UNIVERZA V LJUBLJANI
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NEŽA PALIR

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MAGISTRSKI ŠTUDIJSKI PROGRAM LABORATORIJSKA BIOMEDICINA

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CHARACTERISTICS OF THE NEWLY DISCOVERED MUTATIONS RELATED TO MEDIUM-CHAIN ACYL-COENZYME A DEHYDROGENASE DEFICIENCY

LASTNOSTI NOVO ODKRITIH MUTACIJ POVEZANIH S POMANJKLJIVOSTJO ACIL-KOENCIM A DEHIDROGENAZE SREDNJE DOLGIH VERIG MAŠČOBNIH KISLIN

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The research reported in this thesis was carried out at the Department of Biochemistry and Human Biology and at the Metabolism and Genetics Group of the Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, University of Lisbon, Portugal under the working supervision and the co-mentorship of Assist. Prof. Dr. Maria de Fátima Vieira Ventura and the mentorship of Assoc. Prof. Dr. Matjaž Jeras.

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Statement

I declare that I have created this master thesis independently under supervision of Assist. Prof. Dr. Maria de Fátima Vieira Ventura.

Ljubljana, 2015 Neža Palir

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ABSTRACT

The medium-chain acyl-CoA (MCAD) deficiency is the most common inborn error of fatty acid oxidation. This autosomal recessive disease is characterized by lethargy, hypoglycemia, Reye-like syndrome, coma and death and has been a part of newborn screening in many countries for the past ten years due to a fairly simple preventive therapy. With the inclusion of this deficiency in the newborn screening programs many new sequence variants were identified, including the missense mutations encoding the p.G224R and the p.L238F enzymes, both discovered in The Netherlands.

Our study was set to determine the impact of newly discovered variants on yield and purity grade, oligomeric profile, enzyme activity, kinetic parameters, conformational stability, aggregation profile and aggregation kinetics, as compared to the wild-type MCAD and to p.K304E, being the most frequent variant in the clinical cases of the MCAD deficiency. It is extremely important to estimate the severity degree of a new genotype, because the implemented dietary treatment might as well be a risk for children suffering from MCAD deficiency who readily become overweight.

Our data suggest the need for preventive therapy and close monitoring of patients with mutations encoding the two newly discovered variants. While the p. G224R exerts severely low enzymatic activity with its residual activity being only 2% of that of the wild type, the p.L238F shows structural instability, evidenced by low expression yield due to its intracellular degradation and by the highest loss of the cofactor during the purification process from all of the hMCAD enzymes studied.

Studies clearly indicate a much higher prevalence of the disease after incorporating MCAD deficiency into the newborn screening program, which we believe should be also implemented in Slovenia, considering that the preventive therapy is simple and unexpressive, and that when necessary measures are not taken the clinical outcome can be catastrophic.

Altogether our data shed some additional light on the understanding of the molecular basis of the MCAD deficiency and may contribute to the development of new and effective therapeutic approaches, as well as to better foresee the prognosis of patients with this inherited metabolic disease.

KEYWORDS

Medium-chain acyl-CoA dehydrogenase (MCAD), Medium-chain acyl-CoA dehydrogenase deficiency (MCADD), flavin adenine dinucleotide (FAD), mitochondrial fatty acid β-oxidation (mFAO), misfolded protein, conformational disease.

POVZETEK

Pomanjkljivost acil-koencim A dehidrogenaze srednje dolgih verig maščobnih kislin (MCAD) je najpogostejša prirojena napaka v procesu oksidacije maščobnih kislin. Bolezen se deduje avtosomno recesivno in lahko povzroči vrsto različnih simptomov, kot so: letargija, hipoglikemija, simptomi podobni Rayevemu sindromu, koma in smrt. Otroci s pomanjkljivostjo MCAD pa so tudi nagnjeni k debelosti. Številne države so, predvsem zaradi izredno enostavne preventivne terapije, že vključile testiranje pomanjkljivosti MCAD med presejalne teste za novorojenčke. Pri tem so identificirali veliko novih mutacij, med njimi tudi dve, ki nosita zapis za proteina p.G224R in p.L238F in so ju odkrili na Nizozemskem.

V okviru magistrske naloge smo določili vplive mutacij na čistočo in donos rekombinantno pridobljenega proteina, njegov oligomerni profil, encimsko aktivnost, kinetične parametre, stabilnost terciarne strukture, profil agregacije in agregacijsko kinetiko, in sicer tako, da smo primerjali proteina p.G224 in p.L238F z divjim tipom in z encimom p. K304E, ki je prisoten v skoraj vseh kliničnih oblikah bolezni. Zelo pomembno je namreč določiti potencialno patogene posledice posamezne mutacije, saj se le na osnovi teh podatkov lahko izognemo nepotrebnemu dietnemu zdravljenju, ki med drugim vključuje tudi nočne obroke.

Pri proteinu p.G224R smo odkrili izredno nizko encimsko aktivnost, ki v primerjavi s divjim tipom znaša le 2%, pri p.L238F pa strukturno nestabilnost, ki se odraža predvsem v nizkem donosu med procesom pridobitve rekombinantnih proteinov in v največji izgubi kofaktorja med procesom osamitve proteina. Naši rezultati tako nakazujejo potencialno patogene vplive obeh mutacij in posledično potrebo po preventivni terapiji.

Prevalenca pomanjkljivosti MCAD se je po njeni vključitvi v presejalne teste za novorojenčke izkazala za višjo kot so sprva predvidevali. Zato menimo, da bi morali tudi v Sloveniji slediti trendu iz tujine in uvesti presejalno testiranje pri novorojenčkih tudi za to bolezen, še posebej zato, ker je njeno zdravljenje izredno preprosto in poceni, in ker lahko brez ustrezne preventivne terapije pride do katastrofalnih posledic.

Naši rezultati predstavljajo prispevek k razumevanju molekularnih osnov pomanjkljivosti MCAD in bi lahko prispevali k razvoju novih učinkovitih terapevtskih pristopov. Prav tako omogočajo tudi boljšo prognostično napoved za bolnike s to redno, razmeroma redko presnovno boleznijo.

KLJUČNE BESEDE

Acil-CoA dehidrogenaza srednje dolgih verig maščobnih kislin (MCAD), pomanjkljivost acil-CoA dehidrogenaze srednje dolgih verig maščobnih kislin (MCADD), flavinadenindinukleotid (FAD), mitohondrijska β-oksidacija maščobnih kislin (mFAO), napačno zvitje proteina, konformacijska bolezen.

ABBREVIATIONS

ACAD – acyl-CoA dehydrogenase

bp – base pairs

CACT - carnitine acylcarnitine translocase

cDNA – complementary DNA

CPT1 – carnitine palmitoyltransferase 1

CPT2 – carnitine palmitoyltransferase 2

DCPIP – 2,6-dichlorophenolindophenol

DLS – dynamic light scattering

E. coli – *Escherichia coli*

ETF – electron transfer flavoprotein

FAD – flavin adenine dinucleotide (oxidized)

$FADH_2$ – flavin adenine dinucleotide (reduced)

FT – flow-through

His – histidine

hMCAD – (human) medium-chain acyl-CoA dehydrogenase

HMW – high molecular weight

IMAC – immobilized-metal affinity chromatography

IPTG – Isopropyl β-D-1-thiogalactopyranoside

K_m – *Michaelis Constant*

KPi – phosphate buffer

LB – Luria Bertani

LCAD – long-chain acyl-CoA dehydrogenase

LCEH – long-chain enoyl-CoA hydratase

LCHAD – long-chain 3-hydroxyacyl-CoA dehydrogenase

LCKAT – long-chain 3-ketoacyl-CoA thiolase

MCADD – medium chain acyl-CoA dehydrogenase deficiency

mFAO – (mitochondrial) fatty acid β-oxidation

MCHAD – medium-chain hydroxyacyl-CoA dehydrogenase

MCKAT – medium-chain 3-ketoacyl-CoA thiolase

MTP – mitochondrial trifunctional protein

MS/MS – tandem mass spectrometry

MW – molecular weight

n – number of experiments

NADH – nicotinamide adenine dinucleotide

NBS – newborn screening

ON – overnight

PMS – phenazine methosulfate

PMSF – phenylmethylsulfonyl fluoride

RT – room temperature

SCAD – short-chain acyl-CoA dehydrogenase

SCEH – short-chain enoyl-CoA hydratase

SCHAD – short-chain hydroxyacyl-CoA dehydrogenase

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEC – size-exclusion chromatography

SIDS – sudden infant death syndrome

T_{agg} – temperature of aggregation

Ve – elution volume

VLCAD – very long-chain acyl-CoA dehydrogenase

V_{max} – maximal velocity

Vo – void volume

Wt – wild-type

1. INTRODUCTION

1.1. Fatty acid oxidation

Fatty acids, alongside with carbohydrates and amino acids, are responsible for maintaining energy homeostasis. Besides being a fuel for generation of energy, these substrates are also used as building blocks for the biosynthesis of (macro) molecules. Saturated and unsaturated fatty acids are degraded in the process of mitochondrial fatty acid β-oxidation (FAO) (1). This is an important metabolic pathway for numerous organisms, including humans. FAO is particularly relevant during endurance exercise or fasting, when glucose levels are low, as it becomes the most important energy generating pathway in organs, such as heart, liver and skeletal muscles (2).

While fatty acids of <20 carbon length are metabolized in mitochondria, the peroxisomal pathway is more relevant for degradation of longer chain fatty acids (2). The degradation of fatty acids begins after they enter the cell. Their uptake occurs primarily via membrane proteins, however, passive uptake probably also takes place. Once inside the cell, they are rapidly converted to acyl-CoAs. Since mitochondrial membrane is impermeable to acyl-CoAs, carnitine shuttle intervention, involving three consecutive steps, is required. In the first step, carnitine palmitoyltransferase 1 (CPT1), an enzyme associated with outer mitochondrial membrane, converts acyl-CoAs to acylcarnitines. These are then transported across the inner mitochondrial membrane via carnitine acylcarnitine translocase (CACT). During this process CACT exchanges acylcarnitine for a free carnitine originating from the inside of mitochondria. In the last step acylcarnitines are converted back to their acyl-CoAs counterparts by CPT2, an enzyme located on the inner face of the mitochondrial inner membrane (3). The resulting Acyl-CoA esters are then ready to undergo FAO (4, 5, 6). Both, CPT1 and CPT2 are primarily involved in the import of long-chain acyl-CoAs. Although not yet clarified, it is assumed that short- and medium-chain fatty acids can enter mitochondrial matrix directly. On the other hand, some recent discoveries show that carnitine shuttle can also be involved in the mitochondrial import of medium-chain fatty acyl units (7, 8).

In the course of FAO, acyl-CoA esters undergo four key enzymatic reactions, and, irrespective of their size, they are shortened for two carboxy-terminal carbon atoms which are released as an acetyl-CoA unit, every time one cycle is completed. During the first step of β-oxidation, the acyl-CoA ester is dehydrogenated to *trans*-2-enoyl-CoA, and then the

resulting double bond is hydrated, forming the L-3-hydroxy-acyl-CoA intermediate. This is subsequently dehydrogenated to 3-keto-acyl-CoA (the third step of FAO). Finally, thiolytic cleavage of 3-keto-acyl-CoA produces acetyl-CoA and a two carbon chain-shortened acyl-CoA that reenters the β-oxidation cycle (2, 3, 9). In each cycle one molecule of nicotinamide adenine dinucleotide (NADH) and one molecule of flavin adenine dinucleotide ($FADH_2$) are formed. Both molecules are oxidized within the electron transport chain, thus releasing energy in the form of ATP. The ATP yield increases when the resulting acetyl-CoA exiting the FAO pathway enters the Krebs cycle and the fatty acid metabolism in mitochondria is completed (3). Besides obvious and fundamental contribution of fatty acid metabolism to normal body function, the formation of β-hydroxybutyrate and acetoacetate (ketogenesis), the so-called ketone bodies, from acetyl-CoA in liver, is also very important when glucose levels in blood are low. Namely, ketone bodies are important fuel for most of the tissues, including the brain (10).

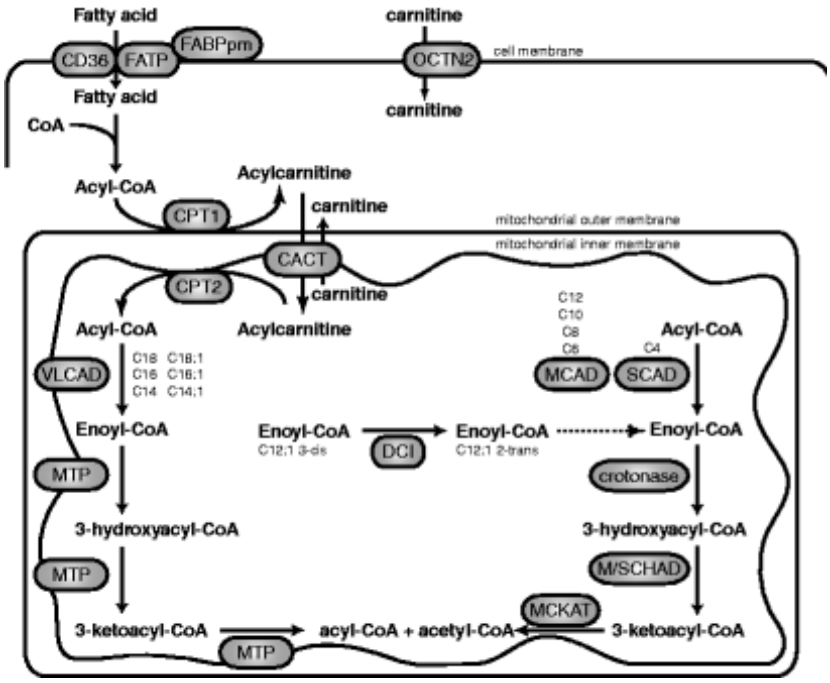

Figure 1: Mitochondrial fatty acid β-oxidation in humans (3). FATP: fatty acid transport proteins, CD36: fatty acid translocase, FABPpm: fatty acid-binding protein, OCTN2: Na^+-dependent organic cation transporter, CPT1: carnitine palmitoyltranferase 1, CACT: carnitine-acylcarnitine translocase, CPT2: carnitine palmitoyltransferase 2, VLCAD: very long chain acyl-CoA dehydrogenase, MCAD: medium chain acyl-CoA dehydrogenase, SCAD: short chain acyl-CoA dehydrogenase, MTP: mitochondrial trifunctional protein, M/SCHAD: medium/short chain hydroxyacyl-CoA dehydrogenase, MCKAT: medium chain 3-ketoacyl-CoA thiolase.

For each of the four steps that complete FAO, different isoforms of a particular enzyme exist, possessing different substrate specificities. Therefore, each isoenzyme used in a specific step depends on the length of the acyl CoA ester undergoing its metabolic pathway (9). Once inside the mitochondria, long-chain acyl-CoAs will first be metabolized by the very long-chain acyl-CoA dehydrogenase (VLCAD) and the mitochondrial trifunctional protein (MTP). The latter exerts hydratase (long-chain enoyl-CoA hydratase- LCEH), dehydrogenase (long-chain 3-hydroxyacyl-CoA dehydrogenase- LCHAD) and thiolase (long-chain 3-ketoacyl-CoA thiolase- LCKAT) activities. Both, VLCAD and MTP are proteins, located on the inner mitochondrial membrane, unlike the enzymes involved in the metabolism of medium and short chain acyl-CoAs, which are situated within the mitochondrial matrix. Medium-chain acyl-CoA dehydrogenase (MCAD) and short-chain acyl-CoA dehydrogenase (SCAD) are involved in the first step of the medium and short chain acyl-CoAs metabolism. The second step depends on the activity of the short-chain enoyl-CoA hydratase (SCEH), also called crotonase, and the third one on the performance of the medium- and short-chain hydroxyacyl-CoA dehydrogenase (M/SCHAD). The cycle is finally completed by the medium-chain 3-ketoacyl-CoA thiolase (MCKAT) (3, 9).

So far 25 enzymes and transport proteins were identified to be involved in the FAO and defects in 22 of them were described as causes of disease in humans. Out of all these defects, the MCAD deficiency was recognized as the most common, with the prevalence at birth between 1:10.000 and 1:27.000 in European countries (7, 11).

1.2. Medium-chain acyl-CoA dehydrogenase (MCAD)

The medium-chain acyl-CoA dehydrogenase (MCAD) is a member of the acyl-CoA dehydrogenase (ACAD) family, a group of enzymes also known as flavoenzymes. They contain flavin adenine dinucleotide (FAD) as a prosthetic group (3). There are so far 9 known members of this family, with 5 of them: SCAD, MCAD, LCAD, VLCAD and VLCAD2, being involved in FAO. As mentioned earlier, MCAD is soluble in mitochondrial matrix and consequently not associated with the membrane (12). This flavoenzyme, required for the initial step of the medium-chain fatty acids metabolism acts upon substrates consisting of chain lengths between 4 and 12 carbons. The first dehydrogenization step yields unsaturated *trans*-2-enoyl-CoA and $FADH_2$, after two hydrogen atoms are released from α, β-carbon atoms (11, 13, 14). The electrons produced

during this step are transferred to the respiratory chain, consisting of a sequence of redox reactions involving the FAD group, electron transfer flavoprotein (ETF) and the ETF dehydrogenase, which finally donates electrons into the respiratory chain via ubiquinone (3).

1.2.1. The medium-chain acyl-CoA dehydrogenase is encoded by the *ACADM* gene

The *ACADM* gene is located in the short (p) arm of the chromosome 1, at position 31, between the base pair 75 724 346 to the base pair 75 763 678 (15). The gene is organized in 21 exons, encoding a 421 amino acid protein in its precursor form (16).

