Untargeted metabolomics of childhood asthma exacerbations from retrospectively collected serum samples

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GCert Business Administration (MasterClass)

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Declaration

I declare that this thesis is my own account of my research and contains, as its main content, work that has not been previously submitted for a degree at any tertiary educational institution.

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Kevin Mendez

Abstract

Asthma is a common chronic inflammatory disease of the airways, affecting 360 million people globally. It is characterised by chronic inflammation, recurrent episodes of bronchoconstriction and mucosal hypersecretion. Symptoms include chest tightness, shortness of breath, coughing and wheezing. Oral corticosteroids used for the treatment of asthma have adverse effects, including bone mineralisation and adrenal suppression. Not all children with acute asthma-like symptoms will have persistent asthma in the future. This is particularly common at a pre-school age. This persistence is only known retrospectively. Identifying children early in their disease course can better direct treatment. Additionally, further understanding of the underlying disease mechanisms can direct novel therapies.

Metabolomics is the systematic study of metabolites in a biological system. Metabolites are low molecular weight biochemical that are reactants, intermediates and end products of biological reactions. They are highly sensitive and are a snapshot of a particular biochemistry and/or pathophysiology. However, they are also highly sensitive to analytical variation. Thus, there were three aims in this study: to assess the impact of potentially limiting factors of retrospectively collected serum samples on metabolomic analysis; to determine whether metabolomics can identify potential biomarkers to distinguish wheeze/asthma exacerbation and control groups; and to determine whether metabolomics-derived biomarkers can identify differences between preschool-aged and school-aged phenotypes.

Serum samples were curated from the Mechanisms of Acute Viral Respiratory Infections in Children (MAVRIC) study. This cohort study recruited children upon presentation to the emergency department at Princess Margret Hospital with acute lower respiratory

illnesses including wheeze/asthma. One–hundred and sixty-one samples were from children with acute wheeze/asthma, and 51 were from healthy controls. Samples were previously stored between 0.8 to 7.9 years at $-80\,^{\circ}\text{C}$. Samples were extracted, derivatised and subsequently analysed using GC-QTOF-MS. SpectralWorks' AnalyzerPro® was used for the deconvolution and untargeted processing of the metabolite data. The quality control-robust spline correction (QC-RSC) algorithm was used for inter- and intra- batch correction. Putative identification of metabolites was made using the NIST (v2.0) library.

Fifty-two metabolites were included in the data analysis, with 24 putatively identified metabolites. The effects of storage time on metabolites were determined via Spearman's correlation. There was a significant difference ($p\text{-value} < 0.05$) in metabolite abundances for succinate, serine and tryptophan; however, the correlation was weak. A two-way Analysis of Variance was performed to compare acute wheeze/asthma vs. healthy, pre-school vs. school age and the associated interactions. Twenty-nine metabolites were found to be significantly different ($p\text{-value} < 0.05$) between the acute wheeze/asthma and the control group. The sub-classes of metabolites include amino acids, fatty acids and sugars. Principal Component Analysis showed a difference in spread between the acute wheeze/asthma and control group. However, there was no clear difference between preschool and school-aged brackets for each group. Arabinofuranose and creatinine were significantly different ($p\text{-value} < 0.05$) between pre-school and school-aged subjects with acute wheeze/asthma. Creatinine is potentially being indicative of higher ASM stress and damage in the school-aged subjects with acute wheeze/asthma.

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List of Conference Abstracts

The work presented in this thesis has been accepted for presentation as follows:

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Full abstract provided in Appendix 1.

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List of Abbreviations

ANOVA	Analysis of variance
ASM	Airway smooth muscle
GC	Gas chromatography
GINA	Global Initiative for Asthma
HPLC	High performance liquid chromatography
IFN	Interferon
IL	Interleukin
LC	Liquid chromatography
n/a	Not applicable
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
NP	No published effect
MAVRIC	Mechanisms of Acute Viral Illness in Children
MS	Mass spectrometry
MSTFA	*N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide
MUC	Mucin gene
MW	Molecular weight
PC	Principal Component
PCA	Principal Component Analysis
PMH	Princess Margret Hospital
QC	Quality control
QC-RSC	Quality control- robust spline correction
Q-TOF	Quadrupole-time of flight
RSD	Relative standard deviation
Th	$CD4^+$ T helper
TIC	Total ion chromatogram
TKI	Telethon Kids Institute
TMS	Trimethylsilyl
TNF	Tumour necrosis factor

List of Units

°C	Degrees Celsius
%	Percentage
min	Minutes
μg	Microgram
μL	Microlitre
mg	Milligram
mL	Millilitre
g	Centrifugal acceleration
RPM	Revolutions per minute
eV	Electron volts
m/z	Mass-to-charge ratio
p-value	Statistical significance level
q-value	Adjusted statistical significance level

Chapter One: Literature Review

1.1. Asthma

Asthma is a common chronic inflammatory disease of the airways, with a global prevalence of 358 million in 2015 (Vos et al. 2016). Chronic inflammation leads to airway restriction through bronchoconstriction, mucosal hypersecretion and airway remodelling (Martinez and Vercelli 2013). Airway restriction during asthmatic symptoms is shown in Figure 1.1. It is characterised by recurrent episodes of chest tightness, shortness of breath, coughing and wheezing (Bousquet et al. 2000). These episodes are referred to as acute asthma, asthma attacks or asthma exacerbations. Morbidity of asthma is high due to the chronic health conditions and treatment side effects (Aagaard and Hansen 2014). There is also a high financial burden on individuals, families and public health systems with an estimated cost of $1.2 billion to Australians in 2015 (Deloitte 2015). Despite the high prevalence and burden, there is no current cure for asthma while treatment options remain limited. The major barrier to developing new management strategies is the heterogeneity of the underlying disease mechanisms.
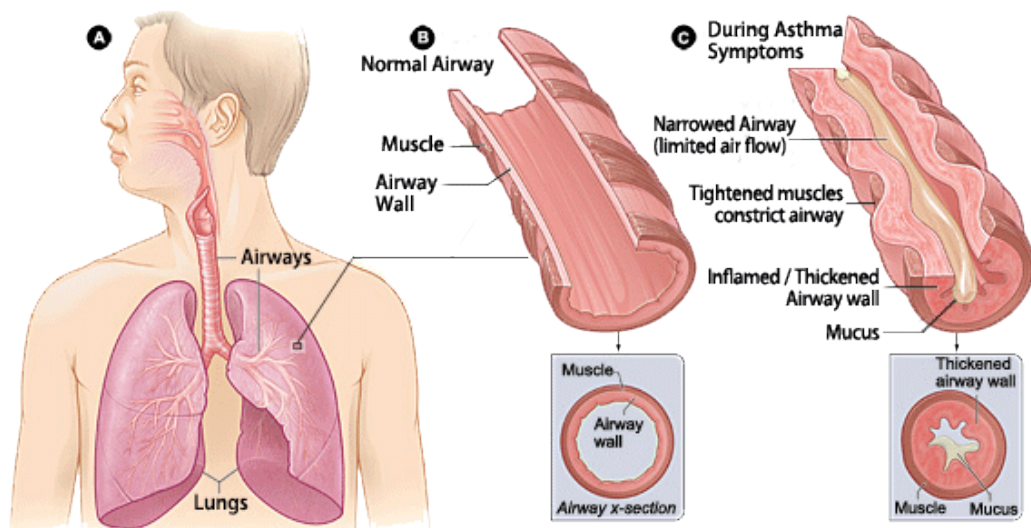
Figure 1.1: A; anatomical position of the airways and lungs. B; section of a normal airway. C; section of an airway during asthmatic symptoms. Reproduced from the National Heart, Lung and Blood Institute (2014).

1.1.1. Definitions

Due to the heterogeneity of asthma, there is a lack of a concise definition. The most widely accepted definition is by the Global Initiative for Asthma (GINA):

> "Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitations." (Global Initiative for Asthma 2017, 14)

This definition is based on common symptoms and identifies the heterogeneous nature of asthma. It does not address underlying pathogenic mechanisms or associated endotypes of asthma (Fahy 2015). The lack of clear definition is further complicated by diagnosis, which is based on a physical examination and lung function test (Franzese 2015). Misdiagnosis is a major issue due to symptoms being shared with other lower respiratory conditions (Heffler et al. 2015). Furthermore, treatment is based on resolving acute symptoms by using non-specific asthma medication, which is ineffective for 5% of patients (Barnes and Woolcock 1998). An improved definition encompassing underlying pathogenic mechanisms and classification of asthma endotypes is needed to develop novel treatment options.

1.1.2. Pathophysiology

Asthma symptoms arise from variable airway obstruction in the bronchi and bronchioles during an inflammatory response to stimuli (Burgel et al. 2009). Bronchoconstriction, mucosal hypersecretion and airway remodelling mediate varying levels of airway

obstruction (Bousquet et al. 2000). The combination of these processes account for the episodic and long-term symptoms associated with asthma.

1.1.2.1.   Inflammation

Inflammation is the hallmark feature of asthma with the involvement of several immune cell types. $CD4^+$ T helper (Th) cells are central to adaptive immunity and play an integral role in asthma. Naïve $CD4^+$ T helper cell are located in peripheral lymphoid tissue. As illustrated in Figure 1.2, the activation of naïve $CD4^+$ T helper cell via antigen-presenting cells cause differentiation, proliferation and release of specific cytokines as interferons (IFN), interleukins (IL) and tumour necrosis factors (TNF) (Alberts 2002). These cytokines directly mediate bronchoconstriction and mucosal hypersecretion. Additionally, these released cytokines recruit other immune cell types including mast cells, eosinophils, neutrophils, dendritic cells and macrophages. These immune cell types can additionally mediate airway obstruction associated with asthma via various released inflammatory mediators.

Asthma exacerbations were previously thought to be caused by Th2 differentiation, resulting in eosinophil recruitment via IL-5 (Cosmi et al. 2011). However, not all asthma present as high Th2 phenotypes (Fahy 2015, Siegle et al. 2011). Th17 mediated responses are also prevalent in severe forms of asthma. Th17 cells recruit neutrophils by secreting the chemoattractant IL-17 (Cosmi et al. 2011). Additionally, combination phenotypes have also been found with high levels of Th2 and Th17 cells (Aujla and Alcorn 2011, Cosmi et al. 2011). Thus, immune cell phenotypes and associated mediators are heterogeneous within asthma.
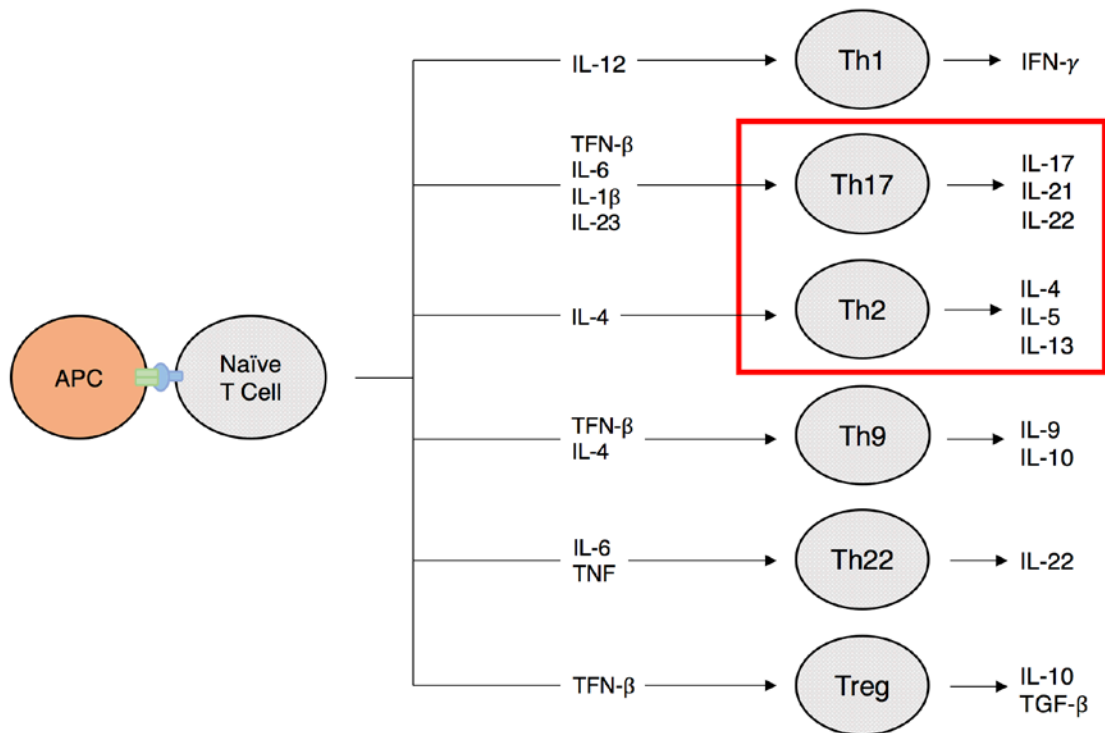
Figure 1.2: Activation and differentiation of naïve Th cell based on cytokines present at the time of antigen presentation by APCs. Activated Th cells secrete various cytokines as illustrated. The red box illustrates Th cell differentiation with established significance to asthma phenotypes. Adapted from Newcomb and Peebles (2013).

1.1.2.2. Bronchoconstriction

Bronchoconstriction is the primary process associated with the airway obstruction of asthma exacerbations (Bousquet et al. 2000). It is caused by the short-term but rapid and severe response of airway smooth muscle (ASM) to stimuli (Black et al. 2012). Stimuli activating ASM receptors initiate down-stream signalling leading to increased intracellular calcium ion concentration, in turn causing bronchoconstriction via excitation-contraction coupling (Pelaia et al. 2008). There are multiple mediators of bronchoconstriction which act on various receptors (Table 1.1). Treatments have been developed to reduce bronchoconstriction by blocking specific receptor activation, though with differing levels of response (Marcello and Carlo 2016). This suggests that there is also heterogeneity in the pathways leading to bronchoconstriction in asthma, and therefore the variable response to treatment.

Table 1.1: Main mediators of bronchoconstriction in asthma and their corresponding binding receptor on ASM. Adapted from Pelaia et al. (2008).

Mediators	Receptor
Acetylcholine	M muscarinic
Adenosine	A_1
Bradykinin	BK_1
Cysteinyl leukotrienes	$CystLT_1R$
Endothelin-1	ET_A/ET_B
Histamine	H_1
Prostaglandin $F_{2\alpha}$	FP
Prostaglandin D_2	DP_2
Serotonin	TP
Thrombin	H_1
Thromoboxanes	TP

1.1.2.3.   Mucosal Hypersecretion

Mucosal hypersecretion also contributes to airway obstruction. It is well documented that airway goblet cell hyperplasia and increased sputum production occur in asthma (Fahy 2002, Rogers 2004, Evans et al. 2009, Izuhara et al. 2009). Airway goblet cells are found in the surface epithelium of conducting airways and secrete mucin glycoproteins; the major molecular constituent of epithelial mucus (Rogers 2003). Mucin glycoproteins are regulated by mucin genes (*MUC*) and form sputum in healthy individuals to trap foreign particles that enter the lungs during normal breathing (Davies et al. 2002). In asthmatics, there is airway goblet cell hyperplasia with overall increased sputum secretion. Sputum is also thicker due to upregulation of *MUC* gene expression (Evans et al. 2009). As shown in Table 1.2, there are numerous mediators. Levels of mucus secretion are highly variable amongst asthmatics. The reasons for this high variability are not fully understood (Niimi 2011).

Table 1.2: Known mediators of airway mucus secretion, goblet cell hyperplasia and *MUC* synthesis/gene expression in asthma. Adapted from (Rogers 2004) with additional information from Kondo et al. (2008), (Yamauchi et al. 2008), Tanabe et al. (2011) and Lin et al. (2014).

Mediators	Secretion	Hyperplasia	*MUC*
Cytokines			
IL-1B	+	NP	NP
IL-6	+	NP	+
IL-9	NP	+	+
IL-13	+	+	+
TNF-α	+	+[a]	+[a]
IL-33	+	+	+
Gases			
Irritant	+	+	+
Nitric oxide	-/+	NP	NP
Inflammatory Mediators			
Bradykinin	+	NP	NP
Cysteinyl Leukotrienes	+	NP	NP
Endothelin	0/+	NP	NP
Histamine	+	+	+
Platelet-Activating Factor	+	+[a]	+[a]
Prostaglandins	+	NP	NP
Proteinases	0/+	+	NP
Purine nucleotides	+	NP	NP

Note: [a]Effects only observed with TNF-α and PAF in combination. + represents positive effect. – represents negative effect. 0 represents no effect. NP represents no published effect.

1.1.2.4. Airway Remodelling

Airway remodelling is the tissue repair response to airway injuries caused by inflammation, particularly due to acute asthma exacerbations. There are numerous structural changes including increased ASM mass, surface epithelial metaplasia, angiogenesis and sub-epithelial fibrosis (Tang et al. 2006, Doherty and Broide 2007, Aceves and Broide 2008, Halwani et al. 2013). These structural changes are associated with poor clinical outcomes. The regulation of the tissue repair mechanisms are highly variable and not well established (Bergeron, Tulic and Hamid 2010). This process can

lead to permanent structural changes in the airways and progressive loss of lung function, measurable by the reduced ability to exhale air in a forced breath (forced expiratory volume) (Pascual and Peters 2009). While airway remodelling occurs throughout an individual's life, some studies have shown it can begin in early childhood (Grol et al. 1999, Bai et al. 2000, Saglani et al. 2007, Lezmi et al. 2015). Airway remodelling is also a normal part of growth so the effects of airway remodelling in children remain controversial. Airway remodelling has no distinct mechanisms, rather it encompasses various types of permanent structural changes. The high complexity and variability have resulted in the lack of treatment focused on repairing airway remodelling.

1.1.3. Aetiology

Aetiology is rarely described in asthma definitions. This combination of complex and partially understood genetic and environmental interactions makes it challenging to isolate specific causes (Ober and Vercelli 2011). These factors and their interactions influence both the progression of asthma and severity of symptoms.

1.1.3.1. Genetic Predisposition

Familial hierarchical clustering and twin studies have shown the strong heritable genetic components of asthma, with heritable estimates between 35% and 95% (Burke et al. 2003, Ober 2011, Thomsen 2014). Known functional changes due to genetic variations include increased sputum production and increased receptor expression of high-affinity immunoglobulin E receptor β on mast cells (Cookson 2004). Genome-wide associated studies have found more than 100 loci harbouring variations associated with asthma (Bossé and Hudson 2006, Vercelli 2008). The function of all the genes associated with asthma are still unknown, with gene-gene and gene-environment interactions not considered in genome-wide associated studies (Ober 2016). Further research is necessary

to understand the complex nature of these interactions and the genetic influences associated with asthma.

1.1.3.2.   Environment Exposure

Asthma exacerbations are often caused by an inflammatory response to an environmental trigger such as bacterial and viral infections, allergen exposure, air pollutants and ambient air temperature changes (Global Initiative for Asthma 2017). Environmental triggers of asthma can act in isolation or in combination. Previous theories were based on specific inflammatory mediators associated with trigger types (Singh and Busse 2006). Siegle et al. (2010) found the interaction between viral infections and allergen exposures were crucial in inducing asthma in murine models. The mechanisms of the interactions between viral infections and allergen exposure are not fully understood (Kloepfer and Gern 2010, Siegle et al. 2010). These poorly understood trigger mechanisms and interactions contribute to substantial heterogeneity in asthma aetiology. This heterogeneity and lack of understanding present a challenge in the development of treatment options.

1.1.4. Treatment

Currently, there is no cure for asthma. Disease management is based on prevention and resolution of symptoms. The lack of knowledge on the disease mechanisms of asthma has resulted in non-specific treatments that are associated with highly variable responses. As highlighted in earlier sections, the multi-factorial and heterogeneous nature of asthma poses many challenges for developing more effective management options. The major drug classes currently used in the management of acute asthma are:

Beta-2 adrenergic agonists: bronchodilators that activate beta-2 adrenoceptors. This inhibits the release of intracellular calcium ions via signalling through cyclic adenosine

monophosphate, resulting in smooth muscle relaxation (Johnson 2001). Short-acting beta-2 adrenergic agonists are used for the initial treatment of asthma exacerbations. Adverse effects include mild tachycardia and tremor (Sears 2002). Resistance is a major issue, leading to the need for additional medication (Barisione et al. 2010).

