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Human Development and Health

Effects of probiotics on markers of immunity and inflammation in the elderly

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by

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Thesis for the degree of Doctor of Philosophy

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Abstract

Faculty of Medicine
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Human life span is increasing steadily. Between 1970 and 2016 global life expectancy rose by an average of 13.5 years for men and 14.8 years for women. Although this is the result of better interventions and preventative strategies, the immune system undergoes fundamental changes throughout the ageing process. Some aspects of innate and acquired immunity decline, termed immunosenescence, while low grade inflammation emerges, termed inflammageing. Together, these make older people more susceptible to infections, poor responses to vaccinations and to many chronic conditions. Ageing also results in changes in the gut microbiota, which plays a role in controlling host immune and inflammatory responses. Conversely, the host immune system has a role in controlling the microbiota. Age-related changes in immunity, inflammation and gut microbiota may be linked. Probiotics are microorganisms, usually bacteria, which are reported to confer health benefits on the host. They act through interaction with the existing microbiota, the gut-associated immune system, and enterocytes. The effect of probiotics on immune function and markers of inflammation have not been well explored in elderly care home residents (ECHRs). In order to prepare for analyses conducted as part of a trial investigating a probiotic intervention in ECHRs, a pilot study investigating the effect of blood storage time on a range of immune and inflammatory biomarkers was conducted. It was identified that some markers need to be measured using fresh blood while others can be measured in blood stored for up to 24 hours, or in some cases for longer. Subsequently, blood samples from a subset of subjects enrolled in a placebo-controlled trial of the combination of two probiotic organisms (*Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium animalis* subsp. BB-12) in ECHRs were used to investigate the effects of the probiotics on a range of markers of innate and acquired immunity, low grade inflammation and the response to seasonal influenza vaccination. None of these markers was significantly affected by the probiotic intervention. Using samples obtained at study entry, it was identified that frailty and age were associated with some markers of low-grade inflammation. In addition to this mechanistical exploration, the *In vitro* epithelial cell line Caco-2 was used as a model of the human epithelial gut to investigate the effects of LGG and BB-12 on barrier function and inflammation. Neither live nor heat-inactivated LGG or BB-12 themselves induced an inflammatory response. Transepithelial Electrical Resistance (TEER) tend to increase more when the live version was used when compared with the heat-inactivated version. Lastly, the exposure to either organism (heat-inactivated) for 24 hours prior to stimulation with an inflammatory cocktail reduced the inflammatory response (assessed by measuring cytokines in the culture medium). LGG and BB-12 were equally effective, however, heat-inactivated organisms did not affect the loss of barrier function caused by the inflammatory cocktail. Research conducted in this thesis indicates that probiotics strains LGG or BB-12 did not influenced immune parameters in ECHRs, whilst the in vitro evidence suggest a possible “local” effect exerted by these strains on epithelial cells. Overall, this area warrants further research throughout a wider exploration of the heterogeneity of the ageing process. Studying different degrees of frailty, and environments is paramount. Likewise, more evidence is required to link localized effects exerted on epithelial cells. Adding an understanding of surrounding cells interacting with gut epithelia will allow to progress the understanding of systemic effects from a local perspective.

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Research Thesis: Declaration of Authorship

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I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly while in candidature for a research degree at this University;
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3. Where I have consulted the published work of others, this is always clearly attributed;
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Signature: Date: 5TH JUNE 2020

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To scientists, dancers and athletes that defeat limits. To those great minds and spirits that have changed the world. To the people that lived, live and will live their own existence with the passion, character, empathy and strength to transform and improve their own reality and by doing so, also improve and inspire others.

Abbreviations

ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
18S	RNA 18s ribosomal pseudogene
ACTB	Actin beta
APC	Allophycocyanin
APCs	Antigen presenting cells
BB-12	Bifidobacterium lactis spp. Animalis BB-12
BSB	Brilliant stain buffer
BV421	Brilliant violet
CACO-2	Colorectal adenocarcinoma cell line
CD	Cluster of differentiation
CFU	Colony forming units
CLDN-1	Claudin-1
DCs	Dendritic cells
DMSO	Dimethyl sulphide
ECHRs	Elderly care home residents
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FBC	Full Blood count
FITC	Fluorescein isothiocyanate
GALT	Gut associated lymphoid tissue
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LGG	Lactobacillus rhamnosus GG
LPS	lipopolysaccharide
MALT	Mucosal-associated immune system
MCP-1	C motif chemokine ligand 2
MFI	Median fluorescence intensity
MOI	Multiplicity of infection
NK	Natural killer cell
OCLDN	Occludin
PAMP	Pattern-recognition receptors
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-cyanine-5
PGN	peptidoglycan
PHA	phytohaemagglutinin
PRRs	Pattern-recognition receptors
RPL13A	ribosomal protein L13a

SDHA	succinate dehydrogenase complex flavoprotein subunit A
TCR	T cell receptor
TEER	Transepithelial electrical resistance
TJ	Tight Junctions
TJP-1	Zonula-1
TLRs	toll-like receptors
TNF	Tumour necrosis factor
VCAM-1	Vascular cell adhesion protein 1
WHO	World health Organization
y	Years
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
ZO-1	Zonula-1

Chapter 1 Introduction: Immune system, gut microbiota and ageing.

1.1 General statement

This research project is in the field of nutrition, immunology and ageing. The overarching hypothesis is that the immune response can be improved, and inflammation modulated in elderly care home residents (ECHRs) using specific probiotic organisms. In addition to using blood samples from the PRINCESS randomised controlled clinical trial to assess immune cell numbers and functions, the project uses an in vitro model of gut epithelial cells to investigate mechanisms that might be involved in the probiotic-host interaction. The research has several aims as follows:

1. To conduct a literature review that brings together knowledge about the science of the ageing process, the immunological and inflammatory modifications that occur with ageing, and the possible effects of probiotics (specifically *Lactobacillus rhamnosus GG* and *Bifidobacterium animalis subsp. lactis BB-12)* on immune function including response to vaccination and susceptibility to infections.
2. To investigate the influence of blood sample storage time prior to processing on markers of immunity and inflammation, to inform measurements to be made as part of a multicentre study.
3. To investigate the effects of the combination of *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. BB-12 on markers of immunity and inflammation in ECHRs.
4. To investigate vitamin D status in ECHRs and how vitamin D is associated with markers of immunity and inflammation.
5. To set up an in vitro model of intestinal inflammation using the Caco-2 cell line.
6. To investigate the effect of *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. BB-12 in an in vitro intestinal inflammation model (Caco-2 cells).

1.2 General overview

Nutritional immunology, so-called *immunonutrition,* is a scientific field integrating the study of certain foods, food components and nutrients in the context of the human immune response and the subsequent clinical outcomes. The modulatory effects of dietary components on the immune system can start in the gastrointestinal system which creates interactions with the endogenous microbiota that reside there. Through better understanding of the mechanisms in which certain nutrients act, as well as describing the nutrition-immune interaction, it has been, and will continue to be, more likely to address public health issues of significance (e.g. food fortification policies and healthy ageing strategies) and to provide better management of certain clinical conditions with an underlying immune or inflammatory cause (e.g. oral tolerance induction as part of the treatment of allergies, treatment of inflammatory bowel diseases, cancer, chronic metabolic diseases).

Among the general population, newborns, children, pregnant and breastfeeding mothers, and older people are at a higher risk of infections. In the elderly, the susceptibility to develop not only infections but a plethora of other immune-associated conditions is more prevalent than in young and middle-aged adults. In the case of newborns this susceptibility is the consequence of a naive immune system, whereas in older people the accumulation of damage and an aged immune system are the underlying causes of their increased risk of diseases. Vulnerable populations can benefit from tailored interventions to optimise the immune responses (e.g. vaccination). Nutritional interventions modulating the immune response can also play a role in improving health outcomes within these groups. Elderly populations and the effects exerted by certain dietary components will be the target of the research herein presented.

Human ageing is a natural process marked by molecular, physiological and anatomical changes with the progression of time. However, it is highly variable and heterogeneous, and it is influenced by both individual and external factors. Genetic background, family history, lifestyle and exposure to environmental challenges are factors that differentiate the ageing process from one person to another. Likewise, external factors including government efforts to improve health services, control in pollution and biomedical interventions can impact positively the ageing process with marked differences geographically. Despite the individual and external factors, there is an overall global trend towards a prolonged life expectancy.

Among the human systems that decline with ageing, the more widely studied are the musculoskeletal, respiratory, cardiovascular and cognitive components. However, the immune system is also extensively affected by ageing, and there seems to be a link in aged individuals between immune decline, the onset of low-grade inflammation and commencement and progression of chronic diseases.

The immune system confers defence against harmful components of the environment, particularly pathogenic organisms. It is complex, involving barrier functions, many cell types that interact with one another, and a myriad of chemical mediators. Age-related immune decline results in increased susceptibility to infection, and poorer vaccination responses. Likewise, it increases the risk of inflammatory conditions and some chronic diseases contributing to the loss of lean mass (muscle and bone) and to frailty. Epithelial barriers (e.g. in the gastrointestinal tract) offer a beneficial microenvironment in which microorganisms, mainly bacteria, can interact with the host immune system. The metabolic products derived from these interactions have several effects on the health of the host. The gut microbiota is also affected by ageing progression suggesting altered microbe-host interactions. In fact, an altered gut microbiota could be one cause of the age-related immune decline. Conversely, a decline in immune competence with ageing may permit the microbiota to change adversely, termed dysbiosis.

Dietary components with adduced immunomodulatory properties such as probiotics may be part of the strategy to prevent, slow or reverse age-related changes and immune competence via gut-associated immune system modulation (GALT). Although the immunomodulatory hypothesis behind the effect exerted by these dietary components has been tested in several in vitro studies and human trials, there are persistent difficulties in the extrapolation of their findings. In vitro studies often offer a partial mechanistic view but fail to replicate real and complete endogenous conditions (e, g. influence of surroundings and different cell types and their metabolic products) and clinical studies tend to be highly heterogeneous with the inclusion of different age groups. Particularly in the ageing scenario, the clinical trial evidence available in the elderly population is insufficient and there are questions that remain.

The central focus of this thesis is the examination of biomarkers of the immune system in elderly care home residents (ECHRs) and whether modulation of the immune system through probiotics can be achieved. To proceed with this investigation, an immunological sub-study derived from a large randomised placebo-controlled clinical

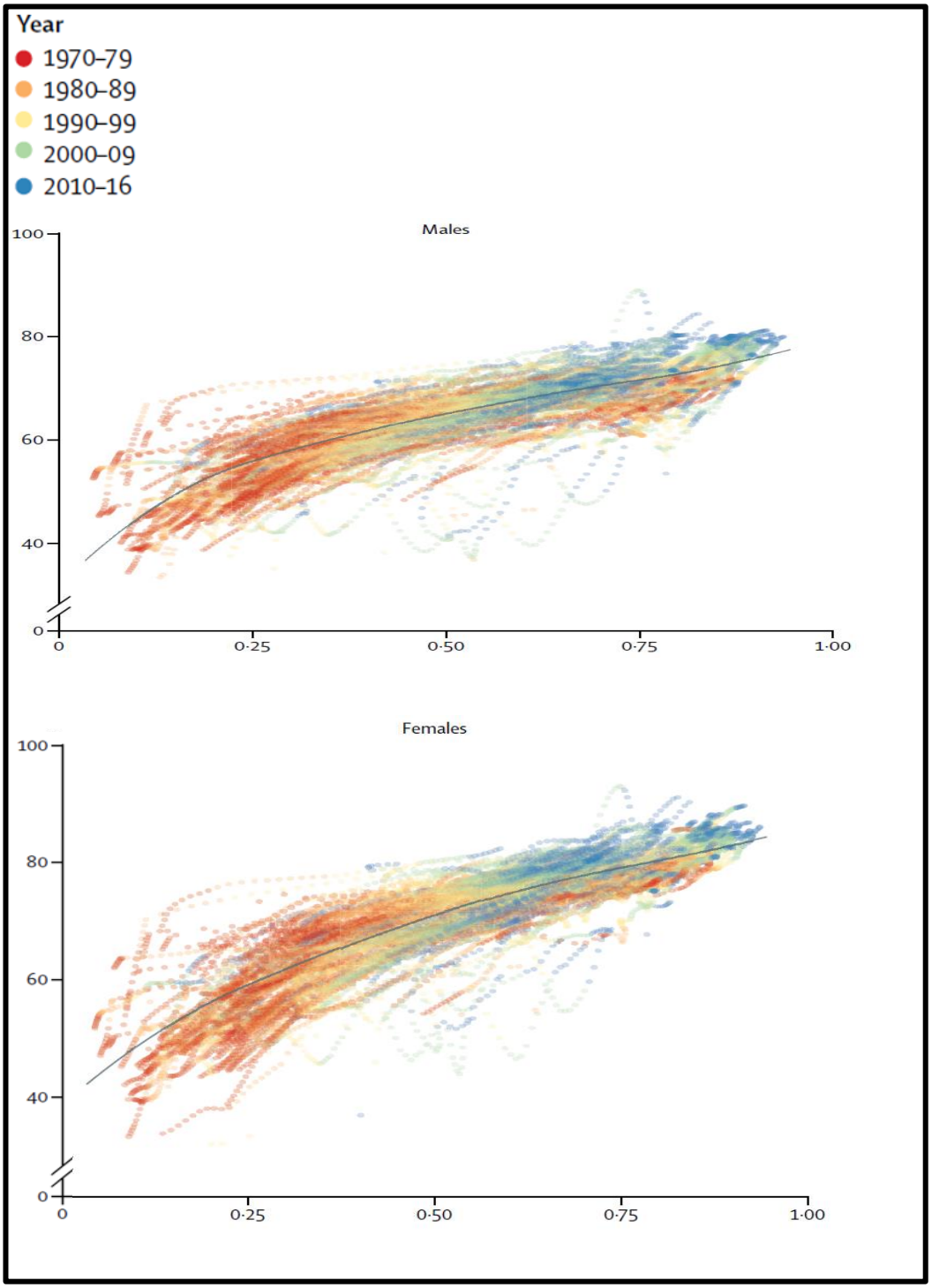
trial (PRINCESS study) conducted in Cardiff and Oxford was performed. The intervention consisted of a combination of the probiotics *Lactobacillus rhamnosus GG* (LGG) and *Bifidobacterium animalis subsp*. BB-12 (BB-12). The immunological sub-study involved the assessment of immune cell phenotypes, immune cell responses *ex vivo*, blood inflammatory markers, and antibody response to influenza vaccination. Due to the nature of this multicentre study, the samples could not be studied in fresh blood. Therefore, a pilot study was performed to ascertain whether the immune outcomes to be measured remained stable over several days in blood kept at room temperature prior to analysis. Furthermore, a mechanistic *in vitro* study of the effect of the specific probiotic organisms on cultured gut epithelial cells was conducted.

This introductory chapter will describe the ageing process, highlighting the importance of older people as a target group that might benefit from nutritional interventions aiming for immune modulation. Afterwards, the components of the immune system, their function and how they change with age will be presented. Vitamin D and its relevance for the immune system in the ageing process will also be described. Subsequently, the probiotic concept, strains of interests and their interactions with healthy ageing will be explored.

1.3 Epidemiology of life expectancy

Life expectancy is increasing. Globally, average life expectancy at birth increased by 13.5 y for men and 14.8 y for women from 1970 to 2016 [1]. There are, however, noticeable differences in life expectancy depending on the region. For example, the lowest average life expectancy can be seen in the Central African Republic (50.2 y), whereas the highest life expectancy is 83.9 y in Japan. Despite these differences, aged populations have increased in most societies: the life expectancy at birth is greater than 80 y for women in 57 countries and in 10 countries for men. Nowadays, living for 60 y and beyond is a possibility for most people according to the World Health Organization (WHO) [2]. Figure 1.1 reflects the global trend towards an increased life expectancy. This figure also shows the differences observed among different geographical regions and between the sexes.

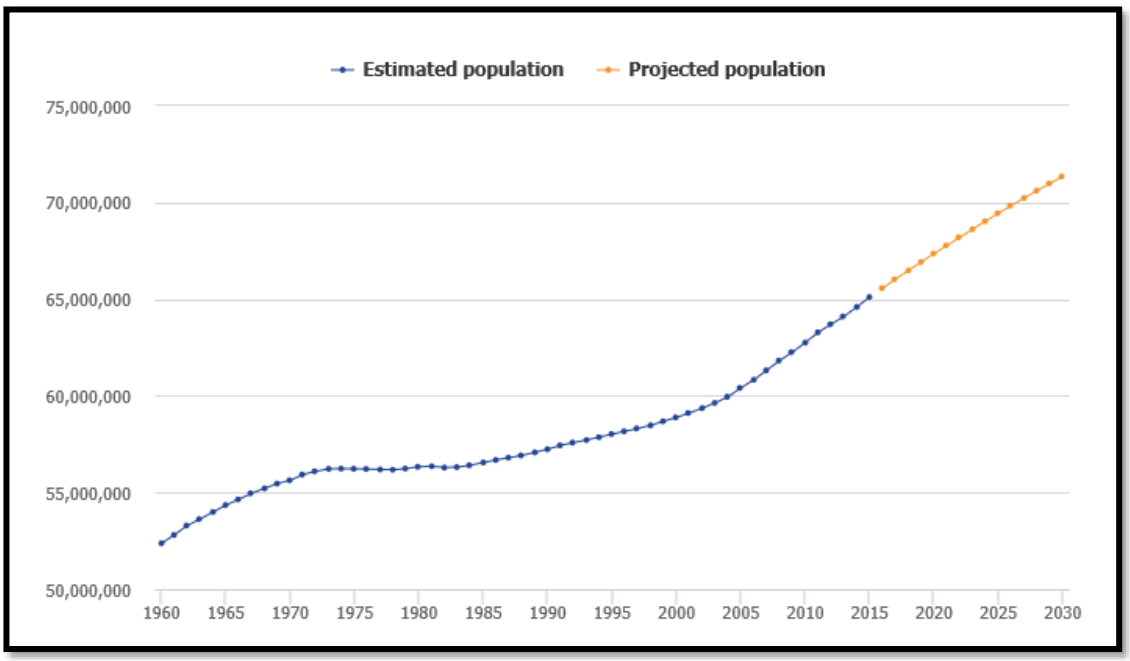
Figure 1.1. Worldwide life expectancy changes from 1970 to 2016 per sociodemographic Index (SDI)



Taken and adjusted from the Global Health Metrics report [3]. The Sociodemographic Index (SDI) is an epidemiological strategy that identifies regions and countries according to their spectrum of development. It is built considering areas like income per capita, average educational levels and fertility rates. The SDI **(x-axis)** is expressed on a scale from 0 to 1, where 0 indicates lower **sociodemographic indexes**, corresponding to countries experiencing higher inequality, and 1 corresponds to wealthier and developed countries. Despite the SDI, there is a general trend towards an increased life expectancy expressed in **years lived (y-axis).** Geographical regions with lower SDI lack data reported in 2010 onwards. This also highlights the importance of good quality systems in public health, particularly aged populations.

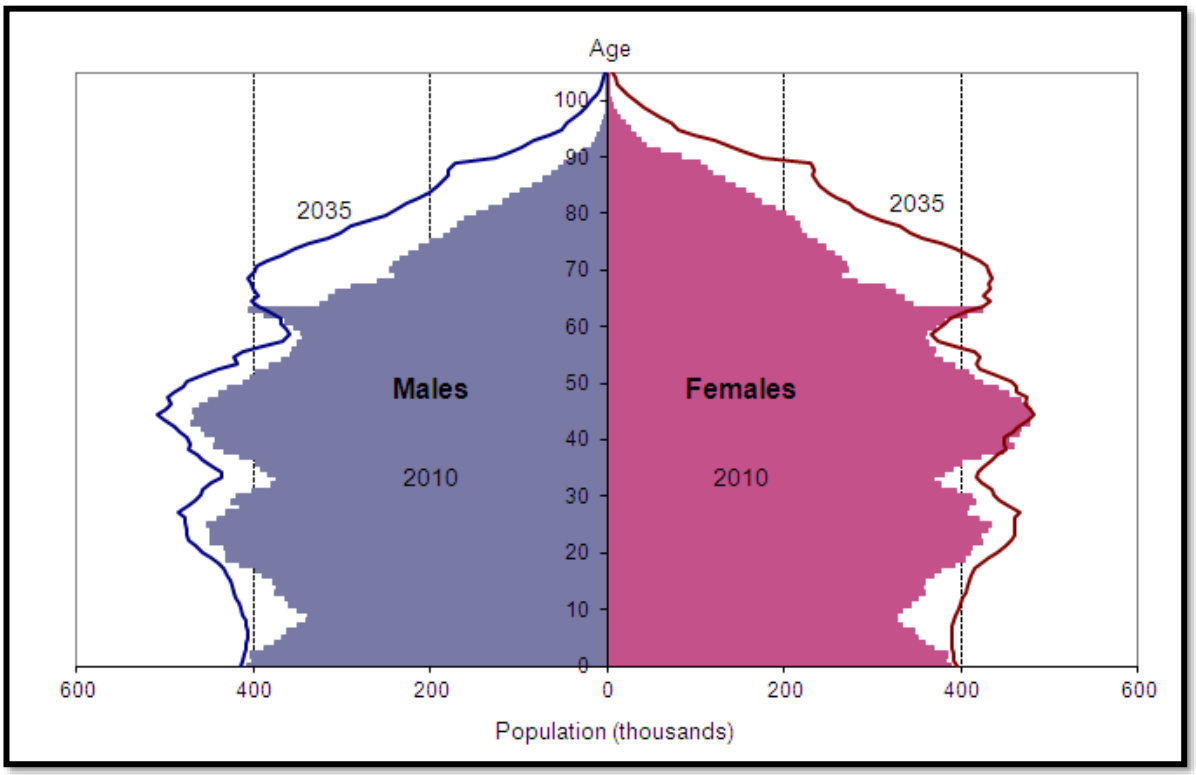
In countries with higher sociodemographic indexes, such as the United Kingdom, it is estimated that the number of elderly people will continue to increase steadily. Figure 1.2 shows the growth of the population in the United Kingdom projected to 2030, which reflects an increase in the total population. Moreover, Figure 1.3 shows the age distribution of the population in United Kingdom in 2010 (males and females) and what it is projected to look like in 2035. The figure shows that already the population over 60 y of age is an important segment [4] and that this sub-group will grow in number by 2035 [5].

Figure 1.2. United Kingdom population estimates and projections, 1960 to 2030



Taken from the Office for National Statistics [6]. The current United Kingdom population is approximately 66,455,000. It is projected that the yearly per cent of increase corresponds to 0.60%, therefore by 2030 it is estimated that the population would have increased by 7%.

Figure 1.3. Estimated and projected age structure of the UK population, mid-2010 and mid-2035.



Taken from the Office for National Statistics [7]. The United Kingdom current median age is 40.3 y. It is estimated that by 2035 the population over 65 y old will correspond to 23.6 per cent of the total population.

The prolongation of the human lifespan has mainly been the result of several public health interventions. These include effective medical interventions like immunisation which have greatly reduced infant and childhood mortality and improvements in living standards with more nutritious diets, cleaner drinking water and improved sanitation [8]. Simultaneously with an enhanced life expectancy, current priorities worldwide go beyond avoiding diseases and reducing mortality rates to include healthier ageing, which is hugely beneficial and cost-effective for health systems and societies [9, 10].

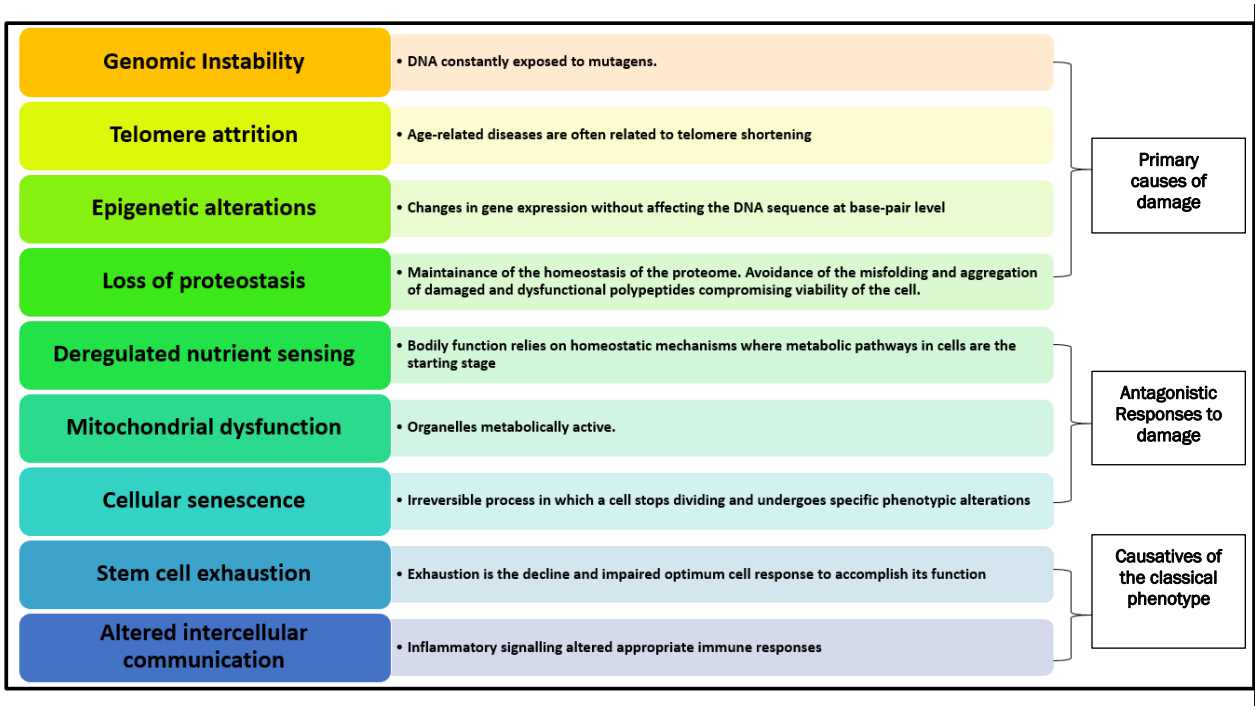
Many strategies focused on maintaining good health during ageing are closely linked to dietary habits [11], physical activity and fitness [12] and social and environmental relationships [13]. All these together seem to shape a healthier phenotype to slow the adverse effects of ageing. Further understanding of the changes that occur during ageing and identifying more effective strategies to promote healthy ageing (i.e. to prevent age-related functional decline and ill health) are important focusses of research.

1.3.1 The biology of ageing

Ageing is a natural and heterogeneous process that is linked to a prolonged lifespan. It affects cells, tissues, organs, and systems. The decline in human functionality with ageing increases the risk of age-associated diseases, often related to low-grade chronic inflammation such as cardiometabolic diseases, altered cognition, and cancer [14]. Ageing also increases the risk of infectious conditions [15-17] and decreases responses to vaccinations with a response of approximately 30% of the population in comparison to young people in which responses are 70% [18].

Starting at a cellular level, there are altered functions due to genomic instability and time-dependent accumulation of damage to the genetic material (i.e. DNA). This is related to cellular senescence, altered mitochondrial functionality and deregulation in nutrient sensing [19]. These changes imply alterations in signalling and metabolic pathways yielding cytokine profiles with a trend towards a pro-inflammatory pattern [20]. Therefore, the naturally-occurring process of ageing drifts towards a modification in compartments of the immune system where a dysregulation rather than an impaired function generates a phenomenon named "*immunosenescence*" [21]. Furthermore, this same environment generates the "*inflammageing*" condition [22], characterised by elevated circulating levels of pro-inflammatory cytokines with a lower production of anti-inflammatory cytokines. The degree of progression of immunosenescence and inflammageing-related processes in the elderly is linked with a poorer prognosis of overall health [20, 23, 24]. Figure 1.4 summarises underlying cellular mechanisms linked to the age-related decline in immune system performance.

Figure 1.4. Interconnections between the hallmarks of ageing and their relationship with the immune response.



Taken and modified from Anuan [25] **Primary causes of damage** are processes produced by external exposure (e.g. diet) and endogenous challenges (e.g. DNA replication errors). These failures yield in cellular and molecular alterations that affect further organ function and systems (e.g. immune system). Where the previous senescent processes occur, there are **antagonistic responses of damage** whose purpose is to provide mechanisms of control (e.g. senescence protecting cells from carcinogenic transformation). These mechanisms are beneficial at regulated intensities, but when they are persistent, then senescent process might be accelerated (e.g. immunosenescence). Lastly, the **causatives of the phenotype** in ageing are the stem cell exhaustion and altered intercellular communication (inflammageing) which are intrinsic mechanisms that directly originate the clinical signs of age and ageing. These causes occur through changes in the communication of cells, organ, and systems.

In fact, the age-associated decline in immune competence is related to the gradual involution of the thymus with reduced output of naive T cells [26], and changes in the bone marrow with increased adipogenesis and reduced production of B cells [27]. Moreover, metabolic and endocrinological changes with ageing including body composition alterations (increased adiposity and loss of muscle mass - sarcopenia), as well as changes in bone density, can yield frailty [11]. These changes are related to a clinical phenotype in the aged individual characterised by impaired organ function and failure to respond effectively to environmental challenges. This naturally-occurring process of decline is heterogeneous due to individual variables including genetic background, lifestyle [28] and environmental influences that shape the nature of the ageing process, immunosenescence and inflammageing patterns [13].

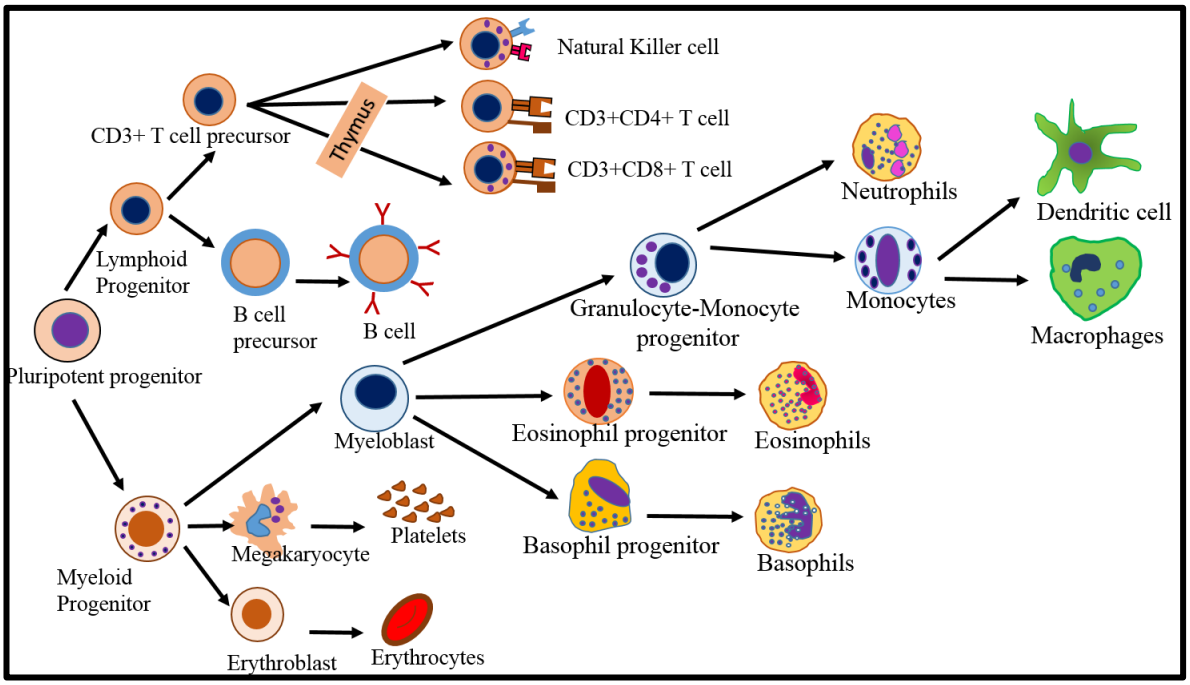
1.4 The immune system

The immune system is an intricate network of cells and chemical mediators and their receptors. When it works and responds in an integrated manner, it can confer protection to the host. Components of this organised system include cells, molecules, and natural barriers that interact in a coordinated way to develop an immune response. Physiological functions of the immune system are commonly related to defence against pathogenic microbes, such as bacteria and viruses as well as fungi and parasites. Additionally, there are also non-infectious substances such as proteins and polysaccharides, and even elements belonging to "self" which are identified as foreign and potentially harmful. These can trigger a variable degree of immune response like those observed in allergies and autoimmunity. The activation of the immune response relies on the origin and optimum maturation of the immune cells as well as their effective communication.

1.4.1 Origin and maturation of immune cells

Immune cells originate in the bone marrow, a process which begins in early in life, *in utero*. The haematopoietic tissue contains pluripotent haematopoietic stem cells, the common ancestral cell not only for immune cells but also other blood cells. These stem cells undergo a differentiation process leading to more specialized immune cells. Figure 1.5 illustrates the differentiation process of immune cells which illustrates the maturation process of B cells (taking place in the bone marrow) and T cells (taking place in the thymus).

Figure 1.5. Differentiation of immune cells and other cell lineages



Modified from Burmester *et al*. [29] and Elgert [30]. The figure illustrates the cell lineage differentiation starting from the pluripotent progenitor which takes place in the bone marrow. Receptors shown in T cells and B cells differ in shape and functionality. Basophils, eosinophils, neutrophils, monocytes and dendritic cells have an important role as phagocytes.

1.4.2 Organization of the immune system

The immune system may be classified into two general components referred to as innate and adaptive. The innate immune system generates an immediate response to a limited number of foreign antigens whereas the adaptive immune system produces a delayed but highly specific response through the recognition of specific foreign antigens. The innate immune component leads to the activation of the adaptive elements in order to mount a fully effective immune response. Therefore, these components respond in an interconnected manner. Further explanation with regards to this interaction will be expanded through this section. Initially, Table 1.1 summarises the main characteristics and components of the immune system according to this classification.

Table 1.1. Characteristics and components of the innate and adaptive immune response.

Characteristic	Innate compartment	Adaptive compartment
Specificity	General structures shared by groups	Antigens of microbial and non-microbial origin
Diversity	Limited	Large: receptors produced by genetic recombination
Barrier function	Skin, mucosa, epithelia, antimicrobial chemicals	Lymphocytes and antibodies at epithelial surfaces
Blood proteins	Complement	Antibodies
Cells	Phagocytes (Macrophages, Monocytes, Neutrophils, Natural Killer (NK) cells)	Lymphocytes (T cells; B cells), Antigen presenting cells (APCs)
Surface markers on cells	A cluster of differentiation and major histocompatibility complexes	A cluster of differentiation and major histocompatibility complexes. Immunoglobulin receptors

Modified from Janeway [31]

1.4.3 Innate Immunity

The main components of innate immunity are physical barriers (Table 1.1.), cells, and biochemical mechanisms which include activation of the complement cascade and chemical humoral factors. Together these components act as the first line of defence against pathogens. The innate response is firstly related to the recruitment of neutrophils, monocytes, macrophages, natural killer (NK) cells, eosinophils and dendritic cells (DCs) to sites of infection, and the subsequent activation of the adaptive immune system.

1.4.3.1 Epithelial barriers: Focus on the intestine as a defensive system

The epithelial barriers in the human body are the skin and the mucosal surfaces of the gastrointestinal, genitourinary and respiratory tracts and are responsible for the exclusion of pathogens present in the external environment. The sites where mucosal surfaces are found are particularly vulnerable to infections as they are thin (often cell monolayers) and permeable barriers which allow the exchange of substances with the external environment [32].

The intestinal epithelium comprises a monolayer formed by the enterocytes. These intestinal epithelial cells are connected by intercellular junctional complexes which consist of the tight junctions, gap junctions, adherence junctions and desmosomes. The intercellular tight junctions are essential for the protective barrier to function (see Chapter 6). Substances can permeate the epithelium by two pathways, through the cells

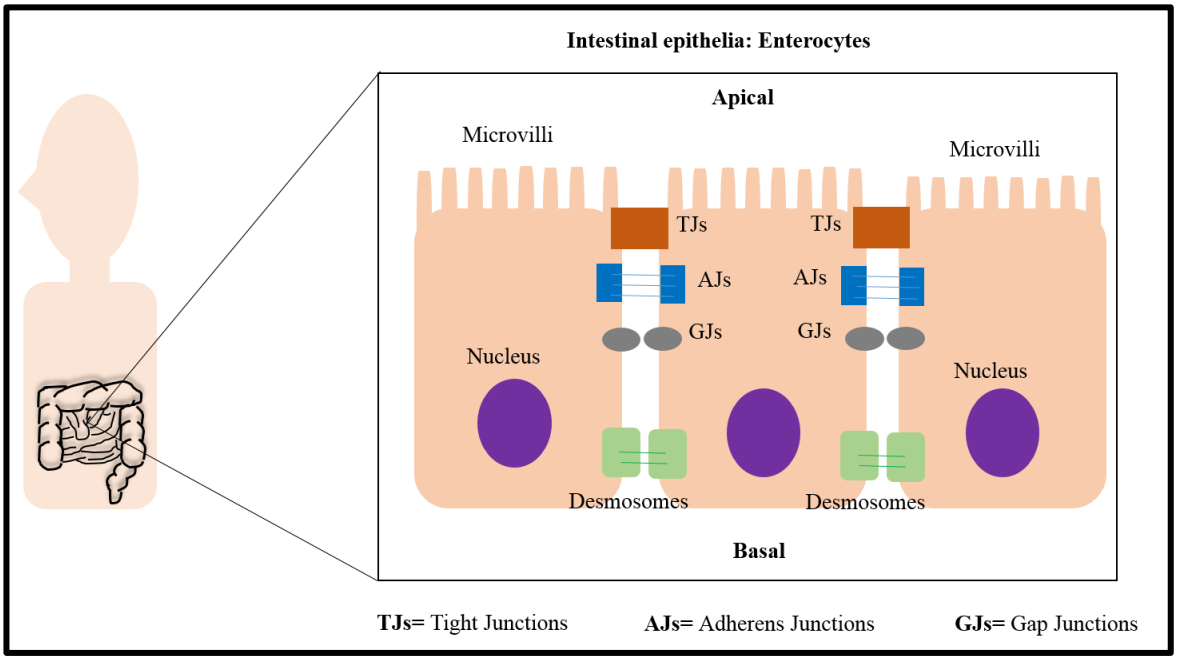
(transcellular) and through the intercellular spaces (paracellular) [32, 33]. When the integrity of the barrier is maintained, it is possible to prevent the entry of microbes. Additional mechanisms of protection consist of releasing peptides with antimicrobial properties into the gut lumen [34].

Enterocytes and their interconnected protein network prevent the translocation of many potentially harmful substances. However, enteric pathogens and pro-inflammatory cytokines may disrupt these tight junctions. The intestinal permeability reflects the state of epithelial tight junctions. Tight junctions selectively regulate the movement of fluids, nutrients, toxins and microbes across the epithelium and thereby act as a semi-permeable barrier.

Barrier epithelia and serosa cavities in the intestine contain lymphocytes including intraepithelial T lymphocytes and a subset of B cells, which recognise and respond to commonly encountered microbes. Dysregulation of epithelial barrier functionality can lead to increased intestinal permeability and bacterial translocation, which contribute to local and systemic immune activation. Another dysregulation is known as dysbiosis (an imbalance among the gut microbiota) that also affects the mucosal immune system, leading to colonisation by pathogenic organisms and consequent disease risk [35].

The integrity of the epithelial junctional complexes is important in determining epithelial barrier properties. Exposure to pro-inflammatory cytokines and deregulation in the expression of junctional complexes can lead to a defect in the epithelial barrier [36] with subsequent clinical consequences associated to increased bacterial translocation and infection. Tissue integrity, organization and differentiation [37] and integration with the associated immune system in the gut, is a crucial first mechanism of host-protection. Figure 1.6 shows how these structures are interconnected starting at a cellular level of the epithelial monolayer.

Figure 1.6. Interconnected structures in the intestinal epithelium monolayer.



This epithelial barrier forms the first mechanism of protection within the innate immune system. Subsequently, the recognition of microorganisms by the pattern recognition receptors (PRRs) expressed in surrounding immune cells, is a mechanism that senses the microbial interactions in the intestinal mucosa in order to trigger a primary response (inflammatory) in the case of microbial invasion.

1.4.3.2 Pattern-recognition receptors (PRRs)

The primary target for the innate immune response is the prevention of systemic dissemination of pathogens. PRRs, by being present in immune cells, are the initiators of this mechanism as these are proteins responsible for sensing the presence of microorganisms by recognising structures highly preserved among microbial species. These highly preserved structures in microbes were originally called pathogen-associated molecular patterns (PAMPs) but are now considered as microbe-associated molecular patterns (MAMPs) because they do not only exist in pathogens.

PRRs are also responsible for recognising endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs). Different families of PRRs have been identified. These include transmembrane proteins such as the toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs).

PRRs are expressed not only in macrophages and DCs but also in various non-professional immune cells. Except for some NLRs, the sensing of MAMPs or DAMPs by PRRs upregulates the transcription of genes involved in inflammatory responses. These genes encode pro-inflammatory cytokines, type I interferons (IFNs) - proteins that confer resistance against many different viruses, inhibit proliferation of normal or malignant cells, and act through immunomodulation of other immune cells -, chemokines, antimicrobial proteins, and proteins involved in the modulation of PRR signalling.

1.4.3.3 Toll-Like receptors (TLRs)

TLRs are a family of PRRs. These proteins are responsible for sensing invading pathogens outside the cell and in intracellular endosomes and lysosomes. TLRs mainly signal through adaptor proteins to activate transcription of proteins involved in host defence. TLRs and their ligands are described in Table 1.2.

Table 1.2. Toll-like receptors (TLRs) and their ligands.

TLR	Localization	Ligand	Origin of the ligand
TLR1	Plasma membrane	Triacyllipoprotein	Bacteria
TLR2	Plasma membrane	Peptidoglycan	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA[1]	Viruses
TLR4	Plasma membrane	Lipopolysaccharide	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR8	Endolysosome	ssRNA[2]	Viruses, bacteria, self
TLR9	Endolysosome	CpG-DNA[3]	Viruses, bacteria, protozoa, self
TRL10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profiling-like-molecule	Protozoa

Modified from Takeuchi [38]. dsRNA[1] (Double-stranded RNA) – Type of non-enveloped viruses capable of infecting humans. ssRNA[2] (Single-stranded RNA) – present in different pathogens. CpG-DNA[3] CpG corresponds to a motif present in the DNA of the organisms that contain or "originate" that ligand.

Once the mechanisms of pathogen recognition are activated, the inflammatory response is triggered (See section 1.6). In brief, the purpose is to attract controlling cells to prevent the systemic dissemination of pathogens.

1.4.3.4 Phagocytosis

The innate immune response relies on phagocytic activity. Phagocytosis is the physical engulfing of particles or cells which is achieved when the cell membrane of the phagocytic cell is placed around the harmful structure to be engulfed. Abnormalities of phagocytosis can result in a variety of clinical disorders as this process is central to

removing pathogens, damaged cells and cellular debris [39]. The phagocytic process starts with the migration of phagocytes to sites of infection, a process known as chemotaxis. After that, an attachment of particles to the cell surface of phagocytes occurs; this usually involves receptors. Subsequently, the ingestion process takes place. Finally, intracellular killing mechanisms are induced. After killing, the ingested structure is digested; this can produce antigens that can be presented by the phagocyte or antigen presenting cells (APCs) to antigen-specific T cells and B cells.

1.4.3.5 Antigen presentation

Antigens are usually peptides, although some other structures, including some lipids, can also be antigenic. Antigens can originate from many sources including foods and microbes. The major histocompatibility complex (MHC) has a fundamental role in controlling immune responses. This assemblage of proteins is responsible for binding foreign peptide fragments (i, e. generated at the end of phagocytosis) and displaying them on the cell surface for recognition by the appropriate T cells. MHC is classified as class I and class II according to the peptide specificities and therefore the binding process. APCs are so-called sentinel cells and their function is to identify and start the immune response towards foreign antigens. DCs have a central role in the antigen presentation. DC maturation results in upregulated expression of MHC and costimulatory molecules and an increased ability to present antigen.

1.4.3.5.1 MHC Class I

MHC I molecules are expressed by all nucleated cells and present protein fragments at the cell surface to T cells expressing CD8. $CD8^+$ T cells can kill cells after recognizing peptides presented to them by MHC I. Antigen presentation through the MHC class I pathway has the purpose of presenting antigenic peptides that are generated inside an infected cell.

1.4.3.5.2 MHC Class II

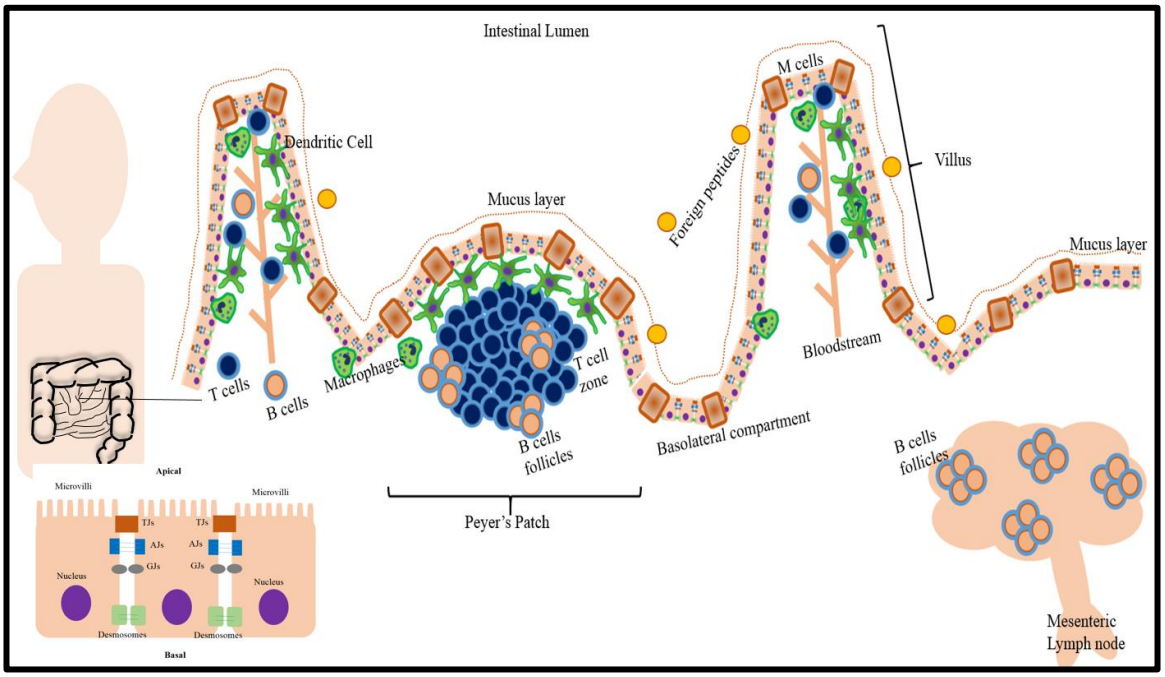
MHC Class II is primarily expressed by professional APCs such as DCs, macrophages and B cells. While MHC I molecules bind to peptides of cytosolic origin, MHC class II molecules bind to peptides that are derived from proteins degraded in the endocytic pathway, and so of extracellular origin [40]. The antigen presentation is mainly to T cells expressing $CD4^+$.

1.5 Interconnection in the immune response: innate and adaptive compartments are integrated

1.5.1 Gut-associated lymphoid tissue (GALT)

Lymphoid tissues are organised structures and sites of immune cell development (e.g. the thymus) and immune cell interactions (e.g. spleen, lymph nodes). The largest collection of lymphoid tissues is in the gut. This mucosa-associated lymphoid tissue (MALT) lining the gut and the integration of immune cells present in the gut is known as GALT (gut-associated lymphoid tissue). Figure 1.7 illustrates the architecture of this structure [41]. Important immune cells within this structure are APCs, T and B cells.

Figure 1.7. Architecture of Gut-Associated Lymphoid Tissue (GALT)



The figure illustrates the interpretation of the architecture of the GALT. The mucous layer that protects the intestine is important in the communication with foreign peptides, including those produced by probiotics, pathogenic bacteria, and foods. Additionally, and probably the most important discrimination that occurs in the gut lumen, is between innocuous (such as food and commensal bacteria) and harmful (pathogens). This process takes place thanks to the integration of the different immune cells and epithelial cells present in the GALT.

1.5.1.1 Mucosal-associated immune system (MALT)

The mucosal immune system has lymphocytic repertoires in the different compartments. The T cells of the gut can be divided into two types. One type participates in conventional T cell responses to foreign antigens. The second class is made up of T cells with unusual surface phenotypes. The receptors of these T cells do not bind to the normal MHC peptide ligands but instead, they bind to several different ligands. Unlike

conventional T cells, these cells may be classified as being at the interface between innate and adaptive immunity. Key roles of T cells are to patrol and survey the body, and contributing in the destruction of cells that express an abnormal phenotype as a result of stress or infection [42].

1.5.2 T Lymphocytes

T cells mediate cytotoxicity, secrete pro- and anti-inflammatory cytokines, and provide help for B cells and other cells involved in the immune response. In commonality with other immune cells, T cell progenitors originate in the bone marrow from hematopoietic stem cells (See Figure 1.5). However, after being exported to the periphery, they migrate to the thymus, giving rise to a large population of immature thymocytes in young individuals.

Initially, these only express the antigen-specific T cell receptor and are CD4 and CD8 double negative. Upon further development in the thymus, most rearrange to produce an antigen-specific T cell receptor (TCR) and become CD4 and CD8 double positive. This is important because T lymphocytes need to be specific. These cells are involved in the recognition of a wide range of antigens and pathogens, “self-antigens” and normal elements within the circulation.

This T cell rearrangement in the thymus involves a reorganisation in the expression of both CD4 and CD8 before further differentiation into single-positive CD4 (MHC class II-restricted) or CD8 (MHC class I-restricted) T cells. This process continues outside the thymus. T cell activation occurs through binding of the TCR to its antigen peptide-MHC complex presented on specialised APCs, particularly DCs.

Lastly, T-cells require co-stimulatory signals, which are delivered through receptors such as CD27 and CD28. CD4 cells are mainly characterised as helper T cells providing help for B cells as well as for CD8 T cells. Most CD8 T cells are cytotoxic and are involved in the lysis of infected cells. Additionally, T regulatory cells (T regs), which inhibit responses of other T cells, are also part of the T cell repertoire.

1.5.2.1 T cell activation and clusters of differentiation

Clusters of differentiation (CD) are cell surface proteins defined on specific cell populations. For example, CD4 and CD8 are an alternative for characterising subsets of T cells according to function, and other CD accomplish different functions according to

the type of cells they are encountered on. Table 1.3 lists common CD, the type of cells they are expressed on and their function.

Table 1.3. Some common CD, type of cells expressing them and function

CD	Cell type(s) expressed on	Function(s)
CD3	T cells	Mediates T cell signal transduction
CD4	T cells, Monocytes, Macrophages and Granulocytes	Initiation or augmentation of the early phase of T cell activation
CD8	T cells, NK cells	Role in the T cell-mediated killing.
CD14	Monocytes, Macrophages, Granulocytes	Mediates the innate immune response to bacterial lipopolysaccharide (LPS)
CD16	T cells, Dendritic cells, NK cells, Macrophages, Monocytes, Granulocytes	Low affinity, antibody binding (IgG1 and 3) and immune response modulation, mediates phagocytosis and antibody-dependent T cell-mediated cytotoxicity
CD19	B cells, Dendritic cells	Assembles with the antigen receptor of B lymphocytes to decrease the threshold for antigen receptor-dependent stimulation
CD20	T cells, B cells	Development and differentiation of B cells into plasma cells
CD25	T cells, NK cells	Expressed by T cells after stimulation; related to CD4+ as an indicator or regulatory T cells and suppressor T cells
CD28	T cells	T cell proliferation and survival. Involved in IL-2 production and Th2 cell development
CD45	T cells, B cells, Dendritic cells, NK cells, Stem cell precursors, Monocytes, Macrophages, Granulocytes	Regulator of T and B cell antigen receptor signalling. Regulator of cell growth and differentiation
CD80	T cells, B cells, Dendritic cells.	Lymphocyte activation
CD86	T cells, B cells, Dendritic cells, Monocytes	Upon Binding of CD28 serves as a costimulatory signal for T cell activation. Binding of CD86 to CD152 (CTLA-4) negatively regulates T cell activation
CD127	T cell, Monocytes, Macrophages	Receptor for IL-7 and thymic stromal lymphopoietin (TSLP)

Modified from Barclay [43]

1.5.3 Th cell subtypes.

When appropriate signals are present, T cells become fully activated and start clonal expansion and differentiation, giving rise to effector cells. Particularly, $CD4^+$ T cells can differentiate into distinct T helper cell subsets (Th cells) with specialised functions once they are activated. Different T helper cell subsets produce specific cytokines that mediate beneficial and sometimes detrimental effects, depending on the context, where infection or disease response should be mounted (see following section). $CD4^+$ T cell

priming relies on signals delivered by the T cell antigen receptor, co-stimulatory receptors and cytokine receptors on the $CD4^+$ T cell surface. These altogether deliver instructive signals that direct T helper cell differentiation or stops the process. In T helper cell differentiation, there is a relevant impact of cell-cell communication on directing T helper cell differentiation [44]. After $CD4^+$ T cells encounter antigen presenting innate immune cells, they are able to differentiate into T helper cells subtypes like Th1, Th2, Th4, Th9, Th17, Th22, Treg, whose diversity is regulated by co-stimulatory molecules and mediators such as interleukin (IL)-4, IL-6, IL-12 and IFN-γ [45]. T helper cells subtypes and their roles in health and disease are described below.

1.5.3.1 Th1 cells

T helper cells type 1 (Th1) cells are a lineage of $CD4^+$ effector T cells responsible for the promotion of cell-mediated immune responses and host defence against intracellular viral and bacterial pathogens. Th1 cells secrete IFN-γ, IL-2, IL-10, and tumour necrosis factor (TNF)-α and β. These cytokines promote macrophage activation, nitric oxide production, and cytotoxic T lymphocyte proliferation, leading to the phagocytosis and destruction of microbial pathogens.

Th1 cells express specific cell surface receptors, including receptors for IL-12 and IL-27. These proteins expressed on the surface not only can be used to distinguish Th1 cells from other T cell subtypes, but also are involved in Th1 cell differentiation and expansion, particularly driven by cytokines that signal through a subset of these receptors, including IL-27, IL-12, and IFN-γ (See table 1.5). Th1 cells are critical for the clearance of intracellular pathogens. However, exaggerated Th1 responses have been found to be associated with autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and type 1 diabetes.

1.5.3.2 Th2 cells

T helper type 2 (Th2) cells provide help for B cells and promote class switching from immunoglobulin (Ig)M to IgG1 and IgE. They also provide host protection against intestinal helminths and extracellular bacteria, in addition, supporting B cell-dependent humoral responses. Th2 cells secrete IL-4, IL-5, IL-9, IL-13 and IL17E/IL-25. Pathological Th2 cell activity is a hallmark of allergic inflammation and asthma.

The balance between Th1 and Th2 cells is important in human health. The difference in cytokine patterns leads to different functions. In general, Th2 cells are helpers for B cell

antibody secretion, particularly IgE responses. On the other hand, Th1 cells are involved in involved in cell-mediated immune responses.

1.5.3.3 Regulatory T cells (T regs)

This type of cell has an important surveillance function which is essential for maintaining a balance in immune responses via controlling a variety of suppressive mechanisms. Regulatory mechanisms may be cell contact-dependent and also mediated by cytokines targeting T cells, B cells and APCs in which the main function is the suppression of autoimmunity as well as inducing and maintaining immune tolerance (relevant in the prevention of undesired uncontrolled immune responses such as allergies)[46].

1.5.3.4 Th17 cells

T helper cells type 17 (Th17) have important roles in mucosal defence. Their differentiation from Th1 and Th2 T cell subtypes is due to IL-17 production and their requirement of the nuclear hormone receptor RORyt and multiple other essential transcription factors (TFs). This T cell subtype is the producer of IL-17A, IL-17F, and IL-22, which protect the mucosa from bacterial and fungal infections and regulate tissue inflammation. Th17 cells have a critical role in autoimmune diseases [47].

1.5.4 B cells

Immature B cells are continuously formed in the bone marrow, exported to the periphery and differentiated to antibody-producing cells when activated by antigen and helped by T cells. Several steps of somatic recombination at the receptor (antibody) loci lead to the production of a unique variable domain in the immunoglobulin functioning as the antigen receptor of each individual B cell. Specifically, B cells undergo clonal selection and expansion so they can reach a degree of specialization to defend the organism from insults and foreign pathogens (bearers of antigens). A variety of different B cell clonotypes (cells sharing phenotypic characteristics) circulate continuously in the blood. Upon direct recognition of foreign antigens and on receiving additional signals from helper T cells, B cells differentiate into plasma cells and produce antibodies specific for the targeted antigen. Later, these B cells will differentiate into memory B cells.

Humoral immune responses are developed when antibodies produced by B cells cause the destruction of extracellular microorganisms, and so prevent the spread of intracellular infections. The activation of B cells and their differentiation into antibody-secreting plasma cells is triggered by an antigen and usually requires Th2 cells (see

section 1.5.3.2). The humoral response starts when an antigen binds to the B cell antigen receptor. Then it is internalized and processed into peptides that activate Th2 cells. Signals from the union between antigen and the T cell induce the B cell to proliferate and differentiate into a plasma cell secreting specific antibodies. These antibodies protect the host by inhibition of toxic effects or infectivity of pathogens by binding to them (neutralization). Also, by coating pathogens, they can enable accessory cells that recognize portions of arrays of antibodies (the constant Fc regions) to ingest and kill the pathogen (opsonisation). Antibodies can also trigger the activation of the complement system. Complement proteins enhance opsonisation, and can directly kill some bacterial cells [31].

1.5.5 Immunoglobulins: antibody production and B cells

Antibodies are antigen-specific immunoglobulins. Immunoglobulins have two heavy and two light chains, but functionally they can be divided according to isotypes; therefore, they can be categorized as IgM, IgG, IgA, IgD, and IgE. Their functionality is related to cell-surface receptors for an antigen which permits cell signalling and cell activation [48]. The binding process of antibodies to a pathogen also includes various types of white cells and a system of blood proteins collectively called complement [49]. The generation of immunoglobulins and their variety is defined at stages of B cell development. Table 1.4 describes the relevant properties of different immunoglobulin classes.

Table 1.4. Immunoglobulins and their relevant properties.

Immunoglobulin class	Heavy chain	Activation of complement	Binds to macrophages and neutrophils	Binds to mast cells and basophils
IgM	M	++++	No	No
IgD	Δ	-	No	No
IgG	Γ	++	Yes	No
IgA	A	-	No	No
IgE	E	-	No	Yes

Modified from Schroeder [48]

1.6 Inflammatory response

Inflammation starts with an increase in the blood flow to the site of infection or injury, continued by an increase in vascular permeability, and the subsequent accumulation of different effector cells at the site. These effector cells are mainly neutrophils, monocytes, and macrophages, which cooperate during the initiation, progression and resolution of inflammation. Overall, the purpose of the inflammatory response is to contribute to the immune defence by recruiting different immune cells to the site of

infection. Regulation of the inflammatory reaction is balanced by the interplay of pro-inflammatory cytokines (See section 1.6.1.), such as IFN-γ, IL-1, IL-6, TNF, transforming growth factor (TGF), IL-11, IL-12, and IL-17, and anti-inflammatory cytokines such as IL-4, IL-10, and IL-13. These proteins act as inflammatory mediators which play a role in the activation and regulation of the activities exerted by the immune cells (see Table 1.5). This response is initiated acutely.

Once pathogenic elements are eliminated, controlled termination of the inflammatory response involves phagocyte-derived signals and the avoidance in the recruitment of neutrophils as well as the clearance of apoptotic neutrophils. Inflammatory mediators are responsible for controlling these activities. Other classes of chemicals which participate in the inflammatory response include vasoactive amines (e.g., histamine and serotonin), various peptides (e.g. vascular cell adhesion molecule (VCAM)), and eicosanoids (e.g., thromboxanes, leukotrienes, and prostaglandins) [50]

1.6.1 Inflammatory mediators: Cytokines

Cytokines are small proteins, glycoproteins or peptides which act like hormones within the immune system, conveying signals between cells. In general, those effects exerted by cytokines could be considered according to main features:

- **Pro-inflammatory cytokines**: These are produced predominantly by activated macrophages and by Th1 cells. They are involved in the up-regulation of inflammatory reactions. Among this family, typical cytokines exerting pro-inflammatory effects are IL-1, IL-2, IL-6, IL-12, IL-15, IL-18, IL-22, IL-23, TNF-α, and IFN-γ.
- **Anti-inflammatory cytokines:** The anti-inflammatory cytokines are a series of immunoregulatory molecules that control the pro-inflammatory cytokine response. Major anti-inflammatory cytokines include IL-1ra, IL-4, IL-10, and TGF-β1.

There are also different classifications of cytokines according to the type of cell that secretes them. For example, cytokines secreted by lymphocytes are named lymphokines, cytokines made by monocytes are named monokines, cytokines with chemotactic activities are named chemokines, and cytokines made by one leukocyte but with effects on others are called interleukins. Overall, they are considered immune mediators due to their variety of functions on the variety of immune cells. Table 1.5 lists immune mediators according to the cell type that produces them and their function.

Table 1.5. Immune mediators in the inflammatory and immune response, main producing cell and main function.

Mediator	Produced by	Function
IL-1	Macrophages, monocytes, fibroblasts, dendritic cells (DCs), T lymphocytes, B lymphocytes, NK cells	Pro-inflammatory cytokine. Related to local and systemic inflammatory responses.
IL-2	T cells	Stimulation of growth and differentiation of T cells, B cells, NK cells, monocytes, macrophages.
IL-3	Activated T cells, eosinophils	Haematopoietic growth factor which stimulates colony formation of erythroid, megakaryocyte, neutrophil, eosinophil, basophil, and monocytic lineages
IL-4	Th2 cells	Exerts pleiotropic effects on many cell types; In T-cells binding of IL-4 induces proliferation and differentiation into Th2 cells. Contributes in B cell survival and enhanced antigen presentation by B cells.
IL-6	T cells, B cells and some non-lymphoid cells including macrophages	Functions in inflammation and maturation of B cells. Induces fever, primarily produced at sites of acute and chronic inflammation.
IL-8	Neutrophils	Major mediator of inflammatory response. Chemotactic factor for neutrophils in sites of infection.
IL-10	Th and Th2 type activated $CD4^+$ and $CD8^+$ cells	Potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1 by activated macrophages; can up-regulate endogenous anti-cytokines and down-regulate pro-inflammatory cytokine receptors.
IL-11	IL-1 stimulated fibroblasts	Stimulates T cell-dependent development of immunoglobulin-producing B cells; supports the proliferation of hematopoietic stem cells and megakaryocyte progenitor cells.
IL-12	Macrophages and dendritic cells, B cells	Allows the differentiation of naïve T cells into Th1 lymphocytes.
IL-13	Activated T cells	An immunoregulatory cytokine produced primarily by activated Th2 cells; involved in several stages of B-cell maturation and differentiation. It up-regulates CD23 and MHC class II expression and promotes IgE isotype switching of B cells; down-regulates macrophage activity, thereby inhibits the production of pro-inflammatory cytokines and chemokines
IL-27	Involved with T cells and Th1 functions	Signalling in naive $CD4^+$ T cells; induces STAT1-dependent expression of the Th1-specific transcription factor
IFN-γ.	Produced by Lymphocytes which have been mitogenically or antigenically stimulated	Classic Th1 cytokine involved in cell- mediated, antiviral and anti-tumour responses. Inhibits Th2 activity and differentiation. Acts on B cell class switching.

IL-1β	Primarily by monocytes and macrophages as well as by nonimmune cells such as fibroblasts and endothelial cells	Important mediator of the inflammatory response. Mediates processes like cell proliferation, differentiation and apoptosis.
TNF-α	Macrophages	Involved in the regulation of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation; implicated in a variety of diseases, including autoimmune diseases, insulin resistance and cancer
IL-1ra		A specific IL-1 receptor antagonist competitively binds to the same receptor as IL-1β but does not transduce a cellular signal, thereby blocking IL-1β mediated cellular changes. Administration of IL-1ra and other anti-inflammatory cytokines have been demonstrated to prevent or attenuate cytokine-mediated inflammatory hyperalgesia
RANTES	T cells, macrophages	Also denominated CCL5; is a pro-inflammatory chemokine that plays a role in controlling the migration and activation of leukocytes
MCP-1	T cells, monocytes, fibroblasts, endothelial cells	Monocyte chemoattractant protein with activity also on basophils. Implicated in pathologies with monocytic infiltrates and atherosclerosis.
MIP-1-α	T cells, B cells, neutrophils and macrophages	Also named as CCL3; macrophage inflammatory protein 1-α, which plays a role in inflammatory responses
TGF-B	Platelets	Transforming Growth factor (TGF)-β; categorised as either anti-inflammatory or pro-inflammatory cytokines, under various circumstances; involved in tissue remodelling, wound repair and haematopoiesis; is also responsible for inhibiting cell growth
IL-18BP	Th cells	Interleukin 18 binding protein, inhibits IL-18 induced IFN-γ production, resulting in reduced T-helper type 1 immune responses; an elevated level is detected in the intestinal tissues of patients with Crohn's disease.

Modified from Callard, and Pigott [51].

1.7 Adaptive Immunity

The adaptive immune response is characterised by its specificity and the ability to respond vigorously. The adaptive immune response is mainly conducted by clonally diverse lymphocytes with different antigen recognition receptors generated by genetic rearrangements.

PRRs expressed in immune cells, bind their ligands present in the corresponding microorganism (See Table 1.2). For instance, the structure of LPS can be recognised by TLR4 in monocytes and macrophages. This process initiates the immune response. The exposure to which ultimately results in the *adaptation* of the immune response, Adaptive immunity results in antigen-specific responses such as T cell activation, clonal expansion and differentiation of naïve cells to effector cells and of B cells to produce specific antibodies to recognise specific antigens. Clonal expansion of antigen-specific lymphocytes should result in the generation of enough antigen-specific cells to combat the pathogen. The retention of a small fraction of antigen-specific cells as memory cells results in a more rapid response to pathogen re-exposure. Adaptive immunity depends on T lymphocytes and B lymphocytes. While cell-mediated immune responses (executed by T cells) control pathogens entering into cells (viruses), the humoral response (executed by B cells) controls extracellular pathogens through antibodies for specific individual antigens.

1.7.1 Immune and inflammatory response: an integrated overview

The overall protection of the host initially involves mechanisms that act as barriers. When these barriers are disrupted, the affected cells can express target molecules that attract sentinel cells. These cells in their role of surveillance are able to identify antigens. When these antigens are from harmful sources, the sources must be quickly removed to avoid extensive damage, and other cells like neutrophils are then attracted to the affected site to continue with the engulfing of invaders and controlling the damage. The inflammatory response uses proteins that act as mediators which control the attraction of neutrophils and other immune cells that act as part of the response. Both, the immune cell migration as well as the production of inflammatory mediators are essential within the mechanisms of defence. However, paradoxical problems arise when this endogenous response is persistent in time (chronic) and when there is a continued activity exerted by these inflammatory mediators and some immune cells. It has been suggested that these uncontrolled and persistent endogenous responses are the underlying basis of many chronic diseases, and likewise, their targeting is the key for prevention and control of many of those prolonged conditions. That persistent low grade of inflammation is a condition linked to ageing.

Interventions addressed towards improvement of the immune response can be done through strategies such as vaccination. Other alternatives are focused on the

improvement of that underlying low-grade inflammation or the chronic inflammation through diet [52, 53] and through the gut microbiota [54].

1.8 Vaccination

A vaccine is a biological preparation that induces protective immunity against a pathogen through inducing adaptive immune responses, particularly immunological memory. The mechanisms by which vaccination triggers an immune response are through the incorporation into the body of a modified pathogenic microorganism. This modification can consist on attenuation or inactivation, that - unlike the disease-causing pathogen in real circumstances - initiates the natural immune response towards the first encounter with the pathogen leading to activation of immune memory through antibody production. A common vaccine, composed of more than one pathogenic microorganisms, in this case viruses, is the influenza vaccine [55].

1.8.1 Influenza vaccination response

Influenza belongs to one group of viruses with a wide variety of antigenically distinct strains causing human disease. Vaccination is used to protect individuals against influenza. This vaccine belongs to the attenuated vaccine group produced from the influenza virus, a single-stranded RNA virus, from which the live attenuated virus vaccine is derived. The seasonal influenza vaccine is designed to protect against the influenza virus-strains which are most likely to spread and cause illness among people during the forthcoming influenza season. Influenza vaccination forms part of a passive immunization scheme where the individual develops a temporary immunity to a particular organism or toxin due to the presence of preformed antibodies, but after their destruction, the individual lacks immunity against that antigen [56]. In the context of influenza, the protective mechanism seems to be conferred through neutralization of antibodies which are believed to correlate with viruses causing infections via mucosal routes. The stimulation of antibodies to the trivalent influenza vaccine is measured by the hemagglutination inhibition assay (HIA), which should be assessed 2 to 6 weeks post vaccination [57].

Considering that influenza viruses are constantly changing, the vaccine composition is reviewed each year and updated as needed. During the year, the World Health Organization (WHO) identifies the presence of viruses and makes recommendations on the composition of the next vaccine. This decision is taken from surveillance in different centres for reference and research on influenza and clinical studies and is made in

February considering the upcoming northern hemisphere's seasonal influenza vaccine and in September for the southern hemisphere's vaccine [58]. Vaccination efficiency is assessed through serological parameters.

1.8.2 Serological parameters

Serological (blood serum) parameters aim to describe the antibody response to influenza vaccine. The state of either having or not having detectable antibodies against a specific antigen in a serologic sample is known as the *serostatus*. Seropositive status means that a person has detectable antibodies, whereas seronegative status means that a person does not have detectable antibodies.

Post-vaccination titration of binding antibody is also an immunological parameter which describes the antibody response to vaccination by indicating the concentration of an antibody determined through the highest dilution at which agglutination of the antigen is present. Specifically, it is assessed by the HIA which measures the relative concentration of specific antibodies.

The protection rate provided by a given vaccine is assessed through the seroprotection indicator. An individual should achieve a hemagglutination-inhibition (HI) titre of equal to or greater than 40. Seroconversion rate corresponds to at least a four-fold increase in HI titre from pre-vaccination [59].

1.9 Microbiota and the gut-associated immune system

The lumen of the gut could be described as part of the external environment as it is in permanent contact with ingested contents. The main immunological function of the epithelium lining the lumen is to act as a barrier against external pathogens (See section 1.5) and to allow entry of nutrients. The gut lumen acts as a microenvironment where a variety of microorganisms ranging from bacteria, viruses and fungi can co-exist with epithelial cells. These microorganisms are known as microbiota. The gastrointestinal immune system must identify and discriminate pathogens from non-pathogens (See Section 1.5.1).

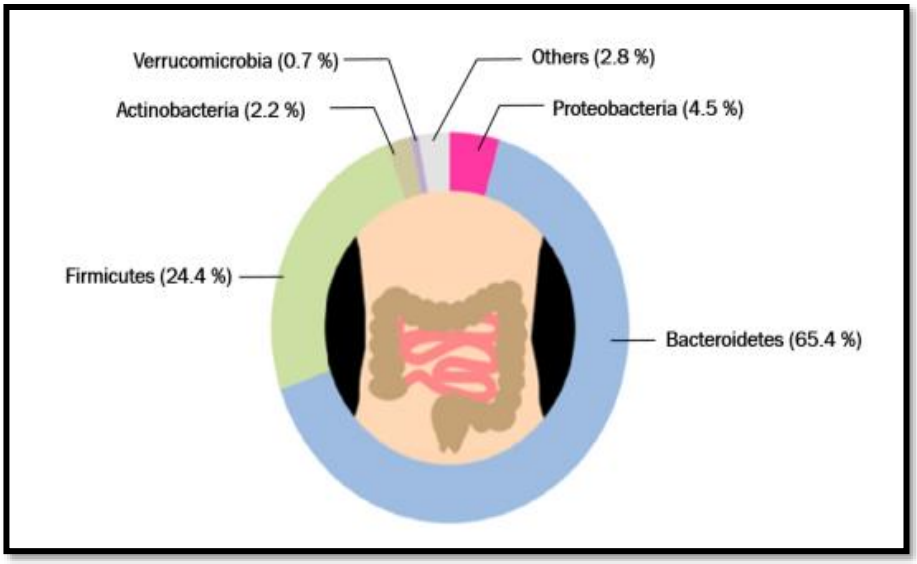
The microbiota composition is affected by different factors including age, pharmaceutical prescription, diet, physical activity and even geographical localization. Gut microbiota interacts actively with the enteric epithelial cells and has a role in the nutritional and immunological functions within the human body. Genes contained within the microbiota are grouped and studied as the human microbiome. It has been

calculated that the intestinal bacteria encode 100-fold more genes than those present in the human genome [60]. The microbiome has been shown to be modified according to individual characteristics and health or disease status. For instance, the microbiome composition found in obese people differs from that of lean individuals [61]; likewise, the microbiota composition from people suffering recurrent episodes of Clostridium associated diarrhoea differs from that of healthy individuals [62]. Findings like these have suggested therapies like faecal microbiota transplantation (FMT) and dietary interventions aiming to improve the microbiota composition by shifting it towards a healthy pattern [63, 64].

FMT aims to provide a wider density and diversity of beneficial bacteria to the gut to optimise metabolism of nutrients and immunological functionality [65]. A healthy donor with an optimum microbiome in the colon (or an ideal microbiota composition) can provide essential microorganisms to a person lacking them. Likewise, dietary interventions aim to provide essential microorganisms through the diet (probiotic consumption with specific strains or combinations of them) and to contribute to the microbiota sustainability through providing fibres and prebiotics (e.g. pectin and fructooligosaccharides) as well as contribute with enterocytic integrity. Large projects like the Human Microbiome Project seek to consolidate datasets to define the most relevant microbial species and their interaction with human health. A single definition of 'healthy microbiota' has not been agreed yet. Overall, it comprises greater diversity, and abundance of species such as Bacteroides spp, Lactobacilli and Bifidobacteria.

This section presents an overview of the microbiota composition in relation to human ageing. The study of different mucosal samples and the microbiota composition according to intestinal subdivisions has been a relevant methodology. However, faeces are the commonest clinical sample collected, as it is easier and less invasive for the individual. Figure 1.8 presents the distribution of main bacterial phyla present in the human gastrointestinal tract under healthy circumstances. Moreover, Table 1.6 presents bacterial phylum classification of relevant species in the human gut microbiota.

Figure 1.8. Relative abundance of main bacterial phyla in the adult human. Microbiome composition under healthy circumstances.



Taken and adapted from Shin, *et al* [66].

Table 1.6. Overview of common bacterial phyla and classification of species comprising the human gut microbiota

Bacterial classification Phylum	Order	Family	Genus / Relevant species in the microbiota
Firmicutes: Gram-positive cell wall structure mainly (Some species might have a pseudo-outer-membrane that causes to stain as Gram-negative)	Lactobacillales	Lactobacillaceae, Streptococcaceae, Enterococcaceae, Leucistic, Carnobacteriaceae, Oeonococcus, Weisella	Enterococcus, Lactobacillus, Lactococcus, Leunostoc, Pediococcus, Streptococcus, Clostridium.
	Bacillales	Staphylococcaceae, Bacillaceae, Listeriaceae, Paenobacillaceae, Macrococcus, Planococcaceae, Bacillales group XII, Alicyclobacillaceae, Bhargaceae	Staphylococcus, Listeria, Pediococcus.
Bacteroidetes: Anaerobic and mostly found in the gastrointestinal tract. Contain clusters of genes associated with polysaccharide utilization. Specialization on the digestion of peptides and polysaccharides. Gram-negative cell wall structure. Either non-motile or motile by gliding.	Bacteroidales, Cytophagales, Flavobacteriales, Sphingobacteriales	Bacteroidaceae, Flavobacteriaceae	Bacteroides fragilis. (*) Bacteroides species are significant clinical pathogens found in most anaerobic infections.
Actinobacteria: Gram-positive bacteria. Exhibits a wide variety of morphologies as well as diverse physiological and metabolic properties, such as the production of extracellular enzymes and the formation of a wide variety of secondary metabolites.	Bifidobateriales	Bifidobacteriaceae	Bifidobacterium.
Proteobacteria Gram-negative with five classes of organisms taxonomically classified. Common human pathogens,	Gammaproteobacteria Epsilonproteobacteria	Pasteurellaceae Enterobscteriaceae	Vibrio cholerae Escherichia coli Helicobacter

Microbe groups information summarised from Lanza *et al*. (Firmicutes) [67], Hahnke *et al*. (Bacteroidetes) [68, 69] and Litvak *et al*. and Stecher *et al*. (Proteobacteria) [70, 71]. Due to the extensive taxonomic classification of these species, only those relevant for humans are presented.

The presence of certain microbes in the gastrointestinal tract shapes an interaction with the mucosal immune system [72]. Some bacterial species are common in certain gastrointestinal diseases, while others have benefits. The difficulty in classifying "healthy microbiota" fully relates to the dynamic behaviour that some bacteria can have. For instance, while the presence of certain bacteria can produce helpful trophic factors for enterocytes, those same bacteria can also overgrow internally. An overview about the most relevant bacterial species in the gut microbiota and their classification is presented in Table 1.7.

Table 1.7. Classification of some harmful and beneficial bacteria in the microbiota.

<table>
<tr><th colspan="2">Harmful</th><th colspan="2">Beneficial</th></tr>
<tr><td rowspan="5">Pathogenic (toxin production) associated with diarrhoea, constipation, infection and systemic effects
Associated with carcinogenic processes
Anaerobic processes associated with putrefaction</td><td>Ps. aeruginosa</td><td rowspan="2">Lactobacillus</td><td rowspan="5">Prevent the growth of harmful bacteria (competing for colonisation, antimicrobial production and lowering pH).
Prevent tumour formation, lower cholesterol.
Reduce gas production
Contribute to digestion and absorption of food components.
Synthesis of vitamins.</td></tr>
<tr><td>Vibrionaceae</td></tr>
<tr><td>Staphylococcus</td><td rowspan="3">Bifidobacterium</td></tr>
<tr><td>Clostridium</td></tr>
<tr><td colspan="2"></td></tr>
<tr><td rowspan="3">May exhibit both harmful and beneficial effects</td><td colspan="2">Methanogens</td><td rowspan="3">May exhibit both harmful and beneficial effects</td></tr>
<tr><td colspan="2">Eubacterium</td></tr>
<tr><td colspan="2">Bacteroides</td></tr>
</table>

Taken and adapted from Gibson [73]

Epithelial cells have a critical role in sensing intestinal bacteria. They are equipped with a variety of PRRs including membrane-bound TLRs. TLRs mainly signal through the adaptor proteins to activate transcription and activation responses of host defence mechanisms (See section 1.4.3.3). The human gut and its interaction with microorganisms living in that environment play a role in the health status of the host, inflammatory processes in the gut and immunological status (i.e. immunoglobulin production) [74]. The "healthy" state of gut microbiota is rather a balance between microbial species. An imbalance (termed dysbiosis) has shown to be a pathological link with a disease state [75]. A healthy gut microbiota balance is also involved in the production of TLR ligands that activate B cells. For example, lipopolysaccharides produced by Gram-negative bacteria are able to activate B cells to produce IgA. Regulatory B cells with anti-inflammatory functions like the production of IL-10 suppress inflammatory T cells and tissue inflammation. Naïve B cell differentiation into regulatory B cells can be induced by IL-1β and IL-6 produced by DCs and tissue cells in mesenteric lymph nodes [76], triggered by the presence of certain bacteria in the gut. Lastly, B cell activation and differentiation are caused by stimulation of the TLRs, and cytokine receptors in the presence of bacterial cell wall components.

1.10 Integration of innate, adaptive, inflammatory and gut-associated immune responses

While innate immunity is a non-specific but faster response, adaptive immunity becomes more specific and vigorous when repeated exposure to the same pathogen occurs. The innate immune response contributes to the control of infections in the host, while the adaptive immune response is more potent and specialised. Throughout an immune and inflammatory response, the innate immune response results in activation of the adaptive immune system through the antigen presentation and generation of inflammatory mediators. Subsequently, effective adaptive immune responses require the induction of regulatory effector T helper cells. T helper cells are induced by antigen-unspecified signals derived from activated APCs [77]. Lymphocytes are activated to generate potent pathogen responses like the production of antibodies and cytotoxic T cells. The production of memory T and B cells is the main feature in the specificity and the efficiency of this response.

These responses can take place in the gut, in the mucosal immune system, which has as a main role the protection and defence of the host. This is a portal of entry to a vast number of foreign antigens in the form of food, amongst other sources. Foreign antigens reaching the lumen of the gut require identification and targeting to be eliminated if harmful. When foreign particles cannot be recognized as nutrients but instead possess structures recognised by APCs (mainly DCs), they activate specific regulatory T cells which produce cytokines to target the recognised foreign particle. Likewise, neutrophils delivered through the circulation can phagocytise foreign particles with the cooperation of macrophages. Neutrophils are highly apoptotic cells, they are responsible for quick responses in the phagocytic process. Likewise, macrophages also support the phagocytic process. They phagocytose dead neutrophils which simultaneously contain engulfed foreign particles. The immune system has evolved mechanisms to avoid a vigorous immune response to food antigens and to detect and kill pathogenic organisms that could enter via the gut.

These efficient responses are altered with the progression of time. The ageing process encounters physical causes like a decreased motility of the intestine, changes in the food consumption pattern and loss of muscular mass (proteolysis), decreased absorptive mechanisms and alteration in the micro-ecosystem found in the intestine. Likewise, the immune system and the response itself are also altered because of the ageing processes. The next section seeks to describe relevant changes seen in the ageing of the immune system.

1.11 The immune system in ageing: Immunosenescence

Dysfunctional consequences of the innate and adaptive immune responses across the ageing process are evident in the increased incidence of infectious diseases associated with decreased vaccine efficacy, and increased prevalence of chronic disease, autoimmune disease and cancer. Underlying immunological mechanisms are partly explained through a decreased tolerance and subsequently increased reactivity towards self-antigens. Understanding specific immunological changes within the elderly population will allow designing interventions targeting crucial changes.

1.11.1 Innate Immunity in ageing

Chemotactic processes, cytokine production and inflammatory mediators, as well as phagocytic responses are the main features of innate immunity altered with ageing.

1.11.1.1 Neutrophils in ageing

Neutrophils are of great importance in age-related diseases, as it has been suggested that the high rate of infections in aged individuals could be the result of neutrophil dysfunction. The comparison of the functions of peripheral blood neutrophils from healthy centenarians with healthy young adults indicates that a progressive impairment of responses accompanies ageing [78]. Blood neutrophil numbers remain unchanged in the elderly; however, the expression of CD16 is reduced (see section1.5.2.1) and, therefore, phagocytosis is affected [79, 80]. In elderly people, neutrophils tend to accumulate at sites of infection, due to a poorer clearance, and this is associated with higher tissue damage [81].

1.11.1.2 Monocytes-macrophages in ageing

The functions of monocytes and macrophages are preserved or even enhanced with the ageing process, where a continuous activation of these cells is usually occurring. Macrophages from aged humans exhibit greater production of prostaglandin (PG) E_2, which induces suppression of T cell functions [82]; and there is also increased production of free radicals [83]. This is relevant during ageing since PGE_2 and its subsequent suppression of T cell function increase susceptibility of the elderly to infections compared to younger individuals. Overall, ageing is characterised by a continuous monocyte activation which induces a permanent suppression of T cell

function [84]. This age-related dysregulation of the immune system forms part of what is known as *inflammageing* (See section 1.12).

1.11.1.3 Eosinophils and basophils in ageing

Overall it is suggested that eosinophil effector functions decline with ageing. These effector functions are associated with degranulation, a cellular process in which cytotoxic molecules are released and serve to control microbes [85]. Modifications in basophils across ageing are not clearly understood. It has been described that basophils support humoral memory responses, through stimulation in a ligand-dependent manner [86].

1.11.1.4 Dendritic cells in ageing

DCs are important in the regulation of adaptive immune responses. DCs control Th1/Th2 ratio and the Th17/Treg polarization, as well as the state of tolerance towards self-antigens. Aged populations tend to present a higher rate in autoimmune disorders, and alterations in DCs might be related [87]. Ageing is characterised by higher exposure to pro-inflammatory cytokines such as TNF-α. This endogenous environment might lead to DC activation altering the antigen uptake capacity dendritic consequences for autoimmunity [88].

1.11.1.5 Natural Killer cells in ageing

Natural killer cells have an important role in the antimicrobial response and the elimination of senescent cells. The mechanisms by which NK cells achieve this task are related to their expression of perforins, which contribute to target cell lysis, as well as their migration [89]. Senescent NK cells seem to have reduced expression in the pattern of perforins and reduced migration ability. This is a plausible explanation of the decreased NK-cell mediated cytolysis in ageing. Likewise, NK cells have a role in inflammatory processes. It has been shown that NK cells from older people have significantly lower production of IFN-γ [90] and of the chemokines MIP-1 α and IL-8.

1.11.2 Adaptive Immunity in ageing

Ageing of the immune system results in a loss of adaptive immune function. There is a decline in the absolute number of B cells and helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells; a typical feature is a low relative level of $CD8^+$ cells, widely accepted as a biomarker of

immune ageing [91] and also considered the most relevant functional change. This decrease in the $CD8^+$ T cell subset and the progressive exhaustion of this compartment leads to the loss of costimulatory molecules that are resistant to the usual apoptotic mechanisms that control the size of memory T cell clones responding to a particular pathogen [92]. These changes are also associated with an increase in levels of inflammatory cytokines, or *"inflammageing",* which may also contribute to the dysregulation of the cell-mediated immune response [93]. Furthermore, there are some age-associated differences in antibody formation via altered capacities for class switching and somatic hypermutation, affecting the humoral responses in the elderly.

1.11.2.1 T cells and cellular-mediated responses in ageing

The main characteristic of aged and differentiated T cells includes telomere erosion, reduced proliferation, a decrease of IL-2 secretion and decreased responsiveness. Phenotypic and functional modifications associated with ageing affect development, differentiation, exhaustion/senescence status, migration, signalling and the general metabolism of T lymphocytes [94]. There is a modification in $CD4^+$ T cells during ageing which prevents them from sustaining effective responses [95]. Likewise, impairment of $CD8^+$ T cells to elicit appropriate responses against pathogens occurs in ageing [96]. Modifications of CD27 and CD28 are a relevant feature in ageing as well. There is higher proportion of more memory cells and fewer naïve T cells. The fact that aged T cells do not display CD28, produces an accumulation of these T cells. Loss of CD28 reflects a stage in the differentiation of CD8 cells from naïve to memory cells and low relative levels of naïve T cells especially CD8 cells [23] [91]. Thymic involution with the progress of age in addition to exposure to a wide variety of pathogens across the life leads to a reduction in the naïve T cells and a relative increase in the proportion of memory T cells [92].

TCR and cytokine secretion patterns are altered in ageing. A large TCR diversity is considered essential for maintaining adequate immune competence to tackle new pathogens. Animal models have shown disturbances in the diversity of TCR repertoire when compared with younger counterparts (study conducted in dogs - [97]). Likewise, in humans, TCR diversity decreases linearly up to 70y [98]. This may contribute to the poor responses to novel infections in the aged [99].

1.11.2.2 B cells and humoral responses in ageing

B cells are important in the secretion of antibodies, and although humoral immune responses are less severely affected by the ageing process than cell-mediated immune

responses, older populations have changes in B cell repertoires which indicate that ageing dysregulates the function and distribution of B cells in the circulation [100]. It has been suggested that changes in the bone marrow and the periphery may result from increased B-cell longevity and decreased B-cell generation in the bone marrow. It is also likely that decreased immunoglobulin levels, particularly of IgM and IgD in the elderly [101], reflect a shift from the naïve B cell compartment toward the memory B cell compartment. The antibody quantity might also be affected, with B cells producing less antigen-specific antibodies with a lower antigen-antibody affinity [102]. Decreased B cell receptor expression is also associated with ageing [103] and loss of diversity in the B cell population impairs B cell responses to new challenges as the available repertoire would be decreased. Table 1.8 summarises the most important age-related changes in innate and adaptive immunity.

Table 1.8. The ageing immune system: key changes in innate and adaptive responses

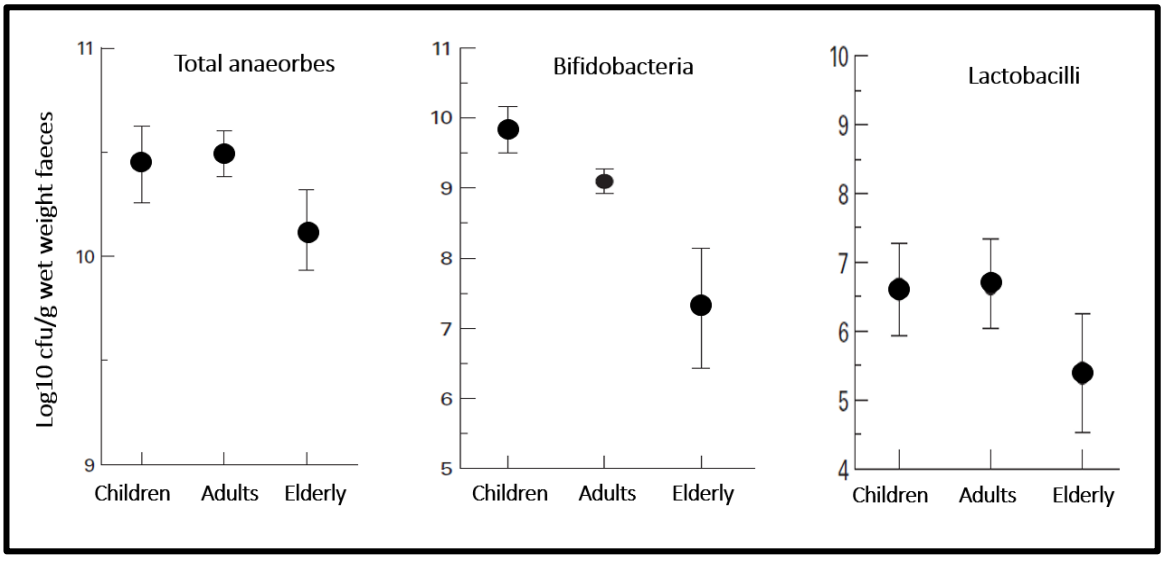
Immune component altered in ageing	*How* it is altered	*What* does it imply in the immune response	Ref.
Innate immunity			
Neutrophils	Number not reduced but phagocytic functionality decreased (neutrophil dysfunction)	Phagocytic mechanisms present a delay in controlling exogenous stimuli. Neutrophils tend to be associated with tissue-damage due to their accumulation and reduced clearance	[104]
Monocytes - macrophages	Enhanced response.	Continues monocyte activation that affects T cell function. Generates T cell response suppression	[105]
Dendritic cells	Reduced response	Reduced recognition of antigens and reduced efficiency in the antigen presentation process	[106]
NK cells	Reduced response	NK cells have a relative increase with a simultaneous decline in naive T cell output. Impaired crosstalk between NK cells and dendritic cells and it is likely that NK cells are not able to execute their functions n neutralizing antigens due to a reduction in the perforin system.	[90]
Adaptive immunity			
B cells	Decreased number and responses	Impaired humoral responses	[107]
T cells	Thymic involution affects the maturation process of T cells.	Absolute count of CD3, CD4 and CD8 T cells decreases with age Th1 to Th2 cytokine production shift Activated peripheral T cells are increased Memory T cells have a decreased IL-2 production and altered signal transduction in TCR (T cell receptor), and therefore become hyporesponsive Naive T cells become hyporesponsive The frequency of Helper cells decreases Cytotoxic T cells decrease in number. A typical feature includes low levels of CD8	[89]

Taken and adjusted from different references and authors as per “Ref.” column indication

1.11.3 The gut microbiome and ageing

Elderly are prone to develop a plethora of diseases, and an alteration in the microbiota may be partially responsible for worsening some conditions. Overall, elderly people are prone to present malnutrition, for instance, which is an important risk factor for diseases (e.g. infection) and an impaired immune response. An imbalance in the gut microbiota leads to decreases in nutrient absorption [108], which enhances the malnutrition process. Simultaneously, the lack of nutrient availability can also impact the nutrients supplied to the microbiome. This negative feedback enhances the microbiome dysfunction and intensifies the probability to develop diseases. Other prevalent conditions in the elderly are altered gut motility, loss of epithelial microvilli, and a decreased muscle mass, which seem to be associated with frailty [11, 109]. Besides the inherent age-associated physiological causes of microbiota dysfunction, it has been shown that the normal intestinal microbiota is affected in older people. A study conducted by Hopkins showed that there is a reduction in two important microbial populations (Bifidobacterium and Lactobacilli) with ageing [110]. It was also found a decline in anaerobic populations in elderly people. These changes are summarised in Figure 1.9.

Figure 1.9. Changes in faecal bacterial populations in different subject groups.

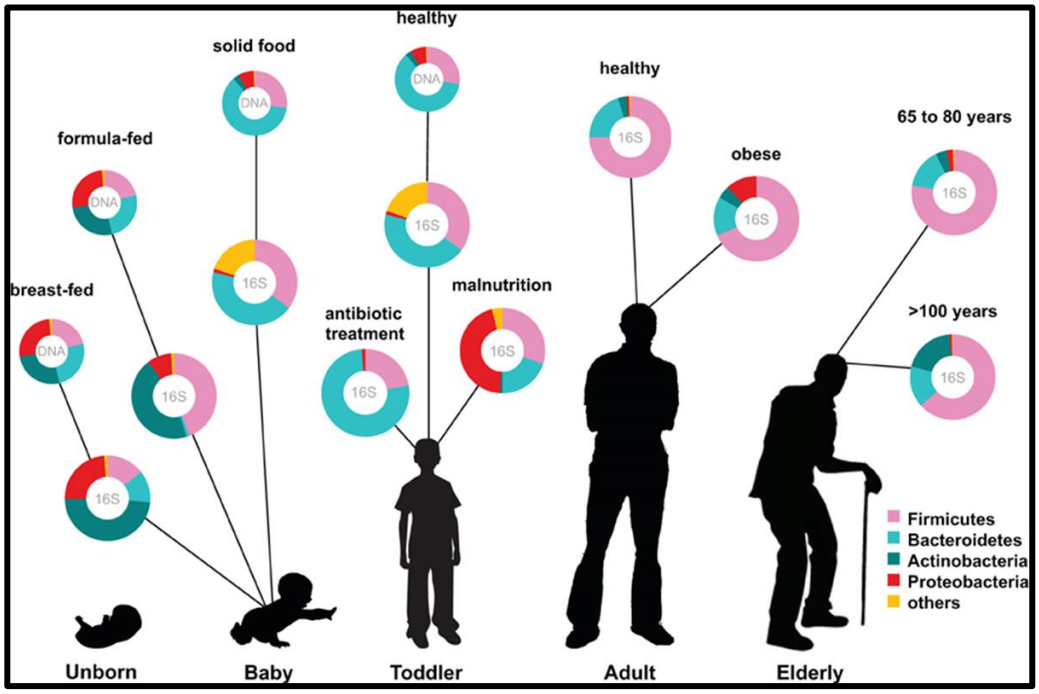


Modified from Hopkins *et al*. [110]. Fresh faecal samples were obtained from elderly participants (n=9) aged 67- 88 y. None of the subjects had a history of antibiotic therapy within the previous two months. Statistical significance was reported ($p<0.05$)

This modification in microbial populations not only consists of a decreased number of lactobacilli and bifidobacteria, but also consists of an increased amount of pathogens like Clostridia (a common pathogen causing diarrhoea) as presented in Figure 1.9 [111].

Another study conducted by Ottman *et al*., summarises the changes in gut microbiota across the lifespan and highlights the decreased diversity in elderly individuals. The maintenance of the gut microbiota and the presence of Bifidobacteria and Lactobacilli are relevant in ageing as these strains contribute to host health status by protecting against colonisation of harmful pathogenic bacteria and also through the release of substances which interact with the enterocytes [112]. Figure 1.10 illustrates the main changes across the lifespan and makes clear those modifications occurring in the ageing process.

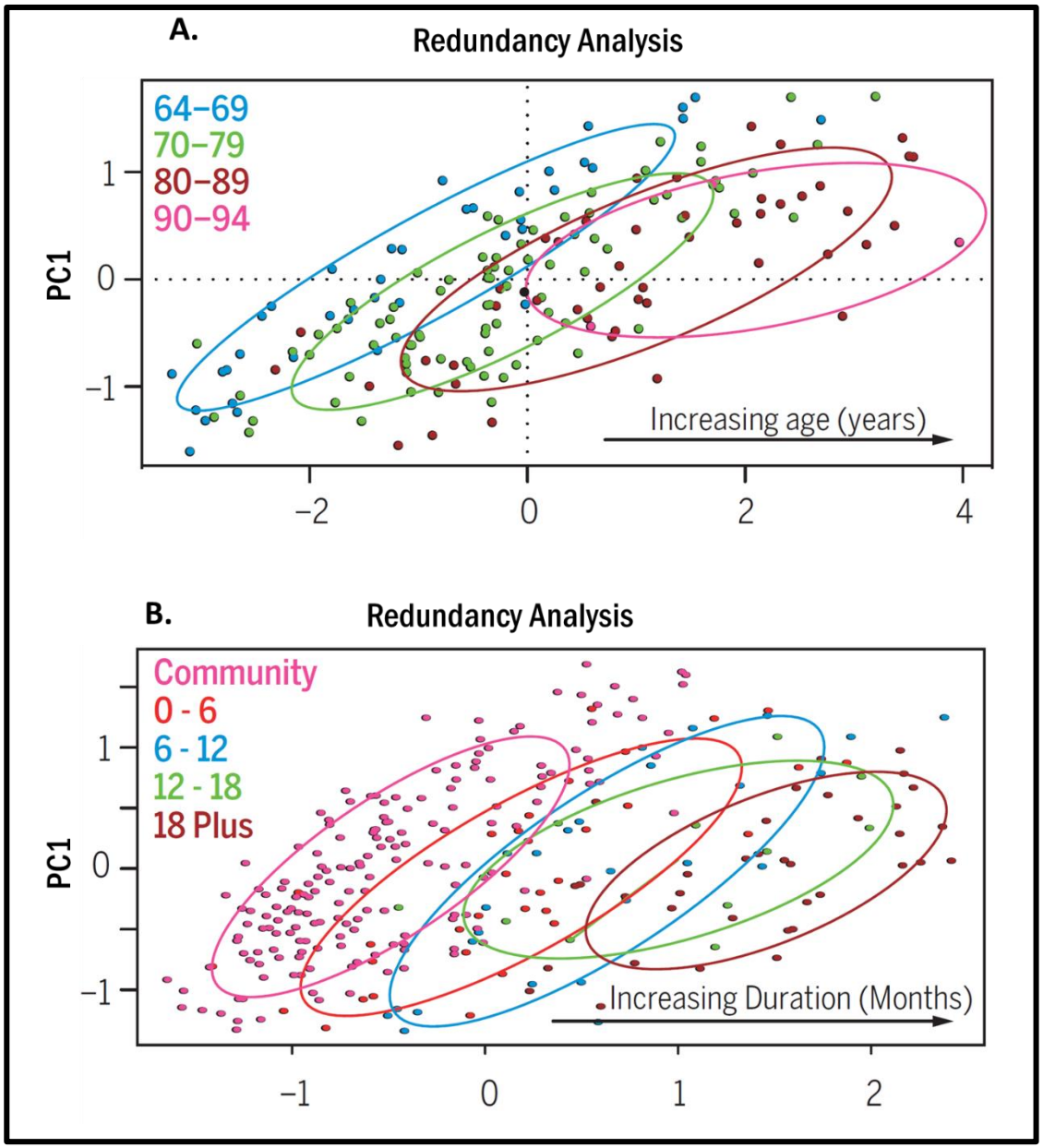
Figure 1.10. Microbiome changes in ageing



Taken from Ottman *et al*. [113] This figure illustrates the change in the microbial composition across the lifespan. Elderly individuals tend to lose diversity in the microbial population and there is an increased contribution of Firmicutes and Bacteroidetes. (DNA= Deoxyribonucleic acid; 16S= Ribosomal sequence used in classification and identification of Archea and bacteria)

It is a common situation that elderly people differ in the *ad libitum* dietary pattern and that their lifestyle is modified often due to their circumstances. The age-related changes in microbiota accelerate when elderly people transfer to care homes [114]. Figure 1.11 shows how the pattern of the microbiome changes with ageing and how it changes with the institutionalisation of old people.

Figure 1.11. Changes in microbiota diversity with ageing and moving into care homes.



Taken from O'Toole [114]. Data presents a redundancy analysis (RDA) plot of microbiota composition and the change with the progression of time. Figure 1.11A reflects the RDA of the microbiota composition and its modification in individuals according to the augmentation in age (n=176). Quadrants reflect the principal component analysis (PC1) to categorise microbiota profiles: (Left superior="Core microbiota" taxa present in the vast majority of healthy individuals where there is diversity in the microbiota composition / Left inferior= "Reduced core microbiota" typically observed in elderly / Right superior= "Diversity-associated" Patterns that tend to be similar and shared among population / Right inferior="Long stay/age-associated" where age-related changes in the microbiota are influenced by environmental factor but with a trend to be homogeneous within the community). The figure reflects the significant change in the microbiota composition from a "Core and reduced core" in the group age from 64-69 y towards a "diversity associated" pattern with the progression of age ($p<0.002$). Figure 1.11B presents the full data set of community and long-term residential care individuals by duration in care (logarithm of days), (n=282) and reflects the significant change in the microbiota composition towards a "diversity associated" and "long stay/age-associated" patterns according to the increased time of stay in the community of residents ($p<0.001$).

1.12 Inflammation in ageing: Inflammageing

The inflammatory process is defined as a localised response with systemic consequences which occurs in reaction to injury or tissue damage and follows two main purposes: the destruction of the harmful agent by enabling a network of molecular and cellular interactions and the repair of the wounded tissue [115]. Throughout the lifespan, the accumulation of damage (at a cellular level), the exposure to different antigenic challenges and lifestyles together with the chronicity of certain diseases can generate an imbalance between pro-inflammatory and anti-inflammatory cytokines which result in the classic low grade chronic pro-inflammatory status that has been described as inflammageing [22]. Healthy ageing seems to be therefore the result of an efficient anti-inflammatory network. Contrariwise, inflammageing is associated with a higher prognosis of inflammatory diseases and therefore an unsuccessful ageing process.

