Exploring the mechanisms underlying the phenotype of MCAD deficiency with Systems Medicine

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Chapter 1

General introduction and outline of the thesis

Introduction

The mammalian body depends on a wide range of cellular and systemic processes. A subset of these processes are physicochemical processes and biochemical reactions that are catalyzed by enzymes and transporters. These processes and reactions fall within the category of metabolism. Ongoing research in mammalian metabolism and metabolic disease has generated major advances in the last decades. This is quite important, as acquired metabolic disease has become a worldwide epidemic due to an unhealthy lifestyle and an aging population [1–4]. Moreover, many more inborn metabolic errors are being discovered due to early screening efforts [5–13]. Understanding metabolism and metabolic disease will improve the chances for effective treatment and the lowering of ever rising healthcare costs. The major question in the field is what are the molecular mechanisms underlying metabolic disease [3,14–17].

Common acquired metabolic diseases

Acquired metabolic diseases are non-communicable, multifactorial diseases that may progress over time [18]. They are one of the most common non-communicable disease groups affecting global health. According to the World Health Organization, the prevalence of overweight and obesity has tripled since 1975 [19]. Particularly obesity, afflicting more than 19% of the Western population (12.5% in the Netherlands and 38% in the US) [20], increases the risk of metabolic diseases, including type 2 diabetes mellitus (T2DM), and nonalcoholic fatty liver disease (NAFLD) [1–4]. T2DM for example, is characterized by insulin resistance of peripheral tissues and impaired insulin secretion by the pancreas upon increased blood glucose levels [21,22]. The resulting high blood glucose levels (hyperglycemia) damage pancreatic cells, B-cells, nerves, and blood vessels, and consequently cause major health problems including cardiovascular diseases, stroke, blindness, neurodegenerative disease, and cancer [21,23–25]. Nonalcoholic fatty liver disease (NAFLD) has a prevalence of over 20%, making it the most common in Western countries [26–29]. The disease can progress from simple lipid accumulation (steatosis) to nonalcoholic steatohepatitis (*i.e.* inflammation of the liver with concurrent fat accumulation in liver) to cirrhosis to hepatocellular carcinoma [30–33]. Common characteristics in these diseases include excessive glucose and fatty-acid supply to the liver, resulting in inflammation and oxidative stress [34–36]. β-cells that are chronically exposed to high glucose and free fatty acid levels, show higher reactive oxygen species (ROS) production. ROS in turn reduces insulin gene expression and insulin content, and activates uncoupling protein 2 (UCP2). The latter lowers ATP production and inhibits glucose-stimulated insulin secretion [37]. Overexpression of antioxidant enzymes has been reported to protect β-cells from dysfunction [37]. ROS also induces insulin resistance of target tissues by inhibiting insulin signal transduction [38,39]. Accordingly, high-fat-induced insulin resistance has been prevented by reducing ROS through antioxidant treatment or overexpression of antioxidant enzymes [37]. Oxidative stress also plays an important role in liver disease progression [40]. This is to be expected, as the liver harbors a lot of processes in which ROS is generated, including protein synthesis [40], mitochondrial respiration and detoxification [40]. Excessive supply of fatty acids in obesity would overwhelm the electron transport chain, leading to excessive ROS production and mitochondrial dysfunction. Mitochondrial dysfunction would in turn amplify excessive ROS production resulting in lipid and protein peroxidation and deregulation of hepatic metabolism in general. In addition,

excessive ROS induces inflammatory cytokines, which in turn exacerbate ROS production and inflammation [15,40,41]. Supplementation of the antioxidant vitamin E to NAFLD patients, has been shown to ameliorate NAFLD at several stages of severity [15]. Aging also makes an individual more prone to metabolic diseases, including loss of muscle mass (sarcopenia), T2DM, NAFLD and cardiovascular disease [42].
In addition, other metabolites are also emerging as relevant contributors in metabolic disease, including specific types of lipids, NAD^+, coenzyme A and amino acids. Below, I will discuss each of these (classes of) metabolites in more detail.

Metabolites and common acquired metabolic disease

Lipid regulators

Lipids are predominantly known as mediators of toxicity and inflammation in metabolic disease. Recently however, studies have identified lipid classes that appear to function in signaling and can induce anti-inflammatory mechanisms. Lipokines, for instance, can function as soluble signals outside the cells. An example is C16:1n7-palmitoleate [43,44]. Palmitoleate synthesis is increased and its blood levels rise as a result of de novo lipogenesis in adipose tissue. This lipid is able to suppress inflammation and liver lipogenesis and stimulates glucose uptake by skeletal muscle [43–46]. In macrophages it alleviates lipotoxicity-induced ER stress and cell death. Another endogenous lipid class consists of fatty-acid hydroxy-fatty acids (FAHFAs). These FAHFAs were identified by lipidomic analysis of adipose tissue and in the circulation of mice with increased *de novo* lipogenesis. A subcategory of these FAHFAs, palmitic-acid-hydroxy-stearic-acids (PAHSAs), lower blood glucose, improve glucose tolerance, stimulate insulin secretion, reduce adipose tissue inflammation and improve insulin tolerance [47]. Finally, resolvins and protectins, both of which are produced by catabolism of omega-3 fatty-acids, have been reported to alleviate obesity-induced insulin resistance and hepatic steatosis [48–50].

NAD^+

NAD^+ does not only play a central role as a redox intermediate in energy and amino acid metabolism, but it is also involved in detoxification, calcium transport, DNA repair, and protein deacylation [51–53]. Its phosphorylated form, $NADP^+$, is essential for lipid biosynthesis [52–54]. In general, an increase in NAD^+ levels improves metabolic homeostasis and prevents or alleviates metabolic disease. NAD^+ plays a protective role in T2DM, as NAD^+-mediated sirtuin1 (SIRT1) signaling improves liver and muscle insulin sensitivity and glucose-stimulated insulin secretion by β-cells. SIRT1 signaling also promotes mitochondrial biogenesis. Administration of NAD^+ precursors have similar effects and can even attenuate high-fat-diet-induced obesity in mice [42]. NAD^+ and SIRT1 play a protective or ameliorating role in NAFLD. Excess lipid supply in the liver decreases hepatic NAD^+ levels, while overexpression of SIRT1 reverses hepatic steatosis and NAD^+ precursors attenuate mitochondrial dysfunction in fatty liver of high-fat-diet-fed mice [42]. NAD^+ has also gained considerable attention due to its role in attenuating aging-related metabolic diseases. NAD^+ decreases during aging as a result of DNA damage, higher expression of the NAD^+-degrading enzyme CD38, and chronic inflammation. The latter reduces the NAD^+-regenerating capacity.

