Review

Diseases caused by defects of mitochondrial carriers: A review

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A B S T R A C T

A strikingly large number of mitochondrial DNA (mtDNA) mutations have been found to be the cause of respiratory chain and oxidative phosphorylation defects. These mitochondrial disorders were the first to be investigated after the small mtDNA had been sequenced in the 80s. Only recently numerous diseases resulting from mutations in nuclear genes encoding mitochondrial proteins have been characterized. Among these, nine are caused by defects of mitochondrial carriers, a family of nuclear-coded proteins that shuttle a variety of metabolites across the mitochondrial membrane. Mutations of mitochondrial carrier genes involved in mitochondrial functions other than oxidative phosphorylation are responsible for carnitine/acylcarnitine carrier deficiency, HHH syndrome, aspartate/glutamate isoform 2 deficiency, Amish microcephaly, and neonatal myoclonic epilepsy; these disorders are characterized by specific metabolic dysfunctions, depending on the physiological role of the affected carrier in intermediary metabolism. Defects of mitochondrial carriers that supply mitochondria with the substrates of oxidative phosphorylation, inorganic phosphate and ADP, are responsible for diseases characterized by defective energy production. Herein, all the mitochondrial carrier-associated diseases known to date are reviewed for the first time. Particular emphasis is given to the molecular basis and pathogenetic mechanism of these inherited disorders.

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1. Introduction

Mitochondrial diseases are hundreds of clinical phenotypes caused by defects in one or more components of the mitochondrial proteome. This proteome consists of more than 1000 proteins that are encoded by either nuclear DNA or mitochondrial DNA (mtDNA). The mitochondrial genome encodes only 13 proteins which are subunits of the four respiratory chain enzyme complexes (I–IV) and ATP synthase complex (V). Both the subunits encoded by mtDNA or nuclear DNA are essential for the activity of these complexes and therefore for the accomplishment of oxidative phosphorylation.

Since 1988, when the first mutations in mtDNA were described [1,2], more than 400 mutations in mtDNA have been identified as being responsible for respiratory chain and oxidative phosphorylation diseases (http://www.mitomap.org). These mutations concern not only mtDNA genes encoding the above-mentioned 13 proteins but also mtDNA genes encoding 2 ribosomal RNAs and 22 transfer RNAs which are part of the protein synthesis machinery present in the mitochondrial matrix. Respiratory chain and oxidative phosphorylation diseases can also be caused by mutations of nuclear DNA genes that encode proteins required for a) the replication, transcription, translation, repair and maintenance of mtDNA; b) the functioning of complexes I–V (the nuclear-coded subunits) and their assembly; c) the import of the substrates of oxidative phosphorylation (see section 4 of this review); and d) the import, modification and insertion of respiratory chain cofactors such as heme, metals and the iron–sulphur clusters. mtDNA-dependent oxidative phosphorylation diseases are due either to large-scale rearrangements including deletions and duplications, which are usually sporadic and invariably heteroplasmic, or to point mutations which may be heteroplasmic or homoplasmic and are transmitted maternally. Nuclear-dependent oxidative phosphorylation disorders can induce secondary multiple mtDNA deletions or loss of mtDNA in affected tissues and follow the Mendelian pattern of inheritance.

Although the primary function of mitochondria is the synthesis of ATP by oxidative phosphorylation, these organelles play many other important roles. They contain several metabolic pathways, such as the citric acid cycle, or parts thereof, are the main site where reactive oxygen species (ROS) are generated, and are fundamental for certain biological processes such as Ca2+ cell signaling, cell proliferation and necrotic or apoptotic cell death. Some enzymes involved in ROS management, in amino acid catabolism and in the biosynthesis of fatty acids, cholesterol, steroid hormones, farnesyl and geranyl side chains, cardiolipin, ubiquinol, heme and urea are located inside mitochondria. Furthermore, mitochondria communicate with other cell compartments by means of transport proteins (carriers) present in the mitochondrial membrane that allow the selective passage of solutes in and out of the mitochondrial matrix. All these functions are accomplished by a large number of proteins which are encoded by the nuclear genome, translated in the cytosol and imported into the mitochondria.

In recent years, the completion of human nuclear genome sequencing and the ongoing identification of nuclear gene function have accelerated the disclosure of several mitochondrial disorders (apart
from oxidative phosphorylation diseases) resulting from mutations in nuclear genes encoding mitochondrial proteins. These include disorders caused by mutations of proteins involved in the citric acid cycle [3], fatty acid β-oxidation [4], cardiolipin metabolism (Barth’s syndrome) [5,6], the utilization of iron (Friedreich’s ataxia, X-linked ataxia and sideroblastic anemia) [7], antioxidant defense [8], protein import (X-linked deafness distonia) [9], network dynamics (fission/fusion) [10] and mitochondrial metabolite carriers (this review). In addition, some cancers are caused by mutations in nuclear genes encoding mitochondrial proteins [11,12]. Several reviews on mitochondrial diseases resulting from defects in the respiratory chain and oxidative phosphorylation have been published [13–19]. The contribution of mtDNA mutations, ROS generation and, in general, mitochondrial dysfunction to cardiovascular disease, diabetes, cancer, aging and aging-related neurodegenerative disorders, such as Parkinson’s and Huntington’s disease, has also been reviewed [12,20–27].

This review highlights for the first time the current molecular, biochemical, clinical and pathophysiological information about all the diseases known thus far to be associated with defects of mitochondrial carriers. As a premise, some features of mitochondrial carriers are summarized.

2. Characteristics and physiological role of mitochondrial carriers

Because mitochondria and cytosol cooperate in a large number of metabolic cellular processes, a continuous and highly diversified flux of metabolites, nucleotides, and cofactors into and out of the mitochondria is needed. The transport of solutes across the inner mitochondrial membrane is catalyzed by a family of nuclear-coded, membrane-embedded proteins called mitochondrial carriers. The carriers are involved in the Krebs cycle, oxidative phosphorylation, modulation of the nucleotide and deoxynucleotide pools in the mitochondrial matrix, the synthesis and breakdown of mtDNA, mtRNA and mitochondrial proteins, fatty acid oxidation, gluconeogenesis, lipogenesis, transfer of reducing equivalents, urea synthesis, amino acid degradation, and other functions shared by cytosol and mitochondria. The central role in cell metabolism of most mitochondrial carriers identified so far (except the pyruvate carrier, PyC) is illustrated in Fig. 1.

