Relevance of metabolic profiles in children with cardiac disease

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Abstract

Fat is primarily a way to store energy. Fat in the form of fatty acids combines with carnitine to form acylcarnitines and these are transported into the cell's mitochondria where the fatty acids are released and metabolised to provide energy. Acylcarnitines can be measured in blood and this reflects concurrent fat metabolic status. This acylcarnitine test, along with several amino acids and endocrine markers is performed in all babies born in New Zealand as part of a routine newborn screening test enabling diagnosis of rare genetic metabolic disorders. This data is stored in the newborn metabolic screening laboratory database at Auckland City Hospital.

The heart has high energy requirements and fat is the main fuel. The failing heart results in disruption to fatty acid metabolism. It has been recently reported that adults with heart failure have plasma acylcarnitine profiles that differ from the normal population with higher levels of long chain fatty acid derivatives.

Babies with severe congenital heart disease can present to hospital in the first two weeks of life. Most of these cases are seen at Starship Children's Hospital in Auckland. Many of these babies will have stressed myocardium. It is possible that these babies will, like their adult counterparts, have abnormal fat metabolism that can be demonstrated by an abnormal acylcarnitine profile.

In addition to congenital heart lesions, the acylcarnitine profiles of children with cardiomyopathy were also reviewed. These children are often admitted to Starship Children's Hospital and have had full metabolic investigations as part of their diagnostic work-up.

The aim of the study was to compare the stored data of the early postnatal metabolic profiles of children with congenital cardiac disease and healthy controls to establish if there is a pattern of metabolites that is similar to that seen in adults with cardiac failure.

The findings highlighted elevation of several short and medium chain acylcarnitines indicative of defects in activities of some enzymes and/or transporters in fatty acid metabolic pathways. The study also identified alterations in blood levels of several amino acids, and endocrine markers. However, this study did not observe difference in long chain acylcarnitine levels reported in adult heart failure. This could be partially due to the general anabolic status of the children cohort.

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Attestation of Authorship

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

Sandra Divanisova
March 2023

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Ethics Approval

Full ethics approval was granted by the AUTEC committee on the 23rd November 2020, application number 20/360.
The Health and Disability Committee (HDEC) approved the ethics application on the 5th October 2020, HDEC ref 20/NTB/167.
Auckland District Health Board Institutional approval was granted on the 23rd October 2020.
The approval letters are attached in Appendix B.

1.0 Literature Review

1.1 Background

The heart muscle is one of the most metabolically active tissues and there is a strong link between myocardial metabolism and cardiac function. Every 13 seconds the heart uses an entire supply of adenosine triphosphate (ATP) utilising mainly free fatty acids as substrates (Piquereau & Ventura-Clapier, 2018). In order for oxidation to occur, long chain fatty acids are transported in the form of acylcarnitines into the mitochondria by a process known as the carnitine cycle (McCoin, Knotts, & Adams, 2015). In addition, amino acids play an essential role in the growth and function of the cardiac tissue (Huang et al., 2011).

The failing cardiac muscle is less efficient at fatty acid oxidation and compensates by increasing energy production from an alternative substrate, glucose. Mitochondrial dysfunction seen in progressive cardiac failure may be reflected by the accumulation of blood long chain acylcarnitines and amino acids (Hunter et al., 2016; Lai et al., 2014; Ruiz et al., 2017). The Auckland metabolic laboratory and the National Metabolic Service clinical team who meet weekly to discuss national cases of potential inborn error of metabolism have noted a number of characteristically elevated acylcarnitine profiles in children with known cardiac disease. These profiles don't reflect any particular genetic metabolic disease but could reflect an abnormality in fat metabolism in patients with cardiac disease.

Congenital heart defects are the most common inherited malformations, with an incidence of between four and 10 per 1,000 live-born infants (Egbe et al., 2014). Only 46% of New Zealand born babies with critical cardiac defects are detected using antenatal mid-trimester anatomy scan. Concerningly, twenty percent of critical cardiac defects are currently diagnosed after discharge from hospital. Late diagnosis can result in hypoxaemia, shock, acidosis and death and it is estimated that four infants die each year in New Zealand as a result of late-diagnosed congenital heart defect (Eckersley et al., 2016). Early detection of heart disease before or immediately after birth is therefore of high importance (Cloete et al., 2019).

The aim of this study is to compare stored data from newborn screening metabolic profiles of babies who have both known and, at the time, unknown neonatal cardiac disease with the normal population. While the study will review the overall cohort, it will also attempt to divide it into broad cardiac categories in order to investigate possible associations between particular cardiac pathologies (outflow track obstruction, high flow lesions, hypoxic lesions) and their metabolic profiles. It will also analyse the profile of children who present later in life to Starship

Children's Hospital with cardiomyopathy. The review will shed light on the metabolic processes that occur in the neonatal congenital heart and childhood cardiomyopathic patients.

1.2 Cardiac metabolism in the healthy heart

The healthy adult heart beats 100,000 times and pumps approximately 7500 litres of blood each day. After blood has travelled through the body, lowering in oxygen, it returns to the heart via the vena cava to the right atrium. From here the blood flows into the right ventricle where it is pumped to the lungs through the pulmonary arteries where it reoxygenates. This oxygenated blood then returns to the left chambers and there it is pumped back out to the body through the aorta (Figure 1) (Mesotten et al., 1998). This extraordinary amount of work requires a constant supply of metabolic substrates along with a large amount of oxygen provided produced by adenosine triphosphate (ATP). The human heart utilises approximately 6kg of ATP daily to support its contractile function. This is 15 times its own weight and results in a nearly complete turnover of the myocardial ATP pool every 10 seconds (Towbin & Jefferies, 2017). The heart consumes more than 30g of fat and 20g of carbohydrates and nearly 35 litres of oxygen every day (Ussher et al., 2016).

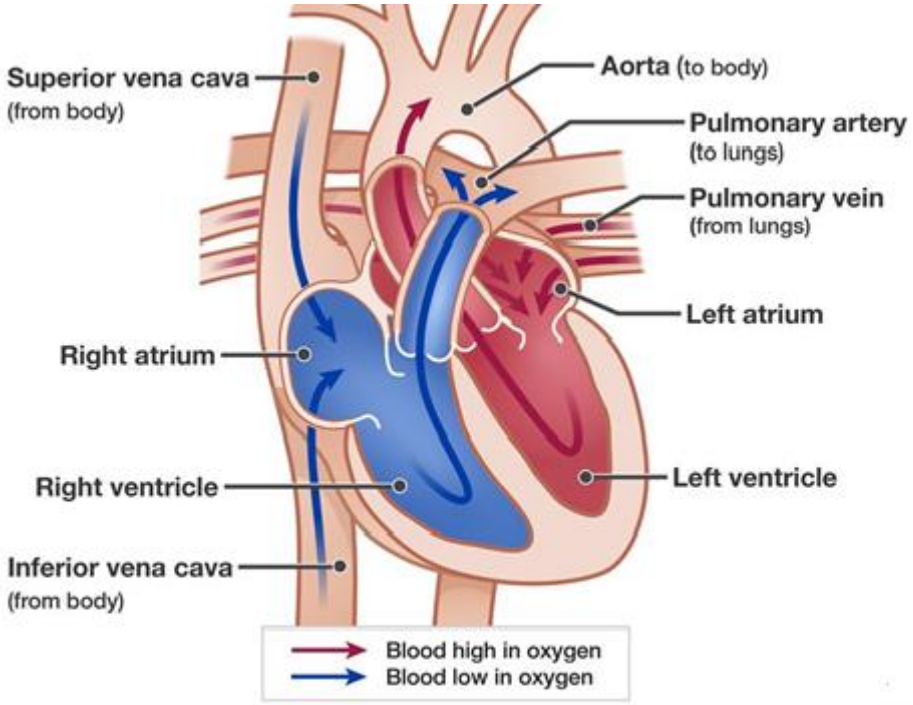

Figure 1. Diagram of the heart muscle.

The heart has two upper (receiving) atrium chambers and two lower (pumping) ventricles. The veins carry blood low in oxygen (blue) into the heart. The arteries carry blood rich in oxygen (red) from the heart to the rest of the body.

Retrieved from https://www.aboutkidshealth.ca/Article?contentid=1577&language=English

1.2.1 Mitochondria and ATP synthesis

Due to the high demand of constant ATP production by oxidative metabolism, the cardiac cells have the highest density of mitochondria, occupying one third of the cell volume of the adult cardiomyocyte in the body. Mitochondria are composed of an outer membrane, an intermembrane space, an inner mitochondrial membrane, and the mitochondrial matrix. The outer membrane is permeable to a variety of small molecules, and the inner membrane is permeable to specific ions only (oxygen, carbon dioxide, and water). The inner membrane forms multiple invaginations known as cristae (Towbin & Jefferies, 2017). The mitochondrion not only produces >95% of ATP for the heart, but also regulates intracellular calcium homeostasis, signalling and cell apoptosis (Kolwicz et al., 2013).

1.2.2 Substrate utilisation and fatty acid oxidation

The heart has limited substrate storage capacity and therefore the balance between substrate demand and oxidation is crucial to its normal function. Almost 70% to 80% of ATP is derived from mitochondrial fatty acids oxidation and thus this membrane-bound cell organelle is often referred to as the powerhouse of the cell. The remaining 20% to 30% is derived from the oxidation of glucose and lactate with a minor contribution from ketones and amino acid oxidation (Sacchetto et al., 2019).

The primary energy source for the healthy human heart is plasma free fatty acids (FFA) bound to albumin (Piquereau & Ventura-Clapier, 2018). The other source is fatty acids derived from hepatic triacylglycerol (TAG) containing very low density lipoproteins (VLDL) and dietary chylomicrons. The normal range for circulating FFA is from 0.2 to 0.6 mM, except in fetal circulation they are found at very low concentration (Lopaschuk et al., 2010). Long chain fatty acids which constitute the major fraction of dietary fatty acids cannot enter the mitochondrion by simple diffusion. To undergo oxidation for energy production in the mitochondria, long chain fatty acids are transported in the form of acyl-coenzyme A (CoA) esters by the ATP-dependant acyl-CoA synthetases (McCoin et al., 2015) (Figure 2).

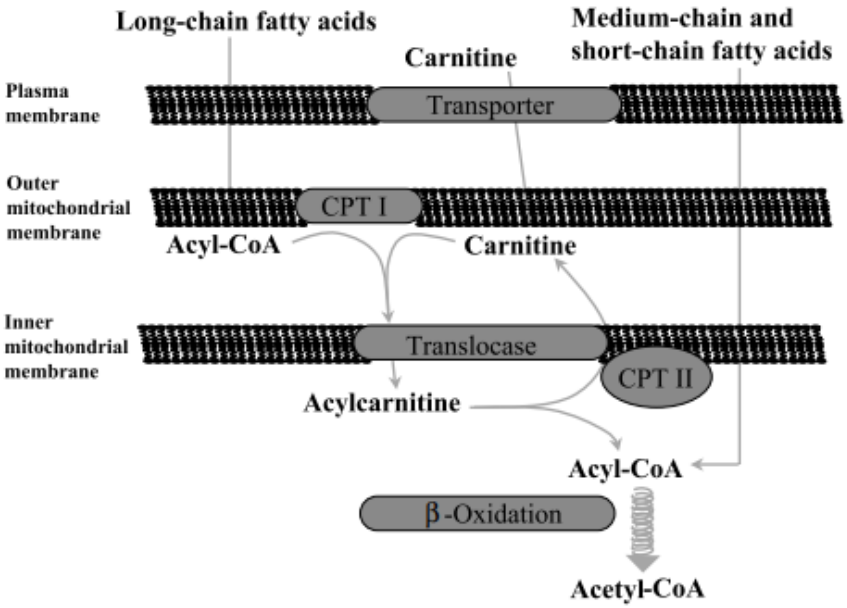

Figure 2. Transport of long chain fatty acids into mitochondria (Guertl et al., 2001).

These acyl-CoA esters are then converted into acylcarnitines and free CoA by carnitine palmitoyl transferase (CPT I) in the outer mitochondrial membrane. The acylcarnitines are then transferred across the inner mitochondrial membrane by the carnitine:acylcarnitine translocase (CACT) transporter protein in exchange for free carnitine. After crossing the mitochondrial inner membrane, the acyl moiety is considered to be committed to complete the oxidation process. The acyl-CoA ester is reformed by the CPTII enzyme (carnitine palmitoyl transferase II) located at the inner mitochondrial membrane (Eaton et al., 1996). The even numbered acyl-CoA fatty acids then undergo β-oxidation to produce the 2-carbon molecule acetyl coenzyme A (acetyl-CoA) which enters the tricarboxylic (TCA) cycle (also known as the Krebs cycle) promoting the synthesis of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) and therefore providing source of electrons for the electron transport chain (ETC) at the inner mitochondrial membrane (Figure 3). This process drives the proton motive force that fuels ATP synthesis (Ussher et al., 2016).

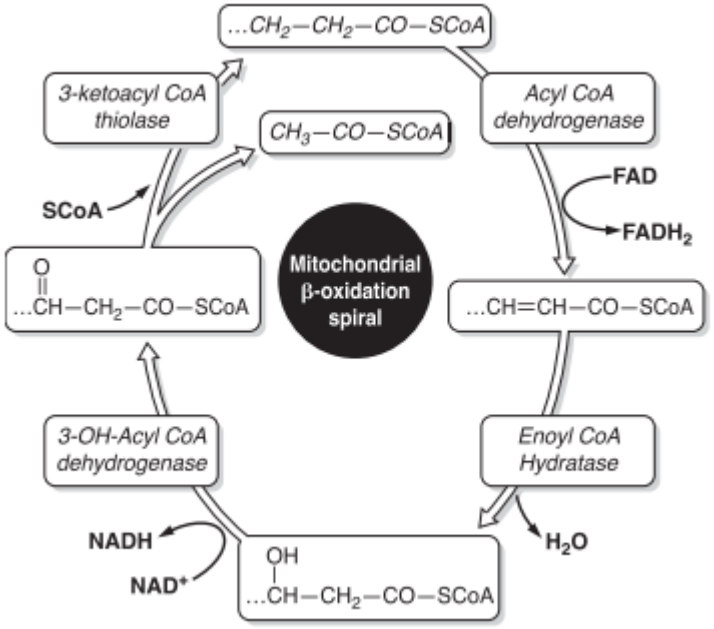

Figure 3. Fatty acid oxidation (FAO) in the heart
The β-oxidation cycle involves four enzymes: i) acyl CoA dehydrogenase ii) Enoyl CoA hydratase iii) 3-hydroxy acyl CoA dehydrogenase iv) 3-ketoacyl CoA thiolase. One cycle results in the synthesis of acetyl-CoA (enters the TCA cycle) and a fatty acyl chain which is two carbons shorter (Lopaschuk et al., 2010).

The mitochondrial β-oxidation enzymatic machinery continues to remove acetyl-CoA from the fatty acyl ester until it is completely oxidised. As a result, a completely oxidised fatty acid molecule, for example oleate or palmitate, generates more acetyl-CoA, NADH and $FADH_2$ and thus much more ATP than the complete oxidation of a glucose molecule (104 ATP from palmitate vs. 31 ATP from glucose oxidation) (Ussher et al., 2016).
The second major fuel of respiration of the heart is carbohydrates, mainly glucose and lactate. When the glucose molecule enters the cell, it undergoes anaerobic glycolysis to produce pyruvate and 2 ATP molecules. Pyruvate derived from glucose and/or lactate enters the mitochondria where it gets converted to acetyl-CoA by pyruvate dehydrogenase. This acetyl-CoA is utilised by the TCA cycle to produce NADH and $FADH_2$ to aid in ATP production. The breakdown of glucose into ATP is called glucose oxidation of glycolysis (Ussher et al., 2016).

1.2.3 Mitochondrial electron transport chain

The mitochondrial electron transport respiratory chain is located in the inner mitochondrial membrane and this is where the ATP is produced. The inner membrane acts as a barrier to positively charged protons resulting in an electrochemical gradient between the matrix containing fewer protons and the intermembrane space with more protons. The inner membrane

also contains a large protein complex called ATP synthase (Complex V) which uses this proton gradient energy to synthesise ATP. These ATP molecules ultimately provide the energy for most of the cell's reactions. If there was no proton flow from one side of the membrane to the other, to rotate the protein subunits, the cell will starve for energy and eventually die. The energy to maintain this electrochemical gradient comes from the oxidative phosphorylation chain. This consists of four complexes I through IV. While complex II doesn't directly pump protons, it promotes proton pumping in complexes III and IV. Complexes I, III and IV directly pump protons from the membrane into the mitochondrial intermembrane space. This proton pumping energy comes from transferring electrons obtained from β-oxidation through complexes I to IV. This is known as the mitochondrial electron transport chain (Figure 4) (Sacchetto et al., 2019).

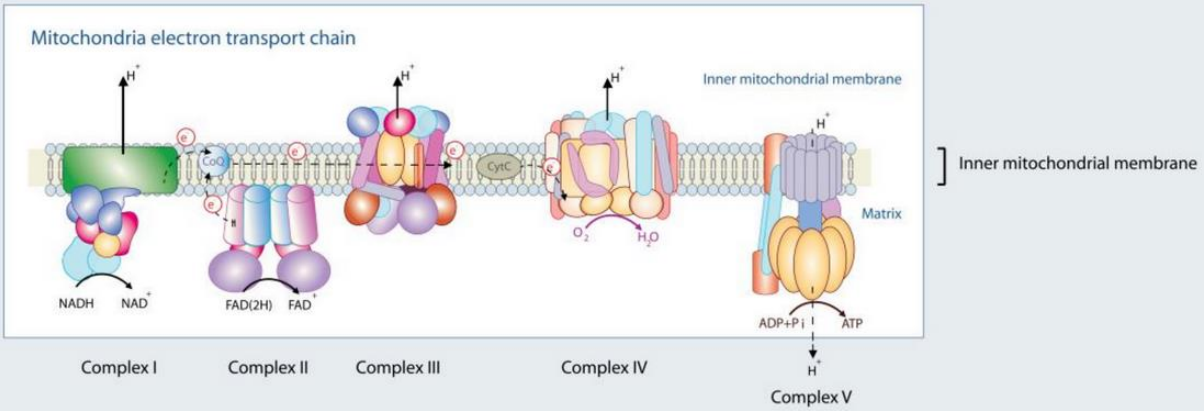

Figure 4. Mitochondrial respiratory chain (Sacchetto et al., 2019)

The mitochondrial respiratory chain consists of 5 multi-polypeptide enzyme complexes:

i) **Complex I**- NADH, a by-product of glucose metabolism deposits 2 high energy electrons passed along a chain of Redox centres (atomic clusters with different affinities to electrons). Energy is released each time the electron is passed between two redox centres. The last redox centre donates 2 electrons to a Coenzyme Q (CoQ) molecule.

ii) **Complex II** – 2 high energy electrons enter the complex via another glucose metabolism by-product, flavin adenine dinucleotide. The electrons are transferred between several redox centres before being donated to CoQ. Complex II doesn't use the energy liberated to pump protons.

iii) **Complex III** – CoQ molecules from complexes I and II donates their electrons to complex III. One electron is recyclable and re-enters complex III later. The other electron is transferred between two redox centres before reaching cytochrome C (CytC) which carries it to complex IV.

iv) **Complex IV** – The electron transport chain ends in this complex where a sequence of reactions involving four electrons converts a molecule of oxygen to 2 molecules of water

v) **Complex V** – ATP synthase drives the production of ATP (Sacchetto et al., 2019).

1.3 Cardiogenesis and foetal development

Cardiogenesis is a highly orchestrated cascade of complex molecular and morphogenetic events combining cells from multiple lineages. Slight perturbations of this process can culminate in life-threatening congenital heart defects (CHD). Being life essential, the heart is the first organ to form to support the rapidly growing embryo. Prior to becoming a four chambered organ, the heart is a simple tubular pump that starts beating around day 23 of embryogenesis and pumps blood in week four. Rightward looping of the heart tube is the initial sign of the break in left-right symmetry in the embryo. Irregularities in the process of cardiac looping are the underlying cause of some CHD. The segments of the primitive looped heart are in a liner pattern and a considerable repositioning is necessary for alignment of the atrial chambers with the appropriate ventricles and also the aorta and pulmonary arteries (Srivastava, 2011). The foetal heart mainly uses glucose and lactate as their concentration in foetal blood is similar to those in newborns and adults (Figure 5). In comparison, the concentrations of fatty acids are very low. The low oxygen and high glycolytic metabolic phenotype enhances the biosynthesis of macromolecules such as cellular lipids, amino acids and nucleotides required for cell growth (Ritterhoff & Tian, 2017).

1.3.1 Neonatal heart

The heart undergoes a metabolic transformation while transitioning from foetal to neonatal life due to exposure to an increased haemodynamic load and oxygen tension. The cardiomyocytes hypertrophy and increase in size and mass. In addition, the intensification of mitochondria biogenesis increases the oxidative capability. In the first week postnatally, the levels of circulating glucose and lactate fall, while the concentration of FFA and TAG increase significantly. As a result, the heart relies on FAO as a major source of ATP synthesis. This metabolic shift is not only due to substrate availability but also by increasing oxygen levels and enhanced heart workload (Ritterhoff & Tian, 2017).

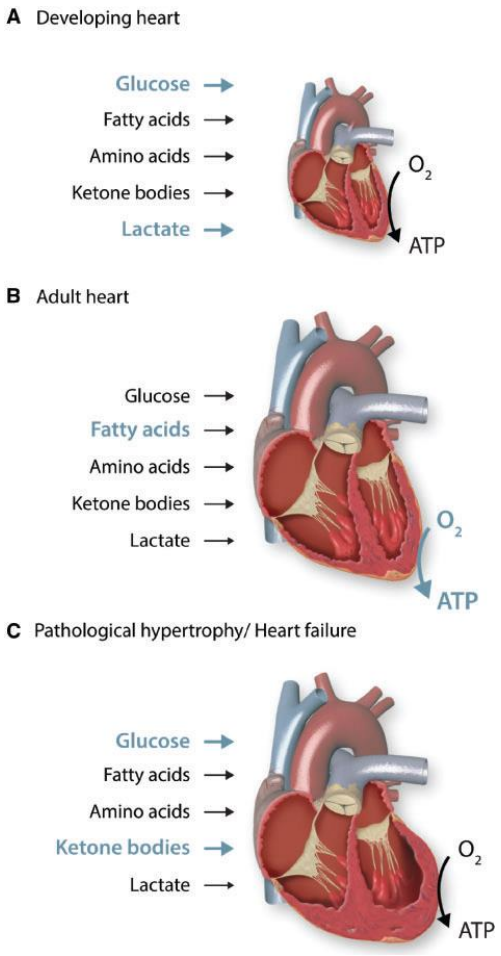

Figure 5. Substrates preference changes in cardiac development and in heart disease
A) In the development phase, the heart greatly relies on aerobic glycolysis and lactate oxidation. This metabolic profile enables biosynthesis of fundamental macromolecular components necessary for cellular proliferation and growth. B) The adult heart is exposed to an enhanced blood flow dynamics and oxygen tension. Fatty acids become the main energy substrate for the mature hearts. C) Heart failure is characterised by a change in fuel metabolism from fatty acids to foetal metabolic profile with increased reliance on glucose. In advanced heart disease, increased use of ketone bodies has been suggested (Ritterhoff & Tian, 2017).