Anticholinergic bronchodilators: bronchodilators that act as antagonists to M muscarinic receptors on ASM (Soler and Ramsdell 2014). This prevents acetylcholine binding to M receptors and associated bronchoconstriction. Inhaled anticholinergics bronchodilators are not used alone, rather typically in conjunction with β_2 adrenergic agonists. This addition is more effective in patients with severe acute asthma exacerbations, suggesting acetylcholine may play a bigger role in more severe forms of asthma (Haydel 2017).

Systemic corticosteroids: synthetic analogues of steroid hormones. Glucocorticoids bind to glucocorticoid receptors, causing the up-regulation of anti-inflammatory proteins and down-regulation of pro-inflammatory proteins and have a plethora of metabolic effects (Oakley and Cidlowski 2013). They are extremely effective as an anti-inflammatory agent usually initiated within the first hour of an asthma exacerbation at emergency departments (EDs). They are most effective as glucocorticoids are key regulators of whole-body homeostasis (McKay and John A. Cidlowski 2003). They are used sparingly in children due to the wide range of associated adverse effects that include: changes in bone metabolism, adrenal suppression, increased skin conditions and multiple deleterious psychiatric effects (Poetker and Reh 2010). Cooper et al. (2015) found 88% of asthma patients taking oral corticosteroids experienced side effects, which led to a high prevalence of compromised adherence to prescribed oral corticosteroids.

1.1.5. Childhood Asthma

Asthma most commonly begins in childhood (Croner and Kjellman 1992, Yunginger et al. 1992). In Australia, the prevalence of asthma in children is 11% (Australian Bureau of Statistics 2015). Accumulated evidence, particularly emphasised by Martinez (2009, 2011) has strongly correlated persistent asthma with disease progression from childhood. Additionally, a longitudinal study from ages 8 to 50 found that 85% of children with severe asthma continued to experience symptoms into adulthood, while only 36% of children with mild asthma followed the same trend (Tai et al. 2014). Persistent childhood asthma that continues into adulthood share environmental triggers and thus potentially underlying mechanisms (de Nijs, Venekamp and Bel 2013). Permanent airway remodelling may begin in early childhood, which makes it is important to treat asthma early to prevent long-term damage, particularly in children with severe forms of asthma.

Conversely, it is equally important to withhold mediations with adverse long-term effects, if the disease is known to not persist. This is a major issue in paediatric emergency departments with the treatment of severe asthmatic symptoms in children using oral corticosteroids (Beigelman, Durrani and Guilbert 2016). Not all children with symptoms of acute asthma will have persistent asthma in the future. These children are characterised with having wheeze, with associate acute wheeze, wheeze attacks or wheeze exacerbations (Brand et al. 2008). The distinguishing characteristic between asthma and wheeze is the lack of chronicity, which is only known retrospectively. Wheeze is common in young children, with the Tuscon Cohort Study of 1246 newborns finding that 48.5% had a wheeze exacerbation before the age of 6 (Martinez et al. 1995). The minimum recommended age of diagnosis of asthma stated by GINA is age 6 (Global Initiative for Asthma 2017, 14). This minimum age may protect pre-school children from being incorrectly diagnosed with asthma. However, this also delays early treatment of pre-

school children with asthma or wheeze that will persist into asthma. To date, there is still a lack of understanding of this disease progression.

1.2.   Metabolomics

Metabolomics is the systematic study of relative metabolite abundance in a biological system (Dunn et al. 2010), as a result of genetic and environmental factors at a given time (Fiehn 2002). Metabolites are reactants, intermediates, and end products of biochemical reactions, typically with a molecular weight (MW) below 1500 Daltons (Dunn et al. 2011). While metabolites are a sensitive snapshot of a particular biochemistry and/or pathophysiology, they are also highly sensitive to analytical variations including, but not limited to, storage, temperature and time (Yin, Lehmann and Xu 2015). With these variations taken into consideration, understanding the roles of significant metabolites can potentially aid in elucidating disease mechanisms and identifying novel therapeutic targets (Emwas et al. 2013). Metabolomics studies can be categorised into two main approaches: targeted and untargeted.

1.2.1.   Metabolomics Approaches

The targeted approach is a bottom-up strategy characterised by the quantification of a defined group of known metabolites (Roberts et al. 2012). Quantification can be made with high specificity and sensitivity (Lämmerhofer and Weckwerth 2013). However, *a priori* knowledge is required to determine the metabolites to target.

The untargeted approach is a top-down strategy characterised by the simultaneous measurement of a wide range of metabolites (Alonso, Marsal and Julià 2015). It is used in metabolomics when there is a lack of *a priori* knowledge, and thus it is used for hypothesis generation. An untargeted approach is used to look at the widest possible range

of metabolites, however due to the range of physicochemical properties, one analytical platform cannot be used to detect all metabolites (Scalbert et al. 2009).

1.2.2. Analytical Tools

There is no single analytical platform that can be used to analyse all metabolites in a given biological sample. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the two most widely used analytical methods of detection in metabolomics (Dunn et al. 2010). NMR can detect metabolites with robustness and reproducibility, but it suffers from relatively low inherent sensitivity with the limit of detection at the nanogram level compared to the picogram level with MS (Pan and Raftery 2007, Villas-Bôas 2007). The greater sensitivity of MS makes it an ideal analytical technique for biomarker discovery.

The principle of MS is to detect ions by their mass-to-charge ratio (*m/z*) and to quantify the ions according to their relative abundance. Mass spectrometers are typically used in combination with chromatography: gas chromatography (GC) or liquid chromatography (LC). This is to enhance resolving power by separating analytes based on their chemical properties prior to ionisation in MS (Wixom and Gehrke 2010). In reference to the differences in column chemistry and design, GC is generally better suited to separate volatile metabolites with low MW, while LC is typically better suited to separate non-volatile metabolites (Suhre 2012). The benefits of GC coupled to MS for the separation of analytes is the reproducibility of elution time. This is used alongside fragmentation patterns for metabolite identification (Schauer et al. 2005).

1.2.2.1. Gas Chromatography

GC provides analyte separation based on elution. The instrument consists of an injector, column and a detector (Figure 1.3). Precise quantities of the sample enter via a syringe into the injector. The solvent and sample are vaporised in the injector into a gaseous phase. Analytes enter the coiled capillary composed of fused silica coated internally with a thin film stationary phase, inside a temperature-controlled oven. Analytes in the gas phase are passed through the column via an inert gas, typically helium (Kitson, Larsen and McEwen 1996). The separation of analytes is based primarily on their volatility and further on their interaction with the stationary phase.
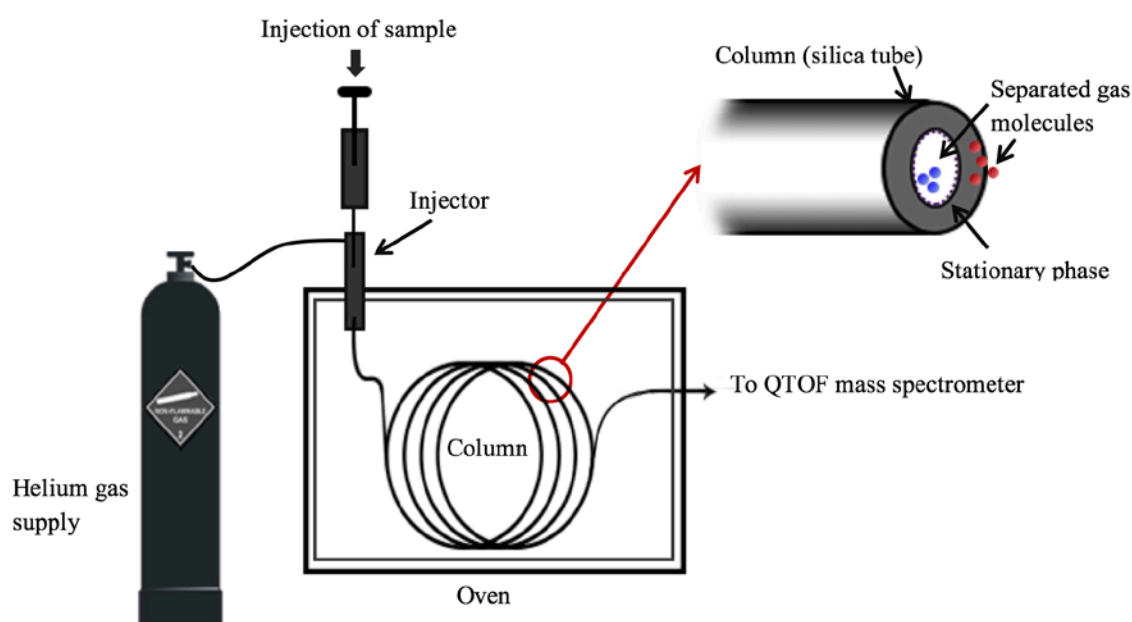
Figure 1.3: Schematic of a gas chromatograph. Adapted from Villas-Bôas (2007).

1.2.2.2. Mass Spectrometry

Mass spectrometers manipulate and detect gas phase ions (Faull et al. 2008). The instrument consists of an ion source, mass analyser and ion detector, maintained under vacuum. Gas phase samples enter the ion source and are immediately ionised. The most common technique for GC-MS is electron ionisation, where analytes are bombarded with

an electron beam commonly at a voltage of 70eV (Harrison 1992). In the mass analyser, ions are separated by *m/z*. The ion detector measures the number of ions by the current as a function of time.

A quadrupole (Q) mass filter and time of flight (TOF) mass analyser in tandem are useful for untargeted metabolomics. The schematic of a MS with a Q-TOF mass analyser is shown in Figure 1.4. The combination of both allows a run in either TOF mode or MS/MS mode. The TOF mode allows full scan spectra with accurate mass and high resolution, with the MS/MS mode in tandem useful for resolving unknown compounds (Chernushevich, Loboda and Thomson 2001).

The quadrupole mass filter only allows ions within a specific *m/z* range to pass through. It consists of four parallel rods, where the specific direct current (DC) and radio frequency (RF) voltage are applied (Villas-Bôas 2007). The DC and RF voltage chosen determines the *m/z* range. Only ions within the specific *m/z* range have the required stable oscillation to pass through (Ekman 2009). This can be used for MS/MS to only allow an ion with a specific *m/z* to pass through, or for TOF mode by increasing voltage to allow for a full MS scan.

The TOF mass analyser separates ions based on the time taken to reach the detector. All ions are accelerated to an identical kinetic energy and thus have a specific velocity through the TOF tube based on mass and charge of the ion. Lighter ions of the same charge will have a higher velocity and ions with a higher charge will have a higher velocity. Due to the high resolution, TOF systems can routinely measure a wide range of ions which is important for an untargeted metabolomics approach.
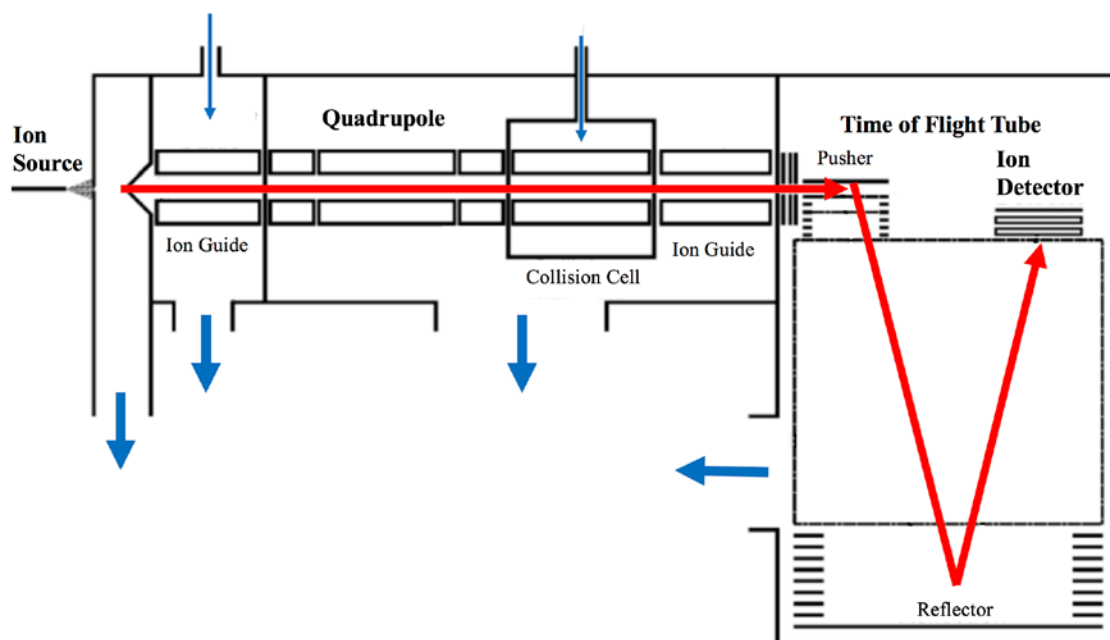
Figure 1.4: Schematic of a mass spectrometer with a quadrupole-time of flight mass analyser. Red arrow represents direction of ions. Blue arrow represents the input and output of gas flow to maintain optimal operating pressure. Adapted from Lee and Reilly (2011).

1.2.3. Pre-Analytical Issues

Metabolites are a sensitive snapshot of a particular biochemistry and/or pathophysiology (Dunn et al. 2010). However, metabolites are also highly sensitive to analytical variation. Pre-analytical issues that can alter metabolite abundance include haemolysis, additional freeze-thaw cycles and storage temperature.

1.2.3.1. Haemolysis

Haemolysis is the rupture of erythrocytes, with intracellular components released into the serum including metabolites and enzymes. As reviewed by Hernandes, Barbas and Dudzik (2017), primary findings on the effects of haemolysis found significant changes in lipids, in particular lysophosphatidylcholines. Haemolysis is visually identifiable as the colour of serum is bright red as opposed to pale yellow. This is due to increased haemoglobin levels. There are numerous factors that can cause haemolysis in blood

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samples, including the type of syringe, site of puncture, size of bore needle, the use of vacuum tubes and proper technique (Heyer et al. 2012). Training and experience are critical in reducing the occurrences of haemolysis, however it is not necessarily avoidable.

1.2.3.2. Additional Freeze-Thaw Cycles

Freezing is a requirement of samples to increase the longevity. Due to the importance of some biological samples, they may be refrozen again and used in supplementary studies. An untargeted LC-MS study found 4 out of 705 metabolites to be significantly associated with freeze-thaw after four freeze-thaw cycles (Yin et al. 2013). Additionally, a targeted LC-MS study found that serum metabolites were generally unaffected by one or two freeze-thaw cycles (Breier et al. 2014). There are also additional factors that have not been considered, including the time spent thawed and the method used. These additional factors warrant further investigation of samples that have been previously used and refrozen.

1.2.3.3. Storage Temperature

Freezing is a critical step in sample handling, with storage conditions having a significant effect on metabolites. Storage at $-80\ °C$ is the current approach taken for long-term storage of bio-fluids (Vaught 2006). Yang et al. (2013) reported differences between plasma stored at $-80\ °C$ for two months and approximately five years. However, Yang et al. (2013) only identified five types of metabolites: lysophosphatidylcholines, lysophosphoethanolamines, acylcarnitines, serotonin and hypoxanthine. No studies have assessed the varying stability of metabolites at $-80\ °C$ (Dunn et al. 2011). As such, issues regarding the stability of metabolites with long-term storage of frozen samples requires additional investigation.

1.2.4. Sample Preparation

Sample preparation is the steps in which the samples are treated prior to instrumental analysis. The serum matrix is complex containing low MW metabolites and higher MW species including proteins and ribonucleic acids. These higher MW species need to be removed, and the metabolites need to be volatile and stable for GC-MS analysis. Additionally, steps including the addition of internal standards and quality control (QC) samples are for quality assurance and to correct for analytical variation.

1.2.4.1. Addition of Internal Standards

Internal standards are chemical compounds with similar chemical properties as the analytes of interest. They are added to samples, sample blanks and calibration standards. This is used for quantification and for the correction of variation during sample preparation (Tan, Boudreau and Lévesque 2012). An internal standard is typically added to samples during the deproteination step in the crash solution (Dunn et al. 2011).

1.2.4.2. Quality Control Samples

In untargeted metabolomics QC samples are used to measure analytical precision, and for inter- and intra- batch correction via QC-based signal correction algorithms. The use of pooled QC samples proposed by Sangster et al. (2006) is routinely used in untargeted metabolomic studies. Pooled QC samples are made from small aliquots of all the biological samples. In small-scale studies, both the relative standard deviation (RSD) and clustering in unsupervised multivariate plots could be used to determine the reproducibility and quality of data prior to statistical analysis. With large-scale metabolomic studies, pooled QC samples can confirm and be used to adjust for analytical drift within and between batches.

1.2.4.3.   Deproteination

Serum and plasma both contain low MW metabolites and high MW species, including proteins and ribonucleic acids. Deproteination is the step to remove these high MW species. Deproteination chemically denatures proteins through the disruption and potential destruction of secondary and tertiary structures causing protein unfolding (Lehninger, Cox and Nelson 2013). Metabolites bound to proteins are released, improving chromatographic separation and MS analysis (Villas-Bôas 2007). A crash solution typically with an organic solvent and internal standard are used, followed by a centrifugation step to separate the precipitated protein and the supernatant containing metabolites (Jiye et al. 2005, Dunn et al. 2011). The precipitated protein is discarded and the supernatant is used for metabolomics analysis.

1.2.4.4.   Lyophilisation

Lyophilisation or freeze-drying is a purification process that removes the solvent, typically water from the sample. Samples are frozen and transferred to the freezer dryer, under vacuum. This method combines deep-freezing and dehydration to cause sublimation of solvent while avoiding heat (Villas-Bôas 2007). The drying step is essential for the removal of water which would hinder the derivatisation of polar compounds (Moldoveanu and David 2015). After lyophilisation, samples could potentially be stored at 4°C for an additional 3 months before derivatisation (Dunn et al. 2011). It is generally considered best practice to store at -80°C.

1.2.4.5.   Derivatisation

Derivatisation improves the volatility and thermal stability of metabolites. Volatile metabolites are generally non-polar with a low MW. For non-volatile metabolites, chemical derivatization can be used to reduce polarity and thus increase volatility. There

are three main types of derivatization reactions: acylation, alkylation and silylation. Acylation reagents react to highly polar functional groups, alkylation reagents target active hydrogens on amines and acidic hydroxyl groups, and silylation reagents react and substitute active hydrogens with silyl group such as trimethylsilyl (TMS) (Grob and Barry 2004). For silylation, N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) is commonly used in combination with methoximation. Methoximation, commonly with *O*-methoxyamine hydrochloride in pyridine solution, converts aldehyde and ketone groups to their methoxime derivatives to stabilise them prior to silylation (Paik and Kim 2004).

1.2.4.6.   Calculation of Retention Index

To compensate for fluctuations in retention time, a retention index is calculated and used instead of a retention time. This is based on the elution of a homologous series of *n*-alkanes added to the sample prior to injection (Dunn et al. 2011). A stable retention index allows analyte comparison to spectral libraries. For example, during GC-MS instrument maintenance, approximately 10 cm of the column is removed from the inlet end. This reduces the retention time of each metabolite while the retention index remains constant. Assuming injection, separation and ionisation parameters are kept constant (Schauer et al. 2005), confident comparisons to libraries can be made based on retention indices.

1.2.5. Data Processing

Data processing is a vital data handling step in the metabolomic workflow where raw mass spectral data is converted to a data matrix with identified metabolites. The data processing workflow includes extraction, deconvolution, alignment, correction and metabolite identification. Extraction involves the processing of the extraction ion chromatogram peaks for every observed mass. Deconvolution involves the construction

of a mass spectrum from the associated peaks at a particular retention time window. Particularly in complex fluids such as serum, multiple metabolites may co-elute. As such the method of deconvolution is the critical step for the correct identification of metabolites. There are multiple software tools for deconvolution with various settings and algorithms, with no clear standardised approach (Katajamaa and Orešič 2007, Lu et al. 2008). Alignment is the correction for elution time shifts, particularly common in larger studies. Additionally, inter- and intra- batch variation due to technical variation requires signal correction in larger studies. The Quality Control- Robust Spline Batch Correction (QC-RSC) utilises pooled QC samples at every x injection to adjust for technical variation (Kirwan et al. 2013). For untargeted GC-MS metabolite identification, libraries such as the National Institute of Standards and Technology mass spectral library (NIST) are a useful resource. The library contains thousands of reference spectra with acquisition information and retention indices. Metabolite identification using commercial or public libraries is putative (Salek et al. 2013).