In man, mitochondrial carriers are encoded by the SLC25 gene family ([28] for a review). Several carriers, such as the ADP/ATP, ATP-Mg/Pi, glutamate and ornithine carriers, have isoforms encoded by different genes, and only the phosphate carrier (PiC) has two alternatively spliced isoforms. The main carriers importing substrates for oxidative phosphorylation are widely distributed in human tissues. In contrast, other carriers are tissue specific and have a limited distribution reflecting their importance in special functions. The majority of mitochondrial carriers catalyze obligatory solute exchange reactions. Those mediating H+–compensated unidirectional substrate flux may also fall into the above category, given that at least PiC has been shown to function in a phosphate/OH− antiport mode. The carnitine–acylcarnitine carrier catalyzes both unidirectional transport of carnitine and the carnitine/acylcarnitine exchange, whereas the uncoupling protein catalyzes uniport as the exclusive transport mode. As their driving force, mitochondrial carriers utilize the concentration gradient

![Fig. 1. Metabolic roles of mitochondrial carriers. The scheme shows 15 carriers catalyzing metabolite transport through the inner mitochondrial membrane. These carriers are involved in: oxidative phosphorylation (AAC, PiC, UCP); oxidation/reduction pathways (AGC, OGC, DIC, CIC, CAC, PyC); homeostasis of the intramitochondrial adenine nucleotide pool (APC); methylation of mtDNA, mtRNA and some intramitochondrial proteins (SAMC); import of the essential coenzyme thiamine pyrophosphate (TPP) required for pyruvate- and oxoglutarate–dehydrogenase complex activities (TPC); and, with partial overlap, amino acid metabolism (AGC, OGC, GC, ODC). The scheme does not show all the metabolic pathways in which the already identified carriers are involved.](image-url)
of the solutes and/or the H⁺ electrochemical potential gradient (ΔµH⁺) generated across the inner mitochondrial membrane by the function of the respiratory chain. Mitochondrial carriers can be divided into electrogenic (or electrophoretic) and electromotive transporters depending on whether their transport reactions result in charge imbalance. The intra-/extra-mitochondrial distribution of solutes transported by electrogenic carriers is regulated by the electrical component of ΔµH⁺, whereas the distribution of solutes carried by the proteins transporting anions together with an equivalent amount of H⁺ (anion/H⁺ symport) or in exchange for OH⁻ (anion/OH⁻ antiport) is regulated by the transmembrane pH gradient [29].

All primary structures of the mitochondrial carrier family members consist of three tandemly repeated homologous domains of about 100 amino acids in length. Each repeat contains two hydrophobic stretches, that span the membrane as α-helices, and a characteristic signature motif P-X-[DE]-X-X-[RK]-[28–30 residues]-[DE]-G-X-X-X-YWLF]-[RK]-G. Based on the sequence features described above and on the accessibility of carriers to peptide-specific antibodies, proteolytic enzymes and impermeable reagents, each mitochondrial carrier monomer has six helices traversing the membrane connected by hydrophilic loops with both the N- and C-termini on the cytosolic side of the inner mitochondrial membrane. In 2003, the 3D structure of a member of this protein family, the ADP/ATP carrier in complex with its inhibitor, carboxyatractyloside, was determined [30]. This structure, whose pseudo-three-fold symmetry [30,31] is in agreement with the fact that each three-fold sequence repeats, consists of six transmembrane α-helices (H1–H6) and three short α-helices (h₁₂, h₃₂ and h₅₀) parallel to the membrane plane on the matrix side [30]. In each repeated domain, the first part of the signature motif P-X-[DE]-X-X-[RK] is located at the end of the odd-numbered transmembrane α-helices, whereas the second part [DE]-G-X-X-X-YWLF]-[RK]-G is located immediately before the even-numbered transmembrane α-helices. The prolines of the signature motif P-X-[DE]-X-X-[RK] sharply kink the odd-numbered transmembrane α-helices, and the charged residues of the three signature motifs form a salt bridge network that closes the water-accessible cavity that is exposed towards the cytosolic side of the mitochondrial membrane and is occupied by the inhibitor.

By using comparative models of carriers with known substrate specificity, a common substrate-binding site was proposed [32]. This site is formed by residues which belong to the three even-numbered transmembrane α-helices and protrude into the cavity at the midpoint of the membrane, one-and-a-half helix turns above the salt bridge network. The significant sequence conservation in the mitochondrial carrier family suggests that the structural fold and transport mechanism are similar for all carriers. Although the conformational changes involved in substrate translocation are not yet known, it is currently thought that translocation might be triggered by substrate-induced rearrangement of the salt bridge network from inter- to intra-domain interactions [33] and references therein.

The human genome encodes about 50 different mitochondrial carriers. In the last decade, a significant advance in the field of mitochondrial transport has been the identification of many mitochondrial carrier family members by over-expression in Escherichia coli and/or Saccharomyces cerevisiae, purification and reconstitution into liposomes for functional characterization by transport assays. Until now, almost 30 human carriers have been characterized in terms of substrate specificity, inhibitor sensitivity, transport mechanism, tissue distribution and kinetic characteristics [28] for a review. [34–37]. Naturally, this extension of the mitochondrial carrier family members has greatly favoured the discovery of their involvement in several diseases.

3. Diseases associated with mitochondrial carriers

Mitochondrial carrier-related diseases are rare errors of metabolism caused by alterations of nuclear genes encoding mitochondrial carriers. The nine known disorders are listed in Table 1. Carrier acronyms throughout the text are those used in ref. [28]; aliases are mentioned in the same reference. These disorders are all transmitted by Mendelian inheritance; with the exception of autosomal dominant progressive external ophthalmoplegia (adPEO), the others are inherited in an autosomal recessive manner.

Mitochondrial carrier-related diseases can be divided into two groups. The first concerns defects of mitochondrial carriers directly linked to oxidative phosphorylation, such as the ADP/ATP carrier and the PiC. The symptomatology of these diseases is characterized by insufficient energy production in tissues where these carriers are expressed and play an important role in oxidative phosphorylation. These diseases should also include adPEO, which is caused by heterogeneous mutations of the ADP/ATP carrier isoform 1 gene (as well as of other nuclear genes), because these mutations cause mtDNA instability.

The second group of diseases is due to alterations of the genes encoding mitochondrial carriers that are required for mitochondrial functions other than oxidative phosphorylation and, in particular, intermediary metabolism. Their symptomatology depends on the metabolism affected and its significance in specific tissues.

4. Diseases due to defects of mitochondrial carriers directly linked to oxidative phosphorylation

Given that the catalytic site of the ATP synthase complex is exposed towards the mitochondrial matrix, the uptake of inorganic phosphate (Pi) and ADP into the mitochondria is essential for the oxidative phosphorylation of ADP to ATP (see Fig. 1). These uptake rates are catalyzed by two distinct carriers, the PiC and the ADP/ATP carrier, respectively; very recently, deficiencies of one isoform of both carriers have been described.