1.4 Metabolic alterations in heart disease

Foetal life evolves in a hypoxic environment with low oxygen saturation in the intrauterine period. After birth, oxygen saturation gradually increases within the first minutes of life (Piquereau & Ventura-Clapier, 2018). However, congenital cardiac defects can lead to low levels of oxygen saturation, volume and pressure overload, and pulmonary hypertension. In neonates with heart defects, cyanosis happens if the defect allows blood low in oxygen from the right side of the heart to enter the left side directly, instead of travelling to the lungs for oxygen. In the left side of the heart, this oxygen-poor blood combines with oxygen-rich blood to be

pumped back out to the body. These low blood oxygen saturation levels may adversely affect these vulnerable neonates (Modi et al., 2006). While metabolic remodelling is an integral part of the pathogenesis of cardiac disease, acute or chronic hypoxia might cause alterations in the metabolic pathways. A striking change is in the shift in substrate metabolism from FAO to glucose and an overall reduced capacity for oxidative metabolism and energy reserve (Figure 5C) (Ritterhoff & Tian, 2017). This may lead to mitochondrial dysfunction reflected by the accumulation of blood long chain acylcarnitines and amino acids detected in adults with progressive cardiac failure. A global perturbed metabolic phenotype has been highlighted in a previous adult study. While these patients had only subtle changes in metabolites reflecting mitochondrial redox status, elevation of all chain length acylcarnitines species in plasma samples indicated a systemic impairment of mitochondrial FAO (Ruiz et al., 2017). The imbalance between the requirement of the myocardium for oxygen and metabolic supplies and their availability, leads to functional, metabolic and morphologic alteration of the cardiac tissue. At cardiomyocyte level, the glucose uptake is decreased and conversion to lactate is increased. Pyruvate is also converted into lactate, thereby increasing cell acidosis. The FAO pathway is also slowed down resulting in less ATP production. All cardiac behaviours are highly ATP-dependent and without ATP, these metabolic perturbations lead to disruption of cell homeostasis, decreased oxygen supply to cardiomyocites, impaired vascularisation, ion imbalance, alterations in membrane structure and ultimately cell death (Hunter et al., 2016; Lai et al., 2014).

Recent reports suggest increased ketone body oxidation in experimental animal models and in human heart failure (Aubert et al., 2016). In addition, amino acid metabolism and in particular branched-chain amino acid (BCAA) catabolism is deranged in pathological cardiac hypertrophy leading to an accumulation of BCAAs. Although the contribution of these substrates to ATP production is limited in the normal heart, their biological role as part of these metabolic pathways in cardiac remodelling warrants further investigation (Huang et al., 2011).
The key metabolites measured in blood circulation within metabolic pathways act as sentinels to reflect changes within a metabolic process are shown in Table 1 (Ussher et al., 2016)

Table 1. Circulating key metabolites measured in heart failure

Metabolite	**Metabolic Perturbation**
Lactate	Glucose metabolism
Leucine, Isoleucine, Valine	BCAA metabolism
Acylcarnitines	Fatty acid oxidation
3-hydroxybutyryl carnitine β-hydroxybutyrate	Ketone body oxidation

1.5 Newborn Bloodspot Screening

1.5.1 Acylcarnitines

Carnitine (3-hydroxy-4-N-trimethylamino butyrate) is a small (molecular weight=161.2 g/mol), water soluble, quaternary nitrogen-containing molecule (Figure 6) synthesized in mammalian tissue in both L- and D- stereoisomer forms, L-carnitine being the biologically active form (Durazzo et al., 2020). It is primarily synthesized in the liver and transported to cardiac and skeletal muscles which are dependent on carnitine for fatty acid oxidation yet unable to synthesize it. Although the human body can synthesize carnitine from the two essential amino acids, lysine and methionine, approximately two thirds of the daily requirements originate from diet, mainly red meats and dairy products. Healthy individuals maintain carnitine homeostasis via endogenous biosynthesis, absorption from exogenous dietary sources and renal reabsorption (Rebouche & Paulson, 1986).

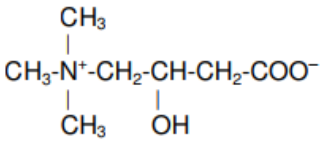

Figure 6. The structural formula of carnitine C7H15NO3 (Durazzo et al., 2020)

In 1955, Friedman and Frankel demonstrated that carnitine can be reversibly acetylated by acetyl-CoA (Friedman & Fraenkel, 1955). In 1959, Fritz observed that carnitine stimulates fatty acid oxidation in liver homogenates, a source of cellular energy in skeletal and heart muscles (Fritz, 1959). These studies led to the finding that carnitine is involved in the transport of long chain fatty acids from the cytosol, across the mitochondrial membranes, towards the mitochondrial matrix where β-oxidation takes place. In 1973, carnitine received further attention when the first carnitine deficient patient was described who required carnitine supplementation to maintain normal energy metabolism (Borum, 1983).

The formation of free carnitines and acylcarnitines in the cell also buffers the potentially toxic build of acyl-CoA metabolites and maintains the optimal activity of mitochondrial TCA cycle-related enzymes. Unlike acyl-CoAs, the acylcarnitines can be transported through the mitochondrial membrane out of the cell to the blood circulation and thus reflect the intracellular pool of the corresponding acyl-CoAs (Ruiz et al., 2017). Defects in oxidative enzymes can result in decreased combustion of fatty acids and accumulation of acylcarnitines (McCoin et al., 2015). Profiles of acylcarnitines and aminoacids in the blood is commonly used as markers of inherited diseases of fatty acid and amino acid metabolism (Rinaldo, Cowan, & Matern, 2008). Recent literature review suggests that over 1000 acylcarnitines have been identified in mass spectrometry-based experiments (Dambrova et al., 2022). However, most commonly measured

acylcarnitine species by clinical laboratories are listed in Table 2 below (Rinaldo et al., 2008). The classification of acylcarnitines is usually based on chemical structure of the variable acyl moiety in their molecule or the length of the carbon chain. Therefore, the acylcarnitines are categorised into four groups: short chain (C2-C5), medium chain (C6-C12), long chain (C13-C20) and very long chain (>C21). Also, chemical groups (e.g., hydroxyl- or carboxyl groups) can substitute the fatty acid moiety in the acylcarnitine, for instance branched dicarboxylic acylcarnitines (3-methylmalonylcarnitine) and long chain hydroxyl acylcarnitines (3-hydroxytetradecenoylcarnitine) (Dambrova et al., 2022).

Table 2. List of common acylcarnitine species

Acylcarnitine	**Chain length**
Free carnitine	-
Acetyl-	C2
Acrylyl-	C3:1
Propionyl-	C3
Formiminoglutamate	-
Isobutyryl-/butyryl-	C4
Tyglyl-	C5:1
Isovaleryl-/2-methylbutyryl-	C5
3-hydroxybutyryl-	C4-OH
Hexanoyl-	C6
3-hydroxyisovaleryl-2-methyl-3-hydroxybutyryl-	C5-OH
Benzoyl	-
Heptanoyl-	C7
3-Hydroxyhexanoyl-	C6-OH
Phenylacetyl-	-
Salicylyl-	-
Octenoyl-	C8:1
Octanoyl-	C8
Malonyl-	C3-DC
Decadienoyl-	C10:2
Decenoyl-	C10:1
Decanoyl-	C10
Methylmalonyl-/succinyl-	C4-DC
3-Hydroxydecenoyl-	C10:1-OH
Glutaryl-/3-hydroxydecanoyl	C5-DC/C10-OH
Dodecenoyl-	C12:1
Dodecanoyl-	C12
3-hydroxydodecenoyl-	C12:1-OH
3-hydroxydodecanoyl-	C12-OH

Tetradecadienoyl-	C14:2
Tetradecenoyl-	C14:1
Tetradecanoyl- (myristoyl-)	C14
3-hydroxytetradecenoyl-	C14:1-OH
3-hydroxytetradecanoyl-	C14-OH
Hexadecenoyl-	C16:1
Hexadecanoyl- (palmitoyl-)	C16
3-hydroxyhexadecenoyl-	C16:1-OH
3-hydroxydecanoyl-	C16-OH
Octadecadienoyl-	C18:2
Octadecenoyl-	C18:1
Octadecanoyl- (stearyl-)	C18
3-hydroxyoctadecadienoyl-	C18:2-OH
3-hydroxyoctadecenoyl-	C18:1-OH
3-hydroxyoctadecanoyl-	C18-OH

1.5.2 Amino acids

Amino acids are the building blocks of polypeptides and ultimately proteins. They are organic molecules that contain both a carboxylic acid (-COOH) and an amino ($-NH_2$) functional group (Figure 7) (Lopez & Mohiuddin, 2022).

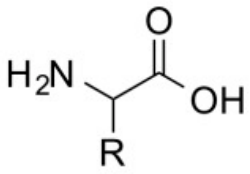

Figure 7. Amino acid generic structure

Proteins are long chains of amino acids linked via peptide bonds. Proteins consist of twenty different amino acids, eleven of each are synthesized endogenously (nonessentials) and nine are acquired from dietary source (essentials) (Table 3). The different R- or chain group determines the unique properties of each amino acid. In addition to being the substrates for protein synthesis, they also serve diverse physiologic functions including regulators of protein turnover, regulators of enzyme activity, transporter of nitrogen, and gluconeogenic substrates and fuel. As well as carrying out these functions, amino acids are also metabolised for energy with their carbon chain being oxidised to produce ATP and carbon dioxide (CO_2). Catabolism of the amino group can produce potentially toxic ammonia but this is broken down via the urea cycle to produce urea and thus nitrogen is excreted in the urine. These losses must be replenished by either synthesis from metabolic intermediates or through diet (Furst, 2008).

Table 3. Classification of amino acids

Essential amino acids	*Nonessential amino acids*
• Valine	• Alanine
• Isoleucine	• Arginine
• Leucine	• Asparagine
• Lysine	• Aspartic acid
• Methionine	• Cystine
• Phenylalanine	• Glutamic acid
• Threonine	• Glycine
• Tryptophan	• Ornithine
• Histidine	• Proline
	• Serine
	• Tyrosine

Unlike most amino acids, the branched chain amino acids (valine, leucine and isoleucine) are initially catabolised outside the liver due to low hepatic activity of the first enzyme (mitochondrial aminotransferase or BCATm) in the BCAA metabolic pathway (Holecek, 2018). During fasting, the first step in BCAA catabolism involves transfer of amino groups to alpha ketoglutarate, followed by amino group transfer to pyruvate producing alanine. Alanine is exported to the liver to support hepatic gluconeogenesis (step 1, Figure 8). In the mitochondria, BCAT enzyme converts the branched-chain amino acids into branched chain α-keto acids (α-ketoisocaproate from leucine, α-ketoisovalerate from valine, and α-keto-β-methylvalerate from isoleucine) which are then oxidised by the branched chain α-keto acid dehydrogenase (BCKD) complex. The BCKD complex is inhibited by BCKD kinase–mediated phosphorylation and increased concentrations of BCAA ketoacids and activated by dephosphorylation catalyzed by protein phosphatase PP2Cm. During fasting, enhanced BCAA catabolism can lead to formation of precursors for the biosynthesis of ketone bodies (step 2, Figure 8). Eventually, the BCAA are metabolised to acetyl-CoA and succinyl-CoA, both substrates for the TCA cycle (step 3, Figure 8) (Kujala et al., 2016).

The branched chain amino acids are mainly catabolised in skeletal and cardiac muscle, renal tissue and the neurons. Their levels rapidly surge in systemic circulation after protein intake and are quickly distributed to muscle tissues and the brain (Holecek, 2018).

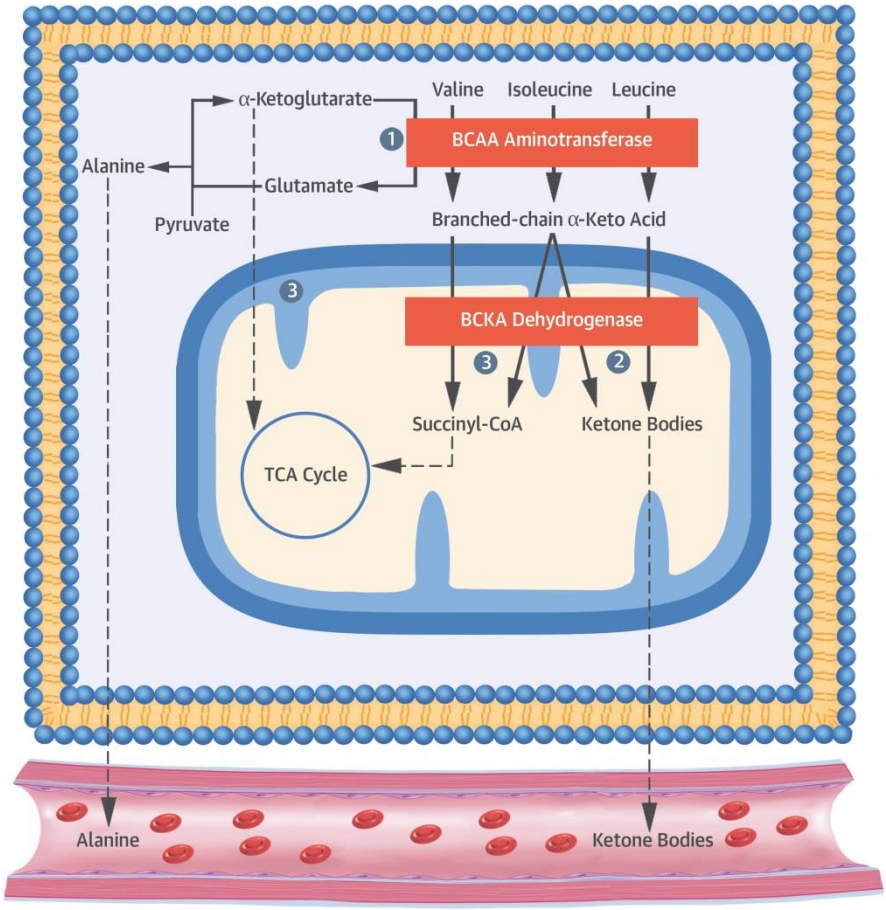

Figure 8. Schematic presentation of BCAA catabolism (Ussher et al., 2016)

1.5.3 Disorders detected through the Newborn Screening Programme

The Newborn bloodspot screening programme is a key and successful public health initiative for the early detection of rare, congenital, and life-threatening diseases. It focuses on disorders for which early recognition enables timely intervention that prevents or minimises irreversible health damage. In New Zealand, newborns are screened for twenty four conditions (Table 4) (Ministry of Health, 2022). Since its beginnings in the 1960s with screening for phenylketonuria, technology innovations have accelerated expansion of newborn screening programmes. A classic example is the introduction of tandem mass spectrometry (MS/MS) which facilitated multiplex biochemical analyses of a number of metabolic disorders (Jansen et al., 2021) .

In New Zealand, simultaneous MS/MS testing of amino acid and acylcarnitines in dried blood spot samples as part of newborn screening began in 2006 (Wilson et al., 2007). Almost all babies born in New Zealand have a heel prick test done at day two of life. This test is a potent tool for the early detection of eighteen metabolic disorders in high-risk newborns. Screening is based on cut-off points for the acylcarnitine and aminoacid metabolites which are trade-offs

between a high detection rate of affected neonates (known as sensitivity) and an acceptable false-positive rate (known as specificity) and represent very high percentiles (e.g. 99.5th or 99.9th). Screening cutoffs do not necessarily indicate reference ranges that can be applied for diagnosis of symptomatic patients (Neto et al., 2012).

Table 4. List of disorders and associated metabolites measured in newborns

Amino Acid Breakdown Disorders		**Metabolites**	
1	Phenylketonuria	Phenylalanine, Tyrosine	Amino acid
2	Maple syrup urine disease	Leucine, Valine	Amino acid
3	Argininosuccinic aciduria (argininosuccinate lyase deficiency)	Citrulline	Amino acid
4	Citrullinaemia (argininosuccinate synthetase deficiency)	Citrulline	Amino acid
5	Glutaric acidaemia type I (glutaryl-CoA dehydrogenase deficiency)	Glutarylcarnitine (C5DC)	Acylcarnitine
6	Homocystinuria (cystathionine beta-synthase deficiency)	Homocysteine, Methionine	Amino acid
7	Isovaleric acidaemia (isovaleryl-CoA dehydrogenase deficiency)	Isovalerylcarnitine (C5)	Acylcarnitine
8	Methylmalonic acidurias (mutase deficiency, CblA, CblB, CblC, CblD defects)	Methylmalonyl carnitine (C3), acetylcarnitine (C2), free carnitine (C0) C3/C2 ratio*, C3/C0 ratio*	Acylcarnitine
9	Propionic acidaemia (propionyl-CoA carboxylase deficiency)	C3, C3/C2 ratio*, C3/C0 ratio*	Acylcarnitine
10	Tyrosinemia Type I	Succinylacetone	Protein
Fatty Acid Oxidation Disorders		**Metabolites**	
1	CACT (carnitine acylcarnitine translocase deficiency)	Hexanoylcarnitine (C16), Octadecenoyl carnitine (C18:1), Octadecanoyl (C18)	Acylcarnitine
2	CPT-I (carnitine palmitoyltransferase-I deficiency)	C16, C18 C0/(C16+C18)*	Acylcarnitine
3	CPT-II (carnitine palmitoyltransferase-II deficiency)	C16, C18:1 (C16+C18:1)/C2*	Acylcarnitine

4	LCHAD (3-hydroxy long-chain acyl-CoA dehydrogenase deficiency)	3-OH hexadecanoyl carnitine (C16OH)	Acylcarnitine
5	TFP (trifunctional protein deficiency)	C16OH	Acylcarnitine
6	MADD (multiple acyl-CoA dehydrogenase deficiency)	C5, Isobutirylcarnitine (C4), Hexanoylcarnitine (C6)	Acylcarnitine
7	MCAD (medium-chain acyl-CoA dehydrogenase deficiency)	Octanoylcarnitine (C8), Decanoylcarnitine (C10) C8/C10 ratio*	Acylcarnitine
8	VLCAD (very-long-chain acyl-CoA dehydrogenase deficiency)	Tetradecenoylcarnitine (C14:1), Tetradecanoylcarnitine (C14)	Acylcarnitine
Other Disorders		**Metabolites**	
1	Congenital Adrenal Hyperplasia (CAH)	17 – Hydroxy Progesterone (17OHP)	Endocrine marker
2	Congenital Hypothyroidism (CH)	Thyroid Stimulating Hormone (TSH)	Endocrine marker
3	Cystic Fibrosis (CF)	Immunoreactive Trypsinogen (IRT)	Pancreatic protein
4	Galactosemias	Total galactose	Carbohydrate
5	Biotinidase Deficiency	Biotinidase	Enzyme
6	Severe Combined Immunodeficiency (SCID)	T-cell receptor excision circles (TREC)	DNA fragments
*calculation of ratio between acylcarnitine species provides a discriminative factor for diagnosis			

1.6 Congenital cardiac defects

Congenital heart defects are the most common inherited malformations, with an incidence of between four and ten per 1,000 live-born infants (Egbe et al., 2014). Only 46% of New Zealand born babies with critical cardiac defects are detected using antenatal mid-trimester anatomy scan. Twenty percent of critical cardiac defects are diagnosed after discharge from hospital. Late diagnosis leads to hypoxaemia, shock, acidosis and death. It is estimated that four babies die each year in New Zealand as a result of late-diagnosed congenital heart defects (Eckersley et al., 2016). Early detection of heart disease before or immediately after birth is therefore of high importance (Cloete et al., 2019). There are some known genetic and environmental factors that can affect the development of heart defects, but the majority of

cases are caused by multiple factors with no specific identifiable trigger. Only about 15% of cases have an identifiable known cause. Maternal conditions such as phenylketonuria and insulin-dependent diabetes can cause congenital heart defects but overall these are uncommon. Other reported risk factors include maternal obesity, rubella infection, febrile illness, use of certain drugs and alcohol during pregnancy and exposure to organic solvents and lithium (Carmona et al., 2013).

There are many different types of congenital heart defect with the most common in this study being Ventricular Septal Defect (VSD), Tetralogy of Fallot (TOF), Transposition of the Great Arteries (TGA), coarctation of the aorta, pulmonary atresia and aortic arch hypoplasia.

1.6.1 Ventricular Septal Defect and Pulmonary Atresia

VSD is the most common congenital heart lesion. The cause is not well understood. VSD is a defect or hole in the wall that separates the heart ventricles (Figure 9). This results in too much blood flowing from the left to the right chamber and to the lungs. This increased flow can lead to cardiac failure and result in increased pulmonary blood pressure. Both complications can lead to difficulties with breathing and feeding, and failure to thrive. A similar presentation albeit often associated with cyanosis can be seen with pulmonary atresia. Here the pulmonary valve is abnormal and cannot be open fully thus restricting the blood flow from the right ventricle to the lung (Hoffman, 2011).

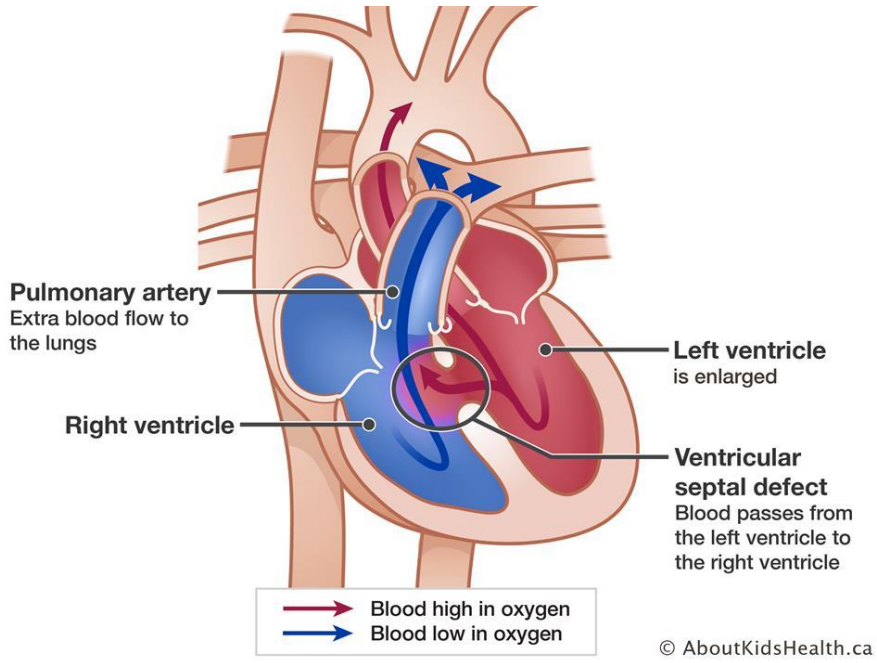

Figure 9. Diagram of a heart with VSD

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1.6.2 Tetralogy of Fallot

About 20-30% of all CHD are caused by defects involving the aortic arch, pulmonary arteries and the cardiac outflow tract. For example, with the CHD known as Tetralogy of Fallot there is a misalignment of the great vessels, the contrunctial septum and the aorta. This results in a ventricular septal defect. In addition, with the associated dysplastic pulmonary valve, the right ventricular muscle becomes hypertrophied. The aortic valve is enlarged and receives blood from both ventricles. This can result in inadequate blood to flow to the lungs and less oxygenated blood to travel to the body therefore causing cyanosis as illustrated in figure 10 (Hoffman, 2011).