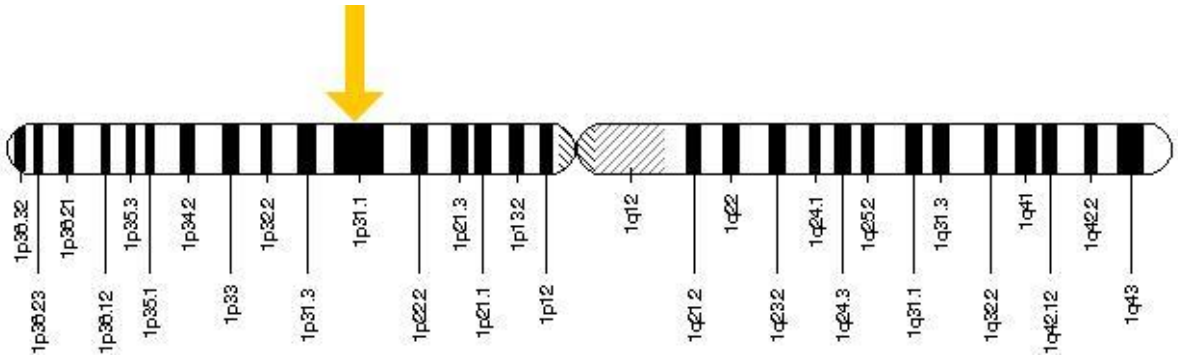

Figure 2: Cytogenetic location of ACADM gene (1p31) on the chromosome 1 is indicated with the yellow arrow (15).

1.2.2. The medium-chain acyl-CoA dehydrogenase (MCAD) structure

This enzyme (MCAD) has been the most extensively studied of all 9 members of the ACAD family, both biochemically and structurally, due to the highest prevalence of the MCAD deficiency out of all inherited fatty acid oxidation disorders. In the structural studies the overall diameter of about 90 Å was determined for its tetrameric structure. Each of its four subunits is formed of three, approximately equally sized domains: the N-terminal α-domain (residues 1-129), the β-domain (residues 130-239) and the C-terminal α-domain (residues 240-396). The core of the tetramer is composed of densely packed α-helices that represent basic structures of the N- and C- terminal domains, whereas the surface of the molecule consists of the middle domain, comprising two orthogonal β-sheets. The catalytic center, i.e. the most important functional part of each enzyme, consists of the substrate and the cofactor FAD binding sites in the MCAD, is mainly formed by the interface between the β-domain and the C-terminal α-domain.

In order to form the mature tetrameric structure of the enzyme, monomers first have to form a dimer. This is accomplished by extensive interactions between the two monomers, which also involve the FAD binding site. The MCAD molecule is actually known to be a dimer of dimers, as its tetrameric structure is formed by stabilization of helix-helix interactions between the two dimers (13, 17).

When human MCAD is synthesized in the cytosol, it is formed as a precursor protein, consisting of 421-amino acid (46,6 kDa) and a leader peptide. It is as such imported into the mitochondrial matrix, where the leader peptide is cleaved at its N-terminus, resulting in a 396 amino acid long protein with a molecular weight of 43,6 kDa (18). The enzyme then needs to be further assembled to reach the mature homotetramer structure. This is achieved by sequential binding of the enzyme to hsp70 and hsp60 mitochondrial chaperones, thereby completing its folding into a native, biologically active form (12, 18).

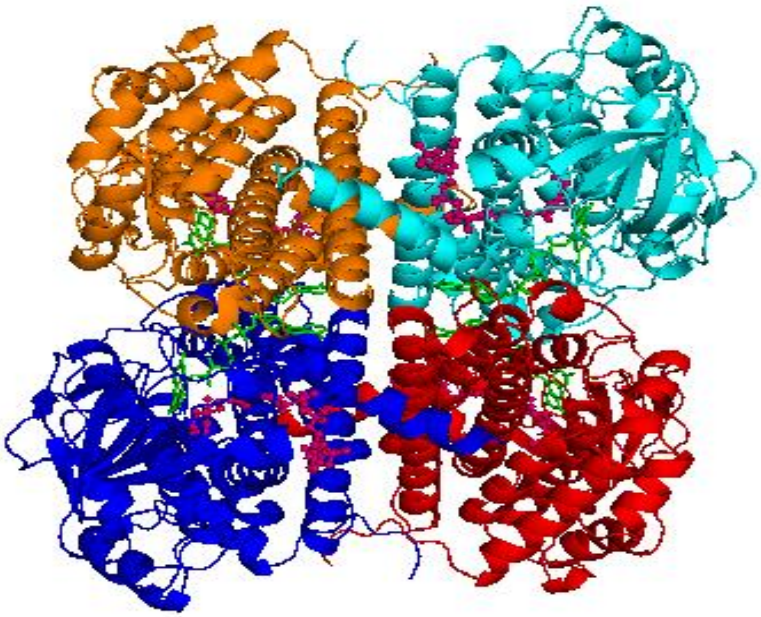

Figure 3: The structure of human MCAD wild-type tetramer (13). The cofactor (FAD) and the substrate (thio-C8-CoA) (stick representation): FAD – green and thio-C8-CoA – pink, respectively. Figure was created with PyMOL™ from PDB 1UDY.

1.2.3. The mechanism of MCAD action

The substrate binding cavity of the MCAD molecule lies between the flavin ring of the FAD cofactor and the carboxyl group of the glutamic acid residue at position 376 (Glu376), which is an ideal arrangement for dehydrogenation reaction. The oxygen of the substrate's carbonyl group (thioester) gets hydrogen bonded to both, the 2'-OH of the

ribityl chain of FAD and the amide nitrogen of the G376 residue. These interactions allow the substrate to precisely bind to the active site of the enzyme and establish optimal catalytic conditions. Mechanistically, the reaction involves proton withdrawal from the α-carbon of the substrate by the Glu376 residue, as well hydride ion transfer from the β-carbon to the FAD (13, 19).

When the substrate is absent, the catalytic cavity is occupied by well-organized water molecules that completely saturate the empty space. Water molecules also play an important role of occupying the remaining space when substrate is present, but only in case when its length is less than the size of the C12-CoA substrate. Namely, this substrate represents a maximum size that can be accommodated by the MCAD molecule. Therefore when C12-CoA substrate occupies the active site all water molecules are removed from the binding cavity. This is the reason why MCAD can catalyze fatty acids with different chain lengths, all the way from C4- to C12-CoA, with the C8- and C10-CoA inducing the highest rate of its enzymatic activity (13).

1.3. The MCAD deficiency (MCADD)

The MCAD deficiency (MCADD), first identified in 1982, is the most common inborn defect of fatty acid oxidation and has therefore been included into the newborn screening programs (NBS) in many countries during past few years (20-23). After MCADD became part of NBS, its estimated birth prevalence increased for 2- to 3-fold (approximately 1:15.000), in comparison to that based only on the number of patients diagnosed following clinical presentation of the disease. This phenomenon was especially noticeable within populations of mostly European origin (24, 25, 26). Namely, after inclusion of MCADD into NBS in Korea and Japan, where around 100.000 newborns were screened, only 2 cases were discovered and it became clear that this metabolic disorder is much less common in populations of the non-European descent (27, 28).

MCADD is a consequence of reduced MCAD activity, causing a variety of complications, such as hypoketotic hypoglycemia, hypotonia, lethargy and vomiting, which can progress to seizures and encephalopathy, severe metabolic crisis with a possible hepatic failure and Reye-like syndrome leading to coma and death (25, 29).

However, some individuals carrying mutations in the *ACADM* gene can be asymptomatic their whole lives while others become affected, which reflects in a variety of symptoms these patients can experience. Usually, clinical presentation of the disease is triggered by

situations requesting high energy demands, various illnesses, prolonged fasting or childhood immunization (25). Even though some individuals with MCADD do not face any related problems in their lives, this disease can be extremely dangerous when patients are not diagnosed in time and properly treated, as many reports have shown their high morbidity and mortality. Some studies even report that death occurred in up to 25% of undiagnosed children which experienced acute metabolic crises and that around 40% of those who survived had permanent neurocognitive impairments (17, 25).

Even though, no cure for the MCADD has been discovered yet, this deficiency has been included into NBS due to a widely accepted belief, that its clinical outcome can be prevented with: a strict diet, low in fat and high in carbohydrates, oral administration of L-carnitine and energy drinks, all providing sufficient levels of blood glucose, as well as with special attention from parents and doctors during the periods of patients' anorexia and infections (25).

1.3.1. Biochemical diagnosis of MCADD

To confirm the presence of MCADD, characteristic metabolites (e.g. acylcarnitines) have to be detected, as it presents clinical signs that are also common to other FAO defects. For this purpose several countries, such as some European, Australia and USA, which have already introduced MCADD to their NBS, use tandem mass spectrometry (MS/MS) as the most sensitive detection method, also allowing blood spot samples taken from newborns to be used for establishing accurate diagnosis.

MCADD is biochemically associated to changes in free fatty acids profiles in plasma, as well as organic acids in urine. Due to the enzymatic block, medium-chain acyl-CoA esters accumulate in mitochondria and can only be metabolized via alternative pathways which lead to the production of medium-chain dicarboxylic acids, ranging from C_6 (adipic acid) to C_{12} (dodecanedioic acid), or to corresponding (ω-1)-hydroxy acids. An alternative metabolic path for clearing accumulated esters is their conjugation with glycine, carnitine and glucuronide and their subsequent elimination from cells in forms of acylglycines (especially hexanoylglycine) that are excreted in urine, or acylcarnitines (C_6-C_{12}) which circulate in plasma (30). The levels of C8-acylcarnitine, the ratios of C8/C10 and C8/C2, and urinary levels of hexanoylglycine, have been recognized as the most reliable MCADD biomarkers (31). It is very important to establish such detection system, which will recognize individuals with *ACADM* gene mutations as MCADD patients, even if they are

still in the asymptomatic stage. It was shown that the acylcarnitine plasma profile can indicate abnormalities in the FAO regardless of the clinical status and before the manifestation of any signs or symptoms, especially when using the detection method of choice, e.g. the highly sensitive MS/MS (30). However, this analytic approach has to be completed by a whole-cell oxidation study, demonstration of patient's reduced MCAD activity in lymphocytes or fibroblasts or by molecular analysis of the *ACADM* gene in order to distinguish between MCADD and other FAO defects. Molecular analysis of *ACADM* gene mutations has become the most frequently used method due to its convenience, as it is relatively rapid and analyses can be performed using filter paper blood spot samples (32).

1.3.2. Molecular analysis of MCADD

More than 100 mutations have been identified so far within the *ACADM* gene, located, as mentioned earlier, on chromosome 1, at position 31. These mutations result in MCADD, which is inherited in an autosomal recessive trait (33). Several reports show that the most common mutation is the c.985A>G, i.e. the adenine to guanine (A→G) transition at the coding position 985 of the MCAD complementary DNA (cDNA), resulting in the substitution of glutamate for lysine at the position 329 of the precursor protein (p.K329E or Lys329Glu), corresponding to the position 304 of the mature protein (p.K304E). It has been reported that 80% of patients diagnosed for MCADD after clinical presentation of the disease have been identified homozygous for the c.985A>G mutation, while another 18% of clinically confirmed patients were heterozygous, carrying one allele with the most common mutation and the other one with a rare, in most of the cases, new *ACADM* mutation. Therefore, the c.985A>G mutation has been linked to almost all clinical manifestations of MCADD and is considered the most pathogenic mutation of the *ACADM* gene within the population of European descent, whereas other genetic variations may predominate within the non-European populations (17, 33). Also in newborns of European descent many new *ACADM* mutations have been identified due to strict implementation of NBS, resulting in severe decline in c.985A>G (p.K304E) mutation prevalence (30-80%), showing a considerably wide genotypic diversity (34). Furthermore, a new term "mild MCAD deficiency" was established, representing a group of newly discovered mutations, which are associated with only milder MCAD protein folding defects that so far have not been linked to any clinical manifestation. In the process of

NBS, the c.199T>C has been identified as the second most frequent mutation, resulting in a p.Y42H enzyme variant. In the majority of patients the p.Y42H protein, like many other mutated MCAD variants, such as p.G224R and p.L238F, is found together with the most common p.K304E mutation (17). Such patients are therefore mostly compound heterozygotes, with the p.K304E being always present in their cells. Because of this, p.K304E, together with p.G224R and p.L238F, represent the main focus of our study.

1.3.3. Genotype-phenotype correlations in MCADD

Patients, who carry the same MCAD mutation, even if they are members of the same family, can present significant differences in clinical phenotypes (22). It is a known fact that patients with the p.K304E mutation can go through their whole lives without metabolic crises, while others can suffer from variety of symptoms, many of them being potentially fatal (14). It is safe to assume that environmental factors, such as diet, stress and intercurrent illnesses have a huge impact on a course of this disease and it is no wonder that many reports claim that a consistent genotype-phenotype correlation does not exist in MCADD. However, it should be pointed out that the existing correlations between the *ACADM* genotype and enzymatic activity have already been observed, proving how important it is to further study this area and try to establish correlations between genotypes and metabolic and enzymatic phenotypes by *in vitro* testing of all MCAD mutant forms (16).

2. PURPOSE OF THE STUDY

Medium-chain acyl-CoA dehydrogenase deficiency has been extensively studied in most European countries, the United States of America and recently also in Japan and Korea, where only some individuals were diagnosed with this disease. By implementing MCADD in the NBS, many novel mutations and the resulting enzyme variants were identified, including p.G224R and p.L238F.

The purpose of this study is to determine functional and structural characteristics of new MCAD variants, identified in the Laboratory of Genetic Metabolic Diseases, Academical Medical Center, University of Amsterdam, in order to elucidate their pathogenic relevance.

The new enzyme variants will be compared to the human wild-type enzyme and p.K304E, the latter being the most common variant in MCADD. The p.K304E variant is associated with about 90% of all clinical presentations and is therefore the most widely studied. Furthermore, both novel mutations were identified in patients which were found to be compound heterozygous for the common c.985A>G (p.K304E) and a novel mutation.

Working plan:

a) Express and purify recombinant hMCAD proteins;

b) Characterize the structure, stability and catalytic function of recombinant hMCAD proteins by assessing:

- their yield and purity grade by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the Bradford method;
- their oligomerization profile by size-exclusion chromatography;
- their enzymatic activity and kinetic parameters by spectrophotometric method;
- their conformational flexibility by limited proteolysis, and
- their thermal stability by dynamic light scattering.

With this information gathered we aim to infer on the potential disease-causing effect of these novel mutations through a better understanding of the protein function and structure. The data obtained would allow us to better improve the prognosis associated with these novel mutations in order to evaluate the need for preventive therapy and enable a better monitoring of MCADD patients, especially those detected by NBS and those who are still in an asymptomatic phase.

3. MATERIAL & METHODS

3.1. Development of expression systems for recombinant hMCAD proteins

To produce considerable amounts of desired recombinant protein we needed a highly efficient expression system. In our case this was a heterologous expression vector, in which cDNA of the mature form of human MCAD was inserted. The final construct was then transferred to the host cell, i.e. the *Escherichia coli* (*E. coli*) strain BL21(DE3) (Invitrogen®, Carlsbad, EUA). Creation of the mutant protein expression vectors was based on the pETMCADd$_{Wt}$ plasmid, previously developed in the laboratory (Fig. 4) from a parental modified pET28(+) expression vector (Stratagene, EUA)) containing 2961 base pairs (bp). This plasmid was cloned with cDNA of the hMCAD wild-type (Wt) (kindly provided by P. Bross, University of Aarhus, Denmark) which holds a hexahistidine sequence (6xHis-tag) at the C-terminus. This sequence was included to enable subsequent protein purification by immobilized-metal affinity chromatography (IMAC) and protein detection by western blotting using antibodies against 6xHis-tag. The first step towards the expression of mutant hMCAD variants, pETMCADd$_{K304E}$, pETMCADd$_{G224R}$ and pETMCADd$_{L238F}$, was creation of suitable plasmids by site-directed mutagenesis reactions, using pETMCADd$_{Wt}$ plasmid (Fig. 4) as a template. This reaction was performed with the *NZY mutagenesis system kit* (NZYTech®, Lda. Portugal) or *QuickChangeII XL* (Agilent Technologies system, USA) and with oligonucleotides designed to mimic cDNA sequence of each specific mutant hMCAD (Annex 1), following the instructions of both manufacturers. According to the indications for each system, the mutated DNA was used to transform competent cells. They prom the resulting colonies, from which plasmid DNA was prepared on a small scale (*Mini-Prep*: ZR PlasmidMiniPrep Classic kit, Zymo Research®, USA). The quantity of DNA was determined at 260 nm by spectrophotometry (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA). The effectiveness of mutagenesis reactions was assessed by direct sequencing in both directions of the plasmid DNA (Stabvida®, Portugal).

All the steps in handling nucleic acids, proteins and cell cultures, were performed according to previously described basic and well established protocols (35).

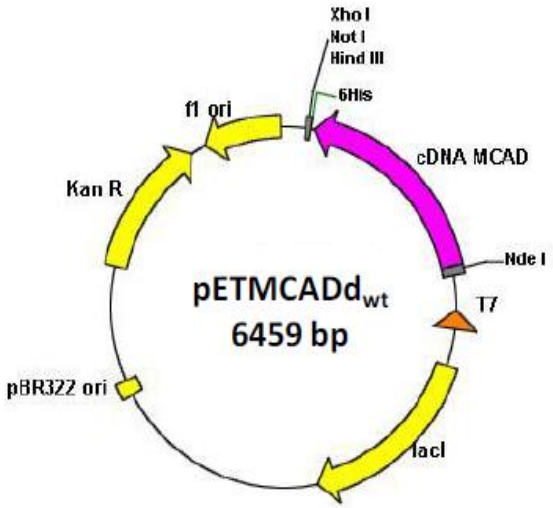

Figure 4: The structure of the expression vector pETMCADd$_{Wt}$.