1.2.6. Metabolomic Studies in Asthma

A diverse range of metabolites has been found in previous metabolomics studies on asthma. A meta-analysis of pathways identified in more than one study is shown in Table 1.4. Overall, the metabolic pathways that have been identified are associated with central energy metabolism alterations (tricarboxylic acid cycle) and immune/inflammatory responses. Hypoxia, amino acid metabolism, lipid metabolism and oxidative stress represent a metabolic intersection between central energy metabolism and inflammation (Kominsky, Campbell and Colgan 2010). Hypoxia signalling is associated with various asthma remodelling and inflammatory mechanisms, including the upregulation of lymphocyte survival factors and increased inflammatory cell infiltration (Ahmad et al. 2012). Amino acid metabolites are known to have various pro- and anti- inflammatory

effects. Lipid mediators can act as inflammatory signalling molecules and can also cause bronchoconstriction via binding to receptors on ASM (Fogarty et al. 2004, Pelaia et al. 2008). Thus, these are potentially important pathways related to the metabolic disease mechanisms of asthma and identifying novel therapeutic targets.

Table 1.3: Meta-analysis containing significant metabolite pathways found in multiple asthma studies. Adapted from Kelly et al. (2017).

Pathways	Bio-fluid	Population	Technique
Amino Acid Metabolism	Plasma[1] Serum[2]	Adults[1,2]	GC-MS[2] MS[1]
Glutamate-Glutamine Cycle; and Glutamate Metabolism	EBC[3] Serum[2]	Adults[2,3] Children[3]	GC-MS[2] NMR[3]
Hypoxia Response Pathways	Plasma[4] Serum[2] Urine[5]	Adults[2,4] Children[5]	GC-MS[2] NMR[4,5]
Immune Pathways	Plasma[1,4,6] Urine[7]	Adults[1,4] Children[6,7]	LC-MS[6,7] MS[1] NMR[4]
Inflammatory Pathways	EBC[8,9] Plasma[1]	Adults[1] Children[8,9]	GC-MS[8] LC-MS[9] MS[1]
Lipid Metabolism	Plasma[4] Serum[10] Urine[11]	Adults[4,10,11]	LC-MS[10,11] NMR[4]
Oxidative Stress	EBC[8,12] Plasma[13] Urine[14]	Adults[14] Children[8,12,13]	GC-MS[8,14] LC-MS[23] NMR[12]
Tricarboxylic Acid Cycle	Serum[2] Urine[5,14]	Adults[2,14] Children[5]	GC-MS[2,14] NMR[5]

Note: [1](Comhair et al. 2015). [2](Chun et al. 2015). [3](Sinha et al. 2012). [4](Jung et al. 2013). [5](Saude et al. 2011). [6](McGeachie et al. 2015). [7](Mattarucchi, Baraldi and Guillou 2012). [8](Caldeira et al. 2012). [9](Montuschi 2009). [10](Ried et al. 2013). [11](Loureiro et al. 2016). [12](Carraro et al. 2007). [13](Fitzpatrick et al. 2014). [14](Loureiro et al. 2014). EBC represents exhaled breath condensate.

The two childhood asthma metabolomics studies using blood samples have been reported from authors Fitzpatrick et al. (2014) and McGeachie et al. (2015). Fitzpatrick et al. (2014) compared mild-to-moderate asthma (n=22) and severe asthma (n=25) and found the glycine, serine and threonine metabolism pathway, the *N*-acylethanolamine and *N*-acyltransferase pathway to be significant. This study lacked a control group and thus only describes differences between severity phenotypes of childhood asthma. McGeachie et al. (2015) used a targeted lipidomics approach on asthma control, comparing usage (n=8) and lack of usage (n=12) of short-acting β_2 agonists within 7 days prior to sampling. No significant pathways were associated with asthma control. Owing to the specific target aims and lack of control groups, both studies lack the ability to understand underlying disease mechanisms.

There is a need for untargeted metabolomics in asthma research to help elucidate underlying disease mechanisms before targeting specific pathways. While metabolomics is a powerful tool for understanding underlying disease mechanisms, irreproducibility arising from biological and analytical sensitivity is a substantial issue. This is further compounded by sampling bias, inadequate sample size, inappropriate analyses and over-fitting (Broadhurst and Kell 2006). Major issues in current metabolomic studies in asthma are the limited sample sizes and lack of findings validation (Amber, Michael and Jessica 2015). There is a need for an untargeted standardised method, particularly for childhood asthma using larger sample sizes to generate suitable libraries of significant metabolites and metabolite pathways.

1.3.   The Mechanisms of Acute Viral Illness in Children Study

The Mechanisms of Acute Viral Illness in Children (MAVRIC) cohort study conducted by the University of Western Australia, in collaboration with Princess Margaret Hospital

(PMH) and the Telethon Kids Institute (TKI) in Perth. As the name suggests, the broad study aim is to investigate viruses causing acute lower respiratory infections, including children with asthma exacerbations and wheeze exacerbations. Children were recruited soon after a presentation to the emergency department at PMH with an acute lower respiratory infection. Serum was collected within 24 hours of presentation and stored at -80 °C. Clinical data collected included age, sex, corticosteroid usage and presence of a viral infection. The control group in this study comprised of children that were healthy children without any respiratory illnesses. Despite its advantages as a cohort, the MAVRIC study was not originally designed with metabolomics in mind. Hence, there are potentially a number of limiting factors that affect the serum samples used in this study, such as storage time of samples (up to 6 years old), number of freeze-thaw cycles and haemolysis of blood prior to serum collection that may impact the outcomes of metabolomics analyses.

1.4.   Research Aims

Serum samples from the MAVRIC cohort study can be used to uncover significant metabolite pathways associated with childhood asthma. As the MAVRIC study was not designed specifically for metabolomics studies, it was important to determine the effects of potential confounding factors on experimental outcomes. The three main research aims were:

1. To assess the impact of potentially limiting factors of the retrospectively collected serum samples on metabolomic analysis.

2. To determine whether metabolomics can identify potential biomarkers to distinguish between acute wheeze/asthma and healthy controls.

3. To determine whether metabolomics-derived biomarkers can identify differences between preschool-aged and school-aged phenotypes.

Chapter Two: Materials and Method

2.1.   Chemicals and Reagents

Acetonitrile, ethanol, heptane and methanol of LC-MS grade, and water of HPLC (High Performance LC) grade were purchased from Thermo Fisher Scientific (Massachusetts, United States of America). Methoxyamine hydrochloride, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), *n*-alkanes (C_{10}, C_{12}, C_{15}, C_{19}, C_{22}, C_{28}, C_{32}, and C_{36}), pyridine and $^{13}C_6$-Sorbitol were purchased from Sigma-Aldrich (New South Wales, Australia).

2.2.   MAVRIC Study Population

Children were recruited upon presentation to the PMH emergency department with an acute lower respiratory infection. Data and bio-fluids were collected within 24 hours according to the MAVRIC protocol and stored for later analysis. Data collected included items from the child's medical record and questionnaires that were administered to at least one parent. These included emergency department physician discharge diagnosis, use of corticosteroids, age and gender. Biofluids collected included serum and nasal fluid. The study was approved by the PMH Human Research Ethics Committee, with parental/guardian written informed consent obtained prior to participation (Human Research Ethics Committee Reference Number: 1761). Emergency department physicians evaluated participants in accordance with published international guidelines (Reddel et al. 2009) and determined each child's diagnosis at discharge. Standard hospital protocols determined the treatment regimen and included inhalation of salbutamol and ipratropium bromide at 20 minute intervals for the first hour, and then oral prednisolone at 1 mg/kg (maximum 40 mg) if respiratory symptoms persisted (Hales et al. 2009, Subrata et al. 2009). Due to the impact of prednisolone on bone health, children under 3

were not given oral corticosteroids. Peripheral blood was collected after initial treatment, with the serum stored at -80 °C according to the laboratory protocol. Viral detection on nasal specimens from each child was primarily based on direct fluorescent antibody testing (Bizzintino et al. 2011). The virus detected were primarily Human Rhinovirus in this study. Additionally, healthy children were recruited as controls including siblings and children at the PMH emergency department with non-chronic and non-respiratory based conditions.

2.3.   **Metabolomics Study**

For this metabolomics study, 215 individual serum samples from the MAVRIC cohort were used (n=164 acute wheeze/asthma diagnosis; n = 51 controls). Samples were selected based on the following two criteria: the diagnosis of acute wheeze/asthma by the emergency department physician and a minimum volume of 186 uL/mL serum was available. Clinical information of the final study population is shown in Table 2.1. Note, three samples (from the acute wheeze/asthma diagnosis group) were excluded from the statistical analysis as noted in Section 3.1.3.

Table 2.1: Clinical information of the final study population (*n*=212).

Clinical Information	Control	Acute Wheeze/Asthma
Subjects, *n*	51	161
Doctor Diagnosed Acute Asthma, ≥6 years old (%)/ <6 years old (%)	0(0%)/0(0%)	32(20.9%)/47(95.7%)
Median age in years (IQR)*	4.4 (2.3-9.3)	3.5 (2.2-6.5)
Gender ratio, Male/Female	25/36	109/52
Virus detected ratio, Yes/No	19/29	132/24
Steroids given ratio, Yes/No	0/51	127/23

* IQR - interquartile range.

2.4.   Sample Randomisation

Randomisation was used to reduce confounding factors in the analytical experimental design. Sample order was randomised during the aliquoting, extraction and derivatisation steps. As such, final instrumental injector order (identical to sample derivatisation order) was primarily based on acute wheeze/asthma or control, and included gender, age, storage time, aliquoting order and extraction order.

2.5.   Sample Preparation

2.5.1. Sample Aliquoting

Minimal volumes were required to be aliquoted from the original serum samples. This is due to the importance of the preservation of serum samples for future MAVRIC cohort studies and publications. In total, 186 μL of serum was used for metabolomic analysis.

At TKI, serum samples were left to thaw in the 4 °C fridge for 15 hours. Samples were vortexed for 5 seconds and then centrifuged for 10 minutes at 9 520 using a Hettich MIKRO 20 centrifuge (Tuttlingen, Germany). Five aliquots of each sample were placed in sterile 1.5 mL microcentrifuge tubes (SSIBio, California, United States of America); one 20 μL aliquot for GC-MS analysis and four 30 μL aliquots for future LC-MS analysis. Additionally, 46 μL of each sample (excluding haemolysed samples) were transferred to a sterile 15 mL centrifuge tube (Sigma-Aldrich, New South Wales, Australia) to make the pooled QC samples. Following the aliquoting of all biological samples, the pooled QC sample was vortexed for 5 seconds, then 20 μL aliquots for GC-MS analysis and 30 μL aliquots for LC-MS analysis were pipetted into sterile 1.5 mL microcentrifuge tubes. Samples were then transferred on dry ice to Murdoch University, South Street Campus and stored at -80 °C.

2.5.2. Metabolite Extraction

The 20 μL serum samples for GC-MS analysis were thawed on ice for 30 minutes. Thirty microliters of $^{13}C_6$-sorbitol (internal standard; 5.0μg/mL) in HPLC-grade water was added and followed by 150 μL of methanol to chemically denature proteins. Samples were agitated at 1400 RPM using an Eppendorf Thermomixer Comfort (New South Wales, Australia) for 10 minutes at 4 °C, then centrifuged at 16 100 g using an Eppendorf 5415R centrifuge (New South Wales, Australia) for 4 minutes at 4 °C. Following this, the supernatant (150 µL) was transferred to a sterile 1.5 mL microcentrifuge tube and 450 μL HPLC-grade water was added. Samples were vortexed for 10 seconds and 75 μL was transferred to a 100 μL glass-insert (Thermo Fisher Scientific, Victoria, Australia). The 100 μL glass-insert was placed inside another sterile 1.5 mL microcentrifuge tube and vacufuged in the Eppendorf Concentrator Plus rotary vacuum concentrator (New South Wales, Australia) for 30 minutes to reduce the methanol content. Samples were frozen on dry ice and freeze-dried using a Labconco Freezone 2.5 Plus freeze-dryer (Missouri, United States of America). After 24 hours, the dried sample extracts were stored at 80 °C until derivatisation.

2.5.3. Metabolite Derivatisation

The dried extracts were treated with 20 µL of methoxyamine hydrochloride in pyridine (20 mg/mL). Extracts were agitated for 90 minutes at 30 °C and 1400 rpm using an Eppendorf Thermomixer Comfort, then centrifuged for 1 minute at 16 100 g using an Eppendorf 5415R centrifuge. MSTFA (40 µL) was then added to the extracts. Samples were agitated further for 30 minutes at 75°C and 300 rpm using the Eppendorf Thermomixer Comfort. The 100 μL glass-inserts containing the samples were then transferred to 2 mL crimp-top vials (Thermo Fisher Scientific, Victoria, Australia). n-alkanes in heptane (5 µL containing 12.5 μg/mL of C_{10}, C_{12}, C_{15}, C_{19}, C_{22} and C_{28};

and 25.0 μg/mL of C_{32} and C_{36}) were added to the samples. All vials were subsequently capped and loaded onto the GC-Q-TOF-MS after 4 hours. The purpose of this 4 hour delay was to ensure completion of the derivatisation reactions prior to the first sample injection.

2.6.   Instrument Analysis

The Agilent 7200 Accurate-Mass Quadrupole Time-of-Flight GC/MS System (Agilent Technologies, California, United States of America) was used for the untargeted metabolomic analysis of the derivatised samples. Each derivatisation batch included an analytical QC at the start, end and after every fifth injection for both inter- and intra- batch correction and to assess analytical precision. Additionally, 3 heptane blanks, 3 derivatisation blanks, 3 extraction blanks and 8 conditioning QC samples were included prior to the run. (Note: heptane blanks contained heptane; derivatisation blanks went through the derivatisation process; and extraction blanks went through the entire process using 20 µL of HPLC-grade water instead of 20 µL serum). The acquisition sequence was run continuously without stops between derivatisation batches.

The method parameters used for GC-MS described below were as per the standard Separation Science and Metabolomics Laboratory protocol for untargeted analysis of serum using GC-QTOF-MS in electron ionisation mode. Six 8 μL aliquots of acetonitrile were used as solvent washes pre- and post- sample injection, with one 2 μL sample wash just prior to injection. 1 μL of derivatised sample was injected into the inlet set at 270°C in splitless mode, with the carrier gas of ultra-high purity helium at a constant flow rate of 0.85 mL/min. The Agilent VF-5-ms fused silica capillary column (0.25 mm ID, 30 m length, 0.25 μm film and 10 m EZ-guard; Agilent Technologies, California, United States of America) was used. The chromatographic method was 23.5 minutes long including a

10 minute solvent delay. Initial oven temperature was 70 °C. A temperature ramp of 15°C/min for 6 minutes and 40 seconds was then applied until a final temperature of 325°C was reached. The transfer line was set at 300°C and the ion source was set to 280°C. Ionisation was achieved with a 70 eV electron beam. The MS has a scan rate of 10 spectral scans per second and scanned ion masses in the range of *m/z* 50 to 700.

2.7.   Data Processing

Raw GC-MS data was converted and imported into Analyzer Pro v5.0 (SpectralWorks, Cheshire, England) for data processing. The parameters for all settings are listed in Appendix 2. Initially, all analytical QC samples were processed. A target component library was made from deconvoluted metabolites found in at least 80% of analytical QC samples. All samples were then processed against this target library and the data matrix was exported as a comma-separated values file. To correct for inter- and intra- batch variation, the QC-RSC algorithm was used (Kirwan et al. 2013). QC samples with uncharacteristic variations in peak area associated with non-systematic technical issues were removed prior to QC-RSC correction (Section 3.1.2).

2.7.1. Exclusion Criteria

Erroneous samples and metabolites were removed prior to statistical analysis. Samples with >20% missing values were initially removed and Principal Component Analysis (PCA) was performed to identify additional erroneous samples (Section 3.1.3). The RSD for the pooled QC samples (RSD_{QC}) and for the biological samples (RSD_{Sample}) was determined for each feature. Metabolites with >20% missing values, RSD_{QC} >30% or a ratio of RSD_{Sample}:RSD_{QC} <1.5 were excluded from further analysis as they were not sufficiently reproducible (Section 3.1.4). Additionally, features that were identified in the extraction and derivatisation blanks were excluded from further analysis (Section 3.1.4).

2.7.2. Metabolite Identification

Features were putatively identified by the comparison of the mass spectra and retention index to the NIST 2011 mass spectral library (v 2.0). Metabolites were identified using the previously published parameters of >700 forward match, >700 reverse match and 20% probability (Abbiss et al. 2015). Features not identified were named Unknown_RetentionTime_RetentionIndex_BasePeak. The list of identified compounds used for statistical analysis is shown in the results Table 3.1.

2.8. <u>Statistical Analysis</u>

The final feature matrix and additional clinical and technical data were combined, in order to complete both univariate and multivariate analysis using RStudio (RStudio Inc., Massachusetts, United States of America). For univariate analysis, missing values were excluded as there was no association (p-value < 0.05) between percent missing across the clinical groups using the chi-squared test (Appendix 3). Spearman's rank correlation coefficient was used to compare feature peak area and storage time. The matrix was log-transformed, and a two-way Analysis of variance (ANOVA) was used to compare acute wheeze/asthma vs. healthy, pre-school vs. school age and the associated interactions. Additionally, adjusted p-values (q-values) to correct for multiple comparisons using the Benjamini-Hochberg method was also reported. For multivariate analysis, the matrix was log-transformed, scaled to unit variance and missing values were imputed using the k-Nearest Neighbour algorithm with k=3. PCA was then used for unsupervised multivariate visualisation.