The inflammatory response directs the components of the immune system to the site of injury or infection. The response initiates with the attraction of phagocytes to the inflammatory site and contact with the triggering agent. There is fusion of the phagosome and lysosome with degranulation of lysosomal contents, the development of an oxidative burst and death and degradation of the agent. The acute phase of the inflammatory process involves pain, heat, redness, swelling and loss of function. The occurrence is common in response to tissue injury produced by trauma or infection, situations that might happen in elderly individuals. When there is a stable low-grade irritation, the inflammation turns into a chronic condition that damages the surrounding tissues. This chronic inflammation is characterized by the persistence and lack of clear resolution, and it is developed when the tissues are unable to neutralize the effects of the harmful agent. The chronic inflammation might impair optimum immune responses, leading to severe tissue deterioration which initially could be asymptomatic. Inflammageing is in general a prevalent condition in the elderly and increases the risk of age-associated diseases based on an inflammatory component [116]

1.13 Influenza vaccination response in ageing

Ageing is associated with an increased susceptibility to infections as the immune system is modified during immunosenescence (See section 1.11). The decline in thymic output of naïve T cells diminishes responses to novel antigens and simultaneously, there are aberrant clonal expansions. Those elements together lead to defects in the T cell

repertoire and poor responses of memory T cells to tackle conserved epitopes of the influenza virus. In general, the immune response to influenza vaccination decreases with age and the rate of seroprotection corresponds to a range from 30% to 70% in the elderly whereas in the young adult it is from 70% to 90% [117]. Although the protection conferred by vaccination is not complete and varies from one individual to another, the vaccine has been found to be associated with the less severe clinical presentation of influenza among the elderly, and therefore vaccination is a strategy which should be reinforced, as well as strategies aimed to improve the efficiency [118]. Although vaccination offers a considerable amount of protection, immunosenescence impairs the optimum efficacy of the vaccine.

1.14 Ageing process overview: Integration of immunosenescence, inflammageing and microbiota modification

There are clear differences in the immune system when comparing older and younger people. Although changes related to ageing are heterogeneous, the identification of biomarkers, hallmarks and their interactions with other factors (e.g. nutritional deficiencies) provide mechanistic insights on ageing progression, but more importantly, can highlight possible interventions. The following section explores factors affecting the ageing immune system.

1.14.1 Factors affecting the aging immune system

There are no well-established methods to predict variations in the immune system, nor definite explanations regarding impaired immune responses to immune challenges (e.g. vaccinations). Common changes as the individual gets older include a decrease in NK cell cytotoxicity, a reduction in both numbers and function of DCs in blood, a lessening in T cell function and expression of CD28, and a decrease in B cell numbers and function, leading to reduced generation of protective antibodies [119].

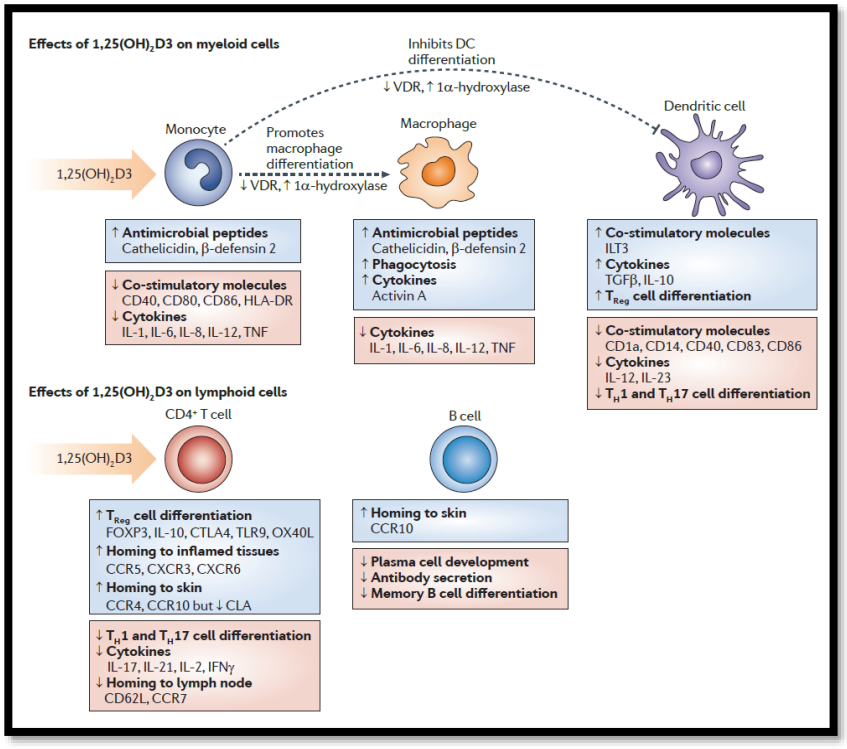
These events constitute a clearer perspective of what *immunosenescence* implies. However, immunosenescence does not appear in every older individual to the same degree. Human immunological diversity is related to several factors. The genetic background seems to be responsible for 25 to 50% of this variation [120]. There are some other factors associated with the age-related-variability of the immune system [121]. It is considered that oxidative stress accelerates telomere shortening and

therefore the DNA damage which will affect the ageing of immune cells and particularly lymphocytes [122]. Some other factors are related to microbiota composition, antibiotic consumption, vaccination history, physical exercise (acute or chronic) as factors that are able to exert an effect on the immune system via gut microbiota due to its close interaction with metabolic modifications [123]. Finally, nutrients have an acknowledged role in the modulation of the function of the immune system [124]. For instance, due to the presence of vitamin D deficiency in the elderly, it has been possible to identify that the actual correction of this deficiency can contribute with the improvement of the immune response [125]. Due to the relevance of this nutrient for the further research presented in this thesis, an exploration of this nutrient is herein presented.

1.14.1.1 Vitamin D and immune function

Vitamin D can be converted to its active form (calcitriol; 1,25-dihydroxyvitamin D3) in immune cells, modulating their differentiation, activation and proliferation [126, 127]. Vitamin D receptors expressed in immune cells are responsive to calcitriol through binding to the nuclear ligand binding domain (LBD) of the vitamin D receptor (nVDR)[128]. nVDR functions as a transcription factor [129]. Mechanisms of interaction between this nutrient and immune cell are summarised in Figure 1.12.

Figure 1.12. 1,25-dihydroxyvitamin D3 and interactions with immune cells and inflammatory responses



Taken from Hart *et al*. [130].

1.15 Probiotics

The intestine is the site of nutrient digestion and absorption. It also has a high microbial content, known as the microbiota (See section 1.9). Finally, the intestinal wall has a high immune cell content (See section 1.5.1). The proximity of microorganism and immune cells means that the gastrointestinal tract is an important site for recognition of pathogens. It is believed that a diverse microbial ecosystem (the gut microbiota) contributes to health (See section 1.9). Understanding the mechanisms involved and the effects of implementing probiotic treatments to elicit a beneficial and long-lasting modification in gut microbiota and the immune system remains a target.

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organisation (WHO) define the term probiotics as "live microorganisms which administered in adequate amounts confer a health benefit on the host"[131]. This general concept establishes some controversies, particularly the term "live" as current evidence indicates that both dead bacteria and individual bacterial components can have some of the effects of the viable organism particularly on the immune response of the host. Additionally, it remains unclear what "adequate amount" implies and in which conditions and life-stages probiotics will have the greatest benefit on health. Those issues highlight the necessity of targeting specific interventions according to age groups [132]. Finally, the impact of those "microorganisms" and whether used alone or in combination will confer the wider effect on immune system and therefore on health is also unclear [133].

As a result of those imprecisions, the International Scientific Association for Probiotics and Prebiotics made a consensus statement on the scope and appropriate use of the term probiotic. It is also suggested to include in the definition of probiotics those "microbial species that have been shown in controlled studies to confer benefits on health", and also claims to keep live cultures traditionally associated with fermented foods, and for which there is no evidence of a health benefit, outside the probiotic concept [134]. The mechanisms of action by which probiotics operate are still under exploration as it not clear whether it is their metabolic activity that leads to the main benefits or their cell wall components. Furthermore, the consensus opinion of the participants in the 4th Triennial Yale/Harvard Workshop on Probiotic Recommendations updated in 2015 proposed that agents such as Bifidobacterium and Lactobacillus, particularly *Lactobacillus rhamnosus GG*, are effective in the treatment of a variety of

clinical conditions [135]. A contribution from this updated consensus is referred to as the effect on gut microbiota where probiotics could have a positive effect as part of the treatment [136].

Probiotic bacteria belong primarily to Lactobacillus and Bifidobacterium genera. These are recognised as species exerting a range of effects, such as pathogen inhibition, immunity activation, vitamin production, anticarcinogenic activity and the formation of antimicrobial compounds which are known as bacteriocins. These effects are also attributed to dietary fibre and the fermentation by the colonic microbiota, including metabolites such as organic acids, for example, lactate and short chain fatty acids, which are the main product of carbohydrate fermentation. These acids lower colonic pH and inhibit the proliferation and activity of harmful microorganisms, which can produce a variety of enzymes converting some dietary compounds into genotoxic, mutagenic and carcinogenic metabolites. The production of antimicrobial substances such as lactic and acetic acid are examples of environmental modification. Some lactobacilli and bifidobacterial strains share carbohydrate-binding specificities with enteropathogens [137].

1.15.1 Probiotics and modulation of the immune system via gut microbiota

Resident microbes are underlying determinants of health and disease. Consumption of probiotics is beneficial in inflammatory intestinal or extra-intestinal diseases. Probiotics are able to restore non-pathogenic intestinal flora, prevent intestinal colonization by pathogenic bacteria [138], and enhance the immune system through increased phagocytosis, cytokine production, and natural killer cell activity [139, 140]. Also, acquired immunity is proposed to be improved through the antibody response [139]. Probiotics can decrease intestinal hyperpermeability [141]. It has been suggested that actions exerted by probiotic interventions also include a strain-specific anti-inflammatory effect [142]

When the effects of three potentially anti-inflammatory probiotic bacteria from three different genera (*Lactobacillus rhamnosus GG, Bifidobacterium animalis ssp. lactis BB-12 and Propionibacterium freudenreichii ssp. Shermanii*) were studied on immune variables, it was found that TNF-α production from *in vitro* cultures of peripheral blood mononuclear cells (PBMCs) was significantly lower in subjects receiving *Lactobacillus rhamnosus GG* when compared with the placebo [143]. The biological importance *in*

vivo of the strain-specific effect and the mechanisms involved remain to be determined [144], but in general, probiotics seem to be a cost-effective alternative for fighting infection, enhancing the immune response and alleviating the symptoms of malnutrition, which are all relevant goals in the elderly [145]. Table 1.9 summarises the mechanisms by which probiotics exert an effect on health via gut, microbiota and enhancement of the immune response.

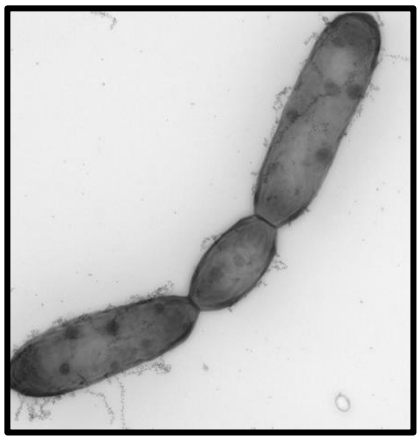
Table 1.9. Relevant mechanisms by which probiotics exert their effects on gut health

Effect on health	Mechanisms	References
Enhancement of the epithelial barrier	• Enhanced expression and redistribution of tight junction (TJ) proteins in the reconstruction of the TJ complex. • TJ structure includes transmembrane proteins such as Occludins and Claudins and intra-cellular connections corresponding to Zonulin. The mechanism involves a restructuring of these proteins as transepithelial measurements increased after a noxious stimulus. • The paracellular permeability likely to occur in the elderly population might be controlled and reduced through the TJ reorganisation.	[146-148]
Increased adhesion to the intestinal mucosa	• Beneficial microorganisms can adhere to gut epithelium and contribute to the exclusion of adherent pathogens by occupying limited binding sites. • Cell-wall of some probiotics can adhere to these particular regions as they express mucus-binding proteins. Those proteins are also associated with aggregation which help with the tissue-associated biofilm formation. • Certain bacteria can penetrate the mucus by swimming or eating their way through	[149-151]
Inhibition of pathogen adhesion	• Competitive exclusion of pathogenic microorganisms. • Reduction of the pH of the gut which affects pathogenic survival. Antimicrobial components such as peroxides are responsible for this modification. • Competition for nutrient metabolism which impairs the survival of pathogens. • Reduced pathogenic microbial count on in vitro adhesion models.	[152]
Production of anti-microorganism substances	• Increased production of bacteriocins (antimicrobial peptides produced by various bacteria including lactic acid bacteria or LAB). • Bacteriocins are able to form pores and inhibit cell wall biosynthesis, and therefore contribute to killing mechanisms for pathogenic microorganisms in the gut without causing toxicity to the host organism.	[153, 154]
Modulation of the immune system (See Chapter 7 and 8)	• Probiotics are able to modulate the immune response with live or dead probiotic bacteria and probiotic-derived components like peptidoglycan fragments. • Probiotic products can be recognized by host cells as those are equipped with recognition receptors. • Release of soluble factors which are able to interact with cells within the immune system	[155]

1.15.2 Lactobacillus rhamnosus GG (LGG)

Lactic acid bacteria, which include the genus Lactobacillus, are recognized as common inhabitants of the gastrointestinal tract and have received attention due to their impact on health [156]. Particularly *Lactobacillus rhamnosus GG* (LGG), isolated from faecal samples by Sherwood Gorbach and Barry Goldwin (explaining the letters "GG" given to this particular strain of Lactobacillus), is a promising probiotic because it is able to exert effects on digestive and gut health and the immune system [157]. Figure 1.13 shows the imaging of this strain.

Figure 1.13. *Lactobacillus rhamnosus* GG examined by transmission electron microscopy



Taken and modified from Kankainen *et al*. [158]. The figure illustrates the identification of pili in LGG, which is a mechanism that mediates its adherence to human intestinal mucus.

Mechanistic approaches support that this strain has an effect on health due to its resistance to acid and bile, subsequent growth and capacity of adhesion to the intestinal epithelial layer, as well as potentiation of anti-inflammatory pathways [159]. Once in the gut, LGG can interact with the host in a number of ways including through adhesive pili or fimbriae (which contribute in the adhesion properties conferred to this strain), lipoteichoic acid molecules, secreted proteins and galactose-rich exopolysaccharides which interact with the host. [160]. A study assessing the inflammatory effects exerted by this strain in an animal model (a neonatal mouse), indicated that its anti-inflammatory potential - calculated through the IL-10/IL-12 ratio as an indicator- could contribute to gut homeostasis [161]. *In vitro* studies using a model of peripheral blood mononuclear cells (PBMCs) have shown that LGG is able to decrease the production of reactive oxygen species (ROS) with subsequent effects on the control of inflammation [162]. Additionally, LGG can regulate the activity of neutrophils and the suggested mechanism is the inhibition of the formation of neutrophil extracellular traps (NETs),

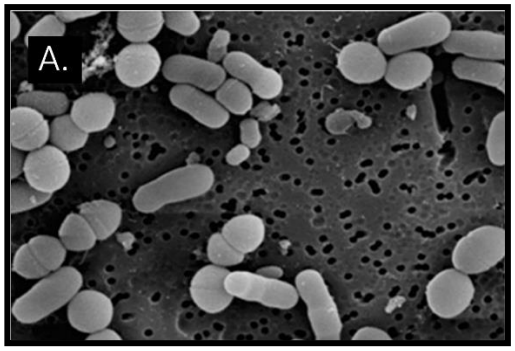
which represent a neutrophil mechanism for capturing, containing and killing microorganisms that in excessive liberation might be related to disease. This was tested using an *in vitro* model of neutrophils derived from murine bone-marrow and the neutrophil-differentiated human cell line d.HL-60 [163].

Finally, there have been some reports about infectious conditions where the microbiological analyses found isolates corresponding to LGG [164]. Nonetheless, the overall evidence indicates neither serious adverse events nor harmful effects of probiotic LGG in healthy elderly, in addition to good tolerability [165]. Novel clinical trials are required for further understanding of how probiotic or non-pathogenic bacteria such as LGG can modulate the immune system and generate health effects in humans.

1.15.3 Bifidobacterium animalis BB-12

Bifidobacterium are Gram-positive, anaerobic, non-spore forming bacteria which are found in the intestinal microbiota. This strain is a probiotic that is widely used, as it has been demonstrated to have beneficial properties for the human health via the digestive tract [166]. Its positive effects are favourable to the host due to its property as a saccharolytic microorganism. It has the ability to use gut-available carbohydrates in fermentative pathways, lowering pH in the gut and enhancing absorptive processes [167]. Moreover, this bacterium produces trophic factors for the enterocytes when reaching the gut [168]. Common challenges for probiotic strains refer to the questionable viability of the organism in the gastric acidity and high bile salt concentrations in the intestine. However, *Bifidobacterium animalis subs. Lactis BB-12* is surrounded by membrane proteins which are thought to confer resistance mechanisms which allow its survival [169]. Figure 1.14 shows the morphological appearance of *Bifidobacterium lactis* BB-12.

Figure 1.14. *Bifidobacterium animalis subs. lactis BB-12*



Taken and modified from Booyens *et al*. [170]. Transmission electron microscopy identifying Bifidobacterial cells shapes with well-defined membranes. Bifidobacterial lack of pili.

Whether probiotics have a beneficial effect in the elderly, and whether these organisms can shape the "healthy" microbiota pattern is not clear. A literature review linked to properties and mechanisms by which these two strains seem to confer an effect *in vivo* will be described in the following section. Other relevant findings in the probiotics field as a result of this literature review will be presented.

1.15.4 Probiotics in ageing

Probiotics may be useful in the prevention and treatment of age-related pathophysiological conditions. Because they can affect diverse sets of immune-regulatory cells at mucosal sites, probiotics seem to influence positively the course of immune-mediated diseases and disorders related to the dysfunction of the intestinal barrier. This may be because probiotics help to maintain the epithelial barrier function and because they can down-regulate inflammatory-associated genes. Probiotics have also shown to improve colonic mucosa and enhance immune responses in macrophages, NK cells, and antigen-specific cytotoxic T-lymphocytes and to modify the release of various cytokines. These effects seem to occur in a strain-specific and dose-dependent manner [171]. -

Probiotics may also interact with a wide variety of cells such as enterocytes, DCs, and Th1, Th2, and Treg cells which are located in the GALT and may modulate the immune response towards pro- or anti-inflammatory action [172]. Many probiotic strains seem to be capable of stimulating the production of IgA by B cells [173]. IgA binds antigens and thereby, limits their access to the epithelium [174]. In the elderly, this effect conferred by probiotics could be especially relevant as IgA by being present in mucosal surfaces can confer protection against infections [175].

Despite the positive findings described above, previous studies suggest some controversy around the risks of consumption of LGG. The most cited cases related to adverse events are reported by Rautio *et al*. [176] and Mackay *et al*. [177]. The first authors reported a case of liver abscess in a 74 y old woman who was admitted to the hospital due to high fever. The gram-staining of a hepatic abscess aspirate and further PCR analyses confirmed the isolation of *L. rhamnosus GG.* Furthermore, the fermentation pattern and enzymatic reaction of the isolate were compatible with a probiotic strain LGG used in dairy products commercialised in the area. The woman also reported daily intake of dairy drinks containing this strain (one-half litre). The second authors reported a case of endocarditis caused by the consumption of capsules containing 2 x 10^9 *L. rhamnosus GG* in a 67 y old patient who also had dental infections self-treated simultaneously with antibiotics. In parallel to the antibiotic treatment, the patient was consuming these probiotic capsules. This authors also included references of at least 58 cases of endocarditis were 50% of the individuals had some form of dental injury or procedure before the diagnosis of endocarditis. These cases have in common immunocompromised elderly individuals in addition to an underlying medical condition (diabetes with poor prognosis in the first case and antibiotic consumption in addition to injury in the second one). Likewise, self-prescribed probiotic consumption was common in these cases. Overall, it has been suggested that the consumption of some strains of probiotics is associated with mortality due to underlying causes such as recurrent infections and different comorbidities (often above 60 y old) indicating that the use of probiotics is not risk-free in vulnerable individuals and highlighting the necessity to evaluate their effects in these patient groups [178].

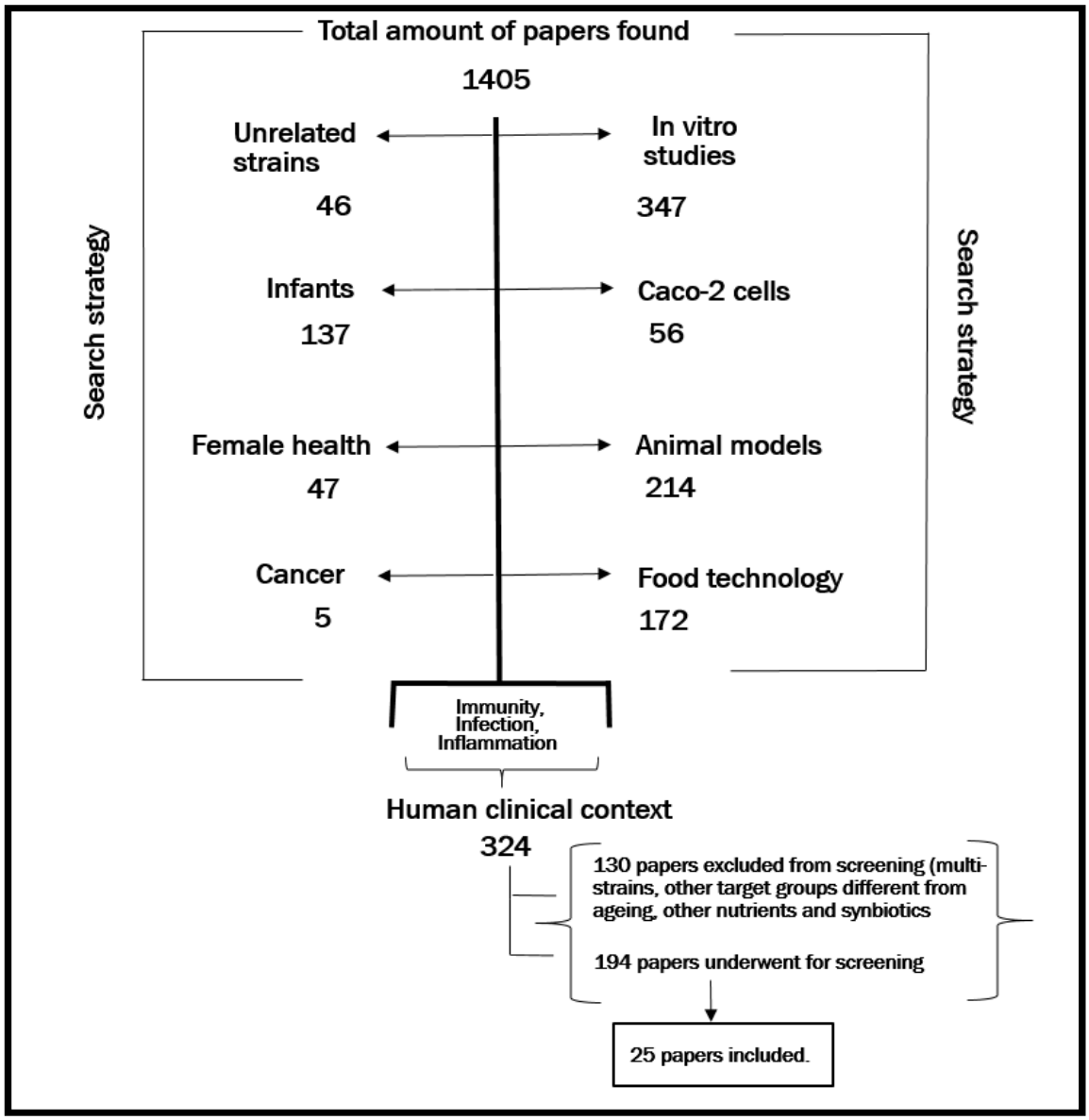
In contrast, a recent study assessing the safety and the tolerability of lactic acid bacteria in 143 human clinical trials found that these strains are safe for consumption in humans [179]. Moreover, a study conducted by Salminen analysed the incidence of adverse events and found 0.3 cases per 100,000 inhabitants per year from 1995 to 2000 in Finland [180]. The authors of this study analysed these findings in the context of increased consumption of LGG in the region. It was suggested that the increased utilisation of LGG did not increase bacteraemia or any other adverse episode associated with this strain [180]. Overall, it can be concluded that probiotics raise different questions, including the dosage of consumption as well as the safety in the elderly population.

1.15.4.1 Literature review of studies of probiotic interventions in the elderly

A literature review on probiotic (LGG and BB-12) use in the elderly was conducted. A semi-systematic approach was taken towards identifying the publications to be included. A first search strategy was conducted using the MeSH terms of interest as follows: (*"Lactobacillus rhamnosus GG AND Bifidobacterium animalis BB-12 OR Bifidobacterium animalis BB-12 AND Elderly OR Old OR Geriatric").* The findings from the search are shown in Figure 1.15.

Subsequently, the search strategy was improved and tailored using the MeSH terms of interest as follows: (*"immunity" OR "immune response" OR "infection" OR "immune system" OR "inflammation" OR "vaccination"* ***AND*** *"elderly" OR "aged" OR "ageing" OR "aging"* ***AND*** *"probiotic" OR "probiotics" OR "Lactobacillus rhamnosus GG" OR "Culturelle" OR "Bifidobacterium animalis BB-12" OR "Bifidobacterium animalis subsp. lactis" OR "Bifidobacterium animalis subsp. BB-12").* Searches were performed using EMBASE (Ovid), Medline (Ovid), BIOSIS Previews (Web of Science) and Cochrane library databases. Filters related to "Human" and "Age: 65+y" were also activated. Search strategies were adjusted according to the tool of research in use (e.g. Mesh terms, specificity or filters). Terms used in this search strategy were tailored to identify evidence related to clinical trials, infection, inflammation and immunity in the geriatric population, which received as part of a treatment or that consumed the specific strains of probiotics corresponding to *Lactobacillus rhamnosus* GG OR/AND *Bifidobacterium animalis* subs. *Lactis* BB-12 exclusively. Papers including a combination of probiotics, micronutrients and other dietary components (e.g. prebiotics) in the probiotic preparation are only included in the chart below if it is possible to discriminate the effect exerted by these two specific probiotic strains. Likewise, papers including a wide range of ages are only presented if the target population (elderly individuals) was considered in the study. Table 1.9 summarises the most important findings that emerged from the tailored search strategy.

Figure 1.15. Findings from the initial search strategy applied.



1.15.4.1.1 Overview of findings

Probiotics exert clinical, immunological and molecular effects related to health. This has been shown through *in vitro* studies, animal models, and clinical trials in humans targeting breastfeeding and pregnancy, newborns, children, healthy adults and the elderly [171, 181-184]. This literature review shows relevant clinical conditions under study relate to mental diseases, oral health, gastrointestinal conditions (microbiota composition, ulcerative colitis, irritable bowel syndrome), cancer (mainly gastric and intestinal), infections (respiratory and gastrointestinal), critical illness, and vaccination

response. Heterogeneity in the results makes it difficult to provide definite answers to aspects such as dosage, and a single strain with the best spectrum of action. Generally, it appears that probiotics are relevant in the management of gastrointestinal conditions. In contrast, their use as a supplement is not encouraged in severely immunocompromised elderly patients, and critically ill patients. Additionally, it was observed that probiotics are a common supplement in the food industry, where research has focused on survival of the species, ideal food matrixes in which probiotics can survive and other food additives (nutrients, fibres, other strains) that might potentiate their effect. Extracting data of studies solely conducted in the elderly was difficult as they are often studied with other younger adults.

A great proportion of studies showed positive effects in what the authors categorised as primary and secondary outcomes. As common primary outcomes, studies examined microbiota composition, bowel movements, NK cell activity, increased anti-inflammatory cytokine production, reduction of oral infections in denture wearers, reduction of pathogens mainly Clostridium, and anti-microbial activity of probiotics co-cultured with pathogenic bacteria. As secondary outcomes there were reports on improvement in the self-perceived quality of life after probiotic consumption. Importantly, many studies included young and elderly populations simultaneously. Considering the aims of the research herein presented, and the elderly as the target population; the following description will highlight relevant findings in human studies including elderly individuals. This evidence will be presented in the context of the probiotic strains of interest.

Addressing ageing, probiotics might act as therapies in common circumstances in the elderly such as infections and gastrointestinal cancer. A study conducted by Myllyluoma *et al*. analysed the effects of a multi-strain probiotic therapy including (1×10^9CFU)/mL of LGG (ATCC 53103), *L. rhamnosus LC705 (DSM 7061), Propionibacterium freudenreichii ssp. shermanii JS (DSM 7067) and Bifidobacterium breve Bb99 (DSM 13692)* in a milk-based fruit drink. It was consumed twice a day during the eradication treatment, and afterwards once daily over the next 3 weeks by subjects *Helicobacter pylori* positive. Mean age of participants was 57.3 y with an age range of 18 to 70 y. At baseline, microbiota composition between *H. pylori*-positive versus *H. pylori*-negative control individuals differed in the number of clostridia and the total number of anaerobes. Prescribed treatment for *H. pylori* infection induces long-term disturbances in the intestinal microbiota and the findings suggest that the probiotic combination contributed to minor changes in the microbiota [185]. Although in this study it is not

possible to identify the strain that exerted the most important benefit, the explanation of the effects observed is a consequence of the multispecies treatment where lactobacilli species were consumed. Also, of interest, it has been shown that colon cancer patients are susceptible to benefits when consuming a symbiotic combination including LGG+BB-12 in addition to a dietary prebiotic (a mixture of long-chain inulin and short-chain oligofructose). Participants aged between 18 and 75 y with a confirmed diagnosis of colon cancer or polypectomized patients were included in a randomized, double-blind, placebo-controlled trial for 12 weeks. Findings suggest that Bifidobacteria and Lactobacillus numbers increased in the faecal count in both cancer and polypectomized patients. Moreover, counts of the pathogen Clostridium were reduced [186]. The evidence showed the positive gastrointestinal effects of supplementation with 1 tablet containing approximately 10^9 CFU of *Lactobacillus rhamnosus* strain GG and 10^9 CFU of Bifidobacterium animalis subsp. lactis BB-12 for 14 weeks. Although this randomized, placebo-controlled study was conducted targeting patients with symptoms of schizophrenia from 18 to 65 y old, the authors reported that patients with an ongoing antipsychotic treatment that simultaneously consumed the mixture of probiotics were less likely to develop gastrointestinal problems including constipation, a common condition in patients under these treatments [187]. These studies have addressed from different angles the positive impact that probiotics (including the strains of interest) might exert in the elderly.

Lastly, returning to concerns about the safety of probiotic consumption in the elderly, Tapiovaara *et al*. developed a study assessing the effect of (*Lactobacillus rhamnosus* GG (LGG) alone or LGG in combination with *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* JS, *Bifidobacterium lactis* BB-12, or *Bifidobacterium breve* 99). The authors categorised the information according to the strains and in the analyses included 1909 individuals. The study concluded that the ingestion of these strains did not seem to cause gastrointestinal disorders, respiratory, thoracic and mediastinal disorders, and infections or infestations in the elderly participants included in this study, thus supporting the absence of adverse events in healthy elderly individuals using probiotics [188].

Findings included in this section were grouped into eight categories which relate to different aspects relevant in the ageing process. These categories correspond to immunological mechanisms, microbiota composition, gastrointestinal infections, vaginal infections, cancer, critical illness, respiratory conditions, and vaccination. Table

1.10 summarises the main findings extracted from the papers (n = 1728) that meet the inclusion criteria for the purpose of this review.

Overall, effects of probiotic (strains LGG and BB-12) tend to differ mainly depending on the condition under examination. With regards to immunological mechanisms, differential effects were observed between young and old populations. Lastly, linked to these immunological mechanisms, it appears that probiotics do not affect pathways related to transplantation as no clinical effects were observed. Moreover, an increased production in IL-8, IL-12 and TNF-α as a result of the probiotic modulation was identified. Subsequently, microbiota composition was found as an important category of study. Studies indicate that bifidobacteria and lactobacilli tend to increase after LGG and BB-12 consumption, moreover, safety was generally assessed as a good outcome within the studies as well as optimum bowel movements. Interestingly, although microbiota improves after probiotic consumption, studies involving participants with gastrointestinal infections shown that these strains are safe and well tolerated, although no effects on modulation of the inflammatory response were observed. Likewise, vaginal and respiratory infections appeared as important categories, but a mixed picture was found. In the vaginal analysis of microbiota, it is not clear whether these strains present an attachment, therefore the mechanism of action is not well established, whilst in the study of respiratory conditions, it was found that viral respiratory infections were reduced in the probiotic group. Beneficial effects might be observed in the absence of adverse effects which were not observed. Lastly, and remarkably, prophylactic administration is not encouraged in critically ill patients due to their underlying immunocompromised condition which can be worsen by the bacteria ingestion.

Table 1.10. Summary of studies of probiotics and infection, inflammation and immunity in ageing

Author and year	Study design (Strains, dosage and duration)	Subject details and Exclusion criteria (ExC)	Findings
Immunological mechanisms			
You *et al* 2014 [189]	Ex vivo/In vitro study. Peripheral blood obtained from healthy donors. Isolation of PBMCs and isolation of low-density cells as a source of human blood-enriched DCs ($1x10^6$cells/ml) Comparison of LPS vs. probiotic strains four probiotics diluted to $1x10^7$CFU/ml: Bifidobacterium longum bv. infantis CCUG 52486, B. longum SP, *Lactobacillus rhamnosus GG (L.GG)* and L. casei Shirota (LcS)	Healthy (young= 20-30y; old=75-75y) ExC: Diabetes needing medication, acquired or congenital immunodeficiencies, autoimmune disease, malignancy, cirrhosis, connective tissue diseases; current use of immunomodulating medication (including oral prednisone and inhaled steroids), self-reported symptoms of acute or recent infection (including the use of antibiotics within last 3 months), alcoholism and drug misuse	✓ Treatment (LPS or probiotics) induced expression of CD40 ($P < 0.01$) and CD80 ($P < 0.001$) in comparison to the untreated control. ✓ All probiotics strain enhanced DC expression of CD40 and CD80, but it was not significant in the case of CD80 in young DC treated with LGG. ✓ The significant influence of age on the expression of CD80 by LGG. CD80 increased in DCs from older donors (but not young). ✓ All strains increased the proportion of TGF-B producing cells in older subjects, but not younger subjects compared with unstimulated DCs. ✓ Induction of TNF-alpha by probiotics significant higher in old DCs compared to young DCs. ✓ All strains induced IL-10 by old DCs. ✓ DCs from older subjects had a significant higher basal expression of IFN-y than young DCs. ✓ Young T cells respond to unstimulated DCs by upregulating CD25. ✓ Older DCs treated with probiotics increased expression of CD25 compared with the control. **No effects:** - Expression of CD86 in DCs was not modulated - No influence of ageing on DCs exerted by other probiotics, except LGG. - Probiotics did not induce TNF- production - T cells from older subjects did not respond to DCs
You 2012 [190]	This in vitro study consisted of the comparison of $2x10^6$ of PBMC which were incubated with B. infantis or B. longum or LGG ($1x10^7$ CFU mL).	Healthy - n=16; (young: 23-30y; older n=65-76y). ExC: As reported in the previous paper	✓ A significant effect of age and treatment on NK cells was observed. ✓ Ex vivo exposure to the probiotic strains (including LGG) increased NK activity in both young and old donors.

			✓ Probiotic stimulation increased the production of IL-8, IL-12 and TNF-alpha in both age groups. ✓ LGG was the least stimulator of IL-10 production. **No effects:** - All probiotics except for LGG enhanced IL-6 production slightly more in older subjects than in young donors. - LGG was the weakest inducer of cytokine production
Gorshein *et al*. 2017 [191]	Randomized clinical trial. Enteric probiotic regimen: LGG Patients that underwent allogeneic stem cell transplantation received LGG	Allogeneic stem cell transplantation - n=20 (25-68y). Control - n=11 No probiotic. ExC: Prior probiotics supplementation within the last 90 days. Participants with a history of inflammatory bowel disease	✓ At 3 months of intervention, 3/20 patients developed grade Ia (Graft-versus-host disease) GVHD, 6/20 experienced grade II GVHD, 3/20 developed extensive cGVHD, 9/20 no disease. 4/20 patients died. ✓ At 12 months of intervention, 3 had limited cGVHD and 5 had extensive cGVHD, 5 no disease, 5 died, 1 underwent second transplantation. **No effects:** - No statistical difference between the two cohorts (probiotic supplement patient and not supplemented). - LGG did not impact the severity of GVHD.
Microbiota composition			
Poutsiaka *et al*. 2017 [192]	Randomized, open-label, two-arm parallel groups prospective study. Consuming probiotic supplement containing BB-12 and LGG ($1x10^9$ CFU/day) for 21 days	Healthy - n=27 (18-70y mean age 42y). ExC: Allergy to milk, consuming probiotics in the 30 days before, lactose intolerance	✓ Viable BB-12 and LGG strains were isolated in tools samples. This data was confirmed by qPCR. ✓ It was possible to identify viable BB-12 and LGG and to recover it from the gastrointestinal tract of healthy humans. ✓ The probiotic supplement was well tolerated **No effects:** - No adverse effects between the probiotic and placebo groups

Kwon *et al* 2015 [193]	A randomized controlled pilot study with LGG 10^{10} CFU during 14 days vs. standard care to prevent MDRO (gastrointestinal multi-drug resistant organism) colonisation in ICU. Probiotic group: 1 capsule (10^{10} cells of LGG) (Culturelle, i-Health, Inc., Cromwell, CT) 2/d	Probiotic - n=30 (Median age 65y from 29 to 82); standard care n=40 (Median age 59y from 32 to 82). ExC: Pregnancy, immunosuppression, HIV with CD4, transplant recipients, chemotherapy, vascular graft, left ventricular assist device, balloon pump, cardiac arrest, cardiac trauma, pancreatitis, endocarditis, rheumatic fever, diarrhoea, GI injury, inability to consent.	✓ No infections related to probiotics consumption. No significant effects: - Between probiotic and SC patients. No difference between died patients. - Acquisition or loss of any (Multi-resistant organism) MDROs (p>0.05). - Overall acquisition of any MDROs between the two groups (10% of probiotic group vs. 15% of SOC group; p=0.72). - No patients in the probiotic group and 7% in the SOC group acquired C. difficile (p=0.50).
Doron 2015 [194]	A randomized, double-blind, placebo-controlled clinical trial to examine the safety and efficacy of administration of the probiotic LGG at $2x10^{10}$ CFU for 14 days for the reduction or elimination of intestinal colonization by VRE.	Placebo - n=6; (53-77y; LGG group n=5 66-90y). ExC: Treatment with antibiotic active against vancomycin-resistant enterococci	No effects: - No differences in vancomycin-resistant enterococci. - Constipation, nausea, bloating, gas, diarrhoea occurred commonly in both groups. No statistical differences observed in those factors.
Solano-Aguilar *et al*. 2016 [195]	28 days of daily LGG. Dose Capsule (1 x 10^{10} UFC) twice/day. During the study until Day 56. *Lactobacillus rhamnosus* GG- (LGG) using RNA-sequencing (RNA-Set)	Participating in a phase I open Label study - n =11 (65-80 y)	✓ 95 differentially expressed genes (DEGs) were detected with a significant difference in gene expression at Day 28 following LGG treatment (FDR<0.1; 77 decreased and 18 increased). ✓ DEGs revealed down-regulation of overlapping genes involved with Cellular movement, Cell to cell signalling interactions, Immune cell trafficking and inflammatory response.

			✓ Pre-treatment transcription levels were restored at 28 days after LGG treatment was stopped.
Eloe-Fadroch *et al*. [196]	Gelatin Capsules with 1 × 10^{10} CFU, twice/day for 28 days L GG ATCC 53103.	n=12 Age (range): 65-80 y	✓ Gut microbiota was modulated by probiotic treatment. ✓ Motility genes increased during LGG administration. ✓ Genes involved in flagellar motility, chemotaxis, and adhesion from Bifidobacterium and the dominant butyrate producers Roseburia and Eubacterium had increased expression during probiotic consumption.
Pedersen *et al*. 2014 [197]	IBS patients assessed fulfilling Rome Ⅲ diagnostic criteria for IBS. Patients classified in 3 IBS subtypes: 6-wk allocated: 1) Low fermentable oligosaccharides, disaccharides, monosaccharides and polyols diet (LFD); 2) LGG Dicoflor60 capsules (Pharmaforce, Hvalsø, Denmark), containing LGG 6billion/capsule twice/day; 3) Normal Danish/Western diet (ND).	n=108 patients (median age 37 y, range: 18-74 y), 90 (73%) females were randomised: 42 to LFD, 41 to LGG and 40 to ND.	✓ Reduction in IBS-severity scoring system(IBS-SSS) (mean ± SD of IBS-SSS) from baseline to week 6 between (LFD) vs (LGG) vs (ND) was: 133 ± 122 vs 68 ± 107, 133 ± 122 vs 34 ± 95, P < 0.01. LGG improved the IBS-SSS ✓ IBS-quality of life showed statistically significant reduction of IBS-SSS in LFD group compared to ND (IBS-SSS score 75; 95%CI: 24-126, P < 0.01) **No effects:** - IBS-quality of life was not modified in LGG compared to ND (IBS-SSS score 32; 95%CI: 18-80, p=0.20).
Ouwehand 2008 [198]	Randomized, double-blind, placebo-controlled, study (Bioferme Oy, Kaarina, Finland) containing *B. animalis* ssp. *lactis* BB-12 (Chr. Hansen Ltd, Hørsholm, Denmark) at levels of about 10^9 CFU day	(84.3±0.98y) Intervention group (*n*=56): Oat-based drink with 10^9 CFU day of *B. longum* 2C (DSM 14579) and 46 (DSM 14583); Placebo group (*n*=67): Product without added probiotic. Control group (*n*=86): Commercial fermented oat drink	✓ Differences between the groups were not significant at the start of the study, but after six months, a significant difference (*P*=0.047) was observed between the placebo group (3.9 species per sample; SD 1.4) and the intervention group (2.8 species per sample; SD 1.4). ✓ *Bifidobacterium longum* was the most frequent species detected (94.2% of the subjects), followed by *B. adolescentis* (63.5%), *B. bifidum* (50.0%), the *B. catenulatum* group (46.2%) and *B. breve* (34.6%). ✓ *Bifidobacterium animalis* (17.3%) and *B. dentium*(9.6%) were less common components of faecal *Bifidobacterium* microbiota. ✓ *B. longum* (including *B. infantis*), *B. adolescentis, B. bifidum* and *B. catenulatum* were the most common species ✓ 6-month consumption of probiotic products resulted in modest increases in the levels of *Bifidobacterium* species **No effects:**

			- serum levels of IL-10 did not change significantly in any of the treatment groups during the study
Gastrointestinal infections			
Hibberd *et al*. 2014 [199]	*Lactobacillus rhamnosus* GG ATCC 53103 in gelatin capsules with 1 × 10 ^{10} CFU, twice/d for 28 d	n=15. Healthy participants. Age: 66-80 y	✓ LGG was safe and well tolerated ✓ 83% of subjects had mild events (no treatment required, no interference with daily activities); 40% of these were considered related to consuming LGG. ✓ Common adverse events were gastrointestinal (bloating, gas, and nausea); 27 were rated as mild and 3 were rated as moderate. ✓ IL-8 decreased significantly in plasma when compared with the baseline and then at day 28. ✓ IL-8 came to normal levels one month after discontinuing LGG. ✓ LGG was recovered in the stool in 11/15 subjects. **No effects:** - No effects on pro-inflammatory cytokines: IFN-y, IL-1b, IL-2, IL-5, IL-8, IL-12p70, TNF-alpha - No effects on anti-inflammatory cytokines: IL-4, IL-10, IL-13.
Ferrie *et al*., 2011 [200]	2 Gelatine capsules/d for 7 days. (LGG 10^{10}+280 mg inulin base (Culturelle) or inulin alone (placebo).	n=36. Consecutive critically ill enterally fed adults with diarrhoea. Patients included received gastric tube feeding in the ICU. Age: (Treatment, 56,2y; Placebo 61,7y)	**No Effects:** - Duration of diarrhoea (Days of diarrhoea since the 1st day of capsule administration.) - The severity of diarrhoea (Loose stools/d for 14 days since 1st capsule). - Average Loose stools/d, over 14 days since the 1st day of capsule administration.) **Adverse effects:** - A trend toward more diarrhoea in the probiotic treatment group. Mean (sd) duration of diarrhoea was 3.83 (2.39) days for the probiotic group and 2.56 (1.85) days for the placebo group (p=0.096).

Manley K *et al*. 2007 [201]	A double-blind, randomised, placebo-controlled trial. Subjects randomly assigned to either a treatment group (receiving 100 g daily of yoghurt containing *Lactobacillus rhamnosus* GG for 4 weeks) or a control group (receiving standard pasteurised yoghurt).	n=11 (vancomycin-resistant enterococci) VRE-positive patients. Mean age: 67 y old.	✓ 11 patients in the treatment group who completed the study cleared VRE. ✓ 3 subjects reverted to VRE positivity after using antibiotics to which LGG is sensitive, while all others remained negative for at least 4 weeks after trial completion. ✓ 12 control subjects completed the study, of whom one cleared VRE and 11 remained VRE-positive. 8 of these 11 patients were subsequently crossed over to receive LGG yoghurt, and all cleared VRE within 4 weeks.
Padilla Ruiz, M., et.al., 2013. [202]	Patients receiving 7 days of (Omeprazole 20 mg, Amoxicillin 1000 mg, Clarithromycin 500 mg). LGG (6x9 UFC)	(n=59) Positive for H. pylori infection (56,6±16,7 y (n=29) Treatment, Placebo (n=30).	No effects: - Side effects (Bloating, diarrhoea, taste disturbance, epigastric discomfort) occurred mainly during the eradication therapy without discontinuation. - Between the 2 groups for individual symptoms.
Holma, et., al. 2010 [203]	Groups: 1)Whole-grain rye bread (minimum 240g/d); 2) LGG (2 x 10^{10} colony-forming units/d)+ whole-grain rye bread (240g/d) 3) LGG (2 x 10^{10} colony-forming units/d) 4) White wheat bread (max.192g/d); 5) Laxatives (usual per participant) for 3weeks.	n=51 constipated adults (47 women, 4 men; 22-78 y)	✓ Rye bread relieved mild constipation and improved colonic metabolism compared with LGG. Not effects: - Colonic metabolism or constipation (measured by bowel movements, softening faeces, easing defecation) by LGG. - SCFA production by LGG consumption measured through an invariable intestinal pH.
Vaginal infections			
Colodner R *et al*. 2004 [204]	1-month administration of 1 or 2 doses per day of yoghurt containing 10^9 CFU of LGG.	n=42 postmenopausal healthy women	No effects: - LGG does not attach well to the vaginal epithelium. - 9.5% (n=4) were colonized with LGG, at a very low number of bacteria. Gastrointestinal tracts of 78.6% (n=33) were colonized. - There were no significant differences between one or two doses daily.

Rossi *et al* 2010 [205]	Prospective open clinical trial.12 and 24 months of treatment	n=40; mean y: 43 (22-70range). Patients treated with vaginal tablets containing 10^6 CFU/tablet	✓ pH values at baseline were 5,02, measured after 12 descended to 4,32 and at 24 months it was 4,1 of therapy. ✓ Itching, vaginal discharge and burning sensation decreased after 12 and 24 months of treatment. ✓ After 24 months of treatment, the reappearance of symptoms was markedly prevented.
Mezzasalma *et al* 2016 [206]	Randomized, double-blind, three arm parallel pilot $5x10^9$ CFU of LGG and $5x10^9$ CFU of BB-12	n=60 pre-menopausal women (18-50y) not suffering from vaginal or urinary infections. Vaginal swabs were collected at four experimental times (0,7,14,21) days of consumption after the first intake	✓ Swabs collected from the vaginal area were tested against C. albicans and E.coli (common pathogens involved in urogenital infections) ✓ The formulation containing *Lactobacillus rhamnosus* and Bifidobacterium animalis subs. Lactis showed stronger growth inhibition measured through the halos around the pathogen colonies tested. ✓ It was found that the species are abundant in the vaginal microbiota. The formulation containing the strains of interest were present after 21 days of consumption.
Cancer			
Osterlund 2007 [207]	Randomly assignment to receive or not *L. rhamnosus* GG (Gelatine capsules 1-$2x10^{10}$ twice/d for 24 weeks of chemotherapy. Consumption either swallowed or dissolved in milk or juice. Guar gum (11g/d) under 2 5-FU-based regimens.	n=150. Diagnosis with colorectal cancer.	✓ Lactobacillus intervention had less grade 3 or 4 diarrhoea (22 vs 37%, P=0.027), reported less abdominal discomfort, needed less hospital care and had fewer chemotherapy dose reductions due to bowel toxicity. ✓ No Lactobacillus-related toxicity was detected. ✓ 10% of patients with LGG had neutropenic infection. 4% of the patients did not have an infection. ✓ 8% of LGG required hospital care for bowel toxicity, compared to 22% in the comparator group. ✓ LGG may reduce the frequency of severe diarrhoea and abdominal discomfort related to 5-FU-based chemotherapy. ✓ None of the patients had LGG growth in blood bacterial cultures.
Critically ill patients			
Barraud 2010	Double-blind, concealed randomized, placebo-controlled trial	n=167; probiotics (n=87, 44 to 74y);	✓ No significant changes in mortality rates in both groups. ✓ There was no effect of treatment on the 90-days mortality rate. It was also observed an absence of the treatment effect.

[208]	in a medical intensive care unit (ICU). Adult patients mechanically ventilated for a period of more than 48 h received enterally administered probiotics (Ergyphilus, 2,9 10^{10} lactic acid bacteria, mostly *Lactobacillus rhamnosus* GG, once a day) or placebo until successful weaning	placebo (n=80,46 to 77y) Intubated, mechanical ventilation for at least 2 days were eligible. ExC: Predicted duration of mechanical ventilation less than 2 days, Age less than 18 y, Pregnancy, Immunosuppression (AIDS, malignant hemopathy, neutrophil count less than 500/ mm^3, cytostatic chemotherapy - past 3 months prior ICU admission), short bowel disease (risk factor for infections), inclusion in another trial.	✓ There was a decline of catheter-related bloodstream infections which was noticed in the probiotic group when compared with the placebo (6,78 vs. 1,84 respectively). ✓ No significant alteration was conferred by probiotics on urinary tract infections, ventilator-associated pneumonia. ✓ Overall there was no impact on mortality among patients mechanically ventilated for more than 2 days. ✓ Despite the acceptable safety profile for the probiotics, daily prophylactic administration of probiotics cannot be encouraged in the critically ill patient notably in non-severe septic patients. **No effects:** - Probiotic treatment did not reduce antibiotic consumption.
Gouriet 2011 [209]	Retrospective study screening for patients admitted to any hospital departments having bacteremia with *L. rhamnosus*	n=28 cases of bacteremia caused by Lactobacillus. 16 cases were caused by LGG	• From the patients resulting positive for the isolation of LGG from the blood. These patients were hospitalized in the oncology department (n=7), critical care unit (n=5), emergency (n=1) • Most common cause was predisposing factors to immunosuppressive therapy in oncology
Respiratory conditions			
Wang 2018 [210]	Randomized, double-blind, placebo-controlled pilot trial. Participants randomized to probiotics received 2	Nursing home residents aged 65 and older (n=196; ; 85.5 +- 7 y)	✓ Viral respiratory infection was confirmed in 14% of the residents within the probiotic group vs. 21.8% episodes in the placebo group. ✓ In the probiotic group, 5% of the participants had Influenza A whilst in the placebo group, 9.4% had Influenza A.

	capsules of LGG 10 billion colony forming units of LGG per capsule daily for 6 months		✓ Noninfluenza respiratory viral infection occurred in 10% of the probiotic group and 13.5% in the placebo group. No effects: - There was no statistically significant difference in confirmed viral respiratory infections between probiotics and placebo group. - There was not a significant difference in the adverse effects (nausea, vomiting, diarrhoea, and other problems).
Morrow 2010 [211]	Enteral probiotic LGG: Capsule $2x10^9$CFU twice/d suspended in sterile, water-based surgical lubricant. Administered as a slurry to the oropharynx. 2nd capsule suspended in sterile water given through the nasogastric tube. The inulin-based placebo was given twice/d. Routine care.	n=146 mechanically ventilated patients. High risk of developing VAP. LGG (n=68); Placebo(n=70)	✓ LGG group significatively less likely to develop microbiologically confirmed VAP compared with patients treated with placebo (40.0 vs. 19.1%; P = 0.007). ✓ Patients treated with probiotics had significantly less Clostridium difficile-associated diarrhoea than patients treated with placebo (18.6 vs. 5.8%; P = 0.02). Duration of diarrhoea per episode was not different between groups (13.2 ± 7.4 vs. 9.8 ± 4.9 d; P = 0.39). ✓ Patients treated with probiotics had fewer days of antibiotics prescribed for VAP (8.6 ± 10.3 vs. 5.6 ± 7.8 d; P = 0.05) and for C. difficile-associated diarrhoea (2.1 ± 4.8 SD d vs. 0.5 ± 2.3 d; P = 0.02). ✓ No adverse events related to probiotic administration were identified.
Cook *et al*. 2016 [212]	Randomized placebo-controlled blinded parallel group trial. Patients allocated to the intervention received 1 × 10^{10} colony-forming units of L. rhamnosus GG (Culturelle, Locin Industries, Ltd) in one capsule suspended in tap water, administered via gastric or duodenal tube twice daily while in the ICU. Patients allocated to placebo received microcrystalline cellulose suspended in tap water, identical in	n =150 ≥18 y of age. Patients expected to be mechanically ventilated for ≥72 hours. excluded patients who: (a) had been mechanically ventilated for more than 72 hours at the time of screening, (b) were immunocompromised (HIV CD4 cells/μl, chronic immunosuppressive medications, prior organ	✓ Study prospect times: study day activities took as an average 9hrs of work per patient (screening, consenting, and enrolling) ✓ Testing of 34 probiotic capsules from 10 centres to monitor the number of CFU of LGG over 25 months. Amount remained stable by being of 10^{10} CFU ✓ Collection of the majority of the samples related to the marker study. Around 4% of the samples were lost. ✓ As clinical outcomes blood infections were present in 19.3%; urinary infections in 12.7% and skin and soft tissue infection (4%) ✓ Antibiotic-associated diarrhoea occurred in 63.3% of the patients. ✓ No serious adverse effects were found ✓ A sub-study on the amount of fun in the capsules demonstrates that they remain among thresholds (10^{10} CFU) for up to 25 months.

	appearance and consistency to the probiotic, and administered similarly	or haematological transplant, absolute neutrophil count	

Tapioovara 2016	A randomized clinical.
	3 groups:
	n=19 Live-LGG;
	n=20 heat inactivated L. rhamnosus GG 10^9CFU;
	n=20 control juice daily for 6 weeks.
		Healthy – (18 to 65 y)
		After 3-week intervention period, an intranasal inoculation with HRV immunotype 39 was performed. Each subject received inoculations of 100 to 300 tissue culture infectious dose (TCID) 50 of HRV in both nostrils. Subjects continued to consume the intervention products for another 3 weeks.
			✓ The effect of life and heat-inactivated LGG consumption on nasopharyngeal HRV load and its association with clinical symptoms during an experimental HRV infection revealed some differences statistically non-significant.
			✓ HRV load positively correlated with the total symptom scores and 2 and 5 days after inoculation.

Vaccination			
Hibberd *et al*, 2014 [199]	Open-label clinical trial 1×10^{10} CFU of LGG. Consumed orally twice per day to elderly volunteers for 28 days	Healthy - n=15. (Age: 66-80 y)	✓ LGG was safe and well tolerated ✓ 83% of subjects had mild events (no treatment required, no interference with daily activities); 40% of these were considered related to consuming LGG. ✓ Common adverse events were gastrointestinal (bloating, gas, and nausea); those were rated from mild to moderate. ✓ IL-8 decreased significantly in plasma when compared with the baseline and then at day 28. ✓ IL-8 came to normal levels one month after discontinuing LGG. ✓ LGG was recovered in the stool in 11/15 subjects. No effects: - Pro-inflammatory cytokines (IFN-y, IL-1b, IL-2, IL-5, IL-8, IL-12p70, TNF-alpha) - Anti-inflammatory cytokines (IL-4, IL-10, IL-13).

1.16 Summary, hypothesis and objectives for the research project

The immune system undergoes many changes across the life span, particularly during ageing. This chapter has described different interconnected mechanisms by which the immune response protects the host. Likewise, the interaction amongst immune components and how these are affected in ageing have been described. It has been highlighted that the impact of decreased immune responses in ageing is observed in poorer response to vaccination and impaired resistance to infectious organisms. Those conditions not only reduce the quality of life in the elderly and increase mortality rates but also translate into higher health costs and burden for society. The evidence herein presented explained relevant mechanisms that play a role in maintaining immune competence in ageing where probiotics have shown to have a role. Probiotics are a suitable strategy to target both intestinal microbiota and host immune defences. These live microorganisms that colonise the lower gut and interact with the intestinal epithelium and with the immune system, may exert benefits according to the specific properties of the strain. LGG and BB-12 are two well-known probiotics. They are known to colonise the lower gut and are believed to interact with the host immune system. They may provide a way to beneficially modulate the immune system in elderly people helping to overcome the consequences of age-related immune decline. However, they are underexplored in the elderly especially with regard to their effects on the immune system.

1.16.1 Hypothesis

According to the existing evidence, it is hypothesized that:

- Consumption of LGG and BB-12 will improve parameters related to the immune system in elderly care homes residents.
- LGG and BB-12 will reduce the response of cultured epithelial cells to an inflammatory stimulus.

1.16.2 Objectives

The overall objectives of this thesis are:

- To investigate the influence of blood storage time on markers of immunity and inflammation.
- To investigate the effects of the combination of *Lactobacillus rhamnosus GG* and *Bifidobacterium animalis* subsp. BB-12 on markers of immunity and inflammation in elderly care home residents.
- To investigate vitamin D status in the elderly care home participants and how vitamin D is associated with markers of immunity and inflammation.
- To set up an *in vitro* model of intestinal inflammation using the Caco-2 cell line.
- To investigate the effect of *Lactobacillus rhamnosus GG* and Bifidobacterium animalis subsp. BB-12 in an *in vitro* intestinal inflammation model.

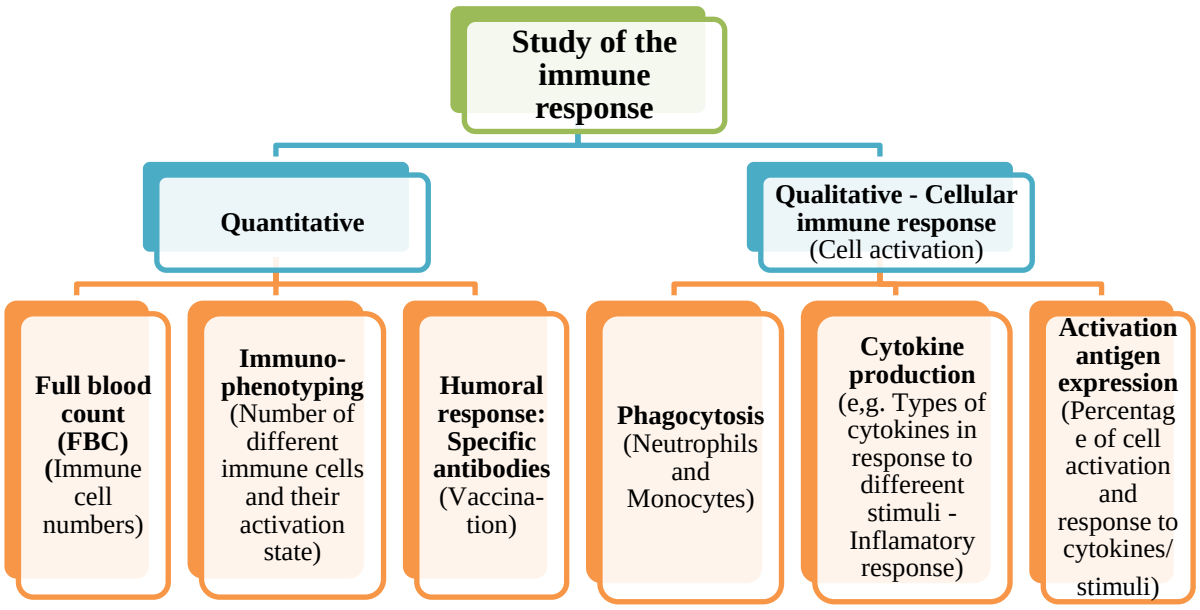
Chapter 2 Effect of delayed processing of peripheral blood on immune and inflammatory biomarker measurements

2.1 Introduction

Assessment of immunological and inflammatory processes is relevant in the study of certain diseases, as well as in the research of the effect of lifestyle and clinical interventions on health. Such investigations may measure relevant immune and inflammatory biomarkers. Such assessment of immune and inflammatory biomarkers and responses might involve additional challenges because of the circumstances in which the measurements need to be made. For example, research and sample-processing settings may be distant from patients and clinical staff (e.g. field studies or multi centre collaborations) so that access to freshly collected samples becomes a challenge.

The immune system can be studied through the assessment of humoral immunity, for example through the detection and quantification of antibodies in the context of infectious agents or vaccination. Alternatively, the study of the immune system might also be conducted by quantifying immune cells through the assessment of immune cell phenotypes and their effector responses [213, 214]. Such experiments take place in specialised laboratory settings. Figure 2.1 summarises a general approach for the study of the immune response in humans.

Figure 2.1. General approach for the study of the immune and inflammatory responses



Adapted from Cunningham-Rundles [215]. Assessment of immune responses from a quantitative and qualitative perspective allows a structured approach to studying the immune response. However, these processes are integrated. For instance, the examination of specific antibodies serves as a surrogate indicator of previous immunization and the B-cell system. Thus, *Humoral immune responses assessed through* immunization might reflect ongoing processes as an indicator of individual progression or immune impairment. Examination of the cellular response might involve the assessment at a cellular activation (e.g. *mitogenic activation* –Stimulation of mitosis and lymphocyte transformation-, *antigenic activation* –Formation of antigens- and *allogenic activation* –Mediators from histocompatibility cell interactions inducing cytotoxic T-lymphocytes in vitro, which can also be quantified). Subsequently, the assessment might include gene activation and protein expression where specific intermediates and cytokines can be indicators of the process. Both categories analysed in the context of an integrated immune response provide a complete overview of this system.

Equally important, the assessment of the human immunological response relates to where (in which compartment) the examination should take place. Blood is widely used in studies seeking to examine the human immune system with one advantage being that its collection is relatively safe. In blood, normal ranges of circulating immune cell numbers have been proposed (See Table 2.1). The peripheral circulating immune cells have been significantly associated with a variety of clinical outcomes and diagnoses (e.g. eosinophil count and respiratory disease progression [216]).

In a research study it may be necessary to make complex and specialized measurements in a designated central laboratory. Where research sites are distant from the central analytical laboratory, there might be a delay of hours to several days between blood collection and the central laboratory receiving the blood sample. The degree to which the delay affects the sample, as well as the optimum time-frame for processing,

are currently underexplored. In such circumstances, it is important to know whether such a delay will influence the outcome of the immune assays being made. The work described in this chapter sets out to identify whether a delay in processing human blood of up to three days affects a range of markers of inflammation and immunity. The immune system was quantitatively described (using full blood counts and the characterisation of immune cell phenotypes) and qualitatively analysed (studying phagocytic responses by neutrophils and monocytes, whole blood cytokine responses to stimulation with toll-like receptor agonists, and antibody titres in response to the seasonal influenza vaccine). Finally, immune mediators and cytokines were measured in plasma. The findings from this research were used to provide evidence of protocols, timings and procedures to set up clinical research including the PRINCESS trial – immunology sub-study, described in chapters 3 to 5.

2.1.1 Hypothesis

It is hypothesized that a specific delay in blood sample processing with storage at room temperature will not have a significant effect on the assessment of immune and inflammatory biomarkers when compared with outcomes from freshly collected blood with immediate processing.

2.1.2 Aim and objectives

The objective of the research described in this chapter is to determine the effect of delayed processing of blood samples on biomarkers of inflammation and innate and acquired immunity. The specific aims are:

- To determine whether delaying the processing of blood samples for 1 day (24 hours after collection), 2 days (48 hours after collection) and 3 days (72 hours after collection) alters the subsequent measurement of seasonal influenza virus vaccine antibody titres, immune phenotypes and immune cell functions compared to the measurements made with freshly processed blood (processed within the first hour after collection).
- To identify whether delayed sample processing is likely to affect any immune assessments to be made as part of the PRINCESS clinical trial – immunology sub-study.
- To select stable immune and inflammatory biomarkers derived from the analyses and translate the findings into the PRINCESS clinical trial – immunology sub-study.

2.2 Methods

2.2.1 Subjects of study

The study received ethical approval from the Southampton Research Biorepository Access Committee (12/NW/0794) and used anonymised human blood samples from 10 healthy individuals who provided written informed consent to participate. Inclusion criteria were age > 18 y, body mass index between 18.5 and 35 kg/m^2, and having received the most recent (by the year in which the study was conducted - 2016) seasonal influenza vaccine: The composition of influenza virus vaccines for use in the 2015-2016 northern hemisphere influenza virus consisted of strains A/California/7/2009 (H1N1)pdm09-like virus; A/Switzerland/9715293/2013 (H3N2)-like virus; and B/Phuket/3073/2013-like virus). Exclusion criteria were a pre-existing chronic disease, malignancy or autoimmune disorder.

2.2.2 Sample handling

The Southampton Research Biorepository provided 60 ml of blood per participant. Samples were provided as blood collected into EDTA as an anticoagulant (2 ml), into lithium heparin as an anticoagulant (6 ml) and as whole blood (2 ml). The different samples collected in different tubes were used for different immune assessments. One set of analyses was performed as soon as the blood was collected from the participant (Day 0 or 0 hr of storage time) and the same set of analyses were performed 24, 48 and 72 hours after blood collection (days 1, 2 and 3). Blood specimens remained stored at room temperature and protected from sunlight. A schematic overview of the process is presented in Figure 2.2.

Figure 2.2. Overview of processing of peripheral blood samples collected into different tubes.

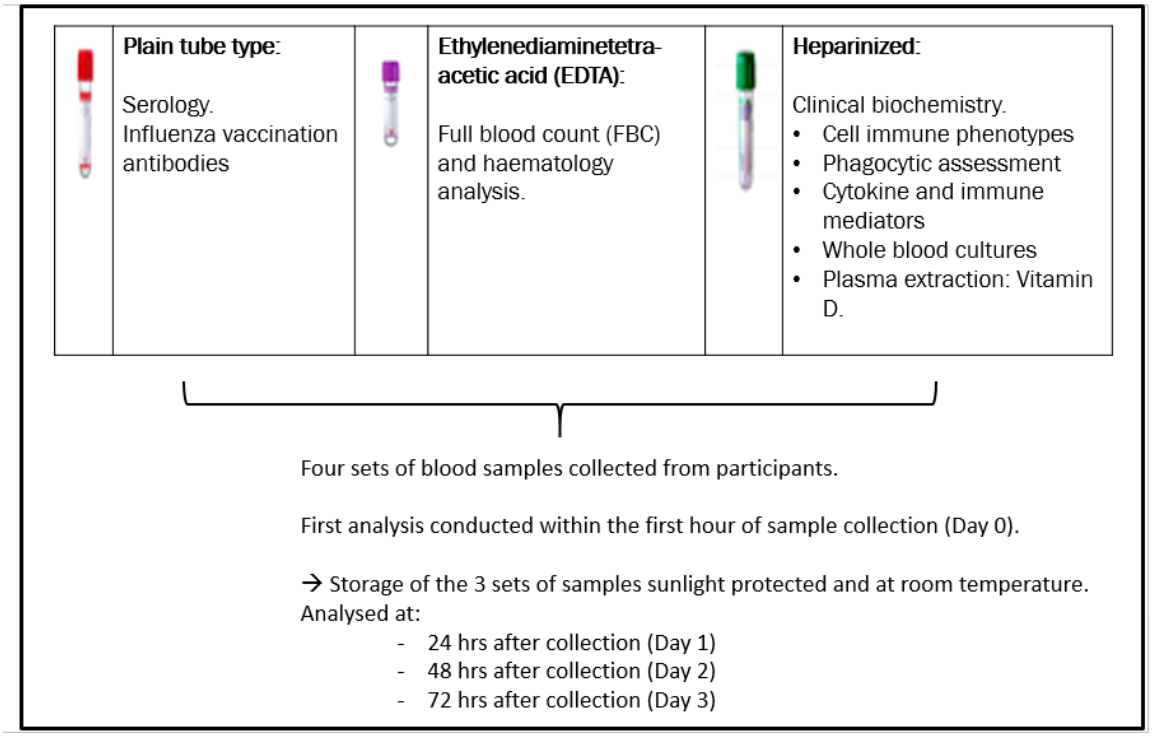


Illustration of the processing of the blood. Serum was isolated by centrifuging 1 ml of the whole blood sample collected from the plain tube. Afterwards, the aliquot of serum was stored at -80^{o}C until further analyses for influenza vaccine antibody titres (Conducted by a Public Health England Laboratory in London – See 2.2.5). Plasma was isolated from the heparinised blood tube by centrifugation. An aliquot of plasma was delivered to the Chemical Pathology Laboratory at Southampton General Hospital to perform Vitamin D analysis (see section 2.2.6). The EDTA tube was delivered to the Chemical Pathology Laboratory at Southampton General Hospital for obtaining the full blood count (see section 2.2.3.1.1). Lastly, an aliquot of the heparinised plasma was stored until further processing and used for the analyses of immunological and inflammatory parameters (See sections for techniques involving flow cytometry 2.2.3.4 and 2.2.3.5 immune mediators sections 2.2.4.1 and 2.2.4.2. These analyses took place in University of Southampton facilities, within the Human Development and Health and Cancer Sciences Academic Units.

2.2.3 Methodological design for the pilot study – Immune and inflammatory study approach

The selected approach to the study of the immune system and inflammatory response followed the scheme presented in Figure 2.1 where a quantitative and qualitative assessment of these responses was performed. The techniques used are organised and presented through protocols as follows:

- Immune cell types in blood using a Full Blood Count methodology (See section 2.2.3.1.1);
- Characterisation of immune cell phenotypes in blood using a flow cytometry-based approach (Immunophenotyping - See section 2.2.3.4);
- Cytokines and other immune mediators in plasma using a magnetic multiplex platform (General description of the technique - See section 2.2.4; Measurement of cytokines and immune mediators in plasma – See section 2.2.4.1.)
- Cellular responses assessed *ex vivo* through the study of the phagocytic activity of neutrophils and monocytes (See section 2.2.3.5) and production of cytokines and immune mediators in whole blood cultures after stimulation with stimulants including lipopolysaccharide (LPS), phytohaemagglutinin (PHA), and peptidoglycan from *Staphylococcus aureus* (PGN) (see section 2.2.4.2);
- Anti-influenza vaccine antibodies in serum (See section 2.2.5).
- Vitamin D levels in plasma (See section 2.2.6).

2.2.3.1 Quantification of immune cells and lymphocytic subsets

2.2.3.1.1 Full Blood count (FBC)

The *Full Blood Count (FBC)* is an assessment performed routinely and automatically for the quantification of immune and other cells in the blood. It has been standardised and allows determination of the proportion of white blood cells, lymphocytes, monocytes, neutrophils, eosinophils, and basophils as well as their number in a determined volume of blood. Parameters like haemoglobin, haematocrit, mean cell volume and mean cell haemoglobin are commonly included in the FBC report. These last parameters are rather associated with medical conditions (e.g. anaemia) and are informative of health status and nutrient deficiencies. Overall, the FBC report indicates values outside the normal

range suggesting ongoing abnormalities (e.g. haematological disorders, infectious processes, etc.).

In the research herein presented, the FBC was performed using peripheral blood collected into EDTA and analysed using a Beckman Coulter Counter, a device that sorts and counts blood particles. Reference values are presented in table 2.1 and were kindly provided by the laboratory [217]. Generally, the principle is based on quantifiable changes in electrical resistance produced by non-conductive particles suspended in an electrolytic solution. This solution passes through an orifice with electrodes which sense the zone through suspended particles. The Beckman Coulter measures the displaced volume as a voltage and the height of the pulses is proportional to the volume of the particle [16]. Table 2.1 lists the components of the FBC, their clinical relevance and the reference values according to the University Hospital Southampton NHS Foundation Trust.

Table 2.1. Clinical relevance, units and reference values of the immune cell populations identified through FBC

FBC parameter	Clinical relevance	Reference value (10*9/L)
Neutrophils	**Neutrophilia** (Increased neutrophil counts) is a common indicator of bacterial infection. The severity of infection is associated with marked neutrophilia. Likewise, it might occur in response to surgery, necrotic processes and certain tumours. **Neutropenia** (Decreased neutrophil counts) might be seen in connective tissue disorders, as a result of certain drug therapies (e.g. cytotoxic chemotherapy). It is also observed in viral infections. Mild-chronic neutropenias are not associated with infection and tend to be rather common.	2.0 - 7.5
Lymphocytes	**Lymphocytosis** is commonly the result of a viral infection (usually in the presence of neutropenia). If persistent, it might be linked to a lymphoproliferative disorder. **Lymphopenia** is usually linked to steroid therapies and immunosuppressive agents. Certain pathologies such as arthritis, systemic lupus and sarcoidosis are also associated with this outcome. Mild-lymphopenia is a relatively common finding and should not trigger further research.	1.5 - 5.0
Monocytes	**Monocytosis** tend to be associated with chronic infection, tuberculosis and is present as response to inflammatory reactions in intestinal diseases as a response to certain carcinomas.	0.2 - 1.0
Eosinophils	**Eosinophilia** is a relatively unusual finding in clinical practice. Causes tend to be related to connective tissue diseases, parasitic infections, neoplasia and allergies (asthma and food allergy).	0.0 - 0.5
Total white cell count (WCC)	**Leukocytosis** is traditionally classified according to the component of white cells that contribute to an increasing number of WCC. Thus, the cause varies but is generally associated with infections, nutritional deficiencies, or cancer.	4 – 11.0
Basophils	**Basophilia** is a relatively unusual finding in clinical practice. An elevation might be associated with an underlying neoplasm, acute myeloid leukaemia, infections (e.g. tuberculosis).	0.0 - 0.1
Platelets	**Thrombocytosis** is common in active chronic infection, inflammation and malignancy. These alterations often occur in the context of elevation of inflammatory markers. **Thrombocytopenia** can be associated with bone marrow failure, due to haematological diseases, presence of bone marrow infiltration and fibrosis.	140 - 400

Taken and modified from Leach [218] and Cambell and Smith [219].

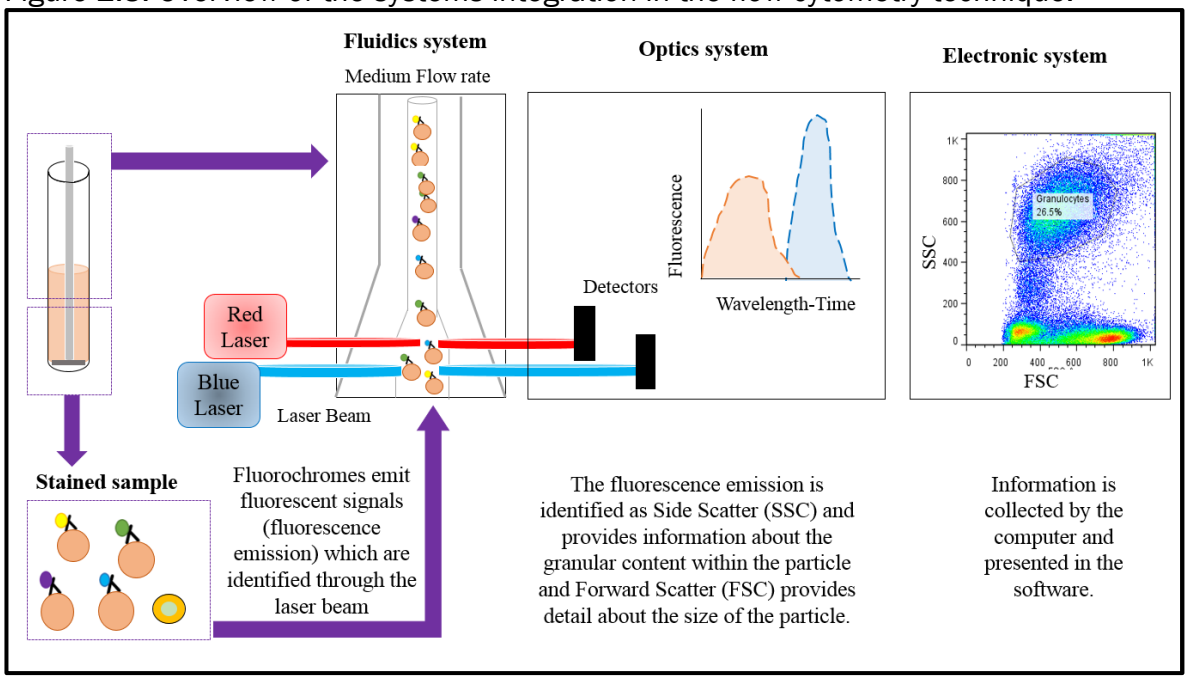
Immunophenotyping is the process of identifying the immune cells in peripheral blood and can be achieved (see section 2.2.3.2) by characterising the different immune cells of interest through the expression of specific cell surface markers using flow cytometry. The *Immunophenotyping* technique identifies immune cell phenotypes according to the "clusters of differentiation" (i.e. markers) expressed on the cell surface (See Table 2.4). Prior to the technique description, principles of the flow cytometry technique will be introduced.

2.2.3.1.2 Flow cytometry technique: Generalities

Flow cytometry is a technique widely used in the identification of immune cell phenotypes, but also can be used to detect fluorescently labelled particles inside targeted cells or intracellular markers. The flow cytometer involves three systems (fluidics, optical and electronic) which are all integrated. The optimum performance of the flow cytometry technique requires the appropriate preparation of the sample, the calibration of the instrument to ensure the quality of the data collection and correct interpretation of results.

The process starts with the fluidics system. This allows the suspension of particles to be analysed, which are transported in a stream past a laser beam. The laser illuminates the particles in the sample stream and reflected and refracted light from the laser is directed through optical filters to the appropriate detectors. The electronics system detects this light and converts it into signals. The detectors collect laser light around the cell/particle termed forward scatter (FSC) giving cell/particle size and light scattered by the cell/particle termed side scatter (SSC) fiving a measure of granularity/complexity. Detectors also collect signals in different emission wavelength band ranges, and in this way can detect different fluorescent signals. The number of different fluorescent signals which can be detected varies between instruments and is dictated by the number of different lasers and detectors within the flow cytometer. The electronic system collects the detected light signals and transforms them into electronic signals to be processed by a computer. Figure 2.3 represents the integration of the three systems.

Figure 2.3. Overview of the systems integration in the flow cytometry technique.



The fluidics system allows the suspended particles/cells to be channelled past the laser beam. The optics system directs reflected and refracted laser light and fluorescent signals (if present). Lastly, the electronic system detects light signals and transforms them into electronic signals to be processed by a computer.

2.2.3.1.2.1 Cytometer settings: Compensation and experimental controls

From a methodological perspective, the first stage in the optimisation of the optic system included running a performance check which was designed to monitor the performance daily and ensure the optimisation of the laser delay. These processes are called cytometer set up and tracking (CS&T) and consist of an automated process which defines instrument (flow cytometer) baseline and sets correct laser delays. The data acquisition consists of the signals captured from the particle passing through two or more laser beams depending on the complexity of the instrument. Data from the first laser need to pass to the subsequent laser, that delay is calculated and automatically adjusted within the optical compensation. This “performance check” monitors changes on the cytometer and is performed according to the manufacturer’s instructions by diluting CS&T microbeads into a suspension inserted in the fluidics system. This analysis provides information related to the cytometer performance and confirms optimum conditions of calibration. These CS&T analyses were performed daily and prior to sample analysis and data acquisition.

Importantly, the sample type and particle size influence the laser adjustment. The cell size, as well as the laser wavelength, alters the scattering behaviour. The light scatters

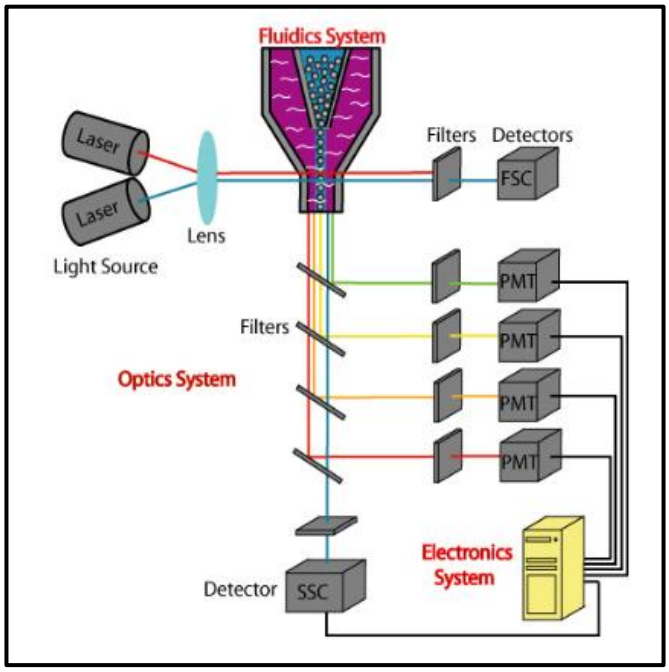
when it hits the particle, and that scatter is measured by two optical detectors (See Figure 2.4):

- Forward scatter (FSC) measures scatter along the path of the laser; thus this parameter allows discrimination of the particles according to their size. The FSC intensity correlates with the diameter of the particle due to the light diffraction around it. The intensity of the voltage is converted into an electrical signal that indicates the diameter of the particle. Immune cell discrimination can, therefore, be based on how large cells are as they will exhibit FSC of different intensities according to their size.

- Side scatter (SSC) measures scatter at a ninety-degree angle relative to the laser and provides information about the internal complexity (granularity) of the particle. Intracellular structures cause the light to either refract or reflect, therefore cellular components will be detected, and immune cell identification can be partly based on the extent of the presence of cytoplasmic granules. This light scatter tends to be weaker than the FSC, and so a photomultiplier tube (PMT) acts as a sensitive optical detector of the signal.

These laser signals are transferred into the electronic system as signals which are informative of the size and granularity of the particles analysed. Additionally, the fluorescence emitted from the particle is informative of the amount in which such particle was present in the sample and thus can be used to quantify the particle of interest. In the development of this research thesis, the compensation was adjusted according to the flow cytometer to be used as well as targeted particles presented in sections 2.2.3.2 about Immunophenotypes and 2.2.3.5 phagocytic assessment. Both techniques were conducted using flow cytometry and their respective compensations will be presented.

Figure 2.4. Forward scatter and side scatter optical system



Taken from Taylor [220]. Illustration of laser scatter and the formation of the forward scatter (FSC) and the side scatter (SSC) signals. Integration with the electronic system as a collector of light signal and conversion into electronic data is the last step used to discriminate among size and granularity of the particles and identify cell populations based on these measurements.

For immunophenotyping using multiple staining (different immune cell phenotypes) a combination of different fluorochromes corresponding to cell surface markers was established as shown in Table 2.4.

The experimental control included the selection of positive regions (stained cells) and negative regions (no targeted particles expected). Unstained or "blank" samples were used to gate these (Laser would not detect fluorescence). Also as part of the experimental controls, each sample was individually tested (per participant) through the use of isotype controls. These controls are antibodies that match the class and type of the antibody targeted (used to detect the required cell type according to the markers expressed on their cell surface) but these isotypes used as controls lack of specificity and help to discriminate among non-specific background signals and the specific antibody signals emitted from the fluorescently labelled particles of interest (See section 2.2.3.2.1). Assessment of the phagocytic process involved the labelling of fluorescently labelled particles (*E. coli* and DNA to differentiate bacteria and leukocytes). The experimental control consisted of a test tube where a higher phagocytic activity was expected and a control tube where it was expected to find a minimal activity. The compensation and controls for this experiment are presented in Table 2.6.

2.2.3.2 Immune cell phenotypes

Immune cell phenotyping is a process of identification and monitoring of the numbers and proportions of the immune cell subsets, their activation state, as well as the changes that occur in response to defined perturbations (for instance interventions aiming to modulate the immune cell number or their activation state). The immunophenotyping process is performed by analysing the expression of surface antigens through the staining and identification of surface proteins referred to as clusters of differentiation (See section 1.5.2.1) using the flow cytometry technique. There are a number of considerations when using this approach. One of these relates to the tissue in which these analyses take place. Ideally, the access to the tissue would allow a realistic (*in situ*) examination of the pathological/prognosis condition. However, costs and risks associated to it make it necessary to include a "liquid biopsy" derived from the blood which acts a surrogate marker of the parameters to be investigated. This principle applies to the methodological procedure herein presented where blood is used as the tissue of study. Another important consideration relates to the leukocyte subpopulation under examination. Depending on the leukocytes of interest, clusters of differentiation need to be selected to prepare the panel design and avoid staining overlapping. In the research herein conducted, this panel is presented in Table 2.4. Likewise, the panel design is directly linked to the flow cytometer in which the analyses will take place. This panel will also set the conditions for the compensation and calibration where the lasers to be involved in the identification of the particles is done.

2.2.3.2.1 Compensation for the immunophenotyping using a multi-staining procedure

Flow cytometer settings requires calibration according to the exact application. The optimization of not only FSC and SSC, but also all the detectors involved in the analysis as well as their voltages was crucial in the establishment of appropriate thresholds of detection. Table 2.2. presents the voltages that were used and the detectors to identify the panel design for the immunophenotyping.

Table 2.2. Voltages of detectors in the analysis of flow cytometry.

Detector (Abbreviation)	Name	Voltage
FSC	Forward scatter	325
SSC	Side scatter	280
FITC	Fluorescein isothiocyanate	540
BV421	Brilliant violet	290
APC	Allophycocyanin	630
PE	Phycoerythrin	490

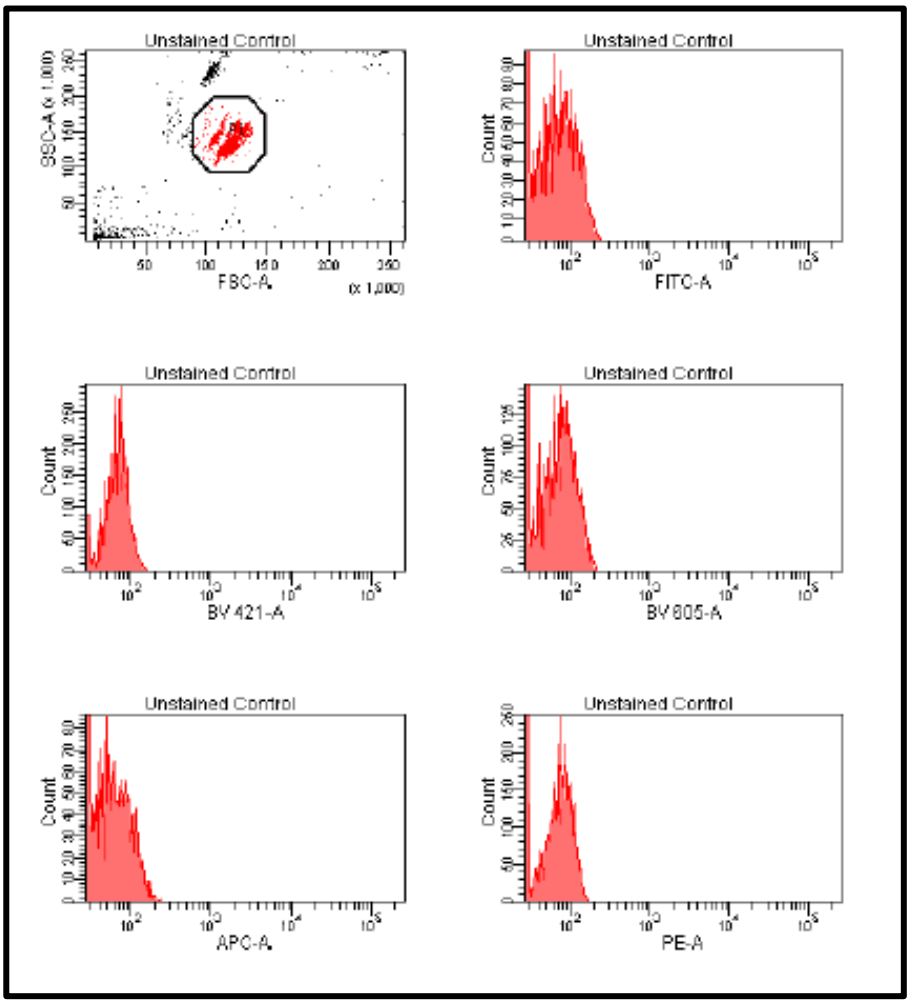
Besides the flow cytometer settings, compensation settings were established for the experiment. This removes the spill over of fluorescent signals into detectors other than the primary detector. The compensation process, therefore, included individual staining of the fluorochromes as described in Table 2.3.

Briefly, these compensation samples were loaded onto the cytometer in order to verify each fluorochrome signal separately. Additionally, this step also included the identification of negative and positive regions (regions where targeted cells will not be found and positive regions where the immune cell of interest would be found). For the discrimination of these regions, micro-particle sets were used (compensation microbeads). These polystyrene microparticles contain a set of anti-mouse antibodies, which can bind to murine immunoglobulin light-chain. They also contain a negative control which has no binding capacity. These microbeads were added to the fluorescent fluorochromes making it possible to obtain positive and negative background fluorescence for the stained population which was used to set up the software prior data acquisition (unstained compensation, Figure 2.5). Figure 2.6 shows the individual compensation of the fluorochromes selected (APC). All parameters followed the same process and plotting proceeded as for these examples.

Table 2.3. Fluorochrome volumes and staining panel used for the flow cytometer compensation.

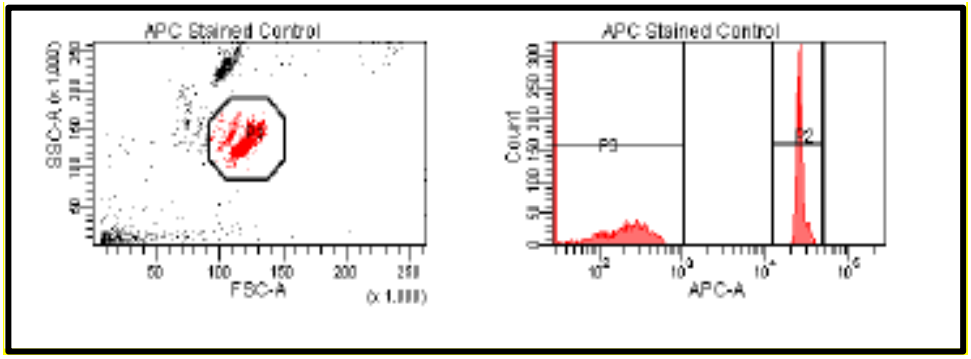
Compensation tube	Antibody stock label	Volume (µl)
1 - Unstained	Unstained	
2 – FITC (Fluorescein isothiocyanate)	CD4 - AF488/FITC	5
3 - BV421 (Brilliant violet)	CD127 - BV421	5
4 – APC (Allophycocyanin)	CD3 - AF647/APC	5
5 - PE (Phycoerythrin)	CD25 - PE	20

Figure 2.5. Unstained compensation and identification of negative regions according to lasers of detection.



The figure illustrates the outlook for the different detectors without staining; thus negative regions. Each diagram reflects the detection in the different lasers where the fluorochromes are aimed to be identified.

Figure 2.6. APC staining process and identification of negative and positive regions.



The figure illustrates the outlook for the APC detector after staining and loading onto the cytometer. Negative and positive regions were also gated. Once parameters were established, samples were only analysed in the positive region.

After ensuring the optimisation of the flow cytometer, as well as the experimental compensation considering the panel design for this research, thresholds and conditions were recorded for the execution of further experiments. All the features presented correspond to the calibration of the LSRF Fortessa flow cytometer. The software used was BD FACSDiva 8.0.1. Once the recruitment started, samples (peripheral blood) were used in the identification of the immune cell phenotypes. After the compensation, the immunophenotyping protocol was performed by using microparticles embedded within polystyrene tubes provided by BD biosciences (called Trucount™ tubes), which are tubes containing bright microbeads that can be used to determine the absolute count of specific leucocytes in a blood sample.

The immunophenotyping technique was carried out using polystyrene test tubes for optical clarity were used to place the blood sample used as controls (unstained and isotype staining in the tubes numbered from 1 to 3 and placed in regular Falcon Round-bottom - FACS tubes), whilst tubes with the fluorescent bead technology were utilised for the blood sample used in the immune staining and phenotype identification (immune population to be identified in the tubes numbered from 4 to 8 and placed in Trucount™ tubes). Overall, the process consisted of adding the indicated monoclonal antibody (according to the panel design) to the blood sample in the respective polystyrene test tubes as shown in Table 2.4. Heparinised whole blood (100 µl) was added to either polystyrene tubes or a Trucount™ as indicated in the table 2.4. Brilliant stain buffer (50 µl) was added to the sample. This staining buffer is used when there are staining reagents conjugated with other fluorescent reagents as interactions may affect data interpretation. Tubes were vortexed for 5 seconds.

Antibody volumes and combinations were added according to the panel design shown on table 2.4. Tubes were covered with aluminium foil and stored in the dark at room temperature for 20 minutes. Lastly, 1 ml of lysing solution was added. The addition of this reagent is crucial as it lyses red blood cells after the immunofluorescent staining process. Red blood cells might interfere with the flow cytometer data acquisition. The colloidal solution was briefly vortexed, and the tubes covered with foil and stored in the dark, at room temperature. Complete lysis of red blood cells occurred after 20 minutes; during this time tubes remained in the previous light-protected conditions.

Where complete lysis had not occurred, an additional 500 µl of lysis solution was added, the tube was vortexed and incubated at room temperature in the dark for a further 20 minutes. The tubes were stored at room temperature in the dark until analysis. Stained samples were analysed within 24 hours (in accordance with the manufacturer's recommendations). A known number of fluorescent beads (as previously described) were counted from the Trucount tubes and then the absolute number of cells was determined by comparing cellular events with the number of positive bead events collected in the gate. For the list of reagents used please see Appendix A – Reagents.

Table 2.4. Panel design for the multiple staining procedure used in the immunephenotype technique.

Tube	Objective/ Cell type	Stain and CD	Immunephenotype	Volume (µl) of antibody used per test
1 FACS	Negative	None	Negative control (without staining)	-
2 FACS	Isotype control	Multiple stain controls	Mouse IgG1 with PE, AF488, AF647, BV421 plus mouse IgG2a with AF488	20(PE) + 5(AF488)+ 5(AF647)+ 5(BV421)
3 FACS	Isotype control	Multiple stain controls	CD3/CD4/ Mouse IgG1 with PE / Mouse IgG1 with BV421 AF647/AF488/PE/BV421	20(PE) + 5(AF488)+ 5(AF647)+ 5(BV421)
4 Trucount	T cells	CD3/CD4/ CD25/CD127 AF647/AF488/PE/BV421	T cells = $CD3^+$ Helper T cells = $CD3^+CD4^+$ Regulatory T cells = $CD3^+CD4^+CD8^-$ $CD25^{HI}CD127^{LO}$	5(CD3)+ 5(CD4)+ 20(CD25)+ 5(CD127)
5 Trucount	B cells	$CD3^-$ $CD19^+CD80^+$ $CD86^+$ AF647/ AF488 / BV421/PE	B cells = $CD3^-CD19^+$ Activated B cells = $CD3^-CD19^+CD80^+$ $CD3^-CD19^+CD86^+$	5(CD3)+ 5(CD19)+ 20(CD80)+ 20(CD86)
6 Trucount	Monocytes	$CD14^+CD80^+$ $CD86^+$ AF488 / BV421/PE	Monocytes = $CD14^+$ Activated Monocytes = $CD14^+CD80^+$ $CD14^+CD86^+$	5(CD14)+ 20(CD80)+ 20(CD86)
7 Trucount	Suppressor T cells	$CD3^+CD8^+CD25^+$ AF647/ AF488 / PE	$CD3^+CD8^+CD25^+$	5(CD3)+ 5(CD8)+ 20(CD25)+
8 Trucount	NK Cells	$CD3^-CD16^+$ AF647/PE	$CD3^-CD16^+$	5(CD3)+ 20(CD16)

AF647= Alexa Fluor 647 - AF488= Alexa Fluor 488 - PE= Phycoerythrin - FITC= Fluorescein isothiocyanate - BV421= Brilliant violet

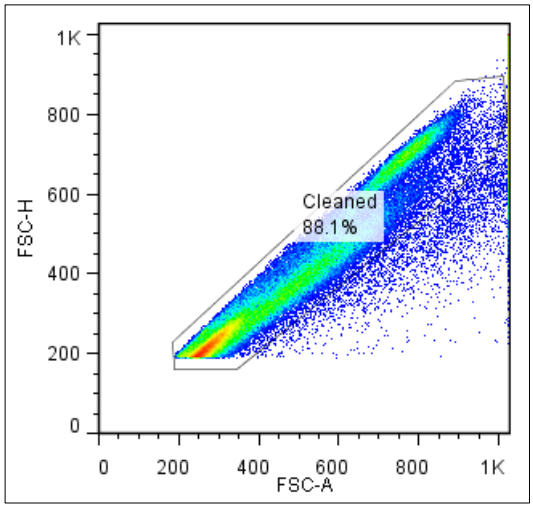
2.2.3.3 Median Fluorescence Intensity (MFI) of CD14⁺ expressed on monocytes and neutrophils

The immunophenotyping process also considered the analysis of the median fluorescence intensity of CD14⁺ expressed by monocytes and macrophages. This cluster of differentiation is a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein (LPB). It is involved in LPS induced tumour necrosis factor-α production. Expression of CD14⁺ increases in monocytes differentiating into macrophages, and in response to LPS, whilst a downregulation can be observed in response to IFN-y [221]

2.2.3.4 Establishing the immunophenotyping – processing the flow cytometry data

Once the protocol for the flow cytometry technique was established (flow cytometer characteristics, laser compensation, and panel design) the following steps involved the correct identification of the population using the electronic system and the appropriate software. Firstly, the gated population was selected by drawing an area around the singlets to delete debris and doublets (population of cells that were clotted when collected by the laser system producing a "noisy" signal that is observed in the graph as an increased size and granularity). This identification is shown in Figure 2.7.

Figure 2.7. Depiction of a flow cytometry profile according to the cleaning process.



Cleaning process. The gated region consists of the cell population that was examined. Debris excluded from the gating was not considered

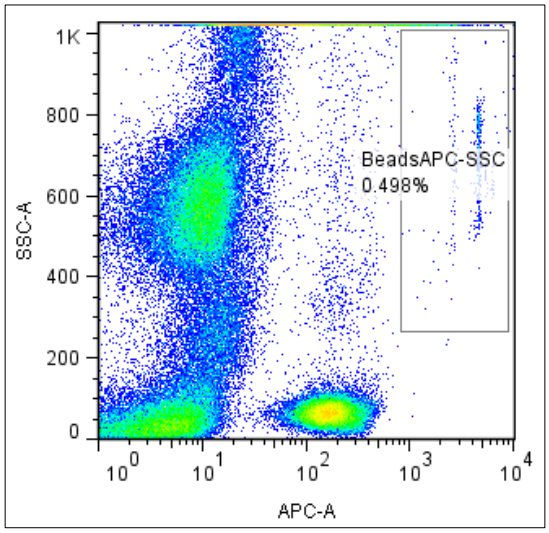
The immune phenotyping process occurred by identifying cells expressing the CD, or combination of CDs, of interest. Table 2.5 lists the immune cell phenotypes examined and the respective CD markers used for identification.

Table 2.5. Definition of cells by immunophenotyping analysis.

Leukocyte subtype	CD expressed.
T cells	$CD3^+$
Helper T cells	$CD3^+CD4^+$
Regulatory T cells	$CD3^+CD4^+CD8^+CD25^+CD127^{+LO}$
Cytotoxic T cells	$CD3^+CD8^+$
Activated cytotoxic T cells	$CD3^+CD8^+CD25^+$
B cells	$CD3^-CD19^+$
Activated B cells	$CD3^-CD19^+CD80^+$
Activated B cells	$CD3^-CD19^+CD86^+$
Monocytes	$CD14^+$
Activated Monocytes	$CD14^+CD80^+$
Activated Monocytes	$CD14^+CD86^+$
NK cells	$CD3^-CD16^+$

The gating process was then continued by the identification of the fluorescent microbeads through a gate which was drawn for all the samples and then individually verified in the software by visual comparison of all of them. The first plot of bead identification is presented in Figure 2.8.

Figure 2.8. Fluorescent microbeads present in the Trucount™ technology. Process of gating and identification.

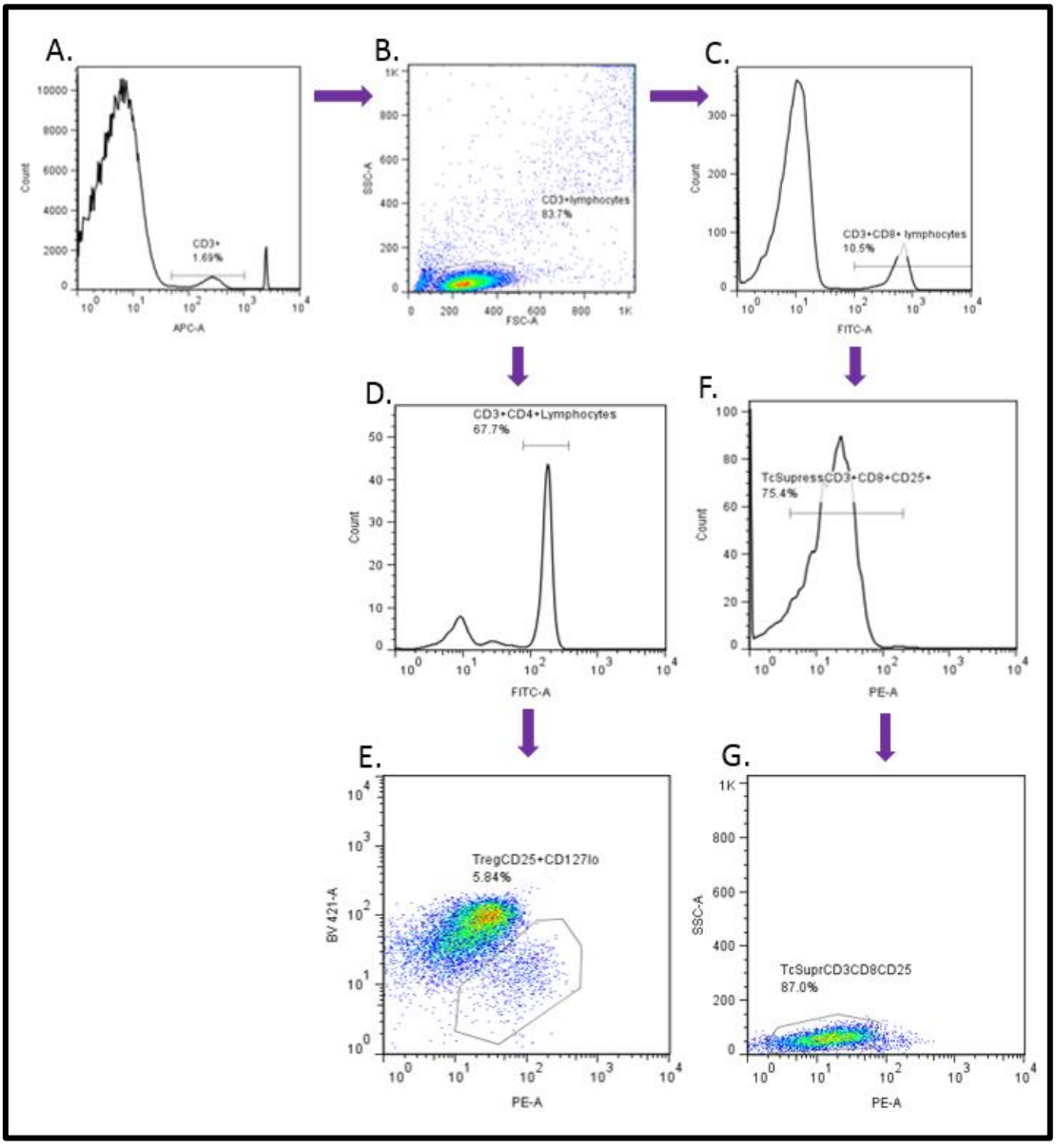


The gating process was done in SSC and APC. The gate was transferred into every tube with beads and the verification that the gate around the beads was properly set was also performed.