4.1. AAC1 deficiency

In 2005, a homozygous mutation (c.368C → A) was found in SLC25A4 gene of a 25-year-old male patient of Slovenian origin [38]. SLC25A4 encodes the heart-/muscle-specific ADP/ATP carrier (AAC1, also termed ANT1). The mutation involves a highly conserved alanine at position 123 that is replaced with an aspartic acid (A123D). In the crystallographic structure of bovine AAC1, A123 protrudes into the central cavity two helix turns above the salt bridge network (slightly above the level of the proposed binding site) and is localized in the third transmembrane segment within the consensus sequence GXXG, which is thought to be involved in high-affinity associations between transmembrane α-helices [39]. The substitution of A123D results in a complete loss of the protein's ability to transport ADP and ATP in reconstituted liposomes [38]. Interestingly, in contrast with the other AAC1 mutations found so far in humans (see 4.4), A123D is the first recessive mutation found in the AAC1 gene.

The patient affected with AAC1 deficiency (OMIM 103220) manifested exercise intolerance, lactic acidosis, hypertrophic cardiomyopathy and a mild myopathy with no PEO. Exercise intolerance with

<table>
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<td>Disorder</td>
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<td>SLC25A4</td>
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<td>Sengers’ syndrome</td>
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<tr>
<td>PIC deficiency</td>
<td>SLC25A3</td>
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<td>adPEO</td>
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<td>CAC deficiency</td>
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Carrier acronyms are taken from ref. [28]; aliases are mentioned in the same reference.
easy fatigability and muscle pain was present since early childhood and ventricular hypertrophy was diagnosed during puberty, indicating that the disease is slowly progressive. Serum lactate levels in fasting conditions and at rest were higher than normal. In the skeletal muscle of the patient, numerous ragged-red fibers and multiple mtDNA large-scale deletions were present (without a reduction of the wild-type mtDNA copy number). The activities of the three mtDNA-dependent respiratory chain complexes (I, III and IV) were lower than normal and citrate synthase activity was much higher than normal, probably due to mitochondrial proliferation. Interestingly, this symptomatology closely resembles that of the AAC1 knock-out mice [40]. As in the mouse model, loss of AAC1 function is compatible with adult life, presumably due to compensation by glycolysis and/or transport activities of AAC isoforms or other adenine nucleotide mitochondrial carriers, such as the ATP-Mg/Pi carrier.

4.2. Sengers’ syndrome

AAC1 deficiency was previously demonstrated in two unrelated patients with Sengers’ syndrome (OMIM 212350), an inherited autosomal recessive disease characterized by congenital cataracts, hypertrophic cardiomyopathy, mitochondrial myopathy and lactic acidosis but not PEO [41]. In the muscle tissues of both patients, the protein content of AAC1 was drastically reduced. Furthermore, adenine nucleotide transport was strongly impaired in liposomes reconstituted with mitochondrial extracts of the patients’ skeletal or heart muscle. No mutation was found in the SLC25A4 gene and involvement of the AAC1 locus was excluded by linkage analysis. The genetic defect underlying Sengers’ syndrome is still unknown. Nevertheless, AAC1 deficiency appears to be causally related to the clinical symptoms and tissue specificity of the disease. It may be that defective transcription, translation or targeting of AAC1 is responsible for this disorder.

4.3. PiC deficiency

In 2007, a homozygous mutation in the gene encoding the PiC (SLC25A3) (c.215G→A) was found in two ill siblings of non-consanguineous Turkish parents (the parents were heterozygous for the same mutation) [42]. The human gene, which is spread over 8.3 kb of DNA, maps to 12q23.1 and contains 9 coding exons; of these, exons 3A and 3B are closely related and spliced alternatively [43]. The resulting isoforms, termed PiC-A and PiC-B, differ in 13 amino acids generated by electron transport. This uptake of Pi into mitochondria at the expense of the pH component of the protonmotive force generated by electron transport. This uptake of Pi into mitochondria is essential for oxidative phosphorylation of ADP to ATP. Like isoforms of other mitochondrial proteins involved in oxidative phosphorylation, PiC-A is abundantly expressed only in heart and muscle, whereas PiC-B is expressed in all tissues [44,45]. The differences between the two PiC isoforms in kinetic properties (Vmax of PiC-B is 3-fold lower than that of PiC-A, and Vmax of PiC-B is 3-fold higher than that of PiC-A) and abundance in tissue (69% of PiC-B per mg protein, and 10% and 8% of PiC-B per mg protein in heart and liver bovine mitochondria, respectively [45]) may account for the variation in reliance of the tissues on oxidative phosphorylation. The ubiquitous PiC isoform B might match the basic energy requirement of all tissues, and isoform PiC-A might become operative to accommodate the higher energy demands associated with contraction of striated muscle fibers.

Interestingly, the c.215G→A mutation found in the two patients is localized in exon 3A and therefore affects the heart-/muscle-specific isoform, PiC-A. In the PiC-A of both patients glycine 72, that is conserved in all known PiC sequences and in most other mitochondrial carriers and is located in the first transmembrane helix three helix turns from the membrane’s cytosolic side, is substituted by a glutamic acid residue; this substitution is deleterious for protein function [42].

PiC deficiency (OMIM 610773) is characterized by muscular hypotonia, progressive hypertrophic cardiomyopathy, elevated plasma lactate levels, and lactic acidosis. In one of the two affected siblings, cyanosis and poor weight gain were also observed. The patients died of heart failure at 4 and 9 months.

In accordance with the tissue specificity of disease symptomatology, the synthesis of ATP by oxidative phosphorylation was defective only in muscle. Indeed, it was found that in heart mitochondria, but not in digitonin-permeabilized fibroblasts, ADP-stimulated respiration of pyruvate was drastically reduced, whereas uncoupler-stimulated respiration was normal [42]. Furthermore, the activities of respiratory chain enzymes, pyruvate dehydrogenase and oligomycin-sensitive ATP-ase were normal. No mutation was detected in the genes of ATP synthase subunits encoded by mtDNA or in SLC25A4 encoding the heart-/muscle-specific isoform of the ADP/ATP carrier. In view of the above-reported results, the disease is due to a defect in the PiC-A-mediated import of Pi into mitochondria, which prevents ATP synthesis by oxidative phosphorylation.

