The Effects of Prebiotic and Probiotic Treatment on Asthma, Inflammation, and Immune Function

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Copyright Statement

List of Abbreviations

AOS	Arabinooligosaccharides
ASM	Airway smooth muscle
B-GOS	*Trans*-galactooligosaccharide
Ca^{2+}	Calcium
CD25	Cluster of differentiation-25
CD3	Cluster of differentiation-3
CD4	Cluster of differentiation-4
cGMP	Cyclic guanosine monophosphate
cNOS	Calcium dependent nitric oxide synthase
CRP	C-reactive protein
CXCL	Chemokine (C-X-C) ligand
DC	Dendritic cells
ECRHS	European Respiratory Health Survey
EIB	Exercise-induced bronchoconstriction
eNOS	Endothelial nitric oxide synthase
EVH	Eucapnic voluntary hyperpnoea
FEV_1	Forced expiratory volume
FOS	Fructooligosaccharide
Foxp3	Forkhead box P3
FSC/FS	Forward scatter
FVC	Forced vital capacity
GALT	Gut-associated lymphoid tissue
GATA3	GATA binding protein 3
GINA	Global Initiative for Asthma
GOS	Galactooligosaccharide
GPCR	G-protein coupled receptor
GPR109A	G-protein coupled receptor 109A
GPR41	G-protein coupled receptor 41

GPR43	G-protein coupled receptor 43
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
ICAM	Intercellular adhesion molecule 1
ICS	Inhaled corticosteroids
IEC	Intestinal epithelial cell
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IL	Interleukin
IL-1	Interleukin-1
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-1β	Interleukin-1β
IL-33	Interleukin-33
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
ISAAC	International Study of Asthma and Allergies
K^+	Potassium
LABA	Long-acting beta-2 agonist
LCn-3PUFA	Long-chain n-3 polyunsaturated fatty acid
LPS	Lipopolysaccharide
M cell	Microfold cell
MAC	Microbiota accessible carbohydrates
MALT	Mucosa-associated lymphoid tissue
MAMP	Microorganisms-associated molecular patterns

MAPK	Mitogen-activated protein kinase
MLN	Mesenteric lymph node
mRNA	Messenger ribonucleic acid
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptors
nNOS	Neuronal nitric oxide synthase
NOD	nucleotide-binding and oligomerisation domain
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PRR	Pattern recognition receptor
pTregs	Peripheral regulatory T cells
SABA	Short acting beta agonist
SCFAs	Short chain fatty acids
SES	Socioeconomic status
SSC/SS	Side scatter
T2D	Type-2 diabetes
TGF-β	Transforming growth factor beta
Th Cell	T helper cells
Th1	T helper 1 cell
Th2	T helper 2 cell
TLR	Toll like receptor
TNFR	Tumour necrosis factor receptor
TNF-α	Tumour necrosis factor alpha
Treg	Regulatory T lymphocytes
tTregs	Thymus derived regulatory T cells
VCAM	Vascular cell adhesion molecule 1
WBC	White blood cells

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Abstract

Asthma affects 5.4 million people in the UK. It is a significant health burden and costs the NHS at least £1.1bn each year. Traditionally treatment of asthma involves pharmacological intervention. However, these methods are not preventative or curative and long-term use can lead to significant side effects and reduced efficacy. The purpose of this thesis was to elucidate the effects of a dietary intervention with prebiotic *trans*-galactooligosaccharide (B-GOS) in Asthma, and to better understand the role of B-GOS in inflammation.

Therefore, this thesis investigated: (i) the effects of prebiotic *trans*-galactooligosaccharide (B-GOS) supplementation on quality of life, control of asthma and markers of systemic inflammation in adults with asthma, and; (ii) prebiotics and synbiotics in the treatment of asthma and changes in associated inflammatory markers: a systematic review and meta-analysis of human and murine trials. Additionally, this thesis assessed the effects of prebiotics *trans*-galactooligosaccharide (B-GOS) treatment on; (iii) biological markers of systemic inflammation in healthy adults and; (iv) peripheral blood mononuclear cells under chronic inflammation

Thirteen studies were included for meta-analyses, eight murine studies and five infant studies. Five treatments from three murine studies indicated that prebiotic treatment (n=3) reduced airway hyper-responsiveness (SMD, -2.19 [-2.91, -1.46] P = 0.001) and synbiotic treatment (n=2) did not (SMD, -0.14 [-0.83, 0.56] P = 0.70). Meta analyses of Five infant studies indicated that prebiotic treatment (n=3) did not reduce cumulative incidence of recurrent wheeze (Risk ratio – 0.85 [95% CI: 0.41, 1.76] P = 0.67) and synbiotic treatment did not (Risk ratio – 0.65 [95% CI: 0.27 – 1.57] P = 0.34). These data provide evidence supporting the beneficial effects of prebiotic treatment in murine models. However, evidence is unsubstantial for prebiotic and synbiotic treatment in infants with asthma.

This Thesis found that 20 days treatment with 3.65g/d of prebiotic B-GOS reduces pro-inflammatory cytokine TNF-α in $CD4^+$ T cells of healthy adults. These findings align with previous work (Williams et al., 2016), and provide further evidence for a potentially protective effect of B-GOS in healthy adults for inflammatory disease risk. Levels of GATA-3 and IL-6 were undetectable, and no differences were observed in $CD4^+$ $CD25^+$ Foxp3 cells, NF-κβ, IL-4 and T cell number after prebiotic treatment.

A novel finding of this thesis was that 20 days treatment with 3.65g/d of prebiotic B-GOS attenuated TNF-α in $CD4^+$ T cells after *in vitro* stimulation of peripheral blood mononuclear cells, demonstrating the protective effect of B-GOS in chronic inflammatory conditions. These results provide scope for future research of B-GOS in clinical populations with inflammatory related disease.

1. Chapter 1 – General Introduction

1.1. Introduction and Rationale

Asthma is a chronic inflammatory disorder of the airways, affecting adults and children of all ages. It usually presents itself in childhood with other atopic conditions such as eczema and hay-fever (Lee and McDonald, 2018). An asthma exacerbation is often triggered by the environment through inhalation of an irritant or allergen, in turn leading to bronchial sensitivity followed by airway inflammation and an increase in mucus production. There is significant heterogeneity in asthma and many individuals will respond differently to various triggers such as pollen, exercise, air temperature, respiratory tract infections, pollution and cigarette smoke (Douglas et al., 2008). Airway obstructions are characterised by smooth muscle contraction, mucus hypersecretion with plug formation and inflammatory cells infiltration; these changes are reversible. However, airway remodelling may occur in chronic asthma patients with hyperplasia, smooth muscle hypertrophy, collagen deposition, basement membrane thickening and epithelial desquamation; resulting in irreversible changes (Hashmi et al., 2021). Currently asthma affects 339 million people worldwide (The Global Asthma Report, 2018). Even with asthma prevalence higher in high income countries, most mortality occurs in low to middle incomes countries (To et al., 2012). The increase in prevalence of asthma has been suggested as a consequence of environmental and lifestyle changes associated with the western lifestyle and not genetic change (Brigham et al., 2015, Kearney, 2010).

Multiple phenotypes of asthma have been characterised in order to better account for the high heterogeneity observed in asthma (Wenzel et al., 2013). However, there is still difficulty in defining the pathology of asthma due to the complexity of immune balancing between phenotypes. The main symptoms contributing to asthma exacerbations are excessive mucous production, airway smooth muscle contraction and inflammation (Hashmi et al., 2021). Airway inflammation is facilitated by infiltration of a variety of immune cells, including eosinophils, B and T lymphocytes, mast cells, macrophages and neutrophils. These immune cells produce

inflammatory cytokines, chemokines, eicosanoids, and histamine, contributing to asthma pathophysiology (Anderson and Kippelen., 2005a).

In 1994, it was highlighted that changes in diet were accompanied by increases in asthma and atopic disease in the UK (Seaton and Brown, 1994, Seaton and Devereux, 2005). They suggested the westernised diet had led to deficiencies in antioxidants from reduced vegetable consumption, increasing the population susceptibility to allergy and asthma. High fat intake in western diets has also been implicated in asthma, in addition to the lack of fibre consumption. Current evidence suggests that a 'Mediterranean diet' rich in fruit, vegetables and fibre, while low in saturated fat, protects against the development of asthma and its symptoms (Thorburn et al., 2014). Moreover, the westernised diet, low in fruit, vegetables and fibre and high in saturated fat, heightens asthma risk through increased systemic inflammation (Guilleminault et al., 2017, Wood et al., 2015). Systemic inflammation is not only associated with Asthma progression, but a number of chronic health conditions such as ischemic heart disease, dementia, cerebrovascular diseases and cancer (McLoughlin et al., 2017). Systemic inflammation results from the chronic activation of the immune system, thus increased activity of immune cells that release pro-inflammatory mediators, such as C-reactive protein (CRP), tumour necrosis factor alpha (TNF-α) and interleukins (i.e. IL-6, IL-1β). Numerous studies have demonstrated a link between reduced dietary fibre intake and biomarkers of systemic inflammation (Ajani et al., 2004, Ma et al., 2008, Bronwyn et al., 2013).

The close relationship of diet and the intestinal microbiome is well known, and the intestinal microbiome has been implicated in modulating several chronic diseases including asthma, inflammatory bowel disease, obesity, type 2 diabetes, cardiovascular disease and cancer (Singh et al., 2017, Frati et al., 2019). Consumption of specific food types leads to shifts in host bacterial genera, affecting immune and metabolic parameters, contributing to disease pathology.

A number of research studies have demonstrated that dietary manipulation through supplementation of prebiotics, 'a substrate that is selectively utilised by host microorganisms conferring a health benefit', may be beneficial in the treatment of infant and adult asthma (Arslanoglu et al., 2012, Ivakhnenko et al., 2013, Williams et al., 2018), and reduce systemic inflammatory biomarkers in multiple inflammatory diseases (McLoughlin et al., 2017). The benefits of synbiotics (prebiotic and probiotics) have also been demonstrated to reduce asthma-like symptoms in infants (van der Aa et al., 2011), and systemic inflammatory biomarkers (McLoughlin et al., 2017). Both pre- and probiotics are able to improve lung function and reduce asthma exacerbations throughout the course of supplementation (Gutowski et al., 2010). These research findings indicate the importance of the gut microbiome and its modulation in not only asthma, but inflammatory disease, and highlight the need to further understand the mechanisms of dietary supplements to improve and manage asthma and inflammatory disease on the whole.

1.2. Research aims

The purpose of this PhD and thesis was to investigate the following research questions:

I. What are the effects of prebiotics and synbiotics in the treatment of asthma and changes in associated inflammatory markers?

The research aims were to evaluate:

- Data from infant asthmatic intervention studies, observing the effects both prebiotics and/or probiotics
- Data from murine studies, observing the effects both prebiotics and/or probiotics in a HDM-induced asthma model.

II. A novel intervention using a prebiotic *trans*-galactooligosaccharide (B-GOS) may influence systemic inflammation through favourable manipulation of the gut microbiome and SCFA action. A randomised double blind, placebo controlled cross-over study will be implemented to analyse the effectiveness of BGOS on specific biological markers of inflammation in healthy adult volunteers.

The research aims were to:

- Determine whether a prebiotic supplement can induce significant changes of specific pro/anti-inflammatory markers and thus be favourable for human health

III. Does prebiotic treatment in healthy adults alleviate chronic inflammation of peripheral blood mononuclear cells stimulated in vitro?

The research aims were to:

- Determine whether prebiotic treatment in healthy adults reduces the release of inflammatory cytokines in PBMCs, following a cellular stimulation to induce chronic inflammation
- Determine the effects of prebiotic treatment at baseline, if any, are maintained following induction of chronic inflammation in PBMCs

2. Chapter 2 – Review of literature

2.1. Asthma

The first case of asthma was reported as breathlessness in the "Code of Hammurabi" 1792-1750 BC: "if a man's lungs pant with his work". In approximately 400 BC, Hippocrates used the term "asthma" to label this 'panting' and respiratory distress, (Keeney, 1964). Asthma refers to airway hyper-responsiveness and tissue remodelling of the airway structure (Murdoch and Lloyd, 2010). Common symptoms include wheezing, breathlessness, chest tightness or a cough, with or without sputum (Kaplan, et al., 2009). Asthma is a generalised umbrella term used to describe these symptoms, however, consisting of many sub-categories, such as exercise induced asthma (EIB), allergic asthma and non-allergic (Holgate, 2010). There are many phenotypes in which asthma may present itself, if we are able to better subtype asthma and identify these phenotypes, we may able to inform both pharmacological and non-pharmacological treatment methods.

2.1.1. Prevalence of Asthma

Asthma is estimated to account for 1 in every 250 deaths worldwide in people of all ages and ethnic backgrounds, with less conservative criteria this estimate is expected to be considerably higher (Masoli, et al., 2004), indicating the importance of treating and identifying this chronic inflammatory syndrome. Asthma prevalence has been reported to increase over the last century, affecting quality of life while also intensifying healthcare costs (Simpson and Sheikh, 2010). In 1989 a programme was introduced, known as the Global Initiative for Asthma (GINA), to raise awareness of increases in asthma incidences among health care workers, the public and government officials. It was then estimated that around 300 million people in the world are effected by asthma using data from the International Study of Asthma and Allergies (ISAAC) and the European Respiratory Health Survey (ECRHS), recorded from 1992-1996 and 1988-

1994, respectively. A correlation was shown between communities adopting 'western lifestyles' and becoming more urbanised, with increased rates of asthma incidence. Over 8 million people in the UK, or 12% of the population, have asthma (National Institute for Health and Care Excellence).

There are many environmental factors that influence the prevalence of asthma, in the United States this prevalence has been reported to vary significantly between certain ethnicities. Asthma severity is higher in Puerto Rican and African American children, demonstrated by a greater frequency of ER visits and mortality rates, in comparison to Caucasians (Forno and Celedon, 2009).

Table 1. 1. The current prevalence of asthma and mortality rates in the United States, in children 0-17 years of age. AI/AN: American-Indian/Alaska Native. ***: no reliable data available. Data obtained from Moorman et al., (2010) and reproduced from Forno and Celedon (2009).

Population	**Current Prevalence (%)**	**Mortality rate (per 1,000,000**
Puerto Ricans	19.2	***
AI/AN	13.0	***
Non-Hispanic blacks	12.7	9.2
Filipino	10.7	***
National average	9.3	2.6
Non-Hispanic whites	8.0	1.3
Mexicans	6.4	1.7
Chinese	5.1	***
Asian Indian	4.4	***

The lowest level of Asthma prevalence appears to be within the Asian community in the US, using data obtained from a Behavioural Risk Factor Surveillance System in 2004, however, it is unclear as to why. For other groups, such as Native and African Americans, socioeconomic status and air quality were considered a driving factor for high levels of asthma (Gorman and Chu, 2009). Similarly, in the United Kingdom, south Asian children were shown to have a lower frequency of symptoms indicative of asthma in comparison to white and black children, with pooled history of wheeze rates for 12 months' of 9.6%, 14.6% and 16.2%, respectively. Additionally, the pooled frequency of children diagnosed with asthma was shown to have an analogous pattern (Netuveli et al., 2005). Asthma is more common in children than adults, with current statistics reporting approximately 6 million children under the age of 18 with asthma in the US. In 2015, 1 in 12 children were diagnosed with the disorder. Furthermore, 3,615 children died from asthma in 2015 and many of these deaths were avoidable with the correct treatment (aafa.org, Accessed on 17.05.2018, cdc.gov, Accessed on 17.05.2018).

Lower socioeconomic status (SES) is associated with asthma ER visits and mortality rates. In 2010 it was shown that 20% of children lived in poverty in the United States, the rates where highest in black and Hispanic children, with 38.2% and 32.3% living in poor conditions, respectively. Conversely, white children only made up 17% of this category (Census.gov, Accessed on 14.05.2018). Indicating a possible correlation between SES, ethnicity and asthma prevalence. There are multiple proposed mechanisms through which a lower SES may influence the development and severity of asthma; one is that low-income children are disproportionately exposed to allergens and pollutants daily, possibly influencing symptoms and diagnoses of asthma (Huang and Boushey, 2013). Indoor allergens are higher in urban households in lower-income areas, in comparison to housing located outside the central business district and suburbs associated with upper/middle class. Moreover, some of the

highest levels of allergens are predicted to be in households with pets and mold/moisture issues (Salo et al., 2008)

Table 1.2. Estimated prevalence and odds ratios predicting the levels of allergens in US homes with standard error and 95% confidence intervals (NSLAH, 1999). To generate this data, a multivariable logistic regression was utilised, the odds ratio was adjusted appropriately for each variable within the table. Over half of the homes studied (51.5%), all had detectable levels of allergens measured. Table reproduced from (Salo et al., 2008).

Family Income	**Percentage (SE)**	**(95% CI)**
$0 – 19,999	22.56 (4.71)	1.84 (0.85, 3.98)
$20,000 – 39,000	23.87 (3.46)	2.44 (1.14, 5.22)
$40,000 – 59,000	11.53 (2.96)	0.72 (0.30, 1.74)
$60,000 +	12.12 (2.96)	1.00
Housing type		
Single family home	19.06 (2.01)	1.94 (1.02, 3.67)
Multi- family home	11.36 (2.32)	1.00
Smoker(s) in household		
Yes	23.47 (2.01)	1.74 (1.19, 2.53)
No	13.21 (2.01)	1.00
Pet(s) in household		
Yes	24.82 (2.55)	2.98 (1.67, 5.31)
No	11.31 (2.19)	1.00
Mold/Moisture problems		
Yes	24.21 (2.63)	2.06 (1.28, 3.30)
No	11.76 (1.80)	1.00

It has been reported that deficiency in vitamin D is associated and possibly responsible for a global increase in the prevalence of allergic diseases (Searing and Leung, 2010). This is due to the immunomodulatory effects of Vitamin D on allergen induced pathways, shaping the adaptive immune response (Mirzakhani et al., 2015). It was shown that a 10-degree change in geographical latitude, from southern to northern regions of the Eastern Seaboard, resulted in a 2% increase of asthma prevalence in adults. Analogous results were obtained using data on Australia (Krstic, 2011). This is due to lack of exposure to the outdoor sun, increasing asthma prevalence.

Advances in diagnosing clusters of populations with similar inducing factors, may allow more specific asthma treatment and prevention, thus reducing the burden economically and helping to manage the inflammatory syndrome on a larger scale.

2.1.2. The Pathogenesis of Asthma

Asthma is a convoluted syndrome, with many unknown biological mechanisms. Various hypotheses have been proposed and are debated regularly to try to elucidate these mechanisms. With improved understanding of asthma pathophysiology, we may be able to diagnose and treat this syndrome more effectively.

The hygiene hypothesis first proposed by Strachan in 1958, who identified an inverse correlation between the number of older siblings and hay fever when observing over 17, 000 British children born in 1958 (Okada et al., 2010). The hypothesis suggests that environmental changes, linked to growing industrialisation, have reduced microbial contact at an early age, and therefore incidence of infections. This may have then given rise to increased frequencies of atopic eczema, asthma and allergic rhinoconjunctivitus. These findings have been considered alongside the Th1/Th2 immune responses, suggesting the hypothesis must be

extended in three respects (Rautava et al., 2004). Due to the short nature of the study in 1958, it is unlikely that the results were due to genetic changes, and therefore favour environmental changes as the cause. Various animal models have been used to assess the 'hygiene hypothesis' and results indicate that the induction of certain pathogens, irrespective to modification of Th1/Th2 balance, may influence the development of autoimmune disease, such as Graves' disease (Nagayama et al., 2004). However, the validity of these models are questionable as they do not mimic the true allergic response (Vivolo Aun et al., 2017). Early versions of the hygiene hypothesis suggest that transmission of infectious agents at a young age may induce allergies, for example, faecal-oral interactions of bacteria such as helicobacter. When it comes to asthma there are also many inconsistent findings, indicating the hygiene hypothesis is unlikely to be the only explanation for increases of asthma incidence in industrialised nations (Ramsey and Celedon 2005).

Furthermore, there is a contradiction associated with industrialisation and cleanliness, as there is increased air pollution and thus biologic material in the air of industrialised countries. It is unclear whether lack of microbial contact and/or air pollution work to induce autoimmune disorders. Occupational asthma is common in developing countries due to rapid industrialisation, attributing 13-15% of asthma cases, in comparison to 6% of cases in less industrialised developing countries (Jeebhay and Quirce, 2007). Many other factors may also work in synergy with one another, suggesting the 'hygiene hypothesis' may need updating to better characterise respiratory syndromes (Bloomfield et al., 2016). Some of these factors include diet, social exposure through sports, time spent indoors, pre-existing conditions, correct use of antibiotics and living conditions. These factors, as well as many others, influence the gut microbiome composition, and if risk assessed could provide a complex framework used to maximise protection against pathogen exposure and transmission of microbes between the public.

The human gut consists of a large and diverse collection of microorganisms, forming the gut microbiome. The gut microbiota coevolve alongside the host to attain a stable environment for bodily functions, such as digestion of nutrients, production of nutrients and vitamins, maintenance of the immune system and defence against pathogens (Koh, et al., 2016). Originally the hygiene hypothesis suggested that 'unhygienic contact' and transmission of infections early in life prevented certain allergies. However, this concept has been adapted in the last few decades and extended to microbiota, linking outdoor exposure, air quality, diet, antibiotic use, pregnancy and genetics to allergic diseases (Shreiner, et al., 2008). By assessing key metabolites of certain microbiota, such as short-chain fatty acids, it has been indicated that gut microbiota is of importance in the immune system and can indirectly modulate lung inflammation. These mechanisms have yet to be elucidated, but the possibility of a gut-lung axis has been speculated, and may have great importance in allergic disease and the asthma syndrome (Koh, et al., 2016; Ege, 2017). The 'Microbiome Hypothesis' can be perceived as an extension to the 'Hygiene Hypothesis', both indicate possible connected mechanisms for asthma pathogenesis.

2.2. Pathophysiology of Asthma

Asthma dates back to near 2700 BC, it was described in ancient history as a 'wind within' and 'noisy breathing', over the years detailed clinical observations have been recorded, enabling better characterisation of the syndrome (Walter et al., 2005). The first clinical description of asthma appeared in a Chinese textbook around 2600 BC, the Greek word 'Asthma', meaning 'to exhale with open mouth' or 'to pant', was first used in the English language in the 1600's (Sakula, 1988). In 1662, asthma was described as 'the lungs are contracted or drawn together' by a Belgian physicist, the first to phenotype asthma. By the 1900s, bronchoconstriction via airway smooth muscle was documented and a mechanism was proposed by Sir John Floyer (Marketos et al., 1982). In the 1970s, there was an asthma breakthrough due to the discovery of fibre-optic bronchoscopy and the utilisation of molecular biology in research. It was then uncovered that Asthma was a chronic inflammatory disorder of the airway, characterised by the chain reaction of immune cell signalling, airway narrowing, excessive mucus production and inflammation (Boushey et., 1980).

Activated inflammatory cells provide a significant contribution to asthma symptoms, infiltrating lung tissue and exhibiting their effects during exacerbations (Figure 1.1.). Eosinophils, phagocytes, neutrophils and mast cells are some of the inflammatory cells responsible for asthma symptoms, as well as airway inflammation and remodelling, provoking normophysiology (Murdoch et al., 2009). This dramatic change in airway structure during asthma exacerbations is known as airway hyper-responsiveness (AHR) and is accompanied by rapid constriction of airway smooth muscle (ASM) (Figure 1.0). Airway smooth muscle has been shown to hypertrophy in asthmatics, leading to increased AHR. In children it was shown bronchial thermoplasty or removal of excess ASM, reduced AHR (Cox et al., 2006). However,

some research shows an inverse correlation between ASM and AHR (Boulet et al., 2000). Airway hyper-responsiveness has been correlated clinically with eosinophil, neutrophil, mast cell and Th2 cell infiltration, emphasising the importance of the underlying allergic airway mechanisms in asthma (Kearley et al., 2009; Siddiqui et al., 2007). These host immune cells are able to adhere to the endothelium of airway cells with virulence, increasing the intensity of AHR through cell adhesion proteins, such as Vascular-cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (NAEPP 2007). Additionally, the influence of multiple chemokines and cytokines prolongs inflammatory cell survival, such as Interluekin-

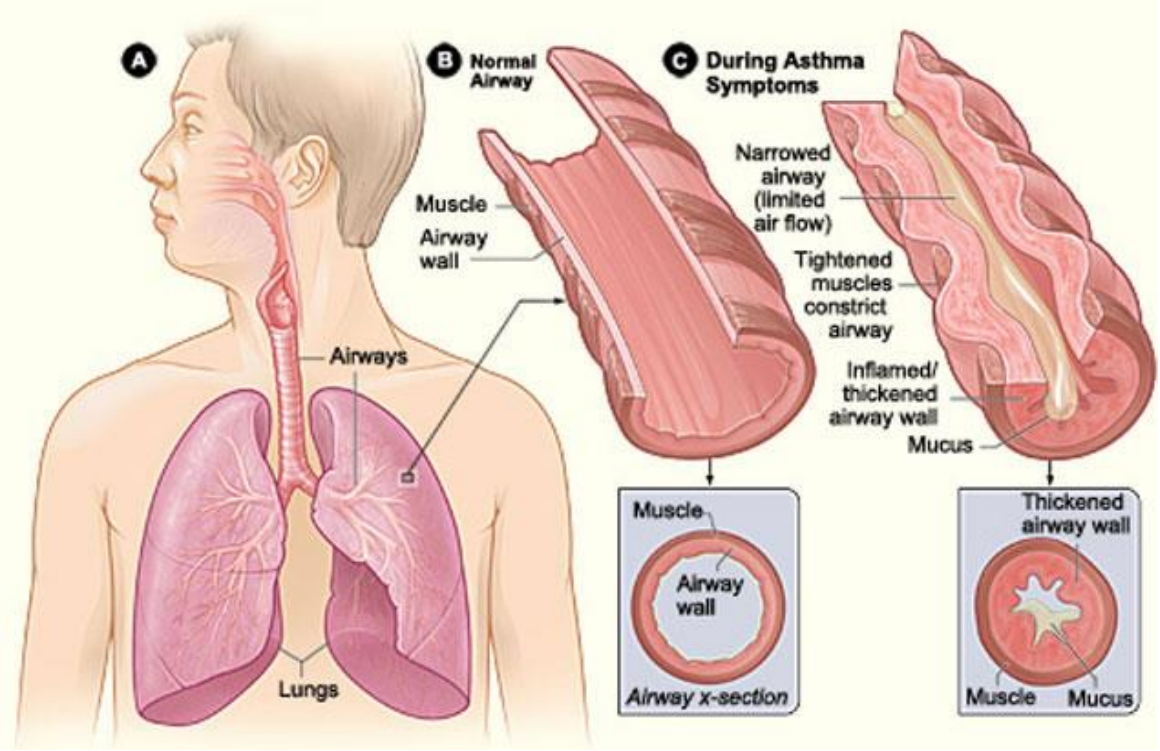

Figure 1.0. Key changes in structure and physiology before and after an asthma exacerbation. Section A shows location of the human lungs and airways, section B shows a cross-section of a normal airway and Section C shows a cross-section of an airway during an asthma exacerbation. (NHLBI)

4 and granulocyte macrophage colony stimulating factor (GM-CSF) (Busse and Lemanske, 2001).

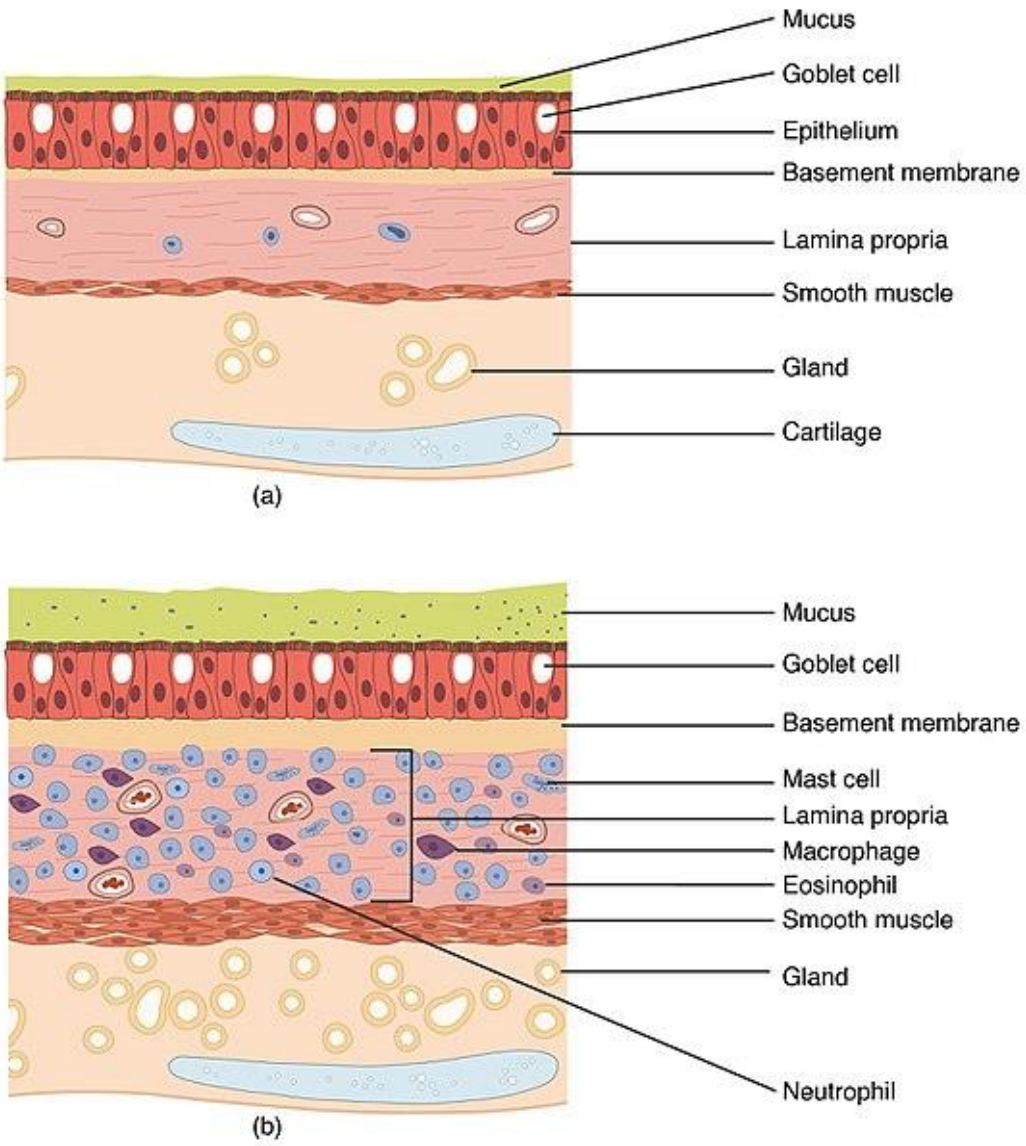

Figure 1.1 Composite figure depicting normal lung tissue (a) and lung tissue with key characteristics of an asthma exacerbation (b). Increased goblet cells, infiltration of mast cells, neutrophils, eosinophils, and macrophages into the lamina propria. Differences of thickness in the basement membrane are observed in the normal lung tissue and asthmatic tissue; this is due to the increased quantity of smooth muscle. Figure adapted from OpenStax College (19/06/2018)

Airway remodelling, inflammation, hyper-reactivity and air flow limitation work in a synergistic manner to induce the symptoms seen in clinical asthma, illustrated in figure 1.3. Many physiological changes in both atopic and non-atopic asthma are shared, however, they are induced via different mechanisms. Microbes such as staphylococci may colonise the airway epithelium in both atopic and non-atopic asthma, these bacteria produce a 'superantigen', and during exacerbation may produce a local response activating B cells and the subsequent production of antigen-specific IgE. This is followed by mast cell sensitisation, airway inflammation and asthma symptoms. These superantigens may also induce T cell expansion, increasing Th2 cell responses while nullifying regulatory T cells. (Pillai et al., 2011).

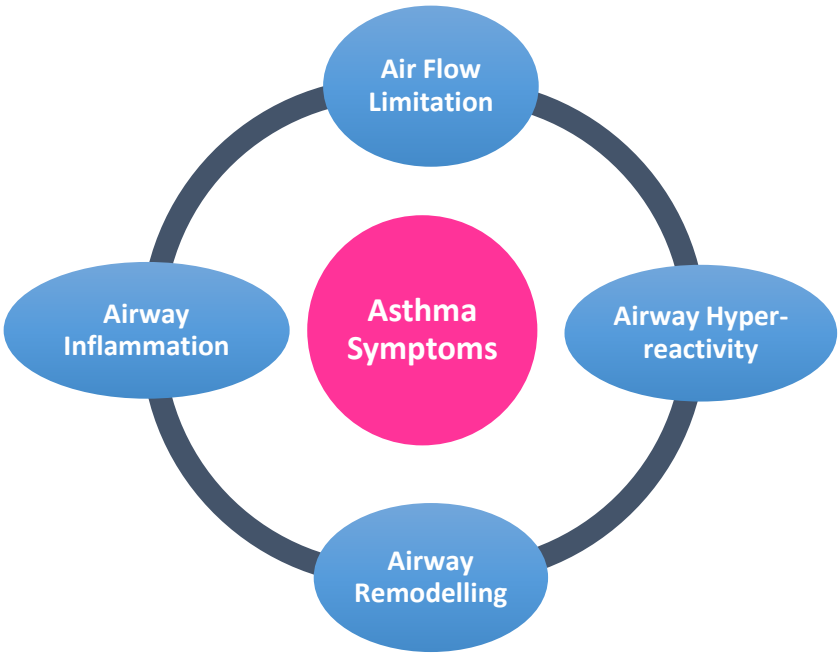

Figure 1.2 Schematic diagram illustrating the various foundations of asthma symptoms. Airflow limitation, AHR, airway inflammation and airway remodelling, are collectively responsible for asthma symptoms. Each characteristic interplays with one another, augmenting asthma exacerbations (Self-made diagram, 2018)

2.2.1. Inflammation and the Immune System

The immune system is a complex interactive network, consisting of cytokines, lymphoid organs, humoral factors and cells. The primary function of the immune system is host defence and it is best demonstrated under instances of pathophysiology, where underactivity results in immunodeficiency and severe infections, and over activity, allergic and autoimmune disease (Parkin and Cohen, 2001). The immune system can be divided into two parts, innate immunity and adaptive immunity. Innate immunity provides an immediate response to foreign material, acting as a first line of defence. Whereas adaptive immunity involves antigen-specific responses through the utilisation and mobilisation of T & B Lymphocytes. The former response is rapid and immediate; however, it can sometimes result in damage of host tissues. The latter is a more specific and precise response, and with subsequent exposure, becomes more rapid and dynamic (Parkin and Cohen, 2001). The mucosal system is part of innate immunity and provides a physical line of defence against pathogens, serving to eliminate them along with several other protection mechanisms. The thin mucosal surfaces of the body are located at sites which are vulnerable to infection, such as the gut, eyes, vagina, nose, mouth, throat and uterus. A cell-to-cell network of epithelial cells produce antimicrobial peptides which are secreted into the mucosal lumen, these peptides are able to kill invading pathogenic bacteria (Nochi and Kiyono, 2006). The human body achieves homeostasis through recognition and elimination of harmful substances or stimuli, such as pathogens. During the removal of foreign invaders, there is a local response of inflammation, followed by damaged tissue reparation.

Inflammation can be characterised as the accumulation of fluid and blood cells, through vasodilation, which in turn causes oxidative stress and ultimately tissue damage (Hunter, 2012). Acute inflammation is a short-term process that occurs in response to tissue injury, it can appear

within minutes or hours and is characterised by the infiltration of leukocytes to the affected region, removal of the stimuli and repair of damaged tissue. Inversely, chronic inflammation is a prolonged, defective and deregulated response, involving a continued active inflammatory response, large amounts of oxidative stress and ultimately tissue destruction (Khansari et al., 2009).

Classic features of acute inflammation include vasodilation, exudation of plasma fluid and leukocyte migration into the site of injury, in some cases the coagulation cascade is also activated. The purpose of vasodilation is to facilitate the transport of inflammatory cells and soluble mediators to the location of injury (Sherwood and Toliver-Kinsky, 2004). Inflammatory-mediated vasodilation is facilitated by nitric oxide (NO) and vasodilatory prostaglandins. NO is produced from the metabolism of l-arginine, through endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS), of which, eNOS and iNOS are produced constitutively, (Sherwood and Toliver-Kinsky, 2004). Furthermore, histamine-induced arteriolar dilation is abrogated in mice which lack the eNOS gene, indicating the significance of this isoform (Pober and Sessa, 2015). PGD_2, PGE_2, PGF_{2a} and prostacyclin (PGI_2) are the primary vasodilatory prostaglandins, these lipid autacoids are derived from arachidonic acid (AA). Cyclooxygenase (COX) catalyses the conversion of AA to prostaglandin H2, prostaglandins facilitate the resolution and promotion of inflammation (Ricciotti and FitzGerald, 2011).

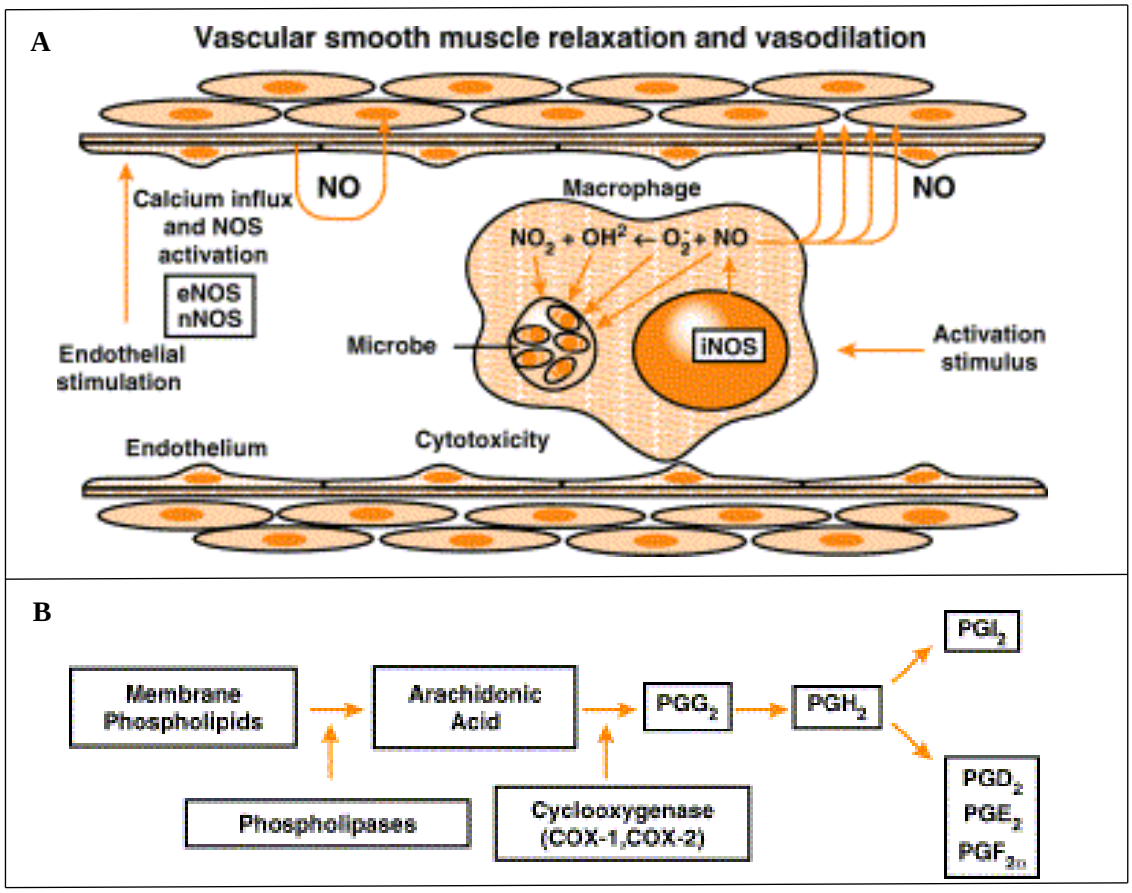

Figure 1.3 VSM relaxation and dilation (A) and the production of vasodilatory prostaglandins (B), composite diagram adapted from Sherwood and Toliver-Kinsky (2004). A, transcellular calcium fluxes regulate the expression of NOS synthase isoforms eNOS and nNOS, indirectly regulating NO production. NO causes VSM relaxation through a cyclic GMP-mediated mechanism, involving endothelial cell and smooth muscle cell cross-talk. B, phospholipase and COX are responsible for the production of the major vasodilatory prostaglandins; PGD_2, PGE_2, PGF_{2a} and PGI_2.

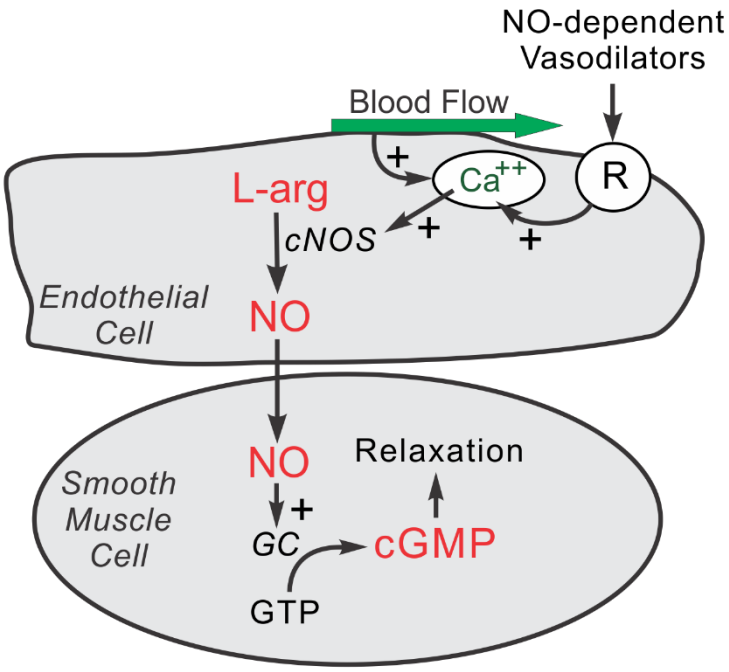

Figure 1.3.1 Cross-talk between endothelial cells and VSM, during vasodilation, adapted from John Dickenson, NTU (2017). L-arg; L-arginine, GC; guanylyl cyclase, GTP; guanosine triphosphate, cGMP; cyclic guanosine monophosphate, cNOS; constitutive nitric oxide synthase. cNOS is a calcium and calmodulin dependent NOS isotype, indicating the importance of calcium signalling in inflammation and thus allergic disease. Following NO binding to GC, cGMP is formed from dephosphorylation of GTP, cGMP acts as a second messenger, facilitating VSM relaxation.

Calcium is crucial for inflammation to occur or not occur, particularly its role is VSM relaxation as shown in figure 1.3.1. Calcium dependent cNOS facilitates NO production and VSM relaxation via cyclic guanosine monophosphate (cGMP). cyclic guanosine monophosphate induces VSM relaxation through multiple mechanisms; increased levels of intracellular cGMP has an inhibitory effect on calcium influx into VSM, thus reduces activation of myosin light chain kinase (MLCK) and therefore maintains 'active relaxation' of the smooth muscle (Carvajal et al., 2000; Lierop et al., 2001). Another mechanism in which cGMP causes relaxation of the VSM is through the activation of K^+ channels, which hyperpolarise smooth muscle, again inhibiting calcium entry into VSM and reducing muscle contraction (Dora and Garland, 2001). The final mechanism of cGMP mediated VSM relaxation is the stimulation of cGMP-dependent protein kinase, which activates myosin light chain phosphatase, leading to dephosphorylation of myosin light chains, inducing relaxation of VSM (Surks, 2007).

Relaxation of VSM allows migration of inflammatory cells to the site of infection or injury, such as white blood cells (WBCs), these inflammatory cells can migrate in the form of fluid exudate. Serous exudate is usually observed in mild inflammation and is formed of low levels of protein, macrophages, lymphocytes, and mesothelial cells (Sherwood and Toliver-Kinksky, 2004). The controlled inflammatory response from mild inflammation protects against infection and is beneficial for human survival, however, dysregulated and unwanted inflammatory responses to certain stimuli can be detrimental to health, such as septic shock.

Initially, the immune response by the innate immune system through the activation of pattern recognition receptors (PRRs), such as, Toll-like receptors (TLRs) and nucleotide-binding oligomerization-domain protein- like receptors (NLRs) (Ting, et al., 2010). Other families of PRRs include C-type lectin receptors (CLRs), Retinoic acid-inducible gene like receptors (RLRs) and NOD-like receptors (NLRs). TLRs detect antigens located on pathogens, called pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) released from damaged cells may also be recognised by PRRs (Takeuchi and Akira, 2010). Upon binding, various intracellular signalling cascades occur orchestrating a host response to infection (Mogensen, 2009). Proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and IL-6 are released, their role to modify vascular endothelial permeability, regulate migration of red blood cells to inflamed tissue and regulate apoptosis (Takeuchi and Akira, 2010). Interleukin-6 and TNF-α are regulated primarily at transcriptional and translational levels, whereas IL-1β is regulated via a two-step mechanism. Expression of pro-IL-1β mRNA is dependent on TLR signalling, whereas, independent of TLR signalling, caspase-1 is able to cleave pro-IL-1β into IL-1β. Caspase-1 is also responsible for the maturation of IL18.

Table 1.3. The four families of pattern recognition receptors (PRRs) and their various ligands; toll-like receptors (TLRs 1-11), retinoic acid-inducible gene (RIG) – receptors (RLRs) (RIG-I, MDA5, LGP2), nucleotide-binding and oligomerisation domain (NOD) – like receptors (NRL) (NOD1 and NOD2) and C-type lectin receptors (CLR) (Dectin-1, Dectin-2 and Mincle). Sin3A associated protein 130, SAP130; lipopolysaccharide, LPS; double-stranded ribonucleic acid, dsRNA; 5-c-phosphate-g-3 deoxyribonucleic acid, CpG-DNA; muramyl dipeptide, MDP. Table adapted from Takeuchi and Akira, 2010.

PRR	Localisation	Ligand	Origin of Ligand
TLR			
TLR 1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR 2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR 3	Endolysosome	dsRNA	Virus
TLR 4	Plasma membrane	LPS	Bacteria, viruses, self
TLR 5	Plasma membrane	Flagellin	Bacteria
TLR 6	Plasma membrane	Diacyl lipoprotein	Bacteria, Viruses
TLR 7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR 9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR 10	Endolysosome	Unknown	Unknown
TLR 11	Plasma membrane	Profillin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5'triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses
LGP2	Cytoplasm	Unknown	RNA Viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

Chemokines are also released following innate immune cell activation, following detection of an infection or tissue injury. Chemokines facilitate leukocyte extravasation from circulation to the designated area through GPCR activation (Newton and Dixit, 2012). T Lymphocytes play a central role in cell-mediated immunity, bearing antigen specific receptors on their surface for recognition of foreign pathogens, they may also recognise host antigens in cases involving autoimmunity. The two main subsets of T-lymphocytes, characterised by cell surface molecules presented, are known as $CD4^+$ T cells or T helper cells and $CD8^+$ T cells or T

regulatory cells. T helper (Th) cells or $CD4^+$ T cells are of great importance within the immune and inflammatory response and they regulate immunity through T cells subsets, innate immune responses and B-cells. T helper cells can further be divided into Th1 (type 1) and Th2 (type 2) cells, both producing specific cytokines (Berger, 2000). Homeostasis between both of these Th cell subsets is crucial for effective immune function. T helper-1 cells produce interleukin 2 (IL-2), interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α), which drive cell-mediated immunity, activating macrophages and protective phagocyte responses; eliminating cancerous cells and fighting viruses. Conversely, Th2 cells, involved in the adaptive immune response, produce IL-4, IL-5, IL-10 and IL-13; leading to eosinophil activation, antibody production and inhibition of macrophages, thus the Th2 response independent of phagocyte protective responses (Deo et al., 2010; Romagnani 2007).

Deregulation of the Th2 response is thought to lead to the development of allergy and asthma, due to increased production of pro-inflammatory cytokines, and thus hyper-responsiveness resulting in immune system dysfunction and a disruption in Th1 and Th2 homeostasis. The hygiene hypothesis has been proposed to enable this dysregulated Th2 response, implicating that lack of exposure to endotoxins and infection alters the Th1/Th2 balance (Barnes, 2001) (see section 2.1). The Th2 pathway is often believed to trigger atopy, allergy and immunoglobulin-E based diseases, however, the mechanisms leading to this imbalance have yet to be characterised (Deo et al., 2010).

Eosinophils play a significant role in allergic inflammation. During an inflammatory event increased cytokine and chemokine signalling lead to chemotaxis of eosinophils to the site of inflammation, they are one of the first cells present following activation of Th2 immunity, inducing airway inflammation by comigrating with other inflammatory cells (Barrett and Austen, 2009). Frequent degranulation leads to the release of cytotoxic products such as eosinophil cationic protein, eosinophil-derived neurotoxin, eosinophil peroxidase and major

basic protein. A mixture of cytokines and chemokines attributing to airway epithelial damage, oedema, over production of mucus and hyperresonsiveness are also produced (Bacharier et al., 1998; Groot et al., 2015).

2.2.2 Airway and Inflammatory Cells

A large variety of inflammatory cells have been implicated in asthma; however, it is uncertain how all of these cell types account for the pathophysiological features of this allergic airway syndrome. It is evident that not one single cell type is responsible for asthma, and that there is a complex mechanism involving interplay between inflammatory cells (Gosset et al., 1992).

Mast cells were first described by Paul Ehrlich in 1878 (Da Silva et al., 2014). They circulate as $CD34^+$ precursors until migration into various tissues where they mature into effector cells. Mast cells are considered multifunctional immune cells and are involved in various allergic diseases. In patients with allergic asthma, inhalation of an allergen results in IgE cross-linkage and mast cell degranulation, leading to rapid release of mast cell mediators, such as proteases, prostaglandins, leukotrienes and histamine (Murray et al., 1985). In asthmatics mast cells localise in the bronchial smooth muscle in bundles, unlike T cells or eosinophils, this factor is likely to be crucial in asthma phenotyping (Bradding et al., 2006). A subcategory of mast cells, MC^T cells, are found predominantly in the lung and store tryptase in their granula, an enzyme released alongside histamine and other chemicals (Reuter et al., 2010). Following release of these mediators vasodilation occurs allowing a late phase response, characterised by infiltration of $CD4^+$ T cells, neutrophils, eosinophils, mast cells and basophils, which induces non-specific AHR (Reuter et al., 2010).

The importance of IgE in mast cell degranulation has been highlighted throughout asthma research, a clinical correlation of IgE, asthma severity and bronchial hyper-responsiveness has been demostrated (Oettgen and Geha, 2001). Interestingly, anti-IgE therapy decreases

circulating IgE levels, but shows minimal clinical improvement in asthmatics who are steroid dependent. Although, anti-IgE therapy does permit a reduction in corticosteroid medication, which may be beneficial for long-term health concerns. Moreover, corticosteroids have little effect on IgE levels and may even augment circulating IgE levels (Kerstjens et al, 1996). Recent evidence suggests mast cells are activated by non-allergic triggers such as cytokines and neuropeptides, and it has been proposed that mitochondrial function may influence mast cell degranulation. Mast cells may also release proinflammatory cytokines, chemokines and growth factors; these findings have led to re-evaluation of mast cells and their role in asthma exacerbation (Barnes 2002).

Macrophages play a key role in immune function, orchestrating the inflammatory response through the production and release of a large range of cytokines. Derived from blood monocytes, macrophages are able traffic into target areas and can be activated by low affinity IgE receptors (FcεRII) (Poulter et al., 1996). Depending on the stimulus, macrophages have the ability to either augment or diminish inflammation. Alveolar macrophages suppress lymphocyte function in normophysiology, however, this function is impaired under asthmatic conditions after allergen exposure (Spiteri et al., 1994). Anti-inflammatory effects of macrophages include the secretion of IL-10 and inhibition of IL-5 secretion from T-lymphocytes, possibly through IL-12 secretion. However, these anti-inflammatory functions are decreased in patients with allergic asthma. Macrophages may also act as APCs (Underhill, 1999). Macrophages that favour inflammation are known as M1 type macrophages, conversely macrophages that specialise in tissue repair are known as M2 type macrophages (Mills 2012). Macrophage M1 cells express mainly proinflammatory cytokines, including TNF-α and IL-1. M2a cells produce IL-3 and IL-4, whereas, M2c cells produce primarily IL-10. Both M2a and M2c have roles in initiation, tissue remodelling and inflammation resolution in the different stages of asthma development (Jiang and Zhu, 2016). The polarisation of alveolar macrophages

(AM) induces either activation of their M1 or M2 type in allergic conditions, the microenvironment also affects the balance of M1/M2 cells through cytokines, chemokines, and immune regulatory cells. The alternative roles of macrophages in asthma may be of great relevance for future research, and intervention on a molecular level may therapeutic potential for different AM phenotypes (Jiang and Zhu, 2016).

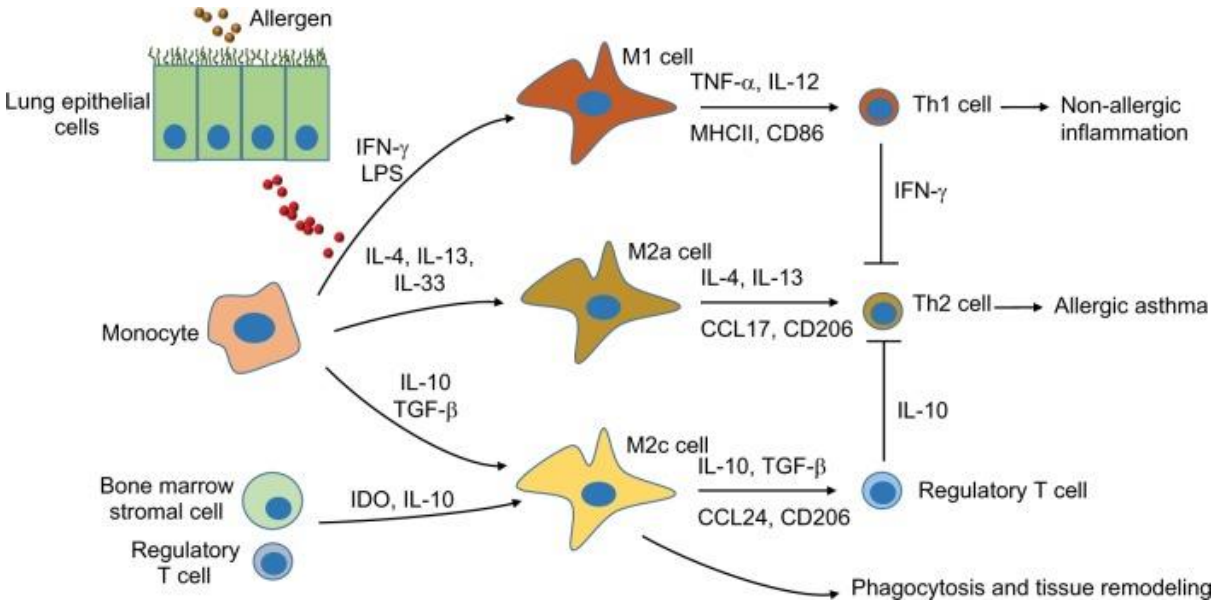

Figure 1.4. Schematic diagram: subtypes of alveolar macrophages (AM) following exposure to an allergen. Interferon gamma, IFN-γ; tumour necrosis factor alpha, TNF-α; interleukin, IL; transforming growth factor beta, TGF-β; T helper cell, Th; multi histocompatibility complex two, MHC II; cluster of differentiation 206, CD206; C-C motif chemokine ligand, CCL; lipopolysaccharide, LPS; M cell, macrophage. Classically activated M1 macrophages can be triggered via IFN-γ and LPS, leading to non-allergic inflammation through TNF-α and IL-12 secretion, and a Th1 cell response. Activated macrophages, or M2 macrophages, are divided into M2a and M2c cells. M2a cells are triggered via IL-4, IL-13 and IL-33, leading to release of IL-4 and IL-13 and a Th2 mediated allergic response. M2c cells induced by IL-10, TGF-β and IDK release IL-10 and TGF-β, thus activating Treg cells leading to inhibition of the Th2 response, similarly to the Th1 response. Figure adapted from Jiang and Zhu, 2016.

Dendritic cells (DCs) are similar to macrophages, however, they have a unique ability which enables them to activate T-lymphocytes and induce an immune response (Banchereau et al., 2000). In mild to moderate asthma dendritic cells facilitate an eosinophilic Th2-mediated response, as well as the Th17-associated neutrophilic response or both combined for a neutrophilic/eosinophilic inflammatory response, as observed in severe asthmatics (Vroman et

al., 2017). The DC family encompasses conventional DCs (cDCs) and plasmacytoid DCs (pDCs), within cDCs exists type 1 cDCs (cDC1s) and type 2 cDCs (cDC2s). In the Th2-mediated inflammatory response cDC1s been demonstrated to attenuate inflammation through production of tolerogenic antigens and mice deficient in CD103 were shown to have reduced allergen uptake in their dendritic cells (Agace et al., 2000). Moreover, cDC2s in asthma possess the ability to induce Th17-cell differentiation in the gut (Schiltzer et al., 2013) and Th2-cell differentiation in allergen-exposed lungs (Furuhashi et al., 2012). Plasmacytoid dendritic cells (pDCs) are able to produce considerable amounts of Interferon alpha (IFN-α) following TLR7 activation (Smit et al., 2006). Unlike other DCs, but similar to cDC1s, pDCs have a tolerogenic function in asthma, and are capable of Treg cell differentiation (Lewkowich et al., 2005). Various mechanisms are elicited by each subtype of DC, some contributing to pathogenesis of asthma and other responses supressing inflammation, indicating a pivotal role for DCs in asthma (Vroman et al., 2017). Moreover, activation of DCs requires the pro-inflammatory transcription factor NF-κβ, TNF-α interacting protein 3/A20 is a deubiquitinating enzyme which negatively regulates NF-κβ (Kool et al., 2011), a potential target in regulating DC-induced inflammation.

White blood cells (WBC) or leukocytes are derived from multipotent hematopoietic stem cells in the bone marrow, the five major types are as follows: neutrophils, lymphocytes, monocytes, basophils and eosinophils.

Eosinophils are multifunctional leukocytes that modulate immune responses via an array of mechanisms, in response to specific stimuli they are drawn to the site of inflammation. In vitro eosinophil migration has been shown to occur through two mechanisms, chemotaxis or directed migration and chemokinesis or random migration, depending on the stimulating cytokine or more specifically chemokine (Schweizer et al., 1996). Following migration, eosinophils may engage receptors for cytokines, immunoglobulins and the complement system, leading to the

release of a large range of proinflammatory cytokines such as: IL-2, IL-4, IL-5, IL-10, IL-12, IL13, IL-16, IL-18, transforming growth factor α or β (TGFα/β), lipid mediation (leukotriene C4 [LTC4] and platelet-activating factor) and chemokines (eotaxin-1 and RANTES) (Kita, 1996; Rothenbery and Hogan, 2006) . These molecules have a variety of roles, including adhesion system upregulation, regulation of cellular trafficking and permeability, constriction of smooth muscle and increased mucus secretion, all of which are proinflammatory responses. Eosinophils may also induce antigen specific responses through antigen presentation and subsequently release effector molecules, such as lipid mediators and granule proteins (Gleich and Adolphson, 1986; Rothenbery and Hogan, 2006).

In asthmatics, accumulation of eosinophils is facilitated by very late antigen-4 (VLA-4), which induces selective migration of eosinophils. Eosinophil adhesion to endothelial cells in the airways is also enabled through ICAM-1 and VCAM-1 protein expression (Pilewski and Albelda 1995). Interestingly, IL-4 has been demonstrated to enhance the expression of VCAM-1 on the endothelium (Lamas et al., 1988). Eosinophilic airway responses require certain growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5, without these factors eosinophils may undergo apoptosis. Clinically, inhibition of IL-5 with anti-IL-5 has been shown to reduce the number of eosinophils in circulation (Park and Bochner, 2010). Furthermore, airway infiltration of eosinophils during allergic inflammation is inhibited entirely following anti-IL-5 treatment, indicating both a unique and critical role for IL-5 in eosinophilic inflammation and AHR in asthma (Possa et al., 2013).

There has been substantial research on the role of eosinophils in allergic disease, and there has been less focus on neutrophils. This may be due to eosinophils being a more prominent cell type in mild to moderate asthma, whereas neutrophils appear to be more common in more severe asthma, with high levels detectable in induced sputum of severe asthmatics (Moore et al., 2013). Neutrophils undergo chemotaxis through the chemical signalling of IL-8,

Leukotriene B4, hydrogen peroxide, C5a and chemotactic peptide (fMLP) (Mayadas et al., 2013), interleukin-8 is elevated in asthmatic patients (Gao et al., 2017). Activated neutrophils are known to release reactive oxygen species (Szuster-Ciesielska et al., 2004), possibly contributing to the chronic inflammation seen in severe asthma. Conversely, AHR is not observed in asthmatic patients with augmented neutrophilic inflammation. It is widely accepted that airway inflammation is instigated via the Th2 response, whereas, neutrophil dominated inflammation can be characterised by low or absent Th2 Cytokines (Gao et al., 2017).

T Lymphocytes (T Cells) are involved in both the antigen specific and acquired immune response. Along with B cells, T cells are the only cells in organisms that are able to recognise and respond specifically when presented with an antigenic epitope (Anaya et al., 2013).

The development and maturation of T cells in mammals is a complex process, it begins in the foetal liver with haematopoietic stem cells (HSC), and HSCs then undergo differentiation into multipotent progenitors in the bone marrow. A subset of these multipotent progenitors instigates gene transcription of recombination activating gene 1 and 2 (RAG1 and RAG2) (Anaya et al., 2013), leading to the formation of common lymphoid progenitors (CLP). Only a fraction of these pluripotent cells migrate to the thymus where they differentiate into early thymic progenitors (ETP) and finally begin to develop into T cells, B cells, Natural killer cells (NKC), DCs and myeloid cells (Schwarz and Bhandoola., 2006).

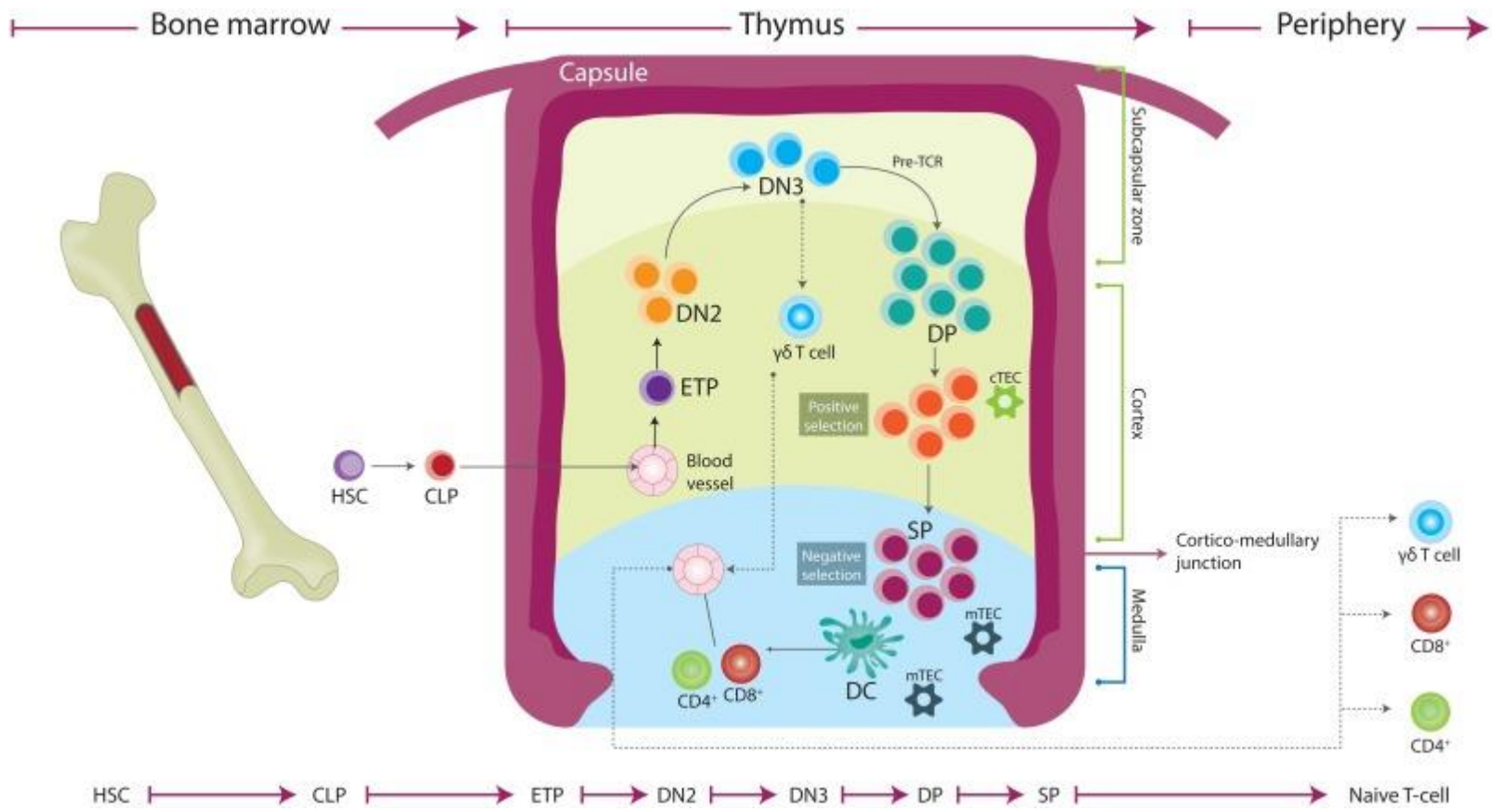

Figure 1.5. Adapted from Moore et al., 2008. An overview of maturation and development of T cells, from the bone marrow to the thymus to the periphery. Abbreviations: DC; Dendritic Cells, HSC; Haematopoietic stem cells, CLP; Common lymphoid progenitors, ETP; Early thymic progenitors, DN; Double negative, SP; Single positive, DP; Double positive, cTECs; Cortical thymic epithelial cells, m TECs; Medullary thymic epithelial cells.

Early thymic progenitors differentiate into double negative (DN) cells that are $CD4^-$ and $CD8^-$, they then differentiate again into DN2 cells and acquire $CD44^+$ and $CD25^+$ receptors, thus losing the potential to become B cells and altering their expressivity to allow cell receptor gene rearrangement (RAG1 & RAG2). Proteins required for T cell receptor (TCR) assembly and CD3 signalling are expressed at the DN3 stage, as well as kinases and phosphatases such as ZAP70, LAT and LCK (Rothenberg et al., 2008). Following TCR assembly, DN3 cells are capable of two differentiation paths, they may express γδ chains leading to the generation of γδ lymphocytes or αβ chains, generating αβ lymphocytes (Vantourout and Hayday, 2013). The former isoform is highly proficient in generating unique antigens, more so than the latter and B cells combined, yet the subpopulation of γδ lymphocytes is mainly comprised of specific subsets, which only recognise a limited number of antigens (Carding and Egan, 2002). Gamma-delta lymphocytes are much less prevalent in the blood than αβ lymphocytes, and are mostly $CD4^-$ $CD8^-$ compared to $CD4^+$ or $CD8^+$, respectively.

Table 1.4. Characteristics of αβ & γδ lymphocytes. Abbreviations: CTL, cytotoxic T lymphocytes; TCR, T cell receptor; iIELs, intestinal intraepithelial T lymphocytes. Table adapted from Carding and Egan, 2002.

CHARACTERISTIC	αβ T CELLS	γδ T CELLS
Antigen-receptor configuration	CD3 complex + αβ TCR	CD3 complex + γδ TCR
Theoretical receptor number	~10^{15}	~10^{20}
Antigen recognition	Peptide + MHC	Protein and non-protein
MHC restriction	Yes	Rare
Phenotype	CD4+ or CD8+	Most are CD4–CD8–; iIELs are CD8(αα)+
Frequency in blood	65–75%	1–5% (25–60% in gut)
Distribution	Blood and lymphoid tissues	Blood, epithelial and Blood and lymphoid tissues
Effector capability	CTLs (CD8+) and cytokine release	CTLs and cytokine release
Function	Immune protection and pathogen eradication	Immunoregulation and immunosurveillance

At the DN3 stage both CD4 and CD8 are expressed by TCRs expressing the β chain, thus becoming DP cells the largest subpopulation of the thymus (Bhandoola and Sambandam, 2006). Cortical thymic epithelial cells (cTEC) segregate these TCRs by presenting self-peptides for binding, Class I (HLA-I) and class II (HLA-II). Any DP TCRs unable to bind to the peptides

undergo apoptosis through the complement system (Noris and Remuzzi, 2013). Interestingly, during apoptosis there is little to no inflammatory response due to the swift removal of cellular fragments from the microenvironment by phagocytic cells (Francelin, 2012). Positive selection allows the differentiation of the DP thymocyte population into either $CD4^{-}$ $CD8^{+}$ or $CD4^{+}$ $C8^{-}$, depending on whether the DP cell recognises HLA-I or HLA-II, respectively (Klein et al., 2009). It is hypothesised that TCR signalling during thymocyte development determines the fate of the cell (Hogquist, 2001). Moreover, abundant high affinity/avidity self-peptides induce the clonal deletion of DP cells, whereas mature T cells that were previously positively selected, are able to survive and differentiate through a small number of low-affinity self-peptides that are presented by self-MHC (major histocompatibility complex) (Francelin, 2012; Starr et al., 2003).

Post positive selection, SP cells enter the medulla of the thymus, where negative selection occurs. Thymocytes are further 'tested' by the release of self-antigens presented by medullary thymic epithelial cells (mTEC) and DCs. Medullary thymic epithelial cells utilise a special epigenetic mechanism by which a certain variation of gene expression occurs, this contributes to low expression of specific genes, such as tissue restricted self-antigens; another name for this mechanism is 'promiscuous gene expression' (Anaya et al., 2013). Single positive cells with a high affinity/avidity for HLA-I or HLA-II are targeted for destruction in order to prevent possible auto-reactivity (Anderson and Takahama, 2012). Following negative selection, cells that survive mature to become naïve T cells and leave the thymus, migrating to the surrounding secondary lymphoid organs, it is here they gain a specialised phenotype and become effector cells. Growing evidence suggest T cell migration/motility is calculable and strategic (Krummel et al., 2016).

As aforementioned, during maturation T cells acquire a T cell receptor responsible for specific antigen (Ag) recognition. The TCR is a multiprotein complex composed of the two variable Ag-binding chains α&β or γδ, see figure 1.6. Each variation of binding chain associates with the invariant accessory proteins CD3γε, CD3δε and CD247ζζ , this is critical for TCR-Ag binding and signalling (Anaya et al., 2013).

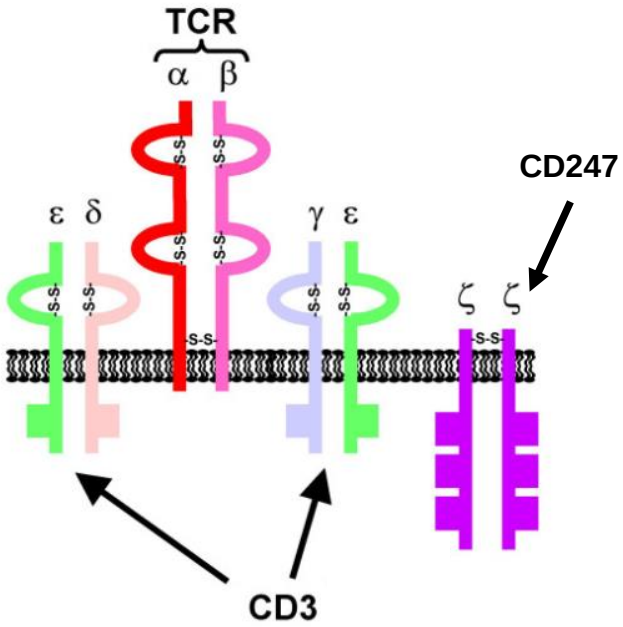

Figure 1.6. Adapted from De Felipe, 2004 (121). Abbreviations; T cell receptor, TCR; cluster of differentiation 3, CD3; cluster of differentiation 247, CD247. The TCR:CD3 complex interacts with the cytoplasmic membrane. The TCR is formed by 2 subunits, each unit secured via two di-sulphide bonds. The other four CD3 proteins assemble in 2 dimers, and the final protein forms CD247.

Alpha and beta chains are highly polymorphic, enabling a diverse recognition of peptides. However, these chains do not recognise Ag in the natural form, but do recognise HLA-I or HLA-II presented Ag. HLA-I molecules consist of 8-10 amino acids and originate intracellularly, whereas HLA-II molecules are composed of 13-25 amino acids and originate extracellularly (Zhang et al., 2017). Alpha and beta CDR-1 and CDR-2 regions of the TCR make contact with both types of HLA (Wucherpfennig et al., 2010), CDR3 α and β bind the central region of the peptide.

The arrangement of TCR genes is crucial in T cell maturation and development, in order to produce a functional TCR the correct DNA must bind and transcribe, β chain gene recombination occurs before the α chain and both processes occur independently. Post positive and negative selection the diversity of the human TCR is estimated at $2x10^7$ (Turner et al., 2006). The TCR recognises short peptide fragments presented by either MHC class I or class II (peptide-MHC complexes) and gene arrangement of the TCR determines antigen specificity. Activation of naïve T cells will only occur if three signals are activated collectively: pMHC-TCR interaction, co-stimulatory signalling, and cytokine initiation of clonal expansion.

After antigen recognition and the subsequent signalling cascade, as shown in Figure 1.8, three vital transcription factors are activated NF-κβ, AP-1 and NFAT. Gene expression and T cell regulation occurs through three mechanisms; the PIP2, DAG and RAS signalling pathway, one for each transcription factor.