Subsequently, the process consisted of examining the cell populations according to the phenotype expressed, which means according to the CD being stained. In this process, the correspondence between the fluorochrome and the channel in which this was

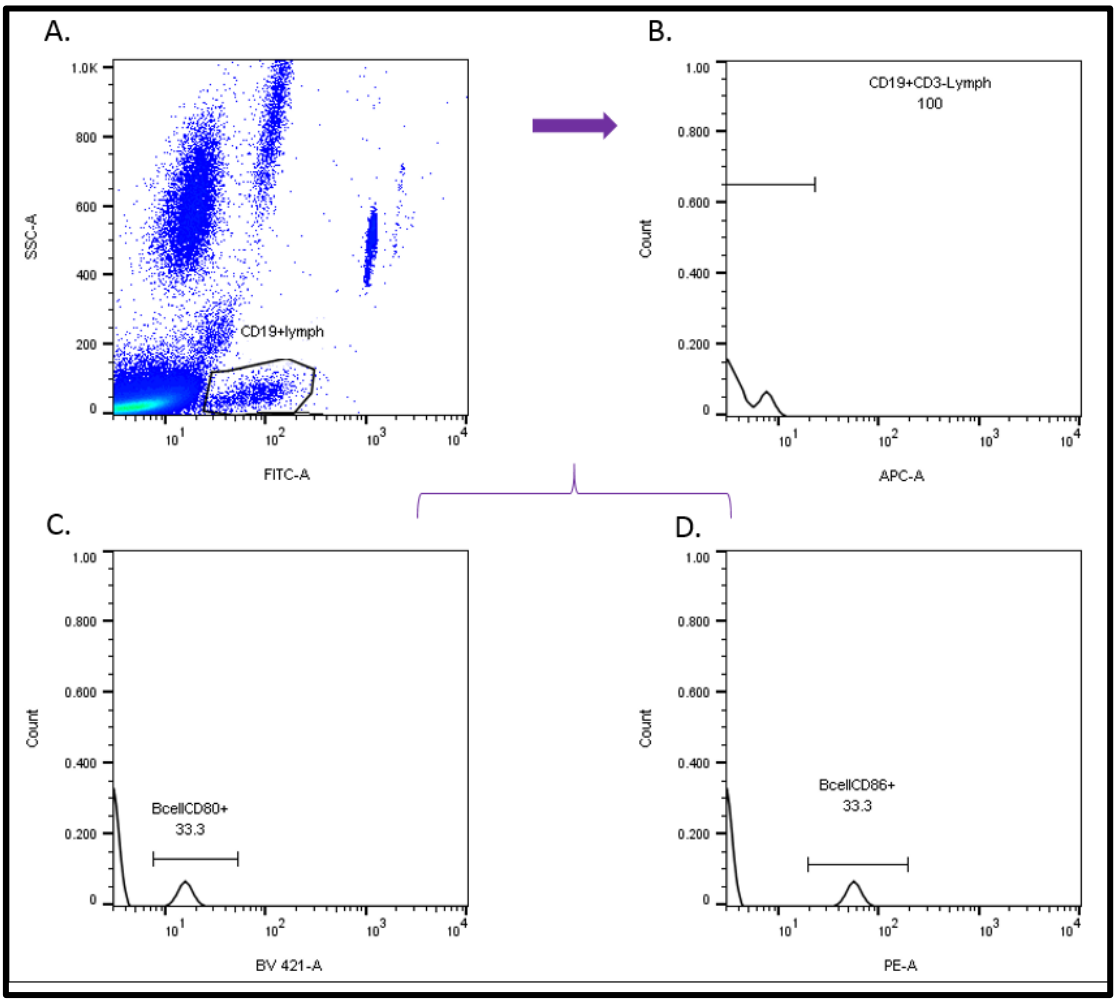
expected to be found were parameters considered to either draw a plot or a histogram. Figure 2.9 to Figure 2.12 illustrate the examination and identification process of the immune phenotypes of interest in this study.

Figure 2.9. Identification of T cells



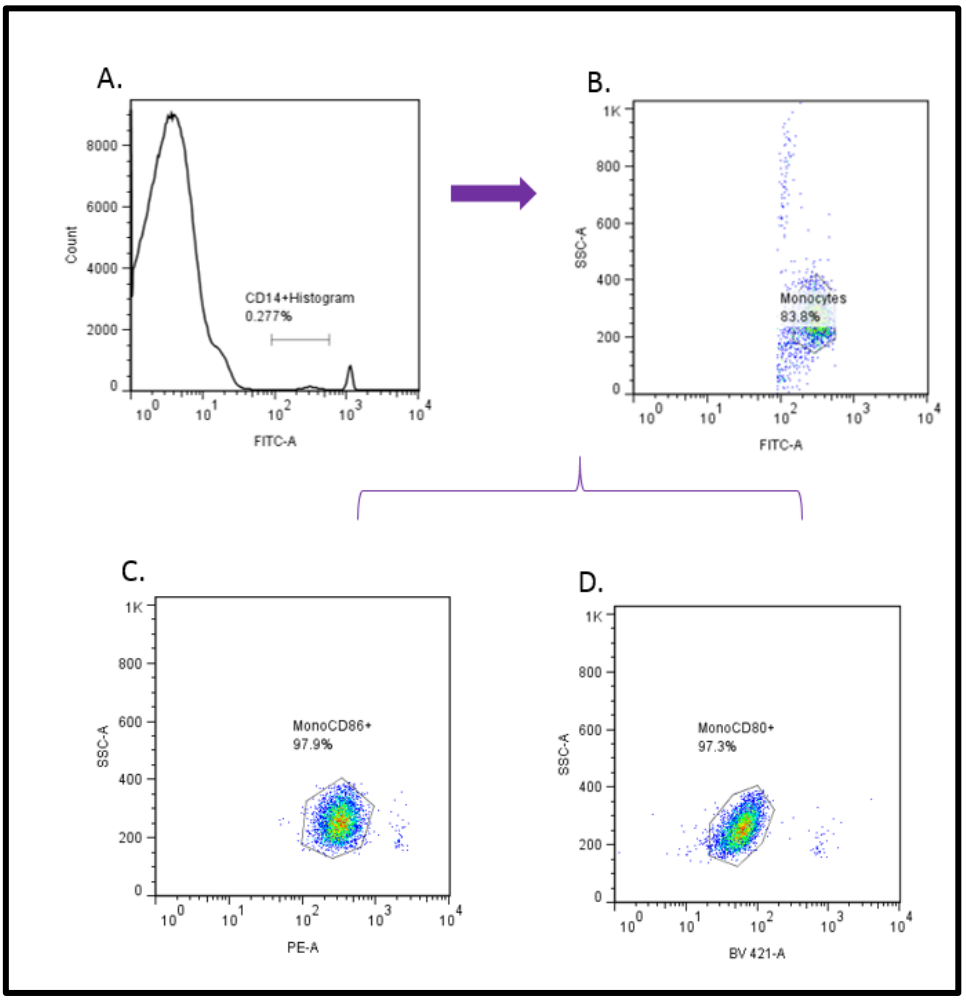
A. Identification of $CD3^+$ population on a APC histogram
B. Draw a lymphocyte gate using a FSC-SSC plot. Identification of **T cells**
C. Draw a $CD3^+CD8^+$ histogram within the gated population using as parameter FITC. Identification of **Cytotoxic T cells**
D. Draw a $CD3^+CD4^+$ histogram within the gated population using as parameter FITC. Identification of **Helper T cells**
E. Plot of BV 421 and PE. Selection of the region in which $CD127^+$ is low and $CD25^+$ is normally expressed. Double check with controls (Appendix data). Draw a gate to select the region. Identification of **Regulatory T cells**
F. Draw a $CD3^+CD8^+CD25^+$ histogram within the gated population using as parameter PE.
G. Plot SSC and PE and draw a gate around the population Identification of **activated Cytotoxic T cells** (also named as Suppressor T cells)

Figure 2.10. Identification of B cells



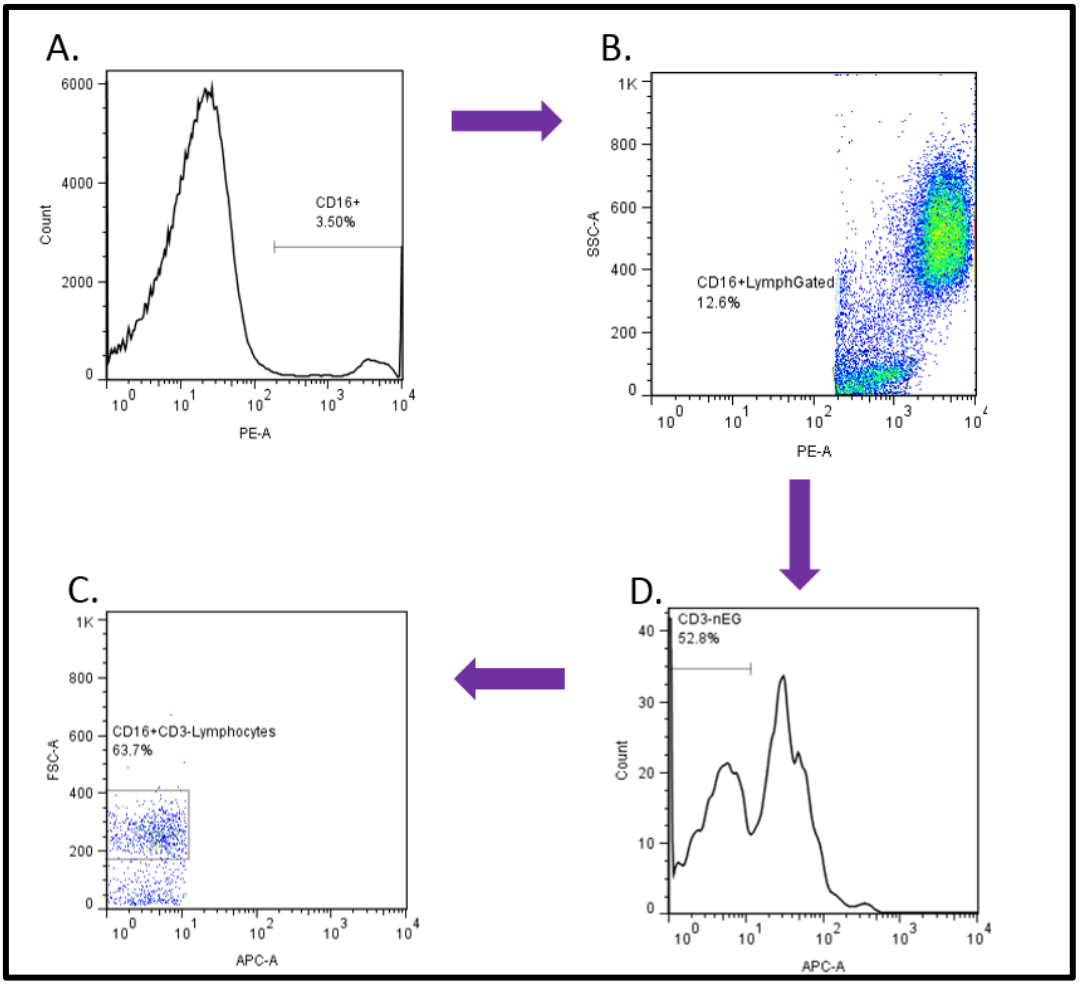
A. Identification of CD19⁺ population on a FIT-C histogram. Draw a lymphocyte gate in the plot.
B. Identify and select the CD3⁻ region within the CD19⁺ population.
C. Within the CD19⁺CD3⁻ population draw a histogram in the parameter BV 421 to select the CD80⁺ population.
D. Within the CD19⁺CD3⁻ population draw a histogram in the parameter PE to select the CD86⁺ population.

Figure 2.11. Identification of Monocytes.



A. Identification of $CD14^{+}$ population on a FIT-C histogram
B. Draw a lymphocyte gate using a SSC and FITC plot. **Monocytes.**
C. Draw a plot around $CD14^{+}CD80^{+}$ population on SSC and PE
D. Draw a plot around $CD14^{+}CD86^{+}$ population on SSC and BV421

Figure 2.12. Identification of NK cells.



A. Identification of $CD16^{+}$ population on a PE histogram
B. Draw a lymphocyte gate using a SSC and PE plot
C. Identification of the population $CD3^{-}$ in the APC area
D. Gate the population and identify it as **NK cells**

The methodological process in immune cell phenotyping also requires consolidation of a data base in which microbeads count collected per sample must be recorded as well as bright beads contained in the tube in which the sample was analysed. To obtain the absolute count of cells of interest per 100 µl of whole blood this formula was used:

(Number of events collected of the cell of interest / Beads collected) * (Total amount of bright beads / 100 µl of whole blood)

2.2.3.5 Phagocytic activity

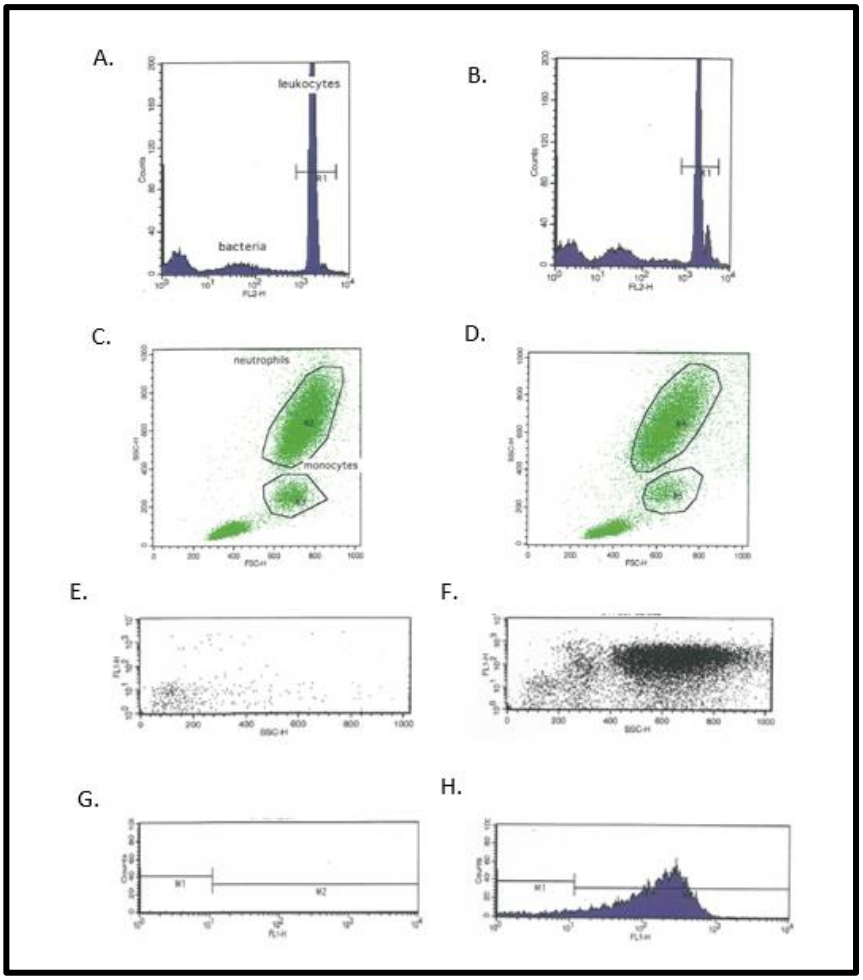
Phagocytosis was assessed in blood neutrophils and monocytes by using a commercially available kit (Phagotest™). This assay is based upon uptake of fluorescently labelled *E. coli* which can be assessed using flow cytometry. 100 µl of heparinised whole blood was incubated with fluorescein-labelled *E. coli* at 37°C for 10 minutes, while the control sample remained on ice. Phagocytosis was stopped by placing the samples on ice and adding 100 µl of a quenching solution which allows the discrimination between attachment and internalisation of bacteria by quenching the FITC fluorescence of surface-bound bacteria without altering the fluorescence of internalised bacteria. Subsequently, 3 ml of a washing solution were added per tube, which was then vortexed and then centrifuged at 1000 rpm (200 g) for 5 minutes. The supernatant was removed, and this washing process was repeated. A lysing step of erythrocytes was done by adding 2 ml of lysing solution for 20 minutes. The sample was then centrifuging at 1000 rpm ~ (200 g) for 5 minutes, followed by the last washing step and a final DNA staining using 200 µl of a DNA staining solution for 10 minutes on ice and protected from light.

The percentage of granulocytes (mainly neutrophils) and monocytes which performed phagocytosis was analysed by using a BD FACSCalibur flow cytometer and Cell Quest Pro software. Both the proportion of cells involved in phagocytosis and their median fluorescence intensity (indicating the number of ingested bacteria per cell) were analysed. Table 2.6 presents parameters of the instrument setting for appropriate data collection and Figure 2.13 shows a typical profile after analysis in the flow cytometer.

Table 2.6. Compensation and parameters in the analysis of phagocytosis.

Parameter	Detector	Voltage	Compensation
P1	FSC	300	N/A
P2	SSC	378	N/A
P3	FL1	471	19.9% FL2
P4	FL2	582	9.0% FL1 – 0% FL3
P5	FL3	150	0% FL2

Figure 2.13. Identification of phagocytic cells by flow cytometry



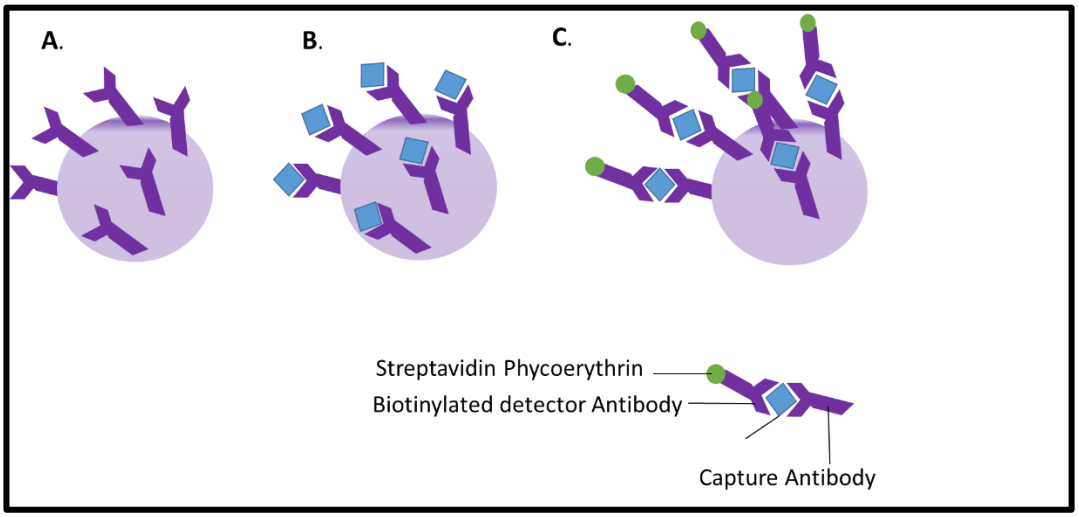
First column of graphs (A, C, E, G) correspond to experimental control (bacteria remained on ice at 4°C). Second column of graphs (B, D, F, H) correspond to test (bacteria were exposed to 37°C for 10 minutes). Figures A and B reflect on their axis the fluorescence of the DNA stain. This DNA selection is the first stage of analysis to allow the selection of leukocytes (population with higher DNA content, thus greater FL2 signal when compared with bacteria and debris) Figures C and D reflect the granularity and the size of the particles on their axis. The graphs show the gated population corresponding to neutrophils and monocytes. Figures E and F correspond to granular content and the FL1 signal fluorescence intensity (from the labelled E.coli bacteria). Figures G and H show FL1 signal fluorescence intensity (from the labelled E.coli bacteria),., which The FI1 signal is higher on figure F and H as phagocytosis occurred at 37°C in the test tube, but minimally at 0°C (control tube).

2.2.4 General principles for the analyses of cytokine and other immune mediator concentrations

General principles of the assay to assess cytokines and other immune mediators rely on the fact that colour coded magnetic microparticles are precoated with specific antibodies against the immune mediators of interest. Components like microparticles, standards and samples were pipetted into wells and the immobilized antibodies were bound to the analytes of interest. After washing the unbound substances, a biotinylated antibody cocktail which is specific to the analytes of interest was added to each well.

Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (streptavidin-PE), was added to each well as it binds to the biotinylated antibody. A final wash removed unbound streptavidin-PE, the microparticles were resuspended in buffer and read using the Bioplex-200 Reader (Bio-Rad). Two spectrally distinct lights illuminated the beads. Analytes were detected, and the second LED determined the magnitude of the PE-derived signal, which was proportional to the amount of analyte bound. See Appendix B to read in detail the protocol followed in this procedure. Figure 2.14 summarises the principle of the experiment. Those values out of range above levels of detection were not used. Table 2.7 presents the minimum detectable values for each analyte. Additional information regarding the kit and reagents preparation is contained in the Appendix.

Figure 2.14. Magnetic microbeads and general description of antibody binding process used to determine the concentration of immune and inflammatory mediators in plasma



A) The antibodies for the capture of antigen-specific particle are bound to microspheres; B) The antigen from the test sample is bound to the capture antibodies; C) The signal is generated by attachment of the labelled detection antibodies.

2.2.4.1 Cytokines and other immune mediators measured in plasma

Cytokines and other immune mediators measured in plasma isolated from the blood on the day of collection or after delayed processing are presented in table 2.7. Plasma aliquots were kept at -20^{o}C until the recruitment of the ten participants was finished (maximum duration 10 months).

Table 2.7. Parameters measured in plasma through Magnetic Luminex Assay and the lower limits of detection.

Immune mediator	Lower limit of detection (pg/ml)
TNF-α	0.8
IL-6	1.0
IL-8	0.8
IL-10	0.5
IL-18BPa	1.9
IL-1 ra/IL-1F3	16.9
RANTES/CCL5	1.8
VCAM-1/CD106	238
TNF RII	6.2
MCP-1/CCL2	3.3
MIP-1α/CCL3	16.0
E-selectin/CD62E	7.4
ICAM-1/CD54	87.9

2.2.4.2 Whole blood cultures

Heparinised whole blood was diluted and cultured in 24 well plastic plates and stimulated with lipopolysaccharide (LPS) from *E. coli* K12 strain, peptidoglycan (PGN) from *Staphylococcus aureus*, which are TLR stimulants, or phytohaemagglutinin (PHA) from *Phaseolus vulgaris* (red kidney bean) which is a T cell stimulant. Whole blood was diluted 1:10 in RPMI 1640 culture medium (Sigma Aldrich), to a final volume of 6 ml. Afterwards, 990 µl were added per well. 10 µl of either medium, LPS, PGN or PHA were also added separately to each well to obtain concentrations of 10 ug/ml, 5 ug/ml and 5 ug/ml, respectively. Cultures were incubated for 24 hours at 37^{o}C at 5% CO_2. After this, the supernatant was collected after centrifuging the plate at 2000 rpm for 5 minutes. Supernatants were stored at -80^{o}C until analysis. Maximum storage time was 10 months. (See Appendix to obtain more information regarding materials and manufacturers providing supplies).

Concentrations of cytokines and other immune mediators produced following stimulation with PHA, LPS and PGN were measured in the supernatants derived from the whole blood cultures and are listed in table 2.8. Minimum detectable concentrations are presented in the table; therefore concentrations out of this range or undetected were not analysed. See Appendix B for additional information regarding the Kit lot and other technical procedures: (i.e., preparation of standards and samples).

Table 2.8. Immune mediators measured through Magnetic Luminex Assay technique as a result of negative and positive stimulations[1] (24 hours incubation) with PHA, PGN and LPS on whole blood cultures.

Immune mediator	Lower limit of detection (pg/mL)
Analytes measured in negative controls and after stimulation with PGN and LPS	
TNF α	1,2
IL-10	1,6
IL-12p70	20,2
IL-6	1,7
IL-1 beta	0,8
Analytes measured in negative controls and after stimulation with PHA	
IL-4 (HS)	2,54
IFN-γ (HS)	0,02
IL-13	2,01
IL-2 (HS)	0,28
IL-5 (HS)	0,12
TNF-α (HS)	0,54

[1]Negative stimulation indicates addition of media instead of stimulants. Positive stimulation indicates stimulation with either PHA, PGN or LPS. (HS) High sensitivity detection kit.

2.2.5 Anti-influenza vaccine antibodies

Measurement of the influenza vaccine antibodies were performed using the haemagglutination inhibition assay (HIA), which was conducted at a Public Health England laboratory in London. This assay is based on the properties of the influenza hemagglutination activity and specificity of antibodies present in the serum sample under analyses. Titrations of targeted samples proceed in two folds 1/10, 1/20, 1/40. After incubation of the samples with the virus of study, red blood cells (RBCs) are added into the assay. RBCs bind to an haemagglutinin glycoprotein on the surface of virus particles and detect inhibition of agglutination (or non-agglutination), so the presence of the virus is inferred through the titration of the sample [222].

2.2.6 Vitamin D analysis

Total vitamin D analysis or 25(OH) vitamin D total assay, was performed on a Beckman Coulter DxI 800 by the Pathology laboratory in the Southampton General Hospital, an automated instrument performing immunoassays on patient samples. This technique is generally based on a two-step competitive binding immunoenzymatic assay where the initial incubation consisted of adding the sample to a reaction vessel with paramagnetic particles coated with sheep monoclonal anti-25(OH) vitamin D antibody. 25(OH) vitamin D is released and bound to the immobilized monoclonal anti-25(OH) vitamin D on the solid phase. Subsequently, a 25(OH) vitamin D analogue-alkaline phosphatase

conjugate is added which competes for binding to the immobilized monoclonal anti-25(OH) vitamin D. After a second incubation, materials bound to the solid phase are held in a magnetic field while unbound materials are washed away. Finally, the chemiluminescent substrate LumiPhos 530 was added and the light generated by the reaction was measured with a luminometer. The light production is inversely proportional to the concentration of 25(OH) vitamin D in the sample where the amount of analyte (25(OH) vitamin D) was determined from a multi-point calibration curve.

2.2.7 Statistical analyses

Normality of data was assessed by visual inspection of histogram distributions and by using the Shapiro Wilk and Kolmogorov-Smirnov tests. Most data were not normally distributed. Statistical analyses conducted on the vaccine titres were performed according to their logarithmic basis using geometric mean and interquartile range. Other parameters were analysed using median and percentiles. Data collation and analysis were performed in SPSS version 22 and Excel Microsoft Office ProPlus. The significance of differences across time was assessed using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks: this test was used as it is applied to non-parametric data and is the equivalent of the ANOVA test for repeated measures with normally distributed data. This test is run on the assumptions that data are ordinal or continuous and that data come from a single group, measured on at least three different occasions. The null hypothesis is that time of storage has no effect and the alternate hypothesis is that time of storage does influence the immune parameters assessed. Where the alternate hypothesis was accepted, parameters where a significant difference was found according to the p value, were examined through pairwise comparisons for Friedman's test. These further comparisons allowed to identify paired samples which were significantly different by comparing all the time points of storage among them. Box plots are used to display median values of data over time of storage as well as percentiles 25 (P_{25}) and 75 (P_{75}) and minimum and maximum ranges. These graphs showcase measurements of central tendency as well as illustrate changes over time. Box plots were drawn using PRISM software version 8.0. Moreover, Individual data are also presented as individual values across the time of storage and are linked among each single participant to reflect specimen's changes across times. These plots were done individually by plotting subject's Individual data using Excel program. In all cases, the significance is indicated by a value for $p < 0.05$. Data out of the range of detection are not presented.

2.3 Results

2.3.1 Effect of delay in blood processing on immune cell populations identified through full blood count analysis

Stability across the timeframe of study was observed for several parameters analysed within the FBC. Comparison between fresh samples (day 0) and samples whose processing was delayed for 24, 48 and 72 hours (days 1, 2, and 3) did not show a significant difference in numbers of lymphocytes, monocytes, eosinophils, basophils, and platelets (See Figure 2.15). The neutrophil and white cell counts showed a degree of effect by delayed processing. Neutrophil count was decreased at day 3 of sample processing when compared with fresh analyses. White cell counts showed an earlier decrease than seen for the neutrophils when delayed blood processing was compared between day 1 and day 2 as well as day 1 and day 3. Significances are shown in Table 2.9. Stability was observed up to 48 hours after sample collection for neutrophil count when compared with day 1 after sample collection and up to 24 hours or day 1 after sample collection for white cell count.

Figure 2.15. Full blood count analysis on fresh blood (0 hr) and blood analysed 24, 48 or 72 hours after collection.

Figure 2.15.1. Neutrophils

Figure 2.15.1-A. Neutrophils – Box Plot

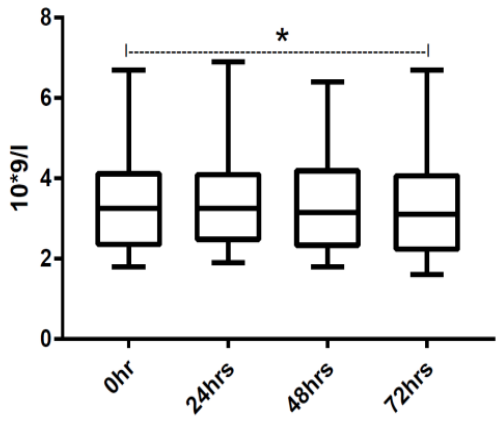
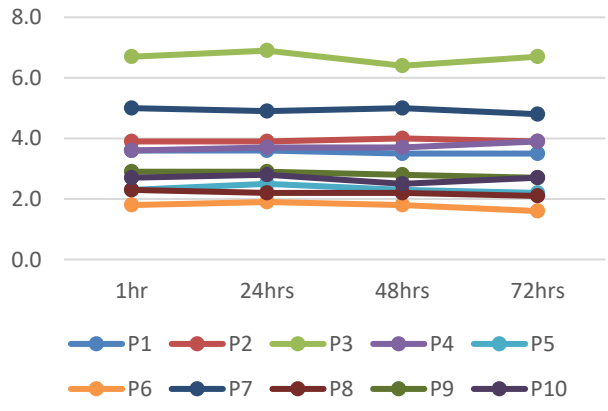


Figure 2.15.1-B. Neutrophils – Individual Data



(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p=0.045**) - Table 2.9 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.436, which indicates lack of statistical significance – Significance (*) was observed. All p values are presented)– Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

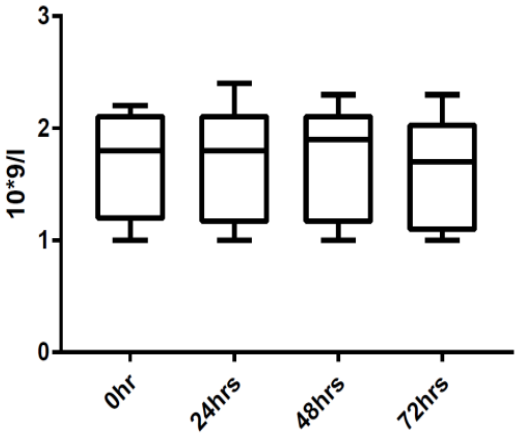
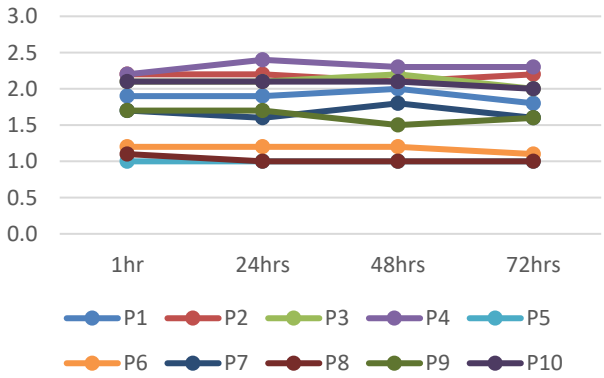
Figure 2.15.2. Lymphocytes	
Figure 2.15.2-A. Lymphocytes – Box Plot	Figure 2.15.2-B. Lymphocytes – Individual Data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.087) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

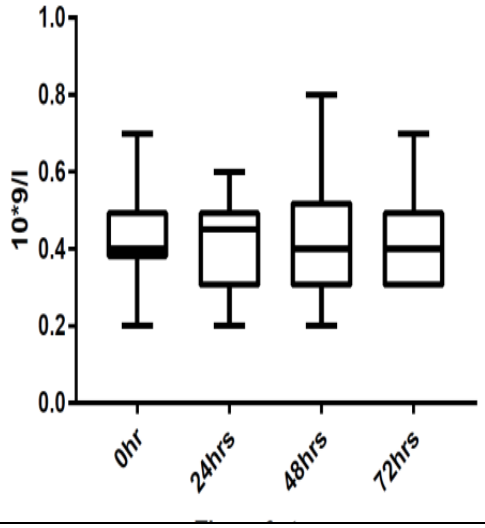
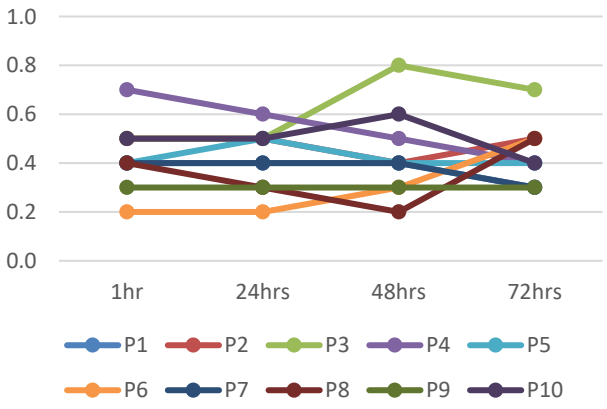
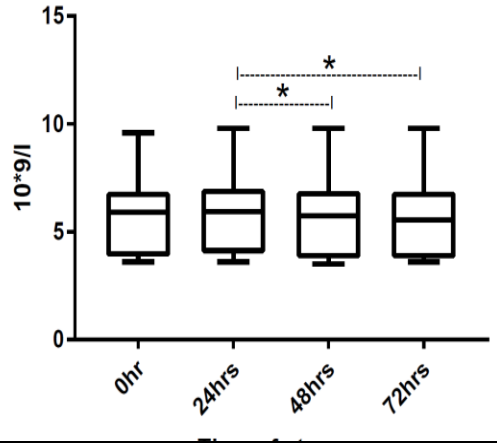
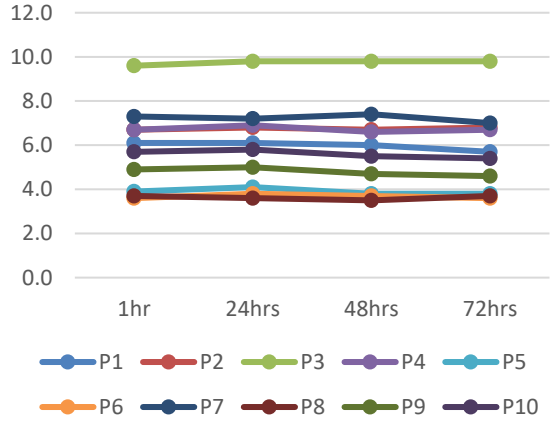
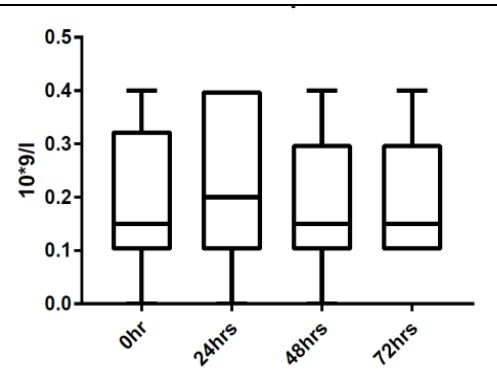
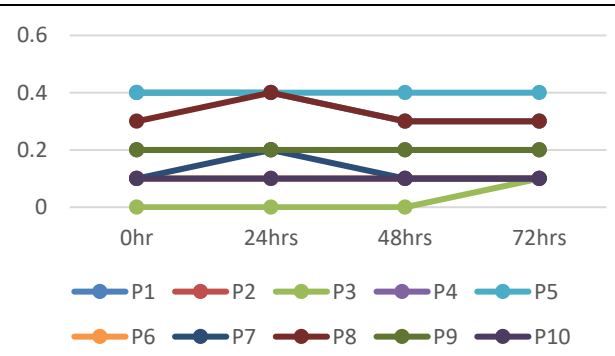
Figure 2.15.3. Monocytes	
Figure 2.15.3-A. Monocytes – Box Plot	Figure 2.15.3-B. Monocytes – Individual Data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.087) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

Figure 2.15.4. White Cell count (WCC)	
Figure 2.15.4-A. WCC – Box Plot	Figure 2.15.4-B. WCC – Individual Data
	

(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p=0.035**) – Table 2.9 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.141, which indicates lack of statistical significance – Significance (*) was observed. All p values are presented)- Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Figure 2.15.5. Eosinophils	
Figure 2.15.5-A. Eosinophils – Box Plot	Figure 2.15.5-B. Eosinophils – Individual Data
	

(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.223) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

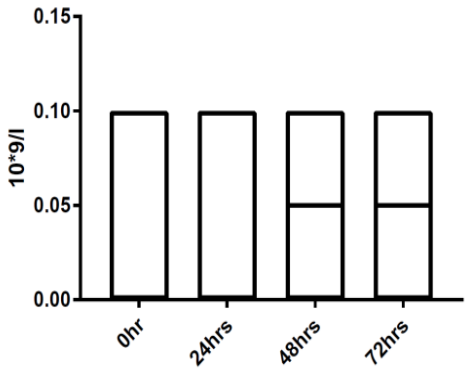
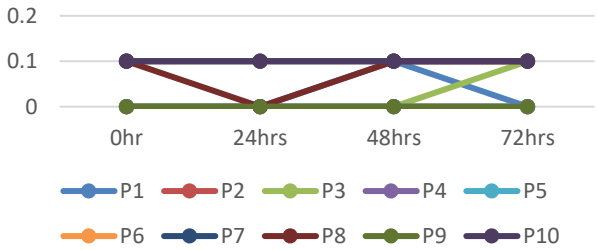
Figure 2.15.6. Basophils	
Figure 2.15.6-A. Basophils – Box Plot	Figure 2.15.6-B. Basophils – Individual Data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.300) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

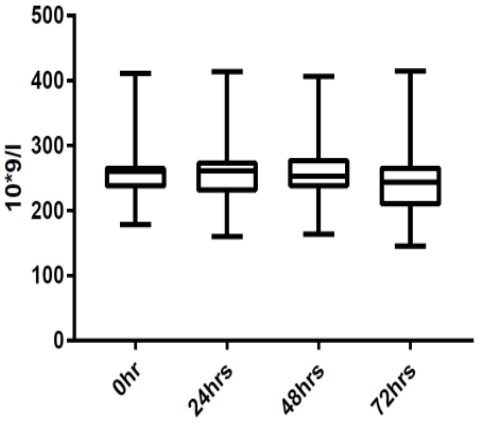
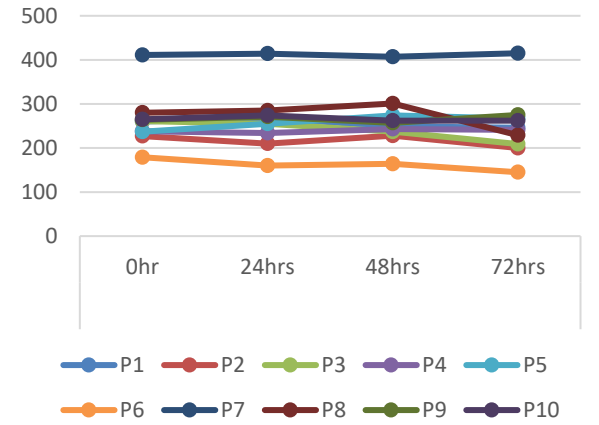
Figure 2.15.7. Platelets	
Figure 2.15.7-A. Platelets – Box Plot	Figure 2.15.7-B. Platelets – Individual Data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.644) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

Table 2.9. Significance of pairwise comparison tests of data for neutrophils and total white cell counts.

	p-value	
Paired comparisons*	Neutrophils	WCC
Fresh sample vs Day 1 of collection	0.436	0.141
Fresh sample vs Day 2 of collection	0.436	0.436
Fresh sample vs Day 3 of collection	0.083	0.299
Day 1 of collection vs Day 2 of collection	0.119	**0.024**
Day 1 of collection vs Day 3 of collection	**0.012**	**0.012**
Day 2 of collection vs Day 3 of collection	0.341	0.795

(*) Paired comparisons or Post-Hoc test derived from the Friedman's test Two-Way Analysis of Variance by Ranks. Identification of paired samples revealing the most significant alteration influenced by time point of delayed processing ($p<0.05$). Bold relate to significance.

2.3.2 Effect of delayed blood processing on imune cell phenotypes

Immune cell phenotypes were categorized according to the absolute count (i.e. number) of cells. The comparison of results in fresh samples (day 0) with those in samples that had delayed processing for 24, 48 and 72 hours (days 1, 2, and 3) did not show a significant effect for most of the cell phenotypes (Figure 2.16). However, activated cytotoxic T cells and NK cells showed a significant effect of time of delayed processing (Table 2.10). In the case of activated cytotoxic T cells, the significant effect was due to the difference between fresh samples and the samples analysed after 72 hrs of collection. NK cells showed a significant effect of delayed processing with a marked decrease at 48 hours after processing.

Figure 2.16. Immunophenotype analysis on fresh blood (0 hr) and blood analysed 24, 48 or 72 hours after collection.

Figure 2.16.1. T Cells

Figure 2.16.1-A. T Cells – Box Plot

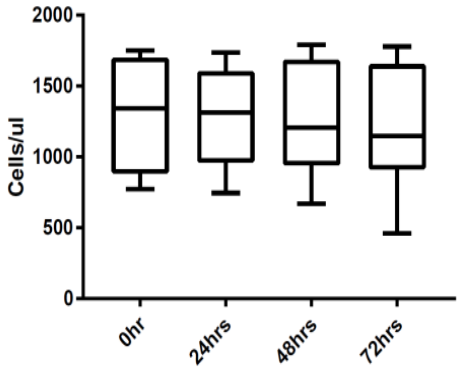
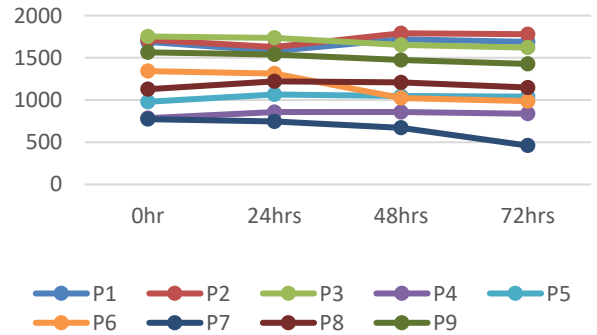


Figure 2.16.1-B. T Cells – Individual Data



(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; $p=0.161$) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Figure 2.16.2. Helper T cells

Figure 2.16.2-A. Helper T cells – Box Plot

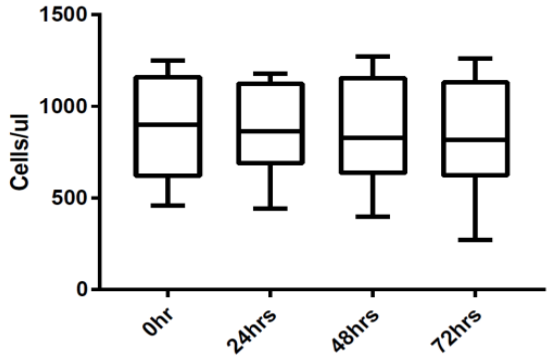
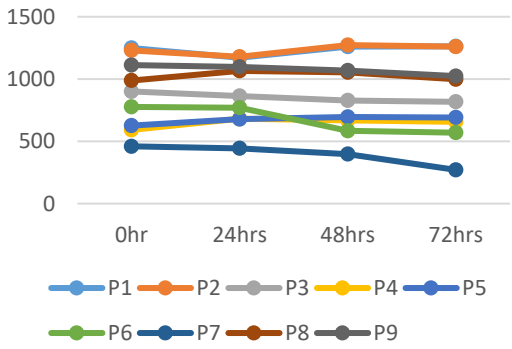


Figure 2.16.2-B. Helper T cells – Individual Data



(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.586) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Figure 2.16.3. Regulatory T cells

Figure 2.16.3-A. Regulatory T cells – Box Plot

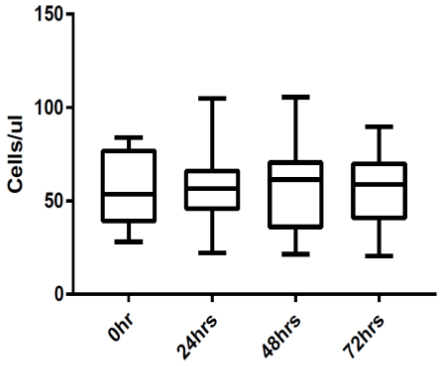
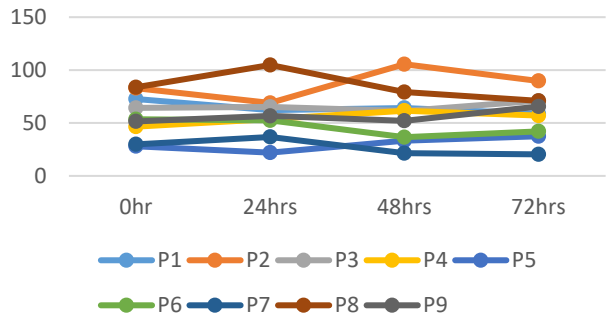


Figure 2.16.3-B. Regulatory T cells – Individual Data



(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.996) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

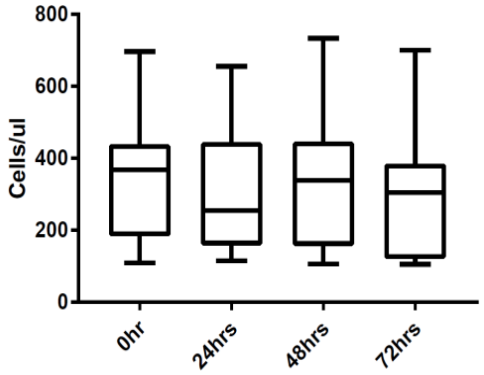
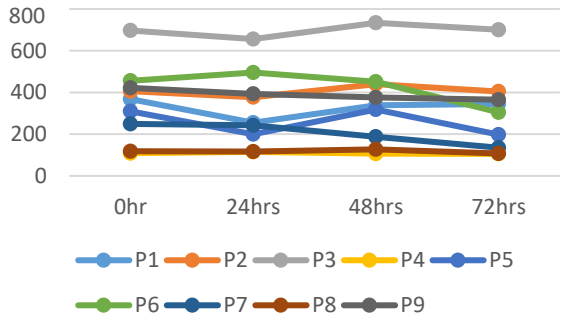
Figure 2.16.4. Cytotoxic T cells	
Figure 2.16.4-A. Cytotoxic T cells – Box Plot	Figure 2.16.4-B. Cytotoxic T cells – Individual Data
	
(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.137) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

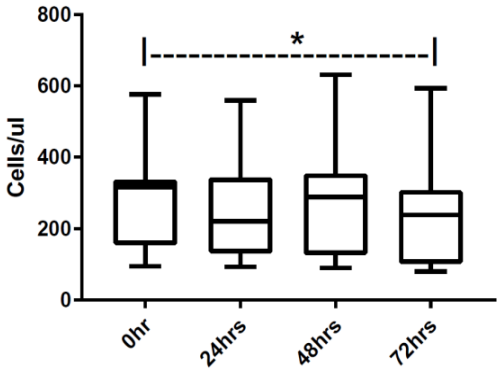
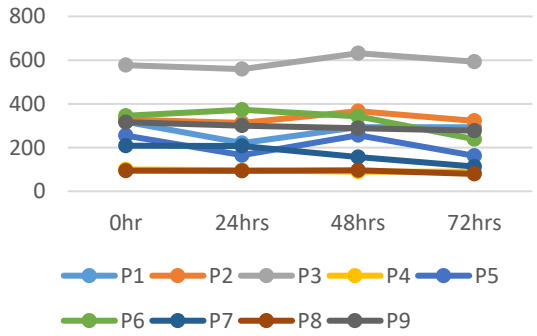
Figure 2.16.5. Activated cytotoxic T cells	
Figure 2.16.5-A. Activated cytotoxic T cells – Box Plot	Figure 2.16.5-B. Activated cytotoxic T cells – Individual Data
	
(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p=0.020**. Table 2.10 shows Pairwise comparisons: Most relevant for this study (*) is among 0 hr and 72 hrs; p=0.003. All p values are presented) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

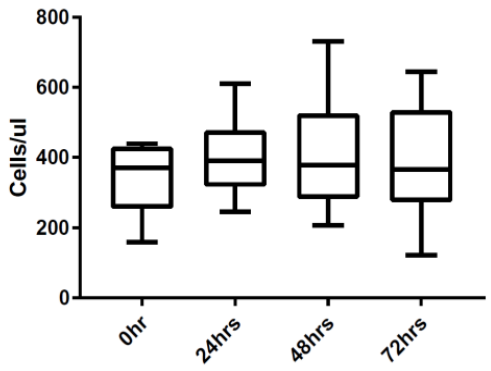
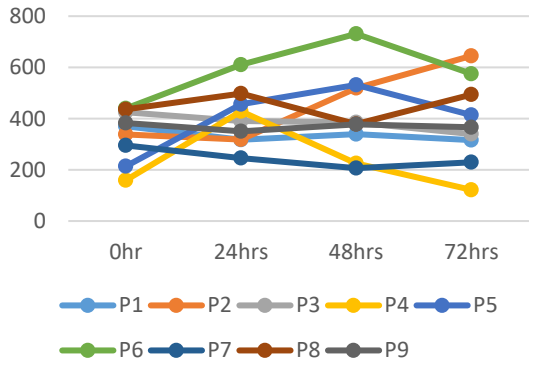
Figure 2.16.6. B cells	
Figure 2.16.6-A. B cells – Box Plot	Figure 2.16.6-B. B cells – Individual Data
	
(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.615) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

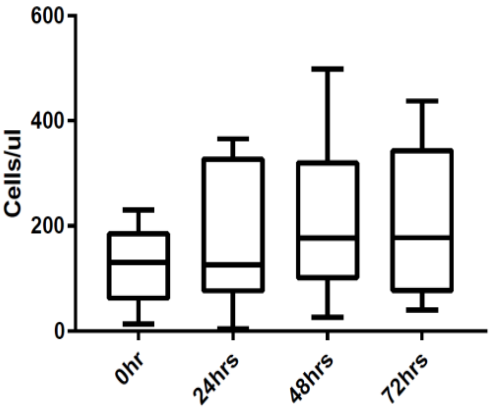
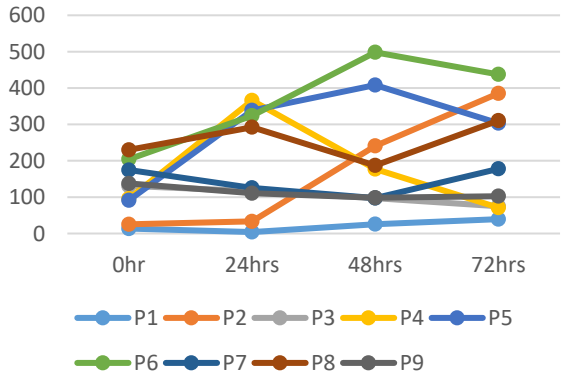
Figure 2.16.7. Activated B cells (CD80^{+} marker)	
Figure 2.16.7-A. Activated B cells (CD80^{+} marker) – Box Plot	Figure 2.16.7-B. Activated B cells (CD80^{+} marker) – Individual Data
	
(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.769) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

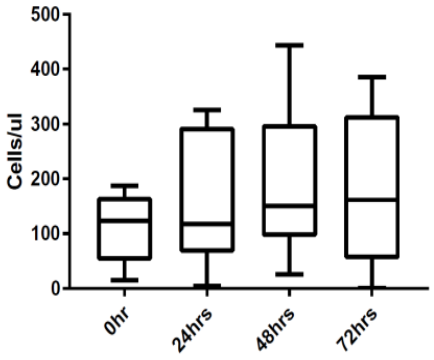
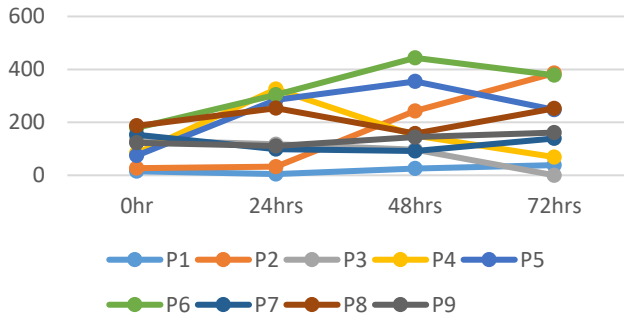
Figure 2.16.8. Activated B cells (CD86^{+} marker)	
Figure 2.16.8-A. Activated B cells (CD86^{+} marker) – Box Plot	Figure 2.16.8-B. Activated B cells (CD86^{+} marker) – Individual Data
	
(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.833) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

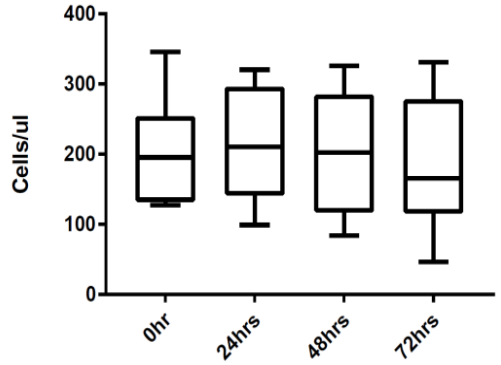
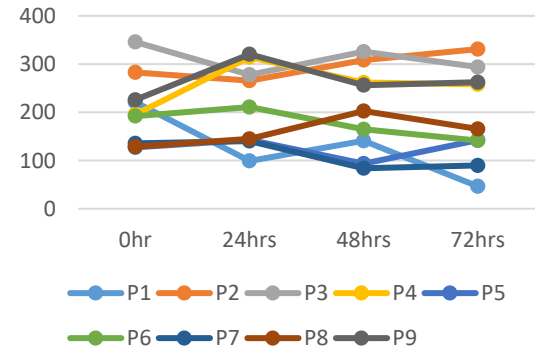
Figure 2.16.9. Monocytes	
Figure 2.16.9-A. Monocytes – Box Plot	Figure 2.16.9-B. Monocytes – Individual Data
	
(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.769) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

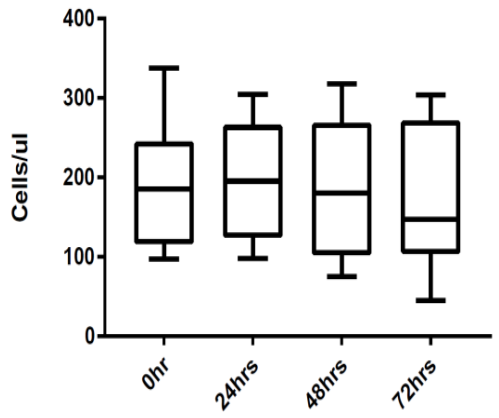
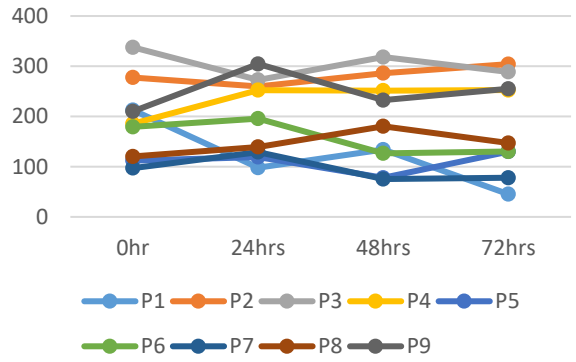
Figure 2.16.10. Activated monocytes (CD80+ marker)	
Figure 2.16.10-A. Activated monocytes (CD80+ marker) – Box Plot	Figure 2.16.10-B. Activated monocytes (CD80+ marker) – Individual Data
	
(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.769) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

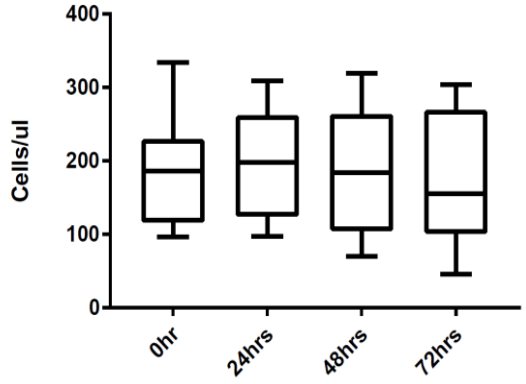
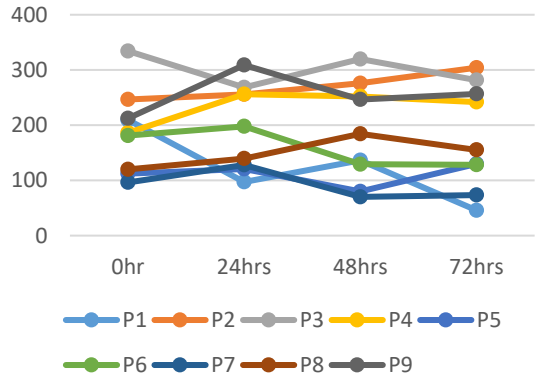
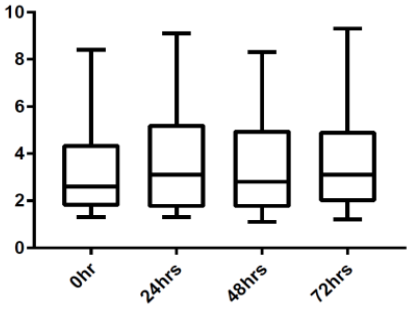
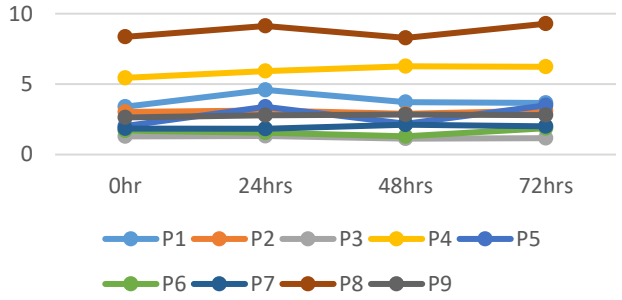
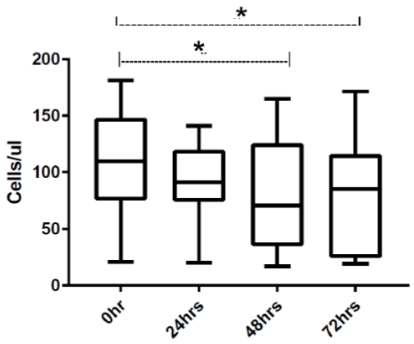
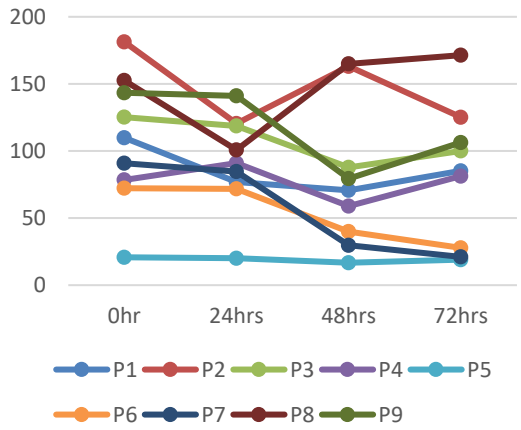
Figure 2.16.11. Activated monocytes (CD86+ marker)	
Figure 2.16.11-A. Activated monocytes (CD86+ marker) – Box Plot	Figure 2.16.11-B. Activated monocytes (CD86+ marker) – Individual Data
	
(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.737) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

Figure 2.16.12. Ratio (Helper T cells: Cytotoxic T cells)	
Figure 2.16.12-A. Ratio (Helper T cells: Cytotoxic T cells) – Box Plot	Figure 2.16.12-B. Ratio (Helper T cells: Cytotoxic T cells) – Individual Data
	

(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.058) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Figure 2.16.13. Natural killer cells	
Figure 2.16.13-A. Natural killer cells – Box Plot	Figure 2.16.13-B. Natural killer cells – Individual Data
	

(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p=0.017**. Table 2.10 shows Pairwise comparisons: Most relevant for this study (*) are among 0 hr and 48 hrs; p=0.002 and 0 hr and 72 hrs; p=0.028. All p values are presented – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Table 2.10. Significance of pairwise comparison tests of data for activated cytotoxic T cells and Natural killer cell counts at each day.

Paired comparisons*	p-value	
	Activated cytotoxic T cells	Natural killer cells
Fresh sample vs Day 1 of collection	0.068	0.100
Fresh sample vs Day 2 of collection	0.465	**0.002**
Fresh sample vs Day 3 of collection	**0.003**	**0.028**
Day 1 of collection vs Day 2 of collection	0.273	0.144
Day 1 of collection vs Day 3 of collection	0.273	0.584
Day 2 of collection vs Day 3 of collection	**0.028**	0.361

(*) Paired comparisons or Post-Hoc test derived from the Friedman's test Two-Way Analysis of Variance by Ranks. Identification of paired samples revealing the most significant alteration influenced by time point of delayed processing ($p<0.05$). Bold relate to significance.

The median fluorescence intensity in the population gated as monocytes and neutrophils was assessed through the analysis of CD14. These measurements remained stable with delayed blood processing (Figure 2.17).

Figure 2.17. Results of MFI of CD14+ on fresh blood (0 hr) and blood analysed 24, 48 or 72 hours after collection.

Figure 2.17. MFI CD14+ - Monocytes

Figure 2.17-A. Monocytes – MFI CD14+ - Box Plot

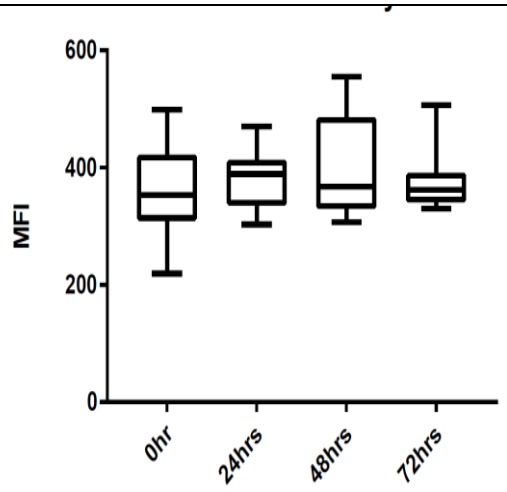
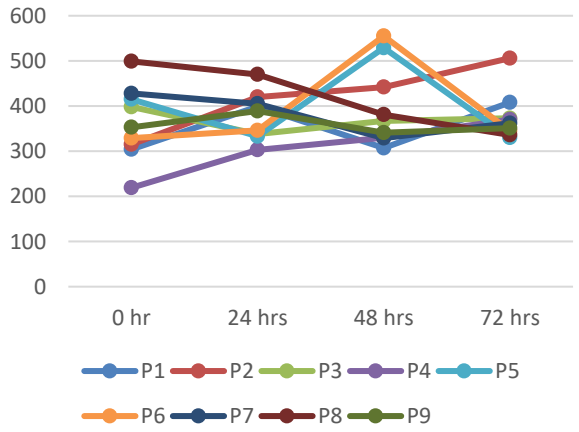


Figure 2.17-B. Monocytes – MFI CD14+ - Individual data



(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.996) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Figure 2.17. MFI CD14+ - Neutrophils

Figure 2.17-A. Neutrophils – MFI CD14+ - Box Plot

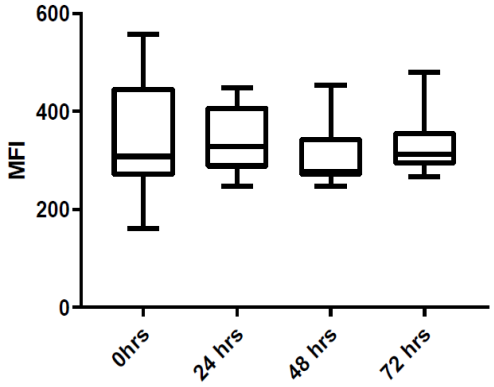
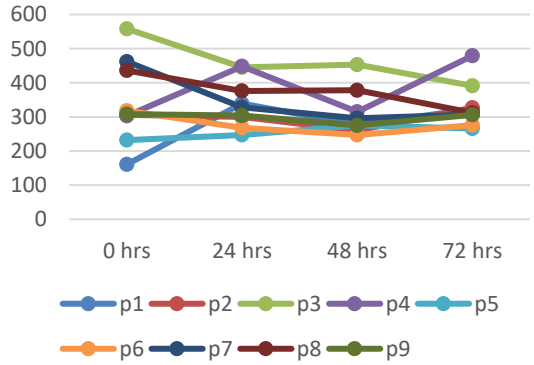


Figure 2.17-B. neutrophils – MFI CD14+ - Individual data



(n=9; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.506) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

2.3.3 Effect of delayed blood processing on phagocytic activity of neutrophils and monocytes

There were no significant differences seen for the percent of neutrophils or monocytes engaged in phagocytosis following delayed processing for 24, 48 and 72 hours (Figure 2.18). However, the exception was the phagocytic activity assessed in neutrophils through the fluorescence intensity (engulfed labelled bacteria) which showed a significant difference with a decrease across time (Figure 2.18). A paired test analysis for this parameter is presented in Table 2.11 which indicates the amount of phagocytosis which each cell engaged in was significantly lower in blood processed at 72 h after collection compared with the fresh sample. This result indicate that phagocytosis measurements should be made for samples analysed up to 48 hours or day 2 after storage.

Figure 2.18. Neutrophil and monocyte phagocytic activity in fresh blood (day 0) or in blood with processing delayed for 24, 48 or 72 hours.

Figure 2.18.1. GMFI of Neutrophils detected with PA

Figure 2.18.1-A. GMFI of Neutrophils detected with PA - Box Plot

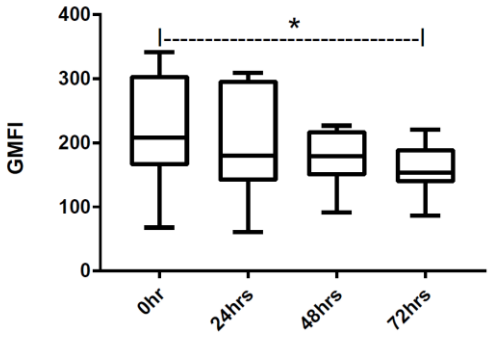
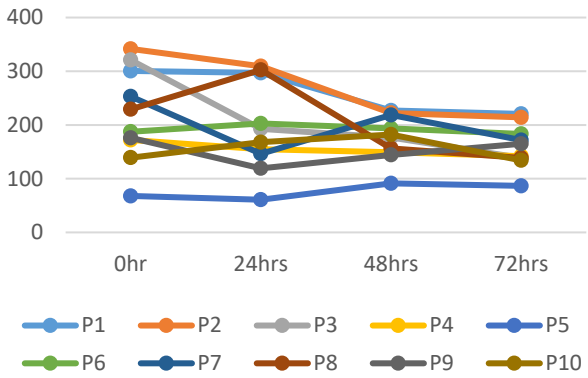


Figure 2.18.1-B. GMFI of Neutrophils detected with PA – Individual data



GMFI= Geometric Fluorescence (n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p=0.026**. Table 2.11 shows Pairwise comparisons: Most relevant for this study (*) are among 0 hr and 72 hrs; p=0.002. All p values are presented) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Figure 2.18.2. GMFI of Monocytes detected with PA

Figure 2.18.2-A. GMFI of Monocytes detected with PA - Box Plot

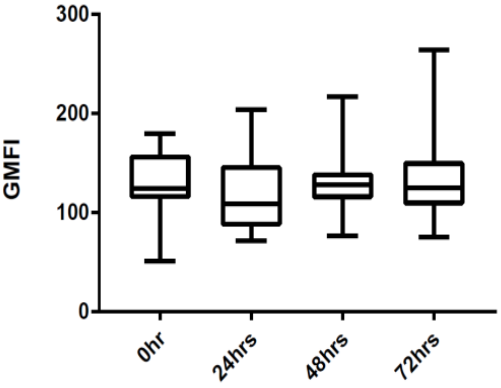
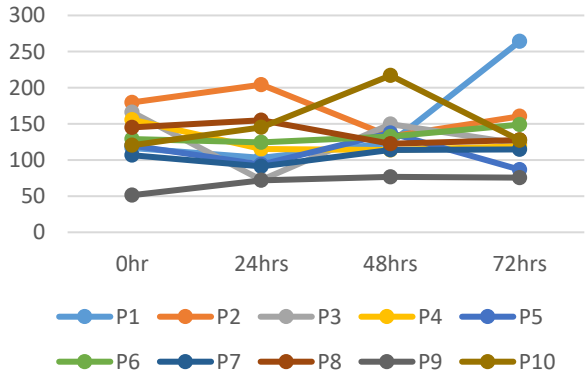


Figure 2.18.2-B. GMFI of Monocytes detected with PA – Individual data



GMFI= Geometric Fluorescence (n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.696) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

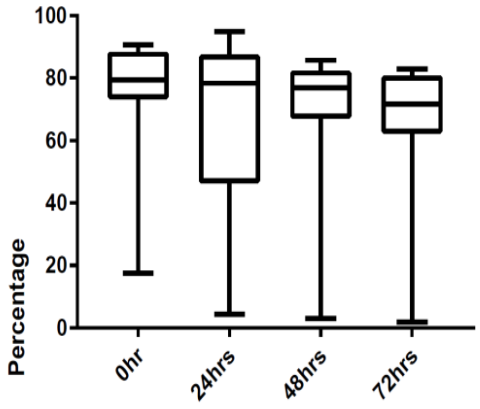
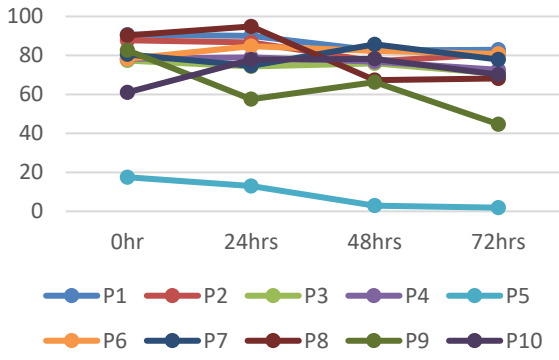
Figure 2.18.3. Percentage of Neutrophils detected with PA	
Figure 2.18.3-A. Percentage of Neutrophils detected with PA - Box Plot	Figure 2.18.3-B. Percentage of Neutrophils detected with PA – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.086) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

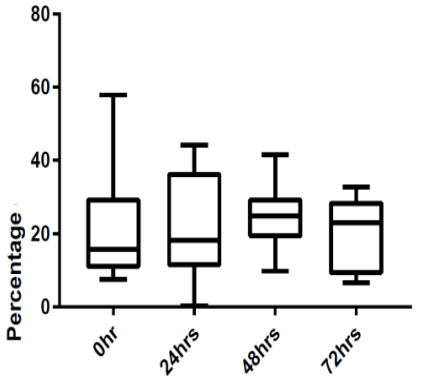
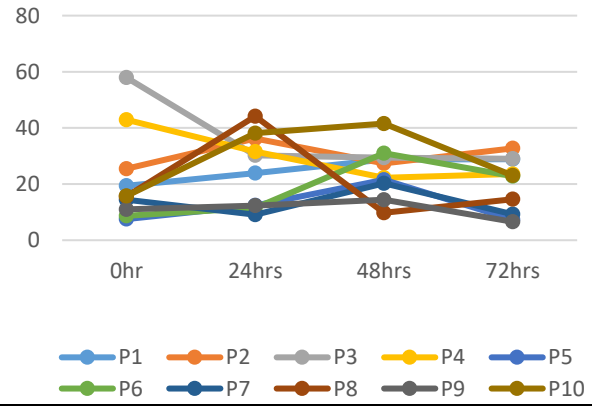
Figure 2.18.4. Percentage of Monocytes detected with PA	
Figure 2.18.4-A. Percentage of Monocytes detected with PA - Box Plot	Figure 2.18.4-B. Percentage of Monocytes detected with PA – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.253) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

Table 2.11. Significance of pairwise comparison tests of data for MFI neutrophil phagocytic activity at each day.

Paired comparisons	p-value
Fresh sample vs Day 1 of collection	0.225
Fresh sample vs Day 2 of collection	0.225
Fresh sample vs Day 3 of collection	**0.002**
Day 1 of collection -vs Day 2 of collection	1.000
Day 1 of collection -vs Day 3 of collection	0.057
Day 2 of collection vs Day 3 of collection	0.057

(*) Paired comparisons or Post-Hoc test derived from the Friedman's test Two-Way Analysis of Variance by Ranks. Identification of paired samples revealing the most significant alteration influenced by time point of delayed processing ($p<0.05$). Bold relate to significance.

2.3.4 Effect of delayed blood processing on the concentration of immune mediators in plasma

Plasma immune mediators were sensitive to delayed processing (See Figure 2.19). Only ICAM-1 showed conclusive stability across the timeframe of blood storage. Specifically, limited stability with blood storage was observed for RANTES, MIP1-α, IL-18BPa and IL-8 which showed a constant increase in concentration with longer delay in blood processing (Figure 2.19). Interestingly, other analytes showed a more stable concentration up to day 1 or 24 hours after delayed processing. Such is the case for MCP-1, TNFRII, IL-1rα, E-selectin, VCAM-1, IL-10, IL-6 and TNF-α (refer to those analytes in Table 2.16).

2.3.4.1 Non stable immune mediators measured in plasma: Significantly increased production at 24 hr in comparison to fresh analyses

RANTES showed to be unstable when compared with samples performed on fresh samples. A similar behaviour was observed for MIP1-α, which showed an increased concentration that became stable when day 3 and 2 were compared as well as day 2 and 1 after collection. However, the concentration of MIP1-α was significantly increased within the first 24 hours after collection (See pairwise comparisons in Table 2.12)

IL-18BPa tended to show an increased concentration that becomes stable with the progress of time; that concentration was significantly different at day 1 or 24 hours in comparison to fresh analyses. Lastly, IL-8 tended to show a markedly increased concentration as the inspection of the box plot indicates. Additionally, the concentration of IL-8 at 48 and 72 hours tended to increase steadily and became significantly different. (See pairwise comparisons in Table 2.12).

2.3.4.2 Immune mediators production in plasma and statistical comparability with analyses performed on freshly processed samples

MCP-1, TNFRII, IL-1ra, E-selectin, VCAM-1, IL-6 and TNF-α tend to show an increased concentration with delayed processing. The effect of delayed processing was particularly marked for IL-1ra. The concentration of these analytes in fresh samples was stable in comparison to that at day 1 or 24 hours as there was no significant difference between these time points (day 0 or fresh analyses and day 1 or 24 hours, refer to significance in Table 2.12. The augmented concentration (refer to table 2.17) became significantly different when day 2 or 48 hours and day 3 and 72 hours of delayed processing are compared with analyses performed on fresh samples.

IL-10 tended to show a steadily increased concentration which was not significantly different from fresh samples in comparison to samples stored for 24 and 48 hours. The increased concentration became significantly different at 72 hours or day 3 after collection.

Figure 2.19. Concentrations of plasma immune mediators analysed on fresh blood (0 hr) and blood analysed 24, 48 or 72 hours after collection.

Variables significantly affected

Figure 2.19.1. RANTES

Figure 2.19.1-A. RANTES - Box Plot

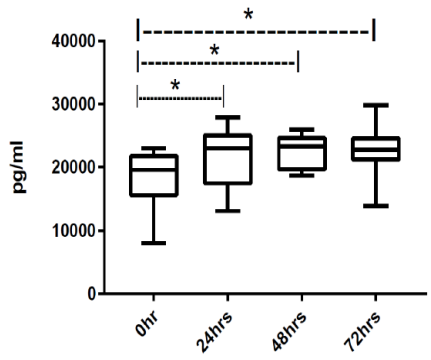
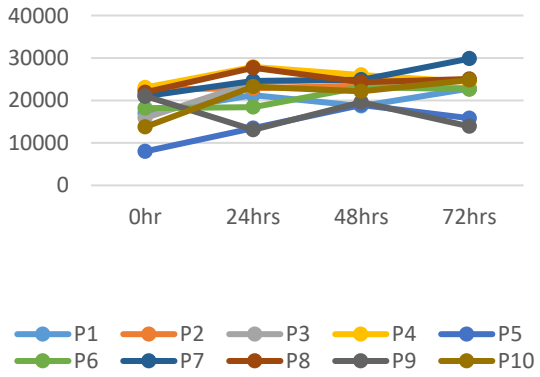


Figure 2.19.1-B. RANTES – Individual data



(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p =0.007** - Table 2.12 shows Pairwise comparisons: Most relevant for this study (*) are among 0 hr and 24 hrs; p=0.015. All p values are presented (Fresh vs 48 and 72 hrs p<0.01) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

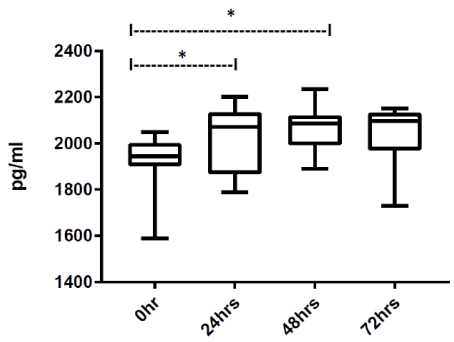
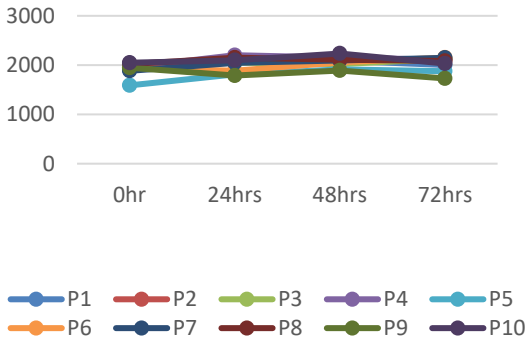
Figure 2.19.2. MIP-1α	
Figure 2.19.2-A. MIP-1α - Box Plot	Figure 2.19.2-B. MIP-1α – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p =0.036** - Table 2.12 shows Pairwise comparisons: Most relevant for this study (*) are among 0 hr and 24 hrs; p=0.015. All p values are presented (Fresh vs 48 hrs p=0.009) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

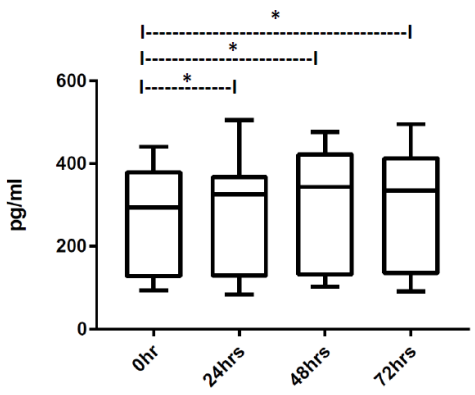
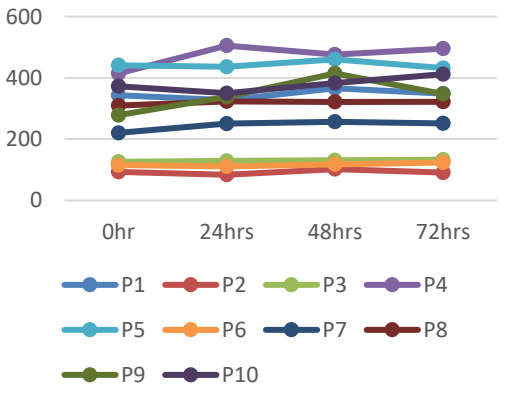
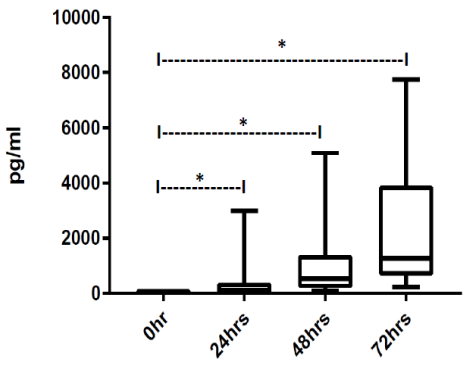
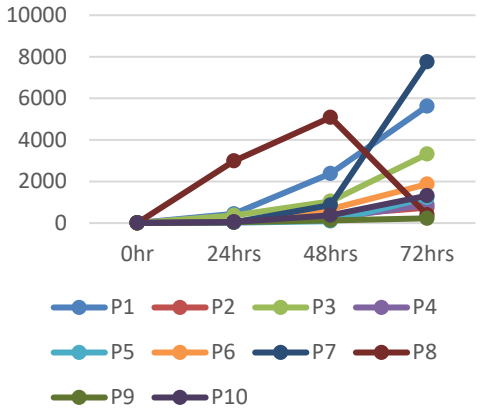
Figure 2.19.3. IL-18Bpa	
Figure 2.19.3-A. IL-18Bpa - Box Plot	Figure 2.19.3-B. IL-18Bpa – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p =0.014** - Table 2.12 shows Pairwise comparisons: Most relevant for this study (*) are among 0 hr and 24 hrs; p=0.046. All p values are presented (Fresh vs 48 and 72 hrs p<0.05) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

Figure 2.19.4. IL-8	
Figure 2.19.4-A. IL-8 - Box Plot	Figure 2.19.4-B. IL-8 – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001** - Table 2.12 shows Pairwise comparisons: Most relevant for this study (*) are among 0 hr and 24 hrs; p=0.05. All p values are presented (Fresh vs 48 and 72 hrs p<0.05) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

Variables relatively stable: Fresh analyses vs. 24 hours

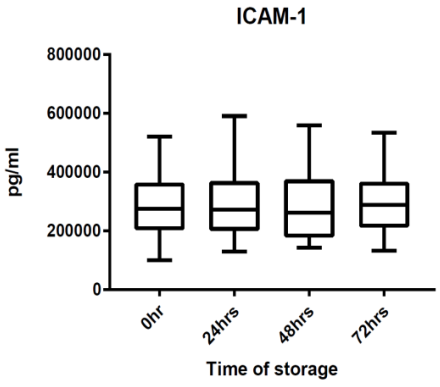
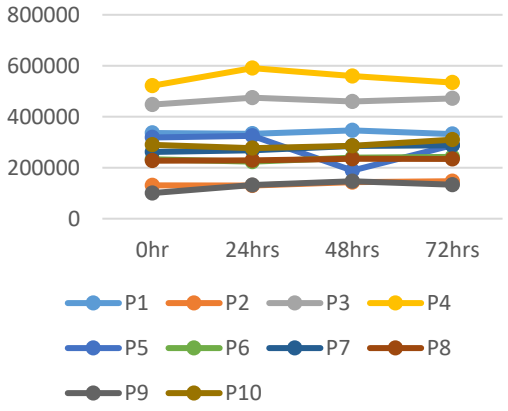
Figure 2.19.5. ICAM-1	
Figure 2.19.5-A. ICAM-1 - Box Plot	Figure 2.19.5-B. ICAM-1 – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p =0.131)– Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

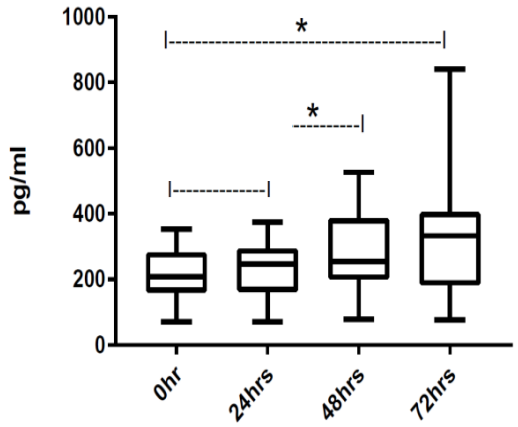
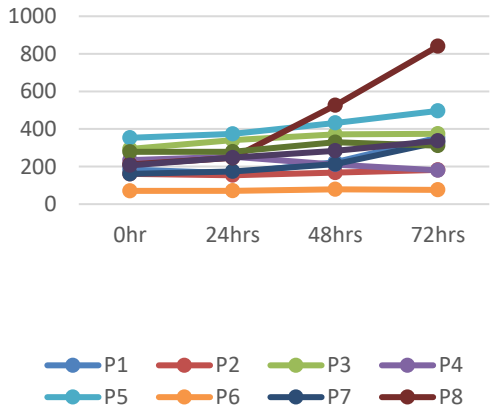
Figure 2.19.6. MCP-1	
Figure 2.19.6-A. MCP-1 - Box Plot	Figure 2.19.6-B. MCP-1 – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001** - Table 2.12 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.299 indicating stability. All p values are presented. Fresh vs 48 hrs (*) and 72 hrs (*) p<0.01 – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

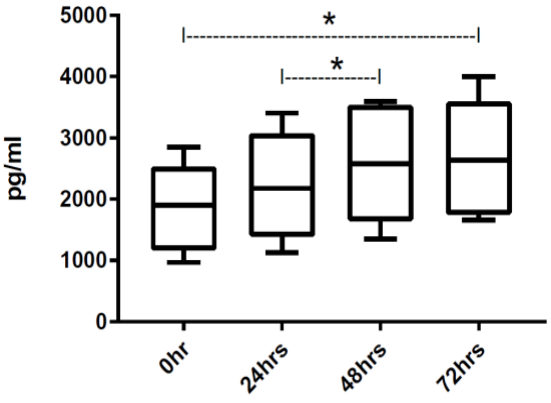
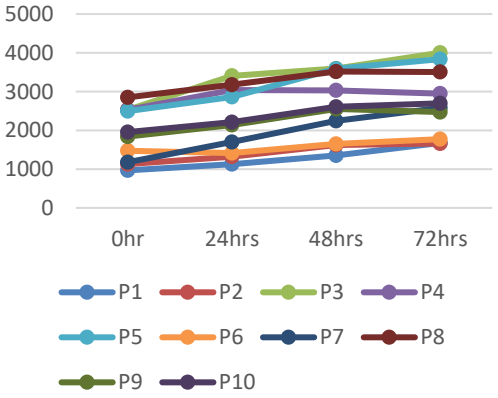
Figure 2.19.7. TNF-RII	
Figure 2.19.7-A. TNF-RII - Box Plot	Figure 2.19.7-B. TNF-RII – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001** - Table 2.12 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.083 indicating stability. All p values are presented. Fresh and 72 hrs (*) p<0.01 – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

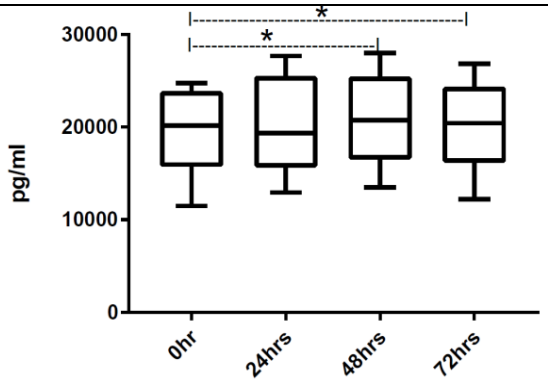
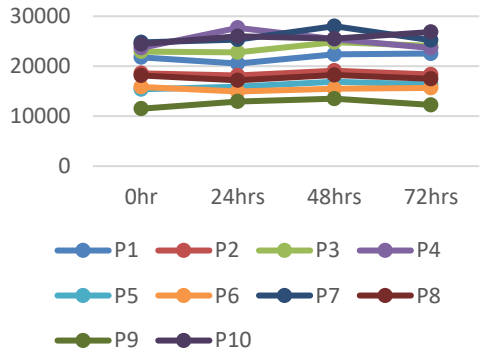
Figure 2.19.8. E- selectin	
Figure 2.19.8-A. E- selectin - Box Plot	Figure 2.19.8-B. E- selectin – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p = 0.048** - Table 2.12 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.057 indicating stability. All p values are presented. Fresh and 48 hrs (*) ; Fresh and 72 hrs (*) p<0.05 – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

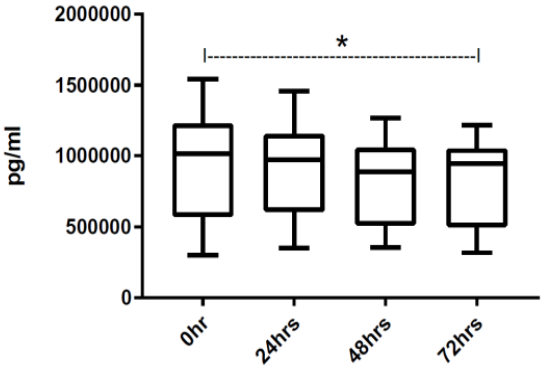
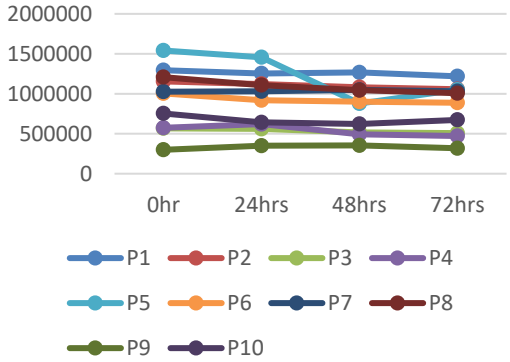
Figure 2.19.9. VCAM-1	
Figure 2.19.9-A. VCAM-1- Box Plot	Figure 2.19.9-B. VCAM-1– Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p = 0.024** - Table 2.12 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.386 indicating stability. All p values are presented. Fresh and 72 hrs (*) p<0.01 – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

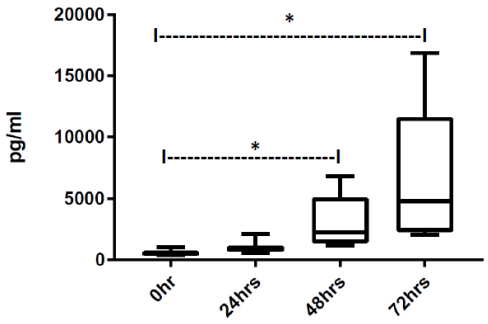
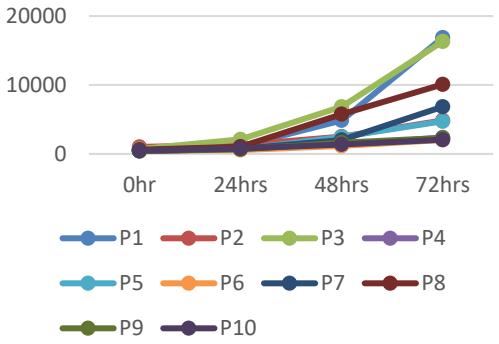
Figure 2.19.10. IL-1ra	
Figure 2.19.10-A. IL-1ra - Box Plot	Figure 2.19.10-B. IL-1ra – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001** - Table 2.12 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.083 indicating stability. All p values are presented. Fresh and 48 hrs (*) and 72 hrs (*) p<0.001 – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

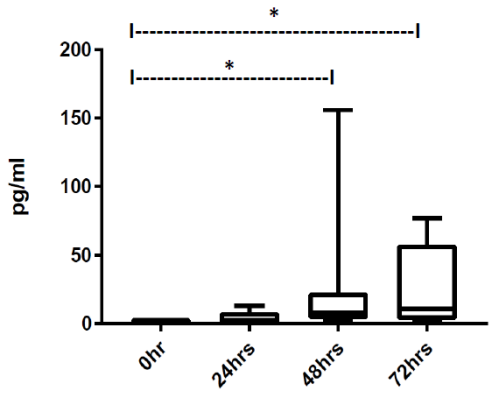
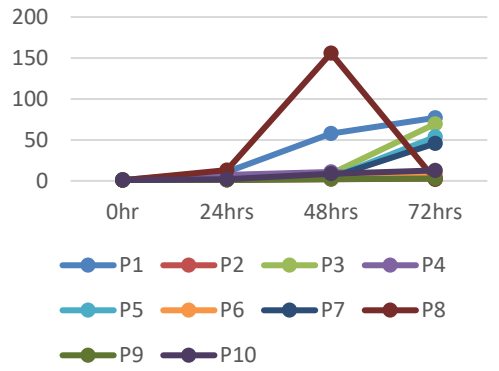
Figure 2.19.11. IL-6	
Figure 2.19.11-A. IL-6 - Box Plot	Figure 2.19.11-B. IL-6 – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001** - Table 2.12 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.083 indicating stability. All p values are presented. Fresh and 48 hrs (*) and 72 hrs (*) p<0.001 – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

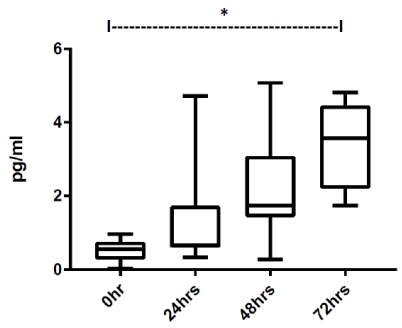
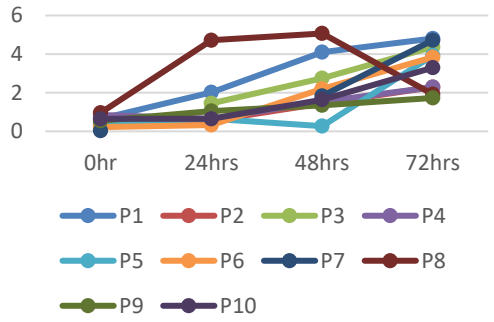
Figure 2.19.12. IL-10	
Figure 2.19.12-A. IL-10 - Box Plot	Figure 2.19.12-B. IL-10 – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001** - Table 2.12 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.245 indicating stability. All p values are presented. Fresh and 72 hrs (*) p<0.001 – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

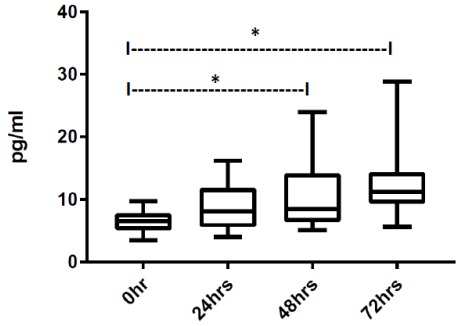
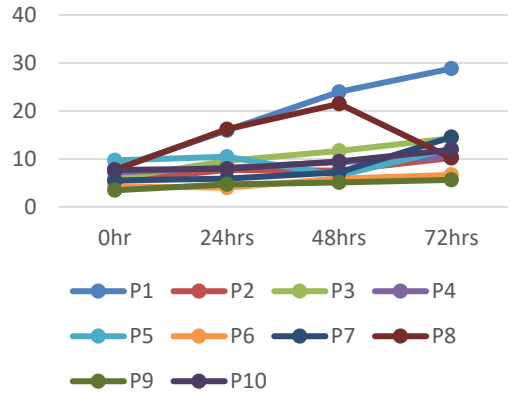
Figure 2.19.13. TNF-α	
Figure 2.19.13-A. TNF-α - Box Plot	Figure 2.19.13-B. TNF-α – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001** - Table 2.12 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.083 indicating stability. All p values are presented. Fresh and 48 hrs (*) and 72 hrs (*) p<0.001 – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

Table 2.12. Significance of pairwise comparison tests of data for plasma immune mediators

Paired comparisons*	p-value					
	MCP-1	RANTES	TNFRII	IL-1ra	MIP1-α	E-selectin
Fresh sample vs Day 1 of collection	0.299	**0.015**	0.083	0.083	**0.015**	0.057
Fresh sample vs Day 2 of collection	**0.003**	**0.002**	**<0.001**	**<0.001**	**0.009**	**0.015**
Fresh sample vs Day 3 of collection	**<0.001**	**0.006**	**<0.001**	**<0.001**	0.057	0.299
Day 1 of collection vs Day 2 of collection	0.057	0.488	0.057	0.083	0.862	**0.015**
Day 1 of collection vs Day 3 of collection	**0.009**	0.729	**0.009**	**<0.001**	0.603	0.299
Day 2 of collection vs Day 3 of collection	0.488	0.729	0.488	0.083	0.488	0.166
Continue Table 2.12 – Other parameters						
Paired comparisons	**IL-18Bpa**	**VCAM-1**	**IL-8**	**IL-10**	**IL-6**	**TNF-α**
Fresh sample vs Day 1 of collection	**0.046**	0.386	**0.053**	0.245	0.083	0.083
Fresh sample vs Day 2 of collection	**0.006**	**0.048**	**0.002**	0.093	**<0.001**	**0.034**
Fresh sample vs Day 3 of collection	**0.024**	**0.003**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Day 1 of collection vs Day 2 of collection	**0.024**	0.386	0.083	0.208	0.057	0.299
Day 1 of collection vs Day 3 of collection	0.083	0.038	**0.019**	**0.054**	0.056	**0.034**
Day 2 of collection vs Day 3 of collection	0.603	0.083	0.225	0.175	0.488	0.083

(*) Paired comparisons or Post-Hoc test derived from the Friedman's test Two-Way Analysis of Variance by Ranks. Identification of paired samples revealing the most significant alteration influenced by time point of delayed processing ($p<0.05$). Bold relate to significance.

2.3.5 Effect of delayed blood processing on the production of immune mediators in whole blood cultures

Unstimulated whole blood cultures did not produce detectable concentrations of most immune mediators analysed: IFN-γ, IL-13, IL-2 and IL-5 were not detected in these cultures (data not shown). Only TNF-α and IL-6 were produced in detectable concentrations in the unstimulated cultures, and production increased steadily and significantly in the case of TNF-α (See Figure 2.20).

Figure 2.20. Concentrations of immune mediators in unstimulated whole blood cultures (UWBC) analysed on fresh blood (0 hr) and blood 24, 48 or 72 hours after collection.

Figure 2.20.1. TNF-α produced UWBC

Figure 2.20.1-A. TNF-α produced UWBC - Box Plot

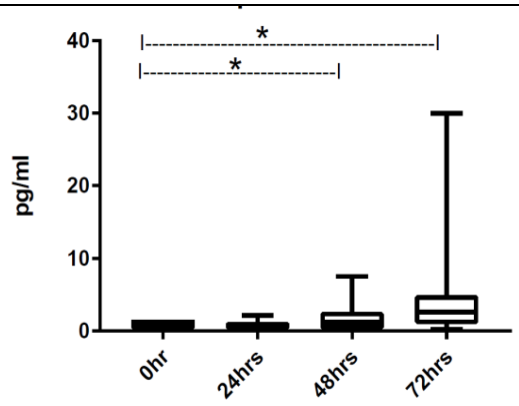
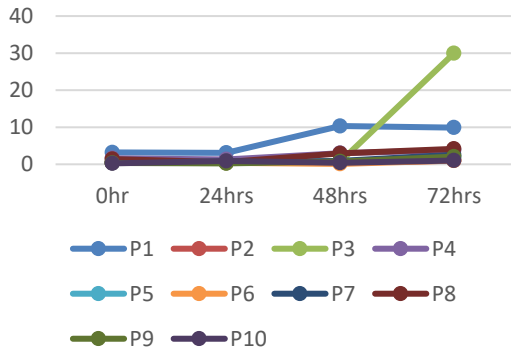
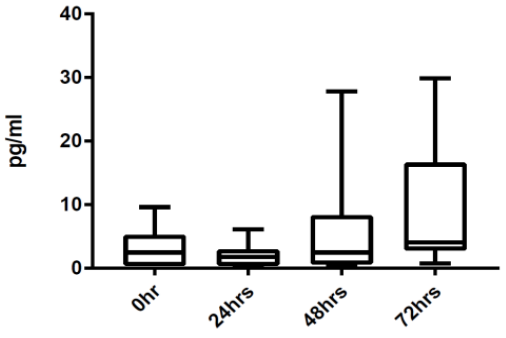
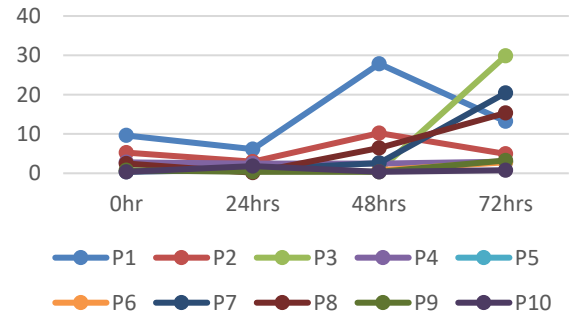


Figure 2.20.1-B. TNF-α produced UWBC



(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001** – UWBC did not produce detectable concentrations for most mediators analysed. Those these UWBC will not be used as controls – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Figure 2.20.2. IL-6 produced UWBC	
Figure 2.20.2-A. IL-6 produced UWBC - Box Plot	Figure 2.20.2-B. IL-6 produced UWBC
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p=0.074** – UWBC did not produce detectable concentrations for most mediators analysed. Those these UWBC will not be used as controls – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

The panel of immune mediators measured in whole blood cultures in response to LPS stimulation was significantly affected by delayed blood processing (See Figure 2.21). These analytes showed variable behaviour. IL-10 production decreased markedly with delay in blood processing, and there was a less marked but also decreased production of TNF-α, IL-12p70, and IL-1β. In the case of IL-6, there was stable production for blood stored for 24 hours (day 1), but the production decreased with longer blood storage time prior to culture.