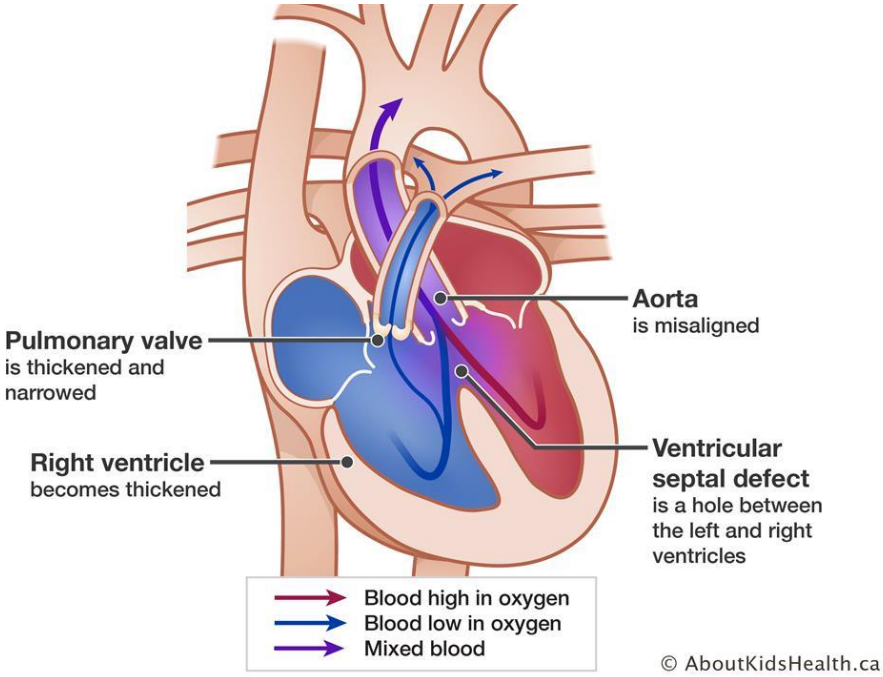

Figure 10. Diagram of a heart with Tetralogy of Fallot
Retrieved from https://www.aboutkidshealth.ca/Article?contentid=1621&language=English

Tetralogy of Fallot represents about 10% of the CHD cases and it is the most common form of cyanotic CHD who survive beyond infancy. Approximately one third of the patients begin to have hypoxic spells by 4 months of age, so elective surgery is normally performed within the first months of life. Treated patients usually survive into adulthood with good quality of life (Hoffman, 2011).

1.6.3 Transposition of the great arteries

This is a serious, rare heart disorder where the two main arteries leaving the heart are reversed. Opposite to the normal heart, the aorta is connected to the right ventricle and the pulmonary artery is connected to the left ventricle causing low oxygen blood to be pumped to the body without passing through the lungs (Figure 11). Survival until corrective surgery is dependent on collateral blood flow via a patent duct or a septal defect thus allowing mixing of the pulmonary and systemic systems (Hoffman, 2011).

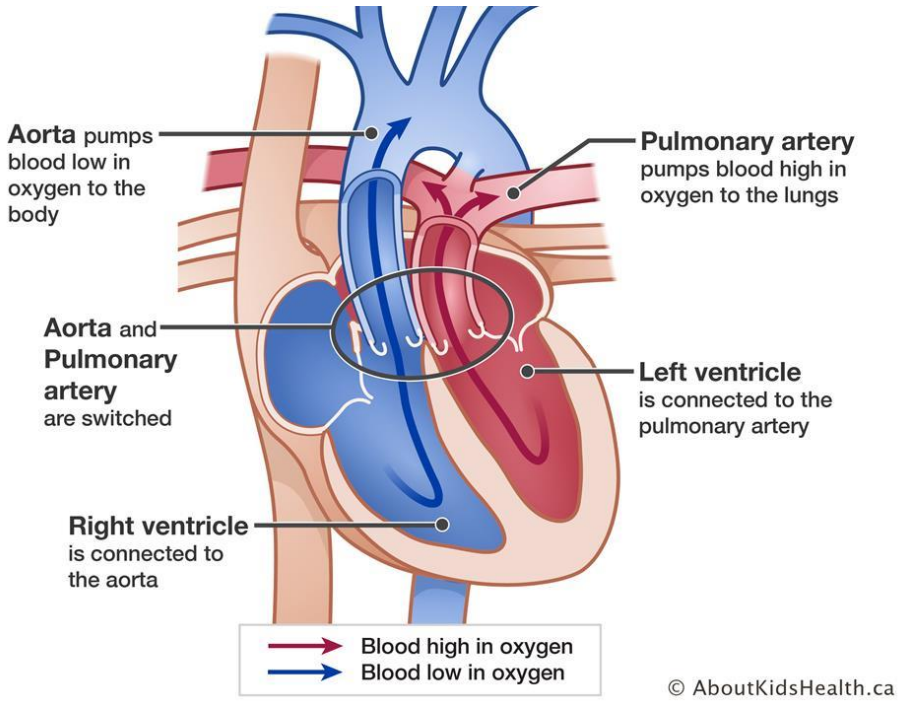

Figure 11. Diagram of a heart with TGA

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1.6.4 Coarctation and hypoplastic aortic arch

Aortic coarctation is narrowing of the aorta, the largest artery carrying oxygen-rich blood from the heart to the body. It forces the heart muscle to pump harder through the aorta. As a result, the blood pressure rises in the left ventricle causing hypertrophy. It can affect any part of the aorta, but most commonly near a blood vessel called the ductus arteriosus that connects the left pulmonary artery to the aorta (Figure 12). Unlike coarctation, in babies with aortic arch hypoplasia, a large segment of the aorta is obstructed with poor blood flow. This requires immediate surgery after birth (Hoffman, 2011).

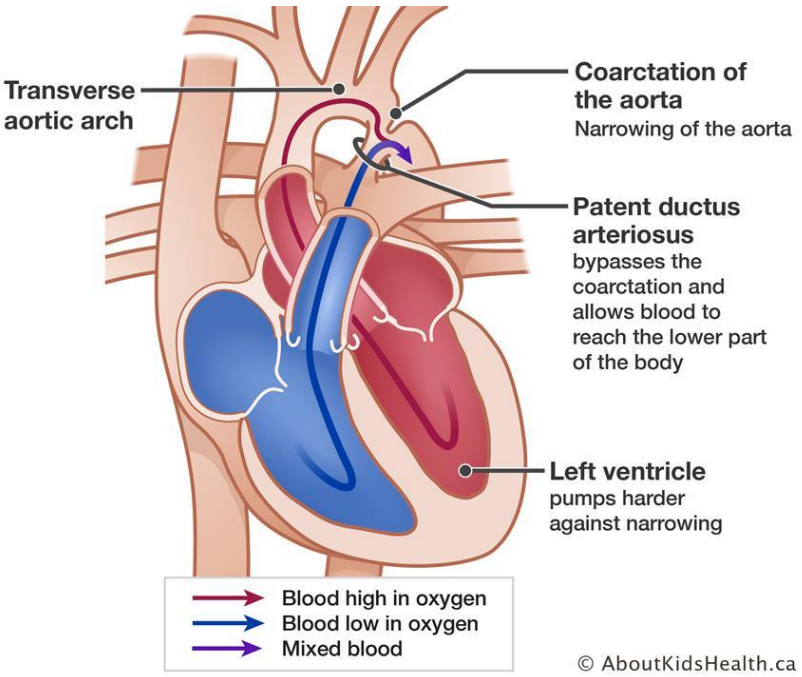

Figure 12. Diagram of a heart with coarctation of the aorta
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1.7 Paediatric Cardiomyopathy

In 1995, the World Health Organisation defined cardiomyopathy as "diseases of the myocardium associated with cardiac dysfunction" (Nordet et al., 1996). It is a rare disease in infants and children. The three subtypes are dilated, hypertrophic and restrictive. Dilated cardiomyopathy is characterised by dilatation or enlargement of the left and/or right ventricle due to heart's pumping dysfunction causing a limited ability to pump blood (Herath et al., 2015). Hypertrophic cardiomyopathy is characterised by abnormally thickened heart walls resulting in blood flow obstruction in and out of the heart (Trongtorsak et al., 2021). Restrictive cardiomyopathy is very uncommon in children. It is characterised by changes in the cardiac muscle walls becoming stiff and leading to impeded blood flow into the heart (Chintanaphol et al., 2022). Paediatric cardiomyopathy may be primary or secondary. Primary cardiomyopathy is due to a genetic variation usually in cardiac structural protein genes, cardiac arrhythmia abnormalities or caused by an external factor causing myocarditis such as virus, bacteria, or toxins. Conditions such as thyroid disease, diabetes, systemic lupus erythematosus, and metabolic disease like Pompe, Fabry, or mitochondrial disease that affect the cardiac muscle in addition to other organ systems are called a secondary cardiomyopathy (Lipshultz et al., 2019). The incidence of cardiomyopathy is approximately 1 in 100,000 children and it is the major indicator for heart transplantation during childhood (Bower et al., 1996; Lipshultz et al., 2019). Common symptoms of cardiomyopathy include dyspnea (shortness of breath), fatigue, chest pain and growth failure. Cardiomyopathy patients are at risk of arrhythmias (irregular

heartbeats). Sometimes it may progress to a congestive heart failure, cardiac arrest, and sudden death (Sozzi et al., 2022).

1.7.1 Acylcarnitine profile used as a diagnostic tool for metabolic disorders and cardiomyopathy

Paediatric cardiomyopathy is a multifactorial and complex disorder driven by several pathophysiologic processes including fibrotic, mechanical and also metabolic changes (Cox, 2007). Metabolic alterations are a common feature due to the impaired mitochondrial function and therefore acylcarnitine profiling is part of the full diagnostic work up for children admitted to Starship hospital with cardiomyopathy (Glamuzina et al., 2019). Acylcarnitine profile analysis primarily detects disorders of inborn error of metabolism (IEM) (Miller et al., 2021). A list of metabolic disorders (mitochondrial fatty acids β-oxidation and organic acids disorders) that may be detected by acylcarnitine analysis is shown in Table 5. More than 40 different inherited metabolic disorders cause cardiomyopathy. Identification of the underlying cause of the cardiomyopathy is crucial for correct diagnosis and improved outcome. Cardiomyopathy may be the presenting or dominant clinical feature and is a major cause of death (Cox, 2007).

Table 5. Metabolic disorders detected by acylcarnitine profile analysis

Fatty Acid Oxidation Disorders	**OMIM***	**Gene**
Carnitine uptake disorder	212140	*SLC22A5*
Carnitine acylcarnitine translocase deficiency**	212138	*SLC25A20*
Carnitine palmitoyltransferase-I deficiency	255120	*CPT1A*
Carnitine palmitoyltransferase-II deficiency**	608836, 600649, 255110	*CPT2*
Short-chain acyl-COA dehydrogenase deficiency (SCAD)	201470	*ACADS*
Medium-chain acyl-CoA dehydrogenase deficiency	201450	*ACADM*
Long chain 3-hydroxy acyl-CoA dehydrogenase deficiency**	609016	*HADHA*
Trifunctional protein deficiency**	609015	*HADHA, HADHB*
Very-long-chain acyl-CoA dehydrogenase deficiency**	201475	*ACADVL*
Multiple acyl-CoA dehydrogenase deficiency	231680	*ETFA, ETFB, ETFDH*
3-hydroxyacyl-CoA dehydrogenase deficiency	231530	*HADH*
Dienoyl-CoA reductase deficiency	616034	*NADK2*

Organic Acid Disorders	OMIM*	Gene
Ethylmalonic encephalopathy	602473	*ETHE1*
Glutaric Acidemia Type I	231670	*GCDH*
Glutamate formiminotransferase deficiency	229100	*FTCD*
3-Hydroxyisobutyryl-CoA hydrolase deficiency	250620	*HIBCH*
3-Hydroxy-3-methylglutaryl-CoA lyase (HMG-CoA lyase) deficiency	246450	*HMGCL*
Isobutyryl-CoA dehydrogenase deficiency	611283	*ACAD8*
Isovaleric Acidemia	243500	*IVD*
B-Ketothiolase deficiency	203750	*ACAT1*
Malonyl-CoA decarboxylase deficiency	248360	*MLYCD*
2-Methylbutyryl-CoA dehydrogenase (short/branched-chain acyl-CoA dehydrogenase (SBCAD) deficiency	610006	*ACADSB*
3-Methylcrotonyl-CoA carboxylase (3-MCC) deficiency	210200, 210210	*MCCC1, MCCC2*
3-Methylglutaconyl-CoA hydratase deficiency	250950	*AUH*
2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency	300438	*HSD17B10*
Methylmalonic acidemia (MMA):		
i Methylmalonyl-CoA mutase deficiency	251000	*MUT*
ii Methylmalonyl-CoA racemase deficiency	251120	*MCEE*
iii Cobalamin metabolism disorders		
cblA	251100	*MMAA*
cblB	251110	*MMAB*
cblC	277400	*MMACHC, PRDX1*
cblD	277410	*MMADHC*
cblF	277380	*LMBRD1*
cblJ	614857	*ABCD4*
cblX	309541	*HCFC1*
Multiple carboxylase deficiency caused by deficiency of:		
i Holocarboxylase synthetase	253270	*HLCS*
ii Biotinidase	253260	*BTD*
Propionyl-CoA carboxylase deficiency (propionic acidemia)	606054	*PCCA, PCCB*
Succinyl-CoA ligase deficiency		
i Mitochondrial DNA depletion syndrome 5	612073	*SUCLA2*
ii Mitochondrial DNA depletion syndrome 9	245400	*SUCLG1*

*Online Mendelian Inheritance in Man **Multisystemic metabolic disorders with cardiac involvement

Note. Adapted from "Laboratory analysis of acylcarnitines, 2020 update: a technical standard of the American College of Medical Genetics and Genomics (ACMG)" by M. J Miller, K. Cusmano-Ozog, D. Oglesbee, and S. Young, 2021, *Genetics in Medicine*, *23*(2), 249-258.

Carnitine and its esters are found and are measured in all biological fluids however concentration levels of different species vary between sample types. Plasma is the preferred specimen type for the diagnostic assessment of patients with suspected metabolic IEM disorders or asymptomatic at-risk individuals (Rinaldo et al., 2008). Dried blood spots are preferred for newborn screening but also have utility in the clinical diagnostic setting (Pitt, 2010). Dried blood spots contain higher concentrations of long chain acylcarnitines and also other species including C2, C3, C4DC, and C5OH, and lower free carnitine in comparison to plasma. Therefore, bloodspots may be the preferred sample type for diagnosing CPT I deficiency, a disorder associated with low levels of long chain acylcarnitine species and increased free carnitine. However, plasma may have better sensitivity for detecting CPT II deficiency and TFP deficiency characterised by elevation of long-chain acylcarnitine levels. Urine samples can be useful in the work up of disorders associated with accumulations of short or medium chain acylcarnitines as these are efficiently excreted in the urine. Therefore, urinary analysis may be useful in detecting glutaric aciduria type I, associated with high concentration of C5DC. In contrast, it is not useful for detecting long chain disorders due to the low solubility of these species (Miller et al., 2021).

1.8 Research Questions and Aim

Knowledge of how hypoxia and ischemia affect acylcarnitines and amino acids is limited (Ahmad et al., 2016; Makrecka-Kuka et al., 2017; Modi et al., 2006). However, evidence of alterations in different ischemic tissues such as the brain underlines the necessity for a better understating of how these metabolic pathways respond to hypoxic insult (Lopez-Suarez et al., 2019). Significant gaps remain in the understanding of hypoxia induced myocardial metabolic derangements in newborns with congenital cardiac disease and cardiomyopathy. Existing reports have been limited to the adult population only (Ruiz et al., 2017).
This study will attempt to characterise the relationship between metabolic profiles in children with known cardiac diseases compared to the normal population to shed light on the metabolic processes that occur in the neonatal congenital heart and childhood cardiomyopathic patients by addressing the following research questions:

1) Do neonates with severe congenital cardiac disease have newborn screening profiles that are different from the general population?

The newborn screening data of babies born with congenital cardiac disease since July 2011 will be reviewed. For the purposes of normal newborn screening and secondary audit, the newborn screening laboratory has already well-established population mean, standard deviation, and cut-off ranges for all acylcarnitines and amino acids. Patients with the very rare inborn errors of metabolism have levels outside of these reference ranges.

The Paediatric Cardiology Service based at Starship Children's Hospital in Auckland holds a database of newborns with congenital cardiac disease. Unless their cardiac disease is a result of an underlying inborn error of metabolism and in which case, they would have been diagnosed on newborn screening, these cardiac babies have a metabolic profile within the set reference intervals. However, it has been shown recently that adults with cardiac failure have acylcarnitine profiles that while being within normal reference ranges and not suggestive of one of the rare inborn errors of metabolism are, as a group, different from the normal population with a characteristic pattern. Thus, we propose that children with congenital cardiac disease as cohort may also have a profile that is different from the general population. While the study will look at the overall cardiac cohort, it will also divide it into broad cardiac categories in order to investigate possible associations between particular cardiac pathologies (systemic outflow track obstruction, pulmonary outflow obstruction, complex lesions, mixed blood flow and other) and their metabolic profiles.

2) Do children with known cardiomyopathy have metabolic profiles that are different from the reference range?
Similar to above, the second group studied is children who are admitted to Starship Children's Hospital with cardiomyopathy. In these children the myocardium may be metabolically stressed and thus they like adults may have a characteristic metabolic profile. Review of the data will establish if there is a link between metabolic profiles and cardiomyopathy.

This study will compare the stored data from newborn screening metabolic profiles of babies who have both known and, at the time, unknown neonatal cardiac disease with the general population. The study will also look at the profile of children who present later in life to Starship Cardiology Service with cardiomyopathy. This may prove to be of some diagnostic or prognostic utility.

2.0 Methodology

2.1 Study design

This study is a retrospective data analysis of the newborn screening and where relevant presentation metabolic profiles of over 500 children who presented with cardiac disease to the Starship Children's Hospital in Auckland between July 2011 and December 2019. The aim was to establish if there is a pattern of metabolites that is similar to that seen in adults with cardiac failure. The reviewed data was compared with data from a control group of neonates with metabolic profile within the normal limits. As they had normal newborn screening and with the known very high sensitivity of screening, they are very unlikely to have an underlying fatty acid oxidation disorder, but may, as a group, because of their abnormal and potentially stressed hearts have metabolic profiles that differ from the general population.

2.2 Sample size and requirements

Five hundred and eighty six National Health Identification (NHI) numbers from children diagnosed with either severe congenital cardiac disease or cardiomyopathy born since July 2011 were provided by the Starship Hospital Cardiac Service.

The first cohort represents 547 newborns with a known severe congenital cardiac disease admitted to Starship Children's Hospital since July 2011 and who had a newborn metabolic screening test analysed shortly after birth. These neonates often presented to hospital in the first two weeks of life with cardiac symptoms. Based on the diagnoses, this group was classified into five different categories: outflow obstruction (aortic arch hypoplasia, coarctation of the aorta), mixed blood flow (transposition of the great arteries, pulmonary atresia with intact ventricular septum), complex presentation (Transposition of great arteries (TGA), Ventricular Septal Defect (VSD), Tetralogy of Fallot (TOF), Single ventricle), pulmonary blood flow (Pulmonary Atresia), and a spectrum of other cardiac diagnoses including arrhythmia, complete heart block, and total anomalous pulmonary venous connection (Table 6).

Table 6. Classification of congenital heart disease presentations

Congenital Heart Disease				
Complex	Mixed Blood Flow	Pulmonary Blood Flow	Systemic Outflow Obstruction	Other
• Transposition of great arteries • Ventricular Septal Defect • TOF • Single ventricle	• Transposition of great arteries • Pulmonary Atresia with Intact Ventricular Septum	• Pulmonary Atresia	• Aortic arch hypoplasia • Coarctation of aorta	• Arrhythmia • Complete heart block • Total anomalous pulmonary venous connection

Over 99% of neonates born in New Zealand have a heel prick test done after day two of life (Ministry of Health, 2022). A few drops of blood dried on filter paper is the preferred sample for newborn metabolic screening as it is less invasive as venous samples and offers a simpler storage and also easier shipping at room temperature (Velden et al., 2013). Twenty four acylcarnitines and seven aminoacid markers are quantitatively measured as part of the newborn screening profile. Fifteen different ratios are calculated from the acylcarnitine or amino acids measured as a discriminative factor for diagnosis. The metabolites are extracted from the samples using methanol and quantification is achieved by adding stable isotope internal standards using tandem mass spectrometry. The sample extract is directly introduced into the electrospray ionisation source without chromatography. The acylcarnitines are analysed by the precursor-ion scan of m/z 85 mode and scanning from m/z 160 to 500. Aminoacids are analysed by neutral loss scan mode where all precursors undergoing the loss of a common fragment are detected (Pitt, 2010).

While the vast majority of babies do not have metabolic conditions, the newborn screening profile reflects day two fat metabolic status. The data is stored in the Newborn Metabolic Screening laboratory database at Auckland Hospital and from July 2011 as part of the consent process for newborn metabolic screening, can be used for research purposes with appropriate ethics approval and permission from the National Screening Unit (Ministry of Health, 2011). The second cohort in this study represents 39 children diagnosed with cardiomyopathy. These children were admitted to Starship Children's Hospital and have had full metabolic investigations as part of their diagnostic work-up. Twenty two of these children have had acylcarnitines measured in their plasma samples and the rest of the children have had acylcarnitines measured in dried blood spot samples at the Auckland Hospital metabolic laboratory.

The metabolic laboratory at Auckland Hospital offers comprehensive metabolic investigations including testing and interpretation of acylarnitine profiles in both plasma and dried blood spots since 2017 (LabPLUS, 2022). While the standard specimen for newborn metabolic screening programmes is whole blood dried on filter paper, both plasma and dried blood spot samples are routinely used by diagnostic metabolic laboratories. The principle method used by the laboratory for quantitative measurement of carnitine and acylcarnitine species is tandem mass spectrometry. This separates and detects each individual species after derivatisation with butylated hydrochloric acid. Firstly, deuterated internal standard made up in methanol is added to each sample to precipitate proteins. The supernatant is then dried down using nitrogen gas and reconstituted in butanolic hydrochloric acid. The sample is then exposed to heat at 60°C for 20 minutes when the active hydrogens in the carboxylic acid groups become butylated. The purpose of the derivatisation step is to enhance analytical sensitivity and also to allow separation of isobaric compounds with the same molecular weight. The sample is then dried down and re-suspended in mobile phase before injected into the triple quadrupole mass spectrometer using flow injection analysis. The liquid sample is analysed by electrospray ionization without chromatographic separation. The acylcarnitine species are separated in the ion source by scanning and filtering ions in the first quadrupole before fragmentation and filtering a common m/z 85 fragment (Rinaldo et al., 2008).
Control groups data derives from 200 newborns with no metabolic disorder. Cardiomyopathy patient data was compared with age related reference ranges established by the Auckland Hospital metabolic laboratory.

2.3 Data management

The National Screening Unit (Ministry of Health) provided access to metabolic screening data to match with the NHI numbers. The data for the patients with cardiomyopathy was acquired through the Information Technology (IT) department at the metabolic laboratory. After linking the databases, the NHI numbers were removed and codes were assigned. The raw and working data was de-identified permanently and transferred electronically in password protected files to ensure appropriate back up and preservation. Data from samples with verified quality were included only. Access was limited to the named investigators in this study.

2.4 Data analysis

Statistical evaluation of the data was performed in consultation with a biostatistician. Generalised Linear Model (GLM) was carried to investigate continuous outcome of interest

between five case groups and control group. Means and standard deviation with 95% confidence interval (CI) were presented. P values less than 0.05 were considered to be statistically significant. Statistical analyses were carried out using SAS 9.4 and R software.

