Investigating the Gut-Lung Axis

Metabolic Profiling and the Effect of SCFAs on Airway Epithelium in Healthy and Asthmatics

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Declaration of Originality

I certify that this thesis, and the research to which it refers, are the products of my own work, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard referencing practices of the discipline.

The following samples or dataset were provided by the other people:

- Collection of bronchoalveolar lavage and sputum samples were performed by Professor Sebastian Johnston's lab.
- 16s rRNA data was collected and sequenced by Professor Sebastian Johnston's, Professor Miriam Mofatt's and Professor William Cookson's lab.
- Batch culture samples were collected by Professor Miriam Mofatt's and Professor William Cookson's lab.
- The culture and treatment of Th2 skewed PBMC cells were performed by Sebastian Holvoet from Nestlé Research Center.
- The samples from the WESSEX severe asthma cohort were collected by the University of Southampton and ^{1}H NMR spectroscopy and UPLC-MS profiling were performed by the National Phenome Centre.
- Collection of plasma and urine samples for the asthma fibre intervention study were performed by Nottingham Trent University.

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Abstract

Asthma is a chronic inflammatory disease characterized by decline in lung function, goblet cell metaplasia and tissue remodeling. Recent years have demonstrated the interplay between the airways and the gut microbiota, showcasing the dampening of airway inflammation by gut-microbial metabolites short-chain fatty acids (SCFAs) in rodent models. No studies have investigated the presence and effect of SCFAs in the airways of asthmatics and limited studies have investigated the metabolic profile of asthmatics and the effect of fibre supplementation.

We demonstrate the presence of SCFAs (mainly acetate and propionate) in the airways of healthy controls and asthmatics, with an increase in acetate in asthmatics. The levels of SCFAs detected in the airways exceeded what has previously been described in peripheral blood. SCFA concentrations in asthmatic airway were correlated with specific airway microbial genera, suggesting a possible production of SCFAs by the airway microbiota. This was further confirmed by *in vitro* batch culture, where airway bacterial species produced higher amount of SCFAs in the presence of mucin, indicating proteolytic activity by the airway microbiota. In order to determine the direct effect, which local SCFA level could have on the airway epithelium, the effect of acetate and propionate were investigated on primary airway epithelium. Both SCFAs promoted a modulation of cytokine gene response upon, stimulation on both apical and basolateral side, which was further enhanced by IL-13 treatment. These results suggest that the microbial-airway-derived SCFAs promote a local change in the inflammatory response *in vitro*, which is different to the response seen with gut-microbial-derived SCFAs in preclinical data.

Furthermore, the metabolic profile of asthmatics was investigate using ^{1}H NMR spectroscopy and UPLC-MS, in order to identify potential urinary biomarkers that would aid in the diagnosis of asthma and sub-populations of asthma. Several urinary biomarkers were found to be either elevated or decreased in asthmatics belonging to purine, polyamine and fatty acid metabolism. Interestingly, a panel of carnitines were able to distinguish between sub-population of severe asthmatics. Lastly, investigating the effect of prebiotic supplementation to individuals with exercise-induced asthma, did not generate any statistically significant effect on SCFAs concentrations nor lung function, possibly due to the low levels of prebiotic supplementation.

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~To my entire family~

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Abbreviations

^{1}H	Hydrogen or proton
ACQ	Asthma Control Questionnaire
ANOVA	Analysis of Variance
AUC	Area Under Curve
BCAA	Branched Chain Amino Acid
BCFA	Branched Chain Fatty Acid
B-GOS	Bimuno-Galacto-Oligosaccharide
BAL	Bronchoalveolar Lavage
BMI	Body Mass Index
CV	Coefficient of Variation
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Damage-Associated Molecular Pattern
ELISA	Enzyme-Linked Immunosorbent Assay
FENO	Fractional Exhaled Nitric Oxide
FEV1	Forced Exhaled Volume in 1 second
FVC	Forced Vital Capacity
FFAR	Free-Fatty Acid Receptor
GC	Gas Chromatography
GM-CSF	Granulocyte-Macrophage Colony –Stimulating Factor
HDAC	Histone Deacetylase
HDM	House Dust Mite

IBD	Inflammatory Bowel Disease
ICAM-1	Intercellular Adhesion Molecule-1
IFN	Interferon
IgE	Immunoglobulin type E
IL	Interleukin
ILC	Innate Lymphoid Cell
LABA	Long-Acting Beta-Agonists
LC	Liquid Chromatography
MHC	Major Histocompatibility
mM	Milimolar
µM	micromolar
mRNA	messenger RNA
MS	Mass Spectrometry
MSI	Metabolomics Standards Initiative
MUC5AC	Mucin 5AC
m/z	mass-to-charge ratio
NMR	Nuclear Magnetic Resonance
OPLS	Orthogonal Projections to Latent Structures
OPLS-DA	Orthogonal Projections to Latent Structures – Discriminant analysis
PAG	Phenylacetylglutamine
PAMP	Pathogen-Associated Molecular Pattern
PAR	Protease-Activated Receptor
PCA	Principal Component Analysis

PLS	Projections to Latent Structures
PLS-DA	Projections to Latent Structures – Discriminant analysis
PRR	Pattern Recognition Receptor
qPCR	Polymerase Chain Reaction
SABA	Short-Acting Beta-Agonist
SCFA	Short Chain Fatty Acid
STOCSY	Statistical Total Correlation Spectroscopy
Th2	T-helper type 2
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
TOF	Time Of Flight
Tregs	T regulatory cells
TSLP	Thymic Stromal Lymphopoitein
UPLC	Ultra-Performance Liquid Chromatography
VIP	Variable Importance in Projection
WHO	World Health Organization

1 BACKGROUND

1.1 Asthma

The estimation of asthmatics around the world, according to World Health Organization (WHO) is 235 million people, where 8 million (12%) of UK's population have asthma [3-5]. Asthma is considered to be a public health problem affecting the whole world regardless of income or level of development [4]. One of the many challenges concerning asthma, are those due to the variation in disease expression and the many factors associated with its aetiology or severity such as ozone, nitric dioxide and air traffic associated pollution [6]. However, there is also a genetic factor that contributes to the development of the disease, with several human genes having been associated with the disease [7]. The complexity and heterogeneity of asthma have resulted in challenges in curing the disease, and asthmatics have to rely on medication to ameliorate the symptoms in the patient's everyday life. Furthermore, the economic burden of asthma is enormous: in the UK, 13 billion pounds are spent annually by the government [8, 9]. It is therefore not a surprise that asthma mortality is higher in low and middle-income countries compared to high-income countries [3].

Asthmatics exhibits different phenotypes due to several different factors and it is therefore important to diagnose asthma using several different invasive clinical tools. A common form of measuring asthma severity is by assessing lung function through spirometry. The volume of air is measured during 1 second (FEV1) and then compared to amount of total air exhaled in one full breath [10]. The same measurements are repeated but the patient is given some form of bronchodilator to assess reversibility of lung function. Final measurements are taken, to assess the level of inflammation in the airways, by measuring fractional exhaled nitric oxide concentration

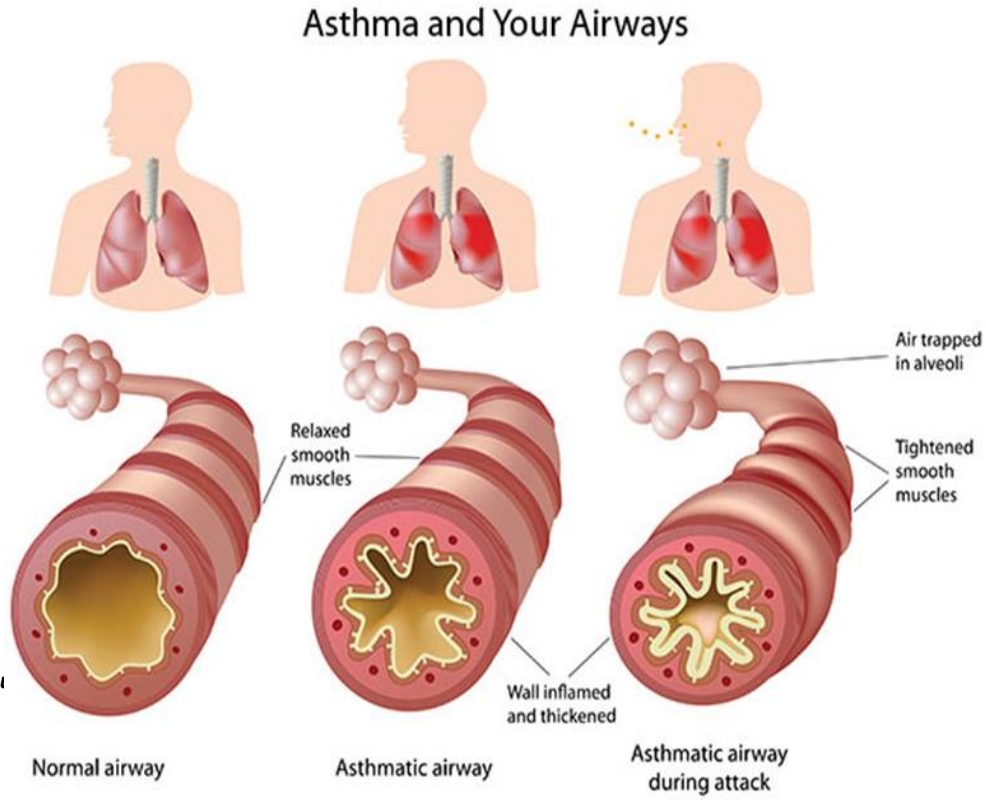
(FE_{NO}). However, these measurements only demonstrate the function and capacity of the lungs and do not define any further phenotypes, such as allergic asthma or adult asthma onset. A skin prick test, with specific allergens, [11] can determine if the subject have allergic asthma, or atopy, which is the majority of asthmatic patients and characterized by an local immunoglobulin type E (IgE) response. In addition to these measurements, other factors such as gender, age, childhood asthma onset and BMI can influence the phenotype of asthma [12, 13].

1.1.1 Asthma Symptoms and Medications

Asthma is a heterogeneous disease with symptom variation within patients as well as between patients, resulting in difficulty targeting the disease through a single mechanism. Common symptoms include wheezing, breathlessness, tight chest and coughing, Symptoms often occur with a peak in the morning and one in the evening. An increase of these symptoms can trigger "asthma attacks", where the patients experience increased heartbeats, dizziness, breathlessness and fainting [14].

Asthma is a chronic inflammatory disease and with time can be characterized by a decline in airway function caused by tightening of smooth muscles, swelling of the bronchial tubes and tissue remodelling [12, 15]. During an asthma attack, the swelling of the airways causes a reduction of airflow in and out of the lungs. There is no complete cure for asthma, however, several drug treatments are available to maintain and relieve asthma symptoms [16-18]. Inhalers used to relieve asthma symptoms contain short-acting beta-agonists (SABAs) and function by rapidly widening the airways making breathing easier. Long-acting beta-agonists (LABAs), or corticosteroids, act to prevent asthma attacks and chronic asthma symptoms by reducing inflammation and sensitivity

in the airways [14]. If the asthma symptoms are severe, oral corticosteroids are often prescribed in combination with previously mentioned medication.



Figu

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1.1.2 Molecular Mechanism and Immunology of Asthma

The severity of asthma can differ between patients, as some have a mild and easily maintained form, while others have a quite severe case where treatments are no longer effective and lung damage is starting to manifest [19]. The abnormal signalling of the immune system, in response to harmless allergens, is one of the reasons behind the development of asthma. Allergic asthma, or atopic asthma, is thought to be a complex disorder involving different pathways with focus on the activation of the allergen-specific Th2 cells and associated cytokines [20]. Atopic asthmatic individuals are thought to have fewer and less functional T regulatory cells (Tregs) [21]. The abnormal signalling of the Tregs, which are responsible for maintaining immune homeostasis in the body, leads to the activation of Th2 cells, which release a variety of cytokines, such as IL-4, IL-5 and IL-13 (figure 2). In normal circumstances, the immune response to an allergen is supposed to be inhibited by the Tregs, whose job is to prevent self-reactivity by keeping the inflammatory markers at a low level [21].

In atopic, or allergic asthma, the allergen comes into contact with surface epithelial layer, which expresses many pattern recognition receptors (PRRs) and responds to different stimulations [22]. Pathogen-associated molecular patterns (PAMPs) respond to microbes, while damage-associated molecular patterns (DAMPs) respond to cell death, tissue damage etc. Other PRRs include NOD-like receptors, C-type lectins and protease-activated receptors (PAR) [23]. House dust mite (HDM) contains allergen that can activate PAR on the epithelium and cause breakage of the tight junctions and damage to the epithelial barrier and subsequently emit an immune response, mimicking allergic asthma [24]. HDM can also activate Toll-Like Receptors (TLRs) which are the most well-studied PRRs in asthma [25].

Upon stimulation of a TLR response, either by an allergen, a cascade of signals emits throughout the cell. Dendritic cells present the allergen antigen through the major histocompatibility (MHC) complex favoring excessive production of the Th2 cytokines IL-4, IL-5, IL-6, IL-9 and IL-13 [20] through differentiation of naïve T cells. These cytokines are involved in activation and differentiation of IgE cells from B lymphocytes (IL-4), increased levels of eosinophils (IL-5), mucus production (IL-9) and airway hyperactivity (IL-13), which directly target the airway epithelium [2, 26].

Another major player in allergic asthma is Thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine, and IL-25 and IL-33, which are secreted by the epithelium upon exposure to allergens or viruses, due to stress or damage [27]. Following secretion, these epithelial alarmins, which have an immunomodulating property drive an allergic Th2 response and are potent activators of mast cells. A subgroup of innate lymphoid cells (ILC), ILC2, are also activated by these epithelial alarmins, and release copious amount of IL-5 and IL-13, recruit eosinophils, promote mucus production and initiates airway hyperresponsiveness [28, 29]

Th2 mediated response is seen in patients with mild to moderate asthma, corresponding to 50% of the asthma population, and has been termed eosinophilic endotype of asthma or atopic asthma [2].

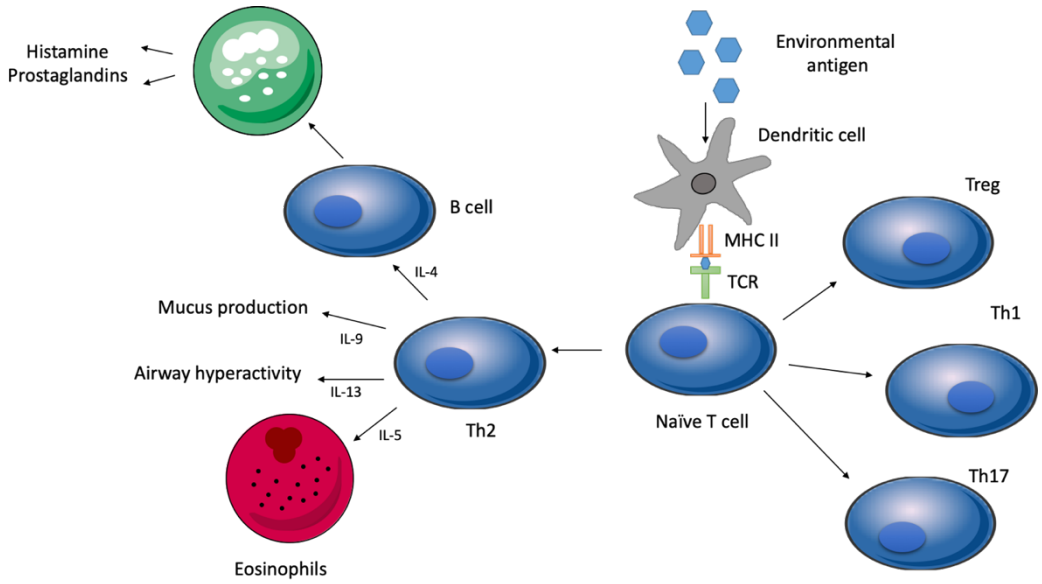


Figure 1.2. Pathway and immune cells involved in eosinophilic asthma.

When in contact with environmental allergens an inflammatory response is emitted and in this case a Th2 mediated response. DC presents the antigen through MHC complex and production and release of certain interleukins is mediated. The interleukins drive the production of eosinophils and IgE drives the production of mast cells, which in turn release several granulocytes promoting the development of chronic inflammation. Figure adapted from [2].

The non-eosinophilic endotype of asthma regulates a macrophage- and neutrophil-dependant pathway resulting in activation of an innate immune response with Th1 and Th17 activation. This pathway is upregulated in steroid-resistant patients [2] and is not clearly understood. The release of CXCL8 is involved in recruiting neutrophils and macrophages, which release pro-inflammatory cytokines such as IL-1β, IFN-γ, TNF-α and GM-CSF. IL-17 is another cytokine involved in non-eosinophilic asthma during neutrophilic inflammation (figure 3) [30]. This consequently leads to structural changes of the airway epithelium, mucus metaplasia and changes in airway smooth muscle mass [2, 21, 31-33]. It is also important to note that the epithelial layers of the airways play an important role as a chemical, immunological and physical barrier in asthma inflammation and are considered to be the first line of defence against allergens.

The impairment of Tregs is of great importance, as the self-reactivity of the immune response is not controlled, resulting in a modulation of both atopic and non-atopic asthma. As previously mentioned, asthmatics are thought to have impaired function and a decreased number of Tregs [21, 34] when compared to non-asthmatics. The transcription factor FOXP3 is constitutively expressed in CD4+CD25+FOXP3+ Tregs and maintains immune homeostasis [35, 36].

However, it is important to note that multiple factors contribute to the development of asthma and continuous research has demonstrated the complexity of the disease where the line between allergic asthma and non-allergic asthma is diminishing.

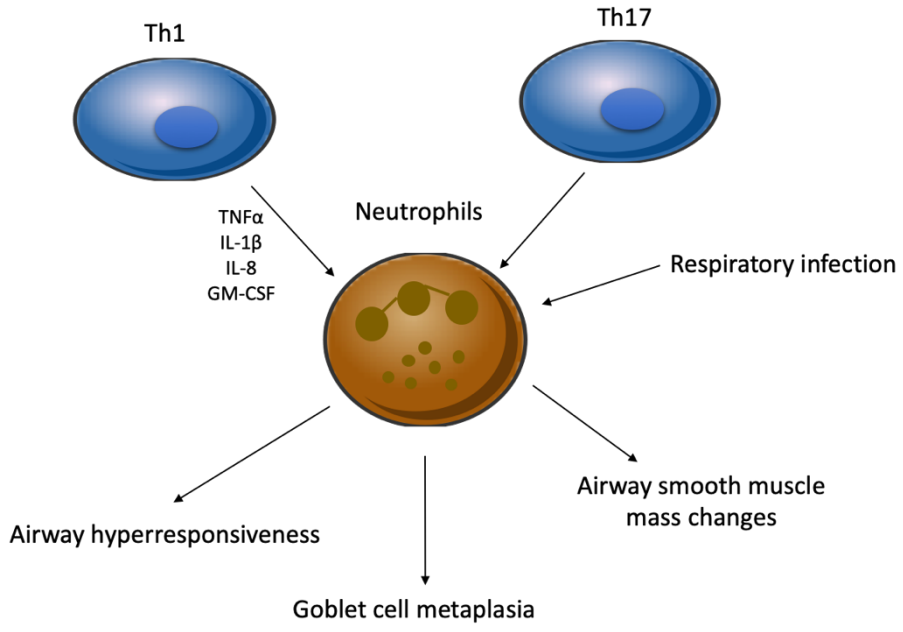


Figure 1.3. Pathway and immune cells involved in non-eosinophilic asthma.

Pathway of non-atopic asthma with increased number of macrophages and neutrophils driving the release of pro-inflammatory cytokines to tissue remodelling. Figure adapted from [2].

1.2 Human Microbiota

The human body supports in the region of 100 trillion microorganisms, 10 times more than human cells, and encodes for 100 times more unique genes than the human genome [37, 38]. Bacteria, archaea, viruses and eukaryotic microbes are all part of the human microbiome, where the bacteria are the most abundant microbes and the ones, which have caught the most attention. The bacteria are found at various locations in the human body, forming a sound ecosystem, which plays a role in human nutrition, health and overall host physiology. Disruption, or major alteration, of the microbial ecosystem will increase susceptibility for a large variety of diseases, resulting in dysbiosis [38].

The acquisition of microbiota starts the moment the infant is delivered [39], or more recently there has been suggestion of some *in utero* acquisition [40]. The colonic microbial composition is associated with mode of delivery (vaginal or caesarean), diet (breast-fed or formula-fed), mother's microbiota [41-43], and will continue to develop until the child reaches the age of 3-4 years [44] after, which the microbiome is thought to be more or less stable. During this early time in life, other factors such as sanitation, use of antibiotics and diet can have prolonged effects on the diversity of the colonic microbial composition, possibly resulting in changes in the immune response, host physiology and the overall health of the child [45].

1.2.1 The Gut Microbiota

The gut microbiota has been studied extensively and has been well-characterized. It is dominated by two phyla, bacteroidetes and firmicutes, with minor phyla present at much lower abundances such as actinobacteria, cyanobacteria, fusobacteria and proteobacteria [38, 46]. The gut microbiota

works in a symbiotic relationship together with the host to regulate host metabolism and immune response, and has been found to play a major role in the pathogenesis of non-communicable diseases such as asthma [47], obesity [48], inflammatory bowel disease (IBD) [49] and diabetes [50]. Many factors, such as diet and environmental factors, can contribute to dysbiosis and alter the richness and abundance of the phyla present in the gut. Clear differences can be observed when comparing the gut microbiota between populations from westernized, African and Mediterranean countries [51, 52], where diets are extremely different between countries. The microbiota in the human body is responsible for regulation of several key metabolic pathways and metabolite generation, including amino acid synthesis, bile acid biotransformation, vitamin production and short-chain fatty acid (SCFA) production [53, 54]. Studies have confirmed a dysfunctional gut microbiota in people with atopic diseases, such as asthma, and further demonstrated that certain microbial species regulate Th1/Th2 balance and the induction of Tregs, which have a major implication in asthma [55]. Furthermore, a change in microbial composition, with an increase in pathogens especially in early life, contributed to the development atopic diseases, food allergy and asthma [56].

1.2.2 The Airway Microbiota

The lungs, have for a long time, been considered to be sterile even though they are constantly exposed to the environment. However, several recent studies have now clearly demonstrated the existence of a distinct microbial population in the airways, which differs in healthy and diseased lungs [57-61]. The lungs, like the gut, are mucosa-lined organs of similar embryological origin, however there is a clear difference in population and dynamics of the microbiota in these two sites. The inhalation and elimination of microorganism form the lungs and regional growth conditions

can determine the airway microbiota, which is lower in total number and diversity compared to the gut microbiota [62]. During respiratory disease processes, such as asthma, an increase in community diversity is seen together with an increase in the relative number of Proteobacteria [59, 61].

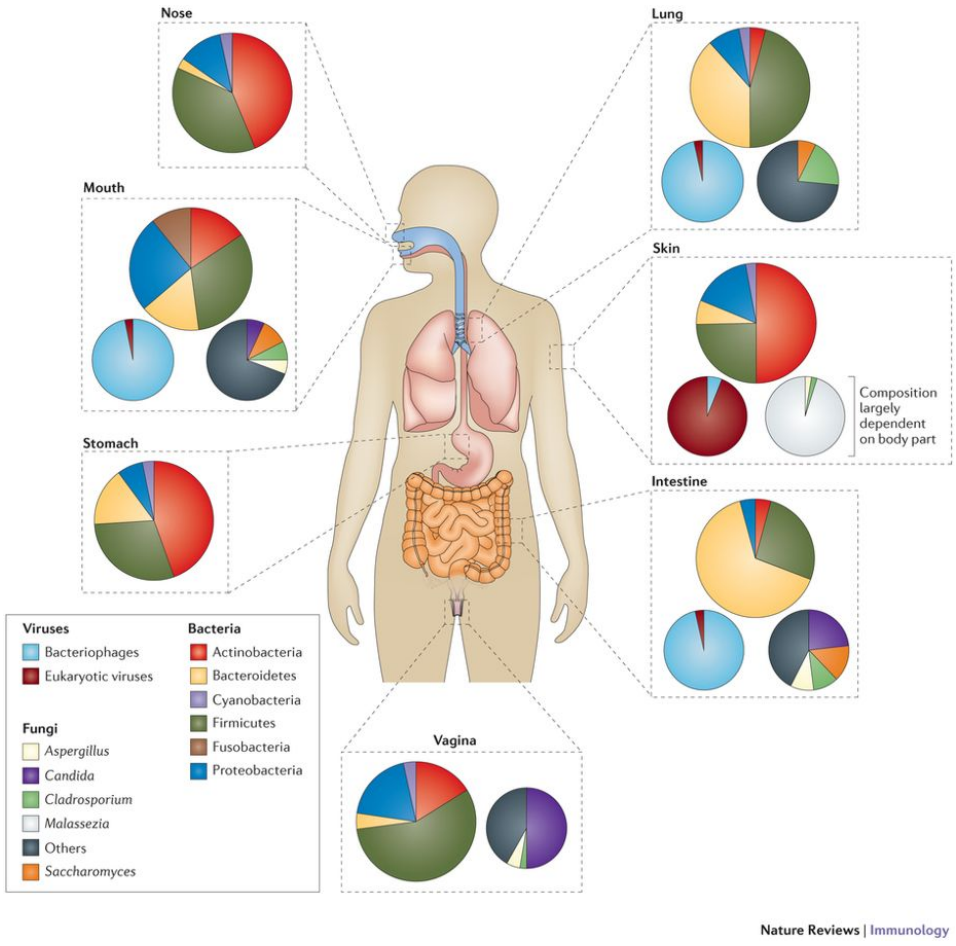


Figure 1.4. The human microbiome.

Microbiome composition is dependent on organ, or body site, and will differ topographically throughout the human body (Figure adapted from [63], with permission).

1.3 The Airway Epithelium

The airway epithelium is the interface between the host and the external environment and the first site of contact for inhaled allergens [23, 64-66]. It is therefore of tremendous importance that the airway epithelium is functioning properly to correctly mediate the transport of the ions, water and fluids to the blood. Furthermore, the airway epithelium is responsible for the production of antimicrobial peptides, proteins, cytokines and chemokines [67] and expresses many pattern recognition receptors (PRRs), whose job is to quickly detect foreign molecules that are released upon cellular stress, damage or death and emit a response [23]. The epithelial barrier prevents allergens and other harmful inhaled substances from entering the airway tissue by communicating with the immune system. It is also of great importance to note that the airway epithelium does not work by itself but rather in conjunction with mesenchymal cells, endothelial cells and the bronchial wall to maintain airway homeostasis and provide air to and from the alveoli [64].

The airway epithelium consists of four major cell types, basal cells, goblet cells, club cells and ciliated cells, in different proportions depending on the location within the lungs (figure 4). Basal cells, which are located closest to the basal lamina, are involved in regeneration upon injury and also serve as a population of stem cells [66]. Club cells are involved in the protection of the bronchial lining by secreting small molecules as well as by cleansing the airways from harmful inhaled substances. Mucus, produced by goblet cells, has the ability to remove pathogens by trapping them in the mucus and removing it by the movement of the ciliated cells.

In asthma, the epithelial lining is damaged (airway remodelling) and it is unable to repair itself resulting in higher susceptibility to allergens [68]. Furthermore, there is an increase in goblet cell

numbers (goblet cell metaplasia) issued from transdifferentiation of ciliated and club cells into goblet cells [23] and resulting in excessive mucus production. Junctional proteins and cytoskeleton, such as E-cadherin, ZO-1 and occludin, holding the epithelial lining, are downregulated in patients with asthma resulting in an impaired barrier [69]. Studies have demonstrated that there are associated genes responsible for integrity and repair of the epithelial layer [31, 32, 70].

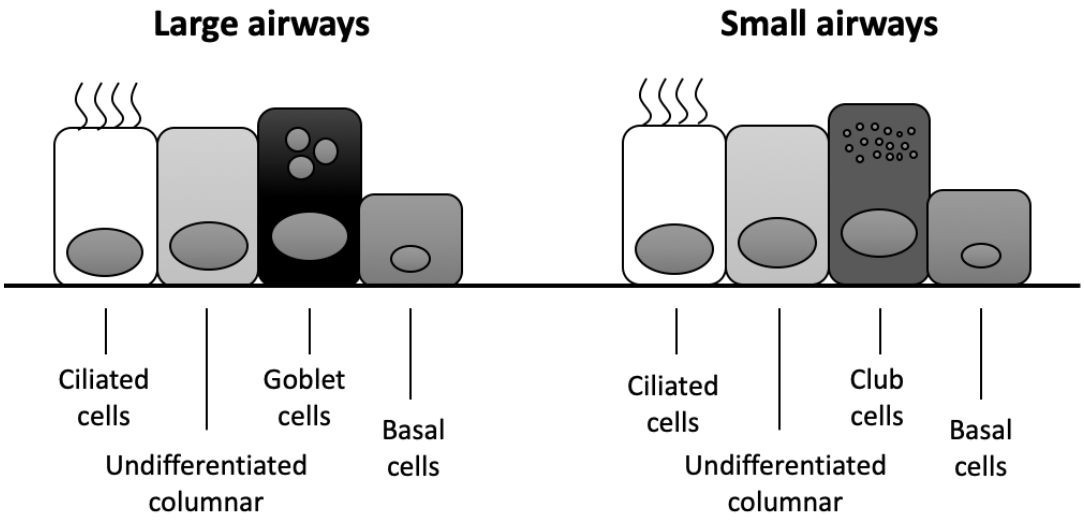


Figure 1.5. Cell composition and structure of the human airways.

The differentiated epithelium will consist of different types of cells depending on the location within the airways. Figure adapted from [64].

1.4 Diet, Fibre and Short Chain Fatty Acids (SCFAs)

Studies investigating the relationship between asthma and diet have found results suggesting that lower prevalence of asthma is associated with diets high in dietary fibre, such Mediterranean diet [71, 72]. In contrast, increased prevalence of asthma has been observed in countries with a westernized diet, which is high in fats and low in dietary fibre [73]. Overall, a decrease of fibre consumption has been observed worldwide [74], which in turn has been shown to increase the prevalence of metabolic disorders [51, 75-79]. The majority of the consumed food is chemically and enzymatically digested and the nutrients are absorbed in the small intestine and further distributed to other organs or tissues. However, humans lack the enzyme required to digest fermentable carbohydrates, such as dietary fibres. This allows dietary fibre to pass unaffected through the small intestine until they reach the colon. The gut microbiota, primarily present in the colon, will ferment fibre to gain ATP as energy, and in turn produce short-chain fatty acids (SCFAs) as waste products to balance redox reactions [44, 78].

SCFAs are fatty acids with aliphatic chains up to 5 carbons, where the most abundant ones are acetate (C2), propionate (C3) and butyrate (C4). Acetate is the SCFA found at highest concentration in peripheral blood, while propionate is readily absorbed by the liver and butyrate by the colonocytes [78]. The concentrations of acetate, propionate and butyrate are approximately 51 mM, 14 mM and 14 mM [80] in faeces, while much lower concentrations are found in peripheral blood, 70 μM, 5 μM and 4 μM respectively [81]. SCFAs are anti-inflammatory [82], promote epithelial integrity [71] and regulate the size and pool of T regulatory cells in the colon [83]. Acetate and propionate are predominately produced by species from the *Bacteroidetes* phylum, which is the phylum dominating the gut microbiota, while butyrate is produced by species

from the *Firmicutes* phylum. Furthermore, studies have shown that a high fibre diet can increase the abundance of *Bacteroidetes* and reduce the abundance of the *Firmicutes* phylum. In contrast, a low-fibre diet was dominated by species from *Proteobacteria* phylum [60, 84].

1.4.1 Formation of SCFAs

The formation of SCFAs have a common pathway (figure 6), derived from non-fermentable carbohydrates that are either hydrolysed through glycolysis or the pentose-phosphate-pathway, depending on the number of carbons in the carbohydrate [77, 85]. The formation of acetate has two possible pathways, either through hydrolysis of acetyl CoA (derived from non-fermentable carbohydrates and is the most common one) or through the Wood-Ljungdahl pathway (one third of acetate is produced from this pathway) that converts carbon dioxide to carbon monoxide, which is then methylated to form acetate [86-88]

Propionate can be produced through 3 different pathways and all three of them are dependent on different types of bacteria (figure 6) [89]. The most common pathway is through the succinate carboxylate pathway, where phosphoenolpyruvate (PEP) gives rise to oxaloacetate, which continues through several steps to succinate, methyl malonate and finally propionate. This pathway exists in species from *Firmicutes* and *Bacteriodetes* families. The acrylate pathway, present in *Veillonellaceae* and *Lachnospiraceae* utilises lactate, which is fermented to acrylate and then propionate, while in the propanodiol pathway, present in *Proteobacteria* and *Lachnospiraceae* family, deoxy sugars are converted to propionaldehyde, propionyl-CoA and finally propionate [89, 90]. The most common pathway to form butyrate, is through conversion of butyryl-CoA to butyrate through the acetyl-CoA pathway, which also produces acetate [78, 86, 89].

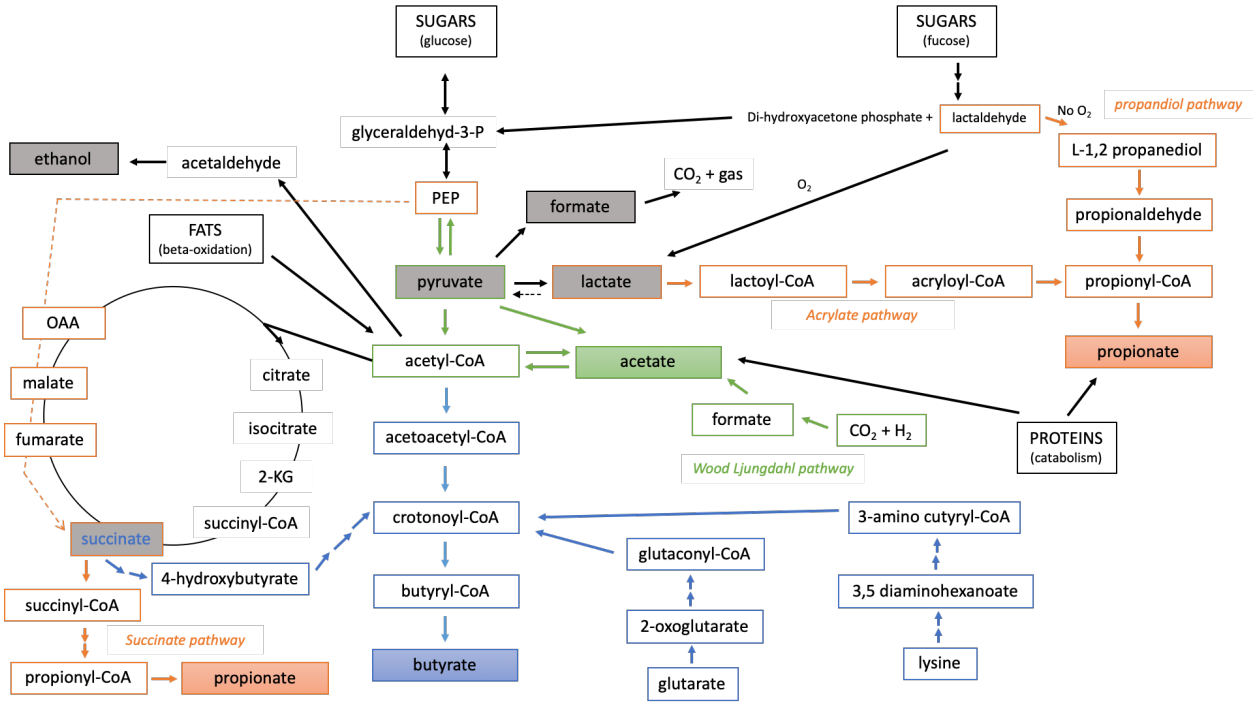


Figure 1.6. Metabolic pathway of end-products acetate, propionate and butyrate.

Acetate can be produced through two pathways, either through hydrolysis of acetyl CoA or through the Wood-Ljungdahl pathway. Propionate also has two pathways, succinate carboxylate pathway, the most common one, and the acrylate pathway. Butyrate is produced thought conversion of butyryl-CoA through the acetyl-CoA pathway. Figure adapted from [85].

1.4.2 Free Fatty Acid Receptor 2 (FFAR2) and Free Fatty Acid Receptor 3 (FFAR3)

The mechanisms of SCFAs are not fully understood, but it has been suggested that SCFAs operate through two principal mechanisms, G-protein-coupled receptors (GPR) via metabolite sensing and gene expression though inhibition of histone deacetylase (HDAC). It is thought that the receptor GPR41/FFAR3 (Free Fatty Acid Receptor 3) has a higher affinity for propionate and butyrate while GPR43/FFAR2 (Free Fatty Acid Receptor 2) for acetate and propionate [84, 91, 92]. Moreover, inhibition of HDAC by SCFAs regulates Foxp3 transcription and function of Tregs in suppressing airway inflammation [84, 93]. Another receptor that has shown to act as a ligand for SCFAs is GPR109A, which is expressed on the luminal side of the colonic epithelium, and immune cells, and has high affinity for butyrate and longer SCFAs [94]. Another receptor includes Olfr78, which is expressed on many tissues, but compared to FFAR2, FFAR3 and GPR109A, much is unknown about this receptor [95].

Activation of FFAR2 is primarily due to acetate and propionate followed by butyrate. Gene expression of *FFAR2* is mainly in intestinal endocrine L-cells, which stimulate the release of glucagon like peptide (GLP)-1 and peptide YY (PYY). GLP-1 and PYY are hormones associated with satiety and reduced hunger signalling to the brain and this has been shown both *in vivo* and *in vitro*, using mice and primary cells [96, 97]. FFAR2 is also expressed in adipose tissue and mice lacking the Ffar2-receptor were shown to develop obesity whilst those overexpressing Ffar2 developed a lean phenotype under normal circumstances. However, these phenotypes were not observed under germ-free conditions or when the mice were treated with antibiotics, indicating that microbial-derived SCFAs were necessary for the phenotype to establish and for maintaining

body energy homeostasis [82]. FFAR2 is also expressed on immune cells such as monocytes and B lymphocytes have been seen to regulate the size and pool of Treg cells [83] through HDAC.

FFAR3 shares 33% homology with FFAR2 when it comes to the amino acid sequence but differs in SCFAs affinity, physiological role and cell distribution [98]. FFAR3 is mainly activated by propionate and butyrate and to a lesser extent acetate. FFAR3, like FFAR2, is also expressed on L cells and involved in the release of GLP-1 and PYY and therefore in energy homeostasis. FFAR3 regulates the activity of the sympathetic nervous system (SNS) and therefore regulates the body energy homeostasis by sensing the nutritional state [88]. FFAR3 is also expressed on immune cells and propionate has been seen to alter bone marrow homeostasis and enhance the number of macrophages and dendritic cell precursors involved in the inflammatory pathway of allergic airway disease [60].

Furthermore, limited studies have investigated the presence of FFAR2 and FFAR3 on airway epithelia, but those who have, have shown a very clear presence of FFAR3 (figure 7b), compared to FFAR2 (figure 7a).

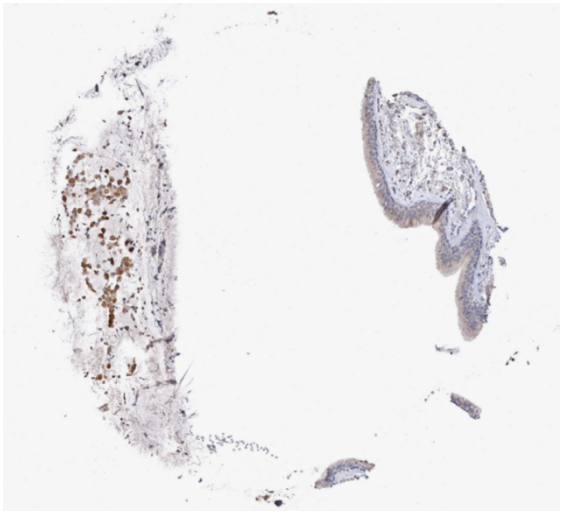
1.4.3 SCFA metabolism *in vitro*

It is well-established that SCFAs have anti-inflammatory properties and play a vital role in the maintenance of colonic homeostasis [71, 82, 83]. *In vitro* studies have demonstrated that pre-treatment of SCFAs, followed by induction of inflammation, inhibited the increase of cytokines and enhanced epithelial barrier in both epithelial cells and colonic cultures [99-103]. Other studies have also shown the suppression of

cytokines when treating macrophages with SCFAs, especially with butyrate [104-107].

However, there are limited studies investigating the effect of SCFAs on airway-derived cell types. In contrast to the results seen with gut-derived cell types, treatment of airway mesenchymal and epithelial cells with SCFAs demonstrated a pro-inflammatory response [108, 109].

a)



b)

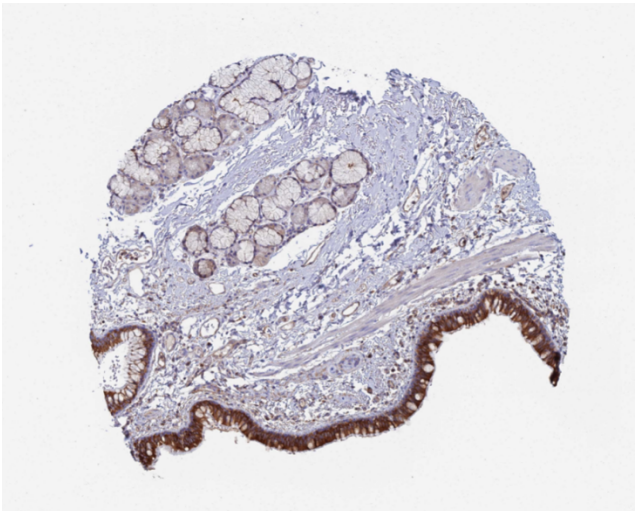


Figure 1.7. Location of SCFAs receptors on airway tissue.

Detection of a) FFAR2 and b) FFAR3 protein on airway epithelial cells. Image credit: Human Protein Atlas. Image available from v19.proteinatlas.org.