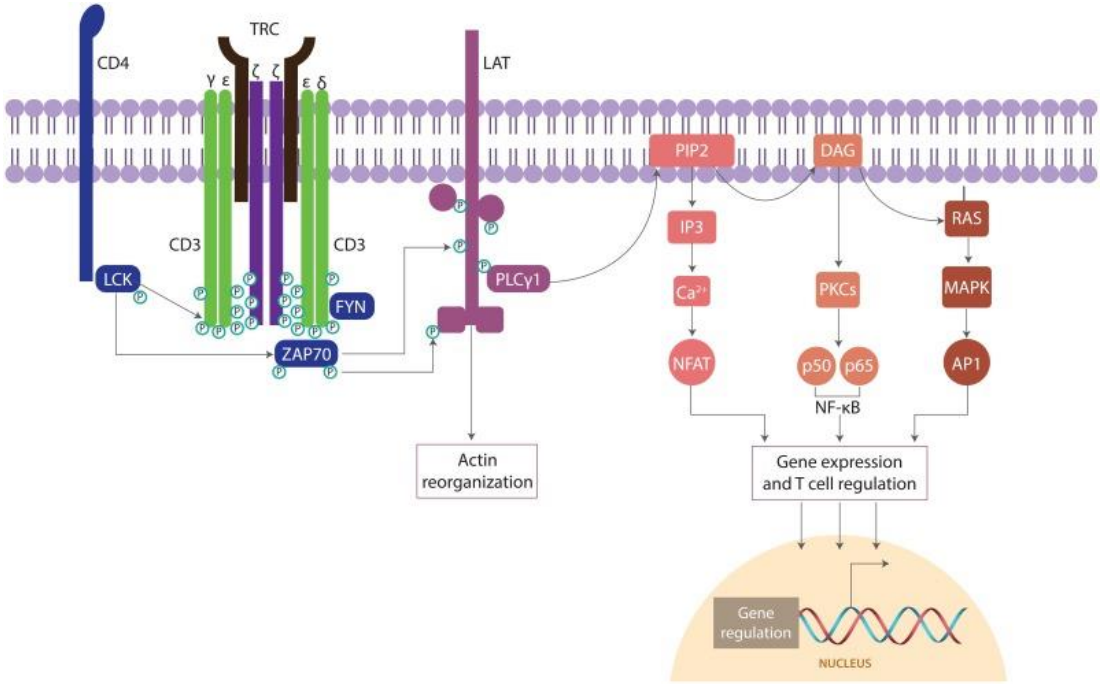

Figure 1.7. Adapted from Brownlie et al., 2013. Abbreviations: LCK, Lymphocyte-specific protein tyrosine kinase; FYN, Src tyrosine kinase; ZAP70, ζ-chain associated protein kinase; LAT, Linker for activation of T cells; PIP2, phosphatidylinositol; PLCγ1, phospholipase C γ1; NFAT, nuclear factor of activated T cells; IP3, inositol triphosphate; PKC, protein kinase C; NF-κβ, nuclear factor kappa beta; DAG, diacylglycerol; RAS, GTP-binding protein; MAPK, mitogen-activated protein kinase and AP1, activator protein 1 transcription factor.

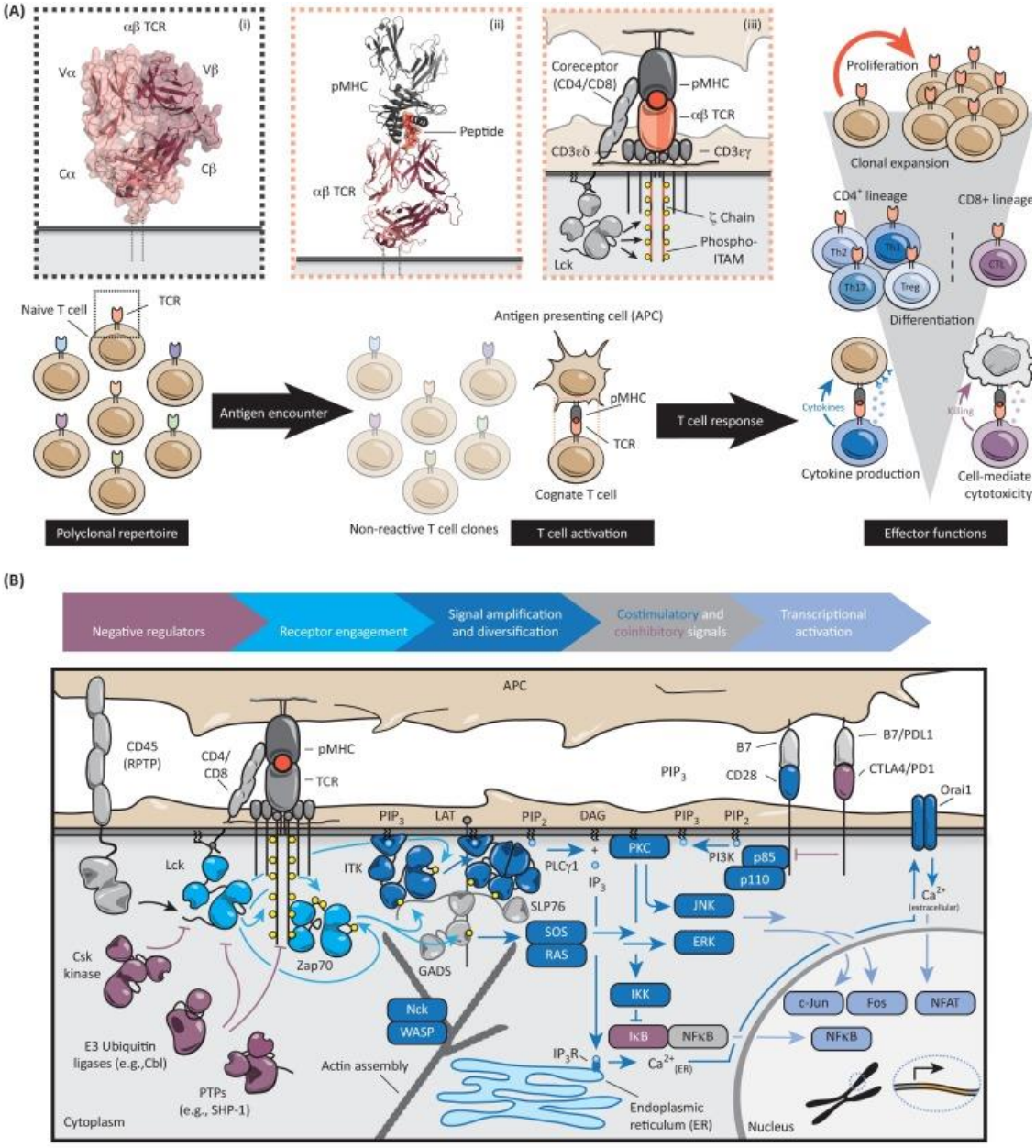

Figure 1.8. Composite overview of TCR antigen recognition. (Ai) TCR Ag-binding chains α&β, the most predominate isotype of TCR. (ii) pMHC-αβ TCR complex, a unique TCR binds to a peptide presented by MHC-I or II. (iii) pMHC- αβ TCR complex, including co-receptor (CD4/CD8) binding and CD3γε, CD3δε chains. TCR binding leads to activation of naïve T cells followed by expansion and differentiation. Effector subsets then utilise their specialist functions to facilitate the adaptive immune response. (B) Each CD3 chain has at least one copy of a signally motif or Immunoreceptor tyrosine based activation motif (ITAM) in its cytoplasmic domain. Various Src Tyrosine kinases associate with ITAMs, such as Fyn and Lck. CD45 is another important molecule in T Cell activation, its cytoplasmic domain holds a tyrosine phosphatase enzyme. Either CD4 or CD8 acts as a co-receptor to the TCR, and binds to its cytoplasmic domain and Tyrosine kinase, Lck. ZAP70, a cytosolic enzyme which facilitates T cell activation. Once TCR-pMHC binding occurs, CD45 activates Fyn through the removal of inhibitory phosphate groups. The activated Fyn kinase then phosphorylates ITAMs on each CD3 chain, allowing the ZAP70 kinase to bind to the phosphorylated CD3ζζ chain. The co-receptor, for example CD4, is capable of binding to the pMHC. This leads to the movement of Lck into close proximity with ZAP70, resulting in Lck phosphorylation and ZAP70 activation. ZAP70 then binds to and activates adaptor proteins, such as LAT, allowing a variety of intracellular signalling mechanisms to follow (Gaud et al., 2018)

Referring to Figure 1.7, NF-κβ activation occurs through the DAG signalling pathway, in this pathway PKC activation leads to the recruitment of the IKK kinase, IKK requires Bc110, MALT1 and Carmal proteins for activation. IKK enables phosphorylation of the IkB inhibitors, allowing translocation of NF-κβ into the nucleus and gene transcription (Siebenlist et al., 2005). NF-κβ stimuli include TCR, BCR, LPS, dsRNA and ROS; NF-κβ target genes include VCAM, ICAM, MHC I, TCRα, β, IkBα, p105, c-Rel, Bcl-XL and IAPs (Oeckinghaus and Ghosh, 2009).

Each transcription factor, NF-κβ, NF-AT and AP-1 promote IL-2 secretion, IL-2Rα expression, cellular adhesion integrins such as CD40L and the expression of various anti-apoptotic proteins involved in death phase regulation (Anaya et al., 2013; Siebenlist et al., 2005). On the surface of the T cell CD25 binds IL2R β and γ chains, resulting in the augmentation of affinity for IL-2 10-fold (Boyman and Sprent 2012). Interleukin-2 is capable of T cell clonal expansion, resulting in a large number of T cells with specific receptors identical to that of the original cell cloned. Furthermore, a pivotal role for IL-15 and IL-21 has been demonstrated in this expansion by enhancing Ag activation, IL-21 has also been demonstrated to promote and sustain $CD8^+$ T cell numbers in mice (Moroz et al., 2004). Following clonal expansion is a death phase, where 90% of the effector cells undergo apoptosis, mediated though the tumour necrosis factor receptor 1 (TNFR-1) pathway, Fas-FasL and CD40-CD40L interactions (Caulfield and Lathem, 2014; Gupta et al., 2005).

Co-stimulatory molecules possess the ability to amplify or diminish TCR complex signalling, indicating their central role in immunological regulation. The expression of these molecules is diverse in both structure and function, and for the most part is context-dependent (Chen and Flies, 2013). Signal modulation by co-stimulation is complex and multileveled, a good example of this is the CD28-B7 co-signalling paradigm. In the two-signal hypothesis it is essential there is both Ag and secondary stimuli for T cell activation (June et al., 1987). Co-stimulatory receptor CD28 is able to bind its ligand B7-1 for signalling, co-inhibitory receptor

cytotoxic T lymphocyte antigen 4 (CTLA4) is also able to bind this ligand, as well as B7-2 (CD86). Constitutive expression of CD28 exists on the surface of naïve $CD8^+$ and $CD4^+$ T cells (Rudd et al., 2009) and following activation is the induction of ICOS and OX-40 (Chen and Flies, 2013). For signalling to occur, B7-1 or B7-2 must bind one of these co-stimulatory receptors on an APC. Following Clonal expansion, co-stimulation, and an immune response, CTLA4 is upregulated and CD28 downregulated by endocytosis (Rudd et al., 2009), resulting in suppression of T cell responses. Modulation of B7-1 & B7-2 expression occurs at low levels by APCs through their constitutive expression of B7-2 to diminish signalling (Hathcock et al., 1994).

Differentiation of $CD4^+$ T cells into specific populations or phenotypes is determined by cytokine microenvironments, concentration and type of Ag, type of APC and its state of activation, levels of co-signalling molecules present and other factors (Anaya et al., 2013). T cells expressing CD4 or T-helper cells (Th cells) are responsible for cytokine production and B cell stimulation for antibody assembly and mobilisation. Six different subsets or phenotypes of Th cells have been identified, Th1, Th2, Th9, Th17, Th22, T cell follicular helpers (Tfh) and regulatory T cells (Treg) (Golubovskaya and Wu, 2016). Many of these Th cell subsets have important roles in inflammation and immune regulation.

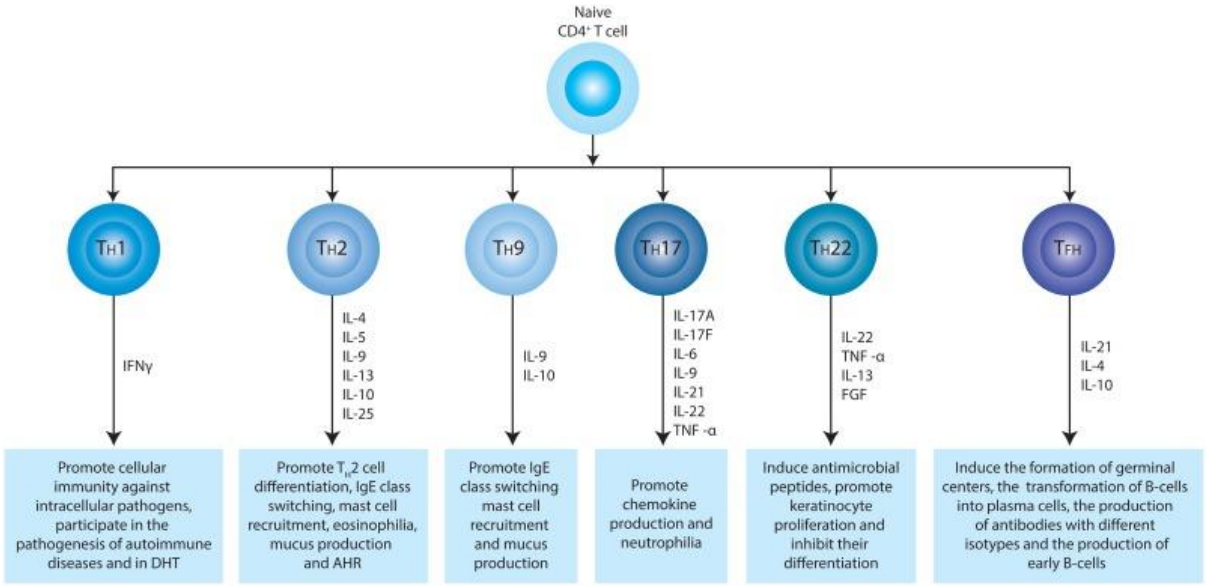

Figure 1.9. CD4 T Cell phenotypes (Lloyd et al., 2010). Abbreviations. TNF-α, tumour necrosis factor alpha; AHR, airway hyperresponsiveness; DTH, delayed hypersensisitivty; FGF, fibroblast growth factor; IFNγ, interferon γ; TGF-β, transforming growth factor beta 1. Regulatory T cells release IL 10 and TGF-β leading to activation of cytotoxic T cell and macrophages as well as B cell secretion of antibodies.

Th1 cell differentiation is initiated by IL-18, IL-12 and type 1 IFN-α & β, these cytokines are secreted by macrophages and DCs following activation by intracellular pathogens. Interleukin-18 signalling leads to the potentiation of IL-12 and augments Th1 phenotype development, IL-18 also regulates the Th2 response, and the overproduction of IL-12 and IL-18 can result in severe inflammatory disorders (Nakanishi et al., 2001). The Th1 response is regulated through the transcription factors STAT4 and T-bet, both are required in order to determine Th1 fate and expressivity (Thieu et al., 2008). Interleukin-2 has been demonstrated to promote longevity of the Th1 subtype along with IL-12Rβ2 (Boyman and Sprent, 2012). T-helper-1 cells produce IL-2, IFNγ, IFNα and IFNβ, they also express the chemokine GPCR gene for IL8, CXCR1 and the transmembrane protein CD161 (Fergusson et al., 2011; Takata et al., 2004).

The Th2 response is induced by allergens and pathogens, hence its importance in asthma pathophysiology. The response is initiated through the secretion of IL-4, IL-11 IL-25 and IL-33 from eosinophils, mast cells and natural killer cells (Fort et al., 2001), followed by activation of GATA-3 and STAT-6 leading to secretion of Th2 cytokines. Moreover, activation of target

genes also results in the augmentation of CCR4 and ICOS expressivity (Ho et al., 2009; Paul et al., 2010; Yoshie and Matsushima, 2014). Th2 cytokines have been studied intensely in recent years in hopes to provide new treatment opportunities for inflammatory disease, including IL-4, IL-5, IL-9 and IL-25 (Barnes, 2001).

Specific Th2 signalling is necessary for B-lymphocytes to switch and produce IgE, mediated by IL-4 and IL13 and CD40 linkage (Poulsen and Hummelshoj, 2007). Following IgE switching, innate immune cells are activated, and mast cells undergo degranulation leading to the release of proteases, cytokines, chemokines, serotonin and histamine (Amin, 2012). These molecules are responsible for contraction of smooth muscle, inflammatory cell migration and an increase in vascular permeability. Furthermore, Th2 cells migrate to both intestinal tissue and the lungs where eosinophil recruitment occurs. Eosinophil recruitment to the airways involves both IL-5 and IL-13, and mast cells are recruited through IL-9 (Pope et al., 2001). Moreover, IL-2 induces IL-4Rα expression and aids conservation of the Th2 phenotype by preserving accessible gene configuration (Boyman and Sprent, 2012).

In asthma, Th2 cells act on smooth muscle to induce mucus production, goblet cell metaplasia and airway hyper-responsiveness. Thus, possibly altering mucin 5AC expression (MUC5AC), a protein-coding gene is linked to hypersecretion of mucus in the airway, specifically chronic obstructive pulmonary disease (Wang et al., 2012). MUC5a is known to be regulated by proinflammatory cytokines IL-1β and IL-17A, both of which are involved in the NF-κβ signalling pathway (Fujisawa et al., 2009).

T-helper-9 cell development is facilitated by TGFβ and IL-4. Th9 cells produce IL-9 and for activation require the transcription factor signal transducer and activator of transcription 6 (STAT6), GATA4, and interferon response factor 4 (IRF4) (Veldhoen et al., 2008). Furthermore, Th9 cells have been demonstrated to produce IL-10 and IL-21, however, their

role in Th9 cellular function has not yet been characterised (Dardalhon et al., 2008). The function of IL-9 has been reviewed extensively in recent years, receptor-signalling leads to downstream activation of STAT1 and STAT5 homodimers, as well as STAT1 and STAT3 heterodimers, resulting in the activation of a series of signalling pathways (Kaplan, 2013). Similar to Th2 in asthma, IL-9 stimulates the release of mast cell products through IL-5 and IL-13, indirectly leading to mucus production, epithelium hyperplasia, contraction of smooth muscle and eosinophilia (Lloyd and Hessel, 2010).

Induced sputum of severe asthmatics has been shown to contain high levels of neutrophils (Jatakanon et al., 1999), Th17 cells, a distinct $CD4^+$ T helper cell subset, are known for the secretion of IL-17, which leads to recruitment of neutrophil granulocytes to the lungs. This recruitment is permitted primarily through CXCL8 (IL-8) production (Pelletier, 2009), and indirectly through the production of IL-6, colony stimulating factors (CSFs) and the chemokines CXCL8, CXCL1 and CXCL5, the latter released from airway epithelial cells (Chesné et al., 2014). Furthermore, IL-17 and Th17 cells may contribute to severe asthma pathophysiology, however, the exact role has yet to be characterised.

Th22 cells are a very specific subset of T helper cells, they are characterised by the secretion of the IL-22 cytokine, and the absence of IL17, IFN-γ and IL-4 secretion (Eyerich and Eyerich, 2015). The primary function of the Th22 subset is protection of the lung and skin epithelial tissue, as well as the regulation of tissue injury and inflammation.

IL-22 function is dependent on the local microenvironment, and cross-talk with other signalling molecules. In the absence of IFN-γ, IL-22 induces migration and proliferation in epithelial cells and inhibits differentiation and reactivity to apoptosis. Additionally, IL-22 has been shown to diminish T cell-mediated cytotoxicity through inhibition of IFN-γ induced MHCI/MHC-II expression (Pennino et al., 2013). Moreover, IL-22 with TNF-β and IL-17 signalling induces

strong innate immune responses (Eyerich and Eyerich 2015). It is probable that Th22 cells act independently of allergen exposure, and are responsible for specific tissue remodelling processes during the pathogenesis of disease. This has been demonstrated in asthma models; however, in the initial phases of asthma inflammation, Th22 cells may have a protective effect on epithelial tissue (Johnson et al., 2013).

T follicular helper cells function as B cell helpers, enabling the development of high affinity antibodies and memory B cells. Maturation of Tfh requires B cell lymphoma 6 (Bcl6), there is still much to be studied regarding Tfh in cell biology and especially its role in allergy and inflammation (Crotty, 2014). A recent study has shown that Tfh cells and their associated markers may have a role in pathogenesis of chronic bronchial asthma, notably through significantly increased BCL-6 in mice, further research is required to elucidate the function of Tfh cells in asthma (Ma et al., 2017).

Regulatory T cells are an important 'cousin cell' of $CD4^+$ T cells, they are responsible for the regulation and balance of systemic inflammation. Tregs can be characterised as $CD4^+$ $CD25^+$ cells and express the IL-2α receptor. However, the defining property of Tregs is the expression of Foxp3, a nuclear transcription factor crucial to Treg development and function. High levels of Foxp3 expression lead to the conversion of $CD4^+$ T cells into Treg cells expressing IL-10, a potent anti-inflammatory cytokine (Iyer and Cheng, 2012). These levels of Foxp3 remain high, possibly in order maintain Treg identity through TGF-β, TCR IL2Rα. Conserved noncoding sequence 2 (CNS2), a Foxp3 gene enhancer located in the introns, maintains this high level of Foxp3 throughout Treg cell life (Zheng et al., 2010). Classification of Tregs is controversial as their suppressive functions are not exclusive and may be elicited by other Th cells such as Th1, Th2, Th17 and Tfh cells (Corthay, 2009). However, in a recent T cell study, 300 base pairs (bp) of approximately 160,000bp DNA methylated regions were demethylated and utilised specifically in Treg cells, indicating a possible epigenetic mechanism in Treg cell development

and maturation (Morikawa et al., 2014). These Treg cell specific demethylated regions (TSDRs) are common in Foxp3, CTLA4 and IL-2Rα genes (Li and Zheng, 2015).

Treg functions that may be important in asthma pathophysiology include Treg cell ability to supress allergy and asthma, regulation of effector cells in the immune response and suppression of T cell activation by weak stimuli (Alpan et al., 2004; Baecher-Allan et al., 2002; Curotto et al., 2009). Treg cells can engage with macrophages via CTLA4-CD80/CD86, inhibiting the secretion of pro-inflammatory cytokines such as IL-1, TNFα and IL6, while also indirectly inhibiting activation of other $CD4^+$ T cells by macrophages. Additionally, Treg cells are able to compete with other $CD4^+$ T cells for IL-2, and able to secrete anti-inflammatory cytokines IL-10 and TGFβ. Furthermore, Treg cells can cause destruction of other T cells via the release of perforin and other granzymes (Cao., et al, 2007).

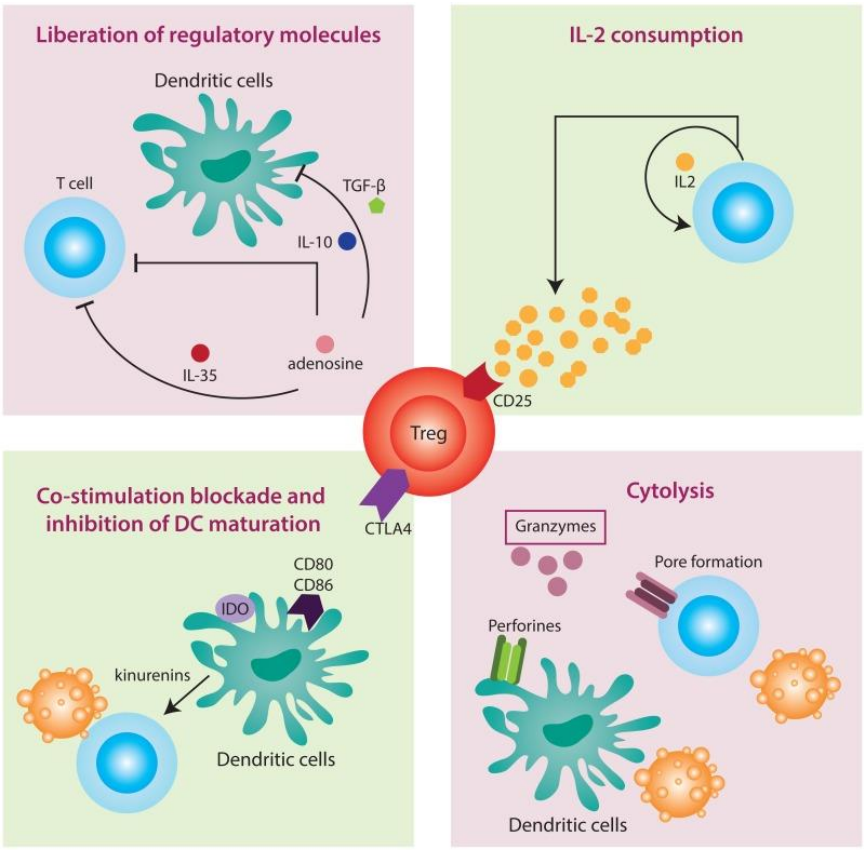

Figure 2.0. An overview of Treg mechanisms and function. Abbreviations, CTLA4: cytotoxic T-lymphocyte antigen 4, TGFβ: Transforming growth factor beta.

The imbalance of Treg cells may be a key component of asthma pathogenesis, however, the topic is convoluted and needs more research. To better understand how Treg development, function and proliferation relates to allergic disease we must improve our understanding of the phenotypic characteristics of Tregs. Such as our knowledge of the five Treg subtypes: thymus-derived Tregs (tTregs) and the peripheral regulatory T cells (pTregs), both $Foxp3^+$, and $Foxp3^-$ subtypes, Tr1, Th3 and $CD8^+$ Tregs (Orozco et al., 2017; Yu et al., 2018).

$CD8^+$ T Cells, once developed are converted into cytotoxic T killer cells (CTLs). These CTLs can induce cellular apoptosis through the liberation of certain cytolytic granules and expression of death receptors. Cytolytic granules utilised by CTLs include proteases, granzymes and granulolysins, which degrade the plasma membrane, and pore-forming proteins or perforins (Anaya et al., 2013). The FasL death receptor is also expressed on $CD8^+$ T cells, and inhibitors of perforins such as, calreticulin and cathepsin G protect the T cell from autolysis (Lieberman, 2003). Dysregulated $CD8^+$ T cell numbers may result in over activity of CTLs and the hyper-production of membrane pores by perforins, leading to osmotic imbalances and the production of ROS, this may contribute to cellular inflammation and damage observed in asthma. Interestingly, asthmatic subjects with the largest decline in lung function were shown to have the highest $CD8^+$ T cell numbers, however, more research is needed on the topic to define a function for $CD8^+$ T cells in asthma (Baraldo et al., 2016)

2.2.3 Cytokines in Asthma and Inflammation

The definition of the word ‘cytokine’ is of Greek origin, ‘kytos’ meaning ‘hollow’ or ‘vessel’ and ‘kinein’ meaning ‘to move’. They are small-secreted peptides and proteins released by various cells types and have a specific role in cell-cell interaction and communication (Zhang et al., 2007). Cytokines have an array of cellular functions including chemotaxis (chemokines),

proliferation, immunomodulation, activation and release of other cytokines or mediators, cellular growth and differentiation, and apoptosis (Foster, 2001).

Cytokines may act on the same cells they are secreted by this is known as ‘autocrine action, if acting on nearby cells and distant cells, it is known as paracrine and endocrine action, respectively. The production and expression of cytokines is highly regulated, therefore they are not stored in cells, with some exceptions, such as TFG-β and platelet-derived growth factor (PDGF). Expression is usually transient and only occurs once a cell is activated by a specific signal. As mentioned, cytokines have an array of cellular functions, eliciting effects to a large variety of cell types. Different cytokines have been demonstrated to overlap in function and activity, acting on a single cell type but showing identical responses, this paradigm is described as ‘cross-talk’. Because of this functional overlap, one factor may compensate for the lack of another factor, however, due to the ubiquitous nature of cytokines it is difficult to study and assess cytokine significance in normophysiology (Foster, 2001).

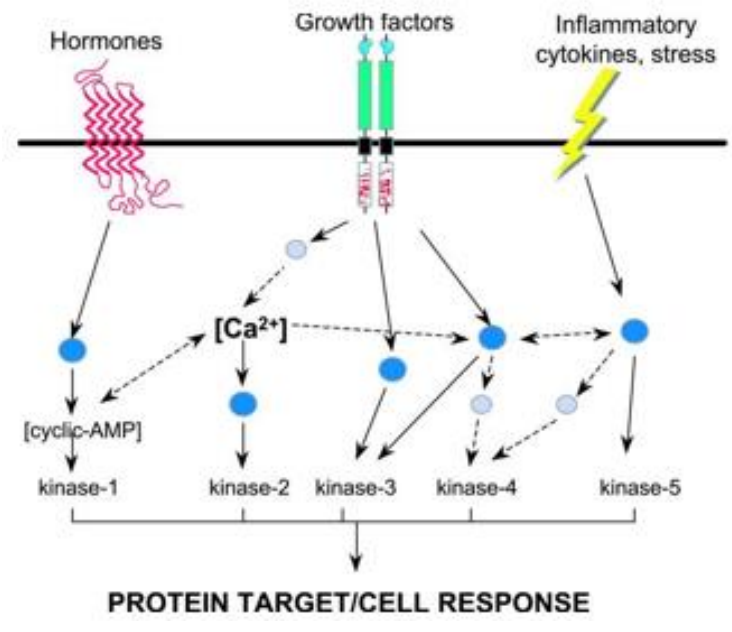

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Figure 2.1. The cross-talk paradigm. Dotted arrows indicate where cross-talk may take place, increases in intracellular calcium ([Ca^{2+}]) and cyclic-AMP leads to cross talk between ligand-receptor interactions and phosphorylation of specific kinases. Circles represent intermediate messengers

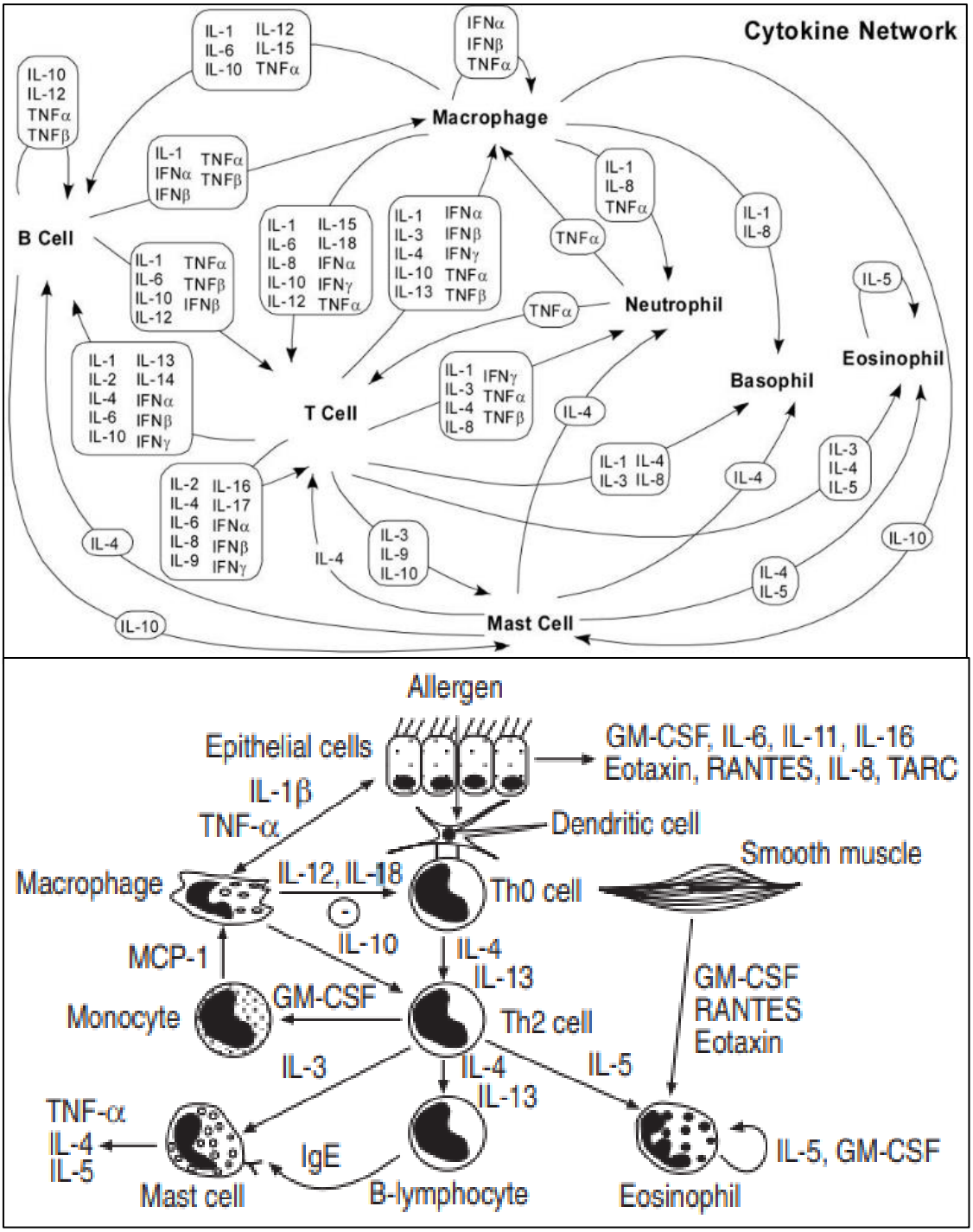

Figure 2.2. Adapted from Zhang and Jianxiong, 2009. **Top:** The cytokine network, various cell types and their intracellular signalling in the immune system. Macrophages present foreign antigen on their cell surface in order to activate B and T cells through specific antigen responses, leading to the secretion of cytokines. Activation and proliferation of neutrophils, eosinophils and basophils, in response to cytokines. **Bottom**: The cytokine network in asthma and how the inflammatory response is orchestrated in response to an allergen. Abbreviations. GM-CSF: granulocyte-macrophage colony-stimulating factor; RANTES: regulated on activated T-cell express and secreted; MCP: monocyte chemotactic protein; TARC: thymus and activated regulated chemokine; EGF: endothelial growth factor; FGF: fibroblast growth factor; IGF: insulin-like growth factor.

Cytokines can be either pro-inflammatory or anti-inflammatory; they may act in a synergistic manner or in an antagonistic way. Usually cytokines are produced and released in a cascade and orchestrate a specific yet complex response to a stimuli.

The pathogenesis of asthma can be described as a two-step process, the first step consisting of Th2 cell development and the second step, Th2-driver allergic airway inflammation. In order to achieve this inflammation a complex network of interactive cytokines are release from inflammatory cells, epithelial cells, smooth muscle cells and fibroblasts. Following the release of these cytokines and chronic inflammation, tissue remodelling may occur, leading to structural alterations (Kips, 2001). Th2-like cytokines including IL-4, -5, -9 and -13, and pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and IL-1β play a major role asthma.

In mice, viral-induced asthma exacerbations induced Th1 and Th2 type neutrophilia and inflammation, respectively, this was associated with IL-1β secretion, a pro-inflammatory cytokine. It was demonstrated that IL-1β signalling was essential for the upregulation of MUC5a, IL-33 and neutrophil chemotactic factors, indicating a role in airway inflammation (Mahmutovic Persson et al., 2018). Certain studies have also investigated genetic polymorphisms in both IL-1β and IL-1Rα that may augment susceptibility to asthma in various populations. A meta-analysis was completed using these studies, and no association was found between the IL-1β (-511C/T) mutation and asthma risk. However, an association was found between IL-1Rα and asthma risk, specifically in Caucasian populations (He et al., 2015). IL-1Rα activation leads to downstream stimulation of transcription factors NF-κβ and activating protein (AP)-1, followed by translocation and activation of specific genes that induce pro-inflammatory cytokines and chemokines. Thus, IL-1α/β and IL-1Rα interaction and regulation is important in asthma and airway inflammation (Mao et al., 2000).

Interleukin-4 can be described as the main cytokine involved in allergic responses and asthma; it is able to stimulate mucus-producing cells, possibly leading to increased expression of Muc5a, as aforementioned (see section 2.2.2). Furthermore, IL-4 stimulates fibroblast production, indicating a possible role airway remodelling in asthma pathogenesis (Doucet et al., 1998; Karim et al., 1999) (Inhalation of human IL-4 recombinant has been demonstrated to induce eosinophilia of the airways in atopic asthma. Additionally, bronchial biopsies have shown increased IL-4 mRNA and protein in the airway mucosa of atopic and some non-atopic asthmatics in comparison to healthy controls (Bradding, 1994). IL-4 binds to the cell surface receptor IL-4R, expressed on a large range of cell types. Two chains form a heterodimer to allow signalling, an IL-4Rα chain and a second γδ chain, which also forms part of the IL-2, -7, -9 and -15 receptors; the IL-4Rα may also form a heterodimer with the IL-13Rα chain (de Vries, 1998). Polymorphisms have been reported in asthmatic IL-4 and IL-4Rα genes, indicating a possible role of epigenetics in cytokine function. Specifically, the IL-4-589T allele, associated with IL-4 gene expression, may have a role in asthma severity. Furthermore, the epithelium of asthmatics express higher, abnormal levels of the IL-4Rα chain (Sandford et al., 2000). The signalling pathway mediating IL-4 responses appears to be STAT-6; and in studies where mice are STAT-6 deficient, IL-4 induced airway eosinophilia, IgE production and AHR are reduced, due to a lack of IL-4 production (Kips, 2001).

Despite its redundancy with granulocyte macrophage colony-stimulating factor (GMCSF) and IL-13, IL-5 appears to be a crucial cytokine involved in eosinophil production, maturation and activation. Recombinants of human IL-5 have been demonstrated to induce eosinophilia in asthmatics, similarly to IL-4 (Shi et al., 1998), while also increasing sputum eosinophil counts and AHR. Positive correlations between IL-5 and asthma severity have also been reported, both at the protein and mRNA level. Interestingly, expression of membrane-bound IL-5Rα chain has been shown to inversely correlate with baseline FEV_1, on the other hand soluble IL-5Rα

chain correlates positively with FEV_1 (Yasruel et al., 1997); ultimately IL-5Rα upregulation may influence eosinophil response.

Increased levels of IL-13 are present in the airways of asthmatics, and its biological functions are like that of IL-4. This is suggested by the sharing of receptor chains, specifically, IL-4Rα binds to IL-13Rα 1 or 2 for signalling. Additionally, both receptors and cytokines are dependent on STAT-6 and GATA-3 pathways (Kips, 2001). Contrary to the redundancy of both IL-13 and IL-4, and their similarities, they both have distinct functions during a Th2 response. IL-13 fails to induce Th2 differentiation following the allergic response, and this is due to a lack of IL-13Rα. Interleukin-13 is thought to have a larger role in secondary Ag exposure, being of importance in IgE synthesis and proliferation, specifically when levels of IL-4 are low (de Vries, 1998). Moreover, recent studies have shown IgE-driven allergies may be best treated via dampening IL-4 production over IL-13(Bao and Reinhardt, 2016). Furthermore, in vitro, IL-13 has been shown to have anti-inflammatory activities and induce lung fibrosis, leading to inhibition of proinflammatory cytokine and chemokine production, and induction of TGFβ1 expression in macrophages (Bou-Gharios and de Crombrugghe, 2008; Punnonen et al., 1999).

In asthma, dysregulated Interleukin (IL)-33 leads to the activation of Th2 cells, eosinophils, basophils, dendritic cells and mast cells, and therefore increases expression of inflammatory cytokines and chemokines (Borish and Steinke, 2010). The exact relation between IL-33 and asthma has not yet been characterised, however, higher levels of IL-33 have been detected in asthmatic patients and it has therefore been suggested as a unique therapeutic target (Momen et al., 2017).

TNF-α is a pro-inflammatory cytokine that acts on TNF-α receptor 1 and 2 (TNFR1 & TNFR2), intracellular signalling leads to NF-κβ activation and transcription. Anti-TNF therapy has shown promise in subjects with asthma, following treatment improvements in quality of life,

lung function and airway hyper-responsiveness has been observed. The mechanism as to how TNF-α elicits its' effects in asthma has not yet been fully elucidated; TNF-α has been proposed to have a direct effect on smooth muscle, through the release of cysteinl-leukotriens LT_{D4} and LT_{C4}. Dysregulation of mast cell mediators also contributes to airway pathophysiology, and TNF-α can directly induce histamine release from mast cells (Cocchiara et al., 1997). Both mechanisms discussed are thought to work synergistically, mast cell/smooth muscle interaction, resulting in bronchoconstriction and AHR (Berry et al., 2007). Moreover, an important factor involved in TNF-α function and signalling is the transcription factor NF-κβ, which may play a vital role in asthma pathogenesis. The TNF signalling pathway and subsequent NF-κβ activation is demonstrated in figure 2.2.1.

As previously discussed, NF-κβ is a protein complex which exists in two heterodimers or classes, it has a crucial role in immune regulation through gene transcription, and dysregulation of this factor is linked to various autoimmune diseases, including cancer and asthma. In asthma, corticosteroid treatment inhibits NF-κβ involvement in a plethora of inflammatory mechanisms, ligand bound glucocorticoid (GC)-receptors (GRs) are able to directly bind NF-κβ supressing its function. Increased nuclear presence and binding of NF-κβ has been detected in the induced sputum of asthmatics, furthermore, mice which lack IκBα, an intermediate NF-κβ signal protein, have attenuated allergic airway inflammation (Schuliga, 2015).

Airway epithelial hyperplasia and metaplasia contribute to mucus abnormalities in asthma through the increased production and storage of mucus. The main components involved in the mucus mechanism are MUC5AC and MUC5B. Both are altered in asthma, leading to changes in gene expression and asthma pathophysiology (Bonser et al., 2017). Altered gene expression changes mucus composition and organisation, contributing to mucus plugging, airway obstruction and goblet cell number, amongst other factors. The over expression of MUC5AC alone appears to not reach a threshold that causes mucus plugging, however, its solo role in

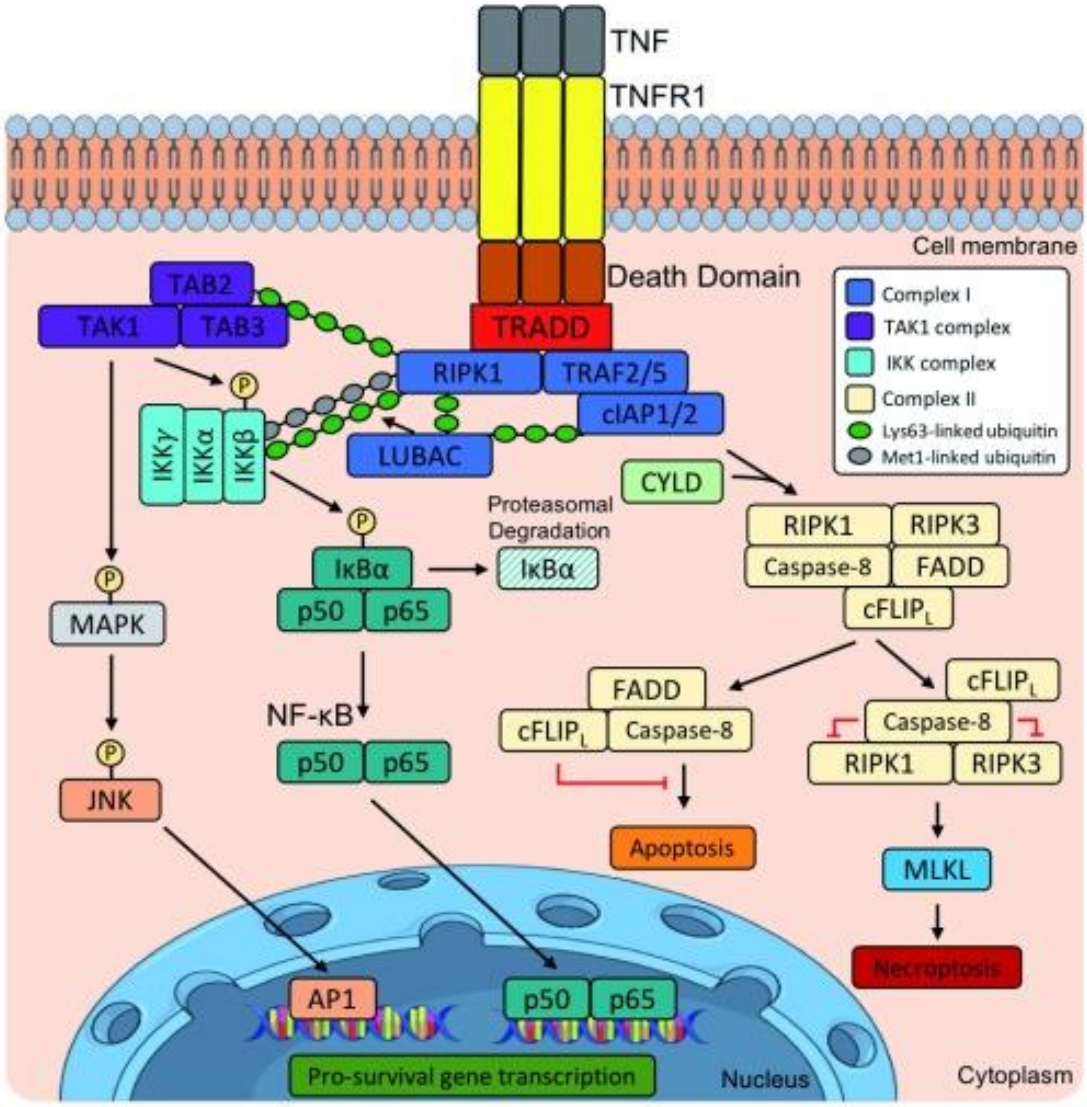

Figure 2.2.1. (Holbrook et al., 2019). Abbreviations; heme-oxidised iron regulatory protein 2 ubiquitin ligase 1, HOIL-1; HOIL-1 interacting protein, HOIP. The signalling pathway of TNFR1. When TNF and TNFR1 bind, TNFR-1 associated death domain (TRADD) is recruited and binds receptor-interacting serine/threonine-protein kinase 1 (RIPK1), TNFR associated factors 2/5 (TRAF2/5), and cellular inhibitor of apoptosis protein ½ (cIAP1/2) forming complex I. The linear ubiquitin chain assembly complex (LUBAC) and cIAP1/2, consisting of HOIL, HOIP and SHARPIN, add the Met1-linked and Lys63 linked polyubiquitin chains, respectively to RIPK1. Leading to stabilisation and amplification of signalling. Transforming growth factor-beta (TGFβ)-activated kinase 1 (TAK1) complex is recruited by Lys63 chains on RIPK1. This complex consists of TGFβ-activated kinase 1 and mitogen-activated protein kinase (MAPK) binding protein 2/3 (TAB2/3) and TAK1. TAK1 phosphorylates MAPK, in turn phosphorylating Jun N-terminal kinase (JNK) leading to translocation of AP1 to the nucleus and transcription of target genes. TAK1 phosphorylates the IkB kinase (IKK) complex, leading to NFκβ translocation to the nucleus and transcription of target genes. RIPK1 undergoes deubiquitination by cylindromatosis tumour suppressor protein deubiquitinase (CYLD), facilitating its dissociation from complex I to form complex IIb, which consists of RIPK1/3, FAS-associated death domains (FADD), Flice-like inhibitory protein long (cFLIP$_L$) and caspase-8. cFLIP$_L$ regulates necrosis and apoptosis through prevention of caspase 8 action.

asthma is thought to increase neutrophil traffic, resulting in lung inflammation and AHR when coupled with smooth muscle contraction (199 Koeppen et al., 2012).

Table 1.5. NF-κβ target inflammatory genes, in various airway cells.

Cell Type	Genes
Th1/Th2 Lymphocytes	Regulated and activation of normal T cell expressed and secreted (RANTES), Eotaxin-1, Interferon-gamma (IFN-γ), Interleukin-2 (IL-2), IL-4, IL-5 and IL-13)
Eosinophils	TNF-α, IL-8, ICAM-1 and leukocyte function-associated antigen-1 (LFA-1)
Neutrophils	IL-8, granulocyte macrophage-colony stimulating factor (GM-CSF), IL-1Rα
Macrophages	IL-8, growth-regulated oncogene-α (GROα) and Monocyte chemotactic protein-1 (MCP-1)
Epithelial cells	Thymic stromal lymphopoietin (TSLP), VCAM-1, ICAM-1, IL-6, IL-8, GM-CSF, chemokine (CXC) ligand (CXCL) 1, RANTES, MCP-1, GROα, eotaxin-1 and MUC5AC.
Smooth Muscle	VCAM-1, ICAM-1, TSLP, CD38, COX-2, IL-6, I-8, CXCL10 (mast cell chemoattractant), RANTES, GM-CSF, MCP-1, GROα, epithelial activating peptide 78 (ENA-78) and neutrophil-activating-protein-2 (NAP-2).

GATA3 is a protein coding gene that controls the expression of a large range of biological genes, it belongs to the GATA family and is crucial for development and function of various cells types, and includes Th2 cells (see section 2.2.2). GATA3 promotes IL-4, IL-5 and IL-13 secretion from human Th2 cells, augments allergic responses, while also inducing naïve T cell differentiation into Th2 cells, demonstrating its potential as a therapeutic target in asthma. The precise mechanism by which GATA3 influences Th2 cytokine expression is unknown, however a direct correlation has been shown with the transcription factor, IL-5 expression and AHR (Nakamura et al., 1999). A potential strategy for asthma treatment may be to block GATA3-induced Th2 activation and differentiation, thereby reducing airway inflammation. However, by blocking Th2 cell induction a Th1 cell generation may be observed (Ray and Cohn., 1999). GATA3 could be perceived as a master regulator in allergic inflammation, due to its role in Th2 regulation, in order to better understand GATA3 function in the pathology of asthma more research is needed to elucidate the exact mechanisms by which it works.

2.2.4 Chemokines in Asthma and Inflammation

Chemokines or chemotactic cytokines are a subgroup in the cytokine family, they function as signalling proteins and their name is derived from their ability to induce chemotaxis of nearby active cells. Other names previously used for chemokines include: SIG cytokine family, SCY cytokine family, SIS cytokine family and the Platelet factor-4 superfamily. Chemokines regulate leukocyte migration through activation of seven-transmembrane rhodopsin-like G protein-coupled receptors (Moser and Willimann, 2004) and leukocyte response to chemokines is determined by the chemokine receptors. Chemokines are able to activate multiple chemokine receptors and chemokine receptors can be activated by more than one chemokine, this system is termed bidirectional promiscuity (Yung and Farber, 2013).

Chemokine family members can be divided into 4 groups, each group determined by ones structure, specifically the spacing of the first two cysteine residues held together by disulphide bonds. A total of 28 Chemokines make up the first group (CC), CCL1-CCL28. An example of the first group is RANTES (CCL5), which functions as a chemoattractant for eosinophils, basophils and T cells that express the chemokine receptor CCR5 (Miller and Mayo., 2017) (see section 2.2.2). RANTES is an important chemokine in asthma pathophysiology, where the 403 G/A polymorphism has been reported as a risk factor following meta-analysis (Wen et al., 2014). The second group, CXC chemokines, possess two N-terminal cysteines separated by an amino acid (X), 17 of these chemokines have been identified in mammals. CXC chemokines can be divided into two categories, those with a specific glutamic acid-leucine-arginine sequence before the first cysteine are described as ELR-positive, and those without this specific sequence are ELR-negative. ELR-positive chemokines are associated with neutrophil migration, may interact with CXCR1 and CXCR2 receptors and are angiogenic, whereas chemokines lacking this amino acid motif act as a chemoattractant for lymphocytes and are angiostatic (Vandercappellen et al., 2008). Interleukin-8, an example of an ELR-positive

chemokine, is elevated in the blood of asthmatics (see section 2.2.2.) and induces neutrophil migration into surrounding tissues from the blood. An ELR-negative cytokine, CXCL13, has been demonstrated to facilitate the allergic airway inflammatory process through induction of $CD4^+$ cell migration (see 2.2.2) and B cell recruitment (Baay-Guzman et al., 2012). The third group are known as C chemokines and they only contain two cysteines, one downstream and one on the N terminal. Only two known cytokines have been found in this group, XCL1 and XCL2. XCL1 is produced by T and natural killer cells during inflammatory responses and is expressed by certain populations of dendritic cells (Lei and Takahama, 2012), the closely related XCL2 has been virtually uncharacterised (Fox et al., 2014). The fourth and final group are CX3C chemokines, they have three amino acids between the cysteine groups, the only discovered member of this group is fractalkine (CXC3CL1), a adhesion molecule and chemoattractant thought to play a role in macrophage trafficking (Lu et al., 2008).

Table 1.6. The four chemokine classes, their receptors, aliases and Uniprot ID.

Uniprot ID	Ligand	Other Biological Name	Receptor
P10147	CCL3	MIP-1a	CCR1
P13236	CCL4	MIP-1β	CCR1, CCR5
P13501	CCL5	RANTES	CCR5
P27784	CCL6	C10, MRP-2	CCR1
P80098	CCL7	MARC, MCP-3	CCR2
P80075	CCL8	MCP-2	CCR1, CCR2B, CCR5
P51670	CCL9/CCL10	MRP-2, CCF18, MIP-1?	CCR1
P51671	CCL11	Eotaxin	CCR2, CCR3, CCR5
Q62401	CCL12	MCP-5	
Q99616	CCL13	MCP-4, NCC-1, Ckβ10	CCR2, CCR3, CCR5
Q16627	CCL14	HCC-1, MCIF, Ckβ1, NCC-2, CCL	CCR1
Q16663	CCL15	Leukotactin-1, MIP-5, HCC-2, NCC-3	CCR1, CCR3
O15467	CCL16	LEC, NCC-4, LMC, Ckβ12	CCR1, CCR2, CCR5, C CR8
Q92583	CCL17	TARC, dendrokine, ABCD-2	CCR4
P55774	CCL18	PARC, DC-CK1, AMAC-1, Ckβ7, MIP-4	
Q99731	CCL19	ELC, Exodus-3, Ckβ11	CCR7
P78556	CCL20	LARC, Exodus-1, Ckβ4	CCR6
O00585	CCL21	SLC, 6Ckine, Exodus-2, Ckβ9, TCA-4	CCR7
O00626	CCL22	MDC, DC/β-CK	CCR4
P55773	CCL23	MPIF-1, Ckβ8, MIP-3, MPIF-1	CCR1
O00175	CCL24	Eotaxin-2, MPIF-2, Ckβ6	CCR3

O15444	CCL25	TECK, Ckβ15	CCR9
Q9Y258	CCL26	Eotaxin-3, MIP-4a, IMAC, TSC-1	CCR3
Q9Y4X3	CCL27	CTACK, ILC, Eskine, PESKY, skinkine	CCR10
Q9NRJ3	CCL28	MEC	CCR3, CCR10
P09341	CXCL1	Gro-a, GRO1, NAP-3, KC	CXCR2
P19875	CXCL2	Gro-β, GRO2, MIP-2a	CXCR2
P19876	CXCL3	Gro-?, GRO3, MIP-2β	CXCR2
P02776	CXCL4	PF-4	CXCR3B
P42830	CXCL5	ENA-78	CXCR2
P80162	CXCL6	GCP-2	CXCR1, CXCR2
P02775	CXCL7	NAP-2, CTAPIII, β-Ta, PEP	
P10145	CXCL8	IL-8, NAP-1, MDNCF, GCP-1	CXCR1, CXCR2
Q07325	CXCL9	MIG, CRG-10	CXCR3
P02778	CXCL10	IP-10, CRG-2	CXCR3
O14625	CXCL11	I-TAC, β-R1, IP-9	CXCR3, CXCR7
P48061	CXCL12	SDF-1, PBSF	CXCR4, CXCR7
O43927	CXCL13	BCA-1, BLC	CXCR5
O95715	CXCL14	BRAK, bolekine	
Q9WVL7	CXCL15	Lungkine, WECHE	
Q9H2A7	CXCL16	SRPSOX	CXCR6
Q6UXB2	CXCL17	DMC, VCC-1	
P78423	CX3CL1	Fractalkine, Neurotactin, ABCD-3	CX3CR1
P47991	XCL1	Lymphotactin a, SCM-1a, ATAC	XCR1
Q9UBD3	XCL2	Lymphotactin β, SCM-1β	XCR1

2.3. The Gut, Immune system and Inflammation

The immune system is a complex network comprised of various proteins, cells, tissues and organs that work synergistically to defend the body from foreign material, as discussed in section 2.2.1. The mucosal associated lymphoid tissue (MALT) plays a crucial role in the adaptive immune system and the gut associated lymphoid tissue (GALT) is included in this. The human gastrointestinal tract (GI) spans 250-400 m^2 and there is an immense collection of bacteria, archaea and eukarya that colonise this area, known as 'gut microbiota'. The gut microbiota has co-evolved with the host over thousands of years to develop a mutually beneficial relationship, the number of microorganisms living in the GI tract is estimated at 10^{14}, with a ratio of near 1:1 for bacterial cells and human cells (Sender et al., 2016). The intestine is the largest lymphoid tissue in the body, its anatomy and interaction with commensal bacteria and immune cells is depicted in figure 2.3 below. Intestinal epithelial cells (IEC) line the entirety of the intestine, separating commensal bacteria from the sterile areas past the lamina propria. This physical barrier is vital for maintenance of intestinal immune homeostasis, as well as immune cell signalling, when exposed to substances found in the intestinal lumen (Artis, 2008). IECs are exposed to various substances such as nutrients, immunoglobulin, goblet cell mucus and commensal bacteria, resulting in epithelial stem cell proliferation and the rise of daughter cells with further potential for proliferation. Moreover, these IECs may differentiate into colonic enterocytes or villous, both capable of nutrient (small intestine) and water (colon) absorption, influencing systemic metabolism. Furthermore, IEC progenitors differentiate into both endocrine cells, secreting enteric hormones and paneth cells which localise at the base of the small intestine in the crypts. Further below is the lamina propria, integrated with stromal cells, T cells, macrophages, B Cells and dendritic cells, all responsible for immune regulation of the gut through various signalling mechanisms involving TLRs (Uematsu et al., 2008)

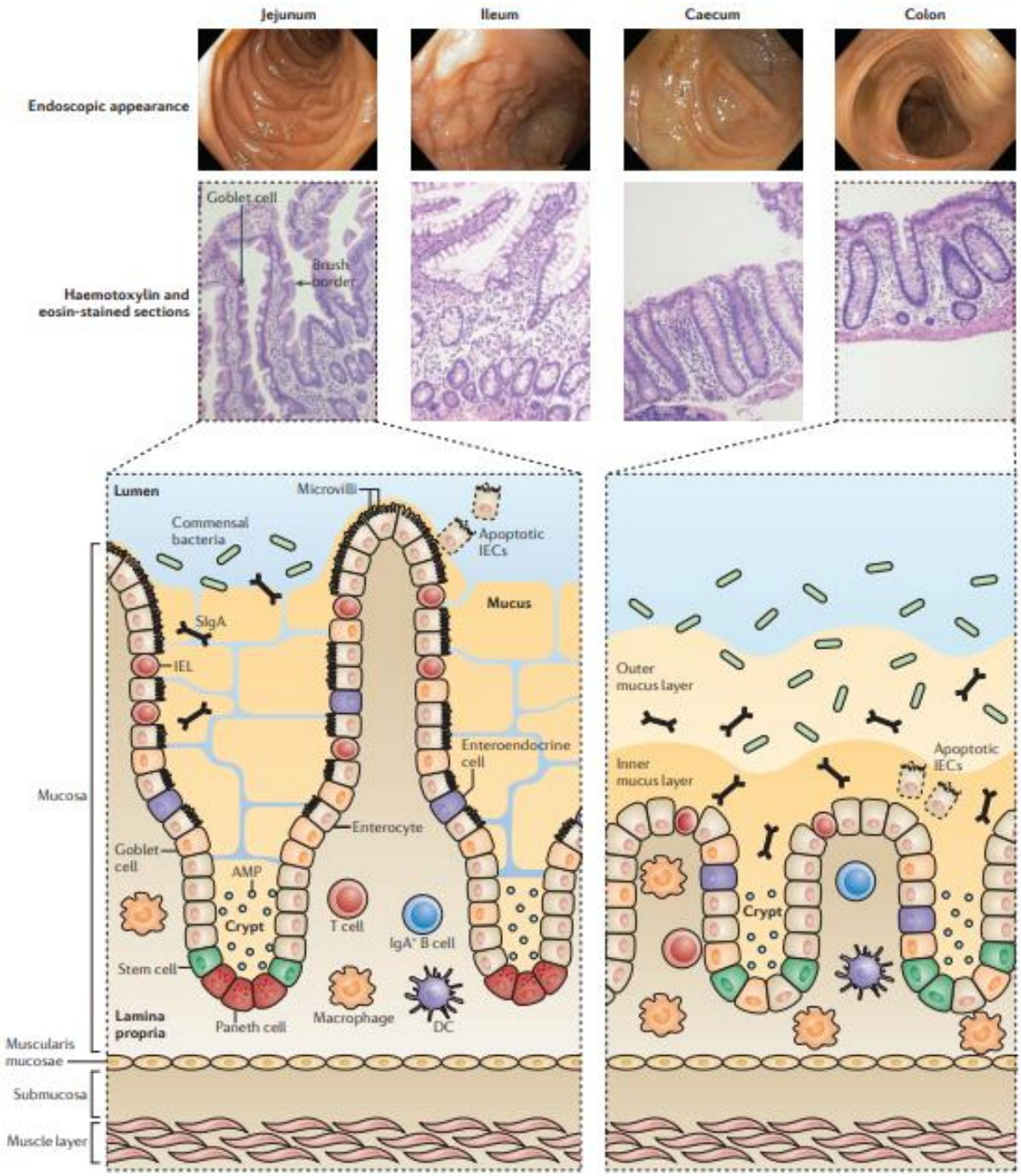

Figure 2.3. The anatomy and physiology of the intestine; endoscopic appearance, haematoxylin and eosin-stained sections and the structure of the mucosa. Abbreviations, IEL; intraepithelial lymphocytes, SIgA; secretory immunoglobulin A, IEC; intestinal epithelial cells, DC; dendritic cells, M cells; microfold cells (Mowat and Agace, 2014).

T cell subsets and dendritic cells (DC) localise between IECs, specialised DCs in the lamina propria are capable of extending and acquiring luminal bacteria, a process dependent on CX3CR1. This allows swift immune responses in order to protect the gut (Artis, 2008). Dendritic cells located in tight junctions are also able to detect changes in commensal bacteria and selectively activate certain immune responses (Coombes and Powrie, 2008). Specialised microfold (M)-cells in the small intestine overlay the peyer's patches, sampling the intestinal lumen, these cells secrete various chemokines crucial in inflammatory and dendritic cell recruitment (Mowat and Agace, 2014).

In addition to GALT, in the GI immune system contains resident microbiota, with over 1000 colonies of known commensal bacteria. These bacteria are part of a complex synergistic system and are essential for immune function and systemic metabolism, as a result of their role, they are crucial for research involving health and disease (Artis, 2008). The latest data reports 1057 intestinal species of Archaea (8), Eukarya (92) and Bacteria (957), based on RNA gene sequencing (Rajilić-Stojanović and Vos, 2014). Microbial composition and colonisation of the GI tract effects the number of intraepithelial lymphocytes, indicating their potential impact on immune regulation and function, through signalling and epigenetics. Host physiology is hyporesponsive to commensal bacteria communities, to allow cohabitation of host and bacterial cells, paradoxically commensal bacteria are required for development and maturation of the peripheral immune system and mucosal function (Macpherson and Harris, 2004). For example, mice developed in germ-free conditions displayed various immunological defects, their Peyer's patches were poorly formed and $CD4^+$ T cells were significantly different when compared with conventional domestic mice. Furthermore, certain TCR isotypes were not present in the germ-free mice, suggesting an epigenetic role for commensal bacteria in T cell variation and differentiation (Smith et al., 2007). These observations as well as many others indicate that molecular mechanisms exist between bacteria and host, allowing basal levels of immune

activation for gene expression programming, this process appears to be crucial in normal development and immune cell function (Artis, 2008).

2.3.1 The Gut Microbiota and Disease

Alterations of commensal bacteria composition in the gut may influence host susceptibility to certain pathologies and immune-mediated disease. Variations may be linked to airway inflammation and asthma, as bacteria-IEC-IEL interaction leads to activation of PPRs, such as TLR, NLRs and RLRs, and subsequently modulation of the immune system. Allergy and asthma pathogenesis/pathophysiology is incompletely understood, commensal bacteria may or may not be key in asthma management/treatment. However, it would be beneficial to research deviations of bacterial composition in healthy controls compared to asthmatics (Huang et al., 2018).

To support the concept that gut microbiota play a crucial role in the immune response it is imperative that we understand the makeup of these bacterial species and how different compositions of bacteria influence disease. Humans typically have 6-7 types of bacteria *phyla*, *actinobacteria*, *firmicutes*, *proteobacteria*, *bacteroidetes*, *cyanobacteria*, *fusobacterial* and less common, v*errucomicrobia*; the first 4 types mentioned are the most dominant. Bacteria make a sizeable contribution to the colonic microbiome, and are present at 60% in dry mass of the faeces (Quigley, 2013). The predominant species in the colon are anaerobic *bacteroides* and anaerobic bacteria in the *Bifidobacterium* genus (*Bifidobacterium bifidum*).

Table 1.7. Adapted from Kenneth, 2012. The range of incidence for various bacteria located in the colon of healthy individuals.

Bacterium	**Range of incidence (%)**
Bacteroides fragilis	100
Bacteroides melaninogenicus	100
Bacteroides oralis	100
Lactobacillus	20-60
Clostridium perfringens	25-35
Clostridium septicum	5-25
Clostridium tetani	1-35
Bifidobacterium bifidum	30-70
Staphylococcus aureus	30-50
Enterococcus faecalis	100
Escherichia coli	100
Salmonella enteritidis	3-7
Klebsiella sp.	40-80
Enterobacter sp.	40-80
Proteus mirabilis	5-55
Pseudomonas aeruginosa	3-11
Peptostreptococcus sp.	?common
Peptococcus sp.	?common

Alterations in the commensal composition of intestinal bacteria has been hypothesised to influence susceptibility, progression, and pathogenesis of immune-related diseases; see sections 2.1.2 for the hygiene and microbiome hypothesis. For example, colonisation of *Clostridium difficile* at the age of 1 month has been associated with asthma at ages of 6-7 years (Penders et al., 2011). Chronic inflammation has been linked to alterations in commensal bacteria in patient groups, as well as airway inflammation, irritable bowel disease, arthritis and enetrocolitus in murine models (Artis, 2008). Furthermore, metal health conditions have even been suggested to influence the gut and its commensal bacteria make up through the bidirectional gut-brain axis, specifically though stress influencing both the CNS and gut microbiota, altering gut epithelium integrity (Collins, 2012).

The evidence for a link between type 2 obesity and changes in gut bacteria is ever-growing, metabolically obese mice were shown to have a shift in their ratio of firmicutes to bacteroidetes, in favour of firmicutes. Conversely lean mice were dominated by bacteroidetes (Turnbaugh et al., 2006), however, these studies have failed to be replicated with human subjects. In fact in humans, a study showed little differences in gut microbiota between type 2 diabetes (T2D) participants and control, although a reduced amount of butyrate-producing bacteria were quantified in the T2D group (Qin et al., 2012). This emphasises the point that the relationship between gut microbiota and disease is not only composition, however, it is the functional capability of the composition that exists. Alterations in short-chain fatty-acid (SCFA) levels have been detected in T2D, known producers of acetate, butyrate and propionate. SCFAs are found at the highest concentrations in the colon and therefore are likely to have a role in shaping immune responses, possibly through influencing the composition and functionality of commensal bacteria (Mowat and Agace, 2014). In atopic eczema, children at the age of 12 months had a significantly decreased diversity of bacteroidetes when compared to healthy controls, and a lower number of proteobacteria which contain lipopolysaccharides (LPS) that when underexposed in infancy increase eczema risk, inferring the hygiene hypothesis (see section 2.1.2) (Bull and Plummer, 2014). This early exposure of LPS is believed to sensitise and calibrate the immune system. Moreover, SCFAs were shown to prevent LPS inflammation in a murine macrophage cell line, RAW264.7, through the NF-κβ signalling system, indicating possible protective role of SCFAs in human physiology (Liu et al., 2012).

Gut microbiota educate the immune system during early life and certain patterns of gut colonisation can be linked with the development of allergic sensitisation. For example *Bifidobacterium bifidum* was the main *Bifidobacterium* species found in breastfed non-allergic infants, whereas *B. longum* and *B. adolescentis* were more prevalent in those who developed allergies (Huang, 2013). In asthma, the early colon colonisation of *bacteroides fragilis* is an

indicator of possible asthma later in life, following results from an asthma predictive index (API) study (Vael et al., 2008). Another study analysed stool samples collected at 1 and 12 months old, the birth cohort was comprised of 411 children with an elevated risk for asthma and 16s rRNA-based denaturing gradient gel electrophoresis (DGGE) was used, allergic sensitisation was inversely correlated with reduced bacterial diversity, estimated by DGGE (Bisgaard et al., 2011).

Overall, it is clear that the development of gut microbiota in infancy is of great important for a strong immune system and reduced risk to certain autoimmune and allergic diseases. Various studies support this statement showing that reduced exposure to a diversity of microbes can negatively impact immune health and disease risk. The mechanisms as to how the gut microbiota regulate immune health remain elusive, and furthermore, the mechanisms of systemic immune regulation by the gut are unknown. Moreover, it is agreed that gut dysbiosis may be a crucial factor in understanding and treating autoimmune disease and certain allergies, such as asthma.

2.3.2. Diet, Gut Microbiota and Asthma

In recent years it has been widely accepted that diet has significant effects on gut bacteria composition. A comparative study looked at the diet of children in rural Africa, Burkina Faso (BF), and European children (EU). In BF diets are high in fibre, similar to early life during the birth of agriculture. Diets of EU children contains considerably less fibre content, resulting in significant differences in gut microbiota between both groups. Children of BF had significant colonisation of *Bacteroidetes* and reduction *Firmicutes* in comparison to EU children (De Filippo et al., 2010). Furthermore, BF levels of SCFAs were significantly higher than EU children and children in rural Africa have much lower levels of asthma incidence compared to the EU. A diet rich in polysaccharides allows the host to maximise their energy intake from

fibre sources while also benefiting from improved inflammatory and immune function as a result. This study and many others suggest that the western diet has had a large impact on diversity and composition of the gut microbiota and infer that this change has led to the increased frequency of atopic disease through impaired immune systems.

Dietary protein directly affects the microbiota composition, a culture-based study showed low counts of *Bifidobacterium adolescentis* and increased *bacteroides* and *clostridia* in subjects with a high level of beef in their diet, meatless diets showed the inverse (Hentges et al., 1977). Many different types of protein have been analysed in order to record changes in gut bacteria composition, overall microbial diversity has been shown to positively correlate with protein consumption. Specifically, pea protein has been reported to increase both *Lactobacillus* and *Bifidobacterium* in the gut, while reducing pathogenic bacterial species *Clostridium perfringens and Bacteroides fragilis* (Singh et al., 2017).

Table 1.8. The effects of protein on gut microbiota, figure adapted from Singh et al., 2017.

	Microbial diversity	*Bifidobacteria*	*Lactobacilli*	*Bacteroides*	*Alistipes*	*Bilophila*	*Clostridia*	*Roseburia*	*Eubacterium Rectale*
Animal protein	↑	↑↓		↑↓	↑	↑	↑	↓	↑↓
Whey protein extract	↑	↑	↑	↓			↓		
Pea protein extract	↑	↑	↑						

Arrow thickness corresponds to relative number of studies supporting the relationship

Plant derived proteins are linked to lower mortality rates than animal protein, with reduced risk of colonic disease, this is due to high levels of fat in animal based diets. Moreover, higher protein intake overall is associated with increased levels of insulin-like growth factor 1 (IGF-1), which is linked to cancer and diabetes.

The western diet is known for its high levels of saturated and *trans*-unsaturated fatty acids, both increase the risk of cardiovascular disease through upregulation of LDL cholesterol and

blood total (Stamler, 2000), other fats such as mono and polyunsaturated are crucial in alleviating the risk of chronic diseases. Fava et al. conducted a study where subjects consumed various fat content, it was noted that lower consumption of fat led to increased levels of *Bifidobacterium* and reduced fasting levels of blood glucose, high fat diets (saturates) increased the number of *Faecalibacterium prausnitzii*. Finally, subjects who consumed high monounsaturated fats did not experience shifts in their bacterial genera, although the total bacteria load and LDL-cholesterol was reduced (Fava et al., 2012).

Table 1.9. Effects of various fat diets on gut microbiota, figure adapted from Singh et al., 2017.

	Lactic acid bacteria[a]	*Bifidobacteria*	*Clostridiales*	*Bacteroides*	*Bilophila*	*Faecalibacterium prausnitzii*	*Akkermansia muciniphila*
High fat	↓		↑	↑			
Low fat		↑					
High saturated fat				↑	↑	↑	
High unsaturated fat	↑	↑					↑

[a] Lactic acid bacteria include Lactobacillus and Streptococcus

In lard-fed mice, increased systemic TLR activation was observed, as well as adipose tissue inflammation and impaired insulin sensitivity, indicating the drastic effect diet can have on biological mechanisms. Increased TLR activation from high fat diets could lead to airway inflammation and possibly the pathogenesis of asthma, as well as other inflammatory disorders.

Carbohydrates have been extensively studied in the scientific world, they can be divided into two categories: digestible and non-digestible. Digestible carbohydrate include starches and sugars, such as fructose, sucrose, lactose and glucose; they are enzymatically degraded within the small intestine and release glucose into the blood stream, prompting an insulin response. Human subjects that consume high amounts of glucose, fructose and sucrose, from date fruits, were shown to have elevated abundance of *Bifidobacterium* and reduced *Bacteroidetes.* Furthermore, lactose lead to similar bacterial shifts and also led to the decrease of *Clostridia*, an IBS related bacterium (Eid et al., 2014). Interestingly, lactose has been reported to increase

beneficial SCFA concentrations, even though it is thought of as a gastrointestinal irritant. Another controversial topic are artificial sweeteners, such as saccharin, sucralose and aspartame.

Table 2.0. Effects of both natural and artificial sugar on gut microbiota, adapted from Singh et al., 2017

	Bifidobacteria	*Bacteroides*	*Clostridia*	*Lactobacilli*
Glucose	↑	↓		
Fructose	↑	↓		
Sucrose	↑	↓		
Lactose	↑	↓	↓	↑
Artificial sweeteners	↓	↑	↓	↓

Artificial sweeteners have recently been reported to induce glucose intolerance, mediating their effect through changes in gut bacterial composition and reducing *Bifidobacterium* concentrations in the colon, it is possible that these sweeteners may be worse for gut health than natural sugars. Furthermore, saccharin fed mice showed intestinal dysbiosis, increased *Bacteroides* and reduced *Lactobacillus reuteri* (Suez et al., 2014). However, this study observed very high concentrations of artificial sweeteners, more studies are required to better conclude the short and long-term effects of these non-nutritional substrates.

