The role of inflammation, gut permeability and body weight in asthma: from molecular to human prebiotic interventions

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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:

- Patients with difficult to control asthma (DCA) were recruited as a part of the WATCH study by clinicians in Southampton. Participants visit, anthropometric measurement, lung function and analysis of blood cytokines were conducted by clinicians in Southampton and made available on a database which the lab group were granted access to.
- Nuclear magnetic resonance (NMR) analysis was conducted by Dr. Elena Hunter's team and results were shared with the research group.

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List of Abbreviations

ACQ-6	Asthma control Questionnaire
AHR	Airway hyperresponsiveness
AT	Adipose tissue
ATP	adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
B-GOS	Bimuno-galacto-oligosaccharide
bNMR	bench top nuclear magnetic field
BSA	Bovine serum albumin
C	Control
CCL4	C-C motif chemokine ligand 4
CCL11	C-C motif chemokine ligand 11
CCL13	C-C motif chemokine ligand 13
CFU	Colony forming units
CVD	Cardiovascular disease
CPMG	Carr-Purcell-Meiboom-Gill
CRP	C-reactive protein
DCA	Difficult to control asthma
DRP1	Dynamin-related protein 1
DMEM	Dulbecco's Modified Eagle Medium
ECAR	Extracellular acidification rate
ECL	Enhanced chemiluminescence

ELISA	Enzyme-linked immunosorbent assays
ELOVL3	Elongation of very long chain fatty acids 3
FBN1	Fibrillin-1
FBS	Serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FEF_{25-75}	Forced expiratory flow at 25-75%
FEF_{75-85}	Forced expiratory flow at 75-85%
FeNO	Fractional exhaled nitric oxide
FEV_1	Forced expiratory volume in 1s
FEV6	Forced expiratory volume in 6s
FFAR	Free fatty acid receptor
FIS1	Mitochondrial fission 1
FVC	Forced vital capacity
GOS	Galacto-oligosaccharide
HADS	hospital anxiety and depression scale
HADS-A	hospital anxiety and depression scale-anxiety subscale
HADS-D	hospital anxiety and depression scale-depression subscale
HARQ	Hull airways reflux questionnaire
^{1}H-bNMR	proton benchtop nuclear magnetic resonance
HDL-C	High-density lipoprotein cholesterol
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
Hr	Hour

HRP	Horseradish Peroxidase
IL	Interleukin
LDL-C	Low-density lipoprotein-cholesterol
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein 1
MFN2	Mitofusin-2
mtSOD2	mitochondrial superoxide dismutase 2
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGAL	Neutrophil gelatinase-associated lipocalin
NMR	Nuclear magnetic field
NRF1	Nuclear respiratory factor 1
OCR	Oxygen consumption rate
OPA1	Optic atrophy 1
OrthoPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal Component Analysis
PEF	Peak expiratory flow
Pen-strep	penicillin-streptomycin-Glutamine
PGC1α	Peroxisome proliferator-activated receptor gamma
ppm	Part per million
POLG	DNA polymerase subunit gamma
qRT-PCR	quantitative real-time polymerase chain reaction

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
SNOT-22	Sino-nasal outcome test
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
Treg	T regulatory cells
TSP	Trimethylsilylpropanoic acid
UCP-1	Uncoupling protein 1
VAT	Visceral adipose tissue
WAT	White adipose tissue
WATCH	Wessex AsThma CoHort of difficult asthma
WBC	White blood cell

WCA	well controlled asthma
WET	Water suppression Enhancement though T1
β-ME	β-mercaptoethanol

List of figures

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List of published abstracts

Parts of this thesis have been published as reviewed abstracts and presented at various conferences:

- **Parenti, C.**, Lad, N., Williams, N. C., Sharpe, G. R., Nelson, C. P., Murphy, A. M. & McTernan, P. G. (2021). Lipocalin 2, a mediator or marker of adipocyte dysfunction? Presented at Society for Endocrinology BES 2021, Edinburgh, UK. Endocrine Abstracts 77, P50.

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Abstract

Asthma is an inflammatory disease of the airways affecting 5.4 million people in the UK. Furthermore, obesity increases the risk of developing asthma is associated with worse symptoms and less response to medication. Both asthma and obesity are characterised by increased systemic inflammation that may be exacerbated by poor gut barrier integrity. Increased gut permeability allows endotoxin such as lipopolysaccharide (LPS) to translocate into the blood stream further increasing inflammation and indirectly exacerbating asthma symptoms. Common strategies to treat asthma are limited to targeting symptoms, rather than of the underlying inflammation. Novel strategies that may help ameliorate inflammation are short chain fatty acids (SCFA), end-products of gut bacteria fermentation of dietary substrates such as prebiotics, which appear to have anti-inflammatory properties and provide benefit at molecular and systemic level.

Therefore, a series of studies have been conducted to evaluate whether SCFA (acetate, butyrate, propionate) can mitigate LPS associated damage in organelles that leads to inflammation (mitochondria) at cellular level. Then, a human prebiotic intervention trial, which could indirectly increase circulating SCFAs level, was conducted to explore whether nutritional supplementation could be effective in reducing asthma symptoms and inflammatory, metabolic and permeability markers. Moreover, the effect of obesity and gut permeability on asthma severity was evaluated in cohorts of patients across asthma disease severity. Finally, to support future disease diagnosis a pilot study was conducted to explore difference in metabolic profiling between patients with and without asthma.

The outcome of these studies demonstrates that SCFA limit LPS induced damage in airway cells. The administration of prebiotics in adults with well-controlled asthma revealed a potential improvement in markers of metabolic health and inflammation. Furthermore, patients with obesity and asthma exhibit greater gut permeability which may represent a contributing factor to disease severity. Finally, through a metabolomic pilot study this thesis has revealed a potential metabolite profile that contributes to cluster separation between asthma and healthy controls.

Taken together the data suggested that increasing circulating SCFA via administration of prebiotic may provide an adjunctive tool to reduce inflammatory and metabolic damage at cellular level and in patients with asthma. Moreover, evaluation of the impact of obesity and gut permeability in asthma suggested that a broader range of approaches are needed to better characterize the pathophysiology of disease and improve future diagnosis and treatment.

Chapter 1: General Introduction

1.1 Introduction and Rationale

Asthma is a chronic inflammatory disease affecting over 262 million people around the world (World Health Organization 2023). In the U.K., it affects 5.4 million people with an estimated cost for the national health service (NHS) of around 1 billion per year (NHS England 2020). It can develop at any stage during life with the majority of people developing asthma before the age of 40 (To et al. 2012). Common symptoms include airways hyperresponsiveness, wheezing and chest tightness. These symptoms can normally be controlled with the use of asthma medications such as corticosteroids and actin β-agonist. However, these treatments are limited to relieve symptoms and long-term use can lead to side effects (Cates et al. 2014; Nelson 2006).

Several triggers can provoke asthma attacks including antigens, cold air, pollen and exercise. Between others, obesity has been recognized as a risk factor for asthma (Peters, Dixon, and Forno 2018; Wong, Forno, and Celedón 2022). Evidence highlight the presence of an alternative phenotype of asthma driven by obesity and characterized by more severe symptoms and low-grade inflammation (Lugogo, Kraft, and Dixon 2010). Moreover, these patients are less responsive to traditional asthma medications, which may further aggravate asthma condition. Therefore, there is a necessity to develop novel therapeutic strategies to prevent symptoms by reducing inflammation at the source rather than just ease symptoms.

The gut microbiota has been recognized as a potential link between obesity and asthma diseases (Gomez-Llorente et al. 2017). Gut bacteria play an essential part in developing immunity and digesting non-digestible dietary substrates (Maslowski et al. 2013).

Dysregulation in gut microbiota, noted in obesity, can lead to reduction of microbial diversity and an increase of proinflammatory bacterial species. This altered microbiota composition leads to lack of protection from pathogenic species which can directly contribute to the disruption the gut barrier, a semi-permeable structure that allow uptake of nutrient and limit access to pathogenic bacteria and molecules (Shu et al. 2023). Alteration of gut barrier, noted as gut permeability, can lead to a translocation of gut derived endotoxin such as lipopolysaccharides (LPS) in the gut mucosa. This can activate the immune cells leading to a proinflammatory cascade that can increase inflammation and as a consequence exacerbate the disease. Several evidence reported an association between obesity and gut permeability (Genser et al. 2018; Patterson et al. 2016; Portincasa et al. 2022). However, limited studies explored the effect of gut permeability in asthma (Benard et al. 1996; Walker et al. 2014) and its not clear whether it plays a role in the severity of the disease. This highlights the need for further studies exploring the effect of the gut barrier impairment on the severity of asthma in order to gain a better understanding of the disease pathophysiology.

At molecular level, mitochondria, a key organelle for cell health, have recently been associated with both asthma and obesity (Bhatraju and Agrawal 2017a). Mitochondria play an important role in the cell acting as a main producer of energy, sensor of threats and stress modulators. Evidence suggest a role of gut microbiota in mitochondria function. The endotoxin LPS appear to negatively affect mitochondria function leading to excessive production of reactive oxygen species (ROS) and down-stream inflammation. On the contrary, the short chain fatty acids (SCFA), end-product of

bacteria fermentation from dietary substrates such as prebiotics, have been shown to beneficially affect mitochondria function by preventing its structural damage and regulating energy homeostasis (Donohoe et al. 2011b; Hu et al. 2020). Dysregulation of mitochondria have been associated with altered metabolic activity, such as insulin resistance and dyslipidaemia which is characteristic of obesity (Ahmed, Sultana, and Greene 2021; Gérard 2016) and recently found associated in patients with asthma (Cottrell et al. 2011a; Gulcan et al. 2009), which may further exacerbate disease. Thus, strategies that increase circulating level of SCFA such as the use of dietary supplementation (e.g. prebiotic), may help to reduce inflammation and ameliorate metabolic health.

Together with altered gut permeability, airway inflammation and mitochondria dysfunction, asthma disease has been correlated with a distinct metabolomic profile compared to healthy controls (Jung et al. 2013; Kelly et al. 2017; Motta et al. 2014), which highlight the complexity of the disease. Metabolomics is the systematic analysis of small molecules including amino acids, carbohydrates and lipids. It represents a powerful tool to increase knowledge of pathological mechanism of disease and improve diagnosis. Current common techniques use high field nuclear magnetic field (NMR) or mass spectroscopy, however the complexity of the equipment's and the high maintenance cost make their use in clinical setting difficult. Therefore, alternative user-friendly techniques and reduced cost (e.g. bench top NMR) require more studies to expand their use in clinical setting and improve future diagnosis.

Considering the essential role of gut microbiota in immune system, it is plausible to assume that favourable manipulation of gut bacteria may have a beneficial effect on disease pathogenesis. Previous evidence in murine and human studies found altered gut and airway microbiota associate with airway diseases including asthma (Hilty et al. 2010; Pragman et al. 2012) as well as altered gut permeability (Benard et al. 1996; Walker et al. 2014). Dietary manipulation of gut microbiota with the use of prebiotics have been demonstrated to be beneficial in ameliorating asthma symptoms and reducing inflammation (McLoughlin et al. 2019a; Williams et al. 2016a). Moreover, recent studies highlight a favourable effect of prebiotics in reducing gut permeability (Krumbeck et al. 2018; Parnell, Klancic, and Reimer 2017a).

Considering the promising initial results shown by previous researches, it would be beneficial to conduct further studies to better understand the effectiveness of manipulating the gut microbiota in improving asthma symptoms, as well as their impact on inflammation, metabolic health and gut permeability.

1.2 Research aims

The purpose of these studies was to:

a) **To assess the efficacy of SCFA in limiting LPS derived damage in airway epithelial cell line.**

The research aim was:

- Evaluate the role of SCFA in preventing mitochondria dysfunction following LPS treatment.

b) Explore the effect of obesity in asthma severity and evaluate whether circulating levels of gut permeability markers differ between the different asthma cohort considered.

Research aims were to:

- Characterize a cohort of patients with difficult to control asthma across a range of BMI, focusing on the impact of obesity on asthma;
- Evaluate differences in concentrations of gut permeability markers in the severity of asthma by investigating cohorts of patients with varying severity of asthma.

c) A pilot study investigating potential metabolic differences between asthma and healthy controls using low-field bench top NMR (bNMR) technique and high field NMR.

Research aim was to:

- Establish if bNMR can discriminate differences in metabolomic profiles between adults with well-controlled asthma and healthy controls compared to high field NMR.

d) Previous research has demonstrated the efficacy of prebiotic administration to ameliorate asthma symptoms and reduce inflammation in EIB participants who undergo physical challenge (Williams et al. 2016)**. Therefore, a double blinded**

placebo controlled randomized study was conducted to evaluate the efficacy of prebiotic administration on asthma, inflammation, and gut permeability in a cohort of adults with well-controlled asthma at resting condition.

Research aims were to evaluate:

- The efficacy of prebiotic supplementation in ameliorating lung function and asthma control;
- If prebiotic B-GOS can favourably affect markers of metabolic health and inflammations;
- The use of B-GOS in strengthening gut barrier through evaluation of markers of gut permeability.

Chapter 2: Review of the Literature

2.1 Asthma pathophysiology

A key feature of asthma is airway hyperresponsiveness (AHR), an over-reaction from the airways to narrow in response to stimuli that would not cause a response in healthy adults. Common triggers for AHR are environmental irritants, cold air, dust, exercise, or allergens. As a result of AHR, bronchoconstriction of the airways occurs as well as an increased production of mucus, airway inflammation and a number of symptoms characteristic of asthma disease including wheezing, chest tightness, breathlessness, and intermittent coughing. Controlling the symptoms with appropriate asthma medication is essential to avoid severe symptoms. If left uncontrolled, a process noted as airway remodelling can occur, which cause structural changes of the airways. Common features of airway remodelling are changes in thickness, mass and volume of cells that compose the airway wall (Saglani and Lloyd 2015). These changes make the airways less flexible, increasing the severity of airway obstruction and AHR. If not treated in time this can progress, leading to permanent airway narrowing, and patients can become less responsive to medication (Postma and Rabe 2015). Patients with severe symptoms are often referred to as having difficult to control asthma, defined as a condition that remain problematic even if treated with high dose inhaled corticosteroids therapies and/or use of frequent oral corticosteroids (British Thoracic Society (BTS) 2019). Characteristics of patients with difficult to control asthma are frequent exacerbations, poor quality of life and elevated morbidities (Sastre et al. 2016a).

Therefore, it is essential to control the pathophysiology of asthma to prevent AHR and airway remodelling, and to ameliorate symptoms.

2.1.1 Phenotypes of Asthma

Asthma diagnosis can be classified as one of two different phenotypes: 'T helper-2 (Th-2)-high' or 'Th-2 low', based on the inflammatory response present in the patient (**Fig. 2.1.1.1**).

The conventional form of asthma is noted as 'Th-2 high' phenotype, or eosinophilic asthma, due to the elevated level of circulating eosinophils in the blood stream. The Th-2 high asthma is associated with high level of Th-2 cytokines (e.g. Interleukin (IL)-4, IL-5, IL-13), elevated sputum eosinophil count, high synthesis of immunoglobulin E (IgE), mucus overproduction and high level of fractional exhaled nitric oxide (FeNO) (Hinks, Levine, and Brusselle 2020; Lambrecht, Hammad, and Fahy 2019). It generally develops during childhood, has a strong genetic influence and patients respond well to medications.

The 'Th-2 low' asthma phenotype is characterized by normal levels of eosinophil blood counts (<2%) and can be further divided into neutrophilic and paucigranulocytic subtypes. Neutrophilic asthma is characterized by high circulating levels of neutrophils and low FeNO while paucigranulocytic asthma exhibits normal levels of eosinophils and neutrophils (Lad et al. 2021).

The most prevalent phenotypes of asthma are eosinophilic and paucigranulocytic. A previous study conducted in a large cohort of 508 patients with asthma found that 42% had eosinophilic asthma, 40% paucigranulocytic and 16% neutrophilic (Schleich et al. 2013). Although neutrophilic asthma is less common, it is often characterized by more severe symptoms and a poor response to traditional asthma medication (Wadhwa et al. 2019). Neutrophilic asthma often develops during adulthood, also noted as late-onset

asthma, and lifestyle factors such as exercise, weight gain and environment are being recognized as potential triggers (Bantulà et al. 2021a; Peters et al. 2018). There is no cure for asthma, but patients with 'Th-2 high' phenotype are generally well responsive to asthma medications such as corticosteroids that keep the diseases under control by reversing airway narrowing and reducing inflammation.

Common asthma medications include inhaled corticosteroids, leukotriene modifiers and short and long-actin β_2 agonists. Corticosteroids and leukotriene modifiers work by reducing the airway inflammatory response induced by leukocytes, such as eosinophils. (Chauhan and Ducharme 2012). The short and long acting β_2-agonists are bronchodilators and work by relaxing the smooth airway cells and widening the airways providing immediate relief (National Institute for Health and Care Excellence (NICE) 2023). However, persistent use of asthma medication can lead to side effect such as headache, tachycardia and muscle cramps, and increased reliance (Cates et al. 2014; Nelson 2006). Current medications focus on reducing, rather than preventing, symptoms. Therefore, there is need for alternative therapies that focus on the prevention both the flaring up of symptoms and the development to a severe form of asthma.

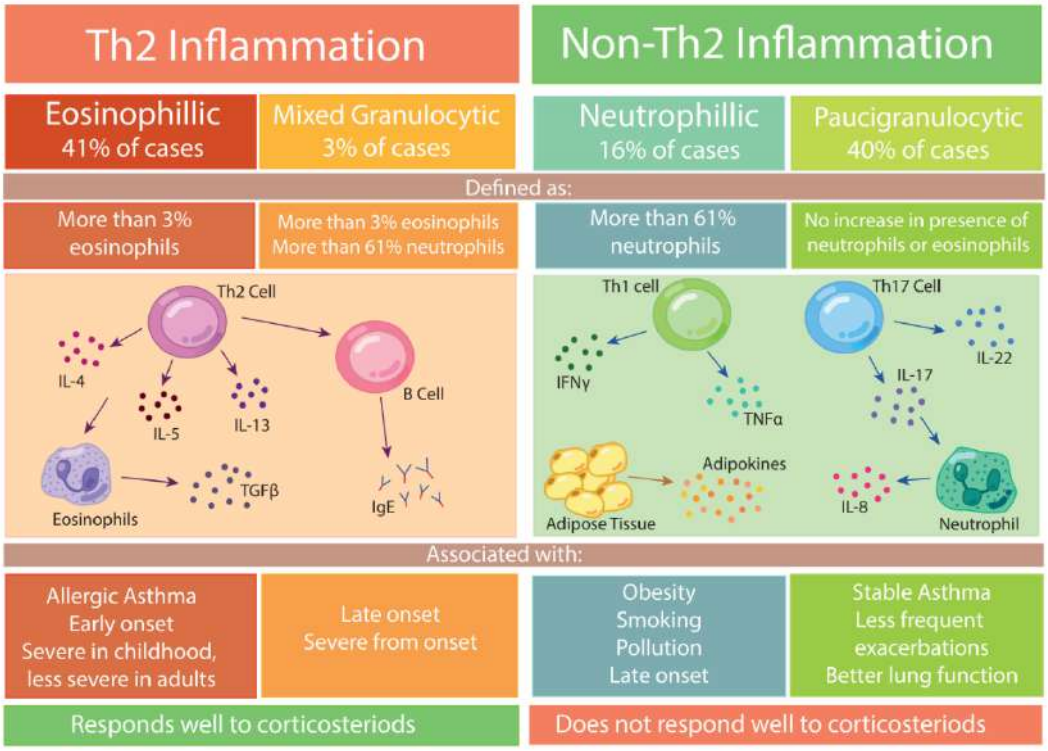

Fig. 2.1.1.1. The phenotypes of Asthma.

The asthma phenotypes classification can be divided based on inflammatory response into T helper cell 2 (Th-2) or non-Th2. The Th-2 inflammatory response or 'Th-2 high' can be eosinophilic (41%) or mixed granulocytic (3%). It is mainly associated with allergic asthma and early onset. The non th-2 inflammation or 'Th-2 low' can be neutrophilic (16%) or paucigranulocytic (40%) and it is associated with late onset. Neutrophilic asthma is associated with lifestyle factors such as obesity and smoking and less response to traditional asthma medication. (Figure from (Lad et al. 2021)).

2.2 Asthma comorbidities: Obesity

Asthma is often associated with other comorbidities that can worsen the condition, including other respiratory diseases (e.g. rhinitis, chronic obstructive pulmonary disease (COPD)), cardiovascular disease, anxiety, and metabolic conditions such as obesity. Patients with obesity appear to develop Th-2 low asthma, mediated by neutrophilic inflammation, and characterized by more severe symptoms, enhanced hospitalization, and poor response to asthma medication (Lugogo et al. 2010).

2.2.1 Obesity

Obesity is a global epidemic affecting over 650 million people worldwide, almost 13% of the global population (World Health Organization, 2020). Since 1975, the obesity prevalence almost tripled worldwide (OECD 2014) and according to recent projections from the World Obesity Atlas, almost 2 billion people will live with obesity by 2035 (World Obesity Atlas 2023). According to these projections, particular concern is observed for United states, Mexico, and U.K. where 58%, 47% and 46% of the population are expected to be obese by 2035, respectively. Moreover, a major study published by the Lancet reported that obesity is responsible for 4.77 million premature deaths worldwide every year, accounting for 8% of global deaths (Stanaway et al. 2018). Of these, about 56,000 deaths are in the U.K. where obesity represents the 4th cause of premature death after smoking, high blood pressure and high sugar intake (Stanaway et al. 2018). The NHS estimated a total annual cost of about £6 billion to treat overweight-obese associated illness and this cost is projected to rise to £9.7 billion by 2050 ("Health Matters: Obesity and the Food Environment - GOV.UK" 2021). The U.K. hospitalization rate directly associated to obesity was over 11,000 in 2019, with an increase of 19%

compared to 2013 (NHS Digital 2020). In the same year, the number of hospitalizations with obesity as a cofactor rose to above 800,000, an increase of +140% compared to the hospitalization rate in 2013 (NHS Digital 2020). This increasing prevalence results in a large economic burden for the healthcare system.

2.2.2 Pathophysiology of obesity

Obesity is defined as an excessive fat accumulation that present risk to health (World Health Organisation (WHO), 2024). It is characterized by low-grade inflammation and as a risk factor of many diseases including diabetes, cardiovascular diseases and metabolic syndrome (Bastien et al. 2014; Frydrych et al. 2018; Ng et al. 2021). Body Mass Index (BMI), calculated by dividing a person's weight by their squared height (expressed in units of kg/m^2), is used to diagnose obesity, with a BMI of more than 30 kg/m^2 considered obese. However, there are several limitations to using only BMI to diagnose obesity, since it does not distinguish between lean and fat mass and does not correlate with visceral fat accumulation. In addition to BMI, the national institute for health and clinical excellence (NICE) recommends the use of waist circumference measurement, indicator of abdominal visceral fat and assessed by placing a tape halfway between the lower rib and the iliac crest of the abdomen, to improve evaluation and diagnosis of obesity. According to the world health organization (WHO) and NICE, the combined use of BMI and waist measurement, helps to estimate the increased health risk of developing diseases including type 2 diabetes, hypertension or heart disease **Table 2.2.2.1** (NICE 2022; World Health Organisation (WHO) 2008).

In most cases, the cause of obesity relies on prolonged excessive calorie intake and low physical activity. Regarding the nutritional aspect, Western diets, noted obesogenic, are

characterized by the consumption of high sugar and fat meals, ultra-processed food, and low consumption of fruit and vegetables and strongly associated with obesity and relative metabolic disorders (Ludwig, Peterson, and Gortmaker 2001; Rouhani et al. 2014). Whilst, sedentary lifestyle contributes to increased weight gain, several studies indicate that physical activity alone is not enough to prevent weight gain (Lee et al. 2010; Moholdt et al. 2014). For examples, a 13-year long study reported that women with a high BMI who had regular physical exercise but no calories restriction in their diet, did not experience weight loss (Lee et al. 2010).

A combined action of healthy balanced diet and physical activity is the most effective way to prevent weight gain and developing obesity and associated metabolic disease.

Table 2.2.2.1. Increased health risk based on combined use of BMI and waist circumference.

Adults with lean BMI (<24.9 kg/m^2) showed no increased risk of developing obesity associated disease, independently of waist circumference. Adults with overweight BMI (25-29.9 kg/m^2), but waist circumference ≤94 cm for man (M) and ≤ 80 cm for women (W) have no increased risk of developing obesity associated disease. Adults with overweight BMI and waist circumference of 94-102 cm or ≥ 102 cm for M, and 80-88 cm or ≥ 88 cm for W, have an increased or high risk of developing obesity associated diseases, respectively. Adults with obesity (BMI ≥30 kg/m^2) but waist circumference ≤94 cm for M, and ≤ 80 cm for W, do not have increased risk of developing obesity associated diseases. Adults with BMI<30 and high (94-102 cm-M; 80-88 cm-F)- or very high (≥ 102 cm-M; ≥ 88 cm-W) waist circumference have a high risk and very high risk to develop obesity associated diseases, respectively (World Health Organisation (WHO) 2008).

BMI (kg/m^2)	BMI classification	Desirable waist circumference (cm)		High waist circumference (cm)		Very High waist circumference (cm)	
		Men	Women	Men	Women	Men	Women
		≤94	≤80	94-102	80-88	≥102	≥88
BMI 18.5-24.9	Lean	No increased risk		No increased risk		No increased risk	
BMI 25-29.9	Overweight	No increased risk		Increased risk		High risk	
BMI ≥30	Obese	No increased risk		high risk		Very high risk	

2.3 The obesity-asthma link

Obesity increases the risk of developing asthma in both adults and children (Peters et al. 2018; Reyes-Angel et al. 2022; Wong et al. 2022). In the USA, 11% of adults with obesity have asthma compared to 7% asthma prevalence in adult with lean BMI (National Centre for Health Statistics 2016). This association is even higher for women with obesity, with 15% suffering with asthma compared to 7% of women with lean BMI (National Centre for Health Statistics 2016). In addition, symptoms in females are more severe than in males resulting in a poorer quality of life, with females three times more likely to be hospitalized for asthma than males (Schatz, Clark, and Camargo 2006). Although numerous studies have been conducted to explain this phenomenon, the underlying mechanism has not yet been clarified. Both genetic and hormonal differences between the sexes have been proposed as possible reasons for this difference (Pignataro et al. 2017).

The association between obesity and asthma appears to be related to systemic chronic inflammation from increased adiposity. The adipose tissue is recognised as an organ that directly contributes to systemic inflammation by secreting inflammatory cytokines such as Interleukin (IL)-6 and Tumor necrosis factor alpha (TNF-α). This is exacerbated further through the secretion of adipokines leptin and adiponectin, which are noted markers of systemic inflammation in addition to their role as hormones. For instance, as a hormone, leptin regulates food intake and energy expenditure by acting on the central nervous system (Stofkova 2009), whilst as an adipokine it induces adipocytes to release pro-inflammatory cytokines such as TNF-α, IL-6, IL-12 and stimulates the activity of th-1 and th-17 cell (Cava & Matarese, 2004; Lord et al. 1998; Martín-Romero et al., 2000; Yu et

al., 2013). On the contrary, adiponectin has anti-inflammatory properties by inhibiting the production of proinflammatory cytokines such as TNF-α, interferon-gamma (INF-γ) and IL-6 (Masaki et al. 2004; Wolf et al. 2004), yet also has a hormonal role in the regulation of glucose level and lipid metabolism (Wada et al. 2022).

Leptin and adiponectin have both been shown to have a relationship with asthma. Increased leptin concentrations were found in a cohort of adolescents with asthma and associated with poor lung function, in particular reduced levels of forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC) and FEV_1/FVC Ratio (Huang et al. 2017). In adults, analysis of visceral fat from patients with asthma revealed elevated levels of leptin associated with AHR (Sideleva et al. 2012). This suggests a major role of leptin in the obese-asthma phenotype.

Patients with obesity showed reduced levels of circulating adiponectin, which was associated with increased levels of proinflammatory cytokines such as IL-6, TNF-α (Achari & Jain, 2017; Esser et al., 2014). In addition, weight loss is associated with increased levels of adiponectin and higher concentrations of anti-inflammatory cytokine IL-10 (Porter Starr et al. 2019; Takao et al. 2021). Although there is limited data on adiponectin levels in asthma patients, children with severe asthma have low levels independent of BMI (Dogru et al. 2015). However, in contrast a recent meta-analysis of 13 studies revealed that lower adiponectin was associated with asthma in adults but not children (Zhang et al. 2017). Further research and understanding of the role of adiponectin in asthma therefore is needed.

2.3.1 Lung mechanics change in obesity

Obesity induces changes in lung mechanics due to the expansion of the adipose tissue that accumulates around the thoracic and abdominal cavities, increasing pressure on the diaphragm and limiting movement of the chest wall, resulting in compression of the lungs (Dixon and Peters 2018). Theses changes can affect lung function, although findings differ as to whether this is associated with BMI or adipose tissue distribution. Previous studies have highlighted a reduced lung function in patients with obesity (Ciprandi et al. 2014; Schachter et al. 2001), while others did not find any association with high BMI (Bildstrup, Backer, and Thomsen 2015; Tang et al. 2022). Other evidence indicated that abdominal fat distribution is more strongly associated to lung function decline than BMI (Leone et al., 2009; Ochs-Balcom et al., 2006). In fact, a large French population-based cohort study of 121,965 participants found that abdominal obesity was a risk factor for lung function decline, independently of BMI (Leone et al. 2009).

Consequently, weight loss has been associated with improvements in asthma symptomatology (Dandona et al. 2014; Dias-Júnior et al. 2014; Nyenhuis, Dixon, and Ma 2018). Therefore strategies that focus on weight reduction including dietary intervention and physical activity should be included in the therapy used for asthma management in patients with obesity.

2.3.2 Systemic inflammation in obesity and asthma

Obesity is associated with low grade inflammation that can in turn affect the severity of asthma. This can be explained in part by pro-inflammatory adipokines, such as leptin, which are released by adipose tissue and are increased during obesity as discussed in

more detail in section **2.3.** In addition, in the obese state, the adipose tissue hypertrophies and becomes infiltrated with proinflammatory macrophages, a type of white blood cell involved in the immune response, which in turn release further pro-inflammatory cytokines. In healthy conditions the number of macrophages are generally below 4%, however in the case of excess adiposity this percentage increases to 12% (Harman-Boehm et al. 2007). Raised macrophage infiltration has been reported before in patients with obesity and asthma and was associated with systemic inflammation (Sideleva et al. 2012). Macrophages drive inflammation via the activation of the nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) pathway by fatty acids, cholesterol, or oxidative stress. This induces the activation of caspase 1 which leads to the type 1 macrophage (M1) maturation. This in turn promotes the secretion of a number of pro-inflammatory cytokines including IL-6, TNF-α and cytokines of Th-17 cells response (**Fig. 2.3.2.1**). The combination of adipokines and macrophages within adipose tissue have an active role in driving systemic inflammation which contribute to many diseases including asthma (Bantulà et al. 2021a; Bastien et al. 2014; Frydrych et al. 2018; Ng et al. 2021; Peters et al. 2018). In fact, analysis of visceral adipose tissue from 12 patients with obesity and asthma revealed an increased expression of leptin and macrophages infiltration, as well as a reduction in adiponectin compared to the control group (Sideleva et al. 2012). Moreover, a murine study revealed that a 12-week high fat diet led to a natural development of the AHR response, a key feature of asthma, without previous exposure to any allergen (Hye Young et al. 2014). AHR did not develop in knock out mice who were NLRP3 deficient (*Nlrp3*$^{-/-}$), suggesting a key role of NLRP3 in the development of AHR in obesity and asthma.

In summary, the evidence suggests that both asthma and obesity are associated with greater levels of systemic inflammation, which is likely to exacerbates symptoms and make disease difficult to control.

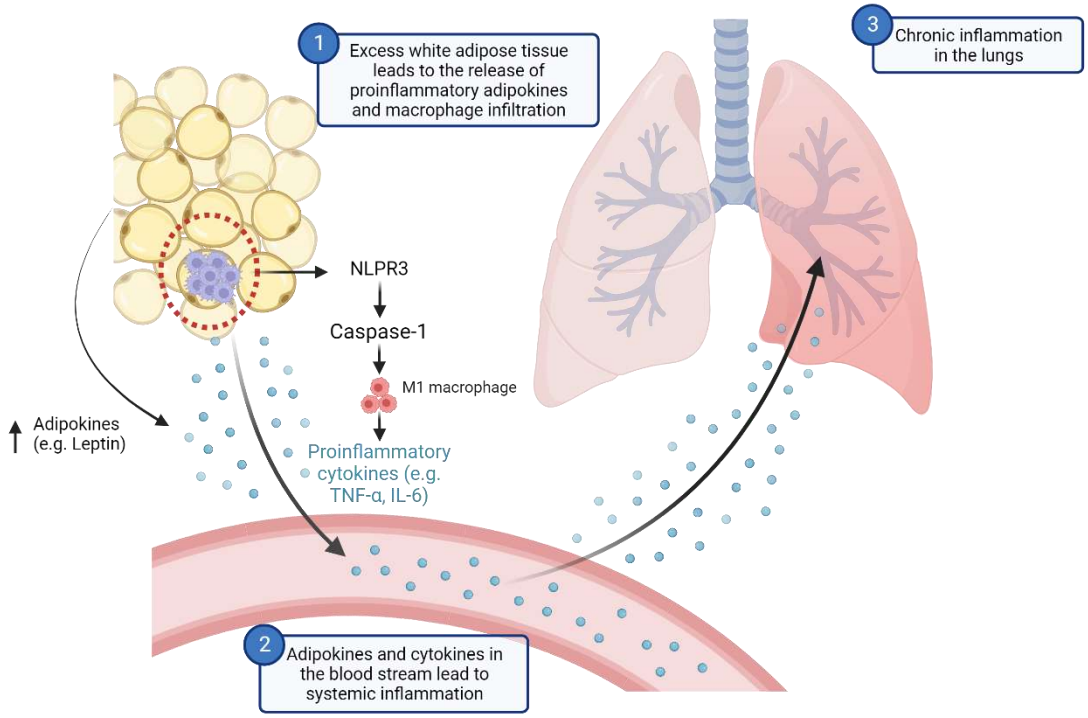

Fig. 2.3.2.1. Schematic representation of obesity-asthma inflammatory link.
Excess of adipose tissue leads to overproduction of the pro-inflammatory adipokine leptin. Additionally, adipocytes become infiltrated with macrophages which promote inflammation via activation of nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) pathway. This in turn leads to the activation of caspase 1 which promotes the maturation of macrophage (M1). This results in the production of proinflammatory cytokines that can translocate in the blood stream and increase inflammation in all organs including lungs. The combination of inflammatory cytokines and adipokines contribute to the exacerbation of disease.

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2.4 The gut-microbiota and link with asthma and obesity

The human body is home to approximately 100 trillion microbes (the human microbiota) which begin colonization of the body from the first breath at birth (Sender, Fuchs, and Milo 2016; Turnbaugh and Gordon 2009). The microbiota represent all the living

microorganisms found in a defined environment such as the gut (Hou et al. 2022). Microorganisms include bacteria, the largest population in our body, yeast, parasites, and viruses. The microbiota is part of the human microbiome. This includes the microbiota, the anatomical sites where they reside, their genome, their microbial metabolites and their surrounding environmental conditions (Berg et al. 2020). The distribution and type of microbes vary greatly between body sites, with the highest concentration of microbes present in our gastrointestinal tract (GI) (Sartor 2008). The colon hosts ~10^{12} microbes, predominantly composed by *Bacteroides*, *Enterobacteriaceae* and *Bifidobacterium*, while the stomach hosts only ~10^{2} microbes divided between *Lactobacillus*, *Candida*, *Streptococcus* and *Helicobacter Pylori* (Sartor 2008).

The gut microbiota has an active role in host health contributing to the formation and education of our immune system, maintaining host energy balance, and digesting non-digestible carbohydrates (Maslowski et al. 2013). Previous studies in germ free (GF) mice, animals with no microorganisms living on or in them, raised in a sterile environment revealed that they had underdeveloped immune system, defects in the gut-associated lymphoid tissue (GALT), a reduced response to pathogens and a consequent susceptibility to infections (Kamada and Núñez 2014; Khosravi et al. 2014; Mazmanian et al. 2005; Round and Mazmanian 2009). GF mice infected with the pathogen *Listeria monocytogenes* failed to develop an immune response to the pathogen. However, following inoculation of complex microbiota, mice were able to restore defect in immune response and were able to develop resistance to the pathogen within 72 hr (Khosravi et al. 2014). This highlights the essential contribution of microbiota in the correct development and function of the immune system.

Alterations in the composition of the gut microbiota community may lead to a loss of protection from the beneficial bacteria, increasing circulating levels of proinflammatory species in the blood stream, which can initiate inflammation (described in further detail in **6.2.5**) (Quigley 2017). Therefore, an ability to alter the gut microbiome to a healthier state and/or reduce the type of bacterial factors entering the circulation may have a positive impact, reducing symptoms and improving the quality of life of patients with obesity and asthma.

2.4.1 Asthma related changes in gut microbiome

Recent evidence highlights that changes in the gut microbiota can influence the immune system and consequently increase susceptibility to allergic disease (Herbst et al. 2011; Hill et al. 2012; Noverr et al. 2005; Russell et al. 2012). A previous murine model demonstrated how manipulation of the commensal bacterial community with 4-weeks antibiotic treatments led to an increase of immunoglobulin E (IgE), and overproduction of Th-2 related cytokines, commonly associated with asthma (Hill et al. 2012). Limited human studies have investigated the gut bacterial alterations in asthma. A recent meta-analysis reported an increased level of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* strains in asthma and chronic obstructive pulmonary disease (COPD) and asthma compared to healthy controls (Chai et al. 2022), suggesting a potential use of microbiota as a marker for diagnosis and treatment of respiratory diseases. Another study found raised level of histamine-secreting bacteria, commonly associated with allergic reaction, in adult with asthma indicating a role of microbiota in the burden of disease (Barcik et al. 2016). Altered microbiota composition was also associated with worse lung function; in fact, researchers observed a lower abundance of

Bacteroides/Firmicutes in adults with asthma and this was strongly associated with reduced lung function, independently of BMI (Begley et al. 2018).

Diet can effectively modify the gut microbiota and affect asthma symptomatology. In an animal study, mice fed with low-fibre diet showed lower levels of circulating SCFA (acetate, 0.2-fold decrease; propionate, 0.8-fold decrease; butyrate, 0.3-fold decrease) greater concentrations of inflammatory cytokines including interleukin (IL)-4, IL-5, 1L-13 and experienced an increase in airway hyper-reactivity (Trompette et al. 2014). In contrast, mice fed with high-fibre diet reported an increase level of SCFAs (acetate, 8.35-fold increase; propionate, 1.45-fold increase; butyrate, 0.64-fold decrease) and an amelioration of asthma symptoms. The beneficial effect of three main SCFAs found in circulation (acetate, propionate, butyrate) was also demonstrated by another murine study where diet enriched with SCFAs (cocktails of 40 mM butyrate, 67.5 mM acetate plus 25.9 mM propionate) led to less severe symptoms of asthma compared to the control group (Cait et al. 2018). The low fibre diet was also associated with reduced richness of gut microbiota and an increase of *Firmicutes* strains, commonly raised in western obesogenic diet (Trompette et al. 2014). This was also found in a different study where mice were exposed to a western high-fat diet (Turnbaugh et al. 2008). In contrast, mice fed with high fibre diet showed a more diverse gut microbiota community with an increase of *Bacteroidaceae* and *Bifidobacteriaceae* strains, which have noted host health benefits (Trompette et al. 2014).

Overall, evidence indicates that gut microbiota composition is associated with respiratory disease. Although limited studies have been conducted in humans, these have shown a potential link between altered gut bacteria composition and asthma

disease. Further studies are needed to confirm these findings. Dietary manipulation of gut composition seems to have beneficial effects on asthma symptoms and therefore warrants further attention.

2.4.2 Obesity related changes in gut microbiome

Several factors can affect the gut microbiota composition such as antibiotic therapy, stress, environmental factors, weight gain and diet. Links between obesity and gut microbiota have been observed by experiments in germ free (GF) mice, where evidence revealed that GF mice were resistant to developing obesity following a western-style high-fat, high-sugar diet (Bäckhed et al. 2007; Rabot et al. 2010). Moreover, the transfer of an obesity-associated pathogen (*Enterobacter cloacae*) extracted from the gut of a volunteer and injected into a GF mouse, induced obesity, and altered glucose tolerance (Fei and Zhao 2013). Similarly, inoculation of microbiota from obese mice donors to GF mice induced an 20% increase in body fat compared to GF mice that received microbiota from lean donors (Turnbaugh et al. 2006), demonstrating the close relationship between microbiota, body fat and obesity. Finally, the microbiota of patients with obesity appear to extract more energy from food compared to the microbiota of patients who are lean, suggesting a role of microbiota in modulating fat storage (Bäckhed et al. 2007; Ridaura et al. 2014; Turnbaugh et al. 2008).

2.5 The gut epithelial barrier in health and disease

The gut epithelial barrier is an essential component of the gastrointestinal tract. It is a physical and immune barrier that allow the uptake of nutrients and immune cells while preventing the passage of pathogens. It is composed of several components: a mucus layer, a single layer of epithelial cells and the lamina propria (a thin layer of connective tissue). The mucus layer contains gut microbiota, secretory immunoglobulin A (IgA) and antimicrobial peptides (AMPs). The single layer of epithelial cells is bound together by a specific group of proteins which form tight junctions, such as claudin and occludin and desmosomes that form stronger bonds, contributing to the integrity of the gut barrier and transporting small molecules. The lamina propria contains immune cells which are involved in the immunological defence of the intestinal barrier. The structure of the gut barrier is represented in **Fig. 2.5.1**.

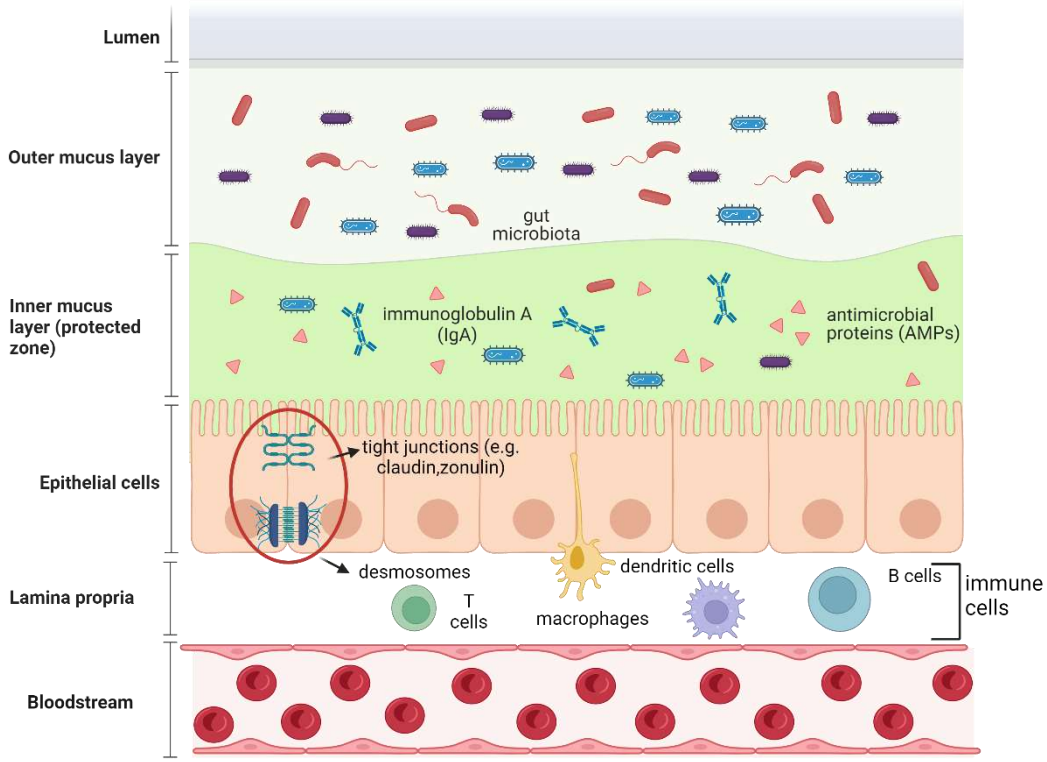


Fig. 2.5.1. Schematic representation of the gut intestinal barrier.
The gut barrier is an essential component of the gastrointestinal tract. It is a semipermeable structure and plays a key role in the uptake of nutrients and immune sensing, while being restrictive against pathogens. It is formed by a mucus layer, a single continuous layer of epithelial cells and a lamina propria underneath. The mucus layer forms a structure overlying the epithelial cells and is composed of antimicrobial peptides (AMPs), secretory IgA molecules (IgA) and gut microbiota. Underneath the mucus layer, there is a continuous monolayer of epithelial cells that is bound together by tight junction (TJ) and desmosomes. The lamina propria contains immune cells (e.g. T cells, B cells, macrophages and dendritic cells) from the adaptive and innate immune system which are essential mechanism of defence in the gut barrier.

The gut microbiota plays an essential role in maintaining the correct function of the gut barrier. In fact, commensal bacteria digest and ferment food that is not being digested by human enzymes, such as dietary fibre, and in turn release anti-inflammatory compounds noted as short chain fatty acids (SCFA). In addition, commensal bacteria play a role of defence against proinflammatory species by competing for nutrients and adhesion sites. Moreover, they can actively eliminate pathogens by releasing

antimicrobial peptides (AMPs), small molecular peptides that contribute to host immune defence, or by releasing a particular virulent factor noted as fucose that limit pathogen reproduction and help to keep the gut barrier intact (Kamada et al. 2013). Finally, some bacteria species such as *Escherichia coli* contribute to strengthening the epithelial barrier by releasing specific signals called indoles which enhance the production of tight junctions (Bansal et al. 2010). In this condition, there is very little bacteria translocation, and that which does occur is rapidly contained by immune cells.

Factors such as diet, antibiotic use and stress can contribute to shift the gut microbiota towards a pathogenic predominance (Bokulich et al. 2016; Doré and Blottière 2015; Karl et al. 2018). Alteration in the gut microbiota towards pathogenic species can lead to prolonged inflammation around the gut barrier. This leads to a disruption in the tight junctions, creating holes around the barrier and compromising its integrity, in a condition noted as 'leaky gut' syndrome (Quigley 2017). Consequently, molecules such as lipopolysaccharides (LPS), an endotoxin located on the surface of gram-negative bacteria, translocate underneath in the submucosal compartment. Here, LPS can activate immune cells such as immune cells which in turn release inflammatory cytokines. Initially, the inflammation is contained in the submucosa, but over time LPS can enter the circulation, turning the inflammation from local to systemic (**Fig. 2.5.2**) (Quigley 2017). In particular, LPS has been recognized to have a key role in systemic inflammation and indirectly in disease decline. LPS is a glycolipid composed of Lipid A, a core oligosaccharide and a distal O-antigen. Lipid A represents the hydrophobic portion of the molecule, it is located in the outer part of gram-negative bacteria, and is responsible for its toxic activity. It contributes to inflammation via interaction with toll like receptor (TLR) 4 that lead to a cascade signalling pathway increasing production of

pro-inflammatory cytokines. Furthermore, binding of LPS to TLR4 can produce a cascade of signalling that reduce tight junction proteins such as zonulin and in consequence increasing gut permeability, promoting the translocation of more endotoxin into the gut mucosa (Tulkens et al. 2020).

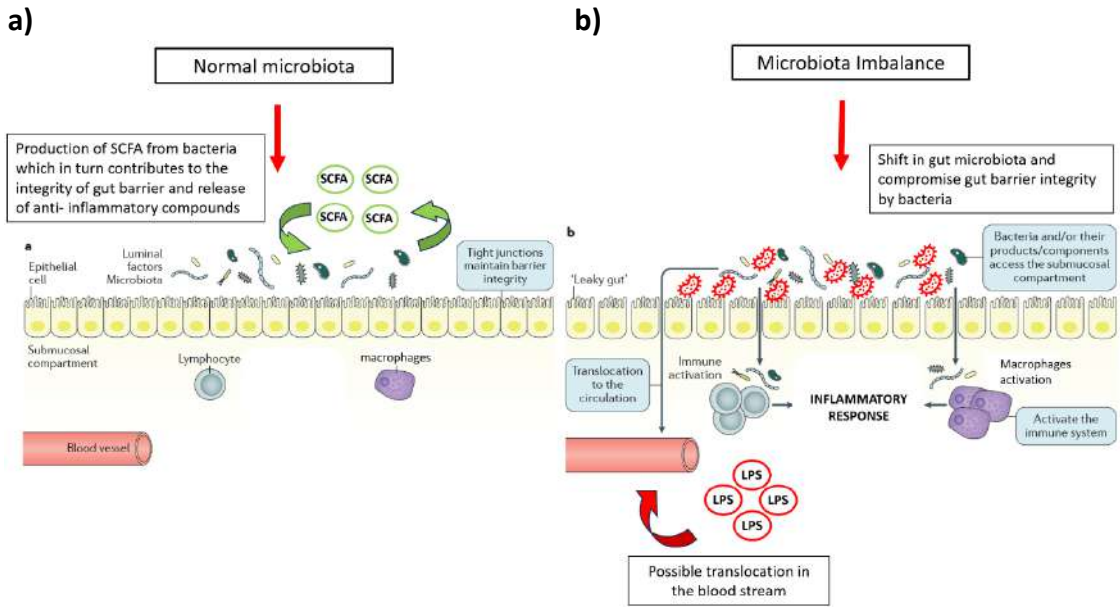


Fig. 2.5.2. Comparison between integral gut barrier a) and impaired gut barrier b).
a) In normal condition the gut barrier is intact, meaning that the monolayer of epithelial cells are tightly attached one to another by tight junction. This prevents translocation of bacteria and/or bacterial components such as LPS into the lamina propria compartment where immune cells are. In this condition there is a balance between commensal and pathogenic species. Commensal bacteria digest non-digestible food such as dietary fibre and produce anti inflammatory compounds noted as short chain fatty acids (SCFA). This in turn provide a range of functions such as strengthening the gut barrier, regulation of immune response and production of antimicrobial peptides (AMPs). Contributing to the correct function of gut barrier.
b) Impairment of gut barrier is characterized by disruption of tight junction integrity which allow bacterial component from altered gut microbiota such as lipopolysaccharides (LPS) to translocate in the lamina propria where activate an immune response, leading to the inflammatory response. If this condition is prolonged over time, LPS can translocate in the systemic circulation and turn inflammation from local to systemic. (Figure adapted from adapted from (Quigley, 2017).

2.5.1 Asthma and gut permeability

To date, there is some limited evidence of impaired gut barrier integrity in patients with asthma. Histological analysis of duodenum intestinal mucosa from patients with bronchial asthma showed a partial atrophy of intestinal villi, small projections that extent on the inner lining of the small intestine and play an essential role in the absorption of nutrient, and an increased infiltration of lymphocytes and eosinophils in the lamina propria compared to the control group (Wallaert et al. 1995). Limited human studies confirmed this view *in vivo*. Altered gut permeability in adults with asthma was reported in a group of 37 patients with moderate asthma which showed elevated level of gut permeability assessed with the use of ethylenediaminetetraacetic acid (CrEDTA) urinary recovery technique, which should reflect the damage of gut mucosa (Benard and Desreumeaux 1996). Similar studies in children and adults with asthma reached the same conclusions (Hijazi et al. 2004; Walker et al. 2014).

Researchers suggested that there may be a defect of the gut mucosa in these patients, but it is not clear if this is a cause of consequence of asthma. Therefore, longer and larger studies are needed to confirm these findings. Whilst studies have suggested increased gut permeability in patients with asthma, there have been limited to no association made to asthma severity assessed measuring lung function, or disease control (Hijazi et al. 2004; Walker et al. 2014).

2.5.2 Markers of gut permeability and asthma

Limited studies have measured biomarkers of gut permeability in asthma. Between others, the biomarkers LPS, LBP, zonulin and the gut inflammatory marker calprotectin have been associated with asthma. Raised level of endotoxin LPS associated with enhanced airway inflammation, AHR and asthma severity (Berger et al. 2015; Curths et al. 2014; Michel et al. 1996; Ren et al. 2019). Furthermore, a murine model of LPS induced exacerbation revealed that these mice had reduced response to corticosteroids, a traditional asthma medication, and an abnormal production of airways inflammatory cytokines including TNF-α, IL-13 (Hadjigol et al. 2020). It has been suggested that LPS is able to cause a shift of inflammatory response from eosinophilic to neutrophilic, associated with more severe asthma (Zhao et al. 2017). Beside *in vitro* and animal studies, little is known about the role of LPS in human asthma cohorts. One of the main difficulties in detecting LPS in blood is due to the low reproducibility and sensitivity of enzyme linked immunosorbent assay (ELISA) kits and high risk of contamination by endogenous endotoxins present in glassware or plastic which can lead to misleading results (Aisha Farhana; Yusuf S. Khan 2023; Stromberg, Mendez, and Mukundan 2017). However, Lipopolysaccharides binding protein (LBP) which play an important role in binding LPS to pattern recognition receptors such as tool like receptor (TLR) or cluster of differentiation 14 (CD14) and activating immune cells, (Schumann 1992; Tsukamoto et al. 2018) has been proved to be al alternative and reliable marker to identify LPS which showed high reliability re-test (Citronberg et al. 2017). Raised concentration of LBP was found in bronchoalveolar cleavage of patients with asthma (Dubin et al. 1996). Another murine study found that LBP-deficient mice did not develop airway bronchoconstriction

following allergen sensitization challenge compared to the control mice which showed a strong airway reactivity (Strohmeier et al. 2001). This suggests an involvement of LBP in asthma pathogenesis. However, studies are limited and there is currently no evidence of the role of LBP in asthma function and control. On the contrary, another marker of intestinal permeability, zonulin, was found upregulated in patients with asthma and associated with disease severity (Baioumy et al. 2021; Kim et al. 2023). However, similarly to LPS, the use of zonulin as a marker of gut permeability has been criticized before due to the non reliability of the commercially available ELISA kits. Several studies criticize the use of zonulin ELISA kits due to failing in detecting the concentration of zonulin and instead detect unknown protein levels that are structurally similar to it but currently not yet identified (Ajamian et al. 2019; Massier et al. 2021; Scheffler et al. 2018; Tatucu-Babet et al. 2020). Therefore, until new methodologies will be developed to correctly identify circulating level of zonulin, it necessary to be cautious when using zonulin as a marker of gut permeability.

Finally, a biomarker initially associated with gut impairment and recently associated with asthma severity is calprotectin. Calprotectin is a calcium and zinc protein of the S100 family, forming complex heterodimer with S100A8 and S100A9 monomers, and it is mainly secreted by neutrophil cells. It is often used as a marker of gut inflammatory disease such as bowel syndrome or colorectal cancer, which are characterized by neutrophilic cell infiltration (Kalla et al. 2016; Moris et al. 2016). In the recent years, calprotectin was also associated with asthma disease. In fact, increased serum and sputum level of calprotectin have been found in patients with asthma and associated with disease severity (Decaesteker et al. 2022; Lee et al. 2020). Moreover, a murine study showed high concentration of calprotectin correlated with the percentage of

neutrophils in the sputum, which may suggest a role of calprotectin in Th-2 low asthma phenotype (Lee et al. 2017). However, studies are limited and therefore further research is needed to further characterize the role of calprotectin in the pathology of asthma.

Evidence of gut permeability in asthma are limited but they suggest an alteration of gut barrier in these patients. Although it is not clear if this is a cause or consequence of the disease pathophysiology. Therefore, more studies with are needed to clarify the role of gut permeability in asthma.

2.5.3 Obesity and gut permeability

There are several studies that reported impaired gut barrier function in obesity (Genser et al. 2018; Pendyala, Walker, and Holt 2012; Wilbrink et al. 2020). Gastrointestinal permeability assessed with lactulose/mannitol test was associated with obese status in 27 adults patients hospitalized for a sleeve gastrectomy. However, following the surgery which resulted in significant weight loss, gut permeability was reversed to lean control levels. This suggest that the surgical procedure and/or weight loss help to improve gut barrier impairment (Wilbrink et al. 2020).

There are several blood circulating markers of gut permeability that are associated with obesity including LPS, LBP, zonulin and the gastrointestinal inflammatory marker calprotectin (Cani 2007; Genser et al. 2018; Lylloff et al. 2017; Trøseid et al. 2013). A recent study conducted in 38 patients with severe obesity revealed an elevated level of LBP in blood circulation together with zonulin, a marker of tight junction, associated with gut barrier impairment (Genser et al. 2018). Similarly, raised concentration of calprotectin was found in patients with obesity (Lylloff et al. 2017; Mortensen et al.