3.2. Expression of recombinant hMCAD proteins

The first step in obtaining recombinant proteins was the transformation of *E.coli* BL21(DE3) cells with 10 ng of plasmid DNA, containing the cDNA of interest. This was accomplished in a dry-bath (Labnet AccuBlock™ digital dry bath) with "heat-shock" (42 °C, 90 sec), which causes bacterial membrane to open and to take in the vector. 1 mL of Luria Bertani (LB) cell culture medium (1% tryptone, 0.5% yeast extract, 0.17 M NaCl, pH 7.0) with 20 mM glucose was added to the cells which were further incubated for 1 hour at 37 °C while being constantly agitated at 150 rpm (Rotabit, JP Selecta). The cells were then cultured on solid LB medium with 1.5% agar containing kanamycin, at a final concentration of 50 µg/mL, and incubated overnight (ON) at 37 °C. After ON incubation an isolated colony was selected and cultured on in an adequate volume of liquid LB medium containing kanamycin (50 µg/mL) at 37 °C with 170 rpm agitation. On the following day the mixture was diluted in a 1/100 ratio with liquid LB medium containing kanamycin (50 µg/mL) and further incubated at 37 °C with170 rpm agitation. Bacterial growth was monitored by spectrophotometry (Hitachi spectrophotometer, model U-2000, Japan) at 600 nm wavelength. After it reached the exponential growth stage (A_{600}>0.6), protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; NZYTtech® Ltd, Portugal) over a period of 21 hours, at 27 °C applying constant 150 rpm agitation. The IPTG is a molecular reagent which mimics lactose that binds to the lac repressor, thereby triggering transcription and translation of T7 RNA polymerase, which recognizes the T7 promotor and rapidly induces transcription of the gene of interest.

As IPTG is not part of any metabolic pathways it is not degraded or used by cells (36). Cells were then isolated by centrifugation (3.220 g, 10 min, 4 °C) (Eppendorf 5810R Centrifuge). The supernatant was discarded and the cell pellet immediately stored at -20 °C for a maximum a month.

3.3. Purification of recombinant hMCAD proteins

The purification of recombinant hMCAD proteins started with suspending bacterial cell pallets in a lysis buffer containing starting buffer (20 mM phosphate buffer (KPi) pH 7,4; 500 mM NaCl), 1mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Aldrich®, EUA) and 1 mg/mL lysozyme, in a volume corresponding to 1/100 of the original cell culture volume. It is important for the lysis buffer to contain a strong buffer such as KPi to overcome the contribution of bacterial lysate, a high ionic strength (500 mM NaCl) to enhance protein solubility and stability, a protease inhibitor such as PMSF, to prevent protein degradation, and lysozyme to destroy the cell wall of bacteria. The lysis buffer was prepared in the absence or presence of 0.1 mg/mL FAD, the hMCAD cofactor (Sigma Aldrich®, EUA).

Before they were incubated for 30 min at 4 °C with lysis buffer, the cells were vortexed in order to re-suspend and thoroughly mix the pellet with the buffer. After this, the resulting suspension was homogenized by sonication at 4 °C with a Vibra Cell sonicator (Sonics, Newtown) during 4 cycles of 60 sec (duty free cycle of 50%) with a 30 seconds interval between each cycle. The soluble protein fraction (crude fraction) was separated from insoluble compounds by centrifuging the cell lysate (12.857 g, 40 min, 4 °C).

hMCAD recombinant proteins were further purified by IMAC (Fig. 5) using Ni_2^+ -NTA resin (Qiagen®, Hilden, Germany). For this purpose, the crude protein fraction was incubated with the resin (4 °C, 60 min, constant agitation), which was pretreated with demineralized water and equilibrated in a lysis buffer containing β-mercaptoethanol (10 mM) as a reducing agent and low levels of imidazole (10 mM) to reduce the number of proteins that bind nonspecifically to the resin. Subsequently the mixture was transformed to a polypropylene column. The first fraction eluted corresponds to the flow-through fraction (FT) and is composed by the contaminant proteins that do not bind to the resin. The elution of proteins of our interest was achieved using the appropriate imidazole gradient, composed of various buffers (20mM KPi, 500 mM NaCl, pH 7,4) containing increasing concentrations of imidazole (2 x 5 ml of 20 mM; 2 x 2.5 ml 40 mM and 5 x 0.5

mL of 500 mM). The inclusion of imidazole (20–40 mM) in the washing buffer increases the stringency of this step and allows the removal of nonspecifically bound proteins more effectively (37). The 6xHis-tagged recombinant hMCAD proteins were finally eluted from the Ni^{2+}-NTA resin with 500mM of imidazole which has a higher affinity for the metal ions than the 6xHis-tag.

Figure 5: Immobilized metal affinity chromatography (IMAC) is used for the purification of recombinant histidine (His)-tagged proteins. The poly-histidine sequences on the target proteins exhibit high affinity towards the Ni^{2+} metal ion which is immobilized on the resin. IMAC using Ni_2^+ -NTA allows highly selective binding of recombinant His-tagged proteins (38).

The eluted fractions were then analyzed by electrophoresis in a denaturing polyacrylamide gel (SDS-PAGE) (see chapter 3.4. for details), to identify the fractions in which the 6xHis-tagged proteins were eluted, as well as to evaluate their purity grades. The eluted fractions containing proteins of interest were additionally purified by dialysis to remove imidazole. For this step fractions containing the recombinant proteins of interest were combined in a dialysis membrane with a molecular weight cutoff ranging from 12-14000 Da (Spectra / Por®4, Spectrum Laboratories, Inc.). They were incubated ON at 4 °C and constant stirring in a dialysis buffer (20 mM Kpi buffer (pH 7,4), 5 mM β-mercaptoethanol, 5% glycerol). The purified proteins were used immediately.

3.4. Protein analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein fractions eluted from the columns during the IMAC step were analyzed with electrophoresis in denaturing polyacrylamide gel (SDS-PAGE) to identify fractions containing the proteins of interest and to assess their purity. The electrophoretic separation was carried out as described previously using a stacking gel with 4% and a separating gel with 12% acrylamide/bisacrylamide, respectively (39). To 20 µL of each protein fraction 4 µL loading buffer (0.24 M Tris-HCl, pH 6.8, 40% glycerol, 8% SDS, 0.02% bromophenol blue, and 25% β-mercaptoethanol) were added before the denaturation step (95 °C, 5 min). The SDS works by disrupting non-covalent bonds in proteins causing their denaturation. β-mercaptoethanol is added to cleave disulfide bonds within or between

proteins and glycerol to weigh down the samples. Samples were applied in the stacking gel in final volumes of 20 µL. The electrophoretic separation was carried out by applying a continuous voltage of 20 mA in the stacking gel and 40 mA for gel separation (EPS 301, Amersham pharmacia biotech). The system was constantly cooled by a cold water flow.

In order to determine the separated protein fractions, gels were placed for at least 20 minutes at room temperature (RT) in a 0.1% solution of Coomassie Brilliant Blue R-250 (Sigma-Aldrich®, USA) prepared in 27% methanol and 18% acetic acid which prevents proteins from being lost by diffusion. Subsequently gels were placed into a solution containing 30% methanol and 10% acetic acid to enable the visualization of protein bands. Molecular weight of protein bands visualized in the gel were inferred from the electrophoretic profile of a defined protein marker *NZYcolour Protein Marker II* (NZYTech®, Lda., Portugal) (Fig. 6) which was applied into SDS-PAGE gels simultaneously with the samples. The purity of each fraction was determined by densitometric analysis using the NIH Image J® 1.43U (*Image Processing and Analysis* in Java) software.

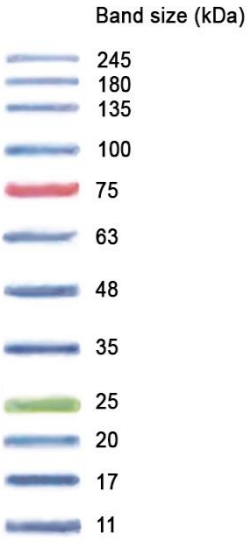

Figure 6: Protein molecular weight marker NZYcolour Protein Marker II (NZYTech®, Lda., Portugal); the molecular weights of separated proteins are shown in kDa.

3.5. Protein quantification with the Bradford method

To determine the concentrations of single proteins following dialysis the colorimetric Bradford method was applied, as described previously (40). Briefly, protein fractions were removed from the dialysis membrane and further centrifuged (9.660 g, 15 min, 4 °C). Then the protein content in the supernatant was determined. Samples were diluted in

advance, based on estimations of their concentrations acquired by qualitative analysis of protein bands intensity observed in SDS-PAGE gel (dilutions of 1 : 100 or 1 : 200). Calibration curve of gradually diluted albumin bovine serum (BSA, Sigma-Aldrich®, USA) (1 to 10 mg/mL) was prepared. A volume of 200 µL of Bradford Bio-Rad Protein Assay (Bio-Rad® Laboratories, Inc., USA) reagent was added to 800 µL of sample or standard solutions and left to incubate for 10 min at RT. The absorbance of sample and standard solutions was then measured at 595 nm. All assays were performed at least in duplicates.

3.6. Determination of oligomeric profile of recombinant hMACD proteins by size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) is a separation technique based on differences in molecular sizes of components. The gel consists of spherical beads containing pores with a specific size distribution. Large molecules cannot enter small pores and are thereby effectively excluded from the column bed volume and thus eluted in the column's void volume. Small molecules diffuse into the pores and their flow through the column is retarded, according to their size. Consequently, molecules are separated based on their size as they pass through the column and are eluted in a decreasing order of their molecular weight (MW). The oligomeric forms of recombinant hMCAD proteins were characterized by analyzing chromatographic profiles of SEC purified proteins. After purification by IMAC, the purified fraction of each protein was centrifuged (10.000 g, 10 min, 4 °C) and then injected into a protein purification system (Fast Protein Liquid Chromatography; *AKTA®primeplus, GE Healthcare, USA*). The separation of protein oligomeric forms was performed on a molecular exclusion column *HiLoad™16/60, Superdex™ 200 prep grade* (cutoff at 10-600 kDa) with a constant flow rate of 0,7 mL/min, until a total volume of 150 mL of eluent (elution buffer: 20 mM KPi, 200 mM NaCl, pH 7,4) was loaded. The particles were detected by UV-spectrophotometry at 280 nm.

The analysis of MW of separated oligomeric species and their subsequent identification was assessed by comparing the ratio of elution volume (Ve) and void volume (Vo) of the recombinant hMCAD protein to the Ve/Vo ratios of protein standards with known MWs (12,4 kDa – 440 kDa): cytochrome C (12,4 kDa), ribonuclease (13,7 kDa), myoglobin (17,6 kDa), ovalbumin (43 kDa), BSA (66 and 132 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa) and apoferritin (440 kDa) (all from Sigma-Aldrich®, St.

Louis, USA). By plotting the logarithms of the known molecular masses of protein standards *versus* their respective Ve/Vo values results in a linear calibration curve. We used Blue Dextran (2000 kDa) to assess the Vo, which is based on the volume of effluent required for the elution of a large molecule, and Tyrosine (0,18 kDa) to determine the total volume of the column.

Thereafter, we proceeded to estimate the amount of each oligomeric form from the areas of various peaks on the chromatogram (PrimeView 5.0, UNICORN 5.01, Amersham Biosciences 2004). The fractions corresponding to the tetrameric form of recombinant hMCAD proteins were combined and concentrated by centrifugation (3.000 g, 10 min, 4 °C), using a filter with a molecular exclusion limit of 50 kDa (Amicon® Ultra, Millipore, USA). The fractions were then used to determine the protein content and their functional and structural characterization.

3.7. Determination of enzymatic activity and kinetic characterization of recombinant hMCAD proteins

The enzymatic activity of purified proteins was assessed spectrophotometrically (Shimadzu UV-1800, Japan), according to a method described previously, using octanoyl-CoA (Sigma-Aldrich®, USA) as a MCAD substrate, phenazine methosulfate (PMS, Sigma-Aldrich®) as an intermediate electron carrier and 2,6-dichlorophenolindophenol (DCPIP, Sigma-Aldrich®) as an artificial electron acceptor (41). The PMS/DCPIP pair allows the cofactor bound to the enzyme MCAD to be re-oxidized after receiving electrons from dehydrogenation reaction between the substrate (octanoyl-CoA) and MCAD. The decrease in the absorbance of DCPIP was followed at 600 nm, because when DCPIP accepts an electron from PMS it is transformed into lauco-DCPIP, which is colourless (42).

The reaction mixture used to determine MCAD enzymatic activity consisted of 20 mM Kpi buffer (pH 7.4), 1,5 mM PMS, 48 µM DCPIP and 33 µM octanoyl-CoA, in a final volume of 1 ml (all indicated values correspond to final concentrations). The initial concentrations of DCPIP and octanoyl-CoA were confirmed spectrophotometrically at 600 nm (ε_{600}= 21,0 $Mm^{-1}cm^{-1}$) and at 259 nm (ε_{259}= 15,9 Mm^{-1} cm^{-1}), respectively, by applying the Lambert-Beer's law. The mixture was mixed well and transported into a cuvette where the baseline was followed for 30 s, after which the reaction was started by the addition of the enzyme (8 µg). The course of reaction was monitored for at least one

minute at RT (25 °C) under constant stirring. Control tests were conducted under the same conditions but in the absence of substrate. The volume of the substrate was replaced with 20 mM Kpi buffer (pH 7,4).

Kinetic parameters of each enzyme sample were determined by assessing the enzymatic activity of hMCAD at increasing substrate concentrations, ranging from 1 to 100 µM (at least 12 different concentrations). The kinetic parameters, such as maximal velocity (V_{max}), *Michaelis Constant* (K_m), K_{cat} and catalytic efficiency (k_{cat}/K_m) were acquired with GraphPad Prism version 6.01 software using non-linear regression of the *Michaelis-Menten* equation or by applying the following equation when substrate inhibition was observed:

$$Y=Vmax*X/(K+(X*(1+X/Ki)))$$

3.8. Determination of the conformational flexibility of recombinant hMCAD proteins by limited proteolysis by trypsin of recombinant hMCAD proteins

To evaluate conformational stability of recombinant hMCAD Wt, p.K304E and new mutant proteins, their proteolysis profiles in the presence of trypsin were evaluated. Limited proteolysis provides an efficient and very sensitive method to assess the protein quality in terms of its stability. Proteins in the folded state with stable domains are usually very resistant to proteolytic degradation because their potential cleavage sites are hardly accessible. On the other hand unstructured, solvent-exposed loops, may be subject to rapid proteolysis. Therefore when using limited amounts of trypsin, properly folded proteins remain largely unaffected whereas those un-/misfolded are cleaved down to more stable fragments (43).

The reaction mixture consisting of 0,3 mg/mL of protein in its tetrameric form and 0,018 mg/mL of trypsin (the final mass ratio of trypsin : enzyme was 1 : 17) was incubated in the SEC buffer (20 mM KPi buffer, 200 mM NaCl, pH 7,4). The assay was performed for 60 min at 37 °C, during which 10 µL aliquots were collected within the period of 0 to 60 min of incubation. The reaction was terminated by addition of trypsin inhibitor to each of the aliquot in a trypsin : inhibitor ratio of 1 : 1,5, by mass. Protein degradation profile was evaluated by electrophoresis on a 12% SDS-PAGE, followed by quantification of the observed changes by densitometric analysis of gels, using the NIH Image J® 1.43U (Image Processing and Analysis, Java) software.

3.9. Determination of the thermal stability of recombinant hMCAD proteins by dynamic light scattering (DLS)

Dynamic light scattering (DLS) was used to evaluate thermal stability of Wt and hMCAD mutants. This method measures the intensity of light scattered by the molecules present in the sample as a function of time. Since all molecules in solution diffuse with Brownian motion (the random movement of microscopic particles suspended in a liquid or gas, caused by collisions with molecules of the surrounding medium) in relation to detector there are interferences which cause changes in transmitted light intensity. The faster the particles diffuse, the faster the intensity of light will change. Molecular diffusion is essentially controlled by the following factors:

- temperature – the higher the temperature the faster the molecules move;
- viscosity of the solvent – the more viscous the solvent the slower is molecular movement;
- the size of molecules – the bigger the molecules, the slower they move.

If the temperature and solvent are constant and known, the variation in the intensity of the scattered light is directly related to the "size" of molecules.

The measurement of movement is performed by quantifying the percentage of light intensity which falls on the particles. According to this principle it is possible to monitor the increase in protein size within suspension with temperature rising and to evaluate protein aggregation in time, at a constant temperature. Thus it is possible to determine the thermal profile and kinetics of aggregation for a given mutated protein, by evaluating its structural stability in comparison to the behavior of the Wt protein under the same conditions (44).

We performed three tests were performed for each of the recombinant proteins studied:

- assessment of thermal stability by measuring its size and scattering intensity as a function of temperature − proteins "melted" under the influence of heat which causes them to denature and leads to massive aggregations;
- determination of aggregation kinetics of recombinant proteins at 37°C;
- determination of aggregation kinetics of recombinant proteins at 42°C.

Each assay was carried out in duplicates.

Before DLS analysis SEC isolated tetramers of recombinant hMCAD proteins were centrifuged (20.000 g, 15 min, 4 °C), diluted to a concentration of 0,15 mg/mL with the SEC buffer in order to prevent the particles from interacting with each other and

disrupting Brownian motion. Samples were filtered through 0,2 μm membranes to remove unwanted particles that could contribute to light scattering and analyzed on The Zetasizer Nano S (Malvern) apparatus, which is capable to measure particles with diameters from 0,3 nm to 10 microns using the patented *NIBS* (non-invasive back scatter) technology.

4. RESULTS

4.1. Expression and purification of recombinant hMCAD proteins: yields and purity

The vectors used for the heterologous expression of the $hMCAD_{Wt}$ protein and p.K304E, p.G224R and p.L238F variants had been already previously developed in the laboratory. The expression vectors for $hMCAD_{Wt}$ and its mutants were inserted in *E.coli* BL21(DE3) cells where recombinant proteins were overexpressed upon induction with 0.5 mM IPTG at 27 °C, for 21h. After collection of cells and their lysis by sonication, soluble fractions of cell lysates were isolated by centrifugation. As these are harsh procedures they may lead to the loss of the MCAD cofactor FAD which is non-covalently bound to the protein structure. The potential loss of FAD during protein purification may compromise the "normal" behaviour of a tested protein, as the co-factor binding is important for its stability and function. In order to assess the true impact of the applied purification procedures of recombinant proteins under study, cell lysis was performed with and without the addition of 0,1 mg/mL FAD into the lysis buffer. Soluble proteins were further purified by the Ni^{2+}-affinity chromatography (IMAC), using the imidazol gradient (0-500 mM).

