The gut microbiota as a novel nutritional target to influence systemic inflammation associated with prevalent health disorders

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Declaration

The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

List of Data Provided and/or Analysis Carried out by Collaborators:

- The Fat on Fire study was previously conducted at University Hospital of Coventry and Warwickshire and University of Warwick. All adipose tissue and blood samples were processed by previous researchers. Analysis relating to gene expression of mitochondrial and adipocyte browning genes were carried out by previous researchers and made available on a database which the lab group were granted access to.

Published work:

The content within Chapter 1 of this thesis has been previously published (further details on page xiv), with the exception of section 1.6 which was added for this thesis.

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List of Abbreviations

2-DG	2-Deoxy-D-Glucose
AbdOmAT	Abdonminal omental adipose tissue
AbdScAT	Abdominal subcutaneous adipose tissue
AHR	Airway hyperresponsiveness
AT	Adipose tissue
B-GOS	Bimuno-galactoligosaccharide
BAT	Brown adipose tissue
BMI	Body mass index
BSA	Bovine serum albumin
CCR2	C Chemokine receptor 2
CIDEA	Cell death inducing DFFA-like effector A
COX4	Cytochrome c oxidase subunit 4
CVD	Cardiovascular disease
CXCL10	C-X-C motif chemokine ligand 10
CXCL8	C-X-C motif chemokine ligand 8
DRP1	Dynamin-related protein 1
ECAR	Extracellular acidification rate
ECL	Enhanced chemiluminescence
ELOVL3	Elongation of very long chain fatty acids 3
FBN1	Fibrillin-1
FBS	Foetal Bovine Serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FEF_{25-75}	Forced expiratory flow at 25-75%
FEF_{75-85}	Forced expiratory flow at 75-85%
FEV1	Forced expiratory volume in 1s
FEV6	Forced expiratory volume in 6s
FFAR	Free fatty acid receptor
FIS1	Mitochondrial fission protein 1
FVC	Forced vital capacity
HDL-C	High-density lipoprotein cholesterol

HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HRP	Horseradish Peroxidase
IkBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IKKγ	Inhibitor of nuclear factor kappa-B kinase subunit gamma
IL	Interleukin
JNK	c-Jun N-terminal kinases
LDL-C	Low-density lipoprotein cholesterol
LPS	Lipopolysaccharide
MAPK8	Mitogen-Activated Protein Kinase 8
MCP-1	Monocyte chemoattractant protein 1
MFN2	Mitofusin-2
MLCK	Myosin Light Chain Kinase
MT-ND1	Mitochondrial NADH-ubiquinone oxidoreductase chain 1
mtATP6	Mitochondrially encoded ATP synthase membrane subunit 6
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRF1	Nuclear respiratory factor 1
OCR	Oxygen comsumption rate
OPA1	OPA1 mitochondrial dynamin like GTPase
OR	Odds ratio
OR4M1	Olfactory Receptor Family 4 Subfamily M Member 1
PAI-1	Plasminogen activator inhibitor 1
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEF	Peak expiratory flow
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PLIN5	Perilipin-5
POLG	DNA polymerase subunit gamma
PPARG	Peroxisome Proliferator Activated Receptor Gamma

PPARGC1A	PPARG Coactivator 1 Alpha
PRC	PPARG related coactivator 1
RIPA	Radioimmunoprecipitation Assay
ROS	Reactive oxygen species
Rot/AA	Rotenone and Antimycin A
SCFA	Short chain fatty acid
SD	Standard deviation
SEM	Standard error of the mean
SLC27A2	Solute carrier 27A
SOD2	Superoxidase dismutase 2
T2DM	Type 2 Diabetes Mellitus
TBS	Tris buffered saline
TFAM	Mitochondrial transcription factor A
TG	Triglycerides
TGF-β1	Transforming growth factor beta 1
Th	T helper cell
TLR-4	Toll-like receptor 4
TNFα	Tumour necrosis factor alpha
UCP-1	Uncoupling protein 1
VAT	Viseral adipose tissue
WAT	White adipose tissue
WBC	White blood cell
β-ME	β-mercaptoethanol

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Publications

Part of this thesis has previously been published as an open access review article:

Lad, N., Murphy, A. M., Parenti, C., Nelson, C. P., Williams, N. C., Sharpe, G. R., & McTernan, P. G. (2021). Asthma and Obesity: endotoxin another insult to add to injury?. *Clinical science (London, England : 1979)*, *135*(24), 2729–2748.

Other publications:

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Abstract

Systemic inflammation is an underlying symptom present in many diseases including Asthma and Obesity. This inflammation may arise internally with the release of pro-inflammatory adipokines such as asprosin, or through increased gut permeability which allows endotoxin, derived from gram-negative bacteria, to cross into the bloodstream and mediate an innate immune inflammatory response. Both asprosin and endotoxin are elevated in conditions of metabolic disease, which share a similar inflammatory profile with Asthma. Currently, Asthma treatments relieve symptoms rather than targeting the trigger that causes the inflammation. Therefore, to target the source of the inflammation, the use of internally derived anti-inflammatory agents, such as short chain fatty acids (SCFAs) may provide a therapeutic answer. SCFAs are produced from the metabolism of dietary substrates (*e.g.* prebiotics) by the gut microbiota, which appear to provide anti-inflammatory health benefits systemically as well as locally.

Therefore, a series of studies addressed the role of SCFAs (acetate, butyrate, and propionate) on endotoxin- and asprosin-induced inflammation in models of Asthma and Obesity using human bronchial epithelial cells and human white adipocyte cells to assess their impact on metabolism. Molecular techniques including Western blot and RT-qPCR, and mitochondrial dynamic analysis were explored to examine metabolism. Furthermore, a human invention trial was conducted to evaluate the use of prebiotics to improve inflammation, lung function, and Asthma symptoms in participants.

The outcomes of these *in vitro* studies suggest that SCFAs have the capacity to mitigate endotoxin and asprosin induced inflammation in airway and adipocyte cells. Whilst *in vivo* studies, where participants with Asthma were given a prebiotic supplementation, showed improvements in serum metabolic markers in. Taken together, these data suggest that increasing SCFA production through dietary interventions may provide a novel management tool to relieve cellular inflammatory damage in patients with Asthma or Obesity-related Asthma to potentially relieve Asthma symptoms over time.

Chapter 1: Introduction

1.1. Health and Economic burden of Asthma

Asthma is an inflammatory disease of the airways affecting 262 million people worldwide (World Health Organisation), with a cost to the US of $81.9 billion per year (Nurmagambetov, Kuwahara and Garbe, 2018), and to Europe of €19.3 billion a year (Nunes, Pereira and Morais-Almeida, 2017). The UK contributes substantially to the European costs, with an annual charge of £4.9 billion, £1.1 billion of which is paid by the National Health Service (NHS) (Mukherjee *et al.*, 2016; Nunes, Pereira and Morais-Almeida, 2017). As such the financial burden for health economies is clear, but as Asthma is often not considered a life-threatening disease in the main part, agents to relieve symptoms have been the main driver, with new therapies less forth coming than other low-grade chronic inflammatory diseases in recent years. This may in part be due to less overall funding given for Asthma research, as the National Institute of Health reported recently that Asthma research received approximately $388 million in funding, compared with billions of dollars of funding for the areas of cardiovascular ($2.5 billion), Diabetes ($1.2 billion), and Obesity ($1.1 billion) research (National Institute of Health, 2021). Despite the reduced comparable funding, Asthma can be a deliberating, life threatening, life-long inflammatory disease with significant quality of life and health economy impact.

1.2. Pathophysiology of Asthma

Asthma may arise as the human airway moves into a state of hypersensitivity, described as airway hyperresponsiveness (AHR), and occurs due to an increased production of inflammatory mediators including histamine and inflammatory cytokines, of which

leukocytes (or white blood cells; WBC) are a source. This AHR results in bronchoconstriction, increased mucus production and leads to symptoms including coughing, wheezing, shortness of breath and chest tightness. Asthma can develop at any stage in life, though it is common to develop Asthma as a child which then becomes less severe as an adult. Several factors can increase the risk of developing Asthma such as genetics, lifestyle, various environmental triggers and allergens. Allergens can include particulate matter such as dust, dust mites, pollutants, smoking and pollen. Lifestyle can become an important risk factor as an adult, as this is typically when triggers such as smoking, weight gain, and exercise can lead to the development of Asthma.

1.3. Phenotypes of Asthma

Asthma severity can range from mild through to moderate and severe. Severe Asthma, defined as Asthma which is uncontrolled or requires multiple therapies in order for it to become controlled (Chung *et al.*, 2014), can be categorised into two inflammatory phenotypes based on the immune cell type present in the patient. The severe phenotypes are T-Helper cell 2 (Th2) mediated and non-Th2 mediated, which indicate whether or not Th2 cells are present as part of the inflammatory response. These severe phenotypes can be further categorised based on the immune cells that are present during an Asthmatic episode (Figure 1.3).

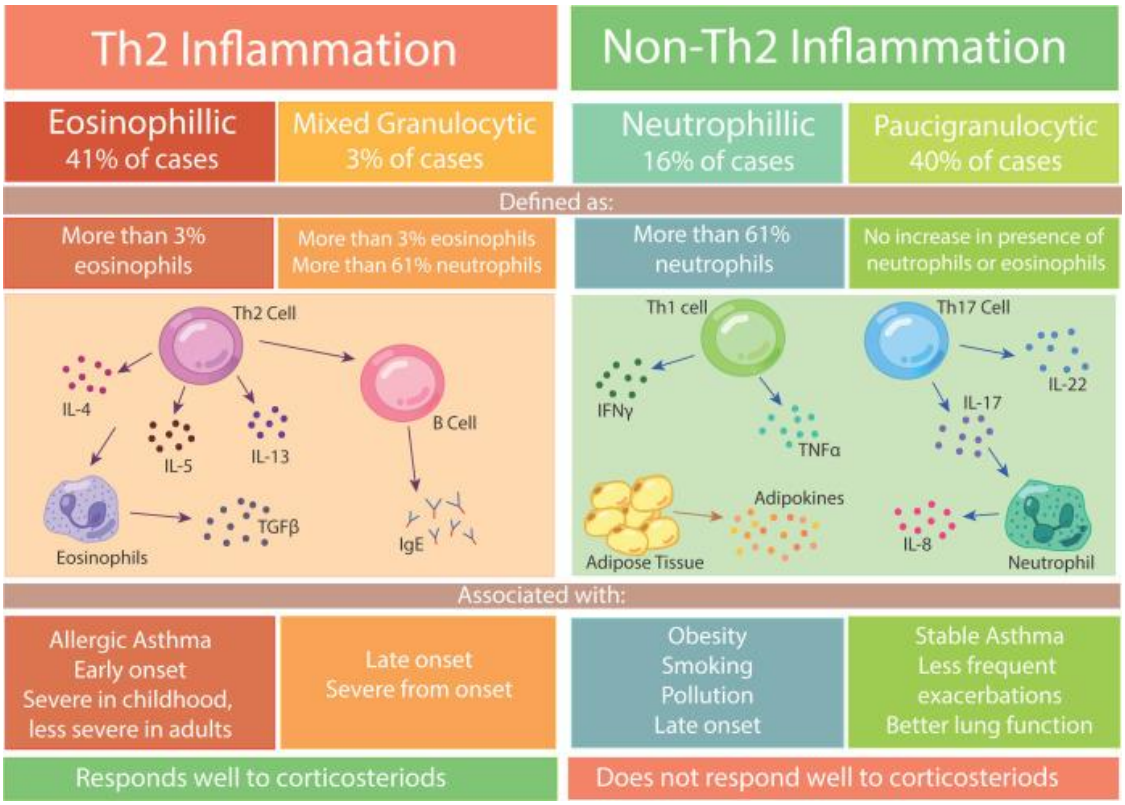

Figure 1.3: The phenotypes of severe Asthma

Inflammation in severe Asthma can be divided into T helper cell 2 (Th2) or Non-Th2. Th2 inflammation can be eosinophilic or mixed granulocytic, and is mainly associated with allergic Asthma and early onset Asthma. Non-Th2 inflammation is either neutrophilic or paucigranulocytic, with the former being mostly associated with lifestyle factors such as Obesity and smoking. Th2 inflammation responds well to corticosteroids, where as non-Th2 inflammation does not respond well to corticosteroids.

Two of the main classes of immune cells that help characterise these severe phenotypes of Asthma are eosinophils and neutrophils. Eosinophils have a bilobed nucleus and can initiate an allergic immune response, which leads to AHR and mucus overproduction in Asthma. Neutrophils are the most abundant WBC, they have a multilobed nucleus and are a type of phagocyte cell, able to engulf bacteria. In Asthma, neutrophils contribute to chronic inflammation through the release of pro-inflammatory cytokines including, IL-8, IL-1β, and TNFα.

The Th2 phenotype can either be defined as eosinophilic Asthma, in which there is an increase in eosinophils, or mixed granulocytic if there is a surge in both eosinophils and neutrophils (Figure 1). The non-Th2 phenotype is associated with neutrophilic Asthma, which is dominated by neutrophils, or is paucigranulocytic, if there is no increase in either eosinophils or neutrophils. It has been established from prior studies that amongst subjects with Asthma, 41% appear to be eosinophilic, 40% paucigranulocytic, 16% neutrophilic and 3% were mixed granulocytic (Schleich *et al.*, 2013). Understanding the type of leukocyte that is activated during an exacerbation is crucial in order to provide the correct Asthma treatments to patients, as 56% of patients fall under the non-Th2 phenotype, which tends to be more poorly controlled and does not respond well to treatments (Esteban-Gorgojo *et al.*, 2018; Tliba and Panettieri, 2019). The non-Th2 phenotype of severe Asthma, which arises due to the neutrophilic immune response, is also associated with corticosteroid resistance (Ray and Kolls, 2017; Wadhwa *et al.*, 2019), corticosteroids being used as a common therapy for most Asthma cases.

In cases where Asthma can be controlled by corticosteroids and leukotriene modifiers, the medication mitigates the inflammatory response and reverses bronchoconstriction. Specifically, the medication reduces inflammation by supressing the activity of leukocytes such as eosinophils, which subsequently leads to the reduction of inflammatory cytokines. Other medicines such as short- and long-acting beta agonists can relax the airway smooth muscle to widen the airway and provide noted relief. However, persistent use of these medications can cause common side effects (1 in 100 people) including muscle cramps, increased heart rate, headaches and feeling unstable, and as such, beta agonists are not always well tolerated by patients (Nelson *et al.*, 2006;

Cates and Cates, 2012; Cates *et al.*, 2014). Since current therapies act predominantly to reduce symptoms, there is a clear need to develop new therapies, that are more well tolerated by patients, to ameliorate inflammation at source rather than targeting the arising symptoms.

1.4. Comorbidities of Asthma

Asthma has several co-morbidities including respiratory diseases such as sinusitis, rhinitis, chronic obstructive pulmonary disease (COPD), and common respiratory viruses (Boulet and Boulay, 2014). Furthermore, metabolic related diseases also increase the risk of Asthma, and often have a shared profile of underlying inflammation.

1.4.1. The role of Obesity in Asthma

Clinical Obesity, defined as an excess accumulation of fat which impairs health and a BMI over 30 kg/m^2, appears to be a critical risk factor for developing Asthma in both children and adults (Forno *et al.*, 2018; Peters, Dixon and Forno, 2018). In the USA, 60% of adults with severe Asthma are obese (Peters, Dixon and Forno, 2018). Furthermore, studies continue to re-affirm that Asthma prevalence is higher in obese adults (11.1%) than in lean adults (7.1%), with the highest prevalence being in obese women (14.6%) compared with obese men (7.1%) (Akinbami and Fryar, 2016). In addition, obese women appear more likely to have frequent exacerbations (To *et al.*, 2018).

Adipose tissue itself has an increased response to inflammatory cells and stimuli, through activated leukocytes and an increase in the release of adipokines *via* other

mediators. Indirectly, it has also been suggested that excess abdominal visceral fat accumulation in Obesity can exacerbate Asthma due do the excess fat causing increased pressure on the diaphragm, which in turn constricts the lungs (Dixon and Peters, 2018; M. S. Yang *et al.*, 2018).

The impact of Obesity to heighten the risk of Asthma is observed in babies born to obese mothers, where they are at a higher risk of developing Asthma with an odds ratio (OR) of 1.31-1.34 (31-34% increase) (Erick Forno *et al.*, 2014; Dumas *et al.*, 2016). This risk is furthered if weight gain in the child continues in infancy, where the risk of developing Asthma is sustained with a similar OR of 1.30 (30% increase) (Popovic *et al.*, 2016; Byberg *et al.*, 2018; Casas *et al.*, 2018). The prevalence of Asthma in children increases proportionally with changes in BMI (Cottrell *et al.*, 2011), with children who are obese exhibiting a 2-fold increased risk in developing Asthma compared with non-obese children (Chen *et al.*, 2013; E Forno *et al.*, 2014).

People with Asthma and Obesity also tend to develop more severe Asthma due to the presence of neutrophilic inflammation and the increased inflammatory response adipose tissue poses compared to lean individuals with Asthma. As such, as the volume of adipose tissue increases, the immune cell composition within the adipose tissue shifts from a Th2 response to a Th1/17 response (Karczewski *et al.*, 2018) with an increased presence of neutrophilic inflammation (Scott *et al.*, 2011). In individuals with Asthma and Obesity, inflammation appears to be predominately driven by the Th1/17 cellular response (Kim *et al.*, 2014; Rastogi *et al.*, 2015; Everaere *et al.*, 2016), leading to less symptomatic control by corticosteroid medication, as they typically target the Th2

response, meaning less well controlled exacerbations and a more severe Asthmatic response (Scott *et al.*, 2011; de Jesus *et al.*, 2018). In addition to inhaled allergens heightening this immune response and inflammation in Asthma, Obesity may further exacerbate this inflammatory response due to the increased level of endotoxin noted in obese patients, which triggers an inflammatory response through activation of Toll-like receptor 4 (Piya, Harte and McTernan, 2013).

1.4.2. The association between Asthma and Diabetes

Diabetes Mellitus is a group of diseases in which there is a high blood glucose concentration. The main types of Diabetes Mellitus are (i) Type 1 Diabetes Mellitus (T1DM), where the immune system damages beta cells in the pancreas, and therefore are unable to produce insulin; (ii) Type 2 Diabetes Mellitus (T2DM), in which not enough insulin is produced or cells do not respond to insulin properly; and (iii) Gestational Diabetes Mellitus (GDM), where high levels of blood glucose are developed during pregnancy. Despite the shared chronic inflammation and common risk factors such as Obesity, there are conflicting reports as to whether patients with Diabetes are at a higher risk of developing Asthma (Ehrlich *et al.*, 2010; Mueller *et al.*, 2013; Baek *et al.*, 2018).

There are limited data exploring the connection between Asthma and Diabetes. It is thought that severe Asthma is related to poor glycaemic control in T2DM patients, which in turn can reduce lung function (Rogala, Bożek and Gluck, 2019; Torres *et al.*, 2021). Furthermore, Diabetes has a negative impact on airway mechanics, which is further exacerbated with the presence of Obesity which also worsens gas exchange within the

lungs (Südy *et al.*, 2021). The underlying inflammation present in diabetic patients may further predispose their risk to respiratory diseases (Moin *et al.*, 2021).

Only one study to date has shown that if a pregnant mother has T2DM or develops GDM, their child has a higher risk of developing Asthma (Martinez *et al.*, 2020). There is evidence that pregnant women with Asthma have a risk of developing T2DM, due to the underlying inflammation that is present (Song *et al.*, 2010). On the other hand, a large-scale study in Korea found no association between Asthma and Diabetes (Baek *et al.*, 2018), and another study showed that the association between Asthma and Diabetes was most likely due to adiposity (Dandona *et al.*, 2014).

1.4.3. The influence of ethnicity on Asthma

Ethnicity may influence the onset and severity of Asthma, particularly in ethnic minority groups due to treatments being based off studies involving predominantly White-Caucasian patients (Pham, Hew and Dharmage, 2021). In the USA, African American patients with Asthma had lower lung function, higher levels of Asthma biomarkers and more instances of uncontrolled Asthma compared to Caucasian Americans (Nyenhuis *et al.*, 2017). A large-scale study undertaken in a Scottish population revealed that South Asian patients with Asthma, in particular those of Pakistani descent, had up to a 50% higher chance of hospitalisation than White Scottish patients, whereas Chinese patients had a 40% lower chance of hospitalisation (Sheikh *et al.*, 2016). Similarly in Australia, migrants of South-East and South Asian ethnicity were more likely to develop Asthma symptoms (Leung *et al.*, 1994; Gibson *et al.*, 2003; Thien *et al.*, 2018).

Combined with the known elevated risk of chronic inflammatory diseases in non-Caucasian adults, the OR of developing Asthma rises if subjects are obese, altered with varying ethnicities: African American (OR 2.9) and Hispanic males (OR 2.7) (Kim and Camargo, 2003) and Indian (OR 1.92) and Chinese women (OR 2.1) (Celedón *et al.*, 2001; Mishra, 2004).

1.5. Molecular links between Asthma and Obesity

1.5.1. The role of Adipokines on Asthma

Beyond several known causes that directly affect lung function such as inhalation of irritants including dust mites, pollen and pollutants, other endogenous mediators of Asthma appear to play their part. Similarly to individuals with Asthma, an underlying systemic inflammation profile is also observed in people during weight gain. These pro-inflammatory cytokines, released from adipose tissue and referred to as adipokines (Taylor, 2021), activate the innate immune response in adipocytes leading to further inflammation (Kusminski *et al.*, 2007; Conde *et al.*, 2011; Mancuso, 2016). As the volume of adipose tissue in the body rises, as in Obesity, there is an associated pro-inflammatory profile of adipokines produced. These adipokines can have a range of systemic cellular and damaging functional organ effects including on lung tissue (Table 1.5.1). As such, adipokines provides an insight into how Obesity can influence Asthma and other chronic diseases.

Adipokine	Effect in Adipose Tissue	Effect in Airway Cells	References
Leptin	Increases lipolysis	Causes Bronchodilation	(Watanabe et al. 2019; Palhinha et al. 2019)
	Promotes adipogenesis	Increases production of chemokines and cytokines - Eotaxin, MCP-1, IL-8, IL-6 and CXCL10	
	Causes the release of pro-inflammatory cytokines including IL-6 and TNFα		
Adiponectin	Increases glucose uptake in fat cells	Increases released of anti-inflammatory cytokine IL-10	(Stern et al. 2016)
	Enhances adipogenesis and lipid storage	Decreases release of pro-inflammatory IL-6 and TNFα	
IL-6	Increases leptin secretion and lipolysis	Promotes ciliogenesis	(Tadokoro et al. 2014; Wueest, Konrad 2018)
	Supresses satiety signals, therefore increasing hunger		
Resistin	Inhibits adiponectin secretion and induces lipolysis	Upregulates mucin production	(Kusminski et al. 2007; Chen et al. 2014; Ikeda et al. 2014)
	Activates innate immune response		
	Regulates expression of PAI-1		
TNFα	Causes mitochondrial dysfunction	Induces apoptosis in cells infected by *Legionella pneumophila*	(Souza et al. 2003; Chen et al. 2010; Kawamoto et al. 2017; Peraldi et al. 1996; Cawthorn, Sethi 2008)
	Alters adipokine production		
	Induces lipolysis		
	Impairs insulin signalling		
Angiotensin	Activates Ca2+ signalling pathways	Angiotensin I converted to angiotensin II in lungs	(Dolgacheva et al. 2016; Than et al. 2017)
	Promotes adipocyte browning		
Visfatin	Involved in brown adipocyte thermogenesis and can decrease UCP-1 expression at high concentrations	Increases mucin production via activation of NF-κB pathway	(Song et al. 2014; Dimitriadis et al. 2019)
MCP-1	Causes insulin resistance and recruits macrophages	Upregulates mucin production through CCR2 receptor	(Dahlman et al. 2005; Monzon et al. 2011)
TGF-β1	Regulates adipocyte browning	Induces PAI-1 expression in airway epithelial cells	(Cho et al. 2015; Wankhade et al. 2018)
PAI-1	Causes inflammation	Causes airway hyperresponsiveness, inflammation and remodelling	(Jo et al. 2018; Liu et al. 2019)
IL-8	Causes insulin resistance via inhibition of Akt phosphorylation	Increases Ca2+ release from airway smooth muscles cells, leads to constriction of airways	(Govindaraju et al. 2008; Kobashi et al. 2009)
IL-10	Prevents adipocyte differentiation and lipid accumulation	Reduces airway inflammation and hyperresponsiveness	(Branchett, Lloyd 2019; Kim, Pyo 2019)
IL-17α	Induced expression of TNFα, IL-6, IL-1β, leptin, and glucose transporter 4	Causes bronchoconstriction and airway hyperresponsiveness	(Kudo et al. 2012; Qu et al. 2016)
IL-1β	Inhibits insulin signalling and glucose transport	Involved in airway cell migration	(White et al. 2008; Bing 2015)
	Increases lipolysis		
	Increases inflammation		

Table 1.5.1: List of the effect of adipokines in adipose tissue and airway cells.

Effect of adipokines on adipose tissue and airway cells which may lead to widespread systemic effects in disease. In adipose tissue this may include increased inflammation, altered lipid and glucose homeostasis and regulation of adipocyte browning. In airway cells, adipokines can control the inflammatory response, airway hyperresponsiveness, bronchoconstriction, bronchodilation and mucin production. In both cell types, these effects can lead to an exacerbation or relief from inflammatory diseases.

Abbreviations in table: C-C Chemokine receptor 2 (CCR2); C-X-C motif chemokine ligand 10 (CXCL10); Interleukin (IL) - 1β, -6, -8, -10, -17α; Tumour necrosis factor alpha (TNFα);

Monocyte chemoattractant protein 1 (MCP-1); Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) Plasminogen activator inhibitor 1 (PAI-1); Transforming growth factor beta 1 (TGF-β1).

Beyond the considered normal function of these adipokines in routine homeostasis such as satiety, blood pressure and glucose regulation, these factors have functions which can impact the health of lung tissue. This occurs when the balance of pro- and anti-inflammatory factors, often mainly derived from adipose tissue, is shifted towards a pro-inflammatory state. From prior studies it has been observed that obese subjects with Asthma have more circulating leptin and a reduction in the anti-inflammatory adipokine, adiponectin, than lean people with Asthma (Sideleva *et al.*, 2012; Nigro *et al.*, 2015; Rastogi *et al.*, 2015; Al-Ayed *et al.*, 2019). This appears important as in patients with Asthma, leptin induces inflammation in lung fibroblasts by enhancing the production of further pro-inflammatory chemokines and cytokines (Watanabe *et al.*, 2019), which appears somewhat supressed when a patient experiences leptin resistance. In contrast, adiponectin can have an anti-inflammatory effect in airway cells by promoting the release of the anti-inflammatory cytokine IL-10 and the inhibition of pro-inflammatory cytokines IL-6 and TNFα (Nigro *et al.*, 2013). However, due to the shift in leptin and adiponectin levels in obese patients with Asthma, the adipokines can mediate more inflammation and AHR.

1.6. The influence of adipokine Asprosin on disease

Asprosin is a recently discovered adipokine which is mainly expressed in white adipose tissue (Romere *et al.*, 2016). It is encoded by exons 65-66 of the FBN1 gene which

transcribes a preprotein called profibrillin, which when cleaved produces the proteins fibrilin-1 and asprosin (Romere *et al.*, 2016). Asprosin has been showed to have varied roles depending on the target tissue, where it has been shown to be damaging in skeletal muscle and pancreas tissues (Jung *et al.*, 2019; T. Lee *et al.*, 2019), but has protective effects in the heart (Z Zhang *et al.*, 2019; Wen *et al.*, 2020).

1.6.1. Asprosin in appetite and glucose regulation

Through activation of the G-protein coupled receptor (GPCR) OLFR734 (Li *et al.*, 2019), asprosin is able to stimulate the production and release of glucose in hepatocytes during a fasted state (Romere *et al.*, 2016). In addition to its glucogenic effects during fasting, asprosin acts as an orexigenic hormone. OLFR734 is also expressed in the AgRP+ neurons in the arcuate nucleus in the hypothalamus, and when activated by asprosin increases the firing rate of the neurons to promote hunger, whilst decreasing the firing rate of the POMC+ neurons to decrease satiety (Duerrschmid *et al.*, 2017). Whilst this process occurs in a healthy, fasted individual, an excess of asprosin in the system may lead to differing effects in different tissues and organs in the body. These preliminary studies were performed in mouse tissues, but it has been identified that the human variant of OLFR734 is OR4M1 (Li *et al.*, 2019; Hoffmann, Xie and Chopra, 2020; Kerslake *et al.*, 2021).

1.6.2. Asprosin in insulin resistance and inflammation

Although asprosin regulates glucose homeostasis in the liver, it also plays a role in insulin resistance. In a primary human skeletal muscle and a mouse derived skeletal muscle cell

line (C2C12), asprosin caused insulin resistance and reduced the uptake of glucose (Jung *et al.*, 2019). In addition, mice treated with asprosin exhibited insulin resistance, increased ER stress and increased inflammation via the release of pro-inflammatory cytokines IL-6, TNFα and MCP-1 (Jung *et al.*, 2019). The actions of asprosin in the skeletal muscle were shown to be mediated by protein kinase C delta (PKCδ) (Jung *et al.*, 2019), which has previously shown to cause insulin sensitivity and inflammation (Kontny *et al.*, 1999; Greene *et al.*, 2004; Li *et al.*, 2015).

Similar effects were shown in a mouse pancreatic β-cell cell line (MIN6), where treatment with asprosin activated the JNK pathway through TLR4 activation (T. Lee *et al.*, 2019). The phosphorylation of JNK was shown to induce apoptosis and phosphorylation of NF-κB, which lead to the release of pro-inflammatory TNFα and MCP-1 (T. Lee *et al.*, 2019). These data suggest that an excess of asprosin in the system due to its increased release with adiposity may possibly be contributing to underlying inflammation and insulin resistance seen in many metabolic related diseases.

1.6.3. Beneficial Effects of Asprosin

In contrast to the damaging pro-inflammatory effects of asprosin, there may also be a protective role for the adipokine. A study by Wen *et al.* showed that although patients with dilated cardiomyopathy had higher circulating asprosin, the patients with higher levels of asprosin had a significantly higher survival rate than patients with lower asprosin levels (Wen *et al.*, 2020). Further *in vitro* studies revealed that in cardiomyocytes, asprosin was in fact able to restore mitochondrial function in hypoxic

conditions, therefore having a protective effect in the heart (Wen *et al.*, 2020). Moreover, asprosin had the same effect to mitochondrial health in mesenchymal stromal cells by reducing reactive oxygen species (ROS) and increasing superoxidase dismutase 2 (SOD2) (Z. Zhang et al. 2019). When mesenchymal stromal cells pre-treated with asprosin were injected into the hearts of mice who suffered a myocardial infarction, the survival rate was improved (Z. Zhang et al. 2019).

1.6.4. Human Studies with Asprosin

Studies in humans have revealed associations between asprosin and an array of diseases. Children with Obesity appear to have a lower level of asprosin than lean children (Long *et al.*, 2019; Corica *et al.*, 2021). In adults this trend is reversed, patients with Obesity had higher circulating asprosin than healthy patients (Ugur and Aydin, 2019; Wang *et al.*, 2019). Furthermore, 6 months after bariatric surgery, asprosin levels were significantly decreased (Wang *et al.*, 2019). The increased asprosin may not be directly related to weight gain and may be due to the hormonal dysregulation seen in Obesity, as increased asprosin is also seen in anorexia nervosa (Hu *et al.*, 2021).

Given the role it plays in glucose homeostasis, it is unsurprising that asprosin levels differ between diabetic patients and those with a normal glucose tolerance (NGT). Patients with type 1 Diabetes (Groener *et al.*, 2019) and type 2 Diabetes Mellitus (T2DM) (X. Li *et al.*, 2018; L. Zhang *et al.*, 2019; Deng *et al.*, 2020; Naiemian *et al.*, 2020; Xinyue Zhang *et al.*, 2020; Zhang, Hu and Zhang, 2020) had higher circulating asprosin than NGT patients. This increase was able to be reversed after treatment with either metformin (Gozel and Kilinc, 2021) or dapagliflozin (Jiang *et al.*, 2021), suggesting that high asprosin levels are

able to be reduced through pharmaceutical intervention. Higher asprosin levels were also observed in pregnant women with gestational Diabetes Mellitus (GDM) in both the maternal and umbilical cord blood (Baykus *et al.*, 2019; Zhong *et al.*, 2020).

Female reproductive hormones also seem to influence asprosin levels in the circulation. Women who take oral contraceptives have lower plasma asprosin levels than those who do not, and the highest plasma asprosin levels were seen during the early follicular phase of the menstrual cycle (Leonard *et al.*, 2020). There also appears to be a correlation between high asprosin and women with polycystic ovary syndrome (PCOS) (X. Li *et al.*, 2018; Alan *et al.*, 2019), however a larger scale study (n=444) saw no difference between PCOS and healthy women (Chang *et al.*, 2019).

Currently, there are no studies looking into the role of asprosin in Asthma or the airways. Given the potential proinflammatory effects of asprosin and the observation that the adipokine is elevated in diseases such as Obesity and T2DM, it appears to be an interesting novel target to study. In addition, there appears to be variable effects depending on the tissue in which asprosin is acting on.

1.7. Molecular links between Asthma and diet

Coupled with weight gain, increasing the systemic release of pro-inflammatory factors is also known to be influenced by diet, which may indirectly lead to Asthma exacerbations. It is understood that insulin resistance and glucose intolerance are associated with severity in Asthma cases (Gulcan *et al.*, 2009; Cottrell *et al.*, 2011; Karampatakis *et al.*, 2017; DeChristopher and Tucker, 2018). It appears that circulating

glucose indirectly causes inflammation, as hyperglycaemia induces oxidative stress which in turn increases inflammation (Sun, Li and Gao, 2014; Chang and Yang, 2016). This inflammatory insult caused by hyperglycaemia can persist in the long term, even after glucose levels are better controlled, due to the 'metabolic memory' that has been observed in cells such as adipocytes (Youssef-Elabd *et al.*, 2012). This is due to the damage caused in the early stages of diabetes which can influence the later prognosis of the disease, so even if the glucose levels are better controlled, the damage can persist due to the 'memory' the cells have. As such, glucose homeostasis appears to represent an important factor in the development of severe Asthma and poor lung function, which may occur as excess glucose increases levels of systemic inflammation.

Furthermore, compared with healthy controls, individuals with Asthma and metabolic syndrome in the form of a combination of Obesity, T2DM, hypertension, and hyperlipidaemia it is observed that these subjects have a 10% decrease in lung function, whilst subjects with Asthma alone had a 6% reduction (Forno *et al.*, 2015). This decrease in lung function combined with the other inflammatory tissue responses could enhance severity of the Asthma response markedly. Whilst it is apparent that other chronic inflammatory conditions can exacerbate Asthma, there has been some suggestion that Obesity alone may not always be sufficient to drive heightened AHR, with a particular study by Karampatakis and colleagues highlighting that only subjects with Obesity and impaired glucose control and/or insulin resistance drove AHR (Karampatakis *et al.*, 2017).

It is understood that other factors such as cholesterol, triglycerides and elevated free fatty acids can also drive inflammation (Hill, Metcalfe and McTernan, 2009; Cardoso and Perucha, 2021), with sustained raised systemic levels postprandially and where ectopic fat deposition challenges arise in Obesity (Karpe, Dickmann and Frayn, 2011; Mizuta *et al.*, 2019). These lipids can induce inflammation in adipose tissue by activating the innate immune system through toll-like receptors (TLRs) (Youssef-Elabd *et al.*, 2012; Rogero and Calder, 2018).

The TLRs themselves form part of a repertoire of germline-encoded pattern recognition receptors (PRRs) to sense inflammatory factors. The major PRRs include Toll-like receptors (TLRs), Nod-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors and C-type lectins. TLRs and NLRs can be activated by a variety of dietary factors in response to Obesity-induced metabolic stress. This stress can arise from nutrient excess, inducing modification of the gut microbiota and increased gut permeability which may trigger an influx of various microbiota-derived pathogen-associated molecular patterns into the circulation that activate their corresponding PRRs in many tissues. Both TLR2 and TLR4 have been shown to sense free fatty acids (FFAs); in addition, ceramides, heat shock proteins and modified LDLs can also activate TLR4. Following activation of TLR2 and TLR4 they can signal through MyD88 dependent and MyD88 independent pathways to activate the NF-κB and MAPK pathways to inhibit insulin signalling through insulin receptor substrate (IRS) serine phosphorylation and to induce the transcription of pro-inflammatory cytokines, such as TNF-α, IL-6, pro-IL-1, and pro-IL-18. Nutrients such as long-chain saturated fatty acids, ceramides, modified LDL, high glucose levels, and cholesterol crystals have been shown to activate NLR-

protein 3, possibly through induction of reactive oxygen species (ROS). NLRP3 then assembles with the adaptor protein, apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and caspase-1 into a multiprotein complex called the inflammasome, which cleaves the inactive precursors of pro-IL-1 and pro-IL-18 to the active forms of IL-1 and IL-18 (Wen *et al.*, 2008; Petnicki-Ocwieja *et al.*, 2009; Harte *et al.*, 2010; Wood, Garg and Gibson, 2011; Sanz and Moya-Pérez, 2014; Wood *et al.*, 2019). Furthermore, FFAs have been shown to directly activate the inflammasome in airway smooth muscle cells, where long chain FAs activate the receptor FFAR1, leading to bronchoconstriction and enhanced Asthma symptoms (Mizuta *et al.*, 2019).

Studies in patients with Asthma also suggest that high serum triglycerides and low-density lipoprotein cholesterol (LDL-C) are also associated with cases of Asthma amongst obese children and adolescents (Cottrell *et al.*, 2011; Rastogi *et al.*, 2015; Ko *et al.*, 2018). Serum triglycerides and LDL-C were also identified to be associated with reduced lung function in adults (Scichilone *et al.*, 2013; Barochia *et al.*, 2015; Scaduto *et al.*, 2018).

Obesity and diet can contribute to an increased inflammatory response, which can lead to cellular damage and dysfunction in lung tissue. However, it appears that diet may not be the sole contributing factor arising from the gut to mediate inflammation in Asthma, as the microbiota may also have a lead role to play.

1.8. Endotoxin induced inflammation

Though dietary micro- and macronutrients have been shown to have an impact on inflammation in disease, endogenous molecules have also been shown to have an impact on inflammation. Endotoxin, referred to as lipopolysaccharide (LPS), is a molecule that consists of Lipid A, which is anchored into the outer membrane of gram-negative bacteria and is responsible for the toxic activity, connected to a polysaccharide which consists of an oligosaccharide core and a distal O-antigen (Figure 1.8.1) (Piya et al. 2013). It is the catalytic activity of the Lipid A component of LPS, which is similar in structure to FFAs, that is able to activate the innate immune cascade via TLRs and mediates an inflammatory response.