1.6 The Gut-Lung Axis

Even though much is known about dietary fibre and SFCAs in the gut, there are still many unknowns regarding the impact of SCFAs on other organs. In recent years, more focus has been on the interplay between the gut and the lungs, the gut-lung axis, in particular the relationship between dietary fibre and asthma. Recent studies have demonstrated that mice fed a high-fibre diet, or acetate and propionate directly, had a suppressed allergic airway inflammation when exposed to house dust mite (HDM, an allergen used to induce airway inflammation) compared to mice fed a low-fibre diet [60, 84]. The mice fed a high-fibre diet did not develop elevated IgE levels, increased eosinophils infiltration or increased cytokine Il-4, IL-5, IL-13 and IL-17A levels compared to the mice fed a low-fibre diet. Changes in gut microbiota were also prominent, where the abundance of high-SCFAs producing bacteria were increased. Other studies have confirmed the same protective nature of SCFAs, particularly propionate, while looking at dendritic cells, which exhibited an impaired ability to activate Th2 effector cells in the lung subsequently inhibiting the development of airway inflammation [60].

While these two studies focused on the impact of gut-derived SCFAs on asthma, neither made elaborate effort to investigate the presence or interaction of SCFAs within the airways. Two other studies investigated the presence of SCFAs in cystic fibrosis patients [110, 111], where acetate and propionate were detected in airway fluids, due to mucus degradation by the airway microbiota [110, 111].

Furthermore, a recent pilot study with asthmatics, demonstrated that one single dose of soluble fibre supplementation, during a meal, reduced airway inflammation after 4 hours, compared to

asthmatic patients with no fibre supplementation [112]. The patients in the study underwent exhaled nitric oxide, spirometry and sputum sampling, which compared to control participants indicated decreased levels of total cell count, neutrophils, macrophages, eosinophils and IL-8 suggesting reduced inflammation. Another study demonstrated improvement of pulmonary function after a 3-week supplementation of a daily dose of 5.5 g of Bimuno-galacto-oligosaccharide (B-GOS) in people with asthma compared to the control group. An attenuation in TNF-α after B-GOS supplementation was also observed [113].

Evidence clearly suggests an interaction between the gut and the lungs and studies have demonstrated the beneficial role of SCFAs in both people with asthma and rodent models. However, not much is known about the airways and what impact SCFAs might have on airway homeostasis.

1.7 *In vitro* Systems and Techniques

There are several limitations present when studying human physiology and pathology in *in vitro* models. Those limitations are mainly due to the inability to investigate what is happening on molecular and cellular level *in vivo*. Another issue is the lack of models to accurately model diseases and test the impact of small molecules and drugs, before they enter the market to ensure that they will not cause any harm to the host. Below is a summary of the *in vitro* systems that will be covered in this thesis.

1.7.1 Immortalized Cell Lines

Many of above-mentioned limitations have been overcome by utilizing cell-lines derived from different organs and hosts depending on research interest. These cell-lines have been genetically modified to never undergo cellular senescence, resulting in indefinite proliferation. This is an extremely useful way to test what compounds and drugs can do to part of host physiology *in vitro*, although there is an obvious need to extend these results to incorporate a system's view of the pathology. In addition, these cell-lines are pure cell population that are often very easy to maintain, very cost-effective, give reproducible results and consistent sampling. The use of cell-lines enables researchers to use, and look for, a wide range of application such as antibody generation, gene function, vaccine development, cell metabolic function, immunology, cell physiology and so forth. The ability to knock out, or silence, parts of the cell can generate a better understanding of the importance of different cell functions and the effect of an external stimulus on cell physiology.

However, there are many limitations present with cell-lines. The main problem is that when genetically modifying their genes, several properties are lost and the cells might start behaving

differently compared to the way they behave *in vivo* [114]. Another limitation is that that many cell-lines are generated from cancers and if the research is non-cancer based, it is difficult to use justify the use of those cells.

1.7.2 Human Primary Cells

The above-mentioned problem was solved by utilizing primary cells, which are cells directly derived from the host with no genetic manipulation. These cells give a better picture of what is happening *in vivo,* and the response observed from these cells when stimulated with certain molecules might differ from cell-lines. It is therefore important that key results, obtained from cell-lines, are performed in primary cells to fully validate the observed results. Further advantage with primary cells, is the ability to differentiate some primary basal cells to different cell types. This is typically useful for primary airway epithelial cells, which can be differentiated to multiple cell types using specific medias. The problem with primary cells is that they are very expensive and if they are patient derived, extremally hard to get a hold off. Furthermore, the media that is needed for the cells to grow is expensive and they require constant and special care to survive for a short time. It is also not possible to passage the cells more than 1-2 times as they will either lose their phenotype or undergo senescence rapidly [115].

However, a major problem with both cell-lines and primary cells is that only one type of cell is utilized at a time. The host consists of multiple type pf cells and they work together to create host homeostasis. This is often not taken into consideration when utilizing both cell-lines and primary cells. Nor can the influence of the microbiome on the system be easily investigated in *in vitro* systems. Therefore, these *in vitro* models serve to answer specific questions that are relevant to the disease but cannot be wholly relied on until tested in the human at the systems level.

1.7.3 Microfluidic Systems

In the last decade, new engineered systems have been developed to integrate the functions that are currently lacking in today's *in vitro* models. The ability to interact with nearby tissue and microvascular endothelium is lacking as well as the dynamic mechanical forces, which are present in many organs [116-118]. A microfluidic chip consists of chambers with the ability incorporate one, two or several cell types that together will model a part of an organ. This will enable scientist to investigate shear stress, mechanical forces and recruitment of immune cells on conditions that are not possible on conventional i*n vitro* work. This technology will enable the ability to investigate the effect of drugs, toxin and other molecules in an environment that consists of physiological conditions that are similar to those in the human body [116, 119]. Furthermore, the ability to completely integrate the relationship between the host and the microbiome is fully feasible [120]. The chip used in this set of studies consists of 2 chambers that are separated by a porous membrane (figure 8). For example, in an airway-chip, airway epithelial cells are grown in the top channel with only air, while airway vascular endothelial cells are grown in the bottom channel in a media suspension [121]. To generate a "breathing" airway-chip, mechanical forces can be introduced in a form of stretch, resembling the movements of a breathing lung. Similar results can be seen in the intestine-chip, which utilizes human derived intestinal organoids forming a monolayer on the top channel while intestinal microvascular endothelial cells are used in the bottom channel. Mechanical forces can also be introduced showcasing the effect of the intestinal movements [122].

While it is immensely useful to be able to utilize specific organ-chips, the technology is moving towards the ability to generate patient specific body-chips and personalized medicine. This technology will enable patient specific drug testing and disease modelling [123, 124].

1.7.4 Batch culture fermentation

Batch fermentation is a widely used *in vitro* technique to study the fermentation activity of bacteria and the production of specific microbial metabolites [125, 126]. The sealed container could contain bacteria from different origin (faecal slurry, airway slurry etc.), depending on research interest, and suitable media for the bacteria to grow in. Samples are then taken at desired time points to investigate change in bacterial diversity, density, media composition or production of specific metabolites. This allows for a better understanding of how the human microbiome can be influenced by diet, drugs or other compounds.

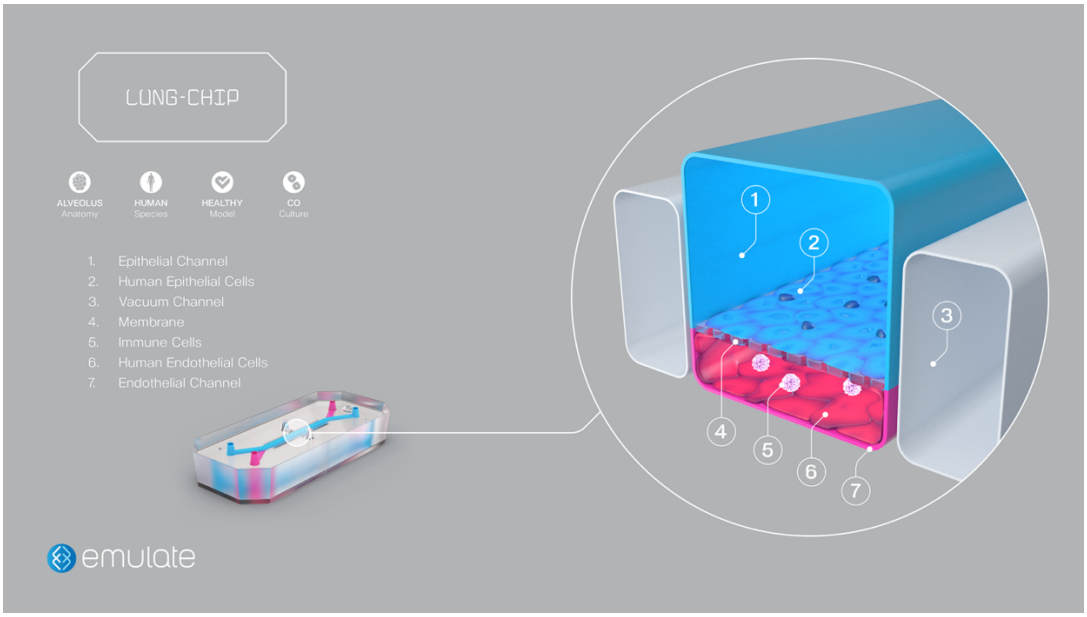


Figure 1.8. Model of the organ chip technology.

Schematic picture of lung chip. Figure adapted from Emulate Inc. https://www.emulatebio.com/.

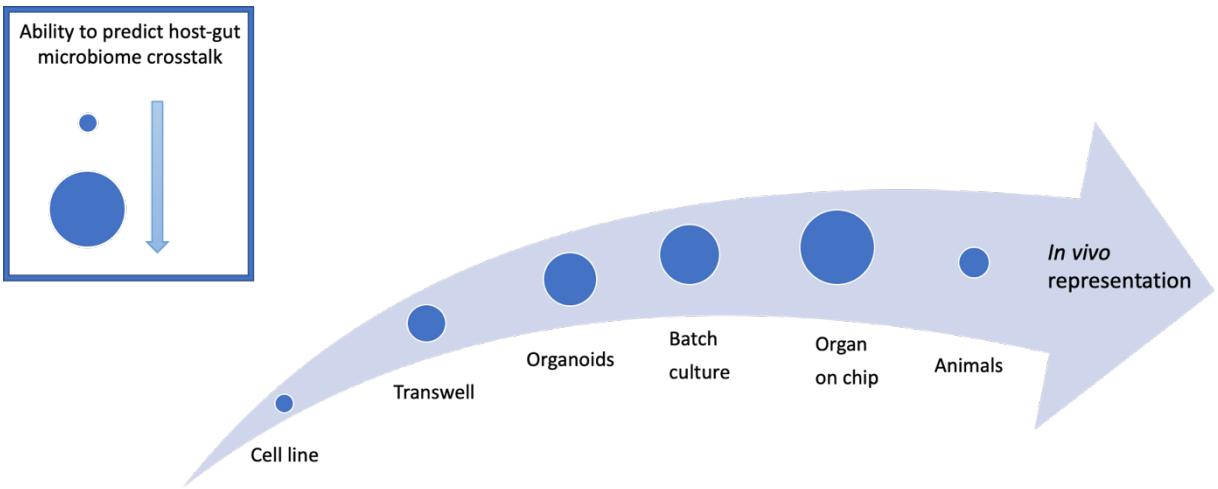


Figure 1.9. Demonstration of several in vitro systems and their in vivo representation.

In this thesis, the following in vitro systems will be used: cell line, transwell, batch cultures and organ on chip. Figure adapted from [1].

1.8 Metabolic Profiling of Asthma

The majority of the research conducted on asthma has been focused on getting a better understanding of the immunological effect of the disease and in turn finding drugs that will target specific parts or pathways. Another way of getting a better understanding of asthma is through, metabolic profiling, or phenotyping. Metabolic profiling involves the measurement of the metabolic processes occurring in biological systems and how these are perturbed by various stimuli, such as a toxin, dietary intake or a disease state [127-129]. A key strength of metabolic profiling, compared to other omics approaches, is that the metabolic changes, that are detected and quantified, are often related to an entire biological system and can give quick information regarding the entire physiology of a system [128]. There has been an increase in use of metabolic profiling to study asthma, which has resulted in observation of several biochemical alterations by profiling urine, serum and exhaled breath condensate [130, 131]. Several studies have outlined metabolites, related to the TCA cycle and enhanced methyl transfer pathway, which were increased or decreased in asthmatic patients compared to controls [132, 133].

Plasma, serum and urine are the most common biofluids used for metabolic profiling and together with cell media and extracts, often analysed by either proton nuclear magnetic resonance (^{1}H NMR) spectroscopy or by liquid, gas, and capillary chromatography coupled to a mass spectrometer. Some of the instruments are more sensitive than others but require more sample preparation, while other are faster and more reproducible but less sensitive. It is therefore important to understand the different techniques and their limitations, to be able to choose the one that is the most appropriate for the type of objective, experiment and sample type.

1.8.1 ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy

A major analytical technique used to characterize the metabolic profile of biofluids is Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR is a technique, based on quantum physics, which exploits a property called nuclear spin and is therefore inherently quantitative. Hydrogen, ^{1}H, possesses this property and can be measured by ^{1}H NMR spectroscopy. ^{1}H is the most abundant element and present in most biological molecules allowing for complete overview of the metabolites present in biofluids. When a magnetic field is applied to the nucleus, the magnetic moment is oriented to the number of possible energy states calculated with the equation: 2I+1, where I is the nuclear spin. Before applying the magnetic field, the orientation of the magnetic moment is random. ^{1}H has a nuclear spin number of 1/2 and, which will result in two possible orientation, one with high energy state (aligned in the opposition to the magnetic field) and the other one with low energy state (aligned with the magnetic field). A NMR signal is observed when there is a change in between the two energy states, which requires an energy transfer. The problem is that the energy transfer is extremally small, which explains why NMR is a very insensitive technique and requires large amount of sample to get a detectable signal. Subsequently, a radiofrequency pulse is applied to the nuclei resulting in a vertical rotation and transition of atoms in the low to the high energy state, and when the pulse stops the nuclei relaxes back to their original position. An oscillating voltage will be released, which is proportional to the number of spins in a molecule, and the sum of these oscillations will be known as free induction decay (FID). The differences between molecules, will be determined by the speed of relaxation and radiofrequency pulse. Finally, the FID is converted into frequency, which in turn is converted into a chemical shift. The chemical shift is scale that references the frequency of a molecule to a standard compound which is defined at 0 parts per million, and to the frequency of the spectrometer. The frequency of

the molecule, and the chemical shift of the molecule, will then be different depending on the electron distribution within a molecule, which is dependent on bond angle, type, length etc. To generate a spectrum, the chemical shift is plotted against the intensity (metabolite abundance) to produce a signal or peak in the ^{1}H NMR spectrum [134, 135].

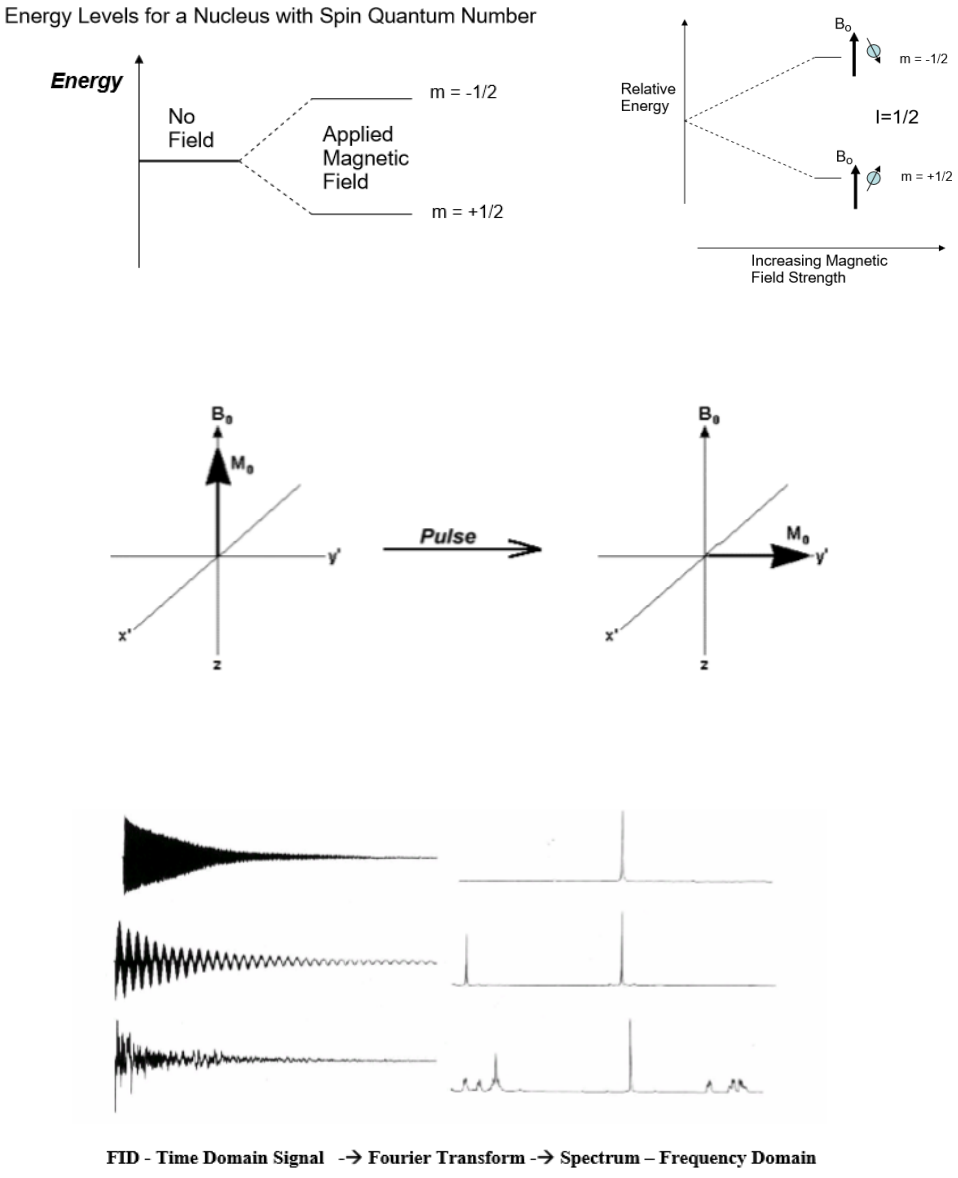


Figure 1.10. Schematic picture of ^{1}H NMR spectroscopy theory.

Magnetic field is applied to the nucleus resulting into two energy states, where a NMR signal is observed when there is a change between the two states. A pulse is applied resulting in rotation of the nuclei, which when released, releases oscillating voltage, which is converted into frequency and then to chemical shift resulting in a peak in the spectra. Figure adapted from https://www.process-nmr.com/principles-of-nmr/

Although 1H has a specific frequency when placed in a magnetic field, local differences in chemical environment cause slight deviations from this frequency and thus each proton environment will have different chemical properties and represent different chemical environments within different metabolites. This information can be used to resolve compositional differences between samples and showcase similarities as well as differences using different databases and further two-dimensional NMR experiments to identify molecules specific to a particular biological group or class [136]. The use of ^{1}H NMR spectroscopy comes with several advantages, one of them being, it provides an overall view of all metabolites present in the sample. Furthermore, the sample is not destroyed and the technique does not rely on ionization radiation. The sample preparation is rapid, simple and inexpensive on consumable basis, the machine gives high throughput and high degree of reproducibility [137]. However, disadvantages include lack of sensitivity, which becomes a problem when the metabolites of interest are present at low concentration and can therefore not be detected.

1.8.2 Chromatography and Mass Spectrometry

Analysing a large variety of samples, consisting of different compounds, often requires the need of some type of separation. Several well-established separation techniques have been developed depending on the nature of the sample and the compounds in need of separation. Separations can be achieved by centrifugation (different densities), chromatography (different interactions within a stationary, liquid or gaseous phase), distillation (liquids with different boiling points), electrophoresis (interaction with a gel) and many more techniques. The use of each technique is based on the property of the sample and the type of compounds that require separation.

Mass spectrometry is an analytical tool used to measure the mass-to-charge (m/z) ratio of ions in a sample or mixture. A mass spectrometer consists of three components, an ion source, a mass analyser and a detector, where each component has several different models and designs. The molecules within the sample of interest, are ionized creating gas-phase ions, in either positive or negative mode, which are then passed through a mass analyser to be separated according to their masses. The separated ions are then passed through a detector generating peaks of different m/z ratio and relative abundance according to the presence of the compound within the sample. Distinct fragmentation pattern can be observed for each compound, which aids in the identification.

1.8.2.1 Ultra Performance Liquid Chromatography – Mass Spectrometry (UPLC-MS)

Liquid chromatography coupled to a mass spectrometer (LC-MS) is widely used tool to analyse a variety of molecules, which are physical and chemical properties, especially in the analysis of complex biofluids such as urine, plasma and serum. This technique is far more sensitive compared to ^{1}H NMR spectroscopy and can detect metabolites at much lower concentrations. Ultra- or high-performance liquid chromatography (UPLC/HPLC) is a separation method where the solvent travels through using high pressure compared to standard LC, where the force of gravity is used [138, 139]. This enables far more sensitive analysis, better resolution and reduced separation time. A common form of UPLC-MS is reversed-phase chromatography, where the column is packed with silica, a non-polar stationary phase, and a polar mobile phase that is often a mixture of water and a solvent. Molecules that are hydrophobic will therefore adhere to the column and will have a higher retention time compared to hydrophilic molecules [140]. After the separation of the molecules, according to polarity, they reach the mass spectrometer, where different types of ionization modes can be applied. One of the most common ones is ESI, electrospray ionization,

where the molecules are ionized by high voltage through electrospray to produced aerosols that are then detected by the detector. This can be done for both positive negative ions and by switching the mode on the mass spectrometer. The computer will at the end compute a 3D chromatogram where high polar molecules will have the shortest retention time and non-polar molecules higher retention time and each retention time will have a spectrum demonstrating the mass/ratio of each ion [141] (figure 1.11).

1.8.2.2 Gas Chromatography – Mass Spectrometry (GC-MS)

Gas chromatography coupled to mass spectrometry (GC-MS) is another analytical tool often used to analyse low molecular and volatile compounds [142]. Utilizing a mobile phase of an inert gas, often helium or nitrogen, the injected sample is carried with the inert gas, without interacting with it, to the stationary phase located inside an oven. The stationary phase is a packed column, several meters long of microscopic layer of liquid or a polymer on solid support. As the sample passes through the stationary phase, the temperature of the oven rises gradually until it reaches 400°C to separate the molecules according to their vapour pressure or affinity to the stationary phase. The separated molecules than reach the quadruple mass analyser, a type of mass spectrometer, where they are fragmented to ions of mass to charge ratio (m/z) and then detected by the detector producing a spectrum with retention time and mass to charge ratio, as describe for UPLC-MS [141] (figure 1.11).

1.8.2.3 Advantages and limitations of metabolomics platforms and data pre-processing

Even though both UPLC-MS and GC-MS are techniques that are sensitive and can detect metabolites at micromolar concentrations, there are several disadvantages. Sample preparation can

be lengthy and not as simple and straightforward as for ^{1}H NMR spectroscopy. The sample is also injected into the machine and can therefore not be used more than once. The availability of several different machines and conditions makes it difficult to compare data across platforms, if the instrument is not identical and the same parameters used. Furthermore, the best way is by analysing the samples by both techniques to cover the advantages of them both and detect all possible metabolites at all concentrations. Due to the volatile nature of SCFAs, and the need to use a sensitive technique due to the low amounts found in biofluids, GC-MS is the preferred method. ^{1}H NMR spectroscopy is an excellent platform to use when there are no specific metabolites of interest, but rather a general profiling is needed. UPLC-MS is the better platform when targeting specific classes of metabolites such as lipids, bile acids, carnitines and if the metabolites of interest are present at low concentration in the biofluid of interest.

Metabolic profiling of biofluids using either ^{1}H NMR, UPLC-MS or GC-MS generates copious amount of data that undergo pre-processing followed by multivariate analysis (described in method 1.9). Pre-processing includes normalization, peak alignment and scaling to ensure that the peaks are aligned as well as eluting at the described time point. Due to the heterogeneity of human samples it is vital that the pre-processing is performed to ensure relative comparison, between samples, and not absolute. Multivariate analysis, described in method 1.9, was performed subsequent to pre-processing.

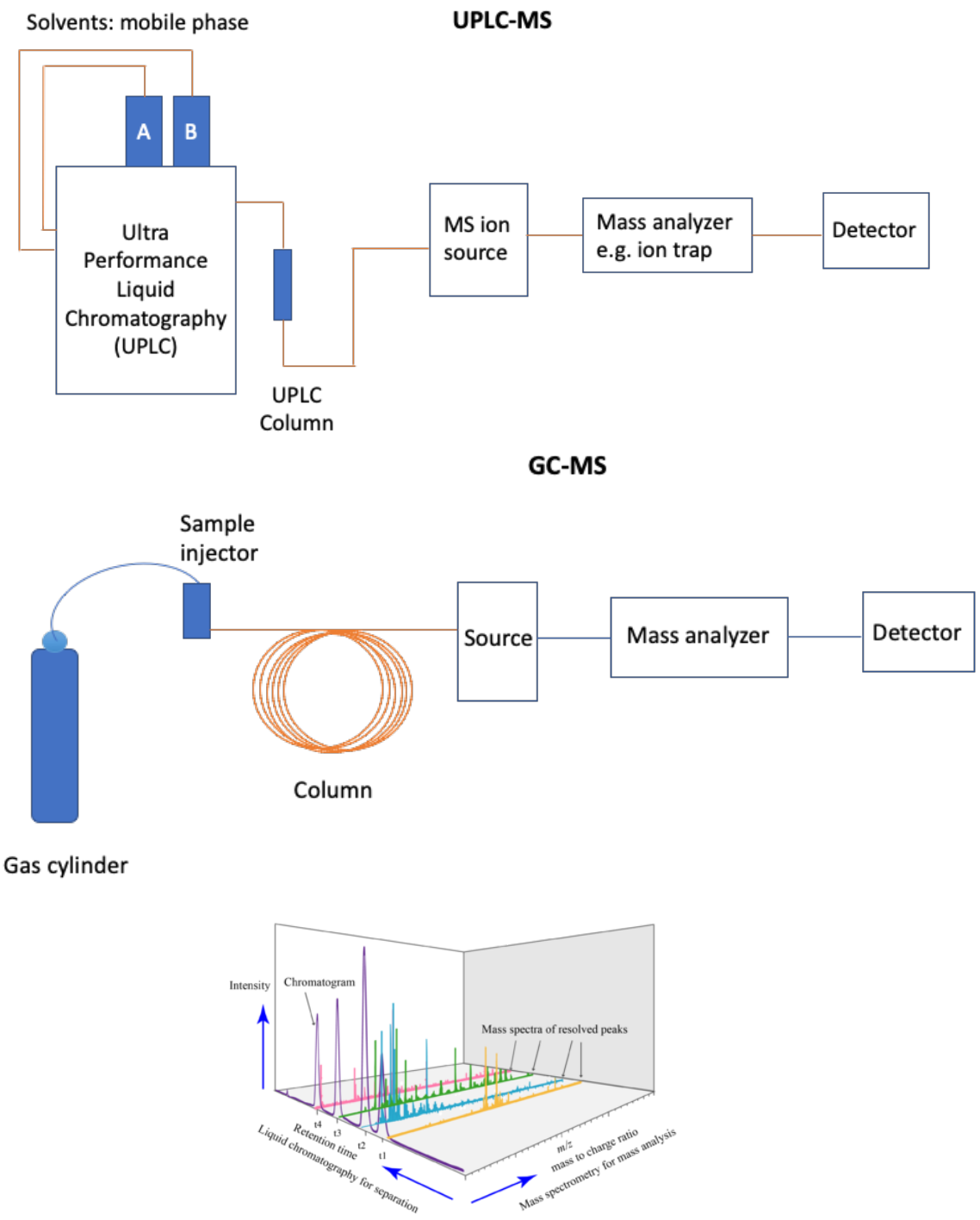


Figure 1.11. Schematic overview of UPLC-MS and GC-MS.

Overview of UPLC-MS setup as well as GC-MS setup. Bottom 3D picture demonstrates the chromatogram acquired together with the mass spectra of the peaks.

1.9 Chemometrics

The data generated from metabolic profiling is typically multi-or megavariate by nature, as hundreds or thousands of molecules can be detected simultaneously and is difficult to interpret. As such, multivariate data analysis techniques are used to elucidate latent patterns within the data associated with a variable of interest (*e.g.* disease presence/absence) [143].

1.9.1 Unsupervised Methods – Principal Component Analysis (PCA)

Principal Component Analysis (PCA) [144] is an unsupervised method that constructs a model of multivariate data with no former information about the dataset. PCA takes the data generated from metabolic profiling and reduces the dimensionality of the data while maintaining the important information regarding the largest source of variation in the data. This is achieved by turning the data into a matrix X, which consists of number of rows (score, describing the sum of the variation in the direction of the object) and number of columns (loadings, describing the variation in the direction of the variable), where the observations in the PCA vary depending on the variation of the matrix, which provides the number of axes [145]. The scores describe the variation of the rows, which for ^{1}H NMR and UPLC-MS spectral data are the different samples, while the loadings describe the variation in the different signals relating to specific metabolites. As the name suggests, there are several components, which can display the variation in the dataset, where each component is a weighted linear combination of the original variables. The first component consists of the largest variance, with subsequent components being orthogonal and explaining progressive less variance than the last. X is then modelled as an object in the multivariate space and together with other objects, corresponding to the number of samples, they form a cloud in this space. As the

metabolic profiling dataset contains multiple variables of different concentrations, it is necessary to centre and scale the cloud of objects. One way of centring the data is by the mean, where the mean square error of the data is minimized, putting all variables on equal footing and looking at relative variation. Scaling the data, for example by unit variance, ensures that each variable is adjusted to its weight.

1.9.2 Supervised Methods – Projections to Latent Structures (PLS) and Orthogonal Projections to Latent Structures (OPLS)

Supervised methods use information regarding the dataset prior to model construction in order to maximise the separation between data classes. Projection to latent structures-discriminant analysis (PLS-DA) [146] is one such approach. In this approach, the metabolic data (e.g. the ^{1}H NMR or UPLC-MS data) forms the descriptor (X) matrix whilst the response variable (e.g. asthma versus healthy status) forms the Y matrix. The ability of the metabolic data to predict or classify the response variable is then assessed. The metabolic variation associated with the response can then be identified and interpreted. The first PLS component, as seen in PCA, maximizes the covariance between the X scores and Y scores, by identifying the scores with high variability and factors with low variability in a linear fashion. However, even though the model is constructed based on the information provided, some systemic variation in X is not related to Y. Orthogonal variation is systemic variation that is not linearly related to the model of interest, for example gender and age when looking at disease vs non-disease. In order to minimize this variation, orthogonal projection is performed, which rotates the PLS model to focus the OPLS model on the effect of interest. This will result in a stricter model, which can describe difference or similarities within the dataset.

The validity of the acquired models, PCA, PLS, OPLS-DA etc. can be determined by examining the R^2X and Q^2Y values. This is important as supervised models are often inclined to over-fitting, which happens when the models have poor predictive ability and predicts noise or random error. The R^2X value describes the variation of the dataset within the model. The closer the value is to 1 the better the model is, whereas a very low R^2X value indicates a poor model, which might be due to increased noise in the dataset or high inherent biological variation within the sample set, as is common in human studies. The Q^2Y parameters describes the ability of the dataset to predict the class of samples, which is calculated by cross-validation. Typically, the dataset is divided up in 7 parts and one part is removed and the "let-out data" is predicted using the remaining parts. The process is then repeated, removing a different seventh of the dataset. This is performed on all parts and then compared to the original dataset and the error is calculated. This error is then converted to a Q^2Y value to make it comparable to the R^2X value. Due to the variation and origin of different datasets, a variety of Q^2Y values will computed. Any model with a Q^2Y value above 0.5 is considered to be a good model, although several studies have used 0.2 as a "good" cutoff metric for human datasets, and the closer the Q^2Y value is to the R^2X value the better the model is. However, the difference between R^2X and Q^2Y should not exceed 0.3 units [147, 148].

A final tool used to determine the validity of a model is by permutation testing. The predictive ability of Q^2Y is calculated by shuffling the dataset, where the Y-observations are random, and the X-matrix is kept intact. A simulated model is generated with new R^2X and Q^2Y values. This permutation testing is done 1000 times where the R^2X and Q^2Y values are ranked. The simulated Q^2Y value is then compared to the real Q^2Y and a significance value is provided ($P<0.05$).

1.10 Metabolite identification

In order to identify metabolites of interest, a range of different analyses and tools are used, which are described here below (figure 1.12).

Metabolic profiling of biofluids using either ^{1}H NMR, UPLC-MS or GC-MS generates copious amount of data. After pre-processing, multivariate analysis is performed and a scores plot is generated, where each dot represent one sample and the distance between the dots portrays how similar the samples are in terms of their metabolic profile. The distance between the samples describes the similarity of their metabolic profile, where shorter distance describes similar profile and longer distance displays more metabolic variation. After producing a valid OPLS-DA model describing metabolic variation between two classes, a loadings plot is generated for the ^{1}H NMR spectroscopy data, displaying the metabolites behind the structure seen in the model. These metabolites are then identified using in-house tools (described below) as well as by application of statistical total correlation spectroscopy (STOCSY) [149]. STOCSY generates a pseudo-two-dimensional spectra that describes the correlation of various peaks across a sample, aiding in the identification of metabolites.

After generating a valid model for UPLC-MS data, significant features causing the separation, were extracted by using the VIP score (Variable Importance in Projection), where a higher VIP score displayed more significance. In combination with Pearson's correlation value (pcorr), the most important features were extracted for further analysis. Univariate analysis (student t-test) was also performed on the healthy and diseased group and the features that were not significant after correction for multiple testing were removed. Thereafter, an in-house correlation script was run to

generate other features that are highly correlated to the features of interest to simplify the metabolic assignment, which was then conducted using HMDB [150] and Metlin [136, 150]. To confirm that the potentially identified metabolite in fact is correct, MS/MS is needed where the parent ion is fragmented to smaller fragments that are then compared to either the sample or the database of the suspected metabolite. Final stage includes addition of a standard of the suspected metabolite to the original sample, followed by UPLC-MS and then comparison of the spiked in metabolite to the metabolite already present in the samples. Correct identification will generate two identical spectrum, which would result in level 1 annotation according to the metabolomics standards initiative (MSI) [151].

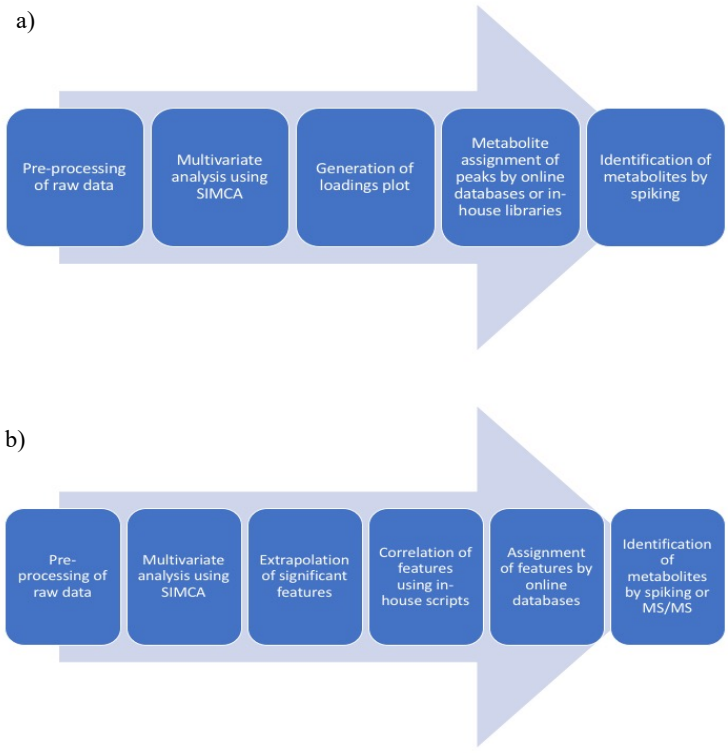


Figure 1.12. Work flow for the identification of metabolites.

In order to identify metabolites that are different between healthy and diseases, or whichever groups that are being compared, several analysis and tools are used. Workflow demonstrated for a) ^{1}H NMR spectroscopy and b) UPLS-MS.

2 MATERIAL AND METHODS

2.1 Targeted SCFA assay using gas chromatography coupled to a mass spectrometer (GC-MS)

2.1.1 Sample preparation of airway samples

Bronchoalveolar (BAL) samples from healthy controls and asthmatics were collected as previously mentioned [152] and kept at -80 °C. Induced sputum collection from asthmatics was performed using 5% saline solution with two-minute tidal breathing method. Samples were divided into portions, one for molecular analysis and one for 16S rRNA gene sequencing. BAL and sputum samples were processed for targeted GC-MS according to previous published method [153]. Two calibration curves were constructed, one for sputum samples consisting of 14 points and one for BAL samples with 12 points, using a synthetic mixture, which contained all 10 fatty acids (Supplementary table 2.2 and 2.3) The samples, together with the calibration curves, were run on 7000D Triple Quadrupole GC-MS (Agilent Technologies, California, US) analysed using Masshunter software to obtain quantitative concentrations.

2.1.2 Sample preparation of airway batch culture samples

Bacterial isolates were stored as stocks on ceramic beads at -80 °C and selected isolates (table 2) were grown back on brain heart infusion (BHI) agar and checked for purity. The single colonies were sub-cultured in different media conditions (table 2). Sampling was conducted after 48 hours and 7 days and growth was checked by turbidity and microscopy. Samples were analysed for

SCFAs using a previously described targeted GC-MS method [153]. A 1.5-fold difference was considered to be of relevance when comparing SCFAs concentrations between media and media with mucus, for each species.

2.1.3 Sample preparation of plasma samples from diet intervention study

Plasma samples were collected from healthy controls and asthmatics who were subjected to two different diets and kept frozen kept at -80 °C. The samples were thawed the day of analysis and run and analysed according to previously published protocol [153].

2.2 Multivariate Association with Linear Models (MaAsLin) of SCFAs data with 16S rRNA data

16S rRNA data, from sputum samples of asthmatics, was generated and analysed as previously described [154]. To gain a better understanding of the interaction of the detected SCFAs in the airways of asthmatics, 16S rRNA data from the sputum samples was used to perform an association model with the SCFAs concentrations, using Multivariate Association with Linear Models (MaAsLin) [155].

2.3 *In vitro* cell culture and assays

2.3.1 Cell culture

2.3.1.1 Submerged Human Normal Bronchial Epithelial cell-line (BEAS-2B)

A human bronchial epithelial BEAS-2B cell line was purchased from American Type Culture Collection (CRL-9609, ATCC, Middlesex, UK), suspended in Keratinocyte media (Thermo Fisher Scientific, Paisley, UK) containing L-glutamine, supplemented with EGF and BPE (Thermo Fisher Scientific, Paisley, UK), and seeded at a density of 2500 cells/cm^2 at 37°C, 5% CO_2 humidified atmosphere. The cells were passaged every 2 days, reaching 70-80% confluency, by trypsinisation with 0.25% Trypsin/0.53mM EDTA solution (Sigma-Aldrich, Poole, UK), centrifuged at 300*g* and seeded at a density of 2.5 x 10^4 cells/ well in a 96-well plate for viability assay and 5 x 10^4 cells/well in a 48-well plate for enzyme immunoassay (ELISA). The cells were treated with 1000, 500, 100, 10, 10 and 0.1 mM of sodium acetate, propionate and butyrate (Sigma-Aldrich, Poole, UK) for 24 h followed by IL-13 for additional 24 hours and thereafter assayed.

2.3.1.2 Submerged Primary Human Normal Bronchial Epithelial cells (NHBE)

Primary human normal bronchial epithelial cells (NHBE), were purchased from American Type Culture Collection (PCS-300-010, ATCC, Middlesex, UK) and cultured in BEGM™ Bronchial Epithelial Cell Growth Medium and BulletKit™ (Lonza, Cologne, Germany) containing 500 ml of bronchial epithelial cell basal medium supplemented with BPE, hydrocortisone, insulin, transferrin, epinephrine, triiodothyronine, retinoic acid and hEGF.

One vial of NHBE cells (Passage 0) containing 10^6 cells was thawed quickly (less than 1 min) at 37°C and then seeded into a T-75 flask with complete BEGM medium. The medium was changed the following day to remove the diluted freezing media. The cells reached 90% confluency after 4 days and were passaged, using Trypsin-EDTA, into 3 new T-75 flasks with a seeding density of 3000 cell/cm^2 and 3 vials containing $5x10^5$ cells were frozen for future use. 24-well plates were coated with 30µg/ml Bovine collagen I (Advanced BioMatrix, US) and incubated at 37°C, 5% CO_2 for 2 hours or O/N. NHBE cells (passage 2) were seeded in a collagen I coated 24-well plate at a density of 40 000 cells/ well in complete BEGM medium and treated with 10 and 1 mM of sodium acetate and propionate for 24 hours followed by 100mg/ml of house dust mite (HDM) (Citeq Biologics, Groningen, Netherlands) for additional 24 hours.

2.3.1.3 Differentiated Primary Human Airway Epithelial cells in transwells

Human airway epithelial cells were purchased from Lonza (CC-2541 LOT 0000517045), Lifeline (FC-0016 LOT 01925, LOT 00656, FC-0035 LOT 04282) and ATCC (PCS-300-010) and cultured in complete BEGM media. Upon reaching 90% confluency the cells were passaged, using TrypLE™ Express Enzyme (1X), no phenol red (Gibco), into 24 transwell plates coated with 100ug/ml of Collagen IV (Sigma-Aldrich, Poole, UK) at a seeding density of 50 000 cells/well with the following ALI media, DMEM/F12 + GlutaMAX (Thermo Fisher Scientific, Paisley, UK) supplemented with 2% Knockout serum (Thermo Fisher Scientific, Paisley, UK), 2% HyClone FetalClone II serum (GE Lifesceinces), 1% Penicillin/Streptomycin (Thermo Fisher Scientific, Paisley, UK) and BEGM™ Bronchial Epithelial Cell BulletKit™ (Lonza, Cologne Germany) containing BPE, hydrocortisone, insulin, transferrin, epinephrine, triiodothyronine and retinoic

acid. 250 µl of media was added to the apical side of the transwell and 750 µl was added to the basolateral side.