4.4. Autosomal dominant progressive external ophthalmoplegia (adPEO)

adPEO is a clinically and genetically heterogeneous disorder that is usually inherited as a dominant trait. It is caused by defects in certain nuclear genes and is characterized by multiple deletions of mtDNA in post-mitotic tissues [46–48]. Therefore, adPEO constitutes an example of nuclear gene defects affecting mtDNA. The mtDNA deletions result in a lack of respiratory chain proteins that lead to defective energy production. adPEO manifests an adult-onset phenotype (typically at 20–40 years of age); however, early onset has also been reported. Typical symptoms of the disorder are PEO due to weakness of the external eye muscles, ptosis, and mild descending myopathy. Addi-
tional variable symptoms include bilateral cataract, sensorineural hypacusia, tremor, ataxia, sensorimotor peripheral neuropathy, generalized muscle weakness, exercise intolerance, depression, parkinsonism and endocrine dysfunction. Serum lactate levels are normal or slightly increased. Muscle biopsies of affected individuals exhibit ragged-red fibers, cytochrome c oxidase-negative fibers, and diminished activity of respiratory chain complexes. Southern blot analysis of muscle DNA reveals the presence of heterogeneous mtDNA species due to multiple large-scale deletions ranging from 3.0 to 8.5 kb in length. mtDNA lesions are present and accumulate in post-mitotic tissues, mainly brain, muscle and heart. This is consistent with the tissue distribution of AAC1 in man, since AAC1 is the predominant isoform of the ADP/ATP carrier in skeletal and heart muscle but is also present in brain.

PEO is one of the most common diseases caused by primary mtDNA deletions. However, more recently, mutations of three nuclear genes have also been shown through linkage analysis to be responsible for adPEO by causing secondary multiple deletions of mtDNA. One of these nuclear genes is SLC25A4 located on chromosome 4q34 and encoding the heart-/muscle-specific mitochondrial AAC1 (OMIM 609283). The other two genes are Twinkle, on chromosome 10q24 encoding an mtDNA helicase (OMIM 606075), and POLG1 on chromosome 15q25 encoding the catalytic subunit of mtDNA-specific polymerase γ (OMIM 157640). Furthermore, at least a fourth locus must be implicated in view of cases that could not be linked to any of the three genes reported above. Nuclear-dependent PEO is usually inherited as an autosomal dominant disorder, but cases with autosomal recessive inheritance

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have been described in families carrying heterozygous mutations in the \textit{POLG1} gene. Mutations in the \textit{POLG1} and \textit{Twinkle} genes are the most frequent cause of PEO, whereas those in the \textit{SLC25A4} gene are rarer. Four heterozygous missense mutations of \textit{AAC1} (A90D, A114P, 198P, D104G) have been found in adPEO families and one (V289M) in a sporadic case. In the crystallographic structure of bovine \textit{AAC1}, these mutations interface with the membrane except for D104G which is located in the cytosolic loop between H2 and H3. All the mutations are near the cytosolic side of the carrier protein, three to four helix turns from the common binding site. \textit{AAC1}-related adPEO is relatively benign because the symptoms are usually limited to skeletal and facial muscles. As for all diseases caused by mutations of mtDNA, treatment of adPEO is symptomatic and includes eye props and ptosis surgery.

The \textit{Twinkle} and \textit{POLG1} genes are directly involved in the repair and replication of mtDNA, whereas \textit{AAC1} catalyzes the exchange of cytosolic ADP for intramitochondrial ATP. Therefore, the mechanism by which \textit{AAC1} mutations cause mtDNA instability is unclear. Several hypotheses have been set forth; the mutations may: modify the transport properties of \textit{AAC1}; alter the intramitochondrial pool of adenine nucleotides causing a shortage of ADP, which may be insufficient for DATP synthesis through the action of ribonucleotide reductase and nucleoside diphosphokinase and, consequently, yield a high error rate of mtDNA polymerase; or cause a defective import of the mutated proteins into mitochondria, leading to misfolded proteins that are more susceptible to ROS or to an increased production of ROS within the mitochondria and damage of mtDNA. To investigate the functional consequences of the human \textit{AAC1} mutations found in adPEO patients, the mutations were introduced at equivalent positions in \textit{AAC2}, the yeast ortholog of human \textit{AAC1} [49]. Interestingly, expression of some of these mutants in \textit{aac2}-defective haploid strains of \textit{S. cerevisiae} caused a growth defect on nonfermentable substrates, a decrease of cytochrome content and reduced respiratory activity, but variable inhibition of ATP and ADP transport as deduced from measurements in liposomes reconstituted with mitochondrial extracts obtained from cells transformed with either each mutant or wild-type \textit{AAC2}. In addition, heteroallelic strains, expressing both the \textit{aac2} mutant allele and wild-type \textit{aac2}, behaved as a recessive trait for oxidative growth and as a dominant trait for mtDNA instability. Therefore, the dominant feature of mtDNA alteration induced by pathogenic \textit{aac2} alleles is similar in yeast and adPEO patients.

5. Diseases due to defects of mitochondrial carriers involved in intermediary metabolism

The diseases of this group known so far are \textit{CAC} deficiency, HHH syndrome, \textit{AGC2} deficiency, Amish microcephaly and neonatal myoclonic epilepsy. They will be reviewed in the following sections in the order in which they have been associated for the first time to alterations of a specific mitochondrial carrier gene.

5.1. \textit{CAC} deficiency

Carnitine–acylcarnitine carrier (\textit{CAC}) deficiency\footnote{Also known as carnitine/acylcarnitine translocase (\textit{CACT}) deficiency.} (OMIM 212138) is the first disease to have been associated with a member of the \textit{SLC25} family, \textit{SLC25A20} [50,51]. This gene, which spans about 42 kb, contains 9 coding exons, maps to chromosome 3p21.31 and encodes the \textit{CAC}, a protein of 301 amino acids. The rat \textit{CAC} was purified in 1990 and 7 years later its cDNA was cloned in rat and man [50,52] and references therein]. \textit{CAC} catalyzes a 1:1 electroneutral exchange between a molecule of acylcarnitine, that enters mitochondria, and a molecule of free carnitine, that exits the organelles (see Fig. 3K of ref. [28]). The \textit{CAC} function is conserved in all eukaryotes, although mammalian \textit{CAC} has a higher affinity for long-chain than for short-chain acylcarnitines and the lowest affinity for free carnitine, in contrast with the fungal carriers [53] and references therein]. In particular, \textit{CAC} is the key component of the carnitine cycle which consists of three steps: i) transfer of acyl groups from acyl-CoA to carnitine through carnitine palmitoyltransferase (\textit{CPT I}), an outer mitochondrial membrane enzyme; ii) \textit{CAC}-mediated transport of acylcarnitines across the inner mitochondrial membrane in exchange for free carnitine; and iii) transfer of acyl groups from acylcarnitines to CoA inside the mitochondria through \textit{CPT II}, an inner mitochondrial membrane enzyme (see Fig. 3K of ref. [28]). By catalyzing the carnitine/acylcarnitine exchange, \textit{CAC} allows the import of fatty acyl moieties into the mitochondria where they are oxidized by the \(\beta\)-oxidation pathway. This pathway is the major source of energy for heart and skeletal muscles during fasting and physical exercise. Besides the exchange, \textit{CAC} is also able to perform unidirectional transport of substrates across the inner mitochondrial membrane but at a much lower rate (about one tenth that of the exchange); nevertheless, unimport of carnitine into carnitine-depleted mitochondria is physiologically important to balance the matrix level of carnitine with that present in the cytosol, a prerequisite for optimal carnitine/acylcarnitine activity. Given that \textit{CAC}-mediated transport is an essential step in the long-chain fatty acid \(\beta\)-oxidation pathway, \textit{CAC} deficiency is a fatty acid \(\beta\)-oxidation disorder that prevents the body from converting long-chain fatty acids into energy.