After purification, protein samples were resolved by denaturing SDS-PAGE. Gels were stained with Coomassie brilliant blue. The intensity of bands are being proportional to the amounts of proteins. Gel electrophoresis provides information on MW and protein charge and allowing the purity of each sample to be determined. Western blot, for all recombinant proteins have already been performed in previous work. The results obtained prove that all the proteins we analyzed as the strongest bands in SDS-PAGE are indeed the proteins of our interest (Fig. 7).

By analyzing the SDS-PAGE profiles (Figs. 7 and 8) we observed that the absence or presence of FAD in the cell lysis buffer does not influence the purity grade of the **Wt** protein, which is approximately 90% in both cases (87,3 ± 7,3% or 88,0 ±7,9%, respectively). Similar results were also obtained for the **p.L238F** mutant (86,0 ± 3,7% to 89,0 ± 4,3%, respectively) (Fig. 8). The differences in purity grades were observed for both **p.K304E** (79,3 ± 7,1% or 71,8 ± 7,1%, respectively) and **p.G224R** (from 70,7 ± 7,7% to 87,3 ± 8,0%) mutant proteins purified in the absence or presence of FAD, but they were not statistically significant.

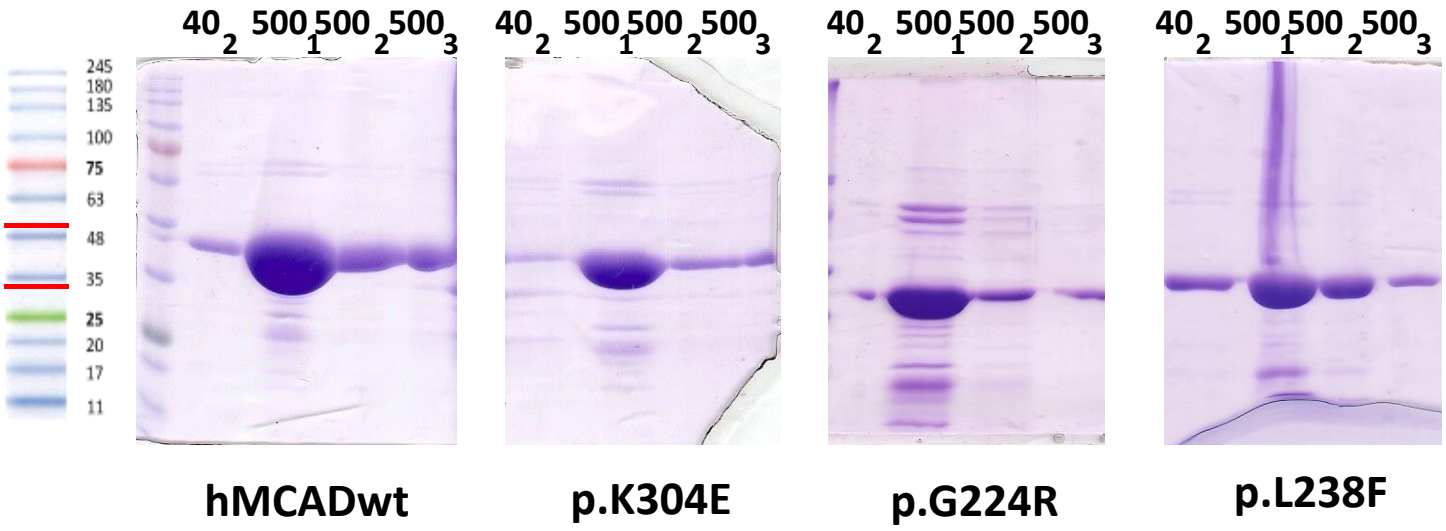

Figure 7: Representative electrophoretic profiles obtained with 12% SDS-PAGE present the second eluted fraction after 40 mM imidazole was applied to the column during IMAC and the first three eluted fractions after 500 mM imidazole was used. IMAC was performed for hMCADwt, p.K304E and p.G224R after their expression in 0,5 L of cell culture and for p.L238F in 2 L of culture, due to low expression levels of this variant. All recombinant hMCAD proteins presented were purified in the presence of FAD.

Nevertheless, the purity grade of the most common variant presented in MCADD, the p.K304E was significantly different from the Wt protein when the purification was initiated in the absence of FAD (79%) ($p < 0,05$). Furthermore, differences between the Wt and variant protein purities only increased when they were purified in the presence of FAD, under these condition we could identify even higher statistically significant difference between the Wt protein and K304E (72%) ($p < 0,001$) (Fig. 7).

The mutant p.G224R showed significant difference in its purity grade in comparison to the Wt protein when purification was carried out in the absence of the cofactor (70%) ($p < 0.001$). This difference however could not be observed when this protein was purified in the presence of FAD, due to the fact that, as already mentioned above, the purity grade of the recombinant protein p.G224R increased, although not significantly (87%) in these conditions, (Fig. 8).

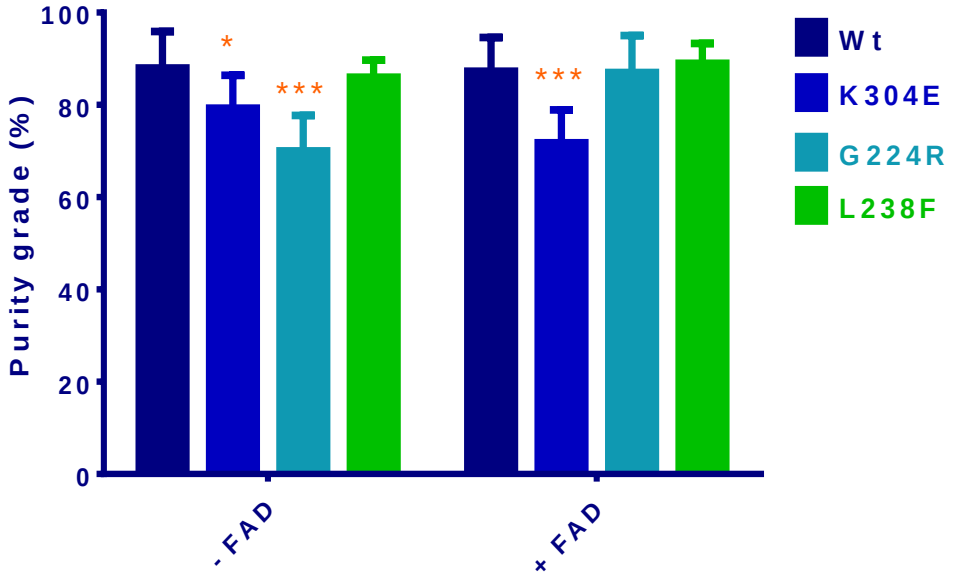

Figure 8: Purity grades were determined for the recombinant Wt and variant hMCAD proteins following IMAC with or without FAD in the lysis buffer. The purity of each protein sample was analyzed with SDS-PAGE and quantified densitometrically with the NIH Image J® 1.43U software (*Image Processing and Analysis* in Java). Significantly different purity grades of mutant forms, as compared with Wt protein were assessed with the student t-test (* $p < 0.05$ and *** $p < 0.001$). The bars represent average results from various independent experiments (n): Wt (n=12), p.K304E (n=6), p.G224R (n=2), and L238F (n=3).

The Wt protein, as well as the p.K304E and the p.G224R variants showed high level of expression when expressed in 0,5 L of culture, while the p.L238F variant was negligible expressed under the same conditions. This justifies the fact that all characterization assays for the p.L238F were performed after its expression in 2 L culture. This could also be the reason why after analysing the electrophoresis gels we could observe that the majority of recombinant proteins were eluting in the first fraction with 500 mM imidazole, whereas the p.L238F variant showed comparable quantities of protein in both, the first and second fraction of 500 mM imidazole (Fig. 7). The eluted fractions of each specific protein were combined and subjected to dialysis, representing an additional step of purification, in order to eliminate the imidazole used in the elution process. Namely, imidazole could diminish the stability of recombinant proteins. It also interferes with the Bradford assay used for the protein content determination. Based on the purities determined within the analysis of electrophoretic protein profiles and on the protein concentration assessed with the Bradford method following dialysis, we were able to infer the yields for each recombinant protein (Fig. 9).

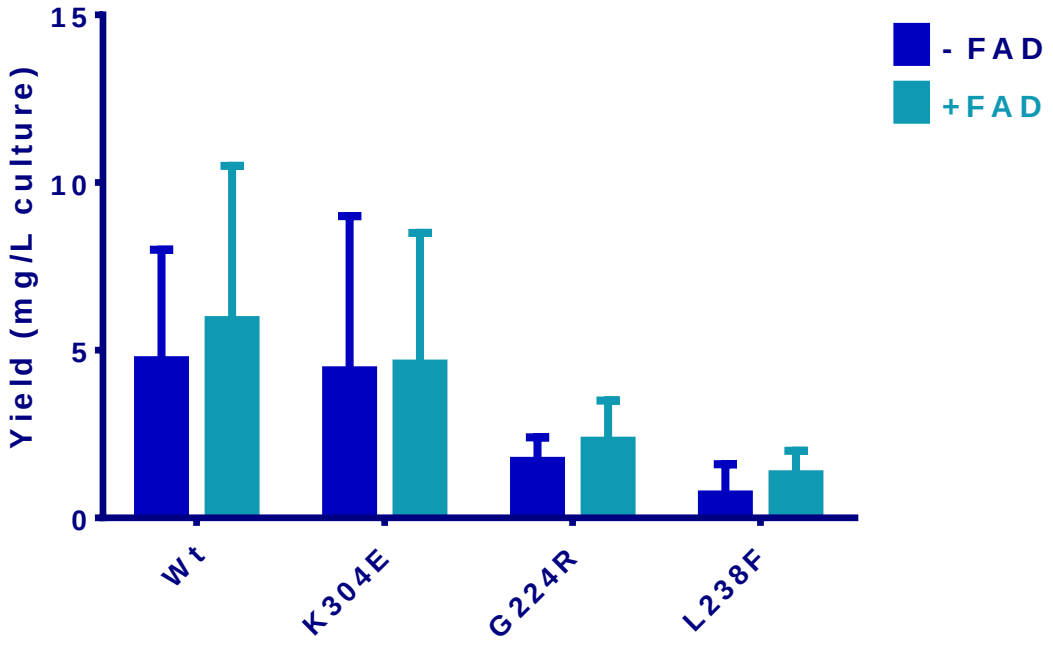

Figure 9: Figure represents the yield of each recombinant protein expressed in the amount (mg) of protein purified from 1 L of culture. The yields of the Wt and hMCAD variant proteins were determined using the Bradford method and the results corrected with the purity grade of each protein sample. Yield was quantified after purification with IMAC in the absence (dark blue bar) and presence (light blue bar) of FAD. Bars show the average results from various independent experiments (n): Wt (n=12), p.K304E (n=6), p.L238F (n=3), and p.G224R (n=2).

The yield of the recombinant **hMCADwt** protein was 4,7 ± 3,3 mg/L of cell culture with a slight increase when purification was carried out in the presence of FAD (5,9 ± 4,6 mg/L) (Fig. 9).

For the **p.K304E** variant we observed a similar yield as in the case of Wt protein (4,4 ± 4,6 mg/L culture). However, the yield increase following the addition of FAD in the purification step was negligible (4,6 ± 4,5 mg/L culture) (Fig. 9). Both **p.G224R** and **p.L238F** proteins showed a similar yield increase patterns when FAD was present during purification, as the yield of p.GRR2R increased from 1,7 ± 0,7 mg/L to 2,3 ±1,2 mg/L and that of p.L238F from 0,7 ± 0,9 mg/L to 1,3 ± 0,7 mg/L (Fig. 9).

4.2. Oligomeric profiles obtained by size exclusion chromatography (SEC) of recombinant hMCAD proteins

Oligomeric profiles of purified recombinant hMCAD proteins in the absence and presence of FAD were obtained by SEC. The identification of the oligomeric species for each protein was accomplished by comparing the results with the calibration curve of standards obtained after their separation with SEC. The analyses of oligomeric profiles showed that

for all recombinant proteins, tetramers are the predominant oligomeric form (Fig. 10). Another common characteristic was s slight alteration in the oligomeric profile when FAD was present during the purification process (Fig. 11).

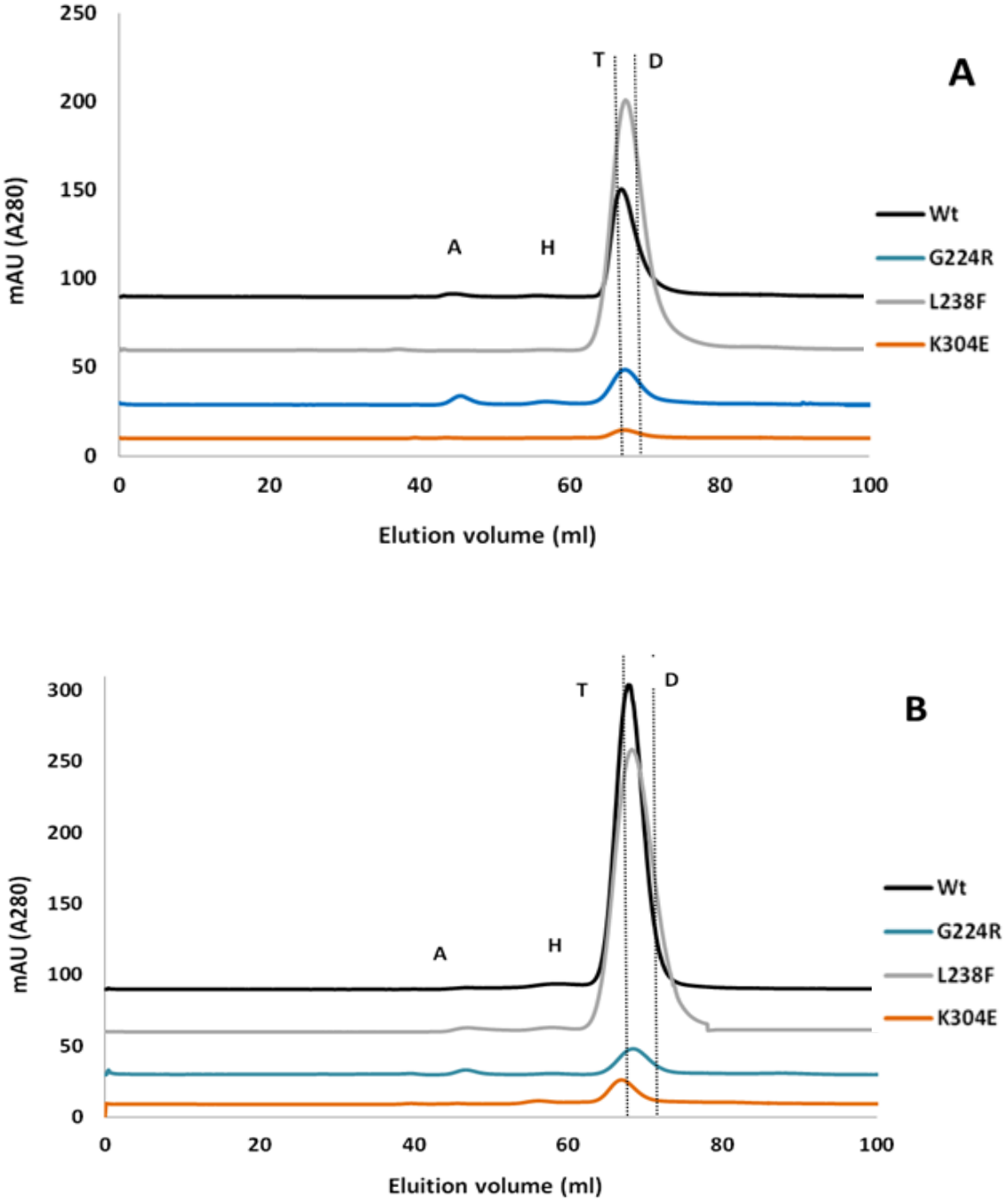

Figure 10: Representative oligomeric profiles obtained by SEC of $hMCAD_{Wt}$ and variant proteins after purification without (A) and with (B) FAD in lysis buffer. A- aggregates (Mr > 1000 kDa); H- high molecular weight forms (HMW - Mr > 400 kDa); T- tetramers (Mr ≈ 170 kDa); D- dimers (Mr ≈ 90 kDa). In order to present all protein profiles simultaneously, the baseline for each protein was normalized by adding to the absorbance data at 280 nm a different constant value for each protein. Dashed lines indicate elution volumes for T and D, respectively. The apparent molecular mass (Mr) was estimated from a calibration curve prepared with protein standards of known molecular masses.

Each fraction corresponding to a particular oligomeric form was determined as the ratio between the area of the corresponding peak and the sum of all peak areas, each representing different oligomeric forms of the protein in question (Table I). In case of the **Wt** protein the presence of FAD during the purification process evoked an oligomerization profile with a minimal increase in the tetrameric fraction, i.e. from 84,2 ± 5,53% to 85,6 ± 6,49% at the expense of a slight decrease in the dimeric fraction (13,5 ± 4,97% to 10,9± 3,25%). Regarding the amount of aggregates, barely noticeable changes in high molecular weight (HMW) forms and monomers were observed (Table I).

The oligomeric profile of the **p.K304E** variant was characterized by a lower percentage of tetramers (61,4 ± 28,8%) in comparison to the Wt protein, which increased (80,4 ± 6,90%) in the presence of FAD during purification. Therefore p.K304E values were comparable to the amounts of tetramers found in the Wt oligomeric profile. Similarly as in the Wt pattern, the content of dimers in the p.K304E variant was also greatly reduced in the presence of FAD (20,9 ± 24,2% to 7,2 ± 2,78%), although not showing statistical significance due to high variability of data. This is probably a consequence of a poor resolution between tetrameric and dimeric fractions. The percentage of HMW forms, aggregates and monomers was significantly higher ($p<0,005$ for HMW and $p<0,0005$ for aggregates and monomers) as compared to the Wt, when purification was accomplished in the absence of FAD. There was no significant difference between amounts of monomers, HMW and aggregates for Wt and the p.K304E when they were purified in the presence of FAD (Table I).

