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A ^{13}C breath test for *in vivo* determination of the metabolic flux in medium-chain acyl-coenzyme A dehydrogenase deficiency

Dissertation

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1 Introduction

Inborn errors of metabolism (IEMs) form a large group of rare genetic diseases causing a disruption of biosynthesis, transport or degradation of substrates within a metabolic pathway (Pugliese *et al.*, 2020; Reijngoud, 2018). Medium-chain acyl-co-enzyme A (CoA) dehydrogenase deficiency (MCADD) is an autosomal recessive disease of the fatty acid oxidation pathway that is dedicated to the large group of IEMs (Pugliese *et al.*, 2020; Wanders *et al.*, 2020). The overall prevalence is 5.3 per 100.000 births across a variety of ethnic groups (Merritt and Chang, 2020). MCADD is genetically caused by variants in the *ACADM* gene, inducing misfolding and de-stabilization of the enzyme medium-chain acyl-CoA dehydrogenase (MCAD) (Jank *et al.*, 2014; Maier *et al.*, 2009a; Nasser *et al.*, 2004). The primary task of MCAD is to break down medium-chain fatty acids when carbohydrates are not available as energy source for the human organism (Maier *et al.*, 2009a; Wanders *et al.*, 2020). If the enzyme is not functioning properly metabolites of medium-chain acyl-CoA intermediates accumulate and cause oxidative damage (Maier *et al.*, 2009a; Wanders *et al.*, 2020). Patients are at risk of hypoketotic crises with hypoglycemia, which can progress to coma and sudden death without timely intervention (Schatz and Ensenauer, 2010). Treatment aim of symptomatic patients in metabolic crisis is the rapid reversal of catabolism (Saudubray *et al.*, 1999; Schatz and Ensenauer, 2010).

MCAD and other fatty acid oxidation disorders as long-chain and very long-chain acyl-CoA dehydrogenase deficiency are detected by newborn screening (NBS) program (Knottnerus *et al.*, 2018; Wanders *et al.*, 2020). Since the implementation of MCADD to the screening program about 20 years ago, improvements in mortality rates from > 20 % to 3.5 %-10 % have been achieved (Anderson *et al.*, 2020; Feuchtbaum *et al.*, 2018; Grosse *et al.*, 2006; Nennstiel-Ratzel *et al.*, 2005).

Disease severity is only estimated by *ex vivo* enzyme activity analysis in lymphocytes or other tissues, as it correlates with the expected clinical phenotype (Derks *et al.*, 2008; Jager *et al.*, 2019; Wanders *et al.*, 2010, 2020). However, the assessment of the current metabolic state in MCADD patients is unsatisfactory as decompensations cannot be predicted. Therefore, this doctoral thesis presents the current state of knowledge of the disease MCADD and highlights a new follow-up method, the ^{13}C breath test for *in vivo* determination of the metabolic flux in MCADD.

1.1 Fatty acids

The 3 major components of nutrients of human nourishment are proteins, carbohydrates and lipids (Stanley and Bennett, 2011; Wanders *et al.*, 2020). Proteins are mainly needed as structural material, but the breakdown of carbohydrates and lipids provides energy to maintain body functions (Stanley and Bennett, 2011; Wanders *et al.*, 2020).

Compared to proteins and carbohydrates, lipids have about twice as much energy value (Tvrzicka *et al.*, 2011). Around 30 % of total energy intake by human nourishment comes from dietary lipids with fatty acids (FA) as their substantial part (Tvrzicka *et al.*, 2011). FA can be used for immediate energy production, or be stored in the liver or in fat tissue. They are usually not found in organisms in their solitary form, but as triglycerides, phospholipids and cholesteryl esters (Tvrzicka *et al.*, 2011). In the form of triglycerides they are a principal source of energy in times of low oral carbohydrate or lipid intake (de Carvalho and Caramujo, 2018; Kremmyda *et al.*, 2011; Tvrzicka *et al.*, 2011).

Additionally, to their role as energy substrates, FA have a number of physiological roles in humans (de Carvalho and Caramujo, 2018; Kremmyda *et al.*, 2011; Tvrzicka *et al.*, 2011). They act as structural and functional components of cell membranes, precursors for lipid mediators, components affecting signal transduction pathways and gene transcription (de Carvalho and Caramujo, 2018; Kremmyda *et al.*, 2011).

1.1.1 Nomenclature

FA are carboxylic acids with a typical RCOOH structure, containing a methyl end, a hydrocarbon chain, and a carboxylic terminus (de Carvalho and Caramujo, 2018; Tvrzicka *et al.*, 2011). Their lengths can vary with carbon chains between 2 and 36 carbon atoms, but most FA have an even number of carbon atoms, as they are synthesized from two-carbon units (Tvrzicka *et al.*, 2011).

FA can be either saturated or unsaturated (Tvrzicka *et al.*, 2011). Saturated FA do not contain any double bonds, while unsaturated FA contain one or more double bonds. The double bonds can have a *cis*- or *trans*-configuration (Tvrzicka *et al.*, 2011). The *cis*-term describes, that the 2 hydrogens at the double bond are on the

same side of the molecule. This leads to a different orientation of the adjoining carbons, which causes a 30° deflection of the carbon chain. The *trans*-term means, that the adjacent 2 hydrogen atoms lie on opposite sides of the chain. Under physiological conditions, double bonds preferably have a *cis*-configuration. Polyunsaturated FA are characterized by pentadiene configuration of double bonds.

FA are represented as schematic formula by IUPAC-IUB commission on biochemical nomenclature (IUPAC-IUB, 1968). (**Figure 1**)

CN:p n-x

CN = total number of carbon atoms

p = number of double bonds

n = methyl terminus

x = position of the first do double bond from the methyl terminus

Figure 1 Schematic formula to name FA by IUPAC-IUB Commission on Biochemical Nomenclature.

For example, the formula for palmitic acid is 16:0, abbreviated C16, as it has 16 carbon atoms and no double bond. The formula for linoleic acid is 18:2n-6, because it has 18 carbon atoms and 2 double bonds. The first double bond is on the sixth carbon atom counted from the methyl end. (**Figure 2**)

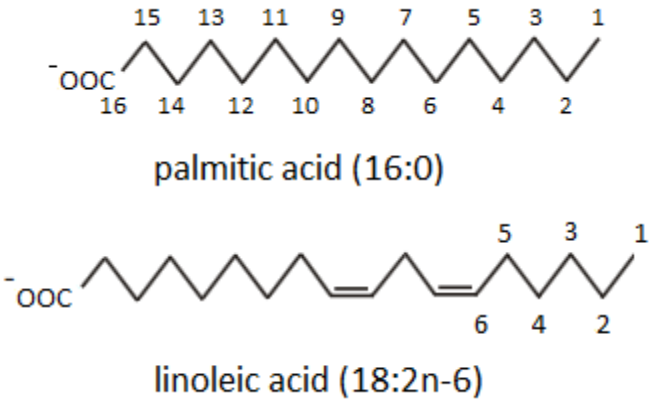


Figure 2 Structural formulas of palmitic and linoleic acid. Adapted from Tvrzicka et al., 2011.

1.1.2 Chain lengths of fatty acids

According to their chain length saturated FA can be divided into short-chain, medium-chain, long-chain and very long-chain FA.

1.1.2.1 Short-chain fatty acids

The group of short-chain FA consists of acetic (C2), propionic (C3), and butyric (C4) acids (Tvrzicka *et al.*, 2011). They are absorbed in the proximal colon, resorbed by portal circulation, and transported to the liver where they are metabolized into glucose (Tvrzicka *et al.*, 2011). Hereby they cover 10-20 % of resting energy expenditure of the human body (Tvrzicka *et al.*, 2011). Particularly butyric acids are used in metabolism, proliferation and restoration of enterocytes and colonocytes (Kremmyda *et al.*, 2011). Further functions of short-chain FA in the colon are the absorption of water, sodium, chloride and bicarbonate, the stimulation of the blood flow through mucous membrane, mucus production, limited reproduction of saprophytic bacteria and putrefication due to decreased acidity (Compher *et al.*, 1997; Tvrzicka *et al.*, 2011).

1.1.2.2 Medium-chain fatty acids

Medium-chain FA enclose caproic (C6), caprylic (C8), and capric (C10) acids (Tvrzicka *et al.*, 2011). They are directly transported by the portal vein (Tvrzicka *et al.*, 2011). Their main function is to act as energy substrates (Tvrzicka *et al.*, 2011). The energy value of medium-chain FA from nourishment is 29 kJ/g (7 kcal/g) (Kremmyda *et al.*, 2011).

1.1.2.3 Long-chain fatty acids

Long-chain FA are lauric (C12), myristic (C14), palmitic (C16), and stearic (C18) acids (Tvrzicka *et al.*, 2011). They are not directly released into the intestinal capillaries, but absorbed into the fatty walls of the intestine villi (Tvrzicka *et al.*, 2011). There is an evidence suggesting that they might have a thrombogenic function, as ingestion of saturated long-chain FA increases levels of low density lipoprotein (LDL)-cholesterol (Astrup *et al.*, 2011). Moreover, they are messengers of prostaglandins, leucotrienes and thromboxanes, which have regulatory, autocrine and

paracrine functions (Kremmyda *et al.*, 2011). Long-chain FA are considered as inflammation-resolving, and thus, fish oil has been characterized as antiinflammatory on rheumatoid arthritis and inflammatory bowel diseases (de Carvalho and Caramujo, 2018). They also act as energy substrates and their energy value from nourishment is 38 kJ/g (9 kcal/g) (Kremmyda *et al.*, 2011). This is higher than the one of medium-chain FA, as the energy value correlates with the chain length of FA.

1.1.2.4 Very long-chain fatty acids

Very long-chain FA include arachidic (C20), behenic (C22), lignoceric (C24), cerotic (C26), montanic (C28), and melissic (C30) acids (Tvrzicka *et al.*, 2011). They react as mechanical isolators, because they are structural components of ceramides, which reduce skin permeability to water (Stanley and Bennett, 2011). Interestingly, significant concentrations are measurable in inherited metabolic diseases as Zellweger syndrome, X-linked adrenoleucodystrophy, Refsum's disease, Menkes' disease (Gotto, 2004). As signal pathways are influenced by genes, nuclear factors and receptors, very long-chain FA, especially arachidic and behenic acids are known to be ligands of these receptors or modulators of gene transcription (de Carvalho and Caramujo, 2018; Kremmyda *et al.*, 2011). Therefore they can significantly influence cell signalling (Chapkin *et al.*, 2008). They have been identified as effective ligands of nuclear receptors of some transcription factors, for example the peroxisome proliferator-activated receptor (PPAR) -α, -β/δ (de Carvalho and Caramujo, 2018; Kremmyda *et al.*, 2011). As ligands of peroxisome PPAR-α, they have a number of pleiotropic effects on lipid and energy metabolism (de Carvalho and Caramujo, 2018; Kremmyda *et al.*, 2011). They are thought to decrease lipogenesis and very low density lipoprotein (VLDL) secretion by suppression of sterol response element binding protein (Astrup *et al.*, 2011). As energy substrates, their energy value from nourishment is equal to long-chain FA 38 kJ/g (9 kcal/g) (Kremmyda *et al.*, 2011).

1.2 Fatty acid oxidation

During periods of reduced caloric intake due to illness or prolonged exercise, most body tissues derive up to 80 % of total energy requirement from FA and fat becomes the major fuel of our body (Zschocke and Hoffmann, 2012). Additionally, the cardiac and the skeletal muscle prefer FA to glucose as a primary source of energy during exercise. The brain is unable to fully oxidize FA. However, it can adapt to metabolize ketone bodies, which are derived from acetyl-CoA and acetoacetyl-CoA, which originate from β-oxidation of fatty acids in the liver (Andrew and Spiekerkoetter, 2016; Merritt and Chang, 2020; Puchalska and Crawford, 2019; Roe and Coates, 1995; Stanley and Bennett, 2011). FA are stored as triglycerides in fat tissue and can be utilized by mitochondrial fatty acid β-oxidation (FAO), which involves 3 major processes (Andrew and Spiekerkoetter, 2016; Merritt and Chang, 2020; Roe and Coates, 1995; Stanley and Bennett, 2011; Wanders *et al.*, 2020). (**Figure 3**)

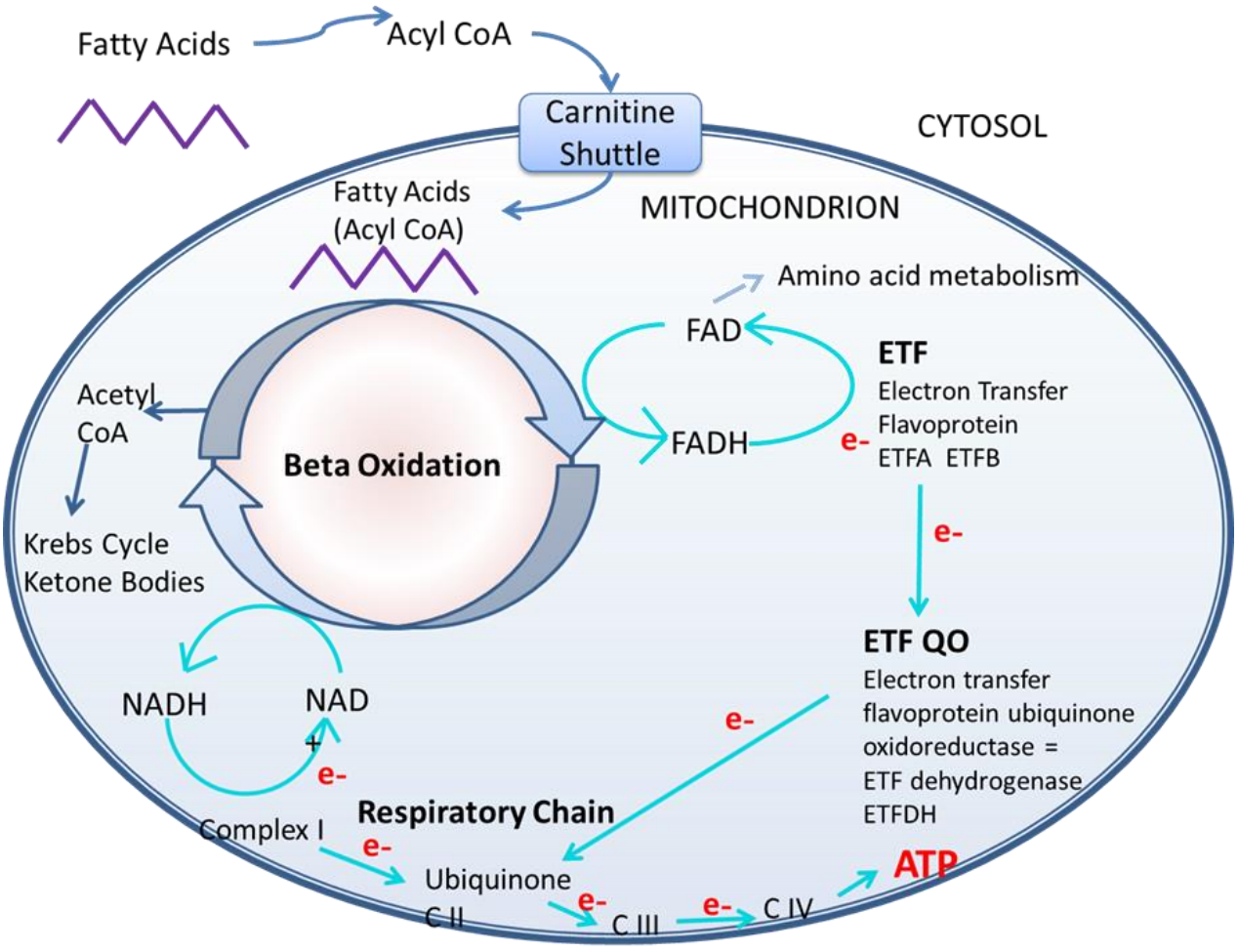


Figure 3 Pathway of mitochondrial fatty acid β-oxidation. Adapted from Andrew and Spiekerkoetter, 2016.

1.2.1 Entry of fatty acids into mitochondria

During times of prolonged fasting FA are released from fat tissue by lipases and activated to acyl-coA esters in the cytoplasm (Andrew and Spiekerkoetter, 2016; Merritt and Chang, 2020; Wanders *et al.*, 2020).

Medium- and short-chain FA enter the mitochondria independently, but a carnitine shuttle system is required for long-chain FA to cross the mitochondrial membrane (Wanders *et al.*, 2020). Firstly, a plasma membrane carnitine transporter is needed to maintain the intracellular supply of carnitine (Wanders *et al.*, 2020). Long-chain FA enter the outer mitochondrial membrane via acyl-CoA-synthetase, while also carnitine enters the membrane (Wanders *et al.*, 2020). Afterwards, transesterification of acyl-CoA is performed by carnitine palmitoyltransferase (CPT) type I prior to mitochondrial translocation by carnitine/acylcarnitine translocase (Wanders *et al.*, 2020). Reesterification of acylcantines to acyl-CoA esters is conducted by CPT type II on the inner mitochondrial membrane (Wanders *et al.*, 2020). (**Figures 3, 4**)

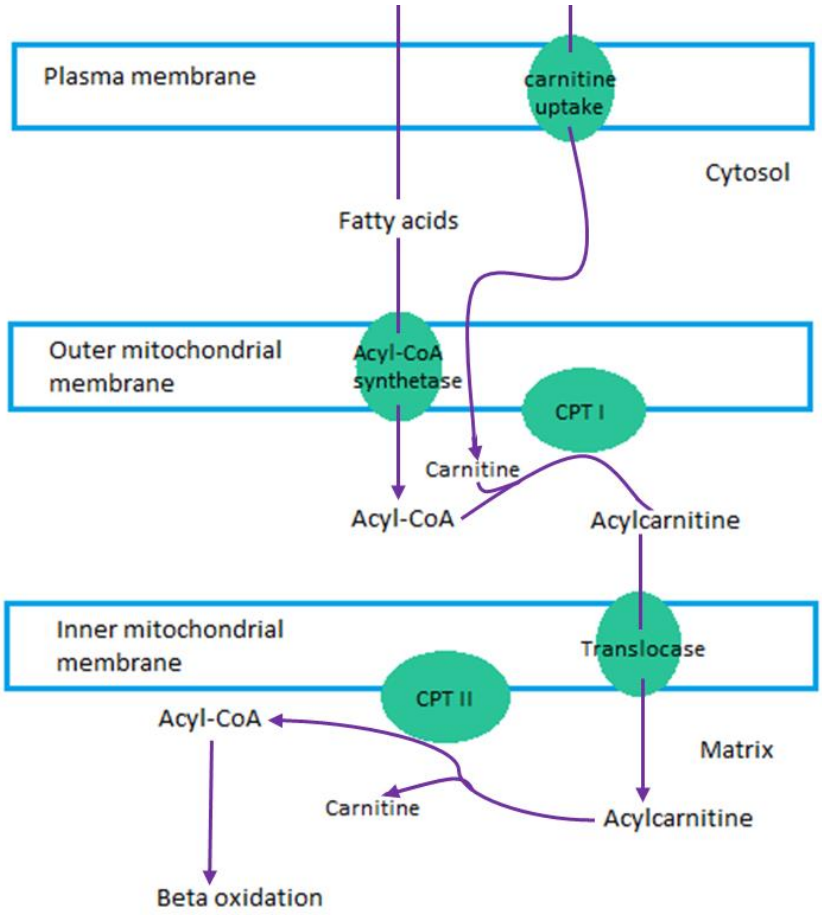


Figure 4 Pathway of carnitine shuttle system for long-chain fatty acids. Adapted from Roe and Coates, 1995.

1.2.2 Mitochondrial fatty acid β-oxidation via spiral pathway

Acyl-CoA esters enter the β-oxidation pathway wherein each turn of the spiral shortens the acyl-CoA by 2 carbon atoms using several chain-length specific enzymes (Wanders *et al.*, 2010, 2020). Most of the long-chain specific enzymes are membrane bound, while medium and short-chain enzymes are located in the matrix (Andrew and Spiekerkoetter, 2016). Briefly, the steps in mitochondrial β-oxidation are dehydrogenation by acyl-CoA dehydrogenase, yielding one flavin adenine dinucleotide (FAD)-H_2, hydration by enoyl-CoA hydratase, dehydrogenation by 3-hydroxyacyl-CoA dehydrogenase, yielding one nicotinamide adenine dinucleotide (NAD)-H and cleavage by thiolase, yielding one acetyl-CoA and a now shortened fatty acid (Wanders *et al.*, 2010, 2020). Acyl-CoA compounds cycle through the β-oxidation pathway as often as it is possible to generate 2-carbon acetyl-CoA fragments (Andrew and Spiekerkoetter, 2016). Acetyl-CoA is the end product of FAO and can be either converted to ketone bodies in the liver or oxidized in the Krebs cycle (Andrew and Spiekerkoetter, 2016; Roe and Coates, 1995). (**Figure 3**) Further degradation by ketone body metabolism or Krebs cycle is explained in the following excursus chapters.

Mitochondrial FAO is the principal pathway for oxidation of FA, although they can also perform alpha- and omega-oxidation (Kunau *et al.*, 1995; Wanders *et al.*, 2003). Both, alpha- and omega-oxidation play an important role in energy production and depend on FAO for further degradation of the FA (Wanders *et al.*, 2010).

FAO is not only performed in mitochondria, but also in peroxisomes (Wanders *et al.*, 2010, 2020; Wanders and Waterham, 2006). Although oxidation in the two organelles proceeds via a similar mechanism, there are major differences, including the involved enzymes, their regulation, and substrates (Wanders *et al.*, 2010). Peroxisomes catalyze the beta-oxidation of a range of FA that is not handled by mitochondria, which includes very-long-chain FA, pristanic acid, and the bile acid intermediates di- and trihydroxycholestanoic acid (Wanders and Waterham, 2006).

1.2.2.1 Excursus: Ketone body metabolism

Ketone bodies play a role in several human metabolic pathways such as FAO, the Krebs cycle, gluconeogenesis, de novo lipogenesis, and biosynthesis of sterols (Puchalska and Crawford, 2019). During times of fasting, they are an important alternative energy fuel for many tissues, including the brain, cardiac and skeletal muscle (Puchalska and Crawford, 2019). They are synthesized predominantly in liver mitochondria, from FAO-derived acetyl-CoA and transported to extrahepatic tissues for terminal oxidation (Stanley and Bennett, 2011).

For ketogenesis in liver mitochondria the isoform of 3-hydroxymethylglutaryl (HMG)-CoA synthase generates HMG-CoA committing condensation of acetoacetyl-CoA and acetyl-CoA (Puchalska and Crawford, 2019). HMG-CoA lyase cleaves HMG-CoA to liberate acetyl-CoA and acetoacetate (Puchalska and Crawford, 2019). The principal ketone bodies are acetoacetate and 3-hydroxybutyrate (Puchalska and Crawford, 2019). They are maintained by the enzyme 3-hydroxybutyrate dehydrogenase in a NAD-coupled reaction (Puchalska and Crawford, 2019; Stanley and Bennett, 2011).

For ketolysis acetoacetate and 3-hydroxybutyrate are released from cells by monocarboxylase transporters (Puchalska and Crawford, 2019). They are conveyed in the circulation to extrahepatic tissues for terminal oxidation. In extrahepatic mitochondrial matrix, acetoacetate is activated by succinyl-CoA 3-oxoacid CoA transferase (SCOT) to acetoacetyl-CoA (Puchalska and Crawford, 2019). This results into an exchange of a CoA-moiety from succinyl-CoA to succinate (Puchalska and Crawford, 2019). The energy released by hydrolysis of acetoacetyl-CoA is greater than the one of succinyl-CoA (Puchalska and Crawford, 2019). A reversible acetyl-CoA thiolase reaction yields two molecules of acetyl-CoA, which can be used for entering the Krebs cycle (Akram, 2014; Anderson *et al.*, 2018; Puchalska and Crawford, 2019; Stanley and Bennett, 2011).

1.2.2.2 Excursus: Krebs cycle

Acetyl-CoA, obtained from amino acids, triacylglycerol, carbohydrates, FAO, and ketone bodies is the primary fuel for the Krebs cycle, also known as tricarboxylic acid cycle (Akram, 2014; Anderson *et al.*, 2018). Main function of the Krebs cycle is the breakdown of acetyl-CoA using several enzymes. Thereby it generates reducing equivalents in form of reduced NADH and $FADH_2$, which are utilized to produce energy in form of ATP in the electron transport chain (Akram, 2014; Anderson *et al.*, 2018).

In the first step of the Krebs cycle acetyl-CoA and oxaloacetate are converted to citrate by the enzyme citrate synthase (Akram, 2014). Citrate is further converted to *cis*-aconitate using the enzyme aconitase and *cis*-aconitate is converted to isocitrate by aconitase (Akram, 2014). After dehydration and rehydration isocitrate is generated, which is then converted to α-ketoglutarate (Akram, 2014). This step produces a reduced NADH (Akram, 2014). An α-ketoglutarate dehydrogenase complex converts α-ketoglutarate to succinyl-CoA, which is further converted to succinate by succinyl-CoA transferase (Akram, 2014). In this reaction guanosine diphosphate (GDP) is converted to guanosine-triphosphate (GTP) (Akram, 2014). Succinate is converted to fumaric acid by succinate dehydrogenase, while FAD is converted to $FADH_2$ (Akram, 2014). Malate is formed by fumarase and dehydrogenated to produce oxaloacetate by the enzyme malate dehydrogenase (Akram, 2014). In this reaction NAD is converted to NADH (Akram, 2014).

In total three NADH, one $FADH_2$, and one GTP are constituted during one round of the Krebs cycle (Akram, 2014; Anderson *et al.*, 2018).

1.2.3 Electron transfer to respiratory chain

Continued enzyme catalysis requires electron transfer down the electron transport chain to the respiratory chain in mitochondria (Wanders *et al.*, 2020). Therefor electrons from NADH or $FADH_2$, resulting from mitochondrial FAO or Krebs cycle metabolism, are pumped from the mitochondrial matrix into the intermembrane space, creating an electrochemical proton gradient across the inner mitochondrial membrane (Fernie *et al.*, 2004). This gradient is occasionally responsible for the mitochondrial membrane potential and allows adenosine triphosphate (ATP) synthase to generate ATP from adenosine diphosphate (ADP) and inorganic phosphate (Fernie *et al.*, 2004; Wanders *et al.*, 2020).

Four membrane-bound complexes have been identified for the transport of electrons in mitochondria. Complex I (NADH coenzyme Q reductase) accepts electrons from the electron carrier NADH, and passes them to coenzyme Q (ubiquinone), which also receives electrons from complex II (succinate dehydrogenase) (Andrew and Spiekerkoetter, 2016; Fernie *et al.*, 2004; Wanders *et al.*, 2020). Electrons can also pass from $FADH_2$ via electron transfer flavoprotein (ETF) and ETF dehydrogenase (ETFQO) to coenzyme Q (Andrew and Spiekerkoetter, 2016; Fernie *et al.*, 2004; Wanders *et al.*, 2020). Thence electrons pass to complex III (cytochrome bc1 complex), which passes them to cytochrome c and afterwards complex IV (cytochrome c oxidase), which uses the electrons and hydrogen ions to reduce molecular oxygen to water (Andrew and Spiekerkoetter, 2016; Fernie *et al.*, 2004; Wanders *et al.*, 2020). Finally, electrons are pumped across the inner mitochondrial membrane to create a proton electrochemical gradient that is required for ATP synthase to phosphorylate ADP to produce ATP, providing energy for cellular processes (Fernie *et al.*, 2004; Sirey and Ponting, 2016; Wanders *et al.*, 2020). (**Figure 3**)

1.3 Medium-chain acyl-Coenzyme A dehydrogenase deficiency

1.3.1 Prevalence

Deficiency of the enzyme medium-chain acyl-CoA dehydrogenase (MCAD) is the most common disorder of mitochondrial FAO (Knottnerus *et al.*, 2018; Matsubara *et al.*, 1986; Merritt and Chang, 2020; Roe and Coates, 1995). The overall prevalence of MCAD deficiency (MCADD [MIM #201450]) is 5.3 per 100.000 births across a variety of ethnic groups (Merritt and Chang, 2020). In Europe the prevalence varies between 3.7 and 20.4 per 100.000 births with the highest proportion in the northern European population (Merritt and Chang, 2020).