Chapter Three: Results

3.1. Data Cleaning

3.1.1. Inclusion of Replicate Samples

There were three biological samples with issues visually identified in their associated total ion chromatogram (TIC) during the instrumental analysis. The TICs of AAS304LD and 681CA (Figure 3.1a) both showed a high baseline shift and missing peaks. The TIC of AAS518CC showed an issue with taller peak heights (Figure 3.2a) and overloading in the peak for the internal standard $^{13}C_6$-sorbitol (Figure 3.2b). As the sample extraction process only required 75 μL of the 600 μL extraction solution, replicate samples were made. Replicate samples of AAS304LD, 681CA and AAS518CC were derivatised and injected at the end of the instrumental run in batch 7. The TICs of these replicate samples did not share the issues previously described (Figure 3.1b, Figure 3.1c, Figure 3.2b and Figure 3.2c). Therefore, the replicate samples were included in the final dataset.
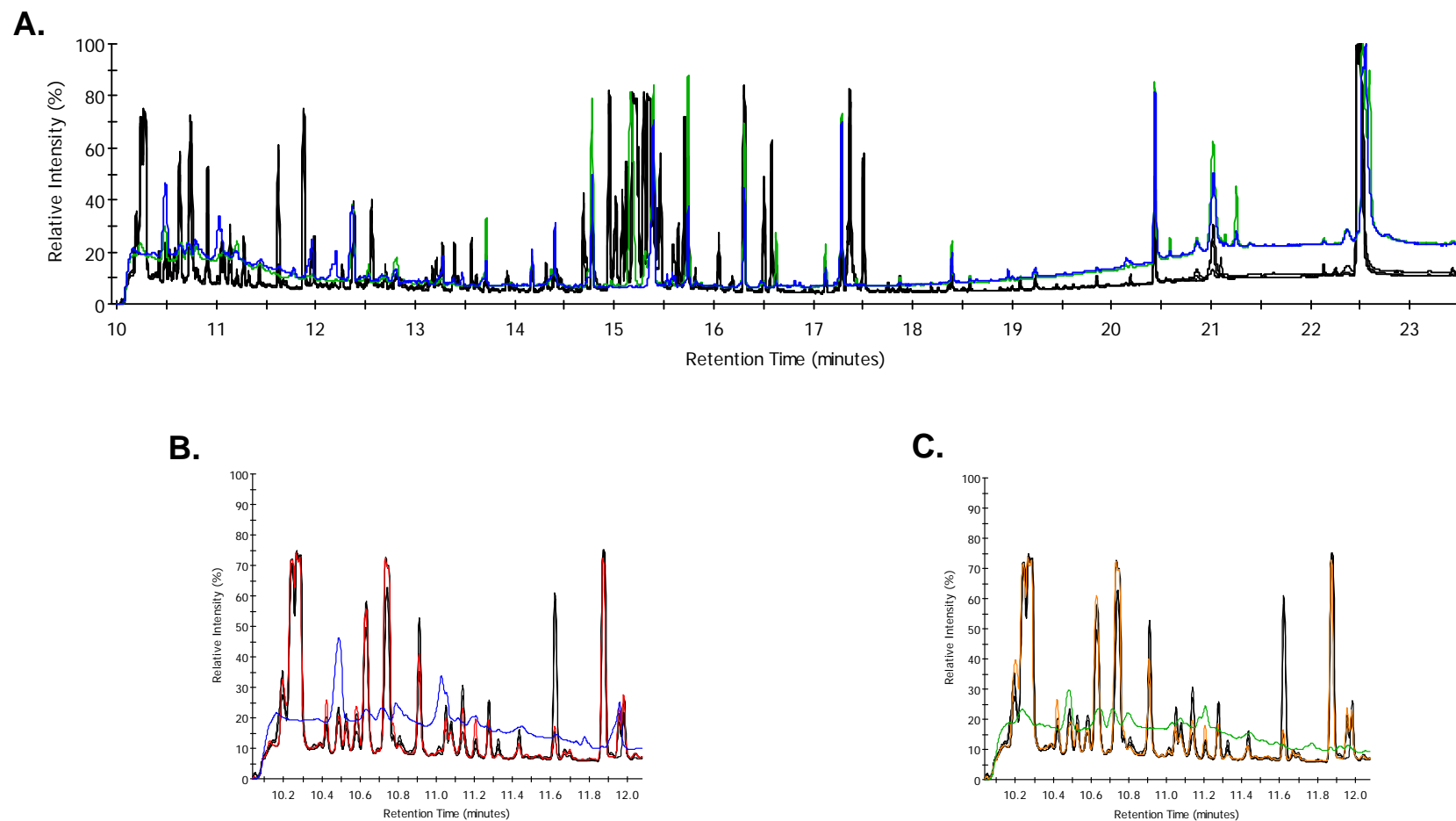
Figure 3.1: Overlay of the TICs for AAS304LD and 681CA. A; TIC of AAS304LD (blue) and 681CA (green) overlaid on QC9 (black), QC36 (black) and QC63 (black). B; TIC of AAS304LD (blue) and replicate AAS304LD (red) overlaid on QC9 (black), QC36 (black) and QC63 (black). C; TIC of 681CA (green) and replicate 681CA (orange) overlaid on QC9 (black), QC36 (black) and QC63 (black).
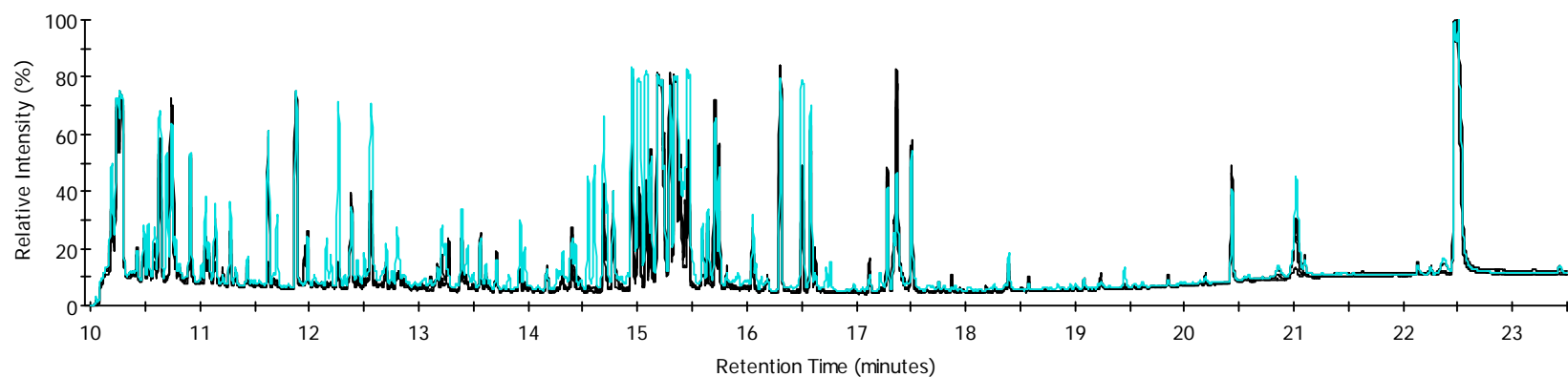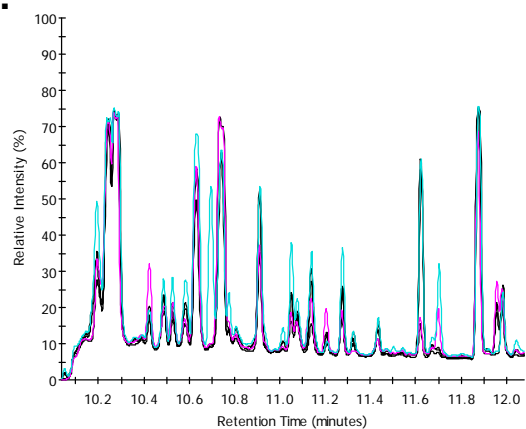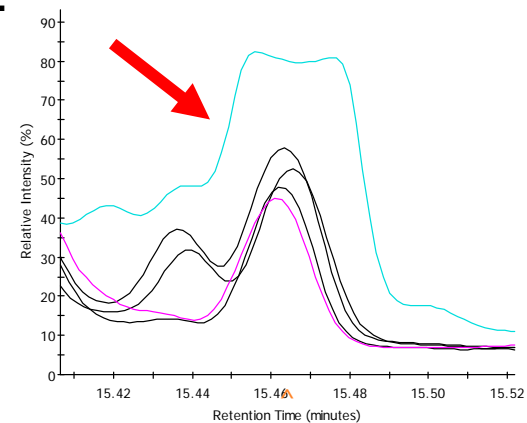
Figure 3.2: Overlay of the TIC for AAS518CC. A; TIC of AAS518CC (cyan) overlaid on QC9 (black), QC36 (black) and QC63 (black). B; TIC of AAS518CC (cyan) and replicate AAS518CC (pink) overlaid on QC9 (black), QC36 (black) and QC63 (black). C; TIC of AAS518CC (cyan) and replicate AAS518CC (pink) overlaid on QC9 (black), QC36 (black) and QC63 (black) for the $^{13}C_6$-sorbitol peak (red arrow).

3.1.2. Exclusion of Quality Control Samples

Analytical QC samples were used for measuring analytical precision, as well as inter- and intra- batch correction via QC-based signal correction algorithms. Due to the latter, analytical QC samples were critically evaluated with possible exclusion determined prior to the finalised batch correction. The TICs of analytical QC samples was overlaid and three analytical QC samples (QC10, QC11 and QC51) appeared to be of poor quality (Figure 3.3a). At close inspection, the TIC of QC10 showed retention time shifts, uncharacteristically tall peak heights as well as additional missing peaks when compared to other analytical QC samples (Figure 3.3b). The TIC of QC11 displayed a systemic issue with high baseline shift and a majority of missing peaks (Figure 3.3c). This is similar to samples AAS304LD and 681CA (Figure 3.1a). The TIC of QC51 showed a reduction in the baseline with uncharacteristically small peak heights when compared to other analytical QC samples (Figure 3.3d). While these three analytical QC samples were identified as potentially erroneous, they were initially included in the first batch correction using the QC-RSC algorithm.
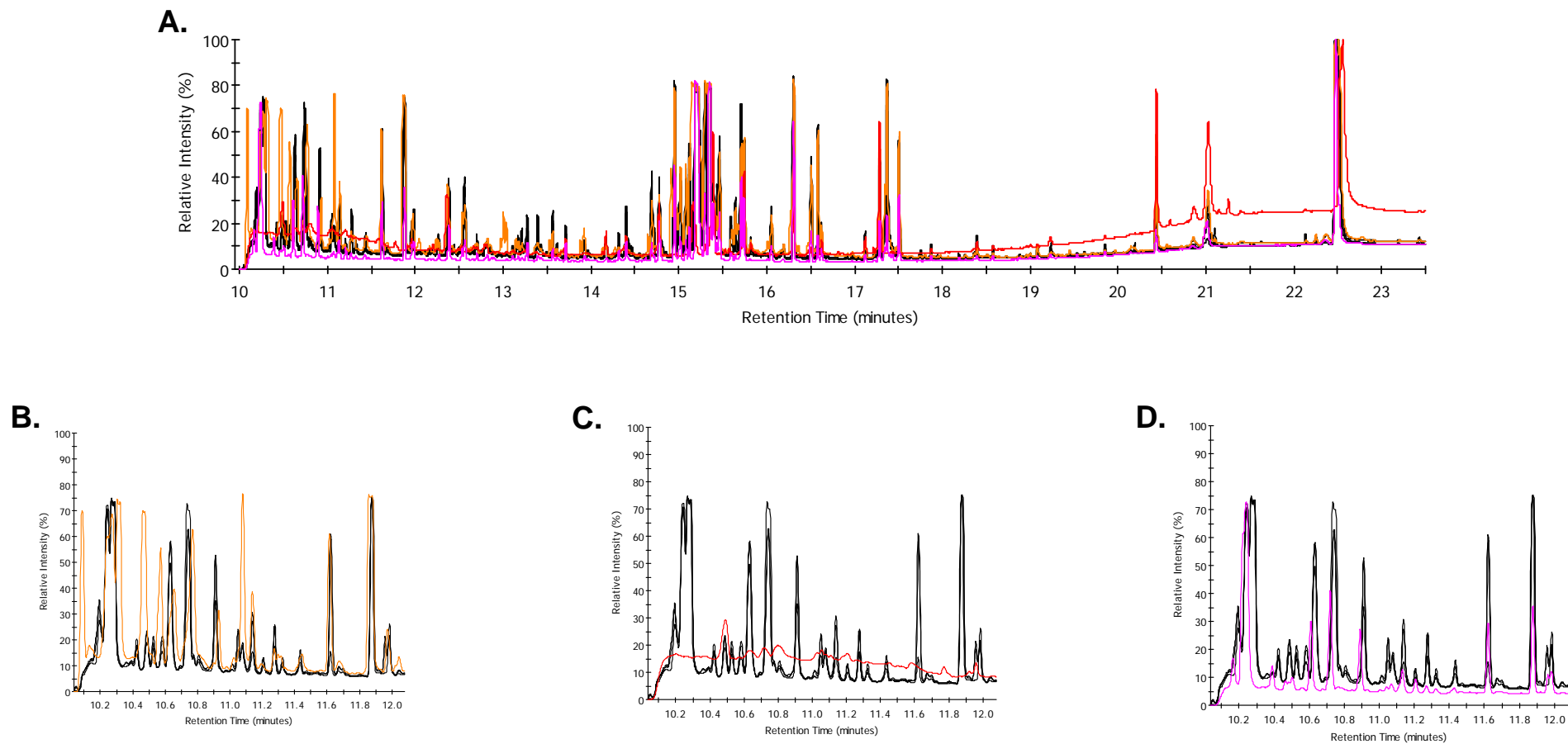
Figure 3.3: Overlay of the TICs for QC10, QC11 and QC51. A; TIC of QC10 (orange), QC11 (red) and QC51 (pink) overlaid on QC9 (black), QC36 (black) and QC63 (black). B; TIC of QC10 (orange) overlaid on QC9 (black), QC36 (black) and QC63 (black). C; TIC of QC11 (red) overlaid on QC9 (black), QC36 (black) and QC63 (black). D; TIC of QC51 (pink) overlaid on QC9 (black), QC36 (black) and QC63 (black).

To assess the impact of QC10, QC11 and QC51 on batch correction, before and after correction plots of peak area (log-scaled) vs. injection order were produced for each metabolite. Figure 3.4 is an example of the effect problem QC samples (QC51) have on batch correction. Figure 3.4a (prior to batch correction) showed the general deviation of peak areas (log-scaled) within and between batches, and the uncharacteristically low peak area for QC51. This low peak area was not shown in samples injected prior to, or after QC51. When QC51 was included, the batch correction over-adjusted for this low peak area which caused a clear upward drift at the end of batch 5 (Figure 3.4b). When QC51 was excluded, the clear upward deviation introduced by this over-correction to QC51 was removed (Figure 3.4c). Given the chromatographic trends and impacts on QC-based signal correction, these identified erroneous QC samples were removed prior to the batch correction that was used for the data analysis.
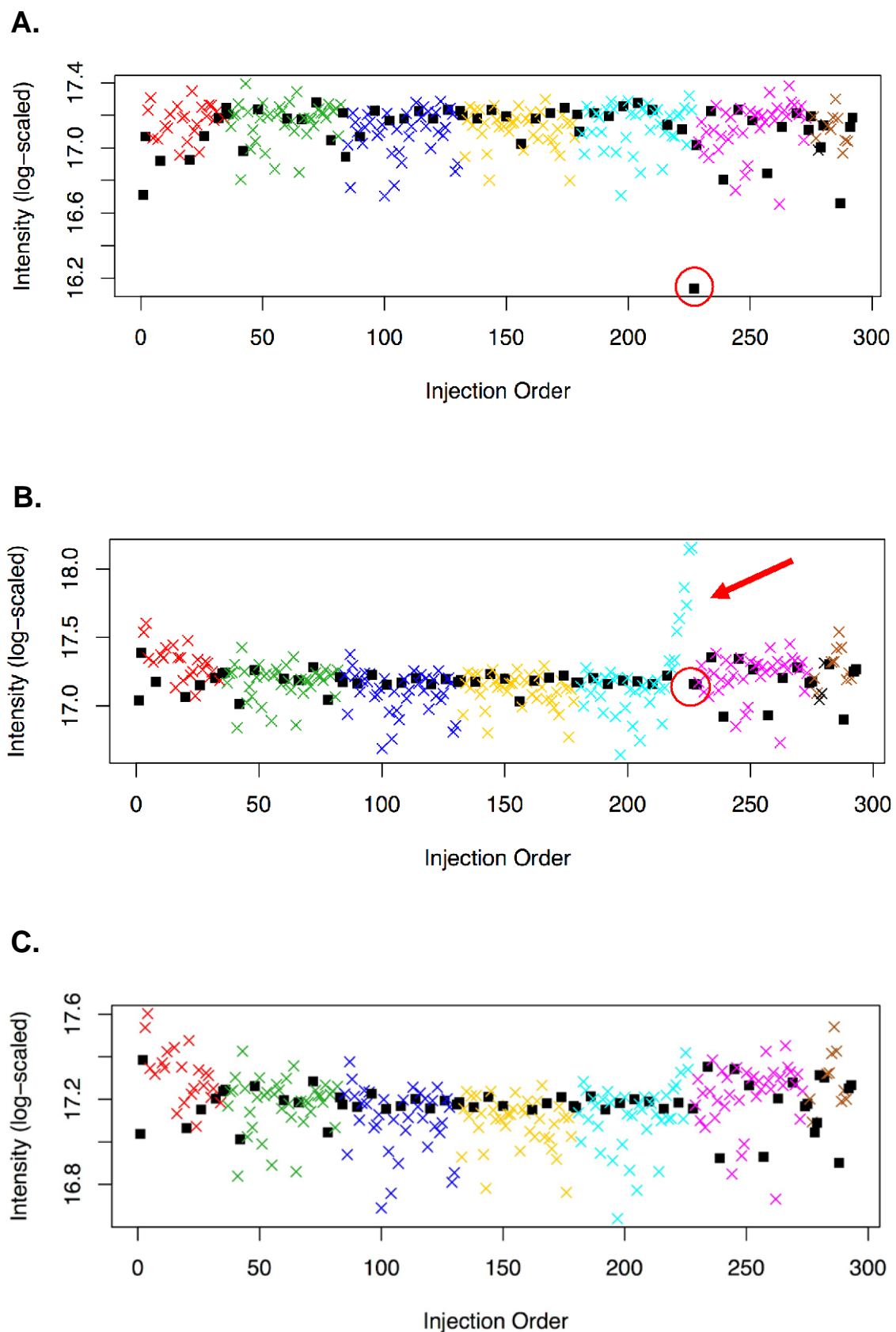
Figure 3.4: Injection order vs. peak area (log-scaled) for Unknown_15.5_1913_147. A; prior to QC correction. B; using QC correction, with the inclusion of QC10, QC11 and QC51. C; using QC correction, with the exclusion of QC10, QC11 and QC51. Red circle indicates QC51. Red arrow indicates the upward trend associated with the batch correction that included QC51.

3.1.3. Exclusion of Samples

As indicated in Section 2.7.1, samples with >20% missing values were excluded. QC10, QC11 and QC51 would have been removed if they weren't previously (as described in Section 3.1.2), with 25.7%, 81.4% and 28.4% missing values respectively. Five biological samples labelled AAS304LD, 476ET, 626PM, 681CA and 704ZA were removed with 80.9%, 20.8%, 24.0%, 76.5% and 43.2% missing values respectively (Clinical information found in Appendix 4). Note, this included AAS304LD and 681CA with issues described in Section 3.1.1, but not their associated replicate samples that had 12.0% and 11.5% missing values respectively. As the samples were retrospectively analysed from an existing cohort and the cohort was not specifically designed for metabolomics, there were potential problems associated with samples. This included 3 haemolysed samples and 2 samples that had been through an additional freeze-thaw cycle compared to all other samples. Visual inspection of the PCA score plot was used to determine whether the 2 replicate samples, 3 haemolysed samples and 2 samples with an additional freeze-thaw should be included or excluded. On inspection of the PCA score plot with all these samples, there was no clear variation observed in Principal Component 1 (PC1) or Principal Component 2 (PC2) from the rest of the samples, nor was there clustering (Figure 3.5). Therefore, there was no clear evidence for the removal of those samples.