After 3 days, air-liquid interphase (ALI) was introduced to the cells by removing the media from the apical side, introducing air, and replacing the media on the basolateral side with 500 ul. Every 2-3 days the apical side was washed with PBS for 10min to remove produced mucus and media on the basolateral side was replaced. At 20-25 days after ALI had been introduced, the cells were treated with either 10 or 1 mM of sodium acetate, sodium propionate or sodium butyrate on the apical or basolateral side for 36 hours. Following 12 hours after addition of sodium acetate, propionate or butyrate 10ng/ml of IL-13 was added to the basolateral side to mimic Th2 inflammation. After additional 24 hours (total of 36 hours of treatment) the media was collected and frozen at -80 °C for ^{1}H NMR analysis and ELISA and the cells were lysed with RLT buffer (Qiagen, Germany) supplemented with 1% of β-mercaptoethanol and stored at -80 °C until further mRNA extraction process.

2.3.1.4 Differentiated Primary Human Airway Epithelial cells (NHBE) in airway-chip

Each microfludic chip with PET membrane was coated with 300 µg/ml collagen IV on the top and bottom channel. Human small airway epithelial cells were purchased from Lifeline (FC-0016 LOT 00656) and seeded in the top channel at a density of 10^{6} cells/chip in complete BEGM media. Complete BEGM media was added to the bottom channel. The media in both the top and bottom channel were replaced and 4 hours and connected to the flow instrument (Emulate Inc. Boston, US) after 6 hours. The flow for the top and bottom channel was 30 µl/hour for the first 3 days. ALI was introduced after 3 days where the media was changed to ALI media and the flow in the top

channel was changed to 0 µl/hour whilst the flow in the bottom channel remained at 30 µl/hour. Media in the bottom channel was changed every 2-3 days. After 20 days of introducing ALI the cells were treated with 10mM of propionate on basolateral side for 36 hours. The media was collected and frozen at -80 °C for ^{1}H NMR analysis and ELISA and the cells were lysed with RLT buffer (Qiagen, Germany) supplemented with 1% of β-mercaptoethanol and stored at -80 °C until further mRNA extraction process.

2.3.1.5 Primary Peripheral blood mononuclear cell (PBMC) skewed towards Th2

Peripheral blood mononuclear cells (PBMCs) were isolated and cultured according to previously published study [156]. Th2 skewed PBMCs were treated with 10, 1 and 0.1 mM of Leucine, Isoleucine and Valine individually and as a cocktail. Levels of IL-5 and IL-10 were quantified using human Enzyme-Linked Immunosorbent Assay (ELISA) Duoset (R&D Systems, Abingdon, UK).

2.3.2 Cell viability using Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit

Media was collected from both BEAS-2B and NHBE and viability was assayed using lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Cambridge Bioscience, Cambridge, UK), according to the manufacturer's protocol. Briefly, the media from the treated cells, negative control (containing no sample) and positive control (containing 10% Triton X-100 solution), were transferred to a clean 96-well plate and 10% of LDH Cytotoxicity Assay reagent was added at incubated for 30 min at 37°C, 5% CO_2 humidified atmosphere and the optical density was read at 450 nm using a spectrophotometer (BioTek). The relative cytotoxicity was calculated by

subtracting the optical density of the negative control from both the experimental sample and positive control.

2.3.3 mRNA extraction, RT-PCR and qPCR

The treated cells were washed with 0.5 ml of PBS, and processed according to Qiagen mRNA extraction kit (Qiagen, Germany). Briefly, the cells were lysed with RLT buffer supplemented with 1% β-mercaptoethanol, collected, vortexed to extract maximum mRNA and then placed on the spin column. The column was washed several times with different buffers to ensure good quality and purity of the mRNA and eluted using RNase free water. The mRNA was frozen at -80°C overnight to ensure stability, and the concentration was measured the following day using Implen nanophotometer (Implen, Germany).

RT-PCR was performed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Thermo Fisher Scientific, Paisley, UK), where 400-1000ng of mRNA was used with the following PCR conditions: step 1 for 10 min at 25°C, step 2 for 90 min at 42°C, step 3 for 15 min at 70°C (DNA Engine Thermal Cycler, Bio-Rad). The cDNA was then frozen at -20°C until processing using qPCR.

qPCR was performed using TaqMan™ Fast Universal PCR Master Mix in MicroAmp™ Optical 96-Well Reaction Plate and run on Step One Plus RT PCR systems (Applied Biosystems, Thermo Fisher Scientific, Paisley, UK) 10 ng of cDNA was utilized for each reaction and probe, and the following human probes were used, GAPDH (Hs02758991_g1), HPRT1 (HS02800695_m1), FFAR2 (Hs00271142), FFAR3 (Hs02519193), (CXCL8/IL-8 (Hs00174103_m1), IL-6 (Hs00174131_m1), CCL26 (Hs00171146_m1), TGF-β (Hs00998133_m1), MUC5AC

(Hs01365616_m1), TUBA1A (HS00362387_m1), CDH1 (Hs01023894_m1), occludin (Hs00170162), ICAM1 (Hs00164932_m1) and VCAM1 (Hs01003372_m1). The following PCR conditions were applied: Holding stage 95°C for 20s followed by Cycling stage step 1, 95°C for 1s and stage 2 60°C for 20s. 40 cycles of amplification were performed. HPRT1 or GAPDH were utilized as housekeeping reference genes. Relative fold change was calculated using $2^{-\Delta\Delta Ct}$ relative to the housekeeping genes and the baseline ($\Delta\Delta Ct$).

2.3.4 Enzyme-Linked Immunosorbent Assay (ELISA)

Previously frozen media was thawed and human Duoset IL-8, IL-6, IL-5, IL-10 and CCL26 ELISA (R&D Systems, Abingdon, UK) were used according to manufactures protocol. Briefly, a 96-well plate was incubated with working concertation of capture antibody overnight in RT, washed 3 times with wash buffer (0.05% Tween-20 in PBS), incubated with blocking buffer (1% BSA in PBS) for 1 hour, washed 3 times and thereafter adding the samples together with working concertation of the standard for 2 h in RT or O/N in 4°C. The plates were then washed 3 times, working concertation of detection antibody added for 2 h in RT, washed 3 times, addition of Streptavidin-HRP to the wells, washed 3 times and addition of substrate solution and finally stop solution to end the reaction. The absorbance was measured at 450nm as well as 540nm (background correction) using a spectrophotometer (BioTek, US). Four-Parameter Logistic Regression and Student t-test were preformed using GraphPad Prism 6.

2.4 Metabolic profiling of cell supernatant and human biofluids

2.4.1 Sample preparation and analysis of cell supernatant using ^{1}H NMR spectroscopy

The previously collected media was thawed and centrifuged for 5 min at 300*g*. Sample preparation as conducted according to urine protocol [157]. Briefly, 180 µl of media was mixed with 20µl of phosphate buffer (1.5M KH_2PO_4, 2mM NaN_3, 1% TSP solution in 100% D_2O, pH 7.4) and vortexed followed by the transfer of 180 µl of the media solution to a 3mm tube and sealed.

Before conducting the analysis on a 600 MHz NMR spectrometer (Bruker Avance III, Bruker Biospin, Germany), machine calibration had to be conducted to ensure implementation of correct parameters [157]. Temperature is a crucial parameter for automatic quantification of metabolites by Bruker's data analysis (Topspin) and the readings from the spectrometer had to be calibrated to show exactly 300K. This was conducted by the use of 99.8% methanol, as the chemical shift of methanol is temperature dependent.

The second calibration parameter is water suppression. Water is present in high quantity in cell samples and the water signal will overshadow the signal of the metabolites. It is therefore important to suppress the water signal as much as possible. The spectrometers performance of water suppression is checked by measuring a standard sample containing 2mM sucrose, in 90% H_2O and 10% D_2O.

The last calibration step is using an external sample, which contains known metabolites that are known to be stable for a long time, to have a background reference for quantification of metabolites in the samples being tested.

The samples were run on 600 MHz ^{1}H NMR spectrometer (Bruker Avance III, Bruker Biospin, Germany) where each experiment consisted of a standard one-dimensional pulse sequence using the first increment of nuclear Overhauser effect (nOe) pulse sequence to achieve water suppression with 64 scans, with a relaxation decay set at 4s where a water pre-saturation pulse was applied. Each spectrum was processed for Fourier transformation, phasing, baseline correction and calibration to TSP (0.00 ppm) using TopSpin (Version 3.2, Bruker Biospin, Germany). The spectral region containing the water signal (4.50-4.90 ppm) was removed from the spectra and probabilistic quotient normalization was applied.

2.4.2 Metabolic profiling of urine samples from controls and asthmatics using ^{1}H NMR spectroscopy and UPLC-MS

Spot urine from healthy controls, mild to moderate and severe asthmatics was collected according to previous published methods [158] and kept at -80 °C. Urine samples were thawed and prepared for ^{1}H NMR spectroscopy and UPLC-MS according to previous published methods [157, 159]. For the UPLC-MS, the urine samples were run using revered-phase chromatography (RPC) positive and negative mode as well as Hydrophilic Interaction Liquid Chromatography (HILIC) positive.

The urine samples and the ^{1}H NMR spectroscopy instrument were prepared as described in section 2.3.1 apart from sample volume, which was 630 µl of urine with 70 µl of buffer in 5 mm tubes. 32 scans were used for the standard 1D experiment and an additional B.I.QUANT-PS experiment was conducted to get quantified concentration of 150 metabolites in each sample.

2.4.3 Metabolic profiling of urine from diet intervention study using ^{1}H NMR spectroscopy

Spot urine was collected early morning before and after a Eucapnic Voluntary Hyperventilation (EVH) test, which is a diagnostic for exercise-induced bronchoconstriction, and frozen at -80°C.

The day of the analysis, urine samples were thawed at room temperature and prepared for ^{1}H NMR spectroscopy as previously described in section 2.3.1 with the exception of sample volume, which was 630 µl of urine with 70 µl of buffer in 5 mm tubes. A total of 32 scans were used for the standard 1D experiment and an additional B.I.QUANT-PS experiment was conducted to get quantified concentration of 50 metabolites in each sample.

2.5 Statistical Analysis

Statistical analysis was performed using Graphpad Prism 6 (GraphPad Software, Inc., CA, US), MATLAB (version R2016b, the Math works, Natick MA, USA), SIMCA 15 (Umetrics, Umea, Sweden) and R studio 1.1447 (The R foundation, New Zealand).

For BAL SCFAs, unpaired, non-parametric t-test was performed. MaAsLin [155] was used for association between SCFAs and 16s rRNA data with a cut-off of FDR adjusted p-value of 0.05. For plasma SCFAs for the crossover intervention study, paired, non-parametric t-test was performed with $P<0.05$ considered as significant.

For the *in vitro* cell work, un-paired parametric t-test was performed with $P<0.05$ considered as significant.

Multivariate analysis was conducted on the ^{1}H NMR spectroscopy and UPLC-MS data using the SIMCA software package, where PCA, OPLS and OPLS-DA models were generated. Models were considered to be valid with positive R^2X and Q^2Y values and a difference of maximum 0.3 units between the two variables. Univariate analysis was conducted on the metabolites from the profiling where a p-value <0.05 were considered significant. Heatmaps were constructed to display the correlation coefficient between the level of identified metabolites and patient metadata using Spearman correlation with R studio 1.1.447 (The R Foundation, New Zealand).

2.6 Metabolite identification in UPLC-MS data and pathway analysis

Following the generation of valid OPLS-DA models, the procedure described in paragraph 1.10 was conducted on the features of interest generating 8 annotations. Three of these putatively annotated compounds could not be purchased, and therefore only classified as level 2 identification. The remaining 5 were purchased and the raw spectra were acquired alongside the standards to confirm complete identification. Pathway analysis was then conducted on the fully identified metabolites, together with associated metabolites, using MetaboNetworks [160]. Metbonetworks utilises metabolic pathways described by Kyoto Encyclopaedia of Genes and Genomes (KEGG) for humans, archaea, fungi and bacteria and displays the shortest metabolic pathway of desired metabolites all together in one map. This allows for pathway interpretation of metabolite of interest, including mammalian and bacterial pathways together.

Chapter 3 Investigating presence of SCFAs in airway fluids of asthmatics and the role of the airway microbiome

Hypothesis

Is it hypothesized that short-chain fatty acids (SCFAs), mainly acetate, are present in the airways of people, with lower levels being present in asthmatics.

Aims

- Examine the presence of SCFAs in control individuals and people with asthma
- Investigate the interaction of SCFAs with the lung microbiome
- Study whether the lung microbiome has proteolytic activity and ability to produce SCFAs from proteins

Objectives

- Targeted GC-MS was used to quantify the amount of SCFAs in two lung fluids, bronchoalveolar lavage (BAL) and sputum from controls and asthmatics.
- Association models between quantified SCFAs in sputum samples of asthmatics and 16s rRNA data were generated and analysed.
- Targeted GC-MS was used to quantify production of SCFAs in batch culture containing previously identified species from sputum samples in protein-rich media.

3.1 Introduction

Asthma is a chronic inflammatory disease characterized by a decline in airway function caused by swelling of the bronchial tubes, goblet cell metaplasia and tissue remodelling [12, 14]. Asthma has been associated with environmental factors [6], genetic factors [7] and host factors such as smoking and obesity [85]. However, in recent years more attention has been brought to the interplay between diet, especially non-digestible carbohydrates, and asthma [60, 71, 72, 84, 161], giving rise to the gut-lung axis [71, 162-164]. Non-digestible carbohydrates undergo fermentation by the colonic microbiota, producing short-chain fatty acids (SCFAs) where the most abundant ones are acetate, propionate and butyrate [44, 78], found in a ratio of 6:2:2 in faeces [78]. Acetate is the SCFA that is found in highest amount in the circulatory system, whereas propionate is readily absorbed by the liver and butyrate by the colonocytes [78]. The average levels of acetate, propionate and butyrate found in faeces are 51mM, 14mM and 14mM respectively [80], while the concentrations in peripheral blood are much lower at 70 μM, 5 μM and 4 μM respectively [81].

Studies have investigated the crosstalk between the gut and the airways. In these studies where rodents were fed either a high-fibre or low-fibre diet, followed by induction of airway inflammation to evaluate the effect of fibre supplementation [60, 84, 165]. The rodents fed a higher-fibre diet did not develop any airway inflammatory symptoms compared to the rodents fed a low-fibre diet. However, what was not investigated was the presence of SCFAs in the airways of the rodents and whether the airway microbiota might play a role in this.

Two studies focusing on cystic fibrosis, have demonstrated that mucus is degraded by the airway microbiota producing SCFAs [110, 111], nevertheless, no studies have investigated the presence

of SCFAs in asthmatics. In this study, I aimed to investigate the presence of SCFAs in two different airway fluids and investigate the interaction of SCFAs with airway microbiota.

3.2 Results

3.2.1 Detection and quantification of SCFAs in airway fluids of controls and asthmatics

To investigate presence of SCFAs in the airways, bronchoalveolar lavage (BAL) and sputum samples were collected from two separate studies, as described in paragraph 2.1.1. BAL samples were collected from controls (n=10) and asthmatics (n=28), processed and run using targeted gas-chromatography coupled to mass spectrometry according to a previously published method [153]. The three main SCFAs were detected in µM concentrations in controls and asthmatics (figure 3.1). Acetate was significantly increased (*P*=0.0308) in asthmatics compared to controls (figure 3.1a). The ratio of acetate, propionate and butyrate in BAL samples was 84:14:2 in controls and 88:11:1 in asthmatics, indicating a higher percentage of acetate and lower percentage of propionate in asthmatics. Isobutyric acid was also detected in BAL samples, however there was no statically significant difference between controls and asthmatics (figure 3.1d). Valerate, Isovalerate, caproate, lactate, 2-hydroxybutyrate and 2-methylbutyrate, were either absent or below detection level in all BAL samples.

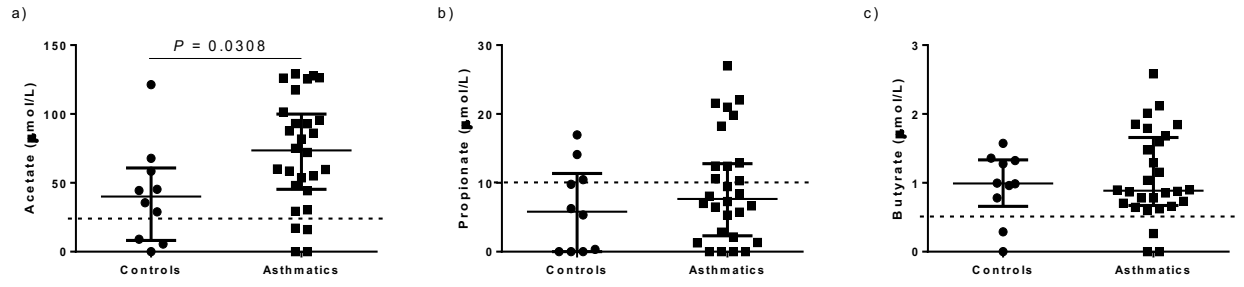


Figure 3.2. SCFAs detected in bronchoalveolar lavage samples from controls and asthmatics, with a significant increase of acetate in asthmatics.

*BAL samples of controls (n=10) and asthmatics (n=28) were analyzed using targeted GC-MS measuring **a)** acetate, **b)** propionate and **c)** butyrate. Dashed lines show the detection limit of the assay. All bars are presented as medians with interquartile range. Unpaired non-parametric two-sided t-test was performed.*

Sputum samples from asthmatics (n=102) were collected and run under identical conditions to the BAL samples [153]. Interestingly, apart from the three main SCFAs (figure 3.2a, 3.2b, 3.2c), which were detected with a ratio of 82:17:1 (acetate, propionate, butyrate), isobutyrate and 2-methylbutyrate were also detected (figure 3.2d, 3.2e). The concentration of SCFAs found in sputum samples were 10 times higher than those previously published to be found in peripheral blood [81]. Valerate, Isovalerate, caproate, lactate, 2-hydroxybutyrate were either absent or below detection level in all sputum samples.

These results demonstrate presence of SCFAs in airway fluids, with an increase in acetate in asthmatics.

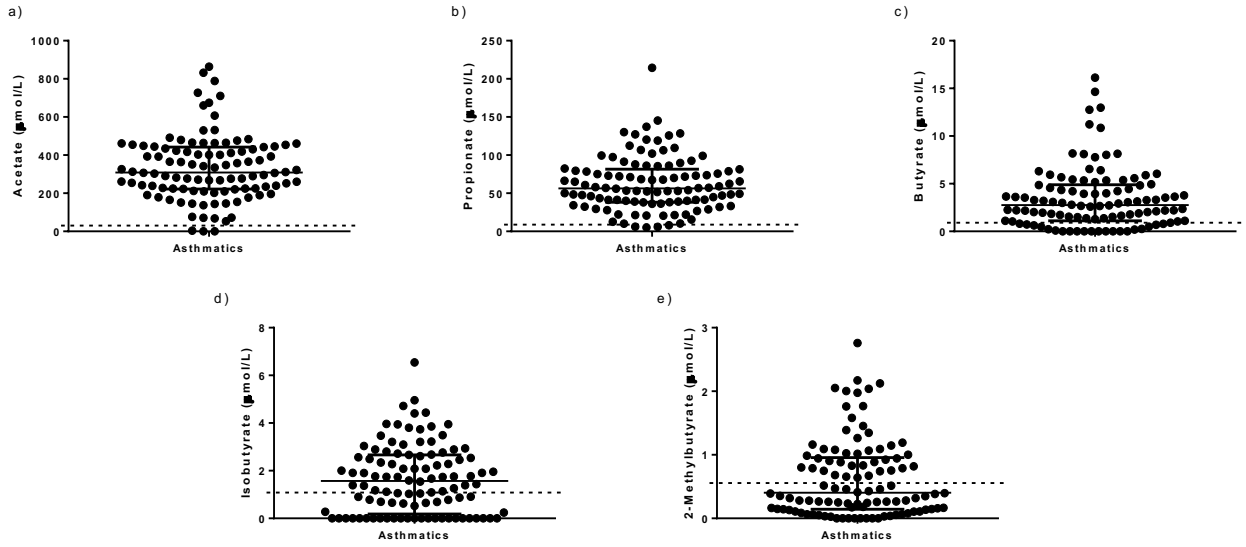


Figure 3.3. SCFAs and BCFAs detected in high concentrations in sputum samples of asthmatics.

*Sputum samples from asthmatics (n=102) were analyzed using targeted GC-MS, measuring **a)** acetate, **b)** propionate, **c)** butyrate, **d)** isobutyrate and **e)** 2-Methylbutyrate. Dashed lines show the detection limit of the assay. All bars are presented as medians with interquartile range.*

3.2.2 Association of sputum SCFAs with microbiota

To get a better understanding of the origin of the detected SCFAs in the airway fluids, 16s rRNA data from the sputum samples [154] were used to perform an association model with the SCFAs data, using Multivariate Association with Linear Models (MaAsLin) [155]. Those bacterial genera that were correlated to a specific SCFA, with a FDR adjusted p-value under 0.05 (q-value) have been summarized in table 1. 2-methylbutyrate had the most correlations with bacterial genera followed by propionate, acetate and isobutyrate. Interestingly, isobutyrate was negatively correlated to all bacteria it was associated with, while acetate was positively correlated to all. Propionate was highly correlated to *Veillonella* spp., *Streptococcus* spp., as was acetate, while both 2-methylbutyrate and isobutyrate were negatively correlated to *Veillonella* spp. (figure 3.3). Many of the species highly correlated to 2-methylbutyric acid were from the *Clostridia* class and from *Neisseria* spp.

This data demonstrates association of airway bacterial genera, some, which have the ability to produce SCFA, with airway SCFA.

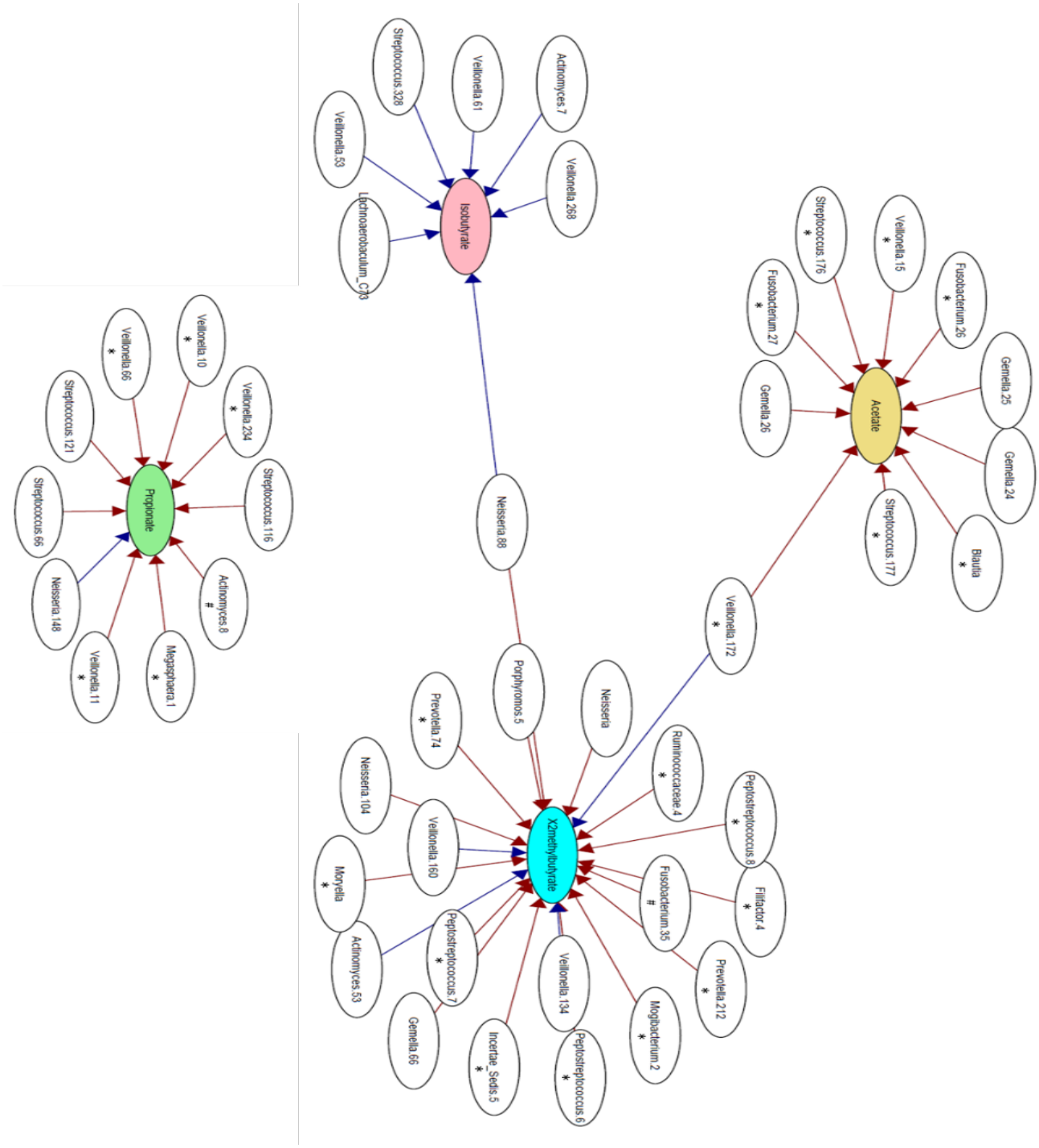


Figure 3.4. Airway SCFAs in asthmatics are associated with SFCAs producing bacteria genera.

*Linear association model was performed between airway SCFAs and airway bacterial genera from asthmatics. Red arrows demonstrate positive association and blue arrows negative association. Each association of one SCFA and one bacterial genus has a FDR corrected p-value<0.05. Genera with * have in previous literature demonstrated the ability to produce the specific SCFAs and genera with # have previously demonstrated the ability to produce other SCFAs.*

3.2.3 Airway species cultured in presence of mucus lead to higher production SCFAs

To validate whether the airway-derived species could produce SCFAs through mucus degradation, selected sputum species were isolated, sequenced and identified followed by culture with and without 1% v/w of mucin (table 2). SCFA concentrations were measured in the supernatant of each species whereupon differences were detected for acetate, propionate, valerate, isovalerate, caproate, lactate, 2-methylbutyrate and 2-hydroxybutyrate, but not for butyrate and isobutyrate. These results suggest that the selected airway species might not produce higher amounts of butyrate or isobutyrate in presence of mucin (figure 3.4c and 3.4d). Four *Prevotella spp.* and *Gemella haemolysans* produced more acetate in presence of mucin (figure 3.4a) compared to cultures containing no mucin. Two of the *Prevotella spp.* also produced more propionate in presence of mucin, together with *Streptococcus constellatus*, *Neisseria flavescens* and *Gemella haemolysans.* Interestingly, the two selected *Neisseria spp.*, *G. haemolysans* and *Veillonela atypica* produced valerate, while caproate was only produced by the *Neisseria spp* (figure 3.4e and 3.4g). Lactate was in general produced by all species, but was only increased with addition of mucin by two *Prevotella spp*. (figure 3.4h). Furthermore, branched SCFAs (isovalerate, 2-methylbutyric acid and 2-hydroxybutirc acid) (figure 3.4f, 3.4i, 3.4j) were increased for only three species, *Prevotella nigrescens, Neisseria subflava* and *G. haemolysans*, demonstrating proteolytic activity of mucin to SCFAs.

These findings suggest that the isolated airway species have the ability to produce SCFAs through mucin degradation.

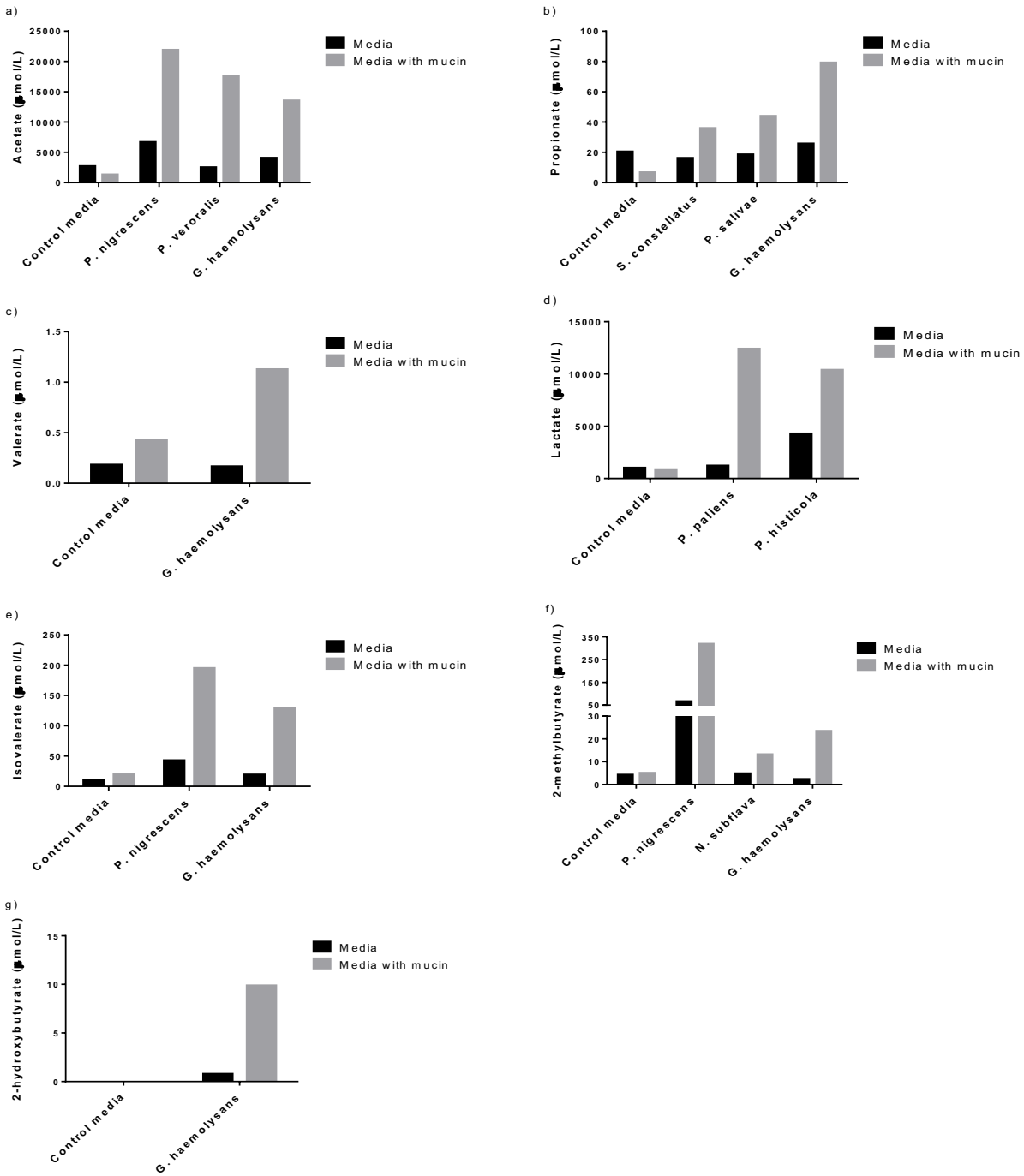


Figure 3.5. Isolated airway bacterial strains form asthmatics produce more SCFAs in presence of mucin.

Airway bacterial strains were isolated from asthmatics and cultured using batch fermentation in presence of only media and media supplemented with 1% w/v mucin (n=1). Samples were taken after 7 days of culture and analyzed using GC-MS measuring ***a)*** *acetate,* ***b)*** *propionate,* ***c)*** *valerate,* ***d)*** *caproate,* ***e)*** *lactate,* ***f)*** *isovalerate,* ***g)*** *2-methylbutyrate,* ***h)*** *2-hydroxybutyrate. Species producing at least 2-fold higher levels of SCFAs in media containing mucin compared to media without mucin were presented and considered to be significant. All bars are presented as medians.*

3.3 Discussion

To our knowledge, this is the first time anyone has reported and quantified SCFAs in the airway fluids of asthmatics. SCFAs were detected in micromolar concentrations in both BAL and sputum, whilst higher amounts of SCFAs were found in BAL samples of asthmatics compared to controls. The amount of SCFAs quantified in sputum samples of asthmatics, suggested that the origin of SCFAs might not be from the gut [81], as the concentrations were 100-fold higher than what those previously reported in peripheral blood. The ratio of acetate, propionate and butyrate, which has been found to be 60:20:20 in faeces [78] and 89:6:5 in peripheral blood [81] was different between controls and asthmatics but also between airway samples. Control BAL samples, had a higher ratio of propionate, 84:14:2, and lower ratio of acetate compared to asthmatics, 88:11:1. Furthermore, comparison between BAL and sputum samples of asthmatics demonstrated that sputum samples had a higher ratio of propionate and lower ratio of acetate, 82:17:1. Even though these ratios were not significantly different, the trend could be due to differences in microbial composition and abundance between the asthmatics and controls, but also location within the airways.

The presence of SCFAs within the airways was further examined by investigating the association between sputum SCFAs and sputum bacteria, where we found several bacterial genera correlated to several SCFAs, with 2-methylbutyric acid demonstrating the highest number of correlations. Several studies have demonstrated presence of airway microbiota [57, 58] as well as a perturbed airway microbiota in asthmatics. Interestingly, Ganesan *et al.* demonstrated that *Lactococcus lactis* had the ability to degrade leucine producing 2-methylbutyric acid *in vitro* [166]. Furthermore, it has been shown that leucine is a component of mucus in the airways [167].

As previously mentioned, two studies focusing on cystic fibrosis, demonstrated that mucus is being degraded by the airway microbiota producing SCFAs [110, 111]. People with asthma, as in people with cystic fibrosis, have excessive mucus production, supporting our results, which identified higher amounts of SCFAs in asthmatics compared to controls. Moreover, the species degrading mucus from the cystic fibrosis patients and producing SCFAs belonged to genera that were highly correlated to the SCFAs found in the sputum samples in the current study. Many of the genera levels that were correlated with specific SCFAs in the sputum samples, have been demonstrated to produce SCFAs in previous literature [168-170].

To further validate the hypothesis of airway-derived SCFAs, several species were isolated from sputum samples of asthmatics and cultured *in vitro* for 7 days in mucin-enriched or non-enriched media. Several species produced more SCFAs in presence of mucin, validating the theory that bacteria in the airways of asthmatics can produce SCFAs using mucin as a substrate. Particularly interesting was that butyrate and isobutyrate concentrations remained unchanged, suggesting that they might not be of great importance for the airways. *Prevotella* nigrescens, *Neisseria subflava* and *Gemella haemolysans* produced more branched chain fatty acids in presence of mucin. Studies have shown that *Prevotella spp.* and *Neisseria spp.* are increased in asthmatics compared to controls [58, 61, 171, 172]. Both *Neisseria flavescens* and *Streptococcus constellatus* produced more propionate in presence of mucin. Interestingly, studies have also demonstrated increased abundance of *Streptococcus spp.* in asthmatic airways [172, 173].

Furthermore, *Gemella haemolysans* produced many different SCFAs and the majority of them were increased upon addition of mucin to the media. Interestingly, not much has been reported about *Gemella spp.* but one study demonstrated increased abundance of *Gemella spp.* in

eosinophilic asthma compared to neutrophilic [174]. It remains to be seen whether *G. haemolysans* plays an important role in asthmatics. Interestingly, *V. atypica* produced copious amount of propionate, but with no significant difference upon addition of mucin. Furthermore, very low levels of lactate were observed compared to other species, which could suggest that propionate is being partly converted from lactate fermentation through the acrylate pathway [89, 90]. Three species also demonstrated their proteolytic activity by producing higher levels of branched chain fatty acids in presence of mucin, further enhancing the hypothesis that the detected airway SCFAs are originating from the airways and not the gut.

Finally, the limitations in this study must be acknowledged, the low number of BAL samples and the absence of controls for sputum samples, which would have been beneficial to do a direct comparison between controls and asthmatics. However, these experiments were novel and demonstrating proof of principle. Furthermore, association between the microbiota and concentration of SCFA concentrations might be considered to be modest, as the bacteria work together as a community. Moreover, the species isolated from the airways of asthmatics were selective and among an excess of 4000 airway OTUs identified by 16s rRNA sequencing [175]. The experiment was only performed once and should be repeated for validation.

3.4 Conclusions

SCFAs were detected in both BAL and sputum samples, where in BAL samples, acetate was significantly increased in asthmatics compare to controls. Association of SCFAs with airway microbiota demonstrated a positive association with SCFAs-producing species suggesting that the SCFAs are being produced in the airways. Further experiments utilizing isolated microbial species from the sputum samples of asthmatics demonstrated production of SCFAs from mucin degradation. Furthermore, the physiological effect of SCFAs in the airways, in particular to the airway epithelium, remains unknown and in need of investigation.

Chapter 4 Exploring the effect of SCFAs on airway epithelium

Hypothesis

It is hypothesized that the airway epithelium plays a role in the inhibition of airway inflammation and that pre-treating the epithelium with SCFAs will inhibit the development of inflammation.

Aims

- Investigate the immunological effect of apical and basolateral stimulation of SCFAs on differentiated airway epithelium
- Examine the physiological difference of propionate stimulation between transwell and airway chips
- Characterize the metabolic differences between apical and basolateral stimulation of SCFAs on differentiated airway epithelium

Objectives

- Treat submerged bronchial airway epithelial cell line (BEAS-2B), submerged and differentiated bronchial (NHBE) and small airway (SAEC) epithelial cells with SCFAs, IL-13 and house dust mite (HDM) to determine cell viability (using lactated dehydrogenase (LDH) assay), presence of receptors, inflammatory cytokines, mucus and cilia production using qPCR and ELISA.

- Apply metabolic profiling of cell supernatant using ^{1}H NMR spectroscopy to determine the metabolic effect of SCFAs and IL-13 stimulation.
- Treat airway chips with propionate to determine differences in cytokine and metabolite response between chip and transwell using qPCR, ELISA and ^{1}H NMR spectroscopy.

4.1 Introduction

The airway epithelium is the first site of contact for inhaled air, which could contain allergens, bacteria and other foreign substances [23, 67]. It is therefore vital that the airway epithelium is correctly functioning to manage foreign substances that are either inhaled or released upon cellular damage or stress. The epithelial barrier together with surrounding cells and bronchial wall work in conjunction to prevent the harmful substances from entering the epithelial tissue by emitting an immune response by releasing cytokines and recruiting immune cells [64].

Airway remodelling occurs in asthmatics, resulting in damage to the epithelial lining and higher susceptibility to allergens, as the epithelium is having a hard time repairing itself [31, 32, 70]. One of the four cell types of the airway epithelium, mucus producing goblet cell, are increased in asthmatics, resulting in excessive mucus production [68]. Furthermore, several proteins such as E-cadherin, ZO-1 and occluding, which are in charge of holding the epithelial lining together are downregulated in asthmatics resulting in an impaired barrier [69].

The beneficial role of SCFAs in metabolic disease is well known [77, 79] as studies have suggested that gut-derived SCFAs are anti-inflammatory [82], promote epithelial integrity [71] and regulate the size and pool of T regulatory cells in the colon [83]. They have also been shown to affect other processes in the body including lipid, glucose and cholesterol metabolism [78]. It has been suggested that SCFAs operate through Free Fatty Receptors, FFAR2 and FFAR3, where FFAR3 has a higher affinity for acetate and propionate while FFAR2 for propionate and butyrate [78]. Moreover, it is not well-known weather the receptors are expressed on the airway epithelium and what role they may play in airway homeostasis.

As shown in chapter 3, we demonstrated the production of airway SCFAs by the airway microbiota. In order to understand the implication of the presence of these metabolites in the airways, we sought to understand their interaction with the airway epithelium.

4.2 Results

4.2.1 Evaluating the effect of SCFAs treatment on submerged BEAS-2B and NHBE cells

To determine optimal concentration of SCFAs, and avoid cytotoxic effect on the cells, lactate dehydrogenase viability assay was utilized. Firstly, human normal bronchial epithelial cell line (BEAS-2B) was dosed with a range of concentrations of acetate and propionate where 500mM of both SCFAs demonstrated a cytotoxic effect (figure 4.1a). Taking into consideration the highest amount of SCFAs detected in sputum samples, and using a concentration that is physiologically relevant, a maximum concentration of 10mM was chosen for both acetate and propionate. Furthermore, the same viability assay was utilized for submerged primary bronchial epithelial cells, where the cells were stimulated with a range of acetate, propionate and butyrate, concentration to a maximum dose of 10mM, which demonstrated no cytotoxic effect for any of the three main SCFAs (figure 4.1b).

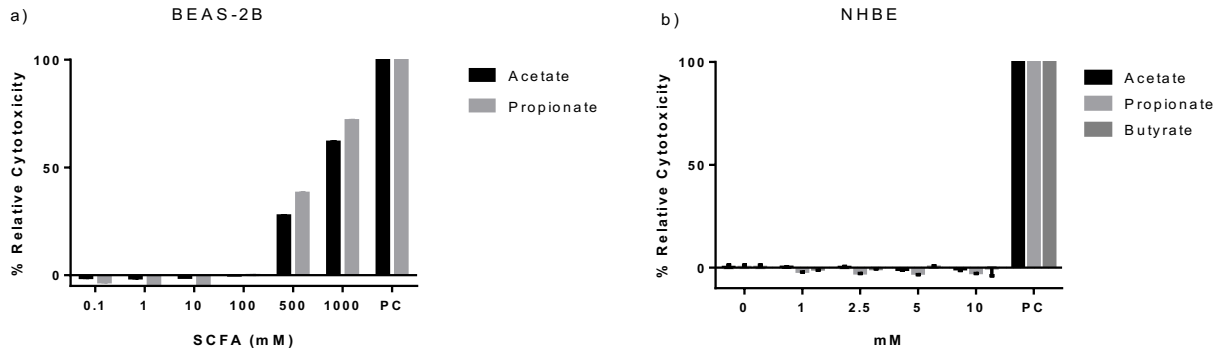


Figure 4.1 Cell viability assay demonstrating, which concentrations of acetate, propionate and butyrate are toxic to the airway epithelium.

LDH cytotoxicity assay was performed to measure the cytotoxic effect of the SCFAs during 36 hours at a range of different concentrations for a) human normal bronchial epithelial cell line (BEAS-2B) and b) human normal primary airway epithelial cell (NHBE). PC – Positive control.

To investigate the impact of SCFAs on BEAS-2B, and whether SCFAs are acting anti-inflammatory, the cells were stimulated with 10 and 1mM for 24 hours. All three SCFAs demonstrated a decrease in IL-6 in a dose-dependent manner (figure 4.2a), while an increase in CXCL8 was observed for 1mM of all three SCFAs followed by a decrease back to baseline for 10mM (figure 4.2b). Additional treatment of 10 ng/ml of IL-13 for another 24h demonstrated the same trend, with an increase at 1mM of SCFAs followed by a decrease back to baseline, for both IL-6 and CXCL8 (figure 4.2c, 4.2d).