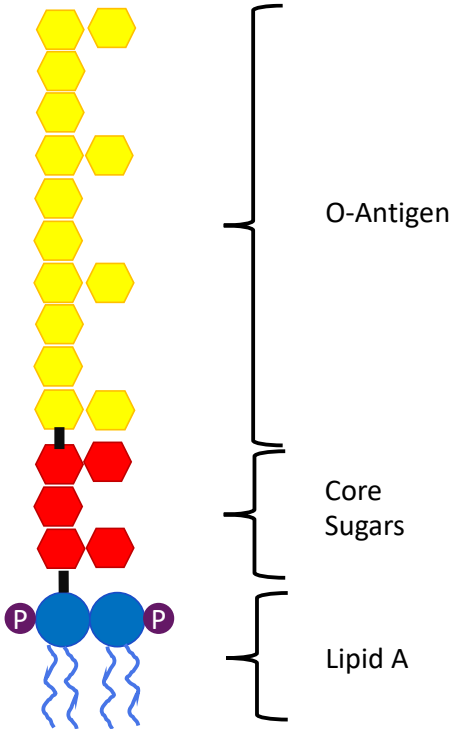

Figure 1.8.1: The structure of a lipopolysaccharide molecule

The basic structure of a lipopolysaccharide (LPS) molecule is comprised of 3 different structural domains. The lipid A component is anchored into the outer membrane of gram-negative bacteria. This is joined to a section of core oligosaccharides, which is attached to a distal O-antigen. The exact composition of each of these domains depends on the strain of the bacteria the LPS is derived from.

Whilst endotoxin predominantly remains in the gut, it is also able to traverse the gut mucosa via several distinct mechanisms (Figure 1.8.2). These mechanisms may arise through endotoxin being attached to chylomicrons, a lipoprotein that usually transports free fatty acids (Ghoshal *et al.*, 2009), or dysfunctional and increased permeability of the gut barrier, which allows increased levels of bacterial extracellular vesicles containing endotoxin to enter the circulation (Tulkens *et al.*, 2020). Several health disorders associated with low grade inflammation have been shown to involve increased intestinal permeability, including inflammatory bowel disease, coeliac disease, Obesity, T2DM, and Asthma (Benard *et al.*, 1996; Hijazi *et al.*, 2004; Cani *et al.*, 2007; Moreira *et al.*, 2012; Walker *et al.*, 2014; Genser *et al.*, 2018).

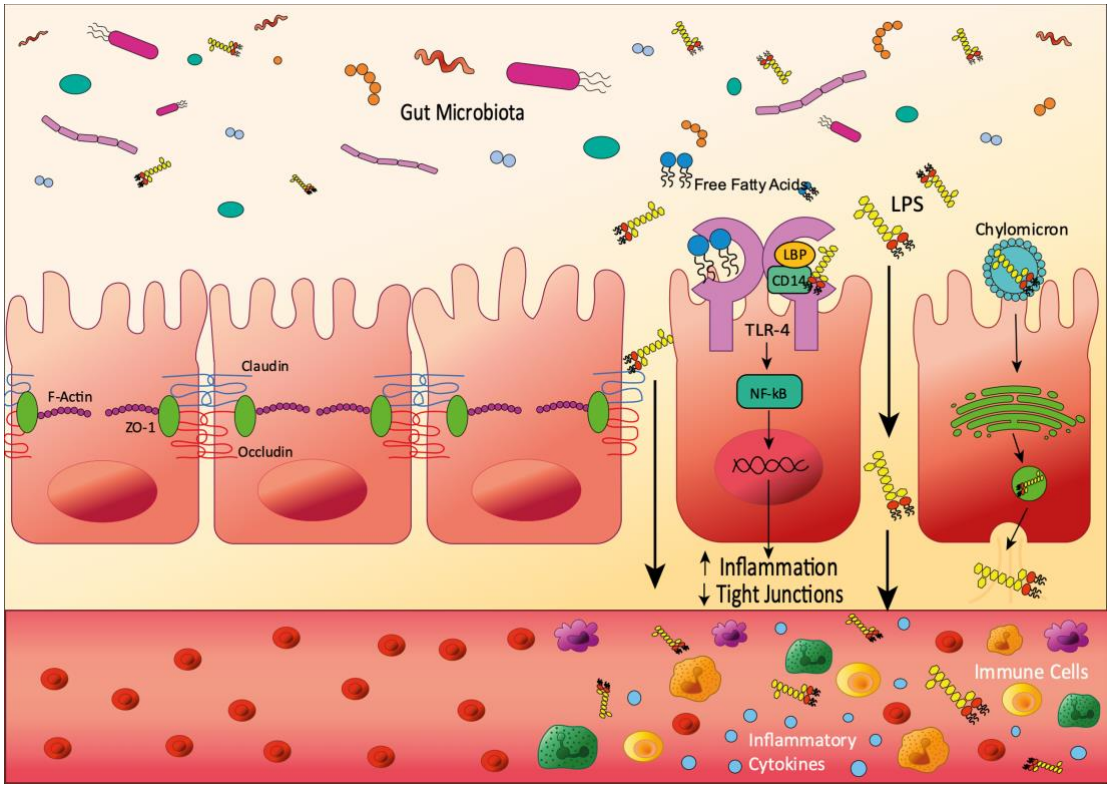

Figure 1.8.2: Leaky gut barrier leads to systemic inflammation.

In healthy patients, the gut epithelia form a barrier, connected by tight junction proteins including claudins, occludins and ZO-1, to prevent molecules in the gut lumen from crossing into the blood. However, in diseases including Asthma and Obesity, tight junctions can become weak, allowing molecules of endotoxin (lipopolysaccharide; LPS) to cross into the circulatory system, which can cause an immune response and inflammation. This can occur

through several mechanisms; firstly, LPS can bind to Toll-like receptor 4 (TLR-4), which activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway and increases the expression of inflammatory cytokines. Secondly, when LPS binds to TLR-4 it can also lead to a signalling cascade that decreases the expression of tight junction proteins, weakening the gut barrier and allowing more LPS to cross. Finally, LPS can be transported into gut cells by chylomicrons, which usually transport fat to the liver and adipose tissues. LPS is taken up by the chylomicrons, they enter the cell and are packaged by the golgi apparatus, before exiting the cells and into the circulatory system via vesicles.

Besides the functional changes to enhance endotoxin entry into the circulation, diet can also enhance the level of endotoxin in the blood stream. The consumption of a high saturated fat meal resulted in increased circulated endotoxin, noted in patients with chronic low-grade inflammation (Harte *et al.*, 2012), as well as healthy adults (Erridge *et al.*, 2007; Lyte, Gabler and Hollis, 2016) and in mouse models (Cani *et al.*, 2007). It is considered that chylomicron release, increases in high fat diets, and leads to increased postprandial endotoxin levels in obese individuals after a high fat meal (Vors *et al.*, 2015). Levels of the associated endotoxin protein CD14 have also been shown to increase during digestion, which coincides with a postprandial peak in IL-6 (Laugerette *et al.*, 2011) and a wider inflammatory response.

Increased circulating endotoxin can then lead to raised inflammation in adipose tissue due to the release of pro-inflammatory cytokines (Creely *et al.*, 2007). Endotoxin may mediate an inflammatory response via TLR-4 through the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Figure 1.8.3). Expression of TLR-4 can be increased by endotoxin itself (Guo *et al.*, 2013). The pro-inflammatory cytokines released then go on to activate an innate immune response through the recruitment of macrophages, neutrophils, and T-cells (Liu *et al.*, 2017). In addition, endotoxin can mediate functional tight junction permeability changes through

this same NF-κB pathway activating the Myosin Light Chain Kinase (MLCK) gene, which then decreases tight junction protein expression to promote a leaky gut (Guo *et al.*, 2015; Nighot *et al.*, 2017, 2019).

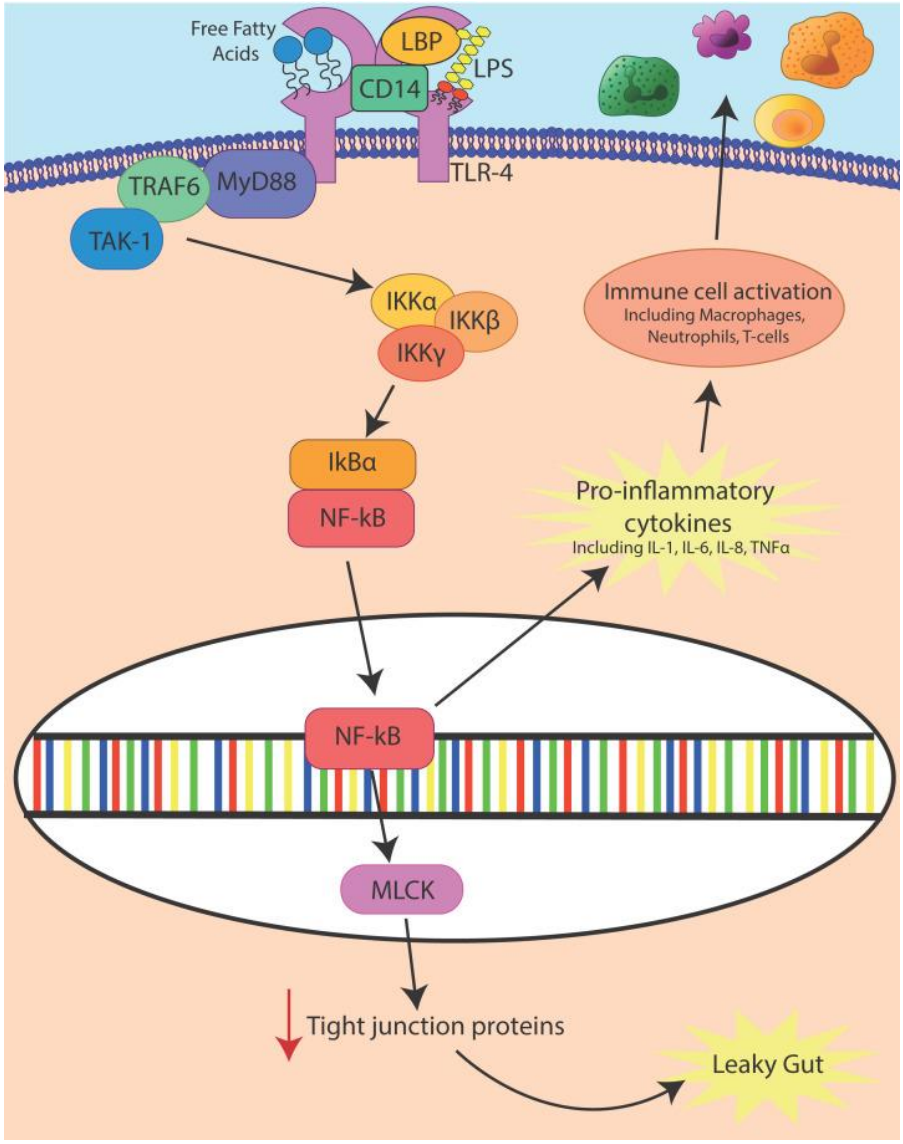

Figure 1.8.3: Endotoxin causes inflammation and leaky gut through activation of NF-κB pathways.

Endotoxin (Lipopolysaccharide; LPS) causes inflammation through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) via Toll-like receptor 4 (TLR-4). LPS is detected by LPS binding protein (LBP). LBP presents the LPS to cluster of differentiation 14 (CD14). CD14 then allows LPS to bind to TLR-4 and activate the NF-κB signalling pathway leading to increased release of inflammatory cytokines. NF-κB also increases transcription of Myosin Light Chain Kinase (MLCK) which decreases the transcription of tight junction proteins, causing the gut barrier to weaken and become leaky.

1.8.1. Endotoxin in Asthma

Endotoxin is known to be associated with both neutrophilic and eosinophilic airway inflammation, AHR and corticosteroid resistance in Asthma (Goleva *et al.*, 2008, 2013; Hauk *et al.*, 2008; Curths *et al.*, 2014; Berger *et al.*, 2015; Ren *et al.*, 2019), enhancing systemic pro-inflammatory cytokines (Blomkalns *et al.*, 2011; Tulkens *et al.*, 2020). In addition, studies have observed that endotoxin is able to increase Th2 cytokine IL-13 secretion and reduce responsiveness to corticosteroid treatment (Hadjigol *et al.*, 2020). Furthermore, it has been suggested that endotoxin is able to cause Asthma phenotypes to shift from eosinophilic to neutrophilic (Zhao *et al.*, 2017), by promoting differentiation of CD4+ cells into Th17 cells rather than Th2 cells (Jiang *et al.*, 2015). This shift would therefore lead to corticosteroid resistant, poorly controlled Asthma and an enhanced severity of the condition.

The level of endotoxin in the environment can vary but generally remains below 10 EU/m^3 in urban and rural areas (Yoda, Tamura and Shima, 2017; Farokhi, Heederik and Smit, 2018; Rolph *et al.*, 2018). When inhaled into the lungs, it can affect a patient's severity of Asthma. Factors including farming and air pollution can increase the ambient endotoxin levels and have been linked to respiratory issues (Barnig *et al.*, 2013; Basinas *et al.*, 2015; de Rooij *et al.*, 2019). It had been considered that early life exposure to endotoxin may be protective against developing allergic Asthma by supressing Th2 inflammatory mechanisms (von Mutius *et al.*, 2000; Kuipers *et al.*, 2003). However recent studies appear to show that infants with a recurrent wheeze have raised levels of endotoxin in their sputum (Xiaoyan Zhang *et al.*, 2020). Furthermore, the apparent

protective action of endotoxin has also been noted to be lost beyond infancy, where endotoxin exposure becomes associated with the onset of Asthma in teenage years (Thorne *et al.*, 2015) and adulthood (Carnes *et al.*, 2017).

It has also been shown that the cellular response to endotoxin is dependent on the structure of the molecule itself (Steimle, Autenrieth and Frick, 2016). Lipid A is typically hexa- or penta-acylated, with hexa-acylated lipid A being able to cause a 100-fold higher immune response than penta-acylated lipid A (Brix *et al.*, 2015). Exposure to the different types of lipid A may also depend on geographical location or climate (Mendy *et al.*, 2018). It has been observed that people living in urban areas appear more likely to be exposed to the penta-acylated lipid A, contained within *Bacteroidetes* and *Prevotella* bacterial species and as a result they are less likely to have Asthma (Lynch *et al.*, 2014; Larsen *et al.*, 2015).

Changes in intestinal permeability have also been reported in Asthma patients (Benard *et al.*, 1996; Hijazi *et al.*, 2004; Walker *et al.*, 2014; Barreto *et al.*, 2015), however a causal link between gut-derived endotoxin, intestinal permeability, and respiratory inflammation has yet to be fully explored. Ultimately, current evidence suggests that both intestinal and systemic inflammation are derived from altered microbiota patterns, where endotoxin may lead to enhance systemic cell pro-inflammatory activation and tissue inflammation (Creely *et al.*, 2007; Benson *et al.*, 2010; Verdam *et al.*, 2013).

1.9. Impact of the microbiota on health

The human microbiota is a collection of all the microorganisms that live within all the tissues and fluids in the body, although it is often confused with the human microbiome, which refers to the genome of the microorganisms. There are more of these microorganisms residing within the human body than our own cells. Current estimates suggest that the average person has approximately 38 trillion microbes inside and on their body, which out numbers our own cells at a ratio of 1.3:1 (Sender, Fuchs and Milo, 2016). Furthermore, it is considered that everyone has their own unique microbial signature, heavily influenced by genetics, environmental and lifestyle factors. This begins prior to birth and the moment of birth, where the altered exposure to microbes coupled with a reduction in *Bifidobacterium* colonisation leads to an increased risk of atopic and inflammatory borne diseases in infants born via c-section delivery (Reyman *et al.*, 2019). This *Bifidobacterium* colonisation can be further influenced by diet, with a plant-based diet associated with increased beneficial strains of *Bifidobacterium* and *Lactobacillus* whilst an animal-based diet is more associated with an increase in *Bacteroides* and *Biophilia* (David *et al.*, 2014; Singh *et al.*, 2017). Furthermore, beyond diet, aging itself has also been shown to shift microbiota diversity in key bacterial genus including an increase in *Bacteroides* and *Enterobacteriaceae* and a decrease in *Bifidobacterium* (Odamaki *et al.*, 2016).

The gut microbiota is most commonly referred to when discussing the link between our health and the bacteria that resides within the body, however there are distinct microbial communities across the human body that are equally as important (Costello *et al.*, 2009). Even within tissues there is diversity, such as the different regions of the

gut, where despite the adjoined locality such as in the duodenum and jejunum the levels of the type of strains vary (Leite *et al.*, 2020). Beyond diversity, the interplay of these communities could impact our health as well as the response patients have to an Asthmatic episode. An optimal healthy bacterial microbiota community should be diverse and well balanced. A reduction in diversity and a bacterial microbiota imbalance, referred to as dysbiosis, can lead to an overgrowth of gram-negative bacteria leading ultimately to the increased release of endotoxin. Dysbiosis, *per se* is known to drive an inflammatory response in the host and is considered a key factor in the development of chronic inflammatory bowel disease, Obesity, T2DM, cardiovascular disease and Asthma (Ley *et al.*, 2005; Schwiertz *et al.*, 2010; Carding *et al.*, 2015; Harris and Chang, 2018; Kitai and Tang, 2018; Chiu *et al.*, 2019; J. J. Lee *et al.*, 2019; Amabebe *et al.*, 2020; Gurung *et al.*, 2020).

1.9.1. Role of the airway and lung microbiota in Asthma

There is emerging evidence that the composition of the airway microbiota plays an active role in the severity of bronchial hyperresponsiveness (Huang *et al.*, 2011) and inflammatory phenotype (Taylor *et al.*, 2018; Pang *et al.*, 2019) in patients with Asthma. It is reported that patients with Asthma have more gram-negative species of bacteria in their airway microbiota compared to those without Asthma (Zhang *et al.*, 2016; J. J. Lee *et al.*, 2019). Furthermore, there is a specific increase in abundance of the gram-negative genus *Moraxella* in the nasal (Depner *et al.*, 2017) and airway microbiota (X. Yang *et al.*, 2018; Zhou *et al.*, 2019; Xiaoyan Zhang *et al.*, 2020) which is also associated with an increased risk of exacerbation. While genes associated with endotoxin biosynthesis are also observed as being raised in the nasal microbiota of young adults with Asthma

compared with those without Asthma (J. J. Lee *et al.*, 2019). There is also a specific increase in the gram-negative phylum Proteobacteria noted in the airway microbiota of patients with Asthma (Zhang *et al.*, 2016; Fazlollahi *et al.*, 2018; X. Yang *et al.*, 2018) with expression of Th17-related genes correlated with this phylum (Huang *et al.*, 2015).

Although both the eosinophilic and neutrophilic phenotypes of Asthma show an increase in gram-negative strains in the lung compared to healthy people, the neutrophilic phenotype was shown to have less bacterial diversity and more pathogenic and opportunistic strains in the airways (Taylor *et al.*, 2018). There is also an increased bacterial load in the neutrophilic phenotype, so these associations are amplified when compared with the eosinophilic phenotype (X. Yang *et al.*, 2018). The increase in endotoxin-producing gram-negative bacterial strains in the airway has been associated with corticosteroid resistance in severe Asthma, due to high activation of the NF-κB pathway via TLR-4 activation (Goleva *et al.*, 2008). Taken together, this suggests that there is an increased potential for airway derived endotoxin to enter the circulation and induce inflammation due to an increase in gram-negative strains observed in patients with Asthma.

1.9.2. Altered Gut Microbiota in People with Asthma

The gut microbiota in chronic low grade inflammatory diseases is associated with a reduced bacterial diversity, leading to a shift towards more gram-negative bacterial strains and therefore more bioavailability of endotoxin, which can mediate the exacerbation of these conditions (Turnbaugh et al. 2006; Bäckhed et al. 2004; Bäckhed et al. 2007; Anhê et al. 2020). Although Asthma is a disease characterised by

inflammation within the airways exacerbated by inhaled allergens and individualised triggers; there is evidence to suggest a role of the gut microbiota in respiratory conditions. The association between the gut microbiota and the airways is termed the gut-lung axis (Marsland, Trompette and Gollwitzer, 2015). Current studies suggests that early bacterial colonisation (and its diversity) of the gut influences whether an infant develops Asthma (Abrahamsson *et al.*, 2014; Arrieta *et al.*, 2015, 2018; Fujimura *et al.*, 2016; Stiemsma *et al.*, 2016; Stokholm *et al.*, 2018). Children with Asthma have been shown to have a reduction in probiotic strains *Bifidobacterium* and *Lactobacilli,* with an increase in harmful strains including *Escherichia coli, Helicobacter pylori, Streptococcus*, and *Staphylococcus,* which appears to result in increased inflammation (Zhang *et al.*, 2018). Reduction in gut bacterial diversity through antibiotic use in pregnancy or early childhood also contributes to this increased risk (Metsälä *et al.*, 2015; Ni *et al.*, 2019). Currently there are limited studies exploring the impact of the gut microbiota on Asthma risk in adults, although a recent pilot study has observed a correlation between opportunistic bacterial strains including *Prevotella* and poor lung function in adults with Asthma (Begley *et al.*, 2018).

Murine studies mirror the importance of early life exposure to diverse microbial environments, as mice raised in a germ-free environment which is free of any bacteria, had an increased risk of developing Asthma symptoms and inflammation via Th2 activation resulting in increased IL-4 and IL-5 production (Qian *et al.*, 2017). Several murine studies using vancomycin, to alter the gut microbiota, have shown subsequently in recolonization that more gram-negative bacteria colonise the gut, and that these mice are at a higher risk of developing Asthma (Russell *et al.*, 2012; Cait *et al.*, 2018; Alhasan

et al., 2020). These studies clearly describe a link between gram-negative bacteria and the risk of developing Asthma with a rise in the availability of endotoxin, and as such a potential for endotoxin-induced inflammation to be a mediator of Asthma. These human and rodent studies also highlight how antibiotic use may increase the risk of developing Asthma. As such, the ability to enhance gut microbiota diversity through diet may be an important method to reduce systemic endotoxin, reduce the arising inflammation and improve the condition of patients with Asthma. As such, modifying the composition of the microbiota by encouraging the growth of beneficial bacteria strains could lead to a reduction of inflammation caused by the harmful bacteria strains. A simple and relatively cheap way of improving the diversity of the microbiota is through diet and may therefore offer a strategy, in part, to reduce inflammation and the arising Asthma symptoms.

1.10. Dietary use of pro/prebiotics as a treatment for Asthma

Therapies that alter the gut microbiota such as pre- and probiotics are becoming more widely accessible and gaining popularity and media attention. Probiotics involve the delivery of live strains of beneficial bacteria to the gut in order to improve the health of the host (Hill *et al.*, 2014). They can be consumed through yoghurt-based drinks, tablets or in fermented foods such as pickled vegetables, kimchi and soy. A prebiotic is a non-digestible carbohydrate which is utilised by bacteria in the gut and alters the composition of the gut microbiota by encouraging the growth of beneficial strains such as *Bifidobacteria* and *Lactobacilli* (Gibson *et al.*, 2017). The main types of prebiotics that are commonly used are inulins, a group of polysaccharides, and galactooligosaccharides

(GOS), an oligosaccharide linked with galactose, both of which are types of plant-derived fibres.

When prebiotics are metabolised by bacteria, the metabolites produced may exert beneficial effects on the host and are termed postbiotics (Klemashevich *et al.*, 2014; Hernández-Granados and Franco-Robles, 2020). The main metabolites are short chain fatty acids (SCFAs), which are classified as fatty acids with fewer than 6 carbon atoms, with acetate (C2), propionate (C3) and butyrate (C4) being the most abundant (Gill *et al.*, 2018). The amount of SCFA produced by bacteria varies depending on the prebiotic used, with GOS leading to the highest rate of production (Holmes *et al.*, 2020). Studies have indicated that SCFAs have an anti-inflammatory effect (Liu *et al.*, 2012; Wang *et al.*, 2017; Theiler *et al.*, 2019), so prebiotics are therefore thought to be beneficial by increasing the levels of SCFAs produced by bacteria.

SCFAs activate free fatty acid receptor 2 (FFAR2) and FFAR3 (also referred to as GPR43 and GPR41 respectively). Both receptors are expressed in leukocytes, endothelial cells and airway smooth muscle and epithelial cells, whilst FFAR3 is also expressed in adipose tissue (Pluznick *et al.*, 2013; M. Li *et al.*, 2018). Expression of FFAR2 and FFAR3 in Asthma patients appears to increase within 4 hours of consuming a high fibre meal (Halnes *et al.*, 2017). FFAR3 has varied effects in different cell types, causing vasodilation in vascular smooth muscle cells (Mizuta *et al.*, 2020). Interestingly, it has been documented that patients with Asthma have a reduced number of total SCFAs compared with healthy individuals, which is thought to arise due to a decrease in the metabolic activity of SCFA producing bacteria (Ivashkin *et al.*, 2019). Notably, a decrease in SCFA-producing

Veillonella in a matter of months following birth has been associated with development of atopic wheeze later in childhood (Arrieta *et al.*, 2015, 2018). Infants with reduced levels of faecal SCFAs were also more likely to develop Asthma and other atopic diseases later in life (Lee-Sarwar *et al.*, 2020). These findings are also confirmed by studies using pregnant mice, showing that reducing SCFA-producing bacteria with vancomycin leads to offspring with severe Asthma symptoms (Alhasan *et al.*, 2020). Therefore, increasing SCFAs could be a potential novel therapy for Asthma, either through diversifying bacterial species, or reducing endotoxin-induced inflammation, mitigating the inflammatory response observed in subjects with Asthma.

Further studies also suggest that SCFAs can increase the expression of tight junction proteins including ZO-1, claudins and occludin, therefore enhancing the intestinal barrier (Peng *et al.*, 2009; Wang *et al.*, 2012; Yan and Ajuwon, 2017; Feng *et al.*, 2018; Nielsen *et al.*, 2018). In addition, SCFAs have also been shown to inhibit damage caused by endotoxin in the gut (Feng *et al.*, 2018; Diao *et al.*, 2019).

Although studies on the use of prebiotics to reduce Asthma are limited, prior studies do suggest they may have beneficial effects. In human interventional trials using inulin and bimuno-galactoligosaccharide (B-GOS) in patients who experienced Asthma or exercise-induced Asthma respectively, both prebiotics were observed to reduce both inflammatory markers and AHR in participants (Williams *et al.*, 2016; McLoughlin *et al.*, 2019). Larger scale studies are needed before detailed conclusions can be made, however these studies along with several murine studies (Verheijden, Akbari, *et al.*,

2015; Verheijden, Willemsen, *et al.*, 2015; Verheijden *et al.*, 2018) highlight the potential of the bifidogenic effect to ease the symptoms of Asthma.

Probiotics may provide a further option for mitigating bacterial dysbiosis in individuals suffering from chronic low-grade inflammation and atopic diseases, where bacterial diversity and levels of beneficial bacterial strains are relatively low. Probiotic intervention trials are complex and fraught with confounders, however studies appear to show that they can reduce inflammation in participants with T2DM (Sabico *et al.*, 2019) as well as in healthy individuals (Burton *et al.*, 2017), although this appears for now to be the case over longer intervention periods rather than acute studies (Leber *et al.*, 2012; Sabico *et al.*, 2017).

There are a small number of studies looking into the use of probiotics in patients with Asthma, and are typically limited to studies in children. The specific use of *Lactobacillus GG* administered to pregnant women, and then to their children for 6 months, demonstrated that the children had a lower risk of developing atopic diseases including Asthma (Kalliomäki *et al.*, 2001). Follow-up studies confirmed that the same children were still at a lower risk of atopic disease 7 years after the original study (Kalliomäki *et al.*, 2003, 2007), although not all such studies have found similar findings (Dotterud *et al.*, 2010; Cabana *et al.*, 2017; Durack *et al.*, 2018). Another study using various strains of Lactobacillus in school aged children showed a reduction in Asthma severity and IgE levels, whilst increasing lung function (Huang, Chie and Wang, 2018). Bifidobacterium administration in adults improved Asthma control and increased the release of anti-inflammatory metabolites (Liu et al. 2021).

The potential challenge with the use of probiotics is that even if the bacteria reach the gut, if they have inadequate food sources such as dietary fibre, which could be due to the hosts poor diet, the bacteria do not colonise sufficiently to exert their beneficial effect. This suggests that there needs to be some form of dietary intervention as well as the probiotic supplement for optimal results. Out of these studies to date, there is evidence to suggest *Bifidobacterium* and *Lactobacillus* are particularly able to reduce endotoxin-induced inflammation and increase tight junction protein expression, therefore improving intestinal permeability (Bergmann *et al.*, 2013; Ling *et al.*, 2016; Ahmadi *et al.*, 2020).

Studies have also sought to administer both pre- and probiotics together, known as synbiotics, to enhance health outcomes in Asthma. Dietary interventions using a mixture of inulin and probiotics has been shown to reduce airway inflammation in participants with Asthma within four hours of consumption (Halnes *et al.*, 2017). Animal studies also reported similar findings, with the synbiotic effect reducing both eosinophil and neutrophil cell counts, inflammation (Sagar *et al.*, 2014) and improving intestinal permeability (Krumbeck *et al.*, 2015). However, conflicting reports have been noted in humans, where pre- and probiotics administered together had no synbiotic effect, even though when given separately they improved intestinal permeability (Krumbeck *et al.*, 2018). This may be due to a number of confounders including competition between probiotics and established bacteria for nutrients, or the participants dietary habits being suboptimal for probiotic growth, so a more controlled diet may be required in future studies.

Insight into systemic endotoxin specifically has shown the levels to be reduced after either pre- or probiotic supplementation, however these studies have been limited to healthy participants (Roberts *et al.*, 2016; Sabico *et al.*, 2019) and patients with chronic metabolic diseases including Obesity and T2DM (Dehghan *et al.*, 2014; Dehghan, Pourghassem Gargari and Asghari Jafar-abadi, 2014; Xiao *et al.*, 2014; Aliasgharzadeh *et al.*, 2015; Parnell, Klancic and Reimer, 2017; Haghighat *et al.*, 2019). Whilst studies in Asthma have not yet considered the therapeutic reduction of endotoxin as a method to reduce symptoms or health outcomes in such patients. Therefore, future Asthma studies including endotoxin as a measurement may give more insight into the role of endotoxin as an instigator of inflammation and how dietary intervention could reduce this inflammation.

1.11. Research Aims & Hypothesis

1.12. Hypotheses

The overall hypothesis for this project is that prebiotics can reduce inflammation in asthma and obesity related asthma. This would occur through the release of SCFAs which exert an anti-inflammatory effect which would then improve asthma symptoms. Prebiotics would also improve overall gut health, therefore reducing the availability of LPS, and preventing the release of LPS into the circulation by improving the gut barrier permeability. Taken together, this would reduce inflammation in patients with asthma and improve their symptoms.

The hypotheses for this research project can be broken down more specifically as follows:

1. Prebiotics are able to reduce inflammation in Asthma and Obesity related Asthma through the anti-inflammatory actions of SCFAs
2. The adipokine asprosin is increased in inflammatory diseases and correlates with cellular dysfunction
3. Asprosin contributes to airway inflammation and cellular dysfunction, which can be mitigated by SCFAs

1.13. Research aims and objectives

In order to confirm or disprove the hypothesis, a range of *in vivo* and *in vitro* techniques were used. Airway and adipocyte cell lines would be used as relevant cell models for asthma and obesity. Each cell lines would be treated with an inflammatory factor (LPS or asprosin) and SCFAs in order to assess whether the SCFAs could mitigate the inflammatory response through the NFκB pathway. This would then be followed by a human nutritional intervention trial in which participants with asthma would be given a prebiotic to see whether it would improve their symptoms, lung function, metabolic markers, and inflammation.

The aims and objectives of this project were broken down as follows:

1. Investigate the role of SCFAs on LPS induced inflammation in airway epithelial and white adipocyte cells
2. Study the role of adipokine asprosin in a patient cohort, and whether it influences cellular processes including adipocyte browning, inflammation, and mitochondria function
3. Explore the potential inflammatory role of the adipokine asprosin in human airway epithelial cells, and whether SCFAs can reduce this effect

4. Further explore the role of prebiotics in Asthma through a human nutritional intervention trial, in order to see if prebiotics can reduce inflammation and improve Asthma symptoms

Chapter 2: Material and Methods

2.1 Cell Culture

2.1.1 Human airway epithelial cell line BEAS2B-R1

2.1.1.1 Cell type and media composition

BEAS2B-R1 cells are an immortalised human airway epithelial cell line taken from the bronchus of a healthy patient, and is commonly used in studies relating to the airways along with inflammatory factors such as LPS (J. Verspohl and Podlogar, 2012; Hou *et al.*, 2021; Si and Zhang, 2021). Cells grow adherently in a monolayer and were cultured in growth medium composed of 500mL Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) and 0.5mg/mL Penicillin-Streptomycin-Glutamine.

2.1.1.2 Passaging and seeding cells

Cells were grown in T75 cell culture flasks (Sarstedt, UK) in 37^{o}C humidified incubator with 5% CO_2. Once the cells reached 100% confluency, they were passaged by gently washing with phosphate buffered saline (PBS) and the addition of 2mL 0.05% trypsin-EDTA. Cells were placed in the incubator for 5 minutes and agitated to detach cells. The trypsin-EDTA was neutralised with 8ml of growth media and cells were then pelleted by centrifugation at 0.3 rcf for 3 minutes. The supernatant was then removed, and the pellet was resuspended in 10ml of growth media. Cells were then transferred into a T75 flask at a dilution of 1:10 in 10mL of media. For 6-well or 96-well cell culture plates, cells were seeded at a density of 100,000 cells/mL.

2.1.2 Human Preadipocyte Chubs-S7 cell line

2.1.2.1 Cell type and media composition

Chub-S7 cells are an immortalised preadipocyte cell line taken from the white adipose tissue of a 33 year old female patient. The cells retain their ability to differentiate into a mature white adipocyte cell type after a 14-day period of differentiation (Darimont *et al.*, 2003).

During the proliferation phase, Chub-S7 cells were cultured in growth medium composed of Phenol-free DMEM supplemented with 10% FBS and 0.5mg/mL Penicillin-Streptomycin-Glutamine. In addition, during the cell differentiation period, cells were grown in differentiation media (days 0-6), nutrition media (days 8-14) and basal media (from day 15). The composition of each cell culture media were as follows:

Differentiation media:

- DMEM/Ham's F-12 phenol-free medium 500mL
- 0.5mg/mL Penicillin-Streptomycin-Glutamine
- Preadipocyte differentiation supplement pack (Promocell #C-39436) (Recombinant Insulin 0.5µg/mL, Dexamethasone 400ng/mL, D-biotin 8µg/mL, Isobutylmethylxanthine (IBMX) 44µg/mL, L-thyroxine 9ng/mL, Ciglitazone 3µg/mL)

Nutrition media:

- DMEM/Ham's F-12 phenol-free medium 500mL
- Adipocyte nutrition supplement pack x1 (Promocell #C39439) (Recombinant human Insulin 0.5µg/mL, Dexamethasone 400ng/mL, D-biotin 8µg/mL, Foetal calf serum 3%)

Basal media:

- DMEM/Ham's F-12 phenol-free medium 500mL
- 0.5% Bovine serum albumin (BSA)

2.1.2.2 Passaging and seeding cells

Cells were grown in T75 cell culture flasks in 37°C humidified incubator with 5% CO_2. Once the cells reached 70-80% confluency, they were passaged by gently washing with PBS and the addition of 2ml 0.05% trypsin-EDTA. Cells were placed in the incubator for 3 minutes and agitated to detach cells. The trypsin-EDTA was neutralised with 8ml of growth media and cells were then pelleted by centrifugation at 0.3 rcf for 3 minutes. The supernatant was then removed, and the pellet was resuspended in 10ml of growth media. Cells were then transferred into a T75 flask at a dilution of 1:2 in 12mL of media.

For 6-well or 96-well cell culture plates, cells were seeded at a density of 50,000 cells/ml. Cells were grown until they reached 100% confluency. At this point, the growth media was replaced with differentiation media (day 0) and replenished every 48 hours between days 0-6. On day 8, the differentiation media was replaced with nutrition media and replenished every 48 hours between days 8-14. On day 15, the nutrition media was replaced with basal media, at which point cells were left for 24 hours in order for the

growth factors to cease having their effect. Treatments were then started from day 16 onwards.

2.2 Treatment of cells

Once BEAS2B-R1 cells had reached 70-80% confluency and on Day 16 of the Chubs-S7 differentiation, cells were ready for treatments. Cells were pre-treated with a SCFA mix 24 hours prior to treatment with an inflammatory factor. The SCFA mix gave a final concentration of 2mM Acetate, 0.25mM Butyrate and 0.25mM of Propionate (each from Sigma, UK), giving a ratio of 80:10:10 which is seen at a physiological level (Jocken et al., 2018; Ktsoyan et al., 2016).

Cells were then treated with either 10ng/mL or 100ng/mL Lipopolysaccharide (LPS; from Escherichia coli O55:B5, Sigma, UK) for 6, 12, and 24 hours in order to induce inflammation. Similarly, cells were also treated with 10ng/mL Asprosin for 6, 12, and 24 hours. The vehicle control for LPS and the SCFA mix was water, and PBS for asprosin, each diluted in media at the same proportion as the treatments.

2.3 Harvesting cells

2.3.1 Protein extraction

2.3.1.1 Extraction of total protein from cells

Protein samples were lysed in 100μL of 1x Radioimmunoprecipitation Assay Buffer (RIPA) containing 2% protease and phosphatase inhibitors. The protease and phosphatase inhibitors were prepared as follows:

- 2mL 1x RIPA buffer (Millipore, UK)
- 2 Roche Complete Mini protease inhibitor cocktail tablets
- 8mg sodium fluoride (NaF, Fisher Scientific)
- 20mg sodium vanadate (Na3VO4, Acros Organics)

Cells were scraped in RIPA buffer on ice and then collected in 1.5mL microfuge tubes. Cells were placed on ice for 30 mins and vortexed every 10 mins to assist with cell lysis. The cells were then pelleted by centrifugation for 15 mins at $16x10^3$ rcf at 4°C. The supernatant containing the protein was then collected and stored at -80°C.

2.3.1.2 Protein quantification

The concentration of protein in each sample was quantified using the Bradford Assay. BSA protein standards were diluted in a range of 0-1mg/mL. Protein samples were diluted 1:20 in deionised water and added to 200μL of 1x Bradford reagent (Bio-rad, UK). All standards and samples were run in triplicate. The absorbance was read at a wavelength of 595nm. A standard curve was created in order to calculate the concentrations of the protein samples. The protein samples were then mixed with 4x Laemmli Buffer and heated at 95°C for 10 mins, then stored at -80°C.

2.3.2 RNA extraction and quantification

RNA was extracted using the Isolate II RNA Mini Kit (Bioline, #BIO-52073). RNA samples were lysed on ice in 350μL Buffer RLY (Bioline, UK) and 3.5μL β-mercaptoethanol (β-ME). The lysate was filtered through an ISOLATE II filter for 1 min at 11,000 rcf. The lysate

was then combined with 350µL ethanol (70%) and transferred to an RNA binding column and centrifuged for 30s at 11,000 rcf. The membrane was desalted by adding 350µL to the column and centrifuging for 1 min at 11,000 rcf. A DNase I reaction mixture was prepared by adding 10µL DNase I to 90µL Reaction Buffer, then 95µL of the DNase I reaction mixture was added to the membrane and incubated at room temperate for 15 mins. Following this, the membrane was washed by first adding 200µL wash buffer RW1 and centrifuging for 30s at 11,000 rcf, then adding 600µL wash buffer RW2 and centrifuging for 30s at 11,000 rcf, and finally adding 250µL wash buffer RW2 and centrifuging for 2mins at 11,000 rcf. The final step resulted in 60µL of RNA being eluted in RNase-free water. In order to calculate the concentration and purity of the RNA samples, 1µL of RNA was measured using the Nanodrop ND-100 (Labtech, UK).