\textit{CAC} deficiency was first described in 1992 by Stanley et al. [54]. It is a severe autosomal recessive, nonpopulation-specific disorder presenting an equal male-to-female ratio. The most affected organs are heart, liver, brain, and skeletal muscles. The disorder manifests with life-threatening episodes of coma upon fasting (due to hypoglycemia, since the liver is unable to produce ketone bodies from fat during fasting and muscles use glucose), cardiomyopathy, cardiac arrhythmia, muscle weakness and abnormal liver function (all likely due to the accumulation of long-chain fatty acids and acylcarnitines that cannot be oxidized). Other symptoms include vomiting, lethargy, hypotonia, weakness, hepatomegaly, cardiac insufficiency, respiratory distress, and seizures. In addition to hypoglycemia, metabolic aberrations in blood include hypoketosis (caused by lack of hepatic fatty acid \(\beta\)-oxidation), acidosis, hyperammonemia (possibly due to a compensatory increase in amino acid oxidation), dicarboxyl aciduria, increased long-chain acylcarnitines, decreased free carnitine, mildly elevated creatine kinase and liver enzyme levels and occasional hypocalcemia. When examined in cultured cells, \textit{CAC} and fatty acid \(\beta\)-oxidation activities are markedly reduced or nearly abolished, although the other carnitine cycle enzymes, \textit{CPT I} and \textit{CPT II}, and \(\beta\)-oxidation enzymes exhibit normal activity.

\textit{CAC} deficiency presents two infantile phenotypes: the first, and most common, with early onset in the neonatal period, and the second milder form with onset in infancy or, less frequently, in childhood. Neonates with the early-onset variety deteriorate rapidly after birth; their mortality rate is high, generally displaying a good correlation between the severe phenotype and null genotype. Infants whose \textit{CAC} retains some residual activity usually have no cardiac involvement and can respond to treatment with medium-chain triglycerides that do not require carnitine to enter the mitochondria. When both phenotypes are untreated, patients with \textit{CAC} deficiency experience hypoglycemic crises that may lead to brain damage, coma and death, or may die from cardiac arrest.

Since the first reported mutation in the \textit{SLC25A20} gene [50], 26 others have been identified (Fig. 2). Interestingly, some missense mutations (D32N, R133W, P230R and D231H; Fig. 2A) regard residues of the signature motifs present in all mitochondrial carriers, and two others (R178Q and G81R; Fig. 2B) concern residues whose equivalents in yeast \textit{CAC} have been proposed to bind the substrate \textit{aac2}, underscoring the importance of the motifs and of the proposed “common substrate-binding site” [32] for the structure and function of these proteins.
Early recognition and treatment is crucial in CAC-deficient patients. CAC deficiency can be identified by newborn screening programs using tandem mass spectrometry, although in severe infantile cases the results of testing might return after the child is already symptomatic. Management and long-term treatment of the CAC patient consist of fasting prevention with frequent meals, a diet rich in carbohydrates, low in lipids (of which about 80% should be provided as medium-chain triglycerides), and supplemented with essential polyunsaturated fatty acids. Patients should have an emergency protocol in place to assure administration of intravenous glucose in case of fever, vomiting or other illness preventing oral intake. Intake of L-carnitine should be avoided, since it transports L- and D-histidine, L-homoarginine and D-isoforms of ornithine, lysine and arginine with the same efficiency as L-isoforms. Secondly, ORC2 has a higher affinity for lysine and arginine and a lower affinity for ornithine and citrulline than does ORC1. Thirdly, ORC1 is expressed at higher levels than ORC2 in all the tissues investigated, particularly in liver, lung, pancreas and testis.

5.2. HHH syndrome

Another error of metabolism is HHH syndrome (OMIM 238970) that is caused by mutations in the SLC25A15 gene [59]. This gene, which maps to band 13q14.1, spans about 23 kb and contains 6 coding exons, encodes isoform 1 of the ornithine carrier, named ORC1. The ornithine carrier was purified from rat liver mitochondria in 1992 and termed ornithine/citrulline carrier for its ability to catalyze a highly active ornithine/citrulline exchange and for its importance in the urea cycle [60]. It was cloned for the first time in S. cerevisiae in 1997 [61]. Later, starting from the yeast ortholog, two human isoforms, ORC1 and ORC2, were identified [59,62]. The two human isoforms were overexpressed and characterized in reconstituted liposomes. Both transport L-isoforms of ornithine, lysine, arginine, and citrulline by a 1:1 substrate exchange or, to a lesser extent, by an exchange of basic amino acids for H⁺; both are inactivated by spermine and spermidine and stimulated by malate and phosphate. However, these isoforms differ in some respects. Firstly, ORC2 has a broader specificity than ORC1, since it transports L- and D-histidine, L-homoarginine and α-isosynths of ornithine, lysine and arginine with the same efficiency as L-isoforms. Secondly, ORC2 has a higher affinity for lysine and arginine and a lower affinity for ornithine and citrulline than does ORC1. Thirdly, ORC1 is expressed at higher levels than ORC2 in all the tissues investigated, particularly in liver, lung, pancreas and testis.

ORC plays a number of important roles in cell metabolism. For example, under conditions of low arginine content in the diet and/or in tissues where argininosuccinate activity is negligible, ornithine produced intramitochondrially from glutamate must be exported to the cytosol to accomplish polyamine biosynthesis. Conversely, when dietary content of arginine is high, the ornithine formed by argininosuccinate hydrolysis in the cytosol must be imported into the mitochondria where it is converted to glutamate and proline by ornithine aminotransferase, an enzyme that is located in the mitochondrial matrix and abundant in liver and kidney. Hepatic ornithine aminotransferase is localized in the pericentral hepatocytes that contain glutamin synthetase and not in the perportal hepatocytes containing the urea cycle enzymes [63]. In perportal hepatocytes, ORC carries out the important function of exchanging cytosolic ornithine for mitochondrial citrulline. By providing this function, ORC links the enzyme activities of urea synthesis in the cytosol to those in the mitochondria and is therefore an essential component of the urea cycle, as previously proposed (see Fig. 3E of ref.