1.3.2 Molecular genetics

MCADD is an inherited autosomal recessive disease (Anderson *et al.*, 2020; Merritt and Chang, 2020). At conception, the child of two carrier parents is at a 25 % risk of being unaffected, a 50 % risk of being heterozygous carrier, and a 25 % risk of being affected. (**Figure 5**)

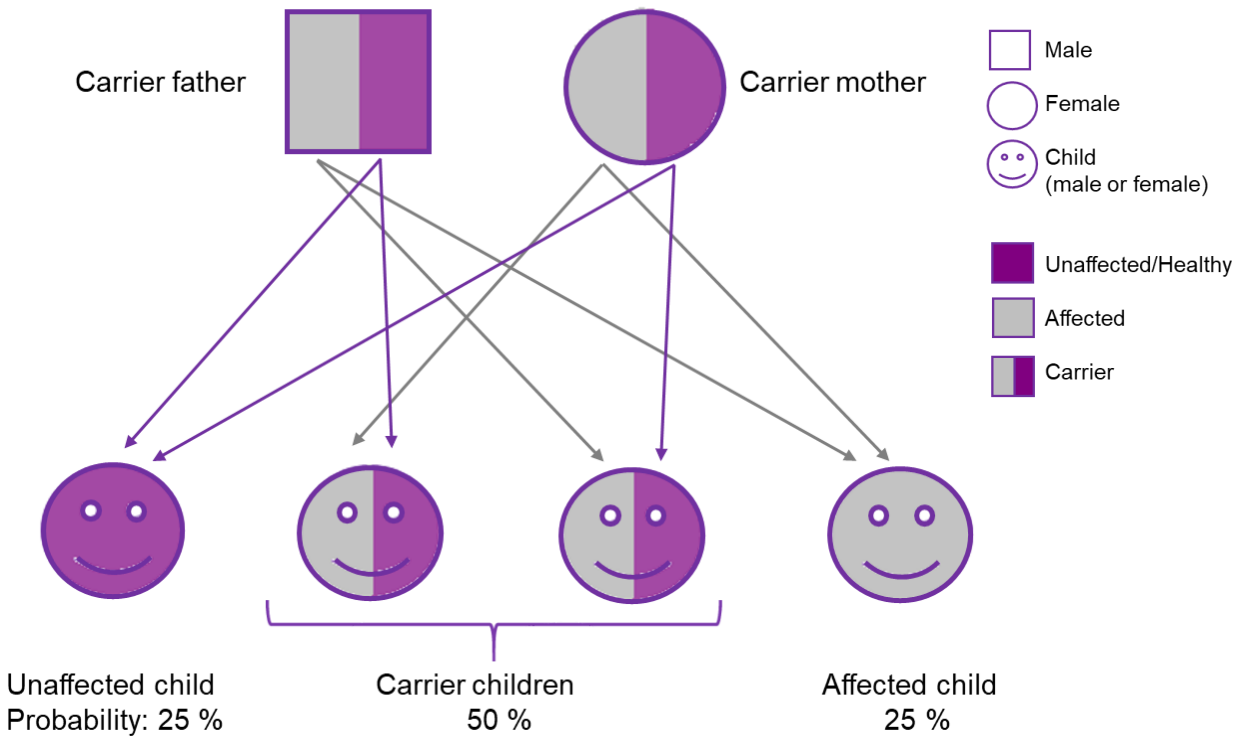


Figure 5 Autosomal recessive inheritance pattern.

MCADD is genetically caused by variants in the *ACADM* gene [MIM #607008] that is located at 1p31 comprising 12 exons which span 44 kb of DNA (Gregersen *et al.*, 2004; Stenson *et al.*, 2017; Zhang *et al.*, 1992). Currently, 183 *ACADM* variants with a wide heterogeneity are known, of those around 70 % are missense/nonsense variants, 12 % are splicing variants and around 8 % are small deletions (Cooper *et al.*, 2020). In European patients with MCADD, 80 % are homozygous for p.[Lys329Glu];p.[Lys329Glu], and a further 18 % are compound heterozygous for p.[Lys329Glu] (Cooper *et al.*, 2020; Gramer *et al.*, 2015; Merritt and Chang, 2020).

1.3.3 Pathophysiology

MCAD is a homotetrameric enzyme of the acyl-CoA dehydrogenase family of flavoproteins, catalyzing the first step of the mitochondrial FAO of medium-chain fatty acids (Bross *et al.*, 1993; Gregersen *et al.*, 2004; Jank *et al.*, 2014; Knottnerus *et al.*, 2018; Maier *et al.*, 2009a; Nasser *et al.*, 2004; O'Reilly *et al.*, 2004). Each subunit is composed of 3 structural domains, labeled as the N-terminal α-domain, the β-domain and the C-terminal α-domain (Maier *et al.*, 2009a). The N- and C-terminal domains shape the core of the tetramer, as they predominantly consist of densely packed α-helices (Kim and Miura, 2004). The middle β-domains are located at the surface of the molecule and comprise 2 orthogonal β-sheets (Kim and Miura, 2004). The catalytic centers consist of the binding sites for the substrate and the natural cofactor FAD (Kim and Miura, 2004). They are mainly formed by the interface between the β-domain and the C-terminal α-domain (Kim and Miura, 2004). Variants in the *ACADM* gene can affect all 3 structural protein domains of the homotetrameric enzyme MCAD (Bross *et al.*, 1993; Jank *et al.*, 2014; Maier *et al.*, 2009a). Side chain modifications lead to a structural change and therefore induce protein misfolding and destabilization. This results in a loss of function of the enzyme (Bross *et al.*, 1993; Jank *et al.*, 2014; Maier *et al.*, 2009a). The ability to catalyze C6 to C10 straight-chain acyl-CoA, as the initial step of the FAO pathway, is deficient. Metabolites of medium-chain acyl-CoA intermediates accumulate and cause oxidative damage. This pathogenic mechanism affects the energy supply to peripheral tissues due to impaired FAO and FAO dependent ketogenesis (Bross *et al.*, 1993; Gregersen *et al.*, 2004; Jank *et al.*, 2014; Knottnerus *et al.*, 2018; Maier *et al.*, 2009a; Nasser *et al.*, 2004; O'Reilly *et al.*, 2004).

1.3.4 Clinical presentation

Patients with MCADD have first been described between the years 1976 and 1983 (Divry *et al.*, 1983; Gregersen *et al.*, 1976, 1983; Kølvraa *et al.*, 1982; Rhead *et al.*, 1983; Stanley *et al.*, 1983). They usually presented within the first 3 months to 5 years of life, but in some cases, manifestation occurred only in adulthood (Rinaldo *et al.*, 2002; Roe and Coates, 1995). The interindividual clinical presentation was very heterogeneous reaching from mild symptoms to sudden unexplained death (Rinaldo *et al.*, 2002; Roe and Coates, 1995). More than 20 years ago, MCADD has been included in newborn screening (NBS) programs all over the world, which significantly reduced morbidity and mortality (Anderson *et al.*, 2020; Maier *et al.*, 2005; Pugliese *et al.*, 2020; Schatz and Ensenauer, 2010; Van Rijt *et al.*, 2016; Wilcken *et al.*, 2007). It is now the second most frequently diagnosed inborn error of metabolism in northern Europe after hyperphenylalaninemia (Chace et al. 2003; Schulze et al. 2003).

1.3.4.1 Prior to newborn screening era

Childhood manifestations

Children were asymptomatic as long as an adequate caloric intake provided enough glucose serving as energy source. An exacerbation of the disease was associated with a decreased energy supply due to a lack of oral food intake, for example in breastfed newborns when mother's breast glands did not produce enough milk in the first days after birth, in children under infection (gastroenteritis or upper respiratory tract infection), while surgery or during prolonged exercise (Merritt and Chang, 2020; Schatz and Ensenauer, 2010; Wilcken, 2010). The most common clinical presentation was an episode of encephalopathy due to accumulation of medium-chain acyl-CoA intermediates inhibiting mitochondrial FAO and inadequate ketone body synthesis (Derks *et al.*, 2006; Gregersen *et al.*, 1976; Iafolla *et al.*, 1994; Stanley *et al.*, 1983; Wilcken *et al.*, 2007). In some cases symptoms were complicated by hypoketotic hypoglycemia as a late sign, metabolic acidosis, liver dysfunction, hepatomegaly, vomiting, dehydration, lethargy and seizures (Iafolla *et al.*, 1994; Saudubray *et al.*, 1999). Similar to Reye syndrome brain edema and hyperammonemia progressed to coma and sudden death (Iafolla *et al.*, 1994; Saudubray *et al.*, 1999). Median age at the initial episode of metabolic decompensation prior to

NBS era was reported as 1 – 1.5 years of age, ranging from the first day of life to school age (Schatz and Ensenauer, 2010). Up to 41% of survivors of metabolic decompensations needed additional hospital admissions during intercurrent illnesses, mostly for prophylactic reasons (Derks *et al.*, 2006; Iafolla *et al.*, 1994; Wilcken *et al.*, 2007). After an uncontrolled metabolic decompensation, some patients developed neurological complications, such as developmental regression, aphasia and attention deficit disorder (Schatz and Ensenauer, 2010). This might have been caused by sustained brain injury following energy undersupply during an acute metabolic event (Andrew and Spiekerkoetter, 2016; Merritt and Chang, 2020; Roe and Coates, 1995). As chronic symptoms muscle weakness, fatigue and poor exercise tolerance have been reported (Derks *et al.*, 2006; Iafolla *et al.*, 1994).

Adult manifestations

Manifestation of MCADD also occurred in adulthood, resulting in a broader phenotypic heterogeneity (Schatz and Ensenauer, 2010). Patients between 16 and 45 years of age presented acutely with multiple organ involvement including neurological, muscular, hepatic, or cardiac symptoms (Boles *et al.*, 1996; Feillet *et al.*, 2003; Mayell *et al.*, 2007; Ruitenbeek *et al.*, 1995). In addition to signs of metabolic decompensation including metabolic acidosis, hyperammonemia, hyperlactacidemia, and hypoglycemia, nearly 50 % of the described patients showed significant elevations of creatine kinase in blood as sign of rhabdomyolysis and myoglobinuria (Boles *et al.*, 1996; Feillet *et al.*, 2003; Mayell *et al.*, 2007; Ruitenbeek *et al.*, 1995).

1.3.4.2 In the screened population

Around 20 years ago, MCADD has been included in NBS programs all over the world, in Germany it was implemented in the year 2002 (Anderson *et al.*, 2020; Maier *et al.*, 2009b; Pugliese *et al.*, 2020; Schatz and Ensenauer, 2010; Wilcken, 2010). With a small blood sample taken by a neonatal heel prick 36 to 72 hours after birth, increased acylcarnitine levels, quantitatively measured with tandem mass spectrometry (MS/MS), can detect disease.

Presymptomatic MCADD patients identified by NBS can be saved from metabolic decompensations and relevant sequelae by disease awareness and instruction provided to the parents (Schatz and Ensenauer, 2010). Effective management includes early initiation of preventative therapy comprising of the avoidance of fasting and high carbohydrate intake at times of metabolic stress (Lindner *et al.*, 2011; Tal *et al.*, 2015; Wilcken *et al.*, 2007).

NBS significantly reduced clinical manifestations and death in MCADD. Improvements in mortality rates from > 20 % to 3.5 %-10 % have been achieved worldwide (Anderson *et al.*, 2020; Feuchtbaum *et al.*, 2018; Grosse *et al.*, 2006; Nennstiel-Ratzel *et al.*, 2005; Pugliese *et al.*, 2020; Wilcken, 2010; Wilcken *et al.*, 2007).

However, NBS does not eliminate neonatal death as there is a general risk of very early metabolic decompensation in the first few days of life prior to NBS or the availability of NBS results, which seems likely to be around 4–5% (Andresen *et al.*, 2012; Lindner *et al.*, 2011; Lovera *et al.*, 2012; Tal *et al.*, 2015; Van Rijt *et al.*, 2016; Wilcken *et al.*, 2007). Postponing the blood sampling of NBS screening beyond the catabolic state of days 2 and 3 of life may yield false negative results and miss diagnosis as metabolic acylcarnitine profiles can be normal in anabolic state (Yusupov *et al.*, 2010). Therefore, it is of great importance to adhere to the time frame of the NBS.

1.3.5 Diagnosis

MCADD is detected by NBS program or suggestive biochemical testing in a previously healthy individual with symptoms and diagnosed by molecular genetics or enzymology (Andrew and Spiekerkoetter, 2016; Merritt and Chang, 2020).

Newborn screening program

Population-based NBS is a public health program that has vastly improved the course of several IEMs or other inherited disorders through early detection (Anderson *et al.*, 2020; Holmes, 2012; Jager *et al.*, 2019; Pugliese *et al.*, 2020; Therrell *et al.*, 2015). Detection provides the possibility of an intervention to alter the clinical course of the disease and to prevent or ameliorate clinical manifestations (Anderson *et al.*, 2020; Jager *et al.*, 2019; Pugliese *et al.*, 2020).

Robert Guthrie, who was working at the Buffalo Children's Hospital in New York in the 1960s, first devised to test newborns 36 to 72 hours after birth by a small blood sample on filter paper for the metabolic disorder phenylketonuria (PKU) (Guthrie, 1969; Guthrie and Susi, 1963; Holmes, 2012). As in many parts of the world, NBS in Europe started ever since, spreading from West to East (Therrell *et al.*, 2015). It is a developing program that continually grew over time. Back in the 1960s, PKU was screened using a culture-based bacterial assay, while the introduction of tandem mass spectrometry (MS/MS) in the 1990s has expanded the number of screened inherited diseases (Holmes, 2012). Many biochemically related disorders can be detected by MS/MS, allowing screening for extremely rare disorders (Chace *et al.*, 2003). At the same time there was growing interest in DNA testing by NBS, which further expanded the detection of even more diseases (Jager *et al.*, 2019; Lindner *et al.*, 2010; McCabe *et al.*, 1987; Tal *et al.*, 2015).

There is no consensus about the included diseases in European countries, leading to a heterogeneity of NBS (Holmes, 2012; Merritt and Chang, 2020).

Currently the NBS in Germany screens for 15 inherited disease groups including:

- congenital hypothyroidism
- adrenogenital syndrome
- biotinidase deficiency
- galaktosaemia
- PKU and hyperphenylalaninaemia
- maple syrup urine disease
- MCADD
- long-chain acyl-CoA dehydrogenase deficiency
- very-long-chain acyl-CoA dehydrogenase deficiency
- disorders of carnitine transport and the carnitine cycle as carnitine-palmitoyl-transferase-I-deficiency, carnitine-palmitoyl-transferase-II-deficiency, and carnitine-acylcarnitine-translocase deficiency
- glutaric aciduria type 1
- isovaleric aciduria
- cystic fibrosis
- tyrosinaemia
- severe combined immunodeficiency

Positive NBS result for MCADD

NBS for MCADD is primarily based on the acylcarnitine profile measurement by MS/MS (Andrew and Spiekerkoetter, 2016; Roe and Coates, 1995). Characteristically increased levels of C8 with lesser elevations of C6 and C10 acylcarnitines are considered positive for MCADD and need further follow-up (Maier *et al.*, 2005, 2009b; Millington *et al.*, 1990; Smith *et al.*, 2010). Secondary ratios, including the C8/C0, C8/C2, C8/C10, and C8/C12 ratios, are also often increased in MCADD and results may be combined with elevated primary markers (Maier *et al.*, 2005, 2009b; Millington *et al.*, 1990; Smith *et al.*, 2010). The positive predictive value for C8 elevations by MS/MS is considered to be high, as false positives are only rarely seen in premature infants and heterozygous carriers for the common variant p.[Lys329Glu] (McCandless *et al.*, 2013).

Secondary carnitine deficiency (C0 deficiency) can be present and reflects a competition between increased acylcarnitine levels and free canitine transport at the plasma membrane (Andrew and Spiekerkoetter, 2016; Roe and Coates, 1995; Stanley *et al.*, 1993). The excess acylcarnitines bind to free carnitine and are renally excreted (Andrew and Spiekerkoetter, 2016; Roe and Coates, 1995; Stanley *et al.*, 1993). This may cause only mildly elevated or normal levels of C8, C6, and C10 acylcarnitines and thereby false negative results (Clayton *et al.*, 1998; Leydiker *et al.*, 2011; Merritt and Chang, 2020). False negatives have been reported in newborns with low free carnitine levels, caused by a previously undiagnosed mothers with MCAD deficiency, maternal carnitine transporter deficiency, or nutritional carnitine deficiency (Aksglaede *et al.*, 2015; Leydiker *et al.*, 2011).

Follow-up testing includes the referral of the newborn and its family to a specialized metabolic center for analysis of plasma acylcarnitines, urine organic acids and acylglycines (Andrew and Spiekerkoetter, 2016; Merritt and Chang, 2020). If the test results support the likelihood of MCADD, additional testing by molecular genetics or enzymology is required to establish the diagnosis (Andrew and Spiekerkoetter, 2016; Merritt and Chang, 2020). All named methods can identify asymptomatic affected patients without the need for provocative tests (Merritt and Chang, 2020).

Acylcarnitine profile in plasma

Acylcarnitine measurements in plasma from a venous blood sample need to be performed to confirm NBS results. A combination of increased primary markers and secondary ratios are often detected in MCADD patients (Maier *et al.*, 2005, 2009b; Millington *et al.*, 1990; Smith *et al.*, 2010). When clinical suspicion of MCAD deficiency remains high and plasma acylcarnitine testing is normal, secondary carnitine deficiency should be considered during the evaluation (Clayton *et al.*, 1998; Leydiker *et al.*, 2011; Merritt and Chang, 2020).

If plasma acylcarnitine analysis is not reliant, urine organic acids or acylglycines should be analyzed to establish diagnosis (Andrew and Spiekerkoetter, 2016; Merritt and Chang, 2020). In ideal circumstances, metabolites in blood and urine are analyzed in times of acute metabolic decompensation, as they might normalize under asymptomatic conditions (Andrew and Spiekerkoetter, 2016; Merritt and Chang, 2020).

Organic acids and acylglycines in urine

Standard urine organic acid profiles are often normal in asymptomatic MCADD patients and only show elevated results in symptomatic patients (Rinaldo *et al.*, 2001). Thereby it might miss detection in MCADD patients, who are compensated well. In symptomatic MCADD patients urine medium-chain dicarboxylic acids, including glycine conjugates including hexanoglycine (C6), octanoglycine (C8) and decanoglycine (C10) may be elevated while ketones are inappropriately low (Gregersen *et al.*, 1976, 1980, 1983; Merritt and Chang, 2020; Rinaldo *et al.*, 1998; Roe and Coates, 1995). Moreover, suberylglycine and dicarboxylic acids (adipic, suberic, sebacic, dodecanedioic, tetradecanedioic) may be increased (Gregersen *et al.*, 1976, 1980, 1983; Merritt and Chang, 2020; Rinaldo *et al.*, 1998; Roe and Coates, 1995).

Urine acylglycine analysis may detect urinary n-hexanoylglycine, 3-phenylpropionylglycine, and suberylglycine even in asymptomatic MCADD patients or patients with a mild or moderate biochemical phenotype (Bennett *et al.*, 1991; Rinaldo *et al.*, 1988, 2001). As acylglycine analysis is more sensitive and specific for identification of asymptomatic MCADD patients, it is also applicable in newborns directly after birth (Bennett *et al.*, 1991; Rinaldo *et al.*, 1988).

During an acute episode of metabolic decompensation in MCADD, large amounts of hexanoylglycine and suberylglycine are detectable by both, urine organic acid and acylglycine analysis (Rinaldo *et al.*, 1998).

Molecular genetics

The field of genetic testing has grown rapidly within the past years (Katsanis and Katsanis, 2017; Wanders *et al.*, 2020). Nowadays genomic technologies can detect genetic variation in patients at high accuracy and reduced cost (Katsanis and Katsanis, 2017). Unfortunately, these technologies are only measured in certain specialized laboratories and not clinically available in many regions (Katsanis and Katsanis, 2017).

When NBS results and other confirmatory findings suggest the diagnosis of MCADD, single-gene or multigene panel testing can be performed to determine the genotype of the patient (Merritt and Chang, 2020).

Single-gene testing identifies small intragenic deletions, insertions, missense, nonsense, and splice site variants after amplification of the DNA coding segments and adjacent regions via polymerase chain reaction (PCR) by Sanger sequencing (Katsanis and Katsanis, 2017; Merritt and Chang, 2020). If only one or no pathogenic variant is found gene-targeted analysis can be used to detect intragenic deletions or duplications (Katsanis and Katsanis, 2017; Merritt and Chang, 2020). In patients with northern European background the common pathogenic variants are p.[Lys329Glu];[Lys329Glu] and p.[Lys329Glu];[Tyr67His] (Andresen *et al.*, 2001; Cooper *et al.*, 2020; Gramer *et al.*, 2015; Wilcken *et al.*, 2007).

A multigene panel test, that includes *ACADM* and other genes of interest, is most likely to identify the genotype of a patient as it looks for variants in several genes at once (Merritt and Chang, 2020).

When the diagnosis of MCADD has not been considered, comprehensive genomic testing, including exome and genome sequencing, can be conducted (Wanders *et al.*, 2020). Both perform fast analysis, have a high fidelity, and relatively low cost (Wanders *et al.*, 2020).

The human exome includes all coding nuclear DNA sequences, while mitochondrial DNA is excluded. It is comprising only 1 % - 2 % of the human genome, but contains the majority of currently recognized variants that might cause diseases (Wallace and Bean, 2018). Exome sequencing identifies the sequence of all protein-coding nuclear genes of the genome. Approximately 95% of the exome can be sequenced with currently available techniques and diagnosis is identified in 20 % - 30 % (Gahl *et al.*, 2012; Lazaridis *et al.*, 2016).

The human genome comprises of coding and noncoding nuclear and mitochondrial DNA sequences. Genome sequencing identifies the sequence of all coding and noncoding nuclear DNA (Wallace and Bean, 2018). This method is more expansive than exome sequencing an diagnostic utility is about the same (Taylor *et al.*, 2015).

In many cases molecular analysis, as targeted sequencing of particular genes or gene panels, or in the form of whole exome or genome sequencing, is performed first after positive acylcarnitine profiling of MCADD (Wanders *et al.*, 2020). Thereby many new variants are diagnosed and further functional analysis, for example enzyme activity assay is needed to verify the consequences (Wanders *et al.*, 2020).

Enzymology

Analysis of acylcarnitines in cultured fibroblasts of MCADD patients involves the incubation of fibroblast cultures with labeled or non-labeled palmitic acid and non-labeled L-carnitine and may reveal increased levels of C6, C8, and C10 levels to confirm diagnosis (Schmidt-Sommerfeld *et al.*, 1998).

Residual enzyme activity of MCAD is usually measured in cultured fibroblasts or lymphocytes from venous blood as enzymes of FAO are expressed in peripheral blood cells (Andrew and Spiekerkoetter, 2016). There is an evidence for a correlation between the residual enzyme activity of MCAD and the expected clinical phenotype of MCADD patients (Derks *et al.*, 2008; Jager *et al.*, 2019; Wanders *et al.*, 2010). Unfortunately, enzyme activity assays of MCAD are only measured in certain specialized laboratories and not clinically available in many regions (Bouvier *et al.*, 2017; Wanders *et al.*, 2010).

Much has been achieved in the past years and many different enzyme activity assays have been set up.

In the beginning, residual enzyme activities were measured fluorimetrically and spectrophotometrically using different dye-reduction assays based on the use of artificial electron acceptors, but it was quickly noticed that these types of assays have a number of disadvantages (Rhead *et al.*, 1983).

The method of choice became the enzyme activity measurement by an ETF reduction assay, using the fluorescence of ETF, which is decreased upon the reduction of ETF (Coates *et al.*, 1985; Hale *et al.*, 1990; Stanley *et al.*, 1983). Hale et al. revealed a cutoff value of residual enzyme activity of 10 % of wild type, showing that patients with MCADD reveal lower values (Hale *et al.*, 1990). ETF has never become commercially available and the assay yields problems in practice as it has to be performed in anaerobic state (Wanders *et al.*, 2010).

Amendt and Rhead introduced a tritium-release assay for MCAD using [2,3-^{3}H]-octanoyl-CoA as substrate (Amendt and Rhead, 1985).

A completely different method using gas chromatography-mass spectrometery was implemented by Kølvraa et al. and variations of others followed (Kølvraa *et al.*, 1982). An extension to this assay was the direct spectrophotometric method for

MCAD, based on the use of ferricenium hexafluorophosphate (Lehman and Thorpe, 1990). The method involved measurement of the 3-phenylpropionyl-CoA mediated reduction of the ferricenium ion at 303 nm (Lehman and Thorpe, 1990). Both methods revealed false results and leaded to artificially high residual activities in MCADD patients (Taylor *et al.*, 1992).

A new method used ferricenium hexafluorophosphate as electron acceptor, followed by the analysis of the products of the MCAD reaction by high performance liquid chromatography (HPLC) coupled to UV-detection or by ultra performance liquid chromatography (UPLC) coupled to MS/MS detection (Wanders *et al.*, 1999). 3-phenylpropionyl-CoA was used as substrate and residual enzyme activity was measured in fibroblasts and lymphocytes (Wanders *et al.*, 2010). Derks et al. entitled this measurement method in leukocytes or lymphocytes as the gold standard to diagnose MCAD deficiency upon an initial positive screening test result (Derks *et al.*, 2008). Enzyme analysis appeared to discriminate within the group of true-positive newborns, allowing the enzymatic identification of even mild phenotypes (Derks *et al.*, 2008). Touw et al. stated that residual MCAD enzyme activities < 10 % of wild type in leukocytes or lymphocytes are associated with clinical symptoms, while residual MCAD enzyme activities ≥ 10 % of wild type do not show clinical symptoms and the necessity to avoid overnight fasting is debatable (Touw *et al.*, 2013).

Octanoyl-CoA as substrate and HPLC coupled MS/MS was used by Tajima et al. and ter Veld et al. to determine residual enzyme activity in MCADD (Tajima *et al.*, 2005, 2008; ter Veld *et al.*, 2009). Sturm et al. used octanoyl-CoA as substrate and HPLC coupled to MS/MS detection and HPLC coupled to UV-detection measurements in lymphocytes and detected that residual MCAD activities < 20 % are clearly disease-causing, activities between 20 % and 30 % need special supervision and follow-up, whereas activities beyond 30 % are associated with asymptomatic disease (Sturm *et al.*, 2012). Bouvier et al analyzed an assay of enzyme activity in fibroblasts using octanoyl-CoA as substrate and HPLC coupled to MS/MS method and suggest that a MCAD activity < 35 % of wild type can be found in MCADD patients (Bouvier *et al.*, 2017).

Conclusions of residual MCAD enzyme activity assays might differ, due to variant test methods.