Non-digestible carbohydrates exist in the form of fibre and resistant starch, both are unable to be digested in the small intestine and travel to the large intestine where they are fermented by the gut microbiota. Dietary fibre intake can be defined as 'microbiota accessible carbohydrates' (MACs) (Singh et al., 2017). MACs are utilised by gut bacteria as an energy source leading to the modification of microbiota composition, of which prebiotics are an example of. Prebiotics are 'non digestible dietary components which benefit host health through selective stimulation of the growth and/or activity of certain microorganisms' (Sonnenburg and Sonnenburg, 2014). Sources of prebiotics include, inulin, unrefined wheat barley, soybeans, raw oats and non-digestible oligosaccharides such as galactooligosaccharides (GOS), fructooligosaccharides

(FOS), arabinooligosaccharides (AOS) (Pandey et al., 2015). In recent years various prebiotics have been studied as treatment for a range of different inflammatory diseases including asthma, a specific prebiotic, *trans*-galactooligosaccharide (B-GOS) has been shown to reduce the severity of hyperpnoea-induced bronchoconstriction, reducing systemic markers of inflammation such as TNFα (Williams et al., 2016). B-GOS is used in the current study, experimental chapter 1, to assess its effect on airway inflammation, asthma quality of life and help define its mechanistic properties. An abundance of research is necessary on these non-digestible carbohydrates in order to fully understand their effect on microbiota composition and how they are beneficial not only asthma, but inflammatory disease in general. Numerous studies suggest that these carbohydrate forms mainly increase *Bifidobacterium* and lactic acid bacteria (Eid et al., 2014; Francavilla et al., 2013; Parvin, 2015; Suez et al., 2014), this increase could be linked with asthma improvements through an unknown mechanism. Furthermore, diets low in the oligosaccharides discussed lead to an overall decrease in bacterial abundance (Halmos et al., 2014) and other non-digestible carbohydrates such as resistant starch and whole grain barley appear to increase abundance of *Ruminococus, E. rectale* and *Roseburia* (Keim and Martin, 2014).

Table 2.1. The effects of Fibre/prebiotics and resistant starch on gut microbiota abundance, adapted from Singh et al., 2017.

	Bacterial abundance	Gene richness	*Lactobacilli*	*Bifidobacteria*	*Clostridia*	*Enterococcus*	*Roseburia*	*Eubacteria*	*Ruminococcus*
Fiber/prebiotics	↑	↑	↑	↑	↓	↑↓			
Resistant starch	↑	↑	↑	↑			↑	↑	↑

Arrow thickness corresponds to relative number of studies supporting the relationship

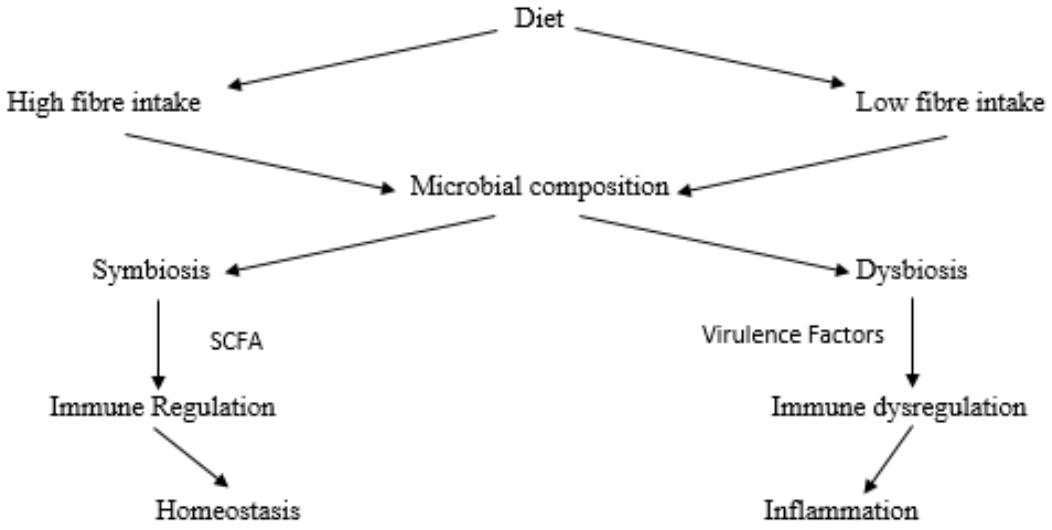

Figure 2.4. The effect of diet on microbial composition through fibre intake and how microbial composition, as well as other environment factors such as genetics, age, hygiene, antibiotic use, infection, inflammation, stress and maternal transfer/early colonisation, effect immune function and host physiology.

Prebiotics, in addition to the discussed effects, are able to produce shifts in immune markers and metabolic function, such as reduce in IL-6, a pro-inflammatory cytokine (Keim and Martin, 2014). Other research has recorded increases in the anti-inflammatory IL-10, following butyrylated high amylose maize starch supplementation (West et al., 2013).

2.3.3 The Gut microbiota as a target for airway inflammation

As discussed previously, it is understood that a balanced and healthy diet can promote the growth of beneficial gut bacteria, leading to changes in the microbiome makeup. These probiotic bacteria are then able to modulate and induce regulatory responses through enteroendocrine cells; such as, epigenetic changes, pulmonary immune responses and innate immunity.

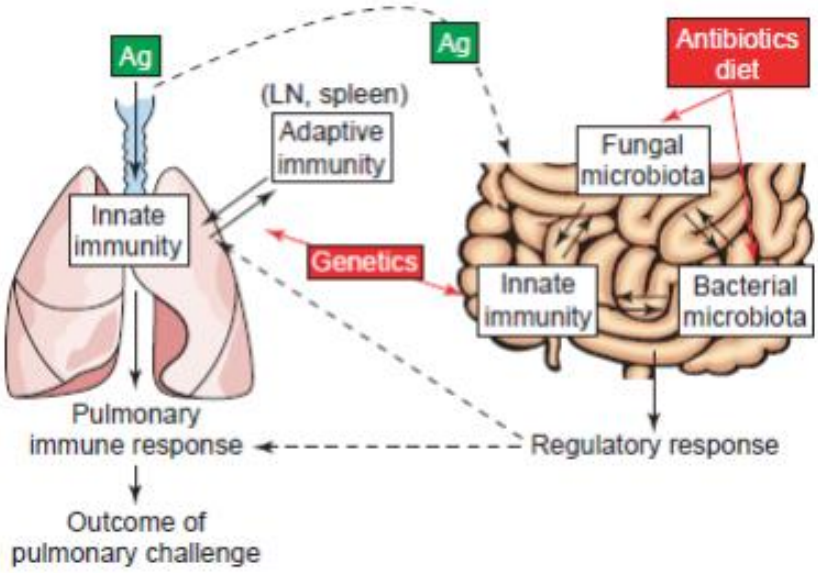

Figure 2.5. The proposed mechanisms of how microbiota may be involved in the modulation of pulmonary immune response (Noverr and Huffnagle, 2004)

Alterations in the gut microbiota, through dietary intervention, influences intestinal epithelial cells (IECs) and dendritic cells within the GALT (see section 2.3). Probiotic bacteria act on dendritic cells (DC) through two mechanisms; DCs located within the lamina propria pass their dendrites through IECs into the lumen of the gut, DCs may also interact directly with bacteria through M cells in the GALT's dome region (Artis, 2008). Probiotic bacteria interact with host cells through their microorganism-associated molecular patterns (MAMPs) that reside on the cell surface. MAMPs interact with host cells, such as DCs, through pattern recognition receptors (PRRs) on their dendrites, leading to TLR activation and subsequently T lymphocyte differentiation, see section 2.2.2 for more on DC function. DCs have a crucial role in the

immune response and asthma, activation of DCs by the microbiota may impact systemic immunity and inflammatory function, ultimately effecting pulmonary function. Morever, the type of IEC determines the molecular response produced, for example Goblet cells will produce mucus following activation (Kim and Ho, 2010).

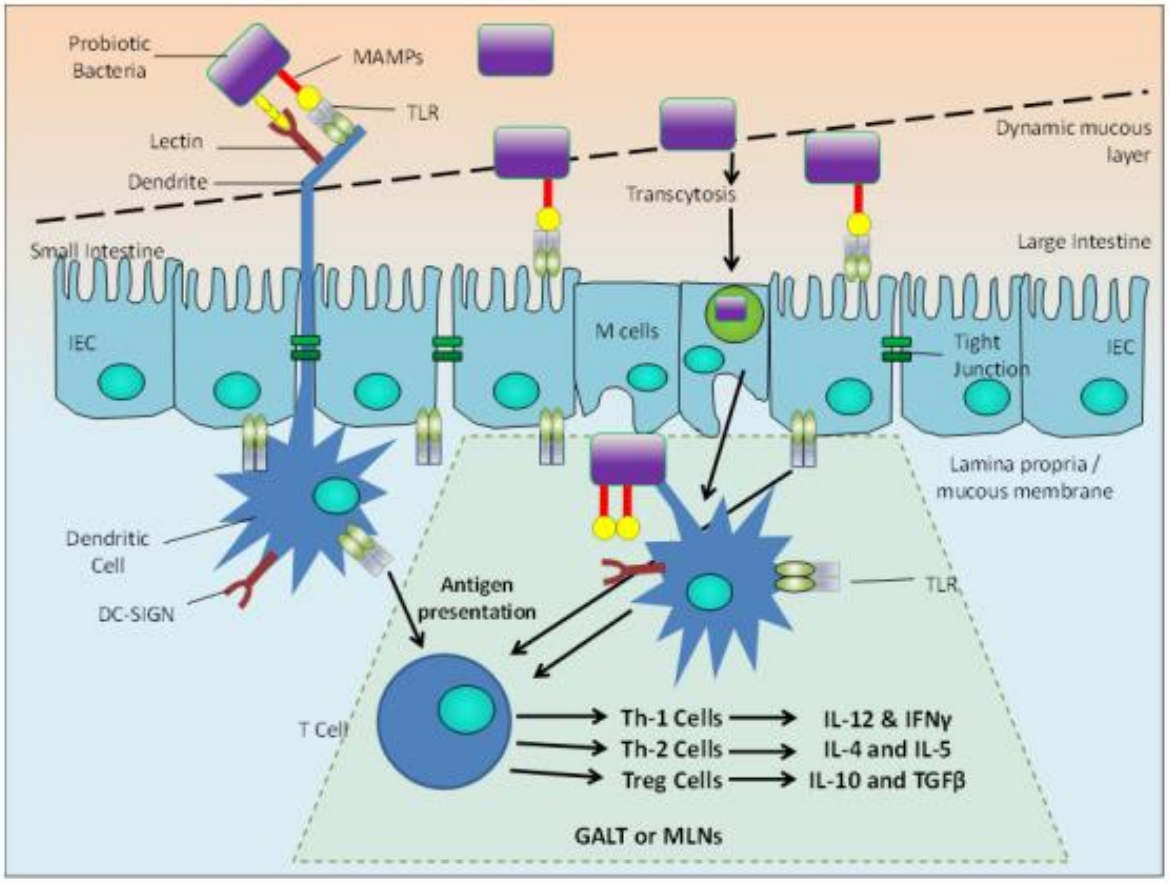

Figure 2.6. The interaction between dendritic cells, intestinal epithelial cells and bacteria within the gut associated lymphoid tissues. These interactions result in T cell activation and differentiation through antigen presentation, followed by the release and production of inflammatory mediators and cytokines. Abbreviations, DC; dendritic cell, TLR; toll like receptor, IEC; intestinal epithelial cell, SIGN; specific intracellular adhesion molecules 3-grabbing non-integrin, IFN-γ; interferon-γ, IL; interleukin, TGFβ; transforming growth factor β, MLNS; mesenteric lymph nodes. Adapted from Leeber et al., (2011).

2.3.4. The airway microbiota and asthma

Similarly to gut microbiota, the human respiratory tract also harbours a local microbiome, the composition of which may play a crucial role in asthma. The lung microbiota must be considered separately from gut microbiota and as its own ecosystem due to some significant composition differences. The most abundant genera of lung bacteria in healthy individuals includes *Prebotella*, *Streptococcus*, *Veillonella*, *Neisseria*, *Haemophilus* and *Fusobacterium*. The main phyla include *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Actinobacteria*. However, there is a high variability between individuals and it remains unclear which lung composition constitutes good lung health, however a shift in microbiota can be seen in patients with asthma (Hooks and O'Malley, 2017).

It is unclear what the function or role the lung microbiome has in allergic disease. A milestone paper produced in 2010 analysed and showed differences in phylum distribution in healthy children and children with severe asthma, specifically at the genus level, *Staphylococcus* and *Haemophilus* appeared to be more abundant in asthma patients and *Prevotella* was reduced (Hilty et al., 2010). Numerous murine studies have shown that depletion of certain gut species following antibiotic intake directly influences lung bacterial composition (Dharmage et al., 2015; Metsala et al., 2015; Russell et al., 2013). Although the mechanism is unknown, it is reasonable to assume that gut bacteria composition can impact lung bacteria composition. This mechanism may be genetic, or through regulatory responses from the IECs in response to bacterial signalling, as discussed in section 2.2.3. This interaction between the gut and lungs is known as the Gut-Lung axis, a bi-directional pathway, best observed by gastro intestinal complications in cases of lung disease (Anand and Mande, 2018). A deep understanding of this axis and its role in asthma is important for the design of treatment, specifically through diet and supplementation strategies.

2.4. Pharmacological methods to treat asthma

At this time there is no known cure for asthma, thus we must treat the symptoms with medication as well as use preventative treatment methods to reduce exacerbations. There are many pharmacological and non-pharmacological methods used to treat asthma, the methods used depend on classification of the patients asthma. The classification of asthma, as discussed previously (see section 1.1.), is debated frequently. Wenzel et al., 2011, proposed that we must cluster patients into specific phenotypic groups in order to produce effective and specialised treatment, opposed to the current guidelines set by the British Thoracic Society. Classifying Asthmatic patients into specific groups remains a convoluted area, therefore the BTS 5 step guidelines are still currently used to determine treatment options.

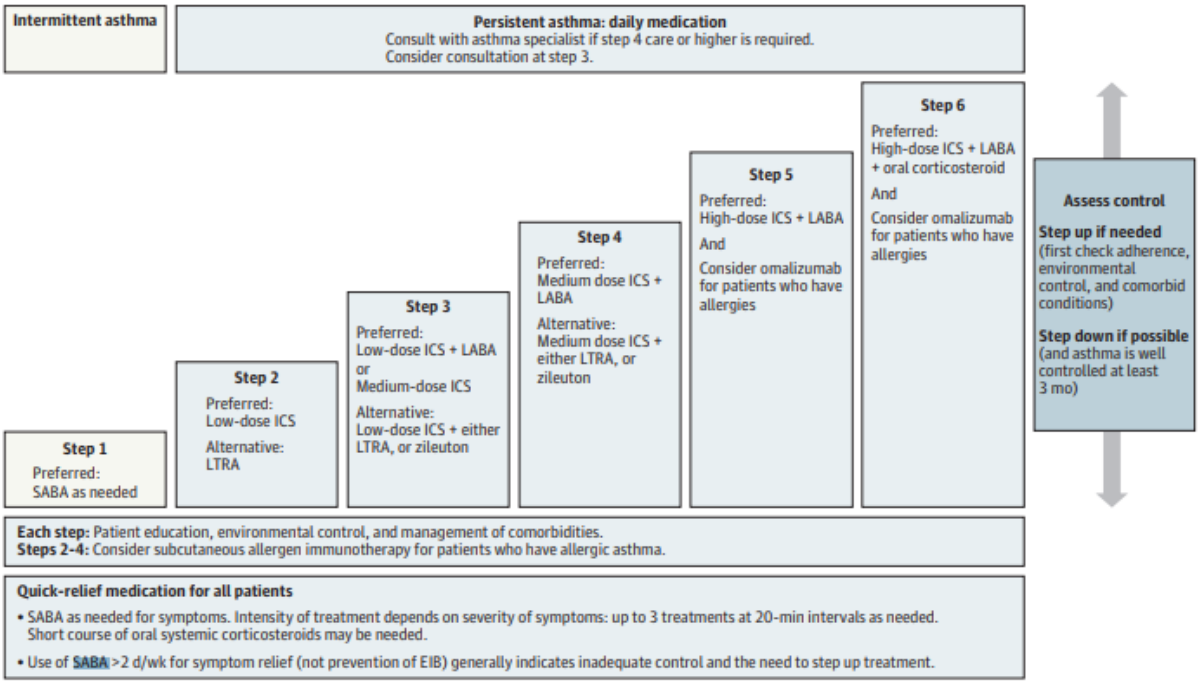

Figure 2.7. The current British thoracic guidelines on asthma management (British Thoracic Society, 2016).

2.4.1 Inhaled β_2-agonists

Short Acting Beta 2 agonists (SABAs) are used in cases of acute asthma, including exercise induced bronchoconstriction, where corticosteroids are ineffective. The usual dosing for SABAs, such as Albuterol, Levalbuterol and Pirbuterol is 2 puffs every 4-6 hours. SABAs modulate bronchodilation, causing as much as a 7-15% increase in FEV_1 (dose dependent) (McCracken et al., 2017), these changes can be effective in asthmatic athletes prior to a period of exercise, through the relaxation of airway smooth muscle and vascular permeability (Kindermann, 2006).

Intensity of SABA treatment is dependent on severity of asthma symptoms, if a patient uses their SABA more than 2 days of a week for symptom relief, it is generally indicated that there is inadequate control, therefore a revaluation of treatment methods is necessary.

Long acting beta 2 agonists (LABAs) include Salmeterol, Formoterol and Vilanterol. LABAs are used alongside inhaled corticosteroids to improve FEV_1. Usual dosing in adults with stable asthma is 2 puffs, twice daily.

2.4.2 Inhaled corticosteroids

Inhaled corticosteroids (ICS) or glucocorticoid steroids are the most effective form of treatment for the control of asthma, even in low doses they suppress airway inflammation. In contrast, they are ineffective at supressing pulmonary inflammation in patients with COPD. As mentioned previously ICS, such as Fluticasone and Budesonide, may be used in conjunction with LABAs for efficacy purposes, specifically in step 3 of the BTS guidelines (refer to figure 2.7.) (Barnes, 2010); this is due to ICS increasing β_2-adrenergic receptor transcription. Inhaled corticosteroid use is associated with the return of the goblet cell/ciliated epithelium ratio to normal, as well as a reduction in inflammatory cell infiltrate, specifically reduced levels of

eosinophils in the lamina propria of the airways. Other effects of corticosteroids are illustrated below in Figure 2.8.

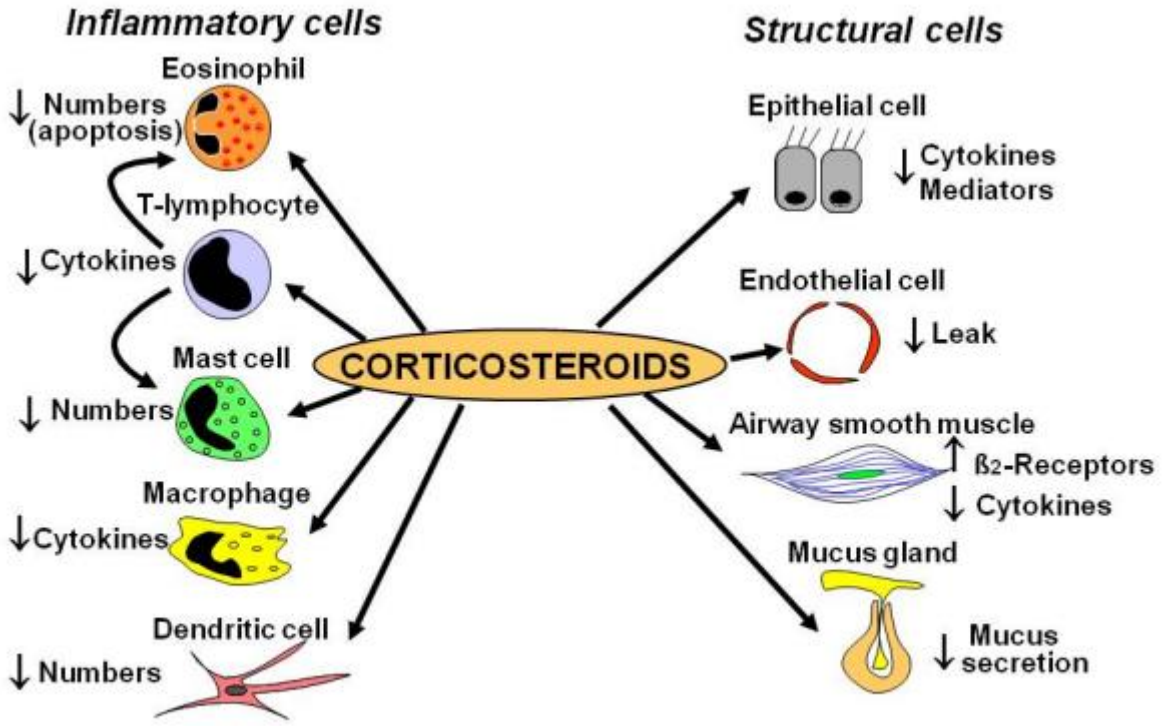

Figure 2.8. The effect of corticosteroids in patients with asthma (Barnes, 2010)

2.4.3. Leukotriene Modifiers

Leukotrienes (LTs), such as cysteinyl LTs (CysLTs) and LTB_4, are potent lipid mediators involved in asthma pathophysiology. In asthma, CysLTs augment airway smooth muscle activity, mucus secretion and microvascular permeability, through the $CysLT_1$ receptor. Oral Leukotriene receptor antagonists (LTRAs) are given to patients with mild asthma to counteract unwanted physiological effects, more severe patients undergo combined LTRAs/ICS treatment (Montuschi, 2010). LTRAs such as Montelukast may be given at 10mg daily in controlled asthmatics, to improve diurnal symptoms and FEV_1 (McCracken, 2017). Reductions in FEV_1, after bronchial provocation challenges, were abridged following montelukast use when

compared to placebo and the treatment was effective in up to 59% of the asthmatic subjects (Rundell, et al., 2005).

2.4.4. Side effects of pharmacological treatment methods of asthma

Inhaled corticosteroid (ICS) treatments are crucial in the treatment of asthma and are used in ever increasing doses. However, long-term use has been associated with the development of osteoporosis through the inhibition of osteoblast function, this can affect approximately ~50% of patients treated with ICS for longer than 12 months (Becker, 2013). This leads to a swift deterioration of bone quality through osteocyte apoptosis, specifically of the femoral head. Furthermore, patients taking ICS undergo adrenal tissue atrophy, as the adrenal cortex does not function normally, thus the prescribed steroid is not only treating inflammation but also altering normal physiological function. Alternate day-steroid therapy may be used for adrenal cortex function on a ‘drug-free-day. (Becker, 2013).

Other treatment methods, such as β2 agonists can cause systemic side effects, including mild tachycardia, tremor, and changes serum potassium and glucose levels. Non-pharmacological effects include hyper-responsiveness to specific and nonspecific stimuli, including exercise, allergens and increased airway inflammation (Sears, 2002).

2.5. Dietary Interventions to Treat Asthma, Inflammation and the Immune System

Non-pharmacological methods are often overlooked in clinical practice, and could be implemented to manage asthma directly, rather than only treating the symptoms and using preventative medication. The focus of the present thesis is to explore a nutritional intervention to aid asthma and inflammation management by observing changes in biological asthma markers.

The role of diet in disease has been studied for decades, research shows that the western diet is paralleled with increased asthma incidences. Western diets mainly consist of highly processed foods leading to a high intake of desserts, sweets, refined grains, processed meat and red meat, saturated fat dairy products and a low intake of fruits and vegetables (Guilleminault et al., 2017). On the other hand, the Mediterranean diet has been associated with a lower prevalence of wheeze and asthma (Nagel et al., 2010). The Med-diet consists of mainly fruit, vegetables, cereals, olive oil, nuts, seeds, olives, white meat, seafood and eggs. Some sweets, potato, processed and red meats are consumed, however, at a much lesser rate than the western diet. A Mediterranean diet contains an abundance of minerals, fibre, vitamins, antioxidants, flavonoids, carotenoids and low energy food content (Slavin and Lloyd, 2012). Fruit and vegetables are also rich in dietary fibre, which is metabolised into short-chain fatty acids (SCFA) that are able to modulate gut bacteria composition and regulate the immune system (see sections 2.3.1 and 2.3.2).

Dietary intake has been shown to regulate systemic inflammation and the Western diet is thought to promote pro-inflammatory responses and immune dysregulation. Contrarily, the Med-diet promotes anti-inflammatory responses due to various factors, such as increased antioxidant levels, which reduce oxidative stress, and low levels of saturated fats, which have been shown to activate TLR4 and the NFkB signalling cascade (Shi et al., 2006) (see section

2.2.4). The various anti-inflammatory effects of the Med-diet have been described in a meta-analysis reporting significant decrease in C-reactive protein plasma levels, IL-6 and ICAM-1, in healthy participants (Schwingshackl and Hoffmann, 2014).

There is evidence that diets consisting of high levels of Vitamin D, E, LC n-3 PUFA (fish oil), fruit, vegetables and fish have a beneficial effect on the risk of asthma and wheeze. These beneficial effects are dependent on diet during specific life stages, for example, there is very strong evidence that vitamin D reduces child asthma risk during pregnancy and LC n-3 PUFA during childhood (Guilleminault et al., 2017). Furthermore, a high fruit and vegetable diet for 14 days has been reported to improve both FEV_1 and FVS, conversely no change was seen in participants with a low fruit and vegetable diet (Wood et al., 2012). Various studies have suggested that Vitamin D intake during pregnancy is associated with a reduction of wheeze in children (Douros et al., 2019). Indicating that vitamin D treatment during pregnancy could be beneficial in reducing asthma risk.

2.5.1 Chronic Inflammatory Disease

Chronic inflammation, also known as slow, long-term inflammation has many causes. Failure to eliminate an agent of acute inflammation such as *tuberculosis*, protozoa, fungi or any other parasite resulting in their survival in host tissue for extended periods of time may lead to chronic inflammation. Chemical exposure at a low, levels for a long period of time, that cannot be eliminated through phagocytosis or enzymatic breakdown may also prolong inflammation. Autoimmune disorders, where host components are recognised as foreign antigen, allow the development of diseases such as rheumatoid arthritis and systemic lupus erythematosus and defects in cells responsible for inflammation may also result in auto-inflammatory conditions. Oxidative stress caused by inflammatory responses and leading to mitochondrial dysfunction

results in advanced glycation end products, free radicals, uric acid and oxidised lipoproteins amongst others, which can result in chronic inflammation (Pahwa et al., 2021).

Chronic inflammatory disease cause the most death in the world and are the greatest threat to human health (WHO, 2021). Three out of five people day worldwide due to chronic inflammatory diseases such as chronic respiratory diseases, heart conditions, cancer, diabetes, obesity and stroke (Peixoto de Barelos et al., 2019, Tsai et al., 2019). Diabetes is the 7th leading cause of death in the US and 9.4% of population had diabetes in 2015. Cardiovascular disease accounts for 31% of deaths worldwide, coronary heart disease accounting for most cardiovascular deaths. Forty-three million Americans suffer with arthritis and joint diseases, and asthma affected more than 24 million people in the US, of that 6 million or more are children. Finally, in 2014 chronic obstructive pulmonary disease was the third most common cause of death in the US (Pahwa et al., 2021). Increased dietary fibre intake has been associated with a decreased risk of colorectal cancer (Aune et al., 2017), coronary heart disease and other cardiovascular disease (Threapleton et al., 2013). It has also been demonstrated that dietary fibre intake has inverse relationship with certain biomarkers of systemic inflammation (McLoughlin et al., 2017). Reducing systemic inflammation through dietary intervention may be a viable method to reduce the risk of developing and treat chronic inflammatory diseases such as asthma, dementia, cerebrovascular diseases, cancer, chronic lower respiratory diseases and ischaemic heart disease (Willerson et al., 2004, Cunningham, 2011).

Markers of systemic inflammation such as high-sensitivity C-reactive protein (CRP) are indicative of inflammation but not a specific marker of chronic inflammation. Pro-inflammatory cytokines such as TNF- α, IL-1β, IL-6 and IL-8 can be measured in order to identify specific causes of chronic inflammation. However, both of these measures are not standardised tests and there are no effective measures to assess and diagnose chronic inflammation in a laboratory setting, diagnoses can only transpire when inflammation occurs

in association with a medical condition (Pahwa et al., 2021). Chronic inflammation a key characteristic in not only Asthma but a range of conditions such as, Alzheimer's disease, cancer, chronic kidney disease, heart disease, inflammatory bowel disease, rheumatoid arthritis, ankylosing spondylitis and type 2 diabetes. A handful of environmental, lifestyle and social factors contribute to systemic chronic inflammation, which is a driving force behind the risk factors for these diseases (Furman et al., 2019).

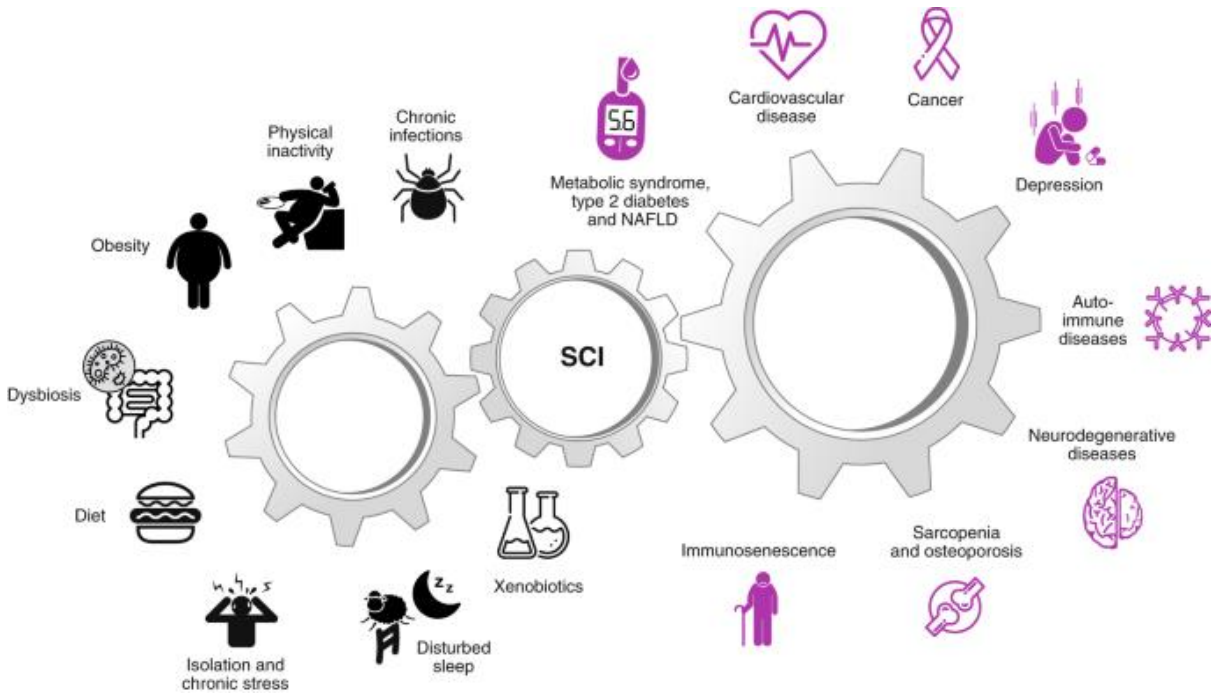

Figure 3.1.2. The causes of low-grade systemic chronic inflammation (SCI) and the consequences for human health and disease (Furman et al., 2019).

Evidence for the association of SCI and disease risk is comprised mainly from RCTs that use anti-inflammatory agents targeting specific pro-inflammatory cytokines such as TNF-α. The implications and evidence for the role of TNF-α in chronic inflammatory disease risk is growing rapidly and the benefits of anti-TNF-α therapy have been demonstrated in both rheumatoid arthritis and Alzheimer's disease (Burska et al., 2015; Camargo et al., 2015). Previously, Williams's et al., demonstrated that dietary treatment with prebiotic B-GOS

reduced plasma TNF-α and CRP in control and patients with exercise-induced bronchoconstriction, highlighting the potential benefits of B-GOS and prebiotic use in chronic inflammatory disease (Williams et al., 2016). As such, the current thesis aims to not only elucidate the effects of B-GOS in asthma, but also assess the effects of B-GOS on markers of systemic chronic inflammation and its potential use in chronic inflammatory diseases.

2.5.2 Prebiotic, Probiotic and Synbiotic Supplementation

Prebiotics are defined as a 'non-digestible food ingredient that beneficially effects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health' (Gibson and Roberfroid, 1995). Over the past few decades, it has been recognised that small changes in dietary habits may allow for positive health values, such as the introduction of prebiotics into the diet, through changes in microbiota composition and host physiology.

The more updated definition of prebiotics is as follows: 'a prebiotic is a selectively fermented ingredient that allows specific changes, both in composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health' (Gibson, et al., 2004). In order for a food ingredient to be classified as a prebiotic it must satisfy various scientific characteristics, the ingredient must therefore:

- Resist gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption
- Be fermented by the intestinal microbiota
- Stimulate the selective growth and/or activity of intestinal bacteria associate with health and well being

Any food ingredient that has the ability to evade digestion in the GI tract has the potential to be a prebiotic, however, only certain carbohydrates have satisfied the requirements of a

prebiotic. Most prebiotic research has been focused on galactooligosaccharides (GOS), fructooligosaccharides (FOS) and inulin (Macfarlane and Cummings, 2006). The most difficult aspect of confirming a food ingredient as a prebiotic relates the third criteria listed above, as in order to measure growth/activity of intestinal bacteria a live faecal sample is needed, in vitro or in vivo work cannot confirm a prebiotic effect (Gibson, et al., 2004). Both FOS and GOS are not substrates for hydrolytic enzymes in the upper region of the digestive tract and are not sensitive to gastric acid, they have also been demonstrated to stimulate growth and activity of intestinal bacteria in the lower regions of the gut (Liu et al., 2017). Following a 7-day treatment period, GOS was shown to generate the greatest prebiotic effects, with a larger bifidogenic effect than short chain FOS (Bouhnik, et al., 2004). Other potential prebiotics show promising data, however, there is not enough to confirm prebiotic effects; potential candidates for prebiotics include glucooligosaccharides, isomaltooligosaccharides, lactosucrose, polydextrose, soybean oligosaccharides and xylooligosaccharides (Bandyopadhyay and Mandal, 2014).

Conversely to probiotics, where live microorganisms must compete with established microbiota communities, prebiotics target bacterial colonies commensal to the large intestine and are arguably a more practical and efficient way to manipulate microbiota. Furthermore, prebiotics may be less likely to cause any GI symptoms than probiotics, as they are simply an energy source, whereas trying to alter the microbiome rapidly through live probiotic bacteria may cause GI discomfort, diarrhoea, clostridium difficile and atopic dermatitis (Islam, 2016). Moreover, a synbiotic may be a more favourable and efficacious approach instead of solely probiotic treatment.

The enzymatic treatment of whey derived lactose by β-galactosidase produces multiple oligomers of varying chain lengths through a multitude of sequential reactions; collectively the intermediate products are known as galactooligosaccharides (Torres et al., 2010). GOS are

comprised of 2-8 saccharide units, one units being a terminal glucose and the remaining units being galactose and disaccharides of 2 units galactose (Tzortzis and Vulevic, 2009). Galactooligosaccharides have a β1/4 linkage, they are stable in acidic environments, at high temperatures, and also have a very low calorific value of 1.7kcal g^{-1}, making them of interest in the food industry (Torres et al., 2010). Many health benefits have been associated with GOS, mainly due to their bifidogenic effect within the lower regions of the gut (Bouhnik, et al., 2004). The Prebiotic supplement used in the current study (B-GOS) was manufactured using a novel strain of *Bifidobacterium Bifidum* (NCIMB 41171) isolated from a healthy volunteer's faecal sample, a common bacterium in healthy individuals. The bacterial strain used expressed β-galactosidase and was utilised to synthesise GOS from lactose (Tzortis and Goulas et al., 2005), into a powder form. The prebiotic is a *trans*-galactooligosaccharide (B-GOS) and a commercially available product.

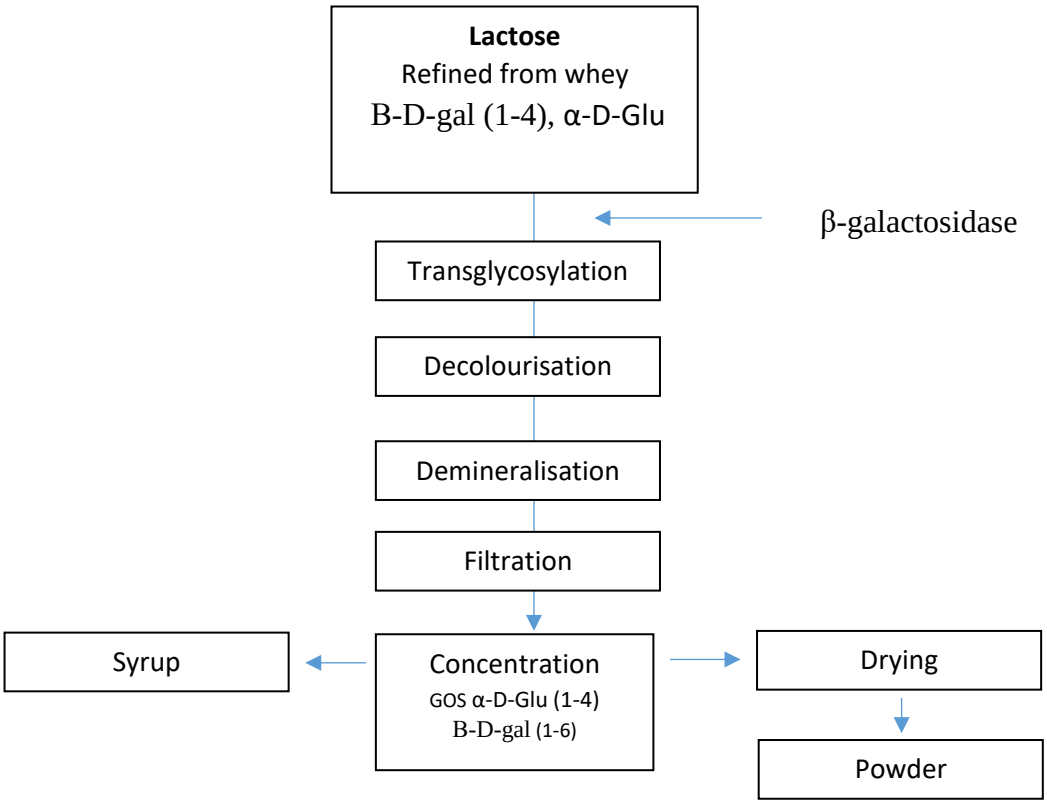

Figure 2.9. Schematic diagram showing the synthesis of GOS from refined whey, GOS; galactooligosaccharides. Adapted from Angus, Smart and Shortt. (2005).

The properties of B-GOS have been studied in a variety of conditions, including *in vivo* work, as well as an *in vitro* study in pigs. *In vivo,* sensitivity assays were used to determine toxic effects of the oligosaccharide mixture on HT29 cells, as well as adhesion assays using the same cell line to observe colony growth. The mixture was added into the diets of male pigs, SCFA concentrations where then analysed from fecal samples, as well as bacterial enumeration and lactic acid production. An in vitro dose study using pigs observed 4 different diets, control, 1.6% GOS, 4% GOS and Inulin. Following a 4-week treatment period pigs were euthanised and the entire large intestine removed, bacteria was quantified in the proximal colon to assess the prebiotic potential of GOS. Pigs fed 4% GOS were shown to have greater numbers of *Bifidobacterium* than control or 1.6% GOS ($P < 0.05$), however, 4% GOS did not differ from pigs fed inulin ($P = 0.533$).

An *In vitro* gut model was used to assess the effects of GOS supplementation on colonic microbiota composition, this model consisted of three man made vessels in a fermenter that represented the proximal, transverse, and distal colon. The total population of bacteria number was unchanged in this model, however, numbers of *Bifidobacterium* and *Lactobacillus* increased significantly (Tzortis and Goulas et al., 2005). The data obtained suggest that B-GOS has a greater and more significant prebiotic potential than inulin. Furthermore, in a 10-week randomised, double-blind, placebo controlled, cross-over human intervention study, the influence of B-GOS on gut microbiota was assessed with a four-week washout period between testing conditions. B-GOS was demonstrated to enhance faecal bacterial populations of *Bacteroides,* but primarily *Bifidobacterium.*

In a clinical study by Williams et al., (2016) ten asthmatic and eight controls completed a double blind, randomised, 8-week prebiotic intervention study with B-GOS. Participants received either the prebiotic or placebo for three weeks, followed by a 2-week washout period and then a crossover. Participants with EIB and asthma experienced attenuation of airway

hyper-responsiveness, along with a reduction of markers associated with airway inflammation and EIB, from baseline to prebiotic. The positive effects of prebiotics have been recorded on numerous occasions, however there is limited research into the effects of prebiotics on asthma patients. Likewise, there is even less knowledge on how prebiotics induce their positive effects on the lungs, and how prebiotics may be able to modulate lung inflammation. In order to potentially use prebiotics as a form of treatment in airway specific diseases, we must fully understand the mechanisms in which the prebiotic elicits its effects.

2.5.3 Prebiotic Mechanism of Action

Prebiotics have been demonstrated to have various positive effects on the body and have been shown to have a beneficial impact on certain auto-immune diseases and various mental health diseases (Clapp et al., 2017). In the current PhD programme, we aim to elucidate how prebiotics effect lung inflammation in asthmatics and systemic inflammation in healthy volunteers. There are different theories for the mechanism of action of prebiotics, however, each of the main theories originate at the metabolism of prebiotics in the colon.

Prebiotics are fermented by bacteria in the colon to form short chain fatty acids (SCFAs); mainly acetate, propionate, and butyrate. The various actions of SCFAs have been discussed previously, particularly the bifidogenic effect of their production, which is thought to affect asthma. However, the actual mechanism of SCFA regulation of asthma is unknown.

The main theories for Prebiotic/SCFA action have been overviewed in figure 3.0. The first, and possibly most supported theory, is that prebiotics induce the proliferation of probiotic bacteria, leading to prominent immunological effects through intestinal epithelial cell signalling (see section 2.3.3.). Immune cells are then transported in the blood to distant sites, i.e. the lungs, where they have a direct effect on immune regulation. A different theory is that SCFAs may

work in a co-dependent manner to activate IECs, leading to augmented immune cell activation followed by their migration to distant sites.

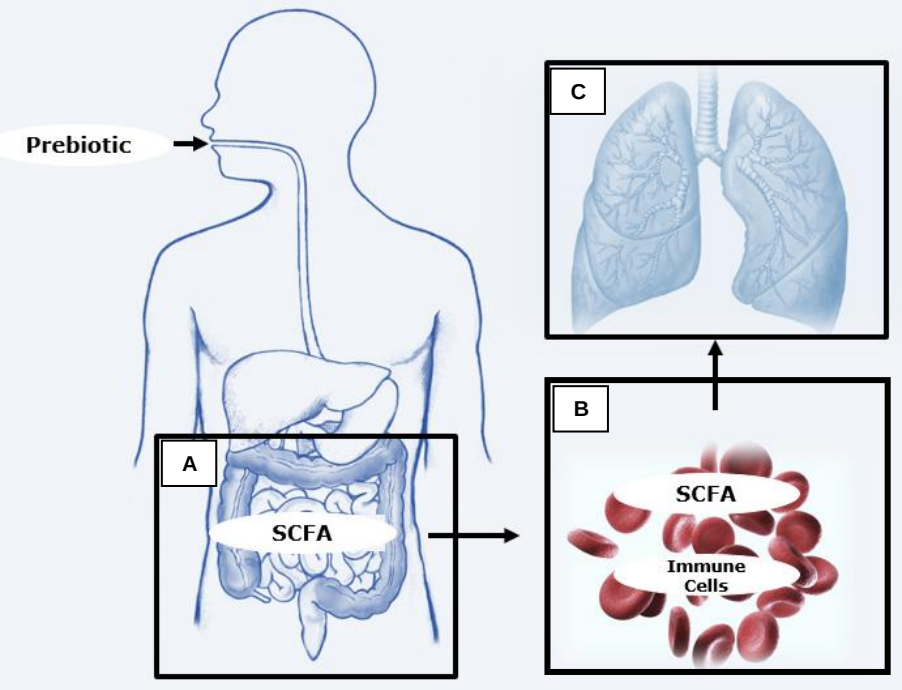

Figure 3.0. Theories for the mechanism of action of prebiotics (A) SCFAs increase the growth of bifidobacteria in the gut. Activation of intestinal epithelial cells (IECs) via bacteria or SCFAs leads to immune cell activation and signalling through the gut associated lymphoid tissue (B) SCFAs may enter the blood in the form of Butyrate, Propionate or Acetate along with activated immune cells (C) Activated Immune cells or SCFAs in the blood may modulate the lung inflammatory response through receptors on the lung epithelium. SCFAs may also activate immune cells in the blood through G-protein coupled receptors 41/43/109A or directly modulate HDAC activity, bypassing cell surface receptors. Self-made schematic, 2019.

Another theory for prebiotic action involves the transport of SCFAs in the blood to distant sites where they can elicit their effects. Following prebiotic treatment, it has been demonstrated that plasma concentrations of acetate, propionate and butyrate levels are somewhat increased. Over 90% of SCFAs are absorbed in the intestinal lumen, with the majority metabolised by colonocytes or transported to the liver through the hepatic portal vein. However, the remaining SCFAs, primarily acetate, enter systemic circulation with the proposed effect of reducing systematic inflammation through G-protein coupled receptor modulation (GPCR41 & 43) and inhibition of histone deacetylase enzymes (McLoughlin et al., 2017). Moreover, SCFAs have been indicated to modify the recruitment leukocytes to inflammatory sites, however, this effect has been demonstrated to vary in different experimental conditions. In vitro studies show

chemotaxis of neutrophils to be dependent on GPR43 (Maslowski et al., 2009), which couples to Gi/o and Gq proteins for signal transduction. GPR43 is expressed primarily by neutrophils and monocytes and SCFAs can bind as a ligand and activate this GPCR, inducing migration (Vinolo et al., 2011). Furthermore, in mice, SCFA metabolite propionate was shown to reduce airway eosinophilia in comparison to mice treated with a saline solution, this effect was demonstrated to be dependent on GPR41 (Tromepette et al., 2014). These results indicate an important role for DCs expression in prebiotic regulation of allergic inflammation.

Histone acetylation is a reaction involved in a diverse range of cellular functions, including inflammatory gene expression, cell proliferation and DNA repair. There are 11 histone deacetylases (HDACs) which are responsible for regulation of inflammatory/immune gene expression. An example is HDAC2, located in lung macrophages, this protein is a key regulator of inflammatory gene expression and forms a complex with NF-κβ to elicit its affect. In asthma patients, corticosteroid treatment supresses histone acetyl transferases to allow recruitment of HDAC2 to the NF-κβ gene complex and subsequently suppression of inflammatory gene expression. HDAC2 is significantly reduced in the blood of severe asthmatics and COPD, resulting in increased oxidative and nitrative stress in the lungs (Barnes, 2009; Bhavsar et al., 2008).

SCFAs, the product of prebiotics, have been demonstrated to elicit specific effects on the intestinal epithelium through the direct inhibition of HDACs and modulation of gene expression. Modulation of gene expression is facilitated through SCFA activation of GPR41, GPR43 and GPR109A, and thus the MAPK signalling pathway; GPR43 and GPR109A can also activate the NF-κβ signalling pathway. SCFA concentration progressively decreases from the proximal colon to the distal end from 70 to 140mmol/l down to 20-70 mmol/l, respectively. Both GPR41 (FFA3) and GPR43 (FFA2) have an EC_{50} of around 0.5mM; potency ranks for

GPR41 are Propionate/butyrate > acetate, and potency ranks for GPR43 are acetate/propionate > butyrate (Brown et al., 2002; Ulven, 2012). GPR109A is expressed mainly on neutrophils, adipocytes and macrophages, there is not yet evidence suggesting it is expressed on epithelial cells, and butyrate is the main ligand for this receptor. SCFAs may pass through the portal vein, and the majority are then metabolised in the liver, however, a small but measureable concentration can be observed in systemic circulation, approximately 0.08mM in peripheral blood (Venegas et al., 2019).

A current hypothesis for SCFA action in asthma and inflammation is that acetate in systemic circulation is capable of modifying HDAC activity of Treg cells located at distant sites, leading to increased Foxp3 expression, enhanced immunosuppression, and regulation of AHR. In a murine study the effects of a high fibre diet on SCFA serum levels were measured, the control diet observed ~170uM of SCFAs, whereas, high fibre observed ~300uM of SCFAs. Acetate levels in the controlled diet and high fibre diet were 150uM and ~260uM, respectively and concentrations of acetate were highest in serum compared with propionate and butyrate, with a ratio of 5:2:1. A concentration of 0.26mmol/l blood acetate, as observed in this murine study, would produce a maximum GPR43 response of 26%, it is unclear whether or not this concentration would produce a meaningful response through epigenetics. Further work is needed in order help uncover the role of SCFAs in systemic circulation. Moreover, it may be the beneficial effects of SCFAs are mediated through direct activation of IECs and/or indirect activation through probiotic bacteria and subsequent immune cell signalling in the blood, as aforementioned. Changes in Foxp3 expression in the lungs through HDAC9 inhibition may be dependent on these mechanisms originating within the GALT and the complex immune cell signalling that follows. (Thorburn et al., 2015).

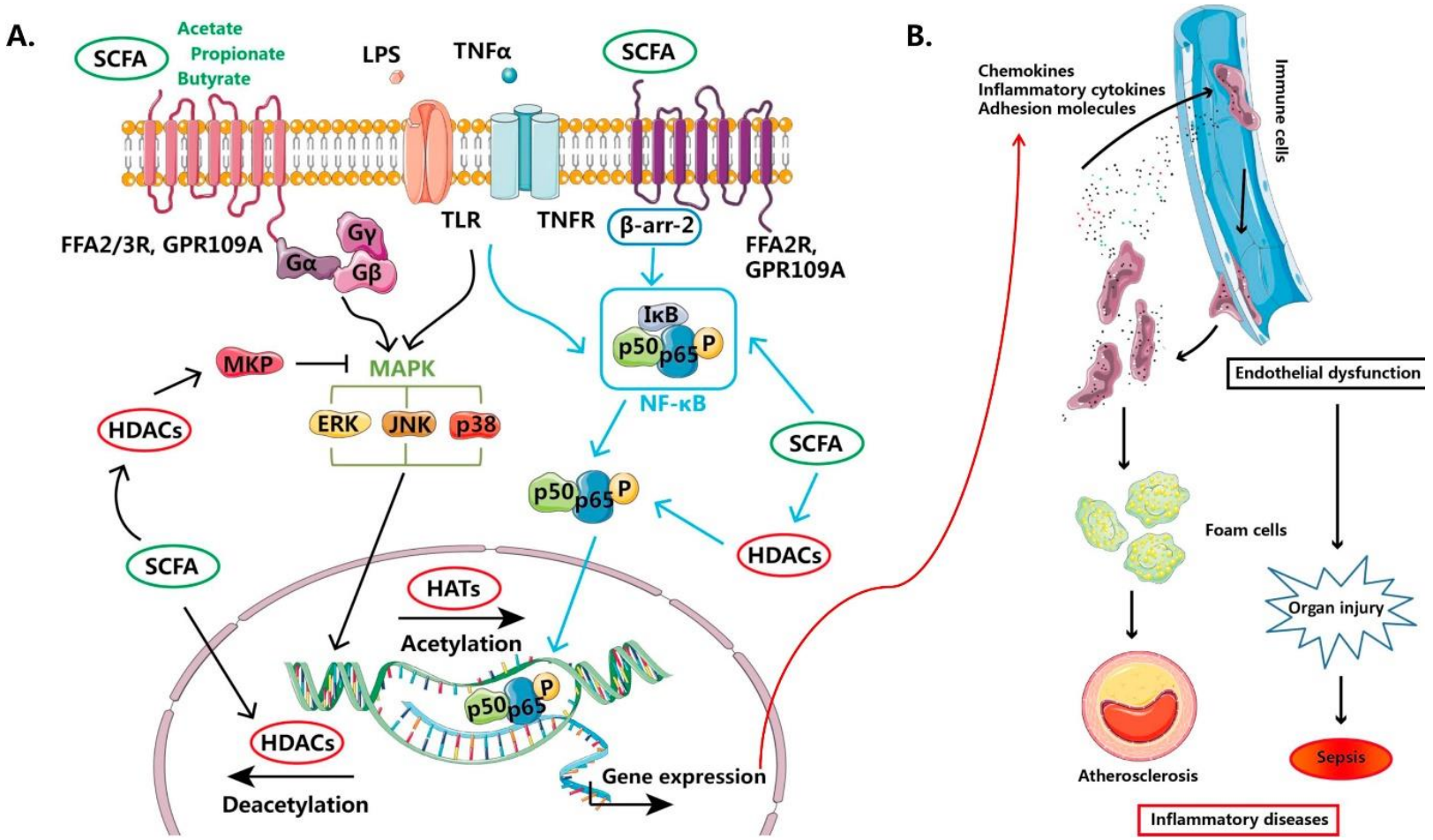

Figure 3.1. (A) The Effects of SCFAs, LPS and TNFα on the intestinal epithelium and inflammatory gene expression. SCFAs, mainly acetate, propionate and butyrate regulate NFkB activation and the MAPK signalling pathway, through FFAR2/3R and GPR109A. SCFAs may directly inhibit HDAC activity and therefore modulate gene expression. (B) The effects of increased chemokine, cytokine, and adhesion molecule expression in the pathophysiology of inflammatory disease (Li et al., 2018)

2.6 General Discussion

The study of the gut microbiome and its role in inflammation is growing exponentially, however, the difficulty of analysing commensal bacteria in humans remains a barrier to progress. To better understand the effects of prebiotic intervention on asthma and inflammation in disease, a more holistic approach may be necessary (Wagenaar et al., 2021). Standardised outcome measures, and the observation of pro-inflammatory markers such as TNF-α, may allow for a better understanding of the effects of prebiotic interventions on shared pathophysiology in patients with multiple chronic inflammatory conditions. A greater number of research studies are necessary for the evaluation and potential use of prebiotics in the treatment of asthma and multiple chronic inflammatory conditions.

Current pharmacological treatment of chronic asthma involves the use of inhaled corticosteroids and bronchodilators, both of which treat asthma symptoms. However, traditional asthma treatment methods are not always effective in providing relief and long-term corticosteroid usage can result in systemic side effects. In order to progress and improve the treatment of asthma we must attempt to target the underlying causes of inflammation. Previous work has demonstrated the effective use of B-GOS in an asthmatic population (Williams et al., 2016), however, more research is required to confirm its effects different asthma phenotypes, as well as uncover its mechanism of action.

Additionally, epidemiological data has demonstrated a parallel increase in asthma with western lifestyle adaptations (Brigham et al., 2015). Similarly, this increase in western lifestyle is also associated with chronic low-grade inflammation, which contributes to the development of many inflammatory diseases. It is likely that one of the main driving factors for the prevalence of inflammatory disease with increased western lifestyles, is alterations in the gut microbiota. Dysbiosis has been recorded extensively in patients with multiple inflammatory conditions,

including asthmatic patients (DeGruttola et al., 2016; Hufnagl et al., 2020). Treatment with prebiotic B-GOS has been previously demonstrated to positively alter the gut microbiota (Vulevic et al., 2013). B-GOS may be a significant therapeutic target in the treatment of asthma, multiple chronic inflammatory conditions, and may also reduce low-grade systemic chronic inflammation and therefore disease risk in healthy individuals. As such, this area requires further investigation to:

- Elucidate whether manipulation of the gut microbiota through prebiotics and synbiotics can provide a significant benefit for patients with asthma
- Determine if B-GOS reduces systemic inflammatory markers in healthy participants and if it has a protective effect during chronic inflammation
- Elucidate the mechanistic actions of B-GOS and its role in inflammation

3. Chapter 3 – General Methods and Method Development

3.1. Participants

Participants were recruited using advertisements around both Nottingham Trent University campuses, leisure centres and gyms, and through the asthma awareness stand ran on Clifton campus (Chapter 4). For each experimental study, all participants were required to fill out a health screen questionnaire (Appendix 1) and to provide written informed consent (Appendix 2 and Appendix 6). All studies obtained approval from the NTU Research Ethics Committee. Participants recorded their diet 24 hours prior to their first visit and a 4-day food diary leading up to visits 2, 3, and 4. On testing days participants abstained from caffeine (24 hours prior), alcohol (48 hours prior), anti-histamines (72 hours prior), food (3 hours prior) and strenuous exercise (24 hours prior). Inclusion and exclusion criteria are outlined in Appendix 3 and Appendix 7 for both intervention studies. In each experimental chapter there is more detailed information on methodology (See sections 4.2, 5.2, 6.2, and 7.2).

3.2. Anthropometric Measurements

Body mass was calculated using a digital scale (AE Adam GFK150 scale, AE Adams, Birmingham, UK) to the nearest 0.01 kg, whilst participants were barefoot. Height measurements were obtained using a stadiometer with participants standing barefoot (SECA stadiometer, SECE, Birmingham, UK) with their heels together, arms relaxed and looking straight ahead (Utkualp and Ercan, 2015).

3.3. Flow Cytometry

Flow cytometry is a method used to measure the optical and fluorescent characteristics of single cells. The underlying principles relate to the scattering of light and fluorescence emission, as a result of particle excitation (usually from a laser). An antibody conjugated with a fluorophore binds to its target, following excitation, fluorescent emission is obtained (stoke shift) and is proportional to the amount of fluorescent probe bound to the cellular component.

Data acquired using flow cytometry is valuable in understanding biophysical, biochemical and molecular characteristics of different particles.

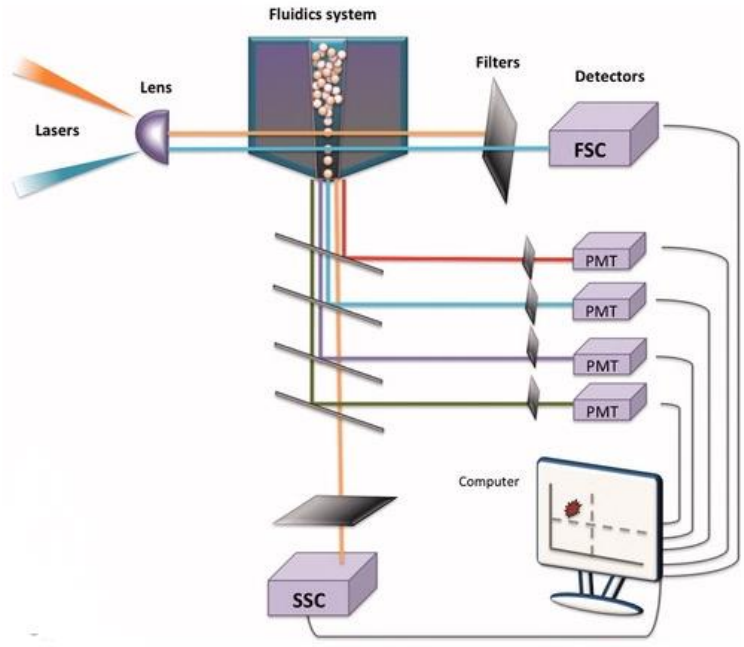

Figure 3.2. Flow cytometry analysis. Abbreviations; FSC, forward scatter; PMT, photo-multiplier tube; SSC, side scatter. Cells undergo intracellular staining with specific antibodies prior to acquiring data. Fluorophore conjugated antibodies bound to specific sites within the sample are excited by a laser, emission is then measured through a detector and particles are quantified.

The fluidic system of the flow cytometer is responsible for transportation of cells for data collection. Sheath fluid containing Phosphate buffered saline, with a pH ranging from 7.0 to 7.4 (10X Clear Flow Sheath Fluid, Leinco, Missouri, US) is injected into the centre chamber of the instrument through pressurised lines. Cells are injected into the chamber at a higher pressure than the sheath fluid, forcing cells to align in single file prior to laser excitation. The machine must be cleaned after use to preserve this process as debris and air bubbles may alter results. Once, organised cells pass through the laser, lasers are lenses are located in the optical bench, in fixed positions. Light is deflected around cells as forward scatter (FSC) and side scatter (SSC). FSC is proportional to cell surface area and SSC to cell granularity (Adan et al., 2016).

In Chapter 6, Flow cytometry was used to quantify a total of 10 markers: Foxp3, TNF-α, NF-κβ, IL-6, CD3, IL4, CD4, CD25, GATA3 and live/dead cells. All methods were optimised using various titration methods, blocking protocols, staining kits and technical software adjustments. Compensation analysis and pilot work were completed in collaboration with the Jon van Geest Cancer Research Centre. The flow cytometry gating procedure involved the identification of live cells using forward scatter and side scatter intensity measurements. Live CD3 cells were then identified, followed by $CD4^+$ cells, and then subsequently $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ cells. The first data point, for each biomarker, of every participant were gated to separate the excitation and emission peaks. The biomarker gate remained constant for each visit after the initial gating procedure, in order to remove any potential bias, protein was quantified as % gated. If there were any clear and obvious errors in gating, from observing excitation and emissions peaks, gates were unlinked from the first data point to allow correction.

3.4 Cell Stimulation

In Chapter 7, a cell stimulation was used to induce chronic inflammation in peripheral blood mononuclear cells. In order to complete this research, the optimal method to stimulate cytokine production was required. Following the recommendation by Wenchao et al., and discussion with the Jon van Geest Cancer Research Centre it was decided a PMA/Ionomycin stimulation would be used in the current study. Wenchao et al., recommended 25 ng/mL PMA and 1 μg/mL ionomycin for a 6hr stimulation on the undiluted whole blood of rats. Their results demonstrated rapid increases of cytokines such as IL-2, IFN-γ, TNF-α, RANTES and TGF-β. In the current study, we were unable to store human tissue as NTU did not have a human tissue authority licence at the time. This meant all testing, including the drawing of blood, was required to be completed in one protocol. The 6hr stimulation recommended by Wenchao resulted in 10% cell death, however, it was an unfeasible incubation due to timing constraints, unless cells could be stored overnight following a stimulation. Overnight storage was piloted,

however, this led to 80% cell death. Growth factors were then used to help proliferate cells overnight and cell death was reduced to 60%. Following multiple changes to methodology it was discovered cell death could not be reduced further post stimulation than 50%, and that flow cytometry data was affected by this high level of cell death. The cell stimulation incubation period was reduced to 5hrs and the final concentration of PMA was 50 ng/ML and ionomycin 1ug/mL, following the testing of multiple concentrations and incubation periods. This 5hr incubation allowed for all testing to be completed in one day, after blood collection at 7-8am.

3.5. Leukocyte Differentiation Analysis

The importance of sub-phenotyping asthma has been acknowledged by researchers for decades, treatment has evolved from identification of asthma subtypes, such as allergen-induced asthma, exercise induced asthma and non-allergic asthma (Hekking and Bel, 2014). Various asthma phenotypes can be distinguished by looking at biomarkers within the blood, such as eosinophil and neutrophil counts, these markers may indicate eosinophilic asthma/non-eosinophilic asthma, or neutrophilic asthma/non-neutrophilic asthma; respectively (Wenzel et al., 1999). It is hypothesised that peripheral blood mononuclear cells, consisting of lymphocytes (T cells, B cells and NK cells), provide insight and reflect asthma severity in children (Grutta et al., 2003). This assumes that asthma is a systemic disease and any abnormalities in the blood indicate airway abnormality. Various subtypes of asthma may respond to specific treatments in different ways, therefore for successful treatment these subtypes must be correctly categorised and identified.

During asthma exacerbations both progenitor cells and hematopoietic stem cells contribute to allergic inflammation. The release of pro-inflammatory cytokines cause differentiation of these progenitor and hematopoietic stem cells, leading to the production of effector cells, which are central in the pathogenesis of asthma. During an asthma exacerbation, effector cells such as

eosinophils and basophils are recruited to the airways, augmenting allergic inflammation (Fischer and Agrawal, 2013). Eosinophils are responsible for secretion of Th2-inducing cytokines (see section *2.2.2)* and function as the effector arm of Th2 immunity (Spencer and Weller, 2010). Th2 induced Eosinophilic inflammation is apparent in the airway lumen of 50% of asthmatics, whereas in severe asthma Th17 cells recruits neutrophils to the airway. Kamath et al., demonstrated that FEV_1 was inversely proportional to neutrophil counts in a group of 59 asthmatics (Kamath et al., 2005).

Blood cell parameters in asthmatic patients can be measured using automated haematology analysers, facilitating differential leukocyte analysis. However, due to a lack of knowledge these automated machines are not being optimally utilised to their full potential, further optimisation and improvements to this method may enable better diagnosis of disease, such as asthma (Chhabra 2018). When attempting to monitor differences in lymphocyte count, reproducibility in crucial. Monitoring differences in blood parameters over a 3 week period will improve the quality and evaluation of Asthma intervention strategies in Chapter 4. Therefore, the aim of the current study was to evaluate the short- (1 day), medium (14 days) and long-(21 days) test-retest reproducibility of the haematology analyser and the variation of whole blood parameters. It was hypothesised that all control participants would receive reproducible leukocyte counts over 21 days. Additionally, whole blood samples were measured repeatedly on testing days at 0, 2, 4, and 6 hrs to assess short term reproducibility of the analyser, as well as the stability of whole blood samples. The rationale for this, was that on test days in Chapter 4, whole blood samples may be analysed at different time points due to uncontrollable variables.

Healthy adult participants were recruited from Nottingham Trent University, Clifton Campus. Throughout the study participants were instructed to continue their habitual lifestyle. Participants abstained from alcohol, caffeine and strenuous exercise for 24 h prior to each

laboratory visit. Participants recorded their diet 24 hours before each visit and consumed 500 mL water. A fasted 4 mL venous blood sample was obtained by a trained phlebotomist (see section 6.2.3).

3.6. Mass Spectrometry

In Chapter 4, pilot work was conducted using the mass spectrometer in The John van Geest Cancer Research Centre (Nottingham, UK). A blood sample was taken and PBMCs isolated using the methodology described in Chapter 6 (Section 6.2.3). A lysis mix was then prepared using sample and RIPA buffer (1:100). After 30 minutes on ice, samples were passed through a 29 Gauge fine needle 20 times, avoiding the introduction of air. The protein was then quantified using BSA protein standard assay. 50uG of protein was taken from the sample and transferred into an Eppendorf tube for sample clean up and protein digestion prior to mass LCMS analysis using the Sciex 5600 or 6600. A total of 1309 different molecules were identified from the single blood sample taken. Mass spectrometry analysis was to be completed in Chapter 4 for each participant sample using proteomic software. This work was piloted in collaboration with Dr. Amanda Miles from John van Geest (JvG), however, due to staffing changes within JvG and logistical aspects it was not implemented into the study.

4. *Chapter 4 – A Study Proposal for Adults with Asthma: The effects of prebiotic trans-galactooligosaccharide (B-GOS) supplementation on quality of life, control of asthma, and markers of systemic inflammation*

4.1. Introduction

This experimental chapter has been presented as a study proposal. Data collection commenced on 27.01.2020, two asthmatic participants completed visit 1 and 2 by 20.02.20. A total of 5 participants were recruited by 13.03.20, however, due to the global pandemic data collection was halted and all laboratories on NTU campus were closed on 19.03.20. Participants who had already began the intervention were unable to finish the study and there was insufficient data for observational analysis. During the COVID-19 lockdown and uncertainty around research, a meta-analysis was conducted and data presented in Chapter 5. Following the easing of restrictions and recommencing of research it became clear the risks of working with asthmatics were significantly high and as consequence, testing unfeasible. Therefore, the study was terminated and restructured to examine the role of B-GOS on systemic inflammation in healthy participants, as presented in Chapter 6.

Asthma is syndrome which accounts for 4 out of 1000 deaths (see section 2.1.1. for Asthma prevalence). This inflammatory disease leads to airway obstruction, hyper-secretion of mucous, airway remodelling and airway hyper-responsiveness (Kudo et al., 2013). The international patterns of asthma prevalence are unknown and current knowledge does not explain causation of the syndrome. Research into primary and secondary interventional strategies, as well as causation studies on asthma are crucial areas that need exploration in order to produce more effective treatment that tackles the underlying pathology of asthma. Research is ongoing, and many studies are currently trying to categorise asthma into various clinical phenotypes in order to better understand asthma and how to personalise treatment to specific groups (Wenzel, 2013). Furthermore asthma medication is not available to many millions of people due to economic factors or poor health care systems. When there is greater understanding of the factors that cause asthma, pharmacological measures can be implemented which reduce the prevalence of

asthma directly, we will then be able to better asthma-management worldwide using cost-effective approaches (Masoli et al., 2004; Nunes et al., 2017).

4.1.1. The Gut Microbiome and the immune response

A considerable amount of asthma research has been directed at the gut microbiome and how this complex microbial ecosystem influences immune responses in the lungs. Certain gut microbes have been linked with increased proportions of T regulatory cells in murine models (Atarashi et al., 2013), specifically *Bifidobacterium*, *Lactobacillus* and *Clostridium* strains have been demonstrated to produce IL-22, leading to the reinforcement of epithelial barriers and a reduction of intestinal permeability. Additionally, *Bifidobacterium* and *Lactobacillus* are capable of dendritic cell activation, also leading to the induction of Treg proliferation. *Bifidobacterium longum* was recently shown to supress T_H17 responses within both the gut and the lung and *Bifidobacterium infantis* (35624) was shown to increase Foxp3 T regulatory cells in peripheral blood while reducing CRP (Konieczna et al., 2011). In addition to *Bifidobacterium*, SCFAs: acetate, propionate, and butyrate, produced by gut bacteria, have been demonstrated to influence the immune response through dendritic cell and T cell modulation. SCFAs can regulate immune cell signalling through ligation of GPCRs and inhibition of HDACs, resulting in epigenetic modifications. Various animal models support the role of the microbiome in the pathology of asthma; antibiotic treatment of mice leads to a sharp reduction in Tregs, and a pronounced Th2 response resulting in increased allergic inflammation (Sudo et al., 2002). Thorburn et al., demonstrated that short chain fatty acid ingestion and a high fibre diet leads to a reduction in a plethora of inflammatory markers in Bronchoalveolar lavage fluid, as well as increased expression of Foxp3 through HDAC inhibition and Treg cell count. Administration of SCFAs have repeatedly been shown to have anti-inflammatory effects in the lungs in murine modes. It is unclear whether SCFAs directly affect lung immune responses or indirectly, through immune signalling in the gut microbiome (Sokolowska et al.,

2018). Moreover, it may be possible to manipulate the gut microbiome and SCFA concentrations to treat asthma.

4.1.2 B-GOS and Asthma

Dysbiosis in the microbiome has been linked with poor respiratory immunity through altered inflammatory responses (Frati et al., 2018). If we are able to manipulate the gut microbiota through dietary supplementation, we may be able to treat asthma using a nutritional intervention, specifically using Prebiotics.

B-GOS is a *trans*-galactooligosaccharide that has been demonstrated to have a bifidogenic effect in the gut (Bouhnik et al., 2004), as well as increase *lactobacillus* populations. B-GOS is fermented to form SCFAs and was manufactured using a novel strain of *Bifidobacterium Bifidum (NCIMB 41171),* isolated using faecal samples from healthy volunteers. This supplement has been studied previously and validated in a variety of conditions. A three stage man made fermenter was used to assess the ability of B-GOS to influence colonisation of *Bifidobacterium* and *Lactobacillus,* significant increases were seen for both at $P<0.0001$ (Tzortis and Goulas et al., 2005). Additionally B-GOS enhanced the faecal bacterial population of *Bifidobacterium* at $P<0.01$.

Moreover, a human, asthma specific, crossover study observed the effects of B-GOS over an 8-week intervention. Participants received either placebo or prebiotic with a 2-week washout between research arms. It was demonstrated that B-GOS reduced airway hyper-responsiveness in exercise induced bronchoconstriction, while also attenuating a range of airway inflammatory markers, such as tumour necrosis factor alpha (Williams et al., 2016). In the study by Williams et al., a total of ten participants with hyperpnoea-induced bronchoconstriction (HIB) and eight participants with no history of asthma were recruited, to form a HIB group and a control group respectively. Participants from the HIB group were on steps 1-3 according to the global

initiative for asthma stepwise approach to asthma control (Masoli et al., 2004). Participants were randomly assigned to receive 5.5g/d of B-GOS or maltodextrin (placebo), and visited the laboratory for baseline, post B-GOS, post washout and post placebo testing. During each of the 4 visits pulmonary lung function was measured pre and post EVH, medication usage was permitted throughout the study apart from before each EVH test. ELISAs were utilised to measure TNF-α and serum chemokines. In the HIB group TNF-α concentration increased by 29% after baseline EVH, the response was the same following EVH testing after placebo treatment. Conversely, after B-GOS treatment TNF-α increases were abolished completely ($p=0.002$), indicating that B-GOS has a protective effect on airway inflammation and reduced airway responsiveness. Furthermore, a reduction in CCL17 from baseline to B-GOS was observed in the HIIB group ($p=0.005$) and a peak fall in PEF after EVH from day 0 of B-GOS treatment to day 21 ($P=0.024$). Additionally peak falls in FEV_1 were significantly reduced from day 0 to day 21 of B-GOS treatment ($p=0.004$). Williams et al., (2016) used 5.5g/day of B-GOS as recommended by the manufacturer. However, the recommended dose of B-GOS is now one 3.65g/day, this is the dose that will be used in the current study.

In order to use B-GOS in the treatment of asthma, we must characterise its effects on the lungs and elucidate how these effects are elicited. If we are to understand the link between the gut microbiome and the lungs, and the role of B-GOS in this axis, we must direct our efforts at the intermediate between these two parameters and observe how blood inflammatory markers change with B-GOS treatment. As such the aim of the current study is to investigate the effects of treatment with prebiotic B-GOS on Asthma and markers of airway inflammation in the blood.