2.4 Western Blot

In order to determine the levels of expression of specific target proteins in the harvested samples, Western Blot analysis was performed. A Spectra™ Multicolor Broad Range Protein Ladder (Thermo Scientific, UK) and 30-50µg of protein sample was loaded onto a 10% polyacrylamide gel. The gel was run initially for approximately 10 mins at 100V to allow for the protein to enter the gel, and then at 140V for approximately 70 mins or until the buffer had run off the bottom of the gel. The gel was then transferred onto an Immobilon-P transfer membrane (Millipore, UK) with 0.45µm pore size by wet transfer for 100 minutes at 100V. The membrane was briefly stained with Ponceau S in order to visualise the protein bands to ensure the transfer had worked. The membrane was then washed in water and blocked in either 5% BSA in tris buffered saline-Tween20 (TBS-T)

or 0.2% I-Block in PBS-T for 1 hour at room temperature. Primary antibodies were then added to the membrane over night at 4°C. The following antibodies were used:

Antibody	Company (Item No.)	Buffer	Blocking	Primary	Secondary
β-Actin	CST (#4970)	PBS	I-Block	1:1000	1:100,000 (R)
NFκB	CST (#8242)	PBS	I-Block	1:1000	1:100,000 (R)
p-NFκB	CST (#3033)	TBS	BSA	1:1000	1:50,000 (R)
IKKβ	CST (#8943)	PBS	I-Block	1:500	1:50,000 (R)
IKKγ	CST (#2685)	PBS	I-Block	1:500	1:50,000 (R)
JNK	CST (#9252)	PBS	I-Block	1:500	1:50,000 (R)
p-JNK	CST (#9251)	TBS	BSA	1:500	1:50,000 (R)
p-IkBα/ IkBα	Novus (NB100-56724)	TBS	I-Block	1:500	1:10,000 (M)

Table 2.4.1: Western blot antibodies and buffers

Details of primary and secondary antibody concentrations and the buffers and blocking solutions used. Abbreviations in table: Cell Signalling Technologies (CST), Rabbit (R), Mouse (M).

The following day, membranes were washed in either TBS-T or PBS-T 3 times for 5 mins. The secondary antibody, which would be either anti-rabbit (Sigma #A9169) or anti-mouse (Cell Signalling Technologies #7076) as stated in table 2.4, was then added for 1 hour at room temperature. The washing step was then repeated, and the membranes incubated briefly in Westar Antares HRP Detection Substrate (Geneflow, UK) which uses enhanced chemiluminescence (ECL) in order to visualise the bands. The membranes were visualised on the GeneGnome XRQ Visualiser (Syngene, UK).

2.5 cDNA Synthesis

In order to synthesise cDNA from extracted RNA, 1ug of RNA in 10µL of nuclease-free H_2O was combined with 1µL random hexamers and 1µL dNTPs (Thermofisher, UK) and

heated at 70°C for 10 mins. Samples were then immediately transferred onto ice for 2 mins. A mastermix was prepared containing the following; 2μL 10x M-MLV reverse transcriptase buffer, 1μL M-MLV (Sigma), 0.5μL RNase OUT (Thermofisher, UK) and 4.5μL H_2O. To each sample, 8μL of this mastermix was added. Finally, samples underwent 1 cycle of 20 mins at 25°C, 50 mins at 37°C and 10 mins at 80°C. The resulting samples were then diluted to 500ng/mL with H_2O and stored at -20°C until use.

2.6 Real Time Quantitative Polymerase Chain Reaction

2.6.1 TaqMan gene expression assays

Gene expression was measured using real time quantitative polymerase chain reaction (RT-qPCR). Each reaction was run in a volume of 20μL, containing 100ng cDNA, 1x housekeeping gene primer (18S), 1x gene of interest primer, 1x TaqMan master mix (Thermofisher, UK) and H_2O. The following TaqMan expression assays (Thermofisher, UK) were used:

Gene	Assay ID
18S	Hs99999901_s1
NFKB1	Hs00765730_m1
MAPK8	Hs01548508_m1
CXCL8	Hs00174103_m1
FBN1	Hs00973198_m1
BECN1	Hs00186838_m1
PPARGC1A	Hs00173304_m1
POLG	Hs01018668_m1
TFAM	Hs00273372_m1
NRF1	Hs00602161_m1
COX4	Hs00971639_m1
mtATP6	Hs02596862_g1

Table 2.6.1: List of TaqMan expression assays

The RT-qPCR was carried out in the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, UK) as follows; 2 mins at 50°C, 10 mins at 95°C, then 40 cycles of 15s at 95°C and 1 min at 60°C.

2.6.2 Gene expression analysis

In order to calculate the expression of each gene relative to the control, the following formula was used:

$$mRNA\ expression = 2^{-(\Delta\Delta Ct)}$$

$$where\ \Delta\Delta Ct = (Gene\ of\ interest - 18S) - Average\ Control$$

2.6.3 Mitochondrial copy number

In order to calculate the mitochondrial copy number, mitochondrial (MT-ND1) and nuclear (BECN1) genes were measured. The copy number was calculated using the following formula:

$$Mitochondrial\ copy\ number = 2^{\Delta Ct}$$

$$where\ \Delta Ct = (BECN1 - 18S) - (MTND1 - 18S)$$

The mitochondrial copy number was then shown as a percentage change compared to the control, calculated as follows:

$$Percentage\ change\ in\ mitochondrial\ copy\ number = \frac{Sample - Control}{Control}\ x\ 100$$

2.7 Seahorse Mitochondrial and Glycolysis stress test assays

In order to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of both the BEAS2B-R1 and Chub-S7 cells, seahorse assays were run on the Seahorse XFe24 analyser (Seahorse Bioscience, Agilent Technologies, UK). BEAS2B-R1 cells were seeded at a density of 20,000 cells/well and Chub-S7 at a density of 10,000 cells/well into Seahorse 24 well plates. Cells were treated as previously described in section 2.2.

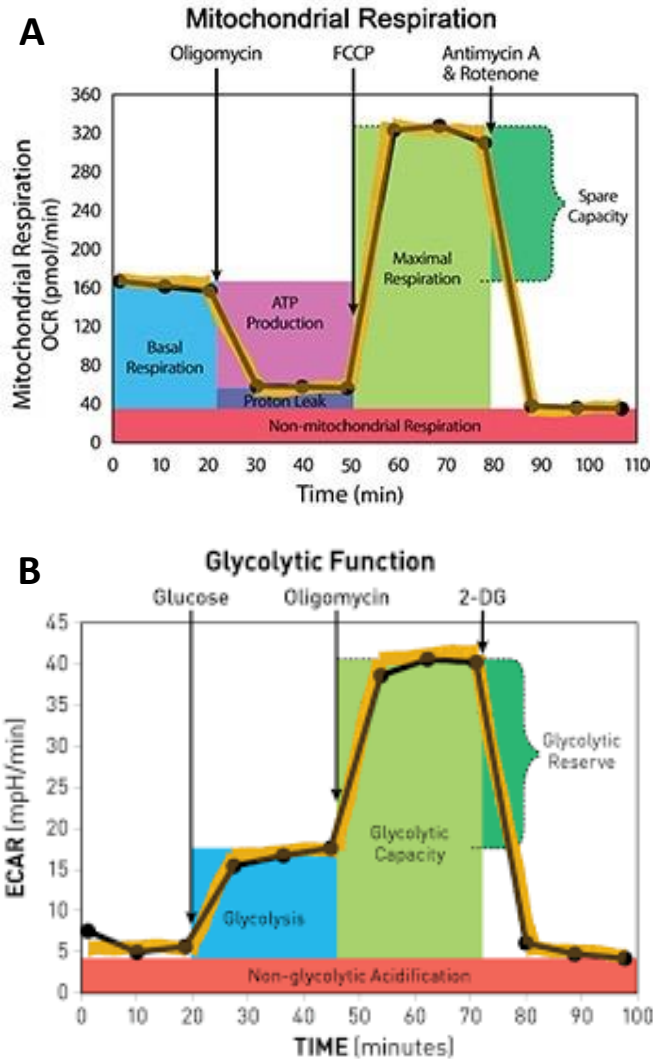

Figure 2.6.3.1: Mitochondrial respiration and glycolytic function analysis on the Seahorse XFe analyser

A) Mitochondrial oxygen consumption rate (OCR) was measured using the mitochondrial stress test. Basal respiration was first measured before adding Oligomycin to calculate the

ATP production and proton leak. FCCP was then added to measure maximal respiration, before Antimycin A & Rotenone was finally added to calculate the spare respiratory capacity and non-mitochondrial respiration. B) The extracellular acidification rate (ECAR) was measured using the glycolysis stress test. Glucose was added to measure the glycolysis, followed by Oligomycin to measure the glycolytic capacity. Finally, 2-DG was added to measure the glycolytic reserve.

The assays were run in Seahorse XF DMEM medium, pH 7.4 supplemented with 10mM Seahorse XF Glucose, 1mM Seahorse XF Pyruvate and 2mM Seahorse XF L-Glutamine (Agilent Technologies, UK). A mitochondria stress test kit and a glycolysis stress test kit (Agilent Technologies, UK) containing each compound needed for each assay was used. The following concentrations were used for each compound in each assay; Mitochondrial stress test - 1.5μM Oligomycin, 2μM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), 0.5μM Rotenone and Antimycin A (Rot/AA), Glycolysis stress test – 10mM Glucose, 1μM Oligomycin, and 50mM 2-Deoxy-D-Glucose (2-DG). The role of each compound in measuring mitochondrial respiration and glycolytic rate is summarised in figure 2.7.

The assay first required a 30 min calibration step, after which the assay plate was inserted. The protocol consisted of 3 cycles of an injection of a compound, mix (3 mins), wait (2 mins) and measure (3 mins). The order in which the compounds were injected was Oligomycin, FCCP then Rot/AA for the mitochondrial stress test, and Glucose, Oligomycin, then 2-DG for the glycolysis stress test.

2.8 Mitochondrial activity analysis

In order to measure the levels of active mitochondria, Mitotracker^{TM} Dye (Thermofisher, UK) was used. The red dye is used to stain the active mitochondria and is dependent on

the membrane potential i.e., the higher the membrane potential, the more red dye can pass through, resulting in a stronger signal. The green dye stains all mitochondria present in the cells, regardless of the membrane potential. Cells were plated in 6 well plates and treated as previously described in section 2.2. Media was removed and cells were washed with PBS. The green and red mitotracker dyes were prepared in serum free, phenol red free media, to give final concentrations of 25μM green and 50μM red. Mitotracker dye was added to cells and incubated for 30 mins at 37°C. Cells were then washed in PBS 3 times, and then 2mL of serum free, phenol red free media was added to cells before imaging. Cells were imaged on the EVOS™ M7000 Imaging System (Thermofisher, UK).

2.9 Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISA) were conducted in order to measure the levels of proteins and cytokines in patient serum and plasma, and condition media from cells. The pro-inflammatory cytokines and adipokines were; Asprosin (Abcam, ab275108, intra-assay cv: <7.9%, sensitivity: 0.92 ng/mL), TNFα (Sigma, RAB0476-1KT, inter-assay cv: <12%, intra-assay cv: <10%, sensitivity: 30 pg/mL), and IL-6 (Sigma, RAB0306-1KT, inter-assay cv: <12%, intra-assay cv: <10%, sensitivity: 3 pg/mL).

2.10 Human Nutritional Trial

2.10.1 Ethical Approval and Trial Summary

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were approved by the Nottingham Trent University Human

Ethics Committee (Register trial 713). All participants provided informed consent having been provided with the participant information sheet for a minimum of 24hrs prior to visit 1.

The study was conducted in a double-blind, placebo-controlled, crossover design, whereby participants with Asthma were provided with a 3.65g/day of the prebiotic Bimuno-Galactooligosaccharide (B-GOS) or 3.65 g/day of taste, and colour matched placebo (Maltodextrin) for 21 days followed by a 14-day wash out period before crossing over to the other intervention. Both supplements where portioned into sachets and double blinding was conducted at site of manufacture (Clasado Ltd). Each supplement was given in powdered form, which would be diluted in a liquid of the participants choice before consumption.

Participants attended the lab on four separate occasions at day 0 and 21 of each intervention. At each visit, participants completed questionnaires relating to their Asthma severity and symptoms, and had their height, weight, hip and waist measurements taken. Participants lung function was assessed (in accordance with ATS/ERS guidelines), and a 40mL blood sample was taken.

2.10.2 Venepuncture for blood serum and plasma collection

Blood serum and plasma samples were collected from each participant at every visit. This was done using a 21G needle and vacutainer tubes. For serum, approximately 5mL of blood was collected in a serum separator tube, incubated at room temperature for

30 mins to allow for clotting, then centrifuged for 15 mins at 1,000 rcf. The serum was aliquoted and stored immediately at -80^{o}C. For plasma, approximately 4mL of blood was collected in a K3 EDTA coated vacutainer tube and immediately centrifuged for 15 mins at 1,500 rcf. The plasma was then aliquoted and immediately stored at -80^{o}C.

2.10.3 Pulmonary Function Test

A spirometry test was conducted at each visit to assess pulmonary function using the Vitalograph Pneumotrac Spirometer with Spirotrac 6 Software (Vitalograph, UK). The spirometer was calibrated with a 3L syringe before each visit. American Thoracic Society (ATS) and the European Respiratory Society (ERS) guidelines state that this test must be performed a minimum of 3 times and a maximum of 8 times in one session (Graham *et al.*, 2019). The 2 largest measurements for FVC and/or FEV_1 must be within 150mL of each other (Graham *et al.*, 2019). The maximum values from the 3-8 taken would be used for further analysis.

Participants completed a flow volume loop manoeuvre stood upright whilst wearing a nose clip. Participants then took a deep breath in, and then strongly exhaled for 6s in order to empty the lungs, followed by a maximal inhalation. The measurements taken from the spirometry test are summarised in table 2.10.3.

Measurement	Abbreviation	Definition
Forced vital capacity	FVC	The volume of air that is able to be displaced from the lungs during maximal effort
Forced expiratory volume in 1s	FEV_1	The amount of air that can be forced out of the lungs in 1 second

Forced expiratory volume in 6s	FEV_6	The amount of air that can be forced out of the lungs in 6 seconds
Peak expiratory flow	PEF	The peak flow of air that is able to be displaced from the lungs during maximal effort
Forced expiratory flow at 25-75%	FEF_{25-75}	The amount of air exhaled in the middle portion of the FVC
Forced expiratory flow at 75-85%	FEF_{75-85}	The amount of air exhaled in the 75-85th percentile of the FVC

Table 2.10.3: Definitions of spirometry measurements

2.10.4 Analysis of blood serum markers

The Pentra C400 (Horiba, UK) was used to analyse biomarkers within the serum of each participant taken at each visit. The following biomarkers were measured in triplicate in each serum sample (all from Horiba, UK); Glucose PAP (1220001668), HDL direct (1220001636), LDL (1220001638), and Triglycerides (1220001640). All samples were run together to avoid variance between runs.

2.11 Fat on Fire study cohort

The Fat on Fire (FOF) study is an ethically approved study in which adipose tissue and blood serum and plasma were collected. A small subset of this study consisting of 126 female patients aged 31.6±6.1 years with a BMI of 27.9±5.9 kg/m^2 was selected by a previous researcher and adipose sample were prepared for gene analysis. In brief, subcutaneous (Sc) and omental (Om) adipose tissue samples were collected during abdominal surgery and flash frozen in liquid nitrogen before being stored at -80^{o}C. RNA was extracted from adipose tissue using the RNeasy lipid tissue kit (Qiagen, Manchester, UK) according to manufacturer's instructions, followed by a DNase digestion step. cDNA was then synthesised following the protocol detailed in section 2.5 and stored at -80^{o}C

until use. Blood serum and plasma samples were collected and prepared as previously described in sections 2.10.2.

2.12 Statistical analysis

Statistical analysis was preformed using GraphPad Prism (Version 9). Data are reported as the mean ± the standard error of the mean (SE) unless otherwise stated. The appropriate statistical test was selected based on the normality of the data, using mainly the One-way ANOVA plus Tukey's post hoc test to determine whether the differences shown between treatment groups were statistically significant, and Pearson r correlation and linear regression to identify correlations between genes. The 2way ANOVA plus Šídák's multiple comparisons test was used for the human intervention trial analysis to compare data before and after each supplement. Statistical significance was considered to be $p<0.05$ and was reported as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Chapter 3: The impact of gut inflammatory and anti-inflammatory factors on inflammation in human airway epithelial and white adipocyte cells

3.1 Introduction

Underlying inflammation is a common link between many chronic diseases, including metabolic disease and Asthma. Asthma is an inflammatory disease of the airways, which is affected by genetics, lifestyle, or environmental triggers leading to comorbidities such as respiratory diseases. In addition, it is apparent that clinical Obesity (BMI over 30Kg/m^2) is a risk factor for the development of Asthma in both children and adults (Forno *et al.*, 2018; Peters, Dixon and Forno, 2018).

Obesity is thought to influence Asthma through multiple mechanisms. Excess visceral adipose tissue in the chest may exacerbate Asthma due to restricted movement of the diaphragm (Dixon, Peters 2018; Yang et al. 2018). Increased weight gain in Obesity increases the volume of adipose tissue stores, and these enlarged adipocytes may in turn release inflammatory factors which may enter the circulation and further increase the underlying inflammation present in Asthma (Conde *et al.*, 2011; Mancuso, 2016; Taylor, 2021). Other mechanisms to influence Asthma may arise as patients with Obesity are reported to have a weaker and more permeable gut epithelial barrier, allowing inflammatory factors from the gut to enter the circulation.

A particular molecule of interest is endotoxin (also known as liposaccharide; LPS), which is found on the outer membrane of gram-negative bacteria (Piya et al. 2013). If endotoxin detaches from the outer membrane, it is able to traverse the gut barrier and enter the circulation (Ghoshal *et al.*, 2009; Tulkens *et al.*, 2020), where it can lead to inflammation through activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway through toll-like receptor 4 (TLR-4) (Creely *et al.*, 2007;

Jang *et al.*, 2014; Liu *et al.*, 2018). Through TLR-4, LPS can also activate the c-Jun N-Terminal kinases (JNK) pathway which signals for cell death in response to inflammation (Hambleton *et al.*, 1996; Tsao *et al.*, 2011; Li *et al.*, 2014; Chang *et al.*, 2021).

Endotoxin release is known to be influenced by the diet, as studies have shown that circulating endotoxin levels are increased after consumption of a high fat meal, which can then lead to systemic inflammation (Cani *et al.*, 2007; Erridge *et al.*, 2007; Laugerette *et al.*, 2011; Harte *et al.*, 2012; Vors *et al.*, 2015; Lyte, Gabler and Hollis, 2016). Endotoxin has been shown to influence cytokine release and immune cell response in Asthma (Goleva *et al.*, 2008, 2013; Hauk *et al.*, 2008; Berger *et al.*, 2015; Zhao *et al.*, 2017; Ren *et al.*, 2019) and is increased in conditions of Obesity (Harte *et al.*, 2012).

Although diet may have a negative impact on inflammation and subsequent disease risk posed by endotoxin and cytokine release, it may also be possible to reverse these effects. A prebiotic is a type of fibre which encourages the growth of beneficial bacteria in the gut (Hill *et al.*, 2014). When metabolised by these bacteria, prebiotics are broken down into metabolites including short chain fatty acids (SCFAs). The main SCFAs produced are acetate, butyrate, and propionate. SCFAs have been shown to have anti-inflammatory effects (Theiler *et al.*, 2019) and the ability to reduce endotoxin-induced damage and inflammation (Liu *et al.*, 2012; Wang *et al.*, 2017; Feng *et al.*, 2018; Diao *et al.*, 2019).

Studies exploring the effect of SCFAs on LPS-induced inflammation *in vitro* are however limited, and no studies to date have examined the effect on airway or adipocyte cell

models. Therefore, the aims of this study were to (1) establish whether LPS induces inflammation in both human airway epithelial and white adipocyte cell lines, (2) evaluate the influence of SCFAs on LPS-induced inflammation, and (3) determine which molecular inflammatory pathways are involved.

3.2 Methods

3.2.1 Cell culture and treatments

Human bronchial epithelial cells (BEAS2B-R1) and human white adipocyte cells (Chub-S7) were cultured in 6-well cell culture plates as previously described in chapter 2.1. Cells were treated once BEAS2B-R1 cells had reached 70-80% confluency and on Day 16 of the Chubs-S7 differentiation. Cells were pre-treated with a SCFA mix 24 hours prior to treatment with an inflammatory factor. The SCFA mix gave a final concentration of 2mM Acetate, 0.25mM Butyrate and 0.25mM of Propionate (each from Sigma, UK), giving a ratio of 80:10:10 which is seen under physiological conditions in the circulation (Jocken *et al.*, 2018). Cells were then treated with either 10ng/mL or 100ng/mL Lipopolysaccharide (LPS; from Escherichia coli O55:B5, Sigma, UK) for 6, 12, and 24 hours in order to induce inflammation.

3.2.2 Protein extraction and quantification

Protein samples were lysed in 100µL of 1x Radioimmunoprecipitation Assay Buffer (RIPA) containing 2% protease and phosphatase inhibitors. Cells were scraped in RIPA buffer on ice and then collected in 1.5mL microfuge tubes. Cells were placed on ice for 30 mins and vortexed every 10 mins to assist with cell lysis. The cells were then pelleted by centrifugation for 15 mins at $16x10^3$ rcf at 4°C. The supernatant containing the protein was then collected and stored at -80°C.

The concentration of protein in each sample was quantified using the Bradford Assay. BSA protein standards were diluted in a range of 0-1mg/mL. Protein samples were

diluted 1:20 in deionised water and added to 200µL of 1x Bradford reagent (Bio-rad, UK). All standards and samples were run in triplicate. The absorbance was read at a wavelength of 595nm. A standard curve was created in order to calculate the concentrations of the protein samples. The protein samples were then mixed with 4x Laemmli Buffer and heated at 95°C for 10 mins, then stored at -80°C.

3.2.3 Western Blot

In order to determine the levels of expression of proteins of interest (see below) in the samples, Western Blot analysis was performed as previously described in chapter 2.4. In brief, 50µg of protein was loaded onto a 10% polyacrylamide gel, which was run at 140V for 90 mins. The gel was then transferred onto an Immobilon-P transfer membrane (Millipore, UK) with 0.45µm pore size for 100 minutes at 100V. The membrane was then washed in water and blocked in either 5% BSA in tris buffered saline-Tween20 (TBS-T) or 0.2% I-Block in PBS-T for 1 hour at room temperature. Primary antibodies were then added to the membrane over night at 4°C. The following antibodies were used:

Antibody	Company (Item No.)	Buffer	Blocking	Primary	Secondary
β-Actin	CST (#4970)	PBS	I-Block	1:1000	1:100,000 (R)
NFκB	CST (#8242)	PBS	I-Block	1:1000	1:100,000 (R)
p-NFκB	CST (#3033)	TBS	BSA	1:1000	1:50,000 (R)
IKKβ	CST (#8943)	PBS	I-Block	1:500	1:50,000 (R)
IKKγ	CST (#2685)	PBS	I-Block	1:500	1:50,000 (R)
JNK	CST (#9252)	PBS	I-Block	1:500	1:50,000 (R)
p-JNK	CST (#9251)	TBS	BSA	1:500	1:50,000 (R)
p-IkBα/ IkBα	Novus (NB100-56724)	TBS	I-Block	1:500	1:10,000 (M)

Table 3.2.3: Western blot antibodies and buffers

Details of primary and secondary antibody concentrations and the buffers and blocking solutions used. Abbreviations in table: Cell Signalling Technologies (CST), Rabbit (R), Mouse (M)

The following day, membranes were washed in either TBS-T or PBS-T 3 times for 5 mins. The secondary antibody, which would be either anti-rabbit (Sigma #A9169) or anti-mouse (Cell Signalling Technologies #7076) as stated in table 3.2.3.1, was then added for 1 hour at room temperature. The washing step was then repeated, and the membranes incubated briefly in Westar Antares HRP Detection Substrate (Geneflow, UK) which uses enhanced chemiluminescence (ECL) in order to visualise the bands. The membranes were visualised on the GeneGnome XRQ Visualiser (Syngene, UK).

3.2.4 RNA Extraction and cDNA synthesis

RNA samples were lysed on ice in 350μL Buffer RLY (Bioline, UK) and 3.5μL β-mercaptoethanol (β-ME). RNA was extracted using the Isolate II RNA Mini Kit (Bioline, #BIO-52073), as previously described in chapter 2.5. The final step resulted in 60μL of RNA being eluted in RNase-free water. In order to calculate the concentration and purity of the RNA samples, 1μL of RNA was measured using the Nanodrop ND-100 (Labtech, UK).

In order to synthesise cDNA from extracted RNA, 1ug of RNA in 10uL of H_2O was combined with 1uL random hexamers and 1μL dNTPs (Thermofisher, UK) and heated at 70^{o}C for 10 mins. Samples were then immediately transferred onto ice for 2 mins. A mastermix was prepared containing the following; 2μL 10x M-MLV reverse transcriptase buffer, 1μL M-MLV (Sigma), 0.5μL RNase OUT (Thermofisher, UK) and 4.5μL H_2O. To each sample, 8μL of this mastermix was added. Finally, samples underwent 1 cycle of 20

mins at 25°C, 50 mins at 37°C and 10 mins at 80°C. The resulting samples were then diluted to 100ng with H_2O and stored at -20°C until use.

3.2.5 Real time quantitative polymerase chain reaction

3.2.5.1 TaqMan gene expression assays

Gene expression was measured using real time quantitative polymerase chain reaction (RT-qPCR). Each reaction was run in a volume of 20µL, containing 100ng cDNA, 1x housekeeping gene (18S), 1x gene of interest, 1x TaqMan master mix (Thermofisher, UK) and H_2O. The following TaqMan expression assays (Thermofisher, UK) were used:

Gene	Assay ID
18S	Hs99999901_s1
NFKB1	Hs00765730_m1
MAPK8	Hs01548508_m1
CXCL8	Hs00174103_m1

Table 3.2.5.1: List of TaqMan expression assays

The RT-qPCR was carried out in the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, UK) as follows; 2 mins at 50°C, 10 mins at 95°C, then 40 cycles of 15s at 95°C and 1 min at 60°C.

3.2.5.2 Gene expression analysis

In order to calculate the expression of each gene relative to the control, the following formula was used:

$$mRNA\ expression = 2^{-(\Delta\Delta Ct)}$$

$$where\ \Delta\Delta Ct = (Gene\ of\ interest - 18S) - Average\ Control$$

3.2.6 Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISA) were conducted in order to measure the levels of pro-inflammatory TNFα (Sigma, RAB0476-1KT) and IL-6 (Sigma, RAB0306-1KT) in conditioned media from Chub-S7 cells.

Conditioned media was removed from cells in a 6-well plate at 6, 12, and 24hrs before they were harvested. The conditioned media was then immediately stored at -80^{o}C until use. Each ELISA was run according to manufacturer's instructions. For the IL-6 ELISA, a 1:2 dilution of the conditioned media was prepared, and for the TNFα ELISA condition media was used undiluted.

3.2.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 9). Data are reported as the mean ± the standard error of the mean (SE) unless otherwise stated. The appropriate statistical test was selected based on the normality of the data, using mainly the Two-way ANOVA plus Tukey's post hoc test to determine significance between treatment groups. Statistical significance was considered to be $p<0.05$ and was reported as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

3.3 Results

3.3.1 The effect of LPS and SCFAs on inflammatory gene expression

The expression of pro-inflammatory genes were measured by RT-qPCR in BEAS2B-R1 cells treated with 100 ng/mL LPS to induce inflammation, with and without a SCFA mixture. LPS increased NFκB expression at 6hrs by 1.8-fold±0.24 ($p<0.05$), but SCFAs did not reduce this expression (Figure 3.3.1.1a). At 12hrs, LPS did not increase NFκB expression compared to the control, but SCFAs reduced NFκB expression by 0.4-fold±0.05 at 12hrs in both the SCFA and LPS+SCFA treatment groups ($p<0.001$) compared to LPS alone. NFκB expression was increased at 24hrs by 2.6-fold±0.23 ($p<0.001$), which SCFAs reduced by 50% ($p<0.05$).

MAPK8 (JNK) was not affected by LPS treatment (Figure 3.3.1.1b). There was a 50% decrease in MAPK8 expression at 12hrs in the LPS in combination with SCFA treatment group compared with the control ($p<0.05$). LPS caused a sharp increase in CXCL8 (IL-8) expression at all time points by over 30-fold ($p<0.0001$) (Figure 3.3.1.1c). This increase was also observed in the LPS in combination with SCFA treatment. The SCFAs were not able to decrease CXCL8 gene expression in the presence of LPS, and also had no effect on CXCL8 expression themselves.

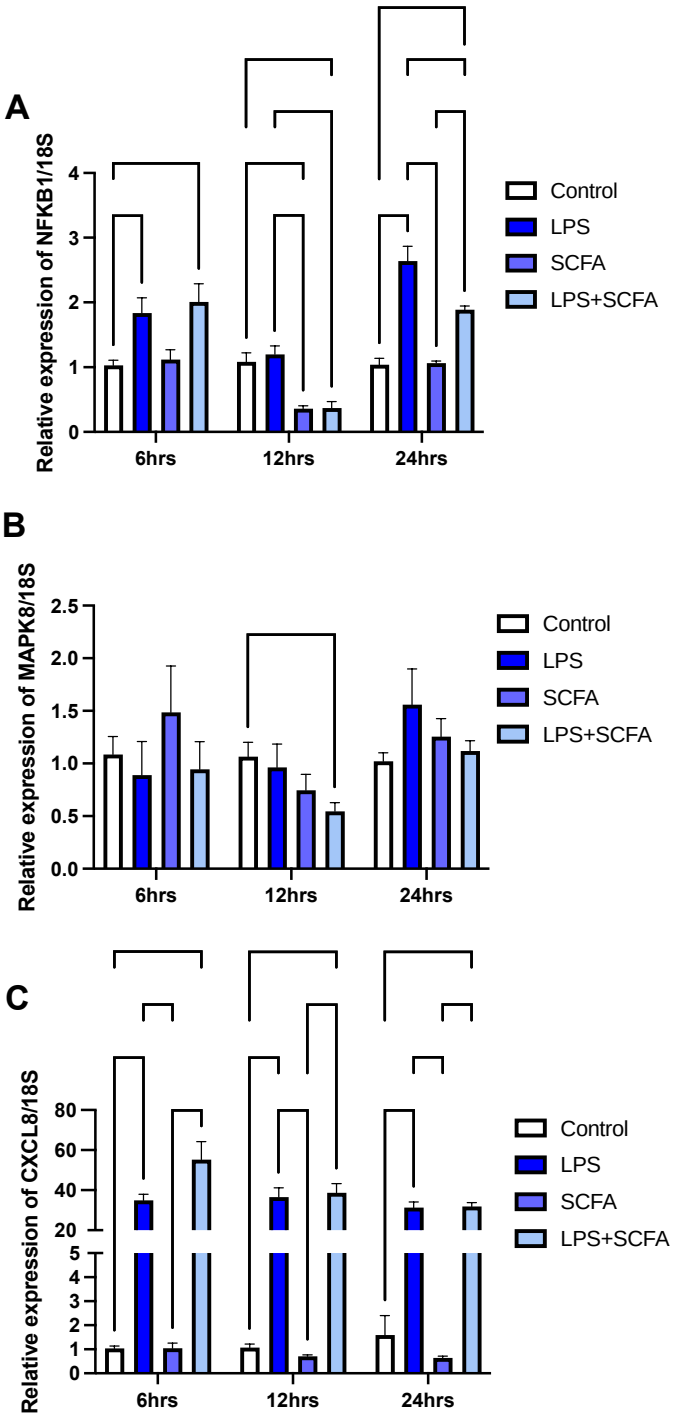

Figure 3.3.1.1: The effect of LPS and SCFAs on inflammatory gene expression in BEAS2B-R1 cells.

BEAS2B-R1 airway epithelial cells treated with 100 ng/mL LPS and SCFA mix for 6, 12, and 24hrs (n=3). Gene expression measured by RT-qPCR for the following inflammatory genes; (A) NFKB1 [NFκB], (B) JNK [MAPK8], (C) IL-8 [CXCL8]. Data is presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post hoc test was performed (p values are displayed as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

The previously described treatments were then administered to Chub-S7 cells, and inflammatory genes were measured (Figure 3.3.2.1). Compared with the control, LPS increased NFKB1 gene expression by 4.6-fold±0.37 at 6hrs ($p<0.0001$), 1.8-fold±0.17 at 12hrs ($p<0.01$), and 1.9-fold±0.15 at 24hrs ($p<0.001$) (Figure 3.3.2.1a). Similarly, CXCL8 gene expression was increased by over 25-fold at each time point in LPS treated cells ($p<0.0001$) (Figure 3.3.2.1c). SCFAs were unable to mitigate the LPS-induced expression of NFKB1 and CXCL8 at any time point. LPS had no effect on MAPK8 in Chub-S7 cells, however at 6hrs LPS appeared to increase MAPK8 expression although this was not significant (Figure 3.3.2.1b).

Each cell line had a different time-dependent response to LPS treatment. Chub-S7 cells had the largest increase at 6hrs, compared with BEAS2B-R1 cells which had more of a pro-inflammatory response at 24hrs. SCFAs appeared to have more of an anti-inflammatory effect in BEAS2B-R1 cells compared with Chub-S7 cells, with BEAS2B-R1 cells showing a decrease in NFKB1 and MAPK8 expression in the LPS+SCFA group.

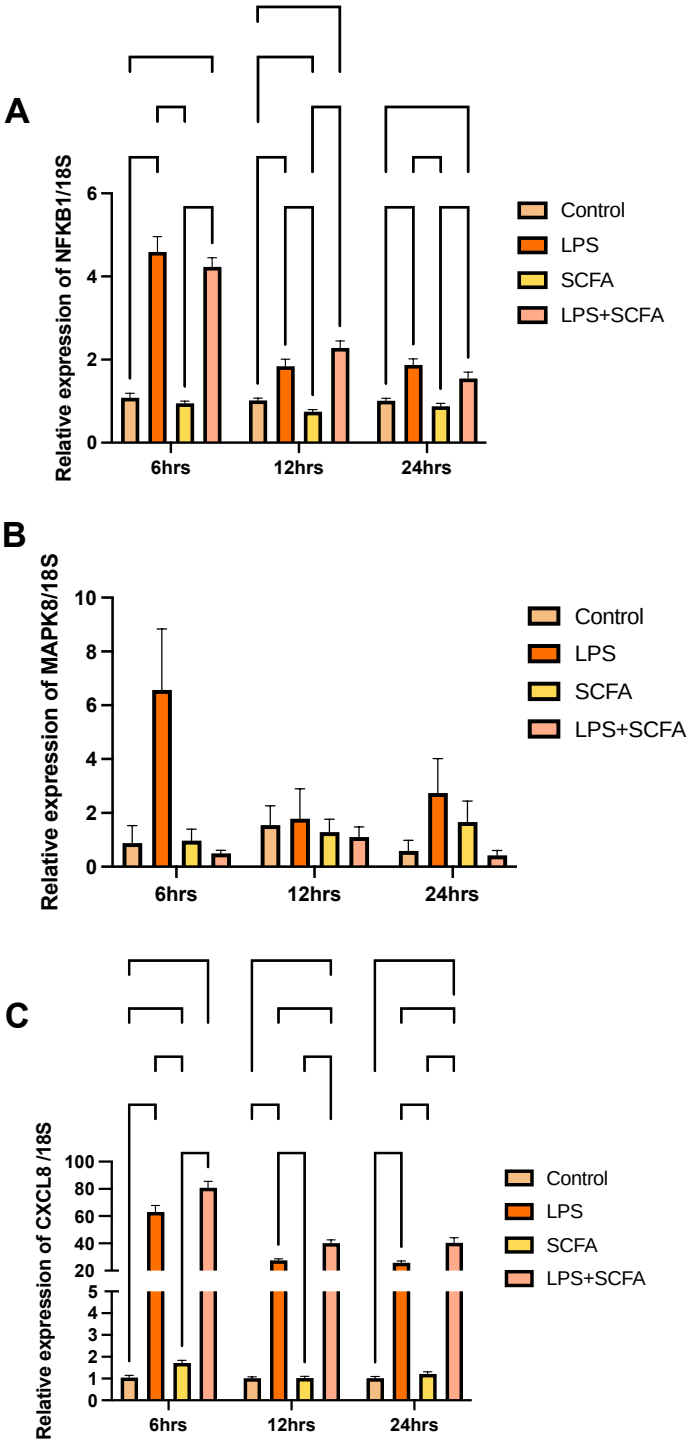

Figure 3.3.1.2: The effect of LPS and SCFAs on inflammatory gene expression in Chub-S7 cells.

Chub-S7 adipocyte cells treated with 100 ng/mL LPS and SCFA mix for 6, 12, and 24hrs (n=6). Gene expression measured by RT-qPCR for the following inflammatory genes; (A) NFKB1 [NFκB], (B) JNK [MAPK8], (C) IL-8 [CXCL8]. Data is presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post hoc test was performed (p values are displayed as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

3.3.2 The effect of LPS and SCFAs on inflammatory protein expression in human airway epithelial cells

The expression of pro-inflammatory proteins in the canonical NFκB pathway were measured by Western blot in BEAS2B-R1 cells treated with 10ng/mL and 100 ng/mL LPS to induce inflammation with or without a SCFA mixture. The varying LPS concentrations were used to determine whether SCFAs could mitigate the effects of LPS on inflammation and if so, at what dose.

LPS did not induce phosphorylation and therefore activation of NFκB in BEAS2B-R1 cells (Figure 3.3.2.1a). At 6hrs, the L100+SCFA treatment group had a 0.5-fold reduction compared to the L10 treatment group ($p<0.05$), however SCFAs were unable to reduce NFκB phosphorylation at any of the other time points. LPS treatment of BEAS2B-R1 cells was also unable to significantly increase IκBα phosphorylation at any time point, though data appeared to show a trend towards a rise (Figure 3.3.2.1b).

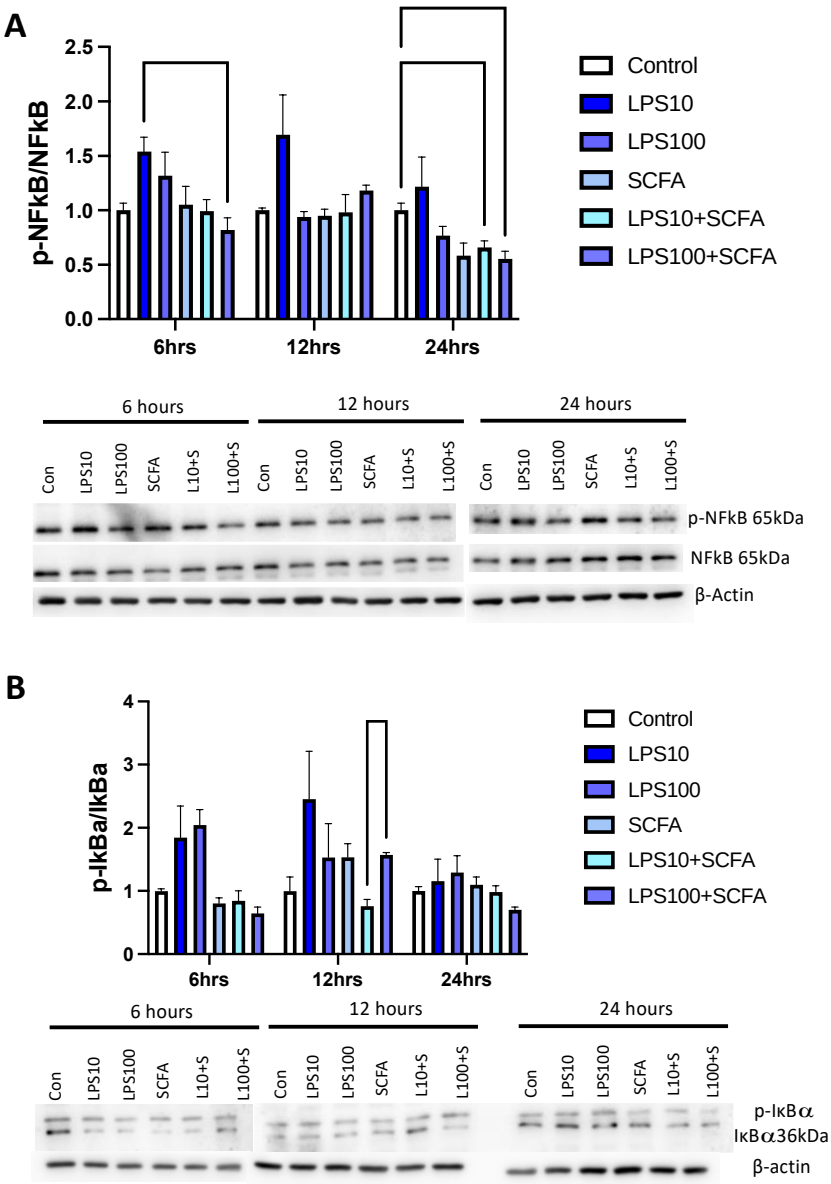

Figure 3.3.2.1: The effect of LPS and SCFAs on NFκB and IκBα expression and phosphorylation in BEAS2B-R1 cells

BEAS2B-R1 airway epithelial cells treated with 10 ng/mL and 100 ng/mL LPS and SCFA mix for 6, 12, and 24hrs (n=4). (a) NFκB and (b) IκBα protein expression and phosphorylation were measured by western blot. Data is presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post hoc test was performed (p values are displayed as follows; * $p<0.05$).