2.5 Ethics

Since patient data was used in this study, the key ethical points to consider were ensuring patient confidentiality. All data had been de-identified prior to study commencement. Although there is a very low risk of harm with this retrospective study, the data studied is still considered sensitive and it was suitably protected.

The study was given full ethics approval by the Auckland University of Technology (AUT) Ethics committee on 23rd November 2020 and the Auckland District Health Board (ADHB) Research Review Committee on the 23rd October 2020.

Furthermore, ethics approval was also obtained from the Health and Disability Ethics Committee through the full review pathway on the 5th October 2020. Permission to use newborn screening retrospective data was attained from the National Screening Unit (Ministry of Health). Stored Guthrie samples were not used in this study.

Māori are the second largest ethnic group in NZ representing about 17% of the total national population (Stats NZ, 2018). It is acknowledged that a portion of this retrospective data analysis included Māori data. The Treaty of Waitangi principles of partnership, participation and protections have been considered. Māori data is considered taonga with special cultural significance. Data has mauri, whairua and whakapapa. Mauri is associated with a person and therefore also their data. Any data or information is a taonga because of these indigenous beliefs. Although ethnicity is not being collected and data was de-identified, we respect Māori cultural values and beliefs and therefore data uses was managed in a highly trusted and protected way to safeguard the privacy and confidentiality from any harmful effects. It is imperative to ground Māori approaches to data and utilise Māori concepts and tikanga as the conceptual basis for appropriate data usage.

The investigators consulted with the ADHB Māori Research Review Committee in a meeting held on the 9th July 2020. The committee accepted the study being appropriate for a New Zealand context. The committee noted that the study will be of significant benefit to all ethnicities in New Zealand.

3.0 Results Analysis

3.1 Data derived from clinical samples

Retrospective stored data from a total of 770 samples were used for this study. According to the cardiac pathology, thirty two samples came from patients with complex presentation, two hundred and thirty six had were diagnosed with systemic outflow obstruction, two hundred and twenty nine presented mixed blood flow pathology, one hundred and fifty three with pulmonary atresia and seventy six with a range of other cardiac diagnoses including arrhythmia, complete heart block, and total anomalous pulmonary venous connection. The concentration of amino acids, acylcarnitines with ratios and endocrine markers in blood spot samples collected in the first few days of age in babies with congenital cardiac disease are presented in Tables 7 to 11. Note that the monohydroxylated (OH) and dicarboxylic acids (DC) carnitine species are isobaric and thus were not separated in this analysis. Tables 12 and 13 show comparison data from cardiomyopathy patients against normal reference ranges.

3.2 Amino acids and acylcarnitine profiles in bloodspot samples

Table 7 shows comparison data of amino acids and acylcarnitine levels in samples from 32 patients with congenital complex cardiac disease presentation. Citrulline, leucine, tyrosine, and valine didn't show significant difference compared with the control group (all $p>0.05$). Significantly higher levels, however, were observed for alanine, methionine with methionine/phenylalanine and phenylalanine/tyrosine ratios ($p<0.001$), and phenylalanine ($p<0.05$).

Free carnitine, C4, C5, C8, and C0/(C16+C18), C8/C10 ratios were found significantly higher in the case group ($p<0.05$). However, C5DC, C14, C14:1, C16 and C18 were significantly lower ($p<0.05$).

Table 7. Data comparison of amino acids and acylcarnitines levels between control subjects and complex cardiac disease (full data shown in appendix A)

	Control Subjects			Patients with Complex CD			
Parameters	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Amino Acids (μM)							
Alanine	200	249.21 (74.8)	238.78 - 259.64	32	308.91 (99.89)	272.90 - 344.93	8.98E-05
Methionine	200	21.12 (5.63)	20.33 - 21.9	32	31.13 (13.99)	26.08 - 36.17	1.04E-11
Phenylalanine	200	63.4 (12.6)	61.64 - 65.16	32	68.91 (22.23)	60.89 - 76.92	0.0441

Acylcarnitines (µM)							
C0-carnitine	200	21.13 (7.41)	20.1 – 22.17	32	25.08 (12.81)	20.46 – 29.70	0.0137
C4-carnitine	200	0.42 (0.17)	0.40 - 0.44	32	0.51 (0.19)	0.44 - 0.58	0.0052
C5-carnitine	200	0.11 (0.05)	0.10 - 0.12	32	0.15 (0.13)	0.10 - 0.20	0.0015
C8-carnitine	200	0.16 (0.05)	0.15 - 0.16	32	0.19 (0.08)	0.16 - 0.22	0.0022
Endocrine markers							
Thyroid Stimulating Hormone	200	1.97 (1.90)	1.70 - 2.23	31	3.06 (1.69)	2.44 - 3.69	0.0026
Ratios							
Met/Phe	200	0.34 (0.09)	0.33 - 0.35	32	0.45 (0.11)	0.41 - 0.49	1.69E-09
Phe/Tyr	200	0.64 (0.21)	0.61 - 0.67	32	0.83 (0.51)	0.65 - 1.01	0.0002
C0/(C16+C18)	200	4.46 (1.79)	4.21 - 4.71	32	7.04 (4.01)	5.60 - 8.49	4.30E-09
C8/C10	200	1.13 (0.38)	1.08 - 1.18	32	1.46 (0.48)	1.29 - 1.64	1.26E-05

Table 8 displays comparison data of amino acids and acylcarnitine levels in samples from 229 patients with congenital mixing cardiac disease presentation with control group. Citrulline and valine didn't show significant difference compared with the control group (all $p>0.05$). Significantly higher levels, however, were observed for alanine, phenylalanine, methionine with met/phe and phe/tyr ratios ($p<0.001$), and leucine ($p<0.05$). Lower levels were noted for tyrosine with unknown clinical significance.

The same findings were identified for the acylcarnitines as with group 1, the mean values for free carnitine, C4, C5, C6 and C8, C0/(C16+C18), C8/C10 ratios were significantly higher. The long-chain acylcarnitine species were found to be significantly lower in the case group ($p<0.05$).

Table 8. Data comparison of amino acids and acylcarnitines levels between control subjects and mixing cardiac disease (full data shown in appendix A)

	Control subjects			Patients with Mixing CD			
Parameters	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Amino acids (µM)							
Alanine	200	249.21 (74.8)	238.78 - 259.64	229	298.21 (104.78)	284.56 - 311.85	<.0001
Leucine	200	148.61 (41.03)	142.88 - 154.33	229	168.69 (85.75)	157.53 - 179.86	0.0026
Methionine	200	21.12 (5.63)	20.33 - 21.9	229	33.04 (16.04)	30.95 - 35.13	<.0001
Phenylalanine	200	63.4 (12.6)	61.64 - 65.16	229	73.61 (23.34)	70.57 - 76.65	<.0001
Acylcarnitines (µM)							
C0-carnitine	200	21.13 (7.41)	20.1 - 22.17	229	23.68 (10.97)	22.25 - 25.11	0.0058
C4-carnitine	200	0.42 (0.17)	0.40 - 0.44	229	0.49 (0.24)	0.46 - 0.52	0.0006
C5-carnitine	200	0.11 (0.05)	0.10 - 0.12	229	0.13 (0.07)	0.12 - 0.13	0.0073
C6-carnitine	200	0.17 (0.08)	0.16 - 0.18	229	0.20 (0.10)	0.18 - 0.21	0.0012
C8-carnitine	200	0.16 (0.05)	0.15 - 0.16	229	0.17 (0.06)	0.16 - 0.18	0.0455
Endocrine markers	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Thyroid Stimulating Hormone	200	1.97 (1.90)	1.70 - 2.23	225	2.76 (3.88)	2.25 - 3.27	0.0085
17-Hydroxy Progesterone	200	4.90 (2.53)	4.55 - 5.25	217	7.46 (9.04)	6.25 - 8.67	0.0001
Ratios	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Met/Phe	200	0.34 (0.09)	0.33 - 0.35	229	0.44 (0.13)	0.43 - 0.46	<.0001
Phe/Tyr	200	0.64 (0.21)	0.61 - 0.67	229	1.05 (0.52)	0.98 - 1.12	<.0001
C0/(C16+C18)	200	4.46 (1.79)	4.21 - 4.71	229	7.97 (6.02)	7.19 - 8.76	<.0001
C8/C2	200	0.01 (0.01)	0.01 - 0.01	229	0.010 (0.007)	0.009 - 0.011	<.0001
C8/C10	200	1.13 (0.38)	1.08 - 1.18	229	1.59 (0.56)	1.51 - 1.66	<.0001

Table 9 presents comparison data of amino acids and acylcarnitine levels in samples from 153 patients with pulmonary outflow obstruction disease with control group. There were no statistically significant differences in citrulline and valine compared with the control group (all $p>0.05$). Significantly higher levels, however, were observed for alanine, leucine, phenylalanine, methionine with met/phe and phe/tyr ratios ($p<0.001$).
Similarly, to the above, free carnitine, C4, C5, C4DC/C5OH, C6, C8, C0/(C16+C18) ratio C8/C10 were found to be significantly higher, but the long chains acylcarnitines were identified as significantly lower than the controls ($p<0.05$).

Table 9. Data comparison of amino acids and acylcarnitines levels between control subjects and pulmonary outflow obstruction (full data shown in appendix A)

	Control subjects			Patients with pulmonary outflow obstruction			
Parameters	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Amino Acids (µM)							
Alanine	200	249.21 (74.8)	238.78 - 259.64	153	321.89 (116.82)	303.23 - 340.55	<.0001
Leucine	200	148.61 (41.03)	142.88 - 154.33	153	172.5 (84.47)	159.01 - 186.0	0.0005
Methionine	200	21.12 (5.63)	20.33 - 21.9	153	34.24 (17.51)	31.44 - 37.03	<.0001
Phenylalanine	200	63.4 (12.6)	61.64 - 65.16	153	73.35 (25.86)	69.22 - 77.48	<.0001
Acylcarnitines (µM)							
C0-carnitine	200	21.13 (7.41)	20.1 - 22.17	153	26.83 (12.29)	24.87 - 28.8	<.0001
C4-carnitine	200	0.42 (0.17)	0.40 - 0.44	153	0.53 (0.24)	0.49 - 0.57	<.0001
C4DC/C5OH-carnitine	200	0.20 (0.10)	0.18 - 0.21	153	0.22 (0.12)	0.20 - 0.24	0.0142
C5-carnitine	200	0.11 (0.05)	0.10 - 0.12	153	0.14 (0.08)	0.13 - 0.15	<.0001
C6 carnitine	200	0.17 (0.08)	0.16 - 0.18	153	0.20 (0.10)	0.19 - 0.22	0.0004
C8 carnitine	200	0.16 (0.05)	0.15 - 0.16	153	0.17 (0.05)	0.16 - 0.18	0.0248
Endocrine markers							
Thyroid Stimulating Hormone	200	1.97 (1.90)	1.70 - 2.23	149	3.21 (3.70)	2.61 - 3.81	<.0001
17-Hydroxy Progesterone	200	4.90 (2.53)	4.55 - 5.25	136	7.02 (7.09)	5.82 - 8.22	0.0001
Ratios							
Met/Phe	200	0.34 (0.09)	0.33 - 0.35	153	0.47 (0.15)	0.44 - 0.49	<.0001
Phe/Tyr	200	0.64 (0.21)	0.61 - 0.67	153	1.12 (0.68)	1.01 - 1.23	<.0001
C0/(C16+C18)	200	4.46 (1.79)	4.21 - 4.71	153	8.57 (6.66)	7.51 - 9.63	<.0001
(C16+C18:1)/C2	200	0.22 (0.14)	0.20 - 0.24	153	0.18 (0.08)	0.17 - 0.19	0.0042
C8/C10	200	1.13 (0.38)	1.08 - 1.18	153	1.64 (0.57)	1.55 - 1.73	<.0001

As shown in table 10, laboratory values for citrulline were similar between patients with systemic outflow obstruction disease (n= 236) and control group. Mean levels for alanine,

leucine, valine, phenylalanine, methionine with met/phe, phe/tyr and leucine/alanine ratios were all statistically higher in the cases compared with controls (p<0.001).
Free carnitine, C4, C5, C6, C8, C0/(C16+C18), C8/C10 ratios were significantly higher in the case group (p<0.05).

Table 10. Data comparison of amino acids and acylcarnitines levels between control samples and systemic outflow obstruction samples (full data shown in appendix A)

	Control subjects			Patients with Systemic outflow obstruction			
Parameters	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Amino Acids (μM)							
Alanine	200	249.21 (74.8)	238.78 - 259.64	234	303.4 (134.57)	286.07 - 320.73	**<.0001**
Leucine	200	148.61 (41.03)	142.88 - 154.33	236	209.06 (215.1)	181.47 - 236.64	**0.0001**
Methionine	200	21.12 (5.63)	20.33 - 21.9	236	40.49 (30.61)	36.56 - 44.41	**<.0001**
Phenylalanine	200	63.4 (12.6)	61.64 - 65.16	236	80.19 (40.22)	75.03 - 85.35	**<.0001**
Valine	200	142.66 (40.51)	137.01 - 148.31	236	162.41 (98.41)	149.79 - 175.03	**0.0083**
Acylcarnitines (μM)							
C0-carnitine	200	21.13 (7.41)	20.1 - 22.17	236	24.64 (10.56)	23.28 - 25.99	**<.0001**
C4-carnitine	200	0.42 (0.17)	0.40 - 0.44	236	0.50 (0.22)	0.47 - 0.53	**<.0001**
C5-carnitine	200	0.11 (0.05)	0.10 - 0.12	236	0.14 (0.09)	0.13 - 0.16	<.0001
C6-carnitine	200	0.17 (0.08)	0.16 - 0.18	236	0.20 (0.09)	0.18 - 0.21	0.0015
C8-carnitine	200	0.16 (0.05)	0.15 - 0.16	236	0.18 (0.07)	0.17 - 0.19	0.0014
Endocrine markers							
Thyroid Stimulating Hormone	200	1.97 (1.90)	1.70 - 2.23	229	2.75 (3.43)	2.30 - 3.19	**0.0044**
17-Hydroxy Progesterone	200	4.90 (2.53)	4.55 - 5.25	221	6.50 (9.64)	5.22 - 7.78	**0.0234**
Ratios							
C0/(C16+C18)	200	4.46 (1.79)	4.21 - 4.71	236	8.71 (8.42)	7.63 - 9.79	**<.0001**
C8/C10	200	1.13 (0.38)	1.08 - 1.18	236	1.54 (0.59)	1.47 - 1.62	**<.0001**

Association between amino acids and acylcarnitine levels in samples from patients with a spectrum of other cardiac diagnoses including arrhythmia, complete heart block, and total anomalous pulmonary venous connection are summarised in Table 11. Mean values for alanine, citrulline, phenylalanine, and valine were similar between the case (n=76) and control group (all p>0.05). Statistically significant higher concentrations for leucine and methionine with met/phe and leu/ala ratios were noted (p<0.05).

Only the short chain acylcarnitines C5, C4DC/C5OH and C0/(C16+C18), C8/C10 ratios were significantly higher in this group (p<0.05).

Table 11. Data comparison of amino acids and acylcarnitines levels between control subjects and patients with "other" congenital cardiac disorders (full data shown in appendix A)

		Control subjects			Patients with "Other" CD		
Parameters	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Amino Acids (μM)							
Leucine	200	148.61 (41.03)	142.88 - 154.33	76	164.86 (75.21)	147.67 - 182.04	**0.0227**
Methionine	200	21.12 (5.63)	20.33 - 21.9	76	30.25 (15.41)	26.73 - 33.77	**<.0001**
Acylcarnitines (μM)							
C4DC/C5OH-carnitine	200	0.20 (0.10)	0.18 - 0.21	76	0.22 (0.10)	0.20 - 0.25	0.0301
C5-carnitine	200	0.11 (0.05)	0.10 - 0.12	76	0.13 (0.07)	0.11 - 0.14	0.0185
Endocrine markers							
Thyroid Stimulating Hormone	200	1.97 (1.90)	1.70 - 2.23	75	3.15 (2.93)	2.47 - 3.82	**0.0001**
Ratios							
Met/Phe	200	0.34 (0.09)	0.33 - 0.35	76	0.45 (0.13)	0.42 - 0.48	**<.0001**
Phe/Tyr	200	0.64 (0.21)	0.61 - 0.67	76	1.04 (0.48)	0.93 - 1.14	**<.0001**
Leu/Ala	200	0.62 (0.15)	0.60 - 0.64	76	0.69 (0.36)	0.61 - 0.77	0.0201
C0/(C16+C18)	200	4.46 (1.79)	4.21 - 4.71	76	9.92 (6.48)	8.44 - 11.40	<.0001
C8/C10	200	1.13 (0.38)	1.08 - 1.18	76	1.66 (0.81)	1.48 - 1.85	<.0001

Box plots for alanine, methionine, C4-carnitine, C14-carnitine, and C14:1-carnitine are shown in the figures below. Box plots show the dispersion and outliers in each data set. The boxes indicate the 1st and 3rd quartiles range, and the central line is the median for each metabolite in the five case groups and the control group. The ends of the whiskers are 1.5 times the interquartile range below the 1st and above the 3rd quartile. The filled dots represent outliers.

Figure 13 shows the median alanine concentration for all five case groups is higher than the control group. The largest spread for distribution was found in the systemic group with 7 outliers (1277, 919, 842, 721, 710, 623, 612). Four outliers were found in the pulmonary group (951, 765, 628, 607), five in the mixing group (749, 701, 634, 622, 585) two in the complex group (598, 512) and one in the other group (694).

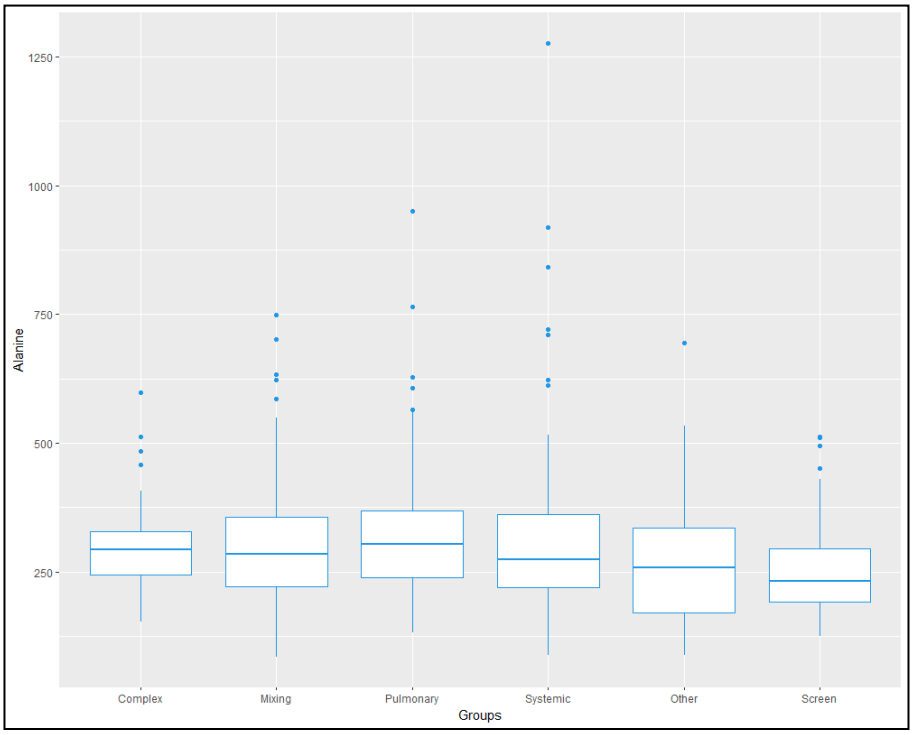

Figure 13. Box plot of the concentration of alanine (µmol/L) in the five different congenital cardiac disease groups and the control group labelled as screen.

Figure 14 shows data comparison of methionine levels. Statistically significant higher levels of methionine in all the case groups were noted. The largest spread of distribution was noted in the systemic case group with a maximum value of 382 µmol/L. This newborn screening sample was collected on day one, admitted to hospital on the same day, and surgery performed on day three.

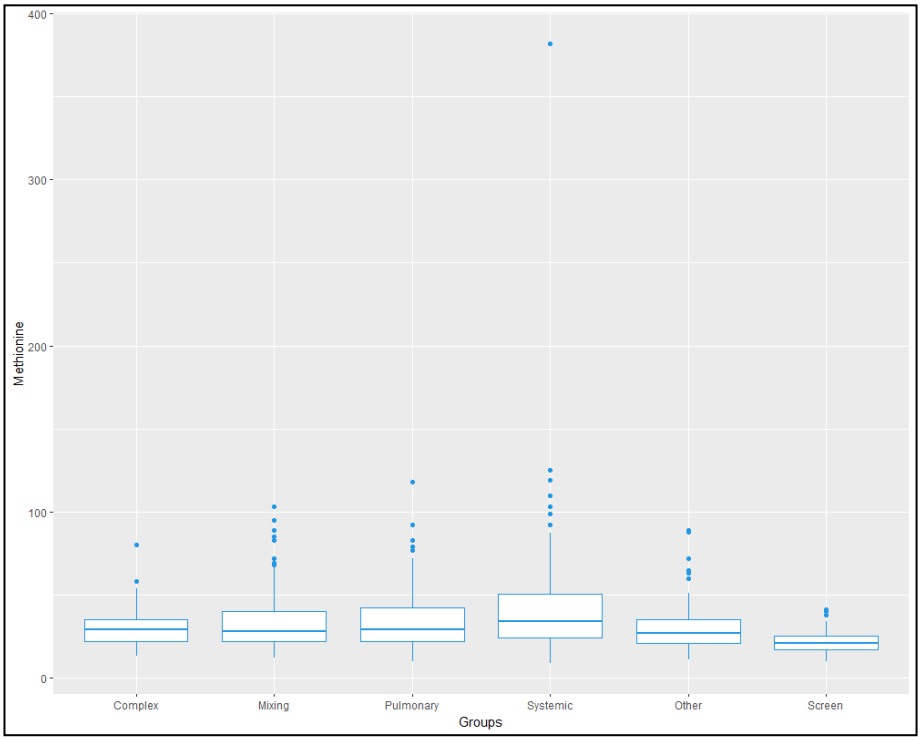

Figure 14. Box plot of the concentration of methionine (µmol/L) in the five different congenital cardiac disease groups and the control group labelled as screen.

Figure 15 represents comparison of C4 acylcarnitine levels between the five different case groups. The largest spread of distribution was found in the systemic group with maximum value of 1.96 µmol/L. Nine outliers were found in the mixing group between 1.02 to 1.63 µmol/L and four outlier point in the systemic group (1.51, 1.23, 1.17, 1.09).