A key marker of airway inflammation is Foxp3, a member of the forkhead transcription factor family. This gene transcription regulator has been implicated in $CD4^+$ Treg expression and ultimately immune tolerance through a multitude of mechanisms (Lu et al., 2017). $CD4^+$ Tregs are critical in controlling inflammation, specifically in hypersensitivity reactions, they do so

through upregulation of immunosuppressive molecules, repression of genes and tissue homing receptors; altogether attenuating the pro-inflammatory response. Two different types of $CD4^+$ $Foxp3^+$ Tregs have been defined. Thymus-derived Tregs (tTregs) also known as natural Tregs (nTregs) regulate tolerance to self-antigens. A second extra-thymic population of Tregs form in secondary lymphoid tissues and are known as peripheral regulatory T cells (pTregs), found mainly in the GI tract and lungs during chronic inflammation (Martin-Orozco et al., 2017). Tregs express several molecules which enable their characterisation, CD25 being one of them; most human $CD4^+CD25^{high}$ express Foxp3. By observing the activity of these Tregs, through Foxp3 expression in response to B-GOS, we may be able to better characterise and understand its mechanism of action.

Principal research objective:

- To determine if adults with asthma display significant changes in pro-inflammatory/anti-inflammatory markers after prebiotic intervention.

Secondary research objectives:

- To identify if adults with asthma reduce the use of reliever medication after prebiotic intervention.
- To identify if perception of asthma control is improved after prebiotic intervention
- To identify if adults with asthma display significant changes in quality of life after prebiotic intervention

4.2. Methods

Study overview

Participants will be randomised (1:1) to receive a prebiotic or a taste and appearance matched placebo. The study will be a double-blind, placebo-controlled, crossover trial consisting of cohort observation through questionnaires and laboratory study.

Participants and recruitment

Fourteen participants will be recruited (Aged 18-50 years) with mild to moderate Asthma to take part in the study. Sample size estimation was calculated with the MGH Biostatistics Centre calculator, using data from Konieczna et al., 2013, which observed changes in PBMC $Foxp3^{+}$ Tregs pre and post *Bifidobacterium infantis* treatment.

Based on a two-sided confidence level of 0.05, a within-subjects standard deviation of 0.9, associated power of 0.9, and a minimal detectable difference in FOXP3 expression in CD25 high CD25 intermediate CD4+ Treg cells from baseline to post-intervention of 1.05% (8.14% ± 0.25 vs. 9.19% ± 0.24; P = 0.003), the estimated sample size was calculated at 14.

Participants will satisfy inclusion and exclusion criteria outlined in the participant information sheet (Appendix 3) and provide written informed consent. All participants will have well controlled asthma and be on steps 1-4 of the stepwise treatment of asthma (National Asthma Education and Prevention Program 2007). Participants will be excluded if their asthma is step 5, based on the British Thoracic Society guidelines. Asthmatic participants will be recruited through research posters (Appendix 4) around NTU Clifton, Brackenhurst and City campuses. Recruitment will also occur through research posters in local leisure centres, and an ‘asthma awareness’ pop-up stand on NTU campuses.

Potential risks and burdens for research participants and how they will be minimised

The exclusion criteria states ‘participants who have been hospitalized in the past 12 months due to asthma attacks’ in order to minimise risk, however, a qualified first aider trained in minimum basic life support (e.g. CPR) will be present during all experimental trials, with a second first aider on standby, able to be on site within 30 seconds. An automated external defibrillator and supplemental oxygen will be available in the technician’s office across the corridor from the laboratory (ERD145). If required, the emergency services will be contacted using the room specific ‘2222’ option from the phone in the technician’s office (ERD145) if a second researcher is present (or using the ‘112’, or ‘999’ option from the researchers mobile whilst remaining with the participant). Directions will be provided to the ambulance crew to park outside Erasmus Darwin building (ERD) reception, with directions through the building to ERD138 provided. ERD138 is down a corridor through a set of 8-10 doors, permitting a wheeled stretcher to be taken through the doors. The distance from the ambulance to the fire doors is ~ 20 metres, consisting of a flat, paved surface. The estimated time of arrival for a car travelling from The Queens Medical Centre to the Erasmus Darwin building is ~ 8 minutes depending on traffic conditions (based on a satellite navigation unit, and 3.5 miles). It is expected that this time should be reduced with an ambulance.

During lung function testing, participants may experience faintness/dizziness due to a high frequency of efforts conducted in a short period. Adequate rest will always be provided with efforts to minimise the risk of adverse effects.

A certain level of discomfort is expected when blood sampling due to the nature of the procedure, however, it will be performed in a clean/well-ventilated laboratory in order to minimise the risk of adverse events. The use of a closed system for sample collection will minimise the risk of spillage/exposure (e.g. BD Vacutainer® Push Button Blood Collection Set

& Pre-Attached Holder). All equipment is regularly inspected, especially prior to the procedure to reduce the risk of an air embolism. Researchers performing venepuncture will have relevant vaccinations/immunisations (e.g. Hepatitis B). The procedure has been risk-assessed and is considered safe by NTU Research Ethics Committee. Each participant is screened for relevant phobias/fears/allergies and blood/airborne infections. An appropriate 21 Gauge needle will be used for sample collection, to minimise bruising, participants will be instructed to elevate their arm in a full extension at shoulder height following sample collection. The amount of blood being taken has been reviewed by Dr Gemma Foulds of the Jon Van Geest Cancer Research Centre.

Although mainly associated with consuming a higher daily dose of prebiotic, or in participants with gastrointestinal disorders, temporary gastrointestinal discomfort may be experienced (e.g. abdominal bloating, flatulence, abdominal cramps/pains, and nausea). Gastrointestinal activity will be monitored throughout the study in order to risk assess dosage, serious issues arising from prebiotic treatment will be discussed within the research team and/or a medical professional, if necessary, to decide whether or not the participant is able to continue in the study.

Participants may need to rearrange personal or business commitments to take part in the trial. To avoid the burden and potential loss of earning, each experimental trial will be organised based on participant availability and convenience. Travel costs will also be reimbursed to all participants.

Before visits 2-5, participants will be asked to refrain from completing any form of strenuous physical activity/exercise 24 hours prior to participation, resulting in a temporary disruption to the participant's regular exercise routine. Experimental trials will always be based on participant convenience, as well as availability.

Arrangements for continued provision of the intervention for participants

The prebiotic supplements provided to participants throughout the trial are available commercially and the necessary information required for purchase will be given to the participant, if requested following study completion. Protocols for access to other nutritional interventions will be explained by the chief investigator in the formal debrief upon trial completion. Results will also be made available following the final participant completing the intervention.

Ethical Approval

The first ethics application for the current study (IRAS 242711- created on 24.01.2018) was reviewed by The East Midlands – Nottingham 2 Research Ethics Committee of the National Health Service, it was strongly suggested that multiple major changes to study were required, and an 'unfavourable opinion' was given on 28.07.2018. Following rejection and recommendations from the ethical committee a new application (IRAS 253339 – Created 22.08.2018) was submitted for ethical review to the Nottingham 1 Research Ethics Committee.

Restriction of asthma medication was removed from the current study. Furthermore, a detailed asthma exacerbation protocol was included and reviewed by Professor Dominick Shaw (Respiratory Medicine Consultant at Nottingham City Hospital) for safety purposes. Additionally, it was advised by Professor Shaw to remove severe asthmatics (Step 5) from our cohort, in order to further reduce risk. Any incidental findings were to be discussed within the research team and if deemed potentially harmful to the participant, this data would then be passed on to participants GPs.

A meeting with the East Midlands - Nottingham 1 Research Ethics Committee was held on 12.03.2019. Following the meeting a 'provisional opinion' was granted on 25.03.2019.

A patient public involvement group was used to evaluate all participant facing documents. Eleven asthmatic patients from the Asthma UK Centre for Applied Research reviewed the study documents on 22.04.19 (Appendix 5), and participant facing documents were changed accordingly (Appendix 3). The study was registered on the ISCRCTN database (36526)

The East Midlands, Nottingham 1 Research Ethics Committee of the Health Research Authority granted ethical approval on 10.06.2019. IRAS application ID: 253339.

Experimental Design and Protocol

The study is a 10-week randomised, double-blind, placebo-controlled crossover design. All participants will continue with their usual diet during the study and will be randomised in a counter-balanced order. The supplement and placebo are given for 21 days:

- *Trans*-galactooligosaccharide (B-GOS) 3.65g (79.5% GOS) (0.15g fat, 1.3g, carbohydrate, 2g fibre, 0.15g salt, 0.05g protein, 2.9g galacto-oligosaccharides)
- Placebo (PLA) – sachet containing maltodextrin (taste and appearance matched polysaccharide)

Supplements are identical in size, shape, taste, and colour; and were blinded by an independent individual at Nottingham Trent University. To standardise participant diets between each testing session, participants will complete a food diary, this is standard for the first two days of each intervention. The supplementation period was chosen based on work by Williams et al 2016., where it was shown that 5.5g/day for 21 days with prebiotic B-GOS reduced HIB severity in adults, as reflected by smaller fall in FEV_1 after an eucapnic voluntary hyperpnoea test. Additionally, the B-GOS supplement used in the current study, was from the same commercial entity 'Bimuno', as in work by Williams et al 2016., and Vulveic et al 2018. Moreover, maltodextrin was also used as a placebo by Williams et al 2016. A two-week washout will separate intervention arms (David et al, 2013). On testing days participants are

asked to not partake in any form of strenuous exercise 24 h prior to testing. Participants are also asked to abstain from antihistamines for 72 hours, alcohol for 48 hours and alcohol for 24 hours, food for 3 hours, medication usage is not restricted. All visits will take less than 30 minutes. The supplement being used, 'Bimuno', is a *trans*-galactooligosaccharide and has a generally recognised as safe (GRAS) certification (GRN000484).

Blood Sampling

Blood samples will be obtained by drawing blood from the antecubital fossa region of the forearm, with a 21-gauge needle (Butterfly Winged Infusion Set, Vacuette, Greiner Bio-One, Kremsmünster, Austria). A total of 100 ml Blood will be collected using 10 ml vacutainer tubes (Plus Blood Collection Tubes, BD Vacutainer, BD, Plymouth, UK).

Pulmonary Function and Dynamic Spirometry

Pulmonary function will be completed on each testing day accurate to the ERS guidelines (Miller, 2005; Wagner, et al., 2005) using a pneumotrac spirometer (Vitalograph, Buckinghamshire, UK). Lung function manoeuvres will be performed by participants wearing a nose clip and standing upright. Three flow-volume loops will be taken to calculate forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1), with the two highest values being within 0.1 L of each other, and the highest taken for analysis (Miller et al., 2005).

Mass Spectrometry

Proteomic analysis will be assessed using mass spectrometry in collaboration with the Jon Van Geest cancer research centre. Protein will be extracted from participant samples and stored for analysis once data collection is completed. Pilot work was completed using PBMC isolates from healthy volunteers and proteins assays using bovine serum albumin (see section 3.6).

5. *Chapter 5 - Prebiotics and synbiotics in the treatment of asthma and changes in associated inflammatory markers: A systematic review and meta-analysis of human and murine trials*

5.1 Introduction

Asthma is a heterogeneous non-communicable disease characterised by chronic inflammation of the airways, manifesting in episodes of reversible airway obstruction and reduced pulmonary function affecting quality of life (Reddel et al., 2009). With approximately 300 million people affected worldwide, and a further 100 million predicted by 2025, asthma remains a major global health burden ("GINA," 2019).

Inhaled corticosteroids and short- and long-acting b_2-agonists are the mainstay of asthma therapy, but they are not curative nor do they modify disease progression. Furthermore, long-term inhaled corticosteroid use has undesirable side effects and adherence is poor, while chronic β_2-agonist use results in tolerance (Barnes, 2010). The development of therapies that modulate the immunopathology of asthma without adverse side effects are therefore desirable.

Ultimately, treatment goals in asthma include a reduction in future exacerbations and control of symptoms. However, some 3 to 5% of asthmatic patients are categorised as severe regardless of maximal treatment (Edris et al., 2019), this figure is expected to increase up to 10% in the United States. Therefore, it is essential to find an alternative treatment approach for asthma, with both a higher efficacy and effectiveness.

In the human body, the gut contains the largest and most diverse quantity of microbes, an estimated total of 10^{11} bacteria reside in the colon alone, making it the most colonised organ of the body (Coleman and Haller, 2018). Immune cell signalling through pattern recognition receptors on the intestinal epithelium is heavily dependent on which bacteria are more prominent within the colon. Accumulating evidence suggests that dysbiosis, an imbalance of organisms in the gut microbiome, is associated with numerous GI tract diseases and has also been shown to have substantial influence on systemic immune function, respiratory health and allergic sensitization (Hansel et al., 2013; Roberfroid et al., 2010); with a shift in gut

microbiome composition in patients with cystic fibrosis, lung cancer, children developing asthma and adults with asthma (Abrahamsson et al., 2014) (Begley et al., 2018) (Madan et al., 2012) (Zhuang et al., 2019). This indicates possible cross-talk between these two mucosal sites, a mechanism which is still undefined and requires attention (termed the gut-lung axis). Thus, it is possible that manipulation of the gut microbiome will provide a potential strategy to positively influence the immunopathology of asthma through reduced exacerbations.

Treatment with exogenous live microorganisms, defined as probiotics, when administered in adequate amounts can confer a health benefit to the host (Hill and Sanders, 2013). Evidence from murine and human trials in asthma suggest probiotics could be effective adjunct treatments to positively influence systemic immune parameters and prevent airway hyper-responsiveness (Karimi et al., 2009; Piotr et al., 2010; Sagar et al., 2014). However, several early meta-analyses conducted on infants failed to observe a significant protective effect of probiotic treatment on the risk of asthma development (Azad et al., 2013; Elazab et al., 2013) . There are potential limitations with probiotic interventions that could contribute to the lack of positive meta-analysis data to date, despite some individual trials displaying positive effects. Delivery of probiotic microorganisms to the host may affect how established microbial communities respond; some probiotic supplements may also not survive transit through the gut or pass the gastric mucosal barrier due to their foreign nature (Slavin, 2013).

Conversely, dietary prebiotics, non-digestible carbohydrates that are selectively utilised by host microorganisms to confer a health benefit, have shown promise in recent work when used alone and in conjunction with probiotics. Moreover, encouraging murine work using allergic models (Verheijden et al., 2015a; Verheijden et al., 2015b; Watanabe et al., 2004), supported by some initial adult human asthma trials (Halnes et al., 2017; Williams et al., 2016) suggests that dietary prebiotics when given in isolation or in combination with a probiotic (a synbiotic) (Verheijden et al., 2016) may reduce airway hyper-responsiveness, inflammation, and disease

severity. Albeit, there is a lack of human trials that observe the effects of prebiotics/synbiotics in asthma.

To our knowledge, this is the first systematic review and meta-analysis to examine the effects of prebiotics and/or synbiotics in the treatment of asthma. Previous review articles have primarily focused on probiotics and asthma in infants (Azad et al., 2013, Elazab et al., 2013), or pre- pro- and synbiotics in systemic inflammation (McLoughlin et al., 2019). However, there is a lack of clarity and various contradictions in the literature regarding asthma and probiotic/prebiotic/synbiotic use. It is essential these contradictions are evaluated for clarification in order to refine future methodology and direction of trials, as well as provide insight into a potential novel asthma treatment.

5.2 Methods

The present study was conducted using the Cochrane Handbook for Systematic Reviewers and followed the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines (Shamseer et al., 2015) and the PICOS (Population, Intervention, Comparator, Outcome and Study type) criteria (Brown et al. 2006)

Search Strategy

The electronic databases Science Direct, PubMed, Scopus, Web of Knowledge and the Cochrane library were searched for articles from the earliest available date to January, 2021. Boolean search parameters were used in order to identify all relevant articles. The search included the following terms: adult, child, mice, murine, prebiotic, synbiotic, galactooligosaccharide, fructooligosaccharide, GOS, FOS, Inulin, Soluble Fibre, Asthma, airway, inflammation, wheeze, hyper-responsiveness. Figure 1 details the search strategy used.

Inclusion and exclusion criteria

The primary research question was Are prebiotics and synbiotics effective for treating asthma and reducing inflammatory markers in humans and mice? The secondary research question was: Are prebiotics or synbiotics more effective?

Trials included examined the effects of prebiotics and synbiotics taken orally on any markers of asthma in humans, independent of age, sex. Included murine trials used varying asthma models, appropriate asthma modelling in mice was regarded as any type or duration of allergen and sensitisation. Randomised controlled trials (RCT's) of any duration were included, as well as trials that administered prebiotic/synbiotic treatments during pregnancy. Exclusion criteria included: in vitro trials, trials examining the effect of prebiotics or synbiotics on inflammation in the absence of asthma, murine trials without sensitisation using an allergen, systematic reviews, narrative reviews and opinion papers. Intervention trials that directly combined

prebiotic/synbiotic with functional ingredients (e.g. whole foods) were also excluded. Trials that employed non-oral administration of the intervention were also excluded due to pharmacokinetic variability

Article Appraisal

Trials found through the search strategy were assessed for relevance (JJ and EG). If there was a disagreement regarding the inclusion of a study, an independent reviewer was consulted (MJ). Each included study was independently assessed by one reviewer (JJ) to determine methodological quality. Each study received a score out of 6 based on the Jadad scale, an additional question was added in order to account for a substantial murine asthma-allergy model. Murine trials with poor ratings due to double/single blinding procedures were not excluded. Trials included in the analysis were also assessed for quality using the Cochrane risk of bias assessment tool (Higgins et al., 2011).

1. adult OR child OR mice OR murine
2. prebiotic OR prebiotic treatment OR galactooligosaccharide OR galactooligosaccharide treatment OR fructooligosaccharide OR fructooligosaccharide treatment OR GOS OR GOS treatment OR FOS OR FOS treatment OR inulin OR inulin treatment OR synbiotic OR synbiotic treatment OR soluble fibre OR soluble fibre treatment OR dietary fibre OR dietary fibre treatment
3. asthma OR airway OR inflammation OR wheeze OR hyper responsiveness OR asthma model OR airway model
4. 1 AND 2 AND 3

Figure 5.1. Examples of search strategies conducted using the scientific databases PubMed, Science Direct, Web of Knowledge, Cochrane library and Scopus. GOS – galactooligosaccharide, FOS – fructooligosaccharide.

Meta-analysis

Meta-analysis was completed using Review Manager 5.3 (Cochrane). The extent of statistical heterogeneity was assessed using the I-squared test (I^2). Heterogeneity was considered substantial ($I^2 \geq 50\%$) or considerable ($I^2 \geq 75\%$) (Higgins et al., 2011) and a random effects model was applied.

Where appropriate, standard error of the mean was converted to standard deviation (SD). For continuous data, analyses were performed on the means and the standard deviations. IL-4, IL-5 and IL-6 data are presented as MD, whereas all other data are presented as standardised mean difference (SMD), with 95% confidence intervals (CI) (Takeshima et al., 2014). SMD values are interpreted as small (0.2) moderate (0.5) and large (0.8) (Higgins and Thompson, 2002). SMD was formulated as Hedges' adjusted g and the minimum significance value was 0.05.

A search across multiple databases revealed there was an insufficient amount of data for meta-analysis in adults.

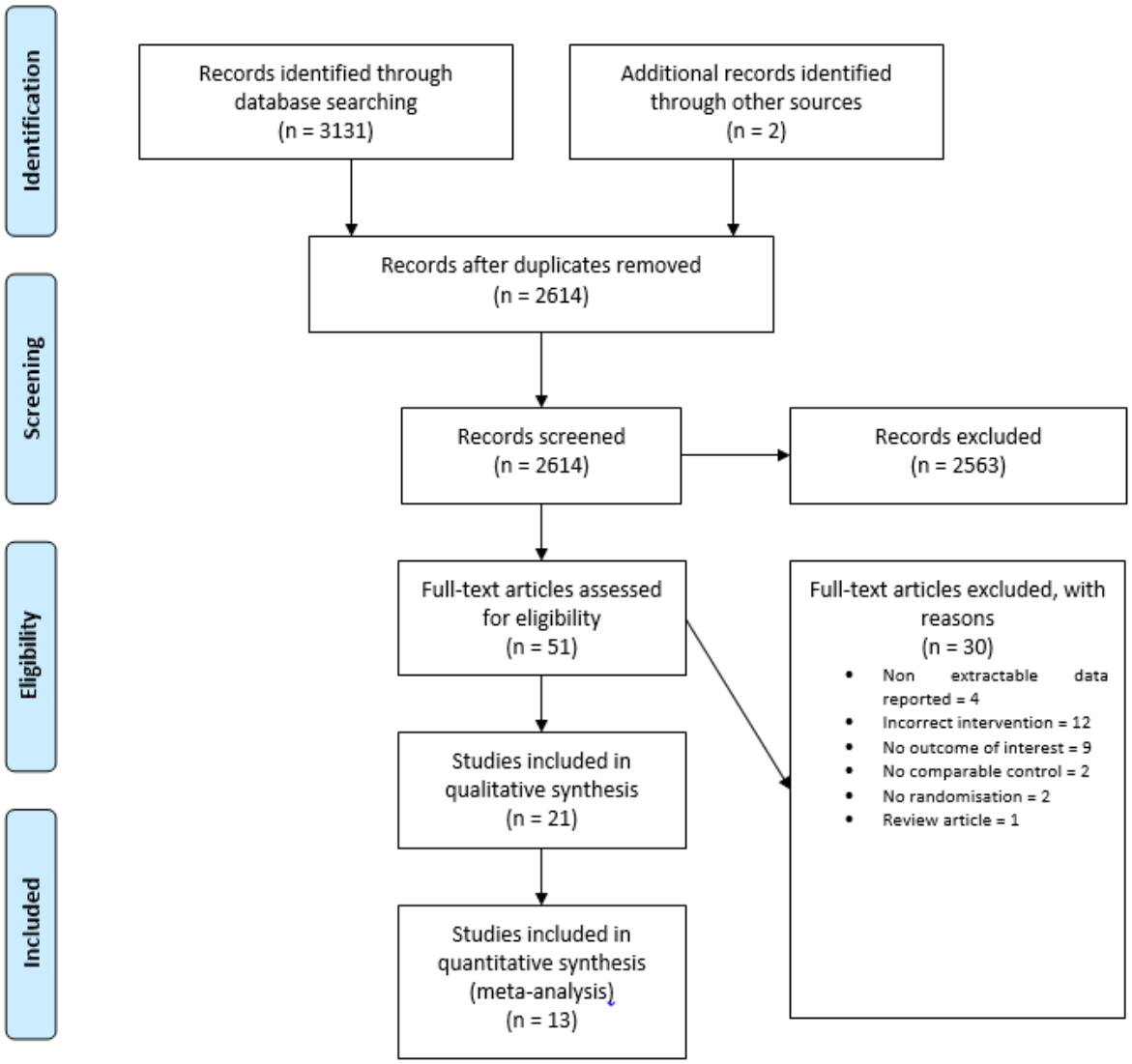

Figure 5.2. Preferred Reporting Items for Systematic Reviews and Meta-analyses flowchart of articles for inclusion in a systematic review of the effect of prebiotics and synbiotics on asthma outcome measures. Seven studies were excluded prior to the quantitative synthesis stage due to non-comparable data. Authors were contacted for raw data, however no response was received.

5.3. Results

Following preferred reporting items for systemic reviews and meta-analyses (Figure 5.2), 21 articles were deemed eligible for qualitative synthesis, and 13 articles were deemed eligible for quantitative analysis (meta-analysis).

Description of Included Murine Trials

Eight murine trials were included in the meta-analysis (Table 5.1), of which one was a randomised controlled trials and seven were described as randomised controlled trials. Sample size in each treatment group ranged from 5 to 9 and the mean JADAD score was 2.3.

Trials used different strains of laboratory mice, including: BALB/c (Verheijden et al., 2015a; Verheijden et al., 2015b; Sagar et al., 2014; Verheijden et al., 2016; Vos et al., 2007), C3HeN (Yasuda et al., 2010) and Brown Norwegian rats (Sonoyama et al., 2005; Watanabe et al., 2004); one study used BALB/c and C57BL/6 offspring (Hogenkamp et al., 2015). Five trials used house-dust mite as an allergy model (Verheijden et al., 2015a; Verheijden et al., 2015b; Verheijden et al., 2016; Yasuda et al., 2010), five trials used ovalbumin (Hogenkamp et al., 2015; Sagar et al., 2014; Sonoyama et al., 2005; Vos et al., 2007; Watanabe et al., 2004) and two trials performed a sensitisation in combination with Bordella Pertusis vaccine (Sonoyama et al., 2005; Watanabe et al., 2004). Initial allergen exposure ranged from day 7 to 63 (median = 20). An airway hyper-responsiveness (AHR) test was conducted in four trials (Hogenkamp et al., 2015; Verheijden et al., 2015a; Verheijden et al., 2016; Vos et al., 2007) between day 14 to 70 (median = 20), with AHR expressed as average airflow resistance (cm H_2O/ml/s) (Hogenkamp et al., 2015; Verheijden et al., 2015a; Verheijden et al., 2016) or enhanced pause parameter (Vos et al., 2007).

Of the eight murine trials, six used a prebiotic treatment (Sonoyama et al., 2005; Verheijden et al., 2015; Verheijden et al., 2016; Vos et al., 2007; Watanabe et al., 2004; Yasuda et al., 2010)

and two (Sagar et al., 2014; Verheijden et al., 2015) used a synbiotic treatment. The control treatment was either formulated by the American institute of Nutrition (AIN-93G) (Sonoyama et al., 2005; Verheijden et al., 2015; Verheijden et al., 2015; Verheijden et al., 2016; Vos et al., 2007; Watanabe et al., 2004), a specified control diet (Yasuda et al., 2010), or was unspecified (Sagar et al., 2014).

In the eight murine trials treatment duration ranged from 13 to 56 days (median = 28). The most common prebiotic treatment was GOS (Sonoyama et al., 2005; Verheijden et al., 2015; Verheijden et al., 2015; Verheijden et al., 2016); other treatments included: FOS (Sonoyama et al., 2005; Yasuda et al., 2010), RAF (Sonoyama et al., 2005; Yasuda et al., 2010), scGOS/lcFOS (Vos et al., 2007), XOS (Sonoyama et al., 2005), scGOS/lcFOS combined with AOS (Vos et al., 2007), and scFOS/lcFOS combined with AOS (Sagar et al., 2014). Two trials used a synbiotic intervention (Sagar et al., 2014; Verheijden et al., 2016), using the probiotic *B breve M16V.* Three trials (Sonoyama et al., 2005; Verheijden et al., 2016; Vos et al., 2007) used two to four prebiotic and/or synbiotic treatments: Sonoyama et al. (Sonoyama et al., 2005) used RAF (Tx1), GOS (Tx2), FOS (Tx3) and XOS (Tx4) treatments, Verheijden et al. (Verheijden et al., 2016) used a GOS/lcFOS/probiotic treatment (Tx1) and a scFOS/lc FOS/probiotic treatment (Tx2), and Vos et al. (Vos et al., 2007) used a scGOS/lcFOS treatment (Tx1) and an 83% scGOS/lcFOS + 17%pAOS treatment (Tx2). Prebiotic treatment doses are detailed in table 5.1.

Effect of prebiotics and synbiotic treatment on murine inflammatory markers and asthma outcome measures

The eight murine trials included in the meta-analysis are summarised in Table 5.1. Sagar et al., (Sagar et al., 2014) and Verheijden et al., (Verheijden et al., 2016) used a synbiotic (probiotic *B breve M-16V*) treatment for 33 and 14 days, respectively, and similar prebiotic mixtures as

aforementioned. Lung IL-6 concentrations were reduced at protein (Verheijden et al., 2016) and mRNA level (Sagar et al., 2014), however, synbiotic doses were double that of the Sagar trials in Verheijden et al., (Verheijden et al., 2016).

Two trials (Sonoyama et al., 2005; Watanabe et al., 2004) reported a decrease lung IL-5 mRNA of 38% and 51% after raffinose treatment, and one trials (Yasuda et al., 2010) reported a decrease in lung IL-5 mRNA and protein after FOS treatment. Moreover, raffinose treatment reduced airway eosinophil counts by 66% (Watanabe et al., 2004), while fructooligosaccharide with *B breve M-16V* treatment reduced BALF eosinophil counts by51% (Sagar et al., 2014). One trials reported a decrease in IL-33 at protein level in the lungs (-63%) and at mRNA level in the small intestine (-18%) (Verheijden et al., 2015), GOS treatment with house-dust mite sensitisation.

Table 5.1. – Summary of included murine trials examining the effects of prebiotics and synbiotics on asthma outcomes

Trial	Design	Murine Strain	Control Diet	Prebiotic, dose	Synbiotic, dose (Probiotic used)	Duration of supplement/ Sensitisation	Effect of intervention on asthma outcomes
Sagar et al. 2014	RCT$^{(-)}$	BALB/c	Diet not specified. "Standard conditions with free access to food and water."	scFOS/lcFOS/AOS Oral gavage, 0.2ml of mixture in PBS, 3x wk, day 22-55.	*B breve M-16V* (10^9 CFU) with maltodextrin used as carrier)	33 days / OVA (10µg on day 0 and 12)	↓Inflammatory cells in BALF,↓Eosinophils ↓IL-1β, IL-6, IL-12 & TNFα.
Sonoyama et al. 2005	RCT$^{(-)}$	Brown Norway rats	*Ad libitum* feeding AIN93G control diet.	GOS, FOS, RAF, XOS was Fed for 7 days (50g/kg of diet) or injected for 13 days (0.5% daily [wt:v]).	*X*	7 - 13 days / OVA (1mg and 0.2ml *Bordetella pertussis* vaccine)	↓Inflammatory cells in BALF,↓ IL-5 (RAF), ↓ airway eosinophilia, Associated with ↓IL4, IL5 mRNA in lungs.
Verheijden et al. 2015a	RCT$^{(-)}$	BALB/c,	AIN93G control diet. Separate control treated with budesonide (oropharyngeally, 500 µg/kg) on days 7, 9 and 11 prior to daily challenge and day 13.	GOS (1% v/w). Vivinal GOS syrup: 59% GOS, 21% Lactose, 19% Glucose and 1% Galactose on dry matter (75%).	*X*	14 days / HDM (Intranasal 1µg on day 0)	↓ CCL5, ↓ IL-13 in lung tissue. 1% GOS prevented development of AHR. ↓ airway eosinophilia.
Verheijden et al. 2015b	RCT$^{(-)}$	BALB/c B6C3F$_1$	*Ad libitum* feeding of AIN93G control diet.	Vivinal GOS (1%v/w) syrup: 59% GOS, 21% Lactose, 19% Glucose and 1%. Galactose on dry matter (75%)	*X*	14 days / HDM (Intranasal 1µg on day 0)	↓IL-33, ↓ST2 mRNA, ↓ IL-33 mRNA associated with Δ's in BALF and intestinal permeability.
Verheijden et al. 2016a	RCT$^{(-)}$	BALB/c	*Ad libitum* feeding of AIN93G control diet.	GOS (1% v/w), Vivinal GOS syrup: 59% GOS, 21% Lactose, 19% Glucose and 1% Galactose on dry matter (75%). FOS/scFOS/lcFOS (1:1, 1% w/w, 95% oligofructose content.	*B breve M-16V* (*$2x10^9$ CFU/g*) was added to each diet. Diets were given 2 wks. prior to sensitisation.	14 days / HDM (Intranasal 1µg on day 0).	↓ IL-6, ↓IL-4, ↓IL-10, ↓IFNγ. Combination of lcFOS/GOS less potent than FBB diet. No significant changes In AHR response.

Table 5.1. *(Continued)*

Trial	Design	Murine Strain	Control Diet	Prebiotic, dose	Synbiotic, dose (Probiotic used)	Duration of supplement/ Sensitisation	Effect of intervention on asthma outcomes
Vos AP et al. 2007	RCT(-)	BALB/cByJIco SPF	Semi purified AIN-93G based diets. All supplemented oligosaccharides replaced carbohydrate content.	One test diet contained 1% (w/w) net oligosaccharides with 83% GOS scGOS/lcFOS and 17% pAOS.	*X*	55 days / OVA Two 10µg intraperitoneal injections.	↓ Inflammatory cells in BALF. Maximal responses of AHR to Methacholine were significantly reduced in both scGOS/lcFOS and scGOS/lcFOS/pAOS diet groups.
Watanabe et al. 2004	RCT(-)	Brown Norway rats	*Ad libitum* feeding of the AIN-93G control diet.	RAF (50g/kg of diet) Rats fed RAF diet for 7 days in Exp.1. RAF injected daily in Expt. 4, alongside RAF diet.	*X*	20 days / OVA (1mg and 0.2ml *Bordetella pertussis* vaccine).	↓IL-4,↓IL-5 (mRNA). ↓ Airway Eosinophilia, following intraperitoneal injection of RAF, ↓total BALF and eosinophil %.
Yasuda et al. 2010	RCT(+)	C3H/Hen	Control diet (/100g/diet), 7.7g moisture. 23.6g croud protein, 5.3g croud fat, 2.9g croud fibers, 6.1g croud carbohydrates, 54.4g soluble nitrogen compound free. Food and water *ad libitum*.	FOS (2.5%) fed *ad libitum* 7 days prior to allergen administration. FOS replaced 2.4% of control diet.	*X*	50 days / HDM (*dermatophagoides farinae - 1* µg)	↓IL-5, ↓ eotaxin (mRNA & protein). ↓IgG, IgE. Eosinophil % counts were significantly reduced in the FOS group following the HDM challenge.

Table 5.2. – Summary of included infant trials examining the effects of prebiotics and synbiotics on asthma outcomes

Trial (country)	Design	Population, n	Prebiotic/dietary intervention, daily dose (g)	Synbiotic, (probiotic) daily dose (g)	Duration/Follow up	Effect of intervention on outcome measures
(i) Moro et al. 2006, Italy	RCT	Atopic dermatitis, 206	Aptamil HA, a hypoallergenic formula with hydrolysed cow milk whey protein was supplemented with GOS (90%) / FOS (10%), 8g/L (Imunofortis) Placebo:8g/L Maltodextrin	*x*	6 months, weaning completed at 5 months with fruit, followed by weaning purees. Follow up and 2 and 5 years.	↓ Recurrent wheeze between groups during the 5 years, but not significant. Cumulative incidence of any allergic manifestation were significantly reduced; allergic rhinoconjuncitivis, urticarial.
(ii) Arslanoglu et al. 2008	RCT	134				
(iii) Arslanoglu et al., 2012	RCT	92				
(i) Kukkonen et al., 2007, Finland	RCT	Atopy risk, 925	GOS (90%)/FOS (10%), 8g/L, 0.8g daily in 20g drops of sugar syrup taken by infants. Placebo infants took sugar syrup without the oligosaccharides.	*B breve Bb-99* $2x10^8$ CFU, *L rhamnosus GG* $5x10^9$ CFU, *L rhamnosus LC705* $5x10^9$ CFU, *Propionibacterium freudenreichii JSS.* 2 $x10^9$CFU. Probiotics were taken in capsule form twice daily by mothers from 36th wk of gestation. Placebo groups took microcrystalline, instead of probiotic. New born infants received the same probiotics/placebo as well as the prebiotic/placebo dose specified.	Mothers took probiotic capsules twice a day from week 34 until birth. Infants received the same probiotics and an additional prebiotic for 6 months. Follow up at 2 and 5 years.	F_eNO did not differ between experimental groups. Airway inflammation unaffected through treatment of prebiotics and probiotics.
(ii) Kuitunen et al., 2009	RCT	891				
(iii) Kukkonen et al., 2011	RCT	131				
Ivakhnenko et al., 2013, Ukraine	RCT	Healthy, 166 .	scGOS(90%)/lcFOS(10%), 8g/L. Formula mix supplemented with oligosaccharides.	X	2 months. Follow up at 18 months.	GOS/FOS treatment ↓ IgE levels, cumulative incidence in recurrent wheezing, and fewer respiratory tract infections

Table 5.2. *(Continued)*

Trial (country)	Design	Population, n	Prebiotic/dietary intervention, daily dose (g)	Synbiotic, (probiotic) daily dose (g)	Duration/Follow up	Effect of intervention on outcome measures
Niele et al., 2012, Germany	RCT	Healthy, 103. 94 participated in follow up study	80% scGOS/lcFOS and 20% pAOS, Increasing dose to a maximum of 1.5g/kg/day. Oligosaccharide mixture added to human milk preterm formula (Nenatal Start).	X	28 days/1 year. Follow up questionnaire 'Prevention and Incidence of Asthma and Mite Allergy Study.	Incidence of allergic disease and bronchial hyper-reactivity was not different in the prebiotic mixture group and the placebo, following the first year of life.
Vanderaa et al., 2011, Netherlands	RCT	Atopic Dermatitis, 82 completed intervention. 75 infants completed the follow up.	scGOS(90%)/lcFOS(10%), 8g/L. Formula was given on demand, with the probiotic.	*B breve M-16V* $1.3x10^9$ CFU/100ml. This study exclusively used a synbiotic.	3 months/18 months	Significant ↓ in wheezing and/or noisy breathing apart from cold in the synbiotic group. Wheezing apart from colds did not reach significance. Asthma medication usage significantly ↓ in synbiotic group.

Meta-analysis of murine trials

Airway hyper responsiveness

For AHR, five treatments from three trials (Verheijden et al., 2015; Verheijden et al., 2016; Vos et al., 2007) contributed data to meta-analysis (Figure 5.3). The overall effect of prebiotic and synbiotic treatment revealed a reduction in AHR (SMD, -1.31 [-2.36, -0.27] $P = 0.010$) with considerable heterogeneity among trials ($I^2 = 76\%$, $P = 0.002$). Subgroup analysis revealed that prebiotic treatment reduced AHR (SMD, -2.19 [-2.91, -1.46] $P = 0.001$) with no statistical heterogeneity among trials ($I^2 = 0\%$, $P = 0.92$), whereas synbiotic treatment did not reduce AHR (SMD, -0.14 [-0.83, 0.56] $P = 0.70$) with no statistical heterogeneity among trials ($I^2 = 0\%$, $P = 0.42$). Moreover, a significant difference between subgroups was found, favouring prebiotic over synbiotic treatment ($P < 0.001$).

Eosinophil, neutrophil and alveolar macrophage counts

For eosinophil and neutrophil counts, eleven different treatments from seven trials (Sagar et al., 2014; Sonoyama et al., 2005; Verheijden et al., 2015; Verheijden et al., 2015; Verheijden et al., 2016; Watanabe et al., 2004; Yasuda et al., 2010) contributed to meta-analysis (Figure 5.5 – A/B). The overall effect of prebiotic and synbiotic treatment revealed a reduction in total eosinophil counts (SMD, -2.36 [-3.37, -1.36] $P < 0.001$) with considerable heterogeneity among trials ($I^2 = 79\%$, $P < 0.001$) (Figure 5.5 - A), and a reduction in total neutrophil counts (SMD, -1.15 [-1.96, -0.33] $P = 0.006$) with substantial heterogeneity among trials ($I^2 = 78\%$, $P < 0.001$) (Figure 5.5 - B). Subgroup analysis revealed that neutrophil and eosinophil counts were reduced after prebiotic treatment ($P < 0.001$) but not after synbiotic treatment ($P = 0.43$ and 0.12, respectively).

For alveolar macrophages, eleven treatments from seven trials (Sagar et al., 2014; Sonoyama et al., 2005; Verheijden et al., 2015a; Verheijden et al., 2015b; Verheijden et al., 2016; Watanabe et al., 2004; Yasuda et al., 2010) contributed to meta-analysis (Figure 5.5). The overall effect of prebiotic and synbiotic treatment revealed a reduction in alveolar macrophage counts (SMD, -1.66 [-2.57, -0.74] $P = 0.0004$) with considerable heterogeneity among trials ($I^2 = 77\%$, $P < 0.001$). Subgroup analysis revealed that alveolar macrophage counts were reduced after prebiotic treatment ($P = 0.001$) but not after synbiotic treatment ($P = 0.41$) (Figure 5.5 – C)

Bronchoalveolar lavage fluid and lymphocyte counts

For BALF, ten different treatments from six trials (Sagar et al., 2014; Sonoyama et al., 2005; Verheijden et al., 2015a; Verheijden et al., 2015b; Verheijden et al., 2016; Watanabe et al., 2004) contributed to meta-analysis. The overall effect of prebiotic and synbiotic treatment revealed a reduction in BALF (SMD, -2.76 [-3.87, -1.64] $P = 0.001$) with considerable heterogeneity among trials ($I^2 = 78\%$, $P = 0.001$). Subgroup analysis revealed that both prebiotic (SMD, -2.71 [-3.68, -1.74] $P < 0.001$) and synbiotic treatments reduced total BALF (SMD, -2.88 [-6.30, 0.54] $P = 0.001$) with considerable heterogeneity among trials ($I^2 = 91\%$, $P = 0.10$) (Figure 5.4 – A). For lymphocyte count, eleven treatments from seven trials (Sagar et al., 2014; Sonoyama et al., 2005; Verheijden et al., 2015a; Verheijden et al., 2015b; Verheijden et al., 2016; Watanabe et al., 2004; Yasuda et al., 2010) contributed to meta-analysis. The overall effect of prebiotic and synbiotic treatment revealed a reduction in lymphocyte count (SMD, -1.61 [-2.37, -0.85] $P = 0.008$) with substantial heterogeneity among trials ($I^2 = 68\%$, $P=0.0008$) (Figure 5.4 – B). Subgroup analysis revealed that lymphocyte count was reduced after prebiotic treatment ($P < 0.001$), but not after synbiotic treatment ($P=0.16$).

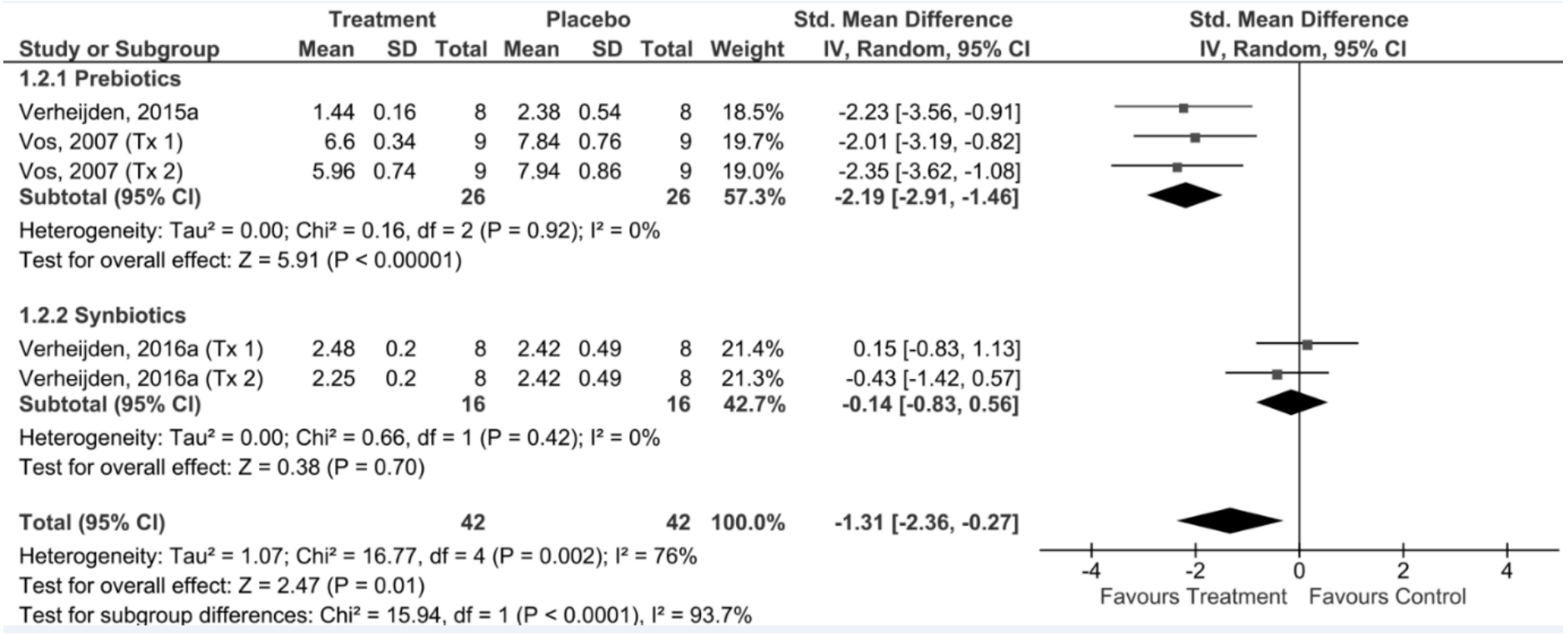

FIGURE 5.3. Forest plot of randomised controlled trials investigating the effects of prebiotic and synbiotic treatment on airway hyper-responsiveness (AHR), subgrouped by treatment method. The pooled effects of AHR are shown as black diamonds. Standard mean difference and 95% CIs were calculated using an inverse variance random effects models and I^2 values were significant at P<0.10.

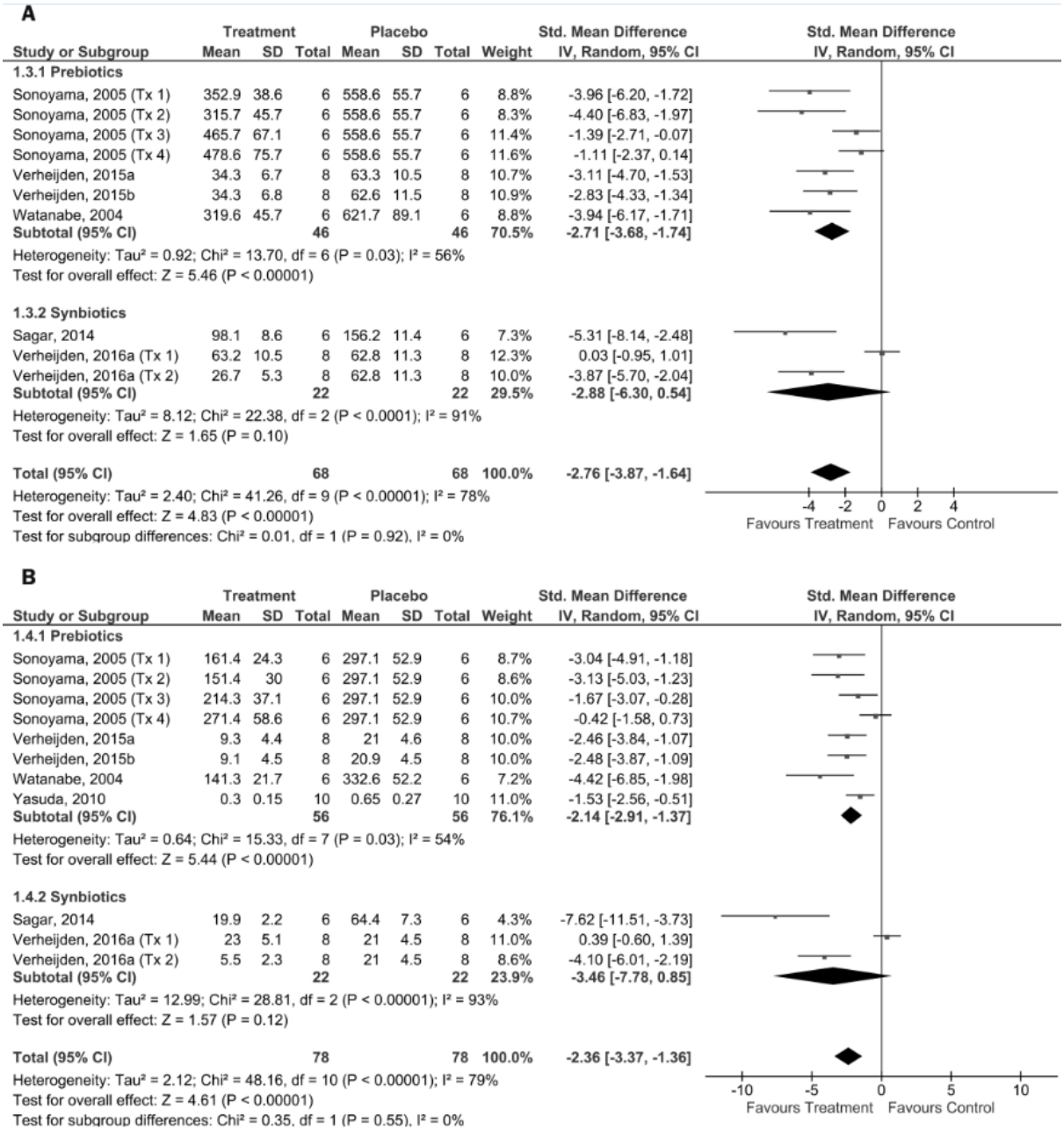

FIGURE 5.4. Forest plot of randomised controlled trials investigating the effects of prebiotic and synbiotic treatment on (**A)** total bronchoalveolar lavage fluid (BALF) and **(B)** Lymphocytes counts, subgrouped by treatment method. The pooled effects of AHR are shown as black diamonds. Standard mean difference and 95% CIs were calculated using an inverse variance random effects models and I^2 values were significant at $P < 0.10$

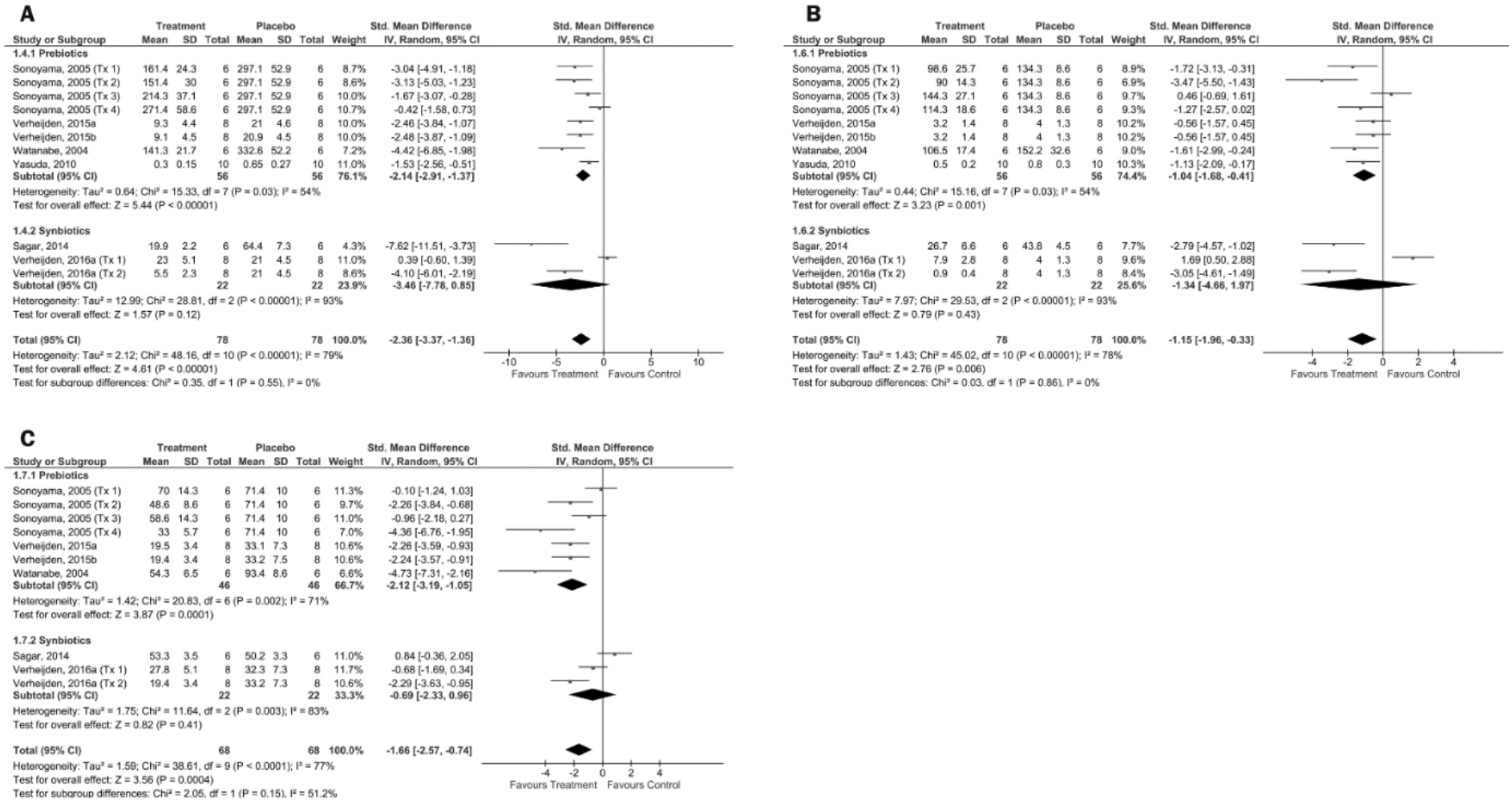

FIGURE 5.5. Forest plot of randomised controlled trials investigating the effects of prebiotic and synbiotic treatment on **(A)** eosinophil**, (B)** neutrophil and **(C)** macrophage counts, subgrouped by treatment method. The pooled effects are shown as black diamonds. Standard mean difference and 95% CIs were calculated using an inverse variance random effects models and I^2 values were significant at P<0.10.

Systemic Inflammatory Markers

A total of 46 markers of systemic inflammation were identified from the murine trials. Common markers, studied in 5 or more trials, included IL-4, IL-5, IL-13 and INFy, with IL-4 recurring the most, followed by IL-5, IL-13 and IFNy. For IL-4, seven treatments from three trials (Sonoyama et al., 2005; Verheijden et al., 2016; Watanabe et al., 2004) contributed to meta-analysis (Figure 5.6 - A). The overall effect of prebiotic and synbiotic treatment revealed a 34% reduction in IL-4 (95% CI [-57, -10] P = 0.004) with considerable heterogeneity among trials (I^2 = 89%, P < 0.001). Subgroup analysis revealed that prebiotic (SMD -20.93% [-39.23, -2.63] P = 0.02) and synbiotic (SMD -61.27% [-107.13, -15.41] P = 0.001) treatments reduced IL-4. For IL-5, nine treatments from five trials (Sonoyama et al., 2005; Verheijden et al., 2015b; Verheijden et al., 2016; Watanabe et al., 2004; Yasuda et al., 2010) contributed to meta-analysis (Figure 5.6 – B). The overall effect of prebiotic and synbiotic treatment revealed a 27% reduction in IL-5 (SMD -27.47 [-40.69, -14.25] P = 0.002) with substantial heterogeneity among trials (I^2 = 67%, P = 0.002*)*. Subgroup analysis revealed that prebiotic (P = 0.005) and synbiotic (P = 0.0003) treatments reduced IL-5. For IL-13, four treatments from three trials (Verheijden et al., 2015a; Verheijden et al., 2015b; Verheijden et al., 2016) contributed to meta-analysis (Figure 5.6 - C). The overall effect of prebiotic and synbiotic treatments revealed a reduction of 24% (SMD -23.97 [-35.42, -12.51] P < 0.001) with considerable heterogeneity among trials (I^2 = 92%, P < 0.0001). Subgroup analysis revealed that prebiotic (P < 0.001) and synbiotic (P < 0.001) treatments reduced IL-13.

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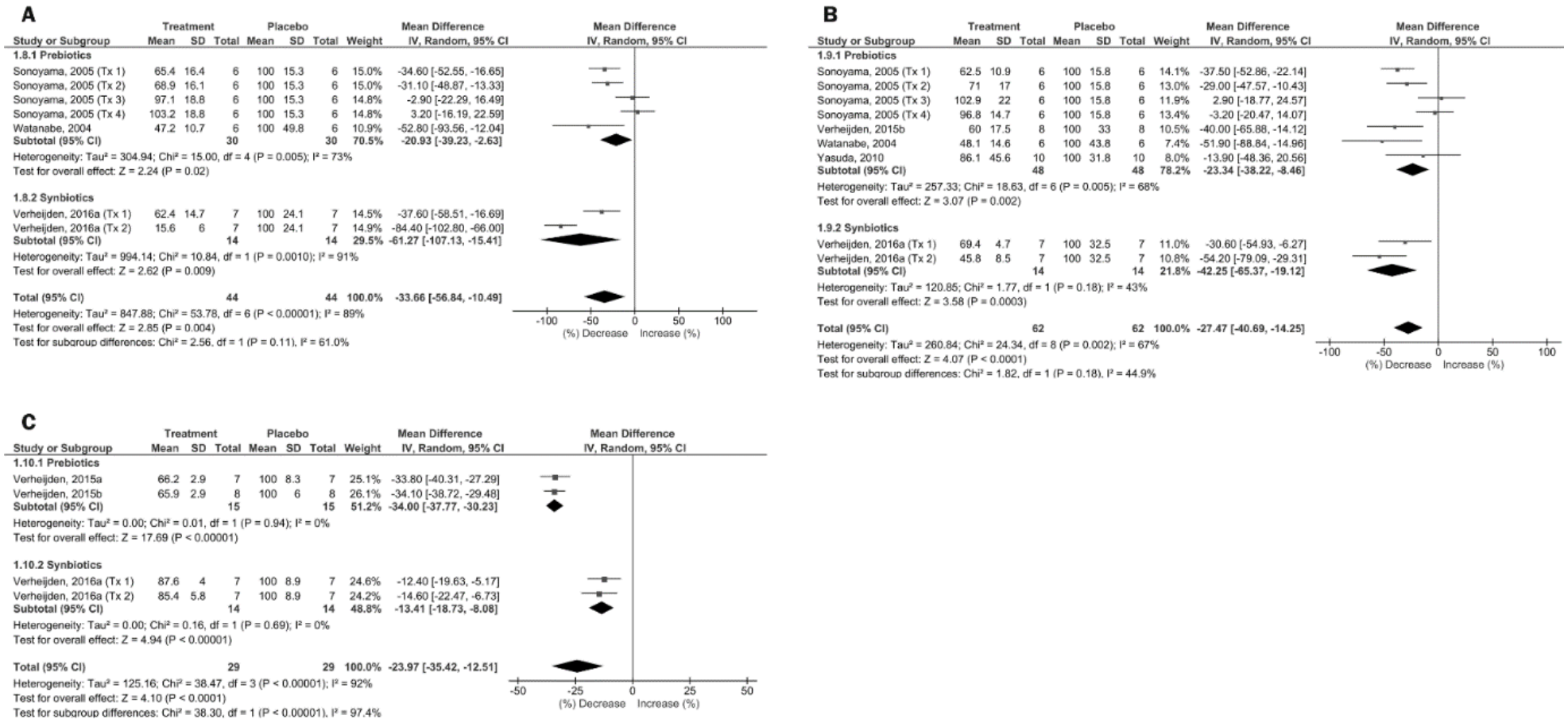


FIGURE 5.6. Forest plot of randomised controlled trials investigating the effects of prebiotic and synbiotic treatment on **(A)** IL-4 **(B)** IL-5 and IL-13 **(C)** concentrations, subgrouped by treatment method. The pooled effects are shown as black diamonds. Standard mean difference and 95% CIs were calculated using an inverse variance random effects models and I^2 values were significant at P<0.10.

Description of included infant population trials

Five studies comprising nine trials examined the effects of prebiotics and synbiotics on infant asthma (Arslanoglu et al., 2008; Arslanoglu et al., 2012; Kukkonen et al., 2007; Kukkonen et al., 2011; Kuitunen et al., 2009; Moro et al., 2006; Niele et al., 2013; Ivakhnenko and Nyankovskyy, 2013; van der Aa et al., 2011), four of which included follow ups (Arslanoglu et al., 2012; Kukkonen et al., 2011; Kuitunen et al., 2009). Arslanoglu et al. completed a follow up after two (Arslanoglu et al., 2008) and five years (Arslanoglu et al., 2012), from the original article by Moro et al., (2006), the trials were from Italy and used the same group of participants. Two follow up trials (Kukkonen et al., 2011; Kuitunen et al., 2009;) were completed following initial research by Kukkonen et al., (2007); the trials were from Finland and also used the same group of participants. Another trial (Ivakhnenko and Nyankovskyy, 2013) was completed in Ukraine and two trials (Niele et al., 2013; van der Aa et al., 2011) from the Netherlands. Seven of the trials (Arslanoglu et al., 2012, 2008; Kukkonen et al., 2011; Kukkonen et al., 2007; Kuitunen et al., 2009; Niele et al., 2013; van der Aa et al., 2011) were randomised, controlled, double blinded and placebo controlled, one trials (Ivakhnenko and Nyankovskyy, 2013) was a randomised controlled trials. The mean JADAD score was 5.5, using a 6-point scale.

A total of 1846 participants were randomised from the original 5 studies (Ivakhnenko and Nyankovskyy, 2013; Kukkonen et al., 2007; Moro et al., 2006; Niele et al., 2013; van der Aa et al., 2011) and sample size per group ranged from 44 to 613 infants. Three of these original trials studied participants at high risk for allergy (Arslanoglu et al., 2008; Kukkonen et al., 2007; Moro et al., 2006; van der Aa et al., 2011), whereas two trials studied healthy participants (Ivakhnenko and Nyankovskyy, 2013; Niele et al., 2013). In two trials vaginal delivery rates were reported as 56% (34) and 68% (Niele et al., 2013) in per protocol statistics and caesarean delivery rates were estimated at 17% in per protocol statistics, reported in one trial (Kukkonen et al., 2007). Male infants ranged from 49-65% of sex in three studies (Arslanoglu et al., 2008;

Kukkonen et al., 2007; Moro et al., 2006; van der Aa et al., 2011). During the neo-natal period, two trials (Kukkonen et al., 2007; Niele et al., 2013) reported parental antibiotic use, 23% and 76% respectively in per protocol statistics; two studies (Arslanoglu et al., 2008; Ivakhnenko and Nyankovskyy, 2013; Moro et al., 2006) did not specify if antibiotics were used, whereas antibiotics use was an exclusion criterion in one trial (van der Aa et al., 2011).

Of the five original infant trials (Ivakhnenko and Nyankovskyy, 2013; Kukkonen et al., 2007; Moro et al., 2006; Niele et al., 2013; van der Aa et al., 2011), three used a prebiotic treatment (Arslanoglu et al., 2008; Ivakhnenko and Nyankovskyy, 2013; Niele et al., 2013) and two a synbiotic treatment (Kuitunen et al., 2009; van der Aa et al., 2011). Treatments were delivered orally in powder or capsule form, with the placebo comprising maltodextrin (Arslanoglu et al., 2008; Moro et al., 2006; Niele et al., 2013) or regular feeding (Kukkonen et al., 2007; Ivakhnenko and Nyankovskyy, 2013; van der Aa et al., 2011). Four of the five trials investigated the effects of postnatal infant treatment (Arslanoglu et al., 2008; Ivakhnenko and Nyankovskyy, 2013; Moro et al., 2006; Niele et al., 2013; van der Aa et al., 2011), whereas one trials investigated the effects of both pre and postnatal treatment (Kukkonen et al., 2007). Treatment durations ranged from 28 days to 6 months. Prebiotic treatments comprised oligosaccharide mixtures, most commonly scGOS/lcFOS (10% FOS) (Arslanoglu et al., 2008; Ivakhnenko and Nyankovskyy, 2013; Moro et al., 2006); scGOS/lcFOS in combination with pAOS was used in one trial (Niele et al., 2013). Four trials (Kukkonen et al., 2007; Ivakhnenko and Nyankovskyy, 2013; Moro et al., 2006; van der Aa et al., 2011) used a prebiotic dose of 8g/L, ad libitum in murine drinking water in one trial (van der Aa et al., 2011) and once daily in two trials (Kukkonen et al., 2007; Niele et al., 2013). However, one trial (Niele et al., 2013) increased daily doses of prebiotic as detailed in table 5.2. One synbiotic trial (Kukkonen et al., 2007) administered a multi-strain probiotic organism (*B breve Bb-99, L rhamnosus GG, L rhamnosus LC705, Propionibacterium freudenreichii JSS),* at a dose ranging from $1.3x10^9$ –

$5x10^9$ CFU, combined with GOS. A second synbiotic trials (van der Aa et al., 2011) administered *B breve M-16V* in combination with scGOS/lcFOS (10% FOS).

Meta-analysis of infant trials

Eight trials (Arslanoglu et al., 2012; Kukkonen et al., 2011; Kuitunen et al., 2009; Ivakhnenko and Nyankovskyy, 2013; Niele et al., 2013; van der Aa et al., 2011) with relevant asthma outcomes were included in meta-analysis. The original trial by Moro et al., was not applicable for meta-analysis, however both follow up trials were included (Arslanoglu et al., 2008; Arslanoglu et al., 2012). Recurrent wheezing was physician diagnosed in six trials (Arslanoglu et al., 2008; Arslanoglu et al., 2012; Kukkonen et al., 2007; Kukkonen et al., 2011; Kuitunen et al., 2009; Ivakhnenko and Nyankovskyy, 2013) and reported by parents in one trial (Niele et al., 2013). Parents reported bronchial hyper-reactivity without specification of exact symptoms was (Niele et al., 2013), recorded criteria included dyspnoea, wheezing, humming breath sounds or a nightly dry cough without rhinitis. For the incidence of recurrent wheeze, five trials contributed data to meta-analysis (Arslanoglu et al., 2012; Kuitunen et al., 2009; Ivakhnenko and Nyankovskyy, 2013; Niele et al., 2013; van der Aa et al., 2011) (Figure 5.7). Overall, prebiotic and synbiotics had no effect on the risk of cumulative incidence of recurrent wheeze (Risk ratio - 0.79 [95% CI: 0.51, 1.22] P = 0.28) and with no heterogeneity among trials (I^2 = 43%; P = 0.14). Subgroup analysis for prebiotic treatment on a total of 533 participants, revealed no change on the risk of cumulative incidence of recurrent wheeze (Risk ratio – 0.85 [95% CI: 0.41, 1.76] P = 0.67) with no heterogeneity among trials (I^2 = 45%; P = 0.16). Similarly, synbiotics treatment on a total of 1313 patients revealed no change (Risk ratio – 0.65 [95% CI: 0.27 – 1.57] P = 0.34) with substantial heterogeneity among trials (I^2= 69%; P = 0.07)

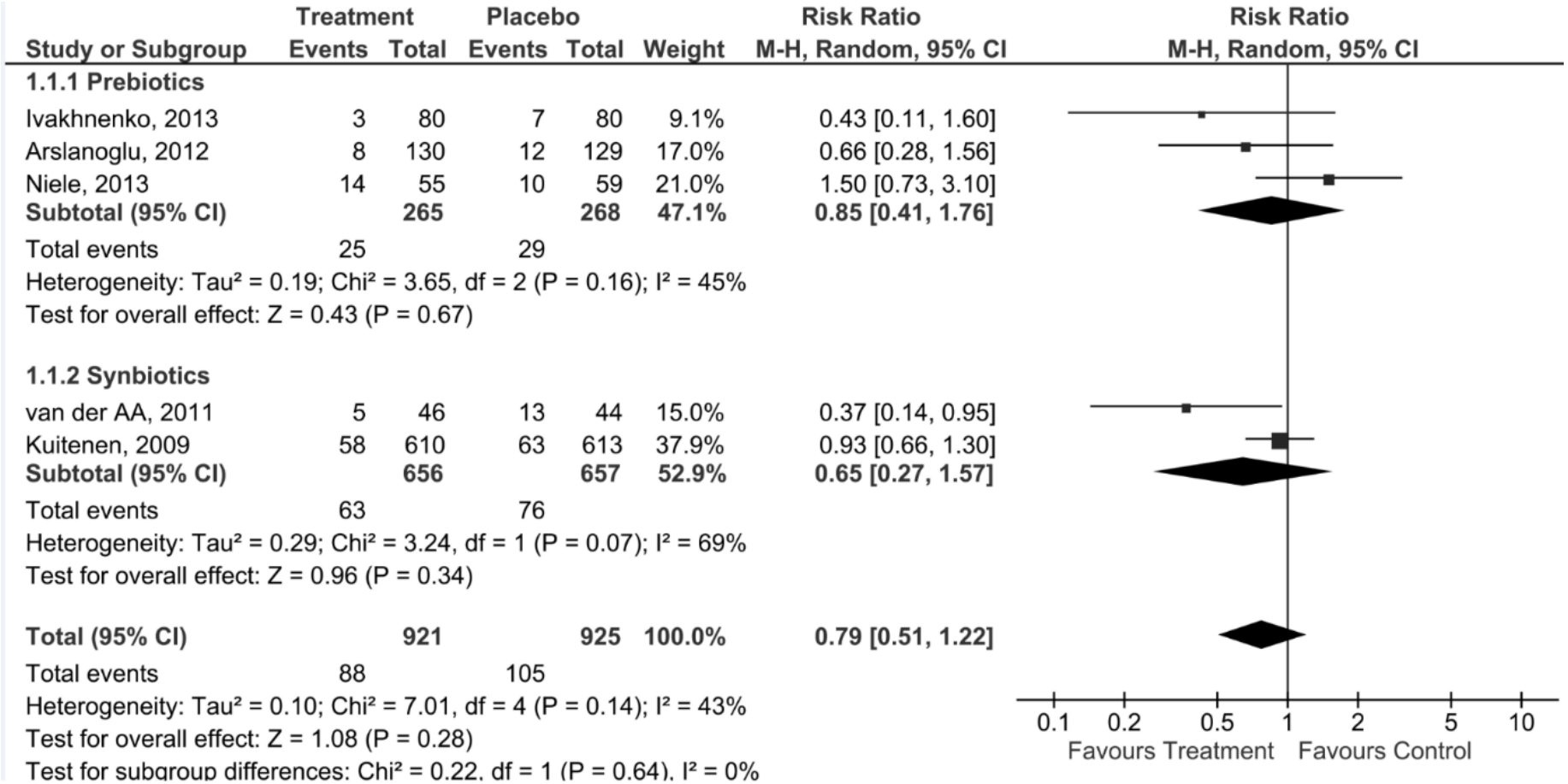


FIGURE 5.7. Forest plot of randomised controlled trials investigating the effects of prebiotic and synbiotic treatment on incidence of cumulative recurrent wheeze in infants, subgrouped by treatment method. The pooled effects are shown as black diamonds. Standard mean difference and 95% CIs were calculated using an inverse variance random effects models and I^2 values were significant at P<0.10.

Subgroup analyses were performed based on follow up duration, for 2 and 5 years. The two year follow up consisted of 623 patients, three prebiotic treatments (Arslanoglu et al., 2008; Ivakhnenko and Nyankovskyy, 2013; Niele et al., 2013) and one synbiotic treatment (van der Aa et al., 2011) revealed no differences between cumulative incidence of recurrent wheeze (Risk ratio - 0.57 [0.25, 1.29] $P = 0.018$) and substantial heterogeneity among trials ($I^2 = 64\%$; $P = 0.04$). When completing subgroup analysis on prebiotic treatments, there was no effect on cumulative incidence of recurrent wheeze (Risk ratio - 0.66 [0.24, 1.84] $P = 0.43$), with substantial heterogeneity among trials ($I^2 = 69\%$; $P = 0.04$). However, synbiotic treatment from one trial reported differences between treatment and placebo for cumulative incidence of recurrent wheeze (Risk ratio - 0.37 [0.14, 0.95] $P = 0.04$); heterogeneity was not applicable. For the follow up duration of 5 years, there was one prebiotic (Ivakhnenko and Nyankovskyy, 2013) and one synbiotic (Kuitunen et al., 2009) treatment, comprising of 1482 patients. No changes were observed on cumulative incidence of recurrent wheeze (Risk ratio - 0.88 [0.65, 1.21] $P = 0.45$) with no heterogeneity among trials ($I^2 = 0\%$; $P = 0.48$), subgroup analysis also reported no changes.

Narrative review on infant trials

Kuitunen et al., (Kuitunen et al., 2009) supplemented synbiotics to caesarean delivered children from birth up to 6 months of age and observed no significant change in Asthma (diagnosis = 2 or more episodes of recurrent wheezing) associated IgE concentrations compared to placebo at a 5 year follow-up (Synbiotic vs Placebo; 7.1% vs 10%; RR: 0.68 [0.21, 2.19]. Another trials by Arslanoglu et al., (Arslanoglu et al., 2012) observed no significant change in the prevalence of persistent wheezing compared to placebo at age 5 (Synbiotic vs Prebiotic, 16% vs. 4.8%), it is also worth noting there was a 64% loss of patients at the 5-year follow-up and results were underpowered. Moreover, three trials evaluated bronchial inflammatory markers, two of which (Kukkonen et al., 2011, p. 20; van der Aa et al., 2011) supplemented synbiotics. Kukkonen et

al., (Kukkonen et al., 2011) reported than F_ENO was not significantly different between the synbiotic and placebo group after 5 years (Synbiotic vs. Placebo; 5.45, [4.3, 7, 3] vs. 5.7 [3.9, 6.8] $P = 0.22$). Conversely, Vanderaa et al., (van der Aa et al., 2011) found no difference in total and house-dust mite specific IgE concentrations following synbiotic treatment. In one trials (Ivakhnenko and Nyankovskyy, 2013) salivary IgA concentrations were reported as significantly increased compared to control standard formula feeding after 2 months ($P<0.05$), and similar to reference breastfeeding. The opposite effects were seen in the same trials for concentrations of alpha defensin HNP1-3, with significantly lower levels in standard formula feeding compared to the treatment and similar levels to reference breastfeeding.

Description of Adult population trials

Three trials, published between 2010 and 2019, examined the effects of prebiotics and/or synbiotics on asthma in adults. The trials were completed in Australia (McLoughlin et al., 2019), United Kingdom (Williams et al., 2016) and the Netherlands (Van De Pol et al., 2011). Sample sizes ranged from 8 to 16 per group and groups were sufficiently randomised; each of the three trials scored 6 on the JADAD scale. Asthma participants were physician diagnosed, one trial (Van De Pol et al., 2011) included participants with house-dust mite allergy, and 59% of participants were female.

One of the three trials used a prebiotic (Williams et al., 2016), one used a synbiotic (Van De Pol et al., 2011) and the other assessed both prebiotics and synbiotics (McLoughlin et al., 2019). Prebiotic was delivered orally in a powder form against a maltodextrin placebo in all trials and intervention durations ranged from 1 to 4 weeks, with a mean duration of 2.6 weeks. Williams et al., (Williams et al., 2016) administered a prebiotic in the form of GOS with a β-galactosidase enzyme (B-GOS) at a dose of 5.5g per day. Conversely, in the synbiotic only trials by Van de pol et al., (Van De Pol et al., 2011) a combination of short chain GOS and long chain FOS, at

a ratio of 9:1, was taken alongside *B breve M16-V* at $2x10^{10}$ CFU in capsule form. Moreover, Mcloughlin et al., (McLoughlin et al., 2019) implemented a 7 day intervention crossover trials supplementing 6g of inulin, twice daily, with and without a multi-strain probiotic capsule daily; the capsule contained *Lactobacillus acidophilus* LA-5 ($7.5x10^9$ CFU), *Lactobacillus rhamnosus* GG ($8.5x10^9$ CFU) and *Bifidobacterium animalis subspecies lactis* BB-12 ($8.75x10^9$CFU).