Delayed blood processing prior to culture did not affect significantly the production of IL-10, IL-6, IL-12p70 or IL-1B in response to PGN (Figure 2.21). TNF-α production in response to PGN was affected by delayed blood processing prior to culture. TNF-α production increased progressively with increasing delay in blood processing and this augmentation became significant for blood that was cultured 48 and 72 hours after collection (See Table 2.13)

Figure 2.21. Concentrations of immune mediators after stimulation of whole blood cultures (SWBC) with LPS, and PGN analysed on fresh blood (0 hr) and blood 24, 48 or 72 hours after collection.

Figure 2.21.1. IL-10 - SWBC

Figure 2.21.1-A. IL-10 – LPS SWBC - Box Plot

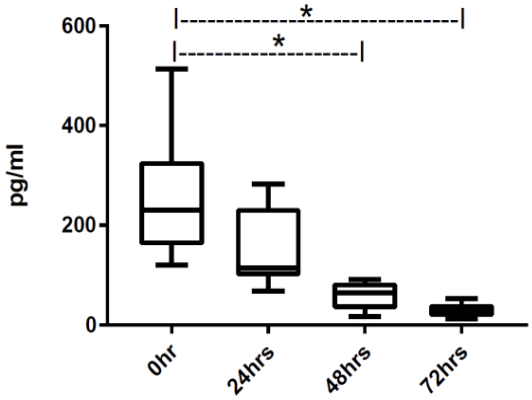
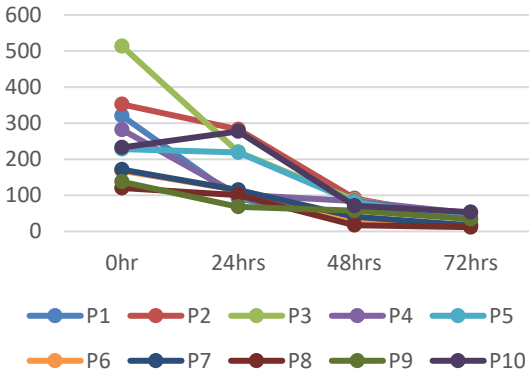


Figure 2.21.1-B. IL-10 – LPS SWBC – Individual data



(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001**) – Table 2.13 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.166 indicating stability. All p values are presented. Fresh and 48 hrs (*) and 72 hrs (*) p<0.001 – Time points indicated on the x axis correspond to time of storage. Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Figure 2.21.1-A. IL-10 – PGN SWBC - Box Plot

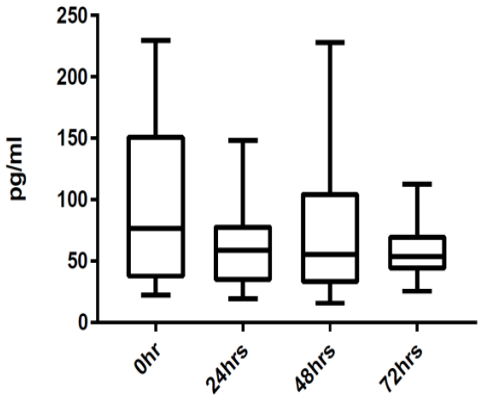
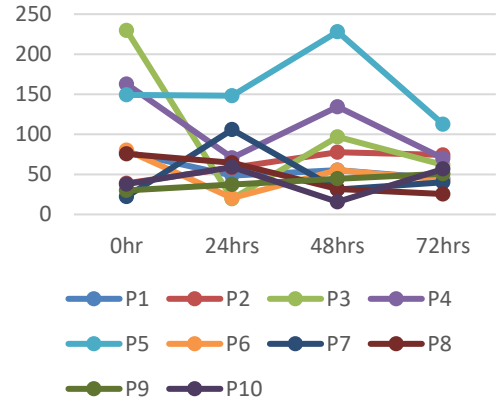


Figure 2.21.1-B. IL-10 – PGN SWBC – Individual data



(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.516)– Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

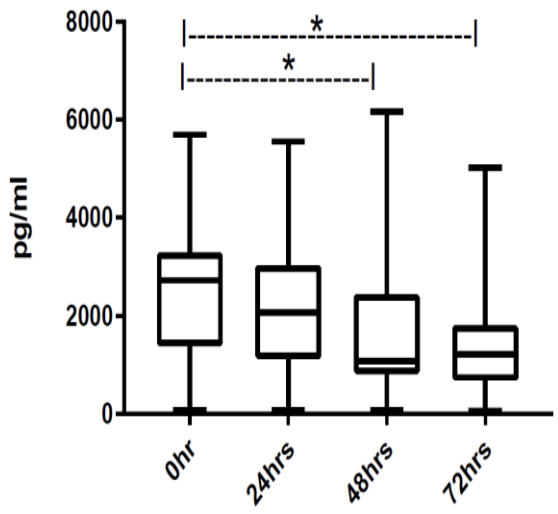
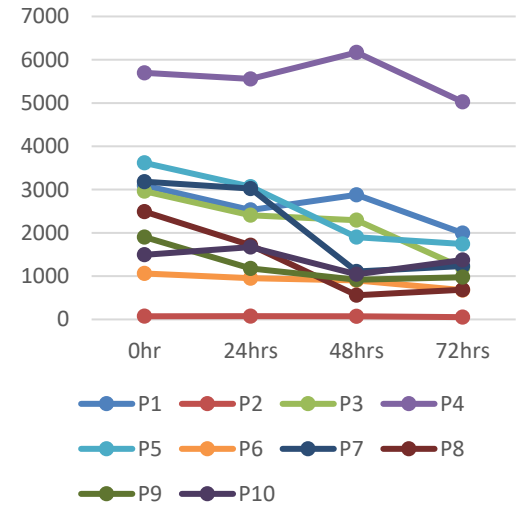
Figure 2.21.2. TNF-α - SWBC	
Figure 2.21.2-A. TNF-α - LPS SWBC - Box Plot	Figure 2.21.2-B. TNF-α - LPS SWBC – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001**) – Table 2.13 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs: p=0.225 indicating stability. All p values are presented. Fresh and 48 hrs (*) and 72 hrs (*) p<0.001 – Time points indicated on the x axis correspond to time of storage. Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

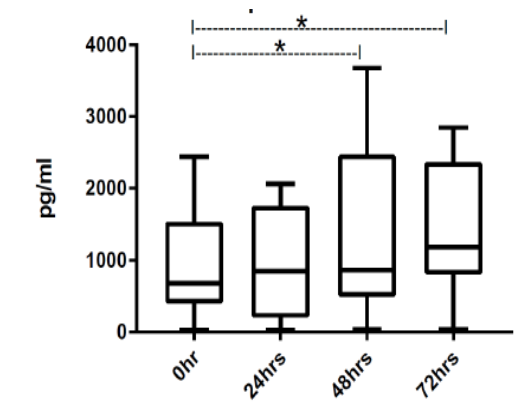
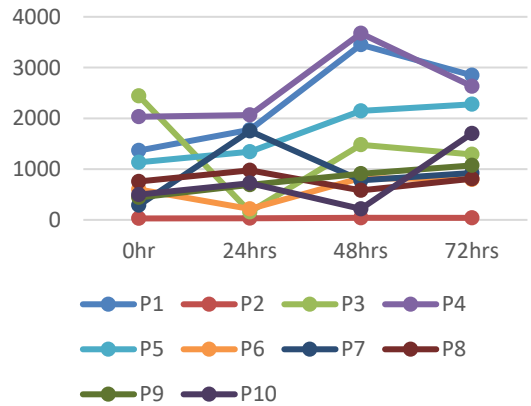
Figure 2.21.2-A. TNF-α - PGN SWBC - Box Plot	Figure 2.21.2-B. TNF-α - PGN SWBC – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p = 0.024**) – Table 2.13 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.561 indicating stability. All p values are presented. Fresh and 48 hrs (*) and 72 hrs (*) p<0.001 – Time points indicated on the x axis correspond to time of storage. Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

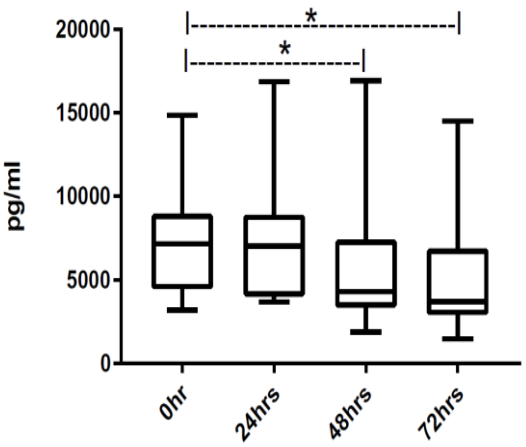
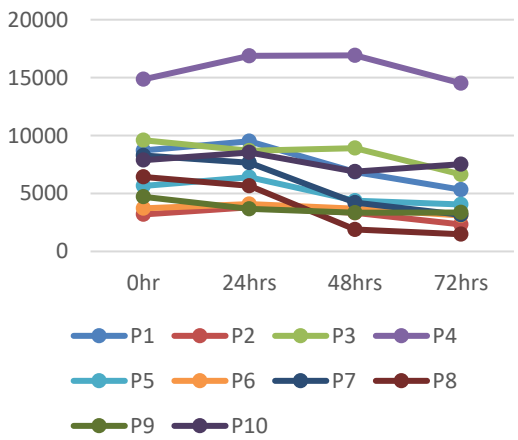
Figure 2.21.3. IL-6 - SWBC	
Figure 2.21.3-A. IL-6 – LPS SWBC - Box Plot	Figure 2.21.3-B. IL-6 – LPS SWBC – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p<0.001**) – Table 2.13 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.726 indicating stability. All p values are presented. Fresh and 48 hrs (*) and 72 hrs (*) p<0.05 – Time points indicated on the x axis correspond to time of storage. Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

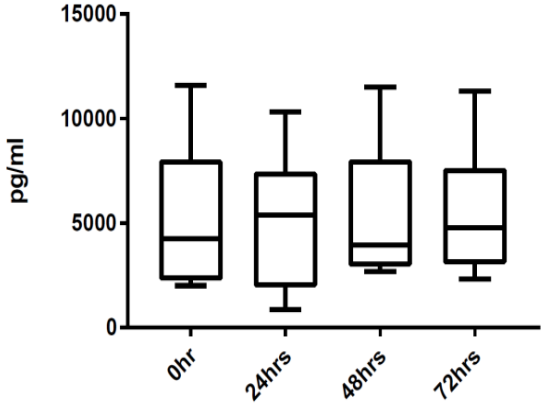
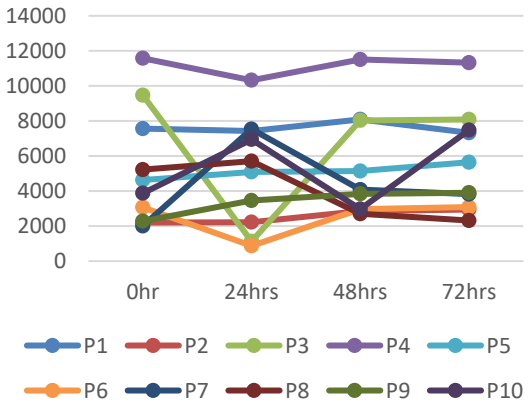
Figure 2.21.3-.IL-6 – PGN SWBC	
Figure 2.21.3-A. IL-6 – PGN SWBC - Box Plot	Figure 2.21.3-B. IL-6 – PGN SWBC – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p =0.615)– Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

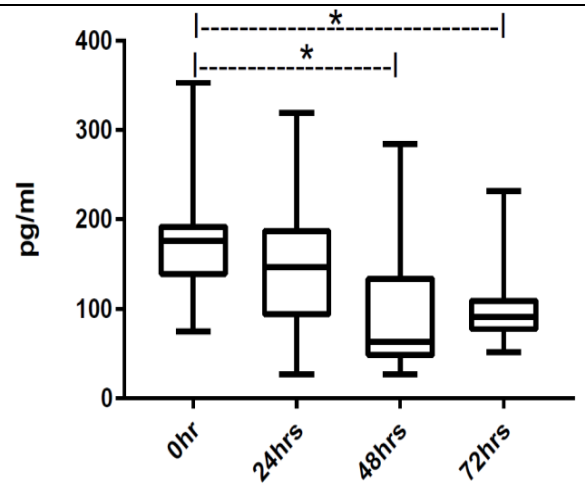
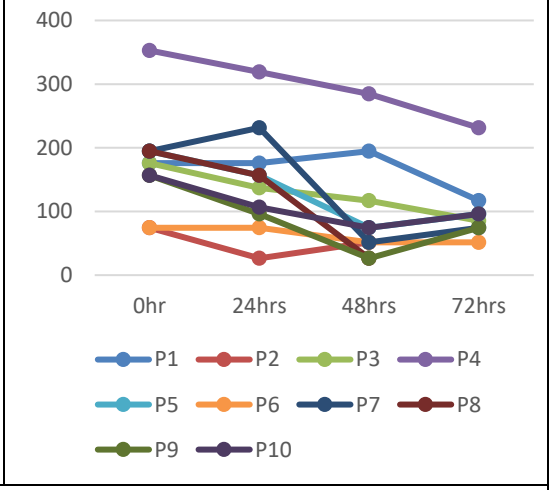
Figure 2.21.4. IL-12p70 - SWBC	
Figure 2.21.4-A. IL-12p70 – LPS SWBC - Box Plot	Figure 2.21.4-B. IL-12p70 – LPS SWBC – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p<0.001**) – Table 2.13 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.439 indicating stability. All p values are presented. Fresh and 48 hrs (*) and 72 hrs (*) p<0.05 – Time points indicated on the x axis correspond to time of storage. Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

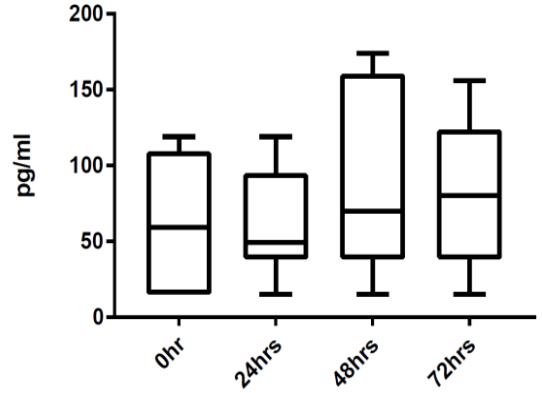
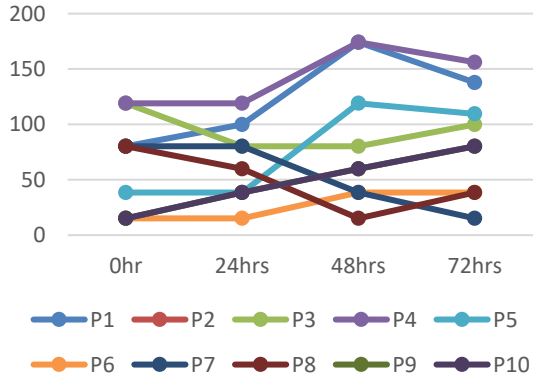
Figure 2.21.4-A. IL-12p70 – PGN SWBC - Box Plot	Figure 2.21.4-B. IL-12p70 – PGN SWBC – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p =0.073)– Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

Figure 2.21.5. IL-1β - SWBC

Figure 2.21.5-A. IL-1β – LPS SWBC - Box Plot

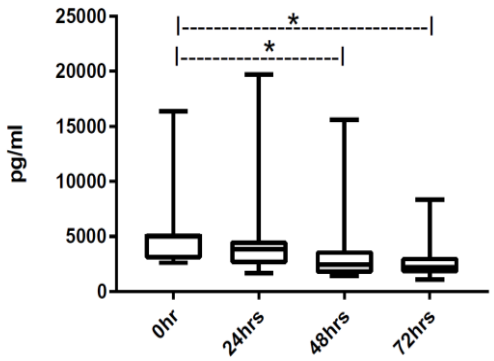
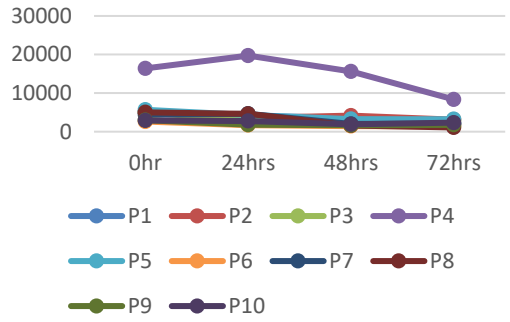


Figure 2.21.5-B. IL-1β – LPS SWBC – Individual data



(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001**) – Table 2.13 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.166 indicating stability. All p values are presented. Fresh and 48 hrs (*) and 72 hrs (*) p<0.01 – Time points indicated on the x axis correspond to time of storage. Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Figure 2.21.5-A. IL-1β – PGN SWBC - Box Plot

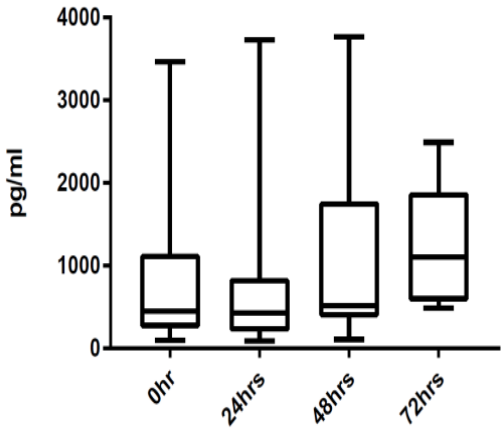
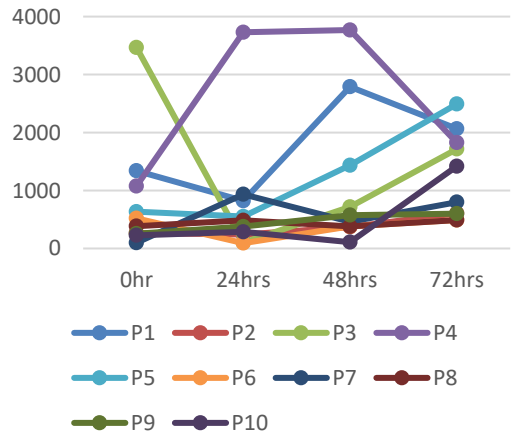


Figure 2.21.5-B. IL-1β – PGN SWBC – Individual data



(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p =0.062)– Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Whole blood cultures were also assessed following stimulation with PHA, a T cell mitogen (See Figure 2.22). The production of IL-13, IL-4, IL-2 and IL-5 was not detected, whilst IFN-γ and TNF-α were produced but were significantly affected by delay in blood processing

prior to culture with a decreased production that is not significant when fresh analyses are compared with analyses at day 1 (See Table 2.13).

Figure 2.22. Concentrations of immune mediators after stimulation of whole blood cultures with PHA analysed on fresh blood (0 hr) and blood 24, 48 or 72 hours after collection.

Figure 2.22.1. IFN-γ PHA SWBC

Figure 2.22.1-A. IFN-γ PHA SWBC - Box Plot

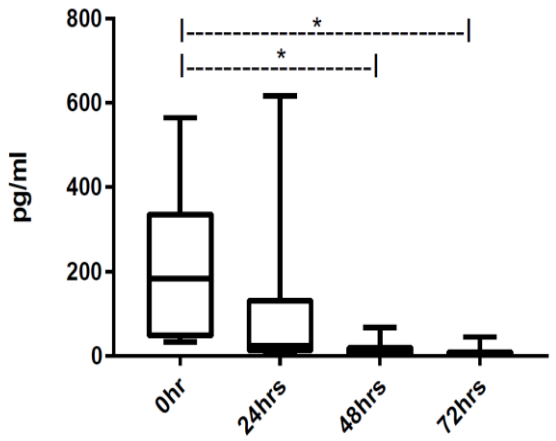
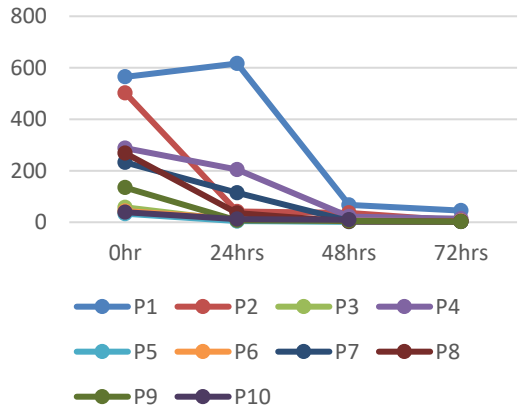


Figure 2.22.1-B. IFN-γ PHA SWBC – Individual data



(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p <0.001**) – Table 2.13 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.201 indicating stability. All p values are presented. Fresh and 48 hrs (*) and 72 hrs (*) p<0.01 – Time points indicated on the x axis correspond to time of storage. Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Figure 2.22.2. TNF-α PHA SWBC

Figure 2.22.2-A. TNF-α PHA SWBC - Box Plot

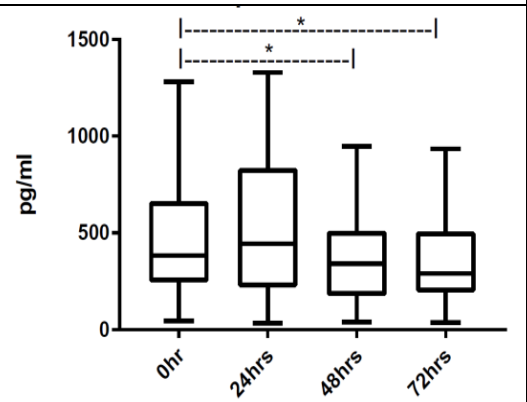
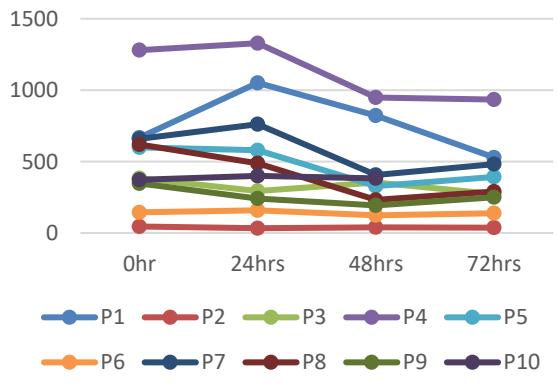


Figure 2.22.2-B. TNF-α PHA SWBC – Individual data



(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; **p=0.024**) – Table 2.13 shows Pairwise comparisons: Most relevant for this study are among 0 hr and 24 hrs; p=0.201 indicating stability. All p values are presented. Fresh and 48 hrs (*) and 72 hrs (*) p<0.01 – Time points indicated on the x axis correspond

to time of storage. Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

Table 2.13. Significance of pairwise comparison tests of data for immune mediators measured in whole blood cultures

Paired comparisons*	p-value							
	TNF-α	IL-10	TNF-α	IL-6	IL-12p70	IL-1B	IFN-γ	TNF-α
Stimulant	PGN	LPS	LPS	LPS	LPS	LPS	PHA	PHA
Fresh sample vs Day 1 of collection	0.561	0.166	0.225	0.729	0.439	0.166	0.201	0.561
Fresh sample vs Day 2 of collection	**0.020**	**0.006**	**0.011**	0.083	**0.016**	**0.002**	**0.002**	**0.020**
Fresh sample vs Day 3 of collection	**0.012**	**<0.001**	**<0.001**	**0.003**	**0.008**	**<0.001**	**<0.001**	**0.012**
Day 1 of collection vs Day 2 of collection	0.081	**<0.001**	0.057	**0.038**	0.026	**0.024**	**0.018**	0.081
Day 1 of collection vs Day 3 of collection	**0.053**	**<0.001**	**0.034**	**0.001**	**0.015**	**<0.001**	**0.021**	**0.053**
Day 2 of collection vs Day 3 of collection	0.846	**0.083**	0.386	**0.083**	0.846	0.729	0.584	0.846

(*) Paired comparisons or Post-Hoc test derived from the Friedman's test Two-Way Analysis of Variance by Ranks. Identification of paired samples revealing the most significant alteration influenced by time point of delayed processing ($p<0.05$). Bold relates to significance.

2.3.6 Effect of delayed blood processing on anti-influenza vaccine antibody titres

Stability across the timeframe of analysis was observed for the anti-vaccine antibody titres (See Figure 2.23). Their comparison in fresh samples (day 0), and after delayed processing for 24, 48 and 72 hours (days 1, 2, and 3) did not show a significant difference when compared with analyses performed on fresh samples.

Figure 2.23. Concentrations of anti-influenza vaccine antibodies analysed in serum isolated from fresh blood (0 hr) and serum isolated from blood analysed at 24, 48 or 72 hours after collection.

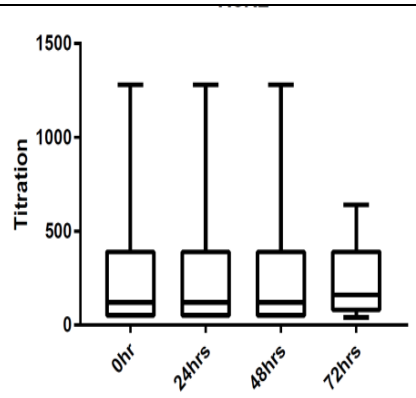
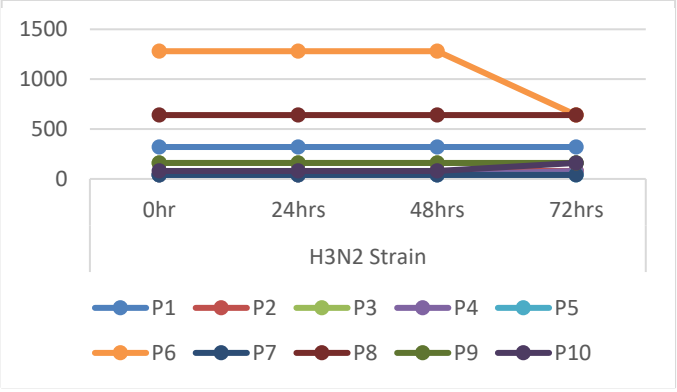
Figure 2.23.1-A. H3N2 Strain - Box Plot	Figure 2.23.1-B. H3N2 – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.468) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

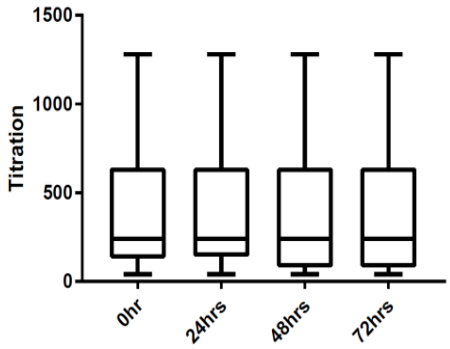
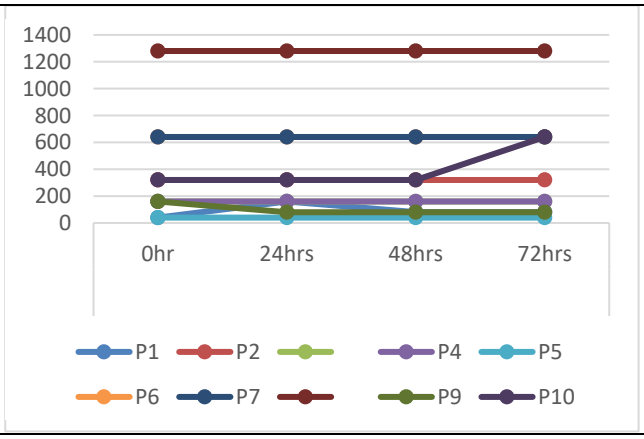
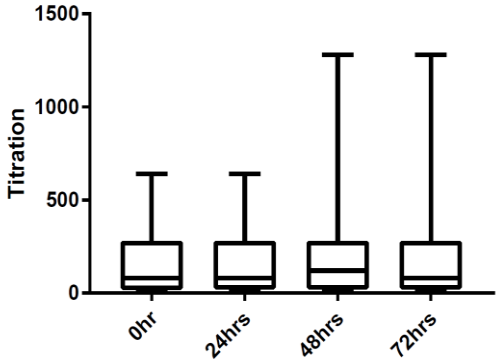
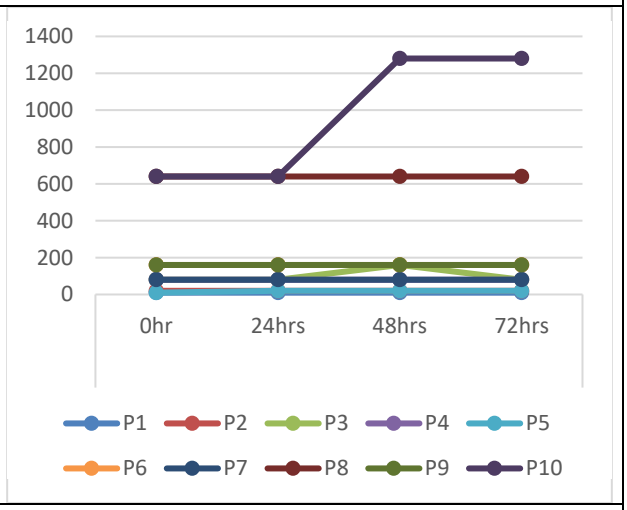
Figure 2.23.2-A. Influenza B Strain - Box Plot	Figure 2.23.2-B. Influenza B – Individual data
	
(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.870) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).	

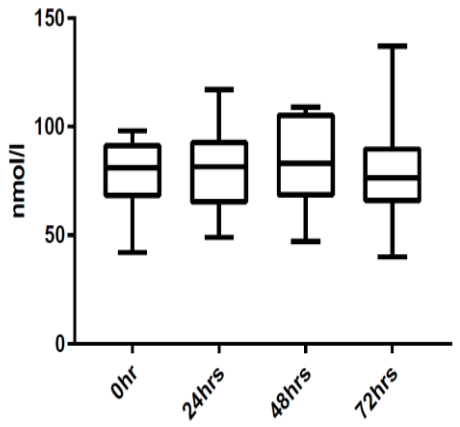
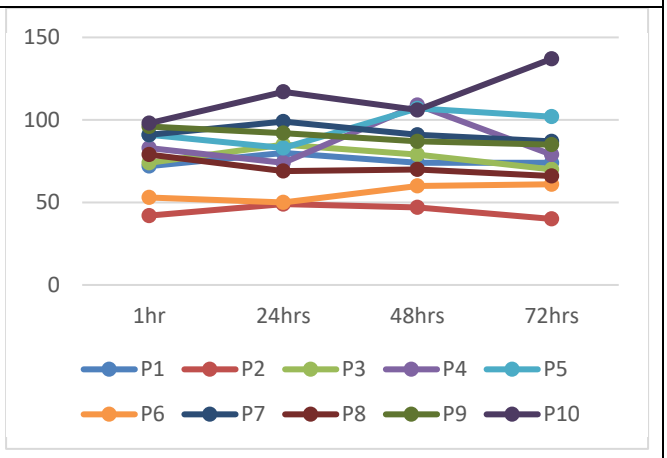
Figure 2.23.3-A. H1N1 Strain - Box Plot	Figure 2.23.3-B. H1N1 – Individual data
	

(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p=0.112) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

2.3.7 Effect of delayed blood processing on plasma vitamin D concentration

Plasma vitamin D concentration was not significantly altered by delayed blood processing (Figure 2.24).

Figure 2.24. Vitamin D concentration analysed in plasma from fresh blood (0 hr) and in plasma from blood stored for 24, 48 or 72 hours after collection.

Figure 2.24-A. Vitamin D - Box Plot	Figure 2.24-B. Vitamin D – Individual data
	

(n=10; Box plot reflects median values, P_{25}, P_{75}, minimum and maximum values. Statistical analyses were conducted using the related-samples Friedman's test Two-Way Analysis of Variance by Ranks; p= 0.457) – Time points indicated on the x axis correspond to time of storage. Individual data are presented to showcase individual values and changes across times of analyses (P=Participant).

2.4 Discussion

The research described in this chapter assessed the influence of delayed blood processing on immune and inflammatory biomarkers [214, 223]. The findings are relevant to human studies focussed on the immune response where there may be a delay in blood processing due to distant settings or participants in different locations from the centre of sample processing. These results are especially relevant to the immune investigations conducted as part of the PRINCESS clinical trial – Immunology sub-study (chapters 3 to 6) in which blood samples collected from elderly residents in Oxford and Cardiff and sent by post to Southampton were used (See Chapter 3 – PRINCESS clinical trial – Immunology sub-study). This research work is therefore used in the discrimination of stable and usable immune and inflammatory parameters and the identification of a time-window for sample processing according to blood sample availability, dictated by arrival at University of Southampton facilities after collection from participants involved in the PRINCESS clinical trial.

The results indicate that a number of the assessed immune biomarkers can be measured in blood kept up to 72 hours after sample collection. These are:

- Full blood cell counts: lymphocytes, monocytes, eosinophils, basophils, platelets.
- Immune cell phenotypes: T cells, helper T cells, cytotoxic T cells, B cells, activated B cells expressing $CD80^+$ and $CD86^+$, monocytes, activated monocytes expressing $CD80^+$ and $CD86^+$, ratio $CD4^+$:$CD8^+$, regulatory T cells, MFI for CD14 expression on monocytes and neutrophils
- Phagocytic activity measured through percentage of active neutrophils and monocytes with phagocytic activity, and GMFI of active monocytes
- Plasma immune mediators: ICAM-1
- Serum anti-influenza vaccine antibodies
- Plasma vitamin D concentration

Additionally, the results indicate that a number of the assessed immune biomarkers can be measured in blood kept up to 48 or 24 hours after sample collection. These are:

- Full blood cell counts: Neutrophils up to 48 hours and total white cell count up to 24 hours.

- Immune cell phenotypes: Activated cytotoxic T cells up to 48 hours; and NK cells up to 24 hours.
- Phagocytic activity measured through GMFI of active neutrophils up to 48 hours.
- Plasma immune mediators: MCP-1, TNFRII, IL-1ra, E-selectin, VCAM-1, IL-6, IL-10 and TNF-α up to 24 hours.
- Whole blood cultures: LPS and PGN stimulated production of IL-10, IL-6, IL-12p70 and IL-1β up to 24 hours and PHA stimulated production of IFN-γ and TNF-α up to 24 hours.

Lastly, plasma RANTES, MIP1-α, IL-18BPa and IL-8 showed to be highly unstable and thus should only be measured when fresh sample is readily available for analysis.

Results will be discussed in the light of two main contributions: Firstly, what is the effect of the delayed sample processing on the immune biomarkers herein analysed in the context of what has been reported by other investigations addressing the issue of delayed sample handling. Secondly, the relevance of these biomarkers for other human studies seeking to assess immune and inflammatory responses, will also be explored. Lastly, and strongly linked to the technical examination of these biomarkers in the laboratory, the examination of the parameters considered in this chapter involves technical concepts related to the haematological biochemistry and the technique execution in the laboratory. Thus, the interassay, intraassay and sensitivity concepts will be examined and an overview of their behaviour in the techniques used in this research will be presented (See section 2.4.3). On important note, the research herein presented did not examined separately these coefficients of variation prior to the commencement of the study. However, consistent and systematic mechanisms to ensure calibration of the instruments (flow cytometry and multiplex ELISA) as well as the use of robust techniques used traditionally in diagnosis (FBC, anti-vaccine titres and vitamin D) served as a basis to implement the protocol used to conduct this study and aimed to detect changes with delayed processing of the blood sample.

2.4.1 Effects of delayed processing on immune and inflammatory biomarkers. Blood handling and haematological chemistry.

Firstly, if FBC is analysed in stored samples interpretation of findings for neutrophils and WCC should be made cautiously since they decrease with increasing storage time. This finding agrees with that reported by Zini [224]. The marked reduction in the WCC

(leukocytes) is likely linked with the significant alteration observed in neutrophils, which are the largest proportion of leukocytes.

Secondly, with regards the immune cell phenotypes, the use of antibodies to detect surface markers showed stability in most parameters (See Figure 2.16 Figure 2.19). However, NK cells showed a significant effect of delayed processing at 48 and 72 hours after blood collection. Stability was only observed within 24 hours after analyses in comparison to fresh samples. Thornthwaite determined the percentages of lymphocytic subtypes from human blood stored at room temperature and observed that T helper and T supressor cells were not significantly altered by overnighyt storage of human blood [225].

A study intending to analyse the phagocytic response of neutrophils in stored blood using heparinised tubes, observed a deterioration in the phagocytic activity of neutrophils as well as in their morphology after two days storage of the blood at 4^{o}C. [226]. Although the study used a different assay system [227] and different storage conditions, the observation that phagocytic activity may not be preserved in stored blood is in accordance with the current research.

Another study aiming to analyse the effect of blood sample "age" at the time of separation on cytokine concentrations in human plasma observed that the concentration of some of the proteins analysed was altered as the samples aged. Measurements pointed to small decreases in E-selectin (2.4% per day) and MCP-1 (13% day) [228]. The current study confirms that concentrations of MCP-1 and E-selectin are unstable in stored human blood, although they increased rather than decreased.

A study aiming to understand the stability of TNF-α, IL-6 and IL-8 in blood samples collected from patients with systemic immune activation found that these biomarkers were stable if samples were stored at room temperature up to 8 hours. These authors also found that the stability can be prolonged if the sample is previously stored at 4^{o}C [229]. The current research did not investigate storage times less than 24 hours, but confirms that concentrations of these biomarkers remain largely unchanged with storage up to 24 hr.

Azis *et al*. observed augmented values of IL-1ra measured in plasma when analysed from samples stored at room temperature. These authors found a significant increase in IL-1ra after 8 hours and thereafter concentrations increased further [230]. Hartweg observed stability of VCAM-1 and ICAM-1 measured in whole blood stored at a

temperature of 4° or 21°C [231]. It is also reported that E-selectin is stable in blood for 2 days [231].

TNF-α and IL-6 could be measured in unstimulated whole blood cultures and concentrations increased progressively with longer storage. This could indicate the presence of an endogenous stimulant in the cultures or could be driven by cell death [232].

The concentrations of several cytokines produced in stimulated cultures declined with longer storage of blood prior to culture. It has been previously described that sample aging at room temperature inhibits LPS-induced monocytic cytokine release in minimally diluted whole blood cultures and the authors also suggest that this reduction can be observed specially in IL-6, IFN-γ and TNF-α [233]. Findings of the current study, which used different experimental conditions, agree with this.

One reason for reduced response with increasing storage of blood is death of responder cells [234]. However it is paradoxical that concentrations in plasma of stored blood increased with increasing storage times whereas cellular responses to challenge decreased. One explanation for this is the concept of "immune exhaustion" where cells that were active "*in vivo*" respond poorly "ex vivo" [235, 236]. LPS induces the production of pro-inflammatory mediators through activation of TLR4 initiated by binding of LPS to CD14 protein anchored in the plasma membrane. CD14 then transfers the LPS to the TLR4/MD-2 complex which dimerizes and triggers MyD88 and the production of pro-inflammatory cytokines and type I IFNs. This signalling is linked with endocytosis of the activated TLR4, which is controlled by CD14 [237]. It is possible that prolonged blood storage causes loss of TLR-4 from the cell membrane. The current study did not assess TLR-4 expression. However, it did assess the relative expression of CD14 on the monocyte and neutrophil populations (seeFigure 2.17.) and it was observed that the relative expression of CD14 did not change significantly on these cell types with the delayed blood processing. Nevertheless, it would be useful to measure TLR-4 expression on the surface of monocytes in blood stored for different periods of time.

2.4.2 Immune and inflammatory parameters assessed: Applications of the findings of this pilot study in assessing immune mechanisms and biomarkers selected in this context of human studies

Biomarkers herein selected are indicators of immune responses and ongoing mechanisms of defence (see Figure 2.1. General approach for the study of the immune and inflammatory responses). This research studied the immune system from both a quantitative perspective (FBC and immune cell phenotypes) and a qualitative perspective (phagocytic responses and immune mediators that form part of the inflammatory response). Cell activation analysed as part of the immunophenotyping and the phagocytic response are important components within the mechanisms involved in the immune response.

2.4.2.1 Quantification of immune cell numbers: FBC and immune cell phenotypes

Quantitative biomarkers such as the number of immune cells measured through FBC and immune cell phenotypes serve to diagnose and follow-up conditions of disease and health. Recent evidence revealed significant associations among blood eosinophils with the prognostic severity of respiratory diseases, and FBC with the prognosis of peripheral arterial diseases [216, 238, 239]. Also, FBC has been associated with the effects of lifestyle habits (e.g. smoking) and an acceleration in the ageing process [240]. Likewise, there is evidence of different immune signatures analysed through $CD4^+$ and $CD8^+$ profiles in relation to the nutritional status [241]. Immunophenotypes are a useful tool to assess the effect of nutritional interventions on immune cell types and markers of cellular activation. The stability of the parameters analysed through FBC (monocytes and neutrophils) was also observed in the immunophenotyping process (MFI analysis in the CD14 expression on both cell types). Additionally, the parameters analysed through FBC were also within ranges of normality when compared with the reference values for FBC, as shown in table 2.1.

Regarding immune cell phenotypes, the results obtained compare well with those of others. Standardised guidelines and reference values for the different immunophenotypes have not been published, but a compilation of different findings, including the results from the current research, is presented in Table 2.14.

Table 2.14. Data on cell immune phenotypes obtained by different research groups, measured in whole peripheral blood from healthy adults with an age range from 18 to 60 y.

Authors and reference	This research (Fresh blood)	Bisset *et al*. 2004 [242]	Wong *et al*. 2013 [243]	Choi *et al*., 2014 [244]	Zhang *et al*. 2015 [245]	Tsegaye *et al*. 2003 [246]	Jentsh-Ullrich *et al*. 2005 [247]	Oladepo *et al*. 2009 [248]	Chang *et al*. 2004 [249]
Sample size	10	70	273	294	268	51	100	2570	232
Country	UK	Switzerland	China	Korea	China	Ethiopia	Germany	Nigeria	Singapore
Immune cell phenotype (mean and range cells/ul)									
CD3⁺	1342 (783-1721)	1140 (558-1817)	1362 (723–2271)	1305 (708-2294)	1303 (762–2043)	1185 (588–2381)	(780-2024)	No data	1550 (796-2679)
CD4⁺	901 (565-1234)	709 (325-1198)	760 (396–1309)	787 (394–1574)	691 (384–1107)	660 (374–1362)	(490-1064)	812 (365-1571)	814 (401-1451)
CD8⁺	368 (116-505)	358 (136-808)	515 (224–1014)	479 (188–830)	469 (198–867)	484 (146–1449)	(170-880)	435 (145–884)	16.0 (6 - 37)
CD4:CD8 ratio	2.6 (1.6-6)	2 (1-5)	1.59 (0.71–2.82)	1.81 (0.8–4.4)	1.63 (0.7–3.3)	1.52 (0.5–3.9)	(0.9-5)	2.3 (0.7–5.3)	1.33 (0.69-2.83)
CD19⁺	370 (203-437)	173 (87-394)	298 (118–645)	NR	203 (69–408)	181 (56–436)	(80-490)	No data	335 (133-714)

The degree of response when immune cells are stimulated is an approach commonly analysed as an immune outcome [213, 214, 223]. Whole blood cultures (WBCs) can be used to assess *ex vivo* immune responses. WBCs have several advantages compared with the study of isolated cell populations: less manipulation of the cells, lower costs, preservation and maintenance of all blood components, reflection of the different cell types at *in vivo* ratios [250]. Thus, WBCs can be considered more physiological than other approaches.

2.4.2.2 Immune mediators measured in plasma: relevance of the parameters selected in this study

The biomarkers selected in this research are relevant to the study of health and disease from an epidemiological perspective and in clinical trials where inflammatory and immune-related processes are being investigated [251].

Many of the cytokines studied have been linked to different roles in health and disease, particularly in the study of inflammatory process related to ageing. IL-6 has generally been attributed roles in the pro-inflammatory pathway, but recent evidence shows that IL-6 acts upon pathways activated during inflammation by inhibiting the production of TNF-α and creating a negative feedback which contributes to terminate TNF-α – inflammatory - response [252]. Evidence in human studies supports that IL-6 mediates positive effects on low-grade inflammation by stimulating the production of IL-10, and – through a more clinical view - it appears that this cytokine stimulates lipid turnover and increases lipolysis, and affects directly the muscles (which has suggested that IL-6 acts as a myokine) [253]. Thus IL-6 is important in the study of geriatric populations, amongst others. IL-10 is mainly produced by monocytes and to a lesser degree by lymphocytes, and its role in human studies has been linked to the prevention of ageing-associated inflammation through the resolution of inflammatory processes and contribution to cellular repair [254, 255]. Also, of importance in biogerontology, clear effects of TNF-α to sarcopenia have been established [256, 257].

The study of adhesion molecules has also been extensive and some epidemiological associations with health outcomes have been achieved. VCAM-1, ICAM-1 and E-selectin have been associated with coronary disease [258] and carcinoma [259]. These molecules belong to two main categories: VCAM-1 and ICAM-1 are intercellular

adhesion molecules whose main function is to facilitate the adhesion and subsequent transmigration of leucocytes. The second category belongs to the selectins where E-selectin is a protein involved in the adhesion cascade in endothelial cells. [260, 261]. Interestingly, adhesion molecules showed good stability in stored blood in comparison to cytokines and chemokines. Findings herein presented and related to the stability shown by adhesion molecules support the findings of Hartweg, *et al*. who showed that VCAM-1 and ICAM-1 are stable in plasma [262].

RANTES and MIP1-A are chemoattractant immune mediators. RANTES is released from lymphocytes and platelets and acts on eosinophils and monocytes, whereas MIP1-A acts on NK cells and monocytes. Their action through binding to corresponding cell surface receptors plays an important role in the regulation of the acute inflammatory response; thus these parameters are informative of acute conditions. These chemokines have been correlated with the progression of some diseases, such as Parkinson's and osteoporosis [263, 264], indicating their importance. A previous study aiming to compare the stability of these chemokines in stored blood also found great variability [265]

2.4.2.3 Immune mediators measured in whole blood in response to stimulation with toll-like receptor (TLR) 2, TLR4 and T cell receptor ligands

TLRs are transmembrane proteins present on the cell surface, and their recognition of different bacterial molecular patterns triggers a signalling cascade resulting in altered gene expression and protein production. LPS is an important component of the cell wall of Gram-negative bacteria and initiates immune responses to limit further infection. The structural pattern of LPS in diverse bacterial strains is recognised by a complex formed by CD14 and TLR4 [266]. PGN is a major component of Gram-positive bacterial cell walls and causes pro-inflammatory cytokine production through TLR2 which is highly expressed on monocytes and T cells [267, 268]. This interaction is essential in bacterial recognition and was effectively demonstrated by the increased production of IL-10, TNF-α, IL-6, IL-12p70, and IL-1B as seen in Figure 2.21 in comparison with the lack of detection of these cytokines in unstimulated whole blood cultures. This figure also reflects the differential patterns of expression induced by LPS and PGN. Storing blood resulted in reduced production of immune mediators in response to LPS compared to the production seen with fresh blood.

PHA was used due to its mitogenic activity to study T cell responses. PHA has the ability to bind to the membranes of T cells and stimulate metabolic activity and cell division [269]. Monocyte are also responsive to PHA stimulation [270]. The declined production of IFN-γ and TNF-α could be related to reduced responsiveness to PHA, cell death, reduced metabolic activity and reduced cell division in the aged sample.

2.4.2.4 Anti-influenza vaccine antibodies

Stability was found in this parameter across times. These results agree with what has been reported by Hodgkinson *et al*., who found that serum antibodies to epitopes from various pathogenic infectious, including viruses can be measured reliably in blood stored at room temperature for at least 6 days [271].

2.4.2.5 Vitamin D

Plasma 25-hydroxy-vitamin D concentrations did not change with blood storage over 72 hours. A study conducted by Drammeh *et al*. was focused on the stability of nutritional biomarkers and defined clinical acceptability as concentrations that varied from 3 to 15% with blood storage. The researchers found that 25-hydroxy-vitamin D measured in serum samples either stored at 32°C or stored at 11°C for over 3 days remained within the percentages of clinical acceptability [272]. The results from this study confirm the stability of 25-hydroxy-vitamin D in stored blood.

2.4.3 Methodological implications for PRINCESS clinical trial – immunology sub-study

Peripheral blood was the tissue sampled to assess immune and inflammatory cell numbers and responses. Main techniques used to describe these findings were flow cytometry, multiplex ELISA, also hemagglutination inhibition test, and automated haematology analysers. The execution of these techniques involves relevant concepts as coefficients of variability (interassay, and intraassay variance) and sensitivity of analyses. These concepts and their behaviour in the techniques used will be discussed according to relevant literature and references reported for these methods. Overall, it was considered that these methodological processes were standardised, and automated in order to produce the piece of research herein presented.

2.4.3.1 Coefficients of variance and sensitivity in the methodological development of techniques used in PRINCESS clinical trial – immunology sub-study.

Coefficients of variation involve establishing reproducibility in the methodological development of techniques, the degree of variation when repeated analyses are conducted can be quantified and that corresponds to the coefficient of variation (CV) indicating precision of the method. Automated methods normally show a minor CV (such is the case of the FBC and vitamin D) whereas methods relying on antibody binding (immunoassays) normally have higher CVs and therefore lower degree of reliability. This reduced reliability is related to the natural intrinsic variations in the cells [273].

2.4.3.1.1 Intra-assay CV

This coefficient quantifies reproducibility of an assay in the short term (for instance in a single day or in a single batch) as well as within assay or run-run variability is conducted by the repeated assay of the same sample within a single process. For immunoassays as the ELISA, it is expected an intra-assay CV lower than 5%, whereas other biochemistry methods might have a CV of or even lower than 3% [273].

2.4.3.1.2 Inter-assay CV

This coefficient quantifies reproducibility of an assay over a longer term (for instance day to day, or week to week). It might also be named assays among variability. The inter-assay CV is measured by repeating the analysis of a sample in consecutive experiments run in the process. It is expected that this analysis will not be larger than the intra-assay CV. Accordingly, the inter-assay CV of an ELISA is generally acceptable if lower than 10%. In other biochemical analyses it is expected than it would be lower than 5% [273].

2.4.3.1.3 Sensitivity

The sensitivity of a method is the proportion of samples (people) known to have a condition which is correctly identified by that method. This corresponds to the number of true positives compared with the sum of the true positives and false negatives (that is all the cases combined) [273].

2.4.3.1.4 Overall implications of these analyses for PRINCESS clinical trial – immunology substudy

Overall, the examination of circulating immune cells in peripheral blood and their responses have been successfully and significantly associated to a variety of clinical conditions as has been described through this chapter. Table 2.15 summarises the findings with regards stable parameters according to delayed processing and serves as a guidance for PRINCESS clinical trial – immunology sub-study.

With regards the analytical parameters related to these variables, literature reports a high degree of reliability (degree in which these measurements are considered accurate) and previous studies have shown validation of the techniques. The analysis of FBC (Section 2.2.3.1.1), and the method used in the analysis of Vitamin D, corresponding to immunoassay (Section 2.2.6), are both automated methods.

The assessment of automated hematology analyzers using fresh human blood has revealed that the maximum CV among instruments were 3.2%, 9,3% and 10.8%, in the analyses of red blood cell count, white blood cell count and platelets (respectively). This study included 114 hospitals where the instrument Beckman coulter in comparison with other automated methods were also included [274]. Validation studies have also been conducted in the study of erythropoiesis by examining red blood size using an automated Beckman-Coulter instrument [275]. Additionally, it has been reported that the access to total 25(OH) vitamin D immunoassay method has an acceptable analytical performance, whereby the CV for vitamin D is low enough to stay within acceptable ranges of analysis. The intra-assay analysis corresponds to 3,3%, the inter-assay analyses corresponds to 5.3% and the total CV for the Unicel vitamin D immunoassay corresponds to 8.3%. These parameters were assessed in a heterogeneous population with different degrees of insufficiency and sufficiency. Thus these methods have been validated [276].

Analyses of the cytokines and other immune mediators produced in both plasma as well as supernatants collected from whole blood cultures (Section 2.2.4) were detected using a multiplex ELISA. A previous report analysing the intra-assay CV found that the study of some cytokines and immune mediators (IL-6, IL-8, IL-10 and MCP-1) ranged from 1.7% to 7.5%, whereas the inter-assay CV for the same analytes ranged 2.4% to 9.6% [277]. The minimum detectable concentrations (sensitivity) were provided by the manufacturer of the kit as presented in the methods (section 2.2.4). Previous calibration

was conducted for the instrument prior to assay running to ensure quality in the assessment.

Similarly, protocols herein presented included calibrations performed to optimise the development of flow cytometry techniques. In the case of the phagotest, four parameters were considered: percentage phagocytizing granulocytes, GeoMean FL1, percentage phagocytizing monocytes and GeoMean FL1 for the latter matter. Respectively, the CV reported by the manufacturer corresponds to 0.2%, 1.5%, 1.1% and 3.6% [278]. In the immunophenotyping there have been validation analysis establishing that inter-assay, intra-assay and inter-operator variations are within acceptable limits (<25% CV). The determination of the inter-assay variability was done by quantifying the cell surface marker expression in duplicate in 3 independent assays on 3 different days. These experiments were replicated by two trained technicians to determine inter-operator differences [279]. Validation techniques in immunophenotyping have also been introduced in other studies, which agree with the CV values reported [280, 281].

Lastly, there is evidence that appropriate standardization among laboratories might lead to consistent HAI assay results [282]. Reproducibility has been achieved in 99.2% to 100% of the laboratories running these assays, whilst classification of seroprotection (titer greater or equal than 40) proved to be accurate in 93.6% or 89.5% of cases [282]. According to the laboratory specification, the antigens used for the individual HAI analyses were egg influenza viruses. Assays were performed using appropriate pre-validated control sera and titres of those controls were used to monitor and validate assay performance according to standard operator procedures indicated by clinical scientist at the public health England laboratory.

Given the previous conditions and the manufacturers indications to proceed with these techniques, it was assumed appropriate validation of the techniques so that our results are summarised in table 2.15 as follows. The outcomes of the research herein presented are useful to other investigations aiming to perform analyses with delayed influence, but these results are important to PRINCESS immunology sub-study.

Table 2.15. Summary and recommendations. Stability of biomarkers of immune and inflammatory responses in stored blood.

Parameter	0 hours (Fresh)	24 hours	48 hours	72 hours
FBC				
Lymphocytes, Monocytes, Eosinophils, Basophils, Platelets.	Ideal			
Neutrophils	Ideal			
White cells	Ideal			
Cell immune phenotypes				
T cells, Helper T cells, Cytotoxic T cells, B cells, Monocytes and markers of activation CD80 and CD86, Ratio $CD4^+$: $CD8^+$, Regulatory T cells	Ideal			
Activated cytotoxic T cells	Ideal			
NK cells	Ideal			
MFI $CD14^+$ neutrophils and monocytes	Ideal			
Phagocytic activity				
Geometric median fluorescence intensity (GMFI) of active monocytes, Percentage of Neutrophils and monocytes gated with phagocytic activity	Ideal			
Phagocytic activity assessed in neutrophils through the geometric median fluorescence intensity	Ideal			
Plasma immune mediators				
ICAM-1	Ideal			
MCP-1, TNFRII, IL-1ra, E-selectin, VCAM-1, IL-10, IL-6 and TNF-α	Ideal			
RANTES, MIP1-α, IL-18BPa and IL-8	Ideal			
Whole blood cultures				
Unstimulated	Not detectable concentrations			
LPS and PGN				
IL-10, IL-6, IL-12p70 and IL-1β	Ideal			
PHA				
IFN-γ, TNF-α	Ideal			
Other parameters				
Influenza antibody titres	Ideal			
Vitamin D	Ideal			

Amber colour indicates time frames in which the corresponding biomarker can be analysed. Red colour indicates time frames that cannot be used for analysis of the corresponding biomarker.

2.5 Conclusions

While freshly analysed blood samples are ideal, the hypothesis proposed in this chapter can be accepted. It is possible to identify specific timeframes in which analyses in blood can be conducted to maintain as minimum variability as possible influenced by delayed processing (summarised in Table 2.15). Findings of this chapter provide evidence on the stability of a range of immune and inflammatory markers analysed in blood stored for up to 3 days prior to processing. It was found that stability is particularly observable in parameters belonging to the FBC, immune phenotyping, phagocytic activity, vitamin D and influenza vaccine strains. A comprehensive summary of specific timeframes is presented in Table 2.15. Moreover, it is possible to use blood samples with delayed processing in the analysis of plasma immune mediators and immune mediators quantified in whole blood cultures following stimulation with LPS, PGN and PHA, but in general the assessment of these immune parameters requires blood processed immediately or within 24 hours. Finally, a small number of immune markers can only be measured in freshly processed blood (Table 2.15) as their variability is significant within 24 hours after collection. Therefore those parameters were not included in this study nor in the following chapters. Findings of this research are relevant in the development of PRINCESS immunology sub-study presented in chapters 3, 4 and 5.

Chapter 3 Characterisation of elderly care home residents involved in the (PRINCESS) – Immunology sub-study: Cross-sectional analyses studying frailty, length of time in care home and age, and their association with biomarkers of immune function

3.1 Introduction

Ageing involves changes for individuals and for society (See Chapter 1). Individual changes include a loss of immune competence, which has been described previously when the concept *immunosenescence* was presented (See section 1.11). This condition results in increased susceptibility to, and prevalence of, infections [283], as well as poor responses to vaccination [284, 285] (See section 1.13). For societies, the aged population represents a more vulnerable sector than younger adults. Older people are more likely to suffer illness and disability (e.g., chronic conditions, infections and hearing or visual loss) and cognitive impairment [286]. The biological definition of the "aged population" and clear-cut thresholds for immune, inflammatory and even biological (e.g. age) biomarkers in the elderly are still raising questions in terms of both disease predictability and success of interventions. This is due to the high heterogeneity in the ageing process. For example, frailty can sometimes be present in people aged over 65 y – considered the traditional age threshold to define "old"- [287], whereas some centenarians show good health conditions corresponding to optimum systemic functioning [288]. Conducting research that unravels and understands the factors priming healthy ageing in order to preserve the quality of life, is currently an important objective for societies.

The clinical and social complexity of the aged population converges when individuals transfer to residential care homes. These settings are common approaches to the care of the aged population where the elderly live as a community that receives required support. There are an estimated 12,525 residential homes in the UK, with more than a quarter of a million people aged 65 y and over living in residential homes in England and Wales [289]. These shared environments provide a setting to extend the current understanding of the ageing process for the old population under these circumstances, in order to identify efficient strategies to improve their quality of life. The heterogeneity of the ageing process entails a challenge –or adds a variable- when studied in different settings. Certainly, the study of the ageing process in institutionalised elderly people will provide more evidence to address efficient interventions for this sector of society [290].

It has been suggested that free-living individuals (i.e. those "living at home") have a significantly better quality of life when compared with nursing home residents [291] and therefore it is important to understand health progression in care home residences [292], considering especially their commonality as an option for the fastest-growing old population.

Elderly tend to show a progressive decline in immune competence which is biologically usual within the ageing process; however, this decline is enhanced in elderly care home residents (ECHRs) who tend to suffer higher rates of infections when compared with free-living individuals of the same age [293]. This creates a problem as antibiotic-resistant microorganisms are a major issue in the treatment of infections and worsen disease and morbidity in ECHRs [294]. Additionally, infections are closely related to higher rates of hospitalisation and more complications [295]. Interestingly, it has been shown that these complications are not necessarily linked to being in a care home, but rather to underlying individual conditions, which triggers geriatric syndromes, so-called chronic conditions. Only 2% of chronic conditions are explained by long-term residence, whereas 50% are explained by self-disability such as visual or hearing impairments, frailty, sarcopenia [296] or chronic diseases [297]. Immunosenescence has been poorly described and studied in the context of frailty and aged individuals in care home residents. Better understanding of immunosenescent changes in ECHRs might help with the objective to improve their quality of life.

In addition to the concept of "years (y) old from birth date" to identify the aged population, frailty has emerged in the past decade as an approach that stratifies the risk of mortality throughout the ageing process in old individuals. Frailty is currently recognized as a "geriatric syndrome" as a consequence of multi-systemic dysregulation [298, 299] which can be identified as a decreased physical reservoir and increased vulnerability to mortality [300]. Frail older adults have a higher risk of adverse health outcomes, including falling, hospitalization, and mortality. Studies have suggested that one of the most important pathways of frailty development is the immune/inflammatory pathway [301]. An inflammatory basis has also been linked to a wide range of chronic diseases of common prevalence within these populations [302, 303].

Likewise, it has been shown that immunosenescent changes are linked to decreased microbial diversity [304] as well as vitamin D deficiency [305, 306] , conditions that are common within ECHRs [307]. Beneficial alterations in the gut microbiota as well as corrections of vitamin D levels [308], have the potential to improve frailty in the aged as they may modulate different aspects of innate [309, 310] and acquired immunity [114, 311]. The reduced intestinal microbial diversity in the elderly also seems to be enhanced by the sub-optimal levels of vitamin D [125]. A comparison among healthy young individuals and healthy old individuals indicates that the gut microbiota might be a target

to decrease the progressive immune decline [312] and subsequently the rate of infections in ECHRs.

It is relevant to examine strategies tailored on modifications in gut bacteria in ECHRs as part of the solution to improve immune responses in ageing. Additionally, exploring other strategies aiming to improve the immune response in the elderly could help them cope with the shared exposure to pathogens in these common environments [313]. Optimum levels of vitamin D might exert a positive impact on the health of ECHRs via underexplored non-classical pathways which involve the immune system [314] and modulation of inflammatory responses via intestinal epithelia [315], and potentially, via gut microbiota composition [125, 316].

Improving the immune and inflammatory response, perhaps via modifying gut microbiota composition, might contribute to reducing the high rate of antibiotic prescription in ECHRs. A retrospective cohort study in the Hampshire Health record found that the annual prescription of antibiotics is almost double among elderly in care homes when compared with the same group adjusted for age, sex and comorbidities but free-living [317].

Interventions aiming at preventing infections in ECHRs, improving their gut microbial composition and their quality of life also seek to reduce high rates of co-morbidities and hospitalisation. Probiotics are live bacteria and may be beneficial to the host by increasing gut microbial diversity by directly competing with pathogenic microorganisms, enhancing the host immune response and therefore reducing infections [318] with improved underlying inflammatory conditions (See section 1.15.4.1).

Previous meta-analyses assessing the consumption of probiotics and health outcomes have been positive. For instance, it has been found that gastrointestinal conditions like infectious diarrhoea and *Clostridium difficile*-associated diarrhoea are reduced by probiotics (RR 0.58, 95% CI 0.51 to 0.65) [319]. Although these authors included eighty-one randomised clinical trials (RCTs) in the meta-analysis, only one of these enrolled elderly participants.

Additional evidence has shown that probiotics might have an impact on common causes of antibiotic-associated diarrhoea and antibiotic prescription in care homes [320, 321]. A systematic review studying the effect of probiotics for preventing acute upper respiratory tract infections (URTIs) found that probiotics were better than placebo in reducing the number of participants experiencing episodes of acute URTIs observed by

mean duration of an episode of acute URTI (mean difference in days (MD) -1.89; 95% CI -2.03 to -1.75, P < 0.001) although the authors considered the quality of evidence to be low [318]. There was also a reduced antibiotic prescription rate for acute URTIs (OR 0.65; 95% CI 0.45 to 0.94), with moderate-quality evidence [318]. Adults in the included studies were mainly aged around 40 y. The reason why the quality of the evidence reported in the RCTs included was generally low or very low was due to poorly conducted trials, unclear blinding and short follow-up.

The lack of understanding about immunosenescent processes in ECHRs and how these are affected or influenced by age, time-length of stay at home residence and frailty are starting to be examined. Describing and understanding these circumstances in care homes will help to suggest strategies to improve the quality of life across the ageing process and specifically in ECHRs. The description of relevant biogerontological conditions such as age, length of stay at care home and frailty in ECHRs, and their interaction with immune and inflammatory biomarkers is the basis for the research study that is described in this chapter.

Probiotics to reduce infections in care home residents (PRINCESS) is a two-arm double-blind individually-randomised controlled trial and is a collaborative study among three centres in the UK (Cardiff, Oxford and Southampton) [322]. Its main purpose was to test the effects of the oral consumption of a combination of two probiotic organisms on cumulative antibiotic administration days for all-cause infections and incidence and duration of infections in ECHRs. The immunology-sub-study conducted in ECHRs was the focus of this thesis and was a piece of research embedded within the PRINCESS trial to provide mechanistic understanding of the effects, if any, of the probiotic intervention. In addition, although the PRINCESS study did not modify vitamin D intake, this mechanistic sub-study also examined the association of immune and inflammatory factors with vitamin D status of the ECHRs; this is due to the promising evidence highlighting the relevance of vitamin D in non-classical pathways related to immune responses [323] and the frequent vitamin D deficiency observed in institutionalised individuals [324].

This chapter presents the characteristics of the participants enrolled in the PRINCESS clinical trial – immunology sub-study and how these are associated with conditions of immunosenescence by relating to assessment of frailty, age and time-length of residence in care homes. Furthermore, the immunology sub-study of the PRINCESS study provided an opportunity to investigate the association between vitamin D status and a range of biomarkers of immunity and inflammation in an elderly population living

in care homes in the UK. Due to the wide range of immunological and inflammatory biomarkers, an analysis of the association of these biomarkers with vitamin D status in the ECHRs community of PRINCESS – immunology sub-study, will also be presented. Maintaining the main target of this research thesis (the probiotic supplementation and immune mechanisms) the effects of the probiotic intervention will be described in chapters 4 and 5, and an *in vitro* model to explore even further the effects of these microorganisms in mechanisms related to immune defence will be presented from chapters 6 to 8.

3.1.1 Hypothesis

The hypothesis being tested is that immunosenescence and inflammatory biomarkers are associated with frailty, time-length of residence in care home and age.

3.1.2 Aim and objectives

The aim of this chapter is to characterise a community of elderly people residing in care homes and participating in the PRINCESS immunology sub-study trial. The objectives of the research herein presented are:

- To describe the characteristics of the participants included in the PRINCESS clinical trial – immunology sub-study through markers of immunosenescence and inflammageing.
- To describe the associations between frailty, length of time in care home and age with markers of immunosenescence and inflammageing.
- To analyse and interpret vitamin D status in elderly care home residents and relate vitamin D status to makers of immunity.

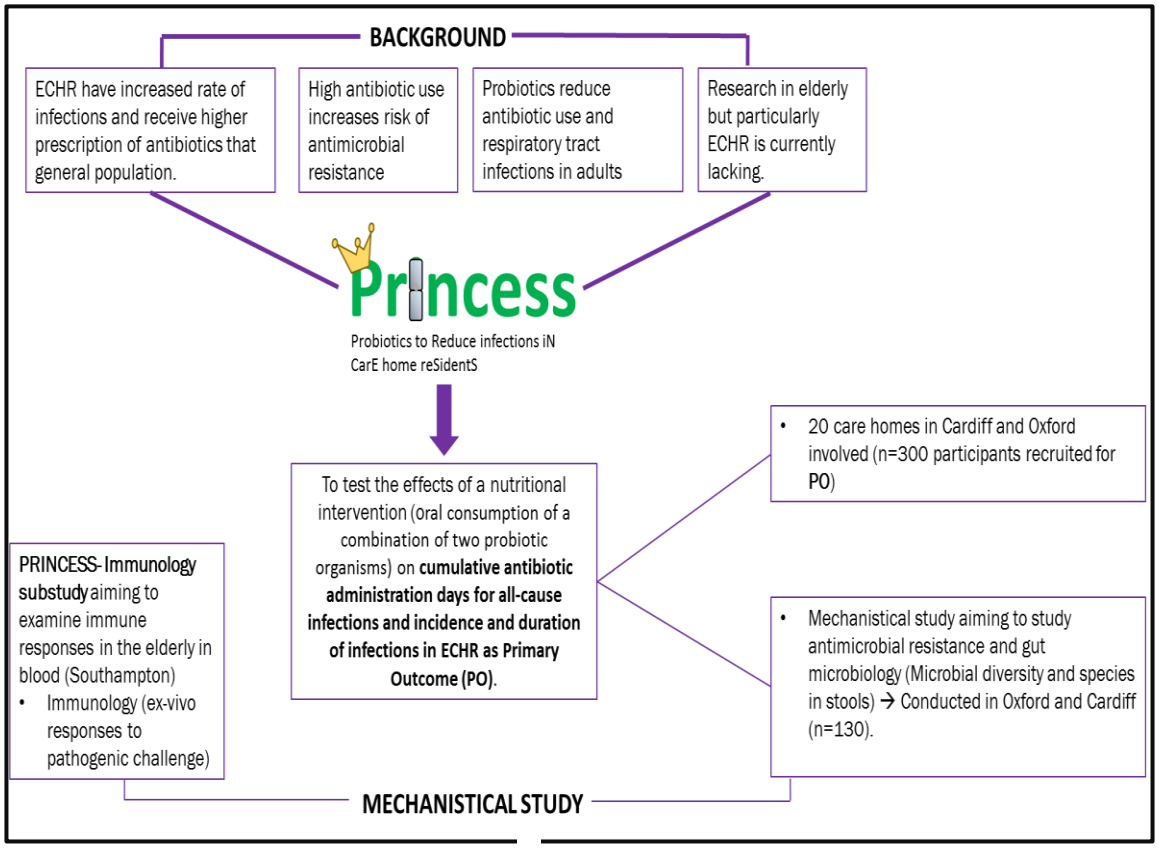
3.2 Methods

3.2.1 The PRINCESS trial

The PRINCESS study was approved by the Research Ethics Committee in Wales REC 3 on 23/10/2015 with reference 15/WA/0306 and was registered as ISRCT16392920. PRINCESS was funded by the National Institute for Health Research (NIHR) and was sponsored by Cardiff University. PRINCESS was a collaboration between Oxford, Cardiff and Southampton (See Figure 3.1).

PRINCESS was a randomized controlled-trial (RCT) investigating the effects of two probiotic strains (combined) versus placebo in ECHRs. The intervention was carried out in care homes in Oxford and Cardiff. Researchers in Oxford and Cardiff carried out the clinical assessments to identify whether possible participants met the inclusion and exclusion criteria (See section 3.2.2). Mechanistic analyses within this clinical trial comprise an assessment of stools, saliva and an immunology sub-study carried out using blood. These analyses allow to extend the understanding of the action of probiotics by correlating the findings with clinical findings at the end of the study. Both Cardiff and Oxford sent blood samples collected from the elderly participants to the University of Southampton where the PRINCESS-immunology sub-study herein described took place. Overall, PRINCESS trial is the first study aiming to assess a probiotic intervention in ECHRs and to integrate clinical outcomes with a deeper understanding of mechanisms involved in influencing clinical outcomes. Figure 3.1 illustrates the integrative process among the background that lead to developing the study and the relevance of the mechanistic study (immunology sub-study) embedded within the research architecture.

Figure 3.1. PRINCESS Clinical trial design and PRINCESS Clinical trial – Immunology sub-study



The flow diagram illustrates the rationale of the study and the relevance of the mechanistic approach. Cardiff and Oxford carry out separately microbiology analyses assessed in stools and saliva (e.g. gut microbiome, presence of oral candidiasis and amount of oral candidiasis). The PRINCESS clinical trial - immunology sub-study conducted in Southampton analysed blood samples which were collected in Cardiff and Oxford and were delivered to the university facilities as soon as possible. External entities specialised in clinical analyses were responsible for the full blood count and vitamin D measurements (Pathology Unit at Southampton General Hospital), and anti-vaccine antibodies (Public Health England Laboratory – London). Ex-vivo analyses were carried out within university facilities. PRINCESS – immunology sub-study was a proof of concept study that aimed to examine immunological pathways assessed in blood and to describe their modifications in comparison to the baseline on any clinical benefits seen and to correlate the findings herein presented with those findings derived from analyses conducted on stools (microbial diversity) and saliva (oral candidiasis). Participants were recruited over a year approximately, time in which baseline data was analysed and collected.

3.2.2 Subjects

Three hundred elderly residents (age > 65 y) from twenty care homes in Cardiff and Oxford were recruited by research nurses.

- Inclusion criteria: Participants living in care homes and providing direct informed consent or consent from a representative if without capacity.

- Exclusion criteria: Severely immunocompromised patients, current consumers of probiotics, and unwillingness to adapt to the study protocol. Temporary admission to the care home was also an exclusion criterion.

The sample size for the PRINCESS clinical trial was based on the finding that antibiotics are prescribed for an average of 17.4 days in ECHRs/year [320]. There was a targeted recruitment for 330 participants (165 per arm, 90% power and 5% significance) to detect a 10% relative reduction in cumulative antibiotic prescription days between arms (absolute reduction to 15.6 days). This number was also calculated based on 30% loss to follow-up due to withdrawal and death. The sample size for the mechanistic sub-study was 130. This was calculated based on the likelihood of obtaining stool, saliva and blood samples at 12 months and assuming that this sample size would provide 90% power at the 5% level of significance to detect a 19% absolute reduction in antimicrobial resistant bacteria and oral Candida; the calculation also assumed a 30% drop-out rate [322].

The immunology – sub-study aimed to recruit 130 participants at baseline and after 1 year of intervention with probiotics or placebo. An approximated drop-out rate of 25% to 30% participants was considered likely. The study aimed to provide immune data for 100 participants at both baseline and study end. The PRINCESS clinical trial – immunology sub-study recruited 184 participants at baseline, thus aiming to recruit 41% above the initial targeted sample size (n=130). Losses were higher than predicted and so the number of participants at a baseline was increased to try to overcome this. The sample size at baseline is different according to the variable under analysis as there were different losses encountered through the baseline recruitment. Section 3.3.2 contains the relevant information in this regard.

3.2.2.1 Anthropometric measurements and frailty assessment

Anthropometric assessment was carried by trained nurses who visited the care homes and were responsible for the assessment of the elderly residents. Middle upper arm circumference was taken as a more accurate measurement of nutritional status than body mass index. Measurement was taken considering humerus middle point. The Clinical Frailty Scale was used to assess frailty of the participants as shown in Table 3.1. This scale uses clinical descriptors and pictographs as a tool to stratify older adults according to the level of vulnerability and therefore frailty. This scale was previously validated in a sample of 2305 older adults from the Canadian Study of Health and Aging

[325]. The information was assessed individually considering the conditions of each participant and was also assessed in collaboration with the care givers.

Table 3.1. Clinical Frailty Scale used in PRINCESS clinical trial.

Category	Description
1- Very fit	Robust, active, energetic, well-motivated and fit. These people commonly exercise regularly and are in the fittest group for their age.
2- Well	Without active disease, but less fit than people in category 1.
3- Well, with treated comorbid disease	Disease symptoms are well controlled compared with those in category 4.
4- Apparently vulnerable	Although not frankly dependent, these people commonly complain of being "slowed up" or have disease symptoms.
5- Mildly frail	With limited dependence on others for instrumental activities of daily living.
6- Moderately frail	Help is needed with both instrumental and non-instrumental activities of daily living.
7- Severely frail	Completely dependent on others for the activities of daily living.
8- Very severely frail	Completely dependent and approaching the end of life.
9- Terminally ill	Life expectancy <6 months

Taken and adjusted from Rockwood *et al* [325].

3.2.3 Probiotic intervention

PRINCESS was a double blind, placebo-controlled, randomised trial of daily consumption of two probiotic organisms (*Lactobacillus rhamnosus GG and Bifidobacterium animalis* BB-12) for one year. The randomisation of participants to the two groups was performed by an external statistician. Participants, nurses and research staff in direct contact with participants and samples were blinded to allocation. The effects of the intervention on outcomes from the immunological sub-study will be presented in chapters 4 and 5.

Participants were asked to take an oral dose of the probiotic mixture (*Lactobacillus rhamnosus GG* and *Bifidobacterium animalis subsp. BB-12*) or a matched placebo once daily for 12 months. The probiotic or placebo (referred to as Study Product) was administered by the care home resident's normal care giver and provided in capsule form. The probiotic intervention contained a total of lactic acid bacteria of 1.3×10^{10} CFU per gram and was provided by Christian Hansen, Denmark. The capsule also contained maltodextrin (587 mg), microcrystalline cellulose (250 mg), silicon dioxide (20 mg) and magnesium stearate (5 mg). The placebo consisted of an opaque capsule containing 180 mg of maltodextrin, microcrystalline cellulose, magnesium stearate and silicon dioxide.

The preferred route of administration was swallowing the whole capsule with water; if that was not possible then the capsule was emptied into a small amount of cold or warm liquid and then swallowed. If neither of the previous options was possible, a final route of administration consisted of opening the capsule and sprinkling its contents onto cold or warm food (not hot food) and then consuming this. Adherence to the intervention was recorded by care home staff and monitored twice per week by research nurses visits.

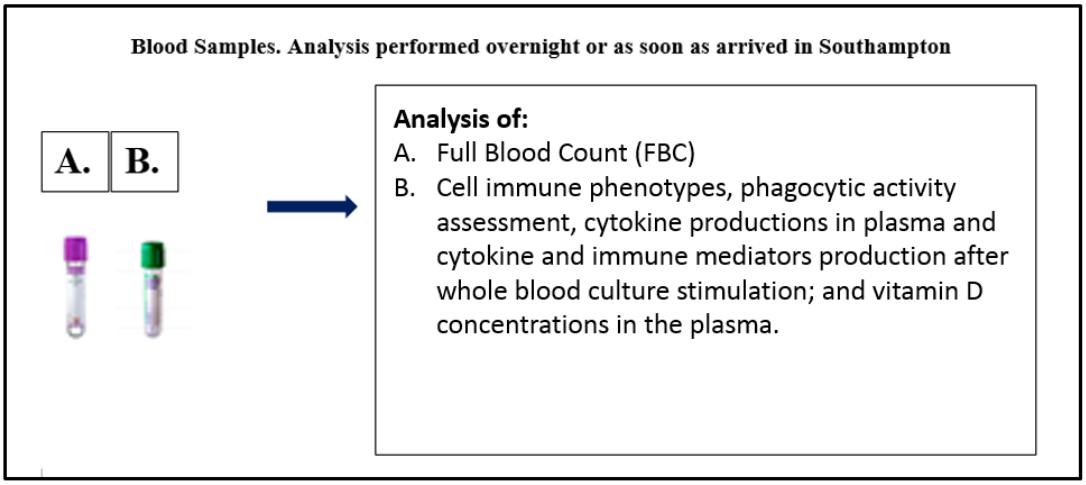
A coding system was also implemented to identify blood samples, so researchers were blinded. Every participant was given an identifier (ID composed of 5 digits) considering care home of recruitment. This code also included initials of the participant, and date of birth.

3.2.4 Blood sample handling – PRINCESS immunology sub-study

Blood samples from Oxford and Cardiff were sent by post to Southampton. They were expected to arrive the day after they were sent. Blood samples (6 ml) were collected into blood collection tubes containing either lithium heparin or ethylenediaminetetraacetic acid (EDTA). Blood samples for measurement of anti-influenza vaccine antibodies in serum were collected without anticoagulant. These samples were collected just prior to the administration of the seasonal influenza vaccination and around 30 days post-vaccination, (This information will be included and expanded in Chapter 4).

Full blood count and vitamin D levels were measured in the Southampton General Hospital, Pathology laboratory division according to the methodology presented in chapter 2 (See section 2.2.6). Seasonal influenza vaccine titres were measured at a Public Health England Laboratory in London (See section 2.2.5). Immune cell phenotypes, phagocytic assessment and whole blood cultures were performed in the facilities at the University of Southampton (See section 2.2.3.2 and 2.2.3.5, respectively). Results previously obtained from the pilot study (See section 2.5) allowed the identification of biomarkers which were not stable when blood processing was delayed after blood collection. Therefore, the day of sample collection and day of sample processing were carefully recorded to ensure that data were only collected within the established time window where the parameter was stable. Figure 3.2 presents the methods and procedures performed to analyse the blood sample collected from the participants recruited. The diagram includes a general scheme of the blood sample handling process.

Figure 3.2. Blood samples. Overview of blood sample collection and general methods performed in the PRINCESS clinical trial.



A. Contained EDTA as an anticoagulant and B. Lithium Heparin.

3.2.5 Experimental considerations

The methodology for blood processing in the PRINCESS clinical trial was established as part of the pilot study (see Chapter 2). There were minor adjustments to some techniques compared to the description in Chapter 2. Where there were differences, they will be described. Techniques that were not modified from the description given in Chapter 2 are not further described here. These include collection of the blood sample into heparin as anti-coagulant and its utilisation in the assessment of phagocytosis and whole blood cultures; the plasma and serum isolation for the study of cytokines, anti-influenza antibodies and vitamin D; and the collection of blood into EDTA as anticoagulant for full blood count (FBC).

The main differences between the pilot study and the PRINCESS clinical trial related to the transportation and delivery of the samples to the laboratory for the immunological analyses. There were also slight modifications in the panel designed and therefore the protocol followed in the identification of the immune cell subsets. Finally, the selection of stable cytokines and immune mediators analysed through the multiplex technique was modified based on the results found in the pilot study.

3.2.5.1 Flow cytometry: Immunophenotyping panel PRINCESS STUDY – immunology sub-study

Immune cell phenotyping used a slightly different panel for the staining with the inclusion of new fluorochromes; these differences consisted of the inclusion of PE-Cy5 and PE-Cy7 to allow a reduced number of tubes (outlooks for plotting were in

correspondence to those described in section 2.2.3.2.1 and the procedure was conducted accordingly). Table 3.2 shows the panel for the compensation. Finally, the parameters of compensation for the flow cytometer are presented in Table 3.3

Table 3.2. Flow cytometer compensation. Panel designed and used in PRINCESS main clinical trial – immunology sub-study

FACS Comp Tubes	Antibody stock label	Volume (ul)
1 - Unstained	Unstained	-
2 - FITC 488	CD4 AF488/FITC	5
3 – BV421	CD 127 BV421	5
4 – BV 605	CD8	5
5 – APC Alexa 647	CD3 AF647/APC	5
6 – PE	CD25/PE	20
7 – PE-Cy5	CD45	20
8 – PE-Cy7	CD14	5

Table 3.3. Parameters in the analysis of Flow cytometer.

Detector	Voltage
FSC	325
SSC	280
FITC	540
BV421	290
BV605	600
APC	630
PE	490
PE-Cy5	520
PE-Cy7	580

Subsequently, the description of the phenotypes targeted in the PRINCESS trial according to the panel designed for flow cytometry in the PRINCESS - immunology study is presented in Table 3.4 and Table 3.5. The process for immune phenotype gating was followed as described in Table 3.4. The outlook of the primary lymphocytic gated population is presented in Figure 3.5. $CD45^+$ was included as a marker within the staining panel as it presents in the immune cells of interest. Other than that, general procedures for compensation and gating in the analysis of the flow cytometry experiments were performed under the same conditions as described in the pilot study (See chapter 2).

Table 3.4. Panel design for staining and flow cytometry in the PRINCESS clinical trial.

Leukocyte subtype	Phenotypes of CD expressed.
T cells	$CD45^{+}/CD3^{+}$
Helper T cells	$CD45^{+}/CD3^{+}/CD4^{+}$
Regulatory T cells	$CD45^{+}/CD3^{+}/CD4^{+}/CD8^{+}/CD25^{+}/CD127^{+LO}$
Cytotoxic T cells	$CD45^{+}/CD3^{+}/CD8^{+}$
Activated cytotoxic T cells	$CD45^{+}/CD3^{+}/CD8^{+}/CD25^{+}$
B cells	$CD45^{+}/CD3^{-}/CD19^{+}$
Activated B cells	$CD45^{+}/CD3^{-}/CD19^{+}/CD80^{+}$
Activated B cells	$CD45^{+}/CD3^{-}/CD19^{+}/CD86^{+}$
Monocytes	$CD45^{+}/CD14^{+}$
Activated Monocytes	$CD45^{+}/CD14^{+}/CD80^{+}$
Activated Monocytes	$CD45^{+}/CD14^{+}/CD86^{+}$
NK cells	$CD45^{+}/CD3^{-}/CD16^{+}$

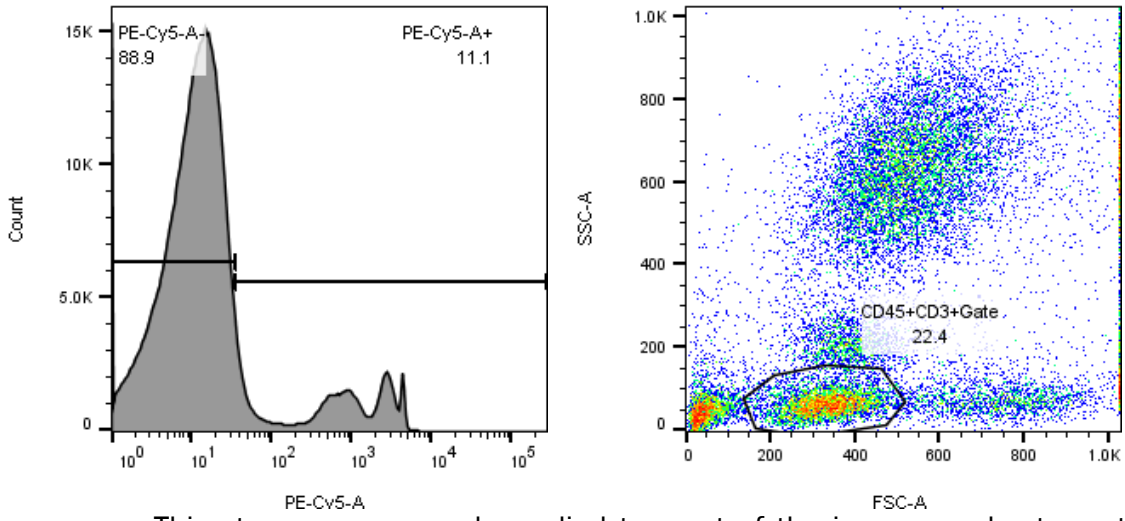
Table 3.5. Panel design and staining for the PRINCESS clinical trial.

Tube	Objective / Cell type	Stain and CD	Immunophenotype	Volume (µl) of antibody used per test
1 FACS	Negative	None	Negative control (without staining)	-
2 FACS	Isotype control	Multiple stain controls	Mouse IgG1 with PE, AF488, AF647, BV421 plus mouse IgG2a with AF488	20(PE) + 5(AF488)+ 5(PE-Cy7)+ 20(PE-Cy5)+ 5(AF647)+ 5(BV421)[1]+ 5(BV605)[2]
3 FACS	Isotype control	Multiple stain controls	CD3/CD4/Mouse IgG1 with PE/Mouse IgG1 with BV421/Mouse IgG1 with BV605/Mouse IgG1 with PE-Cy5	20(PE)+ 5(CD3)+ 5(CD4)+ 5(BV421)[1]+ 20(BV605)[2]+ 5(PE-Cy5)+ 50 (BSB)[3]
4 Trucount	T cells		CD3 (AF647) CD4 (AF488) CD25 (PE) CD127 (BV421) CD8 (BV605) CD45 (PE-Cy5)	5(CD3)+ 5(CD4)+ 20(CD25)+ 5(CD127)+ 5(CD8)+ 20(CD45)+ 50 (BSB) [3]
5 Trucount	B cells Monocytes NK cells		CD19 (AF488) / CD80 (BV421) / CD86 (PE) / CD45 (PE-Cy5) / CD16 (BV605)/ CD14 (PE-Cy7)	5(CD19)+ 20(CD80)+ 20(CD86)+ 20(CD45)+ 5(CD16)+ 5(CD14)+ 50 (BSB)[3]

1) 5 ul for diluted control. Diluted 1 in 4 with BD Pharmingen™ Stain
2) 5 ul for diluted control. Diluted 1 in 2 with BD Pharmingen™ Stain
3) Brilliant staining Buffer

The process used to perform the gating of the cells was followed as described in chapter 2. Adjustments included the addition of $CD45^+$ in the gating process of lymphocytes as described in Figure 3.3.

Figure 3.3. Flow cytometry results plotting for population $CD45^{+}CD3^{+}$. Lymphocytic gating in PRINCESS immunology sub-study



This step was commonly applied to most of the immune subsets as they express CD45+ and CD3+. In the case of Monocytes, B cells, and NK cells the protocol was followed by gating CD45+ and then as presented in chapter 2 (identifying correct fluorescently expressed staining) and following the panel designed as shown in Table 3.5.

3.2.5.2 Cytokine and other immune mediator concentrations: assessment in plasma and whole blood cultures

As described in section 2.2.4, protocols were followed accordingly. Limits of detection for this experiment are provided (the experiments conducted in this chapter included high sensitivity detection kits based on the outcomes from the pilot study). These values are provided in tables 3.6 and Tables 3.7. The list of cytokines and immune mediators included in the study correspond to those identified as stable in outcomes selected from the previous chapter (See section 2.5). The maximum timeframe for keeping the plasma samples under storage at -20°C and whole blood cultures supernatants at -80°C was 22 months (First participant recruited on January 2017, study concluded on October 2018, analyses took place in December 2018).

3.2.5.2.1 Cytokines and other immune mediators measured in plasma

Based upon the findings described in chapter 2, the cytokines and other immune mediators selected were those listed in the Table 3.6. Plasma aliquots isolated from the heparinised blood tube were kept at -20°C until the collection of samples at the post intervention timepoint was finished.

Table 3.6. Inflammatory parameters measured in plasma through Magnetic Luminex Assay and levels of detection.

Immune mediator	Lower limit of detection (pg/ml)
MCP-1	9.9
IP-10	1.18
ICAM-1	87.9
IL-1ra	18
IL-17	1.8
E selectin	18.8
VCAM-1	238
TNFR-II	0.5
TNF α (HS)	0.54
IL-10 (HS)	0.24
IL-12p70 (HS)	2.96
IL-6 (HS)	0.31

(HS) High sensitivity kit. Extended range of detection.

3.2.5.2.2 Cytokines and other immune mediators measured in stimulated whole blood cultures

Supernatants were stored at -80°C. Concentrations of cytokines and other immune mediators produced in response to stimulation with PHA, LPS and PGN were measured in the supernatants derived from the whole blood cultures and are listed in Table 3.7. Results from the stability study demonstrated that no immune mediators were stable beyond a one day delay in processing. Therefore results are shown for those immune mediators which were stable when processing occurred within one day after collection. Minimum detectable concentrations are shown.

Table 3.7. Immune mediators measured through Magnetic Luminex Assay as a result of negative and positive stimulations (24 hours incubation) of whole blood cultures with PHA, PGN and LPS.

Immune mediator	Lower limit of detection (pg/mL)
Analytes measured in negative controls and after stimulation with PGN and LPS	
TNF α	0.62
IL-10	2.93
IL-12p70	2.39
IL-6	0.38
IL-1 beta	0.25
Analytes measured in negative controls and after stimulation with PHA	
IFN-γ (HS)	0.4
TNF-α (HS)	1.2

(HS) High sensitivity detection kit.

3.2.6 Statistical analysis

Normality of data was assessed by visual inspection of histogram distributions and by using the Shapiro Wilk and Kolmogorov-Smirnov tests. Data were not normally distributed. Thus, data are presented using median, interquartile range and percentiles. The methodological design for the research herein presented is a cross-sectional study. Therefore the univariate analysis includes a descriptive analysis by characterising categorical variables in frequencies and percentages. The bivariate analysis includes the Kruskal-Wallis test and analyses of correlations between frailty, time-length residence in the care home and age. Where significant ($p<0.05$) differences were detected according to the Kruskal-Wallis test, box plot diagrams were used and statistical pairwise comparisons were performed among categories to test for significance. The final step was a multivariate analysis where linear regression models were used to expand the interaction among markers of *immunosenescence* with the variables of interest by testing the level of association among the dependent variables. Data collation and analysis were performed in SPSS version 22, Microsoft Excel and PRISM software. Data outside the appropriate time of arrival were considered missing data (immune mediators measured in plasma and from supernatants collected from whole blood cultures had a reduced sample size – n=33 due to timing of sample arrival).

3.3 Results

3.3.1 Characteristics of the participants

General characteristics of study participants at study entry are shown in Table 3.8. This analysis proceeds over the total of participants recruited which provided consent and were enrolled in the study (n=179). Any baseline differences between arms of intervention will be presented in chapters 4 and 5. The population had a mean±SD age of 85.3 y±(7.5) and the majority had been approximately over a year residing in the care home at the time of study commencement, mean±SD of years living at care home corresponded to 1.89 y±(2.16). Considering the middle upper arm circumference as an indicator of nutritional status, 27% of participants were below the threshold of 25 cm indicating a sub-optimum status. Body mass index (BMI) was not used as an indicator of nutritional status given difficulties to collect height with precision, and frail status in a great proportion of the population. were able to provide samples to be enrolled in the PRINCESS immunology sub-study.

Figure 3.4

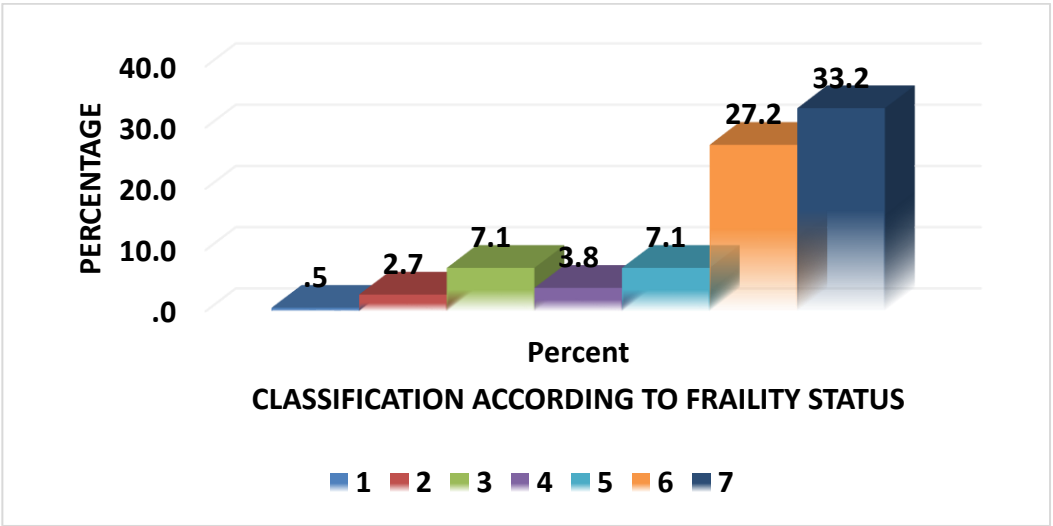
Table 3.8. Baseline characteristics of participants recruited into the PRINCESS - immunology sub-study.

Variable	Median	p10	p90
Age (y)	86.4	74.4	94.1
Time at care home residence (y)	1.2	0.2	4.2
Weight (kg)	64.0	47.8	88.6
Middle upper arm circumference (cm)	27.2	22.8	32.0

(n=184) participants initially consent their participation in this study. (y)= Years; Percentile (p) 10 and Percentile (p) 90

Subsequently, Figure 3.4 shows the relative distribution of Frailty index (n=151) at the level of progressive impairment physically and cognitively. Overall, declined activities were observed in 59% of the population but there were no terminally ill subjects (categories 8 and 9 in the frailty scale) were able to provide samples to be enrolled in the PRINCESS immunology sub-study.

Figure 3.4. Relative distribution of participants according to frailty status.



Frailty score is ranked as follows: **1 = *Fittest category for their age* (n=1):** Active and energetic; **2 = *Well* (n=5)**: Absent symptomatology of disease but less active; **3 = Managing well (n=13):** Medical problems under control but not active; **4 = Vulnerable (n=7):** Symptoms that limit activities; **5 = Mildly frail (n=13)**: Impairment of daily activities; **6 = Moderately frail (n=50):** Progressive impairment and declined activities); **7 = Severely frail (n=61):** Completely dependent cognitively or physically. Not terminally ill or participants beyond scale 8 were recruited into the immunology sub-study.

3.3.1.1 Vitamin D status in the ECHRs

Median values and IQR of plasma vitamin D according to classification is as follows: Osteomalacia (Median: 8.5 nmol/l – IQR:17); Proximal myopathy (Median: 12 nmol/l – IQR:24); "Deficiency" (Median: 39 nmol/l – IQR:20), "Insufficiency" (Median: 41 nmol/l – IQR:11), and "Sufficiency" (Median: 61 nmol/l – IQR:37). The results of the vitamin D categorisation in ECHRs according to age participating in the PRINCESS immunology sub-study are presented in Table 3.9.

Table 3.9. Plasma concentrations of 25(OH) Vitamin D categorisation according to age classification in ECHRs participating in PRINCESS immunology sub-study.

	Vitamin D classification (%)					
Age	Osteomalacia	Proximal myopathy	Deficiency	Insufficiency	Sufficiency	Total
65 - 79 y old	0	3	9	1	7	**20**
80 - 89 y old	2	4	20	4	18	**49**
> 90 y old	2	4	8	5	11	**31**
Total	**4**	**11**	**38**	**11**	**36**	**100**

Categories used to classify vitamin D status were established according to Lips and the establishment of more strict diagnostic criteria in the elderly. The suggested categorisation is as follows: "Osteomalacia" (>10nmol/l), "Proximal myopathy" (10-12 nmol/l). These diagnoses can also be grouped within "Deficiency" (<25nmol/l), "Insufficiency" (25 and 50 nmol/l), and "Sufficiency" (>50 nmol/l) [326]

3.3.2 Blood Sample analyses: Baseline characterisation of the population recruited in PRINCESS trial immunology sub-study. Immune and inflammatory biomarkers

3.3.2.1 Preliminary considerations: sample availability and sample size

A total of 184 participants were consented to participate and therefore were recruited in the baseline for PRINCESS clinical trial – immunology sub-study. Participants included within the immunology sub-study consented to provide blood, saliva and stools as part of the main clinical trial. Southampton only received, processed and analysed blood. From these 184 participants, it was not possible to obtain baseline blood sample for 4 participants (blood samples were available only post-intervention), and so these participants were excluded from the study. 1 participant provided the sample but then asked to destroy the material collected. These losses of 2.7% led to 179 participants

being included within the baseline count. Subsequently, 6.6% further reduction occurred due to late sample arrival. Table 3.10 shows frequency of arrival of blood samples in Southampton. According to the findings from the pilot study, samples arriving outside 3 days after collection were disposed of.

Table 3.10. Time-frame and frequency of blood sample arrival in Southampton

Arrival: day post sample collection	Frequency	Percent
1	114	63.7
2	47	26.3
3	6	3.4
Total (Usable samples)	**167**	**93.4**
>4	12	6.6
Total	179	100.0

Subsequently, within the recruited participants and those whose blood sample was sent and arrived in Southampton within optimum time-frame (n=167) there was difficulty in bleeding 19 participants (11.4%), thus leading to a lower volume of sample provided and limiting some of the analyses. EDTA collector tube used for FBC analyses was obtained for n=153, whereas for the heparinised blood collector tube enough volume to conduct all of the analyses was available from fewer participants (n=148). Final sample size is presented accordingly from tables 3.11 to table 3.15. This section will indicate sample availability, and the descriptive statistics. Sample size also includes sample viability according to outcomes presented in chapter 2.

3.3.2.2 Full Blood Count (FBC) in elderly care home residents

Variables considered in the FBC analyses were neutrophils, lymphocytes, monocytes, eosinophils, basophils, total white cells (white cell count: WCC) and platelets as shown in table 3.11. Reference values as well as their clinical relevance are presented in table 2.1 in chapter 2. Overall, participants in the immunology sub-study exhibited a normal FBC according to the reference values used for the population.

Table 3.11. Descriptive statistics of the full blood count results for participants in the immunology sub-study

Variable	n (Samples available)	Median	p10	p90
		FBC (10*9/l)		
Neutrophils	151	4.5	2.90	7.2
Lymphocytes	157	1.6	0.9	2.5
Monocytes	158	0.6	0.3	0.9
Eosinophils	153	0.1	0.1	0.3
Basophils	153	0.1	0	0.1
White cell count	109	7.4	5.1	10.5
Platelets	158	268	191	390

Descriptive statistics is shown using median and percentile (p)= p10 and p90. n values correspond to sample availability considering usability (delayed processing – as shown in Table 2.15)

3.3.2.3 Immunophenotypes in elderly care home residents

The panel designed for PRINCESS immunology sub-study lead to the characterisation of 13 immune cell lymphocytic populations and monocytes with their activated state. Results are presented in Table 3.12.

Table 3.12. Descriptive statistics of immunophenotypes in the blood of participants in the immunology sub-study

Variable	n (Samples available)	Median	p10	p90
		Immune cell phenotypes (cells/µl)		
T cells	148	1249	875	1726
T cytotoxic	148	648	402	1005
Activated T cytotoxic	142	224	126	367
T regs	148	40	16	191
T helper	148	859	304	1391
Ratio $CD4^+$:$CD8^+$	148	1.3	1	1.8
Monocytes	148	500	255	820
Monocytes Activated ($CD80^+$)	148	152	36	379
Monocytes Activated ($CD86^+$)	148	106	20	275
NK cells	98	81	49	116
B cells	148	221	102	342
B cells Activated ($CD80^+$)	148	119	68	213
B cells Activated ($CD86^+$)	148	118	72	220

Descriptive statistics is shown using median and percentile (p)= p10 and p90. n values correspond to sample availability considering usability (delayed processing – as shown in Table 2.15)

3.3.2.4 Phagocytic function in elderly care home residents

The phagocytic function of neutrophils and monocytes is presented as the percentage of cells engaging in phagocytosis (gated as percentage) and the number of bacteria ingested per active phagocyte (the geometric median fluorescence intensity). Table 3.13 shows the descriptive findings for these parameters.

Table 3.13. Descriptive statistics of phagocytosis by neutrophils and monocytes from the blood of participants in the immunology sub-study

Variable	n (Samples available)	Median	p10	p90
Percentage of neutrophils gated with phagocytic activity	147	83.9	64.6	91.6
Geometric median fluorescence intensity (GMFI) of active neutrophils	142	256.8	158.6	378.5
Percentage of monocytes gated with phagocytic activity	147	29.9	13.6	47.9
Geometric median fluorescence intensity (GMFI) of active monocytes	147	182.1	105.9	295.9

Descriptive statistics is shown using median and percentile (p)= p10 and p90. n values correspond to sample availability considering usability (delayed processing – as shown in Table 2.15)

3.3.2.5 Immune mediators measured in plasma in elderly care home residents

The sample size of the inflammatory panel was significantly reduced due to instability of the immune mediators measured in plasma (as indicated in chapter 2). Therefore table 3.14 reflects outcomes and findings for the samples arriving within the established time range.

Table 3.14. Descriptive statistics of immune mediators in plasma from the participants in the immunology sub-study

Variable	n (Samples available)	Median	p10	p90
ICAM-1 (ng/ml)	95	385.9	207.6	763.5
IL-1ra (pg/ml)	95	1558.9	705.1	4644.4
E-Selectin (ng/ml)	95	22.8	11.3	39.8
VCAM-1 (ng/ml)	95	791.2	431.6	1390.8
MCP-1 (pg/ml)	95	355.5	165.0	690.6
IP-10 (pg/ml)	95	151.7	74.6	284.5
IL-17A (pg/ml)	95	0.9	0.6	6.9
TNFRII (pg/ml)	95	4072.4	2118.9	7963.1
IL-6 (pg/ml)	96	4.4	1.7	20.4
IL-10 (pg/ml)	96	0.6	0.1	1.8
TNF-α (pg/ml)	96	17.7	9.2	26.4

Descriptive statistics is shown using median and percentile (p)= p10 and p90. n values correspond to sample availability considering usability (delayed processing – as shown in Table 2.15)

3.3.2.6 Immune mediators measured in whole blood cultures in elderly care home residents

Whole blood cultures were affected by prolonged blood storage (Chapter 2). Additionally, when the volume of the blood sample collected from the participant was significantly less than intended a prioritisation protocol was used which further reduced the sample size for these outcomes. The supernatant collected from whole blood cultures was used to determine the production of IL-10, TNF-α, IL-6, IL-12p70, IL-1β following stimulation with LPS or PGN as described in chapter 2. Additionally, the production of IFN-γ and TNF-α was measured following the whole blood stimulation with PHA. Results are presented in table 3.15.

