavioural factors immediately prior to immune challenge may be particularly salient (Dhabhar, 2002; Dhabhar & Viswanathan, 2005; Edwards, Burns, Carroll, et al., 2007), suggests improving positive affect immediately prior to vaccination may enhance influenza vaccination responses in older adults.

To begin developing a novel intervention to enhance vaccine effectiveness in older adults, it is important to consider the context in which such an intervention would need to be delivered. Vaccines are predominately delivered in primary care settings, typically GP surgeries. While little empirical data exists relating to vaccine administration practices, it is common for surgeries to administer the majority of annual influenza vaccinations in specific time-slots, where patients either drop-in or are assigned an appointment to attend. These sessions can involve the vaccination of hundreds of patients, with many patients in the GP surgery for only a matter of minutes. Multi-session, lengthy or staff-intensive interventions are therefore unlikely to be of particular utility or be deemed suitable for adoption in this context. Therefore, for a novel intervention to demonstrate utility and acceptability it would be required to be brief, single-session, and potentially self-deliverable.

Medical Research Council guidance on complex intervention development notes that identifying existing literature, ideally in the form of a systematic review, represents a critical early step in the intervention development process (Craig et al., 2008). This allows researchers to learn from what has been done previously and identify features of previous interventions that may increase efficacy or practicality of a new intervention. In line with this

guidance, this chapter presents a systematic scoping review of the existing literature on brief, positive affect enhancing interventions on immunity. A systematic scoping review is methodologically very similar to a traditional systematic review with a view key differences. Where a systematic review seeks to definitively answer a very specific and relatively narrow question, a systematic scoping review is more preliminary and exploratory in nature. Typically, the aims of scoping reviews are to assess the size, quality or general nature of prior research on a topic. This can be particularly useful in relatively nascent or diverse areas of research or where many study designs are employed. Scoping reviews can inform future research by providing a broad qualitative synthesis of knowledge in that area, highlighting knowledge gaps, methodological issues and populations in which there is a paucity of research. In addition, scoping reviews are comparatively more pragmatic than traditional systematic reviews and are most appropriate where time or resources are limited. This is because traditional systematic reviews often go to great lengths to be exhaustive (e.g., searching grey literature, hand searching specific journals or reference lists) and require multiple researchers duplicating efforts to ensure completeness. In contrast, a scoping review is comparatively rapid, and can be completed by a single researcher.

Given the inevitable entanglement of the psychological concepts positive affect, negative affect and stress – the current review takes a broad approach, focusing on the effects of 'mood enhancing' interventions on immunity. The primary aims of this review were to (1) identify the size and nature of the existing literature base, (2) to assess the quality of this literature (3) to identify the types of interventions that both increase positive mood and show evidence of enhanced immunity and (4) gauge their appropriateness for use in primary care.

8.2 ***Methods***

8.2.1 Search Strategy

Systematic electronic searches were conducted of EMBASE, PsycINFO, PsycARTICLES, and MEDLINE from each databases earliest records to 14th August 2016. A comprehensive overview of the search terms employed are presented in Appendix G. In brief, the searches included synonym terms relating to (1) intervention/manipulation (e.g., *modulat*, induc*)* (2) mood (e.g., *emotion*, happ*),* and (3) immunity (e.g., *immun*, cytokine*).* Medical subheadings (MeSH) were used when possible. Hand-searches of previous reviews of potential relevance (Brod, Rattazzi, Piras, & D'Acquisto, 2014; Chanda & Levitin, 2013; Denson, Spanovic, & Miller, 2009; Gangrade, 2011; Gick, 2011; Gruzelier, 2002; Martin, 2001) were also conducted to identify additional articles not picked up by the electronic searches.

8.2.2 Inclusion and Exclusion criteria

Studies were included where they appeared in a peer-reviewed journal presenting primary research reporting a single session intervention/manipulation demonstrating an improvement in mood and measuring some aspect of immunity. To be included, immune measures had to be conducted for both pre- and post-intervention or compared to a suitable control group. Studies which did not include a sufficient manipulation check (i.e., demonstrate an improvement in mood/positive affect because of the intervention) were excluded. A small number of studies did not include a true manipulation check but asked participants to report on the nature of the intervention (e.g., rate the funniness of a comedy clip). Given the scoping nature of this review, these studies were included. Studies

focusing exclusively on endocrine outcomes (e.g., cortisol), animal studies, and conference proceedings were excluded. For pragmatic reasons, inclusion was limited to English language articles.

8.2.3 Quality Assessment

Study quality was assessed by the Effective Public Health Practice Project (EPHPP) quality assessment tool for quantitative studies (Thomas, Ciliska, Dobbins, & Micucci, 2004). This tool (see Appendix H) is designed to assess quality for any quantitative study design and assesses six primary domains: selection bias, study design, confounders, blinding, data collection method, and withdrawals/dropouts. Each of these domains is graded individually as strong, moderate, or weak which are then combined into an overall global rating. A study receives a global rating of 'Strong' if none of the six individual domains are graded as weak, 'Moderate' if only one is coded as weak, and 'Weak' if two or more domains are assessed as weak. The EPHPP also includes items relating to intervention integrity and analyses however as these are not used to assessing the global rating of study quality they were not assessed in the present review. The tool is recommended by the Cochrane Collaboration as an alternative to their own risk of bias tool that is primarily designed for randomised controlled trials only (Higgins & Green, 2011). A comprehensive guidance document for completing the EPHPP is used in conjunction with the tool to improve consistency in quality judgements.

8.3 ***Results***

8.3.1 Study Selection Process

The flow of studies through the review process is presented in Figure 8-1. After duplicates were removed, 3,704 articles were screened with 107 full-texts examined for eligibility. From these 28 articles were identified as suitable for inclusion in the review. As some of these articles presented multiple studies and crossover designs, in total 31 individual studies were included (hereafter designated 'k') reporting 38 interventions (hereafter designated 'i') that resulted in an improvement in mood. The most common reasons for exclusions at the full-text retrieval stage were not including an assessment of successful mood manipulation (n = 32) and the intervention lasting longer than a single session (n = 24).

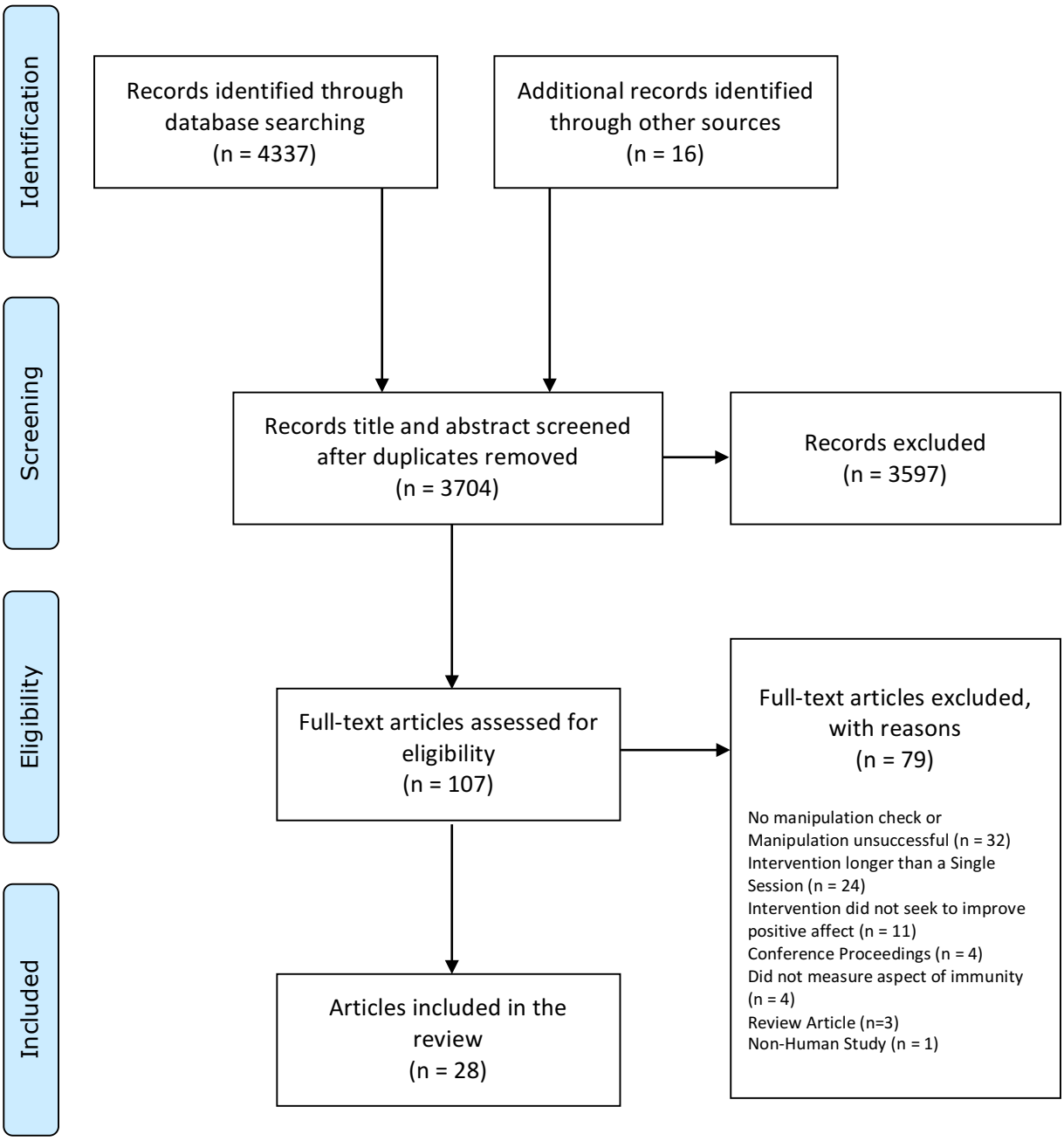


Figure 8-1: PRISMA flow diagram of study selection process

8.3.2 Studies Characteristics

The most common origin of studies was the USA (k=12), with a large number from Japan (k=9). The remaining studies were conducted in the UK (k=3), Canada (k=3), Australia (k=1), South Korea (k=1), Germany (k=1), and Israel (k=1). Most commonly studies adopted a crossover design (k=18;

58.1%), with participants receiving more than one intervention (with a gap between) in a randomised order. Eight studies included a cohort design, with participants just receiving the positive mood intervention, and six were randomised controlled trials (RCTs). Sample sizes were generally small, ranging from 5 to 123 with a mean of 33.6. Participants were most frequently adults self-selected from the general population (k=12; 38.7%) although a substantial proportion recruited from exclusively student samples (k=9; 29%). Only one study explicitly recruited older adults (Koyama et al., 2009), with one study conducted in children (Hewson-Bower & Drummond, 1996). The remaining studies recruited from more specific populations including singers, method actors, yoga practitioners, couples or patient groups with an existing health condition.

8.3.3 Intervention Characteristics

Interventions were varied, although by far the most common was a comedy film or audiotape (i=15; 39.5%). Music making in the form of group drumming (i=3) or singing (i=4) were also popular intervention forms. The remaining interventions included relaxation with or without immune suggestions (i=3), mental recall of positive autobiographical events (i=2), pleasant or memory retrieving odours (i=2), acting out an imagined positive experience (i=2), Qi therapy + rest (i=2), Yoga (i=1), watching film clips of attractive celebrities (i=1), hugging and kissing a romantic partner (i=1), and writing about self-congruencies (i=1). Interventions were most commonly group based (i=20; 52.6%) with the remaining administered individually (i=12) or in couples (i=1). No information was reported as to the nature of intervention administration in five cases. Interventions ranged from 90 seconds to 150 minutes in duration, although were typically 60 minutes or under (i=23; 60.5%).

8.3.4 Immunological Outcomes & Intervention Effects

Table 8.1 provides a brief description of the immune parameters measured in the included studies and their function. Immune parameters were measured in a variety of ways including absolute levels, percentage and ratio in comparison to other immune parameters. Further, multiple studies examined *in-vitro* responses to stimulation (e.g., with mitogen), with the stimulating agent examined at multiple effector-to-target concentrations. Most studies measured multiple immune outcomes (k=21; 67.7%). The number of immune outcomes ranged across studies from 1 to 19, with a mean of 4.7 per study. The most common immune outcome measures related to secretory IgA (S-IgA) concentration or flow rate (k=12) and NK cell count or activity (k=11). Other immune outcomes were only measured in only a few or single studies.

Table 8.1: Immune parameters measured in included studies

Immune Parameter	**Description/Function**
Albumin	Abundant protein present in serum, often used as a comparator against other immune parameters (e.g., Secretory IgA to Albumin Ratio).
B-Cells	A type of cell important in clearing extracellular pathogens. Pre-cursor to antibody secreting plasma cells.
C-reactive protein	Protein thought to assist complement binding to foreign pathogens aiding phagocytosis.
CD-3	T-cell co-receptor used as a marker for T-cell population.
CD-4	T-cell co-receptor expressed on helper T cells. Interacts with MHC on antigen-presenting cells.
CD-8	T-cell co-receptor expressed on cytotoxic T cells. Interacts with MHC on antigen-presenting cells.
CD-16	Molecule expressed on natural killer cells. Binds to IgG antibodies to activate cell-mediated cytotoxicity.
CD-19	Molecule expressed on B-cells. Promotes B-cell response to antigens.

Immune Parameter	Description/Function
CD-56	Molecule expressed on natural killer cells and natural killer T cells. Multiple functions in cell-adhesion.
CD-57	Molecule expressed on natural killer cells. Indicates cell maturation and decreased sensitivity to cytokines.
Granulocytes	A sub-group of leukocytes including neutrophils, eosinophils, and basophils. Multiple functions including infection clearance through phagocytosis and triggering inflammatory response.
IFN-γ	Cytokine involved in antiviral activity and stimulation of macrophages.
IL-1β	Cytokine involved in activation of B-cells, T-cells, and macrophages.
IL-2	Cytokine involved in proliferation and/or activation of B-cells, T-cells, and NK cells.
IL-3	Cytokine involved in production of various blood cell types, and proliferation of pluripotent stem cells.
IL-4	Cytokine involved in activation of B-cells
IL-5	Cytokine involved in activation of eosinophils
IL-6	Cytokine involved in inflammatory response, activation of B- and T-cells.
IL-10	Cytokine involved in suppression of macrophage function and activation of B cells.
Leukocytes	A broad grouping of white blood cells with an array of functions involved in the protection against infection.
Lymphocytes	A sub-type of leukocytes including B-cells and T-cells. Multiple functions in protecting against infection including secretion of antibodies, cell lysis and cytokine production.
Natural Killer Cell	A type of cell involved in clearing virally-infected cells.
Neutrophil Superoxide Anions	Produced by neutrophil cells, with toxic effects on invading pathogens.
Neutrophils	A type of cell that can ingest and destroy invading pathogens.
Secretory-IgA	Antibody involved in protection against pathogens on mucosal surfaces.
Secretory-IgE	Antibody involved in protection against parasites. Released in allergic reactions to certain stimuli (allergens).
Secretory-IgG4	Sub-type of IgG antibodies, involved in inflammatory response. Released in allergic reactions to certain stimuli (allergens).
Soluble IL-6 Receptor	Secreted form of IL-6 receptor which plays an important role in inflammatory responses.
T-cells	A type of cell important in cell mediated immunity.
TNF-α	Cytokine involved in activation of macrophages and granulocytes.

Note: CD= Cluster of Differentiation; IL= Interleukin; Ig= immunoglobulin.

Table 8.2 summarises the findings of studies included in the review. The majority of studies reported at least one significant immunological change as a result of the intervention or in the intervention arm compared to a comparator (k=27; 87.1%). Four studies reported no significant effects of the intervention on immune outcomes (Bennett, Zeller, Rosenberg, & McCann, 2003; Futterman, Kemeny, Shapiro, Polonsky, & Fahey, 1992; Labott et al., 1990; Wachi et al., 2007) although one of these did show between group differences in means but did not include appropriate significance testing (Labott et al., 1990). Of the 12 studies that measured secretory IgA, ten reported a significant increase in S-IgA concentration or flow rates as a result of the interventions (Beck, Cesario, Yousefi, & Enamoto, 2000; Dillon et al., 1985; Hewson-Bower & Drummond, 1996; Hucklebridge et al., 2000; Kreutz, Bongard, Rohrmann, Hodapp, & Grebe, 2004; Lefcourt, Davidson-Katz, & Kueneman, 1990; McClelland & Cheriff, 1997; Perera, Sabin, Nelson, & Lowe, 1998), one reported a significant pre-to-post increase in S-IgA concentration but no greater than that observed in neutral comparators (Harrison et al., 2000), and one demonstrated an increase in S-IgA concentration but did not assess the significance of the change (Labott et al., 1990).

Studies that measured some aspect of NK cell count or activity, were less consistent with five studies demonstrating increased NK cell counts or activity following the intervention (Futterman et al., 1994; Jung, Shin, Kim, Shin, & Lee, 2006; Matsunaga et al., 2008; Strauman, Woods, Schneider, Kwapil, & Coe, 2004; K. Takahashi et al., 2001) five finding no effect (Bennett et al., 2003; Futterman et al., 1992; Knapp et al., 1992; Koyama et al., 2009; Wachi et al., 2007), and one study finding no significant changes to NK cell activity from before to immediately after the intervention,

but significantly greater increases than the control group (Bittman, Berk, Felten, & Westengard, 2001).

Table 8.2: Summary of studies included in the review

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Beck et al. (2000), USA Crossover	Choral Singers 41, 46.4 (NR), 25-62, 51.6%	a) Morning Choral Singing Rehearsal, 150 mins, Group b) Afternoon Choral Singing Rehearsal, 150 mins, Group c) Choral Singing Performance, 90 mins, Group	S-IgA Concentration (1)	Note: Manipulation check consisted of asked participants about the emotions experienced during singing. Immediately following all conditions, S-IgA concentrations significantly increased from before the intervention. This increase was greatest in the performance condition (c).
Bennett et al. (2003), USA RCT	General Population 33, NR (NR), NR, 100%	Comedy film, NR, Group	NK cell cytotoxicity (1)	Note: Control condition comprised viewing a neutral film. No significant differences in NK cell cytotoxicity change between participants who viewed the comedy film compared the control.

Bittman et al. (2001) USA RCT	General Population 60; NR (NR); 48%	Drumming; 60 mins; Group	NK cell activity at effector to target ratio of 6:1; NK cell activity at effector to target ratio of 12:1; NK cell activity at effector to target ratio of 25:1; NK cell activity at effector to target ratio of 50:1; Lymphokine-activated Killer Activity with IL-2 at 12:1; Lymphokine-activated Killer Activity with IL-2 at 25:1; Lymphokine-activated Killer Activity with IFN-γ at 12:1; Lymphokine-activated Killer Activity with IFN-γ at 25:1; Baseline for IFN-γ stimulated at 12:1; Baseline for IFN-γ stimulated	Note: Control condition comprised reading quietly. Significant increases from before to immediately after intervention in Baseline for IFN-γ stimulated at 12:1, Baseline for IFN-γ stimulated at 25:1, Lymphokine-activated Killer Activity with IFN-γ at 12:1, and Lymphokine-activated Killer Activity with IFN-γ at 25:1 There were no significant changes in other immune outcomes. Compared to controls the intervention arm had significantly larger increases from before to immediately after the intervention in NK cell activity at effector to target ratio of 6:1; NK cell activity at effector to target ratio of 12:1; Baseline for IFN-γ stimulated at 12:1, Baseline for IFN-γ stimulated at 25:1, Lymphokine-activated Killer Activity with IFN-γ at 12:1, and Lymphokine-activated Killer Activity with IFN-γ at 25:1. There were no significant differences between groups in other immune outcomes.