Figure 3.3.2.2a shows that in BEAS2B-R1 cells at 6hrs, 100ng/mL LPS treatment was able to increase IKKβ protein expression by 1.9-fold±0.18 ($p<0.05$) which was subsequently reduced by SCFA treatment ($p<0.01$). At 12hrs, SCFAs reduced LPS-induced IKKβ

expression when treated with 10ng/mL LPS ($p<0.05$), but this effect was not present by 24hrs. LPS and SCFAs had no effects on IKKγ expression across all time points (Figure 3.3.2.2b).

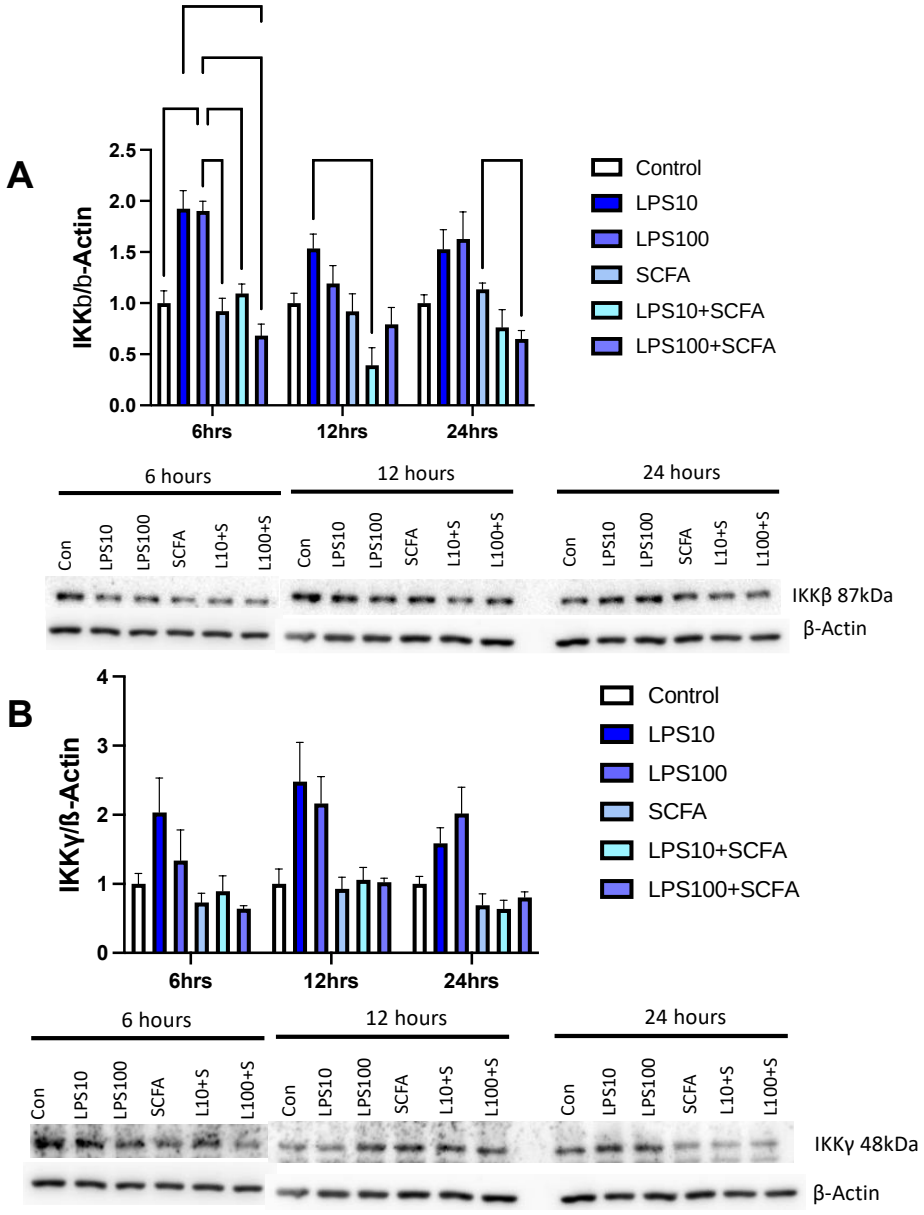

Figure 3.3.2.2: The effect of LPS and SCFAs on IKKβ and IKKγ expression in BEAS2B-R1 cells

BEAS2B-R1 airway epithelial cells treated with 10 ng/mL and 100 ng/mL LPS and SCFA mix for 6, 12, and 24hrs (n=4). IKKβ and IKKγ protein expression were measured by western blot. Data is presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post hoc test was performed (p values are displayed as follows; * $p<0.05$, ** $p<0.01$).

LPS and SCFA treatment had no significant effects on JNK54 phosphorylation (Figure 3.3.2.3), although there was a trend towards an increase in JNK phosphorylation at 24hrs in cells treated with 100ng/mL LPS (p=n.s.). At 12 and 24hrs, phosphorylation of JNK46 in the L100+S treatment group was reduced by 50% compared to 100 ng/mL LPS alone ($p<0.05$).

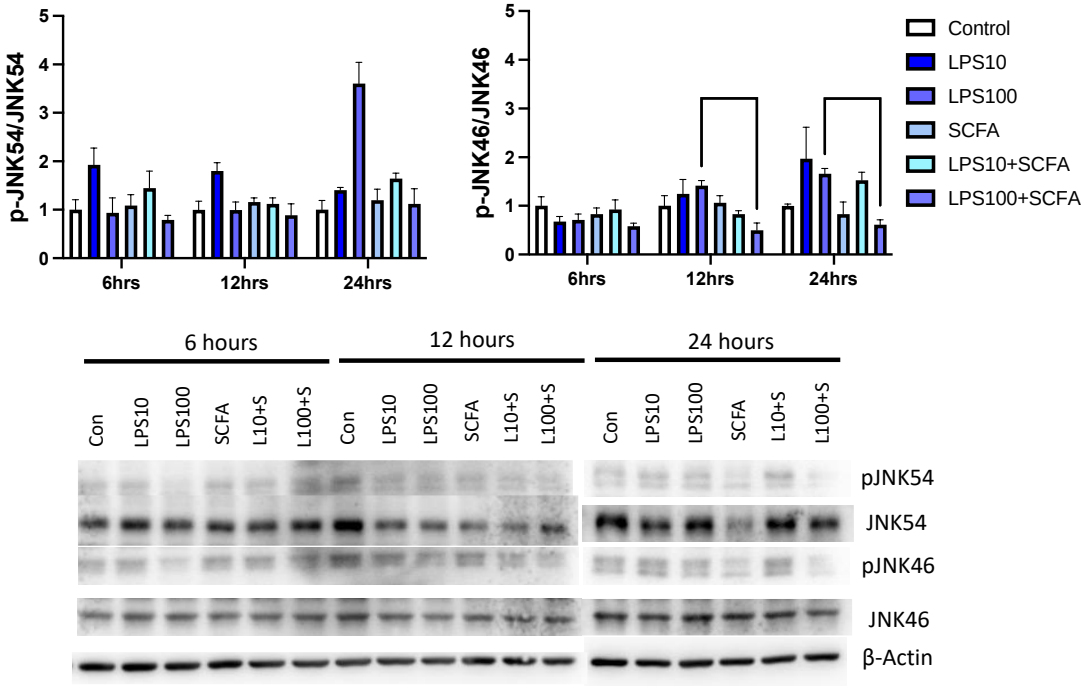

Figure 3.3.2.3: The effect of LPS and SCFAs on JNK54 and JNK46 expression and phosphorylation in BEAS2B-R1 cells

BEAS2B-R1 airway epithelial cells treated with 10 ng/mL and 100 ng/mL LPS and SCFA mix for 6, 12, and 24hrs (n=3). JNK54 and JNK46 protein expression and phosphorylation were measured by western blot. Data is presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post hoc test was performed (p values are displayed as follows; * $p<0.05$).

There was no significant difference between the 10ng/mL and 100ng/mL LPS treatments in terms of increasing the inflammatory response. To provide the cells with a suitable

induction of inflammation that could be mitigated at times by SCFAs, subsequent studies maintained the higher level of 100ng/mL LPS.

3.3.3 The effect of LPS and SCFAs on inflammatory protein expression in human adipocyte cells

The expression of pro-inflammatory proteins in the NFκB pathway were measured by western blot in Chub-S7 cells treated with 100 ng/mL LPS to induce inflammation and a SCFA mixture. As shown in Figure 3.3.3.1, LPS increased NFκB phosphorylation by 4-fold at 6hrs (p=n.s.) and 24hrs ($p<0.05$). This phosphorylation was reduced by SCFAs at 12hrs ($p<0.01$) and 24hrs ($p<0.05$).

There was no increase in expression of IKKβ or IKKγ (Figure 3.3.3.2) or JNK54 and JNK46 phosphorylation (Figure 3.3.3.3) in the presence of LPS. There was a significant decrease in IKKβ expression at 12hrs in the LPS+SCFA group compared to the control ($p<0.01$) and LPS ($p<0.05$) groups. Whilst in contrast, at 6hrs in the LPS+SCFA group, there was a 1.7-fold±0.13 increase of JNK54 phosphorylation compared with the control ($p<0.05$).

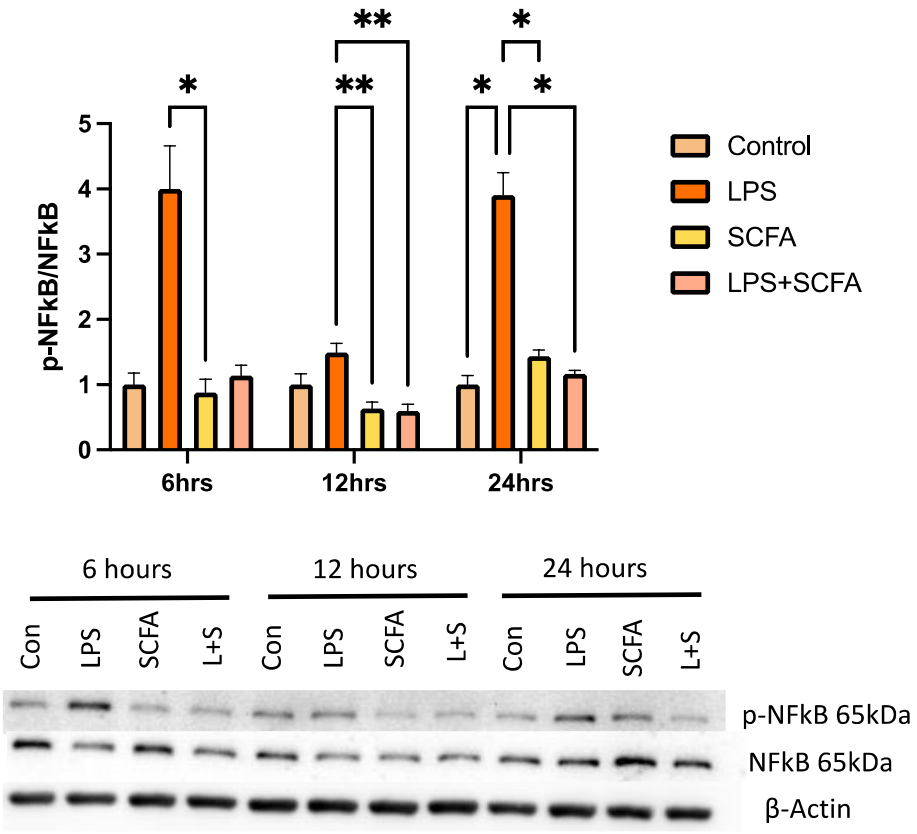

Figure 3.3.3.1: The effect of LPS and SCFAs on NFκB expression and phosphorylation in Chub-S7 cells

Chub-S7 cells treated with 100 ng/mL LPS and SCFA mix for 6, 12, and 24hrs (n=5). NFκB protein expression and phosphorylation were measured by western blot. Data is presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post hoc test was performed (p values are displayed as follows; * $p<0.05$, ** $p<0.01$).

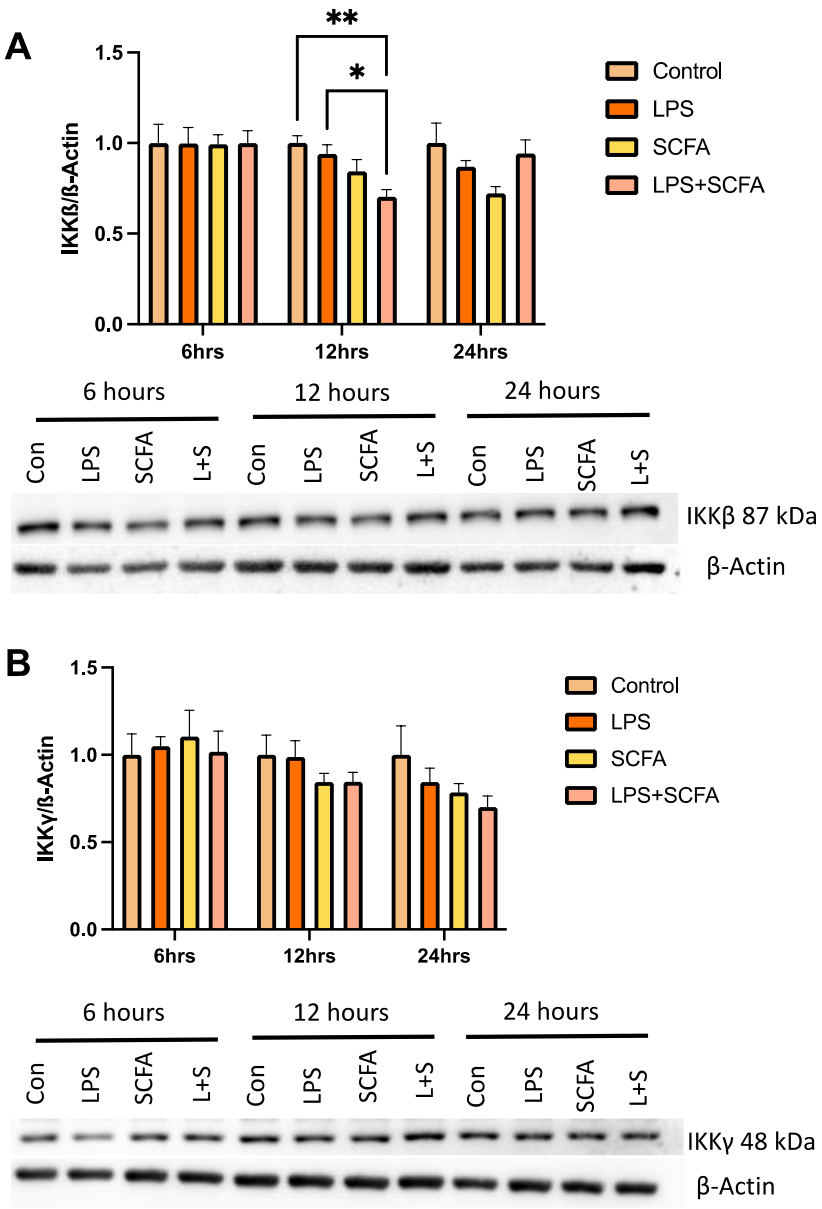

Figure 3.3.3.2: The effect of LPS and SCFAs on IKKβ and IKKγ expression in Chub-S7 cells

Chub-S7 adipocyte cells treated with 100 ng/mL LPS and SCFA mix for 6, 12, and 24hrs (n=5). IKKβ and IKKγ protein expression were measured by western blot. Data is presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post hoc test was performed (p values are displayed as follows; * $p<0.05$, ** $p<0.01$).

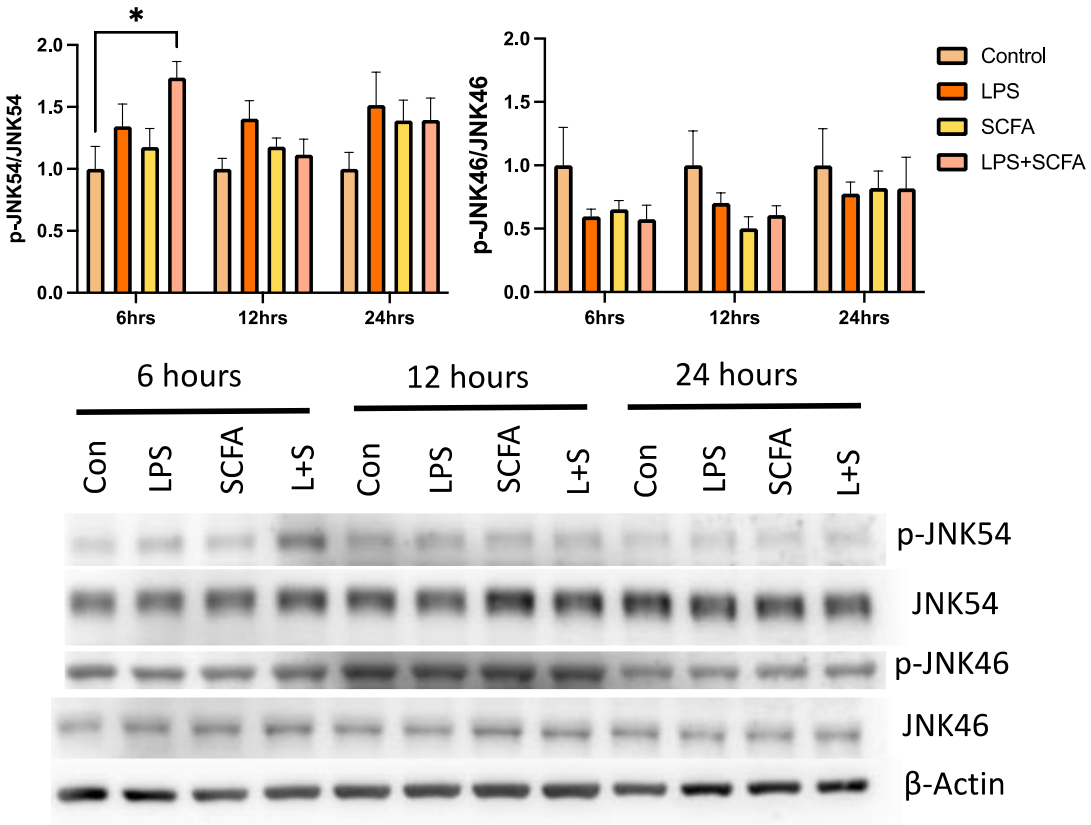

Figure 3.3.3.3: The effect of LPS and SCFAs on JNK54 and JNK46 expression and phosphorylation in Chub-S7 cells

Chub-S7 adipocyte cells treated with 100 ng/mL LPS and SCFA mix for 6, 12, and 24hrs (n=4-5). JNK54 and JNK46 protein expression and phosphorylation were measured by western blot. Data is presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post hoc test was performed (p values are displayed as follows; * $p<0.05$).

3.3.4 The effect of LPS and SCFAs on inflammatory cytokine levels in the conditioned media of human adipocyte cells

Levels of inflammatory cytokines IL-6 and TNFα were measured by ELISA in the conditioned media of Chub-S7 cells which had been treated with SCFAs and LPS for 6, 12, and 24hrs. SCFAs were able to reduce LPS-induced IL-6 release by 57% at 24hrs (LPS; 3.53±0.21ng/ml vs LPS+SCFA; 1.54±0.06ng/ml; $p<0.05$) (Figure 3.3.4.1a). No significant differences relating to TNFα release were observed across all time points (Figure

3.3.4.1b), however the data trended towards a reduction in TNFα release in cells treated with LPS compared with the control.

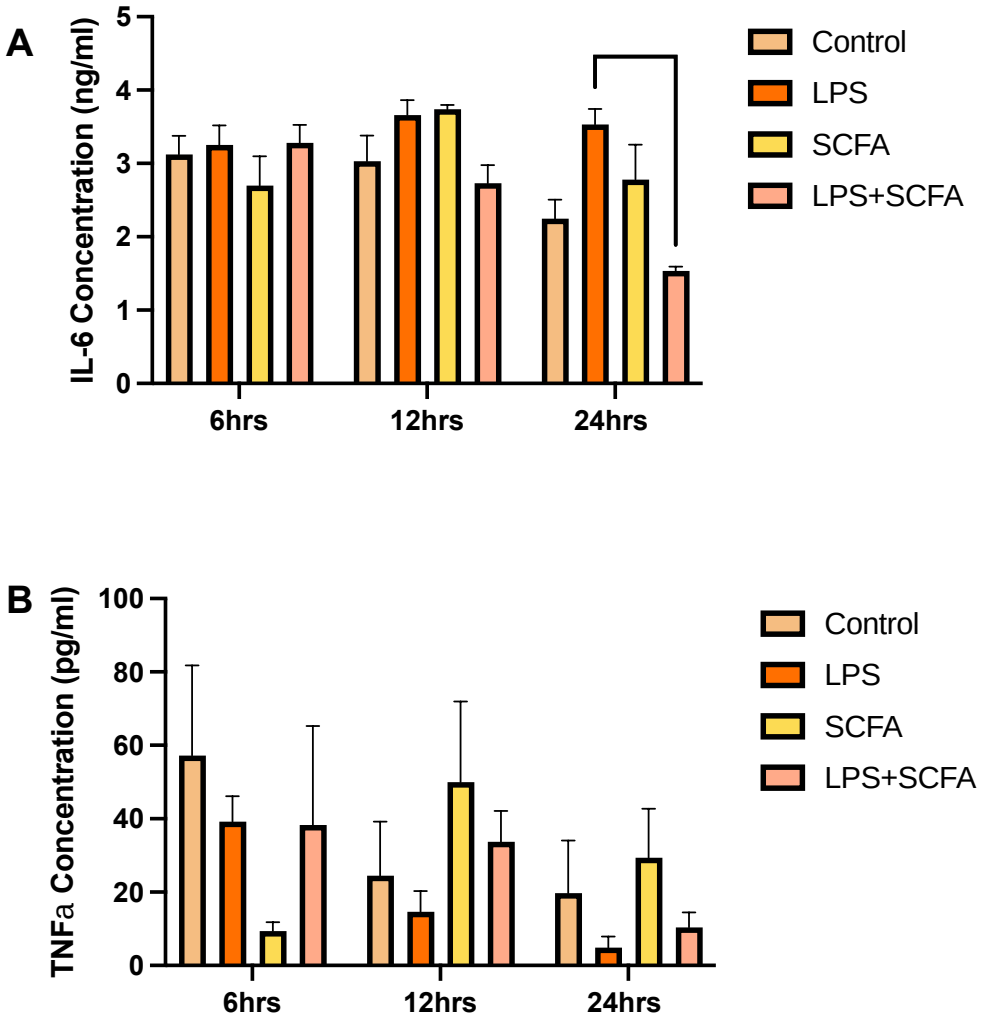

Figure 3.3.4.1: The effect of LPS and SCFAs on inflammatory cytokines measured in the condition media of Chub-S7 cells

Chub-S7 adipocyte cells treated with 100 ng/mL LPS and SCFA mix for 6, 12, and 24hrs (n=3). Levels of (A) IL-6 and (B) TNFα were measured in condition media by ELISA. Data is presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post hoc test was performed (p values are displayed as follows; * $p<0.05$).

3.4 Discussion

This chapter explores the potential impact of gut derived endotoxin (LPS) as a mediator of inflammation in airway epithelial cells and white adipocyte cells, and whether SCFAs could mitigate this endotoxin induced inflammation. Principally, these studies sought to use *in vitro* human model systems to (1) establish whether LPS induced inflammation in a human airway epithelial and white adipocyte cell lines, (2) examine the capacity of SCFAs to mitigate LPS-induced inflammation, and (3) explore the extent of the involvement of inflammatory pathways.

3.4.1 The effect of LPS and SCFAs on inflammatory gene expression

Studies have previously shown circulating levels of LPS to be increased in patients with inflammatory and metabolic disease (Harte *et al.*, 2010, 2012; Lyte, Gabler and Hollis, 2016). The current study offers insight into how circulating LPS may enhance inflammation in airway and adipocyte cells. Gene expression data showed that LPS was able to increase pro-inflammatory genes NFKB1 and CXCL8 in both the BEAS2B-R1 and Chub-S7 cell lines. LPS was not able to increase expression of MAPK8, suggesting that the increased inflammation appears to be mediated through the NFκB pathway. This has been previously shown in BEAS2B-R1 cells, rat lung tissue, and human adipocytes, where LPS activates NFκB through the TLR-4 receptor pathway (Creely *et al.*, 2007; Liu *et al.*, 2020; Hou *et al.*, 2021; Si and Zhang, 2021).

Interestingly the ability of SCFAs to mitigate the inflammatory effects posed by LPS appeared to be cell type specific, as within these gene expression studies, SCFAs were

only able to reduce LPS-induced NFκB expression in the BEAS2B-R1 cells. There were no anti-inflammatory effects observed in the Chub-S7 cells. Previous studies have shown that SCFAs can reduce LPS-induced inflammation, although these studies were in intestinal barrier cells (Feng *et al.*, 2018), endothelial cells (Li et al. 2018), and macrophage-like cells (Liu *et al.*, 2012; Wang *et al.*, 2017). These studies were not looking directly at gene expression as they measured secreted protein expression. This suggests that although the effects of SCFAs were not observed in the gene expression data, they may instead alter protein activation in order to reduce inflammation within these cell lines. Furthermore, many studies use only 1 SCFA alone, rather than a mixture as in this study. Using a mixture of acetate, butyrate, and propionate is more physiologically relevant as they are the most abundant SCFAs, but the use of all 3 may dampen the individual effects which may explain why the data in this study did not show anti-inflammatory effects across each gene and cell line. This consideration of subtle changes based on SCFA use is reflected in a study which showed that acetate can reduce LPS-induced IL-8 levels, but butyrate and propionate did not (Li et al. 2018).

3.4.2 The effect of LPS and SCFAs on inflammatory protein expression

Further analysis explored the influence of LPS and SCFA on protein expression in airway cells. In BEAS2B-R1 cells, LPS caused an acute (defined as under 12hrs) response from IKKβ, and a more chronic (defined as over 12hrs) response from JNK46, both of which were mitigated by SCFAs. A trend towards a chronic response appeared to be shown with JNK54 (p=ns), but NFκB activation was not observed. This was an unexpected finding, as a previous study using BEAS2B-R1 cells had shown that 100ng/mL LPS is able

to cause NFκB translocation to the nucleus, which occurs as a result of phosphorylation (Hou *et al.*, 2021). Furthermore, it has been shown that LPS can increase activation of NFκB in BEAS2B-R1 cells, however the concentration of LPS utilised in these studies was often substantially higher (up to 25μg/mL) than that used in the present study (Muthumalage and Rahman, 2019; Liu *et al.*, 2020; Si and Zhang, 2021).

Additionally, IKKβ is known to phosphorylate the p65 subunit of the NFκB complex at S536 (Christian, Smith and Carmody, 2016), so given that IKKβ expression was increased at 6hrs, it was expected that there should have been phosphorylation of p65. Since there was no p65 phosphorylation observed, it suggests that there could be a different factor involved that may be blocking NFκB phosphorylation in this cell line, since gene expression data did show an increase in expression of NFκB in response to LPS (Figure 3.3.1.1a). In order to activate NFκB and allow translocation to the nucleus, an increase in IκBα phosphorylation must occur to allow it to dissociate from NFκB. Data in Figure 3.3.2.1b trends towards this, as there is no increase in IκBα phosphorylation in the L100 group, which may have prevented NFκB activation. Also, this study only looked at phosphorylation at S536 on the p65 subunit, but there are many other phosphorylation sites. IKKβ has been shown to phosphorylate at S468 which inhibits NFκB activity (Christian, Smith and Carmody, 2016), so this may have occurred.

Chub-S7 cells had a heightened response to LPS compared to the BEAS2B-R1 cells, showing an activation of NFκB in response to LPS treatment, which was reduced by SCFAs at 12 and 24hrs. There was also a reduction in LPS-induced IKKβ expression at

12hrs in response to SCFAs, whilst JNK54 phosphorylation was increased at 6hrs in the LPS+SCFA treatment group. Although this increase in JNK54 phosphorylation may be unexpected, it could be due to SCFAs activating the innate immune response to induce apoptotic signalling and the inflammasome in order to respond to damage, which has been previously shown (Tang *et al.*, 2011; Matthews, Howarth and Butler, 2012; Tsugawa *et al.*, 2020).

The different responses to LPS in each cell line may be due to their location in the body. Given that BEAS2B-R1 cells are airway epithelial cells, they would typically be constantly exposed to the outside environment and given that LPS is ubiquitous, if the airways always reacted with a pro-inflammatory response to LPS exposure, the patient would be in a constant state of high inflammation which would have detrimental effects to health. This is reflected in studies stating that early exposure to endotoxin can reduce the instance of Asthma, due to suppression of Th2 inflammatory mechanisms (von Mutius *et al.*, 2000; Kuipers *et al.*, 2003). Inflammatory response also depends on the strain of endotoxin, where strains derived from species such as *Bacteroidetes* and *Prevotella* are less likely to be associated with Asthma (Lynch *et al.*, 2014; Larsen *et al.*, 2015). This is compared to the Chub-S7 cells as a representative of adipose tissue which is an internal organ, so would most likely only be in contact with circulatory LPS derived from the gut. This is reflected in the fact that the BEAS2B-R1 treated with LPS only increased NFκB protein phosphorylation by around 2-fold compared to 4-fold in the Chub-S7 cells.

3.4.3 The effect of LPS and SCFAs on inflammatory cytokine release in human white adipocyte cells

Activation of the NFκB pathway leads to the release of pro-inflammatory cytokines, which give an indication of the levels of inflammation present in the cell. These cytokines may include IL-6, IL-8, TNFα, and MCP-1. IL-6 can be anti-inflammatory in response to acute inflammation. A previous study showed that an increase in IL-6 can decrease plasma levels of endotoxin-induced TNFα (Starkie *et al.*, 2003). This is reflected in figure 3.3.4.1 where at 24hrs, LPS increased IL-6 levels in Chub-S7 condition media, but a reduction in TNFα appeared to be observed (p=ns). It could be that this potential decrease in TNFα is due to the increase of IL-6. The decrease in TNFα may also be due to a potential decrease in pJNK46 (p=ns), given that JNK activation leads to an increase in TNFα.

In contrast, SCFAs appeared to increase TNFα levels (Figure 3.3.4.1b), despite the idea that these metabolites are anti-inflammatory (Wang *et al.*, 2017; Diao *et al.*, 2019; Theiler *et al.*, 2019). There is evidence that at lower levels, 2mM acetate and 0.2mM of butyrate and propionate, SCFAs can increase TNFα levels in PBMCs (Mirmonsef *et al.*, 2012). Furthermore, an acute increase in pJNK54 appeared to occur in the LPS+SCFA treatment group (Figure 3.3.3.3), and with JNK being known to increase TNFα levels, this may have also been a contributing factor to increased TNFα levels in the presence of SCFAs. Since JNK is involved in cell death signalling, this may be part of a defence mechanism rather than a cytotoxic effect (Tang *et al.*, 2011; Matthews, Howarth and Butler, 2012; Tsugawa *et al.*, 2020).

3.4.4 The role of SCFAs in inflammation

SCFAs are usually described as anti-inflammatory. Previous studies relating to this area tend to look at the anti-inflammatory role of SCFAs in gut epithelial cell lines (Liu *et al.*, 2012; Perdijk *et al.*, 2019) or in animal models (Lu *et al.*, 2016; Diao *et al.*, 2019). There are no studies to date looking into the role of SCFAs in inflammation in either a human airway epithelial or adipocyte cell line, therefore this study provides novel findings.

Within this study, SCFAs were able to reduce LPS-induced inflammation at gene level for NFκB and protein level for NFκB, IKKβ, IL-6 and JNK46 however, this effect was not clear for the proteins and genes investigated within the JNK and NFκB pathways. This may be partly due to the nature of the administration of the treatments, as cells were treated once with SCFAs, compared to in a human where there would be a steady stream of SCFAs being produced and released from the gut microbiota as a result of the metabolism of dietary prebiotics. It may be that in these animal/human models, it is the accumulation of SCFAs which exert an anti-inflammatory effect, rather than just one dose. This is reflected in studies in which prebiotics are administered to human participants with Asthma over 1-3 weeks, which would allow steady SCFA production and a reduction in inflammation (Williams *et al.*, 2016; McLoughlin *et al.*, 2019).

There are different theories about how SCFAs reduce inflammation. One way is through the release of anti-inflammatory cytokines such as IL-10 (Pujari and Banerjee, 2021), and another possibility is through blocking NFκB activity (Liu *et al.*, 2012; Tayyeb *et al.*, 2020), which this study focused on. In BEAS2B-R1 cells, this was shown through a reduction in IKKβ protein expression (Figure 3.3.2.2a), which led to a reduction of NFκB

phosphorylation (Figure 3.3.2.1a). SCFAs also reduced IκBα phosphorylation (Figure 3.3.2.1b) which meant that it remained bound to NFκB, which would reduce its activity. Although these results were not consistent throughout the study, there appeared to be an effect of SCFAs on NFκB signalling. Further exploring the effects of SCFAs on anti-inflammatory cytokines such as IL-10, or other cytokines downstream of NFκB would be of interest given the evidence of this in the literature (Vinolo *et al.*, 2011; Pujari and Banerjee, 2021), however this was beyond the scope of this study.

3.4.5 Conclusion

This study showed that inflammatory responses to LPS were dependant on cell line, with more of an inflammatory response shown in the Chub-S7 adipocyte cells compared with the BEAS2B-R1 airway epithelial cells. SCFAs were able to mitigate some of this inflammation through varied pathways. BEAS2B-R1 responded through blocking NFκB activation, and Chub-S7 appeared to activate an innate immune response through JNK activation. These data suggest that increasing SCFA levels in patients with inflammatory diseases such as Asthma and Obesity may be an effective treatment, which would be possible through dietary interventions that would improve gut health.

Chapter 4: The influence of Obesity and adipokine asprosin on adipocyte browning, inflammation, and mitochondrial dysfunction in human adipose tissue

4.1 Introduction

Adipose tissue is an endocrine organ whose classical roles include storage of lipids for energy and protecting vital organs. Adipose tissue (AT) is also associated with an increased risk of metabolic diseases such as type 2 Diabetes Mellitus (T2DM) and cardiovascular disease (CVD). There are various fat depots, with the main depots being either subcutaneous or visceral, which can be further broken down into more specific depots based on type and location. Subcutaneous adipose tissue (ScAT) typically lays just below the skin epidermis and is located mainly around the hips and thighs. ScAT has been considered to pose a lower metabolic risk compared with visceral adipose tissue (VAT) which is has a higher health risk (Hill, Solt and Foster, 2018). VAT lines the lower abdomen and encompasses the mesenteric AT, perirenal AT and the omentum, which has been of interest in metabolic health research. As the size and morphology of adipose tissue changes with increasing adiposity, it contributes towards the development of systemic inflammation, insulin resistance, and glucose impairment (Belligoli *et al.*, 2019; Longo *et al.*, 2019). The adipokines and biomarkers associated with metabolic risk are observed to correlate with increasing abdominal omental adipose tissue (AbdOmAT) (O'Connell *et al.*, 2010), with more recent analysis of abdominal subcutaneous adipose tissue (AbdScAT) also highlighting the metabolic risk this depot confers as well (Piya et al. 2013; Patel, Abate 2013; Abate et al. 2013).

Within the last 20 years, multiple studies have highlighted that adipose tissue releases a number of secreted factors, referred to as adipokines, which appear to influence a number of processes within the body including appetite, innate immune response and metabolic function (Taylor, 2021). Additionally, it is also established that during periods

of adipose tissue expansion, there is associated changes in adipokine release. Previous studies have highlighted that in people with Obesity, there is a shift in the balance from anti-inflammatory to pro-inflammatory adipokines, which may lead to an increased systemic inflammatory response (Unamuno *et al.*, 2018). Furthermore, in altered states such as Obesity, these adipokines can propagate cellular damage such as inflammation across different tissues in the body, contributing to metabolic dysfunction. As such, by understanding the functions of adipose tissue and the role of adipokines, insight can be gained into how excess weight gain can influence other chronic diseases such as Asthma and type 2 Diabetes Mellitus (T2DM).

Beyond their influence on systemic inflammation, adipokines can influence metabolic processes within adipose tissue. These processes include adipocyte browning, in which white adipose tissue (WAT) shifts towards a brown adipose tissue (BAT) phenotype. A range of genes relating to lipid homeostasis are upregulated in BAT including Cell death inducing DFFA-like effector A (CIDEA), Elongation of very long chain fatty acids 3 (ELOVL3), Perilipin-5 (PLIN5), and Solute carrier 27A (SLC27A2). Adipocyte browning can be influenced by adipokines such as angiotensin, visfatin, and TGF-β1 (Dolgacheva *et al.*, 2016; Wankhade *et al.*, 2018; Dimitriadis *et al.*, 2019).

Mitochondria also play an important role in adipose tissue, especially in BAT due to their higher level of energy expenditure. Mitochondrial dynamics are complex and there are many genes involved which relate to various aspects of mitochondrial function. These include the biogenesis genes (Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α), Nuclear respiratory factor 1 (NRF1), Mitochondrial

transcription factor A (TFAM), and PPARG related coactivator 1 (PRC)), fission and fusion genes (Dynamin-related protein 1 (DRP1), Mitochondrial fission protein 1 (FIS1), OPA1 mitochondrial dynamin like GTPase (OPA1), and Mitofusin-2 (MFN2)), electron transport chain genes (*e.g.* Cytochrome c oxidase subunit 4 (COX4), and Superoxide dismutase 2 (SOD2) which removes reactive oxygen species (ROS)). Studies have shown that mitochondria function can be influenced by anti-inflammatory and pro-inflammatory adipokines such as, adiponectin and TNFα (Chen et al. 2010; Iwabu et al. 2010; Nakajima et al. 2019).