SIRT1 overexpression or administration of NAD^+ precursors have been reported to protect against age-related diabetes, cancer, and mitochondrial dysfunction [42].

Coenzyme A

Coenzyme A has also gained renewed interest in the metabolic field in recent years [55–60]. CoA activates fatty acids for oxidation and chain elongation. It is also an essential coenzyme for *de novo* lipid synthesis, detoxification of xenobiotics, the TCA cycle, and the catabolism of amino acids. In these pathways, CoA can be involved as free CoA (from here on referred to as CoASH, because of its characteristic thiol (–SH) group) or as a thioester intermediate. It was recently shown that CoA levels affect the phenotype of obesity and T2DM. For example, deletion of the CoA biosynthesis enzyme *PANK1* causes lower serum insulin levels, higher glucose tolerance and insulin sensitivity, and lower fatty-acid oxidation rates in mice [55]. The *ob/ob* T2DM mouse model has deregulated CoA metabolism and abnormally high liver CoA levels, which coincides with high blood glucose and insulin levels (hyperglycemia), insulin resistance and increased gluconeogenesis [61,62]. Deletion of *PANK1* in this mouse model attenuated the diabetic phenotype with lower total CoA levels, reduced mitochondrial β-oxidation and gluconeogenesis, normalized glucose levels, and lower insulin levels. The mice, however, remained obese, insulin resistant and hepatosteatotic [55]. This suggests that lowering the CoA levels and fatty-acid oxidation rate may have simply reduced the gluconeogenesis rate and thereby the insulin production, which does not completely resolve the fatty-acid excess issues and might even exacerbate lipid toxicity-related problems.

Another player in coenzyme A metabolism involved in metabolic disease is Vanin 1. It catalyzes the degradation of pantetheine, an intermediate in CoA biosynthesis and degradation, to pantothenate (Vitamin B_5), a Coenzyme A synthesis precursor and PANK substrate. A byproduct of this reaction is cysteamine, an antioxidant. Vanin 1 appears to have both inflammatory and cytoprotective effects, depending on the conditions [63]. In relation to T2DM, Vanin 1 was able to protect pancreatic islet beta cells against from streptozotocin-induced death *in vitro*, and its deletion has been associated with higher incidence of diabetes and more severe inflammatory inflammation of the islets of Langerhans (insulitis) in the non-obese diabetic (NOD) mouse model [64].

Amino acids

Already several decades ago, elevated serum amino acid levels have been associated with obesity and insulin resistance [65]. However, recent advances in metabolomics techniques have sparked renewed interest in the relation between amino acids and metabolic disease. Particularly, circulating branched-chain amino acids (BCAAs), methionine, and the aromatic amino acids phenylalanine and tryptophan have been linked to obesity and insulin resistance. Animal models and humans with obesity and insulin resistance show lower levels of enzymes involved in BCAA catabolism in white adipose tissue (WAT) and lower hepatic activity of the second enzyme in the BCAA catabolism pathway, BCAA dehydrogenase complex (BCKDH). In addition, it was reported that BCAA overload increases acyl-carnitine accumulation in rat muscle [44,66]. Methionine restriction was reported to reduce adiposity, increase insulin sensitivity and attenuate hepatosteatosis in mice [44]. Phenylalanine and tryptophan appear to be predictive markers for T2DM [44].

Advances in approaches and methods to study acquired metabolic diseases

Bioinformatic approaches

Phenotype- or trait-related bioinformatics approaches, such as genome-wide associated studies (GWAS), are often used to discover mechanisms underlying acquired metabolic disease [67]. With ever-growing sample sizes in population studies and with the development of faster and more detailed sequencing techniques, more gene-trait associations have been found. These associations, however, do not necessarily imply biological function or causality. Additional experiments and methods would be required to unravel mechanisms underlying the investigated traits [67]. Recent advances have been made in the use of additional "-omics" data in GWAS. Furthermore, so-called "pathway analysis" allows biological interpretation based on biological pathway- , networks-, and gene ontologies (GOs) and/or disease biomarker gene sets [68].

Experimental models

A wide range of experimental models have been developed over the years to study acquired metabolic diseases. Most widely used are mouse models and *in vitro* cell culture models. For example, dietary, chemical and genetic mouse models have been developed for both T2DM and NAFLD [14,69,70]. Metabolic disease originates from a complex interplay between different organelles, cells and tissues of an organism, which often develops over time to a pathological presentation. Therefore, studying the whole mouse as opposed to individual cells or body fluids, may offer insight into mechanisms underlying metabolic disease that are currently unattainable in humans. However, mouse models might not be fully translatable to humans [14,69]. For example, several NAFLD mouse models showed gene expression patterns that were so different from those of humans patients that no conclusions regarding disease stage could be translated from mice to humans [71]. More recently, human *in vitro* and *ex vivo* cell or tissue models, where care is taken to uphold the differentiated organ function, have also been used to study acquired metabolic disease [69,72]. These include tissue slices, cultured primary human cells, co-culture of different cell types of a specific tissue, patient-derived human pluripotent stem cells (hPSCs) and genome-edited human cells [69,72–76].

Analytical methods and data analysis

In the past decades, also major advances were made in the analytical field. This has enabled "multi-omics" research, which encompasses large-scale analysis of different levels of regulation [14,21,69,77–80]. Notably, RNA sequencing and mass spectrometry (MS) techniques used for proteomics, lipidomics and metabolomics have enabled high-throughput, quantitative determination of gene expression and protein, small metabolite and lipid concentrations [5,6,77–83]. Considering the large datasets generated by these techniques, methods were also developed to interpret the data and translate them to biological function. An example for the interpretation of RNA sequencing results is gene-set-enrichment analysis (GSEA) [84]. Herein, the expression of genes is evaluated at the level of functionally relevant *sets* of genes. These sets contain genes that have a biological function, chromosomal location or associated phenotypes in common. By evaluating gene sets, biological functions can be more easily linked to molecular mechanisms. Other often used pattern recognition techniques

are Principle Component Analysis (PCA), hierarchical clustering and classification. For all these types of data analysis, a range of graphical-user-interphase (GUI) online tools and freely-downloadable software has been developed, based on common programming languages, such as R. This makes processing and interpretation of omics data more easily accessible and user-friendly. Popular software and online tools include the java-based GSEA desktop software GSEA-P, and the R-based Clustvis and Metaboanalyst [84–87]. Finally, comprehensive omics databases are a crucial aid in interpreting vast amounts of generated data. Next to the widely known databases such as KEGG [88], GenBank [89], Pubchem [90] and Uniprot [91], two human-specific databases, the "Human Protein Atlas" [92] and the "Human Metabolome Database" (HMDB) [93,94] are relevant for the metabolic disease field. The "Human Protein Atlas" is an easy to use Web-based database that provides a combination of quantitative mRNA expression- and immunohistochemistry-based relative protein concentration data for 32 different human tissues and organs, covering >90% of the putative protein-coding genes [92]. HMDB is the largest and most comprehensive web-based human metabolomic database. It contains information about human metabolites, reactions, and enzymes, with links to metabolic pathways, reference and pathologic concentrations, disease associations, reference spectra and other related omics databases. These two databases were sufficiently complete and structured to enable the construction of computational models of metabolism based on these databases [95–100].