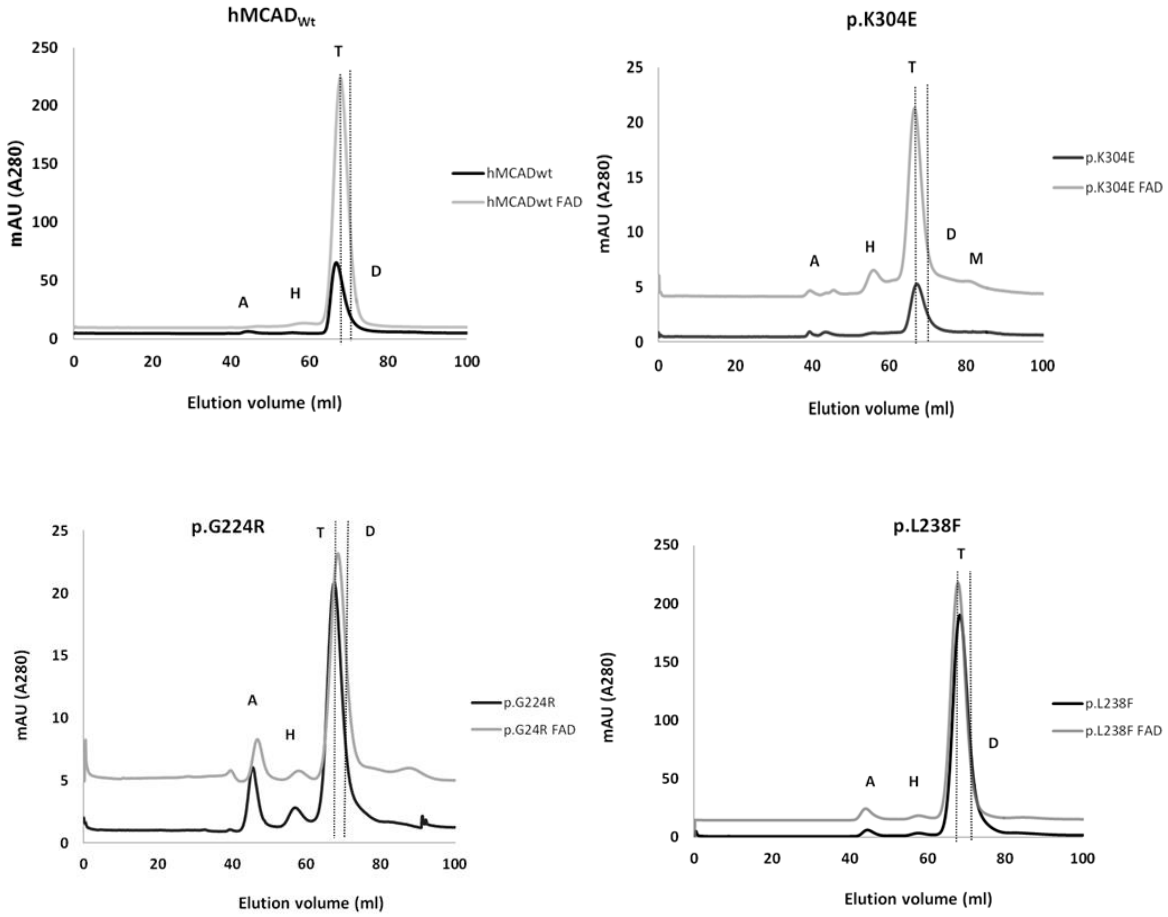

Figure 11: Oligomeric profiles of the Wt and variant recombinant hMCAD proteins, purified in the absence (black line) and the presence (gray line) of the cofactor FAD. A- Aggregates (Mr > 1000 kDa); H- High Molecular Weight Forms (HMW - Mr > 400 kDa); T- Tetramers (Mr ≈ 170 kDa); D- Dimers (Mr ≈ 90 kDa); Monomers (Mr > 40 kDa). Dashed lines indicate volume where T and D start to elute. In order to present both (without and with FAD) oligomeric profiles simultaneously, the baseline in each case was normalized by adding to the absorbance data at 280 nm a different constant value. The apparent molecular mass (Mr) was estimated from a calibration curve prepared with protein standards of known molecular masses.

The presence of the cofactor also alters oligomerization profile of the **p.G224R** variant in the same way as observed for the Wt and p.K304E, i.e. with an increase in the amount of the tetrameric fraction (66,6 ± 12,2% to 75,8 ± 16,9%) and a decrease in the percentage of dimers (from 16,4 ± 10,7% to 10,9 ± 3,25%). The tetrameric form content was significantly lower in comparison to Wt, in the absence of FAD ($p<0,05$), while the contents of aggregates ($p<0,005$) and HMW forms ($p<0,05$) were significantly higher. The content of monomers in the absence, as well as in the presence of FAD, during IMAC, were significantly different between Wt and p.G224R ($p<0,05$) (Table 1).

Table I: Distribution of oligomeric forms of the Wt protein and variant forms of hMCAD obtained by SEC in the absence (A) and presence (B) of FAD during purification. Each oligomeric form is presented in %, where 100% is the total sum of all peaks for a specific protein.

A	**Oligomeric profile (%)**				
Protein	**Aggregates**	**HMW**	**Tetramers**	**Dimers**	**Monomers**
hMCADwt	1,57 ± 1,63	0,85 ± 1,25	84,2 ± 5,53	13,5 ± 4,97	0,37 ± 0,89
p.K304E	7,81 ± 3,50***	6,26 ± 5,09**	61,4 ± 28,8	20,9 ± 24,2	5,23 ± 1,46***
p.G224R	8,11 ± 5,97**	4,74 ± 5,09*	66,6 ± 12,2*	16,4 ± 10,7	0,00*
p.L238F	0,97 ± 1,04	0,29 ± 0,41	81,2 ± 0,65	17,5 ± 0,79	4,22 ± 4,88

B	**Oligomeric profile (%)**				
Protein	**Aggregates**	**HMW**	**Tetramers**	**Dimers**	**Monomers**
hMCADwt	2,05 ± 3,36	0,67 ± 0,21	85,6 ± 6,49	10,9 ± 3,25	0,76 ± 1,32
p.K304E	9,56 ± 6,46	1,67 ± 2,89	80,4 ± 6,90	7,20 ± 2,78	1,02 ± 1,76
p.G224R	5,53 ± 6,99	2,09 ± 1,51	75,8 ± 16,9	10,9 ± 3,25	4,05 ± 4,03*
p.L238F	1,49 ± 2,00	0,39 ± 0,68	70,0 ± 9,61	22,7 ± 9,58	5,44 ± 5,95*

Statistical significance was determined using Student unpaired t-test, comparing variant and Wt protein forms of hMCAD (* $p<0,05$; ** $p<0,005$; *** $p<0,0005$). Each independent experiment (n) was performed in a duplicate. Data represent the averages with standard deviations (SD) of hMCADwt (n=11), p.K304E (n=4), p.G224R (n=2) and p.L238F (n=2). Aggregates (Mr > 1000 kDa); High Molecular Weight Forms (HMW - Mr > 400 kDa); Tetramers (Mr ≈ 170 kDa); Dimers (Mr ≈ 90 kDa); Monomers (Mr > 40 kDa). The apparent molecular mass (Mr) was estimated from a calibration curve prepared with protein standards of known molecular masses.

In case of the **p.L238F** variant, the content of the tetrameric form without FAD being present during the purification process (81,2 ± 0,65%) was the most comparable to that of the Wt among all the variants studied. Strangely, the content of tetramers decreased to 70,0 ± 9,61% when FAD was added to the lysis buffer. This was only observed with the p.L238F variant. In order to understand this phenomenon, we determined all the others oligomeric forms under the studied condition B (with FAD) in comparison to condition A (without FAD) which were increased. A significant difference ($p<0,05$) in the amount of monomers between the p.L238F mutant (5,44 ± 5,95%) and the Wt (0,76 ± 1,32%) was observed when FAD was present during the purification process (Table I).

4.3. Determination of enzymatic activities of recombinant hMCAD proteins

The measurement of enzymatic activity of hMCAD requests a reaction mixture composed of Kpi buffer, PMS/DCPIP redox pair and MCAD octanoyl-CoA substrate, spiked with the protein of interest. Electrons resulting from dehydrogenation of the substrate (octanoyl-CoA) reduce the cofactor FAD, bound to the enzyme, which is then further reoxidised by the PMS/DCPIP redox pair. These two components function as artificial electron acceptors, mimicking the role of the ETF protein associated with acyl-CoA dehydrogenases in reoxidation of FAD. The enzymatic activity was assayed in terms of reduction of DCPIP, as the final electron acceptor, reflected in decrease of absorbance measured at 600 nm. The enzymatic activity of proteins was determined with Shimadzu UVProbe 2.1 software, as Δabsorbance/min. The results obtained were adjusted with factor 5,9524 (1/ $\varepsilon_{600}$of DCPIP /8 µg protein). The enzymatic activities measured in the absence of substrate were used as blanks to correct for the non-enzymatic reduction of DCPIP.

The enzymatic activity of the recombinant **$hMCAD_{Wt}$** protein was 1,190 ± 0,470 µmol min^{-1} mg^{-1}, but when it was purified in the presence of FAD, the activity greatly increased (1,730 ± 0,400 µmol min^{-1} mg^{-1}), although this was not statistically significant (Fig.12).

The most common mutation found in MCAD, **p.K304E**, had a significantly lower enzymatic activity ($p<0,05$) in comparison to the wild type protein, when FAD was not added to the lysis buffer, showing only 44,4% residual activity. Contrary to the Wt, the specific enzymatic activity of p.K304E decreased when the protein was purified with FAD (0,528 ± 0,331 to 0,430 µmol min^{-1} mg^{-1}) causing even a bigger gap between Wt and p.K304E enzymatic activities, as under this condition 24,8% residual activity of the letter was determined (Fig. 12).

For the variant **p.G224R** a significantly lower activity ($p<0,05$) (0,373 ± 0,150 µmol min^{-1} mg^{-1}) was observed, as compared to the Wt protein when the purification step was carried out in the absence of FAD, with the variant protein exerting only 31,3% of residual activity. The results differed even more when the proteins were purified in abundance of cofactor. Namely, the activity of the variant p.G224R under this condition significantly decreased ($p<0,05$) (0,034 ± 0,037 µmol.min^{-1}.mg^{-1}), showing only 2,0% of residual activity (Fig. 12).

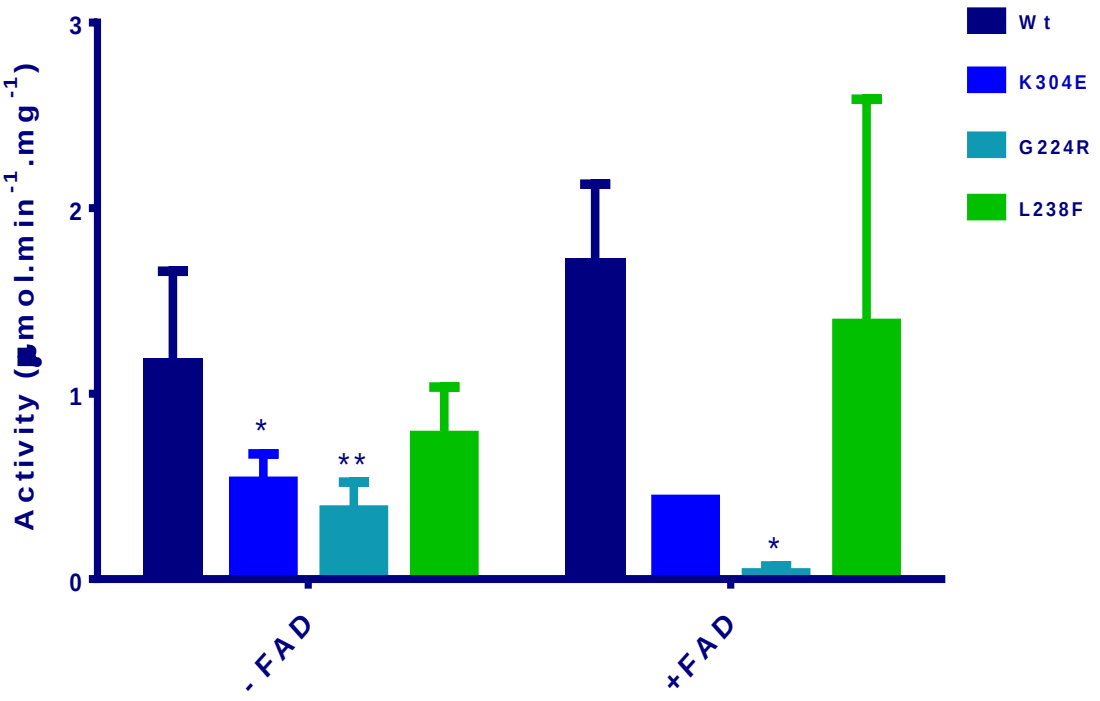

Figure 12: Enzymatic activity of recombinant proteins. Statistical differences between the Wt and its mutant forms were determined by using the Student t-test: * p <0,05 and ** p <0,005. Number of experiments (n) for each recombinant hMCAD protein: Wt-FAD (n=12); Wt+FAD (n=2); K304E-FAD (n=5); K304E+FAD (n=1); G224R-FAD (n=4); G224R+FAD (n=2); L238F-FAD (n=2); L238F+FAD (n=2).

When **p.L238F** protein was assayed we observed a residual activity which was the closest to that of the Wt (65,1%), when purified without FAD (0,775 ± 0,260 µmol min^{-1} mg^{-1}). The same pattern as seen with the Wt variant was also observed with p.L238F. In the presence of FAD during the purification step its enzymatic activity increased to 79,7% residual activity, as compared to its activity measured in the previous condition (the absence of FAD) (Fig. 12).

4.4. Kinetic parameters of recombinant hMCAD proteins

Apart from determining specific enzyme activity for each protein studied we also evaluated their kinetic parameters, such as: maximal velocity (V_{max}), *Michaelis Constant* (K_m), K_{cat} and catalytic efficiency (K_{cat}/K_m). Determinations of these parameters were performed similarly as their enzymatic activities, by following the change in absorbance of the DCPIP at 600 nm, for at least 1 min. The reaction was carried out in the presence of purified protein (8 µg) and increasing concentrations of the substrate, octanoyl-CoA (1-100 µM). The proteins used were purified in the absence of the cofactor FAD and

subsequently separated in their oligomeric forms by SEC. Only tetrameric forms were used to determine kinetic parameters of the recombinant hMCAD proteins.

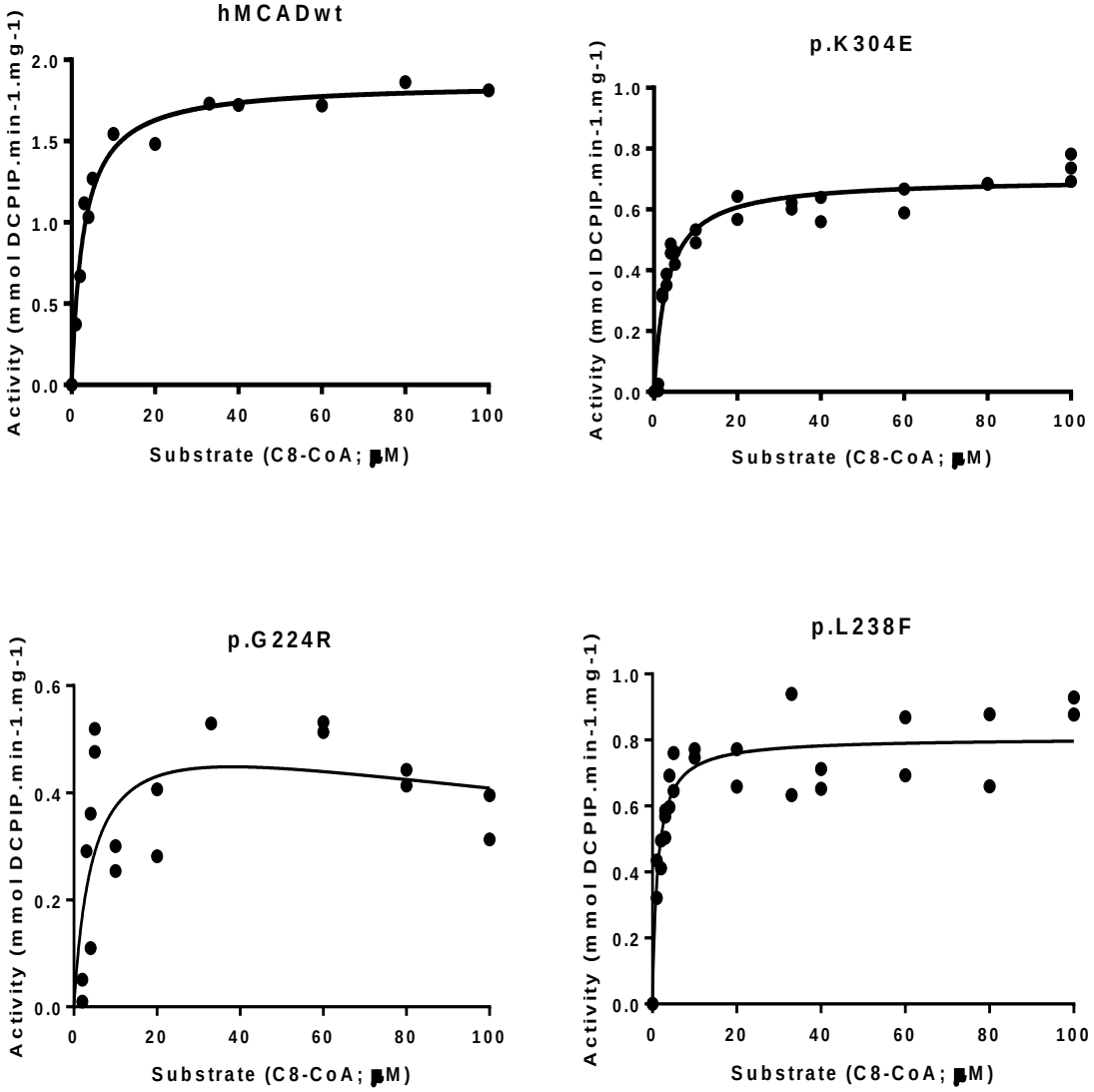

Figure 13: Figures represent enzymatic kinetics of recombinant hMCAD proteins, all purified in the absence of FAD. All reactions were performed at RT (25°C) for 1 min with C8-CoA substrate ranging from 1 to 100 µM. Decreases of DCPIP absorbance were evaluated by spectrophotometry and the resulting experimental data were processed using GrapPad Prism6® software. All curves were fitted by non-linear regression of the Michaelis-Menten equation, except for the mutant p.G224R for which, due to its substrate inhibition, the experimental data were fitted using the V_{max}*X/(K+(X*(1+X/K_i))) equation. Data were obtained from two independent experiments for Wt and a single reaction for hMCAD mutants. Each point represents average of duplicate measurements.