1.3.6 Management and treatment

1.3.6.1 Asymptomatic patients

The management of asymptomatic patients with MCADD involves to provide an adequate oral caloric intake and to avoid periods of fasting (Derks *et al.*, 2007; Merritt and Chang, 2020; Roe and Coates, 1995; Stanley and Bennett, 2011). The guideline of the British inherited metabolic disease group (BIMDG) advocates a maximum safe fasting time for well and asymptomatic children according to their age. Children under 4 months of age are allowed to fast for maximal 6 hours, children 4 to 8 months of age can fast up to 8 hours, children from 8 to 12 months of age for maximal 10 hours and children from 12 months of age and onwards for maximal 12 hours (Dixon, 2009). Newborns require feeding every 2 to 3 hours and may receive breast milk or standard infant formulas to meet standard infant feeding demands. Breast feeding is feasible, but during the first days of life, formula may be given if the supply of breast milk is reduced (Merritt and Chang, 2020). Solid foods may be offered to children over 4 months of age (Derks *et al.*, 2007). A diet high in carbohydrates and relatively low in fat is recommended (Derks *et al.*, 2007). Toddlers may receive milk as a bedtime snack or 2g/kg uncooked cornstarch, a source of complex carbohydrates for a sufficient glucose supply overnight (Derks *et al.*, 2007; Merritt and Chang, 2020; Roe and Coates, 1995). Medium-chain-triglycerides (MCT), as MCT oil or coconut oil should be avoided, because they contain high amounts of medium-chain fatty acids that cannot be oxidized by the deficient enzyme MCAD (Derks *et al.*, 2007; Merritt and Chang, 2020; Roe and Coates, 1995). Also, overfeeding should be prevented as MCADD patients do not need extra calories (Derks *et al.*, 2007; Merritt and Chang, 2020).

Oral L-carnitine supplementation is discussed controversially, but can be considered as an add-on treatment option (Merritt and Chang, 2020). It has been advocated for the management of MCADD patients to reverse secondary carnitine deficiency (de Moraes *et al.*, 2020; Roe and Coates, 1995; Zschocke and Hoffmann, 2012). Moreover, it displays an alternative conjugation pathway for the removal of potentially toxic metabolites which accumulate under metabolic crisis (de Moraes *et al.*, 2020; Roe and Coates, 1995). 20-100 mg/kg/day of carnitine is recommended as oral supplementation to correct a secondary carnitine deficiency (Merritt and Chang, 2020; Zschocke and Hoffmann, 2012).

1.3.6.2 Symptomatic patients

Symptomatic MCADD patients are individuals under metabolic crisis with insufficient energy supply to peripheral tissues due to impaired FAO and FAO dependent ketogenesis (Aldubayan *et al.*, 2017). This can be caused by infections, for example respiratory tract infections or gastroenteritis, with reduced oral caloric intake. The treatment aim of symptomatic patients in metabolic crisis is the reversal of catabolism and thereby prevention of hypoglycemia (Gramer *et al.*, 2015; Janeiro *et al.*, 2019). Drinks with an appropriate amount of glucose should be started early, when first symptoms of illness occur and continued every 2 hours (Aldubayan *et al.*, 2017; Merritt and Chang, 2020). If the patient does not improve or even deteriorates, hospital admission is needed urgently (Aldubayan *et al.*, 2017; Andrew and Spiekerkoetter, 2016; Merritt and Chang, 2020). A prompt intravenous fluid substitution with a bolus of 2 ml/kg containing 25 % glucose should be started in hospital. Intravenous substitution should be continued with a 10 % glucose infusion providing at least 10-12 mg/kg/min of glucose (Aldubayan *et al.*, 2017; Andrew and Spiekerkoetter, 2016; Merritt and Chang, 2020). This corresponds to the physiological hepatic glucose production level (Stanley and Bennett, 2011). Infusion therapy should be started without delay and even if hypoglycemia is not present, as it is a late event in MCADD. (Andrew and Spiekerkoetter, 2016)

1.3.6.3 Long-term follow-up

As detection by NBS is mostly established in presymptomatic newborns, patients appear entirely healthy, despite having a risk of metabolic decompensation under metabolic stress (Schatz and Ensenauer, 2010). Thereby the risk and need for preventative measures may be easily undervalued (Schatz and Ensenauer, 2010). Severe metabolic crises during lifetime can still potentially lead to sudden death (Dessein *et al.*, 2010; Janeiro *et al.*, 2019; Pugliese *et al.*, 2020; Wanders *et al.*, 2020; Yusupov *et al.*, 2010). To prohibit a severe course of the disease all patients should be considered at risk of developing clinical manifestations and require individualized follow-up by experienced clinicians at specialized centers (Arnold *et al.*, 2010; Janeiro *et al.*, 2019; Schatz and Ensenauer, 2010). It is necessary that patients carry their emergency pass at all times including a detailed management plan and telephone number of their metabolic clinician (Schatz and Ensenauer, 2010).

1.3.7 Genotype-phenotype correlation

Inclusion of MCADD into the NBS program has vastly improved the clinical course through early detection of highly but also only slightly increased medium-chain acylcarnitine levels (Andresen *et al.*, 2001; Maier *et al.*, 2005; Smith *et al.*, 2010; Zschocke *et al.*, 2001). Thereupon, molecular genetics detected many new homozygous and compound heterozygous genotypes of MCADD patients (Andresen *et al.*, 2001; Maier *et al.*, 2005; Smith *et al.*, 2010; Zschocke *et al.*, 2001). The question, whether a correlation between genotype and phenotype of patients can be established, is due to the rapid development of genomic technologies nowadays more present than in the past. In the literature contradicting statements can be found.

Andresen et al. argue, that there might be a genotype-phenotype correlation for several genotypes (Andresen *et al.*, 2001). Homozygosity for p.[Lys329Glu];p.[Lys329Glu] is associated with a severe phenotype, a diminished enzymatic activity, and altered protein folding, while the variant p.[Lys329Glu];[Tyr67His] appears to be benign, since it has never been detected in the homozygous state in a symptomatic patient (Andresen *et al.*, 2001; Andrew and Spiekerkoetter, 2016; Gramer *et al.*, 2015; Waddell *et al.*, 2006). Homozygous patients of p.[Lys329Glu];[Lys329Glu] showed the highest C8 values in the NBS screening and often presented with symptoms after birth (Arnold *et al.*, 2010; Bentler *et al.*, 2017; Gramer *et al.*, 2015; Waddell *et al.*, 2006).

Andrew and Spiekerkoetter state that there is no clear genotype-phenotype correlation (Andrew and Spiekerkoetter, 2016). In patients with MCADD, the severity of an exacerbation correlates with the exposure to metabolic stress, but less with the genotype (Andrew and Spiekerkoetter, 2016). Patients with the same genotype can have completely different courses of disease development, ranging from being asymptomatic to premature death (Andrew and Spiekerkoetter, 2016).

The C8/C10 ratio can discriminate between mild and severe phenotypes, but does not allow the transfer of the results to other patients with an equal genotype (Maier *et al.*, 2005, 2009b; Millington *et al.*, 1990; Smith *et al.*, 2010).

The benchmark method to assess disease severity is the *ex vivo* enzyme activity assay analysis in cultured lymphocytes or fibroblasts using the HPLC method, as residual enzyme activity correlates with the expected clinical phenotype (Derks *et al.*, 2008; Jager *et al.*, 2019; Wanders *et al.*, 2010, 2020). This method is time-consuming and not available in most countries or not measured routinely, which indicates that new methods for the assessment of severity of disease and follow-up of patients are needed to get a more precise and personalized picture of each individual MCADD patient and for further genotype-phenotype discussions.

1.4 ^{13}C breath test

The ^{13}C-breathtest is a non-invasive method to assess enzyme metabolism *in vivo.* After oral application of ^{13}C-labelled stable isotopes, its subsequent metabolism to ^{13}carbon dioxide ($^{13}CO_2$) by a specific enzyme can be quantified in exhaled air (Braden, 2009; Braden *et al.*, 1994, 2007; Haisch *et al.*, 1994; Rating and Langhans, 1997). The stable isotope^{13}C differs by only one neutron from the naturally more common^{12}C-atom (Braden, 2009). The mass difference between $^{13}CO_2$ and $^{12}CO_2$ can be measured as ratio of $^{13}CO_2/^{12}CO_2$ by infrared spectroscopy (Braden, 2009; Braden *et al.*, 1994, 2007; Haisch *et al.*, 1994; Rating and Langhans, 1997). Results are labelled as cumulative ^{13}C recovery rate and determination over a defined time period allows indirect estimation of the activity level of a specific enzyme (Braden, 2009; Braden *et al.*, 1994, 2007; Haisch *et al.*, 1994; Rating and Langhans, 1997).

Breath tests with specific stable isotopes were developed for different indications. The currently known most sensitive breath test uses ^{13}C-urea as substrate to detect a helicobacter pylori infection in the stomach or duodenum (Braden, 2009; Haisch *et al.*, 1994; Rating and Langhans, 1997).

The substrates ^{13}C-bicarbonate and ^{13}C-octanoic acid are used to analyze gastric emptying and provide reliable, non-radiating alternatives to scintigraphy (Braden, 2009; Rating and Langhans, 1997).

Hepatic function in acute and chronic liver disease, also before and after transplantation and enzyme induction can be tested with ^{13}C-aminopyrine, ^{13}C-caffeine and ^{13}C-methacetin (Braden, 2009; Braden *et al.*, 1994; Rating and Langhans, 1997).

^{13}C-ketoisocaproic acid is known to be used for differentiation between cholestatic and cirrhotic conditions (Braden, 2009; Rating and Langhans, 1997).

Substrates as ^{13}C-lactose and ^{13}C-sucrose are used to identify maldigestion of carbohydrates and ^{13}C labelled mixed triglycerides for maldigestion of lipids (Braden, 2009; Rating and Langhans, 1997).

The breath test with ^{13}C-mixed triglycerides, ^{13}C-triolein, ^{13}C-trioctanoin, ^{13}C-tripalmitin, ^{13}C-hiolein and ^{13}C-cholesterol-octanoate can measure the exocrine pancreatic function by intraluminal activity of pancreatic enzymes (Braden, 2009).

As an example for metabolic diseases, a ^{13}C breath test is already an applied method in PKU [MIM #261600] patients for follow-up assessment (Muntau *et al.*, 2002; Okano *et al.*, 2004, 2007; Treacy *et al.*, 1997; Turki *et al.*, 2015). PKU is an inborn autosomal recessive disease, also detected by NBS, that results in a deficient hydroxylation of phenylalanine to tyrosine by the enzyme phenylalanine hydroxylase (Blau *et al.*, 2010; Muntau *et al.*, 2002; Pugliese *et al.*, 2020; Zurflüh *et al.*, 2008). Clinically it can lead to impaired cognitive development resulting from a neurotoxic effect of hyperphenylalaninemia (Blau *et al.*, 2010; Muntau *et al.*, 2002; Pugliese *et al.*, 2020; Zurflüh *et al.*, 2008). Hence, phenylalanine blood levels are measured regularly (Blau *et al.*, 2010; Muntau *et al.*, 2002; Zurflüh *et al.*, 2008). The breath test is performed using ^{13}C-phenylalanine (Muntau *et al.*, 2002; Okano *et al.*, 2004, 2007; Turki *et al.*, 2015). Results of healthy individuals serve as baseline values for comparison with affected patients (Muntau *et al.*, 2002; Okano *et al.*, 2004, 2007; Turki *et al.*, 2015). Differences between healthy individuals, heterozygous carriers and affected patients (homozygous and compound heterozygous) with decreased breath test results in the respective order have been shown (Muntau *et al.*, 2002; Okano *et al.*, 2004, 2007; Turki *et al.*, 2015). An improvement of the cumulative ^{13}C recovery under therapy can be seen in breath test results of tetrahydrobiopterin-responsive patients (Muntau *et al.*, 2002; Okano *et al.*, 2004, 2007; Turki *et al.*, 2015).

In MCADD, breath tests have been performed after oral administration of ^{13}C-butyric, ^{13}C-octanoic and ^{13}C-palmitic acids (Jakobs *et al.*, 1997). The *in vivo* oxidation of all tested substrates was similar to results of healthy controls and has limited diagnostic use for the enzyme MCAD (Jakobs *et al.*, 1997). A ^{13}C breath test for MCADD has not been established as a diagnostic or follow-up tool yet.

2 Work hypothesis and specific aims

2.1 Hypothesis

Our study hypothesizes that the *in vivo* metabolic flux of ^{13}C-labelled phenylpropionic acid (^{13}C-PPA) can be measured by a non-invasive breath test, providing indirect conclusions about the enzyme function of MCAD.

The combination of the 3 biochemical approaches, namely biochemical acylcarnitine profiles, *ex vivo* enzyme activity assay in lymphocytes and *in vivo* metabolic flux by breath test, will enable the establishment of a more precise and personalized picture of each individual MCADD patient.

2.2 Specific aims

Aim 1: Validation of the *in vivo* ^{13}C-PPA breath test in healthy controls.

Approach: Application of the ^{13}C-PPA breath test in healthy controls with respect to the metabolism and kinetics of the ^{13}C labelled substrate. Analysis of inter- and intraindividual variability.

Aim 2: Validation of the *in vivo* ^{13}C-PPA breath test as a reproducible method in MCADD patients.

Approach: Application of the ^{13}C-PPA breath test in MCADD patients (homozygous or compound heterozygous genotype) and their parents (heterozygous carriers). Comparison of the results to established biochemical methods (C8/C10 ratio, MCAD activity in lymphocytes).

Aim 3: Analysis of potential cluster groups of phenotypes of participants.

Approach: Performance of unbiased hierarchical cluster analysis (HCA), ROC analysis and cutoff value determination.

3 Patients und methods

3.1 Study design and patient recruitment

The data of this thesis are based on a prospective multi-center study and were collected from the beginning of 2017 until the end of 2019. Study participants were recruited from the University Children's Hospitals in Hamburg, Leipzig and Münster, Germany.

The *in vivo* metabolic flux was analyzed using a ^{13}C breath test. Following oral application of ^{13}C-PPA as substrate for the MCAD enzyme, $^{13}CO_2$ was determined in exhaled air for 180 minutes. Breath test measurements were evaluated by the analysis of acylcarnitine profiles in blood and MCAD enzyme activity in lymphocytes from venous blood samples.

The study was approved by the Ethical Committee of the Hamburg Chamber of Physicians (Registered Trial PV5388). All participants or their legal custodies consented their participation in the study prior to commencement.

3.2 Study visit schedule

Recruitment	Participants were recruited by telephone.
	Consent form was sent by email and participants received an introduction into the study program by telephone. They were allowed to ask questions.
Study visit	After a detailed explanation of the study program, participation was consented by signature. Children over 7 years were asked to sign their own consent form in addition to an extra consent form for their legal custodies.
	Venous blood samples were taken: - 2 blood spot fields on filter paper for analysis of acylcarnitines. - 2.5 – 10 ml (depending on the age) of heparin blood samples for enzyme activity measurement in lymphocytes.

	Reference breath test sample was taken, subsequently ^{13}C labelled substrate was given orally. Thereafter, breath samples were taken every 20 minutes for 180 minutes.
End of the study	Evaluation of the ^{13}C breath test, acylcarnitine levels, and lymphocyte activity assay.
	Patients were informed about their results.

3.3 Study population

Genetic analysis of all MCADD patients was previously performed by conventional Sanger sequencing independently to our study project.

The study population consisted of 40 participants, 11 patients homozygous for the variant p.[Lys329Glu];[Lys329Glu], one homozygous patient for the variant c.[244insT*104];[244insT*104], 13 compound heterozygous patients, 9 heterozygous carriers of p.[Lys329Glu] and 6 healthy controls. (**Table 1**)

Of this group, 7 compound heterozygous patients were recruited from the University Children's Hospital Leipzig (PD Dr. med. Skadi Beblo) and one homozygous patient for the variant c.[244insT*104];[244insT*104] was recruited from the University Children's Hospital Münster (Prof. Dr. med. Frank Rutsch). All other patients, heterozygous carriers and healthy controls are tied to the University Children's Hospital Hamburg (Headed by Prof. Dr. med. Ania Muntau).

Table 1 Study population. *Only for lymphocyte enzyme activity measurements we extended our population by 12 buffy coats.

	Homozygous patients	Compound heterozygous patients	Heterozygous carriers	Healthy controls	Healthy buffy coats*
Number of participants	12	13	9	6	12
Genotype (number of partici-pants)	p.[Lys329Glu];[Lys329Glu] (n=11) c.[244insT*104];[244insT*104] (n=1)	p.[Lys329Glu];c.[387+1delG] (n=1) p.[Lys329Glu];[Ile78Thr] (n=1) p.[Lys329Glu];[Lys197Glu] (n=2) p.[Lys329Glu];[Met155Thr] (n=1) p.[Lys329Glu];[Ser245Leu] (n=1) p.[Lys329Glu];[Thr351Ile] (n=1) p.[Lys329Glu];[Arg206Cys] (n=2) p.[Lys329Glu];[Ile416Thr] (n=1) p.[Lys329Glu];[Leu107Ser] (n=1) p.[Lys329Glu];[Tyr67His] (n=2)	p.[Lys329Glu]; Wild type (n=9)	Wild type (n=6)	Wild type (n=12)
Mean age (years)	14	14	43	34	34
Gender (females : males)	5 : 7	8 : 5	4 : 5	3 : 3	6 : 6

3.4 Diagnostics

3.4.1 ^{13}C-PPA breath test

Participants were instructed to fast at least 3 hours prior to the breath test and a fasting breath sample, serving as a reference, was collected prior to the administration of the tracer. Then, 5 mg/kg of ^{13}C-PPA (^{13}C-phenylpropionic acid, ^{13}C-PPA; 99% atom enrichment, Euroisotop, Cambridge Isotope Laboratories, Inc) were dissolved in 20 – 30 ml of apple or orange juice in order to mask its slightly acidic flavour and odour. The tracer was administered orally.

In vivo, ^{13}C-PPA is metabolized by the enzyme MCAD to cinnamoyl-CoA. After hydratation, dehydrogenation and thiolysis to acetyl-CoA the release ^{13}C-PPA labelled $^{13}CO_2$ was measured in exhaled air (Mao *et al.*, 1994; Rinaldo *et al.*, 1990; Yao and Schulz, 1993). (**Figure 6**)

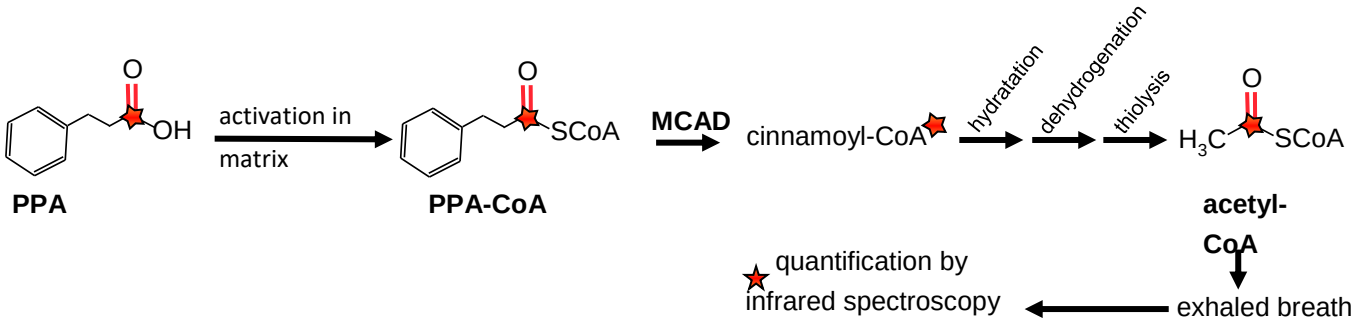


Figure 6 Schematic presentation of the *in vivo* metabolic flux of ^{13}C-PPA.

Quantification was performed by the Infrared Isotope Analyser 3 (IRIS 3, Kibion, Bremen, Germany) in the laboratories of University Children's Research headed by Prof. Dr. med. Søren Gersting, University Medical Center Hamburg-Eppendorf. IRIS 3 is equipped with a non-dispersive infrared-spectrometer (NDIR) using a broadband light source and acoustic-optical detectors, which are sensitive to the wavelengths where gases are IR-absorbing.

Breath samples were collected every 20 minutes over a period of 180 minutes (aluminium bags, Kibion, Bremen, Germany). After 30 minutes of testing, the participants were allowed to eat.

In total, 10 breath samples per participant were collected throughout the course of the experiment. The filled breath bags were connected to the ports on the front panel of the IRIS 3 system and the breath samples were pumped through the measurement unit for $^{13}CO_2$ and $^{12}CO_2$ by a membrane pump. (**Figure 7**)

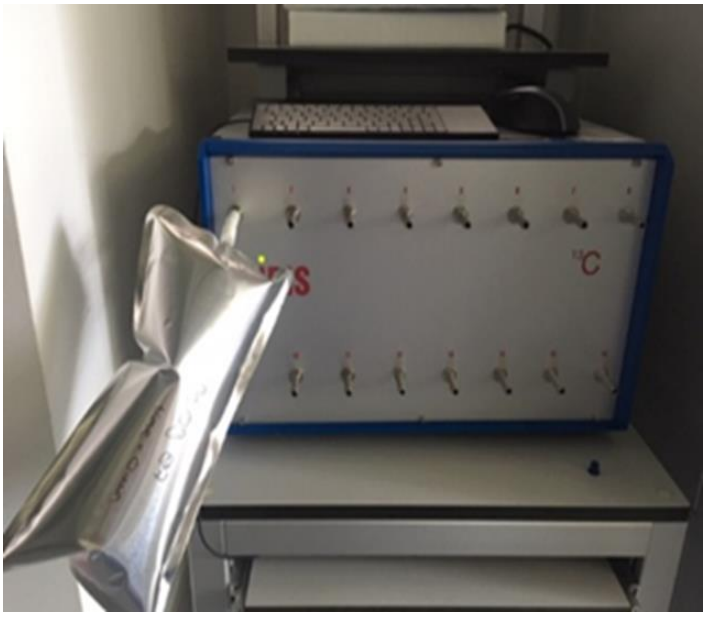


Figure 7 Photographic representation of an aluminium breath test bag connected to the ports of IRIS 3.

Two detectors, which are individually sensitive for the absorption spectra of $^{13}CO_2$ and $^{12}CO_2$ measured the $^{13}CO_2/^{12}CO_2$ ratio. Results were available after 20 minutes and are presented as the total amount of cumulative ^{13}C recovery rate after 180 minutes (Braden, 2009; Braden *et al.*, 1994, 2007; Haisch *et al.*, 1994; Rating and Langhans, 1997).

3.4.2 Acylcarnitine analysis

Acylcarnitines were analysed in dried blood spots on filter paper (**Figure 8**) by MS/MS flow injection method (La Marca *et al.*, 2003; Millington *et al.*, 1990). Analysis was performed by in the metabolic laboratories of the University Children's Hospital headed by Prof. Dr. med. René Santer, University Medical Center Hamburg-Eppendorf.

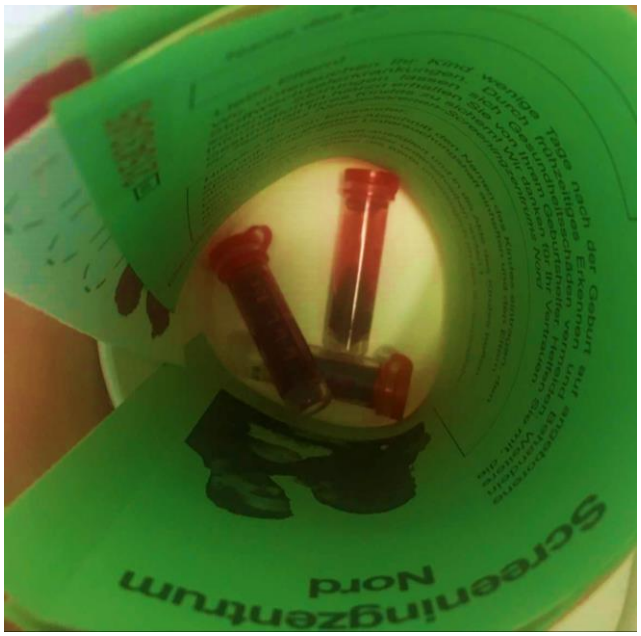


Figure 8 Dried blood spots and venous blood samples.

Samples were prepared according to the protocol described in the NeoGram AAAC Tandem Mass Spectrometry Kit (Perkin Elmer, USA). Analysis was performed on Quattro Micro tandem mass spectrometer, operating in positive ionization mode, coupled with AllianceHT 2795 HPLC system (Waters, USA). The concentrations of acylcarnitines were calculated using the appropriate internal standards and MassLynx MS software (Waters, USA). The accuracy and precision of acylcarnitine determinations were validated by the analysis of low and high control materials (provided with the kit).

Results were available after 1-2 days through our metabolic lab information system.

3.4.3 Lymphocyte enzyme activity assay

Determination of the MCAD activity assay was performed in the laboratories of University Children's Research headed by Prof. Dr. med. Søren Gersting, University Medical Center Hamburg-Eppendorf. Enzyme activity was measured in lysed lymphocytes that were isolated from venous heparin blood samples. In newborns 2.5 ml, in toddlers 2.5-5 ml, in primary school kids 5-7.5 ml and in teenagers 7.5-10 ml of heparin blood were taken for performance of the assay. An HPLC-based assay was performed, using 3-phenylpropionyl-CoA as substrate (Derks *et al.*, 2008; Touw *et al.*, 2012, 2013; Wanders *et al.*, 1999, 2010, 2020; Yao and Schulz, 1993).

Lymphocytes of the collected blood samples were enriched by using Bicoll separating solution (Biochrome) according to the manufacturer's protocols. The enriched lymphocyte fraction was resuspended in 200 µl of 20 mM HEPES buffer, supplemented with a protease inhibitor cocktail (complete mini, Roche). Lymphocytes were lysed four times by repeated thaw/freeze cycles. Protein determination was performed using the standard Bradford assay and samples were adjusted to 0.6 mg protein per ml with 20 mM HEPES buffer, supplemented with protease inhibitor cocktail.

For MCAD activity determination 20 µl of the sample was added to a V-shaped 96-well plate (Greiner). The reaction was started by adding 15 µl of the assay mix (8.5 µl 535 mM TRIS-HCl, pH 8; 6 µl 5.8 mM ferricenium hexafluorophosphate (Sigma), and 0.5 µl 10 mM 3-phenylpropionyl-CoA (Amsterdam University Medical Center, metabolic laboratory)) per sample, followed by incubation for 30 minutes at 37°C. The reaction was terminated by adding 3.5 µl 2N HCl. After 5 minutes, the sample was neutralized by adding 3.5 µl 2M KOH. For HPLC analysis 3.5 µl 10 mM cysteine and 10.5 µl acetonitrile (both Sigma) were added.

10 µl of each sample was injected to HPLC (DIONEX 3000, Thermo Fisher) under a constant flow rate of 1 ml/min of 90 % buffer A (16.9 mM Na2HPO4 and NaH2PO4 and 10 % acetonitrile) and 10 % buffer B (16.9 mM Na2HPO4 and NaH2PO4 and 70 % acetonitrile) by using an ODS-2 hypersil column (ThermoFisher). The cinnamoyl-CoA peak was detected by UV-Vis light (308 nm) within a retention time of 2 to 2.5 minutes. MCAD enzyme activities are expressed as µM cinnamoyl-CoA as and data was processed and stored using Chromeleon software (ThermoFisher).

Peak areas were converted into cinnamoyl-CoA concentrations according to the measured standard sample set (0.3125 µM up to 160 µM of cinnamoyl-CoA (Amsterdam University Medical Center, metabolic laboratory) in 20 mM HEPES buffer).

Experiments were performed in triplicates.

Results were available after 1-2 weeks and are expressed as percentage from healthy controls.

3.5 Dataset

^{13}C-PPA breath test and acylcarnitine analysis

39 of 40 participants consented to the *in vivo* ^{13}C breath test and the analysis of acylcarnitines in blood. Due to neurological impairment, one participant carrying the compound heterozygous variant for p.[Lys329Glu];[Lys197Glu] was not able to follow the breath test instructions. One participant carrying the homozygous variant c.[244insT*104];[244insT*104] declined blood sampling.

Lymphocyte enzyme activity assay

In total, 32 of 40 participants consented to a MCAD enzyme activity analysis in lymphocytes. Because of a limited number of data on healthy controls (n = 2), we extended our study population in this category by 12 healthy controls using buffy coats of routine blood sampling. Of the 12 homozygous participants, only one with the variant c.[244insT*104];[244insT*104] declined blood sampling and all compound heterozygous participants volunteered blood for the analysis. Moreover, we did not obtain blood for the lymphocyte analysis of 3 heterozygous carriers.