Additionally, based on the PCA score plot (Figure 3.6), there were two other potentially erroneous samples with a PC1 score less than -15 and greater than 15. The sample with the PC1 score less than -15 was identified as AAS518CC, previously described in Section 3.1.1 as having issues regarding peak heights, thus confirming the exclusion. The sample with a PC1 score >15 was identified as AAS327DD. Based on the TIC for this sample, there was no clear issue that could explain this score beyond expected biological variation and therefore the sample was not removed (Figure 3.7).
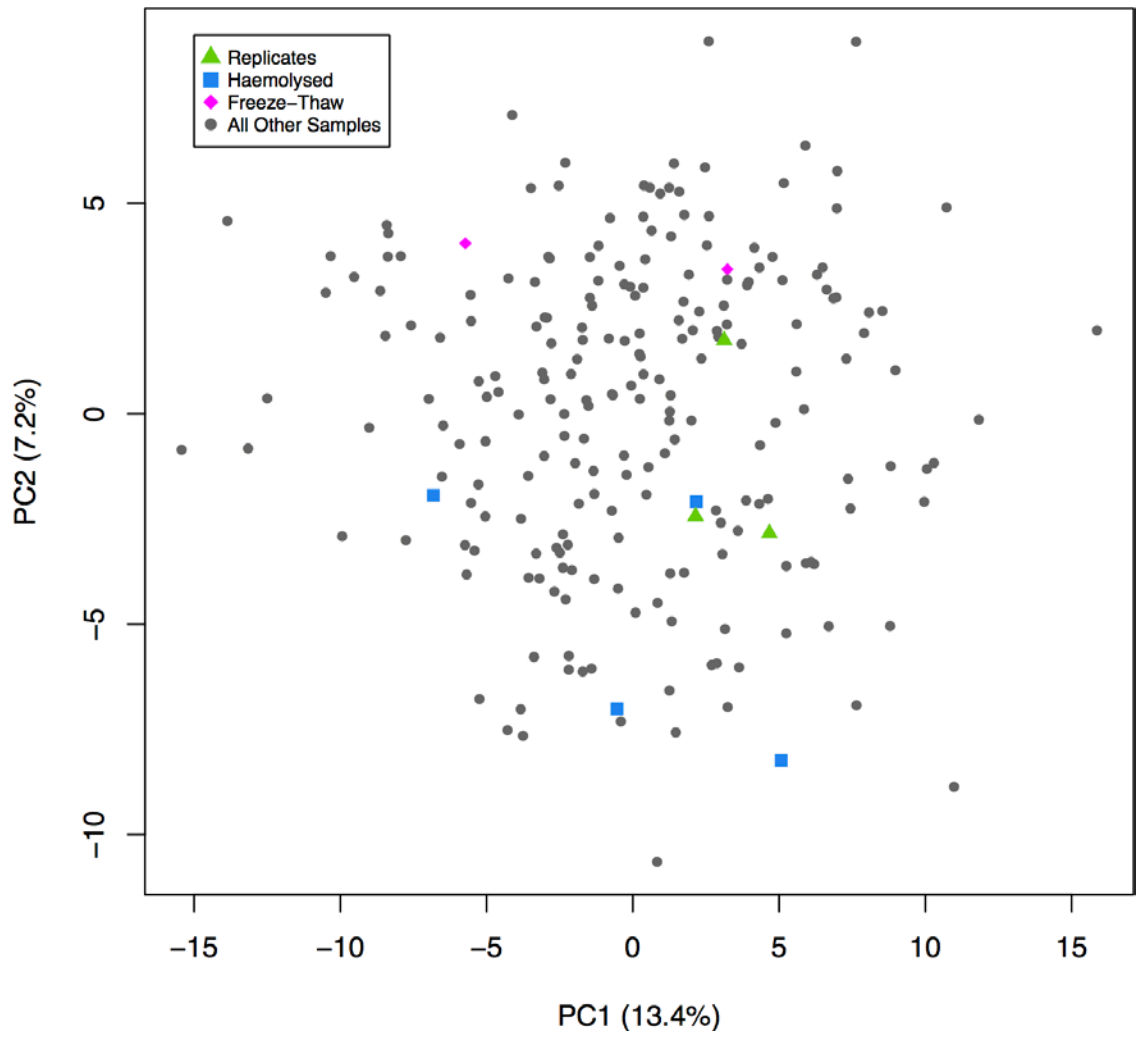
Figure 3.5: Principal component score plot of PC1 vs. PC2 for samples after the exclusion of AAS304LD, 476ET, 626PM, 681CA and 704ZA (>20% missing values). Replicate samples are green. Additional freeze-thaw samples are pink. Haemolysed samples are blue. All other samples are grey.
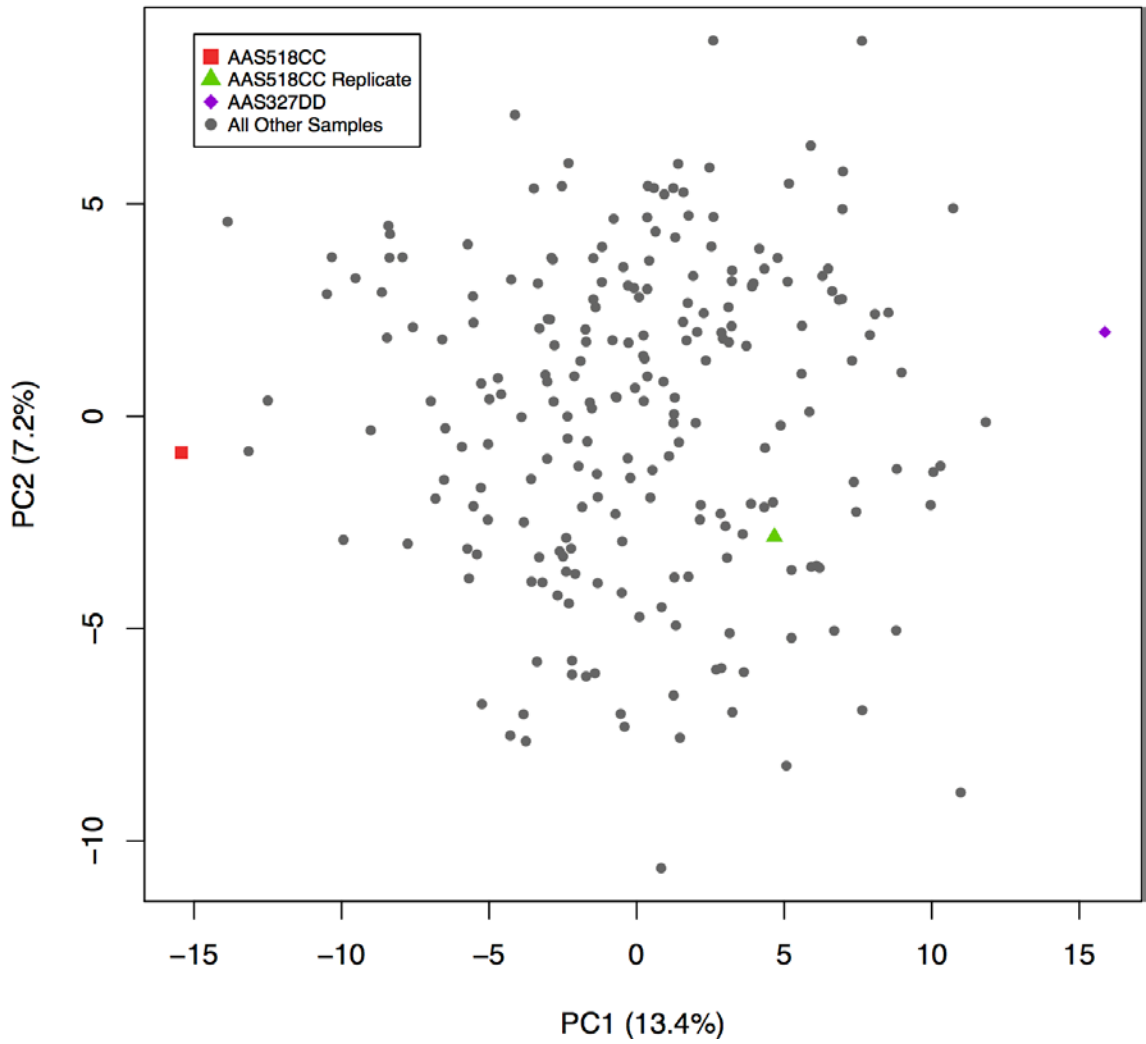
Figure 3.6: Principal component score plot of PC1 vs. PC2 for samples after the exclusion of AAS304LD, 476ET, 626PM, 681CA and 704ZA (>20% missing values). AAS518CC is red. AAS518CC replicate is green. AAS327DD is purple. All other samples are grey.
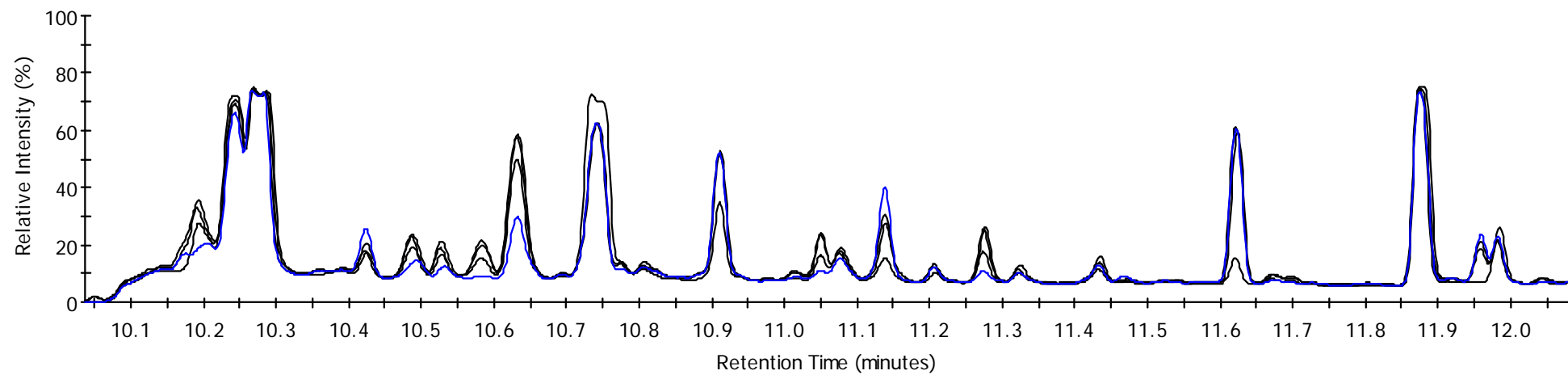
Figure 3.7: Overlay of the TIC for AAS304LD (blue) against QC9 (black), QC36 (black) and QC63 (black).

3.1.4. Exclusion of Features

As previously stated (Section 2.7.1), features with >20% missing values, RSD_{QC} >30%, or a ratio of $RSD_{Sample}:RSD_{QC}$ <1.5 were excluded from further analysis, as they were not sufficiently reproducible. One hundred and eighty-three features were initially identified and processed as described in Section 2.7. Twenty-three features were excluded based on >20% missing values (Appendix 5). Thirty-four features were excluded based on RSD_{QC} >30% (Appendix 6). Twenty-three features were then excluded based on a ratio of $RSD_{Sample}:RSD_{QC}$ <1.5 (Appendix 7). After these exclusion criteria, 103 features remained. A number of these were features that were included with no biological relevance. Thus, features that were found in the extraction and derivatisation blanks were also excluded. As a consequence, 52 features remained since 51 features were found in the blanks and were subsequently removed. Of these final 52 features, 24 were putatively identified using NIST. The final list of features and the identified metabolites are shown in Table 3.1 (Supplementary information provided in Appendix 8).

Table 3.1: Compound information for final list of metabolites/features. Information from data processing; compound number (based on order of retention time), retention time, retention index and base peak. Match criteria (forward match, reverse match, NIST probability and retention index) and CAS number was provided from NIST match. RSD$_{QC}$ is also provided.

Compound Number	Compound Name	Compound Class	Retention Time	Retention Index	Base Peak	Forward Match	Reverse Match	NIST Probability (%)	NIST Retention Index	CAS number	RSD$_{QC}$
003	Serine 2TMS	Amino acid	10.19	1208	116	886	834	95.5	1158	70125-39-2	26.0
008	Unknown_10.27_1218_301	Unknown	10.27	1218	301						07.1
012	Isoleucine 2TMS	Amino acid	10.49	1247	158	898	913	69.3	1272	7483-92-3	11.5
014	Threonine 2TMS	Amino acid	10.53	1253	073	920	926	92.5	1193	7536-82-5	14.3
015	Proline 2TMS	Amino acid	10.58	1260	142	911	919	93.5	1258	7364-47-8	15.6
017	Unknown_10.63_1267_086	Unknown	10.63	1267	086						06.9
019	Unknown_10.67_1272_129	Unknown	10.67	1272	129						21.0
032	Serine 3TMS	Amino acid	11.05	1323	204	885	892	30.4	1322	64625-17-8	19.2
035	Unknown_11.13_1333_188	Unknown	11.13	1333	188						23.5
037	Unknown_11.16_1337_147	Unknown	11.16	1337	147						26.8
040	Threonine 3TMS	Amino acid	11.28	1353	073	907	912	96.4	1357	7537-02-2	16.8
047	Unknown_11.52_1385_176	Unknown	11.52	1385	176						07.6
050	Unknown_11.55_1389_243	Unknown	11.55	1389	243						20.6
053	beta-Alanine 3TMS	Amino acid	11.70	1410	248	863	863	93.3	1434	55255-77-1	24.9
059	Unknown_12.11_1464_155	Unknown	12.11	1464	155						16.7
060	Succinate 3TMS	Carboxylic acid	12.16	1470	073	755	755	63.0	1390	38166-11-9	23.5
064	meso-Erythritol, 4TMS	Polyhydric Alcohol	12.27	1485	073	913	915	37.6	1427	n/a	14.9
071	Unknown_12.45_1509_084	Unknown	12.45	1509	084						25.6
072	Unknown_12.50_1516_176	Unknown	12.50	1516	176						22.1
075	Unknown_12.57_1525_292	Unknown	12.57	1525	292						11.3
081	Creatinine 3TMS	Amino Acid	12.80	1556	115	763	795	48.8	1592	n/a	29.7
082	Phenylalanine 1TMS	Amino Acid	12.85	1562	120	848	854	97.4	1547	2899-42-5	21.1
083	Unknown_12.87_1564_166	Unknown	12.87	1564	166						26.0

085	Unknown_13.04_1587_142	Unknown	13.04	1587	142						28.8
086	Unknown_13.95_1589_174	Unknown	13.05	1589	174						19.5
087	Ribofuranose 4TMS	Monosaccharide	13.11	1597	217	781	825	24.7	1671	n/a	13.9
096	Phenylalanine 2TMS	Amino Acid	13.39	1635	218	902	904	81.8	1710	2899-52-7	22.0
100	Unknown_13.50_1649_073	Unknown	13.50	1649	073						23.3
102	Unknown_13.62_1664_073	Unknown	13.62	1664	073						18.8
106	Arabitol 5TMS	Polyhydric Alcohol	13.93	1706	073	869	869	23.0	1746	35-21-2	13.7
109	Arabinofuranose 4TMS	Monosaccharide	14.32	1758	217	830	868	24.0	1671	55399-49-0	19.0
112	Citric acid 4TMS	Tricarboxylic Acid	14.69	1808	273	896	896	98.2	1944	14330-97-3	14.6
113	Ornithine 4TMS	Amino acid	14.72	1811	142	817	818	58.6	1702	n/a	25.2
119	Unknown_15.08_1860_073	Unknown	15.08	1860	073						21.0
121	Unknown_15.21_1877_059	Unknown	15.21	1877	059						09.8
122	Unknown_15.36_1897_158	Unknown	15.36	1897	158						11.1
123	Unknown_15.37_1898_160	Unknown	15.37	1898	160						08.0
125	Mannitol 6TMS	Polyhydric Alcohol	15.42	1905	319	792	806	48.5	2066	14317-07-8	14.4
130	Unknown_15.64_1941_073	Unknown	15.64	1941	073						27.7
134	Unknown_16.05_2006_204	Unknown	16.05	2006	204						16.9
135	Inositol 6TMS	Polyhydric Alcohol	16.08	2011	217	780	780	43.3	2194	14251-18-4	18.1
136	Palmitoleic acid 1TMS	Fatty Acid	16.19	2027	117	784	784	68.6	1995	n/a	21.7
138	Unknown_16.47_2072_204	Unknown	16.47	2072	204						26.1
139	*Myo*-Inositol 6TMS	Polyhydric Alcohol	16.51	2078	217	922	923	80.5	2194	2582-79-8	12.7
147	Linoleic acid TMS	Fatty Acid	17.35	2212	081	802	805	69.5	2202	56259-07-5	18.0
148	Tryptophan 3TMS	Amino acid	17.39	2220	202	920	930	64.0	2257	55429-28-2	13.8
157	Unknown_18.26_2387_091	Unknown	18.26	2387	091						21.8
161	Unknown_18.96_2519_427	Unknown	18.96	2519	427						21.4
174	Unknown_20.72_2855_221	Unknown	20.72	2855	221						29.2
178	Gastrodigenin 2TMS	Benzyl Alcohol	21.30	2966	221	717	879	67.8	1803	n/a	17.9
182	Unknown_22.47_3190_329	Unknown	22.47	3190	329						24.3
183	Unknown_22.47_3190_107	Unknown	22.47	3190	107						05.4

3.1.5. Result of Data Cleaning

The exclusion of sample and feature outliers, and the inter- and intra- batch correction were necessary prior to statistical analysis. This is demonstrated in the PCA score plot (PC1 vs. PC2) for the data matrix prior to data cleaning (Figure 3.8). In this initial PCA score plot, PC1 explained 13.8% of the variance and PC2 explained 9.7% of the variance. Additionally, PC1 clearly showed at least one analytical QC sample outlier, specifically with a PC1 value less than -30. There was also a large spread in the analytical QC samples in both PC1 and PC2. Following data cleaning, PC1 explained 26.4% of the variance and PC2 explained 10.5% of the variance (Figure 3.9). Additionally, there were no clear outliers in QC samples with PC1 or PC2. PC1 did not cluster analytical QC samples, showing that the variance described in PC1 is mostly associated with technical variation. The analytical QC samples were clustered in PC2 with a large spread of biological samples, showing the biological variation.
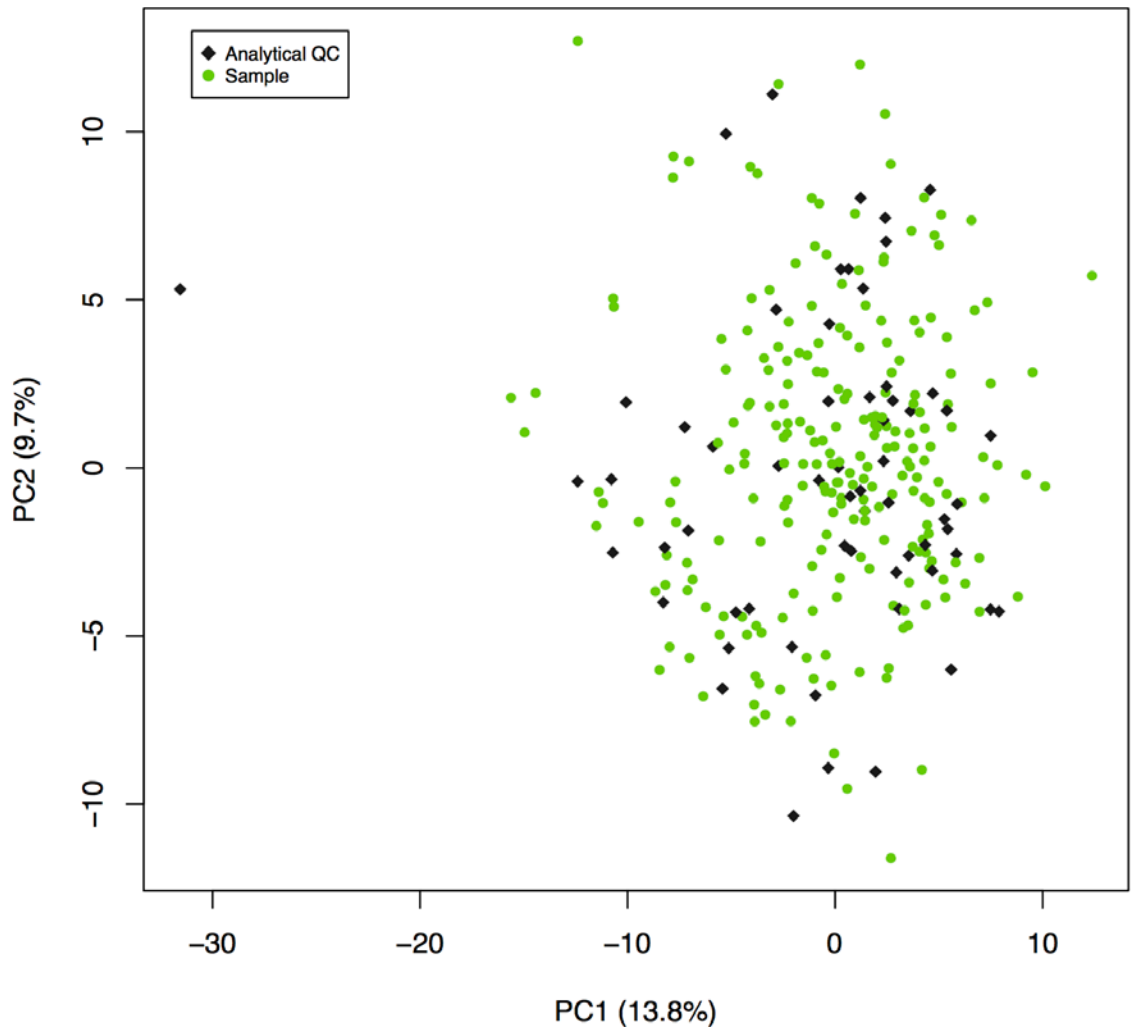
Figure 3.8: Principal component score plot of PC1 vs. PC2 for the data matrix prior to the removal of metabolite outliers, feature outliers and inter- and intra- batch correction. Analytical QC samples are black. Biological samples are green.
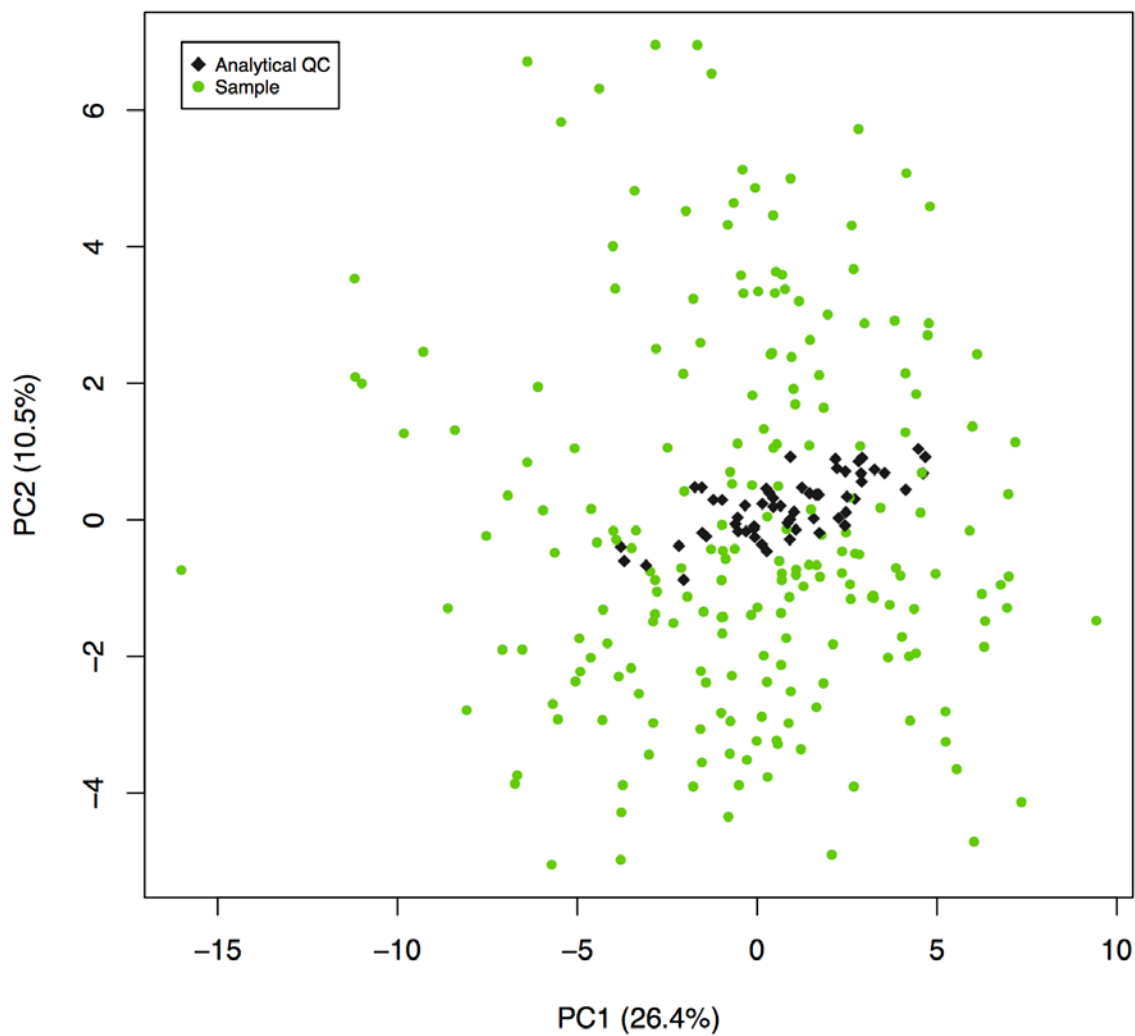
Figure 3.9: Principal component score plot of PC1 vs. PC2 for the data matrix after the removal of metabolite outliers, feature outliers and inter- and intra- batch correction. Analytical QC samples are black. Biological samples are green.