All three trials (McLoughlin et al., 2019; Van De Pol et al., 2011; Williams et al., 2016) reported lung function (FEV_1) and inflammatory gene and/or protein expression. Sputum cell counts, including changes in white blood cells, were measured in two of the trials (McLoughlin et al., 2019; Van De Pol et al., 2011); and one trials (McLoughlin et al., 2019) observed changes in expression of GPR41, GP43 and HDAC9. The characteristics and outcome measures of the three included trials are outlined in table 5.3. Williams et al., demonstrated a significant increase in FEV_1 following a bronchoprovocation challenge. Following prebiotic treatment, peak fall in FEV_1 post EVH increased by 40% (P = 0.004) and peak fall of PEF post EVH (P = 0.024) increased by 11%. Furthermore, prebiotic treatment decreased systemic markers of inflammation, serum TNF-α (P = 0.002), CCL17 (P = 0.005) and CRP (P = 0.015*)*. Moreover, CCL11, IgE and F_ENO were not significantly changed. In the synbiotic trials (Van De Pol et al., 2011), baseline FEV_1 was unchanged (P = 0.603), however following synbiotic treatment morning PEF increased (P = 0.003) but not vening PEF (P = 0.11). Moreover, there was reduction in serum IL-5 (P=0.034), and IL-4 + IL-13 (P=0.046) from house-dust mite stimulated peripheral blood mononuclear cells. Other markers including IL-10, Il-12p40/p70, IFN-γ and Foxp3 were unchanged, as well as sputum eosinophil and neutrophil counts (Van De Pol et al., 2011). Conversely, Mcloughlin et al., (McLoughlin et al., 2019) demonstrated that a soluble fibre (inulin) intervention reduced sputum eosinophil counts significantly (P = 0.006), along with sputum HDAC9 gene expression (P = 0.008).

Table 5.3. – Summary of adult trials examining the effects of prebiotics and synbiotics on asthma outcomes

Study (country)	Design	Population, n	Prebiotic/dietary intervention, daily dose (g)	Synbiotic, (probiotic) daily dose (g)	Relevant outcome measures
Mcloughlin et al., 2019 Australia	RCT, Double blind, three way crossover	Physician diagnosed asthmatics, 17	Soluble fibre (Inulin) at 12g per day, through oral administration (powder form)	*Lactobacillus acidophilus* LA-5 ($7.5x10^9$ CFU), *Lactobacillus rhamnosus* GG ($8.5x10^9$ CFU) and *Bifidobacterium animalis subspecies lactis* BB-12 ($8.75x10^9$CFU).	Sputum cell counts, inflammatory gene expression; GPR41/43, HDAC9. FEV_1, ACQ-6
Williams et al., 2016 United Kingdom	RCT, double blind, crossover	Asthma (hyperpnoea-induced bronchoconstriction), 10	GOS with a β-galactosidase enzyme at 5.5 g per day, through oral administration (powder form)	X	Inflammatory markers; CC17, TNFα and CRP.PEF, FEV_1
Vandepol, 2010 Netherlands	RCT, double blind, parallel design	Asthma and house dust mite allergy, 30 (15 in both control and synbiotic groups)	scGOS/lcFOS (9:1), 8g per day, oral administration in powder form, combined with synbiotic and Imunofortis.	*B breve M-16V* $10x10^{10}$. This study exclusively used a synbiotic only.	FEV_1, PEF, markers of inflammation (IL-5) following PC20 methacholine challenge/HDM allergen challenge. Sputum cell counts

5.4. Discussion

This review examined the evidence of prebiotic and synbiotic treatment on asthma in human and murine trials. Meta-analysis of early life prebiotic and synbiotic treatment, in infants, found no protection against the development of recurrent wheezing up to 5 years of age. However, meta-analysis of murine trials revealed prebiotic treatment, but not synbiotic, reduced macrophages, neutrophils, eosinophils, overall lymphocyte counts and airway hyper-responsiveness. Moreover, prebiotic and synbiotic treatment reduced bronchoalveolar lavage fluid, IL-4, IL-5 and IL-13. The effectiveness of prebiotics over synbiotics for the treatment of multiple asthma related outcome measures were demonstrated in the current chapter, although the positive effects of synbiotics were still observed and are not to be dismissed.

The current review can be considered an update of the Cochrane review in 2011, 'early life prebiotic use for prevention of allergic diseases' (Osborn and Sinn, 2007) and 'Prebiotics: Mechanisms and Preventative Effects in Allergy' (Brosseau et al., 2019).

In adults, two trials reported improvements in lung function parameters. Specifically, prebiotics improved both FEV_1 and PEF (Williams et al., 2016); synbiotics improved only PEF (Van De Pol et al., 2011). A more recent adult synbiotic study reported a reduction in sputum eosinophil count post intervention (McLoughlin et al., 2019); this data is conflicted with the meta-analysis of murine trials, as prebiotic treatment reduced eosinophil count but synbiotic treatment did not. Similarly, Meta-analysis of murine trials reported attenuation in AHR severity following both prebiotic and synbiotic treatment. Meta-analysis of murine trials revealed that prebiotic, but not synbiotic, reduced AHR severity, BALF, and white blood cell count.

Various asthma specific markers changed in murine models following synbiotic and prebiotic treatment; perhaps the clearest change was reported in eosinophil counts. All murine trials favoured treatment over control, except from one treatment from a single study (Verheijden et al., 2016). In the study by Verheijden et al., two treatments were used, a mixture of GOS/FOS

(9:1) with *B breve M16V* (Tx1) and FOS/*B breve M16V* (Tx2). Tx2 combination treatment appeared to be more effective at reducing eosinophil counts, than Tx1, highlighting possible differences in the synergistic efficacy of these prebiotic combinations and challenging the conventional formula (Macfarlane et al., 2007). Conversely, in the study by Sonoyama et al., GOS reduced eosinophil counts more than FOS did, although this was without synbiotic treatment. Moreover, the Verheijden Tx1 treatment (Verheijden et al., 2016) appeared to be less effective than Tx2 and favored the control for neutrophils, lymphocytes and airway hyperresonsiveness.

It has previously been reported that prebiotic treatment does not prevent infant asthma (Osborn and Sinn, 2007); asthma was diagnosed by incidence of 3 or more episodes of recurrent wheezing from two trials enrolling 373 infants (Arslanoglu et al., 2008; Westerbeek et al., 2010). The current meta-analysis added an extended 5 year follow up (Arslanoglu et al., 2012) and an additional trials with 160 infants (Ivakhnenko and Nyankovskyy, 2013) to recurrent wheeze data. However, it was concluded that there is still insufficient evidence to recommend the use of prebiotics in infants for prevention of the asthma outcome that is recurrent wheeze. Similarly, two synbiotic intervention trials also indicated no protective effect on recurrent wheeze (Kuitunen et al., 2009; van der Aa et al., 2011) for a sample size of 1313 infants. Three trials (Arslanoglu et al., 2008; Ivakhnenko and Nyankovskyy, 2013; van der Aa et al., 2011) reported a protective effect with the use of both synbiotic and prebiotics up to a 2 year follow up. However, this effect was not observed at a 5 year follow up (Ivakhnenko and Nyankovskyy, 2013; Kuitunen et al., 2009). Gut microbiota colonisation is most susceptible to manipulation at early infancy and *Bifidobacterium* are dominant initially (Arboleya et al., 2015; Barrett et al., 2013; Taft et al., 2018); therefore, this may explain why prebiotic and synbiotics did not have a durable effect after the treatment period.

In one trial (Ivakhnenko and Nyankovskyy, 2013) at 2 months of age, *Bifidobacterium* levels were parallel in both the breastfed group and prebiotic formula fed group; however, *Bifidobacterium* were unstable in the breastfed group, and began to decline as infants aged (Harmsen et al., 2000; Voreades et al., 2014). Gut bacteria stability is achieved at 1-2 years of age, with a permanent adult-like structure in the third year of life (Koenig et al., 2011; Yatsunenko et al., 2012). Furthermore, a separate study recently reported that gut microbiota are not fully established until 5 years of age (Cheng et al., 2016), highlighting the importance of prebiotic and synbiotic treatment duration. A 5 year treatment duration may therefore achieve a prolonged protective effect on recurrent wheeze in infants and further studies are warranted to examine this possibility. Moreover, supplementing earlier in pregnancy to mothers with high risk of atopy may contribute to fetal colonisation, which can occur as early placenta or amniotic fluid forming stages (Collado et al., 2016), where $CD4^+$, $CD8^+$ T cell activity is apparent (Spencer et al., 1986).

The present study encompasses a small number of adult trials in comparison to murine and infant trials, without more adult trials it is challenging to assess prebiotic and/or synbiotic treatment in an adult Asthma population. Accordingly, meta-analysis of adult trials was not completed due to a lack of data and excessive methodological variability between trials. Moreover, adult trials reported changes in FEV_1, PEF and eosinophil counts following prebiotic and/or synbiotic treatment. It appears the use of a bronchoalveolar challenge elucidates the physiological differences between treatment and control groups (Williams et al., 2016), allowing a demonstration of the prebiotic mechanism of action, through silent epigenetic changes. Halnes, et al, observed changes in genetic markers such as GPR43 and GPR41 and McLoughlin et al., measured HDAC activity in peripheral blood mononuclear cells. By uncovering the prebiotic mechanism of action, we can better experimental design, increase the

number and quality of adult asthma trials. However, at this stage there is an insufficient data to recommend prebiotic use for attenuation of asthma outcomes in adults.

It remains unclear how prebiotics elicit their immune-regulatory effect. Prebiotics have been demonstrated to increase the quantity of certain bacteria species in the gut, in turn altering immune cell signalling in the intestinal lumen (Vulevic et al., 2015). SCFAs are also able to influence immune cells through G protein –coupled receptor GPR41, GPR43, and GPR109a, leading to activation of signal cascades (Ang and Ding., 2016). Moreover, SCFAs are absorbed by substrate transporters such as MCT_1 and $SMCT_1$ promoting cellular metabolism (Parada Venegas et al., 2019).

Over 90% of SCFAs are absorbed in the intestinal lumen, the majority of which are metabolised by colonocytes or transported to the liver. However, the remaining SCFAs, primarily acetate, enter systemic circulation with the proposed effect of reducing systematic inflammation through G-protein coupled receptor modulation (GPCR41 & 43) and inhibition of histone deacetylase enzymes (McLoughlin et al., 2019). In vitro trials have reported that chemotaxis of neutrophils is GPR43 dependent (Maslowski et al., 2009) and SCFAs can bind directly to and activate GPR43 (Vinolo et al., 2011). Furthermore it has been reported that SCFAs reduce airway eosinophilia, this effect was dependent on GPR41 (Theiler et al., 2019). Modulation of gene expression is facilitated through SCFA activation of GPR41, GPR43 and GPR109A, and thus the MAPK signalling pathway; GPR43 and GPR109A can also activate the NF-κβ signalling pathway.

SCFA concentration progressively decreases from the proximal colon to the distal end from 70 to 140mmol/l down to 20-70mmol/l, respectively (Holtug et al., 1992). Both GPR41 (FFA3) and GPR43 (FFA2) have an EC_{50} of around 0.5mM; potency ranks for GPR41 are Propionate/butyrate > acetate, and potency ranks for GPR43 are acetate/propionate > butyrate

(Brown et al., 2003; Ulven, 2012). An important measurement to consider when researching prebiotics is how many SCFAs are in the blood following administration, and what effect do different concentrations have on physiological responses and genetic change. Improved understanding of prebiotic and synbiotic mechanisms will inform research, increasing the quality and quantity of trials on prebiotic and synbiotic treatment of asthma, therefore, allowing a more informative meta-analysis in the future.

Existing research on prebiotic and synbiotic treatment of asthma has number of limitations, heterogeneity among trials, study duration, supplement formulation, dosage, asthma outcome variables and an all-round limited number of trials included in each meta-analysis. Due to limited data, particularly in humans, many asthma outcome measures could not be assessed, and therefore the overall effectiveness of prebiotics as a treatment for asthma could not be determined. Furthermore, there is a lack of data on background dietary intake in both infants and adults, thus it is difficult to assess the effects of treatment independently, specifically in the infant trials of the present study (Table. Despite these limitations, to our knowledge, this is the first systematic review to perform a comprehensive evaluation on all available evidence on the effect if prebiotics and synbiotics on asthma outcomes in both murine models and human participants.

In summary, it has been demonstrated that there is a variety of evidence supporting the benefits of prebiotic and synbiotic treatment for certain asthma outcome measures in both murine models and humans. Early life treatment was not effective against recurrent wheeze development in children up to 5 years, however evidence in adult and murine trials suggests lung function parameters can be improved, perhaps more so with prebiotic treatment. An increased number of high-quality trials are needed in order for these parameters to be categorically assessed, particularly adult human trials measuring both physiological and epigenetic change in response to an asthma specific challenge.

Chapter 6 – The effects of prebiotic *trans*-galactooligosaccharide (B-GOS) treatment on biological markers of systemic inflammation in healthy adults

6.1 Introduction

Inflammation is a biological response to harmful stimuli such as pathogens, toxic compounds and irradiation or damaged cells. In normophysiology, acute inflammation efficiently minimises injury or infection and results in restoration of tissue homeostasis. However, in a disease state uncontrolled inflammation may become chronic, leading to tissue damage and cell death (Chen et al., 2017). Living with low-grade systemic inflammation can often lead to the damaging of healthy cells, tissues, and organs, over time leading to DNA damage, internal scarring, and the development of both mental and physical disease. The strongest evidence for the association of systemic chronic inflammation (SCI) and disease risk comes from RCTs that test drug therapies or biologics that target pro-inflammatory biomarkers, such as TNF-α and IL-1β (Furman et al., 2019). Anti-TNF-α therapy has been demonstrated to improve insulin sensitivity in RA and reduce insulin resistance, the risk for developing Alzheimer's disease was also significantly lower in patients with RA treated with Anti-TNF-α (Chou et al., 2016). However, we must look for alternatives to pharmacological treatments in order to better treat the underlying causes of SCI and inflammatory disease risk.

The prevalence of chronic inflammatory disease is increasing worldwide (Molodecky et al., 2012), and environmental factors such as diet and the commensal microbiota have been identified as key variables. Consumption of a 'Westernised' diet is associated with increased risk for many diseases, such as IBD (Uranga et al., 2016) and asthma (Leiria et al., 2015). The westernised or low fibre diet is characterised by a large quantity of proteins (from fatty processed meats), saturated fats, sugar, alcohol, refined grains, and corn derived fructose syrup, accompanied by reduced consumption of fruit and vegetables (Tilg and Moschen, 2015; Uranga et al., 2016). Diets rich in fibres and low in fats reduce the risk of allergy, obesity, metabolic syndrome and cardiovascular diseases (Shoelson et al., 2007). The link between diet,

nutrients and immunity is hidden within a complex network of signalling, to better our understanding, this network needs to be considered along with microbial composition, lifestyle and genetic background. Understanding the relationship between the gut microbiome and the immune system will drive the discovery of novel nutritional interventions to benefit disease pathology.

The gastrointestinal (GI) microbiota is comprised of thousands of bacterial species which colonise the mucosal surface of the gut (Thursby and Juge, 2017). These intestinal bacteria regulate the gut associated lymphoid tissue (GALT), in which cells modulate immune responses through the regulation of T lymphocytes. T lymphocytes are responsible for the release of both pro-inflammatory and anti-inflammatory cytokines and chemokines, and they play a pivotal role in the pathology of inflammatory disease (Betancourt et al., 2017). In chronic inflammatory disease cytokine and chemokine release is uncontrolled, resulting in persistent tissue inflammation. This is common for the onset of dementia, cerebrovascular diseases, cancer, chronic lower respiratory diseases and ischaemic heart disease (Chen et al., 2017). The increase of chronic inflammatory disease seen in western societies over the past decade may be associated with alterations in GI microbiota in response to poor diet, increased antibiotic use and changes in lifestyle, which leads to dysbiosis of the GI tract and an increased risk to multiple diseases (DeGruttola et al., 2016).

The gut microbiota are responsible for fermentation of substrates such as, intestinal mucus and dietary fibres. This fermentation allows growth of specific microbes that produce three main SCFAs; butyrate, propionate and acetate. Butyrate maintains energy homeostasis through intestinal gluconeogenesis and is capable of inducing cancer cell apoptosis in the colon (De Vadder et al., 2014). It is also crucial for epithelial cell oxygen consumption, enabling β oxidation, which mitigates dysbiosis by maintaining oxygen balance in the gut (Byndloss et al., 2017). Propionate moves to the liver and regulates satiety and gluconeogenesis through free

fatty acid receptors (FFARs) in the gut (Besten et al., 2013). Acetate is the most abundant SCFA and critical for bacteria growth, it is active in peripheral tissues where it is used in lipogenesis and cholesterol metabolism (see section 2.5.3 for more on SCFAs). In murine models, high production of SCFAs have been demonstrated to regulate hormones and reduce food intake (Lin et al., 2012), and is also correlated with reduced insulin resistance and prevalence of type-2-diabetes (Zhao et al., 2018). Manipulation of the gut microbiome through SCFA production, may help treat inflammatory disease. Specifically consuming fermentable dietary fibre, such as a prebiotic, may modulate bacteria growth through SCFA production, and regulate host interaction, reducing risk of inflammatory disease.

In 1995, Gibson and Roberfroid defined prebiotics as 'non-digested food components that, through the stimulation of growth and/or activity of a single type or a limited number of microorganisms residing in the gastrointestinal tract, improve the health condition of a host' (Gibson and Roberfroid, 1995). Later in 2004, prebiotics were defined as 'selectively fermented components allowing specific changes in the composition and/or activity of microorganisms in the GI tract, beneficial for host health and wellbeing' (Gibson and Probert, 2004). Finally in 2017, ISAPP defined a prebiotic as 'a substrate that is selectively utilised by host microorganisms conferring a health benefit' (ISAPP, 2017). Prebiotics are broken down into SCFAs via fermentation in the gut. It has been demonstrated that prebiotic and/or probiotic intake in multiple diseases is associated with a reduction in certain inflammatory biomarkers, such as TNF-α, CRP and interleukins (McLoughlin et al., 2017), although the underpinning mechanisms remain unclear.

Acetate, butyrate and propionate (SCFAs) are the end products of anaerobic fermentation of dietary fibre by the intestinal microbiota. Various factors influence SCFA production; for example, oligosaccharide soluble fibres such as fructo-oligosaccharides (FOS) produce higher yields than longer chain polysaccharides such as pectin (Besten et al., 2013). Following

fermentation of dietary fibre, SCFAs lower colonic pH which is associated with the growth of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* (Venegas et al., 2019). Interestingly, the ingestion of these bacteria or others, known as probiotics, has been demonstrated to benefit the host by temporarily altering the colonic microbiome (Hemarajata et al., 2013). Similarly, synbiotics, a mixture of both prebiotics and probiotics has demonstrated host benefits through alteration of microbiota (Besten et al., 2013). It has been estimated that more than 90% of SCFAs are absorbed within the intestinal lumen, the majority of which are metabolised by colonocytes, remaining SCFAs are transported to the liver via the hepatic portal vein. Some SCFAs, especially acetate, may enter the systemic circulation and facilitate a reduction in systemic inflammation through GPR41, GPR43, inhibition of histone deacetylase enzymes and histone acetyltransferase activation (Sydney and Denu., 2021; Tromepette et al., 2019).

Research into prebiotic use for general health and nutrition is growing, unlike probiotics, prebiotics are resistant to acids, proteases, and bile salts in the GI tract, one of many favourable attributes (see section 2.5.2 onwards for prebiotics and B-GOS). Current research provides evidence to support the use the prebiotics in multiple disease states and demonstrate the systemic anti-inflammatory effects of prebiotic treatment (McLoughlin et al., 2017). Through SCFA modulation, galacto-oligosaccharides display a protective effect against high fat/western-style diets in mice (Dai et al., 2019) and induce positive change in the human gut microbiome (Depeint et al., 2008; Johnstone et al., 2021; Vulevic et al., 2015). Multiple studies have also demonstrated the use of B-GOS in individuals without disease, despite the limited research on this topic, there is a potential benefit (Vulevic et al., 2015; Williams et al., 2015). Williams et al., demonstrated a reduction of TNF-α in both control and EIB participants, after B-GOS treatment. Moreover, in a study by Vulevic et al., a reduction in IL-1β was observed, along with increases in IL-8 and IL-10 after B-GOS treatment. The significantly greater

bifidogenic effect of B-GOS over other GOS products is also worth mentioning, highlighting its level of functionality and selectivity as a prebiotic (Depeint et al., 2008).

Strategies for advancing the early prevention and treatment of SCI are essential for improving public health and mitigating disease risk. As such the aim of the current study was to investigate the effectiveness of prebiotic B-GOS, A galacto-oligosaccharide food supplement, on biological markers of systemic inflammation in healthy adults.

6.2 Methods

6.2.1. Participants

Sixteen healthy male participants volunteered for the study and provided written informed consent (Appendix 6). Participants were screened for eligibility in accordance with the inclusion/exclusion criteria detailed in the participant information sheet (Appendix 7). Two participants were excluded from the trial, one for antibiotic use and the other due to travel logistics; leaving a total of 14 participants (age 26 ± 1.8 yr; height 1.8 ± 0.08 m; body mass 77 ± 7.6 kg) (Figure 6.0). The sample size of 14 participants was calculated in Chapter 4 (see section 4.2), research reagents were purchased based on this number. As the study in Chapter 4 was stopped due to COVID-19, the research reagents were utilised in the current study, where there was only enough research reagents for 14 participants to complete the intervention, funding constraints meant this number could not be adapted. All recruiting and interactions with volunteers was conducted by Mr Jacob Jayaratnasingam.

Throughout the study participants were instructed to continue their habitual lifestyle. Participants abstained from alcohol, caffeine and strenuous exercise for 24 h prior to each laboratory visit in order to reduce blood parameter variables and inflammation (Onsa, 2010; Tauler et al., 2013)

6.2.2. Experimental Design and Protocol

The study was conducted according to guidelines in the Declaration of Helsinki and all procedures were approved by the Nottingham Trent University Human Invasive Ethical Reviews Committee (approval no. 585). The present study adopted a randomised, double-blind, placebo controlled cross-over design over nine weeks per participant. Each participant maintained their normal diet and were randomised to receive one of the following supplements (powder form) for 20 days:

- *Trans*-galactooligosaccharide (B-GOS) 3.65g (79.5% GOS) (0.15g fat, 1.3g, carbohydrate, 2g fibre, 0.15g salt, 0.05g protein, 2.9g galacto-oligosaccharides)
- Placebo (PLA) – sachet containing maltodextrin 3.65g (taste appearance matched polysaccharide)

Supplements were identical in size, shape, taste and colour. Prior to each testing session dietary intake was monitored by a food diary and participants completed a gastrointestinal symptoms questionnaire (Gaskell, 2018). At Day 0 and Day 21 of each experimental arm participants attended the laboratory between 08:00 h and 09:00 h. Thus, participants attended the laboratory the day after their final sachet was ingested to avoid any possible acute effects of the prebiotic (Halnes et al., 2016). Fasted blood samples were collected on the morning of each visit by a trained phlebotomist, after ingestion of 500ml water. A two-week washout separated intervention arms, analogous with another B-GOS intervention study (Williams et al., 2017), two-weeks is a suitable time period for the gut microbiota to return to baseline levels (David et al, 2013; Depeint et al., 2008). No bacteriology was carried out due to funding constraints.

6.2.3 Blood collection and preparation

Blood samples were obtained by drawing blood from the antecubital fossa region of the forearm, with a 21-gauge needle (Butterfly Winged Infusion Set, Vacuette, Greiner Bio-One, Kremsmünster, Austria). Blood was collected using 5ml vacutainer tubes (Plus Blood Collection Tubes, BD Vacutainer, BD, Plymouth, UK). After collection of 10 mL whole blood, peripheral blood cells were separated from platelet-rich plasma using a series of centrifugation cycles. Blood was decanted into falcon tubes and then phosphate buffered saline (Lonza, Basel, Switzerland) was added at a 1:1 dilution (PBS). Once mixed, the contents were aliquoted into Leucosep tubes pre centrifuged with 15 mL Ficoll Paque (Sigma Aldrich, Missouri, US), and then PBS was added for a 1:1 dilution. Leucosep tubes were centrifuged for 30 minutes at 800 x g, followed by transfer of media into a 50 mL falcon tube and another 1:1 dilution with PBS.

Falcon tubes were centrifuged for 10 minutes at 600 x g, supernatant was discarded, and pellet resuspended with PBS, followed by a final centrifugation cycle for 10 minutes at 400 x g, supernatant was discarded and pellet resuspended in 1 mL of RPMI 1640 (Sigma Aldrich, Missouri, US). Cell counts were completed using a 1 in 25 dilution of Trypan-blue and a haemocytometer. The concentration of PBMCs obtained was adjusted to $1x10^6$/ml and transferred to 12x75mm FACS tubes. Samples were incubated in RPMI 1640 at 40 degrees Celsius for 5hr with Brefeldin A (10ug/ml; Sigma Aldrich, Missouri, US), this incubation was for logistical purposes (see section 7.2.2). After incubation cells were permeablised with True-Nuclear™ transcription factor buffer set (BioLegend) and stained for flow cytometry. Specific antibodies conjugated with a fluorophore were utilised to quantify Foxp3 (Thermofisher Scientific), TNF-α (BioLegend), NF-kβ (BD Biosciences), IL-4 (BD Biosciences), CD3 (BioLegend), IL-6 (BioLegend), CD4 (BioLegend), CD25 (BioLegend), GATA3 (BioLegend), and live/dead cells (BioLegend).

6.2.4. Statistical analysis

Data were analysed using statistical software GraphPad for Windows version 9.00 (GraphPad Software., San Diego, CA, USA). Data were examined for normality using the Shapiro-Wilk test. Normally distributed data (CD3, CD4, and Foxp3) were analysed using a two-way repeated-measures ANOVA and Bonferroni adjusted paired *t* tests. If data were not normally distributed (IL-4, TNF-α, NF-kβ, $CD4^+$ $CD25^+$) the non-parametric Friedman test was used with a mixed effects model and Dunn's correction for multiple comparisons. Two-way repeated measures ANOVAs were used to analyse the effects of treatment (Prebiotic vs Placebo) time (Day 0 vs Day 21) and time x treatment with between group analysis (Pre Placebo, Post Placebo, Pre Prebiotic, Post Prebiotic). Data are presented as mean values with standard deviations with statistical significance set at $P<0.05$.

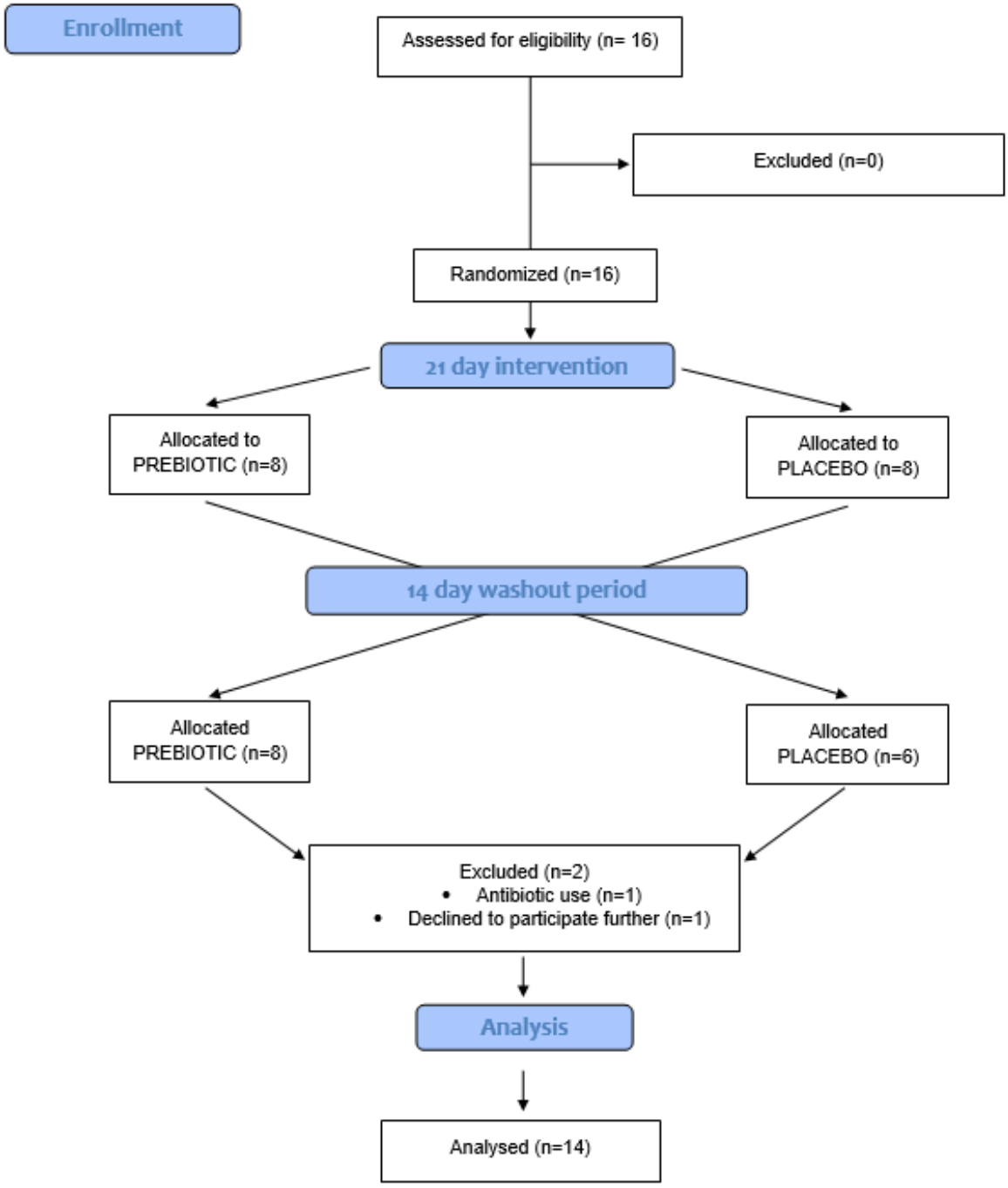


Figure 6.0 CONSORT diagram of participant flow throughout the study

6.3. Results

Gating methods for flow cytometry are presented in Figure 6.1, more information can be found on gating methods in section 3.4. No differences in total $CD4^+$ $CD25^+$ were found across the intervention (3.15 ± 0.246), a Friedman statistic of 2.914 was rendered, which was not significant (P=0.405). For the Gaskell questionnaire there were no main effects of treatment on participant's GI symptoms (P>0.999). Moreover, the completion rate for the food diary was 37.5%, which was considered insufficient for further analyses.

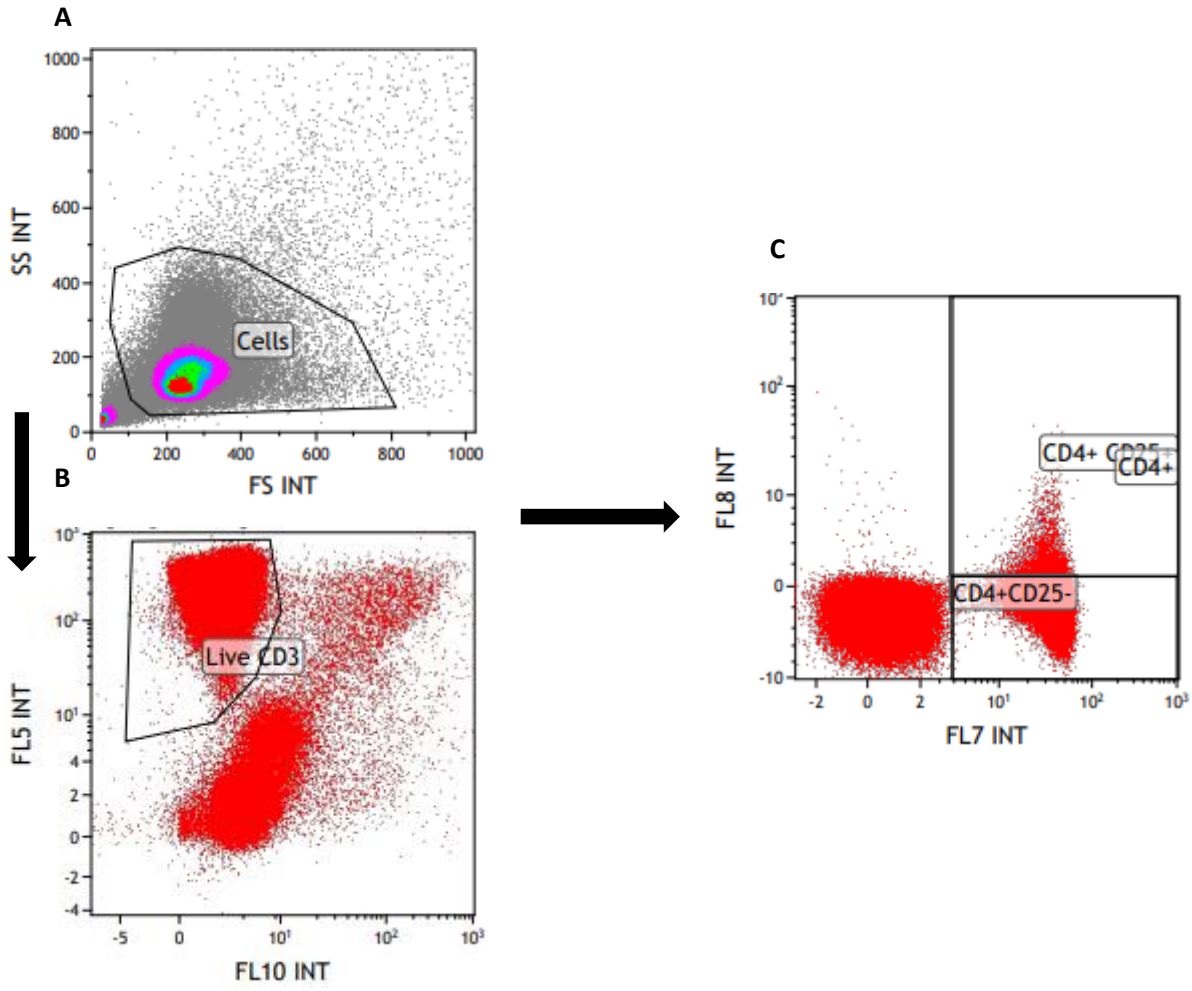


Figure 6.1. Gating method for flow cytometry analysis of peripheral blood mononuclear cells. **(A),** live single cell gating; **(B),** live CD3 gating; **(C),** gating of $CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ cells. Abbreviations; SS INT, side scatter intensity; FS INT, forward scatter intensity; FL, fluorophore; CD, cluster of differentiation.

For $CD4^+$ T cells, there was a main effect of time (P=0.0179), but no main effect of treatment (P=0.578) or treatment-time interaction (P=0.817). Bonferroni's multiple comparisons test showed no differences between testing pre and post placebo (P=0.281), or pre and post prebiotic (P=0.512). For all measures, there were no differences between treatments at baseline (P > 0.999).

For CD4+ CD25+ FoxP3 cells, there were no main effects of treatment (P=0.118) or time (P=0.679), and no treatment-time interaction effect (P=0.351). There were no differences in CD4+ CD25+ $FoxP3^+$ T cells after prebiotic treatment (P>0.999) (Figure 6.2 - A).

For NF-kβ in $CD4^+$ T cells a Friedman statistic of 3.77 was rendered, which was not significant (P=0.286), and no differences were observed after prebiotic treatment (P=0.269). Similarly, for NF-κβ in CD4+ CD25+ T cells a Friedman statistic of 1.32 was rendered, which was not significant (P=0.725), and no differences were observed after prebiotic treatment (>0.999) (Figure 6.2 - B).

For TNF-α in $CD4^+$ T cells a Friedman statistic of 10.29 was rendered, which was significant (P=0.016). TNF-α was reduced by 29.7% after prebiotic treatment (P=0.017) (Figure 6.3). Conversely, for TNF-α in $CD4^+CD25^+$ T cells a Friedman statistic of 1.187 was rendered, which was not significant (P=0.756), and TNF-α was unchanged after prebiotic treatment (P>0.999).

For IL-4 in $CD4^+$ T cells a Friedman statistic of 4.25 was rendered, which was not significant (P=0.236). IL-4 was unchanged after prebiotic treatment (P=0.497) (Figure 6.4). For IL-4 in $CD4^+CD25^+$ T cells a Friedman statistic of 1.66 was rendered, which was not significant (P=0.645). IL-4 was also unchanged after prebiotic treatment (P>0.999).

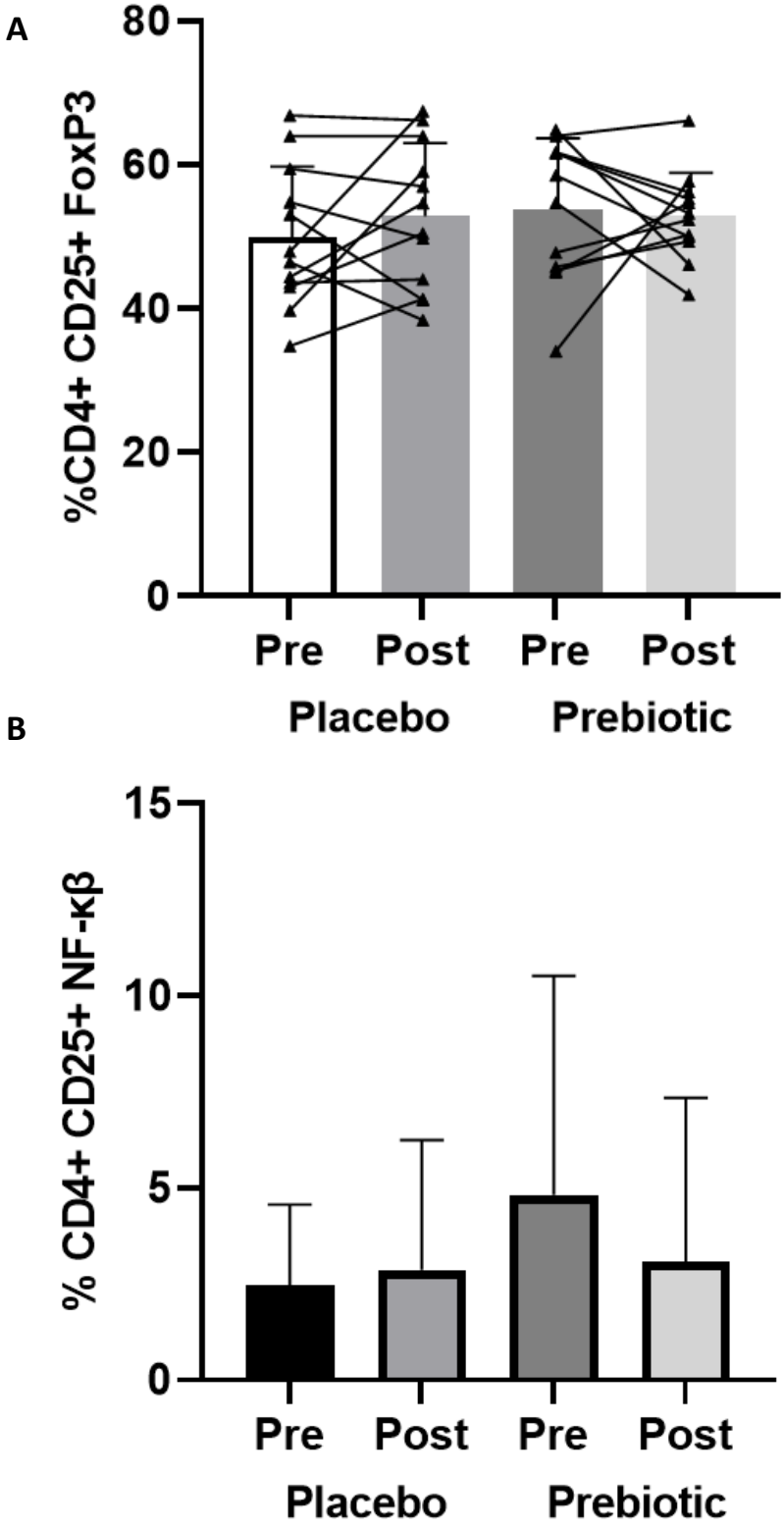


Figure 6.2. The effects of 20 days treatment with prebiotic B-GOS on **(A)** NF-κβ and **(B)** Foxp3 in the peripheral blood mononuclear cells of healthy adult participants. Results are shown as % mean difference ± SD.

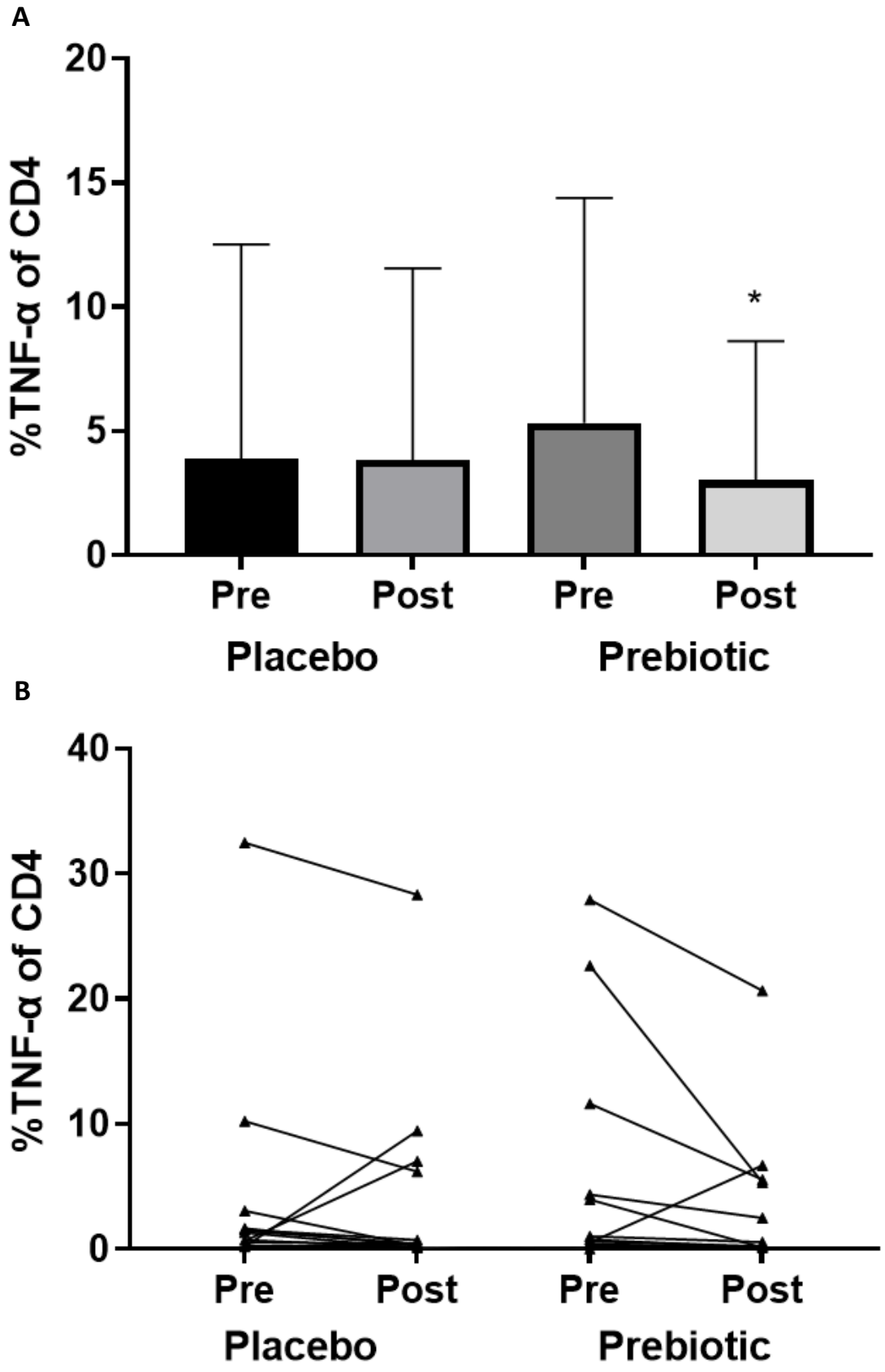


Figure 6.3. The effects of 20 days treatment with prebiotic B-GOS on TNF-α in $CD4^+$ T cells in the peripheral blood mononuclear cells of healthy adult participants. * denotes a significant difference between pre and post prebiotic conditions $P < 0.05$. **(A)** results are shown as % mean difference ± SD. **(B)** results are shown as individual participant responses to prebiotic B-GOS.

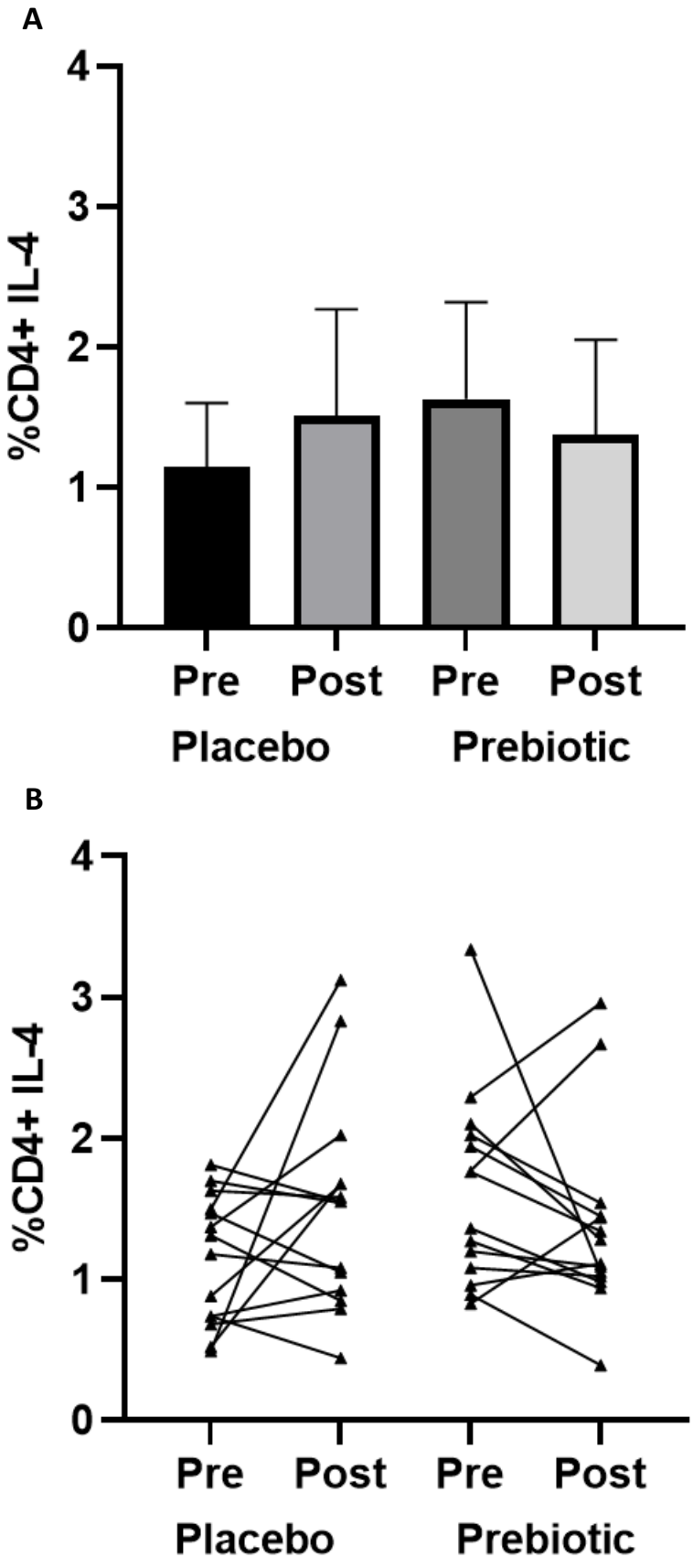


Figure 6.4. The effects of 20 days treatment with prebiotic B-GOS on Interleukin-4 in $CD4^+$ T cells in the peripheral blood mononuclear cells of healthy adult participants. **(A)** results are shown as % mean difference ± SD. **(B)** Individual responses to prebiotic B-GOS.

6.4. Discussion

6.4.1. Main Findings

Twenty days treatment with 3.65g of prebiotic B-GOS (80% GOS) resulted in a significant reduction of TNF-α in $CD4^+$ T cells of healthy adults; this affect was not observed in $CD4^+CD25^+$ T cells. In contrast, B-GOS did not affect IL-4 concentrations in PBMCs, $CD3^+$, $CD4^+$ and $CD4^+$ $CD25^+$ T cell counts, or protein expression of transcription factors Foxp3 and NF-κβ.

6.4.2. Cytokine response to B-GOS

TNF-α was reduced in Th cells by 29.7% after prebiotic treatment, although this result was not replicated in Tregs. The findings are consistent with a recent meta-analysis, where TNF-α was reduced following oligosaccharide treatment in multiple studies on type 2 diabetes mellitus (McLoughlin et al., 2017). In one study from the meta-analysis, an 8-week treatment with oligofructose -enriched inulin led to a 19.8% decrease of plasma TNF-α in women with type 2 diabetes (Dehghan et al., 2014). The reduction of $CD4^+$ TNF-α observed in the present study after B-GOS treatment is consistent with the 18.6% reduction of plasma TNF-α previously observed in adults with asthma who also consumed, daily, 3.65g of B-GOS for 3 weeks (Williams et al. 2015). It is worth highlighting that in the latter study the final B-GOS dose was ingested on the same day as blood sampling, which may have elicited an acute effect of B-GOS ingestion and mediated the reduction in TNF-α (Halnes et al., 2017). The results of the present study therefore suggest that the reduction in TNF-α was not due to an acute effect, since blood samples were taken 1 day after the final dose. Conversely, a study examining the effects B-GOS treatment over 10 weeks, in elderly persons aged 65-80 years, observed no differences in TNF-α (Vulevic et al, 2015). A larger dose of 5.5g daily was used and a 10 week intervention,

as opposed to 3 weeks in the current study. Moreover, it was also found that B-GOS treatment led to increased IL-10, IL-8, NKT activity, CRP and reduced IL-1β. However, this increase in CRP was biologically insignificant and much lower than CRP values associated with negative effects (Vulevic et al., 2015). These differences in IL-10, Il-8, IL-1β and NKT activity, as well as other biomarkers which were not measured, may compensate for the lack of change observed in TNF-α through a functional overlap (Foster, 2001). These findings along with the present study's findings, highlight the anti-inflammatory effects of B-GOS, the potential benefits of B-GOS as a prebiotic to enhance both the gut microbiome and immune system, and that the effects of B-GOS may be age-dependent. In the same study by Vulevic, it was demonstrated there were no effects of time or intervention on gastrointestinal symptoms (GIS), analogous results were obtained in the current study (Vulevic et al., 2015).

Baseline PBMC concentrations of IL-4 did not change in response to prebiotic treatment in Th and Treg cell populations of healthy adults. The role of IL-4 in hypertension and cardiovascular disease is well documented (Kassem et al., 2020), supplementation of IL-4 in rats has been demonstrated to improve pathophysiological mechanisms involved in hypertension, specifically through Th2 numbers and inflammatory regulation (Cottrell et al., 2018). Moreover, IL-4 has an important role in allergic inflammation, where ligation of IL-4R enables eosinophil migration to the lungs through expression of eotaxin (Steinke and Borish, 2001), while also inhibiting eosinophil apoptosis in addition to promoting eosinophilic inflammation. IL-4 is increased in PBMCs of atopic asthmatics (Leonard et al., 1997), and regulating this altered cytokine production may prove beneficial in the treatment of chronic inflammation in respiratory disease. In one study, PBMC isolates from allergic patients were treated with probiotics, including *L.casei*, *L. lactis* and *L. plantarum*, resulting in a decrease in IL-4 secretion (Toh et al., 2012). Similar findings have also been reported in HDM murine models with raffinose treatment which reduce IL-4 and IL-5 mRNA (Watanabe et al., 2004), and

fructooligosaccharide treatment which reduce IL-4, IL-10 and IFNγ (Verheijden et al., 2015). Verheijden et al., also demonstrated in HDM mice that the protective effect of galactooligosaccharide treatment was abolished following the depletion of Tregs, indicating the importance of Tregs in the prebiotic mechanism of action. However, there is contradictory evidence in the literature and another study in HDM mice showed no influence of fructooligosaccharides on IL-4 or IFNγ concentrations (Yasuda et al., 2010). Moreover, the findings of the current study are not in a clinical population and an inflammatory challenge may be necessary to observe any veiled reductions in IL-4 after B-GOS treatment.

Pre/probiotics have been demonstrated to regulate T cell balance in both human and murine research (Kim et al., 2018, Kwon et al., 2010), facilitating Tregs in the prevention of excessive inflammation (Kang et al., 2020). In the current study, the B-GOS mediated decrease of TNF-α in Th cells may have prevented excessive Treg inflammation through TNFR2 signalling (Jung et al., 2019), regulating NF-κβ expression through both TNFR1&2, thus regulating the release of Treg TNF-α. However, there are discrepancies on the interactions between TNF-α and Treg cells which need elucidating. Though some data suggests TNF-α augments the suppressive function of Tregs (Chen at al., 2008; Chen and Oppenheim, 2010), other studies demonstrate TNF-α reduces this suppressive function (Nagar et al., 2010; Valencia et al., 2006) and that TNF-α appears to reduce Foxp3 expression in Tregs through TNFR2 signalling (Valencia et al., 2006) and the canonical NF-κβ pathway (Nagar et al., 2010).

TNFRs are a crucial target for successful treatment of multiple inflammatory disorders such as obesity, rheumatoid arthritis, psoriasis and vasculitis; anti-TNF treatments confirm this stance. Manipulation of TNF-α and its downstream targets, allows for alterations in immune profile and T cell balance which may benefit patients with chronic inflammatory diseases. In type two diabetes inhibition of TNF-α improves both insulin resistance and lipid profiles, reducing the risk of cardiovascular disease (Popa et al., 2007). Multiple studies have shown that a TNF-α

gene polymorphism may affect its transcription and expression, and is associated with many diseases such as ankylosing spondylitis, type 1 diabetes, type 2 diabetes, rheumatoid arthritis (RA), sarcoidosis and silicosis (Huang et al., 2020; Koch et al., 2001).

6.4.3. Transcription factor expression after B-GOS

In the current study, NF-κβ was unchanged after prebiotic treatment, possibly due to a reduction in TNF-α and thus reduced TNFR1/TNFR2 signalling, as aforementioned. The TNF receptor superfamily facilitate the NF-κβ non-canonical pathway signalling pathway, this pathway is slow but persistent but dysregulated NF-κβ activity is directly related to inflammatory disease as well as cancers (Yu et al., 2020). With the correct stimuli, NF-κβ signalling may also occur through the canonical pathway, this pathway induces quick but transient transcriptional changes. Ligation of IL-1R and TLR4, in the canonical NF-κβ pathway, leads to deubiquitination of Nemo, phosphorylation of IkBα and thereby the release of NF-κβ for translocation (Lotze and Tracey, 2005); prebiotic treatment has been demonstrated to reduce LPS and IL1-β, both ligands of IL-1R and TLR4 (Hardy et al., 2013; Savignac et al., 2016; Vulevic et al., 2013).

Regulatory T cells are characterised by stable expression of 'the master regulator' Foxp3, the role of which is to supress the function of NFAT and NF-κβ and thus downstream cytokine production (Kim, 2009). NF-κβ is also capable of regulating Foxp3 and has been demonstrated to directly promote transcription in natural T cell development; pro-inflammatory cytokines such as TNF-α and IL-6 are also involved, but they negatively regulate transcription and expression (Lu and Pan., 2017). A recent study observed an increase $CD25^{+}Foxp3^{+}$ Treg frequency following synbiotic treatment in a murine model of HDM-Induced asthma. The synbiotic group had increased Tregs compared to both the prebiotic group and asthmatic group

(Ghiamati et al., 2020). However, in the current study $CD4^+CD25^+$ Foxp3 remained unchanged after B-GOS treatment, possibly due to the normophysiology of healthy participants.

6.4.4. SCFA signalling

It has been hypothesised that the prebiotic mechanism of action involves activation of GPR41 & GPR43 through SCFA signalling **(See section 2.5.3)**. Interestingly, in a study on RA, activation of GPR43 reduced the activation of the NF-κβ signalling pathway, other key inflammatory factors were also suppressed, such as IL-6, IL8, ICAM-1, VCAM-1 and ROS. These findings suggest a promising role for GPR43 in RA treatment as well as other inflammatory conditions, however, more human studies are needed to elucidate the protective or causative roles of both GPCRs before being considered as drug targets (Ang and Ding, 2016; Zhang et al., 2020). In vitro it was demonstrated propionate and butyrate are able to dose-dependently induce histone acetylation by activating histone acetyltransferases (HATs), instead of HDAC inhibition (Thomas and Denu., 2021), thereby regulating acetylation/deacetylation of p65 and the canonical NF-κβ signalling pathway through indirect inhibition of TNFR signalling (Li et al., 2018).

6.4.5. Limitations of analysis

The main limitation of the current study was that stool samples were not collected for analysis and the bifidogenic properties of B-GOS could not be quantified. As a consequence, we are unable to establish a causal relationship of B-GOS treatment and the reduction of TNF-α in healthy adults. However, previous research has demonstrated the significant bifidogenic characteristics of B-GOS (Vulevic et al., 2013). In order to understand the beneficial effects of B-GOS and prebiotic treatment it is essential to elucidate the mechanism of action. Moreover, another limitation of the current study was that there was no measurements of SCFAs in the blood. Measuring SCFA concentration in the blood would provide clarity of the B-GOS

mechanism of action and help us to understand whether prebiotic treatment has a direct effect through SCFA signalling, or indirectly affects the immune response through modulation of the gut microbiota.

Moreover, due to the ubiquitous nature of cytokines and the regulatory mechanisms within the human body, assessing cytokine significance in normophysiology has its challenges. A functional overlap may occur where the lack of one factor, such as TNF-α, may be compensated by one or more other factors. In order to assess whether B-GOS has a true impact on the inflammatory response, further study of B-GOS in pathophysiological conditions is required.

6.4.6. Conclusion

For the first time, the current study demonstrates that a 20 day intervention of prebiotic B-GOS can successfully reduce pro-inflammatory cytokine TNF-α in $CD4^+$ T cells of healthy adults. This observed reduction of TNF-α post prebiotic treatment, coupled with the well documented bifidogenic effect of B-GOS (Fanaro et al., 2009; Grimaldi et al., 2016; Vulevic et al., 2015), suggests that changes in the commensal bacteria may significantly alter the immune response in healthy adults. The present study also provides further evidence that the manipulation of commensal bacteria using prebiotic supplementation can influence inflammatory responses beyond the gastrointestinal tract. Moreover, this finding implicates a potentially protective effect of B-GOS for inflammatory disease risk, through TNF-α modulation and thus TNFR signalling. There is still limited research on the effects of prebiotic treatment and GOS in healthy / disease-free individuals, and a direction for future work may be to therefore observe these biomarkers of systemic inflammation using different cell stimulation techniques to better elucidate TNFR signalling pathways, while also characterising the mechanism of SCFAs.

Chapter 7 – The effects of prebiotic *trans*-galactooligosaccharide (B-GOS) on peripheral blood mononuclear cells under chronic inflammation

7.1. Introduction

During T-cell stimulation, interaction between the TCR and peptide-MHC induces TCR cluster formation with other cell surface receptors, followed by activation of signalling pathways and transcriptional T cell programming. Naïve T cell activation depends on a specific second signal contributed mainly by professional APCs in secondary lymphoid organs, ligation of non-polymorphic cell surface receptors and major co-stimulator CD28. In contrast to naïve T cells, memory T cell activation is independent of professional APCs, providing specific antigen can be presented by MHC molecules on non-professional APCs (Skapenko et al., 2005).

$CD8^+$ T cells respond to pathogen-encoded peptides presented by MHC Class I molecules on infected cells or APCs. This leads to the release of an array of antimicrobial molecules and effector pathway signalling, in-turn there is cytolysis of infected cells or recruitment of other immune cells. The amount of antigen is directly related to the magnitude of inflammatory response and expansion of specific $CD8^+$ T cells (Haring et al., 2006).

$CD4^+$ T cells, the main focus of the current study, play an important role in inflammatory disease and autoimmunity. As well as their key role as helper cells, $CD4^+$ T cells drive autoimmune diseases and allergies, as discussed previously (see section 2.2.2.6.3). The extensive number of $CD4^+$ T cell subsets play a complex role in immune-mediated inflammatory disease. IFN-γ producing T_h1 cells have been considered contributors to autoimmune organ-specific diseases such as multiple sclerosis and type 1 diabetes, also both T_h1 and T_h17 cells are involved in psoriasis, a chronic inflammatory disease of the skin and joints. In Allergic diseases, T_h2, T_h9 and T_h17 cell subsets secrete cytokines after activation. In an allergy induced murine model, T_h2 cells have been demonstrated to secrete IL-4, IL-5 and IL-13, as well as increase eosinophil count through chemotaxis and mucus production in the airway (Verheijden et al., 2015). T_h9 cells produce IL-9, inducing mast cell proliferation and preventing apoptosis, contributing to asthma airway pathophysiology. Furthermore, a

correlation of T_h9 cell number and AHR severity has been demonstrated in BALB/c mice (Kerzerho et al., 2012). As well as eosinophilia of the airway in asthma, neutrophils are also recruited through IL-17 secreting T_h17 cells, a possible cause of steroid resistant airway inflammation (McKinley et al., 2008). T_h17 cells are also capable of IL-22 secretion, for the protection and proliferation of epithelial barriers in the gut and lungs. Interestingly, in humans there is a population of $CD4^+$ T cells known as T_h22 cells, these cells can produce IL-22 without the characteristics of T_h17 cells (Cui et al., 2019).

B cells contribute to both host protection and the inflammatory response through the production and secretion of antibodies. T_{fh} cells, categorised by surface expression of CXCR5 (Crotty, 2011), augment this action and support expansion and differentiation of B cells through co-stimulatory binding of CD40 and CD40L (Kazanietz et al., 2019). Moreover, T_{fh} cells also secrete IFN-γ, IL-4 and IL-17. Patients with systemic lupus erythematous have a larger quantity of IL-17 producing T cells, an activator of B cells, leading to increased antibody production and kidney infiltration (Crispin et al., 2008).

In distinction to the transient life of effector T cells, regulatory T cells or Tregs are crucial for the prevention of autoimmune disease, regulation of self-tolerance and inflammatory modulation. As discussed previously (see section 2.2.1) the importance of Foxp3 in the expansion of Treg cells has been demonstrated in mice and humans (Rudensky., 2011; Verheijden et al., 2015). There are two divisions of Tregs, thymus-derived (tTreg) and peripherally induced (pTreg); both can be identified through the markers Neuropilin 1 and Helios (Hirahara and Nakayama, 2016). However, the latter has been proposed to lack expression of Helios, allowing potential differentiation between the subsets (Skadow et al., 2019). In allergic inflammation pTregs are active in the gut and lung, especially at mucosal surfaces, supressing excessive antigen specificity through the suppression of effector T cell induction and proliferation (Yadav et al., 2013).

The $CD4^+$ T cell subsets described previously are all subject to further differentiation through extrinsic factors, specifically cytokines, which activate signal transducers and activators of transcription (STATs). T cell receptor and STAT signalling is crucial for the activation of subset specific transcription factors (O'Shea et al., 2011). T_{reg} differentiation is led by transcription factor and master regulator Foxp3 (Rudensky., 2011). As discussed in chapter 6, NF-κβ, activated by the cytokine TNF-α through TNFR signalling, also modulates T_{reg} development through the regulation of Foxp3 expression (Lu and Pan., 2017). The depth and complexity of $CD4^+$ T cells provides clarity and cause for the heterogeneity observed in inflammatory disease, understanding this complex network of cellular signalling will aid in the future development of therapeutics.

The role of Short-chain fatty acids (SCFAs), acetate, propionate and butyrate in the gut associated lymphoid tissue (GALT) have previously discussed previously (see section 6.4.4). SCFAs signal through GPR41, GPR43 and GPR109a, both GPR41 and GPR43 can be absorbed into any cell type (Biere et al., 2011; Binder., 2010) and GPR43 can be highly expressed on effector T cells (Arpaia et al., 2013). Independently of GPR41 or GPR43, SCFAs may promote T-cell differentiation toward IL-17, IL-10 and IFN-γ producing T cells. This differentiation is facilitated by histone deacetylase (HDAC) inhibition, specifically, augmented acetylation of p70 S6 kinase and rS6 phosphorylation (Park et al., 2014). Moreover, SCFAs can bypass the cell surface to exert their effects. Inhibition of HDAC by SCFAs increases T_{reg} cell suppressive function, promotes Foxp3 gene expression and enhances the heat shock response, enabling cell survival under stress (Beier et al., 2011). Interestingly, induction of $Foxp3^+$ T cells by SCFAs can be facilitated even in a low TCR-activation condition (Park et al., 2014), suggesting a potential benefit of prebiotic treatment in normophysiology.

Moreover, butyrate can reprogramme NF-κβ in colon cancer cells through inhibition of HDAC inhibitor trichostatin A (TSA), in these butyrate-differentiated cells TNF-α activation is

supressed and IL-1β activation is not. This finding highlights the potential benefits of SCFAs in cancer-related inflammation and a role for HDAC inhibitors in inflammatory disease, as well as their use in cancer (Mottomal et al., 2015). (Place et al., 2005). Conversely, it is possible that in vivo SCFAs in GALT affect non-T cells such as epithelial cells, leading to downstream affects through systemic distribution of cells from lymph nodes. More specifically, butyrate can bind GPR109A on macrophages and DCs, indirectly inducing IL10 producing T cells and $Foxp3^+$ T cells (Park et al., 2014).

In the previous chapter baseline changes in cytokine secretion and transcription factor expression were measured after prebiotic treatment in healthy adults. Due to their interplay and ability to compensate for one another, cytokines are difficult to study in normophysiology (Foster, 2001). Thus, the reduction of TNF-α in Chapter 6 after prebiotic treatment, may not be reflective of the true inflammatory response to B-GOS (Kollias et al., 1991). Therefore, in the current study, cell stimulations with PMA/Ionomycin were utilised to imitate chronic inflammation *in vitro,* using peripheral blood mononuclear cells from the intervention in the previous chapter. The current study aims to:

- Elucidate the effects of prebiotic B-GOS in chronic inflammatory conditions.
- Observe whether the reduction of TNF-α observed in chapter 6 is maintained after a cell stimulation with PMA and ionomycin

7.2. Methods

7.2.1. Experimental design and protocol

The current study is as an extension of chapter 6 and the experimental design (see section 6.2.2) and blood preparation (see section 6.2.3) are analogous. In the current study, in order to observe the effects of prebiotic B-GOS in chronic inflammation, cells were stimulated with PMA and ionomycin; details on cell stimulation method development can be found in the general methods (see section 3.4).

7.2.2. *in vitro* assessment of cytokine production and expression of transcription factors

For assessment of T cell transcription factor expression and cytokine secretion, PBMCs were stimulated with PMA (50ng/ml; Sigma Aldrich, Missouri, US), ionomycin (1ug/ml; Sigma Aldrich, Missouri, US) and Brefeldin A (10ug/ml; Sigma Aldrich, Missouri, US) was added, cells were incubated for 5hrs. After stimulation, cells were stained with anti-CD3 (BioLegend), anti-CD25 (Biolegend) and anti-CD4 (Biolegend). A True-Nuclear^{TM} transcription factor buffer set (BioLegend) was used to permeablise cells according to manufacturer's instructions, after specific Abs for Foxp3 (ThermoFisher Scientific), NF-κβ (BD Bioscience), TNF-α (BioLegend), IL-4 (BD Bioscience), IL-6 (BioLegend) and GATA-3 (BioLegend) were used, analogous to Chapter 6.

PMA and ionomycin are potent stimulatory molecules that do not require TCR activation for Treg cell development. PMA activates protein kinase C activation, while ionomycin facilitates Ca^{2+} transport, these actions have been demonstrated to upregulate $CD25^{+}$ expression and thus Treg phenotype (Majowicz et al., 2012). However, in the current study there is insufficient incubation time for PMA/ionomycin to induce changes in T-cell proliferation and differentiation (June et al., 1987). Therefore, any T cell expansion is likely to be independent of cell stimulation and due to prebiotic treatment with B-GOS.

7.2.3. Statistical analysis

Data were analysed using statistical software GraphPad for Windows version 9.00 (GraphPad Software., San Diego, CA, USA). Data were examined for normality using the Shapiro-Wilk test. Normally distributed data (CD3, CD4, and Foxp3) were analysed using a two-way repeated-measures ANOVA and Bonferroni adjusted paired *t* tests. If data were not normally distributed (IL-4, TNF-α, NF-kβ, $CD4^+$ $CD25^+$) the non-parametric Friedman test was used with a mixed effects model and Dunn's correction for multiple comparisons. Two-way repeated measures ANOVAs were used to analyse the effects of treatment (Prebiotic vs Placebo) time (Day 0 vs Day 21) and time x treatment with between group analysis (Pre Placebo, Post Placebo, Pre Prebiotic, Post Prebiotic). Data are presented as mean values with standard deviations with statistical significance set at $P<0.05$.

7.3. Results

For CD4+ T cells there were no main effects of treatment (P=0.812) or time (P=0.112), and no treatment-time interaction effect (P=0.548). No differences in $CD4^+CD25^+$ T cells were found across the intervention, a Friedman statistic of 1.11 was rendered, which was not significant (P=0.775). For all measures, there were no differences between treatments at baseline ($P \geq 0.999$).

For TNF-α in $CD4^+$ T cells a Friedman statistic of 10.29 was rendered, which was significant (P=0.0088). TNF-α was reduced by 13.9% after prebiotic treatment (P=0.004) (Figure 7.1). Conversely, for TNF-α in $CD4^+CD25^+$ T cells a Friedman statistic of 1.187 was rendered, which was not significant (P=0.756) and TNF-α was unchanged after prebiotic treatment (P>0.999).

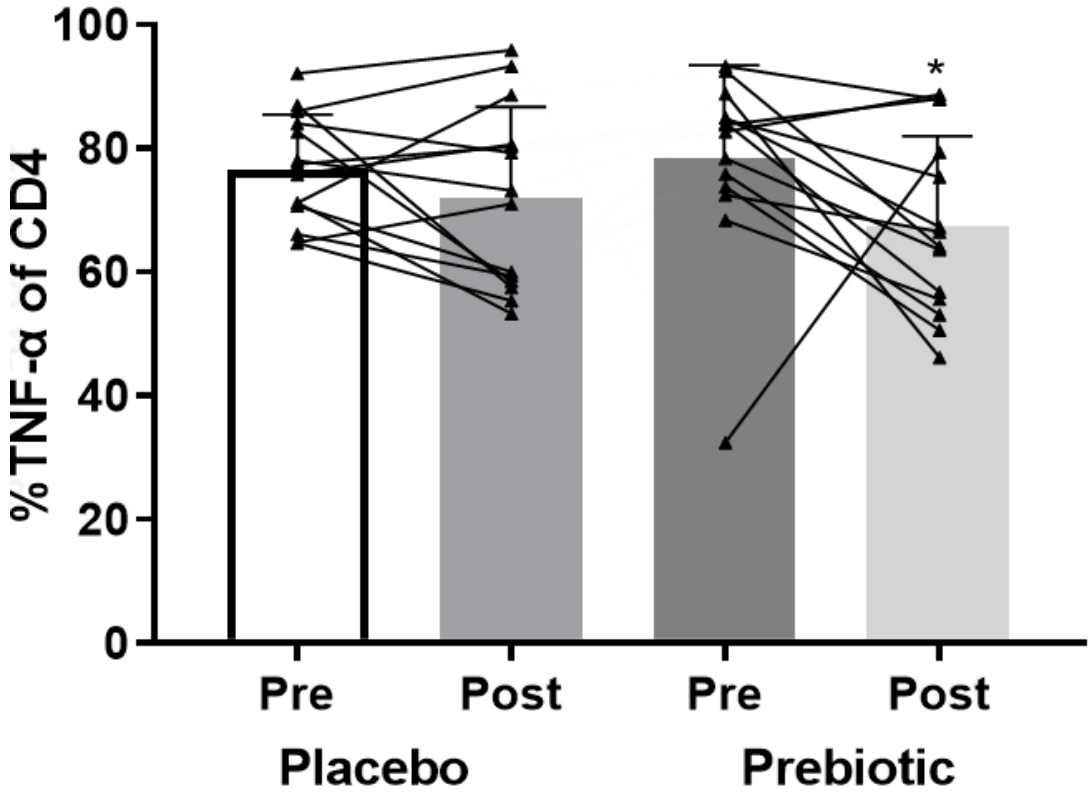


Figure 7.1. The effects of 20 days treatment with B-GOS on TNF-α in $CD4^+$ T cells in the peripheral blood mononuclear cells of healthy adult participants following an inflammatory challenge with PMA/Ionomycin. Results are shown as % mean difference ± SD. * denotes a significant difference between testing conditions (P > 0.05).

For CD4+ CD25+ FoxP3 cells, there were no main effects of treatment (P=0.158) or time (P=0.849), and no treatment-time interaction effect (P=0.723). There were no differences in CD4+ CD25+ FoxP3^{+} T cells after prebiotic treatment (P>0.999) (Figure 7.2).