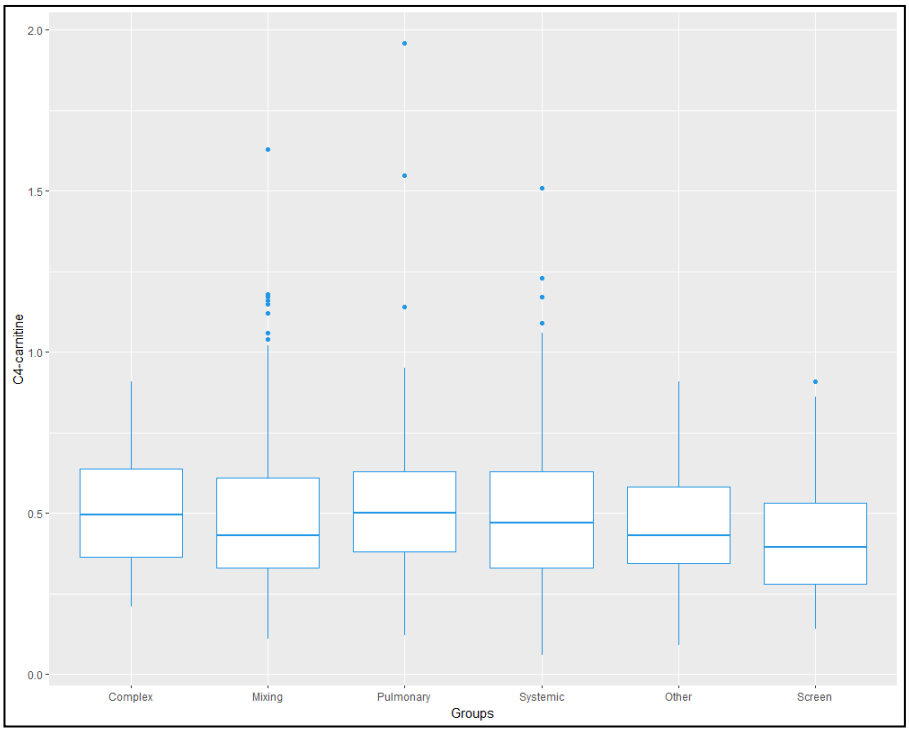

Figure 15. Box plot of the concentration of C4 acylcarnitine in the five different congenital cardiac disease groups and the control group labelled as screen.

Figure 16 shows the comparison of C14:1 acylcarnitine levels among the different cardiac groups. While C14:1 level was found to be generally lower in the case groups versus the control group, there was one extreme outlier in the systemic cardiac case group, C14:1=1.58 µmol/L. This newborn screening sample was collected on day three, admitted to hospital on day 4, and surgery was performed on day 8.

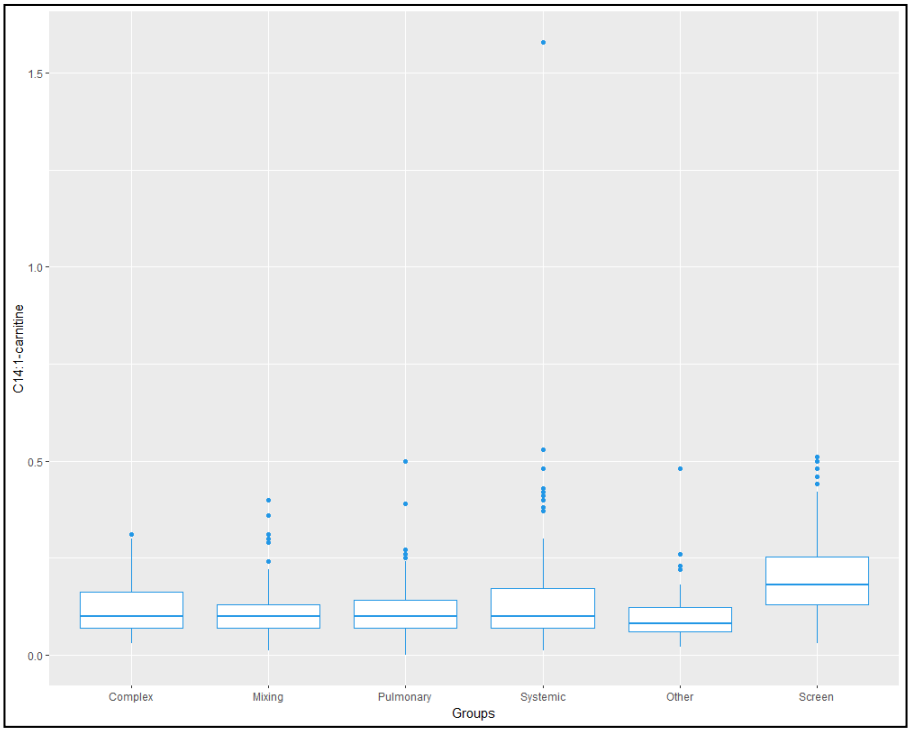

Figure 16. Box plot of the concentration of C14:1 acylcarnitine (µmol/L) in the five different congenital cardiac disease groups and the control group labelled as screen.

Figure 17 demonstrates the comparison of C14 acylcarnitine levels amongst the five case groups. Although C14 level was found to be generally lower in all the case groups in comparison to the control group, two extreme outliers were found in the systemic group (1.06, 0.75), one in the pulmonary group (0.74), and one in the other group (0.74).

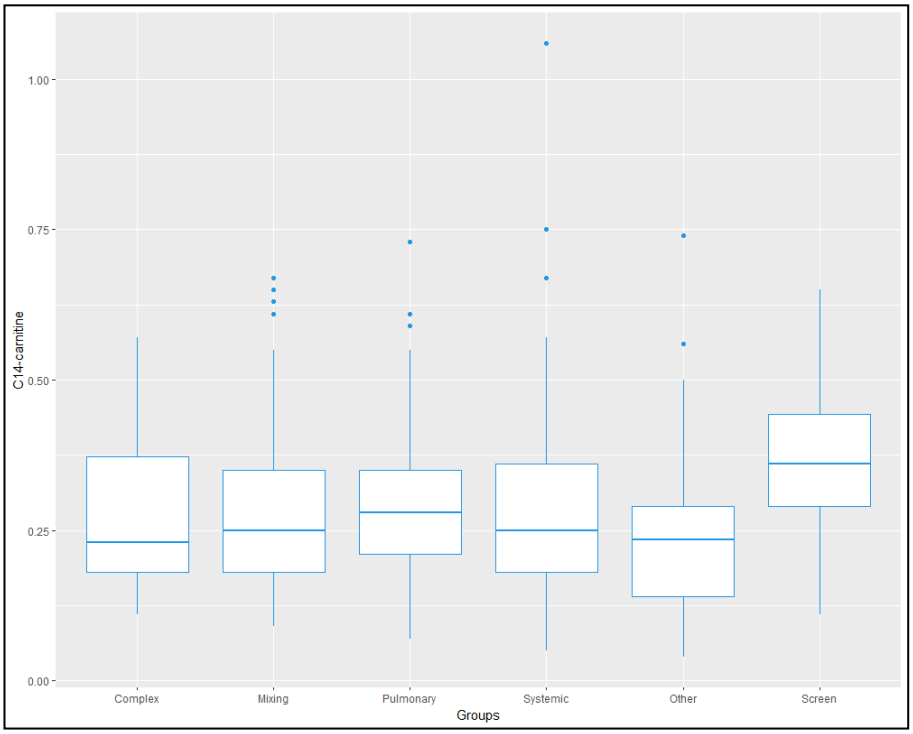

Figure 17. Box plot of the concentration of C14 acylcarnitine (µmol/L) in the five different congenital cardiac disease groups and the control group labelled as screen.

3.3 Endocrine markers in bloodspot samples

Mean concentration levels of thyroid stimulating hormone were found to be significantly higher in all five groups of patients with congenital heart defects and also in the newborn screening data of the cardiomyopathy group ($p<0.05$). The second endocrine hormone measured in newborns, 17-hydroxyprogesterone, was significantly higher in groups 2, 3 and 4 only ($p<0.05$).

3.4 Acylcarnitine profiles in bloodspot and plasma samples in cardiomyopathy patient cohort

Figure 18 shows that both C8:1 and C4OH acylcarnitines were significantly increased in 37.5% (9/24) samples from cardiomyopathy patients when compared with normal reference ranges. C12:1 was raised in 29.2% (7/24) and C4 was found to be increased in 33.3% (8/24) samples.

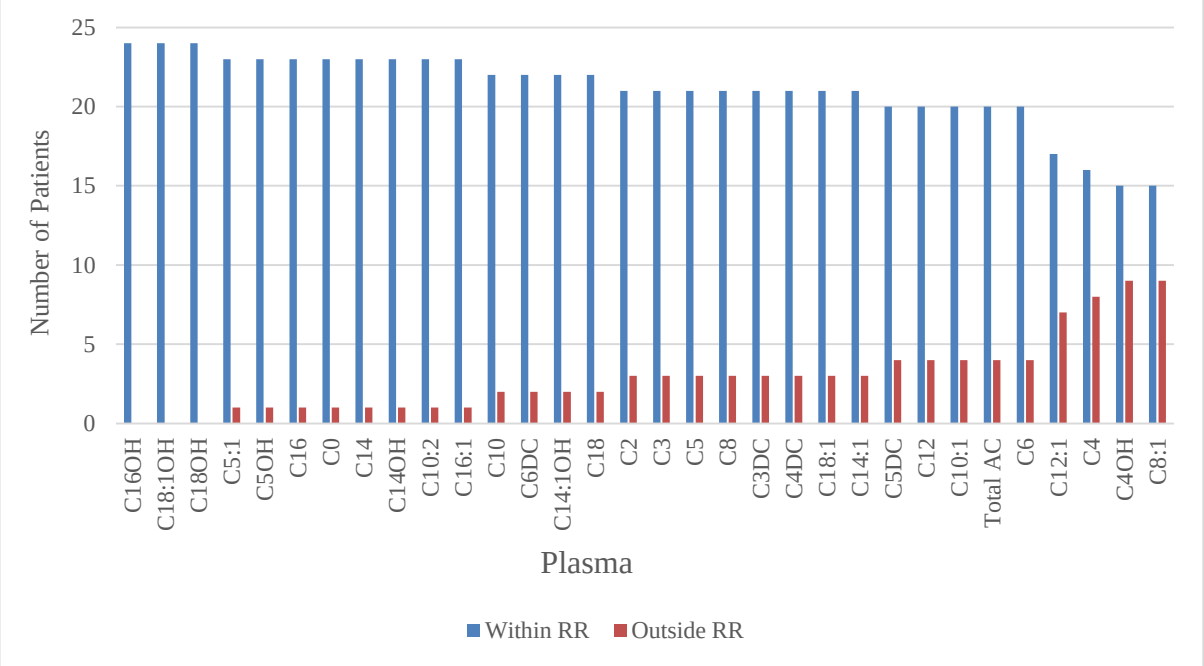

Figure 18. Twenty four plasma samples from children with cardiomyopathy compared with reference ranges

Acylcarnitine chain lengths are denoted by the numbers. Acylcarnitine species that represent monohydroxylated (OH) or dicarboxylic acid (DC) species are also shown. Full results are shown in Table 12.

Table 12. Plasma sample data compared with reference ranges

Plasma Sample Data

					Within RR	Above RR
	N	Mean (SD)	95% CI	Reference Range (RR) µmol/L	N (%)	N (%)
Age (days)	24	1719 (2937)	439 - 3328			
Birth weight (g)	17	3110 (532)	2836 - 3383			
Gestational Age (weeks)	17	38.35 (1.32)	37.67 - 39.03			
Total AC (µM)	24	23.18 (22.50)	13.48 – 32.89	5.0-27.0	20 (83.3)	4 (16.7)
C0-carnitine	24	40.32 (15.86)	33.48 – 47.16	15.0-70.0	23 (95.8)	1 (4.2)
C10-carnitine	24	0.21 (0.15)	0.14 - 0.27	0.0-0.50	22 (91.7)	2 (8.3)
C10:1-carnitine	24	0.14 (0.08)	0.10 - 0.17	0.0-0.20	20 (83.3)	4 (16.7)
C10:2-carnitine	24	0.02 (0.02)	0.02 - 0.03	0.0-0.05	23 (95.8)	1 (4.2)
C12-carnitine	24	0.12 (0.11)	0.07 - 0.16	0.0-0.20	20 (83.3)	4 (16.7)
C12:1-carnitine	24	0.11 (0.09)	0.06 - 0.14	0.0-0.10	17 (70.8)	7 (29.2)
C14-carnitine	24	0.09 (0.09)	0.05 - 0.12	0.0-0.20	23 (95.8)	1 (4.2)
C14:1-carnitine	24	0.11 (0.10)	0.06 - 0.15	0.0-0.20	21 (87.5)	3 (12.5)
C14:1OH-carnitine	24	0.02 (0.01)	0.02 - 0.03	0.0-0.05	22 (91.7)	2 (8.3)
C14OH-carnitine	24	0.02 (0.02)	0.01 - 0.03	0.0-0.05	23 (95.8)	1 (4.2)
C16-carnitine	24	0.21 (0.20)	0.12 - 0.29	0.0-0.50	23 (95.8)	1 (4.2)
C16:1-carnitine	24	0.08 (0.08)	0.04 - 0.10	0.0-0.10	23 (95.8)	1 (4.2)
C16OH-carnitine	24	0.01 (0.01)	0.01 - 0.02	0.0-0.10	24 (100)	0 (0)
C18-carnitine	24	0.07 (0.09)	0.03 - 0.10	0.0-0.10	22 (91.7)	2 (8.3)
C18:1-carnitine	24	0.30 (0.49)	0.09 - 0.51	0.0-0.35	21 (87.5)	3 (12.5)
C18:1OH-carnitine	24	0.01 (0.01)	0.00 - 0.01	0.0-0.05	24 (100)	0 (0)
C18OH-carnitine	24	0.01 (0.01)	0.0 - 0.01	0.0-0.05	24 (100)	0 (0)
C2-carnitine	24	15.7 (14.23)	10.5 – 27.11	4.0-24.0	21 (87.5)	3 (12.5)
C3-carnitine	24	0.63 (0.41)	0.45 - 0.80	0.0-1.00	21 (87.5)	3 (12.5)
C3DC-carnitine	24	0.10 (0.13)	0.04 - 0.15	0.0-0.15	21 (87.5)	3 (12.5)
C4-carnitine	24	0.45(0.37)	0.29 - 0.61	0.0-0.65	16 (66.7)	8 (33.3)
C4DC-carnitine	24	0.07 (0.06)	0.04 - 0.10	0.0-0.10	21 (87.5)	3 (12.5)
C4OH-carnitine	24	0.41 (0.81)	0.06 - 0.75	0.0-0.15	15 (62.5)	9 (37.5)
C5-carnitine	24	0.18 (0.15)	0.11 - 0.23	0.0-0.30	21 (87.5)	3 (12.5)
C5:1-carnitine	24	0.02 (0.03)	0.01 - 0.03	0.0-0.10	23 (95.8)	1 (4.2)
C5DC-carnitine	24	0.10 (0.08)	0.06 - 0.14	0.0-0.30	20 (83.3)	4 (16.7)
C5OH-carnitine	24	0.03 (0.02)	0.02 - 0.03	0.0-0.10	23 (95.8)	1 (4.2)
C6-carnitine	24	0.12 (0.10)	0.07 - 0.16	0.0-0.20	20 (83.3)	4 (16.7)
C6DC-carnitine	24	0.11 (0.12)	0.05 - 0.16	0.0-0.20	22 (91.7)	2 (8.3)
C8-carnitine	24	0.17 (0.12)	0.11 - 0.22	0.0-0.30	21 (84.5)	3 (12.5)
C8:1-carnitine	24	0.35 (0.26)	0.23 - 0.46	0.0-0.40	15 (62.5)	9 (37.5)

Figure 19 displays data from twenty samples collected from cardiomyopathy patients. Similar to the congenital cardiac cohort, C4OH acylcarnitine is shown to be increased and outside of the reference ranges in 20% (4/20) samples.

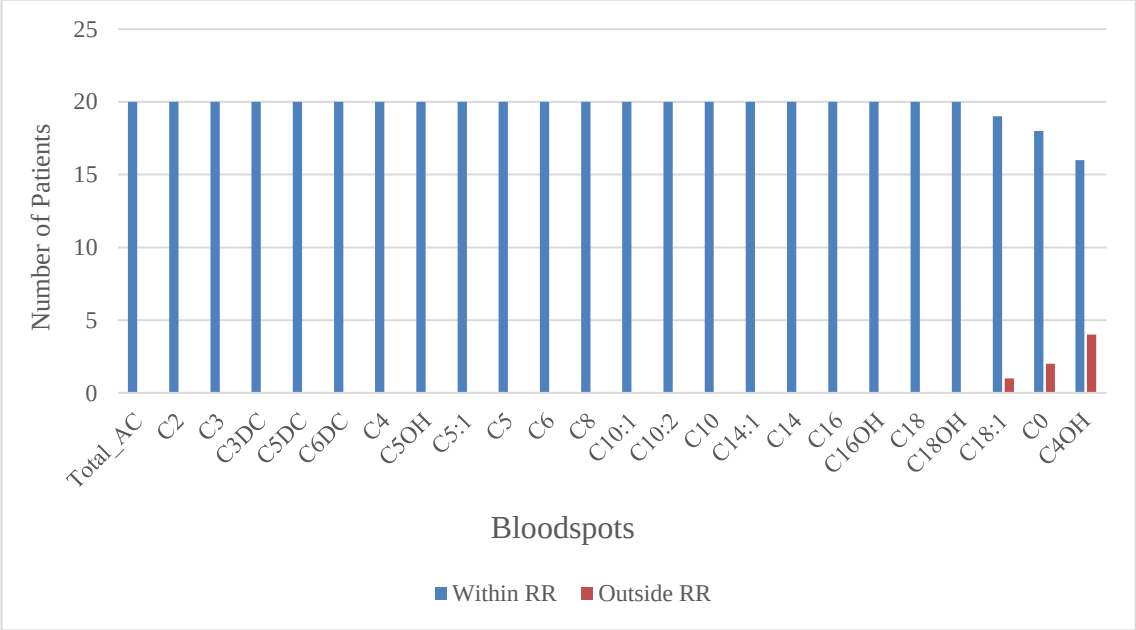

Figure 19. Twenty bloodspot samples from children with cardiomyopathy collected after 8 days of age compared with reference ranges.

Acylcarnitine chain lengths are denoted by the numbers. Acylcarnitine species that represent monohydroxylated (OH) or dicarboxylic acids (DC) species are also shown. Full results are shown in Table 13.

Table 13. Dried bloodspot data compared with reference ranges

	Dried Bloodspot Sample Data					
	N	Mean (SD)	95% CI	RR (µmol/L)	Within RR	Above RR
					N (%)	N (%)
Age (days)	20	1716 (5049)				
Birth weight (g)	20	3056 (734)				
Gestational age (weeks)	20	38 (4)				
Total Acylcarnitine (µM)	20	33.76 (14.47)	26.81 - 40.71	15-70	20 (100)	0 (0)
C0 - carnitine	20	40.62 (17.15)	32.39 – 48.86	0.0-70	18 (90)	2 (10)
C2 - carnitine	20	25.64 (11.97)	19.90 – 31.39	0.0-50	20 (100)	0 (0)
C3 - carnitine	20	1.60 (1.00)	1.13 – 2.09	0.4-4.7	20 (100)	0 (0)
C3DC - carnitine	20	0.07 (0.03))	0.06 – 0.10	0.0-0.2	20 (100)	0 (0)
C5DC - carnitine	20	0.07 (0.04)	0.06 – 0.11	0.0-0.2	20 (100)	0 (0)
C6DC - carnitine	20	0.09 (0.03)	0.08 – 0.12	0.0-0.4	20 (100)	0 (0)
C4 - carnitine	20	0.3 (0.11)	0.25 – 0.35	0.0-0.6	20 (100)	0 (0)
C4OH - carnitine	20	0.50 (0.68)	0.17 – 0.84	0.0-0.45	16 (80)	4 (20)
C5OH - carnitine	20	0.21 (0.08)	0.16 – 0.24	0.0-0.4	20 (100)	0 (0)
C5:1 - carnitine	20	0.02 (0.01)	0.01 – 0.02	0.0-0.1	20 (100)	0 (0)
C5 - carnitine	20	0.18 (0.08)	0.14 – 0.22	0.0-0.4	20 (100)	0 (0)
C6 – carnitine	20	0.08 (0.03)	0.07 – 0.11	0.0-0.2	20 (100)	0 (0)
C8 - carnitine	20	0.09 (0.03)	0.07 – 0.10	0.0-0.2	20 (100)	0 (0)
C10:1 - carnitine	20	0.10 (0.03)	0.08 – 0.11	0.0-0.2	20 (100)	0 (0)
C10:2 - carnitine	20	0.02 (0.01)	0.01 – 0.02	0.0-0.1	20 (100)	0 (0)
C10 - carnitine	20	0.10 (0.05)	0.08 – 0.13	0.0-0.3	20 (100)	0 (0)
C14:1 - carnitine	20	0.08 (0.04)	0.06 – 0.12	0.0-0.3	20 (100)	0 (0)
C14 - carnitine	20	0.17 (0.08)	0.13 – 0.21	0.0-0.65	20 (100)	0 (0)
C16 - carnitine	20	1.20 (0.49)	0.97 – 1.44	0.0-0.4	20 (100)	0 (0)
C16OH - carnitine	20	0.02 (0.01)	0.01 – 0.02	0.0-0.1	20 (100)	0 (0)
C18:1 - carnitine	20	1.58 (0.82)	1.20 – 1.98	0.0-3.4	19 (95)	1 (5)
C18 - carnitine	20	0.67 (0.26)	0.54 – 0.79	0.0-1.3	20 (100)	0 (0)
C18OH - carnitine	20	0.01 (0.01)	0.01 – 0.02	0.0-0.1	20 (100)	0 (0)

Table 14 represents newborn screening data from 30 cardiomyopathy patients collected within the first 48 hours after birth. Marked difference in two amino acids, alanine ($p<0.05$) and methionine ($p<0.001$) were observed and higher levels of C2, C4 acylcarnitines. TSH was also significantly raised when compared to healthy controls.