Authors (Year of publication) Origin Study Design	Sample Type Sample Size, Mean age (SD), Range, Female%	Intervention Description, Duration, Mode of Delivery	Immune outcome(s) measured (n)	Main Findings
			at 25:1; IL-2; IFN-γ; Leukocyte count (13)	
Dillon et al. (Dillon et al., 1985), USA Crossover	Students 10, 22.9 (NR), 20-36, 60%	Comedy film, 30 mins; Group	S-IgA Concentration (1)	Note: Manipulation check consisted of rating the funniness of the film clips viewed. This study also included a didactic videotape condition. S-IgA concentration increased significantly from before to immediately following the comedy film intervention. There was no change in S-IgA concentration following the viewing a neutral control film.
Futterman et al. (1992) USA Crossover	Method Actors 5, 31.2 (NR), 25-38, 40%	Acting out a happiness scenario; <120 mins, Individual	NK Cell Activity; CD-3; CD-4; CD-8; CD-16; CD56; CD57 (7)	No significant changes in any of the immune outcomes from pre-to-post intervention.

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Futterman et al. (1994) USA Crossover with Control Group	Method Actors (General Population Controls) Actors: 16, 35 (NR), 24-47, 0% Controls: 9, 29.4 (NR), 18-43, 11%	a) Acting out a euphoric happiness scenario; <120 mins, Individual b) Acting out a relaxed happiness scenario; <120 mins, Individual	NK cell cytotoxicity; Lymphocyte response to high doses of PHA; Lymphocyte response to low doses of PHA; CD-3%; CD-4%; CD-8%; CD-16%; CD-56%; CD57% (9)	Note: This study also included two negative mood induction conditions. The controls acted as baseline comparators for immune assessments. In all induced mood states (positive and negative combined) significant increases in CD-8%, CD-16%, CD-56%, CD-57% and NK cell cytotoxicity from pre-to-post mood inductions. No significant effects on remaining immune parameters. Significant differential lymphocyte responses to high doses of PHA were observed between positive and negative mood inductions, with responses increased in both positive inductions but decreased in negative inductions. No differences in any other immune outcome. No immune outcomes changed in the neutral control group.

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Hall et al. (1992) USA Cohort	General Population 19, 51 (NR), 22-81; 36.8%	Relaxation with immune based imagery, 45 mins, Individual	Leukocyte count; lymphocytes; T-cells; B-cells; Lymphocyte response to PHA; Lymphocyte response to Con-A; Lymphocyte response to Pokeweed (7)	Significant increases in leukocyte count and lymphocyte response to Pokeweed. No significant effects on other immune outcomes.
Harrison et al. (Harrison et al., 2000) UK Crossover	Students 30, Males: 21.07 (1.83) Females: 20.93 (0.96), NR, 50%	a) Comedy film, 10 mins, NR b) Exciting film, 10 mins, NR	S-IgA concentration; S-IgA Secretion rate (2)	Note: Manipulation check involved rating of film clips. This study also included neutral film and rest conditions. All film conditions (including neutral) induced a significant increase in s-IgA secretion rate compared to resting. No significant differences between intervention type. No difference between conditions in S-IgA concentration.

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Hewson-Bower & Drummond (1996) Australia RCT	Children with and without recurrent URT infections 90, Children with URT infections: 9.4 (NR), 8-12, 44.4% Healthy Children: 9.7 (NR), 8-12, 44.6%	a) Relaxation, 25 mins, Group b) Relaxation with immune suggestions, 25 mins, Group	S-IgA concentration; S-IgA:Albumin Ratio (2)	Note: Control condition comprised group conversation. S-IgA concentration increased significantly immediately following both relaxation conditions compared to controls. No effects on S-IgA:Albumin Ratio in any condition. No differences between healthy children and those with recurrent URT infections.
Hucklebridge et al. (2000) UK Crossover	Students 19, 19.7 (NR), NR, 100%	Mental Recall and writing about happy life experience, 10 Mins, Individual	S-IgA concentration; S-IgA secretion rate (2)	Note: Manipulation check consisted of self-report of how emotional participants found the recall. This study also included a negative mood induction condition. Significant increase in S-IgA secretion rate immediately following happiness induction from pre-intervention. No effect on S-IgA concentration.

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Jung et al. (2006) South Korea RCT	General population 24, Qi touch therapy + rest group: 25 (5), NR, 0% Qi non-touch therapy + rest group: 26 (3), NR, 0%	a) Qi touch therapy + rest, 70 mins, Individual b) Qi non-touch therapy + rest, 70 mins, Individual	Superoxide anions produced by neutrophils; NK cell cytotoxicity (2)	At 10 mins following both interventions NK cytotoxicity was significantly increased from pre-intervention. At 1-hour post-intervention, this remained significant only in Qi non-touch therapy + rest condition. Superoxide anion production by neutrophils was increased in Qi non-touch therapy + rest condition at 10 minutes post-intervention from pre-intervention but not at 1 hour. No change in superoxide anion production at any time point in Qi-touch therapy + rest condition.
Kiecolt-Glaser et al. (2010) USA Crossover	Regular yoga practitioners 50, 41.32 (10.33), 30-65; 100%	Yoga, 75 mins, Group	IL-6; Soluble IL-6 receptor, TNF-α, C-reactive protein, LPS stimulated IL-6 production, LPS stimulated TNF-α production (6)	Note: This study also included movement control and passive film control conditions. Significant increase in Soluble IL-6 receptors immediately following yoga and the following morning, compared to pre-intervention. TNF-α significantly increased the following morning after yoga but not immediately after the intervention. No effects on other immune outcomes reported.

Authors (Year of publication) Origin Study Design	Sample Type Sample Size, Mean age (SD), Range, Female%	Intervention Description, Duration, Mode of Delivery	Immune outcome(s) measured (n)	Main Findings
Kimata (2004, study 1) Japan Cohort	Patients with atopic dermatitis having atopic kertoconjuctivitis and healthy general population 48; Patients: 27 (NR), 22-43 General population: 26 (NR), 20-41, 50%	Comedy Film, NR, Group	Japanese cedar pollen-specific IgE; Japanese cedar pollen-specific IgG4; Japanese cedar pollen-specific IgA (3)	In general population patients no significant effects of intervention as immune outcomes undetectable in tears. In patients with atopic dermatitis having atopic kertoconjuctivitis, there was a significant decrease in Japanese cedar pollen-specific IgE and IgG4 and significant increase in IgA immediately following and at 2 hours following the intervention compared to baseline, but not at 4 hours.
Kimata (2004, study 2) Japan Cohort	Patients with atopic dermatitis having atopic kertoconjuctivitis 15, NR (NR), NR, NR	Comedy Film, NR, Group	Japanese cedar pollen-specific IgE; Japanese cedar pollen-specific IgG4; Japanese cedar pollen-specific IgA (3)	Note: Manipulation check consisted of observer checking for laughter during the intervention. It is unclear from published manuscript whether these patients were a subset of Kimiata (2004, study 1). There was a significant decrease in IgE and IgG4 and increase in IgA immediately following the intervention but not at 2 or 4 hours post-intervention.

Authors (Year of publication) Origin Study Design	Sample Type Sample Size, Mean age (SD), Range, Female%	Intervention Description, Duration, Mode of Delivery	Immune outcome(s) measured (n)	Main Findings
Knapp et al. (1992) USA Cohort	General population 20, NR (NR), NR, 50%	Mental recall and discussion of positive life interval with interviewer, NR, Individual	Lymphocyte response to moderate dose PHA; Lymphocyte response to low dose PHA; Lymphocyte response to Con-A; Lymphocyte response to Pokeweed; NK cell activity, leukocyte count, PMN cells, lymphocytes, monocyes, CD-3%; CD-4%, CD-8% (12)	Significant increase in lymphocyte response to low dose PHA immediately following intervention. No effects on other immune outcomes.

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Koyama et al. (2009) Japan Cohort	General population young and older adults; Younger adults: 27, 27.9 (8.4), NR, 70.4% Older adults: 27,70.3 (2.9), NR, 55.6%	Drumming, 60 mins, Group	NK cell activity; leukocyte count; neutrophil count; lymphocyte count; T-cell count; B-cell count; CD-4 count; CD-8 count; CD-4:CD8 ratio; Naive T-cell count; Memory T-cell count; NK-cell count; IFN-γ production; IL-2 production; IL-4 production; IL-6 production; IL-10 production (18)	In young adults there were no significant changes to any of the immune outcomes from before to after the intervention. In older adults there were significant increases in lymphocyte count, T-cell count, CD-4 count, Memory T-cell count, IFN-γ production, and IL-6 production. There were no significant changes in any other immune outcome.
Kreutz et al. (2004) Germany Crossover	Amateur choir members 31, 56.9 (14.8), 29-74, 74.2%	Singing, 60 mins, Group	S-IgA:Albumin Ratio (1)	Note: This study also included a listening to music condition; however, manipulation check revealed this did not alter mood. S-IgA:Albumin ratio increased significantly from before to after the intervention.

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Labott et al. (1990) USA RCT with Crossover	Students who considered themselves expressive 39, 21.6 (NR), 18-40, 100%.	a) Expressing emotions during a Comedy Film, 28 mins, NR b) Inhibiting emotions during a comedy Film, 28 mins, NR	S-IgA concentration (1)	Note: A negative mood induction condition (negative film) and control group (two neutral films) were included in this study. Participants asked the inhibit emotions condition showed increases in S-IgA concentration from before to after the comedy film, however the significance of this change was not assessed. In the expressing emotions condition there were no differences in S-IgA concentration from before to after the comedy film.
Lefcourt et al. (1990, study 1) Canada Cohort	Students 45, NR (NR), NR, 100%	Comedy Audiotape, 30 mins, Group	S-IgA Concentration (1)	Note: Manipulation check consisted of participants rating funniness of the comedy audiotape. Significant increase in S-IgA concentration from before to immediately following intervention.
Lefcourt et al. (1990, study 2) Canada Cohort	Students 34, NR (NR), NR, 100%	Comedy Film, 30 mins, Group	S-IgA Concentration (1)	Note: Manipulation check consisted of participants rating funniness of the comedy film. Significant increase in S-IgA concentration from before to immediately following intervention.

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Lefcourt et al. (1990, study 3) Canada Cohort	Students 41, NR (NR), NR, 48.7%	Comedy Audiotape, 30 mins, Group	S-IgA Concentration (1)	Significant increase in S-IgA concentration from before to immediately following intervention.
Matsunaga et al. (Matsunaga et al., 2008) Japan Crossover	General population 12, NR (NR), 20-29, 0%	Film containing actresses chosen by participants as attractive, 4 mins, Individual	NK cell activity (1)	Note: A neutral control film was included as a condition in this study. Significant increase in NK cell activity from before to immediately post intervention. No change from before to after control film.
Matsunaga et al. (2011a) Japan Crossover	General population 23; NR (NR), 21-38, 78.3%	Odour selected by participant to invoke positive autobiographical memory, 90 seconds, Individual	IL-2; IL-4; IL-6; IL-10; TNF-α (5)	Note: A neutral control odour condition was included as a condition in this study. No significant changes on any immune outcome from before to after odour intervention. IL-2 was significantly lower immediately following self-selected odour intervention compared to immediately following control odour.

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Matsunaga et al. (2011b) Japan Crossover	Romantic Couples 14, NR (NR), 21-38, 50%	Kissing and hugging partner, 60 mins, Couples	IL-6, TNF-α, IFN-γ (3)	Note: Control condition involved reading quietly separately. IFN-γ significantly decreased from before to immediately after intervention. No changes in IL-6 or TNF-α.
Matsunaga et al. (2013) Japan Crossover	General population who self-report the ability to retrieve autobiographical odour memories; 10, NR (NR), 20-35, 70%	Odour selected by participant to invoke positive autobiographical memory, 3 x 60 seconds, Individual	IL-2, IL-5, IL-6, IL-10, TNF-α, IFN-γ (6)	Note: A neutral control odour condition was included in this study. Significant decreases in TNF-α and IFN-γ immediately following self-selected odour compared to immediately following control odour. No differences in other immune outcomes.
McClelland & Cheriff (1997) USA Crossover	General population 18, NR (NR), NR, 44.5%	Comedy film, 25 mins, Group	S-IgA Concentration (1)	Note: The manipulation check consisted of rating the funniness of the film clips. A neutral control film condition was included in this study. Significant increase in S-IgA concentration from before to immediately after intervention.

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Mittwoch-Jaffe et al. (1995) Israel RCT	General Population 123, NR (NR), NR, 52%	Comedy film, 45 mins, Group	IL-1b, IL-2, IL-3, Il-6, TNF-α (5)	Note: Comparison group watched a horror film for matched period of time. Significant increase in IL-2, IL-3, and significant decrease in TNF-α from before to immediately following comedy film intervention. No changes on IL-1b and IL-6.
Perera et al. (1998) UK Crossover	Students 15, 25 (NR), 20-36, 40%	Comedy film, 30 mins, Group	S-IgA concentration; S-IgA secretion rate (2)	Note: A neutral control film was included as a condition in this study. Significant increase in S-IgA concentration and secretion rates from before to immediately following comedy film. S-IgA concentration significantly decreased immediately following control film

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Strauman et al. (2004) USA Crossover	Students with high and low self-discrepancy 32, Low-discrepant: 19.51(1.58), NR, 100% High-discrepant-19.87 (1.41), NR, 100%	Writing about self-congruencies, 20 mins, Individual	Leukocyte count; lymphocyte count; lymphocyte %; neutrophil count; neutrophil %; CD-3 count; CD-3 %; CD-4 count; CD-4 %; CD-8 count; CD-8%; NK cell count; NK cell %; CD-19 count; CD-19%; NK cytotoxicity at effector to target ratio of of 100:1; NK cytotoxicity at effector to target ratio of of 50:1; NK cytotoxicity at effector to target ratio of of 25:1; lysis per 1000 NK cells (19)	Note: A negative mood induction condition (writing about self-discrepancies) and control group (two neutral films) were also part of this study. In both high and low discrepant students, there were significantly higher leukocyte, lymphocyte, CD-3, CD-4, CD-8, and NK cell counts immediately following self-congruency intervention compared to immediately following control condition. NK cell count increases were significantly higher in high discrepant compared to low discrepant students. In high discrepant students, lysis per 1000 NK cells was also significantly lower immediately following self-congruency intervention compared to immediately following control condition.

Authors (Year of publication) **Origin** **Study Design**	**Sample Type** **Sample Size, Mean age (SD), Range, Female%**	**Intervention Description, Duration, Mode of Delivery**	**Immune outcome(s) measured (n)**	**Main Findings**
Takahashi et al. (2001) Japan Crossover	General Population 21, NR (NR), 18-26, 0%	Comedy film, 75 mins, NR	NK cell activity at effector to target ratio of of 20:1; NK cell activity at effector to target ratio of of 10:1; CD-16%; CD-56%; CD-57%; leukocyte count; (6)	Note: A neutral control film was included as a condition in this study. Significant increase in NK cell activity at effector to target ratio of 20:1 from before to immediately after comedy film intervention, but not following control film. No effects on other immune outcomes reported.
Wachi et al. (2007) Japan Crossover	Yamaha Employees 40, 38.4 (8.4), NR, 0%	Drumming, NR, Group	NK cell activity at effector to target ratio of of 25:1; NK cell activity at effector to target ratio of of 12.5:1; NK cell activity at effector to target ratio of of 6.25:1; CD-56%, Leukocyte count (5)	Note: A control condition consisting of quite reading was included in this study No significant changes in any of the immune outcomes assessed as a result of the intervention.

Note: SD= Standard Deviation; Mins= Minutes; NR= Not Reported; S-IgA = Secretory Immunoglobulin Isotype A; NK= Natural Killer; RCT= Randomised Controlled Trial; IL= Interleukin; INF= Interferon; CD= Cluster of Differentiation; PHA= phytohaemagglutinin; Con-A= Concanavalin A; URT= Upper Respiratory Tract; TNF= Tumor necrosis factor; LPS = Lipopolysaccharide; PMN = polymorphonuclear.

8.3.5 Quality Assessments

A summary of quality assessment ratings for each of the EPHPP individual domain and global quality ratings are shown for each study in Table 8.3. In terms of global quality ratings, 1 study was classified as strong, 23 as moderate, and 7 as weak. This is suggestive of moderate-poor overall quality for this literature. Further examination of the individual quality domains shows selection bias was an issue for nearly all studies. In the selection bias domain all but one study was classified as weak, indicating participants were either self-selecting or that the sample selection processes were not adequately reported, with one classified as moderate. In the study design domain, six studies where classified as strong (indicating they were RCTs or clinical controlled trials), 25 as moderate, and none as weak. Confounders were often controlled for by the crossover nature of many study designs resulting in 25 studies being classified as strong in this domain, none as moderate, and 6 identified as weak. All studies received a moderate rating for the blinding domain, in the majority of cases because assessor and participant blinding was not mentioned. With regards data collection method, 11 studies were categorised as strong - meaning that outcome measures (immune) had been demonstrated as both valid and reliable – with the remaining 20 studies classified as moderate due to not reporting reliability statistics for the outcome measures or failing to refer to previous reports of the measures reliability. Most studies reported minimal or no attrition during the studies, meaning that for the withdrawals and dropouts domain 28 studies were classified as strong, two as moderate, and only one as weak.

Table 8.3: Summary of quality assessments

Authors (Year of publication)	Selection Bias	Study Design	Confounders	Blinding	Data Collection Method	Withdrawals and Dropouts	Global Study Quality Rating
Beck et al. (2000)	Moderate	Moderate	Strong	Moderate	Moderate	Moderate	Strong
Bennet et al. (2003)	Weak	Strong	Weak	Moderate	Strong	Strong	Weak
Bittman et al. (2001)	Weak	Strong	Weak	Moderate	Moderate	Strong	Weak
Dillon et al. (1985)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate
Futterman et al. (1992)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate
Futterman et al. (1994)	Weak	Moderate	Weak	Moderate	Moderate	Strong	Weak
Hall et al. (1992)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate
Harrison et al. (2000)	Weak	Moderate	Strong	Moderate	Strong	Strong	Moderate
Hewson-Bower & Drummond (1996)	Weak	Strong	Strong	Moderate	Strong	Strong	Moderate
Hucklebridge et al. (2000)	Weak	Moderate	Strong	Moderate	Strong	Strong	Moderate
Jung et al. (2006)	Weak	Strong	Weak	Moderate	Moderate	Strong	Weak
Kiecolt-Glaser et al. (2010)	Weak	Moderate	Strong	Moderate	Strong	Weak	Weak
Kimiata (2004, Study 1)	Weak	Moderate	Strong	Moderate	Strong	Strong	Moderate
Kimiata (2004, Study 2)	Weak	Moderate	Strong	Moderate	Strong	Strong	Moderate
Knapp et al. (1992)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate
Koyama et al. (2009)	Weak	Moderate	Weak	Moderate	Moderate	Strong	Weak
Kreutz et al. (2004)	Weak	Moderate	Strong	Moderate	Strong	Strong	Moderate
Labott et al. (2010)	Weak	Strong	Weak	Moderate	Strong	Strong	Weak
Lefcourt et al. (1990, Study 1)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate
Lefcourt et al. (1990, Study 2)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate
Lefcourt et al. (1990, Study 3)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate

Authors (Year of publication)	Selection Bias	Study Design	Confounders	Blinding	Data Collection Method	Withdrawals and Dropouts	Global Study Quality Rating
Matsunaga et al. (2008)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate
Matsunaga et al. (2011a)	Weak	Moderate	Strong	Moderate	Strong	Strong	Moderate
Matsunaga et al. (2011b)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate
Matsunaga et al. (2013)	Weak	Moderate	Strong	Moderate	Strong	Strong	Moderate
McClelland & Cheriff (1997)	Weak	Moderate	Strong	Moderate	Moderate	Moderate	Moderate
Mittwoch-Jaffe et al. (1995)	Weak	Strong	Strong	Moderate	Moderate	Strong	Moderate
Perera et al. (1998)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate
Strauman et al. (2004)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate
Takahashi et al. (2001)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate
Wachi et al. (2007)	Weak	Moderate	Strong	Moderate	Moderate	Strong	Moderate

8.4 ***Discussion***

The present review was conducted to identify and assess the existing literature relating to brief mood improving interventions on immunity. Specifically, the review aimed to (1) identify the size and nature of the existing literature base, (2) to assess the quality of this literature, and (3) to identify the types of interventions that have demonstrated mood improvements, and gauge their appropriateness for use in primary care. Findings relating to each of these aims are discussed in turn below.