2009). A murine study found that calprotectin contributed to increase low-grade inflammation via interaction with TLR-4 (Nagareddy et al. 2014).

Diet can play a major role on the circulation of endotoxin in the blood stream. The first evidence of gut damage was reported in a murine study where they were fed for 4 weeks with a high fat diet (HFD) which led to a significant reduction in *Bifidobacterium* that protect the gut barrier and increased levels of circulating LPS (Cani et al., 2007). A later study investigated the effect of 15 weeks HFD in a murine model and reported elevated gut permeability in the region of jejunum and colon using a Ussing chamber technique, which measure gut barrier function of living tissue (Stenman, Holma, and Korpela 2012). The strong effect of diet in gut barrier impairment was confirmed in several human studies, which reported elevated post-prandial level of endotoxin LPS was post HF meal (Erridge et al. 2007; Harte et al. 2012; Lyte, Gabler, and Hollis 2016).

2.6 The gut-lung axis hypothesis

For long time lungs have been considered sterile, but with the introduction of next generation sequencing was possible to detect microbial DNA in the lungs of both healthy and ill patients (Bassis et al. 2015; Morris et al. 2013). However, as opposite of intestinal tract that host 10^{13} bacteria (Sender et al. 2016), the respiratory tract host between 10^{2} (lungs) – 10^{6} (oropharynx) bacteria, being the least populated surface in the human body (Man, De Steenhuijsen Piters, and Bogaert 2017). Interestingly, both intestine and respiratory tract present similar predominant bacteria composition of *Firmicutes* and *Bacteroidetes* (Erb-Downward et al. 2011; Robles Alonso and Guarner 2013). Alteration in the airway microbiota has been reported in several airways' disease such as asthma

and chronic obstructive pulmonary disease (COPD) (Huang et al., 2011; Pragman et al., 2012; Segal et al., 2013). In particular, patients with asthma showed a reduction in Bacteroides and an increase in Firmicutes and Proteobacteria compared to healthy controls (Hilty et al. 2010; Sze et al. 2012).

Several evidence reported that the gut and airway microbiota develop at the same time after birth and that they are in constant crosstalk (Madan et al. 2012). In fact, it has been observed that some bacteria species appear in the intestinal tract before appearing in the airways tract, suggesting a role of micro aspiration of gut bacteria into the lungs (Madan et al. 2012). Moreover, study conducted in a population of children with asthma revealed an impaired gut microbiota with an overproduction of *Clostridium* compared to the control group, which suggest a role in the asthma disease (Stiemsma et al. 2016).

External factors, such as changes in diet or weight gain has been proved to impact both airway and gut microbiota and affect both systems (**Fig. 2.6.1**) (Madan et al. 2012). This is supported by evidence linking altered gut microbiota composition with airway diseases, including asthma, chronic obstructive pulmonary disease (COPD) (Hufnagl et al. 2020; Li et al. 2021).

Shift in the gut bacteria towards harmful bacteria may led to the alteration of gut barrier and consequent translocation of endotoxin LPS in the blood stream which in turn can drive inflammation via activation of Toll like receptors and worsen the disease (described in more detail in section **2.5**). Another mechanism is via production of SCFA, which are the end-product of fibre fermentation from gut bacteria. SCFA have been proved to have anti-inflammatory properties by either activating free fatty acids

receptors (FFARs) or by inhibiting histone deacetylase (HDAC) which can contribute to ameliorate disease (described in more detail in section **2.7.1**).

Taken together this data show a correlation between the gut and airway microbiota and the association between microbiota disbalance and numerous airways diseases pointing out that the microbiota has a potential central role in the development of these diseases.

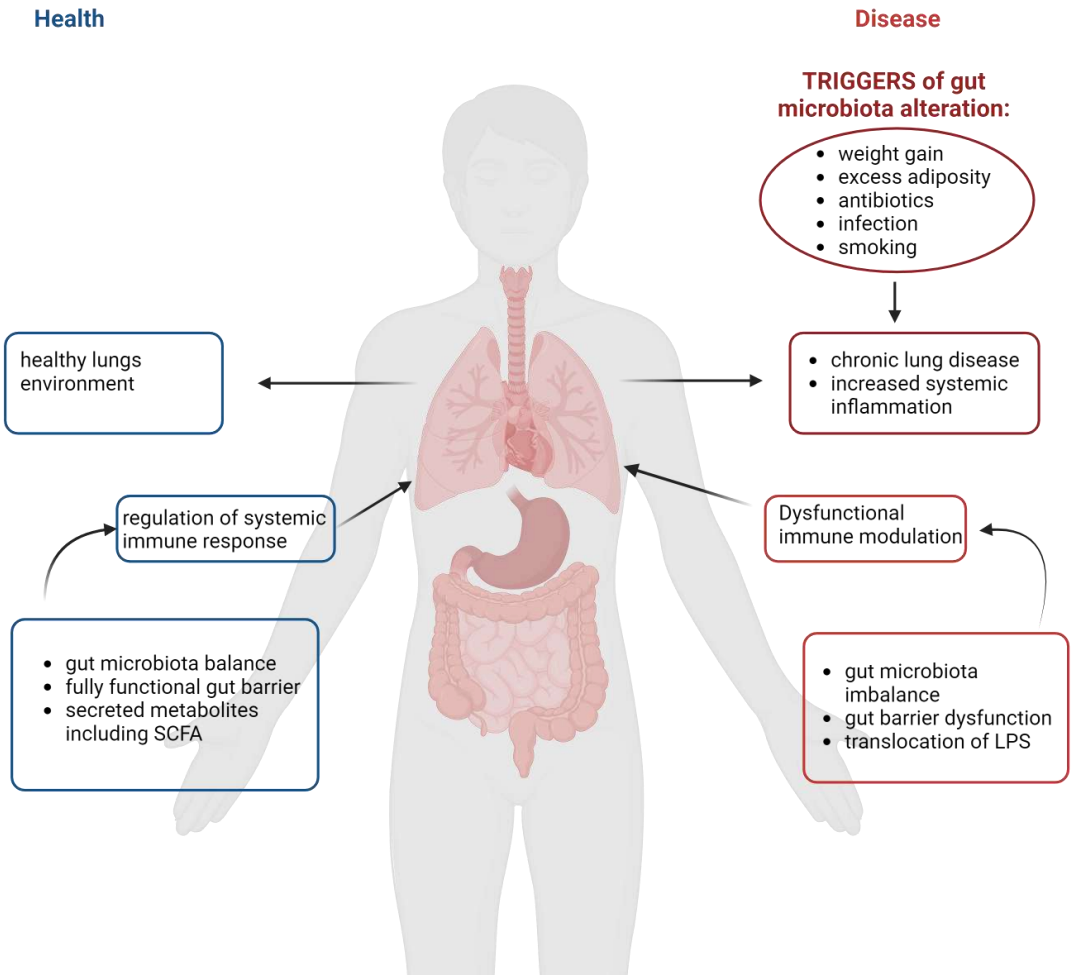


Fig. 2.6.1. The Gut-lung axis hypothesis.
During health condition there is a balance in the gut microbiota and a fully functional gut barrier which contributes to maintaining a homeostatic immune response. Commensal bacteria produce metabolites such as short-chain fatty acids (SCFA) that contribute to the regulation of immune responses and strengthening of gut barrier.
When there is an impairment on the gut microbiota due to several factors including weight gain, stress, antibiotic use, this leads to an altered immune response. Impaired gut microbiota is associated with disruption of gut barrier and translocation of endotoxin including LPS in the blood circulation. This can cause systemic inflammation and a further outgrowth of opportunistic pathogens, which can lead to chronic inflammation in distal organs including lungs.

2.6.1. Molecular link between obesity and asthma: mitochondria dysfunction

A key molecular link with between obesity and asthma diseases is mitochondrial dysfunction. Mitochondria are key organelles that play an essential role for cellular health and survival. They represent the powerhouse of the cell and have important roles in regulating stress signalling, sensing danger and combating cytotoxicity. (Bhatraju and Agrawal 2017a). Mitochondrial dysfunction, together with systemic inflammation and abnormal metabolic activity have been associated with obesity. Additionally, recent evidence suggest that mitochondrial dysfunction may induce a proinflammatory cascade which is critical for asthma development and exacerbations (Bhatraju and Agrawal 2017a). Evidence of mitochondrial dysfunction in asthma was first reported in the analysis of the bronchial epithelium from a cohort of children with asthma (Konrádová et al. 1985). Murine research revealed that mice with mitochondrial dysfunction exhibit an increased bronchial hyperresponsiveness and greater number of eosinophils in the bronchoalveolar lavage fluid (Aguilera-Aguirre et al. 2009).

Mitochondria are essential for cellular respiration and are sensitive to changes such as nutritional overload, stress and other triggers including LPS or toxins. As a response to these triggers, mitochondria secrete reactive oxygen species (ROS) to limit cell damage and reduce pathogen infection mainly via activation of NLRP3 or TLR pathway (West, 2017). If prolonged, this excessive production of ROS from the mitochondria can lead to a proinflammatory cascade that promote mitochondrial dysfunction, cell damage and ultimately worsening of disease.

Evidence demonstrates that LPS can alter mitochondria morphology which in turn can led to mitochondria dysfunction. Mitochondria are dynamic organelles, and they continuously undergo fusion and fission. Balance of fusion and fission processes are essential for the correct mitochondria division and reorganization of their components in order to maintain healthy mitochondria. A shift towards upregulated fission processes leads to mitochondria fragmentation, while upregulated fusion processes leads to mitochondria elongation and swelling (**Fig. 2.6.1.1**) (van der Bliek, Shen, and Kawajiri 2013).

Therefore, alteration of fission/fusion balance can significantly alter mitochondria structure, leading to the accumulation of permanently damaged mitochondria which in turn promote ROS overproduction (Buck et al. 2016). Studies demonstrated that LPS can significantly shift the balance of these processes towards excess of fission process, leading to mitochondria fragmentation (Nair et al. 2019a). This in turn is associated with reduced mitochondrial activity and ROS over production. As a response to this damage, cells can activate several compensatory strategies including mitophagy (elimination of damaged mitochondria) and biogenesis (production of new mitochondria) (Widdrington et al. 2018a).

High fat diet and excess calories, noted obesogenic, can lead to the mitochondria dysfunction. In fact, similarly to LPS-induced damage, overfeeding is also associated with mitochondria fragmentation (Liesa and Shirihai 2013). This condition is linked with metabolic abnormalities such as insulin resistance, a protective mechanism activated by cells to prevent further damage to mitochondria by reducing insulin sensitivity, and therefore limiting nutrient uptake (Martin and McGee 2014). Such metabolic

abnormalities are seen in obesity and can represent an increased risk of asthma as described in more detail in section **2.6.2**.

Strategies that target mitochondria such as repair via scavenger of reactive oxygen species (ROS) or replacement of damaged mitochondria through upregulation of biogenesis may be beneficial for ameliorating asthma and obesity (Qian et al. 2022). Between others, nutritional strategies showed promising results in regulating mitochondrial activity, via interaction with microbiota (Donohoe et al. 2011b; Khalil et al. 2022; Memme et al. 2021). In fact, evidence suggests that SCFA, end-product of bacterial fermentation of dietary fibre, play a role in the regulation of mitochondrial homeostasis. In particular, butyrate, one of the most abundant SCFA together with acetate and propionate, can enter the Krebs cycle and reduce NAD+ to NADH, in a process that produces ATP, regulating energy production (Donohoe et al. 2011b). *In vitro* conducted using colonocytes from germ free (GF) mice, lacking SCFAs, reported a reduced level of $NADH/NAD^+$ which resulted in reduced ATP production in the mitochondria (Donohoe et al. 2011b). Another *in vitro* study revealed that the SCFAs, acetate and butyrate, prevented mitochondria structure damage in β-cells, previously treated with a cell stressor noted as streptozotocin, by preventing the reduction of fission/fusion protein. These proteins are essential regulators of mitochondrial dynamics and are strongly associated with their bioenergetics (Hu et al. 2020).

This suggests that increased levels of circulating SCFAs may be a potential way to improve mitochondrial dysfunction and indirectly contribute to the amelioration of disease.

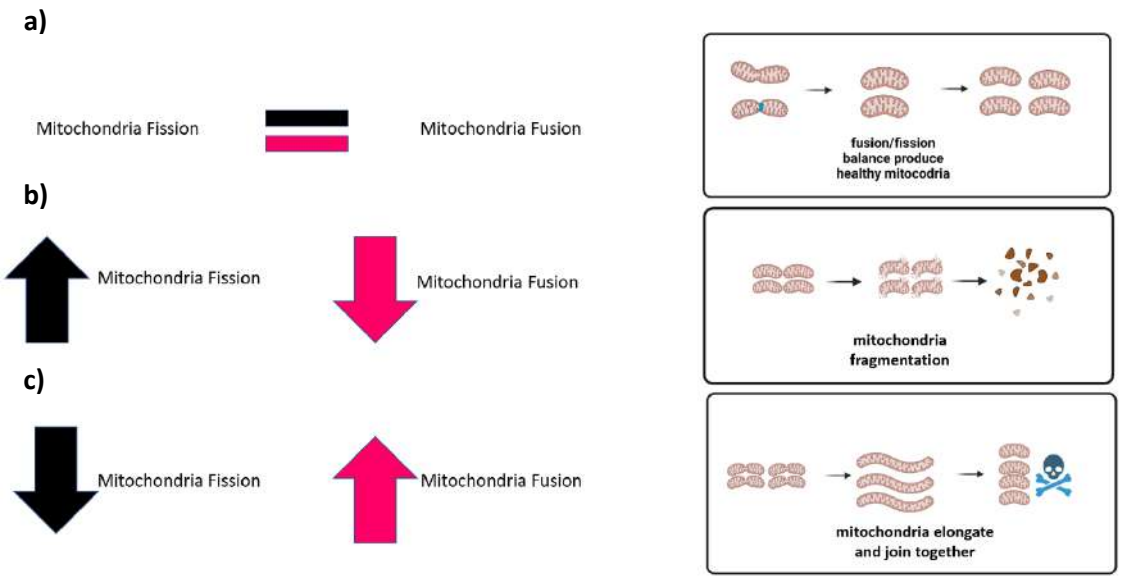


Fig. 2.6.1.1. Fission/fusion processes in mitochondria.
Mitochondria are dynamic organelles that are constantly rearranging themselves; a balance of fission and fusion events are essential for the correct functionality of mitochondria. a) Under homeostatic conditions, there is a balance of fusion and fission events and mitochondria undergo under a correct division. b) An upregulation of fission processes and a downregulation of fusion processes results in fragmentated mitochondria, increasing autophagy and leading to cells' death. c) A downregulation of fission and upregulation of fusion processes leads to large, elongated mitochondria which fuse together, inducing cell death.

2.6.2. Metabolic dysfunction in obesity and asthma

Metabolic dysfunction, noted in obesity, has recently been associated with increased asthma severity (Cottrell et al., 2011a; Husemoen et al., 2008; Karampatakis et al., 2017; Singh et al., 2015). Impaired glucose tolerance and insulin resistance have been associated with asthma exacerbations, including lung function decline and/or airway hyperresponsiveness in both children and adults (Cottrell et al. 2011b; Forno et (Hill et al. 2014)t al. 2009; Karampatakis et al. 2017). Altered glucose metabolism may drive inflammation in several ways including via activation of reactive oxygen species (ROS) and cleavage of transforming growth factor (TGF)-β which in turn stimulates the release of pro-inflammatory cytokines (Johnson, Justin Milner, and Makowski 2012). Moreover,

studies in mice revealed that metabolic abnormalities, including insulin resistance, were associated with airway hyperresponsiveness (AHR), independently of BMI (Singh et al., 2015). Similarly, in humans, insulin resistance was associated with risk of developing asthma in adults and adolescents (Forno et al., 2015; Husemoen et al., 2008; Singh et al., 2013).

Together with glucose impairment, obesity is associated with altered lipid metabolism including cholesterol and triglycerides. This was shown to drive inflammation by directly activating membrane-associated pattern recognition receptors (PRRs), including the toll-like receptor (TLR) family, or by acting as agonists for members of the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors, which are involved in the regulation of pro-inflammatory cytokines, such as TNF-α and IL-6 (Andersen 2022; Cardoso and Perucha 2021; Hill et al. 2008; Köberlin, Heinz, and Superti-Furga 2016). This can in turn affect asthma severity. In fact, recent studies found elevated circulating level of triglycerides and low-density cholesterol (LDL) were associated with lung function decline in a cohort of patients with asthma (Barochia et al. 2015; Scichilone et al. 2013). Interestingly, raised levels of triglycerides and impaired glucose levels were associated with asthma, independently of BMI, in a cohort of children (Cottrell et al. 2011a). This is interesting because it may suggest that metabolic abnormalities can represent a predisposing factor for developing asthma, regardless of BMI.

Evidence indicate that metabolic dysfunction may further exacerbate asthma symptoms. However, it appears that obese status is not always a cause of metabolic alteration in asthma, indicating that this may be a consequence of asthma

pathophysiology. Further work is required to establish the role of multimorbidity in asthma severity.

2.7 Dietary strategies to target the gut microbiota to improve host health

Common dietary strategies to target the gut microbiota are prebiotics, probiotics, synbiotic (a combination of both) and postbiotic to manipulate the gut microbiota have seen a surge in their popularity and interest in their potential to support a range of disease management.

Probiotics are defined as 'live microorganism that confer health benefit to the host when assumed in adequate amount' (Hill et al. 2014). Prebiotic are defined as 'a non-digestible substrate that can be selectively used by the host microbes and induce health benefit' (Gibson et al. 2017). A combination of both noted as a synbiotic are defined as a combination of live microorganisms and substrates used by the gut microbiota and that confer a health benefit to the host (Swanson et al., 2020). Lastly, postbiotic are recently new and defined as preparation of inanimate microorganisms and/or their components that confers a health benefit on the host (Salminen et al. 2021).

2.7.1 Prebiotics and dietary fibre

A dietary strategy to target the gut microbiota could be to increase fibre intake. The latest recommendation from the Scientific advisor committee on Nutrition is to increase the daily fibre intake to 30 grams per day, which can be achieved with the consumption of 5 daily portion of fruits and vegetables (Scientific Advisory Committee on Nutrition (SACN) 2015). However, the last finding from the National Diet and Nutrition Survey

Results showed that the average fibre consumption in the U.K. is about 19.7 grams per day, which is 33% less than the recommended daily intake (Public Health England 2020).

Although not all dietary fibre can be classed as prebiotic. There are a number of proposed prebiotic fibre often available in the form of powder or tablets which can be added to drinks and food such as yoghurt or smoothie bowl. Common noted prebiotics are fructo-oligosaccharides (FOS), inulin and galacto-oligosaccharides (GOS) which have demonstrated effect in promoting the growth of beneficial species of bacteria such as Lactobacillus and/or Bifidobacterium species (Gibson et al. 2017). FOS and Inulin are group of fructans while GOS is an oligosaccharide linked with a galactose. Prebiotics cannot be digested by human enzymes, and they reach the gastrointestinal tract indigested. Here they are metabolized by the bacteria as source of energy and as end-product of their digestion they produce metabolites that exert a beneficial effect in the host. The main metabolites produced by bacteria are short chain fatty acids (SCFA) which are fatty acids with less than 6 atoms of carbons. The most abundant SCFA are Acetate (C2), propionate (C3) and butyrate (C4), with the highest distribution present in the colon (Macfarlane and Macfarlane 2003). Other SCFA are produced by the gut microbiota, such as formate, valerate, caproate, iso-butyrate, 2 methyl-butyrate and isovalerate, but in lesser amount (Macfarlane and Macfarlane 2003). Depending on the prebiotic used, bacteria can produce various amount of SCFA, with GOS previously demonstrating the greatest production (Holmes et al. 2020) . The SCFA produced are then absorbed by epithelial cells by either H^+-dependent monocarboxylate transporters (MCTs), through binding to G protein-coupled receptors (GPCRs), also noted as free fatty acid receptors (FFARs), or by inhibiting histone deacetylase activity (HDAC) (Silva, Bernardi, and Frozza 2020). Activation of GPRCRs receptors and/or HDAC inhibition is

essential for modulate SCFA function. The 2 main GPCRs involved in humans are GPR-41 and GPR-43. Both receptors are present in airways epithelial cells, smooth cells and leukocytes with GPR-43 being expressed in the adipose tissue as well; they are the main receptors for acetate but can also recognize propionate and butyrate (Tan et al. 2014). Enhanced expression of these receptors has been found in sputum of patients with asthma following a high fibre meal and associated with amelioration in airway inflammation (Halnes et al. 2017). Interestingly, experiment in mice fed with low fibre diet have found reduced level of circulating SCFA and association with increased allergic reaction by activating Th-2 cell mediated inflammation pathways, suggesting a role of SCFA in the disease (Trompette et al. 2014). Reduced levels of SCFA have been found in the faeces of 44 patients with bronchial asthma compared to healthy controls, which suggest a reduced metabolic activity from SCFA producing bacteria (Ivashkin et al. 2019).

Although only a small fraction of SCFA reach the blood stream, their potential beneficial effect of system and peripheral organs have been reported. In particular, butyrate appears to influence systemic inflammation through the modulation of T regulatory (Treg) cells, which regulate the immune response and reduce proinflammatory cytokines production, playing a key role in maintaining homeostasis (Arpaia et al. 2013; Haghikia et al. 2015; Smith et al. 2013) (**Fig. 2.7.1.1**). Furthermore, *in vitro* and murine studies demonstrated that SCFA were able to limit the LPS induced inflammation by attenuating the expression of proinflammatory cytokines including TNF-α, IL-6 (Liu et al. 2012; Wang et al. 2017). These findings suggest that prebiotics are beneficial by increasing the levels of SCFAs produced by bacteria.

Furthermore, *In vitro* studies demonstrated a potential role of SCFA in ameliorating gut barrier by increasing the expression of tight junction such as occludin, zonulin-1 and claudin (Peng et al. 2009; Wang et al. 2012; Yan, Ajuwon 2017; Feng et al. 2018; Nielsen et al. 2018). In addition, experiment conducted using Caco-2 cells monolayers exposed to acetate, propionate and butyrate in combination with LPS, significantly improved transepithelial permeability damage induced by LPS and increased the expression of tight junctions occludin and claudin (Feng et al. 2018a).

Therefore, dietary prebiotic strategies that enhances the production of SCFA may be a potential adjunctive tool to mitigates asthma exacerbations.

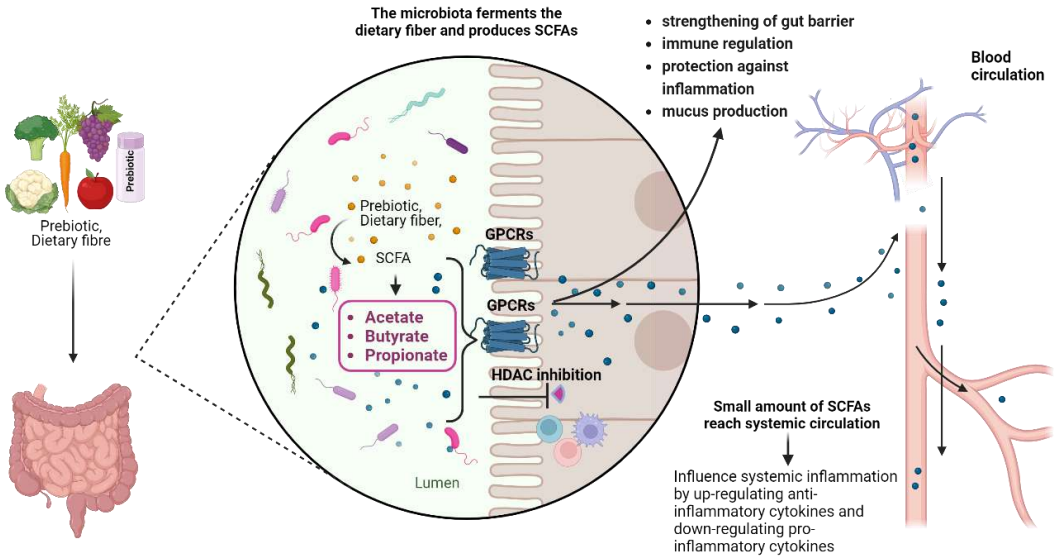


Fig. 2.7.1.1. The mechanism of action of SCFA.
The short-chain fatty acids (SCFA) are the main metabolites produced by the gut microbiota via fermentation of indigestible food components such as dietary fibre, resistant starch or prebiotics. SCFA are then absorbed by epithelial cells, mainly via H^+-dependent monocarboxylate transporters (MCTs), through binding to G protein-coupled receptors (GPCRs) or by inhibiting histone deacetylases.
SCFAs play an important role in regulating intestinal mucosal immunity, barrier integrity and function. The SCFA can translocate in the blood stream and influence systemic inflammation by activating T regulatory cells (Treg) differentiation and by regulating the secretion of cytokines.

2.7.2 Prebiotic interventions in asthma and obesity

Limited animal studies have been conducted investigating the use of prebiotics in obesity and asthma which demonstrate some encouraging findings. In a murine model of allergic asthma, mice fed a diet enriched with 1% Galacto-oligosaccharide (GOS) for two weeks demonstrated to prevent the development of airway hyperresponsiveness, reduced pulmonary eosinophilic inflammation and concentration of proinflammatory cytokine IL-13 (Verheijden et al. 2015).

Other studies showed how prebiotic can be used as additional tool to prevent inflammation and weight gain. Van Hul et al. showed that mice models fed with HFD supplemented with the prebiotic fructo-oligosaccharides had reduced level of the endotoxin LPS and lower levels of systemic inflammatory cytokines compared to mice with HFD only (Van Hul et al. 2020). A recent study showed that 16 weeks supplementation of GOS in rat fed with western diet showed reduced weight gain, amelioration of insulin tolerance and lower circulating level of cholesterol compared to the control group (Mistry et al. 2020).

In humans, limited studies are present on regards the use of prebiotic supplement in obesity an asthma, however they showed promising beneficial effect in the amelioration of diseases. Evidence demonstrated the efficacy of prebiotics in reducing marker of systemic inflammation in overweight and obese patients (Morel et al. 2015; Vulevic et al. 2013a). In particular, Morel et al. found out that two weeks of dose dependent prebiotic administration of GOS (6 g, 12 g, 18 g per day) in 88 overweight adults led to a led to a significant reduction of the appetite (greater reduction in the group who received the highest dose of prebiotic), which it may suggest a potential contribution of the prebiotic in maintaining weight loss over the time dose-dependent (Morel et al. 2015). Moreover, a reduction of plasma concentration of the endotoxin LPS was reported compared to the placebo group (Morel et al. 2015). Similarly, a 40% reduction of LPS levels was observed in a study investigating the effect of 12 weeks administration of prebiotic oligofructose (7 g, 3 times a day) in a cohort of 37 adults who were overweight or obese (Parnell, Klancic, and Reimer 2017b).

In asthma, evidence showed that 7 days dietary intervention trial of 12 g Inulin per day in 16 participants with stable asthma, significantly reduced level of sputum eosinophils, involved in airway inflammation and asthma control (McLoughlin et al., 2019). Moreover, Williams et al. showed for the first time in humans that three weeks of 5.5 g daily dose of prebiotic Bimuno Galacto-oligosaccharide (B-GOS) supplement reduced inflammation and asthma associated exacerbation in a cohort of 10 adults with exercise induced bronchoconstriction (EIB) (Williams et al. 2016).

This suggests a promising role of prebiotic in reducing inflammation and ameliorating asthma symptomatology. Although more studies in larger scale population are needed to reach a final conclusion on the role of prebiotic in asthma.

2.7.3 Non-fibre prebiotics and their role in obesity asthma

There are other dietary components that are also potential prebiotic such as polyphenols (e.g. resveratrol, curcumin, quercetin) and omega-3 polyunsaturated fatty acids (n-3 PUFAs). Several evidence suggest a beneficial effect of non-fibre prebiotic in reducing and/or maintain weight loss (Albracht-schulte et al. 2020; Anhê et al. 2015; Boccellino and D'Angelo 2020).

Regarding asthma, studies investigating the role of non-fibre prebiotic in asthma are mixed. Numerous reports have suggested that consuming omega-3 fatty acids or polyphenols reduced the occurrence of asthma, the prevalence of symptoms related to asthma, or levels of exhaled nitric oxide (NO), while also enhancing lung function in adults (R. Barros et al. 2011; Kompauer et al. 2008; Patchen et al. 2023; Wu et al. 2024). In a four-week randomized, double-blind, placebo-controlled trial, 22 adults with

symptomatic asthma were given either oral purple passion fruit peel (PFP) extract (150 mg/day), a novel blend of flavonoids containing quercetin, or placebo pills. The study demonstrated a reduction in coughing and wheezing following supplementation compared to the placebo group (Watson et al. 2008). A 20 years epidemiological study in 4162 young adults revealed that high intake of n-3 PUFA prevented asthma onset (Li et al. 2013). However, other studies found no beneficial effect of supplementation with non-fiber prebiotic in asthma disease (Lang et al. 2019; McKeever et al. 2008; Smith et al. 2017; Woods et al. 2004). A recent study investigating the effect of 24 weeks supplementation of fish oil 3-PUFA (4g/day) in 98 young adults who were obese and/or overweight and with uncontrolled asthma found no effect in the amelioration of asthma symptomatology, assessed with the use of asthma control questionnaire (ACQ-6) (Lang et al. 2019). In another randomized, double-blinded, placebo-controlled study, soy isoflavones were supplemented (50 mg, twice a day) to a cohort of 193 adults and adolescents (aged 12 years and older) with symptomatic asthma for 24 weeks, alongside standard control medications. However, there was no observed improvement in lung function, as assessed by spirometry, compared to the placebo group. (Smith et al. 2017).

In summary, while research has shown varying results, further investigation is needed to clarify their potential role of non fibre prebiotic in asthma management. Future studies should focus on elucidating the mechanisms of action, conducting larger clinical trials and assessing their impact on various asthma-related outcomes.

2.7.4 Probiotics interventions in obesity and asthma

The use of probiotic may be a further option to manipulate gut microbiota and increase the number of beneficial bacteria and improving microbiota diversity. This indirectly may play a role in reducing systemic inflammation.

In obesity, *in vitro* studies demonstrated the efficacy of the probiotic *Bifidobacterium* ($1x10^8$ colony forming units (CFU)) improving gut permeability by preventing the disruption of tight junction (TJ) proteins (e.g. occludin, claudin-3 and zonula occludens) and reducing cytokines associated to systemic inflammation such as TNF-α and IL-6 model in a model of gut cells (Ca-co2) treated with LPS (Ling et al. 2016). Similarly, a subsequent *in vivo* study from the same research group showed that *Bifidobacterium* somministration in mice previously exposed to LPS, ameliorated gut barrier dysfunction induced by LPS and reduced circulating levels of pro-inflammatory cytokines TNF-α and IL-6 (Ling et al. 2016).

Limited human clinical studies patients with obesity have been conducted so far, but results are encouraging. An 8-week treatment with the yogurt enriched with bacteria *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium lactis* ($1x10^7$ CFU/mL each) was associated with reduced levels of inflammatory cytokines in a group of adult with obesity (n=25) (Zarrati et al. 2014). Conversely, a study conducted in 50 adolescents revealed that 12 weeks supplementation of probiotics strain *Lactobacillus salivarius* ($1x10^{10}$ CFU/g) led to no significant changes in inflammatory markers compared to the control group (Gøbel et al. 2012).

Limited studies have been conducted in asthma and the majority of them have been conducted in children or infants. Previous studies demonstrate that probiotics administration of *Lactobacillus* ($1x10^8$ CFU) for 9 months to a year at early life and/or school age children could either decrease asthma symptoms or reduce its incidence later in life (Abrahamsson et al. 2007; West, Hammarström, and Hernell 2009). However, follow up studies reported no difference in the prevalence of respiratory diseases between children who received probiotics and whom who received placebo during their infancy, suggesting that the effect of probiotics treatment was only transient (Abrahamsson et al. 2013; West, Hammarström, and Hernell 2013). The first study in 15 adults with moderate asthma revealed that 1 month supplementation with yogurt containing *Lactobacillus acidophilus, Lactobacillus bulgaricus, and Streptococcus thermophilus* ($7.6x10^8$, $3.4x10^8$, and $3.7x10^8$ bacteria/g, respectively) showed no changes in airway inflammation and asthma control (Wheeler et al. 1997). However, recent study conducted in 29 patients with stable asthma demonstrated that 3 months supplementation of probiotic *Bifidobacterium Lactis* Probio-M8 (one sachet per day of 2 g; $3.0x10^{10}$ CFU/sachet/day) together with the use of corticosteroid preventative inhaler, Symbicort Turbuhaler, improved asthma control and led to increase of circulating metabolites associated with anti-inflammatory properties including tryptophan and sphingomyelin compared to the placebo group using Symbicort Turbuhaler only (A. Liu et al. 2021). Similarly, 8-weeks supplementation of multistrains of probiotic containing 7 different strains of bacteria including *Lactobacillus*, *Bifidobacterium* and *Streptococcus* (minimum of $3x10^9$ CFU/g of each strain) in a cohort of 40 patients with moderate to severe asthma showed a significant improvement of

lung function and reduced circulating levels of pro-inflammatory cytokines such as IL-6, IL-17 and Treg cells (Sadrifar et al. 2023).

So far, few studies have been conducted to evaluate the potential benefit of probiotic in both obesity and asthma. Different strains were used to conduct the studies and both animals and clinicals trials differ for study length. Also, there is a lack of follow up studies that prevent us to know what are the long-term effect of probiotics somministration. More studies, longer trials and more homogeneity in the strains used are needed to evaluate the potential benefit of probiotics in both obesity and asthma. Furthermore, possible problematic of probiotic supplementation are associated with nutritional status of the host. In order to exert their beneficial properties, it is essential that probiotics have access to adequate nutritional sources, in the form of dietary fibre. Therefore, it is fundamental to have an adequate diet in parallel with probiotic supplementation to obtain optimal results.

2.7.5 Synbiotic interventions in obesity and asthma

Studies have tried to use a combination of prebiotic and probiotics, noted as synbiotic to test whether they can have a synergistic effect in ameliorating the disease, although findings are controversial.

A recent study conducted in a cohort of 94 adults with obesity showed that a 3-week dietary intervention trial with the prebiotic GOS had a beneficial effect in improving gut permeability. However, when the GOS was used in synergism with the probiotic *Bifidobacterium lactis BB-12* ($1x10^9$ CFU), no effects were seen (Krumbeck et al. 2018).

In asthma, a murine asthma model supplemented with 1% fructo-oligosaccharides and probiotic *Bifidobacterium breve* M-16V ($2x10^9$ CFU) for two weeks demonstrated effective in reducing airway eosinophilia in lungs and concentration of proinflammatory cytokines including IL-6, IL-10, IL-4 when compared to control (Verheijden et al. 2016). Amelioration of asthma symptoms and airways associated inflammation was also reported in a murine study following supplementation with a combination of prebiotic fructo-oligosaccharides and probiotic *Bifidobacterium* ($1x10^9$ CFU) (Sagar et al. 2014). In humans, a dietary intervention using a mix of prebiotic Inulin and probiotic *Lactobacillus* ($1x10^8$ CFU) led to a reduction of inflammation in a cohort of 29 patients with stable asthma (Halnes et al. 2017). Similarly, a cohort of 29 patients with asthma that received 4-week supplementation with synbiotic composed of prebiotic (90% short-chain galacto-oligosaccharides, 10% long-chain fructo-oligosaccharides) and the probiotics *Bifidobacterium breve* M-16V ($1x10^{10}$ CFU), significantly reduced the production of Th-2 associated cytokines and improve peak respiratory flow (Van De Pol et al. 2011). Conversely, other studies showed no synbiotic effect when prebiotic and probiotics were administrated together (Krumbeck et al., 2018; McLoughlin et al., 2019). In fact, supplementation of prebiotic Inulin ameliorated asthma condition in 17 adults when administrated alone but showed no effect in combination with multi strain probiotic containing *Lactobacillus* and *Bifidobacterium* (>$7.0x10^9$ each CFU) (McLoughlin et al., 2019).

This could be due to antagonistic effect for competition of nutrients in the gut or suboptimal conditions for probiotic absorption due to poor participants' diet.

Despite the promising findings of prebiotic and probiotic supplementation on gut barrier integrity and endotoxin levels, no study considered the use of prebiotic to reduce circulating level of endotoxin and/or ameliorate gut permeability in asthma cohort. Therefore, studies should consider including these markers as a possible outcome measure of dietary intervention in patients with asthma.

2.7.6 Postbiotic in obesity and asthma

Postbiotic are recently new generation of supplement, which is gaining growing interest due to their potential beneficial effects for the host. The international scientific association of probiotics an prebiotics (ISAPP) defined postbiotic as a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host (Salminen et al. 2021). Postbiotic may have a number of beneficial effect for the host such as boosting immune system, lower the risk of respiratory infections, and help with weight loss (Byrne et al. 2015a; Chambers, Morrison, and Frost 2015; Kotani et al. 2010; Di Sabatino et al. 2005).

At the moment there are no studies looking at the effect of postbiotic on asthma, however one study addressed a potential beneficial effect of postbiotic on the amelioration of atopic dermatitis disease, commonly associated with asthma. The study administered the postbiotic (heat-killed *Lactobacillus paracasei* K71) to 34 adults suffering with atopic dermatitis for 8 weeks and showed a significant reduction in the severity of disease compared to the placebo group (Moroi et al. 2011).

More evidences are available regarding the effect of postbiotics in obesity. Recent studies have shown that postbiotic SCFA and in particular butyrate and propionate, help

reduce body weight by suppressing the hunger signal in animal studies (Byrne et al. 2015b; Goswami, Iwasaki, and Yada 2018). Another postbiotic, KetoA, a metabolite produced from linoleic acid by lactic acid bacteria in the gut, showed promising results in reducing body weight by increasing energy expenditure in a mice model (Canfora et al. 2019).

Initial studies on the efficacy of postbiotic showed promising host health benefit, however large-scale studies in humans are needed in order to evaluate their beneficial metabolic effects.

Chapter 3: Material and Methods

3.1. Cell culture

3.1.1 Human epithelial airway cells culture and treatments used

Human bronchial epithelial airway cell line (BEAS2B-R1) are an immortalised cell line taken from the bronchus of healthy volunteers. This cell line has been validated as a good model of airway biology studies and to evaluate airways damage induced by inflammation and/or lipopolysaccharides (LPS) insult (Hou et al. 2021; Si and Zhang 2021).

Cells were seeded on to 6 well plates, unless otherwise specified, and grown until 80% confluent in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, U.K., #41965-039) with 10% of foetal bovine serum (FBS, Labtech, USA, #FCS-SA/500) and 0.5 mg/mL penicillin-streptomycin-Glutamine (Pen-strep) (Thermofisher, U.K., #10378016), referred to as cell culture media. Once confluent, cells were treated at 6, 12 and 24 hr with or without Lipopolysaccharides (LPS, from Escherichia coli O55:B5, Sigma, U.K. # L2880-10MG) as follow:

- LPS 10 ng/mL
- LPS 100 ng/mL
- Short Chain Fatty Acids (SCFA) mix (details of the mix below)
- LPS 10 ng/mL + SCFA
- LPS 100 ng/mL + SCFA

In addition to the above treatments, cells were pre-treated with SCFA mix for 24hr and then treated with:

- LPS 10 ng/mL +SCFA
- LPS 100 ng/mL+ SCFA

The SCFA mix was prepared at a final concentration of Acetate 2 mM (Sigma, U.K., #S5636-250G), Butyrate 0.25 mM (Sigma, U.K.#303410-100G), Propionate 0.25 mM (Sigma, U.K., #P1880-100G) in a ratio of 80:10:10 respectively, which is the highest molar ratio found in the peripheral venous blood in healthy individual (Ktsoyan et al. 2016). This ratio was chosen to reflect human physiological condition.

Treatments details are listed in the **Table 3.1.1.1**.

Following the initial western blot experiments, all subsequent investigations were conducted using the higher dose of LPS (100 ng/mL) and cells pre-treated cells with SCFA.

Table 3.1.1.1. Treatments and concentration used.
(LPS = Lipopolysaccharides).

Treatment	**Stock solution**	**Final concentration**	**Supplier**	**Catalogue Number**
LPS	1 mg/mL	100 ng/mL	Sigma	L2880-10MG
Acetate	200 mM	2 mM	Sigma	S5636-250G
Propionate	200 mM	0.25 mM	Sigma	P1880-100G
Butyrate	200 mM	0.25 mM	Sigma	303410-100G

3.1.2 Protein extraction and quantification

Cultured epithelial airway cells ($1x10^5$; BEAS2B-R1) were harvested using 250 µL of Radioimmunoprecipitation assay (RIPA) lysis buffer (Millipore U.K., #20-188) containing 2% of protease and phosphatase inhibitors (reagent preparation was listed in **Table 3.1.2.1**). Samples were then vortexed and left on ice for 30 minutes, spinning the samples down every 10 minutes. The samples were then centrifuged at $16x10^3$ rfc for 15 minutes, aliquoted and stored at -80 °C until assayed.

Protein concentrations of sample were quantified using a Bradford assay. To carry out this assay, a calibration curve was prepared using a bovine serum albumin (BSA, Sigma, #A9418-10G), starting from a stock solution of 1 µg/mL, with increasing quantities of BSA from 1 µg to 8 µg. One blank reference sample was also prepared.

Bio-rad protein assay reagent (Bio-Rad Laboratories, U.K., #5000006), 1 mL, was added to each test tube and then 2 µL of samples were added. Samples were then immediately vortexed to allow the reaction to develop. All samples were assayed in triplicate using a spectrophotometer (Tecan, infinite M plex, Switzerland) at a wavelength of 596 nm, from which the relative absorbance of each sample was obtained based on the amount of total protein. The standard curve generated was used to quantify the protein concentrations.

Table 3.1.2.1. Protease and phosphatase inhibitors preparation.
(RIPA= Radioimmunoprecipitation assay).

Solution	Volume used	Company	Catalogue number
1x RIPA buffer	2 mL	Millipore U.K.	20-188
Roche complete Mini protease inhibitor cocktails	2 tablets	Sigma, U.K.	11836153001
Sodium Fluoride (NaF)	8 mg	Fisher Scientific	10528070
Sodium orthovanadate (Na3VO4)	20 mg	Acros Organics	WZ-88219-57

3.1.3 Western Blot analysis

Western blotting analyses were used to detect the expression of specific proteins in the harvested BEAS2B-R1 cells. Proteins present in the samples were separated based on their molecular weight by electrophoretic separation in the polyacrylamide gel and were subsequently transferred onto a nitrocellulose membrane. A primary antibody was used to detect the protein of interest which in turn was recognized by a secondary antibody to which an enzyme such as horseradish peroxidase (HRP) has been conjugated to enable its detection. The use of an enhanced chemiluminescence (ECL) substrate for HRP allowed detection of the amount of antigen by using an imagining method. The luminescence arising from its conjugation to the antibody complex, is directly proportional to the amount of protein of interest present. The luminescence can be captured quantitively using a sensitive system such Syngene Genesys with image acquisition software. A schematic representation of the Western blotting reaction is shown in **Fig. 3.1.3.1.**

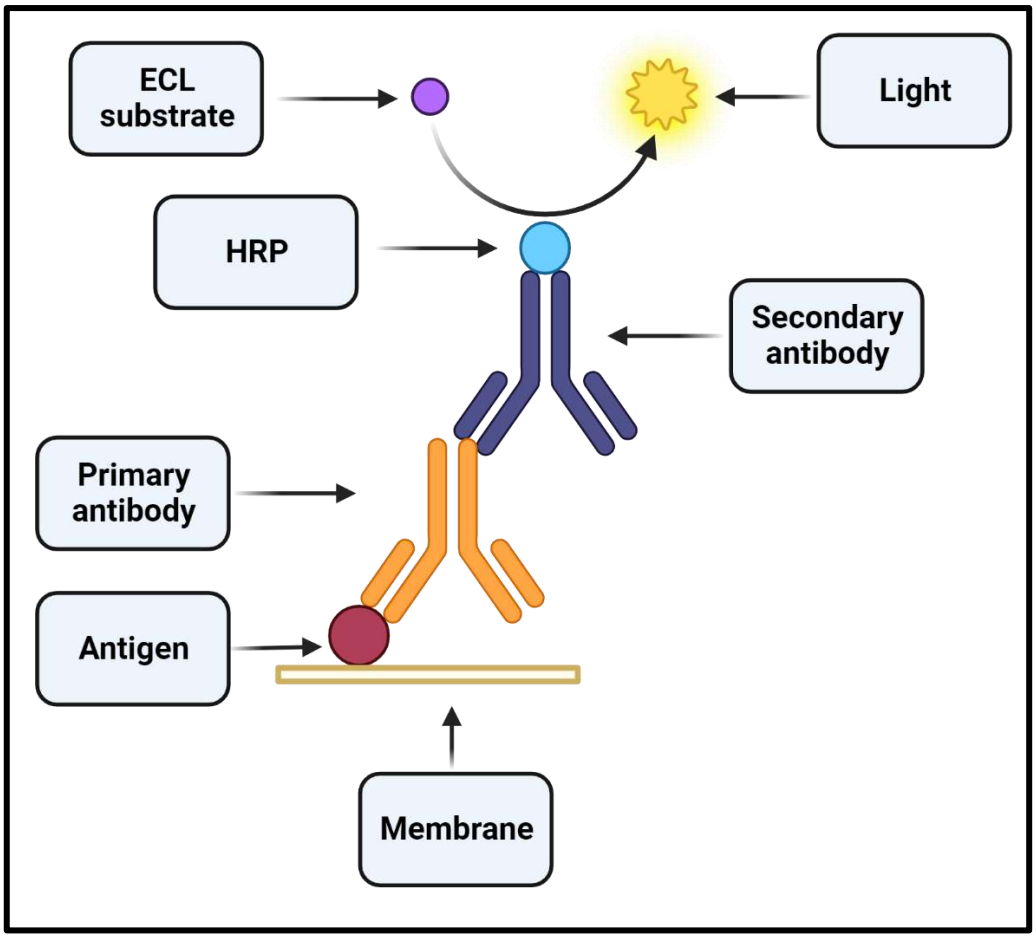


Fig. 3.1.3.1. Schematic representation of Western Blot reaction.
Primary antibody specifically binds the protein of interest. Following an incubation period, a secondary antibody was added to the membrane which specifically recognizes the primary antibody and to which an enzyme horseradish peroxidase (HRP) is linked. The addition of an enhanced chemiluminescence (ECL) substrate allowed the detection of the antigen by imaging method.

Briefly, once the protein concentration of each BEAS2B-R1 sample was determined through Bradford assay, each extracted protein treatment was subsequently prepared to be loaded onto the polyacrylamide gel. Each sample loaded on to the Western blot contained 5 µL of Laemmli buffer (Thermofisher Scientific, U.K., #J60015), 20-30 µg of protein with a variable volume of sterile water up to a volume of 20 µL. All samples were then heated at 95°C for 10 minutes and then left on ice for 5 minutes to completely denature the proteins. After carrying out these steps, the samples were loaded onto polyacrylamide gel. Running samples through the gel separates the protein of interest

based on its molecular weight by separation in the electric field. The polyacrylamide gels were prepared at a final concentration of either 8, 10 or 12.5%, based on the protein molecular weight. Subsequently, samples were loaded into the gel, together with the protein ladder (Thermofisher, U.K., #11862124). The electrophoretic run took place by setting the constant voltage to 100 V for the first 15/20 minutes and subsequently, when the ladder entered the running gel, this was adjusted to 120 V for each gel and left until the marker had run off the bottom of the gel. Once the migration of the samples was complete, the separated proteins were transferred onto a nitrocellulose membrane with 0.45 µm pore (Merck, Germany, #IPVH00010) by wet transfer for 90 minutes at 100V. At the end of the transfer, the membranes were briefly stained with Ponceau S to ensure the transfer has worked properly and then washed in water to remove any trace of the dye. Subsequently, membranes were incubated in either 0.2% I-BLOCK (Thermofisher, U.K., #T2015) or 5% Tris Buffered Saline Tween 20 (TBS-T), based on the antibody manufacturer's instruction, with the purpose to use the solutions to block the non-specific protein-binding sites across the membrane surface.

The primary antibody for the protein of interest was then diluted into either 0.2% I-BLOCK or 5% BSA TBST together with the membrane of interest and the samples were placed at 4°C overnight under constant agitation. The concentration of primary antibodies utilised for Westen blotting were optimised noting the guidelines of the manufacturer with similar cellular protein extracts (**Table 3.1.3.1**).

Following an overnight incubation (approximately 12 hr), the primary antibody was removed, and 3 washes consisting of 10 minutes each in either PBST or TBS-T were performed. Subsequently, the secondary antibody was diluted in either 0.2% I-BLOCK or

5% BSA TBS-T and added to the membrane for 60 minutes under constant agitation at room temperature. Following this period, the secondary antibodies were removed and a further 3 washes were performed in PBST or TBS-T as previously described. Finally, the proteins of interest were detected using a Bio-Rad chemiluminescence detection kit (ECL, GeneFlow, #K1-0096), mixed in a 1:1 ratio prior to addition to the membrane. Subsequently, the image was immediately acquired using Syngene G-BOX with GeneSys image acquisition software.

Table 3.1.3.1. Primary antibodies used and related dilution for primary and secondary antibodies.
(SOD-2 = superoxide dismutase 2; FIS1 = mitochondrial fission 1; DPR1 = Dynamin-1-like protein; MNF2 = Mitofusin-2; OPA1 = optic atrophy 1; TFAM= Mitochondrial transcription factor A; POLG = DNA polymerase subunit gamma; NRF1 = Nuclear respiratory factor 1; PGC1-α = Peroxisome proliferator-activated receptor-gamma coactivator- alpha; β-actin = beta-actin).

Primary antibody	Primary antibody Dilution	Secondary antibody dilution	Supplier	Catalogue Number
SOD-2	1:1000	Anti-Rabbit 1:100.000	Cell-Signaling	13194S
FIS1	1:1000	Anti-Rabbit 1:50.000	Abcam	ab156865
DPR1	1:500	Anti-Rabbit 1:100.000	Cell-Signaling	5391S
MNF2	1:500	Anti-Rabbit 1:50.000	Cell-signaling	83667S
OPA1	1:500	Anti-Rabbit 1:50.000	Cell-signaling	80471S
TFAM	1:1000	Anti-Rabbit 1:100.000	Cell-signaling	8076S
POLG	1:1000	Anti-Rabbit 1:100.000	Cell-signaling	13609S
NRF1	1:1000	Anti-Rabbit 1:100.000	Cell-signaling	69432S
PGC1-α	1:1000	Anti-Rabbit 1:5.000	Cell-signaling	2178S
β-Actin	1:5000	Anti-Rabbit 1:100.000	Cell-signaling	4970S

3.1.4 RNA extraction and qPCR

For RNA extraction, cells were lysed in 350 µL Buffer RLY (Bioline, U.K., #BIO-52079) containing 3.5 µL β-mercaptoethanol (β-ME) (Sigma, U.K.). RNA was extracted from cell culture samples using Isolate II RNA Mini Kit (Bioline, U.K., #BIO-52073) according to manufacturer's instructions.

Briefly, the cell lysate was filtered through an ISOLATE II filter for 1 minute at 11,000 rcf. Then 350 µL ethanol (70%) was added to the lysate and the mix was transferred to an RNA binding column and centrifuged for 30 seconds at 11,000 rcf. The membrane was desalted by adding 350 µL to the column and centrifuging for 1 minute at 11,000 rcf. The next step was preparing the DNase I reaction mixture by adding 10 µL DNase I to 90 µL Reaction Buffer, then 95 µL of the DNase I reaction mixture was added to the membrane and incubated at room temperate for 15 minutes. Next, the membrane was washed by adding 200 µL wash buffer RW1 and then it was centrifuged for 30 seconds at 11,000 rcf, then 600 µL wash buffer RW2 was added and centrifuged again for 30 seconds at 11,000 rcf. Finally, 250 µL wash buffer RW2 was added and centrifuged for 2 minutes at 11,000 rcf. The final step consisted in 60 µL of RNA being eluted in RNase-free water. Finally, the concentration and purity of the RNA samples was calculated by measuring 1 µL of RNA using the Nanodrop ND-100 (Labtech, UK).

Synthesis of cDNA was performed using 100 ng RNA, 1 µL of random hexamers (Fisher Scientific, U.K., #SO142), and 1 µL of dNTP (Fisher Scientific, U.K., #R0191) per sample. Each tube was left at 70°C for 10 minutes and then transferred immediately to ice for 2 minutes. A master mix solution composed of 2 µL 10x reverse transcriptase (RT) buffer (Merck, Germany, #M1302-40KU), 1 µL M-MLV (Merck, Germany, #M1302-40KU), 0.5

µL Rnase OUT (Fisher Scientific U.K., #10777019) and 4.5 µL ultra-pure distilled water were added per sample to make a total volume of 20 µL. Samples were then incubated in a thermocycler at 25°C for 20 minutes, 37°C for 50 minutes and 80°C for 10 minutes to obtain cDNA.

Gene expression was performed using quantitative real-time polymerase chain reaction (qRT-PCR). Each reaction was prepared to 20 µL final volume containing Taqman Universal PCR mastermix (Applied Biosystems, U.K., #4304437),1 µL sample cDNA and specific commercially available Taqman gene expression assay (ThermoFisher Scientific, U.K., **Table 3.1.4.1**). All samples were assayed in triplicate and multiplexed using 18S (ribosomal Ribonucleic acid (RNA)) as a pre-optimised control probe. Reactions were carried out at 50ºC for 2 minutes,95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds and then 60°C for 1 minute according to the manufacturer's instructions. For data analysis, gene expression was calculated based on the following formula:

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

Where ΔΔCt= (Gene of interest- 18s)-Average of control.

Table 3.1.4.1. Genes considered and catalogue number.
(FIS1 = mitochondrial fission 1; OPA1 = optic atrophy 1; 18s = Ribonucleic acid (RNA) subunit 18s).

Gene	Gene assay ID	Company	Catalogue number
FIS1	Hs00211420_m1	Thermofisher	#4351370
OPA1	Hs01047013_m1	Thermofisher	#4351370
18s	Hs99999901_s1	Thermofisher	#4351368

3.1.5. Nanolive imaging

Nanolive microscope was used to visualize a single live cells and study mitochondria morphological changes with time.

For imaging the BEAS2BR1 cells were seeded, in a high glass bottom µ-Dish 35mm, (Thistle Scientific, U.K., #41122107) at concentration of 25,000 cells/mL and left to grow until 80% confluent. Once ready, the cells were treated with either 100 ng/mL of LPS or SCFA mix or a combination of these for either 6, 12, or 24 hr.

After treatment, cell culture media was replaced with 50 nM of Mitotracker Red (Invitrogen™, Thermofisher Scientific, U.K., #M7512) and 25 nM of Mitotracker Green (Invitrogen™, Thermofisher Scientific, U.K., #M7514), diluted in a phenol free, FBS free media (DMEM.F-12, Gibco, U.K., #11039-021), and cells were incubated for 25 minutes at 37°C in CO_2 incubator. Next, cells were washed 3 times with media and imaged using Nanolive microscope (Nanolive SA, 3-D cell explorer, Switzerland). Analysis of the images were performed using image J software following Stuart lab protocol (Valente et al. 2017).

3.1.6. EVOS imaging

Cells were seeded at the concentration of 25,000 cells/mL on a 6 well-plate, grown and treated with 100 ng/mL of LPS or SCFA or combination of both for either 6, 12 or 24 hr. Following their respective incubations Mitotracker Green FM (25 nM; Invitrogen™,Thermo Fisher, U.K., #M7514) and Mitotracker Red (50 nM; Invitrogen™, Thermo Fisher, U.K., #M7512) were added into the phenol-free media (DMEM/F-12, Gibco, U.K.,#11039-021) without FBS, and left in the incubator for 25 minutes at 37°C.

Cells were subsequently washed three times with media and imaged using EVOS™ M7000 Imaging System (Thermofisher, UK). A minimum of 50 cells per condition were analysed. Data were then reported as a ratio between the relative red fluorescence and the relative green fluorescence, to provide a measurement of the mitochondria activity according to the following formula:

$$Mitocondria\ activity = \frac{[Red\ fluorescence - cell\ background]}{[Green\ fluorescence - cell\ background]}$$

3.1.7. Mitochondria stress test

Mitochondrial respiration rate was measured using a Seahorse XF24 Extracellular Flux analyser (Seahorse Bioscience, Agilent technologies). For this assessment BEAS2B-R1 cells were seeded in a 24-well plate (Seahorse Bioscience, USA, #100777-004) at density of 20,000 cells per well and grown. One experimental group was pre-treated with SCFA mix on the day the cells were seeded. After 24 hr, cells were treated for 6, 12 or 24 hr with LPS 100 ng/mL or SCFA mix or a combined treatment of LPS 100 ng/mL and SCFA mix. Each experimental group consisted of 5 replicates and the assay was repeated on 1-3 separate occasions. Cells were assessed using an assay media made from combining Seahorse XF DMEM medium, pH 7.4, (Seahorse Bioscience, USA, #103575-100) supplemented with Sodium Pyruvate (1 mM, Agilent technologies, USA, #103578-100), Glucose (10 mM, Agilent technologies, USA, #103577-100) and L-Glutamine (2 mM, Agilent Technologies, USA, #103579-100). BEAS2-BR1 cell media was then replaced with assay media and incubated for 1 hr at 37°C in the absence of CO2 before being assayed.