Table 3.15. Descriptive statistics of immune mediators in cultures of whole blood from participants in the immunology sub-study

Variable	n (Samples available)	Median	p10	p90
Immune mediators measured in whole blood cultures – LPS stimulation				
IL-10 (pg/ml)	86	2428	473	10780
TNF-α (pg/ml)	86	13231	3358	32884
IL-6 (ng/ml)	86	47.6	15.7	87.2
IL-12p70 (pg/ml)	86	24.9	11.6	118.7
IL-1β (pg/ml)	86	4090	1476	14588
Immune mediators measured in whole blood cultures – PGN stimulation				
IL-10 (pg/ml)	86	468	90	2049
TNF-α (pg/ml)	86	3391	564	11334
IL-6 (ng/ml)	86	42.4	11.9	100.6
IL-12p70 (pg/ml)	86	14.3	5.3	64.0
IL-1β (pg/ml)	86	318	29	1448
Immune mediators measured in whole blood cultures – PHA stimulation				
IFN-γ (pg/ml)	86	5.2	0.2	55.1
TNF-α (pg/ml)	86	1846	658	3472

Descriptive statistics is shown using median and percentile (p)= p10 and p90. n values correspond to sample availability considering usability (delayed processing – as shown in Table 2.15)

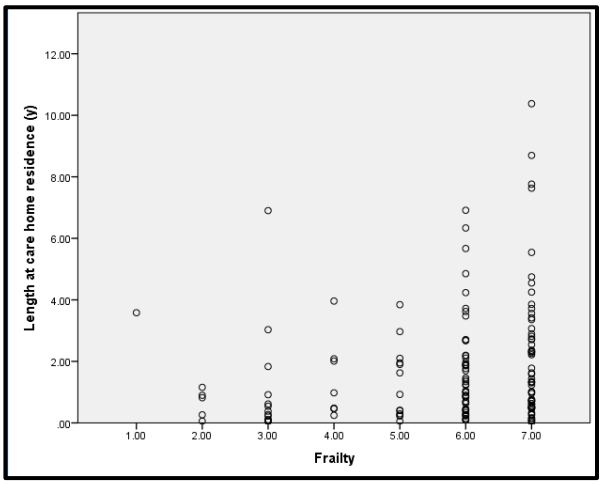
3.3.3 Cross-sectional analyses investigating the relationship between immune biomarkers and age, frailty and length of stay in the care home

This section presents the results by considering the following main variables: age as current indicator of ageing process calculated as y from date of birth, frailty calculated according to the clinical frail scale and length of stay as y of residence in the care home at the time of recruitment. There was a significant positive correlation between length of stay at care home residence and frailty (Person correlation=0.168; p=0.041). Age was not significantly correlated with either length of stay at care home residence or frailty. These results are presented in the bivariate analyses section 3.3.3.1 in Figure 3.5 to Figure 3.7 .

3.3.3.1 Bivariate analyses: correlations

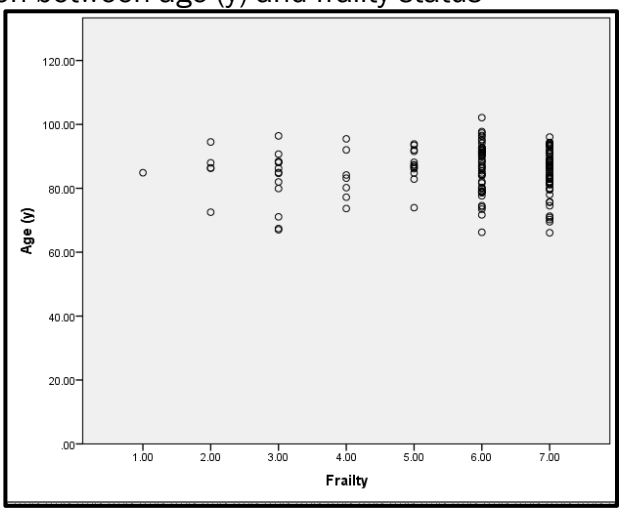
There was a significant positive correlation between length of stay in the care home and frailty (Pearson correlation=0.168; p=0.041) (Figure 3.5). Age was not significantly correlated with either length of stay in the care home residence or frailty (See Figure 3.6 and Figure 3.7).

Figure 3.5. Correlation between length at care home residence (y) and frailty status



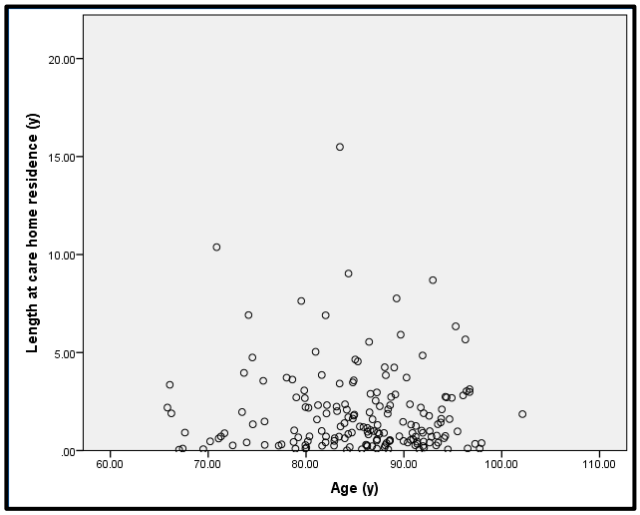
Frailty score (n=151) is ranked as follows: 1 = Fittest category for their age (active and energetic); 2 = Well (absent symptomatology of disease but less active); 3 = Managing well (medical problems under control but not active); 4 = Vulnerable (symptoms that limit activities); 5 = Mildly frail (impairment of daily activities); 6 = Moderately frail (progressive impairment and declined activities); 7 = Severely frail (completely dependent cognitively or physically. Not terminally ill). Pearson correlation=0.168; p=0.041.

Figure 3.6. Correlation between age (y) and frailty status



Age (n=167) was collected at the baseline when the participant was recruited and data is analysed in y. Frailty score is ranked as follows: 1 = Fittest category for their age (active and energetic); 2 = Well (absent symptomatology of disease but less active); 3 = Managing well (medical problems under control but not active); 4 = Vulnerable (symptoms that limit activities); 5 = Mildly frail (impairment of daily activities); 6 = Moderately frail (progressive impairment and declined activities); 7 = Severely frail (completely dependent cognitively or physically. Not terminally ill). Pearson correlation=0.056; p=0.497.

Figure 3.7. Correlation between length at care home residence (y) and age (y)



Length of stay at care home was recorded as months or years from starting to live in care home (n=167). Pearson correlation=-0.049; p=0.522

3.3.3.2 Multivariate analyses: linear regression model

Following the bivariate analyses described in the previous section, a linear regression model was used to analyse the contribution of age, frailty and length of residence in the care home to the various immune parameters as the dependent variables. To fit the regression model it was necessary to log transform the variables. Frailty was a significant associated factor to platelets measured through FBC (Adjusted mean: 0.51; 95% CI: 0.18 to 0.85; p<0.01). Other than that, none of the log transformed variables was found to contribute significantly to the individual components of the FBC or the immune cell phenotypes. There were no associations among the phagocytic activity and the variables under examination, as shown from table 3.16 to table 3.18. In contrast, log transformed age, frailty and length of residence in the care home contributed to the concentrations of immune mediators measured in plasma or produced in whole blood cultures (table 3.19, and table 3.20).

Age was significantly associated with monocytic activation measured through expression of CD86 (Adjusted mean: 2.78; 95% CI: 0.869 to 4.699; p<0.01), IP-10 (Adjusted mean: 1.77; 95% CI: 0.61 to 2.93; p<0.01) and TNFRII (Adjusted mean: 1.76; 95% CI: 0.61 to 2.93; p<0.01). Frailty was a significant contributing factor to log transformed IL-1ra (Adjusted mean: 0.43; 95% CI: 0.001 to 0.867; p=0.05), E-selectin (Adjusted mean: 0.35; 95% CI: 0.049 to 0.66; p=0.024), MCP-1 (Adjusted mean: 0.23; 95% CI: -0.08 to 0.55; p=0.026) and IP-10 (Adjusted mean: 0.32; 95% CI: 0.012 to

0.636; p=0.042). Furthermore, log transformed frailty was also a significant contributor to log transformed IL-10 production as a result of PGN stimulation of whole blood cultures (Adjusted mean:-0.79; 95%CI: -1.53 to -0.045; p=0.038). Lastly, log transformed length of stay in the care home was a contributing factor to the plasma concentration of MCP-1 (Adjusted mean:-0.10; 95% CI: 0.013 to 0.189; p=0.026).

Table 3.16 Linear regression model for age, length in the care home and frailty to predict the components of FBC in ECHRs in PRINCESS-immunology sub-study

Immune parameter	Covariates	Adjusted mean	95% Confidence Interval		p-value
			Lower Bound	Upper Bound	
Neutrophils (n=151)	Age	0.109	-0.608	0.826	0.764
	Length in care home	-0.044	-0.097	0.008	0.098
	Frailty	0.049	-0.144	0.242	0.616
Lymphocytes (n=157)	Age	-0.114	-1.002	0.775	0.801
	Length in care home	-0.021	-0.086	0.044	0.530
	Frailty	-0.057	-0.296	0.181	0.636
Monocytes (n=158)	Age	-0.279	-0.984	0.425	0.435
	Length in care home	-0.029	-0.081	0.022	0.263
	Frailty	-0.046	-0.236	0.143	0.628
WBC (n=109)	Age	-0.111	-0.787	0.564	0.744
	Length in care home	-0.043	-0.093	0.006	0.087
	Frailty	0.068	-0.113	0.250	0.456
Platelets (n=158)	Age	0.359	-0.877	1.595	0.567
	Length in care home	-0.044	-0.135	0.046	0.336
	Frailty	0.520	0.188	0.852	**0.002**

Variables were log transformed to fit the regression model. Missing data were adjusted in the model to be analysed pairwise (analysis to be run on cases where there is data). Covariables age, length in care home and Frailty were considered independent variables. Parameter under examination was considered dependent variable. Model identifies the effect of independent variables on the variable under analysis and method followed was “enter” where independent variables are analysed simultaneously. Lack of significance p>0.05. Bold indicates significance.

Table 3.17 Linear regression model for age, length in the care home and frailty to predict immune cell phenotypes in ECHRs in PRINCESS-immunology sub-study

Immune parameter	Covariates	Adjusted mean	95% Confidence Interval		p-value
			Lower Bound	Upper Bound	
T cells (n=148)	Age	-0.140	-0.632	0.352	0.574
	Length in care home	0.008	-0.028	0.044	0.660
	Frailty	-0.009	-0.141	0.123	0.897
T cytotoxic (n=148)	Age	0.360	-0.350	1.071	0.318
	Length in care home	-0.027	-0.079	0.025	0.315
	Frailty	0.124	-0.067	0.315	0.201
Activated T cytotoxic (n=142)	Age	-0.283	-1.229	0.664	0.556
	Length in care home	0.036	-0.033	0.105	0.307
	Frailty	0.155	-0.099	0.409	0.229
T regs (n=148)	Age	0.834	-0.787	2.455	0.311
	Length care home	-0.089	-0.208	0.030	0.141
	Frailty	0.078	-0.358	0.513	0.724
T helper (n=148)	Age	0.702	-0.435	1.839	0.224
	Length in care home	-0.028	-0.111	0.056	0.515
	Frailty	0.208	-0.097	0.513	0.180
Ratio $CD4^+$:$CD8^+$ (n=148)	Age	0.471	-0.363	1.304	0.266
	Length in care home	-0.006	-0.067	0.055	0.853
	Frailty	0.120	-0.104	0.344	0.293
Monocytes (n=148)	Age	0.028	-0.800	0.855	0.948
	Length in care home	-0.021	-0.081	0.040	0.502
	Frailty	0.017	-0.206	0.239	0.881
Monocytes $CD80^+$ (n=148)	Age	1.309	-0.444	3.062	0.142
	Length in care home	-0.065	-0.194	0.063	0.317
	Frailty	0.175	-0.296	0.646	0.463
Monocytes $CD86^+$ (n=148)	Age	2.784	0.869	4.699	**0.005**
	Length in care home	-0.034	-0.175	0.106	0.628
	Frailty	-0.213	-0.727	0.302	0.415
NK cells (n=98)	Age	-0.021	-0.812	0.770	0.958
	Length in care home	-0.016	-0.075	0.042	0.574
	Frailty	0.035	-0.177	0.248	0.742
B cells (n=148)	Age	-0.066	-0.911	0.778	0.877
	Length in care home	0.048	-0.014	0.110	0.130
	Frailty	-0.123	-0.350	0.104	0.285
B cells CD80+ (n=148)	Age	0.438	-0.366	1.242	0.284
	Length in care home	0.018	-0.041	0.077	0.542
	Frailty	-0.139	-0.355	0.077	0.204
B cells CD86+ (n=148)	Age	0.062	-1.216	1.341	0.923
	Length in care home	0.025	-0.069	0.118	0.603

Frailty	-0.185	-0.529	0.158	0.288

Variables were log transformed to fit the regression model. Missing data were adjusted in the model to be analysed pairwise (analysis to be run on cases where there is data). Covariables age, length in care home and Frailty were considered independent variables. Parameter under examination was considered dependent variable. Model identifies the effect of independent variables on the variable under analysis and method followed was “enter” where independent variables are analysed simultaneously. Lack of significance $p>0.05$.

Table 3.18 Linear regression model for age, length in the care home and frailty to predict phagocytic activity in ECHRs in PRINCESS-immunology sub-study

Immune parameter	Covariates	Adjusted mean	95% Confidence Interval		p-value
			Lower Bound	Upper Bound	
Phagocytic Activity (%) Neutrophils (n=147)	Age	-0.083	-0.495	0.330	0.693
	Length in care home	-0.016	-0.046	0.014	0.300
	Frailty	-0.037	-0.148	0.074	0.510
Phagocytic Activity (GMFL) Neutrophils (n=142)	Age	0.144	-0.459	0.747	0.637
	Length in care home	0.024	-0.020	0.068	0.290
	Frailty	0.035	-0.127	0.197	0.669
Phagocytic Activity (%) Monocytes (n=147)	Age	0.351	-0.610	1.312	0.472
	Length in care home	0.002	-0.069	0.072	0.959
	Frailty	-0.132	-0.390	0.126	0.314
Phagocytic Activity (GMFL) Neutrophils (n=147)	Age	0.160	-0.595	0.916	0.675
	Length in care home	0.015	-0.041	0.070	0.602
	Frailty	-0.120	-0.323	0.083	0.246

Variables were log transformed to fit the regression model. Missing data were adjusted in the model to be analysed pairwise (analysis to be run on cases where there is data). Covariables age, length in care home and Frailty were considered independent variables. Parameter under examination was considered dependent variable. Model identifies the effect of independent variables on the variable under analysis and method followed was “enter” where independent variables are analysed simultaneously. Lack of significance p>0.05

Table 3.19 Linear regression model for age, length in the care home and frailty to predict plasma immune mediators ECHRs in PRINCESS-immunology sub-study

Immune parameter	Covariates	Adjusted mean	95% Confidence Interval		p-value
			Lower Bound	Upper Bound	
ICAM-1 (n=95)	Age	0.185	-1.013	1.382	0.760
	Length in care home	0.024	-0.064	0.112	0.589
	Frailty	0.197	-0.124	0.519	0.226
IL-1ra (n=95)	Age	0.275	-1.338	1.889	0.735
	Length in care home	0.085	-0.034	0.203	0.158
	Frailty	0.434	0.001	0.867	**0.050**
E Selectin (n=95)	Age	0.252	-0.887	1.391	0.661
	Length in care home	0.016	-0.067	0.100	0.697
	Frailty	0.355	0.049	0.661	0.024
MCP-1 (n=95)	Age	-0.400	-1.601	0.800	0.510
	Length in care home	0.101	0.013	0.189	**0.026**
	Frailty	0.235	-0.088	0.557	0.152
IP-10 (n=95)	Age	1.772	0.610	2.933	**0.003**
	Length in care home	0.066	-0.019	0.151	0.126
	Frailty	0.324	0.012	0.636	**0.042**
TNF-RII (n=95)	Age	1.762	0.601	2.924	**0.003**
	Length in care home	0.007	-0.078	0.092	0.874
	Frailty	0.122	-0.190	0.434	0.440
IL-6 (n=96)	Age	-0.601	-2.949	1.748	0.613
	Length in care home	0.091	-0.081	0.263	0.296
	Frailty	-0.452	-1.083	0.179	0.158

IL-10 (n=96)	Age	0.568	-1.805	2.940	0.636
	Length in care home	0.149	-0.024	0.323	0.091
	Frailty	-0.398	-1.035	0.240	0.218
TNF-α (n=96)	Age	1.183	-0.338	2.705	0.126
	Length in care home	0.042	-0.069	0.154	0.452
	Frailty	-0.095	-0.503	0.314	0.646
VCAM-1 (n=95)	Age	1.193	0.128	2.257	**0.029**
	Length in care home	0.021	-0.057	0.099	0.595
	Frailty	0.094	-0.192	0.380	0.516

Variables were log transformed to fit the regression model. Missing data were adjusted in the model to be analysed pairwise (analysis to be run on cases where there is data). Covariables age, length in care home and Frailty were considered independent variables. Parameter under examination was considered dependent variable. Model identifies the effect of independent variables on the variable under analysis and method followed was “enter” where independent variables are analysed simultaneously. Lack of significance $p>0.05$. Bold indicates significance.

Table 3.20 Linear regression model for age, length in the care home and frailty to predict immune mediators produced in whole blood cultures in ECHRs in PRINCESS-immunology sub-study

Immune parameter	Covariates	Adjusted mean	95% Confidence Interval		p-value
			Lower Bound	Upper Bound	
PGN IL-10 (n=86)	Age	0.058	-2.724	2.839	0.967
	Length in care home	-0.009	-0.213	0.195	0.929
	Frailty	-0.792	-1.539	-0.045	**0.038**
PGN TNF-α (n=86)	Age	0.728	-2.022	3.478	0.600
	Length in care home	0.003	-0.198	0.205	0.975
	Frailty	-0.353	-1.092	0.386	0.345
PGN IL-6 (n=86)	Age	1.182	-1.278	3.642	0.341
	Length in care home	0.036	-0.144	0.216	0.691
	Frailty	-0.275	-0.936	0.386	0.410
PGN IL1beta (n=86)	Age	1.589	-2.124	5.303	0.398
	Length in care home	0.043	-0.229	0.315	0.755
	Frailty	-0.892	-1.890	0.105	0.079
LPS IL-10 (n=86)	Age	0.521	-2.251	3.294	0.710
	Length in care home	0.010	-0.193	0.214	0.920
	Frailty	-0.105	-0.849	0.640	0.781
LPS TNF-α (n=86)	Age	0.445	-1.501	2.391	0.651
	Length in care home	0.035	-0.108	0.177	0.631
	Frailty	0.290	-0.233	0.813	0.273
LPS IL-6 (n=86)	Age	0.931	-0.696	2.558	0.258
	Length in care home	0.003	-0.116	0.123	0.956
	Frailty	0.079	-0.358	0.517	0.718

LPS IL12p70 (n=86)	Age	0.250	-1.797	2.296	0.809
	Length in care home	0.029	-0.121	0.179	0.699
	Frailty	-0.229	-0.778	0.321	0.411
LPS IL1beta (n=86)	Age	1.691	-0.690	4.072	0.161
	Length in care home	0.026	-0.149	0.200	0.772
	Frailty	0.188	-0.452	0.827	0.560
PHA IFN-γ (n=86)	Age	1.433	-3.707	6.572	0.581
	Length in care home	0.180	-0.197	0.557	0.345
	Frailty	0.058	-1.323	1.438	0.934
PHA TNF-α (n=86)	Age	0.113	-1.433	1.658	0.885
	Length in care home	-0.037	-0.151	0.076	0.516
	Frailty	0.018	-0.397	0.433	0.930

Variables were log transformed to fit the regression model. Missing data were adjusted in the model to be analysed pairwise (analysis to be run on cases where there is data). Covariables age, length in care home and Frailty were considered independent variables. Parameter under examination was considered dependent variable. Model identifies the effect of independent variables on the variable under analysis and method followed was “enter” where independent variables are analysed simultaneously. Lack of significance p>0.05. Bold indicates significance.

3.3.3.3 Categorical classification of variables and statistical analysis across categories

Results presented in this section are an examination of age and length of stay at care home as categorical data and their relationship with the measured immune parameters. These variables followed a non-parametric distribution so therefore the Kruskal-Wallis test was used to analyse the behaviour of the immune outcomes of interest across categories of variables of interest. Age was categorised into three groups: “65” which comprises younger old participants from 65 y old inclusive until 79 y old; “80” which comprises octogenarian participants in their eighth decade of life and “90” which comprises participants in their ninth decade of life and one centenarian participant that was grouped within this category. Length of stay in care home was categorised as “0.5” for those participants residing at the care home for less than 6 months; “1” and “2” for participants residing 1 and 2 y respectively and “3” for participants residing 3 and 4 y due to the number of participants residing with this length of time; and lastly “5” was used to group participants living for 5 y or more at the care home. Results of these analyses are presented from table 3.21 to table 3.25. Box plots are also used to illustrate the behaviour of the variable across categories.

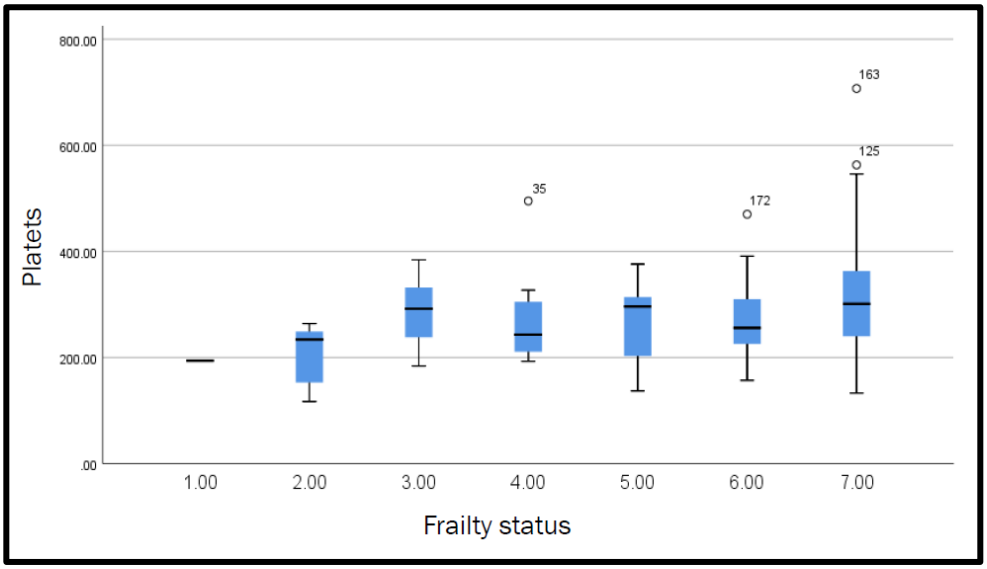
In the examination of the FBC it was observed that frailty was significantly associated with platelet numbers as shown in table 3.20. Figure 3.8 shows a progressive increase in platelet numbers with worsening of frailty status. No other FBC parameters were found to be significantly correlated with age, frailty or length in the care home.

Table 3.21 P values for the relationship between components of FBC and categories of age, frailty status and length in the care home in ECHRs in the immunology sub-study

Variable	Age	Frailty	Length in care home
Neutrophils	0.894	0.527	0.125
Lymphocytes	0.404	0.410	0.404
Monocytes	0.083	0.543	0.290
Platelets	0.813	0.024	0.847
WBC	0.797	0.145	0.347

Categorical data Age, Frailty and Length in care home were analysed across continuous data described as variable, using Kruskal-Wallis test. Significance is shown using p values<0.05. Sample size is shown in section 3.3.3.2.

Figure 3.8 Box plot of platelet numbers (Platelets; 10*9/l) across categories of frailty



Platelets and Frailty status (n values given according to categories of Frailty) – [“1“, n=1; “2“, n=5; “3“, n=13 ; “4“, n=7 ; “5“, n=13 ; “6“, n=46; “7“, n=68]. Kruskal-Wallis test p=0.024. Significantly different categories were Frail status 2 “Well” and 7 “Severely frail” (p=0.009) and 6 “Moderately frail” and 7 (p=0.01).

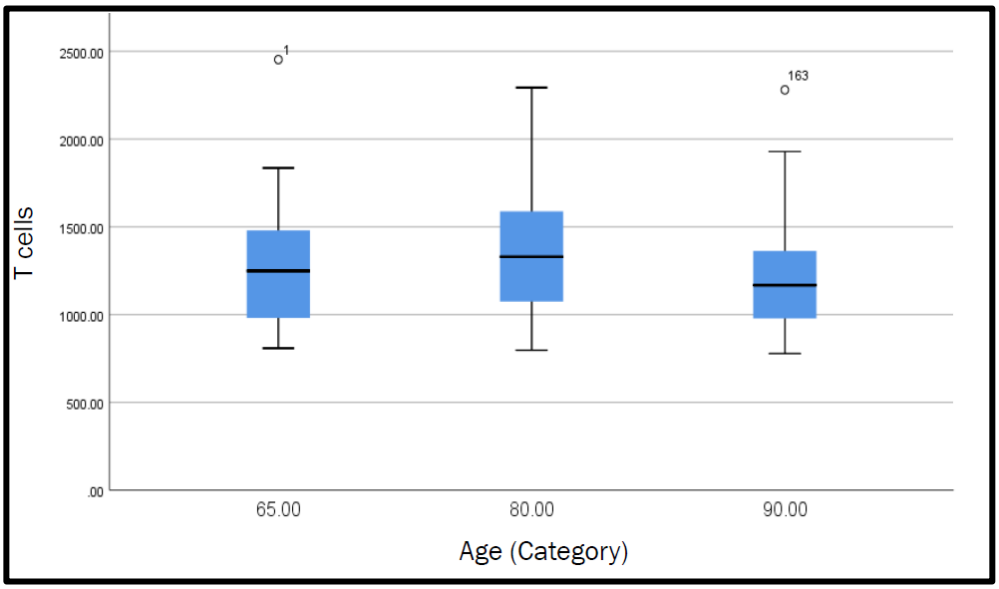
In the examination of the immune cell phenotypes it was observed that age was significantly negatively associated with T cells (P=0.041) as shown in table 3.22. Figure 3.9 shows a progressive decline in T cell mean absolute count across age, where the “nonagenarian” group has a lower value compared with the younger groups. B cells also appeared to be positively associated with length of stay in the care home (p=0.046). Figure 3.12 shows the behaviour of B cells across categories of time of stay in care home. No other parameters were found to be significantly correlated with age, frailty or length in care home.

Table 3.22 P values for the relationship between blood immunophenotypes and categories of age, frailty status and length in the care home in ECHRs in the immunology sub-study

Variables	Age	Frailty	Length CH
Tcells	0.041	0.603	0.846
Tcyt	0.801	0.527	0.852
Activated Tcyt	0.580	0.503	0.781
Tregs	0.536	0.451	0.288
Thelp	0.298	0.702	0.804
Ratio CD4+:CD8+	0.220	0.256	0.780
Monocytes	0.490	0.871	0.929
Monocytes CD80	0.826	0.207	0.690
Monocytes CD86	0.069	0.435	0.328
NK cells	0.782	0.818	0.105
B cells	0.877	0.068	0.046
B cells CD80	0.773	0.219	0.286
B cells CD86	0.624	0.435	0.186

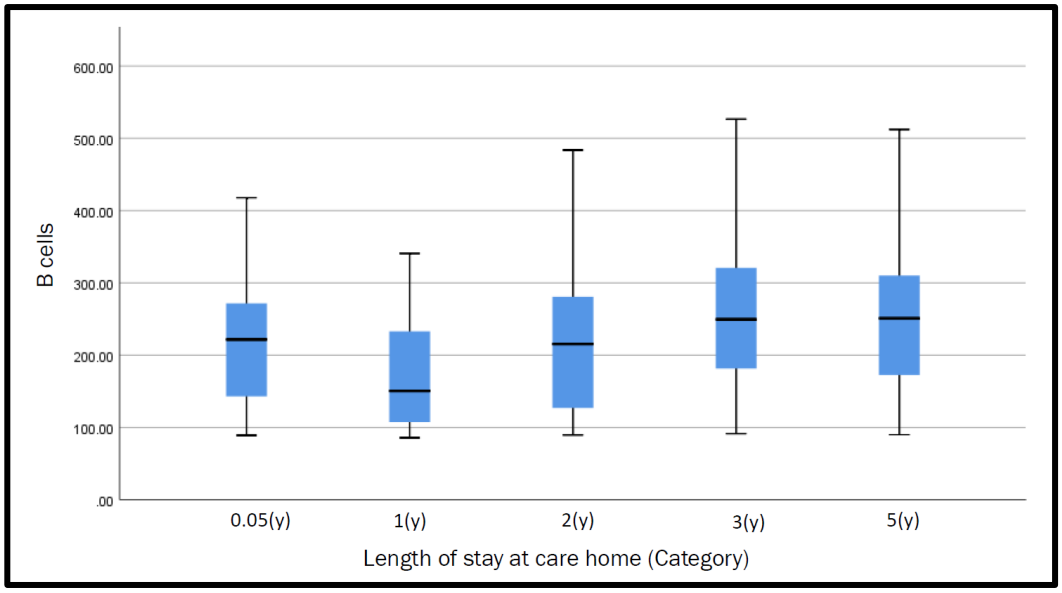
Categorical data Age, Frailty and Length in care home were analysed across continuous data described as variable, using Kruskal-Wallis test. Significance is shown using p values<0.05. Sample size is shown in section 3.3.3.2.

Figure 3.9 Box plot of T cell numbers (cells/µl) across categories of age



T cells and Age (n values given according to categories of Age) – ["65",n=28 ; "80", n=77; "90", n=43]. Kruskal-Wallis test (T cells and Age; p=0.041). Significantly different categories correspond to (Age 80 "Octogenarians" and 90 "nonagenarians"; p=0.012)

Figure 3.10. Box plot of B cell numbers (cells/µl) across categories of length of stay in care home.



B cells and Length of stay at care home (n values given according to categories of Length of stay at care home) – ["0.05(y)",n=45 ; "1(y)", n=25; "2(y)", n=29; "3(y)", n=35; "5(y)", n=10]. Kruskal-Wallis test (B cells and Length at care home (LCH); p=0.046). Significantly different categories correspond to (1 year LCH and 3 y LCH; p=0.003)

Phagocytic function was not significantly associated with age, frailty or length of time in care home (table 3.23).

Table 3.23 P values for the relationship between neutrophil and monocyte phagocytic activity and categories of age, frailty status and length in the care home in ECHRs in the immunology sub-study

Variables	Age	Frailty	Length CH
Phagocytic Activity (%) Neutrophils	0.593	0.780	0.920
Phagocytic Activity (GMFL) Neutrophils	0.257	0.825	0.602
Phagocytic Activity (%) Monocytes	0.084	0.125	0.336
Phagocytic Activity (GMFL) Monocytes	0.818	0.341	0.256

Categorical data Age, Frailty and Length in care home were analysed across continuous data described as variable, using Kruskal-Wallis test. Significance is shown using p values<0.05. Sample size is shown in section 3.3.3.2.

Table 3.24 shows the significant association between age with VCAM-1 (p=0.033), IP-10 (p=0.037) and TNFRII (p=0.001) and Figure 3.11, Figure 3.12, and Figure 3.13 show that the nonagenarian group had higher concentrations of these immune mediators than their younger counterparts. MCP-1 (p=0.002) and IP-10 (p=0.046) showed a significant correlation with frailty and Figure 3.14 and Figure 3.15 show higher concentrations with frailty status 5. The concentrations of these two mediators tend to

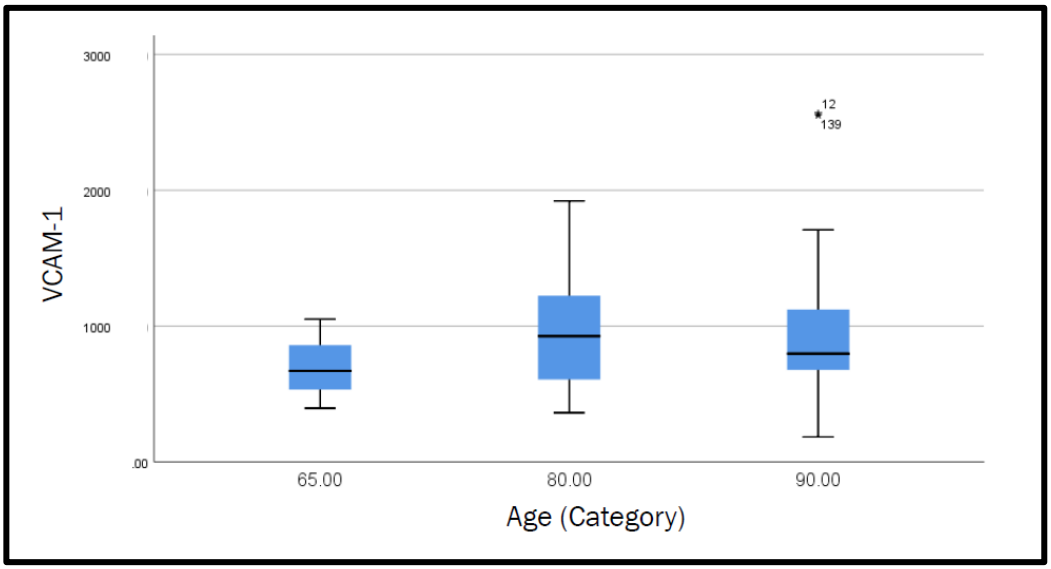
decrease with a more severe frailty status. IL-1ra (p=0.02) and E-selectin (p=0.036) were significantly associated with length in care home as shown in table 3.23. Figure 3.16 and Figure 3.17 show that the concentration of this mediator tended to increase towards 2 y of living in the care home in the case of IL-1ra, whereas the peak concentration of E-selectin tended to occur towards the first year.

Table 3.24 P values for the relationship between plasma immune mediator concentrations and categories of age, frailty status and length in the care home in ECHRs in the immunology sub-study

Variables	Age	Frailty	Length CH
ICAM-1	0.979	0.079	0.310
IL-1ra	0.488	0.286	**0.020**
E-Selectin	0.372	0.156	**0.036**
VCAM-1	0.033	0.508	0.165
MCP-1	0.478	0.002	0.180
IP-10	0.037	0.046	0.130
TNFRII	0.001	0.957	0.563
IL-6	0.599	0.597	0.281
IL-10	0.977	0.426	0.524
TNF-α	0.374	0.548	0.743

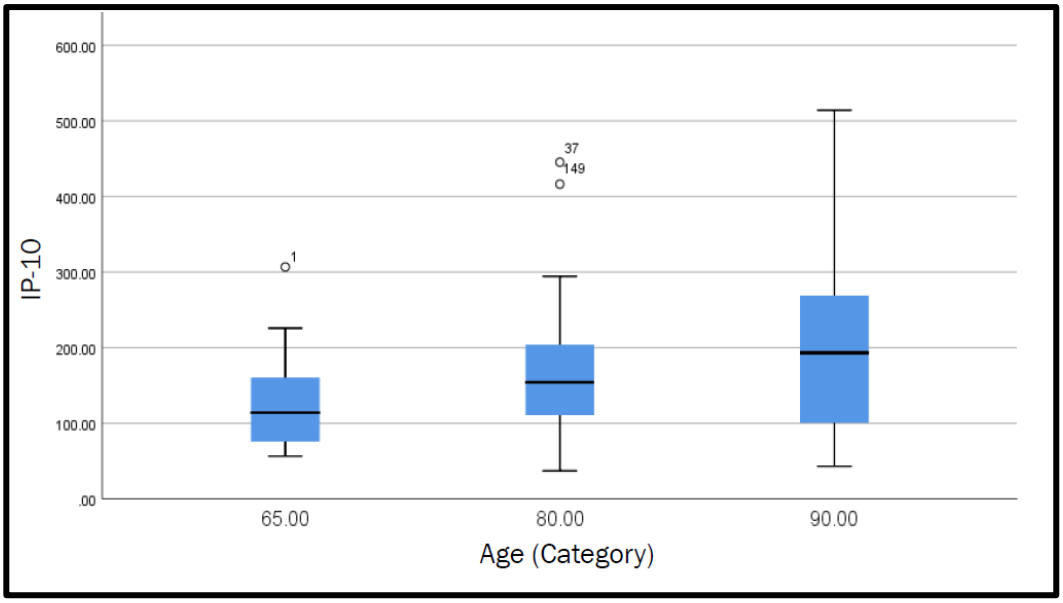
Categorical data Age, Frailty and Length in care home were analysed across continuous data described as variable, using Kruskal-Wallis test. Significance is shown using p values<0.05. Sample size is shown in section 3.3.3.2.

Figure 3.11 Box plot of plasma VCAM-1 (pg/mL) concentration across categories of age



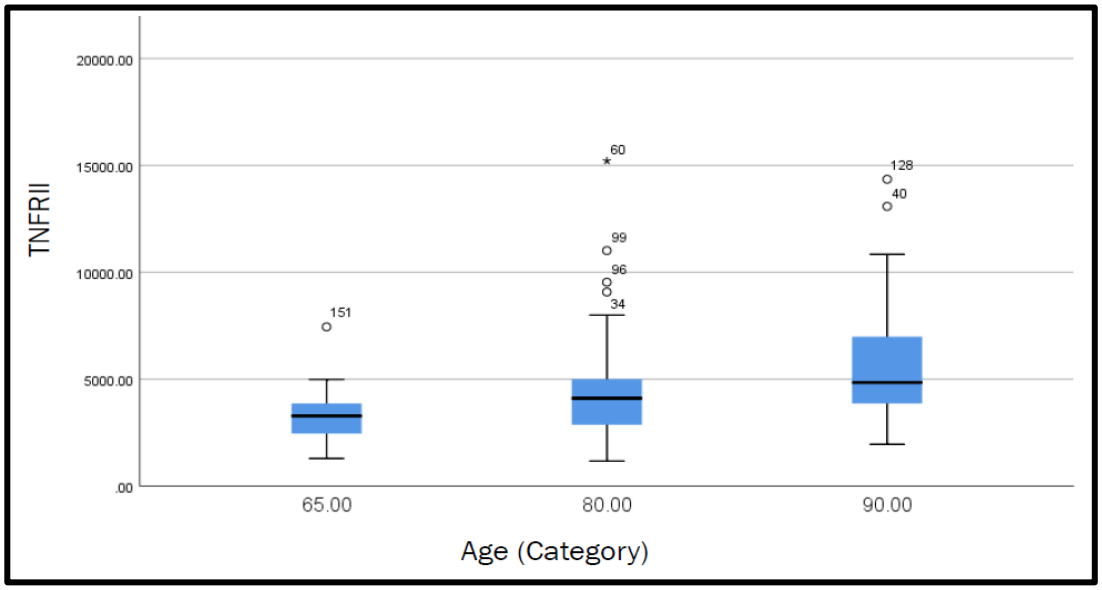
VCAM-1 and Age (n values given according to categories of Age) – ["65",n=22 ; "80", n=46; "90", n=27]. Kruskal-Wallis test (VCAM-1 and Age; p=0.033). Significantly different categories correspond to (Age 65 "65 to 79 y old" and 80 "octogenarians"; p=0.010 / 65 and 90 "nonagenarians"; p=0.044)

Figure 3.12 Box plot of plasma IP-10 (pg/mL) concentration across categories of age



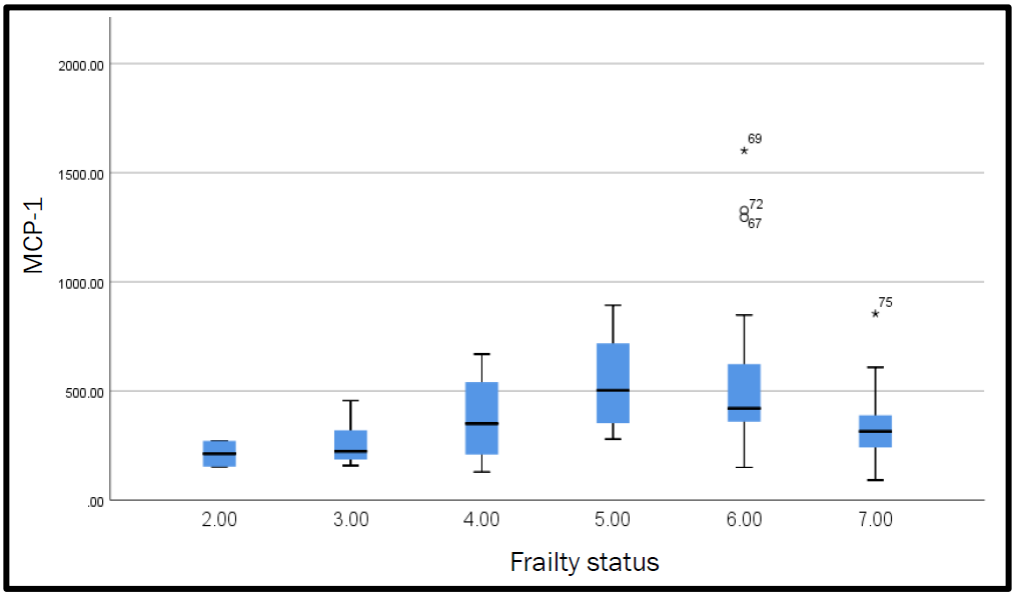
IP-10 and Age (n values given according to categories of Age) – ["65",n=22 ; "80", n=46; "90", n=27]. Kruskal-Wallis test (IP-10 and Age; p=0.037). Significantly different categories correspond to (Age 65 "65 to 79 y old" and 90 "nonagenarians"; p=0.011)

Figure 3.13 Box plot of plasma TNF-RII (pg/mL) concentration across categories of age



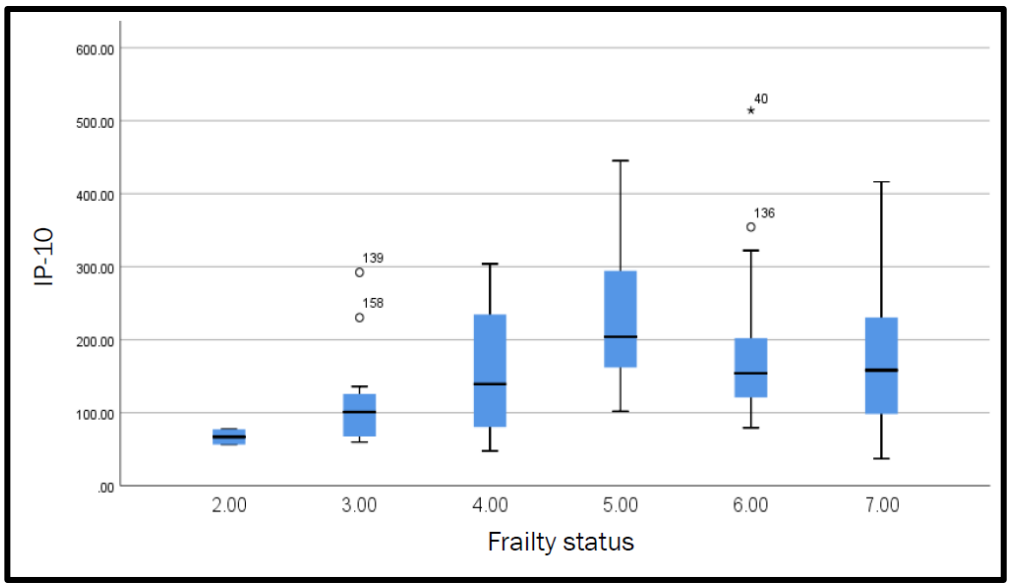
TNF-RII and Age (n values given according to categories of Age) – ["65",n=23 ; "80", n=46; "90", n=26]. Kruskal-Wallis test (TNF-RII and Age; p=0.001). Significantly different categories correspond to (Age 65 "65 to 79 y old" and 90 "nonagenarians"; p=0.001 / 80 "octogenarians" and 90 ; p=0.019)

Figure 3.14 Box plot of plasma MCP-1 (pg/mL) concentrations across categories of frailty



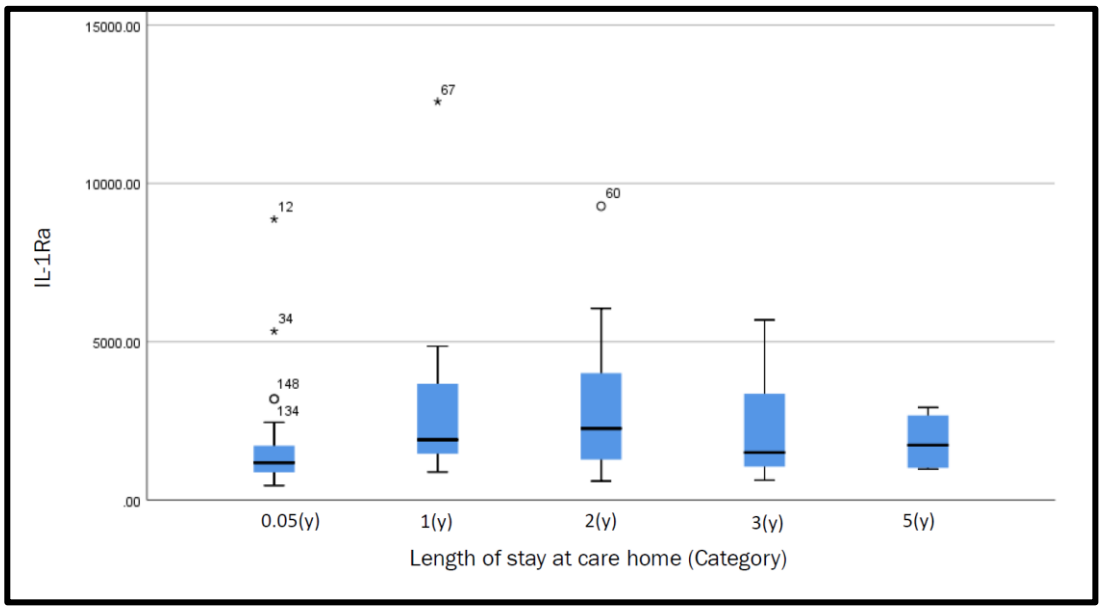
MCP-1 and Frailty status (n values given according to categories of Frailty) – ["1", n=0; "2", n=3; "3", n=10; "4", n=4 ; "5", n=6 ; "6", n=32 ; "7", n=36]. Kruskal-Wallis test (MCP-1 and Frailty; p=0.002). Significantly different categories correspond to (Frail status 2 "Well" and 6 "Moderately frail"; p=0.037 / 2 and 5 "Mildly frail"; p=0.02 / Frail status 3 "well, but comorbid disease" and 6; p=0.001/ 3 and 5; p=0.007 / Frail status 7 "Severely frail" and 6; p=0.005 / 7 and 5; p=0.03)

Figure 3.15 Box plot of plasma IP-10 (pg/mL) concentration across categories of frailty



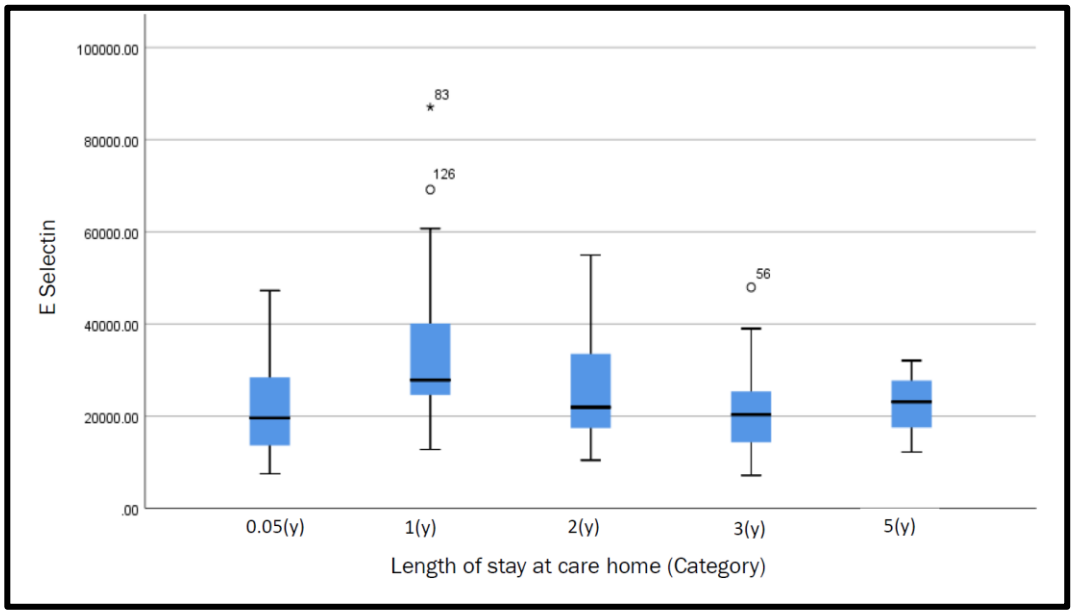
IP-10 and Frailty status (n values given according to categories of Frailty) – ["1", n=0; "2", n=3; "3", n=10; "4", n=4 ; "5", n=6 ; "6", n=32 ; "7", n=36]. Kruskal-Wallis test (IP-10 and Frailty; p=0.046). Significantly different categories correspond to (Frail status 2 "Well" and 7 "Severely frail"; p=0.046 / 2 and 6 "Moderately frail"; p=0.034 / 2 and 5 "Mildly frail"; p=0.01 / Frail status 3 "well, but comorbid disease" and 6; p=0.03/ 3 and 5; p=0.013)

Figure 3.16 Box plot of plasma IL-1ra (pg/mL) concentration across categories of length in care home



IL-1Ra and Length of stay at care home (n values given according to categories of Length of stay at care home) – ["0.05(y)",n=35 ; "1(y)", n=14; "2(y)", n=20; "3(y)", n=21; "5(y)", n=4]. Kruskal-Wallis test (IL-1ra and Length at care home (LCH); p=0.02). Significantly different categories correspond to (6 months LCH and 1 year LCH; p=0.01 / 6 months LCH and 2 y LCH; p=0.003)

Figure 3.17 Box plot of plasma E-selectin (pg/mL) concentration across categories of length in care home



E Selectin and Length of stay at care home (n values given according to categories of Length of stay at care home) – ["0.05(y)",n=35 ; "1(y)", n=14; "2(y)", n=20; "3(y)", n=21; "5(y)", n=4]. Kruskal-Wallis test (E-selectin and Length at care home (LCH); p=0.036). Significantly different categories correspond to (0.05(y)LCH and 1-year LCH; p=0.003 / 1 year LCH and 3 y LCH; p=0.006).

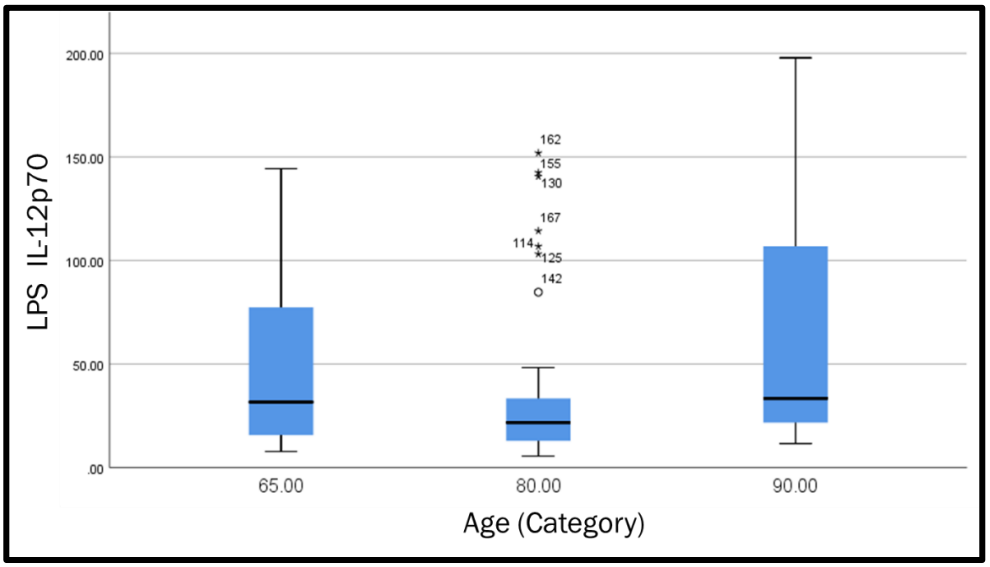
Table 3.25 shows that age was significantly associated with the production of IL12p70 induced by LPS stimulation of while blood cultures (p=0.042); Figure 3.18 seems to show a "U" shape behaviour in this analyte where the production in the nonagenarian and the younger group is higher but it tended to decrease in the octogenarian group. IL-10 induced by PGN and LPS was significantly associated (p = 0.029 and p=0.024, respectively) with frailty and the assessment of the box plots in Figure 3.19 and Figure 3.20 indicates an increased production at stage 3 of frailty. Lastly, TNF-α produced as consequence of stimulation of whole blood with LPS had a significant association with frailty (p=0.033). However, the box plot (Figure 3.21) does not seem to show a clear trend. No associations were identified with the analysis of length of stay in care home.

Table 3.25 P values for the relationship between immune mediator concentrations in whole blood cultures and categories of age, frailty status and length in the care home in ECHRs in the immunology sub-study

Variables	Age	Frailty	Length CH
PGN IL-10	0.237	**0.029**	0.480
PGN TNF-α	0.182	0.300	0.209
PGN IL-6	0.760	0.560	0.779
PGN IL-1Beta	0.224	0.299	0.207
LPS IL-10	0.141	**0.024**	0.966
LPS TNF-α	0.180	**0.033**	0.705
LPS IL-6	0.341	0.178	0.875
LPS IL12p70	**0.042**	0.094	0.590
LPS IL-1Beta	0.069	0.434	0.248
PHA IFN-γ	0.378	0.850	0.385
PHA TNF-α	0.936	0.560	0.322

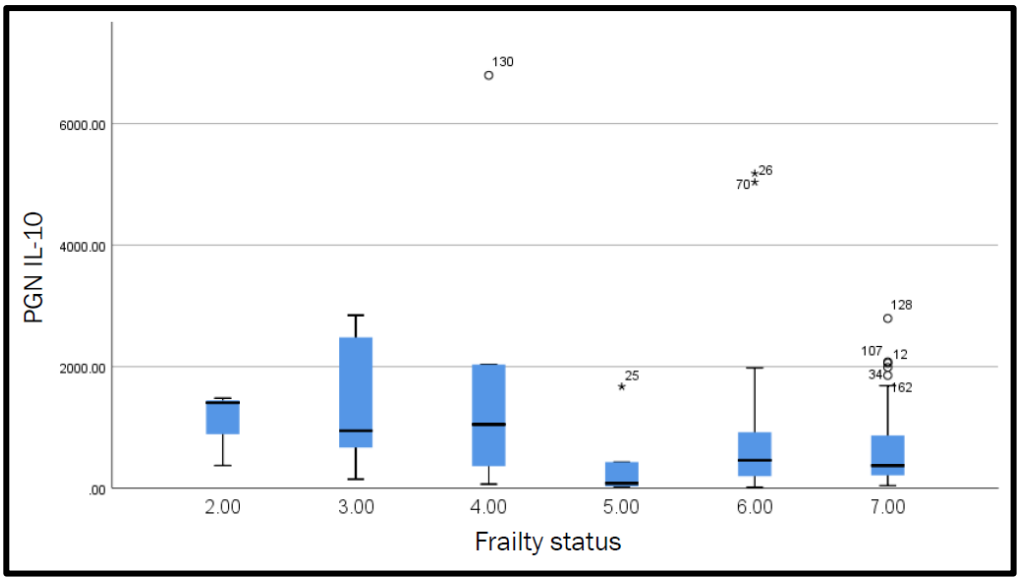
Categorical data Age, Frailty and Length in care home were analysed across continuous data described as variable, using Kruskal-Wallis test. Significance is shown using p values<0.05. Sample size is shown in section 3.3.3.2.

Figure 3.18 Box plot of IL12p70 (pg/mL) in LPS stimulated whole blood cultures across categories of age



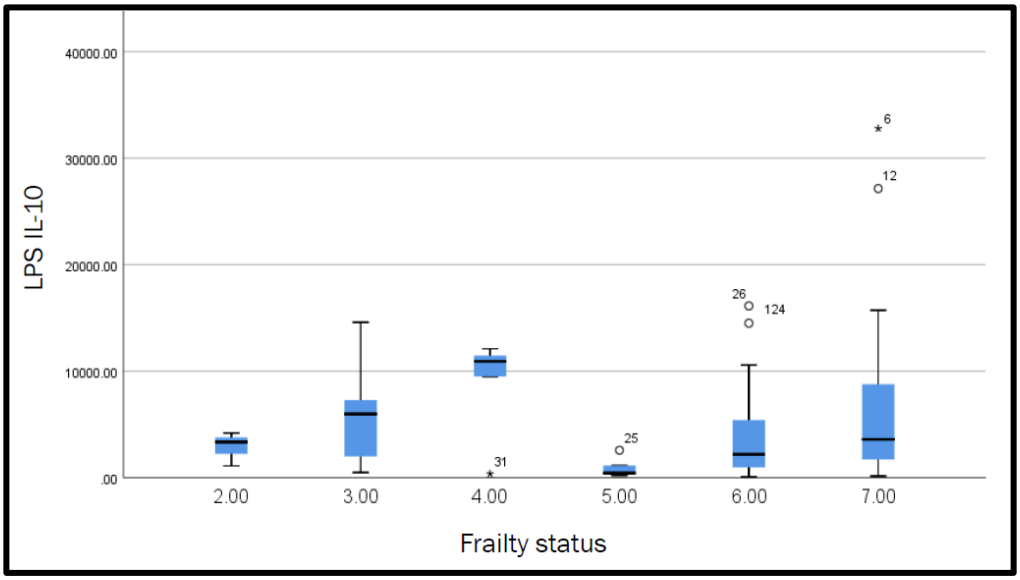
LPS IL-12p70 and Age (n values given according to categories of Age) – ["65",n=23 ; "80", n=47; "90", n=26]. Kruskal-Wallis test (IL12p70 and Age; p=0.042). Significantly different categories correspond to (Age 80 "octogenarians" and 90 "nonagenarians"; p=0.014)

Figure 3.19 Box plot of IL-10 (pg/mL) in PGN stimulated whole blood cultures across categories of Frailty



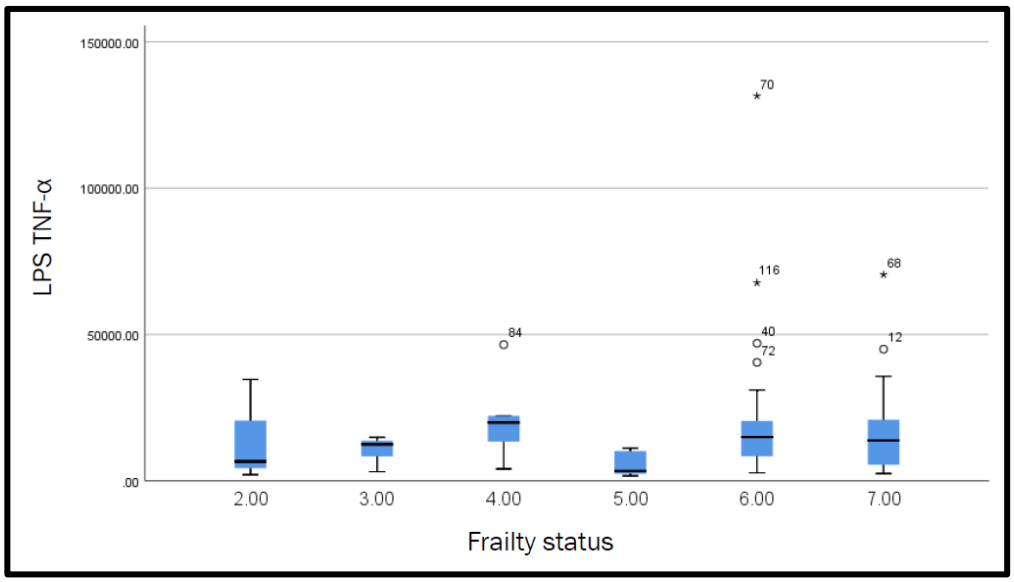
PGN IL-10 and Frailty status (n values given according to categories of Frailty) – ["1", n=0; "2", n=3; "3", n=11; "4", n=5 ; "5", n=6 ; "6", n=31 ; "7", n=36]. Kruskal-Wallis test (PGN - IL-10 and Frailty; p=0.029)Significantly different categories correspond to (Frail status 5 "Mildly frail" and 4 "Apparently vulnerable"; p=0.039 / 5 and 2 "Well"; p=0.046 / 5 and 3 "well, but comorbid disease"; p= 0.002 / 7 "Severely frail" and 3; p=0.018 / 6 "Moderately frail" and 3; p=0.019)

Figure 3.20 Box plot IL-10 (pg/mL) in LPS stimulated whole blood cultures across categories of Frailty



LPS IL-10 and Frailty status (n values given according to categories of Frailty) – ["1", n=0; "2", n=3; "3", n=11; "4", n=5 ; "5", n=6 ; "6", n=31 ; "7", n=36]. Kruskal-Wallis test (LPS - IL-10 and Frailty; p=0.024). Significantly different categories correspond to (Frail status 5 "Mildly frail" and 3 "well, but comorbid disease"; p=0.006 / 5 and 4 "Apparently vulnerable"; p=0.002 / 5 and 6 "Moderately frail"; p=0.027 / 5 and 7 "Severely frail"; p=0.004)

Figure 3.21 Box plot TNF-α (pg/mL) in LPS stimulated whole blood cultures across categories of Frailty



LPS TNF-α and Frailty status (n values given according to categories of Frailty) – ["1", n=0; "2", n=3; "3", n=11; "4", n=5 ; "5", n=6 ; "6", n=31 ; "7", n=36]. Kruskal-Wallis test (LPS – TNF-α and Frailty; p=0.033). Significantly different categories correspond to (Frail status 5 "Mildly frail" and 4 "Apparently vulnerable"; p=0.007 / 5 and 6 "Moderately frail"; p=0.003 / 5 and 7 "Severely frail"; p=0.005)

3.3.3.4 Cross-sectional analysis of vitamin D and its association with outcomes of immune and inflammatory parameters in ECHRs – immunology sub-study.

As previously indicated, it was an objective of the PRINCESS – immunology sub-study to study whether plasma vitamin D levels among ECHRs were associated with immunological and inflammatory parameters, as part of the understanding of the immune phenotype of the participants and associations with vitamin D levels. As shown in table 3.9. sample size corresponded to n=100. Table 3.9 also shows categories used in the analysis of vitamin D. The analyses presented in Table 3.26 to Table 3.30 reveal that the categorical classification of vitamin D concentrations suggested by Lips [326] (See Table 9) was not associated with any of the immune or inflammatory biomarkers. Further analyses aiming to analyse vitamin D as a continuous variable found that frailty, length of stay at care home or age were not associated with the plasma vitamin D levels measured in ECHRs.

Table 3.26 P values for the relationship between components of the FBC and categorical classification of Vitamin D status in ECHRs in PRINCESS-immunology sub-study

Variables	p-value of association with Vitamin D*
Neutrophils	0.650
Lymphocytes	0.662
Monocytes	0.061
Platelets	0.874
WBC	0.388

(n=100). Vitamin D was analysed as categorical variable and analysed across continuous variables. Kruskal-Wallis test was used and statistical difference was considered if $p<0.05$.

Table 3.27 P values for the relationship between immune cell phenotypes and categorical classification of Vitamin D status in ECHRs in PRINCESS-immunology sub-study

Variables	p-value of association with Vitamin D*
T cells	0.527
T cytotoxic	0.553
Activated T cytotoxic	0.920
T regs	0.318
T helper	0.108
Ratio $CD4^+$:$CD8^+$	0.199
Monocytes	0.515
Monocytes $CD80^+$	0.224
Monocytes $CD86^+$	0.397
NK cells	0.782
B cells	0.473
B cells $CD80^+$	0.386
B cells $CD86^+$	0.944

(n=100). Vitamin D was analysed as categorical variable and analysed across continuous variables. Kruskal-Wallis test was used and statistical difference was considered if $p<0.05$.

Table 3.28 P values for the relationship between phagocytic activity and categorical classification of Vitamin D status in ECHRs in PRINCESS-immunology sub-study

Variables	p-value of association with Vitamin D*
Phagocytic Activity (%) Neutrophils	0.863
Phagocytic Activity (GMFL) Neutrophils	0.448
Phagocytic Activity (%) Monocytes	0.589
Phagocytic Activity (GMFL) Monocytes	0.170

(n=100). Vitamin D was analysed as categorical variable and analysed across continuous variables. Kruskal-Wallis test was used, and statistical difference was considered if $p<0.05$.

Table 3.29 P values for the relationship between immune mediators measured in plasma and categorical classification of Vitamin D status in ECHRs in PRINCESS-immunology sub-study

Variables	p-value of association with Vitamin D*
ICAM-1	0.128
IL-1ra	0.355
E-Selectin	0.105
VCAM-1	0.108
MCP-1	0.102
IP-10	0.490
TNFRII	0.381
IL-6	0.460
IL-10	0.731
TNF-α	0.519

(n=100). Vitamin D was analysed as categorical variable and analysed across continuous variables. Kruskal-Wallis test was used, and statistical difference was considered if $p<0.05$.

Table 3.30 P values for the relationship between immune mediators measured in whole blood cultures and categorical classification of Vitamin D status in ECHRs in PRINCESS-immunology sub-study

Variables	p-value of association with Vitamin D*
PGN IL-10	0.616
PGN TNF-α	0.481
PGN IL-6	0.280
PGN IL-1β	0.848
LPS IL-10	0.111
LPS TNF- α	0.919
LPS IL-6	0.662
LPS IL12p70	0.670
LPS IL-1 β	0.195
PHA IFN-γ	0.810
PHA TNF-α	0.166

(n=100). Vitamin D was analysed as categorical variable and analysed across continuous variables. Kruskal-Wallis test was used and statistical difference was considered if $p<0.05$.

Table 3.31. Linear regression model for age, length at care home residence and frailty to predict vitamin D status.

Immune parameter	Covariates	Adjusted mean	95.0% Confidence Interval for B		p-Value
			Lower Bound	Upper Bound	
Vitamin D	Age	0.314	-0.510	1.137	0.452
	Length care home	0.496	-5.192	6.185	0.863
	Frailty	0.062	-5.014	5.137	0.981

(n=100) Vitamin D analysed as continuous variable. Covariates analysed as categorical variables.

3.4 Discussion

Findings presented in this chapter described aspects of innate and acquired immunity in a group of ECHRs. The outcomes herein described correspond to the baseline data characterisation of the participants involved in the PRINCESS immunology sub-study. Further chapters (4 and 5) will present data and analysis corresponding to the post-intervention period. The relationship of age, frailty and length of stay at care home residence was analysed in the context of immune and inflammatory outcomes measured in whole blood, plasma and whole blood cultures. The detailed statistical analyses were conducted through bivariate and multivariate approaches.

3.4.1 Cross-sectional integrative analyses: Immune and inflammatory biomarkers in the population recruited for the PRINCESS immunology sub-study and their association in the context of ageing, frailty and length of stay at care home

3.4.1.1 FBC parameters: analysis of associations

Overall, participants involved in the PRINCESS immunology sub-study had blood immune cell numbers, determined as part of the FBC, within the normal range [217]. Perhaps the only remarkable finding corresponds to platelet numbers which were high for 10% of participants (> 460 x 10*9/l) and lymphocyte count below normality as shown through the p10. The exact threshold at which platelet numbers become a marker of chronic inflammation has not been clearly defined, but it has been suggested that high platelet numbers are related to chronic conditions such as endothelial dysfunction in

older people through enhanced monocyte-platelet aggregation [327, 328]. The increased count of platelets in elderly individuals has also been discussed to be related with atherosclerotic plaque formation [329]. No other remarkable finding was observed, generally indicating that the ECHRs showed normality according to FBC analysis.

Cross-sectional analyses conducted through the categorical classification of frailty showed that the median count of platelets increased significantly across categories of frailty. These findings were also confirmed through the modelling, where frailty emerged as a significant contributory factor over age and length of stay at care home. Fuentes *et al*. found that platelet oxidative stress is a novel target of cardiovascular risk in frail older people [330]. Over a time-frame of 8 y, Starr and Deary observed increased numbers of platelets in individuals initially aged over 79 y old [331]. The current study found no association of platelets with age. However, increased platelet numbers could be a marker of morbidity and an indicator of disease progression and mortality through increased frailty, as the findings herein presented suggest. Platelets trigger leukocyte adhesion which favours their aggregation. The mechanism seems to be linked to platelet-induced production of adhesion molecules [332, 333].

3.4.1.2 Immunophenotypes: analysis of associations

Immune cell sub-populations determined by immunophenotyping - using cell-subset specific stains through flow cytometry - yielded outcomes within the expected ranges for the elderly population. Although there are no accepted normal ranges for these parameters in the elderly, a comparative study conducted by Tavares *et al*, [334]. reported immunophenotypic values in the elderly that agree with the values herein reported.

Examination of the immune cell phenotypes shows that age was significantly negatively associated with T cell numbers. There was a progressive decline in T cell absolute count across age, where the “nonagenarian” group had a lower value compared with younger elderly groups. It has been shown that NK cells and T cells tend to decline steadily with the ageing process [335]. At 90 y old and beyond there is a marked decline in production of “fresh” naive T cells by the thymus and a more restricted T cell receptor repertoire which leads to weaker activation of T cells in aged persons [336].

Another emergent parameter linked with age corresponds to activated monocytes expressing CD86. The linear regression model showed that age was associated with activated monocytes over frailty and length of stay at care home. Stefan *et al*.

demonstrated that monocytes expressing CD86 were increased in elderly individuals. The conclusions proposed by Stefan *et al*. suggest that this disturbance is a consequence of immunosenescence as this trait appeared in both a cohort of elderly individuals with dementia and in healthy age-matched controls [337]. Although in the results presented in this chapter the model showed that age is a significant contributory factor to activated $CD86^+$ monocytes, the direction of this association was not examined as not significant associations were detected among the categorical analyses of age. The fact that age appeared as the most important contributory factor with activation of monocytes is consistent with the inflammatory state that was detected in ECHRs. In support of this conclusion, high levels of immune mediators and pro-inflammatory cytokines were found in this population (see Sections 3.4.1.4 and 3.4.1.5). Epidemiological evidence of this association was also observed in a large cohort study (n=627) where the monocyte count was found as an independent predictor of cardiovascular risk using the Framingham risk score [338].

Lastly, immunophenotyping showed that B cells were associated with length of stay at care home. The direction of association did not show a clear pattern. Initially, it appears that ECHRs go through a progressive B cell decline in the first year, and afterwards, B cell counts appear to recover. The most profound change appeared to be in the first year of residence at the care home. It has been described that B cells remain constant throughout adult life to only decrease in very old individuals [339]. A reduction in B cells could result in an inability to produce some antibodies [103, 107]. No other components of the immunophenotyping were found to be significantly correlated with age, frailty or length at care home.

3.4.1.3 Phagocytic function: analysis of associations

Overall, there are no established expected ranges or cut offs for normal phagocytic function. It has been reported that phagocytic function declines with age which leads to a failure in recognising foreign antigenic particles as well autologous senescent cells [340, 341]. In this study, phagocytic function of neutrophils and monocytes was not significantly associated with age, frailty or time length at care home. These findings do not confirm what has been shown by others where phagocytic function, especially in neutrophils, declined with age [342, 343]. A study comparing the neutrophil phagocytic process in three age groups (21-36, 38-56, and 62-83 y) found a significant age-dependent reduction in the number of phagocytized *E. coli* [344]. A major difference with the current study is that all participants were in the upper age group studied by

Wenisch *et al*. [63]. Hence Wenisch and collaborators were investigating a much wider age range than in the current study and it is possible that beyond 60 y of age, or so, there is no alteration in phagocytic activity of neutrophils and monocytes.

3.4.1.4 Inflammatory and immune mediators biomarkers measured in plasma: analysis of associations

A number of circulating markers of immunity and inflammation have been associated with different chronic conditions (e.g. cardiovascular disease and dementia [345, 346]). Recently, Marzetti *et al*. proposed an inflammatory profiling that they called the "cytokinome" that presents a characterisation of different cytokines and immune mediators in people with physical frailty and sarcopenia [347]. The importance of inflammatory profiling, particularly in frailty and ageing, is to provide an insight into endogenous processes and to suggest a window to preventive strategies. It has generally been shown that pro-inflammatory cytokines are increased in aged individuals which represents the inflammageing condition. Those pro-inflammatory mediators that consistently appear to be increased in aged individuals include acute phase proteins such as CRP, cytokines such as TNF-α, IL-6 and IL-8 and adhesion molecules such as ICAM-1 and VCAM-1 [348, 349].

3.4.1.4.1 Baseline findings in ECHRs participating in PRINCESS immunology sub-study and comparisons with other studies investigating immune mediators in the circulation

The adhesion molecules ICAM-1 and VCAM-1 were higher in ECHRs when compared with other studies including elderly participants, whereas E-selectin was not notably different. Median plasma ICAM-1 was 385.9 ng/mL in the current study. Another study conducted by Sesso et al. in male participants aged from 40 to 80 y found comparable ICAM-1 values in both hypertensive cases and healthy controls [350]: 311.4 ± 67.6 ng/mL in the hypertensive cases and 305.1 ± 54.2 ng/mL in the healthy cases. Liu et al. reported ICAM-1 values of 307 (250-381) ng/mL in frail elderly subjects [351]. In the current study median plasma VCAM-1 was 791.2 ng/mL. The study conducted by Hoke et al. found median values of 646 (IQR: 460-837) ng/mL [352]. In the current study, median plasma E-selectin was 22.8 ng/mL. A study conducted by Kasza et al. analysed E-selectin in a population of diabetics and healthy persons aged 61.71 ± 12.31 y old. Mean concentrations in the two groups were 32.95 ng/mL and 26.55 ng/mL [353]. The relevance of particularly ICAM-1 and VCAM-1 as biomarkers for frailty has also been

confirmed in a study examining frailty biomarkers, whereby these two biomarkers might be candidates with potential prognostic and therapeutic potential [349].

Median plasma MCP-1 reported by Beyer *et al*. [354] was 767.5 pg/mL whereas the median value measured in PRINCESS - immunology sub-study was 355 pg/mL. This value is closer to that reported by Liu *et al*., who found among phenotypically frail and elderly individuals a level of MCP-1 of 415 (345-501) pg/mL [351]. Associations relating to MCP-1 and frailty agree with the findings from the Singapore longitudinal aging study, in which, despite lower levels being reported (238.8 pg/mL) [355], this chemokine showed an association with frailty in the elderly supporting the hypothesis of systemic inflammation as core basis in the biology of frailty.

Beyer *et al*. [354] also reported median values of TNF-α and IP-10 of 6.4 pg/mL and 50 pg/mL, respectively. The values herein reported were slightly higher at 17.7 and 151.7 pg/mL, respectively. The higher level observed for TNF-α agrees with findings reported by Marzetti *et al [347]*. Physically frail and sarcopenic old women and men showed high values of TNF-α (37.5 pg/mL and 32.7 pg/mL, respectively).

An important study conducted by Liu *et al*., aiming to characterise biomarkers associated with frailty, reported the cross-sectional associations among the frail phenotype in individuals older than 60 y. In addition to ICAM-1 and MCP-1, Liu also studied levels of IL-6, and TNFR-II. Their values were 3.42 (2.15-6.48) pg/mL and 3.1 (2.5-4.4) ng/mL [351]. TNFRII was found at median plasma values of 4.1 ng/mL in PRINCESS - immunology sub-study whereas the geometric mean value of TNFR-II was reported to be 5.0 ng/mL by Bruunsgaard *et al* [356]. IL-6 was found in median plasma values of 4.4 pg/mL in this PRINCESS – immunology sub-study, values which are comparable with Forsey et al., who reported median values in older individuals of 5.98 pg/mL [357]. IL-6 has been correlated with low walking speed and declined strength in muscles in frail elderly [358]. The Singaporean longitudinal study in aging also analysed the soluble receptor IL-6R as a marker of the IL-6 activity, and found an association with frailty. This receptor is negatively associated with bone density [355]; thus potentially the alteration in the IL-6 pathway is linked to osteological disturbances leading to the frail phenotype. Although inflammation consists of an interplay among pro and anti-inflammatory cytokines, that dichotomist dynamic might be slightly simplistic as some cytokines might behave as pro and anti-inflammatory depending on the exact concentrations, and timings of production within the immune response [359]. The activation of inflammatory pathways with elevation of inflammatory biomarkers such as

IL-6 has been considered a pathophysiological feature of frailty and IL-6 together with TNF-alpha have been closely related with the acute-phase inflammatory response [360], the effects of these cytokines in frailty require further exploration.

PRINCESS - immunology sub-study found median plasma values of IL-1ra corresponding to 1558.9 pg/mL whereas Ferrucci et al., reported mean values of 154 (CI: 131-180) pg/mL in "long lived persons" corresponding to people above 90 y old [361]. Another study conducted by Jylha et al., reported increased mean values of 340 (CI: 261-420) pg/mL in nonagenarians [362]. Within the different reports examining biomarkers related to frailty and ageing, IL-1ra has not been widely explored. However, IL-1ra has been associated with increased risk of osteoporosis and fractures [363], important factors determining the frail phenotype.

IL-10 was found in median plasma values of 0.6 pg/mL in the current study. These values were much lower than the values reported by Forsey et al., who found circulating values of 6.13 pg/mL in elderly above 90 y old [357]. This finding is important as it has been described that IL-10 can be reduced in sarcopenic older adults [348]. In the ECHRs herein studied, it appeared an advanced state of frailty for more than half of the population, reflecting also a sarcopenic condition.

3.4.1.4.2 Cross-sectional integration of plasma immune mediators in ECHRs and integration with age, frailty and length of stay at care home.

The cross-sectional analyses herein presented included different statistical approaches. Age and frailty emerged as significant factors in the population of ECHRs which showed to be associated with some inflammatory biomarkers. Age and frailty are different concepts and their differentiation becomes clearer in healthy and free-living older populations (e.g. centenarians having good health conditions [288]). However, in care homes, it appears that the differentiation between age and frailty is less clear, as frailty is a common condition present in care home residents regardless of age. Correlation analyses presented in this research did not show significant association between age and frailty, probably due to the high proportion of individuals with a degree of vulnerability or frailty as shown through a frailty index above 4 (around 64% as shown in were able to provide samples to be enrolled in the PRINCESS immunology sub-study (Figure 3.4). Other authors, however, agree with the findings herein presented where age and frailty were factors associated with biomarkers used in inflammageing [364-366].

Contrarily, length of stay at care home appeared associated with inflammatory biomarkers measured in plasma to a less degree as fewer biomarkers showed significant associations. Moreover, length of stay at care home appeared to be positively and significantly correlated. This remarkable aspect reflects an important dynamic among length of stay and the progression in the condition of vulnerability in elderly people. Due to its relevance, this will be discussed in more depth in section 3.4.2.

Age showed significant associations with IP-10, TNFRII and VCAM-1. The findings reported in this cross-sectional study agree with Cardoso *et al*., who have recently proposed that there are some pathways upregulated in ageing and age-related diseases and that relate to frailty syndrome [349]. Likewise, frailty showed to be associated to IP-10. Cardoso *et al*. have suggested that genes encoding adhesion molecules such as VCAM-1, among other inflammatory markers are related to frailty [349]. It has been described that these analytes are associated with progression of diseases in a non-diabetic population [367].

Beyer et.al. suggest that inflammation is related to muscle wasting and therefore a worse frailty status. They found that in a population of 33 geriatric individuals, those with higher MCP-1 show a significantly lower grip strength and lower lean body mass [354]. In the current study, MCP-1 was significantly associated with an advanced status of frailty in ECHRs. Recently, MCP-1 has been suggested as a potential biomarker of biological ageing in an animal model of accelerated ageing [368]. A mouse model with failure in DNA repair showed higher plasma levels of MCP-1 when matched with the normal mouse model. In that research, the findings were later confirmed in a study conducted in humans by measuring plasma MCP-1 in non-frail elderly individuals (n=27) versus elderly frail individuals (n=36) and identifying a significantly higher concentration in frail individuals [368]. The current findings obtained in a larger sample of individuals in residential care homes (n=95) add evidence for MCP-1 as an important biomarker in frailty. Elevated MCP-1 levels may reflect a greater number of senescent cells in the circulation [369].

Other variables where frailty appeared as a significant contributory factor over age and length of stay at care home – identified through the regression model - were IL-1ra and E-selectin. IL-1ra has been linked by other researchers as an independent risk factor of morbidity and mortality in the elderly [366]. Remarkably, the categorical analyses showed that length of stay in care homes was significantly associated with plasma concentrations of IL-1ra and E-selectin. Levels of IL-1ra were increased in participants

residing at care homes for over 2 y, whilst E-selectin seems to be higher in participants resident for a year. These inflammatory patterns were also observed in the analysis of covariances through the regression model, where inflammation and upregulation of the expression of adhesion molecules was present in frailty as shown by others [370, 371]. The relevance of these biomarkers in ECHRs is also supported through the correlation analyses where a positive and significant correlation was observed with frailty and length of stay at care homes.

Overall, these findings confirm what has been described by other authors where inflammation, either low grade or chronic, is a common cause of morbidity and mortality [372, 373]. Our findings support an association of inflammation with frailty in ECHRs. Inflammageing has been suggested as a predictor of frailty in elderly people [374]. Edvardsson *et al*. have demonstrated that inflammatory markers are related to reduced survival in a follow-up study for one year with frail ECHRs [375]. The population recruited in the study showed a high degree of inflammation when compared with existing literature. Similar outcomes are likely to be found in other care home residents.

3.4.1.5 Inflammatory and immune mediators measured in whole blood cultures following TLR-2 and TLR-4 stimulation with PGN and LPS and T cell stimulation with PHA.

3.4.1.5.1 Baseline findings in ECHRs participating in PRINCESS immunology sub-study and comparisons with other studies investigating whole blood cultures in the elderly

Threshold values in the levels of immune mediators produced in whole blood cultures have not been determined. However, the value of these *ex vivo* cultures has been discussed (See 2.4.2.3). Generally, it has been established that TLR-4 is stimulated by LPS and that TLR-2 is stimulated by PGN. The activation of these receptors leads to increased production of TNF-α and IL-6 [267, 376]. The current findings show that IL-10, TNF-α and IL-1B were potently induced by LPS in comparison to PGN. LPS induced median production values 5 fold higher for IL-10, 3.9 fold higher for TNF-α and almost 12 fold higher for IL-1B when compared with PGN. Furthermore, a superior production of IL-12p70 was induced by LPS when compared with PGN, but the difference was less (two-fold). Lastly, IL-6 was similarly induced by both PGN and LPS. PHA stimulates T cells. The production of TNF-α following PHA stimulation was lower than with LPS and PGN. The potent effects exerted by LPS agree with what has been shown by others [377]. The

association of health and TLR responsiveness, particularly TLR4, in ECHRs has not been widely explored. However, a meta-analysis comparing the TLR4 polymorphisms with risk of cancer reported a degree of association with the prevalence of overall cancer when data were analysed as healthy controls versus cases [378]. Furthermore, McFarlin *et al*. have suggested that TLR4 appears to have a role in regulating the linkage between cytokine production (IL-1β and TNF-α) and physical active lifestyle regardless of age. In this study a group of old (60-80 y old) and young individuals (18-30 y old) were categorised as "active" or "inactive". There were significantly higher levels of IL-1β and TNF-α in the inactive group in both young and old people [379]. McFarlin also reported lower expression of TLR4 in the active group. Similar observations were reported in a group of older women exposed to regular training [380]. The current findings certainly suggest an active TLR4 pathway in the ECHRs according to the cytokine production detected in the cultures following LPS stimulation.

3.4.1.5.2 Cross-sectional integration of immune mediators analysed in whole blood cultures in ECHRs and integration with age and frailty

IL-10 induced by PGN and LPS was in both cases significantly associated with frailty and the statistical pairwise comparison revealed a significantly lower production in both cases at stage 5 – "middle frail". Additionally, TNF-α produced in response to the stimulation with LPS also showed a significant association with frailty. Consistent with the findings for IL-10, TNF-α also showed the lowest production at frailty stage 5. The statistical modelling also confirmed that frailty was significantly associated with IL-10 production as result of PGN stimulation. IL-10 is an anti-inflammatory cytokine that counter balances pro-inflammatory responses [381]. The ECHRs appeared to show an imbalance in IL-10 and TNF-α with a marked lower production of IL-10 and increased TNF-α in a condition of middle frailty. Lastly, age was significantly associated with the production of IL12p70 induced by LPS stimulation. The production of IL12p70 in the "nonagenarian" was higher than in the "octogenarian" group. This biomarker has also been linked to other diseases such as Alzheimer's disease [382].

3.4.2 Integrative analyses: relevance of age, frailty and length of stay at care home residences

There was a positive association between frailty and length of stay at care home residence, as described previously [383]. This is a concern since frailty has been linked to increased mortality in the elderly [287], and care home residences are often a choice

for elderly people that cannot look after themselves. Results herein presented (Figure 3.5) show a significant and positive association whereby the longer stay at the care home correlates with an increased frailty level. This is likely attributable to the state in which elderly people reach the care home residence initially, meaning that they are often vulnerable when they transfer to these institutions. With the progress of time, the degree of frail condition only seems to be worse. This is an important biogerontological finding which might be related to the environment [293, 297]. Strategies aiming to offer social and physical support are relevant, and these findings seem to highlight people at prolonged stay at care home as requiring special attention. Preventive strategies could also address newly elderly members to try and preserve their health to avoid frailty. Another study aiming to characterise inflammatory mediators and lymphocytic subset in frail older individuals found that the inflammatory mediators, particularly IL-6 and TNF-RII, progressed with frailty severity [384]. The examination of inflammatory parameters and immune mediators could offer an overview of the frailty condition in those participants relocating to institutionalised homes.

It seems important for care homes to focus on reducing frailty and to monitor those likely to have long term residence. There is a need to develop better support for those individuals that have been living in care homes for prolonged times. This might include focusing on avoiding frailty through maintenance of optimum physical activity levels [296] and nutritional strategies focussed on addressing the low-grade inflammatory condition [385].

The observation that age itself was not related to either frailty or length of stay at care home highlights the heterogeneity of the ageing process. The traditional categorisation of “old” as above 65 y from date of birth to define the “elderly” population does not always indicate negative outcomes on health. For example, it has been shown that centenarians can have good health [288]. Thus, rather than focusing only on age to screen an old individual, frailty, and time length in care home are important predictors of decline in general health.

3.4.3 Plasma vitamin D measured in ECHRs and associations with immune and inflammatory parameters.

Commonly used cut offs for vitamin D status are “Deficiency” (<25nmol/l), “Insufficiency” (25 and 50 nmol/l), and “Sufficiency” (>50 nmol/l). These cut offs are

based upon findings in young or middle aged adult populations. Older people may require different cut offs because they may show signs of lower vitamin D status at higher concentrations that in younger adults [386].Corrective measures are a priority in this population. Categories used to classify vitamin D status were established according to Lips and the necessity of establishing more strict diagnostic criteria in the elderly [326]. Deficiency, or levels below 25 nmol/l are considered chronic deficiency and may cause proximal myopathy or increased bone turnover. These conditions are closely related to frailty, and thus vitamin D status may be a useful parameter in the study of frailty [387]. Whether that categorisation is also related to immune and inflammatory parameters has not been explored, but it has an importance in frailty as it examines bone health. In the population of ECHRs studied, over 50% of the population showed 25-hydroxy-vitamin D deficiency assessed in plasma.

Clinical evidence of the immune and inflammatory role of vitamin D has been obtained through different types of study. The NHANES evaluated 8655 individuals including some who were elderly and identified an association between low plasma levels of vitamin D and metabolic diseases with an inflammatory component [388]. It has also been shown that deficiencies in vitamin D intake are associated with inflammatory disturbances. An association between low serum vitamin D and an imbalance between “pro-inflammatory” IL-6 and anti-inflammatory IL-10 was detected in a sample of 957 elderly Irish subjects [389]. That inflammatory disturbance has also been confirmed by Ritterhouse *et al*. who found an elevated level of C-reactive protein, IL-6 and TNF-alpha in the context of vitamin D deficiency in 774 apparently “healthy” Americans with different ethnic backgrounds [390]. There was also an inverse correlation between an inflammatory biomarker (C-reactive protein) and 25 OH vitamin D in a group of 923 elderly patients in the Netherlands. This correlation was stronger in individuals with inflammatory diseases [391]. Lastly, in the studies focused on associations, the InCHIANTI study found an inverse correlation between serum IL-6 and vitamin D in the elderly. The authors of the study suggest vitamin D as an anti-inflammatory immunomodulator [392]. Furthermore, seasonal variations have also added evidence of the vitamin D status and prevalence of diseases, especially infectious pathologies. For instance, seasonal changes across the year have shown a suppression of anti-inflammatory cytokines in winter periods whereas their production appeared to increase in summer [393-395]. These findings suggest the importance of vitamin D in controlling inflammation. The vitamin D categorisation herein presented has been linked to bone health, which is closely linked to frailty. Although no associations were found, further

exploration of vitamin D linked with immune parameters would be of great interest and value. Further methodological designs are necessitated, especially those focused on causality roles among vitamin D status (not only in plasma but binding proteins and cellular receptors) and immune outcomes

3.5 Conclusions

The hypothesis proposed in this chapter can be accepted as immunosenescence and inflammageing are associated to some extent with outcomes in frailty, length of stay at care home and age. Analyses herein conducted describe the association of frailty, age and to a lesser extent, length of stay at care home, with immune parameters (particularly cell numbers in blood) in a community of ECHRs. Overall, it was found that associations between frailty and age were significant and largely based upon increased production of inflammatory immune mediators rather than disturbances at the immune cell count level. The outcomes also reflect the relevance of frailty and its severity as highly informative of the inflammageing process in ECHRs.

A positive relationship between frailty and platelet count was seen. From the inflammageing perspective, frailty is a significant contributor variable for increased levels of IL-1ra and E-Selectin. MCP-1 and IP-10 are significantly linked with frailty. Finally, the analyses of the cytokines produced in whole blood cultures indicate that the production of IL-10 and TNF-α following TLR stimulation are significantly associated with frailty. Age appears as a significantly associated factor for activated monocytes. Age appeared as a significant contributory factor for IP-10, TNFRII, and VCAM-1, and lastly, the length of stay at care home appeared as a significant contributor for MCP-1, VCAM-1, IL-1ra and E-selectin. Phagocytic function did not show any significant associations with the parameters under investigation age, frailty or length of stay in the care home and the same was true for analyses conducted on Vitamin D.

Chapter 4 PRINCESS immunology sub-study: Effect of probiotic intervention in elderly care home residents on immune cell numbers and phenotypes and response to the seasonal influenza vaccine

4.1 Introduction

Chronic conditions are of concern in gerontology. There is a less effective immune response due to the process of immunosenescence and the presence of persistent inflammation (Chapter 1). These changes have been described among the biomarkers of ageing and tend to be heterogeneous among individuals [25, 396].

There are many ways to measure the immune system and immune responses [214, 223]. Many of these relate to measurements made in blood. The full blood count (FBC) is used with reference to establish values for blood cell counts and can be used to indicate clinical conditions such as infections (Chapter 2). FBC is perhaps the most common routine investigation to quantify and study the immune system; however, FBC is a crude measure of the types of immune cells present. Flow cytometry is a technique that has gained relevance in the clinical context as it allows identification of immune cell subtypes in more detail. This include an examination of activation markers, different T cell and B cell subsets and the level of expression of certain markers of differentiation which can be correlated with clinical findings. Flow cytometry has allowed the development of immunophenotyping. There are general guidelines to conduct and perform immunophenotyping by flow cytometry [397], but in general there are no reference values. With ageing there is decreased *de novo* generation of naive T and B cells and accumulation of memory cells [398, 399]. Moreover, there is a common observation of decreased absolute numbers of T cells in blood with ageing [400]. Lastly, ageing has been shown to be associated with decreased vaccine responses due to reduced production of memory B cells and subsequently new antibodies [399, 401].

Age-related changes in the immune cell phenotypes and FBC, both analysed in peripheral blood in humans, have been associated with the prognosis of some common conditions in the elderly. For instance the eosinophilic counts have been associated to infectious diseases [238, 402, 403] and platelets have been linked to cardiovascular diseases [404]. Neutrophils and white blood cells have also been linked to immunological disturbances and are considered as pathophysiological contributors to atherosclerotic diseases and dementia [405-407].

Disturbances in the proportion of immune cells as well as leukocytic subsets and cell surface markers (clusters of differentiation) in the elderly have been described using the FBC and immunophenotypes through different stages of the aging process (e.g., young old and centenarians). That detailed examination of B cell subsets has provided useful

information with regards functionality of B cells which has been linked to decreased antibody production [401, 408] and so the characteristic poor response towards influenza vaccination [409]. Influenza is associated with increased mortality of the geriatric population [410, 411] and according to the World Health Organization (WHO), seasonal influenza comprises an epidemic with higher virulence than many other microorganisms [412]. Older adults (> 65 y of age) - particularly vulnerable to influenza illness and infections [413] - present a reduced ability to respond to some antigens and environmental influences lead to increased susceptibility to mortality. Influenza vaccination provides benefits for older adults against influenza; however, vaccine effectiveness is lower than in younger adults [119]. The use of high-dose vaccines such as the quadrivalent influenza vaccine enhances the protection conferred to the old, and these are currently the best strategy in the control of influenza [414]. A systematic review and meta-analysis assessing the efficacy and safety of a high-dose influenza vaccine in elderly adults described that high-dose influenza vaccination is better at preventing influenza infections when compared with the standard dose vaccine [415].

Co-adjuvant strategies to confer immune protection in the geriatric population may involve the use of probiotics [416, 417]. *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. BB-12, which have been studied in the elderly, seem to provide interesting findings in terms of health outcomes of aged individuals. These benefits refer to improved constipation [418], reduced diarrheal episodes caused by pathogenic organisms [419] and even psychological benefits [420, 421]. However, randomized clinical trials in the elderly are underrepresented in the clinical evidence currently available, as confirmed through the literature search presented in chapter 1. A plausible explanation for the lack of evidence is the concern about immunocompromised individuals and their tolerability of the probiotic. The research herein presented seeks to address the lack of evidence by providing the first part of the results from a randomised clinical trial – PRINCESS immunology sub-study - which aims to describe the effects of a probiotic intervention on immune parameters measured including FBC, immune cell phenotypes, and the response to an immune challenge (quadrivalent influenza vaccine) in ECHRs.

4.1.1 Hypothesis

The overall hypothesis being tested is that probiotics enhance immune function in elderly care home residents. In order to test this hypothesis, there is a need to measure a range of biomarkers of immune function. In this chapter indicators of immune status revealed through FBC and immune phenotyping will be reported. It is expected that FBC will be maintained within ranges of normality whereas it is expected that some parameters of the immune phenotyping will be improved (particularly NK cells and increased markers of activation). Furthermore, it is hypothesized that the vaccine-specific antibody response following seasonal influenza vaccination will be improved in the group that was allocated to probiotics compared to the control group.

4.1.2 Aim and objectives

The aim of the research described in this chapter was to compare the effects of a probiotic intervention with a placebo-controlled intervention on markers related to immune function in ECHRs. The objectives were to assess the following immune biomarkers in the community participating in PRINCESS clinical trial immunology sub-study:

- Whole blood numbers of neutrophils, lymphocytes, monocytes, total white cells, eosinophils, and basophils (identified through full blood cell count);
- Lymphocytic and monocytic subsets identified through immune cell phenotyping.
- Vaccine-specific antibody response following seasonal influenza vaccination.

4.2 Methods

4.2.1 Subjects

Inclusion and exclusion criteria are described in section 3.2.2. From the participants included in the PRINCESS – Immunology sub-study, only paired samples (i.e. samples pre- and post-intervention) are included within the analyses herein conducted. Participants enrolled in this study were recruited over a year and received the most recent seasonal influenza vaccine for the year 2017. The influenza virus vaccines for use in the northern hemisphere in 2017-2018 consisted of strains A/Michigan/45/2015 (H1N1)pdm09- like virus, A/Hong Kong/4801/2014 (H3N2)-like virus, and B/Brisbane/60/2008-like virus. A quadrivalent vaccine that also included

B/Phuket/3073/2013-like virus was used in the PRINCESS study. A descriptive analysis comparing mean of age vs mean of age in the subgroup indicates that in the subgroup – (population included chapter 4 and 5) mean age ± SD in this subgroup corresponds to 86.2 ± (0.86). The time spent at care home was 1.5(y) ± (0.21). Moreover, no differences were observed in the distribution of sex and frailty when subgroup analyses were compared with the general group.

4.2.2 Follow up

The probiotic intervention, selected route of administration and compliance were closely supervised by researchers, as mentioned in section 3.2.1. Assessment of adverse events was performed by the study nurses. Only participants that continued in the intervention groups are herein reported. Information related to participant loss was recorded (section 3.2.6)

4.2.3 Measurement of immune parameters

4.2.3.1 Full Blood count - Immune cell numbers

The methodology that was utilised for the FBC was as described in section 2.2.3.1.1 2

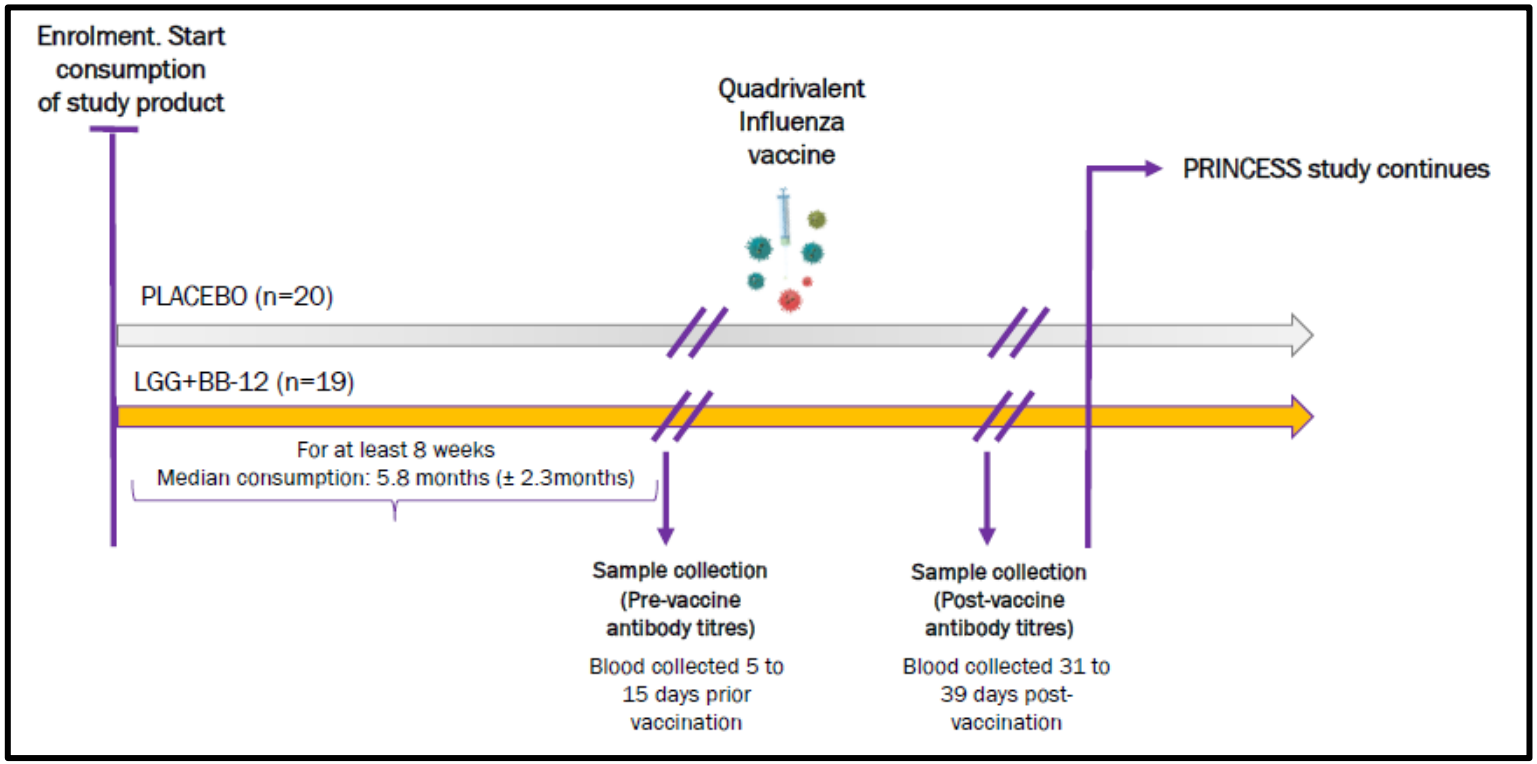
4.2.3.2 Immunophenotyping

In general the approach used for immune cell phenotyping was the same as that described in section 2.2.3.2.1 A modification was made as described in section 3.2.5.1. This modification was seeking to optimise the number of tubes to be stained to make the data acquisition in the flow cytometer more efficient. Procedures related to the flow cytometer compensation and gating process were as described.

4.2.3.3 Anti-influenza vaccine antibodies

The methodology used was as described in section 2.2.5. The flu-vaccine sub-analysis included thirty-nine participants taken from the PRINCESS-Immunology sub-study. All participants in the PRINCESS study who started the probiotic intervention remained consuming the probiotic intervention or the placebo for least 8 weeks and received the quadrivalent influenza vaccine were included (Figure 4.1)

Figure 4.1. Overview of the design of the sub-study investigating response to seasonal influenza vaccination.