These results suggest that SCFAs might not be acting in an anti-inflammatory manner.

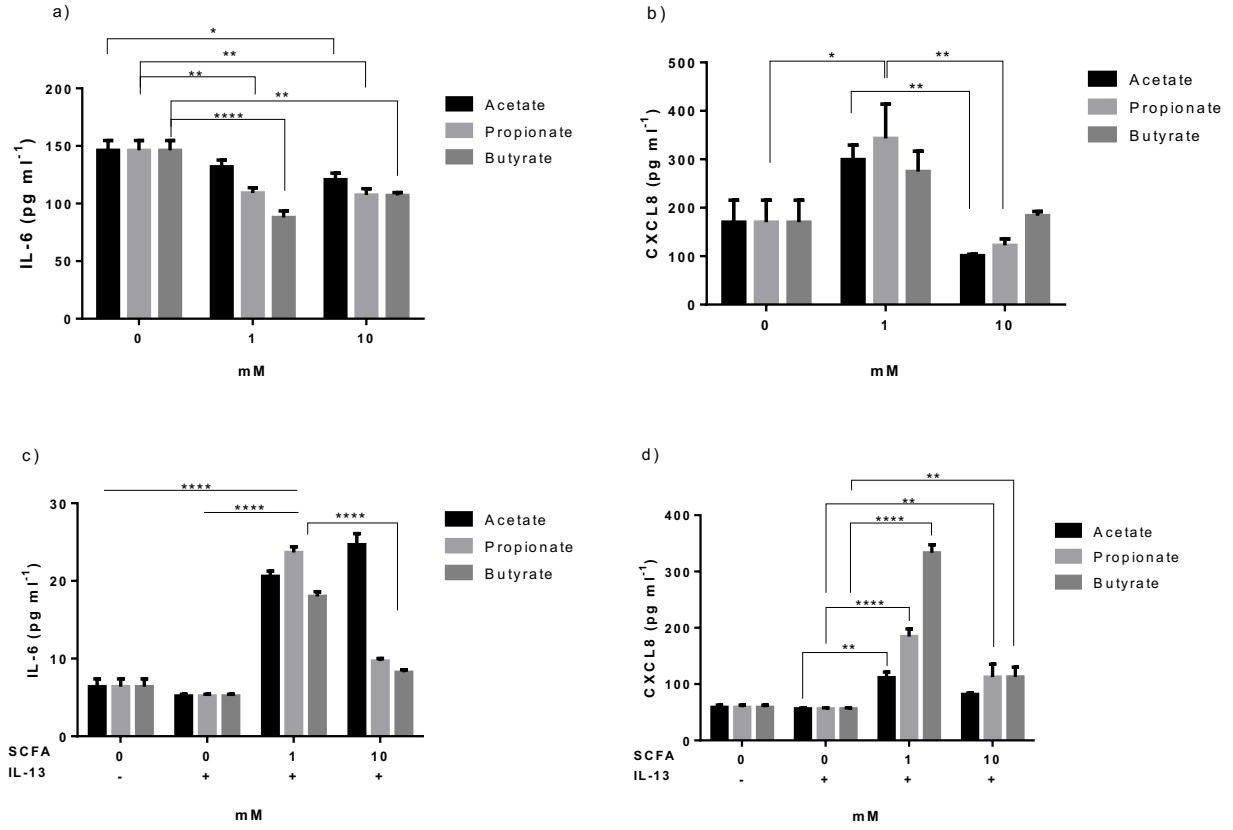


Figure 4.2. Mixed results observed when treating BEAS-2B with SCFAs.

Protein levels of treatment of SCFAs for 36 hours on BEAS-2B, ***a)*** *IL-6 and* ***b)*** *CXCL8. Protein levels of treatment of SCFAs for 12 hours followed by addition of IL-13 for another 24 hours,* ***c)*** *IL-6,* ***d)*** *CXCL8. All bars are presented as triplicates and medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

In order to investigate whether the same response was observed with submerged primary cells derived from human airways, we performed the same experiment with the most abundant SCFAs, acetate and propionate, on primary bronchial epithelial cells (NHBE), where propionate showed the greatest difference. Interestingly, the SCFAs receptors FFAR2 and FFAR3 were detected on the airway epithelium and decreased in a dose-dependent manor for propionate, with no difference observed for acetate (figure 4.3a, 4.3b). Eotaxin-3, IL-6 and CXCL8 were all increased when stimulated with 10mM of propionate, but no differences were observed for 1mM of propionate or for acetate (figure 4.3c, 4.3d, 4.3e). These results suggest that acetate and propionate are having a modulating effect.

Additional treatment of house dust mite (HDM), an allergen used to develop airway inflammation in mice, for 24 hours did not exhibit different results but rather enhanced what was already seen with only SCFA treatment (figure 4.4a, 4.4b, 4.4c, 4.4d, 4.4e).

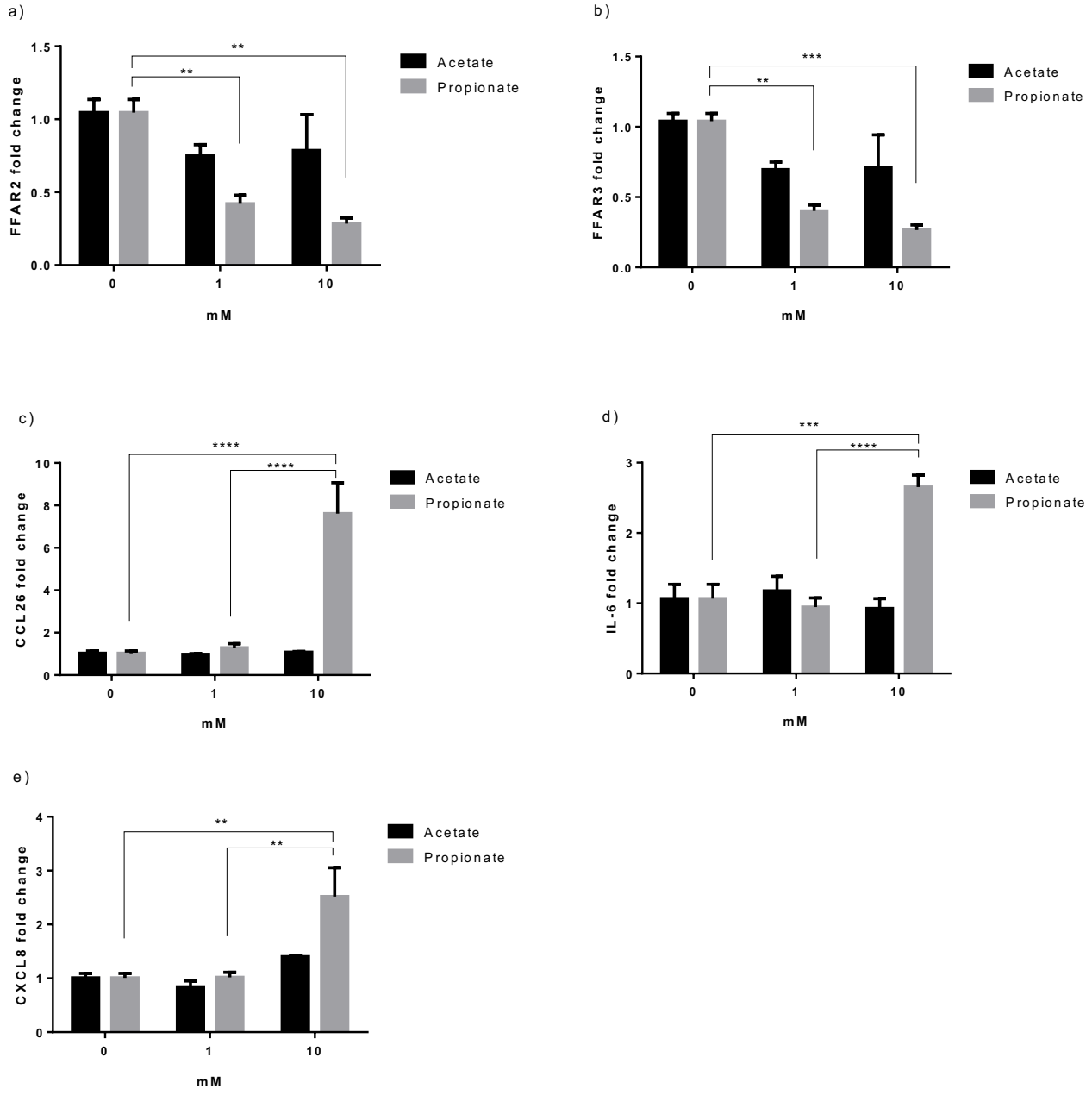


Figure 4.3. Propionate, and not acetate, promotes an immunomodulatory response and decrease in SCFAs receptors when treated on submerged airway epithelium.

Gene expression of treatment of acetate and propionate for 36 hours on submerged airway epithelium, ***a)*** *SCFAs receptor FFAR2,* ***b)*** *SCFAs receptor FFAR3,* ***c)*** *CCL26,* ***d)*** *IL-6 and* ***e)*** *CXCL8. All bars are presented as triplicates and medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

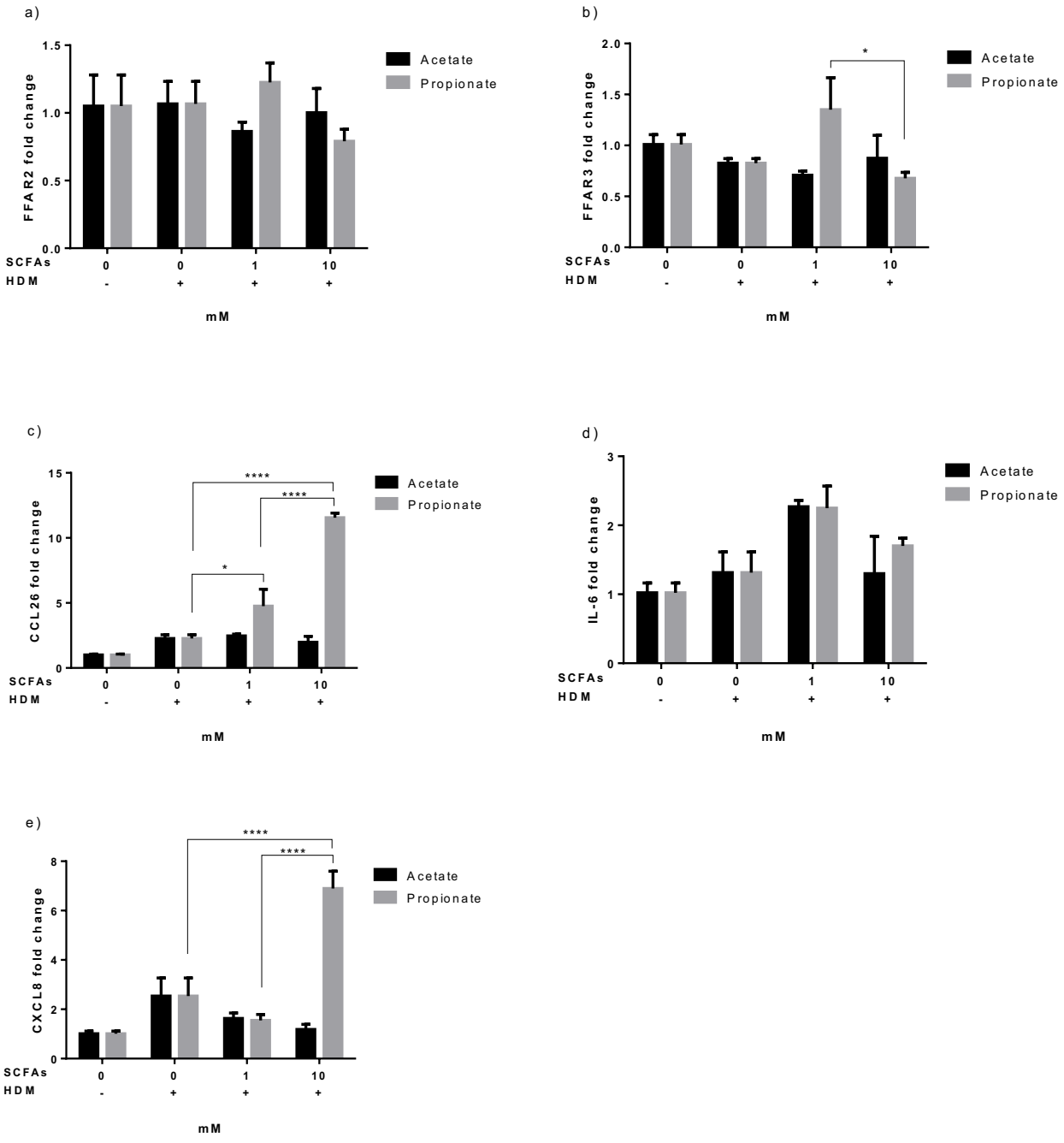


Figure 4.4. Propionate, and not acetate, promotes an immunomodulatory response and decrease in SCFAs receptors when treated on submerged airway epithelium.

Gene expression of treatment of acetate and propionate for 12 hours, followed by HDM for 24 hours, on submerged airway epithelium, ***a)*** *SCFAs receptor FFAR2,* ***b)*** *SCFAs receptor FFAR3,* ***c)*** *CCL26,* ***d)*** *IL-6 and* ***e)*** *CXCL8. All bars are presented as triplicates and medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

4.2.2 Treatment of differentiated bronchial epithelium with acetate and propionate demonstrates an immunomodulatory response

In order to investigate whether differentiated primary airway epithelial exhibited the same response, upon stimulation with acetate and propionate, as submerged cells did, primary bronchial epithelial (NHBE) cells were cultured at air-liquid interphase (ALI) between 18-22 days to develop a fully differentiated epithelium. The epithelial cells were incubated with acetate and propionate on either apical (directly on the cells) or the basolateral (in the media) side for 36 hours. FFAR2 and FFAR3 gene expression was detected in airway epithelial cells (figure 4.5a, 4.5b, 4.6a, 4.6b). Apical stimulation demonstrated a trend (however, it wasn't statistically significant) towards decreased expression of the receptors with increased concentration of acetate and propionate, while no difference was seen for basal stimulation. IL-6 and CXCL8 were increased with either apical or basolateral stimulation with acetate or propionate (4.5d, 4.5e, 4.6d, 4.6e). However, Eotaxin-3 mRNA was only increased for 10mM of basolateral propionate (figure 4.5c) and not for apical propionate at any concentration (figure 4.6c). A trend towards decreased levels of mucus with 10mM of propionate incubated on either side of the epithelium was observed (figure 4.5f, 4.6f), while there was a significant increase of MUCA5C at 1mM of acetate incubated on the basolateral side (figure 4.6f), which decreased down to baseline at 10mM. Cilia production was unchanged for apical stimulation (figure 4.5g) but decreased with increasing levels of basolateral acetate and propionate. However, this decrease was only statistically significant for propionate (figure 4.6g).

These results suggest that acetate and propionate act in an immunomodulatory manner when treated to airway epithelial cells.

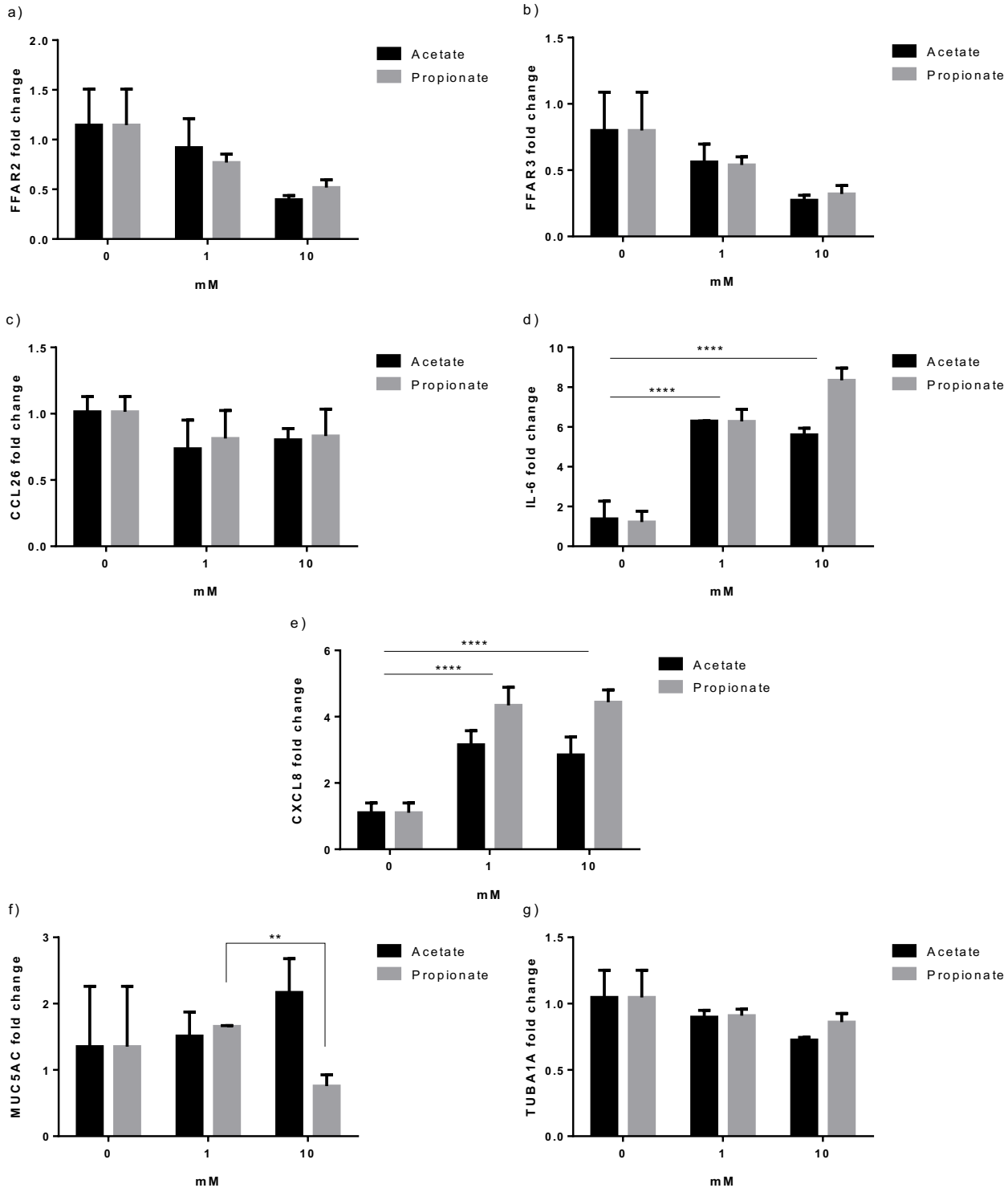


Figure 4.5. Acetate and propionate modulate gene expression, through elevation of IL-6 and CXCL8 but not CCL26, when treated on the apical side of the airway epithelium.

*Gene expression of treatment of acetate and propionate for 36 hours on the apical side of the differentiated airway epithelium, a) SCFAs receptor FFAR2, b) SCFAs receptor FFAR3, c) CCL26, d) IL-6, e) CXCL8, f) MUC5AC and g) TUBA1A. Each bar represents triplicates and the experiment was repeated 3 times. All bars are presented as medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

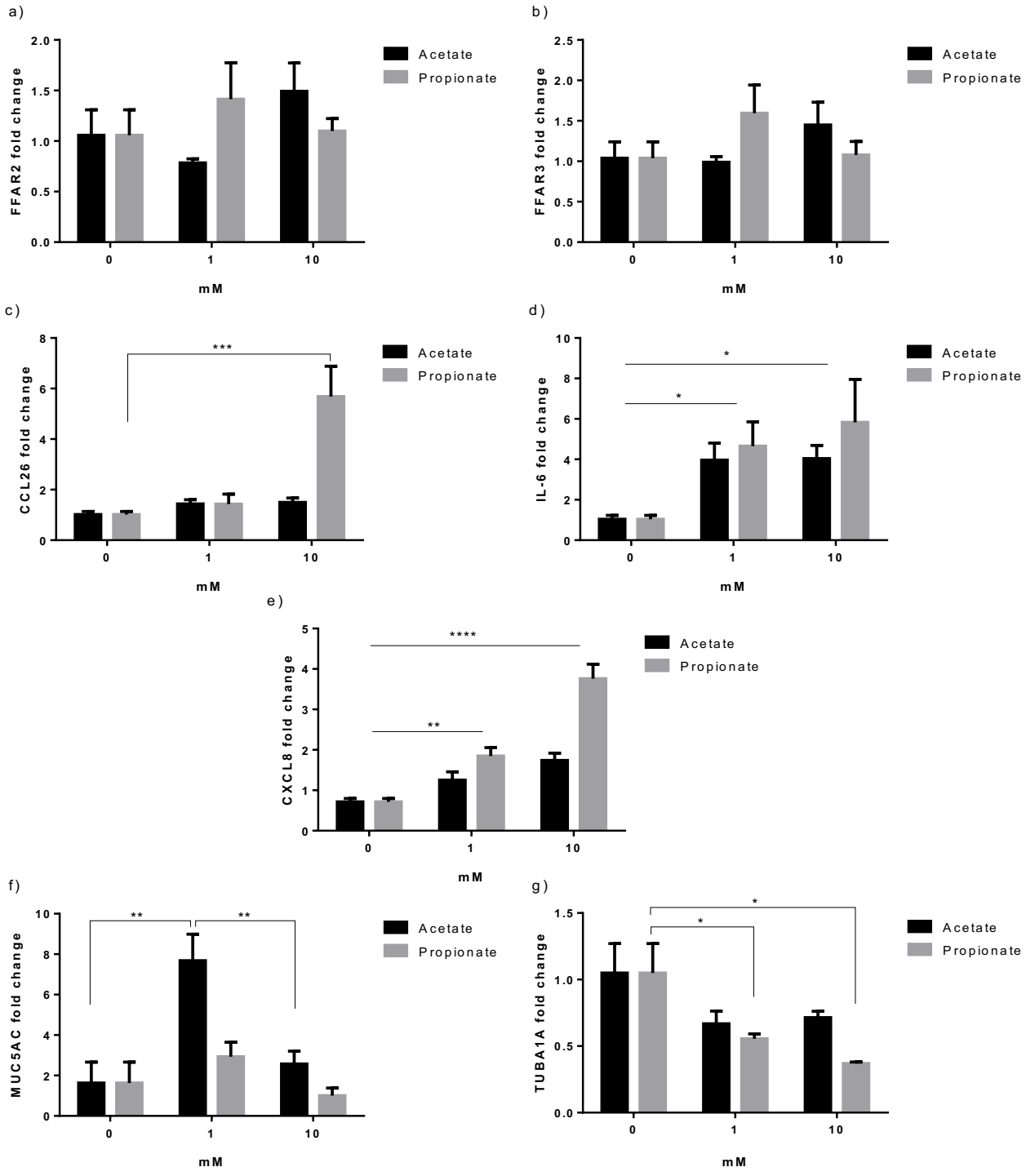


Figure 4.46. Acetate and propionate promote an immunomodulatory response, through elevation of IL-6 and CXCL8, and only propionate for CCL26, when treated on the basolateral side of the airway epithelium.

Gene expression of treatment of acetate and propionate for 36 hours on the basolateral side of the differentiated airway epithelium, ***a)*** *SCFAs receptor FFAR2,* ***b)*** *SCFAs receptor FFAR3,* ***c)*** *CCL26,* ***d)*** *IL-6,* ***e)*** *CXCL8,* ***f)*** *MUC5AC and* ***g)*** *TUBA1A. Each bar represents triplicates and the experiment was repeated 3 times. All bars are presented as medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001*

Furthermore, to evaluate the possible anti-inflammatory potency of SCFAs in allergic airway condition, the epithelial cells were pre-treated with SCFAs on apical or basolateral side for 12 hours followed by IL-13 for additional 24 hours. The receptors emitted the same response, as with only SCFAs, however, some gene expressions were significant decreased for 10mM of propionate on both sides of the epithelium (figure 4.7a, 4.7b, 4.8a, 4.8b). Both IL-6 and CXCL8 were significantly increased, in a dose-dependent manner, on both sides of the epithelium compared to only IL-13 treated cells (figure 4.7d, 4.7e, 4.8d, 4.8e). IL-13 treatment elevated gene expression of Eotaxin-3 on both the apical or basolateral side of the epithelium (figure 4.7c, 4.8c), with a slightly greater increase with addition of acetate and propionate on the basolateral side (figure 4.8c). Mucus production was increased and peaked at 1mM in response to stimulation of both acetate and propionate on both sides of the epithelium (figure 4.7f, 4.8f). 10mM of propionate brought MUC5AC production back to baseline, which was significant with apical stimulation (figure 4.7f). Cilia production was significantly decreased for 1mM and 10mM of propionate on apical side (figure 4.7g) and the trend was the same for basolateral stimulation (figure 4.8g). To conclude, modulation of gene expression was only observed with the addition of IL-13 and not without it.

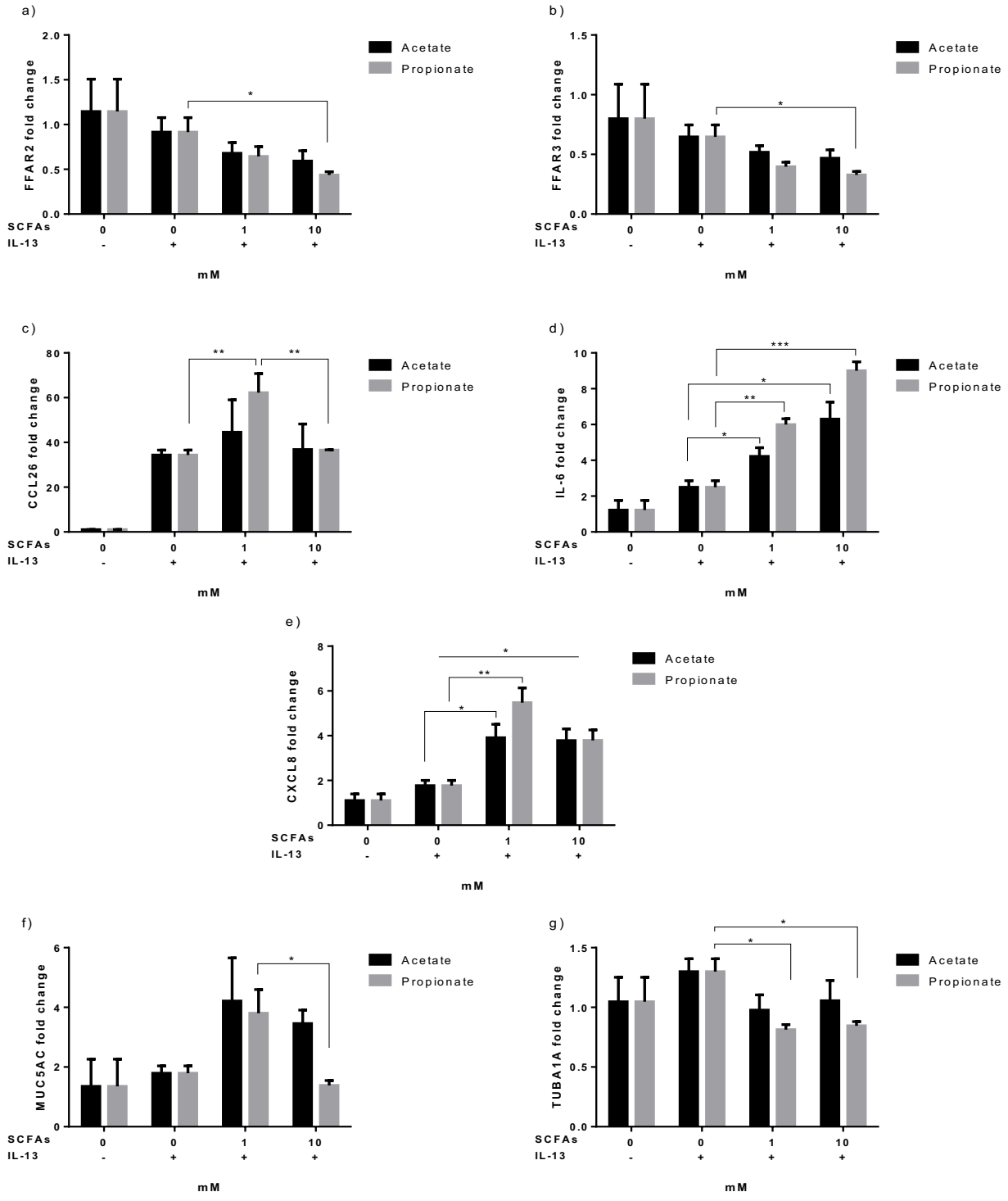


Figure 4.47. Additional treatment of IL-13 enhances the immunomodulatory response and decreases expression of the SCFAs receptors.

Gene expression of treatment of acetate and propionate for 12 hours on the apical side of the differentiated airway epithelium, followed by addition of IL-13 for another 24 hours, ***a)*** *SCFAs receptor FFAR2,* ***b)*** *SCFAs receptor FFAR3,* ***c)*** *CCL26,* ***d)*** *IL-6,* ***e)*** *CXCL8,* ***f)*** *MUC5AC and* ***g)*** *TUBA1A. Each bar represents triplicates and the experiment was repeated 3 times. All bars are presented as medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01, *** P<0.001.*

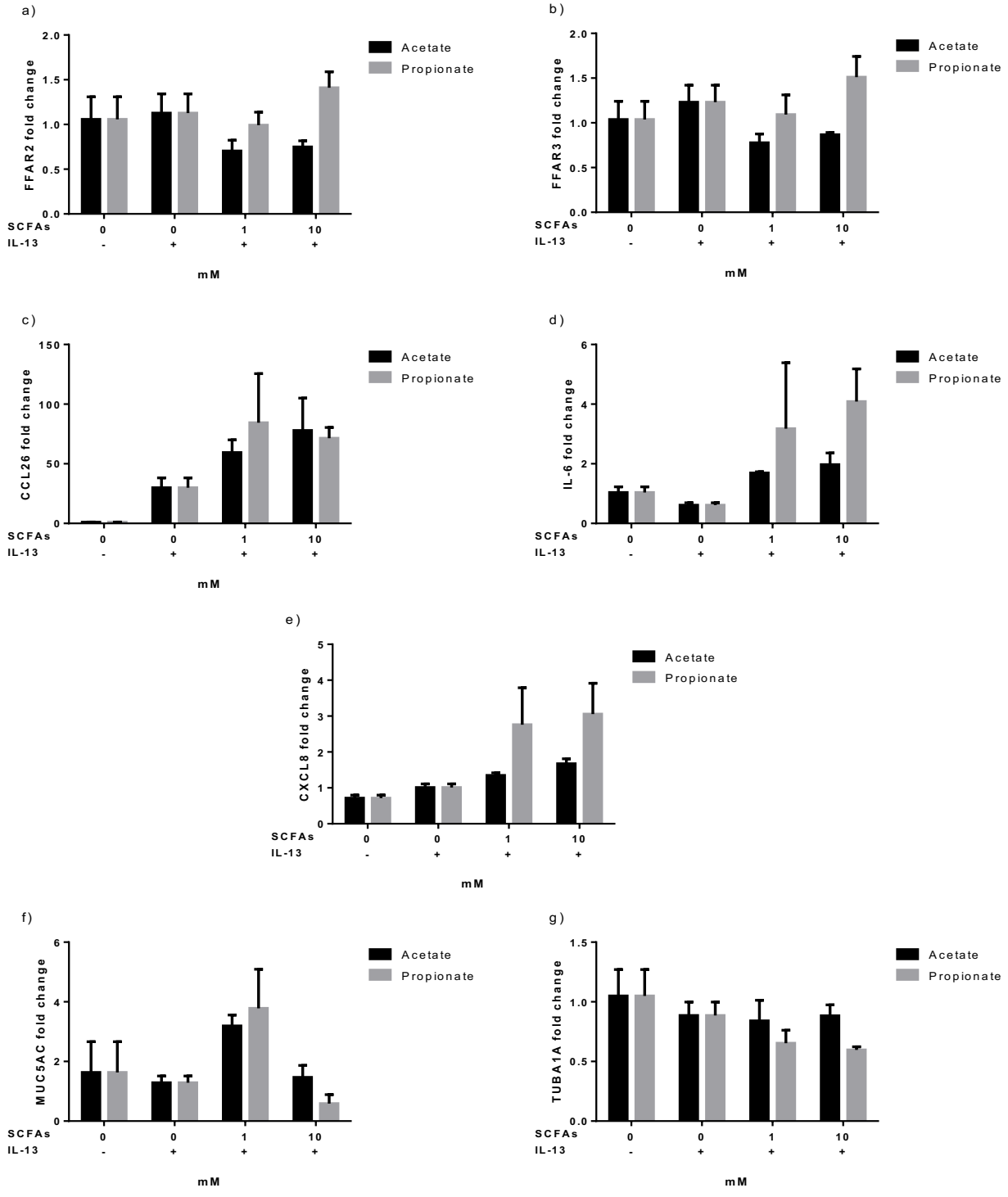


Figure 4.48. Additional treatment of IL-13 did not demonstrate a statistically significant increase of IL-6 and CXCL8, when acetate and propionate were treated on the basolateral side of the airway epithelium.

Gene expression of treatment of acetate and propionate for 12 hours on the apical side of the differentiated airway epithelium, followed by addition of IL-13 for another 24 hours, a) SCFAs receptor FFAR2, b) SCFAs receptor FFAR3, c) CCL26, d) IL-6, e) CXCL8, f) MUC5AC and g) TUBA1A. Each bar represents triplicates and the experiment was repeated 3 times. All bars are presented as medians with interquartile range. Unpaired parametric two-sided t-test was performed.

In order to investigate if the response of the proteins are in line with what is seen for the mRNA, the media was analysed for cytokines levels for previously described experiments. Interestingly, when investigating the response of apical and basolateral stimulation of Eotaxin-3, IL-6 and CXCL8, slightly different results were observed. Stimulation of both acetate and propionate on both sides of the epithelium increased IL-6 levels (figure 4.9b, 4.9e 4.10b), apart for 10mM of basolateral propionate together with IL-13 (figure 4.10e). Only dosing with apical acetate demonstrated an immunomodulatory response for CXCL8, but propionate simulation remained unchanged (figure 4.9c). Interestingly, basolateral stimulation of acetate and propionate demonstrated a decrease in CXCL8 levels (figure 4.10c). Eotaxin-3 remained mostly unchanged. To conclude, mixed results were observed when investigating the protein response for apical or basolateral treatment of acetate or propionate, suggesting that these experiments need to be performed using several donors to conclude definite results.

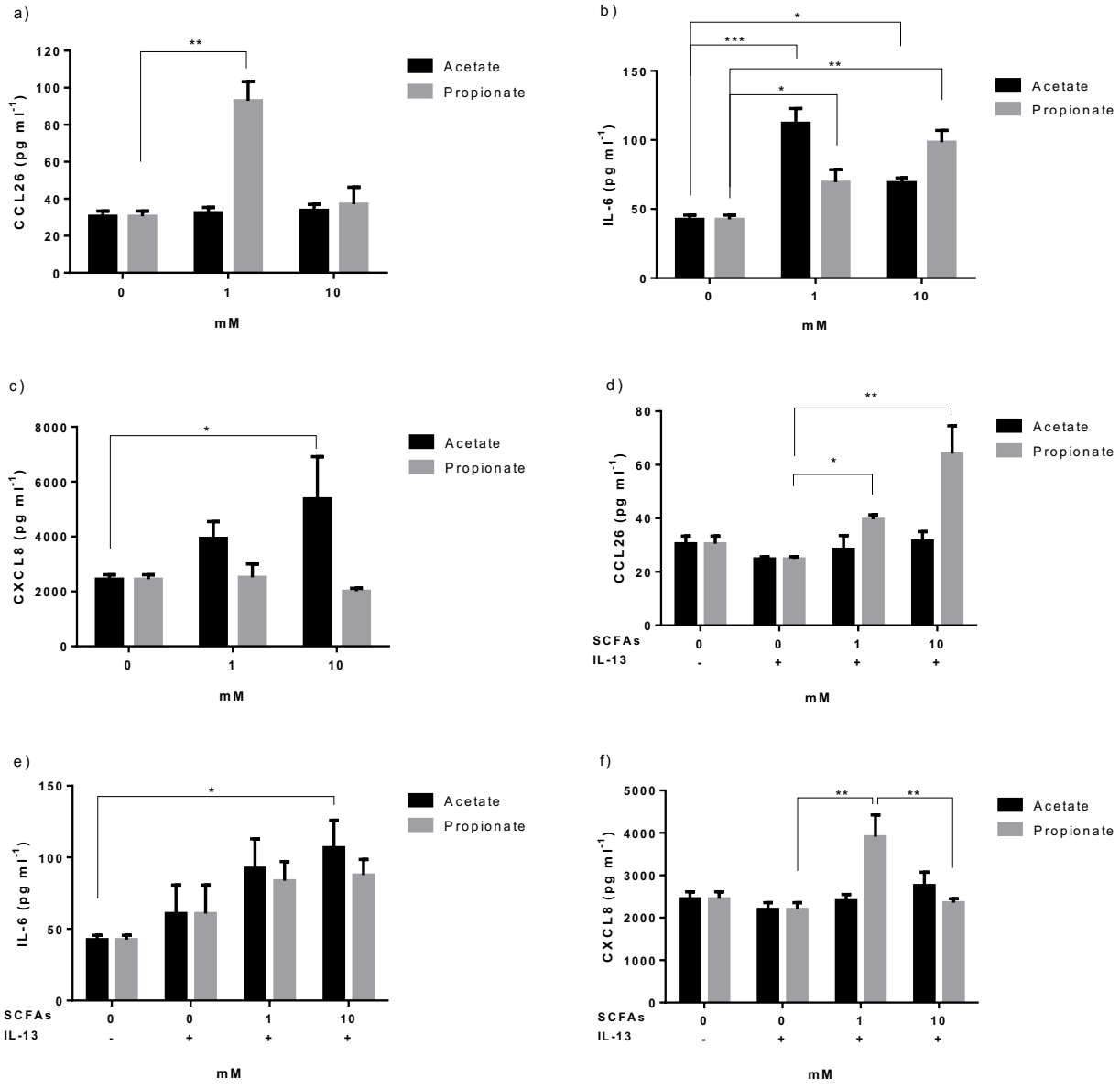


Figure 4.49. Acetate and propionate promote an immunomodulatory response, through elevation of IL-6, CXCL8 and CCL26, when treated on the apical side of the airway epithelium.

Protein levels of treatment of acetate and propionate for 36 hours on the apical side of the differentiated airway epithelium, ***a)*** *CCL26,* ***b)*** *IL-6 and* ***c)*** *CXCL8. Protein levels of treatment of acetate and propionate for 12 hours on the apical side of the differentiated airway epithelium, followed by addition of IL-13 for another 24 hours,* ***d)*** *CCL26,* ***e)*** *IL-6 and* ***f)*** *CXCL8.All bars are presented as triplicates and medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01, *** P<0.001.*

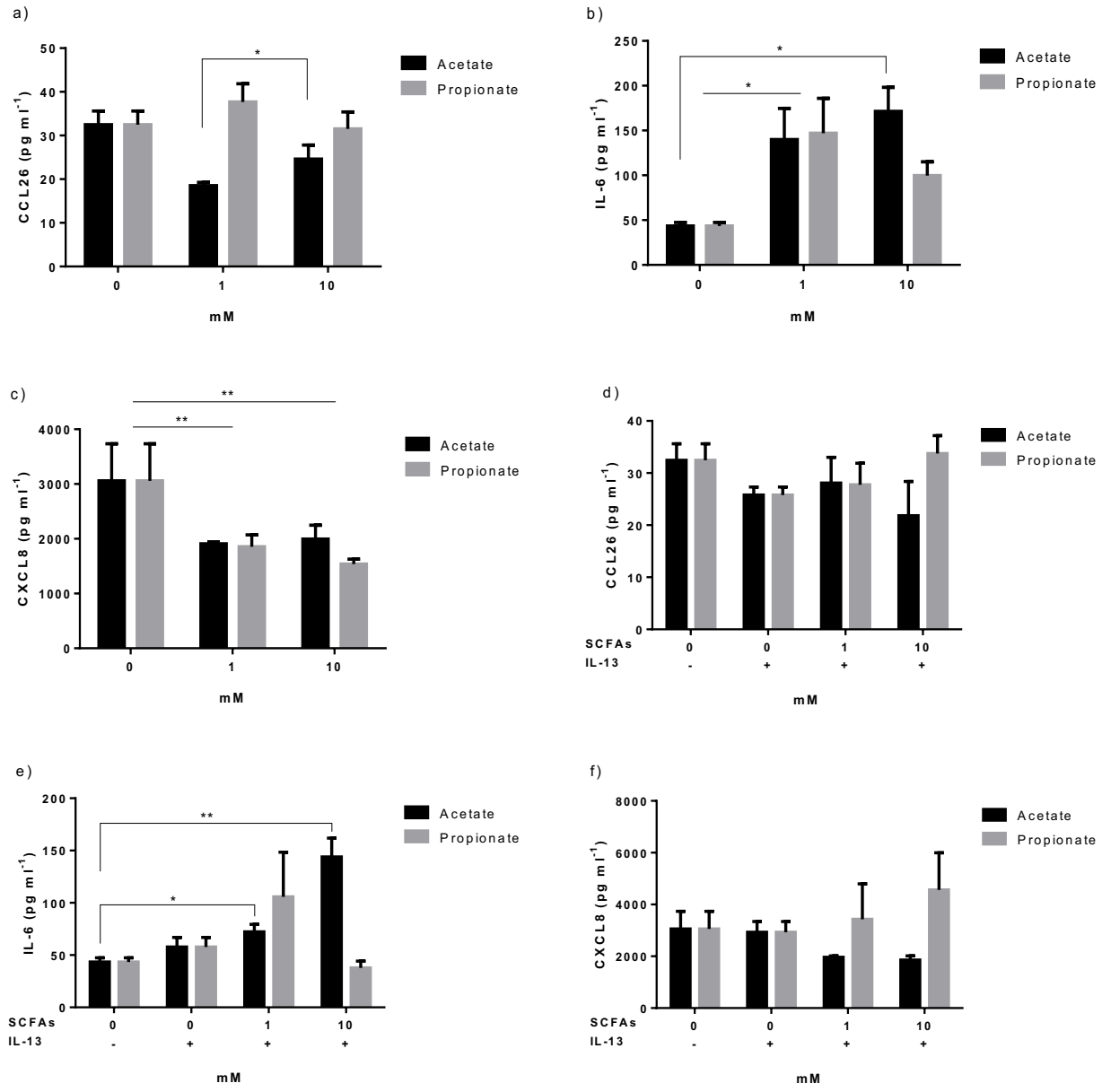


Figure 4.50. Mixed results are observed when acetate and propionate are treated on the basolateral side of the airway epithelium.