Table 14. Data from newborn screening cardiomyopathy case group compared with control group

	Control subjects			Newborn screening Cardiomyopathy case group			
Parameters	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Amino Acids (µM)							
Alanine	200	249.21 (74.8)	238.78 - 259.64	30	295 (143)	242 - 348.31	**0.0074**
Valine	200	142.66 (40.51)	137.01 - 148.31	30	137 (67.8)	111.4 - 162.06	0.5011
Leucine	200	148.61 (41.03)	142.88 - 154.33	30	151 (78.2)	121.5 - 179.86	0.8244
Phenylalanine	200	63.4 (12.6)	61.64 - 65.16	30	69.73 (22.63)	61.28 - 78.19	**0.0244**
Tyrosine	200	110.41 (43.4)	104.35 - 116.46	30	100.87 (77.31)	72 - 129.74	0.3215
Methionine	200	21.12 (5.63)	20.33 - 21.9	30	30.43 (18.4)	23.56 - 37.3	**<.0001**
Citrulline	200	18.58 (9.25)	17.29 - 19.87	30	17.67 (14.78)	12.15 - 23.19	0.6454
Acylcarnitines (µM)	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Total AC	200	35.17 (10.35)	33.73 - 36.61	30	41.12 (16.45)	34.97 - 47.26	**0.0078**
C0-cartinine	200	21.13 (7.41)	20.1 - 22.17	30	24.41 (12.89)	19.6 - 29.22	**0.0453**
C2-carnitine	200	25.92 (8.74)	24.7 - 27.13	30	31.25 (13.9)	26.06 - 36.44	**0.0047**
C3-carnitine	200	2.27 (0.89)	2.14 - 2.39	30	2.52 (1.56)	1.94 - 3.10	0.1949
C4-carnitine	200	0.42 (0.17)	0.40 - 0.44	30	0.57 (0.28)	0.47 - 0.67	**<.0001**
C5-carnitine	200	0.11 (0.05)	0.10 - 0.12	30	0.13 (0.07)	0.11 - 0.16	**0.0153**
C3DC/C40H	119	0.19 (0.24)	0.14 - 0.23	19	0.28 (0.34)	0.11 - 0.44	0.1422
C6-carnitine	200	0.17 (0.08)	0.16 - 0.18	30	0.21 (0.11)	0.17 - 0.25	**0.021**
C4DC/C5OH	200	0.20 (0.10)	0.18 - 0.21	30	0.20 (0.09)	0.17 - 0.24	0.7101
C5DC-carnitine	200	0.16 (0.07)	0.15 - 0.17	30	0.14 (0.09)	0.10 - 0.17	0.0862
C8-carnitine	200	0.16 (0.05)	0.15 - 0.16	30	0.18 (0.06)	0.16 - 0.20	**0.0133**
C10-carnitine	200	0.15 (0.06)	0.14 - 0.16	30	0.13 (0.05)	0.11 - 0.14	0.0233
C14-carnitine	200	0.37 (0.11)	0.35 - 0.38	30	0.36 (0.15)	0.31 - 0.42	0.7665
C16-carnitine	200	3.94 (1.14)	3.78 - 4.10	30	4.14 (1.46)	3.60 - 4.69	0.3924
C16OH-carnitine	200	0.03 (0.02)	0.03 - 0.03	30	0.03 (0.02)	0.03 - 0.04	0.3345
C18-carnitine	200	1.00 (0.27)	0.96 - 1.04	30	1.03 (0.51)	0.84 - 1.23	0.6077
Endocrine markers							
17OHP	200	4.90 (2.53)	4.55 - 5.25	30	4.77 (2.54)	3.82 - 5.72	0.7879
TSH	200	1.97 (1.90)	1.70 - 2.23	30	3.00 (3.49)	1.70 - 4.30	**0.0154**

Ratios	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Leu/Ala	200	0.62 (0.15)	0.60 - 0.64	30	0.55 (0.23)	0.46 - 0.63	0.0257
Leu/Phe	200	2.37 (0.55)	2.29 - 2.45	30	2.21 (1.03)	1.82 - 2.59	0.1961
Phe/Tyr	200	0.64 (0.21)	0.61 - 0.67	30	0.88 (0.41)	0.73 - 1.04	**<.0001**
Met/Phe	200	0.34 (0.09)	0.33 - 0.35	30	0.42 (0.12)	0.37 - 0.46	**<.0001**
C0/(C16+C18)	200	4.46 (1.79)	4.21 - 4.71	30	5.05 (2.40)	4.15 - 5.94	0.1115
C16/C2	154	0.16 (0.11)	0.15 - 0.18	26	0.15 (0.05)	0.13 - 0.17	0.4824
C3/C2	200	0.09 (0.04)	0.09 - 0.10	30	0.08 (0.03)	0.07 - 0.10	0.2200
C3/Met	200	0.11 (0.05)	0.11 - 0.12	30	0.10 (0.05)	0.08 - 0.11	0.0659
C3/C16	200	0.6 (0.25)	0.57 - 0.64	30	0.63 (0.31)	0.51 - 0.74	0.6810
C3/C0	200	0.11 (0.04)	0.11 - 0.12	30	0.11 (0.06)	0.09 - 0.13	0.5863
C8/C2	200	0.01 (0.01)	0.01 - 0.01	30	0.01 (0.01)	0.01 - 0.01	0.8209
C8/C10	200	1.13 (0.38)	1.08 - 1.18	30	1.59 (0.48)	1.41 - 1.77	**<.0001**
C16OH/C16	200	0.01 (0.0)	0.01 - 0.01	30	0.01 (0.0)	0.01 - 0.01	0.2443

4.0 Discussion

Substrate metabolism is a crucial element of cellular health and existence overall. Both anabolic and catabolic metabolic reactions are essential to support all the cellular functions to keep the cell, organ, and organism thriving. When cells in the body generate energy in the form of ATP, they catabolise the various energy sources available, either from endogenous energy stores (primarily glycogen or triacylglycerols), or from exogenous substrates circulating in the body (carbohydrates, fatty acids, amino acids and ketones). In contrast, when cells need to build complex molecules for example proteins for growth or to perform cellular processes like contraction, they consume ATP, releasing the energy required to support these functions. Although glucose and fatty acids are the major contributors to overall ATP production, most cells are also capable of metabolising amino acids and ketone bodies to acetyl-CoA for oxidative energy metabolism. The healthy heart derives virtually all ATP production from mitochondrial oxidative metabolism. The metabolic flexibility of the heart is demonstrated by its ability to rapidly switch its pattern of fuel utilisation in order to adapt to its substrate availability, whereby all energy substrates can be consumed to generate ATP (Ussher et al., 2016).

Interestingly, over the last few decades, there has been growing evidence that the normal patterns of cardiac substrate metabolism are deranged in heart failure and other cardiovascular pathologies (Ahmad et al., 2016; Shah et al., 2010; Yu et al., 2018). The heart undergoes reprogramming of its fuel preference to a foetal format, shifting toward glucose, away from the chief energy substrate for the normal adult heart, fat. The capacity of the mitochondria to generate ATP is also compromised during the development of heart failure (Lai et al., 2014) . Studies conducted in animal models and in humans have shown that the high energy phosphate stores are reduced in failing hearts (Ingwall & Weiss, 2004). The potential role of metabolic disturbances as causal in heart failure is also supported by the observations that genetic defects in mitochondrial fuel catabolism and ATP synthesis cause cardiomyopathy (Bennett, Rinaldo, & Strauss, 2000). Indeed, alterations in cardiac energy metabolism assessed by using positron emission tomography (PET), or cardiac magnetic resonance imaging have been observed in patients with heart failure. Derangement in cardiac metabolism often leads to the accumulation or loss of specific metabolites and these changes in the metabolic pathways can be reflected in the circulation. Therefore, the ability to accurately identify changes in these metabolites and to use this information to detect changes in a specific metabolic pathway adds to the panel of diagnostic information available to physicians. Fortunately, advancements in metabolomics-based technology are enabling detection of these metabolites with high sensitivity and specificity. However, metabolomics only provides a snapshot of metabolic flux through a particular biochemical process and the reason for rise in concentration may not be readily

apparent. Thus, predictions of deranged metabolic pathways based on blood samples alone must be approached with caution. Despite the limitations, detection of metabolite levels related to a common metabolic pathway in the blood can yield significant insight into potential disturbance of energy metabolism to enhance the understanding of disease pathobiology. Furthermore, these biomarkers may have clinical utility in regard to predicting (screening biomarkers) and detecting (diagnostic biomarkers) disease (Ussher et al., 2016).

The purpose of this study was to compare the early postnatal amino acid and acylcarnitine profiles of children with congenital cardiac disease and healthy controls to establish if there is a pattern of metabolites that is similar to that seen in adults with cardiac failure.

4.1 Amino acid metabolism in congenital heart disease

Amino acids are basic protein units that play key cardioprotective roles in cardiac energy metabolism (Jauhiainen et al., 2021; Modi et al., 2006). They are a source of acetyl-CoA thus contributing to NAD and $FADH_2$ synthesis and are able to convert glutamine and glutamate to free radical scavengers. The heart is quite resilient being able to maintain function under conditions of prolonged stress, ischemia or hypoxia. Deprived of FAO by the lack of oxygen supply, cardiac cells derive energy from amino acids due to their potential for non-oxidative metabolism and low contribution to acidic and reactive by-products. Some amino acids are easier converted to metabolic intermediates than others, for example cardiac cells are unable to metabolize the aromatic amino acids (AAAs) whereas alanine is synthesised in abundance under even the most favourable conditions. Others like glutamine, glutamate and the BCAAs are preferentially used as metabolites in the Krebs cycle during anoxia and ischemia. Of note, while amino acid metabolism has an important role and provides a measure of protection against ischemia, it is not able to sustain the cardiac enormous energy demand for extended periods (Drake et. al., 2012).

In this study, the amino acid metabolite profile of newborns with congenital heart disease were characterised by increased alanine, methionine, phenylalanine and leucine levels in blood. Analysis of the stored data from the complete cohort of 547 newborns with congenital cardiac disease revealed a clear increase in circulating levels of alanine consistent with the finding reported by Turer et al (2009). The effect was similar across all five CHD groups and also the newborn screen profile of the cardiomyopathy group. Patients with congenital cardiac disease usually exhibit fast heart rate and increased total body energy expenditure. The oxygen deprived muscle increases glucose consumption and produces lactate to increase anaerobic production of energy. The amino acid alanine is, like lactate, released by the heart during hypoxia and

ischemia confirmed in patients with chronic heart ischemic heart disease and exercise-induced ischemia. Elevated alanine production levels are thought to be the result of increased substrate availability of pyruvate and transamination of glutamate as the nitrogen donor in the alanine transaminase reaction. The end product of anaerobic metabolism of glutamate is succinate and synthesis of succinate would restore the oxidation-reduction balance of the glycolytic pathway disturbed by the synthesis of alanine instead of lactate. This metabolic signature is consistent with compromised glucose oxidation and subsequent diversion of pyruvate away from TCA cycle and into lactate dehydrogenase and alanine transaminase reactions. However, acute alanine production levels may not necessary reflect the rate of glycolytic pyruvate synthesis since ischemia and thus lactate production could be the cause of the release of intracellular stores of alanine (Drake et al., 2012). In the field of metabolic diseases, elevated alanine is often seen as a potential marker of chronic lactic acidosis with alanine forming through transamination of pyruvate and pyruvate and lactate being in equilibrium. Blood lactate concentrations were not reviewed and included in this study, however, the study by Yu et al (2018) has shown significant increases of lactate associated with abnormal cardiac structure, lack of blood supply, increased anaerobic glycolysis and lactic acidosis. Ingested carbohydrate is metabolised to pyruvate which enters the Krebs cycles in the mitochondria. However, during anaerobic metabolism of glucose, pyruvate does not enter the mitochondria but instead is reduced to lactic acid. Usually lactate and pyruvate concentrations are in equilibrium with the lactate to pyruvate ratio being 10:1. A ratio greater than 20:1 indicates a reduced redox state of the cell. Lactate concentration in blood is determined by the pyruvate concentration, with blood hydrogen ion and cellular NADH/NAD ratio. In hypoxia, the mitochondria are unable to generate NAD thus the NADH/NAD ratio increases and results in an increase of the lactate/pyruvate ratio. Lactic acidosis results when the blood lactate concentration is increased but the blood pH is low. In newborns with lactic acidosis, structural congenital heart disease should always be considered (Stern, 1994).

Additionally, methionine levels were also significantly increased in agreement with results of a previous report (Yu et al., 2018). Accumulation of methionine and related metabolites may be associated with possible oxidative stress and secondary accumulation of S-adenosylhomocysteine which results in inhibition of DNA methyltransferase reactions, altered gene expression and DNA hypomethylation demonstrated to be significantly low especially in patients with TOF (Hobbs et al., 2005).

Moreover, Shah et al. (2010) confirmed independent association between elevation in plasma BCAAs and cardiovascular disease risk. A recent study by Zhang et al (2021) found that patients with coronary heart disease had increased levels of isoleucine, valine, leucine, tyrosine and phenylalanine. The effective BCAAs metabolic pathway leads to degradation of BCKAs

into acetyl-CoA and succinyl-CoA, the final product that enters the TCA cycle for ATP synthesis. The underlying reason for the adverse effects of BCAA defects in the heart are not well understood however it has been suggested that elevated BCAA in the stressed myocardium can result in cardiac autophagy obstruction, suppression in the regulation of cellular bioenergetics, and hypertrophy. In addition, BCKAs have direct impact on mitochondrial function and viability of the cardiomyocytes. Dilated cardiomyopathy associated with defects in the mitochondrial respiratory chain is a common complication in propionic aciduria and methylmalonic aciduria patients. Liver transplant in these patients can reverse the symptoms of cardiomyopathy and metabolic stress implying that systemic induction of BCKAs may trigger cardiac dysfunction (Huang et al., 2011).

Furthermore, this study also found increased levels of phenylalanine and phenylalanine/tyrosine ratio (aromatic amino acids) in patients with congenital heart disease. This indicated a disturbance in the AAAs metabolism. Phenylalanine is an essential amino acid which gets hydrolysed to tyrosine by phenylalanine hydroxylase. Research suggests that nitrotyrosine, product of tyrosine nitration, is a potential oxidative stress biomarker associated with inflammation and cell damage (Zhang et al., 2021). The healthy heart is unable to metabolise phenylalanine and tyrosine, which makes them good markers for protein turnover assessments. Disproportionate uptake of aromatic amino acids to other amino acids is an indication that the amino acids are not being used for protein synthesis but have come from proteolysis. Protein degradation during oxygen deprivation is manifested as an increase in the degree of aromatic amino acid release from the cardiac muscle. Heart ischemia and cell damage is expected to be accompanied by disruptive amino acid flux during and post ischemic episodes (Drake et al., 2012).

4.2 Fatty acid metabolism in heart disease

Carnitine is involved in the metabolism of fatty acids, therefore in energy production. Alterations in the level of circulating acylcarnitines indicate a disturbance of fatty acid oxidation metabolism (Rinaldo et al., 2008).

A research study by Ruiz et al. (2017) assessed carnitine and acylcarnitine profiles using blood samples collected from adult patients with heart failure exhibiting depressed left ventricular ejection fraction. The study confirmed 22-79% elevated acylcarnitines of all chain lengths indicative of a global impairment of mitochondrial FAO in one or more tissues including the heart. Unlike the outcome for the adult cohort, this study observed an increase in two of the short chain acylcarnitines, isovaleryl (C5) and butyrylcarnitine (C4). Short-chain acyl-CoA

dehydrogenase is the most vulnerable enzyme which catalyses the first step in mitochondrial fatty acid oxidation. Elevated C4 is the result of the dysfunction of this enzyme (Lopez-Suarez, et al., 2019). Alternatively, this might be due to possible changes in the enzyme catalysing the conversion of acyl-CoA to acylcarnitines specifically carnitine acetyltransferase for the short-chain acylcarnitines or proteins involved in cellular acylcarnitines efflux such as monocarboxylate transporter 9, SLC22 and SLC16 (McCoin et al., 2015).

Along with C4 acylcarnitine, a significant difference with hydroxybutyrylcarnitine (C4OH) was also found in patients with cardiomyopathy. This metabolite reports on mitochondrial levels of β-hydroxybutyryl-CoA, an intermediate in ketone oxidation and product of FAO, suggesting that ketone metabolism may have an important role in cardiac metabolic pathology (Turer et al., 2009). Little is known about ketone body metabolism in the failing human heart, but a previous study by Aubert et al. (2016) demonstrated that the failing rodent heart oxidises ketone bodies as an alternative source for oxidative ATP energy synthesis and observed accumulation of C4OH and C2 acylcarnitines in the failed heart. This could reflect increased ketone oxidation rates associated with greater ketone body delivery in heart failure or a "bottleneck" downstream of ketone fuel input to the TCA cycle (Aubert et al., 2016). Recently, hydroxybutyrylcarnitine levels were shown to be elevated in heart failure patients (Ruiz et al., 2017). As noted, monohydroxilated acylcarnitine species are commonly used as biomarkers of defects in long chain 3-hydroxyacyl-CoA dehydrogenase, and patients with LCHAD gene mutations show muscle weakness and cardiomyopathy (Miller et al., 2021). However, hydroxy acylcarnitines could also be metabolised through omega oxidation in the endoplasmic reticulum instigated by cytochrome *P*-450 enzymes. The role of the endoplasmic reticulum in the pathogenesis of cardiac disease has not been fully explored in the literature to date (Ruiz et al., 2017).

Furthermore, higher levels of C8 were observed in four of the case groups. Octanoyl-CoA is one of the main products from peroxisomal β-oxidation and it is exported to the mitochondria for complete oxidation. Accumulation of C8 acylcarnitine may also serve as an indicator of impaired β-oxidation. A research study by Strand et al. (2017) demonstrated an association between accumulation of even chained acylcarnitines (C6 and C8) and the risk of cardiovascular events.

Finally, elevated levels in acetylcarnitine (C2) in the early postnatal period of cardiomyopathic children could also reflect a mismatch between acetyl-CoA formation from both mitochondrial FAO and glucose oxidation, and its utilisation in the TCA cycle (Ruiz et al., 2017). It is noteworthy that changes in circulating acylcarnitines may also be associated with defective glucose metabolism as it has been previously reported (Makrecka et al., 2014).

Notably, the findings in neonates and children in this study do not support the results of previous studies in adults. Ahmad et al (2016) study of 453 ambulatory adult patients with chronic end-stage heart failure observed significant elevation in long chain acylcarnitines (C16 and C18) in plasma. These are derivatives of the most abundant dietary long chain fatty acids, palmitic and oleic acid, respectively and esterified to carnitine. Functionally, long chain acylcarnitines are facilitating transfer of long chain fatty acids into the mitochondria for β-oxidation. Although the underlying mechanism associated with long chain acylcarnitines in heart failure is unclear, one explanation derives from abnormalities seen in rare Mendelian genetic disorders of the carnitine shuttle, particularly CPT II and CACT deficiencies. Both of which are associated with skeletal and cardiac myopathy (Longo, Frigeni, & Pasquali, 2016). While usually short-lived, they accumulate in a state of ineffective FAO which may be due to defects in mitochondrial enzymes or increased FAO relative to TCA flux. Long chain acylcarnitines have been previously linked to insulin resistance and premature death from heart failure or as a result of kidney dysfunction, a prominent feature of these patients (Ruiz et al., 2017). In addition to the impaired carnitine shuttle leading to mitochondrial dysfunction and compromised β-oxidation, the accumulation of long chain acylcarnitines in blood may reflect a shift toward increased myocardial glucose oxidation with down-regulation of fatty acid oxidation (Lai et al., 2014).

Moreover, in utero, the foetus receives a supply of substrates through the placenta that are required for growth, deposition of fuel stores essential after birth, and for energy metabolism. This continuous transplacental supply is abruptly disrupted at birth and newborns must swiftly adapt to the change in major energy substrate from glucose transferred via the placenta, to fat released from adipose tissue stores and ingested with milk feeds. These changes include an endocrine stress response by insulin and glucagon driving metabolic changes such as breakdown of glycogen, fat, and proteins, and FAO that generates ketone bodies. Immediately after birth, energy requirements are met by glucose derived from glycogenolysis and gluconeogenesis, and to a lesser extent from lactate. After a few hours, fat metabolism becomes more prominent. While energy metabolism is in a physiologically catabolic state in the first days of life, breaking down fat, an anabolic state emerges in normal babies in the process of adaptation to extra uterine life. In heart failure though, the opposite is observed, a decreased use of fatty acids by the heart, and increased glucose oxidation (Piquereau & Ventura-Clapier, 2018). Alternatively, irrespective of the nature of the cardiac defect, malnutrition is common among newborns with CHD. They are prone to malnutrition due to decreased energy intake and malabsorption, and increased energy requirements caused by enhanced metabolism, heart failure, and infections (Piquereau & Ventura-Clapier, 2018). It is suspected that these newborns spent their first days of life in hospital being actively fed with high-energy infant formulas that can assist in achieving nutrition target and promote anabolism. This is in contrast to the control

group who, as healthy newborns, are often relatively fasted as it is normal for newborn babies to be in negative energy balance and lose weight in the first weeks of life. Indeed the upper limit of normal reference range of long chain acylcarnitines in the first few days of life is much higher than that of children. Therefore, the failure of this study to replicate the elevated levels of long chain acylcarnitines seen in adult heart failure could be partially due to the general anabolic status of the cohort.

In addition, energy metabolism in the myocardium in neonates with CHD at the time of the newborn screening is likely to be, in most instances quite different from the myocardium of an adult with heart failure. Many CHD babies are quite stable cardiovascularly and in conditions such as the large septal defects, Tetralogy of Fallot and the outflow tract obstructions it is not until later in life, ideally by which time corrective surgery would have occurred, that the patients experience heart failure. It would thus be interesting to repeat the study in a cohort of older infants whose hearts were more clinically stressed.

Of note, the long chain acylcarnitines were elevated in a small percentage of individual patients with CHD and cardiomyopathy. One suspects these patients had a degree of myocardial stress or failure. Unfortunately, this study did not receive ethical approval to look at individual patients detailed medical records as this would have required parental consent, something beyond the scope of the study.

4.3 Endocrine responses to critical illness

Critical illness such as heart disease triggers immediate biological stress responses comprising of endocrine and metabolic adaptations and alterations within the hypothalamic-pituitary axis. One of the main characteristics is the reactivation of the foetal heart metabolism including the preference of glucose metabolism over fatty acids as the main energy substrate. These vulnerable newborns are exposed to many cardiac and surgical stresses and hemodynamic instability may result in hypoxic insults. The foetal reprogramming leads to a “low energy state” that protects the strained myocardium upon stress (Boonen & Berghe, 2014).

Both fasting and acute illness have an immediate effect on circulating levels of thyroid hormones. Generally, plasma levels of triiodothyronine (T3) decrease as a result of inactivation of thyroid hormone in peripheral tissues but TSH levels have been shown to rise preceding the onset of recovery from severe illness and post-surgery (Boonen & Berghe, 2014). The present study revealed that CHD patients have increased levels of thyroid stimulating hormone compared to the control group. TSH is a biomarker for congenital hypothyroidism usually

diagnosed early by newborn bloodspot screening. Congenital hypothyroidism (CH), characterised by elevated TSH levels, occurs when the thyroid gland fails to develop or function properly and early detection prevents intellectual impairment and optimises growth and development. Common mechanisms involved in the cardiac and thyroid tissues development supports the strong association between hypothyroidism and congenital heart defect in this paediatric cohort (Passeri et al., 2011). Observational studies (Bak et al., 2018; Passeri et al., 2011) report that CHD patients have a 10-fold higher risk for CH than the general newborn population but there was no significant correlation between TSH levels and CHD type or disease severity. Another possible link is the positive correlation between low oxygen saturation in complex heart disease with elevated TSH and CH (Passeri et al., 2011).

Congenital adrenal hyperplasia (CAH) is a rare autosomal recessive disorder in which there is impaired cortisol production due to a deficiency in one of the enzymes required for cortisol synthesis. The lack of enzymatic function leads to a declined cortisol secretion and increased adrenocorticotropic hormone (ACTH) via the classic endocrine feedback loop mechanism. More than 90% of the CAH cases are caused by deficiency in the adrenal 21-hydroxylase enzyme, in which there is impaired conversion of 17OHP to 11-deoxycortisol and progesterone to deoxycorticosterone, essential steps in the pathway to cortisol and aldosterone secretion. The diagnostic biomarker is 17OHP which is grossly elevated in 21-hydroxylase deficiency. The androgen production excess causes virilisation, the hallmark of this disease. Additionally, classic CAH cases also have aldosterone deficiency which can lead to salt-wasting crisis. If left untreated, CAH can result in significant morbidity and mortality, so early diagnosis and treatment is essential. Advances in newborn bloodspot screening techniques have allowed for earlier diagnosis of CAH (Trapp et al., 2013). It has been suggested that bloodspot screening samples should not be collected before 24 hours of age to eliminate placental influence on 17OHP levels. A study by Murphy et al (1983) reported that non-adrenal disorders including severe congenital heart disease or other surgical conditions can increase 17OHP levels above the threshold for healthy term neonates. The cause of increased 17OHP levels in stressed newborns is unclear however a response to stress is not the only explanation because of the low cortisol levels measured in some sick neonates. Cortisol levels in term neonates fall at 1 week after birth and then rise gradually towards adult ranges during year one. Similar changes in corticosteroid binding globulin levels follow throughout this time and reflect liver maturation. This process may be postponed in the preterm and sick neonates. Because cortisol and cortisone are bound to corticosteroid binding globulin, this may be the cause of divergence in early postnatal blood 17OHP levels and cortisol in some stressed preterm neonates (Murphy, 1983).