As well as the classic adipokines which are well characterised, new adipokines are still being discovered. This allows for new mechanisms and links to be uncovered to further improve our understanding of the influence of adiposity on diseases and cellular functions. A recently discovered adipokine asprosin is involved in appetite regulation and glucose homeostasis (Romere *et al.*, 2016). Asprosin has been shown to be elevated in Obesity and T2DM (Ugur and Aydin, 2019; Wang *et al.*, 2019; Deng *et al.*, 2020; Naiemian *et al.*, 2020; Xinyue Zhang *et al.*, 2020). The exact consequences of raised asprosin in the circulation is not fully established, however there is evidence that asprosin has pro-inflammatory effects in tissues including the pancreas and skeletal muscle (Jung et al. 2019; Lee et al. 2019).

Given the limited data currently available into the role of asprosin in metabolic disease, this chapter sought to evaluate whether asprosin influenced adipocyte functions in conditions such as Obesity. The main aims of this study were to determine whether (1) weight gain affected circulating levels of asprosin, (2) adipose tissue depots and weight

gain effected the gene expression of asprosin, and (3) the influence asprosin has on adipocyte browning, mitochondria function, and inflammation.

4.2 Methods

4.2.1 Subject and clinical data

In this study, fasted blood was collected along with abdominal subcutaneous (AbdSc) and omental (AbdOm) adipose tissue (AT) from a group of white Caucasian females (n=130, female, 31.6±6.1 yrs, BMI 27.9±5.9 Kg/m^2). A sub-group of this study was selected and grouped by BMI: lean (n=44, 32.1±5.4 yrs, BMI 22.1±1.9 Kg/m^2), overweight (n=49, 31.4±7 yrs, BMI 27.4.1±1.3 Kg/m^2), and obese (n=37, 31.2±5.8 yrs, BMI 35.2±4.7 Kg/m^2). Participants with cancer, thyroid disorders, on steroids or on medication considered to alter inflammatory status, including thiazolidinediones, were excluded from the study. Ethical approval was obtained from the local research ethics committee and all patients gave written consent. All patients were examined in the morning after an 8-10hr overnight fast and venous blood samples were obtained. Anthropometric data including weight, height, and BMI measurements were recorded along with biomarkers as well as glucose and lipids.

Additional serum samples were taken from a cohort of bariatric patients with Obesity and T2DM (n=32, 52.1±9.0yrs, 43.5±7.3Kg/m^2). Serum samples were taken pre-bariatric surgery, and all patients were taking metformin.

4.2.2 *In vivo* biochemical profile assessment

Lipid and glucose levels were undertaken through the pathology laboratories at University Hospital of Coventry and Warwickshire (UHCW). In brief, the routine blood tests included glucose and a standard lipidemic/cholesterol profile (TGs, HDL and LDL).

4.2.3 RNA extraction and Asprosin Gene expression analysis

RNA was previously extracted from adipose tissue samples using RNeasy lipid tissue kit (Qiagen, Manchester, UK) according to manufacturer's instructions, followed by a DNase digestion step. cDNA was synthesised using reverse transcriptase reagents (Bioline, London, UK).

Gene expression of the asprosin gene (Fibrilin-1; FBN1) was measured using real time quantitative polymerase chain reaction (RT-qPCR). Each sample was run in duplicate using the TaqMan gene expression assays (Thermofisher, UK) FBN1 (Hs00973198_m1) and housekeeping gene 18S (Hs99999901_s1). The RT-qPCR was carried out in the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, UK).

In order to calculate the relative expression of FBN1 in each sample, the following formula was used:

$$FBN1\ expression = \frac{1}{\Delta Ct}$$

$$where\ \Delta Ct = FBN1 - 18S$$

4.2.4 Measuring levels of Asprosin in serum samples

Levels of serum asprosin was measured using the one step Human Asprosin ELISA kit (ab275108; intra-assay %CV=7.9, [Abcam, Abingdon, UK]) according to manufacturer's instructions. Serum samples were diluted 1:2 in assay diluent for them to fall within the standard curve.

4.2.5 Statistical analysis

Statistical analysis was preformed using GraphPad Prism 9. Power analyses was carried out to determine the sample size, these calculations were performed using the programme G*Power version 3.1.9.2. Data is reported as the mean ± either the standard deviation (SD) or the standard error of the mean (SEM). For all data, a normality test was performed to check the distribution of the data. For analysis of patient characteristics, a Multiple Mann-Whitney test was performed as the data was non-parametric. Gene expression and serum asprosin data was normally distributed, so a One-way ANOVA plus a post-ad hoc analysis using Tukey was used to compare significance between BMI groups. Pearson r correlation and linear regression was used to identify correlations between genes. Statistical significance was considered to be $p<0.05$ and was reported as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

4.3 Results

4.3.1 Patient Characteristics

Participants were grouped based on BMI (n=130); lean (BMI: 18.5-24.9 Kg/m^2), overweight (BMI: 25-29.9 Kg/m^2) and obese (BMI: 30 Kg/m^2 or over). An additional cohort of patients with Obesity + T2DM was also used (n=32). Participant characteristics including age, glucose and lipid levels were shown below (Table 4.3.1). Participants in the FOF cohort did not have T2DM, had a fasting level of glucose within the normal range (<7.8 mmol/L). Patients from the obese+T2DM cohort were all on metformin which may affect some of the measurements shown in table 4.3.1.

	Lean		Overweight		Obese		Obese+T2DM	
n	44		49		37		32	
	Average	±SD	Average	±SD	Average	±SD	Average	±SD
Age (years)	32.1	5.4	31.4	7.0	31.2	5.8	52.1$^{\$\$\$\$}$	9.0
BMI (Kg/m^2)	22.1	1.9	27.4****	1.3	35.2********$^{\#\#\#\#}$	4.7	43.5$^{\%\%\%\%}$	7.3
Insulin (pmol/L)	39.8	28.3	65.4****	39.1	84.6****	53.6	27.8$^{\$\$\$\$}$	16.1
Glucose (mmol/L)	3.6	0.6	3.7	0.6	3.9	0.8	8.9$^{\$\$\$\$}$	2.6
LDL (mmol/L)	4.6	1.2	4.5	1.3	4.2	1.2	2.9$^{\$\$\$\$}$	0.7
HDL (mmol/L)	1.7	0.4	1.5*	0.4	1.5**	0.4	1.0$^{\$\$\$\$}$	0.2
Triglycerides (mmol/L)	3.1	1.1	3.2	0.9	3.2	1.2	2.0$^{\$\$\$\$}$	1.1
HOMA-IR	1.0	0.8	1.6***	1.0	2.2****	1.8	11.8$^{\$\$\$\$}$	8.1

Table 4.3.1: General characteristics of patients based on BMI categories.

Anthropometric data in each of the groups are presented as the mean ± standard deviation (SD). The 3 groups were categorised by BMI and T2DM status; lean (BMI 18.5-24.9 Kg/m^2), overweight (BMI 25-29.9 Kg/m^2), obese (BMI 30 Kg/m^2 or over), and obese+T2DM. Statistical analysis between groups was performed using Multiple Mann-Whitney tests (p-values displayed as follows; lean vs overweight, lean vs obese *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, overweight vs obese $^{\#\#\#\#}$p<0.0001, obese+T2DM vs all groups $^{\$\$\$\$}$p<0.0001, obese+T2DM vs overweight and obese groups $^{\%\%\%\%}$p<0.0001).

4.3.2 Serum levels of Asprosin

Circulating asprosin levels within the cohort were measured (Figure 4.3.2.1). No differences in asprosin level were shown based on a change in weight alone, although there appeared to be a trend towards a rise in asprosin as BMI increased. In the

presence of T2DM, there was an increase in serum asprosin compared to the lean and overweight groups.

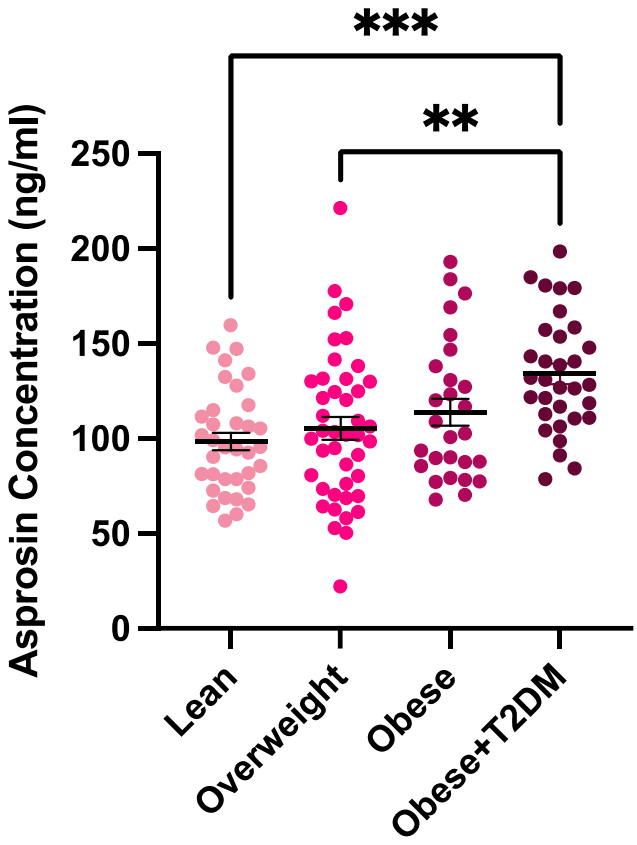

Figure 4.3.2.1: Levels of asprosin in the serum.

Serum asprosin levels split by weight and T2DM status (Lean, Overweight, Obese, and Obese+T2DM). Data is presented as individual data points along with the mean ± standard error of the mean (SEM). One-way ANOVA plus Tukey was performed for statistical analysis between each group (p-values displayed as follows; **p<0.01 and ***p<0.001).

4.3.3 Gene expression of asprosin in human adipose tissue

Gene expression of asprosin was examined in paired abdominal omental (AbdOmAT) and subcutaneous (AbdScAT) adipose tissue (Figure 4.2). Findings from this study determined that in AbdScAT, FBN1 expression decreased in the obese group compared with both the lean and overweight groups by 11.4% and 17.1% respectively. This was

not observed in AbdOmAT, and no significant differences were observed between groups.

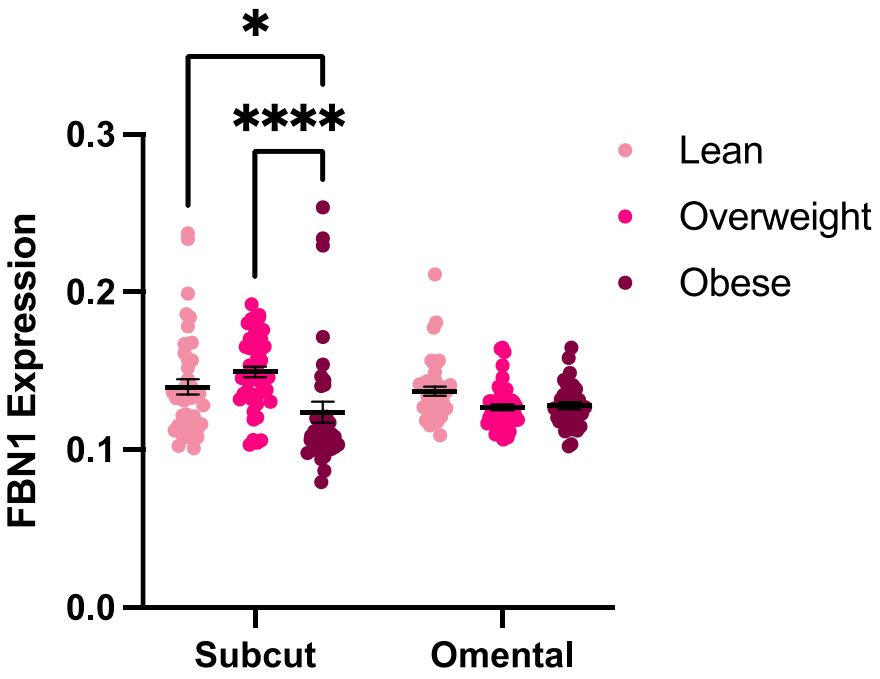

Figure 4.3.3.1: Gene expression of asprosin in abdominal subcutaneous and omental adipose tissue.

Expression of the asprosin gene, FBN1, in AbdScAT and AbdOmAT. Expression levels were compared between each group; Lean, Overweight, and Obese. Data is presented as individual data points along with the mean ± standard error of the mean (SEM). Statistical analysis using One-way ANOVA plus Tukey was performed (p values are displayed as follows; * $p<0.05$, **** $p<0.0001$).

4.3.4 Associations between asprosin and browning, mitochondrial, and inflammatory genes

Expression of FBN1 was analysed for correlations with genes involved in adipocyte browning, mitochondrial processes, and inflammation. In AbdScAT (Table 4.3.4.1), FBN1 expression was negatively correlated with adipocyte browning genes CIDEA, ELOVL3 and PLIN5. FBN1 expression also appeared to have an impact on mitochondrial function, through positive correlations with mitochondrial biogenesis genes PRC and NRF1, a

reduction in mitochondria function through COX4, and mitochondrial fusion shown by a reduction in MFN2. Whilst there was a positive correlation with SOD2 and MCP1.

	Gene	Pearson r	P Value	
Browning	CIDEA	-0.2488	0.0055	**
	ELOVL3	-0.2203	0.0144	*
	PLIN5	-0.2577	0.004	**
	SLC27A	-0.1525	0.0909	ns
Mitochondrial	GPR120	-0.08463	0.3461	ns
	MFN2	-0.4067	<0.0001	****
	OPA1	-0.1185	0.1864	ns
	FIS1	0.06045	0.5014	ns
	DRP1	0.1	0.2651	ns
	PGC1α	-0.05175	0.5649	ns
	COX4	-0.4634	<0.0001	****
	NRF1	0.1804	0.0433	*
	PRC	0.2921	0.0009	***
	SOD2	-0.195	0.0287	*
	TFAM	-0.02726	0.7619	ns
Inflammation	IL6	0.005927	0.3915	ns
	MCP1	0.08274	0.0011	**
	IL1B	0.00049	0.8056	ns
	TNFα	0.001976	0.6211	ns

Table 4.3.4.1: Associations between asprosin and genes relating to adipocyte browning, mitochondria, and inflammation in subcutaneous adipose tissue.

Correlation analysis was performed between asprosin, and genes related to adipocyte browning (CIDEA, ELOVL3, PLIN5 and SLC27A2), mitochondria (GPR120, MFN2, OPA1, FIS1, DRP1, PCG1α, COX4, NRF1, PRC, SOD2 and TFAM) and inflammation (IL6, MCP1, IL1B and TNFα). The correlation is presented as Pearson r along with the p value, which are displayed as follows; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Asprosin appeared to have a depot specific effect as further analysis in AbdOmAT (Table 4.3.4.2) highlighted that mitochondrial genes MFN2 and COX4, and inflammatory IL1B were significantly correlated with an increase in FBN1 expression. All significance was lost between FBN1 and adipocyte browning genes.

	Gene	Pearson r	P value	
Browning	CIDEA	-0.09187	0.3142	ns
	ELOVL3	-0.1489	0.1003	ns
	PLIN5	-0.1308	0.1579	ns
	SLC27A	-0.02192	0.8106	ns
Mitochondrial	GPR120 A2	0.06691	0.4585	ns
	MFN2	0.2205	0.0142	*
	OPA1	0.1479	0.0997	ns
	FIS1	-0.01335	0.8825	ns
	DRP1	0.1145	0.2036	ns
	PGC1α	0.07908	0.3807	ns
	COX4	0.2398	0.0071	**
	NRF1	0.1081	0.2301	ns
	PRC	-0.06862	0.447	ns
	SOD2	-0.07727	0.3917	ns
	TFAM	0.094	0.2991	ns
Inflammation	IL6	-0.04379	0.6277	ns
	MCP1	-0.09659	0.28	ns
	IL1B	-0.2293	0.0104	*
	TNFα	0.0313	0.73	ns

Table 4.3.4.2: Associations between asprosin and genes relating to adipocyte browning, mitochondria, and inflammation in omental adipose tissue.

Correlation analysis was performed between asprosin and genes related to adipocyte browning (CIDEA, ELOVL3, PLIN5 and SLC27A2), mitochondria (GPR120, MFN2, OPA1, FIS1, DRP1, PCG1α, COX4, NRF1, PRC, SOD2 and TFAM) and inflammation (IL6, MCP1, IL1B and TNFα). The correlation is presented as Pearson r along with the p value, which are displayed as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

4.4 Discussion

This chapter sought to investigate the influence of asprosin as a contributing factor to metabolic dysfunction within adipose tissue depots. The key ambitions of this study were to examine (1) the effect of weight gain and metabolic state on circulating asprosin levels, (2) the influence of asprosin gene expression in AT depots and on weight gain; and (3) the effect of asprosin on adipocyte browning, mitochondrial function, and inflammation.

4.4.1 Asprosin expression and serum

Previous studies suggest that asprosin levels increase with weight gain (Ugur and Aydin, 2019; Sünnetçi Silistre and Hatipoğl, 2020) noting that examining participants without T2DM across adiposity displayed only a trend towards an increase but this did not attain significance. This current study adds to the conflicting data on asprosin and the influence of weight gain (Corica *et al.*, 2021; Shabir *et al.*, 2021). This observed disparity between asprosin and BMI across human studies is not uncommon, where adipokines are considered to have both anti-inflammatory and pro-inflammatory roles, such as IL-6 and TNFα, (Hunter and Jones, 2015; Duffles *et al.*, 2019; Uciechowski and Dempke, 2020) as well as the study cohorts being influenced by co-founders such as BMI and HOMA-IR in those participants examined that were non-T2DM.

As prior studies have mainly addressed asprosin in participants with T2DM, these studies also sought to extend and affirm previous insights on the influence of asprosin in participants with T2DM. In studies examining asprosin levels, where BMI and glucose

intolerance/insulin resistance have been evaluated, asprosin levels are increased (Corica et al. 2022; Wang et al. 2018; Zhang et al. 2020), which suggests BMI alone is not the key factor in the elevation of asprosin. These current studies highlighted that T2DM status was an important influence on increasing asprosin, although the participants with T2DM were also class II-III obese (BMI over 35 Kg/m^2).

Expression of the asprosin gene FBN1 was shown to decrease with weight gain (figure 4.3.3.1). This was not anticipated given that previous studies have shown that serum asprosin increases with BMI, and that FBN1 is expressed in adipose tissue, so it would be assumed that gene expression would also increase with BMI. However, currently there are no other studies looking at asprosin gene AT expression in patient cohorts. In metabolic diseases including Obesity, it is well established that hormones and adipokines relating to appetite and glucose regulation for example can be dysregulated, leading to dysfunction and changes in expression, which may also be the case for asprosin (Coppack, 2001; Unamuno *et al.*, 2018). Asprosin itself is considered an orexigenic hormone involved in appetite control, so as hormones in Obesity become dysregulated, asprosin regulation may also be affected. Interestingly in other diseases where appetite and hormones are dysregulated such as anorexia nervosa and bulimia, asprosin is noted to be increased (Mitchell and Crow, 2006; Hu *et al.*, 2021, 2022). In physiological circumstances asprosin upon secretion from adipose tissue travels to the liver, where it promotes hepatic glucose release through OR4M1, an olfactory G-protein-coupled receptor. However, in conditions such as anorexia nervosa and NAFLD, where the liver is known to be compromised, asprosin may remain unmetabolised,

which may in part explain the increase in the circulation in such conditions (Liu et al. 2021; Ke et al. 2020).

4.4.2 Asprosin effect on adipocyte browning gene expression

Further investigation in the associations between asprosin and genes related to adipocyte browning identified depot specific differences. In AbdScAT, significant negative correlations were observed between asprosin and CIDEA, ELOVL3, and PLIN5, suggesting asprosin could reduce adipocyte browning. Given that asprosin is increased with adiposity, whilst browning is decreased, these negative correlations would affirm with the current theories within the literature. Furthermore, as WAT tends to act as an energy reservoir and releases adipokines which may be pro-inflammatory, if asprosin keeps the AbdScAT in the WAT phenotype, it could serve to increase the pro-inflammatory state through the release of more adipokines which mediate inflammation.

Interestingly, despite the potential pro-inflammatory nature of asprosin and that of AbdOmAT, there were no correlations between asprosin and browning genes; this lack of correlation, may be due to the OmAT being much more pro-inflammatory in nature, even within AT from lean individuals, and less likely to promote beige adipocytes or be influenced by asprosin.

4.4.3 Asprosin effect on mitochondria gene expression

Asprosin had further depot specific effects with mitochondrial gene expression. In AbdScAT, asprosin expression was negatively correlated with COX4, and MFN2 suggesting reduced mitochondrial function. There was also a positive correlation between asprosin and PRC and NRF1 suggesting an increase in mitochondrial biogenesis. Taken together asprosin appears to lead to reduced mitochondria function, as well as an increase in the production of mitochondria. This may be a compensatory mechanism, as the cell may need to produce more mitochondria to mitigate those mitochondria present in the cell which are dysfunctional and not working adequately for the cells metabolic requirements. A similar mechanism has been observed for adipocyte cells treated with a pro-inflammatory mediator of cellular damage, lipopolysaccharide (LPS), where the damage caused by LPS seemed to be masked due to the over production of mitochondria, as the cell increases mitochondria production to support the cell meet its normal respiratory demand (Widdrington *et al.*, 2018). This may be the case for asprosin, though mechanistic work would need to be undertaken to examine this further.

In AbdOmAT, no correlations were observed between asprosin and mitochondrial genes except for COX4 and MFN2, though the correlation was weaker than observed in AbdScAT. As with the browning genes, asprosin appears to have less of an effect on AbdOmAT compared with AbdScAT. Interestingly, whilst mitochondrial activity is considered to be higher in OmAT than ScAT (Kraunsøe *et al.*, 2010), this is lost with weight gain, as mitochondrial gene expression appears downregulated in OmAT (Christe *et al.*, 2013; Lindinger *et al.*, 2015). These depot specific differences may be due to increased metabolic activity in AbdOmAT compared with AbdScAT, which may mask the

impact asprosin has on mitochondrial activity. Furthermore, as AbdOmAT is anatomically close to vital organs with a high turn-over of lipids, it may have more of an metabolic influence, as such this depot may also be more resilient to intermittent damage posed by the intestine as a way of protecting these vital organs from metabolic damage.

Given that this cohort was all female, sex-related differences may have had an impact on these findings. Studies with female mice have been shown to be less susceptible to weight gain related metabolic dysfunction than male mice, and are more able to adapt to change for energy intake needs during mitochondrial respiration (MacCannell *et al.*, 2021). Also, for a given BMI and age, prior to the menopause, women are at reduced risk of metabolic disease compared to men, with central Obesity being the leading cause for this increased risk and as this cohort were all pre-menopausal this may have also influenced the findings.

4.4.4 Asprosin effect on inflammatory related genes

In AbdScAT, asprosin was associated with inflammation through a positive correlation with MCP1 and a negative correlation with SOD2. SOD2 removes ROS from cells, so a decrease in SOD2 would relate to an accumulation of ROS in cells, leading to more damage which an increase in asprosin may appear to enhance. MCP1 recruits immune cells to the site of inflammation, so a positive correlation with asprosin suggests that there is an increase inflammation. No effect was shown between asprosin and IL-6 or TNFα in either depot. Noting that only a female cohort was used in this study, another recent study showed that only male mice showed increased inflammation in response

to reduced adipocyte browning (Mills *et al.*, 2022). Had this cohort included male patients, there may have been some relation between asprosin and the inflammatory genes, given that this study showed that asprosin correlated with reduced adipocyte browning.

This data also suggests that asprosin may have an anti-inflammatory effect in AbdOmAT rather than pro-inflammatory, due to a negative correlation with IL-1B. Prior studies with asprosin indicate tissue specific effects where asprosin appears protective effect in the heart (Zhang et al. 2019; Wen et al. 2020), but damaging in skeletal muscle and the pancreas (Lee et al. 2019; Jung et al. 2019). Given the differing correlations between asprosin and inflammatory factors in these differing adipose tissue depots, it would be insightful to further examine the potential pro-/anti-inflammatory role of asprosin in an *in vitro* cell model.

4.4.5 Conclusions

Overall, it appears as though asprosin may have a negative effect on the levels of genes involved with various cellular functions in abdominal human adipose tissue. This is particularly observed in AbdScAT, where an increase in asprosin leads to a reduction in the expression of adipocyte browning genes and altered mitochondrial gene expression. Furthermore, adiposity may increase these effects, as with increasing AbdScAT asprosin may have more potential to exert these effects as noted in participants with Obesity and T2DM. Further *in vitro* mechanistic work must be undertaken to evaluate how asprosin influence cellular dysfunction in adipocytes.

Chapter 5: The role of adipokine Asprosin on inflammation and mitochondrial dysfunction in airway epithelial cells

5.1 Introduction

Asthma is an inflammatory disease of the airways which can be influenced by lifestyle, genetics, and environmental triggers. The inflammation present may be exacerbated by numerous allergen triggers or by systemically produced cellular factors. Whilst it is understood that certain molecules produced by white adipose tissue, referred to as adipokines (Taylor, 2021), can mediate inflammatory responses in various tissues around the body, studies have shown that these same adipokines can influence inflammation in Asthma (Sideleva *et al.*, 2012; Watanabe *et al.*, 2019). Two particularly well characterised adipokines in mammals are leptin, which is often associated with causing a pro-inflammatory response (Watanabe *et al.*, 2019) and adiponectin, known to mediate an anti-inflammatory response (Nigro *et al.*, 2013). It is understood that an increase in volume of adipose tissue through weight gain can also exacerbate Asthma, which may arise through increased adipokine production, as patients with Obesity and Asthma have raised leptin and decreased adiponectin levels compared with lean patients with Asthma (Sideleva *et al.*, 2012; Nigro *et al.*, 2015; Rastogi *et al.*, 2015; Al-Ayed *et al.*, 2019). Additionally other adipokines, including those recently discovered, may also have an impact on Asthma, derived from an increase in white adipose tissue.

Asprosin is one of those recently discovered adipokines which is mainly produced by white adipose tissue (Romere *et al.*, 2016), but its role inflammation is unclear. Asprosin is coded by exons 65-66 of the Fibrilin-1 (FBN1) gene, which produces a preprotein called profibrillin which is then cleaved to produced fibrilin-1 and asprosin. Despite assumptions in the literature that asprosin is pro-inflammatory, due to it being raised in patients with inflammatory diseases (X. Li *et al.*, 2018; Ugur and Aydin, 2019; Deng *et*

al., 2020; Ke *et al.*, 2020; Naiemian *et al.*, 2020; Xinyue Zhang *et al.*, 2020; Yuan *et al.*, 2020), there is little research related to this area. The main role of asprosin identified to date is that it promotes glycolysis in the liver through activation of the Olfactory Receptor Family 4 Subfamily M Member 1 (OR4M1) receptor in response to hunger signals (Romere *et al.*, 2016), but studies have now started to explore the molecular role of asprosin in other tissues, with both pro- and anti-inflammatory effects being observed.

Current studies have shown that asprosin causes inflammation in pancreatic and skeletal muscle cells (Lee et al. 2019; Jung et al. 2019), and induces insulin resistance and ER stress in the skeletal muscle of mice (Jung *et al.*, 2019). Studies also indicate that asprosin has protective effects within the heart by restoring mitochondrial function, reducing reactive oxygen species (ROS), and increasing superoxidase dismutase 2 (SOD2) (Wen et al. 2020; Zhang et al. 2019). This suggests that asprosin may have beneficial effects on mitochondria, although these effects may be tissue dependant as asprosin has been shown to increase ROS in the brain (Wang *et al.*, 2022). Asprosin has also been shown to directly activate the JNK and NFκB pathways through TLR-4 (Lee et al. 2019), the same mechanism noted for with LPS. LPS mediates systemic inflammation as it traverses the gut intestinal wall to enter the circulation and facilitate cellular inflammation via the TLR pathway. It appears that asprosin may also act as a potential inflammatory mediator, similar to LPS, and the pro-inflammatory actions of asprosin could be ameliorated by the anti-inflammatory effects of short chain fatty acids (SCFA) produced by the fermentation of prebiotics by gut bacteria. SCFAs can then transverse into the circulation and reduce inflammation, where it can exert its anti-inflammatory

effects through blocking cellular NFκB activity (Vinolo *et al.*, 2011; Liu *et al.*, 2012; Tayyeb *et al.*, 2020; Yue *et al.*, 2022).

Currently, there is only one study that has investigated the role of asprosin in the airways, suggesting patients with obstructive sleep apnoea syndrome have raised asprosin (Ding *et al.*, 2021), although this study along with most other *in vivo* studies did not explore factors to reduce the potential damaging effect of asprosin. Given the novelty of this adipokine, and the lack of studies exploring asprosin in Asthma or any *in vitro* studies examining the function or dysfunction caused within the airways, these current studies sought to explore the role of asprosin within the airways. Specifically, this study sought to investigate how asprosin may influence lung tissue and provide a molecular link between Obesity and Asthma by exploring (1) whether asprosin could induce inflammation in airway epithelial cells, (2) if SCFAs were able to mitigate any inflammation caused, and (3) the role of asprosin in mitochondrial function in airway epithelial cells.

5.2 Methods

5.2.1 Cell culture and treatments

Human airway epithelial cells (BEAS2B-R1) were cultured in 6-well cell culture plates as previously described in chapter 2.1. BEAS2B-R1 cells were treated once they had reached 70-80% confluency. Cells were pre-treated with a SCFA mix with a final concentration of 2mM Acetate, 0.25mM Butyrate and 0.25mM of Propionate (each from Sigma, UK), giving a ratio of 80:10:10, considered a systemic physiological level in humans (Jocken *et al.*, 2018). Cells were then treated with 10ng/mL Asprosin for 6, 12, and 24 hours. PBS was used as a vehicle, therefore control cells were treated to give a final concentration of 1% PBS.

5.2.2 Protein extraction and quantification

Protein samples were lysed in 100μL of 1x Radioimmunoprecipitation Assay Buffer (RIPA) containing 2% protease and phosphatase inhibitors. Cells were scraped in RIPA buffer on ice and then collected in 1.5mL microfuge tubes. Cells were placed on ice for 30 mins and vortexed every 10 mins to assist with cell lysis. The cells were then pelleted by centrifugation for 15 mins at $16x10^3$ rcf at 4^{o}C. The supernatant containing the protein was then collected and stored at -80^{o}C.

The concentration of protein in each sample was quantified using the Bradford Assay. BSA protein standards were diluted in a range of 0-1mg/mL. Protein samples were diluted 1:20 in deionised water and added to 200μL of 1x Bradford reagent (Bio-rad, UK). All standards and samples were run in triplicate. The absorbance was read at a

wavelength of 595nm. A standard curve was created in order to calculate the concentrations of the protein samples. The protein samples were then mixed with 4x Laemmli Buffer and heated at 95°C for 10 mins, then stored at -80°C.

5.2.3 Western Blot

In order to determine the levels of protein expression in the samples, Western Blot analysis was performed as previously described in chapter 2.4. In brief, 50µg of protein was loaded onto a 10% polyacrylamide gel which was run at 140V for 90 mins. The gel was then transferred onto an Immobilon-P transfer membrane (Millipore, UK) with 0.45µm pore size for 100 minutes at 100V. The membrane was then washed in water and blocked in either 5% BSA in tris buffered saline-Tween20 (TBS-T) or 0.2% I-Block in PBS-T for 1 hour at room temperature. Primary antibodies were then added to the membrane over night at 4°C. The following antibodies were used:

Antibody	Company (Item No.)	Buffer	Blocking	Primary	Secondary
β-Actin	CST (#4970)	PBS	I-Block	1:1000	1:100,000 (R)
NFκB	CST (#8242)	PBS	I-Block	1:1000	1:100,000 (R)
p-NFκB	CST (#3033)	TBS	BSA	1:1000	1:50,000 (R)
JNK	CST (#9252)	PBS	I-Block	1:500	1:50,000 (R)
p-JNK	CST (#9251)	TBS	BSA	1:500	1:50,000 (R)
IKKβ	CST (#8943)	PBS	I-Block	1:500	1:50,000 (R)

Table 5.2.1: Western blot antibodies and buffers

Details of primary and secondary antibody concentrations and the buffers and blocking solutions used. Abbreviations in table: Cell Signalling Technologies (CST), Rabbit (R), Mouse (M)

The following day, membranes were washed in either TBS-T or PBS-T 3 times for 5 mins. The anti-rabbit secondary antibody (Sigma #A9169) was then added for 1 hour at room temperature. The washing step was then repeated, and the membranes incubated briefly in Westar Antares HRP Detection Substrate (Geneflow, UK) which uses enhanced

chemiluminescence (ECL) in order to visualise the bands. The membranes were visualised on the GeneGnome XRQ Visualiser (Syngene, UK).

5.2.4 RNA Extraction and cDNA synthesis

RNA samples were lysed on ice in 350μL Buffer RLY (Bioline, UK) and 3.5μL β-mercaptoethanol (β-ME). RNA was extracted using the Isolate II RNA Mini Kit (Bioline, #BIO-52073) as previously described in chapter 2.5. The final step resulted in 60μL of RNA being eluted in RNase-free water. In order to calculate the concentration and purity of the RNA samples, 1μL of RNA was measured using the Nanodrop ND-100 (Labtech, UK).

In order to synthesise cDNA from extracted RNA, 1ug of RNA in 10uL of H_2O was combined with 1uL random hexamers and 1μL dNTPs (Thermofisher, UK) and heated at 70^oC for 10 mins. Samples were then immediately transferred onto ice for 2 mins. A mastermix was prepared containing the following; 2μL 10x M-MLV reverse transcriptase buffer, 1μL M-MLV (Sigma), 0.5μL RNase OUT (Thermofisher, UK) and 4.5μL H_2O. To each sample, 8μL of this mastermix was added. Finally, samples underwent 1 cycle of 20 mins at 25^oC, 50 mins at 37^oC and 10 mins at 80^oC. The resulting samples were then diluted to 100ng with H_2O and stored at -20^oC until use.

5.2.5 Real time quantitative polymerase chain reaction

5.2.5.1 TaqMan gene expression assays

Gene expression was measured using real time quantitative polymerase chain reaction (RT-qPCR). Each reaction was run in a volume of 20µL, containing 100ng cDNA, 1x housekeeping gene (18S), 1x gene of interest, 1x TaqMan master mix (Thermofisher, UK) and H_2O. The following TaqMan expression assays (Thermofisher, UK) were used:

Gene	Assay ID
18S	Hs99999901_s1
NFKB1	Hs00765730_m1
MAPK8	Hs01548508_m1
CXCL8	Hs00174103_m1
BECN1	Hs00186838_m1
MT-ND1	Hs02596873_s1
PPARGC1A	Hs00173304_m1
POLG	Hs01018668_m1
TFAM	Hs00273372_m1
NRF1	Hs00602161_m1
COX4	Hs00971639_m1
mtATP6	Hs02596862_g1

Table 5.2.2: List of TaqMan expression assays

The RT-qPCR was carried out in the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, UK) as follows; 2 mins at 50°C, 10 mins at 95°C, then 40 cycles of 15s at 95°C and 1 min at 60°C.

5.2.5.2 Gene expression analysis

In order to calculate the expression of each gene relative to the control, the following formula was used:

$$mRNA\ expression = 2^{-(\Delta\Delta Ct)}$$

$$where\ \Delta\Delta Ct = (Gene\ of\ interest - 18S) - Average\ Control$$

5.2.5.3 Mitochondria copy number analysis

In order to calculate the mitochondria copy number, mitochondrial (MT-ND1) and nuclear (BECN1) genes were measured. The copy number was calculated using the following formula:

$$Mitochondria\ copy\ number = 2^{\Delta Ct}$$

$$where\ \Delta Ct = (BECN1 - 18S) - (MTND1 - 18S)$$

The mitochondria copy number was then shown as a percentage change compared to the control, calculated as follows:

$$Percentage\ change\ in\ mitochondria\ copy\ number = \frac{Sample - Control}{Control}\ x\ 100$$

5.2.6 Seahorse mitochondrial and glycolytic stress assays

Mitochondrial function was assessed using the Seahorse XFe analyser (Seahorse Bioscience, Agilent Technologies, UK). The oxygen consumption rate (OCR) was measured using a mitochondrial stress test which represented the mitochondrial respiration capacity. The extracellular acidification rate (ECAR) was measured using a glycolysis stress test which represented the glycolytic capacity. BEAS2B-R1 cells were seeded at a density of 10,000 cells/well into Seahorse 24 well plates. Cells were treated as previously described in section 5.2.1.

The assay was run in Seahorse XF DMEM medium, pH 7.4 supplemented with 10mM Seahorse XF Glucose, 1mM Seahorse XF Pyruvate and 2mM Seahorse XF L-Glutamine (Agilent Technologies, UK). A mitochondrial stress test kit and a glycolysis stress test kit (Agilent Technologies, UK) containing each compound needed for each assay was used.

The following concentrations were used for each compound in each assay; Mitochondrial stress test - 1.5μM Oligomycin, 2μM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), 0.5μM Rotenone and Antimycin A (Rot/AA), Glycolysis stress test – 10mM Glucose, 1μM Oligomycin, and 50mM 2-Deoxy-D-Glucose (2-DG).

The assay first required a 30 min calibration step, after which the assay plate was inserted. The protocol consisted of 3 cycles of an injection of a compound, mix (3 mins), wait (2 mins) and measure (3 mins). The order in which the compounds were injected was Oligomycin, FCCP then Rot/AA for the mitochondrial stress test, and Glucose, Oligomycin, then 2-DG for the glycolysis stress test.

5.2.7 Mitochondrial activity analysis

In order to measure the levels of active mitochondria, Mitotracker™ Dye (Thermofisher, UK) was used. Green mitotracker dye is used to stain all mitochondria within the cell, whereas the red dye only stains active mitochondria, as it relies on the membrane potential in order to pass the membrane and stain the mitochondria. The green and red mitotracker dyes were prepared in serum free, phenol red free media, to give final concentrations of 25μM green and 50μM red. Cells were plated in 6 well plates and treated as previously described in section 5.2.1, then incubated with the mitotracker dyes for 30 mins at 37°C. Cells were then washed in PBS 3 times, and then 2mL of serum free, phenol red free media was added to cells before imaging. Cells were imaged on the EVOS™ M7000 Imaging System (Thermofisher, UK).