The protocol consisted in an initial 30 minutes calibration step and 30 minutes equilibration step, followed by 3 repeated cycle steps for each compound injected: 3 minutes mix, followed by 2 minutes wait and 3 minutes measure, in accordance with the manufacturer's instructions. The compounds used, injected in the listed order, at the start of each cycle were: Oligomycin, Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) ad Rotenone/antimycin (Seahorse Bioscience, Mitochondria stress kit, #103010-100).

Reagents were injected to make a final solution of 1.5 µM Oligomycin, 2 µM FCCP and 0.5 µM Rotenone/Antimycin. WAVE software (Seahorse bioscience, Agilent technologies) was used to calculate oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The data were normalized by the basal respiration of each experimental group and shown as a percentage of change of the baseline to account for cell number variability between wells.

3.2 Human Trials

3.2.1. Investigation of body weight and gut permeability across asthma severity: The NTU-WATCH collaboration study-Ethics and study design.

The Nottingham Trent University (NTU) -Southampton study was approved by the Ethics Committee of NTU (ethics protocol: #689). All study participants provided written and informed consent in accordance with the Declaration of Helsinki.

NTU received a database and blood samples previously collected from patients who were clinically evaluated to suffer from severe asthma by a GP and collected with the

Wessex AsThma CoHort of difficult asthma (WATCH) study at Southampton University Hospital (https://clinicaltrials.gov/ct2/show/NCT03996590). Patients had a body mass index (BMI) between 18-50.4 kg/m^2 and most of them were overweight or obese. Blood samples (n=98) were collected from patients as described in **3.3.3** and serum and plasma were stored at -80°C until use. Samples were then transferred to NTU on dry ice and stored at -80°C until required. An anonymised participant database of the WATCH samples clinical information was maintained on the NTU DataStore. The DataStore project folder was protected by a multi-layer password system, meeting all necessary General Data Protection Regulation (GDPR) safeguards in accordance with relevant ethical approval guidance and good clinical practice.

At NTU, further recruitment of participants with a GP diagnosis of well-controlled asthma (n=26) and participants with no underlying diseases, referred as controls (n=34) was undertaken. As with the WATCH participants, all study participants recruited within NTU provided written and informed consent in accordance with the Declaration of Helsinki. Each participant completed an informed consent form and a self-reported health screen questionnaire to confirm that the inclusion and exclusion criteria were met. There were differences in the inclusion and exclusion criteria between the WATCH and NTU participants. The inclusion criteria for the WATCH participants was to be on "high dose therapies" and/or "continuous or frequent use of oral steroids", according to the BTS Adult Asthma Management Guidelines 2016. The exclusion criteria was to be diagnosed with asthma but not use "high dose therapies" and/or "continuous or frequent use of oral steroids", according to the BTS Adult Asthma Management Guidelines 2016. These criteria can be found in the published protocol of the WATCH study (Azim et al. 2019). The NTU participants were required to have a body mass index

(BMI) of 18-34.9, be a non-smoker, not being admitted to hospital during the past 12 months for your asthma, not suffering of pulmonary hypertension, embolism, or other pulmonary heart disease, have not taken antibiotics in the past 3 months and had a history or current evidence of gastrointestinal disease (e.g. chronic constipation, diarrhoea, irritable bowel syndrome, Crohn's disease). A full list of inclusion and exclusion criteria can be found in **Appendix 1: WATCH participant information sheet and consent form**. Pulmonary function, anthropometric measurement, asthma-controlled questionnaire (ACQ-6) and venous blood samples was assessed during the visit.

3.2.2. Human Nutritional intervention trial

The study was approved by the Nottingham Trent University Human Ethics Committee (ethics application: #675), and all procedures were conducted in accordance with the Declaration of Helsinki.

The study was a double-blinded, placebo-controlled cross-over design whereby participants with asthma were randomly assigned to consume either the prebiotic Bimuno containing 2.9 g galacto-oligosaccharide per day for 21 days in the form of one sachet (3.65 g) of Bimuno® Daily (Clasado Biosciences Ltd) or one placebo sachet containing 3.65 g of maltodextrin (Clasado Biosciences Ltd) for 21 days. This was followed by 14 days washout period before switching to the other treatment. The prebiotic and placebo were given to participants in single dose sachets and were in the form of a white powder that could be dissolved in liquids.

Participants with a GP diagnosis of well-controlled asthma (n=18: 4 males; 14 females) volunteered to take part in the study and provided written informed consent. A full list of the inclusion and exclusion criteria are listed in **Appendix 2: Prebiotic trial participant information sheet and consent form**. The main inclusion criteria were that participants were diagnosed with well-controlled asthma by a GP, had a BMI between 18-35 kg/m^2, were non-smokers, had no gastrointestinal diseases and had not consumed antibiotics in the past 3 months. Two participants declined to participate further. Three participants did not meet the inclusion criteria and therefore were excluded. Of the initial 18 participants recruited, a total of 13 participants successfully completed the study.

Participants visited the laboratory on 4 different occasions (days 0, 21, 35 and 56) and pulmonary function, anthropometric measurements and venous blood samples was assessed during each visit. Asthma related questionnaires (ACQ-6, Nijmegen, SNOT-22, Hull airways reflux, HADS, Epworth questionnaires) were completed at each visit. Blood collection and questionnaires were described in section **3.3**.

3.3 Human Trial Measurements

3.3.1. Anthropometric measurements

Body mass was measured to the nearest 0.1 kg using calibrated electronic scales (SECA 877 Scale, SECA, U.K.), wearing lightweight clothing and barefoot. Height was measured with a stadiometer (SECA stadiometer, SECA, U.K.). Participants stood bare foot with heels together with arms by their side. Hip and waist circumference were measured using a medical tape. Waist measurement was taken halfway between the lower rib and

the iliac crest point, and hip circumference as the widest point. Participants were instructed to breathe out gently while the measurements were taken.

Waist to hip ratio was calculated by dividing the waist by the hip circumference and used to estimate body fat distribution.

3.3.2. Pulmonary functions

Pulmonary function was conducted in accordance with the American Thoracic Society (ATS) and European Respiratory Society (ERS) guidelines (Miller et al. 2005) using a spirometer (Pneumotrac; Vitalograph, U.K.) calibrated with a 3L syringe. Participant's body mass, height, age, sex, ethnicity and smoker status were entered in the spirometry software in order to determine the personal predicted lung capacity value based on the predicted equation of Quanjer et co-workers and approved by the European Respiratory Society (Quanjer et al. 2013).

Participants performed the test in a standing position and wearing a nose clip.

Maximal flow-volume loops to determine absolute and percentage predicted forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), peak expiratory flow rate (PEF), forced expiratory flow rate from 25-75 of FVC (FEF_{25-75}), forced expiratory flow rate 75-85 of FVC (FEF_{75-85}) and FEV_1/FVC ratios were performed by the participants. Before the test, participants were instructed to inhale maximally, place their mouth around the mouthpiece filter and exhale as quickly as possible for six seconds. After six seconds participants were instructed to rapidly inhale to full inspiration, keeping the mouth on the mouthpiece. During each visit, participants performed a minimum of three flow-volume loops for pulmonary function measures. The test was accepted when the two highest values for the measurements of FEV_1 and FVC were within 0.150 L, with

the highest values taken for subsequent analysis (Graham et al. 2019). Data were reported as a % of the predicted lung capacity values.

3.3.3. Blood sampling

Venous blood was drawn from a vein in the antecubital fossa region of the elbow using a 23-gauge butterfly needle (Becton Dickinson (BD) Vacutainer Safety Lok Blood Set 23g x 7" Tubing, U.K.).

At each visit, blood (8 mL) was drawn into a Ethylenediaminetetraacetic acid (EDTA) plasma vacutainer (BD, U.K.) for the measurement of cytokines in blood plasma, and a further 10 mL into a serum separating tube (BD, U.K.) for the measurement of cytokines in blood serum.

EDTA plasma vacutainers were gently inverted and immediately centrifuged for 15 minutes at 1,500 g at 4°C. The plasma layer was aliquoted and subsequently stored at -80°C until analysis. Serum separating tubes were gently inverted and left to stand for 30 minutes at room temperature and then centrifuged for 15 minutes at 1,000 g at 4°C. The serum layer was then aliquoted and stored at -80°C until analysis. After blood sampling, the butterfly needle was removed, and firm pressure was applied on the puncture site to avoid hematoma.

Blood samples were collected, processed and stored in the exact same way from WATCH and NTU participants.

3.3.4. Enzyme-linked Immunosorbent assays

Commercially available enzyme-linked immunosorbent assays (ELISAs) were used to determine concentrations of cytokines from human blood serum and plasma samples. ELISAs were used to quantify lipopolysaccharides binding protein (LBP; Hycult Biotech, #HK315), Calprotectin (Bio-techne; #DS8900), and neutrophil gelatinase-associated lipocalin (NGAL) (Bio-techne; #DLCN20) levels in serum/plasma samples. All ELISAs were performed according to the manufacturers' instructions. Samples, reagents, and standards were prepared using the appropriate dilutions as instructed by the manufacturer (**Table 3.3.4.1**). For calprotectin and NGAL, the standards and samples were introduced into each well containing the capture antibody and incubated at room temperature to allow the antigen to bind with the capture antibody. The plate was washed with wash buffer and then the enzyme-linked polyclonal antibody, specific for the protein of interest, was added and the plate was incubated at room temperature under constant stirring. The plate was then washed again before adding the substrate solution and colours developed in proportion to the amount of protein bound in the initial step. Finally, the stop solution was added to stop the reaction and the plate was read immediately on a plate reader (Tecan Infinite M plex, Switzerland).

For the LBP assay, the same steps were followed but before adding the linked antibody, a biotinylated tracer was added to the plate (to bind the captured LBP antibody) and incubated at room temperature under constant stirring.

According to manufacturer's, the lower limits of detection for the assays were 4.4 ng/mL for LBP, 0.086 ng/mL for Calprotectin and 0.012 ng/mL for NGAL. The intra-assay and

inter-assay coefficient of variation (CV) were 2.2% and 4.1% for LBP, 3.4% and 4.6% for Calprotectin and 3.7% and 6.5% for NGAL, respectively.

All samples were analysed in duplicate on one 96-well plate.

Table 3.3.4.1. Elisa assays kit, catalogue number and relative dilution used.
(LBP= lipopolysaccharide binding protein; NGAL= neutrophil gelatinase-associated lipocalin).

Marker considered	Company	Catalogue number	Sediment used	Dilution
LBP	Hycult Biotech	HK315	Plasma	1:2000
Calprotectin	Bio-techne	DS8900	Plasma	1:100
NGAL	Bio-techne	DLCN20	Serum	1:40

3.3.5. Analysis of blood plasma inflammatory cytokines

Blood cytokine levels (pg/mL) were measured in plasma from 98 WATCH study participants using a human magnetic Luminex® multiplex assay for C-C Motif Chemokine Ligand 3 (CCL3), Chemokine Ligand 4 (CCL4), Chemokine Ligand 11 (CCL11), Interleukin (IL)-4, IL-5, IL-6, IL-8, Periostin, Chitinase-3like-1, Neutrophil gelatinase-associated lipocalin (NGAL), Leptin, Adiponectin, Granzyme A, Granzyme B and Tumor Necrosis Factor-Alpha (TNF-α) according to the manufacturer's instructions (R&D Systems, Bio-Techne Ltd, Abington, UK).

3.3.6. Nutritional intervention trial: serum markers analysis

A Pentra C400 (Horiba, U.K.) was used to measure serum biomarker (mmol/L) from participants with asthma who attended the prebiotic intervention trial to assess changes in biomarkers between each visit. The measured biomarkers were Glucose (Horiba, U.K.,

#1220001668), high-density lipoprotein (HDL) direct (Horiba, U.K., #1220001636), low-density lipoprotein (LDL) (Horiba, U.K., #1220001638), total cholesterol (Horiba, U.K., #A11A01634), C-reactive protein (CRP) (Horiba, U.K., #A11A01611) and Triglycerides (Horiba, U.K., #1220001640).

All samples were measured in duplicate and run together to avoid batch variations. Variation between repeats was <0.01 mmol/L.

3.3.7. Self-reported questionnaires:

3.3.7.1 Asthma Control Questionnaire (ACQ)-6

The ACQ-6 test is a validated questionnaire designed to assess asthma control and participants had to recall their symptoms over the past week (Juniper et al. 1999).

The ACQ-6 includes six items which are scored on a 6-point scale (0 = never/no symptoms/none; 6 = all the time/very severe symptoms/totally limited) and a total maximum score of 36. The total raw score is then divided by the number of items in the questionnaire (6) and results were used for analysis.

Optimal cut-offs to identify poorly controlled asthma were set at a score ≥1.5 and those deemed to represent well controlled asthma were set at a score ≤0.75 with a minimal significant difference being a 0.5 change in score (Juniper et al. 1999). The questionnaire has shown good test reproducibility in stable asthma patients (intraclass correlation coefficient = 0.9) (Juniper et al. 1999).

The ACQ-6 questionnaire is displayed in **Appendix 4.1: Asthma control questionnaire (ACQ-6)**.

3.3.7.2 Nijmegen questionnaire

The Nijmegen score questionnaire is a validated tool used for screening symptoms associated with dysfunctional breathing pattern and for hyperventilation syndrome in asthmatics from mild to severe asthma conditions (Grammatopoulou et al. 2014). It comprises of a16 symptoms checklist to be answered on a 0 to 4 scale (0=never; 4=very often) and a total score of 64. A score above 23 suggests a positive diagnosis of hyperventilation.

The Nijmegen questionnaire is displayed in **Appendix 4.2: Nijmegen questionnaire.**

3.3.7.3 The Hull airway reflux questionnaire

The Hull airways reflux questionnaire is used to assesses symptoms associated with airways reflux. It is used by clinicians to diagnose non-acid gaseous reflux impacting on the airway leading to bronchospasm and shortness of breath. It is self-administered and comprises 14 questions with a maximum score of 70. Each question is rated on a scale (0= no problem; 5=severe/frequent problem). The average score for healthy people is 4 out of 70 and the upper limit of normal is 13, whereas a score higher than 13 indicate presence of airway reflux (Morice, Spriggs, and Bell 2011).

The Hull airways questionnaire can be found in **Appendix 4.3: Hull airway reflux questionnaire.**

3.2.7.4 Sino-nasal outcome test (SNOT)-22

The sino-nasal outcome test is a self-reported questionnaire with an outcome measure for patients with chronic rhinosinusitis. It was originally used and validated to distinguish patients with Chronic rhinosinusitis and to correlate high score SNOT-22 score with reduced quality of life (Hopkins et al. 2009). The SNOT-22 assesses a patient's symptoms over the preceding 2-week period, which increases its ability to detect significant clinical changes over time.

More recently the SNOT-22 questionnaire was used as a widespread outcome assessment tool to evaluate patient self-reported outcomes measure in diseases such as allergic rhinitis (Husain et al. 2020), obstructive sleep apnoea (OSA) (Bengtsson et al. 2020), nasal septum deviation (Buckland, Thomas, and Harries 2003) and asthma (C. Huang et al. 2017). However, the SNOT-22s usefulness in clinical practice is limited to a snapshot in time. The SNOT-22 consists of 22 questions ranging from 0 ('no problem') to 5 ('problem as bad as it can be'), with the total score of 110.

Any value above 8 is considered abnormal and indicates worse symptoms (Questionnaire cut-off scores are listed in **Table 3.3.7.4.1)**.

The SNOT-22 is displayed in **Appendix 4.4: SNOT-22 questionnaire.**

Table 3.3.7.4.1. SNOT-22 related score and severity of disease.

SNOT-22 score	outcome
0-7	normal
8-20	mild
21-50	moderate
>50	severe

3.3.7.5 Hospital Anxiety and Depression Scale (HADS questionnaire)

The HADS is a self-reported questionnaire to detect anxiety disorders and depression among patients in nonpsychiatric hospital clinics (Zigmond and Snaith 1983). It is useful to detect states of depression and anxiety as well as the severity of emotional disorder (Zigmond and Snaith 1983). It was initially developed for hospital setting use but numerous studies conducted around the world have confirmed its validity for the general population use, as confirmed by a review of 747 papers that used HADS in various settings (Bjelland et al. 2002). It is divided into an Anxiety subscale (HADS-A) and a Depression subscale (HADS-D) both containing seven questions each.

For each section, the normal value range is between 0-7, a value between 8-10 is considered borderline, while a subscale scores above 11 is considered abnormal and may require medical attention.

The HADS questionnaire is displayed in **Appendix 4.5: HADS questionnaire**.

3.2.7.6 Epworth scale

Epworth scale is a validated test used to measure sleepiness. It was developed by Murry W. Johns in 1991 and it provides a measurement of the subject's general level of daytime sleepiness (Johns 1991). The Epworth scale evaluates if the participants experience excessive sleepiness that may require clinical attention. The scale has been correlated with respiratory disturbances and obstructive sleep apnoea, which are diseases frequently correlated with asthma (Dixit 2018).

The questionnaire comprises 7 items with a total score ranging from 0 to 24. In healthy adults the normal value of sleepiness is below 10, while values above 16 represent an abnormal day time sleepiness. The detailed cut-off values are listed in **Table 3.3.7.6.1.** The Epworth scale is displayed in **Appendix 4.6: Epworth questionnaire**.

Table 3.3.7.6.1. Epworth scale interpretation scale.

Epworth scale interpretation:	
0-5	lower normal daytime sleepiness
6-10	normal daytime sleepiness
11-12	mild excessive daytime symptoms
13-15	moderate excessive daytime symptoms
16-24	severe excessive daytime symptoms

3.4. Nuclear Magnetic Resonance (NMR)

Metabolomic analysis of human plasma samples was conducted using a bench top low-field Nuclear Magnetic Resonance (bNMR).

NMR is a chemistry technique that uses magnetic field and electromagnetic frequencies to study molecular structure. There are different methods of NMR used, but the most common and the one considered in this study was ^{1}H NMR due to the high abundance of ^{1}H isotope in the atoms of molecules. There are several types of NMR available, but they can be divided in two main categories: high field and low-field NMR. High-field NMR operates at the frequency of above 300MHz offering both high sensitivity and resolution spectrum that allows accurate identification of compounds in samples. Although, the high cost and the complexity of the equipment limit its use (Castaing-Cordier, Bouillaud,

et al. 2021). Low-field NMR operates at a frequency between 40 and 100mHz; it is less sensitive and has lower resolution than high field NMR, which results in a poorly resolved spectra making difficult to distinguish between metabolites (Castaing-Cordier, Ladroue, et al. 2021). Although advanced data processing and optimised pulse sequences have made it possible to improve structural and quantitative information obtained from the spectrum (Castaing-Cordier, Bouillaud, et al. 2021; Giberson et al. 2021). Other advantages of low-field NMR are its contained cost in terms of maintenance and support cost and a minimal samples preparation.

The bNMR, considered in this study, used a low-field frequency of 60MHz and had the advantage of being compact and therefore requiring a minimal space.

There are three main components to consider when looking at the spectrum: chemical shift, integration, and spin-spin coupling.

Chemical shift provides information about the chemical environment of a protons. If the spectrum is more downfield, this means that the proton is closer to the electronegative atoms. If the peak is upfield, the proton is further way from the electronegative elements (Fig.1).

Chemical shift is expressed in part per million (ppm) and common reference peak used is Trimethylsilylpropanoic acid (TSP) that has a chemical shift of 0.

Samples's integration represents the area under the curve of the peak which give information on regards how many chemically equivalent protons are generating that peak.

Spin-spin coupling represent how the orientation of neighbouring nuclei affect the magnetic field causing a splitting of the signal for each nucleus in two or more lines. The number of splitting generated indicates the number of chemically bonded nuclei in the vicinity of the observed nucleus. A schematic NMR spectrum is represented in **Fig.3.4.1.**

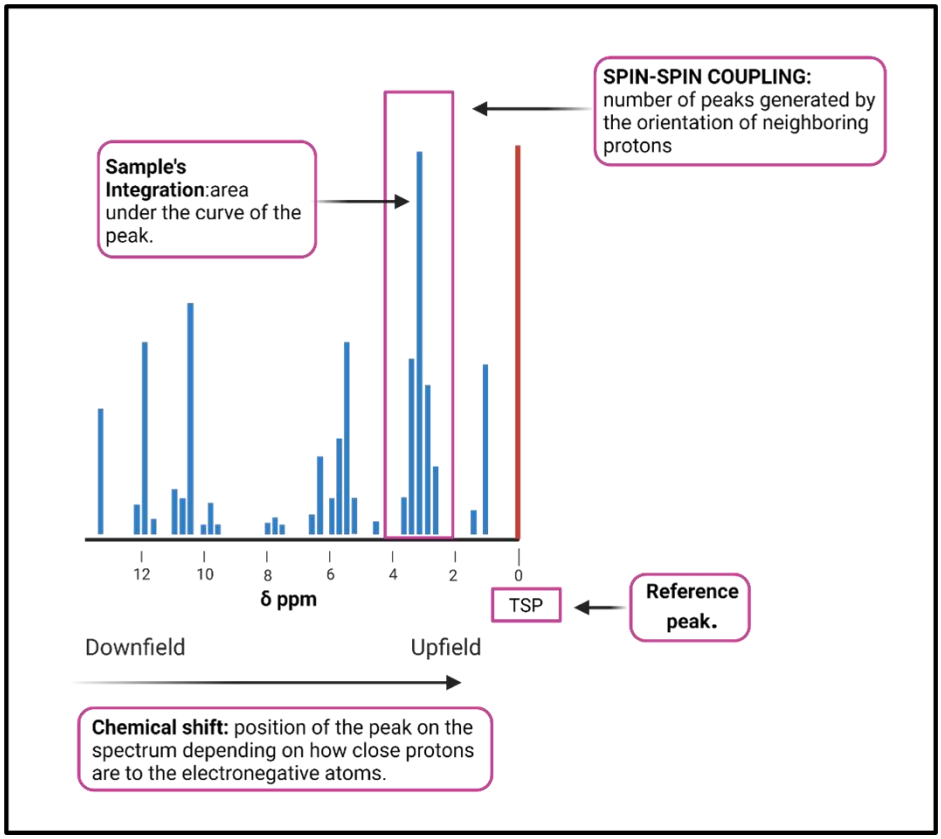


Fig.3.4.1. Schematic representation of NMR spectra.
Schematic representation of NMR spectra showing the three major component of the peak: spin-spin coupling, samples' integration, and chemical shift. The scale is expressed in δ, which represents frequency of the signal- the frequency of the spectrophotometer. Other abbreviations are part per million (ppm) and Trimethylsilyl propanoic acid (TSP).

3.4.1. Samples preparation for bNMR and analysis

A total of 12 human plasma samples, 6 belonging to subjects with no underlying disease and 6 with well-controlled asthma, were collected from participants as described in section **3.3.3** and stored at -80°C until use. Before proton benchtop nuclear magnetic

resonance (^{1}H-bNMR) experiment, samples were thawed on ice until defrosted. Then, 450 µL of each plasma sample was mixed with 50 µL of Trimethylsilylpropanoic acid (TSP) (Acros organics, U.K.) (0.05% w/v), 25 µL of Sodium azide (Sigma Aldrich, U.K.) (18.8mg in 10 mL Deuterium) (Thermofisher Scientific) and 25 µL of Phosphate Buffer (7.1pH) (Sigma Aldrich, U.K.). The mixture was then transferred into an 8" NMR tube (WILMAD 5mm 100mHz NMR benchtop tube, U.S.A., #WG-BTNMR-8) for analysis. Following bNMR spectrophotometer (X-pulse 60MHz, Oxford Instruments, U.K.) calibration, each sample spectra was acquired using the pulse sequence noted as (Water suppression Enhancement though T1) WET-180°- Carr-Purcell-Meiboom-Gill (CPMG). The WET °180 was used for water suppression, while CPMG was a spin-echo pulse sequence used to reduce protein signals. Each sample were run on the ^{1}H-bNMR for 23 hr using 4096 scans at the acquisition time of 3.14 seconds. Once completed, the spectra were binned into 0.01 part per million (ppm) wide regions and the regions of the spectrum containing no signal were removed. The area considered were between 0-4 ppm regions as they contained the majority of peaks and, therefore, used for statistical analysis.

The data was processed using Mestrenova software where baseline correction, phase correction and reference alignment was completed. Metabolites identification was conducted using human metabolome database (HMDB) website and existing literature. Metabolites comparison between bNMR and high field NMR were conducted by visual inspection of the stacked spectra. Statistical analysis was conducted using MetaboAnalyst 5.0. Data were normalized by sum and filtered by the mean intensity value. Pareto scaling was applied and then Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OrthoPLS-DA) were performed.

To confirm results obtained from low-field bNMR, plasma samples were analysed using high-field NMR (HF-NMR) (400MHz, Oxford Instruments, Jeol consol). Once the samples was prepared (the same sample prepared and used for the bNMR was re-used for the HF-NMR), they were loaded onto the autosampler of the 400MHz spectrometer. The samples followed an automatic quick shim (adjusting the resolution of the signal by optimizing the homogeneity of the magnetic field), tuning and matching (optimises the efficiency of the radio frequency pulses and maximises sensitivity). The parameters and exact pulse sequence used for bNMR was used for HF-NMR (WET-180°-CPMG with 4096 scans at the acquisition time of 3.14 seconds).

The data obtained were then processed and statistically analysed with the same method described above for bNMR data. In addition, to assess differences in metabolites levels between groups, a t-test was performed on selected metabolites.

3.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.5.1 software, unless otherwise specified. All graphs were reported as mean±standard error of the mean (SEM) unless otherwise specified. Statistical analysis test analysis was based on normality test using Shapiro-Wilks criteria.

For cell culture work, mainly one-way ANOVA plus Tukey's post hoc test or independent samples t-test were used to determine difference between the treatments used.

For the WATCH-NTU collaboration study, multiple linear regression and Pearson r correlation was used to identify correlation between BMI and inflammatory cytokines

and lung function. To determine association between BMI and/or gut permeability and lung function, one-way ANOVA plus Tukey's post hoc test or Kruskal-Wallis non-parametric test were used, based on normality test outcome. Comparison between two groups was performed using unpaired t-test or Mann-Whitey test, based on normality test outcome.

For the NMR pilot study, data were analysed using MetaboAnalyst 5.0. Statistical analysis was performed as descripted in section **3.4.1**. Briefly, following data normalization, PCA and OrthoPLS-DA analysis were used to identify difference in the spectra region between participants with asthma and healthy controls.

For the prebiotic human intervention trial, mainly two-way ANOVA plus Šidàk multiple comparison test was used to compare results before and after the supplementation. Data were presented as a mean±Standard Deviation (SD) unless otherwise specified.

The % of change between day 0 and day 21 for each parameter was calculated as follow:

$$\%change = \frac{(Outcome\ day\ 21 - outcome\ day\ 0)}{Outcome\ day\ 21} * 100$$

For human studies, data analysed with the ANOVA test, partial eta squared (η^2) effect sizes was calculated as:

$$\eta^2 = \frac{Sum\ of\ the\ squares\ beetween\ groups\ (SSE)}{Sum\ of\ the\ squared\ total\ (SST)}$$

Effect size for Kruskal-wallis test was Epsilon squared (ϵ^2), calculated as follow:

$$\epsilon^2 = \frac{H}{(n^2 - 1)/(n + 1)}$$

Where

H= outcome value obtained from the Kruskal-Wallis test.

N=Total sample size.

Cohen d (d) effect sizes were calculated for variables assessed by unpaired t-test as:

$$Cohen\ d\ (d) = \frac{Mean\ Value\ group\ 1\ (M1) - Mean\ Value\ group\ 2\ (M2)}{SDpooled}$$

Where pooled standard deviation (SD):

$$\sqrt{\frac{SD1^2 + SD2^2}{2}}$$

Statistical significance was considered to be $p<0.05$, and reported as *$p<0.05$, **$p<0.01$, **$p<0.001$; **** $p<0.0001$.

Chapter 4: The role of LPS and SCFA on mitochondria morphology and function in airway cells

4.1 Introduction

Mitochondria are essential organelles that are involved in several processes including regulation of adenosine triphosphate (ATP) production, autophagy, key process that remove damaged material from the cell, and stress responses (Shen et al. 2022). This makes mitochondria essential to a cell's life by regulating its growth, homeostasis, and movement. Mitochondria are dynamic organelles that are constantly reorganising themselves by fission, fusion and biogenesis processes, and autophagy; a balance of these processes are essential to maintain a healthy mitochondrion (Wai and Langer 2016). Dysregulation of mitochondrial function can compromise the cell, which may promote excessive reactive oxygen species (ROS) production and inflammation. ROS are generated as byproducts of oxidative phosphorylation in the mitochondria. They play a dual role as destructive and constructive species. Specifically, ROS are involved in many redox-governing activities of the cells for the preservation of cellular homeostasis (for example protection against invaders, regulation of autophagy) (Bardaweel et al. 2018). However, ROS overproduction has been reported to result in oxidative stress, a deleterious process involved in the damage of cell structures that is linked with various disease states including asthma (Bhatraju and Agrawal 2017a; Mabalirajan and Ghosh 2013). ROS overproduction can lead to oxidative damage to mitochondrial proteins, membranes and DNA, impairing the ability of mitochondria to synthesize ATP and to carry out their metabolic functions, including the Krebs acid cycle and fatty acid oxidation (Bardaweel et al. 2018).

Altered mitochondrial metabolism has recently been linked with asthma disease. In fact, an aberrant mitochondria metabolism can negatively affect oxidative stress, airway

contractility, apoptosis, and proliferation, all of which can exacerbate the disease (Bhatraju and Agrawal 2017b). Additionally, altered mitochondria function have been associated with low metabolic activity which is a key characteristic of obesity (Bhatraju and Agrawal 2017b).

The gut microbiota can also positively or negatively affect mitochondrial activity and production of ROS (Hu et al. 2020; Saint-Georges-Chaumet et al. 2015a). One of the mechanisms by which the microbiota may increase healthy mitochondrial function is through the release of short chain fatty acids (SCFA), the end-product of intestinal microbial fermentation of carbohydrates. Prior studies using the SCFA, butyrate, one of the major SCFA found in humans, along with propionate and acetate, has shown that it may regulate mitochondrial activity by rescuing oxidative phosphorylation and improving ATP levels. In the study conducted by Donohoe et al., they observed a reduction in tricarboxylic acid (TCA) cycle activity in germ-free (GF) mice, assessed by transcriptomic and proteomic data(Donohoe et al. 2011a). The TCA cycle plays a crucial role in cellular energy metabolism by converting NAD+ to NADH, which fuels the electron transport chain for ATP production via oxidative phosphorylation. The diminished TCA cycle activity in GF colonocytes led to a lower NADH/NAD+ ratio in mitochondria.

To directly attribute this energy deficit to the absence of gut microbes, the researchers colonized GF mice with microbiota from control mice. Additionally, they investigated the specific impact of butyrate, by colonizing GF mice with a butyrate-producing bacterial strain called *Butyrivibrio fibrisolvens* (Diez-Gonzalez et al. 1999; Rumney et al. 1995).

Their experiments demonstrated that both complete microbiota and colonization with *B. fibrisolvens* rescued oxidative phosphorylation in colonocytes compared to GF mice.

Importantly, this restoration of microbial colonization, especially with butyrate-producing bacteria, led to improved ATP levels in the colonocytes (Donohoe et al. 2011a).

Another in vitro study using β-cells investigated the role of acetate on cell metabolism. Hu et all. treated β-cells with 1mM of acetate and evaluated its effect on oxygen consumption rate (OCR), indicator of mitochondrial respiration, compared to control group. Data showed an increase of 22% of OCR compared to control cells, assessed using a mitochondria stress test kit (Hu et al. 2020). Furthermore, acetate appears to reduce the production of ROS in cells during cellular induced stressed conditions (Hu et al. 2020).

However, in cases where there is an imbalance in the microbiota population there can be a rise in gut-derived lipopolysaccharide (LPS) which can enter the circulation and initiate inflammation throughout the body, turning inflammation from local to systemic. LPS can induce the release of ROS from mitochondria, contributing to an increase in the inflammatory response and cellular oxidative stress (Saint-Georges-Chaumet et al. 2015b). Following an increase in ROS production, mitochondria release superoxide dismutase 2 (mtSOD2) to limit the damage caused by the oxidative stress (Ning et al. 2021).

Yet beyond LPS-induced mitochondrial functional effects, recent studies have observed LPS to modify mitochondria morphology with the overexpression of fission processes which in turn leads to mitochondrial fragmentation in at least primary neuronal immune cells, microglia (Nair et al. 2019b). In contrast, SCFAs appear to mitigate LPS-induced change in fission and fusion ratio, protecting mitochondrial structure as observed with

in vitro study using a combination of human islet cells and mouse insulinoma cell line MIN6 (Hu et al. 2020). Fusion and fission processes are essential to regulate mitochondrial division, hence fundamental to maintain mitochondrial quality and function. In order to regulate the mitochondrial division, balance of fission and fusion process are essential (van der Bliek et al. 2013). An alteration in fission/fusion ratio could significantly affect mitochondria structure leading to mitochondria fragmentation and the possible accumulation of irreversibly damaged mitochondria, which in turn may lead to excessive reactive oxygen species (ROS) and pro-inflammatory cytokine production (Andrieux et al. 2021; Liesa and Shirihai 2013).

One mechanism through which the mitochondria can respond to a gut derived LPS insult is by increasing biogenesis and autophagy of mitochondria. This mechanism is used to replace the damaged mitochondria with new ones to clear damaged material and allow the cell to perform its normal function as observed in a recent study investigating the effect of LPS in monocytic cells (Widdrington et al. 2018b).

Although the mechanism of action of LPS and SCFA has been studied in several cell types, little is known about the effect of LPS and SCFA on airway cells (BEAS2B-R1) and how this may provide a mechanism for how gut-derived LPS may impact lung function.

AIMS OF THE STUDY

Thus, the aim of this chapter was to:

- Evaluate whether in airway cells LPS and/or SCFA could affect mitochondrial functionality (expression of fusion and fission genes and proteins) and

morphology (by imaging live mitochondria to determine if alterations in mitochondrial branching and size occur).

- Determine how airway cell mitochondrial function may be compromised by LPS and rescued by SCFA treatments (acetate, butyrate and propionate mix at a ratio of 80:10:10 (2mM:0.25mM:0.25mM)).

  Define whether mitochondrial biogenesis is enhanced by LPS or SCFA treatments.

4.2 Material and Methods

4.2.1 Cell culture: BEAS2B-R1 cells

The human epithelial lung cell line BEAS2B-R1 was cultured in a growth media composed of 500 mL Dulbecco's Modified Eagle Medium (DMEM) cell culture media (Gibco U.K.) containing 10% Foetal Bovine Serum (FBS) and 0.5 mg/mL penicillin-streptomycin-Glutamine (Pen strep), referred as cell culture media. Briefly, $1x10^5$ cells were seeded on to 6-well plates unless otherwise specified and grown to 80% confluence. Once confluent, cells were treated for 6, 12 or 24 hr with either 10 or 100 ng/mL of LPS with or without SCFA mix composed of acetate, butyrate, and propionate at a ratio of 80:10:10 (2mM:0.25mM:0.25mM).

In addition to the above treatments, 2 plates were pre-treated with SCFA for 24hr and then treated with:

- LPS 10 ng/mL +SCFA
- LPS 100 ng/mL+ SCFA

Following the initial results obtained with the Western Blot analysis, all subsequent experiments were conducted using a high dose of LPS (100 ng/mL), SCFA and the SCFA pre-treated cells treated with LPS 100 ng/mL and SCFA at 6, 12 and 24 hr.

4.2.2 Protein extraction and Western blot

For protein extraction, $1x10^5$ BEAS2B-R1 cells were collected in 250 µL of 1x radioimmunoprecipitation assay buffer (RIPA) (Millipore U.K.), with dissolved protease and phosphatase inhibitor solutions (Roche Complete Mini protease inhibitor cocktail

tablets) and sodium fluoride (NaF, Fisher Scientific, U.K.) and sodium vanadate (Na_3VO_4, Acros Organics, U.K.).

A Bio-Rad detergent protein assay kit (Bio-Rad Laboratories, USA) and plate reader (Infinite m plex, U.K.) were used to quantify protein concentrations.

As previously described in section **3.1.3** for Western blot analyses, 20-30 µg of protein was loaded onto a polyacrylamide gel, transferred on to a nitrocellulose membrane and then incubated with a primary antibody diluted in either 0.2% I-block phosphate buffer saline -tween (PBS-T) or 5% BSA 1x tris-buffer saline tween (TBST) at 4ºC overnight. The next day, the primary antibody was removed, and the membrane was then washed 3 times with PBS-T or TBS-T. The secondary antibody was then added and incubated for 60 minutes under constant stirring. A chemiluminescence detection system (ECL Plus, GeneFlow, U.K.) was used to visualize protein bands, and densitometry was conducted using ImageJ Software. Equal protein loading was confirmed by examining ß-actin protein expression.

Primary antibodies utilized were listed in **Table 3.1.3.1**.

4.2.3 RNA isolation and qRT-PCR

For RNA extraction, BEAS2B-R1 cells were harvested by adding 350 µL of RNA lysis buffer (RLY) (Bioline U.K.) and 3.5 µL β-mercaptoethanol (β-ME) (Sigma) to each well. RNA was extracted from cell culture samples using Isolate II RNA Mini Kit (Bioline U.K., #BIO-52073) according to manufacturer's instructions. Synthesis of cDNA was carried using 1 µg of RNA as described in section **3.1.4**.

Gene expression was assayed through quantitative real-time polymerase chain reaction (qRT-PCR). Each reaction was prepared to 20 µL final volume containing Taqman Universal PCR mastermix (Applied Biosystems, U.K.), 1 µL sample cDNA, a specific Taqman gene expression primer of interest (Applied Biosystems, UK) and 20x 18s (ribosomal RNA) as a control. The primer sequences and commercial catalogue numbers used in this project are listed in **Table 3.1.4.1**. All samples were assayed in triplicate. The assay reaction was performed according to the manufacturer instructions at 50 °C for 2 minutes, 95 °C for 10 minutes and then 40 cycles of 95 °C for 15 seconds and then 60 °C for 1 min.

The data were analysed using ΔCt that was calculated by subtracting the difference between 18s and the target gene.

Finally, gene expression was calculated as: $2^{-\Delta\Delta Ct}$

where *ΔΔCt*= (Gene of interest- 18s)-Average of control

4.2.4 Mitochondria Stress Test

Seahorse XF24Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies) was used to measure mitochondrial respiration rate. Bronchial epithelial cells BEAS2B-R1 were seeded onto 24-well plates (Seahorse Bioscience, Agilent technologies) at a density of 20,000 cells/well, grown until 80% confluent and then treated for 6, 12 or 24 hours with or without LPS 100 ng or SCFA.

Each experimental group was assayed in 5 replicates on at least 2 separate occasions.

Seahorse buffer media, prepared as instructed by the manufacturer, was used to run the experiment. The mitochondria stress test consisted of a 30 minute calibration step and a 30 minutes equilibrium step, followed by cycles repeated 3 times: 3 minutes mix, 2 minutes wait and 3 minutes measure. The order of the reagents injected for each cycle were Oligomycin, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and Rotenone/Antimycin (Agilent technologies, Mitochondria stress kit). The reagents were injected at the final concentration of 1.5 µM for Oligomycin, 2 µM FCCP and 0.5 µL Rotenone/Antimycin, according to manufacturer's instruction.

The WAVE software calculated Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) (Seahorse Bioscience, Agilent Technologies).

4.2.5 Mitochondrial activity analysis

To determine mitochondrial activity, fluorescent imaging of live cells was conducted using a EVOS microscope.

BEAS2B-R1 cells were seeded at the concentration of 25,000 cells/mL in a 6 well-plate and left to grow until 80% confluent. The following day, cells were treated with either LPS 100 ng, SCFA or a combination of them for 6, 12 or 24 hr. Once ready, DMEM cell culture media (Gibco U.K.) was replaced with serum free, phenol-free media (DMEM, F-12, Gibco, U.K.) containing 50 nM Mitotracker red (Invitrogen, Thermofisher Scientific, U.K.) and 25 nM of Mitotracker green (In vitrogen, Thermofisher Scientific, U.K.). Cells were incubated for 25 minutes at 37°C. Following this, cells were washed 3 times using with serum free, phenol-free media and live imaged using an EVOS microscope.

Images are shown at 20x magnification and are representative of 3 independent experiments and a minimum of 100 cells analysed of each experimental group. To quantify mitochondrial activity, ratios between red Mito tracker staining cells and green Mito tracker-stained cells were calculated using ImageJ.

4.2.6 Nanolive Imaging

Analysis of the mitochondrial morphology network was performed through live cell imaging using a Nanolive microscope. BEAS2B-R1 cells were seeded at the concentration of 25,000 cells/mL in a µ-Dish 35 mm, high Glass Bottom (Thistle Scientific, U.K.) and left to grow until 80% confluent. Cells were then treated for 6, 12 or 24 hr with or without LPS.

After completion of treatment, culture media was replaced with 50 nM of Mitotracker Red (Invitrogen, Thermofisher Scientific) and 25 nM of Mitotracker Green (In vitrogen, Thermofisher Scientific) and cells were incubated for 25 minutes at 37 °C. Cells were then washed 3 times with serum-free, phenol-free media (DMEM F-12, Gibco, U.K.,) and imaged using Nanolive microscope (Nanolive SA, 3-D cell explorer). Morphological assessment of mitochondria was conducted using Nanolive images with image J software as previously described by Stuart lab (A. J. Valente et al. 2017). The data evaluated were: mitochondria number, area, branches, average branches length and fragmentation (ratio between mitochondria area and perimeter). Images are shown at 20x magnification and are representative of 3 independent experiments and a minimum of 30 cells counted for each experimental group.

4.2.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.5.1 software. Data analysis was based on normality test outcome, assessed by using Shapiro-Wilks test criteria. All graphs are reported as mean±standard error of the mean (SEM) unless otherwise specified. Comparison between multiple groups were made using one way ANOVA analysis plus Tukey's post hoc test. Comparison between two groups were made with independent samples t-test, if passed the normality distribution test. If not, analysis was conducted using a Kruskal-Wallis non-parametric test. Statistical significance was considered to be $p<0.05$ and reported as follow: *<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.3 Results

4.3.1 LPS altered mitochondria fusion/fission genes

A preliminary analysis was performed to look at gene expression of one fission gene, mitochondrial fission 1 (FIS1), and one fusion gene, optic atrophy 1 (OPA1), following treatment of BEAS2-BR1 cells with 100 ng/mL of LPS with or without SCFA for 6, 12 or 24 hr.

LPS treated cells showed a significant overexpression of the fission gene, FIS1, at 6 hr (*p<0.05) and 12 hr (**p<0.01) compared to the basal level, while it remained elevated at 24hr but not statistically significant **(Fig. 4.3.1.1-**b), d), f)). Cells treated with SCFA alone resulted in an overexpression of FIS1 at 6 hr (****p<0.0001) compared to control, while it returned to baseline levels at 12 hr and 24 hr. SCFA pretreated cells in combination with LPS ((P)LPS100+SCFA) exhibited a level of FIS1 similar to the control level in all the timepoints considered.

LPS did not change the expression of the fusion gene OPA1 at any of the timepoints considered, although OPA1 tended to be more highly expressed at 24 hr compared to baseline (**Fig. 4.3.1.1-Fig. 4.3.1.2** -a), c), e)). SCFA alone had no significant effect on the expression of OPA1 at the considered timepoints. However, SCFA tended to increase the expression of OPA1 at 6 hr compared to the control. Pre-treating cells in combination with LPS led to an overexpression of the OPA1 gene at 12hr (**p<0.01) compared to the control group, while no other changes were reported at the other timepoints (**Fig. 4.3.1.1-**-a), c), e)).

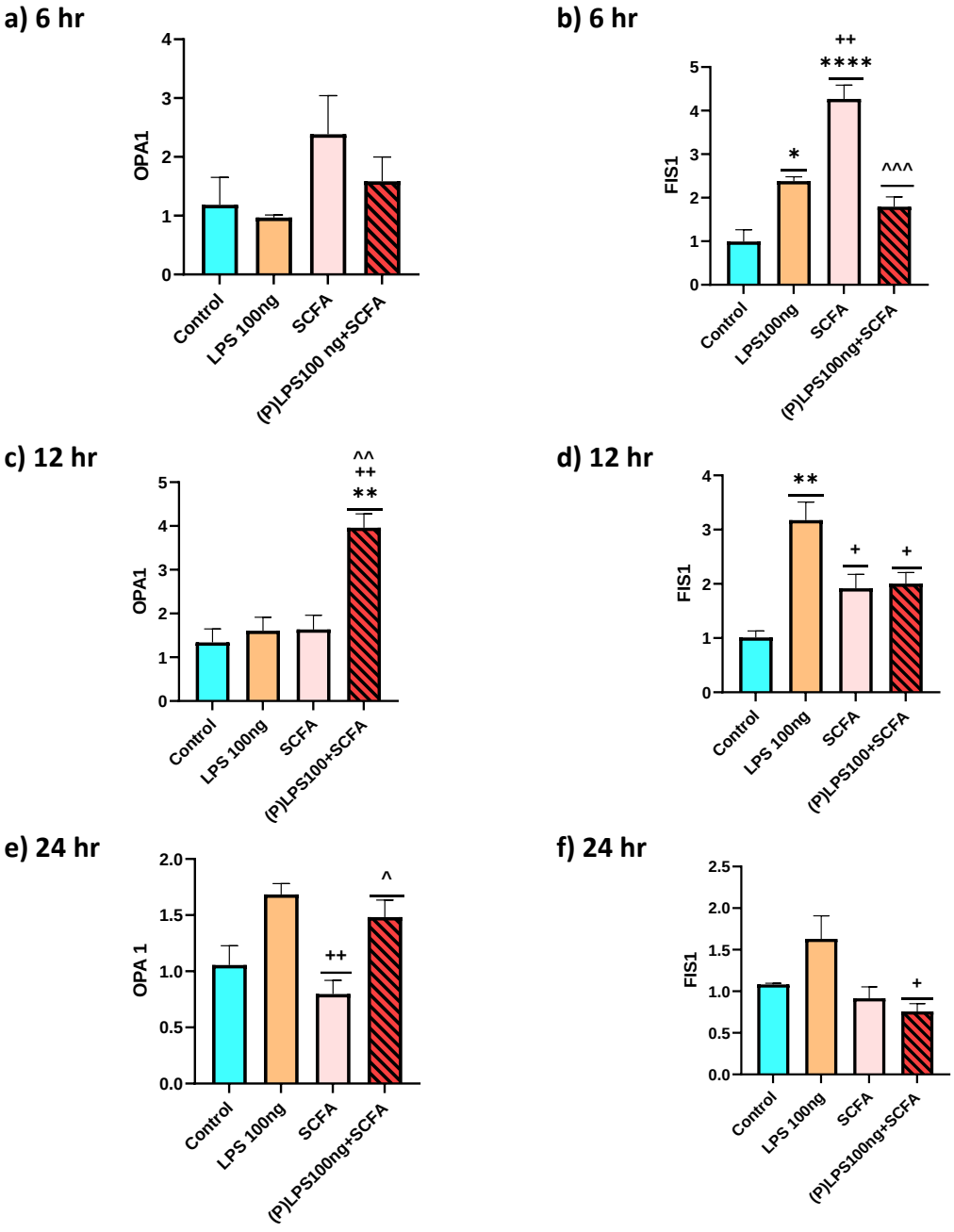


Fig. 4.3.1.1. FIS1 and OPA1 gene expression in BEAS2B-R1 cells treated with LPS ± SCFA. Gene expression was assessed for FIS1 and OPA1 at 6 hr (a, (b), 12 hr (c, d), 24 hr (e, f) (n=3)). Data are presented as the mean with standard error of the mean (SEM). Statistical analysis was performed using one way ANOVA plus Tukey's post hoc analysis. p-values are displayed as follows: (*=control vs other treatments; += LPS 100ng vs other treatment; ^=SCFA vs other treatments) *p<0.05; **p<0.01,***p<0.001.

A ratio analysis between FIS1/OPA1 was performed to evaluate if there was an alteration in the fission/fusion levels and the results were reported as a % of change from the control group. LPS treated cells showed a 200% increase in the FIS1/OPA1 ratio at 6 hr and 12 hr compared to the basal level (6hr, 12hr: *p<0.05) (**Fig. 4.3.1.2**-a) b)). At 6hr, although not significant, combined treatment of LPS and SCFA appeared to partially restore FIS1/OPA1 ratio to basal levels (p=ns, **Fig. 4.3.1.2**-a)). At 12 hr, combined treatment of LPS and SCFA significantly reduce FIS1/OPA1 ratio compared to LPS (**p<0.01) (**Fig. 4.3.1.2**-b)). No differences in FIS1/OPA1 ratio were observed at 24 hr (p=ns, **Fig. 4.3.1.2**-c)).

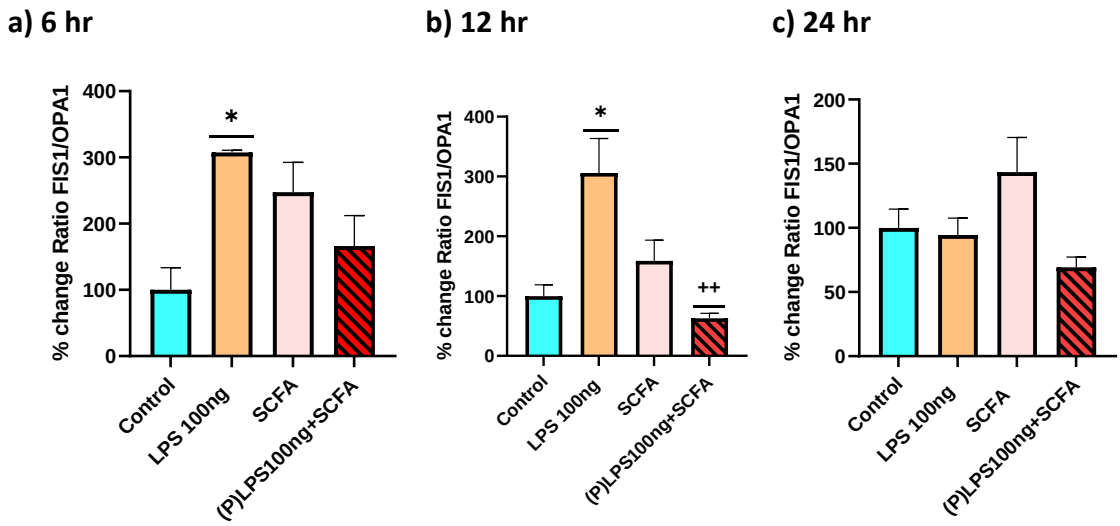


Fig. 4.3.1.2. Ratio summary for FIS1/OPA1 gene expression in BEAS2B-R1 cells treated with LPS ± SCFA.

Summary graph of ratio analysis between fission protein FIS1 and fusion genes OPA1 at a) 6, b)12, c)24 hr (n=3). Data are presented as the mean with standard error of the mean (SEM). Statistical analysis was performed using one way ANOVA plus Tukey's post hoc test. p-values are displayed as follows: (*=control vs other treatments; += LPS 100ng vs other treatment) *p<0.05; **p<0.01, ***p<0.001.

4.3.2 LPS induced imbalance in mitochondrial fusion/fission protein expression

Protein expression studies were conducted to confirm findings obtained with the gene expression analysis observed in **4.3.1**. To best evaluate the effect of LPS on mitochondrial structure, 2 mitochondrial fission proteins (FIS1, DPR1) and 2 fusion proteins (OPA1, MNF2) were measured in airway epithelial cells (BEAS2B-R1) using different concentration of LPS. Cells were treated with low or high doses of LPS (10 ng/mL or 100 ng/mL respectively), with or without SCFA, for 6, 12 or 24 hr.

4.3.2.1 Changes in mitochondrial fusion proteins following LPS treatment

Cells treated with 100 ng/mL of LPS showed a trend of decreasing the level of fusion protein, mitofusin 2 (MNF2) at 6 hr (p=ns), while it led to an increase at 12 hr (p=ns) and 24 hr (**p<0.01) compared to basal levels. Although not significant, cells treated with 100 ng/mL of LPS treated cells showed an increased trend in the concentration level of the fusion protein OPA1 at all the time points considered. No significant differences were observed for OPA1 and MNF2 following treatment with the lower concentration of LPS (10 ng/mL) with or without SCFA. (P)LPS100+SCFA treated cells, reduced levels of MNF2 at 6 hr (*p<0.05) compared to control cells, while it was restored to basal levels at 12 hr and 24 hr. Similar basal levels of OPA1 were observed in the (P)LPS100+SCFA treated cells at all the timepoints considered (**Fig.4.3.2.1; Fig.4.3.2.2; Fig.4.3.2.4**).

4.3.2.2 Changes in mitochondrial fission proteins following LPS treatment

Cells treated with 100 ng/mL of LPS showed no changes in the fission proteins dynamin related protein (DPR1) and mitochondrial fission protein FIS1 compared to control levels at 6 hr, while an increase of both were observed at 12 hr (FIS1, DPR1: p=ns) and 24 hr (DPR1: ns, FIS: ***p<0.001) compared to basal levels. (P)LPS100 ng+SCFA treated cells showed a decreased level of FIS1 (**p<0.01) and un-changed level of DPR1 at 6 hr compared to basal levels, while no changes were observed at 12 hr or 24 hr (p=ns). No significant changes were observed for DPR1 and FIS1 with the lower concentration of LPS (10 ng/mL) with or without SCFA (p=ns) (**Fig.4.3.2.1; Fig.4.3.2.2**; **Fig.4.3.2.3).**

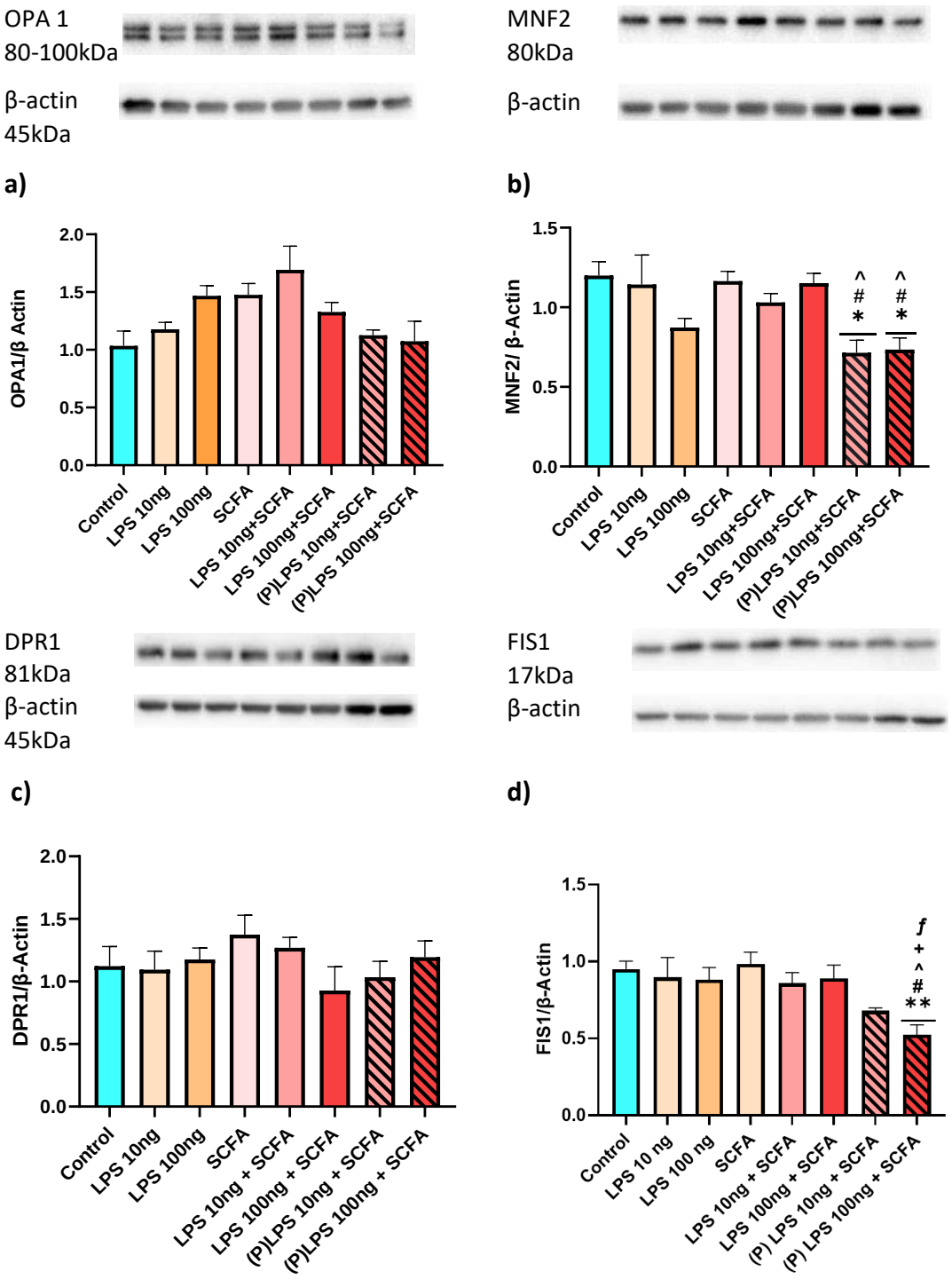


Fig.4.3.2.1. Level of fission/fusion proteins in BEAS2B-R1 cells treated with LPS ± SCFA at 6 hr timepoint.