Systems biology and computational modeling

Systems biology, in the widest sense of the word, from correlation analysis to detailed computational modeling of metabolic dynamics is a powerful tool to gain insight into metabolism and acquired metabolic diseases [101–104]. Genome-scale metabolic and dynamic models of mammalian metabolism are getting increasing attention [95–100,105–110]. Genome-scale metabolic models are stoichiometric models that contain all known biochemical and transport reactions in the modeled network. Constraint-based analysis of these models enables the estimation of relative flux distributions through the network, based on a steady-state assumption and often also other constraints, such as reaction thermodynamics, nutrient supply, gene expression, protein concentration or other omics data [111]. Consequently, these models can reveal which cellular metabolic pathways may contribute to an observed metabolic state or disease. They are also used to identify biomarkers of disease. An example is *iHepatocytes2322*, a genome-scale metabolic model of the human hepatocyte, which was used to identify serine deficiency in NAFLD [99]. Our group has curated the human metabolic model Recon2 [96] to accurately represent the entire flavoproteome [112]. Recently, the metabolism of 20 organs, six sex organs, six blood cells, the gastrointestinal lumen, systemic blood circulation, and the blood-brain barrier have been coupled into a single whole-body model (Harvey and Harvetta) [113].

A limitation of stoichiometric models is that they lack kinetic information. Dynamic metabolic models are often represented as systems of ordinary differential equations for the variable metabolites of the modeled pathways. The ordinary differential equations include all the reactions and transport processes in the model that consume or produce the metabolite of interest at the modeled intracellular location [111,114]. Reaction and transport rates are preferably based on detailed kinetic knowledge of enzyme-catalyzed-, transporter-mediated and physicochemical processes inside the cell [111]. Dynamic models predict metabolite time

courses, but also steady state concentrations and fluxes. They enable metabolic control analysis, and sensitivity analysis in general. Metabolic control analysis (MCA) is the analysis of the sensitivity of model variables, such as fluxes and metabolite concentrations, to modulation of the rate of a reaction or transport process. MCA allows a generalized theoretical analysis of the relation between sensitivity coefficients. An important outcome of MCA is that flux control can be distributed over all the steps in the system and depends on the metabolic state of the system [115–118]. This is in contrast to the classical and often erroneous concept of one "rate-limiting" step with full flux control. In MCA, also the sensitivity of model variables to external effectors can be calculated. External effectors are properties in the system that are not controlled by the modeled system. These include cofactor concentrations that have been set to a constant value or enzyme affinities and catalytic capacities that have been set to a constant value [119]. Where classical MCA is focused mostly on the analysis of steady state, there are many extensions that deal with dynamics, which may be relevant to gain insight into mechanisms underlying disease progression over time. An important extension allows to quantify the contribution of individual variable regulators such as PTM-mediated regulation and metabolites to the change of reaction rates over time [120]. This is important, since it gives detailed insight into the etiology of a pathological condition. It is important to note however, that the outcomes depend on the level of detail and accuracy of the dynamic model. Therefore, a functional phenomenon discovered *in silico*, should be validated *in vitro or in vivo* [111].

Examples of dynamic models of metabolism and acquired metabolic disease include models of hepatic glucose metabolism with hormonal regulation [121], mitochondrial energy metabolism [107,122], zonation of hepatic energy metabolism, insulin resistance and steatosis [123], glucose and amino acid-induced insulin secretion in a pancreatic β-cell [124], metabolic syndrome and dyslipidemia [125], and mitochondrial fatty-acid oxidation [108]. The simulations generated with these models suggest that we are currently able to gain considerable insight into the workings of metabolism and metabolic disease, especially when used in combination with multi-omics data.

Inborn errors of metabolism/Inherited metabolic diseases

Inborn errors of metabolism (IEM) are inherited diseases primarily caused by pathogenic mutations in genes that code for metabolic enzymes or transport proteins. IEMs have been classified into 1) intoxication diseases, including amino-acidopathies and organic acidurias, 2) inborn errors of energy metabolism, including mitochondrial respiratory-chain and fatty-acid oxidation deficiencies, and 3) diseases related to impaired degradation or synthesis of complex molecules, such as glycogen storage disease and congenital disorders of glycosylation [126]. Some of these defects fall into multiple classes. Collectively, the IEM prevalence was 1/2400 in Germany 2003 and 1/1894 in Italy in 2015 [6,9,10,18,102,127]. One of the most common IEMs is medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (MCADD) [8–10,18,102,127]. The MCADD prevalence at birth was 1/8300 in the Netherlands between 2007 and 2015 [128] and 1/9093 in Italy in 2015 [6]. MCAD is an enzyme of the mitochondrial fatty-acid oxidation pathway. Especially newborns and children can present with a serious illness and disease related to IEMs. Consequently, early diagnosis and treatment before the onset of symptoms is important to prevent severe permanent damage or death [10,102,127].