8.4.1 Size and Nature of the Literature

The review identified a moderate-sized literature of 31 studies presenting one or more single-session mood improving interventions and measuring an aspect of immunity. The majority of studies adopted crossover or cohort designs, which are generally considered less rigorous tests of an intervention than RCTs (D. Evans, 2003) - of which there were six. Sample sizes were, in general, relatively small – indicating the need for more substantial RCTs in the future. Nearly all studies examined immune changes immediately post-intervention, with only two studies measuring aspects of immunity in a later follow-up. As such, there is a lack of evidence regarding how transient the effects of mood-enhancing interventions on immunity are. This is relevant for the development of positive affect interventions to enhance vaccination responses because, based on current evidence, there is little to guide how close to vaccination such an intervention would need to be administered to give immunological benefits.

Despite the above weaknesses in this literature, it is striking that nearly all mood enhancing interventions were associated with at least one immunological change. While the clinical relevance of these changes was not

demonstrated in any of the included studies, most of the immunological changes observed (e.g., increase in S-IgA) can be broadly seen as indicative of enhanced immune function. The consistency of the relationship observed between increasing positive affect and enhanced immunity, regardless of the intervention employed to achieve the affect improvement, leads further credibility to the hypothesis that a brief positive affect enhancing intervention could improve vaccination responses through inducing short-term immunological change.

With regards the primary focus of this thesis on vaccination in the elderly, it is noteworthy that no studies included in this review investigated the effects of a mood enhancing intervention on response to an in-vivo immune challenge, such as vaccination. Indeed, all but two studies (Kimata, 2004 studies 1 & 2) considered fairly non-specific markers of immunity, such as S-IgA secretion and NK cytotoxicity. Most of the immune outcomes investigated in the identified studies form part of the cascade of innate and adaptive immunological processes that follows vaccination (see 1.3.2). Yet due to the redundancy and complexity inherent in the immune response to potential foreign pathogens, it is hard to determine the importance of these changes to individual components measured in isolation to the outcomes of immune challenge (e.g., antibody formation) as a whole. While increases in non-specific aspects of immunity are likely indicative of enhanced in-vivo immune responses to challenge (Mantis, Rol, & Corthésy, 2011; Ogata et al., 2001), there is a clear need for additional research examining the effects of mood enhancing interventions on immune challenge more directly. Further, only one included study examined the effects of mood enhancing interventions on older adults (Koyama et al., 2009). Given the wide-ranging decline in immunological competence known to occur in later life (discussed at length in Chapter one), it is unclear whether the immune enhancing

effects of brief mood improving interventions shown in younger adults are generalisable to older adults.

8.4.2 Quality of the Literature

Overall study quality was weak-to-moderate, with all studies at some risk of selection bias. Future studies in this area would benefit from more directed recruitment strategies, to ensure participants are representative of the target population. Examining the other domains assessed by the EPHPP study quality instrument, the reviewed studies were typically well designed to control for potential confounding factors and, due to their brief nature, showed little evidence of problematic attrition. Reporting standards were generally poor, with few studies providing details about recruitment processes, study blinding, and the reliability of immunological outcome measures. Calls made over recent decades, across research domains, to improve recording standards appear to have had little impact in this area.

Looking beyond the included studies, it is noteworthy that 32 potentially relevant articles were excluded from the present review because of not demonstrating their 'mood enhancing' intervention enhanced mood. The absence of such manipulation checks limit the insight that can be gained from those studies, as it is not possible to ascertain whether any immunological changes resulting from the interventions are related to mood enhancement or caused by some other factor. For future studies in this area, including a satisfactory manipulation check should be considered essential, as has been recommended for all positive psychological interventions (J. L. Smith, Harrison, Kurtz, & Bryant, 2014).

8.4.3 Types of Interventions & Suitability for Primary Care

A wide variety of interventions that enhanced positive affect were identified in this review. Fifteen of 38 interventions lasted longer than 60 minutes, making them poor candidates for primary care adoption in their current form. Further, some interventions relied on specialist equipment (e.g., odour generation) or skilled facilitators (e.g., yoga, group drumming) that would likely make such interventions relatively costly to implement. However, the most common interventions of watching or listening to a comedy programme, mental recall of positive memories, and listening to music have greater potential. While the length of most of these interventions in their current form (often over 30 minutes) exceeded the length of time many patients spend in primary care settings when receiving vaccination, shortened versions of these interventions drawing on their central components could potentially be administered, or self-administered, in, or in advance of attending, primary care. Indeed, there is no reason why such strategies could not be combined in a single intervention, although there is no evidence at this stage this would be a more effective approach. Further research is needed relating to the minimum required length of a positive mood intervention to induce immune changes, as well as whether larger mood enhancement is associated with larger immune effects.

8.4.4 Strengths and Limitations

The present scoping review adopted pre-determined inclusion criteria and systematic searches of multiple electronic databases to reduce any possible selection bias. Studies were not excluded on the basis of design-type to maximise the identification of relevant research evidence and the quality of included studies were assessed using an established standardised tool. However, this review did not seek to retrieve and include relevant

unpublished articles (the so-called grey literature). The rationale behind this omission is that unpublished articles have not successfully gone through the peer-review process – thus, it is not possible to be certain of the articles quality and veracity. Yet, it is important to acknowledge that in excluding this literature the influence of any publication bias could be exacerbated – as interventions demonstrating no immune effects may have been less likely to be published.

The present review is also unable to answer two particularly pertinent questions in relation to the development of novel positive affect interventions to enhance vaccine responses in older adults. First, because the review is narrative in nature, as opposed to quantifying effects, it is not able to examine whether interventions that resulted in larger increases in mood are associated with larger immunological effects than interventions with smaller effects on mood. While ostensibly this question could be examined via meta-analyses, the poor reporting standards and lack of consistency among immune outcomes meant this such an analysis would have included too few studies to be meaningful or reliable.

Second, due to the lack of studies including older adult samples, it is not possible to draw conclusions regarding the effect of brief mood-enhancing interventions in the elderly. Further research, both quantitative and qualitative in nature, would be useful in this regard. As the present review adopted a 'scoping' methodology, it is possible that not all studies examining the effects of mood enhancing interventions on immunity were identified. However, given the consistency in findings of the included studies it would appear unlikely that any missed studies would significantly alter the conclusions drawn. By not exploring grey literature however, the present review was unable to explore the potential of publication bias. Given this

review identified a considerable number of small sample studies showing positive effects, but comparatively few showing null findings, the issue of publication bias is worthy of further consideration in any future review.

8.5 ***Chapter Summary***

This chapter has presented the findings of a systematic scoping review of mood enhancing interventions on immunity. A moderate-sized, but low quality, literature was identified with a paucity of studies examining the effects of such interventions on immune challenges or focusing on older adults. The vast majority of studies reported enhanced non-specific immunological outcomes as a result of intervention driven mood improvements, regardless of the specifics of the intervention. In terms of suitability for primary care, many the existing wide array of interventions were typically unsuitable for use in their current form for use primary care (e.g., too time consuming, requiring specialist staff, equipment or space). However, the assumed 'active ingredients' of these interventions (e.g., humour, positive memory triggering, music) could potentially be re-packaged in a self-deliverable format more suitable for use in, or immediately prior to attending, primary care settings.

Chapter 9: General Discussion

Chapter Synopsis

This final chapter presents a synthesis of findings described in this thesis, discussing some of the clinical, theoretical and methodological implications of the findings. In addition, some brief reflections on the challenges of conducting the research are offered, with an emphasis on alternative approaches that would be considered if the work was to be replicated. Finally, a series of recommendations for future research are outlined.

9.1 *Introduction*

This thesis aimed to investigate the influence of multiple psychological and behavioural factors on antibody outcomes following vaccination, with a view towards informing the development of brief, novel interventions to improve vaccination effectiveness. Specifically, due to the disproportionate burden of infectious diseases and reduced vaccine efficacy amongst older adults (Chen et al., 2009; K. Christensen et al., 2009; Feng et al., 2012; Pitman et al., 2003) that was discussed at length in Chapter 1, the emphasis of this thesis has been in this population – although findings may have broader relevance to any groups with sub-optimal vaccination outcomes (e.g., pregnant women, those with immunosuppressive illnesses).

Building on a large and growing body of evidence that psychological and behavioural factors can impact on health, immunity, and vaccination responses (reviewed in Chapters 2 & 3), this thesis has presented the findings from a longitudinal prospective observational cohort study of older adults (the BeHIVE-65 Study). The BeHIVE-65 study employed a repeated measures diary-based methodology, examining multiple psychological and

behavioural influences on short and long-term antibody responses to influenza vaccination (Chapters 4, 6, and 7). Prior to this, research in this area was generally limited to focusing on a single psychological or behavioural factor per study and over-relied on the use of single time-point retrospective measures that were open to systematic biases. In addition, previous research had not explicitly examined the time-points in relation to vaccination at which psychological and behavioural influences were most potent, or whether an individual's variability in psychological experience over time predicted vaccination outcomes. The BeHIVE-65 study was designed to address these issues, whilst also employing a novel microarray assay - which was developed and validated specifically for this research as part of this thesis (see Chapter 5) - for the measurement of influenza-specific serum IgG antibodies that boasts significant advantages over previously used assays.

Based on the intriguing finding that greater positive affect on the day of vaccination was associated with increased vaccine-induced antibody responses and protection in older adults following influenza vaccination, this thesis also presented an early step in the intervention development process, through a systematic scoping review of brief positive mood enhancing interventions on immunity (Chapter 8). Together, these studies were conducted to act as a springboard for the development of new, evidence-based interventions to improve vaccination outcomes, particularly in older adults.

Here, in the final chapter of this thesis, after a short re-iteration of the main research findings, the contributions of this work are discussed in relation to its clinical, theoretical and methodological implications. Further, this chapter will contain some brief reflections on the challenges encountered in

conducting the research contained in this thesis, highlighting recommendations for alternative approaches were the work to be replicated. Finally, the chapter ends with a series of possibilities for future work in this area that build on the work presented in this thesis.

9.2 ***Summary of Findings***

9.2.1 Development of a Microarray Assay for Influenza-specific IgG

Chapter 5 presented a series of optimisation and replication experiments designed to refine and validate a novel multiplex protein microarray assay to quantify influenza-specific IgG antibodies in human sera. The case of need for a novel microarray assay for identifying influenza-specific antibodies stems from limitations with previously used simplex assays such as the hemagglutination inhibition assay (HAI) and the Enzyme-linked immunosorbent assay (ELISA). Specifically, these assays only allow one analyte (e.g., one type of antibody) to be examined per assay/well - meaning the volumes of sample, antigens, reagents, and consumables required are significant limiting factors on study sample sizes, due to their substantial cost, labour requirements, and low throughput. This issue is of significant importance for studies in this field, which have historically received criticism for an over-reliance on small, often under-powered, studies (Segerstrom & Smith, 2012). In contrast, multiplex protein microarrays – use of which is becoming increasingly common in other areas of basic science (e.g., Negm et al., 2015; Selvarajah et al., 2014; Tighe, Negm, Todd, & Fairclough, 2013) – allow multiple analytes to be quantified in a single processing of a sample, whilst having superior dynamic ranges, increased reliability and allowing for the inclusion of multiple internal quality control measures (Negm et al., 2015).

The primary findings of the experiments presented in Chapter 5 were that:

(1) The assay had increased reliability when performed on commercially produced Aminosilane coated slides compared to in-house produced Aldehyde coated slides.

(2) The developed microarray assay could distinguish between positive and negative control samples for antibodies against all eight different influenza strains tested.

(3) The optimal dilution of serum and antigens for quantifying serum IgG against the 2014/15 northern hemisphere influenza vaccination were 1:8000 and 50µg/ml respectively.

(4) The novel microarray assay had excellent intra-assay reliability and acceptable inter-assay reliability – with analysed samples correlating well with results from ELISA.

It was concluded from these experiments that the developed assay is a reliable method for quantifying influenza-specific IgG in serum. Demonstrating the utility of this assay and the protocols developed represent a significant methodological advancement for researchers interested these outcomes. The multiplex, microarray assay is superior to existing assays, offering much higher throughput, lower costs per analyte and greater quality control that make the assay ideal for use in large sample studies.

9.2.2 BeHIVE-65 Study

The BeHIVE-65 study was an intensive longitudinal, prospective observational cohort study that aimed to explore whether (1) long-term (modifiable and non-modifiable) participant characteristics, (2) behavioural and psychological factors in the period immediately prior to and following

vaccination, and (3) intra-individual variability in psychological factors predicted short- (4 weeks post-vaccination) and long-term (16 weeks post-vaccination) IgG antibody outcomes following influenza vaccination in older adults. While there were many novel aspects to this study – its principle contribution was to examine the relative influence of multiple psychological (positive affect, negative affect, perceived stress) and behavioural factors (sleep, diet, physical activity) on vaccination responses that had previously been examined separately.

In contrast to previous research, most of the demographic, clinical, behavioural and psychological long-term factors measured at baseline including age, gender, BMI, number of medications prescribed, habitual physical activity performance, and perceived stress over the past month were not associated with post-vaccination IgG antibody responses. However, correlational and hierarchical regression analyses showed that older adults with greater ill-health, as defined by reporting a greater number of diagnosed illnesses, had poorer long-term responses to the H1N1 strain of the vaccination.[10] This means that at the time where influenza viruses are most prevalent in the general population, older adults with greater ill-health are less likely to be protected than healthier peers. In addition to the above, two surprising observations relating to long-term behavioural influences were made: that older adults who reported having a habitual diet that met recommended intake levels for selenium had lower short-term antibody responses in all strains compared to those who did not, and that older adults who habitually engaged in flexibility training had poorer long-term H3N2 antibody responses than those who did not. In the case of the former, it was

[10] The H1N1 strain of the 2014/15 northern hemisphere influenza vaccination was found to be the least immunogenic antigen included in the vaccine, in that participants had lower overall levels post-vaccination and smaller vaccine-induced increases in this compared to other strains (see Chapter 6). This is noteworthy because as discussed throughout this thesis, previous literature has demonstrated that behavioural and psychological influences on vaccination response are typically only evident in response weakly immunogenic antigens due to ceiling effects (e.g., Edwards et al., 2012).

argued in 6.4 that given the weight of existing evidence that selenium plays an important role in the optimal immune function (for a review see Hoffmann & Berry, 2008) this finding may be a spurious artefact of the data - a conclusion supported by the observed lack of congruence between food frequency questionnaire measures and daily diary measures of selenium in this study. With regards the latter, while some previous literature has also reported poorer antibody responses among older adults undertaking flexibility training when compared to cardiovascular training (e.g., Woods et al., 2009) it has been argued that the observed negative association may reflect frailty or other health issues that encourage older adults to engage in flexibility training, rather than flexibility training being somehow immunosuppressive.

Considering behavioural and psychological factors measured during the six-week period around vaccination (2 weeks prior and 4 weeks following), it was found that in contrast to previous literature, most of the investigated factors (including physical activity, sleep duration and efficiency, and many dietary factors) did not significantly predict short or long-term antibody responses following vaccination in this sample. While this was contrary to predictions, it has been speculated in this thesis that this divergence likely stems from the unusual scenario encountered in the BeHIVE-65 study of participants being vaccinated with identical vaccine strains in two consecutive years, leading to ceiling effects obscuring any weak-to-moderate psychological and behavioural influences. Ceiling effects have previously been shown to limit the observation of psycho-behavioural influences on vaccine responses (e.g., Edwards et al., 2012) and thus it is likely that all observed associations between psychological and behavioural factors and antibody responses in the BeHIVE-65 study were somewhat smaller than they would be in a more 'typical' scenario (where components

of the vaccine change year-on-year). Despite this preponderance of unexpected null findings, a series of statistical approaches reliably found explicit positive affect to be associated with, and independently predicts, both short- and long-term antibody responses in the weakest immunogenic strain (H1N1) above and beyond significant demographic and clinical determinants. Prior to this study, positive affect had received only minimal attention in relation to vaccine responses – although previous literature relating to immune function implicated positive affect as an important influence on immunity (S. Cohen et al., 2006; Dillon et al., 1985; Lutgendorf et al., 2001; Marsland et al., 2006; Stone et al., 1994). Crucially, the findings presented in this thesis, suggest that of all the psycho-behavioural factors measured (many of which have received considerably more research attention), explicit positive affect has the greatest impact on post-vaccination antibody responses and is therefore the most appropriate target for a non-pharmacological intervention designed to improve vaccination outcomes in older adults.

When exploring the issue of timing (specifically when psycho-behavioural factors exert their greatest influence on vaccination outcomes), correlational analyses found that, in general, there was only minimal, and inconsistent, differences in associations between pre- and post-vaccination measures of psycho-behavioural factors with short- and long-term antibody outcomes following vaccination. However, it was found that the day of vaccination was a particularly salient time for psychological factors, with hierarchical multiple regression analyses finding that explicit positive affect on this day was a stronger predictor of both short and long-term H1N1 antibody responses to vaccination than measures taken over the complete diary period. This broadly accords with an earlier finding from Edwards and colleagues (2006), which found modulation of psychological factors on the day of vaccination

(in that case mental stress) can influence immune responses sufficiently to impact on long-term vaccination responses. This suggests there may be considerable utility in targeting brief, positive mood enhancing interventions on the day of vaccination.

Finally, the BeHIVE-65 study was the first study in this area to examine the role of intra-individual variability in psychological measures over the complete diary period and its associations with vaccination outcomes. In line with research in other areas (Eid & Diener, 1999; Wang et al., 2012), intra-individual variability in psychological factors over-time was shown to be a characteristic largely distinct from a participant's average levels on those factors. While intra-individual variability in psychological factors was not associated with short-term antibody outcomes at 4 weeks post-vaccination, correlational analyses showed associations between greater variability in perceived stress measures and poorer long-term (16 weeks post-vaccination) antibody responses for all strains of the vaccination. Hierarchical regression analyses extended this latter finding, showing that intra-individual variability in perceived stress remained a significant independent predictor of long-term antibody responses in two of the three strains, even when average levels of perceived stress was also accounted for in the model. This finding implicates variability in psychological experience as an important, albeit frequently overlooked, predictive factor in vaccination, and, more broadly, immunological and health outcomes.

9.2.3 Scoping Review

Based on one of the most intriguing findings from the BeHIVE-65 study that explicit positive affect on the day of vaccination was the strongest psycho-behavioural predictor of both short- and long-term H1N1 antibody

responses, a systematic scoping review was conducted of previous brief 'mood enhancing' interventions on immunity. A moderate sized, but low quality, body of literature was identified with a notable paucity of studies in older adults and trialled interventions that were sufficiently brief and practical for broader adoption. Further, no studies included an in-vivo immunological challenge that was comparable to vaccination. However, an encouraging finding was that over 85% of studies reported at least one change in an immunological outcome (typically considered to be enhancements) because of intervention-driven mood improvements, regardless of the specifics of the intervention. These findings are congruent with the conclusion drawn from the BeHIVE-65 study that positive affect is a potent immune modifier and points to the possible utility of mood-enhancing interventions to improve vaccination responses. While it was concluded that the specific interventions reviewed were generally unsuitable for broad adoption in their present form, it was argued that the 'active ingredients' of these interventions (e.g., humour, positive memory triggering, music) could potentially be re-packaged in a brief, potentially self-deliverable, format more suitable for use in, or immediately prior to attending, primary care settings.

9.3 ***Clinical Implications***

The primary clinical implication of the above work relates to the conclusion that while there is now a considerable body of evidence that psychological and behavioural factors can influence immunity and vaccination responses - positive affect appears to be one of the most important of such influences. Clinicians should, as a minimum, take note that a vaccine recipient's mood can be an important determinant of the procedures relative success. Some patients (e.g., those with clinical and sub-clinical low mood) are less likely

to be protected following vaccination, thus opening them to greater risk of later infection and its associated consequences. Crucially however, clinicians should note that short-term positive affect was shown in the systematic scoping review to be modifiable and that brief changes in affect were sufficient to impact on immune dynamics.