After analyzing enzymatic activities with increasing concentrations of the substrate we confirmed that the majority of proteins actually follow the *Michaelis-Menten* equation. The kinetic parameters for the proteins were determined by non-linear regression of the *Michaelis-Menten* equation (V_{max}*X/(K_m+X)), with the exception of p.G224R protein

which seemed to present substrate inhibition, and therefore the equation V_{max}*X/(K+(X*(1+X/K_i))) was used instead.

If we compared kinetic parameters of the **p.K304E** variant with those obtained for the Wt we found that it showed reduced catalytic efficiency (65,8%). This variant had lower V_{max} (0,70 ± 0,02 µmol min^{-1} mg^{-1}) in comparison to Wt (1,86 ± 0,05 µmol min^{-1} mg^{-1}) and a slightly weaker substrate affinity (Table II).

The biggest difference in kinetic parameters between the Wt and the studied hMCAD variants was observed with the **p.G224R** protein. This variant had a greatly reduced maximum velocity (only 0,56 ± 0,12 µmol min^{-1} mg^{-1}) and substrate affinity (4,59 ± 3,71 µM), as compared to the Wt. The recombinant p.G224R protein also showed about 3 times lower K_{cat} value, which together with a high K_m results in 18,6% of catalytic efficiency compared to the Wt (Table II).

Table II: Analyses of kinetic parameters for the recombinant hMCAD proteins.

hMCAD	V_{max} (µmol min^{-1} mg^{-1})	K_m (µM)	K_{cat} (min^{-1})	Catalytic efficiency (K_{cat}/K_m; µM^{-1} min^{-1})
hMCADwt	1,86 ± 0,05	2,82 ± 0,34	82,1	**29,0**
p.K304E	0,70 ± 0,02	3,13 ± 0,47	31,0	**9,9**
p.G224R	0,56 ± 0,12	4,59 ± 3,71	24,6	**5,4**
p.L238F	0,81 ± 0,03	1,23 ± 0,24	35,6	**29,1**

Data were analyzed with the GrapPad Prism6® software to determine the V_{max} and K_m parameters using non-linear regression of the Michaelis-Menten equation for all of the proteins except the p.G224R. Due to its apparent substrate inhibition, the equation V_{max}*X/(K+(X*(1+X/K_i))) for the p.G224R variant was used instead. K_{cat} was determined based on the molecular weight of the hMCAD subunit (44,2 kDa). Data represent the mean ± SD of duplicates from two independent reactions for $hMCAD_{Wt}$ and single reactions performed in duplicates for the variant proteins.

The **p.L238F** variant showed identical catalytic efficiency and even greater substrate affinity than the Wt, but on the other hand its maximum velocity and K_{cat} were 2,3 times lower than in the case of Wt. However, V_{max} and K_{cat} values of p.L238F were still the most comparable to those of Wt (Table II).

4.5. Assessment of conformational flexibility of recombinant hMCAD proteins by limited proteolysis with trypsin

The principle of limited proteolysis is the incubation of a given protein with a relatively low concentration of trypsin. In our case we used a ratio trypsin : enzyme = 1 : 17, by

mass to determine each protein is conformation stability. Proteolysis was performed at 37 °C for 60 min and during this period the reaction was stopped with the addition of trypsin inhibitor on different time points in order to evaluate the rate of protein degradation (Fig. 14). In this process only the tetrameric forms of recombinant hMCAD proteins, obtained by SEC after purification in the presence of FAD, were used. Following their digestion with the protease, the samples were analyzed by SDS-PAGE to quantify the changes in the intensities of protein bands in time, which is directly proportional to the starting amount of the full-length protein (Fig. 14).

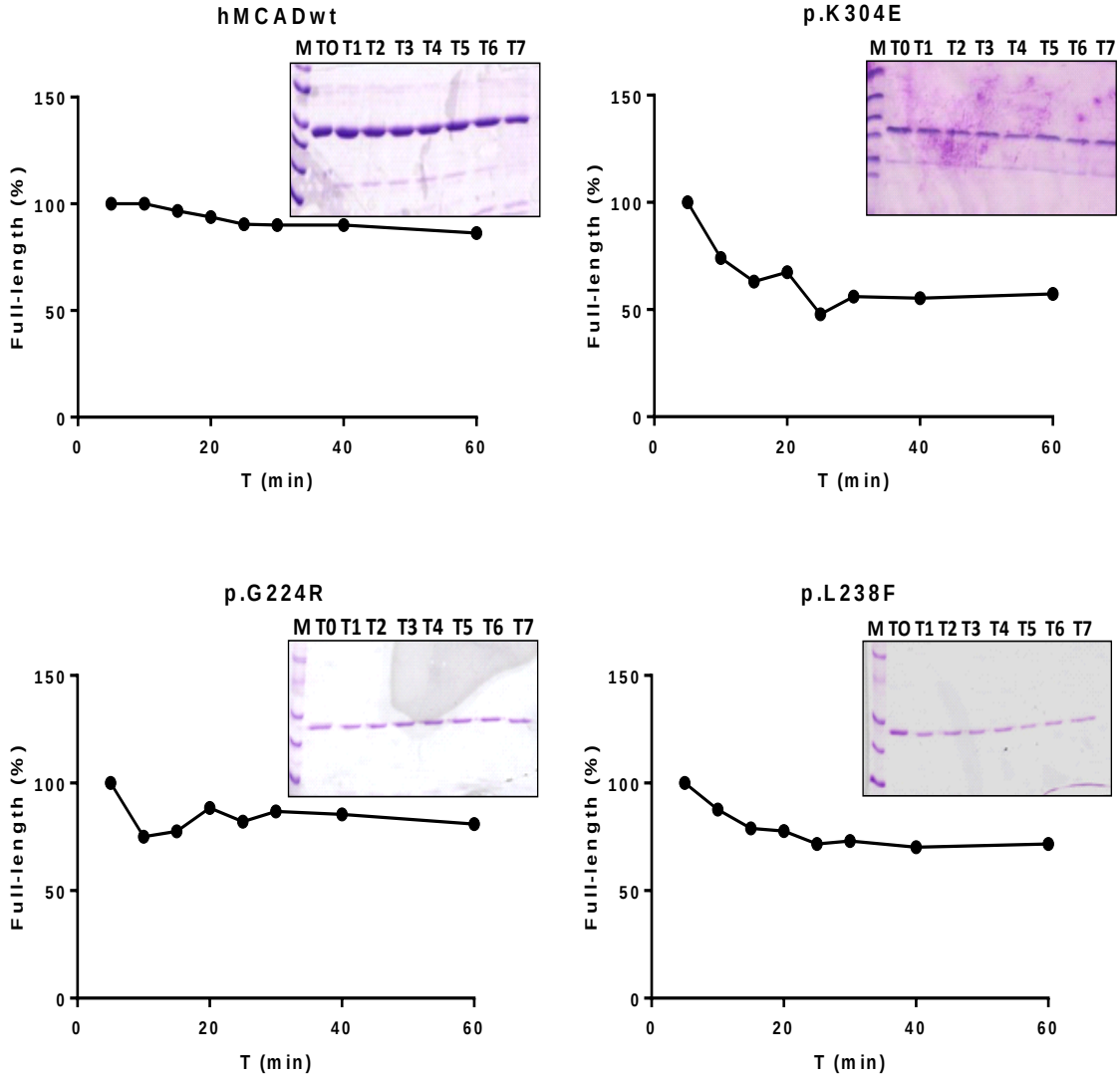

Figure 14: Degradation profiles for each recombinant hMCAD protein were obtained during limited proteolysis by trypsin. At the indicated time points reaction aliquots were collected, denatured and applied to the 12% SDS-PAGE. The changes in electrophoretic profiles were quantified by densitometry of protein bands (ImageJ® 1.43U NIH). M− *NZYTech LMW Protein Marker* (NZYTech®, Portugal Lda.); T0− 5 min without trypsin; T1− 10 min with trypsin; T2− 15 min; T3− 20 min; T4− 25 min; T5− 30 min; T6− 40 min and T7− 60 min.

From these data we determined the rates of protein degradation, which are represented by half-life (i.e. time required for 50% of the protein to be degraded, considering that T0 was normalized to 100% of the full-length protein). Due to the fact that all proteins, except the p.K304E variant, were not degraded to 50% during 60 min, data are presented alternatively, i.e. as percentages of degradation within 50% of reaction time, which is 30 min (Table III).

Table III: Amounts of residual (full-length) proteins of interest following 30 min exposure to trypsin in a ratio trypsin : enzyme = 1 : 17, by mass. The value of T0 was normalized to 100% of a full-length protein.

hMCAD protein	Wt	p.K304E	p.G224R	p.L238F
Residual protein/30 min (%)	90,0	56,1	86,8	73,0

The analysis of degradation profiles lead us to conclusion that **Wt** with a residual amount of 90,0% and the **p.G224R** variant with 86,8% are quite resistant to proteolysis under these conditions proving their high protein conformational flexibility. Also a reasonably high proteolytic stability was observed for the **p.L238F** variant (73,0%). The most common variant in MCAD deficiency, the **p.K304E**, was between more susceptible to proteolysis, as it was degraded to 56,1% after being in contact with trypsin for 30 min (Table III, Fig. 14).

4.6. Assessment of the aggregation profile and aggregation kinetics of recombinant hMCAD proteins by dynamic light scattering (DLS)

Small particles, such as our enzymes of interest, are subjected to Brownian motion in solution. This is a random movement of particles due to bombardment by solvent molecules surrounding them. It is known that the larger the particle, the slower the Brownian motion will be. With DLS, the speed at which particles are diffusing because of the Brownian motion is determined. This is achieved by measuring the rate at which the intensity of scattered light fluctuates when particles are irradiated with a monochromatic laser source. The faster scattered light fluctuates, the smaller are the particles. Light intensity data are collected using an autocorrelator to determine their size. DLS analysis uses the Stokes-Einstein equation to relate the velocity of a particle in solution to its hydrodynamic radius:

$$D=kT/6\pi\eta a$$

D is diffusion velocity of the particle, **k** is Boltzmann's constant, **T** is temperature, **η** is viscosity of the solution and **a** is hydrodynamic radius of a particle. According to the Stokes-Einstein equation the diffusion velocity is inversely proportional to the radius of a particle (45).

To complete protein stability studies the recombinant hMCAD proteins studied were purified in the absence and presence of FAD, only the tetrameric fractions, which were isolated by SEC were used in our analyses. At first, determinations of proteins thermal aggregation profiles was performed by increasing temperature (20-70 °C), thereby causing proteins to denature and aggregate at specific temperatures, being specified as temperatures of aggregation (T_{agg}). In a second approach, two assays were completed in order to determine the kinetics of each protein aggregation, i.e. at 37 and at 42 °C.

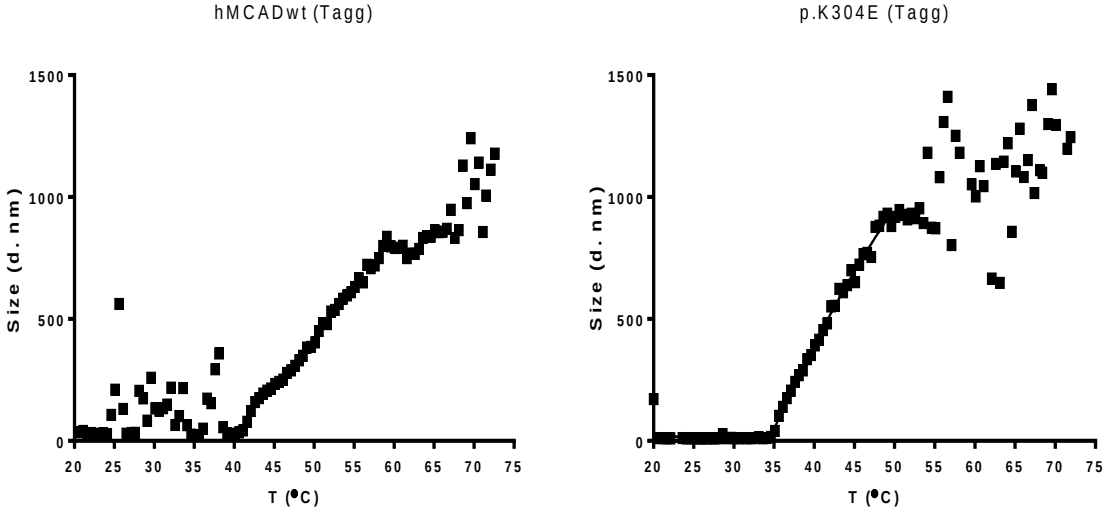

Figure 15: Thermal aggregation profiles for the hMCADWt and its variant p.K304E, using DLS technique are presented. The tetrameric forms of each protein used (0,15 mg/mL) were obtained by SEC with addition of FAD. Data was presented for the recombinant hMCAD proteins showing the biggest difference between their Tagg. Graphs were prepared with the GrapPad Prism6® software, helping us to assess at which temperature the proteins start to aggregate.

The analysis of thermal aggregation profile of **hMCAD$_{Wt}$** protein revealed that it starts to aggregate at around 41 °C, irrespective of the addition of FAD during the process of its purification (absence of FAD - T_{agg} = 41,6 °C; presence of FAD - T_{agg} = 40,6 °C) (Table IV).

On the other hand the thermal aggregation profile of the most common variant presented in MCADD, **p.K304E** showed much lower T_{agg} in comparison to Wt (36,5 °C when FAD was not present in the lysis buffer). The temperature of its aggregation was even lower,

when the cofactor was present (35,1 °C), indicating a less stable structure of the p.K304E as compared to the Wt (Table IV, Fig 15).

The aggregation temperature for the **p.G224R** variant did not differ whether FAD was used or not during the purification process and in it was lower than in case of the Wt protein (≈37,5 °C) (Table IV).

When the cofactor was not present in the lysis buffer, the **p.L238F** variant had even lower T_{agg} (36,2 °C) than the p.K304E, but the situation reversed when FAD was used during the purification process. In case of the p.K304E T_{agg} was even lower when FAD was present during its purification, while for the p.L238F variant much higher T_{agg} was observed, which was comparable to that of the Wt protein (Table IV).

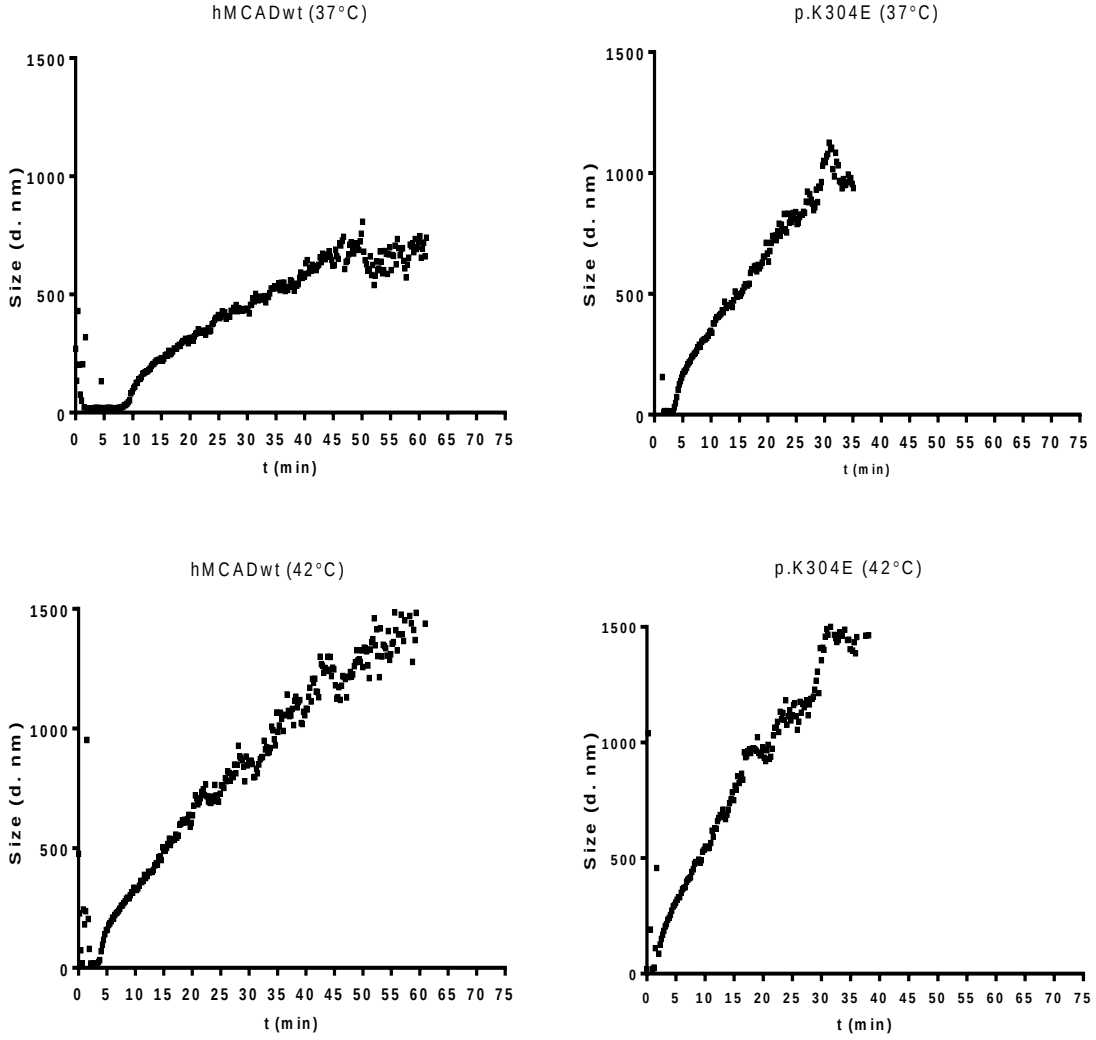

Figure 16: Kinetics of thermal aggregation of recombinant proteins hMCADWt and p.K304E, both purified in the presence of FAD. The graphs were generated with the GrapPad Prism6® software. Kinetics was determined by DLS at 37 and 42 °C. Graphs show increasing size of aggregation as a function of time at the constant temperatures (37 and 42 °C).