Protein levels of treatment of acetate and propionate for 36 hours on the basolateral side of the differentiated airway epithelium, ***a)*** *CCL26,* ***b)*** *IL-6 and* ***c)*** *CXCL8. Protein levels of treatment of acetate and propionate for 12 hours on the basolateral side of the differentiated airway epithelium, followed by addition of IL-13 for another 24 hours,* ***d)*** *CCL26,* ***e)*** *IL-6 and* ***f)*** *CXCL8.All bars are presented as triplicates and medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01.*

4.2.3 Treatment of differentiated bronchial epithelium with butyrate exhibits a slightly different response than acetate and propionate

In order to evaluate the response of all three main SCFAs, and determine if they behave in a similar manner, stimulation of butyrate to the airway epithelium was also investigated. Treatment of butyrate in the same approach as previously described for acetate and propionate, demonstrated a slightly different response. No difference was seen for the receptors when only dosing with SCFAs (figure 4.11a, 4.11b, 4.12a, 4.12b). However, addition of IL-13 demonstrated a trend towards increased expression of both receptors (figure 4.13a, 4.13b, 4.14a, 4.14b). Basolateral stimulation of 1mM with butyrate further enhanced the increase in expression, but a significant decrease was observed at levels of 10mM (figure 4.14a, 4.14b). Expression of eotaxin-3 exhibited the same response for butyrate as previously seen for acetate and propionate (figure 4.11c, 4.12c, 4.13c, 4.14c). Both apical and basolateral stimulation of 10mM of butyrate demonstrated decreased expression of IL-6 (figure 4.11d, 4.12d), as did CXCL8 for apical stimulation (figure 4.11e). However, basolateral stimulation of 10mM butyrate demonstrated a clear increase of CXCL8 level (figure 4.12e). Mucus and cilia production were both decreased at 10mM of butyrate on both apical and basolateral side of the epithelium (figure 4.11f, 4.11g, 4.12f, 4.12g, 4.13f, 4.13g, 4.14f, 4.14g). In conclusion, butyrate stimulation promotes a slightly different response compared to acetate and propionate stimulation. However, it is important to note that the concentration of butyrate is considerably higher to what is typically encountered *in vivo*, suggesting that these results are perhaps not physiologically correct.

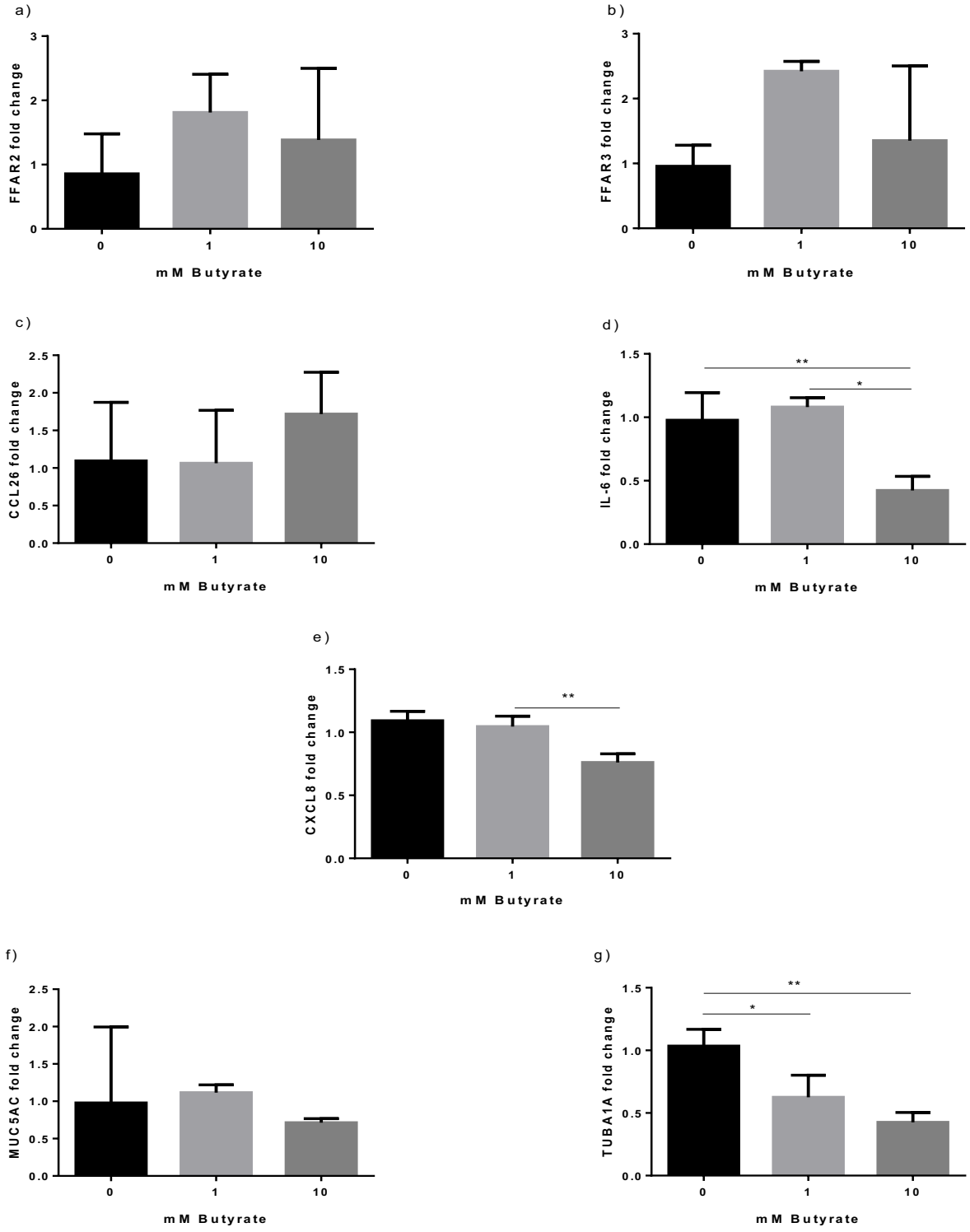


Figure 4.51. Butyrate promotes mixed results when treated on the basolateral side of the airway epithelium.

Gene expression of treatment of butyrate for 36 hours on the basolateral side of the differentiated airway epithelium), ***a)*** *SCFAs receptor FFAR2,* ***b)*** *SCFAs receptor FFAR3,* ***c)*** *CCL26,* ***d)*** *IL-6,* ***e)*** *CXCL8,* ***f)*** *MUC5AC and* ***g)*** *TUBA1A. All bars are presented as triplicates and medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01, *** P<0.001.*

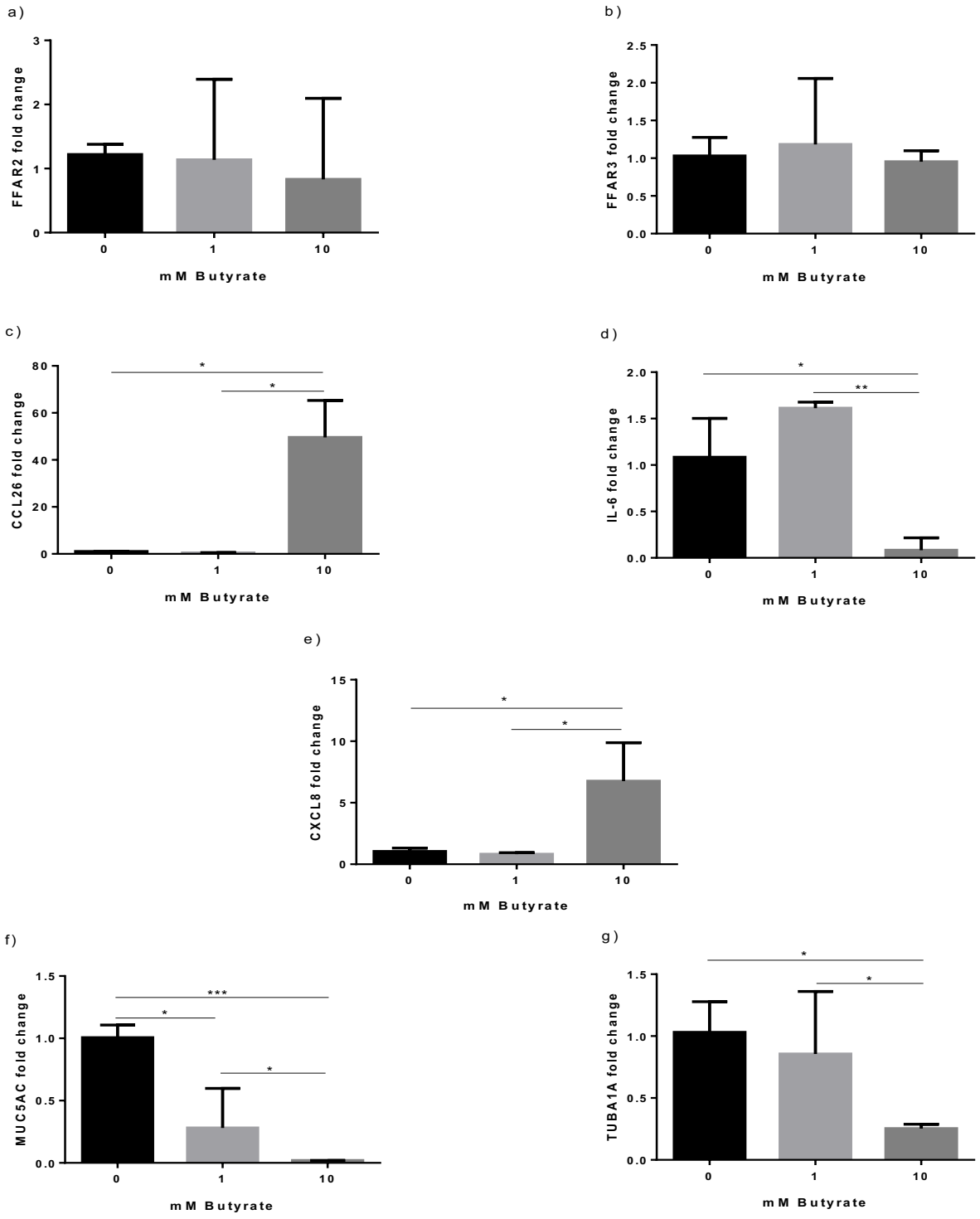


Figure 4.52. Butyrate promotes an anti-inflammatory response, through decrease of IL-6 and CXCL8 but not CCL26, when treated on the apical side of the airway epithelium.

Gene expression of treatment of butyrate for 36 hours on the apical side of the differentiated airway epithelium), ***a)*** *SCFAs receptor FFAR2,* ***b)*** *SCFAs receptor FFAR3,* ***c)*** *CCL26,* ***d)*** *IL-6,* ***e)*** *CXCL8,* ***f)*** *MUC5AC and* ***g)*** *TUBA1A. All bars are presented as triplicates and medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01.*

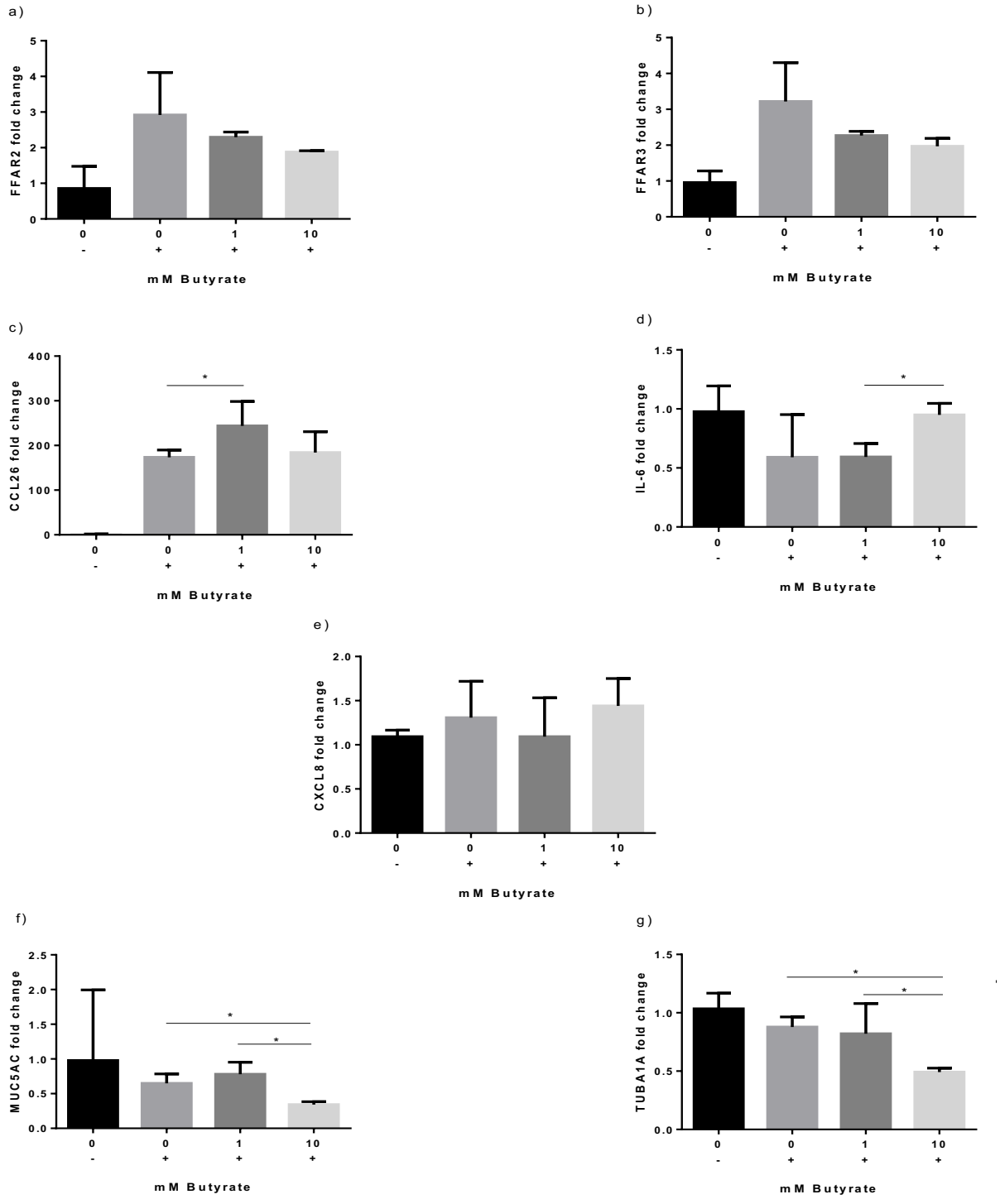


Figure 4.53. No major inflammatory differences observed when treating with IL-13 in addition to butyrate on apical side of the epithelium.

Gene expression of treatment of butyrate for 12 hours on the apical side of the differentiated airway epithelium, followed by addition of IL-13 for another 24 hours, ***a)*** *SCFAs receptor FFAR2,* ***b)*** *SCFAs receptor FFAR3,* ***c)*** *CCL26,* ***d)*** *IL-6,* ***e)*** *CXCL8,* ***f)*** *MUC5AC and* ***g)*** *TUBA1A. Each bar represents triplicates and the experiment was repeated 3 times. All bars are presented as medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05.*

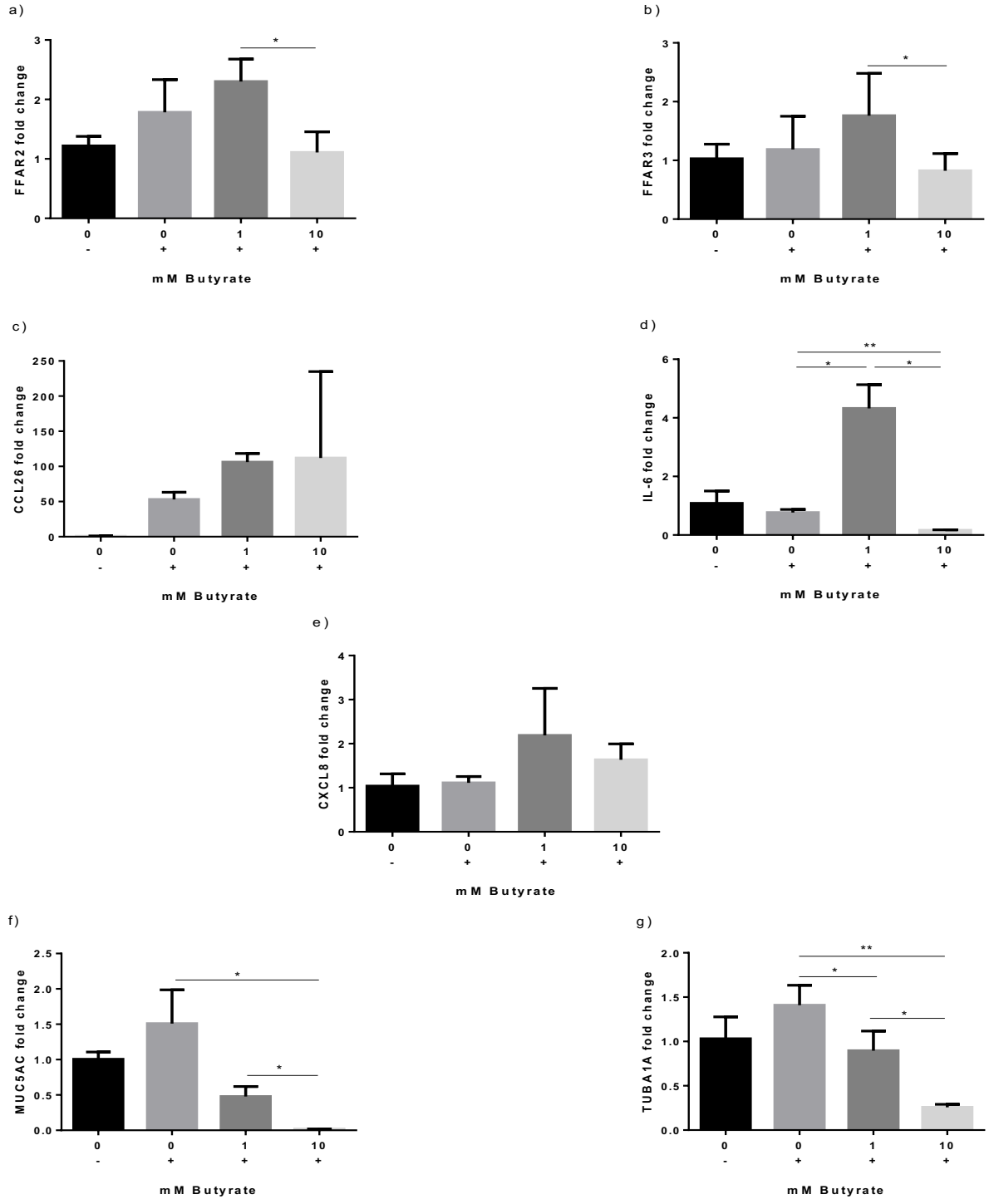


Figure 4.54. Decreased expression of the receptors and decreased mucus and cilia production when treating with IL-13 in addition to butyrate on basolateral side of the epithelium.

*Gene expression of treatment of butyrate for 12 hours on the apical side of the differentiated airway epithelium, followed by addition of IL-13 for another 24 hours , **a)** SCFAs receptor FFAR2, **b)** SCFAs receptor FFAR3, **c)** CCL26, **d)** IL-6, **e)** CXCL8, **f)** MUC5AC and **g)** TUBA1A. Each bar represents triplicates and the experiment was repeated 3 times. All bars are presented as medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01.*

4.2.4 Differences in inflammatory profile between transwells and an airway chip

In order to evaluate the response of a static transwell in comparison with a microfluidic chip, which has media flowing continuously on the basolateral side of the membrane, primary small airway epithelial cells were utilized to compare treatment of 10mM of propionate. In comparison to transwells, the airway chip had a constant flow of media in the basolateral compartment, which was supplemented with propionate. The SCFAs receptors were expressed in both systems, but no differences were observed for the treatment or system (figure 4.15a, 4.15b). As previously seen, Eotaxin-3 was increased with 10mM propionate and this was the case for the airway chip as well (figure 4.15c). However, IL-6 behaved differently between transwell and chip (figure 4.15d). In transwells, there was a clear increase in IL-6, as previously seen, but in the chip, there was a significant decrease of IL-6 for 10mM of propionate. For CXCL8, there was an increase for both transwell and chip, however the increase was much lower for the chip than transwell (figure 4.15e). Mucus and cilia production were both decreased in transwells and chip and no difference were seen between the two systems (figure 4.15f, 4.15g). In conclusion, the response of 10 mM of propionate generated the same response for both the chip and the transwell with exception for IL-6. These results suggest that the chip might need to be more complex to mimic what is happening *in vivo*.

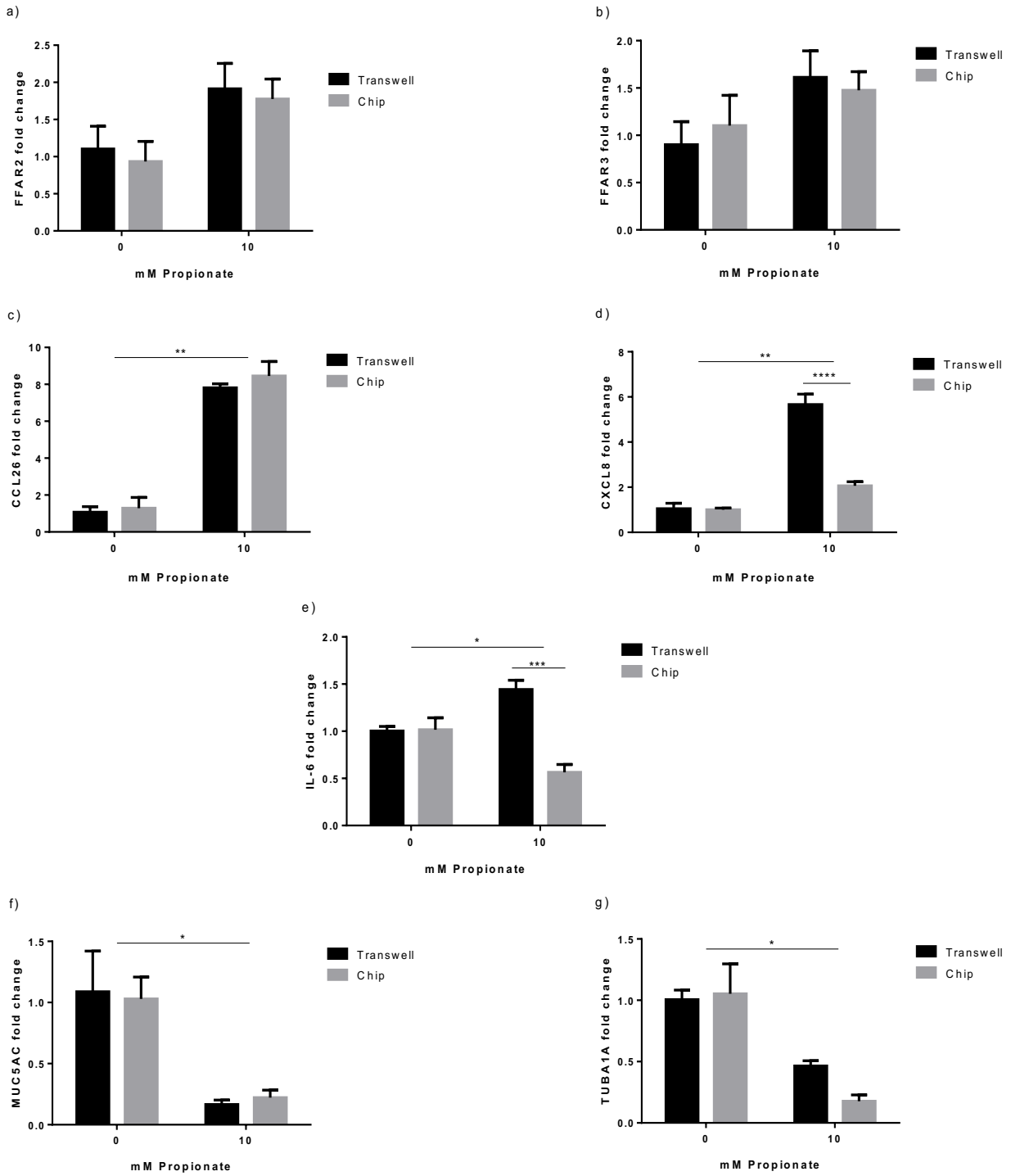


Figure 4.55. Comparison of treatment of 10mM of propionate between airway-chip and transwell demonstrate a stronger inflammatory response in the transwell.

*Gene expression of treatment of propionate for 36 hours on the basolateral side of the differentiated airway epithelium in transwell and chip, a) SCFAs receptor FFAR2, b) SCFAs receptor FFAR3, c) CCL26, d) IL-6, e) CXCL8, f) MUC5AC and g) TUBA1A. All bars are presented as triplicates and medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

4.2.5 Induction of inflammation promotes the release of branched chain amino acids

Next, we sought to understand the difference in metabolism when treating the cells with acetate and propionate, and subsequently with and without IL-13. The metabolic content of the media from the experiment previously described in paragraph 4.2.1.3 was run using ^{1}H NMR spectroscopy and 22 selected metabolites were indicated to either have been used by the cells, produced or not changed at all (supplementary table 4.1). Stimulation with acetate, followed by a comparison of with or without IL-13, did not exhibit many differences in metabolite profiles. There was a significant decrease for glutamine (figure 4.16b) with induction of inflammation for 1mM of basolateral acetate as well as a relative decrease of glucose (figure 4.16b) and choline (figure 4.16b) with 10mM of basolateral acetate.

Interestingly, 1mM of apical propionate treatment followed by IL-13 elicited increased levels of branched chain amino acids (BCAA), glucose, glutamine, lysine, threonine and phenylalanine (figure 4.17a) compared to non-IL-13 treated cells. Additionally, basolateral stimulation of 10mM of propionate increased the levels of BCAA for IL-13-treated cells (figure 4.17b).

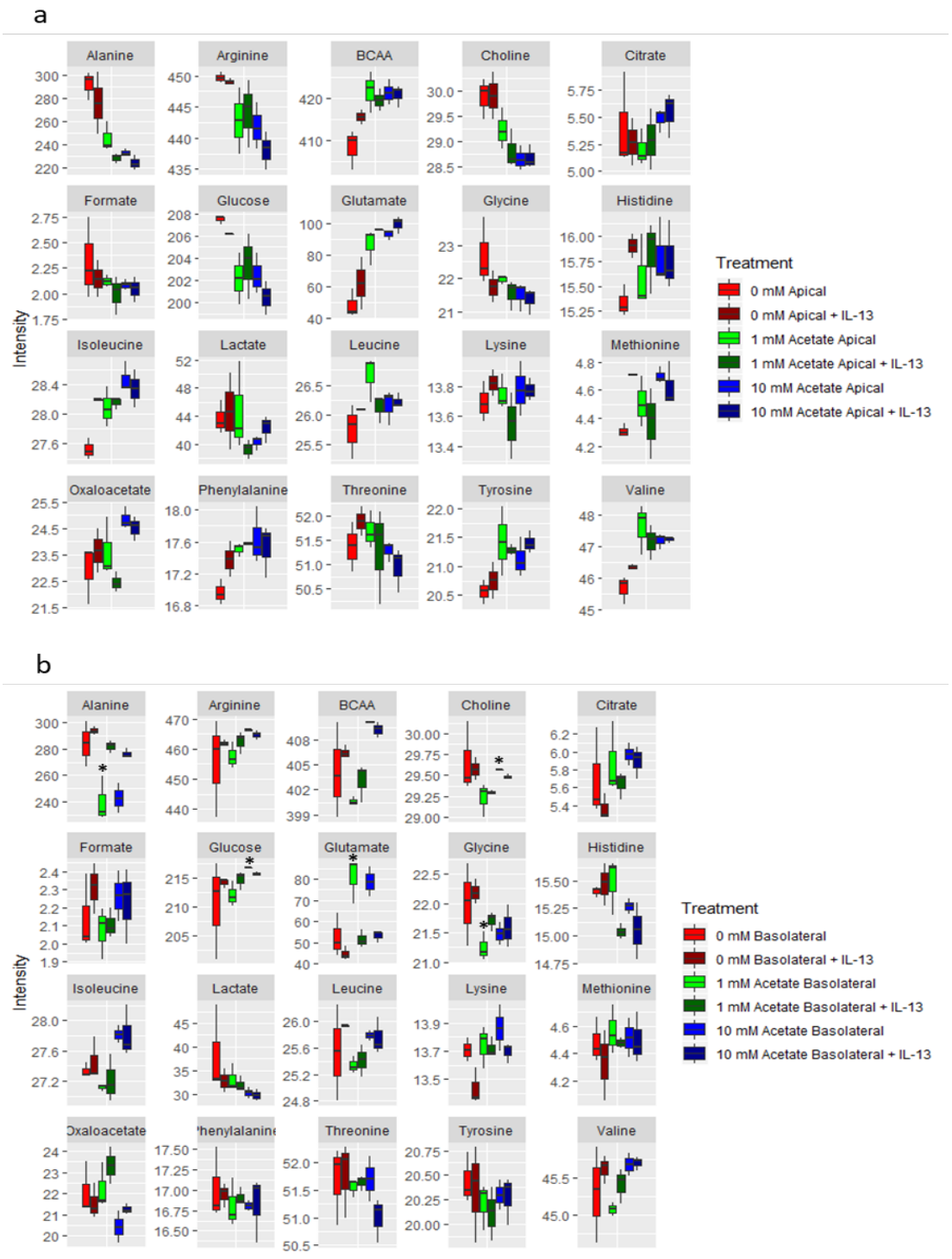


Figure 4.56. Metabolic profiling of cell supernatant for apical and basolateral stimulation of acetate followed by IL-13 using ^{1}H Nuclear Magnetic Resonance Spectroscopy.

22 selected metabolites were peak fitted, where each metabolite was compared with and without addition of IL-13 on top of acetate stimulation. ***a)*** *Apical or* ***b)*** *basolateral stimulation of acetate for either 36 hours or 12 hours followed by addition of IL-13 for additional 24 hours. All bars are presented as medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05.*

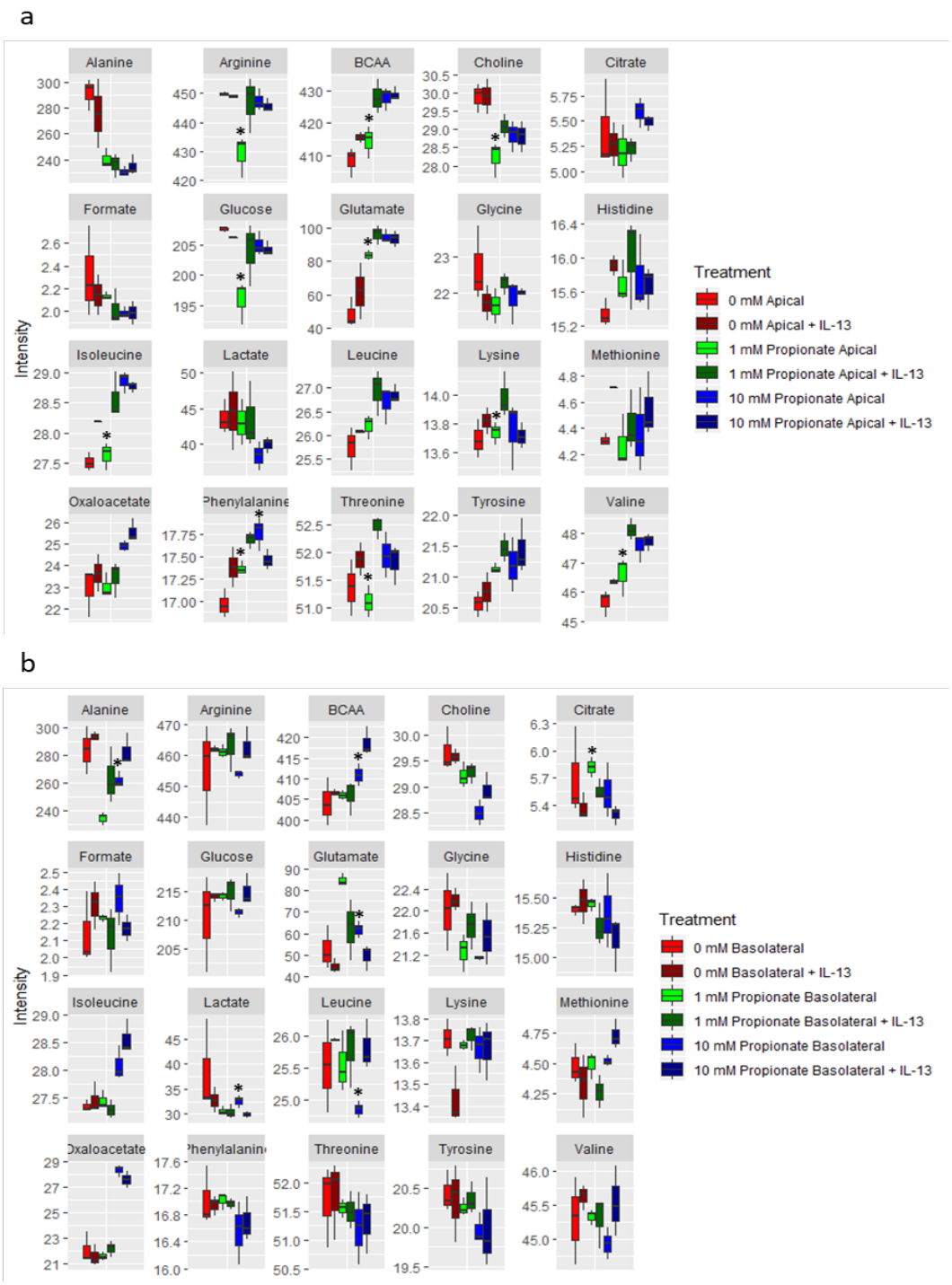


Figure 4.57. Metabolic profiling of cell supernatant for apical and basolateral stimulation of propionate followed by IL-13 using ^{1}H Nuclear Magnetic Resonance Spectroscopy.

*22 selected metabolites were peak fitted, where each metabolite was compared with and without addition of IL-13 on top of propionate stimulation **a)** Apical or **b)** basolateral stimulation of propionate for either 36 hours or 12 hours followed by addition of IL-13 for additional 24 hours. All bars are presented as medians with interquartile range. Unpaired parametric two-sided t-test was performed, * P<0.05*

The same experiment was conducted for basolateral stimulation of propionate in transwells and airway chips. The same 22 metabolites were indicated to either have been used by the cells, produced or not changed at all (supplementary table 4.2). Interestingly, a higher production of alanine, choline, lactate, lysine and tyrosine (figure 4.18a) were seen in transwells compared to the chip at baseline, but no differences were observed for treatment of 10mM propionate. Higher levels of BCAA were seen for 10mM of propionate in transwells, partially driven by isoleucine (figure 4.18a). Phenylalanine was utilized more by the transwells, but no differences in concentration were seen with the addition of propionate (figure 4.18a). However, the chip treated with propionate utilized phenylalanine at a higher degree compared to non-treated cells. No differences were seen for oxaloacetate apart from an increase when treating with propionate in the transwell (figure 4.18a).

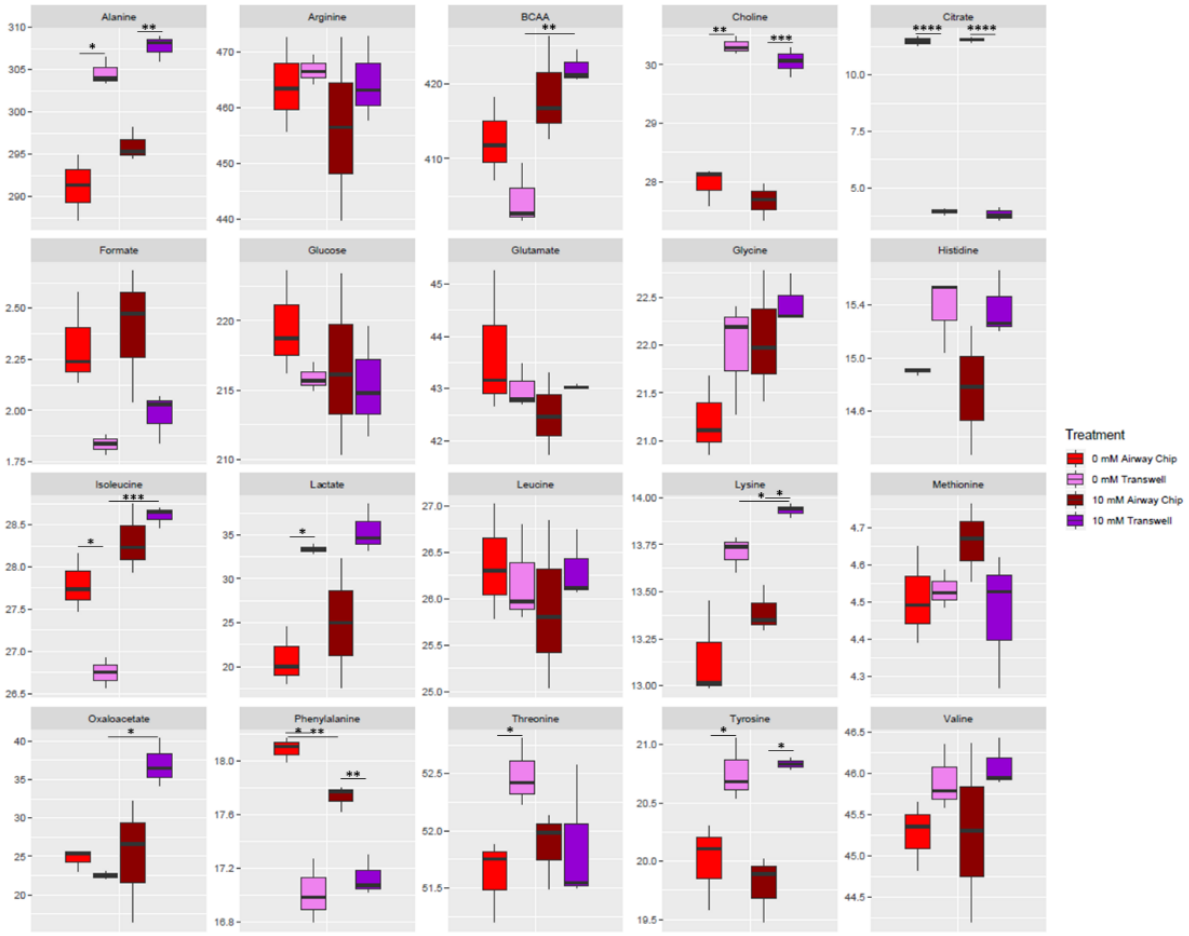


Figure 4. 58. Metabolic profiling of cell supernatant for stimulation of 10mM of propionate in chip and transwell using ^{1}H Nuclear Magnetic Resonance Spectroscopy.

*22 selected metabolites were peak fitted, where each metabolite was compared between chip and transwell. All bars are presented as medians with interquartile range. Unpaired parametric two-sided t-test was performed, P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

4.2.6 Treatment of peripheral blood mononuclear cells with branched chain amino acids promotes an anti-inflammatory response

In order to investigate the effect of BCAAs on circulating cells, isolated PBMCs (from 4 donors) were cultured and skewed towards a Th2 response according to previously published methods [156]. The cells were treated with 10, 1 and 0 mM of leucine, isoleucine and valine separately as well as a cocktail mix all together and analysed for IL-10 and IL-5. An increase in IL-10 was observed for 10mM of the cocktail mix (figure 19a), but not for 10 mM of each BCAAs individually. Furthermore, a trend towards decrease of IL-5 was seen for 10 mM of Leucine (figure 19f), but not for other BCAAs or for the mix. These results suggest that BCAAs could promote an anti-inflammatory response, but further investigations are needed to conclude on this.