Common principles underlying IEMs and acquired metabolic diseases

IEMs are typically monogenic, single-enzyme deficiencies. Nevertheless, IEMs often display a spectrum of disease phenotypes, even within a subset of individuals with the same mutation. Therefore, IEMs have been viewed to display complex traits, not unlike common acquired, multifactorial diseases [18,102,129]. It has been proposed that modifying factors, including environmental factors (such as diet and ambient temperature [130]), epigenetic factors and other genes, may contribute to the disease presentation [18]. In addition, phenotypes similar to those of acquired metabolic diseases, for instance hepatosteatosis, overweight and diabetes, have been reported in IEMs [18,131,132]. IEMs may even be considered as part of the same metabolic disease spectrum. On the one end of the spectrum the defective gene of the IEM together with modifying genes and other factors determine the disease presentation, often at young age. On the other end of the spectrum multiple genes together with lifestyle and history determine the development over time of acquired metabolic disease [18]. It is thus in both cases the cumulative effect of different gene variants plus external factors that result in the observed phenotype. This is not unlike the distribution of flux control over many enzymes in a metabolic network. Consequently, the contribution of a genetic variation to the phenotype determines whether it is identified as an inborn error or not. This also means that “weaker mutations" in an otherwise healthy individual (represented by a combination of genetic variants that result in the phenotype being denoted as "healthy") would not be easily detected. A weaker mutation would be a mutation that on its own would not result in a clinical phenotype. An example is a GWAS showing association of single-nucleotide polymorphisms (SNPs) at the ACADM locus with blood C8-carnitine levels, a biomarker for MCADD [18,133]. Finally, IEMs result in a more pathological phenotype compared to the weaker mutation phenotype (some similar to acquired metabolic disease) and might therefore be more vulnerable to additional weakness-increasing variances in other genes and other factors. Therefore, modifying factors (including genes) that contribute to the development and progression of acquired metabolic diseases can be more easily identified when studying IEMs. Similarly, differences in gene expression between environmentally-challenged IEM and control groups could point to (mal-)adaptations in modifier genes. Since particularly newborn and children with IEMs can present with very severe symptoms, it is very important to understand the etiology of disease in IEMs. Additionally, the study of IEMs may give insights in mechanisms that are also relevant for acquired metabolic disease. On the other hand, research in acquired metabolic disease can aid in understanding IEMs. Thus, methods to study both types of disease would include the use of omics approaches and computational modeling. In fact, newborn screening efforts use MS-based techniques for targeted metabolomics, notably for the measurement of plasma amino acid, plasma acyl-carnitine and urine organic acid profiles. This has greatly increased the number of IEMs discovered in the last 20 years [6,10,18,134]. This also led to the identification of weaker mutations for which it was unclear if they give rise to disease. For example, after the introduction of newborn screening for mFAO deficiencies, several variants of the *ACADM* gene were discovered with more than 10% residual activity, for which the need for treatment is disputed [134]. This would be in line with *ACADM* SNPs that associate with C8-carnitine levels in healthy individuals [18,133].

Many metabolites that play a role in acquired metabolic diseases have also been identified as key modifiers in IEMs. These include NAD^+, amino acids, and coenzyme A. NAD^+ has been

implicated in IEMs because of its ability to activate sirtuins (SIRTs) [135–137]. SIRTs can deacetylate and deacylate proteins, including enzymes, which may result in changes in the catalytic properties of enzymes [135–142]. For example SIRT3-catalyzed deacetylation of long-chain- and very-long-chain acyl-CoA dehydrogenases (LCAD and VLCAD, respectively) and 3-Hydroxy-3-Methylglutaryl CoA Synthase 2 (HMGCS2) oxidation increases the activity of these enzymes [139,140]. HMGCS2 is involved in ketogenesis. SIRT3 also deacetylates MCAD at the same conserved lysine residue near the active site that is acetylated in VLCAD and LCAD [140]. To our knowledge it has, however, not been investigated whether this affects the catalytic capacity of MCAD. The role of SIRTs and NAD^+ in IEMs makes them potential therapeutic targets [137,143,144]. Altered amino acid levels have also been reported in IEMs other than the aminoacidopathies. Coenzyme A, which is also a cofactor in several IEMs has recently gained considerable attention as a modifier in IEMs, either via its free form (CoASH) or its esterified form (CoA ester) [59,60] .

Computational modeling and omics in IEMs

Computational modeling, in combination with the use of omics techniques would also be an important tool in order to gain insight into the mechanisms underlying disease presentation in IEMs. Indeed, a Recon 1-derived [145] genome-scale stoichiometric model of the human small intestinal epithelial cells correctly predicted the known biomarkers in 72% of the 36 simulated IEMs [146], while a model of the human hepatocyte correctly predicted about half of the 38 simulated IEMs [99]. After our group curated the flavoproteome in Recon2.2, the model reached 74% accuracy in biomarker prediction for 45 flavoproteome-related IEMs [112]. Computational dynamic modeling of IEMs is rare. Our group has, however, generated a dynamic model of mitochondrial fatty-acid oxidation which could predict the biochemical phenotype of MCADD and multiple acyl-CoA dehydrogenase deficiency [104,107,147]. To model metabolic adaptations in IEMs and even the phenotype of individual patients, the incorporation of omics data will be important. It has been proposed that a combination of high-throughput omics analysis in combination with computational modeling could provide for faster and more accurate diagnoses and subsequent treatment [148].

Mitochondrial fatty-acid β-oxidation (mFAO) and mFAO deficiency

From the previous sections it is evident that mitochondrial fatty-acid oxidation is an important pathway in both acquired and inborn metabolic disease. Studying mFAO could therefore reveal valuable insights into the mechanisms underlying both types of diseases.

Mitochondrial fatty-acid oxidation, more specifically mitochondrial fatty-acid β-oxidation is the cyclic oxidation of activated fatty acids (acyl-CoA esters) to acetyl-CoA. The “β” in “β-oxidation” relates to the fact that the bond between the α (the second carbon) and β carbon (third carbon) of the activated fatty-acid is broken during each round of the oxidation cycles, thereby generating acetyl-CoA and a shortened acyl-CoA ester. mFAO is particularly important during prolonged periods of fasting or high energy demand, such as exercise and cold exposure. In these conditions, mFAO in the liver plays a prominent role by providing ATP for gluconeogenesis and precursors for ketogenesis. This enables the liver to provide the peripheral tissues with glucose and ketone bodies as fuel. We will therefore look into hepatic mFAO biochemistry in more detail (Figure 1).

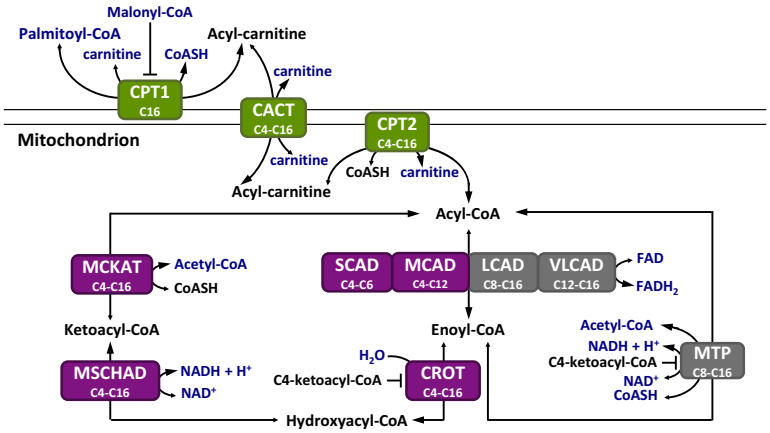

Figure 1. Schematic representation of the rodent mFAO pathway. Green: enzymes participating in the carnitine cycle; purple: enzymes participating in the short-chain branch (*i.e.* the CROT-M/SCHAD-MCKAT branch); grey: enzymes participating in the medium-and long-chain branch (*i.e.* the MTP branch). All processes are reversible and the size of the arrowheads indicates the net direction of the reactions.