While a further systematic and rigorous research programme is required to establish whether such brief changes to immune dynamics because of mood enhancement are sufficient to meaningfully improve vaccination responses and how to best achieve this – there seems minimal downside to implementing some of these initial findings into current practices. GP surgeries could consider how to make the relatively humdrum, or even anxiety-provoking, experience of waiting for and being vaccinated more enjoyable. Many primary care settings can present waiting patients with positive and uplifting stimuli through routinely placed information boards, monitors and sound systems. However, anecdotal and personal experience suggests patients are more likely to be exposed to relatively negative stimuli (e.g., adverts for funeral parlours, lists of worrying disease symptoms). Making minor changes during influenza vaccination season such as these would have little foreseeable downside for patients, while possibly conferring health benefits. More formally, these findings provide a compelling rationale for the development, and ultimately implementation, of brief mood-enhancing interventions that could be delivered on the day of vaccination with the aim of enhancing vaccine effectiveness. Such interventions could, in the first instance, be targeted at populations considered to be at risk of sub-optimal outcomes (e.g., older adults with low mood).

The finding that intra-individual variability in perceived stress was associated with poorer long-term antibody responses to influenza vaccination in older

adults also has potential clinical importance. It extends the rather general previous observations that greater variability in psychological factors are associated with physical health outcomes (e.g., Chan et al., 2016; Hardy & Segerstrom, 2016) to more specific immunological effects. While clinically, these findings may point to the possible benefit of pioneering interventions that encourage greater stability in psychological factors – any such recommendations at this stage are premature. As a relatively understudied area, the viability of developing interventions to reduce intra-individual variability is unknown. Further, it is not self-evident that high levels of stability in psychological factors is desirable given a significant body of evidence demonstrating associations between emotional inertia (reduced psychological reactions to positive and negative stimuli/events) and poorer mental health (Koval, Kuppens, Allen, & Sheeber, 2012; Kuppens et al., 2012; Kuppens, Allen, & Sheeber, 2010). At this stage, considerable additional research is needed to explore intra-individual variability in psychological factors both in terms of its consequences and causes. For example, the BeHIVE-65 study was not designed to establish whether increased variability in psychological factors were trait-like participant characteristics or instead a reflection of more variable environmental and social situations. Indeed, recent evidence from twin studies have suggested that variability in affect may be heritable, although for differing degrees for positive and negative affect variability (Zheng, Plomin, & von Stumm, 2016). Therefore, while intriguing, the finding that intra-individual variability in perceived stress was associated with poorer long-term antibody responses to influenza vaccination in older adults needs greater examination before any changes to clinical practice can be recommended.

Importantly, while the BeHIVE-65 study examined psycho-behavioural influences on influenza vaccination specifically – there is every expectation

that these findings have clinical relevance for other vaccinations discussed in this thesis such as Herpes Zoster (shingles), Hepatitis B, and Pneumococcal. The effectiveness of all of these vaccines are understood to be limited in groups with underlying immune dysregulation such as the elderly (Chen et al., 2009; Mor & Cardenas, 2010) resulting in significant individual, societal, and economic burden.

The confidence in the present findings relevance to other vaccines stems from the review of previous literature presented in Chapter 3 which, in general, showed little evidence of differences in psycho-behavioural influences based on specific vaccination under examination. Indeed, given the proposed mechanisms by which factors such as positive affect influence vaccination outcomes described in Chapter 2 (through wide-ranging but relatively non-specific changes to multiple immune cell dynamics) it would be expected that any psycho-behavioural influences on vaccination outcomes are not antigen specific (other than their relative immunogenicity – see above). A small caveat to this is that some vaccine antigens (e.g., Hepatitis B) are more likely to be novel to the host immune system than is the case for influenza antigens – to which most are exposed to regularly throughout the life-span. However, based on the evidence reviewed in this thesis, a reasonable speculation is that psycho-behavioural influences would, if anything, be comparatively stronger in primary exposure vaccines such as Hepatitis B than for influenza. Studies that have employed the use of such vaccines have regularly found significant impacts of psycho-behavioural influences in populations that generally have robust immune responses (e.g., young adults) despite the expectation that ceiling effects might obscure such findings (e.g., Burns et al., 2002; Glaser et al., 1992; Jabaaij et al., 1993; Lange, 2003; Lange et al., 2011). While additional research addressing this issue more directly would be beneficial, the existing

evidence would suggest all vaccines are likely influenced by psycho-behavioural factors. Therefore, developing brief, positive affect enhancing interventions may have broader uses than just to enhance influenza vaccination outcomes in older adults.

9.4 ***Theoretical Implications***

While this thesis is situated within the research tradition of psychoneuroimmunology (PNI), and more broadly within a biopsychosocial framework, it has not sought to explicitly examine or test a named theoretical model. However, that is not to say that the findings from this work do not contribute to theoretical understandings of psycho-behavioural influences on immunity and vaccination responses. First, it is worth acknowledging that while PNI has been considered a field of enquiry and long-standing research tradition in this thesis, some researchers present PNI as a theory or model (Greenberg, 2003; Nokes & Kendrew, 1990), albeit that these same researchers tend leave this 'model' relatively ill-defined. Sidestepping this issue of academic semantics, it is sufficient to say the findings presented in this thesis adds to the body of literature in support of the main tenants of psychoneuroimmunology: that psychological experiences and behaviour can have immunological consequences. While the BeHIVE-65 study demonstrated this in terms of significant observational associations (most notably for positive affect and vaccination responses), the experimental studies synthesised in the systematic scoping review provide additional, and more direct evidence in support of PNI 'theory'.

Further, as discussed in Chapters 1 & 2, within PNI the popularity of examining psychological and behavioural influences on vaccination is not purely because of the clinical relevance of vaccination outcomes in

preventing disease. Rather its popularity extends from the immunological underpinnings of vaccination – which is an facsimile of natural disease exposure without the associated risks (Phillips, 2012). The implication of this is that the findings presented in this thesis in respect to vaccination outcomes, also extend understanding in relation to psychological and behavioural influences on our responses to naturally occurring immune challenges that can cause infection.

One more defined theoretical model to which the findings presented in this thesis speak to is the Allostatic Load model (McEwen, 1998; McEwen & Stellar, 1993). This model, which was introduced briefly in 3.3.1, posits that 'wear and tear' is caused to the body due to repeated attempts to adapt (allostasis) to psychological and physiological stress, ultimately leaving it more vulnerable to disease. Primate models have provided some of the most robust support for these concepts - for example blood pressure increases that occur in following interactions between monkeys in unstable dominance hierarchies are associated with accelerated arterial plaque formations (Manuck, Kaplan, Adams, & Clarkson, 1988). With respect to this thesis, the finding that greater intra-individual variability in perceived stress is associated with poorer long-term antibody responses to influenza vaccination is consistent the Allostatic Load model. Specifically, the repeated activation of physiological allostatic systems by having highly variable perceived stress levels causes a degradation to immune cells (and their associated functions) over time, which ultimately leads to a reduced ability to respond to antigenic challenge. Interestingly, high average levels of perceived stress (which have more traditionally been the focus of studies examining the Allostatic Load model) were not found to be a significant predictor of antibody responses to influenza vaccination in older adults. As such, future research examining the predictive utility of the allostatic load

model may be advised to consider variability, as well as simply chronicity, in stress.

9.5 *Methodological Implications*

A key methodological contribution of the work conducted in this thesis to the literature is the development and validation of the novel microarray assay described in Chapter 5. This is significant for two main reasons. First, on a pragmatic level, the protocols developed and subsequently published (Ayling et al., 2017) can be adopted in full by other researchers interested in measuring IgG antibodies to influenza vaccination for whatever research purpose, in a large number of samples, with confidence that findings correlate well with previous assays. With minimal adaptation, these protocols should also be suitable for future versions of the seasonal influenza vaccination, in addition to the strains contained in the 2014/15 northern hemisphere vaccine. Second, and arguably most importantly, the demonstration and successful use of this assay within the BeHIVE-65 study will hopefully serve as a justification for greater adoption of protein microarray assays within PNI, which has so far been slow in embracing this emergent area of assay technology. This is most likely due to a lack of familiarity with the methods and its wide-ranging benefits over commonly used simplex assays such as ELISA. For PNI, one of the most significant benefits of using microarray assays is the increased capacity for conducting larger scale studies that were previously cost-prohibitive. Beyond this, it is worth emphasising that adoption of microarray methods allow researchers in PNI to ask and answer a host of novel questions regarding psychological-behavioural-brain-immune mechanisms and changes over time. This is because protein microarray assays allow researchers to measure many hundreds of proteins in a single processing of a tiny amount of liquid sample

– something not practical when using traditional simplex assays. For example, microarray assays could be used to track levels of an array of brain-immune signalling cytokines taken repeatedly over time during a positive affect intervention. Such a design would provide considerable insight into the underlying immunological mechanisms and processes that co-occur with affect change – potentially providing insight for new interventions both psycho-behavioural and pharmacological. Considering this potential, this thesis has only used protein microarrays in a relatively rudimentary way – barely scratching the surface of this technological development and its possible utility in this area of research. However, it will hopefully serve as a starting point for greater use of these methods in PNI.

A further methodological implication of this work is in highlighting the need for, and benefits of, research employing repeated momentary measures or diary-based designs – such as those employed in the BeHIVE-65 study – to understand psycho-behavioural influences on immunity and/or vaccination. Throughout this thesis, the argument has been made that prior research in this area has over-relied on single-time point retrospective measures of psychological and behavioural factors. The concern with such measures is that, while simple to use, they are underpinned by the assumption that individuals can reliably recall experiences and actions over a prolonged period. This assumption has been widely challenged (e.g., Bradburn et al., 1987; N. Schwartz & Sudman, 1995) with considerable evidence demonstrating such measures are open to systematic biases (e.g., Redelmeier & Kahneman, 1996; Shiffman et al., 2008; Teasdale & Russell, 1983). Findings presented in this thesis add further weight to these concerns, with disparities evident between what participants reported retrospectively and diary measures (e.g., some aspects of diet were significantly different when assessed by retrospective food frequency

measures compared to regular food diaries). Given these limitations with single-time point retrospective measures, the greater adoption of intensive longitudinal methods including ecological momentary assessment and diary-based studies would improve the quality of the current literature in this area.

One highly novel finding of the work presented in this thesis was that variability in perceived stress over time was a significant predictor of long-term antibody responses to vaccination. This association was largely independent of average levels of perceived stress which did not significantly predict antibody outcomes. Crucially, beyond the clinical implications of these findings discussed above, this highlights the importance, and indeed benefits, of looking beyond average levels of measured psychological states where possible (e.g., in intensive longitudinal designs). Within healthcare research broadly, intra-individual variability in psychological experiences have only relatively recently begun to be examined as possible determinants of health outcomes, but are showing considerable predictive value. Recent evidence has found variability in psychological experience is associated to both self-reported and objectively measured health outcomes (Chan et al., 2016; Hardy & Segerstrom, 2016). For example, in a study of over 15,000 Chinese adults, Chan et al. (2016) found positive affect variability across a day was a significant independent predictor of self-reported health status as well as having a clinical diagnosis of depression or angina. The examination of intra-individual variability as an influence on immunity in the context of vaccination in this thesis will hopefully act as a catalyst for others to further investigate this issue.

9.6 ***Reflections, Challenges and Recommendations***

Many limitations with specific aspects of the research programme presented in this thesis have been discussed at length in earlier chapters, which will not be repeated here. However, this section describes some brief reflections conducting the research, its challenges and changes that would be advised if the work was to be replicated.

9.6.1 Recruitment

The BeHIVE-65 study, in its initial conception, aimed to recruit a sample of 200 older adults – although this included an expectation of moderately high attrition due to the intensive nature and length of the study. However, disappointingly only 138 eligible older adults were ultimately recruited for the study, although it is noteworthy that very low attrition rates across the study were achieved (<3% at 6 weeks post-vaccination). While one cause of this lower than anticipated recruitment was discussed in Chapter 7 (one of four recruitment sites sent out letters to ineligible participants) - a further challenge was the relatively narrow time window in which influenza vaccinations are offered (typically from the end of September-January, with the clear majority being vaccinated before the end of October). This means that contacting participants after October to boost recruitment numbers was unlikely to be successful – as most eligible patients are vaccinated by this time. In hindsight, beginning recruitment earlier in the calendar year would have been advisable – and maximised the chances of achieving the desired sample size.

9.6.2 The cost-benefit balance of repeated measures designs

In this chapter, and indeed throughout this thesis, the argument for greater use of intensive, repeated measures designs has been strongly put forward. However, it is important for researchers to carefully balance the implicit advantages of these designs (reduced recall biases, exploration of intra-individual variability etc.) against ethical and pragmatic considerations. In terms of ethical issues, the primary concern with intensive repeated measures designs is participant burden. Where measurement occasions are overly frequent or long – intensive repeated measures designs may place unreasonable demands (both in terms of time and effort) on participants who typically receive minimal personal benefits from research participation. Further, overly burdensome study designs may also present data integrity problems relating to missing data, attrition, response fatigue and poor recruitment (Rolstad, Adler, & Rydén, 2011). In terms of pragmatic considerations - the collection, recording, and analysis of intensive repeated measurement studies can be challenging and time-consuming. For example, in the BeHIVE-65 Study, all participants chose to complete diary measures in a traditional pen and paper manner. These responses then had to be transferred into appropriate computer software packages prior to analyses. This was particularly time-consuming for dietary measures – as one 24-hour food diary can take around 30 minutes to code appropriately onto dietary computer software with each participant completing up to 18 across the study. Overall, the coding of this dietary data alone took 3 researchers, 3 months to complete. While such issues can be addressed in some populations by collecting measures using mobile phone apps or other electronic means – this requires researchers to have access to appropriate expertise, equipment, and secure storage mechanisms and may not be suitable or appropriate in older adult populations.

On balance, if the BeHIVE-65 were to be replicated a small number of changes relating to measurement would be advised. Informal discussions with participants and personal observations indicated that completing 24-hour food diaries was the most burdensome aspect of the study (both from the perspective of participant and researcher). Given the relative concordance between the retrospective food frequency questionnaires and 24-hour food diary estimates of dietary intake (with the notable exceptions of selenium and vitamin E), using the former alone may have been sufficient. Conversely, given the relative low levels of internal reliability found for negative affect measures during the diary period – employing a longer version of the chosen scale (I-PANAS-SF) throughout may have improved this reliability and allowed greater confidence in the findings relating to negative affect.

9.6.3 Measuring Implicit Affect

In section 4.2.5, the case for measuring implicit as well as explicit affect in the BeHIVE-65 study was made – because both theoretical models and empirical evidence pointed to the limitation of explicit measures alone as only capturing a portion of affective experience. In the BeHIVE-65 study, a measure of implicit affect (IPANAT) was included throughout the diary period – but was ultimately unusable due to high levels of missing data and reports of participants completing the measure 'randomly'. While there is limited choice for measures of implicit affect, IPANAT was chosen for use in the BeHIVE-65 study because of published reports of its excellent reliability (Quirin, Kazén, & Kuhl, 2009), as well as its successful recent use in a diary-based study examining psychological influences on in-vitro fertilisation success (Massey, 2015). However, these studies were conducted in younger

adults – compared to the older adult focus of the BeHIVE-65 study. While feedback from a patient and public involvement group did not highlight any issues with this measure, a more extensive piloting procedure may have revealed the challenges with using this measure with older adults. Had this been identified, an alternative approach to measuring implicit affect would have been adopted (e.g., word-stem completion). More broadly, a programme of qualitative research exploring the acceptability of different implicit affect measures (and possibly the development of new measures) for older adults would be beneficial for future research.

9.7 ***Future Research***

The research presented in this thesis points to the possible utility of brief, positive affect enhancing interventions to improve the effectiveness of vaccinations in older adults. However, the systematic scoping review described in Chapter 8 found a paucity of intervention studies conducted in this population. Further, existing interventions were found to be relatively lengthy or require specialist equipment making them unlikely to be fit-for-purpose in a resource-stretched NHS. Therefore, there is considerable scope for a structured programme of research seeking to develop, pilot and refine brief positive affect enhancing interventions that are not only effective in improving vaccination outcomes, but also acceptable to patients and feasible for wider implementation.

The Medical Research Council (MRC) guidelines on developing and evaluating complex interventions (Craig et al., 2008) provide a useful and systematic structure around which a brief positive affect enhancing intervention to enhance vaccination responses can be developed. Broadly, the MRC guidelines can be divided into 4 interrelated stages: (1)

development (identifying the evidence base, identifying/developing theory, modelling process and outcomes); (2) feasibility/piloting (testing procedures, estimating recruitment/retention, determining sample size); (3) evaluation (assessing effectiveness, understanding change process, assessing cost-effectiveness); and (4) implementation (dissemination, surveillance and monitoring, long-term follow-up). While these stages are sequential, information gathered at one stage may necessitate returning to an earlier stage to refine and improve the intervention. Below, a brief description of a future programme of research following these stages is provided:

Development Stage

The first step to developing a complex intervention involves identifying existing evidence relating to similar work and interventions (Craig et al., 2008). Throughout this thesis and in particularly the systematic scoping review presented in Chapter 8, the existing evidence base relating to psychological and behavioural influences on vaccination responses has been reviewed. The systematic scoping review led to the understanding that brief positive mood enhancing interventions tend to influence immune function – but that existing approaches may not be suitable for adoption in primary care settings.

The underlying processes and theory relating to how improving positive affect might influence vaccination responses has been discussed throughout the latter part of this thesis. As such the 'identifying/developing theory' step has to some extent been met (although it has been noted that further work is needed on the specific biological mechanisms by which such influences are manifested). To model intervention processes and outcomes, a particularly beneficial next step would be to conduct in-depth qualitative

research with key stakeholders. Specifically, the acceptability of different intervention approaches to improve positive affect in older adults could be explored through semi-structured focus groups. Topics of interest would include: (1) whether there are pre-existing strategies used by older adults to improve their mood in the short-term; (2) whether intervention components identified from the systematic scoping review (e.g., comedy, uplifting music, positive images) would be considered acceptable for them; and (3) what would assist or prevent them engaging in such interventions. Further, these focus groups could explore possible delivery mechanisms (electronic, face-to-face) for positive affect interventions close to vaccination. Alongside these patient focused groups, interviews with health care professionals, commissioners, and other stakeholders could be conducted – with a focus on more pragmatic issues relating to the barriers for adopting such interventions. This modelling of potential outcomes and their acceptability to key stakeholders is key to ensuring at an early stage that the intervention itself, and the outcomes from any future evaluation work, would be appropriate for future implementation if the intervention demonstrated sufficient benefit.

Feasibility/Piloting Stage

Findings from the qualitative research described above, in addition to findings from the systematic scoping review presented in Chapter 8, could then be drawn together to produce an intervention. While it is not possible to be prescriptive at this stage – based on previous literature, the intervention could draw upon multiple mood manipulation techniques (e.g., comedy clips, uplifting music, mental recall, viewing positive images) and possibly be delivered in a digital or audio-visual package suitable for the age of the target population. While speculative at this stage, it may be most feasible if the intervention was designed to be 'self-administered' by vaccine recipients on the day of vaccination.

To assess the feasibility and possible utility of any developed intervention, a feasibility randomised controlled trial could be conducted, comparing the immune function and response to influenza vaccination in older adults who received the intervention against a usual care, or matched (non-positive) control. The main aims of a feasibility trial would be to ensure the developed intervention induced change in positive affect (as measured by self-report measures) and gather information needed for designing an appropriately powered definitive evaluation of this intervention. To be more specific, a feasibility trial would allow detailed data to be collected regarding: recruitment uptake and retention levels, engagement with the intervention and associated outcome measures, missing data levels, and the utility of multiple different methods (electronic and manual) of collecting non-surrogate outcome data (e.g., self-reports of flu-like illness symptoms and health service utilisation). Initial effect size estimates relating to the interventions effects on mood, surrogate-immune outcomes (e.g., antibody levels), and non-surrogate outcomes (flu-like illnesses) could be calculated from this feasibility trial to help determine the necessary sample size for a

definitive evaluation trial. Finally, further post-feasibility trial qualitative work with participants and health care professionals involved in the feasibility trial would also allow the intervention to be further refined ahead of a full-scale evaluation.