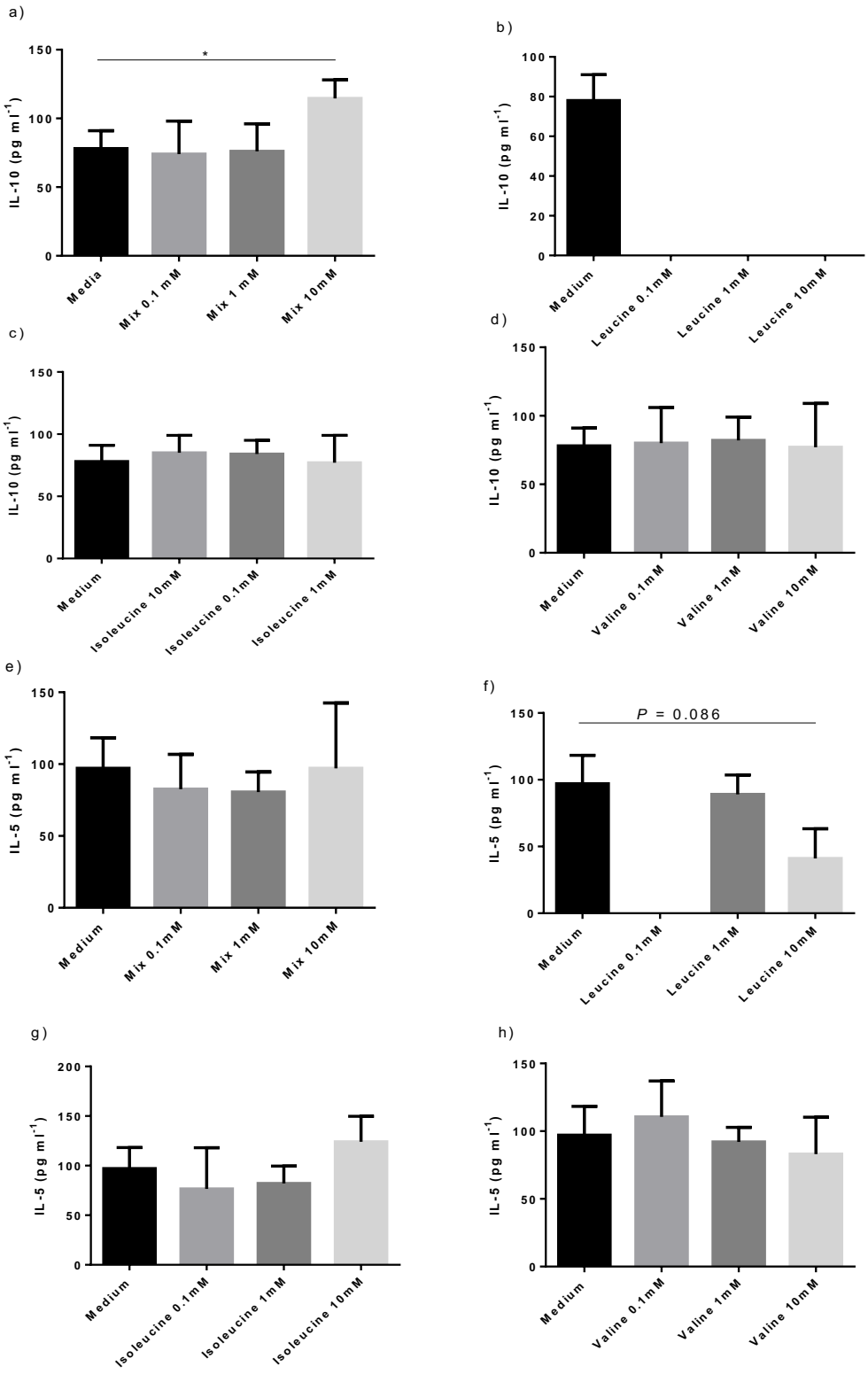


Figure 4.59. Treatment of BCAAs on Th2 skewed PBMCs demonstrate a trend towards anti-inflammatory properties.

a)-d) *IL-10 and* ***e)-h)*** *IL-5 were measured upon treatment of Th2 skewed PBMCs with a cocktail of BCAAs, Leucine, Isoleucine and Valine. Nothing was detected for IL-10 for the 3 Leucine concentrations. All bars are presented as medians with interquartile range. Unpaired parametric two-sided t-test was performed, P<0.05.*

4.3 Discussion

The airway epithelium is the first site of contact for inhaled air, pathogens and other foreign molecules and it is crucial that the epithelial layer and barrier is fully functional in order to maintain lung homeostasis [65, 68, 70, 176]. The epithelium is the interplay between the adaptive and innate immune system, and upon exposure to foreign molecules, or objects, emits an response recruiting immune cells to the site of contact [177]. Previous literature has demonstrated the anti-inflammatory effect of gut-derived SCFAs in rodents, where mice on a high-fibre diet did not exhibit elevated inflammatory response upon house-dust mite stimulation, compared to the mice fed a low-fibre diet [60, 84, 165]. To get a better understanding of the role of airway-derived SCFAs I sought to understand their effect on the epithelium.

Initial investigations using a human normal bronchial epithelial cell line demonstrated a decrease in IL-6 but an increase in CXCL8 at 1mM of all three SCFAs. The increase at 1mM was observed for both IL-6 and CXCL8 with addition of IL-13, but a decrease back to baseline was observed at 10mM. Interestingly, a clear immunomodulatory response was observed when primary bronchial epithelial cells were treated with acetate and propionate. This is interesting since previous studies that have demonstrated the anti-inflammatory properties of gut-derived SCFAs on immune cells and allergic airway inflammation. The effect of apical and basolateral stimulation of acetate and propionate was investigated where similar results were observed apart from eotaxin-3. Interestingly, eotaxin-3 was only elevated in basolateral stimulation of 10mM of propionate and not apically. Eoxtaxin-3 is a chemoattractant for eosinophils, which play a key role in eosinophilic asthma [178-180]. Moreover, eosinophils play a big role in infections [181, 182] and are recruited

through release of eotaxin-3 in the bloodstream. This could explain why higher level (10mM) of propionate only on basolateral side, and not on apical safe, stimulate its expression.

The results point towards a more prominent effect of propionate stimulation compared to acetate. Interestingly, both the SCFAs receptors were downregulated upon exposure to acetate and propionate. Looking at Protein Atlas, it is shown that FFAR3 is expressed more on both apical and basolateral side of the epithelium compared to FFAR2. Since FFAR3 has a higher affinity for propionate, it could explain why propionate has a more prominent effect than acetate.

Furthermore, MUC5AC mRNA expression increased at 1mM and decreased back to baseline at 10mM while cilia production went down with increased concentration of acetate and propionate. It is known that asthmatics have excessive mucus production due to the inflammation going on, hence this suggests that the immunomodulatory response of SCFAs are promoting mucus production. However, a high concentration of acetate and propionate might signal “infection”, thus making the cells produce less mucus in order to decrease any available substrate and allow clearance for the immune cells to handle the invasion.

Treatment with butyrate exhibited a very different result compare to acetate and propionate, where apical treatment of butyrate demonstrated an anti-inflammatory response while basolateral treatment revealed mixed results. Decreased expression of mucus and cilia production with butyrate treatment followed by IL-13, which is in accordance to what was previously seen with acetate and propionate. However, it is known that butyrate is very potent at low concentrations and the concentrations used for the experiments are much higher than those encountered *in vivo*. This raises the question as to whether the results seen are physiologically correct or just a product of enhanced levels of butyrate.

In order to evaluate the effect metabolic activity of IL-13 treatment, in combination with acetate and propionate respectively, together with differences in metabolic activity ^{1}H NMR spectroscopy of the cell supernatant was conducted. Interestingly, IL-13 increased the release of BCAAs when pre-treating with propionate. Also, an increase of BCAAs was observed at 1 mM of propionate on apical side, but required 10mM when treating on basolateral side. This could be due to a higher sensitivity observed on the apical side compared to the basolateral side. To understand the reason begin release of BCAAs, we treated Th2 skewed peripheral blood mononuclear cells (PBMCs) to a range of BCAAs concentrations, which demonstrated a trend towards an anti-inflammatory response when treating with a cocktail mix of all 3 BCAAs. This could suggest that metabolites released from the airway epithelium are trying to regulate the already high inflammatory response by secreting anti-inflammatory cytokines.

Limitations are present in this study. Using more donors to culture the airway epithelial cells would have further validated the results, especially when dosing with butyrate. It would also have been interesting to dose the epithelium with a cocktail of all 3 SCFAs and to see whether similar results were obtained. Furthermore, an interesting experiment would have been stimulating with either 2-methylbutyrate or 2-hydroxybutyate, as they were present in the airway samples, shown in the beginning. The use of the airway chip would have also added more value if the endothelial cells would have been present together with mechanical movements to mimic a breathing chip. In the future it would be interesting to incorporate bacteria with the epithelium to investigate whether the bacteria would break done the mucus and produce SCFAs. Finally, the use of eosinophils, or perhaps macrophages or neutrophils, to investigate the effect of treatment of BCAAs might have generate more accurate results.

4.4 Conclusions

Treatment with acetate and propionate on both the apical and basolateral side of the epithelium demonstrated an immunomodulatory response, which was further enhanced with the treatment of IL-13. Propionate treatment on the basolateral side of the epithelium demonstrated increased expression of CCL26 and additional treatment of IL-13 demonstrated a decreased expression of FFAR2 and FFAR3. Furthermore, metabolic profiling of propionate and IL-13 treatment demonstrated a release of BCAAs compared to non-IL-13 treated cells. Treatment of PBMCs with BCAAs demonstrated a slight increase of IL-10 showcasing an anti-inflammatory response. These results suggest a new role of SCFAs, which when airway-derived act in a different immunomodulatory manner compared to gut-derived SCFAs acting more anti-inflammatory shown in the preclinical data. Further research is needed to elucidate the interplay between gut-derived and airway-derived SCFAs in asthmatics.

Chapter 5 Metabolic profiling of urine in asthmatic cohort

Hypothesis

It is hypothesised that the urinary metabolic profile is different between asthmatic and control individuals, but also between sub-types of asthmatics.

Aims

- Investigate the urinary metabolic profile of control participants, mild to moderate asthmatics and severe asthmatics to identify potential biomarkers that will aid in asthma diagnosis.

Objectives

- Metabolic profiling, multivariate and univariate statistics of urine of above-mentioned cohort using ^{1}H NMR spectroscopy and UPLC-MS.

5.1 Introduction

Asthma is a chronic inflammatory disease characterised by a decline in airway function caused by swelling of the bronchial tubes and tissue remodelling [12, 14]. Due to the heterogeneity of asthma and the range of different phenotypes that the disease exhibits, it is difficult to diagnose asthma and the level of severity with a single test [6, 7]. The most common form of diagnosis involves both spirometry and prick test [10-13, 20], both being invasive and, in many cases, not sufficient to diagnose the majority of asthma endotypes.

Metabolic profiling involves the measurement of the metabolic processes occurring in an entire biological system and how these are perturbed by various stimuli, such as a toxin, dietary intake or a disease state [127-129]. The identification of metabolites, or biomarkers, that differ between healthy and disease individuals could aid in the diagnosis of a disease or distinguish sub-types with a certain population. Several studies have been conducted in asthmatics investigating their metabolic profile to identify various metabolic processes that might differ in the disease state. Studies have been performed in both children [131, 133, 183-188] and adults [130, 189-195] investigating a range of biofluids such as urine [185, 186, 193, 196], serum and plasma [133, 184, 195] and exhaled breath condensate [130, 131, 183, 187-191, 194]. The majority of metabolites that have been suggested to be altered in asthmatics are involved in amino acid metabolism, glutamate-glutamine cycle, TCA cycle, immune pathways, lipid metabolism and oxidative stress [197].

However, due to small sample size and the heterogeneity of the disease, the variability between these studies is large and metabolites often found to be different in asthmatics are often also connected to various other diseases.

The aim of this study is to identify potential biomarkers in urine of adults, which will (1) differ between healthy controls and people with asthma, but also (2) between different endotypes of asthma using two different untargeted approaches.

5.2 Results

5.2.1 Clinical information of the WESSEX severe asthma cohort

377 participants were enrolled in this study, with the majority being severe asthmatics. No differences in age were observed between any groups. There was a significant increase in age and BMI between healthy controls and severe asthmatics, as well as between mild to moderate and severe asthmatics. A difference in percentage forced vital capacity (%FVC) before dilator and in ACQ6 values were observed. FeNO50 was different comparing healthy controls with both severities of asthma ($P = 0.0041$, $P = 0.0048$).

Table 5.1. Clinical information of the WESSEX severe asthma cohort.

Data show as mean with standard error of mean (SEM). Unpaired non-parametric two-sided t-test was performed.

	Healthy controls	**Mild to moderate asthmatics**	**Severe asthmatics**
Participants % (n)	19 (72)	18 (66)	63 (239)
Sex % women (n)	61 (44)	55 (36)	66 (157)
Age	36.17 ± 1.50	40.05 ± 1.62****	48.05 ± 0.89****
BMI	24.81 ± 0.51	29.65 ± 0.72****	31.29 ± 0.50****
Atopic % (n)	-	82 (54)	72 (172)
FeNO50	21.46 ± 2.01	34.87 ± 4.27**	34.92 ± 2.53**
%FVC before dilator	104.96 ± 1.36	94.23 ± 2.26****	70.28 ± 1.65****
%FVC after dilator	-	102.71 ± 2.06	78.07 ± 1.71****
FEV_{rev}	-	-	13.10 ± 0.96
ICS dose (µg)	-	304.62 ± 39.43	2052.9 ± 67.15****
Prednisolone dose (mg)	-	-	5.93 ± 0.73
ACQ6	0.025 ± 0.0092	0.91 ± 0.095****	2.81 ± 0.076****

5.2.1 UPLC-MS data demonstrates metabolic differences between control and people with mild to moderate and severe asthma

To determine metabolic differences between control, mild to moderate asthmatics and severe asthmatics, spot urine was collected and run using UPLC-MS [159] (methods paragraph 2.4.2) Applying multivariate analysis (methods paragraph 2.5) on the data indentified clear metabolic differences between controls and severe asthmatics, as well as, between mild to moderate and severe asthmatics (figure 5.1a, 5.1b). No difference was observed between controls and mild to moderate asthmatics (data not shown). Interestingly, an OPLS model demonstrated metabolic differences for depression, which was also associated with increased asthma severity (figure 5.2a, 5.2b). The models were cross validated, compared for R^2X and Q^2Y values (supplementary table 5.1) and permutation testing (1000 permutations) was performed (figure 5.1c, 5.1d, 5.2c).

These data suggest metabolic differences seen between severe asthmatics and controls and also with mild to moderate asthmatics, but not between controls and mild to moderate asthmatics.

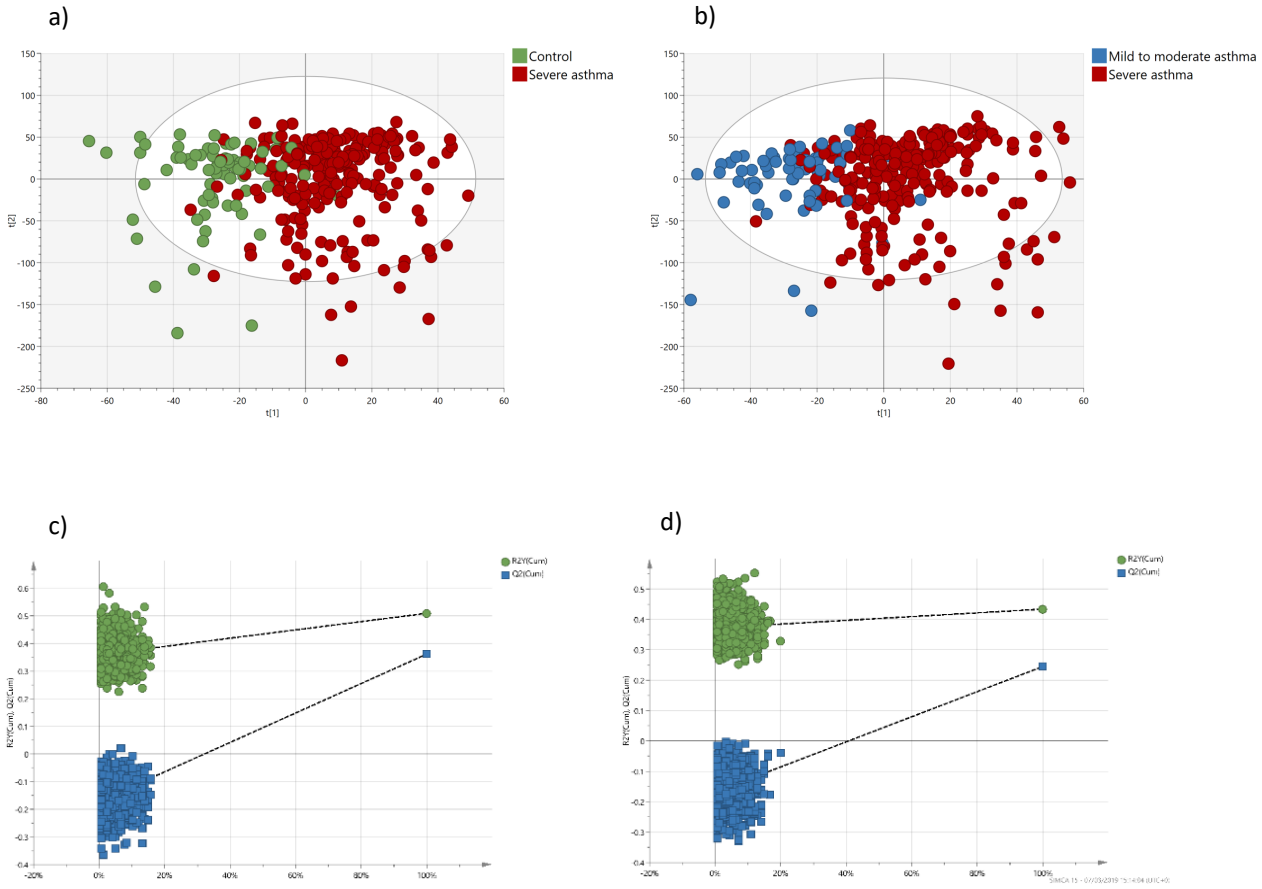


Figure 5.1. Metabolic differences between controls, mild to moderate and severe asthmatics.

OPLS-DA analysis demonstrate two distinct groups between ***a)*** *controls and severe asthmatics (R^2X = 0.509, Q^2Y = 0.362)* ***b)*** *mild to moderate and severe asthmatics (R^2X = 0.434, Q^2Y = 0.245). Permutation test (999 permutations) demonstrate valid models between* ***c)*** *controls and severe asthmatics and* ***d)*** *mild to moderate and severe asthmatics.*

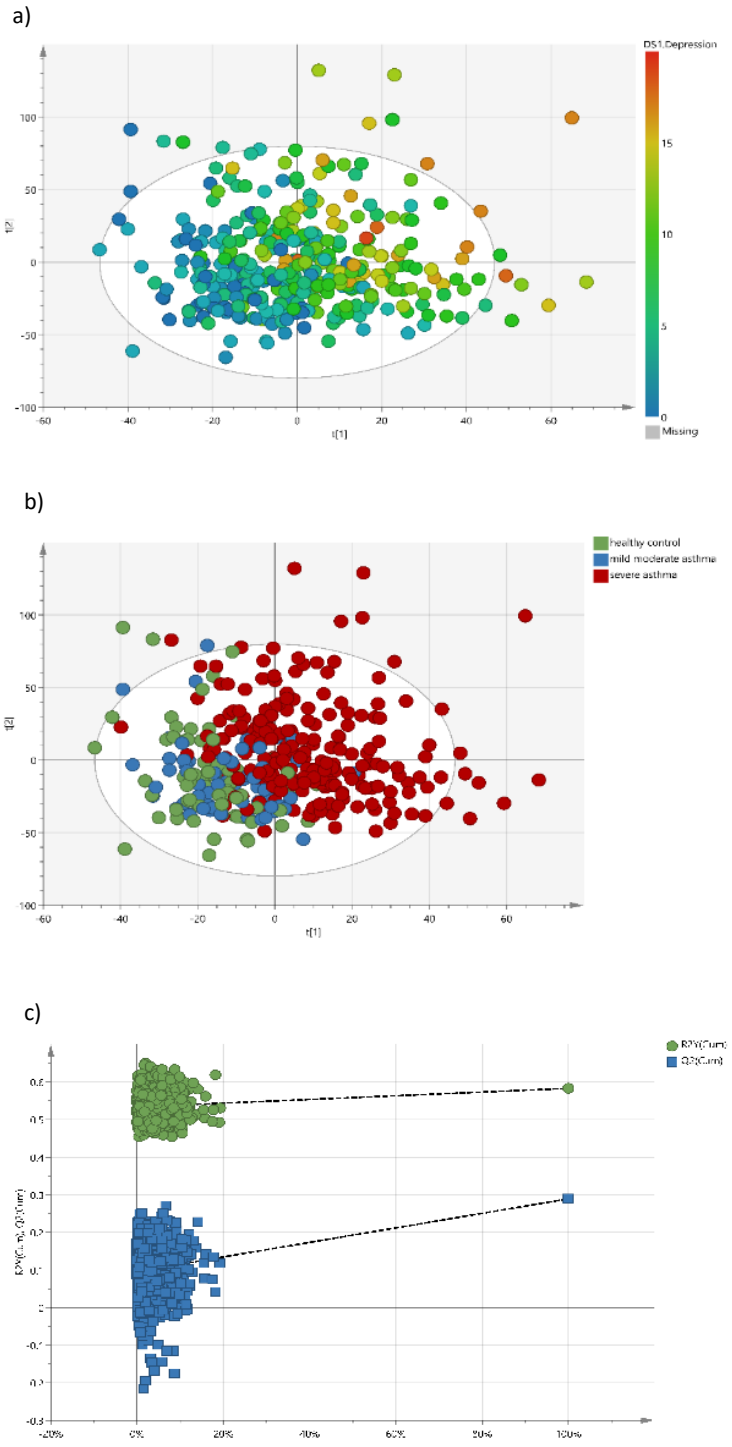


Figure 5.5. Metabolic differences associated with depression, which is also associated with increased asthma severity.

*OPLS model was generated for **a)** depression and b) coloured according to the groups (R^2X = 0.406, Q^2Y = 0.137). Permutation test (999 permutations) demonstrate valid model for **c)** depression.*

5.2.2 Identification of discriminant metabolites between controls and asthmatics in UPLC-MS data

To determine, which metabolites were causing the metabolic differences between the groups, the flow chart explained in section 1.10 was applied. VIP score (Variable Importance in Projection) was used for each model where the features with the highest VIP (cut-off at 2.1) were selected. To aid in the identification of the features extracted using VIP scores, each mass of interest was run through an in-house correlation script generating a spectrum, displaying all the masses highly correlated to the mass of interest (figure 5.3). MS/MS was conducted on selected features of interest, which would generate fragmentation patterns to aid in the identification of selected features. Further selection of important features was conducted by univariate analysis, distribution across all samples and ROC curves, which resulted in 13 features of interest (figure 5.4, table 5.1).

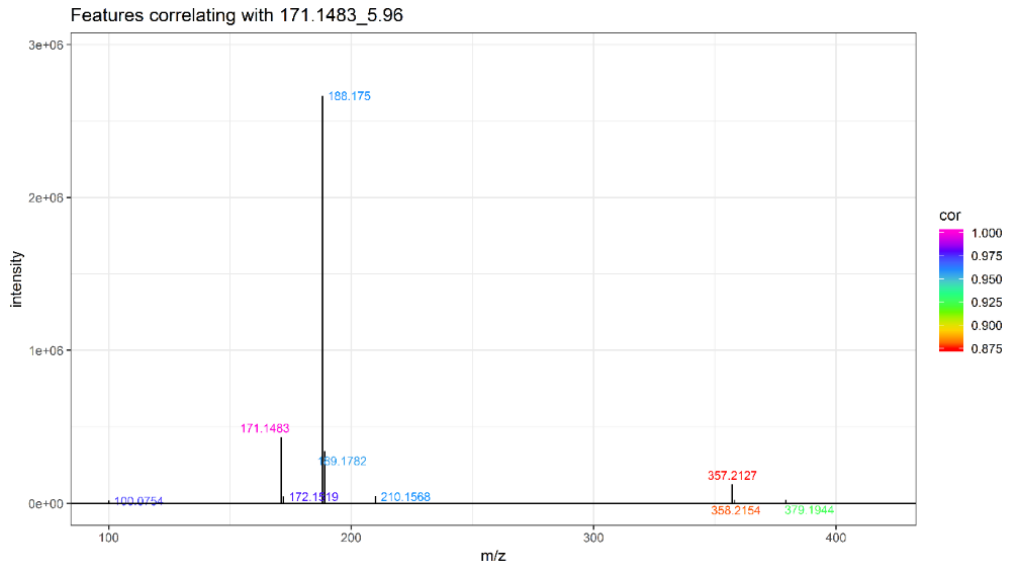


Figure 5.6. Correlation plot demonstrating the correlation between the mass of interest and other associated masses using an in-house script.

Table 5.2. Features of interest, from the multivariate models of the UPLC-MS data, and their ability to predict asthma through ROC curves and AUC values.

Features of interest	Mode	AUC % Healthy vs severe asthma	AUC % Mild to moderate asthma vs severe asthma
0.69_274.0915	RPOS	70.35	69.76
1.40_240.0492	RPOS	62.70	59.17
1.84_284.0746	RPOS	61.84	59.84
2.35_180.0874	RPOS	68.78	69.54
2.47_254.1249	RPOS	65.93	61.80
3.58_264.1123	RPOS	50.08	57.63
5.25_493.16	RNEG	64.73	71.25
0.62_396.08	HPOS	62.91	64.39
1.87_185.12	HPOS	61.84	57.27
2.43_313.08	HPOS	67.33	64.59
4.10_312.21	HPOS	62.40	61.79
5.96_188.17	HPOS	64.29	63.80
6.34_558.37	HPOS	67.78	71.09

Tentative assignments were generated by comparing correlation, MS/MS and raw spectra with online databases (Metlin and HMDB). In total, eight features were tentatively assigned, *N*-acetylneuraminic acid, beta-citryl-L-glutamic acid, xanthosine, N2,N2-dimethylguanosine, phenylacetylglutamine, *N*-acetylisoputreanine-y-lactam, decadienoylcarnitine and N1-acetylspermidine (Table 5.2). Interestingly, all of the features were increased in asthmatics apart from two unknowns and decadienoylcarnitine. To fully identify the metabolites, available standards (*N*-acetylneuraminic acid, xanthosine, N2,N2-dimethylguanosine, phenylacetylglutamine, N1 -acetylspermidine) were run alongside the samples and compared for

100% spectral identity. Those metabolites that were not available as standards, were compared to online databases and published literature [136, 150, 198, 199]. The five available standards were correctly matched by retention time and fragmentation pattern (Supplementary figure 5.1).

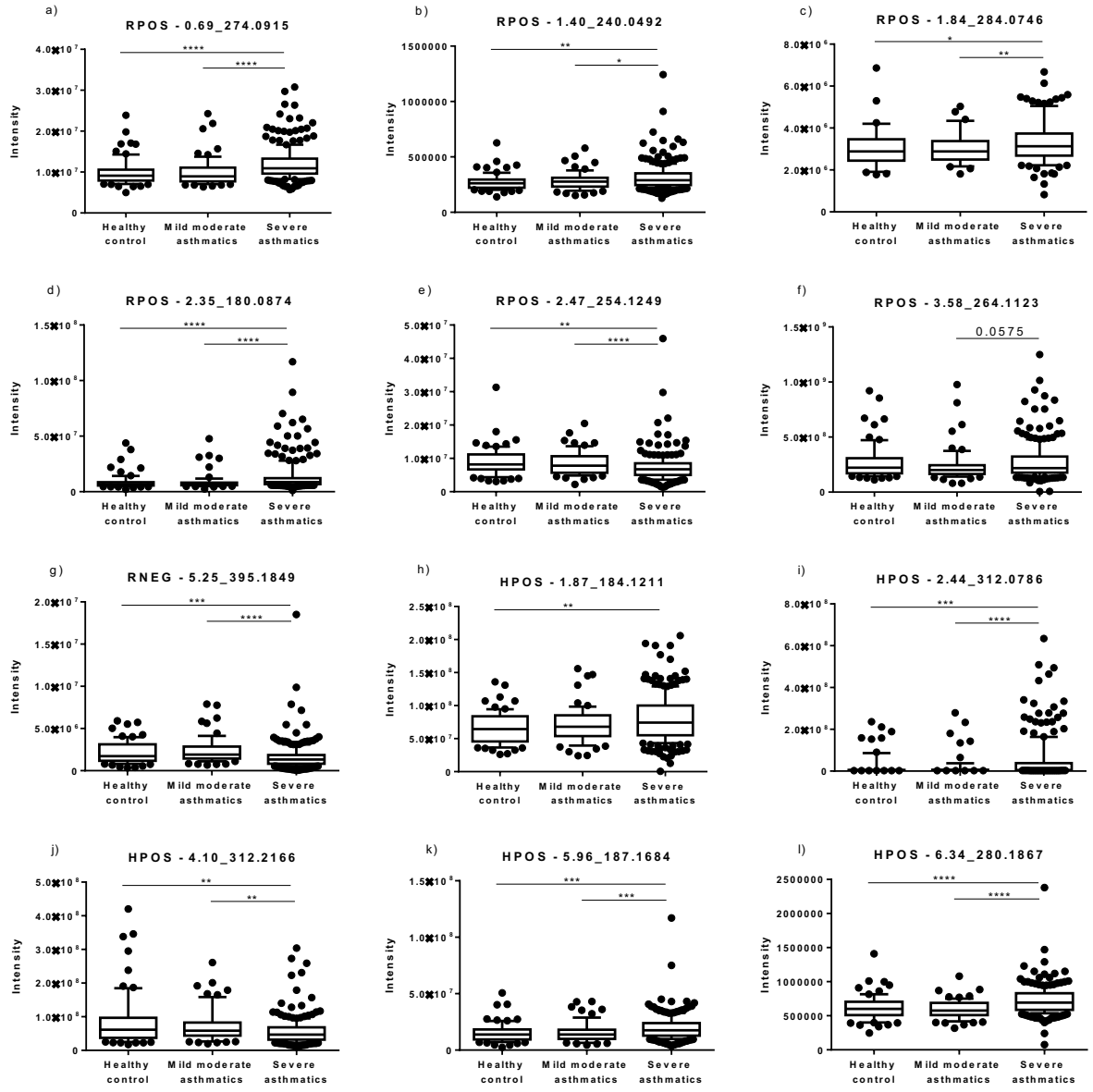


Figure 5.7. Univariate analysis on significant metabolites from the UPLC-MS data between healthy controls, mild to moderate and severe asthmatics.

*Significant metabolites found at a specific retention time and mass-to-charge ratio for a-f) revered-phase positive, g) reversed-phase negative and h-l) HILIC positive. All bars are presented as medians with 5-95 percentile. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

Table 5.3. Identified features of interest, from the multivariate models of the UPLC-MS data, and whether they are increased or decreased in asthma.

Features of interest	Mode	Tentative ID	High (+) or low (-) in asthma	Metabolism
0.69_274.0915	RPOS	*N*-acetylneuraminic acid	+	Amino sugar and nucleotide metabolism
1.40_240.0492	RPOS	Beta-citryl-L-glutamic acid	+	Glutamine and glutamate metabolism
1.84_284.0746	RPOS	Xanthosine	+	Purine metabolism
2.35_180.0874	RPOS	N2,N2-dimethylguanosine	+	Purine metabolism
2.47_254.1249	RPOS	Unknown 1	-	Unknown
3.58_264.1123	RPOS	Phenylacetylglutamine	+	Phenylalanine metabolism
5.25_493.16	RNEG	Unknown 2	-	Unknown
0.62_396.08	HPOS	Unknown 3	+	Unknown
1.87_185.12	HPOS	*N*-acetylisoputreanine-γ-lactam	+	Polyamine metabolism
2.43_313.08	HPOS	Unknown 4	+	Unknown
4.10_312.21	HPOS	Decadienoylcarnitine	-	Amino acid and fatty acid metabolism
5.96_188.17	HPOS	N1-acetylspermidine	+	Polyamine metabolism
6.34_558.37	HPOS	Unknown 5	+	Unknown

5.2.3 Biological interpretation and pathway analysis of identified metabolites in asthmatics from UPLC-MS data

To get a better biological understanding of the metabolites and their implication in asthma, the identified metabolites, together with the five unknowns, were correlated to the asthma clinical data and presented as a heatmap (figure 5.5a). Interestingly, decadienoylcarnitine together with two unknowns were negatively correlated to all asthma severity measurements, while the rest were all positively correlated. Phenylacetylglutamine was negatively correlated to BMI and positively correlated to diet score, which is reasonable as it is a microbial metabolite and has been seen to be associated with leaner individuals [44, 200]. Interestingly, the metabolites that were positively correlated with asthma severity were also positively correlated to depression, which was also observed in the multivariate models (figure 5.2). To determine whether other metabolites, related or in the same pathway as the ones identified, behaved in similar way, several metabolites were extracted from the chromatogram and plotted together in a heatmap (figure 5.5b). Surprisingly, many of the carnitines behaved in similar manner and were negatively correlated with asthma severity. Several carnitines were negatively correlated to CEACAM-5 levels in sputum. CEACAM-5 is an adhesion molecule that has been shown to be upregulated in asthma [201]. Furthermore, N2,N2-diemethylguanosine and xanthosine were also positively correlated with asthma severity and both are involved in purine metabolism, which has previously been found to be altered in asthmatics [195, 202]. *N*-acetylisoputreanine-y-lactam and N1-acetylspermidine were also increased with asthma severity. Previous studies have shown an increase in polyamines in asthmatics, but this is the first study to identify such changes in the urine samples of humans [203, 204].

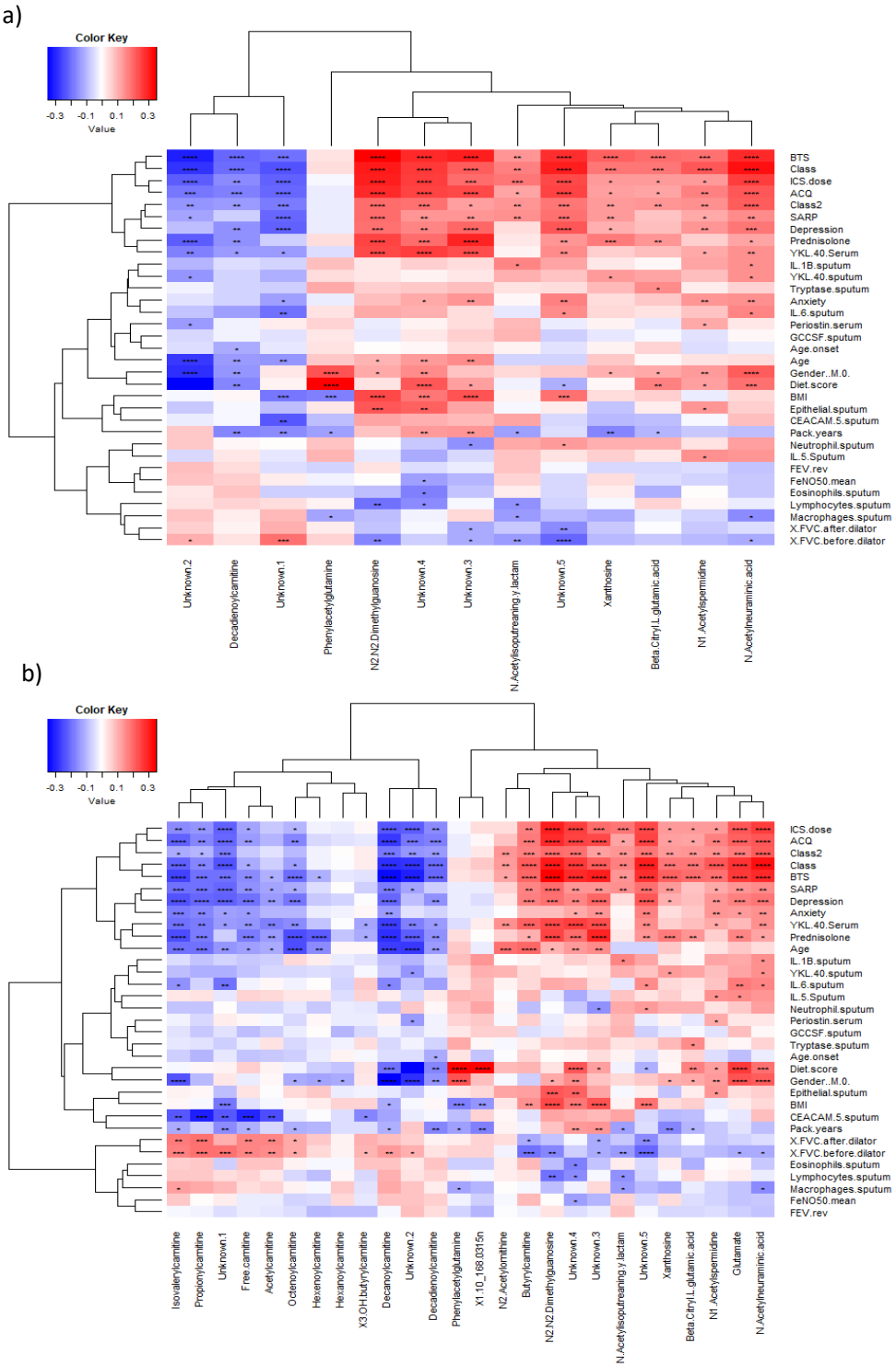


Figure 5.8. Spearman correlation of metabolites with clinical patient data, viewed as a heatmap, where red is positive association and blue is negative.

*a) Heatmap demonstrated correlation of metabolites of interest with metadata while b) includes metabolites associated with metabolites of interest. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

To further evaluate the association with these metabolites with asthma, the metabolites were corrected for sex, age and BMI and thereafter plotted as a heatmap (Supplementary figure 5.2). The same effect seen in figure 5.5 remained, however the correlation value was slightly decreased. Moreover, to determine, which metabolites could have the ability to predict asthma, a multiple regression model was performed on the metabolites presented in figure 5.5. Apart from the unknowns that were significant in the regression model, phenylacetylglutamine, *N*-Acetylisoputreanine-y-lactam and five carnitines were also significant in the model, suggesting that they could play an important role asthma (Supplementary table 5.2).

To examine the pathways of the identified metabolites, each metabolite, that had an available pathway in KEGG, was imported into Metabonetwork [160] (figure 5.6). N2,N2-dimethylguanosine was not available in KEGG, but guanosine and deoxyguanosine were, suggesting that they could be part of the same pathway of purine metabolism. Xanthosine, also involved in purine metabolism, was closely situated in the network suggesting that it could be related to N2,N2-dimethylguanosine and behaving in similar matter. Acetylisoputreanine-y-lactam was not found as part of any pathway in KEGG, however one study demonstrated the relation between this metabolite and N1-acetylspermidine, which was found in KEGG [198]. Both of these metabolites were closely related and part of polyamine metabolism, which has been shown to play a role in asthma. *N*-acetylornithine glutamic acid and beta-citrylglutamate were also closely situated to N1-acetylspermidine suggesting that they could all be related. N-acetylneuraminic acid were separated from the rest of the metabolites as were the carnitines that were available in the KEGG pathway (figure 5.6) (Method paragraph 2.6).

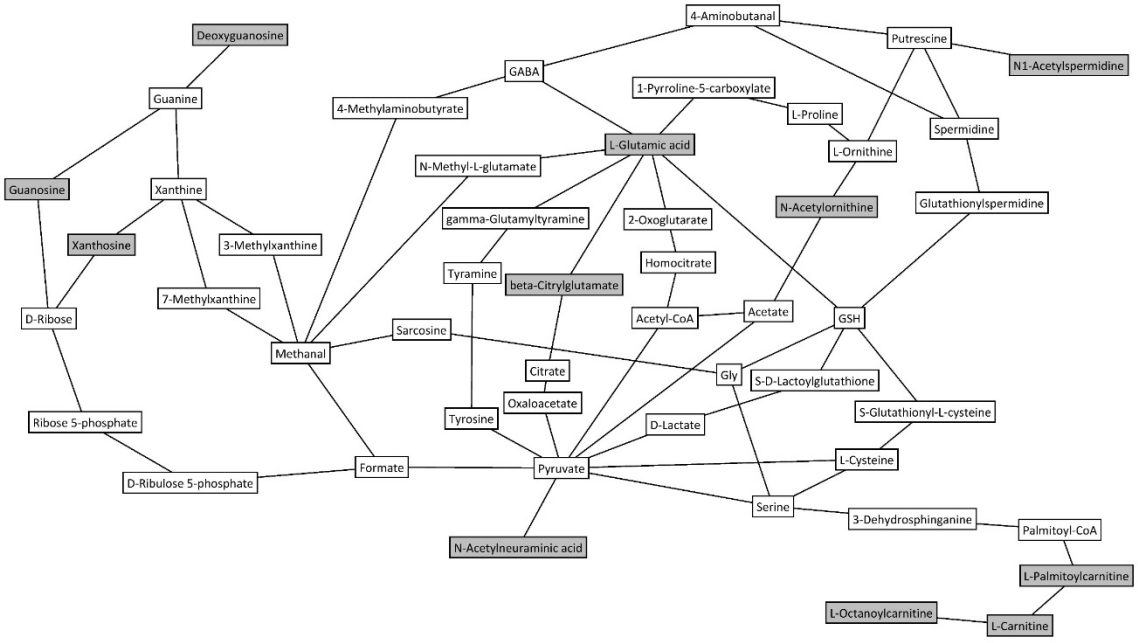


Figure 5.18. Metabonetwork of significant metabolites, which are available in KEGG.

Metabolites with grey shading were manually added as a base to the network, while the ones in white are metabolites important to generate a network.

5.2.4 Ability of identified metabolites from UPLC-MS data to distinguish sub-population within the severe asthmatics

To investigate whether sub-populations were present within the group of severe asthmatics, the identified UPLC-MS metabolites were compared with BTS score 4 and 5 (figure 5.7). The majority of the carnitines were significantly lower in asthmatics with BTS score 4 compared to BTS score 5, and the significance increased with increased carbon chain of the carnitines (figure 5.7). These results are comparable to the results previously described, where carnitines were higher in controls compared to asthmatics. Furthermore, both xanthosine and N2,N2-dimethylguanosine were

significantly increased with BTS 5 compared to BTS 4 suggesting that these two metabolites are increased with asthma severity, the opposite to what was seen with the carnitines.

These results suggest that certain metabolites can distinguish sub-populations within severe asthmatics.

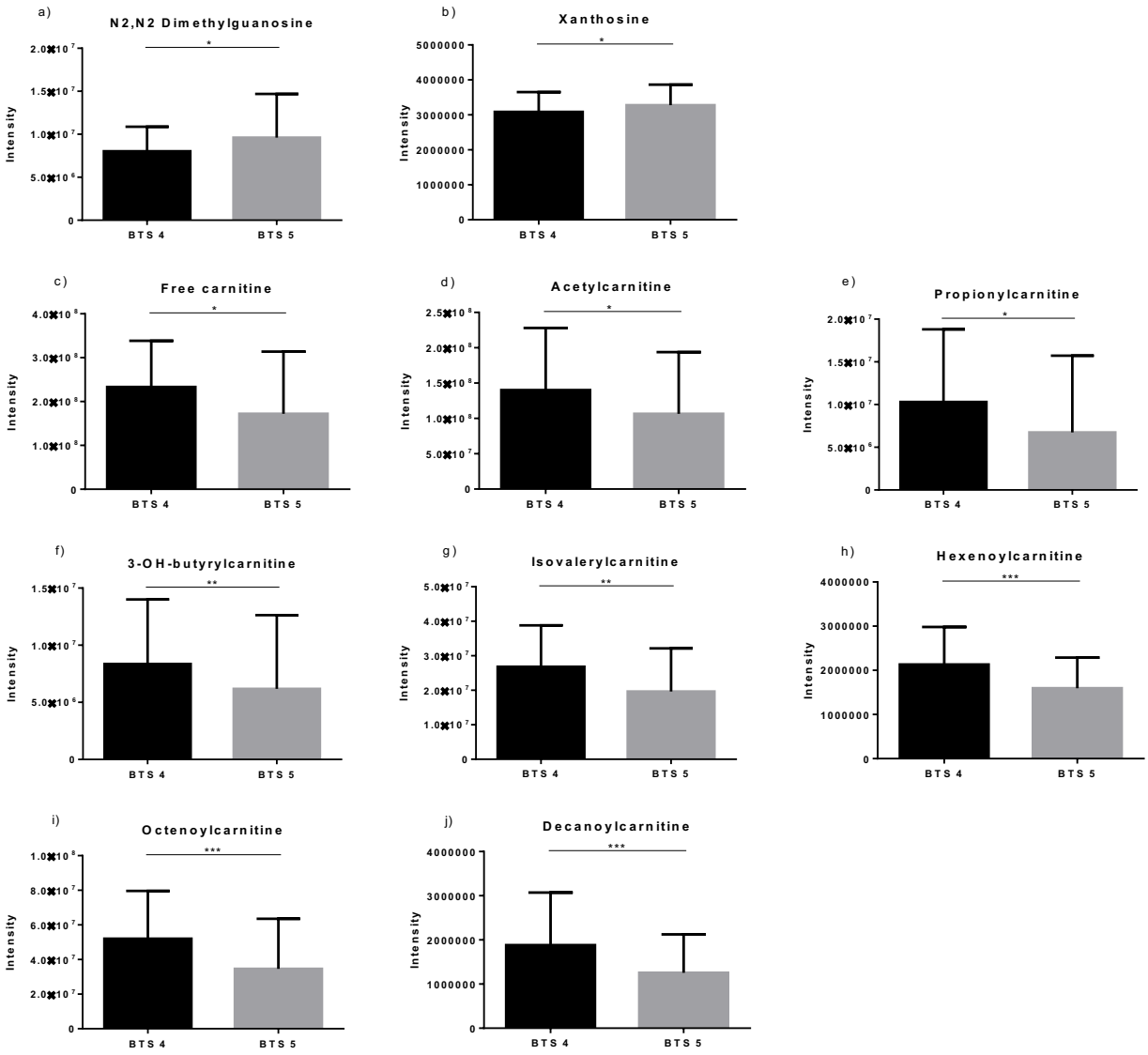


Figure 5.19. Identified metabolites differ within the severe asthmatic cohort and could contribute to the distinguish subpopulations within severe asthmatics.