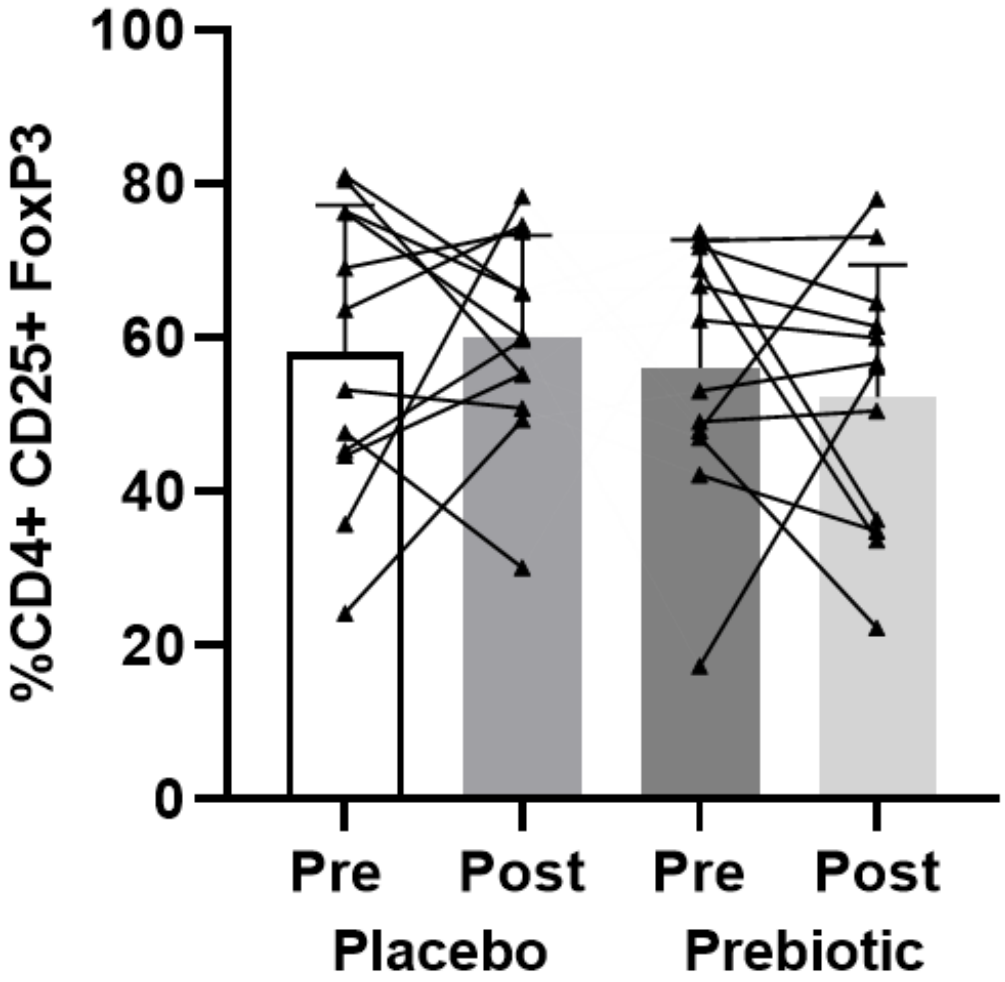


Figure 7.2. The effects of 20 days treatment with B-GOS on $CD4^{+}CD25^{+}$ Foxp3 regulatory T cells in the peripheral blood mononuclear cells of healthy adult participants following an inflammatory challenge with PMA/Ionomycin. Results are shown as % mean difference ± SD.

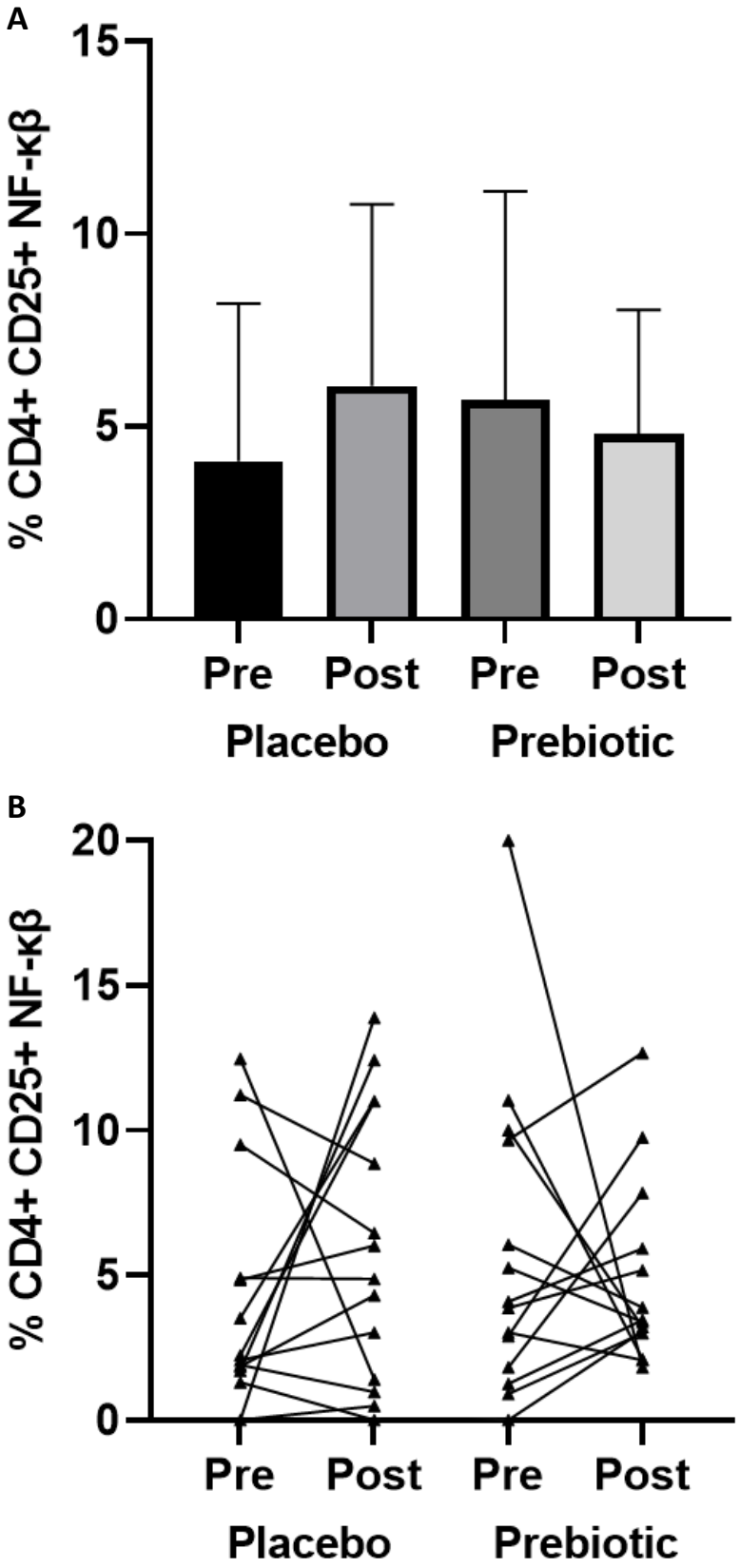


Figure 7.3. The effects of 20 days treatment with prebiotic B-GOS on NF-κβ in $CD4^+$ T cells in the peripheral blood mononuclear cells of healthy adult participants following an inflammatory challenge with PMA/Ionomycin. **(A)** results are shown as % mean difference ± SD. **(B)** results are shown as individual participant responses to prebiotic B-GOS.

For, transcription factor NF-κβ in $CD4^+$ T cells, A Friedman statistic of 4.03 was rendered, which was not significant (P=0.257). No differences were observed after prebiotic treatment (P=0.269) (Figure 7.3) and there were no differences between pre placebo vs pre prebiotic (P=0.657). Similarly, for NF-κβ in $CD4^+$ $CD25^+$ T cells a Friedman statistic of 1.31 was rendered, which was not significant (P=0.725). No differences were observed after prebiotic treatment (>0.999).

For IL-4 in $CD4^+$ T cells a Friedman statistic of 6.58 was rendered, which was not significant (P=0.087) and IL-4 was unchanged after prebiotic treatment. For IL-4 in $CD4^+$ $CD25^+$ T cells and a Friedman statistic of 4.28 was rendered, which was not significant (P=0.232) and there were no differences in IL-4 after prebiotic treatment (P=0.572).

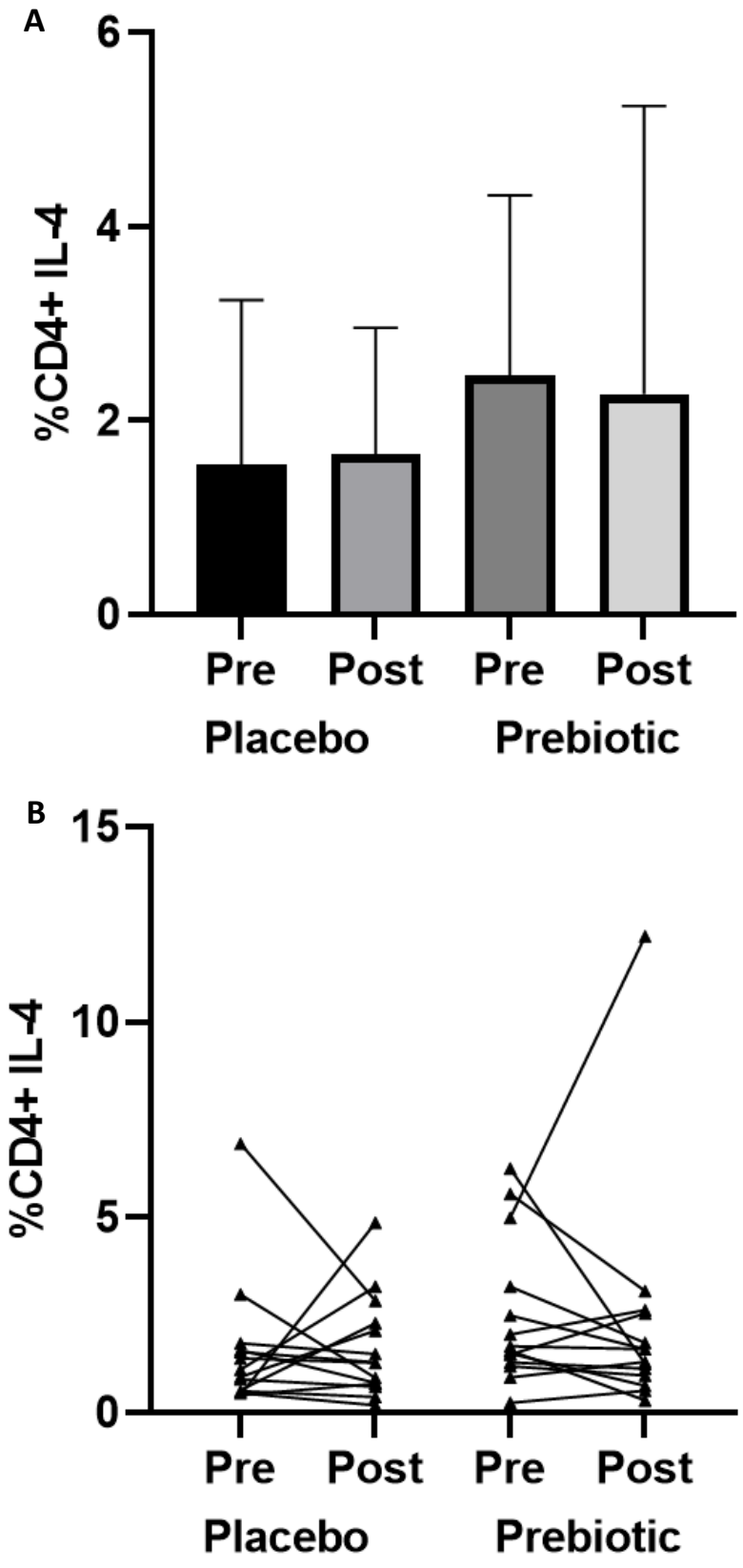


Figure 7.4. The effects of 20 days treatment with prebiotic B-GOS on interleukin-4 in $CD4^+$ T cells in the peripheral blood mononuclear cells of healthy adult participants following an inflammatory challenge with PMA/Ionomycin. **(A)** results are shown as % mean difference ± SD. **(B)** results are shown as individual participant responses to prebiotic B-GOS.

7.4. Discussion

7.4.1. Main findings

Twenty days supplementation with 3.65g of B-GOS (80% GOS) resulted in a significant reduction of TNF-α in $CD4^+$ T cells of healthy adults stimulated with PMA/ionomycin. No differences were observed in transcriptional factors Foxp3 and NF-κβ after prebiotic treatment. There were no differences of IL-4 concentrations in $CD4^+$ and $CD4^+$ $CD25^+$ T cells and no differences in the total number of $CD4^+$ and $CD4^+$ $CD25^+$ T cells after prebiotic treatment.

7.4.2. The inflammatory response to prebiotic B-GOS

In Chapter 6, $CD4^+$ TNF-α was reduced by 43.3% after prebiotic treatment. In the current study $CD4^+$ TNF-α was also reduced by 13.9% after prebiotic treatment and a 5hr cell stimulation with PMA (50ng/ml) and Ionomycin (1ug/ml). This reduction in TNF-α demonstrates that 20 days treatment with prebiotic B-GOS can modulate the immune response through the regulation of $CD4^+$ T cells.

Since the identification of TNF-α in 1975 (Carswell et al., 1975), a vast array of functions across a diverse number of cell types has been documented. In recent years, its potential target has been recognised for the treatment of inflammatory diseases such as ankylosing spondylitis, Crohns disease, rheumatoid arthritis, and psoriasis (Holbrook et al., 2019). TNF signalling is heavily regulated by post-translational ubiquitination, a vital mechanism for cellular homeostasis and thus normophysiology. Following activation of TNFRs, ubiquitin (Ub) chains are assembled, attached to specific substrate, and modulate protein function, and this process is reversible by the deubiquitinase (DUB) enzymes. Ubiquitination sites K48 and K63 are essential for NF-κβ activation and signalling (Wu et al., 2018). The activation of complex I is dependent on ubiquitination of RIPK1, and involves the linear ubiquitin chain assembly complex (LUBAC) followed by a complex cascade of cell signalling. Activation of complex I

ultimately leads to pro-survival signalling, inflammatory responses, and proliferation of immune cells. Following activation of NF-κβ, complex IIa is inhibited by cellular FLICE-like inhibitory protein long ($cFLIP_L$), preventing caspase-8 activation and thus apoptosis (Holdbrook et al., 2019). When RIPK1 is not ubiquitinated, complex IIb is formed, leading to apoptosis through cleavage of pro-caspase 8. For this process to occur, cylindromatosis tumour suppressor protein DUB enzyme deubiquitinates RIPK1, facilitating the dissociation from complex I and negatively regulating NF-κβ (Holdbrook et al., 2019; Kovalenko et al., 2003).

A20 is a particular protein of interest, essential for regulation of the TNF signalling pathway, and it contains DUB and E3 ligase domains. It is involved in removal of K48/K46-RIP1 linked Ub chains, inhibition of LUBAC-IKKγ interaction upon TNF stimulation (Tokunaga et al., 2012) and has various other roles disrupting NF-κβ signalling. Interestingly, there is evidence of increased A20 gene expression in patients with IBD and IECs with increased sensitivity to TNF-mediated-cell death. Additionally, A20 has been demonstrated to cause deubiquitination of RIPK3 in T cells, protecting them from necroptosis (Onizawa et al., 2015).

Boks et al., (2014) demonstrated that inhibition of TNF signalling through anti- TNF-α biologicals leads to priming and expansion of $CD4^+$ T cells toward IL-10 producing regulatory T cells, and the reduction of IFNγ, a signature proinflammatory cytokine involved in the innate and adaptive immune response. However, in the current study the reduction observed in TNF-α was not accompanied by an increase in $Foxp3^+$ regulatory T cells as demonstrated by Boks et al., and no significant changes were observed in any other inflammatory markers. As TNFR2 is a vital factor for sustained expression and function of Foxp3 Tregs, reduced TNF-α and therefore TNFR2 signalling may explain why Foxp3 remained constant throughout testing conditions (Chen et al., 2013). This also provides caution for the use of B-GOS in some autoimmune disorders, as similarly to some forms of anti-TNF therapy it may cause exacerbation through Treg modulation (Caminero et al., 2011). Conversely, B-GOS does not

cause any common (10% of patients) adverse effects associated with anti-TNF agents such as headaches, infusion reactions, rashes, upper respiratory tract infections, nausea, abdominal pain, anaemia, and diarrhoea (Gerriets et al., 2021), highlighting the benefits and safety of this food supplement.

In the current study, IL-4 concentrations were unchanged after prebiotic treatment. Conversely, a study examining the effects of B-GOS observed increases of IL-4 and IL-6 in the hippocampus of rats, after a 3-week treatment (15g/l ad libitum) (Yang et al., 2018). There were also no differences in IL-4 concentrations between pre placebo, post placebo and pre prebiotic; this was unexpected as a PMA activates pathways that induce IL-4 transcription and mRNA stabilisation (Guo et al., 2009). Moreover, in vivo, and in vitro study of mice, ionomycin stimulation produces IL-4 and IFN-γ in Th1 and Th2 cells, enhances p38 phosphorylation and activates calmodulin-dependent kinase IV, inducing AP-1 (Guo et al., 2009). In allergy, TCR stimulation also leads to phosphorylation of p38, activated p38 is necessary for IL-4 and IFN-γ production (Guo et al., 2009). Interestingly, acetate treatment of microglia primary culture has been demonstrated to attenuate cell signalling through p38 MAPK and NF-κβ phosphorylation (Silva et al., 2020).

In a study by Bothur et al., stimulation of C57BL/6 cells with PMA/ionomycin downregulated Foxp3 expression in iTregs, due to transcriptional/translational modifications (Bothur et al., 2015). In the current study, cell stimulation showed no differences in $CD4^+$ $CD25^+$ $Foxp3^+$ cell number at baseline, as expected with a 5 hr incubation. However, there were also no differences in $CD4^+$ $CD25^+$ $Foxp3^+$ cell number after prebiotic treatment. As discussed in Chapter 6, SCFAs are capable of bypassing cell surface receptors, inhibiting HDAC and upregulating Foxp3, thereby increasing the suppressive function and number of Tregs (Wang et al., 2019). This effect coupled with the downregulation of Foxp3 by PMA/Ionomycin and reduced TNF-α provides a possible reason for the absence of change observed in the current study Moreover,

future research should utilise primary cell culture techniques for 24 or 48 hr after PMA/ionomycin stimulation, similarly to Bothur et al., to better characterise the role of prebiotic B-GOS in inflammation and its effects on T cell differentiation.

7.4.3. The proposed mechanism of action of prebiotic bacteria and SCFAs on systemic inflammation

SCFAs, the metabolites of prebiotics, generate the growth of *Bifidobacterium* (Depeint et al., 2008) which maintain intestinal homeostasis through the 'training' of the immune system (Rakoff-Nahoum et al., 2004). These *Bifidobacterium* possess specific TLR ligands, and are capable of TLR modulation and initiation of Th1 and Treg led immune responses (Kalliomaki et al., 2010). As a result, Treg modulation of effector T cells leads to the attenuation of inflammation (Figure 7.5). This mechanism may be involved in the reduction of TNF-α after prebiotic treatment in the current study, however, there were no differences were observed in Foxp3 Tregs. Moreover, probiotic treatment has been demonstrated to increase $Foxp3^+$ Tregs in inflamed regions, supressing immune disorders (Kwon et al., 2010). Therefore, it may be possible that SCFAs interfere with commensal bacteria signalling. Future work should investigate the mechanisms of SCFAs in more detail and aim to elucidate their effects on T cell immunology.

The gastrointestinal associated lymphoid tissue is the largest mass of lymphoid tissue in the human body and includes the lamina propria, intraepithelial lymphocytes, mesenteric lymph nodes, Peyer patches and isolated lymphoid follicles. Some of these regions contains antigen presenting cells such as dendritic cells, capable of responding to changes in commensal bacteria after prebiotic treatment.

Intestinal epithelial cells are the initial contact point of commensal bacteria in the GALT and provide a barrier between the intestinal lumen and the rest of the body. However, the contents

of the intestinal lumen are constantly assessed by specialised areas of the GALT including the Peyers patches of the small intestine, lymphoid follicles in the lamina propria and microfold cells in the intestinal tract (Figure 7.5.). These assessments are crucial to regulate the immune response and enable the differentiation between harmful pathogenic bacteria and beneficial bacteria (McLoughlin and Mills., 2011). Therefore, by increasing the mass of beneficial bacteria through prebiotic treatment, we may be able to augment beneficial probiotic bacteria signalling with IECs through TLR activation. In turn, inducing cytokine production, growth factor synthesis and ultimately facilitating intestinal homeostasis (Rakoff-Nahoum et al., 2004).

IECs are multifunctional, in addition to their interaction with probiotic bacteria, they are also able to interact with dendritic cells through surface toll-like receptors and subsequently secrete thymic stromal lymphopoietin lymphopotein (TSLP) and TGF-β. TSLP has been implicated in a variety of allergic diseases, such as asthma and AD, chronic inflammatory diseases, such as COPD and celiac disease, various autoimmune disorders and several types of cancer (Varrichi et al., 2018). TSLP regulates DC expression of IL-12, and promotes the production of IL-10. Interleukin-10 enhances naïve T cell differentiation and Treg proliferation (Figure 7.5.), shifting the balance towards regulatory mechanisms instead of pro-inflammatory responses (Iliev et al., 2009), supporting the reduction in TNF-α in the current study after prebiotic treatment. Additionally, *Bifidobacterium lactis* and *longum* have been demonstrated to reduce TNF-α in humans (Bernini et al., 2016; Groeger et al., 2013; Meng et al., 2017), while also shifting the balance of Th cells to a Th1/Th2 response over Th17 (Ruiz et al., 2017). Moreover, a Bifidobacteria mixture of *B.bifidum*, *B. longum*, *B lactis* and *B. breve* in mice was shown to modulate the gut bacteria in a regulatory T cell dependent manner, enhancing the IL-10 mediated suppressive functions of intestinal Tregs (Sun et al., 2020).

Dendritic cells are able sample contents of the gut lumen through extension of their dendrites between gaps in the IECs known as tight junctions (Figure 7.6). Pathogen recognition receptors

(PRRs) on the dendritic cell surface facilitate immune responses to pathogen and microorganisms associated molecular patterns (PAMPs and MAMPs), such as nucleotide-binding oligomerisation like receptors expressed on commensal bacteria and pathogens (Lebeer et al., 2010). In the Peyers patches, microfold (M) cells located in the follicle associated epithelium are able to internalise bacteria, presenting them to dendritic cells via transcellular pores for dendrites to reach through. Additionally, low molecular weight soluble material may be transported by small intestine goblet cells, as well as retro-transport of immune complexes, such as IgG, across the epithelium and for presentation to dendritic cells (Mazzini et al., 2014; Yoshida et al., 2004). However, very little is understood about the functional role of each sampling method and their effect on inflammation (Stagg., 2018). Moreover, treatment with prebiotic B-GOS increases beneficial probiotic bacteria (Depeint et al., 2008), theoretically leading to increased interaction of dendritic cells and antigen presented material. This in turn induces Treg cell expansion and differentiation from naïve T cells, leading to a more balanced immune response in response to chronic inflammation (McLoughlin and Mills, 2011; McLoughlin et al., 2017). $CD103^+$ dendritic cells can differentiate naïve T cell to inducible $CD4^+$ $CD25^+$ $Foxp3^+$ T cells or Treg cells (Coombes et al., 2007). This mechanism is dependent on TGF-β and retinoic acid (RA), a dietary metabolite of vitamin A. Interestingly, it was demonstrated that silencing RA receptor signalling in intestinal macrophages is associated with a reduction in TNF-α in Crohns disease (Abdelhamid and Luo., 2018).

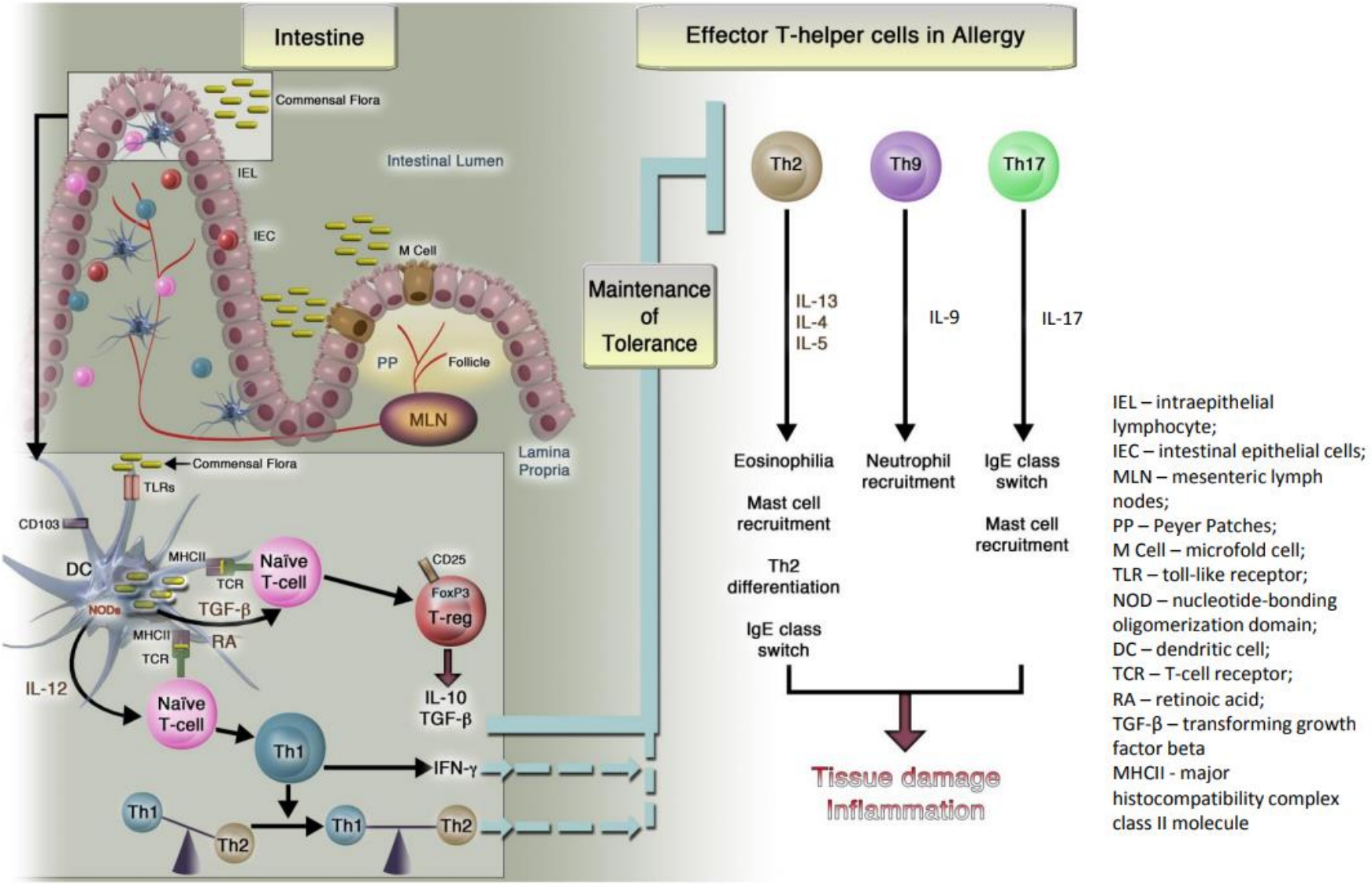


Figure 7.5 The effect of the gut microbiota on the immune system and the associated inflammatory responses. (McLoughlin and Mills., 2011)

Although in the current and previous chapter there were no differences in baseline and stimulated $CD4^+$ $CD25^+$ $Foxp3^+$ cells after prebiotic treatment, it is possible under long-term chronic inflammation or in individuals with autoimmune diseases, this would not be the case. It is well documented that in inflammatory diseases such as asthma, individuals typically show a low expression of $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells (Lin et al., 2008; Provoost et al., 2009). Enhanced *Bifidobacterium bifidum*, a target for B-GOS, are associated with increased $CD4^+$ $Foxp3^+$ T cells in isolated mesenteric lymph node (MLN) and supress the progression of experimental atopic dermatitis, rheumatoid arthritis and inflammatory bowel disease (Kwon et al., 2010). Beneficial gut bacteria increase Foxp3 expression and Treg cell number in the GALT (Round and Mazmaniam.,2010) and MLN, while also increasing lymphatic migration of activated dendritic cells and Treg cells (Hampton and Chtanova., 2019). It is reasonable to suggest that treatment with B-GOS may positively modulate the immune system in inflammatory disease, through the aforementioned mechanisms.

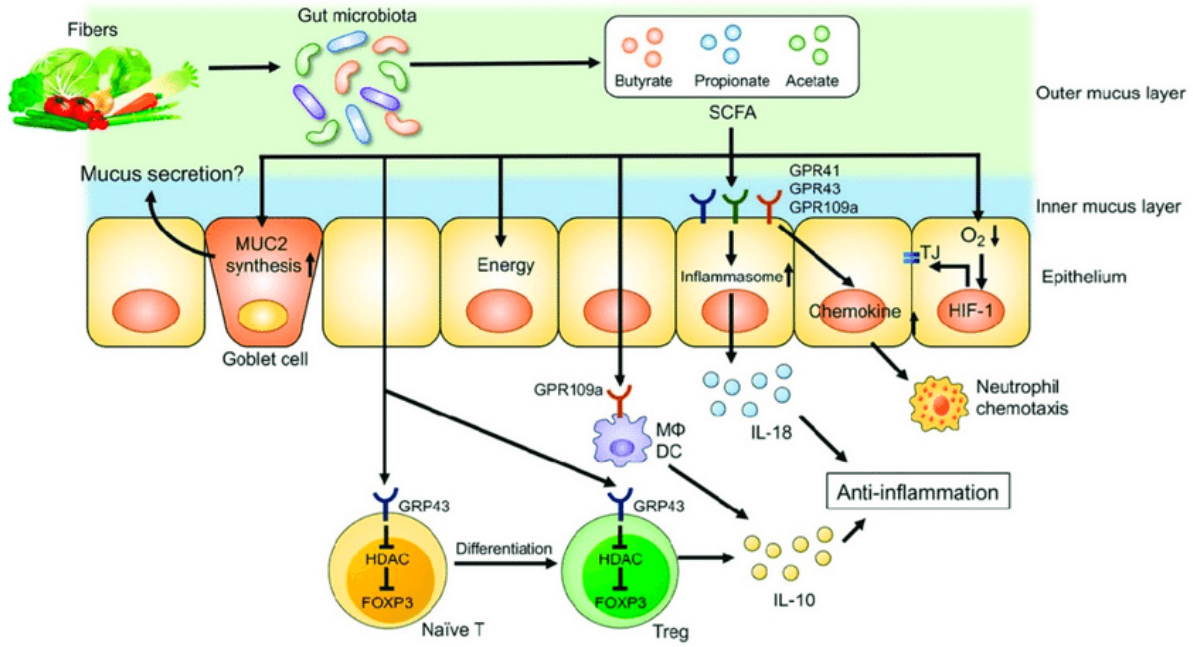


Figure 7.6. The function of prebiotic fibre-derived short-chain fatty acid (SCFA) in intestinal homeostasis. DC; dendritic cells, Foxp3; forkhead box protein 3, HDAC; histone deacetylases, Mφ; macrophages, TJ; tight junctions. As well as a source of energy for colonocytes, SCFAs signal through G-protein-coupled receptors on the surface of intestinal epithelial cells, promoting Treg cell differentiation and production of anit-inflammatory cytokines, such as Interleukin (IL)-10 and IL-18 (Keshteli et al., 2019).

In contrast to probiotic bacteria signalling, the by-products of B-GOS are also able to elicit immunomodulatory effects. Acetate, propionate, and butyrate can have a direct or indirect effects on cellular processes such as, differentiation, gene expression and proliferation. They exert their effects through passive diffusion into cells, dedicated transporters facilitate this action e.g. sodium-coupled Monocarboxylate transporter 1 and Monocarboxylate transporter 1. It is well documented that SCFAs are inhibitors of histone deacetylases (HDACs) (Licciardi et al., 2011), these enzymes are responsible for the removal of acetyl functional groups, allowing histone to wrap DNA more 'tightly' and generally suppressing gene transcription. HDACs have increased expression in many types of cancers and have been identified as a smart target for inhibition (Chen et al., 2016). Propionate and butyrate have a higher HDAC inhibiting activity than Acetate, with acetate being the highest concentration in peripheral blood (20-150 μM), followed by propionate (1-13 μM) and butyrate (1-12 μM) (Chang., 2021). These concentrations are deemed considerable enough to affect host cells. Interestingly, SCFAs are also able to activate histone acetyltransferases or HATs, which are enzymes responsible for the addition of an acetyl group from acetyl coenzyme A to form ε-*N*-acetyl lysine, in general, augmenting gene expression. On entry to host cells, propionate and butyrate are rapidly metabolised into acetyl coenzyme A by Acyl-Coa synthetases to facilitate HAT activation (Thomas and Denu., 2021). HDACs and HATs regulate Treg cell function through the inhibition of HDAC6, HDAC9 and Sirtuin-1, which in turn augments Treg suppressive function through preservation of Foxp3 lysine ε-acetylation and thus reduced ubiquitination, degradation and increased DNA binding (Beier et al., 2011). Additionally, SCFAs are ligands for GPR41, GPR43 and GPR109a (Brown et al., 2003; Thorburn et al., 2014). GPR41 and 43 are expressed in human adipocytes, PBMCs and IECs, they have been implicated in multiple chronic inflammatory disorders including asthma, obesity, colitis, and arthritis. However, the role of these GPCRs is varying between studies and a causative or protective role is yet to be

established (Ang and Ding, 2016). SCFA activation of GPR41 and GPR43 in IECs has been shown to produce cytokines and chemokines in mice and culture, additionally, GPR41 and GPR43 were essential for intestinal recruitment of leukocytes and effector T cell activation (Kim et al., 2013). GPR109a has been demonstrated to function as a tumour suppressor within the colon (Thangarju et al., 2009), as well as play a key role in vascular inflammation, particularly in atherosclerosis (Chai et al., 2013). Additionally, GPR109a is highly expressed on DCs, which play a key role in T cell activation and differentiation (Docampo et al., 2019).

In pigs, SCFA concentrations are at their highest in the proximal colon (70-140 mM) in comparison to the distal colon (20-70 mM), SCFA levels in the liver and blood are much lower than the intestine, with concentrations of 0.08 mM in peripheral blood (Holtug et al., 1992), inferring SCFA signalling mainly occurs through intestinal epithelial cells. However, the detection of SCFAs in peripheral blood alone implies that they have systemic functions (Topping and Clifton., 2001; Venegas et al., 2019). It is possible SCFAs induce their anti-inflammatory systemic effects directly, traveling to distant sites and signalling with GPR41, GPR43 and GPR109a, which in turn leads to immune cell activation, differentiation (Docampo et al., 2019; Kwon et al., 2010). Additionally, SCFAs may modulate HDACs or HATs to elicit their immunomodulatory effects (Biere et al., 2011; Thomas and Denu, 2021).

In Summary, the exact mechanism of B-GOS remains elusive, it is not known whether the immunomodulatory effects observed following prebiotic treatment are due to SCFA signalling or the indirect signalling of Bifidobacteria generated through B-GOS ingestion (Vulveic, 2015). However, it is likely that both mechanisms are responsible for the enhancement of microbial and systemic immunity and may prove beneficial in the treatment of chronic inflammatory disease.

7.4.4. Limitations of analysis

A more in-depth assay would need to be undertaken to confirm the effects of B-GOS treatment on $CD4^+$ $CD25^+$ $Foxp3^+$ cells, with various stimulation time points and further culture/expansion of T cell subsets. Perhaps the next step would be to measure HDAC and GPR41/GPR43 activity in response to B-GOS. Future work should observe phosphorylation of the p65 subunit, allowing the measurement of any transient changes in p65 phosphorylation and thus NF-κβ in response to B-GOS (Chan et al., 2013). Another restraint of the current study was specific sample storage conditions due to HTA licensing, many study-design decisions were based around this, amongst other variables such as funding and COVID-19.

7.4.5. Conclusion

For the first time this study shows that a 20-day treatment of the prebiotic B-GOS significantly reduces TNF-α in $CD4^+$ T cells following in vitro stimulation of peripheral blood mononuclear cells, indicating a protective effect of B-GOS under inflammatory conditions. These results provide scope for application of B-GOS in healthy participants to mitigate inflammation, and future research of B-GOS in clinical populations with chronic inflammatory disease.

8. Chapter 8 – General Discussion

8.1. Introduction

Asthma concerns have been increasing over the past few decades. It has become one of the most common diseases, which for many has a significant effect on quality of life. Asthma affects over 300 million people worldwide and it is likely that by 2025 400 million people will have asthma. Prevalence appears to be higher in high income countries, although most mortality occurs in lower income countries (Stanojevic et al., 2012). The most common form of asthma is atopic, affecting 70-90% of children and 50% of adults (Unsworth, 2011). Despite recent advances in treatment methods, the disease still holds a considerable financial burden on the NHS, costing £1.1 billion a year (Asthma UK Accessed on 03.03.2022). There are still gains to be made improving patient education, diagnostic approaches and management of individual cases of asthma. By understanding different asthma phenotypes and how to differentiate between them we may be able to achieve this.

Traditionally asthma treatment targets symptoms alone, β2-agonists and corticosteroids are both currently used and the long-term use of the latter can have systemic side effects. Furthermore, more than 50% of asthmatics suffer most due to poor medication adherence (Barnes, 2010). It is estimated that 90% of deaths caused by asthma are preventable, and that hospital admission is avoidable with the correct asthma management techniques (Asthma UK Accessed on 03.03.2022). These statistics alone highlight the limitations with current treatment strategies and provide a strong rationale for the development of novel treatment strategies that not only encourage adherence, but also target the underlying inflammatory response and immune response. Moreover, epidemiological data suggests that asthma risk in children has been increased by the Western dietary pattern (Carey et al., 1996), and that the Western diet is positively associated with increased frequency of asthma exacerbations in adults (Varraso et al., 2009). Recent research has suggested that low fibre intake, typical of the Western diet, is associated with increased risk of asthma, chronic respiratory symptoms, and markers of

systemic inflammation (Saeed et al., 2019). Additionally, in healthy individuals, dietary fibre, specifically prebiotic intake, is associated with low levels of systemic inflammatory markers, markers which are linked to not only asthma, but various inflammatory and cardiovascular diseases (McLoughlin et al., 2017; Yunsheng et al., 2009). To establish the use of a novel nutritional intervention strategy in asthma and inflammatory disease, it is also important to understand the mechanism of action in normophysiology.

These questions instigated the rationale for this research thesis. A summary of the key findings is detailed below.

Chapter 5 *– A variety of evidence was demonstrated to support the benefits of prebiotic and synbiotic treatment for certain asthma outcome measures in both murine models and humans. Early life treatment was not effective against recurrent wheeze development in children up to 5 years, however evidence in adult and murine trials suggests lung function parameters can be improved, perhaps more so with prebiotic treatment than synbiotic treatment. An increased number of high-quality trials are needed in order for these parameters to be categorically assessed, particularly adult human trials measuring both physiological and epigenetic change in response to an asthma specific challenge.*

Chapter 6 *– showed for the first time that 20 days of treatment with 3.65g of prebiotic B-GOS (80% GOS) resulted in a significant reduction of TNF-α in $CD4^+$ T cells of healthy adults; this effect was not observed in $CD4^+CD25^+$ T cells. In contrast, B-GOS did not affect IL-4 concentrations, $CD3^+$, $CD4^+$ and $CD4^+$ $CD25^+$ T cell counts, or protein expression of transcription factors Foxp3 and NF-κβ. This suggests that B-GOS modulates systemic inflammation and immune function in healthy adults.*

Chapter 7 *– showed for the first time that a 20day treatment of the prebiotic B-GOS significantly reduces TNF-α in $CD4^+$ T cells following in vitro stimulation of peripheral blood*

mononuclear cells. This suggests B-GOS may favourably manipulate the commensal gut bacteria leading changes in systemic inflammation and immune function under chronic inflammation. These results provide scope for future research on B-GOS and highlight the potential use of B-GOS in clinical populations with chronic inflammatory disease

8.2. Experimental findings and recommendations

Prior to performing a meta-analysis on 'Prebiotics and synbiotics in the treatment of asthma' and investigating the use of prebiotic B-GOS in healthy populations, the aim of the current thesis was to investigate the effects of B-GOS in mild to severe asthmatic. Chapter 4 required ethical approval from the East Midlands Nottingham Health Research Authority, to ensure ethical standards were upheld for protection of clinical subjects from harm and to preserve subjects' rights (NHS – Health Research Authority accessed on 04.03.2022). The aim of this study was to determine if B-GOS reduced baseline FEV_1 and biomarkers of airway inflammation in the blood, while also to observe if the intervention had a genuine effect on asthma quality of life (QOL), asthma control and medication adherence. These additional measures allow for greater confidence when assessing interventions that aim to reduce asthma severity. Understanding these results and their relationship with biomarkers markers of airway inflammation, along with FEV_1, may improve the monitoring and evaluation of asthma management and intervention strategies. In Chapter 4, another aim was to try and better understand asthma phenotyping, and specifically the effect of B-GOS on both eosinophilic and neutrophilic asthma (Wenzel et al., 2013). In order determine asthma severity and identify phenotype characteristics, differential leukocyte analysis was performed with a haematology analyser. In a pilot study, the test-retest reproducibility of this analyser was assessed over a short- (1 day), medium (14 days) and long (21 days) time period (See section 3.5). Understanding different asthma phenotypes is crucial for understanding asthma pathophysiology, and will help develop novel treatment strategies, improving asthma

management and prevention (Pembrey et al., 2018). Moreover, all participant facing documentation from chapter 4 was reviewed in a patient public involvement feedback report from the Asthma UK Centre for Applied Research. Although there were no data outcomes from, Chapter 4 improved study design ability for Chapter 6 and 7 and provided insight into the way clinical asthmatics view novel nutritional interventions as a form of asthma treatment.

Novel therapeutic strategies for the treatment of asthma and associated inflammatory markers are warranted, and an area that has gained considerable interest is the use of prebiotic, probiotic and synbiotic nutritional interventions (McLoughlin et al., 2017; Williams et al., 2016). Prebiotic interventions, such as B-GOS, aim to attenuate the release of pro-inflammatory mediators, and regulate T cell expansion/differentiation through modulation of the gut microbiota. These alterations are thought to concur with the beneficial effects observed in asthmatic populations following prebiotic or synbiotic treatment. Dietary intake of prebiotics and synbiotics can result in increased concentrations of SCFAs along with the growth of beneficial commensal bacteria. Thus, impacting the inflammatory immune response indirectly through enhanced intestinal epithelial cell signalling (Vieira et al., 2013) and directly through SCFA signalling (He et al., 2020).

Previous research in murine trials has utilised both prebiotic (Sonoyama et al., 2005; Verheijden et al., 2015; Verheijden et al., 2016; Vos et al., 2007; Watanabe et al., 2004; Yasuda et al., 2010) and synbiotic (Sagar et al., 2014; Verheijden et al., 2015) treatment for asthma outcome measures and associated inflammatory markers. In Chapter 5, meta-analysis of this data revealed that prebiotic treatment reduces airway hyper-responsiveness but synbiotic treatment does not. Indicating that prebiotic use may be more effective in reducing asthma outcomes in murine models, while also highlighting the efficacy of the prebiotic compounds GOS and FOS. Additionally, meta-analysis revealed both prebiotic and synbiotic treatment resulted in significant reductions of IL-4, IL-5 and IL-13, the largest of which was IL-4 with a

34% decrease, a key cytokine involved in asthma pathology. Neutrophil, eosinophil, alveolar macrophage, and lymphocyte counts were reduced after prebiotic treatment, possibly due to the by-products of prebiotic fermentation, SCFAs. These findings implicate a role for SCFAs in lymphocyte function and locomotion in asthma-like conditions. Moreover, Theiler et al., demonstrated the attenuation of allergic airway inflammation by limiting eosinophil trafficking through butyrate treatment. These findings from Chapter 5 highlight the potential of SCFAs and their precursors as a promising treatment strategy in allergic inflammatory disease.

In chapter 5, five infant studies comprised of 9 different experimental trials examined the effects of prebiotic (Arslanoglu et al., 2008; Ivakhnenko and Nyankovskyy, 2013; Niele et al., 2013) and synbiotic treatment (Kuitunen et al., 2009; van der Aa et al., 2011) on infants with asthma. Meta-analysis of this data revealed that prebiotic and synbiotic treatment had no effect on the risk of cumulative incidence of recurrent wheeze. Furthermore, subgroup analysis on a 2 year and 5 year follow up also revealed no differences in cumulative incidence of recurrent wheeze. Three adult studies examined the effects of prebiotic and synbiotic treatment on asthma, however there was insufficient data for meta-analysis. Williams et al., demonstrated 5.5g/day of prebiotic B-GOS for 21 days significantly increased FEV_1 in adults with HIB following EVH testing, while also recording a 18.9% decrease in TNF-α of healthy adults. Similar to murine data, synbiotic treatment did not produce the same effects and baseline FEV_1 was unchanged in adults with allergic asthma (Van De Pol et al., 2011). However, with the bronchoprovocation method used by Williams et al., different results may have been obtained. Mcloughlin et al., observed a decrease in sputum eosinophil counts and sputum HDAC9 gene expression following inulin treatment, a promising finding for asthma research. However, the heterogeneity in outcome measures across these three studies makes data incomparable. Furthermore, there was substantial differences in the intervention used, Van De Pol et al., used a combination of scGOS and lcFOS (9:1) alongside *B breve M16-V* at $2x10^{10}$ CFU in capsule

form, whereas Mcloughlin et al., utilised 6g of inulin with or without Lactobacillus *acidophilus* LA-5 at $7.5x10^9$ CFU.

Recommendations based on the findings of Chapter 5 are that prebiotic treatment of asthma may potentially be a beneficial strategy in treating adults with asthma. The murine data shows a beneficial reduction in airway inflammation after prebiotic and synbiotic treatment. Recurrent wheeze in infants is not improved after prebiotic or synbiotic treatment, suggesting further studies are required to confirm the efficacy of both prebiotics and synbiotics on asthma outcomes in infants. However, data in adults shows promising effects of prebiotic and synbiotic treatment of EIB and asthma. Finally, much of the data suggests that prebiotics may be more effective than synbiotics for the treatment of asthma outcomes, possibly due to the competitive inhibition of SCFA signalling by probiotic bacteria signalling, though this notion needs investigating.

There is much heterogeneity in the nutritional interventions and outcome measures from the adult studies discussed. In order to assess the efficacy and use of prebiotics for prevention of asthma outcomes in adults, more comparable human research is required, perhaps using prebiotics alone.

Systemic chronic inflammation can lead to variety of inflammatory diseases, collectively they are the largest causes of mortality and disability worldwide, responsible for over 50% of all deaths (Furman et al., 2020). Chronic low-grade inflammation and unresolved inflammatory responses are likely to contribute to early stages of disease development (Minihane et al., 2015). Specifically, low grade inflammation can lead to chronic diseases such as asthma, cardiovascular disease, type 2 diabetes, and obesity (Lad et al., 2021). Chronic inflammation of the airways is a key part of asthma pathogenesis and characterises the disease, even during asymptomatic phases (Locksley, 2019). Recent evidence suggests that risk of developing

chronic inflammation can be altered in early development and throughout life with nutritional intervention and thus modulation of commensal bacteria (Furman et al., 2020). Vulevic et al., examined the effect of 5.5g/d B-GOS in elderly persons aged 65-80 years, following treatment with prebiotic B-GOS beneficial bacteria *Bacteroides* and bifidobacteria were increased, differences in IL-10, IL-8 and IL-1β were observed, however, contrary to Chapter 6, TNF-α was unchanged. It is worth highlighting that B-GOS is a commercially available product and in recent years has been optimised and its efficacy increased, the recommended dose is now one 3.65g/d.

In Chapter 6, TNF-α concentrations were reduced in $CD4^+$ T cells following 3.65g/d of B-GOS for 20-days, these results both conflict (Vulevic et al., 2013; Vulevic et al., 2015) and support previous research (Vulevic et al., 2008; Williams et al., 2016). No differences were observed for IL-4 and NF-κβ (p65) concentrations, or $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells. In contrast to previous work, the current study did not supplement prebiotic B-GOS on the testing day to avoid any acute effects (Halnes et al., 2017).

Tumour necrosis factor-alpha binds to TNF receptor (TNFR) 1 and 2 to elicit an extensive spectrum of cellular effects. Both signalling pathways lead to activation of nuclear factor-kappaβ (NF-κβ) and subsequently cell proliferation, tissue regeneration and inflammation. (Holbrook et al., 2019). The activation of the TNFR2 signalling by TNF-α may lead to regulatory T cell expansion, and a suppressive effect. However, TNFR2 signalling may also act in a pro-inflammatory manner, inducing apoptosis (Complex IIa/IIb) or necroptosis and inflammation (Complex IIc). TNFR1 is ubiquitously expressed on nearly all cells, whereas TNFR2 has limited expression and it mainly found on Tregs. Previously it was thought that TNF-α impairs Treg activity through TNFR2 signalling (Lin et al., 2008), however this notion has been questioned. TNFR2 expression and signalling leads to expansion and proliferation of immunosuppressive Tregs, a subset with the highest suppressive capacity (Salomon et al.,

2018). Thus, indicating a potentially immunosuppressive effect for TNF-α through its interaction with TNFR2, and a potential novel therapeutic target for allergic inflammation (Ahmad et al., 2018). The mechanisms of TNF signalling are poorly understood and often paradoxical. TNF is initially expressed as mTNF, once cleaved by proteolytic enzymes it is released in its soluble form (sTNF). mTNF has a high affinity for TNFR1 whereas, sTNF has a high affinity for TNFR2. Preventing or enhancing the cleavage of mTNF to sTNF may be key to understanding the role TNF signalling in inflammation and a viable approach to modulate Tregs (Chen and Oppenheim, 2016). A recommendation for future research is to observe differences in TNFR2 expression and TNF-TNFR interaction in response to treatment with B-GOS, in order to better understand the function of TNF-α and modulation of $Foxp3^+$ $CD4^+$ $CD25^+$ T cells in inflammation (Chen et al., 2010).

For the first time, Chapter 6 shows that 20-day intervention of prebiotic B-GOS can successfully reduce pro-inflammatory cytokine TNF-α in $CD4^+$ T cells of healthy adults. This reduction of TNF-α after prebiotic treatment, coupled with the well documented bifidogenic effect of B-GOS (Fanaro et al., 2009; Grimaldi et al., 2016; Vulevic et al., 2015), suggests that changes in the commensal bacteria may significantly alter the immune response in healthy adult participants. While also providing further evidence that the manipulation of commensal bacteria through nutritional intervention influences inflammatory responses beyond the gastrointestinal tract.

In Chapter 7, peripheral blood mononuclear cells from healthy adults were stimulated *in vitro* to assess the effects of prebiotic B-GOS on the chronic inflammatory response. This method was developed in order to determine the optimal stimulant, concentration, incubation period and thus testing conditions. Different incubation periods were piloted for various concentrations of PMA/Ionomycin (see section 3.4). PMA and ionomycin are effective at increasing cytokine production without inducing significant damage to immune cells.

Moreover, ionomycin is able to bypass the T cell receptor, activating intracellular pathways directly (Wenchao et al., 2013), and allowing transcription of NF-κβ and Foxp3. When assessing cytokine release, it is important the stimulation used is able to produce maximal concentrations of cytokines at the lowest dose possible, and in the shortest time period to avoid variability in immune cell function outside of the body (House, 1999). The optimal testing conditions were found to be 5hs with PMA at 50ng/ml and Ionomycin at 1ug/ml. Understanding the optimal testing conditions for *in vitro* inflammatory challenges is crucial for improving the quality of data obtained and allows for greater confidence when evaluating nutritional interventions.

In acute inflammation, NF-κβ is regulated at its normative level through negative feedback loops, during chronic inflammation the persistence of stimuli may outperform this inhibitory feedback loop leading constitutive activation of NF-κβ (Hoesel and Schmid, 2013). Maguire et al., demonstrated the activity of NF-κβ in response to PMA/Ionomycin in PBMCs, it was found that phosphorylated p65 (P-p65) expression increased following treatment with PMA/ionomycin, expression levels of P-p65 remained above baseline 60 minutes post treatment and total p65 was unchanged. In the same study, a TNF-α stimulation led to a greater increase in P-p65 than the PMA/ionomycin stimulation, P-p65 declined to baseline 40 minutes post treatment and total p65 was unchanged. In Chapter 6, total p65 was unchanged after B-GOS treatment, and in Chapter 7, total p65 also remained unchanged, as expected. Additionally, there were no differences of p65 quantity between Chapter 6 and Chapter 7. These findings indicate that prebiotic B-GOS has no effect on total p65 expression in PBMCs. However, it is likely that B-GOS affects the phosphorylation of the p65 subunit through the modulation of TNF-α. Recommendations for future work are to observe the effects of B-GOS on NF-κβ phosphorylation, enabling better understanding of B-GOS mechanism of action.

In Chapter 6 it was demonstrated that B-GOS reduced TNF-α by 43.3% in $CD4^+$ T cells. In Chapter 7, after cell stimulation, B-GOS reduced TNF-α by 13.9%. This reduction after stimulation with PMA/ionomycin indicates a protective role for B-GOS in chronic inflammation. The regulation of TNF-α expression is complex and tissue/stimulus specific (Sullivan et al., 2007), TNF gene transcription is regulated by nucleoprotein complexes or enhanceosomes (Falvo et al., 2000). Nuclear factor of activated T cells (NFATs) facilitate enhanceosome formation and thus regulate TNF gene transcription (Torgerson et al., 2009). Interestingly, $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells can repress transcription of cytokine genes through NFAT2 (Falvo et al., 2000). Moreover, SCFAs may modulate NF-κβ/p65 signalling through HDAC3 inhibition, reducing TNF-α expression (Zhu et al., 2010). The effects of B-GOS on systemic inflammation are poorly understood. To better characterise, understand and evaluate the mechanisms involved, more in-depth *in vitro* studies are required, utilising primary cell culture and T cell differentiation analysis.

For the first time, Chapter 7 shows that 20-day intervention of prebiotic B-GOS can successfully reduce pro-inflammatory cytokine TNF-α in $CD4^+$ T cells after stimulation with PMA/ionomycin. This reduction of TNF-α after prebiotic treatment, provides further evidence that the manipulation of commensal bacteria, through prebiotic treatment, reduces markers of systemic inflammation (McLoughlin et al., 2017; Vulevic et al., 2008; Williams et al., 2016) and that prebiotics may be a novel nutritional approach for attenuating chronic inflammation.

8.3. Limitations

The specific limitations of each study are discussed in the relevant chapter. With regards to Chapter 4 and 5, a limitation of assessing asthma populations is the high heterogeneity across varying asthma severities and the associated phenotyping difficulties (Wenzel et al., 2013). The infant studies used for meta-analysis in Chapter 5 have relatively large cohorts from different

countries, which may influence asthma phenotype through diet, lifestyle and economic background, leading to heterogeneity. Furthermore, asthma is characterised as 'recurrent wheeze' in infant studies and diagnosed by a different physician depending on the study. Moreover, prebiotic type, dose and control diet vary in each study and many participants dropped out before completing their follow up visit. Evidence from adult asthma studies suggest that asthma lung function parameters can be improved with prebiotic treatment. However, a larger number of these studies are required to confirm this with meta-analysis. Despite these limitations, the findings of Chapter 5 provide powerful insights into prebiotic and synbiotic intervention for the treatment of asthma and associated inflammatory markers.

Chapter 6 and 7 show the successful use of prebiotic B-GOS in reducing TNF-α, a key marker of inflammation, in $CD4^+$ T cells, in both normophysiology and chronic inflammatory conditions *in vitro*. In order to support or refute these findings, larger replication studies should be conducted. Normality testing failed for several inflammatory markers in Chapter 6 and 7, possible due to sample size, increasing participant number this will allow for more representative data and reduce heterogeneity. Finally, the measurements of NF-κβ p65 phosphorylation, TNF-TNFR receptor interactions and gene regulation of TNF-α would provide further insight into the mechanistic actions of B-GOS and the role of SCFAs in inflammation.

8.4. Significance of findings and direction for future research

The findings of Chapter 5 provide significant scope for research of prebiotics in adults with asthma, in order to reduce asthma exacerbations, improve FEV_1 and reduce asthma associated inflammatory markers. Chapter 4 provides a framework for one of these future studies, considering asthma control, medication adherence and asthma quality of life. Well-controlled asthma should not pose as a barrier to quality of life and prebiotic intervention is easily

implemented with no adverse effects. The findings of Chapter 6 and 7, demonstrate the benefits of prebiotics in healthy adults to reduce systemic inflammation, and support the study of prebiotics in multiple inflammatory diseases. This thesis expands our knowledge of B-GOS mechanism of action and shows for the first that B-GOS can significantly attenuate the pro-inflammatory marker TNF-α in healthy participants at baseline and following a chronic inflammatory challenge *in vitro*. This thesis provides future research direction, which will improve our understanding of the mechanistic and therapeutic properties of prebiotics and synbiotics in the treatment of asthma, and prebiotic B-GOS in reducing inflammation

- Meta-analysis of murine trials revealed the benefits of prebiotics and synbiotics in the treatment of asthma outcome measures and associated inflammatory markers. Prebiotic treatment significantly reduced airway hyper-responsiveness and while synbiotics did not. Both prebiotic and synbiotic treatment significantly reduced multiple inflammatory cytokines; IL-4, IL-5, IL-13, and inflammatory cell counts; neutrophil, eosinophil, alveolar macrophage and lymphocyte.
- Conversely, meta-analysis of infant trials revealed that prebiotic and synbiotic treatment had no effect on the risk of cumulative incidence of recurrent wheeze. However, adult studies examining the prebiotic and synbiotic treatment of asthma demonstrated improvements in FEV_1, reduced sputum eosinophil counts and reduced pro-inflammatory marker expression.
- A greater number of prebiotic intervention studies in adults with asthma are necessary for meta-analysis and will inform future asthma treatment strategies.
- A more standardised approach to prebiotic intervention methodology in infants and adults with asthma will reduce heterogeneity and allow for more comparability between studies.
- Furthermore, asthma quality of life should be measured more frequently in nutritional intervention studies, to better characterise the effects of prebiotics.

In Chapter 6 and 7, we have shown that for the first time an intervention with the prebiotic B-GOS reduces a key marker of systemic inflammation in healthy adults at baseline and during a chronic inflammatory challenge *invitro*. In order to elucidate the exact mechanisms of B-GOS in inflammation, future research studies should focus on:

- A 3.65g·d^{-1} B-GOS dose was given during the intervention in Chapter 6, the dose was not given on the testing day to avoid an acute response. Therefore, the reduction of TNF-α in peripheral blood mononuclear cells after B-GOS treatment is likely to be caused by genetic changes. Thus, an important development would be to measure phosphorylated p65 in response to B-GOS.
- The reduction of TNF-α shown in Chapter 7, demonstrates a potential therapeutic role for B-GOS in patients with chronic inflammatory disease. In order to confirm this, future research should focus on prebiotic intervention for the treatment of chronic inflammatory disease.
- There are multiple methods to uncover the mechanism of action of prebiotic B-GOS. The collection of faecal samples would allow confirmation that any changes of systemic inflammatory markers or study specific measures are due to changes in the commensal bacteria
- Furthermore, the measurements of SCFA concentration in the blood following B-GOS administration should be collected. The action of these SCFAs on G-protein coupled receptors 41, 43 and 109a, histone deacetylases and/or histone acetyltransferases should be measured at the site of interest.
- Recent research has demonstrated the association of TNF-TNFR2 signalling with the expansion and increased suppressive function of $CD4^+$ $CD25^+$ Foxp3 regulatory T cells (Salomon et al., 2018). Conversely, it has been demonstrated that TNFR1 signalling is associated with allergic inflammatory responses (Ahmad et al., 2018). Through the

observation of the TNFR1/TNFR2 signalling pathway we may better our understanding of inflammation in response to B-GOS treatment.

- The effect of prebiotics on lymphatic cell migration and T cell differentiation in the gut associated lymphoid tissue is poorly understood and should be measured in future research
- There is also scope for other populations to be studied, such as healthy adult females, with the correct methodology in place to account for the menstruation cycle.

8.5. Conclusion

In conclusion, this thesis provides novel evidence for the efficacy of prebiotic and synbiotic use in treatment of asthma and changes in associated inflammatory markers, and the efficacy of the prebiotic B-GOS as an agent to reduce inflammation in healthy adults. We have shown for the first time that treatment with B-GOS is effective at reducing marker of systematic inflammation TNF-α at baseline in healthy participants and under chronic inflammation conditions *in vitro*. The implication of these novel findings provide scope for research of prebiotics, such as B-GOS from the current programme of work, in healthy participants to alleviate inflammation and in multiple chronic inflammatory disorders. The data obtained increases evidence the commensal bacteria and SCFAs have a significant role in systemic inflammation, systemic immune function and pose a potential therapeutic target for health and disease.

Reference List

Abdelhamid, L. and Luo, X., 2018. Retinoic Acid, Leaky Gut, and Autoimmune Diseases. Nutrients, 10(8), pp.1016.

Abrahamsson, T. R., Jakobsson, H.E., Andersson, A.F., Björkstén, B., Engstrand, L., Jenmalm, M.C., 2014. Low gut microbiota diversity in early infancy precedes asthma at school age. Clin Exp Allergy 44, pp.842–850.

Adan, A., Alizada, G., Kiraz, Y., Baran, Y., & Nalbant, A., 2016. Flow cytometry: basic principles and applications. Critical Reviews In Biotechnology, 37(2), pp.163-176.

Agace, W., Higgins, J., Sadasivan, B., Brenner, M. and Parker, C., 2000. T-lymphocyte–epithelial-cell interactions: integrin αE(CD103)β7, LEEP-CAM and chemokines. Current Opinion in Cell Biology, 12(5), pp.563-568.

Ai, W., Li, H., Song, N., Li, L. and Chen, H., 2013. Optimal Method to Stimulate Cytokine Production and Its Use in Immunotoxicity Assessment. International Journal of Environmental Research and Public Health, 10(9), pp.3834-3842.

Ajani, U., Ford, E. and Mokdad, A., 2004. Dietary Fiber and C-Reactive Protein: Findings from National Health and Nutrition Examination Survey Data. The Journal of Nutrition, 134(5), pp.1181-1185.

Ali, F., 2011. Does this patient have atopic asthma?. Clinical Medicine, 11(4), pp.376-380.

Alpan, O., Bachelder, E., Isil, E., Arnheiter, H. and Matzinger, P., 2004. 'Educated' dendritic cells act as messengers from memory to naive T helper cells. Nature Immunology, 5(6), pp.615-622.

Amin, K., 2012. The role of mast cells in allergic inflammation. Respiratory Medicine, 106(1), pp.9-14.

Anand, S. and Mande, S., 2018. Diet, Microbiota and Gut-Lung Connection. Frontiers in Microbiology, 9.

Anaya, J., Shoenfeld, y., Rojas-Villarraga, A., 2013. Autoimmunity: From Bench to Bedside. El Rosario University Press, 5.

Anderson, G. and Takahama, Y., 2012. Thymic epithelial cells: working class heroes for T cell development and repertoire selection. Trends in Immunology, 33(6), pp.256-263.

Ang, Z. and Ding, J., 2016. GPR41 and GPR43 in Obesity and Inflammation – Protective or Causative?. Frontiers in Immunology, 7.

Arboleya, S., Sánchez, B., Milani, C., Duranti, S., Solís, G., Fernández, N., de los Reyes-Gavilán, C. G., Ventura, M., Margolles, A., Gueimonde, M., 2015. Intestinal Microbiota Development in Preterm Neonates and Effect of Perinatal Antibiotics. The Journal of Pediatrics 166, pp.538–544.

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P., Liu, H., Cross, J., Pfeffer, K., Coffer, P. and Rudensky, A., 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature, 504(7480), pp.451-455.

Arslanoglu, S., Moro, G.E., Boehm, G., Wienz, F., Stahl, B., Bertino, E., 2012. Early neutral prebiotic oligosaccharide supplementation reduces the incidence of some allergic manifestations in the first 5 years of life. J. Biol. Regul. Homeost. Agents 26, 49–59.

Arslanoglu, S., Moro, G.E., Schmitt, J., Tandoi, L., Rizzardi, S., Boehm, G., 2008. Early Dietary Intervention with a Mixture of Prebiotic Oligosaccharides Reduces the Incidence of Allergic Manifestations and Infections during the First Two Years of Life. The Journal of Nutrition 138, pp.1091–1095.

Artis, D., 2008. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nature Reviews Immunology, 8(6), pp.411-420.

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., & Nishikawa, H. et al., 2013. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature, 500(7461), pp.232-236.

Aune, D., Chan, D., Lau, R., Vieira, R., Greenwood, D., Kampman, E. and Norat, T., 2011. Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. BMJ, 343(nov10 1), pp.d6617-d6617.

Azad, M. B., Coneys, J. G., Kozyrskyj, A. L., Field, C.J., Ramsey, C.D., Becker, A.B., Friesen, C., Abou-Setta, A.M., Zarychanski, R., 2013. Probiotic supplementation during pregnancy or infancy for the prevention of asthma and wheeze: systematic review and meta-analysis. BMJ 347, pp.f6471–f6471.

Baay-Guzman, G., Huerta-Yepez, S., Vega, M., Aguilar-Leon, D., Campillos, M., Blake, J., Benes, V., Hernandez-Pando, R. and Teran, L., 2012. Role of CXCL13 in Asthma. Chest, 141(4), pp.886-894.

Bacharier LB, Jabara H, Geha RS., 1998. Molecular mechanisms of immunoglobulin E regulation. Int Arch Allergy Immunol; 11(5), pp. 257–269.

Baecher-Allan, C., Viglietta, V. and Hafler, D., 2002. Inhibition of Human CD4+CD25+high Regulatory T Cell Function. The Journal of Immunology, 169(11), pp.6210-6217.

Banchereau J, Briere F, Caux C., 2000. Immunobiology of dendritic cells. Annu Rev Immunol: 2(18), pp.767–811.

Bao, K., & Reinhardt, R. L., 2015. The differential expression of IL-4 and IL-13 and its impact on type-2 immunity. Cytokine, 75(1), pp.25-37.

Baraldo, S., Turato, G., Cosio, M. and Saetta, M., 2016. Which CD8+T-cells in asthma? Attacking or defending?. European Respiratory Journal, 48(2), pp.287-290.

Baran, J., Kowalczyk, D., Oz˙óg, M. and Zembala, M., 2001. Three-Color Flow Cytometry Detection of Intracellular Cytokines in Peripheral Blood Mononuclear Cells: Comparative Analysis of Phorbol Myristate Acetate-Ionomycin and Phytohemagglutinin Stimulation. Clinical Diagnostic Laboratory Immunology, 8(2), pp.303-313.

Barbera Betancourt, A., Lyu, Q., Broere, F., Sijts, A., Rutten, V., & van Eden, W., 2017. T Cell-Mediated Chronic Inflammatory Diseases Are Candidates for Therapeutic Tolerance Induction with Heat Shock Proteins. Frontiers In Immunology, 8.

Barcelos, I., Troxell, R. and Graves, J., 2019. Mitochondrial Dysfunction and Multiple Sclerosis. Biology, 8(2), p.37.

Barnes PJ., 2001. Th2 cytokines and asthma: an introduction. Respiratory Research. 2 (2), pp.64-65.

Barnes PJ., 2002. Are mast cells still important in asthma? Rev Fr Allergol Immunol Clin, 42(1), pp20–27.

Barnes, P., 2009. Histone deacetylase-2 and airway disease. Therapeutic Advances in Respiratory Disease, 3(5), pp.235-243.

Barnes, P., 2010. Inhaled Corticosteroids. Pharmaceuticals, 3(3), pp.514-540.

Barrett N. A, Austen KF., 2009. Innate cells and T helper 2 cell immunity in airway inflammation. Immunity; 3(1),pp.425–437

Barrett, E., Kerr, C., Murphy, K., O'Sullivan, O., Ryan, C.A., Dempsey, E.M., Murphy, B.P., O'Toole, P.W., Cotter, P.D., Fitzgerald, G.F., Ross, R.P., Stanton, C., 2013. The individual-specific and diverse nature of the preterm infant microbiota. Arch Dis Child Fetal Neonatal Ed 98, pp.334–340.

Becker D. E., 2013. Basic and clinical pharmacology of glucocorticosteroids. Anesthesia progress, 60(1), pp.25–32.

Begley, L., Madapoosi, S., Opron, K., Ndum, O., Baptist, A., Rysso, K., Erb-Downward, J.R., Huang, Y.J., 2018. Gut microbiota relationships to lung function and adult asthma phenotype: a pilot study. BMJ Open Respir Res 5, p.324.

Berger, A., 2000. Science commentary: Th1 and Th2 responses: what are they?. BMJ, 321(7258), pp.424-424.

Bernasconi, E., Pattaroni, C., Koutsokera, A., Pison, C., Kessler, R., Benden, C., Soccal, P., Magnan, A., Aubert, J., Marsland, B. and Nicod, L., 2016. Airway Microbiota Determines Innate Cell Inflammatory or Tissue Remodeling Profiles in Lung Transplantation. American Journal of Respiratory and Critical Care Medicine, 194(10), pp.1252-1263.

Bernini, L., Simão, A., Alfieri, D., Lozovoy, M., Mari, N., de Souza, C., Dichi, I. and Costa, G., 2016. Beneficial effects of Bifidobacterium lactis on lipid profile and cytokines in patients

with metabolic syndrome: A randomized trial. Effects of probiotics on metabolic syndrome. Nutrition, 32(6), pp.716-719.

BERRY, M., BRIGHTLING, C., PAVORD, I. and WARDLAW, A., 2007. TNF-α in asthma. Current Opinion in Pharmacology, 7(3), pp.279-282.

Berthon, B., Macdonald-Wicks, L., Gibson, P. and Wood, L., 2013. Investigation of the association between dietary intake, disease severity and airway inflammation in asthma. Respirology, 18(3), pp.447-454.

Bhandoola, A. and Sambandam, A., 2006. From stem cell to T cell: one route or many?. Nature Reviews Immunology, 6(2), pp.117-126.

Bhavsar, P., Ahmad, T., & Adcock, I., 2008. The role of histone deacetylases in asthma and allergic diseases. Journal Of Allergy And Clinical Immunology, 121(3), pp.580-584.

Binder, H., 2010. Role of Colonic Short-Chain Fatty Acid Transport in Diarrhea. Annual Review of Physiology, 72(1), pp.297-313.

Bisgaard, H., Li, N., Bonnelykke, K., Chawes, B., Skov, T., Paludan-Müller, G., Stokholm, J., Smith, B. and Krogfelt, K., 2011. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. Journal of Allergy and Clinical Immunology, 128(3), pp.646-652.e5.

Bloomfield, S. F., Rook, G. A., Scott, E. A., Shanahan, F., Stanwell-Smith, R., Turner, P.. 2016. Time to abandon the hygiene hypothesis: new perspectives on allergic disease, the human microbiome, infectious disease prevention and the role of targeted hygiene. Perspect Public Health, 136(4), pp.213–224.

Boks, M., Kager-Groenland, J., Mousset, C., van Ham, S. and ten Brinke, A., 2014. Inhibition of TNF receptor signaling by anti-TNFα biologicals primes naïve CD4+ T cells towards IL-10+ T cells with a regulatory phenotype and function. Clinical Immunology, 151(2), pp.136-145.

Bonser, L. R., & Erle, D. J., 2017. Airway Mucus and Asthma: The Role of MUC5AC and MUC5B. Journal of clinical medicine, 6(12), p.112.

Borish, L. and Steinke, J., 2010. Interleukin-33 in Asthma: How Big of a Role Does It Play?. Current Allergy and Asthma Reports, 11(1), pp.7-11.

Bothur, E., Raifer, H., Haftmann, C., Stittrich, A., Brüstle, A., Brenner, D., Bollig, N., Bieringer, M., Kang, C., Reinhard, K., Camara, B., Huber, M., Visekruna, A., Steinhoff, U., Repenning, A., Bauer, U., Sexl, V., Radbruch, A., Sparwasser, T., Mashreghi, M., Wah Mak, T. and Lohoff, M., 2015. Antigen receptor-mediated depletion of FOXP3 in induced regulatory T-lymphocytes via PTPN2 and FOXO1. Nature Communications, 6(1).

Bou-Gharios, G., & de Crombrugghe, B., 2008. Type I Collagen Structure, Synthesis, and Regulation. Principles Of Bone Biology, pp.285-318.

Bouhnik, Y., Raskine, L., Simoneau, G., Vicaut, E., Neut, C., Flourié, B., Brouns, F. and Bornet, F., 2004. The capacity of nondigestible carbohydrates to stimulate fecal bifidobacteria in healthy humans: a double-blind, randomized, placebo-controlled, parallel-group, dose-response relation study. The American Journal of Clinical Nutrition, 80(6), pp.1658-1664.

Boulet, L.P., Turcotte, H., Laviolette, M., Naud, F., Bernier, M.C., Martel, S., Chakir, J., 2000. Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids. Am. J. Respir. Crit. Care Med, 162, pp.1308–1313

Boushey, HA., Holtzman, MJ., Sheller, JR., Nadel, JA., 1980. Am Rev Respir Dis. 121(2), pp.389-413.

Bousquet J., Jeffery P. K., Busse W. W., Johnson M., Vignola A. M., 2000. Asthma. From bronchoconstriction to airways inflammation and remodeling. Am. J. Respir. Crit. Care Med.161, pp.1720–1745

Boyman, O. and Sprent, J., 2012. The role of interleukin-2 during homeostasis and activation of the immune system. Nature Reviews Immunology, 12(3), pp.180-190.

Bradding, P., Roberts, J., Britten, K., Montefort, S., Djukanovic, R., Mueller, R., Heusser, C., Howarth, P. and Holgate, S., 1994. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. American Journal of Respiratory Cell and Molecular Biology, 10(5), pp.471-480.

Bradding, P., Walls, A. and Holgate, S., 2006. The role of the mast cell in the pathophysiology of asthma. Journal of Allergy and Clinical Immunology, 117(6), pp.1277-1284.

Brigham, E., Kolahdooz, F., Hansel, N., Breysse, P., Davis, M., Sharma, S., Matsui, E., Diette, G. and McCormack, M., 2015. Association between Western diet pattern and adult asthma: a focused review. Annals of Allergy, Asthma & Immunology, 114(4), pp.273-280.

Brosseau, C., Selle, A., Palmer, D.J., Prescott, S.L., Barbarot, S., Bodinier, M., 2019. Prebiotics: Mechanisms and Preventive Effects in Allergy. Nutrients 11, p.1841.

Brown, A., Goldsworthy, S., Barnes, A., Eilert, M., Tcheang, L., & Daniels, D. et al., 2002. The Orphan G Protein-coupled Receptors GPR41 and GPR43 Are Activated by Propionate and Other Short Chain Carboxylic Acids. Journal Of Biological Chemistry, 278(13), pp.11312-11319.