Data are presented as the mean with standard error of the mean (SEM) (n=3). Statistical analysis was performed using one way ANOVA plus Tukey's post hoc test. p-values are displayed as follows: (*=control vs other treatments; += LPS 100ng vs other treatment; ^=LPS 10ng vs other treatments; ƒ=(P)LPS+SCFA vs treatments; #=LPS100ng+SCFA vs treatment) *p<0.05; **p<0.01, ***p<0.001.

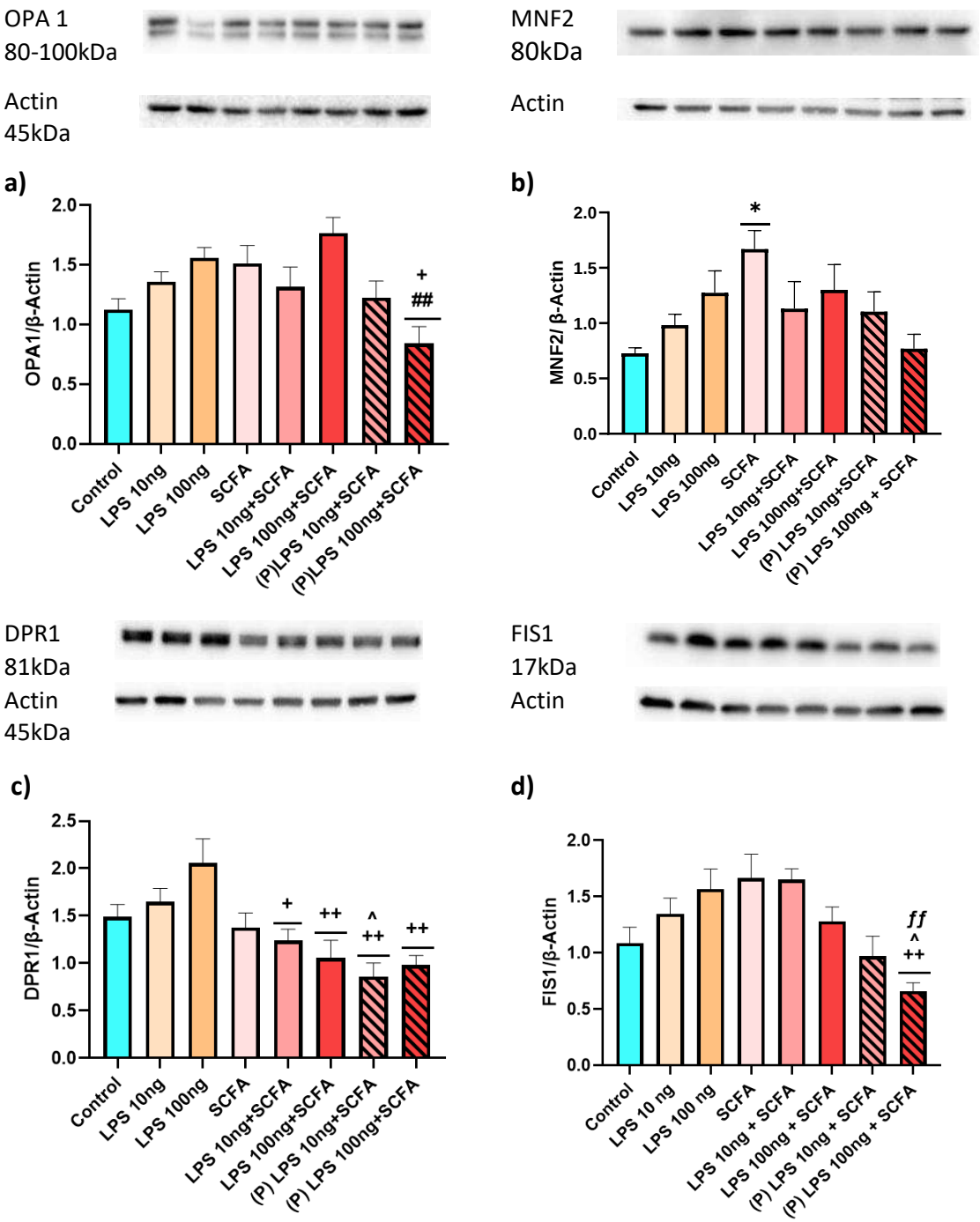


Fig.4.3.2.2. Level of fission/fusion proteins in BEAS2B-R1 cells treated with LPS ± SCFA at 12 hr timepoint.

Data are presented as the mean with standard error of the mean (SEM) (n=3). Statistical analysis was performed using one way ANOVA plus Tukey's post hoc test. p-values are displayed as follows: (*=control vs other treatments; += LPS 100ng vs other treatment; ^=LPS 10ng vs other treatments; ƒ=(P)LPS+SCFA vs treatments; #=LPS100ng+SCFA vs treatment) *p<0.05; **p<0.01, ***p<0.001.

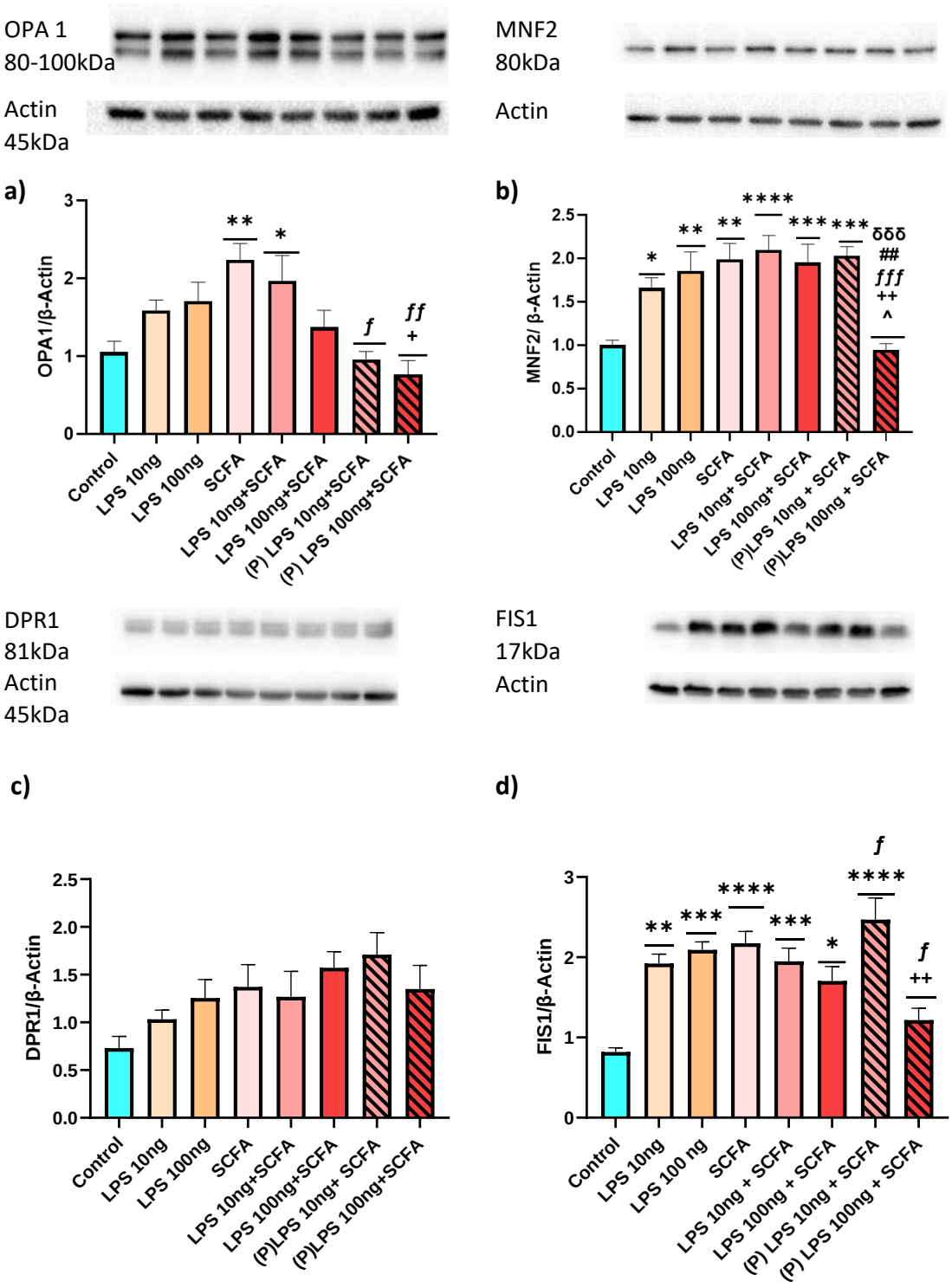


Fig.4.3.2.3. Level of fission/fusion proteins in BEAS2B-R1 cells treated with LPS ± SCFA at 24 hr timepoint.

Data are presented as the mean with standard error of the mean (SEM) (n=3). Statistical analysis was performed using one way ANOVA plus Tukey's post hoc test. p-values are displayed as follows: (*=control vs other treatments; += LPS 100ng vs other treatment; ^=LPS 10ng vs other treatments; ƒ=(P)LPS+SCFA vs treatments; #=LPS100ng+SCFA vs treatment; δ=(P)LPS10+SCFA) P-values: *p<0.05; **p<0.01, ***p<0.001.

4.3.2.3 Changes in fission/fusion ratio

A ratio between the fission protein, FIS1, and the fusion protein, OPA1, was undertaken to measure the overall level of fission and fusion proteins for each treatment. Data were expressed as a % of change from the control (**Fig.4.3.2.4**).

Results were not significant but trend in the data showed that 100 ng/mL LPS treated cells showed a reduction of 30% in the FIS1/OPA1 ratio compared with the control at 6 hr, while an increase of 40% and 60% of FIS1/OPA1 ratio was observed at 12 hr and 24 hr respectively, compared to the basal level (**Fig.4.3.2.4**-a) b) c)).

Cells treated with only SCFA did not lead to any changes at the considered timepoints compared to the basal level.

Pre-treating cells with SCFA appeared to mitigate the effect observed with LPS 100 ng/mL reducing FIS1/OPA1 ratio by 30% at both 12 and 24 hr and restoring basal level of FIS/OPA1 Ratio, however data were not statistically significant (p=ns). No significant differences were observed with 10 ng/mL of LPS with or without SCFA (**Fig.4.3.2.4**-b), c)).

The highest dose of LPS 100 ng and the SCFA pre-treated cells caused the greatest effect on mitochondria dynamics. Therefore, all other experiments for this study have been performed using these treatments.

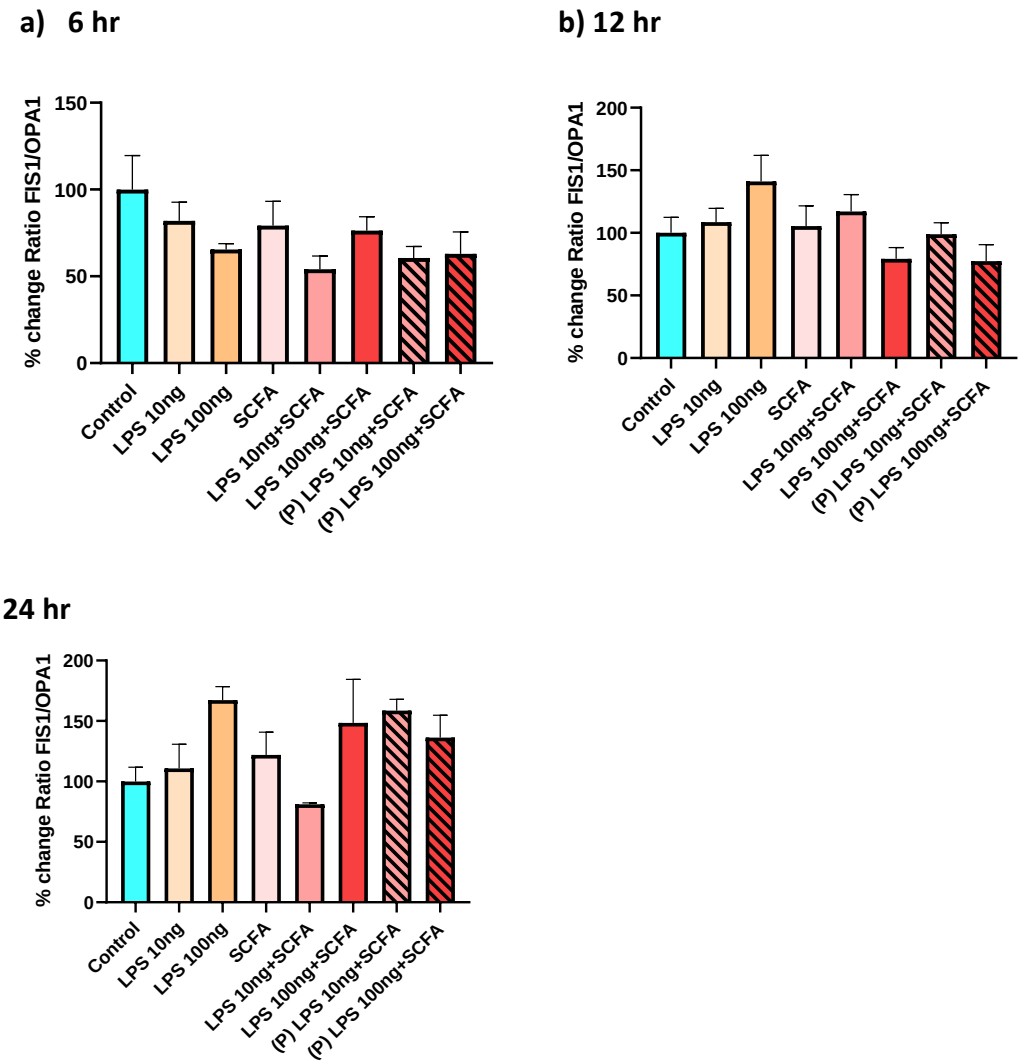


Fig.4.3.2.4. Ratio between fission protein FIS1 and fusion protein OPA1 in BEAS2B-R1 cells treated with LPS ± SCFA at 6,12 and 24 hr.

Summary graph of ratio analysis between fission protein FIS1 and fusion protein OPA1 at a) 6, b)12, c)24 hr. Data are presented as the mean with standard error of the mean (SEM) (n=3). Statistical analysis was performed using one way ANOVA plus Tukey's post hoc analysis. Data were not significant (p=ns).

4.3.3 The effect of LPS on mitochondria morphology

To look at changes in the morphological network of the mitochondria, treated cells were live imaged using a nano live microscope. These images showed the nucleus of single cells and the mitochondrial network around it (**Fig. 4.3.3.1**). Results were not significant (p=ns) (**Fig. 4.3.3.1**).

Initially at 6hr, mitochondria in LPS treated cells tended to be 30% more fragmentated (ratio between mitochondria area and perimeter) and 60% larger in size than the control group (p=ns) (**Fig. 4.3.3.1** a)). In the SCFA treated cells, there were no change in size and fragmentation compared to the basal level, however a 30% increase in the number and length of branches were observed (**Fig. 4.3.3.1** c) d)). The LPS+SCFA group mitochondria tended to be larger in size and with a higher number of branches, both with a 40% increase compared with the control group (p=ns). Fragmentation and branch length remained similar to control levels (p=ns) (**Fig. 4.3.3.1**).

No major changes were noted at 12 hr. At 24 hr, visually, the mitochondria in the control appeared elongated and highly interconnected, which are signs of a healthy mitochondria network. In the LPS treated cells, alterations in the mitochondria network were observed. Mitochondria were more fragmentated and less organized than the control group, as showed by the annotation (**Fig.4.3.3.3**).

Similarly to what had been observed in the Nanolive images, quantitative analysis of the cells at 24 hr revealed that LPS-treated cells showed a trend towards having more fragmented (**Fig. 4.3.3.3.**, b)) and smaller mitochondria (**Fig. 4.3.3.3**, a)) compared with the control group.

In addition, no significant changes were observed in the number of mitochondrial branches between the LPS and control groups. However, % change analysis showed a trend of 30% increase in mitochondrial branches in both SCFA and (P)LPS+SCFA treated cells compared to the control (p=ns). Moreover branch length was approximately 30% shorter in the LPS treated cells compared to the control group, while LPS+SCFA partially restored branch length, showing a 20% branches length increase compared with the LPS treated cells (p=ns) (**Fig. 4.3.3.3.**, d)).

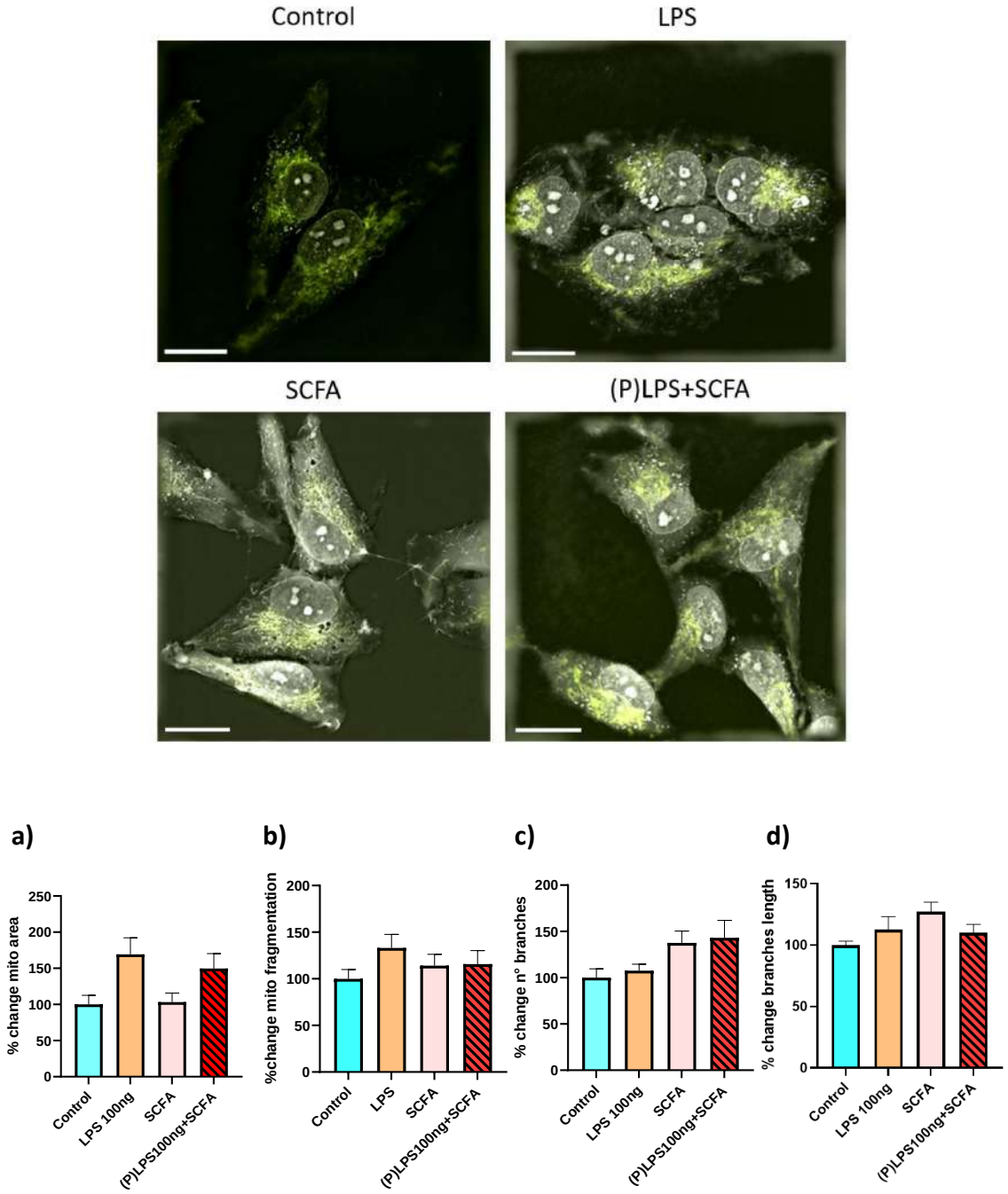


Fig. 4.3.3.1. Nanolive images and quantitative analysis of mitochondria morphology in BEAS2B-R1 cells treated with LPS ± SCFA at 6 hr timepoint.

Representative images of Mito tracker green-stained BEAS2B-R1 cells after 6 hr treatment. Images are representative of 30-40 images taken in 3 independent experiments. Images are shown at 40x magnification, scale bar represents 20µm. Images were analysed using Image J software to determine a) mitochondria area; b) mitochondria fragmentation; c) number of mitochondria branches; d) branch length. Data are shown as a % of change from the control and reported as mean with standard error of the mean (SEM). Data were analysed using one-way ANOVA plus Tukey's post hoc analysis. Data were not significant (p=ns).

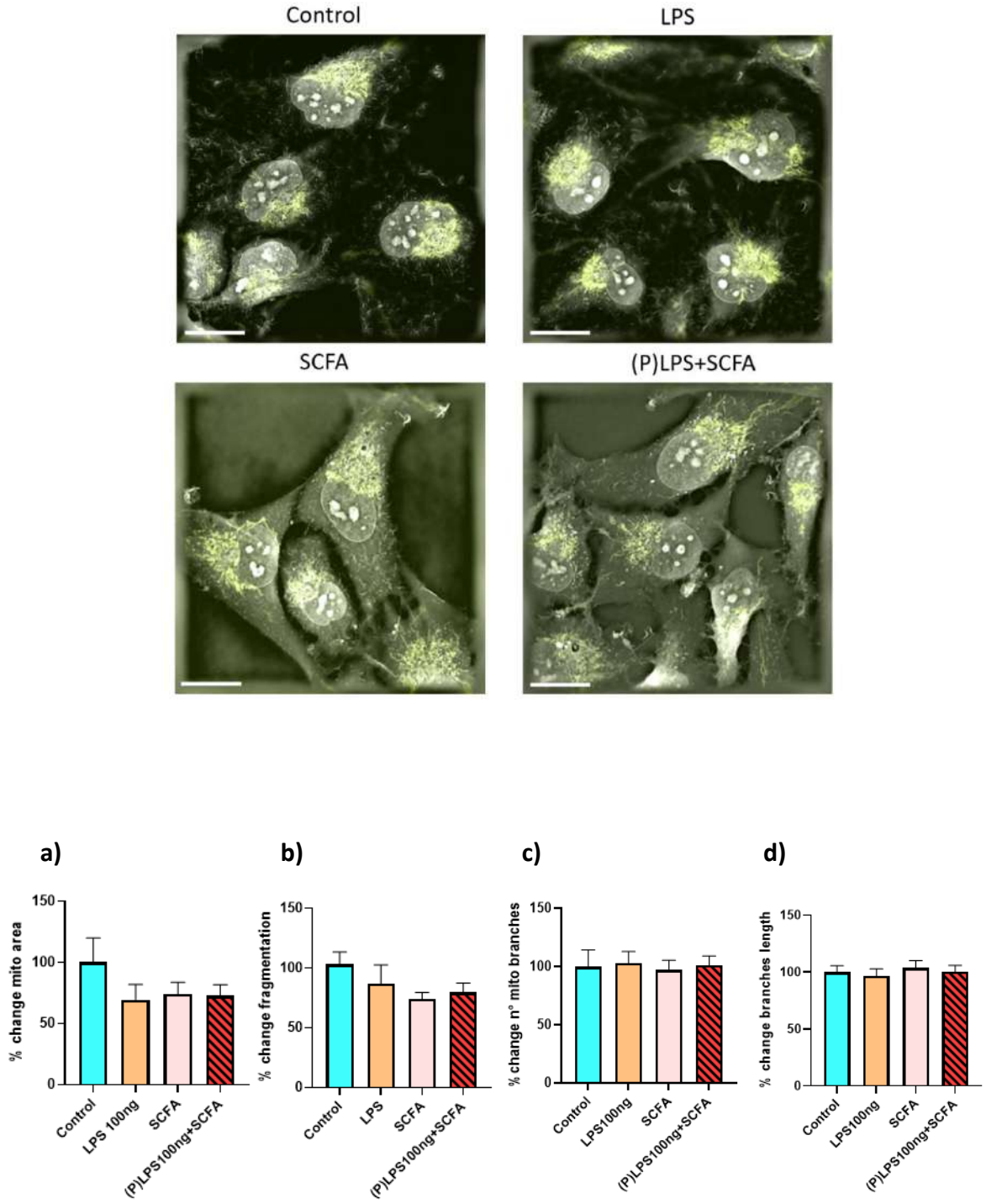


Fig. 4.3.3.2. Nanolive images and quantitative analysis of mitochondria morphology in BEAS2B-R1 cells treated with LPS ± SCFA at 12 hr timepoint.

Representative images of Mito tracker green-stained BEAS2B-R1 cells after 6 hr treatment. Images are representative of 30-40 images taken in 3 independent experiments. Images are shown at 40x magnification, scale bar represents 20µm. Images were then analysed using Image J software to determine a) mitochondria area; b) mitochondria fragmentation; c) number of mitochondria branches; d) branches length. Data are shown as a % of change from the control and reported as mean with standard error of the mean (SEM). Data were analysed using one-way ANOVA plus Tukey's post hoc analysis. Data were not significant (p=ns).

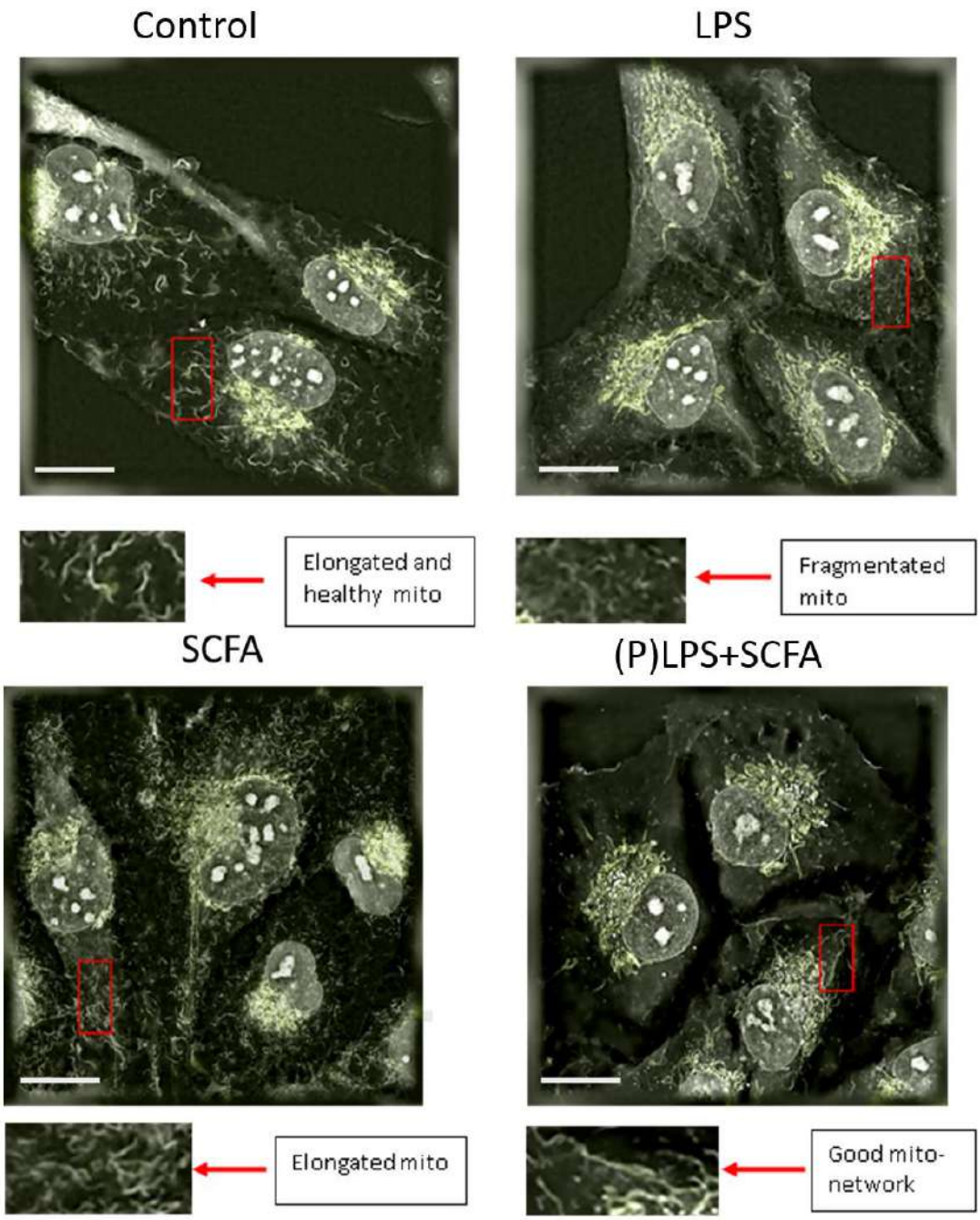


Fig. 4.3.3.3. Nanolive images of mitochondria morphology in BEAS2B-R1 cells treated with LPS ± SCFA at 24 hr timepoint.
Representative images of Mito tracker green-stained BEAS2B-R1 cells after 24 hr treatment. Images are representative of 30-40 images taken in 3 independent experiments. Images are shown at 40x magnification, scale bar represent 20µm. The insert shows a higher magnification detail of part of the main image.

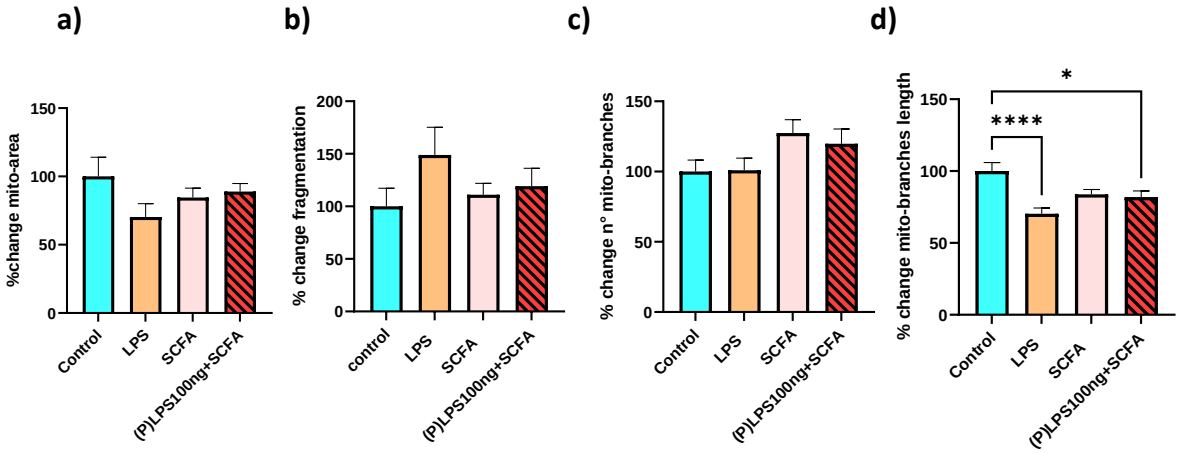


Fig. 4.3.3.4. Nanolive quantitative analysis of mitochondria morphology in BEAS2B-R1 cells treated with LPS ± SCFA at 24 hr timepoint.
Images were then analysed using Image J software to determine a) mitochondria area; b) mitochondria fragmentation; c) number of mitochondria branches; d) branches length. Data are shown as a % of change from the control and reported as mean with standard error of the mean (SEM). Data were analysed using one-way ANOVA plus Tukey's post hoc analysis. P values are displayed as follows: *p<0.05; **p<0.01, ***p<0.001.

4.3.4 SOD2 protein expression

Mitochondrial SOD2 (mtSOD2) is a protein noted to be a scavenger of ROS and it is released by the mitochondria following an insult in the cell (Lu et al. 2015; Ning et al. 2021). The expression of mtSOD2 protein was increased following treatment with low and high doses of LPS, with the highest increase observed using 100ng of LPS at 6 (***p<0.001), 12 (****p<0.0001) and 24 hr (****p<0.0001) timepoints (**Fig. 4.3.4.1**). Combined treatment of LPS 100ng and SCFA led to a significant reduction of mtSOD2 levels compared to LPS (100ng) treated cells at 12 hr (***p<0.001) and 24 hr (****p<0.0001) (**Fig. 4.3.4.1** b) c)). At 6hr, mtSOD2 expression remained elevated in all treated groups, except SCFA treated cells (**Fig. 4.3.4.1**-a)). No changes in mtSOD2 was observed in the SCFA treated cells compared to the baseline level at all considered timepoint (**Fig. 4.3.4.1**-a)b)c)).

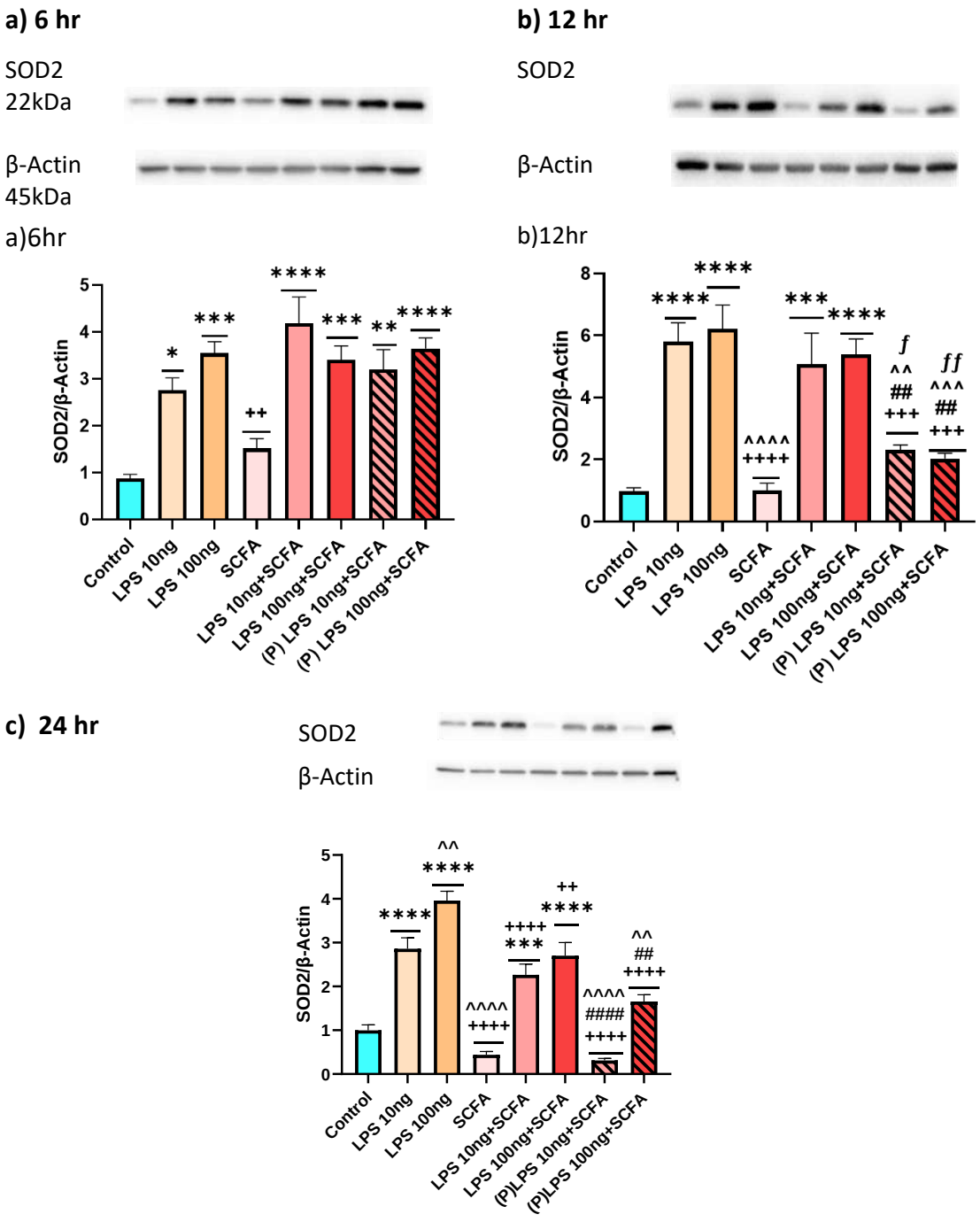


Fig. 4.3.4.1. mtSOD2 expression in BEAS2BR1 cells treated with LPS ± SCFA.
Data are presented as the mean with standard error of the mean (SEM) (n=4). Statistical analysis was performed using one way ANOVA plus Tukey's post hoc test. p-values are displayed as follows: (*=control vs other treatments; ^=LPS10ng vs other treatments; += LPS 100ng vs other treatment; ƒ=LPS10ng+SCFA vs treatments; #=LPS100ng+SCFA vs treatment) *p<0.05; **p<0.01, ***p<0.001.

4.3.5 The effect of LPS on mitochondrial activity

To determine whether mitochondrial activity was affected by LPS or SCFA treatments, cells were live imaged using two mitochondria dyes. The red dye, MitoTracker Red, stained the mitochondria dependent on membrane potential, while Green MitoTracker stained all mitochondrial independently of membrane potential.

LPS led to reduced mitochondrial activity at 6hr (*$p<0.05$), and 24hr (**$p<0.01$) compared to the control, while SCFA mitigated the effect induced by LPS, improving mitochondrial potential when combined with LPS at 6 hr (**$p<0.01$) (**Fig. 4.3.5.4**). Although results were not significant, combined treatments appeared to increase mitochondrial activity at 24 hr (Fig. 4.3.5.4-a, c)). Treatment with SCFA only appeared to reduce mitochondrial activity at 24 hr compared to the control cells, but to a lesser extent then LPS treated cells (*$p<0.05$) (**Fig. 4.3.5.4**-c)).

No differences were observed between treatments at the 12 hr timepoint (**Fig. 4.3.5.4**-b).

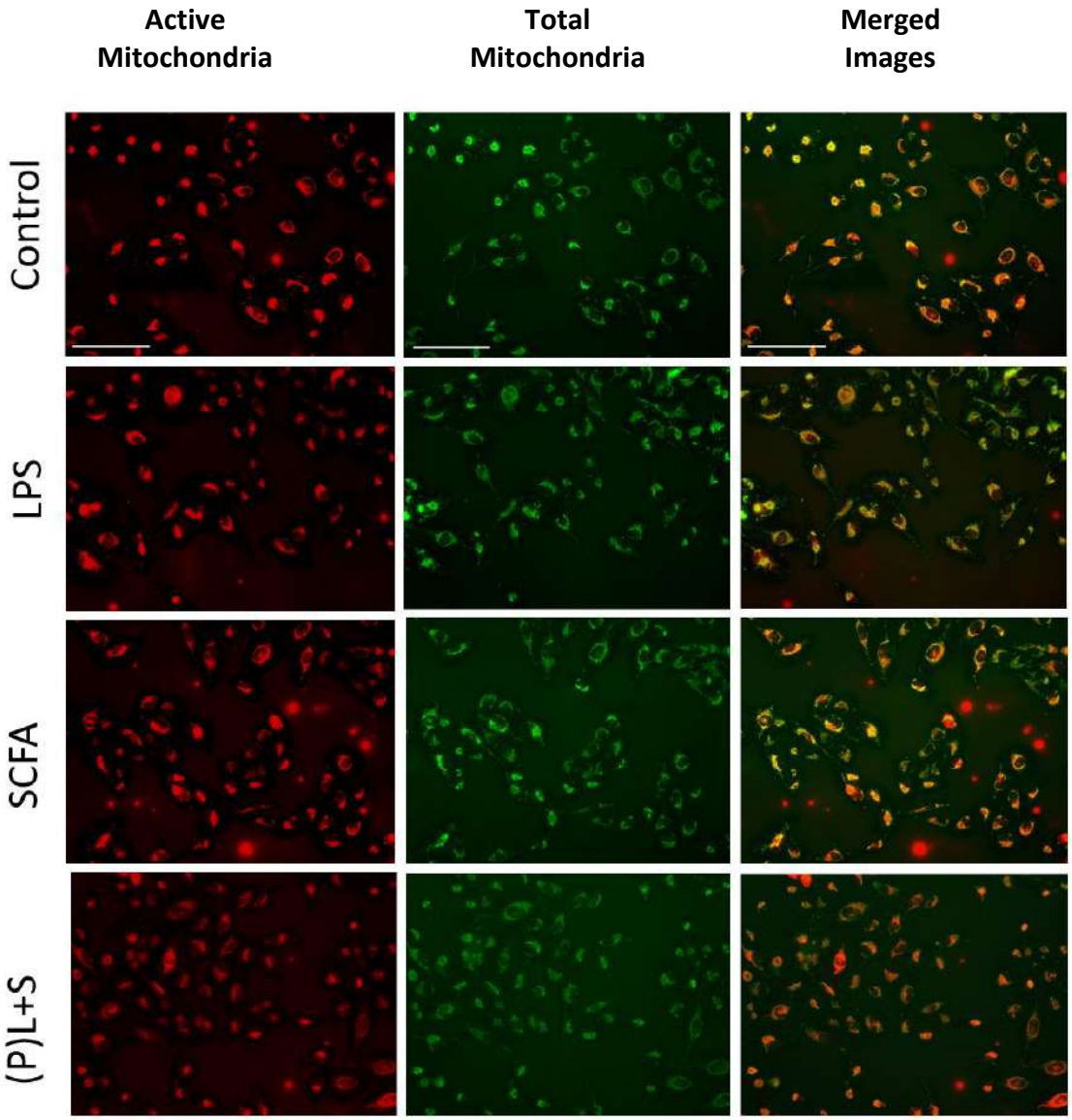


Fig. 4.3.5.1. Mitochondria activity in BEAS2BR1 cells at 6 hr.
BEAS2B-R1 airway epithelial cells treated with either LPS100 ng/mL, SCFA or LPS+SCFA at 6 hr (n=150 cells per treatment). Images are shown at 20x magnification, scale bar represents 150µm. Cells were stained with Mitotracker dyes to distinguish the total mitochondria (green) and the active mitochondria (red). Experiments were conducted on 3 separate occasions.

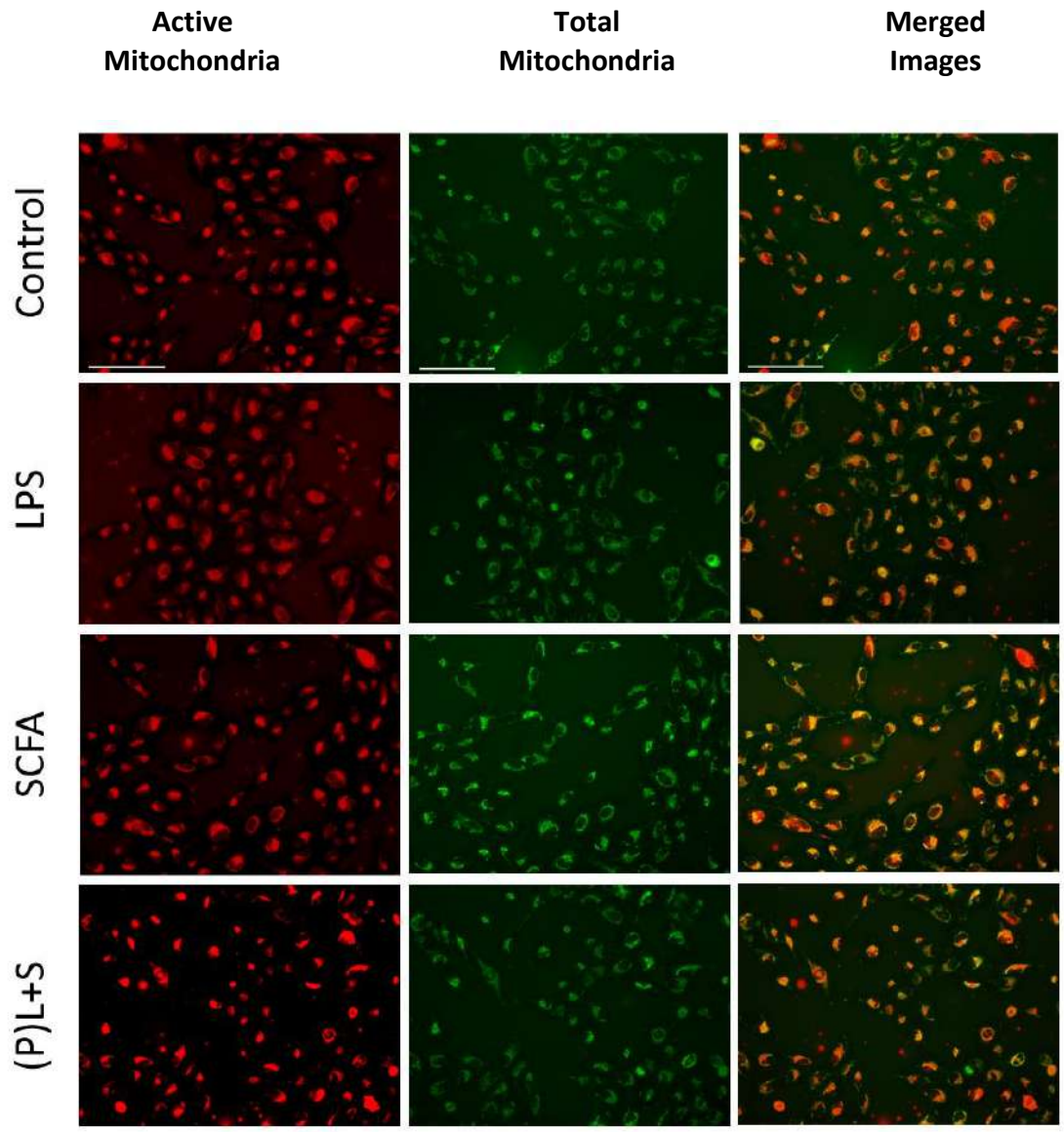


Fig. 4.3.5.2. Mitochondria activity in BEAS2BR1 cells at 12 hr.
BEAS2B-R1 airway epithelial cells treated with either LPS100 ng/mL, SCFA or LPS+SCFA at 12 hr (n=150 cells per treatment). Images are shown at 20x magnification, scale bar represents 150µm. Cells were stained with Mitotracker dyes to distinguish the total mitochondria (green) and the active mitochondria (red). Experiments were conducted on 3 separate occasions.

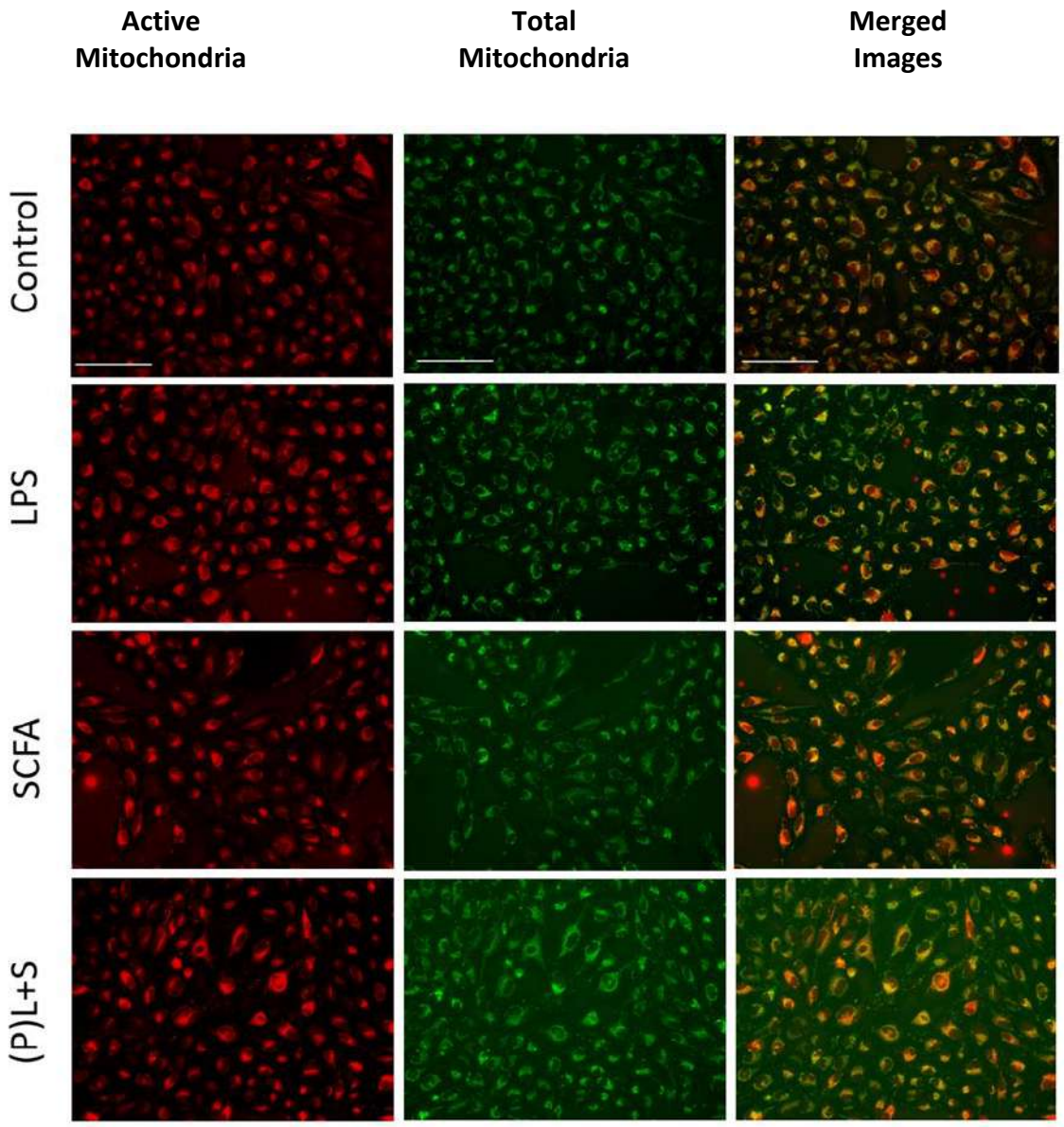


Fig. 4.3.5. 3. Mitochondria activity in BEAS2B-R1 cells at 24 hr.
BEAS2B-R1 airway epithelial cells treated with either LPS100 ng/mL, SCFA or LPS+SCFA at 24 hr (n=150 cells per treatment). Images are shown at 20x magnification, scale bar represents 150µm. Cells were stained with Mitotracker dyes to distinguish the total mitochondria (green) and the active mitochondria (red). Experiments were conducted on 3 separate occasions.

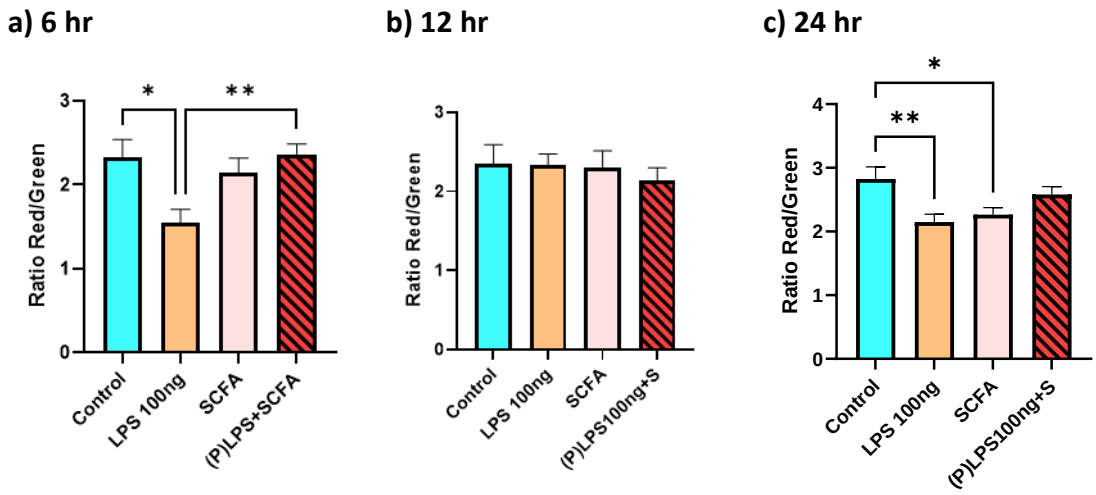


Fig. 4.3.5.4. Mitochondrial activity in BEAS2BR1 cells treated with LPS ± SCFA at 6, 12 and 24 hr.

BEAS2B-R1 airway epithelial cells treated with either LPS100 ng/mL, SCFA or LPS+SCFA at 6, 12, and 24 hr (n=150 cells per treatment). Cells were stained with Mitotracker dyes to distinguish the total mitochondria (green) and the active mitochondria (red). Experiments were conducted on 3 separate occasions. Statistical analysis using one way ANOVA plus Tukey's post hoc test was performed and data are shown as mean with SEM. (p values are displayed as follows; *p<0.05; **p<0.01).

4.3.6 The effect of LPS on mitochondrial function

To understand the implications of the LPS± SCFA treatments when mitochondria are under stress, the Seahorse Mito Stress Test was performed using live BEAS2B-R1 cells to measure basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) that are respectively indicators of aerobic and anaerobic respiration. Briefly, the test provides key measurements to assess mitochondrial respiration by systematically injecting 3 compounds that deteriorate the electron transport chain. The first compound was Oligomycin that inhibits the ATP synthase, then FCCP that destroys the membrane potential and lastly rotenone/antimycin A that inhibits the complex I action.

There were no differences in either OCR or ECAR between control, LPS, SCFA or LPS/SCFA treated cells at any time point (**Fig. 4.3.6.1; Fig. 4.3.6.2**).