Age and gender distribution

The mean age of all 40 included participants, buffy coats excluded, was 23.6 years with a wide range between 4 and 52 years. The gender proportion of females and males was 1:1. (**Table 1**)

3.6 Statistics

For statistical analysis and presentation of the data, the programs Microsoft Excel, GraphPad Prism (version 8.3.0), and R (version 3.6.1) with the amplifications tidyverse (version 1.2.1), ggplot2 (version 3.2.1), ROCR (version 1.0-7) were used.

Anonymized tables were set up and frequency tables were created with Microsoft Excel. Descriptive statistics enabled a general overview of the age, gender and genotype distribution of our study cohort.

Moreover, results of acylcarnitine analysis, lymphocyte assay results and breath test results are displayed in tables. Means and standard deviation of several genotype groups (homozygous, heterozygous carriers, compound heterozygous and healthy wild type individuals) were determined. These Microsoft Excel tables were used for further analysis of other statistical programs.

For graphical presentation of the data GraphPad Prism (version 8.3.0) was used to constitute the relation of two variables or to show participants results grouped by their genotype in boxplots.

The program R (version 3.6.1) with its amplifications represented the correlation of two variables using rank analysis by Spearman's correlation coefficient and performed unbiased hierarchical cluster analysis (HCA). Results of cluster groups were then presented as means with standard deviation, which were calculated with Microsoft Excel. An unpaired t-test was performed by GraphPad Prism (version 8.3.0) to describe the significance of HCA group distinctions. Receiver-operated-curve (ROC) and subsequent cutoff value determination were calculated by the program R (version 3.6.1) with its amplifications.

A p-value of less than 0.05 ($p \leq 0.05$) was considered as statistically significant.

4 Results

4.1 The ^{13}C-PPA breath test in healthy controls

4.1.1 Metabolic capacity

For analysis of the *in vivo* metabolic flux of ^{13}C-PPA in healthy controls, we performed the breath test in 6 healthy participants after a fasting time of 3 hours. **Figure 9** shows the baseline values of three female and three male healthy controls (wild type participants). The slope of the curves indicates a saturation curve where cumulative ^{13}C recovery rates were measured every 20 minutes. After 180 minutes cumulative ^{13}C recovery rates of healthy controls were 38.18±3.35 % (mean ± standard deviation (SD)). Interindividual comparison detected higher rates for healthy females (n = 3, 40.9±1.95 % (mean ± SD)) than for healthy males (n = 3, 35.47±1.45 % (mean ± SD)). (**Table 2**)

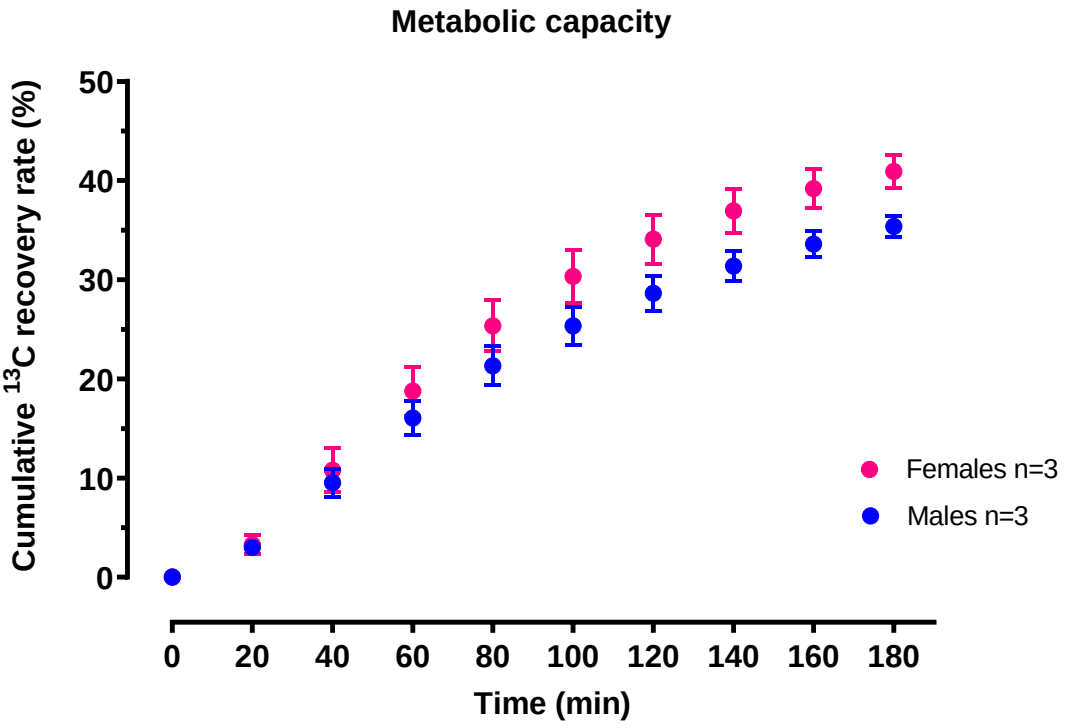


Figure 9 Metabolic capacity of the ^{13}C breath test in healthy females and males.

Table 2 Mean ± SD results of wild type participants representing the cumulative ^{13}C recovery rates after 180 minutes.

	Cumulative ^{13}C recovery rates (%), (number of participants)		
	All	Females	Males
Wild type	38.18±3.35 (6)	40.90±1.95 (3)	35.47±1.45 (3)

4.1.2 Kinetics

Figure 10 represents the kinetics of the ^{13}C breath test in healthy controls. After substrate ingestion kinetic results displayed a rapid increase of ^{13}C exhalation per hour. Mean ± SD peak doses of 25.4±1.87 % were reached at t_{max} = 53±12 minutes in healthy females and 20.6±2.26 % at t_{max} = 40±0 minutes in healthy males. Thereafter, ^{13}C recovery gradually decreased.

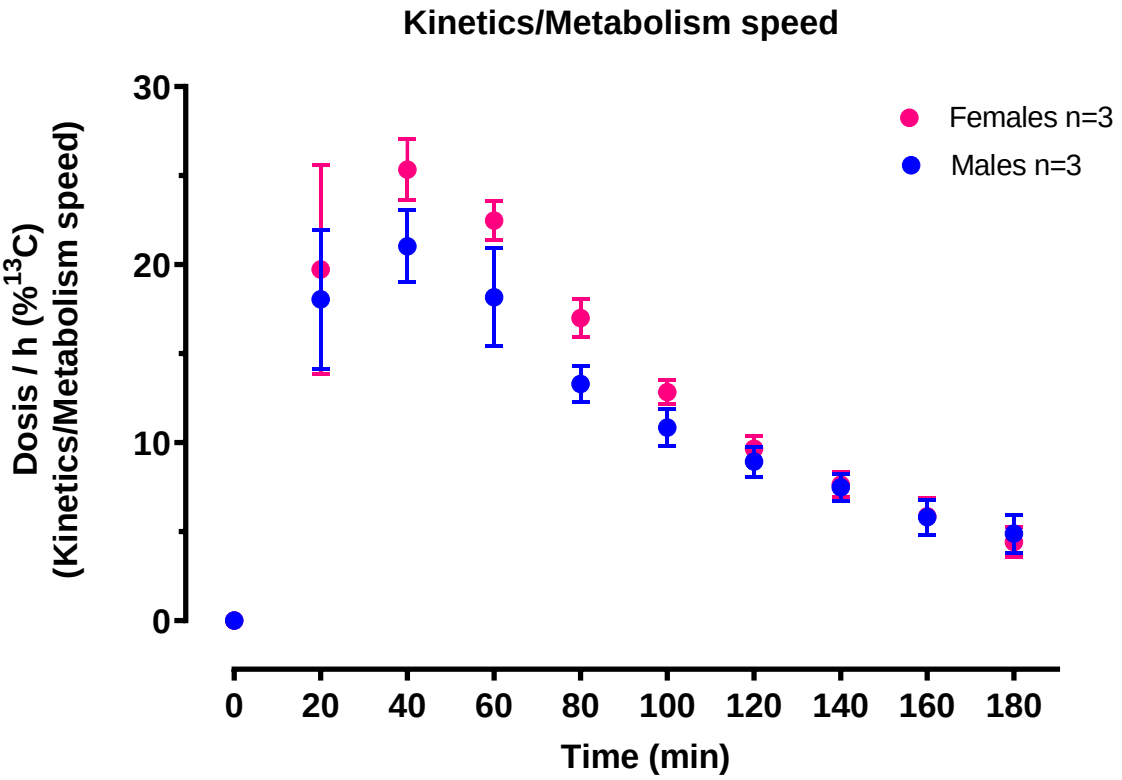


Figure 10 Kinetics/Metabolism speed of the ^{13}C breath test in healthy females and males.

4.1.3 Inter- and intraindividual variability

To analyze the impact of a 3-hour fasting time prior to the test, breath tests of two healthy controls (one male, one female) were performed at different time points after food ingestion. Results are shown in **Table 3.** Cumulative ^{13}C recovery rates of 26.5±1.41 % (mean ± SD) were observed directly after food intake, whereas after a fasting time of 3 hours higher recovery rates of 37.5±1.41 % (mean ± SD) and after an overnight fast of 12 to 15 hours highest results of 40.3±2.76 % (mean ± SD) were observed. All results showed higher ^{13}C recovery rates in females than males.

Table 3 Performance of the breath test at different time points after food ingestion, Mean ± standard deviation (SD)

	Cumulative ^{13}C recovery rates [%], (number of participants)		
Performance of the breath test	**Directly after food ingestion**	**3 hours after food ingestion**	**After an overnight fast of 12-15 hours**
Wild type females and males	26.5±1.41 (2)	37.5±1.41 (2)	40.3±2.76 (2)
Wild type female	27.5 (1)	38.5 (1)	42.2 (1)
Wild type male	25.5 (1)	36.5 (1)	38.3 (1)

To test for intraindividual variability, two healthy participants (one female, one male) performed the breath tests at three different days. Mean ± SD results are listed in **Table 4** and show cumulative ^{13}C recovery rates of 37.6±0.79 % in the female participant and 35.6±0.79 % in the male participant combining the measurements of day 1, 2, and 3.

Table 4 Intraindividual variability, Mean ± standard deviation (SD)

	Cumulative ^{13}C recovery rates [%], (number of participants)			
Performance of the breath test 3 hours after food ingestion	**Day 1**	**Day 2**	**Day 3**	**Mean ± SD (Day 1+2+3)**
Wild type females and males	37.5±1.41 (2)	36.2±1.63 (2)	36.2±1.2 (2)	36.6±1.31 (2)
Wild type female	38.5 (1)	37.3 (1)	37.0 (1)	37.6±0.79 (1)
Wild type male	36.5 (1)	35.0 (1)	35.3 (1)	35.6±0.79 (1)

4.2 The ^{13}C-PPA breath test in MCADD patients

Table 5 and **Figure 11** describe results of participant subgroups of the cumulative ^{13}C recovery rates. Healthy controls were henceforward labelled as wild type participants/controls. The subgroups are categorized as wild type participants, heterozygous carriers for p.[Lys329Glu], homozygous patients for p.[Lys329Glu];[Lys329Glu], one homozygous patient for c.[244insT*104];[244insT*104] and compound heterozygous patients.

Results of the cumulative ^{13}C recovery rates of 9 heterozygous carriers for p.[Lys329Glu] varied between 34.51±7.0 % (mean ± SD). There was no detectable difference between genders, but compared with wild type results, mean values were lower and SD varied considerably. (**Table 5, Figure 11**)

The 24 affected homozygous and compound heterozygous patients showed cumulative ^{13}C recovery rates of 19.22±8.45 % (mean ± SD) and thereby lower results than wild type participants and heterozygous carriers. Subdivision into homozygous and compound heterozygous patients represented rates of 13.95±3.86 % (mean ± SD) in 11 homozygous patients for p.[Lys329Glu];[Lys329Glu], 15.1 % in the homozygous patient for c.[244insT*104];[244insT*104], and 24.4±8.79 % (mean ± SD) in all 12 compound heterozygous patients. (**Table 5, Figure 11**)

Table 5 Mean ± SD results of participant subgroups representing the cumulative ^{13}C recovery rates.

	Cumulative ^{13}C recovery rates (%), (number of participants)		
	All	Females	Males
Wild type participants	38.18±3.35 (6)	40.90±1.95 (3)	35.47±1.45 (3)
Heterozygous carriers for p.[Lys329Glu]	34.51±7.00 (9)	34.24±8.73 (4)	34.85±5.36 (5)
Patients homozygous for p.[Lys329Glu];[Lys329Glu]	13.95±3.86 (11)	12.50±4.11 (5)	15.15±3.53 (6)
Patients homozygous for c.[244insT*104];[244insT*104]	15.1 (1)	-	15.1 (1)
Compound heterozygotes	24.40±8.79 (12)	24.30±6.21 (8)	20.68±6.21 (4)

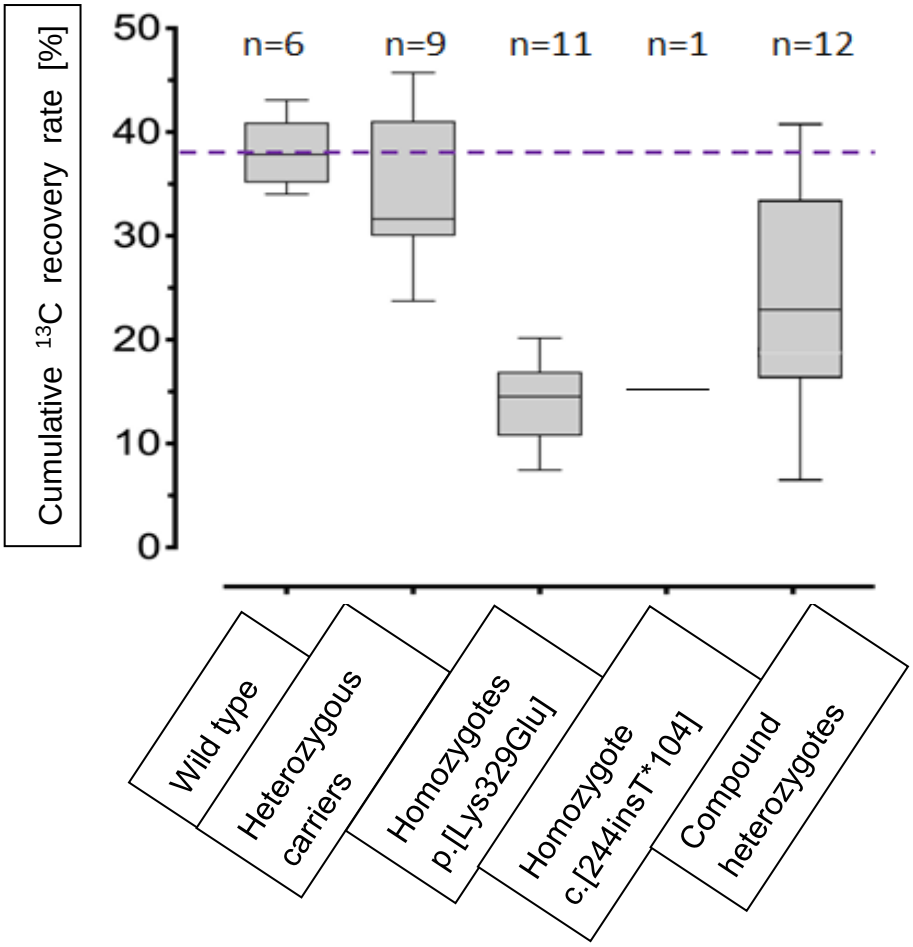


Figure 11 Results of participant subgroups presenting cumulative ^{13}C recovery rates by box plots. ─Mean of wild type controls.

4.3 Correlation of breath test results, C8/C10 ratios and MCAD activities in lymphocytes

4.3.1 Spearman's correlation coefficient

Mean ± SD of the C8/C10 ratios was 0.72±0.49 in wild type participants, 0.98±0.54 in heterozygous carriers, 10.37±5.30 in homozygous patients, and 6.13±4.25 in compound heterozygous patients. (**Table 6**)

Results of enzyme activity assays in lymphocytes revealed 17.88±3.37 µM (mean ± SD) in wild type participants, 11.47±6.67 µM (mean ± SD) in heterozygous carriers, 0.86±1.83 µM (mean ± SD) in homozygous patients and 2.92±5.34 µM (mean ± SD) in compound heterozygous patients. (**Table 6**)

Data of cumulative ^{13}C recovery rates were presented in the previous chapter. (**Table 6**)

Table 6 Mean ± SD results of participant subgroups representing C8/C10 ratios, , MCAD activities in lymphocytes [µM] and cumulative ^{13}C recovery rates [%].

	C8/C10 ratios	**MCAD activities in lymphocytes**	**Cumulative ^{13}C recovery rates**
Wild type participants	0.72±0.49 (6)	17.88±3.37 (14)	38.18±3.35 (6)
Heterozygous carriers for p.[Lys329Glu]	0.98±0.54 (9)	11.47±6.67 (6)	34.51±7.00 (9)
Patients homozygous for p.[Lys329Glu];[Lys329Glu]	10.37±5.30 (11)	0.86±1.83 (11)	13.95±3.86 (11)
Patients homozygous for c.[244insT*104];[244insT*104]	-	-	15.1 (1)
Compound heterozygotes	6.13±4.25 (13)	2.92±5.34 (13)	24.40±8.79 (12)

We correlated the *in vivo* measured breath test results of all participant subgroups with biochemical determination of C8/C10 acylcarnitine ratios and *ex vivo* enzyme activity assays in lymphocytes.

Rank correlation analysis by Spearman's correlation coefficient revealed a strong relation between C8/C10 ratios and cumulative ^{13}C recovery rates in breath test (Spearman -0.67, p-value = 0.000008) as well as between MCAD activities in lymphocytes and cumulative ^{13}C recovery rates in the breath test (Spearman 0.65, p-value = 0.000108). The observed correlation was gender independent and is shown in **Figures 12 and 13**.

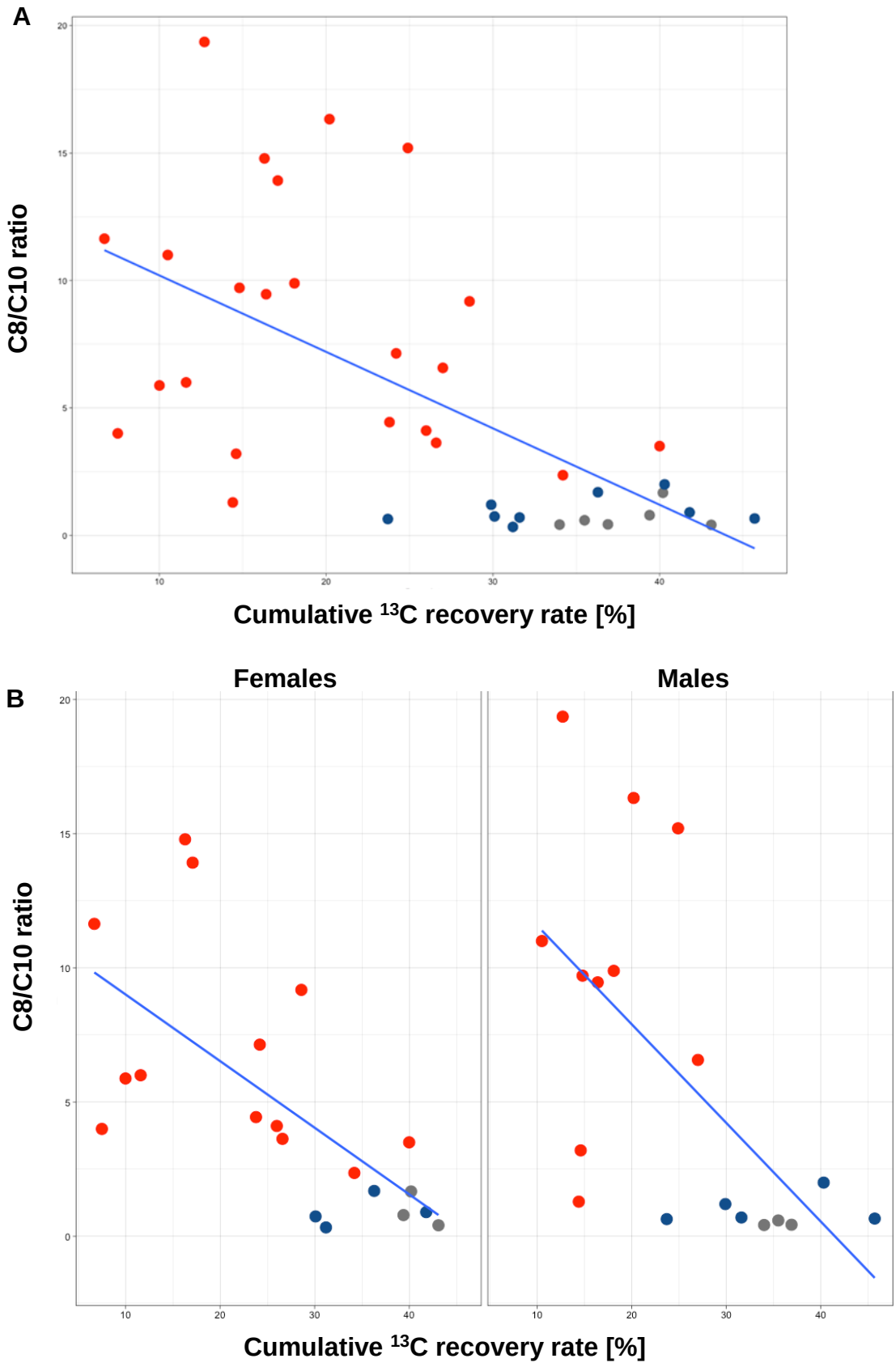


Figure 12 A Correlation of C8/C10 ratio and cumulative ^{13}C recovery rate in breath tests (—Spearman -0.67, p-value = 0.000008). **B** Gender distribution.

● MCADD patient (homozygous or compound heterozygous)

● Heterozygous carrier ● Healthy control/wild type

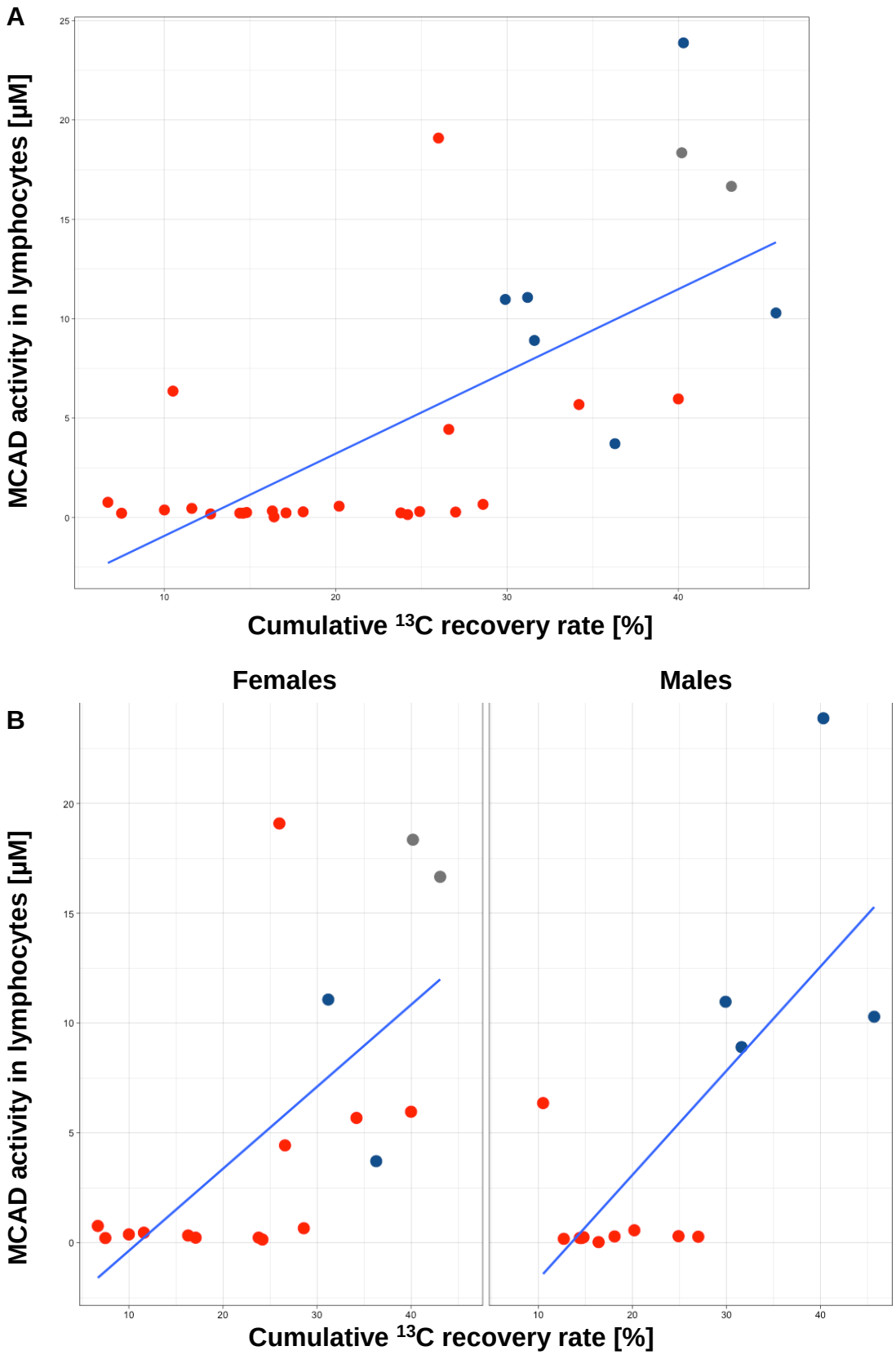


Figure 13 A Correlation of MCAD activity in lymphocytes and cumulative ^{13}C recovery rate in breath tests (—Spearman 0.65, p-value = 0.000108). **B** Gender distribution. ● MCADD patient (homozygous or compound heterozygous) ● Heterozygous carrier ● Healthy control/wild type

4.3.2 Hierarchical cluster analysis

4.3.2.1 Phenotype based clustering

We described the phenotypes of all 40 participants by three different testing methods, namely the cumulative ^{13}C recovery rate, the C8/C10 ratio, and the MCAD activity in lymphocytes. In the next step, we performed unbiased hierarchical cluster analysis (HCA) of this dataset to determine potential cluster groups of phenotypes.

HCA was performed in 31 participants (2 healthy controls, 6 heterozygous carriers of p.[Lys329Glu], 11 homozygous patients for p.[Lys329Glu];[Lys329Glu], and 12 compound heterozygous patients with p.[Lys329Glu] on one allele), who had full datasets of C8/C10 acylcarnitine ratio, lymphocyte enzyme activity assay and ^{13}C breath test.

Results of each participant are shown in **Figure 14, Table 7**. Participants are listed by their anonymized ID and the color code indicates the affiliation to a cluster group.