5.0 Conclusions

This study examined the relationship between metabolic profiles in children with known cardiac disease compared to the normal population. The findings highlighted elevation of short (C4 and C5) and medium chain acylcarnitines (C6 and C8) indicative of defects in activities of some enzymes and/or transporters in fatty acid metabolic pathways. Elevated levels of C4OH-carnitine and C2-carnitine observed in cardiomyopathy suggest switch to ketone body metabolism as an adaptive response to stress. The study also identified alterations in blood levels of branch chain amino acids, methionine, phenylalanine and endocrine markers (TSH and 17OHP) associated with possible oxidative stress and protein degradation during oxygen deprivation. Elevation in alanine concentration is often related to chronic lactic acidosis. However, this study did not observe any difference in long chain acylcarnitine levels reported in adult heart failure. This could be partially due to the general anabolic status of the children cohort which is not in the scope of the project.

Moreover, cardiomyopathies demonstrate a leading cause of death and hospitalization worldwide, with a major economical and societal impact. Despite the typical morphological disease-specific features, cardiac energy alterations can be an incidental or minor finding, often discovered during multisystemic evaluation. Further research in the development of cardiomyopathies could advance diagnostic approaches and develop early therapies to improve patients' quality of life.

5.1 Study limitations and future directions

The strengths of the study include its relatively large sample size of sample data from newborns with congenital heart disease from several geographical locations in New Zealand. To the best of our knowledge, there are no other similar studies carried out on children with congenital cardiac disease or cardiomyopathy to compare data with available in the literature. The mechanisms linking circulating levels of metabolic analytes to congenital heart disease are still to be elucidated.

This study also has several limitations. As with any research project, the results are often limited because of time and financial constraints. Since this study was undertaken as part of a university Master's degree, the sample number was restricted due to time limitations and clinical characteristics of the study participants as the baseline examination were not available and are not included.

The number of data available for the cardiomyopathy group was relatively small.
With new, improved methods available, many additional plasma metabolites such as energy-related metabolites including lactate, pyruvate, ketone bodies (acetoacetate and β-hydroxybutyrate), and TCA cycle intermediates (citrate, succinate, fumarate, and malate) can be measured, which can be of importance for cardiac disease. Also, prospective study design with detailed data collection over a long period of follow-up, and the same experimental setup for metabolite quantification across multiple studies can be of benefit.

For ethical reasons there was no access to the patients' medical records. Thus the degree of cardiac compromise for each patient was unknown. It seems likely that babies who were sicker at the time of newborn screening sampling are likely to have different metabolic profiles. For instance cardiologists make use of a group of medical call inotropes to support a failing myocardium and it would be fascinating to review the metabolic profiles of those babies that were receiving these medications. Likewise this study did not have information on the feeding status of the cohort. It is presumed that compared to the general population, or control group, they were, as a group, relatively anabolic due to active feeding. This would strongly influence the metabolic profiles, especially the long chain acylcarnitine levels which rise during fasting. Finally, the reported associations may represent signals due to other metabolites or other factors. This was a study on peripheral metabolite profiles and thus cannot assume that they represent myocardial metabolism, but rather reflect overall changes in metabolism. Therefore, future studies should explore if these metabolites play a causal role in cardiac disorders in neonates and children.

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7.0 Appendices

7.1 Appendix A

Table 7. Comparison of amino acids and acylcarnitines levels between control subjects and complex cardiac disease (complete data)

	Control Subjects			Patients with Complex CD			
Parameters	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Amino Acids (μM)							
Alanine	200	249.21 (74.8)	238.78 - 259.64	32	308.91 (99.89)	272.90 - 344.93	**8.98E-05**
Citrulline	200	18.58 (9.25)	17.29 - 19.87	32	19.22 (8.34)	16.21 - 22.23	0.7137
Leucine	200	148.61 (41.03)	142.88 - 154.33	32	158.56 (89.49)	126.3 - 190.83	0.3001
Methionine	200	21.12 (5.63)	20.33 - 21.9	32	31.13 (13.99)	26.08 - 36.17	**1.04E-11**
Phenylalanine	200	63.4 (12.6)	61.64 - 65.16	32	68.91 (22.23)	60.89 - 76.92	**0.0441**
Tyrosine	200	110.41 (43.4)	104.35 - 116.46	32	98.72 (42.87)	83.26 - 114.17	0.1580
Valine	200	142.66 (40.51)	137.01 - 148.31	32	130.13 (65.02)	106.68 - 153.57	0.1413
Acylcarnitines (μM)	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Carnitine and short-chain acylcarnitines							
Total Acylcarnitine	200	35.17 (10.35)	33.73 - 36.61	32	35.11 (12.63)	30.56 - 39.67	0.9775
C0-carnitine	200	21.13 (7.41)	20.1 - 22.17	32	25.08 (12.81)	20.46 - 29.70	**0.0137**
C2-carnitine	200	25.92 (8.74)	24.7 - 27.13	32	26.75 (10.82)	22.85 - 30.66	0.6274
C3-carnitine	200	2.27 (0.89)	2.14 - 2.39	32	2.31 (1.12)	1.90 - 2.71	0.8093
C3DC/C4OH-carnitine	119	0.19 (0.24)	0.14 - 0.23	24	0.16 (0.13)	0.10 - 0.21	0.5554
C4-carnitine	200	0.42 (0.17)	0.40 - 0.44	32	0.51 (0.19)	0.44 - 0.58	**0.0052**
C4DC/C5OH-carnitine	200	0.20 (0.10)	0.18 - 0.21	32	0.20 (0.10)	0.16 - 0.24	0.7249
C5-carnitine	200	0.11 (0.05)	0.10 - 0.12	32	0.15 (0.13)	0.10 - 0.20	0.0015
C5DC-carnitine	200	0.16 (0.07)	0.15 - 0.17	32	0.11 (0.06)	0.08 - 0.13	8.13E-05
C5:1-carnitine	200	0.02 (0.02)	0.01 - 0.02	32	0.011 (0.010)	0.007 - 0.014	0.0400
Medium-chain acylcarnitines							
C6-carnitine	200	0.17 (0.08)	0.16 - 0.18	32	0.20 (0.09)	0.17 - 0.23	0.0558
C8-carnitine	200	0.16 (0.05)	0.15 - 0.16	32	0.19 (0.08)	0.16 - 0.22	0.0022

C10-carnitine	200	0.15 (0.06)	0.14 - 0.16	32	0.14 (0.08)	0.11 - 0.17	0.4869
C10:1-carnitine	200	0.07 (0.04)	0.07 - 0.08	32	0.079 (0.057)	0.058 - 0.099	0.3979
C10:2-carnitine	200	0.02 (0.02)	0.02 - 0.02	32	0.028 (0.014)	0.022 - 0.033	0.0862
Long-chain acylcarnitines							
C14-carnitine	200	0.37 (0.11)	0.35 - 0.38	32	0.28 (0.13)	0.23 - 0.32	2.69E-05
C14:1-carnitine	200	0.20 (0.09)	0.19 - 0.21	32	0.13 (0.07)	0.10 - 0.15	3.10E-05
C16-carnitine	200	3.94 (1.14)	3.78 - 4.10	32	3.22 (1.41)	2.71 - 3.73	0.0015
C16:1-carnitine	95	0.25 (0.09)	0.23 - 0.27	4	0.31 (0.10)	0.16 - 0.46	0.1850
C16OH-carnitine	200	0.03 (0.02)	0.03 - 0.03	32	0.024 (0.013)	0.020 - 0.029	0.1881
C16:1OH-carnitine	95	0.05 (0.02)	0.05 - 0.06	4	0.070 (0.018)	0.041 - 0.099	0.0756
C18-carnitine	200	1.00 (0.27)	0.96 - 1.04	32	0.79 (0.25)	0.69 - 0.88	3.86E-05
C18:1-carnitine	200	1.26 (0.52)	1.19 - 1.34	32	1.10 (0.41)	0.95 - 1.24	0.0841
C18OH-carnitine	95	0.01 (0.01)	0.01 - 0.02	4	0.013 (0.005)	0.005 - 0.020	0.6513
C18:1OH-carnitine	95	0.02 (0.01)	0.02 - 0.02	4	0.015 (0.010)	0.0 - 0.031	0.3137
Endocrine markers	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Thyroid Stimulating Hormone	200	1.97 (1.90)	1.70 - 2.23	31	3.06 (1.69)	2.44 - 3.69	**0.0026**
17-Hydroxy Progesterone	200	4.90 (2.53)	4.55 - 5.25	31	5.74 (5.37)	3.77 - 7.71	0.1546
Ratios	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Met/Phe	200	0.34 (0.09)	0.33 - 0.35	32	0.45 (0.11)	0.41 - 0.49	**1.69E-09**
Leu/Phe	200	2.37 (0.55)	2.29 - 2.45	32	2.25 (0.84)	1.95 - 2.55	0.3089
Phe/Tyr	200	0.64 (0.21)	0.61 - 0.67	32	0.83 (0.51)	0.65 - 1.01	**0.0002**
Leu/Ala	200	0.62 (0.15)	0.60 - 0.64	32	0.53 (0.28)	0.43 - 0.64	0.0117
C3/Met	200	0.11 (0.05)	0.11 - 0.12	32	0.080 (0.045)	0.064 - 0.096	0.0006
C0/(C16+C18)	200	4.46 (1.79)	4.21 - 4.71	32	7.04 (4.01)	5.60 - 8.49	**4.30E-09**
(C16+C18:1)/C2	200	0.22 (0.14)	0.20 - 0.24	32	0.17 (0.05)	0.15 - 0.19	0.0458
C14:1/C16	200	0.05 (0.02)	0.05 - 0.06	32	0.043 (0.025)	0.034 - 0.052	0.0371
C3/C2	200	0.09 (0.04)	0.09 - 0.10	32	0.09 (0.04)	0.07 - 0.10	0.6290
C3/C0	200	0.11 (0.04)	0.11 - 0.12	32	0.10 (0.07)	0.08 - 0.13	0.2809
C16/C2	154	0.16 (0.11)	0.15 - 0.18	30	0.13 (0.04)	0.11 - 0.14	0.0672
C8/C2	200	0.01 (0.01)	0.01 - 0.01	32	0.009 (0.006)	0.007 - 0.011	0.0983
C16OH/C16	200	0.01 (0.0)	0.01 - 0.01	32	0.009 (0.006)	0.007 - 0.011	0.1049
C8/C10	200	1.13 (0.38)	1.08 - 1.18	32	1.46 (0.48)	1.29 - 1.64	1.26E-05
C3/C16	200	0.60 (0.25)	0.57 - 0.64	32	0.82 (0.48)	0.65 - 0.99	0.0001

Table 8. Comparison of amino acids and acylcarnitines levels between control subjects and mixing cardiac disease (complete data)

	Control subjects			Patients with Mixing CD			
Parameters	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Amino acids (µM)							
Alanine	200	249.21 (74.8)	238.78 - 259.64	229	298.21 (104.78)	284.56 - 311.85	**<.0001**
Citrulline	200	18.58 (9.25)	17.29 - 19.87	229	18.53 (9.82)	17.25 - 19.81	0.9593
Leucine	200	148.61 (41.03)	142.88 - 154.33	229	168.69 (85.75)	157.53 - 179.86	0.0026
Methionine	200	21.12 (5.63)	20.33 - 21.9	229	33.04 (16.04)	30.95 - 35.13	**<.0001**
Phenyalanine	200	63.4 (12.6)	61.64 - 65.16	229	73.61 (23.34)	70.57 - 76.65	**<.0001**
Tyrosine	200	110.41 (43.4)	104.35 - 116.46	229	82.76 (48.63)	76.42 - 89.09	<.0001
Valine	200	142.66 (40.51)	137.01 - 148.31	229	141.79 (58.07)	134.23 - 149.36	0.8600
Acylcarnitines (µM)	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Carnitine and short chain acylcarnitines							
Total Acylcarnitine	200	35.17 (10.35)	33.73 - 36.61	229	29.33 (13.34)	27.59 - 31.06	<.0001
C0-carnitine	200	21.13 (7.41)	20.1 - 22.17	229	23.68 (10.97)	22.25 - 25.11	**0.0058**
C2-carnitine	200	25.92 (8.74)	24.7 - 27.13	229	21.65 (11.03)	20.22 - 23.09	<.0001
C3-carnitine	200	2.27 (0.89)	2.14 - 2.39	229	2.24 (1.24)	2.08 - 2.40	0.7857
C3DC/C4OH-carnitine	119	0.19 (0.24)	0.14 - 0.23	176	0.12 (0.15)	0.10 - 0.15	0.0072
C4-carnitine	200	0.42 (0.17)	0.40 - 0.44	229	0.49 (0.24)	0.46 - 0.52	**0.0006**
C4DC/C5OH-carnitine	200	0.20 (0.10)	0.18 - 0.21	229	0.21 (0.10)	0.19 - 0.22	0.2088
C5-carnitine	200	0.11 (0.05)	0.10 - 0.12	229	0.13 (0.07)	0.12 - 0.13	0.0073
C5DC-carnitine	200	0.16 (0.07)	0.15 - 0.17	229	0.11 (0.07)	0.10 - 0.11	<.0001
C5:1-carnitine	200	0.02 (0.02)	0.01 - 0.02	229	0.016 (0.017)	0.013 - 0.018	0.2586
Medium-chain acylcarnitines							
C6-carnitine	200	0.17 (0.08)	0.16 - 0.18	229	0.20 (0.10)	0.18 - 0.21	0.0012
C8-carnitine	200	0.16 (0.05)	0.15 - 0.16	229	0.17 (0.06)	0.16 - 0.18	0.0455
C10-carnitine	200	0.15 (0.06)	0.14 - 0.16	229	0.12 (0.05)	0.11 - 0.12	<.0001
C10:1-carnitine	200	0.07 (0.04)	0.07 - 0.08	229	0.061 (0.042)	0.056 - 0.067	0.0185
C10:2-carnitine	200	0.02 (0.02)	0.02 - 0.02	229	0.027 (0.024)	0.024 - 0.030	0.0241

Long-chain acylcarnitines							
C14-carnitine	200	0.37 (0.11)	0.35 - 0.38	229	0.27 (0.12)	0.26 - 0.29	<.0001
C14:1-carnitine	200	0.20 (0.09)	0.19 - 0.21	229	0.11 (0.06)	0.10 - 0.11	<.0001
C16-carnitine	200	3.94 (1.14)	3.78 - 4.10	229	2.83 (1.33)	2.66 - 3.01	<.0001
C16:1-carnitine	95	0.25 (0.09)	0.23 - 0.27	41	0.22 (0.09)	0.19 - 0.25	0.0610
C16OH-carnitine	200	0.03 (0.02)	0.03 - 0.03	229	0.021 (0.013)	0.020 - 0.023	<.0001
C16:1OH-carnitine	95	0.05 (0.02)	0.05 - 0.06	41	0.055 (0.029)	0.046 - 0.064	0.3702
C18-carnitine	200	1.00 (0.27)	0.96 - 1.04	229	0.76 (0.34)	0.71 - 0.80	<.0001
C18:1-carnitine	200	1.26 (0.52)	1.19 - 1.34	229	0.97 (0.51)	0.90 - 1.04	<.0001
C18OH-carnitine	95	0.01 (0.01)	0.01 - 0.02	41	0.006 (0.007)	0.004 - 0.008	<.0001
C18:1OH-carnitine	95	0.02 (0.01)	0.02 - 0.02	41	0.015 (0.010)	0.012 - 0.018	0.0080
Endocrine markers	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Thyroid Stimulating Hormone	200	1.97 (1.90)	1.70 - 2.23	225	2.76 (3.88)	2.25 - 3.27	**0.0085**
17-Hydroxy Progesterone	200	4.90 (2.53)	4.55 - 5.25	217	7.46 (9.04)	6.25 - 8.67	**0.0001**
Ratios	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Met/Phe	200	0.34 (0.09)	0.33 - 0.35	229	0.44 (0.13)	0.43 - 0.46	**<.0001**
Leu/Phe	200	2.37 (0.55)	2.29 - 2.45	229	2.32 (0.96)	2.20 - 2.45	0.5308
Phe/Tyr	200	0.64 (0.21)	0.61 - 0.67	229	1.05 (0.52)	0.98 - 1.12	**<.0001**
Leu/Ala	200	0.62 (0.15)	0.60 - 0.64	229	0.60 (0.31)	0.56 - 0.64	0.3369
C3/Met	200	0.11 (0.05)	0.11 - 0.12	229	0.076 (0.046)	0.070 - 0.082	<.0001
C0/(C16+C18)	200	4.46 (1.79)	4.21 - 4.71	229	7.97 (6.02)	7.19 - 8.76	**<.0001**
(C16+C18:1)/C2	200	0.22 (0.14)	0.20 - 0.24	229	0.19 (0.08)	0.18 - 0.20	0.0124
C14:1/C16	200	0.05 (0.02)	0.05 - 0.06	229	0.043 (0.027)	0.040 - 0.046	0.0001
C3/C2	200	0.09 (0.04)	0.09 - 0.10	229	0.11 (0.05)	0.10 - 0.12	<.0001
C3/C0	200	0.11 (0.04)	0.11 - 0.12	229	0.10 (0.06)	0.10 - 0.11	0.0162
C16/C2	219	0.14 (0.06)	0.13 - 0.15	154	0.16 (0.11)	0.15 - 0.18	0.0156
C8/C2	200	0.01 (0.01)	0.01 - 0.01	229	0.010 (0.007)	0.009 - 0.011	<.0001
C16OH/C16	200	0.01 (0.0)	0.01 - 0.01	229	0.009 (0.007)	0.008 - 0.010	0.0035
C8/C10	200	1.13 (0.38)	1.08 - 1.18	229	1.59 (0.56)	1.51 - 1.66	<.0001
C3/C16	200	0.60 (0.25)	0.57 - 0.64	229	0.89 (0.55)	0.82 - 0.96	<.0001

Table 9. Comparison of amino acids and acylcarnitines levels between control subjects and pulmonary outflow obstruction (complete data)

	Control subjects			Patients with pulmonary outflow obstruction			
Parameters	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Amino Acids (µM)							
Alanine	200	249.21 (74.8)	238.78 - 259.64	153	321.89 (116.82)	303.23 - 340.55	**<.0001**
Citrulline	200	18.58 (9.25)	17.29 - 19.87	153	19.86 (9.08)	18.41 - 21.31	0.1940
Leucine	200	148.61 (41.03)	142.88 - 154.33	153	172.5 (84.47)	159.01 - 186.0	0.0005
Methionine	200	21.12 (5.63)	20.33 - 21.9	153	34.24 (17.51)	31.44 - 37.03	**<.0001**
Phenylalanine	200	63.4 (12.6)	61.64 - 65.16	153	73.35 (25.86)	69.22 - 77.48	**<.0001**
Tyrosine	200	110.41 (43.4)	104.35 - 116.46	153	76.03 (29.86)	71.26 - 80.80	<.0001
Valine	200	142.66 (40.51)	137.01 - 148.31	153	139.44 (61.6)	129.6 - 149.28	0.5547
Acylcarnitine (µM)	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Carnitine and short-chain acylcarnitines							
Total Acylcarnitine	200	35.17 (10.35)	33.73 - 36.61	153	33.78 (14.54)	31.46 - 36.1	0.2948
C0-carnitine	200	21.13 (7.41)	20.1 - 22.17	153	26.83 (12.29)	24.87 - 28.8	**<.0001**
C2-carnitine	200	25.92 (8.74)	24.7 - 27.13	153	25.31 (12.09)	23.38 - 27.24	0.5856
C3-carnitine	200	2.27 (0.89)	2.14 - 2.39	153	2.52 (1.53)	2.27 - 2.76	0.0539
C3DC/C4OH-carnitine	119	0.19 (0.24)	0.14 - 0.23	123	0.11 (0.14)	0.09 - 0.14	0.0037
C4-carnitine	200	0.42 (0.17)	0.40 - 0.44	153	0.53 (0.24)	0.49 - 0.57	**<.0001**
C4DC/C5OH-carnitine	200	0.20 (0.10)	0.18 - 0.21	153	0.22 (0.12)	0.20 - 0.24	0.0142
C5-carnitine	200	0.11 (0.05)	0.10 - 0.12	153	0.14 (0.08)	0.13 - 0.15	<.0001
C5DC-carnitine	200	0.16 (0.07)	0.15 - 0.17	153	0.12 (0.07)	0.10 - 0.13	<.0001
C5:1-carnitine	200	0.02 (0.02)	0.01 - 0.02	153	0.015 (0.011)	0.013 - 0.017	0.1934
Medium-chain acylcarnitines							
C6 carnitine	200	0.17 (0.08)	0.16 - 0.18	153	0.20 (0.10)	0.19 - 0.22	0.0004
C8 carnitine	200	0.16 (0.05)	0.15 - 0.16	153	0.17 (0.05)	0.16 - 0.18	0.0248
C10 carnitine	200	0.15 (0.06)	0.14 - 0.16	153	0.11 (0.04)	0.11 - 0.12	<.0001
C10:1 carnitine	200	0.07 (0.04)	0.07 - 0.08	153	0.069 (0.036)	0.064 - 0.075	0.6672
C10:2 carnitine	200	0.02 (0.02)	0.02 - 0.02	153	0.029 (0.017)	0.027 - 0.032	<.0001
Long-chain acylcarnitines							
C14 carnitine	200	0.37 (0.11)	0.35 - 0.38	153	0.29 (0.12)	0.27 - 0.31	<.0001
C14:1 carnitine	200	0.20 (0.09)	0.19 - 0.21	153	0.11 (0.07)	0.10 - 0.12	<.0001