3.2. <u>Statistical Analysis</u>

3.2.1. Storage Time

In this study, efforts were made to ensure both the wheeze/asthma exacerbation group and the control group were matched for storage time. However, there was a greater range of storage time for the wheeze/asthma exacerbation group. The median and interquartile-range of storage time were 3.8 (2.8-5.2) years for the wheeze/asthma group, and 3.4 (2.9-4.1) years for the control group. The Spearman's rank correlation coefficient was used to assess the relationship between storage time and peak area (Table 3.3). Seven features were found to be significantly (p-value < 0.05) correlated with storage time. Of these, 4 were identified metabolites including serine (both 2TMS and 3TMS products), succinate and tryptophan. While statistically significant, the Spearman's Rho values were between -0.14 and 0.22. This is only a minimal to weak correlation with storage time.

Table 3.2: Spearman's rank correlation between metabolite abundance and storage time.

Compound Number	Compound Name	Spearman's Rho	p-value	q-value
161	Unknown_18.96_2519_427	0.50	1E-12	8E-11
102	Unknown_13.62_1664_073	-0.22	0.002	0.04
060	Succinate 3TMS	0.22	0.003	0.06
100	Unknown_13.50_1649_073	0.20	0.01	0.08
003	Serine 2TMS	0.16	0.02	0.19
032	Serine 3TMS	0.14	0.05	0.37
148	Tryptophan 3TMS	-0.14	0.05	0.37
113	Ornithine 4TMS	0.12	0.08	0.53
109	Arabinofuranose 4TMS	-0.11	0.10	0.59
134	Unknown_16.05_2006_204	-0.10	0.16	0.74
081	Creatinine 3TMS	-0.10	0.16	0.74
136	Palmitoleic acid 1TMS	0.11	0.17	0.74
015	Proline 2TMS	-0.09	0.20	0.81
112	Citric acid 4TMS	-0.09	0.23	0.81
017	Unknown_10.63_1267_086	-0.08	0.25	0.81
085	Unknown_13.04_1587_142	-0.08	0.26	0.81
083	Unknown_12.87_1564_166	-0.08	0.28	0.81
122	Unknown_15.36_1897_158	-0.08	0.29	0.81
086	Unknown_13.95_1589_174	-0.07	0.30	0.81
130	Unknown_15.64_1941_073	-0.07	0.31	0.81
008	Unknown_10.27_1218_301	-0.07	0.33	0.81
139	*Myo*-Inositol 6TMS	0.06	0.36	0.86
138	Unknown_16.47_2072_204	-0.06	0.38	0.86
053	beta−Alanine 3TMS	0.06	0.40	0.87
135	Inositol 6TMS	0.05	0.44	0.88
019	Unknown_10.67_1272_129	-0.06	0.45	0.88
157	Unknown_18.26_2387_091	0.05	0.46	0.88
147	Linoleic acid TMS	0.05	0.49	0.90
174	Unknown_20.72_2855_221	0.05	0.50	0.90
106	Arabitol 5TMS	-0.05	0.53	0.91
119	Unknown_15.08_1860_073	-0.04	0.54	0.91
071	Unknown_12.45_1509_084	0.04	0.56	0.91
123	Unknown_15.37_1898_160	-0.04	0.61	0.93
125	Mannitol 6TMS	0.04	0.61	0.93
047	Unknown_11.52_1385_176	0.03	0.65	0.96
037	Unknown_11.16_1337_147	0.03	0.69	0.96
035	Unknown_11.13_1333_188	-0.03	0.70	0.96
096	Phenylalanine 2TMS	0.03	0.71	0.96
050	Unknown_11.55_1389_243	0.02	0.79	0.96
072	Unknown_12.50_1516_176	0.02	0.80	0.96
059	Unknown_12.11_1464_155	-0.02	0.80	0.96
082	Phenylalanine 1TMS	0.02	0.81	0.96
182	Unknown_22.47_3190_329	0.01	0.85	0.96
014	Threonine 2TMS	0.01	0.85	0.96
040	Threonine 3TMS	0.01	0.85	0.96
075	Unknown_12.57_1525_292	-0.01	0.88	0.96
121	Unknown_15.21_1877_059	0.01	0.88	0.96
178	Gastrodigenin 2TMS	-0.01	0.92	0.96
087	Ribofuranose 4TMS	0.00	0.95	0.96
012	Isoleucine 2TMS	0.00	0.96	0.96
183	Unknown_22.47_3190_107	0.00	0.96	0.96
064	meso-Erythritol, 4TMS	0.00	0.96	0.96

3.2.2. Acute Wheeze/Asthma vs. Controls

The univariate analysis comparing the wheeze/asthma exacerbation group and the controls was performed using an ANOVA and median fold-change as previously described (Section 2.8). The output of this analysis is shown in Table 3.3. Twenty-nine compounds were found to be significantly different (p-value < 0.05) between the wheeze/asthma exacerbation group and the control group. Of those 29 features, 14 were putatively identified. The amino acids threonine, ornithine, proline, serine, tryptophan and isoleucine were decreased in abundance for the wheeze/asthma group compared with the control group. There was an increased relative abundance of the monosaccharides arabinofuranose and ribofuranose, the polyhydric alcohol mannitol, the carboxylate succinate and the fatty acids linoleic acid and palmitoleic acid.

Table 3.3: Two-way ANOVA and median fold-change output. Variable One: wheeze/asthma exacerbation (case) vs. controls. Variable Two: age under 6 vs. age 6 and over. Q-value: correction for multiple comparisons using the Benjamini and Hochberg Procedure.

Compound Number	Compound Name	Fold-Change (Case/Control)	Control/Case p-value	Control/Case q-value	Fold-Change (≥6/<6 years)	Age p-value	Age q-value	Interaction p-value	Interaction q-value
040	Threonine 3TMS	0.60	3E-09	2E-07	0.83	0.03	0.20	0.76	0.97
109	Arabinofuranose 4TMS	1.58	1E-08	4E-07	1.06	0.48	0.74	0.04	0.46
113	Ornithine 4TMS	0.61	1E-07	2E-06	0.96	0.49	0.74	0.62	0.97
014	Threonine 2TMS	0.64	2E-07	2E-06	1.08	0.11	0.30	0.91	0.98
087	Ribofuranose 4TMS	1.41	9E-07	1E-05	1.33	0.17	0.39	0.24	0.89
138	Unknown_16.47_2072_204	1.58	1E-06	1E-05	1.04	0.57	0.78	0.02	0.46
037	Unknown_11.16_1337_147	1.40	3E-06	2E-05	1.26	0.003	0.07	0.49	0.97
015	Proline 2TMS	0.67	7E-06	4E-05	0.94	0.08	0.25	0.11	0.66
134	Unknown_16.05_2006_204	1.31	8E-06	5E-05	1.25	0.33	0.58	0.03	0.46
072	Unknown_12.50_1516_176	0.65	1E-04	5E-05	0.84	0.36	0.62	0.97	0.98
083	Unknown_12.87_1564_166	0.74	2E-04	0.001	1.19	0.30	0.55	0.27	0.89
019	Unknown_10.67_1272_129	1.35	3E-04	0.001	0.95	0.07	0.24	0.04	0.46
125	Mannitol 6TMS	2.64	4E-04	0.001	0.88	0.62	0.80	0.96	0.98
130	Unknown_15.64_1941_073	1.22	4E-04	0.001	0.97	0.15	0.38	0.11	0.66
071	Unknown_12.45_1509_084	0.79	0.001	0.002	1.04	0.10	0.30	0.34	0.89
085	Unknown_13.04_1587_142	0.77	0.001	0.002	0.98	0.23	0.46	0.23	0.89
032	Serine 3TMS	0.84	0.001	0.003	1.02	0.04	0.21	0.73	0.97
148	Tryptophan 3TMS	0.81	0.002	0.004	1.00	0.64	0.80	0.82	0.97
060	Succinate 3TMS	1.40	0.002	0.005	1.11	0.72	0.80	0.48	0.97
003	Serine 2TMS	0.87	0.01	0.01	0.95	0.07	0.24	0.67	0.97
136	Palmitoleic acid 1TMS	2.05	0.01	0.01	0.75	0.01	0.07	0.57	0.97
121	Unknown_15.21_1877_059	1.10	0.01	0.01	0.97	0.70	0.80	0.52	0.97
012	Isoleucine 2TMS	0.91	0.01	0.03	1.35	0.43	0.72	0.64	0.97

059	Unknown_12.11_1464_155	0.77	0.02	0.03	1.13	0.69	0.80	0.67	0.97
157	Unknown_18.26_2387_091	1.22	0.02	0.04	0.93	0.57	0.78	0.76	0.97
174	Unknown_20.72_2855_221	0.81	0.02	0.04	1.08	0.20	0.44	0.52	0.97
017	Unknown_10.63_1267_086	0.94	0.02	0.04	0.83	0.90	0.91	0.98	0.98
147	Linoleic acid TMS	1.29	0.03	0.05	0.81	0.01	0.11	0.81	0.97
050	Unknown_11.55_1389_243	1.18	0.04	0.08	0.78	0.03	0.20	0.70	0.97
086	Unknown_13.95_1589_174	1.23	0.05	0.08	0.93	0.24	0.46	0.43	0.97
106	Arabitol 5TMS	1.11	0.06	0.10	0.91	0.74	0.81	0.46	0.97
122	Unknown_15.36_1897_158	1.05	0.08	0.14	1.06	0.58	0.78	0.16	0.82
135	Inositol 6TMS	0.81	0.10	0.16	0.99	0.50	0.74	0.09	0.66
035	Unknown_11.13_1333_188	0.90	0.14	0.22	1.16	0.01	0.07	0.58	0.97
008	Unknown_10.27_1218_301	0.91	0.15	0.22	1.02	0.04	0.21	0.55	0.97
182	Unknown_22.47_3190_329	1.06	0.17	0.24	1.00	0.86	0.89	0.71	0.97
178	Gastrodigenin 2TMS	0.92	0.19	0.26	1.00	0.23	0.46	0.91	0.98
119	Unknown_15.08_1860_073	1.07	0.28	0.38	0.62	0.85	0.89	0.05	0.46
053	beta-Alanine 3TMS	0.92	0.41	0.55	1.07	0.49	0.74	0.93	0.98
102	Unknown_13.62_1664_073	1.07	0.45	0.59	0.99	0.05	0.24	0.22	0.89
081	Creatinine 3TMS	1.11	0.50	0.64	1.00	0.05	0.24	0.05	0.46
064	meso-Erythritol, 4TMS	1.11	0.53	0.65	0.41	0.98	0.98	0.39	0.92
096	Phenylalanine 2TMS	1.16	0.63	0.76	1.08	0.70	0.80	0.62	0.97
047	Unknown_11.52_1385_176	1.00	0.66	0.78	0.93	0.17	0.39	0.17	0.83
183	Unknown_22.47_3190_107	1.00	0.67	0.78	0.75	0.12	0.31	0.88	0.98
161	Unknown_18.96_2519_427	1.11	0.72	0.81	1.02	0.001	0.06	0.33	0.89
112	Citric acid 4TMS	1.08	0.80	0.87	0.97	0.07	0.24	0.32	0.89
139	*Myo*-Inositol 6TMS	0.97	0.81	0.87	0.64	0.08	0.25	0.29	0.89
082	Phenylalanine 1TMS	1.07	0.87	0.92	0.90	0.67	0.80	0.97	0.98
100	Unknown_13.50_1649_073	0.98	0.94	0.96	0.93	0.01	0.07	0.82	0.97
075	Unknown_12.57_1525_292	1.04	0.95	0.96	1.00	0.53	0.76	0.31	0.89
123	Unknown_15.37_1898_160	1.00	0.96	0.96	0.99	0.69	0.80	0.37	0.92

The wheeze/asthma exacerbation group and the control group were visually compared using a PCA (Figure 3.10). PC1 showed no separation of groups. PC2 showed a variation in metabolite profile between the two groups. There was a small spread in PC2 for the control group, with a larger spread in PC2 for the wheeze/asthma group. Additionally, there was also a small overlap between the wheeze/asthma group and control group samples in PC2.
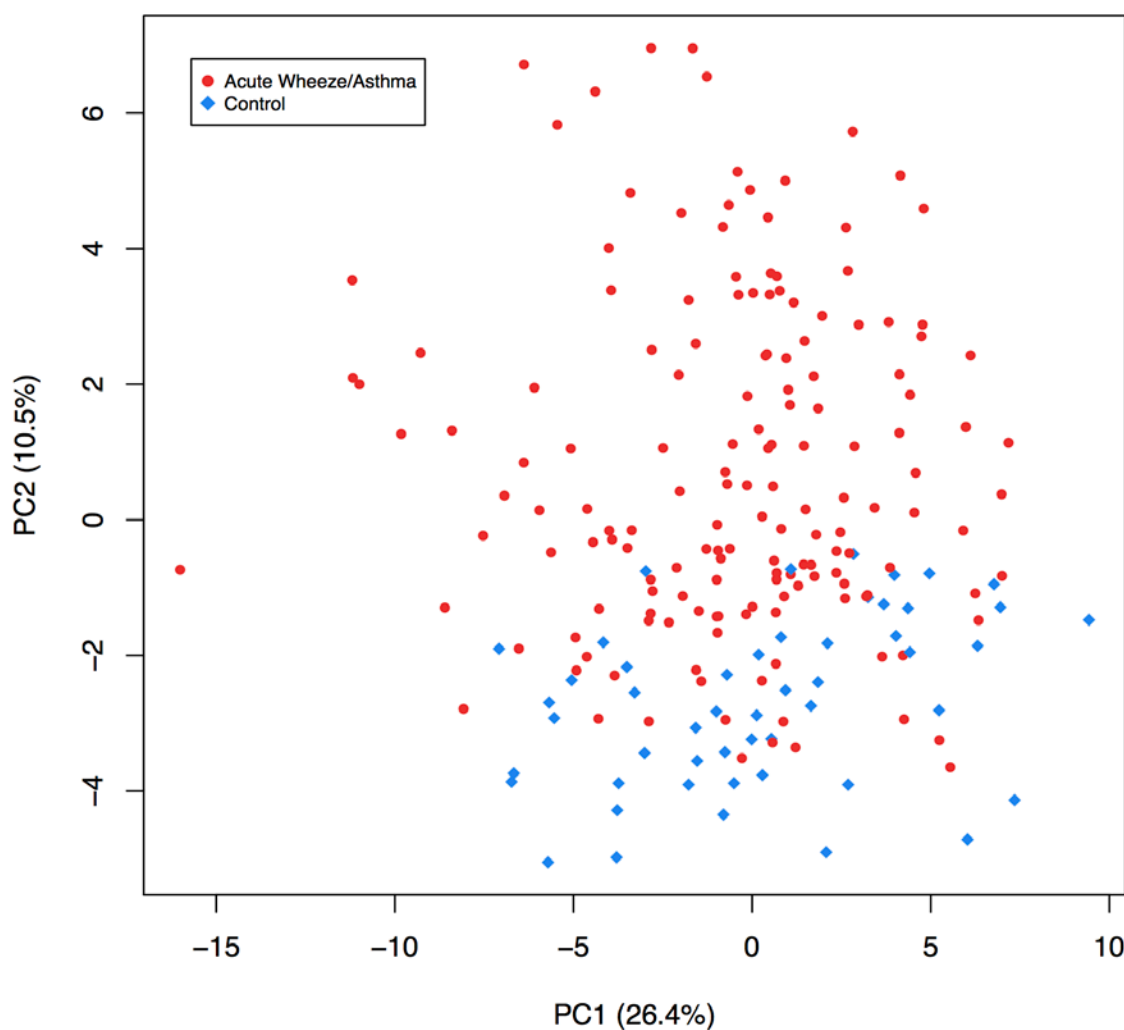
Figure 3.10: Principal component score plot of PC1 vs. PC2 for all biological samples. Acute wheeze/asthma group is coloured red. Control group is coloured blue.

3.2.3. Preschool-aged vs. School-aged Acute Wheeze/Asthma

To determine if there was a difference of acute wheeze/asthma based on age, a two-way ANOVA was used as previously described (Section 2.8). The age brackets of less than 6 years old and greater than (and including) 6 years old were used due to GINA regulations on the minimum age (6 years) on asthma diagnosis (Section 1.1.5). The significant metabolites were determined by the interaction term of the two-way ANOVA. Five features were found to be significantly associated with a p-value less than 0.05 (Table 3.3). Two were identified as arabinofuranose and creatinine. For arabinofuranose, there was a decreased abundance with age for the control group (negative gradient), and increased abundance with age for the acute wheeze/asthma group (positive gradient) (Figure 3.11). Additionally, there was a difference in metabolite abundance between all the groups. For creatinine, the metabolite abundance was similar between the age brackets for the acute wheeze/asthma group (0 gradient) (Figure 3.12). For the control group, there was a decreased abundance with the ≥6 years old age bracket. Note, unlike in Figure 3.11, there is overlap suggesting slight variations in metabolite abundance.