Fatty acids originating from the white adipose tissue (WAT) typically have a chain length of 16 or 18 carbon atoms. They are taken up into the liver and activated by condensation with CoASH. This activation is catalyzed by acyl-CoA synthetases or fatty acid transfer proteins (FATPs) and generates acyl-CoA esters [149,150]. The acyl-CoA esters are subsequently taken up into the mitochondria for oxidation to acetyl-CoA. The acetyl-CoA can subsequently be oxidized for ATP production or used as precursor for ketogenesis. Uptake of acyl-CoA esters into the mitochondrial matrix occurs via the carnitine shuttle. This shuttle consists of the enzymes carnitine palmitoyltransferase I (CPT1), Carnitine-acyl-carnitine translocase (CACT), and carnitine palmitoyltransferase II (CPT2). Herein, the cytosolic acyl-CoA is converted to acyl-carnitine, by consuming L-carnitine and releasing CoASH. The cytosolic acyl-carnitine is subsequently translocated into the mitochondria through CACT in exchange for mitochondrial L-carnitine. Once in the mitochondria, the acyl-carnitine is converted back to acyl-CoA by CPT2. The acyl-CoA molecule subsequently undergoes fatty-acid beta-oxidation. Mitochondrial fatty-acid beta-oxidation (mFAO) occurs in repetitive four-reaction cycles (Figure 1). In each round, the acyl-CoA ester is shortened by two carbon atoms and one acetyl-CoA is produced [151–153]. Different cycles are catalyzed by the same set of enzymes. This means that each mFAO enzyme catalyzes the conversion of fatty-acid derivatives of a range of chain lengths [108,153]. Enzymes that catalyze the conversion of multiple substrates are called promiscuous. Since each mFAO enzyme is promiscuous, the different substrates and resulting products compete for binding to the active site. There are nevertheless different isoenzymes for each reaction. The first reaction of each cycle is catalyzed by different acyl-CoA dehydrogenase isoenzymes: the Very-Long-, Long-, Medium-, and Short-Chain Acyl-CoA Dehydrogenases VLCAD, LCAD, MCAD, and SCAD) (ACADs) (E.C. 1.3.99.3 and E.C.1.3.8.1). Each of these isoenzymes accepts a range of acyl-CoA substrates of different, overlapping chain lengths. Rodents express all four acyl-CoA dehydrogenase isoenzymes in the liver, while in human liver LCAD is lowly expressed [80] and of limited importance for hepatic mFAO [153]. In rodents, chain-length specificities of the ACADs are 12-16, 8-16, 4-12 and 4-6 carbon

atoms for VLCAD, LCAD, MCAD and SCAD, respectively, while in humans the chain-length specificities are 6-16, 4-16 and 4-6 for VLCAD, MCAD and SCAD, respectively. Since the isoenzymes have overlapping chain-length specificities, they compete for their substrates and products. Together with the cyclic nature of the pathway, the vast biochemical competition makes the mFAO biochemically particularly complex. The product of the acyl-CoA dehydrogenases is enoyl-CoA (Figure 1). Enoyl-CoA esters of 8 or more carbon atoms can be further metabolized by a single enzyme with 3 activities Mitochondrial Trifunctional Protein (MTP). I refer to this as the medium-and long-chain or MTP branch of the mFAO pathway. Alternatively, the enoyl-CoA ester can undergo further chain-shortening catalyzed by 3 separate enzymes: enoyl-CoA hydratase or crotonase (CROT, E.C. 4.2.1.74 and E.C. 4.2.1.150), hydroxyacyl-CoA dehydrogenase (M/SCHAD, E.C. 1.1.1.211 and E.C. 1.1.1.35) and medium-chain 3-keto-acyl-CoA thiolase (MCKAT, E.C. 2.3.1.16)), respectively. Acyl-CoA esters of all chain lengths can be oxidized via this branch. I refer to this as the short-chain branch or the CROT-M/SCHAD-MCKAT branch. The reactions catalyzed by M/SCHAD and MCKAT require the coenzymes NAD^+ and CoASH, respectively. M/SCHAD oxidizes hydroxyacyl-CoA to ketoacyl-CoA with NAD^+ as the electron acceptor. This reaction is reversible and operates close to equilibrium [108] due to its high catalytic capacity. It has a very small equilibrium constant ($2.17 \cdot 10^{-4}$) and therefore requires a very low product over substrate ratio to run in the forward direction. In the last reaction of the cycle, catalyzed by MCKAT, CoA is consumed and ketoacyl-CoA is split into an acyl-CoA ester that is two carbon shorter and acetyl-CoA. MCKAT has a high equilibrium constant of 10^3 [153]. This allows to maintain low ketoacyl-CoA concentrations. Notably, CROT, M/SCHAD and MCKAT also catalyze the final three reactions of the catabolism of isoleucine: together, these enzymes convert the isoleucine catabolism intermediate metabolite tiglyl-CoA (i.e. 2-methylbut-2-enoyl-CoA) to 2-methyl-3-hydroxybutyryl-CoA, subsequently to 2-methylacetoacetyl-CoA and then to propionyl-CoA and acetyl-CoA [154–157]. This results in extra substrate competition for these enzymes.

Another complexity in the mFAO pathway is subcellular localization of the enzymes. While CPT1, CACT, CPT2, VLCAD and MTP are located at the inner mitochondrial membrane, LCAD, MCAD, SCAD, CROT, M/SCHAD, and MCKAT are usually considered soluble and localized in the mitochondrial matrix [153]. However, experimental evidence in rat liver mitochondria has shown that LCAD, MCAD and SCAD can also be physically associated to the membrane-bound oxidative phosphorylation (OXPHOS) supercomplexes [158]. It was suggested that their intracellular localization may be condition dependent [158]. CROT, M/SCHAD and MCKAT have also been shown to bind directly or indirectly to the mitochondrial membrane (with substantial C4 activity at the membrane for all these enzymes) [159]. Interestingly, substantially higher respiration-linked oxidation of butyryl-CoA, butenoyl-CoA and hydroxybutyryl-CoA has been reported in gently-disrupted compared to completely-disrupted mitochondria [160]. Furthermore, physical associations between M/SCHAD and NADH dehydrogenase (Complex I), M/SCHAD and the ketogenesis enzyme HMGCS2 (Complex I) and between MCKAT and the acetyl-CoA-consuming TCA cycle enzyme citrate synthase have been demonstrated [161–163]. This suggests an efficient mechanism for the reoxidation of NADH, the regeneration of CoASH from acetyl-CoA by citrate synthase or the ketogenic pathway for instance and removal of C4-ketoacyl-CoA into ketogenesis. Thus, potentially mFAO soluble enzymes may spatially organize in such a manner that higher fatty-acid oxidation rates can be achieved, particularly in conditions of high-energy demand.