![Fig. 2. Mutations in patients with CAC deficiency. (A) Lateral view of the structural-homology model of human CAC (in cartoon representation) showing the positions of the 9 amino acid substitutions found to date in CAC deficiency. (B) A view of the proposed substrate-binding site from the cytoplasmic side. The carnitine substrate is shown in stick representation. (C) List of type and number of mutations found to date in patients with CAC deficiency. All mutations are from the HGMD database (http://www.hgmd.cf.ac.uk/ac/index.php). The colour code for the transmembrane α-helices is as follows: H1, red; H2, orange-green; H3, light green; H4, dark green; H5, light blue; and H6, blue. Purple surfaces highlight the salt bridge network between residues K35 and D231, R234 and E132, and K135 and D32.](image-url)
HHH syndrome may present at any age from the neonatal phase to adulthood. It usually manifests in early childhood, with 10% of patients presenting in the neonatal period. The most common symptoms of the disorder are episodes of confusion, lethargy, and coma due to hyperammonemia, and neurologic features such as mental retardation, spastic paraplegia and seizures. Interestingly, two of the disease-causing mutations, R275Q and E180K, are expressed in liver at a much lower level. De novo mutations in HHH syndrome, since ORC2 has a lower affinity for ornithine and citrulline and is expressed in liver at a much lower level. Deficiency of ORC1 in patients with HHH syndrome supports this interpretation.

As indicated by its name, HHH syndrome is characterized by hyperammonemia, hyperornithinemia and homocitrullinuria. These metabolic alterations result from a defect in ORC1 as do many clinical symptoms of the disease. Hyperammonemia is due to impairment of the urea cycle at the level of ORC1. Defective ORC1 does not allow export of ornithine from the cytosol to the mitochondria and, consequently, impedes the function of intramitochondrial ornithine transcarbamoylase that condenses carbamoyl phosphate and ornithine to form citrulline. Accumulation of ornithine in the cytosol explains hyperornithinemia and leads to an increased production of polyamines. In the absence of ornithine inside the mitochondria, carbamoyl phosphate accumulates and either condenses with lysine to form homocitrulline, leading to homocitrullinuria, or enters the cytosolic pyrimidine biosynthetic pathway, leading to increased excretion of orotic acid and uracil. Other laboratory findings include increased levels of glutamine, alanine, liver transaminases and alkaline phosphatase.

HHH syndrome is an autosomal recessive disease and has a pan-ethnic distribution with a higher proportion of cases reported in Canada (a founder mutation located in Quebec), Italy, and Japan. The male-to-female ratio is approximately 2:1. Since its first description in 1969 by Shih et al. [67], 71 additional cases have been reported. In Fig. 3, all the 33 naturally occurring mutations in ORC1 known to date are listed, including 16 from Dr. Dionisi-Vici that are yet unpublished. Many of the mutations have been functionally analyzed in reconstructed liposomes and found to inactivate the carrier[62,68]. Interestingly, two of the disease-causing mutations, R275Q and E180K, concern residues involved in the proposed substrate-binding site,

Fig. 3. Mutations in patients with HHH syndrome. (A) Lateral view of the structural-homology model of human ORC1 (in cartoon representation) showing the positions of the 14 amino acid substitutions found to date in HHH syndrome. The transmembrane α-helices are coloured as reported in Fig. 2. Purple surfaces highlight the salt bridge network between residues K34 and D231, K234 and E128, and K131 and D31. (B) A view of the proposed substrate-binding site from the cytoplasmic side. The ornithine substrate is shown in stick representation. (C) List of type and number of mutations found thus far in patients with HHH syndrome. Seventeen mutations were taken from the HGMD database (http://www.hgmd.cf.ac.uk/ac/index.php); the remainder was obtained through the courtesy of Dr. Dionisi-Vici.
whereas T32R and P126R residues belonging to the signature motifs of the mitochondrial carrier family (Fig. 3). Furthermore, 6 of the 14 missense mutations affect residues protruding toward the cavity occupied by the entering substrate in the cysticotic conformation of the carrier (Fig. 3).

Early diagnosis and treatment of HHH syndrome are important to prevent neurologic and cognitive deterioration. However, diagnosis is often delayed as a result of non-specific symptoms. Differential diagnosis is based mainly on biochemical characteristics: persistently elevated plasma ornithine levels 5 to 10 times higher than normal, which distinguishes the disorder from other urea cycle defects; the presence of homocitrulline and orotic acid in urine; and episodic or postprandial hyperammonemia, differentiating HHH syndrome from gyrate atrophy (OMIM 258870) which is caused by ornithine transaminase deficiency. Measurement of carrier activity in cultured fibroblasts [59] and in reconstituted liposomes followed by genetic testing constitutes evidence of the disease.

Treatment of HHH syndrome is similar to that of other urea cycle defects and is based mainly on a low-protein diet. It is usually accompanied by supplementation of citrulline and ornithine (to reduce ammonia), although the long-term side effects of hyperornithinemia are not known. Arginine, sodium benzoate and sodium phenylbutyrate have also been administered to decrease ammonia levels. Treated patients exhibit good metabolic control and very infrequent relapses of hyperammonemia. The mortality rate for the disease is low.

5.3. AGC2 deficiency

Aspartate/glutamate carrier isoform 2 (AGC2) deficiency is a highly widespread autosomal recessive disease in the Far East and Southeast Asia with a carrier prevalence of about 1 in 70 individuals and a male-to-female ratio of 2:4:1 [69]. AGC2 deficiency was also detected in the Middle East, including Israel. More recently, some cases have been found in the US and the United Kingdom, pointing towards a pan-ethnic distribution. AGC2 deficiency has been extensively studied by Dr. Saheki in Japan who identified SLC25A13 as the gene responsible for the disease by means of homology mapping and positional cloning [70]. The SLC25A13 gene, spanning about 201 kb, maps to chromosome 7q21.3 and consists of 18 exons. Subsequently, it was found that the gene product of SLC25A13, previously termed citrin, and its closely related human homolog, aralar, function as Ca2+-binding sites, which make up their regulatory portion. In fact, the activity of AGC1 and AGC2 is stimulated by Ca2+ on the external side of the inner mitochondrial membrane, since this stimulation is not affected by ruthenium red, a specific inhibitor of Ca2+ entry into mitochondria [71]. Furthermore, by using mitochondrial targeted luciferase to monitor ATP formation, a surplus production of ATP was observed in the mitochondria of agonist-stimulated cells overexpressing AGC1 or AGC2 but not in stimulated cells overexpressing the C-terminal domains lacking the Ca2+-binding sites. Notably, the intramitochondrial Ca2+ level was the same in cells overexpressing either the entire protein (AGC1 or AGC2) or each of the two C-terminal domains alone [74]. Therefore, cytosolic Ca2+ activates mitochondrial oxidative metabolism through its binding to the N-terminal domain of AGC.