4.2.4 Statistical analyses

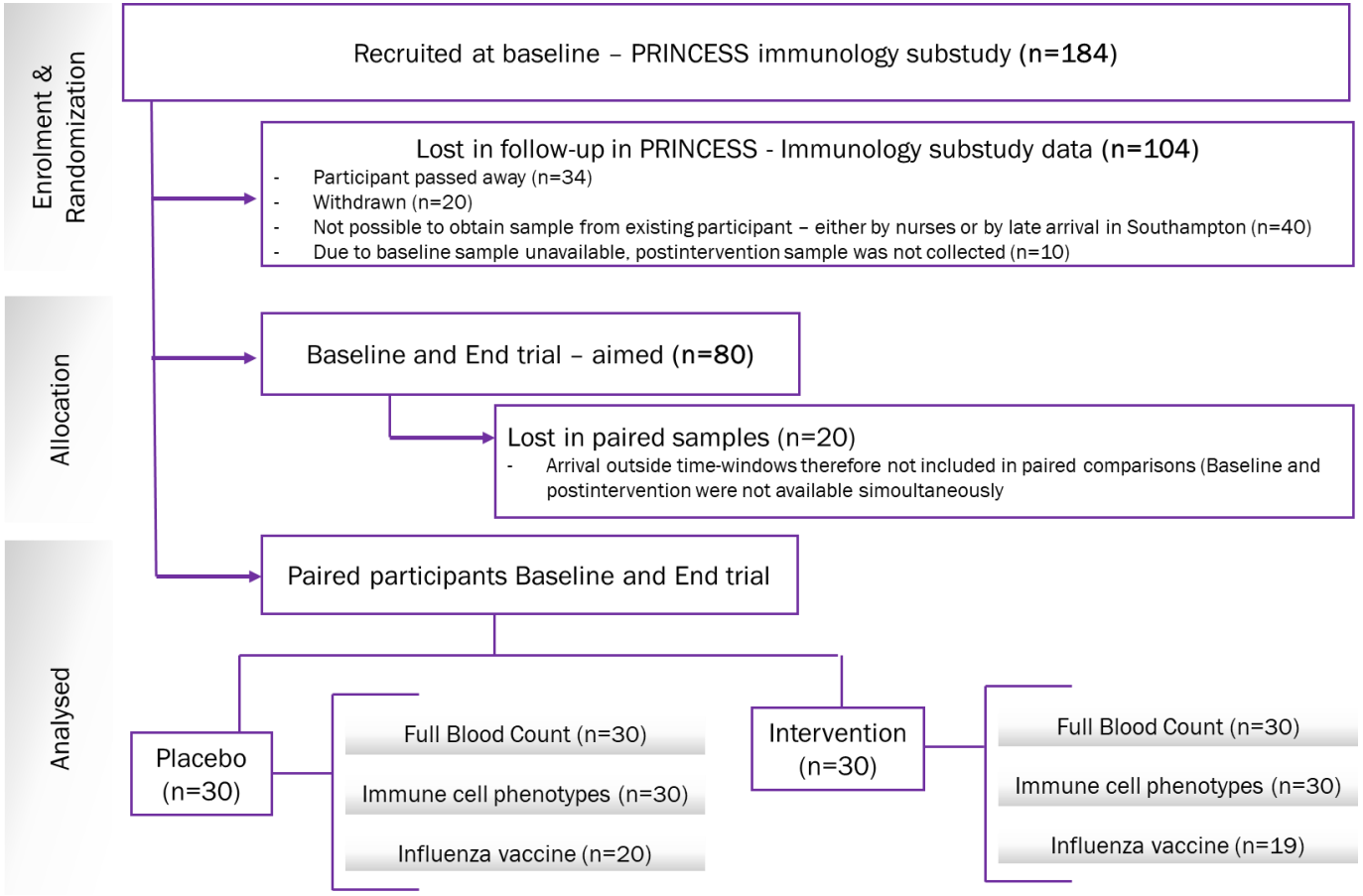
Only available data were analysed; no imputation was performed. Analyses of normality were conducted using the Kolmogorov- Smirnov test and by visualisation of the Gaussian distribution in the histogram plot. Descriptive statistics for all of the variables are presented as median and 25th and 75th percentiles. Non-parametric parameters were log transformed to fit a regression model where analyses were adjusted by allocation (trial arm - either placebo or probiotic), sex and baseline measurement through the analysis of covariance (ANCOVA). Variables that did not fit the model assumptions were analysed through the Mann-Whitney or Kruskal Wallis tests if the variable was numerical, non-parametric and the comparison was performed across groups (placebo or probiotic). Significant effects of the post-intervention outcome were defined as p-values <0.05. Differences between subgroup of analysis were compared with the overall sample using the related-samples Wilcoxon signed rank test.

4.3 Results

4.3.1 Recruitment and loss to follow up in PRINCESS trial – immunology sub-study: FBC, immune cell phenotypes and response to influenza vaccination

Recruitment into the full PRNCESS trial was challenging. It was initially planned to recruit 330 participants into the full PRINCESS trial and 120 participants into the PRINCESS immunology-sub-study; finally 305 participants were recruited into the full PRINCESS trial and 184 into the immunology sub-study considering losses. Subsequently some blood samples did not arrive within the window identified in chapter 2 and so were excluded and some end of study samples were not collected for various reasons. As a result, the number of paired samples (i.e, from individuals at both baseline and post intervention) was 71 for the variables described in this chapter. 38.6% of the participants who entered the trial were lost by/at the end of the trial. Reasons for the losses are identified in Figure 4.2.

Figure 4.2. Consort diagram illustrating number of participants involved at the different stages of the PRINCESS immunology sub-study and showing sample size for full blood count, immune cell phenotyping and influenza vaccination components.



4.3.2 Effect of probiotic intervention on whole blood immune cell numbers measured by Full Blood Count in ECHRs

There was no effect of the probiotic intervention on the numbers of neutrophils, lymphocytes, monocytes, WBC, platelets, eosinophils and basophils in the blood (See Table 4.1). References values are also included in this table for comparison. From the covariates used in the statistical model, baseline value was a significant predictor of the post intervention outcome ($P<0.001$).

Maximum values observed for eosinophils and basophils were above the upper limit of the respective reference values. A more detailed examination of these two parameters highlighted that 2 participants had high eosinophil values at both baseline and post intervention, while 1 participant started with normal values and developed higher values in the post intervention period, this latter participant was allocated in the probiotic group. The same probiotic-allocated participant developed high basophil values post intervention. Two of these 3 participants were residents in the same care home.

Table 4.1. Effects of a probiotic intervention on components of the FBC in elderly care home residents.

Parameter and reference value (10*9/L)	Placebo (n=30)	Probiotic (n=30)	Covariables	Adjusted mean difference (CI 95%)			p-value
Descriptive statistics categorised according to allocation			**ANCOVA –Neutrophils (LogTr)**				
Neutrophils (2.0 - 7.5)	Mean± SE	Mean± SE	Trial Arm	0.006	(-0.058	0.069)	0.861
Baseline	4.8 (0.4)	4.6 (0.4)	Sex	0.017	(-0.047	0.081)	0.593
Post-intervention	4.4 (0.3)	4.7 (0.3)	Baseline	0.520	(0.348	0.691)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA – Lymphocytes (LogTr)**				
Lymphocytes (1.5 - 5.0)	Mean± SE	Mean± SE	Trial Arm	0.018	(-0.047	0.082)	0.583
Baseline	1.4 (0.1)	2.4 (0.5)	Sex	0.007	(-0.055	0.068)	0.826
Post-intervention	1.5 (0.1)	2.5 (0.6)	Baseline	0.796	(0.651	0.940)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA - Monocytes (LogTr)**				
Monocytes (0.2 - 1.0)	Mean± SE	Mean± SE	Trial Arm	-0.023	(-0.104	0.058)	0.566
Baseline	0.5 (0.0)	0.6 (0.0)	Sex	-0.020	(-0.099	0.060)	0.623
Post-intervention	0.5 (0.0)	0.6 (0.1)	Baseline	0.665	(0.403	0.928)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA – WCC (LogTr)**				
WCC (4 – 11.0)	Mean± SE	Mean± SE	Trial Arm	0.014	(-0.035	0.064)	0.563
Baseline	6.9 (0.4)	8.0 (0.6)	Sex	0.006	(-0.044	0.055)	0.824
Post-intervention	7.1 (0.4)	8.2 (0.7)	Baseline	0.702	(0.535	0.868)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA – Platelets (LogTr)**				
Platelets (140 - 400)	Mean± SE	Mean± SE	Trial Arm	0.019	(-0.021	0.060)	0.347
Baseline	281.6 (15.5)	291.7 (22.5)	Sex	-0.008	(-0.054	0.037)	0.718
Post-intervention	267.4 (21.2)	284.0 (15.4)	Baseline	0.832	(0.668	0.995)	<0.001
Descriptive statistics categorised according to allocation			**Mann Whitney Test - Factor (Allocation)**				
Eosinophils (0.0 - 0.5)	Mean± SE	Mean± SE	**Eosinophils**	p-Value			
Baseline	0.2 (0.0)	0.2 (0.0)	Post-intervention	0.816			

Post-intervention	0.2 (0.0)	0.3 (0.0)		
Basophils (0.0 - 0.1)	**Mean± SE**	**Mean± SE**	**Basophils**	**p-Value**
Baseline	0.0 (0.0)	0.1 (0.0)	**Post-intervention**	**0.688**
Post-intervention	0.1 (0.0)	0.1 (0.1)		

Descriptive statistics for FBC obtained from data without normalisation. Data shown as mean±(SE) and extracted from Individual data. ANCOVA modelling used in log transformed (LogTr) variables and used to determine effects of trial arm (probiotic or placebo): p-value obtained from the adjusted analysis of covariance (ANCOVA): adjusted for allocation, sex, baseline value. Post-intervention variable considered dependent outcome. Significant levels were defined as p-values <0.05. Eosinophils and basophils received a different statistical treatment to determine significance due to the nature of the variable (values including zero) thus the Mann Whitney test was used. Significant levels were defined as p-values <0.05.

4.3.3 Effect of probiotic intervention on immunophenotypes in the blood of ECHRs

Immune cell phenotypes were determined as absolute cell count in whole blood. Data are expressed as total counts and are presented in Table 4.2. There was no effect of the probiotic intervention on T cells ($CD45^+/CD3^+$), helper T cells ($CD45^+/CD3^+/CD4^+$), regulatory T cells ($CD45^+/CD3^+/CD4^+/CD8^+/CD25^+/CD127^{+LO}$), cytotoxic T cells ($CD45^+/CD3^+/CD8^+$), activated T cytotoxic cells ($CD45^+/CD3^+/CD8^+/CD25^+$), ratio $CD4^+$:$CD8^+$, NK cells ($CD45^+/CD3^-/CD16^+$), B cells ($CD45^+/CD3^-/CD19^+$), activated B cells ($CD45^+/CD3^-/CD19^+/CD80^+$ and $CD45^+/CD3^-/CD19^+/CD86^+$), monocytes ($CD45^+/$ $CD14^+$), or activated monocytes ($CD45^+/$ $CD14^+/CD80^+$ and $CD45^+/$ $CD14^+/CD86^+$). The baseline values of these parameters were significantly correlated with the post- intervention values.

Table 4.2. Effects of a probiotic intervention on blood immunophenotypes of elderly care home residents.

Parameter (cells/µl)	Placebo (n=30)	Probiotic (n=30)	Covariables	Adjusted mean difference (CI 95%)			p-value
Descriptive statistics categorised according to allocation			**ANCOVA – T cells (LogTr)**				
T cells	Mean± SE	Mean± SE	Trial Arm	0.03	(-0.02	0.08)	0.26
Baseline	1298.3 (50.8)	1337.8 (78.5)	Sex	0.04	(-0.01	0.10)	0.12
Post-intervention	1387.7 (64.9)	1539.1 (89.4)	Baseline	0.65	(0.41	0.90)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA - T helper (LogTr)**				
T helper	Mean± SE	Mean± SE	Trial Arm	0.01	(-0.06	0.07)	0.79
Baseline	886.3 (64.3)	912.4 (82.4)	Sex	0.05	(-0.02	0.11)	0.17
Post-intervention	985.8 (72.6)	1014.5 (79.8)	Baseline	0.79	(0.66	0.93)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA - T regs (LogTr)**				
T regs	Mean± SE	Mean± SE	Trial Arm	0.02	(-0.08	0.12)	0.71
Baseline	87.2 (15.4)	85.7 (14.3)	Sex	-0.05	(-0.16	0.06)	0.34
Postintervention	80.1 (13.5)	84.1 (13.4)	Baseline	0.79	(0.67	0.92)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA - T cytotoxic (LogTr)**				
T cytotoxic	Mean± SE	Mean± SE	Trial Arm	-0.01	(-0.06	0.04)	0.67
Baseline	631.2 (35.2)	767.0 (33.3)	Sex	-0.04	(-0.08	0.00)	0.07
Postintervention	717.3 (38.8)	803.1 (37.9)	Baseline	0.74	(0.58	0.90)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA - Activated T cytotoxic (LogTr)**				
Activated T cytotoxic	Mean± SE	Mean± SE	Trial Arm	-0.02	(-0.09	0.05)	0.59
Baseline	230.9 (18.2)	270.0 (19.2)	Sex	-0.02	(-0.10	0.05)	0.48
Postintervention	278.5 (14.1)	282.3 (20.5)	Baseline	0.10	(-0.05	0.26)	0.19
Descriptive statistics categorised according to allocation			**ANCOVA - RatioCD4⁺:CD8⁺ (LogTr)**				
RatioCD4⁺:CD8⁺	Mean± SE	Mean± SE	Trial Arm	0.02	(-0.05	0.10)	0.56
Baseline	1.4 (0.1)	1.2 (0.1)	Sex	0.09	(0.02	0.16)	0.02

Postintervention	1.4 (0.1)	1.3 (0.1)	Baseline	0.76	(0.56	0.95)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA - NK cells (LogTr)**				
NK cells	Mean± SE	Mean± SE	Trial Arm	0.04	(-0.04	0.12)	0.33
Baseline	80.8 (5.3)	82.4 (5.8)	Sex	0.03	(-0.05	0.11)	0.46
Postintervention	72.6 (4.9)	79.2 (4.2)	Baseline	0.33	(0.04	0.63)	0.03
Descriptive statistics categorised according to allocation			**ANCOVA - B cells (LogTr)**				
B cells	Mean± SE	Mean± SE	Trial Arm	-0.04	(-0.09	0.01)	0.10
Baseline	221.2 (20.1)	240.1 (19.8)	Sex	0.02	(-0.02	0.07)	0.34
Postintervention	224.3 (21.4)	232.3 (20.1)	Baseline	0.84	(0.73	0.95)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA - B cells CD80$^+$ (LogTr)**				
B cells CD80$^+$	Mean± SE	Mean± SE	Trial Arm	0.00	(-0.05	0.05)	0.96
Baseline	137.8 (11.2)	148.5 (16.3)	Sex	-0.01	(-0.05	0.04)	0.81
Postintervention	126.9 (11.6)	142.7 (17.1)	Baseline	0.86	(0.74	0.98)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA - B cells CD86$^+$ (LogTr)**				
B cells CD86$^+$	Mean± SE	Mean± SE	Trial Arm	-0.02	(-0.07	0.03)	0.52
Baseline	141.7 (12.0)	155.8 (17.1)	Sex	-0.03	(-0.08	0.02)	0.23
Postintervention	140.1 (14.0)	151.6 (17.8)	Baseline	0.88	(0.76	1.01)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA – Monocytes (LogTr)**				
Monocytes	Mean± SE	Mean± SE	Trial Arm	-0.02	(-0.11	0.07)	0.60
Baseline	447.6 (31.5)	577.6 (37.7)	Sex	0.00	(-0.09	0.08)	0.92
Postintervention	504.1 (41.4)	569.9 (40.0)	Baseline	0.80	(0.57	1.02)	<0.001
Descriptive statistics categorised according to allocation			**ANCOVA - Monocytes CD80$^+$ (LogTr)**				
Monocytes CD80$^+$	Mean± SE	Mean± SE	Trial Arm	0.02	(-0.11	0.16)	0.75
Baseline	154.8 (20.5)	164.2 (25.9)	Sex	-0.07	(-0.21	0.07)	0.30
Postintervention	112.9 (18.3)	120.4 (23.1)	Baseline	0.71	(0.52	0.91)	<0.001

Descriptive statistics categorised according to allocation			ANCOVA - Monocytes CD86^{+} (LogTr)				
Monocytes CD86^{+}	Mean± SE	Mean± SE	Trial Arm	0.07	(-0.14	0.28)	0.50
Baseline	130.8 (17.9)	122.0 (19.5)	Sex	0.04	(-0.17	0.25)	0.71
Postintervention	110.9 (19.5)	124.4 (22.3)	Baseline	0.87	(0.65	1.08)	<0.001

Descriptive statistics for immunephenotypes obtained from data without normalisation. Data shown as mean±(SE) and extracted from Individual data. ANCOVA modelling used in log transformed (LogTr) variables and used to determine effects of trial arm (probiotic or placebo): p-value obtained from the adjusted analysis of covariance (ANCOVA); adjusted for allocation, sex, baseline values. Post-intervention variable considered dependent outcome. Significant levels were defined as p-values <0.05.

4.3.4 Effect of probiotic intervention on the anti-seasonal influenza virus vaccine antibody response in ECHRs

4.3.4.1 Antibody titres at baseline and pre-vaccination

Vaccine-specific antibodies were measured in serum. Protection against the seasonal influenza virus through vaccination is described as seroprotection (Antibody titre equal to or greater than 40 at commencement of the study - i.e. baseline and pre-influenza vaccination-). A high proportion of the population was already seroprotected. (See section 1.8.2). A comprehensive categorisation according to strain and serostatus is shown in Table 4.3

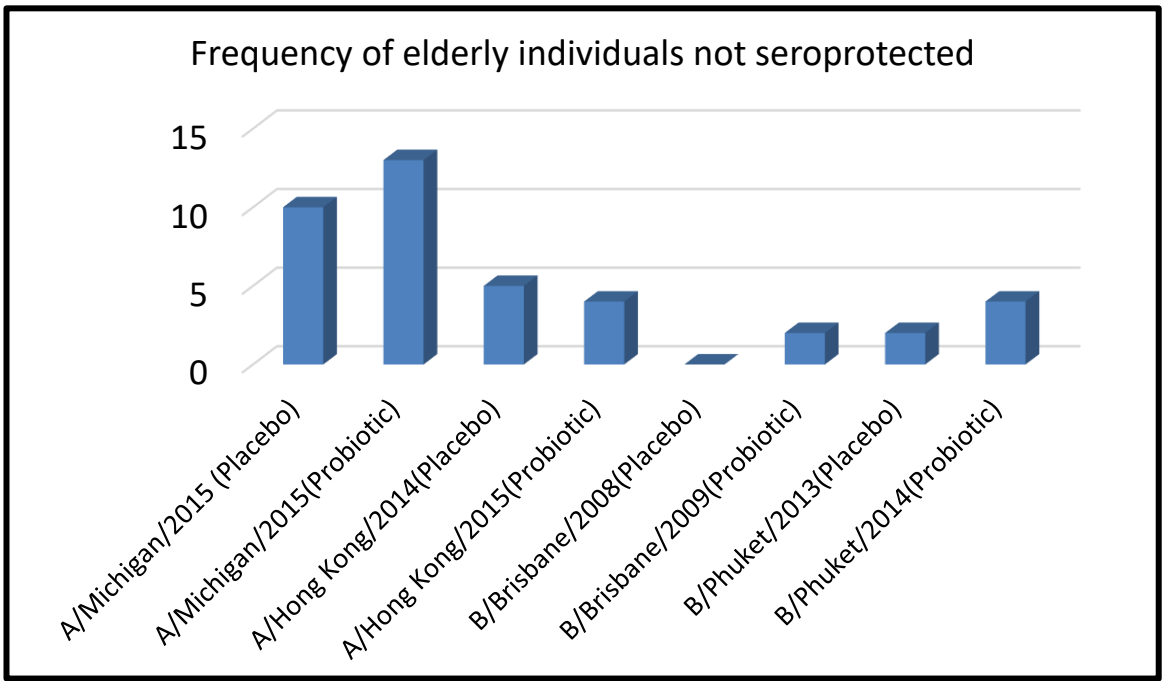
Table 4.3. Seroprotection status pre- and post-vaccination

	Seroprotection status	Pre-vaccine		Post-vaccine	
		n	%	n	%
HAI1_A/Michigan/2015	Negative	23	59.0	12	30.8
	Positive	16	41.0	27	69.2
	Total n		39		
HAI1_A/Hong Kong/2014.	Negative	9	23.1	4	10.3
	Positive	30	76.9	35	89.7
	Total n		39		
HAI1_B/Brisbane/2008	Negative	2	5.1	1	2.6
	Positive	37	94.9	38	97.4
	Total n		39		
HAI1_B/Phuket/2013.	Negative	6	15.4	1	2.6
	Positive	33	84.6	38	97.4
	Total n		39		

Sample size per strain included in the table as shown as “Total n”. Percentages of seroprotection and no seroprotection statuses are presented per strain used in the quadrivalent vaccine. Descriptive analyses are presented. Percentage describes the proportion of individuals according to seroprotection status both positive (Antibody titres≥40) and negative (Antibody titres≤40)

Statistical differences among the no seroprotected status in the pre-vaccination period was analysed. Except for B/Brisbane/2008 (Placebo) where 100% of the individuals were seroprotected, median values were calculated per influenza vaccine strain and either placebo or probiotic: Median values were 10 units of antibody titres and confirmed the negative seroprotection status. No significant differences among placebo and probiotic were found per strain. Figure 4.3. shows the frequency of elderly individuals not seroprotected per strain and trial arm.

Figure 4.3. Pre-vaccine analyses of not seroprotected individuals according to either placebo or probiotic in PRINCESS-immunology sub-study.



Frequency of individuals who were not seroprotected (indicated by having an antibody titre of <40) prior to vaccination – Median value: 10 antibody titres. related-samples Wilcoxon signed rank test. P values as follows: A/Michigan/2015 (p=0.214); A/Hong Kong/2015 (p=1.00); B/Brisbane/2008 (p=0.157); B/Phuket/2014 (p=0.276).

4.3.4.2 Antibody titres pre and post-vaccination

Many individuals showed increased antibody titres post-vaccination. However, there was no effect of the probiotic on the anti-influenza vaccine titres in the post-vaccine period when compared with the placebo group (figure 4.4 to figure 4.7).

Figure 4.4. Anti-influenza vaccine titres for the strain A/Michigan/2015 according to trial arm of intervention

Figure 4.4-A. Anti-influenza vaccine titres for the strain A/Michigan/2015 according to trial arm of intervention – Box Plot

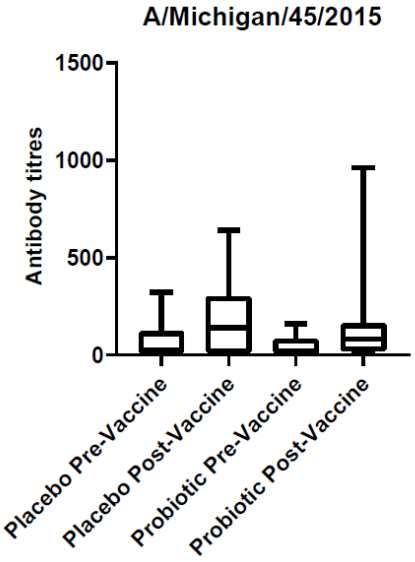
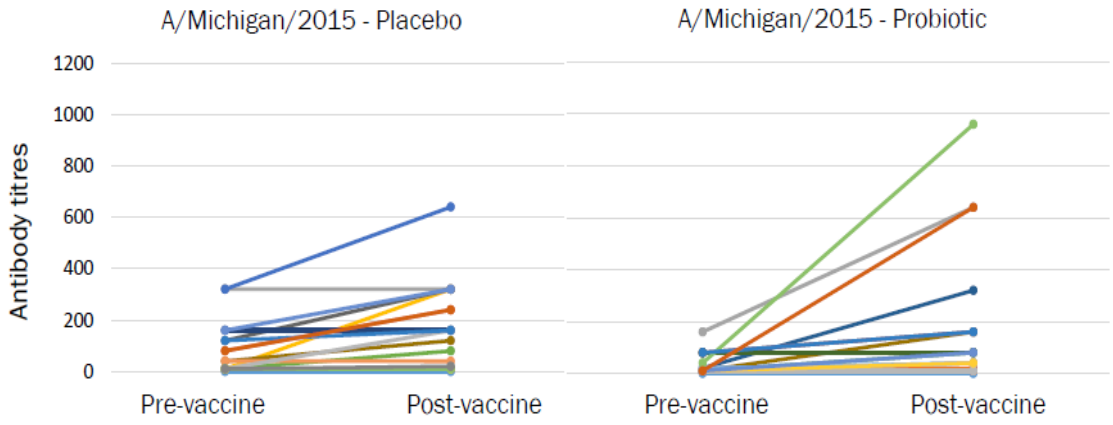


Figure 4.4-B. Anti-influenza vaccine titres for the strain A/Michigan/2015 according to trial arm of intervention – Individual Data



(Placebo n=20; Probiotic n=19) - Individual data: Each line represents one participant according to allocation and levels pre and post vaccination. Statistical significance of the post vaccine antibody titres between groups was determined using the Mann-Whitney test; no significant difference was found (p=0.967).

Figure 4.5. Anti-influenza vaccine titres for the strain A/Hong Kong/2014 according to trial arm of intervention

Figure 4.5-A. Anti-influenza vaccine titres for the strain A/Hong Kong/2014 according to trial arm of intervention – Box Plot

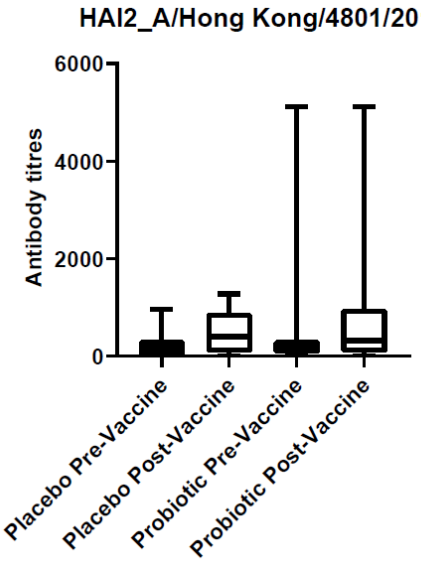
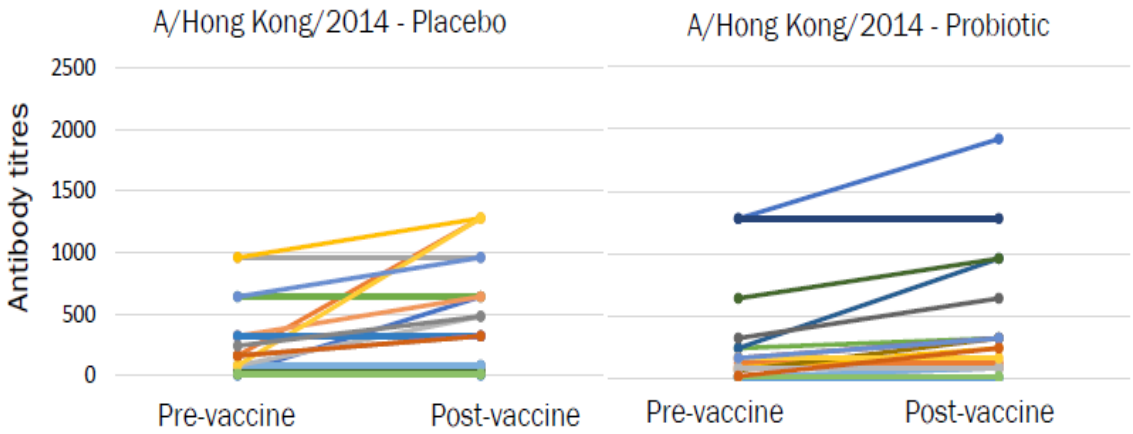


Figure 4.5-B. Anti-influenza vaccine titres for the strain A/Hong Kong/2014 according to trial arm of intervention – Individual Data



(Placebo n=20; Probiotic n=19) Each line represents one participant according to allocation and levels pre and post vaccination. Statistical significance of the post vaccine antibody titres between groups was determined using the Mann-Whitney test; no significant difference was found (p=0.708).

Figure 4.6. Anti-influenza vaccine titres for the strain B/Brisbane/2008 according to trial arm of intervention

Figure 4.6-A. Anti-influenza vaccine titres for the strain B/Brisbane/2008 according to trial arm of intervention – Box Plot

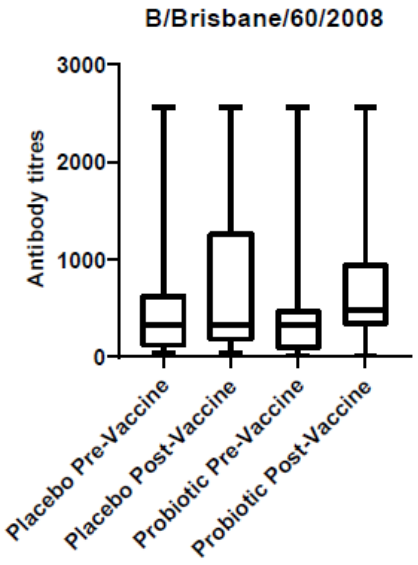
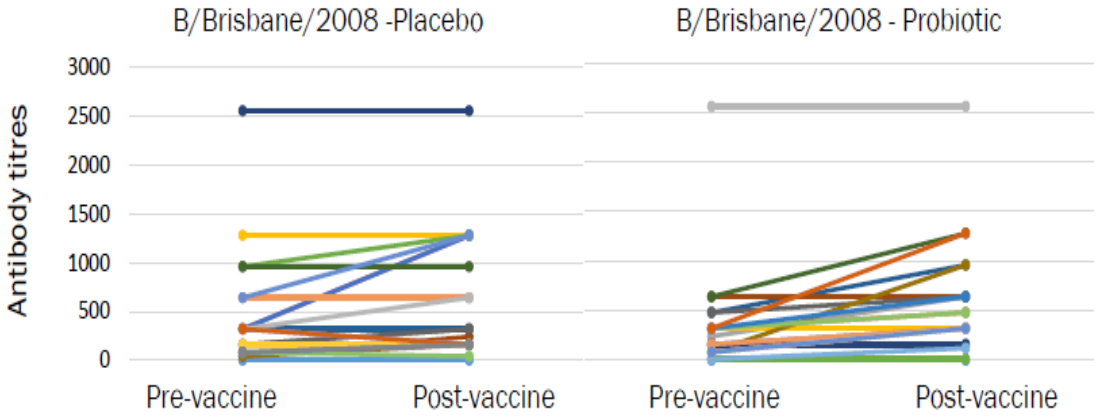


Figure 4.6-B. Anti-influenza vaccine titres for the strain B/Brisbane/2008 according to trial arm of intervention – Individual Data



(Placebo n=20; Probiotic n=19) Each line represents one participant according to allocation and levels pre and post vaccination. Statistical significance of the post vaccine antibody titres between groups was determined using the Mann-Whitney test; no significant difference was found (p=0.901).

Figure 4.7. Anti-influenza vaccine titres for the strain B/Phuket/2013 according to trial arm of intervention

Figure 4.7-A. Anti-influenza vaccine titres for the strain B/Phuket/2013 according to trial arm of intervention – Box Plot

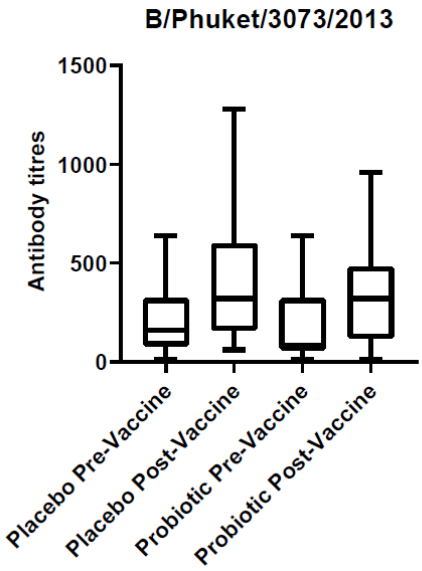
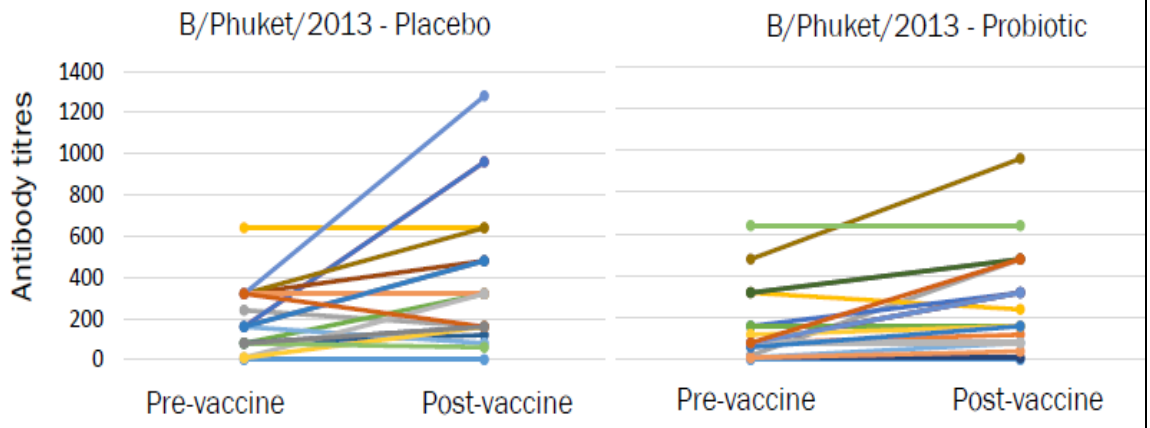


Figure 4.7-B. Anti-influenza vaccine titres for the strain B/Phuket/2013 according to trial arm of intervention – Individual Data

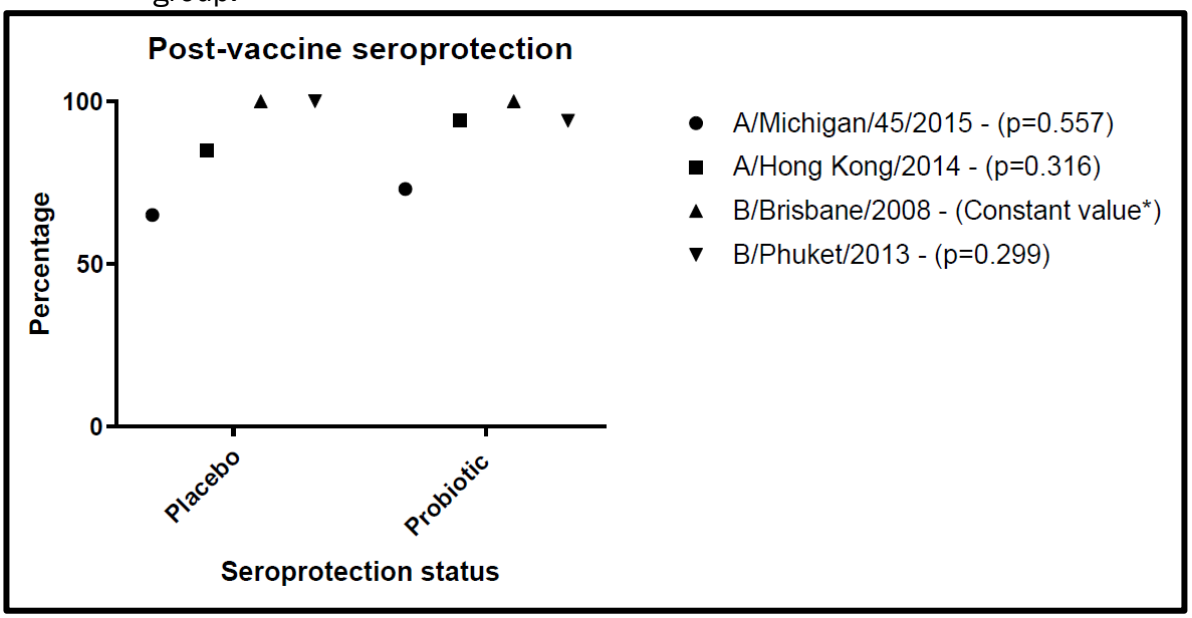


(Placebo n=20; Probiotic n=19) Each line represents one participant according to allocation and levels pre and post vaccination. Statistical significance of the post vaccine antibody titres between groups was determined using the Mann-Whitney test; no significant difference was found (p=0.569).

4.3.4.3 Seroprotection post-vaccination

As expected, there was an increased proportion of participants seroprotected in the post-vaccination period as shown in Table 4.3. There was no significant difference in seroprotection between treatment groups when strains in the quadrivalent influenza vaccine were analysed as shown in Figure 4.8.

Figure 4.8. Post-vaccination seroprotection status (percentage seroprotected) for the quadrivalent anti-influenza virus vaccine strains according to allocation group.



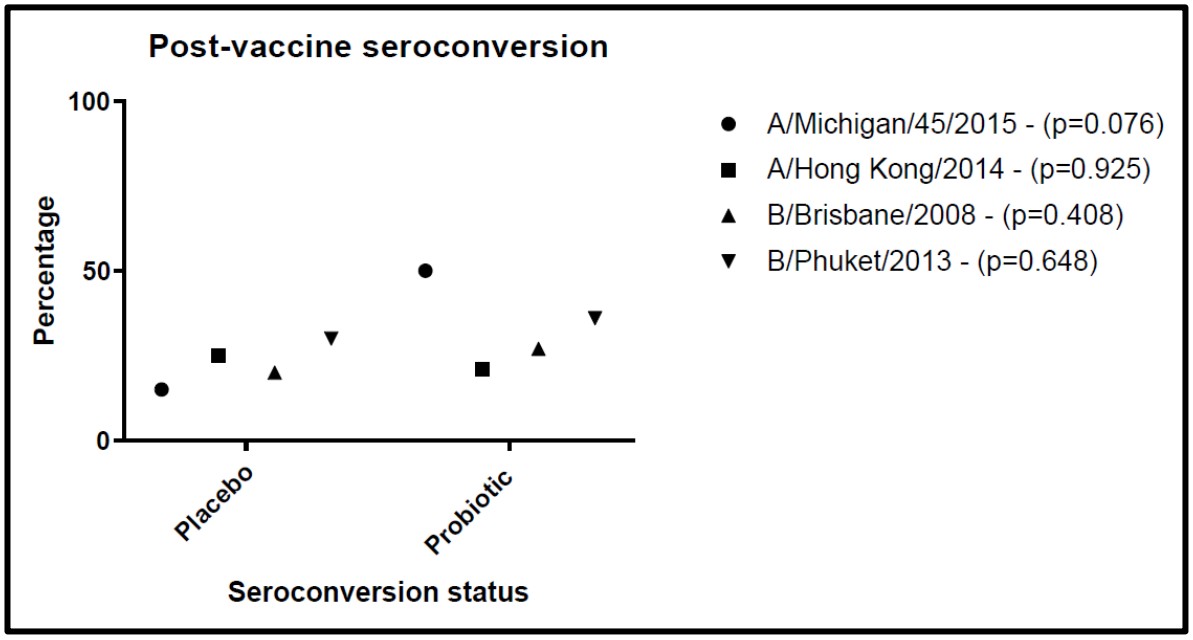
The figure illustrates the percentage of subjects that were seroprotected in the post-vaccination period according to intervention group Placebo (n=20) Probiotic (n=19). Statistical analyses and discrimination of significance was conducted using Chi-Square test analysing percentages of positive seroprotection and comparing these across placebo and probiotic intervention. Significant differences were not observed. In the analyses for the B/Brisbane strain, percentages remained constant. Kruskal-Wallis test was also carried out by considering the Individual value and comparing distributions among both groups. No statistical significance was found (p=0.657).

4.3.4.4 Seroconversion

Seroconversion is defined as the percentage of subjects showing at least a four-fold increase in antibody titre post-vaccination compared with pre-vaccination.

Seroconversion was not different between probiotic and placebo groups (Figure 4.9).

Figure 4.9. Post-vaccine seroconversion for the quadrivalent anti-influenza virus vaccine strains according to allocation group.



The figure illustrates the percentage of subjects that seroconverted in the post-vaccination period according to intervention group Placebo (n=20) Probiotic (n=19). Statistical analyses and discrimination of significance was conducted using Chi-Square test analysing percentages of positive seroconversion and comparing these across placebo and probiotic intervention. Significant differences were not observed.

4.4 Discussion

The findings presented in this chapter indicate that a probiotic combination consisting of *Lactobacillus rhamnosus GG* (LGG) and *Bifidobacterium lactis subs. BB-12* (BB-12) does not exert a significant effect on blood immune cell numbers or subtypes or on responses to seasonal influenza vaccination in ECHRs.

Conducting this multicentre clinical trial identified some difficulties that reduced the availability of samples at the end of the trial to less than half of those available at the beginning. The anticipated loss of participants due to death and withdrawal (estimated at 30%) was realised (32% loss). Such losses must be considered when designing long term intervention trials in vulnerable elderly populations. Importantly, also a further 30% of participants were lost to follow-up because of unforeseen difficulties in collecting end of intervention blood samples. Despite these losses, the sample size achieved in this study is comparable to those of other studies testing probiotic interventions in the elderly [422-425].

It was expected that the probiotic combination of LGG and BB-12 provided to the ECHRs in the PRINCESS - immunology study would improve aspects of the immune system

when compared with the placebo. The hypothesis proposed that lead to the research described in this chapter was based in evidence which has described that LGG and BB-12 are able to modulate immune responses in humans [426, 427], in animal models [428, 429] and *in vitro* [430, 431]. Additional analyses conducted in Cardiff by Mandy Wotton (microbiologist and researcher in the PRINCESS study who was in charge of tissues other than blood collected in the study) indicate that in the probiotic group it was possible to recover higher counts of LGG and BB-12 in faeces (data not shown). Thus both probiotics increased their presence in the gut as per recovery in faecal sample. However, no significant effects on blood immune cell numbers or sub-types or response to seasonal influenza vaccination were observed in the group that followed the intervention when compared against the placebo.

4.4.1 Effect of probiotic intervention on Full Blood Count in ECHRs

According to the analyses of the FBC, at baseline both groups had numbers of neutrophils, monocytes, lymphocytes, WCC and platelets within the normal range, and the same pattern remained in the post-intervention period. However, there were 3 cases of eosinophilia in the post-intervention period. From these 3 cases, 2 participants also had increased basophils in the baseline period (one allocated to placebo and the other allocated to probiotic intervention). The remaining participant started with normal baseline values and was allocated to the probiotic intervention. It is not possible to link the outcomes for the latter participant as causally associated to the LGG+BB-12 intervention. The participant that started with normal values and later developed increased values, although allocated to probiotic intervention; was in the same care home as the participant with increased eosinophil values at baseline and allocated to placebo. This might suggest some environmental conditions (e.g. a particular infectious agent) in the care home that could result in increased eosinophils in some individuals. A study conducted by Haran has demonstrated that there is relative stability among the "signature microbiota" shared among ECHRs. Residents living in closer physical locations tend to share similar strains of organisms [432].

There is evidence supporting an "eosinophilic syndrome" associated with probiotic supplementation. A case report described marked peripheral blood eosinophilia which developed "in a close temporal association with the recent consumption of probiotics" [433]; the two women aged 55 and 65 y were diagnosed with "eosinophilic syndromes". Their medical history reported relative normality without any evident cause of previous

eosinophilia or infections but the recent consumption of a new probiotic brand. In that case report there is hypothesized a link between the probiotic consumption (strain was not identified due to the unclear report given by the patients) and the eosinophilic condition which was assumed to be developed because of exposure to immunogenic substances (assumed to be due to the probiotic consumption). A latter counter-report by Ouwehand suggests that the lack of identification of the probiotic strain makes it incorrect to ascribe such eosinophilic effects to "probiotics" as confirmatory biopsy was not presented as part of the evidence. Additionally, the eosinophilic episode was observed 2 to 4 weeks after the probiotic consumption, a period by which probiotics tend to be cleared from the system [434].

Altogether, this observation points to the critical importance of addressing correctly suitable recipients for probiotic interventions and populations at a higher risk. Due to the relevance of these immune outcomes in the elderly, further investigations are required.

The analysis of covariance showed that the baseline status of neutrophils, monocytes, lymphocytes, WCC and platelets was highly significantly associated with the status at the end of intervention (see Table 5.2). This is an agreement with evidence indicating that the components of the FBC tend to remain stable in one individual despite heterogeneity between individuals [343, 435, 436]. Of course, an infection would perturb these values. It has been described that WCC count in the elderly is clinically useful predicting long term survival, as this parameter is a marker of systemic inflammation [437]. This has also been described for neutrophil values [438].

4.4.2 Effect of probiotic intervention on immunophenotypes in ECHRs

The rationale behind the use of probiotics (and specifically LGG+BB-12) is that they are capable of affecting the immune system through actions on immune cells located in the intestinal wall via communication with TLRs on the enterocytes as well as direct contact with immune cells [439-441]. The link to systemic immune cells is that cells may leave the gut-associated lymphoid tissue and circulate in the bloodstream [442]. It has been shown that the consumption of Bifidobacterium by elderly volunteers (aged 63–84 y) for nine weeks yields an increased number of blood helper T cells ($CD4^+$) and activated ($CD25^+$) T cells as well as NK cells [443]. Additionally, it has been described through a double-blind clinical trial, that healthy individuals (41–81 y; median age: 60 y) exhibited increased NK cell activity following an intervention with *B. lactis* when compared with

the control group (low-fat milk as carrier alone) [444]. These studies show that elderly participants are susceptible to immune enhancement as an action exerted by probiotics. The *Bifidobacterium* strain used in the previously described studies corresponds to HN019.

Although probiotics seem to enhance aspects related to cellular immunity in humans, especially in elderly subjects [445, 446], the findings of the current chapter do not support this effect for the combination of LGG and BB-12. Changes in cellular immunity are often seen in the activation and function of NK cells and cytotoxic T-lymphocytes. This study found that the absolute count of NK cells remained unchanged in the placebo and probiotic groups. Another randomised clinical trial conducted in healthy volunteers (aged 23 to 62 y) who received a fermented milk product containing BB-12 at a dosage of $1x10^{10}$ CFU/day for 3 weeks did not find significant effects on lymphocyte subsets after the BB-12 supplementation period [447]. The current study agrees with these findings although PRINCESS immunology sub-study provided a higher dose (1.3×10^{10} CFU) for a much longer period of time; neither the current study nor the earlier one observed changes in numbers of T cells, B cells, suppressor and cytotoxic T cells and NK cells [447]. It has been suggested in animal models that consumption of LGG induced activation of $CD4^+$ T cells with the subsequent expansion of Tregs, a mechanism that seems to be mediated through butyrate production in the gut [448]. The findings reported here suggest no effects of LGG+BB-12 on the Treg subset pool.

The immune cell phenotyping also comprises a description of the B cell subset which, in conjunction with the responses against the quadrivalent influenza vaccine, aimed to understand the impact on humoral immunity conferred by the consumption of a combination of LGG+BB-12 in the ECHRs. Antibody production is a surrogate indicator of B cell function and it has been described that B cells do not change numbers with age progression, but rather they suffer an impairment in their ability to produce antibodies [449]. It has been shown that reduced gene expression encoding for immunoglobulin class switch recombination as well as altered mechanisms of somatic hypermutation (involved in antibody production by B cells) have a detrimental impact on humoral immune responses [401] with the subsequent reduced memory responses with new antigenic challenges and thus poorer responses to vaccination and to new infections. The assessment of these parameters in the elderly participants indicate that the supplementation with the combination of LGG+BB-12 did not alter B cell numbers.

4.4.3 Effect of probiotic intervention on Influenza vaccination in ECHRs

The current study did not find significant effects of probiotics as none of the parameters measured (antibody titres, seroprotection and seroconversion) were enhanced in the intervention group when compared with the placebo. A noticeable, but non-significant finding, relates to increased seroconversion for the strain A/Michigan/2015 which appears to show an increased response post-vaccine in the probiotic group. The effect of the probiotic on the other viral strains could not be easily assessed due to the high level of seroprotection prior to vaccination, particularly for the strains B/Brisbane and B/Phuket Figure 4.7

The high seroprotection status at baseline and prior to vaccination could have two explanations: either some subjects had already had influenza viral infection with the strains explored, or some of the subjects were still seroprotected from the previous influenza vaccine (2016/2017), whose composition also included A/Hong Kong 2014 H3N2-like virus and B/Brisbane 2008.

It has been described that Lactobacilli and Bifidobacteria are able to enhance mucosal B cell responses and modulate systemic antibody responses in an animal model [451]. It has also been described that the presence of probiotics increases the expression of TLR4 and TLR2 and such changes in pattern recognition receptors can modulate IgA responses in the small intestine [452]. Moreover, a systematic review conducted in humans and analysing the effects of probiotic supplementation on antibody titres following influenza vaccination, found that supplementation with probiotics may enhance influenza antibody titres, importantly, this systematic review and meta-analysis of RCTs was conducted in a large and heterogeneous group of adults including middle-aged adults and included studies of a large variety of probiotic strains which included *Lactobacillus fermentum, casei, plantarum, GG, paracasei, Bifidobacterium longum* and even heat-treated lactic acid bacteria. The authors of this study conclude that it is difficult to discriminate the effects on immune responses towards influenza vaccination in elderly participants although they suggest that probiotics might be a useful strategy [453].

Boge conducted a pilot study in 68 healthy adults (mean age 84 y) in nursing homes and then conducted a confirmatory study in 222 elderly residents in the same setting. It was found that the consumption of a product containing a strain of Lactobacillus (*L. casei*) for 7 weeks in the pilot and then 13 weeks in the main study improved response

to influenza vaccination in the probiotic group compared to the control group by measuring antibody titres and quantifying seroconversion and seroprotection rates. Analyses, although non-significant, revealed that the antibody response was higher in the probiotic group [454]. Likewise, Bosh also conducted a study in 15 healthy adults aged 65 to 85 y in nursing homes, by quantifying antibody titres following an intervention consisting of a strain of Lactobacillus (*L. plantarum*) at a dosage of $5x10^9$ CFU/day for 3 months and found an immunostimulating effect quantified through increased levels of influenza-specific IgA and IgG antibodies [455]. Lastly, another study conducted in hospitalized elderly participants with mean age of 81 y and receiving heat-treated lactic acid bacteria found that the increased presence of Bifidobacterium in the gut was associated with enhanced and positive changes in the antibody titre against the influenza vaccine using strains H1N1 and H3N2 [456]. Thus, it is likely that some probiotic strains, through their effect on microbiota composition, could exert a positive effect on seroprotection and seroconversion in the population. Confirmatory studies are required to extend mechanisms associated with this immune effect. Also a better identification of strains, dosage and duration, especially for the elderly should remain target of study.

4.5 Conclusions

The overall hypothesis related to probiotics as enhancers of the immune function in ECHRs shall be rejected as there were no statistically significant effects exerted by probiotics. A positive finding is that probiotics did not alter the profile in the FBC which indicates parameters that remained within stability. Probiotics did not exert any effect in the immune phenotyping when compared against the placebo intervention. Conclusions point towards a lack of effect exerted by a probiotic intervention consisting of LGG plus BB-12 as these did not have any effect on the parameters analysed not only in the FBC, or blood immunophenotypes, but also in the response towards a quadrivalent version of the influenza vaccine in ECHRs. Interestingly, baseline measurements of FBC parameters and blood immunophenotypes were highly correlated with the end of intervention measurements which indicates that there is high stability of these outcomes over one year of follow-up in these elderly participants. Overall, there was no effect of the probiotic intervention on the response to seasonal influenza vaccination, although there was a non-significant enhancement of seroconversion in response to the A/Michigan strain in the vaccine. The next chapter will further explore the impact of the probiotic intervention on innate and acquired immune cell responses.

Further discussions related to sample size and power calculation will be expanded in chapter 9, general discussions.

Chapter 5 PRINCESS immunology sub-study. Effect of probiotics on biomarkers of immunity in elderly care home residents: Analyses of phagocytic function, immune mediators in plasma and production of immune mediators in whole blood cultures

5.1 Introduction

Immunosenescence and inflammageing, result in altered functionality of immune cells as well as disproportionate inflammatory responses in the elderly [302, 347, 457]. The nature of the ageing process involves physiological and morphological changes that directly affect the components of immunity as well as the inflammatory processes. Because bone marrow and thymus change with ageing, it is likely that immune cell components and their functionality also change [458]. In addition, phagocytic responses tend to decline with aging [341, 459] and this can be associated with higher susceptibility to infections [342].

Aged-related low-grade of inflammation and increased concentration of many inflammatory mediators and markers in the bloodstream [460, 461] might also be linked to physiological and age-related modifications in the gut. Aging progresses with changes in microbiota composition. Higher amounts of proteobacteria found in elderly individuals [462] can increase locally the production of pro-inflammatory cytokines by DCs, which results in a higher local inflammation [109] and dysbiosis [463]. This has overall been linked with higher systemic inflammation [71], worsening the inflammageing condition.

Probiotics have been shown to modify the human microbiota [464]. It has been suggested that by improving the microbial composition and gut functionality, systemic benefits can be achieved by reducing the production of pro-inflammatory cytokines by immune cells such as DCs, monocytes and lymphocytes present in the MALT [465]. The study of the effects of probiotics on the microbiota of elderly individuals suggests that they are safe and that they have a positive impact in the management of common conditions such as constipation [466] [467]. The influence of probiotics on aspects of immune function and low-grade inflammation in the elderly has been little studied in elderly care home residents (ECHRs). It has been hypothesized that age-associated changes in intestinal microbiota and interventions such as probiotics for healthy ageing, might contribute with the improvement of microbiota composition and improve inflammation by reducing the production of cytokines locally and systemically [468-470]. The effect of probiotics on markers of immunity and inflammation require wider exploration in ECHRs. PRINCESS immunology sub-study is a randomised, double-blind, placebo-controlled, clinical trial aimed at studying aspects of immune function (phagocytic responses and production of immune mediators) and markers of inflammation in ECHRs.

5.1.1 Hypothesis

The overarching hypothesis being tested is that the combination of the probiotics LGG and BB-12 will enhance immune function in elderly care home residents. In this chapter the following hypothesis will be tested:

- Probiotics (LGG and BB-12) will increase phagocytosis of blood neutrophils and monocytes
- Probiotics (LGG and BB-12) will decrease circulating concentrations of a panel of inflammatory markers
- Probiotics (LGG and BB-12) will enhance the response of cultured whole blood to a range of immune stimulants.

5.1.2 Aim and objectives

The aim of this research is to compare the effects of a probiotic (LGG + BB-12) intervention with placebo on the outcome variables of interest. The objectives are to assess the following immune parameters:

- Phagocytic function in neutrophils and monocytes
- Inflammatory profile measured in plasma
- Immune mediators produced by whole blood in response to 3 different stimulants.

5.2 Methods

5.2.1 Subjects

Inclusion and exclusion criteria are presented in section 3.2.2. From the participants included in the PRINCESS – Immunology sub-study, only paired samples were included within the analyses herein conducted. Missing data were handled according to the statistical methodology (See section 4.2.4).

5.2.2 Measurement of immune parameters

5.2.2.1 Phagocytosis of *E. coli* by neutrophils and monocytes

The methodology used to assess phagocytosis has been described in 2.2.3.5

5.2.2.2 Assessment of the concentrations of inflammatory markers in plasma

The methodology used to measure the concentrations of inflammatory markers in plasma has been described in section 3.2.5.2.1

5.2.2.3 Assessment of the whole blood responses following stimulation with LPS, PGN and PHA

The methodology used has been described in section 3.2.5.2.2

5.2.3 Statistical methodology

The statistical procedures performed are described in section 4.2.4

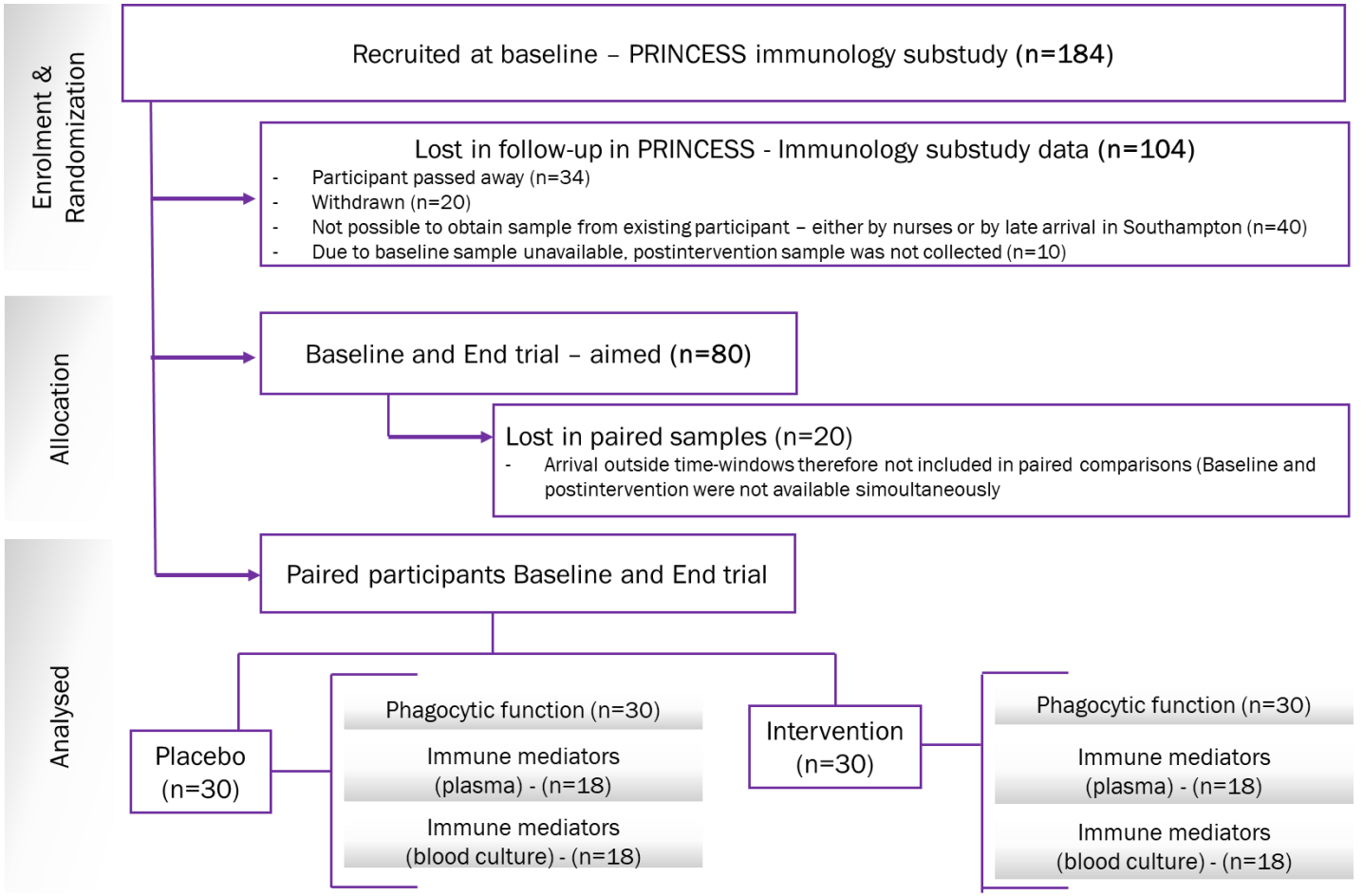
5.3 Results

Overall, there was no effect of the probiotic intervention on any of the immune parameters under investigation in the research described in this chapter.

5.3.1 Recruitment and loss to follow up in PRINCESS trial – immunology sub-study: Phagocytic function, immune mediators measured in plasma and immune mediators measured in whole blood culture.

A greater proportion of samples were lost than expected due to the time-frame of arrival or the small sample volume that was collected. Figure 5.1 shows the flow of participants and samples through the study.

Figure 5.1. Consort diagram illustrating number of participants involved at the different stages of the PRINCESS immunology sub-study and showing sample size for assessment of phagocytic function, immune mediators measured in plasma and whole blood cultures



5.3.2 Phagocytic function of neutrophils and monocytes

No significant effects on phagocytosis were observed with the probiotic intervention when compared with the placebo group as shown in Table 5.1.

Table 5.1. Phagocytic function of neutrophils and monocytes according to trial arm

Parameter	Placebo (n=30)	Probiotic (n=30)	Covariables	Adjusted mean difference (CI 95%)			p-Value
Descriptive statistics categorised according to Trial Arm			**ANCOVA - (PA) Neutrophils (%) (LogTr)**				
Phagocytic activity (PA) Neutrophils (%)	Mean± SE	Mean± SE	Trial Arm	0.006	(-0.028	0.039)	0.727
Baseline	78.7 (1.8)	81.2 (1.4)	Sex	0.024	(-0.010	0.058)	0.158
Post-intervention	81.8 (2.1)	83.1 (2.5)	Baseline	0.207	(0.028	0.386)	0.025
Descriptive statistics categorised according to Trial Arm			**ANCOVA - (PA) Neutrophils (GMFL) (LogTr)**				
Phagocytic activity (PA) Neutrophils (GMFL)	Mean± SE	Mean± SE	Trial Arm	0.014	(-0.052	0.081)	0.666
Baseline	272.8 (10.2)	243.4 (9.5)	Sex	0.057	(-0.008	0.123)	0.084
Post-intervention	247.7 (11.4)	245.2 (14.8)	Baseline	0.398	(0.145	0.650)	0.003
Descriptive statistics categorised according to Trial Arm			**ANCOVA - (PA) Monocytes (%) (LogTr)**				
Phagocytic activity (PA) Monocytes (%)	Mean± SE	Mean± SE	Trial Arm	0.032	(-0.069	0.134)	0.524
Baseline	33.9 (1.6)	28.3 (1.4)	Sex	0.001	(-0.098	0.100)	0.979
Post-intervention	29.6 (1.9)	29.5 (2.1)	Baseline	0.333	(0.100	0.566)	0.006
Descriptive statistics categorised according to Trial Arm			**ANCOVA - (PA) Monocytes (GMFL) (LogTr)**				
Phagocytic activity (PA) Monocytes (GMFL)	Mean± SE	Mean± SE	Trial Arm	0.028	(-0.048	0.104)	0.465
Baseline	182.3 (8.7)	184.3 (11.0)	Sex	-0.013	(-0.089	0.063)	0.738
Post-intervention	174 (9.5)	184.9 (11.6)	Baseline	0.039	(-0.176	0.254)	0.070

Descriptive statistics for phagocytic assessment obtained from data without normalisation. .Data shown as mean±(SE) and extracted from Individual data. ANCOVA modelling used in log transformed (LogTr) variables and used to determine effects of trial arm (probiotic or placebo):p-Value obtained from the adjusted analysis of covariance (ANCOVA). Adjusted for Trial Arm; Sex; baseline values. Post-intervention variable considered dependent outcome. Significant levels were defined as p-values <0.05. GMFL=Geometric Mean Fluorescence intensity

5.3.3 Plasma immune mediators

No significant effects on plasma immune and inflammatory mediator concentrations were observed with the probiotic intervention as shown in Table 5.2.

Table 5.2. Concentrations of plasma immune mediators assessed according to Trial Arm.

Parameter	Placebo (n=18)	Probiotic (n=18)	Covariables	Adjusted mean difference (CI 95%)			p-Value
Descriptive statistics categorised according to trial arm			ANCOVA - ICAM-1 (LogTr)				
ICAM-1 (ng/ml)	Mean± SE	Mean± SE	Trial arm	0.042	-0.032	0.117	0.252
Baseline	459.8 (41.8)	424.7 (33.1)	Gender	0.066	-0.01	0.142	0.087
Post-intervention	424.8 (64.4)	440.1 (53.2)	Baseline	0.916	0.740	1.092	<0.0001
Descriptive statistics categorised according to trial arm			ANCOVA - IL-1ra (LogTr)				
IL-1ra (ng/ml)	Mean± SE	Mean± SE	Trial arm	0.032	-0.148	0.212	0.718
Baseline	2.24 (0.34)	2.23 (0.27)	Gender	0.120	-0.062	0.303	0.189
Post-intervention	2.49 (0.68)	2.52 (0.44)	Baseline	0.761	0.496	1.026	<0.0001
Descriptive statistics categorised according to trial arm			ANCOVA - E-Selectin (LogTr)				
E-Selectin (ng/ml)	Mean± SE	Mean± SE	Trial arm	0.011	-0.108	0.129	0.853
Baseline	25.0 (2.2)	25.1 (1.9)	Gender	-0.032	-0.150	0.087	0.591
Post-intervention	24.2 (2.7)	25.4 (3.8)	Baseline	0.757	0.523	0.991	<0.0001
Descriptive statistics categorised according to trial arm			ANCOVA - VCAM-1 (LogTr)				
VCAM-1 (ng/ml)	Mean± SE	Mean± SE	Trial arm	-0.003	-0.120	0.115	0.964
Baseline	1040.4 (74.5)	803.8 (52.6)	Gender	0.025	-0.092	0.141	0.671
Post- intervention	1151.5 (199.5)	913.9 (108.0)	Baseline	1.013	0.693	1.334	<0.0001
Descriptive statistics categorised according to trial arm			ANCOVA - MCP-1 (LogTr)				
MCP-1 (ng/ml)	Mean± SE	Mean± SE	Trial arm	0.073	-0.047	0.193	0.224
Baseline	0.4 (0.04)	0.4 (0.03)	Gender	0.049	-0.074	0.172	0.423
Post-intervention	0.4 (0.05)	0.4 (0.04)	Baseline	0.670	0.348	0.991	<0.0001
Descriptive statistics categorised according to trial arm			ANCOVA - IP-10 (LogTr)				

IP-10 (ng/ml)	Mean± SE	Mean± SE	Trial arm	-0.002	-0.162	0.158	0.981
Baseline	0.2 (0.01)	0.2 (0.01)	Gender	-0.008	-0.176	0.16	0.923
Post-intervention	0.1 (0.02)	0.1 (0.01)	Baseline	0.409	-0.016	0.834	0.059
Descriptive statistics categorised according to trial arm			ANCOVA - IL-17A (LogTr)				
IL-17A (ng/ml)	Mean± SE	Mean± SE	Trial arm	0.076	-0.191	0.342	0.567
Baseline	2.3 (0.5)	2.5 (0.4)	Gender	-0.183	-0.445	0.08	0.166
Post-intervention	3.0 (0.8)	3.1 (0.7)	Baseline	0.353	0.041	0.664	0.028
Descriptive statistics categorised according to trial arm			ANCOVA - TNFR-II (LogTr)				
TNFR-II (ng/ml)	Mean± SE	Mean± SE	Trial arm	0.016	-0.075	0.106	0.728
Baseline	4.0 (0.3)	5.0 (0.4)	Gender	0.070	-0.019	0.159	0.120
Post-intervention	3.3 (0.4)	4.0 (0.5)	Baseline	0.685	0.473	0.898	<0.0001
Descriptive statistics categorised according to trial arm			ANCOVA - IL-6 (LogTr)				
IL-6 (pg/ml)	Mean± SE	Mean± SE	Trial arm	-0.006	-0.299	0.287	0.966
Baseline	7.9 (1.5)	12.5 (4.2)	Gender	0.049	-0.246	0.344	0.738
Post-intervention	9.8 (3.4)	6.6 (1.6)	Baseline	0.199	-0.221	0.62	0.340
Descriptive statistics categorised according to trial arm			ANCOVA - IL-10 (LogTr)				
IL-10 (pg/ml)	Mean± SE	Mean± SE	Trial arm	-0.098	-0.369	0.172	0.463
Baseline	0.9 (0.2)	0.9 (0.1)	Gender	0.342	0.065	0.62	0.017
Post-intervention	0.7 (0.1)	0.7 (0.2)	Baseline	0.639	0.361	0.917	<0.0001
Descriptive statistics categorised according to trial arm			ANCOVA - IL-12p70 (LogTr)				
IL-12p70 (pg/ml)	Mean± SE	Mean± SE	Trial arm	0.154	-0.285	0.593	0.448
Baseline	1.5 (0.3)	1.6 (0.2)	Gender	-0.087	-0.554	0.381	0.685
Post-intervention	1.2 (0.4)	1.6 (0.4)	Baseline	0.268	-0.607	1.143	0.506
Descriptive statistics categorised according to trial arm			ANCOVA - TNF- α (LogTr)				
TNF-α (pg/ml)	Mean± SE	Mean± SE	Trial arm	0	-0.076	0.077	0.991

Baseline	17.0 (1.1)	19.0 (1.6)	Gender	0.052	-0.03	0.135	0.205
Post-intervention	17.1 (1.2)	17.4 (1.2)	Baseline	0.626	0.325	0.927	<0.0001

Descriptive statistics for plasma immune mediators obtained from data without normalisation. Data shown as mean±(SE) and extracted from Individual data. ANCOVA modelling used in log transformed (LogTr) variables and used to determine effects of trial arm (probiotic or placebo): p-Value obtained from the adjusted analysis of covariance (ANCOVA). Adjusted for Trial Arm; Sex; baseline values. Post-intervention variable considered dependent outcome. Significant levels were defined as p-values <0.05.

5.3.4 Immune mediators measured in whole blood culture following stimulation with LPS, PGN and PHA

Immune mediators produced in whole blood culture stimulated with LPS, PGN and PHA did not show any significant effect with the probiotic intervention when compared with the placebo group (Table 5.3, Table 5.4, Table 5.5; respectively).

Table 5.3. Concentrations of immune mediators assessed in supernatants from whole blood cultures (WBC) stimulated with LPS according to Trial Arm.

Parameter	Placebo (n=18)	Probiotic (n=18)	Covariables	Adjusted mean difference (CI 95%)			p-Value
Descriptive statistics categorised according to Trial Arm			**ANCOVA – IL-10 LPS stimulated WBC (LogTr)**				
IL-10 (ng/ml)	Mean± SE	Mean± SE	Trial Arm	-0.044	(-0.301	0.213)	0.730
Baseline IL-10	4.3 (1.7)	4.2 (1.4)	Sex	-0.127	(-0.388	0.134)	0.328
Post-intervention IL-10	3.7 (0.7)	3.9 (0.8)	Baseline	0.376	(0.128	0.625)	0.004
Descriptive statistics categorised according to Trial Arm			**ANCOVA – TNF-α LPS stimulated WBC (LogTr)**				
TNF-α (ng/ml)	Mean± SE	Mean± SE	Trial Arm	-0.051	(-0.28	0.179)	0.655
Baseline TNF-α	10.9 (2.3)	13.7 (2.6)	Sex	-0.099	(-0.326	0.129)	0.384
Post-intervention TNF-α	11.4 (1.9)	12.1 (2.4)	Baseline	0.249	(-0.053	0.551)	0.103
Descriptive statistics categorised according to Trial Arm			**ANCOVA – IL-6 LPS stimulated WBC (LogTr)**				
IL-6 (ng/ml)	Mean± SE	Mean± SE	Trial Arm	-0.037	(-0.209	0.135)	0.664
Baseline IL-6	40.2 (5.3)	47.6 (6.9)	Sex	-0.081	(-0.256	0.094)	0.351
Post-intervention IL-6	43.8 (4.6)	45.7 (6.5)	Baseline	0.261	(-0.036	0.557)	0.083
Descriptive statistics categorised according to Trial Arm			**ANCOVA – IL-1β LPS stimulated WBC (LogTr)**				
IL-1β (ng/ml)	Mean± SE	Mean± SE	Trial Arm	0.095	(-0.205	0.395)	0.522
Baseline IL-1β	3.8 (0.8)	6.4 (2.8)	Sex	-0.274	(-0.576	0.029)	0.075
Post-intervention IL-1β	4.3 (0.7)	15.1 (8.8)	Baseline	0.171	(-0.238	0.581)	0.399

Descriptive statistics for immune mediators assessed in supernatants from whole blood cultures stimulated with LPS obtained from data without normalisation.Data shown as mean±(SE) and extracted from Individual data. ANCOVA modelling used in log transformed (LogTr) variables and used to determine effects of trial arm (probiotic or placebo): p-Value obtained from the adjusted analysis of covariance (ANCOVA). Adjusted for Trial Arm; Sex; baseline values. Post-intervention variable considered dependent outcome. Significant levels were defined as p-values <0.05.

Table 5.4. Concentrations of immune mediators assessed in supernatants from whole blood cultures stimulated with PGN according to Trial Arm.

Parameter	Placebo (n=18)	Probiotic (n=18)	Covariables	Adjusted mean difference (CI 95%)			p-Value
Descriptive statistics categorised according to Trial Arm			**ANCOVA – IL-10 PGN stimulated WBC (LogTr)**				
IL-10 (pg/ml)	Mean± SE	Mean± SE	Trial Arm	-0.103	-0.349	0.142	0.396
Baseline IL-10	345.6 (57.6)	583.3 (132.5)	Sex	-0.272	-0.524	-0.02	0.035
Post-intervention IL-10	577.9 (93.4)	504.0 (98.9)	Baseline	-0.354	-0.614	-0.094	0.009
Descriptive statistics categorised according to Trial Arm			**ANCOVA – TNF-α PGN stimulated WBC (LogTr)**				
TNF-α (pg/ml)	Mean± SE	Mean± SE	Trial Arm	-0.123	-0.407	0.162	0.386
Baseline TNF-α	2376.6 (726.4)	3765.8 (1002.9)	Sex	-0.114	-0.393	0.166	0.413
Post-intervention TNF-α	2675 (494.9)	2129.7 (407.4)	Baseline	-0.061	-0.352	0.23	0.672
Descriptive statistics categorised according to Trial Arm			**ANCOVA – IL-6 PGN stimulated WBC (LogTr)**				
IL-6 (ng/ml)	Mean± SE	Mean± SE	Trial Arm	-0.12	-0.401	0.16	0.388
Baseline IL-6	36.2 (7.2)	70.9 (17.7)	Sex	-0.274	-0.556	0.009	0.057
Post-intervention IL-6	54.9 (7.3)	54.0 (10.3)	Baseline	-0.148	-0.457	0.162	0.339
Descriptive statistics categorised according to Trial Arm			**ANCOVA – IL-1β PGN stimulated WBC (LogTr)**				
IL-1β (pg/ml)	Mean± SE	Mean± SE	Trial	-0.244	-0.639	0.15	0.216
Baseline IL-1β	165.7 (45.1)	321.4 (123.0)	Sex	-0.137	-0.54	0.266	0.492
Post-intervention IL-1β	339.7 (93.8)	214.2 (60.7)	Baseline	-0.049	-0.316	0.218	0.711

Descriptive statistics for immune mediators assessed in supernatants from whole blood cultures stimulated with PGN obtained from data without normalisation. Data shown as mean±(SE) and extracted from Individual data. ANCOVA modelling used in log transformed (LogTr) variables and used to determine effects of trial arm (probiotic or placebo): p-Value obtained from the adjusted analysis of covariance (ANCOVA). Adjusted for Trial Arm; Sex; baseline values. Post-intervention variable considered dependent outcome. Significant levels were defined as p-values <0.05.

Table 5.5. Concentrations of immune mediators assessed in supernatants from whole blood cultures stimulated with PHA according to Trial Arm.

Parameter	Placebo (n=18)	Probiotic (n=18)	Covariables	Adjusted mean difference (CI 95%)			p-Value
Descriptive statistics categorised according to Trial Arm			**ANCOVA – IFN-γ PHA stimulated WBC (LogTr)**				
IFN-γ (pg/ml)	Mean± SE	Mean± SE	Trial Arm	0.045	-0.777	0.867	0.912
Baseline IFN-γ	23.2 (17.1)	21.3 ()	Sex	0.079	-0.713	0.870	0.841
Post-intervention IFN-γ	18.5 (9.4)	16.5 ()	Baseline	0.383	-0.019	0.785	0.061
Descriptive statistics categorised according to Trial Arm			**ANCOVA – TNF-α PHA stimulated WBC (LogTr)**				
TNF-α (pg/ml)	Mean± SE	Mean± SE	Trial Arm	-0.024	-0.197	0.148	0.776
Baseline TNF-α	1342.5 (155.8)	2013.2 ()	Sex	0.015	-0.150	0.179	0.854
Post-intervention TNF-α	1804.2 (223.5)	2094.9 ()	Baseline	0.298	-0.015	0.610	0.061

Descriptive statistics for immune mediators assessed in supernatants from whole blood cultures stimulated with PHA obtained from data without normalisation.Data shown as mean±(SE) and extracted from Individual data. ANCOVA modelling used in log transformed (LogTr) variables and used to determine effects of trial arm (probiotic or placebo): p-Value obtained from the adjusted analysis of covariance (ANCOVA). Adjusted for Trial Arm; Sex; baseline values. Post-intervention variable considered dependent outcome. Significant levels were defined as p-values <0.05.

5.4 Discussion

Inflammageing and the increased concentrations of inflammatory markers in the bloodstream of the elderly is undoubtedly associated with lower quality of life, frailty -as demonstrated in chapter 3, and mortality [471]. It has been suggested that the correction of the persistent inflammatory condition can be achieved via gut microbiota and diet, with a protective role conferred by probiotics [472]. In fact it has been described that probiotics can control and even reverse some inflammatory conditions [473, 474]. Furthermore, probiotics may influence health through increased immune activity measured such as an augmented phagocytic response of monocytes and granulocytes [443, 475-477]. Despite evidence of the potential positive effects exerted by probiotics, the current study did not detect any significant effect exerted by the combination of LGG and BB-12 on any of the immune or inflammatory markers assessed in a community of ECHRs.

Phagocytosis of foreign pathogens and of apoptotic cells (so-called autologous debris) is mediated by different sets of phagocytic receptors to induce a pro- or anti-inflammatory response. Phagocytosis of infectious pathogens is modulated through TLRs and this leads to release of pro-inflammatory cytokines. In contrast, apoptotic cells or cellular debris are internalized through different receptors such as phosphatidylserine which trigger immunosuppressive signalling with the release of anti-inflammatory cytokines [341]. Heat-inactivated *L. rhamnosus* has been shown to enhance activation of TLRs in human macrophages [478]. The findings herein presented related to the phagocytic function indicate that probiotics (or at least the combination of LGG and BB-12) do not increase the phagocytic activity of monocytes or neutrophils in ECHRs.

Median ICAM-1 concentration was decreased by 10% post-intervention in the probiotic group. Although this finding was not significant, this adhesion molecule is responsible for monocyte adhesion which is a process induced in low-grade inflammation and that has been linked to common conditions in the elderly like frailty [349, 479], as was confirmed in the cross-sectional analysis presented in chapter 3. The disruption of the production of ICAM-1 exerts a protection on the vascular system by attenuating the pathophysiological process of cardiovascular diseases which involves vascular inflammation, as described by Lee [479]. Methods to inhibit vascular inflammation through inhibition of ICAM-1 would help in reducing common causes of mortality in the elderly. The association of ICAM-1 with frailty suggests that leukocyte migration and

inflammation cascade activation might contribute to frailty, in addition to monocyte/macrophage-mediated immuno-inflammation [479]. Studies *in vitro* have shown anti-inflammatory effects and mechanisms of single and combined probiotics. Although *L. acidophilus* did not decrease ICAM-1 concentrations by its own, the strain enhanced the inhibitory efficacy of *B. animalis* subsp. lactis as the researchers reported VCAM-1 reduction [480].

Owehand *et al*. conducted a study in 55 institutionalised elderly individuals, and showed that the serum levels of IL-10 decreased in those supplemented with BB-12 for 6 months while there was no effect on TNF-alpha concentrations [481]. The lack of effect of BB-12 on TNF-α agrees with the lack of effect of the combination of LGG and BB-12 in the current study. However, their observation of decreased IL-10 suggests that a modest anti-inflammatory effect might be induced by BB-12. This was not seen in the current study. It is possible that the presence of LGG mitigates the effect of BB-12. This requires further investigation.

The cytokines and other immune mediators measured in plasma and after stimulation of whole blood cultures with different immune stimulants have various roles in inflammation and host defence. Overall no significant effect of the probiotic intervention was observed on any of these mediators.

It has been suggested that probiotic bacteria can modify pattern-recognition receptor expression as well as the cytokine profile in a human macrophage model challenged with LPS [482]. Other studies have demonstrated that BB-12 is able to influence the maturation of dendritic cells to a similar or even higher degree than LPS measured by surface expression markers [483]. Cell-free supernatant only had a weak or no effect on maturation of dendritic cells, meaning that the direct interaction with the organism was partially responsible for the outcomes observed. Expression of cytokines varied to a great extent depending on the strain, however, BB-12 demonstrated induction of IL-12 and TNF-α to a high degree and IL-10 to a low degree. In PBMCs, BB-12 induced high levels of IL-10, IFN-γ and TNF-α [483]. Other studies have shown that BB-12 is able to induce all cytokines tested (IL-1β, IL-6, IL-10, IL-12 and IFN-γ) in a dose-dependent manner [484]. Studies in animal models have found that LGG is able to decrease TNF-alpha production induced by LPS in murine macrophages [485]. Karamese *et al*. used a rat model and administered a mixture of Lactobacillus and Bifidobacterium species to evaluate their immunomodulatory effects [486]. This research showed that IL-10 was upregulated whilst TNF-alpha and IL-6 were downregulated. Differences between the

findings of the current study and of these previous studies most likely relate to the different experimental models used.

5.5 Conclusion

The overall hypothesis related to probiotics as enhancers of the immune function in ECHRs shall be rejected as there were no statistically significant effects exerted by probiotics on the immune parameters examined in this chapter. The current study indicates that the combination of LGG and BB-12 at a total dose of 1.3 x 10^{10} CFU per gram through approximately one year of consumption does not significantly affect ex vivo monocyte or neutrophil phagocytosis, circulating markers of inflammation or whole blood responses to different immune stimulants in ECHRs. Further discussions related to sample size and power calculation will be expanded in chapter 9, general discussions.

Chapter 6 Caco-2 cells as an *in vitro* model of intestinal barrier inflammation and permeability

6.1 Introduction

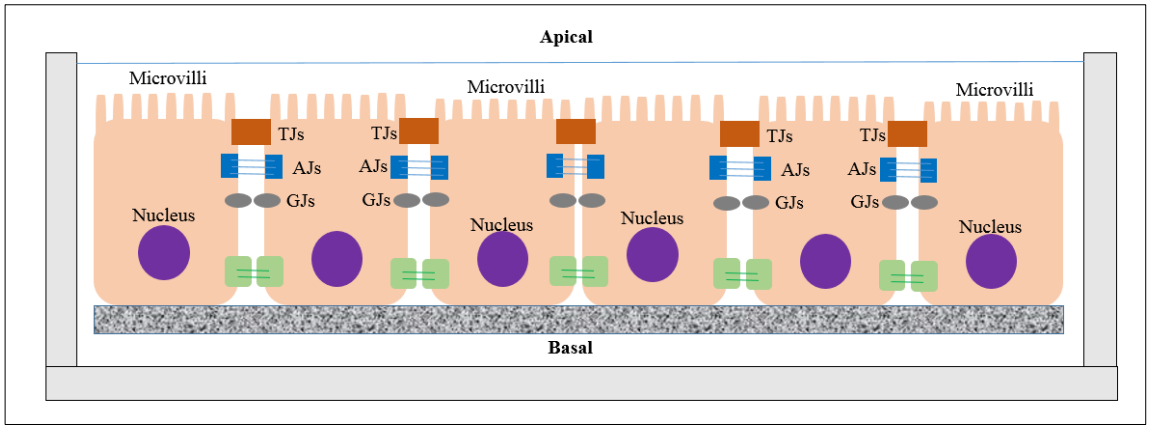
Clinical trials are crucial methodological designs to understand the effect of nutrients on the human body -and particularly the immune system- with the purpose of advancing the efficiency of public health and medical interventions as well as preventive strategies. Understanding the interaction of nutrients and the immune system is a scientific process that starts through the comprehensive study of one of the most important defensive and protective barriers for the body: the gut epithelium. Extensive evidence has shown that a disrupted gut barrier is the starting point that allows pathogenic bacterial translocation [487, 488] and impairs absorption of nutrients [489]; such disruption has an impact on the immune response [471, 490].

Relevant clinical data have provided evidence for the link between a dysfunctional gut and an altered immune response in both certain diseases and the ageing process. For instance, people with Crohn's disease – a gastrointestinal inflammatory disease - often present with co-current immune disturbances. Crohn's patients suffer from gut epithelial barrier disruption, noticeable by damage to intercellular tight junctions [491], and this decreased mucosal integrity exposes the mucosal immune system to bowel contents. It has been identified through biopsies that these patients present with an accumulation of Th1 type T-cells and local production of pro-inflammatory cytokines [492]. Moreover, Crohn's disease proceeds with a disproportionate immune reaction whereby high serum and mucosal concentrations of pro-inflammatory cytokines such as TNF-alpha have been described [493]. Crohn's disease together with ulcerative colitis are categorised as inflammatory bowel diseases (IBD) which have as a commonality a local imbalance among pro-inflammatory and anti-inflammatory cytokines together with neutrophil and monocyte disturbance where secretion of oxygen radicals and enzymes leads to tissue damage [494]. The understanding of IBD in the context of ageing is also relevant as the population of aged individuals with this diagnosis has been increasing, possibly as a reflection of this fast-growing group and the increased number of aged persons [495]. Knowledge about the physiological changes in the geriatric group has also led to the understanding that decreased mucus production, shortened microvilli and the generalised immune changes (See chapter 1) are linked to the alterations observed in the epithelial tissue of the gut. *In vitro* experiments can help to expand the mechanistic understanding of the processes involved. Gut epithelial models and their disruption in response to inflammatory stimuli which mimic the local burden of cytokines seen in different gut-associated diseases are useful in this regard. More expanded and complex

models can address the interaction of the gut epithelium and immune cells to understand their bidirectional communication and integrate this work in the context of gut biopsies, clinical trials and clinical evidence. However, a first step commences with the identification of the effects exerted by inflammation in these cells.

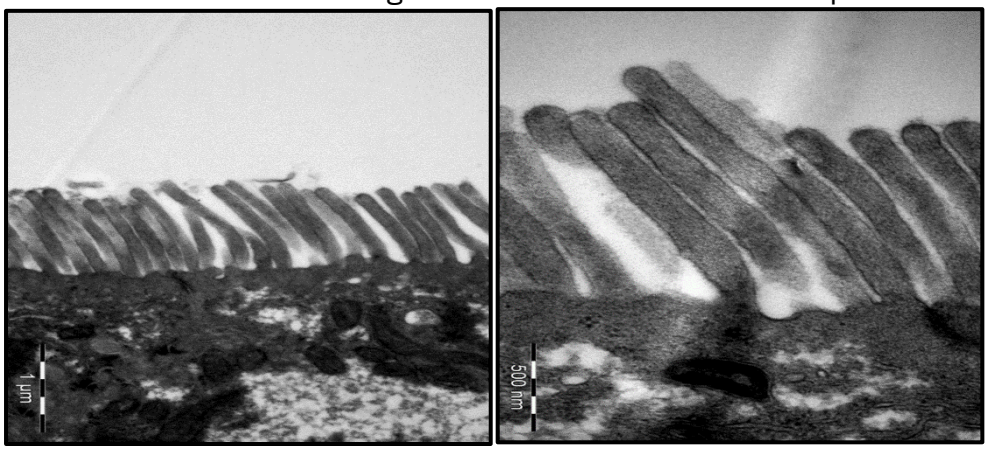
The Caco-2 cell line is derived from a human colorectal adenocarcinoma and has been extensively used in the study of the human gut epithelium since the cells form an epithelial monolayer [496-498]. This single monolayer mimics the human intestinal epithelium as it retains many of the properties of the intestinal barrier. This culture system differentiates to a morphology similar to the human small intestine as cells grow as a monolayer and then become polarised with apical and basolateral sides, as the diagrammatic representation in Figure 6.1 reflects. On the apical – so-called, luminal side - of the monolayer, the brush border microvilli extend perpendicularly from the surface of the monolayer. The cells also develop tight junctions and brush border microvilli as shown in Figure 6.2 and Figure 6.3 through transmission electron microscopy imaging (G. Salim, 2018 unpublished data). The disruption of the gut epithelial monolayer is related to the damage of structural proteins in the tight junctions (See Figure 6.4 to identify Tight Junctions (TJs) structural interplay) which leads to increased transcellular permeability.

Figure 6.1. Diagrammatic representation of the Caco-2 cell culture system as a model of the gut epithelium.



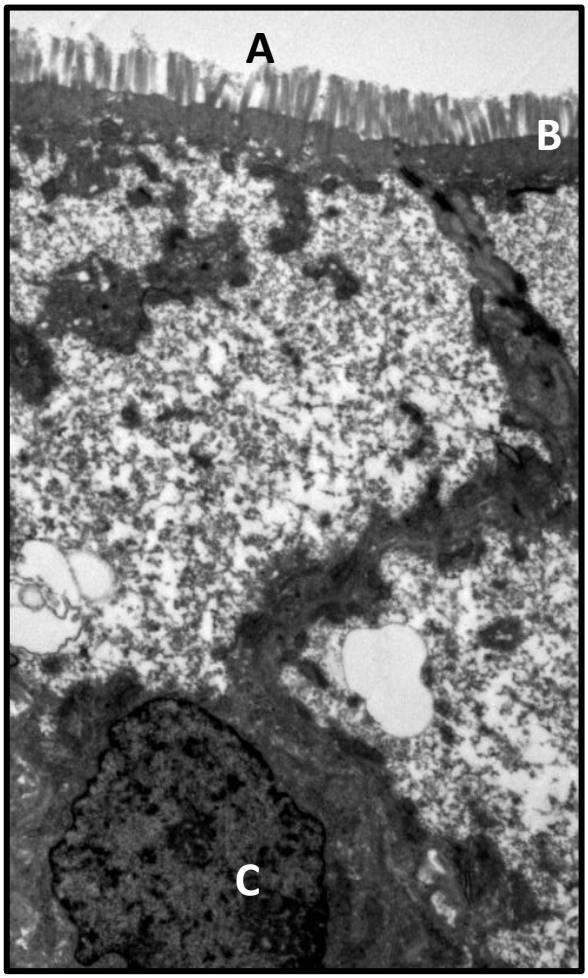
TJs= Tight Junctions; AJs= Adherens Junctions; GJs= Gap Junctions. In the culture system, cells are placed over a membrane pre-coated with collagen. The apical side is where treatments are applied and mimics the luminal side of the intestine; therefore microvilli are in contact with nutrients accessing the body via the digestive system. Products as a result of these treatments are released into the basal side and can be collected in the media, which mimics the production of soluble proteins released into the bloodstream and circulation.

Figure 6.2. Transmission electron microscopy imaging of mature and differentiated Caco-2 cells showing the microvilli structure on the apical side.



Taken from Salim, G. 2018 (Data non-published).

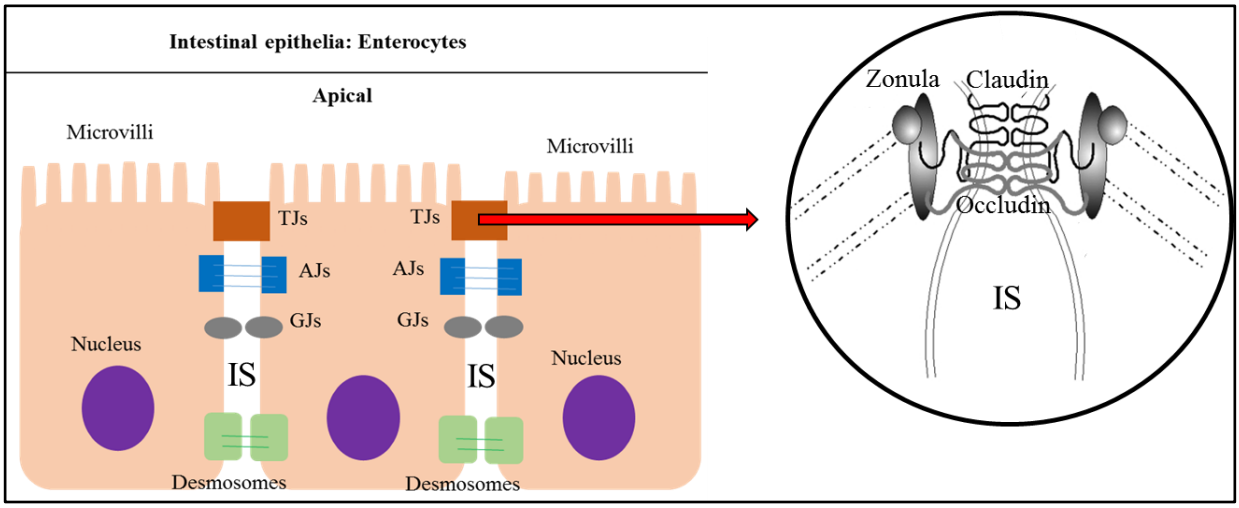
Figure 6.3. Transmission electron microscopy imaging of Caco-2 cell structure



Taken from Salim, 2016 (unpublished). A. Luminal or apical side. B. Microvilli. C. Nucleus.

Tight junctions seem to be the first structure affected when the epithelial gut barrier is disrupted [499]. Likewise, it has been suggested that TJ proteins, particularly claudin (See Figure 6.4) seem to be affected during the ageing process [500]. The disruption of the integrity of the epithelial monolayer has been studied and linked to the pathogenesis of common diseases in the elderly [501-503]. Understanding the causes leading to this gut epithelial disruption is a crucial step to develop efficient strategies to intervene and contribute in gut epithelial regeneration as it has an impact on absorptive processes and finally, health. Figure 6.4 illustrates the relevant proteins forming these junctional complexes.

Figure 6.4 Tight junctions and proteins involved



TJs= Tight Junctions; AJs= Adherens Junctions; GJs= Gap Junctions; IS= Intercellular space. Diagram adapted from Arrieta *et al*. [504].

6.1.1 Tight Junctions (TJs)

When the junctional complexes between adjacent cells allow the appropriate exchange of substances, and when the interconnection among enterocytes is stable, the gut barrier remains under optimum conditions of integrity. The most apical junctional complex corresponds to the TJs which are composed of transmembrane proteins, these are the claudins and occludins. Also in this structure, the primary cytoplasmic actin-binding proteins are the zonula proteins. Overall, TJs accomplish as a main purpose the regulation and control of the passage of ions, water, and macromolecules through the intercellular space - so-called paracellular space [505]. The interconnection of these proteins allows the accomplishment of this function.

An examination of the main structures composing the TJs has allowed discrimination and identification of different proteins, which have an interconnected function. It has been suggested that claudins are the most important components as they regulate the gate role of TJs by restricting passage of molecules according to their size. The analysis of the structure of claudins has revealed that these structures are integrated by transmembrane domains: two large extracellular loops and a cytoplasmic tail. Together they regulate paracellular selectivity and mediate claudin dimerization between adjacent cells so that the junction can be sealed and the paracellular transport can be regulated [506, 507]. Interlinked with claudins, it is possible to find occludins, which are essential for TJ assembly by mediating the interaction of occludins between adjacent cells. Claudins also have a tail oriented towards the cytoplasm so that they mediate protein-protein interactions. The barrier formation of TJs binds several cytoplasmic proteins including zonula proteins [508]. Lastly, the zonula-occludens conform a group of proteins that are structurally similar with two domains whose function is mediating protein-protein interactions [509].

Optimal gut function and an optimal gut epithelial barrier maintenance are relevant in the elderly as this population often presents with malnutrition derived from poor absorption, sarcopenia and a generalised decline in health status. The optimum status of the gut can also allow better communication with the microbiota and with the dietary components. Understanding strategies to re-generate the gut epithelia is relevant as well in the elderly population.

The previous chapters of this thesis have described findings from the PRINCESS trial particularly with regard to immune and inflammatory biomarkers. The PRINCESS trial used a combination of two probiotics as the intervention was clearly targeting gut dysbiosis in the older participants. Any positive (i.e. health promoting) effects exerted by probiotics may involve interaction of the probiotic organisms with the gut epithelium, although the mechanisms have not been clearly unravelled. Some hypotheses suggest that this could be via direct physical contact and strengthening of TJs or through the production of chemicals which act as trophic factors for enterocytes (e.g. butyrate) or bacteriocins which control the growth of pathogenic bacteria, as well as controlling local inflammation which would have an impact systemically. The gut epithelium as an extensive protective barrier is considered part of the innate immune system, but its role in adaptive immunity is also crucial. The mucosa-associated lymphoid tissue (MALT) is key to the interaction of both the gut microbiota and the gut epithelium with the immune

system and is closely connected with the gut epithelium. 70% of the body's total amount of immune cells are present in the gut [510]. The gut-associated lymphoid system is dispersed in the epithelium (see section 1.5.1) and remarkably, gut epithelial cells themselves can mount responses in the presence of microbial signals, for example by producing inflammatory cytokines while in parallel avoid those responses in the context of nutrients and non-harmful dietary components. In the context of the PRINCESS study and the overall aim to unravel the mechanisms by which probiotics exert an effect on the host, it is of interest to investigate whether the probiotic organisms being used in the PRINCESS study have a direct influence on gut epithelial cells. In order to do this the Caco-2 cell model needed to be established and the response of Caco-2 cell monolayers to an inflammatory stimulus needed to be identified. These are the topics of the research described in this chapter. Later chapters (7 and 8) will investigate the effect of probiotics on the Caco-2 monolayer inflammatory response.

6.1.2 Hypothesis

- It is hypothesized that a useful *in vitro* model of Caco-2 cells can be established by identifying the optimum stage to induce an inflammatory response in the model and a relevant inflammatory insult.
- It is also hypothesized that the inflammatory stimulus will reduce the expression of functional genes related to tight junctions as part of the mechanism that induces barrier disruption.

6.1.3 Aim and objectives

The aim of this research was to establish an inflammatory model of gut epithelial cells. The objectives of this research project are:

- To compare transepithelial and inflammatory responses of Caco-2 cells to two different inflammatory cocktails.
- To establish a protocol that allows conducting molecular experiments in the best conditions to identify RNA purity and integrity and to obtain cDNA and optimum handling conditions to conduct PCR analyses.
- To assess the expression of three genes encoding proteins that form tight junctions and their alteration as a consequence of the inflammatory stimulus.

6.2 Methods: Cell culture system

6.2.1 Caco-2 cells as a monolayer of the gut epithelium

Caco-2 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC), a Culture Collection of Public Health England. Previous research in the field has identified that Caco-2 cells are highly affected by external conditions in which they are cultured (e.g., temperature, cell density, passage) [511, 512]; therefore, maintenance of similar conditions of culture in every experiment is of critical importance, as well as to have an objective measurement to track the progress or growth of the cell system, the status in which the monolayer is at optimum conditions to perform experiments, and to achieve replicability.

The optimum condition to use the Caco-2 cell monolayer is when integrity in the epithelial tissue has been achieved. To identify this, the measurement of the transepithelial electrical resistance (TEER) is performed. The TEER is a quantitative technique to measure the integrity of tight junctions in cell culture models; it uses two electrodes to calculate the ohmic resistance through the application of a voltage signal [513]. Researchers in the field have suggested that this model exhibits an augmentation in TEER values after passage 36 and a decrement in TEER values after passage 60; therefore the passage should be also established and identified within the system and be kept consistent [514]. According to this, a protocol was established for Caco-2 cells cultured from passages 48 to 56 to be used.

Caco-2 cells were grown using Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 1% nonessential amino acids, 2% L-glutamine, and 1% penicillin streptomycin as antibiotics, at 37 °C in an atmosphere of 5% CO_2 and 95% air. Caco-2 cells were cultured following a sequential series of stages:

- *Thawing cells:* Cells were resuscitated from liquid nitrogen by transferring the vial containing the cells to dry ice, exposing the vial for 2 minutes to pre-heated water at 37 °C and then centrifuging at 1500 rpm (300g) for 5 minutes using warmed media to wash off DMSO and other toxic components; this process was conducted as rapidly as possible as this ensured a prompt transfer of cells into the incubator and their optimum growth.
- *Subculturing and splitting:* Cells were grown in 75 cm^2 flasks and passaged until 50% of confluence was reached. Afterwards, cells were seeded in culture plates

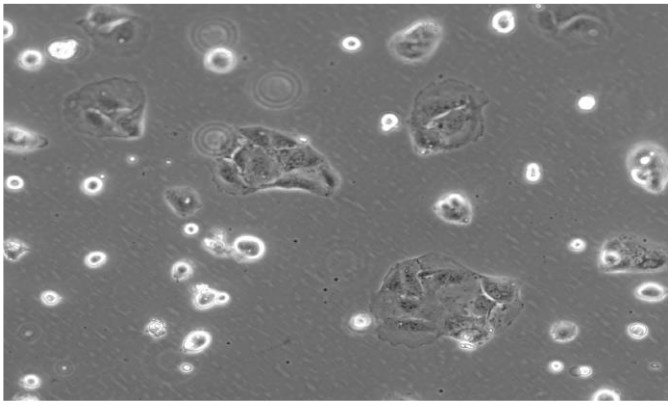
on a 12 transwell insert plate pre-coated with collagen. Inserts were 6.5 mm^2 with 0.4 µm clear pore size (Costar 3460). Considering the transwell insert diameter, the insert membrane growth area was 0.33 cm^2.

- *Preparation of transwells:* prior to seeding cells on transwell membranes, the membranes were pre-coated with 100 µl of a solution of sterile water and 1% collagen. This solution was homogeneously distributed and kept for 3 hours of incubation. Subsequently, the solution was carefully removed and supplemented media was added to both apical and basolateral sides. Transwells were placed in a 37^oC incubator overnight to neutralise the membrane. Cells were transferred to the transwells after this process by removing the "neutralising" media and adding new pre-warmed supplemented media. All of the experiments were performed within the same range of passages. The experiment was performed in 12 insert transwell plates at a density of 7 x 10^4 cells/well.
- *Experiments were performed*: Monolayer integrity was followed through TEER measurements. The volume of supplemented medium added per well was 1500 µl on the basolateral side and inside the transwell (apical side) the volume added was 500 µl. During the process of cell growth, the medium was changed every other day and the TEER was measured to verify viability and monolayer formation.

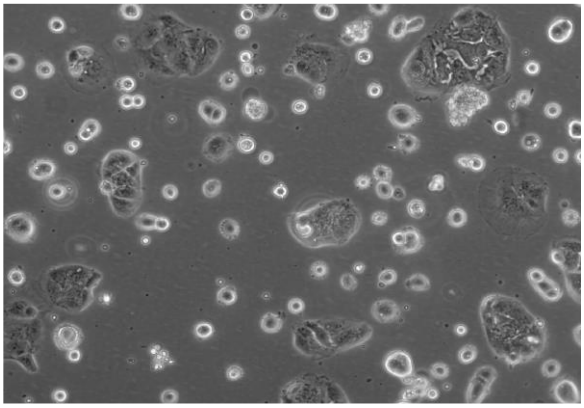
6.2.1.1 Caco-2 cell culture system and growth: Microscopy imaging of the process related to culture and cell growth

The Caco-2 cell culture process was documented following the cells from the initiation of cultures using cells resuscitated from liquid nitrogen (Figure 6.5-A) and their growth once cell attachment was observed in the flask (Figure 6.5-B). Once cells reached the confluent stage to be transferred onto the membranes, cells were trypsinised and transferred to pre-coated membranes as shown in figure 6.5-C. Lastly, cells reached the optimum stage to be treated and the monolayer imaging was captured in figure 6.5-D. Imaging was captured with an EVO microscope and 10 x augmentation at brightfield. No additional staining or fluorescence were used. This procedure was performed with the help of the technician Kate Parry. The overall results are presented in figure 6.5.