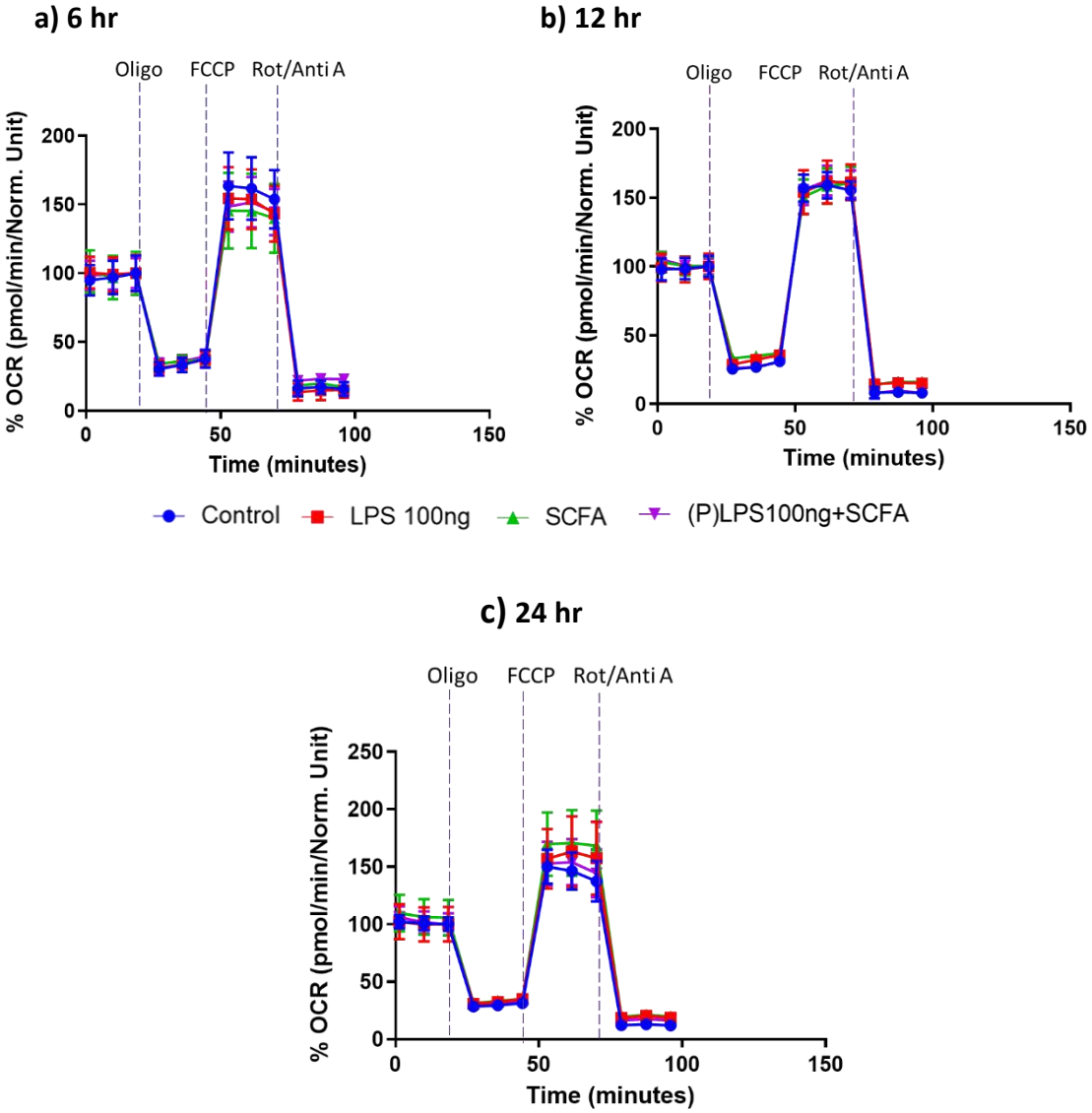


Fig. 4.3.6.1.The oxygen consumption rate (OCR) of BEAS2BR1 cells treated with either LPS100 ng, SCFA or a combination of both for a) 6 hr, b) 12 hr, c) 24 hr.

The oxygen consumption rate (OCR) was measured using a mitochondrial Stress test on a Seahorse analyser XFe analyser (6 hr,12 hr: n=2; 24 hr: n=3). The compounds used in this assay were: 1.5 µM, Oligomycin (Oligo), 2µM, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and 0.5µM, Rotenone and Antimycin A (Rot/AA). Data are presented as the mean±standard error of the mean (SEM). Statistical analysis using one way ANOVA plus Tukey's post-hoc test was performed (p=ns).

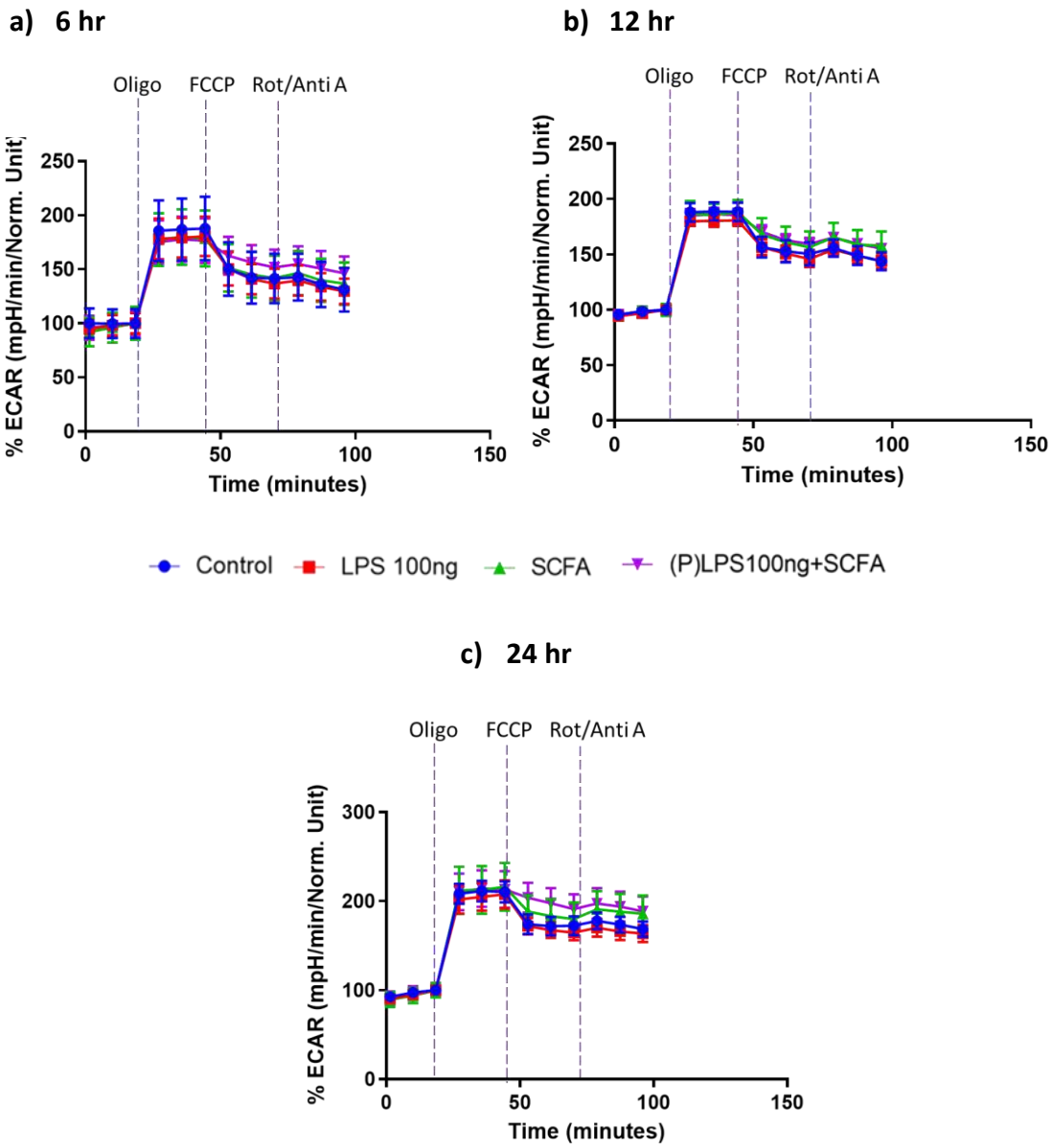


Fig. 4.3.6.2. The extracellular acidification rate (ECAR) of BEAS2BR1 cells treated with either LPS100ng, SCFA or combination of both for a)6 hr, b)12 hr, c) 24 hr.

The extracellular acidification rate (ECAR) was measured using a mitochondrial Stress test on a Seahorse analyser Xfe analyser (6 hr,12 hr: n=2; 24 hr: n=3). The compounds used in this assay were: 1.5 µM, Oligomycin (Oligo), 2µM, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and 0.5µM, Rotenone and Antimycin A (Rot/AA). Data are presented as the mean±standard error of the mean (SEM). Statistical analysis using one way ANOVA plus Tukey's post-hoc test was performed (p=ns).

4.3.7 Investigation of the effect of LPS on mitochondrial biogenesis-related protein expression

To further investigate the role of LPS in the regulation of mitochondria function, mitochondrial biogenesis proteins have been investigated in BEAS2B-R1 cells at 6, 12 and 24 hr.

The mitochondrial biogenesis-related proteins considered were: mitochondrial transcription factor A (TFAM), nuclear respiratory factor (NRF1), DNA polymerase gamma (POLG) and peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α).

LPS treated cells showed an increase in TFAM, a key regulator of mitochondria biogenesis, at 6 hr (*p<0.05) and again at 24 hr (***p<0.001) compared to basal level (Fig. 4.3.7.1). LPS also tended to increase levels of other mitochondrial biogenesis markers, NGF1 and POLG, at 6 hr (NGF1, POLG: ns) and again at 24 hr (NGF1: *p<0.05, POLG: ns) (**Fig. 4.3.7.1**).

SCFA treated cells showed a significant decrease in TFAM (**p<0.01) at 12hr and an increase in TFAM (***p<0.001) at 24hr compared to the baseline level. Moreover, SCFA tended to increase level of NRF1 and POLG at 6, 12 and 24 hr compared to baseline level, but results were not significant (**Fig. 4.3.7.1**).

Combined treatment of LPS+SCFA led to a decreased level of TFAM at 6 hr (####p<0.0001), 12 hr (##p<0.01) and 24 hr (####p<0.0001) compared to LPS treated cells. A similar trend was observed with NGF1 and POLG at all the considered timepoints, although only NGF1 at 24 hr was statistically significant (##p<0.01).

No changes were observed in PGC1-α, a mitochondria transcription factor, over time with any treatments (**Fig. 4.3.7.1**).

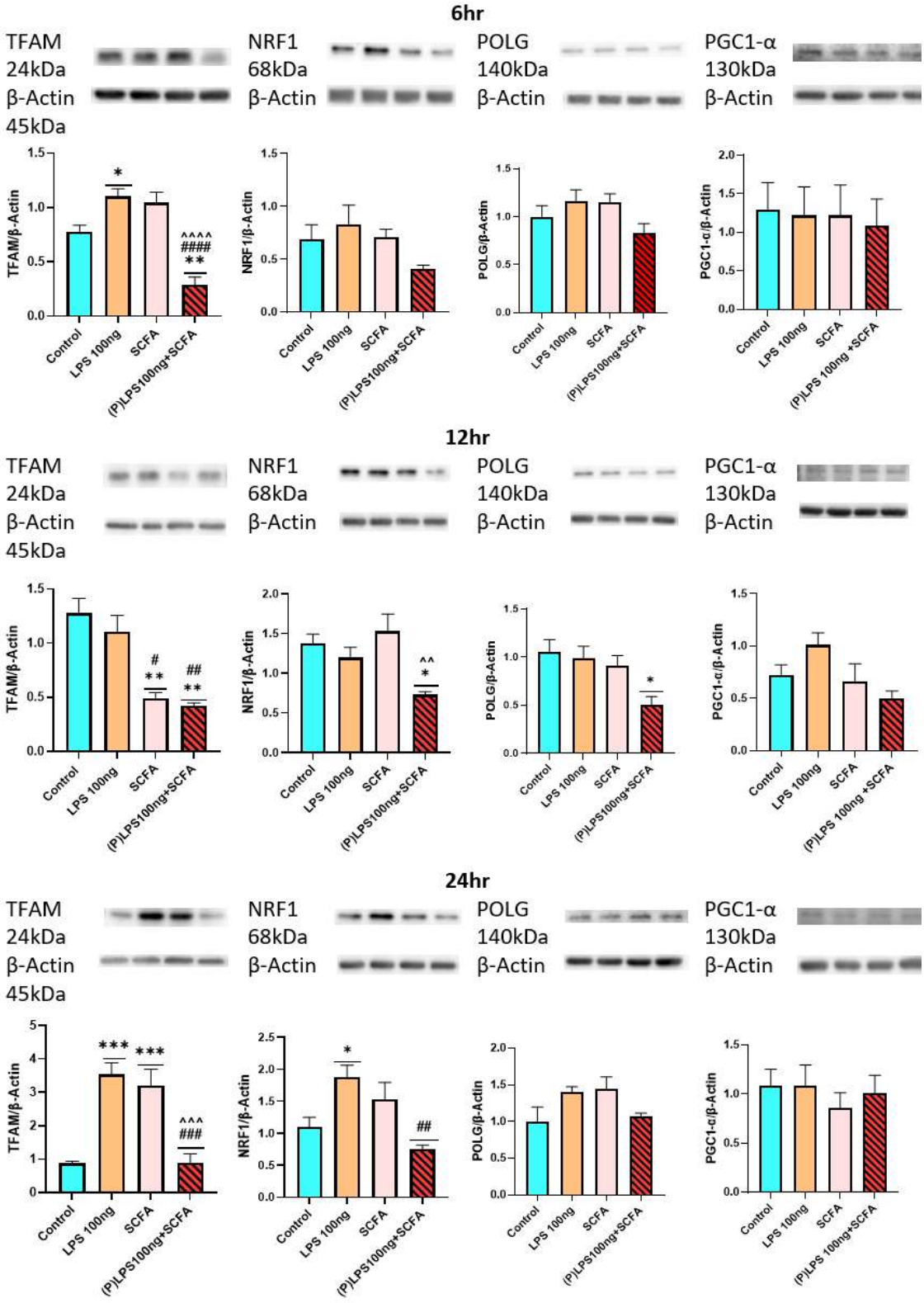


Fig. 4.3.7.1. Levels of biogenesis proteins in BEAS2BR1 cells treated with LPS ± SCFA at 6, 12, 24 hr timepoints.

Data are presented as the mean with standard error of the mean (SEM) (n=3). Statistical analysis was performed using one way ANOVA plus Tukey's post hoc test. p values are displayed as follows: (*=control vs other treatments; #=LPS100ng vs other treatments; ^=SCFA vs other treatments;) *p<0.05; **p<0.01, ***p<0.001.

4.4 Discussion

In this chapter, it was hypothesized that LPS could affect mitochondrial morphology and function in airways cells, while SCFA may help limit the negative effects induced by LPS. To investigate this, airway epithelial cells, BEAS2B-R1, were treated with LPS with and without SCFA, and mitochondrial structure and function was assessed by several different approaches. From these investigations, this study suggested that: 1) LPS induced mitochondrial dysfunction by altering mitochondrial dynamics and activity; 2) it induced an overexpression of mtSOD2; 3) SCFA partially mitigated the LPS-induced damage on mitochondria.

4.4.1 The effect of LPS and SCFA on mitochondrial dynamics

Mitochondrial health is maintained by a balance of dynamic related proteins and genes (van der Bliek et al. 2013). Alteration of these can lead to fragmented and damaged mitochondria which in turn can lead to proinflammatory cytokine production and excessive ROS production from mitochondria (Andrieux et al. 2021; Liesa and Shirihai 2013).

This study reported that LPS induced an acute overexpression on the fission gene 1 (FIS1) at 6 and 12 hr, while combined treatments of LPS and SCFA appeared to restore it to basal level.

When looking at protein expression, LPS (100 ng/mL) tend to increase the fission/fusion ratio at 12 and 24 hr, whist combined treatment with SCFA partially restored basal level

of mitochondria dynamics proteins. No acute change was reported in proteins expression at 6 hr.

Discrepancies between protein and gene expression results can be explained by the various post-transcriptional mechanisms that regulate and control protein function (Santoni 2017; Liu, Beyer, and Aebersold 2016a; Tang and Amon 2013; Wethmar, Smink, and Leutz 2010; McManus, Cheng and Vogel 2015). Protein synthesis requires time and during the process the transcript can change affecting the protein level with a temporal delay (Liu, Beyer, and Aebersold 2016b). In addition, spatial variations in protein transport physically separate protein from the transcript from which it was originally synthesized (Liu et al. 2016b). These processes strongly influence the relationship between protein expression and mRNA level and may explain the delay that we observed with respect to protein expression.

Overall, LPS tend to induce an overexpression of fission protein, FIS1 at 12 and 24 hr.

Chronic LPS-induced damage was also confirmed via live imaging which revealed a trend towards a more fragmentated and smaller mitochondria network compared to the control group. In contrast, SCFA appeared to protect mitochondria from fragmentation, by partially restoring a basal mitochondria network. A recent study reported that SCFA helped to prevent alteration of fusion and fission proteins, protecting mitochondria in stressful conditions in β-cells (Hu et al. 2020), similarly to what was found in this study with airway cells.

This suggests that cells were able to cope with LPS insult at the early stage but over time the LPS-induced chronic damage began to affect mitochondria dynamics.

4.4.2 The effect of LPS and SCFA on mitochondrial function

Alteration in mitochondria structure, following a triggering insult such as LPS, can lead to ROS overproduction which in turn could impair the ability of mitochondria to produce ATP, damage mitochondrial proteins and affect a range of mitochondria function (Murphy, 2009).

MtSOD2 is an enzyme located in the mitochondrial matrix that plays a key role as a scavenger of ROS (Ning et al. 2021). MtSOD2 functions include the conversion of superoxide dismutase, which represent the vast majority of the ROS produced and are highly toxic for cells, to hydrogen peroxide, a less reactive enzyme that passively diffuses away from mitochondrial matrix (Flynn and Melovn 2013). This process is extremely important for cell's health as it prevents the production of highly aggressive ROS such as peroxynitrite which can lead to mitochondria dysfunction as described before.

LPS treated cells exhibited an acute and chronic overexpression of mtSOD2 compared to control. Combined treatment with both LPS and SCFA returned MtSOD2 levels to basal levels. ROS are released from the cells as a response to various insults occurring in cells such as inflammation response, bacteria, xenobiotics, stress. Between those, LPS is one stimulus that is known to increase ROS production (Ali et al. 2020; Qu et al. 2019). Previous research found high expression of mtSOD2 in rat primary microglia that were previously treated with LPS (Ishihara et al. 2015). Moreover, mtSOD2 increased the tolerance to ROS production in the same cell lines (Ishihara et al. 2015). Therefore, from the data obtained in the present study, it can be speculated that the high expression of mtSOD2 was an attempt from the cell to deal with the ROS production from LPS. In fact, cells treated with SCFA appeared to return levels of mtSOD2 to control levels. Previous

studies reported that acetate and butyrate, the main SCFA present in the human body, reduced ROS generation in β-cells (Hu et al. 2020). A reduction in the ROS level could explain the reduced mtSOD2 expression in the LPS+SCFA treated group. Although ROS levels were not investigated during this project, further studies that take ROS production into consideration are needed to better elucidate this pathway.

Mitochondrial respiration was not affected by LPS or SCFA, but considering that it is an essential function, it can be speculated that cells activate a range of compensatory strategies to meet cellular demands. A previous study reported similar findings in adipose cells line; cells treated with LPS showed an increase in mitochondrial biogenesis protein as a compensatory mechanism to cope with LPS insult, which led to an overall increase in mitochondrial oxidation rate (Widdrington et al. 2018a). In the present study, airway cells appeared to activate the same compensatory mechanism to mitigate the LPS-induced stress as reflected in a reduced mitochondrial activity, acutely and chronically, and by enhanced mitochondrial biogenesis as was seen with the significant increase of TFAM and NRF1. NRF1 is a transcription factor that activate TFAM which in turn is an essential mtDNA protein that regulates mitochondria transcription initiation and replication (Campbell, Kolesar, and Kaufman 2012).

The activation of the NRF1/TFAM pathway may lead to a production of new mitochondria that do not compromise mitochondrial respiration but lead to less active mitochondria as seen with the cell imaging.

4.4.3 Conclusions

Taken together, this study has shown that LPS induced a chronic damage in mitochondrial dynamics that can be partially restored by SCFA. The cells responded to the LPS insult by increasing mtSOD2 levels to cope with ROS production and by producing new mitochondria to meet the mitochondrial respiration requirement yet the mitochondria in the airway cells still appear to be less active and responsive to LPS stimuli.

The LPS induced damage on mitochondria may exacerbate inflammation and therefore may represent a contributing factor to the worsening of asthma symptoms.

Chapter 5: Characterising a cohort of patients with difficult to control asthma: Impact of BMI on asthma severity and inflammation

5.1 Introduction

Asthma is a chronic disease of the airways, and is characterised by airway hyper-responsiveness, wheezing, breathlessness, and chest tightness (World Health Organization 2020). The U.K. has one of the highest rates of asthma in Europe with approximately 1 in 12 adults diagnosed. This results in an estimated cost for the National Health Service (NHS) of at least £1 billion per annum (NHS England 2020). Obesity is a commonly reported comorbidity of asthma. Obesity is a metabolic disease characterised by excess body fat accumulation and low-grade inflammation; it is associated with several comorbidities including asthma (Bantulà et al. 2021a; Gibeon et al. 2013; Gomez-Llorente et al. 2017; Peters et al. 2018). In recent years, the association between asthma and obesity has gained growing interest as it has been established that people with obesity and asthma have exacerbated asthma symptoms, poorer disease control and are less responsive to medication compared with people who are lean with asthma (Lugogo et al. 2010).

Patients with asthma and obesity can show an alternative phenotype, characterized by low eosinophils and high neutrophil cell counts in the blood, with greater concentrations of T helper (Th)-17 cytokines and systemic low-grade inflammation, noted as “Th-2 low” or neutrophilic asthma (Hinks et al. 2020). Furthermore, patients with asthma and obesity are 4-6 times more likely to be hospitalised for asthma than patients with a lean BMI (Vortmann and Eisner 2008). The underlying mechanism by which obesity affects asthma is still poorly understood. Therefore, there is a greater need to understand inflammatory and phenotypic characteristics of patients with obesity and asthma to help support future disease management strategies.

5.1.1. The WATCH-NTU collaboration study

In an attempt to better understand the characteristics of patients with obesity and asthma a collaboration study was established between NTU and the Wessex AsThma CoHort (WATCH) of difficult asthma. The WATCH is an ongoing prospective study started in 2015 and conducted by researchers and clinicians at Southampton University Hospital. The aim of the WATCH cohort is to give a better understanding of mechanisms underlying difficult asthma representing a valuable tool to improve disease management. “Difficult to control asthma” is defined by the British Thoracic Society (BTS) as a condition that remains problematic even with the treatment of high dose inhaled therapies and/or it requires frequent or continuous use of oral corticosteroids (British Thoracic Society (BTS) 2019). The asthma of these patients is characterized by regular exacerbations, poor quality of life and increased morbidity/mortality (Sastre et al. 2016b; Smith et al. 1997).

To date, the WATCH study team have recruited over 500 patients and collected anthropometric and detailed clinical health data, disease-related questionnaires, lung function testing, and collection of serum and plasma biological samples (Azim et al. 2020). Thus, the NTU-Southampton study draws upon data and blood samples previously collected from patients with severe asthma across a range of body mass index (BMI) from the WATCH team at Southampton University Hospital.

AIMS OF THE STUDY

The aims of this collaborative study were to characterize a subset of the WATCH cohort examining patients with difficult to control asthma to investigate the contribution of BMI and fat mass (FM) on 1) lung function and asthma control; 2) biomarkers of systemic inflammation.

5.2 Material and Methods

5.2.1. Ethics and study design

The collaboration study between NTU and the WATCH team was approved by the NTU Human Invasive Ethics Committee (protocol: #689), and developed in accordance with the WATCH clinical trial NHS ethical approval (https://clinicaltrials.gov/ct2/show/NCT03996590). Following a material transfer agreement, NTU received ninety-eight serum and plasma samples previously collected from patients with difficult to control asthma across a range of BMIs and associated data from the WATCH database. Samples were shipped to NTU via courier and stored at -80°C until use. The database was transferred to a NTU Data Store via project folder, protected with password and all necessary safeguards in accordance with any relevant laws, statutes, regulations, and good practice.

5.2.2. Participant recruitment

Patients attending the University Hospital Southampton Foundation Trust (UHSFT) Difficult Asthma Clinic were recruited through an approach by a clinician for enrolment to the WATCH Study. To take part in the study the patients had to be diagnosed with difficult to control asthma and be able to provide informed consent (Appendix 1: WATCH participant information sheet and consent form). Asthma is defined as difficult to control when patients exhibit symptoms such as severe breathlessness requiring clinical attention, even if managed with “high dose therapies” and/or “continuous or frequent use of oral steroids”, according to the British Thoracic Society (BTS) Adult Asthma Management Guidelines 2016 (British Thoracic Society (BTS) 2019). If the patient met

the inclusion criteria and agreed to take part in the study, they were invited to attend one research visit. The visit involved the participant completing an asthma-related questionnaire, anthropometric measurements (height, weight, body composition), lung function assessment and venous blood sample collection.

5.2.3. Anthropometric measurements

Participants body mass was measured to the nearest 0.1 kg using calibrated electronic scales (SECA, Birmingham, UK), wearing lightweight clothing and barefoot. Participants' height was measured with a stadiometer (SECA, Birmingham, UK). Participants stood bare foot with heels together with arms by their side. Body mass index (BMI) was calculated as weight (kg)/height $(m)^2$. BMI categories were listed in **Table 5.2.3.1**.

A Medical Body Composition Analyser (SECA, Hamburg Germany) was used to analyse body composition of patient with difficult to control asthma based on Bioelectrical Impedance Analysis (BIA), and Fat mass (FM) was recorded.

Table 5.2.3.1. Participant's body mass index (BMI) categories and number of participants per group.

	Body mass index (BMI)-categories (kg/m^2)	**Number of participants per group**
LEAN	<25	18
OVERWEIGHT	25-30	28
OBESE	≥30	52

5.2.4. Lung Function

Lung function parameters of forced vital capacity (FVC), forced expiratory volume in one second (FEV_1) and the ratio of the forced expiratory volume in the first one second to the forced vital capacity (FEV_1/FVC Ratio) were assessed using a spirometer (Vitalograph, U.K.) as described in section **3.3.2**. Patients were asked, where possible, to refrain from using their bronchodilator inhaler for 12 hr before performing the test. On arrival, patients inhaled a 200 μg dose of their bronchodilator therapy via a spacer device 15-minutes before performing a pulmonary function test, registered as a "post-bronchodilator" measurement. If subjects were unable to withhold regular inhaler therapy prior to visit, then spirometry results were recorded as "post-bronchodilator". Data were reported as a % of the predicted calculated value. Predicted values were calculated based on participants' height, weight, age, sex and ethnicity according to the modelling equation approved by the European Respiratory Society (ERS) (Quanjer et al. 2013).

5.2.5. Asthma Control Questionnaire (ACQ)-6

The ACQ-6 test is a validated questionnaire to assess symptoms of asthma control (Juniper et al. 1999). The ACQ-6 includes six items which are scored on a 6-point scale (0 = never/no symptoms/none; 6 = all the time/very severe symptoms/totally limited) that assess symptoms and the rescue by bronchodilator use over the previous week. The total maximum score of the questionnaire is 36. The total raw score was then divided by the 6 items and results were used for analysis. Optimal cut-offs to identify poorly controlled asthma are set at a score ≥1.5 and well controlled asthma is set at a score ≤0.75 (Juniper et al. 2005).

5.2.6. Blood collection

Venous blood was drawn from a vein in the antecubital fossa region of the forearm using a 23-gauge butterfly needle (BD Vacutainer Safety Lo k Blood Set 23g x 7" Tubing, Plymouth, UK). Approximately 18 mL of blood was drawn and 8 mL of it was immediately transferred into a EDTA plasma vacutainer (BD, Plymouth, UK) for the subsequent assessment of plasma cytokines, the remaining 10 mL was transferred into a serum separating tube (BD, Plymouth, UK) for the assessment of serum cytokines. Tubes were gently inverted and centrifuged as described in section **3.3.3** and safely stored in the -80°C freezer until use. After blood sampling, the needle was removed, and medical tissue was applied under pressure to prevent a superficial hematoma.

5.2.7. Analysis of blood plasma inflammatory cytokines

Blood cytokine levels (pg/mL) were measured in plasma from 98 WATCH study participants using a human magnetic Luminex® multiplex assay for, C-C Motif Chemokine Ligand 3 (CCL3), Chemokine Ligand 4 (CCL4), Chemokine Ligand 11 (CCL11), Interleukin (IL)-4, IL-5, IL-6, IL-8, Periostin, Chitinase-3like-1, Neutrophil gelatinase-associated lipocalin (NGAL), Leptin, Adiponectin and Tumor Necrosis Factor-Alpha (TNF-α) according to the manufacturer's instructions (R&D Systems, Bio-Techne Ltd, Abington, UK).

5.2.8. Enzyme linked immunosorbent assay (ELISA)

Blood serum Lipocalin-2 (NGAL) was measured using enzyme linked immunosorbent assays (ELISAs) (Bio-techne, #DLCN20). ELISA's were performed according to the manufacturer's instruction as described in section **3.3.4**. According to manufacturer's claim the sensitivity of the assay was 0.04 ng/mL. The intra-assay coefficient of variation was 3.7% and the inter-assays coefficient of variation was 6.4%. All samples were analysed in duplicate on one 96-well plate.

5.2.9. Statistical analysis

Statistical analyses were performed using Graphpad Prism 9.5.1 software. Data were reported as mean ± standard deviation (SD), unless otherwise specified. Data were examined for normality according to the Shapiro-Wilks criteria. Correlation between BMI, FM and lung function, ACQ-6, inflammatory cytokines were performed using correlation matrix and r Pearson analysis.

The 98 participants were grouped based on their BMI into three groups (Lean: BMI <25 kg/m^2; Overweight: BMI 25-30 kg/m^2; Obese: BMI ≥30 kg/m^2) or two groups for asthma management questionnaire (ACQ-6 score_low or ACQ-6 score_high). Cut-off score for ACQ-6 group division was 1.5.

Between group differences in lung function were assessed using one-way ANOVA plus post hoc Tukey's multiple comparison test. To assess variation of ACQ-6 score across BMI or FM, an unpaired t-test was performed. Differences between BMI and inflammatory cytokines were conducted based on normality test using either one-way

ANOVA plus post hoc Tukey multiple comparison test or Kruskal-Wallis test plus post hoc Dunn's multiple comparison test.

For data analysed from the two- way ANOVA, partial eta squared (η^2) effect sizes were calculated as:

$$\eta^2 = \frac{Sum\ of\ the\ squares\ beetween\ groups\ (SSE)}{Sum\ of\ the\ squared\ total\ (SST)}$$

Effect size for Kruskal-wallis test was Epsilon squared (ϵ^2), calculated as follow:

$$\epsilon^2 = \frac{H}{(n^2 - 1)/(n + 1)}$$

Where:

H= value obtained from the Kruskal-Wallis test.

N=Total number of samples

Cohen d (d) effect sizes were calculated for variables assessed by unpaired t-tests as:

$$Cohen\ d\ (d) = \frac{Mean\ Value\ ACQ6high(M1) - Mean\ Value\ ACQ6low(M2)}{SDpooled}$$

Where pooled standard deviation (SD):

$$\sqrt{\frac{SD1^2 + SD2^2}{2}}$$

5.3 Results

5.3.1. Participants characteristics

A total of 98 patients (n=69 female; n=29 male) from the WATCH cohort were included in the current study. The mean age at recruitment was 48.3±15yo with an average BMI of 31±7.11kg/m^2. Detailed demographics of patients are presented in **Table 5.3.1.1** and **Table 5.3.1.2**.

Table 5.3.1.1. General characteristics of participants.
(BMI=body mass index; FM= fat mass).

	Number of patients	**Mean**	**SD**
Demographics			
Total participant enrolled	98		
- female	69		
-male	29		
Age at the enrolment(yr)	98	48.3	15
Age at asthma diagnosis(yr)	88	26	19
BMI (kg/m^2)	98	31	7.11
-lean (kg/m^2)	18	22.6	1.7
-overweight (kg/m^2)	28	27.3	1.4
-obese (kg/m^2)	52	36.6	5.4
FM	97	36	14
Smoker Status	98		
-smoker	4		
-non smoker	48		
-ex-smoker	46		

Table 5.3.1.2. General asthma related information of patients from WATCH cohort. (GORD=gastro-oesophageal reflux disease; FEV_1=forced expiratory volume in 1 second; FVC=forced vital capacity; ACQ6= asthma control questionnaire).

	Number of patients	Mean	SD	% missing data
Comorbidities				
Patients with history of Rhinitis	49			
Patients diagnosed with GORD	60			
Patients diagnosed with Depression	29			
Patients diagnosed with Anxiety	18			
Patients diagnosed with Dysfunctional Breathing	44			
Patients diagnosed with Vocal Cord Dysfunction	13			
Health care utilization:				
Been in Intubated	16			
≥1 Admission to hospital in the last 12 months	67			
≥1 Asthma attacks in the last 12 months	73			
Asthma Treatments				
Use of long-term oral corticosteroids	26			
Biologic therapy	16			
Lung function Test Results:				
Post Bronchodilator use $\%FEV_1$	66	78	22	31.3
Post Bronchodilator use %FVC	65	91	18	32
Post Bronchodilator use $\%FEV_1$/FVC Ratio	65	68	17	32
Questionnaire:				
ACQ6 score	93	2.51	1.27	4.9

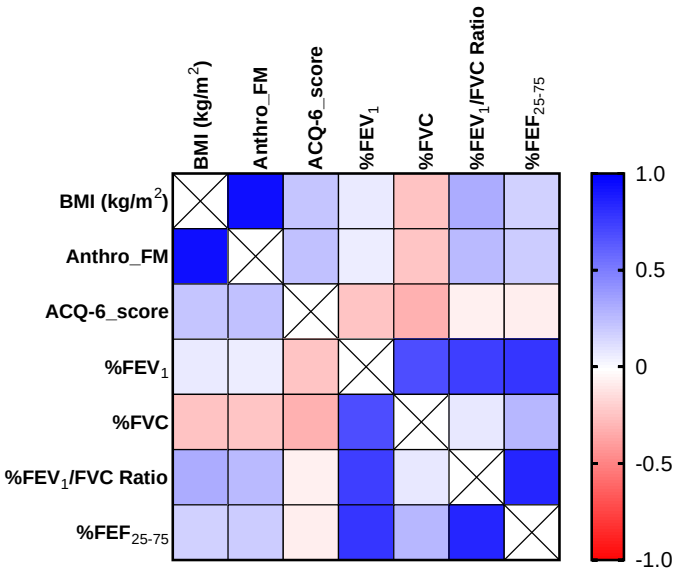
5.3.2. The role of BMI on lung function and asthma symptoms

A correlation analysis was undertaken between BMI, fat mass (FM) and lung function parameters and asthma management (ACQ-6) (**Fig. 5.3.2.1**). Correlation analysis showed a significant positive relationship between BMI, FM with $\%FEV_1$/FVC ratio (BMI: r=0.32, **p<0.01; FM: r=0.27, *p<0.05). There was no relationship between BMI, FM

with %FEV_1, %FVC or %FEF_{25-75}. For asthma control, both BMI and FM were positively correlated with ACQ-6 score (BMI: r= 0.23, *p<0.05; FM: r= 0.24, *p<0.05).

One-way ANOVA analysis was conducted to investigate differences in lung function between the three BMI groups (**Fig. 5.3.2.2**). The analysis revealed that the overweight asthma group had a greater %FEV_1 compared to the lean (*p<0.05; η^2=0.10). %FVC was lower in the obese asthma group compared to the overweight asthma group (*p<0.05; η^2=0.15). No between group differences were observed for %FEV_1/FVC Ratio (p=ns; η^2=0.10).

a)



b)

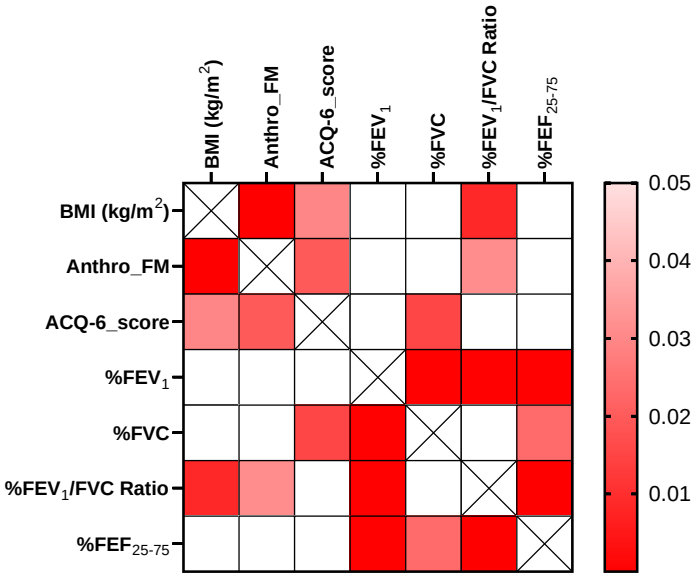


Fig. 5.3.2.1. Heatmap correlation matrix showing relationship between BMI, FM and lung function, asthma control.

a) r squared heat map for BMI and lung function parameters. Blue squares represented a positive correlation, values close to 1 were strongly correlated. Values between 0 to -1, indicated negative correlation between the parameters considered. b) p-value heat map. The white squares represent non-significant p value. Red squares represent significant p value. The darker the red, the stronger the significance was (measured as p values: p=0-p=0.05; n=98).

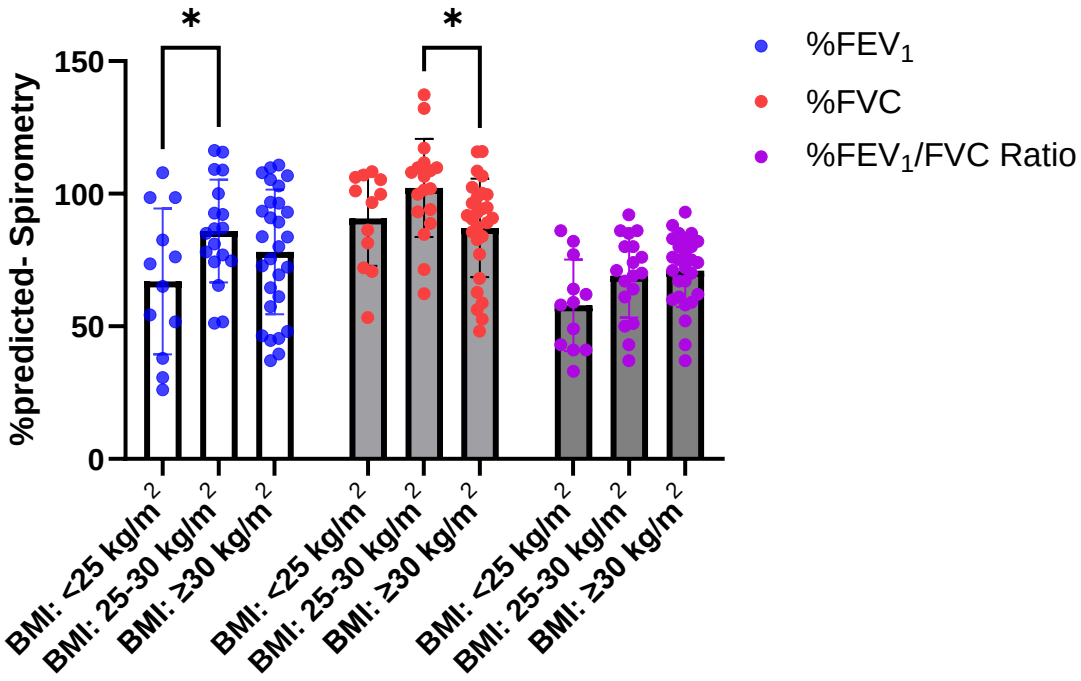


Fig. 5.3.2.2. The role of BMI on lung function.
The role of BMI on $\%FEV_1$ (blue dots; η^2=0.10), %FVC (red dots; η^2=0.15), $\%FEV_1$/FVC Ratio (purple dots; η^2=0.10). Data were displayed as mean±standard deviation (SD). Statistical analysis was performed using one-way ANOVA plus post hoc Tukey's multiple comparison test. P values are showed as follows: *p<0.05. (lean BMI: n=15; overweight BMI: n= 18; obese BMI: n=28).

For asthma control, participants were grouped depending on their ACQ-6 score (ACQ-6 score low<1.5; ACQ-6 score_high≥1.5), and subsequent independent T-tests revealed that patients with high ACQ-6 score had higher BMI and FM than patients with a low ACQ-6 score (BMI,:**p<0.01, d=0.87; FM:**p<0.01, d=0.93) (**Fig. 5.3.2.3**).

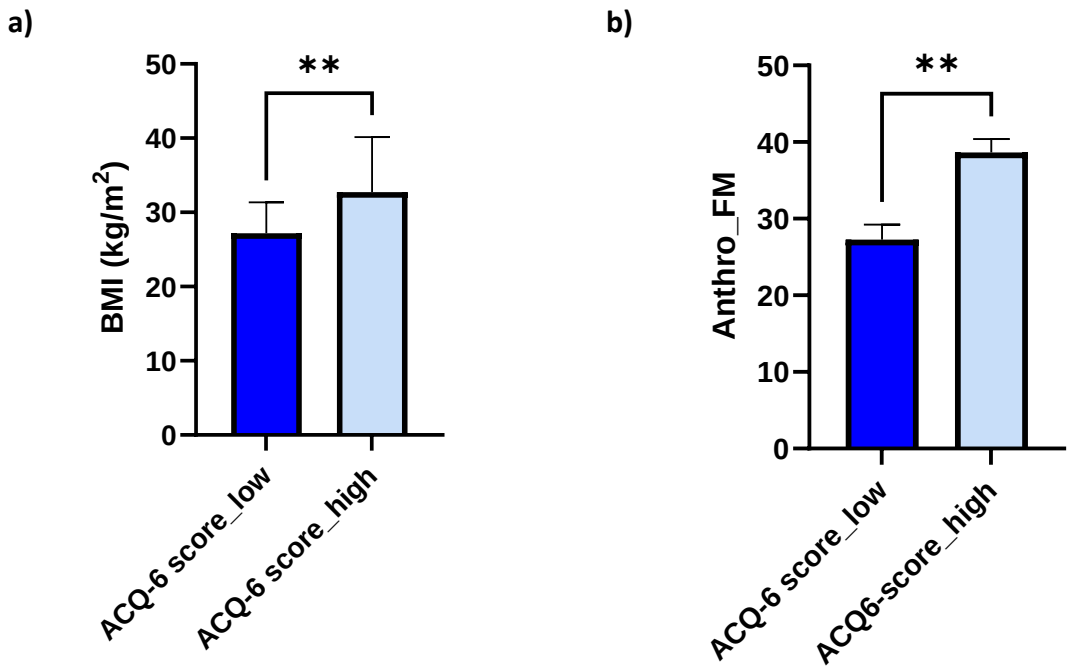


Fig. 5.3.2.3. The role of BMI and FM on asthma control.
The role of BMI a) and FM b) on asthma control assessed with ACQ-6 questionnaire. Data were reported as mean±standard deviation (SD). Statistical analyses were performed using un-paired t-test. P-value was showed as follow: *p<0.05, **p<0.01; ***p<0.001. BMI: Cohen d=0.87; FM: Cohen d= 0.93. (ACQ-6_low group: n= 19; ACQ-6_high group: n=74).

5.3.3. The role of BMI on inflammatory markers

Correlation matrix was conducted to establish relationships between BMI, FM with the blood biomarkers of inflammation (**Fig. 5.3.3.1**). Patients with both high BMI and FM were significantly associated with increased concentrations of plasma inflammatory cytokines: Granzyme A (BMI: r=0.34, **p<0.01; FM: r=0.30, **p<0.01), IL-4 (BMI: r=0.33, *p<0.05; FM: r=0.34, *p<0.05), IL-6 (BMI: r= 0.48, ****p<0.0001; FM: r=0.45, ****p<0.0001), IL-5 (BMI: r=0.64,****p<0.0001; FM: r= 0.68, ****p<0.0001), chemokine ligands 4 (CCL4) (BMI: r=0.39, ***p<0.0001; FM: r=0.40, ***p<0.001) and Leptin (BMI: r=0.55,****p<0.0001, FM: r=0.53, ****p<0.0001). However, no correlations were observed between BMI, FM with NGAL, adiponectin, CCL3, CCL11, IL-4, Periostin, CCL2, Chitinase3like1, Granzyme B, IL-8, TNF-α (p=ns).

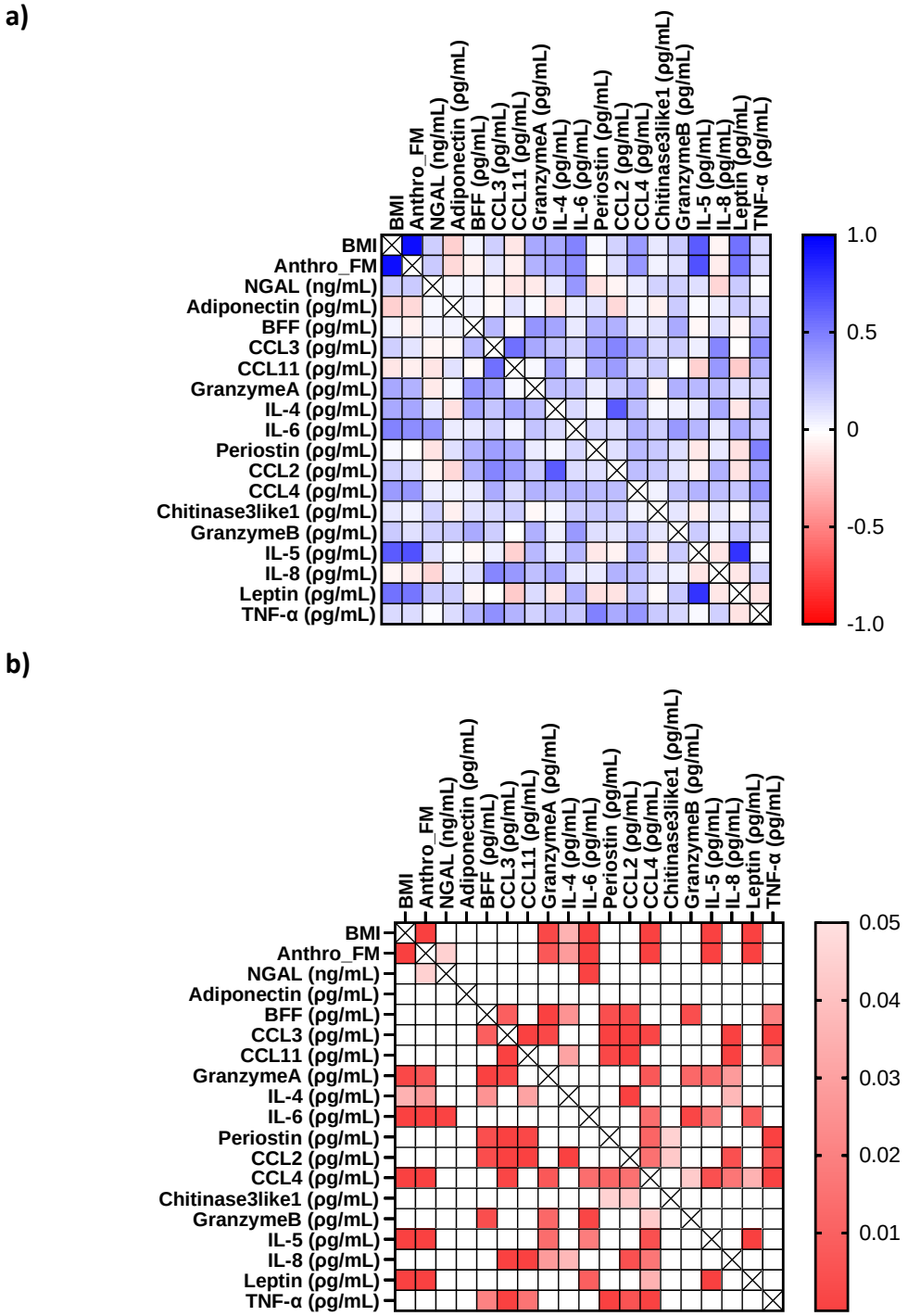
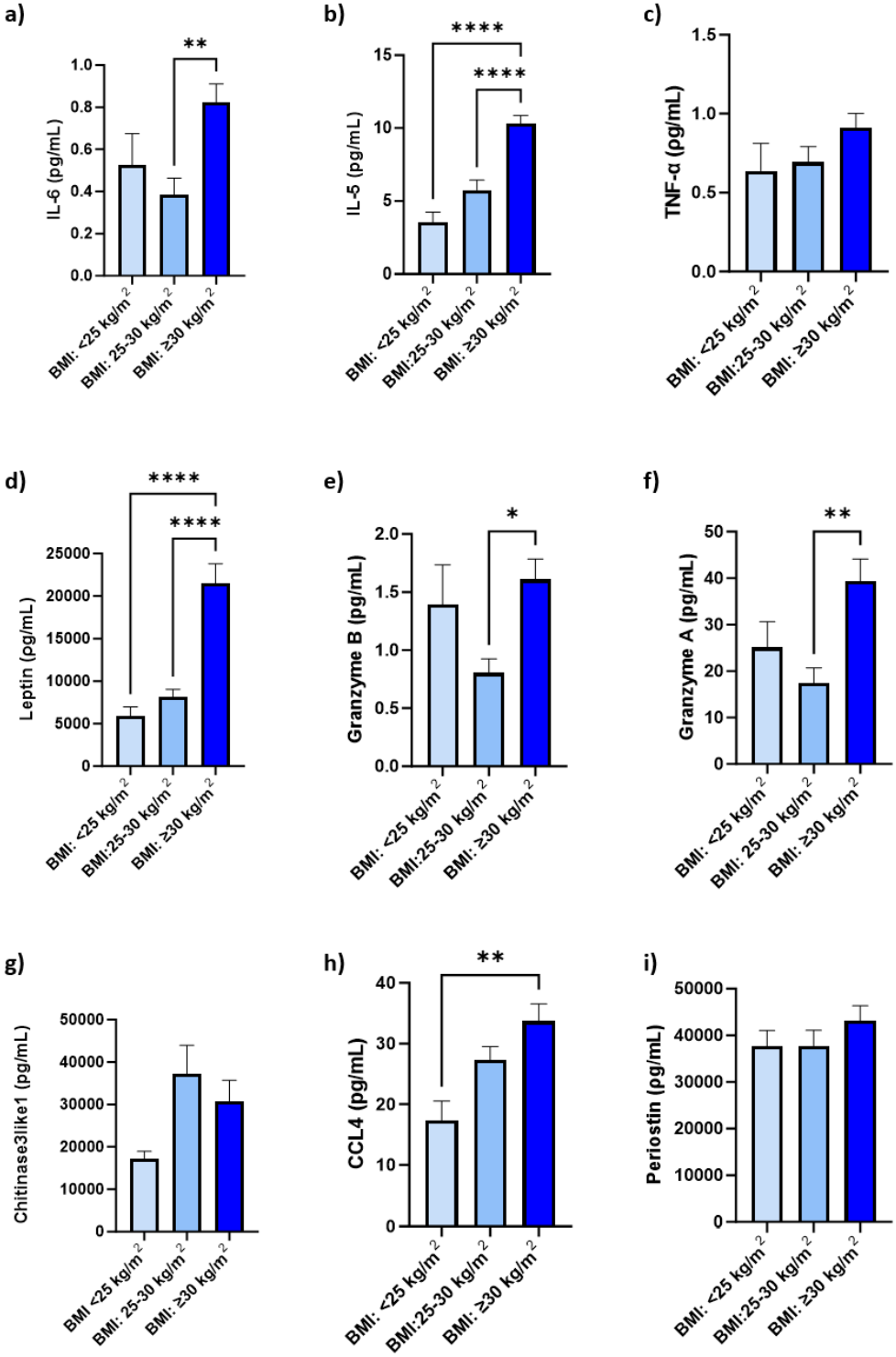


Fig. 5.3.3.1. Heatmap correlation matrix showing relationship between BMI, FM and inflammatory cytokines.

a) r squared heat map for BMI, FM and markers of inflammation. Blue squares represented a positive correlation with values close to 1 were strongly correlated. Values between 0 to -1, indicated negative correlation between the parameters considered. b) p-value heat map. The white squares represented non-significant p value. Red squares showed significant p value. The darker the red, the stronger the significance was (measured as p values: p=0-p=0.05; n=70 except IL-4; IL-4=41).

To evaluate the differences in biomarker concentrations between BMI groups a series of one-way ANOVA was performed (**Fig. 5.3.3.2**).This analysis revealed that patients with asthma and obesity had a greater concentrations of: IL-5 (****$p<0.0001$; $\eta^2=0.4$), IL-6 (**$p<0.001$; $\eta^2=0.15$), Leptin (****$p<0.0001$; ($\epsilon^2 =0.50$), Granzyme A (**$p<0.01$; ($\epsilon^2 =0.15$), Granzyme B (*$p<0.05$; ($\epsilon^2 =0.10$) and CCL4 (**$p<0.01$; $\eta^2=0.15$) compared to the patients with lean and overweight BMI. However, no major differences in level of inflammatory markers were observed between patients with lean BMI and patients with an overweight BMI.


a)
IL-6 (pg/mL)
**
BMI: <25 kg/m2
BMI: 25-30 kg/m2
BMI: ≥30 kg/m2
b)
IL-5 (pg/mL)
****
****
BMI: <25 kg/m2
BMI:25-30 kg/m2
BMI: ≥30 kg/m2
c)
TNF-α (pg/mL)
BMI: <25 kg/m2
BMI:25-30 kg/m2
BMI: ≥30 kg/m2
d)
Leptin (pg/mL)
****
****
BMI: <25 kg/m2
BMI:25-30 kg/m2
BMI: ≥30 kg/m2
e)
Granzyme B (pg/mL)
*
BMI: <25 kg/m2
BMI:25-30 kg/m2
BMI: ≥30 kg/m2
f)
Granzyme A (pg/mL)
**
BMI: <25 kg/m2
BMI: 25-30 kg/m2
BMI: ≥30 kg/m2
g)
Chitinase3like1 (pg/mL)
BMI <25 kg/m2
BMI: 25-30 kg/m2
BMI: ≥30 kg/m2
h)
CCL4 (pg/mL)
**
BMI: <25 kg/m2
BMI:25-30 kg/m2
BMI: ≥30 kg/m2
i)
Periostin (pg/mL)
BMI: <25 kg/m2
BMI:25-30 kg/m2
BMI: ≥30 kg/m2

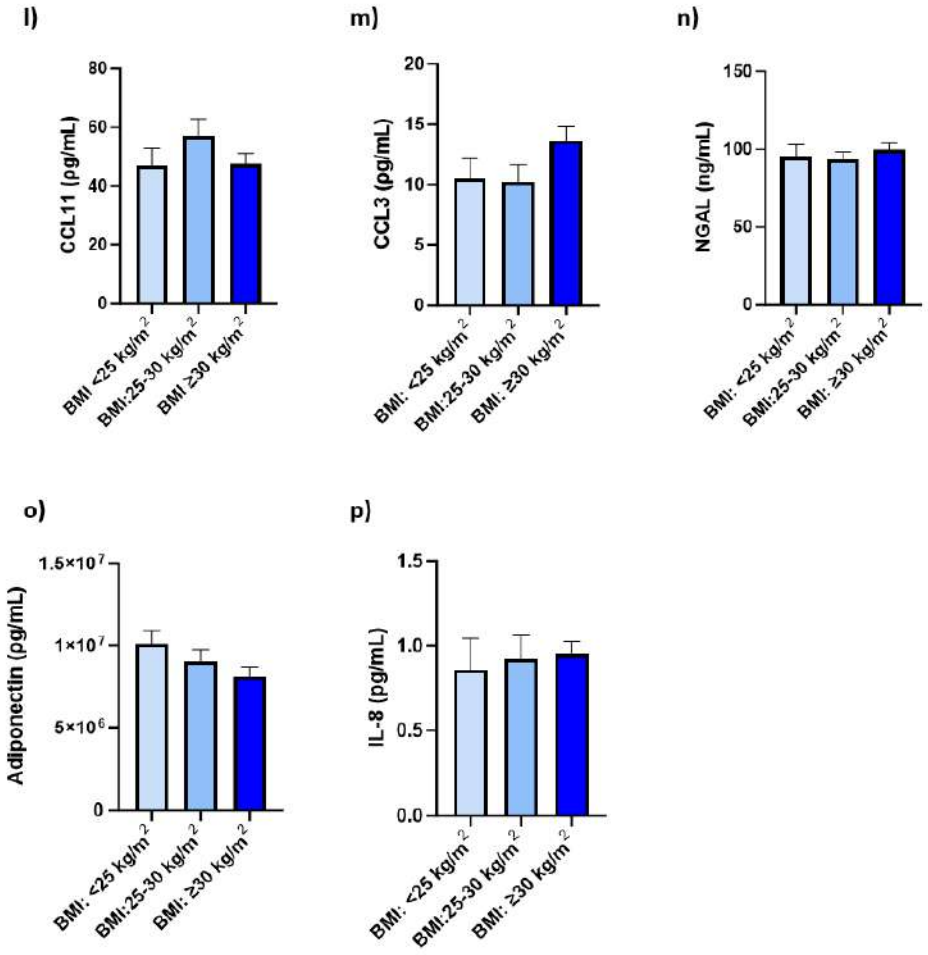


Fig. 5.3.3.2. The role of BMI in markers of inflammation.
The effect of BMI on systemic inflammation was evaluated with the following cytokines: a) Interleukin (IL)-6 (η2=0.15), b) IL-5 (η2=0.40), c) Tumor Necrosis Factor (TNF)-α (η2=0.10), d) Leptin (ϵ2 =0.50), e) Granzyme B (ϵ2 =0.10), f) Granzyme A (ϵ2 =0.15), g) Chitinase3like1 (η2=0.05), h) chemokine CC motif ligand 4 (CCL4) (η2=0.15), i) Periostin (η2=0.05), l) CCL11 (η2=0.05), m) CCL3 (η2=0.05), n) Neutrophil gelatinase-associated lipocalin (NGAL) (η2=0.01), o) Adiponectin (ϵ2 =0.10), p) IL-8 (ϵ2 =0.05). Data are shown as a mean±standard error of the mean (SEM). One-way ANOVA plus post hoc Tukey multiple comparison test or Kruskal-Wallis plus post hoc Dunn's multiple comparison test were performed, based on normality test. P values were showed as follow: *<0.05, **p<0.01, ***p<0.0001, ****p<0.0001. (Lean BMI <25 kg/m2: n=13; overweight BMI 25-30 kg/m2 group: n=19; obese BMI ≥30kg/m2: n=39.