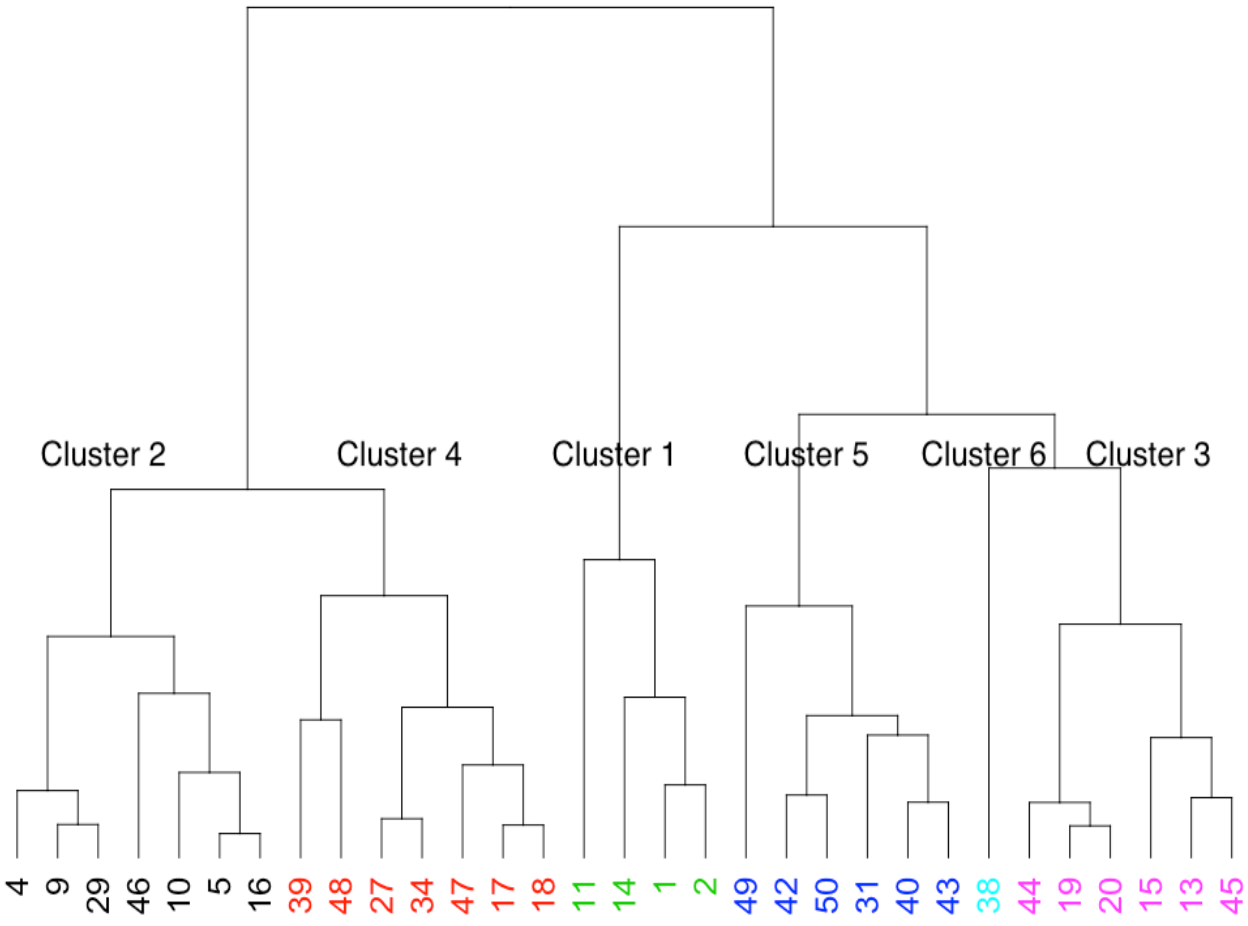


Figure 14 Hierarchical cluster analysis performed with data of 31 participants.

Table 7 Hierarchical cluster analysis showing cluster groups and results of C8/C10 ratio, MCAD activity in lymphocytes [µM], and cumulative ^{13}C recovery rate in breath test [%]. f = female, m = male. Color code indicates the affiliation to a cluster group.

Id	Cluster	Allele 1	Allele 2	Gender	Age	C8/C10 ratio	MCAD activity in lymphocytes	Cumulative ^{13}C recovery rate
1	1	Wild type (WT)	WT	f	35	1.67	18.35	40.2
2	1	WT	WT	f	31	0.41	16.66	43.1
11	1	p.[Lys329Glu]	WT	m	51	0.66	10.29	45.7
14	1	p.[Lys329Glu]	WT	m	40	2	23.88	40.3
4	2	p.[Lys329Glu]	p.[Lys329Glu]	m	20	9.89	0.2837	18.1
5	2	p.[Lys329Glu]	p.[Lys329Glu]	f	22	13.92	0.2285	17.1
9	2	p.[Lys329Glu]	p.[Lys329Glu]	m	16	9.71	0.25	14.8
10	2	p.[Lys329Glu]	p.[Lys329Glu]	m	16	16.33	0.5653	20.2
16	2	p.[Lys329Glu]	p.[Lys329Glu]	f	8	14.79	0.328	16.3
29	2	p.[Lys329Glu]	p.[Ile78Thr]	m	29	9.46	0.02683	16.4
46	2	p.[Lys329Glu]	p.[Lys329Glu]	m	9	19.36	0.1763	12.7
13	3	p.[Lys329Glu]	p.[Tyr67His]	f	4	2.36	5.681	34.2
15	3	p.[Lys329Glu]	p.[Tyr67His]	f	13	3.5	5.963	40
19	3	p.[Lys329Glu]	WT	f	46	0.33	11.07	31.2
20	3	p.[Lys329Glu]	WT	m	52	1.2	10.97	29.9
44	3	p.[Lys329Glu]	WT	m	37	0.7	8.906	31.6
45	3	p.[Lys329Glu]	WT	f	32	1.69	3.711	36.3
17	4	p.[Lys329Glu]	p.[Lys329Glu]	f	18	6	0.455	11.6
18	4	p.[Lys329Glu]	p.[Lys329Glu]	f	21	5.88	0.3763	10
27	4	p.[Lys329Glu]	p.[Lys329Glu]	m	6	3.2	0.213	14.6
34	4	p.[Lys329Glu]	p.[Lys197Glu]	m	14	1.29	0.216	14.4
39	4	p.[Lys329Glu]	c.[387+1delG]	f	7	11.64	0.7624	6.7
47	4	p.[Lys329Glu]	p.[Lys329Glu]	f	13	4	0.212	7.5
48	4	p.[Lys329Glu]	p.[Lys329Glu]	m	7	11	6.357	10.5
31	5	p.[Lys329Glu]	p.[Leu107Ser]	f	11	3.63	4.431	26.6
40	5	p.[Lys329Glu]	p.[Thr351Ile]	f	6	4.44	0.2307	23.8
42	5	p.[Lys329Glu]	p.[Met155Thr]	m	18	6.57	0.2717	27
43	5	p.[Lys329Glu]	p.[Ser245Leu]	f	19	7.14	0.1438	24.2
49	5	p.[Lys329Glu]	p.[Arg206Cys]	m	18	15.2	0.2967	24.9
50	5	p.[Lys329Glu]	p.[Arg206Cys]	f	28	9.18	0.6577	28.6
38	6	p.[Lys329Glu]	p.[Ile416Thr]	f	8	4.11	19.09	26

4.3.2.2 Comparison of groups of clusters

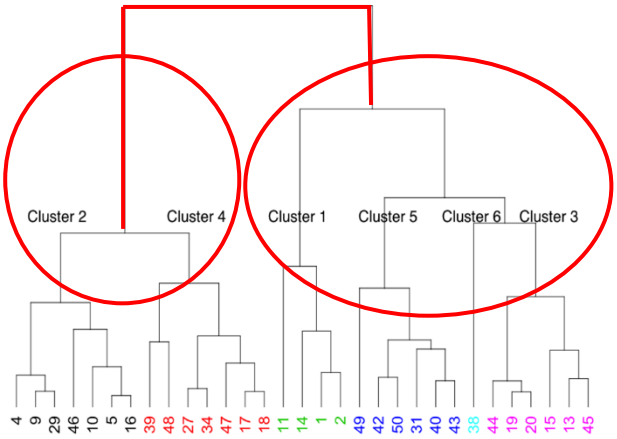
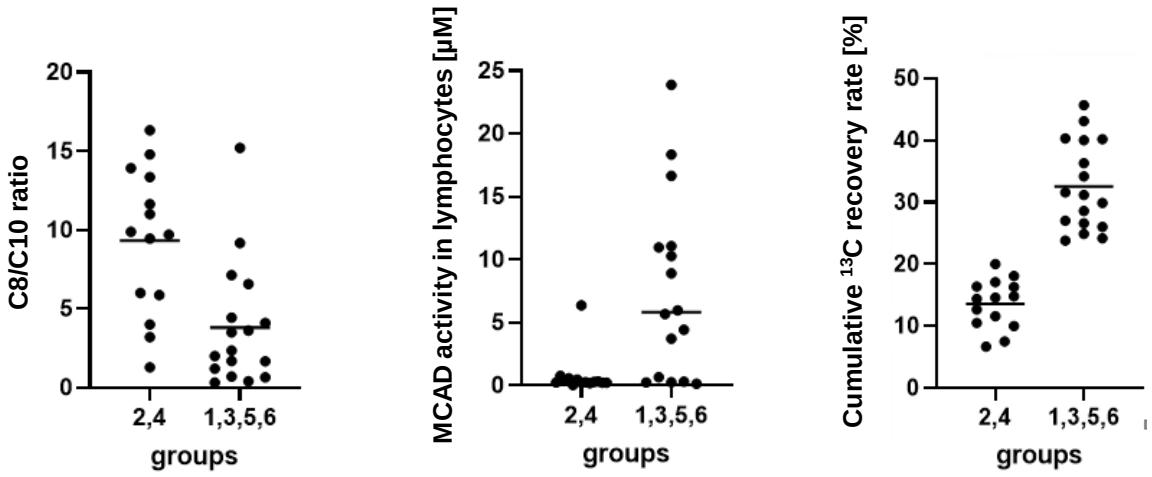


Figure 15 First distribution by HCA.

Analysis divided our cohort of 31 participants into 6 cluster groups. On the top level HCA separated cluster groups 2 and 4 on the one hand and 1, 3, 5, and 6 on the other hand. (**Figure 15**) Differences between these two groups of clusters were analyzed using unpaired t-test. Groups 2 and 4 showed significantly higher results of C8/C10 ratios, lower results for MCAD enzyme activities in lymphocytes and for cumulative ^{13}C recovery rates in breath test. C8/C10 acylcarnitine ratios were 9.32±4.63 (mean ± SD) in cluster groups 2 and 4, and 3.81±3.89 (mean ± SD) in cluster groups 1, 3, 5, and 6. MCAD activities in lymphocytes were 0.75±1.57 µM (mean ± SD) in cluster groups 2 and 4, and 8.27±7.57 µM (mean ± SD) in cluster groups 1, 3, 5, and 6. Cumulative ^{13}C recovery rates were 13.62±3.96 % (mean ± SD) in cluster groups 2 and 4 and 32.56±7.13 % (mean ± SD) in cluster groups 1, 3, 5, and 6. The greatest distinction between group 2, 4 and 1, 3, 5, 6 is shown in results of cumulative ^{13}C recovery rate. (**Figure 16**)



Unpaired t-test: Groups 2, 4 versus (vs.) groups 1, 3, 5, 6

P value: 0.0012 0.0018 < 0.0001

Figure 16 Unpaired t-test representing differences between groups 2 and 4 on the one hand and groups 1, 3, 5, and 6 on the other.

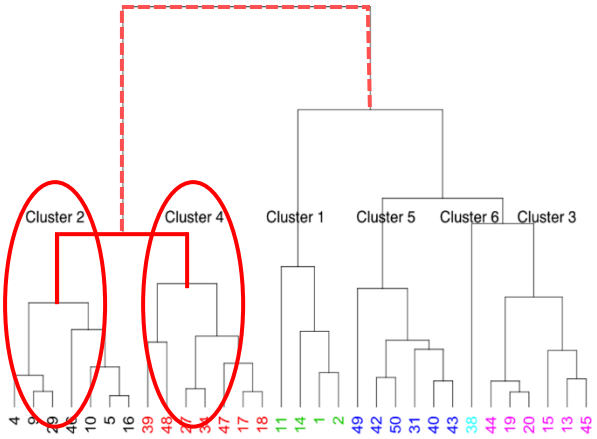
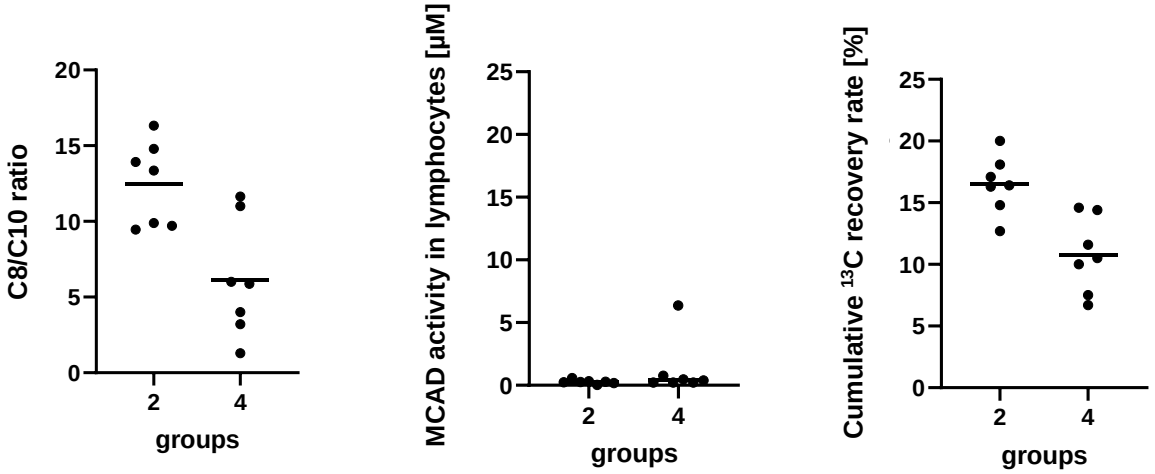


Figure 17 Distribution between groups 2 and 4 by HCA.

Next we aimed to delineate differences between cluster groups 2 (color code: black) and 4 (color code: red). (**Figure 17**) The highest C8/C10 ratios were 13.4±3.82 (mean ± SD) in group 2, while cluster group 4 results were 6.14±3.89 (mean ± SD). Lowest MCAD enzyme activities were displayed by cluster group 2 with 0.27±0.16 µM (mean ± SD), cluster group 4 revealed the third lowest results with 1.23±2.27 µM (mean ± SD). Additionally, cluster group 4 showed the lowest results in breath test of 10.76±3.07 % (mean ± SD), followed by cluster group 2 with 16.49±2.38 % (mean ± SD). MCAD activity measurements in lymphocytes did not show significant differences between these groups. The greatest distinction between groups 2 and 4 is shown in results of cumulative ^{13}C recovery rate. (**Figure 18**)



Unpaired t-test: Group 2 vs. group 4

P value: 0.0043 0.2852 0.0020

Figure 18 Unpaired t-test representing differences between groups 2 and 4.

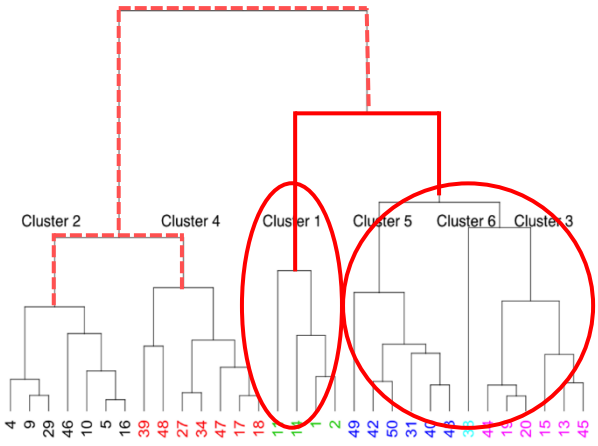
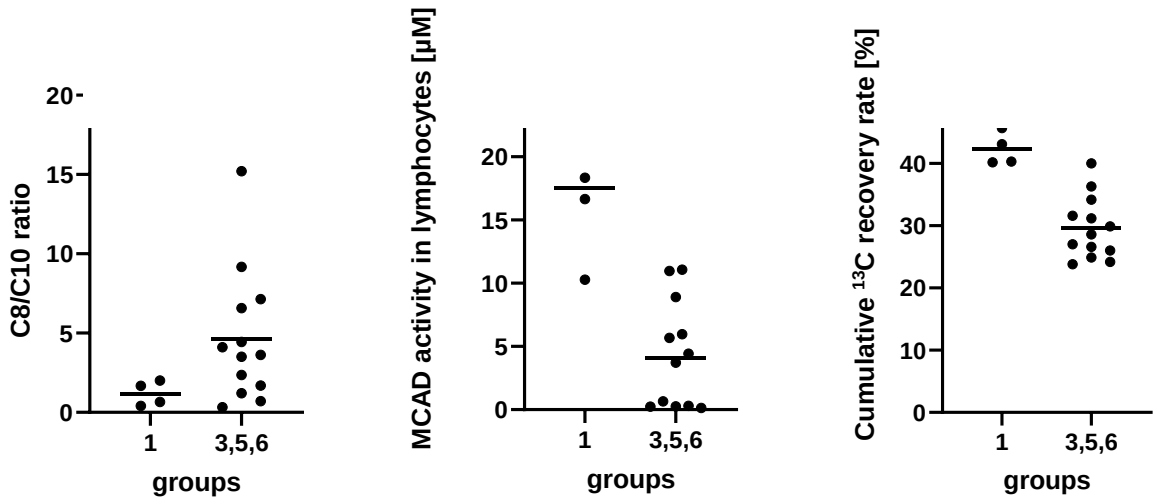


Figure 19 Distribution between group 1 and groups 3, 5, 6 by HCA.

Based on HCA, cluster groups 1, 3, 5, and 6 were further divided into cluster group 1 (color code: green) on the one side and cluster groups 3, 5, and 6 on the other side. (**Figure 19**) Cluster group 1 showed the lowest C8/C10 ratios of 1.18±0.77 (mean ± SD), the second highest MCAD enzyme activities in lymphocytes of 17.3±5.6 µM (mean ± SD), and the highest cumulative ^{13}C recovery rates of 42.3±2.62 % (mean ± SD), while cluster groups 3, 5, and 6 revealed C8/C10 ratios of 4.62±4.13 (mean ± SD), MCAD enzyme activities in lymphocytes of 5.49±5.75 (mean ± SD), and cumulative ^{13}C recovery rates of 29.56±4.96 %. The greatest distinction between these groups is found in results MCAD activity in lymphocytes and cumulative ^{13}C recovery rate. (**Figure 20**)



Unpaired t-test: Group 1 vs. groups 3, 5, 6

P value: 0.1262 0.0002 0.0002

Figure 20 Unpaired t-test representing differences between group 1 and groups 3, 5, 6.

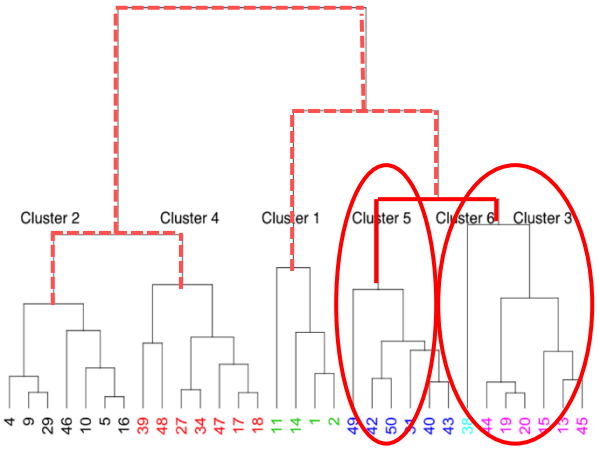
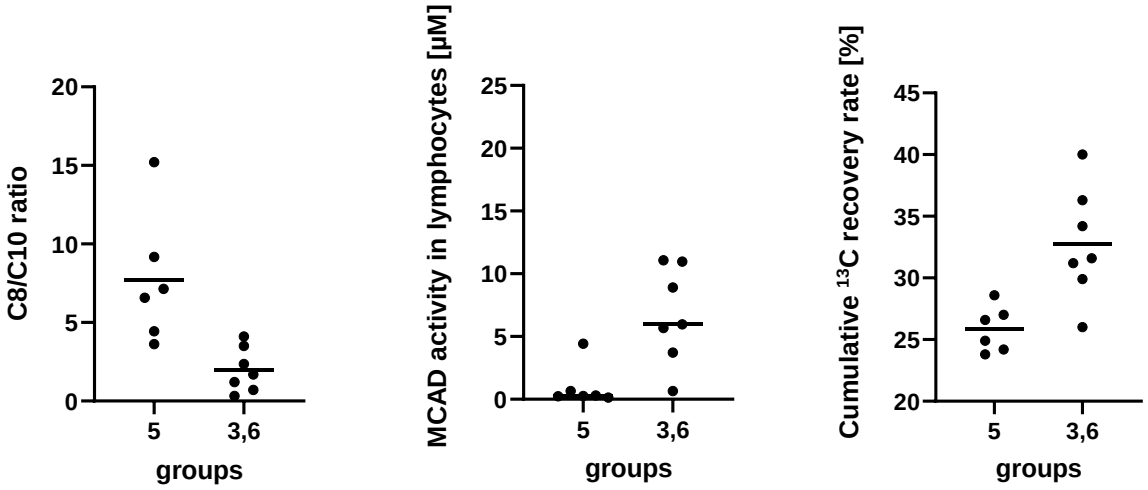


Figure 21 Distribution between group 5 and groups 3, 6 by HCA.

In groups 3, 5, and 6 the HCA further distinguished group 5 (color code: blue) on the one side and groups 3 and 6 on the other side. (**Figure 21**) Cluster group 5 showed C8/C10 ratios of 7.69±4.18 (mean ± SD), the second lowest MCAD enzyme activities of 1.01±1.69 µM (mean ± SD), and cumulative ^{13}C recovery rates of 25.8±1.86 % (mean ± SD), while cluster groups 3 and 6 revealed C8/C10 ratios of 1.98±1.42 (mean ± SD), MCAD enzyme activities of 9.34±5.12 (mean ± SD), and cumulative ^{13}C recovery rates of 32.74±4.56 % (mean ± SD). All 3 of the measured parameters differed significantly between cluster group 5 and cluster groups 3 and 6. (**Figure 22**)



Unpaired t-test: Group 5 vs. groups 3, 6

P value: 0.0058 0.0066 0.0054

Figure 22 Unpaired t-test representing differences between group 5 and groups 3, 6.

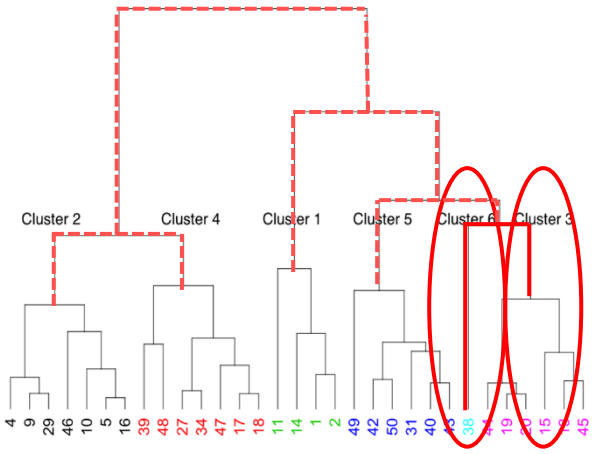


Figure 23 Distribution between groups 3 and 6

Finally, we analyzed the differences between groups 3 (color code: turquoise) and 6 (color code: pink) (**Figure 23**). Group 3 demonstrated the second lowest C8/C10 ratios with 1.63±1.16 (mean ± SD), the third highest MCAD activities in lymphocytes with 7.72±3.05 µM (mean ± SD), and the second highest results of cumulative ^{13}C recovery rate of 33.87±3.79 % (mean ± SD). Group 6 featured a C8/C10 ratio of 4.11, the highest MCAD activity in lymphocytes of 19.09 µM, and a cumulative ^{13}C recovery rate 26 %. (**Figure 24**) As cluster group 6 consists of only one participant, t-test evaluation could not be performed.

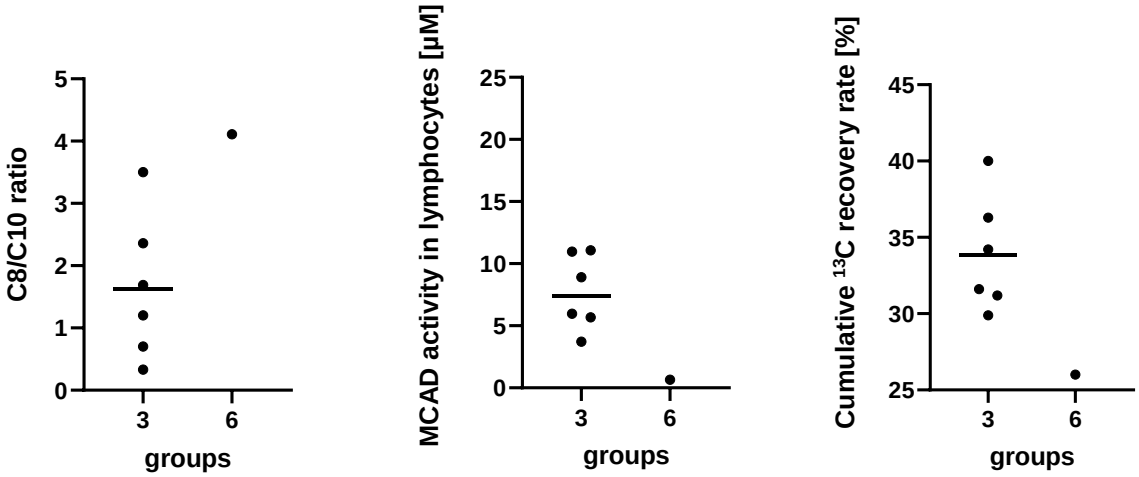


Figure 24 is representing differences between groups 3 and 6.

4.3.2.3 Summary HCA

Mean ± SD results of all cluster groups were analyzed for the categories of C8/C10 ratio, MCAD activity in lymphocytes, and cumulative ^{13}C recovery rate in breath test. (**Table 8**)

Table 8 Mean ± standard deviation (SD) of C8/C10 ratios, MCAD activities in lymphocytes [µM], and cumulative ^{13}C recovery rates in breath test [%] of the cluster groups of HCA.

	C8/C10 ratio		MCAD activity in lymphocytes		Cumulative ^{13}C recovery rate	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Cluster 1	1.18	0.769	17.3	5.6	42.3	2.62
Cluster 2	13.4	3.82	0.266	0.164	16.49	2.38
Cluster 3	1.63	1.16	7.72	3.05	33.9	3.79
Cluster 4	6.14	3.89	1.23	2.27	10.76	3.07
Cluster 5	7.69	4.18	1.01	1.69	25.8	1.86
Cluster 6	4.11	-	19.1	-	26	-

HCA and unpaired t-test indicate, that genotypes of cluster groups 2 and 4 represent patients carrying a more severe phenotypes than the ones of cluster groups 1, 3, 5, and 6, as evidenced by testing parameters C8/C10 ratio, enzyme activity in lymphocytes, and breath test.