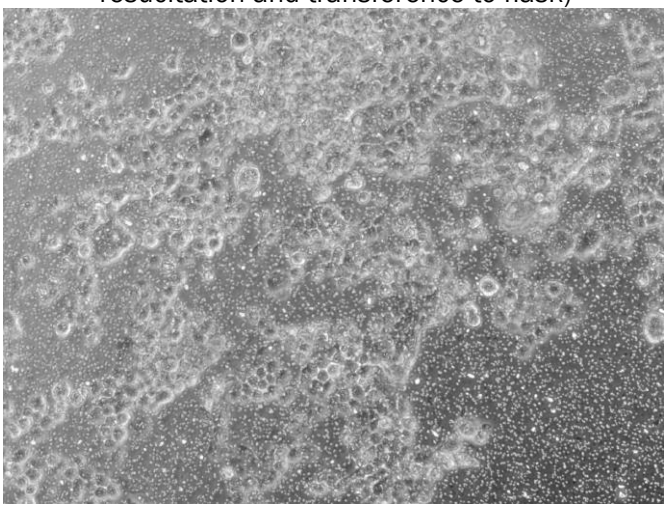
Figure 6.5. Identification of the Caco-2 cell growth process: microscopy imaging.



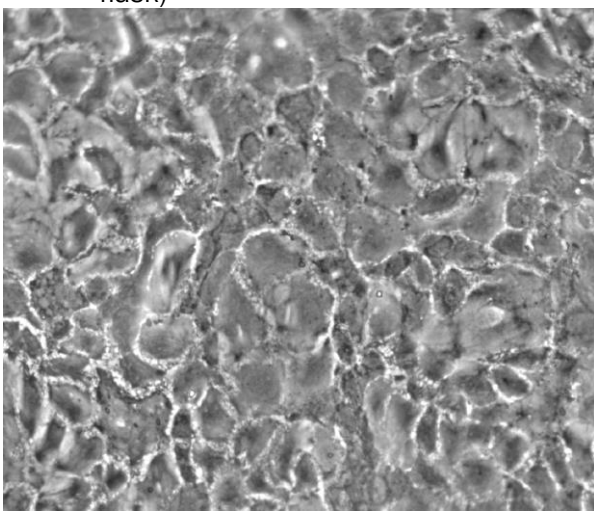
A) 10x image taken using EVO microscope brightfield flask T75cm3. – Day 1 (After caco-2 resucitation and transference to flask)



B) 10x high-density cell attachment flask T75 cm3. - Day 3 (Caco-2 attachment in flask)



C) 10x collagen cell monolayer 50% confluence.- Day 1 (Caco-2 attachment into the collagen membrane)

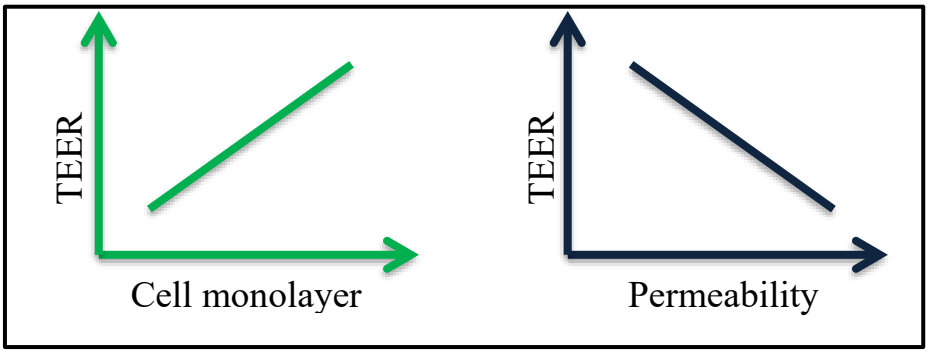


D) 20x confluent monolayer (19 days after cellular growing)

6.2.2 Caco-2 model and transepithelial electrical resistance (TEER) measurements

Transepithelial electrical resistance (TEER) is a common measure to assess the stability and integrity of Caco-2 cells grown on the membrane described in section 6.2.1. TEER is a surrogate marker of the tight junction state and acts as an indicator of physical structures and properties of epithelial cultures. Figure 6.6 shows the overall behaviour of TEER measurements to discriminate permeability or optimum conditions in the cell system [512].

Figure 6.6. TEER behaviour according to monolayer integrity.



Schematic representation of TEER behaviour in the monolayer. Increased TEER values indicate less permeability and a highly confluent monolayer. When TEER values decrease it might indicate decreased cell viability due to cell death and increased permeability.

The establishment of the model, as well as the TEER measurements, were tracked according to the protocol established by Natoli *et al* [512]. Caco-2 cells are an *in vitro* model, where cells behave in an adherent manner and attach to the surface on which they growth. Therefore, from the initial subculturing process in flasks to the transfer and seeding into the transwells, cells require a careful trypsinisation process which consisted of adding a pre-heated trypsin solution for a maximum period of 8 minutes. Once cells were completely detached from the surface of growth they were transferred and seeded onto the pre-coated collagen membranes in the transwell plates when they reached 50% of confluence. For the actual measurement of the TEER, an epithelial voltohmeter (EVOM) was used. A test resistor provided by the manufacturers was used to calibrate the instrument at 1000Ω. The instrument uses a pair of electrodes (chopsticks) which are inserted in the transwell by placing one of the electrodes in the baseline culture media and the shorter electrode on top of the actual membrane where cells are seeded by avoiding cell membrane disruption. Once calibration was ensured, the resistance was measured and recorded as $\Omega.cm^2$. Electrodes must be kept in a fixed position at a 90° angle to the plate insert. Maintenance of both the machine and electrodes was according to manufacturer's instructions.

For the first experimental phase in which the model was being established (see sections 6.3.16.3.1 and 6.3.2), TEER values were recorded 3 times per week to monitor cell-growth and assurance of the epithelial monolayer integrity. Further experiments relied on this process to have occurred and so TEER values were recorded once the monolayer was at the stage to be stimulated and/or treated (chapters 7 and 8) and once the experiment was terminated.

The methodological development of the research described in this chapter consisted of an initial standardisation phase involving the assessment of TEER from day 3 to day 24 to monitor cell stability according to the procedure previously explained. The medium was changed on alternate days. This experiment allowed establishing a culture system to set up the desired inflammatory model.

6.2.3 Exploratory study: Effect of inflammatory stimuli on Caco-2 cells

Considering that the inflammatory response to be studied should be induced through a physiologically relevant stressor, the establishment of the model included different stimulatory treatments whose purpose was to induce a response in the cells. It has been established that an inflammatory model should follow these criteria [515]:

- ✓ The inflammatory response should be induced through a physiologically relevant stressor.
- ✓ The model should resemble intestinal inflammatory processes as closely as possible. Therefore, no substantial or permanent destruction of the Caco-2 barrier should occur.
- ✓ The system ideally should recover itself without additional manipulation of the culture.
- ✓ A TEER reduction by at least 20-25% compared to the Caco-2 monoculture should be obtained.

6.2.3.1 Physiological conditions of stimulation

According to the previous literature [515], the stimulation of the monolayer involves the appropriate identification of physiological-like conditions. This involves parameters related to a) The stage in which the monolayer should receive the physiological stimulus to allow a recovery process; b) The nature of the stimulus used to generate the response; c) The time-length in which the physiological stimuli should remain in the cell "environment"; and, d) The "side" or compartment of the transwell that generates a response.

In order to address the aspect "a)", previous work has suggested that Caco-2 cells reach an optimum stage of confluence at day 21 after transwell-seeding [512], but likewise, previous work has suggested that the formation of the monolayer is highly susceptible to cell density, passage number and temperature [514]. Therefore, the methodological development of this model aimed to compare the monolayer stability using cell

passages from 48 to 56 for 17, 19 and 21 days of cell growth. Secondly, to address aspect “b)”, previous work conducted in our group used individual cytokines (TNF- α, IFN-γ and IL-1β) to generate an inflammatory response, each at different concentrations. Additionally, it was also attempted to explore the response of the model using a “priming” system whereby cells were first stimulated with a cytokine for a certain period of time (4 hours and 6 hours) and subsequently, a cytokine “cocktail” (a term used to describe a combination of different inflammatory stimuli) was added to the system. This experimental development failed to show great stability as it seems that taking out cells from the incubator altered the system and increased permeability due to alteration of temperature (data not shown).

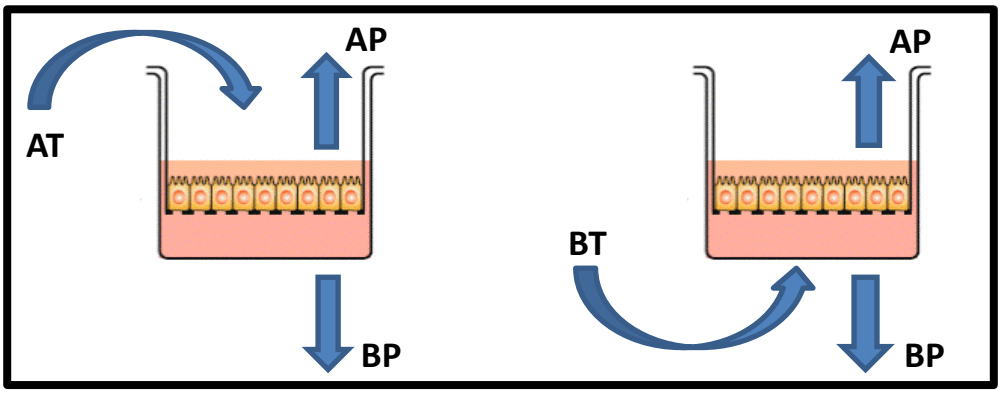
The methodological design to set up the inflammatory model presented in this research initiated with the combination of the three cytokines (TNF- α, IFN-γ and IL-1β) at two different concentrations (5 ng/ml; 50 ng/ml and 5 ng/ml; respectively and serial dilutions were prepared to reach the second cocktail at a concentration of 1 ng/ml; 10 ng/ml and 1 ng/ml; respectively). Subsequently, and to address aspect “c)” the time-length to explore the inflammatory response was previously assessed by exploring responses of the model after 12, 24 and 48 hours of stimulation (data not shown); the time point that was chosen and was considered optimum for this *in vitro* investigation was 24 hours. Lastly, and in order to decide for aspect d) previous research has been conducted in the apical and basolateral compartments (See Table 6.1). The compartment on the basolateral side mimics the bloodstream and thus facilitates the assessment of cytokines and other soluble immune mediators that are released from that basolateral compartment. That is physiologically relevant as it allows to assess the response exerted by the immune cells; thus, this was targeted as the main side to induce inflammation. It was also interesting to explore the Caco-2 cell response when cells received apical stimulation, so inflammation was also induced at 21 days of cell growth just to compare the disruption from that side.

Table 6.1. Summary of the conditions used to investigate Caco-2 cell inflammatory responses

Reference	Author	Stimulus	Localization of stimulus
[516]	Shen *et al*. 2017	TNF-α (10 ng/ml)	Basolateral side
(non-published data)	G. Salim, 2016	TNF-α (10 ng/ml)	Basolateral side
[517]	Moran *et al*. 2012	TNF-α (100 ng/ml)	Basolateral side
[518]	Alhamoruni *et al*. 2011	IFN-γ (10 ng/ml) → TNF-α (10 ng/ml)	Basolateral side
[519]	Ou *et al*. 2009	TNF-α; IL-1β; IFN-γ	Basolateral side
[520]	Wang *et al*. 2006	IFN-γ (10 ng/ml) → TNF-α (2.5 ng/ml)	Basolateral side
[521]	Ma *et al*. 2004	TNF-α (10 ng/ml)	Basolateral side
[522]	Ferrari *et al*. 2016	TNF-α (50 ng/ml)	Apical and Basolateral side
[523]	Van de Wall *et al*. 2010	LPS; TNF-α; IL-1β; IFN-γ (isolated and cocktail)	Apical and Basolateral side
[524]	Kasper *et al*. 2016	LPS; TNF-α; IL-1β; IFN-γ	Apical side

Both apical and basolateral stimulation have been explored as sites to provoke the response of the cells; therefore, it was aimed to stimulate the system as shown in Figure 6.7 at 21 days of cell growth. It appeared from the literature search that basolateral stimulation offered a more relevant physiological alternative as the pathophysiology of different intestinal disorders is caused by the load of inflammatory stimulants, in the form of immune mediators, soluble proteins or pro-inflammatory cytokines present in the bloodstream, resembling the real-condition of cytokine production and accumulation as a result of the respective immune cells activation.

Figure 6.7. Caco-2 cell monolayer culture. Apical and basolateral stimulation and collection of supernatants for inflammatory mediator analysis.



AT= Apical treatment; **AP**= Apical production; **BP**= Basal production; **BT**= Basolateral treatment. This model as presented was used in Caco-2 cells grown for 21 days of time as it reflects the maximum confluence and epithelial barrier integrity. Monolayers grown for 17 and 19 days were stimulated from the basolateral side only (BT).

6.2.3.2 Inflammatory panel assessed.

The analysis of an inflammatory panel consisting of the assessment of the production (i.e. concentrations) of cytokines, adhesion molecules and chemokines was implemented. The methodology used was multiplex Luminex following the principles and protocols presented in chapter 2.

In the setup of the experimental conditions, the analytes were measured in cell culture supernatants collected on the basolateral and apical sides of Caco-2 cell monolayers once the experiment was terminated. The media collected were stored at -80°C for a maximum period of one month when the luminex analysis was carried out. Reagents were freshly prepared and diluted the same day of the assay. Likewise, serial dilutions were prepared according to manufacturer's instructions to develop the standard curve. Immune mediators under examination and the conditions for these particular analyses (standard curve ranges and sensitivity analysis) are presented in Table 6.2.

Table 6.2. Inflammatory panel assessed in media from Caco-2 cell monolayers

Immune mediators	Standard curve (pg/mL)	Sensitivity (pg/mL)
Cytokines		
IL-6	4.8 - 1,154	1.7
IL-8	5.2 - 1,255	1.8
IL-18	10.1 - 2,460	1.93
Chemokines		
MIG	586 - 142,400	23.8
IP-10	2.8 - 690	1.18
Adhesion molecules		
ICAM-1	6888 - 1,673,860	87.9
VCAM-1	7781 - 1,890,780	238
E-Selectin	334 - 81,270	18.8
Growth factor		
VEGF	8.4 - 2,051	2.1

MIG = Monokine induced by gamma interferon (CXCL9); IP-10 = C-X-C Motif chemokine ligand 10 (CXCL10); VEGF = Vascular endothelial growth factor; ICAM -1= Intercellular adhesion molecule 1; VCAM-1 = Vascular cell adhesion molecule 1; E-selectin = Endothelial cell selectin.

6.2.4 Molecular experiments analysing the effects of an inflammatory insult on junctional genes expression in differentiated caco-2 cells

The expression of genes encoding proteins associated to TJs was assessed. The genes (and official full name and symbol) under examination correspond to human occludin (OCLN), claudin (CLND) and zonula or tight junction protein (ZO-1 or TJP-1). Targeted genes were customised and provided by Primer design. The experimental development of this section involved RNA extraction (Section 6.2.4.1), DNA conversion (Section 6.2.4.2) and real time polymerase chain reaction (RT-qPCR - Section 6.2.4.3).

6.2.4.1 RNA extraction and quality assurance

Transwells containing confluent Caco-2 cells were washed with ice-cold PBS three times after removal of apical and basolateral media. This process ensured the removal of dead cells. The PBS and the “washing” was gently performed by adding 500 µl in the apical side and 1000 µl in the basolateral side. Subsequently, cells were removed by using

100 µl of a solution of thioglycerol + ice cold BL buffer (guanidine thiocyanate) following the instructions provided with the ReliaPrep™ RNA cell miniprep system. The suspended cell pellet was collected and transferred to a sterile Eppendorf tube containing 100% isopropanol. The pellet + isopropanol was vortexed for 5 seconds and immediately placed on ice. A series of washing procedures using minicolumns to retain other cellular components and separate them from RNA was followed. The washing procedure was followed using RNA washing solution. The process also involved DNase to remove DNA and obtain purified RNA. After the series of washes and centrifugations, the final stage consisted of the transfer of the minicolumn containing the RNA to an elution tube where nuclease-free water was added to the membrane in the minicolumn and finally centrifuged. RNA was obtained and placed in a tube in dry ice to continue with the procedure.

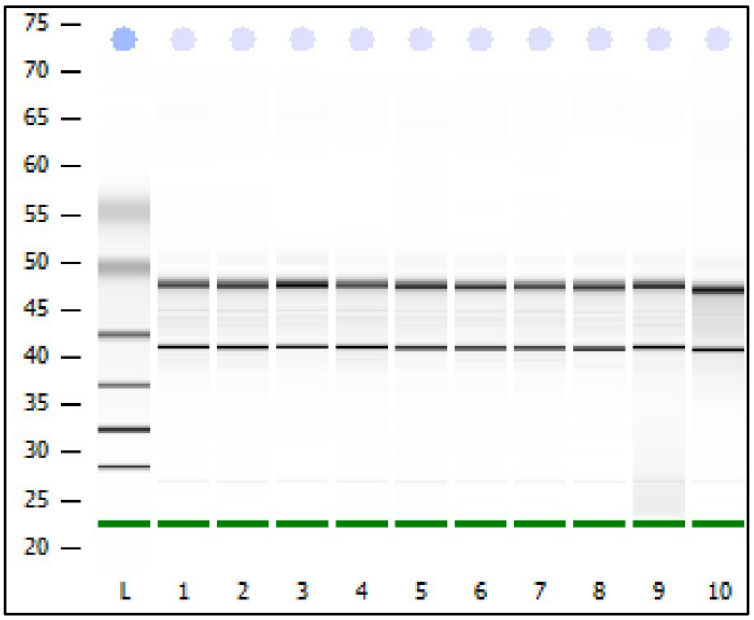
6.2.4.1.1 Total cellular RNA analysis: quantification

Once RNA was extracted from all the experiments intended to be analysed, an analysis using the NanoDrop™ technology was performed. This technology allows a sample to be pipetted directly onto an optical measurement surface. NanoDrop™ uses surface tension to hold micro-volumes of the sample in a specific position during the measurement. Once the measurement was completed, the surface was required to be cleaned with a wipe. Results include the amount (ug) of RNA measured in 1 µl of the sample collected in the previous step and the level of purity.

6.2.4.1.2 Total cellular RNA analysis of quality

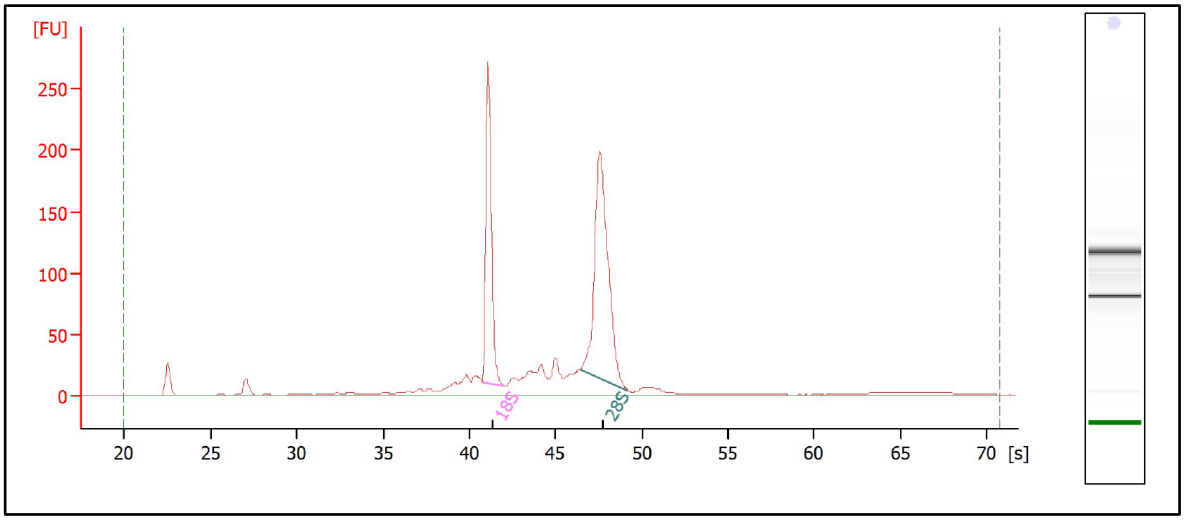
In addition to the quantification of RNA obtained, it was necessary to identify RNA integrity, a measure of the quality of the RNA extracted. This process was performed using the Agilent Bioanalyzer. The Bioanalyzer instrument is an automated electrophoresis tool used to determine the quality of biomolecules. The digital data obtained indicates the sizing, quantitation, integrity, and purity of the RNA. Figure 6.8 illustrates the output of a run and figure 6.9 indicates how the electropherogram summary looks. Once the integrity of the RNA collected was ensured, it was placed back on ice and immediately after that, the cDNA conversion process commenced.

Figure 6.8. Digital Electrophoresis Run summary graph



L=Ladder, used as control whereby its run allowed the identification of other molecular weights corresponding to other RNAs run in the sample. Numbers correspond to the samples under analysis. Clear bands indicate clean RNA extraction and integrity of the nucleic acid. This also indicates lack of contamination with other particles.

Figure 6.9. Electropherogram summary



Example taken from samples analysed. The electropherogram produces a RIN score. This corresponds to a RNA integrity number whose aim is to avoid subjective interpretation in RNA quality control. It is calculated by taking the entire electrophoretic trace and classifies the eukaryotic total RNA based on a numbering system from 1 to 10, where 1 corresponds to the most degraded profile and 10 corresponds to the most intact RNA [525]. This facilitates interpretation of the electropherogram and comparison among samples.

6.2.4.2 DNA extraction: complementary DNA (cDNA)

Once the quality and the quantity of RNA were ensured, the cDNA synthesis process was developed following GoScript™ Reverse Transcriptase to extract cDNA from total RNA. This process was followed according to manufacturer's instruction, but generally it relies on the synthesis of DNA from a single strand of the RNA previously collected from the cells.

The process initiates with the calculation of RNA to be collected to obtain a final volume of 20 ul of cDNA. In this calculation, it was also incorporated a volume of nuclease free-water. Once these volumes were calculated and added into sterile tubes, 1 ul of random primers (as provided by the detection kit) were added to the tubes to be analysed. This mix was placed in a Thermocycler at 70°C for 5 minutes. Lastly, and while this incubation process occurred, a reaction mix consisting on $MgCl_2$ (2 µl), PCR nucleotide mix (1 µl), RNA sin (1 µl) and reverse transcriptase (1 µl) was prepared; this was centrifuged for 10 seconds and gently vortexed and transferred to ice-cold temperature once the incubation was concluded. The last incubation consisted of 25°C for 5 minutes/42°C for 60 minutes/70°C for 15 minutes and lastly, 4°C to cool down the reaction. Samples were placed on ice and transferred to -80°C until the subsequent analysis of PCR took place.

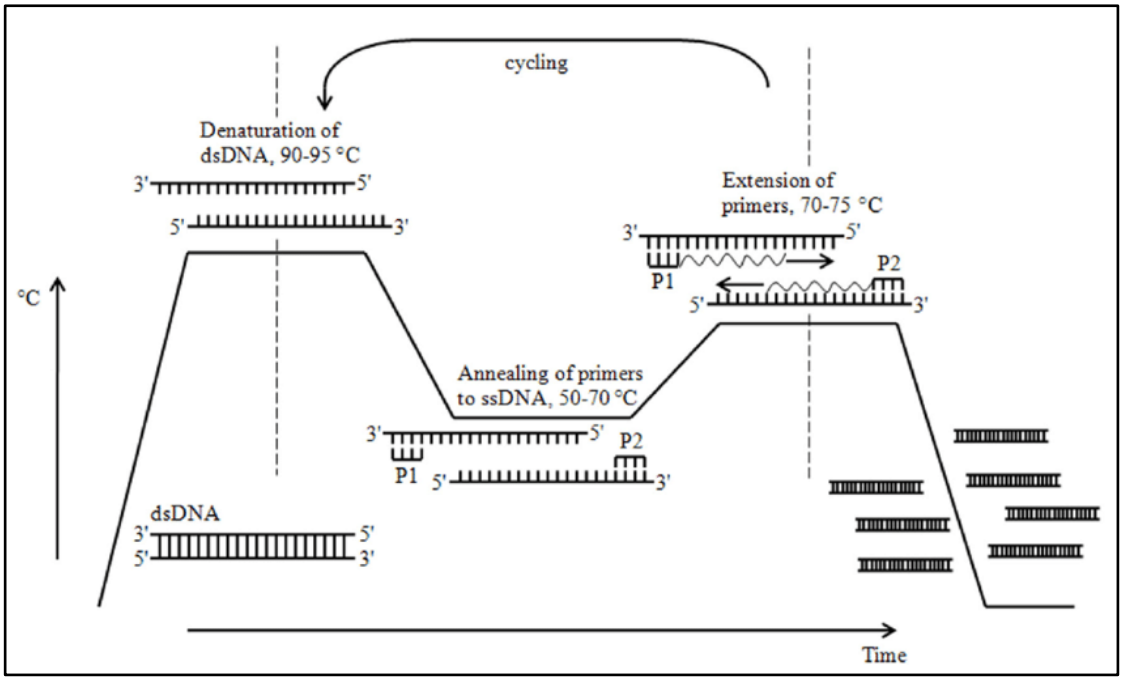
6.2.4.3 Analysis of targeted gene expression using Real-time Polymerase Chain Reaction (RT-qPCR) using TaqMan technology.

PCR is a technique for DNA replication by targeting a specific DNA sequence to be selectively and exponentially amplified. The principle of this technique involves a primer mediating an enzymatic amplification of DNA through the ability of the DNA polymerase to synthesize a strand of cDNA based on a template. The DNA polymerase elongates the terminal 3' by adding more nucleotides and generating an extended region of double-stranded DNA. Overall, the process involves many rounds of DNA synthesis, which require the following components: cDNA template, primer DNA polymerase and nucleotides. Primers are used to target the section to be amplified and thereafter, DNA synthesis is developed through the region between primers.

Each round of DNA synthesis is named a cycle and involves serial steps: denaturation, annealing of primers and primer extension. These occur under temperature cycling. Products of each cycle are used as templates for further DNA synthesis which yields in

the amplification of the targeted sequence. The overall development of the process is presented in figure 6.10.

Figure 6.10. Schematic of the PCR amplification process.

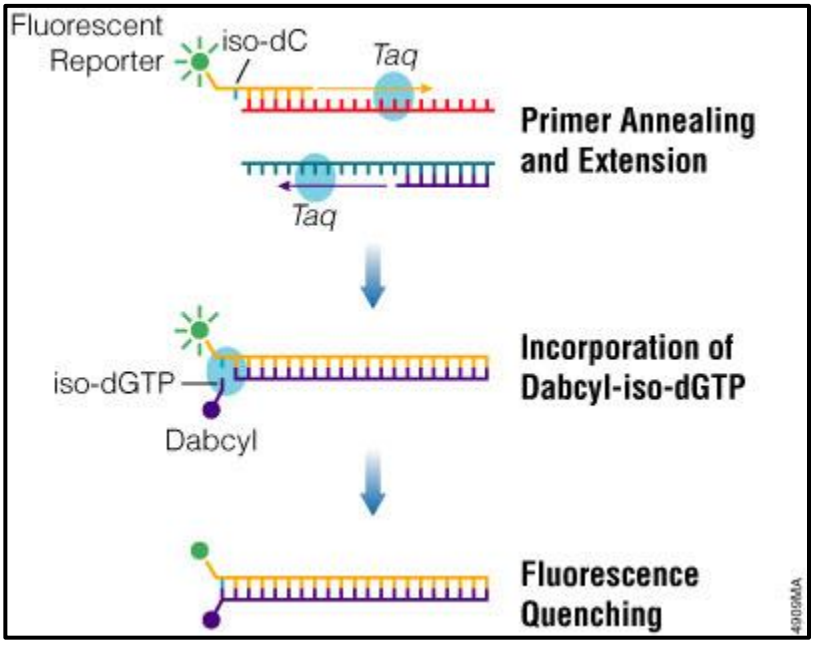


Taken from Strandgren [526]

The technique of quantitative reverse transcriptase PCR (RT-qPCR) is used to measure the proportional increase in the amount of cDNA within each PCR cycle. Once the quality and quantity of RNA were ensured, the specific mRNAs were transcribed from the targeted genes. Reverse transcriptase then forms complimentary DNA which is then called cDNA by using RNA as a template. This gives cDNA for every RNA extracted in the same proportion as they are present in the cell lysate or in the sample under analysis. Specific primers which are only annealed to the cDNA specific to the gene of interest measure the point at which the amount of amplified DNA reaches a threshold level (known as Ct Value). The earlier the amount of DNA within a sample reaches the threshold level in comparison to the other samples, the more cDNA template was present and thus more RNA was expressed in the original sample. Levels of cDNA can be measured by use of a dye that specifically binds to the double-stranded DNA as it is the case in SYBR-Green technologies; alternatively, it can be performed in addition to the probe, as it is the case in Taqman. Particularly, TaqMan probes are a technology that is based on the activity of a Taq polymerase to cleave a dual-labelled probe by using a fluorescence emitting dye (in the development of the actual experiment it was called FAM) that is bound to one end of a short DNA sequence with a molecule of quencher

(TAMRA) at the other. While these molecules are bound in close proximity to each other, no fluorescence is emitted. Once probes bind to specific sites and Taq DNA polymerase uses its exonuclease activity. The mechanism by which this technique functions is shown in Figure 6.11.

Figure 6.11. TaqMan (Hydrolysis) probes



Taken from PCR amplification Promega corporation

To conduct the RT-qPCR, general manufacturer's instruction were followed. Briefly, RNA and cDNA were extracted in anticipation. Samples were always kept on ice for defrosting. Dilution of samples occurred at a ratio 1:10 using nuclease free water. Subsequently a master mix was prepared using a master mix solution (Precision Plus 2x qPCR master mix), nuclease free water and primers for the targeted genes. Subsequently the plate was loaded with the diluted sample of cDNA and the master mix solution. Experiments were run in an instrument for Real-Time PCR (Applied Biosystems 7500 Fast real-time PCR system) using FAM as reporter and TAMRA as quencher. The instrument ran the analyses in 3 stages: Hold (for 50°C for 2 min and then at 95°C for 20 secs), subsequently the cycles were run in the enzyme activation stage at a temperature of 95°C for 2 min, then denaturation at 95°C for 10 sec and finally the data collection at 60°C for 60 sec. Data were analysed using qBase software.

6.2.4.3.1 Reference ("housekeeping") genes

Previous work on Caco-2 cells has proposed candidate reference genes for RT-qPCR studies based on cell differentiation [527]. Additional work has resulted in a careful selection of reference genes required for reliable performance [528]. Table 6.3. presents a summary of the reference genes selected according to the literature search.

Table 6.3. Reference genes used to run the qPCR-RT experiments.

Official Symbol	Function	Primer sequence
ACTB	Cytoskeletal structural protein	fw: CTGGAACGGTGAAGGTGACA rv: AAGGGACTTCCTGTAACAATGCA
GAPDH	Oxidoreductase in glycolysis and gluconeogenesis	fw: GGAGTCCACTGGCGTCTTCAC rv:GAGGCATTGCTGATGATCTTGAGG
SDHA	Electron transporter in the Krebs cycle and respiratory chain	fw: TGGGAACAAGAGGGCATCTG rv: CCACCACTGCATCAAATTCATG
YWHAZ	Signal transduction by binding to phosphorylate serine residues	fw: CTTTTGGTACATTGTGGCTTCAA rv: CCGCCAGGACAAACCAGTAT
RPL13A	Ribosomal proteins. Also involved in the repression of inflammatory genes as a component of the IFN-y activated inhibitor translation inhibition	fw: CGAAAGCATCTTGAGAGGAACA rv: TCGAGCCAAACGGTGAATC
18S	RNA 18S	fw: AGAAACGGCTACCACATCCA rv: CACCAGACTTGCCCTCCA

Based on findings presented by Jacob *et al*. [528] and Piana *et al* [527]. Selection of reference genes is critical for data normalization. These researchers have proposed a set of suitable and reliable reference genes in human Caco-2 cells.

6.2.5 Statistical analysis

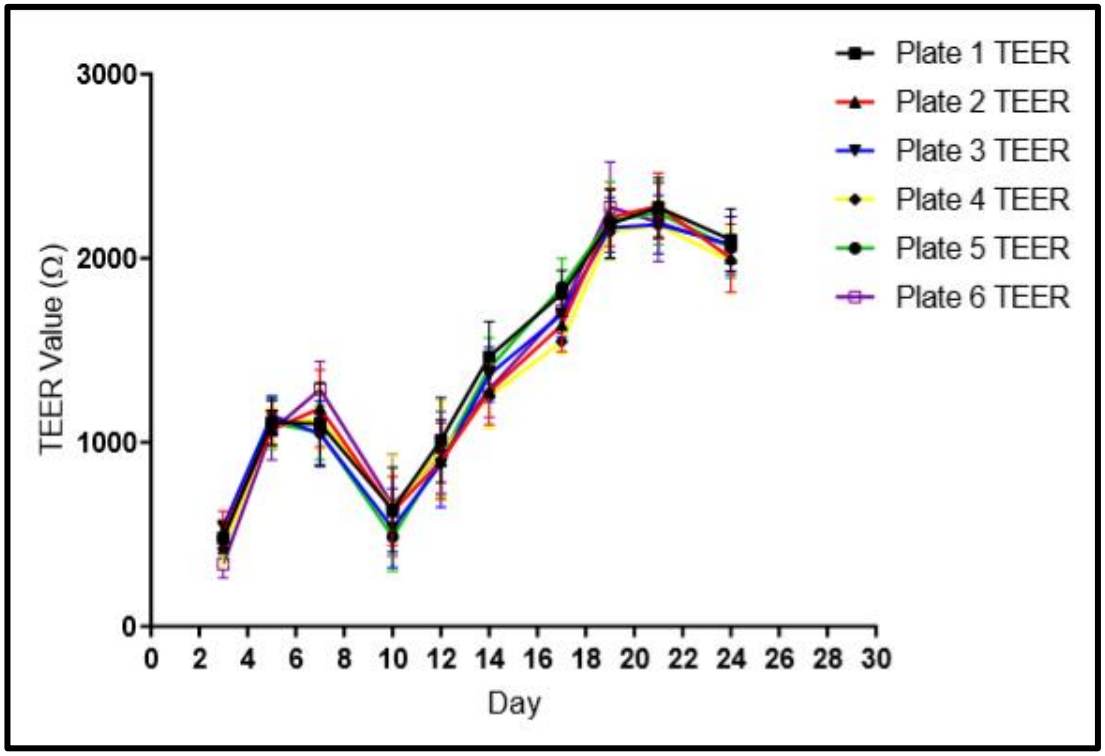
Initially, data were compared between time points to test if a combination of stimulus produced a significant change in TEER for 24 hours at days 17, 19 and 21. The inflammatory panel presented in section 6.3.2 was analysed by comparing basolateral production with the control without stimulation and then apical side against the control without stimulation. Test used was two-way Analysis of variance by ranks (ANOVA) using as factor concentrations and the days in which the production was assessed. Analysis was performed using SPSS version 22. A value for $p < 0.05$ was considered to indicate statistical significance. The molecular analysis and the selection on the optimal number of reference genes was conducted by using geNorm software. This analysis indicates the number of reference genes that must be included in the analysis.

6.3 Results

6.3.1 Establishment of the Caco-2 cell model and transepithelial electrical resistance (TEER) measurements

To assess the repeatability in the TEER measurements and how they change during Caco-2 cell culture, measurements were made twice per week when media change was performed. This standardization process was conducted using transwell plates in triplicate for a total n=6. Measurements were recorded for 24 days when a reduction corresponding to a 7% change in TEER was observed in relation to day 21 of cell growth. The observed reduction was attributed to cell overgrowth, cell detachment from the membrane and cellular death, reasons that lead to termination of the experiment. Moreover, it was also observed that the monolayer was stable at days 19 and 21 offering a time-window in which there is cell confluence and TEER stability. Results are illustrated in Figure 6.12.

Figure 6.12. Repeatability TEER values and how they change with culture time



(n=6 - plates) Data are mean (±SEM) corresponding to inserts per plate where cell growth took place.

6.3.2 Exploratory study. Effects of inflammatory cytokines on the Caco-2 monolayer.

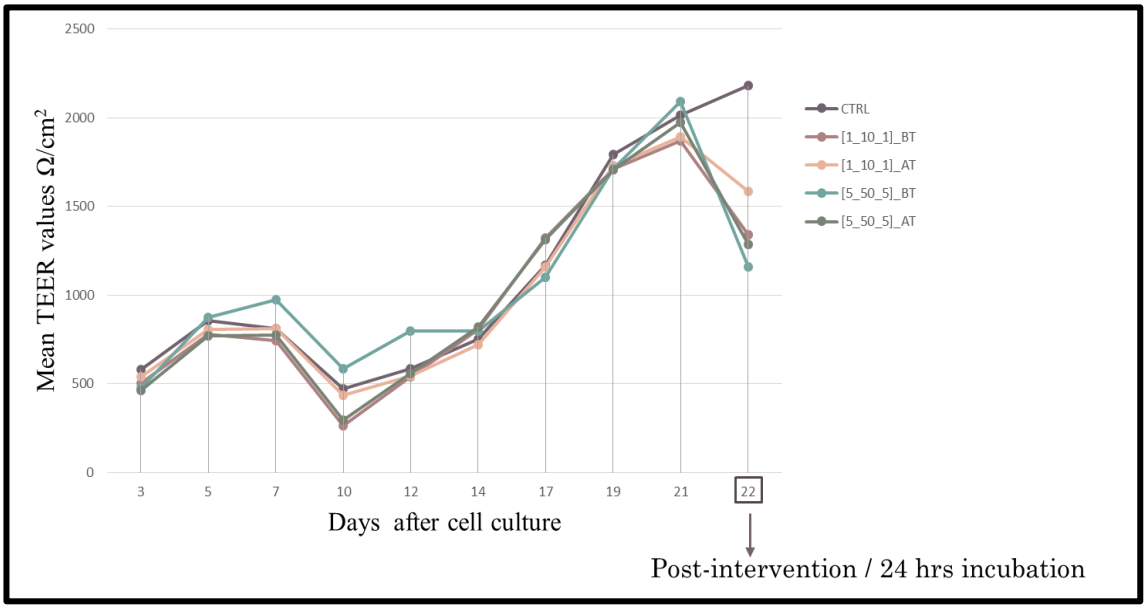
After establishing and verifying the Caco-2 cell growth process, monolayers were stimulated with two different cytokine cocktails: a lower concentration consisting of TNF-α (1 ng/ml); IFN-γ (10 ng/ml); IL-1β (1 ng/ml) and a higher concentration: TNF-α (5 ng/ml); IFN-γ (50 ng/ml); IL-1β (5 ng/ml). Overall, the lower concentrated cocktail reduced the TEER in a less dramatic manner than the higher concentration. From these findings, the basolateral stimulation using the cocktail TNF-α (1 ng/ml); IFN-γ (10 ng/ml); IL-1β (1 ng/ml) at 19 days induced a less drastic reduction in TEER measurements from the 3 time-points compared. Thus, this was the stimulus selected to induce inflammation in the cells.

6.3.2.1 Transepithelial resistance (TEER) of Caco-2 cell monolayers after exposure to cytokine cocktails on either apical or basolateral sides after 21 days of culture, and TEER after exposure on basolateral side at 19 and 17 days of culture

This experiment allowed the identification of the optimum cocktail concentration. Additionally, it allowed to explore the TEER responses depending on side of application of inflammatory stimuli at 21 days, and the TEER responses exerted when the inflammatory response was induced from the basolateral side at 19 and 17 days. Lastly, the TEER measurement also allowed to explore the effects of the same inflammatory cocktail in a time course stimulation at 19 days of cell growth. 19 days was the time frame selected as it showed the least reduction in the TEER values as shown in the following section.

At 21 days the basolateral treatment using the lower cocktail concentration reduced TEER by 43.1% whereas the higher cocktail concentration reduced TEER by 49.5% when compared with the control (Figure 6.13).

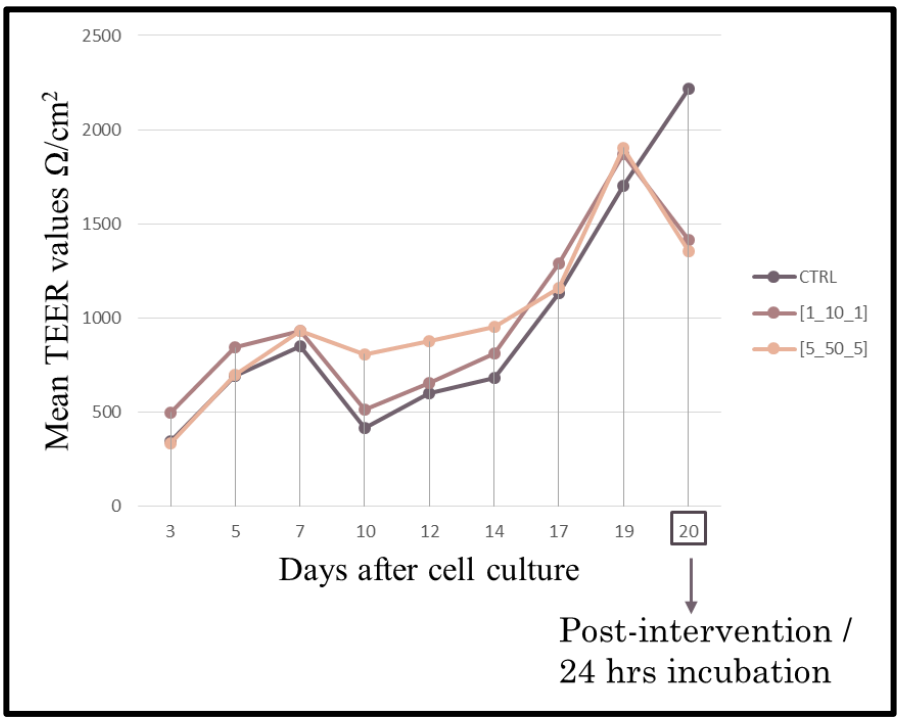
Figure 6.13. TEER of Caco-2 cell monolayers stimulated with cytokine cocktails on either apical of basolateral sides after 21 days of growth



Data shown as mean TEER values. Graph shows decreased TEER values when controls (n=4) are compared with the four conditions of treatment. The cocktail preparation consisting on TNF-α (1 ng/ml); IFN-γ (10 ng/ml); IL-1β (1 ng/ml) coded in the graph as [1_10_1] reflect a minor TEER reduction when compared against the higher concentration used in the model, coded in the graph as [5_50_5] which consisted on a cytokine combination of TNF-α (5 ng/ml); IFN-γ (50 ng/ml); IL-1β (5 ng/ml). Effects exerted by these cocktails indicate a statistically significant reduction in TEER values which are observed among CTRL and BT=Basolateral treatment using the concentration [5_50_5] (n=4; p=0.003) and CTRL and AT=Apical treatment concentration [5_50_5] (n=4; 0.007). Likewise, it is shown that some differences among CRTL and BT= Basolateral treatment concentration [1_10_1] (n=4; p=0.074) whereas CTRL and AT= Apical treatment concentration [1_10_1] (n=4; p=0.371).

At 19 days the lower concentration cocktail yielded a reduction of 36.3% compared with 40.4% for the higher concentration as shown in Figure 6.14

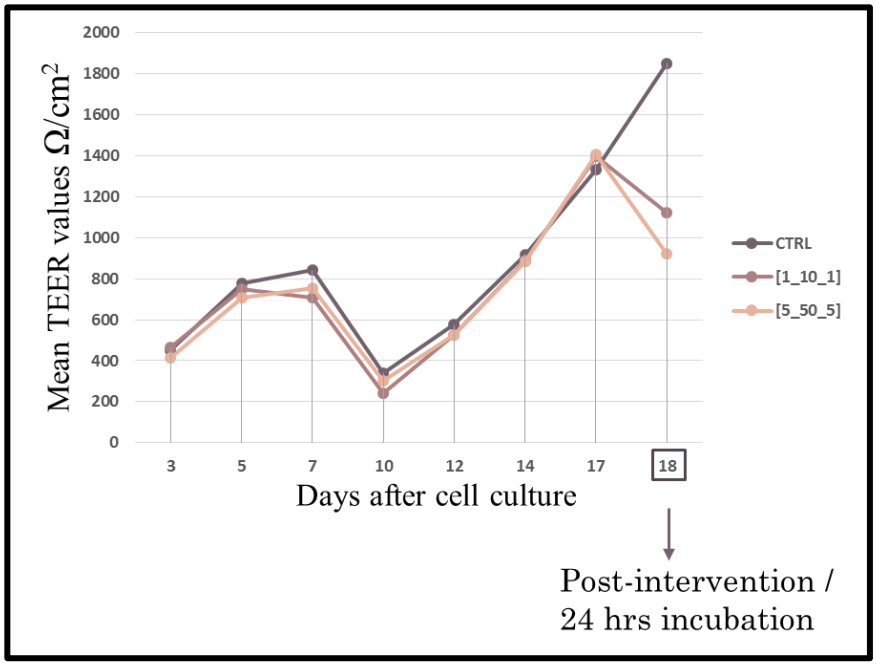
Figure 6.14. TEER of Caco-2 cell monolayers stimulated with cytokine cocktails on either apical of basolateral sides after 19 days of growth



Data shown as mean TEER values. Graph shows decreased TEER values when controls (n=4) are compared with TNF-α (1 ng/ml); IFN-γ (10 ng/ml) coded in the graph as [1_10_1] (n=4; p=0.157); and IL-1β (5 ng/ml) and TNF-α (5 ng/ml); IFN-γ (50 ng/ml); IL-1β (5 ng/ml) coded in the graph as [5_50_5] (n=4; p=0.014).

Lastly, at 17 days of cell growth, the lower cocktail concentration induced a reduction of 44.7% when compared with the control; whilst the higher cocktail concentration reduced TEER by 51.3% as shown in Figure 6.15

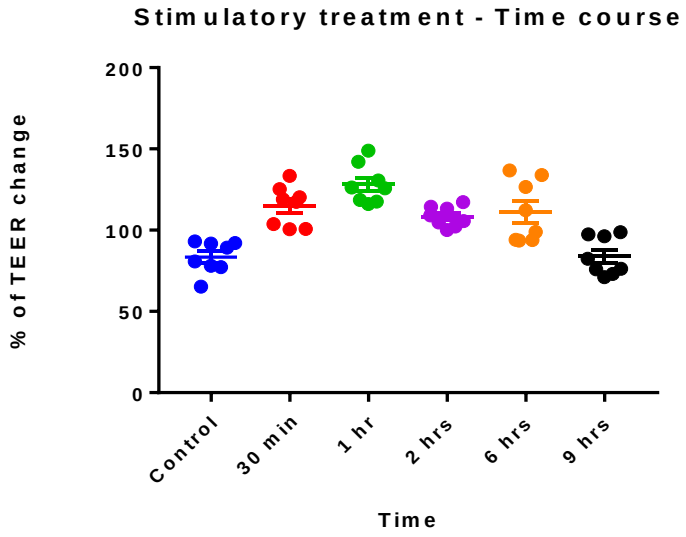
Figure 6.15. TEER of Caco-2 cell monolayers stimulated with cytokine cocktails on either apical of basolateral sides after 17 days of growth



Data shown as mean TEER values. Graph shows decreased TEER values when controls (n=4) are compared with TNF-α (1 ng/ml); IFN-γ (10 ng/ml); IL-1β (1 ng/ml) coded in the graph as [1_10_1] (n=4; p=0.157); also decreased values are observed when controls are compared with TNF-α (5 ng/ml); IFN-γ (50 ng/ml); IL-1β (5 ng/ml) coded in the graph as [5_50_5] (n=4; p=0.014).

As shown earlier in figure 6.14, stimulating the culture at 19 days yielded a minor reduction in the TEER stimulation when compared with 17 and 21 days; thus a time-course stimulation from the basolateral side was induced at earlier time points at 19 days of cell growth to assess the change in the TEER parameter. These results are shown in figure 6.16.

Figure 6.16. TEER change measurement

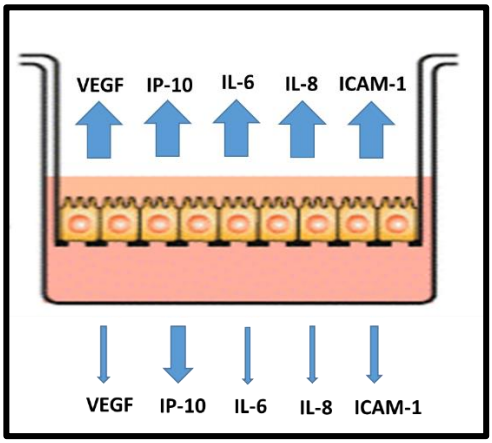


TEER values post-stimulation were corrected considering the baseline TEER value. Mean (±SEM) (n=8). Values correspond to positive percentage of increase from baseline. TEER positive area. Percentage of TEER change statistically significant ($p<0.001$)

6.3.3 Inflammatory mediator concentrations produced by Caco-2 cell monolayers apically and basolaterally after exposure to cytokine cocktails on basolateral side after 17, 19 and 21 days of culture

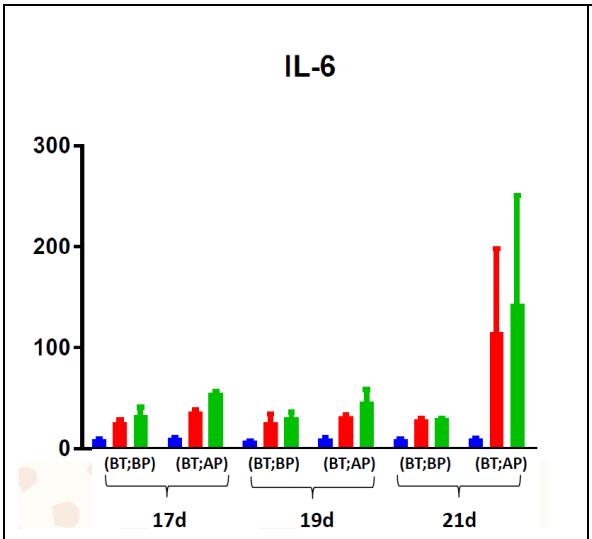
The inflammatory panel herein analysed was assessed in the basolateral and apical media as a result of the stimulation of the cells from the basolateral compartment. Comparisons against the controls without cocktail were performed. The lower concentration of the cocktail was used. Figure 6.17 summarises the findings: overall it was observed that the production of the cytokines IL-6 and IL-8 and of the immune mediators IP-10, VEGF and ICAM-1 can be detected in both compartments. The chemokine MIG and the adhesion molecules VCAM-1 and E-Selectin were poorly produced in the cultures, thus data are not shown.

Figure 6.17 Schematic representation of immune mediators produced on the apical and basolateral sides of Caco-2 cells when the inflammatory stimulation is induced from the basolateral side.

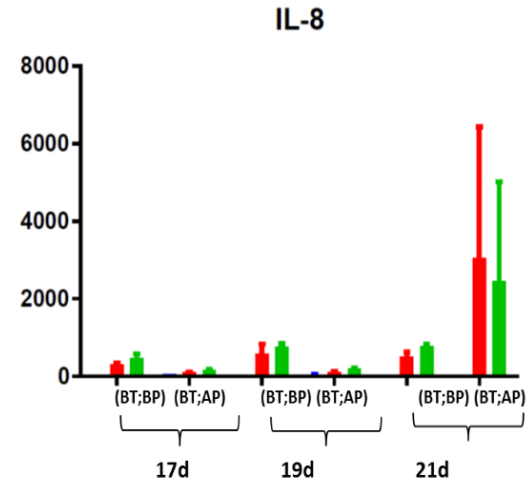


In the cytokine assessment, it was observed that IL-6 was produced on both apical and basolateral sides of the cell monolayers with particularly high apical production for 21 day cultures stimulated on the basolateral side. IL-6 production was higher with the higher cocktail concentration. A similar pattern was seen for IL-8. IL-18 was not induced differentially by the cytokine cocktails. Results are condensed and presented in Figure 6.18

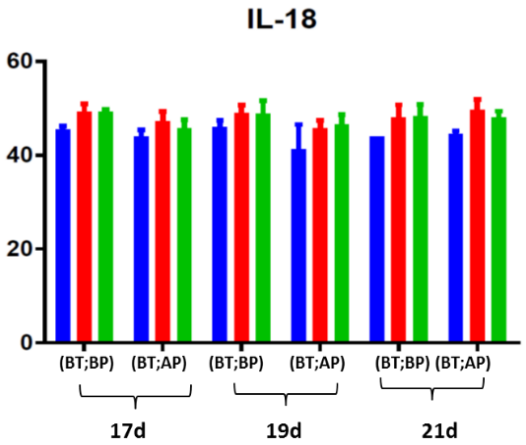
Figure 6.18. Concentrations of cytokines in the apical and basolateral media of 17, 19 and 21 day Caco-2 monolayers stimulated with cytokine cocktails from the basolateral side.



BP=Basal production vs. control (p=0.002)
AP=Apical production vs. control (p<0.001)



BP=Basal production vs. control (p<0.001)
AP=Apical production vs. control (p<0.001)



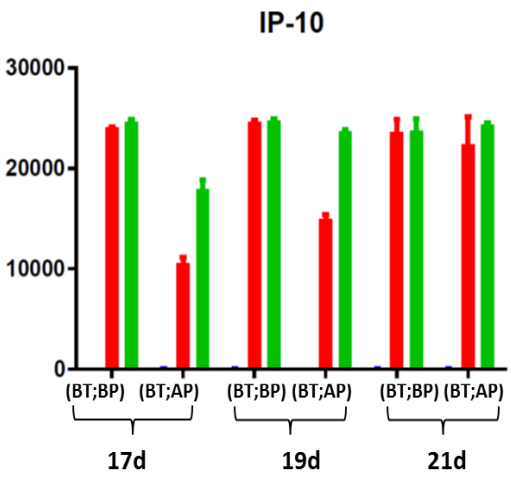
BP=Basal production vs. control (p=0.187)
AP=Apical production vs. control (p=0.166)

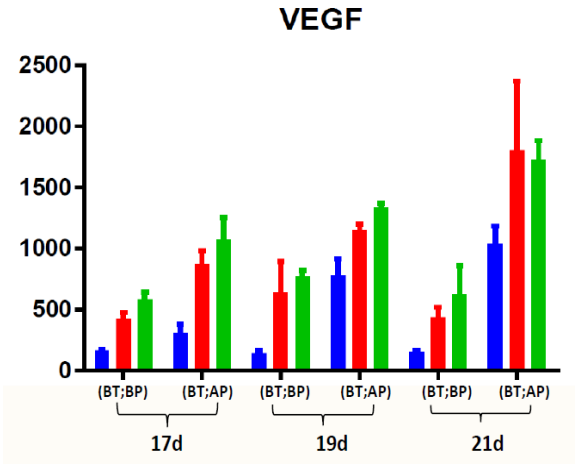
Colour coding: **Blue**= Control treatment (n=4); **Red**= Cocktail concentration 1/10/1 (n=4); **Green**= Cocktail concentration 5/50/5 (n=4). X axis indicates treatments added (pg/ml): **BT**= Basal treatment; **BP**= Basal production; **AP**= Apical production.

Subsequently, the assessment of immune mediators IP-10 and VEGF was performed. IP-10 production was comparable in cultures stimulated on the basolateral side and controls at 17, 19 or 21 days. Cocktail concentration had little effect on IP-10 production except that it was higher for the higher concentration cocktail on the apical side following basolateral stimulation of 17 day and 19 day cultures. Production of VEGF depended upon side of cytokine stimulation, side of measurement, age of cultures and to a lesser

extent cytokine cocktail concentration. MIG expression was not detected. Results are condensed and presented in Figure 6.19

Figure 6.19. Concentrations of soluble immune mediators in the apical and basolateral media of 17, 19 and 21 day Caco-2 monolayers stimulated with cytokine cocktails from the basolateral side.

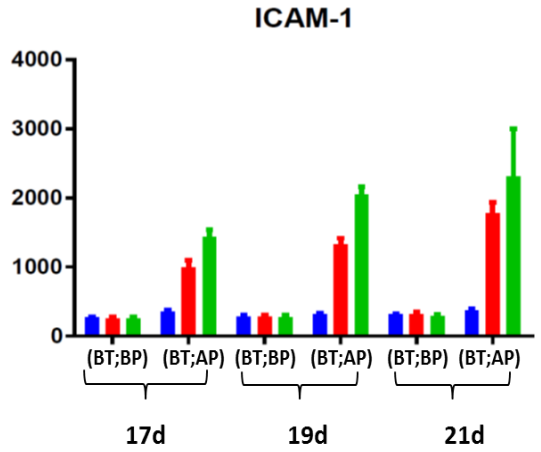




BP=Basal production vs. control (p=0.066) **AP**=Apical production vs. control (p=0.046)	**BP**=Basal production vs. control (p=0.056) **AP**=Apical production vs. control (p=0.066)
Colour coding: **Blue**= Control treatment (n=4); **Red**= Cocktail concentration 1/10/1 (n=4); **Green**= Cocktail concentration 5/50/5 (n=4). X axis indicates treatments added: **BT**= Basal treatment; **BP**= Basal production; **AP**= Apical production.	

Lastly, from the immune mediators assessed, only ICAM-1 was produced in the monolayers following the basolateral stimulation. VCAM-1 and E-Selectin were not produced in detectable amounts. Results are condensed and presented in Figure 6.20

Figure 6.20 Concentrations of adhesion molecules in the apical and basolateral media of 17, 19 and 21 day Caco-2 monolayers stimulated with cytokine cocktails from the basolateral side.



BP=Basal production vs. control (p=0.105)
AP=Apical production vs. control (p=0.044)

Colour coding: **Blue**= Control treatment (n=4); **Red**= Cocktail concentration 1/10/1 (n=4); **Green**= Cocktail concentration 5/50/5 (n=4). X axis indicates treatments added: **BT**= Basal treatment; **BP**= Basal production; **AP**= Apical production.

6.3.4 Effects of the inflammatory cocktail TNF-α (1 ng/ml); IFN-γ (10 ng/ml); IL-1β (1 ng/ml) on gene expression of OCLN, CLDN-1and TJP-1

When the reference gene expression was explored at day 21 when the monolayer confluence was reached, and also at day 19 which was the time-window selected to conduct the experiments, it was observed that SDHA and YWAHSZ were consistently stable and so these two genes were used as the housekeeping gene. Overall, the analysis of the gene expression of the targeted genes in the tight junctions (OCLN, CLDN-1and TJP-1) did not change. Results are presented in the following section.

6.3.4.1 Quality of RNA isolated from Caco-2 monolayers

6.3.4.1.1 Total cellular RNA analysis: quantification

This process consisted of identifying the quality of the RNA extracted by quantifying the amount extracted (ng/ul) as well as identifying the purity of that extract. Generally, an absorbance ratio in the A260/280 parameters of around 2 and absorbance ratio of A260/230 of between 2.0 and 2.2 are considered to indicate good RNA purity. Table 6.5 shows data for these measurements of RNA quality for samples isolated from Caco-

2 cell monolayers after 21 days of growth and then cytokine cocktail stimulation for 24 hours. These data indicate that good quality RNA had been isolated.

Table 6.4. Markers of the quality of RNA isolated from 21 day Caco-2 cell monolayers after stimulation with cytokine cocktails for 24 hours.

Cell treatment	ng/ul	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}/A_{230}
Control	283	7.075	3.394	2.08	2.04
Apical side treatment Cocktail [1/10/1]	343.8	8.595	4.099	2.10	2.07
Basal side treatment Cocktail [1/10/1]	358.3	8.957	4.340	2.06	1.99
Apical side treatment Cocktail [5/50/5]	326.7	8.167	3.928	2.08	2.06
Basal side treatment Cocktail [5/50/5]	375.8	9.396	4.590	2.05	1.96

(A_{260}=Absorbance 260; A_{280}=Absorbance 280)

RNA extraction was also conducted in cell cultures grown for 19 days when the inflammatory response was induced from the basolateral side in a time-course manner. Table 6.6 shows the results of the extraction conducted at this time course.

Table 6.5. Amount and quality of RNA extracted from caco-2 cells after 19 days of culture when inflammatory response was induced from the basolateral side in a time course stimulation manner.

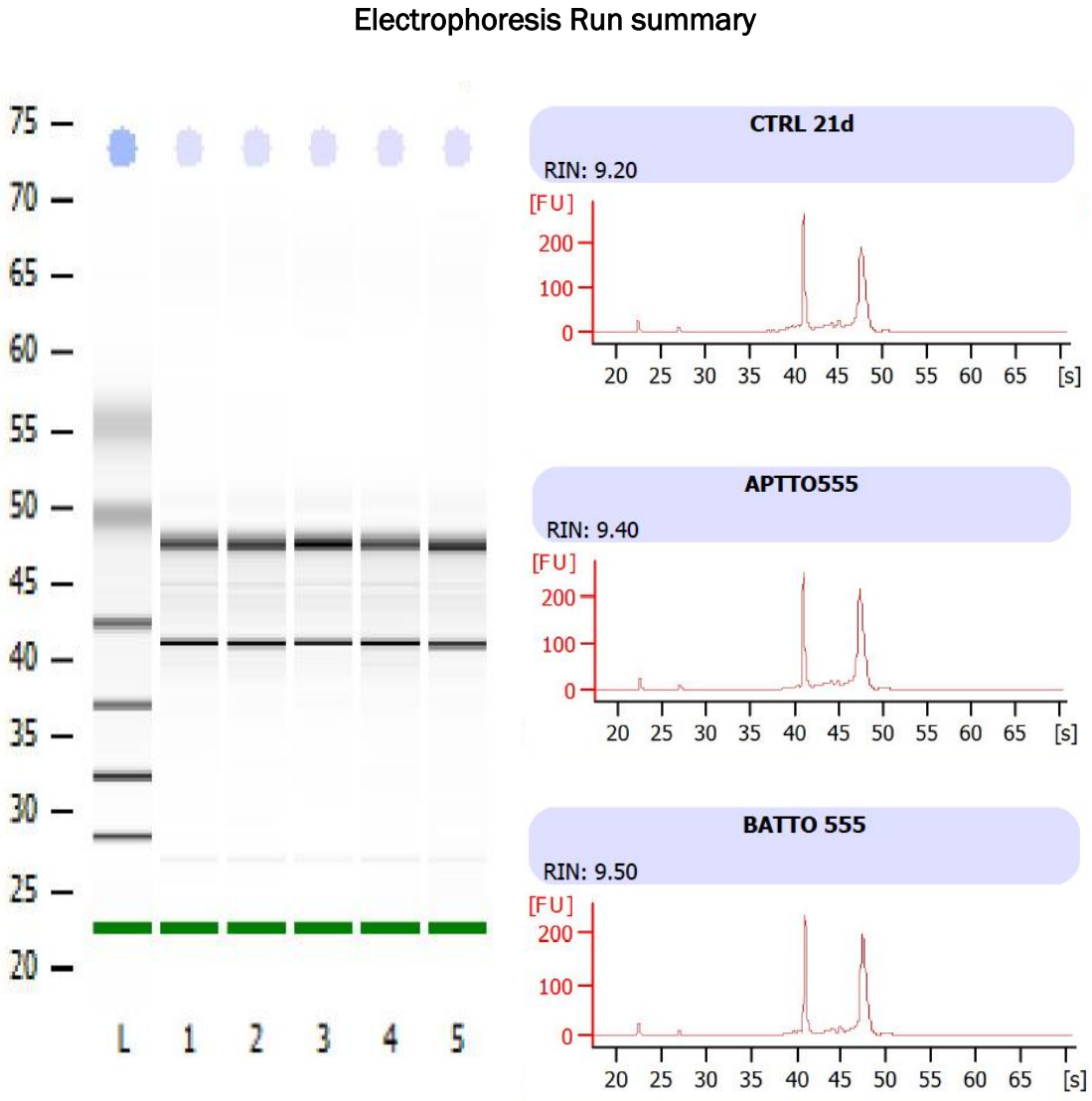
Samples	ng/ul	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}/A_{230}
CTRL	277.83	3.86	1.841	2.10	1.28
30 min	297.06	5.438	2.640	2.06	1.61
1 hr	255.53	5.973	2.899	2.06	1.71
2 hrs	247.23	6.388	3.052	2.09	1.85
6 hrs	273.68	5.499	2.619	2.10	1.61
9 hrs	324.91	3.836	1.833	2.09	1.65

(A_{260}=Absorbance 260; A_{280}=Absorbance 280)

6.3.4.1.2 Total cellular RNA analysis of quality

RNA extraction requires a second quality checkpoint which consists of identifying the integrity of the RNA extracted. This technique was performed in collaboration with Melissa Doherty from the molecular laboratory at University of Southampton. The output of this technique indicates that there is high quality RNA extracted when RNA integrity scores (RIN) are above 8. This was the case for the experiments presented in Figure 6.21 and Figure 6.22.

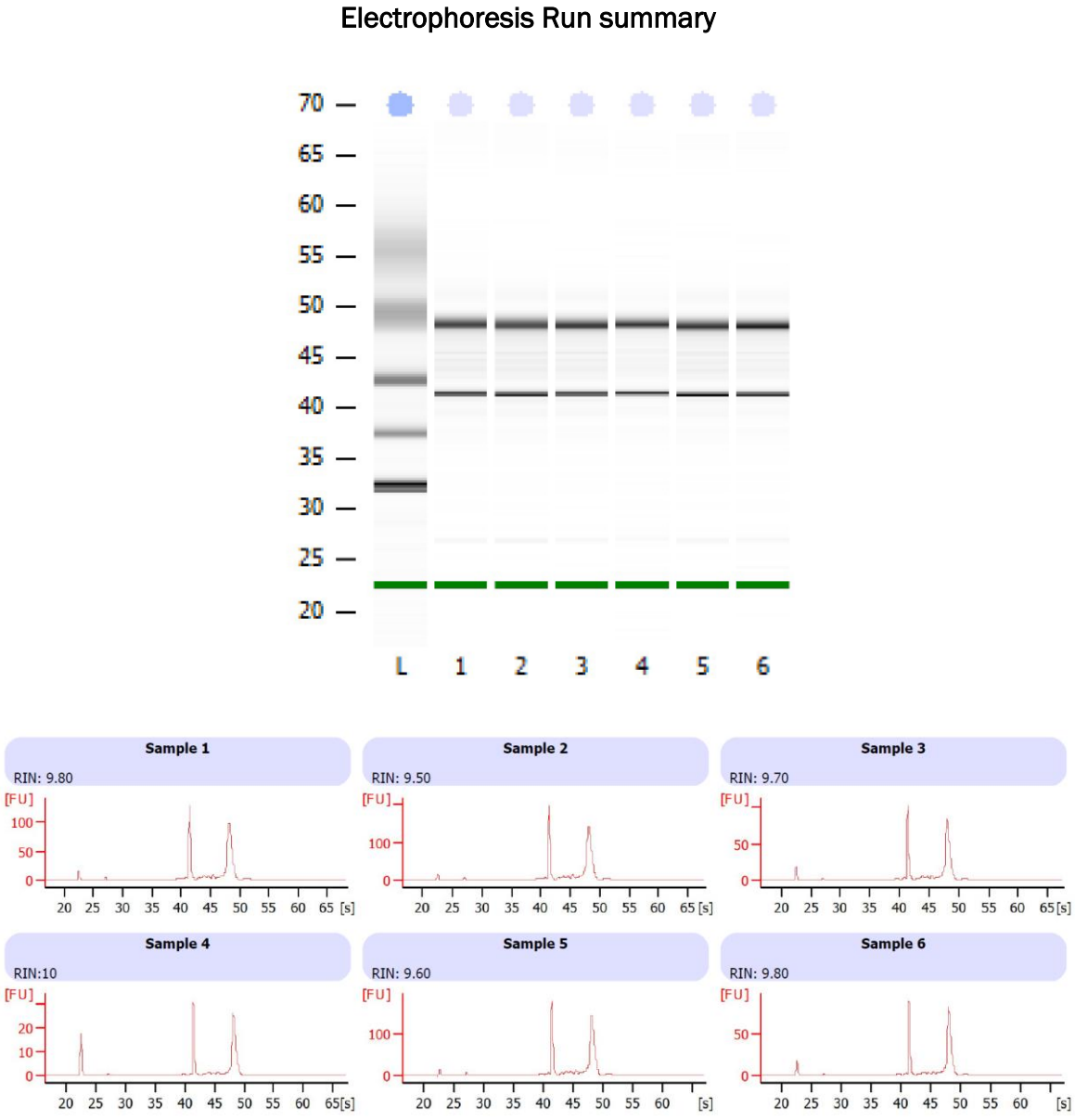
Figure 6.21 Quality of RNA extracted at 21 days of Caco-2 cell growth when inflammatory response was induced from the basolateral and apical sides. Outputs using Agilent Bioanalyzer technology.



L=Ladder; 1=Control; 2=Apical side treatment Cocktail [1/10/1]; 3= Basal side treatment Cocktail [1/10/1]; 4= Apical side treatment Cocktail [5/50/5]; 5=Basal side treatment Cocktail [5/50/5]. (RIN scores for all experimental conditions were above 8 which indicates quality in the RNA extracted. Representative samples are shown).

Quality of RNA extracted at 19 days of Caco-2 cell growth when inflammatory response was induced in a time course manner from the basolateral sides is shown in figure 6.22.

Figure 6.22 Quality of RNA extracted at 19 days of Caco-2 cell growth when inflammatory response was induced in a time-course manner from the basolateral side. Outputs using Agilent Bioanalyzer technology.



L=Ladder; 1=Control; 2=30 min; 3= 1hr; 4= 2hrs; 5=6hrs 6=9hrs. (RIN scores for all experimental conditions were above 8 which indicates quality in the RNA extracted. Representative samples are shown).

6.3.4.2 Analysis of reference genes using RT-qPCR in the Caco-2 cell culture model exposed to TNF-α; IFN-γ and IL-1β

Reference gene expression was analysed in the experimental conditions under examination. Gene expression in response to exposure to the inflammatory cocktail was assessed using two approaches: exposing Caco-2 monolayers grown for 21 days to the two cytokine cocktails for 24 hours when the inflammatory response was induced from the apical and basolateral side using the low and high cocktail concentrations (Figure 6.23) and exposing monolayers grown for 19 day to the cytokine cocktails for 30 minutes, 1 hour, 2 hours, 6 hours and 9 hours to induce inflammatory response from the basolateral side (Figure 6.24). Both approaches yielded the same finding: two reference genes were consistently stable, these were SDHA and YWAZ.

Figure 6.23 Average expression stability of candidate reference genes following the inflammatory stimulation for 24 hrs of incubation at 21 days of cell culture growth

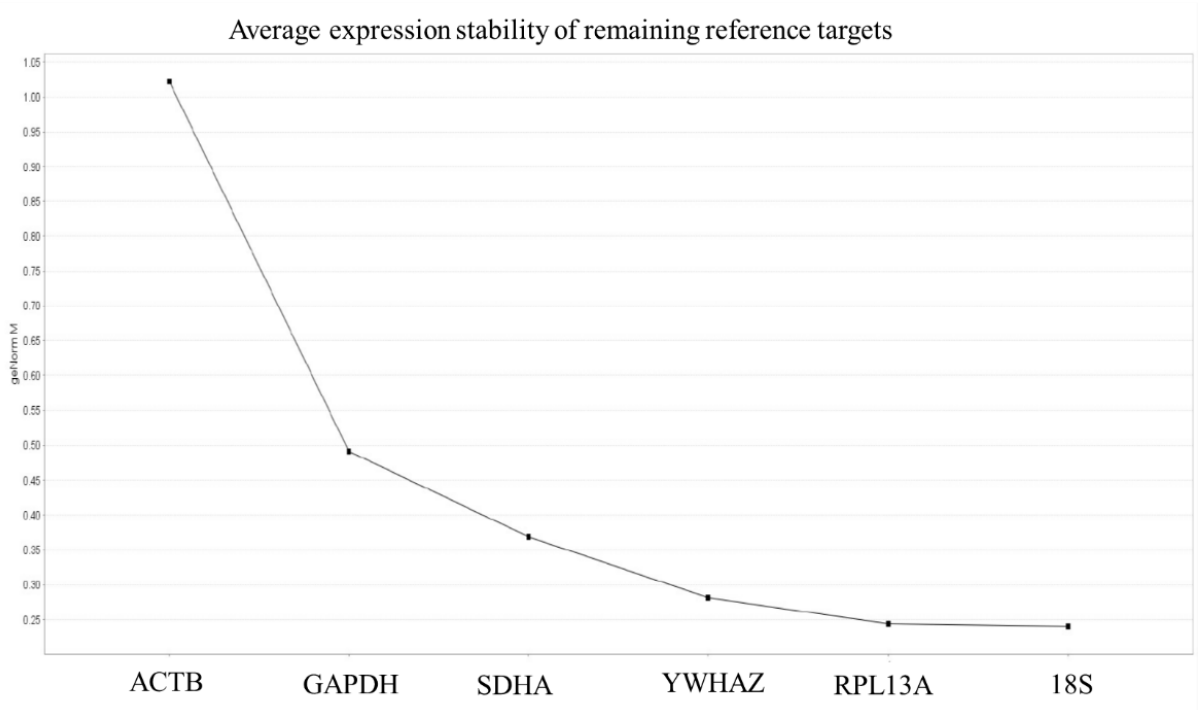
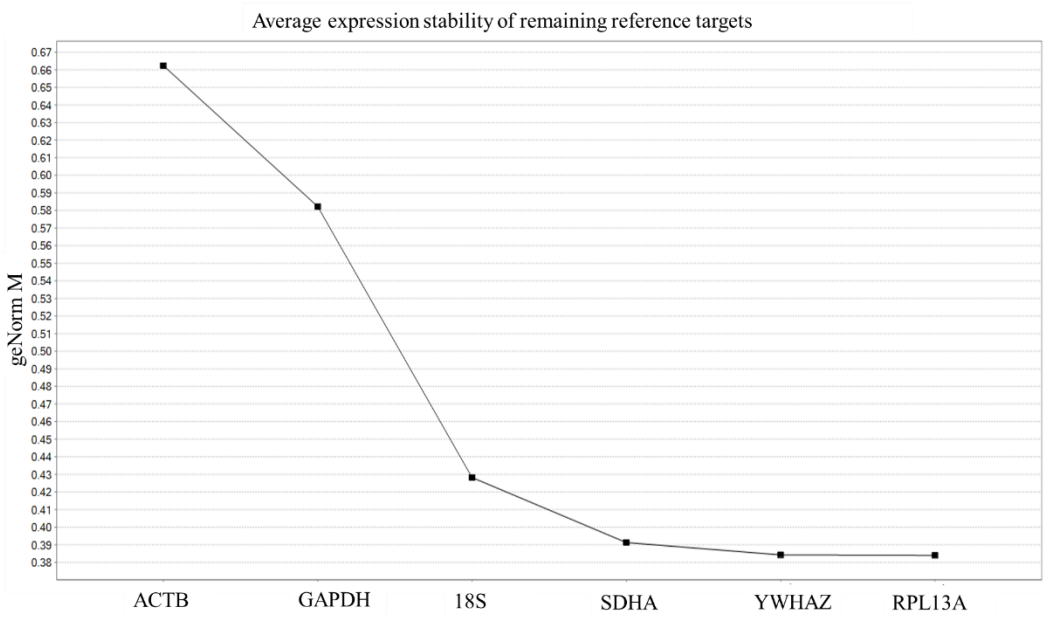


Figure 6.24 Average expression stability of candidate reference genes following the inflammatory stimulation for 30 min and,1,2,6 and 9 hrs of incubation at 19 days of cell culture growth.

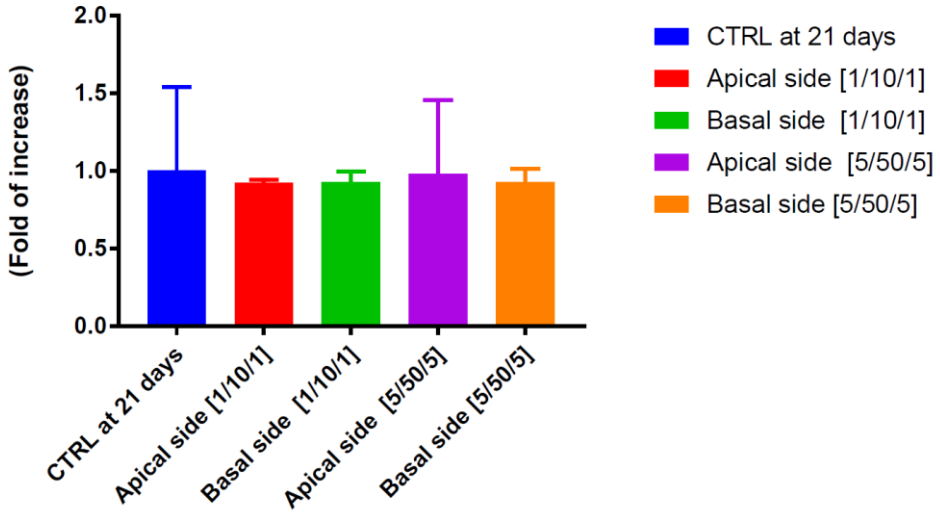


6.3.5 Targeted gene expression analysis of junctional complexes (TJ): Relative mRNA gene expression of (OCLDN), ZO-1 (TJP-1), claudin-1 (CLDN-1) in Caco-2 cells determined by RT-qPCR

Occludin, zonula and claudin are important structural genes encoding for junctional complexes. The results herein presented indicate that the inflammatory stimuli added to the cells did not yield to changes in the relative mRNA gene expression of the targeted genes. The relative mRNA expression of occludin (OCLDN), zonula-1 (ZO-1 or TJP-1) and claudin (CLDN) was not affected by cytokine cocktail exposure of 21 day Caco-2 monolayers regardless of the side of treatment application and the cytokine concentrations as shown in Figure 6.25

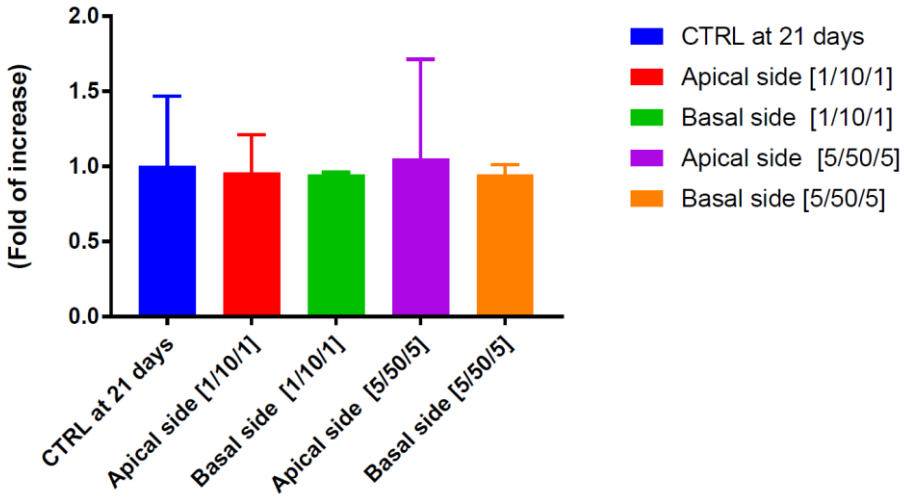
Figure 6.25 Relative mRNA expression of OCLDN, ZO-1 and CLDN-1 in differentiated Caco-2 cells exposed to inflammatory stimulus at 21 days.

OCLDN



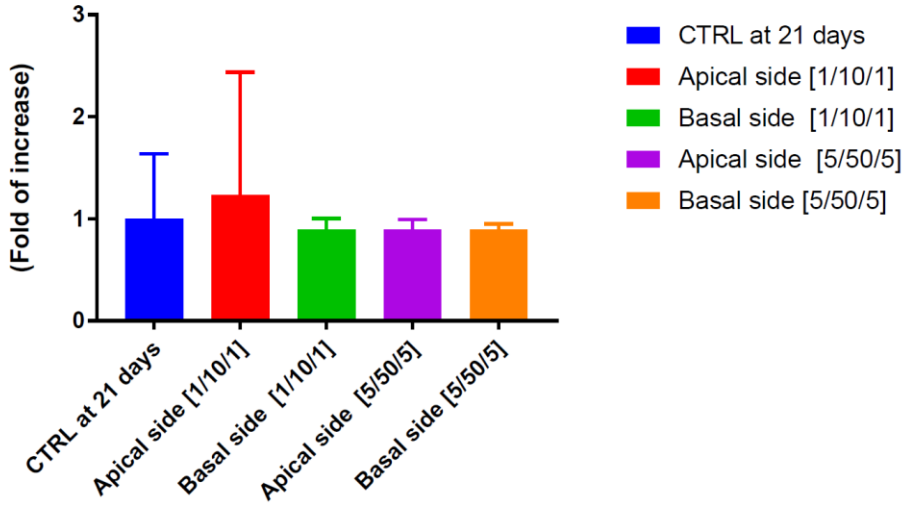
Mean (±SEM) (n=4). Gene expression of occludin in cells exposed to inflammatory cocktail or control following 24 hours of incubation. Ct values for occludin expression were normalised to SDHA YWAZ and 18 s. Two way ANOVA control and time point vs conditions of interest. Differences in fold of expression not statistically significant. Related Samples Two-Way Analysis of Variance (ANOVA (p>0.05)

CLDN-1



Mean (±SEM) (n=4). Gene expression of claudin in cells exposed to inflammatory cocktail or control following 24 hours of incubation. Ct values for claudin expression were normalised to SDHA YWAZ and 18 s. Two way ANOVA control and time point vs conditions of interest. Differences in fold of expression not statistically significant. Related Samples Two-Way Analysis of Variance (ANOVA (p>0.05)

TJP-1

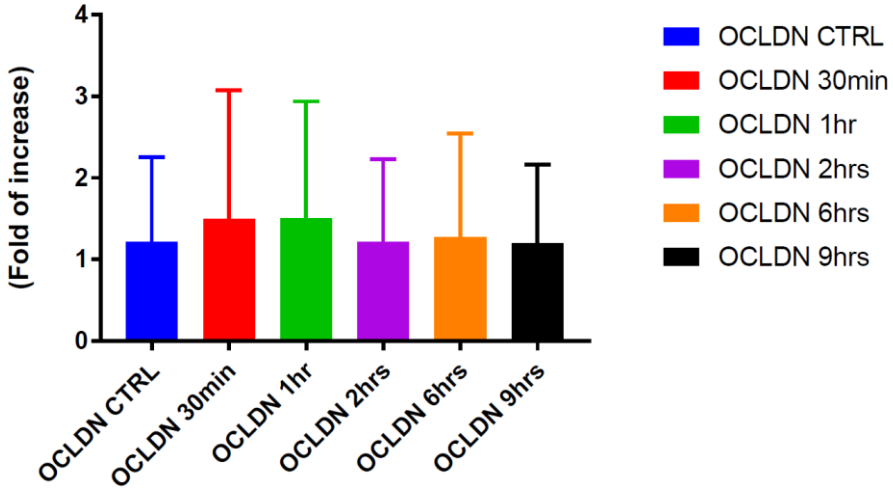


Mean (±SEM) (n=4). Gene expression of zonula (TJP-1) in cells exposed to inflammatory cocktail or control following 24 hours of incubation. Ct values for zonula expression were normalised to SDHA YWAZ and 18 s. Two-way ANOVA control and time point vs conditions of interest. Differences in fold of expression not statistically significant. Related Samples Two-Way Analysis of Variance (ANOVA ($p>0.05$)

Moreover, the relative mRNA expression of OCLDN, ZO-1 and claudin was examined at earlier time points after inducing an inflammatory response (30 min, 1, 2, 6 and 9 hours) from the basolateral side at 19 days of cell growth. Results shown in figure 6.26 indicate no significant changes in the expression of targeted genes.

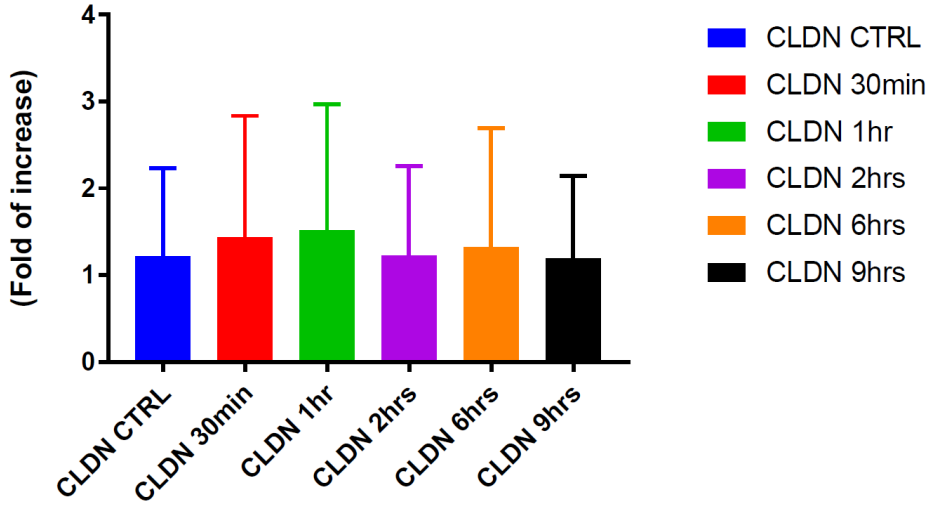
Figure 6.26 Relative mRNA expression of OCLDN, ZO-1 and CLDN-1 in differentiated Caco-2 cells exposed to inflammatory stimulus at 19 days.

OCLDN

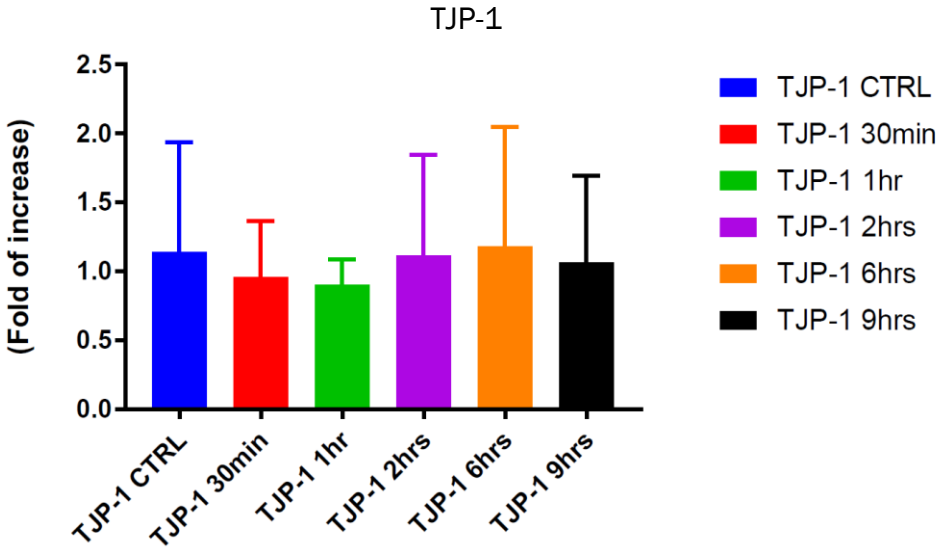


Mean (±SEM) (n=8). Gene expression of occludin in cells exposed to inflammatory cocktail or control following 24 hours of incubation. Ct values for occludin expression were normalised to SDHA YWAZ and RPL13. Two way ANOVA control and time point vs conditions of interest. Differences in fold of expression not statistically significant. Related Samples Two-Way Analysis of Variance (ANOVA ($p>0.05$)

CLDN-1



Mean (±SEM) (n=8). Gene expression of claudin in cells exposed to inflammatory cocktail or control following 24 hours of incubation. Ct values for claudin expression were normalised to SDHA YWAZ and RPL13. Two way ANOVA control and time point vs conditions of interest. Differences in fold of expression not statistically significant. Related Samples Two-Way Analysis of Variance (ANOVA ($p>0.05$)



Mean (±SEM) (n=8). Gene expression of zonula in cells exposed to inflammatory cocktail or control following 24 hours of incubation. Ct values for zonula expression were normalised to SDHA YWAZ and RPL13. Two way ANOVA control and time point vs conditions of interest. Differences in fold of expression not statistically significant. Related Samples Two-Way Analysis of Variance (ANOVA ($p>0.05$)

6.4 Discussion

The intestinal epithelial barrier is the first mechanism of immune defence, since it separates the endogenous environment from the "internal" exogenous environment. However, this is a simplistic view since it emphasises only separation and omits more complex interactions that the intestinal epithelium has with, amongst others, the host immune system. Disease and ageing both affect the physical and immunological properties of the intestinal epithelial barrier (Chapter 1). The altered intestinal barrier properties in turn are likely to impact age-associated conditions like inflammageing [474]. Thus, the study of intestinal barrier integrity and functionality is important in understanding both the transition from health to disease and age-related changes. Furthermore, such studies are important to identify novel preventive and therapeutic strategies and their mechanism(s) of action. Some aspects of gut epithelial biology can be studied *in vitro*, since cells such as Caco-2 cells form monolayers *in vitro* that reflect some of the gut's *in vivo* properties, including nutrient transport. The research presented in this chapter used the Caco-2 cell culture system to establish a model of gut epithelial inflammation that can be used to test the effects of probiotics (described in chapters 8 and 9) as a parallel *in vitro* investigation to the PRINCESS immune sub-study (described in chapters 3 to 6). The research described in this chapter identifies that Caco-2 cell

monolayers are capable of mounting a significant inflammatory response involving the release of cytokines, chemokines and the adhesion molecule ICAM-1 in response to exposure to an inflammatory cocktail that mimics the pattern of cytokines initially released by monocytes and T-cells in the initiation of an immune response towards microbial infections or to a classic inflammatory stimulus like LPS released from Gram negative bacteria: a mix of TNF-α, IFN-γ and IL-1β was used as the stimulant. The research also indicates that the structural properties and integrity of the epithelial barrier are highly sensitive to such an inflammatory insult, as the TEER measurements are affected rapidly. Intriguingly however, there was little effect on expression of genes encoding TJ proteins in response to the inflammatory cocktail. This may indicate that the loss of barrier function that was observed does not relate to down-regulation of TJ proteins as might be expected, although there may be other explanations for this apparent disparity.

6.4.1 Establishing the Caco-2 cell monolayer model and selection of the inflammatory cocktail

The Caco-2 cell monolayer model was established according to the literature and the procedure indicated in section 6.2.2. TEER values observed are in accordance with what has been previously described. A TEER value ranging from 1400 to 2400Ω.cm^2 indicates a "tight" classification of the epithelial barrier [529]. TEER values ranging from 300-400 Ω. cm^2 indicate an "intermediate" permeability barrier, and values from 50-100 Ω.cm^2 indicate a "leaky" permeability barrier [513]. In the current research, TEER time course is consistent with reports in the literature [513]. At 21 days of Caco-2 cell growth and basolateral treatment with the cocktail TNF-α (1 ng/ml); IFN-γ (10 ng/ml); IL-1β (1 ng/ml) for 24 hours TEER was reduced by 43.1%. The reduction seen with 19 day cultures was fairly similar (36.3%), indicating that either time point is suitable to assess any potential anti-inflammatory effect of probiotics or other agents. This timeframe of 19-21 days is in accordance with what other researchers have identified [530].

The initial selection of cytokines used in the cocktail composition to induce the inflammatory response was based on previous research showing that these cytokines individually and in various combinations can elicit inflammatory responses in Caco-2 monolayers (Table 1.5) These cytokines are also relevant to ageing since older people often show higher circulating concentrations of these cytokines than younger adults [531] and the production of TNF-α, IFN-γ and IL-1β by T cells and monocytes assessed

in vitro is higher in a healthy aged population (mean: 79.6 +/- 7.5 y) when compared with healthy young controls (mean: 24.6 +/- 3.1 y) [531]. Thus, considering two important aspects (firstly, that ageing is associated with increased inflammatory activity measured in the blood due to immune disturbances, and secondly, that IL-1β and TNF-α are mainly produced by monocytes and macrophages and that IFN-γ is produced mainly by T lymphocytes -important immune cells in the GALT) the selection of these cytokines as part of the cocktail is biologically relevant to the cross-talk of immune and epithelial cells in the context of the gut.

6.4.2 Barrier integrity assessment and immune mediators assessed in Caco-2 cell monolayer supernatants

The observations made here agree with evidence indicating that TEER increases progressively as long as a differentiated morphology and strengthening of junctional complexes in the Caco-2 monolayer occurs [512]. TEER values indicate that TJs were retained during the cell growth and incubation process. The findings herein presented also indicate that an inflammatory cocktail consisting of TNF-α (1 ng/ml), IFN-γ (10 ng/ml) and IL-1β (1 ng/ml) exerts an effect on the cell monolayer, measurable through the decreased TEER values and the increased production of the cytokines IL-6, IL-8 and IL-18. Additionally, the production of the immune mediators IP-10 and VEGF was also detected as well as the adhesion molecule ICAM-1. Interestingly, these mediators were produced with either apical or basolateral stimulation and were produced on both the apical and basolateral sides of the monolayer, although different patterns were evident for the different mediators (as shown in Figure 6.17). The inflammatory cocktail dosage selected and the time-frame of introduction into the system as well as the compartment of the culture chamber into which they are introduced, comprise a reproducible *in vitro* inflammatory model with which to explore the production of relevant immune mediators and *in vitro* strategies to preserve and recover epithelial barrier integrity. It is worth noting that the chemokine MIG and the adhesion molecules E-selectin and VCAM-1 were not produced in this cell monolayer system.

Of important consideration, preliminary experiments conducted in our group (data not shown) using these cytokines individually and in different concentrations support the conclusion that the cocktail is the optimal inflammatory stimulus for the Caco-2 cells. The special focus was the basolateral side as it resembles the physiological side corresponding to the bloodstream; however responsiveness in the apical side was also

examined as treatments added in this side will be examined in chapters 7 and 8. The inflammatory panel assessment was focused on the basolateral side due to its physiological relevance. Of particular interest, 21 days of cell growth has been reported as a stage were the monolayer reaches maximum confluence. However, if extended cultures are intended, it appears that the monolayer confluence starts to be lost as seen through the decreased TEER values (see Figure 6.6). Due to the intended use of this epithelial cell culture model, 19 days was chosen to set up the model to assess the inflammatory response adding the cocktail in the basolateral compartment to assess the responsiveness of the cells to further treatments (chapters 7 and 8).

6.4.2.1 Cytokine production in the *in vitro* model. Translation of findings into the biogerontological field

Importantly in this culture system, it was possible to assess the production of relevant inflammatory cytokines, and these findings are translatable in the context of ageing and the development of models for its biological study. IL-6 and IL-8 production were detected in the basolateral and apical media of this enterocyte model and their production was significantly higher when compared with the control.

In the *in vivo* context, IL-6 and IL-8 are important cytokines in the understanding of ageing as it has been described that aged individuals often present a limited control over their production with increased concentrations in the bloodstream [532-534]. IL-18 is linked to the inflammatory process that increases during aging, as it is implicated in the pathogenesis of atherosclerosis as well as autoimmune diseases [535]. The unregulated production of IL-6 has been linked to chronic inflammatory diseases, chronic infectious processes, osteoporosis and autoimmune conditions -common conditions in aged individuals- as this pleiotropic cytokine targets different cell types and possesses biological activity in an endocrine, paracrine and autocrine manner and, from an immunological perspective, IL-6 is secreted by macrophages, which tend to be overly active in ageing and thus contributing with increased levels of IL-6 [536]. IL-8 has been found to be produced by neutrophils and macrophages, immune cells which often present a disturbance with the progress of age [537]. Strategies aimed to identify how to control the production of these inflammatory cytokines might contribute to the control of the inflammageing process. The intestinal epithelial barrier might confer a site to achieve this control, and the establishment of the Caco-2 inflammatory model offers a system to continue with the study of this.

Vitkus *et al*. have found that the combination of IL-1β and TNF-α induces a synergistic enhancement of IL-6 secretion in Caco-2 cells [538]. The current findings indicate that IFN-γ can be added to the inflammatory cocktail of cytokines to examine IL-6 production in a differentiated cell culture system. Using this *in vitro* model Sonnier *et al*. have described that TNF-α induces an increased production of IL-8 using a concentration of 100 ng/ml [539]. The findings herein presented suggest that an inflammatory cocktail using a much lower concentration of TNF-α is capable to induce a measurable response in IL-8 production.

Lastly, although IL-18 was not produced in differential amounts between the unstimulated and cytokine stimulated Caco-2 monolayers, IL-18 is produced by macrophages and dendritic cells. Both these cell types are a crucial part of the mucosal immunity. Incorporating these cells in co-culture into the system might allow a more in depth study of IL-18. Additionally, it has been suggested that IL-18 is produced by the intestinal epithelium although it is still unclear which physiological conditions induce its production [540]. The findings herein presented confirm that an inflammatory cocktail consisting on TNF-α, IL-1β and IFN-γ does not promote IL-18 production, although it is produced by the monolayers. Interestingly, it has been previously described that IFN-γ is a limiting agent of the biological functions of IL-18 as IFN-γ is able to induce IL-18Bpa, thus limiting the release of IL-18 [541]. The system IL-18/IL-18Bpa was not a target of study in the current experiments, but future research could examine the role of IL-18Bpa in the response to the cytokine cocktail and also whether a cocktail without IFN-γ might have a different effect on IL-18 than a cocktail with IFN-γ.

6.4.2.2 IP-10 and VEGF production in the *in vitro* model. Translation of findings into the biogerontological field

Two relevant immune mediators were successfully induced following the stimulation of Caco-2 cell monolayers with the inflammatory cocktail. IP-10 is chemokine which has pleiotropic immune effects as it can stimulate monocytes, NK cells and T cell migration as well as modulate the expression of adhesion molecules. It has been demonstrated through a cohort study that serum values of IP-10 are gradually upregulated with the progression of age, as its concentration was almost doubled in elderly participants from 76 to 80 y old when compared with the age group from 40 to 50 y old [542]. Setting up an inflammatory model using enterocytic cells as herein presented might be a suitable strategy to study the pathways in which IP-10 is involved. The inflammatory cocktail was

able to induce the production of IP-10 both basolaterally and apically in significant amounts when compared with the control conditions. From an enteric perspective, IP-10 has been implicated in the pathophysiology of Crohn's disease [543]. Thus, this *in vitro* model offers a way to study strategies to control its contribution to inflammageing and to intestinal disease. IP-10 is inducible by IFN-γ [544], perhaps explaining its high production in this model.

VEGF belongs to the growth factor family. The significant expression of the vascular endothelial growth factor in the Caco-2 model confirms the close cross-talk among epithelial cells and endothelial cells. This supports what has been recently described in the area of gut endothelial cells as part of a second line of immune defence mechanism [545]. Enterocytes can produce VEGF to exert an effect on intestinal endothelial cells. VEGF has been implicated in pathologies such as gastric cancer and recently, pharmacological strategies have been exploring anti-VEGF treatments in the treatment of gastric cancer. These analyses are being conducted in Caco-2 cells [546]. Co-adjuvant therapies targeting the intestine are therefore crucial and the model herein proposed offers an alternative to study the production of VEGF apically and basolaterally.

6.4.2.3 ICAM-1 production in the *in vitro* model. Translation of findings into the biogerontological field

Lastly in the inflammatory panel exploration, ICAM-1 was produced in increased amounts on the apical side of the monolayers when the inflammatory stimulatory cocktail was added basolaterally. In contrast, the adhesion molecules VCAM-1 and E-selectin were not detected in the inflammatory model. The expression of ICAM-1 on human intestinal epithelial cells has been described previously in the context of increased levels of pro-inflammatory cytokines locally present in the gut mucosa [547].

6.4.3 Molecular analysis: Change in epithelial integrity but not in junctional gene expression

The modifications in the TEER values following a pro-inflammatory stimulation were not accompanied by significant modification in the gene expression of the junctional proteins zonula, occludin and claudin. This lack of effect on these genes is surprising as it has been shown that the components of the inflammatory cocktail separately are able to induce disruption in TJs as well as in TEER values [517, 548].

The quality of RNA obtained from the Caco-2 monolayers was good. With regards the reference genes, SDHA and YWHAZ appeared as the most stable reference genes when assessing the targeted gene expression. SDHA, whose official full name is succinate dehydrogenase complex flavoprotein subunit A, encodes for a protein that corresponds to a main catalytic subunit of the protein succinate-ubiquinone oxidoreductase. That protein is a complex of the mitochondrial respiratory chain. The protein is localized in the inner membrane of the mitochondria [549]. YWHAZ, whose official full name is tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta, is a gene that encodes a protein that mediates the signal transduction by binding phosphoserine-containing proteins [550].

PCR experiments were conducted according to the MIQE guidelines (minimum information for publication of quantitative real-time PCR experiments) [551]. The RT-qPCR was conducted using geNorm (Primer design) which allowed to identify the specific reference genes for the analysis of RT-qPCR. The geometric mean of these reference genes was used to quantify the relative expression of the targeted genes.

It has been reported that TNF-α at a concentration of 10 ng/ml is able to induce a reduction in TEER through a decreased expression of zonula [552]. Another study reported that TNF-α at concentrations of 10 or 100 ng/ml did not affect the expression of occludin mRNA. Western blotting confirmed that TNF-α did not affect the expression of the non-phosphorylated occludin protein, these results were analysed in a time-course manner at 0, 4, 8, 16 and 24 hours of treatment with the cytokine [553]. The findings of the current study suggest that an effect of inflammation on TEER can occur without influencing expression of the genes encoding junctional proteins. The proteins themselves were not measured in this research. It is possible that protein levels were affected by inflammation through mechanisms not involving altered gene expression, assessed as mRNA levels. For example, the inflammatory stimulus could have inhibited junctional protein synthesis, promoted junctional protein degradation or altered a mechanism of protein modification that affected the junctional protein function. Those aspects could be assessed in future work.

6.5 Conclusion

The hypothesis related to the establishment of a reproducible Caco-2 monolayer can be accepted as an inflammatory model was developed, reflected in TEER values changes ranging from a tight barrier to a decrease in permeability. An inflammatory cytokine

cocktail was shown to reduce TEER by around 40% in monolayers previously grown for 19 or 21 days. In response to that cocktail the monolayers produced a range of cytokines, chemokines, growth factors and adhesion molecules in a time dependent manner. These appeared on both apical and basolateral sides of the monolayer in response to stimulation on each side and each mediator had an individual pattern of appearance. In contrast to the effects of inflammation on TEER and inflammatory mediator production, there was no effect on expression of junctional protein genes. Nevertheless, it is considered that a reproducible model of intestinal epithelial inflammation has been established. This will be used to investigate the stimulatory effects of probiotics and the preventive and restorative effects of their heat-inactivated version in the next two chapters.