Additionally, through the two-way ANOVA, 10 features were found to be significantly associated with the age bracket. Four were identified as serine, threonine, linoleic acid and palmitoleic acid (Table 3.3). There was increased relative abundance with the higher age bracket for the amino acid serine. There was a decreased relative abundance of the amino acid threonine, and the fatty acids linoleic acid and palmitoleic acid.
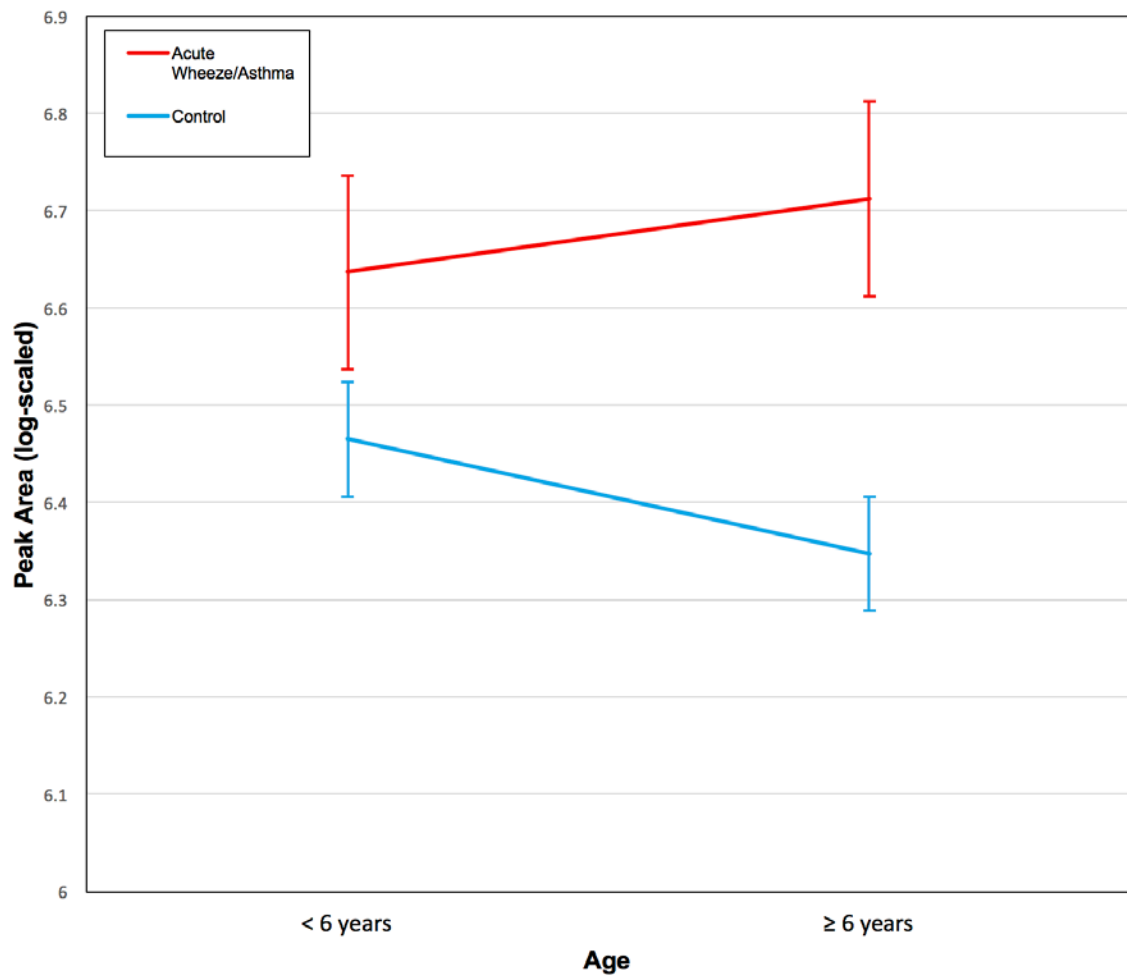
Figure 3.11: Plots of peak area (log-scaled) with mean and 95% confidence intervals for acute wheeze/asthma vs. control with age bracket for arabinofuranose. Acute wheeze/asthma group is coloured red. Control group is coloured blue.
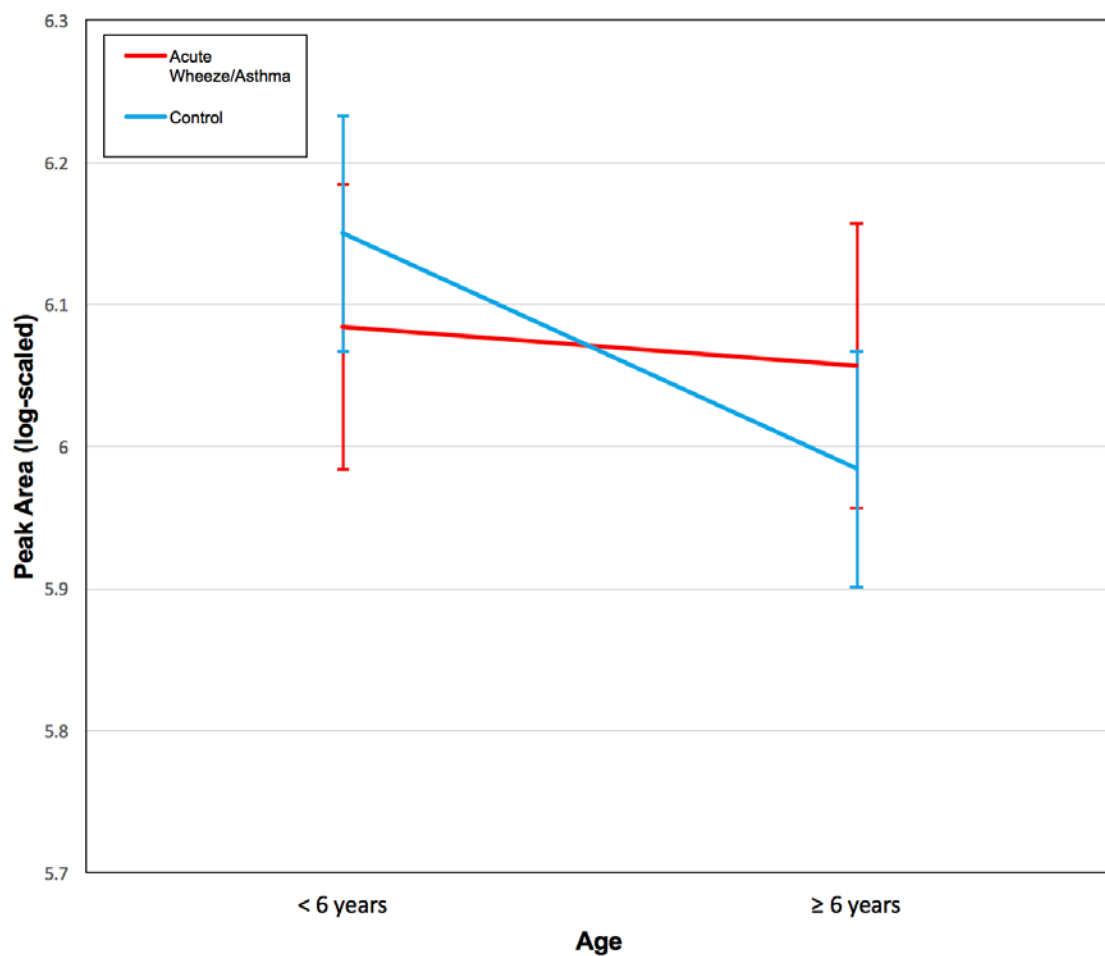
Figure 3.12: Plots of peak area (log-scaled) with mean and 95% confidence intervals for acute wheeze/asthma vs. control with age bracket for creatinine. Acute wheeze/asthma group is coloured red. Control group is coloured blue.

The difference between the wheeze/asthma exacerbation group and control group for the brackets of less than 6 years old and greater than (and including) 6 years old was visually compared using PCA (Figure 3.13). The PCA score plot (PC1 vs. PC2) compared children with wheeze/asthma that were less than years old at recruitment, children with wheeze/asthma that were greater than (and including) 6 years old, control children that were less than years old and control children that were greater than (and including) 6 years old (Figure 3.11). There is minimal variation found, with no clear distinction to separate these groups based on age.
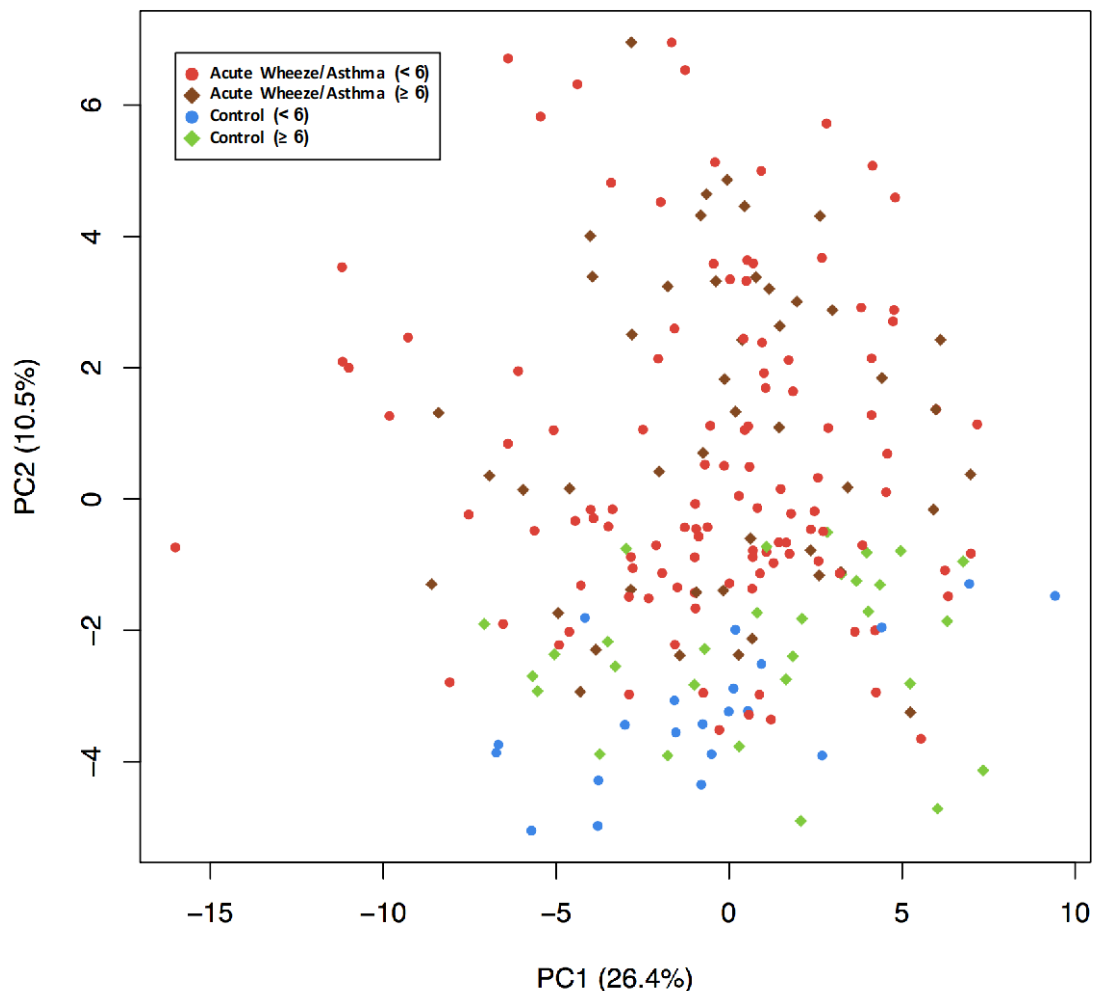
Figure 3.13: Principal component score plot of PC1 vs. PC2 for all biological samples. Wheeze/asthma exacerbation group; < 6 years old are coloured red and ≥ 6 years old are coloured brown. Control group; < 6 years old are coloured blue and ≥ 6 years old are coloured green.

Chapter Four: Discussion

There were three main aims that this research addressed: to assess the impact of potentially limiting factors of retrospectively collected serum samples on metabolomic analysis; to determine whether metabolomics can identify potential biomarkers to distinguish between wheeze/asthma exacerbation and control groups; and to determine whether metabolomics-derived biomarkers can identify differences between preschool-aged and school-aged phenotypes. The three potentially limiting factors of the samples in the study were haemolysis, additional freeze-thaw cycles and storage time. Haemolysis and additional freeze-thaw cycles were not found to be significant. Serine, succinate and tryptophan abundance was found to be weakly correlated with storage time. Twenty-nine metabolites were found to be significantly different (p-value < 0.05) between the acute wheeze/asthma group and the control group. Fourteen metabolites were putatively identified including amino acids, monosaccharides, fatty acids, a carboxylate and a polyhydric alcohol. There was no clear difference on the PCA score plot between the acute wheeze/asthma groups based on the age-cut at 6 years (Figure 3.13). However, there were five metabolites that were identified as statistically significant, these were arabinofuranose and creatinine.

4.1. Potential Limiting Factors of Retrospectively Collected Samples

4.1.1. Haemolysis

As haemolysis is the lysis of erythrocytes resulting in the release of intracellular components, the release of both metabolites and enzymes was expected to alter the abundance of various metabolites. Multiple studies have confirmed that there are metabolite abundance variations associated with haemolysis (Denihan et al. 2015, Yin et al. 2013, Kamlage et al. 2014). Three of the samples were haemolysed in this study. Due

to the small proportion of haemolysed samples, the removal (if required) would result in a minimal loss of statistical power. However, there was no clear indication of the need to remove these samples (Section 3.1.3). There are two possible explanations that can align the findings of this study with that of previous studies and the current understanding of asthma; either haemolysis does not affect the metabolites in this study, or the effect on the metabolite abundance is minimal. Previous studies have found significant changes primarily in lipids which form part of the plasma membrane including phosphatidylcholines and lysophsophatidylcholines (Denihan et al. 2015, Yin et al. 2013). Lipids are not well suited to this untargeted GC-MS analysis given their physicochemical properties, with only palmitoleic acid and linoleic acid identified. Denihan et al. (2015) found a significant difference in 4 amino acids for haemolysis including serine with a minimally associated fold-change of 0.9. This variation in metabolite abundance caused by haemolysis may be overshadowed by other factors in this study, such as the acute wheeze/asthma. An additional targeted LC-MS analysis is needed to analyse the effect of haemolysis on lipids.

4.1.2. Additional Freeze-Thaw Cycle

Freezing is a requirement of samples to increase their longevity, with samples required to be refrozen and thawed for supplementary studies. Two samples in this study went through an additional freeze-thaw cycle as they were used in another study of the MAVRIC cohort. As a small proportion of samples went through an additional freeze-thaw cycle, the removal (if required) would have resulted in a minimal loss in statistical power. However, there was no clear indication of the need to remove these samples based on the PCA score plot (Section 3.1.3). Previous studies on additional freeze-thaw cycles support the lack of impact of the additional freeze-thaw cycles (Yin et al. 2013, Breier et al. 2014, Anton et al. 2015). However, the reason may be the lack of effect on the specific

metabolites identified or the minimal effect on metabolites abundance compared to other factors, such as the acute wheeze/asthma. Another issue is the lack of additional information regarding the time or method of thawing. Regardless, future LC-MS analysis performed on these samples may indicate that the additional freeze-thaw cycle has a greater impact on the stability of the metabolites identified.

4.1.3. Storage Time

There are various studies on the effect of short-term storage (months), however the effects of long-term storage (years) on metabolite abundance is limited. One study using untargeted LC-MS found a difference in abundance of lysophsophatidylcholines, phosphoethanolamines, acylcarnitines, serotonin and hypoxanthine between 2-month and 5-year batches of plasma from healthy volunteers (Yang et al. 2013). These serum samples curated from the MAVRIC cohort were stored between 0.8 and 7.9 years at -80°C. The correlation between storage time and metabolite abundance was found to be statistically significant (p-value < 0.05) for three metabolites; succinate, serine (2TMS and 3TMS) and tryptophan. While statistically significant, the low Spearman's Rho values indicated weak correlations. This suggests that impact of storage time is minimal compared to other sources of variation, which in this case is between acute wheeze/asthma and the control group. There are no current studies that indicate whether there is an impact of long-term storage time on succinate, serine and tryptophan. Additionally, the metabolites identified by Yang et al. (2013) were not identified in this GC-MS analysis, due to their physicochemical properties. Further LC-MS analysis is needed to determine if storage time has a greater effect on lipids. Regardless, the samples curated were matched for storage time to minimise the potential effects.

4.2. Acute Wheeze/Asthma vs. Controls

Twenty-nine of the 52 metabolites analysed were found to be significantly different (p-value < 0.05) between the acute wheeze/asthma group and the control group. This large difference in metabolite profile corresponded to a difference in the spread in the PC2 shown in the PCA score plot (Section 3.2.2). Fourteen of the 29 metabolites identified including the amino acids threonine, ornithine, proline, serine, tryptophan and isoleucine were significantly decreased in the acute wheeze/asthma group. Additionally, there was a significant increased relative abundance of the monosaccharides arabinofuranose and ribofuranose, the polyhydric alcohol mannitol, the carboxylate succinate and the fatty acids linoleic acid and palmitoleic acid.

The decreased abundance of serine and threonine levels in acute wheeze/asthma group align with a previous study on childhood asthma. In this study, the glycine, serine and threonine metabolism pathways were significantly decreased in the severe form of asthma (Fitzpatrick et al. 2014). The role of threonine in asthma is not known and there is no established role of threonine in inflammation. The decreased abundance of serine may be due to the biosynthesis of the antioxidant glutathione, found to be associated with airway macrophage functional impairment in severe childhood asthma (Fitzpatrick, Jones and Brown 2012).

Ornithine and proline are known to play roles in cell proliferation and collagen synthesis. Both have been previously hypothesised to cause airway remodelling and hyper-responsiveness via synthesis through arginase (Maarsingh, Zaagsma and Meurs 2008). Arginase activity has been previously established to be associated with inflammation, as the inhibition has been found to prevent air remodelling and inflammation in guinea pig models of chronic obstructive pulmonary disease (Pera et al. 2014).

Tryptophan metabolism has been linked to multiple disease mechanisms. The important pathways of tryptophan include the synthesis of serotonin and kynurenine. Indoleamine 2,3-dioxygenase, an enzyme in the kynurenine pathway has been implicated in immune tolerance and T cell regulation (Meisel et al. 2004, Chen et al. 2016, Bauer et al. 2005). However, previous studies on tryptophan, kynurenine or indoleamine 2,3-dioxygenase have not shown a significant difference in abundance in asthmatics compared to healthy controls (Adkins et al. 2015, van Aalderen 2012). Unlike in this study, previous studies have not used samples from children with the acute form of asthma.

There was an increased abundance in the two monosaccharides identified as arabinofuranose and ribofuranose. The term furanose is defined as carbohydrates that include a five-membered ring with four carbon atoms and one oxygen. Arabinofuranose is the furanose form of arabinose, a sugar used as a sweetener. Ribofuranose is the furanose form of ribose, synthesised by the body. Ribose is a vital component as derivatives are major components of deoxyribonucleic acid, ribonucleic acid, and adenosine mono-, di- and tri- phosphates. Additionally, it is noted that ribofuranose can be synthesised from arabinofuranose. Decreased levels of arabinose have been reported in induced asthma studies in mice (Ho et al. 2013). The decreased levels of arabinose could potentially be related to an unidentified biochemical pathway relevant to asthma. However, as arabinose is a sweetener, it could also be linked to varying levels of food consumption or the current state of fasting in the children.

The polyhydric alcohol mannitol was found to be significantly increased in the acute wheeze/asthma group. Polyhydric alcohols such as mannitol are derived from sugars. Like arabinose, mannitol is a sweetener so it may also be an indication of the level of food consumption or current fasting state. Unlike arabinose, mannitol has been

established to induce bronchoconstriction in asthmatics (Romberg, Tufvesson and Bjermer 2017, Sverrild et al. 2016). Due to this airway responsiveness to mannitol, studies have proposed inhaled mannitol to be a suitable challenge test for asthma when spirometry is near normal (White et al. 2016, Parra Pérez et al. 2015). However, there is no evidence to suggest that the test was not performed on the children in this study.

Succinate is a key citric acid cycle intermediate produced in the mitochondria. Succinate has multiple biological roles, such as an intermediate involved in making adenosine triphosphate, and as a signalling molecule in signal transduction, reactive oxygen species and hypoxia (Tretter, Patocs and Chinopoulos 2016). Additionally, succinate is linked to inflammation as it induces IL-1 beta, an important inflammatory mediator (Tannahill et al. 2013). Increased levels of succinate have been found in a previous study in mild persistent asthma (Chun et al. 2015). However, as succinate plays multiple roles, more information regarding the metabolic pathways are needed.