Groups 2 and 4 showed high results in C8/C10 ratios, low MCAD activity in lymphocyte assays, and low cumulative ^{13}C recovery rates. Group 2 and 4 differed by results of C8/C10 ratios, they were significantly higher in group 2, and cumulative ^{13}C recovery rates, which was significantly higher in group 2. The MCAD activity in lymphocytes did not yield significant results. Therefore, group 2 represented a more severe phenotype than group 4 in C8/C10 ratios, showed a tendency towards decreased MCAD activities in lymphocytes without statistical significance, and revealed less severe phenotypes in breath test. A clear distinction of severity was not possible. (**Figure 25**)

Group 1 of HCA represented patients carrying a very mild phenotype, because group 1 showed lowest results in C8/C10 ratios, second highest results in MCAD

activity in lymphocyte assays, and highest results in breath test. Group 1 and groups 3, 5, 6 differed significantly in results of MCAD activities in lymphocytes and cumulative ^{13}C recovery rate results. (**Figure 25**)

Patients mapping to group 5 showed in comparison to groups 3, 6 higher results in C8/C10 ratios, lower results in MCAD activities in lymphocytes, and lower results in breath test. All three measured parameters differed significantly between cluster group 5 and cluster groups 3 and 6. Cluster group 5 represented patients carrying a more severe phenotype. (**Figure 25**)

Group 6 revealed compared to group 3 higher results in C8/C10 ratio and lower results in MCAD activity in lymphocytes and breath test. Therefore, group 6 is ranked as more severe than group 3. (**Figure 25**)

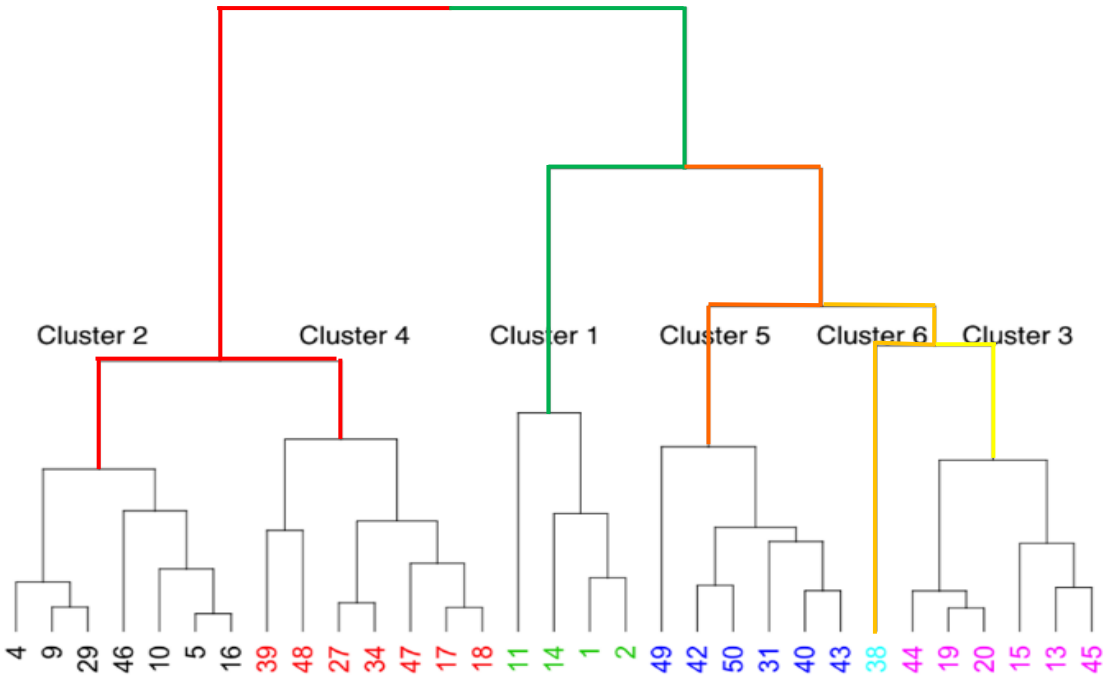


Figure 25 HCA with groups of severities analyzed by unpaired t-test.
Participants phenotype from severe to mild:
— severe
—
—
—
— mild

4.3.3 Cutoff values

For differentiation of cluster groups by cutoff values, analysis was performed comparing all participants results (C8/C10 ratios, MCAD activities in lymphocytes and cumulative ^{13}C recovery rates) using receiver-operated-curve (ROC) and area under the curve (AUC). (**Table 9**)

Table 9 Areas under the curve (AUCs) for C8/C10 ratio, MCAD activity in lymphocytes and cumulative ^{13}C recovery rate. - = not significant.

AUC	C8/10 ratio	MCAD activity in lymphocytes	Cumulative ^{13}C recovery rate
Homozygotes vs. Compound heterozygotes	-	-	0.8322
WT/Heterozygotes vs. Homozygotes/Compound heterozygotes	0.9806	0.9667	0.9361

4.3.3.1 Acylcarnitines

Primary analytes C0, C6, C8, C10:1, C12, and secondary ratios C8/C0, C8/C2, C8/C10 and C10:1 differed between healthy controls and affected patients. No statistically significant differences were found for the primary analytes C8:1, C8DC, C10, C10:2, and C10DC. (**Table 10**)

Table 10 Areas under the curve (AUCs) for acylcarnitine levels. - = not significant.

AUC	C0	C6	C8	C8:1	C8DC	C10	C10:1	C10:2	C10DC	C12
WT/Heterozygotes vs. Homozygotes/Compound heterozygotes	0.9333	0.9097	0.6514	-	-	-	0.8625	-	-	0.8097

AUC	C8/C0 ratio	C8/C2 ratio	C8/10 ratio	C8/12 ratio
WT/Heterozygotes vs. Homozygotes/Compound heterozygotes	0.9800	0.9606	0.9806	0.9806

When comparing all primary acylcarnitine analytes and their secondary ratios for division between healthy controls and affected patients AUCs for the C8/C10 and C8/C12 ratios showed highest results of 0.981 ($p \leq 0{,}0001$). (**Table 10**)

Further analysis of both ratios included the calculation of the effect size by the following formula:

$$effect\ size = \frac{mean_{affected} - mean_{healthy}}{\sqrt{\frac{SD_{affected}{}^2 + SD_{healthy}{}^2}{2}}}$$

The effect size for C8/C10 ratio was 1.97 and for C8/C12 ratio 1.72. The higher result for C8/C10 ratio revealed it as the most valid acylcarnitine parameter to distinguish between healthy controls and affected patients in our cohort. This has been reported before (Maier *et al.*, 2005, 2009b; Smith *et al.*, 2010).

In order to find best discrimination between both groups a cutoff value of 2.36 demonstrated a sensitivity of 91.6 % and a specificity 100 %. (**Figure 26**)

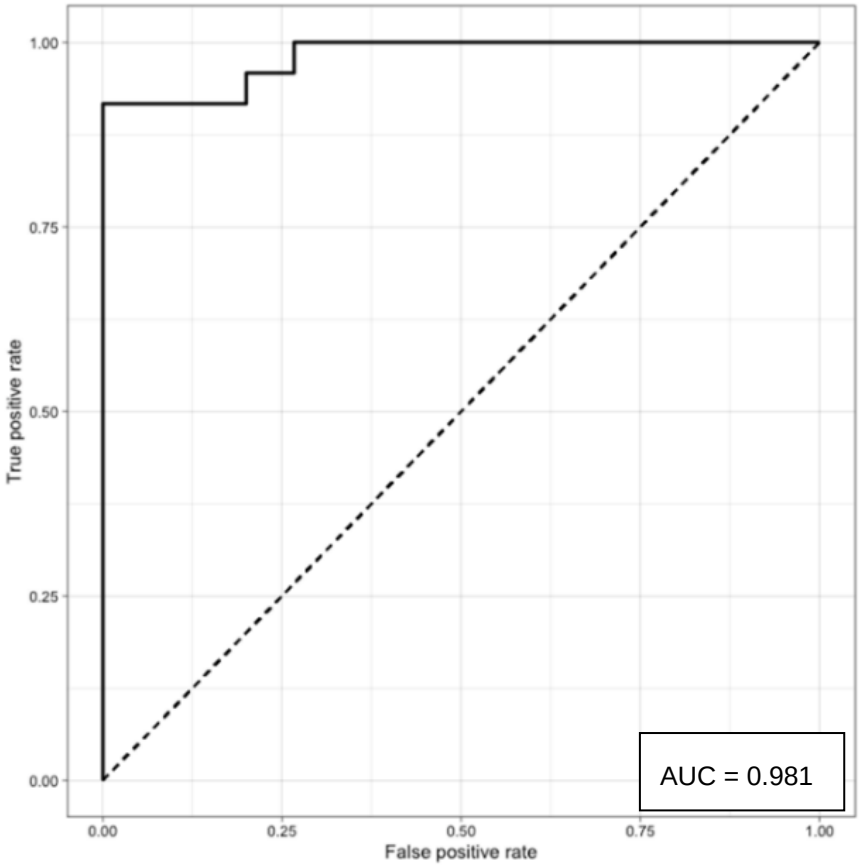


Figure 26 ROC curve of C8/C10 ratio of wild type/heterozygous carriers and MCADD patients.

4.3.3.2 MCAD activity in lymphocytes

Best discrimination between healthy participants and affected patients in MCAD activities in lymphocytes was shown by a cutoff value of 6.35 µM (= 35.57 % of wild type) demonstrating a sensitivity of 95.8 % and a specificity of 87.5 % (AUC 0.967). (**Table 9**, **Figure 27**)

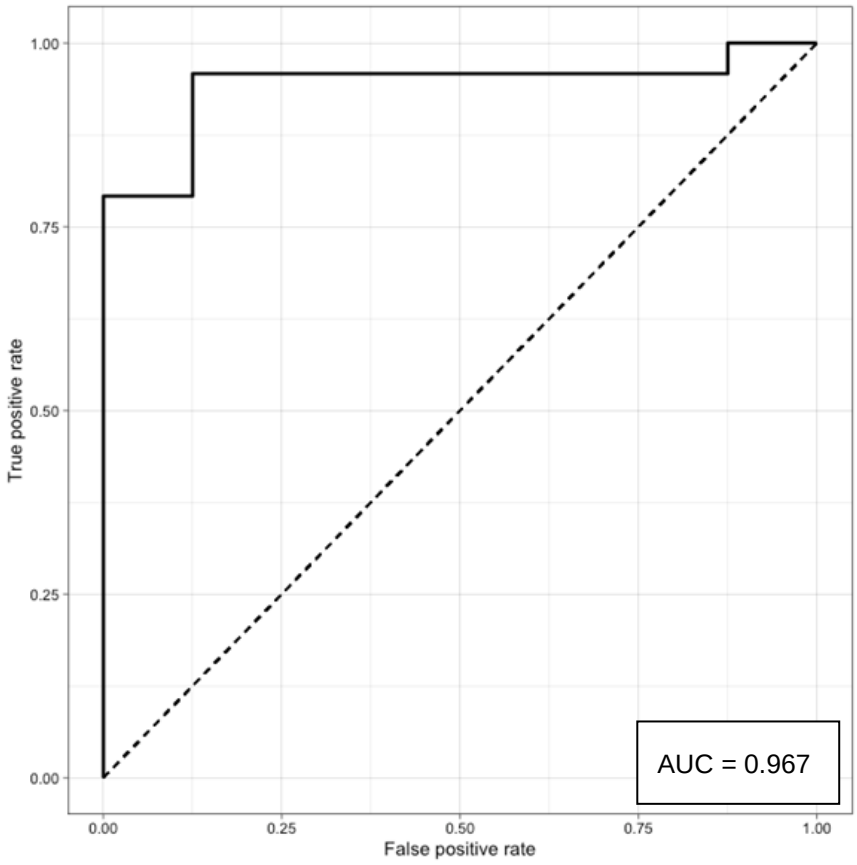


Figure 27 ROC curve of MCAD activity in lymphocytes of wild type/heterozygous carriers and MCADD patients.

4.3.3.3 ^{13}C Breath test

Best discrimination between healthy participants and affected patients in ^{13}C breath test was shown by a cutoff value of 28.6 % demonstrating a sensitivity of 91.7 % and a specificity of 93.3 % (AUC 0.936). (**Table 9**, **Figure 28**)

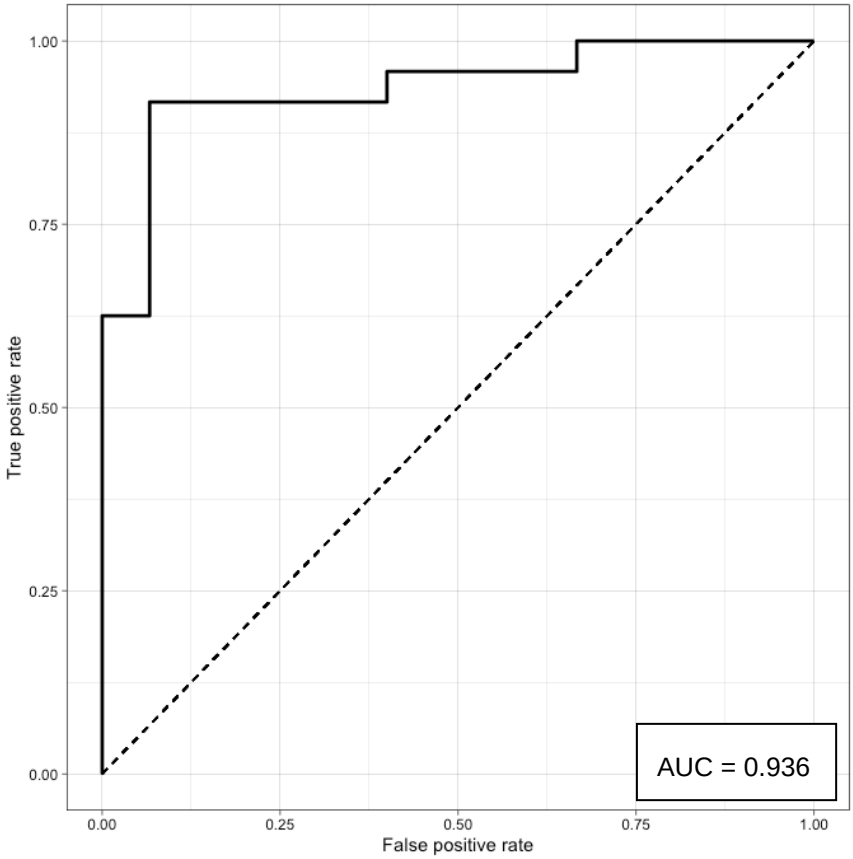


Figure 28 ROC curve of cumulative ^{13}C recovery rate in breath test of wild type/heterozygous carriers and MCADD patients.

Only the ^{13}C breath test allowed further discrimination in the group of affected patients between homozygous and compound heterozygous patients. The cutoff value of 20.3 % demonstrated a sensitivity of 82 % and a specificity of 100 % (AUC 0.832). (**Table 9**, **Figure 29**)

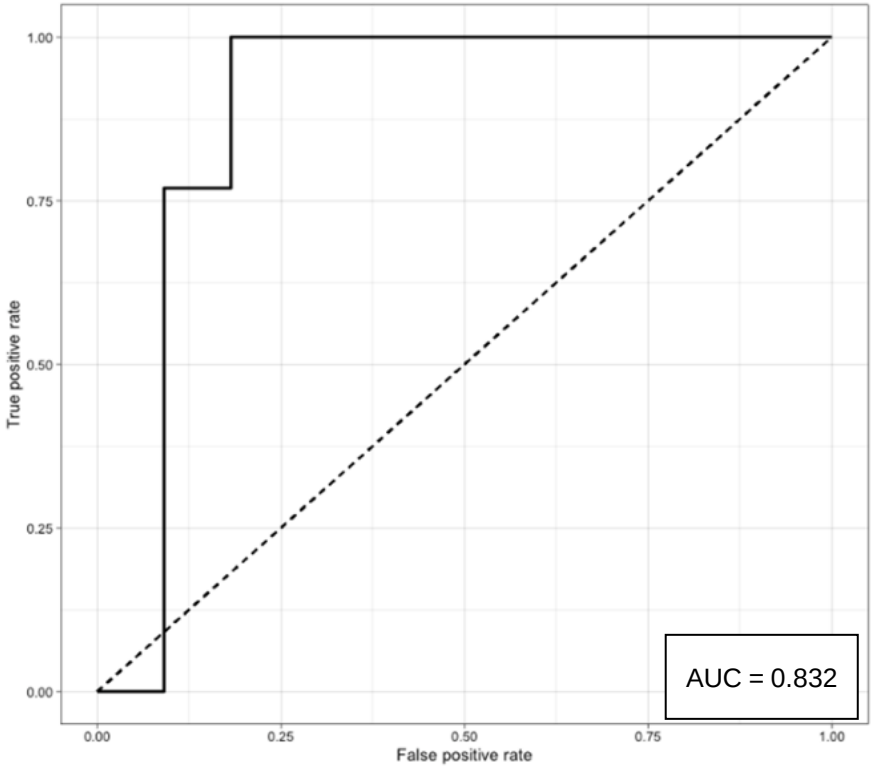


Figure 29 ROC curve of cumulative ^{13}C recovery rate in breath test of homozygous and compound heterozygous patients.

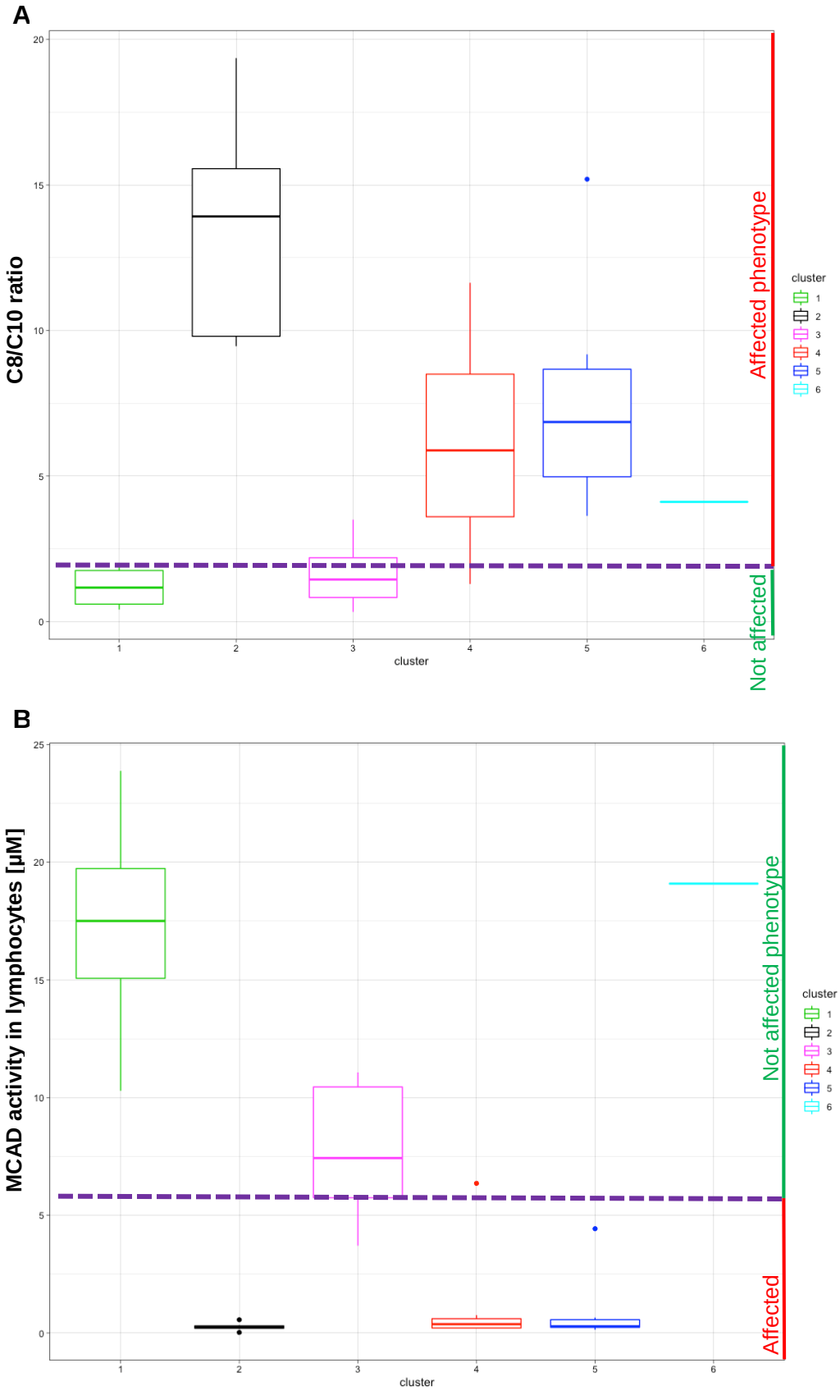
4.3.3.4 Summary of cutoff values

Summarizing cutoff results, all three diagnostic tools are able to detect a disease phenotype. C8/C10 ratio values above 2.36, MCAD activity in lymphocytes below 35.57 % of wild type and breath test results below 28.6 % detected affected individuals.

The ^{13}C breath test was identified as the only parameter that allowed for discrimination of three groups of severity in our cohort. Breath test values above 28.6 % grouped healthy individuals (wild type or heterozygous carriers), while results lower than 28.6 % categorized affected patients. These patients were further categorized into a moderate phenotype, by results greater than 20.3 % and a severe phenotype by results lower than 20.3 %.

4.3.4 Consolidation of hierarchical cluster analysis and cutoff value results

A combination of the results of HCA and cutoff values is shown in **figure 30**.



Cutoff 2.36 (between WT/heterozygous carriers and affected patients)

Cutoff 6.35 µM = 35.57 % of WT (between WT/ heterozygous carriers and affected patients)

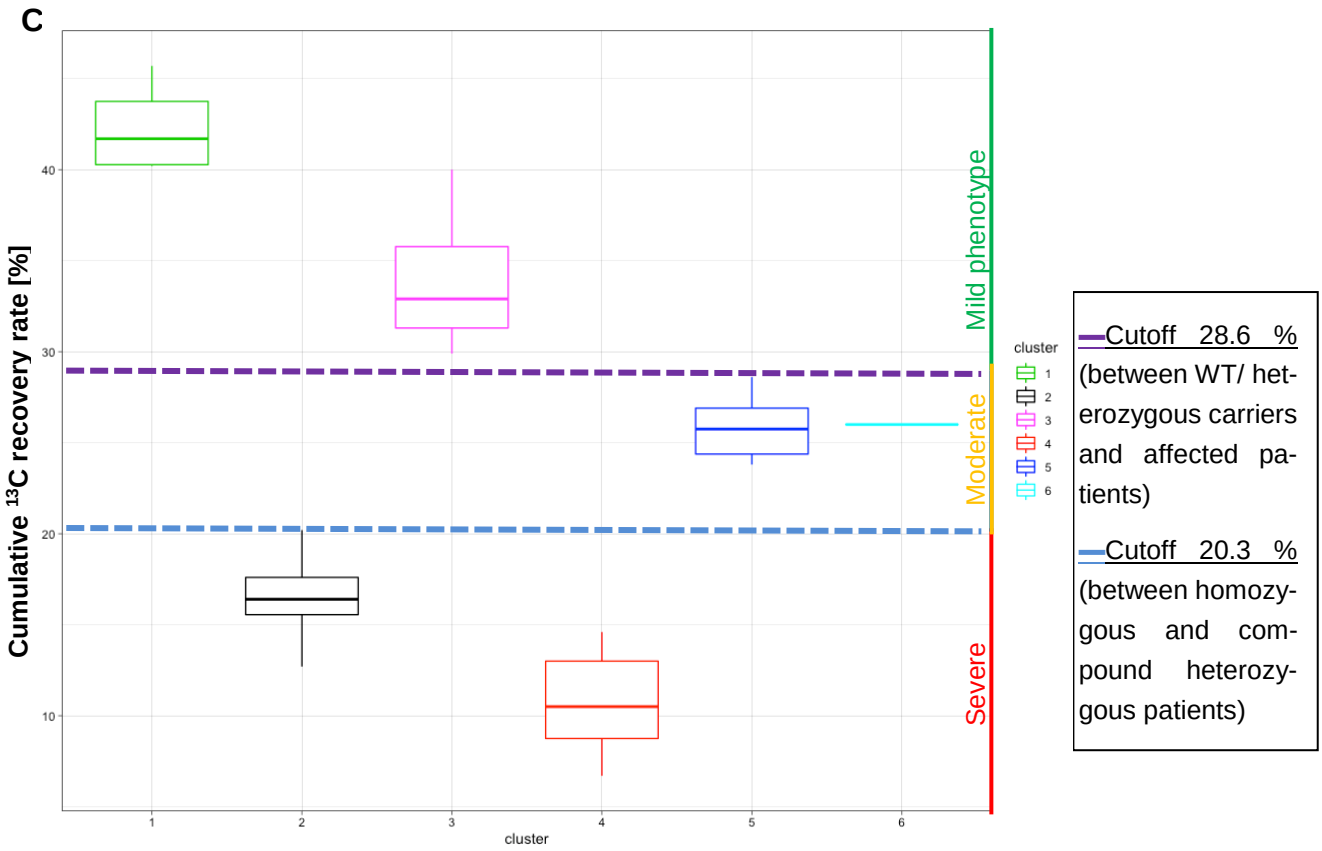


Figure 30 Hierarchical cluster analysis and cutoff values. **A** C8/C10 ratio, **B** MCAD activity in lymphocytes, **C** cumulative ^{13}C recovery rate.

We were able to detect cutoff values for all three diagnostic tools to discriminate between healthy and affected participants when single outliers were ignored.

In C8/C10 ratio cluster groups 1 and 3 were categorized as containing healthy individuals. Groups 6, 4, 5, and 2 represented affected patients, while group 6 demonstrated the mildest and group 2 the severest phenotype.

In MCAD activity in lymphocytes cluster groups 6, 1, and 3 contained healthy individuals. Groups 4, 5, and 2 represented affected patients, while group 4 displayed the mildest and group 2 the severest phenotype.

Except for the fact that cluster group 6 represented a mildly affected participant in C8/C10 ratio and a healthy individual in MCAD activity in lymphocytes, the results of both diagnostic tools represent the same results of disease affection for the cluster groups 1, 3 and 4, 5, 2.

In ^{13}C breath tests, we also identified a value for discrimination within the group of affected patients. Cluster groups 1 and 3 represented healthy individuals. Groups 6 and 5 demonstrated moderate phenotypes and groups 2 and 4 were identified to represent severe phenotypes.

Groups 1 and 3 include the genotypes of:
- 2 wild type controls
- 6 heterozygous carriers of p.[Lys329Glu]
- 2 compound heterozygous patients for p.[Lys329Glu];[Tyr67His]

Groups 6 and 5 comprise of:
- 5 compound heterozygous patients for p.[Lys329Glu];[Met155Thr], p.[Lys329Glu];[Ser245Leu], p.[Lys329Glu];[Leu107Ser], p.[Lys329Glu];[Thr351Ile], p.[Lys329Glu];[Ile416Thr] and 2 compound heterozygous patients for the variant p.[Lys329Glu];[Arg206Cys]

Genotypes of groups 2 and 4 consist of:
- 11 patients homozygous for p.[Lys329Glu];[Lys329Glu]
- 3 compound heterozygous patients for p.[Lys329Glu];[Ile78Thr], p.[Lys329Glu];[Lys197Glu] and p.[Lys329Glu];c.[387+1delG]

4.3.5 Consolidation of individual genotype and cutoff value results

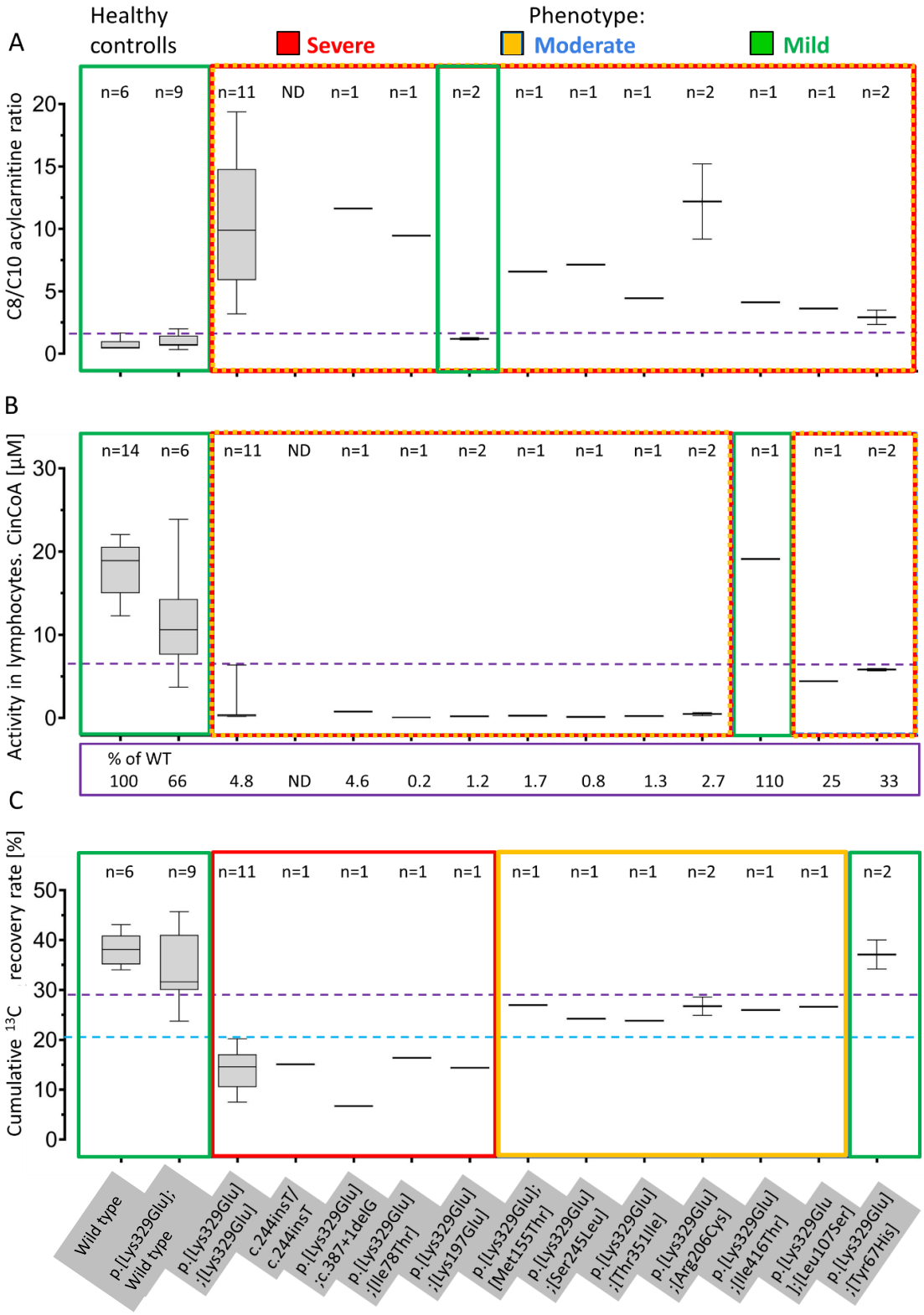


Figure 31 Complex analysis of the MCADD patient population in our study.