Evaluation & Implementation Stages

Once an intervention has been developed and demonstrated to be feasible with some evidence of possible benefit, a full-scale evaluation of the intervention to assess its effectiveness would likely be appropriate. One possible form this could take would be in a large multi-site randomised controlled trial (either individual-based or a cluster trial depending on the nature of the intervention). The primary outcome of such a trial would be the most clinically relevant non-surrogate outcome – likely incidence of flu-like symptoms (assessed via medical records or self-report). Surrogate immune outcomes (e.g., antibody levels) could be measured in a sub-group of participants, but it would likely be cost-prohibitive to conduct antibody testing for all participants (assuming >1000 participants). In addition to such a trial examining the interventions effectiveness, MRC guidelines highlight the importance of assessing the processes involved in of the intervention delivery. For example, fidelity would need to be assessed across research sites to ascertain whether the intervention was delivered as intended. This could be done through independent observations and self-monitoring methods (e.g., Spillane et al., 2007). Further, it is important that cost-effectiveness of the intervention be assessed using a health economic evaluation, considering possible reductions in health service utilization because of the intervention.

The final stage of the MRC guidelines on complex intervention concerns the implementation and long-term monitoring of interventions demonstrated to

be effective (both clinically and cost-effective). While comments about this stage of the proposed research programme is highly speculative, it would be important to continually monitor whether the intervention had longer term benefits over subsequent influenza seasons, improved responses to other vaccinations, or benefited populations beyond older adults. Further, the intervention content itself would likely need updating over-time as videos and music that improve mood in older adults may evolve.

9.8 ***Concluding Remarks***

The proportion of older adults sufficiently protected following vaccination against infectious diseases is troublingly low. For influenza vaccination, less than 50% of those vaccinated will be sufficiently protected to prevent infection. The individual, societal, and economic costs of this are substantial and growing alongside an aging population which is expected to double by 2050. This thesis has argued for, reviewed, and presented novel empirical evidence in support of, psycho-behavioural interventions to improve this situation. There is growing recognition amongst healthcare professionals, commissioners, and other relevant stakeholders that psychological and behavioural factors can be important determinants of health outcomes. However, evidence supporting the wider adoption of non-pharmacological interventions for many conditions – in particular those with immunological aetiologies – is lacking. The work conducted for this thesis makes a small, but significant, step in this regard. Crucially, the evidence presented in this thesis highlights the need for higher quality, robust research that extends understanding of links between behaviour, psychology, social factors, and physiology; coupled with pragmatic and mindful intervention development that minimises additional burden on an already stretched health care system.

9.9 *Chapter Summary*

This chapter has presented the key findings of the research conducted as part of this thesis and discussed them in terms of their clinical, theoretical and methodological implications. It has argued that while the findings point to a need for the development, and ultimately implementation, of positive affect improving interventions, implementing small changes to the vaccination experience (e.g., presenting uplifting stimuli) may provide benefits in primary care. The generalisability to other vaccines and natural infection exposure were discussed, as well as how findings provide partial support for the central tenants of psychoneuroimmunology and the Allostatic Load model. Next, the methodological contributions of the conducted research were highlighted including the development of the novel microarray assay, demonstrating the benefits of using intensive longitudinal designs, and the potential utility of exploring intra-individual variability in psychological experience as a health determinant. In terms of future research, a programme of mixed methods research has been suggested to develop, and trial, acceptable and feasible positive affect improving interventions to improve vaccine effectiveness in older adults.

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Appendix A

Appendix A-1: A Lay Summary of Original BeHIVE-65 Proposal Provided to Patient and Public Involvement Group

Behavioural Approaches to Optimising Vaccinations in the over-65s

Background

Those over the age of 65 are vulnerable to the flu virus and, as such, are offered the influenza vaccination (flu jab) during autumn/winter months. How well the vaccine works is dependent on the quality of a person's immune system; something that decreases with age. Therefore, the over 65, who are at greater risk of catching the flu, are also at risk of the flu jab not protecting them as well as it could.

Why are we carrying out this research?

Research has found that behavioural factors, including a person's mood, diet, and levels of physical activity can improve their immune function and response to a flu jab, providing a better protection. Through observation, we aim to find out which of these factors (mood, diet, and physical activity) leads to the best improvement in a person's response to the flu jab, as well as when would be the best time to deliver an intervention that would improve these factors in a person (i.e. before or after receiving the flu jab). By carrying out this research, we hope to get a clearer understanding of how mood, diet, and physical activity affect the success of a vaccine, which, in turn, may lead to new methods of improving the response to the flu jab.

What would be involved for participants?

We aim to recruit 200 participants aged 65-85 years old from primary care (i.e. GP surgery). We would not recruit individuals who are too frail to take part, have been diagnosed with dementia, or with a low level of English. Individuals will receive a written invitation to take part, which will be sent by their GP. The main part of the study will last 8 weeks in total. With a further brief follow up at 3 months. Participants will be compensated for their involvement in the study (amount to be decided).

At the start of the study, general (demographic) information will be collected, such as gender, marital status, and any medications you are already taking. We will also conduct a detailed assessment of mood (using questionnaires), diet (using questionnaires and body measurements, such as weight, height and waist circumference) and physical activity (by wearing a pedometer or, for a few participants, an Actiheart device over 3 successive days – see next page).

Over the 8 week period, diet (what one ate in the past 24 hours) and current mood will be assessed by conducting a short interview on the telephone at pre-arranged times, twice a week (16 telephone calls in total). Physical

activity will continue to be measured by wearing a pedometer or Actiheart device over 3 successive days per week (24 days in total).

After 4 weeks, participants will have a small blood sample taken (approx.. 3 teaspoons), receive a standard dose of the influenza vaccination (the flu jab), as well as having some body measurements taken, completing a dietary questionnaire, and recalling what they ate in the past 24 hours.

At the end of the 8 week period (4 weeks after your flu jab), participants will have a further small blood sample taken, some body measurements taken, complete a dietary questionnaire, and recall what they ate in the past 24 hours.

3 Months after the flu jab, a final small blood sample will be taken to examine the long term effects of the vaccine.

Pedometer device:

Actiheart device:

Appendix B

Appendix B-1: Research Ethics Committee Approval Letter

NHS
Health Research Authority
NRES Committee East Midlands - Nottingham 1
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0115 8839697

28 May 2014

Mr Paul Cartledge
Head of Researcdh Grants and Contracts, Research Innovation Services
King's Meadow Campus, Lenton Lane
Nottingham
NG7 2NR

Dear Mr Cartledge,

Study title:	**Behavioural and Psychological Factors Associated with Optimal Influenza Vaccination Response in Older Adults**
REC reference:	**14/EM/0201**
Protocol number:	**14045**
IRAS project ID:	**141313**

Thank you for your letter of 27th May 2014. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 22 May 2014.

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Participant information sheet (PIS)	1.1	23 May 2014

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only)	1 August 2013 - 31 July 2014 (Henderson Corporate)	31 July 2013
Letter from sponsor	University of Nottingham	28 April 2014
Non-validated questionnaire [Participant Questionnaire Booklet - Baseline]	1.0	16 April 2014
Other [Investigator's CV]	Jonathan Mark Garibaldi	
Other [GP Letter]	1.0	16 April 2014
Other [Food Diary - Instructions and Assistance]	1.0	16 April 2014

Other [Investigator's CV]	Patrick Jason Tighe	28 April 2014
Other [Dept of Primary Care Health Science - NIHR Funding Letter]		28 March 2013
Other [Investigator's CV]	Dr Heather Buchanan	25 April 2014
Other [Investigator's CV]	Kavita Vedhara	02 October 2012
Other [Diagram of Participant Participation]	1.0	28 April 2014
Other [Investigator's CV]	Dr Lucy Fairclough	28 April 2014
Other [Investigator's CV]	Thomas David Bowden	23 April 2014
Other [Invitation Letter]	1.0	16 April 2014
Other [Investigator's CV]	Kieran Ayling	24 April 2014
Other [Email - University MRC Doctoral Training Grant]		23 October 2012
Participant consent form	1.0	16 April 2014
Participant information sheet (PIS)	1.1	23 May 2014
REC Application Form	141313/602314/1/655	28 April 2014
Research protocol or project proposal	1.0	16 April 2014
Validated questionnaire [Participant Questionnaire - Visit 2 - 4 weeks after your flu jab]	1.0	16 April 2014
Validated questionnaire [Participant Questionnaire - 4 Month Follow Up]	1.0	16 April 2014
Validated questionnaire [Participant Questionnaire - Food Frequency Questionnaire]	1.0	16 April 2014
Validated questionnaire [Participant Questionnaire - Before your flu jab - Weeks 1 and 2]	1.0	16 April 2014
Validated questionnaire [Participant Questionnaire Booklet - Visit 1 - Flu Vaccination]	1.0	16 April 2014
Validated questionnaire [Participant Questionnaire - After your flu jab - Weeks 3, 4, 5 and 6]	1.0	16 April 2014

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

14/EM/0201 **Please quote this number on all correspondence**

Yours sincerely,

Ms Rachel Nelson
REC Assistant

E-mail: NRESCommittee.EastMidlands-Nottingham1@nhs.net

Appendix C

Appendix C-1: BeHIVE-65 Invitation Letter

Local letter head to be added

Dear {insert patients *name*},

The BeHIVE-65 Study

We are a team of doctors , researchers and scientists conducting a landmark scientific study into the flu vaccine in the over 65 s with The Division of Primary Care at The University o f Nottingham. We are very interested in making vaccines as effective as possible and we are contacting you to invite you to take part in a research study called The BeHIVE-65 Study (Behaviours that Help Improve Vaccine Efficacy in the over 65 s).

Each year, up to 20,000 people in the UK die from complications of the flu virus, and around 90% of these are in those aged over 65. In part, this is because the flu vaccine is less effective in older adults than younger adults due to ag eing of the immune system. However, we believe we can help to improve this situation and are determined to find ways to improve the number of people protected against flu.

You have probably heard people say that they get ill when they are run-down or stressed. But did you know that your lifestyle and emotional well-being can also influence how effective vaccines are?

Research has shown that how you eat, exercise and sleep, as well as how stressed you are, can affect how your body responds to vaccines like the flu jab. In this study, we are asking for your help to find out which of these factors (diet, exercise, sleep, mood, etc.) has the greatest impact on vaccine response in those aged over 65 and at what time (before or after vaccination) they are most important. This will help us develop ways to improve the vaccines success in the future.

We are inviting all people who attend {insert GP practice name}, who are aged over 65 and received last year's annual influenza vaccination (the flu jab). Many other GP practices around Nottingham are also involved and we are aiming for around 200 participants like you to take part in this research.

What does the research involve?

If you agree to take part, you will be asked to attend your local GP practice to complete a questionnaire and have some measurements taken (e.g., weight, height) on four occasions during the next few months. The first of which will be approximately 2 weeks before you receive this year's flu jab. We will also take small blood samples (approximately 2-3 teaspoons) on three of these occasions to see how well the vaccine has worked for you .

Over the six weeks around the time you receive the vaccine (two weeks before and four weeks after), we will also ask you to wear a pedometer (a small device that clips on your waist band or belt to measure steps) for three days each week and provide some information about things like your diet and recent mood. You will be able to provide this information in a manner of your choosing: either by filling in a booklet provided to you at the start of the study; through online surveys delivered via email; or we can call you at a pre-arranged time.

Local letter head to be added

I have included a detailed information booklet about the study with this letter for you to read.

If you are interested in taking part, or hearing more about the research, please complete and return the reply slip at the bottom of this letter.

Alternatively, you can contact one of our researchers on the contact details below:

Kieran Ayling - Telephone: {insert number} email: kieran.ayling@nottingham.ac.uk

Thom Bowden – Telephone: {insert number} email: thomas.bowden@nottingham.ac.uk

We understand that some individuals may have health problems and might feel anxious about taking part in a research project. The research team have experience of working with older people. We will work with you to suit your individual needs, e.g. by assisting you with completion of questionnaires or allowing a family member or carer can be present to help answer questions on your behalf.

Thank you.

Yours sincerely,

{Insert Research Lead GP Name & Signature here}

On behalf of The BeHIVE-65 Study Team

Reply Slip - {Insert GP Surgery Name}

Yes, I would be interested in hearing more about the BeHIVE-65 study and potentially taking part.

Please complete the following contact details

Name:

Contact Telephone Number(s) or Email:

Once you have filled in the contact details on this slip, please return it to us in the FREEPOST envelope provided. A researcher will be in contact with you once this reply slip is received.

Appendix D

Appendix D-1: Rapid Assessment of Physical Activity

PART G

Physical activities are those where you move and increase your heart rate above its resting rate, whether you do them for pleasure, work, or transportation.

Using the examples shown below, please answer the following questions about the amount and intensity of physical activity you usually do by placing a tick (✓) in the appropriate box. The intensity of the physical activity is related to the amount of energy you use to do these activities.

Examples of physical activity intensity levels:

Light activities • your heart beats slightly faster than normal • you can talk and sing	 Walking Leisurely  Stretching  Vacuuming or Light Yard Work
Moderate activities • your heart beats faster than normal • you can talk but not sing	 Fast Walking  Aerobics Class  Strength Training  Swimming Gently
Vigorous activities • your heart rate increases a lot • you can't talk or your talking is broken up by large breaths	 Stair Machine  Jogging or Running  Tennis, Racquetball, Pickleball or Badminton

	YES	NO
I rarely or never do any physical activity.	☐	☐
I do some light or moderate physical activities, but not every week.	☐	☐
I do some light physical activity every week.	☐	☐
I do moderate physical activities every week, but less than 30 minutes a day or 5 days a week.	☐	☐
I do vigorous physical activities every week, but less than 20 minutes a day or 3 days a week.	☐	☐
I do 30 minutes or more a day of moderate physical activities, 5 or more days a week.	☐	☐
I do 20 minutes or more a day of vigorous physical activities, 3 or more days a week.	☐	☐
I do activities to increase muscle strength, such as lifting weights or calisthenics, once a week or more .	☐	☐
I do activities to improve flexibility, such as stretching or yoga, once a week or more.	☐	☐

Appendix D-2: EPIC-Norfolk Food Frequency Questionnaire

Please estimate your average food use as best you can, and answer every question—do not leave ANY lines blank.

PLEASE PUT A TICK (✓) ON EVERY LINE

FOOD AND AMOUNTS	AVERAGE USE LAST YEAR								
MEAT AND FISH (medium serving)	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew or casserole									
Beefburgers									
Pork: roast, chops, stews or slices									
Chicken or other poultry, e.g. turkey									
Bacon									
Ham									
Corned beef, Spam, luncheon meats									
Sausages									
Savoury pies, e.g. meat pie, pork pie, pasties, steak & kidney pie, sausage roles									
Liver, liver paté, liver sausage									
Fried fish in batter, as in fish and chips									
Fish fingers, fish cakes									
Other white fish, fresh or frozen, e.g. cod, haddock, plaice, sole, halibut									
Oily fish, fresh or canned, e.g. mackerel, kippers, tuna, salmon, sardines, herring									
Shellfish, e.g. crab, prawns, mussels									
Fish roe, taramasalata									

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOOD AND AMOUNTS	AVERAGE USE LAST YEAR								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls									
Brown bread and rolls									
Wholemeal bread and rolls									
Cream crackers, cheese biscuits									
Crispbread e.g. Ryvita									
CEREALS (one bowl)									
Porridge, Readybrek									
Breakfast cereal, e.g. cornflakes, muesli, etc.									
POTATOES, RICE AND PASTA (medium serving)									
Boiled, mashed, instant or jacket potatoes									
Chips									
Roast potatoes									
Potato salad									
White rice									
Brown rice									
White or green pasta, e.g. spaghetti, macaroni, noodles									
Wholemeal pasta									
Lasagne, moussaka									
Pizza									

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOOD AND AMOUNTS	AVERAGE USE LAST YEAR								
SOUPS, SAUCES, AND SPREADS	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Vegetable soups (bowl)									
Meat soups (bowl)									
Sauces, e.g. white sauce, cheese sauce, gravy (tablespoon)									
Tomato ketchup (tablespoon)									
Pickles, chutney (tablespoon)									
Marmite, Bovril (teaspoon)									
Jam, marmalade, honey (teaspoon)									
Peanut butter (teaspoon)									
DRINKS									
Tea (cup)									
Coffee, instant or ground (cup)									
Coffee, decaffeinated (cup)									
Coffee whitener, e.g. Coffee-mate (teaspoon)									
Cocoa, hot chocolate (cup)									
Horlicks, Ovaltine (cup)									
Wine (glass)									
Beer, lager or cider (half pint)									
Port, sherry, vermouth, liqueurs (glass)									
Spirits, e.g. gin, brandy, whisky, vodka (single)									
Low calorie or diet fizzy soft drinks (glass)									
Fizzy soft drinks, e.g. Coca Cola, lemonade (glass)									
Pure fruit juice (100%), e.g. orange, apple juice (glass)									
Fruit squash or cordial (glass)									

PLEASE PUT A TICK (✓) ON EVERY LINE

FOOD AND AMOUNTS	AVERAGE USE LAST YEAR								
DAIRY PRODUCTS AND FATS	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream (tablespoon)									
Double or clotted cream (tablespoon)									
Low fat yoghurt or fromage frais (125g carton)									
Full fat or Greek yoghurt (125g carton)									
Dairy desserts (125g carton)									
Cheese, e.g. cheddar, brie, edam (medium serving)									
Cottage cheese, low fat soft cheese (medium serving)									
Egg as boiled, fried, scrambled, etc. (one)									
Quiche (medium serving)									
Low calorie, low fat salad cream (tablespoon)									
Salad cream, mayonnaise (tablespoon)									
French dressing (tablespoon)									
Other salad dressing (tablespoon)									
The following on bread or vegetables									
Butter (teaspoon)									
Block margarine, e.g. Stork, Krona (teaspoon)									
Polyunsaturated margarine (tub), e.g. Flora, sunflower (teaspoon)									
Other soft margarine, dairy spreads (tub), e.g. Blue Band, Clover (teaspoon)									
Low fat spread (tub), e.g. Outline, Gold (teaspoon)									
Very low fat spread (tub) (teaspoon)									

PLEASE PUT A TICK (✓) ON EVERY LINE

FOOD AND AMOUNTS	AVERAGE USE LAST YEAR								
SWEETS AND SNACKS (medium serving)	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Sweet biscuits, chocolate, e.g. digestive (one)									
Sweet biscuits, palin, e.g. Nice, ginger (one)									
Cakes, e.g. fruit, sponge, home baked									
Cakes, e.g. fruit, sponge, ready made									
Buns, pastries, e.g. scones, flapjacks, home baked									
Buns, pastries, e.g. croissants, doughnuts, ready made									
Fruit pies, tarts, crumbles, home baked									
Fruit pies, tarts, crumbles, ready made									
Sponge puddings, home baked									
Sponge puddings, ready made									
Milk puddings, e.g. rice, custard, trifle									
Ice cream, choc ices									
Chocolate, singles or squares									
Chocolate snack bars, e.g. Mars, Crunchie									
Sweets, toffees, mints									
Sugar added to tea, coffee, cereal (teaspoon)									
Crisps or other packet snacks, e.g. Wotsits									
Peanuts or other nuts									

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOOD AND AMOUNTS	AVERAGE USE LAST YEAR								
FRUIT (for seasonal fruit marked *, please estimate your average use when the fruit is in season)	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Apples (1 fruit)									
Pears (1 fruit)									
Oranges, satsumas, mandarins (1 fruit)									
Grapefruit (half)									
Bananas (1 fruit)									
Grapes (medium serving)									
Melon (1 slice)									
* Peaches, plums, apricots (1 fruit)									
* Strawberries, raspberries, kiwi fruit (medium serving)									
Tinned fruit (medium serving)									
Dried fruit, e.g. raisins, prunes (medium serving)									
VEGETABLES—Fresh, frozen or tinned (medium serving)									
Carrots									
Spinach									
Broccoli, spring greens, kale									
Brussels sprouts									
Cabbage									
Peas									
Green beans, broad beans, runner beans									
Marrow, courgettes									
Cauliflower									

Please check that you have a tick (✓) on every line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOOD AND AMOUNTS	AVERAGE USE LAST YEAR								
Vegetables—Fresh, frozen, or tinned **(medium serving)**	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Parsnips, turnips, swedes									
Leeks									
Onions									
Garlic									
Mushrooms									
Sweet peppers									
Beansprouts									
Green salad, lettuce, cucumber, celery									
Watercress									
Tomatoes									
Sweetcorn									
Beetroot									
Coleslaw									
Avocado									
Baked beans									
Dried lentils, beans, peas									
Tofu, soya meat, TVP, Vegeburger									

Please check that you have a tick (✓) on EVERY line

YOUR DIET LAST YEAR, continued

2. Are there any OTHER foods which you ate more than once a week?

YES ☐ NO ☐

IF YES, please list below

Food	Usual serving size	Number of times eaten per week

3. What type of milk did you most often use? (select one only)

Full cream, silver	☐	Semi-skimmed, red/white	☐
Skimmed/blue	☐	Channel Islands, gold	☐
Dried milk	☐	Soya	☐
Other (specify)	☐	None	☐

4. How much milk did you drink each day, including milk with tea, coffee, cereals, etc.?

None	☐	Three quarters of a pint	☐
Quarter of a pint	☐	One pint	☐
Half a pint	☐	More than one pint	☐

5. Did you usually eat breakfast cereal (excluding porridge and Readybrek mentioned earlier)?

YES ☐ NO ☐

IF YES, which brand and type of breakfast cereal, including muesli, did you usually eat? List the one or two types most often used.