5.2.8 Statistical Analysis

Statistical analysis was preformed using GraphPad Prism (version 9). Data are reported as the mean ± the standard error of the mean (SE) unless otherwise stated. The appropriate statistical test was selected based on the normality of the data, using mainly the Two-way ANOVA plus Tukey's post-hoc test to compare significance between treatment groups. Statistical significance was considered to be $p<0.05$ and was reported as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

5.3 Results

5.3.1 The effect of asprosin and SCFAs on inflammation in human airway epithelial cells

BEAS2B-R1 cells were pre-treated with a SCFA mix for 24hrs, then treated with 10ng/mL asprosin for 6, 12, and 24hrs. The effect of asprosin on inflammation was first assessed by measuring the expression of NFKB1 and CXCL8 (IL-8) in these samples. At 6hrs, asprosin increased expression of NFKB1 by 2-fold$\pm$0.26 ($p<0.05$) and CXCL8 by 1.9-fold$\pm$0.21 ($p<0.05$) compared with the control. SCFAs did not decrease asprosin-induced NFKB1 expression at any timepoint, however at 24hrs there was a decrease in both the SCFA and Asprosin in combination with SCFAs treatment groups compared with control ($p<0.05$). At 24hrs, SCFAs were able to decrease asprosin-induced CXCL8 expression compared with asprosin alone ($p<0.05$). Asprosin did not increase MAPK8 expression, however asprosin in combination with SCFAs increased expression ($p<0.0001$)

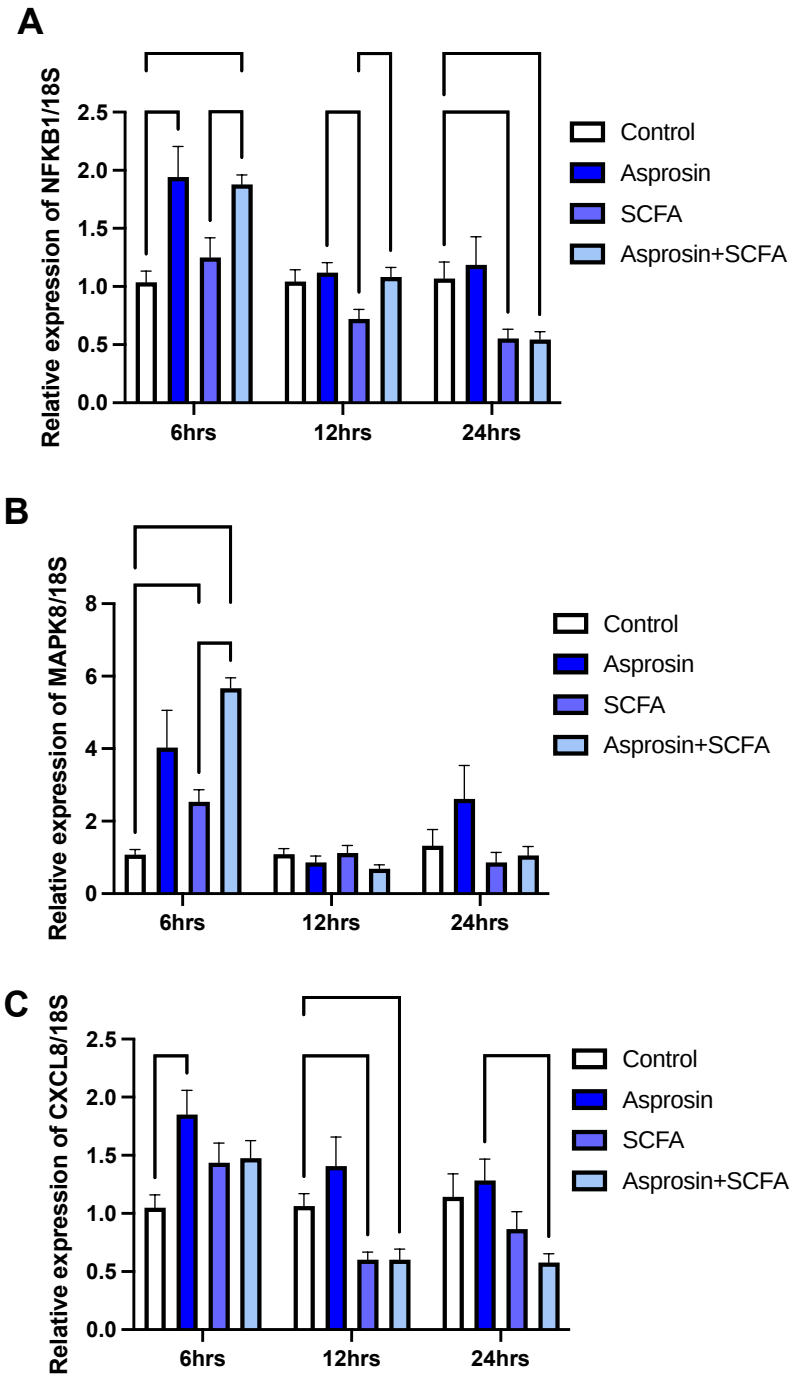

Figure 5.3.1.1: The effect of Asprosin and SCFAs on inflammatory gene expression in BEAS2B-R1 cells.

BEAS2B-R1 airway epithelial cells treated with 10 ng/mL asprosin with and without a SCFA mix for 6, 12, and 24hrs (n=6). Gene expression was measured by RT-qPCR for the following inflammatory genes; (A) NFKB1 [NFκB], (B) MAPK8 (JNK46), and (C) IL-8 [CXCL8]. Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post-hoc test was performed (p values are displayed as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

The effect of asprosin on inflammatory protein expression and phosphorylation was then measured by Western blot in BEAS2B-R1 cells. Asprosin alone was unable to increase NFκB phosphorylation, however at 12 and 24hrs there was a decrease in the Asprosin+SCFA group compared to asprosin alone (12hrs $p<0.001$; 24hrs $p<0.01$), suggesting SCFAs were able to mitigate NFκB activation, a key intracellular signaller of inflammation. This was also shown with IKKβ, which an upstream activator of NFκB, where there was a decrease in expression at 12 and 24hrs in the Asprosin with SCFA group ($p<0.05$).

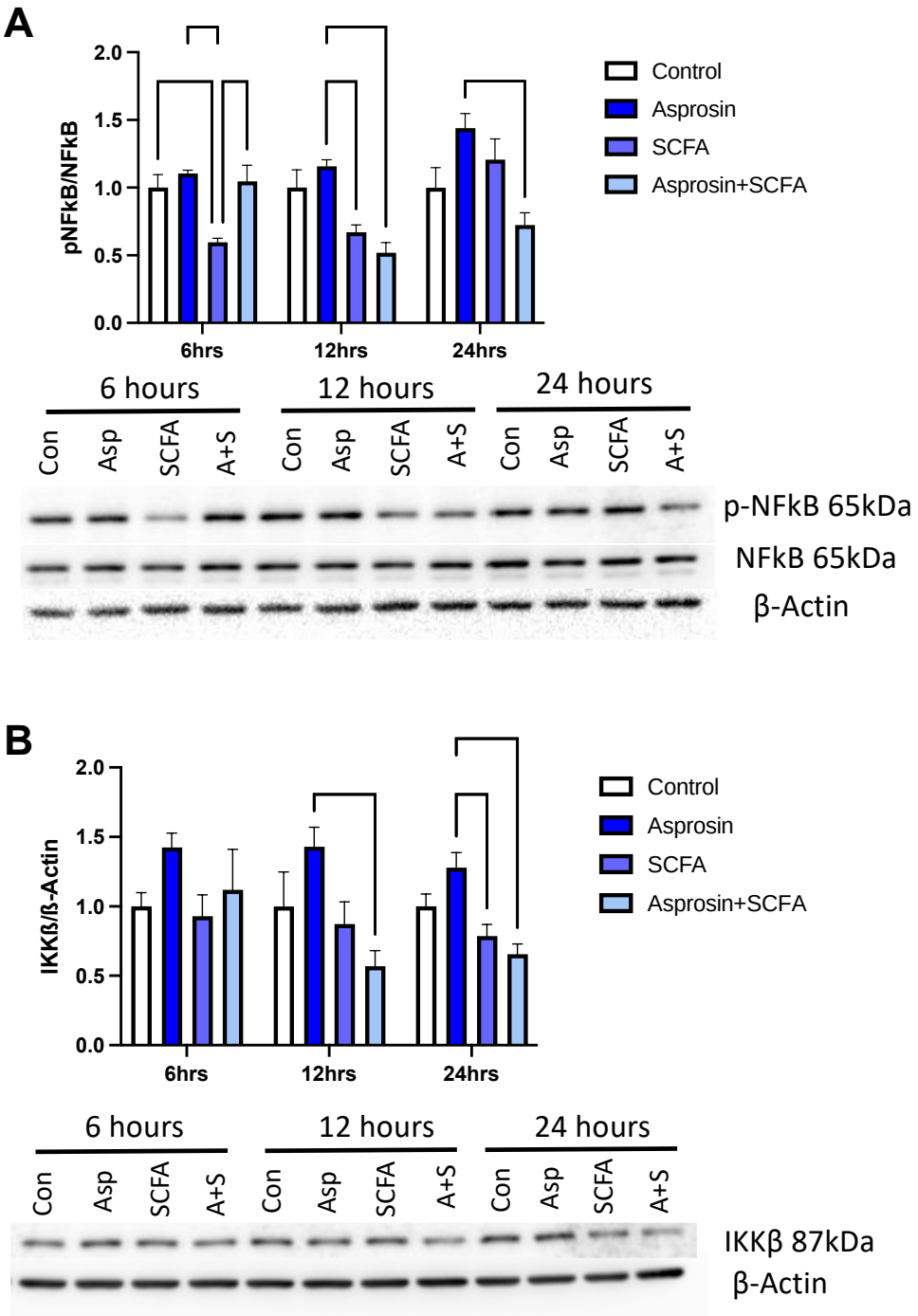

Figure 5.3.1.2: The effect of Asprosin and SCFAs on NFκB phosphorylation and IKKβ protein expression and in BEAS2B-R1 cells

BEAS2B-R1 airway epithelial cells treated with 10 ng/mL asprosin and SCFA mix for 6, 12, and 24hrs. (a) NFκB phosphorylation (n=5) and (b) IKKβ protein expression (n=4) were measured by western blot. Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post-hoc test was performed (p values are displayed as follows; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

Asprosin and SCFAs had no apparent effect across time on JNK54 phosphorylation in BEAS2B-R1 cells. Asprosin treated cells showed no change in JNK46 phosphorylation, whilst SCFAs increased JNK46 phosphorylation by 2-fold±0.1 ($p<0.01$) at 6 hrs, and again at 24hrs by 1.5-fold±0.09 ($p<0.05$).

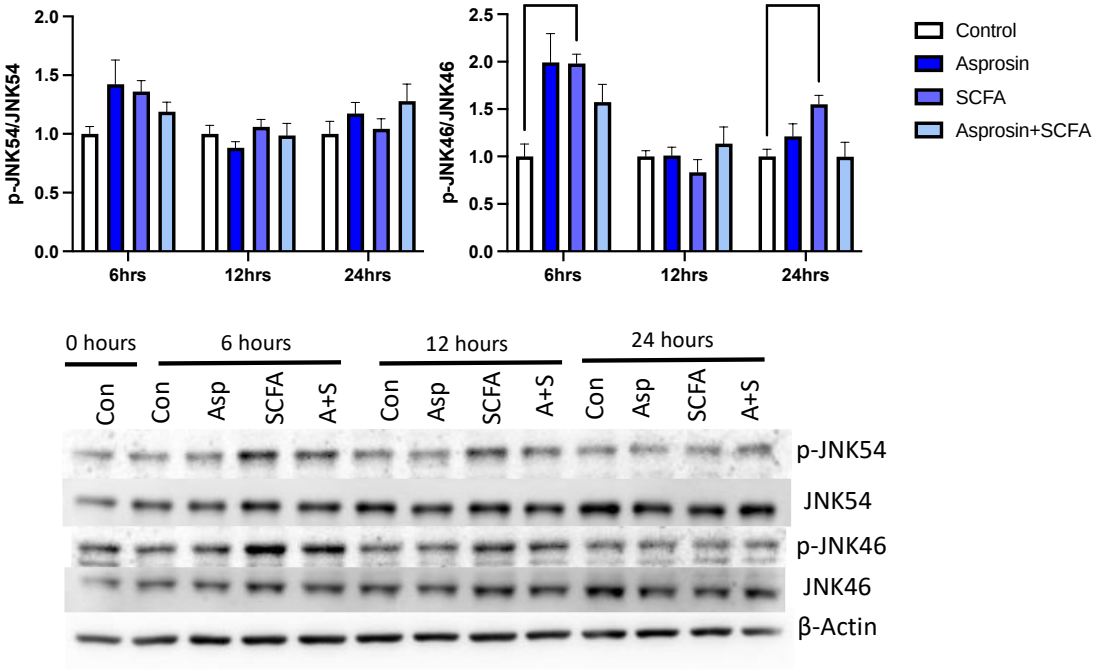

Figure 5.3.1.3: The effect of Asprosin and SCFAs on JNK54 and JNK46 phosphorylation in BEAS2B-R1 cells

BEAS2B-R1 airway epithelial cells treated with 10 ng/mL asprosin and SCFA mix for 6, 12, and 24hrs (n=4). JNK46 and JNK54 phosphorylation was measured by western blot. Data is presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post-hoc test was performed (p values are displayed as follows; * $p<0.05$, ** $p<0.01$).

5.3.2 The effect of asprosin on mitochondrial health and function in human airway epithelial cells

Mitochondrial function was assessed using a mitochondrial stress test and glycolysis stress test assay on the Seahorse XFe analyser. Cells were treated with 10ng/mL asprosin for 6, 12, and 24hrs before each assay was run. There was no difference in OCR between the control and asprosin treated cells at any time point. At 6 and 12hrs, asprosin treated cells appeared to have a higher glycolytic capacity, which returned to control levels at 24hrs.

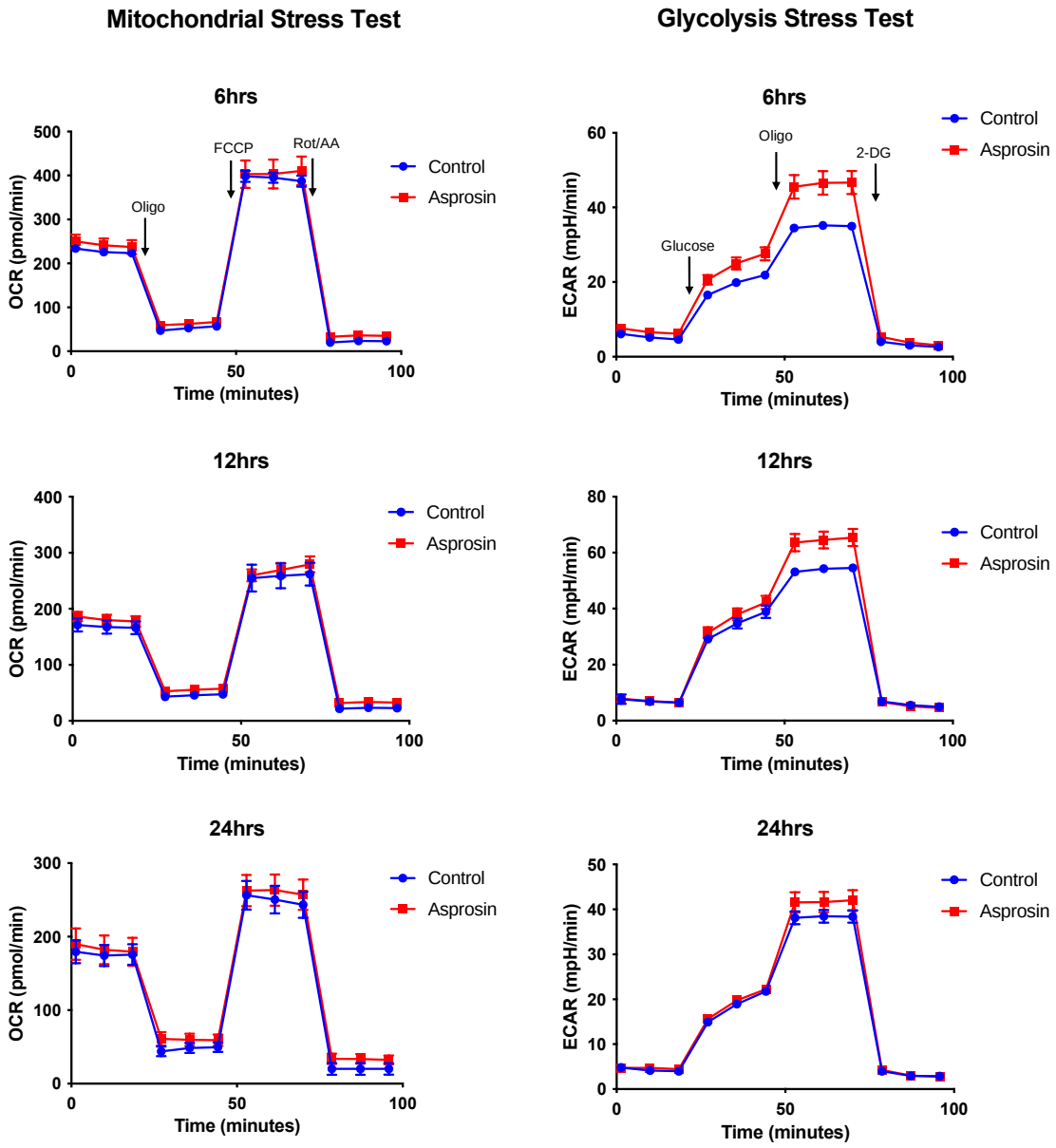

Figure 5.3.2.1: The effect of asprosin on the oxygen consumption rate and extracellular acidification rate of BEAS2B-R1 cells

BEAS2B-R1 airway epithelial cells treated with 10 ng/mL asprosin for 6, 12, and 24hrs. The oxygen consumption rate (OCR) was measured using a mitochondrial stress test (6 and 12hrs n=1; 24hrs n=3), and the extracellular acidification rate (ECAR) was measured using a glycolysis stress test (n=1) on the Seahorse XFe analyser. Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post-hoc test was performed (p=ns).

To further investigate the role of asprosin in regulating mitochondrial function, genes relating to mitochondria biogenesis and function were measured in BEAS2B-R1 cells treated with 10ng/mL asprosin for 24hrs. Asprosin decreased expression of biogenesis genes PPARG ($p<0.05$), POLG ($p<0.01$), TFAM ($p<0.05$), and NRF1 ($p<0.01$), and functional genes COX4 ($p<0.05$) and mtATP6 ($p<0.05$) by at least half compared to the control.

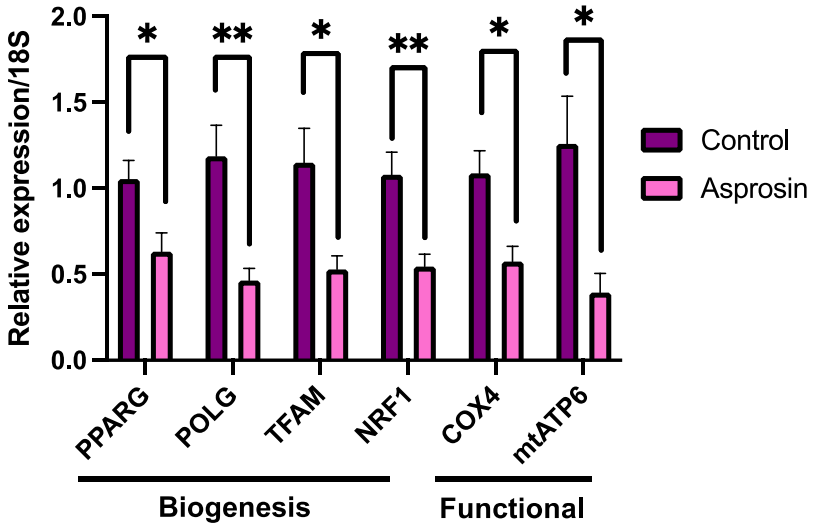

Figure 5.3.2.2: The effect of Asprosin on mitochondrial gene expression in BEAS2B-R1 cells

BEAS2B-R1 airway epithelial cells treated with 100 ng/mL asprosin 6, 12, and 24hrs (n=6). The expression of the following genes were measured by RT-qPCR; PPARG, POLG, TFAM, NRF1, COX4, and mtATP6. Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post-hoc test was performed (p values are displayed as follows; * $p<0.05$, ** $p<0.01$).

The change in mitochondria copy number was also calculated using the expression of both mitochondrial (MT-ND1) and nuclear (BECN1) genes. Initially at 6hrs there was a 58% decrease in copy number compared to the control when cells were treated with asprosin. By 12hrs, the copy number increases by 5%, and further increases by 10% at 24hrs ($p<0.05$).

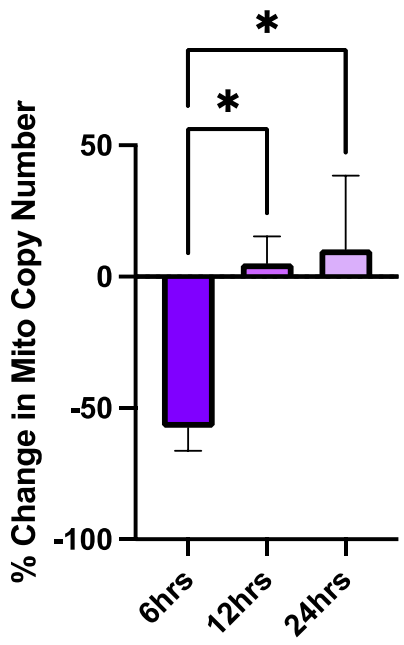

Figure 5.3.2.3: The effect of Asprosin on mitochondrial copy number in BEAS2B-R1 cells

BEAS2B-R1 airway epithelial cells treated with 100 ng/mL asprosin for 6, 12, and 24hrs (n=6). The mitochondrial copy number was calculated using the gene expression of BECN1 and mtND1, measured by RT-qPCR. Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis using Two-way ANOVA plus Tukey's post-hoc test was performed (p values are displayed as follows; * $p<0.05$).

Mitochondrial activity was measured using Mitotracker dye in BEAS2B-R1 cells. Cells were stained green for total mitochondria, and red for the active mitochondria. The red dye will only penetrate the mitochondrial membrane at an active membrane potential, whereas the green dye will penetrate the membrane regardless of the membrane potential. At 6hrs there was no difference in the number of active mitochondria between treatment groups. At 12hrs there was a modest increase in active mitochondria in asprosin treated cells (Control; 1.22±0.03 vs Asprosin 1.37±0.05; $p<0.01$), but by 24hrs there was a substantial decrease of 51% in active mitochondria in the asprosin treated cells (Control; 1.51±0.06 vs Asprosin 0.74±0.03; $p<0.0001$).

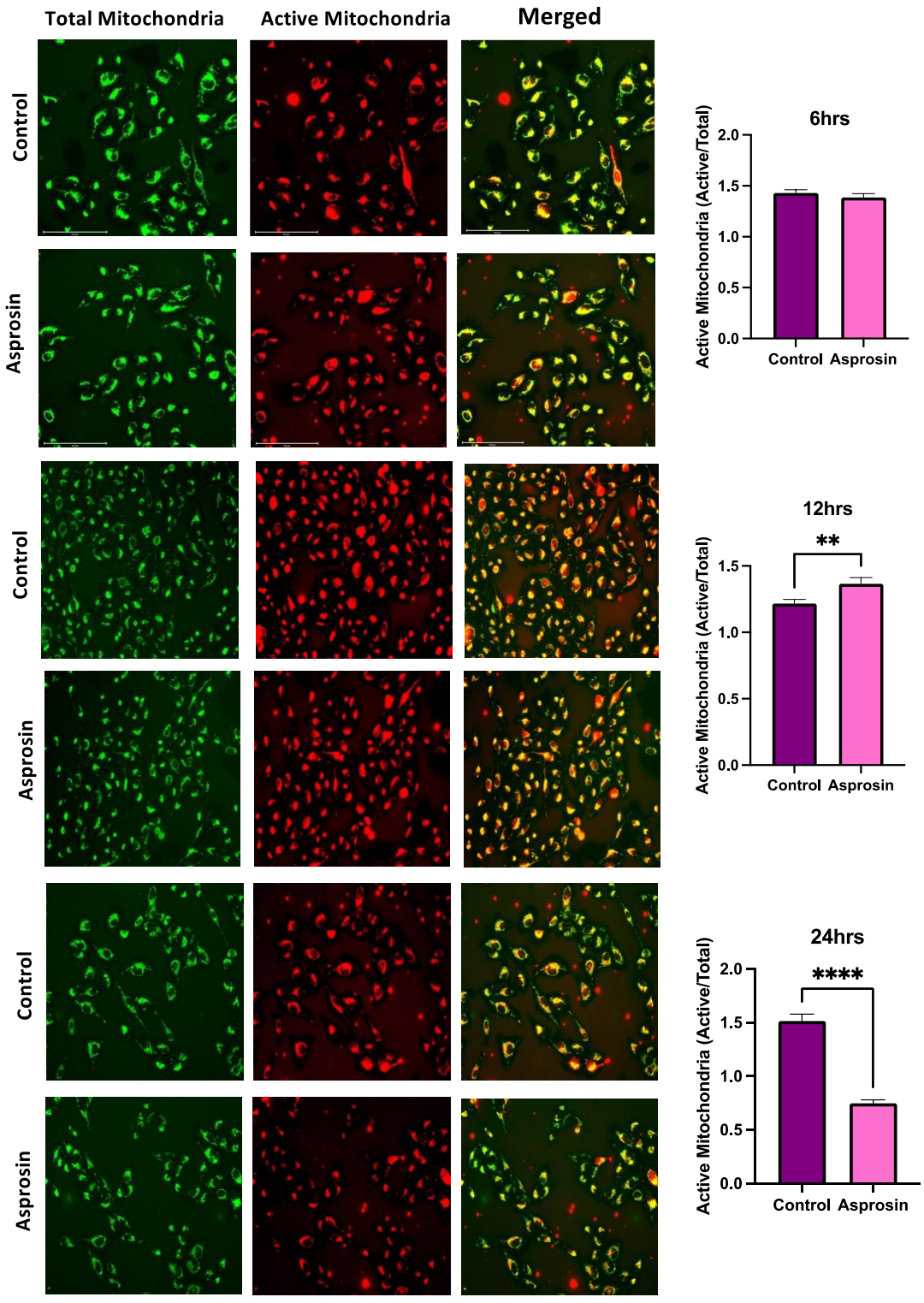

Figure 5.3.2.4: The effect of asprosin on mitochondrial activity in BEAS2B-R1 cells

BEAS2B-R1 airway epithelial cells treated with 100 ng/mL asprosin 6, 12, and 24hrs (n=100). Cells were stains with Mitotracker dye to distinguish the total mitochondria (green) and the active mitochondria (red). Statistical analysis using an unpaired t test was performed (p values are displayed as follows; ** $p<0.01$, **** $p<0.0001$).

5.4 Discussion

This novel study assessed the role of asprosin to mediate inflammatory responses in airway epithelial cells and its effect on mitochondrial function, as mechanisms that may lead to asprosin contributing to cellular damage in lung tissue to further exacerbate Asthma in Obesity. The main findings of this study were that in BEAS2B-R1 cells (1) asprosin induced inflammation, (2) had a negative impact on mitochondrial health (3) SCFAs could mitigate the asprosin-induced inflammation.

5.4.1 The effect of asprosin and SCFAs on inflammation in human airway epithelial cells

The role of asprosin in inflammation is currently not well characterised. This study showed that asprosin induced inflammation in BEAS2B-R1 airway epithelial cells through the NFκB pathway. Asprosin treatment induced an acute increase in the gene expression of NFKB1 and CXCL8. SCFAs were able to reduce the chronic asprosin-induced expression of CXCL8, and appeared to reduce NFKB1 expression, with the level of expression being brought down to the same levels as to that of SCFAs alone. The ability of SCFAs to mitigate asprosin-induced inflammation was reflected in protein data, with a reduction of NFκB phosphorylation and IKKβ expression shown in the asprosin combined with SCFAs group at 12 and 24hrs. This contributes to the understanding that SCFAs reduce inflammation by reducing NFκB activity (Vinolo *et al.*, 2011; Liu *et al.*, 2012; Tayyeb *et al.*, 2020; Yue *et al.*, 2022).

Asprosin did not alter the gene expression or protein activation of JNK54 or JNK46, suggesting that asprosin mainly activates inflammation through the NFκB pathway. At 6hrs, SCFAs increased gene expression and protein phosphorylation of JNK46. Though this may initially seem like SCFAs are contributing to cellular damage, there is evidence that SCFAs can activate an innate immune response in order to respond to cellular damage (Tang *et al.*, 2011; Matthews, Howarth and Butler, 2012; Tsugawa *et al.*, 2020). Whilst there is some evidence that low levels SCFAs, as used in this study, may induce inflammation (Mirmonsef *et al.*, 2012), so this may be the case in the airway epithelial cell line.

Although it would be expected that an increase in pro-inflammatory gene expression would translate into an increase in inflammatory protein cascades, asprosin was unable to activate NFκB. So although there may be an increase in NFκB in the cell, it is unable to be activated and translocate to the nucleus and increase the expression of pro-inflammatory cytokines. The asprosin dose used within this study may not have been high enough to induce inflammation within a cellular model. Previous *in vitro* studies have used higher doses of asprosin than physiologically relevant (Lee et al. 2019; Jung et al. 2019), which may be to compensate for other inflammatory proteins present in the blood to enhance the carrying and activation of asprosin. Our current study used a more physiologically relevant concentration of asprosin, noted from *in vivo* studies (Wang *et al.*, 2018; Ke *et al.*, 2020; Naiemian *et al.*, 2020; Zhang, Hu and Zhang, 2020). Those with metabolic disease have a much higher circulating concentration of asprosin than healthy patients (Ugur and Aydin, 2019; Wang *et al.*, 2019), but despite this, our *in vitro* studies found that the pro-inflammatory effects of asprosin were mitigated by

SCFAs at lower concentrations. This suggests that increasing systemic levels of SCFAs by taking prebiotics for example, could be beneficial to patients with Asthma and Obesity in order to relieve airway inflammation.

5.4.2 The effect of asprosin on mitochondrial function in human airway epithelial cells

Adipokine dysregulation occurs in metabolic-related diseases such as Obesity, which can lead to tissue-specific effects on cellular functions. For example, reduced adiponectin levels can lead to reduced mitochondrial function in adipocytes (Eun *et al.*, 2007) and monocytes (Iwabu et al. 2010). Leptin can be protective in neuronal cells by balancing mitochondrial dynamics (Cheng *et al.*, 2020), but can decrease mitochondrial respiration in the liver (Holmström *et al.*, 2013). Prior studies tend to focus on mitochondria copy number as an indicator of mitochondrial health, however an increase or decrease in copy number doesn't simply indicate the health of the cells. An increase in copy number could indicate that cells are healthy and functioning normally but may also be increased in response to damage. On the other hand, a lower copy number may be due to the cells not being able to make more mitochondria, or that there are enough mitochondria to supply energy to the cells and there is no need for more. It is therefore important to measure other aspects of mitochondrial function and health to give a better overall view.

Previous studies have stated that patients with lung diseases including Asthma tend to have a higher mitochondrial copy number than healthy patients (Carpagnano *et al.*,

2017; Cocco *et al.*, 2020; Mori *et al.*, 2022). However, there are a wide range of diseases where a lower mitochondrial copy number is present, including neurodegenerative diseases and various cancers (Pyle *et al.*, 2016; Sravya *et al.*, 2020; Filograna *et al.*, 2021). This study sought to use flux analysis, gene expression, cellular imaging as well as copy number to further investigate mitochondrial health and its dynamics.

Mitochondrial genes were measured to determine whether there were any transcriptional changes in response to asprosin. All genes relating to mitochondrial biogenesis (PPARG, POLG, TFAM, and NRF1) and function (COX4 and mtATP6) were reduced by 24hrs after asprosin treatment. A previous review has highlighted mitochondrial fission and fusion imbalance in Asthma (Caldeira *et al.*, 2021), so these data may suggest that asprosin may further contribute to this dysfunction. In addition, the mitochondrial copy number initially decreased, then increased over time. This suggests that asprosin may have initially damaged the mitochondria, so the cell responded by increasing turnover, but chronic damage over time began to affect the dynamics of the mitochondria.

Mitochondrial flux analysis showed that asprosin increased the cells glycolytic capacity at 6 and 12hrs. This was to be expected given that asprosin is involved in glycolysis signalling (Romere *et al.*, 2016). The ECAR level dropped back down to control levels by 24hrs. This time-dependant oscillation in ECAR suggests that chronic damage and inflammation over time impaired asprosin's ability to carry out its cellular functions properly. However, as previously mentioned asprosin signals for glycolysis in the liver in response to hunger signals as part of a whole organism (Romere *et al.*, 2016). Given that

in our study this was in a closed cellular system, it suggests a tissue-dependant role for asprosin in airways cells which leads to cellular damage; since glycolysis is more inefficient than oxidative phosphorylation and can lead to increased extracellular acidification which can be toxic to cells. Therefore, an increase in circulatory asprosin would allow it to travel to other tissues and induce glycolysis, causing further cellular damage. This damage may then contribute to the cellular dysfunction and inflammation seen in the lungs of Asthma patients.

The OCR remained unchanged over time, though a similar finding was shown with adipocyte cells treated with LPS where the OCR remained unchanged, but underlying mitochondrial mechanisms were overcompensating to meet cellular demands (Widdrington *et al.*, 2018). This idea of a compensatory mechanism was reflected in this study, where along with reduced mitochondrial biogenesis- and function-related gene expression, there was a reduction in mitochondrial activity over time.

Taken together, these data suggest that asprosin causes chronic mitochondrial damage in airway epithelial cells over time, in contrast to previous studies in cardiac cells suggesting a protective role for asprosin (Zhang et al. 2019; Wen et al. 2020). In order to meet the cells respiratory demands more mitochondria may be produced, however they may be of lower quality, which leads to reduced mitochondrial activity and increased turnover. The chronic inflammatory damage and increased glycolysis caused by asprosin may also be contributing to the impaired cellular functions, highlighting the tissue dependant damaging role of asprosin in airway cells.

5.4.3 Conclusions

This study is the first to suggest a pro-inflammatory role for asprosin in BEAS2B-R1 airway epithelial cells through activation of the NFκB pathway, which was mitigated by SCFAs. As well as its influence on inflammation, asprosin had a negative impact on mitochondrial health, shown by a reduction in biogenesis, function, and activity over time. Together, these data highlight asprosin as a novel inflammatory adipokine which is able to induce cellular dysfunction and may represent a further contributing factor to why weight gain may exacerbate Asthma symptoms in patients with Obesity and Obesity with type 2 Diabetes. Targeting therapies to reduce pro-inflammatory adipokines such as asprosin may help treat these chronic inflammatory conditions.

Chapter 6: The effect of prebiotic bimuno-galactooligosaccharide on inflammation, lung function, and metabolic markers in Asthma

6.1 Introduction

Airway inflammation present in Asthma leads to airway hyperresponsiveness (AHR). During AHR, symptoms such as bronchoconstriction, wheezing, and chest tightness occur. Although it is common to develop Asthma during childhood with an observed reduction in severity into adulthood (Ilmarinen *et al.*, 2020; Toppila-Salmi *et al.*, 2021), Asthma can develop at any stage of life, due to an array of factors. These may include exposure to allergens, viruses, and environmental triggers, but as an adult, lifestyle becomes an important influence on Asthma severity. Adult onset Asthma may develop as a result of smoking, exercise, or weight gain. These factors may increase production of pro-inflammatory cytokines and cause airway damage, which may in turn increase the likelihood of Asthma onset or increase severity. These triggers may also influence internal risk factors, where lifestyle may cause alterations of the human microbiota which may further influence the risk of Asthma.

Beyond smoking, exercise and weight gain the composition of the human gut microbiota is considered to influence various diseases, where a shift towards more harmful gut bacteria strains have been shown to drive inflammation and exacerbate conditions including chronic inflammatory bowel disease, Obesity, T2DM, cardiovascular disease and Asthma (Ley *et al.*, 2005; Schwiertz *et al.*, 2010; Carding *et al.*, 2015; Harris and Chang, 2018; Kitai and Tang, 2018; Chiu *et al.*, 2019; J. J. Lee *et al.*, 2019; Amabebe *et al.*, 2020; Gurung *et al.*, 2020). The composition of both the gut and airway microbiota are known to influence Asthma, where an increase in harmful gram-negative bacteria can lead to increased inflammation and AHR (Turnbaugh *et al.*, 2006b; Huang *et al.*, 2011; Taylor *et al.*, 2018; J. J. Lee *et al.*, 2019; Pang *et al.*, 2019).

Due to the associated risk posed by an altered gut microbiota, studies have considered manipulating the composition of the gut microbiota through diet to improve health. The main dietary supplements are probiotics, live strains of beneficial bacteria consumed through foods such as yoghurts and fermented food (Hill *et al.*, 2014), and prebiotics, types of fibre which have been shown to increase the number of beneficial bacteria in the gut (Gibson *et al.*, 2017). Studies exploring the effects of prebiotics on relieving Asthma symptoms in patients are limited, however initial studies have shown that prebiotics can reduce systemic inflammatory markers including C-reactive protein (CRP), TNFα, and eosinophils and AHR measured through spirometry (Williams *et al.*, 2016; McLoughlin *et al.*, 2019). Murine studies have also shown that prebiotics have the ability to reduce inflammatory markers and AHR (Verheijden, Akbari, *et al.*, 2015; Verheijden, Willemsen, *et al.*, 2015; Verheijden *et al.*, 2016, 2018).

As such due to the potential impact the gut microbiota may have on inflammation and disease, a human nutritional intervention trial was conducted to consider the influence of a prebiotic on participants with Asthma. The prebiotic Bimuno-Galactooligosaccharide (B-GOS) was used for this study, after being previously used in a study within the lab group with asthmatic participants and was shown to improve markers of inflammation and lung function (Williams *et al.*, 2016; Parker *et al.*, 2023). The principle aims of the study were to establish whether prebiotics can alter the gut microbiota to (1) reduce systemic inflammation, (2) reduce the levels of asprosin, a pro-inflammatory adipokine, as a potential molecular link between weight gain and

exacerbation of Asthma and, (3) provide additional benefits in participant's lung function and Asthma symptoms.

6.2 Methods

6.2.1 Ethical Approval and Study Design

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were approved by the Nottingham Trent University Human Ethics Committee (Register trial 713). All participants provided informed consent following provision of participant information sheet for a minimum of 24 hrs prior to visit 1 (Appendix 1). At the time of this thesis, the study was still ongoing and being conducted by PhD student Cristina Parenti.

The study was conducted in a double-blind, placebo-controlled, crossover design, whereby participants with Asthma were provided with a 3.65g/day of the prebiotic Bimuno-Galactooligosaccharide (B-GOS) or 3.65 g/day of taste, and colour matched placebo (Maltodextrin) for 21 days. This was followed by a 14-day wash out period, which has been shown to be a sufficient amount of time for the effects of the prebiotics to cease (Roberfroid, 2005) before crossing over to the other intervention (figure 6.2.1). Both supplements where portioned into sachets and double blinding was conducted at site of manufacture (Clasado Ltd).

Participants attended the lab on four separate occasions at day 0 and 21 of each intervention. At each visit, participants completed questionnaires relating to their Asthma severity and symptoms, and had their height, weight, hip and waist measurements taken. Participants lung function was assessed (in accordance with ATS/ERS guidelines), and a 40mL blood sample was taken.

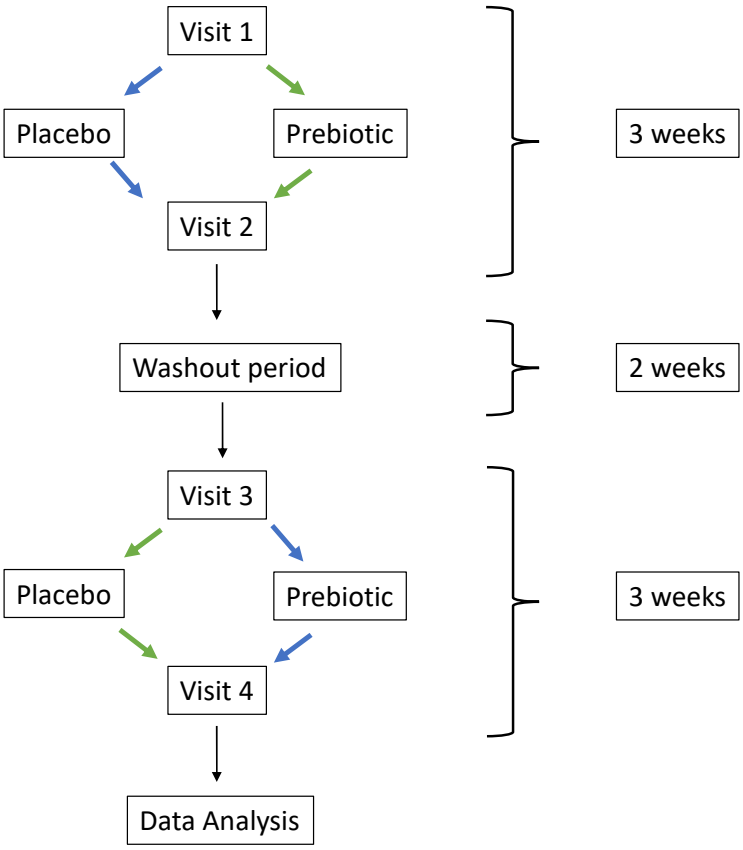

Figure 6.2.1.1: Prebiotic intervention trial design

Summary of the double-blind, placebo-controlled, crossover study design for the prebiotic intervention trial. Participants would be randomly allocated their first supplement, then would follow the path of either the blue or green arrow to completion.