mFAO deficiency

Pathological inborn errors have been identified for all enzymes of the mFAO pathway [153,164]. If untreated, mFAO-deficient individuals run a risk of severe, life-threatening hypoketotic hypoglycemia during fasting or energy-demanding conditions. VLCAD, MCAD, and SCAD can in principle take over each other's role, due to their overlapping substrate specificities [109,153], albeit with lesser affinity and at the expense of capacity for their own function. Furthermore, a deficiency of VLCAD inhibits mFAO already in the beginning, while in MCAD deficiency and SCAD deficiency the first cycles of the β-oxidation can proceed normally. Thus, when MCAD or SCAD are deficient, some Gibbs energy can still be harvested from a partial β-oxidation. This may explain why SCAD-deficient (SCADD) children, who would only be deficient in the last mFAO cycle, often have only minor symptoms [153] and why the severity of disease presentation generally increases from SCADD to MCADD to VLCADD [153]. Based on reported clinical presentation, MCADD may be regarded as less severe than VLCADD and more severe than SCADD [153,165]. VLCADD has been associated with hypoketotic hypoglycemia, cardiomyopathy, cardiac arrythmias and conduction defects, skeletal myopathy and rhabdomyolysis. MCADD has been predominantly associated with hypoketotic hypoglycemia. SCADD has no clear pathological phenotype and is generally regarded as a deficiency with questionable clinical significance [153,165,166]. However, as previously indicated for IEMs in general, there is a large heterogeneity in disease presentation between mFAO-deficient individuals with the same enzyme deficiency, and even with the same genetic mutation [134,153,165,167–171].

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency

MCAD deficiency (OMIM 201450) is the most common mFAO defect, with an observed prevalence of up to 1 in 6600 newborns [11,130]. In untreated individuals MCADD has a disease severity that ranges from completely asymptomatic to fatal. Apart from having a clinical relevance in its own right, MCADD may therefore also offer insights in the modifying factors of mFAO function. This would be important for understanding other mFAO deficiencies and metabolic disease in general.

Individuals homozygous for the loss-of-function c.985A>G mutation in the *ACADM* gene, encoding MCAD, account for 77% of the cases [134]. This mutation causes a change from lysine to glutamate at position 304 (p.K304E) in the mature MCAD. This results in impaired protein folding, decreased protein stability and severely impaired MACD enzyme activity [172–175]. Children in this group run a severe risk of life-threatening low blood glucose and ketone bodies concentrations (hypoketotic hypoglycemia) [153,164]. Additional clinical symptoms include overweight, muscle pain, muscle fatigue, a lowered exercise tolerance, hepatomegaly, temperature instability, hypothermia, drowsiness or lethargy that may develop into coma or even sudden death [130,131,165]. Biochemically, MCADD is characterized by accumulation of dicarboxylic acids, acyl-glycines and acyl-carnitines of carbon-chain length C6-C10 and a higher ratio of C8 over C10 chains in plasma and urine [165,170,176–183]. MCADD individuals with a residual MCAD activity higher than 10 % of controls showed a milder biochemical and clinical phenotype [134]. However, not all variability between individuals can be explained by residual enzyme activity. Even a subset

of children homozygous for the c.985A>G mutation remains asymptomatic throughout life [11,130,131,134,171,184–187]. Notably, MCAD-deficient children oxidize medium-chain fatty acids normally under fed conditions [188] and they are able to fast for 18-24 hours [131,153,189,190]. The extent to which the youngest MCADD infants (0-2 years old) are able to fast is, however, currently unclear. It is currently thought that specifically a combination of prolonged fasting and an additional trigger, such as airway or gastrointestinal infections, alcohol use and cold-exposure, increase the risk of metabolic crisis and low blood ketone and glucose levels [130,164,169,183,191]. This shows that the etiology of the disease presentation is clearly not completely understood.

Mouse models of mFAO deficiency

To understand the etiology of mFAO deficiencies, several mouse models have been generated, including a VLCAD-KO, LCAD-KO, MCAD-KO and SCAD-KO mice [153]. In these mouse models, the LCAD-KO mouse model has the most severe phenotype, with hepatosteatosis, cardiac hypertrophy and dysfunction, triglyceride and ceramide accumulation, and fasting-induced hypoketotic hypoglycemia [153]. The LCAD-KO mouse is considered a closer representation of the human VLCAD deficiency than the VLCAD-KO mouse model. As indicated above, in rodents VLCAD catalyzes the C16-C12 dehydrogenation while LCAD catalyzes a broader range of C16-C8. In contrast, in humans the concentration of LCAD is very low and human VLCAD catalyzes C16-C6 dehydrogenation. Disease severity in ACAD-KO mice is highest in LCAD-KO mice, followed by MCAD- and SCAD-KO mice and subsequently VLCAD-KO mice [153]. It seems that VLCAD is more or less dispensable in mice, due to the presence and broad specificity of LCAD. All the ACAD-KO mice have been shown to be cold-intolerant [153,192–194]. Even in the relatively mild VLCAD-KO mouse [195] fasting in combination with cold exposure resulted in lower blood glucose concentrations than in WT mice [196–198].

To date, MCAD-KO mouse models of two different backgrounds have been generated [109,192,199]. Initially, an MCAD-KO mouse model was generated in a C57BL/6NTac & 129P2/OlaHsd mixed background [192]. The mouse showed several of the human MCADD-associated disease characteristics, including higher neonatal death rate, lower C8-acyl-CoA oxidation capacity, higher urinary medium-chain organic acid levels, and higher serum levels of medium-chain acyl-carnitine levels compared to wild type (WT) mice. 24 hours of fasting resulted in hepatosteatosis in the MCAD-KO but not in the WT mice. This corresponds to hepatic steatosis and higher blood NEFA levels seen in symptomatic MCADD patients, [130,164,169,189,191]. However, fasting did not induce blood glucose and ketone body level differences between the MCAD-KO and WT mice. Fasting in combination with cold exposure was lethal or led to unrecoverably low body temperatures in the KO, but not in the WT mice [192]. Hypoglycemia has, however, never been reported in MCAD-KO mice [80,192,199]. Hepatic microarray analysis was done in MCAD-KO mice that were either fasted for 12 hours or given lipopolysaccharide (LPS) to induce an acute-phase immune response. WT and MCAD-KO showed marked differences in expression of genes involved in glucose metabolism [199]. Several years later, the MCAD-KO mouse model was backcrossed to a C57BL/6J pure-background. Also, this pure-background MCAD-KO mouse showed higher medium-chain acyl-carnitine levels in serum and a lower C8-acyl-CoA oxidation capacity than the WT after 12 hours of fasting. In addition, the phenylproprionyl-CoA oxidation capacity was also lower in these KO mice compared to their WT littermates. The capacity of phenylproprionyl-CoA

oxidation is a diagnostic marker for MCADD in the clinic, because phenylproprionyl-CoA is exclusively oxidized by MCADD, while the endogenous acyl-CoA substrates can also be oxidized by the other ACADs. Furthermore, also in the C57BL/6J background the blood glucose and ketone body levels were similar in the MCAD-KO and WT mice after 12 hours of fasting [109]. These results may suggest that the MCAD-KO might not be a useful model for the acute hypoglycemia observed in symptomatic MCADD patients. However, this mouse might have not been sufficiently challenged to mimic the triggers that cause hypoglycemia in humans. During cold exposure glucose has not been reported [192].