Brownlie, R. and Zamoyska, R., 2013. T cell receptor signalling networks: branched, diversified and bounded. Nature Reviews Immunology, 13(4), pp.257-269.

Bull, M. J., & Plummer, N. T., 2014. Part 1: The Human Gut Microbiome in Health and Disease. Integrative medicine (Encinitas, Calif.), 13(6), pp.17-22.

Burska, A., Sakthiswary, R. and Sattar, N., 2015. Effects of Tumour Necrosis Factor Antagonists on Insulin Sensitivity/Resistance in Rheumatoid Arthritis: A Systematic Review and Meta-Analysis. PLOS ONE, 10(6), e0128889.

Busse, WW., Lemanske, RF., 2001. Advances in Immunology N Engl J Med, 344, pp.350-362.

Byndloss, M., Olsan, E., Rivera-Chávez, F., Tiffany, C., Cevallos, S., Lokken, K., Torres, T., Byndloss, A., Faber, F., Gao, Y., Litvak, Y., Lopez, C., Xu, G., Napoli, E., Giulivi, C., Tsolis, R., Revzin, A., Lebrilla, C. and Bäumler, A., 2017. Microbiota-activated PPAR-γ signaling inhibits dysbiotic Enterobacteriaceae expansion. Science, 357(6351), pp.570-575.

Camargo, C., Justus, F., Retzlaff, G., Blood, M. and Schafranski, M., 2015. Action of anti-TNF-α drugs on the progression of Alzheimer's disease: A case report. Dementia & Neuropsychologia, 9(2), pp.196-200.

Caminero, A., Comabella, M. and Montalban, X., 2011. Tumor necrosis factor alpha (TNF-α), anti-TNF-α and demyelination revisited: An ongoing story. Journal of Neuroimmunology, 234(1-2), pp.1-6.

Cao, X., Cai, S., Fehniger, T., Song, J., Collins, L., Piwnica-Worms, D. and Ley, T., 2007. Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance. Immunity, 27(4), pp.635-646.

Carding, S. and Egan, P., 2002. γδ T cells: functional plasticity and heterogeneity. Nature Reviews Immunology, 2(5), pp.336-345.

Carey, O., Cookson, J., Britton, J. and Tattersfield, A., 1996. The effect of lifestyle on wheeze, atopy, and bronchial hyperreactivity in Asian and white children. American Journal of Respiratory and Critical Care Medicine, 154(2), pp.537-540.

Carswell, E., Old, L., Kassel, R., Green, S., Fiore, N. and Williamson, B., 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proceedings of the National Academy of Sciences, 72(9), pp.3666-3670.

Carvajal, J., Germain, A., Huidobro-Toro, J. and Weiner, C., 2000. Molecular mechanism of cGMP-mediated smooth muscle relaxation. Journal of Cellular Physiology, 184(3), pp.409-420.

Caulfield, A. and Lathem, W., 2014. Disruption of Fas-Fas Ligand Signaling, Apoptosis, and Innate Immunity by Bacterial Pathogens. PLoS Pathogens, 10(8), e1004252.

Chai, J., Digby, J. and Choudhury, R., 2013. GPR109A and Vascular Inflammation. Current Atherosclerosis Reports, 15(5).

Chen, H., Zhao, Y. and Zhao, T., 2015. Histone Deacetylases and Mechanisms of Regulation of Gene Expression. Critical Reviews in Oncogenesis, 20(1-2), pp.35-47.

Chen, L., & Flies, D. B., 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nature reviews. Immunology, 13(4), pp.227-42.

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X. and Zhao, L., 2017. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget, 9(6), pp.7204-7218.

Chen, X. and Oppenheim, J., 2010. TNF-α: An Activator of CD4+FoxP3+TNFR2+ Regulatory T Cells. Current Directions in Autoimmunity, pp.119-134.

Chen, X. and Oppenheim, J., 2016. Paradoxical effects of targeting TNF signalling in the treatment of autoimmunity. Nature Reviews Rheumatology, 12(11), pp.625-626.

Chen, X., Subleski, J., Kopf, H., Howard, O., Männel, D. and Oppenheim, J., 2008. Cutting Edge: Expression of TNFR2 Defines a Maximally Suppressive Subset of Mouse CD4+CD25+FoxP3+ T Regulatory Cells: Applicability to Tumor-Infiltrating T Regulatory Cells. The Journal of Immunology, 180(10), pp.6467-6471.

Chen, X., Wu, X., Zhou, Q., Howard, O., Netea, M. and Oppenheim, J., 2012. TNFR2 Is Critical for the Stabilization of the CD4+Foxp3+ Regulatory T Cell Phenotype in the Inflammatory Environment. The Journal of Immunology, 190(3), pp.1076-1084.

Cheng, J., Ringel-Kulka, T., Heikamp-de Jong, I., Ringel, Y., Carroll, I., de Vos, W.M., Salojärvi, J., Satokari, R., 2016. Discordant temporal development of bacterial phyla and the emergence of core in the fecal microbiota of young children. ISME J 10, pp.1002–1014.

Chesné, J., Braza, F., Mahay, G., Brouard, S., Aronica, M. and Magnan, A., 2014. IL-17 in Severe Asthma. Where Do We Stand?. American Journal of Respiratory and Critical Care Medicine, 190(10), pp.1094-1101.

Chhabra G., 2018. Automated hematology analyzers: Recent trends and applications. Journal of laboratory physicians, 10(1), pp.15–16.

Chou, R., Kane, M., Ghimire, S., Gautam, S. and Gui, J., 2016. Treatment for Rheumatoid Arthritis and Risk of Alzheimer's Disease: A Nested Case-Control Analysis. CNS Drugs, 30(11), pp.1111-1120.

Clapp, M., Aurora, N., Herrera, L., Bhatia, M., Wilen, E., & Wakefield, S., 2017. Gut microbiota's effect on mental health: The gut-brain axis. Clinics and practice, 7(4), pp.987.

Cocchiara, R., Bongiovanni, A., Albeggiani, G., Azzolina, A., Lampiasi, N., Di Blasi, F. and Geraci, D., 1997. Inhibitory effect of neuraminidase on SP-induced histamine release and TNF-α mRNA in rat mast cells: evidence of a receptor-independent mechanism. Journal of Neuroimmunology, 75(1-2), pp.9-18.

Coleman, O.I., Haller, D., 2018. Bacterial Signaling at the Intestinal Epithelial Interface in Inflammation and Cancer. Front. Immunol. 8, p.1927.

Collado, M.C., Rautava, S., Aakko, J., Isolauri, E., Salminen, S., 2016. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Sci Rep 6, p.23129.

Collins, S., Surette, M. and Bercik, P., 2012. The interplay between the intestinal microbiota and the brain. Nature Reviews Microbiology, 10(11), pp.735-742.

Coombes, J. and Powrie, F., 2008. Dendritic cells in intestinal immune regulation. Nature Reviews Immunology, 8(6), pp.435-446.

Corthay A., 2009. How do regulatory T cells work?. Scandinavian journal of immunology, 70(4), pp.326-36.

Cottrell, J., Amaral, L., Harmon, A., Cornelius, D., Cunningham, M., Vaka, V., Ibrahim, T., Herse, F., Wallukat, G., Dechend, R. and LaMarca, B., 2019. Interleukin-4 supplementation improves the pathophysiology of hypertension in response to placental ischemia in RUPP rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 316(2), pp.165-171.

Cox, G., Miller, J.D., McWilliams, A., FitzGerald, J.M., Lam, S., 2006. Bronchial thermoplasty for asthma. Am. J. Respir. Crit. Care Med, 173, pp.965–969

Crispín, J., Oukka, M., Bayliss, G., Cohen, R., Van Beek, C., & Stillman, I. et al., 2008. Expanded Double Negative T Cells in Patients with Systemic Lupus Erythematosus Produce IL-17 and Infiltrate the Kidneys. The Journal Of Immunology, 181(12), pp.8761-8766.

Crotty, S., 2011. Follicular Helper CD4 T Cells (TFH. Annual Review Of Immunology, 29(1), pp.621-663.

Crotty S., 2014. T follicular helper cell differentiation, function, and roles in disease. Immunity, 41(4), pp.529-42.

Cui, G., 2019. TH9, TH17, and TH22 Cell Subsets and Their Main Cytokine Products in the Pathogenesis of Colorectal Cancer. Frontiers In Oncology, 9.

Cunningham, C., 2011. Systemic inflammation and delirium: important co-factors in the progression of dementia. Biochemical Society Transactions, 39(4), pp.945-953.

Curotto de Lafaille, M. A., Muriglan, S., Sunshine, M. J., Lei, Y., Kutchukhidze, N., Furtado, G. C., Wensky, A. K., Olivares-Villagómez, D., … Lafaille, J. J., 2001. Hyper immunoglobulin E response in mice with monoclonal populations of B and T lymphocytes. The Journal of experimental medicine, 194(9), pp.1349-59.

Da Silva, E. Z. M., Jamur, M. C., & Oliver, C., 2014. Mast Cell Function: A New Vision of an Old Cell. Journal of Histochemistry and Cytochemistry, 62(10), pp.698–738.

Dabbagh, K., Takeyama, K., Lee, H., Ueki, I.F., Lausier, J.A., and Nadel., J.A., 1999. IL-4 Induces Mucin Gene Expression and Goblet Cell Metaplasia In Vitro and In Vivo. J Immunol, 162(10), pp.6233-6237.

Dai, Z., Feng, S., Liu, A., Wang, H., Zeng, X. and Yang, C., 2019. Protective effects of α-galacto-oligosaccharides against a high-fat/western-style diet-induced metabolic abnormalities in mice. Food & Function, 10(6), pp.3660-3670.

Dardalhon, V., Awasthi, A., Kwon, H., Galileos, G., Gao, W., Sobel, R. A., Mitsdoerffer, M., Strom, T. B., Elyaman, W., Ho, I. C., Khoury, S., Oukka, M., … Kuchroo, V. K., 2008. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. Nature immunology, 9(12), pp.1347-1355.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., … Turnbaugh, P. J., 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature, 505(7484), pp.559–563.

De Felipe, P., 2004. Genetic Vaccines and Therapy, 2(1), p.13.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J., Massart, S., Collini, S., Pieraccini, G. and Lionetti, P., 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Sciences, 107(33), pp.14691-14696.

De Groot, J., ten Brinke, A. and Bel, E., 2015. Management of the patient with eosinophilic asthma: a new era begins. ERJ Open Research, 1(1), pp.00024-2015.

De Vries, J., 1998. The role of IL-13 and its receptor in allergy and inflammatory responses. Journal Of Allergy And Clinical Immunology, 102(2), pp.165-169.

De Vadder, F., Kovatcheva-Datchary, P., Goncalves, D., Vinera, J., Zitoun, C., Duchampt, A., Bäckhed, F. and Mithieux, G., 2014. Microbiota-Generated Metabolites Promote Metabolic Benefits via Gut-Brain Neural Circuits. Cell, 156(1-2), pp.84-96.

DeGruttola, A., Low, D., Mizoguchi, A. and Mizoguchi, E., 2016. Current Understanding of Dysbiosis in Disease in Human and Animal Models. Inflammatory Bowel Diseases, 22(5), pp.1137-1150.

Dehghan, P., Pourghassem Gargari, B. and Asghari Jafar-abadi, M., 2014. Oligofructose-enriched inulin improves some inflammatory markers and metabolic endotoxemia in women with type 2 diabetes mellitus: A randomized controlled clinical trial. Nutrition, 30(4), pp.418-423.

Den Besten, G., van Eunen, K., Groen, A., Venema, K., Reijngoud, D., & Bakker, B., 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal Of Lipid Research, 54(9), pp.2325-2340.

Deo, S. S., Mistry, K. J., Kakade, A. M., & Niphadkar, P. V., 2010. Role played by Th2 type cytokines in IgE mediated allergy and asthma. Lung India : Official Organ of Indian Chest Society, 27(2), pp.66–71.

Depeint, F., Tzortzis, G., Vulevic, J., I'Anson, K. and Gibson, G., 2008. Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of Bifidobacterium bifidum NCIMB 41171, in healthy humans: a randomized, double-blind, crossover, placebo-controlled intervention study. The American Journal of Clinical Nutrition, 87(3), pp.785-791.

Devereux, G. and Seaton, A., 2005. Diet as a risk factor for atopy and asthma. Journal of Allergy and Clinical Immunology, 115(6), pp.1109-1117.

Dharmage S. C., Lodge C. J., Lowe A. J., Allen K. J., 2015. Antibiotics and risk of asthma: a debate that is set to continue. Clinical Experiments in Allergy. 45, pp.6–8

Docampo, M., Stein-Thoeringer, C., Lazrak, A., Burgos da Silva, M., Cross, J. and van den Brink, M., 2018. Expression of the Butyrate/Niacin Receptor, GPR109a on T Cells Plays an Important Role in a Mouse Model of Graft Versus Host Disease. Blood, 132(Supplement 1), pp.61-61.

Doeing, D. and Solway, J., 2013. Airway smooth muscle in the pathophysiology and treatment of asthma. Journal of Applied Physiology, 114(7), pp.834-843.

Doogue, M. and Polasek, T., 2013. The ABCD of clinical pharmacokinetics. Therapeutic Advances in Drug Safety, 4(1), pp.5-7.

Dora, K. and Garland, C., 2001. Properties of smooth muscle hyperpolarization and relaxation to K+ in the rat isolated mesenteric artery. American Journal of Physiology-Heart and Circulatory Physiology, 280(6), pp.H2424-H2429.

Doucet, C., Brouty-Boyé, D., Pottin-Clémenceau, C., Canonica, G. W., Jasmin, C., & Azzarone, B., 1998. Interleukin (IL) 4 and IL-13 act on human lung fibroblasts. Implication in asthma. The Journal of clinical investigation, 101(10), pp.2129-39.

Douros, K., Thanopoulou, M., Boutopoulou, B., Papadopoulou, A., Papadimitriou, A., Fretzayas, A. and Priftis, K., 2019. Adherence to the Mediterranean diet and inflammatory markers in children with asthma. Allergologia et Immunopathologia, 47(3), pp.209-213.

Edris, A., De Feyter, S., Maes, T., Joos, G., Lahousse, L., 2019. Monoclonal antibodies in type 2 asthma: a systematic review and network meta-analysis. Respir. Res. 20, p.179.

Ege, M., 2017. The Hygiene Hypothesis in the Age of the Microbiome. Annals of the American Thoracic Society, 14(5), pp.S348-S353.

Eid, N., Enani, S., Walton, G., Corona, G., Costabile, A., Gibson, G., Rowland, I. and Spencer, J., 2014. The impact of date palm fruits and their component polyphenols, on gut microbial ecology, bacterial metabolites and colon cancer cell proliferation. Journal of Nutritional Science, 3.

Elazab, N., Mendy, A., Gasana, J., Vieira, E.R., Quizon, A., Forno, E., 2013. Probiotic Administration in Early Life, Atopy, and Asthma: A Meta-analysis of Clinical Trials. PEDIATRICS 132, pp.666–676.

Eyerich, K., & Eyerich, S., 2015. Th22 cells in allergic disease. Allergo journal international, 24(1), pp.1-7.

Falvo, J., Tsytsykova, A. and Goldfeld, A., 2010. Transcriptional Control of the TNF Gene. Current Directions in Autoimmunity, pp.27-60.

Falvo, J., Uglialoro, A., Brinkman, B., Merika, M., Parekh, B., Tsai, E., King, H., Morielli, A., Peralta, E., Maniatis, T., Thanos, D. and Goldfeld, A., 2000. Stimulus-Specific Assembly of Enhancer Complexes on the Tumor Necrosis Factor Alpha Gene Promoter. Molecular and Cellular Biology, 20(6), pp.2239-2247.

Fanaro, S., Marten, B., Bagna, R., Vigi, V., Fabris, C., Peña-Quintana, L., Argüelles, F., Scholz-Ahrens, K., Sawatzki, G., Zelenka, R., Schrezenmeir, J., de Vrese, M. and Bertino, E., 2009. Galacto-oligosaccharides Are Bifidogenic and Safe at Weaning: A Double-blind Randomized Multicenter Study. Journal of Pediatric Gastroenterology & Nutrition, 48(1), pp.82-88.

Faoud, I.T., 2011. The Journal of the American Osteopathic Association, 111(1), pp.11-17.

Fava, F., Gitau, R., Griffin, B., Gibson, G., Tuohy, K. and Lovegrove, J., 2012. The type and quantity of dietary fat and carbohydrate alter faecal microbiome and short-chain fatty acid excretion in a metabolic syndrome 'at-risk' population. International Journal of Obesity, 37(2), pp.216-223.

Fergusson, J., Fleming, V. and Klenerman, P., 2011. CD161-Expressing Human T Cells. Frontiers in Immunology, 2.

Fischer, K. and Agrawal, D., 2013. Hematopoietic Stem and Progenitor Cells in Inflammation and Allergy. Frontiers in Immunology, 4.

Forno, E., & Celedón, J. C., 2009. Asthma and Ethnic Minorities: Socioeconomic Status and Beyond. Current Opinion in Allergy and Clinical Immunology, 9(2), pp.154–160.

Fort, M., Cheung, J., Yen, D., Li, J., Zurawski, S., Lo, S., Menon, S., Clifford, T., Hunte, B., Lesley, R., Muchamuel, T., Hurst, S., Zurawski, G., Leach, M., Gorman, D. and Rennick, D., 2001. IL-25 Induces IL-4, IL-5, and IL-13 and Th2-Associated Pathologies In Vivo. Immunity, 15(6), pp.985-995.

Foster J. R., 2001. The functions of cytokines and their uses in toxicology. International journal of experimental pathology, 82(3), pp.171-92.

Foster, J., 2008. The functions of cytokines and their uses in toxicology. International Journal of Experimental Pathology, 82(3), pp.171-192.

Fox, J. C., Nakayama, T., Tyler, R. C., Sander, T. L., Yoshie, O., & Volkman, B. F., 2014. Structural and agonist properties of XCL2, the other member of the C-chemokine subfamily. Cytokine, 71(2), pp.302-11.

Francavilla, R., Calasso, M., Calace, L., Siragusa, S., Ndagijimana, M., Vernocchi, P., Brunetti, L., Mancino, G., Tedeschi, G., Guerzoni, E., Indrio, F., Laghi, L., Miniello, V., Gobbetti, M. and De Angelis, M., 2012. Effect of lactose on gut microbiota and metabolome of infants with cow's milk allergy. Pediatric Allergy and Immunology, 23(5), pp.420-427.

Francelin, C., 2012. Apoptosis and the Developing T Cells. Journal of Clinical & Cellular Immunology, 01(S3).

Frati, F., Salvatori, C., Incorvaia, C., Bellucci, A., Di Cara, G., Marcucci, F., & Esposito, S., 2018. The Role of the Microbiome in Asthma: The Gut–Lung Axis. International journal of molecular sciences, 20(1), p.123.

Fujisawa, T., Velichko, S., Thai, P., Hung, L. Y., Huang, F., & Wu, R., 2009. Regulation of airway MUC5AC expression by IL-1beta and IL-17A; the NF-kappaB paradigm. Journal of immunology (Baltimore, Md. : 1950), 183(10), pp.6236-6243.

Furman, D., Campisi, J., Verdin, E., Carrera-Bastos, P., Targ, S., Franceschi, C., Ferrucci, L., Gilroy, D., Fasano, A., Miller, G., Miller, A., Mantovani, A., Weyand, C., Barzilai, N., Goronzy, J., Rando, T., Effros, R., Lucia, A., Kleinstreuer, N. and Slavich, G., 2019. Chronic inflammation in the etiology of disease across the life span. Nature Medicine, 25(12), pp.1822-1832.

Furuhashi, K., Suda, T., Hasegawa, H., Suzuki, Y., Hashimoto, D., Enomoto, N., Fujisawa, T., Nakamura, Y., Inui, N., Shibata, K., Nakamura, H. and Chida, K., 2012. Mouse Lung CD103+and CD11bhighDendritic Cells Preferentially Induce Distinct CD4+T-Cell Responses. American Journal of Respiratory Cell and Molecular Biology, 46(2), pp.165-172.

Gao, H., Ying, S., & Dai, Y. 2017. Pathological Roles of Neutrophil-Mediated Inflammation in Asthma and Its Potential for Therapy as a Target. Journal of immunology research, 3743048.

Gaud, G., Lesourne, R. and Love, P., 2018. Regulatory mechanisms in T cell receptor signalling. Nature Reviews Immunology, 18(8), pp.485-497.

Gerriets, V., Goyal, A., Khaddour, K., 2022. Tumor Necrosis Factor Inhibitors. Treasure Island (FL), StatPearls. Available from: https://www.ncbi.nlm.nih.gov/books/NBK482425/

Ghiamati Yazdi, F., Zakeri, A., Ark, I., Leusink-Muis, T., Braber, S., Soleimanian-Zad, S., & Folkerts, G., 2020. Crude Turmeric Extract Improves the Suppressive Effects of Lactobacillus rhamnosus GG on Allergic Inflammation in a Murine Model of House Dust Mite-Induced Asthma. Frontiers In Immunology, 11.

Gibson, G. and Roberfroid, M., 1995. Dietary Modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics. The Journal of Nutrition, 125(6), pp.1401-1412.

Gibson, G., Probert, H., Loo, J., Rastall, R. and Roberfroid, M., 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutrition Research Reviews, 17(2), pp.259-275.

Gleich GJ, Adolphson CR., 1986. The eosinophilic leukocyte: structure and function. Adv. Immunol. 39(1), pp.177–253.

Global Strategy for Asthma Management and Prevention. GINA., 2019.

Golubovskaya, V. and Wu, L., 2016. Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy. Cancers, 8(3), p.36.

Gorman, B. and Chu, M., 2009. Racial and ethnic differences in adult asthma prevalence, problems, and medical care. Ethnicity & Health, 14(5), pp.527-552.

Gosset P, Tsicopoulos A, Wallaert B, Joseph M, Capron A, Tonnel AB., 1992. Tumor necrosis factor alpha and interleukin-6 production by human mononuclear phagocytes from allergic asthmatics after IgE-dependent stimulation. Am Rev Respir Dis, 146, pp.768-774.

Grimaldi, R., Cela, D., Swann, J., Vulevic, J., Gibson, G., Tzortzis, G. and Costabile, A., 2016. In vitro fermentation of B-GOS: impact on faecal bacterial populations and metabolic activity in autistic and non-autistic children. FEMS Microbiology Ecology, 93(2), p. 233.

Groeger, D., O'Mahony, L., Murphy, E., Bourke, J., Dinan, T., Kiely, B., Shanahan, F. and Quigley, E., 2013. Bifidobacterium infantis35624 modulates host inflammatory processes beyond the gut. Gut Microbes, 4(4), pp.325-339.

Guilleminault, L., Williams, E. J., Scott, H. A., Berthon, B. S., Jensen, M., & Wood, L. G., 2017. Diet and Asthma: Is It Time to Adapt Our Message?. Nutrients, 9(11), p.1227.

Guo, L., Urban, J., Zhu, J. and Paul, W., 2008. Elevating Calcium in Th2 Cells Activates Multiple Pathways to Induce IL-4 Transcription and mRNA Stabilization. The Journal of Immunology, 181(6), pp.3984-3993.

Gupta, S., Su, H., Bi, R., Agrawal, S. and Gollapudi, S., 2005. Immunity & Ageing, 2(1), p.12.

Hackett, T., 2012. Epithelial–mesenchymal transition in the pathophysiology of airway remodelling in asthma. Current Opinion in Allergy and Clinical Immunology, 12(1), pp.53-59.

Halmos, E., Christophersen, C., Bird, A., Shepherd, S., Gibson, P. and Muir, J., 2014. Diets that differ in their FODMAP content alter the colonic luminal microenvironment. Gut, 64(1), pp.93-100.

Halnes, I., Baines, K., Berthon, B., MacDonald-Wicks, L., Gibson, P. and Wood, L., 2017. Soluble Fibre Meal Challenge Reduces Airway Inflammation and Expression of GPR43 and GPR41 in Asthma. Nutrients, 9(1), p.57.

Hampton, H. and Chtanova, T., 2019. Lymphatic Migration of Immune Cells. Frontiers in Immunology, 10.

Han, Y., Blatter, J., Brehm, J., Forno, E., Litonjua, A. and Celedón, J., 2013. Diet and asthma: vitamins and methyl donors. The Lancet Respiratory Medicine, 1(10), pp.813-822.

Han, Y., Forno, E., Holguin, F. and Celedón, J., 2015. Diet and asthma. Current Opinion in Allergy and Clinical Immunology, 15(4), pp.369-374.

Hansel, T.T., Johnston, S.L., Openshaw, P.J., 2013. Microbes and mucosal immune responses in asthma. Lancet 381, pp.861–873.

Hardy, H., Harris, J., Lyon, E., Beal, J. and Foey, A., 2013. Probiotics, Prebiotics and Immunomodulation of Gut Mucosal Defences: Homeostasis and Immunopathology. Nutrients, 5(6), pp.1869-1912.

Haring, J., Badovinac, V., & Harty, J., 2006. Inflaming the CD8+ T Cell Response. Immunity, 25(1), pp.19-29.

Harmsen, H.J.M., Wildeboer–Veloo, A.C.M., Raangs, G.C., Wagendorp, A.A., Klijn, N., Bindels, J.G., Welling, G.W., 2000. Analysis of Intestinal Flora Development in Breast-Fed and Formula-Fed Infants by Using Molecular Identification and Detection Methods: Journal of Pediatric Gastroenterology and Nutrition 30, pp.61–67.

Hathcock, K. S., Laszlo, G., Pucillo, C., Linsley, P., & Hodes, R. J., 1994. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. The Journal of experimental medicine, 180(2), pp.631-40.

He, J., Zhang, P., Shen, L., Niu, L., Tan, Y., Chen, L., Zhao, Y., Bai, L., Hao, X., Li, X., Zhang, S. and Zhu, L., 2020. Short-Chain Fatty Acids and Their Association with Signalling Pathways in Inflammation, Glucose and Lipid Metabolism. International Journal of Molecular Sciences, 21(17), p.6356.

He, Y., Peng, S., Xiong, W., Xu, Y. and Liu, J., 2015. Association between Polymorphism of Interleukin-1beta and Interleukin-1 Receptor Antagonist Gene and Asthma Risk: A Meta-Analysis. The Scientific World Journal, 2015, pp.1-9.

Hekking, P. and Bel, E., 2014. Developing and Emerging Clinical Asthma Phenotypes. The Journal of Allergy and Clinical Immunology: In Practice, 2(6), pp.671-680.

Hemarajata, P. and Versalovic, J., 2012. Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. Therapeutic Advances in Gastroenterology, 6(1), pp.39-51.

Hentges, D., Maier, B., Burton, G., Flynn, M., Tsutakawa, K., 1977. Effect of a High-Beef Diet on the Fecal Bacterial Flora of Humans. American Association for Cancer Research, 37(2)

Higgins, J.P.T., Altman, D.G., Gotzsche, P.C., Juni, P., Moher, D., Oxman, A.D., Savovic, J., Schulz, K.F., Weeks, L., Sterne, J.A.C., Cochrane Bias Methods Group, Cochrane Statistical Methods Group, 2011. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. BMJ 343, pp.5928–5928.

Higgins, J.P.T., Thompson, S.G., 2002. Quantifying heterogeneity in a meta-analysis. Statist. Med. 21, pp.1539–1558.

Hill, C., Sanders, M.E., 2013. Rethinking "probiotics." Gut Microbes 4, pp.269–270.

Hilty, M., Burke, C., Pedro, H., Cardenas, P., Bush, A., Bossley, C., Davies, J., Ervine, A., Poulter, L., Pachter, L., Moffatt, M. and Cookson, W., 2010. Disordered Microbial Communities in Asthmatic Airways. PLoS ONE, 5(1), pp.8578.

Ho, I. C., Tai, T. S., & Pai, S. Y., 2009. GATA3 and the T-cell lineage: essential functions before and after T-helper-2-cell differentiation. Nature reviews. Immunology, 9(2), 125-35.

Hogenkamp, A., Thijssen, S., van Vlies, N., Garssen, J., 2015. Supplementing Pregnant Mice with a Specific Mixture of Nondigestible Oligosaccharides Reduces Symptoms of Allergic Asthma in Male Offspring. The Journal of Nutrition 145, pp.640–646.

Hogquist, K., 2001. Signal strength in thymic selection and lineage commitment. Current Opinion in Immunology, 13(2), pp.225-231.

Holbrook, J., Lara-Reyna, S., Jarosz-Griffiths, H. and McDermott, M., 2019. Tumour necrosis factor signalling in health and disease. F1000Research, 8, p.111.

Holgate, S., 2010. A Brief History of Asthma and Its Mechanisms to Modern Concepts of Disease Pathogenesis. Allergy, Asthma and Immunology Research, 2(3), p.165.

Holscher, H., 2017. Dietary fiber and prebiotics and the gastrointestinal microbiota. Gut Microbes, 8(2), pp.172-184.

Holtug, K., Rasmussen, H. and Mortensen, P., 1992. An in vitro study of short-chain fatty acid concentrations, production and absorption in pig (Sus scrofa) colon. Comparative Biochemistry and Physiology Part A: Physiology, 103(1), pp.189-197.

Hooks, K. and O'Malley, M., 2017. Dysbiosis and Its Discontents. American Scoiety for Microbiology, 8(5).

House, R., 1999. Theory and Practice of Cytokine Assessment in Immunotoxicology. Methods, 19(1), pp.17-27.

Huang, C., Wang, J., Zheng, X., Chen, Y., Zhou, R., Wei, H., Sun, R. and Tian, Z., 2018. Commensal bacteria aggravate allergic asthma via NLRP3/IL-1β signaling in post-weaning mice. Journal of Autoimmunity, 93, pp.104-113.

Huang, R., Zhao, S., Li, Y., Liu, F., Gong, Y., Xing, J. and Xu, Z., 2020. Association of tumor necrosis factor-α gene polymorphisms and coronary artery disease susceptibility: a systematic review and meta-analysis. BMC Medical Genetics, 21(1).

Huang, Y. and Boushey, H., 2013. The Bronchial Microbiome and Asthma Phenotypes. American Journal of Respiratory and Critical Care Medicine, 188(10), pp.1178-1180.

Huang, Y., 2013. Asthma Microbiome Studies and the Potential for New Therapeutic Strategies. Current Allergy and Asthma Reports, 13(5), pp.453-461.

Hunter, P., 2012. The inflammation theory of disease: The growing realization that chronic inflammation is crucial in many diseases opens new avenues for treatment. EMBO Reports, 13(11), pp.968–970.

Islam S. U., 2016. Clinical Uses of Probiotics. Medicine, 95(5), e2658.

Ivakhnenko, O.S., Nyankovskyy, S.L., 2013. Effect of the specific infant formula mixture of oligosaccharides on local immunity and development of allergic and infectious disease in young children: randomized study. Pediatria Polska 88, pp.398–404.

Iyer, S. S., & Cheng, G., 2012. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Critical reviews in immunology, 32(1), pp.23-63.

Jatakanon, a., Uasuf, c., Maziak, w.,Llim, s., Chung, k. and Barnes, p., 1999. Neutrophilic Inflammation in Severe Persistent Asthma. American Journal of Respiratory and Critical Care Medicine, 160(5), pp.1532-1539.

Jeebhay M.F., Quirce S. 2007. Occupational asthma in the developing and industrialised world: a review. Int. J. Tuberc. Lung Dis. 11, pp.122–133.

Jiang, Z., & Zhu, L., 2016. Update on the role of alternatively activated macrophages in asthma. Journal of Asthma and Allergy, 9, pp.101–107.

Johnson, J. R., Nishioka, M., Chakir, J., Risse, P. A., Almaghlouth, I., Bazarbashi, A. N., Plante, S., Martin, J. G., Eidelman, D., … Hamid, Q., 2013. IL-22 contributes to TGF-β1-mediated epithelial-mesenchymal transition in asthmatic bronchial epithelial cells. Respiratory research, 14(1), 118.

Johnstone, N., Milesi, C., Burn, O., van den Bogert, B., Nauta, A., Hart, K., Sowden, P., Burnet, P. and Cohen Kadosh, K., 2021. Anxiolytic effects of a galacto-oligosaccharides prebiotic in healthy females (18–25 years) with corresponding changes in gut bacterial composition. Scientific Reports, 11(1).

June, C. H., Ledbetter, J. A., Gillespie, M. M., Lindsten, T., & Thompson, C. B., 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. Molecular and cellular biology, 7(12), pp.4472-81.

June, C., Ledbetter, J., Gillespie, M., Lindsten, T. and Thompson, C., 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. Molecular and Cellular Biology, 7(12), pp.4472-4481.

Jung, M., Lee, J., Kwak, J. and Shin, E., 2019. Tumor Necrosis Factor and Regulatory T Cells. Yonsei Medical Journal, 60(2), p.126.

Kalliomäki, M., Antoine, J., Herz, U., Rijkers, G., Wells, J. and Mercenier, A., 2010. Guidance for Substantiating the Evidence for Beneficial Effects of Probiotics: Prevention and Management of Allergic Diseases by Probiotics. The Journal of Nutrition, 140(3), pp.713S-721S.

Kalluri, R., & Weinberg, R. A., 2009. The basics of epithelial-mesenchymal transition. The Journal of clinical investigation, 119(6), pp.1420–1428.

Kamath, A., 2005. Is the neutrophil the key effector cell in severe asthma?. Thorax, 60(7), pp.529-530.

Kang, L., Oh, E., Cho, C., Kwon, H., Lee, C., Jeon, J., Lee, H., Choi, S., Han, S., Nam, J., Song, C., Jung, H., Kim, H., Park, E., Choi, E., Kim, J., Eyun, S. and Yang, S., 2020. 3′-Sialyllactose prebiotics prevents skin inflammation via regulatory T cell differentiation in atopic dermatitis mouse models. Scientific Reports, 10(1).

Kaplan M. H., 2013. Th9 cells: differentiation and disease. Immunological reviews, 252(1), pp.104-15.

Kaplan, A., Balter, M., Bell, A., Kim, H. and McIvor, R., 2009. Diagnosis of asthma in adults. Canadian Medical Association Journal, 181(10), pp.210-220.

Karimi, K., Inman, M.D., Bienenstock, J., Forsythe, P., 2009. Lactobacillus reuteri –induced Regulatory T cells Protect against an Allergic Airway Response in Mice. Am J Respir Crit Care Med 179, pp.186–193.

Kassem, K., Ali, M. and Rhaleb, N., 2019. Interleukin 4: Its Role in Hypertension, Atherosclerosis, Valvular, and Nonvalvular Cardiovascular Diseases. Journal of Cardiovascular Pharmacology and Therapeutics, 25(1), pp.7-14.

Kazanietz, M., Durando, M., & Cooke, M., 2019. CXCL13 and Its Receptor CXCR5 in Cancer: Inflammation, Immune Response, and Beyond. Frontiers In Endocrinology, 10.

Kearley, J., Buckland, K.F., Mathie, S.A., Lloyd, C.M., 2009. Resolution of allergic inflammation and AHR is dependent upon disruption of the T1/ST2-IL-33 pathway. Am. J. Respir. Crit. Care Med, 179, pp.772–781.

Kearney, J., 2010. Food consumption trends and drivers. Philosophical Transactions of the Royal Society B: Biological Sciences, 365(1554), pp.2793-2807.

Keeney, E., 1964. The history of asthma from Hippocrates to meltzer. Journal of Allergy, 35(3), pp.215-226.

Keim, N. and Martin, R., 2014. Dietary Whole Grain–Microbiota Interactions: Insights into Mechanisms for Human Health. Advances in Nutrition, 5(5), pp.556-557.

Kenneth, T., 2012. The Normal Bacterial Flora of Humans. Todar's Online Textbook of Bacteriology, 10, pp.1-5.

Kerstjens, H., Kauffman, H. and Postma, D., 1996. Corticosteroids and IgE. Journal of Allergy and Clinical Immunology, 97(1), pp.138.

Kerzerho, J., Maazi, H., Speak, A., Szely, N., Lombardi, V., & Khoo, B. et al., 2013. Programmed cell death ligand 2 regulates TH9 differentiation and induction of chronic airway hyperreactivity. Journal Of Allergy And Clinical Immunology, 131(4), pp.1048-1057.

Keshteli, A., Madsen, K. and Dieleman, L., 2019. Diet in the Pathogenesis and Management of Ulcerative Colitis; A Review of Randomized Controlled Dietary Interventions. Nutrients, 11(7), p.1498.

Key, T., Bradbury, K., Perez-Cornago, A., Sinha, R., Tsilidis, K. and Tsugane, S., 2020. Diet, nutrition, and cancer risk: what do we know and what is the way forward?. BMJ, p.m511.

Khansari, N., Shakiba, Y. and Mahmoudi, M., 2009. Chronic Inflammation and Oxidative Stress as a Major Cause of Age- Related Diseases and Cancer. Recent Patents on Inflammation & Allergy Drug Discovery, 3(1), pp.73-80.

Kim, C., 2009. FOXP3 and Its Role in the Immune System. Advances In Experimental Medicine And Biology, pp.17-29.

Kim, C., 2021. Control of lymphocyte functions by gut microbiota-derived short-chain fatty acids. Cellular & Molecular Immunology, 18(5), pp.1161-1171.

Kim, H., Hong, R., Choi, E., Yu, K., Kim, N., Hyeon, J., Cho, K., Choi, I. and Yun, C., 2018. A Probiotic Mixture Regulates T Cell Balance and Reduces Atopic Dermatitis Symptoms in Mice. Frontiers in Microbiology, 9.

Kim, M., Kang, S., Park, J., Yanagisawa, M. and Kim, C., 2013. Short-Chain Fatty Acids Activate GPR41 and GPR43 on Intestinal Epithelial Cells to Promote Inflammatory Responses in Mice. Gastroenterology, 145(2), pp.396-406.e10.

Kim, Y. and Ho, S., 2010. Intestinal Goblet Cells and Mucins in Health and Disease: Recent Insights and Progress. Current Gastroenterology Reports, 12(5), pp.319-330.

Kindermann, W., 2006. Inhaled 2 agonists and performance in competitive athletes. British Journal of Sports Medicine, 40(Supplement 1), pp.i43-i47.

Kips, J., 2001. Cytokines in asthma. European Respiratory Journal, 18(1), pp.24-33.

Kita H., 1996. The eosinophil: a cytokine-producing cell? J. Allergy Clin. Immunol. 97(1), pp.889– 92

Klein, L., Hinterberger, M., Wirnsberger, G. and Kyewski, B., 2009. Antigen presentation in the thymus for positive selection and central tolerance induction. Nature Reviews Immunology, 9(12), pp.833-844.

Koch, W., Kastrati, A., Böttiger, C., Mehilli, J., von Beckerath, N. and Schömig, A., 2001. Interleukin-10 and tumor necrosis factor gene polymorphisms and risk of coronary artery disease and myocardial infarction. Atherosclerosis, 159(1), pp.137-144.

Koenig, J.E., Spor, A., Scalfone, N., Fricker, A.D., Stombaugh, J., Knight, R., Angenent, L.T., Ley, R.E., 2011. Succession of microbial consortia in the developing infant gut microbiome. Proceedings of the National Academy of Sciences 108, pp.4578–4585.

Koeppen, M., McNamee, E. N., Brodsky, K. S., Aherne, C. M., Faigle, M., Downey, G. P., Colgan, S. P., Evans, C. M., Schwartz, D. A., … Eltzschig, H. K., 2012. Detrimental role of the airway mucin Muc5ac during ventilator-induced lung injury. Mucosal immunology, 6(4), pp.762-75.

Koh, A., De Vadder, F., Kovatcheva-Datchary, P. and Bäckhed, F., 2016. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. Cell, 165(6), pp.1332-1345.

Kollias, G., Douni, E., Kassiotis, G. and Kontoyiannis, D., 1999. The function of tumour necrosis factor and receptors in models of multi-organ inflammation, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease. Annals of the Rheumatic Diseases, 58(Supplement 1), pp.i32-i39.

Konieczna, P., Groeger, D., Ziegler, M., Frei, R., Ferstl, R., & Shanahan, F. et al., 2011. Bifidobacterium infantis35624 administration induces Foxp3 T regulatory cells in human peripheral blood: potential role for myeloid and plasmacytoid dendritic cells. Gut, 61(3), pp.354-366.

Kool, M., van Loo, G., Waelput, W., De Prijck, S., Muskens, F., Sze, M., van Praet, J., Branco-Madeira, F., Janssens, S., Reizis, B., Elewaut, D., Beyaert, R., Hammad, H. and Lambrecht, B., 2011. The Ubiquitin-Editing Protein A20 Prevents Dendritic Cell Activation, Recognition of Apoptotic Cells, and Systemic Autoimmunity. Immunity, 35(1), pp.82-96.

Kovalenko, A., Chable-Bessia, C., Cantarella, G., Israël, A., Wallach, D. and Courtois, G., 2003. The tumour suppressor CYLD negatively regulates NF-κB signalling by deubiquitination. Nature, 424(6950), pp.801-805.

Krishnan JA, Lemanske RF, Canino GJ, et al., 2012. Asthma Outcomes: Asthma Symptoms. The Journal of allergy and clinical immunology, 129(3 0), pp.S124-S135.

Krstić, G., 2011. Asthma Prevalence Associated with Geographical Latitude and Regional Insolation in the United States of America and Australia. PLoS ONE, 6(4), e18492.

Krummel, M. F., Bartumeus, F., & Gérard, A., 2016. T cell migration, search strategies and mechanisms. Nature reviews. Immunology, 16(3), pp.193-201.

Kudo, M., Ishigatsubo, Y. and Aoki, I., 2013. Pathology of asthma. Frontiers in Microbiology, 4(263), pp.1-3.

Kuitunen, M., Kukkonen, K., Juntunen-Backman, K., Korpela, R., Poussa, T., Tuure, T., Haahtela, T., Savilahti, E., 2009. Probiotics prevent IgE-associated allergy until age 5 years in cesarean-delivered children but not in the total cohort. Journal of Allergy and Clinical Immunology 123, pp.335–341.

Kukkonen, A.K., Kuitunen, M., Savilahti, E., Pelkonen, A., Malmberg, P., Mäkelä, M., 2011. Airway inflammation in probiotic-treated children at 5 years: FENO in probiotic-treated children. Pediatric Allergy and Immunology 22, pp.249–251.

Kukkonen, K., Savilahti, E., Haahtela, T., Juntunen-Backman, K., Korpela, R., Poussa, T., Tuure, T., Kuitunen, M., 2007. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: A randomized, double-blind, placebo-controlled trial. Journal of Allergy and Clinical Immunology 119, pp.192–198.

Kwon, H., Lee, C., So, J., Chae, C., Hwang, J., Sahoo, A., Nam, J., Rhee, J., Hwang, K. and Im, S., 2010. Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proceedings of the National Academy of Sciences, 107(5), pp.2159-2164.

La Grutta, S., Gagliardo, R., Mirabella, F., Pajno, G., Bonsignore, G., Bousquet, J., Bellia, V. and Vignola, A., 2003. Clinical and Biological Heterogeneity in Children with Moderate Asthma. American Journal of Respiratory and Critical Care Medicine, 167(11), pp.1490-1495.

Lad, N., Murphy, A., Parenti, C., Nelson, C., Williams, N., Sharpe, G. and McTernan, P., 2021. Asthma and obesity: endotoxin another insult to add to injury?. Clinical Science, 135(24), pp.2729-2748.

Lamas, M., Mulroney, C., Schleimer, R., 1988. Studies of the adhesive interaction between purified human eosinophils and cultured vascular endothelial cells. J Immunol, 140, pp.1500–1510.

Lebeer, S., Claes, I., Tytgat, H., Verhoeven, T., Marien, E., von Ossowski, I., Reunanen, J., Palva, A., de Vos, W., De Keersmaecker, S. and Vanderleyden, J., 2011. Functional Analysis of Lactobacillus rhamnosus GG Pili in Relation to Adhesion and Immunomodulatory Interactions with Intestinal Epithelial Cells. Applied and Environmental Microbiology, 78(1), pp.185-193.

Lee, J. and McDonald, C., 2018. Review: Immunotherapy improves some symptoms and reduces long-term medication use in mild to moderate asthma. Annals of Internal Medicine, 169(4), p.JC17.

Lei, Y. and Takahama, Y., 2012. XCL1 and XCR1 in the immune system. Microbes and Infection, 14(3), pp.262-267.

Leiria, L., Martins, M., & Saad, M., 2015. Obesity and asthma: beyond TH2 inflammation. Metabolism, 64(2), pp.172-181.

Leonard, C., Tormey, V., Burke, C. and Poulter, L., 1997. Allergen-induced Cytokine Production in Atopic Disease and Its Relationship to Disease Severity. American Journal of Respiratory Cell and Molecular Biology, 17(3), pp.368-375.

Lewkowich, I., Herman, N., Schleifer, K., Dance, M., Chen, B., Dienger, K., Sproles, A., Shah, J., Köhl, J., Belkaid, Y. and Wills-Karp, M., 2005. CD4+CD25+T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. The Journal of Experimental Medicine, 202(11), pp.1549-1561.

Li, M., van Esch, B., Wagenaar, G., Garssen, J., Folkerts, G. and Henricks, P., 2018. Pro- and anti-inflammatory effects of short chain fatty acids on immune and endothelial cells. European Journal of Pharmacology, 831, pp.52-59.

Li, X., & Zheng, Y., 2015. Regulatory T cell identity: formation and maintenance. Trends in immunology, 36(6), pp.344-53.

Licciardi, P., Ververis, K. and Karagiannis, T., 2011. Histone Deacetylase Inhibition and Dietary Short-Chain Fatty Acids. ISRN Allergy, 2011, pp.1-8.

Lieberman, J., 2003. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nature Reviews Immunology, 3(5), pp.361-370.

Lin, H., Frassetto, A., Kowalik Jr, E., Nawrocki, A., Lu, M., Kosinski, J., Hubert, J., Szeto, D., Yao, X., Forrest, G. and Marsh, D., 2012. Butyrate and Propionate Protect against Diet-Induced Obesity and Regulate Gut Hormones via Free Fatty Acid Receptor 3-Independent Mechanisms. PLoS ONE, 7(4), e35240.

Liu, F., Li, P., Chen, M., Luo, Y., Prabhakar, M., Zheng, H., He, Y., Qi, Q., Long, H., Zhang, Y., Sheng, H. and Zhou, H., 2017. Fructooligosaccharide (FOS) and Galactooligosaccharide (GOS) Increase Bifidobacterium but Reduce Butyrate Producing Bacteria with Adverse Glycemic Metabolism in healthy young population. Scientific Reports, 7(1).

Liu, T., Li, J., Liu, Y., Xiao, N., Suo, H., Xie, K., Yang, C. and Wu, C., 2012. Short-Chain Fatty Acids Suppress Lipopolysaccharide-Induced Production of Nitric Oxide and Proinflammatory Cytokines Through Inhibition of NF-κB Pathway in RAW264.7 Cells. Inflammation, 35(5), pp.1676-1684.

Lloyd, Clare M and Edith M Hessel., 2010 “Functions of T cells in asthma: more than just T(H)2 cells” Nature reviews. Immunology vol. 10(12), pp.838-48.

Locksley, R., 2010. Asthma and Allergic Inflammation. Cell, 140(6), pp.777-783.

Lotze, M. and Tracey, K., 2005. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nature Reviews Immunology, 5(4), pp.331-342.

Lu, L., Barbi, J., & Pan, F., 2017. The regulation of immune tolerance by FOXP3. Nature Reviews Immunology, 17(11), pp.703-717.

Lu, P., Li, L., Kuno, K., Wu, Y., Baba, T., Li, Y., Zhang, X. and Mukaida, N., 2008. Protective Roles of the Fractalkine/CX3CL1-CX3CR1 Interactions in Alkali-Induced Corneal Neovascularization through Enhanced Antiangiogenic Factor Expression. The Journal of Immunology, 180(6), pp.4283-4291.

Ma, X., Sun, Z., Zhai, P., Yu, W., Wang, T., Li, F., & Ding, J., 2017. Effect of follicular helper T cells on the pathogenesis of asthma. Experimental and therapeutic medicine, 14(2), pp.967-972.

Ma, Y., Hébert, J., Li, W., Bertone-Johnson, E., Olendzki, B., Pagoto, S., Tinker, L., Rosal, M., Ockene, I., Ockene, J., Griffith, J. and Liu, S., 2008. Association between dietary fiber and markers of systemic inflammation in the Women's Health Initiative Observational Study. Nutrition, 24(10), pp.941-949.

Macfarlane, G.T., Steed, H., Macfarlane, S., 2007. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J Appl Microbiol 0.

Macfarlane, S., Macfarlane, G. and Cummings, J., 2006. Review article: prebiotics in the gastrointestinal tract. Alimentary Pharmacology and Therapeutics, 24(5), pp.701-714.

Macpherson, A. and Harris, N., 2004. Interactions between commensal intestinal bacteria and the immune system. Nature Reviews Immunology, 4(6), pp.478-485.

Madan, J.C., Koestler, D.C., Stanton, B.A., Davidson, L., Moulton, L.A., Housman, M.L., Moore, J.H., Guill, M.F., Morrison, H.G., Sogin, M.L., Hampton, T.H., Karagas, M.R., Palumbo, P.E., Foster, J.A., Hibberd, P.L., O'Toole, G.A., 2012. Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: interaction between intestinal and respiratory tracts and impact of nutritional exposures. mBio 3.

Mahmutovic Persson, I., Menzel, M., Ramu, S., Cerps, S., Akbarshahi, H. and Uller, L., 2018. IL-1β mediates lung neutrophilia and IL-33 expression in a mouse model of viral-induced asthma exacerbation. Respiratory Research, 19(1).

Mao, X., Kawai, M., Yamashita, T., Enomoto, T., Dake, Y., Sasaki, S., Kataoka, Y., Fukuzumi, T., Endo, K., Sano, H., Aoki, T., Kurimoto, F., Adra, C., Shirakawa, T. and Hopkin, J., 2000. Imbalance Production between Interleukin-1β (IL-1β) and IL-1 Receptor Antagonist (IL-1Ra) in Bronchial Asthma. Biochemical and Biophysical Research Communications, 276(2), pp.607-612.

Marketos S, Ballas C., 1982. Bronchial asthma in medical literature of Greek antiquity. Hist Sci Med 17, pp.35–39

Martín-Orozco, E., Norte-Muñoz, M., & Martínez-García, J., 2017. Regulatory T Cells in Allergy and Asthma. Frontiers in pediatrics, 5, p.117.

Maslowski, K., Vieira, A., Ng, A., Kranich, J., Sierro, F., Di Yu, Schilter, H., Rolph, M., Mackay, F., Artis, D., Xavier, R., Teixeira, M. and Mackay, C., 2009. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature, 461(7268), pp.1282-1286.

Masoli, M., Fabian, D., Holt, S. and Beasley, R., 2004. The global burden of asthma: executive summary of the GINA Dissemination Committee Report. Allergy, 59(5), pp.469-478.

Mathieu, E., Escribano-Vazquez, U., Descamps, D., Cherbuy, C., Langella, P., Riffault, S., Remot, A. and Thomas, M., 2018. Paradigms of Lung Microbiota Functions in Health and Disease, Particularly, in Asthma. Frontiers in Physiology, pp.9.

Mayadas, T. N., Cullere, X., & Lowell, C. A., 2013. The multifaceted functions of neutrophils. Annual review of pathology, 9, pp.181-218.

Mazzini, E., Massimiliano, L., Penna, G. and Rescigno, M., 2014. Oral Tolerance Can Be Established via Gap Junction Transfer of Fed Antigens from CX3CR1+ Macrophages to CD103+ Dendritic Cells. Immunity, 40(2), pp.248-261.

McCracken, J., Veeranki, S., Ameredes, B. and Calhoun, W., 2017. Diagnosis and Management of Asthma in Adults. JAMA, 318(3), p.279.

McKenzie, C., Tan, J., Macia, L. and Mackay, C., 2017. The nutrition-gut microbiome-physiology axis and allergic diseases. Immunological Reviews, 278(1), pp.277-295.

McKinley, L., Alcorn, J., Peterson, A., DuPont, R., Kapadia, S., & Logar, A. et al., 2008. TH17 Cells Mediate Steroid-Resistant Airway Inflammation and Airway Hyperresponsiveness in Mice. The Journal Of Immunology, 181(6), pp.4089-4097.

McLoughlin, R., Berthon, B., Jensen, M., Baines, K. and Wood, L., 2017. Short-chain fatty acids, prebiotics, synbiotics, and systemic inflammation: a systematic review and meta-analysis. The American Journal of Clinical Nutrition, ajcn156265.

McLoughlin, R., Berthon, B.S., Rogers, G.B., Baines, K.J., Leong, L.E.X., Gibson, P.G., Williams, E.J., Wood, L.G., 2019. Soluble fibre supplementation with and without a probiotic in adults with asthma: A 7-day randomised, double blind, three way cross-over trial. EBioMedicine 46, pp.473–485.

Meng, H., Ba, Z., Lee, Y., Peng, J., Lin, J., Fleming, J., Furumoto, E., Roberts, R., Kris-Etherton, P. and Rogers, C., 2015. Consumption of Bifidobacterium animalis subsp. lactis BB-12 in yogurt reduced expression of TLR-2 on peripheral blood-derived monocytes and pro-inflammatory cytokine secretion in young adults. European Journal of Nutrition, 56(2), pp.649-661.

Metsälä J., Lundqvist A., Virta L. J., Kaila M., Gissler M., Virtanen S. M., 2015. Prenatal and post-natal exposure to antibiotics and risk of asthma in childhood. Clinical Experiments in Allergy 45, pp.137–145.

Miller, M. C., & Mayo, K. H., 2017. Chemokines from a Structural Perspective. International journal of molecular sciences, 18(10), pp.2088

Miller, M., 2005. Standardisation of spirometry. European Respiratory Journal, 26(2), pp.319-338.

Mills, CD., 2012. "M1 and M2 Macrophages: Oracles of Health and Disease". Critical Reviews in Immunology. 32 (6), pp.463–88.

Minihane, A., Vinoy, S., Russell, W., Baka, A., Roche, H., Tuohy, K., Teeling, J., Blaak, E., Fenech, M., Vauzour, D., McArdle, H., Kremer, B., Sterkman, L., Vafeiadou, K., Benedetti, M., Williams, C. and Calder, P., 2015. Low-grade inflammation, diet composition and health: current research evidence and its translation. British Journal of Nutrition, 114(7), pp.999-1012.

Mirzakhani, H., Al-Garawi, A., Weiss, S. and Litonjua, A., 2014. Vitamin D and the development of allergic disease: how important is it?. Clinical & Experimental Allergy, 45(1), pp.114-125.

Mogensen, T., 2009. Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. Clinical Microbiology Reviews, 22(2), pp.240-273.

Moldoveanu, B., Otmishi, P., Jani, P., Walker, J., Sarmiento, X., Guardiola, J., … Yu, J., 2009. Inflammatory mechanisms in the lung. Journal of Inflammation Research, 2, 1–11.

Molodecky, N., Soon, I., Rabi, D., Ghali, W., Ferris, M., & Chernoff, G. et al., 2012. Increasing Incidence and Prevalence of the Inflammatory Bowel Diseases With Time, Based on Systematic Review. Gastroenterology, 142(1), pp.46-54.

Momen, T., Ahanchian, H., Reisi, M., Shamsdin, S. A., Shahsanai, A., & Keivanfar, M., 2017. Comparison of Interleukin-33 Serum Levels in Asthmatic Patients with a Control Group and Relation with the Severity of the Disease. International journal of preventive medicine, 8, p.65.

Montuschi, P., 2010. Role of Leukotrienes and Leukotriene Modifiers in Asthma. Pharmaceuticals, 3(6), pp.1792-1811.

Moore, W. C., Hastie, A. T., Li, X., Li, H., Busse, W. W., Jarjour, N. N., Wenzel, S. E., Peters, S. P., Meyers, D. A., Bleecker, E. R., National Heart, Lung, and Blood Institute's Severe Asthma Research Program (2013. Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. The Journal of allergy and clinical immunology, 133(6), 1557-63.

Moore, W., Meyers, D., Wenzel, S., Teague, W., Li, H., Li, X., D'Agostino, R., Castro, M., Curran-Everett, D., Fitzpatrick, A., Gaston, B., Jarjour, N., Sorkness, R., Calhoun, W., Chung, K., Comhair, S., Dweik, R., Israel, E., Peters, S., Busse, W., Erzurum, S. and Bleecker, E., 2010. Identification of Asthma Phenotypes Using Cluster Analysis in the Severe Asthma Research Program. American Journal of Respiratory and Critical Care Medicine, 181(4), pp.315-323.

Moorman, JE., Rudd, RA., Johnson, CA., et al., 2007. National Surveillance for Asthma - United States, 1980–2004. Morb Mortal Wkly Rep Surveill Summ, 56(8), pp.18–54.

Morikawa, H., Ohkura, N., Vandenbon, A., Itoh, M., Nagao-Sato, S., Kawaji, H., Lassmann, T., Carninci, P., Hayashizaki, Y., Forrest, A. R., Standley, D. M., Date, H., Sakaguchi, S., 2014. Differential roles of epigenetic changes and Foxp3 expression in regulatory T cell-specific transcriptional regulation. Proceedings of the National Academy of Sciences of the United States of America, 111(14), pp.5289-5294.

Moro, G., 2006. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Archives of Disease in Childhood 91, pp.814–819.

Moroz, A., Eppolito, C., Li, Q., Tao, J., Clegg, C. and Shrikant, P., 2004. IL-21 Enhances and Sustains CD8+ T Cell Responses to Achieve Durable Tumor Immunity: Comparative Evaluation of IL-2, IL-15, and IL-21. The Journal of Immunology, 173(2), pp.900-909.

Moser, B., & Willimann, K., 2004. Chemokines: role in inflammation and immune surveillance. Annals of the rheumatic diseases, 63(2), pp.84-89

Mottamal, M., Zheng, S., Huang, T. and Wang, G., 2015. Histone Deacetylase Inhibitors in Clinical Studies as Templates for New Anticancer Agents. Molecules, 20(3), pp.3898-3941.

Mowat, A. and Agace, W., 2014. Regional specialization within the intestinal immune system. Nature Reviews Immunology, 14(10), pp.667-685.

Murdoch, J. and Lloyd, C., 2010. Chronic inflammation and asthma. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 690(1-2), pp.24-39.

Murray JJ, Tonnel AB, Brash AR, Roberts LJ, 2nd, Gosset P, Workman R., 1985. Prostaglandin D2 is released during acute allergic bronchospasm in man. Trans Assoc Am Physiciansm, 98, pp.275–280.

Nagar, M., Jacob-Hirsch, J., Vernitsky, H., Berkun, Y., Ben-Horin, S., Amariglio, N., Bank, I., Kloog, Y., Rechavi, G. and Goldstein, I., 2010. TNF Activates a NF-κB–Regulated Cellular Program in Human CD45RA– Regulatory T Cells that Modulates Their Suppressive Function. The Journal of Immunology, 184(7), pp.3570-3581.

Nagayama, Y., McLachlan, S., Rapoport, B. and Oishi, K., 2004. Graves' Hyperthyroidism and the Hygiene Hypothesis in a Mouse Model. Endocrinology, 145(11), pp.5075-5079.

Nagel G, Weinmayr G, Kleiner A (2010. Effect of diet on asthma and allergic sensitisation in the International Study on Allergies and Asthma in Childhood (ISAAC. Phase Two Thorax, 5(65), pp.16-522.

Nakamura, Y., Ghaffar, O., Olivenstein, R., Taha, R., Soussi-Gounni, A., Zhang, D., Ray, A. and Hamid, Q., 1999. Gene expression of the GATA-3 transcription factor is increased in atopic asthma. Journal of Allergy and Clinical Immunology, 103(2), pp.215-222.

Nakanishi, K., Yoshimoto, T., Tsutsui, H. and Okamura, H., 2001. INTERLEUKIN-18 REGULATES BOTH TH1 AND TH2 RESPONSES. Annual Review of Immunology, 19(1), pp.423-474.

National Asthma Education and Prevention Program (NAEPP), Third Expert Panel on the Diagnosis and Management of Asthma. Expert Panel Report 3: Guidelines for the Diagnosis and Management of Asthma. Bethesda (MD): National Heart, Lung, and Blood Institute (US); 2007

Netuveli, G., Hurwitz, B., Levy, M., Fletcher, M., Barnes, G., Durham, S. and Sheikh, A., 2005. Ethnic variations in UK asthma frequency, morbidity, and health-service use: a systematic review and meta-analysis. The Lancet, 365(9456), pp.312-317.

Newton, K. and Dixit, V., 2012. Signaling in Innate Immunity and Inflammation. Cold Spring Harbor Perspectives in Biology, 4(3), a006049.

Niele, N., van Zwol, A., Westerbeek, E.A., Lafeber, H.N., van Elburg, R.M., 2013. Effect of non-human neutral and acidic oligosaccharides on allergic and infectious diseases in preterm infants. Eur J Pediatr 172, pp.317–323.

Nochi, T. and Kiyono, H., 2006. Innate Immunity in the Mucosal Immune System. Current Pharmaceutical Design, 12(32), pp.4203-4213.

Noris, M., & Remuzzi, G., 2013. Overview of complement activation and regulation. Seminars in nephrology, 33(6), pp.479-492.

Noverr, M. and Huffnagle, G., 2004. Regulation of Candida albicans Morphogenesis by Fatty Acid Metabolites. Infection and Immunity, 72(11), pp.6206-6210.

Nunes, C., Pereira, A. M., & Morais-Almeida, M., 2017. Asthma costs and social impact. Asthma research and practice, 3, 1.

Oeckinghaus, A., & Ghosh, S., 2009. The NF-kappaB family of transcription factors and its regulation. Cold Spring Harbor perspectives in biology, 1(4), a000034.

Oettgen, H. and Geha, R., 2001. IgE regulation and roles in asthma pathogenesis. Journal of Allergy and Clinical Immunology, 107(3), pp.429-441.

Okada, H., Kuhn, C., Feillet, H. and Bach, J., 2010. The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. Clinical & Experimental Immunology, 160(1), pp.1-9.

Onizawa, M., Oshima, S., Schulze-Topphoff, U., Oses-Prieto, J., Lu, T., Tavares, R., Prodhomme, T., Duong, B., Whang, M., Advincula, R., Agelidis, A., Barrera, J., Wu, H., Burlingame, A., Malynn, B., Zamvil, S. and Ma, A., 2015. The ubiquitin-modifying enzyme A20 restricts ubiquitination of the kinase RIPK3 and protects cells from necroptosis. Nature Immunology, 16(6), pp.618-627.

Osborn, D.A., Sinn, J.K., 2007. Probiotics in infants for prevention of allergic disease and food hypersensitivity. Cochrane Database of Systematic Reviews.

O'Shea, J., Lahesmaa, R., Vahedi, G., Laurence, A., & Kanno, Y., 2011. Genomic views of STAT function in CD4+ T helper cell differentiation. Nature Reviews Immunology, 11(4), pp.239-250.

Pahwa R, Goyal A, Bansal P, et al., 2022. Chronic Inflammation. Treasure Island (FL): StatPearls. Available from: https://www.ncbi.nlm.nih.gov/books/NBK493173/

Pandey, K., Naik, S. and Vakil, B., 2015. Probiotics, prebiotics and synbiotics- a review. Journal of Food Science and Technology, 52(12), pp.7577-7587.

Parada Venegas, D., De la Fuente, M.K., Landskron, G., González, M.J., Quera, R., Dijkstra, G., Harmsen, H.J.M., Faber, K.N., Hermoso, M.A., 2019. Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. Front. Immunol. 10, p.277.

Park, J., Kim, M., Kang, S., Jannasch, A., Cooper, B., Patterson, J. and Kim, C., 2014. Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR–S6K pathway. Mucosal Immunology, 8(1), pp.80-93.

Park, Y. M., & Bochner, B. S., 2010. Eosinophil survival and apoptosis in health and disease. Allergy, asthma & immunology research, 2(2), pp.87-101.

Parkin, J. and Cohen, B., 2001. An overview of the immune system. The Lancet, 357(9270), pp.1777-1789.

Parvin, S., 2015. Nutritional Analysis of Date Fruits (Phoenix dactylifera L.) in Perspective of Bangladesh. American Journal of Life Sciences, 3(4), p.274.

Paul, W. E., & Zhu, J., 2010. How are T(H)2-type immune responses initiated and amplified?. Nature reviews. Immunology, 10(4), pp.225-35.

Pelaia, G., Vatrella, A., Busceti, M., Gallelli, L., Calabrese, C., Terracciano, R. and Maselli, R., 2015. Cellular Mechanisms Underlying Eosinophilic and Neutrophilic Airway Inflammation in Asthma. Mediators of Inflammation, 2015, pp.1-8.

Pelletier, M., Maggi, L., Micheletti, A., Lazzeri, E., Tamassia, N., Costantini, C., Cosmi, L., Lunardi, C., Annunziato, F., Romagnani, S. and Cassatella, M., 2009. Evidence for a cross-talk between human neutrophils and Th17 cells. Blood, 115(2), pp.335-343.

Pembrey, L., Barreto, M., Douwes, J., Cooper, P., Henderson, J., Mpairwe, H., Ardura-Garcia, C., Chico, M., Brooks, C., Cruz, A., Elliott, A., Figueiredo, C., Langan, S., Nassanga, B., Ring, S., Rodrigues, L. and Pearce, N., 2018. Understanding asthma phenotypes: the World Asthma Phenotypes (WASP) international collaboration. ERJ Open Research, 4(3), pp.00013-2018.

Pennino, D., Bhavsar, P., Effner, R., Avitabile, S., Venn, P., Quaranta, M., Marzaioli, V., Cifuentes, L., Durham, S., Cavani, A., Eyerich, K., Chung, K., Schmidt-Weber, C. and Eyerich, S., 2013. IL-22 suppresses IFN-γ–mediated lung inflammation in asthmatic patients. Journal of Allergy and Clinical Immunology, 131(2), pp.562-570.

Pilewski, J., Albelda, S., 1995. Cell adhesion molecules in asthma: homing activation and airway remodelling. Am J Respir Cell Mol Biol, 12(1), pp.1–3.

Prathap, P., Corrigan, C.J., Ying, S., 2011. Airway Epithelium in Atopic and Nonatopic Asthma: Similarities and Differences. ISRN Allergy, PMC 195846.

Piotr, G., Madaliński, K., Grek, M., Dmenska, H., Syczewska, M., Michałkiewicz, J., 2010. Clinical immunology Effect of orally administered probiotic strains Lactobacillus and Bifidobacterium in children with atopic asthma. Centr Eur J Immunol 35, pp.233–238.

Place, R., Noonan, E. and Giardina, C., 2005. HDAC inhibition prevents NF-κB activation by suppressing proteasome activity: Down-regulation of proteasome subunit expression stabilizes IκBα. Biochemical Pharmacology, 70(3), pp.394-406.

Pluznick, J., 2017. Microbial Short-Chain Fatty Acids and Blood Pressure Regulation. Current Hypertension Reports, 19(4).

Pober, J. S., & Sessa, W. C., 2015. Inflammation and the Blood Microvascular System. Cold Spring Harbor Perspectives in Biology, 7(1), a016345.

Popa, C., Netea, M., van Riel, P., van der Meer, J. and Stalenhoef, A., 2007. The role of TNF-α in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. Journal of Lipid Research, 48(4), pp.751-762.

Pope, S., Brandt, E., Mishra, A., Hogan, S., Zimmermann, N., Matthaei, K., Foster, P. and Rothenberg, M., 2001. IL-13 induces eosinophil recruitment into the lung by an IL-5– and eotaxin-dependent mechanism. Journal of Allergy and Clinical Immunology, 108(4), pp.594-601.

Possa, S. S., Leick, E. A., Prado, C. M., Martins, M. A., & Tibério, I. F., 2013. Eosinophilic inflammation in allergic asthma. Frontiers in pharmacology, 4, pp.46.

Poulsen, L. and Hummelshoj, L., 2007. Triggers of IgE class switching and allergy development. Annals of Medicine, 39(6), pp.440-456.

Poulter LW, Burke CM., 1996. Macrophages and allergic lung disease. Immunobiology, 195, pp574–587.

Provoost, S., Maes, T., Van Durme, Y., Gevaert, P., Bachert, C., Schmidt-Weber, C., Brusselle, G., Joos, G. and Tournoy, K., 2009. Decreased FOXP3 protein expression in patients with asthma. Allergy, 64(10), pp.1539-1546.

Punnonen, J., Carballido, J., Aversa, G., & de Vries, J., 1998. Interleukin 13 and its Receptor. Encyclopedia Of Immunology, pp.1489-1492.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W., Guan, Y., Shen, D., Peng, Y., Zhang, D., Jie, Z., Wu, W., Qin, Y., Xue, W., Li, J., Han, L., Lu, D., Wu, P., Dai, Y., Sun, X., Li, Z., Tang, A., Zhong, S., Li, X., Chen, W., Xu, R., Wang, M., Feng, Q., Gong, M., Yu, J., Zhang, Y., Zhang, M., Hansen, T., Sanchez, G., Raes, J., Falony, G., Okuda, S., Almeida, M., LeChatelier, E., Renault, P., Pons, N., Batto, J., Zhang, Z., Chen, H., Yang, R., Zheng, W., Li, S., Yang, H., Wang, J., Ehrlich, S., Nielsen, R., Pedersen, O., Kristiansen, K. and Wang, J., 2012. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature, 490(7418), pp.55-60.

Quigley E. M., 2013. Gut bacteria in health and disease. Gastroenterology & hepatology, 9(9), 560-9.

Rajilić-Stojanović, M., & de Vos, W. M., 2014. The first 1000 cultured species of the human gastrointestinal microbiota. FEMS microbiology reviews, 38(5), pp.996-1047.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S. and Medzhitov, R., 2004. Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis. Cell, 118(2), pp.229-241.

Ramsey, C. and Celedon, J., 2005. The hygiene hypothesis and asthma. Current Opinion in Pulmonary Medicine, 11(1), pp.14-20.

Rautava, S., Ruuskanen, O., Ouwehand, A., Salminen, S. and Isolauri, E., 2004. The Hygiene Hypothesis of Atopic Disease–An Extended Version. Journal of Pediatric Gastroenterology and Nutrition, 38(4), pp.378-388.

Ray, A., & Cohn, L., 1999. Th2 cells and GATA-3 in asthma: new insights into the regulation of airway inflammation. The Journal of clinical investigation, 104(8), pp.985-93.

Reddel, H.K., Taylor, D.R., Bateman, E.D., Boulet, L.-P., Boushey, H.A., Busse, W.W., Casale, T.B., Chanez, P., Enright, P.L., Gibson, P.G., de Jongste, J.C., Kerstjens, H.A.M., Lazarus, S.C., Levy, M.L., O'Byrne, P.M., Partridge, M.R., Pavord, I.D., Sears, M.R., Sterk, P.J., Stoloff, S.W., Sullivan, S.D., Szefler, S.J., Thomas, M.D., Wenzel, S.E., American Thoracic Society/European Respiratory Society Task Force on Asthma Control and Exacerbations, 2009. An official American Thoracic Society/European Respiratory Society statement: asthma control and exacerbations: standardizing endpoints for clinical asthma trials and clinical practice. Am. J. Respir. Crit. Care Med. 180, pp.59–99.

Reuter, S., Stassen, M., & Taube, C., 2010. Mast Cells in Allergic Asthma and Beyond. Yonsei Medical Journal, 51(6), pp.797–807.

Ricciotti, E., & FitzGerald, G. A., 2011. Prostaglandins and Inflammation. Arteriosclerosis, Thrombosis, and Vascular Biology, 31(5), pp.986–1000.

Roberfroid, M., Gibson, G.R., Hoyles, L., McCartney, A.L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam, V., Davicco, M.-J., Léotoing, L., Wittrant, Y., Delzenne, N.M., Cani, P.D., Neyrinck, A.M., Meheust, A., 2010. Prebiotic effects: metabolic and health benefits. Br. J. Nutr. 104 Suppl 2, pp.1-63.

Rodriguez, A., Vaca, M., Oviedo, G., Erazo, S., Chico, M., Teles, C., Barreto, M., Rodrigues, L. and Cooper, P., 2011. Urbanisation is associated with prevalence of childhood asthma in diverse, small rural communities in Ecuador. Thorax, 66(12), pp.1043-1050.

Romagnani, S., 2007. Th1/Th2 cells. Inflammatory Bowel Diseases, 5(4), pp.285-294.

Rothenberg, EV., Moore, JE., Yui MA. 2008. Launching the T Cell-lineage developmental programme. Nat Rev Immunol. 8, pp.9–21.

Rothenberg, M. and Hogan, S., 2006. THE EOSINOPHIL. Annual Review of Immunology, 24(1), pp.147-174.

Rudd, C. E., Taylor, A., & Schneider, H., 2009. CD28 and CTLA-4 coreceptor expression and signal transduction. Immunological reviews, 229(1), pp.12-26.

Rudensky, A., 2011. Regulatory T cells and Foxp3. Immunological Reviews, 241(1), pp.260-268.

Ruiz, L., Delgado, S., Ruas-Madiedo, P., Sánchez, B. and Margolles, A., 2017. Bifidobacteria and Their Molecular Communication with the Immune System. Frontiers in Microbiology, 8.

Rundell, K., Spiering, B., Baumann, J. and Evans, T., 2005. Bronchoconstriction Provoked by Exercise in a High-Particulate-Matter Environment is Attenuated by Montelukast. Inhalation Toxicology, 17(2), pp.99-105.

Russell S. L., Gold M. J., Willing B. P., Thorson L., Mcnagny K. M., Finlay B. B., 2013. Perinatal antibiotic treatment affects murine microbiota, immune responses and allergic asthma. Gut Microbes, 4, pp.158–164.

Saeed, M., Gribben, K., Alam, M., Lyden, E., Hanson, C. and LeVan, T., 2020. Association of Dietary Fiber on Asthma, Respiratory Symptoms, and Inflammation in the Adult National Health and Nutrition Examination Survey Population. Annals of the American Thoracic Society, 17(9), pp.1062-1068.

Sagar, S., Morgan, M.E., Chen, S., Vos, A.P., Garssen, J., van Bergenhenegouwen, J., Boon, L., Georgiou, N.A., Kraneveld, A.D., Folkerts, G., 2014. Bifidobacterium breve and Lactobacillus rhamnosus treatment is as effective as budesonide at reducing inflammation in a murine model for chronic asthma. Respir Res 15, 46.

Sakula, A., 1987. A history of asthma. The FitzPatrick lecture. J R Coll Physicians London. 22, pp.36–44.