A C8/C10 acylcarnitine ratio, **B** MCAD activity in lymphocytes, **C** Cumulative ^{13}C recovery rate
(n = number of participants, ND = not determined, – – cutoff for distinction between healthy controls and affected patients, – – cutoff for distinction between homozygous and compound heterozygous patients).

4.4 Supplement

Table 11 Raw data collected for the study.

allele 1	allele 2	gender (female or male)	age (years)	free carnitine (10-70 µmol/l)	C2 (10-500 µmol/l)	C6 (0-0.18 µmol/l)	C8 (0.01-0.4 µmol/l)	C8/C0	C8:1 (0 - 0.3 µmol/l)	C8/C2	C8DC (0 - 0.14 µmol/l)	**C8/C10**	C8/C12	C10 (0 - 0.34 µmol/l)	C10:1 (0 - 0.13 µmol/l)	C10:2 (0 - 0.1 µmol/l)	C10DC (0 - 0.35 µmol/l)	C12 (0.02 - 0.3 µmol/l)	**MCAD activity in lymphocytes (µM)**	**MCAD activity in lymphocytes (% of WT)**	**Cumulative 13C recovery rate (%)**
WT	WT	f	35.00	36.70	15.26	0.18	0.15	0.00	0.22	0.01	0.07	1.67	1.25	0.09	0.11	0.06	0.06	0.12	18.35	103.09	40.20
WT	WT	f	31.00	26.90	10.05	0.16	0.13	0.00	0.09	0.01	0.04	0.41	1.30	0.32	0.06	0.06	0.09	0.10	16.66	93.18	43.10
WT	WT	f	35.00	44.00	15.69	0.05	0.33	0.01	0.14	0.02	0.07	0.79	1.57	0.42	0.11	0.03	0.00	0.21	-		39.40
WT	WT	m	37.00	29.80	7.37	0.11	0.10	0.00	0.04	0.01	0.04	0.43	0.77	0.23	0.11	0.04	0.00	0.13	-		36.90
WT	WT	m	32.00	29.40	13.68	0.06	0.05	0.00	0.05	0.00	0.01	0.42	0.83	0.12	0.03	0.04	0.10	0.06	-		34.00
WT	WT	m	34.00	36.30	89.22	0.06	0.13	0.00	0.06	0.00	0.01	0.59	1.18	0.22	0.11	0.05	0.14	0.11	-		35.50
WT (buffy)	WT (buffy)	m	23.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	22.05	123.88	-
WT (buffy)	WT (buffy)	m	25.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20.43	114.26	-
WT (buffy)	WT (buffy)	m	25.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20.91	116.95	-
WT (buffy)	WT (buffy)	m	34.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20.45	114.37	-
WT (buffy)	WT (buffy)	m	44.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	21.54	120.47	-
WT (buffy)	WT (buffy)	m	67.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20.45	114.37	-
WT (buffy)	WT (buffy)	f	20.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12.28	68.68	-
WT (buffy)	WT (buffy)	f	26.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12.94	72.37	-
WT (buffy)	WT (buffy)	f	29.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	19.46	108.84	-
WT (buffy)	WT (buffy)	f	31.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14.01	78.35	-
WT (buffy)	WT (buffy)	f	39.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15.31	85.63	-
WT (buffy)	WT (buffy)	f	40.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15.42	86.24	-
p.[Lys329Glu]	WT	m	51.00	43.80	16.02	0.16	0.23	0.01	0.19	0.01	0.10	0.66	1.15	0.35	0.10	0.04	0.21	0.20	10.29	57.55	45.70
p.[Lys329Glu]	WT	f	45.00	31.70	10.40	0.10	0.19	0.01	0.12	0.02	0.06	0.90	1.90	0.21	0.10	0.03	0.13	0.10	-		41.80
p.[Lys329Glu]	WT	m	40.00	37.90	15.11	0.11	0.34	0.01	0.15	0.02	0.06	2.00	4.25	0.17	0.12	0.07	0.13	0.08	23.88	133.56	40.30
p.[Lys329Glu]	WT	f	46.00	37.30	14.35	0.05	0.08	0.00	0.07	0.01	0.05	0.33	1.00	0.24	0.06	0.02	0.00	0.08	11.07	61.91	31.20
p.[Lys329Glu]	WT	m	52.00	38.00	13.17	0.08	0.12	0.00	0.10	0.01	0.05	1.20	1.33	0.10	0.10	0.05	0.00	0.09	10.97	61.35	29.90
p.[Lys329Glu]	WT	m	45.00	30.00	20.00	0.22	0.27	0.01	0.11	0.01	0.04	0.64	3.00	0.42	0.15	0.05	0.20	0.09	-		23.70
p.[Lys329Glu]	WT	f	41.00	18.90	9.03	0.14	0.14	0.01	0.11	0.02	0.04	0.74	2.80	0.19	0.11	0.04	0.08	0.05	-		30.10
p.[Lys329Glu]	WT	m	37.00	37.30	11.58	0.07	0.16	0.00	0.20	0.01	0.07	0.70	5.33	0.23	0.10	0.06	0.1	0.03	8.91	49.83	31.60
p.[Lys329Glu]	WT	f	32.00	37.30	8.36	0.18	0.22	0.01	0.16	0.03	0.00	1.69	3.14	0.13	0.20	0.01	0.17	0.07	3.71	20.74	36.30
p.[Lys329Glu]	p.[Lys329Glu]	m	20.00	14.90	6.28	0.23	1.78	0.12	0.25	0.28	0.06	9.89	25.43	0.18	0.33	0.11	0.02	0.07	0.28	1.57	18.10
p.[Lys329Glu]	p.[Lys329Glu]	f	22.00	16.80	9.22	0.69	7.24	0.43	0.29	0.79	0.10	13.92	144.80	0.52	1.51	0.11	0.06	0.05	0.23	1.29	17.10
p.[Lys329Glu]	p.[Lys329Glu]	m	16.00	22.20	6.90	1.04	9.42	0.42	0.21	1.37	0.03	9.71	942.00	0.97	1.10	0.15	0.05	0.01	0.25	1.40	14.80
p.[Lys329Glu]	p.[Lys329Glu]	m	16.00	18.30	7.79	0.64	6.37	0.35	0.18	0.82	0.04	16.33	159.25	0.39	0.82	0.09	0.05	0.04	0.57	3.19	20.20
p.[Lys329Glu]	p.[Lys329Glu]	f	8.00	16.20	7.06	0.70	2.07	0.13	0.14	0.29	0.04	14.79	51.75	0.14	0.44	0.04	0.01	0.04	0.33	1.85	16.30
p.[Lys329Glu]	p.[Lys329Glu]	f	18.00	11.70	7.01	0.26	0.60	0.05	0.11	0.09	0.06	6.00	15.00	0.10	0.10	0.04	0.03	0.04	0.46	2.57	11.60
p.[Lys329Glu]	p.[Lys329Glu]	f	21.00	7.10	6.32	0.25	1.00	0.14	0.07	0.16	0.01	5.88	50.00	0.17	0.45	0.06	0.02	0.02	0.38	2.13	10.00
p.[Lys329Glu]	p.[Lys329Glu]	m	6.00	5.80	2.83	0.06	0.32	0.06	0.11	0.11	0.07	3.20	6.40	0.10	0.08	0.00	0	0.05	0.21	1.17	14.60
p.[Lys329Glu]	p.[Lys329Glu]	m	9.00	34.10	5.15	1.02	2.13	0.06	0.16	0.41	0.03	19.36	71.00	0.11	0.57	0.06	0	0.03	0.18	1.01	12.70
p.[Lys329Glu]	p.[Lys329Glu]	f	13.00	3.60	3.17	0.04	0.08	0.02	0.05	0.03	0.00	4.00	4.00	0.02	0.09	0.00	0.06	0.02	0.21	1.17	7.50
p.[Lys329Glu]	p.[Lys329Glu]	m	7.00	11.00	4.36	0.40	1.76	0.16	0.07	0.40	0.05	11.00	44.00	0.16	0.25	0.04	0.05	0.04	6.36	35.57	10.50
c.244insT, p*104	c.244insT, p*104	m	8.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	17.35	15.10
p.[Lys329Glu]	p.[Lys197Glu]	m	14.00	22.30	-	0.33	0.81	0.04	0.10	-	0.03	1.29	9.00	0.63	0.27	0.03	0.03	0.09	0.22	1.23	14.40
p.[Lys329Glu]	p.[Lys197Glu]	m	10.00	29.20	-	0.31	0.30	0.01	0.28	-	0.07	1.11	6.00	0.27	0.30	0.04	0.04	0.05	0.22	1.23	-
p.[Lys329Glu]	p.[Ile78Thr]	m	29.00	14.40	6.95	0.22	1.23	0.09	0.11	0.18	0.04	9.46	24.60	0.13	0.38	0.04	0.07	0.05	0.03	0.17	16.40
p.[Lys329Glu]	p.[Ile416Thr]	f	8.00	14.20	7.97	0.24	0.78	0.05	0.07	0.10	0.04	4.11	7.80	0.19	0.17	0.07	0.21	0.10	19.09	106.77	26.00
p.[Lys329Glu]	c.387+1delG	f	7.00	16.40	3.96	0.52	4.89	0.30	0.35	1.23	0.01	11.64	244.50	0.42	0.55	0.05	0	0.02	0.76	4.25	6.70
p.[Lys329Glu]	p.[Ser245Leu]	f	19.00	21.70	3.47	0.57	1.50	0.07	0.15	0.43	0.03	7.14	50.00	0.21	0.17	0.05	0.11	0.03	0.14	0.78	24.20
p.[Lys329Glu]	p.[Thr351Ile]	f	6.00	33.60	6.04	0.53	2.71	0.08	0.20	0.45	0.09	4.44	33.88	0.61	1.03	0.13	0.17	0.08	0.23	1.29	23.80
p.[Lys329Glu]	p.[Arg206Cys]	m	18.00	22.50	4.82	0.27	2.28	0.10	0.14	0.47	0.00	15.20	57.00	0.15	0.42	0.07	0	0.04	0.30	1.68	24.90
p.[Lys329Glu]	p.[Arg206Cys]	f	28.00	23.90	4.90	0.44	2.57	0.11	0.12	0.52	0.08	9.18	32.13	0.28	0.51	0.02	0	0.08	0.66	3.69	28.60
p.[Lys329Glu]	p.[Met155Thr]	m	18.00	28.40	3.98	0.33	2.89	0.10	0.16	0.73	0.03	6.57	41.29	0.44	0.69	0.07	0.13	0.07	0.27	1.51	27.00
p.[Lys329Glu]	p.[Leu107Ser]	f	11.00	15.30	12.50	0.23	0.58	0.04	0.13	0.05	0.12	3.63	3.63	0.16	0.15	0.03	0.19	0.16	4.43	24.78	26.60
p.[Lys329Glu]	p.[Tyr67His]	f	4.00	28.20	9.83	0.15	0.33	0.01	0.12	0.03	0.10	2.36	3.67	0.14	0.08	0.04	0.02	0.09	5.68	31.77	34.20
p.[Lys329Glu]	p.[Tyr67His]	f	13.00	24.00	57.67	0.47	1.32	0.06	0.15	0.02	0.02	3.50	18.86	0.37	0.47	0.04	0.00	0.07	5.96	33.33	40.00

5 Discussion

5.1 Set-up of the ^{13}C-PPA breath test

To date, follow-up methods in patients with MCADD are in most countries limited to acylcarnitine measurements in blood and urine analysis of organic acids or acylglycines (Merritt and Chang, 2020). Elevations of these biomarkers can detect disease and discriminate between the mild phenotype of p.[Lys329Glu];p.[Tyr67His] and the severe phenotype of p.[Lys329Glu];p.[Lys329Glu] (Andresen *et al.*, 2001; Andrew and Spiekerkoetter, 2016; Gramer *et al.*, 2015; Waddell *et al.*, 2006).

In many cases molecular analysis, as targeted sequencing of particular genes or gene panels, or in the form of whole exome or genome sequencing, is performed first after positive acylcarnitine profiling of MCADD (Wanders *et al.*, 2020). Thereby many new variants are diagnosed and further functional analysis is needed to verify the consequences (Wanders *et al.*, 2020).

The benchmark method to assess disease severity is the *ex vivo* enzyme activity assay analysis in cultured lymphocytes or fibroblasts using the HPLC method, as residual enzyme activity correlates with the expected clinical phenotype (Derks *et al.*, 2008; Jager *et al.*, 2019; Wanders *et al.*, 2010, 2020). The liver plays a key role in fatty acid synthesis and lipid circulation through lipoprotein synthesis and is thereby the main site for lipid metabolism (Nguyen *et al.*, 2008). Enzymes of FAO are expressed and measured in peripheral lymphocytes or fibroblasts, but not routinely in liver cells, because of the invasiveness of the method (Andrew and Spiekerkoetter, 2016). Unfortunately activity assays are only measured in certain specialized laboratories and are not available in many regions of the world (Bouvier *et al.*, 2017; Wanders *et al.*, 2010).

All named methods do not provide a detailed insight into the current state of the metabolic pathway (Derks *et al.*, 2008; Jager *et al.*, 2019; Maier *et al.*, 2005, 2009b; Wanders *et al.*, 2010). Particular in rarely described compound heterozygous patients, the workup of a genotype-phenotype correlation is weak (Anderson *et al.*, 2020).

Aiming to bridge this gap of knowledge, we established a simple, time-saving and non-invasive ^{13}C breath test for patients with MCADD that measures the current *in vivo* metabolic flux of ^{13}C-PPA by MCAD for 180 minutes.

Analysis of cumulative ^{13}C recovery rate of female and male healthy controls provided baseline values measuring the metabolic flux of ^{13}C-PPA through the MCAD pathway. Results of male participants were slightly lower than female results. Also kinetics displayed a lower value of maximal ^{13}C exhalation in males. Several criteria as gastrointestinal transit or resorption of PPA, diet, hormones, physical activity and fitness, illness, medication and the human microbiome might have an influence on PPA metabolism (Huttenhower *et al.*, 2012). Healthy individuals differ remarkably in the composition of microbes occupying the gut (Huttenhower *et al.*, 2012). Much of this diversity is still unexplained, although diet, environment, host genetic factors and early microbial exposure have been analyzed (Huttenhower *et al.*, 2012). Intra-individual breath test results differed inessentially. Breath test results after an overnight fast were higher than those directly after or 3 hours after food ingestion. Fatty acid oxidation is performed in times of prolonged fasting. We hypothesize that the longer an individual fasts, the higher exhaled cumulative ^{13}C recovery rate can be expected. To harmonize results we defined a fasting time prior to the test. As newborns and young MCADD patients should have a regular caloric intake, we chose 3 hours as justifiable fasting time.

After determination of baseline values of healthy controls, we performed the ^{13}C breath test with heterozygous carriers of p.[Lys329Glu] and detected lower ^{13}C recovery rates with a mean of 34.51 %. These results suggest a lower *in vivo* metabolic flux of ^{13}C-PPA in heterozygous carriers, likely a consequence of the metabolic influence of one disease affected allele. In addition, we observed a high standard deviation in these results. A deviation by 7 % may be a clinically insignificant decrease, as heterozygous carriers are usually asymptomatic of the disease. Reason for this variance might be a different enzyme activity and metabolic flux in heterozygous carriers of our cohort.

Homozygous patients of p.[Lys329Glu];[Lys329Glu] and c.[244insT*104];[244insT*104] were found to have the lowest values in breath test, which might underline the disease severity grade.

Compound heterozygous patients with p.[Lys329Glu] on one allele and another MCADD causing variant on the other allele showed the greatest variability, which suggests that the breath test phenotype relies on the genotype of both alleles.

We determined the biochemical phenotypes of 40 participants, including healthy controls, heterozygous carriers of p.[Lys329Glu], homozygous and compound heterozygous patients by methods of cumulative ^{13}C recovery rate, C8/C10 acylcarnitine ratio and MCAD activity in lymphocytes. Based on rank correlation analysis using Spearman's correlation coefficient we demonstrated a strong correlation between results of biochemical C8/C10 acylcarnitine ratio, *ex vivo* enzyme activity assay in lymphocytes and *in vivo* metabolic flux in ^{13}C breath test. This indicates, that there is a monotonic association between all three methods which correlate well with each other and underlines the reproducibility of the new test method, the ^{13}C breath test.

We performed unbiased hierarchical cluster analysis (HCA) to analyze potential cluster groups of phenotypes and were able to detect 6 cluster groups. Subsequently, we defined cutoff values to differentiate disease severity between these groups. All three diagnostic tools, C8/C10 acylcarnitine ratio, *ex vivo* measured MCAD activity in lymphocytes and cumulative ^{13}C recovery rate in breath test, were able to detect a disease phenotype. The ^{13}C breath test was identified as the only parameter that allowed for discrimination of 3 groups of severity in our cohort. It also distinguished between phenotypes of affected patients, identifying moderate and severe phenotypes.

5.2 Advantages and limitations of the ^{13}C-PPA breath test

Our findings gave evidence that the *in vivo* ^{13}C-PPA breath test can discriminate among phenotypes of different severity in MCADD. The ^{13}C breath test can detect disease, but also differentiates moderate from severe phenotypes. Therefore, the ^{13}C breath test not only provides a diagnostic tool to determine the patients underlying phenotype. In addition, the ^{13}C breath test may serve as a tool to estimate the current metabolic state in our patients.

Baseline breath test values were set up for healthy controls and heterozygous carriers. In order to determine the lower threshold value of the breath test for MCADD patients with no residual enzyme activity, we examined one patient with the homozygous variant for c.[244insT*104];[244insT*104]. The insertion of the variant is assumed to result in a frameshift and premature termination codon in the mutant MCAD mRNA (Korman *et al.*, 2004). Misspliced MCAD mRNA encodes a non-functional protein, resulting in a total lack of the functional MCAD enzyme (Korman *et al.*, 2004). Unfortunately, it was not possible to characterize the homozygous variant for c.[244insT*104];[244insT*104] by enzyme activity test, as we did not collect blood from the patients, but we expect a total lack of the functional MCAD enzyme. Breath test results revealed a cumulative ^{13}C recovery rate of 15.1 %. As other homozygous and compound heterozygous patients revealed lower results in breath test, further analysis of the present variant is needed and further patients with a frameshift variation leading to a premature termination codon should be tested by breath test.

The ^{13}C-PPA breath test can be performed in any routine clinical environment. The slightly acidic flavour and odour of the substrate ^{13}C-PPA can be covered by sweet juice. Breath test bags are easily filled and even in newborns and toddler's breath test can be performed using a mask to catch exhaled air. Results are available within 20 minutes after test performance and allow a direct interpretation by metabolic experts and enable rapid adaption of the patient's current treatment regime. Therefore, it is the fastest method, compared to C8/C10 ratio and MCAD activity in lymphocytes. The breath test can be performed when first symptoms occur and before symptoms exacerbate. This may help to identify metabolic stress at an earlier stage and thus prevent more significant decompensations. If the ^{13}C breath test is used

for a routine control, breath samples do not have to be measured straight after breath test performance. They can be stored up to 2 weeks in a dark place at room temperature (around 20°C).

However, the cumulative ^{13}C recovery rate is not an equivalent of MCAD enzyme activity. Numerous influencing factors, such as gastrointestinal transit or resorption of PPA, diet, hormones, physical activity and fitness, illness, medication and the human microbiome might have an influence on PPA metabolism, which may lead to variation in breath test results between individuals.

5.3 Correlation of individual genotypes to phenotypic data reported in the scientific literature

In this study, we validated the results of the C8/C10 ratio, the MCAD activity in lymphocytes and the ^{13}C breath test in MCADD patients and aimed to describe their current biochemical phenotypes. We differed between mild, moderate and severe phenotypes. The ^{13}C breath test did not only detect disease, but also differentiated moderate from severe phenotypes.

Previous studies have come to the conclusion that in MCADD, correlation between genotype and clinical phenotype is not straightforward (Gramer *et al.*, 2015; Maier *et al.*, 2005; Waddell *et al.*, 2006)

We compared our results with individual genotypes and phenotypic data reported in the scientific literature.

5.3.1 Genotypes classified as associated with healthy or mild phenotypes

Groups 1 and 3 of HCA represented mild phenotype. Both groups include the genotypes of:

- 2 wild type controls
- 6 heterozygous carriers of p.[Lys329Glu]
- 2 patients compound heterozygous for p.[Lys329Glu];[Tyr67His]

These genotypes were classified as mild phenotypes by all 3 diagnostic methods. They had a low C8/C10 ratio (< 2.36), a high MCAD activity in lymphocytes (> 35.57 % of wild type), and high results of cumulative ^{13}C recovery rate in the breath test (> 28.6 %).

Wild type controls

Wild type controls set the baseline value of 100 % MCAD enzyme activity in lymphocytes measured by HPLC-UV or HPLC-ESI-MS/MS using octanoyl-CoA as substrate (Sturm et al., 2012; Zschocke et al., 2001). In this group measurements varied between 57 and 114 % (Sturm et al., 2012; Zschocke et al., 2001).

In our study cohort, wild type controls revealed C8/C10 ratios of 0.72±0.49 (mean ± SD), MCAD activities in lymphocytes of 100±18,88 % (mean ± SD) and cumulative ^{13}C recovery rates in breath test of 38.18±3.35 % (mean ± SD). Our results correspond to the phenotype of healthy subjects as described in literature (Bouvier *et al.*, 2017; Sturm *et al.*, 2012; Touw *et al.*, 2013; Wanders *et al.*, 2010; Zschocke *et al.*, 2001).

Heterozygous carriers of p.[Lys329Glu]

Heterozygous carriers of p.[Lys329Glu] on one allele and a healthy second allele regarding MCADD are described to have C8 levels < 0.6 µmol/l, C8/C10 levels < 1.5 and an enzyme activity in lymphocytes that varies between 12 and 87 % of wild type measured by HPLC-UV or HPLC-ESI-MS/MS using octanoyl-CoA as substrate (Smith et al., 2010; Sturm et al., 2012; Zschocke et al., 2001).

Our results showed C8/C10 levels of 0.98±0.54 (mean ± SD), a MCAD activity in lymphocytes between of 64.16±37.32 % of wild type (mean ± SD) and a cumulative ^{13}C recovery rate of 34.51±7.00 % (mean ± SD). Our results correspond to the phenotype of heterozygous carriers as described in literature (Smith et al., 2010; Sturm et al., 2012; Zschocke et al., 2001).

Compound heterozygotes of p.[Lys329Glu];[Tyr67His]

Patients compound heterozygous for p.[Lys329Glu];[Tyr67His] are known to have low C8 levels (0,5-2 µmol/l) and low C8/C10 values (2-4) (Smith et al., 2010; Sturm et al., 2012; Zschocke et al., 2001).

Enzyme activity using octanoyl-CoA as substrate and measured by HPLC-UV or HPLC-ESI-MS/MS varied between 16 and 49 % of wild type (Smith et al., 2010; Sturm et al., 2012; Zschocke et al., 2001).

Median residual MCAD enzyme activity in patients with this genotype was 31 % of wild type using the specific substrate phenylpropionyl-CoA (Touw et al., 2012).

Allele-specific enzyme activities for variant Tyr67His proteins in Japanese patients detected almost normal levels of 62.5 % of wild type using octanoyl-CoA as substrate (Hara et al., 2016).

To date, this genotype is the second most diagnosed alteration in MCADD, which was not identified in clinically symptomatic patients yet (Gramer *et al.*, 2015; Maier *et al.*, 2005; Sturm *et al.*, 2012; Zschocke *et al.*, 2001).

In our cohort, the genotype revealed a C8/C10 ratio of 2.93±0.81 (mean ± SD), which is slightly above the cutoff of 2.36. MCAD enzyme activity in lymphocytes was 32.55±1.1 % of wild type (mean ± SD) and the breath test showed a cumulative ^{13}C recovery rate of 37.1±4.1 % (mean ± SD). Our results correspond to the phenotype of compound heterozygotes for p.[Lys329Glu];[Tyr67His] as described in literature (Gramer *et al.*, 2015; Hara *et al.*, 2016; Maier *et al.*, 2005; Smith *et al.*, 2010; Sturm *et al.*, 2012; Touw *et al.*, 2012; Zschocke *et al.*, 2001).

5.3.2 Genotypes classified as associated with moderate phenotypes

Cluster groups 5 and 6 were designated as moderate phenotypes. Genotypes consisted of patients compound heterozygous for:

- p.[Lys329Glu];[Met155Thr]
- p.[Lys329Glu];[Ser245Leu]
- p.[Lys329Glu];[Thr351Ile]
- p.[Lys329Glu];[Leu107Ser]
- p.[Lys329Glu];[Ile416Thr]
- p.[Lys329Glu];[Arg206Cys]

All genotypes showed a moderate presentation in the ^{13}C breath test (> 20.3 %, < 28.6%), but differed in the presentation of C8/C10 ratio and MCAD activity in lymphocytes.

Compound heterozygotes of p.[Lys329Glu];[Arg206Cys]

Patients carrying p.[Lys329Glu];[Arg206Cys] showed a C8:0 value of 7.2 and 12 % MCAD activity in lymphocytes measured by HPLC-UV and HPLC-ESI-MS/MS using octanoyl-CoA as substrate (Sturm et al., 2012).

Our results revealed a severe phenotype of C8/C10 ratio of 12.19±4.26 (mean ± SD) and MCAD activity in lymphocytes was 2.69±1.42 % of wild type (mean ± SD).

However, the ^{13}C-PPA breath test showed a moderate phenotype of 26.75±2.61 % (mean ± SD). Our results do not fully correspond to the phenotype of compound heterozygotes for p.[Lys329Glu];p.[Arg206Cys] as described in literature, because MCAD activity in lymphocytes was notably lower in our cohort (Sturm et al., 2012).

Compound heterozygotes of p.[Lys329Glu];[Met155Thr]

The genotype p.[Lys329Glu];[Met155Thr] has been described as severe variant with 3 % of MCAD activity of wild type measured in lymphocytes using octanoyl-CoA as substrate (Sturm et al., 2012).