Linoleic acid and palmitoleic acid were found to be significantly increased in the wheeze/asthma exacerbation group. Linoleic acid is an essential fatty acid used in the synthesis of arachidonic acid, which is a precursor to some prostaglandins, leukotrienes and thromboxane. These are known mediators of both bronchoconstriction and mucosal hypersecretion (Section 1.1.2). Additionally, the linoleic acid metabolite known as 13-*S*-hydroxyoctadecadienoic acid has been known to drive severe asthma, especially the Th2 phenotype (Mabalirajan et al. 2013). Palmitoleic acid is an essential fatty acid which is a common constituent of glycerides in human adipose tissue. Palmitoleic acid is linked to common chronic diseases due to the associated anti-inflammatory properties leading to deceased IL-6 and TNF (Souza et al. 2017). The abundance of palmitoleic acid has been previously found to be highly correlated with inhaled corticosteroid usage (Reinke et al.

2017). All children in this study were on inhaled corticosteroids due to the severity of the wheeze/asthma exacerbation, which could explain the high abundance of palmitoleic acid.

4.3. <u>Preschool-aged vs. School-aged Acute Wheeze/Asthma</u>

For this analysis, children were classified based on two age brackets; less than 6 years old and greater than (and including) 6 years old. As previously stated, current GINA recommendations have set the minimum age for asthma diagnosis at age 6. Five metabolites were found to be statistically significant between these two groups when accounting for potential age effects (Section 3.2.3). Two of the metabolites were identified; creatinine and arabinofuranose. While statistically significant (p-value < 0.05), there was no clear metabolite separation in the PCA score plot between the less than 6-year-old group and the greater than (and including) 6-year-old group with acute wheeze/asthma (Figure 3.14).

Arabinofuranose was found to increase with the higher age bracket for the acute wheeze/asthma group and decrease for the control group. Additionally, in the lower age bracket the abundance of arabinofuranose was higher in the acute wheeze/asthma group. Arabinofuranose is the furanose form of arabinose, and as previously stated in Section 4.2, arabinose has been reported in induced asthma studies in mice (Ho et al. 2013). However, as it is a sweetener, it could potentially be indicative of the level of food consumption or the current state of fasting in the children.

Creatinine was also found to be statistically significant (p-value < 0.05). In the control group, there was a decreased creatinine abundance with age. In the acute wheeze/asthma group, there was a similar level of creatinine abundance between the two age brackets.

The creatinine abundance of the acute wheeze/asthma group and control group below 6 years was approximately the same. Creatinine is the breakdown product of creatine phosphate which is synthesized in the muscle from arginine, glycine and methionine (Brosnan, da Silva and Brosnan 2011). Creatine kinase, which catalyses the conversion of creatine, has been shown to be higher in asthmatics (Alberts, Williams and Ramsdell 1986). Creatine kinase and creatinine levels are related to muscle stress leading to muscle damage. This could indicate that ASM stress and/or damage is insignificant in the acute wheeze/asthma group under 6, that there is a cumulative and permanent damage associated with asthma over time, or that there is a variation in the pathophysiology between acute wheeze and acute asthma.

The lack of clear distinction in the PCA score plot may indicate that such age groups are arbitrary. This could suggest that wheeze and asthma share the same or similar pathophysiology. Alternatively, this could indicate that an age bracket is not suitable. As previously mentioned, whether the children have wheeze or asthma (or wheeze that develops into asthma) is only known retrospectively (Section 1.1.5). Specific follow up studies on the children in this study may prove to be of vital importance as the analysis of this data matrix could be based on true wheeze and true asthma. Potentially, arabinofuranose or creatinine could be of importance to novel asthma diagnosis.

4.4. <u>General Conclusion</u>

While the MAVRIC cohort was not designed for metabolomic analysis, there were no issues regarding pre-analytical variation that impacted this study. Haemolysis and additional freeze-thaw cycles were not found to be significant issues; however, it could impact future studies. This could be due to the lack of impact on the types of metabolites identified using untargeted GC-MS, or the low level of effect combined with the low sample size. Regardless, this suggests that for future studies, these factors would not lead to automatic exclusion. The amino acids serine, succinate and tryptophan were found to be significantly correlated with storage time. However, the Spearman's Rho value was small, suggesting a minimal impact of storage time compared to other sources of variation in metabolite abundance. This is an important breakthrough that would suggest that other bio-banked samples could be used without the major impact of long-term storage time on metabolites.

There were issues from the MAVRIC cohort that were discovered through this study. These were in regard to the nature of the source of the metabolite abundances of the monosaccharides, arabinofuranose and ribofuranose, and the polyhydric alcohol mannitol. While they may be related to the pathophysiology of asthma, this cannot be assumed. There is the potential that this variation in abundances is directly related to food intake or the fasting state of the patient. As such, food intake is a significant factor in this metabolomic analysis. This calls for the need for more specific questions to be included in questionnaires that can be given to the children who are recruited into the cohort study.

The major finding of this study is that there is a clear differentiation in metabolite profile between the wheeze/asthma exacerbation group and the control group. Further research, particularly in amino acid and lipid metabolism would be beneficial to improve the

understanding of disease mechanisms. Lipids in particular are more readily identified in LC‑MS analysis and the future untargeted LC-MS analysis on this population may uncover vital information regarding the further understanding of the pathophysiology of asthma and subsequent novel treatments.

With regards to the age brackets of under 6 years, and over (and including) 6 years for the acute wheeze/asthma group, there was little difference in the PCA score plot. Arabinofuranose was found to be significant between preschool and school‑aged wheeze/asthma, however the reason may be in regard to fasting. Creatinine was an interesting and unexpected discovery. Creatinine blood tests are readily available and thus further analysis on creatinine and the associate pathways may be an important contributing tool in the differentiation between acute asthma and acute wheeze.

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Appendix 1: Abstract for the Scottish Metabolomics Network Symposium, 2nd-3rd November. Glasgow, Scotland.

TITLE
Trends of TMS-derivatised serum metabolites analysed by GCqTOF MS: observations from a metabolomics-based childhood asthma study

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INTRODUCTION
Asthma is a common, chronic inflammatory disease of the airways which varies widely in cause and severity. Asthma attacks are characterised by chest tightness, shortness of breath, coughing and wheezing and the underlying pathophysiology for which remains unknown. The Mechanisms of Acute Viral Respiratory Infections in Children (MAVRIC) study is a cohort study which recruited children upon presentation to the emergency department at Princess Margaret Hospital for Children with acute lower respiratory illness including wheeze and/or asthma. The aim of the present study was to determine serum metabolites which were reproducible over multiple GCqTOF MS acquisition batches with a further aim to determine metabolite markers of wheeze/asthma.

METHODS
The methods used are depicted in Figure 1. Briefly, serum metabolites were extracted and in 7 batches and analysed as their TMS derivatives using the Agilent 7200 quadrupole time-of-flight GC/MS system without inlet, column or source maintenance. TOF-MS data were imported into SpectralWorks' AnalyzerPro® for deconvolution and untargeted processing of the metabolite data. Pooled analytical quality control samples were initially processed, with 183 metabolites found in >80% of the samples. All samples were subsequently processed against the generated library.

RESULTS

Figures 2 and 3 show samples and QC samples before and after QC-RSC correction. After applying a quality control-robust spline correction (QC-RSC) algorithm for batch correction, fifty-two features had an RSD within the range 5.4 – 29.7%. Twenty-four of these features were putatively identified using the National Institute of Standards and Technology (NIST v2.0) mass spectral library. Putatively identified features are shown in Table 1.

CONCLUSION

AnalyzerPro provides a robust platform for the visualisation, deconvolution and alignment of GC-MS data. Further, AnalyzerPro encompasses a simplified and automated identification platform by integrating the NIST Mass Spectral Database for EI GC-MS. Of the 52 features deemed reproducible, twenty-four were putatively identified with ease. These metabolites are being further investigated for significant differences between the wheeze/asthma serum samples and serum from healthy subjects.

Appendix 2: Settings for Data Processing

Setting	Corresponding Value
Minimum Masses	4
Mass Range	50-700
Area Threshold	5000
Height Threshold	0.01%
Width Threshold	0.01 Minutes
Tailing	0%
Signal to Noise	10
Scan Window	1
Resolution	Minimum
Smoothing	9
Retention Index	RI File and RI Method
Target Library Building Retention Time	0.1 Minutes

Appendix 3: Chi-Squared Test between Missing and Non-Missing Values

Compound Number	Compound Name	p-value	q-value
060	Succinate 3TMS	0.10	1.00
035	Unknown_11.13_1333_188	0.18	1.00
071	Unknown_12.45_1509_084	0.20	1.00
014	Threonine 2TMS	0.23	1.00
123	Unknown_15.37_1898_160	0.31	1.00
106	Arabitol 5TMS	0.31	1.00
147	Linoleic acid TMS	0.36	1.00
085	Unknown_13.04_1587_142	0.36	1.00
100	Unknown_13.50_1649_073	0.44	1.00
003	Serine 2TMS	0.53	1.00
082	Phenylalanine 1TMS	0.54	1.00
087	Ribofuranose 4TMS	0.62	1.00
075	Unknown_12.57_1525_292	0.67	1.00
178	Gastrodigenin 2TMS	0.67	1.00
059	Unknown_12.11_1464_155	0.69	1.00
050	Unknown_11.55_1389_243	0.72	1.00
109	Arabinofuranose 4TMS	0.75	1.00
161	Unknown_18.96_2519_427	0.77	1.00
017	Unknown_10.63_1267_086	0.80	1.00
122	Unknown_15.36_1897_158	0.80	1.00
138	Unknown_16.47_2072_204	0.81	1.00
112	Citric acid 4TMS	0.83	1.00
113	Ornithine 4TMS	0.83	1.00
053	Beta-Alanine 3TMS	0.87	1.00
119	Unknown_15.08_1860_073	0.89	1.00
182	Unknown_22.47_3190_329	0.93	1.00
157	Unknown_18.26_2387_091	0.98	1.00
183	Unknown_22.47_3190_107	0.99	1.00
008	Unknown_10.27_1218_301	1.00	1.00
015	Proline 2TMS	1.00	1.00
019	Unknown_10.67_1272_129	1.00	1.00
032	Serine 3TMS	1.00	1.00
037	Unknown_11.16_1337_147	1.00	1.00
040	Threonine 3TMS	1.00	1.00
047	Unknown_11.52_1385_176	1.00	1.00
072	Unknown_12.50_1516_176	1.00	1.00
083	Unknown_12.87_1564_166	1.00	1.00
086	Unknown_13.95_1589_174	1.00	1.00
102	Unknown_13.62_1664_073	1.00	1.00
121	Unknown_15.21_1877_059	1.00	1.00
125	Mannitol 6TMS	1.00	1.00
130	Unknown_15.64_1941_073	1.00	1.00
134	Unknown_16.05_2006_204	1.00	1.00
135	Inositol 6TMS	1.00	1.00
136	Palmitoleic acid 1TMS	1.00	1.00
139	*Myo*-Inositol 6TMS	1.00	1.00
148	Tryptophan 3TMS	1.00	1.00
174	Unknown_20.72_2855_221	1.00	1.00
012	Isoleucine 2TMS	n/a	n/a
064	meso-Erythritol, 4TMS	n/a	n/a
081	Creatinine 3TMS	n/a	n/a
096	Phenylalanine 2TMS	n/a	n/a

Appendix 4: Clinical Information for the Excluded Samples

ID	Group	Age	Gender	Diagnosis	Viral	Steroid Given
476ET	Acute wheeze/asthma	3.3	Female	Acute asthma	Yes	Yes
626PM	Acute wheeze/asthma	6.8	Female	Acute asthma	No	Yes
704ZA	Acute wheeze/asthma	3.1	Female	Acute wheeze	Yes	Yes

Appendix 5: Feature Exclusion (>20% missing values)

Compound Number	Retention Time	Retention Index	Base Peak	% missing value
001	10.05	1189	147	28.5
006	10.26	1217	147	25.3
016	10.62	1266	174	20.6
029	10.97	1313	073	27.4
031	11.01	1318	245	24.9
046	11.50	1383	147	20.2
057	12.07	1458	244	24.2
062	12.20	1475	073	24.9
065	12.28	1487	071	29.6
079	12.79	1554	073	29.6
094	13.27	1618	355	29.6
097	13.41	1637	206	59.9
099	13.45	1642	217	35.0
107	14.06	1723	277	43.3
128	15.50	1918	217	41.5
141	16.69	2107	117	38.6
150	17.43	2227	357	20.2
152	17.74	2286	163	40.8
154	17.80	2298	217	64.6
156	17.98	2332	188	63.2
166	19.26	2577	217	39.4
169	19.62	2645	174	33.2
171	19.96	2711	361	27.1

Appendix 6: Feature Exclusion (RSD$_{QC}$)

Compound Number	Retention Time	Retention Index	Base Peak	RSDqc
009	10.34	1229	341	33.1
041	11.32	1359	191	33.1
045	11.48	1380	242	41.5
056	11.98	1447	117	31.5
066	12.32	1492	073	48.9
073	12.53	1519	069	35.9
074	12.53	1520	221	31.7
076	12.63	1533	165	75.9
088	13.13	1599	239	32.8
090	13.17	1605	235	47.0
091	13.21	1611	156	42.9
093	13.24	1615	246	30.7
111	14.43	1773	156	61.1
115	14.85	1829	156	31.4
116	15.00	1849	117	34.9
118	15.03	1853	174	51.3
126	15.44	1908	156	38.6
129	15.59	1933	218	42.9
131	15.67	1945	117	44.1
132	15.82	1968	147	44.4
140	16.63	2096	221	31.8
145	17.22	2190	056	55.3
146	17.24	2193	073	33.2
153	17.76	2290	073	37.6
155	17.87	2313	281	30.2
158	18.39	2412	129	55.5
160	18.89	2506	221	32.6
162	19.00	2528	055	57.7
167	19.45	2613	361	96.8
168	19.54	2630	221	32.9
172	20.43	2799	281	31.4
177	21.10	2928	340	105.7
180	22.25	3148	237	32.6
181	22.37	3171	311	33.4

Appendix 7: Feature Exclusion (RSD$_{Sample}$:RSD$_{QC}$)

Compound Number	Retention Time	Retention Index	Base Peak	RSD$_{Sample}$:RSD$_{QC}$
004	10.20	1210	179	1.32
010	10.39	1235	071	1.25
018	10.65	1269	069	1.44
021	10.72	1278	213	1.47
022	10.75	1283	117	1.27
023	10.76	1284	209	0.99
024	10.81	1290	069	1.42
028	10.91	1304	184	1.24
030	10.99	1315	071	1.35
036	11.14	1334	073	1.13
048	11.52	1386	174	1.31
049	11.53	1387	057	1.11
052	11.68	1407	184	1.04
054	11.87	1431	147	1.23
067	12.36	1498	174	1.44
068	12.38	1500	057	1.29
069	12.39	1502	174	1.19
089	13.15	1602	221	1.23
105	13.92	1705	282	1.12
114	14.84	1828	174	1.33
133	15.98	1994	174	1.36
164	19.21	2568	149	1.37
173	20.44	2801	057	1.29

Appendix 8: Missing Values (%), RSD$_{QC}$ and RSD$_{Sample}$:RSD$_{QC}$ for the Final List of Metabolites.

Compound Number	Compound Name	Compound Class	Retention Time	Retention Index	Base Peak	Missing Value (%)	RSD$_{QC}$	RSD$_{sample}$:RSD$_{QC}$
003	Serine 2TMS	Amino acid	10.19	1208	116	01.44	26.0	1.54
008	Unknown_10.27_1218_301	Unknown	10.27	1218	301	01.81	07.1	2.88
012	Isoleucine 2TMS	Amino acid	10.49	1247	158	00.36	11.5	3.93
014	Threonine 2TMS	Amino acid	10.53	1253	073	03.61	14.3	4.21
015	Proline 2TMS	Amino acid	10.58	1260	142	01.08	15.6	3.73
017	Unknown_10.63_1267_086	Unknown	10.63	1267	086	06.14	06.9	2.03
019	Unknown_10.67_1272_129	Unknown	10.67	1272	129	17.33	21.0	2.24
032	Serine 3TMS	Amino acid	11.05	1323	204	04.69	19.2	2.92
035	Unknown_11.13_1333_188	Unknown	11.13	1333	188	00.72	23.5	3.27
037	Unknown_11.16_1337_147	Unknown	11.16	1337	147	00.36	26.8	3.05
040	Threonine 3TMS	Amino acid	11.28	1353	073	12.27	16.8	3.36
047	Unknown_11.52_1385_176	Unknown	11.52	1385	176	04.33	07.6	2.58
050	Unknown_11.55_1389_243	Unknown	11.55	1389	243	02.89	20.6	6.29
053	beta-Alanine 3TMS	Amino acid	11.70	1410	248	16.61	24.9	2.35
059	Unknown_12.11_1464_155	Unknown	12.11	1464	155	14.08	16.7	4.19
060	Succinate 3TMS	Carboxylate	12.16	1470	073	02.53	23.5	2.93
064	meso-Erythritol, 4TMS	Polyhydric Alcohol	12.27	1485	073	07.22	14.9	2.13
071	Unknown_12.45_1509_084	Unknown	12.45	1509	084	00.36	25.6	2.91
072	Unknown_12.50_1516_176	Unknown	12.50	1516	176	04.33	22.1	7.24
075	Unknown_12.57_1525_292	Unknown	12.57	1525	292	00.00	11.3	2.37
081	Creatinine 3TMS	Amino Acid	12.80	1556	115	00.36	29.7	2.35
082	Phenylalanine 1TMS	Amino Acid	12.85	1562	120	01.81	21.1	2.32
083	Unknown_12.87_1564_166	Unknown	12.87	1564	166	02.17	26.0	2.75
085	Unknown_13.04_1587_142	Unknown	13.04	1587	142	05.05	28.8	2.68

086	Unknown_13.95_1589_174	Unknown	13.05	1589	174	11.55	19.5	3.23
087	Ribofuranose 4TMS	Monosaccharide	13.11	1597	217	00.36	13.9	2.33
096	Phenylalanine 2TMS	Amino Acid	13.39	1635	218	08.30	22.0	5.09
100	Unknown_13.50_1649_073	Unknown	13.50	1649	073	08.30	23.3	4.59
102	Unknown_13.62_1664_073	Unknown	13.62	1664	073	14.08	18.8	3.91
106	Arabitol 5TMS	Polyhydric Alcohol	13.93	1706	073	01.81	13.7	3.37
109	Arabinofuranose 4TMS	Monosaccharide	14.32	1758	217	06.14	19.0	2.61
112	Citric acid 4TMS	Tricarboxylic Acid	14.69	1808	273	00.36	14.6	2.67
113	Ornithine 4TMS	Amino acid	14.72	1811	142	09.39	25.2	3.11
119	Unknown_15.08_1860_073	Unknown	15.08	1860	073	16.25	21.0	1.84
121	Unknown_15.21_1877_059	Unknown	15.21	1877	059	06.14	09.8	1.70
122	Unknown_15.36_1897_158	Unknown	15.36	1897	158	03.61	11.1	1.64
123	Unknown_15.37_1898_160	Unknown	15.37	1898	160	15.52	08.0	10.44
125	Mannitol 6TMS	Polyhydric Alcohol	15.42	1905	319	01.08	14.4	1.73
130	Unknown_15.64_1941_073	Unknown	15.64	1941	073	00.72	27.7	2.04
134	Unknown_16.05_2006_204	Unknown	16.05	2006	204	00.72	16.9	2.73
135	Inositol 6TMS	Polyhydric Alcohol	16.08	2011	217	02.17	18.1	4.91
136	Palmitoleic acid 1TMS	Fatty Acid	16.19	2027	117	14.44	21.7	5.07
138	Unknown_16.47_2072_204	Unknown	16.47	2072	204	09.03	26.1	2.76
139	*Myo*-Inositol 6TMS	Polyhydric Alcohol	16.51	2078	217	03.97	12.7	2.31
147	Linoleic acid TMS	Fatty Acid	17.35	2212	081	02.17	18.0	3.75
148	Tryptophan 3TMS	Amino acid	17.39	2220	202	01.81	13.8	3.58
157	Unknown_18.26_2387_091	Unknown	18.26	2387	091	01.44	21.8	2.29
161	Unknown_18.96_2519_427	Unknown	18.96	2519	427	12.27	21.4	5.44
174	Unknown_20.72_2855_221	Unknown	20.72	2855	221	00.36	29.2	1.78
178	Gastrodigenin 2TMS	Benzyl Alcohol	21.30	2966	221	07.58	17.9	1.90
182	Unknown_22.47_3190_329	Unknown	22.47	3190	329	06.14	24.3	2.11
183	Unknown_22.47_3190_107	Unknown	22.47	3190	107	09.03	05.4	2.02

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