MCAD deficiency *in silico*

The phenotypic heterogeneity, complexity, and interconnectivity of mFAO warrant a systems biology approach. This should help to unravel how the interplay between disease mutation, modifying factors, and additional triggers determine whether or not patients are at risk of hypoketotic hypoglycemia [18]. Genome-scale stoichiometric models of MCAD deficiency show promise for the identification of biomarkers [112,200]. However, the kinetic complexity of the mFAO pathway outlined above, cannot be properly represented in stoichiometric models.

The kinetic model developed by our group does not only contain the stoichiometry of the pathway, including the reactions in all the mFAO cycles, but also the promiscuity of the enzymes [108]. This promiscuity results in competition between all substrates and products of an enzyme, such that they inhibit each other's conversion competitively. Allosteric inhibition of CPT1 by malonyl-CoA was also incorporated as well as conservation of coenzyme A in the mitochondrial matrix [108]. The latter stems from the assumption that CoA biosynthesis takes place at a slower timescale than the mFAO. A consequence is that any sequestration of CoA in CoA esters leads to a corresponding drop in free CoASH. The kinetic parameters were taken from *in vitro* enzyme kinetics available in the literature. The model simulates the complete oxidation of cytosolic palmitoyl-CoA (C16-acyl-CoA) to acetyl-CoA. It has been experimentally validated *ex vivo* by measuring 30-minute acyl-carnitine time courses in isolated rat liver mitochondria [108]. Human and mouse versions of the model were constructed later, by fitting parameters to relevant mouse and human datasets [109].

Model simulations results showed that increasing palmitoyl-CoA concentrations did not lead to saturation of the mFAO flux, as would be expected, but led to a steep decline of the mFAO flux [108]. It was shown that this was caused by acyl-CoA ester accumulation and CoASH depletion and did not occur if the enzyme promiscuity was computationally removed [108]. In the murine version of the model deletion of MCAD made the model more susceptible to the flux decline at high substrate concentrations [109]. The flux decline has not yet been validated experimentally. The human version of the model has been related to patient data, however [109]. MCAD deficiency showed, not surprisingly, accumulation of medium-chain acyl carnitines, just as in patients [109,170,176–178,182,183]. What was more surprising was that the elevated levels of a broad range of acyl-carnitines in multiple acyl-CoA dehydrogenase, required the enzyme promiscuity [109]. The model reproduced the plasma acyl-carnitine profiles of two individual patients and correctly predicted their disease severity based on mFAO flux, but only if enzyme promiscuity was included [109].

Altogether, the results suggest that enzyme promiscuity makes the mFAO pathway intrinsically sensitive to substrate overload and that some mFAO deficiencies may aggravate

this risk. The mechanism underlying the relation between enzyme promiscuity and flux decline at high substrate concentrations had not been addressed before and will be analyzed in **Chapter 2** of this thesis. In **Chapter 4** I will address how surrounding pathways affect mFAO function. Both studies will help to understand how modifying factors may affect disease presentation.

Outline of this thesis: a systems medicine approach for MCAD deficiency

In my thesis I have taken a systems medicine approach to get a better insight in the complex etiology of MCADD. My overarching question has been what elicits hypoketotic hypoglycemia in some MCAD and other ACADD patients on the one hand, but also what protects them. I would like to understand on the one hand which modifying factors within and outside the pathway may push metabolism from an apparently healthy state into a detrimental trajectory towards hypoketotic hypoglycemia. On the other hand, I aim to identify the factors that under some conditions and/or in some patients keep the system safely away from this dangerous state. In an integrative approach, I take into account adaptations in gene expression as well as the role of surrounding pathways, nutrients, cofactors and internal metabolites. To this end I take a computational approach in **Chapters 2 and 5** and an experimental approach in **Chapters 3, 4, and 6**. In the first chapters I focus on rodents, but in **Chapter 5** I shift completely to human MCADD and other ACAD deficiencies. In this section I will briefly explain the approach that I will take in each Chapter.

In **Chapter 2** I will focus on the problem of the steep decline of flux as a function of an increasing concentration of the mFAO substrate palmitoyl-CoA. Although it has never been proven that this decline underlies the hypoketotic hypoglycemia in patients, it is a serious possibility. It had been shown before that the enzyme promiscuity was essential for this behavior, but the mechanism was not understood. All work in this chapter will be done in the previously described computational model of mFAO in rat mitochondria. I used essentially three methods of analysis. First, I will use Metabolic Control Analysis [115–118] to quantify which enzymes control the mFAO flux and how this changes as a function of the palmitoyl-CoA concentration. Second, I will look into the role of promiscuity of each individual enzyme separately, by either removing it in only the enzyme of interest, or removing it in all enzymes but the enzyme of interest. Together these two approaches will pinpoint which enzymes are the key players. Finally, I will inspect the time courses of the metabolites and enzyme rates in more detail. The time courses in combination with the regulatory strength of each metabolites (analyzed according to [120] will elucidate the causal chain of events that caused the flux decline.

In **Chapter 3** I will take the opposite approach and ask the question which genes or pathways could protect the metabolic network against hypoketotic hypoglycemia. To this end I will subject the MCAD-KO mice to mildly challenging conditions (12-24 hrs of fasting and different diets). Under these conditions the partial loss of mFAO capacity does not trigger hypoglycemia or other overt metabolic phenotypes. I will present a combination of RNASeq and bioinformatics to identify potential compensatory pathways. In view of the mild adaptations, I will not only use classical gene-set enrichment analysis, but also present a new approach to identify more subtle adaptations. Considering the importance of NAD^+ and CoA I will include tailor-made gene sets to study their role.

In **Chapter 4** I will take a more drastic approach by subjecting the mice to a combination of fasting and cold stress, which is a severely energy-demanding condition. Until now, this has been the only condition reported to cause a severe phenotype in MCAD-KO mice [192]. However, a biochemical characterization of MCAD-KO mice under cold stress, including blood glucose levels, has never been reported. In this chapter I will focus on the role of the liver. Interestingly, I managed to study the mice in a dynamic condition where body temperature was declining rapidly and I found for the first time that plasma glucose was lower in MCAD-KO mice than WT. I will show how comprehensive metabolomic analysis, including lipidomics, reveals strong metabolic adaptations as well as a key role of liver mFAO during cold stress.