5.4 Discussion

The characterisation of a subset of the WATCH cohort of difficult to control asthma established that increasing BMI and FM: 1) were associated with poorer asthma control; 2) were associated with greater concentrations of systemic inflammatory blood biomarkers; 3) there was no clear relationship between BMI and FM with lung function.

5.4.1. The role of BMI on lung function and asthma control

Analysis of this cohort demonstrated that higher BMI and FM were not correlated to worse lung function, but they were significantly associated with poorer asthma control, evaluated by the ACQ-6 questionnaire. Association between lung function and BMI is controversial. Several studies have previously reported an association between obesity and poor lung function (Biring 1999; Ciprandi et al. 2014; Gibeon et al. 2013) while others reported no association (Bildstrup et al. 2015; Klepaker et al. 2021; Tang et al. 2022). Indeed, in the current study the lean asthma group had lower %FEV_1 predicted values compared to patients who were overweight. This may be indicative of high Th-2 driven asthma predominating in lean asthma patients who exhibit poor asthma control. As demonstrated in **Fig. 5.3.2.2**, this may have been driven by three very severe and poorly controlled patients in this group. On the contrary, patients with obese BMI exhibited a lower %FVC compared to the overweight patients, but a similar level to participants with lean BMI. This suggest that higher BMI may not be involved in lung function decline as previously reported (Bildstrup et al. 2015). However, spirometry may not be the most sensitive method to detect lung dysfunction driven by obesity. Salome et al. investigated lung function mechanics in a cohort of patients with asthma with and without obesity and showed no difference in spirometry parameters between groups.

However, the obese group had higher respiratory system resistance and reduced respiratory system reactance, a measure of airways closure, when assessed via oscillometry technique (Salome et al. 2008). This was then confirmed in similar subsequent studies (Al-Alwan et al. 2014; Chapman et al. 2014). Researchers concluded that obesity may be involved in peripheral airway closure and a standard method such as spirometry may not be able to detect this change. Therefore, a combined use of spirometry and other methods that evaluate airway narrowing such us impulse oscillometry may be a more sensitive way to evaluate lung dysfunction. Moreover, most previous studies conducted lung function tests prior to bronchodilator use, while the WATCH cohort performed pulmonary function assessments post-bronchodilator use. The β_2 agonist bronchodilators used by patients will relax the airway smooth muscles allowing widening of the airways, which is likely to have reduced any potential between group differences. Finally, a review paper by Dixon and Peters concluded that body fat distribution had a stronger association with lung function than BMI (Dixon and Peters 2018). However, the current study failed to demonstrate any relationship between FM and lung function. This may be due to the low participant numbers and uneven distribution of participants across BMI groups in the current cohort.

Although there were no functional differences between groups, BMI and FM were strongly associated with the subjective reporting of asthma control assessed with ACQ-6 score questionnaire. The current data supports a previous study conducted in 382 patients with mild or severe asthma demonstrating that those with higher BMI had poorer asthma control which was independent from asthma severity, age, sex and asthma treatment (Lavoie et al. 2006). Similar findings were also reported in a later study conducted in a cohort of 508 patients with severe asthma (L. L. Barros et al. 2011).

In both studies the BMI was associated with declining asthma control, but lung function was unaffected. This may suggest a direct role of obesity in lack of asthma control and poor quality of life, independent of a decline in lung function. One mechanism through which BMI may affect asthma control may be via mediation of markers of systemic inflammation such as Leptin, IL-5 and IL-13 (Zhang et al. 2018). In the present study, increased plasma levels of Leptin and IL-5 were strongly correlated with BMI and FM, supporting the theory that obesity and greater adiposity may play a key role in the lack of asthma control. As a further contribution to this hypothesis, weight loss has been reported as a successful strategy for the amelioration of asthma symptoms and reduction of medication used (Bantulà et al. 2022; Eneli, Skybo, and Camargo 2008; Maniscalco et al. 2008). The findings of the present study contribute to previously published literature supporting the theory that BMI and greater adiposity may be a contributing factor in the decline of asthma control.

5.4.2. The role of BMI on systemic inflammation

Significant correlations were identified between patients with high BMI and FM with greater concentrations of proinflammatory cytokines IL-5, CCL4, Granzyme A, Granzyme B, IL-6, and Leptin.

The cytokines IL-5, Granzyme A, Granzyme B and CCL4 are markers of eosinophilic inflammation involved in the classic form of asthma known as Th-2 asthma (Annoni et al. 2015; Desai et al. 2014; Tschopp et al. 2006). However, the cytokine IL-5, commonly associated with systemic inflammation, was elevated in a previous cohort of patients with obesity and severe asthma (Boulet 2013; Desai et al. 2014), similarly to what was found in the present study. Additionally, granzyme B, generally associated with airway

inflammation, was recently shown to be expressed by visceral adipose tissue in patients with obesity and associated with adiposity derived inflammation (Cimini et al. 2020). Therefore, this suggests that although a subgroup of the WATCH patients examined in the present study may have exhibited a Th-2 high phenotype of asthma, the strong correlations between some of the Th-2 cytokines considered with high BMI and FM, indicate a contributing role of adiposity in the expression of airway inflammatory cytokines.

Greater BMI and FM were also significantly correlated with high levels of IL-6 and Leptin, commonly associated with Th-2 low asthma phenotype. Both IL-6 and Leptin are mostly released by the white adipose tissue and associated with low grade inflammation (Sideleva et al. 2012; Sutherland et al. 2008). Previous reports support that IL-6 could play a major role in the pathogenesis of asthma (Marini et al. 1992; Rincon and Irvin 2012; Yokoyama et al. 2012), while high level of Leptin was associated with airway hyperresponsiveness and reduced asthma control (Shore et al. 2005; Zhang et al. 2018). This suggests that the current cohort the patients with obesity and asthma showed low grade inflammation which may contribute to poorer asthma control. Although not reaching significance, patients with obesity and asthma demonstrated a trend with a medium effect size for greater circulating levels of TNF-α. The pro-inflammatory cytokine TNF-α is involved with systemic inflammation in obesity (Alzamil 2020; Popko et al. 2010) and lung function impairment in asthma (Lecube et al. 2011), suggesting a role of obesity in driving inflammation in asthma.

The strong correlation between FM, BMI and IL-6, Leptin and the trend of TNF-α support the theory that obesity may contribute to the worsening of asthma disease via mediation of systemic inflammation.

Overall, this study contributes to the developing understanding that increasing BMI and FM will worsen asthma control, especially in a cohort of difficult-to-control asthma patients. Although BMI did not influence lung function directly, it was associated with poorer asthma control. BMI was also associated with increased circulating levels of proinflammatory cytokines involved in both Th-2 high (IL-5, Granzyme A, Granzyme B and CCL-4) and Th-2 low (IL-6 and Leptin) asthma phenotypes. This suggests a key role for obesity in asthma, perhaps independent of being Th-2 low or Th-2 high driven and highlight the complexity of the interaction between obesity and asthma which goes beyond the simple phenotype classification. Llorente et al. suggested that other factors such as inflammation, shifts in the microbiota community and alteration of metabolic status such as insulin resistance can all contribute to the pathophysiology of asthma (Gomez-Llorente et al. 2017). The combination of the underlying inflammation going on in both asthma and obesity may contribute to the worsening of disease. In addition, obesity is a complex disease associated with metabolic disorders such as impaired glucose, insulin resistance and/or cholesterol level which may likely impact asthma control too (Cardoso and Perucha 2021; Cottrell et al. 2011b; DeChristopher and Tucker 2018; Forno et al. 2015; Hill et al. 2008; Karampatakis et al. 2017). Moreover, obesity is associated with increased circulation of endotoxin such as LPS via gut barrier disruption which may further enhance inflammation and poor asthma control (Clemente-Postigo et al. 2019; Tulkens et al. 2020) Therefore a more integrated approach that takes into consideration the effect of obesity and asthma on metabolic impairment and gut barrier

integrity may support developments in understanding disease complexity and help to support future disease management strategies.

5.4.3. Conclusion

In conclusion, characterisation of a subset of the difficult to control WATCH cohort showed that BMI and FM were correlated with poor asthma control and increased inflammatory cytokines related to Th-2 high and Th-2 low inflammation. This suggests a direct role for obesity on asthma control via mediation of systemic inflammation. This provides evidence to suggest that dietary strategies, and physical activity that target weight loss may have a beneficial effect on asthma control and therefore warrant future investigation.

Chapter 6: Comparison of gut permeability markers in mild well-controlled and difficult to control asthma cohorts

6.1 Introduction

Asthma is an inflammatory disease of the airways characterized by transient bronchoconstriction, wheezing and chest tightness. It can be generally managed with the use of asthma medications, although several factors such as lifestyle, environmental triggers and genetics can worsen asthma symptoms and lead to severe asthma condition. Obesity increases the risk of developing asthma and patients with obesity and asthma have worse symptoms, poorer disease control and are less responsive to medication (Bantulà et al. 2021a). Asthma in patients with obesity is characterized by higher levels of markers of T helper (Th)-17 inflammation, normal level of Th-2 cytokines, elevated neutrophil blood cell counts and are associated with neutrophilic airway inflammation. This differs from the conventional form of asthma, characterized by elevated Th-2 cytokines and elevated eosinophils blood cells.

Obesity is also linked with changes in the gut microbiota composition (Gérard 2016; Gomes, Hoffmann, and Mota 2018; Liu et al. 2021). The gut microbiota represents the collection of microorganisms that populate the human gastro-intestinal tract and actively contributes to developing a healthy immune system, helping food digestion and supporting energy harvest (Maslowski et al. 2013). In healthy conditions, bacteria are confined in the intestinal lumen of the gastrointestinal tract by the gut barrier. The intestinal barrier represents a semi-permeable structure that prevents proinflammatory bacteria species and molecules from reaching the lamina propria where the immune system cells are located. Alteration in the gut microbiota composition can lead to the disruption of gut barrier and subsequent translocation of proinflammatory bacteria and

related fragments, such as Lipopolysaccharides (LPS) into the gut lumen which in turn increase systemic inflammation (Quigley 2017). Limited studies investigated biomarkers of gut permeability in asthma. Between others, the biomarkers lipopolysaccharides binding protein (LBP) and calprotectin have been recently associated with asthma.

LBP plays an important role in binding LPS to pattern recognition receptors such as tool like receptor (TLR), which is essential for initiating inflammation (Schumann 1992; Tsukamoto et al. 2018). Raised plasma concentration of LBP has been previously reported in bronchoalveolar cleavage of patients with asthma (Dubin et al. 1996), but there is currently no evidence for a role of LBP in asthma function and control.

The gut inflammation marker noted as calprotectin has been recently linked with asthma severity (Decaesteker et al. 2022; Lee et al. 2020). Calprotectin is a protein secreted from neutrophils and macrophages, with known antimicrobial and pro-inflammatory properties, but its role in the inflammation response is still not well characterized. Additional information on LBP and Calprotectin can be found in section **2.5.2**.

Studies often group patients with severe or mild well-controlled asthma conditions together making harder to assess which factors contribute to disease severity. Therefore, the present study aims to compare markers of gut permeability in two separate cohorts of asthma based on asthma disease progress: a cohort with well-controlled asthma and a cohort of patients with difficult-to-control asthma.

AIMS OF THE STUDY

This study aimed to test whether plasma concentrations of gut permeability markers, (LBP and Calprotectin) differed across the whole cohort recruited in terms of BMI and lung function.

A further aim of the study was to explore whether plasma concentrations of gut permeability markers differed in the three populations:

1. mild well-controlled asthma
2. difficult to control asthma
3. healthy controls.

6.2 Material and Methods

6.2.1 NTU-WATCH Study Design

The NTU-Southampton study was approved by the NTU Ethics Committee (protocols: #689). All procedures were conducted in accordance with the Declaration of Helsinki.

NTU received 98 serum and plasma samples previously collected from patients with difficult to control asthma across a range of BMIs and their related database from the WATCH at Southampton University Hospital (Ethical approval: https://clinicaltrials.gov/ct2/show/NCT03996590). Samples were shipped to NTU via courier and stored at -80°C until use. The database is stored in the NTU Data Store and protected by password with all necessary safeguards in accordance with any relevant laws, statutes, regulations and good practice.

A total of 67 participants were recruited at NTU: 28 participants with well-controlled asthma and 39 healthy controls. From the initial cohort of participants, 60 subjects met the criteria and were used for analysis as showed in the consort chart in **Fig. 6.2.1.1**.

In accordance with the Declaration of Helsinki all participants were provided with a participant information sheet at least 24 hr before commencing the study. All participants completed a self-reported health screen questionnaire to assess whether inclusion or exclusion criteria were met. A full list of inclusion and exclusion criteria can be found in Appendix 1: WATCH participant information sheet and consent form. Briefly, a GP asthma diagnosis was required, and participants were asked to refrain from asthma medication 24 hr before the testing session. There were differences in the inclusion and exclusion criteria between the WATCH and NTU participants, highlighted in 3.2.1.

Participants from the WATCH cohort were defined as patients with difficult to control asthma (DCA) and referred to as such from now on. Asthma is defined as difficult to control when patients continue to be symptomatic following the use of high dose inhaled corticosteroids therapies and/or use of frequent oral corticosteroids (British Thoracic Society (BTS) 2019). This type of asthma is characterized by frequent exacerbations, poor quality of life and elevated morbidities (Sastre et al. 2016a).

Participants recruited at NTU were defined as mild well-controlled asthma (WCA) and referred to as such from now on. Well-controlled asthma participants were on steps 1 to 5 based on the British Thoracic Society guidelines (British Thoracic Society (BTS) 2019).

Healthy control (C) participants were defined as participants with no underlying diseases evaluated with the use of the health screen questionnaire given to the participants during the visit (health screen questionnaire can be found in **Appendix 3: Self-reporting Health screen**).

Participants recruited at NTU attended the lab for one visit lasting approximately 60 minutes for the collection of blood samples, spirometry, anthropometry and completion of asthma related questionnaires.

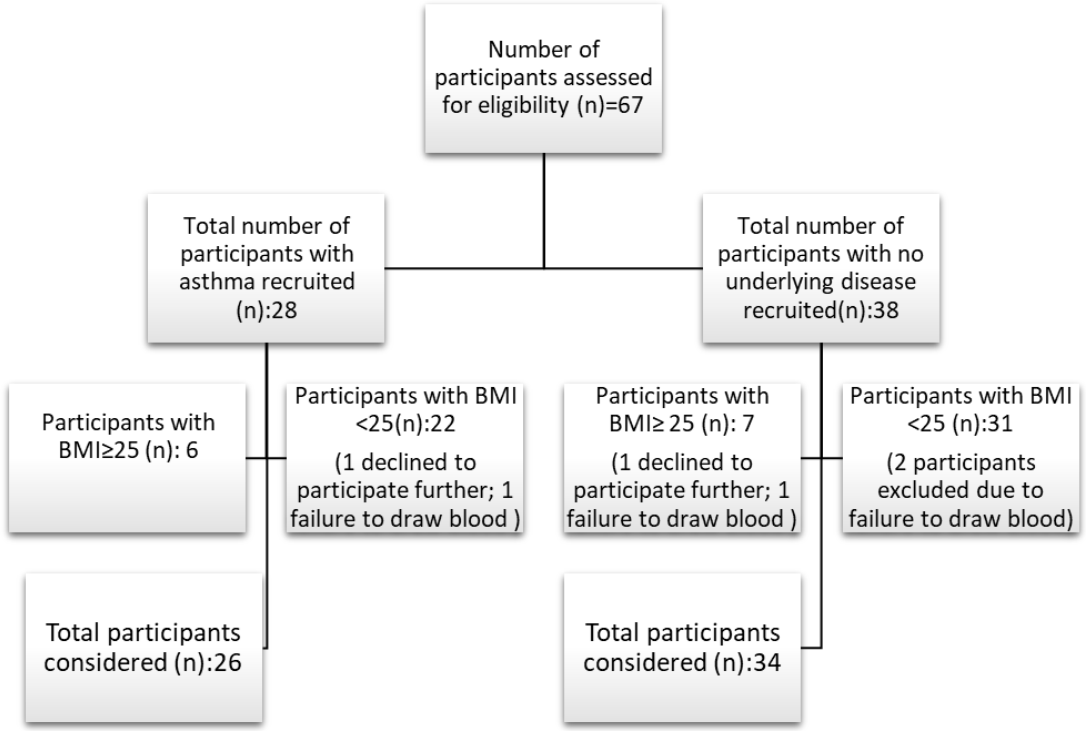


Fig. 6.2.1.1. Consort chart for participants recruited at NTU.

6.2.2 Anthropometric measurements

Participants' body mass was measured to the nearest 0.1 kg using calibrated electronic scales (SECA 877 Scale, SECA, Birmingham, UK), wearing lightweight clothing and barefoot while their height was measured using a stadiometer (SECA stadiometer, SECA, Birmingham, UK). BMI was then calculated as mass (kg)/height $(m)^2$.

6.2.3 Pulmonary function

Pulmonary function was conducted in accordance with the ATS/ERS guidelines using a spirometer (Pneumotrac; Vitalograph, Buckingham, UK) calibrated with a 3L syringe

(Miller et al. 2005). Participants performed the test a minimum of 3 times in a standing position and wearing a nose clip.

Participant's mass, height, age, sex, ethnicity and smoking status were entered in the Spirometry software to calculate their personal predicted lung function based on the predictive equations of Quanjer and co-workers (Quanjer et al. 2013).

Participants performed maximal flow-volume loops to determine absolute and percentage predicted forced vital capacity (%FVC), forced expiratory volume in 1 second (%FEV_1), forced expiratory flow rate from 25-75% of FVC (%FEF_{25-75}), forced expiratory flow rate 75-85% of FVC (%FEF_{75-85}) and %FEV_1/FVC ratio. Before the test, participants were instructed to inhale to their maximum capacity, place their mouth around the mouthpiece filter and to exhale as quickly as possible for six seconds. After six seconds participants were instructed to rapidly inhale to full inspiration.

Participants performed a minimum of three flow-volume loops for pulmonary function measures. The two highest values for the measure of FEV_1 had to be within 0.150 L and the highest values were taken for the analysis. Participants with asthma performed spirometry twice: an initial test was performed on the arrival, before the use of the inhaler, and a second test was performed 15 minutes after using their inhaler and registered as a "post-bronchodilator" measurement. The dose inhaler used was 200 µg, inhaled via a spacer device between tests.

6.2.4 Blood collection

Blood was drawn from a vein in the antecubital fossa region of the elbow using a 23-gauge butterfly needle (BD Vacutainer, Plymouth, UK).

For plasma, 8 mL of whole blood was collected and immediately transferred into an EDTA plasma vacutainer (BD, Plymouth, UK), gently inverted and immediately centrifuged for 15 minutes at 1500g at 4°C. For serum, 10 mL of whole blood was collected and transferred into a serum separating tube (BD, Plymouth, UK), gently inverted and left to clot for 30 minutes at room temperature, begore centrifugation for 15 minutes at 1000g at 4°C. After centrifugation, plasma and serum layers were then aliquoted and subsequently stored at -80°C until use. After blood sampling the butterfly needle was removed, and firm pressure was applied upon the puncture site to avoid hematoma.

6.2.5 Enzyme linked Immune sorbent assays

Commercially available enzyme linked immune-sorbent assays (ELISAs) were used to determine concentrations of proteins from human blood serum and plasma samples.

ELISAs were used to quantify lipopolysaccharides binding protein (LBP) (Hycult Biotech) and Calprotectin (R&D System). All ELISAs were performed according to the manufacturer's instructions. Samples, reagents, and standards were prepared using the appropriate dilutions as instructed by the manufacturer.

According to the manufacturer, the lower limits of detection for the assays were 4.4 ng/mL for LBP and 0.086 ng/mL for Calprotectin. The intra-assay and inter-assay coefficient of variation (CV) were 2.2% and 4.1% for LBP, 3.4% and 4.6% for Calprotectin, respectively. All samples were analysed in duplicate on one 96-well plate.

6.2.6 Asthma control Questionnaire (ACQ-6)

The ACQ-6 test is a validated questionnaire used to assess symptoms of asthma control (Juniper et al. 1999). It is composed of six questions about asthma symptoms experienced during the previous week and inhaler use. Questions included frequency of asthma symptoms upon waking, awakening due to asthma during the night, frequency of breathlessness and wheezing, limitation of daily activity due to asthma and use of inhaler. The ACQ-6 includes six items which are scored on a 6-point scale with a total score of 36. Final participant score was obtained by dividing the total raw score by 6 (number of items in the questionnaire). Optimal cut-offs to identify poorly controlled asthma were set at a score ≥1.5 (Juniper et al. 2005).

6.2.7 Statistical Analysis

Statistical analyses were performed using Graphpad Prism 9.5.1 software. Data were tested for normality using the Shapiro-Wilks criteria. Differences in asthma control between mild well-controlled asthma and difficult to control asthma cohorts were assessed using independent samples t-tests. Correlation between LBP, Calprotectin and BMI, lung function was performed using correlation matrix and r Pearson analysis. Correlation between LBP, Calprotectin and ACQ-6 score were performed using simple linear regression.

Differences between gut permeability markers and lung function were conducted based on a normality test using Kruskal-Wallis test plus post hoc Dunn's multiple comparison test. Data were reported as mean ± standard deviation (SD), unless otherwise specified.

A further Mann-Whitney non-parametric test was conducted between groups to evaluate differences in plasma concentration of calprotectin or LBP.

Effect size used for ANOVA test was eta squared (η^2) and calculated as follows:

$$\eta^2 = \frac{Sum\ of\ the\ squares\ beetween\ groups\ (SSE)}{Sum\ of\ the\ squared\ total\ (SST)}$$

Effect size for Kruskal-Wallis test was Epsilon squared (ϵ^2), calculated as follow:

Where:

$$\epsilon^2 = \frac{H}{(n^2 - 1)/(n + 1)}$$

H= value obtained from the Kruskal-Walli test.

N=Total samples' size.

Effect size for the Mann-Whitney test was Cohen d (d) and calculated as follow:

$$Cohen\ d\ (d) = \frac{Mean\ Value\ (WCA\ or\ DCA) - Mean\ Value\ (C)}{SDpooled}$$

Where pooled standard deviation (SD):

$$\sqrt{\frac{SD1^2 + SD2^2}{2}}$$

6.3 Results

6.3.1 Participant characterization

Participants were split into three groups based on the presence and control of asthma: healthy controls (C), participants with well-controlled asthma (WCA) and participants with difficult to control asthma (DCA) (**Table 6.3.1.1**).

Table 6.3.1.1. Participant characterization of healthy controls, well-controlled asthma and difficult to control asthma cohorts.

	Healthy Controls (C)		**Participants with well-controlled asthma (WCA)**		**Participants with difficult to control asthma (DCA)**	
n=	34		26		98	
	Average	**SD**	**Average**	**SD**	**Average**	**±SD**
Age(yr)	27.5	±5	26	±8	48	±15
Sex	13F;21M		18F;8M		69F;29M	
BMI(kg/m^2):						
Lean BMI (kg/m^2) <25	(n=29)21.9	±1.6	(n=21)23.1	±1.7	(n=18)22.6	±1.7
Overweight BMI (kg/m^2) 25-30	(n=4)28.4	±1.4	(n=5)28.0	±1.80	(n=28)27.3	±1.4
Obese BMI(kg/m^2) ≥30	(n=2)30.2	±0.17	-	-	(n=52)36.7	±5.4
Age at Asthma diagnosis(yr)	-	-	11.6	±9.9	24	±19.6
	%	%missing data	%	%missing data	%	%missing data
Asthma medication:						
Oral Corticosteroids (tablets)	-	-	0	0	26%	0
Biological treatment	-	-	0	0	16%	0
Inhaler Corticosteroids	-	-	100%	0	57%	0
Smoker Status	100%non-smoker	-	100% non-smokers	-	94% non-smokers; 4% smokers	2%
Diagnosis of Anxiety	100%No	-	25%Yes; 75%No	-	13%Yes; 56%No	30%
Diagnosis of Depression	100% No	-	10%Yes; 90%No	-	32% Yes; 50%No;	18%

6.3.2 Assessment of lung function and asthma control of healthy controls, well-controlled and difficult to control asthma groups.

One way ANOVA analysis was used to investigate lung function of the different groups (**Fig. 6.3.2.1**). In the DCA group, all lung function parameters (%FVC, $\%FEV_1$, $\%FEV_1/FVC$, $\%FEF_{25-75}$) were significantly lower compared to the control group (%FVC: **p<0.01; $\%FEV_1$: ****p<0.0001; $\%FEV_1/FVC$: ****p<0.0001; $\%FEF_{25-75}$: ****p<0.0001). In the DCA group, lung function parameters were significantly lower than WCA group (%FVC: p=ns; $\%FEV_1$: ***p<0.001; $\%FEV_1/FVC$: ****p<0.0001; $\%FEF_{25-75}$: **p<0.01). No differences in lung function were observed between healthy controls and the WCA group **Fig. 6.3.2.1**.

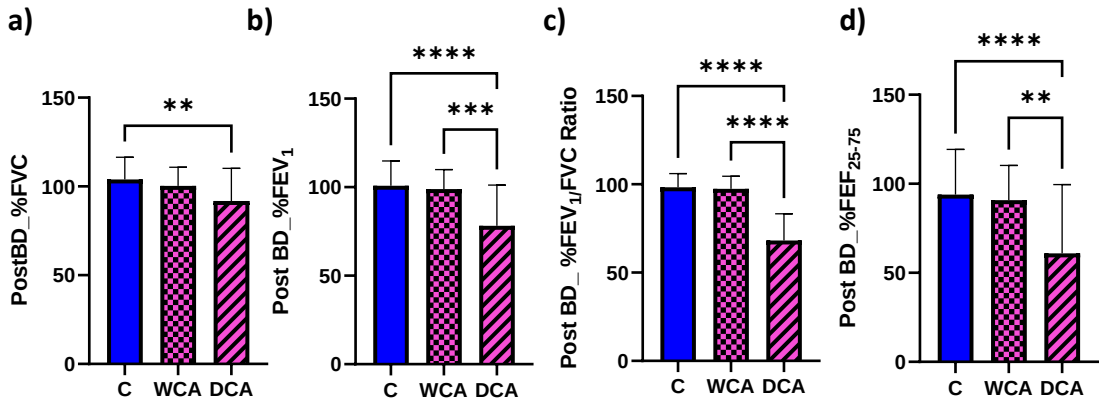


Fig. 6.3.2.1. Lung function assessment of healthy controls (C), well-controlled (WCA) and difficult to control asthma (DCA) cohorts.
Lung function characterization of C, WCA and DCA asthma assessed by spirometry and with the evaluation of a) %FVC (η^2=0.2), b) $\%FEV_1$ (η^2=0.3), c) $\%FEV_1/FVC$ (η^2=0.4), d) $\%FEF_{25-75}$ (η^2= 0.2), Data are shown as a mean±standard deviation (SD). One-way ANOVA plus post hoc Tukey multiple comparison test was performed. P values are shown as follows: **p<0.01, ***p<0.0001, ****p<0.0001. -(Healthy control: n=34; well-controlled asthma: n=26; difficult to control asthma: n=98.

Patients with DCA had a significantly higher ACQ-6 score compared to WCA group (****p<0.0001; d=1.63) (**Fig. 6.3.2.2**).

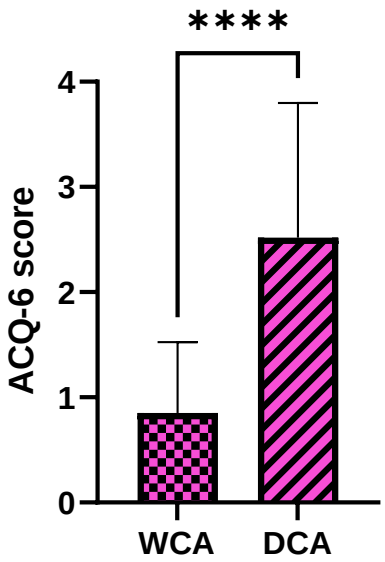


Fig. 6.3.2.2. Differences in ACQ-6 asthma score between participants with well controlled asthma (WCA) and difficult to control asthma (DCA).
Statistical analysis was performed using an independent samples t-test. p-value was shown as follows: ****p<0.0001. Data were reported as mean±standard deviation (SD). Cohen d=1.63; (ACQ-6_well controlled asthma group: n= 26; ACQ-6_difficult to control asthma: n=91).

6.3.3 The role of LBP and Calprotectin in BMI and lung function

A correlation analysis was undertaken between LBP, Calprotectin and BMI and lung function parameters for all 3 groups considered (healthy controls, well-controlled asthma and difficult to control asthma) (**Fig. 6.3.3.1**). Significant strong correlation was found between LBP and BMI and between calprotectin and BMI (LBP: r=0.49, ****p<0.0001; Calprotectin: r=0.36, ****p<0.0001). Circulating levels of LBP were negatively correlated with lung function parameters: $\%FEV_1$ (r=0.27, **p<0.01), %FVC (r=0.23, **p<0.01), $\%FEV_1$/FVC Ratio (r=0.31, ***p<0.001) and $\%FEF_{25-75}$ (r=0.24, *p<0.05).

There was no relationship between Calprotectin and lung function parameters %FEV_1, %FVC, %FEV_1/FVC Ratio or %FEF_{25-75} (p=ns) (**Fig. 6.3.3.1**).

The high BMI was negatively correlated with lung function parameters FEV_1 (r=0.23, **p<0.01), %FVC (r=0.35, ****p<0.0001), %FEV_1/FVC Ratio (r=0.31, ***p<0.001). No correlation was observed between BMI and %FEF_{25-75} (r=0.11, p=ns) (**Fig. 6.3.3.1**).

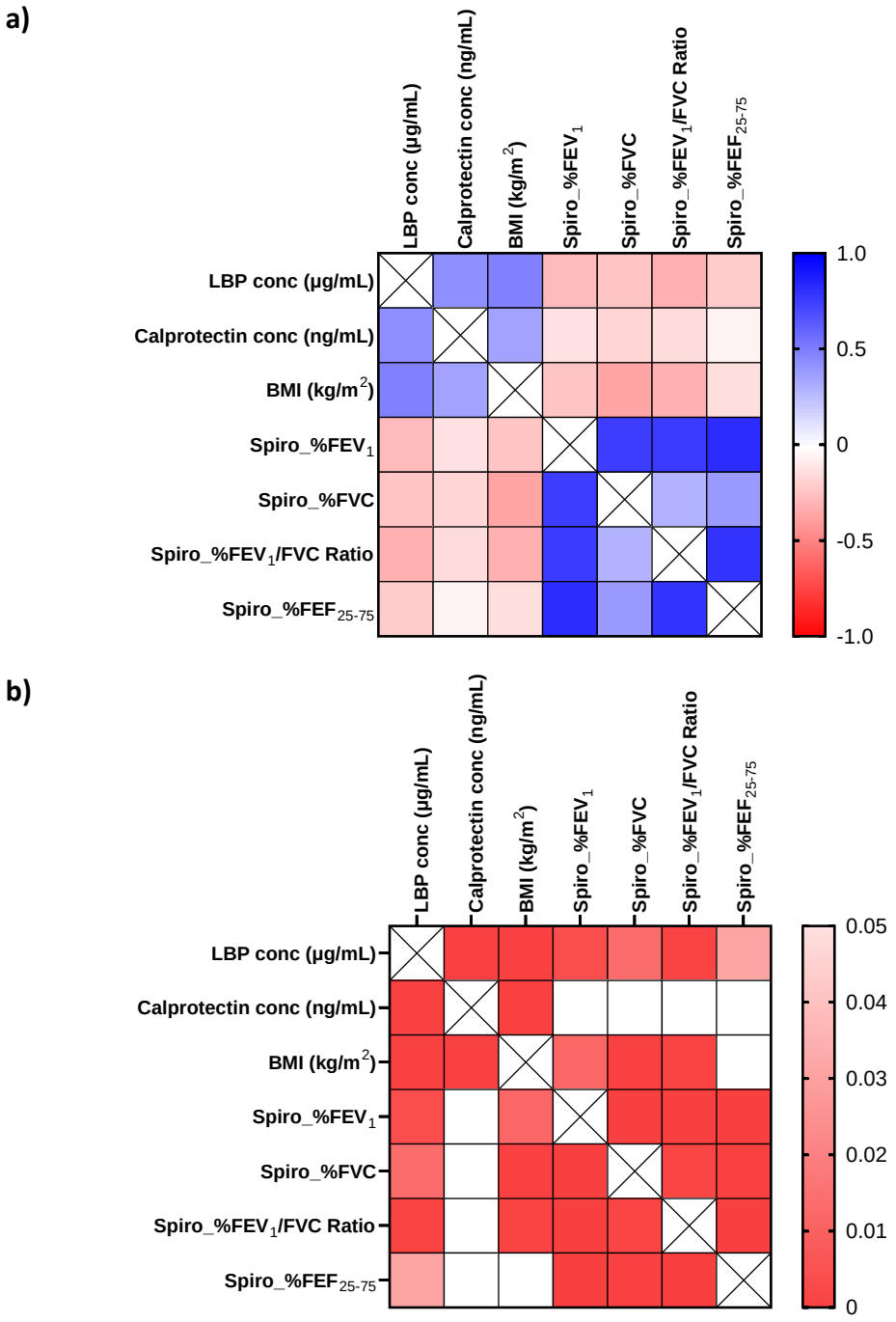


Fig. 6.3.3.1. Heatmap correlation matrix showing relationship between LBP, Calprotectin and BMI and lung function parameters in all 3 groups considered (Difficult to control asthma, well-controlled asthma and healthy controls).

a) r squared heat map for LBP, Calprotectin and BMI and lung function parameters. Blue squares represented a positive correlation with values close to 1 were strongly correlated. Values between 0 to -1, indicated a negative correlation between the parameters considered. b) p-value heat map. The white squares represented non-significant p values. Red squares show significant p values. The darker the red, the stronger the significance was (measured as p values: p=0-p=0.05; n=158).

6.3.4 The simple linear regression analysis between LBP, Calprotectin and asthma control

A simple regression analysis was conducted between LBP, Calprotectin and the ACQ-6 score to evaluate correlation in asthma control for the asthma groups (difficult to control and well-controlled asthma). The circulating level of LBP was positively correlated with ACQ-6 score (r=0.06, **p<0.01) while there was no correlation between Calprotectin and ACQ-6 score (r=0.02, p=ns) (**Fig. 6.3.4.1**).

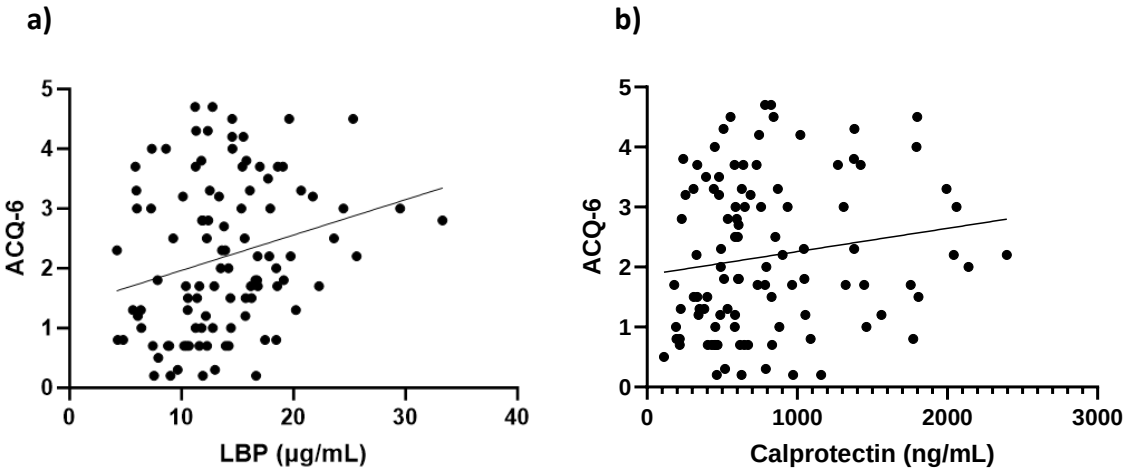


Fig. 6.3.4.1. Simple linear regression analysis showing correlation between a) LBP, b) Calprotectin and ACQ-6 score in asthma groups.
Correlation analysis between a) LBP (r=0.06; **p<0.01), b) Calprotectin (r=0.02, p=ns) and ACQ-6 score in the asthma groups. (n= 111). p-value was shown as follows: **p<0.01.

6.3.5 Differences in LBP and Calprotectin biomarkers between controls, well-controlled asthma and difficult to control asthma groups.

The concentration of gut permeability biomarkers was evaluated across the 3 groups (**Fig. 6.3.5.1**). Kruskal-Wallis analysis revealed significantly greater concentrations of LBP and calprotectin in the DCA group compared to the control group (LBP: ****p<0.0001; calprotectin: ***p<0.001). No differences were seen between DCA and WCA groups (p=ns, d=0.3).

The Mann-Whitney test revealed statistical differences in LBP levels between C and WCA (**p<0.01) and the Cohen d effect size between groups was 0.97. No significant difference was shown for Calprotectin between WCA and C group (p=ns), but an effect size of 0.5 was observed.

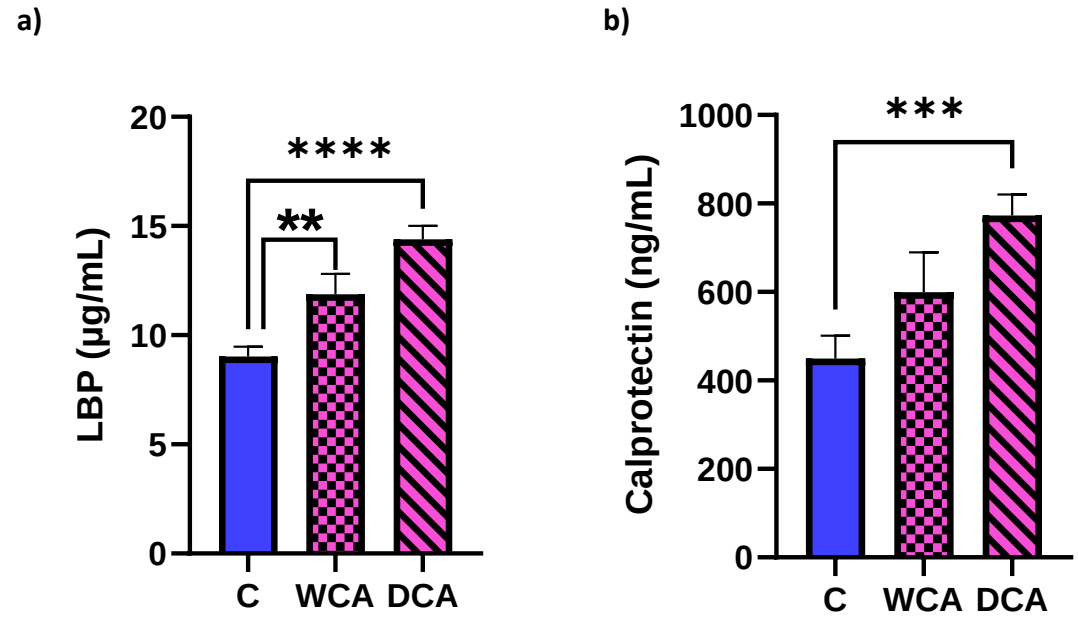


Fig. 6.3.5.1. Level of gut permeability markers, LBP and Calprotectin in healthy controls (C), well-controlled asthma (WCA) and difficult to control asthma (DCA) cohorts.
The serum level of a) LBP (ϵ^2 =0.20) and b) Calprotectin (ϵ^2 =0.10) in the three groups considered. Data are reported as a mean±standard error of the mean (SEM). Statistical analysis was performed using Kruskal-Wallis plus post hoc Dunn's multiple comparison test. Next, Mann-Whitney analysis was conducted between pair-group. p-value was shown as follows: **p<0.01; ***p<0.001; ****p<0.0001. (Healthy controls: n=34; well-controlled asthma group: n= 26; difficult to control asthma: n=91).

6.4 Discussion

The main findings of this study were that: 1) Raised plasma concentration of LBP was negatively correlated with lung function in the whole population considered and positively correlated with poor asthma controls in the asthma groups; 2) LBP and Calprotectin were significantly raised in the DCA cohort compared to the controls group; 3) Raised concentrations of both LBP and Calprotectin were positively correlated with high BMI.

6.4.1 The role of gut permeability markers in lung function and asthma control

The gut permeability marker LBP was negatively correlated with lung function and positively correlated with poor asthma control in the whole population considered. On the contrary, no correlations were observed between calprotectin and lung function or asthma control.

No previous study looked at the role of LBP in lung function or asthma control. However, a previous murine study reported a role for LBP in sensitizing alveolar macrophages to LPS during bronchoconstriction, process following antigen sensitization (Strohmeier et al. 2001). This suggests a contributing role of LBP in asthma exacerbation that in turn may enhance severity of asthma. On the contrary, in this study, calprotectin did not correlate with lung function decline or asthma control. This is in contrast with findings from two recent studies where raised concentrations of serum calprotectin were found to be negatively correlated with lung function, specifically with lower FEV_1/FVC Ratio, in a cohort of patients with severe asthma (Decaesteker et al. 2022; Lee et al. 2020).

However, to date there are few studies exploring the role of gut permeability markers in obesity and asthma and therefore more studies are needed to clarify their role in the disease.

In the present study, raised plasma concentrations of both LBP and calprotectin were observed in patients with DCA compared to C group, suggesting a link between severity of asthma and gut permeability. No previous study reported an association between difficult to control asthma and raised LBP levels, while there is previous evidence of raised concentrations of calprotectin in difficult to control asthma (Decaesteker et al. 2022; Quoc et al. 2021). High level of LBP was found in the WCA group compared to the C group. Additionally, although not significant, trends in the data indicated a raised levels of Calprotectin in the WCA group compared to C group. This may suggest an initial damage of the gut barrier when the asthma is well controlled, which then further deteriorates as the disease progresses. Histological analysis of duodenum intestinal mucosa from patients with bronchial asthma revealed architectural abnormalities of gut mucosa and increased infiltration of lymphocytes and eosinophils compared to the control group (Wallaert et al. 1995). Limited human studies have supported this view, finding high levels of gut permeability in asthma patients using various methods including the dual sugar test or a chromium 51-labeled ethylenediaminetetraacetatic acid (CrEDTA) urinary recovery test (Benard et al. 1996; Hijazi et al. 2004; Walker et al. 2014). However, it is unclear whether gut mucosal defects are a result of, or cause of, asthma. The present study found a trend towards raised levels of LBP and calprotectin in the WCA group compared to C, supporting the view that gut mucosal defects can

potentially worsen with the severity of asthma. This may be due to more frequent asthma exacerbation or asthma attack in the DCA group or due to enhanced systemic inflammation or a combination of both that may affect gut barrier and consequently increase circulating gut permeability markers. However, due to uneven group distribution between WCA (n=26) and DCA (n=98) groups, modest changes in gut integrity in the WCA group may have been more difficult to detect. Therefore, from the data obtained in this study, it's not possible to elucidate whether gut permeability is a cause or consequence of asthma. Future studies should look to recruit larger cohort of participants across the severity of asthma and use interventions trial such as prebiotic supplementations to see if it has a positive impact on asthma.

6.4.2 The role of gut permeability in obesity and asthma

Both LBP and Calprotectin markers were positively correlated with high BMI. The link between obesity and asthma is well known (Bantulà et al. 2021a; Gibeon et al. 2013; Wipt and George 2012). This may be due to low grade inflammation, excess adipose tissue around the lungs and/or alteration of markers of metabolic health, which can in turn affect disease decline as discussed in more detail in **Chapter 5**: Characterising a cohort of patients with difficult to control asthma: Impact of BMI on asthma severity and inflammation**.**

The association between obesity and impaired gut barrier has been reported in several animal and human studies (Boutagy et al. 2016; Cani 2007; Dey et al. 2019; Fuke et al. 2019). Obesity appears to influence the composition of the gut microbiota, shifting the gut microbiota community towards harmful bacteria and affecting the gut barrier integrity, which in turn may worsen diseases including asthma (Hufnagl et al. 2020; Li et al. 2021; Martel et al. 2022). Raised plasma concentrations of LBP and calprotectin were

strongly correlated with high BMI, which in turn was negatively correlated with lung function. This may suggest a key role for obesity in driving gut permeability and leading to disease severity. However, raised levels of gut permeability markers in the systemic circulation may in turn further contribute to increased systemic inflammation and consequently influence severity of asthma as observed with the correlation analysis between LBP and lung function and ACQ-6 score.

Due to recruitment limitations, patients with obesity represented the majority of the participants recruited (n=52) and they all suffer with DCA, while participants with WCA formed a smaller group (n=26), and 20 of them had a lean BMI. This is likely to have considerably affected the results. Therefore, future studies need to consider larger cohort across a range of BMI to further elucidate the role of gut permeability in asthma severity.

Considering the impact of high BMI on asthma, a potential strategy to improve the disease may be weight loss. Several studies have reported an improvement in spirometry indices following weight loss (Pakhale et al. 2015; Santos et al. 2019; Sekine et al. 2021). This could be due to a reduction in mechanical impairment of adipose tissue surrounding the lungs or due to a reduction in systemic inflammation (Dixon and Peters 2018).

Physical activity and weight loss nutritional strategies can help to reach this goal. Regarding the nutritional approach, a diet rich in fruits and vegetables and use of supplementation (e.g. prebiotics, probiotics) may be beneficial for host health through the production of short chain fatty acids (SCFA), end-products of bacterial fermentation.

An increased level of circulating SCFAs, particularly butyrate, has been reported to have potential beneficial effects in reducing systemic inflammation and protecting the gut barrier from impairment as described in detail in section **2.7.1** ((Hamer et al. 2009; Sahagún et al. 2015; Ye et al. 2018).

6.4.3. Conclusion

Taken together these results suggest that gut barrier impairment may be present when disease is well controlled but more pronounced in difficult to control asthma. However, BMI appears to be the main driver of gut permeability and the major contributor to asthma severity. Therefore, weight loss and dietary strategies that target the intestinal barrier could be potential way to improve disease and worth it future investigations.

Chapter 7: Pilot study on metabolomics: Comparative methods to identify metabolites in plasma of adults with well-controlled asthma

7.1 Introduction

Asthma is a chronic disease of the airways, and several inflammatory pathways are involved which may contribute to a variation of its clinical phenotypes (Payne et al. 2001; Van Den Toorn et al. 2001). Several studies have argued that asthma, particularly the obesity-associated asthma, is driven by an altered metabolic status (Cottrell et al. 2011c; Singh et al. 2013).

Metabolomics is the study of small molecules, noted as metabolites, within cells, tissue or biofluids such as blood or urine. Metabolites can be direct reporters of diseases because their abundance in biofluids might be directly associated with the underlying pathological mechanism of the disease (Gerszten and Wang 2008). Thus, the use of metabolomics represents a powerful technique to unravel disease mechanism, monitoring and diagnostics. The main techniques used for metabolomic analysis are mass spectroscopy which can be used in conjunction with gas chromatography, liquid chromatography or supercritical fluid chromatography and nuclear magnetic resonance (NMR), which is the one taken in consideration in the present study.

There are many types of NMR, but it can generally be divided into high and low-field. The high-field NMR offers high sensitivity and resolution spectra that allow accurate recognition of compounds in the samples. It has been used in several studies to assess metabolomic differences between subjects with asthma and healthy controls demonstrating the powerful use of this technique for clinical purposes (Jung et al. 2013; Saude et al. 2009; Xu, Panettieri, and Jude 2022). A study investigating metabolic profile of 39 patients with asthma using high field NMR revealed an increased serum levels of histamine and histidine, correlated with immune response, compared to the control

group (Jung et al. 2013).In addition glucose, arginine and acetate were decreased, associated with impaired metabolic function, common in asthma (Jung et al. 2013). However, the high maintenance cost and complexity of the equipment make its use in health care setting impractical (Castaing-Cordier, Bouillaud, et al. 2021).

The low-field NMR is less sensitive and has lower resolution than high-field NMR, which results in a poorly resolved spectra making it challenging to distinguish between metabolites (Castaing-Cordier, Ladroue, et al. 2021). However, advances in data processing and optimized pulse sequences have made it possible to improve structural and quantitative information obtained from a spectrum (Castaing-Cordier, Bouillaud, et al. 2021; Giberson et al. 2021).

The bench top NMR (bNMR), which uses low-field frequency (45-100MHz), has been developed ~15 years ago and has several advantages over high-field NMR. For instance, bNMR is portable, more compact and user-friendly compared to high-field NMR. Due to its reduced dimensions and related low-cost, the bNMR has the potential to be used at locations (e.g. in clinics and bedside) where high-field NMR would have been financially burdensome or physically impractical. The bNMR has a broad range of applications. It was firstly used in metabolomic studies in a cohort of patients with diabetes to study biomarkers associated with the disease (Percival et al. 2019). The use of data processing made it possible to discriminate between healthy and diabetic subjects (Percival et al. 2019). This unveils the potential role of bNMR for the clinical use in terms of diagnosis.

AIM OF THE STUDY

The aim of this pilot study was to establish if bNMR can be used to discriminate differences in metabolomic profiles between healthy controls and participants with well-controlled asthma and whether there was a cluster separation between groups. Therefore, evaluate whether bNMR technique could offer qualitative analysis of samples at a reduced cost compared to high-field NMR.

7.2 Methods

7.2.1. Participants recruitment

The study was approved by the NTU Ethics Committee (protocols: #689) and all procedures were conducted in accordance with the Declaration of Helsinki. A total of 12 participants were recruited for the study, of which 6 had asthma. All study participants were provided with the participant information sheet a minimum of 24 hr before taking part in the study and completed a self-reported health screen on arrival. If participants met the inclusion criteria, they were enrolled to the study. Briefly, participant with well-controlled asthma must had a prescription from a GP and they were asked to refrain from asthma medication 24 hr before the testing session. All participants had to be not smokers and refrain from consuming caffeine in the 24 hr before the visit. A full list of inclusion and exclusion criteria can be found **in Appendix 1: WATCH participant information sheet and consent form**.

The study was conducted in collaboration with Medical Technologies Innovation Facility (MTIF) at NTU campus where bench top nuclear magnetic field (bNMR) and high field NMR analysis were conducted.

7.2.2. Blood samples collection

Venous blood was drawn from a vein in the antecubital fossa region of the elbow using a 23-gauge butterfly needle (BD Vacutainer, Plymouth, UK).

An 8 mL of whole blood was collected and immediately transferred into a EDTA plasma vacutainer (BD, Plymouth, UK) gently inverted and immediately centrifuged for 15

minutes at 1500g at 4°C. Following the centrifugation, plasma aliquoted and subsequently stored at -80°C until use. After blood sampling the butterfly needle was removed, and firm pressure was applied upon the puncture site to avoid hematoma.

7.2.3. Nuclear Magnetic Resonance analysis

Before proton benchtop nuclear magnetic resonance (^{1}H-bNMR) experiment, samples were thawed on ice until defrosted. Then, 450 µL of each plasma sample was mixed with 50µL of Trimethylsilylpropanoic acid (TSP) (Acros organics, U.K.) (0.05% w/v), 25µL of Sodium azide (Sigma Aldrich, U.K.) (18.8mg in 10ml Deuterium) (Thermofisher Scientific) and 25µL of Phosphate Buffer (7.1pH) (Sigma Aldrich, U.K.).

The mixture was then transferred into an 8" NMR tube (WILMAD 5mm 100mHz NMR benchtop tube, U.S.A., #WG-BTNMR-8) for analysis.

Following bNMR spectrophotometer (X-pulse 60MHz, Oxford Instruments, U.K.) calibration, each sample spectra was acquired using the pulse sequence noted as (Water suppression Enhancement though T1) WET-180°- Carr-Purcell-Meiboom-Gill (CPMG). More information on the pulse sequence can be found in **3.4.1**. Once completed, the spectra were binned into 0.01 part per million (ppm) wide regions and the regions of the spectrum containing no signal were removed. The area considered were between 0-4 ppm regions as they contained the majority of peaks and, therefore, used for statistical analysis. In order to confirm the results obtained with bNMR, were analysed using high-field NMR (400MHz, Oxford Instruments, Jeol consol).

7.2.4. Statistical analysis

The data was processed using Mestrenova software where baseline correction, phase correction and reference alignment was completed. Metabolites identification was conducted using human metabolome database (HMDB) website and existing literature. Comparison between NMR spectrum peaks obtained from participants with asthma and controls were based on visual evaluation of the spectra. Comparison between bNMR and high field NMR was conducted by visual inspection of the spectra.

Statistical analysis was conducted using MetaboAnalyst 5.0. Data were normalized by sum and filtered by the mean intensity value. Pareto scaling was applied and then Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OrthoPLS-DA) were performed.

The high-field NMR (400MHz, Oxford Instruments, Jeol consol) data obtained were processed and statistically analysed with the same method described above for bNMR data. In addition, to assess differences in metabolites levels between groups, an unpaired t-test was performed on selected metabolites.

Due to limited numbers of participants all analysis were qualitative.

7.3 Results

7.3.1. Participants characteristics

A total of 12 participants, 6 controls and 6 with well-controlled asthma have been considered for the pilot study. Participants from the two groups have been matched based on their BMI and age (**Table 7.3.1.1**). An uneven sex distribution was present in the two groups due to limited number of participants available at the time of the analysis.

Table 7.3.1.1. **General characteristics of subjects considered for the bNMR pilot study.** Anthropometric data in each of the groups are presented as the mean ± standard deviation (SD). F=female; M=male.

	Healthy controls		Subjects with asthma	
n	6		6	
	Average	SD	Average	SD
Age(yr)	29	±3	24	±4
Sex	2F; 4M	n/a	4F; 2M	n/a
BMI (kg/m^2)	21.3	±1	21.6	±1.6

7.3.2. Evaluation of metabolites in participants with asthma using bNMR

The analysis of the spectra led to the identification of several metabolites, summarized in **Table 7.3.2.1**. The preliminary observations from the stacking spectra of participants with or without asthma revealed that the asthma group appeared to have an increased levels of the Glycine, Creatine, Histidine, Bulk sugars, Glutamate, Citrate, Acetate, Alanine, Lactate, Branched-Chain Amino Acids (BCAAs) and Cholesterol compared to the control group (**Fig. 7.3.2.1**).

Table 7.3.2.1. Summary table of the identified metabolites from spectra of 60MHz ^{1}H bNMR. Metabolites were identified by visual observation of spectra using HMDB website and existing literature. The metabolites ID represents the metabolite identified on the spectra. Black arrow (↑) represented metabolites that appeared increased in the asthma group (control: n=6; participants with asthma: n=6).