By analyzing the aggregation profiles at 37 and 42 ºC, we were able to determine at which time points the hMCAD proteins start to denature and consequently aggregate and thereby infer their kinetics of aggregation.

We estimated that the tetramers of the **hMCADwt** protein purified in the absence of FAD began to aggregate after 9,6 ± 0,2 min at 37 °C, and similarly so when FAD was used (9,3 ± 0,4 min). At 42°C the Wt protein began to aggregate at 3,8 ± 0,1 min, when purified in the absence of FAD and at 3,7 ± 0,3 min when FAD was applied (Table IV, Fig. 16).

The aggregation kinetics at 37 °C showed that the **p.K304E** variant in the presence, as well as in the absence of FAD behaves similarly, showing only 0,3 minutes difference under these two different conditions. However, its aggregation kinetics differed strongly in comparison to the Wt, which started to aggregate 2,5 times later. The presence or absence of FAD also did not cause any difference in its aggregation at 42 °C. In this case the p.K04E variant also started to aggregate earlier than the Wt protein (1,9 ± 0,1 min) (Table IV, Fig 16).

Table IV: Thermal stabilities of recombinant hMCAD proteins were analyzed by DLS. Temperatures of aggregation (T_{agg}) and time points at which the studied proteins started to aggregate (t_{lag}) at 37 and 42 °C were determined.

hMCAD	FAD	T_{agg} (°C)	37 °C	42 °C
			t_{lag} (min)	t_{lag} (min)
hMCADwt	-	41,6	9,6 ± 0,2	3,8 ± 0,1
	+	40,6	9,3 ± 0,4	3,7 ± 0,3
p.K304E	-	36,5	3,9 ± 0,1	1,9 ± 0,1
	+	35,1	3,6 ± 0,1	1,9 ± 0,1
p.G224R	-	37,4	7,6 ± 0,1	3,6 ± 0,2
	+	37,5	6,0 ± 0,4	2,9 ± 0,1
p.L238F	-	36,2	3,4 ± 0,2	1,7 ± 0,1
	+	39,1	8,8 ± 0,2	3,2 ± 0,1

T_{agg} was assessed in one experiment and t_{lag} in two independent assays.

The **p.G224R** variant (in the absence of FAD) began to aggregate earlier than the Wt protein, i.e. after 7,6 ± 0,1 min at 37 °C, while at 42 °C its T_{agg} (3,6 ± 0,2 min) was comparable to that of the Wt. The addition of FAD during purification caused the p.G224R variant to aggregate faster than the Wt protein, at both temperatures studied (Table IV).

The hMCAD variant **p.L238F** was the only enzyme we have studied that started to aggregate later at both temperatures, when FAD was used in its purification process,

which was comparable to the Wt protein. However, in the absence of FAD, the p.L238F variant started to aggregate sooner than the Wt, i.e. at 3,4 ± 0,2 min (37 °C) and 1,7 ± 0,1 min (42 °C) (Table IV).

5. DISCUSSION

The medium-chain acyl-CoA dehydrogenase deficiency (MCADD) is the most common defect in a group of inherited acute life-threating disorders of mitochondrial fatty acid oxidation. Usually, the clinical presentation of MCADD is related to fasting periods and increased metabolic stress during infancy, which may lead to acute symptoms, such as drowsiness or lethargy which may lead into coma. At its first presentation, about 25% of patients die during a metabolic crisis, however the prognosis is excellent when the diagnosis of MCADD is established in time and proper therapeutic measurements are taken (46, 47, 48). Based on this information and the fact that preventive therapy is simply based on the avoidance of fasting and on high carbohydrate intake, many countries have decided to introduce MCADD into their NBS. The latter revealed that the MCADD prevalence is much higher than expected regarding only clinically detected patients (Table V) and the presence of boarder and more complex spectrum of enzyme mutations (11).

Table V: Comparison of MCADD prevalence based upon clinically detected cases and following newborn screening estimates in selected countries (25).

Country	Prevalence of clinically detected cases (A)	Prevalence from newborn screening (B)
England	1:30.700	1:12.700
Southern Germany	1:38.000	1:13.100
Australia	1:47.300	1:21.300

Interestingly, since the inclusion of MCADD into NBS programs, the frequency of homozygote patients presenting the c.985A>G mutation, forming the mature protein K304E, decreased from 80% to 30-80% and many novel rare mutations have been discovered. In the majority of cases, DNA mutation analyses revealed that the newborns who are not homozygous for the c.985A>G point mutation, are in fact compound heterozygotes for the c.985A>G and another mutation, not yet being reported previously in clinically ascertained patients or a completely novel mutation (46). Among the newborns screened in the Laboratory of Genetic Metabolic Diseases, Academical Medical Center of the University of Amsterdam, more than 15 novel mutations were discovered, including those resulting in the p.G224R and p.L238F proteins, which were functionally characterized in this study.

It is still controversial whether the subjects with novel mutations will be at risk later in life, as this does not always mean that such genetic alterations are the ones responsible for the onset of MCADD. Therefore it needs to be determined whether a new mutation results in a protein with such changes at its functional and/or structural level, which may result in a:

- complete loss of function;
- impaired enzymatic activity;
- conformational alterations leading to its subsequent degradation by the quality control system of a cell (proteasome);
- conformational changes causing abnormalities in its folding, especially when exposed to higher temperatures.

In vitro characterization of each new mutation, focusing on the assessment of the characteristics, is of utmost importance for foreseeing the prognosis of a patient carrying such mutated protein. In our study we did this by determining oligomeric profiles, residual enzymatic activities, kinetic parameters and structural stabilities by using limited proteolysis and DLS of selected recombinant hMCAD variants. It is extremely important to estimate the degree of severity imposed by each new genotype, because the dietary treatment implemented for decreasing the likelihood of a metabolic derangement might itself be a risk. Namely the pre-pubertal children with MCADD can readily become overweight, which brings a set of new life-threatening complications. Therefore, dietary treatment encouraging increased food consumption and prescribing late-evening meals with bedtime snacks must be applied with caution and only when necessary.

Our focus was to study novel mutations with the *ACADM* gene encoding the p.G224R and p.L238F variant proteins. All the assays performed were done in parallel for the Wt protein and the p.K304E form of hMCAD, in order to provide a frame of reference. We compared the results obtained for the p.G224R and p.L238F proteins against those for the p.K304E and the hMCADwt, with the latter representing the “normal” behavior or “normal” results. The p.G224R and p.L238F variants were also compared to those of p.K304E, due to a fact, that the most severely affected MCADD patients have either one or two copies of the c.985A>G missense mutation (46).

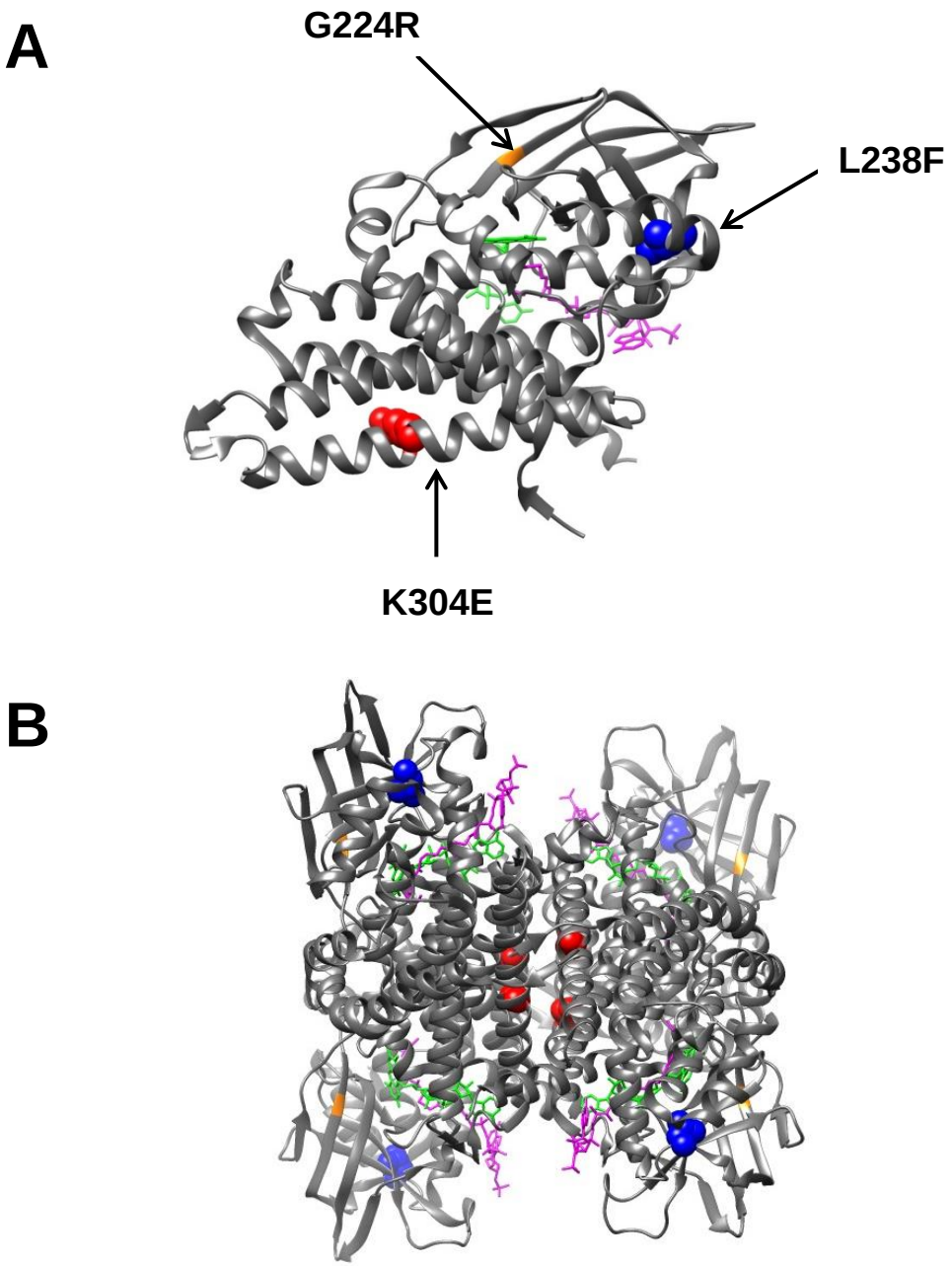

Figure 17: Structural representation of: (A) the hMCAD wild-type monomer showing localization of these mutations (space filling representation): p.K304E – red; p.G224R – orange; p.L238F – blue. The FAD cofactor and substrate (stick representation); FAD – green and thio-C8-CoA – magenta, respectively. (B) The hMCAD wild-type tetramer and localizations of studied mutants. Figures were created with the UCSF Chimera version 1.8 from PDB 1UDY software.

Results of assays determining characteristics of the p.K304E protein differ a lot in all so far published studies, proving how important it is to compare different mutated proteins either to the Wt or to another well-defined mutant which are expressed, purified, separated and analyzed under the same conditions (17, 49, 50, 51). We are convinced, that the only useful information is obtained from the relation between the Wt protein and the mutated

variant studied. This approach was used for comparing experimental data of the p.K304E and the Wt in our study with already reported results.

5.1. The p.K304E variant

The c.985A>G point mutation is considered a severe mutation, encoding for the mature protein K304E (also referred to as its precursor form p.K329E). About 80% of symptomatic MCADD patients of European origin are homozygous for this mutation, so it is no wonder that this protein is the most extensively characterized hMCAD variant.

The mutation is located in the C-terminal α-domain (Fig. 18) of the hMCAD protein and is often reported to affect helix-helix interactions that are crucial for tetramer assembly (17). This fact is additionally supported with the oligomeric profile data gathered in our study, where we observed that the p.K304E protein contained a much lower percentage of the tetrameric form (61,4%) in comparison to the Wt (84,2%) and a much higher amount of dimers and monomers, when purified in the absence or presence of FAD. Modifications in interactions crucial for tetramerization may lead to protein misfolding and aggregation, which we actually observed as significantly higher percentage of aggregates in p.K304E, as compared to the Wt protein, in particular under stressed conditions. In fact, faster aggregation rate in respect to the hMCADwt protein was demonstrated when analyzing the p.K304E enzyme by DLS: about 4 min at 37 ºC and 2 min at 42 ºC for the p.K304E variant vs. about 10 min at 37 ºC and 4 min at 42 ºC for the hMCADwt protein.

Using this approach, we were able to identify the temperature of aggregation, at which the protein starts to denature and aggregate. This was determined to be approximately at 36 °C for the p.K304E variant, which is 5 °C earlier than in the case of the Wt, actually falling within the frame of normal body temperature. The exact same difference (5 °C) between the temperatures at which the hMCADwt and the p.K304E variant begin to aggregate has already been reported before (52). It is also known that the p.K304E protein is highly unstable and our results confirm this. Besides thermal instability the unstable structure of the p.K304E protein was additionally shown by limited proteolysis by trypsin, where within the 50% reaction time (30 min) its full length reduced to 56,1%, which is much lower, as compared to the hMCADwt (90,0%). The data on the structural instability of the p.K304E variant gathered in our study are therefore in accordance to previous findings (26, 55, 56).

When determining the functional characteristics of the p.K304E protein we identified 24,8% of its residual activity when it was purified in the presence of FAD. The enzymatic activity of the p.K304E variant was compared to the hMCADwt, with the activity of the latter being set to 100%. Similar results were also observed in many others studies, where the reported residual activity of the p.K304E variant was between 5-40% (24, 29, 49, 52).

The kinetics of the hMCAD catalyzed reaction with octanoyl-CoA as the substrate and DCPIP as the final electron acceptor were studied with each of the purified proteins. One of the kinetic parameters to be determined according to the Michaelis-Menten equation was the V_{max}, which reflects how fast the enzyme can catalyze the reaction. The V_{max} of the p.K304E variant was only one third of the Wt, which is once again in accordance with previous results (53). For the K_m parameter the value of 2,82 µM was determined for the hMCADwt, which also compares well to previously published result of 3.4 µM, as well as to the K_m value determined in our study for the p.K304E variant (49, 50). This proves that the affinity of this variant protein towards octanoyl-CoA is comparable to that of the hMCADwt. On the other hand its catalytic efficiency was found to be about three times lower than that of the hMCADwt.

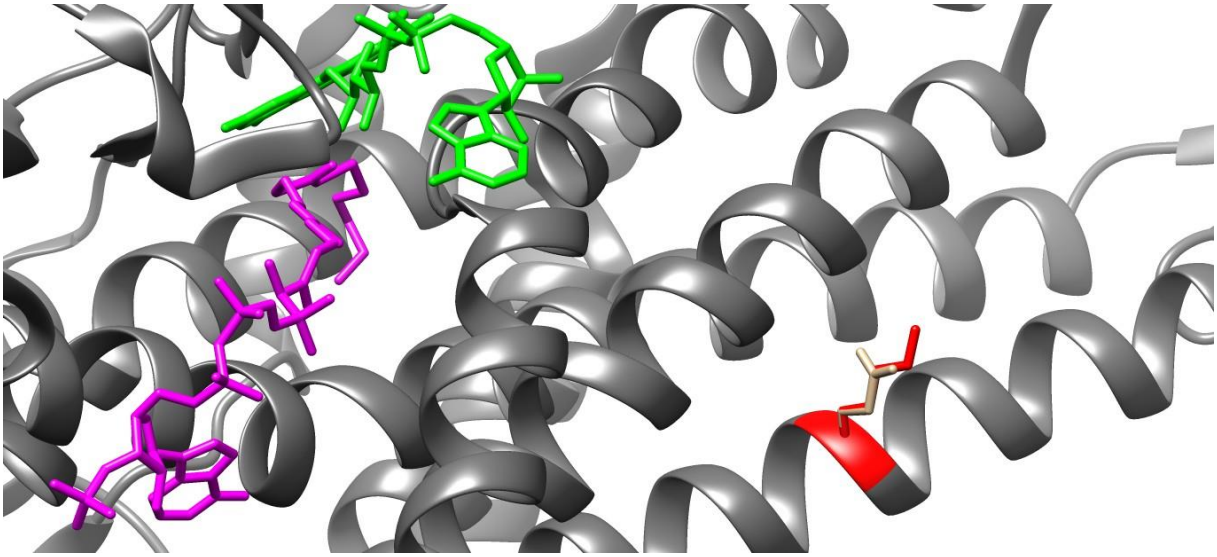

Figure 18: Representation of the p.K304E variant. Red color represents the Wt residue and beige the p.K304E residue upon the presence of mutation, cofactor and substrate (stick representation) are also presented: FAD in green and thio-C8-CoA in magenta, respectively. The figure was created with the UCSF Chimera version 1.8 from PDB 1UDY software.