Brand, e.g. Kellogg's	Type, e.g. cornflakes

6. What kind of fat did you most often use for frying, roasting, grilling, etc.? (select one only)

Butter ☐ Solid vegetable fat ☐
Lard/dripping ☐ Margarine ☐
Vegetable oil ☐ None ☐

IF YOU USED VEGETABLE OIL, please give the type, e.g. corn, sunflower

☐

7. What kind of fat did you most often use for baking cake, etc.? (select one only)

Butter ☐ Solid vegetable fat ☐
Lard/dripping ☐ Margarine ☐
Vegetable oil ☐ None ☐

IF YOU USED MARGARINE, please give the name or type, e.g. Flora, Stork

☐

8. How often did you eat food that was fried at home?

Daily ☐ Less than once a week ☐
1-3 times a week ☐ Never ☐
4-6 times a week ☐

9. How often did you eat fried food away from home?

Daily ☐ Less than once a week ☐
1-3 times a week ☐ Never ☐
4-6 times a week ☐

10. What did you do with the visible fat on your meat?

Ate most of the fat ☐ Ate as little as possible ☐
Ate some of the fat ☐ Did not eat meat ☐

11. How often did you eat grilled or roast meat?

☐☐ times a week

12. How well cooked did you usually have the grilled or roast meat?

Well done/dark brown ☐ Lightly cooked/rare ☐
Medium ☐ Did not eat meat ☐

13. How often did you add salt to food while cooking?

Always ☐ Rarely ☐
Usually ☐ Never ☐
Sometimes ☐

14. How often did you add salt to any food at the table?

Always ☐ Rarely ☐
Usually ☐ Never ☐
Sometimes ☐

15. Did you regularly use a salt substitute, e.g. LoSalt?

YES ☐ NO ☐

IF YES, which brand? ☐

16. During the course of last year, on average, how many times a week did you eat the following foods?

Food type	Times per week	Portion size
Vegetables (not including potatoes)		medium serving
Salads		medium serving
Fruit and fruit products (not including fruit juice)		medium serving or 1 fruit
Fish and fish products		medium serving
Meat, meat products and meat dishes (including bacon, ham and chicken)		medium serving

17. Have you taken any vitamins, minerals, fish oils, fibre, or other food supplements during the past year?

YES ☐ NO ☐ DON'T KNOW ☐

IF YES, please complete the table below. If you have taken more than 5 types of supplement, please put the most frequently consumed brands first.

VITAMIN SUPPLEMENTS	AVERAGE FREQUENCY—tick one box per line to show how often on average you consumed supplements										
Name and brand **Please list full name, brand and strength**	Dose No. of pills, capsules or teaspoons consumed	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Appendix D-3: Mini Nutritional Assessment

PART B

Please answer each of the following questions to the best of your knowledge by placing a tick (✓) next to the appropriate answer.

Has your food intake declined over the past 3 months due to a loss of appetite, digestive problems, chewing or swallowing difficulties?

- Severe decrease in food intake ☐
- Moderate decrease in food intake ☐
- No decrease in food intake ☐

Have you experience weight loss during the last 3 months?

- Weight loss greater than 3kg (6.6lbs) ☐
- I do not know ☐
- Weight loss between 1 and 3 kg (2.2 and 6.6lbs) ☐
- No weight loss ☐

How would you class your mobility status?

- I am bed or chair bound ☐
- I am able to get out of chair or bed, but not go out ☐
- I go out ☐

Have you suffered psychological stress or an acute disease in the past 3 months?

- Yes ☐
- No ☐

Do you take more than 3 prescription drugs per day?

- Yes ☐
- No ☐

Do you have pressure sores or skin ulcers?

- Yes ☐
- No ☐

How many full meals do you eat daily?

- 1 meal ☐
- 2 meals ☐
- 3 meals ☐

Do you eat or drink at least one serving of dairy products (e.g. milk, cheese, yoghurt) per day?

- Yes ☐
- No ☐

Do you eat two or more servings of legumes (e.g. beans, peas, nuts) or eggs per week?

- Yes ☐
- No ☐

Do you eat meat, fish, or poultry every day?

- Yes ☐
- No ☐

Do you consume two or more servings of fruit or vegetables per day?

- Yes ☐
- No ☐

How many cups of fluid, e.g. water, juice, coffee, tea, milk, do you consume per day?

- Less than 3 cups ☐
- 3 to 5 cups ☐
- More than 5 cups ☐

How would you class your ability to eat?

- I cannot eat without assistance ☐
- I can feed myself, but with difficulty ☐
- I can feed myself without a problem ☐

How do you view your nutritional status?

- I think I am malnourished ☐
- I do not know ☐
- I do not think I am malnourished ☐

When comparing yourself to other people your age, how do you consider your health?

- My health is not as good as others ☐
- I do not know ☐
- My health is as good as others ☐
- My health is better than others ☐

Appendix D-4: Perceived Stress Scale (10 Item Version)

PART H

The following questions will ask you about your thoughts and feelings during the past month. For each question, please answer by circling the appropriate number, whereby:

Never	Almost Never	Sometimes	Fairly Often	Very Often
0	1	2	3	4

	Never → Very Often				
In the last month, how often have you been upset because of something that happened unexpectedly?	0	1	2	3	4
In the last month, how often have you felt that you were unable to control the important things in your life?	0	1	2	3	4
In the last month, how often have you felt nervous and 'stressed'?	0	1	2	3	4
In the last month, how often have you felt confident about your ability to handle your personal problems?	0	1	2	3	4
In the last month, how often have you felt that things were going your way?	0	1	2	3	4
In the last month, how often have you found that you could not cope with all the things you have to do?	0	1	2	3	4
In the last month, how often have you been able to control irritations in your life?	0	1	2	3	4
In the last month, how often have you felt that you were on top of things?	0	1	2	3	4
In the last month, how often have you been angered because things were outside of your control?	0	1	2	3	4
In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?	0	1	2	3	4

Appendix D-5: Epic-Norfolk Food Diary

PART A

Please keep a food diary for everything you eat and drink today. Please refer to the booklet titled 'Food Diary Instructions and Assistance' to help you complete this.

DATE: / / DAY OF THE WEEK:

BEFORE BREAKFAST		
Food/Drink	**Description and Preparation**	**Amount**

BREAKFAST		
Food/Drink	**Description and Preparation**	**Amount**

MID MORNING—between breakfast and lunch time		
Food/Drink	**Description and Preparation**	**Amount**

LUNCH		
Food/Drink	**Description and Preparation**	**Amount**

TEA—between lunch time and the evening meal		
Food/Drink	**Description and Preparation**	**Amount**

EVENING MEAL		
Food/Drink	**Description and Preparation**	**Amount**

LATER EVENING—up to last thing at night		
Food/Drink	**Description and Preparation**	**Amount**

ANYTHING ELSE? Between meal snacks and drinks NOT already written before		
Food/Drink	**Description and Preparation**	**Amount**
Chocolate		
Toffees, sweets		
Crisps, peanuts		
Other snacks		
Beer, wine		
Sherry, spirits		
Other cold drinks		
Tea, coffee		
Other hot drinks		
Ice cream		
Anything else?		

Space to write in the recipe or ingredients of any homemade dishes, take-away meals, etc., that you have mentioned, but not described previously. Where applicable, please list amounts of ingredients and brand names. Please indicate the amount or proportion actually consumed by yourself.

END OF FOOD DIARY FOR THIS DAY

Appendix D-6: Adapted Items from the Pittsburgh Sleep Quality Diary

PART D

Below are some questions asking about your sleep. When answering the questions, please remember the questions are asking about last night and this morning.

What time did you go to bed last night?

How long, in minutes, did it take for you to fall asleep?

What time did you get up this morning?

How many hours of actual sleep did you get last night? This might be different from the number of hours you spent in bed.

Appendix D-7: International Positive and Negative Affect Schedule Short Form (Daily Response Example)

PART C

This questionnaire lists a number of words that people often use to describe different feelings and emotions. Read each item and then tick (✓) the appropriate answer in the space next to the word.

Please indicate the extent to which you feel this way right now, that is, at the present moment.

	Very slightly or not at all	A little	Moderately	Quite a bit	Extremely
1) Afraid	☐	☐	☐	☐	☐
2) Nervous	☐	☐	☐	☐	☐
3) Inspired	☐	☐	☐	☐	☐
4) Alert	☐	☐	☐	☐	☐

Appendix D-8: Implicit Positive and Negative Affect Test (Daily Response Example)

PART B

The following words are from an artificial language. They are intended to express various moods. In all languages, there are words that help to express their meanings by the way they sound (e.g., the word 'rattle' almost sounds like something that rattles). For each of the following words, please rate how well each artificial word expresses the different moods underneath them by placing a tick (✓) in the relevant box (e.g. to what extent does the word 'FILNU' convey each of the following moods: happy, helpless, energetic, tense, cheerful, inhibited?). When doing this, please try not to think too much about it and let yourself be guided by your spontaneous feelings.

TUNBA

	Doesn't fit at all	Fits somewhat	Fits quite well	Fits very well
Happy	☐	☐	☐	☐
Helpless	☐	☐	☐	☐
Energetic	☐	☐	☐	☐
Tense	☐	☐	☐	☐
Cheerful	☐	☐	☐	☐
Inhibited	☐	☐	☐	☐

TALEP

	Doesn't fit at all	Fits somewhat	Fits quite well	Fits very well
Happy	☐	☐	☐	☐
Helpless	☐	☐	☐	☐
Energetic	☐	☐	☐	☐
Tense	☐	☐	☐	☐
Cheerful	☐	☐	☐	☐
Inhibited	☐	☐	☐	☐

PART E

The following questions will ask you about your thoughts and feelings during the past few days. For each question, please answer by circling the appropriate number, whereby:

Never	Almost Never	Sometimes	Fairly Often	Very Often
0	1	2	3	4

	Never → Very Often				
In the last few days, how often have you felt that you were unable to control the important things in your life?	0	1	2	3	4
In the last few days, how often have you felt confident about your ability to handle your personal problems?	0	1	2	3	4
In the last few days, how often have you felt that things were going your way?	0	1	2	3	4
In the last few days, how often have you felt difficulties were piling up so high that you could not overcome them?	0	1	2	3	4

Appendix D-10: International Positive and Negative Affect Schedule Short Form (Day of Vaccination)

PART B

This questionnaire lists a number of words that people often use to describe different feelings and emotions. Read each item and then tick (✓) the appropriate answer in the space next to the word.

Please indicate the extent to which you feel this way right now, that is, at the present moment.

	Very slightly or not at all	A little	Moderately	Quite a bit	Extremely
1) Upset	☐	☐	☐	☐	☐
2) Hostile	☐	☐	☐	☐	☐
3) Alert	☐	☐	☐	☐	☐
4) Ashamed	☐	☐	☐	☐	☐
5) Inspired	☐	☐	☐	☐	☐
6) Nervous	☐	☐	☐	☐	☐
7) Determined	☐	☐	☐	☐	☐
8) Attentive	☐	☐	☐	☐	☐
9) Afraid	☐	☐	☐	☐	☐
10) Active	☐	☐	☐	☐	☐

Appendix D-11: Implicit Positive and Negative Affect Test (Day of Vaccination)

PART A

The following words are from an artificial language. They are intended to express various moods. In all languages, there are words that help to express their meanings by the way they sound (e.g., the word 'rattle' almost sounds like something that rattles). For each of the following words, please rate how well each artificial word expresses the different moods underneath them by placing a tick (✓) in the relevant box (e.g. to what extent does the word 'FILNU' convey each of the following moods: happy, helpless, energetic, tense, cheerful, inhibited?). When doing this, please try not to think too much about it and let yourself be guided by your spontaneous feelings.

SAFME

	Doesn't fit at all	Fits somewhat	Fits quite well	Fits very well
Happy	☐	☐	☐	☐
Helpless	☐	☐	☐	☐
Energetic	☐	☐	☐	☐
Tense	☐	☐	☐	☐
Cheerful	☐	☐	☐	☐
Inhibited	☐	☐	☐	☐

VIKES

	Doesn't fit at all	Fits somewhat	Fits quite well	Fits very well
Happy	☐	☐	☐	☐
Helpless	☐	☐	☐	☐
Energetic	☐	☐	☐	☐
Tense	☐	☐	☐	☐
Cheerful	☐	☐	☐	☐
Inhibited	☐	☐	☐	☐

TUNBA

	Doesn't fit at all	Fits somewhat	Fits quite well	Fits very well
Happy	☐	☐	☐	☐
Helpless	☐	☐	☐	☐
Energetic	☐	☐	☐	☐
Tense	☐	☐	☐	☐
Cheerful	☐	☐	☐	☐
Inhibited	☐	☐	☐	☐

TALEP

	Doesn't fit at all	Fits somewhat	Fits quite well	Fits very well
Happy	☐	☐	☐	☐
Helpless	☐	☐	☐	☐
Energetic	☐	☐	☐	☐
Tense	☐	☐	☐	☐
Cheerful	☐	☐	☐	☐
Inhibited	☐	☐	☐	☐

BELNI

	Doesn't fit at all	Fits somewhat	Fits quite well	Fits very well
Happy	☐	☐	☐	☐
Helpless	☐	☐	☐	☐
Energetic	☐	☐	☐	☐
Tense	☐	☐	☐	☐
Cheerful	☐	☐	☐	☐
Inhibited	☐	☐	☐	☐

SUKOV

	Doesn't fit at all	Fits somewhat	Fits quite well	Fits very well
Happy	☐	☐	☐	☐
Helpless	☐	☐	☐	☐
Energetic	☐	☐	☐	☐
Tense	☐	☐	☐	☐
Cheerful	☐	☐	☐	☐
Inhibited	☐	☐	☐	☐

Appendix E

Table E.1: Missing data levels for multi-item psychological pre- and post-imputation

Measure	Pre-Imputation Missing (n)	Post-Imputation Missing (n)
Positive Affect		
Week 1 Day 1	16	12
Week 1 Day 2	13	12
Week 1 Day 3	18	12
Week 2 Day 1	18	16
Week 2 Day 2	18	16
Week 2 Day 3	20	16
Week 3 Day 1	23	17
Week 3 Day 2	19	17
Week 3 Day 3	22	17
Week 4 Day 1	19	18
Week 4 Day 2	20	18
Week 4 Day 3	22	18
Week 5 Day 1	20	20
Week 5 Day 2	22	20
Week 5 Day 3	20	20
Week 6 Day 1	20	20
Week 6 Day 2	21	20
Week 6 Day 3	24	20
Day of Vaccination	14	14
Negative Affect		
Week 1 Day 1	16	11
Week 1 Day 2	13	11
Week 1 Day 3	18	11
Week 2 Day 1	19	17
Week 2 Day 2	19	17
Week 2 Day 3	21	17
Week 3 Day 1	23	18
Week 3 Day 2	19	18
Week 3 Day 3	23	18
Week 4 Day 1	19	18
Week 4 Day 2	20	18
Week 4 Day 3	21	18
Week 5 Day 1	20	20
Week 5 Day 2	22	20
Week 5 Day 3	20	20
Week 6 Day 1	20	10
Week 6 Day 2	20	10
Week 6 Day 3	24	10
Day of Vaccination	14	14
Perceived Stress		
Week 1 Day 1	11	10
Week 1 Day 2	13	11
Week 1 Day 3	14	13
Week 2 Day 1	20	17

Week 2 Day 2	19	17
Week 2 Day 3	17	15
Week 3 Day 1	19	17
Week 3 Day 2	21	19
Week 3 Day 3	20	18
Week 4 Day 1	20	20
Week 4 Day 2	19	18
Week 4 Day 3	19	19
Week 5 Day 1	24	23
Week 5 Day 2	26	23
Week 5 Day 3	21	19
Week 6 Day 1	21	21
Week 6 Day 2	24	21
Week 6 Day 3	26	24
Baseline (over the past month)	18	6

Appendix F

Table F.1: Correlations (Pearson's unless otherwise indicated) between demographic and clinical factors with non-adjusted antibody levels at 4 weeks post-vaccination

	H1N1	H3N2	B
Age	-.157	-.149	-.063
Gender[+]	.028	.071	.132
Illnesses	-.103	-.002	-.065
Medication	-.029	-.020	-.013

*p<.05, **p<.01, ***p<.001
[+] Point-biserial correlation. Positive correlations indicates females have greater antibody levels

Table F.2: Correlations (Pearson's unless otherwise indicated) between long-term modifiable participant characteristics with non-adjusted antibody levels at 4 weeks post-vaccination

	H1N1	H3N2	B
Perceived Stress	-.065	-.053	.033
BMI[‡]	**.183[t]**	.121	.055
Calories[+]	-.061	-.137	.024
Zinc[+]	-.145	-.041	.066
Selenium[+]	**-.242***	**-.338****	**-.182[t]**
Vitamin A[+]	-.169	**-.185[t]**	-.017
Nutritional Status[~]	.069	.186	.114

[t]p<.1, *p<.05, **p<.01, ***p<.001
[‡] Biserial Correlation. Positive correlations indicate those with a healthy BMI have greater antibody levels
[+] Biserial Correlation. Positive correlations indicate those with intakes meeting recommended levels have greater antibody levels
[~] Biserial Correlation. Positive correlations indicate those with adequate nutrition have greater antibody levels

Table F.3: One-way ANOVAs for categorical long-term modifiable participant characteristics on non-adjusted antibody levels at 4 weeks post-vaccination.