Our results showed a severe phenotype in C8/C10 ratio and MCAD activity in lymphocytes, but a moderate phenotype in ^{13}C breath test. C8/C10 ratio was 6.57, MCAD activity was 1.5 % of wild type and breath test results was 27 %. In C8/C10 ratio and MCAD activity in lymphocytes our results correspond to the phenotype of p.[Lys329Glu];[Met155Thr] as described in literature (Sturm et al., 2012). The moderate phenotype in breath test does not correspond to the severe phenotype described in literature.

Compound heterozygotes of p.[Lys329Glu];[Ser245Leu]

Patients carrying the variants p.[Lys329Glu];[Ser245Leu] were described as a moderate phenotype associated with a C8 level of 1.3 µmol/l and a C8/C10 ratio of 2.9 (Smith et al., 2010).

MCAD activity in homozygous state of p.[Ser245Leu];[Ser245Leu] was 10 % of wild type using the specific substrate phenylpropionyl-CoA and high pressure liquid chromatography (Zschocke et al., 2001).

Our results point to a notable increase of C8/C10 ratio to 7.14, to a very low amount of MCAD activity in lymphocytes of 0.78 % of wild type and a moderate presentation by cumulative ^{13}C recovery rate of 24.2 %. The phenotype of p.[Lys329Glu];[Ser245Leu] differs from previous reports in higher C8/C10 ratio and lower MCAD activity in lymphocytes (Smith *et al.*, 2010; Zschocke *et al.*, 2001). Our results point to a more severe phenotype.

Compound heterozygotes of p.[Lys329Glu];[Thr351Ile]

The genotype p.[Lys329Glu];[Thr351Ile] was first reported in 2015 and may lead to a moderate phenotype. The C8 value of two patients was 3.72±0.73 µmol/l (Drendel *et al.*, 2015).

Our results revealed a C8/C10 ratio of 4.44, a severe phenotype of MCAD activity in lymphocytes of 1.29 % of wild type, and a moderate cumulative ^{13}C recovery rate of 23.80 %. Our results of acylcarnitines correspond to the phenotype of p.[Lys329Glu];[Thr351Ile] as described in literature (Drendel *et al.*, 2015).

Compound heterozygotes of p.[Lys329Glu];[Leu107Ser]

p.[Lys329Glu];[Leu107Ser] was described as a moderate phenotype with C8 levels of 2.1 µmol/l and a C8/C10 ratio of 4.8 (Smith et al., 2010).

Our results showed a moderately increased C8/C10 ratio of 3.63, MCAD activity in lymphocytes was 24.78 % of wild type, and the cumulative ^{13}C recovery rate was 26.6 %. This genotype shows the full picture of a moderate phenotype. Our results correspond to the phenotype of p.[Lys329Glu];[Leu107Ser] as described in literature (Smith et al., 2010).

Compound heterozygotes of p.[Lys329Glu];[Ile416Thr]

The genotype p.[Lys329Glu];[Ile416Thr] is known associated with a mild phenotype with normal C8 values of 0.5 µmol/l and a C8/C10 ratio of 1.8 (Smith et al., 2010).

Allele-specific enzyme activities for variant Ile416Thr proteins in Japanese patients detected almost normal levels of 86.9 % of wild type using octanoyl-CoA as substrate (Hara et al., 2016).

Our study revealed a slightly increased C8/C10 ratio of 4.1, a MCAD activity in lymphocytes of 106.8 % of wild type, and a cumulative ^{13}C recovery rate of 26 % in breath test. Although results of MCAD activity are above 100 % of wild type, this patient only reached 26 % in the cumulative ^{13}C recovery rate, which indicates a moderate phenotype. Our results correspond to the phenotype of p.[Lys329Glu];[Ile416Thr] as described in literature (Hara *et al.*, 2016; Smith *et al.*, 2010).

5.3.3 Genotypes classified as associated with severe phenotypes

Due to our HCA results groups 2 and 4 were classified as severe phenotypes. Genotypes of these groups included:
- 11 patients homozygous for p.[Lys329Glu];[Lys329Glu]
- 3 patients compound heterozygous for p.[Lys329Glu];[Ile78Thr], p.[Lys329Glu];[Lys197Glu] and p.[Lys329Glu];c.[387+1delG]

Except for the genotype p.[Lys329Glu];[Lys197Glu], all other genotypes of this group demonstrated a severe presentation by all 3 diagnostic methods. They showed high C8/C10 ratios (> 2.36), low MCAD activities in lymphocytes (< 35.57 % of wild type), and low results in cumulative ^{13}C recovery rate (< 20.3 %). Although the C8/C10 ratio was nearly normal for the genotype p.[Lys329Glu];p.[Lys197Glu], results of MCAD activity and breath test revealed a severe phenotypic presentation.

Homozygotes of p.[Lys329Glu];[Lys329Glu]

In literature reports, 80 % of European MCADD patients are homozygous for p.[Lys329Glu];[Lys329Glu] (Andresen *et al.*, 2001; Cooper *et al.*, 2020; Gramer *et al.*, 2015; Merritt and Chang, 2020; Wilcken *et al.*, 2007). Thereby, this genotype represents the highest frequency in diagnosed MCADD patients (Andresen *et al.*, 2001; Cooper *et al.*, 2020; Gramer *et al.*, 2015; Merritt and Chang, 2020; Wilcken *et al.*, 2007).

Patients homozygous for p.[Lys329Glu];[Lys329Glu] have been described previously as severe phenotypes, presenting with early neonatal symptoms, high C8 (> 2 µmol/l) and C8/C10 (> 4) NBS values above conventional levels (Andresen *et al.*, 2001; Arnold *et al.*, 2010; Bentler *et al.*, 2017; Smith *et al.*, 2010; Sturm *et al.*, 2012; Touw *et al.*, 2012; Waddell *et al.*, 2006; Zschocke *et al.*, 2001). Residual activities measured by HPLC-UV or HPLC-ESI-MS/MS range from 0 to 8 % of healthy controls (Andresen *et al.*, 2001; Arnold *et al.*, 2010; Bentler *et al.*, 2017; Smith *et al.*, 2010; Sturm *et al.*, 2012; Touw *et al.*, 2012; Waddell *et al.*, 2006; Zschocke *et al.*, 2001).

Allele-specific enzyme activities for variant Lys329Glu proteins in Japanese patients have revealed levels of 28.6 % of wild type using octanoyl-CoA as substrate (Hara et al., 2016).

In our cohort, we determined a C8/C10 ratio of 10.37±5.30 (mean±SD), which is markedly above the normal range. In addition, these patients showed cumulative ^{13}C recovery rates of 14.04±3.70 % (mean±SD). For MCAD activity in lymphocytes values between 1.01 and 35.57 % of wild type, equivalent to 4.8±10.22 % of wild type (mean±SD) were measured. Only one patient revealed an enzyme activity of 35.57 %, while the other 10 examined patients showed results of 1.74±0.73 %. There is no clear explanation for the high MCAD activity result in lymphocytes of this one homozygous patient, as C8/C10 ratio was increased at 11.0 and ^{13}C breath test result was very low at 10.5 %. This patient was one of 3 siblings. When comparing to the other 2 siblings with the same genotype, both, the breath test and acyl-carnitines, demonstrated similar levels and it was only the lymphocyte activity that was incongruous. To investigate this outlying result, we will remeasure the enzyme activity in lymphocytes on the occasion of the next clinical routine visit. For patients homozygous for the common p.[Lys329Glu];[Lys329Glu] genotype, our results correspond well to phenotypic data described in literature reports (Andresen *et al.*, 2001; Arnold *et al.*, 2010; Bentler *et al.*, 2017; Cooper *et al.*, 2020; Gramer *et al.*, 2015; Hara *et al.*, 2016; Merritt and Chang, 2020; Smith *et al.*, 2010; Sturm *et al.*, 2012; Touw *et al.*, 2012; Waddell *et al.*, 2006; Wilcken *et al.*, 2007; Zschocke *et al.*, 2001).

Homozygotes of c.[244insT*104];[244insT*104]

For the genotype c.[244insT*104];[244insT*104], Maier et al. described a highly elevated C8 value of 19.98 µmol/l and a C8:C10 ratio of 10.4 (Maier et al., 2005). Thereby it was characterized as severe phenotype (Maier *et al.*, 2005).

Our dataset collected for this genotype is incomplete as blood samples are missing. The cumulative ^{13}C recovery rate was low with 15.1 %, which also indicates a severe phenotype. Thus, our results correspond to the phenotype of c.[244insT*104];[244insT*104] as described in literature (Maier *et al.*, 2005).

Compound heterozygotes of p.[Lys329Glu];[Ile78Thr]

Compound heterozygotes for p.[Lys329Glu];p.[Ile78Thr] have been described as severe phenotypes (Andresen *et al.*, 2001; Derks *et al.*, 2014; Smith *et al.*, 2010; Sturm *et al.*, 2012).

The phenotype of these patients is associated with a considerable increase of C8 concentration of > 2.0 µmol/l, C8/C10 ratios of > 4 and a decrease in MCAD enzyme activities of wild type (Andresen *et al.*, 2001).

It showed an activity in lymphocytes of 0 % of wild type as measured by HPLC-UV or HPLC-ESI-MS/MS using octanoyl-CoA as substrate (Sturm et al., 2012).

Expression studies that were using the specific substrate phenylpropionyl-CoA described a median residual MCAD enzyme activity of 2 % of wild type for p.[Lys329Glu];[Ile78Thr], and 3 % of wild type for the homozygous genotype of p.[Ile78Thr];[Ile78Thr] (Touw et al., 2012).

Our results showed an increased C8/C10 ratio of 9.46, a very low MCAD activity in lymphocytes of 0.17 % of wild type and a very low cumulative ^{13}C recovery rate of 16.4 %. Therefore, our results correspond to the phenotype of p.[Lys329Glu];[Ile78Thr] as described in literature (Andresen *et al.*, 2001; Derks *et al.*, 2014; Smith *et al.*, 2010; Sturm *et al.*, 2012; Touw *et al.*, 2012).

Compound heterozygotes of p.[Lys329Glu];[Lys197Glu]

The compound heterozygous genotype p.[Lys329Glu];p.[Lys197Glu] has not been described in the scientific literature yet.

C8/C10 ratios of 1.20±0.13 (mean±SD) were nearly normal, MCAD activities in lymphocytes were only 1.23±0 % of wild type (mean±SD), and cumulative ^{13}C recovery rate was only measured in one patient and showed a low result of 14.4 %. Thereby our data suggest a severe phenotypic presentation of the genotype p.[Lys329Glu];[Lys197Glu].

Compound heterozygotes of p.[Lys329Glu];c.[387+1delG]

The variant c.[387+1delG] is a splice mutation, expected to disrupt RNA splicing and likely results in an absent or disrupted protein product (Bentler et al., 2017; Maier et al., 2005; Sturm et al., 2012).

As a compound heterozygous variant with c.[387+1delG];[1229T>G] it results in an enzyme activity of 0-7 % of wild type measured by HPLC-UV or HPLC-ESI-MS/MS in lymphocytes (Sturm et al., 2012).

In our patient carrying the genotype p.[Lys329Glu];c.[387+1delG] we a severe biochemical phenotype was found. C8/10 ratio was 11.64, MCAD activity in lymphocytes was 4.25 % of wild type, and cumulative ^{13}C recovery rate was only 6.7 %. Our results correspond to the phenotype of c.[387+1delG];[1229T>G] as described in literature. The phenotype p.[Lys329Glu];c.[387+1delG] has not been described in scientific literature reports before.

5.4 Conclusion

In conclusion, the ^{13}C breath test not only provides a diagnostic tool to determine the patients underlying phenotype, but may serve as a tool to estimate the current metabolic state in our patients.

We propose that the combination of all three diagnostic tools, namely the C8/C10 ratio, the MCAD activity in lymphocytes, and the ^{13}C breath test, allows favorable functional testing at multiple levels. The combination of these three biochemical approaches will enable the establishment of a more precise picture of MCADD, which specifies our knowledge of risk assessment.

Especially for patients with compound heterozygous genotypes or a genotype that has not been described in literature reports before, the combination of all three tools might give a clue to the phenotype that can be expected.

We described the phenotypes of the compound heterozygous genotypes p.[Lys329Glu];p.[Lys197Glu] and p.[Lys329Glu];c.[387+1delG] by C8/C10 ratio, MCAD activity in lymphocytes, and cumulative ^{13}C recovery rate as severe phenotypes, that have not been described in scientific literature before.

For compound heterozygous genotypes p.[Lys329Glu];[Thr351Ile], p.[Lys329Glu];[Leu107Ser], p.[Lys329Glu];[Ile416Thr], and for the homozygous genotype c.[244insT*104],[244insT*104], the phenotype has only been reported by literature reports based on acylcarnitine levels. Except for the patient with the homozygous genotype, who only performed the breath test, we present a full picture of C8/C10 ratio, MCAD activity in lymphocytes, and cumulative ^{13}C recovery rate in breath test that gives a deep insight into the metabolic pathway of the disease.

5.5 Future perspectives

Further participants need to be tested to validate the breath test in more detail:

- Healthy controls

To date, only 6 participants functioned as a healthy control group in the ^{13}C-PPA breath test. Mean age of these participants was 34 years and ranged from 31 to 37 years. The future plan is to include continuing healthy control group participants that cover all age groups, especially those of children between 0 and 18 years.

Moreover, baseline values of healthy controls with febrile infections are needed to understand whether the cumulative ^{13}C recovery rate is decreased under metabolic stress.

To study the influence of the microbiome, healthy newborns are also planned to be tested by breath test in the first year of life, where the microbiome is known to be not colonized like adult-type colonic flora (Bhala et al., 1993). The development of a microbiome from birth follows dynamic changes and is dependent on gestational age, mode of delivery, diet, hormones, antibiotics and many more influence factors. After birth, there seems to be a decrease in gut alpha diversity and by one week of age, the gut microbiota is already very similar to that in a month-old baby. Afterwards and until the age of 2 to 3 years it develops rapidly until an adult-like composition and stability is established. (Bokulich et al., 2016; Gordon et al., 2012; Hill et al., 2017) The ^{13}C-PPA breath test can technically be performed in newborns, using a mask that is connected to the breath test aluminum bags. But the current study does not answer the question whether a breath test in newborns interferes with the dynamic changes of the gut flora.

- Patients

Further study of our MCADD patient cohort with repeated breath tests is required to determine the intraindividual variablitity, both, in the well or steady state, but also in metabolic stress and decompensation, for example during an intercurrent infection with fever or other stressing conditions. Results of children with MCADD should be interpreted with age specific reference values of healthy controls and their own baseline results.

Integration of the ^{13}C-PPA breath test as diagnostic tool might improve management and treatment of MCADD patients. Therefore, the metabolic department of the Children's Hospital in Hamburg may become the reference center for Germany and Europe. Other metabolic centers can be taught by correspondents of our metabolic team how to perform the breath test and breath samples can be sent to our department in Hamburg by express delivery. As breath samples can be stored for 14 days an express delivery is only needed for patients with an urgent question.

The University Medical Center Groningen in the Netherlands is currently recruiting for a fasting tolerance study in MCADD-infants at the ages of 2 to 6 months, because data on this age group are absent (Derks, 2018). The *in vivo* ^{13}C-PPA breath test may be an additional tool to estimate the current metabolic situation in MCADD patients under fasting conditions.

The University Children's Hospital of Pittsburgh in the United States has completed a phase 1 study, testing the drug Ravicti, consisting of glycerol phenylbutyrate in adult patients with MCADD and at least one copy of the p.[Lys329Glu] variation. Ravicti is currently approved in patients with urea cycle disorders providing an alternative for ammonia excretion. Previous research suggests that it may also be effective as a chaperone to stabilize the enzyme in MCADD. (Kormanik *et al.*, 2012; Vockley, 2013) The *in vivo* ^{13}C-PPA breath test may help to test the efficacy of new therapeutic approaches.

6 Abstract

6.1 English

Background: Deficiency of medium chain acyl-coenzyme A (CoA) dehydrogenase (deficient enzyme: MCAD, disease: MCADD) leads to a reduced ability to catalyze C6 to C10 straight-chain acyl-CoA as the initial step of the mitochondrial fatty acid oxidation. This pathogenic mechanism impairs the energy supply to peripheral tissues predisposing affected individuals to become symptomatic in situations of decreased oral carbohydrate intake. Patients are at risk of hypoketotic crises with hypoglycemia, which can progress to coma and sudden death. MCADD is detected by medium-chain acylcarnitine profiles and disease severity is estimated by *ex vivo* enzyme activity analysis in lymphocytes. However, the assessment of the current metabolic state in MCADD patients is unsatisfactory.

Methods: The *in vivo* metabolic flux was analyzed by using a ^{13}C breath test. Following oral application of ^{13}C-phenylpropionic acid (^{13}C-PPA) as substrate for the MCAD enzyme, $^{13}CO_2$ was determined in exhaled air for 180 minutes. Breath test measurements were accompanied by the analysis of acylcarnitine profiles and MCAD enzyme activity in lymphocytes. The study population consisted of 11 patients homozygous for the variant p.[Lys329Glu];[Lys329Glu], 1 patient homozygous for the variant c.[244insT*104];[244insT*104], 13 compound heterozygous patients, 9 heterozygous carriers, and 6 healthy controls.

Results: Results of C8/C10 ratio and MCAD activity in lymphocytes revealed a strong correlation to breath test results in our study cohort (Rank correlation analysis by Spearman's correlation coefficient). Cutoff values of 2.36 for C8/C10 ratio, 35.57 % of wild type for MCAD activity in lymphocytes and 28.6 % for cumulative ^{13}C recovery rate in breath test were calculated to distinguish between affected and healthy participants. Only the ^{13}C breath test delivered a second significant cutoff value of 20.3 % to divide between severely and moderately affected patients.

Discussion: We show that an *in vivo* metabolic flux analysis by ^{13}C-PPA breath test promises to serve as an additional tool to determine the current metabolic state in MCADD patients. Hence, we conclude that the combination of all 3 diagnostic tools (C8/C10 ratio, MCAD activity in lymphocytes, ^{13}C-PPA breath test) allows favorable functional testing at multiple levels.

6.2 Deutsch

Hintergrund: Ein Mangel des Enzyms mittelkettige Acyl-Coenzym A-Dehydrogenase (Enzym: MCAD, Erkrankung: MCADM) führt zu einer Störung im Fettstoffwechselabbau, bei der die mittelkettigen Fettsäuren C6-C10 nur unzureichend verwertet werden können. Durch längere Nahrungspausen, fieberhafte Infekte oder chirurgische Eingriffe kann es im Rahmen der katabolen Stoffwechsellage zu schweren hypoketotischen Krisen mit Hypoglykämie, Koma und plötzlichem Tod kommen. MCADM wird durch eine Erhöhung der mittelkettigen Acylcarnitine im Blut diagnostiziert. In Lymphozyten kann die Restaktivität des Enzyms MCAD gemessen werden und Rückschlüsse auf den Schweregrad der Erkrankung liefern. Dennoch erlauben die vorliegenden Biomarker keine Risikoeinschätzung der aktuellen Stoffwechsellage bei MCADM Patienten.

Methoden: Wir untersuchten einen Atemtest, für den die Probanden ^{13}C markiertes Phenylpropionat (^{13}C-PPA) oral einnahmen. Dieses wird durch das Enzym MCAD verstoffwechselt. In der Ausatemluft wurde markiertes $^{13}CO_2$ über eine Zeit von 180 Minuten in Intervallen gemessen. Der ^{13}C-PPA Atemtest wurde mit den herkömmlichen Methoden, einer Analyse der Acylcarnitine und der Bestimmung der MCAD Enzymaktivität in Lymphozyten verglichen. Die Studienkohorte bestand aus 11 Patienten, die homozygot für die Variante p.[Lys329Glu];[Lys329Glu] waren, 1 homozygoten Patienten für die Variante c.[244insT*104];[244insT*104], 13 compoundheterozygoten Patienten, 9 heterozygoten Anlageträgern und 6 gesunden Kontrollen.

Ergebnisse: Die Ergebnisse der C8/C10-ratio und der MCAD-Aktivität in Lymphozyten zeigten eine starke Korrelation zu den ^{13}C-Atemtestergebnissen in unserer Studienkohorte (Rangkorrelationsanalyse nach Spearman). Grenzwerte von 2.36 für die C8/C10-ratio, 35.57 % vom Wildtyp für die MCAD-Aktivität in Lymphozyten und 28.6 % für den ^{13}C-Atemtest konnten zwischen MCADM Patienten und gesunden Probanden unterscheiden. Nur der ^{13}C-PPA Atemtest ergab einen zweiten signifikanten Grenzwert von 20.3 %, um zwischen phänotypisch schwer und moderat betroffenen Patienten zu unterscheiden.

Diskussion: Der ^{13}C-PPA-Atemtest stellt eine zusätzliche Testmethode zur Bestimmung der aktuellen Stoffwechsellage bei MCADM Patienten zur Verfügung. Die Kombination aller 3 Methoden (C8/C10 ratio, MCAD-Aktivität in Lymphozyten, ^{13}C-PPA Atemtest) ermöglicht die Beurteilung der aktuellen Stoffwechsellage eines Patienten auf mehreren Ebenen.

7 Figures

8 Tables

9 Abbreviations

ADP	Adenosine-diphosphate
ATP	Adenosine-triphosphate
AUC	Area under the curve
BIMDG	British inherited metabolic disease group
C2	Acetic acid
C3	Propionic acid
C4	Butyric acid
C6	Caproic acid
C8	Caprylic acid
C10	Capric acid
C12	Lauric acid
C14	Myristic acid
C16	Palmitic acid
C18	Stearic acid
C20	Arachidic acid
C22	Behenic acid
C24	Lignoceric acid
C26	Cerotic acid
C28	Montanic acid
C30	Melissic acid
°C	Centigrade
CO_2	Carbon dioxide
CoA	Coenzyme A
CPT	Carnitine palmitoyltransferase
DNA	Deoxyribonucleic Acid
ETF	Electron transfer flavoprotein
ETFQO	Electron transfer flavoprotein ubiquinone oxidoreductase or electron transfer flavoprotein dehydrogenase
f	Female
FA	Fatty acids
FAD	Flavin adenine dinucleotide

FAO	Fatty acid β-oxidation
GDP	Guanosine-diphosphate
GTP	Guanosine-triphosphate
HCA	Hierarchical cluster analysis
HMG	3-hydroxymethylglutaryl
HPLC	High performance liquid chromatography
IEM	Inborn error of metabolism
IRIS	Infrared Isotope Analyser
LDL	Low density lipoprotein
m	Male
MCAD	Medium chain acyl-coenzyme A dehydrogenase
MCADD	Medium chain acyl-coenzyme A dehydrogenase deficiency
MCADM	Mangel des Enzyms mittelkettige Acyl-Coenzym A-Dehydrogenase
MS/MS	Tandem mass spectrometry
NAD	Nicotinamide adenine dinucleotide
NBS	Newborn screening
NDIR	Non-dispersive IR-spectroscopy
PCR	Polymerase chain reaction
PKU	Phenylketonuria
pp	Pages
PPA	Phenylpropionic acid
PPAR	Peroxisome proliferator-activated receptor
RNA	Ribonucleic Acid
ROC	Receiver-operated-curve
SCOT	Succinyl-CoA 3-oxoacid CoA transferase
SD	Standard deviation
UPLC	Ultra performance liquid chromatography
VLDL	Very low density lipoprotein
vs.	Versus
WT	Wild type

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12 Curriculum vitae

PERSONAL DATA

Surame:	Neumann, geb. Seggewies
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WORK EXPERIENCE

Since July 2020	Specialist in Pediatric Medicine
Since September 2017	Specialization in Pediatric Metabolic Medicine University Children´s Hospital Hamburg Director, Univ.-Prof. Dr. med. Ania C. Muntau
April 2017 – July 2020	Pediatric resident, Kinder-UKE University Children´s Hospital Hamburg Director, Univ.-Prof. Dr. med. Ania C. Muntau
May 2015 - April 2017	Pediatric resident in Pediatric Cardiology and Pediatric Intensive Care, University Heart Center, Hamburg Director, Prof. Dr. Rainer Kozlik-Feldmann

MAJOR TEACHING RESPONSIBILITIES

April 2017 – May 2019	Organization of pediatric lectures, update of teaching database, exams, attend our module meetings D2, D3, examination of the teaching budget tables, bedside instruction of medical students, residents, pediatric fellows

SECONDARY EDUCATION AND CLASSIFICATION

1.10.09- 1.12.14	Studies of Human Medicine, University of Graz, Austria Final exams: grade A, Stipend for high marks Title: Dr. med. univ. (Diploma programme)
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DOCTORAL THESIS

External validation of the derived neutrophil to lymphocyte ratio as a prognostic marker on a large cohort of pancreatic cancer patients
Supervisors: Mag. rer. nat. Dr. med. univ. Martin Pichler and Prof. PD Dr. med. univ. et scient. med. Armin Gerger
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EDUCATION AND CLASSIFICATION

2000 – 2007	Gymnasium Paulinum Münster, Germany 16.06.2007 Final exams: grade A, Ephasis: Mathematics, Physics 06.2007 – 06.2008 Award for a membership of DPG (deutsche physikalische Gesellschaft)
1998 – 2000	Kardinal-von-Galen Gymnasium Hiltrup, Germany
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RESEARCH PRESENTATIONS AND PUBLICATIONS

Oral presentations by invitation:

1. First data of clinical presentation of siblings with diagnosed marfan-syndrome: many but not all. February 2016 DGPK/ DGTHG, Leipzig
2. Riboflavin non-responsive multiple acyl-CoA dehydrogenase deficiency (MADD) with early severe cardiomyopathy: Favorable long-term outcome on D,L-3-hydroxybutyrate

(OHB) supplementation. February 2020 DGPK/DGTHG, Wiesbaden

Poster presentations:

1. A severe neonatal phenotype associated with a pathogenic *C12orf65* variant: a disorder of mitochondrial translation and protein synthesis. September 2019 SSIEM, Rotterdam
2. An *in vivo* ^{13}C breath test as diagnostic tool to determine the residual metabolic flux in medium-chain acyl-CoA dehydrogenase deficiency. September 2019 SSIEM, Rotterdam
3. Riboflavin non-responsive multiple acyl-CoA dehydrogenase deficiency (MADD): favorable long-term outcome of a severe neonatal presentation on D,L-3-hydroxybutyrate supplementation. September 2019 SSIEM, Rotterdam
4. Arginase 1 deficiency – a clinical, molecular genetic, and histopathologic study in two patients with liver transplantation. August 2018 SSIEM, Athens
5. The quotient of sinus valsalvae and sinotubular junction – an additional and independent parameter to classify the aortic affection of pediatric patients with Marfan-syndrome? July 2017 WCPCC, Barcelona
6. Transition of pediatric patients with Marfan-syndrome to adult care: an important factor to prevent exacerbation? July 2017 WCPCC, Barcelona
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Co-authorship in publications:

1. Renner S et al. (2019) Next-generation Sequencing of 32 Genes Associated With Hereditary Aortopathies and Related Disorders of Connective Tissue in a Cohort of 199 Patients. Genet Med. 21(8):1832-1841.
2. Stotz M et al. (2015) The lymphocyte to monocyte ratio in peripheral blood represents a novel prognostic marker in patients with pancreatic cancer. Clin Chem Lab Med. 53(3):499-506.
3. Szkandera J et al. (2014) Validation of C-reactive protein levels as a prognostic indicator for survival in a large cohort of pancreatic cancer patients. Br J Cancer. 110(1):183-8.
4. Szkandera J et al. (2013) External validation of the derived neutrophil to lymphocyte ratio as a prognostic marker on a large cohort of pancreatic cancer patients. PLoS One. 8(11):e78225.
5. Stotz M et al. (2013) Increased neutrophil-lymphocyte ratio is a poor prognostic factor in patients with primary operable and inoperable pancreatic cancer. Br J Cancer. 109(2):416-21.

Co-authorship in books:

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ADDITIONAL INFORMATION

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13 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift:

Hamburg, 01.11.20 

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