*All bars are presented as medians with interquartile range. Unpaired parametric two-sided t-test was performed, P<0.05, ** P<0.01, *** P<0.001.*

5.2.5 BMI is increased with asthma severity, but no difference was seen in diet score between controls and asthmatics

To explore possible associations between asthma and diet, BMI and diet score were compared between controls and asthmatics. The diet score was obtained from the metabolic profile of urine samples according to previously published tools [205], where a high score indicates a healthy diet and a low score a less healthy diet. A higher BMI was found with increased asthma severity (figure 5.8a), however the diet score remained unchanged between the groups (figure 5.8b). To further understand the link between metabolites, diet and BMI, metabolites associated with the gut microbiota and diet were compared between the groups. Interestingly, many microbial and dietary metabolites were decreased with asthma severity (figure 5.9), suggesting disease interaction with the gut microbiota. Furthermore, arginine (figure 5.9i) was increased with asthma severity. N1-acetylspermidine, identified using UPLC-MS, is a product of arginine degradation, which was also increased with asthma severity. These results indicate that there could be a possible link between asthma severity, diet and gut microbiome.

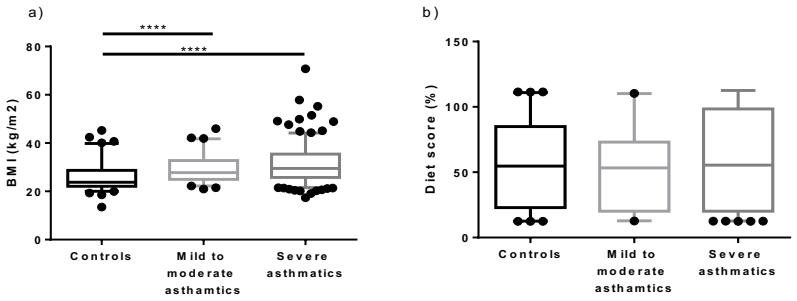


Figure 5.21. Increased BMI with increased asthma severity but not due to dietary profile.

*a) Difference in BMI between the groups and b) difference in diet score, generated from 1H NMR spectroscopy data from urine samples. All bars are presented as medians with 5-95 percentile. Unpaired parametric two-sided t-test was performed, **** P<0.0001.*

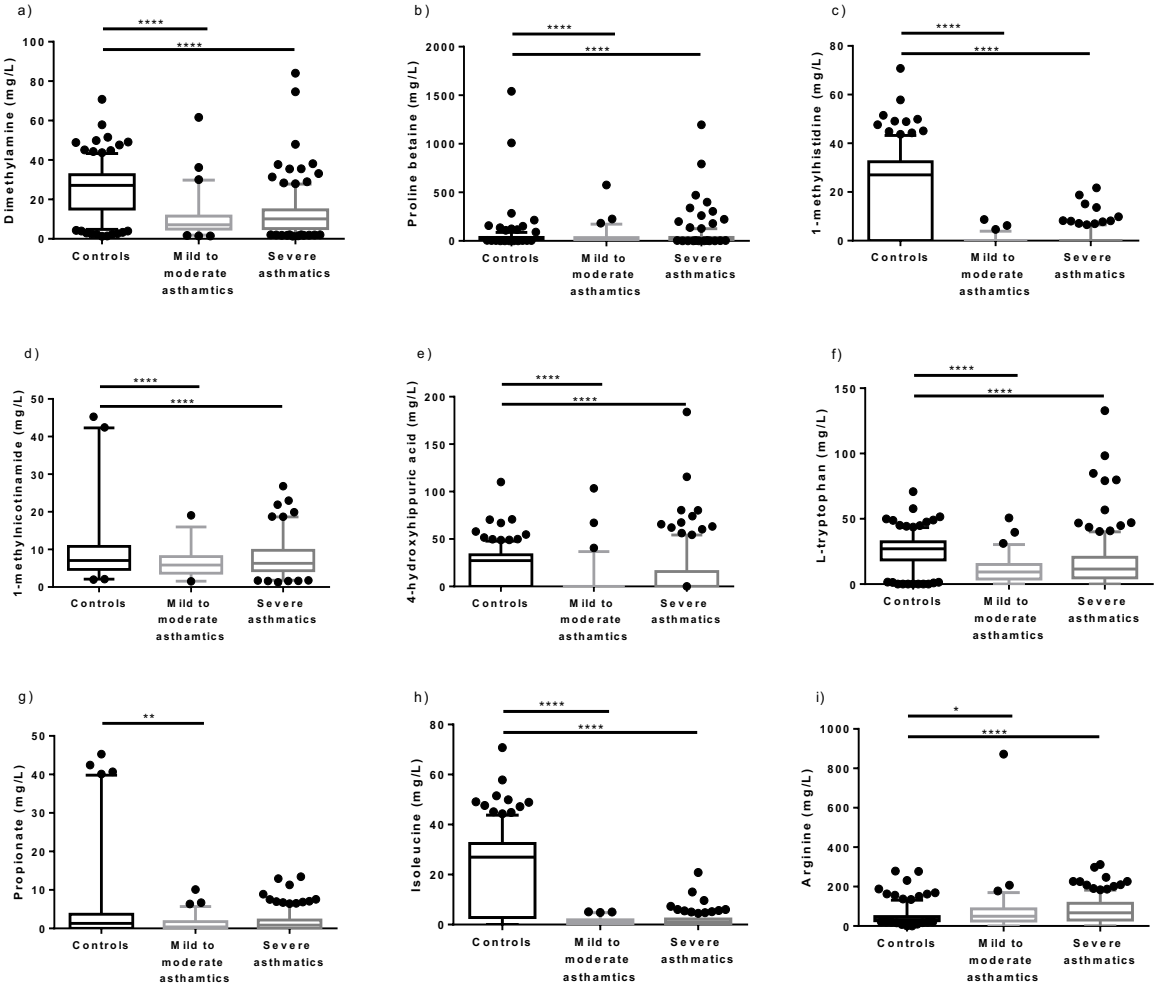


Figure 5.20. Dietary and microbial metabolites differ between healthy controls and asthmatics.

*Dietary and microbial metabolites were extracted from the ^{1}H NMR spectroscopy using Brukers quantification tool (B.I.QUANT-PS) and compared between the groups. All bars are presented as medians with 5-95 percentile. Unpaired parametric two-sided t-test was performed, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

5.3 Discussion

It is difficult to diagnose asthma with one single test and several forms of examinations are needed to not only diagnose asthma, but also to determine its severity. The tools and diagnostic kits that are available are quite invasive and difficult to perform and the results are often not conclusive. The use of metabolic profiling could aid in the development of potential biomarkers that could distinguish asthmatics from non-asthmatics as well as the severity of asthma by one single urine test.

Metabolic profiling of urine from a cohort consisting of 72 healthy controls, 66 mild to moderate and 239 severe asthmatics using UPLC-MS, demonstrated metabolic differences between controls and severe asthmatics and between mild to moderate and severe asthmatics. No differences were seen between controls and mild to moderate asthmatics, suggesting that those two groups were metabolically similar. Further analysis required to elucidate the significant metabolites that were causing the separation between the groups, resulted in the annotation of five metabolites compared to standards, three metabolites identified using online databases and previously published spectra with no available standards and five metabolites remained unknown.

One of the metabolites confirmed with standards, *N*-acetylneuraminic acid, has not been previously associated with asthma, however studies have suggested that the metabolite attenuates high-fat-diet-induced inflammation in rats. Furthermore, exposure to the closely related metabolite *N*-glycolylneuraminic acid in children prevented the development of airway inflammation and colitis [206, 207]. Secondly, elevation of N1-acetylspermidine has been observed in the airways of rodents with airway inflammation [203, 204, 208, 209], but no investigations have been made

to distinguish the metabolites in human airways or urine. Furthermore, identification of acetylisoputreanin-y-lactam, which is in the same pathway as N1-acetylspermidine and positively correlated with asthma severity, clearly suggesting that polyamine metabolism could play an important role in asthma and airway inflammation [203]. Glutamic acid and beta-citryl-glutamic acid were both closely related to polyamine pathway analysis and behaved in similar fashion.

Particularly interesting was that decadienoylcarnitine, which was negatively correlated with asthma severity. Further analysis demonstrated that many other carnitines followed a similar trend, further validating the negative correlation. Furthermore, multiple linear regression model demonstrated that five carnitines were significant and could possibly have the ability to predict asthma. Further analysis in severe asthmatics, demonstrated lower levels of carnitine in severe asthmatics with a BTS score of 5 compared to BTS score 4, further proving that carnitine levels are decreased with increased asthma severity. Previous literature has demonstrated that L-carnitine was decreased in children with asthma [210] and supplementation of L-carnitine improved asthma control in children [211]. Furthermore, supplementation of L-carnitine to murine models demonstrated a decrease in airway inflammation and improved oxygen saturation [212]. A strong negative correlation of several carnitines was seen for CEACAM-5. This correlation was weakened when the dataset was corrected for age, gender and age. CEACAM-5 is an adhesive molecule expressed on the airway epithelium and involved in many cellular functions such as signalling, proliferation and repair response [201]. The molecule has been shown to be elevated in IL-13 driven inflammation in serum samples [213] and could play a role in airway infection [214, 215]. CEACAM-5 has in previous literature been suggested as a therapeutic target, which is particularly interesting as many carnitines were seen to be negatively correlated to this protein. This could further indicate that both CEACAM-5 and carnitines are particularly important in

asthma. Carnitines are involved in the transport of fatty acids to the mitochondria for oxidation [216]. Carnitine deficiency leads to the accumulation of fatty acids in organs such as the heart, brain and skeletal muscle and will remain there resulting in severe consequences [217]. These results suggest that carnitines could play an important role in asthma physiology.

Xanthosine and N2,N2-dimethylguanosine are both involved in purine metabolism and were seen to be positively correlated with asthma severity. These metabolites could play a role in tRNA regulation, but limited studies have seen any interaction with these metabolites and asthma. Oral supplementation of adenosine, and potentially guanosine, to asthmatics demonstrated bronchoconstriction [218] suggesting that both adenosine and guanosine could be worsening the asthma symptoms, in agreement with our findings. A recent study [219] identified N2,N2-dimetylguanosine as a marker for decreased lung capacity, which is something that asthmatics have. As guanosine is part of nucleotide metabolism, this could suggest that something in that pathway could be dysfunctional in asthmatics.

Finally, phenylacetylglutamine, a microbial metabolite, was not statistically significant between the groups, but was a metabolite that was able to predict asthma according to the multiple regression model, which included analysis of all metabolites. Studies have demonstrated that specific bacterial species modulate different immunological aspects in asthma and that certain species could contribute to the development of asthma [55]. This could suggest that the gut microbial metabolites could be involved in asthma.

An untargeted approach was used to identify metabolites that could discriminate individuals with asthma from healthy controls. Several metabolites were found that had not been reported in

humans previously. Glutamic acid has previously been reported as a biomarker in asthma and we were able to identify other metabolites that are part of the same pathway.

To determine whether diet could have an effect on asthma severity, a diet score was calculated for each participant using a previously published tool [205]. No differences were seen between controls and asthmatics, but an increase in BMI was seen with increased asthma severity. Previously published literature has suggested a potential link between asthma and BMI, but the cause behind this remains unclear [85, 161]. It is difficult to determine whether obesity is a consequence of asthma or the other way around. As severe asthmatics have a hard time controlling their asthma and difficulty breathing, the consequence could be less exercise decreasing the energy expenditure. This would lead to higher fat accumulation and increase in BMI. Another possibility could be that an increase in BMI could lead to higher fat mass and difficulty breathing. This puts a heavier strain on the airways, which have to work harder. Studies have shown that obese people have 7-10% higher chance of developing asthma per unit BMI increase [220]. It is therefore difficult to determine whether the biomarkers found to distinguish between controls and severe asthmatics are due to the asthma and not obesity. However, the majority of the identified biomarkers where not strongly associated with BMI, suggesting that those biomarkers are involved with asthma pathogenesis and not obesity.

Further analyses were undertaken to determine whether microbial and dietary-related metabolites could be different between the groups. Interestingly, nine microbial and dietary metabolites were decreased with asthma severity suggesting that diet and gut microbiota could play a role in asthma. Previous literature has suggested that asthmatics have perturbed gut as well as airway microbiota [55, 221-224]. Decrease of microbial metabolites (seen in figure 5.9), tryptophan, propionate,

dimethylamine, 4-hydroxyhippuric acid and 1-methylnicotinamide, seen in this study, strongly suggests that there is a change is gut microbiota, which could potentially contribute to disease pathogenesis. However, as the gut microbiota was not investigated for these individuals, it is difficult to conclude whether a change in gut bacterial species is related to asthma pathogenesis. Variation in three dietary metabolites suggests that diet could play a role in asthma. An increase in arginine was seen with asthma severity. This increase is consistent with the increase in N1-acetylspermidine and N-acetylisoputreanine-y-lactam as they are produced from arginine degradation [209]. Moreover, arginine degradation can result in two pathways, where one results in the production of nitric oxide and citrulline while the other enhances the production of polyamines through arginase [225, 226]. Asthmatics are known to have increased levels of exhaled nitric oxide, which is a marker for airway inflammation [227] and increased levels of polyamine has been shown to contribute to airway remodeling, something that is also present in asthmatic airways. Both UPLC-MS and ^{1}H NMR spectroscopy data suggests that arginine degradation and polyamine production is increased in asthmatics patients and could be potential biomarkers for asthma.

Several other limitations present in this study include the use of only one cohort. Even though the cohort we used was significantly larger than previously reported cohorts, we lacked the ability to validate the identified metabolites in another separate cohort. Cross-validated models were used together with permutation test, for model validity, and ROC curves with multiple linear regression to further strengthen our identified metabolites.

5.4 Conclusion

Several metabolites were identified as biomarkers for asthma. Polyamine metabolism was upregulated in asthmatics and identified in both UPLC-MS and ^{1}H NMR spectroscopy datasets, which included L-arginine and N1-acetylspermidine. Several carnitines were downregulated in asthmatics and could be a potential target for asthma. Furthermore, N2,N2-dimethylguansoien and xanthosine, both belonging to purine metabolism were seen to be upregulated in asthmatics providing another set of biomarkers. Finally, a decrease in several microbial and dietary metabolism were seen in asthmatics suggesting a change in gut microbiota compared to controls. Several metabolites were identified as potential biomarkers for asthma, which could both diagnose the disease and disease phenotypes. However, further studies are needed to validate these results in other cohorts.

Chapter 6 Investigating the effect of fibre supplementation to exercise induced bronchoconstriction – pilot study

Hypothesis

It is hypothesised that supplementation of fibre will improve lung function, increase circulating short-chain fatty acids (SCFAs) and alter the metabolic profile of individuals with exercise-induced bronchoconstriction (EIB).

Aims

- Examine the ability of fibre supplementation to modulate lung function, circulating SCFAs and the metabolic profile of EIB positive and negative individuals.

Objectives

- Quantify and identify differences in SCFAs concentrations between EIB positive and negative individuals provided with diets with high fibre and control supplementation.
- Metabolic profiling, multivariate and univariate statistics and diet analysis of urine of above-mentioned cohort using ^{1}H NMR spectroscopy

6.1 Introduction

The crosstalk between the gut and the lungs has gained tremendous interest the last couple of years. It has been shown that people with respiratory disorders often have, and have a higher prevalence of developing, gut disorders such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) [162]. It has also been shown that people with gut disorders, who have increased intestinal permeability, often have some type of respiratory problem. Furthermore, there is clear evidence suggesting immunological crosstalk between these two sites. One common factor for all these observations is the microbiome [228]. It has been shown that administration of a high fibre diet to rodents hindered the development of allergic airway inflammation, compared to low fibre fed rodents. Fibre was broken down by the gut microbiota producing SCFAs, which enhanced the development of immune cells and subsequently impaired the ability to activate Th2 cells [60]. Moreover, SCFAs promoted the production of T regulatory cells as well as upregulation of the FOXP3 promoters, which in turn inhibited histone deacetylase (HDAC), inhibiting the development of allergic airway inflammation [84].

However, only two studies have investigated the effect of fibre supplementation in asthmatics. One study investigated the effect of one single fibre supplementation and the other a 3-week supplementation [112, 113]. Both studies demonstrated improved lung function together with attenuation of inflammatory markers.

Furthermore, exercise-induced asthma (EIA) or exercise-induced bronchoconstriction (EIB) is a common occurrence with athletes where exercise causes the airways to narrow and constrict leading to coughing, shortness of breath, tightening of the chest and wheezing [229]. EIA can be

diagnosed by measuring the level of airway restriction and inflammation, which occurs in response to increased breathing rates. A positive response in hyperpnoea-induced bronchoconstriction (HIB) corresponds to a drop in approximately 13% in FEV_1, when measuring at rest at two consecutive time points [230].

While some studies have exhibited positive results of fibre supplementation to asthmatics, additional studies are needed to elucidate the effect of fibre on HIB positive individuals and how this affects host response, immunology and metabolism. The aim of this pilot study is to investigate the effect of 3-week 3.4g Bimuno-galactooligosaccharide (B-GOS) supplementation on HIB positive and negative individuals on host response and metabolism.

6.2 Results

6.2.1 Patient information

Of the 16 participants, 7 were HIB positive and 9 were HIB negative. 12.5% of the HIB positive participants were women, while 33.3% of the HIB negative were women. The average age for the HIB positive were 23.57 ± 2.01 and 22.67 ± 1.13 for HIB negative and BMI was 22.64 ± 0.52 and 23.60 ± 0.87 respectively. FEV1 at baseline and after HIB test was lowered with 23% for HIB positive individuals and 10% for HIB negative (table 6.1).

Table 6.1. Characteristics of HIB positive and HIB negative individuals participating in this pilot study.

	HIB negative	**HIB positive**
Participants (n)	9	8
Sex % women (n)	33.33 (3)	12.5 (1)
Age	22.67 ± 1.13	23.57 ± 2.01
BMI	23.60 ± 0.87	22.64 ± 0.52
FEV_1 baseline (L)	4.06 ± 0.31	4.11 ± 0.30
Predicted FEV_1 (%)	92.56 ± 2.96	91.67 ± 4.22
FEV_1 post EVH (L)	3.67 ± 0.28	3.17 ± 0.32
$FEF_{25\%-75\%}$ baseline (L)	4.93 ± 0.34	3.66 ± 0.47
Predicted $FEF_{25\%-75\%}$ (%)	86.44 ± 9.00	77.57 ± 8.49
$FEF_{25\%-75\%}$ post EVH (L)	4.57 ± 0.30	2.30 ± 0.42
Intervention adherence (%)	X – 98, L – 99	X – 98, L – 95

6.2.2 No change in plasma SCFAs, after supplementation of prebiotic, in HIB positive and negative individuals

To evaluate the change in SCFAs with prebiotic supplementation in both HIB positive and negative individuals, plasma samples were taken at baseline and after supplementation and analysed for SCFAs using a targeted GC-MS method [153] (method paragraph 2.1.3). Acetate, propionate, butyrate, valerate, lactate and 2-hydroxybutyrate were detected, while isobutyrate, isovalerate, caproate and 2-methylbutyarte were not. No significant change was seen for any of the SCFAs for either HIB positive or negative individuals after prebiotic supplementation (figure 6.1). There was a trend towards increased levels of acetate in HIB positive individuals (figure 6.1a), but it did not reach statistical significance.

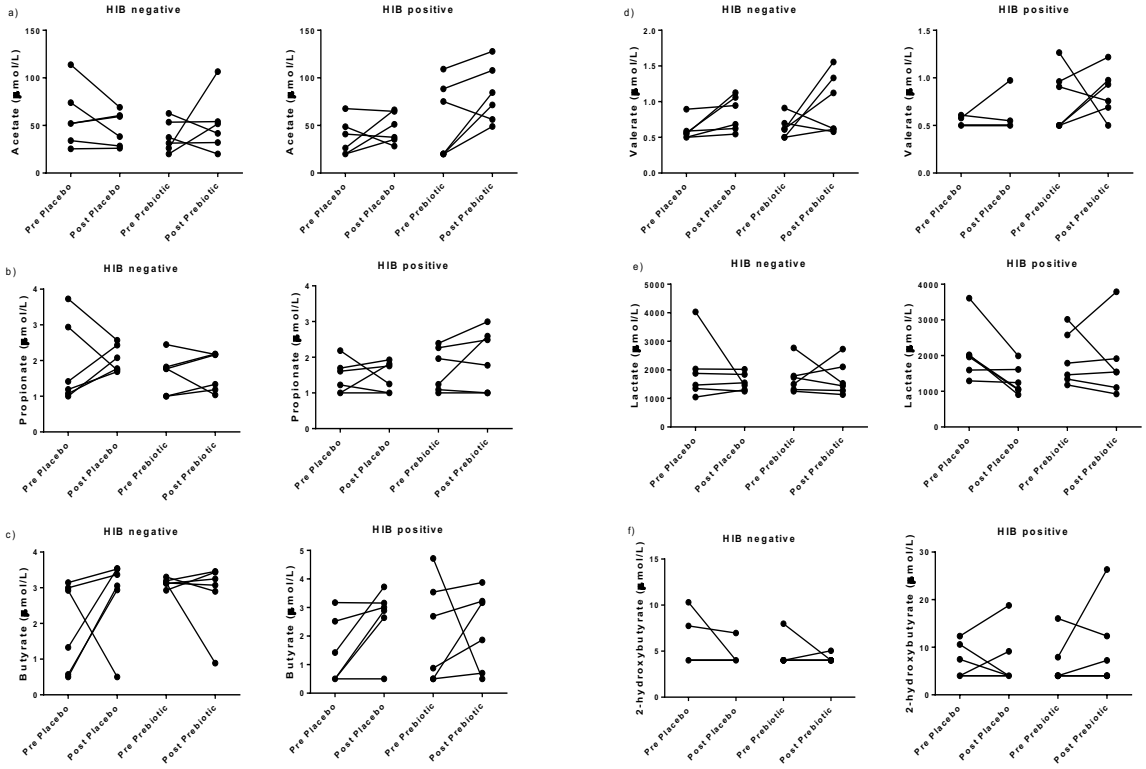


Figure 6.1. No differences observed in circulating SCFAs levels after each intervention for both HIB positive and negative participants.

a) Acetate, b) propionate, c) butyrate, d) valerate, e) lactate and f) 2-hydroxyburyrate. Data shown as each individual sample before and after each intervention. Unpaired parametric two-sided t-test was performed.

In order to determine if the supplements had an effect on the SCFA concentrations in the plasma samples of the participants, irrespective of being HIB positive or negative, the data were combined and compared between the diets. No significant differences were observed for the different SCFAs for either the placebo or the prebiotic, suggesting that the supplement did not have an impact on circulating SCFA concentrations (figure 6.2).

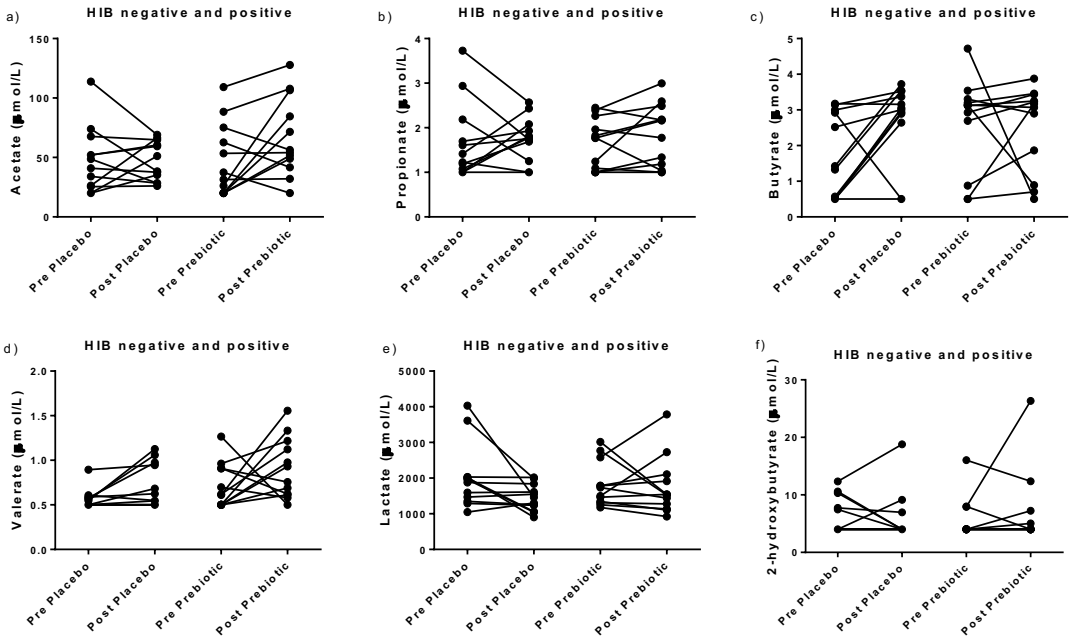


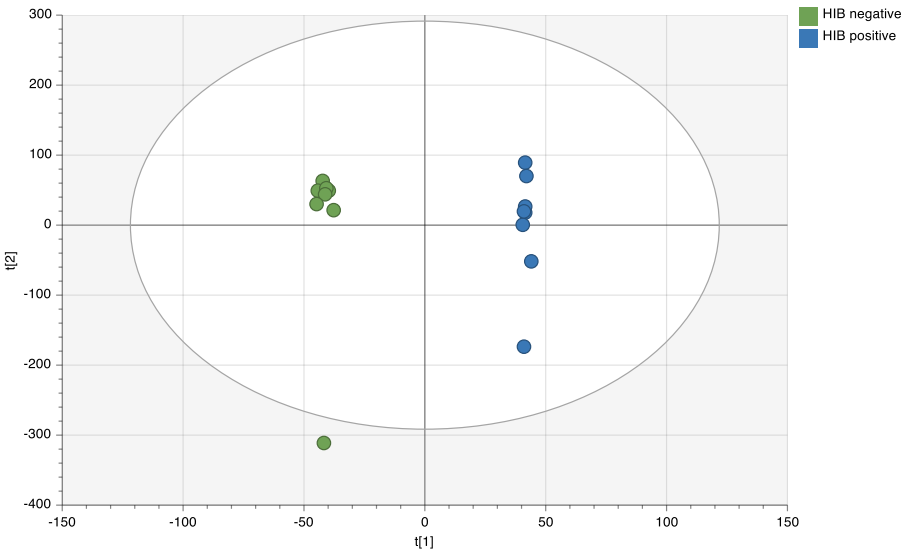
Figure 6.2. No differences observed in circulating SCFAs levels after each intervention for all participants.

a) Acetate, b) propionate, c) butyrate, d) valerate, e) lactate and f) 2-hydroxyburyrate. Data shown as each individual sample before and after each intervention. Unpaired parametric two-sided t-test was performed.

6.2.3 Metabolic difference between HIB positive and negative individuals after prebiotic supplementation was not observed in multivariate models.

To evaluate whether the prebiotic supplement had an effect on the metabolism of HIB positive and negative individuals, metabolic profiling using ^{1}H NMR spectroscopy (method paragraph 2.4.3), was performed on spot urine samples collected in the morning. Multivariate analysis (method paragraph 2.5), using unsupervised and supervised analysis, did not exhibit any differences between HIB positive and negative individuals at baseline. No differences were seen comparing the baseline and post-prebiotic urine profiles for either HIB positive or HIB negative individuals (all models yielded negative R^2X and R^2Y values). However, a model was generated between HIB positive and negative individuals after prebiotic supplementation, with positive R^2X and R^2Y values, but this change was not statically significant (figure 6.3).

a)



b)

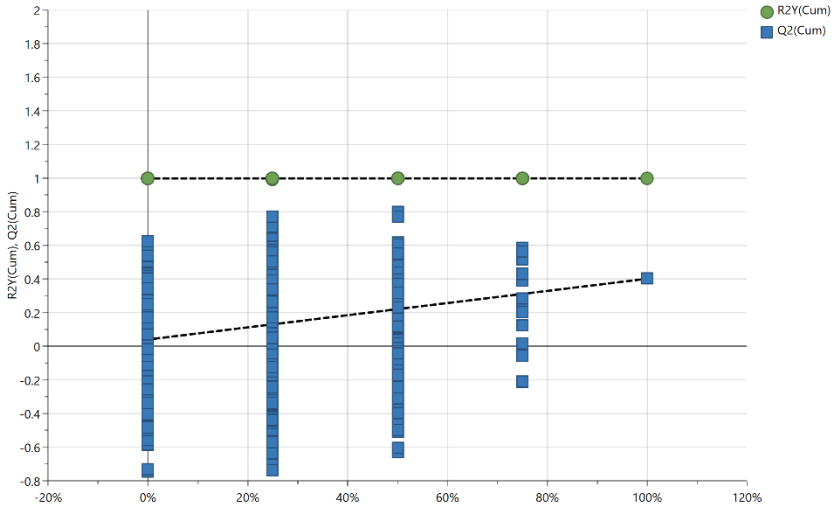


Figure 6.3. OPLS-DA model comparing HIB positive and negative individuals after prebiotic supplementation.

a) The generated model is valid (R^2X = 0.998, Q^2Y = 0.401), but not statistically significant (CV-ANOVA P=0.63). b) Permutation test *(999 permutations)* performed on the model showcasing non-significance.

Next, the participant's diets were examined to observe if they changed during the supplementation period. A previous study [205] has developed a tool to measure how healthy each individual's diet can be based on a single urine test, with a high percentage demonstrating a healthy diet. No difference in diet score could be observed between HIB positive and negative individuals before and after each supplement (figure 6.4a) and no difference was seen when combining both groups (figure 6.4b).

These results once again demonstrate that the supplement did not have an effect on HIB positive and negative individuals with regards to their metabolic profile and their diet score.

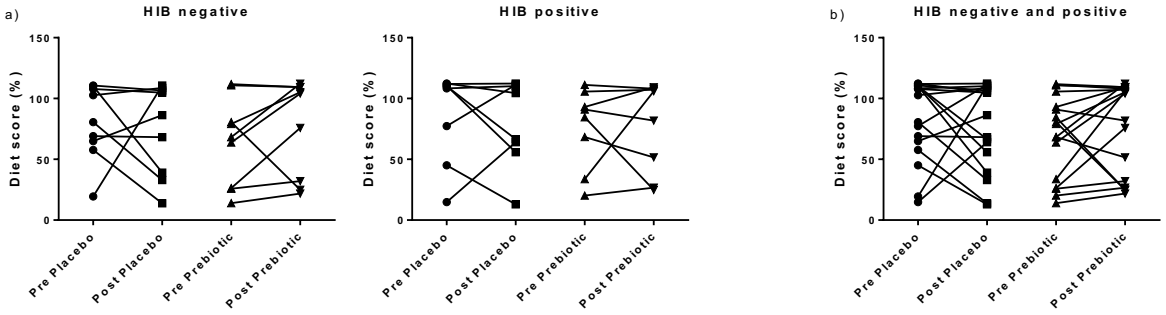


Figure 6.4. No difference in diet score after supplementation for neither HIB negative nor positive individuals.

Diet score for a) HIB negative b) HIB positive and c) both combined.

6.2.4 No difference in metabolites between HIB positive or negative individuals after each intervention, apart from two microbial metabolites.

To determine if any individual urinary metabolites changed across the study, univariate analysis was performed on selected metabolites extracted using the Bruker B.I.QUANT tool. No major differences were seen. A trend was noted for a reduction in urinary acetate in HIB positive individuals after prebiotic supplementation (figure 6.5b) and an increase in hippurate for HIB negative after prebiotic supplementation.

These results could suggest a functional change in the gut microbiota following supplementation, although those differences were not significant.

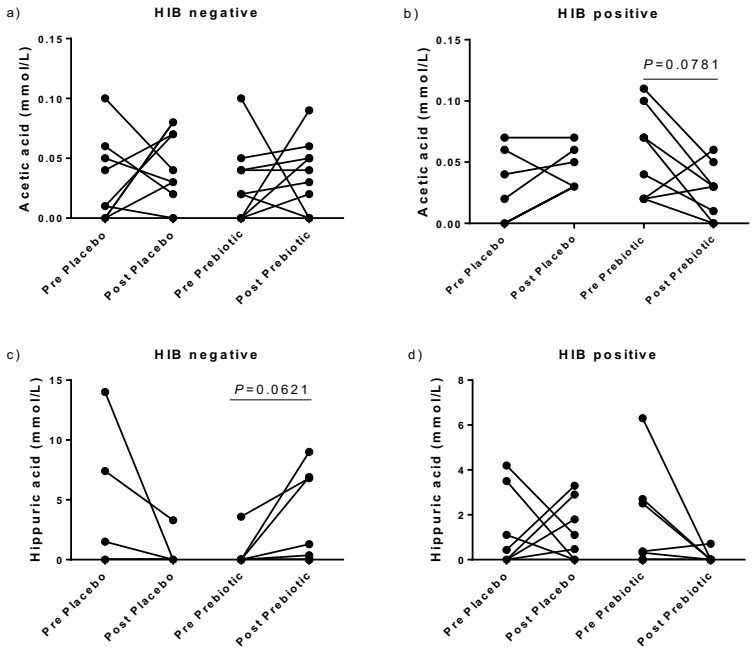


Figure 6.5. Trend towards change in gut microbial metabolites in both HIB negative and positive individuals.

Metabolites were extracted from the 1H NMR spectroscopy data and compared between supplement, demonstrating differences in a) acetic acid and b) hippuric acid. Paired nonparametric two-sided t-test was performed.

6.3 Discussion

In the last few years major focus has been on the interplay between diet and the airways and how intake of specific dietary components can modulate the airways [60, 71, 73, 84, 112, 165, 229, 231-234]. Especially interesting is the effect of dietary fibre on asthmatics and how intake of fibre prevented the development of airway inflammation in rodent models [60, 84]. Only two studies have investigated the effect of fibre supplementation to asthmatic individuals and results have shown a slight increase in lung function and attenuation of inflammatory markers [112, 113]. However, the study that noted increased lung function only investigated the acute effect of fibre supplementation and did not investigate the long-term consequences if this supplementation. One study has investigated the association of SCFAs, particular propionate and butyrate, with allergy in children and demonstrated that children with high levels of propionate and butyrate in their faeces, when they are 1 years old, are less likely to develop asthma between the ages 3-6 years [165].

In this crossover pilot study, daily intake of 3.6 g of fibre, or placebo, was randomly assigned to individuals who tested positive for exercise induced asthma (HIB) (n=8) and those who tested negative (n=9). Each participant was randomly assigned one supplement for 4 weeks followed by a washout period of 2 weeks and then crossed over to the second supplement for additional 4 weeks. SCFAs analysis was conducted on plasma samples to evaluate the production of these metabolites comparing diets and groups. Due to sampling issues there were only plasma samples from 7 HIB positive individuals. Previous studies have shown that fibre supplementation should increase circulating SCFAs, especially acetate levels [235]. However, this was not seen for any of the individuals in both groups on the prebiotic diet. There was a trend towards increased acetate

levels, but this did not reach statistically significance. One of the reasons we didn't see any change with the prebiotic supplement, could be that the amount of GOS was only 3.4 g, which is relatively low compared to other studies that have used up to 30 g of fibre supplement. For this reason, we cannot conclude that fibre supplementation did not have an effect, but rather that the amount needed to see a difference may be greater. Another reason for not seeing any difference of the supplement could be the change was missed and not "collected" in the sample that was taken. Another limitation present in this study is that breath hydrogen was not collected. The levels of breath hydrogen could have been used to assess compliance, as the gut microbiota releases hydrogen during fermentation of fibre [236].

Another aim of the study was to determine whether fibre supplementation could have an effect on the metabolic profile of the participants. Previous studies have demonstrated that diet can have an extreme impact on the metabolism of individuals [237-240]. ^{1}H NMR spectroscopy was performed on spot urine samples and analysed using multivariate and univariate analysis. No metabolic differences were seen with unsupervised methods; however, a valid model was generated between HIB positive individuals before and after prebiotic supplementation, but this was not statistically significant. To determine if the diets, for the two groups, changed between the two supplementation periods, a diet score was calculated using previously published tool, which is based on an intervention study investigating differences in urinary biomarkers between less healthy and healthy diets according to WHO guidelines [205]. However, no difference was seen neither between the groups nor between the diets, once again suggesting that the fibre supplement was not high enough to make a difference.

Univariate analysis on single metabolites was conducted to try and elucidate whether there were any differences at all for the metabolites. Interestingly, there was a trend decrease in urinary acetic acid after prebiotic supplementation, but this did not reach statistical significance. Prebiotic supplementation should generate higher levels of faecal and circulating acetate, but perhaps the decrease in urinary acetate could be due to better absorption in presence of prebiotic supplementation. Another speculation could be that HIB positive individuals are less affected by the fibre supplementation compared to the HIB negative ones and require a higher dose to actually see an increase in urinary acetic acid. It is important to note that in this study valid multivariate models can be generated, but when performing statistical analysis, the majority of the models are not valid, which are indicated with a p-value higher than 0.05. Furthermore, when a "trend" is implied, this indicates that the majority of the samples are following the same trend by either decreasing or increasing, but they do not reach statistical significance, possibly due to the low sample number or outliers.

The increase seen in hippuric acid, which was observed for HIB negative individuals but not HIB positive, could be due to alteration of the gut microbiota, which is more responsive for change with prebiotic supplementation for HIB negative compared to HIB positive. The question remains whether a higher dose of prebiotic would have generated similar results for the HIB positive individuals.

Furthermore, the collection of faeces would have been interesting as it would have told us whether the fibre supplementation had an impact on the gut microbiota and whether HIB positive have a perturbed microbiota compared to HIB negative. It would also been interesting to compare the

microbiota between the gut and the airways as well as the SCFAs concentration to provide us with further information about the interplay between these two sites.

Lastly, as this was a pilot study, the numbers were low but perhaps slightly different results would have been observed if the study would have had more participants.

6.4 Conclusion

There is a growing interest surrounding the gut-lung axis and the effect of fibre on airway inflammation. Here, we tried to address this growing interest by a longitudinal pilot study looking at the effect of daily prebiotic supplementation to individuals with exercise induced asthma (HIB positive). There was a metabolic difference between HIB positive and negative individuals after prebiotic supplementation but not at baseline. Furthermore, no change in plasma SCFAs were observed after the intervention. In conclusion, this pilot study was a good attempt on addressing the many of the questions regarding inflammation, lung function and fibre, but several study components need to be addressed to generate any results.

Chapter 7 Concluding remarks

The framework for this thesis was set from the work of Trompette *et al*. [60] and Thorburn *et al.* [84], demonstrating that fibre supplementation to rodents followed by induction of airway inflammation, resulted in no development of elevated levels of inflammatory associated immune cells or cytokines. These results set the context for the gut-lung axis, which demonstrated in a clear way that SCFAs, derived from gut fermentation by the microbiota, inhibited the development of airway inflammation. It is well known that dietary fibre is beneficial to the host, and two pilot studies did demonstrate a trend towards improvement of lung function together with attenuation of inflammatory markers in asthmatics with fibre supplementation [112, 113]. Another study in children, demonstrated that high levels of propionate and butyrate found in faeces of 1 year old children, had a lower risk of developing asthma at the ages of 3-6 years compared to those with lower levels [165].

The focus of much work done on the gut-lung axis has been focused around the gut-derived SCFAs and effort has been put on investigating whether there is an interaction of gut-derived SCFAs with the actual airways. In **Chapter 3**, the presence of SCFAs in airway fluids was investigated. Surprisingly, the majority of the SCFAs were detected in both bronchoalveolar lavage (BAL) samples as well as sputum samples of both healthy controls and asthmatics. Another surprise was that acetate concentrations were elevated in asthmatics, which at first glance would be considered a bit unusual as SCFAs are considered beneficial to the host and should therefore be present at a higher concentration in healthy individuals. The second surprise was that the levels of SCFAs detected in sputum samples of asthmatics demonstrated higher levels than those described to be found in peripheral blood. This raised the question to whether the detected SCFAs might not be

gut-derived but rather airway-derived. This hypothesis was also further strengthened with the publication of two papers in cystic fibrosis, culturing airway species in presence of mucus and the results demonstrated that those species had the ability to break down mucus and produce SCFAs [110, 111]. Luckily, there was 16s rRNA sequencing data for the sputum samples, so a linear association model was performed between the genera and the SCFAs concentrations. Many positive associations were seen with acetate, propionate and 2-methylbutyrate, with the highest number of associations for 2-methylbutyrate. 2-methylbutyrate can be produced through proteolytic activity, which further supported that it could be degradation of mucus. Interestingly, one paper has shown the production of 2-methylbutyrate from leucine and another paper has shown that leucine in one of the components of mucin [166, 167]. To further validate these findings, an *in vitro* batch culture experiment was set up with selected species from the sputum samples of asthmatics, belonging to the genera found in the association model, and cultured in presence and absence of mucin. An increase in SCFAs was quantified for selected species in presence mucin, strongly suggesting that the SCFAs detected in the airways are airway-derived and not gut-derived, yet these data are preliminary. Furthermore, as asthmatics have inflamed airways and excessive mucus production, the elevated levels of acetate found in the BAL samples would make sense as the airway microbiota has more mucus to break down subsequently producing higher levels of SCFAs. As the *in vitro* batch culture were done on species isolated form asthmatic sputum samples, it would have been interesting to see if the same species from sputum samples of healthy controls behaved in a similar fashion. This would have demonstrated whether the findings are species dependent or disease dependant. What was also interesting was that butyrate was not found to be elevated in the *in vitro* batch culture, suggesting that perhaps that the airway bacterial species do

not produce higher amount of butyrate in presence mucin and acetate and propionate are more important than butyrate in the airways.