6.2.2 Participant recruitment

Participants with a GP diagnosis of well-controlled Asthma, defined as Steps 1 to 5 based on British Thoracic Society guidelines July 2019 (Scottish Intercollegiate Guidelines Network., British Thoracic Society. and Healthcare Improvement Scotland., 2019), were recruited, aged 18-50 years old, with a BMI between 18.5-35 kg/m^2. A full list of the exclusion criteria can be found in appendix 1, but the main exclusions were that the participants did not have any known heart, pulmonary, or gastrointestinal diseases, be lactose intolerant, or have consumed any pre- or probiotics in the past month, did not

eat more than 1-2 portions of oily fish or take omega-3 supplements regularly, had not taken antibiotics in the 3 months prior to beginning the trial, and was not pregnant.

Recruitment was conducted through displaying of posters around the main buildings of both NTU City and Clifton campuses. The trial was advertised on social media via twitter, including on the NTU research and marketing twitter profiles. In person recruitment took place in lectures and lab classes for undergraduate and postgraduate biosciences students. A summary of participant recruitment is shown in figure 6.2.2.

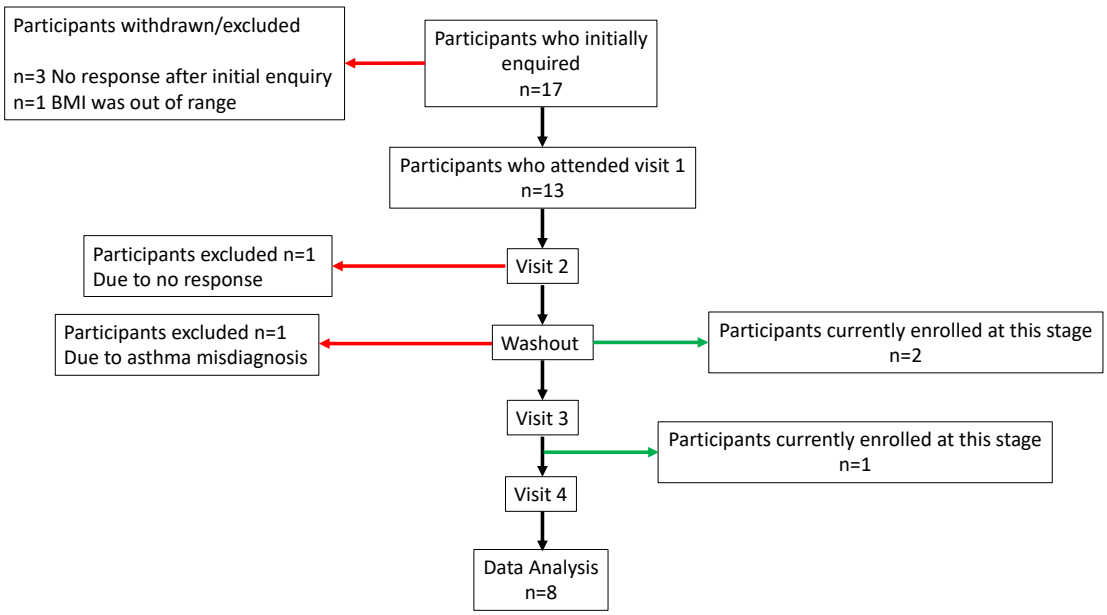

Figure 6.2.2.1: CONSORT Flow Diagram for the prebiotic intervention trial

CONSORT flow diagram showing the total number of participants recruited for the prebiotic intervention trial. Red arrows represent participants who were excluded or withdrawn from the trial, and the green arrows represent those who were currently enrolled on the study but had not fully completed the trial. Of the initial 17 people who enquired, 13 made it to the first visit and 8 have fully completed the trial. A total of 4 participants were excluded after initial enquiry, and a further 2 were excluded during the trial intervention period.

6.2.3 Health screen and questionnaires

At visit 1, participants completed the health screen questionnaire to identify any health conditions which may affect their eligibility of the trial, their Asthma diagnosis, and whether they had been diagnosed with COVID-19. If at this stage any health problems were identified which would exclude them from participating in the trial, the participants were withdrawn.

At each visit, participants were asked to complete the Asthma control questionnaire (ACQ6) in order to assess their Asthma severity (Juniper *et al.*, 1999). The baseline ACQ6 score at visit 1 was used to categorise each participants Asthma severity as follows; Mild ≤ 0.75, Moderate >0.75 to <1.5, and Severe ≥ 1.5. Further to this, participants also completed questionnaires to assess their quality of life in relation to Asthma symptoms (Appendix 2). These questionnaires included; the Nijmegen questionnaire for the assessment of hyperventilation, in which a score over 23 indicates hyperventilation syndrome (van Dixhoorn and Duivenvoorden, 1985), Hull airway reflux questionnaire (HARQ) to assess airway reflux and cough sensitivity within the last month, indicated by a score of over 14 (Morice *et al.*, 2011), Sino-nasal outcome test-22 (SNOT22) to evaluate the effect of nasal disorders (Hopkins *et al.*, 2009) with severity defined as the following; Mild ≤ 20, Moderate >20 to <50, and Severe ≥ 50, Hospital anxiety and depression scale (HADS) which evaluates both anxiety and depression on a scale in which 0-7 is normal, 8-10 is a moderate, and 11-21 is a case for diagnosis (Zigmond and Snaith, 1983), and finally the Epworth sleepiness scale questionnaire to assess daytime sleepiness (Johns, 1991), where a score of 0-10 is normal, 11-12 is mild, 13-15 is moderate, and 16-24 is severe sleepiness.

6.2.4 Venepuncture for blood serum and plasma collection

At each visit resting blood serum and plasma samples were collected using a 21G needle and vacutainer tubes from the antecubital vein. For serum, approximately 5mL of blood was collected in a serum separator tube, incubated at room temperature for 30 mins to allow for clotting, then centrifuged for 15 mins at 1,000 x g. The serum was aliquoted and stored immediately at -80^{o}C. For plasma, approximately 4mL of blood was collected in a K3 EDTA coated vacutainer tube and immediately centrifuged for 15 mins at 1,500 x g. The plasma was then aliquoted and immediately stored at -80^{o}C.

6.2.5 Pulmonary Function Test

A spirometry test was conducted at each visit to assess pulmonary function using the Vitalograph Pneumotrac Spirometer with Spirotrac 6 Software (Vitalograph, UK). Pulmonary function was conducted in accordance with American Thoracic Society (ATS) and the European Respiratory Society (ERS) guidelines, performed a minimum of 3 times and a maximum of 8 times in one session (Graham *et al.*, 2019). A pulmonary function manouvere was deemed acceptiable ifthe three largest values for FVC and/or FEV_1 were within 150mL of each other (Graham *et al.*, 2019).

Participants completed a flow volume loop manoeuvre where they took a deep breath in, and then strongly exhaled for 6s in order to empty the lungs, followed by a maximal inhalation. The maximum values from the pulmonoary function tests were taken for subsequent analysis and are summarised in table 6.2.5.

Measurement	Abbreviation	Definition
Forced vital capacity	FVC	The volume of air that is able to be displaced from the lungs during maximal effort
Forced expiratory volume in 1s	FEV_1	The amount of air that can be forced out of the lungs in 1 second
Forced expiratory volume in 6s	FEV_6	The amount of air that can be forced out of the lungs in 6 seconds
Peak expiratory flow	PEF	The peak flow of air that is able to be displaced from the lungs during maximal effort
Forced expiratory flow at 25-75%	FEF_{25-75}	The amount of air exhaled in the middle portion of the FVC
Forced expiratory flow at 75-85%	FEF_{75-85}	The amount of air exhaled in the 75-85th percentile of the FVC

Table 6.2.1: Definitions of spirometry measurements

6.2.6 Analysis of blood serum markers

The Pentra C400 (Horiba, UK) was used to analyse biomarkers within the serum of each participant taken at each visit. The following biomarkers were measured in triplicate in each serum sample (all from Horiba, UK); Glucose PAP (1220001668), HDL direct (1220001636), LDL (1220001638), and Triglycerides (1220001640). All samples were ran concurrently to avoid variance through serial runs. The variance between samples ran in triplicate was calculated to be <0.001 mmol/L, therefore any singular runs were deemed to be accurate.

6.2.7 Analysis of pro-inflammatory cytokines in the serum

Enzyme-linked immunosorbent assays (ELISA) were conducted in order to measure the levels of Asprosin (Abcam, ab275108, intra-assay cv: <7.9%, sensitivity: 0.92 ng/mL),

TNFα (Sigma, RAB0476-1KT, inter-assay cv: <12%, intra-assay cv: <10%, sensitivity: 30 pg/mL), and IL-6 (Sigma, RAB0306-1KT, inter-assay cv: <12%, intra-assay cv: <10%, sensitivity: 3 pg/mL) in blood serum/plasma of each participant pre and post each supplement.

6.2.8 Statistical Analysis

In order to determine the minimum sample size for statistical significance, the Massachusetts General Hospital Biostatistics sample size calculator was used, based off data outcomes previously reported in a prior B-GOS intervention study (Williams *et al.*, 2016). The minimum sample size was calculated to be 13 participants. Statistical analysis was preformed using GraphPad Prism 9. Data is reported as the mean ± the standard error of the mean (SE) unless otherwise stated. The appropriate statistical test was selected based on the normality of the data, using mainly the 2way ANOVA plus Šídák's multiple comparisons test to compare results before and after each supplement. Statistical significance was considered to be $p<0.05$ and was reported as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

6.3 Results

6.3.1 Participant characteristics

A total of 13 participants were recruited to the trial, of which 8 fully completed the trial, 3 are currently enrolled, and 2 excluded. The eight participants who had fully completed the trial were used for analysis, with their baseline characteristics summarised in table 6.3.1. Results according to BMI categorised participants as lean (n=5) and overweight (n=3), however due to the known inaccuracies for BMI classifications in relation to health, waist to hip ratio was used as a secondary measurement of health risk factor. The waist to hip ratio showed that of the 8 participants, 7 were healthy and 1 was at a moderate risk. Participant adherence to each supplement was 97.6% for maltodextrin and 97% for B-GOS.

	Total	**Male**	**Female**
n	8	2	6
	Mild	**Moderate**	**Severe**
Asthma Severity	3	4	1

Parameters	**Average**	**±SD**
Age (Years)	28.8	5.9
Weight (Kg)	69.1	8.4
BMI (Kg/m²)	25.1	2.6
Waist/Hip Ratio	0.77	0.06

Table 6.3.1: General characteristics of participants

A total of 8 participants were recruited onto the trial. Data within the table are baseline measurements at visit 1. Asthma severity was defined using the ACQ6 scores as follows; Mild $\leq$0.75, Moderate >0.75 to <1.5, and Severe $\geq$1.5. Anthropometric data is presented as the mean $\pm$ standard deviation (SD).

6.3.2 The effect of prebiotics on Asthma symptoms and lung function

Assessment of lung function was performed using spirometry before and after consumption of each supplement (figure 6.3.2.1). Overall changes for each supplement are summarised in figure 6.3.2.2. There was no change in pulmonary function from day 0 to day 21 of each intervention, or between B-GOS and maltodextrin. A decrease in FEF_{25-75} appeared to be shown with both supplements, but a larger decrease after maltodextrin. Differences between each supplement was shown in PEF data, where maltodextrin caused a 4.9%±3.3% decrease in PEF, whereas B-GOS led to a slight increase of 0.9%±7.1%. The most pronounced difference was shown in FEF_{75-85}, where B-GOS caused a 14.6%±16.7% increase as opposed to maltodextrin which only showed a 0.4%±5.5% increase. However, as shown in figure 6.3.2.1f, the large increase after B-GOS is most likely due to large increases noted in two of the participants.

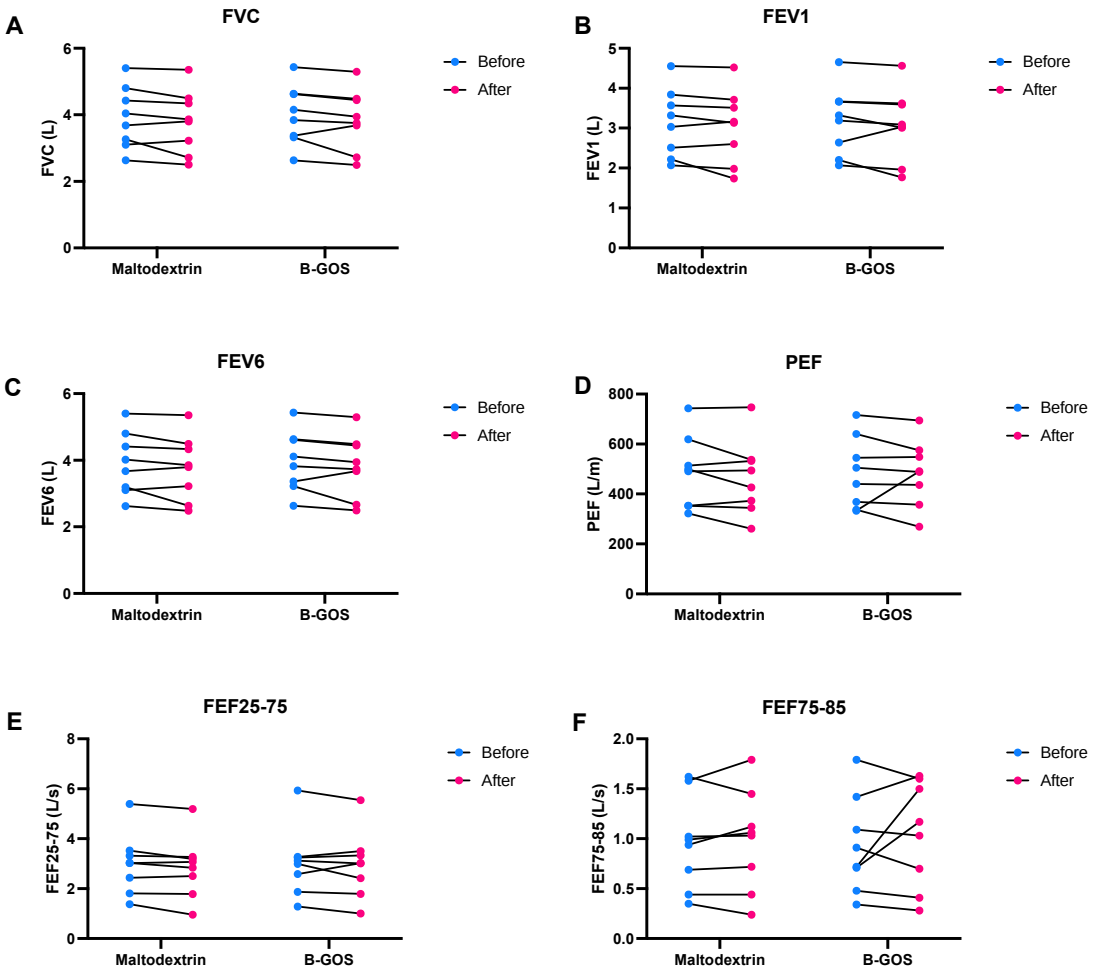

Figure 6.3.2.1: The effect of prebiotics on lung function in participants with Asthma

Lung function was assessed by spirometry before and after consuming a prebiotic or placebo for 3 weeks (n=8). The following measurements were taken; A) FVC, B) FEV1, C) FEV6, D) PEF, E) FEF_{25-75}, and F) FEF_{75-85}. Data is displayed as before and after each supplement for each individual participant. Statistical analysis was preformed using 2-way ANOVA plus Šídák's multiple comparisons test, data was not significant (p=ns).

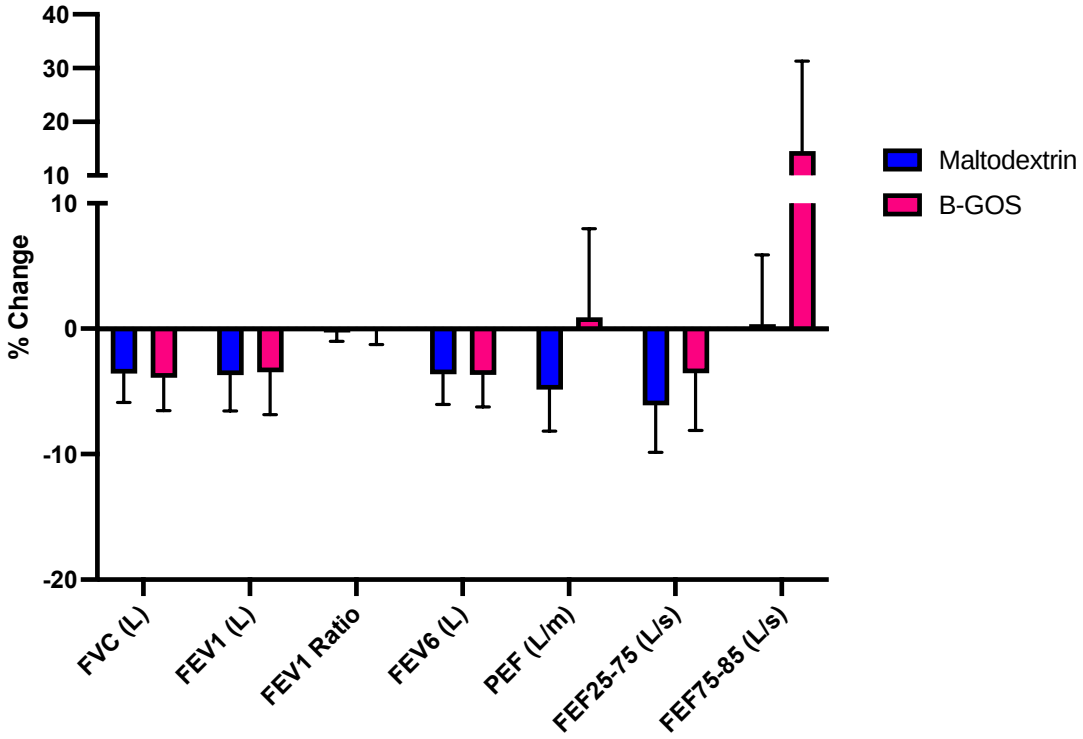

Figure 6.3.2.2: The percentage change in lung function after prebiotic supplementation

Lung function was assessed by spirometry before and after consuming a prebiotic or placebo for 3 weeks (n=8). Data is displayed the mean percentage change after prebiotic or placebo consumption, ± standard error of the mean (SEM). Statistical analysis was preformed using 2-way ANOVA plus Šídák's multiple comparisons test, data was not significant (p=ns).

Asthma symptoms and control were self-reported through questionnaires at each visit (figure 6.3.2.3). Overall changes in score are summarised in figure 6.3.2.4. There was no significant difference in questionnaire scores within and between each intervention. Maltodextrin showed the greatest change in score, with an average decrease of 3.9±0.6 points in the Nijmegen score, 7.4±3.8 in the SNOT22 score, 2.1±1.5 in the HADS anxiety score, and 1±1.1 in the Epworth score. In addition, of the 4 participants who were initially categorised as having a moderate nasal disorder before Maltodextrin, their scores reduced to mild after the supplement. There was also a small increase in HARQ score of 0.9±2.9.

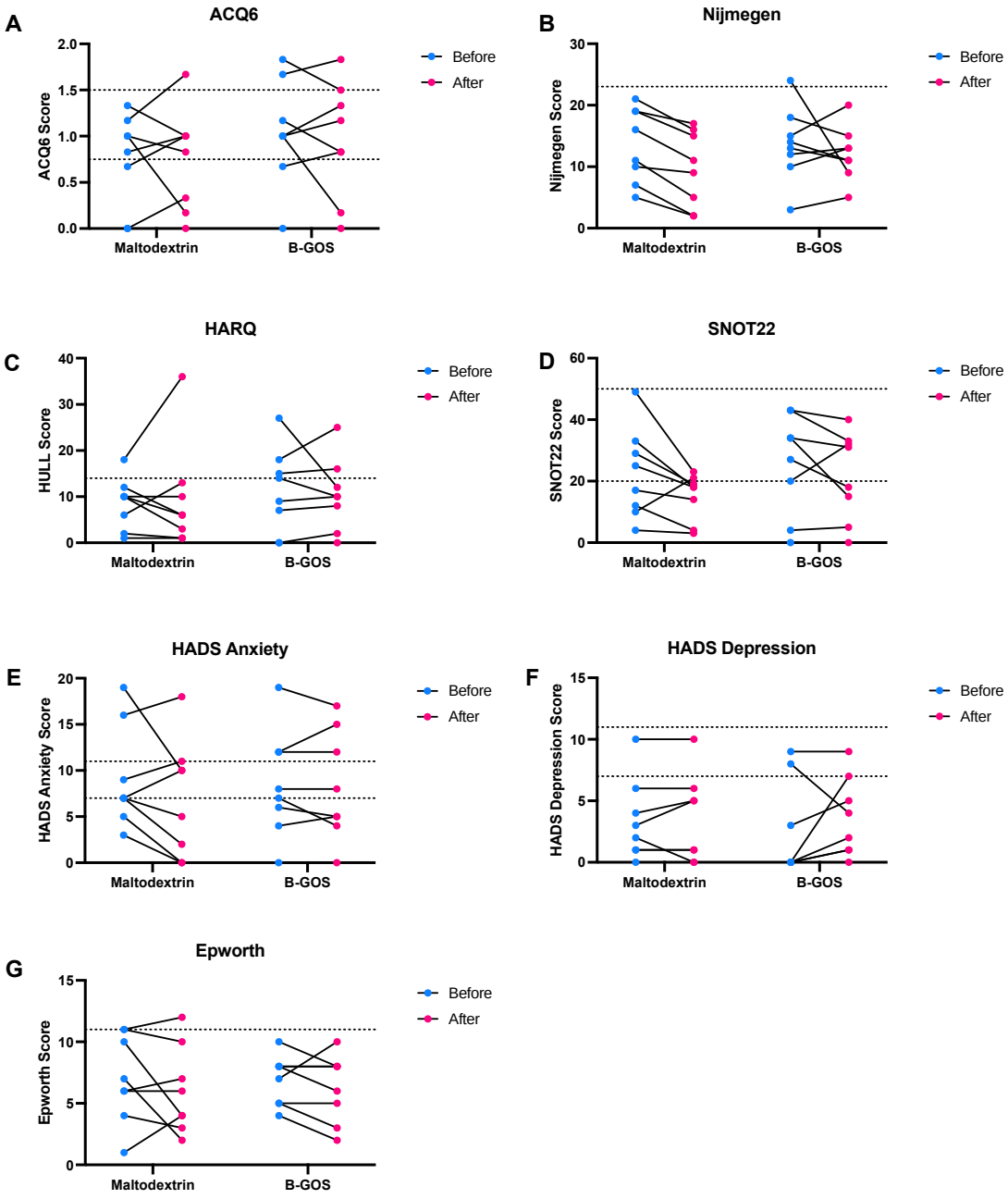

Figure 6.3.2.3: The effect of prebiotics on self-reported Asthma symptoms and severity

Questionnaires were completed by participants at each visit (n=8). The following questionnaires were used to determine the effect of prebiotics on self-reported Asthma symptoms and severity; A) ACQ6, B) Nijmegen, C) HARQ, D) SNOT22, E) HADS Anxiety, F) HADS Depression, and G) Epworth sleepiness scale. Data is displayed as before and after each supplement for each individual participant. Score thresholds for severe cases are represented by a line for each questionnaire. Statistical analysis was preformed using 2-way ANOVA plus Šídák's multiple comparisons test, data was not significant (p=ns).

B-GOS had smaller effects on the questionnaire scores, with an average decrease of 1.5±2.1 in the Nijmegen score, 0.9±2.3 in the HARQ score, and 0.6±0.63 in the Epworth score. There was also an average increase of 1.1±1 in the HADS depression score.

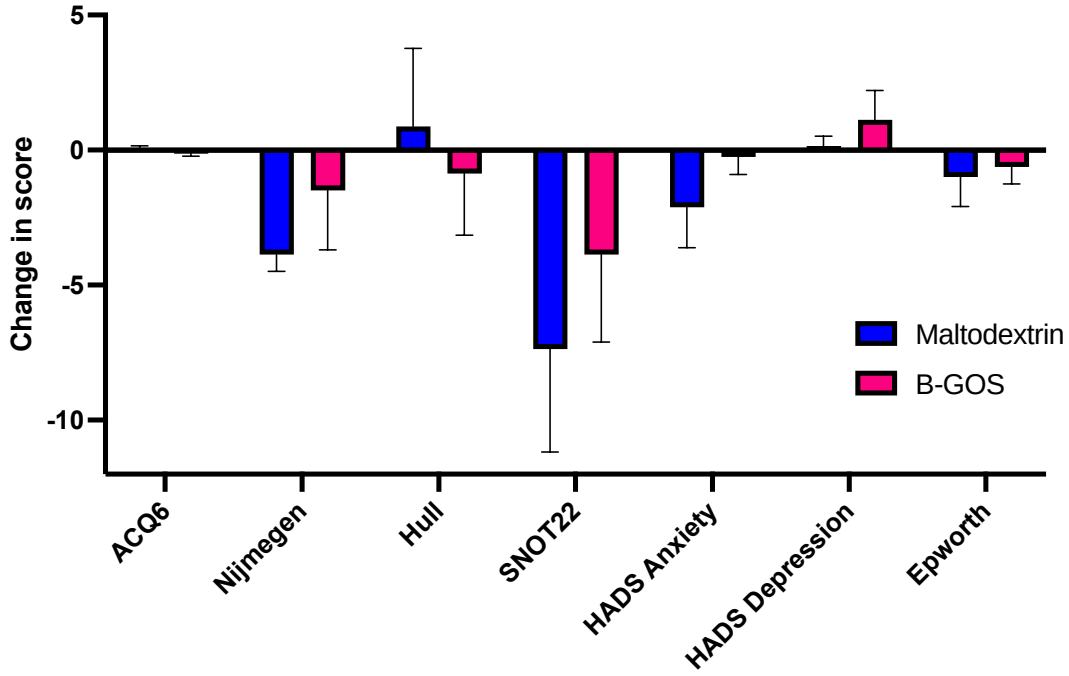

Figure 6.3.2.4: The change in score of self-reported Asthma symptoms and severity after prebiotic supplementation

Asthma symptoms and severity were assessed through questionnaires at each visit (n=8). Data is displayed the mean change in score after prebiotic or placebo consumption, ± standard error of the mean (SEM). Statistical analysis was preformed using 2-way ANOVA plus Šídák's multiple comparisons test, data was not significant (p=ns).

6.3.3 The effect of prebiotics on health and pro-inflammatory markers in serum

Serum glucose, cholesterol (HDL, and LDL), and triglycerides were measured pre and post consumption of the prebiotic and placebo (Figure 6.3.3.1). There was no difference in metabolic markers between each intervention group or between days 0 and 21 for

each supplement. The trends in the data suggest that there was a decrease in triglycerides and LDL and an increase in HDL after consumption of both the prebiotic and placebo. Greater differences were shown by B-GOS, where HDL increased by 9.6%±8% compared to 7.1%±3.5% with maltodextrin, and LDL decreased by 5%±9.8% compared to 1.8%±3.6% with maltodextrin. Triglycerides decreased by 8% for both the prebiotic and placebo. Glucose levels increased by 13%±10.9% after consumption of Maltodextrin, and decreased by 16%±8.1% after B-GOS.

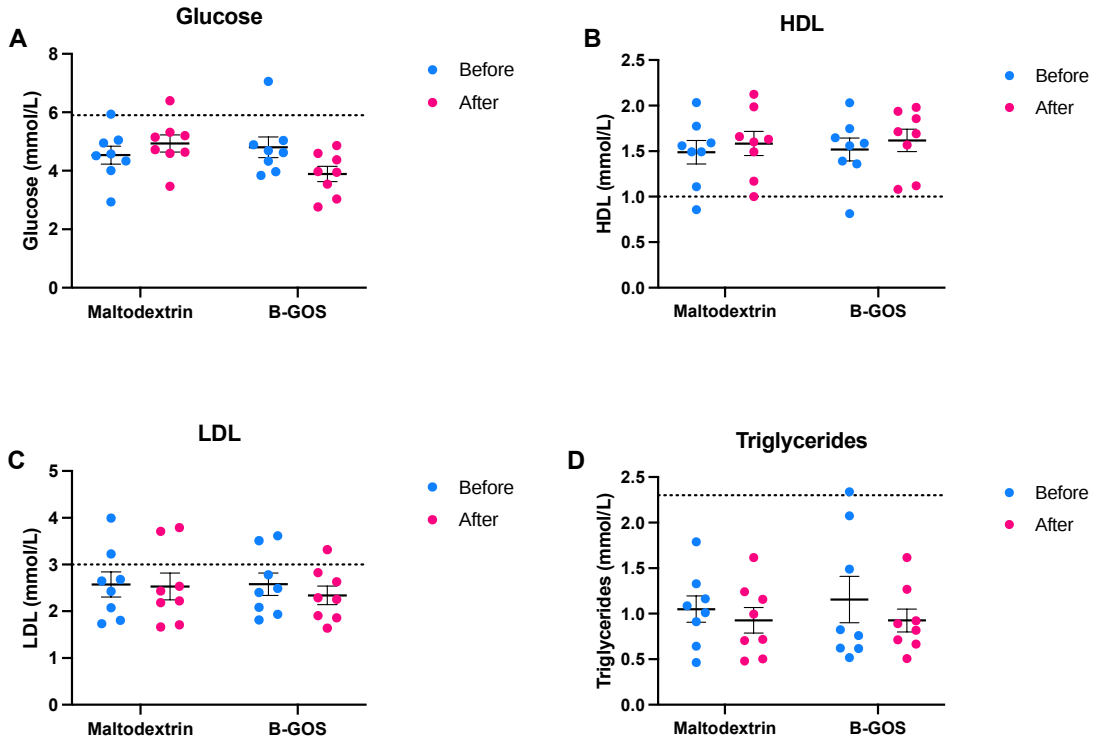

Figure 6.3.3.1: The effect of prebiotics on blood serum markers in participants with Asthma

Levels of the following markers were measured in the serum of participants before and after consumption of the prebiotic and placebo (n=8); A) Glucose, B) HDL, C) LDL, and D) Triglycerides. Data is displayed the mean ± standard error of the mean (SEM). Threshold levels for high glucose, triglycerides and LDL, and low HDL are represented by a line on each graph. Statistical analysis was preformed using 2-way ANOVA plus Šídák's multiple comparisons test, data was not significant (p=ns).

Circulating levels of IL-6, TNFα, and asprosin were measured in serum samples before and after consumption of each supplement (Figure 6.3.3.2). There was no difference in the levels of pro-inflammatory markers after consuming each supplement, or between treatment groups. Serum asprosin increased slightly after both maltodextrin and B-GOS, by 4.4% and 7.2% respectively. TNFα increased by 19.2% after maltodextrin and decreased by 18.4% after B-GOS. IL-6 increased by 44% after B-GOS and had a very small decrease of 4.8% after maltodextrin.

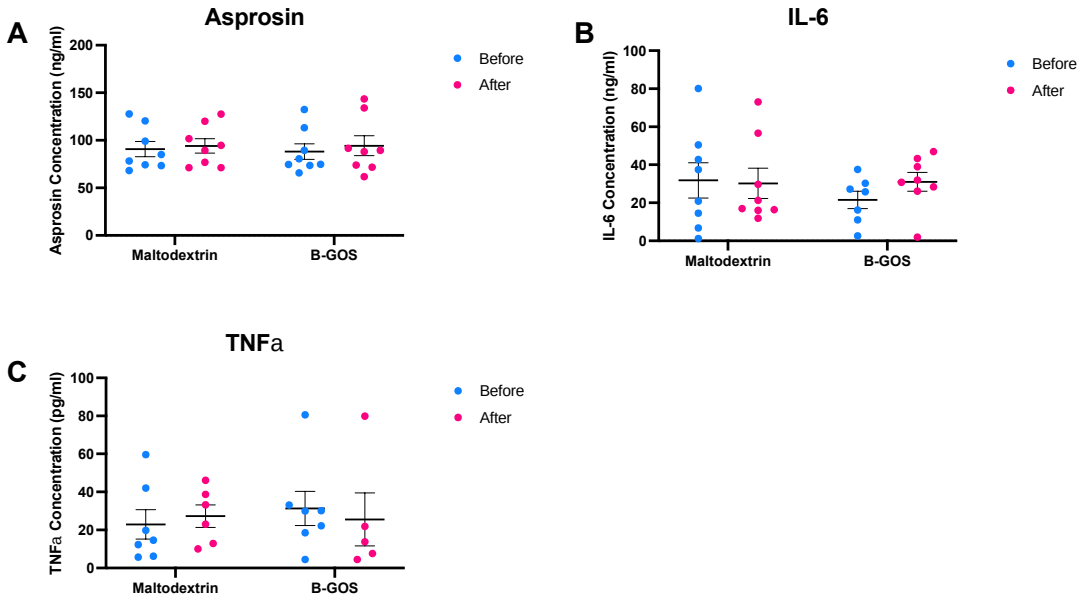

Figure 6.3.3.2: The effect of prebiotics on serum inflammatory markers in participants with Asthma

Inflammatory markers were measured in the serum of participants before and after consumption of a prebiotic and placebo (n=8). The following inflammatory markers were measured; A) Asprosin, B) IL-6, and C) TNFα. Data is displayed the mean ± standard error of the mean (SEM). Statistical analysis was preformed using 2-way ANOVA plus Šídák's multiple comparisons test, data was not significant (p=ns).

6.4 Discussion

This study presents the findings of the prebiotic intervention trial. At this stage, 8 participants completed both branches of the trial and were used within this thesis to explore the effect of prebiotics on health, noting that due to exclusion and drop-outs the number in the trial fell below the original target within the time constraints of this thesis. Initial trends of this data show that prebiotics 1) beneficially altered serum HDL, LDL, triglyceride, and glucose levels, 2) reduced inflammatory marker TNFα, and 3) did not appear to improve lung function and Asthma symptoms.

6.4.1 The effect of prebiotics on lung function and symptoms in participants with Asthma

The current study sought to investigate whether B-GOS could improve lung function and Asthma related symptoms. Given that this cohort were well-controlled Asthmatics and there was no challenge such as exercise or allergen exposure prior to testing, there was little change in lung function and self-reported symptoms. Previously an improvement in lung function in response to a 3-week prebiotic invention was shown, but this was after a eucapnic voluntary hyperpnoea challenge in order to induce exercise-induced bronchoconstriction (Williams *et al.*, 2016). The trend in these participants with Asthma is that they achieve lower values for PEF, $FEF_{25-75,}$ and FEF_{75-85}, and data showed a slight improvement of 0.9% in PEF and 14.6% in FEF_{75-85} after B-GOS. The change in FEF_{75-85} reported was most likely due to a small group of participants achieving a larger improvement in their post-prebiotic spirometry tests. However given that the

intervention period was only 3 weeks, this data shows early changes and may suggest that prolonged prebiotic use could improve lung function.

Asthma symptoms such as hyperventilation, nasal disorders which may affect breathing, and the severity were reported at each visit through questionnaires. The prebiotic did not seem to improve any of these factors. It had previously been reported that B-GOS can improve anxiety and depression (Johnstone *et al.*, 2021), but this was not reported within this study. Although questionnaires are a simple way of reporting symptoms, there are several issues with the data collected from them. The questionnaires were completed at each visit however they were not specific to Asthma in some cases, therefore other factors such as illness, hay fever, and the time of year were not taken into account for the questionnaires relating to breathing, nasal, and sinus disorders. Given the short invention period of 3 weeks, any seasonal illness or allergies would have influenced the data, compared to a long intervention period. Similarly, the HADS questionnaire related to general feelings of anxiety and depression, so may have been influenced by the participants life at the time, for example stress relating to work or if they had been on holiday recently. Since the questionnaires were self-reported on a basic numerical scale, it meant that each participants own idea of what they would report as mild to severe could vary for each individual. Therefore, although questionnaires were a good way to understand the effect of the prebiotics on each participants symptoms, it was difficult to compare when looking at the cohort as a whole, and could be more useful to examine trends of increases and decreases instead.

6.4.2 The influence of prebiotics on inflammatory and metabolic markers in participants with Asthma

Supplements that alter the gut microbiota composition such as pro- and prebiotics have previously been reported to reduce inflammation and improve markers of metabolic health (Aliasgharzadeh *et al.*, 2015; Williams *et al.*, 2016; Burton *et al.*, 2017; Sabico *et al.*, 2017, 2019; Ghiamati Yazdi *et al.*, 2019; McLoughlin *et al.*, 2019). Due to the small sample size the current study was unable to reach significance for changes in metabolic markers of health. However, there were some encouraging changes following the B-GOS intervention that warrant further investigation where B-GOS appeared to improve serum HDL, LDL, triglyceride, and glucose levels, and reduced TNFα.

Following 21-days of B-GOS Asthma patients were tending to show a 16% reduction in serum glucose levels. Although this did not reach significance in eight Asthma participants it would be beneficial given that impaired glucose homeostasis can cause AHR and inflammation (Gulcan *et al.*, 2009; Cottrell *et al.*, 2011; Chang and Yang, 2016; Karampatakis *et al.*, 2017). Conversely maltodextrin tended to raise serum glucose and TNFα. Whereas there was a trend for an 18.4% reduction in TNFα following B-GOS, which has been previously reported (Williams *et al.*, 2016). Mechanisms as to how prebiotics reduce TNFα have been investigated, where it has been suggested that prebiotics do this indirectly when digested by probiotics, resulting in the production of SCFAs which inhibit TNFα release through inhibition of immune cells (Pujari and Banerjee, 2021).

Serum IL-6 appeared to be raised by 44% following B-GOS consumption (p=n.s.), however this may not directly relate to increased inflammation. Prior studies investigating the influence of IL-6 provide conflicting reports, with studies often highlighting tissue dependent pro-inflammatory nature as well as its anti-inflammatory beneficial role in other cells. Studies with IL-6 have reported the cytokine to increase insulin secretion and improve glucose metabolism (Glund *et al.*, 2007; Suzuki *et al.*, 2011; Timper *et al.*, 2017), which would lead to increased cellular uptake of glucose in a healthy individual, and therefore lower blood glucose levels. This suggests that the increased IL-6 observed within this study may be contributing to beneficial molecular mechanisms.

Asthma patients have previously been shown to exhibit higher concentrations of LDL and lower HDL (Yiallouros *et al.*, 2014; Su *et al.*, 2018; Azofra *et al.*, 2019). These studies suggested that the increase in LDL may be due to the use of inhaled corticosteroids. Although serum levels of metabolic markers such as cholesterols and glucose are not typically considered as an outcome for studies within a lean Asthma cohort, although not reaching statistical significance this study highlights that a 3-week prebiotic intervention is sufficient time to see improvement in metabolic markers in this cohort, with a 9.6% increase in HDL, 5% decrease in LDL, and 16% decrease in glucose. Given that raised glucose and cholesterols are associated with the onset of metabolic related diseases, and may contribute to inflammation, it is beneficial to improve levels of these markers in order to prevent the development of further disease. Furthermore, it may also suggest that prebiotics could improve these markers within a cohort of patients with Asthma and Obesity, a demographic which would be of interest in future studies.