Metabolites ID	Metabolites	Regions (ppm)	Metabolites upregulated compared to control
1	Glycine	3.5-3.0	↑
2	Bulk sugars	3.5-3.0	↑
3	Creatinine, Histidine	3.0-2.5	↑
4	Glutamate	3.0-2.5	↑
5	Citrate	3.0-2.5	↑
6	Acetate	2.0-1.5	↑
7	Alanine	1.5-1.0	↑
8	Lactate	1.5-1.0	↑
9	BCAAs: Valine, Leucine, Isoleucine	1.0-0.5	↑
10	Cholesterol	1.0-0.5	↑

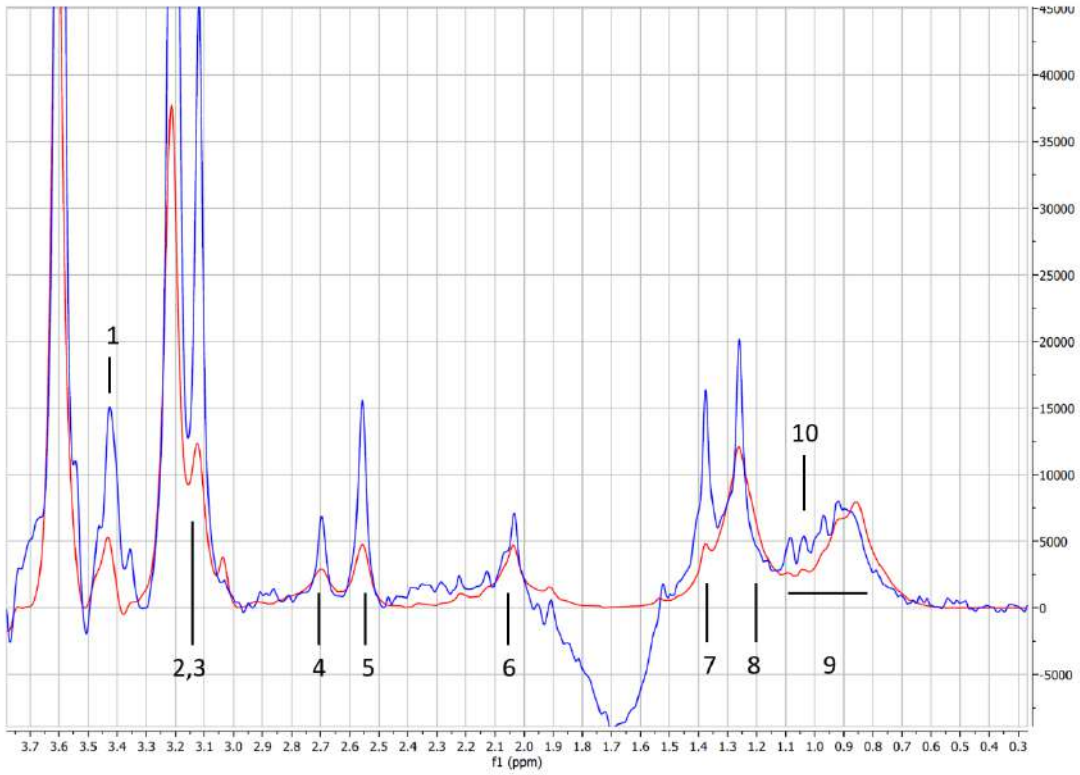


Fig. 7.3.2.1. Representative one dimensional spectra from 60MHz ^{1}H bNMR of plasma from a) control (red line); b) participant with asthma (blue line).
The stacked spectra are representative of 1 healthy control participant (red line) and 1 participant with asthma (blue line). Metabolites identified in the spectra were: 1) Glycine, Methanol; 2) Bulk sugars; 3) Creatinine, Histidine; 4) Glutamate; 5) Citrate; 6) Acetate; 7) Alanine; 8) Lactate; 9) BCAACs; 10) Cholesterol.

To confirm the data obtained with the bNMR, plasma samples were analysed using high-field NMR (400 MHz, Oxford Instruments). The analysis of the spectra confirmed the identification of the metabolites detected with bNMR (**Fig. 7.3.2.2**). The observational evaluation of the spectra from control and asthma groups confirmed the trend observed with the bNMR with several metabolites who appeared increased in participants with asthma. Participants with asthma appeared to have an elevated level of Histidine, Creatinine, Citrate, BCAAs and Cholesterol compared to the control group. Moreover, participants with asthma had a peak that appeared elevated in the region of Glycine or

Glucose or Methanol. The peaks of these metabolites were very close to each other, and this made it difficult to evaluate which metabolite they belonged to. The peaks belonging to acetate, glutamate, alanine and lactate were unchanged between the two groups considered.

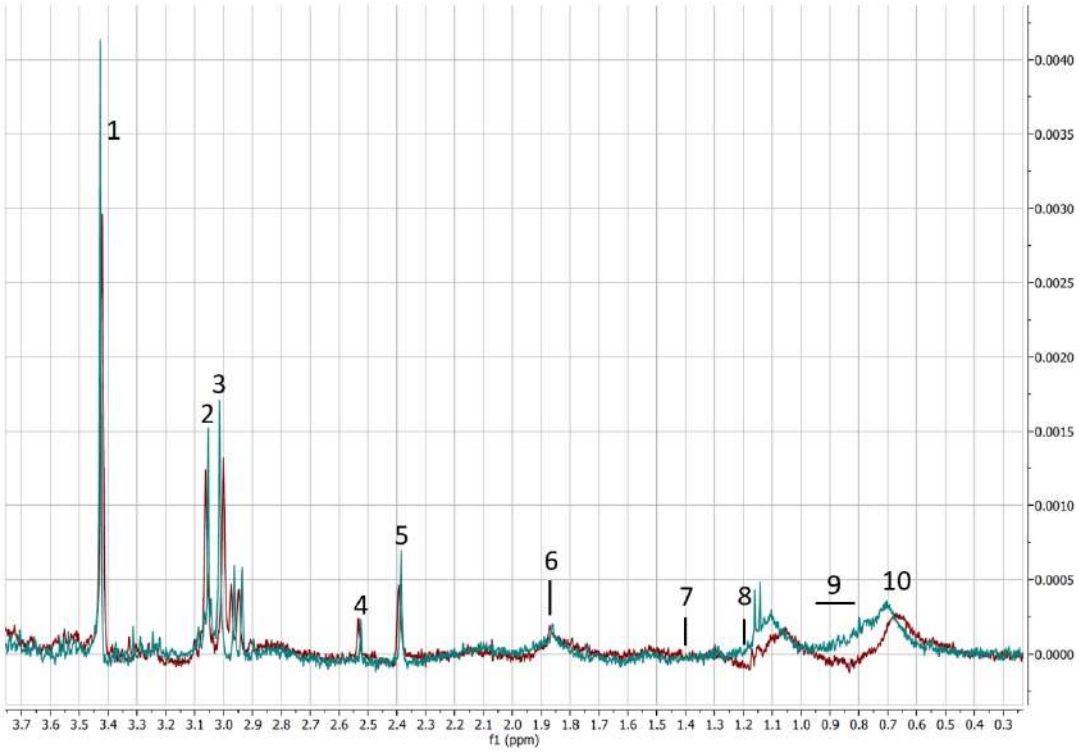
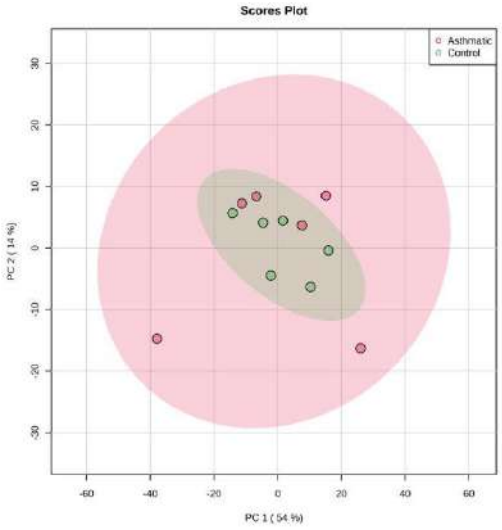


Fig. 7.3.2.2. Representative one dimensional spectra from 400MHz ^{1}H high field NMR of plasma from a) control (red line); b) participant with asthma (blue line).
The stacked spectra are representative of 1 healthy control participant (red line) and 1 participant with asthma (blue line). Metabolites identified in the spectra were: 1) Glycine, Glucose, Methanol; 2) Histidine; 3) Creatinine; 4) Glutamate; 5) Citrate;6) Acetate 7) Alanine; 8) Lactate; 9) BCAAs; 10) Cholesterol.

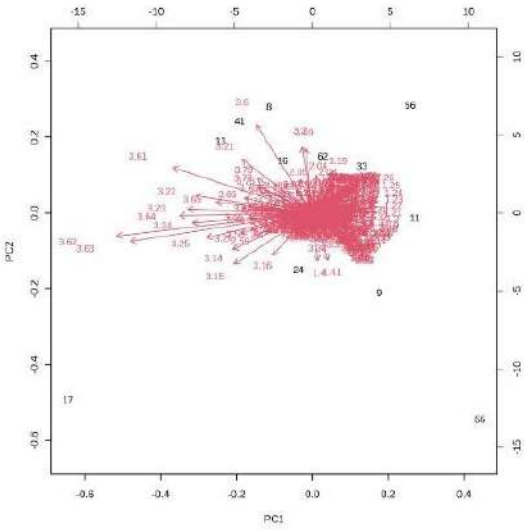
7.3.3. Cluster analysis between control and participants with asthma using bNMR

Principal Component Analysis (PCA) was used to identify the pattern difference between healthy controls and participants with asthma, based on group samples variation in the spectra (**Fig. 7.3.3.1**). The PCA conducted was not significant. Although initial observation revealed a separation between the control and the asthma groups across the spectrum (**Fig. 7.3.3.1** a, c, e; p=ns). The vector graphs represented the values on the spectra that caused the pattern difference between the considered groups (**Fig. 7.3.3.1** b, d, f).

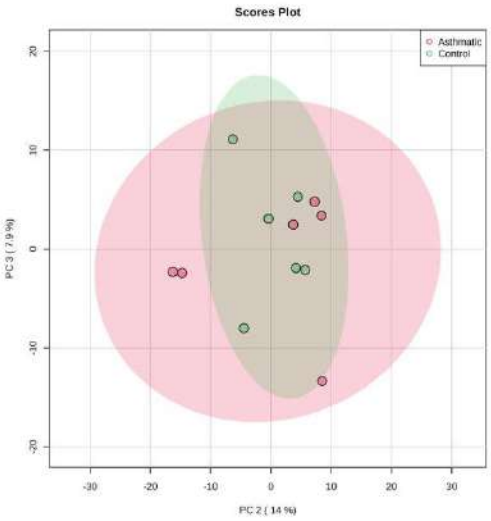
a)


Scores Plot
Asthmatic
Control
PC 2 (14 %)
PC 1 (54 %)

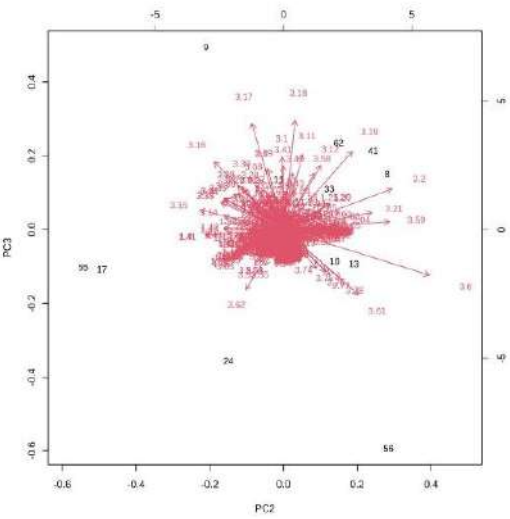
b)


PC2
PC1

c)


Scores Plot
Asthmatic
Control
PC 3 (7.9 %)
PC 2 (14 %)

d)


PC3
PC2

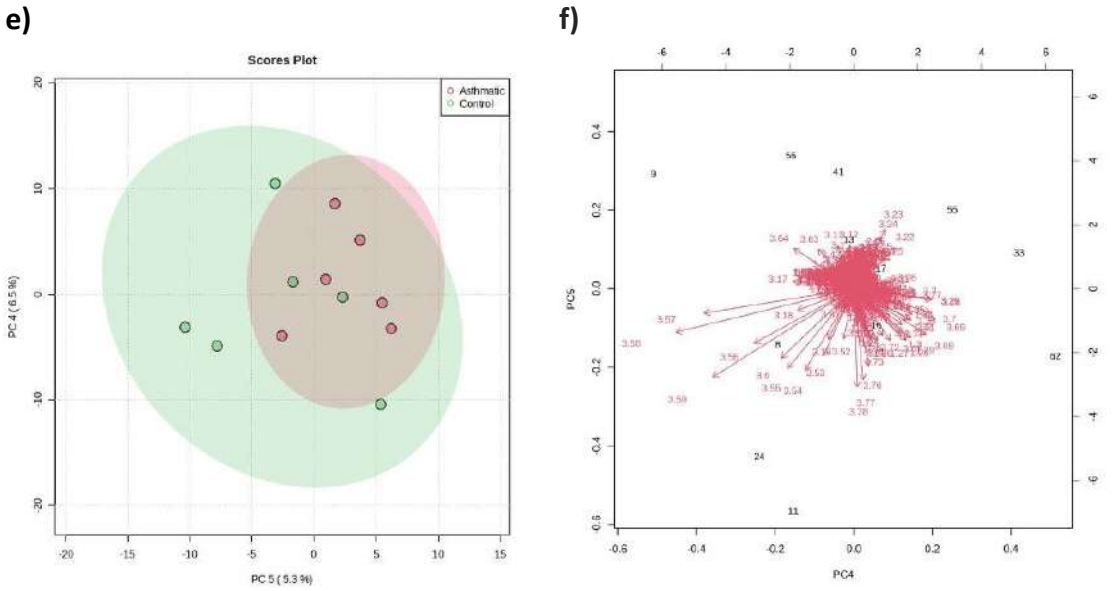


Fig. 7.3.3.1. Principal component analysis (PCA) score plot a) c) e) and vectors graphs b), d), f) derived from the ^{1}H bNMR spectra of plasma obtained from control subjects (green circles) and participants with asthma (red circles).
The preliminary analysis of the bNMR spectra showed a cluster separation between healthy controls and participants with asthma. (Healthy control: n=6; participants with asthma: n=6). Result was not significant (p=ns).

Further analysis was conducted to determine which regions caused separations between the two groups considered. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) revealed no statistical differences between groups. Although, initial observation revealed a potential cluster separation between the control and asthmatic groups (p=ns) (**Fig. 7.3.3.2**). The Variable Importance in Projection (VIP) plot (**Fig. 7.3.3.2** b) obtained from the OrthoPLS-DA indicated which metabolites were responsible for the cluster separation between group and showed tended to be upregulated (red squares) or downregulated (blue squares) in the spectra (**Fig. 7.3.3.2** b; p=ns). According to the VIP plot, metabolites related to the regions of BCAAs, cholesterol, glutamine and histidine (1.0-0 ppm) appeared to be increased in the group of participant with asthma (p=ns) (**Fig. 7.3.3.2** b).

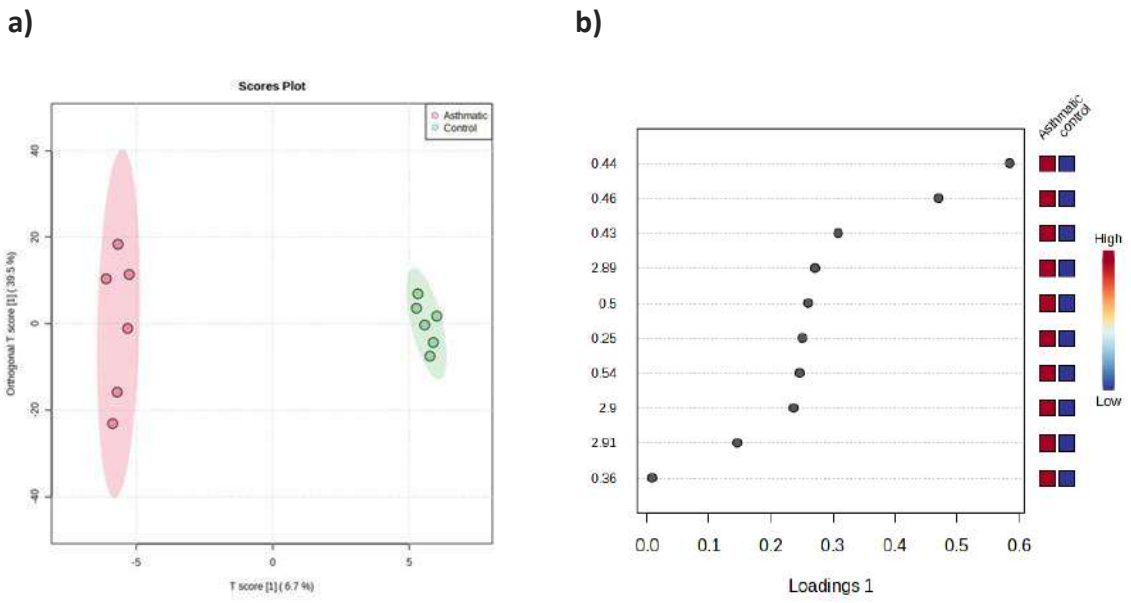


Fig. 7.3.3.2. Orthogonal partial least squares discriminant analysis (OPLS-DA) score plot a) and Variable Importance in Projection (VIP) plot b) derived from the ^{1}H bNMR spectra of plasma obtained from control subjects (green circles) and participants with asthma (red circles).
a) OrthoPLS-DA showed a clear cluster division between healthy controls and subjects with asthma. b) VIP plot represented the variables that influenced the predictive components. (Healthy control: n=6; participants with asthma: n=6). Results were not significant (p=ns).

7.3.4. Data analysis from high-field NMR spectra

Statistical analysis on the high-field spectra confirmed the cluster separation trend observed with the bNMR between control and asthma group (p=ns) (**Fig. 7.3.4.1**). There were no statistical differences between groups. Although trend in data from the PCA and OPLS-DA analysis revealed a potential cluster separation between groups. The VIP score plot obtained from the OPLS-DA revealed that the metabolites responsible for cluster separations between groups tended to be lower in the asthma group compared to the control. Only one region that appeared to be upregulated in the asthma group was related to Glycine or Glucose or Methanol region (3.65-3.90 ppm). The metabolites most involved in cluster separation were Lactate (1-1.3 ppm), BCAAs or Cholesterol

(0.65-0.85ppm), Acetate (1.8-2.0 ppm), Glycine or Glucose or Methanol (3.65-3.90 ppm) (p=ns) (**Fig. 7.3.4.1** d). An unpaired t-test was then performed on these metabolites to assess differences between the two groups considered (**Fig. 7.3.4.1**). The analysis revealed no differences between groups but trend in data showed a reduction of Lactate, BCAAs or Cholesterol (it can be any of these metabolites due to peaks being close to each other, that made difficult to identify the specific metabolite); Acetate; Glycine or Glucose or Methanol (peaks close to each other) metabolites in the asthma groups compared to the control (p=ns) (**Fig. 7.3.4.2**).

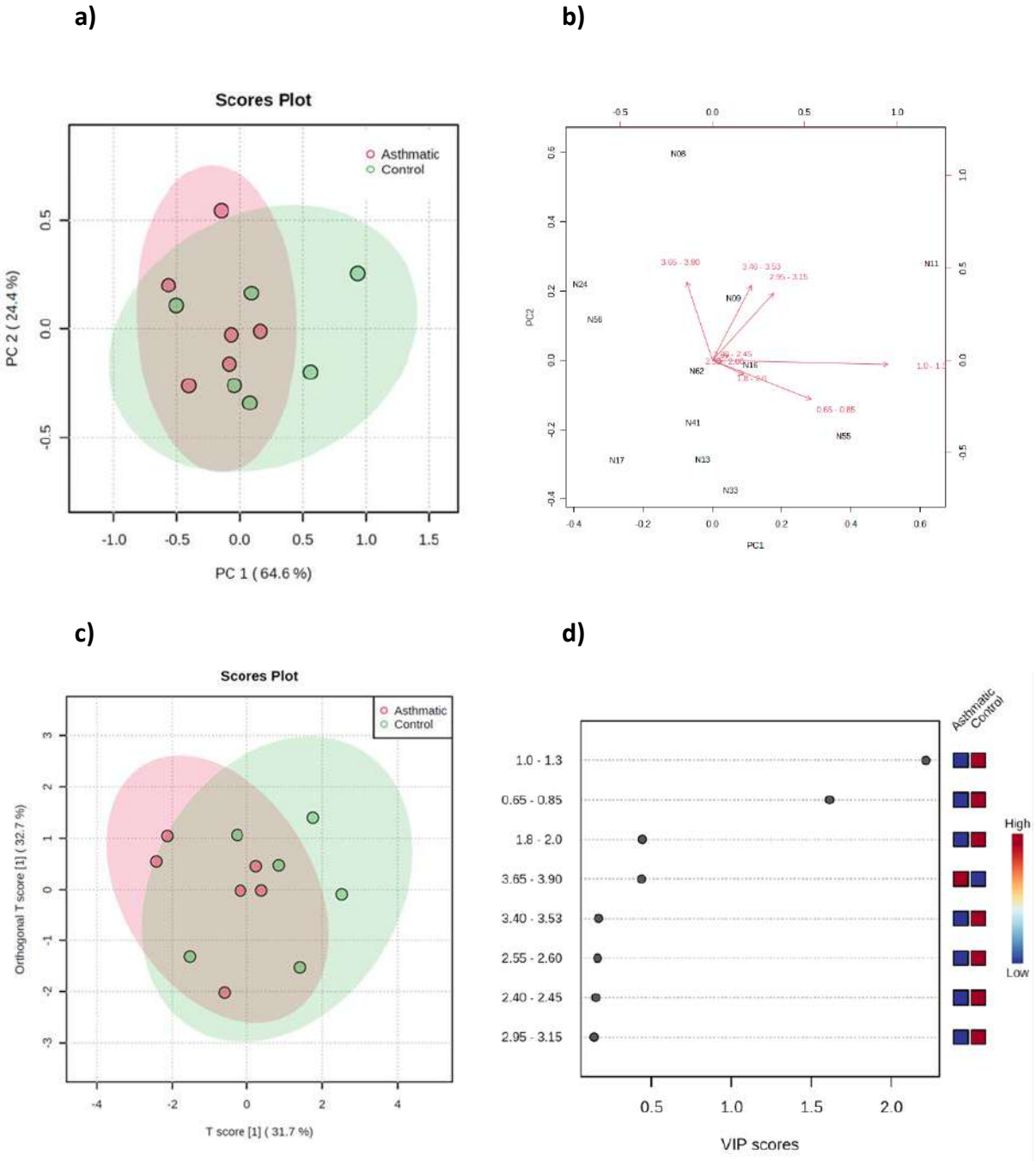


Fig. 7.3.4.1. Principal component analysis (PCA) score plot a), vectors graph b), Orthogonal partial least squares discriminant analysis (OPLS-DA) score plot c) and Variable Importance in Projection (VIP) plot d) derived from the ^{1}H bNMR spectra of plasma obtained from control subjects (green circles) and participants with asthma (red circles).

The preliminary analysis of the bNMR spectra showed a cluster separation between healthy controls and participants with asthma. VIP plot represented the variables that influenced the predictive components (healthy control: n=6; participants with asthma: n=6). Results were not significant (p=ns).

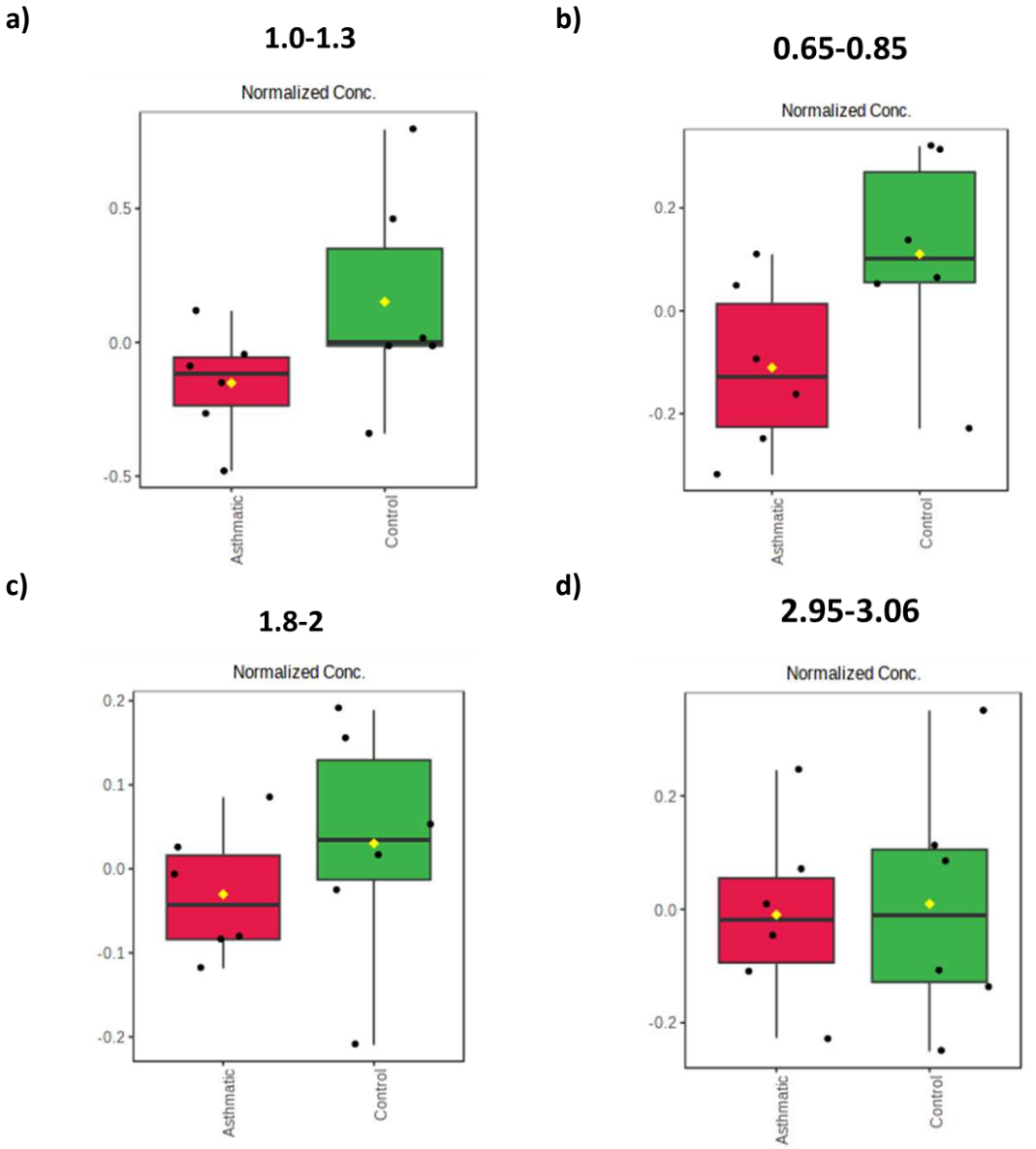


Fig. 7.3.4.2. Differences in metabolites levels in participants with asthma and control. The metabolites considered were: a) Lactate (1.3-1.0 ppm); b) BCAAs or Cholesterol (0.65-0.85 ppm); c) Acetate (1.8-2.0 ppm); d) Glycine or Glucose or Methanol (2.95-3.06 ppm). Data were reported as mean±standard deviation (SD). Statistical analyses were performed using un-paired t-test. (healthy control: n=6; participants with asthma: n=6). Results were not significant (p=ns).

7.4 Discussion

The preliminary results obtained from the analysis of 12 plasma samples with the bNMR showed: 1) comparative identification of metabolites between bNMR and high field NMR; 2) no changes in metabolite concentrations were observed in the asthma group compared to the control group; 3) potential cluster separation between groups.

Metabolic profiling of plasma samples successfully identifies metabolites involved in asthma pathophysiology including histamine, BCAAs, methanol, glycine, acetate and cholesterol.

Initial observation from the metabolic profiling of plasma samples, confirmed with high-field NMR, revealed that participants with asthma tend to have upregulated levels of histamine, BCAAs, methanol, glucose, glycine, creatine, acetate, citrate and cholesterol compared to the control group. The metabolite histidine is an essential amino acid, and it is responsible for the production of histamine which is the key player in allergic reactions in asthma (Ohtsu 2012). Similarly, high BCAAs level are often correlated to allergic reaction in asthma (Chiu et al. 2020). Raised levels of this metabolites are common in allergic phenotype of asthma, driven by Th-2 response. Therefore, the trend observed in this study may be an indicator to distinguish the asthma phenotype.

Impaired lipid profile has been linked with asthma severity and the preliminary observation from this study reported raised level of cholesterol which may be an early marker of metabolic impairment. However, evidence suggested that this effect may be due to the use of inhaled corticosteroids (Su et al. 2018; Yiallouros et al. 2014; Azofra et

al. 2019). Other studies using high-field NMR technique revealed metabolites such as alanine, methanol, acetate and glycine were associated with asthma pathways (Jung et al. 2013; Motta et al. 2014; Saude et al. 2011).

If confirmed in large cohort, the observations conducted in the present study may contribute to understand the underlying mechanism of asthma disease or provide a better method for diagnosis or monitoring patients.

The PCA revealed a possible cluster separation between control and asthma groups. A previous study revealed that the differences between groups were caused by a lower level of metabolites such as histamine, glucose, valine and leucine in the asthma group (Jung et al. 2013). In the present study, analysis conducted in bNMR and high-field NMR led to opposite results showing a tendency in upregulation of metabolites in the asthma group with bNMR and a downregulation with high-field NMR. In addition, the metabolites responsible for the cluster separation were different in the two techniques used. The method used is prone to overfitting and on both occasions the results were not significant, therefore no conclusion can be made at this stage. An increased number of participants could help gain a better understanding of technique and disease. Recent studies showed a distinct metabolic profile between biofluids such as blood and urine, therefore an integrated approach would be ideal to evaluate metabolic pathways underlying asthma disease (Chiu et al. 2020; Kelly et al. 2017). Moreover, if cluster separation will be confirmed in large cohort, this will open ideas for the potential use of bNMR in clinical settings for several applications such as diagnosis discrimination, as previously reported (Percival et al. 2019).

7.4.1 Limitation and Future works

The limited number of participants considered for this pilot study were not sufficient to reach statistical significance between groups. The next stage of this study will be to analyse plasma samples from a large cohort of 98 patients with difficult to control asthma, previously recruited as part of this PhD project, using both bNMR and high field NMR. This will provide a better understanding of use of bNMR for metabolites identification and metabolic discrimination between asthma and control.

7.4.2 Conclusion

These preliminary results obtained with the analysis of the small cohort considered showed promising potential for the use of bNMR in metabolomic analysis. If confirmed in larger cohorts, these results can contribute to help with monitoring and diagnostics of disease.

Chapter 8: The effect of a 21-day dietary prebiotic supplement on asthma management, gut permeability and biomarkers of health in adults with well-controlled asthma

8.1 Introduction

The pathophysiology of asthma is characterized by airway hyperresponsiveness (AHR), airway remodelling, and airway inflammation (Global Initiative for Asthma 2022). Based on symptoms, asthma can be defined as well-controlled or difficult to control. Well-controlled asthma is defined as a condition characterized by low frequency of symptoms including wheezing, shortness of breath or coughing, no limitation on daily activity including exercise and infrequent exacerbations (BTS 2014). If symptoms are not monitored, adults with well-controlled asthma can develop asthma attack and/or more severe condition similar to patients with difficult to control asthma. Therefore, it is important to monitor the symptoms and keep the disease under control. Triggers such as smoke, exercise, stress, weight gain or irritants, can cause airways hyperresponsiveness, an over-reaction from the airways causing bronchoconstriction in patients with asthma (Stoodley et al. 2019; Vernon et al. 2012). If left untreated, these symptoms can exacerbate and cause airway obstructions and/or permanent airway remodelling, such as airway hypertrophy and membrane thickening which can limit responsiveness to common asthma medications (Suzanne Bollmeier 2017).

Alteration of gut microbiota composition is considered to influence various diseases including asthma. Changes in the composition of the gut microbiota can lead to a shift towards an overgrowth of harmful bacteria commonly associated with increased inflammation, which in turn may exacerbate a number of diseases including obesity and asthma (Amabebe et al. 2020; Chiu et al. 2019; Gurung et al. 2020; Sankararaman et al. 2023). One mechanism by which harmful bacteria may contribute to systemic

inflammation is by altering the gut barrier, a semipermeable structure that separates the intestinal lumen from the body. High levels of *Firmicutes* and *Proteobacteria* strains are associated with altered tight junctions (essential proteins that hold together the epithelial cells that make up the gut barrier) expression, leading to altered trans-epithelial permeability (Allam-Ndoul et al. 2020). This alteration of the gut barrier allows gut bacteria fragments such as lipopolysaccharides (LPS) to translocate across the gut mucosa and promote inflammation through activation of the nuclear factor kappa B (NFkB) pathway via toll-like receptor 4 (TLR-4) (Lad et al. 2021). Moreover, LPS stimulation of TLR-4 can activate an inflammatory cascade that can lead to reduced transcription of the tight junction proteins, which can further increase gut barrier damage and allow more LPS to cross (Nighot et al. 2017, 2019). From here LPS may enter the systemic circulation and turn inflammation from local to systemic.

Raised serum concentrations of LPS-binding protein (LBP), which plays a key role in enhancing the sensitivity of immune cells to LPS (Hailman et al., 1994), and calprotectin, a marker of gut inflammation, are observed in metabolic conditions such as obesity and linked with systemic inflammation (Calcaterra et al. 2018; Gonzalez-Quintela et al. 2013; Moreno-Navarrete et al. 2012; Mortensen et al. 2009). Similarly, studies have reported high levels of circulating serum calprotectin in patients with asthma compared to controls (Lee et al. 2020; Decaesteker et al. 2022), while the concentration of LBP was found to be significantly elevated in bronchoalveolar fluid of patients with asthma compared to control (Dubin et al. 1996), and associated with chronic airway response in murine models (Strohmeier et al. 2001).

In addition to gut barrier impairment, asthma has been associated with metabolic dysfunction, characterized by insulin resistance and altered lipid profile (Barochia et al. 2015; Cottrell et al. 2011a; Singh et al. 2015). A murine study reported an association between impaired glucose metabolism, insulin resistance and greater AHR (Singh et al. 2015;). Studies demonstrating this link are limited and further study is warranted.

Prevention strategies such as dietary interventions that target the gut microbiota may be beneficial to improve health and reduce the risk of developing severe symptoms of asthma. Common supplements include probiotics, defined as a live microorganisms that confer health benefit to the host when consumed in adequate amounts (Hill et al. 2014); and prebiotics, substrates that are selectively utilized by host microorganisms conferring a health benefit (Gibson et al. 2017). Prebiotics are a dietary strategy to target the gut microbiota, resulting in increased activity and abundance of beneficial microbes such as *Lactobacillus* or *Bifidobacterium* (Gibson et al. 2017). When prebiotics are metabolized by gut bacteria, short chain fatty acids (SCFA) are produced as an end-product which can have beneficial effects on human health (Gibson et al. 2017). Limited studies have investigated the role of prebiotics in adults with asthma (McLoughlin et al. 2019a; Williams et al. 2016a). It has been shown that 3 weeks supplementation with 5.5 g of prebiotic Bimuno-galacto-oligosaccharides (B-GOS) attenuated airways hyperresponsiveness and reduced markers of airway inflammation such as TNF-α, CRP and eosinophil levels (Williams et al. 2016). Similar findings have been reported in murine studies, where a 14-day diet supplemented with 1% prebiotic galacto-oligosaccharides (GOS) led to reduced markers of airway inflammation and prevented airway hyperresponsiveness in allergic asthma models (Verheijden et al. 2016;

Verheijden et al. 2015). Prebiotics can also indirectly contribute to the maintenance of gut barrier integrity through an increase in the circulating level of SCFA. Previous studies using the human intestinal epithelial cell line, CaCo-2, demonstrated that the SCFA butyrate, can improve gut barrier integrity by enhancing tight junction assembly via inhibition of nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) inflammasome and activation of AMP-activated protein kinase (AMPK) (Feng et al. 2018b; Peng et al. 2009; Wang et al. 2020). Other studies demonstrated a direct effect of prebiotics in enhancing gut barrier integrity via activation of the AMPK pathway, independently of surrounding microbiota (Akbari et al. 2017; Wongkrasant et al. 2020). In these studies, researchers pre-treated a Caco-2 cell monolayer with different types and concentration of prebiotics, specifically GOS, inulin and fructo-oligosaccharides (FOS), before challenging the cells with a viral compound to induce gut permeability. The prebiotic GOS showed the most protective results on gut barrier by increasing tight junction assembly, in a concentration dependent manner. Researchers concluded that the effect of different prebiotics on gut barrier may be due to the particular molecular structure of the prebiotic and/or its concentration (Akbari et al. 2017). These studies are limited to cell work and further investigations are needed. So far, no studies have investigated the effect of prebiotic on gut permeability in adults with asthma.

Finally, prebiotic supplementation (Inulin, Oligofructose) was also associated with improved markers of metabolic health including glucose, triglycerides and cholesterol (Aliasgharzadeh et al. 2015; Sabico et al. 2017, 2019). However, studies are limited to cohorts of patients with obesity and diabetes, and none examined the effect of prebiotic administration in patients with asthma.

AIMS OF THE STUDY

Therefore, a prebiotic intervention trial was conducted in a double-blind placebo control cross-over design in a cohort of adults with mild well-controlled asthma. The aims of the study were to evaluate if 3 weeks supplementation with 2.9 g/day of commercially available prebiotic B-GOS would: 1) influence plasma concentrations of LBP and calprotectin; 2) improve serum biomarkers of health (glucose, cholesterol, triglycerides) and inflammation (CRP); and/or 3) improve lung function and asthma control.

8.2 Material and Methods

8.2.1 Experimental design

The study was approved by the Nottingham Trent University Human Ethics Committee, and all procedures were conducted in accordance with the Declaration of Helsinki.

A total of 18 adults with asthma (4 male, 14 female) volunteered for the study and provided written informed consent. Inclusion of participants was dependent upon a diagnosis of mild well-controlled asthma by a GP and a BMI ranging between 18-35 kg/m^2. A full list of inclusion and exclusion criteria can be found in **Appendix 2: Prebiotic trial participant information sheet and consent form**. Two participants declined to participate further. Three participants did not meet the inclusion criteria and therefore were excluded from the study. A total of 13 participants completed the study.

Following a double blind, placebo controlled cross over design, participants were assigned to either the prebiotic intervention of 2.9 g galacto-oligosaccharide per day for 21 days in the form of one sachet (3.65 g) of Bimuno® Daily (Clasado Biosciences Ltd) (n= 13) or one placebo sachet (3.65 g maltodextrin) (Clasado Biosciences Ltd) (n= 13) for 21 days (**Fig. 8.2.1.1**). Participants visited the lab on four different occasions (days 0, 21, 35 and 56). At each visit, anthropometric measurements (height, weight, hip, waist circumferences) were collected, pulmonary function assessed, asthma-related questionnaires completed, and venous blood samples were collected.

Adherence to the supplement was calculated as follows:

$$\%supplement\ taken = \frac{N°\ days\ supplement\ was\ taken}{N°\ days\ supplementation\ (21)} * 100$$

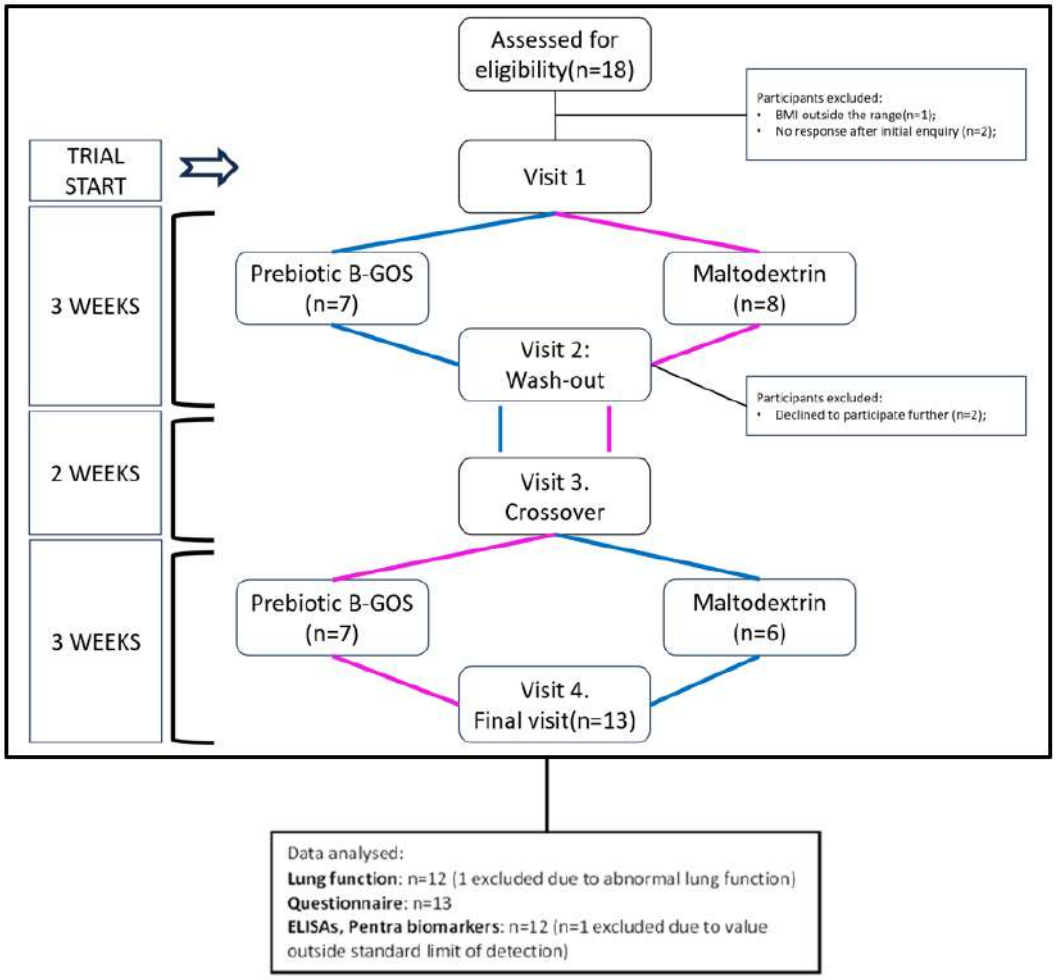


Fig. 8.2.1.1. CONSORT flow chart for the prebiotic intervention trial.
A total of 18 adults with mild well-controlled asthma were initially recruited to the study. Of the initial 18 participants, 15 made it to the first visit and 13 completed the trial. A total of 5 participants were excluded for not meeting criteria or declined to participate further.

8.2.2 Anthropometric measurement

Participants' height, body mass and wait hip circumference were measured at each visit.

Participants' body mass was measured to the nearest 0.1 kg using calibrated electronic scales (SECA 877 Scale, SECA, UK), wearing lightweight clothing and barefoot while their height was measured using a stadiometer (SECA stadiometer, UK). BMI was then calculated as weight (kg)/height $(m)^2$.

Hip and waist circumference were measured using a medical tape. Waist was taken halfway between the lower rib and the iliac crest point, and hip circumference as the widest point. Participants wore light clothes and were instructed to breath out gently while the measurements were taken. Waist to hip ratio was calculated by dividing the waist by the hip circumference and used to estimate body fat distribution (National Institute for Health and Care Excellence (NICE) 2022).

8.2.3 Blood collection

Venous blood was collected at each visit. Resting blood was drawn from a vein in the antecubital fossa region in the forearms using a 23-gauge butterfly needle (BD Vacutainer, Plymouth, UK) and serum and plasma were collected. For serum, 5 mL of blood was collected in a serum separating tube (BD, Plymouth, UK), gently inverted and left to clot for approximately 30 minutes. The tube was then centrifuged at 1,000g for 15 minutes at 4°C. For plasma, 4 mL of blood was collected into an EDTA tube (BD, Plymouth, UK), gently inverted, and immediately centrifuged at 1,500g for 15 minutes at 4°C. Following centrifugation, both serum and plasma were aliquoted and stored in

the -80°C freezer until use. After blood sampling, the needle was removed, and medical tissue was applied under pressure to prevent a superficial hematoma.

8.2.4 Pulmonary function

Pulmonary function was assessed via spirometry. Participants performed maximal flow-volume loops to determine absolute and percentage predicted: forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), peak expiratory flow rate (PEF), forced expiratory flow rate from 25-75 of FVC (FEF_{25-75}), forced expiratory flow rate 75-85 of FVC (FEF_{75-85}) and FEV_1/FVC ratios. Each participant performed a minimum of 3 tests and if the values were within 0.150 L of each other, the highest value was taken for the analysis. Data were reported as a % of the predicted calculated value. Predicted values were calculated based on participants' height, weight, age, sex and ethnicity based on equation approved by the European Respiratory Society (Quanjer et al. 2013). Pulmonary function was conducted in accordance with the procedures outlined in section **3.3.2.**

8.2.5 Blood serum markers analysis

Pentra C400 (Horiba, U.K.) was used to assess the concentration of serum biomarkers of health. These biomarkers were glucose, high-density lipoprotein (HDL), low-density lipoprotein (LDL), total Cholesterol, C-reactive protein (CRP) and triglycerides (Horiba, U.K.). All samples were measured in duplicate and run together to avoid batch variations. Variation between repeats was <0.01 mmol/L.

8.2.6 Enzyme linked immunosorbent assay (ELISA)

Plasma LBP and Calprotectin were measured on each visit using enzyme linked immunosorbent assays (ELISAs) (LBP: Hycult; Calprotectin: Bio-techne). ELISAs assays were performed according to the manufacturer's instructions. The lower limits of detection for the assays were 4.4 ng/mL for LBP and 0.086 ng/mL for Calprotectin. The intra-assay coefficient of variation was 2.2 % for LBP and 3.4% for Calprotectin. All samples were analysed in duplicate on one 96-well plate for both assays. Further details of these procedures are outlined in **3.3.4.**

8.2.7 Self-reported questionnaires:

8.2.7.1 Asthma control questionnaire (ACQ)-6

The ACQ-6 test is a self-reported asthma symptoms questionnaire, validated and designed by Juniper, O'byrne, Guyatt, Ferrie, & King to assess asthma control (Juniper et al. 1999). The ACQ-6 includes six questions referring to asthma control symptoms (frequency of night-time awakening due to asthma, symptoms on morning-waking, daily activities limitation, breathlessness and wheeze frequency, and rescue by short-acting β2-agonist use), scored on a 6-point scale (0 = never/no symptoms/none; 6 = very severe symptoms). The total maximum score is 36. The total participant score was then divided by the number of items in the questionnaire to obtain the final participant score. Un-controlled asthma is set at a cut-off score of ≥1.5, while well-controlled asthma is set at score of ≤0.75, with a minimal change in score of 0.5 (Juniper et al. 2005).

8.2.7.2 Nijmegen questionnaire

The Nijmegen score questionnaire screens for symptoms associated with dysfunctional breathing pattern, for hyperventilation syndrome in asthma patients from mild to severe asthma conditions (Grammatopoulou et al. 2014). The questionnaire is composed of a 16-symptom checklist using a 0 to 4 scale (0=never; 4=very often) giving a total score of 64. A score above 23 suggests a positive diagnosis of hyperventilation.

8.2.7.3 Hull airways reflux questionnaire

The Hull airways reflux questionnaire (HARQ) is a validated tool used to assess symptoms associated with airways reflux. It is used by clinicians to diagnose non-acid gaseous reflux impacting on the airway, leading to bronchospasm and shortness of breath. It consists of 14 questions each rated on a scale of 0 to 5 (0=no problem; 5= severe/frequent problem) with a maximum score of 70. The upper limit of normal is 13 out 70, whereas a score higher than 13 indicates the presence of airway reflux (Morice et al. 2011).

8.2.7.4 Sino-nasal outcome test

The Sino-nasal outcome test (SNOT-22) is a self-reported questionnaire with outcome measures to assess rhinosinusitis. The SNOT-22 assesses a patient's symptoms during the previous 2-week period, and it is marked out of a total score of 110. Normal values

are between 0 and 7, mild values are >8 and ≤20, moderate values >20 and severe symptoms are over>50.

8.2.7.5 Hospital anxiety and depression scale

The hospital anxiety and depression scale (HADS) is a self-reported questionnaire, as developed by Zigmond and Snaith in 1983, to detect anxiety disorders and depression among patients in nonpsychiatric hospital clinics (Zigmond and Snaith 1983). Despite its initial use in clinical setting, many studies confirmed its validity for the general population use (Bjelland et al. 2002). It is currently used to detect states of depression and anxiety and to assess the severity of emotional disorder. It is divided into an Anxiety subscale (HADS-A) and a Depression subscale (HADS-D) each containing seven questions. Normal values range is between 0 and 7 for each section, while values between 8 and 10 are considered borderline and scores above 11 are considered abnormal.

8.2.7.6 Epworth scale

The Epworth scale is a validated test used to measure sleepiness. It was developed by Murry W. Johns in 1991 and it provides a measurement of the subject's general level of daytime sleepiness (Johns 1991). It is correlated with respiratory disturbance and obstructive sleep apnoea, which is a disease frequently correlated with asthma (Dixit 2018). The scale ranges from 0 to 24, with normal values below 7 and severe symptoms considered above 16.

8.2.8 Statistical analysis

The minimum sample size was calculated as 12 participants. This was made using the Massachusetts General Hospital Biostatistics sample size calculator, based on results previously obtained from a B-GOS intervention trial in adults with exercise induced asthma (Williams et al. 2016a). A minimum sample size of 12 was calculated using the outcome of a change in serum CRP following prebiotic intervention. Using previously published data showing changes in serum CRP following prebiotic interventions a sample size calculation revealed that with a power = 0.95 and α=0.05 a sample size of 12 would be required to detect a significant reduction (>1.5mg/L) of serum CRP in asthma patients. Accounting for a 15-20% dropout of participants a final sample size of 14 was the initial target.

Data analyses were performed using Graph Pad Prism 9.5.1. Data were tested for normality and then two-way ANOVA plus Sidàk multiple comparison test was used to compare changes between treatments pre and post intervention. Data were expressed as a mean±standard deviation (SD) unless otherwise specified. At day 0 of each treatment, baseline differences for all the parameters considered were evaluated using a paired samples t-test.

Effect size was conducted using Cohen's d and calculated as follow:

$$Cohen's\ d\ (d) = \frac{Mean\ value\ day\ 21\ (M2) - Mean\ value\ day\ 0\ (M1)}{pooled\ SD}$$

Where pooled standard deviation (SD)=

$$\left[\sqrt{\frac{(SD1^2 + SD2^2)}{2}}\right]$$

The % of change between day 0 and day 21 for each parameter was calculated as follow:

$$\%change = \frac{(Outcome\ day\ 21 - outcome\ day\ 0)}{Outcome\ day\ 21} * 100$$

The % change analysis was then performed using two-way ANOVA plus Sidàk multiple comparison test.

Statistical significance was considered to be $p<0.05$ and was reported as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

8.3 Results

8.3.1 Participants characterization

A total of 18 participants were recruited, of which 13 successfully completed the trial. All participants had mild well-controlled asthma and their anthropometric characteristics are summarized in **Table 8.3.1.1**. Participant adherence to supplement was 98.4% for B-GOS and 97.7% for maltodextrin.

Table 8.3.1.1. Participants characterization.
A total of 13 adults (n= 9 female (F); n=4 male (M)) with well-controlled asthma were recruited in the study. Waist circumference cut-off : low: <94cm (M), <80cm (F); high: 94-102cm (M), 80-88cm (F); very high:≥102cm (M), ≥88cm (F) (Moody and Neave 2020). Cut-off for waist/hip ratio: increased risk of developing metabolic disease: >1 (M), >0.85 (F) (National Institute for Health and Clinical Excellence (NICE) 2006). The data in the table represents baseline measurements collected at visit 1. Data is presented as a mean±standard deviation (SD).

Parameters	Average	SD
Age (yr)	27	6
Mass (kg)	70.46	8.17
Height (m)	1.67	0.09
BMI (kg/m^2)	25.36	2.22
Waist circumference (cm)-F	77.80	6.01
Waist circumference (cm)-M	84.0	2.20
Hip circumference (cm)-F	103.90	4.13
Hip circumference (cm)-M	101.2	2.21
waist/hip ratio-F	0.74	0.04
waist/hip ratio-M	0.83	0.02

8.3.2 Changes in gut permeability markers following B-GOS supplementation

At day 0, there was no difference in the concentration of LBP between conditions (day 0 maltodextrin 11.1±4.3 µg/mL vs day 0 B-GOS 10.2±4.3 µg/mL; p=ns). Likewise there

was no difference in the concentration of calprotectin at day 0 (day 0 maltodextrin 715±500 ng/mL vs day 0 B-GOS 458±299 ng/mL, p=ns). Descriptive data measurements each gut permeability marker at day 0 and day 21, pre and post treatments, % change post treatments and their normative threshold level, can be found in Error! Reference source not found..

At day 21, no changes were observed in circulating LBP following either B-GOS supplementation (p=ns, d=0.08) or maltodextrin (p=ns, d=0.21) (**Fig. 8.3.2.1**-a)). Similarly, calprotectin levels were unchanged after treatment with either maltodextrin (p=ns, d=0.14) or B-GOS (p=ns, d=0.17). (**Fig. 8.3.2.1**-b).

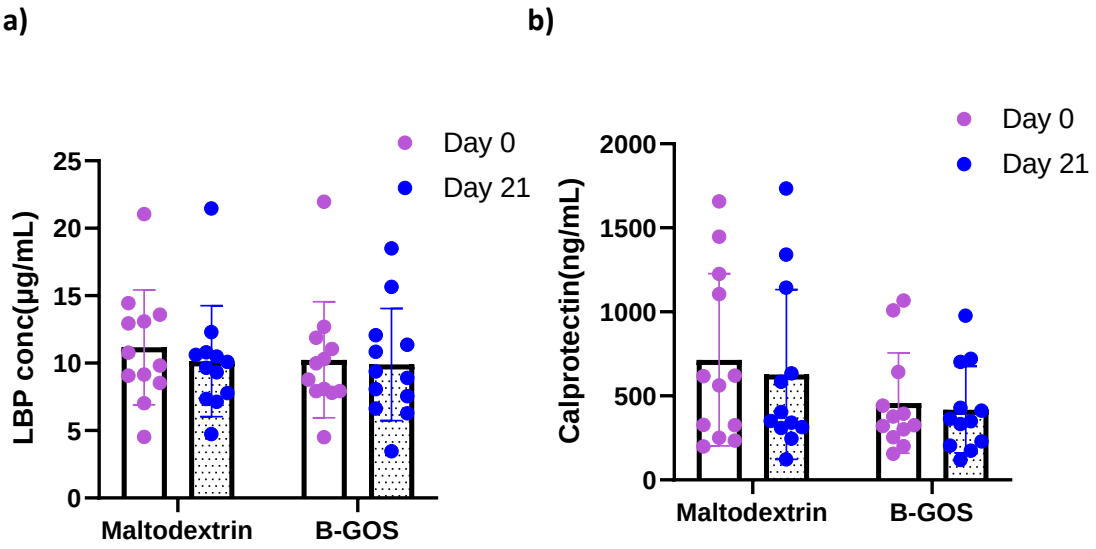


Fig. 8.3.2.1. Effect of prebiotic on gut permeability markers in asthma.
Plasma concentration of selected gut biomarkers was assessed at each visit, before (Day 0) and after (Day 21) supplementation with either maltodextrin or B-GOS. The gut permeability markers assessed were a) LBP, b) Calprotectin. Graphs display participants' gut permeability marker levels before and after supplementation with either maltodextrin or B-GOS. Statistical analysis performed was two-way ANOVA plus Sidàk multiple comparison test. Data were expressed as a mean±Standard Deviation (SD). Data were not significant (p=ns, d<0.40) (n=13).

Table 8.3.2.2. 1. Descriptive measurements of gut permeability markers before and after supplementation, % change and normative threshold levels.
Circulating level of gut permeability markers were assessed at each visit, before and after supplementation with either maltodextrin or B-GOS. Data are expressed as mean±standard deviation (SD). Lipopolysaccharide binding protein (LBP).

Gut permeability markers	Threshold levels	Maltodextrin day 0 (mean±SD)	Maltodextrin-day 21 (mean±SD)	% change-Maltodextrin	B-GOS-day 0 (mean±SD)	BGOS-day 21 (mean±SD)	% change- B-GOS
LBP (µg/mL)	<15	11.07±4.10	9.98±3.98	-6.30%	10.24±4.30	9.90±4.16	-3.00%
Calprotectin (ng/mL)	<1,600	714.51±512.75	627.22±505.46	-1.50%	457.62±298.68	418.00±257.75	-1.40%

8.3.3 The effect of prebiotic on serum biomarkers of health

Serum glucose, cholesterol (HDL, LDL, Total), triglyceride and CRP were measured at day 0 and day 21 of each intervention. At day 0 of each treatment there were no differences in baseline levels of the health markers (p=ns).

Changes between day 0 and 21 was evaluated with 2-way ANOVA analysis and it revealed no statistical differences in metabolic biomarkers with either maltodextrin or B-GOS (p=ns) (**Fig. 8.3.3.1**). Descriptive data measurements each biomarkers at day 0 and day 21, pre and post treatments, and their normative threshold level, can be found in **Table 8.3.3.2 1**.

Although not significant, the percentage change analysis demonstrated that both maltodextrin and B-GOS supplementation reduced circulating triglycerides level by ~5% (Maltodextrin: p=ns, d=0.2; B-GOS: p=ns, d=0.30). Circulating glucose levels appeared to increase by 6% with maltodextrin (p=ns, d=0.20) compared to B-GOS leading to a 7% reduction (p=ns, d=0.60). Circulating CRP levels appeared to increase by 2% with maltodextrin (p=ns, d=0.30), while it was reduced by 12% with B-GOS (p=ns, d=0.20). Maltodextrin tended to increase HDL level by 2% (p=ns, d=0.03), whereas B-GOS caused an increase of 7% (p=ns, d=0.20). Circulating LDL and total cholesterol levels remained unchanged between treatments.