Salo, P. M., Arbes, S. J., Crockett, P. W., Thorne, P. S., Cohn, R. D., & Zeldin, D. C., 2008. Exposure to multiple indoor allergens in US homes and relationship to asthma. The Journal of Allergy and Clinical Immunology, 121(3), pp.678–684

Salomon, B., Leclerc, M., Tosello, J., Ronin, E., Piaggio, E. and Cohen, J., 2018. Tumor Necrosis Factor α and Regulatory T Cells in Oncoimmunology. Frontiers in Immunology, 9.

Sandford, A., Chagani, T., Zhu, S., Weir, T., Bai, T., & Spinelli, J. et al., 2000. Polymorphisms in the IL4 , IL4RA , and FCERIB genes and asthma severity. Journal Of Allergy And Clinical Immunology, 106(1), pp.135-140.

Savignac, H., Couch, Y., Stratford, M., Bannerman, D., Tzortzis, G., Anthony, D. and Burnet, P., 2016. Prebiotic administration normalizes lipopolysaccharide (LPS)-induced anxiety and cortical 5-HT2A receptor and IL1-β levels in male mice. Brain, Behavior, and Immunity, 52, pp.120-131.

Schlitzer, A., McGovern, N., Teo, P., Zelante, T., Atarashi, K., Low, D., Ho, A., See, P., Shin, A., Wasan, P., Hoeffel, G., Malleret, B., Heiseke, A., Chew, S., Jardine, L., Purvis, H., Hilkens, C., Tam, J., Poidinger, M., Stanley, E., Krug, A., Renia, L., Sivasankar, B., Ng, L., Collin, M., Ricciardi-Castagnoli, P., Honda, K., Haniffa, M. and Ginhoux, F., 2013. IRF4 Transcription Factor-Dependent CD11b+ Dendritic Cells in Human and Mouse Control Mucosal IL-17 Cytokine Responses. Immunity, 38(5), pp.970-983.

Schuliga M., 2015. NF-kappaB Signaling in Chronic Inflammatory Airway Disease. Biomolecules, 5(3), pp.1266-83.

Schwarz, B. and Bhandoola, A., 2006. Trafficking from the bone marrow to the thymus: a prerequisite for thymopoiesis. Immunological Reviews, 209(1), pp.47-57.

Schweizer, R., van Kessel-Welmers, B., Warringa, R., Maikoe, T., Raaijmakers, J., Lammers, J. and Koenderman, L., 1996. Mechanisms involved in eosinophil migration. Platelet-activating factor-induced Chemotaxis and interleukin-5-induced chemokinesis are mediated by different signals. Journal of Leukocyte Biology, 59(3), pp.347-356.

Schwingshackl, L. and Hoffmann, G., 2014. Mediterranean dietary pattern, inflammation and endothelial function: A systematic review and meta-analysis of intervention trials. Nutrition, Metabolism and Cardiovascular Diseases, 24(9), pp.929-939.

Searing, D. and Leung, D., 2010. Vitamin D in Atopic Dermatitis, Asthma and Allergic Diseases. Immunology and Allergy Clinics of North America, 30(3), pp.397-409.

Sears, M., 2002. Adverse effects of β-agonists. Journal of Allergy and Clinical Immunology, 110(6), pp.S322-S328.

Seaton, A., Godden, D. and Brown, K., 1994. Increase in asthma: a more toxic environment or a more susceptible population?. Thorax, 49(2), pp.171-174.

Sender, R., Fuchs, S., & Milo, R., 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS biology, 14(8), e1002533.

Sherwood, E. and Toliver-Kinsky, T., 2004. Mechanisms of the inflammatory response. Best Practice & Research Clinical Anaesthesiology, 18(3), pp.385-405.

Shi, H., Kokoeva, M. V., Inouye, K., Tzameli, I., Yin, H., & Flier, J. S., 2006. TLR4 links innate immunity and fatty acid-induced insulin resistance. The Journal of clinical investigation, 116(11), pp.3015–3025.

SHI, H., XIAO, C., ZHONG, D., QIN, S., LIU, Y., & LIANG, G. et al., 1998. Effect of Inhaled Interleukin-5 on Airway Hyperreactivity and Eosinophilia in Asthmatics. American Journal Of Respiratory And Critical Care Medicine, 157(1), pp.204-209.

Shoelson, S., Herrero, L., & Naaz, A., 2007. Obesity, Inflammation, and Insulin Resistance. Gastroenterology, 132(6), pp.2169-2180.

Shreiner, A., Huffnagle, G. and Noverr, M., 2008. The "Microflora Hypothesis" of allergic disease. Advances in Experimental Medicine and Biology, 635, pp.113-134.

Siddiqui, S., Hollins, F., Saha, S., Brightling, C.E., 2007. Inflammatory cell microlocalisation and airway dysfunction: cause and effect? Eur. Respir. J. 30, pp.1043–1056

Siebenlist, U., Brown, K. and Claudio, E., 2005. Control of lymphocyte development by nuclear factor-κB. Nature Reviews Immunology, 5(6), pp.435-445.

Silva, Y., Bernardi, A. and Frozza, R., 2020. The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. Frontiers in Endocrinology, 11.

Simpson, C. and Sheikh, A., 2010. Trends in the epidemiology of asthma in England: a national study of 333,294 patients. Journal of the Royal Society of Medicine, 103(3), pp.98-106.

Singh, R., Chang, H., Yan, D., Lee, K., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T., Bhutani, T. and Liao, W., 2017. Influence of diet on the gut microbiome and implications for human health. Journal of Translational Medicine, 15(1).

Skadow, M., Penna, V., Galant-Swafford, J., Shevach, E., & Thornton, A., 2019. Helios Deficiency Predisposes the Differentiation of CD4+Foxp3− T Cells into Peripherally Derived Regulatory T Cells. The Journal Of Immunology, 203(2), pp.370-378.

Skapenko, A., Leipe, J., Lipsky, P., & Schulze-Koops, H., 2005. Arthritis Research & Therapy, 7(Suppl 2), p.4.

Slavin, J. L., & Lloyd, B., 2012. Health benefits of fruits and vegetables. Advances in nutrition (Bethesda, Md.), 3(4), pp.506–516.

Slavin, J., 2013. Fiber and prebiotics: mechanisms and health benefits. Nutrients 5, pp.1417–1435.

Smit, J., Rudd, B. and Lukacs, N., 2006. Plasmacytoid dendritic cells inhibit pulmonary immunopathology and promote clearance of respiratory syncytial virus. The Journal of Experimental Medicine, 203(5), pp.1153-1159.

Smith, K., McCoy, K. and Macpherson, A., 2007. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Seminars in Immunology, 19(2), pp.59-69.

Sokolowska, M., Frei, R., Lunjani, N., Akdis, C. A., & O'Mahony, L., 2018. Microbiome and asthma. Asthma research and practice, 4, 1.

Sonnenburg, E. and Sonnenburg, J., 2014. Starving our Microbial Self: The Deleterious Consequences of a Diet Deficient in Microbiota-Accessible Carbohydrates. Cell Metabolism, 20(5), pp.779-786.

Sonoyama, K., Watanabe, H., Watanabe, J., Yamaguchi, N., Yamashita, A., Hashimoto, H., Kishino, E., Fujita, K., Okada, M., Mori, S., Kitahata, S., Kawabata, J., 2005. Allergic Airway Eosinophilia Is Suppressed in Ovalbumin-Sensitized Brown Norway Rats Fed Raffinose and α-Linked Galactooligosaccharide. The Journal of Nutrition 135, pp.538–543.

Spencer, J., Dillon, S.B., Isaacson, P.G., MacDonald, T.T., 1986. T cell subclasses in fetal human ileum. Clin Exp Immunol 65, 553–558.

Spencer, L. A., & Weller, P. F., 2010. Eosinophils and Th2 immunity: contemporary insights. Immunology and cell biology, 88(3), pp.250–256.

Spiteri MA, Knight RA, Jeremy JY, Barnes PJ, Chung KF., 1994. Alveolar macrophage-induced suppression of peripheral blood mononuclear cell responsiveness is reversed by in vitro allergen exposure in bronchial asthma. Eur Respir J, 7, pp1431–1438.

Stagg, A., 2018. Intestinal Dendritic Cells in Health and Gut Inflammation. Frontiers in Immunology, 9.

Stamler, J., 2000. Relationship of Baseline Serum Cholesterol Levels in 3 Large Cohorts of Younger Men to Long-term Coronary, Cardiovascular, and All-Cause Mortality and to Longevity. JAMA, 284(3), pp.311.

Starr, T., Jameson, S. and Hogquist, K., 2003. POSITIVE ANDNEGATIVESELECTION OFT CELLS. Annual Review of Immunology, 21(1), pp.139-176.

Steinke, J. and Borish, L., 2001. Respiratory Research, 2(2), p.66.

Subbarao, P., Mandhane, P. and Sears, M., 2009. Asthma: epidemiology, etiology and risk factors. Canadian Medical Association Journal, 181(9), e181-190.

Suez, J., Korem, T., Zeevi, D., Zilberman-Schapira, G., Thaiss, C., Maza, O., Israeli, D., Zmora, N., Gilad, S., Weinberger, A., Kuperman, Y., Harmelin, A., Kolodkin-Gal, I., Shapiro, H., Halpern, Z., Segal, E. and Elinav, E., 2014. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. Nature, 514(7521), pp.181-186.

Sullivan, K., Reddy, A., Dietzmann, K., Suriano, A., Kocieda, V., Stewart, M. and Bhatia, M., 2007. Epigenetic Regulation of Tumor Necrosis Factor Alpha. Molecular and Cellular Biology, 27(14), pp.5147-5160.

Sun, S., Luo, L., Liang, W., Yin, Q., Guo, J., Rush, A., Lv, Z., Liang, Q., Fischbach, M., Sonnenburg, J., Dodd, D., Davis, M. and Wang, F., 2020. Bifidobacteriumalters the gut microbiota and modulates the functional metabolism of T regulatory cells in the context of immune checkpoint blockade. Proceedings of the National Academy of Sciences, 117(44), pp.27509-27515.

Surks, H., 2007. cGMP-Dependent Protein Kinase I and Smooth Muscle Relaxation: A Tale of Two Isoforms. Circulation Research, 101(11), pp.1078-1080.

Szuster-Ciesielska, A., Hryciuk-Umer, E., Stepulak, A., Kupisz, K. and Kandefer-Szerszeń, M. 2004. Reactive oxygen species production by blood neutrophils of patients with laryngeal carcinoma and antioxidative enzyme activity in their blood. Acta Oncologica, 43(3), pp.252-258.

Taft, D.H., Liu, J., Maldonado-Gomez, M.X., Akre, S., Huda, M.N., Ahmad, S.M., Stephensen, C.B., Mills, D.A., 2018. Bifidobacterial Dominance of the Gut in Early Life and Acquisition of Antimicrobial Resistance. mSphere 3, e00441-18

Takata, H., Tomiyama, H., Fujiwara, M., Kobayashi, N. and Takiguchi, M., 2004. Cutting Edge: Expression of Chemokine Receptor CXCR1 on Human Effector CD8+ T Cells. The Journal of Immunology, 173(4), pp.2231-2235.

Takeshima, N., Sozu, T., Tajika, A., Ogawa, Y., Hayasaka, Y., Furukawa, T.A., 2014. Which is more generalizable, powerful and interpretable in meta-analyses, mean difference or standardized mean difference? BMC Med Res Methodol 14, p.30.

Takeuchi, O. and Akira, S., 2010. Pattern Recognition Receptors and Inflammation. Cell, 140(6), pp.805-820.

TAULER, P., MARTÍNEZ, S., MORENO, C., MONJO, M., MARTÍNEZ, P. and AGUILÓ, A., 2013. Effects of Caffeine on the Inflammatory Response Induced by a 15-km Run Competition. Medicine & Science in Sports & Exercise, 45(7), pp.1269-1276.

Theiler, A., Bärnthaler, T., Platzer, W., Richtig, G., Peinhaupt, M., Rittchen, S., Kargl, J., Ulven, T., Marsh, L., Marsche, G., Schuligoi, R., Sturm, E. and Heinemann, A., 2019. Butyrate ameliorates allergic airway inflammation by limiting eosinophil trafficking and survival. Journal of Allergy and Clinical Immunology, 144(3), pp.764-776.

Thieu, V. T., Yu, Q., Chang, H. C., Yeh, N., Nguyen, E. T., Sehra, S., & Kaplan, M. H., 2008. Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination. Immunity, 29(5), pp.679-90.

Thomas, A., Lemanske, R. and Jackson, D., 2011. Approaches to stepping up and stepping down care in asthmatic patients. Journal of Allergy and Clinical Immunology, 128(5), pp.915-924.

Thomas, S. and Denu, J., 2021. Short-chain fatty acids activate acetyltransferase p300. 22(10), e72171

Thorburn, A., Macia, L. and Mackay, C., 2014. Diet, Metabolites, and "Western-Lifestyle" Inflammatory Diseases. Immunity, 40(6), pp.833-842.

Thorburn, A., McKenzie, C., Shen, S., Stanley, D., Macia, L., & Mason, L. et al., 2015. Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites. Nature Communications, 6(1).

Threapleton, D., Greenwood, D., Evans, C., Cleghorn, C., Nykjaer, C., Woodhead, C., Cade, J., Gale, C. and Burley, V., 2013. Dietary fibre intake and risk of cardiovascular disease: systematic review and meta-analysis. BMJ, 347(2), pp6879-6879.

Thursby, E. and Juge, N., 2017. Introduction to the human gut microbiota. Biochemical Journal, 474(11), pp.1823-1836.

Tilg, H., & Moschen, A., 2015. Food, Immunity, and the Microbiome. Gastroenterology, 148(6), pp.1107-1119.

Ting, J., Duncan, J. and Lei, Y., 2010. How the Noninflammasome NLRs Function in the Innate Immune System. Science, 327(5963), pp.286-290.

To, T., Stanojevic, S., Moores, G., Gershon, A., Bateman, E., Cruz, A. and Boulet, L., 2012. Global asthma prevalence in adults: findings from the cross-sectional world health survey. BMC Public Health, 12(1).

Toh, Z., Anzela, A., Tang, M. and Licciardi, P., 2012. Probiotic Therapy as a Novel Approach for Allergic Disease. Frontiers in Pharmacology, 3.

Tokunaga, F., Nishimasu, H., Ishitani, R., Goto, E., Noguchi, T., Mio, K., Kamei, K., Ma, A., Iwai, K. and Nureki, O., 2012. Specific recognition of linear polyubiquitin by A20 zinc finger 7 is involved in NF-κB regulation. The EMBO Journal, 31(19), pp.3856-3870.

Topping, D. and Clifton, P., 2001. Short-Chain Fatty Acids and Human Colonic Function: Roles of Resistant Starch and Nonstarch Polysaccharides. Physiological Reviews, 81(3), pp.1031-1064.

Torres, D., Gonçalves, M., Teixeira, J. and Rodrigues, L., 2010. Galacto-Oligosaccharides: Production, Properties, Applications, and Significance as Prebiotics. Comprehensive Reviews in Food Science and Food Safety, 9(5), pp.438-454.

Trompette, A., Gollwitzer, E., Yadava, K., Sichelstiel, A., Sprenger, N., & Ngom-Bru, C. et al., 2014. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nature Medicine, 20(2), pp.159-166.

Tsai, D., Riediker, M., Berchet, A., Paccaud, F., Waeber, G., Vollenweider, P. and Bochud, M., 2019. Effects of short- and long-term exposures to particulate matter on inflammatory marker levels in the general population. Environmental Science and Pollution Research, 26(19), pp.19697-19704.

Turnbaugh, P., Ley, R., Mahowald, M., Magrini, V., Mardis, E. and Gordon, J., 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature, 444(7122), pp.1027-1031.

Turner, S., Doherty, P., McCluskey, J. and Rossjohn, J., 2006. Structural determinants of T-cell receptor bias in immunity. Nature Reviews Immunology, 6(12), pp.883-894.

Tzortzis, G. and Vulevic, J., 2009. Galacto-Oligosaccharide Prebiotics. Prebiotics and Probiotics Science and Technology, pp.207-244.

Tzortzis, G., Goulas, A., Gee, J. and Gibson, G., 2005. A Novel Galactooligosaccharide Mixture Increases the Bifidobacterial Population Numbers in a Continuous In Vitro Fermentation System and in the Proximal Colonic Contents of Pigs In Vivo. The Journal of Nutrition, 135(7), pp.1726-1731.

Uematsu, S., Fujimoto, K., Jang, M., Yang, B., Jung, Y., Nishiyama, M., Sato, S., Tsujimura, T., Yamamoto, M., Yokota, Y., Kiyono, H., Miyasaka, M., Ishii, K. and Akira, S., 2008. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nature Immunology, 9(7), pp.769-776.

Ulven, T., 2012. Short-chain free fatty acid receptors FFA2/GPR43 and FFA3/GPR41 as new potential therapeutic targets. Frontiers In Endocrinology, 3.

Underhill, D. M., Bassetti, M., Rudensky, A., & Aderem, A., 1999. Dynamic Interactions of Macrophages with T Cells during Antigen Presentation. The Journal of Experimental Medicine, 190(12), pp.1909–1914.

Uranga, J., López-Miranda, V., Lombó, F., & Abalo, R., 2016. Food, nutrients and nutraceuticals affecting the course of inflammatory bowel disease. Pharmacological Reports, 68(4), pp.816-826.

Utkualp, N., & Ercan, I., 2015. Anthropometric Measurements Usage in Medical Sciences. BioMed research international, p.404261.

Vael, C., Nelen, V., Verhulst, S., Goossens, H. and Desager, K., 2008. Early intestinal Bacteroides fragilis colonisation and development of asthma. BMC Pulmonary Medicine, 8(1).

Valencia, X., Stephens, G., Goldbach-Mansky, R., Wilson, M., Shevach, E. and Lipsky, P., 2006. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood, 108(1), pp.253-261.

Van De Pol, M.A., Lutter, R., Smids, B.S., Weersink, E.J.M., Van Der Zee, J.S., 2011. Synbiotics reduce allergen-induced T-helper 2 response and improve peak expiratory flow in allergic asthmatics: Effect of synbiotics in allergic asthmatics. Allergy 66, pp.39–47.

Van der Aa, L.B., van Aalderen, W.M.C., Heymans, H.S.A., Henk Sillevis Smitt, J., Nauta, A.J., Knippels, L.M.J., Ben Amor, K., Sprikkelman, A.B., the Synbad Study Group, 2011. Synbiotics prevent asthma-like symptoms in infants with atopic dermatitis: Synbiotics prevent asthma-like symptoms. Allergy 66, pp.170–177.

Van Lierop, J., Wilson, D., Davis, J., Tikunova, S., Sutherland, C., Walsh, M. and Johnson, J., 2001. Activation of Smooth Muscle Myosin Light Chain Kinase by Calmodulin. Journal of Biological Chemistry, 277(8), pp.6550-6558.

Van Nimwegen, F., Penders, J., Stobberingh, E., Postma, D., Koppelman, G., Kerkhof, M., Reijmerink, N., Dompeling, E., van den Brandt, P., Ferreira, I., Mommers, M. and Thijs, C., 2011. Mode and place of delivery, gastrointestinal microbiota, and their influence on asthma and atopy. Journal of Allergy and Clinical Immunology, 128(5), pp.948-955.e3.

Vandercappellen, J., Van Damme, J. and Struyf, S., 2008. The role of CXC chemokines and their receptors in cancer. Cancer Letters, 267(2), pp.226-244.

Vantourout, P. and Hayday, A., 2013. Six-of-the-best: unique contributions of γδ T cells to immunology. Nature Reviews Immunology, 13(2), pp.88-100.

Varraso, R., Kauffmann, F., Leynaert, B., Le Moual, N., Boutron-Ruault, M., Clavel-Chapelon, F. and Romieu, I., 2009. Dietary patterns and asthma in the E3N study. European Respiratory Journal, 33(1), pp.33-41.

Varricchi, G., Pecoraro, A., Marone, G., Criscuolo, G., Spadaro, G., Genovese, A. and Marone, G., 2018. Thymic Stromal Lymphopoietin Isoforms, Inflammatory Disorders, and Cancer. Frontiers in Immunology, 9.

Veldhoen, M., Uyttenhove, C., van Snick, J., Helmby, H., Westendorf, A., Buer, J., Martin, B., Wilhelm, C. and Stockinger, B., 2008. Transforming growth factor-β 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9–producing subset. Nature Immunology, 9(12), pp.1341-1346.

Verheijden, K.A.T., Willemsen, L.E.M., Braber, S., Leusink-Muis, T., Jeurink, P.V., Garssen, J., Kraneveld, A.D., Folkerts, G., 2016. The development of allergic inflammation in a murine

house dust mite asthma model is suppressed by synbiotic mixtures of non-digestible oligosaccharides and Bifidobacterium breve M-16V. Eur J Nutr 55, pp.1141–1151.

Verheijden, Kim A. T., Willemsen, L.E.M., Braber, S., Leusink-Muis, T., Delsing, D.J.M., Garssen, J., Kraneveld, A.D., Folkerts, G., 2015b. Dietary galacto-oligosaccharides prevent airway eosinophilia and hyperresponsiveness in a murine house dust mite-induced asthma model. Respir. Res. 16, p.17.

Verheijden, Kim A.T., Akbari, P., Willemsen, L.E.M., Kraneveld, A.D., Folkerts, G., Garssen, J., Fink-Gremmels, J., Braber, S., 2015a. Inflammation-Induced Expression of the Alarmin Interleukin 33 Can Be Suppressed by Galacto-Oligosaccharides. Int Arch Allergy Immunol 167, pp.127–136.

Verheijden, Kim AT, Braber, S., Leusink-Muis, T., Thijssen, S., Boon, L., Kraneveld, A.D., Garssen, J., Folkerts, G., Willemsen, L.E., 2015. Regulatory T Cell Depletion Abolishes the Protective Effect of Dietary Galacto-Oligosaccharides on Eosinophilic Airway Inflammation in House Dust Mite–Induced Asthma in Mice. The Journal of Nutrition 146, pp.831–837.

Vieira, A., Teixeira, M. and Martins, F., 2013. The Role of Probiotics and Prebiotics in Inducing Gut Immunity. Frontiers in Immunology, 4.

Vinolo, M. A., Rodrigues, H. G., Nachbar, R. T., & Curi, R., 2011. Regulation of inflammation by short chain fatty acids. Nutrients, 3(10), pp.858–876.

Vivolo, M. A., Bnamichi-Santos, R., Arantes-Costa, F.M, Kalil. J., Giavina-Bianchi, P., 2017. Animal models of asthma: utility and limitations. Journal of Asthma and Allergy 7(10), pp.293-301.

Voreades, N., Kozil, A., Weir, T.L., 2014. Diet and the development of the human intestinal microbiome. Front. Microbiol. 5.

Vos, A.P., van Esch, B.C., Stahl, B., M'Rabet, L., Folkerts, G., Nijkamp, F.P., Garssen, J., 2007. Dietary supplementation with specific oligosaccharide mixtures decreases parameters of allergic asthma in mice. International Immunopharmacology 7, pp.1582–1587.

Vroman, H., Hendriks, R. and Kool, M., 2017. Dendritic Cell Subsets in Asthma: Impaired Tolerance or Exaggerated Inflammation?. Frontiers in Immunology, 8.

Vulevic, J., Juric, A., Walton, G., Claus, S., Tzortzis, G., Toward, R. and Gibson, G., 2015. Influence of galacto-oligosaccharide mixture (B-GOS) on gut microbiota, immune parameters and metabonomics in elderly persons. British Journal of Nutrition, 114(4), pp.586-595.

Walter MJ, Holtzman MJ. A., 2005. Centennial History of Research on Asthma Pathogenesis. American Journal of Respiratory Cell and Molecular Biology, 32(6), pp.483-489.

Wang, B., Yao, M., Lv, L., Ling, Z. and Li, L., 2017. The Human Microbiota in Health and Disease. Engineering, 3(1), pp.71-82.

Wang, G., Xu, Z., Wang, R., Al-Hijji, M., Salit, J., Strulovici-Barel, Y., Tilley, A., Mezey, J. and Crystal, R., 2012. Genes associated with MUC5AC expression in small airway epithelium of human smokers and non-smokers. BMC Medical Genomics, 5(1).

Wang, H., 2010. Alcohol, inflammation, and gut-liver-brain interactions in tissue damage and disease development. World Journal of Gastroenterology, 16(11), p.1304.

Wang, L., Beier, U., Akimova, T., Dahiya, S., Han, R., Samanta, A., Levine, M. and Hancock, W., 2018. Histone/protein deacetylase inhibitor therapy for enhancement of Foxp3+ T-regulatory cell function posttransplantation. American Journal of Transplantation, 18(7), pp.1596-1603.

Watanabe, H., Sonoyama, K., Watanabe, J., Yamaguchi, N., Kikuchi, H., Nagura, T., Aritsuka, T., Fukumoto, K., Kasai, T., 2004. Reduction of allergic airway eosinophilia by dietary raffinose in Brown Norway rats. Br J Nutr 92, pp.247–255.

Wen, D., Du, X., Nie, S. P., Dong, J. Z., & Ma, C. S., 2014. Association between RANTES gene polymorphisms and asthma: a meta-analysis. PloS one, 9(6), e90460.

Wenzel, J. and Siddall, M., 1999. Noise. Cladistics, 15(1), pp.51-64.

Wenzel, S., 2013. Complex phenotypes in asthma: Current definitions. Pulmonary Pharmacology & Therapeutics, 26(6), pp.710-715.

West, N., Christophersen C., Pyne., D, Crips., A, Conlon., M, and Topping., D., 2013. Butyrylated starch increases colonic butyrate concentration but has limited efects on immunity in healthy physically active indi- viduals. Exercise Immunology Review, 19(1), pp.102-119.

Westerbeek, E.A., van den Berg, J.P., Lafeber, H.N., Fetter, W.P., Boehm, G., Twisk, J.W., van Elburg, R.M., 2010. Neutral and acidic oligosaccharides in preterm infants: a randomized, double-blind, placebo-controlled trial. The American Journal of Clinical Nutrition 91, pp.679–686.

Willerson, J. and Ridker, P., 2004. Inflammation as a Cardiovascular Risk Factor. Circulation, 109(21).

Williams, N., Johnson, M., Shaw, D., Spendlove, I., Vulevic, J., Sharpe, G. and Hunter, K., 2016. A prebiotic galactooligosaccharide mixture reduces severity of hyperpnoea-induced bronchoconstriction and markers of airway inflammation. British Journal of Nutrition, 116(05), pp.798-804.

Williams, N.C., Johnson, M.A., Shaw, D.E., Spendlove, I., Vulevic, J., Sharpe, G.R., Hunter, K.A., 2016. A prebiotic galactooligosaccharide mixture reduces severity of hyperpnoea-induced bronchoconstriction and markers of airway inflammation. Br J Nutr 116, pp.798–804.

Wood, L., Garg, M., Smart, J., Scott, H., Barker, D. and Gibson, P., 2012. Manipulating antioxidant intake in asthma: a randomized controlled trial. The American Journal of Clinical Nutrition, 96(3), pp.534-543.

Wood, L., Shivappa, N., Berthon, B., Gibson, P. and Hebert, J., 2014. Dietary inflammatory index is related to asthma risk, lung function and systemic inflammation in asthma. Clinical & Experimental Allergy, 45(1), pp.177-183.

Wu, Y., Kang, J., Zhang, L., Liang, Z., Tang, X., Yan, Y., Qian, H., Zhang, X., Xu, W., Mao, F., 2018. Ubiquitination regulation of inflammatory responses through NF-κB pathway. Am J Transl Res, 10(3), pp.881-891.

Wucherpfennig, K. W., Gagnon, E., Call, M. J., Huseby, E. S., & Call, M. E., 2010. Structural biology of the T-cell receptor: insights into receptor assembly, ligand recognition, and initiation of signaling. Cold Spring Harbor perspectives in biology, 2(4), a005140.

Yadav, M., Stephan, S., & Bluestone, J., 2013. Peripherally Induced Tregs – Role in Immune Homeostasis and Autoimmunity. Frontiers In Immunology, 4.

Yasruel, Z., Humbert, M., Kotsimbos, T., Ploysongsang, Y., Minshall, E., & Durham, S. et al., 1997. Membrane-bound and soluble alpha IL-5 receptor mRNA in the bronchial mucosa of atopic and nonatopic asthmatics. American Journal Of Respiratory And Critical Care Medicine, 155(4), pp.1413-1418.

Yasuda, A., Inoue, K.-I., Sanbongi, C., Yanagisawa, R., Ichinose, T., Yoshikawa, T., Takano, H., 2010. Dietary Supplementation with Fructooligosaccharides Attenuates Airway Inflammation Related to House Dust Mite Allergen in Mice. Int J Immunopathol Pharmacol 23, pp.727–735.

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R.N., Anokhin, A.P., Heath, A.C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J.G., Lozupone, C.A., Lauber, C., Clemente, J.C., Knights, D., Knight, R., Gordon, J.I., 2012. Human gut microbiome viewed across age and geography. Nature 486, pp.222–227.

Yoshida, M., Claypool, S., Wagner, J., Mizoguchi, E., Mizoguchi, A., Roopenian, D., Lencer, W. and Blumberg, R., 2004. Human Neonatal Fc Receptor Mediates Transport of IgG into Luminal Secretions for Delivery of Antigens to Mucosal Dendritic Cells. Immunity, 20(6), pp.769-783.

Yoshie, O. and Matsushima, K., 2014. CCR4 and its ligands: from bench to bedside. International Immunology, 27(1), pp.11-20.

Yu, H., Lin, L., Zhang, Z., Zhang, H. and Hu, H., 2020. Targeting NF-κB pathway for the therapy of diseases: mechanism and clinical study. Signal Transduction and Targeted Therapy, 5(1).

Yu, Y., Ma, X., Gong, R., Zhu, J., Wei, L., & Yao, J., 2018. Recent advances in CD8+ regulatory T cell research. Oncology letters, 15(6), pp.8187-8194.

Yung, S. and Farber, J., 2013. Chemokines. Handbook of Biologically Active Peptides, pp.656-663.

Zhang, C., Chang, J., Wu, W., Deng, Y., Zhou, P., Jiang, W., Wang, C. and Huang, F., 2020. Activation of GPR43 suppresses TNF-α-induced inflammatory response in human fibroblast-like synoviocytes. Archives of Biochemistry and Biophysics, 684, e108297.

Zhang, J. M., & An, J., 2007. Cytokines, inflammation, and pain. International anesthesiology clinics, 45(2), pp.27-37.

Zhang, Q., Cox, M., Liang, Z., Brinkmann, F., Cardenas, P., Duff, R., Bhavsar, P., Cookson, W., Moffatt, M. and Chung, K., 2016. Airway Microbiota in Severe Asthma and Relationship to Asthma Severity and Phenotypes. PLOS ONE, 11(4), e0152724.

Zhang, Y. H., Xing, Z., Liu, C., Wang, S., Huang, T., Cai, Y. D., & Kong, X., 2017. Identification of the core regulators of the HLA I-peptide binding process. Scientific reports, 7, p.42768.

Zhao, L., Zhang, F., Ding, X., Wu, G., Lam, Y., Wang, X., Fu, H., Xue, X., Lu, C., Ma, J., Yu, L., Xu, C., Ren, Z., Xu, Y., Xu, S., Shen, H., Zhu, X., Shi, Y., Shen, Q., Dong, W., Liu, R., Ling, Y., Zeng, Y., Wang, X., Zhang, Q., Wang, J., Wang, L., Wu, Y., Zeng, B., Wei, H., Zhang, M., Peng, Y. and Zhang, C., 2018. Gut bacteria selectively promoted by dietary fibers alleviate type 2 diabetes. Science, 359(6380), pp.1151-1156.

Zheng, Y., Josefowicz, S., Chaudhry, A., Peng, X. P., Forbush, K., & Rudensky, A. Y., 2010. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature, 463(7282), pp.808-12.

Zhu, H., Shan, L., Schiller, P., Mai, A. and Peng, T., 2010. Histone Deacetylase-3 Activation Promotes Tumor Necrosis Factor-α (TNF-α) Expression in Cardiomyocytes during Lipopolysaccharide Stimulation. Journal of Biological Chemistry, 285(13), pp.9429-9436.

Zhuang, H., Cheng, L., Wang, Y., Zhang, Y.-K., Zhao, M.-F., Liang, G.-D., Zhang, M.-C., Li, Y.-G., Zhao, J.-B., Gao, Y.-N., Zhou, Y.-J., Liu, S.-L., 2019. Dysbiosis of the Gut Microbiome in Lung Cancer. Front Cell Infect Microbiol 9, 112.

Appendix 1 – Health Screen Questionnaire

Health screen

Name or Number _____

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present,** do you have any health problem for which you are:

(a) on medication, prescribed or otherwise Yes ☐ No ☐

(b) attending your general practitioner Yes ☐ No ☐

(c) on a hospital waiting list Yes ☐ No ☐

2. **In the past two years,** have you had any illness which require you to:

(a) consult your GP Yes ☐ No ☐

(b) attend a hospital outpatient department Yes ☐ No ☐

(c) be admitted to hospital Yes ☐ No ☐

3. **Have you ever** had any of the following?

(a) Convulsions/epilepsy Yes ☐ No ☐

(b) Asthma Yes ☐ No ☐

(c) Eczema Yes ☐ No ☐

(d) Diabetes Yes ☐ No ☐

(e) A blood disorder Yes ☐ No ☐

(f) Head injury Yes ☐ No ☐

(g) Digestive problems Yes ☐ No ☐

(h) Heart problems Yes ☐ No ☐

(i) Problems with bones or joints Yes ☐ No ☐

(j) Disturbance of balance / coordination Yes ☐ No ☐

(k) Numbness in hands or feet Yes ☐ No ☐

(l) Disturbance of vision Yes ☐ No ☐

(m) Ear / hearing problems Yes ☐ No ☐

(n) Thyroid problems Yes ☐ No ☐

(o) Kidney or liver problems Yes ☐ No ☐

(p) Allergy to nuts, alcohol etc. Yes ☐ No ☐

(q) Any problems affecting your nose e.g. recurrent nose bleeds Yes ☐ No ☐

(r) Any nasal fracture or deviated nasal septum Yes ☐ No ☐

4. **Has any,** otherwise healthy, member of your family under the age of 50 died suddenly during or soon after exercise? Yes ☐ No ☐

5. Are there any reasons why blood sampling may be difficult? Yes ☐ No ☐

6. Have you had a blood sample taken previously? Yes ☐ No ☐

7. Have you had a cold, flu or any flu like symptoms in the last Month? Yes ☐ No ☐

COVID19

8. Do you think you have had COVID-19? Yes ☐ No ☐

9 If YES, was this confirmed via a swab test? Yes ☐ No ☐

10. If YES, was this confirmed via an anti-body test? Yes ☐ No ☐

11. State the dates over which you had COVID-19 symptoms:

FROM _____ TO _____

NB Please note that in the 7-day period prior to any visit to the University to undertake a trial in a research study or to visit a University research facility YOU WILL NEED TO COMPLETE a COVID-19 symptom questionnaire. Please DO NOT come to the University if you have not completed this questionnaire and the member of research staff supervising the research study has not confirmed you should attend.

If you have answered YES to any question above, please describe briefly (e.g. to confirm problem was/is short-lived, insignificant or well controlled.)

_____

Females Only:

12 Are you currently on any form of contraceptive Yes ☐ No ☐

If yes, please state _____

13 Have you been taking the form of contraception stated above for at least the last 6 months?

Yes ☐ No ☐

If known, please state consumption duration _____

14 Have you had a consistent menstrual cycle (e.g. between 26-30 days) for at least the last 3 months?

Yes ☐ No ☐

Infant History Questions:

15. Were you born early, on-time, or late in relation to your due date?

If known, please specify:

16. Were you classified as a low weight at birth?

If known, please specify and state your birth weight:

17. What was your mode of birth? (if known)

Natural Birth ☐ Caesarean Birth ☐

If known, please specify (Caesarean Only): Elective ☐ Emergency ☐

18. What was your infant feeding method? (if known)

Breast Milk ☐ Formula Milk ☐ Both ☐

Antibiotic Questions:

19. Have you taken antibiotics in the past 3 months?

Yes ☐ No ☐

20. Have you taken antibiotics in the past 12 months?

Yes ☐ No ☐

21. Did you/your mother experience exposure to antibiotics during infancy/pregnancy?

Yes ☐ No ☐

If known, please specify:

Appendix 2 – Chapter 4 Informed Consent

IRAS Project ID: 253339

Participant ID Number:

CONSENT FORM

Study Title: The Effects of Prebiotic Supplementation on Quality of Life, Control of Asthma, and Markers of Systemic Inflammation in Adults with Asthma. A Double-Blind, Placebo-Controlled, Crossover Trial.

Chief Investigator: Dr Michael Johnson

Researchers: Mr Jacob Jayaratnasingam & Mr Robert Needham

	Initials
1. I confirm that I have read the participant information sheet dated: (Version 2.0, Date: 09/04/19) for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to leave at any time without giving any reason, without my medical care or legal rights being affected.	☐
3. I understand that information collected about me, and blood samples collected from me will be used to support future research studies undertaken by the research team stated in the participant information sheet.	☐
4. I agree to take part in the above study.	☐

Participant Name: _____ Date:_____ Signature:_____

Researcher Name: _____ Date:_____ Signature:_____

Appendix 3 – Chapter 4 Participant Information Sheet

NOTTINGHAM TRENT UNIVERSITY

Participant Information Sheet
(version 2.0. 24/04/19)

IRAS Project ID: 253339

International Standard Randomised Controlled Trial Number (ISRCTN): 11358769

Title of Study: The Effects of Prebiotic Supplementation on Quality of Life, Control of Asthma, and Markers of Systemic Inflammation in Adults with Asthma. A Double-Blind, Placebo-Controlled, Crossover Trial.

Patient Friendly Title of Study: Compared to a placebo, does feeding the good bacteria in the gut with a prebiotic supplement improve asthma symptoms, medication use, quality of life, and lung inflammation?

Chief Investigator: Dr Michael Johnson

Researchers: Mr Jacob Jayaratnasingam & Mr Robert Needham

Institution: Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS

We're inviting you to take part in our study. Before deciding, please read the information below carefully. It explains why we're doing the research and what your involvement would be. To help you make a decision, one of our researchers can discuss this information sheet with you. Talk to others about the study if you wish. Taking part is voluntary. You may choose not to take part, or leave the study at any time without giving a reason.

What is the purpose of the study?

Asthma is a chronic condition that affects the airways, i.e. the small tubes that carry air in and out of the lungs. Bacteria living in our gut affects our immune system and possibly asthma. Therefore, increasing the 'good bacteria' in the gut by eating a 'prebiotic' supplement might benefit people with asthma. A prebiotic is 'food' for the good bacteria. This is different from a probiotic, which is the actual live bacteria and which you'll often see in some yoghurts. In 2016 we published a study in the *British Journal of Nutrition*, which showed that when adults with asthma took a prebiotic supplement, their airways did not narrow / constrict as much during a breathing challenge test. We'd now like to know if there are other benefits to individuals with asthma. Therefore, we'll look at whether a prebiotic supplement benefits people with asthma by affecting their symptoms, lung

function, medication use, quality of life, and the chemicals in the blood that tell us how bad the inflammation is in your lungs.

Can I take part in this study?

To take part, you have to:

- Be 18-50 years of age at the date of your first laboratory visit.
- Have a body mass index (BMI) between 18.5-25 $kg \cdot m^2$ (we'll work this out for you using your height and body weight).
- Be a non-smoker.
- Have been on stable asthma treatment for 3 months, and do not take oral steroid tablets as part of your treatment.
- Have a **current** medication prescription from your GP (e.g. maintenance and reliever inhalers).
- Satisfy the researchers that you are willing and able to follow all trial requirements.
- Have told the researchers about any nutritional supplements you take in case taking them rules you out of the study.

Unfortunately, you cannot take part if any of the following apply to you:

- You have asthma but do not have a **current** medication prescription from your GP (e.g. maintenance and reliever inhalers).
- You take oral steroid tablets as part of your asthma medication.
- You regularly take Omega-3 supplements, and/or eat high levels of Omega-3 (e.g. more than 1-2 portions of oily fish such as salmon or mackerel a week).
- You take aspirin or other non-steroidal anti-inflammatory drugs such as ibuprofen once a day on 5 or more days of the week.
- Females only: You are pregnant or planning a pregnancy during the time of the study.
- You've taken, in the 4 weeks before signing the consent form, a prebiotic and/or probiotic supplement, or laxatives or drugs that loosen stools and increase bowel movements.
- You're currently taking a daily dose of anti-histamine, which you can't temporarily stop for 72 hours before each testing session without exacerbation of symptoms.
- You follow a vegetarian or vegan diet.
- You've been previously diagnosed with chronic obstructive pulmonary disease (COPD), emphysema, chronic bronchitis, or similar respiratory (breathing-related) illness.
- You've been admitted to hospital during the past 12 months due to your asthma.
- You've a history of heart failure, pulmonary hypertension, embolism, or other pulmonary heart disease.

- You've a history of recurrent chest infections.
- You've had an acute infection in the last four weeks, and/or major operation in the past four months.
- You react badly to gastrointestinal drugs.
- You've taken antibiotics in the past 3 months.
- You've a history or current evidence of gastrointestinal disease (e.g. chronic constipation, diarrhoea, irritable bowel syndrome, Crohn's disease).
- You've recently taken part in other research projects. Please notify us so we can see if this excludes you from the study.
- You are or you believe you may be lactose intolerant.
- You regularly take antioxidant supplements, such as beta-carotene, vitamin A, vitamin C, vitamin E, lutein and selenium. Standard multivitamin and mineral supplements are ok; however, if a single supplement (e.g. Vitamin C) is more than the recommended daily DRV's (dietary reference values) you need to check this with the researchers.

Study location and time commitment

We'll ask you to visit our laboratory in the Erasmus Darwin Building at Nottingham Trent University's Clifton Campus five times over a 9-week period. Visit 1 will last about 1 hour, and visits 2-5 will last about 1 hour and 30 minutes (7-9 hours in total).

What will I have to do if I take part in this study?

You can continue with your regular diet during the study. You don't have to restrict your asthma medication - just write down your medication usage during the 24 hours before each visit. **YOU MUST have your asthma reliever medication with you for all visits**. **You need to drink 500ml of water 1-hour before each visit**. If required, females will do a pregnancy test at the start of each visit. After visit 2, we'll give you a prebiotic supplement or a taste/appearance-matched dummy supplement (i.e. a placebo that has no effect). You need to take the supplement for 3-weeks. Then, after a 2-week "washout" (no supplement) period, you'll take the other supplement for 3 weeks. Visits 2-5 take place at the start and at the end of each 3-week period.

Visit 1: Initial consultation and familiarisation

We'll check that you have your asthma reliever mediation with you, and check that you're suitable for the study. Then we'll tell you all about the study and answer any questions you have. If you want to go ahead and take part, we'll ask you to sign a written consent form. We'll ask you to complete a health screen and health history questionnaire, and we'll measure your height and body weight to work out your body mass index. You can practice

lung function assessment. For this, you'll breathe out through a mouthpiece as hard as you can for 6 seconds. Finally, we'll explain what you need to do for visit 2.

Visits 2-5: Main experimental visits

We'll ask you to record your diet for the 24 hours before visit 2. We also need you to complete a 4-day food diary during the four days leading up to visits 3, 4 and 5. We'll explain how to do this during visit 1. When you arrive at the laboratory, we'll check you have your asthma reliever medication with you and we'll collect your 24-hour (visit 2) or 4-day (visits 3-5) food diary. You'll then complete a health screen and health history questionnaire, and give written consent for us to take your blood. We'll then measure your body weight and lung function and take the blood sample. Finally, we'll ask you to complete some questionnaires that check your asthma control, your quality of life, how you're using your asthma medication, and how well you're sticking to the nutritional supplement timetable.

General testing restrictions

General testing restrictions and their duration apply to all visits (see Table 1).

Table 1. General Testing Restriction Guidelines

	How long to avoid (Before each visit)
Anti-Histamines	72 Hours
Caffeine	24 Hours
Alcohol	48 Hours
Food	3 Hours
Strenuous exercise	24 Hours

What nutritional supplements will I be given in this study?

We'll ask you to consume two powder-based nutritional supplements. One will be a prebiotic, and the other a taste/appearance-matched placebo containing maltodextrin. Neither you nor the researchers will know which supplement you are taking at any time – this is only revealed at the end of the study. The prebiotic contains galactooligosaccharides, lactose (up to 16%), glucose and galactose (see Table 2). It's gluten free and doesn't contain artificial flavourings or colourings, or genetically modified organisms. The prebiotic supplement is GRAS (generally recognised as safe) certified (GRN: 000484).

Table 2. Nutritional information for prebiotic supplement.

	Per sachet (3.65g)	Per 100g
Energy	41.5 kj/10.1kcal	1138kj/276kcal
Fat	<0.1g	<0.1g
(of which are saturates)	<0.0g	<0.1g
Carbohydrates	2.0g	42.1g
(of which are sugars)	1.0g	15.6g
Fibre	2.0g	53.4g
Protein	0.0g	0.1g
Salt	<0.1g	2.0g
Galactooligosaccharides	2.9g	79.7g

You'll take one supplement sachet around the same time each morning. The supplements can be dissolved in tea, coffee, fruit juice, water, or other drink. You need to take the supplements in the same way throughout the study. We won't carry on giving you the supplements after the study, but we can tell you where to buy similar products.

Which supplement will I take first?

You won't know because in this type of study it's important that we don't bias the results in any way. So at the start of the study we'll use a computer programme to randomly allocate you to your first supplement. We won't find out which one this is until the study is completed.

How will my blood be taken and stored?

We'll ask you to tell us of any fears/phobias (e.g. of blood or needles) or allergies (e.g. Elastoplast, alcohol, latex). During each visit, a trained phlebotomist will take up to 100ml of blood using a needle inserted into a vein. Before we take your blood, we will make sure you are comfortable and give you time to ask any questions you may have. We'll store your blood samples in the John Van Geest Cancer Research Centre at Nottingham Trent University. We intend to perform additional tests on your blood (and the cells in your blood) as part of future research studies. Please tell us if you do not want us to use your blood samples in this way, in which case we'll securely destroy your samples after this study.

What are my responsibilities?

If you are not comfortable with any of the procedures, please tell us as soon as possible. We need you to complete all documents accurately, and follow all instructions / guidelines throughout the study. You need to take the nutritional supplements as instructed. If you miss taking any sachets, don't compensate by taking multiple sachets. Just get back on to the instructed dosing timetable and write down any discrepancies. Let us know if you haven't followed any of these instructions, and/or if there are changes that affect you taking part in this study (e.g. antibiotics use).

What are the potential benefits of taking part?

The prebiotic supplement may improve your lung function and asthma symptoms. We can also give you information regarding your lung function.

What are the possible risks and disadvantages of taking part?

You may experience discomfort when we take your blood. However, only trained phlebotomists will take your blood. Therefore, the risk of bruising and swelling is low. Performing spirometry can make you cough and feel light-headed, although these symptoms don't last long. The prebiotic supplement may cause some temporary gastrointestinal discomfort (e.g. abdominal bloating, flatulence, abdominal cramps/pains, nausea). However, this is mainly down to taking a higher daily dose of prebiotic, or because you already have a gastrointestinal disorder. If you keep getting gastrointestinal discomfort, stop taking the supplement and contact us immediately.

How will we use the results from the study?

We'll present the findings of this study at a scientific conference, and publish the results in a scientific journal. We'll anonymise all data so you can't be identified in any report or publication. You'll receive a copy of the final findings, as well as your own results if you wish to have these.

Will my information be kept confidential?

Yes. We'll keep all information that we collect from you strictly confidential and store it in a locked office at Nottingham Trent University, and on password-protected computers/user accounts there. We'll use a secure online server that only the researchers can access. We'll assign you a unique code so that your information and data are anonymised. Your information may be retained for up to 5-10 years, but will be disposed of securely when no longer required, in line with Data Protection Legislation. We don't destroy your signed informed consent form.

Females only: What if I become pregnant during the study?

Unfortunately, you'll have to leave the study. We'll ask females to do a pregnancy test at the start of each visit. If you know that you are not able to bear children then you will not have to do a pregnancy test – we just ask that you tell us this at the start of the study.

How are we avoiding biased sampling (i.e. participant recruitment)?

We'll avoid biased sampling by recruiting participants from a variety of places, including all Nottingham Trent University campuses, the student union, local leisure centres, and gyms.

What safeguarding mechanisms are in place?

A qualified first aider trained in minimum basic life support (e.g. CPR) will be there during all visits, with a second first aider on standby and able to be on site within 30 seconds. An automated external defibrillator and supplemental oxygen is on hand. If required, emergency services will be called out. The travel time from The Queens Medical Centre to Nottingham Trent University is about 8 minutes. The ambulance crew can park outside our building, and the laboratory can be acccessed with a wheeled stretcher.

What if we find something considered abnormal?

The researchers will discuss any abnormal findings. Then, if we think it necessary and with your consent, we'll notify your GP so that they can follow this up.

Injury and trial complaints procedure / independent complaints service

In the event of injury/illness caused by negligence by the research team, you may contact the Research Sponsor for independent advice: Professor Barbara Pierscionek, Associate Dean for Research, Nottingham Trent University (contact details are provided in the next section).

What if there is a problem?

If you have any concerns regarding any aspect of this study, speak to one of the researchers who will strive to answer your questions – their contact details are found at the end of this information sheet. If you aren't satisfied, you can make a formal complaint to our research sponsor Professor Barbara Pierscionek, Associate Dean for Research, Nottingham Trent University: Email: Barbara.Pierscionek@ntu.ac.uk TEL: 0115 84 83738

Travel Reimbursement

We don't pay you to take part in this study, but we will pay travel expenses according to Nottingham Trent University's policy - £0.25 per mile for travel by car.

Who has reviewed the study?

The East Midlands, Nottingham 1 Research Ethics Committee has reveiewed the study.

Contact details

Researchers
Name: Jacob Jayaratnasingam
Telephone: 0115 8483362
Email: Jacob.Jayaratnasingam2014@ntu.ac.uk

Name: Robert Needham
Telephone: 0115 8483362
Email: Robert.needham2016@my.ntu.ac.uk

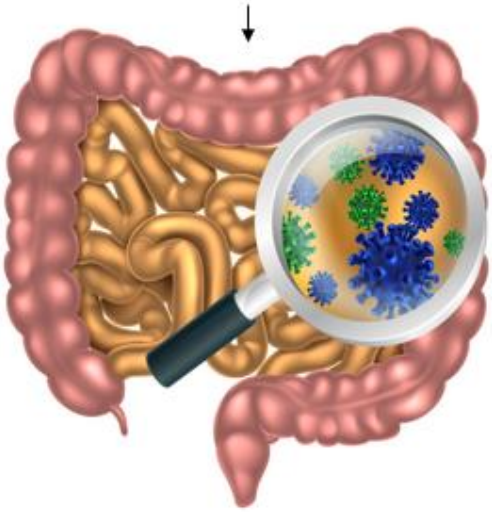
Chief Investigator
Name: Dr Michael Johnson
Telephone: 0115 8483362
Email: Michael.johnson@ntu.ac.uk

Appendix 4 – Chapter 4 Participant Information Poster

Inflammation Research:

Volunteers Needed

Can the Gut influence the body?



Can supplementing prebiotics (dietary fibre) increase the activity of beneficial gut bacteria, regulate systemic inflammation, improve inflammatory markers in the blood and overall health?

YOU COULD HELP US FIND OUT!

We complete blood tests, questionnaires and provide prebiotic supplements

Aged 18-50? Do you want to help?

please get in touch!

Jacob Oliver Jaya (PhD Student)
Nottingham Trent University
Jacob.Jayaratnasingam2014@my.ntu.ac.uk

Appendix 5 – Chapter 4 PPI Feedback Report

Patient and Public Involvement Feedback Report

For

The Effects of Prebiotic Supplementation on Quality of Life, Control of Asthma, and Markers of Systemic Inflammation in Adults with Asthma. A Double-Blind, Placebo-Controlled, Crossover Trial

Compiled by: Tracy Jackson

PPI reviewer one

I have had a look over the documents and feel that they are certainly lay friendly and detailed. However I do wonder how many people will be prepared to take supplements while they are already trying to cope with their asthma. The researchers may have a better view of this than me. I hope that this is helpful

PPI reviewer two

I've just had a look at the documents and here is my feedback:

1. Design – I think the documents could do with some colour to make them a bit more interesting to look at. Especially as the PIS is 9 pages long, some heading in different colours/font sizes would help break up the text. Or make the tables in a different colour.

2. Views on the project – I think it's a great idea to look into other ways of helping people with asthma outside of the traditional medication and there are lot of asthma sufferers hesitant about taking steroid inhalers and would like other methods of trying to help their asthma. I'd personally be very interested in seeing the outcome of the study having only just started taking supplements. My only issue is the restrictions around who can take part, I fit all the inclusion criteria but unfortunately a lot of the exclusion criteria so wouldn't be able to take part. I'm a bit worried it might exclude lots of people with asthma who might want to take part?

3. Consent form – mentions 'biological samples' – would everyone know what this means? Might be good to specify blood samples etc.

Hope that helps – happy to answer any questions

PPI reviewer three

I have just been through all the documents you attached and feel that Dr Johnson and his team who produced them have done an extremely thorough job of covering all the necessary bases and then some.

The only useful comments I feel I can offer are:

that in the Participant Information Sheet "wash out period" is not explained when it first appears though it is set out on Page 5.

I wondered, too, whether the water intake asked for should be highlighted as the "taking your inhaler/medication with you on visits" is?

There is quite a lot asked of participants, not in terms of overall time, but in terms of what they will experience etc. so it will require a certain level of understanding in participants and some may balk at it too.

Otherwise I think everything is set out and explained and covered well.

I hope comments, small as they are, are useful.

PPI reviewer four

Thank you for sharing these. From my perspective, the information is very clear and I think the project is an exciting one. I would have appreciated a paragraph at the beginning which explains a little more about why they think this is a good experiment to do from a patient perspective. I understand that they don't want to prejudice people's ideas before they join the study, but are they the first people to try this? Have others done this and found interesting results? It would give me more context as to why I would want to be a participant.

PPI reviewer five

I have spent some time reviewing all the materials for this study. Overall I am impressed with the language level of all parts of the documentation. I have set out my comments below. I would have liked to see the researchers say that each participant would receive a copy of the final findings not just their own results.

RESEARCH STUDY Invitation Letter

Nice and short. Despite the complex study title, the letter explains the project clearly in simple [not simplistic] language.

PARTICIPANT INFORMATION SHEET.

Again clear language. Like the early mention of willingness for members of the team to talk to potential participants.

Purpose section. Again clear. Possibly need another sentence explaining what prebiotic actually is [although here are details in a later section on nutrition supplement]

Overall the information sheet uses straightforward language in all sections.

Blood section. Gives a thorough explanation. I like the clear optional to agree/ refuse to have blood samples used in future research. This demonstrates respect for participants.

Responsibility section for participants. Easy to understand.

Potential benefits / risks / etc.

Again all clearly spelt out. Strategy if participants experience persistent discomfort, which is good to see.

Results. It is important the study demonstrates how information will be stored and when it will be disposed of securely. Participants have to have confidence in the use, storage and disposal of data. The study sets its strategy well.

Safeguarding Mechanisms. Liked the planning which had gone into providing these safeguards.

Possible abnormal results. Again pleased to see that there is a strategy to reassure and help participants.

Complaints procedure. Clearly set out.

BLOOD SAMPLE CONSENT FORM. Simple and easy to fill in.

PPI REQUEST FORM. Provides clear explanation of Prebiotics. Language straightforward. Well set out form.

PARTICIPANT CONSENT FORM. Concise and clear.

Hope these comments are of use.

PPI reviewer six

This reviewer's comments apply to Appendix 1 – comments highlighted in appendix in yellow

I have read through all five of the documents supplied in relation to the proposed study and my comments on each paper are as follows:- Paper 1 Research Study Invitation

A very clear document and I am sure that lay people receiving the invitation would have little difficulty with it.

Paper 2 Blood Sample Consent

Whilst this paper is necessary, there is a much greater level of detail related to the blood sampling, its use and subsequent testing within the participant information paper. Is there a need to duplicate some of that information in this document?

Paper 3 Participant Consent

As for Paper 1 above

Paper 4 Participant Information

I have some significant concerns regarding this paper. It is very lengthy (9 pages long) which few lay people will want to read in full particularly at the outset. Whilst the information supplied is very complete and informative, I suspect that it would be very important to be careful on the timing of its issue.

A prospective participant could be readily "put off" by the level of detail supplied and length of the document. Can a much shorter summary be provided at the outset and only where a participant seeks more information, then he/she can be given the level of detail they require.

I am majorly concerned that the inclusion and exclusive qualifications are so restrictive that locating the required number of participants will be more than difficult to achieve.

I attach a copy of this paper with areas that I find difficult highlighted.

Paper 5 PPI request form As for Paper 1 I hope that these comments are useful

PPI reviewer seven

This reviewer's comments apply to Appendix 2 – comments highlighted in appendix in yellow Just a few points, I've made them directly on two of the sheets … just about clarity, and consistency of language. But a wider observation, and I'm no scientist so please disregard this optioning if not helpful … It's hard to believe that a few (2?) extra grams of fibre (even assuming all the fibre in the supplement is of the prebiotic type) will be enough to have any meaningful impact. Humans evolved eating close to 100 g per day for optimum health, so will 2g really move any dial, medically? Is it statistically significant? I have witnessed people up their intake 10 – 20g per day (including myself), just in food, and without any difficulty, and my understanding is even that level is only just starting to move a dial in terms of worthwhile, measurable benefits. Hope this is helpful is some way …

PPI reviewer eight

I think the research project is definitely one that would be interesting to see the results and should hopefully be beneficial. After looking at the attached documents I think the consent form may need reworking. The font in some areas is rather small and I find this can be off putting. A more generic clearer layout thought the documents would be helpful in my opinion. Hope that helps.

PPI reviewer nine

I've been through all the documents. Firstly, here are my thoughts on the study itself. The study looks interesting. Maybe it's a bit ambitious, trying to find out so many things in one go. I'm puzzled as to why there's such a low upper age limit on who can participate. (Perhaps older people tend to have other things wrong with them?) But if you're limited to people of working age, will it be possible to get enough people who can take all that time off for all the visits? The researcher didn't say how many people he would be looking for, or how and where he planned to find them. Also, some women patients might be averse to having to take a pregnancy test at each visit. It's a bit of a slap in the face if you know you can't have children for whatever reason. And some women will have already been through the menopause, had gynaecological surgery which would obviate any testing, or may be on HRT. To ask these women to do a pregnancy test each visit is a bit of a waste of time, so maybe there should be a way of identifying them beforehand. And of course, with all the questionnaires to fill in, people unable to read print are excluded, unless some sort of provision is made.

Now for the actual paperwork. Most of the documents are fine; the consent forms and invitation letter are brief and easy to understand. The participant information sheet, however, is extremely long. It's understandable, though it might be an idea to indicate that they've already done research that shows prebiotics affect narrowing of the airways. Some people may not make a connection between gut bacteria and asthma, so without a mention of the previous research may assume the study is of the "interesting but academic" category. But my main concern is that the information sheet is just too much reading. I don't know what can be left out. (Not everyone will bother with or understand ethical approval.) I wonder how many people will either give up halfway through and forget it, or decide to take part in the study without reading all the way through and then get caught out, for example, by not having read the exclusion criteria. More time wasted. Again, I think the sheet is understandable, but maybe it could be made more concise?
I hope all this helps, and makes sense even. As always, contact me (or the researcher can contact me) if something doesn't make sense, etc.

PPI reviewer ten

This reviewer's comments apply to Appendix 3 – comments highlighted in appendix in yellow
It's always interesting to see studies which look at using existing medications in new ways. All the docs you've sent me look pretty good but I think there's scope to make them a bit more user friendly without going too informal.
Invitation Letter
Just a few changes suggested here to simplify things and make the letter a bit more personal and inviting. After all, the idea is to get volunteers!
Patient Info Sheet
This is well laid out and has a good explanation of what the trial is all about. But I have made some suggestions to help the reader through what is a long document.
There's plenty of active voice, which is great to see - but I've suggested even more (e.g. 'Your blood samples will be stored…' becomes 'We'll store your blood samples…'. It does help the sense of narrative which is so important in a long document.
I've simplified quite a lot so that there is less 'friction' for the reader e.g. 'Finally, we will ask you to complete a series of questionnaires that focus on asthma control, quality of life, asthma medication use and nutritional supplement adherence.' becomes 'Finally, we'll ask you to complete a series of questionnaires that check your asthma control, your quality of life, how you're using your asthma medication and how well you're sticking to the nutritional supplement timetable.' A bit longer perhaps, but easier to read I think. BTW, the overall word count is down.
I've split some sections into more paragraphs as some were rather long.

I've introduced some contractions (mainly 'changing 'we will' to 'we'll'). I think they are friendlier and help the flow without being too informal. For emphasis, I've left some in e.g. the exclusion criteria in the 'Can I take part in this study' section.

I rearranged the exclusion criteria so that there is an explanation of what gastrointestinal disease is when it's first mentioned.

I've tried to take words like 'exclusion criteria' out because although most people will understand their meaning, not everyone will and it's not really everyday language. Plus it's not that friendly - no one likes being told they are excluded. Let them down gently!

Overall I don't think my version is as precise, but I don't think it misleads and crucially it is easier to read. You know my mantra - The easier a document is to read, the more informed the consent.

BTW, under the 'possible risks' section the acronym NTU is used. Will the reader know what this means?

Consent forms

Just a couple of minor tweaks.

Hope some of my suggestions go down well and make the final versions.

PPI reviewer eleven

This reviewer's comments apply to Appendix 4 – comments highlighted in appendix in yellow
I have attached my comments for the study looking at prebiotics to improve asthma control.

The two consent forms were ok. Although they appeared really similar and I wonder if participants might think they have two copies of the one consent form and may not fill them both in.
Also first impressions of the patient information sheet…. I was put off it due to the sheer amount of text on each page and then the number of pages too.
I think the concept of the study is valid etc. I don't think they have taken into account how labour intensive it will be for the participants having to go back and forth to the hospital, do lung function. In an ideal world gathering all that data would be great but part

Appendix 6 – Chapter 6 Informed Consent

IRAS Project ID: 253339

Participant ID Number:

CONSENT FORM

Study Title: The Effects of Prebiotic Supplementation on Gastrointestinal Symptoms and Markers of Systemic Inflammation in Healthy Adults. A Double-Blind, Placebo-Controlled, Crossover Trial.

Chief Investigator: Dr Michael Johnson

Researchers: Mr Jacob Jayaratnasingam

	Initials
1. I confirm that I have read the participant information sheet dated: (Version 2.0, Date: 14/10/20) for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to leave at any time without giving any reason, without my medical care or legal rights being affected.	☐
3. I understand that information collected about me, and blood plasma samples collected from me will be used at a later date as stated in the participant information sheet.	☐
4. I agree to take part in the above study.	☐

Participant Name: _____ Date:_____

Signature:_____

Researcher Name: _____ Date:_____ Signature:_____

Appendix 7 – Chapter 6 Participant Information Sheet

NOTTINGHAM TRENT UNIVERSITY

Participant Information Sheet

(version 2.0. 14/10/20)

Title of Study: The Effects of Prebiotic Supplementation on Gastrointestinal Symptoms and Markers of Systemic Inflammation in Healthy Adults. A Double-Blind, Placebo-Controlled, Crossover Trial.

Patient Friendly Title of Study: Compared to a placebo, does feeding the good bacteria in the gut with a prebiotic supplement improve gastrointestinal symptoms and inflammation in the body.

Chief Investigator: Dr Michael Johnson

Researchers: Mr Jacob Jayaratnasingam & Mr Robert Needham

Institution: Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS

We're inviting you to take part in our study. Before deciding, please read the information below carefully. It explains why we're doing the research and what your involvement would be. To help you make a decision, one of our researchers can discuss this information sheet with you. Talk to others about the study if you wish. Taking part is voluntary. You may choose not to take part, or leave the study at any time without giving a reason.

What is the purpose of the study?

Bacteria living in our gut affects our immune system and possibly health. Therefore, increasing the 'good bacteria' in the gut by eating a 'prebiotic' supplement might benefit people's immune function. A prebiotic is 'food' for the good bacteria. This is different from a probiotic, which is the actual live bacteria and which you'll often see in some yoghurts. In 2016 we published a study in the *British Journal of Nutrition*, which showed that when adults with asthma took a prebiotic supplement, their airways did not narrow / constrict as much during a breathing challenge test. We'd now like to know if there are other benefits. Therefore, we'll look at whether a prebiotic supplement benefits people by affecting the chemicals in the blood that tell us about inflammation in the body.

Can I take part in this study?

To take part, you have to:

- Be 18-50 years of age at the date of your first laboratory visit.
- Have a body mass index (BMI) between 18.5-25 $kg \cdot m^2$ (we'll work this out for you using your height and body weight).
- Be a non-smoker.
- Satisfy the researchers that you are willing and able to follow all trial requirements.
- Have told the researchers about any nutritional supplements you take in case taking them rules you out of the study.

Unfortunately, you cannot take part if any of the following apply to you:

- You regularly take Omega-3 supplements, and/or eat high levels of Omega-3 (e.g. more than 1-2 portions of oily fish such as salmon or mackerel a week).
- You take aspirin or other non-steroidal anti-inflammatory drugs such as ibuprofen once a day on 5 or more days of the week.
- Females only: You are pregnant or planning a pregnancy during the time of the study.
- You've taken, in the 4 weeks before signing the consent form, a prebiotic and/or probiotic supplement, or laxatives or drugs that loosen stools and increase bowel movements.
- You're currently taking a daily dose of anti-histamine, which you can't temporarily stop for 72 hours before each testing session without exacerbation of symptoms.
- You follow a vegetarian or vegan diet.
- You've been previously diagnosed with chronic obstructive pulmonary disease (COPD), emphysema, Asthma, chronic bronchitis, or similar respiratory (breathing-related) illness.
- You've a history of heart failure, pulmonary hypertension, embolism, or other pulmonary heart disease.
- You've a history of recurrent chest infections.
- You've had an acute infection in the last four weeks, and/or major operation in the past four months.
- You react badly to gastrointestinal drugs.
- You've taken antibiotics in the past 3 months.
- You've a history or current evidence of gastrointestinal disease (e.g. chronic constipation, diarrhoea, irritable bowel syndrome, Crohn's disease).

- You've recently taken part in other research projects. Please notify us so we can see if this excludes you from the study.
- You are or you believe you may be lactose intolerant.
- You regularly take antioxidant supplements, such as beta-carotene, vitamin A, vitamin C, vitamin E, lutein and selenium. Standard multivitamin and mineral supplements are ok; however, if a single supplement (e.g. Vitamin C) is more than the recommended daily DRV's (dietary reference values) you need to check this with the researchers.

Study location and time commitment

We'll ask you to visit our laboratory in the Erasmus Darwin Building at Nottingham Trent University's Clifton Campus five times over a 9-week period. Visit 1 will last about 1 hour, and visits 2-5 will last about 1 hour and 30 minutes (7-9 hours in total).

What will I have to do if I take part in this study?

You can continue with your regular diet during the study. **You need to drink 500ml of water 1-hour before each visit**. If required, females will do a pregnancy test at the start of each visit. After visit 2, we'll give you a prebiotic supplement or a taste/appearance-matched dummy supplement (i.e. a placebo that has no effect). You need to take the supplement for 3-weeks. Then, after a 2-week "washout" (no supplement) period, you'll take the other supplement for 3 weeks. Visits 2-5 take place at the start and at the end of each 3-week period.

Visit 1: Initial consultation and familiarisation

We'll check that you're suitable for the study. Then we'll tell you all about the study and answer any questions you have. If you want to go ahead and take part, we'll ask you to sign a written consent form. We'll ask you to complete a health screen and health history questionnaire, and we'll measure your height and body weight to work out your body mass index. Finally, we'll explain what you need to do for visit 2.

Visits 2-5: Main experimental visits

We'll ask you to record your diet for the 24 hours before visit 2. We also need you to complete a 4-day food diary during the four days leading up to visits 3, 4 and 5. We'll explain how to do this during visit 1. When you arrive at the laboratory we'll collect your 24-hour (visit 2) or 4-day (visits 3-5) food diary. You'll then complete a health screen and health history questionnaire, and give written consent for us to take your blood. We'll then measure your body weight and take the blood sample. Finally, we'll ask you to complete

some questionnaires about how well you're sticking to the nutritional supplement timetable and your gastrointestinal symptoms.

General testing restrictions

General testing restrictions and their duration apply to all visits (see Table 1).

Table 1. General Testing Restriction Guidelines

	How long to avoid (Before each visit)
Anti-Histamines	72 Hours
Caffeine	24 Hours
Alcohol	48 Hours
Food	3 Hours
Strenuous exercise	24 Hours

What nutritional supplements will I be given in this study?

We'll ask you to consume two powder-based nutritional supplements. One will be a prebiotic, and the other a taste/appearance-matched placebo containing maltodextrin. Neither you nor the researchers will know which supplement you are taking at any time – this is only revealed at the end of the study. The prebiotic contains galactooligosaccharides, lactose (up to 16%), glucose and galactose (see Table 2). It's gluten free and doesn't contain artificial flavourings or colourings, or genetically modified organisms. The prebiotic supplement is GRAS (generally recognised as safe) certified (GRN: 000484).

Table 2. Nutritional information for prebiotic supplement.

	Per sachet (3.65g)	Per 100g
Energy	41.5 kj/10.1kcal	1138kj/276kcal
Fat	<0.1g	<0.1g
(of which are saturates)	<0.0g	<0.1g
Carbohydrates	2.0g	42.1g
(of which are sugars)	1.0g	15.6g
Fibre	2.0g	53.4g
Protein	0.0g	0.1g
Salt	<0.1g	2.0g
Galactooligosaccharides	2.9g	79.7g

You'll take one supplement sachet around the same time each morning. The supplements can be dissolved in tea, coffee or water. You need to take the supplements in the same way throughout the study. We won't carry on giving you the supplements after the study, but we can tell you where to buy similar products.

Which supplement will I take first?

You won't know because in this type of study it's important that we don't bias the results in any way. So at the start of the study we'll use a computer programme to randomly allocate you to your first supplement. We won't find out which one this is until the study is completed.

How will my blood be taken and stored?

We'll ask you to tell us of any fears/phobias (e.g. of blood or needles) or allergies (e.g. Elastoplast, alcohol, latex). During each visit, a trained phlebotomist will take up to 10ml of blood using a needle inserted into a vein. Before we take your blood, we will make sure you are comfortable and give you time to ask any questions you may have. We will process samples, on the day in the John Van Geest Cancer Research Centre at Nottingham Trent University. Blood plasma will be stored as we intend to perform additional tests on your plasma cells as part of the current research study.

What are my responsibilities?

If you are not comfortable with any of the procedures, please tell us as soon as possible. We need you to complete all documents accurately, and follow all instructions / guidelines throughout the study. You need to take the nutritional supplements as instructed. If you miss taking any sachets, don't compensate by taking multiple sachets. Just get back on to the instructed dosing timetable and write down any discrepancies. Let us know if you haven't followed any of these instructions, and/or if there are changes that affect you taking part in this study (e.g. antibiotics use).

What are the potential benefits of taking part?

The prebiotic supplement may improve your overall immune health.

What COVID-19 safety measures are there in place?

All paperwork will now be completed online to minimise contact as well as daily completion of a COVID symptom questionnaires leading up to each laboratory visit. PPE will be provided for both researcher and participant; this includes both a surgical mask and visor for blood sampling.

What are the possible risks and disadvantages of taking part?

You may experience discomfort when we take your blood. However, only trained phlebotomists will take your blood. Therefore, the risk of bruising and swelling is low. Performing spirometry can make you cough and feel light-headed, although these symptoms don't last long. The prebiotic supplement may cause some temporary gastrointestinal discomfort (e.g. abdominal bloating, flatulence, abdominal cramps/pains, nausea). However, this is mainly down to taking a higher daily dose of prebiotic, or

because you already have a gastrointestinal disorder. If you keep getting gastrointestinal discomfort, stop taking the supplement and contact us immediately.

How will we use the results from the study?

We'll present the findings of this study at a scientific conference, and publish the results in a scientific journal. We'll anonymise all data so you can't be identified in any report or publication. You'll receive a copy of the final findings, as well as your own results if you wish to have these.

Will my information be kept confidential?

Yes. We'll keep all information that we collect from you strictly confidential and store it in a locked office at Nottingham Trent University, and on password-protected computers/user accounts there. We'll use a secure online server that only the researchers can access. We'll assign you a unique code so that your information and data are anonymised. Your information may be retained for up to 5-10 years, but will be disposed of securely when no longer required, in line with Data Protection Legislation. We don't destroy your signed informed consent form.

Females only: What if I become pregnant during the study?

Unfortunately, you'll have to leave the study. We'll ask females to do a pregnancy test at the start of each visit. If you know that you are not able to bear children then you will not have to do a pregnancy test – we just ask that you tell us this at the start of the study.

How are we avoiding biased sampling (i.e. participant recruitment)?

We'll avoid biased sampling by recruiting participants from a variety of places, including all Nottingham Trent University campuses, the student union, local leisure centres, and gyms.

What safeguarding mechanisms are in place?

A qualified first aider trained in minimum basic life support (e.g. CPR) will be there during all visits, with a second first aider on standby and able to be on site within 30 seconds. An automated external defibrillator and supplemental oxygen is on hand. If required, emergency services will be called out. The travel time from The Queens Medical Centre to Nottingham Trent University is about 8 minutes. The ambulance crew can park outside our building, and the laboratory can be acccessed with a wheeled stretcher.

What if we find something considered abnormal?

The researchers will discuss any abnormal findings. Then, if we think it necessary and with your consent, we'll notify your GP so that they can follow this up.

Injury and trial complaints procedure / independent complaints service

In the event of injury/illness caused by negligence by the research team, you may contact the Research Sponsor for independent advice: Professor Barbara Pierscionek, Associate Dean for Research, Nottingham Trent University (contact details are provided in the next section).

What if there is a problem?

If you have any concerns regarding any aspect of this study, speak to one of the researchers who will strive to answer your questions – their contact details are found at the end of this information sheet. If you aren't satisfied, you can make a formal complaint to our research sponsor Professor Barbara Pierscionek, Associate Dean for Research, Nottingham Trent University: Email: Barbara.Pierscionek@ntu.ac.uk TEL: 0115 84 83738

Travel Reimbursement

We don't pay you to take part in this study, but we will pay travel expenses according to Nottingham Trent University's policy - £0.25 per mile for travel by car.

Who has reviewed the study?

The East Midlands, Nottingham 1 Research Ethics Committee has reveiewed the study.

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