6.4.3 Asprosin in an Asthma cohort

This study is the first to this date to measure serum asprosin levels within a cohort of patients with Asthma and after prebiotic intervention. Asprosin levels did not appear to have a significant change following either placebo or prebiotic use, but small increases of 3.7% and 7% shown after the placebo and prebiotic respectively. This was to be expected since the cohort comprised of a majority of lean, healthy participants, so did not have high levels of asprosin pre-intervention. It has been noted previously that increased serum asprosin relies on the presence of insulin resistance or glucose intolerance (Wang *et al.*, 2018; Xinyue Zhang *et al.*, 2020; Corica *et al.*, 2022), which this cohort did not exhibit. Expanding the trial to allow for a wider range of BMI and the presence of T2DM may allow clearer changes to be seen.

Asprosin is known to be an orexigenic hormone which increases with hunger (Duerrschmid *et al.*, 2017). This may have influenced the concentrations reported in this study, as participants were asked to refrain from eating in the hours prior to each visit. Since hunger levels can vary, an increase in serum asprosin may be due to this rather than directly from the prebiotic.

6.4.4 Conclusion

Preliminary data from this double-blind, placebo controlled, crossover study showed that the prebiotic B-GOS had the potential to improve the metabolic markers glucose, triglycerides, HDL, and LDL, and to reduce pro-inflammatory cytokine TNFα. The initial

data from this study indicates that prebiotics may improve the health of healthy, lean participants with Asthma, and could prevent the onset of metabolic diseases related to increased glucose and cholesterols. Finally, these outcomes would be beneficial to explore further within a cohort with Asthma and Obesity, in order to improve their metabolic health.

Chapter 7: Final discussion and conclusions

7.1 Discussion

This thesis investigated the role of a potential dietary therapeutic, such as prebiotics, and how these agents could be used to reduce the chronic inflammation seen in weight gained induced Asthma. The premise of the thesis explored how short chain fatty acids (SCFAs), produced by beneficial gut bacteria in response to prebiotics, may have particular actions on tissues such as adipose and airway epithelial cells. As such this thesis examined the potential benefits of SCFA on health through a combination of *in vitro* cell work and *in vivo* human studies.

The gut was considered a target for such therapy as inflammatory agents such as gut derived endotoxin (also lipopolysaccharide; LPS) is known to be increased in people with Obesity (Harte *et al.*, 2012), with endotoxin established to enter the circulation and mediate an innate immune response, exacerbated in weight gain (Creely *et al.*, 2007). Furthermore, as the innate immune cascade can enhance pro-inflammatory adipokine release, these studies explored the role of the adipokine asprosin as a potential molecular link between Asthma and Obesity.

Prior to the studies of this thesis, there were limited insights examining the effect of prebiotics in patients with Asthma (Williams *et al.*, 2016; McLoughlin *et al.*, 2019) therefore, the current studies addressed a gap in the literature by examining the potential link between the gut, innate immunity, and tissue damage. Specifically, these studies explored cellular damage and recovery in adipocyte and airway epithelial cells through both *in vitro* molecular cell work with a human nutritional intervention trial

involving participants with Asthma to ascertain the circulatory, tissue and health benefits to participants.

The initial studies in this thesis investigated whether SCFAs could mitigate LPS-induced inflammation in both airway epithelial and white adipocyte cells. This study highlighted that SCFAs were able to reduce LPS-induced inflammation in each cell line. However, the airway cells appeared to have a lower inflammatory response to LPS treatment compared with the adipocyte cells. This may have been due to the resilience the airway cells may have acquired given that in the body, they are in constant contact with the outside environment, so would encounter many inflammatory stimuli including various strains of LPS and allergens. If airway cells responded with a more heightened response to all pro-inflammatory stimuli easily, the airways would be in a constant state of inflammation influx that would be damaging to cellular system and human health.

Other allergens exacerbating Asthma in the lungs has been explored, but the concept that gut derived LPS, and adipokines such as asprosin derived from adipose tissue, increased in obese conditions to exacerbate Asthma had not been examined. Within this thesis, initial studies examined whether circulatory asprosin was associated with weight gain and cellular dysfunction in two adipose tissue depots from a cohort of females with a range of BMIs. Circulating asprosin levels were shown to be raised as BMI increased, which was significantly increased in patients with Obesity and type 2 Diabetes Mellitus (T2DM). These findings affirmed previous studies where insulin resistance and T2DM status appeared to be an important factor for raised circulating asprosin levels (Wang *et al.*, 2018; Xinyue Zhang *et al.*, 2020; Corica *et al.*, 2022).

In addition to circulating asprosin levels, gene expression data, examined the influence of asprosin on mitochondria and browning. In these studies, asprosin showed that there was an abdominal AT depot specific effect, where in subcutaneous abdominal adipose tissue (ScAbdAT), increased asprosin gene expression correlated with a reduction in browning and mitochondrial genes. These data suggested that with an increase in ScAbdAT, there was a potential to increase asprosin expression, which may lead to a reduction in adipocyte browning and mitochondrial function. These findings were of particular interest as they had not been previously reported, so were further explored on a molecular level within a cellular model.

As Obesity is known to exacerbate Asthma conditions, the concept of how asprosin may also impact on airway cells was explored. From the literature, asprosin appears to have tissue dependent effect showing both pro-inflammatory damaging effects (Jung *et al.*, 2019; T. Lee *et al.*, 2019; Huang *et al.*, 2022) and protective effects (Z Zhang *et al.*, 2019; Wen *et al.*, 2020). To date no studies have reported the influence of asprosin on airway cells. As such, studies examined the role of asprosin in airway cells in order to determine whether it may represent a molecular link between Obesity and exacerbation of Asthma through asprosin-induced damage to airway cells. The data from this thesis showed that asprosin did mediate a pro-inflammatory effect in airway epithelial cells, through activation of NFκB, which was mitigated by SCFAs. This mechanism appeared to be the same as shown previously in LPS treated cells, and also in *in vitro* asprosin studies (Huang *et al.*, 2022). The exact mechanism of how SCFAs reduce inflammation is still unknown,

but data from this thesis contributes to the concept that SCFAs may block NFκB activation (Tayyeb *et al.*, 2020).

Asprosin was also shown to mediate mitochondrial dysfunction in airway epithelial cells, through a reduction in biogenesis and functional genes, reflecting the data previously shown within the adipose tissue depots within the patient cohort. The effect of asprosin on the airway cells led to an increase in mitochondrial turnover, a reduction in activity, and an increase in glycolysis. Whilst it has previously been noted that asprosin signals for glycolysis in the liver in response to hunger signals (Romere *et al.*, 2016), an increase in glycolysis within the airway cells could lead to further damage due to an increased acidification of cellular body. Taken with the inflammation data, it appears that asprosin causes damage to the airways, which would be exacerbated with weight gain as the amount of adipose tissue increases, which increases the potential for more asprosin to be released into the circulation. This study also highlights the tissue specific role for asprosin in airway cells, contributing to the role in damage shown by others (Jung *et al.*, 2019; T. Lee *et al.*, 2019; Huang *et al.*, 2022) rather than any noted protective effects in other tissues (Z Zhang *et al.*, 2019; Wen *et al.*, 2020).

With the knowledge gained from previous studies, the concept of gut microbiota modulation to produce anti-inflammatory compounds such as SCFAs was moved into a human nutritional intervention trial to confirm whether prebiotics were able to reduce inflammation and Asthma symptoms in participants with Asthma. Although the prebiotic had little effect on lung function, Asthma symptoms, and inflammatory markers, it was able to reduce glucose, HDL, LDL, and triglycerides within the serum,

which has yet to be reported in the literature. The intervention period within the trial was relatively acute, so preliminary data suggests that prebiotics may improve metabolic markers, but a longer intervention period could improve other Asthma symptoms over time. Furthermore, given that participants in this trial were young, lean, well-controlled Asthmatics and some changes were seen in glucose and cholesterol levels, this would be encouraging for future trials involving participants with Obesity and Asthma.

7.2 Limitations

The biggest limitation of these studies was the impact that the COVID-19 pandemic and lockdowns had on the timeline of this project. Within the first year of the PhD, there was no lab access for 6 months, followed by another 9 months of restricted access. This meant that experiments had to be prioritised in order to complete the main experiments needed for this thesis. Ideally, the first year of the PhD would have been used to optimise times and concentrations in each experiment, to then develop the project further in subsequent years, but this was not possible due to time.

The pandemic also impacted the human trial, as during this time the NHS were prioritising COVID related research therefore it was no longer possible to apply for NHS ethics within the time frame of this PhD. Due to this, the trial had to be adapted in order to comply with NTU ethics, meaning there were limitations on which samples and measurements could be taken. Recruitment began in January 2022, and although the best efforts were made to complete the trial within 3 years, it was not possible so only preliminary data from the first 8 participants could be used. Given that the recruitment

effort was somewhat limited in options, due to the lack of NHS ethics meaning it was not possible to recruit through local GP surgeries and hospitals, participants were mainly recruited through the university, which again caused issues due to the long summer holidays where students were away from campus. Given the demographic of a university campus, most participants were young, healthy, and with a lean BMI. This meant that the effect of adiposity on Asthma, and the effect of prebiotics on participants with Obesity and Asthma could not be as widely studied as had been planned.

To ensure that prebiotics had been metabolised by the gut microbiota, it had initially been suggested to either measure SCFAs levels in the circulation, or indirectly measured bacterial fermentation through gaseous compounds in the breath. However, it was felt that this was beyond the scope of the study and not feasible within the time frame. These measurements would have strengthened the data and would be of interest in the future once the technology is available.

Data on the cellular effects of asprosin are very limited in the literature, with the study within chapter 5 being the first to look at the effect of asprosin in the airways. As such there was limited information regarding concentrations and timings when treating cells. The treatments in this study were chosen based on physiological levels noted in the circulation, which would be most appropriate given that this would be the main way asprosin would be delivered to the airways. It would however had been better to have used a range of concentrations in order to optimise the concentration rather than only choosing one concentration. Although other published studies have used a much higher dose, the studies did show asprosin had effects at a lower concentration.

7.3 Future Work

The data within this study has opened up a number of further directions to explore in future experiments. Firstly, the asprosin studies would be of interest to repeat in the Chub-S7 cell line given the data showed *in vitro* in the airway cells. These experiments could also be repeated in different airway cell type, given that airway smooth muscle cells also play an important role in airway hyperresponsiveness in Asthma. It would therefore be good to use an airway smooth muscle cell line, before moving into either primary or immortalised cells from patients with Asthma.

In order to strengthen these asprosin studies, it would be beneficial to explore the mechanism of how asprosin causes inflammation further. Although asprosin typically acts through its own receptor OR4M1 (Romere *et al.*, 2016), it may be causing an inflammatory response through TLR4, as established in the literature with LPS. Therefore, experiments involving the inhibition or knockdown of TLR4 would likely be able to confirm this mechanism, and strengthen the evidence that asprosin induces inflammation through NFκB in airway epithelial and adipocyte cells. Following on from this, it would also be of interest to have an airway-adipocyte co-culture model, to see how the cells communicate and how the adipocytes can influence the airway cells. This would allow a deeper exploration into the role of Obesity in Asthma and would strengthen the previous data shown within this thesis.

Given the encouraging preliminary data from the human intervention trial, it would be of interest to expand the trial to a wider range of participants, mainly patients with a wider range of BMIs. This would be possible with NHS ethics, which would also allow

recruitment of participants through local GP surgeries and hospitals. NHS ethical approval would also allow for more measurements to be taken, including a body composition scan using the DEXA scanner.

7.4 Final Conclusions

The gut can be used as a target to reduce inflammation mediated by LPS or a downstream mediator of inflammation such as asprosin, and as such the use of a prebiotic may be beneficial to reduce inflammation caused by LPS and asprosin within the airways by increasing the levels of SCFAs within a patient. This study was the first to show that asprosin had a pro-inflammatory effect in the airways and caused mitochondrial dysfunction. This dysfunction was reflected within a patient cohort, where asprosin correlated with mitochondrial dysfunction as well as reduced adipocyte browning in AbdScAT. Finally, the administration of a prebiotic to healthy, well-controlled participants with Asthma did not improve lung function or symptoms but trended towards an improvement in serum levels of glucose, HDL, and LDL.

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Appendix

Appendix 1: Participant Information sheet and consent form

Participant information sheet

The effect of prebiotic supplementation on Asthma control and markers of systemic inflammation

Chief Investigator: Dr Neil Williams
Researchers: Miss Nikita Lad & Miss Cristina Parenti
Institution: Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS
We would like to invite you to take part in our research study. Before you make a decision, please carefully read the information below, which explains why the research is being done and what your involvement would be. To help you decide, one of our research team can discuss this information sheet with you. Talk to others about the study if you wish. Participation is voluntary. You may choose not to take part or withdraw from the study at any time without giving a reason.

Brief Introduction:

Many prevalent health disorders (including Asthma and Obesity) cause patients to have increased levels of systemic inflammation. A potential cause for the increased inflammation could be due to the types and activity of the bacteria that reside in the gut (gut microbiota). Evidence suggests that in Obesity and Asthma, the gut becomes more permeable, allowing harmful bacterial fragments to enter the circulation and increase levels of inflammation. However, the human gut is also home to many strains of beneficial bacteria, such as Bifidobacterium and Lactobacilli, which can exert health benefits and reduce systemic inflammation.

Dietary prebiotics, a form of non-digestible carbohydrate, are shown to encourage the growth and activity of beneficial bacteria and can confer a health benefit. Recent nutritional research into prebiotics has highlighted their potential to improve gastrointestinal health, and have an effect on the immune system to help fight infection, and may be involved in inflammatory processes and conditions. Recent work from our laboratory has shown that three weeks of a daily dietary prebiotic supplement can reduce Asthma severity and inflammatory markers. It could be hypothesised that prebiotics may help modulate systemic inflammation in Asthma and Obesity, improving disease management.

This study aims to assess the effects of a 3-week prebiotic supplementation period on Asthma control and markers of systemic inflammation.

Study Requirements:

You will be required to visit our laboratory on five separate occasions over a 9-week period, one familiarisation visit lasting approximately 1 hour and four experimental trials lasting approximately 90 minutes. During visit 1 you will become familiarised with pulmonary function (to assess your lung function), and the questionnaires to be used throughout the study. Also, height, weight, BMI, hip/waist ratio and body composition will be measured in all 5 visits. During visits 2-5 you will have measures of pulmonary function, have a 100ml blood sample and 1.8ml saliva taken. Visits 2 and 3, and visits 4 and 5 will be separated by three weeks in which you will take a daily (3.6g/d) prebiotic (Bimuno-galacto-oligosaccharide) or placebo (maltodextrin) for this duration. Visits 3 and 4 will be separated by two weeks in which you will not consume any supplements. Prior to each visit you must not brush your teeth, or use mouth wash within 60-minutes of arrival to the lab.

Inclusion Criteria:

To be eligible to take part, you must satisfy the following criteria:

- Be 18-50 years of age at the date of your first visit.
- Have a body mass index (BMI) of 18.5-35 kg·m2 (we can work this out for you using your height and body weight).
- Be a non-smoker.
- Your Asthma is defined as Steps 1 to 5 based on British Thoracic Society guidelines (we can tell you which step you are on based on the Asthma medication you use).
- You must have a current medication prescription from your GP if diagnosed with Asthma (e.g. maintenance and reliever inhalers).
- You must in the researcher's opinion, be able and willing to follow all trial requirements.
- You must disclose any nutritional supplements you take to the researcher, to determine whether these may be considered as 'exclusion criteria'

Exclusion Criteria:

Unfortunately, you will not be able to take part if any of the following apply to you:

- You suffer from Asthma but do not have a current medication prescription from your GP (e.g. maintenance and reliever inhalers)
- Unable to refrain from Asthma medication, e.g. standard inhaled corticosteroids (12 hours), long-acting inhaled corticosteroids (24 hours), combined inhaled corticosteroids and long acting β_2-agonists therapy (24 hours), and leukotriene modifiers (96 hours) prior to each testing session.
- You regularly consume 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 of the 7 days of the week.
- *Females only:* You are pregnant or planning a pregnancy during the time of the study (on each visit you will be asked to complete a pregnancy test if you are within childbearing age).
- You have consumed prebiotics and/or probiotics (supplements), drugs that affect gastrointestinal mobility or laxatives in the 4 weeks before signing the consent form.
- You have been previously diagnosed with chronic obstructive pulmonary disease (COPD), emphysema, chronic bronchitis, or similar respiratory (breathing-related) illness.
- You have been admitted to hospital during the past 12 months for your Asthma.
- You have a history of heart failure, pulmonary hypertension, embolism, or other pulmonary heart disease.
- You have a history of recurrent chest infections.
- You have had an acute infection in the last four weeks, and/or major operation in the past four months.
- You have a history of gastrointestinal drug reaction.
- You have taken antibiotics in the past 3 months.
- You have a history or current evidence of gastrointestinal disease (e.g. chronic constipation, diarrhoea, irritable bowel syndrome, Chrohn's disease).
- You have recently taken part in other research projects. Please notify the chief investigator.
- 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 acceptable; however, if a single antioxidant supplement (e.g. Vitamin C), is more than the recommended daily DRV's this must be checked with the chief investigator.

Restrictions During Testing:

Participants with Asthma will be allowed to continue the use of medication throughout the duration of the study. However, for us to assess airway inflammation during each visit you will be required in the lead up to each visit (2-5) to refrain from:

- Using short acting β_2-agonists for at least 12h before each testing session
- Inhaled long acting β_2-agonists for 48h before testing sessions
- Taking antihistamines for 48h before testing sessions
- Taking inhaled corticosteroids for 4 days before testing sessions
- Taking leukotriene modifiers for 4 days before testing sessions
- Ingesting caffeine and alcohol for 24h prior to testing sessions

- Ingestion of food and carbonated drinks 2 hours prior to testing sessions
- Physical exercise for 24h prior to testing sessions
- Use of mouth-rinse and brushing your teeth 60 minutes prior to testing sessions

These are standard requirements for diagnosing/assessing Asthma and airway inflammation. However, **if you struggle to control your symptoms during this period, then you must resume your medication immediately and subsequently will not be allowed to take part.** The reduction in medication use may have a very short-term impact on your exercise performance that will be reversed following the testing session and when you take your medication again.

If you have a previous diagnosis of Asthma but fail to present your reliever/rescue medication to the research team on arrival to the laboratory you will not be able to complete that testing session. You will be invited back on another agreed date and asked to bring your medication with you. Furthermore, if you have a previous diagnosis of Asthma but no current prescription for a reliever/rescue inhaler then you will be excluded from the study until you can present to the research team a current prescription.

Location:

Erasmus Darwin building, Room 138, Clifton Campus, Clifton Lane, Nottingham Trent University, NG11 8NS.

Testing Protocol:

Visit 1: Initial consultation and familiarisation

The chief investigator will explain what participation would involve, and how data obtained from participants will be used/stored. The procedures for documenting adverse/serious adverse events throughout the study will be explained. You will have the opportunity to ask any questions/raise any concerns regarding taking part. If satisfied, you will be asked to provide written informed consent to participate, and to complete a health screen and history questionnaire, both of which will be reassessed at each visit by the chief investigator and/or Miss Nikita Lad and/or Miss Cristina Parenti. This visit will begin by confirming you have your Asthma reliever medication with you and checking that you are suitable for the study. We will then determine your body composition through waist and hip circumference, height, body weight, and body mass index (BMI). You will have the opportunity to practice spirometry to assess your lung function. This requires you to breathe out through a mouthpiece as hard as possible for 6 seconds. This measurement is similar to a peak flow assessment, which you may have completed with your GP. The investigators will explain how the nutritional supplements will be administered, and how/when to complete certain questionnaires/scales throughout the study. These will include a questionnaire to assess your perceptions of Asthma control, and adherence to your Asthma medication/the nutritional supplements. The procedures for collecting blood samples during visits 2-5 will be

explained. We will explain what you need to do in preparation for your second laboratory visit. An overview of the study design is depicted in Figure 1.0 below.

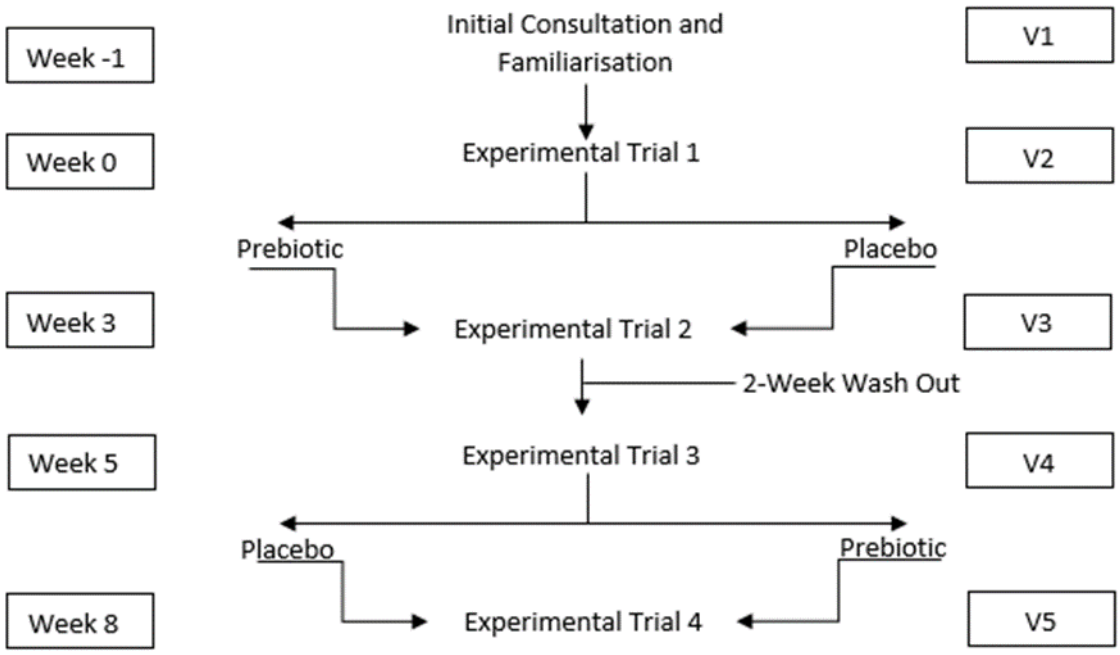

Figure 1.0 Schematic diagram illustrating study design and duration. V = Visit Number.

Visits 2-5: Main experimental visits

Please see Figure 1.1 below, showing an overview of experimental procedures during each experimental trial.

UPON ARRIVAL	ASTHMA ASSESMENT	END
Check Health Status	Resting Lung Function	Questionnaires
Present Food Diary	Blood Sample	
Self-Report Scales	Anthropometric data collection	

START → **END**

- Measurements of body composition analysis, waist and hip circumference, height, body weight, BMI, and lung function will be conducted during each experimental trial as per visit 2.
- You will present your 24-hour diet log (if visit 2,3, 4 or 5) and reliever medication to the chief investigator upon arrival, or 4 day food diary (if visits 3 or 5)
- You will be asked to complete a questionnaire to assess Asthma control, quality of life, and a scale to monitor Asthma medication/nutritional supplement adherence.

- You will be asked to provide a resting blood sample and saliva sample, and the same procedure will be followed during visits 2-5.

We will ask you to record your diet for the 24 hours preceding visit 2. We will also ask you to complete a 4-day food diary during the four days leading up to visits 3, 4 and 5. The procedures for recording your diet will be explained during visit 1. On arrival to the laboratory we will confirm that you have your Asthma reliever medication with you and collect your 24-hour (visit 2) or 4-day (visits 3-5) food diary. You will then complete health screen and health history questionnaires and provide written consent for us to take your blood and saliva. We will then measure your body weight, hip ad waist circumference, body analysis composition, lung function and take a blood sample and salivary sample. 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.

Blood sampling

Blood samples will be collected using a needle inserted into a vein in the mid-arm by trained personnel following sterile protocols (similar procedure to blood donation). The procedure will be explained in full before sample collection. You will be asked to provide written informed consent for the procedure to be conducted. You will be asked to state any fears/phobias (e.g. blood/needles), or allergies (e.g. elastoplast, alcohol, latex) before blood samples are collected. Up to 100ml of blood will be taken during each visit. This will be used to assess markers of immune function and inflammation. Blood samples will be stored securely in the Erasmus Darwin Building at Nottingham Trent University, Clifton Campus. Blood samples from the study will also be used for future In-vitro PhD research.

Nutritional Intervention

Between visits 2 and 3, and visits 4 and 5 participants you will be required to consume a total of 7.2 g/d of either Bimuno Galactooligosaccharide Prebiotic (CLASADO Biosciences) or maltodextrin (taste, sight and smell matched placebo) (CLASADO Biosciences) for 3 weeks. You will be required to consume a total of two powdered sachets per day (one 3.6g in the morning and one 3.6g in the evening) which can be reconstituted in water, tea, or coffee. You will be randomly assigned to which supplement you take during the first supplementation period. After a 2-week wash-out period you will commence on the opposite supplement for a further 3 weeks. The study will be conducted in a double-blind fashion, so neither you or the researchers will be aware of which supplement was taken during each supplementation period until all participants have completed the study.

Pulmonary function

You will be asked to perform three repeatable measures of pulmonary function. This will involve you producing a maximal exhalation proceeded by a sharp maximal inhalation

into a mouthpiece for the measurement of lung function parameters (forced vital capacity (FVC); forced expiratory volume over 1 second (FEV_1); peak expiratory flow (PEF)).

4-day weighed food diary

Between the familiarisation visit (visits 1) and visits 2, within both supplementation periods, and during the wash-out period you will be asked to complete a 4-day food diary. This will require you during one weekend day and three weekdays to weigh your daily food intake and document the time of consumption, brand of food, cooking methods used and weight of food within a food diary. It is important you maintain your normal dietary habits throughout the study and provide as much detail as possible within the food diary to ensure we can perform to most accurate analysis on your diet.

Data handling

All information you provide, and data collected during the study will be anonymised. Data and information will not be kept for any longer than 5 years.

After Participation: Formal Debrief

During your final visit, the chief investigator will explain how data obtained from you will be used (e.g. research publications, conference presentations), and how you will be able to access any publications/reports of the research. Procedures for withdrawing yourself and/or your data following trial completion will be explained.

Participant Responsibility

You are kindly asked to complete all documents accurately, and to follow all control measures/testing restrictions/intervention guidelines throughout the study. If completed accurately, the information from this study may help develop new methods of managing Asthma for people in the future. Consume the nutritional supplements as instructed. If sachets are missed, do not compensate by consuming multiple sachets. Proceed with the instructed dosing schedule and document any discrepancies. Please notify the chief investigator of any deviations from these instructions, and/or changes relevant to the inclusion/exclusion criteria (e.g. antibiotics prescription).

Potential Benefits

You will undergo an in-depth personalised assessment of Asthma. This involves information regarding your lung function, and the assessment of FEV_1 'forced expiratory volume, which means the amount of air a person can forcefully exhale in one second. Other lung measurements will also be recorded. In terms of the nutritional supplements, no specific benefits are anticipated. However, it is possible Asthmatic symptoms may be reduced.

Potential Risks to You

Slight discomfort may occur during venepuncture, all investigators taking blood samples however will be fully trained and will take the up most care. Although very rare some individuals may feel symptoms of gastrointestinal discomfort such as bloating and abdominal cramps during the prebiotic supplementation period.

If at any point you decide to withdraw from the study your data will be destroyed

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Participant Statement of Consent to Participate in the Investigation Entitled:

"*The effect of prebiotic supplementation on the control of Asthma and markers of systemic inflammation"*

1) I, _____ agree to partake as a participant in the above study.
2) I understand from the participant information sheet, which I have read in full, and from my discussion(s) with Miss Nikita Lad & Miss Cristina Parenti that this will involve me visiting the Nottingham Trent University Sports Science Laboratories on five separate occasions. This will involve me performing pulmonary function assessment and providing a blood sample. Height, weight, BMI, ip/waist ratio and body composition will be measured. Additionally, I will be required to complete questionnaires on Asthma control, Asthma quality of life, medication adherence, and gastrointestinal symptoms. I will also be required to consume two supplements (B-GOS and maltodextrin) daily in two separate three week periods.
3) It has also been explained to me by Miss Nikita Lad & Miss Cristina Parenti that the risks and side effects which may result from my participation are as follows: Slight discomfort may also occur during venepuncture, all investigators taking blood samples however will be fully trained and will take the up most care. Although very rare some individuals may feel symptoms of gastrointestinal discomfort such as bloating and abdominal cramps during the prebiotic supplementation period.
4) I confirm that I have had the opportunity to ask questions about the study and, where I have asked questions, these have been answered to my satisfaction.
5) I undertake to abide by University regulations and the advice of researchers regarding safety.
6) I am aware that I can withdraw my consent to participate in the procedure at any time and for any reason, without having to explain my withdrawal and that my personal data will be destroyed.
7) I understand that any personal information regarding me, gained through my participation in this study, will be treated as confidential and only handled by individuals relevant to the performance of the study and the storing of information thereafter. Where information concerning myself appears within published material, my identity will be kept anonymous.
8) I confirm that I have had the University's policy relating to the storage and subsequent destruction of sensitive information explained to me. I understand that sensitive information I have provided through my participation in this study, in the form of questionnaires, blood samples, exhaled breath condensate samples or other measures taken throughout the study will be handled in accordance with this policy.
9) I understand that I will not be informed of any genotype.
10) I understand that as part of this study I will be consuming a supplement. I am aware that elite sports people (i.e. international or national standard) may undergo either

out-of or in-competition (or both) doping tests and appreciate that the supplement being studied could be contaminated with a substance that appears on the banned lists.

11) I confirm that I have completed the health questionnaire and know of no reason, medical or otherwise that would prevent me from partaking in this research.

12) I confirm that I have completed the COVID-19 questionnaire and know of no reason, or otherwise that would prevent me from partaking in this research.

13) I confirm that I am not affected by COVID-19 or not to be subject to a mandatory quarantine period.

14)At moment, I do not experience any of the COVID-19 related symptoms, described in the COVID-19 questionnaire.

Participant signature: Date:

Independent witness signature: Date:

Primary Researcher signature: Date:

Appendix 2: Questionnaires

Appendix 2.1: Asthma Control Questionnaire

Asthma Control Questionnaire ©

Circle the number of the response that best describes how you have been during the past week

1. On average, during the past week, how often were you woken by your Asthma during the night?

0 Never
1. Hardly ever
2. A few minutes
3. Several time
4. Many times
5. A great many times
6. Unable to sleep because of Asthma

2. On average, during the past week, how bad were your Asthma symptoms when you woke up in the morning?

0 No symptoms
1. V mild symptoms
2. Mild symptoms
3. Moderate symptoms
4. Quite severe symptoms
5. Severe symptoms
6. Very severe symptoms

3. In general, during the past week, how limited were you in your activities because of your Asthma?

0 Not limited at all
1. V slightly limited
2. Slightly limited
3. Moderately limited
4. V limited
5. Extremely limited
6. Totally limited

4. In general, during the past week, how much shortness of breath did you experience because of your Asthma?

0 None
1. A very little
2. A little
3. A moderate amount
4. Quite a lot
5. A great deal
6. A very great deal

5. In general, during the past week, how much of the time did you wheeze?

0 Not at all
1. Hardly any of the time
2. A little of the time
3. Moderate amount of time
4. A lot of the time
5. Most of the time

6. All of the time

6. On average, during the past week, how many puffs of short-acting bronchodilator (e.g Ventolin) have you used on each day?

0 None
1. 1-2 puffs most days
2. 3-4 puffs most days
3. 5-8 puffs most days
4. 9-12 puffs most days
5. 13-16 puffs most days
6. More than 16 puffs most days

Appendix 2.2: Nijmegen Questionnaire

	Never 0	Rarely 1	Sometimes 2	Often 3	Very Often 4
Chest pain					
Feeling tense					
Blurred vision					
Dizzy spells					
Feeling confused					
Faster or deeper breathing					
Short of breath					
Tight feelings in chest					
Bloated feeling in stomach					
Tingling fingers					
Unable to breathe deeply					
Stiff fingers or arms					
Tight feelings round mouth					
Cold hands or feet					
Palpitations					
Feeling of anxiety					

Appendix 2.3 : HULL Airway Reflux Questionnaire

HULL AIRWAY REFLUX QUESTIONNAIRE

Name:

D.O.B:_____ UN: _____

DATE OF TEST:

Please circle the most appropriate response for each question

Within the last MONTH, how did the following problems affect you? 0 = no problem and 5 = severe/frequent problem						
Hoarseness or a problem with your voice	0	1	2	3	4	5
Clearing your throat	0	1	2	3	4	5
The feeling of something dripping down the back of your nose or throat	0	1	2	3	4	5
Retching or vomiting when you cough	0	1	2	3	4	5
Cough on first lying down or bending over	0	1	2	3	4	5
Chest tightness or wheeze when coughing	0	1	2	3	4	5
Heartburn, indigestion, stomach acid coming up (or do you take medications for this, if yes score 5)	0	1	2	3	4	5
A tickle in your throat, or a lump in your throat	0	1	2	3	4	5
Cough with eating (during or soon after meals)	0	1	2	3	4	5
Cough with certain foods	0	1	2	3	4	5
Cough when you get out of bed in the morning	0	1	2	3	4	5
Cough brought on by singing or speaking (for example, on the telephone)	0	1	2	3	4	5
Coughing more when awake rather than asleep	0	1	2	3	4	5
A strange taste in your mouth	0	1	2	3	4	5

TOTAL SCORE_____ /70

Appendix 2.4: SNOT22 Questionnaire

I.D.:_____ **SINO-NASAL OUTCOME TEST (SNOT-22)** DATE:_____

Below you will find a list of symptoms and social/emotional consequences of your rhinosinusitis. We would like to know more about these problems and would appreciate your answering the following questions to the best of your ability. There are no right or wrong answers, and only you can provide us with this information. Please rate your problems as they have been over the past two weeks. Thank you for your participation. Do not hesitate to ask for assistance if necessary.

1. Considering how severe the problem is when you experience it and how often it happens, please rate each item below on how "bad" it is by circling the number that corresponds with how you feel using this scale: →	No Problem	Very Mild Problem	Mild or slight Problem	Moderate Problem	Severe Problem	Problem as bad as it can be		5 Most Important Items
1. Need to blow nose	**0**	**1**	**2**	**3**	**4**	**5**		○
2. Nasal Blockage	**0**	**1**	**2**	**3**	**4**	**5**		○
3. Sneezing	**0**	**1**	**2**	**3**	**4**	**5**		○
4. Runny nose	**0**	**1**	**2**	**3**	**4**	**5**		○
5. Cough	**0**	**1**	**2**	**3**	**4**	**5**		○
6. Post-nasal discharge	**0**	**1**	**2**	**3**	**4**	**5**		○
7. Thick nasal discharge	**0**	**1**	**2**	**3**	**4**	**5**		○
8. Ear fullness	**0**	**1**	**2**	**3**	**4**	**5**		○
9. Dizziness	**0**	**1**	**2**	**3**	**4**	**5**		○
10. Ear pain	**0**	**1**	**2**	**3**	**4**	**5**		○
11. Facial pain/pressure	**0**	**1**	**2**	**3**	**4**	**5**		○
12. Decreased Sense of Smell/Taste	**0**	**1**	**2**	**3**	**4**	**5**		○
13. Difficulty falling asleep	**0**	**1**	**2**	**3**	**4**	**5**		○
14. Wake up at night	**0**	**1**	**2**	**3**	**4**	**5**		○
15. Lack of a good night's sleep	**0**	**1**	**2**	**3**	**4**	**5**		○
16. Wake up tired	**0**	**1**	**2**	**3**	**4**	**5**		○
17. Fatigue	**0**	**1**	**2**	**3**	**4**	**5**		○
18. Reduced productivity	**0**	**1**	**2**	**3**	**4**	**5**		○
19. Reduced concentration	**0**	**1**	**2**	**3**	**4**	**5**		○
20. Frustrated/restless/irritable	**0**	**1**	**2**	**3**	**4**	**5**		○
21. Sad	**0**	**1**	**2**	**3**	**4**	**5**		○
22. Embarrassed	**0**	**1**	**2**	**3**	**4**	**5**		○

2. Please mark the most important items affecting your health (maximum of 5 items)_____ ↑

Appendix 2.5: HADS Questionnaire

Hospital Anxiety and Depression Scale (HADS)

Tick the box beside the reply that is closest to how you have been feeling in the past week.
Don't take too long over you replies: your immediate is best.

D	A		D	A	
		I feel tense or 'wound up':			**I feel as if I am slowed down:**
	3	Most of the time	3		Nearly all the time
	2	A lot of the time	2		Very often
	1	From time to time, occasionally	1		Sometimes
	0	Not at all	0		Not at all
		I still enjoy the things I used to enjoy:			**I get a sort of frightened feeling like 'butterflies' in the stomach:**
0		Definitely as much		0	Not at all
1		Not quite so much		1	Occasionally
2		Only a little		2	Quite Often
3		Hardly at all		3	Very Often
		I get a sort of frightened feeling as if something awful is about to happen:			**I have lost interest in my appearance:**
	3	Very definitely and quite badly	3		Definitely
	2	Yes, but not too badly	2		I don't take as much care as I should
	1	A little, but it doesn't worry me	1		I may not take quite as much care
	0	Not at all	0		I take just as much care as ever
		I can laugh and see the funny side of things:			**I feel restless as I have to be on the move:**
0		As much as I always could		3	Very much indeed
1		Not quite so much now		2	Quite a lot
2		Definitely not so much now		1	Not very much
3		Not at all		0	Not at all
		Worrying thoughts go through my mind:			**I look forward with enjoyment to things:**
	3	A great deal of the time	0		As much as I ever did
	2	A lot of the time	1		Rather less than I used to
	1	From time to time, but not too often	2		Definitely less than I used to
	0	Only occasionally	3		Hardly at all
		I feel cheerful:			**I get sudden feelings of panic:**
3		Not at all		3	Very often indeed
2		Not often		2	Quite often
1		Sometimes		1	Not very often
0		Most of the time		0	Not at all
		I can sit at ease and feel relaxed:			**I can enjoy a good book or radio or TV program:**
	0	Definitely	0		Often
	1	Usually	1		Sometimes
	2	Not Often	2		Not often
	3	Not at all	3		Very seldom

Please check you have answered all the questions

Scoring:
Total score: Depression (D) _____ Anxiety (A) _____
0-7 = Normal
8-10 = Borderline abnormal (borderline case)
11-21 = Abnormal (case)

Appendix 2.6: Epworth Sleepiness Scale Questionnaire

Epworth Sleepiness Scale[11]

How likely are you to nod off or fall asleep in the following situations, in contrast to feeling just tired? This refers to your usual way of life in recent times.

Even if you haven't done some of these things recently, try to work out how they would have affected you. It is important that you answer each question as best you can.

Use the following scale to choose the most appropriate number for each situation.

	Would never nod off 0	Slight chance of nodding off 1	Moderate chance of nodding off 2	High chance of nodding off 3
Sitting and reading				
Watching TV				
Sitting, inactive, in a public place (e.g., in a meeting, theater, or dinner event)				
As a passenger in a car for an hour or more without stopping for a break				
Lying down to rest when circumstances permit				
Sitting and talking to someone				
Sitting quietly after a meal without alcohol				
In a car, while stopped for a few minutes in traffic or at a light				

Add up your points to get your total score. A score of 10 or greater raises concern: you may need to get more sleep, improve your sleep practices, or seek medical attention to determine why you are sleepy.