We found that some functional characteristics of the most common mutant p.K304E were comparable to those of the Wt, while some of those were 2-3 times lower, like the enzyme activity. If the p.K304E mutant would have been structurally stable, the residual activity and kinetic parameters would be sufficient enough for its normal functional efficiency,

possibly insuring asymptomatic status of MCADD in a well-controlled patient. However, under stress conditions such as increasing temperature, the p.K304E variant started to aggregate faster than the hMCADwt, reinforcing the conviction that the p.K304E may undergo misfolding in certain cellular conditions leading to progressive loss of its activity and to MCADD. Currently there are many ongoing investigations searching for compounds that may act as protein stabilizers chaperones, which would, when administered to the patient, promote the rescuing of the p.K304E variant native conformation of the and stabilize its structure, therefore restoring its activity. New reports claim a discovery of a chaperone which can increase p.K304E activity by 154% (55). Misfolded proteins with some residual activity, such as the p.K304E, present the basis for studying the mechanisms of action of chaperones. Therefore the search for molecules that can stabilize the p.K304E variant is an excellent approach to design therapeutic strategies for MCADD patients harboring this common mutation.

5.2. The p.G224R variant

The c.745G>A point mutation within the *ACADM* gene encodes the mature protein variant p.G224R. The result of this mutation leads to a really harsh substitution of amino acid residues, as glycine, the smallest of the 20 amino acids, commonly found in proteins, is replaced with arginine. This mutation affects β-domain of the MCAD polypeptide, which is exposed at the surface of the enzyme and comprises two orthogonal β-sheets. Even though one study has reported that conformational changes and destabilization are most pronounced in mutations mapping the β-domain of the MCAD, our results show comparable structural characteristics between the Wt and the p.G224R variant when analyzing protein stability by limited proteolysis and increasing temperature, thereby proving that it is impossible to equate the impacts of different mutations affecting the same domain of the enzyme (17). Conformation flexibility determined by limited proteolysis, following 30 min of protein exposure to trypsin, showed that the residual amount of full-length protein was 86,8%, being equivalent to that of the Wt (90,0%) and was 30% higher than in the case of the most pathogenic mutated variant, the p.K304E. Furthermore, when analyzing proteins by DLS, we evaluated the kinetics of their aggregation and found out that at 37°C the p.G224R variant starts to aggregate after approx. 6 min, i.e. 3 min earlier than the Wt and 2,5 minutes later than the p.K304E variant. Under the conditions which mimic the fever in human body (42 °C), the p.G244R

variant started to aggregate at approx. 3 min also substantially comparable to Wt. The temperature of aggregation of this variant was at 37,5 °C, which is 2,5 °C higher than in case of the p.K304E variant, which is known for being very unstable.

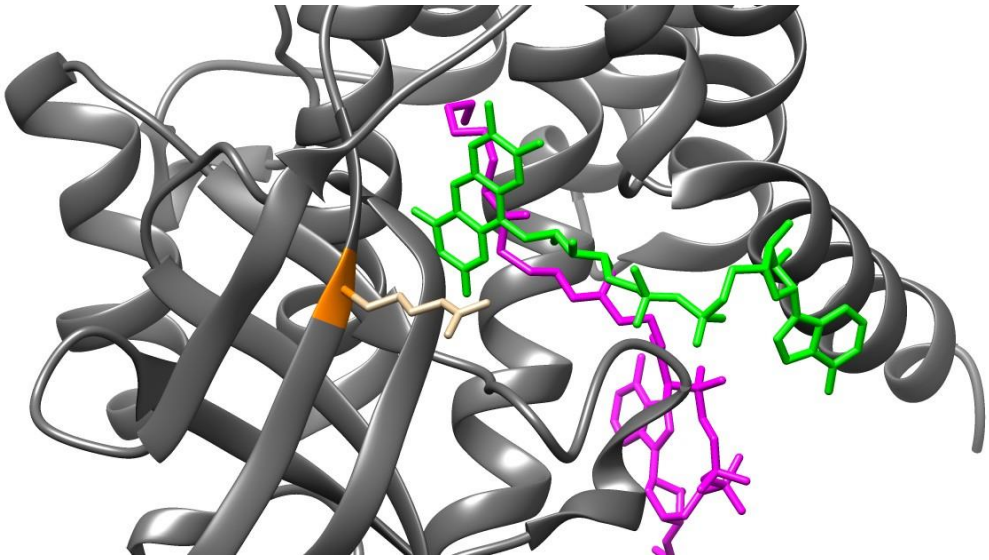

Figure 19: Representation of the p.G224R variant. Orange color represents the Wt residue and beige the p.G224R residue upon the presence of mutation, cofactor and substrate (stick representation) are also presented: FAD in green and thio-C8-CoA in magenta, respectively. The figure was created with the UCSF Chimera version 1.8 from PDB 1UDY software.

Out of all analyzed enzymes the p.G224R demonstrated the lowest residual enzymatic activity, which was only 2% when the FAD was present in the lysis buffer, while the most pathogenic variant showed a 25% activity. When determining the kinetic parameters of the p.G224R protein, we used the V_{max}*X/(K+(X*(1+X/K_i))) and not the *Michaelis-Menten* equation, due to an observed inhibition of its activity in the presence of increasing concentrations of the substrate. The substrate inhibition, which occurred at higher substrate concentrations was about 20% of that found with all known enzymes. It is primarily induced by the binding of more than one substrate molecule to the active site which is meant just for binding one of them (56). This may actually explain the very low residual activity of the p.G224R variant. By adapting our measurements to the appropriate equation mentioned before, we determined that the catalytic efficiency of the p.G224R variant was 6-times reduced in comparison to the Wt enzyme and even 2-times lower than that of the p.K304E variant. The parameter K_{cat} or the "turnover number" which reveals the number of times each enzyme's site converts substrate to a final product per unit of time, was the lowest of all enzymes studied (24,6 min^{-1}). This mutation also affected the maximum velocity and substrate affinity, which were again the most reduced among all

the studied enzymes. The reason for the lowest affinity of the p.G224R variant may be hidden in the fact that catalytic centers consisting of substrate binding sites are mainly formed by the interface between the β-domain and the C-terminal α-domain, where the p.G224R amino acids substitution actually occurs (17).

Contrary to the protein resulting from the most frequent mutation, i.e. K304E variant, the p.G224R variant has a reasonably stable structure. However its catalytic parameters are significantly reduced, due to the severity of the glycine to arginine substitution. The effect of this substitution was especially evident in a very strong reduction of its residual activity being less than 5%, making it a classical MCADD, according to the German algorithm (23). Our results suggest that the MCADD patients with the mutation encoding for the p.G224R variant are in need for dietary treatment and close clinical monitoring.

5.3. The p.L238F variant

The c.789A>C point mutation in the *ACADM* gene encoding the p.L238F protein also leads to substitution of amino acid residues in the β-domain, as in the case of the p.G224R variant. The result of this missense mutation is that the highly hydrophobic and aliphatic leucine is substituted with phenylalanine, having similar characteristics in size and hydrophobicity (Fig. 20). As already mentioned above, amino acid substitutions in the β domain are associated with particularly unstable proteins which are prone to severe conformational distortions. Such changes can induce even degradation of the protein. In fact, this possibility could explain a low yield of the purified p.L238F variant. We have verified that this low purity was not due to the loss of protein in the insoluble fraction during the purification process. Therefore, we were obliged to use a 4-times larger volume of cell culture in comparison to other proteins studied in order to achieve sufficient amount of protein needed to carry out the whole set of planned assays.

The hypothesis that the p.L238F is a highly unstable protein was additionally confirmed by observing the influence of the FAD addition of in to the lysis buffer. Namely, the presence of FAD during cell lysis improved the outcome of subsequent assays, suggesting a considerable loss of FAD during the purification process and consequently weaker interactions between the cofactor and the binding site in this variant. This can be explained by the position of the p.L238F mutation, which is situated on the FAD binding site. The highest difference between the absence and presence of FAD during purification process was observed in DLS analysis. Actually, in the presence of FAD we could observe that

aggregation temperature and aggregation kinetics at 37 and 42 °C were equivalent to those of the Wt, however when FAD was not added to the lysis buffer we detected even lower thermal stability than in the case of the very unstable p.K304E variant. Additionally, limited proteolysis by trypsin, completed after p.L238F purification in the presence of FAD, resulted in 73% of the residual full-length protein, which is almost 20% more than in the case of the p.K304E variant.

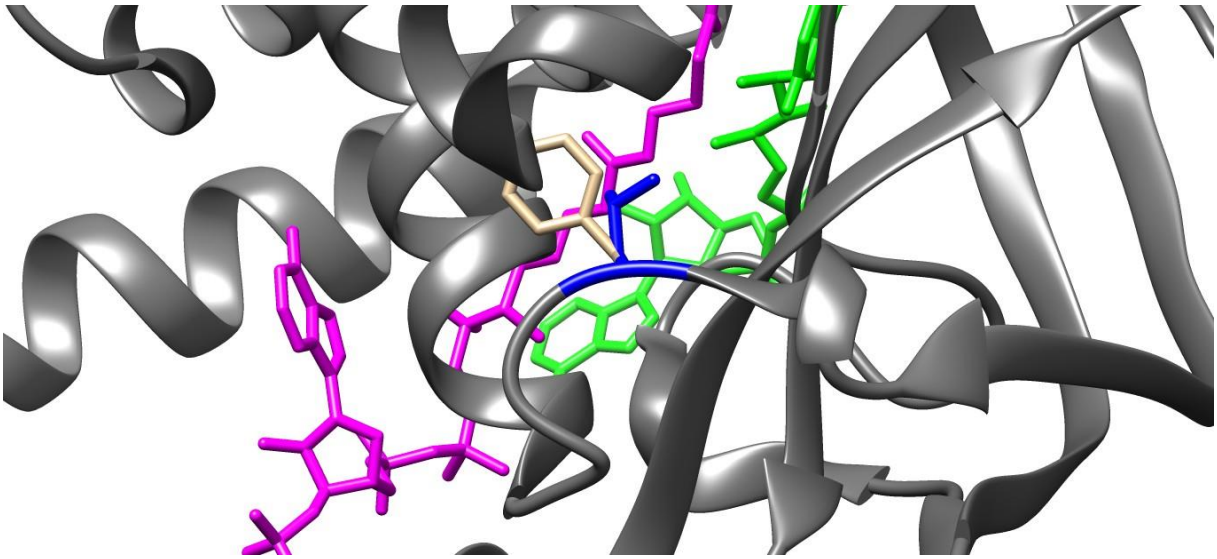

Figure 20: Representation of the p.L238F variant. Blue color represents the Wt residue and beige the p.L238F residue upon the presence of mutation, cofactor and substrate (stick representation) are also presented: FAD in green and thio-C8-CoA in magenta, respectively. The figure was created with the UCSF Chimera version 1.8 from PDB 1UDY software.

Even though this protein was poorly expressed, it displayed 80% residual activity, which is by far the highest out of all the three hMCAD variants studied. Furthermore, its kinetic parameters were highly comparable to those of the Wt, especially the catalytic efficiency which was calculated to be approx. 29 μM^{-1} min^{-1} for both enzymes. Surprisingly, the affinity towards the substrate was more than 2-times higher than in the case of the Wt, showing a remarkable functional characteristic of the p.L238F variant.

Our results show really high functional characteristics and also conformational stability of the p.L238F variant, but only when FAD was present during the purification process. Of all three mutations studied, the results obtained for the p.L238F variant clearly indicate that this variant is subjected to the highest loss of FAD during the purification process, suggesting that it easily loses this cofactor, being crucial for the protein folding and oligomerization, as already reported previously (57). This may also explain considerable lower expression levels presented by this mutant, as conformational alterations can also

cause severe protein degradations *in vivo* by the cellular quality control system. The results gathered in our study suggest that a close monitoring of patients with the p.L238F variant having a risk of metabolic decompensation, cannot be disregarded.

5.4. Influence of presence of FAD cofactor in the purification process on hMCAD proteins

The process used for purification of proteins is usually harsh and aggressive, especially when they are already structurally altered. Therefore we set out to determine how strong is the bond between the enzyme variant and their cofactor, which would give us an insight regarding their stability. Furthermore, while circumventing the loss of FAD during the purification process by supplementing it to the lysis buffer, we were also able to observe the impact of its absence on functional and structural characteristics of each of the proteins studied. The biggest increase in residual activity when FAD was added to the lysis buffer was seen in the case of the p.L238F variant, where we determined a 15% increase of its residual activity in comparison to the hMCADwt protein. Furthermore, in case of the p.L238F protein the temperature of aggregation increased for 3 °C when FAD was added during its purification and the time point at which it started to aggregate was postponed from 3,4 min to 8,8 min at 37 °C and from 1,7 min to 3,2 min at 42 °C. On the other hand, this enzyme was also the only one whose amount of tetramers reduced following the addition of FAD into purification process. However this was probably due to a poor resolution between tetrameric and dimeric fractions in the chromatography.

Interestingly, for both, the p.K304E and the p.G224R variant we measured a decrease in residual activity when FAD was present in the lysis buffer. This can be attributed to lower amounts of dimers and higher amounts of tetramers under this condition, indicating that dimers are functionally more effective forms of these enzymes. However, this outcome may also result from technical pitfalls related to, for example pipetting, and should be further studied, as enzymatic activities of p.K304E and p. G224R, both being purified in the presence of FAD, was only performed once for the first (p.K304E) and twice for the second (p.G224R) enzyme variant.

5.5. The medium-chain acyl-CoA dehydrogenase deficiency in Slovenia

A recent report states that there are no MCADD patients in Slovenia (58). However because of variable and nonspecific clinical presentation, MCADD is often not recognized

and consequently, may be underdiagnosed at the population level, especially when considering the absence of newborn screening for this pathology in a particular country (25). Furthermore, sudden death in previously healthy children and adults who were undergoing caloric restricted diet for different reasons, have been described in numerous cases of undiagnosed MCADD. Sudden death can occur as early as in the first days of life. Such deaths may often be misascribed to "sudden infant death syndrome" (SIDS) (7). Therefore, there is a possibility that the cause of death being attributed to SIDS, may be in fact a consequence of MCADD. Another possibility why there are still no registries of MCADD patients in Slovenia is, that the potentially existing patients are asymptomatic, especially as in the mentioned study reports only symptomatic patients were included (58). Plausibly, a study analyzing stored blood specimens from randomly chosen newborns would give much more accurate conclusions about the MCADD frequency in Slovenia. An example of this comes from one of the studies performed abroad, where around 100.000 stored blood spot specimens were analyzed and 8 children with MCADD were positively identified. Out of this 8 cases 1 has died, 3 had episodes of encephalopathy, 2 others had episodes of hypoglycemia in infancy and remaining 2 remain asymptomatic (25).

We believe that in Slovenia NBS should be expanded from only phenylketonuria and congenital hypothyroidism to also other metabolic disorders, especially MCADD, as the most prevalent disorder of mitochondrial β-oxidation that requires a fairly simple preventive therapy.

6. CONCLUSION

Our study was designed to estimate pathogenic potential of two novel mutations related to MCADD which were discovered with NBS. These mutations resulted in the p.G224R and the p.L238F variants. We prepared recombinant proteins and compared them to both, the Wt protein and the most prevalent and clinically presented p.K304E variant. Immediately at the beginning we faced a problem of a very low yield in case of the p.L238F variant which we resolved by performing recombinant protein expression in the increased amount of the cell culture.

For the p.G224R variant a very low residual enzyme activity was determined, as well as inferior kinetic parameters, while the p.L238F variant showed functional characteristics that were comparable to those of the Wt. However, analyses of the p.L238F variant showed that we are dealing with a very unstable protein subjected to the highest loss of FAD cofactor during purification process, among all of the hMCAD enzymes studied.

Our most surprising discovery was decreased residual enzyme activities of p.K304E and p.G224R variants, when FAD was used during the purification process. Under these conditions we observed an increase in the tetrameric and a decrease in the dimeric forms of both mutant enzymes, suggesting a higher activity of dimers. The question whether these contradictory results are reflecting possible methodologic or performance problems of the enzymatic assays used or a possibility that dimers indeed possess higher activity, still remains unanswered. The possibility that dimers actually have enzymatic activity was confirmed in additional experiments (data not shown), although we must have in mind that the results from activity measured could be due to contamination of dimeric with tetrameric forms, which requires further investigation. Additionally determination of kinetic parameters of enzyme variants purified in the presence of FAD was not performed in our study.

Another phenomenon that should be investigated in the near future is the interallelic complementation, which has already been observed in other inherited metabolic disorders, but was never addressed in studies of MCADD. Both novel mutations that we studied in our work were discovered in patients being compound heterozygous for the c.985A>G and one of these new mutations. With the knowledge that the MCAD molecule is a dimer of dimers, the possibility of interactions between subunits encoded by two different mutant alleles should not be overlooked, together with the possibility that these interactions could

result in positive or negative complementation, and consequently in higher or lower activity and/or stability compared to the corresponding homotetramer. This could explain difficulties establishing a genotype-phenotype correlations in MCADD.

Hopefully, our functional and structural characterization of selected enzymatic variants, resulting from novel mutations identified in patients during NBS, together with additional data gathered for the most pathogenic p.K304E protein variant contribute to a better understanding of the molecular basis of MCADD. This could lead to development of new and more efficacious therapeutic approaches and to better assessment of the need and magnitude of preventive therapy for the MCADD, most common inherited defect in metabolism of fatty acids.

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Annex 1. Oligonucleotides designed to mimic cDNA sequence of specific mutant hMCAD in site-directed mutagenesis reactions

Oligonucleotides [(a)]	cDNA position	Sequence (5'-3')
K304E_F	1396-1435	GCT GGC TGA AAT GGC AAT GGA AGT TGA ACT AGC TAG AAT G
K304E_R	1396-1435	ATT CTA GCT AGT TCA ACT TCC ATT GCC ATT TCA GCC AGC
G224R_F	1147-1186	CGA TGT TCA GAT ACT AGA AGA ATT GTC TTC GAA GAT GTG
G224R_R	1147-1186	CAC ATC TTC GAA GAC AAT TCT TCT AGT ATC TGA ACA TCG
L238F_F	1201-1236	GCCT AAA GAA AAT GTT TTC ATT GGT GAC GGA GCT GG
L238F_R	1201-1236	CC AGC TCC GTC ACC AAT GAA AAC ATT TTC TTT

[(a)]F-forward, R-reverse.