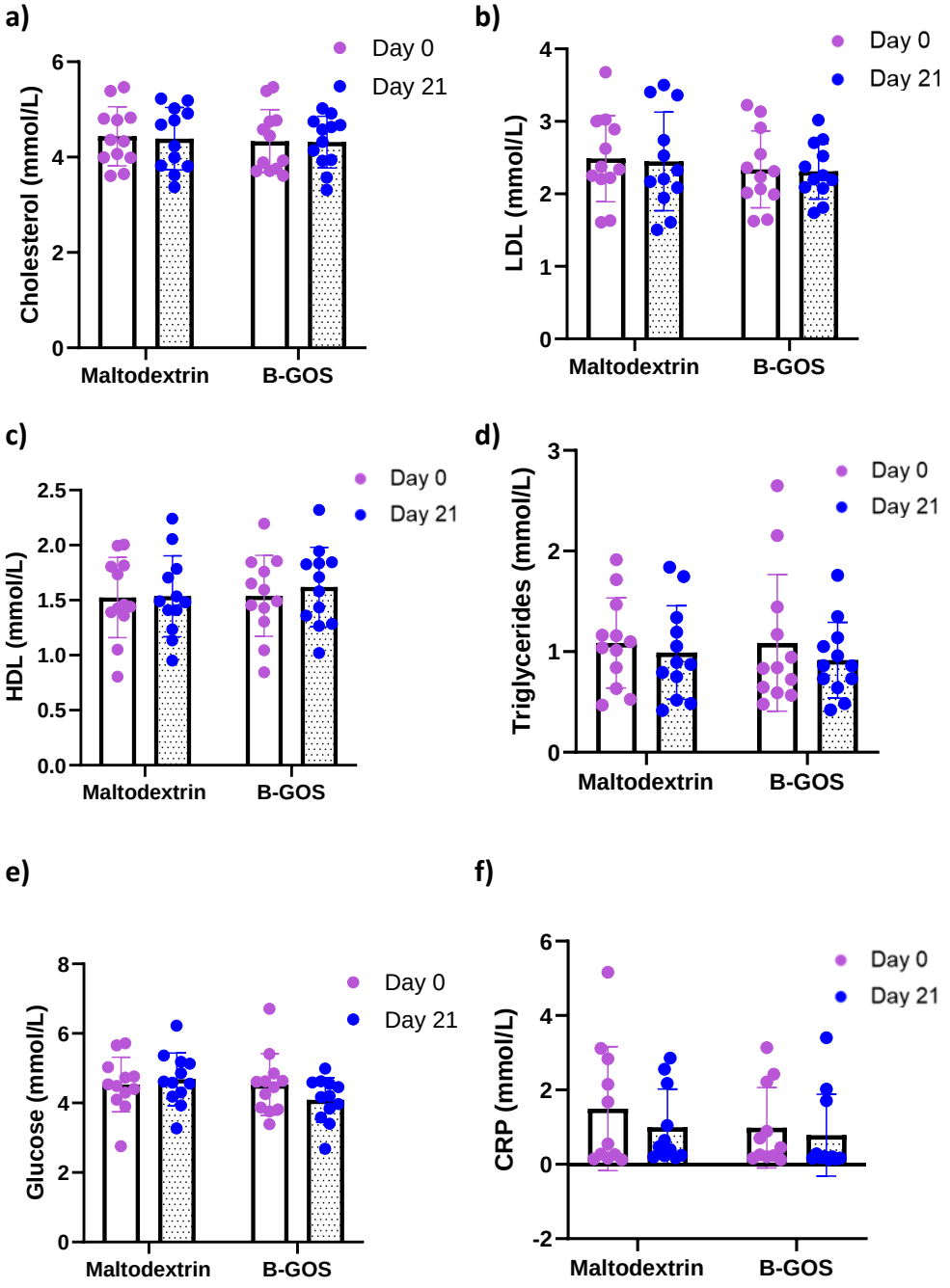


Fig. 8.3.3.1. Effect of prebiotic on health markers in asthma.
Circulating levels of selected biomarkers were assessed at each visit, before (Day 0) and after (Day 21) supplementation with either maltodextrin or B-GOS. The health markers considered were a) Total Cholesterol, b) LDL, c) HDL, d) Triglycerides, e) Glucose, f) CRP. Graphs display participants' biomarker levels before and after supplementation with either maltodextrin or B-GOS. Statistical analysis performed was two-way ANOVA plus Sidàk multiple comparison test. Data were expressed as a mean±standard deviation (SD). Data were not significant (p=ns, d<0.40) (n=12).

Table 8.3.3.2 1Descriptive measurements of health biomarkers before and after supplementation, % change and normative threshold levels.

Circulating level of health biomarkers were assessed at each visit, before and after supplementation with either maltodextrin or B-GOS. Data are expressed as mean±standard deviation (SD). Cholesterol, low-density protein (LDL), Cholesterol, high-density protein (HDL), C-reactive protein (CRP).

Blood Biomarker	Threshold levels	Maltodextrin- day 0 (mean±SD)	Maltodextrin-day 21 (mean±SD)	% change-Maltodextrin	B-GOS-day 0 (mean±SD)	BGOS-day 21 (mean±SD)	% change-B-GOS
Cholesterol (mmol/L)	<6	4.38±0.63	4.35±0.64	-1.00%	4.34±0.63	4.27±0.54	0.50%
LDL (mmol/L)	<3	2.44±0.60	2.40±0.67	-2.00%	2.32±0.51	2.27±0.40	-1.00%
HDL (mmol/L)	<1	1.52±0.37	1.55±0.37	2.00%	1.54±0.37	1.62±0.37	7.00%
Triglycerides (mmol/L)	<2	1.09±0.45	1.01±0.47	-7.00%	1.08±0.68	0.92±0.38	-5.80%
Glucose (mmol/L)	<6	4.53±0.79	4.72±0.76	6.00%	4.53±0.88	4.08±0.64	-7.00%
CRP (mmol/L)	<10	1.5±1.66	1.00±1.03	2.40%	0.99±1.09	0.78±1.10	-12%

8.3.4 The effect of prebiotic on lung function

Lung function was assessed using spirometry measurements at day 0 and day 21 of both interventions. There was no difference in day 0 pulmonary function values (p=ns) between interventions, confirming that participants had stable asthma, no exacerbation and there was no carry over effect after the two-week washout period.

Descriptive data measurements each lung function parameter at day 0 and day 21, pre and post treatments, % of change and their normative threshold level, can be found in **Table 8.4.3.2. 1**.

Two-way repeated measures ANOVAs revealed no significant changes in %FEV_1, FVC, FEV_1/FVC, PEF, FEF_{25-75}, FEF_{75-85} within (day 0 and 21), or between interventions (p=ns) (**Fig. 8.3.4.1**). Although not significant, the % of change analysis showed a 12% improvement in %FEF_{75-85} following B-GOS supplementation with a small/medium effect size (p=ns, d=0.30), while there was no change after maltodextrin (p=ns, d=0.06) (**Error! Reference source not found.**).

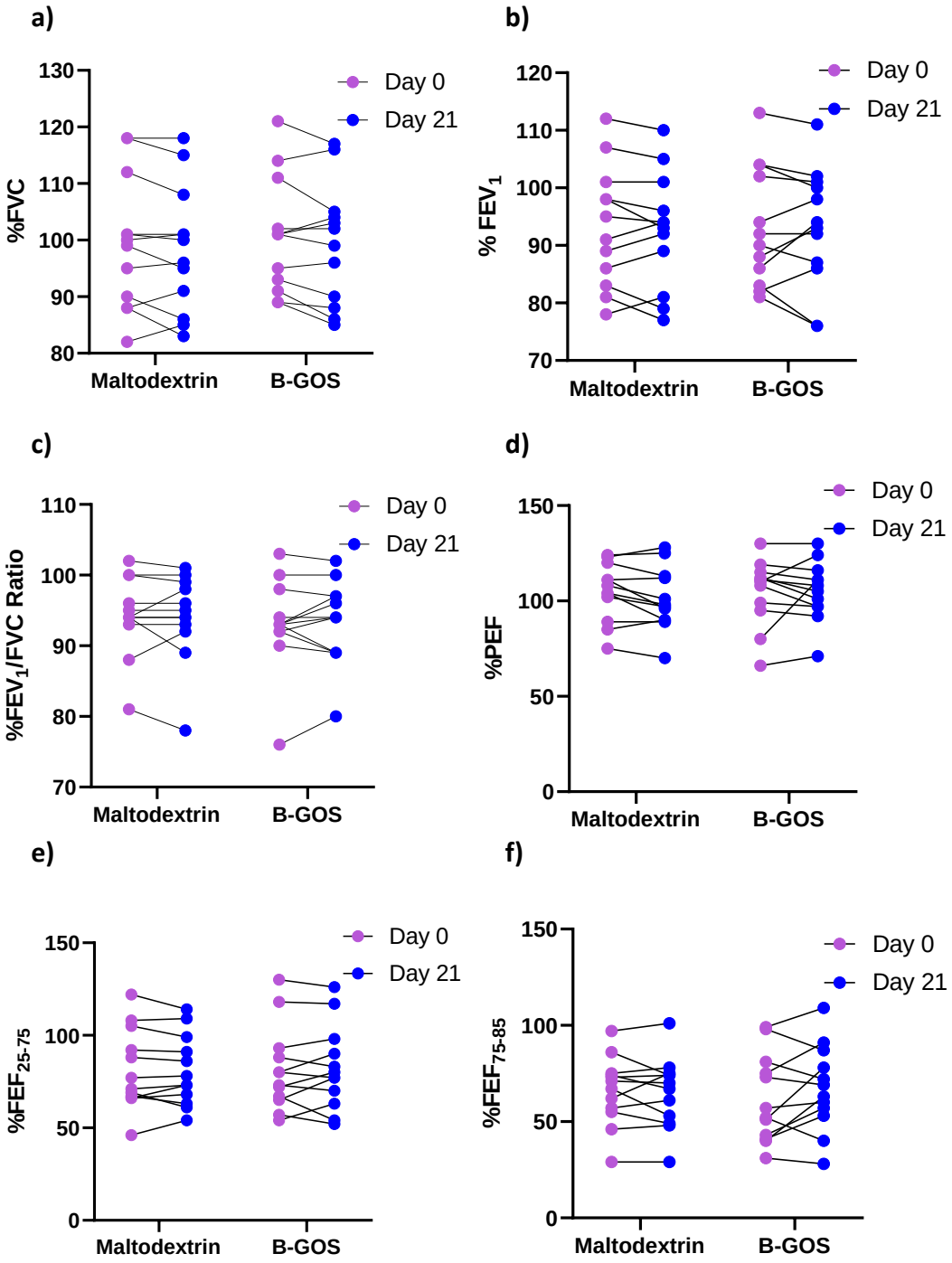


Fig. 8.3.4.1. The effect of prebiotic on lung function of participants with asthma.
Lung function was assessed with spirometry at each visit before (Day 0) and after (Day 21) supplementation with either prebiotic B-GOS or maltodextrin. Data measured were a) FVC, b) FEV_1, c) FEV_1/FVC Ratio, d) PEF, e) FEF_{25-75}, f) FEF_{75-85}. Graphs represent data for each participant before and after the maltodextrin and B-GOS supplementation. Statistical analysis used was two-way ANOVA plus Sidàk multiple comparison test. Data are displayed as a mean±standard deviation (SD). Data were not significant (p=ns; d<0.40) (n=12).

Table 8.4.3.2. 1. Descriptive measurements of lung function parameters before and after supplementation, % change, and normative threshold levels

Lung function was assessed with spirometry at each visit before and after supplementation with either maltodextrin or B-GOS. Data are expressed as a % of change of either maltodextrin or prebiotic B-GOS ± standard deviation (SD). Forced expiratory volume in one second (FEV1), forced vital capacity (FVC), peak expiratory flow (PEF), forced expiratory flow between 25% and 75% (FEF25-75), forced expiratory flow between 25% and 75% (%FEF75-85).

Spirometry-parameters	Threshold levels (%)	Maltodextrin- day 0 (mean± SD)	Maltodextrin-day 21 (mean± SD)	% change-Maltodextrin	B-GOS-day 0 (mean± SD)	BGOS-day 21 (mean± SD)	% change-B-GOS
%FEV1	<80	93.25±10.50	92.60±10.10	0.70%	93.25±9.80	93±10.10	-0.20%
%FVC	<80	99.30±11.80	97.01±11.30	-0.80%	100±10.00	99.30±10.80	-1.40%
%FEV1/ FVC ratio	<80	94.30±6.60	94.10±6.60	-0.40%	93.20±6.60	94.40±5.90	0.50%
%PEF	<80	10.5.10± 15.25	100.80±16.40	-4.00%	105.00±17.60	105.30±15.50	0.20%
$\%FEF_{25-75}$	<80	81.00±22.00	81.00±19.30	0.00%	78.10±25.10	78.80±25.20	0.00%
$\%FEF_{75-85}$	<80	66.00±18.10	65.00±18.50	-1.00%	61.00±23.00	68.10±22.40	-12%

8.3.5 The effect of prebiotic on asthma symptoms questionnaires

The effects of prebiotic on self-reported asthma symptoms and asthma associated symptoms were measured through questionnaires, assessed at each visit, and scores were used for analysis (**Fig. 8.3.5.1**). There were no differences in baseline score between interventions at day 0 (p=ns).

Descriptive data measurements each asthma related questionnaires at day 0 and day 21, change in score and their normative threshold level, can be found in **Table 8.3.5.2. 1.**

No changes were observed at day 0 and day 21 of prebiotics or maltodextrin in self-reported symptoms of asthma (ACQ-6) (Maltodextrin, B-GOS: p=ns, d=0.1). The

hyperventilation score assessed through the Nijmegen questionnaire, went down by 2 points following the supplementation of both maltodextrin (p=ns, d=0.30) and B-GOS (p=ns, d=0.20) but this did not reach significance. The HARQ score went up by 3 points following maltodextrin, (p=ns, d=0.30), while it went down by 2 points after B-GOS administration (p=ns, d=0.20). A slight decrease in the nasal disorder questionnaire score, SNOT-22, was observed with both supplements but a larger decrease with B-GOS (maltodextrin: -2 points, p=ns, d=0.10; B-GOS: -5 points, p=ns, d=0.30). Next, the HADS-A score was slightly reduced with maltodextrin (-2 points, p=ns, d=0.20), while no changes were observed after B-GOS supplementation (p=ns, d=0.10). Finally, no differences in scores were observed in HADS-D (maltodextrin: p=ns, d=0.1; B-GOS: p=ns, d=0.2) and Epworth (maltodextrin: p=ns, d=0.2; B-GOS: p=ns, d=0.1) with either treatment.

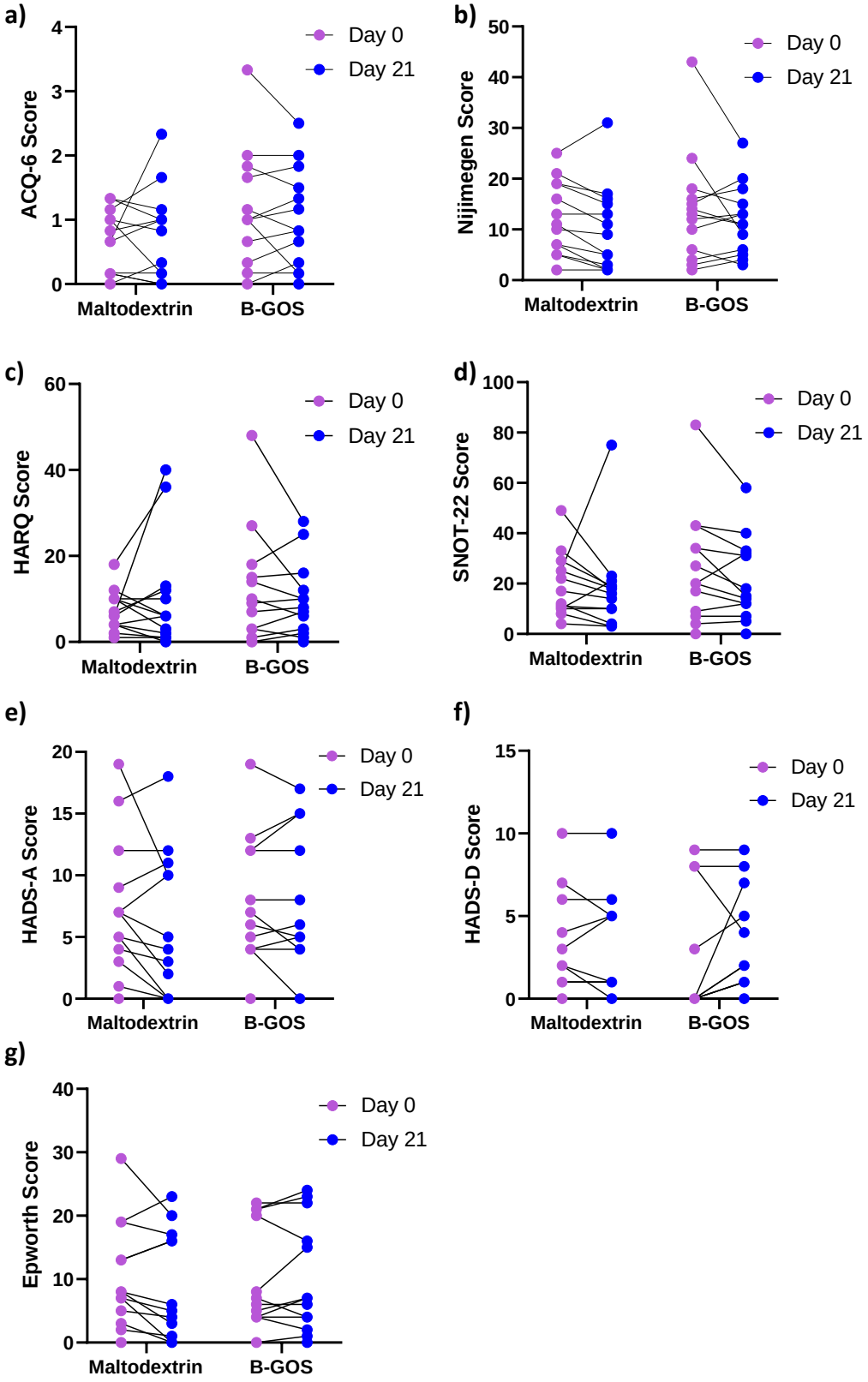


Fig. 8.3.5.1. The effect of prebiotic on asthma symptoms.
Questionnaires were completed by participants at each visit. The questionnaires used were a) ACQ-6, b) Nijmegen, c) HARQ, d) SNOT-22, e) HADS-A, f) HADS-D, g) Epworth. Graphs display participants scores before (Day 0) and after (Day 21) supplementation with either maltodextrin or B-GOS. Statistical analysis performed was two-way ANOVA plus Sidàk multiple comparison test. Data were expressed as a mean±standard deviation (SD). Data were not significant (p=ns, d<0.40) (n=13).

Table 8.3.5.2. 1. Descriptive measurements of asthma related questionnaire **before and after supplementation. % change and normative threshold levels.**
Asthma related questionnaires were assessed at each visit, before and after supplementation with either maltodextrin or B-GOS. Data are expressed as mean change in score of either maltodextrin or prebiotic B-GOS±standard deviation (SD). Hull Airways Reflux questionnaire (HARQ), Sino-nasal Outcome Test (SNOT-22), Hospital Anxiety and Depression Scale-Depression (HADS-D), Hospital Anxiety and Depression *Scale- Anxiety (HADS-A).*

Questionnaires	Threshold levels	Maltodextrin day 0 (mean±SD)	Maltodextrin-day 21 (mean±SD)	Change in score-Maltodextrin (mean±SD)	B-GOS-day 0 (mean±SD)	B-GOS-day 21 (mean±SD)	Change in score-B-GOS (mean±SD)
ACQ-6	<1.5	0.65±0.50	0.74±0.72	0.1±0.40	1.09±0.95	1.02±0.78	-0.1±0.6
Nijmegen	<23	12.31±7.17	10.08±8.43	-2.23±3.16	13.85±10.85	11.93±6.93	-1.92±6.55
HARQ	<13	7.23±4.68	10.46±12.94	3.23±11.34	11.92±13.48	9.85±8.71	-2.1±7.53
SNOT-22	<7	19.61±12.57	18.00±18.43	-1.61±17.73	26.23±22.21	18.25±16.44	-4.92±9.52
HADS-D	<7	2.92±3.04	2.77±3.11	-0.15±1.10	2.85±3.18	3.08±3.20	0.23±2.24
HADS-A	<7	10.23±8.19	8.54±8.51	-1.53±3.33	9.38±8.38	10.08±8.76	-0.24±1.92
Epworth	<10	7.00±2.94	6.00±3.11	-1±2.90	6.62±1.76	6.00±2.58	-0.62±1.80

8.4 Discussion

The aim of this study was to evaluate if 21 days of daily intake of prebiotic B-GOS in a cohort of mild well-controlled asthma could improve lung function and asthma control, influence plasma concentration of gut permeability markers (LBP and calprotectin), improve serum biomarkers of health (glucose, cholesterol, triglycerides) and inflammation (CRP).

This study showed that 21 days daily intake of prebiotic B-GOS did not affect pulmonary function, asthma related symptoms or circulating biomarkers of gut permeability. Similarly, no changes in health biomarkers (glucose, cholesterol, triglycerides) or systemic inflammation (CRP) were observed, following B.GOS supplementation.

The gut microbiota has a key role in the development of the gut immune system and in the development of immune tolerance (McLoughlin and Mills 2011). Associations between altered gut microbiota and respiratory disease have been highlighted in the past (Begley et al. 2018; Cait et al. 2018; Chai et al. 2022; Russell et al. 2012). Therefore, it is plausible that prebiotic B-GOS may have a beneficial effect on asthma symptoms and improvements in metabolic health. It was previously reported that a 5.5 g/day B-GOS intervention successfully reduced the level of bronchoconstriction and systemic inflammation in a cohort of physically active patients with exercise induced bronchoconstriction (EIB) (Williams et al. 2016a). The aim of this study was to evaluate if daily dose of B-GOS could be used to improve lung function and affect gut permeability and markers of metabolic health in mild well-controlled asthma patients at resting conditions.

Furthermore, the previous study used a 5.5 g/day B-GOS dose (Williams et al. 2016) which has been shown to have a potent Bifidogenic effect, increasing the number of beneficial bacteria at the expense of the less beneficial ones, as well as leading to a reduction in pro-inflammatory cytokines (Vulevic et al. 2008). In contrast, the present study used a 2.9 g/day dose of B-GOS to match the existing commercially available dose of prebiotic.

Findings from this study increases the knowledge of B-GOS in cohort of patients with mild well-controlled asthma. However, further investigations are needed to confirm these findings.

8.4.1 Effect of prebiotic on blood biomarkers of gut permeability

This was the first study to investigate the effect of a prebiotic on intestinal permeability in a cohort of adults with mild well-controlled asthma. The results obtained in this study reported no changes in gut permeability markers. However, baseline levels of LBP and calprotectin were not elevated at rest in this cohort. Normal circulating levels for LBP ranges from 5-15 μg/mL (Gonzalez-Quintela et al. 2013; Zweigner et al. 2001) while serum calprotectin levels range from 100-1,600 ng/mL (Jarlborg et al. 2020) with only one participant exceeding this level at day 0. This suggests that there was little to no impairment in the gut barrier of the mild well-controlled asthma patients in the current study and therefore little room for improvement. This may be due to young age of participants, lean BMI or mild well-controlled asthma status which may have impacted changes. It also suggests that gut permeability may not be an early marker of change in mild well-controlled asthma.

However, high levels of circulating gut permeability markers have been found in metabolic conditions such as obesity (Martel et al. 2022; Calcaterra et al. 2018; Moreno-Navarrete et al. 2012). The use of prebiotics in improving circulating levels of gut marker was demonstrated in a cohort of adults that were overweight or obese, in which 12 weeks prebiotic supplementation resulted in a 40% reduction of circulating levels of LPS (Parnell et al. 2017a). Another study showed that 3-weeks prebiotic treatment in a cohort of adults with obesity ameliorated colonic permeability, but it did not reduce circulating levels of LPS and LBP (Krumbeck et al. 2018). These studies demonstrated a beneficial effect of prebiotic supplementation in improving gut permeability levels and therefore may suggest that patients with abnormal gut permeability resulting from comorbidities such as obesity, may benefit from prebiotic supplementation and therefore warrant future investigation.

8.4.2 Effect of prebiotics on blood biomarkers of metabolic and inflammatory health

B-GOS supplementation did not significantly affect blood biomarkers of health. An initial trend towards reduced circulating levels of glucose (by 7% with a large effect size of 0.60), triglycerides (by 5% with a small effect size of 0.30), HDL (by 6% with a small effect size of 0.20) and circulating CRP (by 12% with a small affect size of 0.20) following B-GOS supplementation was observed. However, these were only small changes and therefore further analysis are needed to confirm the trend observed in this study.

B-GOS supplementation showed a small reduction in circulating glucose levels from day 0 to day 21. However, participants were not screened for pre-diabetes condition and

therefore it cannot be excluded that elevated concentration of glucose were due to this condition.

Previous studies showed an association between impaired glucose levels and worse asthma symptoms such as airway hyperresponsiveness and inflammation (Cottrell et al. 2011b; Gulcan et al. 2009; Karampatakis et al. 2017). The prebiotic B-GOS can selectively stimulate the growth of *Bifidobacterium* (Depeint et al. 2008) and it has been demonstrated that some of these species are specialized in the digestion of carbohydrates, therefore suggesting a potential beneficial effect in improving carbohydrate digestion within the gut and ameliorating glucose uptake (Hosaka et al. 2020; Kikuchi, Ben Othman, and Sakamoto 2018; Milani et al. 2015; Zhu et al. 2018). In the present study, there were no significant changes in glucose levels following B-GOS treatment.

Similarly, higher concentrations of cholesterol LDL and lower levels of cholesterol HDL have been reported before in participants with asthma (Azofra et al. 2019; Su et al. 2018). In the present study the LDL and total cholesterol levels were unchanged, but a tendency of a 7% increase in HDL was reported following B-GOS supplementation. Previous studies demonstrated improvements in markers of metabolic health such glucose, cholesterol and triglycerides in obesity and type 2 diabetes diseases following supplementation with prebiotics or probiotics for two or more months (Sabico et al. 2019; Aliasgharzadeh et al. 2015; Sabico et al. 2017). The present study showed a potential improvement in glucose levels and HDL after 21 days of B-GOS supplementation, which may suggest a potential beneficial effect of prebiotics on improving markers of metabolic health. Therefore, further investigations into the role of B-GOS in a cohort of participants with asthma and obesity are warranted.

High circulating CRP levels are commonly associated with airway inflammation and systemic inflammation in asthma (Kony et al. 2004; Takemura et al. 2006; Williams et al. 2016a). A previous study reported a reduction in serum CRP concentration following 3-weeks supplementation with 5.5 g/day B-GOS in a cohort of asthma participants with EIB (Williams et al. 2016a). In contrast to the present study, Willliams et al. conducted the trial in a specific phenotype cohort of EIB and used a larger dose of B-GOS (5.5 g/day), which may have contributed to the differences in CRP levels seen between studies.

CRP is also a well-known marker of systemic inflammation in metabolic diseases such as obesity (Thomson et al. 2003; de Jesus et al. 2018; Barros et al. 2011; Bantulà et al. 2021). Previous research reported a reduction in plasma CRP concentration in a cohort of adults with obesity, following B-GOS (5.5g/day) supplementation (Vulevic et al. 2013b). However, the reduction in plasma concentration of CRP was significant only after prolonged use of B-GOS (12 weeks), while no difference compared to placebo was observed after the first 6 weeks of B-GOS administration (Vulevic et al. 2013b). This may suggest that prolonged use of prebiotic and higher dose could have potential benefit in reducing markers of systemic inflammation and should be investigated further in asthma.

Moreover, in the present study baseline CRP levels were all within physiological range (<10 mmol/L) (Wium-Andersen et al. 2013). This suggests that there was no systemic inflammation in the mild well-controlled asthma patients in the current study and therefore little room for improvement. This may be due to young age of participants, lean BMI or mild well-controlled asthma status which may have impacted changes.

8.4.3 The effect of prebiotics on lung function and asthma symptoms

The 21 days of supplementation with B-GOS did not significantly affect resting lung function. Even if not significant, the % of change analysis showed an increase of 12% in the $\%FEF_{75-85}$ parameter with a 0.3 effect size, following B-GOS supplementation. The $\%FEF_{75-85\ is}$ an indicator of small airways dysfunction and is used mostly to assess obstructive respiratory diseases such as chronic obstructive pulmonary disease (COPD) (Chattopadhyay et al. 2007; Pittman et al. 2012; Sorbello et al. 1981). That said, increased peripheral airway resistance, which can lead to bronchoconstriction or wheeze in patients with asthma (Dekerlegand et al. 2007), was increased in patients with mild asthma with apparently normal lung function compared to a control group (Martin 2002) and correlated with poorer quality of life (Takeda et al. 2010) and increased asthma exacerbation (Veen et al. 2000). Increased airway resistance was an important observation because it demonstrated that there is a disease activity even in mild well-controlled asthma patients with no apparent lung function impairment. Although not statistically significant, the results obtained from the present study suggest a potentially protective effect of B-GOS on the function of small airways that could be indirectly mediated by an increase in circulating SCFA, which have anti-inflammatory properties, and/or due to an increase in beneficial *Bifidobacterium*, following B-GOS supplementation.(Vulevic et al. 2013b; Williams et al. 2016a) Further investigations are needed to confirm the potential beneficial effect of B-GOS on lung function and to disclose its mechanistic effects.

Beyond the trend in amelioration of $\%FEF_{75-85}$, no other improvement in lung function was found in the present study. This contrasts with previous findings that demonstrated

an improvement in lung function in adults with hyperpnoea-induced bronchoconstriction (HIB), a surrogate for exercise-induced bronchoconstriction, following 3-weeks supplementation with B-GOS (Williams et al. 2016a). However, unlike the current study, participants in Williams et al. (2016) used higher dose of prebiotic (5.5 g/day) and underwent a bronchoprovocation challenge noted as eucapnic voluntary hyperpnoea (EVH) to induce bronchoconstriction and airway inflammation. This challenge resulted in a reduction in pulmonary function and increases in inflammation, which may have provided a window of opportunity to see improvements as a result of B-GOS. The participants in the current study were only tested at rest and had very mild well-controlled asthma as demonstrated by resting %FEV1 values of >80%, potentially limiting any small effects that may be seen from a dietary B-GOS intervention. It could be speculated that dietary prebiotics may only show effects during periods of asthma exacerbations, when the airways are exposed to a trigger (e.g. exercise, allergens etc.).

Asthma severity and related symptoms were assessed at each visit with questionnaires. The prebiotic B-GOS did not significantly improve any of these factors. However, there was a statistical trend (with a small effect size) towards a reduction in nasal disorder (SNOT-22) score, an asthma associated symptom, following B-GOS supplementation. Questionnaires are an easy way to report a broad range of symptoms, although there are several issues that may affect the results. Questionnaire scores could have been affected by external factors such as stress, hay fever or illness. Considering the 3-week supplementation period, any of these factors could have influenced the self-reporting of symptoms. With self-reporting questionnaires there is potential bias, and alterations

in perceptions of symptoms at the specific time the participants completed the questionnaire. Although questionnaires are a useful tool to detect changes in symptoms, the relatively short supplementation period and the well-controlled asthma cohort being studied made it difficult to evaluate changes.

8.4.4 Conclusion

The results of this double-blind, placebo controlled cross-over study did not show improvement in markers of metabolic health, inflammation and/or lung function in mild well-controlled participants with asthma, following B-GOS supplementation. Therefore, it would be of interest to explore the effects of B-GOS in a cohort of patients with asthma and obesity.

Chapter 9: General Discussion and conclusions

9.1 Introduction

Asthma represents a rising public health and financial burden. In the U.K., 4.3 million adults suffer of asthma with an annual cost for the NHS of around 1 billion pound per year (NHS England 2020). Several factors can increase the risk of developing asthma. Between others obesity appear to be associated with a more severe subtype of asthma characterized by severe exacerbation and less response to traditional medication (Lugogo et al. 2010; Peters et al. 2018; Wong et al. 2022). Obesity is characterized by systemic low-grade inflammation and altered metabolic profile which can contribute to the severity of asthma. However, the underlying mechanism by which obesity affects asthma is still poorly understood. Therefore, there is a greater need to understand phenotypic characteristics of patients with obesity and asthma to help support future disease management strategies.

Dysregulation in the gut microbiota, known in obesity, has been previously linked with asthma exacerbation (Ashique et al. 2022; Li et al. 2021; Sokolowska et al. 2018). Specifically, alteration of gut bacteria composition towards harmful species may lead to the disruption of gut barrier and potential translocation of gut derived endotoxin such as lipopolysaccharides (LPS) in the blood circulation. This in turn can activate a pro-inflammatory cascade pathway leading to systemic inflammation as well as alteration of metabolic health markers. Greater understanding of the role of LPS at molecular and human level are needed to evaluate the consequence of gut barrier disruption on asthma severity.

Long term use of medication can cause common side effects including headaches, muscle cramps and increased heart rate and for these reasons asthma medications are

not always well tolerated by patients (Cates et al. 2014; Cates and Cates 2012; Nelson 2006). Moreover, asthma treatments focus on reducing symptoms by supressing the activity of leukocytes such as eosinophils, which subsequently leads to reduction of inflammation and reversal of bronchoconstriction, rather than preventing them (Chauhan and Ducharme 2012; National Institute for Health and Care Excellence (NICE) 2023). Therefore alternative strategies need to be investigated. Nutritional supplements such as the use prebiotic has been showed to improve asthma symptoms and reduce inflammation (McLoughlin et al., 2019; Williams et al., 2016). However, no human study investigated the effect of prebiotics in strengthening gut barrier in cohort of asthma.

These gaps in the literature build up the rationale for this PhD thesis. A summary of key finding of the thesis are detailed below.

Chapter 4 evaluated the role of short chain fatty acids (SCFA), end-product of prebiotic fermentation by gut bacteria, in airway epithelial cells to study their mechanism of action in preventing LPS-induced damage in a particular pathway leading to inflammation (mitochondria dysfunction). In particular, airway cells responded to the LPS insult by increasing mtSOD2 levels to cope with ROS production and by producing new mitochondria to meet the mitochondrial respiration requirement yet the mitochondria in the airway had structural damage and were less responsive to LPS stimuli. SCFAs partially restored LPS-induced chronic mitochondrial structural damage in airway cells.

Chapter 5 aimed to characterize a cohort of difficult to control asthma and investigate the role of BMI on asthma severity. This chapter found high BMI positively correlated with poor asthma control and risen plasma concentration of inflammatory cytokines related to Th-2 high and Th-2 low phenotype. This may suggest a key role of obesity in driving asthma severity via mediation of inflammation.

Chapter 6 aimed to assess whether levels of gut permeability markers differed between participants with well-controlled asthma and difficult to control asthma across a range of BMIs were considered. Trend in data reported an increased plasma concentration of gut permeability markers, LBP and calprotectin, in participants with well-controlled asthma suggesting an impairment of gut barrier when the disease is well-controlled which further deteriorate with the progress of disease. However, obesity appeared to be the main driver of asthma severity, while gut permeability is likely to be a contributing factor to the disease.

Chapter 7 was a pilot study to compare the use of two metabolic techniques: low-field bench top nuclear magnetic field (bNMR) and high field NMR. The aim was to evaluate whether bNMR could be used as an alternative method to high field NMR to discriminate metabolic profile between participants with and without asthma at a reduced cost. Preliminary findings from this chapter revealed a potential cluster separation between asthma and control group. If confirmed in large cohorts of participants, bNMR could be used as an additional tool to facilitate disease diagnosis.

Chapter 8 evaluated the role of 2.9 g/day of B-GOS prebiotic supplementation for 21 days in asthma to assess whether it reduced asthma symptoms, improved metabolic health and help strengthening gut barrier. Prebiotic administration did not affect lung

function or asthma control, but it showed a potential beneficial effect in improving markers of metabolic health (CRP and glucose) in participants with well-controlled asthma. This result paves the way for extending the study in a cohort of participants with obesity and asthma which may benefit more of the supplementation.

9.2 Experimental findings

The potential effect of gut microbiota manipulation on inflammation and asthma management was investigated using a combination of *in vitro* and human *in-vivo* trials. Previous findings reported promising results of prebiotic supplementation in ameliorating asthma symptoms and improving airway inflammation (McLoughlin et al. 2019a; Williams et al. 2016b), but none have yet explored the role of prebiotics in strengthening the gut barrier. Therefore chapter 8 of this thesis investigated for the first time the effect of dietary prebiotics on biomarkers of gut barrier integrity as well as asthma symptoms, systemic inflammation and metabolic health in participants with well controlled asthma. In parallel with this, chapter 4 evaluated the mechanistic activity of SCFA, (the metabolic by-products of prebiotic fermentation) at molecular level using airway cells to assess their effect in limiting LPS-related damage.

In chapter 4, initial experiments aimed to evaluate the role of SCFA in reducing the harmful effects of LPS on mitochondria structure and function. Mitochondria are essential organelles that respond to negative stimulus by producing reactive oxygen specie (ROS) to protect cells from damage, although an aberrant mitochondrial response may lead to a proinflammatory cascade that in turn worsening the disease (Bhatraju and Agrawal 2017a). Findings from this study revealed that SCFA was able to partially protect

mitochondria from structural and functional decline. In fact, cells treated with LPS had a reduced mitochondria activity which in turn can make mitochondria less responsive to stimulus such as LPS. Cells treated with SCFA appeared to limit the mitochondrial activity decline as evaluated with live imaging. In addition, SCFA protected mitochondria structure from fragmentation by partially restoring levels of mitochondrial dynamic proteins to control levels. However, mitochondria were able to cope with LPS stimulus by enhancing the production of the anti-inflammatory compound, superoxide dismutase 2 (mtSOD2), and by increasing the production of new mitochondria. This allowed the cells to carry on essential activity as observed with the mitochondria respiration assay. This could be explained by the natural resistance of this airways cell that they may have acquired due to their position in the epithelium of the lungs which expose them to external environmental agents. This means epithelial airway cells are in constant contact with all sorts of inflammatory stimulus such allergens and LPS strains. As a response to this, cell probably developed some sort of resilience to this stimulus to avoid being in a constant inflammatory state which in turn could significantly affect cellular system and human health. However, no previous studies have demonstrated so, therefore future investigations are needed to confirm this hypothesis.

The next step was to evaluate the role of obesity in asthma severity. To do so, chapter 5 aimed to characterize a cohort of difficult to control asthma cohort across a range of BMI. It appeared that patients exhibited common characteristics from Th-2 low and Th-2 high phenotypes suggesting a complex pathophysiological interaction between obesity and asthma. This can be a result of low-grade inflammation and the metabolic dysregulation ongoing in both diseases (Forno et al. 2015; Husemoen et al. 2008; Sideleva et al. 2012; Singh et al. 2015). In fact, the metabolic pilot study (Chapter 7)

revealed a potential cluster separation between participants with well-controlled asthma and healthy controls within a lean BMI. This may suggest an underlying metabolic dysfunction in asthma which can further deteriorate disease progression. Therefore, a broader approach that takes in consideration the impact of obesity, systemic inflammation and metabolic dysregulation is needed to better understand the pathophysiology of asthma and improve diagnosis and treatment.

To further evaluate the role of gut and LPS-induced damage in asthma severity, chapter 6 investigated whether concentrations of gut permeability markers differ between participants with well-controlled and difficult to control asthma across a range of BMI. Due to uneven group distribution, it was not possible to reach a final conclusion and therefore warrant further investigation. However, trend in data suggest an impairment of gut barrier when the disease is well controlled, and a further progress with disease severity. Moreover, the findings do suggest that the combination of asthma and obesity is associated with a more permeable gut barrier which likely exacerbates disease severity and asthma control. Therefore, strategies that target gut barrier such as the use of prebiotic may be a beneficial for these patients.

After the initial molecular evaluation of the role of SCFA on airways cells and the further human study analysis highlighting the importance of the gut barrier in asthma and obesity, the final study of this thesis (chapter 8) explored the effect of gut microbiota manipulation on asthma with the dietary prebiotic intervention trial. The aim of the study was to evaluate the effect of daily intake of 2.9 g of prebiotic B-GOS for 21 days

on asthma control, asthma function, metabolic health and gut permeability in a cohort of participants with well-controlled asthma. The results from the current study did not show any amelioration on lung function, asthma control and gut permeability. However, B-GOS did demonstrate a potential to reduce circulating levels of glucose (by 7%) and C-reactive protein (CRP) (by 12%). These results did not reach significance but considering that the cohort had very mild well-controlled asthma and the vast majority of participants were young, healthy and within lean BMI, it was promising to see these changes. Similarly, the fact that there were no changes in lung function, asthma control and gut permeability was probably due to the cohort studied. Thus, a cohort of patients with more severe symptoms and impaired gut barrier such as the difficult to control asthma cohort (e.g. chapter 5) may benefit more from adjunctive treatment with prebiotic supplementation, and this warrant further studies.

9.3 Limitation

Due to Covid pandemic, the initial plan of this study was significantly affected. For the first 9 months of the PhD, access to the laboratory was restricted and limited staff members were allowed. This meant that the first year, which should have been used to learn molecular techniques in the laboratory and optimize the concentration of LPS and SCFA for cells treatment to further develop experimental plans in subsequent years, was not possible due to time constraints.

Both human studies, were affected by Covid pandemic. The initial studies plan required NHS ethics which would allow recruitment of patients from hospital and including the use of other technique such as (dual x-ray absorptiometry) DEXA to allow deep evaluation of body composition on asthma disease. Due to Covid and time limitation this

was not possible and both studies were approved with a simplified protocol via NTU human invasive ethics committee instead, following considerable delay. This limited the recruitment process to a university population, which were young, healthy and wit with a lean BMI.

9.4 Future work

The results obtained in this thesis provide initial evidence for a number of future experiments. Firstly, it would be interesting to replicate the experiments performed using airway cells in different cell lines such as adipose cells and human colon epithelium cells line. This would be beneficial to explore the mechanism of action of SCFA in different compartment and gain better insight of the complex interaction between gut, obesity and asthma disease.

In order strengthened the results obtained from the WATCH-NTU study, it would be beneficial to continue recruitment of participants with well-controlled asthma with a broad range of BMIs. This will allow better evaluation of the role of gut permeability in asthma severity. In addition, considering the potential cluster separation noted with the metabolomic pilot study, it would be of interest to expand this analysis to the rest of the cohorts studied from patients with difficult to control and well-controlled asthma. This will allow for comparisons to be made in metabolomic profiles which could provide evidence for future treatment options and targets.

Given the promising results obtained from the prebiotic study, it would be beneficial to expand the recruitment to patients with asthma who are overweight or obese. This will allow further evaluation of the role of SCFA in obese state. Ideally, DEXA measurement could be introduced for a more in-depth assessment of body composition and its association with disease severity. Furthermore, it would be of interest exploring prebiotic supplementation in a cohort of patients with difficult-to-control asthma, especially during exacerbations, as this may provide more room for the prebiotic to exert its effects.

This would be possible by submitting the study for NHS ethics, which will also allow for the recruitment of participants from hospitals settings.

9.5 Final conclusion

At molecular level, the SCFA showed a beneficial effect in limiting LPS related damage by protecting mitochondria dysfunction in airway cells. In the human study, characterization of a cohort of patients with difficult to control asthma revealed a complex pathophysiology and suggest that an integrated approach that takes into account the impact of systemic inflammation and metabolic dysfunction is needed to enable a better strategy of treatments. Furthermore, combination of obesity and asthma appeared to alter gut barrier which in turn can further contribute to disease severity. Finally, the prebiotic supplementation to adults with well-controlled asthma did not improve lung function and asthma control but showed the potential to improve glucose and CPR levels.

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Appendix

Appendix 1: WATCH participant information sheet and consent form

Participant information sheet

"Investigating NGAL and markers of gut permeability and damage across asthma severity and adiposity."

Chief Investigator*:* Dr Neil Williams

Researcher: Miss Cristina Parenti

Institution: Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS

Invitation

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.

Invitation and brief summary

Obesity and asthma are common chronic diseases requiring urgent action. Asthmatic obese patients show an alternative type of asthma associated with poor asthma control, worsening symptoms and reduced response to medication. Systemic inflammation and the gut microbiota could be potential treatment targets for asthma and obesity. Therefore, greater understanding of disease markers that can distinguish between asthma severity and levels of obesity are warranted.

The aims of this study are to:

1) Investigate biomarkers of gut health and damage between asthma severity and obesity.
2) Investigate the concentration of a novel blood inflammatory biomarkers between asthma severity and obesity.

Study Requirements

You will be required to visit our laboratory on one occasion. The visit will last approximately 1 hour. During the visit you will have measures of lung function, have up to 30 ml blood sample taken and conduct anthropometry.

In the morning of your visit, you must have taken and received a negative test result for COVID-19 using a lateral flow test. These can be collected from the research team, or you can book into the NTU campus testing here https://myntuac.sharepoint.com/sites/LateralFlowTesting

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 18-34.9 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 3 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 β2-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.
- 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, Crohn's disease).
- You have recently taken part in other research projects. Please notify the chief investigator.
- 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.
- You were previously hospitalised for COVID-19 in the last 4 months.
- You are continuing to suffer from symptoms associated with "Long-COVID".

Location

Erasmus Darwin building, Room 256, Clifton Campus, Clifton Lane, Nottingham Trent University, NG11 8NS.

Restrictions During Testing:

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.

Testing Protocol:

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 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 waist and hip circumference, height, body weight, and body mass index. 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 to complete the asthma questionnaires/scale. These will include a questionnaire to assess your perceptions of asthma control, and adherence to your asthma medication. The procedures for collecting the blood sample will be explained.

Experimental visit:

An overview of experimental procedures can be found below in figure 1.1.

UPON ARRIVAL	ASTHMA ASSESMENT	END
Check Health Status	Resting Lung Function	Questionnaires
Anthropometric measurement	Blood Sample	

START → **END**

- Measurements of body waist and hip circumference, height, body weight, BMI, and lung function will be conducted during each experimental trial.
- You will be asked to complete a questionnaire to assess asthma control, quality of life, and a scale to monitor asthma medication adherence.
- You will be asked to provide a resting blood sample.

On arrival to the laboratory, we will confirm that you have your asthma reliever medication with you. You will then complete health screen and health history questionnaires and provide written consent for us to take a blood sample. We will then measure your body weight and lung function and take a blood 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 use.

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 100 ml of blood will be taken during each visit. This will be used to assess markers of inflammation and gut permeability. 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.

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 (FEV1); peak expiratory flow (PEF).

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

After the 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.

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.

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.

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

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Appendix 2a

Participant Statement of Consent to Participate in the Investigation Entitled:

"Investigating NGAL and markers of gut permeability and damage across asthma severity and adiposity – A WATCH Cohort Collaboration Study".

1) I, *[……]* agree to partake as a participant in the above study.
2) I understand from the participant information sheet (Dated… Version…), which I have read in full, and from my discussion(s) with *[Neil Williams and/or Cristina Parenti]* that this will involve me *[performing pulmonary function and have blood samples taken. Also, height, weight, BMI, hip/waist ratio and body composition will be measured during one visit to the laboratory]*
3) It has also been explained to me by *[Neil Williams and/or Cristina Parenti]* that the risks and side effects that may result from my participation are as follows: *Slight discomfort may occur during venepuncture, all investigators taking blood samples however will be fully trained and will take the up most care.*
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 and that my medical care or legal rights will not be affected.
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, anthropometric data and pulmonary function data.]* will be handled in accordance with this policy.
9) 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.
10) If appropriate) I understand that the information collected about me will be used to support other research in the future and may be shared anonymously with other researchers.
11). (If appropriate) I agree to my General Practitioner being informed of my participation in the study. / I agree to my General Practitioner being involved in the study, including any necessary exchange of information about me between my GP and the research team.
12) It has been explained to me that there may be additional risks arising from the current COVID pandemic. I have read the NTU recommendations for undertaking 'Research with human participants' and undertake to abide by the special measures which have been explained to me for this study together with such Government Guidelines that are at the time prevailing.

Participant signature: Date:

Independent witness signature: Date:

Primary Researcher signature: Date:

Appendix 2: Prebiotic trial 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 *Lactobacillus*, 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 dexa body scan, 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.

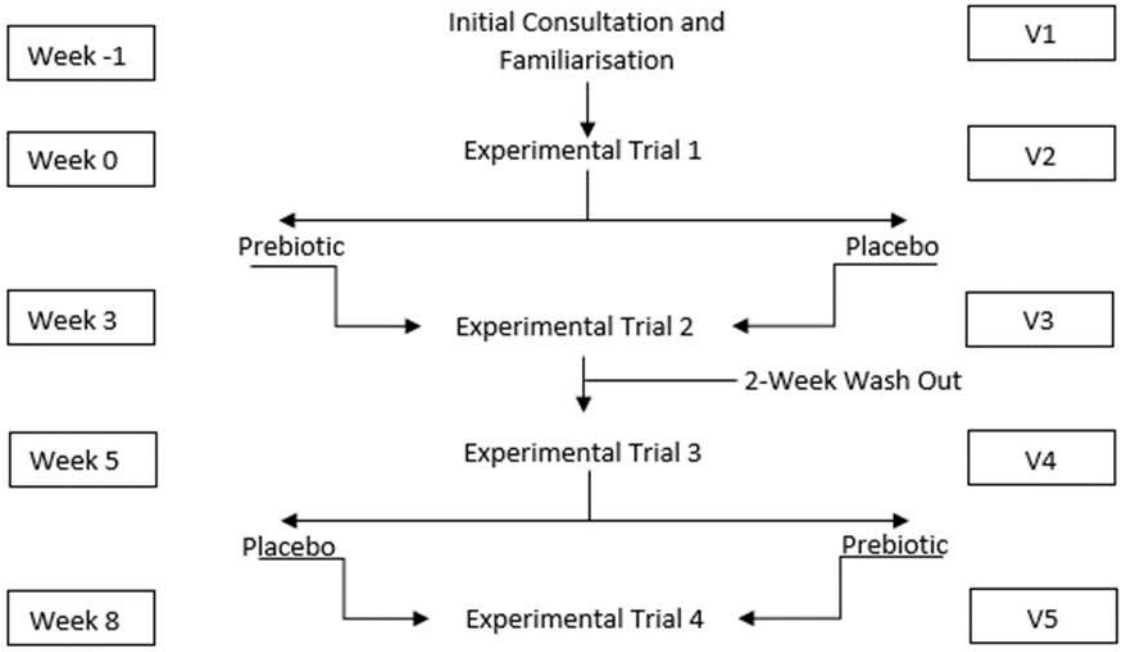

Figure 1.0 Schematic diagram illustrating study design and duration. V = Visit Number.

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	

- 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.

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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 Galacto-oligosaccharide 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 nor 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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Contacts:

Appendix 2a

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 separates 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 3: Self-reporting Health screen

Health screen

Name or Number .....

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present,** do you have any health problem for which you are:

(a) on medication, prescribed or otherwise Yes ☐ No ☐

(b) attending your general practitioner Yes ☐ No ☐

(c) on a hospital waiting list Yes ☐ No ☐

2. **In the past two years,** have you had any illness which require you to:

(a) consult your GP Yes ☐ No ☐

(b) attend a hospital outpatient department Yes ☐ No ☐

(c) be admitted to hospital Yes ☐ No ☐

3. **Have you ever** had any of the following?

(a) Convulsions/epilepsy Yes ☐ No ☐

(b) Asthma Yes ☐ No ☐

(c) Eczema Yes ☐ No ☐

(d) Diabetes Yes ☐ No ☐

(e) A blood disorder Yes ☐ No ☐

(f) Head injury Yes ☐ No ☐

(g) Digestive problems Yes ☐ No ☐

(h) Heart problems Yes ☐ No ☐

(i) Problems with bones or joints Yes ☐ No ☐

(j) Disturbance of balance / coordination Yes ☐ No ☐

(k) Numbness in hands or feet Yes ☐ No ☐

(l) Disturbance of vision Yes ☐ No ☐

(m) Ear / hearing problems Yes ☐ No ☐

(n) Thyroid problems Yes ☐ No ☐

(o) Kidney or liver problems Yes ☐ No ☐

(p) Allergy to nuts, alcohol etc. Yes ☐ No ☐

(q) Any problems affecting your nose e.g. recurrent nose bleeds Yes ☐ No ☐

(r) Any nasal fracture or deviated nasal septum Yes ☐ No ☐

4. **Has any,** otherwise healthy, member of your family under the age of 50 died suddenly during or soon after exercise? Yes ☐ No ☐

5. Are there any reasons why blood sampling may be difficult? Yes ☐ No ☐

6. Have you had a blood sample taken previously? Yes ☐ No ☐

7. Have you had a cold, flu or any flu like symptoms in the last Month? Yes ☐ No ☐

8. Have you ever tested positive for COVID Yes ☐ No ☐

Women only (delete if not applicable)

8. Are you pregnant, trying to become pregnant or breastfeeding?Yes ☐ No ☐

If YES to any question, please describe briefly if you wish (e.g. to confirm problem was/is short-lived, insignificant or well controlled.) .....
.....
.....

COVID-19

1. Have you ever been exposed to someone with documented or suspected COVID-19 infection (such as co-workers. Family members or others)? Yes ☐ No ☐

2. Have you ever been tested with COVID-19? Yes ☐ No ☐

3. If so, how this test was performed? (If you never been tested, please jump to question n.16)

(a)A swab in your nose or throat Yes ☐ No ☐

(b)A spat in a cup/tube Yes ☐ No ☐

(c)A finger-prick blood test Yes ☐ No ☐

(d)A blood test, done using a needle Yes ☐ No ☐

Other, please specify…….

14. Where was your test performed?

(a)At home Yes ☐ No ☐

(b)Drive-through regional testing centre Yes ☐ No ☐

(c)Hospital (not drive through) Yes ☐ No ☐

(d)GP Yes ☐ No ☐

(e)Chemist/Pharmacy Yes ☐ No ☐

Other, please specify…….

15. What was the result of this test?

(a)Negative Yes ☐ No ☐

(b)Positive Yes ☐ No ☐

(c)Not clear/failed Yes ☐ No ☐

16. Have you been hospitalized for COVID-19? Yes ☐ No ☐

17. If so, please specify the duration of your stay……

18. **If you had COVID-19**, which symptoms did you experience?

(a) Fever Yes ☐ No ☐

(b) Fells chills or shivers Yes ☐ No ☐

(c) Persistent cough (coughing a lot more than an hour or 3 more coughing episodes in 24 hours) Yes ☐ No ☐

(d) Unusual fatigue Yes ☐ No ☐

(e) Headache Yes ☐ No ☐

(f) Felt nausea or experience vomiting Yes ☐ No ☐

(g) Dizziness or light-headedness Yes ☐ No ☐

(h) Unusual shortness of breath or have trouble breathing Yes ☐ No ☐

(i) Sore or painful throat Yes ☐ No ☐

(j) Loss of smell/taste Yes ☐ No ☐

(k) Unusually hoarse voice Yes ☐ No ☐

(l) Unusual chest pain or tightness in your chest Yes ☐ No ☐

(m) Unusual abdominal pain or stomachache Yes ☐ No ☐

(n) Diarrhoea Yes ☐ No ☐

Other symptom, please specify……..

19. How long did you experience symptoms? Please specify.
……

20. **At moment,** do you experience any of the symptoms? Yes ☐ No ☐

21. **If so,** please specify which one…….

22. If you have been previously diagnosed with COVID-19, are you currently experiencing illness associated with “Long COVID” and continuation of symptoms highlighted above in question 19?

……

ASTHMA SPECIFIC QUESTIONNAIRES

23. Age of diagnosis:…….
24. Have you ever been hospitalized for Asthma? Yes ☐ No ☐
25. Have you ever been diagnosed with Rhinitis? Yes ☐ No ☐
26. Have you ever been diagnosed with GORD? Yes ☐ No ☐
27. Have you ever been diagnosed with Depression? Yes ☐ No ☐
28. Have you ever been diagnosed with anxiety? Yes ☐ No ☐
29. Have you ever been diagnosed with Dysfunctional Breathing? Yes ☐ No ☐
30. Have you ever been diagnosed with VCD? Yes ☐ No ☐
31. Asthma medication:

    Name:…….

Dose……

Appendix 4: Questionnaires

Appendix 4.1: Asthma control questionnaire (ACQ-6)

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?

   0Never

   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?

   0No 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?

   0Not 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?

   0None

   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?

0Not 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?

0None

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 4.2: Nijmegen questionnaire

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 4.3: 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 4.4: SNOT-22 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 4.5: 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 4.6: Epworth 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.