C16 carnitine	200	3.94 (1.14)	3.78 - 4.10	153	3.09 (1.46)	2.86 - 3.32	<.0001
C16:1 carnitine	95	0.25 (0.09)	0.23 - 0.27	36	0.21 (0.10)	0.17 - 0.24	0.0221
C16OH carnitine	200	0.03 (0.02)	0.03 - 0.03	153	0.022 (0.014)	0.020 - 0.024	0.0003
C16:1OH carnitine	95	0.05 (0.02)	0.05 - 0.06	36	0.066 (0.036)	0.053 - 0.078	0.0048
C18 carnitine	200	1.00 (0.27)	0.96 - 1.04	153	0.82 (0.34)	0.77 - 0.88	<.0001
C18:1 carnitine	200	1.26 (0.52)	1.19 - 1.34	153	1.15 (0.52)	1.06 - 1.23	0.0364
C18OH carnitine	95	0.01 (0.01)	0.01 - 0.02	36	0.006 (0.007)	0.004 - 0.009	<.0001
C18:1OH carnitine	95	0.02 (0.01)	0.02 - 0.02	36	0.015 (0.011)	0.011 - 0.019	0.0069
Endocrine markers	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Thyroid Stimulating Hormone	200	1.97 (1.90)	1.70 - 2.23	149	3.21 (3.70)	2.61 - 3.81	**<.0001**
17-Hydroxy Progesterone	200	4.90 (2.53)	4.55 - 5.25	136	7.02 (7.09)	5.82 - 8.22	**0.0001**
Ratios	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Met/Phe	200	0.34 (0.09)	0.33 - 0.35	153	0.47 (0.15)	0.44 - 0.49	**<.0001**
Leu/Phe	200	2.37 (0.55)	2.29 - 2.45	153	2.40 (0.94)	2.25 - 2.56	0.6578
Phe/Tyr	200	0.64 (0.21)	0.61 - 0.67	153	1.12 (0.68)	1.01 - 1.23	**<.0001**
Leu/Ala	200	0.62 (0.15)	0.60 - 0.64	153	0.57 (0.30)	0.53 - 0.62	0.0637
C3/Met	200	0.11 (0.05)	0.11 - 0.12	153	0.083 (0.051)	0.074 - 0.091	<.0001
C0/(C16+C18)	200	4.46 (1.79)	4.21 - 4.71	153	8.57 (6.66)	7.51 - 9.63	**<.0001**
(C16+C18:1)/C2	200	0.22 (0.14)	0.20 - 0.24	153	0.18 (0.08)	0.17 - 0.19	0.0042
C14:1/C16	200	0.05 (0.02)	0.05 - 0.06	153	0.043 (0.031)	0.038 - 0.048	0.0010
C3/C2	200	0.09 (0.04)	0.09 - 0.10	153	0.10 (0.04)	0.10 - 0.11	0.0133
C3/CO	200	0.11 (0.04)	0.11 - 0.12	153	0.10 (0.05)	0.09 - 0.11	0.0020
C16/C2	154	0.16 (0.11)	0.15 - 0.18	143	0.13 (0.06)	0.12 - 0.14	0.0032
C8/C2	200	0.01 (0.01)	0.01 - 0.01	153	0.008 (0.006)	0.007 - 0.009	0.0270
C16OH/C16	200	0.01 (0.0)	0.01 - 0.01	153	0.009 (0.008)	0.008 - 0.010	0.0327
C8/C10	200	1.13 (0.38)	1.08 - 1.18	153	1.64 (0.57)	1.55 - 1.73	<.0001
C3/C16	200	0.60 (0.25)	0.57 - 0.64	153	0.97 (0.72)	0.86 - 1.09	<.0001

Table 10. Data comparison of amino acids and acylcarnitines levels between control samples and systemic outflow obstruction samples (complete data)

	Control subjects			Patients with Systemic outflow obstruction			
Parameters	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Amino Acids (µM)							
Alanine	200	249.21 (74.8)	238.78 - 259.64	234	303.4 (134.57)	286.07 - 320.73	**<.0001**
Citrulline	200	18.58 (9.25)	17.29 - 19.87	236	19.94 (9.06)	18.78 - 21.11	0.1212
Leucine	200	148.61 (41.03)	142.88 - 154.33	236	209.06 (215.1)	181.47 - 236.64	**0.0001**
Methionine	200	21.12 (5.63)	20.33 - 21.9	236	40.49 (30.61)	36.56 - 44.41	**<.0001**
Phenylalanine	200	63.4 (12.6)	61.64 - 65.16	236	80.19 (40.22)	75.03 - 85.35	**<.0001**
Tyrosine	200	110.41 (43.4)	104.35 - 116.46	236	78.06 (45.0)	72.29 - 83.83	<.0001
Valine	200	142.66 (40.51)	137.01 - 148.31	236	162.41 (98.41)	149.79 - 175.03	**0.0083**
Acylcarnitines (µM)	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Carnitine and short-chain acylcarnitines							
Total Acylcarnitine	200	35.17 (10.35)	33.73 - 36.61	236	32.11 (14.23)	30.29 - 33.94	0.0119
C0-carnitine	200	21.13 (7.41)	20.1 - 22.17	236	24.64 (10.56)	23.28 - 25.99	**<.0001**
C2-carnitine	200	25.92 (8.74)	24.7 - 27.13	236	24.02 (11.71)	22.52 - 25.52	0.0599
C3-carnitine	200	2.27 (0.89)	2.14 - 2.39	236	2.37 (1.32)	2.20 - 2.54	0.3491
C3DC/C4OH-carnitine	119	0.19 (0.24)	0.14 - 0.23	184	0.17 (0.22)	0.13 - 0.20	0.4482
C4-carnitine	200	0.42 (0.17)	0.40 - 0.44	236	0.50 (0.22)	0.47 - 0.53	**<.0001**
C4DC/C5OH-carnitine	200	0.20 (0.10)	0.18 - 0.21	236	0.21 (0.10)	0.19 - 0.22	0.2651
C5-carnitine	200	0.11 (0.05)	0.10 - 0.12	236	0.14 (0.09)	0.13 - 0.16	<.0001
C5DC-carnitine	200	0.16 (0.07)	0.15 - 0.17	236	0.12 (0.06)	0.11 - 0.12	<.0001
C5:1-carnitine	200	0.02 (0.02)	0.01 - 0.02	236	0.017 (0.016)	0.015 - 0.019	0.6311
Medium-chain acylcarnitines							
C6-carnitine	200	0.17 (0.08)	0.16 - 0.18	236	0.20 (0.09)	0.18 - 0.21	0.0015
C8-carnitine	200	0.16 (0.05)	0.15 - 0.16	236	0.18 (0.07)	0.17 - 0.19	0.0014
C10-carnitine	200	0.15 (0.06)	0.14 - 0.16	236	0.13 (0.08)	0.12 - 0.14	0.0012
C10:1-carnitine	200	0.07 (0.04)	0.07 - 0.08	236	0.080 (0.061)	0.072 - 0.088	0.0841
C10:2-carnitine	200	0.02 (0.02)	0.02 - 0.02	234	0.030 (0.022)	0.028 - 0.033	<.0001

Long-chain acylcarnitines							
C14-carnitine	200	0.37 (0.11)	0.35 - 0.38	236	0.28 (0.13)	0.26 - 0.30	<.0001
C14:1-carnitine	200	0.20 (0.09)	0.19 - 0.21	236	0.14 (0.13)	0.12 - 0.15	<.0001
C16-carnitine	200	3.94 (1.14)	3.78 - 4.10	236	2.91 (1.51)	2.71 - 3.10	<.0001
C16:1-carnitine	95	0.25 (0.09)	0.23 - 0.27	46	0.18 (0.11)	0.15 - 0.21	<.0001
C16OH-carnitine	200	0.03 (0.02)	0.03 - 0.03	236	0.026 (0.016)	0.023 - 0.028	0.0593
C16:1OH-carnitine	95	0.05 (0.02)	0.05 - 0.06	46	0.050 (0.030)	0.041 - 0.059	0.7877
C18-carnitine	200	1.00 (0.27)	0.96 - 1.04	236	0.78 (0.40)	0.73 - 0.83	<.0001
C18:1-carnitine	200	1.26 (0.52)	1.19 - 1.34	236	1.07 (0.60)	1.0 - 1.15	0.0005
C18OH-carnitine	95	0.01 (0.01)	0.01 - 0.02	46	0.006 (0.006)	0.004 - 0.007	<.0001
C18:1OH-carnitine	95	0.02 (0.01)	0.02 - 0.02	46	0.015 (0.008)	0.013 - 0.018	0.0049
Endocrine markers	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Thyroid Stimulating Hormone	200	1.97 (1.90)	1.70 - 2.23	229	2.75 (3.43)	2.30 - 3.19	**0.0044**
17-Hydroxy Progesterone	200	4.90 (2.53)	4.55 - 5.25	221	6.50 (9.64)	5.22 - 7.78	**0.0234**
Ratios	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Met/Phe	200	0.34 (0.09)	0.33 - 0.35	236	0.49 (0.15)	0.47 - 0.50	**<.0001**
Leu/Phe	200	2.37 (0.55)	2.29 - 2.45	236	2.51 (0.90)	2.40 - 2.63	0.0503
Phe/Tyr	200	0.64 (0.21)	0.61 - 0.67	236	1.23 (0.67)	1.15 - 1.32	**<.0001**
Leu/Ala	200	0.62 (0.15)	0.60 - 0.64	236	0.70 (0.37)	0.66 - 0.75	0.0031
C3/Met	200	0.11 (0.05)	0.11 - 0.12	236	0.072 (0.053)	0.065 - 0.079	<.0001
C0/(C16+C18)	200	4.46 (1.79)	4.21 - 4.71	236	8.71 (8.42)	7.63 - 9.79	**<.0001**
(C16+C18:1)/C2	200	0.22 (0.14)	0.20 - 0.24	236	0.18 (0.07)	0.17 - 0.19	0.0003
C14:1/C16	200	0.05 (0.02)	0.05 - 0.06	236	0.052 (0.040)	0.047 - 0.057	0.8472
C3/C2	200	0.09 (0.04)	0.09 - 0.10	236	0.11 (0.05)	0.10 - 0.11	0.0006
C3/C0	200	0.11 (0.04)	0.11 - 0.12	236	0.10 (0.05)	0.10 - 0.11	0.0075
C16/C2	154	0.16 (0.11)	0.15 - 0.18	217	0.13 (0.05)	0.12 - 0.14	0.0002
C8/C2	200	0.01 (0.01)	0.01 - 0.01	236	0.010 (0.008)	0.009 - 0.011	<.0001
C8/C10	200	1.13 (0.38)	1.08 - 1.18	236	1.54 (0.59)	1.47 - 1.62	**<.0001**
C16OH/C16	200	0.01 (0.0)	0.01 - 0.01	236	0.011 (0.009)	0.010 - 0.012	<.0001
C3/C16	200	0.60 (0.25)	0.57 - 0.64	236	0.98 (0.67)	0.89 - 1.07	<.0001

Table 11. Comparison of amino acids and acylcarnitines levels between control subjects and patients with "other" congenital cardiac disorders (complete data)

		Control subjects			Patients with "Other" CD		
Parameters	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Amino Acids (µM)							
Alanine	200	249.21 (74.8)	238.78 - 259.64	76	267.34 (115.71)	240.90 - 293.78	0.1270
Citrulline	200	18.58 (9.25)	17.29 - 19.87	76	16.41 (8.14)	14.55 - 18.27	0.0731
Leucine	200	148.61 (41.03)	142.88 - 154.33	76	164.86 (75.21)	147.67 - 182.04	**0.0227**
Methionine	200	21.12 (5.63)	20.33 - 21.9	76	30.25 (15.41)	26.73 - 33.77	**<.0001**
Phenylalanine	200	63.4 (12.6)	61.64 - 65.16	76	66.58 (23.78)	61.15 - 72.01	0.1523
Tyrosine	200	110.41 (43.4)	104.35 - 116.46	76	71.05 (27.51)	64.77 - 77.34	<.0001
Valine	200	142.66 (40.51)	137.01 - 148.31	76	145.96 (54.68)	133.47 - 158.46	0.5853
Acylcarnitines (µM)	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Carnitine and short-chain acylcarnitines							
Total Acylcarnitine	200	35.17 (10.35)	33.73 - 36.61	76	24.76 (12.95)	21.8 - 27.72	<.0001
C0-carnitine	200	21.13 (7.41)	20.1 - 22.17	76	22.96 (10.25)	20.62 - 25.31	0.1025
C2-carnitine	200	25.92 (8.74)	24.7 - 27.13	76	18.36 (10.54)	15.96 - 20.77	<.0001
C3-carnitine	200	2.27 (0.89)	2.14 - 2.39	76	1.70 (1.03)	1.46 - 1.94	<.0001
C3DC/C4OH-carnitine	119	0.19 (0.24)	0.14 - 0.23	65	0.12 (0.12)	0.09 - 0.15	0.0383
C4-carnitine	200	0.42 (0.17)	0.40 - 0.44	76	0.46 (0.18)	0.42 - 0.50	0.1020
C4DC/C5OH-carnitine	200	0.20 (0.10)	0.18 - 0.21	76	0.22 (0.10)	0.20 - 0.25	0.0301
C5-carnitine	200	0.11 (0.05)	0.10 - 0.12	76	0.13 (0.07)	0.11 - 0.14	0.0185
C5DC-carnitine	200	0.16 (0.07)	0.15 - 0.17	76	0.10 (0.06)	0.09 - 0.11	<.0001
C5:1-carnitine	200	0.02 (0.02)	0.01 - 0.02	76	0.015 (0.013)	0.012 - 0.018	0.3765
Medium-chain acylcarnitines							
C6-carnitine	200	0.17 (0.08)	0.16 - 0.18	76	0.19 (0.08)	0.17 - 0.20	0.1147
C8-carnitine	200	0.16 (0.05)	0.15 - 0.16	76	0.17 (0.06)	0.16 - 0.18	0.0654
C10-carnitine	200	0.15 (0.06)	0.14 - 0.16	76	0.11 (0.04)	0.10 - 0.12	<.0001
C10:1-carnitine	200	0.07 (0.04)	0.07 - 0.08	76	0.069 (0.042)	0.059 - 0.078	0.6804
C10:2-carnitine	200	0.02 (0.02)	0.02 - 0.02	76	0.030 (0.024)	0.025 - 0.036	0.0013

Long-chain acylcarnitines							
C14-carnitine	200	0.37 (0.11)	0.35 - 0.38	76	0.25 (0.13)	0.22 - 0.28	<.0001
C14:1-carnitine	200	0.20 (0.09)	0.19 - 0.21	76	0.10 (0.07)	0.08 - 0.12	<.0001
C16-carnitine	200	3.94 (1.14)	3.78 - 4.10	76	2.28 (1.29)	1.98 - 2.57	<.0001
C16:1-carnitine	95	0.25 (0.09)	0.23 - 0.27	13	0.17 (0.08)	0.12 - 0.22	0.0030
C16OH	200	0.03 (0.02)	0.03 - 0.03	76	0.023 (0.012)	0.020 - 0.025	0.0061
C16:1OH	95	0.05 (0.02)	0.05 - 0.06	13	0.039 (0.020)	0.027 - 0.051	0.0521
C18	200	1.00 (0.27)	0.96 - 1.04	76	0.63 (0.30)	0.56 - 0.70	<.0001
C18:1	200	1.26 (0.52)	1.19 - 1.34	76	0.87 (0.44)	0.77 - 0.97	<.0001
C18OH	95	0.01 (0.01)	0.01 - 0.02	13	0.005 (0.005)	0.001 - 0.008	0.0009
C18:1OH	95	0.02 (0.01)	0.02 - 0.02	13	0.013 (0.013)	0.006 - 0.021	0.0238
Endocrine markers	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Thyroid Stimulating Hormone	200	1.97 (1.90)	1.70 - 2.23	75	3.15 (2.93)	2.47 - 3.82	**0.0001**
17-Hydroxy Progesterone	200	4.90 (2.53)	4.55 - 5.25	71	5.25 (6.65)	3.68 - 6.83	0.5259
Ratios	N	Mean (SD)	95% CI	N	Mean (SD)	95% CI	p
Met/Phe	200	0.34 (0.09)	0.33 - 0.35	76	0.45 (0.13)	0.42 - 0.48	**<.0001**
Leu/Phe	200	2.37 (0.55)	2.29 - 2.45	76	2.53 (0.87)	2.33 - 2.72	0.0744
Phe/Tyr	200	0.64 (0.21)	0.61 - 0.67	76	1.04 (0.48)	0.93 - 1.14	**<.0001**
Leu/Ala	200	0.62 (0.15)	0.60 - 0.64	76	0.69 (0.36)	0.61 - 0.77	0.0201
C3/Met	200	0.11 (0.05)	0.11 - 0.12	76	0.064 (0.043)	0.054 - 0.074	<.0001
C0/(C16+C18)	200	4.46 (1.79)	4.21 - 4.71	76	9.92 (6.48)	8.44 - 11.40	<.0001
(C16+C18:1)/C2	200	0.22 (0.14)	0.20 - 0.24	76	0.19 (0.08)	0.17 - 0.21	0.0759
C14:1/C16	200	0.05 (0.02)	0.05 - 0.06	76	0.054 (0.042)	0.045 - 0.064	0.5792
C3/C2	200	0.09 (0.04)	0.09 - 0.10	76	0.10 (0.04)	0.09 - 0.11	0.1560
C3/CO	200	0.11 (0.04)	0.11 - 0.12	76	0.080 (0.04)	0.07 - 0.09	<.0001
C16/C2	154	0.16 (0.11)	0.15 - 0.18	74	0.13 (0.05)	0.12 - 0.15	0.0281
C8/C2	200	0.01 (0.01)	0.01 - 0.01	76	0.013 (0.009)	0.010 - 0.015	<.0001
C16OH/C16	200	0.01 (0.0)	0.01 - 0.01	76	0.012 (0.008)	0.010 - 0.014	<.0001
C8/C10	200	1.13 (0.38)	1.08 - 1.18	76	1.66 (0.81)	1.48 - 1.85	<.0001
C3/C16	200	0.60 (0.25)	0.57 - 0.64	76	0.86 (0.51)	0.74 - 0.98	<.0001

7.2 Appendix B Ethics Approval

Health and Disability Ethics Committees

Health and Disability Ethics Committees
Ministry of Health
133 Molesworth Street
PO Box 5013
Wellington
6011

0800 4 ETHICS
hdecs@health.govt.nz

05 October 2020

Mrs Sandra Divanisova
14 Canary Pl
Unsworth Heights
Auckland 0632

Dear Mrs Divanisova,

Re:	**Ethics ref:**	**20/NTB/167**
	Study title:	Relevance of metabolic profiles in children with cardiac disease

I am pleased to advise that this application has been *approved* by the Northern B Health and Disability Ethics Committee. This decision was made through the HDEC-Full Review pathway.

Summary of outstanding ethical issues

The main ethical issues considered by the Committee which require addressing by the Researcher are as follows.

- The Committee wishes to thank you for the information that you have provided.

Conditions of HDEC approval

HDEC approval for this study is subject to the following conditions being met prior to the commencement of the study in New Zealand. It is your responsibility, and that of the study's sponsor, to ensure that these conditions are met. No further review by the Northern B Health and Disability Ethics Committee is required.

Standard conditions:

1. Before the study commences at *any* locality in New Zealand, all relevant regulatory approvals must be obtained.

2. Before the study commences at *each given* locality in New Zealand, it must be authorised by that locality in Online Forms. Locality authorisation confirms that the locality is suitable for the safe and effective conduct of the study, and that local research governance issues have been addressed.

After HDEC review

Please refer to the *Standard Operating Procedures for Health and Disability Ethics Committees* (available on www.ethics.health.govt.nz) for HDEC requirements relating to amendments and other post-approval processes.

Your next progress report is due by 05 October 2021.

Participant access to ACC

The Northern B Health and Disability Ethics Committee is satisfied that your study is not a clinical trial that is to be conducted principally for the benefit of the manufacturer or distributor of the medicine or item being trialled. Participants injured as a result of treatment received as part of your study may therefore be eligible for publicly-funded compensation through the Accident Compensation Corporation (ACC).

Please don't hesitate to contact the HDEC secretariat for further information. We wish you all the best for your study.

Yours sincerely,

Chairperson
Northern B Health and Disability Ethics Committee

Encl: appendix A: documents submitted
appendix B: statement of compliance and list of members

23 November 2020

Fabrice Merien

Faculty of Health and Environmental Sciences

Dear Fabrice

Re Ethics Application: **20/360 Relevance of metabolic profiles in children with cardiac disease**

Thank you for providing evidence as requested, which satisfies the points raised by the Auckland University of Technology Ethics Committee (AUTEC).

Your ethics application has been approved for three years until 23 November 2023.

Standard Conditions of Approval

1. The research is to be undertaken in accordance with the Auckland University of Technology Code of Conduct for Research and as approved by AUTEC in this application.
2. A progress report is due annually on the anniversary of the approval date, using the EA2 form.
3. A final report is due at the expiration of the approval period, or, upon completion of project, using the EA3 form.
4. Any amendments to the project must be approved by AUTEC prior to being implemented. Amendments can be requested using the EA2 form.
5. Any serious or unexpected adverse events must be reported to AUTEC Secretariat as a matter of priority.
6. Any unforeseen events that might affect continued ethical acceptability of the project should also be reported to the AUTEC Secretariat as a matter of priority.
7. It is your responsibility to ensure that the spelling and grammar of documents being provided to participants or external organisations is of a high standard and that all the dates on the documents are updated.

AUTEC grants ethical approval only. You are responsible for obtaining management approval for access for your research from any institution or organisation at which your research is being conducted and you need to meet all ethical, legal, public health, and locality obligations or requirements for the jurisdictions in which the research is being undertaken.

Please quote the application number and title on all future correspondence related to this project. For any enquiries please contact ethics@aut.ac.nz. The forms mentioned above are available online through http://www.aut.ac.nz/research/researchethics

The AUTEC Secretariat

Auckland University of Technology Ethics Committee

Unisys Building 650 Great South Road Penrose
Private Bag 92 522 Wellesley Street
Auckland 1141 New Zealand
T+64 9 580 9000

16 November 2020

Sandra Divanisova Auckland District Health Board
Level 3 Building 31 LabPlus
Dear Sandra

Request for Information: Antenatal and Newborn Screening Programme
Thank you for submitting your request to obtain permission to review retrospective newborn metabolic screening data of babies born with congenial cardiac disease between 2011 and 2020.

I can confirm that the Newborn Metabolic Screening Programme technical working group (TWG) are happy to approve your request.

I wish you all the best with your study and look forward to receiving updates for the TWG meetings, when requested, and a copy of the findings once the study is completed.

Yours sincerely

Jasmine Plimmer

Jasmine Plimmer
Manager, Antenatal and Newborn Screening Team
National Screening Unit

Auckland DHB
Research Office
Level 14, Support Bldg
Auckland City Hospital
PB 92024, Grafton, Auckland
Phone: 64 9 307 4949 Extn. 23854
Fax: 64 9 307 8913
Email: mwoodnorth@adhb.govt.nz
Website: www.adhb.govt.nz/ResearchOffice

23rd October 2020

Sandra Divanisova **Institutional Approval**

Newborn Screening LabPlus Auckland City Hospital

Dear Sandra

Re: Research project A+ 8944 (20/NTB/167) Relevance of metabolic profiles in children with cardiac disease

The Auckland DHB Research Review Committee (ADHB-RRC) would like to thank you for the opportunity to review your study and has given approval for your research project.

Your Institutional approval is dependent on the Research Office having up-to-date information and documentation relating to your research and being kept informed of any changes to your study. It is your responsibility to ensure you have kept Ethics and the Research Office up to date and have the appropriate approvals. ADHB approval may be withdrawn for your study if you do not keep the Research Office informed of the following:

- Any communication from Ethics Committees, including confirmation of annual ethics renewal
- Any amendment to study documentation
- Study completion, suspension or cancellation

More detailed information is included on the following page. If you have any questions please do not hesitate to contact the Research Office.

Yours sincerely

On behalf of the ADHB Research Review Committee Dr Mary-Anne Woodnorth
Manager, Research Office
Auckland DHB

27 July 2020

Sandra Divanisova

Auckland District Health Board Auckland

Re: Relevance of metabolic profiles in children with cardiac disease

The study is a retrospective study using anonymous data which does not have ethnicity data included. As per the title the investigator is seeking to determine whether there is a relationship between metabolic profiles in children and cardiac disease.

The current study may lead to a more in-depth study in the investigators research journey.
A Māori approval is not required for this study. However, I have provided a review and thanks for coming to talk to us. We wish you well.

Heoi ano

H.A Wihongi

Ngā mihi mahana

Dr Helen Wihongi

Director | Maori Health Research | He Kamaka Waiora

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helen.wihongi@waitematadhb.govt.nz **Waitemata and Auckland District Health Board**