	H1N1			H3N2			B		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Aerobic	4,131	0.611	.656	4,131	0.838	.504	4,132	1.173	.326
Strength and Flexibility	3,132	0.118	.950	3,132	0.996	.397	3,133	0.159	.924

[t]p<.1, *p<.05, **p<.01, ***p<.001

Table F.4: Hierarchical multiple regression analysis for long-term modifiable participant characteristics predicting non-adjusted H1N1 antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.704	1.005	
Age	-0.026	0.014	-.165
Gender	0.045	0.150	.026
R^2 =.028, *F*(2,131)=1.900, *p*=.154			
Step 2			
(Constant)	2.827	0.988	
Age	-0.026	0.013	-.163
Gender	0.102	0.149	.059
Selenium	-0.368	0.149	**-.211***
R^2=.072, ΔR^2=.044, *F*(3,130)=3.352, *p*=.021*, Δ*F*(1,130)=6.108, *p*=.015*			
Step 3			
(Constant)	2.847	0.981	
Age	-0.027	0.013	**-.172***
Gender	0.061	0.150	.035
Selenium	-0.369	0.148	**-.212***
BMI	0.261	0.153	.145
R^2=.092, ΔR^2=.020, *F*(4,129)=3.271, *p*=.014*, Δ*F*(1,129)=2.882, *p*=.092			

*p<.05, **p<.01, ***p<.001

Table F.5: Hierarchical multiple regression analysis for long-term modifiable participant characteristics predicting non-adjusted H3N2 antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	4.896	1.245	
Age	-0.031	0.017	-.158
Gender	0.139	0.185	.064
R^2 =.030, *F*(2,132)=2.047, *p*=.133			
Step 2			
(Constant)	5.130	1.199	
Age	-0.031	0.016	-.158
Gender	0.229	0.180	.106
Selenium	-0.616	0.180	**-.285****
R^2=.110, ΔR^2=.080, *F*(3,131)=5.382, *p*=.002, Δ*F*(1,131)=11.719, *p*=.001*****			
Step 3			
(Constant)	4.995	1.221	
Age	-0.029	0.017	-.147
Gender	0.236	0.181	.109
Selenium	-0.587	0.186	**-.272****
Vitamin A	-0.124	0.197	-.055
R^2=.112, ΔR^2=.003, *F*(4,130)=4.117, *p*=.004, Δ*F*(1,130)=0.395, *p*=.531**			

*p<.05, **p<.01, ***p<.001

Table F.6: Hierarchical multiple regression analysis for long-term modifiable participant characteristics predicting non-adjusted B antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.960	1.085	
Age	-0.011	0.015	-.062
Gender	0.242	0.161	.129
R^2 =.021, *F*(2,132)=1.431, *p*=.243			
Step 2			
(Constant)	3.080	1.075	
Age	-0.011	0.015	-.062
Gender	.228	0.161	.154
Selenium	-.315	0.161	-.168
R^2=.049, ΔR^2=.028, *F*(3,131)=2.243, *p*=.086, Δ*F*(1,131)=3.806, *p*=.053			

*p<.05, **p<.01, ***p<.001

Table F.7: Correlations (Pearson's unless otherwise indicated) between psychological and behavioural factors over the complete diary period with non-adjusted antibody levels at 4 weeks post-vaccination

	H1N1	H3N2	B
Explicit Positive Affect	**.239*** **	.120	.103
Explicit Negative Affect[+]	-.042	-.067	-.026
Perceived Stress	**-.164[t]**	-.098	-.050
Physical Activity	.081	.105	.005
Sleep Duration	-.083	-.012	-.081
Sleep Efficiency	-.002	-.056	-.072
Calories	.137	.090	.133
Zinc	.023	-.060	.061
Vitamin E	.073	-.100	.057

[t]p<.1, *p<.05, **p<.01, ***p<.001
[+]indicates non-parametric correlation calculated (Spearman's rho)

Table F.8: Hierarchical multiple regression analyses predicting non-adjusted H1N1 antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.551	1.001	
Age	-0.024	0.014	-.158
Gender	0.117	0.146	.071
R^2 =.031, *F*(2,123) = 1.976, *p*=.143			
Step 2			
(Constant)	1.656	1.022	
Age	-0.024	0.013	-.156
Gender	0.058	0.143	.035
Explicit Positive Affect	0.138	0.048	**.249****
R^2=.092, ΔR^2=.061, *F*(3,122)=4.117, *p*=.008, Δ*F*(1,122)=8.169, *p*=.005****			
Step 3			
(Constant)	1.851	1.079	
Age	-0.024	0.013	-.156
Gender	0.063	0.144	.038
Explicit Positive Affect	0.119	0.059	**.215***
Perceived Stress	-0.022	0.039	-.061
R^2=.094, ΔR^2=.003, *F*(4,121)=3.155, *p*=.017*, Δ*F*(1,121)=0.336, *p*=.563			

*p<.05, **p<.01, ***p<.001

Table F.9: Correlations (Pearson's unless indicated otherwise) between psycho-behavioural factors as measured during the pre-vaccination, post-vaccination, and complete diary period with non-adjusted antibody levels at 4 weeks post-vaccination

	H1N1			H3N2			B		
	Pre	Post	Complete	Pre	Post	Complete	Pre	Post	Complete
Explicit Positive Affect	**.211***	**.260****	**.239****	.122	.114	.120	**.151^{t}**	.081	.103
Explicit Negative Affect^{+}	-.030	-.050	-.042	.005	-.080	-.067	-.014	-.001	-.026
Perceived Stress	-.119	-.120	**-.164^{t}**	-.085	-.057	-.098	-.028	.011	-.050
Physical Activity	.082	.073	.081	.127	.084	.105	-.019	.001	.005
Sleep Duration	-.080	-.079	-.083	-.039	.000	-.012	-.039	-.115	-.081
Sleep Efficiency	-.084	-.090	-.002	-.113	-.078	-.056	.005	-.016	-.072
Calories$^{\sim}$	.123	.031	.137	.129	-.123	.090	.116	-.040	.133
Zinc$^{\sim}$	-.013	.039	.023	.015	-.014	-.060	.072	.105	.061
Vitamin E$^{\sim}$	.077	.094	.073	-.073	-.144	-.100	.003	.025	.057

$^{t}p<.1$, $*p<.05$, $**p<.01$, $***p<.001$

$^{+}$ Non-parametric correlation (Spearman's rho)

$^{\sim}$ Biserial correlation. Positive correlations indicate those with intakes meeting recommended levels have greater antibody levels

Pre= Pre-vaccination period (from 2 weeks prior to vaccination to point of vaccination); Post= Post-vaccination period (from point of vaccination to 4 weeks after vaccination); Complete= Complete diary period (from 2 weeks prior to vaccination to 4 weeks after vaccination).

Table F.10: Hierarchical multiple regression analysis for pre-vaccination measurements of psycho-behavioural factors predicting non-adjusted H1N1 antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.327	0.984	
Age	-0.021	0.013	-.140
Gender	0.087	0.145	.054
R^2 =.023, *F*(2,123) = 1.450, *p*=.239			
Step 2			
(Constant)	1.533	1.023	
Age	-0.020	0.013	-.132
Gender	0.027	0.144	.017
Explicit Positive Affect	0.116	0.049	**.210***
R^2=.066, ΔR^2=.043, *F*(3,122)=2.856, *p*=.040*, Δ*F*(1,122)=5.562, *p*=.020*			

*p<.05, **p<.01, ***p<.001

Table F.11: Hierarchical multiple regression analysis for pre-vaccination measurements of psycho-behavioural factors predicting non-adjusted B antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.623	1.138	
Age	-0.006	0.016	-.037
Gender	0.271	0.168	.144
R^2 =.022, *F*(2,123) = 1.414, *p*=.247			
Step 2			
(Constant)	2.050	1.199	
Age	-0.006	0.015	-.032
Gender	0.228	0.169	.121
Explicit Positive Affect	0.084	0.058	.131
R^2=.039, ΔR^2=.017, *F*(3,122)=1.653, *p*=.181, Δ*F*(1,122)=2.105, *p*=.149			

*p<.05, **p<.01, ***p<.001

Table F.12: Hierarchical multiple regression analysis for post-vaccination measurements of psycho-behavioural factors predicting non-adjusted H1N1 antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.615	1.012	
Age	-0.025	0.014	-.165
Gender	0.154	0.149	.093
R^2 =.037, *F*(2,119) = 2.265, *p*=.108			
Step 2			
(Constant)	1.722	1.032	
Age	-0.025	0.013	-.163
Gender	0.102	0.146	.061
Explicit Positive Affect	0.137	0.048	**.250****
R^2=.098, ΔR^2=.062, *F*(3,118)=4.287, *p*=.007, Δ*F*(1,118)=8.062, *p*=.005****			

*p<.05, **p<.01, ***p<.001

Table F.13: Correlations (Pearson's unless otherwise indicated) between affect on the day of vaccination and non-adjusted antibody levels at 4 weeks post-vaccination

	H1N1	H3N2	B
Explicit Positive Affect	**.318*****	**.190***	**.202***
Explicit Negative Affect$^+$	-.133	-.083	-.103

^{t}p<.1, *p<.05, **p<.01, ***p<.001
$^+$indicates non-parametric correlation calculated (Spearman's rho)

Table F.14: Hierarchical multiple regression analysis for affect on the day of vaccination predicting non-adjusted H1N1 antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.550	1.035	
Age	-0.024	0.014	-.155
Gender	0.119	0.149	.072
R^2 =.030, *F*(2,120)=1.841, *p*=.163			
Step 2			
(Constant)	1.108	1.079	
Age	-0.018	0.014	-.115
Gender	0.044	0.145	.027
Explicit Positive Affect (Day of Vaccination)	0.058	0.017	**.299*** **
R^2=.115, ΔR^2=.086, *F*(3,119)=5.178, *p*=.002, Δ*F*(1,119)=11.528, *p*<.001*** **			

Table F.15: Hierarchical multiple regression analysis for affect on the day of vaccination predicting non-adjusted H3N2 antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	5.234	1.345	
Age	-0.037	0.018	**-.178***
Gender	0.241	0.194	.111
R^2 =.045, *F*(2,120)=2.825, *p*=.063			
Step 2			
(Constant)	4.259	1.452	
Age	-0.032	0.018	-.157
Gender	0.191	0.194	.088
Explicit Positive Affect (Day of Vaccination)	0.039	0.023	.154
R^2=.068, ΔR^2=.023, *F*(3,119)=2.886, *p*=.039*, Δ*F*(1,119)=2.918, *p*=.090			

Table F.16: Hierarchical multiple regression analysis for affect on the day of vaccination predicting non-adjusted B antibody levels at 4 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.833	1.184	
Age	-0.009	0.016	-.052
Gender	0.267	0.170	.142
R^2 =.023, *F*(2,120)=1.420, *p*=.246			
Step 2			
(Constant)	1.841	1.272	
Age	-0.005	0.016	-.028
Gender	0.216	0.170	.114
Explicit Positive Affect (Day of Vaccination)	0.040	0.020	**.180***
R^2=.054, ΔR^2=.031, *F*(3,119)=2.278, *p*=.083, Δ*F*(1,119)=3.925, *p*=.050*			

Table F.17: Correlations (Pearson's unless otherwise indicated) between intra-individual variability and non-adjusted antibody levels at 4 weeks post-vaccination

	H1N1	H3N2	B
Explicit Positive Affect	.102	.083	.138
Explicit Negative Affect	-.023	.021	.061
Perceived Stress	.021	-.021	.070

[t] *p*<.1, **p*<.05, ***p*<.01, ****p*<.001

Table F.18: Correlations (Pearson's unless indicated otherwise) between demographic and clinical factors with non-adjusted antibody levels at 16 weeks post-vaccination

	H1N1	H3N2	B
Age	-.149	-.077	-.038
Gender[+]	.039	.091	-.036
Illnesses	-.143	-.024	.027
Medication	-.076	-.120	-.031

*p<.05, **p<.01, ***p<.001
[+] Point-biserial correlation. Positive correlations indicates females have greater antibody levels

Table F.19: One-way ANOVAs for categorical demographic factors on non-adjusted antibody levels at 16 weeks post-vaccination

	H1N1			H3N2			B		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Income	7,109	0.976	.452	7,109	0.683	.686	7,109	0.372	.917
Marital Status	4,115	1.375	.247	4,115	1.823	.129	4,115	0.492	.741
Education	3,114	0.762	.518	3,114	1.997	.118	3,114	0.354	.786

*p<.05, **p<.01, ***p<.001

Table F.20: Correlations (Pearson's unless indicated otherwise) between long-term modifiable participant characteristics with non-adjusted antibody levels at 16 weeks post-vaccination

	H1N1	H3N2	B
Perceived Stress	-.139	-.111	-.034
BMI[‡]	**-.186[t]**	**-.253***	**-.200[t]**
Calories[+]	.034	-.074	-.037
Iron[+]	-.166	-.002	-.006
Zinc[+]	-.038	.103	.015
Selenium[+]	**-.190[t]**	-.160	-.123
Vitamin A[+]	-.129	-.098	-.093
Vitamin E[+]	-.062	-.117	.117
Nutritional Status[~]	-.092	-.052	.020

[t]p<.1, *p<.05, **p<.01, ***p<.001
[‡] Biserial Correlation. Positive correlations indicate those with a healthy BMI have greater antibody levels
[+] Biserial Correlation. Positive correlations indicate those with intakes meeting recommended levels have greater antibody levels
[~] Biserial Correlation. Positive correlations indicate those with adequate nutrition have greater antibody levels

Table F.21: One-way ANOVAs for categorical long-term modifiable participant characteristics on non-adjusted antibody levels at 16 weeks post-vaccination

	H1N1			H3N2			B		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Aerobic	4,115	0.548	.701	4,115	0.400	.809	4,10.224[+]	0.810	.546
Strength and Flexibility	2,117	1.138	.324	**2,117**	**3.917**	**.023***	2,117	1.944	.148

[t]p<.1, *p<.05, **p<.01, ***p<.001
[+]Brown-Forsythe test

Table F.22: Hierarchical multiple regression analysis for long-term modifiable participant characteristics predicting non-adjusted H1N1 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.504	1.160	
Age	-0.023	0.016	-.134
Gender	0.101	0.176	.053
R^2 =.021, *F*(2,116)=1.246, *p*=.291			
Step 2			
(Constant)	2.554	1.153	
Age	-0.022	0.016	-.130
Gender	0.141	0.177	.074
Selenium	-0.284	0.178	-.147
R^2=.042, ΔR^2=.021, *F*(3,115)=1.689, *p*=.173, Δ*F*(1,115)=2.540, *p*=.114			
Step 3			
(Constant)	2.195	1.297	
Age	-0.021	0.016	-.125
Gender	0.161	0.180	.084
Selenium	-0.301	0.181	-.156
BMI	0.011	0.017	.057
R^2=.045, ΔR^2=.003, *F*(4,114)=1.353, *p*=.255, Δ*F*(1,114)=0.372, *p*=.543			

*p<.05, **p<.01, ***p<.001
Note: 1 multivariate outlier was excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

Table F.23: Hierarchical multiple regression analysis for long-term modifiable participant characteristics predicting non-adjusted H3N2 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	3.490	1.354	
Age	-0.010	0.018	-.054
Gender	0.196	0.205	.094
R^2 =.012, *F*(2,103)=0.630, *p*=.534			
Step 2			
(Constant)	2.083	1.516	
Age	-0.006	0.018	-.031
Gender	0.241	0.203	.115
BMI	0.038	0.019	.192
R^2=.048, ΔR^2=.036, *F*(3,102)=2.714, *p*=.169, Δ*F*(1,102)=3.845, *p*=.053			
Step 3			
(Constant)	2.697	1.506	
Age	-0.009	0.018	-.049
Gender	0.256	0.199	.123
BMI	0.029	0.019	.148
Flexibility vs None	-0.541	0.230	**-.227***
R^2=.097, ΔR^2=.049, *F*(4,101)=2.726, *p*=.033*, Δ*F*(1,101)=5.536, *p*=.021*			

*p<.05, **p<.01, ***p<.001
Note: 1 multivariate outlier was excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

Table F.24: Hierarchical multiple regression analysis for long-term modifiable participant characteristics predicting non-adjusted B antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	β
(Constant)	2.808	1.263	
Age	-0.006	0.017	-.035
Gender	-0.069	0.192	-.033
R^2 =.002, F(2,116)=0.131, p=.877			
Step 2			
(Constant)	1.826	1.409	
Age	-0.004	0.017	-.023
Gender	-0.021	0.193	-.010
BMI	0.029	0.019	.144
R^2=.022, ΔR^2=.020, F(3,115)=0.879, p=.455, ΔF(1,115)=2.370, p=.126			

*p<.05, **p<.01, ***p<.001

Note: 1 multivariate outlier was excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

Table F.25: Correlations (Pearson's unless indicated otherwise) between non-adjusted antibody levels at 16 weeks post-vaccination and psycho-behavioural factors over the complete diary period

	H1N1	H3N2	B
Explicit Positive Affect	**.280****	.095	.103
Explicit Negative Affect^{+}	**-.164^{t}**	**-.208***	-.076
Perceived Stress	**-.224***	**-.199***	-.103
Physical Activity	.065	-.019	-.043
Sleep Duration	-.014	-.025	-.037
Sleep Efficiency	.011	-.112	-.037
Calories$^{\sim}$	.059	-.008	.067
Iron$^{\sim}$	.052	-.106	.093
Zinc$^{\sim}$	.061	-.117	.031
Vitamin E$^{\sim}$	-.145	**-.306****	-.112

^{t}p<.1, *p<.05, **p<.01, ***p<.001

$^{+}$indicates non-parametric correlation calculated (Spearman's rho)

$^{\sim}$Biserial Correlation. Positive correlations indicate those with intakes meeting recommended levels have greater antibody levels

Table F.26: Hierarchical multiple regression analysis for non0adjusted H1N1 antibody levels at 16 weeks post-vaccination and psycho-behavioural factors over the complete diary period

Step 1	B	SE B	*β*
(Constant)	2.796	1.260	
Age	-0.028	0.017	-.154
Gender	0.113	0.188	.058
R^2 =.027, *F*(2,105)=1.477, *p*=.223			
Step 2			
(Constant)	1.349	1.301	
Age	-0.024	0.017	-.133
Gender	0.067	0.182	.034
Explicit Positive Affect	0.186	0.061	**.285****
R^2=.108, ΔR^2=.080, *F*(3,104)=4.189, *p*=.008, Δ*F*(1,104)=9.377, *p*=.003****			
Step 3			
(Constant)	2.031	1.393	
Age	-0.026	0.017	-.145
Gender	0.088	0.182	.045
Explicit Positive Affect	0.135	0.071	.208
Perceived Stress	-0.063	0.047	-.146
R^2=.123, ΔR^2=.015, *F*(4,103)=3.613, *p*=.008, Δ*F*(1,103)=1.792, *p*=.184**			
Step 4			
(Constant)	2.478	1.484	
Age	-0.026	0.017	-.145
Gender	0.073	0.183	.037
Explicit Positive Affect	0.146	0.072	**.224***
Perceived Stress	-0.042	0.053	-.097
Explicit Negative Affect	-0.257	0.291	-.092
R^2=.130, ΔR^2=.007, *F*(5,102)=3.040, *p*=.013*, Δ*F*(1,102)=0.778, *p*=.380			

**p*<.05, ***p*<.01, ****p*<.001

Note: 4 multivariate outliers were excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

Table F.27: Hierarchical multiple regression analysis for non-adjusted H3N2 antibody levels at 16 weeks post-vaccination and psycho-behavioural factors over the complete diary period

Step 1	B	SE B	*β*
(Constant)	3.959	1.346	
Age	-0.018	0.018	-.092
Gender	0.269	0.201	.129
R^2 =.025, *F*(2,105)=1.373, *p*=.258			
Step 2			
(Constant)	4.947	1.325	
Age	-0.027	0.018	-.141
Gender	0.451	0.201	**.216***
Vitamin E	-0.668	0.206	**-.315****
R^2=.115, ΔR^2=.089, *F*(3,104)=4.189, *p*=.005, Δ*F*(1,104)=10.483, *p*=.002****			
Step 3			
(Constant)	6.445	1.484	
Age	-0.029	0.018	-.152
Gender	0.446	0.198	**.213***
Vitamin E	-0.727	0.205	**-.343*****
Explicit Negative Affect	-0.578	0.274	**-.194***
R^2=.151, ΔR^2=.037, *F*(4,103)=4.597, *p*=.002, Δ*F*(1,103)=4.462, *p*=.037***			
Step 4			
(Constant)	6.186	1.480	
Age	-0.029	0.017	-.153
Gender	0.452	0.196	**.216***
Vitamin E	-0.681	0.205	**-.321****
Explicit Negative Affect	-0.356	0.303	-.119
Perceived Stress	-0.077	0.047	-.166
R^2=.173, ΔR^2=.022, *F*(5,102)=4.277, *p*=.001, Δ*F*(1,102)=2.693, *p*=.104**			

*p<.05, **p<.01, ***p<.001

Note: 4 multivariate outliers were excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

Table F.28: Correlations (Pearson's unless indicated otherwise) between non-adjusted antibody levels at 16 weeks post-vaccination and independent variables as measured during the pre-vaccination period