The major function of AGC is to supply aspartate to the cytosol. This is particularly important in hepatocytes that have a negligible capacity of taking up aspartate from the blood. In hepatocytes, AGC plays a fundamental role in urea synthesis by supplying aspartate to arginosuccinate synthetase (ASS), a urea cycle enzyme that is localized in the cytosol and condenses citrulline and aspartate to produce arginosuccinic acid. The reactions of the urea cycle, their subcellular localization and site of aspartate involvement are shown in Fig. 3E of ref. [28]. Besides urea synthesis, cytosolic aspartate is necessary for gluconeogenesis from reduced substrates and for protein, purine and pyrimidine synthesis. Cytosolic aspartate is also very important for the oxidation of cytosolic NADH+H+ since AGC is a key component of the malate–aspartate shuttle that transfers the reducing equivalents of NADH+H+ from cytosol into mitochondria (see Fig. 3H of ref. [28]). In this shuttle, AGC again provides the cytosol with aspartate; in the cytosol, aspartate is transaminated to oxaloacetate which is reduced by cytosolic malate dehydrogenase to malate, leading to the oxidation of cytosolic NADH+H+. Malate enters the mitochondria via the oxoglutarate carrier and in the matrix is reduced to oxaloacetate by mitochondrial malate dehydrogenase, resulting in the reduction of intramitochondrial NAD+. Subsequently, oxaloacetate is transaminated to aspartate by mitochondrial aspartate aminotransferase, completing the cycle. It should be emphasized that the malate–aspartate shuttle operates in the direction of transferring reducing equivalents from cytosol to mitochondria because the reaction catalyzed by AGC is unidirectional and irreversible. By oxidizing cytosolic NADH, the malate–aspartate cycle allows aerobic glycolysis and alcohol metabo- lism to occur. It is important to note that the glycero–3-phosphate shuttle, which is the main physiological alternative mechanism for the oxidation of cytosolic NADH, exerts low activity in human liver (whereas it is very active in murine liver).

Because AGC2 is the only isoform, or at least the most prevalent isoform, of this carrier expressed in liver, lack of its function in this tissue explains many symptoms of the disease. AGC2 deficiency causes two age-dependent phenotypes: neonatal intrahepatic cholestasis caused by citrin (AGC2) deficiency (NICCD, OMIM 605814) and adult-onset type II citrullinemia (CTLN2, OMIM 603471). Type II citrullinemia usually manifests in adults between the ages of 20 and 50 years. A shortage of aspartate in the cytosol of hepatocytes causes citrullinemia, hypoprolineemia, and recurrent episodes of hyperammonemia responsible for the observed encephalopathy and neuropsychiatric symptoms (e.g., sudden aberrant behavior, disturbance of consciousness, disorientation, delirium, tremor, convulsive seizures and coma). The inhibition of cytosolic NADH oxidation, with the consequent increase in the NADH/NAD+ ratio, causes a distaste for carbohydrates, a preference for fat and especially for proteins as well as an incapacity to consume alcohol. In fact, in patient dietary surveys carbohydrates average only ~34% of total caloric intake, whereas proteins average ~23%. In particular, a strong desire for beans and nuts, which are...
particular rich in aspartate and asparagine, has been described. Citrullinemia and hyperammonemia are also due to a unique feature of the disease, i.e., a variable liver-specific reduction in ASS protein and activity with no alterations in either its gene or hepatic ASS mRNA levels [75]. Conversely, in type I citrullinemia (CTLN1, OMIM 215700) the gene for ASS is mutated, leading to ASS deficiency in all tissues [76]. It is not yet clear why the level of hepatic ASS is low in CTLN2. It may be that critical aspartate levels are required to make ASS active or to stabilize the enzyme. Patients also manifest fatty liver most likely because they oxidize cytosolic NADH+H+ but yet another pathway, the citrate–malate shuttle (see Fig. 3D of ref. [28]), whose normal function is to synthesize fatty acids by transferring acetyl-coenzyme A from the mitochondrial matrix to the cytosol. In fact, the key enzyme of the citrate–malate shuttle, the citrate carrier, exports citrate from mitochondria to the cytosol where it is cleaved to acetyl-coenzyme A and oxaloacetate by ATP-citrate lyase. Furthermore, some patients develop hyperlipidemia which likely results from the functioning of the citrate–malate shuttle and from the accumulation of glycerol-3-phosphate due to poor activity of the glycerol-3-phosphate cycle in human liver. Other laboratory findings are increased arginine and pancreatic secretory trypsin inhibitor levels, an elevated threonine-to-serine ratio, and a decreased ratio between branched-chain amino acids (BCAAs) to aromatic amino acids (AAAs) in patient sera. Finally, pancreatitis and hepatocellular carcinoma occur with a higher incidence in CTLN2 patients. Adult-onset type II citrullinemia is progressive and patients usually die from hyperammonemic encephalopathy and complications of brain edema. Given the poor prognosis of the adult AGC2-deficiency phenotype, patients should consider undergoing liver transplantation.

The neonatal phenotype, NICCD, was recognized more recently by Saheki and co-workers [77]. Affected children commonly present transient intrahepatic cholestasis, fatty liver, hepatomegaly, growth retardation, citrullinemia, aminoacidemias (elevated methionine, threonine, tyrosine, phenylalanine, lysine and arginine), an increased threonine-to-serine ratio and galactose concentration, ketotic hypoglycemia, and hypoproteinemia. Some patients present with hepatitis, jaundice, decreased coagulation factors, hemolytic anemia and bleeding diathesis as a result of liver dysfunction and hypoproteinemia. High levels of plasma α-fetoprotein have been detected in NICCD but not in CTLN2. The neonatal phenotype of AGC2 deficiency is usually benign. Symptoms disappear by the age of 12 months; afterwards, these patients become seemingly healthy (or manifest non-specific symptoms) but display a preference for protein- and lipid-rich foods and an aversion for foods high in carbohydrate content. One or several decades later, some AGC2-deficient individuals go on to develop cirrhosis in their liver. The clinical spectrum of AGC2 deficiency has recently been expanded with the report of infantile presentation characterized by failure to thrive and bleeding diathesis and no evidence of neonatal intrahepatic cholestasis [78].