In **Chapter 5**, I will switch to human mFAO. A limitation of the existing computational model is that it simulates mFAO in isolation. In reality mFAO depends strongly on the activities of surrounding pathways. In this chapter I will revisit the conversion of the rat model to a human version, making use of extensive literature data. More importantly, I will extend the model with pathways that affect the availability of CoASH. The resulting model is almost twice as large as the original model. I will test various existing and hypothetical interventions to get insight in the strength of potential modifying factors and treatment options.

In **Chapter 6**, I will study skin fibroblasts derived from symptomatic and asymptomatic MCADD patients *in vitro*. As previously indicated, within the subpopulation of individuals with a homozygous loss-of-function c.985A>G mutation in the MCAD-encoding ACADM gene, symptoms can range from fatal hypoketotic hypoglycemia early in life to a complete lack of symptoms throughout life [7–16]. The reason behind the heterogeneity in symptoms in this subpopulation is still unclear. Therefore, I will investigate whether there are metabolic or proteomic adaptations in cultured skin fibroblasts of MCADD individuals that distinguish symptomatic from asymptomatic MCADD individuals.

Finally, in **Chapter 7**, the General Discussion, I will evaluate my progress and discuss limitations and next steps. Specifically, I will discuss possibilities to link my systems medicine approach to patient data or *in vitro* studies based on patient-derived cells.

Summarizing, I will address the following questions in this PhD thesis.

1. What is the underlying mechanism that links enzyme promiscuity to the flux decline that is observed at high substrate concentrations in the rat computational model of mFAO? (**Chapter 2**)
2. Which metabolic pathways contribute to the robustness of MCAD-KO mice under fed and fasting conditions on a high- or low-fat diet? (**Chapter 3**)
3. How does hepatic metabolism respond to severe energetic stress in cold-exposed, fasted MCAD-KO mice? (**Chapter 4**)
4. How do the pathways surrounding the mFAO, notably those that affect CoASH, modulate the phenotype in a computational model of ACAD-deficient human cells and can be the impact of interindividual variation and various treatment options? (**Chapter 5**)
5. Do cultured fibroblasts of symptomatic or asymptomatic MCADD individuals show adaptations in their acyl-carnitine profiles or mitochondrial proteome? (**Chapter 6**)

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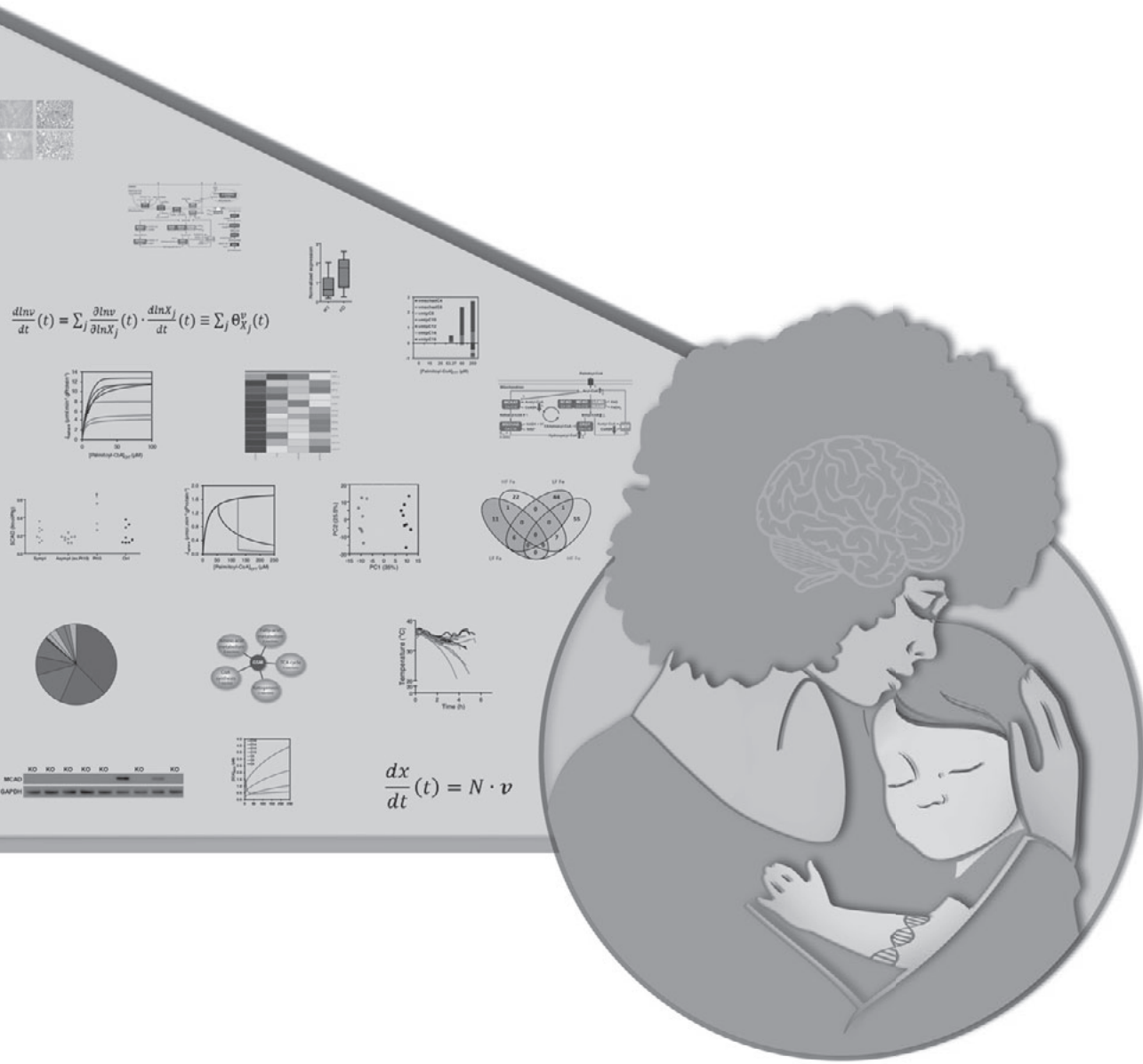
$\frac{dlnv}{dt}(t) = \sum_j \frac{\partial lnv}{\partial lnX_j}(t) \cdot \frac{dlnX_j}{dt}(t) \equiv \sum_j \theta^v_{X_j}(t)$
KO KO KO KO KO KO KO
MCAD
GAPDH
$\frac{dx}{dt}(t) = N \cdot v$