Chapter 7 Effect of heat inactivated and live probiotics on Caco-2 cells

7.1 Introduction

The definition provided by the World Health Organisation (WHO) is that probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (See section 1.15). This concept makes reference to "live organism" because it is assumed that probiotic organisms need to be live to interact metabolically with the host, particularly the gut epithelial tissue and the mucosal immune system, in order to produce any health benefits [554]. Live probiotic bacteria have shown clinical benefits (See section 1.15.4.1). These include induction of remission of active ulcerative colitis and alleviation of symptoms of patients with gastrointestinal disease through control of pathogenic intestinal bacterial overgrowth and therefore prevention of infections [555, 556]. Relatively recent evidence has suggested that probiotics can act beyond the gastrointestinal tract through neuroimmunomodulatory actions resulting from their live metabolic state and the release of molecules that have neuroactive actions, which has been postulated in the concept of the gut-brain axis [440]. These are examples of live probiotics exerting an effect on health.

Despite this evidence, less clarity is available on the health effect of probiotics in the elderly, who often tend to have poorer health status, or an immunocompromised condition linked to frailty (See section 3.5). This situation is aggravated in institutionalised care or nursing homes (See section 1.14.1). Available evidence has suggested that introducing live bacteria to improve different aspects of health in the elderly could be an efficient strategy. This rationale is based on the mechanisms of action through which probiotics act and therefore, probiotics could also contribute in the management of common comorbidities in the elderly. For instance, through improving surfaces of absorption in the epithelial gut [557] which theoretically could improve malnutrition, or by improving the general innate immune response [558]. These mechanisms, proved in animals [559, 560] or *in vitro* studies [561] have not also been equally successful demonstrated *in vivo,* particularly in the elderly. Thus, there is mixed evidence that probiotics might be a useful strategy in the management of malnourished elderly [562], or frailty in long-term facilities [563] or intestinal infections [564]. Other evidence incline towards avoiding probiotic therapeutic usage in immunocompromised individuals [565].

General safety of probiotics in humans has been assumed and accepted, particularly for the strains LGG and BB-12 [566-570]. However, there are still safety concerns, particularly in the elderly for different reasons. Firstly, there is lack of evidence of

research conducted solely on aged individuals, as often studies include adults with a wide age range (See literature review conducted on Chapter 1). Secondly, there are reports that probiotic bacteria belonging to the genera lactobacilli, mainly, might have caused systemic infections [571]. Other case report studies of elderly individuals seem to indicate that elderly individuals are prone to suffer deleterious effects from probiotics, particularly LGG due to its rapid growth rate, which apparently creates microbial collections in liver biopsies. The hypothesis was confirmed by a study where the bacterial isolates from the product consumed corresponded with the bacterial strain identified in liver tissue [572]. These reports and the immunocompromised status in which elderly individuals are found, raise questions related the safety of probiotics in the elderly.

Some of the effects assumed for probiotics on health are linked to metabolic products of bacterial metabolism. However, it also appears that some of the components present in the bacterial strain are responsible for the benefits observed on health, for instance the lipoteichoic acid in the cell wall [376, 573, 574]. It might be that the physical contact and interaction with the gut epithelium is an initial mechanism in which effects on health start to be triggered, prior any interaction through metabolic products. Therefore, inactivated organisms could also be functional and may have health benefits. Animal models have shown that both live and heat-killed organisms have significant anti-inflammatory effects through the reduction of IL-6, and IL-23, in an experimental model of colitis [575]. *In vitro* studies examining the effect exerted by Bifidobacterium in its heat-killed state showed that its action was comparable with the anti-inflammatory results exerted by the live (active) strain. This experiment consisted of comparing the production of IL-8 and IL-10 in immune cells collected from patients with ulcerative colitis. Both strains showed anti-inflammatory effects [576]. Another study addressing the effects exerted by LGG in Caco-2 cells, observed that LGG can inhibit IL-8 production, even when cells are treated with structural material of LGG [430]. In humans, particularly vulnerable subgroups, heat-inactivated probiotics may be a safer alternative to live organisms [577]. The rationale for the use of inactivated and even killed microorganisms has come from studies showing that the interactions of the host with the bacterial cell wall might enhance immune responses in the gut epithelium through interactions of the microbe-associated molecular patterns (MAMPs) with the Toll-like receptors (TLRs) and other mucosal pattern recognition receptors (PRRs) likely mediating some of the beneficial responses to non-viable bacteria [559, 578, 579].

In addition to considerations related to immunomodulatory effects of probiotics, it seems likely that any probiotic preparation contains non-viable and even dead organisms [580]. Non viability and death could result through prolonged storage and different organisms might be more susceptible than others. LGG and BB-12 are anaerobic organisms and this condition cannot always be ensured in a given matrix. Probiotic inactivation can also happen during the gastrointestinal passage of the microorganism. Thus, individuals are exposed to inactive organisms and it is important to see whether they have effects that might be beneficial or detrimental to the host.

Researchers have suggested that the Caco-2 model might be a suitable alternative to assess the interaction among the microorganisms and the gut epithelia [581]. The inactivation of the microorganism *in vivo* might occur due to environmental conditions in which the probiotics are kept. Laboratory-developed techniques to generate non-viable bacteria include acid digestion, which would mimic the probiotic being affected by bile acids [582]. Other approaches include heat-inactivation. Heat inactivation has shown to be a condition in which the heat-killed microorganism might beneficial to the host without causing harm [583].

The research described in this chapter addressed conditions to heat-inactivate the probiotic strains LGG and BB-12 and then explored the inflammatory response that these inactivated microorganisms might exert on the Caco-2 epithelial cell model.

7.1.1 Hypothesis

It is hypothesized that LGG and BB-12 can be heat inactivated. Likewise, it is hypothesized that both the heat- inactivated LGG and BB-12 version as well as the live version will exert a comparable degree of inflammatory responses in Caco-2 cell monolayers.

7.1.2 Aim and objectives

The aim of this chapter was to identify optimum conditions to heat-inactivate LGG and BB-12 and to compare these inactivated strains and their live version with an experimental control (untreated). Objectives of the research described in this chapter were to:

- Identify the optimum temperature and duration required to heat-inactive LGG and BB-12;

- Identify the effects of live and heat inactivated LGG and BB-12 on TEER in Caco-2 cell monolayers;
- Identify the inflammatory responses of Caco-2 cell monolayers exposed to live and heat inactivated LGG and BB-12;
- Compare the inflammatory responses of Caco-2 cell monolayers exposed to live and heat inactivated LGG and BB-12 with an experimental control (untreated)

7.2 Methods

These experiments were conducted at the Christian Hansen R&D laboratories in Denmark. Training and access to the facilities were provided by the research staff, Dr Anja Wellenjaus and the technician Christine Rasmussen. LGG and BB-12 were kindly provided by Christian Hansen laboratories and are the same strains that were offered to the participants in the PRINCESS clinical trial. LGG and BB-12 were heat inactivated by exposure to different heating times in order to identify an optimum condition to stop microbial growth. Previous findings obtained by the laboratory (data not published) have shown that a water-bath temperature of 70^{o}C for 5 minutes is able to heat inactivate LGG and BB-12 as evidenced by complete inhibition of growth. A water-bath temperature of 60^{o}C for 5 minutes does not completely kill either strain.

Overall, the methodology used involved growing LGG and BB-12 in MRS bouillion pH 6.5, pre-filled in 10 mL tubes. Subsequently, a selection of the late exponential phase was performed; the MRS bouillion used to grow the cells was washed away and after adjusting the count of probiotic organisms, the strains were resuspended in the Caco-2 media (grown according to methodology established by Natoli *et al*. [512]) and as described in section 6.2 to be used in later experiments. The TEER was performed at a multiplicity of infection (MOI) of 1:225. MOI corresponds to the ratio of targets (Caco-2 cells) in relation to agents (bacterial strains, LGG and BB-12) using live and heat-inactivated microorganisms. To heat-inactivate the microorganism, bacterial cultures were counted and cultured to reach a late exponential growth, early stationary phase. Later, the strains were heat-killed in pre-heated water at 62^{o}C for 0 min, 2 min, 4 min, 6 min and 8 min. Subsequently, a 10-fold dilution series of all the heat-treated samples was performed. This was conducted to prepare a deep-seed in MRS agar and count the CFU after 2 days to confirm the killing-curves. Once this step was completed, the successfully heat-inactivated strains were transferred to the confluent monolayer containing Caco-2 cells to compare the TEER and inflammatory responses analyzing

TEER behavior at 10 and 22 hours of growth. Subsequently, the inflammatory responses were explored.

7.2.1 Bacteria and related preparations

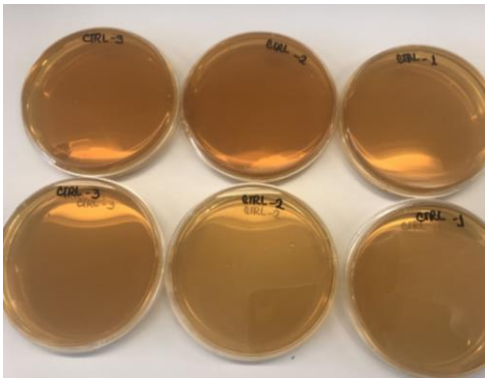
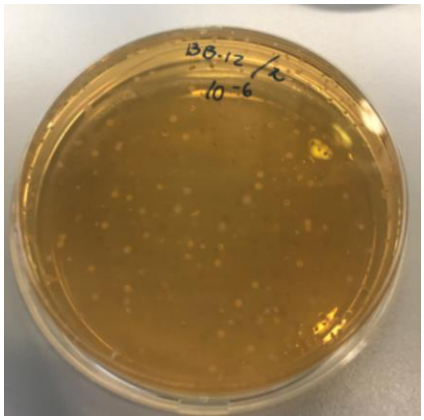
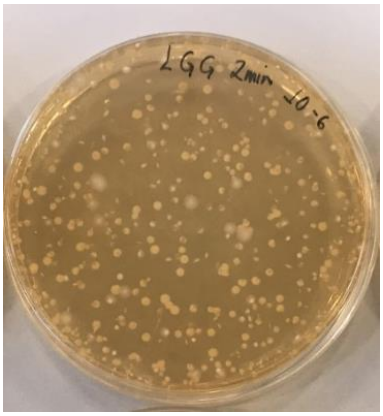
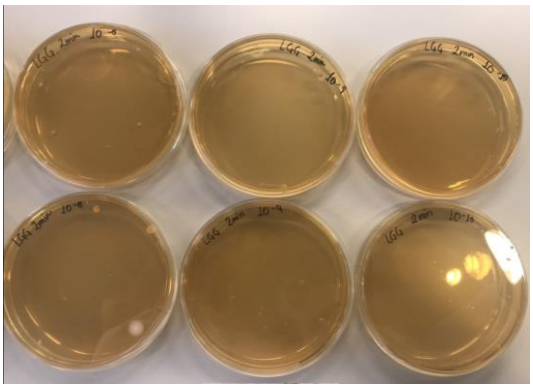
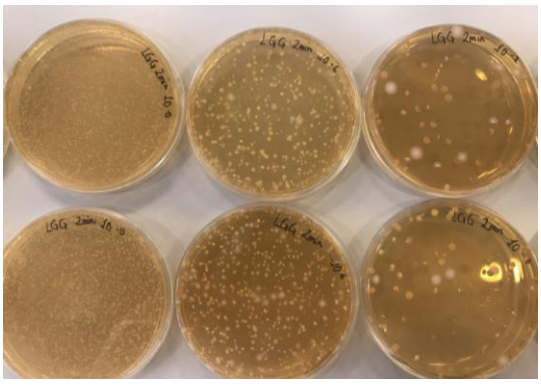
The strains used to perform these experiments are the same that were provided in the PRINCESS clinical trial to the elderly participants: BB-12 (**Genus:** *Bifidobacterium* **Species:** *animalis* **Subspecies:** *lactis*) and LGG (**Genus**: *Lactobacillus* **Species:** *rhamnosus*). The process started by collecting the strains from the -80°C freezer and transferring them to the pre-filled tubes containing 10 ml of MRS bouillion pH 6.5 (0.05% CyHCL). These strains require anaerobic conditions; therefore, the tubes were placed in chambers containing oxygen-removal bags. The chambers were placed in an incubator at 4°C overnight. The following day the incubator temperature was adjusted to 37°C and strains were left overnight. Once this period was completed, the growth curve was prepared by identifying the bacterial microorganisms at their optimum growth phase, which corresponds to late exponential and early stationary phase and was identified using spectrophotometry and clear semi-micro cuvettes. Serial dilutions from the agar were used by collecting 100 uL from each growth tube (Dilution factor = 10x). Absorbance was recorded and exponential phase of growth was chosen to subsequent culture.

To perform the heat-killing and inactivation verification, pre-warmed MRS agar was prepared in serial dilutions and poured into plates which were swirled gently in a circular motion to evenly distribute bacteria. Once agar solidification occurred the plates were transferred to an incubator at 37◦C for 2 days. Cell count was verified by checking the dilutions at which the cells stopped growing (See section 7.2.3.)

7.2.2 Bacterial strain growth in agar

Confirmation of bacterial growth was performed through seeding in agar and further incubation in anaerobic conditions to identify cell growth, if any. The process was conducted as presented in Figure 7.1., when a temperature of 62.3◦C was used for both strains. As shown in this figure, LGG continued to exhibit a high growth rate, thus the temperature was increased to 70◦C, where absence of growth was found.

Figure 7.1. LGG and BB-12 heat-inactivation process observed through seeding in agar.

<table>
<tr><td colspan="2">Experimental controls</td></tr>
<tr><td colspan="2">  </td></tr>
<tr><td>BB-12 CFU counting</td><td>LGG CFU counting</td></tr>
<tr><td>  </td><td>  </td></tr>
<tr><td>Cell count process</td><td>Cell count process</td></tr>
<tr><td>  </td><td>  </td></tr>
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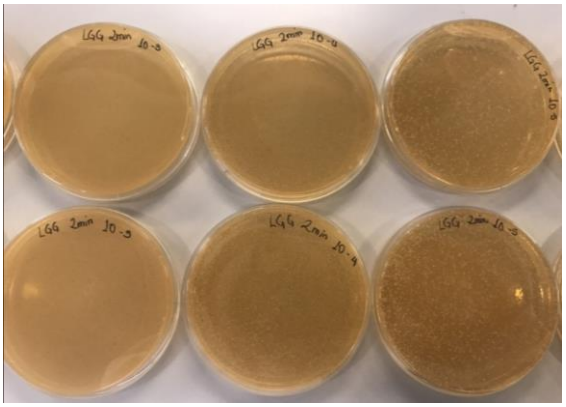
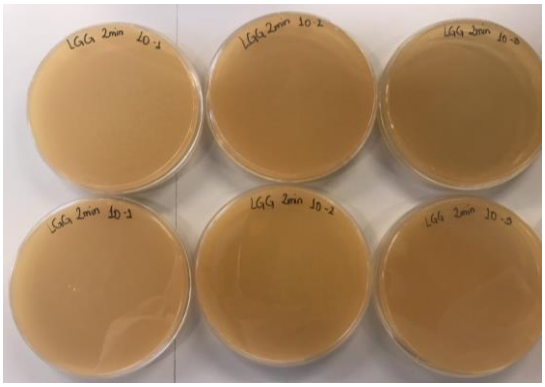




The observation of the agar and subsequent translation of findings required a process of quantification of colonies for both strains (LGG and BB-12) according to the different dilutions and the different times for heat-inactivation to identify rate of growth. This methodology consisted of counting the number of colony forming units (CFUs). Table 7.1. confirms that BB-12 exposed to 62.3°C for 6 and 8 minutes was optimum to achieve heat-inactivation. For LGG the conditions that allowed optimum inactivation were 70°C for 1 minute.

Table 7.1. CFUs for LGG and BB-12 grown in different temperatures for different times.

Conditions	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Temperature=62.3°C						TNTC	76	12	1	0
BB-12 0 min						TNTC	99	8	0	0
Temperature=62.3°C	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	40	1	0	0
BB-12 2 min	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	38	3	0	0
Temperature=62.3°C	TNTC	TNTC	102	11	2	0	0	0	0	0
BB-12 4 min	TNTC	TNTC	145	13	1	0	0	0	0	0
Temperature=62.3°C	0	0	1	0	0	0	0	0	0	0
BB-12 6 min	0	0	0	0	0	0	0	0	0	0
Temperature=62.3°C	0	0	0	0	0	0	0	0	0	0
BB-12 8 min	0	0	0	0	0	0	0	0	0	0
Temperature=70°C	0	0	0	0	0	0	0	0	0	0
LGG 1 min	0	0	0	0	0	0	0	0	0	0
Control media	0	0	0							
	0	0	0							
TNTC = Too numerous to count										

Finally, Table 7.2. in section 7.3.1. presents concluding conditions of heat-inactivation selected for each organism once the experiment was repeated and optimised for LGG. It was observed that BB-12 can be heat inactivated at 62.3°C for 6 minutes, whereas for LGG minimum conditions of heat-inactivation were 70°C for 3 minutes. These conditions of heat-inactivation were used to conduct experiments related to the assessment of inflammatory responses.

7.2.3 Immune mediators measured in Caco-2 following probiotic or heat-inactivated treatments

This experiment was conducted following the panel design in the preliminary inflammatory assessment as shown in section 6.2.3.2.

7.3 Results

Firstly, optimum conditions to achieve heat inactivation of BB-12 was 62.3^{o}C for 6 minutes, while optimum conditions of heat inactivation for LGG was 70^{o}C for 1 minute as shown in Table 7.2.

7.3.1 CFU count and heat treatment

Optimum heat-inactivation times correspond to those in which there was a null growth of CFU. Table 7.2 shows the effect of heating LGG or BB-12 on their viability.

Table 7.2. Effect of heat-treatment for different times on LGG and BB-12 counts.

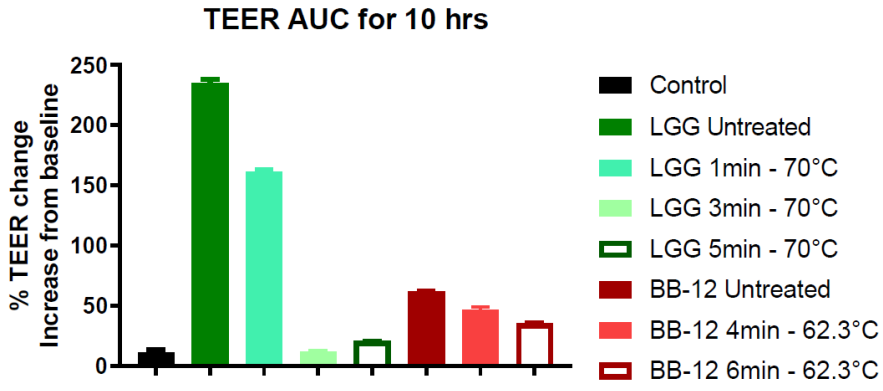
Strain	Heat-treatment at 62.3^{o}C	CFU/mL
BB-12	**0 minutes**	**9.4*10^{8}**
BB-12	2 minutes	3.9*10^{8}
BB-12	**4 minutes**	**1.2*10^{5}**
BB-12	**6 minutes**	**0**
BB-12	8 minutes	0
LGG	0 minutes	7.5*10^{8}
LGG	2 minutes	6.8*10^{8}
LGG	4 minutes	3.9*10^{8}
LGG	6 minutes	1.1*10^{8}
LGG	8 minutes	2.2*10^{7}
Strain	Heat-treatment at 70^{o}C	CFU/mL
LGG	**0 minutes**	**8.9*10^{8}**
LGG	**1 minute**	**5.5*10^{8}**
LGG	**3 minutes**	**0**
LGG	**6 minutes**	**0**

Bold indicates conditions selected to perform experiments.

7.3.1.1 TEER assessment in differentiated Caco-2 cells.

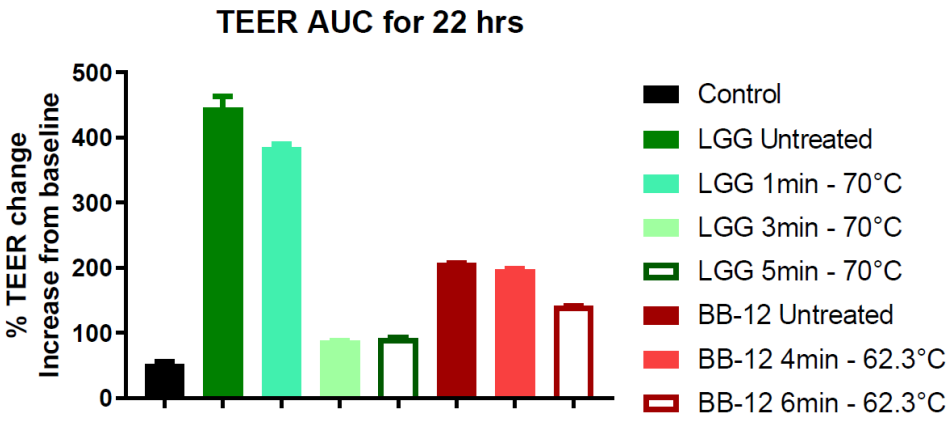
Apical application of live LGG and BB-12 increased TEER markedly, with the most pronounced effect seen with LGG (Figure 7.2 and Figure 7.3). Heat-inactivated organisms had a much smaller effect on TEER. The enhancement of TEER decreased as inactivation increased. The percentage of change in comparison to experimental control (without any probiotic or heat-inactivated microorganism) indicates that both strains were efficient in increasing the TEER. Live LGG was the most efficient strain in increasing the TEER (more than 2-fold at 10 hours; Figure 7.2), with the value almost doubling at 22 hours (Figure 7.2). Likewise, LGG heat-inactivated for 1 minute was efficient in increasing TEER values. The heat-inactivated versions at 10 and 22 hours did not differ from controls. Lastly, BB-12 in its live and untreated state was also effective in increasing TEER values (Figure 7.3). This increment was more prominent when the experiment was terminated at 22 hours. Inactive BB-12 was effective in increasing TEER values when compared with control.

Figure 7.2. TEER change after bacterial strain inclusion on the apical side at 10 hours.



Data are presented as mean and SD (n=3 per condition tested and control). Percentage of TEER increase is shown on the y-axis. These percentages were derived from correcting the augmentation observed with the baseline TEER value prior starting the experiment and calculating the percentage of increase. TEER values were measured at 10 hours after treatment. Experimental control (untreated – without probiotic) was used as factor control to conduct One-way Analysis of Variance - ANOVA using the factor control vs conditions of treatment p value <0.0001. Significance of change of conditions tested p<0.0001 (LGG and BB-12 untreated; LGG 1 min; BB-12 4 and 6 min). Lack of significance was observed for LGG untreated for 3 min when compared against factor.

Figure 7.3 TEER change after bacterial strain inclusion on the apical side at 22 hours.

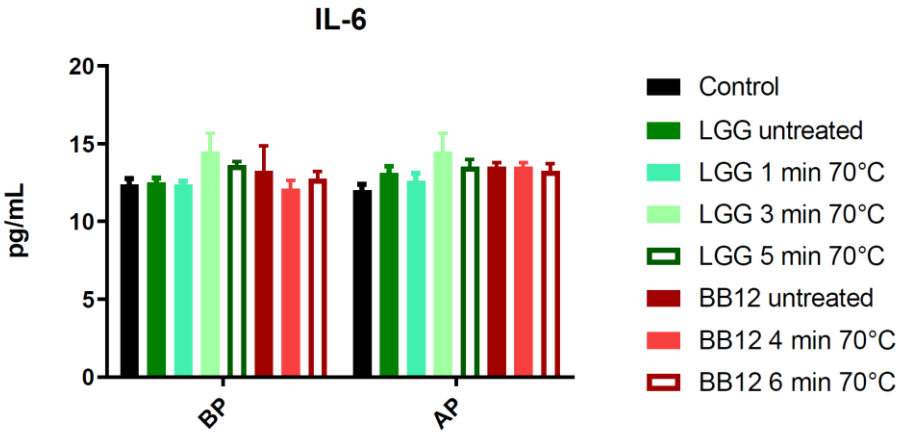


Data are presented as mean and SD (n=3 per condition tested and control). Percentage of TEER increase is shown on the y-axis. These percentages were derived from correcting the augmentation observed with the baseline TEER value prior starting the experiment. TEER values were measured at 22 hours after treatment. Experimental control (untreated – without probiotic) was used as factor control to conduct One-way Analysis of Variance - ANOVA using the factor control vs conditions of treatment: p value <0.0001 All conditions revealed significance of change when compared with control p<0.0001.

7.3.1.2 Effect of live and heat inactivated LGG and BB-12 on inflammatory mediator production by Caco-2 cell monolayers

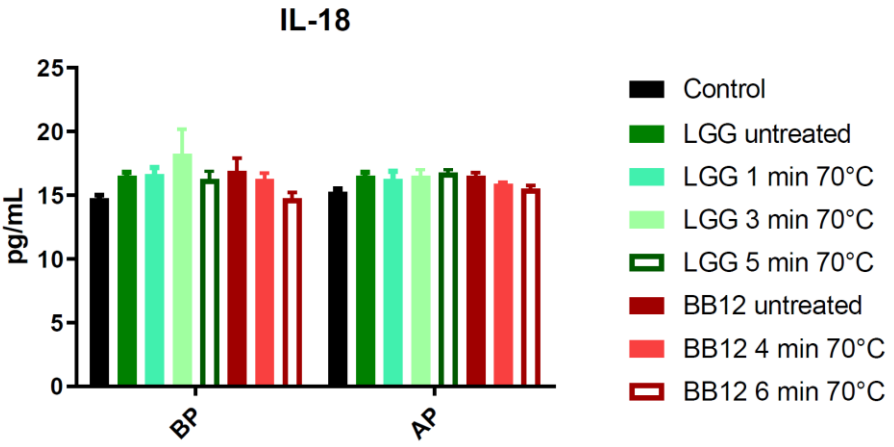
Caco-2 cell monolayers were exposed to live or heat inactivated LGG and BB-12 on the apical side and six inflammatory mediators (IL-6, IL-18, IL-8, IP-10, VEGF, ICAM-1) were measured on both the apical and basolateral sides. There was no effect of the organisms (live or inactivated) on the concentrations of IL-6 (Figure 7.4), IL-18 (Figure 7.5), ICAM-1 (Figure 7.9) or VEGF (Figure 7.8) measured on either side. While there was no significant effect on IL-8 production, there was a trend for BB-12, especially when live, to increase this mediator on the apical side (Figure 8.3). Similarly there was a trend for BB-12 either live or heat inactivated to increase IP-10 on the apical side (Figure 8.5.).

Figure 7.4 IL-6 concentration (pg/mL) on the apical and basolateral sides of Caco-2 monolayers incubated with live or heat inactivated LGG and BB-12 for 22 hr on the apical side.



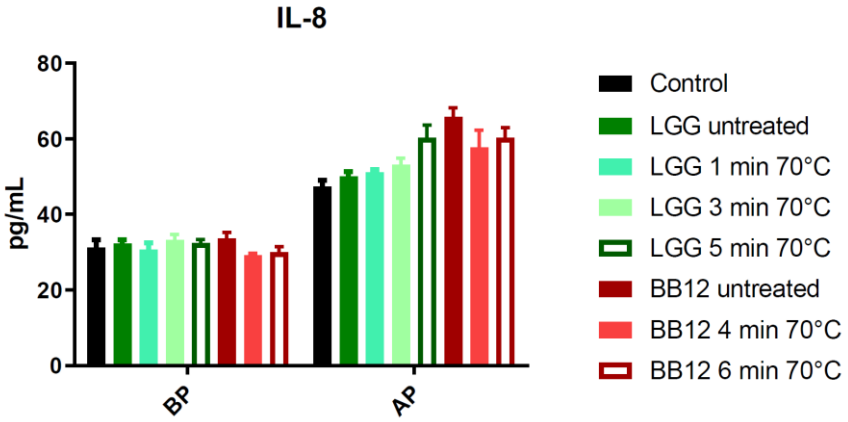
Mean (±SEM) (n=4). IL-6 concentration in medium of cells treated with live or heat inactivated LGG or BB-12 for 22 hours. One-way Analysis of Variance - ANOVA using as factor control (untreated – without probiotic) vs conditions of treatment. Analyses of production on the apical and the basolateral side were conducted separately. IL-6 concentrations were not significantly different ($p>0.05$) from control experiments.

Figure 7.5 IL-18 concentration (pg/mL) on the apical and basolateral sides of Caco-2 monolayers incubated with live or heat inactivated LGG and BB-12 for 22 hr on the apical side.



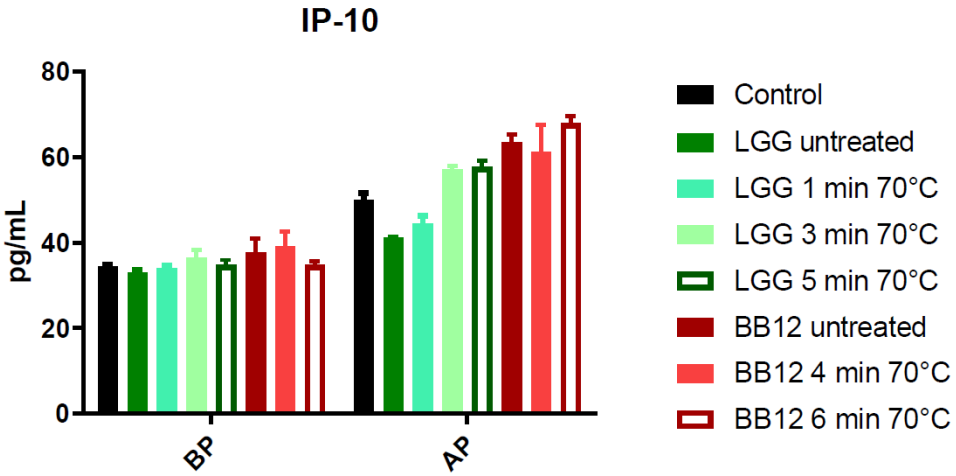
Mean (±SEM) (n=4). IL-18 concentration in medium of cells treated with live or heat inactivated LGG or BB-12 for 22 hours. One-way Analysis of Variance - ANOVA using as factor control (untreated – without probiotic) vs conditions of treatment. Analyses of production on the apical and the basolateral side were conducted separately. IL-18 concentrations were not significantly different ($p>0.05$) from control experiments.

Figure 7.6 IL-8 concentration (pg/mL) on the apical and basolateral sides of Caco-2 monolayers incubated with live or heat inactivated LGG and BB-12 for 22 hr on the apical side.



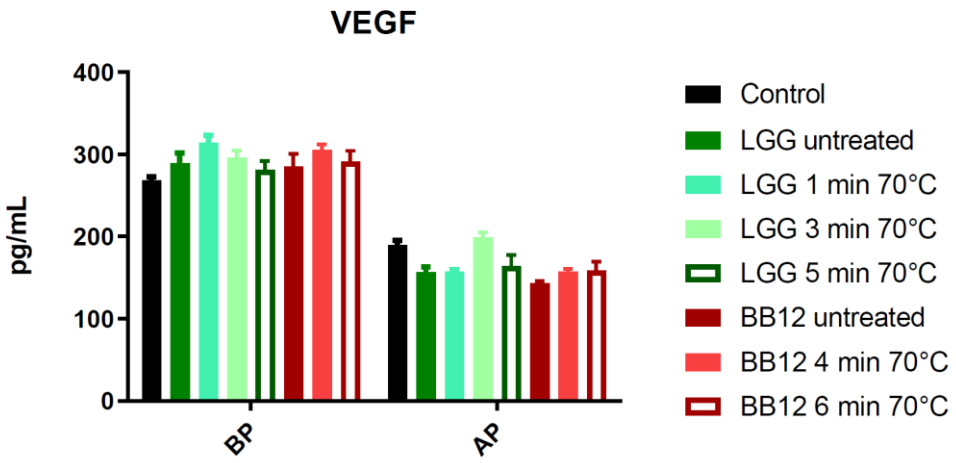
Mean (±SEM) (n=4). IL-8 concentration in medium of cells treated with live or heat inactivated LGG or BB-12 for 22 hours. One-way Analysis of Variance - ANOVA using as factor control (untreated – without probiotic) vs conditions of treatment. Analyses of production on the apical and the basolateral side were conducted separately. IL-8 concentrations were not significantly different ($p>0.05$) from control experiments.

Figure 7.7. IP-10 concentration (pg/mL) on the apical and basolateral sides of Caco-2 monolayers incubated with live or heat inactivated LGG and BB-12 for 22 hr on the apical side.



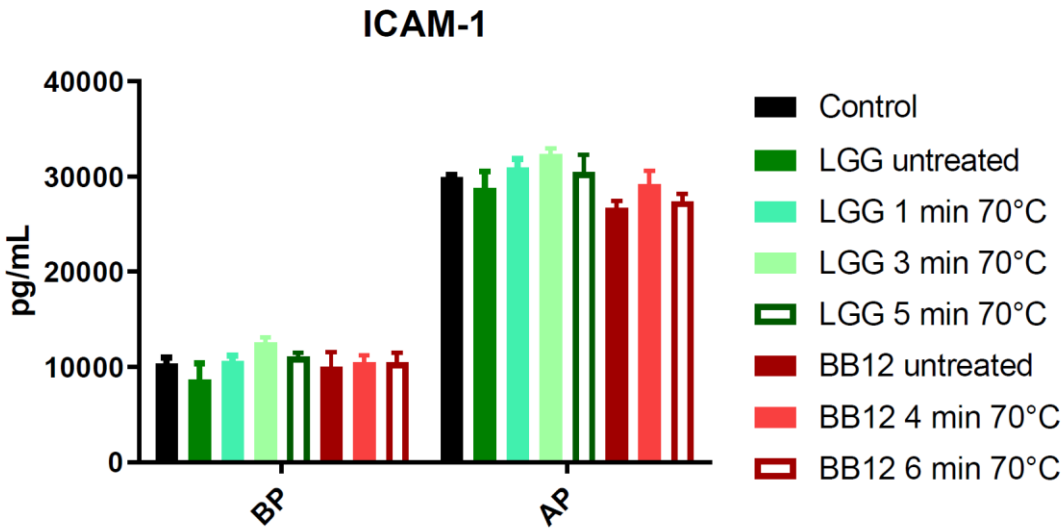
Mean (±SEM) (n=4). IP-10 concentration in medium of cells treated with live or heat inactivated LGG or BB-12 for 22 hours. One-way Analysis of Variance - ANOVA using as factor control (untreated – without probiotic) vs conditions of treatment. Analyses of production on the apical and the basolateral side were conducted separately. IP-10 concentrations were not significantly different ($p>0.05$) from control experiments.

Figure 7.8. VEGF concentration (pg/mL) on the apical and basolateral sides of Caco-2 monolayers incubated with live or heat inactivated LGG and BB-12 for 22 hr on the apical side.



Mean (±SEM) (n=4). VEGF concentration in medium of cells treated with live or heat inactivated LGG or BB-12 for 22 hours. One-way Analysis of Variance - ANOVA using as factor control (untreated – without probiotic) vs conditions of treatment. Analyses of production on the apical and the basolateral side were conducted separately. VEGF concentrations were not significantly different ($p>0.05$) from control experiments.

Figure 7.9. ICAM-1 concentration (pg/mL) on the apical and basolateral sides of Caco-2 monolayers incubated with live or heat inactivated LGG and BB-12 for 22 hr on the apical side.



Mean (±SEM) (n=4). ICAM-1 concentration in medium of cells treated with live or heat inactivated LGG or BB-12 for 22 hours. One-way Analysis of Variance - ANOVA using as factor control (untreated – without probiotic) vs conditions of treatment. Analyses of production on the apical and the basolateral side were conducted separately. ICAM-1 concentrations were not significantly different ($p>0.05$) from control experiments.

7.4 Discussion

In the research described in this chapter, LGG and BB-12 were successfully heat-inactivated. Subsequently live and heat inactivated organisms were placed in the apical side of Caco-2 cell monolayers. Both live LGG and live BB-12 increased TEER values in Caco-2 cell monolayers at 10 and 22 hours of culture, indicating a strengthening of the epithelial monolayer when compared with the control conditions. This suggests a direct benefit from the live organisms on the epithelium. In contrast, the heat-inactivated organisms had much less effect on TEER, although it was enhanced. This suggests that for an optimal beneficial effect on the epithelium live organisms are required. The difference in effect between live and heat-inactivated organisms suggests that there may be two separate mechanisms of interaction of these bacteria with the epithelium, one physical (and seen with heat-inactivated organisms) and one metabolic (and so requiring live organisms). Alternatively there may be a single physical mechanism but the bacterial structures involved in that interaction are affected by the heat treatment. Lastly, analysis of production of a panel of immune mediators by Caco-2 cells found no effect of the organisms whether live or heat inactivated and no differences between strains. Outcomes of this research suggest that live organisms are more effective in increasing tight junctions measured through TEER on gut epithelial cells than the heat inactivated version. It was also observed that neither live organisms nor their heat-inactivated version elicit a differential effect on immune mediator production in this in vitro model when compared with the control without treatment (bacterial strains).

Probiotics produce a number of metabolic products such as bacteriocins, acetaldehydes, and short-chain fatty acids which contribute with the maintenance of enterocytes through biochemical pathways that keep enterocytes live and also avoid apoptosis [584, 585]. This allows probiotics to exert biological activity not only by inhibiting the pathogenic growth of microbes in the host (bacteriocins), but through the strengthening of tight junctions, as described by others [506, 586, 587] and as suggested by the enhancement of TEER seen in the current research. The biological basis for any effect of heat-killed organisms has been described as an activity exerted through components in their cell walls, such as lipoteichoic acids [588] and peptidoglycan [589]. According to the findings presented in this research, it is likely that the effects in strengthening the tight junctions are inferior when exerted by the heat-inactivated organisms. To put this another way, TJ strengthening is highest when exerted by the metabolically active probiotic organisms.

The active interaction between the bacterial strains and the host mucosal immune system and enterocytes differs and is specific according to bacterial properties. The differences observed between LGG and BB-12, confirming the observations of others [590, 591], are likely due to intrinsic properties of each organism, such as the presence of the pili in LGG [592], composition of the cell wall with components such as lipoteichoic acid [574] and the presence of proteins which contribute to coping with stress conditions such as heat-inactivation. Such proteins are also identified as WhiB-like proteins [593]. One of the strengths of this work is that it compares the effects exerted by live and heat-inactivated strains on epithelial integrity through TEER measurements. This work confirms the efficiency of the probiotic LGG in increasing TEER values, most likely through the strengthening of tight junctions, when compared with BB-12. This work also adds evidence to how those "intrinsic" properties of the bacteria can be affected depending on the conditions in which the microorganism is used. The current study used shorter times of heat-inactivation for BB-12 and LGG (6 minutes and 1 minute) than used by other researchers [594].

These results showed a strong effect of LGG when compared with either BB-12 or its heat-inactivated version. One of the mechanisms by which LGG seems to interact with epithelial cells in a more effective manner than BB-12 is through its pili structure, mainly because the pili allow closer interaction with the enterocytes, while BB-12 lack this structure [595]. The findings may indicate that the pili structure in the viable microorganism is partially responsible for a better interaction with the enterocytes. A possible mechanism explaining reduction in TEER strengthening using LGG inactivated could be that the heat treatment might potentially reduce pili's ability to function. Although it has been suggested that the heat-inactivation does not destroy this structure [595], it might potentially reduce its ability to function. This deserved further exploration. Another mechanism of action of the live version refers to the release of trophic factors that interact with enterocytes (e.g. butyrate). The results of this thesis might suggest that those metabolic products are more important than the components of the LGG cell wall.

Although it has been claimed that these strains in their inactivated form are safer than the active form in immunocompromised individuals [596], precisely due to their lack of metabolic activity and lack of potential overgrowth, the findings from the TEER measurements indicate that heat-inactivated microorganisms have reduced interaction with epithelial cells. The lack of effect of LGG and BB-12 on inflammatory mediator production even when live, suggests that the nature of the interaction that increases

TEER does not enhance or suppress the inflammatory response of gut epithelial cells. *In vivo*, the gut barrier includes a significant immune cell component which might respond differently from the epithelial cells [597]. Therefore, further *in vitro* experiments could explore these other interactions by adding immune cells like dendritic cells into a co-culture system with the Caco-2 cells. Inflammatory responses to probiotic bacteria may require leucocyte-epithelial cell cross-talk [598]. Clinical studies have been able to find benefits from heat-inactivated strains including LGG and BB-12 [599]. Through the reduction of bowel movement, abdominal pain, and the improvement in the stool consistency, a multicentre randomized trial found that a heat-inactivated strain of lactobacillus was able to help patients with chronic diarrhoea [599]. Additional studies suggest that BB-12 can reduce cariogenicity of *Streptococcus mutans* [600]. Mechanisms however, have not been entirely understood and animal models have suggested that it is via the stimulation of dendritic cells and T cell proliferation. It therefore appears that the enterocytes are susceptible of modification (strengthening) by LGG. This interaction is likely to modulate the gut-associated lymphoid tissue and local immune response [601-603].

The current study did not test mechanisms associated with direct contact of probiotic organisms with the epithelial cells or metabolites produced in cultures or whether the enhancement of TEER relates to an increase in junctional proteins. The current study tested a ratio of bacteria to epithelial cells (1000:1) in junctional strengthening through TEER, and a fixed ratio of bacteria to epithelial cells of 10:1. Caco-2 cell model was responsive to the live version of the bacteria, but when dosage was reduced and used live and inactivated no differences in the inflammatory panel were seen when the organisms were added into the system.

Six inflammatory mediators were measured on both apical and basolateral sides of the monolayers. There was no significant effect of either organism, live or heat-inactivated on the concentrations of these mediators. There were some trends seen for live BB-12 and the production of some mediators on the apical side. However, the overall null findings suggest that the organisms have neither a pro- nor anti-inflammatory effect in this culture system. The number of observations correspond to n = 4, so these outcomes could be expanded with an increased number of experimental conditions.

With regards the immune mediators produced, no differences were detected between the two bacterial strains. Although Caco-2 cells offer a partial view of the response exerted by an *in vitro* enterocyte model, translating these findings into the *in vivo* context

can be a challenge. Incorporating relevant physiological aspects of the gut might offer an improved way to study the interaction with heat-inactivated microorganisms. One important condition that probiotics should have is to resist the flushing effects of the peristaltic movements. In the *in vitro* context cells are in permanent contact with the live or heat-inactivated microorganism as the system remains static, but others have reported that if the adhesion fails, the microorganism is flushed away. However some conditions, like those common in the elderly tend to suggest reduced peristaltic movements [604]

7.5 Conclusion

Overall, the hypothesis proposed can be accepted. LGG and BB-12 can be heat inactivated using minimum times of heat- exposure (LGG: heat-exposure at 70˚C for 3 minutes and BB-12: heat-exposure at 62.3˚C for 6 minutes). Likewise, it can be accepted that both the heat- inactivated LGG and BB-12 version as well as the live version exerted a comparable degree of inflammatory responses in Caco-2 cell monolayers, when compared against an experimental control (untreated) and as far as these results are concerned. However and remarkably, TEER was greatly improved only by both live organisms, suggesting enhanced TJs; whereas heat-inactivated organisms had a weaker effect compared to the live version. This might suggest that heat inactivation interferes with metabolic mechanisms that enhance junctional complexes in the epithelial cells. Finally, under the conditions used, neither live nor heat-inactivated LGG or BB-12 induced differentially inflammatory mediator production, suggesting a divergence of TEER and TJs on one hand and activation of inflammatory pathways on the other. The precise mechanism of interaction of the probiotics and epithelial cells warrants further investigation. Additionally, it would be valuable adding other immune cells into the in vitro model to deepen the understanding of inflammatory stimuli and influences on immune mediator production.

Chapter 8 Heat inactivated version of probiotics as a preventive strategy in an *in vitro* model of gut epithelial inflammation

8.1 Introduction

Both live and non-viable or inactive probiotic organisms can interact with enterocytes and immune cells [578, 596, 605]. In the previous chapter it was demonstrated that neither live nor inactive LGG and BB-12 induced a differential production of immune mediators by Caco-2 cells when they were incorporated into the system once the Caco-2 cells reached a confluent and mature state (chapter 7). In the previous piece of research, Caco-2 cells were exposed to the bacteria without any other previous or subsequent stimulation. Inactivated organisms could potentially interact with the host gut epithelium or immune cells through surface structures and potentially protect or contribute to the recovery of damaged epithelia.

Peptidoglycans and lipoteichoic acid from LGG have been shown in animal models to increase the production of IgA in the small intestine as well as to increase the number of activated dendritic cells in the intestine [606]. These cell wall components can cross the intestinal epithelial cell barrier and M cells in Peyer's patches and can also be recognised by host cells through recognition receptors like TLRs [607]. This mechanism confers the ability to modulate the innate and adaptive immune response in the body. In the case of BB-12, heat-killed organisms can be effective in the improvement of bloating reported by patients with irritable bowel syndrome [608]. Similar positive findings have been reported for LGG where it has been suggested that this organism can modulate inflammatory responses by decreasing the production of pro-inflammatory cytokines [609].

It is possible that heat-inactivated microorganisms can act in a more direct manner with the host as genetic material inside the bacteria and cell wall components are readily available. The loss of viability of the microorganism might make it prone to lysis and subsequently interact with enterocytes and immune cells more directly. Theoretically, these could be part of the benefits conferred to the host [605]. Live probiotic organisms in food products lose viability over time and this loss can be accelerated in some storage conditions (e.g. at room temperature compared to at 4^{o}C); hence exact numbers of live organisms in a food at time of consumption are not known. From an industrial perspective, heat-killed microorganisms, if they were active, would be easier to standardize, transport and store as the requirements would be different from those required for live organisms. Also, and more importantly, some immunocompromised patients for whom the role of (live) probiotics is not very clear, and they may even be

harmful, could benefit from the use of inactivated organisms. Therefore the biological properties of inactivated probiotic organisms need to be fully explored.

The purpose of the research presented in this chapter was to identify whether heat-inactivated LGG and BB-12 could have a role in preventing cultured enterocytes (Caco-2 cells) being damaged by an inflammatory insult. This experimental treatment on caco-2 cells is referred to as preventative properties.

8.1.1 Hypothesis

It is hypothesized that heat-inactivated LGG and BB-12 either alone or in combination are able to prevent the damaging effects of an inflammatory cocktail in an intestinal epithelial model.

8.1.2 Aim and Objectives

The objectives of the research presented in this chapter were to:

- Identify epithelial layer integrity through the assessment of TEER when heat-inactivated LGG and BB-12 are used as a preventive strategy.
- Identify whether LGG and BB-12 are able to reduce the inflammatory response of Caco-2 cells to a classical inflammatory stimulus when the stimulus is added to the cells after the treatment with the heat-inactivated organism (probiotics used as preventative or enterocyte-barrier protective strategy).
- Identify whether LGG and BB-12 act better in combination than individually to control the Caco-2 cell inflammatory response in the preventative experimental design.

8.2 Methods

8.2.1 Experimental design

Caco-2 cell cultures were established as described in section 6.2. The cytokine cocktail used to stimulate the cells was as described in section 6.3.4. TEER was measured as described in section 6.2.3. Concentrations of inflammatory mediators in the culture medium were measured as described in section 6.2.3.2.

The preventive experiment involved treating Caco-2 cell monolayers at 19 days once maturity and differentiation was reached with heat-inactivated LGG and BB-12 alone at a ratio of epithelial cells to probiotic organism (MOI) of 1:10 or in combination at a ratio of 1:10 as well (i.e. the same ratio of organisms to epithelial cells was maintained). After 24 hours of co-incubation with the heat-inactivated strains, cells were stimulated by adding the cytokine cocktail into the basolateral medium to induce the inflammatory response and test how different it would be from experimental controls (Caco-2 cells only exposed to the cytokine cocktail). Cells were incubated for an additional 24 hours with the inflammatory cocktail in the basolateral side without removal of the organisms that were previously added.

8.2.2 Statistical analysis

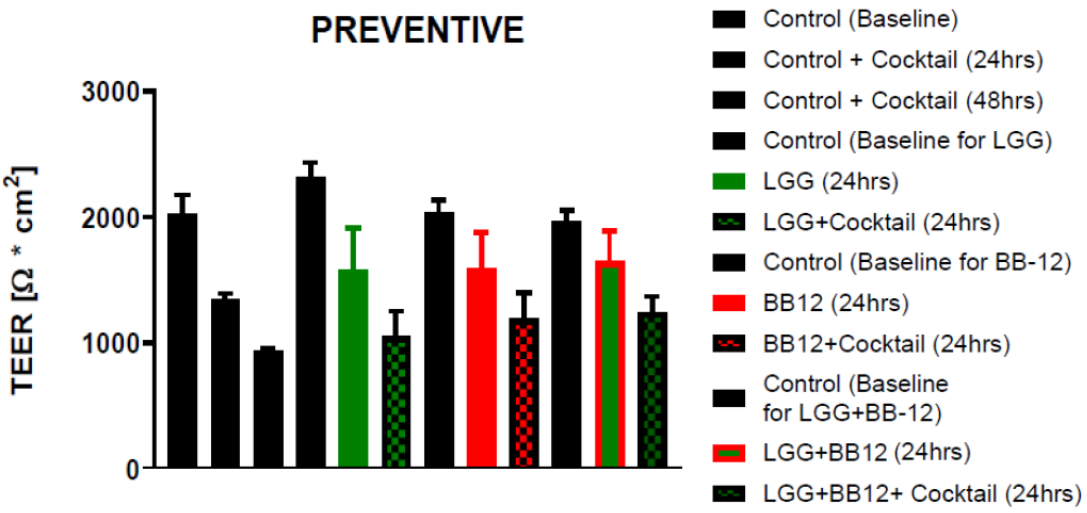
The experimental design included three different experiments, and each experiment was conducted in triplicate (n=9 per variable). The test used to determine statistical significance was One-way Analysis of Variance (ANOVA) using as factor control vs conditions of treatment. Where the ANOVA was significant Dunnet's test was used for-post-hoc analysis of differences between control and treatment. Statistical analysis was performed using SPSS Version 22 and GraphPad PRISM 7.

8.3 Results

8.3.1 Epithelial integrity assessment: TEER as an indicator of the effect exerted by preventive and restorative treatments

Figure 8.1 confirms that exposure of Caco-2 cell monolayers to a cytokine cocktail causes a decline in TEER, in this case by about 40% after 24 hours. This is consistent with findings in earlier chapters. It is also seen that addition of heat inactivated LGG or BB-12 or their combination for 24 hours prior to stimulation with the cytokine cocktail did not alter TEER (Figure 8.1).

Figure 8.1.TEER measurements following the preventive experimental design



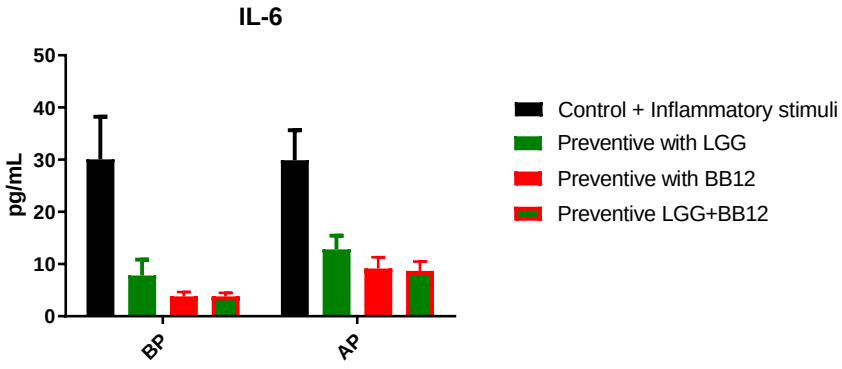
Data are mean (±SEM) (n=9). TEER values were measured at baseline (control), after 24 hours after adding treatment (LGG or BB-12 or LGG+BB-12) and after 24 hours after adding the cocktail stimulation. Comparison of TEER in control conditions - prior starting any treatment – Control (Baseline alone; for LGG; for BB-12; and for LGG+BB-12) were not significantly different among them ($p=0.25$). For LGG: Control + inflammatory cocktail vs. end of experiment + inflammatory cocktail ($p<0.0001$). For BB-12: Control + inflammatory cocktail vs. end of experiment ($p=0.03$). Control LGG+BB-12 + inflammatory cocktail vs. end of experiment ($p=0.02$). Comparisons among final TEER values among experimental conditions vs. control $p=0.481$.

8.3.2 Inflammatory panel to assess potential preventive and restorative treatments with heat inactivated LGG and BB-12 in the epithelial model of inflammation

Concentrations of IL-6, IL-8, IL-18, IP-10 and VEGF on both basolateral and apical sides were used to monitor the response of Caco-2 cells exposed to an inflammatory cocktail on the basolateral side. Both LGG and BB-12 reduced the response of some of these inflammatory mediators when added either prior to or after the inflammatory cocktail.

IL-6 concentrations were significantly lower on both basolateral and apical sides when LGG or BB-12 or their combination was added 24 hours prior to (preventive) or 24 hours after (restorative) the inflammatory cocktail (Figure 8.3). Effects of LGG and BB-12 were not significantly different.

Figure 8.2. IL-6 concentrations (pg/mL) in the basolateral and apical medium after stimulation of Caco-2 cells with an inflammatory cocktail with exposure to LGG or BB-12 or their combination 24 hours before or 24 hours after the cocktail.

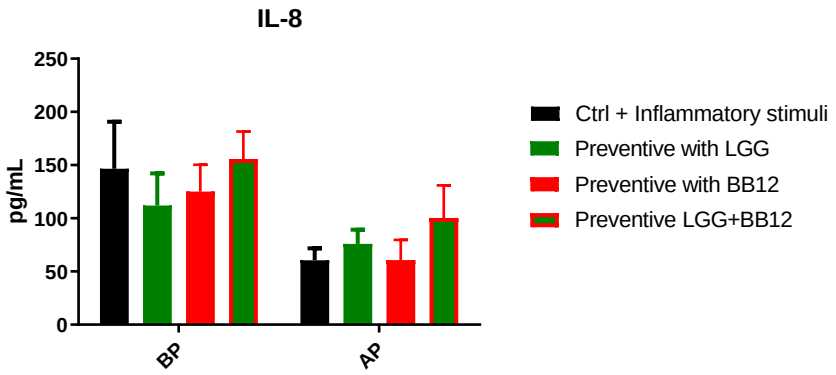


Data are mean (±SEM) (n=9). IL-6 concentrations in basolateral (BP) and apical (AP) medium of Caco-2 cells treated with heat-inactivated LGG or BB-12 or LGG+BB-12 (control was exposed to inflammatory cocktail). Statistical test used was one-way ANOVA using as control factor the control+inflammatory stimuli. Comparisons were performed by discriminating among BP with its control and AP and its control.

IL-6 concentrations were statistically significant from control in both basolateral and apical sides. Basolateral (BP) side post-hoc test analysis (Dunnett's multiple comparison test vs control) for preventive approach: LGG p=0.0005; BB-12 p=<0.0001; LGG+BB-12 p<0.0001. Apical (AP) side post-hoc test analysis (Dunnett's multiple comparison test vs control) for preventive approach: LGG p=0.009; BB-12 p=0.0012; LGG+BB-12 p=0.0009.

IL-8 concentrations were not affected by exposure to LGG or BB-12 either before or after the inflammatory cocktail (Figure 8.4).

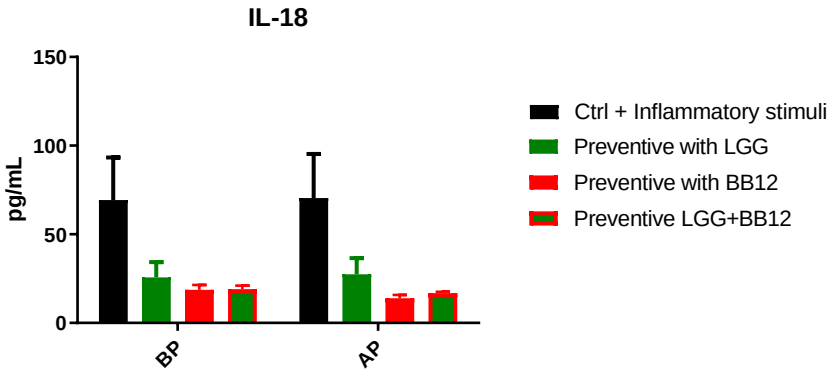
Figure 8.3. IL-8 concentrations (pg/mL) in the basolateral and apical medium after stimulation of Caco-2 cells with an inflammatory cocktail with exposure to LGG or BB-12 or their combination 24 hours before or 24 hours after the cocktail.



Data are mean (±SEM) (n=9). IL-8 concentrations in basolateral (BP) and apical (AP) medium of Caco-2 cells treated with heat-inactivated LGG or BB-12 or LGG+BB-12 (control was exposed to inflammatory cocktail) . Statistical test used was one-way ANOVA using as control factor the control+inflammatory stimuli. Comparisons were performed by discriminating among BP with its control and AP and its control.
IL-8 concentrations were not statistically significant from control experiments in both basolateral (BP) and apical (AP) sides.
Post-hoc test analysis using Dunnett's multiple comparison test revealed in both BP and AP that comparing against controls not significant differences in p values were found neither in the preventive nor restorative treatments using LGG, BB-12, or LGG+BB-12 ($p>0.05$).

IL-18 concentrations were significantly lower on the basolateral side when LGG or BB-12 or their combination was added 24 hours prior to (preventive) or 24 hours after (restorative) the inflammatory cocktail (Figure 8.5). Effects of LGG and BB-12 were not significantly different. There was no effect of the organisms on IL-18 concentration on the apical side.

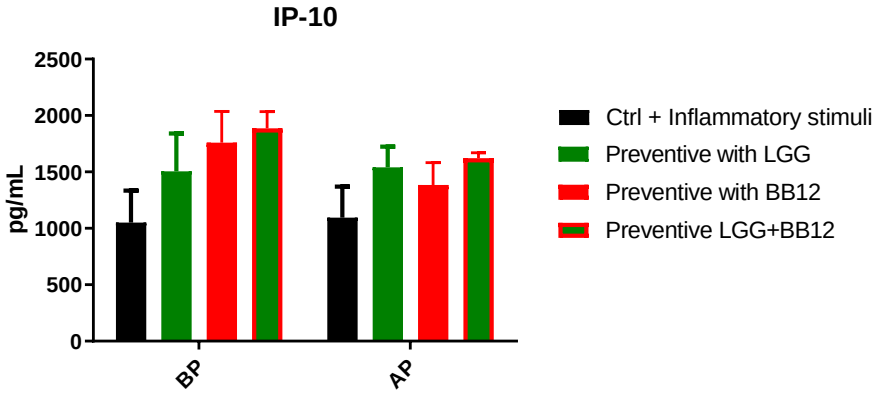
Figure 8.4 IL-18 concentrations (pg/mL) in the basolateral and apical medium after stimulation of Caco-2 cells with an inflammatory cocktail with exposure to LGG or BB-12 or their combination 24 hours before or 24 hours after the cocktail.



Data are mean (±SEM) (n=9). IL-18 concentrations in basolateral (BP) and apical (AP) medium of Caco-2 cells treated with heat-inactivated LGG or BB-12 or LGG+BB-12 (control was exposed to inflammatory cocktail). Statistical test used was one-way ANOVA using as control factor the control+inflammatory stimuli. Comparisons were performed by discriminating among BP with its control and AP and its control.
IL-18 concentrations were statistically significant from control experiments in both basolateral (BP) and apical (AP) sides.
Post-hoc test analysis using Dunnett's multiple comparison test revealed in BP and comparing against controls the following p values were using LGG (p=0.03*), BB-12 (p=0.008**), LGG+BB-12 (p=0.008*) and in the AP LGG (p=0.03*), BB-12 (p=0.003*), LGG+BB-12 (p=0.004*).

IP-10 concentrations were significantly higher on the basolateral, but not the apical, side when LGG or BB-12 or their combination was added 24 hours after (restorative) the inflammatory cocktail (Figure 8.6). Effects of LGG and BB-12 were not significantly different.

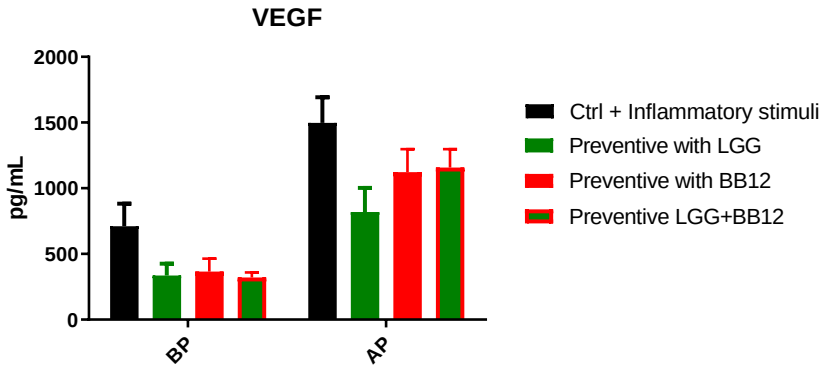
Figure 8.5. IP-10 concentrations (pg/mL) in the basolateral and apical medium after stimulation of Caco-2 cells with an inflammatory cocktail with exposure to LGG or BB-12 or their combination 24 hours before or 24 hours after the cocktail



Data are mean (±SEM) (n=9). IP-10 concentrations in basolateral (BP) and apical (AP) medium of Caco-2 cells treated with heat-inactivated LGG or BB-12 or LGG+BB-12 (control was exposed to inflammatory cocktail). Statistical test used was one-way ANOVA using as control factor the control+inflammatory stimuli. Comparisons were performed by discriminating among BP with its control and AP and its control.
Post-hoc test analysis using Dunnett's multiple comparison test revealed in BP and comparing against controls a lack of significance in p values in the preventive treatment using LGG, or BB-12 but there was significance when control were compared against LGG+BB-12 (p=0.04*). Lack of significance was observed in the AP when comparing control against LGG, BB-12, and LGG+BB-12 (p>0.05).

VEGF concentrations were significantly lower on the basolateral side when LGG or BB-12 or their combination was added 24 hours prior to (preventive) the inflammatory cocktail (Figure 8.7). There was no restorative effct when the organisms were added on the basolateral side. VEGF concentrations were significantly lower on the apical side when LGG, but not BB-12, was added either prior to or after the inflammatorey cocktail (Figure 8.7).

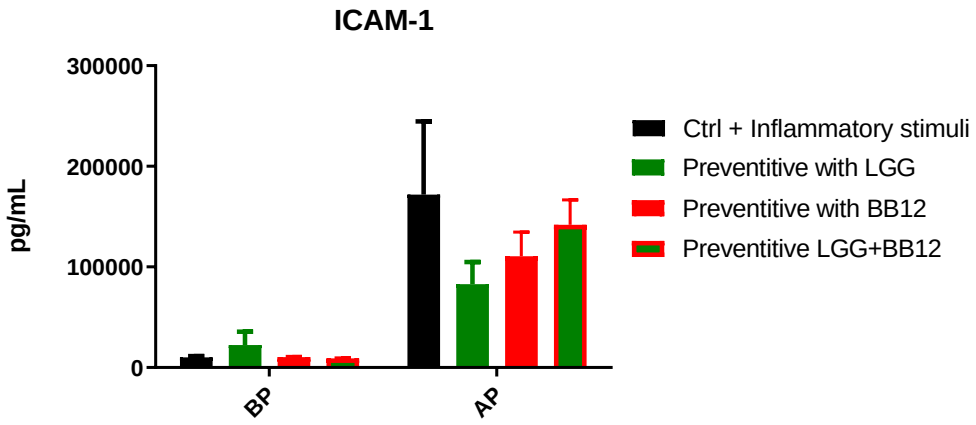
Figure 8.6 VEGF concentrations (pg/mL) in the basolateral and apical medium after stimulation of Caco-2 cells with an inflammatory cocktail with exposure to LGG or BB-12 or their combination 24 hours before or 24 hours after the cocktail.



Data are mean (±SEM) (n=9). VEGF concentrations in basolateral (BP) and apical (AP) medium of Caco-2 cells treated with heat-inactivated LGG or BB-12 or LGG+BB-12 (control was exposed to inflammatory cocktail). Statistical test used was one-way ANOVA using as control factor the control+inflammatory stimuli. Comparisons were performed by discriminating among BP with its control and AP and its control.
Post-hoc test analysis using Dunnett's multiple comparison test revealed lack of effect of LGG, BB-12 or LGG+BB-12 when these were compared against control ($p>0.05$).
AP also revealed a lack of signigicant effects when control were compared against BB-12 and LGG+BB-12 but significant differences were observed when control was compared with LGG ($p=0.005^*$).

ICAM-1 concentrations were significantly lower on the apical side when LGG was added 24 hours after (restorative) the inflammatory cocktail (Figure 8.8).

Figure 8.7 ICAM-1 concentrations (pg/mL) in the basolateral and apical medium after stimulation of Caco-2 cells with an inflammatory cocktail with exposure to LGG or BB-12 or their combination 24 hours before or 24 hours after the cocktail..



Data are mean (±SEM) (n=9). ICAM-1 concentrations in basolateral (BP) and apical (AP) medium of Caco-2 cells treated with heat-inactivated LGG or BB-12 or LGG+BB-12 (control was exposed to inflammatory cocktail).). Statistical test used was one-way ANOVA using as control factor the control+inflammatory stimuli. Comparisons were performed by discriminating among BP with its control and AP and its control. ICAM-1 expression were not statistically significant from control experiments in basolateral side (BP) or in the (AP) side ($p>0.05$) sides.

8.4 Discussion

The research herein conducted presents mixed findings with regards the effects exerted by the heat-inactivated organisms LGG and BB-12 on the inflammatory response of Caco-2 cells stimulated with a cocktail of cytokines. The findings are summarised in Table 8.1.

Table 8.1. Qualitative summary of the effects exerted by the heat inactivated probiotics in the preventive and restorative models.

	Preventive		
	LGG	BB-12	LGG+BB-12
TEER	↔	↔	↔
IL-6 (BP- AP)	↓↓↓	↓↓↓	↓↓↓
IL-8 (BP- AP)	↔	↔	↔
IL-18 (BP)	↓↓↓	↓↓↓	↓↓↓
IP-10 (BP)	↔	↔	↔
VEGF (BP)	↓↓	↓↓	↓↓
ICAM-1 (AP)	↓	↓	↓

Firstly, the findings herein presented failed to identify an improvement in epithelial barrier integrity (determined by TEER) exerted by the heat-inactivated LGG or BB-12 or their combination when used as a “preventive” strategy. In contrast, it was possible to observe altered responses of the Caco-2 cells with both preventive and restorative approaches through the measurement of inflammatory mediators on both basolateral and apical sides of the cultures. The effects observed were selective according to the mediator and the side at which the measurements were made. IL-8 was not affected but each of the other five mediators was affected. Generally, the concentrations of those mediators affected was reduced by the heat inactivated organisms, although IP-10 was increased. IL-6 and IL-18 were most strongly affected and were equally affected by LGG and BB-12.

Because the response of TEER to the inflammatory cocktail was unaffected by the heat inactivated organisms, it cannot be concluded that they enhance epithelial barrier integrity. Other researchers have suggested that heat-inactivated microorganisms enhance epithelial barrier integrity through increased expression of zonula-occludens and therefore increase junctional complexes [610]. The mentioned research was conducted on an animal model of colitis, where an examination of the mucus layer was also performed. It is plausible that the mechanism behind this benefit was the stimulation of mucus-producing cells by heat-inactivated LGG, so that the increased integrity was observed via increased mucus production. Further experiments using the Caco-2 cell model could be performed by either adding mucus-producing cells to examine their interaction or by testing the model by adding a mucus layer and assessing the interaction with the monolayer. It has been shown that a reduction in pro-inflammatory cytokine production by Caco-2 cells is a contributory factor in the

protection of the barrier and reduction of its disruption [611]. The current study identified a reduction in cytokine production but no effect on integrity as assessed by TEER and so indicates that these two responses may not always be linked.

The pathway by which microorganisms modulate the immune response is via their microbe-associated molecular patterns (MAMPs) [612]. Heat-inactivated bacteria preserve these, as they are mainly cell wall components such as LPS, peptidoglycans, lipoteichoic acids, and so on. Because of the temperature used to heat inactivate LGG and BB-12 it is likely that the organisms are still intact [605, 613]. Research has suggested that MAMPs can be recognised by intestinal epithelial cells via TLRs, whereby an induction in the signalling cascade can result in the production of cytokines, chemokines and immune cell activation [439]. However, the findings of the previous chapters indicate that heat inactivated LGG and BB-12 themselves do not trigger such responses in this model.

The results herein presented showed that the heat inactivated organisms caused a reduction in the appearance of IL-6 on both basolateral and apical sides of the Caco-2 cells whether added before or after the cytokine cocktail. On the basolateral side, a marked reduction in IL-6 concentration of 60% was measured with the preventive treatment using LGG. The preventive treatment using BB-12 and LGG+BB-12 also resulted in a reduction of 80% when compared with the cytokine cocktail alone condition. Effects were smaller but still significant on the apical side. The observations suggest that the heat inactivated LGG and BB-12 can both prevent the IL-6 response to stimulation and reverse an elevated IL-6 response. TNF-alpha and IL-1β, components of the stimulating cytokine cocktail, activate the transcription factors that induce IL-6 gene expression [538]. The observed reduction in IL-6 production suggests that the Caco-2 cell response to the heat inactivated bacteria interferes with that signalling pathway. IL-6 is a pleiotropic cytokine with several effects on the immune response in the gut [614] as IL-6 receptors are expressed in intestinal cells [615]. This cytokine has been found in gut biopsies of patients with inflammatory disease and coeliac disease as well as in healthy controls. In these biopsies the protein was predominantly found in enterocytes [616]. These observations implicate IL-6 in intestinal damage and disease. However, other investigations have shown that IL-6 is also implicated in tissue repair, as some therapies blocking the effects of IL-6 cause damage to the intestine [617]. Thus, although the effects of the heat inactivated organisms on IL-6 production may be viewed as anti-inflammatory and therefore of benefit, a clear conclusion cannot be made.

IL-18 was decreased by LGG and BB-12 on the basolateral, but not apical, side in both preventive and restorative approaches. Inflammasomes are a complex of proteins that emerge during infections or tissue damage; they are induced by inflammatory cytokines [618]. Inflammasomes rapidly activate the release of IL-18 [618, 619]. The observations made here suggest that the Caco-2 cell response to the heat inactivated bacteria interferes with the signalling pathway that leads to inflammasome activation or activity. Generally, IL-18 is relevant in the signalling within intestinal epithelial cells that activates further immune responses [620].

IP-10 appearance was enhanced on the basolateral side of the epithelial cells when the restorative approach was used. IP-10 is a chemokine that can be induced by enterocytic stimulation with inflammatory cytokines [621] as confirmed in earlier chapters. The current observations suggest a priming of IP-10 production by the heat inactivated organisms.

VEGF was decreased on the basolateral side by both organisms and on the apical side by LGG in the preventive approach. Release of VEGF by epithelial cells indicates a relationship with and regulation of endothelial cells [22]. VEGF has shown to be increased in patients with active inflammatory bowel disease, Chron's disease and ulcerative colitis[622].

ICAM-1 release on the apical side was decreased by heat inactivated LGG in the preventive approach only. LGG has been shown to inhibit NF-kB activation which led to reduced expression of ICAM-1 in enterocytes, suggesting that LGG might interact with epithelial cells to regulate ICAM-1. [623, 624]. However, animal models have indicated that the anti-inflammatory effects of LGG are the result of the interaction with mucosal immune cells not only the epithelium, thus requiring further exploration.

The recent discovery of the expression of TLRs on the basolateral as well as the apical sides of enterocytes [439] reinforces the relevance of the epithelial barrier not only as a mechanism of defence but also of recognition of bacterial strains and their components. It is likely that the first interaction taking place in the epithelial barrier is key in the anti-inflammatory pathway. Components present in LGG and BB-12 could prime a certain degree of tolerance since the very first contact by decreasing the inflammatory response but the different mechanisms observed could implicate that there are anti-inflammatory and pro-inflammatory pathways that are counterbalancing the effects observed.

8.5 Conclusions

The results presented in this chapter show that heat inactivated LGG and BB-12 can reduce the inflammatory response of Caco-2 cells later exposed to a cytokine cocktail. Therefore the hypothesis can be partially accepted. Contrarily, the TEER mechanism as surrogate marker of epithelial strengthening did not improve, thus a full recovery of the model was not achieved. These findings might be part of the strategies indicating that the heat-inactivation can potentially contribute in the prevention of intestinal inflammation. There seems to be little difference between LGG and BB-12 and the combination was not more effective than either organism alone. Thus they may be able to treat intestinal inflammation to a certain extent. Intriguingly the clear effect of the heat inactivated organisms of the inflammatory response was not translated into an effect on epithelial integrity as assessed by TEER. This model can work as a primary attempt, but further studies are encouraged to incorporate other elements into the system as surrounding immune cells and mucus to study the integrative interaction among these cells and probiotic strains.

Chapter 9 General discussion and conclusions

9.1 Summary of findings

The purpose of the research described in this thesis was to evaluate whether the combination of the probiotics *Lactobacillus rhamnosus GG* and *Bifidobacterium animalis BB-12* could improve parameters related to immune and inflammatory function. This was examined *in vivo* through a clinical trial focused on immunological and inflammatory mechanisms in ECHRs. This human research was supported by more mechanistic studies conducted in a model of the gut epithelial barrier using Caco-2 cells *in vitro.* Because the state in which probiotics reach the intestine and are incorporated into the system could not be controlled in the main trial, the experiments with probiotics and Caco-2 cells were conducted using both live and heat inactivated LGG and BB-12. Using heat-inactivated organisms might be especially relevant to immunocompromised patients.

The *in vivo* approach involved analysing immune and inflammatory biomarkers using blood: immune parameters assessed were blood immune cells and immune mediators, blood immune cell responses measured *ex vivo*, and the *in vivo* response to an immunological challenge (influenza vaccination). Overall this represented a broad evaluation of *in vivo* and *ex vivo* immune status and of both innate and acquired immunity to explore a comprehensive overview of the immune and inflammatory responses in the elderly. The probiotic intervention assessed did not exert significant benefits on any of the immune and inflammatory markers examined.

The *in vitro* approach involved co-culture of the two probiotic organisms with the gut epithelial Caco-2 cell line, with a focus of the effects of heat-inactivated organisms. The main outcomes were barrier function and production of inflammatory mediators. The live version of both probiotics were able to strengthen tight junctions in the epithelial barrier when compared with their heat-inactivated organisms. Furthermore, the inflammatory response was not differentially induced with live or heat-inactivated strains. When the protective effects of the heat-inactivated strains were tested by prior exposure to Caco-2 cells for 24 hours before the inflammatory stimulus they were able to reduce inflammatory mediator production but did not affect the decline in the epithelial barrier.

9.2 PRINCESS Clinical trial – immunology sub-study. An *in vivo* strategy aiming to study immunomodulatory properties of probiotics in ECHRs

The findings presented in this thesis addressed immunological mechanisms of probiotic action in what was presented as PRINCESS – immunology sub-study. The PRINCESS clinical trial is the first collaborative study that pursued a clinical and mechanistic understanding of how probiotics can affect a community of ECHRs.

9.2.1 Stability study for PRINCESS immunology study

Conducting feasibility studies is important in clinical trials. The pilot study (Chapter 2) not only assessed the stability of immune and inflammatory biomarkers when blood samples must be stored through a period, but this study was also used to assess the feasibility of conducting the PRINCESS-immunology sub-study. It was found that the majority of immune markers can be assessed in blood that is stored at room temperature for up to 3 days or 72 hours after sample collection [422, 423, 625, 626]. However, it was observed that immune mediators measured in plasma as well as immune mediators measured in stimulated whole blood cultures were highly unstable and making these measurements requires processing of fresh blood [627]. This is a relevant consideration for other studies in the immunology field as these markers are only reliable if analysed in appropriate settings close to patients. The findings from this pilot study also allowed to ensure the optimum time-frame to carry out the measurements and analysis in the immunology sub-study.

9.2.2 Cross-sectional analysis. Study of inflammatory and immunological associations with frailty, age and length of stay in ECHRs.

There was a significant association among some immune and inflammatory markers measured, especially immune mediators in blood and plasma, and frailty. These findings highlight the relevance of frailty, but also age, in the context of ECHRs in association with inflammageing.

Studies have shown that frailty – considered as a geriatric syndrome - predicts disability and mortality in the elderly [628]. Findings from the work presented in this thesis also highlight the relevance that age, length of stay at the care home and frailty possess in terms of their independent association with inflammatory and immune parameters

(Chapter 3). Identifying relevant markers associated with frailty and optimising the screening of older individuals in the care home residences might contribute to the better targeting of aged individuals at risk. This research thesis contributes by presenting evidence of the association of some immune mediators in plasma with frailty status in ECHRs. Other cross-sectional studies recently conducted agree with the relevance of some of the immune mediators herein encountered and their significant association with frailty, and suggest that the profiling of inflammatory molecules as well as immune mediators within the "cytokinome" are important in the biogerontological field and the identification of personalised interventions [347].

Since the concept of frailty was proposed, associations to study plausible causality from an immune perspective has been superficially studied in ECHRs. The approach used to study the immune system (See Figure 2.1) indicates that inflammageing is closely associated with different stages of frailty in ECHRs. Sean *et al*. conducted a cross-sectional logistic regression analysis on a community of 1106 disabled women aged from 65 to 101 y. The authors reported that high monocyte counts were associated with frailty [629] . The current study did not see this association, although there was a trend for such an association; the difference to the earlier study (or the weaker association) may be because the subjects were younger in the current study and the sample size was smaller.

In addition to this, the findings in this thesis revealed a significant association between platelets and frailty, indicating that platelets possess relevance in the context of frailty and may be a useful marker in "younger" elderly communities. Platelets have been associated with chronic conditions and it is likely that they also have a biological role in the *"inflamm-aged"* and frail phenotype [330, 630]. Mechanisms require further exploration to identify pathways of causality.

Furthermore, the multivariate analysis and linear regression model used to determine the independent contribution of age, length at care home and frailty to the variety of biomarkers analysed, revealed that length at care home presents the most important contribution to the WBC count (See table 3.15). No other immune cell quantification (immunophenotypes and FBC) revealed other associations with frailty, but rather with several markers of inflammaging.

Wilson et al. have recently suggested that inflammation and frailty are linked as factors that increase sarcopenia, immunosenescence and thus mortality risk in the elderly

[301]. Bektas et al. also suggest that inflammation and ageing are related, by highlighting the relevance of the environment in which ageing occurs [631]. As inflammatory processes occur first, it might be that inflammaging is the first and most common response in frail individuals before other immunosenescent changes appear as prominently.

The cross-sectional analysis conducted in the current research supports links amongst markers related to inflammaging and frailty through a number of immune mediators measured in plasma and whole blood culture. Secondarily, age also appeared linked to some of these parameters and length of stay was only correlated to a few. Concentrations of VCAM-1, IP-10 and TNFR-II were significantly associated with age. The mean concentration of these parameters tended to increase with age, being higher in the nonagenarian group (individuals greater or equal than 90 y old) when compared with the younger group. Moreover, MCP-1 and IP-10 were significantly associated with frailty. Actually, they were highest in the frailty status 5 group. IL1Ra and E-selectin were significantly associated with length of stay in care home residences.

The response of some markers following the whole blood culture immune stimulation was also related to age and frailty. The production of IL12p70 in response to LPS increased significantly with age, and the production of IL-10 following stimulation with PGN and LPS and of TNF-alpha following the stimulation LPS was also significantly different across the different categories of frailty. There was a significant association between the concentration of IL-10 produced following stimulation with PGN and frailty status. These findings show that individuals with a frail category of 3 to 5 corresponding to “Well, with treated comorbid disease” to “Mildly frail” seem to show a highly responsive inflammatory system that might contribute to their co-morbidities and chronic conditions [632]. In contrast, participants with a more severe frail status might present with another immunosenescence status where the immune cells are too exhausted to be responsive to external stimuli.

Finally, the immunophenotypes and the examination of T cells also showed a significant association with age. The T cell absolute count decreased in the nonagenarian group. Findings described throughout this cross-sectional study correspond to what has been described as T cell senescence and T cell exhaustion. Senescent cells exhibit a pro-inflammatory profile by secreting higher levels of pro-inflammatory cytokines whereas exhausted T cells are unable to proliferate and to secrete cytokines upon stimulation [400, 633]. The whole blood culture findings seem to support these findings.

9.2.3 Probiotics LGG and BB-12: Intervention study in ECHRs

Ageing is characterised by a decline in immune functionality [634], with a progressive increase in low-grade inflammation [635]. The ageing process in care home residences tends to progress with higher rates of respiratory infections as well as gastroenteritis and influenza [293]. It has been suggested that probiotics might be a possible intervention for vulnerable populations like aged individuals living in care homes [636]. However, the findings herein reported do not support this hypothesis as an intervention consisting of LGG and BB-12 did not exert any impact on innate or adaptive immune or inflammatory responses. Frailty, other co-morbidities and chronic inflammation were already present in this community. It is possible to suggest based on the findings of this research that elderly people who are already presenting frailty might not benefit from probiotic interventions.

A systematic review and meta-analysis including four RCTs in adults with an average age of 74.8 y old suggests that probiotics are relatively effective in elevating immunogenicity by influencing seroprotection in response to seasonal influenza vaccination [637]. The authors reported significant findings in the seroprotection in titres for H1N1 strain (with an odds ratio (OR) of 1.88 and a 95% confidence interval (CI) of 1.06–3.33). In the analysis of the H3N2 strain, the seroprotection rate (OR) was 3.11 (95% CI = 1.25–7.71). Other influenza vaccine strains assessed did not yield significant changes, and seroconversion rates were not significantly affected by the intervention. Of the four included studies, two used *Lactobacillus casei*, another one used *Lactobacillus paracasei* and one used LGG at a dosage 1 x 10^{10} CFU twice daily [450]. Potentially, probiotic interventions can confer health benefits if consumed by younger or healthy people or people with specific gastrointestinal problems, but their therapeutic use in populations which are already fragile and with other co-morbidities seems not to be significant.

Commensal bacteria and probiotics are recognised through ligation via TLRs as well as in mesenteric lymph nodes in DCs. These can direct T helper responses towards Th1 or Th2 regulatory patterns and subsequently influence cell-mediated immunity [638] and humoral immunity [639, 640] through improvement of responses against vaccination [641]. TLRs are the pathway by which probiotics - through contact with the intestine - can exert effects systemically. Older people show altered patterns of gut microbiota and [113] age-related changes may be accelerated in residential care homes [293]. Because of the link between gut microbiota and the host immune system, through

improving the microbiota of older people, probiotics may have benefits in terms of enhanced reduced infections and antibiotic use and improved vaccination responses. A multisite, randomised, placebo-controlled trial conducted in Canada provided LGG daily for 6 months at a total daily dosage of 20 billion CFU to nursing homes residents aged 65 years and above [210]. The objective of the study was to prevent pneumonia. The study recruited 108 participants in the probiotic arm and 101 in the placebo arm. Within the outcomes assessed, LGG did not alter influenza or other viral respiratory infections [210]. Evidence for effects of BB-12 on the immune response of elderly individuals is not clear [642] but BB-12 has been shown to have a role in the management of constipation among nursing home residents [321]. LGG and BB-12 also appear to be useful in the management of gastrointestinal conditions such as ulcerative colitis [643] and irritable bowel disease [644] in older people, and an increased presence of lactobacilli and bifidobacteria is linked with decreased presence of streptococci and other pathogens [645].

Some of the different responses in diverse individuals undergoing through probiotic supplementation are related to the genetic variation in the host [646]. This finding was concluded through a double-blind placebo-controlled study in healthy volunteers to determine mucosal responses to different probiotics including LGG [646]. Investigators clustered the transcriptomes per person (instead of considering solely the different interventions) and found person-to-person variation in gene expression. Moreover, the different probiotic bacteria used in the intervention induced differential gene-regulatory networks and pathways in the human proximal small intestinal mucosa. The concept of *immunobiography* – the personal history of antigenic exposure, environment, immunisation and immune diseases - [647] and the overall state of immunosenescence and inflammageing can also explain differential responses in different elderly communities.

It was found that probiotics exert little to no effect on the immune biomarkers assessed in the community of elderly care home residents studied herein; however, it is biologically plausible that the probiotics and even the non-viable or inactive strains used in this trial are able to impact gut barrier integrity as they interact with the enterocytes. Although effects on the immune system were not seen, other studies have suggested that these strains can modulate immune parameters in younger populations [648, 649].

9.2.3.1 Probiotics and gut microbiota

Findings about the microbiome have highlighted its interaction with the immune system response through immune mediators and control of pro-inflammatory processes [650-652]. The PRINCESS clinical trial included a sub-study examining changes on the gut microbiota through the study of faeces after the study intervention, and it is a further (future) intention to explore these findings in the context of the immune response systemically. Microbiome data or faecal composition are not within the scope of the research herein conducted.

9.2.3.2 Methodological calculations and sample size. Retrospective power calculation approach.

As discussed in chapters 4 and 5, the findings of the research herein conducted do not seem to point that a combination of probiotics LGG+BB-12 are effective in "boosting" the immune response in ECHRs. The biological explanation behind could be that the frail condition in which these individuals were found could impair the response as they were already under an "inflammaged" condition and rather vulnerable. It could be argued as well that the microbiota was also altered given the frail condition as it has been described elsewhere [653, 654]. However, another relevant methodological reason for the lack of effect could be referred to the sample size and the reduced power achieved in comparison to the initial target.

The current research included 30 participants per trial arm whose baseline and postintervention data were paired, so a total of 60 subjects were included for most variables (immune mediators analysed in plasma and in whole blood cultures were more unstable thus paired samples were reduced to 18 therefore 36 subjects). The initial target of PRINCESS immunology sub-study was having 50 participants per trial arm with baseline and postintervention data paired) thus a total of 100 individuals. The sample reduction observed in this research can be examined in the light of the power of the study and the risk of type II error, which means accepting wrongly the lack of positive findings given the underpowered sample size.

To conduct an effective analysis of the power of the study a retrospective power calculation was incorporated. This is relevant to determine the probability of correctly rejecting the null hypothesis, meaning lack of effect given the actual experimental sample size. These analyses can also be used to predict a better sample size in upcoming studies of this nature. Calculations were performed on PS Power and sample

size calculations software which was used on its Version 3.0, January 2009 Copyright © 1997-2009 by William D. Dupont and Walton D. Plummer [655, 656]. Standard deviations were calculated on the basis of the differences among postintervention and baseline. Table 9.1 contains a selection of primary outcomes per category of immune parameters analysed throughout this research to examine the power according sample size including an alpha of 0.05 in the analysis.

Table 9.1. Table of sample size power calculation

Variable	n	Calculated power of actual study (%)	Ideal sample size to achieve an accepted power of 80%
Neutrophils (FBC)	60	28.6	168
T cells (immunophenotyping)	60	37.7	127
Monocytes (immunophenotyping)	60	44.0	109
Phagocytic activity in Monocytes	60	57.0	84
Average of immune-related parameters with a sample size of 60 subjects of study	**41.8%**		**122**
TNF-α (Detected in plasma)	36	39.5	73
TNF- α (Detected in whole blood cultures PGN stimulated)	36	39.5	73
TNF- α (Detected in whole blood cultures LPS stimulated)	36	49.9	58
TNF- α (Detected in whole blood cultures PHA stimulated)	36	28.5	101
Average of immune mediators markers measured in plasma and in whole blood cultures with a sample size of 36 subjects of study	**39.4%**		**76**

Variables to build this chart were considered based on their biological relevance in the ageing process. Neutrophils have been linked to prognosis of certain atherosclerotic and chronic processes in the elderly people, likewise such is the case of monocytes and their phagocytic activity [657]. TNF-alpha measured in plasma and in whole blood cultures exposed to different immunostimulants has also demonstrated to be involved in different biochemical pathways within the inflammaging process [658, 659]. The sample size achieved in FBC, immunophenotyping and phagocytic analyses was bigger when compared with the sample size achieved in the immune mediators analyses, however the calculated power of the study was overall 40.6% indicating that at least 100 participants would have been required to increase the power of the study to 80%.

Outcomes and methodological design for this study are relevant for further mechanistical and proof-of-concept studies. Given the parameters herein analysed, it can be assumed that n=100 participants can provide a better power of the study from a statistical perspective.

In addition to the statistical perspective it is essential to consider what can be feasible in a population of vulnerable and frail ECHRs. Our study clearly found that the rate of deaths was higher than the expected 30%, and that the nature of the collaborative study reduced sample usability due to long distances and delayed processing. Further studies aiming to study similar populations and also happening under similar circumstances (multiple research centres with a central laboratory) might be benefited from the events herein identified. Thus, these studies could aim for sample sizes above 137 subjects if considering mechanistical immunological studies in addition to variables such as death (increase sample size in 30%) and in addition to lost sample usability (over 30%).

On that basis the analysis of the retrospective statistical power calculation and sample size indicates that the power of the study varies slightly with the immune-related parameters were analysed in contrast with the immune mediators measured in plasma or in whole blood cultures. In the case of immune mediators an overall analysis indicates that at least 122 participants would have been required to achieve a minumim 80% of study power. In the case of immune mediators losses correspond to approximately 39% thus at least 76 participants would have been needed to achieve a minimum 80% of study power.

Given these circumstances it is possible to say that these results should not be extrapolated to the general population of ECHRs as this study is underpowered. However, high rates of deaths in this frail population might indicate that the condition of vulnerability and advanced state of frailty are not good time windows to offer immunomodulatory interventions. Based on the outcomes herein identified it is reasonable to suggest that better immunomodulatory alternatives can start either in earlier stages when ECHRs are admitted to institutions and that earlier preventative strategies should start before progression of frailty.

9.3 *In vitro* study using LGG and BB-12 and Caco-2 cells

Overall it was observed that live LGG and BB-12 promote epithelial barrier recovery, whereas there is less benefit for probiotics that are heat-inactivated. This may indicate that metabolic products of the probiotic organisms are involved in ensuring epithelial integrity, although this was not investigated in the current research. Heat killed LGG and BB-12 could reduce the inflammatory response of Caco-2 cells in response to a cytokine cocktail. It has been indicated that the proinflammatory cytokines are able to promote TJ permeability: TNF-α, IL-1β and IFN-γ are able to act as epithelial barrier disrupters and are produced in increased amounts ageing [660]. The current research indicates that LGG and BB-12 may have a role in maintaining epithelial integrity in older people.

From a molecular perspective, no significant modification in the gene expression of structural genes was observed with the live probiotics even though they improved barrier function. This requires further exploration.

9.4 Final conclusions

The findings of this research do not support that a probiotic combination consisting of LGG + BB-12 affects the immune response of ECHRs. The PRINCESS trial immunology sub-study identifies several key points:

Biomarkers indicative of the ongoing inflammatory process are associated with frailty. Since the appearance of this concept, it is widely recognised that frailty on its own is suggestive of chronic conditions.

It is likely that probiotic interventions in the aged population will not provide them with an extensive benefit as they already presenting an inflammatory state that does not seem to be counterbalanced by the ascribed anti-inflammatory activity of probiotics. Potentially, these fragile individuals can see a benefit of probiotic interventions if they receive this intervention before the chronic condition appears and the inflammatory process gets established.

The *in vitro* work conducted as part of this research shows that live LGG and BB-12 can directly improve the gut epithelia barrier but heat-inactivated organisms cannot. In contrast the latter can protect the epithelium from mounting an inflammatory response.

9.5 Study strengths and limitations

This research project has several strengths. Firstly, it is conducted in a sector of the population that is fast-growing globally, older people and more specifically an understudied group of older people, ECHRs. With little known about probiotics and ECHRs, this research filled a research gap. The pilot study was conducted to carefully identify the stability of a range of immune markers in stored human blood and informed the conduct of the main human trial enabling a multicentre collaboration where laboratory outcomes could be assessed alongside the primary and secondary clinical outcomes. The protocols implemented in the pilot as well as in the PRINCESS immunology sub-study measured a range of markers reflecting innate and cell mediated parameters aiming to study broadly the inflammatory and immune response. These included responses to vaccination, regarded as the most valid marker of immune function in human trials.

This study also aimed to provide mechanistic evidence using an *in vitro* model of enterocytes to assess the interaction with the same probiotics as used in the clinical trial. The inflammatory response of the cultured epithelial cells was measured along with their integrity. A variety of inflammatory markers on both basolateral and apical sides were studied using both live and heat-inactivated microorganisms. The inflammatory cocktail used to induce inflammation and disrupt integrity in this model is relevant to ageing.

It is also acknowledged that this study also has limitations. In the clinical trial – immunology sub-study - not all samples arrived within the time frame that was expected and so not all samples could be used. Thus, sample size was smaller than initially anticipated. This was particularly true for the study of immune mediators in plasma and in whole blood cultures. Additionally, the findings herein presented are likely to incur in a type II error by accepting a null hypothesis due to reduced sample size. This valuable statistical and methodological approach requires the biological perspective behind that seems to point that high drop-out rates are likely to happen in frail populations and therefore earlier interventions, with a strong preventative focus, and wider sample sizes to achieve better power are necessary.

One limitation of the pilot study is that it did not investigate all the possible environmental factors that might affect sample stability. For example, effects of

temperature and agitation were not assessed. Lastly, the main limitation of *in vitro* studies using Caco- 2 cells is the absence of immune cells and lack of mucus.

9.6 Future work

The findings of the current study suggest future research. Firstly, although the probiotic intervention did not affect immune markers in the PRINCESS study, it is worthwhile to investigate other potential health effects of probiotics in ECHRs such as on gastrointestinal and mental health. Furthermore, probiotic intervention as a preventive strategy prior to occurrence of frailty should also be investigated.

The pilot study could be extended by investigating other factors that might affect stability of blood samples including storage temperature and agitation. These factors mimic other exposures that samples might encounter in a multicentre study or field research. Also, although NK cell numbers were explored, NK cell activity was not examined in the current research, either in the pilot study or in the immunology sub-study

The research involving the in vitro model of Caco-2 cells could be extended by using co-culture to examine the influence of probiotics on interactions between the gut epithelium and immune cells; several types of immune cell would be of interest including monocytes, macrophages, lymphocytes, and B cells. Furthermore, co-culture with mucus producing goblet cells would take the model closer to the *in vivo* situation. The in vitro model could also be used to get a better understanding of the effect of probiotics on molecular biology of epithelial cells, for example tight junction proteins and inflammatory signalling pathways including inflammasome formation.

Appendix A Reagents

BD BIOSCIENCES					
Name	**Code**	**Size**	**Vol per test**	**Storage**	**Application**
Stain Buffer (FBS)	554656	500ml	-	4°C	Flow cytometry
Brilliant stain Buffer	563794	5 ml	50 ul	4°C	Flow cytometry
Lysing Buffer	555899	100ml	2 ml	4°C	Flow cytometry
Alexa Fluor 488 Mouse IgG2a, K Isotype control	557703	100 tests	5 ul	4°C	Flow cytometry/ Isotype control
PE Mouse Anti-Human CD25	555432	100 tests	20 ul	4°C	Flow cytometry
Alexa Fluor 488 Mouse-Anti Human CD14	557700	100 tests	5 ul	4°C	Flow cytometry
BV421 Mouse Anti-Human CD80	564160	100 tests	5 ul	4°C	Flow cytometry
Alexa Fluor 488 Mouse Anti- Human CD8	557696	100 tests	5 ul	4°C	Flow cytometry
Alexa Fluor 488 Mouse IgG1 k Isotype control	557702	100 test	5 ul	4°C	Flow cytometry Isotype control
Alexa Fluor 647 Mouse IgG1 k Isotype control	557714	100 test	5 ul	4°C	Flow cytometry Isotype control
Alexa Fluor 488 Mouse Anti- Human CD19	557697	100 tests	5 ul	4°C	Flow cytometry
PE Mouse Anti-Human CD86	555658	100 tests	20 ul	4°C	Flow cytometry
Alexa Fluor 488 Anti-Human CD4	557695	100 tests	5 ul	4°C	Flow cytometry
BV421 Mouse Anti-Human CD127	562436	100 tests	5 ul	4°C	Flow cytometry
Alexa Fluor 647 Anti-Human CD3	557706	100 tests	5 ul	4°C	Flow cytometry
PE Mouse IgG1, k Isotype Control	555749	100 tests	20 ul	4°C	Flow cytometry Isotype control
PE Mouse Anti-Human CD16	555407	100 tests	20 ul	4°C	Flow cytometry

Anti-Mouse Ig, k/Negative Control Compesation Particles Set	552843	6 ml		4°C	Flow cytometry
TrueCount tubes™	340334	100 tubes per pack	According to experiment design	Room Temperature	Flow cytometry
PHAGOTEST™	341060	100 tests per kit	Manufacturer's instruction	4°C (Reagents supplied with a preservative. Storage following period shown on the packaging label)	Flow cytometry
BD™ Cytometer Setup and Tracking Beads	641319	3ml	1 drop into filtered water	4°C	Flow cytometry
BD™ CompBeads	552843	6ml	1 drop into calibration tube	4°C	Flow cytometry
All reagents need storage undiluted at 4°C and protected from prolonged exposure to light. Do not freeze					
INVIVOGEN					
Name	**Code**	**Size**	**Vol per test**	**Storage buffer**	**Application**
LPS-EK Lipopolysaccharide from E. Coli K 12 strain TLR4 ligand	Tlrl-eklps	5mg standard lipopolysaccharide from E. Coli K 12 (LPS-EK) 1,5 ml endotoxin – free water	Working concentration: 1ng-10mcg/ml	-20°C	Whole Blood culture/ Stimulant

PGN-SA Peptidoglycan from Staphylococcus aureus: TLR2 ligand	Tlrl-pgns2	5mg peptidoglycan from S. aureus (PGN-SA)		-20°C	Whole Blood culture/ Stimulant
SIGMA-ALDRICH					
Name	**Code**	**Size**	**Vol per test**	**Storage buffer**	**Application**
Lectin from Phaseolus vulgaris (red kidney bean)	L1668			2 to 8*C	Whole Blood culture/ Stimulant
RPMI-1640 Medium	R0883	500ml	5.4ml	4° C	Whole Blood culture
Dulbecco's Modified Eagle's Medium – High Glucose. Without L-glutamine (Media)	D6546	500ml	According to protocol	-20°C	Cell culture (Media)
Dulbecco's Phosphate Buffered Saline with MgCl2 and CaCl2. Liquid, sterile-filtered, suitable for cell culture.	D8662	500ml	50ml to be diluted in media	-20°C	Cell culture
L-Glutamine Solution 200 mM	59202C	100ml	5ml to be diluted in media	-20°C	Cell culture
1X sterile, sterile-filtered BioReagent suitable for cell culture 0.5g porcine trypsin and 0.2g EDTA 4Na per litre of Hanks Balanced salt solution	T3924	100ml	According to protocol	-20°C	Cell culture
Penicillin streptomycin				-20°C	
Bio-Techne, R&D Systems Europe Ltd					
Name	**Code**	**Size**	**Vol per test**	**Storage buffer**	**Application**
Human Magnetic Luminex Screening Assay	LXSAHM	1 assay		4° C	Multiplex ELISA
Thermo fisher scientific					
Name	**Code**	**Size**	**Vol per test**	**Storage buffer**	**Application**
TaqMan™ Gene Expression Master Mix – 5ml	4369016	5ml	According to experiment design	4° C	PCR
Primer Design					

Name	Code	Size	Vol per test	Storage buffer	Application
OCLDN-Target gene	DD-CustomGOI Inv	Reconstitute according to manufacturer's instruction (rxn)	According to experiment design	4◦ C	PCR
TJP-1- Target gene	DD-CustomGOI	Reconstitute according to manufacturer's instruction (rxn)	According to experiment design	4◦ C	PCR
CLDN1- Target gene	Inv	Reconstitute according to manufacturer's instruction (rxn)	According to experiment design	4◦ C	PCR
Reference genes		Reconstitute according to manufacturer's instruction (rxn)	According to experiment design	4◦ C	PCR
PROMEGA					
Name	**Code**	**Size**	**Vol per test**	**Storage buffer**	**Application**
GoScript™ Reverse Transcriptase	A5003	100 reactions	According to manufacturer's instruction	4◦ C	cDNA synthesis
ReliaPrep™ RNA Cell Miniprep system	Z6011	100 reactions	According to manufacturer's instruction	4◦ C	RNA extraction

Appendix B Magnetic Luminex assay

Magnetic Luminex Assay - Analytes produced from plasma	
Kit Lot.	1443207 (Human plasma) – 1443204 (Whole blood culture)
Kit catalog number	LXSAHM-9 - LXSAHM-5
Microparticle mix Lot number	1443208
Biotin Antibody Mix Lot Number	1443209
Analytes and Standard Cocktail Lot information	According to manuacturer's instruction
Sample preparation	According to protocol, serum and plasma samples require at least 2-fold dilution. Suggested 2-fold dilution is 75ul of Calibrator diluent RD6-52.
Reagent preparation	Reagents were brought to room temperature before use. However, dilutions were prepared when the reagent was required. The standards provided differ depending on the analytes analysed. Standards were reconstituted starting with a gentle agitation during 15 minutes, and then upon reconstitution, each standard cocktail is a 10x concentrate.
Calibrator diluent	RD6-52
Diluted microparticle cocktail preparation	1. Centrifuge the Microparticle Cocktail vial for 30 seconds at 1000xg 2. Dilutions of the microparticle-Cocktail using diluent RD2-1. Calculations done considering the total of wells to be used
Diluted Biotin Antibody cocktail preparation	1. Centrifuge the Biotin Antibody Cocktail vial for 30 seconds at 1000xg prior to removing the cap 2. Vortex the vial without invert the vial 3. Dilute the Biotin Antibody Cocktail in Diluent RD2-1. Mix gently.
Streptavidin-PE preparation	Recommendations from the protocol include using a polypropylene amber bottle or a polypropylene test tube wrapped with alumina foil. Protect the Streptavidin PE from light during handling and storage. 1. Centrifuge the vial 2. Gently vortex 3. Dilute the Streptavidin-PE concentrate to a 1X concentration by adding 220ul uf Streptavidin-PE Concentrate to 5,35ml of wash Buffer.
Instrument settings	
Bio-Rad-Plex analyser. - Assign the microparticle region for each analyte being measured - 50 events/bead - Minimum events. 0 - Flow rate. 60 ul/minute	

- Sample size. 50ul
- Collect median fluorescence intensity

Assay procedure

1. Prepare all reagents and standards and only use after preparation. Allow them to chill at room temperature prior use
2. Resuspend microparticle cocktail by vortexing. Add 50ul of the microparticle cocktail
3. Add 50ul of sample per well. Samples were diluted 2 folds
4. Cover with a foil plate sealer and incubate (usually 2 hous - but review manufacturer's instruction) at room temperature on a microplate shaker (750 rpm)
5. Using the magnetic device, accommodate the microplate. 3 washes were applied by ensuring the magnet to the bottom of the microplate. Allow 1 minute before removing the liquid, filling each well with Wash Buffer. 1 minute was temporized before removing the liquid again. Removal of liquid is essential for good performance.
6. Add 50ul of diluted biotin antibody cocktail to each well. Securely cover with a foil plate sealer and incubated at room temperature (usually 1 hour- but review manufacturer's instruction).
7. Repeat 3 washes timing 1 minute before each one.
8. Resuspend the microparticles by adding 100ul of wash buffer to each well. Incubate for 2 minutes on the shaker set at 750rpm.
9. Read within 90 minutes.

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