	H1N1	H3N2	B
Explicit Positive Affect	**.299****	.141	**.159^t**
Explicit Negative Affect$^+$	-.127	**-.174^t**	-.072
Perceived Stress	**-.193***	**-.169^t**	-.063
Physical Activity	.087	.008	-.020
Sleep Duration	.028	-.018	.010
Sleep Efficiency	.047	-.070	.045
Calories$^{\sim}$	.168	.147	.092
Iron$^{\sim}$	.213	-.025	.217
Zinc$^{\sim}$	-.025	-.035	.006
Vitamin E$^{\sim}$	.016	-.127	-.075

^{t}p<.1, *p<.05, **p<.01, ***p<.001

$^+$indicates non-parametric correlation calculated (Spearman's rho)

$^{\sim}$Biserial Correlation. Positive correlations indicate those with intakes meeting recommended levels have greater antibody levels

Table F.29: Correlations (Pearson's unless indicated otherwise) between non-adjusted antibody levels at 16 weeks post-vaccination and independent variables as measured during the post-vaccination period

	H1N1	H3N2	B
Explicit Positive Affect	**.306****	.112	.103
Explicit Negative Affect[+]	**-.178[t]**	**-.199***	-.041
Perceived Stress	**-.179[t]**	-.126	-.051
Physical Activity	.057	-.018	-.049
Sleep Duration	-.036	.003	-.054
Sleep Efficiency	.004	-.031	.013
Calories[~]	.016	-.108	-.003
Iron[~]	-.047	-.177	-.014
Zinc[~]	-.019	-.019	-.008
Vitamin E[~]	**-.190[t]**	**-.329****	-.182

[t]p<.1, *p<.05, **p<.01, ***p<.001
[+]indicates non-parametric correlation calculated (Spearman's rho)
[~]Biserial Correlation. Positive correlations indicate those with intakes meeting recommended levels have greater antibody levels

Table F.30: Hierarchical multiple regression analysis for pre-vaccination measurements of psycho-behavioural factors predicting non-adjusted H1N1 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	β
(Constant)	2.432	1.255	
Age	-0.022	0.017	-.121
Gender	0.046	0.187	.023
R^2 =.015, F(2,107)=0.823, p=.442			
Step 2			
(Constant)	1.028	1.353	
Age	-0.022	0.016	-.123
Gender	0.000	0.118	.000
Explicit Positive Affect	0.230	0.064	**.327*****
R^2=.122, ΔR^2=.106, F(3,106)=4.891, p=.003, ΔF(1,106)=12.844, p=.001*****			
Step 3			
(Constant)	1.130	1.342	
Age	-0.022	0.016	-.124
Gender	0.003	0.179	.002
Explicit Positive Affect	0.221	0.075	**.315****
Perceived Stress	-0.010	0.046	-.024
R^2=.122, ΔR^2=.000, F(4,105)=3.648, p=.008, ΔF(1,105)=0.048, p=.827**			

*p<.05, **p<.01, ***p<.001
Note: 1 multivariate outlier was excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

Table F.31: Hierarchical multiple regression analysis for pre-vaccination measurements of psycho-behavioural factors predicting non-adjusted H3N2 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	3.782	1.381	
Age	-0.015	0.019	-.075
Gender	0.223	0.209	.104
R^2 =.017, *F*(2,104)=0.885, *p*=.416			
Step 2			
(Constant)	4.111	1.499	
Age	-0.014	0.019	-.070
Gender	0.203	0.212	.095
Explicit Negative Affect	-0.171	0.299	-.057
R^2=.020, ΔR^2=.003, *F*(3,103)=0.695, *p*=.557, Δ*F*(1,103)=0.328, *p*=.568			
Step 3			
(Constant)	4.096	1.482	
Age	-0.017	0.019	-.086
Gender	0.263	0.212	.122
Explicit Negative Affect	0.063	0.322	.021
Perceived Stress	-0.097	0.053	-.194
R^2=.051, ΔR^2=.031, *F*(4,102)=1.377, *p*=.247, Δ*F*(1,102)=3.374, *p*=.069			

*p<.05, **p<.01, ***p<.001
Note: 4 multivariate outliers were excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

Table F.32: Hierarchical multiple regression analysis for pre-vaccination measurements of psycho-behavioural factors predicting non-adjusted B antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.492	1.319	
Age	-0.003	0.018	-.014
Gender	-0.056	0.199	-.027
R^2 =.001, *F*(2,108)=0.051, *p*=.951			
Step 2			
(Constant)	1.678	1.393	
Age	-.001	0.018	-.007
Gender	-.086	0.198	-.042
Explicit Positive Affect	0.118	0.069	.163
R^2=.027, ΔR^2=.026, *F*(3,107)=0.995, *p*=.398, Δ*F*(1,107)=2.881, *p*=.093			

*p<.05, **p<.01, ***p<.001

Table F.33: Hierarchical multiple regression analysis for post-vaccination measurements of psycho-behavioural factors predicting non-adjusted H1N1 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.853	1.300	
Age	-0.028	0.018	-.155
Gender	0.091	0.192	.047
R^2 =.026, *F*(2,100)=1.327, *p*=.270			
Step 2			
(Constant)	1.393	1.327	
Age	-0.026	0.017	-.145
Gender	0.093	0.184	.048
Explicit Positive Affect	0.203	0.064	**.301****
R^2=.116, ΔR^2=.090, *F*(3,99)=4.339, *p*=.006, Δ*F*(1,99)10.120, *p*=.002****			
Step 3			
(Constant)	1.826	1.337	
Age	-0.030	0.017	-.168
Gender	0.177	0.188	.091
Explicit Positive Affect	0.205	0.063	**.304****
Vitamin E	-0.332	0.192	-.169
R^2=.142, ΔR^2=.026, *F*(4,98)=4.068, *p*=.004, Δ*F*(1,98)=2.994, *p*=.087**			
Step 4			
(Constant)	1.944	1.423	
Age	-0.030	0.017	-.168
Gender	0.181	0.190	.093
Explicit Positive Affect	0.194	0.079	**.286***
Vitamin E	-0.325	0.195	-.165
Perceived Stress	-0.013	0.051	-.030
R^2=.143, ΔR^2=.001, *F*(5,97)=3.236, *p*=.010, Δ*F*(1,97)=0.063, *p*=.802**			
Step 5			
(Constant)	2.714	1.519	
Age	-0.031	0.017	-.174
Gender	0.167	0.189	.086
Explicit Positive Affect	0.223	0.082	**.330****
Vitamin E	-0.364	0.196	-.185
Perceived Stress	0.036	0.062	.084
Explicit Negative Affect	-0.451	0.323	-.161
R^2=.160, ΔR^2=.017, *F*(6,96)=3.049, *p*=.009, Δ*F*(1,96)=1.955, *p*=.165**			

**p*<.05, ***p*<.01, ****p*<.001

Note: 3 multivariate outliers were excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

Table F.34: Hierarchical multiple regression analysis for post-vaccination measurements of psycho-behavioural factors predicting non-adjusted H3N2 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	3.996	1.408	
Age	-0.018	0.019	-.093
Gender	0.265	0.208	.126
R^2 =.024, *F*(2,100)=1.240, *p*=.294			
Step 2			
(Constant)	4.916	1.375	
Age	-0.026	0.019	-.136
Gender	0.437	0.205	**.208***
Vitamin E	-0.680	0.21	**-.320****
R^2=.118, ΔR^2=.094, *F*(3,99)=4.415, *p*=.006, Δ*F*(1,99)=10.528, *p*=.002****			
Step 3			
(Constant)	6.142	1.527	
Age	-0.028	0.018	-.142
Gender	0.440	0.203	**.209***
Vitamin E	-0.695	0.208	**-.326****
Explicit Negative Affect	-0.501	0.284	-.165
R^2=.145, ΔR^2=.027, *F*(4,98)=4.162, *p*=.004, Δ*F*(1,98)=3.120, *p*=.080**			

*p<.05, **p<.01, ***p<.001

Note: 3 multivariate outliers were excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

Table F.35: Correlations (Pearson's unless indicated otherwise) between affect on the day of vaccination with non-adjusted antibody levels at 16 weeks post-vaccination

	H1N1	H3N2	B
Explicit Positive Affect	**.365*****	.124	.158
Explicit Negative Affect$^+$	**-.162^t**	-.158	-.057

^{t}p<.1, *p<.05, **p<.01, ***p<.001

$^+$indicates non-parametric correlation calculated (Spearman's rho)

Table F.36: Hierarchical multiple regression analysis for psychological factors on the day of vaccination predicting non-adjusted H1N1 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.106	1.290	
Age	-0.017	0.018	-.094
Gender	0.026	0.189	.013
R^2 =.009, *F*(2,103)=0.469, *p*=.627			
Step 2			
(Constant)	-0.328	1.359	
Age	-0.004	0.017	-.020
Gender	-0.039	0.177	-.020
Explicit Positive Affect	0.086	0.022	**.369*** **
R^2=.139, ΔR^2=.130, *F*(3,102)=5.469, *p*=.002, Δ*F*(1,102)=15.337, *p*<.001*** **			
Step 3			
(Constant)	0.109	1.383	
Age	-0.002	0.017	-.013
Gender	-0.050	0.178	.026
Explicit Positive Affect	0.086	0.022	**.370*** **
Explicit Negative Affect	-0.055	0.063	-.082
R^2=.145, ΔR^2=.007, *F*(4,101)=4.287, *p*=.003, Δ*F*(1,101)=0.777, *p*=.380**			

*p<.05, **p<.01, ***p<.001

Note: 4 multivariate outliers were excluded from this analyses based on having a Mahalanobis distance above the critical χ^2 threshold.

Table F.37: Correlations (Pearson's unless indicated otherwise) between intra-individual variability and non-adjusted antibody levels at 16 weeks post-vaccination

	H1N1	H3N2	B
Explicit Positive Affect	-.151	-.008	-.048
Explicit Negative Affect	.018	.026	.127
Perceived Stress	**-.294****	**-.200***	**-.185^t**

^{t}p<.1, *p<.05, **p<.01, ***p<.001

Table F.38: Hierarchical multiple regression analysis for intra-individual variability of psychological factors predicting non-adjusted H1N1 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	1.827	1.287	
Age	-0.014	0.017	-.084
Gender	0.162	0.197	.084
R^2 =.015, *F*(2,95)=0.714, *p*=.492			
Step 2			
(Constant)	1.980	1.239	
Age	-0.014	0.017	-.084
Gender	0.114	0.190	.059
IIV Perceived Stress	-0.325	0.111	**-.288****
R^2=.097, ΔR^2=.082, *F*(3,94)=3.377, *p*=.022*, Δ*F*(1,94)=8.588, *p*=.004**			
Step 3			
(Constant)	2.301	1.249	
Age	-0.015	0.017	-.090
Gender	0.100	0.189	.052
IIV Perceived Stress	-0.305	0.111	**-.270****
Perceived Stress (LGM-Intercept)	-0.067	0.044	-.149
R^2=.119, ΔR^2=.022, *F*(4,93)=3.146, *p*=.018*, Δ*F*(1,93)=2.313, *p*=.132			

**p*<.05, ***p*<.01, ****p*<.001

Table F.39: Hierarchical multiple regression analysis for intra-individual variability of psychological factors predicting non-adjusted H3N2 antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.269	1.402	
Age	-0.010	0.019	-.052
Gender	0.339	0.215	.160
R^2 =.029, *F*(2,95)=1.424, *p*=.246			
Step 2			
(Constant)	3.479	1.385	
Age	-0.010	0.019	-.052
Gender	0.304	0.213	.143
IIV Perceived Stress	-0.233	0.124	-.188
R^2=.064, ΔR^2=.035, *F*(3,94)=2.145, *p*=.100, Δ*F*(1,94)=3.513, *p*=.064			
Step 3			
(Constant)	3.813	1.398	
Age	-0.011	0.019	-.058
Gender	0.290	0.212	.137
IIV Perceived Stress	-0.211	0.124	-.170
Perceived Stress (LGM-Intercept)	-0.069	0.049	-.142
R^2=.084, ΔR^2=.020, *F*(4,93)=2.217, *p*=.084, Δ*F*(1,93)=2.002, *p*=.160			

**p*<.05, ***p*<.01, ****p*<.001

Table F.40: Hierarchical multiple regression analysis for intra-individual variability of psychological factors predicting non-adjusted B antibody levels at 16 weeks post-vaccination

Step 1	B	SE B	*β*
(Constant)	2.533	1.438	
Age	-0.004	0.020	-.020
Gender	0.021	0.220	.010
R^2 =.001, *F*(2,95)=0.025, *p*=.976			
Step 2			
(Constant)	2.643	1.422	
Age	-0.004	0.019	-.020
Gender	-0.013	0.219	-.006
IIV Perceived Stress	-0.232	0.127	-.185
R^2=.035, ΔR^2=.034, *F*(3,94)=1.121, *p*=.344, Δ*F*(1,94)=3.314, *p*=.072			
Step 3			
(Constant)	2.774	1.448	
Age	-0.004	0.019	-.022
Gender	-0.019	0.220	-.009
IIV Perceived Stress	-0.223	0.129	-.178
Perceived Stress (LGM-Intercept)	-0.027	0.051	-.055
R^2=.038, ΔR^2=.003, *F*(4,93)=0.907, *p*=.464, Δ*F*(1,93)=0.288, *p*=.593			

*p<.05, **p<.01, ***p<.001

Appendix G

EMBASE, Medline and PsycINFO and PsycArticles were searched in August 2016 – using the below search strategy.

1. manipulat*.ti,ab.
2. modulat*.ti,ab.
3. Interven*.ti,ab.
4. induc*.ti,ab.
5. modif*.ti,ab.
6. elicit*.ti,ab.
7. stimuli.ti,ab.
8. Film*.ti,ab.
9. video*.ti,ab.
10. movie*.ti,ab.
11. music*.ti,ab.
12. hypno*.ti,ab.
13. laugh*.ti,ab.

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14. *Emotional States/
15. mood*.ti,ab.
16. positive affect*.ti,ab.
17. emotion*.ti,ab.
18. happ*.ti,ab.

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19. immune*.ti,ab.
20. cytokine*.ti,ab.
21. neuroimmun*.ti,ab.
22. antibod*.ti,ab.
23. *Immunology/ or *IMMUNE SYSTEM/
24. exp CYTOKINES/
25. exp ANTIBODIES/
26. 19 or 20 or 21 or 22 or 23 or 24 or 25

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27. exp animals/ not humans.sh.
28. humour.ti,ab.
29. humor.ti,ab.
30. humorous.ti,ab.
31. humourous.ti,ab.

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32. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 28 or 29 or 30 or 31

33. 14 or 15 or 16 or 17 or 18 or 28 or 29 or 30 or 31

34. 19 or 20 or 21 or 22 or 23 or 24 or 25

35. 32 and 33 and 34

36. 35 not 27

37. limit 36 to (peer reviewed journal and english language)

Appendix H

QUALITY ASSESSMENT TOOL FOR QUANTITATIVE STUDIES

COMPONENT RATINGS

A) SELECTION BIAS

(Q1) Are the individuals selected to participate in the study likely to be representative of the target population?

1 Very likely
2 Somewhat likely
3 Not likely
4 Can't tell

(Q2) What percentage of selected individuals agreed to participate?

1 80 - 100% agreement
2 60 – 79% agreement
3 less than 60% agreement
4 Not applicable
5 Can't tell

RATE THIS SECTION	STRONG	MODERATE	WEAK
See dictionary	1	2	3

B) STUDY DESIGN

Indicate the study design

1 Randomized controlled trial
2 Controlled clinical trial
3 Cohort analytic (two group pre + post)
4 Case-control
5 Cohort (one group pre + post (before and after))
6 Interrupted time series
7 Other specify _____
8 Can't tell

Was the study described as randomized? If NO, go to Component C.

No Yes

If Yes, was the method of randomization described? (See dictionary)

No Yes

If Yes, was the method appropriate? (See dictionary)

No Yes

RATE THIS SECTION	STRONG	MODERATE	WEAK
See dictionary	1	2	3

C) CONFOUNDERS

(Q1) Were there important differences between groups prior to the intervention?

1 Yes
2 No
3 Can't tell

The following are examples of confounders:

1 Race
2 Sex
3 Marital status/family
4 Age
5 SES (income or class)
6 Education
7 Health status
8 Pre-intervention score on outcome measure

(Q2) If yes, indicate the percentage of relevant confounders that were controlled (either in the design (e.g. stratification, matching) or analysis)?

1 80 – 100% (most)
2 60 – 79% (some)
3 Less than 60% (few or none)
4 Can't Tell

RATE THIS SECTION	STRONG	MODERATE	WEAK
See dictionary	1	2	3

D) BLINDING

(Q1) Was (were) the outcome assessor(s) aware of the intervention or exposure status of participants?

1 Yes
2 No
3 Can't tell

(Q2) Were the study participants aware of the research question?

1 Yes
2 No
3 Can't tell

RATE THIS SECTION	STRONG	MODERATE	WEAK
See dictionary	1	2	3

E) DATA COLLECTION METHODS

(Q1) Were data collection tools shown to be valid?

1 Yes
2 No
3 Can't tell

(Q2) Were data collection tools shown to be reliable?

1 Yes
2 No
3 Can't tell

RATE THIS SECTION	STRONG	MODERATE	WEAK
See dictionary	1	2	3

F) WITHDRAWALS AND DROP-OUTS

(Q1) Were withdrawals and drop-outs reported in terms of numbers and/or reasons per group?

1 Yes
2 No
3 Can't tell
4 Not Applicable (i.e. one time surveys or interviews)

(Q2) Indicate the percentage of participants completing the study. (If the percentage differs by groups, record the lowest).

1 80 -100%
2 60 - 79%
3 less than 60%
4 Can't tell
5 Not Applicable (i.e. Retrospective case-control)

RATE THIS SECTION	STRONG	MODERATE	WEAK	
See dictionary	1	2	3	Not Applicable

G) INTERVENTION INTEGRITY

(Q1) What percentage of participants received the allocated intervention or exposure of interest?

1 80 -100%
2 60 - 79%
3 less than 60%
4 Can't tell

(Q2) Was the consistency of the intervention measured?

1 Yes
2 No
3 Can't tell

(Q3) Is it likely that subjects received an unintended intervention (contamination or co-intervention) that may influence the results?

4 Yes
5 No
6 Can't tell

H) ANALYSES

(Q1) Indicate the unit of allocation (circle one)

community organization/institution practice/office individual

(Q2) Indicate the unit of analysis (circle one)

community organization/institution practice/office individual

(Q3) Are the statistical methods appropriate for the study design?

1 Yes
2 No
3 Can't tell

(Q4) Is the analysis performed by intervention allocation status (i.e. intention to treat) rather than the actual intervention received?

1 Yes
2 No
3 Can't tell

GLOBAL RATING

COMPONENT RATINGS
Please transcribe the information from the gray boxes on pages 1-4 onto this page. See dictionary on how to rate this section.

A	**SELECTION BIAS**	**STRONG**	**MODERATE**	**WEAK**	
		1	2	3	
B	**STUDY DESIGN**	**STRONG**	**MODERATE**	**WEAK**	
		1	2	3	
C	**CONFOUNDERS**	**STRONG**	**MODERATE**	**WEAK**	
		1	2	3	
D	**BLINDING**	**STRONG**	**MODERATE**	**WEAK**	
		1	2	3	
E	**DATA COLLECTION METHOD**	**STRONG**	**MODERATE**	**WEAK**	
		1	2	3	
F	**WITHDRAWALS AND DROPOUTS**	**STRONG**	**MODERATE**	**WEAK**	
		1	2	3	Not Applicable

GLOBAL RATING FOR THIS PAPER (circle one):

1	STRONG	(no WEAK ratings)
2	MODERATE	(one WEAK rating)
3	WEAK	(two or more WEAK ratings)

With both reviewers discussing the ratings:

Is there a discrepancy between the two reviewers with respect to the component (A-F) ratings?

No Yes

If yes, indicate the reason for the discrepancy

1	Oversight
2	Differences in interpretation of criteria
3	Differences in interpretation of study

Final decision of both reviewers (circle one):

1	**STRONG**
2	**MODERATE**
3	**WEAK**