Next, we wanted to understand the reason behind the presence of SCFAs in the airway, so in **Chapter 4**, we sought to investigate the effect SCFAs in airway epithelium. If the airway microbiota is breaking down mucus to gain energy resulting in the production of SCFAs, the epithelium would be the first site of contact for these metabolites. The main results are centred on acetate and propionate as they are the most abundant ones in the airways, were found in the association model and elevated in the *in vitro* batch culture results. Airway differentiated epithelium was treated with acetate and propionate on both apical and basolateral side. The SCFAs receptors, FFAR2 and FFAR3, were detected on airway epithelium and there was a trend towards decreased expression of the receptors with propionate stimulation, which was significant, when inflammation was induced with IL-13. Literature has shown that FFAR3 is expressed more on both sides of the epithelium whilst FFAR2 is much less expressed.

Literature investigating the effect of SCFAs have shown their anti-inflammatory effect, in preclinical settings, and it was a slight surprise when overproduction of immunomodulatory cytokines were found for the airway epithelial cells stimulated with SCFAs. IL-6 and CXCL8 were both elevated when treating the epithelium with acetate and propionate on both sides, with propionate having a slightly stronger effect. It was further proven that propionate was more potent when investigating the effect of CCL26, an eosinophil chemoattractant, which was elevated when treating with 10mM of propionate on basolateral side and not apical and with no change for acetate. It is interesting to speculate that high dose of acetate and propionate, in the airways, could be perceived as pathogenic microbial derived molecules making the epithelium emit an

immunomodulatory response to control the danger signal. The reason for the high levels of CCL26 on the basolateral side could be that the epithelium wants to recruit eosinophils through the circulating system, which is done on the basolateral side. Furthermore, FFAR3 is expressed more on the airway epithelium and has a higher affinity for propionate, which could explain why propionate has a more prominent effect than acetate when investigating some of the cytokine levels. These results clearly suggest that SCFAs interacting with the airway epithelium are acting in an immunomodulatory manner, which has not been seen in the gut or on immune cells. SCFAs may behave in a different way depending on their dose and location throughout the body. A limitation that was not accounted for was the transport of SCFAs from one side to the other. Perhaps it would have been beneficial to measure the amount of SCFAs on both sides of the epithelium, as well as within the cell, to get a better understanding of the transport mechanisms and potential effects on the results we observed.

Furthermore, the same experiments were conducted with butyrate and different results were seen. High levels of butyrate were acting in a more anti-inflammatory manner, but it is important to note that the effect was seen at 10mM of butyrate. This concentration is not physiologically correct, especially in the airways, suggesting that this effect is not observed *in vivo*.

Further investigations on the immunomodulatory response was done by exploring the metabolic content on the basolateral side of the epithelium. What was particularly interesting, was that treatment with propionate followed by IL-13, promoted the release of branched chain amino acids, with inflammation, compared to treatment with only propionate. This effect was seen at 1mM of propionate on apical side compared to 10mM on basolateral side, suggesting that perhaps the cells are more prominent to reaction on the apical side of the epithelium. In order to determine the reason

behind the release of BCAAs, Th2 skewed PBMCs were treated with a range of BCAAs concentration. A decrease in IL-5 and an increase of IL-10 suggesting an immunomodulatory effect. As the PBMCs are Th2 skewed, they are already emitting a pro-inflammatory response, mimicking asthma or in our case an IL-13 induced response. This could be a regulatory mechanism by the cells, meaning as inflammation is running high, anti-inflammatory cytokines are released to try and dampen inflammation to maintain homeostasis. However, as asthmatics have inflamed lungs, inflammation is ongoing and together with mucus metaplasia results in a loop that was described in **Chapter 3** and continued in **Chapter 4**. These 2 chapters are illustrated in figure 7.1. The reason immune cells are acting pro- and anti-inflammatory is due to that we did not screen for all immune cells are they could be behaving in both ways.

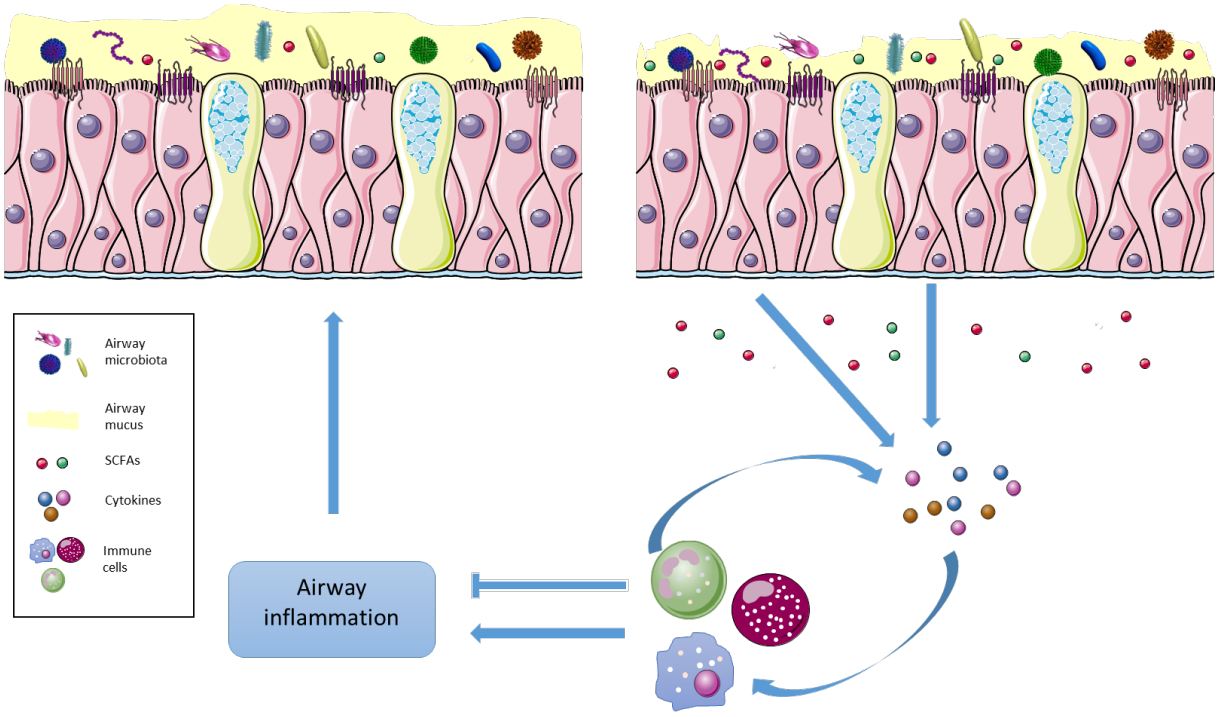


Figure 7.1. Model of microbial-airway-derived SCFAs and their immunomodulatory response.

The airway microbiota breaks down to mucus for energy and produces SCFAs as by-products which when in contact with the airway epithelium emits an immunomodulatory response, secreting some pro-inflammatory cytokines and other metabolites. This results in the recruitment of immune cells and mono nuclear cells which can either act in an anti-inflammatory manner or pro-inflammatory. As asthmatics have mucus metaplasia more SCFAs can be produced by the airway microbiota and there is a constant cascade of inflammation on in on in the airway.

In **Chapter 5**, I sought to get a better understanding regarding biomarkers associated with asthma. Asthma is a hard disease to characterize and it requires many different invasive tests to determine whether an individual has the disease or not. Furthermore, the variability within the disease itself is huge and several endotypes are present within a class of asthma. This study aimed to explore non-invasive ways of identifying biomarkers related to asthma using urine samples. Interestingly, no differences were observed between healthy controls and mild to moderate asthmatics suggesting that these are very similar metabolically. However, differences were observed both between healthy controls and severe asthmatics and between mild to moderate and severe asthmatics. Further investigations demonstrated that the difference was driven by several metabolites and after further analysis a subpart of these were identified. Two of them belong to purine metabolism and were elevated in severe asthmatics. Purine metabolism has been previously described to be altered in asthma, but not many studies have investigated this [202]. What is particularly interesting is that two metabolites were both metabolites of interest and are both involved in purine metabolism, further strengthening the hypothesis that purine metabolism is important in asthma. Furthermore, comparing subgroups, within severe asthmatics, these two metabolites were further elevated in the more severe subgroup, suggesting that these two metabolites could have the potential of distinguishing within subpopulations of asthmatics.

Two other metabolites that were also elevated in asthma are part of polyamine metabolism and studies in rodents have demonstrated elevated levels in both urine and serum but these findings have not been shown in human urine [204, 208, 209]. Polyamine metabolism has been associated with activation of eosinophils, contraction of airway smooth muscle cells, induce oxidative burst in neutrophils and induce histamine release [203]. Furthermore, arginine degradation gives rise to ornithine, spermidine and other metabolites in the polyamine pathway and this metabolite was

increased in severe asthmatics from the ^{1}H NMR spectroscopy data, further supporting this hypothesis. These metabolites could be of great interest for further validation as asthma biomarkers. One other metabolite, which was also very interesting belong to the carnitine family. This carnitine was downregulated in asthmatics, and when comparing several other carnitines, it was demonstrated that they all behaved in similar fashion. As for the metabolites in purine pathway, carnitines were also able to distinguish between sub-groups of severe asthmatics. Furthermore, 8 carnitines had this ability and the longer the carnitine chain was, the higher the statistical significance. This could also suggest that different pathways are involved depending on the length of the carnitines. Carnitines are involved in fatty acid oxidations are transporters of long-chain fatty acids to the mitochondria for β-oxidation to gain energy [216]. Other studies have demonstrated carnitine deficiency in asthmatics and that supplementation improves asthma control in both humans and rodents [210-212]. Carnitines seem to be very interesting biomarkers and perhaps a target for asthma diagnosis.

Another finding in this cohort was the direct association between asthma and BMI, which was significantly elevated with increased asthma severity. Previous studies have suggested that there might be a link between obesity and asthma [85, 161]. However, there was no difference in diet score between the groups indicating that the healthiness of the diets might not play a major role in asthma. Furthermore, looking at dietary and microbial metabolites there was a decrease of these metabolites with increased asthma severity indicating that there might be a change in gut microbiota. Previous studies have demonstrated an altered gut microbiota in asthmatics [221, 224], but faecal samples were not collected in this study to verify these finding. Unfortunately, we were not able to validate these results in an external cohort to be able to determine that the potential

biomarkers found are consistent across multiple asthma cohorts. However, this study had a large number of participants and the metabolite were partly validated using a multiple regression model.

To tie all of these projects together, **Chapter 6** was a pilot study investigating the effect of fibre supplementation to people with exercises-induced asthma (EIA). A prebiotic was administered to individuals who tested positive for EIA and those who tested negative, followed by a washout period and a control supplement. Unfortunately, most of the results in this study were negative. This was probably due to the low amount of prebiotic supplement (3.4g of GOS), where most studies use around 30g to see a difference. Another major limitation was that several samples that could have aided in the analysis were not collected. Breath hydrogen, which is a measurement for gut fermentation by the gut-microbiota, faecal samples, which would have indicated whether the prebiotic supplement altered the gut microbiota. Also, if there is an interest in the gut-lung axis, airway samples would have been important to collect. There was no improvement in lung function nor in circulating cytokines. The hypothesis and idea behind the study was very good but the amount of prebiotic was not enough, and the participant number was also very low.

In conclusion, in this thesis SCFAs have been shown to be present in the airways, possibly though production by the airway microbiota, and when in contact with the airway epithelium induce an immunomodulatory response. Biomarkers related to asthma pathogenesis and difficulty have been identified and could serve as potential diagnostic tools to be able to diagnose asthma as well as sub-groups within asthma. And finally, a high level of prebiotic is necessary to modulate the effect in asthmatics and to be able to further investigate the role of the gut-lung axis.

Chapter 8 Future perspectives

Several different researchers, including myself, have tried to elucidate some portion of the mechanism and potential role of SCFAs in asthmatics. In this thesis, the following bullet points are the main findings:

- SCFAs are present in the airways
- Acetate is present in higher concentration in asthmatics compared to controls
- SCFAs concentrations in sputum samples of asthmatics are higher than what has been previously described in peripheral blood
- SCFAs detected in airway samples could possibly be due to mucus degradation by the airway microbiota
- Acetate and propionate have an immunomodulating effect on the airway epithelium, which is different from what has been seen with the gut epithelium
- SCFAs receptors, FFAR2 and FFAR3, are expressed on the airway epithelium
- The airway epithelium secretes elevated levels of branched chain amino acids when treated with propionate and IL-13
- Several urinary metabolites were found to be different between controls and asthmatics including polyamines, purines and carnitines
- Carnitine levels were different between sub-populations of severe asthmatics
- The amount of fibre supplementation needs to be higher, than what was given in this study, to possibly see a change in lung function and in circulating levels of SCFAs in people with exercise-induced asthma

Papers have shown parts of how the gut-lung axis is working but what everyone has failed to do is to actually demonstrate the entire gut-lung axis in humans. The study that everyone has not yet achieved is to do is to enrol asthmatics in a study where they are supplemented with a daily dose of high levels of fibre for a prolonged time. As we have seen in our final study, the dose of fibre has to be sufficiently high to actually modulate an effect. What is then needed is to measure the levels of SCFAs in three different biological matrices: faeces, serum and sputum before and after the supplementation period. It would also be valuable to collect nasal and oral swabs, both for eliminating contamination upon sputum induction, but also to measure both SCFA concentrations and microbiota. These measurements can then answer the question whether SCFAs in the airways are affected by the levels in the gut. Furthermore, isolating and analysing the gut and airway microbiota would answer the question whether SCFAs supplementation can modulate the airway microbiota and whether the gut microbiota can crosstalk with the airway microbiota. Using shotgun metagenomics, instead of 16S sequencing, would generate functional information and species resolution, adding considerably more information to the study. This would also answer the question whether the anti-inflammatory effect of SCFAs seen in the gut is overpowering the immunomodulation seen in the airways. Finally, measuring lung function and inflammatory markers, both in circulating system and in the airways, could answer the question whether asthma is being resolved.

To validate some of the results seen in my thesis another batch culture experiment, with isolated airway bacterial species, could be important. The bacteria should be supplemented with airway mucus and sampled continuously, to check for bacterial growth and pH differences. A decrease in pH could indicate production of fatty acids. Another experiment could be done in germ-free mice, where airway fluids could be sampled to check for presence of SCFAs. This could be compared to

germ-free mice inoculated intranasally with airway bacteria and mice with an airway microbiome and in theory SCFAs production. At the same time, stimulation with HDM intranasally could promote airway inflammation and potential differences can be observed. The problem with this is the volume of fluid within a mouse lung, which is quite small and can cause detection problems for SCFAs quantification. Another issue is that the signalling happening from the gut and whether the inoculated airway bacteria stays in the airways. Perhaps some sort of labelling could be useful.

To conclude, in this thesis I have introduced new topics regarding the gut-lung axis with more focus on the airways. With these exciting results I hope that we can be one step closer in getting a complete understanding of asthma and how the disease can be modulated by diet and the microbiota.

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Appendix

Table S2.1. Characterisation of the asthmatic cohort (n=102) from where the sputum samples were taken for the SCFAs and microbiome analysis.

Data demonstrated as mean and standard error of mean (SEM).

Clinical characteristics	**Asthmatics (n=102)**
Gender Male n (%)	34 (46)
Female n (%)	40 (54)
Age (years)	39.51±1.52
Age asthma onset (years)	15.04±1.63
Ex-smokers n (%)	9 (12)
ACQ-7 (units)	1.04±0.10
ICS dose (µg/day)	347.1±46.65
FEV1	2.99±0.09
FVC	4.10±0.12

Table S2.4. Calibration curve for targeted fatty acid assay using GC-MS. 14 points were constructed for the analysis of sputum samples. All concentrations are in µmol/L.

Point	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Acetic acid	3200	2000	1600	1000	640	320	160	80	32	12.8	6.4	3.2	1.6	0.8
Propionic acid	640	400	320	200	128	64	32	16	6.4	2.56	1.28	0.64	0.32	0.16
Butyric acid	20	12.5	10	6.25	4	2	1	0.5	0.2	0.08	0.04	0.02	0.01	0.005
Valeric acid	4	2.5	2	1.25	0.8	0.4	0.2	0.1	0.04	0.02	0.01	0.005	0.0025	0.00123
Isovaleric acid	40	25	20	12.5	8	4	2	1	0.4	0.16	0.08	0.04	0.02	0.01
Hexanoic acid	10	6.25	5	3.125	2	1	0.5	0.25	0.1	0.04	0.02	0.01	0.005	0.0025
Isobutyric acid	20	12.5	10	6.25	4	2	1	0.5	0.2	0.08	0.04	0.02	0.01	0.005
Lactic acid	40000	25000	20000	12500	8000	4000	2000	1000	400	160	80	40	20	10
2-methyl-butyric acid	20	12.5	10	6.25	4	2	1	0.5	0.2	0.08	0.04	0.02	0.01	0.005
2-hydroxy butyric acid	2000	1250	1000	625	400	200	100	50	20	8	4	2	1	0.5

Table S2.5. Calibration curve for targeted fatty acid assay using GC-MS. 12 points were constructed for the analysis of BAL samples. All concentrations are in µmol/L.

Point	1	2	3	4	5	6	7	8	9	10	11	12
Acetic acid	800	500	400	250	160	80	40	20	8	3.2	1.6	0.8
Propionic acid	80	50	40	25	16	8	4	2	0.8	0.32	0.16	0.08
Butyric acid	20	12.5	10	6.25	4	2	1.00	0.50	0.20	0.08	0.04	0.02
Valeric acid	4	2.5	2	1.25	0.80	0.40	0.20	0.10	0.04	0.02	0.01	0.005
Isovaleric acid	40	25	20	12.5	8	4	2	1.00	0.40	0.16	0.08	0.04
Hexanoic acid	10	6.25	5	3.125	2	1.00	0.50	0.25	0.10	0.04	0.02	0.01
Isobutyric acid	20	12.5	10	6.25	4	2	1.00	0.50	0.20	0.08	0.04	0.02
Lactic acid	40000	25000	20000	12500	8000	4000	2000	1000	400	160	80	40
2-methyl-butyric acid	20	12.5	10	6.25	4	2	1.00	0.50	0.20	0.08	0.04	0.02
2-hydroxy butyric acid	2000	1250	1000	625	400	200	100	50	20	8	4	2

Table S3.1. 16s rRNA data was correlated to SCFAs data obtained from sputum samples from asthmatics, using Multivariate Association with Linear Models (MaAsLin).

Association data presented with coefficient value and a cutoff with a FDR adjusted p-value <0.05.

Metabolite	Phylum	Class	Organism	Family	Genus	Coefficient	Q.value
Acetate	Firmicutes	Bacilli	Bacillales	Family_XI_Incertae_Sedis	Gemella.24	1.61E-05	0.026691
Acetate	Firmicutes	Bacilli	Bacillales	Family_XI_Incertae_Sedis	Gemella.25	5.36E-06	0.042498
Acetate	Firmicutes	Bacilli	Bacillales	Family_XI_Incertae_Sedis	Gemella.26	3.74E-06	0.033001
Acetate	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus.176	7.76E-06	0.009678
Acetate	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus.177	1.82E-05	0.041741
Acetate	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	1.41E-05	0.045535
Acetate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.15	4.82E-06	0.044608
Acetate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.172	1.02E-05	0.00397
Acetate	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium.26	3.72E-06	0.042498
Acetate	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium.27	2.64E-05	0.004891
Propionate	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces.8	4.52E-05	0.032586
Propionate	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus.116	4.38E-05	0.041045
Propionate	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus.121	0.000162	0.00397
Propionate	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus.66	3.87E-05	0.028112
Propionate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Megasphaera.1	3.48E-05	0.04191
Propionate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.10	6.71E-05	0.036172
Propionate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.11	2.80E-05	0.028112
Propionate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.234	0.000101	0.015798
Propionate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.66	0.000193	0.008184
Propionate	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Neisseria.148	-0.00217	0.044608
Isobutyrate	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces.7	-0.00087	0.042498
Isobutyrate	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus.328	-0.00109	0.029558
Isobutyrate	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnoaerobaculumhuman_oral_bacterium_C73	-0.00161	0.042498
Isobutyrate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.268	-0.00355	0.042498
Isobutyrate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.53	-0.00132	0.042498
Isobutyrate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.61	-0.00081	0.042498
Isobutyrate	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Neisseria.88	-0.00092	0.023091
2-methylbutyrate	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces.53	-0.01531	0.046485
2-methylbutyrate	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromodaceae	Porphyromos.5	0.021195	0.025199
2-methylbutyrate	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella.212	0.001627	0.042178
2-methylbutyrate	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella.74	0.003246	0.041741
2-methylbutyrate	Firmicutes	Bacilli	Bacillales	Family_XI_Incertae_Sedis	Gemella.66	0.002216	0.026691
2-methylbutyrate	Firmicutes	Clostridia	Clostridiales	Family_XIII_Incertae_Sedis	Incertae_Sedis.5	0.001928	0.015798
2-methylbutyrate	Firmicutes	Clostridia	Clostridiales	Family_XIII_Incertae_Sedis	Mogibacterium.2	0.003971	0.045535
2-methylbutyrate	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Moryella	0.003583	0.005346
2-methylbutyrate	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Filifactor.4	0.002154	0.00397
2-methylbutyrate	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus.6	0.017649	0.015798
2-methylbutyrate	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus.7	0.002235	0.023091
2-methylbutyrate	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus.8	0.003331	0.046089
2-methylbutyrate	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae.4		0.008926	0.008184
2-methylbutyrate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.134	-0.00183	0.023091
2-methylbutyrate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.160	-0.15048	0.015798
2-methylbutyrate	Firmicutes	Negativicutes	Selenomodales	Veillonellaceae	Veillonella.172	-0.00214	0.042178
2-methylbutyrate	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium.35	0.068278	0.017136
2-methylbutyrate	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Neisseria	0.001737	0.017136
2-methylbutyrate	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Neisseria.104	0.001078	0.015798
2-methylbutyrate	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Neisseria.88	0.00255	0.004891

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Table S3.2. The following species were isolated from the sputum samples of asthmatics and cultures in vitro.

Each species was culture in selective basal medium as well as medium supplemented with 1% w/v of mucin. Sampling was conducted after 48 hours and 7 days and growth was checked by turbidity and microscopy

Phylum	**Genus**	**Species**	**Medium 1**	**Medium 2**
Firmicutes	Streptococcus	*S. parasanguinis*	BHI	BHI with 1% w/v Mucin
		S. salivarius	BHI	BHI with 1% w/v Mucin
		S. constellatus	BHI	BHI with 1% w/v Mucin
Proteobacteria	Neisseria	*N. subflava*	BHI	BHI with 1% w/v Mucin
		N. flavescens	BHI	BHI with 1% w/v Mucin
Firmicutes	Gemella	*G. haemolysans*	BHI	BHI with 1% w/v Mucin
Firmicutes	Veillonella	*V. parvula*	BHI	BHI with 1% w/v Mucin
		V. atypica	Thioglycolate	Thioglycolate with 1% w/v Mucin
Bacteroidetes	Prevotella	*P. veroralis*	Thioglycolate	Thioglycolate with 1% w/v Mucin
		P. salivae	Thioglycolate	Thioglycolate with 1% w/v Mucin
		P. histicola	Thioglycolate	Thioglycolate with 1% w/v Mucin
		P. nigrescens	Thioglycolate	Thioglycolate with 1% w/v Mucin
		P. pallens	Thioglycolate	Thioglycolate with 1% w/v Mucin
Fusobacteria	Fusobacterium	*F. periodonticum*	Thioglycolate	Thioglycolate with 1% w/v Mucin

Table S4.1. Metabolic profile using ^{1}H NMR spectroscopy of cell suspension in transwell.

22 selected metabolites are shown to be either produced by the cells, taken up by the cells or unchanged.

Metabolites	Produced by the cells	Used by the cells
Alanine	X	
Arginine		X
BCAA	X	X
Choline	X	
Citrate		X
Formate	X	
Glucose		X
Glutamate		X
Glycine	X	
Histidine	X	
Isoleucine	X	X
Lactate	X	
Leucine		X
Lysine	X	
Methionine	-	-
Oxaloacetate		X
Phenylalanine		X
Threonine		X
Tyrosine	X	X
Valine	X	X

Table S4.2. Metabolic *profile using ^{1}H NMR spectroscopy of cell suspension in airway chip.*

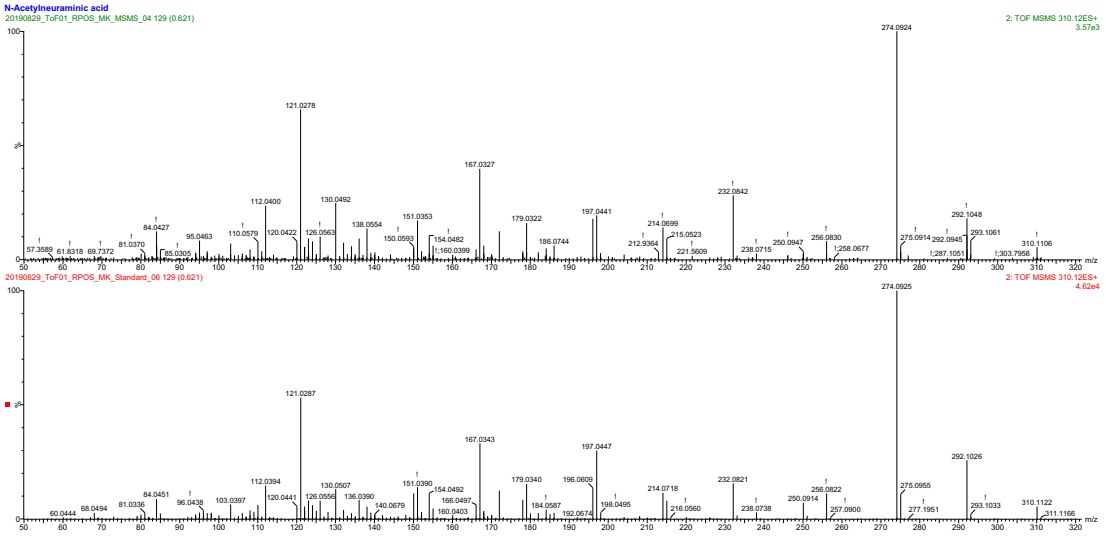
22 selected metabolites are shown to be either produced by the cells, taken up by the cells or unchanged

Metabolites	Produced by the cells	Used by the cells
Alanine	X	
Arginine		X
BCAA		X
Choline	X	
Citrate	X	X
Formate	X	
Glucose		X
Glutamate		X
Glycine	X	
Histidine	X	X
Isoleucine	X	X
Lactate	X	
Leucine		X
Lysine	X	
Methionine	-	-
Oxaloacetate	X	X
Phenylalanine		X
Threonine	X	X
Tyrosine	X	X
Valine		X

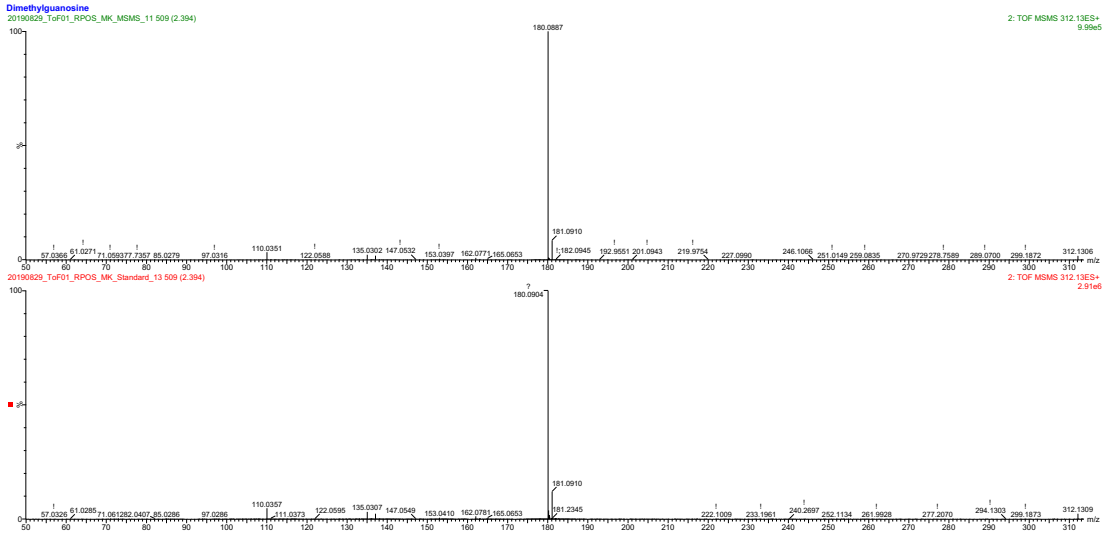
Table S5.1. R^2X and Q^2Y values for the models generated from the UPLC-MS data.

	R2Y	Q2Y
RPOS		
Healthy and Severe	0.509	0.362
Mild and Severe	0.434	0.245
Depression	0.406	0.137
RNEG		
Healthy and Severe	0.532	0.393
Mild and Severe	0.456	0.251
Depression	0.535	0.191
HPOS		
Healthy and Severe	0.501	0.311
Mild and Severe	0.427	0.237
Depression	0.398	0.149

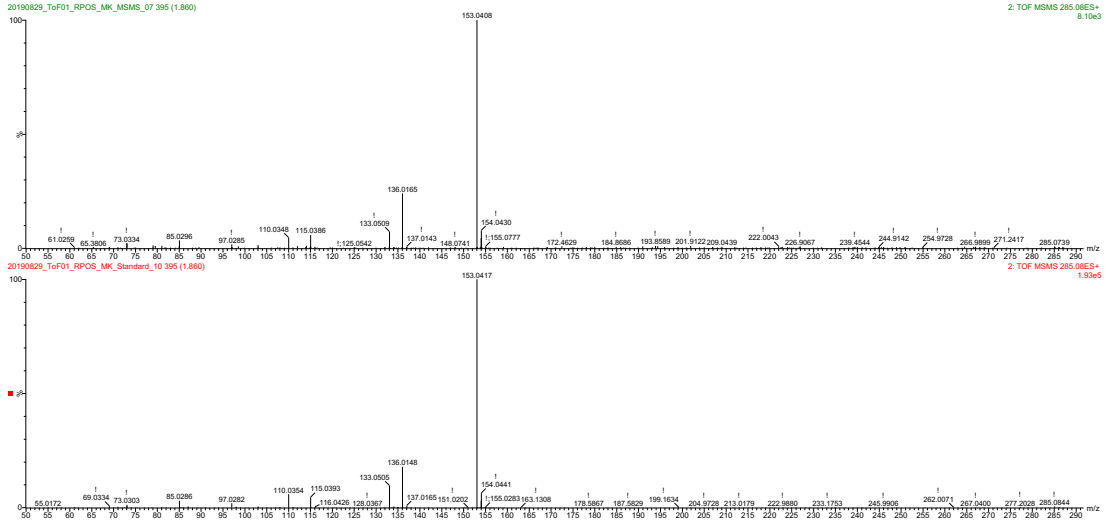
N-Acetylneuraminic acid



Dimethylguanosine



Xanthosine



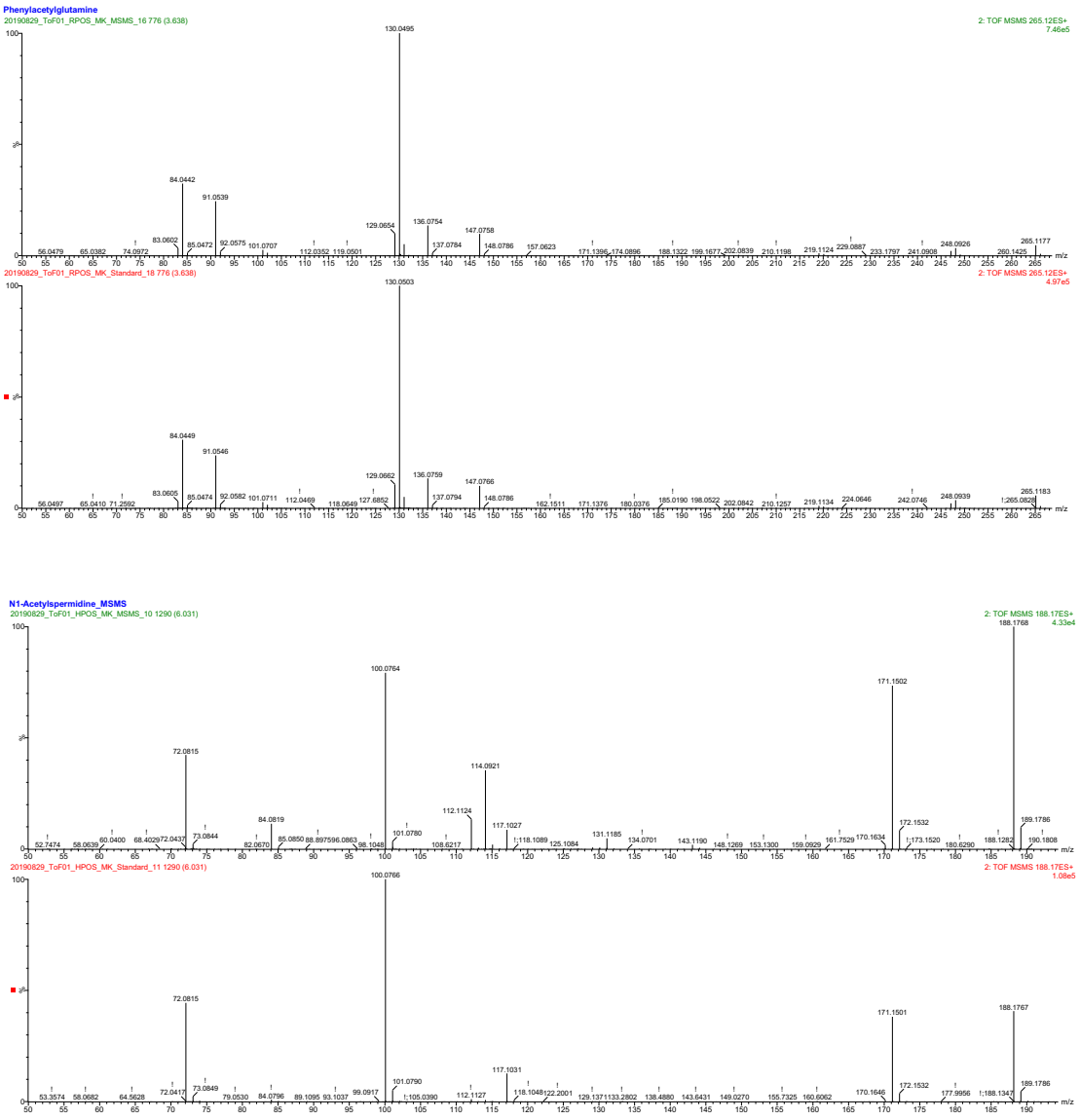


Figure S5.1. MS/MS on standard together with sample, in parallel, to confirm complete identification of the metabolite of interest.

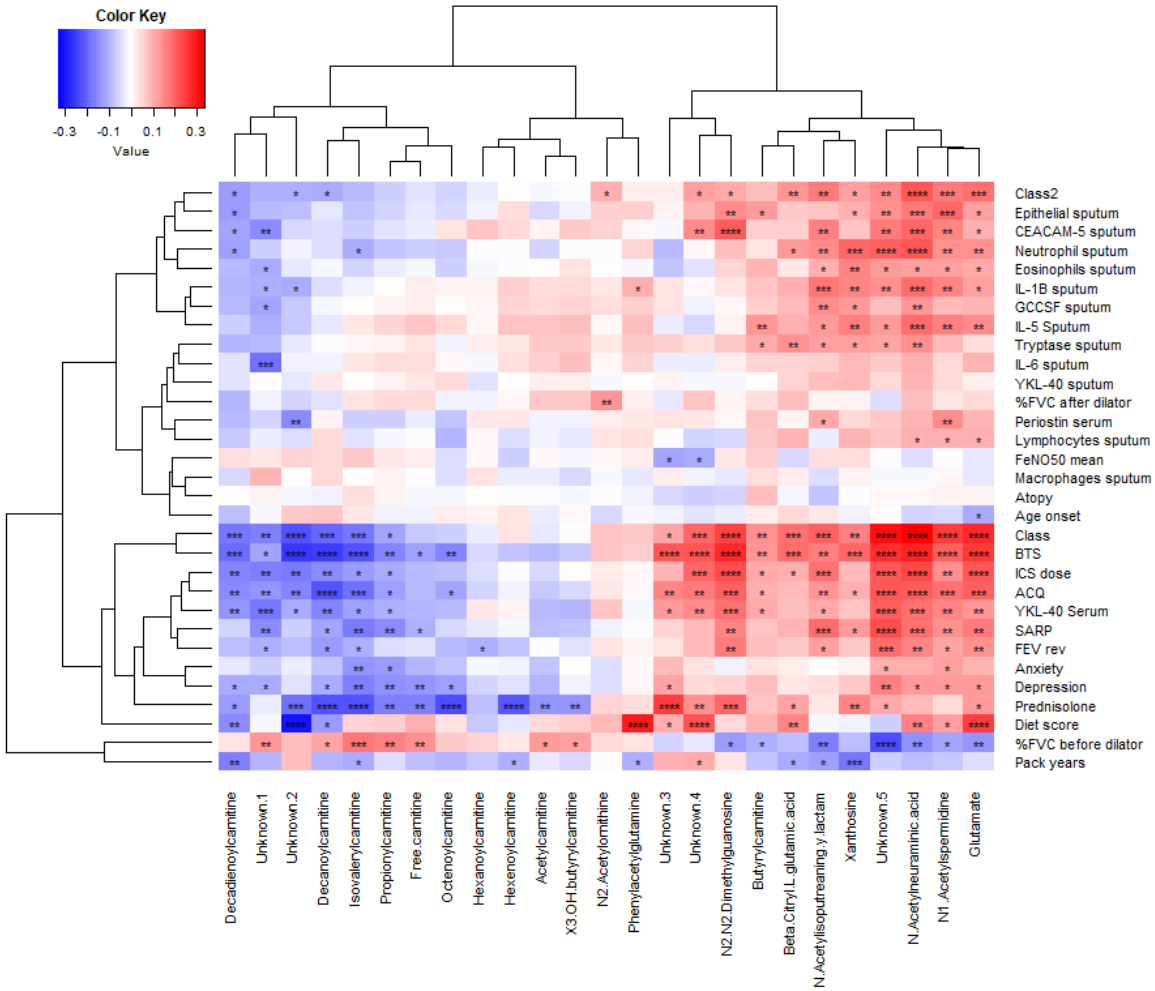


Figure S5.2. Correlation of metabolites of interest with clinical metadata, corrected for sex, age and BMI.

*Spearman correlation of corrected data presented as a heatmap. Red colour indicates positive correlation and blue colour indicate negative association. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

Table S5.2. Linear regression model on metabolites of interest.

[#] *P <0.1, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

Metabolites	Estimate	Std. Error	t value	Pr(>|t|)	Signif.
(Intercept)	**0.8386583375816**	**0.2120178036132**	**3.956**	**0.00009243**	****
N.Acetylneuraminic.acid	-0.0000000053916	0.0000000191830	-0.281	0.778831	
Beta.Citryl.L.glutamic.acid	0.0000004737087	0.0000004390189	1.079	0.281323	
Xanthosine	-0.0000000755859	0.0000000616775	-1.226	0.221210	
Dimethylguanosine	0.0000000081845	0.0000000066432	1.232	0.218773	
Unknown.1	**-0.0000000162561**	**0.0000000087577**	**-1.856**	**0.064263**	**#**
Phenylacetylglutamine	**-0.0000000015644**	**0.0000000003334**	**-4.692**	**0.00000388**	****
Unknown.2	-0.0000000352490	0.0000000246955	-1.427	0.154371	
Unknown.3	**0.0000032143939**	**0.0000012854058**	**2.501**	**0.012852**	*****
N.Acetylisoputreaning.y.lactam	**0.0000000068832**	**0.0000000015321**	**4.493**	**0.00000957**	****
Unknown.4	-0.0000000000577	0.0000000009678	-0.060	0.952496	
Decadienoylcarnitine	**-0.0000000022546**	**0.0000000007351**	**-3.067**	**0.002329**	****
N1.Acetylspermidine	0.0000000007637	0.0000000053199	0.144	0.885928	
Unknown.5	**0.0000006143106**	**0.0000002241454**	**2.741**	**0.006446**	****
Acetylcarnitine	0.0000000005829	0.0000000007056	0.826	0.409359	
Free.carnitine	0.0000000004579	0.0000000005435	0.842	0.400113	
Propionylcarnitine	-0.0000000055899	0.0000000057035	-0.980	0.327723	
Butyrylcarnitine	**0.0000016345843**	**0.0000007350233**	**2.224**	**0.026796**	*****
Isovalerylcarnitine	-0.0000000018759	0.0000000018500	-1.014	0.311299	
X3.OH.butyrylcarnitine	0.0000000031235	0.0000000034866	0.896	0.370932	
Hexenoylcarnitine	**0.0000001227460**	**0.0000000361324**	**3.397**	**0.000759**	*******
Hexanoylcarnitine	**0.0000000080694**	**0.0000000045521**	**1.773**	**0.077154**	**#**
Octenoylcarnitine	**-0.0000000052405**	**0.0000000014089**	**-3.720**	**0.000232**	*******
Decanoylcarnitine	**-0.0000000676370**	**0.0000000220558**	**-3.067**	**0.002333**	****
Glutamate	0.0000000358803	0.0000000259985	1.380	0.168439	
N2.Acetylornithine	0.0000000125477	0.0000000228558	0.549	0.583362	