In the compensatory period (which corresponds to the transition from NICCD to the onset of CTLN2), affected individuals live with metabolic defects that mainly involve liver, i.e., a high cytosolic NADH/NAD+ ratio and impairment of urea synthesis. It is likely that in the AGC2-deficient liver, partial oxidation of cytosolic NADH is accomplished by the low activity of the glycerol-3-phosphate NADH shuttle and by the citrate–malate cycle, as mentioned above. Given that in patient liver the export of aspartate from the mitochondria is nearly null and aspartate is virtually not taken up by the blood, ureagenesis is dependent on aspartate production in the cytosol. Aspartate can be produced in the cytosol in two was: from asparagine by the action of asparaginase and from oxaloacetate by cytosolic aspartate aminotransferase. Asparagine can be synthesized by asparagine synthetase or can be supplied from the diet via the portal venous blood. Oxaloacetate can be formed from fumarate, a product of the urea cycle enzyme argininosuccinate lyase, by the successive actions of cytosolic fumarase and malate dehydrogenase. In both cases serious drawbacks emerge, i.e., the production of 1 mol of ammonia per each mol of aspartate provided by asparagine and the generation of 1 mol of NADH+H+ per each mol of aspartate provided by oxaloacetate originating from malate (see Fig. 6B of ref. [79]). Therefore, in the AGC2-deficient patient, the deficit of ureagenesis is due not only to a decreased availability of aspartate to ASS but also to an increased cytosolic NADH/NAD+ ratio as a consequence of the defective malate–aspartate NADH shuttle and of aspartate production from fumarate, as mentioned above.

SLC25A13 is the only gene known to be associated with AGC2 deficiency. The first mutations observed in SLC25A13 led to either truncation of the protein or extensive deletions causing AGC2 deficiency [80]. More recently, missense mutations were also detected. Until now, no significant correlation has been observed between SLC25A13 mutation types and the age of CTLN2 onset. Forty-four mutations have been found thus far in patients with AGC2 deficiency, 24 of which have not yet been published (T. Saheki, personal communication). All together, 14 missense mutations have been found. The newly identified mutation R588Q that inhibits transport of glutamate and aspartate in reconstituted liposomes by about 90% [81] regards a residue belonging to the proposed common substrate-binding site formed by R588, R492, E404 and K405 — residues equivalent to those proposed to bind the substrate in yeast AGC1 (R792, R692, E600 and K601) [32]. R588 corresponds to R288 of the bovine oxoglutarate carrier, to R294 of the yeast AAC2, and to R276 of the murine UCP1 which also do not tolerate replacement with other amino acids [82] and references therein) [82]. R588 also corresponds to R275 of the human ORC1; its substitution with Q causes HH1 syndrome (see 5.2).

To examine the consequences of the R588Q mutation, docking of glutamate was performed using the residues of the common substrate-binding site of the human AGC2 [83]. Docking with the human wild-type AGC2 shows the substrate directly on top of the salt bridge network interacting with R588 and the charged residues K453 and K353 of the network (Fig. 4A). In the case of the R588Q mutant, the substrate is positioned far from the salt bridge network at the level of the binding site (Fig. 4B). Therefore, lack of R in position 588 impede the interaction of the substrate with the salt bridges causing loss of activity. It is tempting to speculate that residue R588 functions as a mobile side-chain that transports the substrate onto the salt bridges, determining their aperture and the substrate’s progression through the protein toward the matrix.

The pathophysiology of AGC2 deficiency was recently investigated by elegant studies conducted on knock-out mice [84]. SLC21A13−/− mice exhibited a marked decrease in mitochondrial aspartate transport and aspartate shuttle activities in vitro. In liver perfused with ammonia, the rate of urea production was drastically reduced and the lactate-to-pyruvate ratio, which reflects the cytosolic NADH/NAD+ redox state, was increased. Under these conditions, both the deficit in ureagenesis and increase in the NADH/NAD+ ratio were partially corrected by the administration of asparagine that enters hepatocytes and is hydrolyzed to aspartate. Likewise, pyruvate (but not aspartate or citrate) reduces the deficit in ureagenesis and decreases the NADH/NAD+ ratio in the liver-perfused system. Furthermore, in liver perfused with lactate (but not pyruvate) glucoseoneogenesis was diminished. These results demonstrate that hepatic cytosolic aspartate is rate-limiting in SLC25A13−/− mice. However, AGC2 knock-out mice did not manifest the key symptoms of AGC2 deficiency present in man. The mice exhibited neither hyperammonemia and changes in amino acid levels even following administration of protein-rich diets nor hypoglycemia upon fasting.

The most likely explanation for the lack of CTLN2-like phenotype in the AGC2 knock-out mice is the high activity of the glycerol-3-phosphate cycle in their liver. Consequently, Saheki et al. generated mice with a combined disruption of the AGC2 gene and the gene for mitochondrial glycerol-3-phosphate dehydrogenase, a key component of the glycerol phosphate cycle [85]. Double knock-out mice
may often provoke CTLN2 symptoms. At present, liver transplantation treatment of hyperammonemia and brain edema [85]. Alcohol intake have been observed after administration of glycerol or fructose for the carbohydrate diets. Likewise, severe episodes of hyperammonemia upon consumption of high-hyperammonemia. In contrast, CTLN2-affected patients manifest episodes of urea cycle disorders. Individuals with urea cycle enzyme defects are AGC2 de

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of urea cycle disorders. Individuals with urea cycle enzyme defects are AGC2 de

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most of the features of the human AGC2-deficient phenotype.

AGC2 deficiency should be suspected in children with hypoglycemia, hepatomegaly, growth retardation or failure to thrive. In addition, AGC2 deficiency should also be considered in children presenting with elevated blood galactose levels. It is important to confirm the diagnosis of AGC2 deficiency, as treatment of these patients highly contrasts that of urea cycle disorders. Individuals with urea cycle enzyme defects are given protein-restricted, high-caloric diets to prevent episodes of hyperammonemia. In contrast, CTLN2-affected patients manifest severe episodes of hyperammonemia upon consumption of high-carbohydrate diets. Likewise, severe episodes of hyperammonemia have been observed after administration of glycerol or fructose for the treatment of hyperammonemia and brain edema [85]. Alcohol intake and the use of anti-inflammatory and analgesic drugs and/or surgery may often provoke CTLN2 symptoms. At present, liver transplantation is the only effective treatment for type II citrullinemia. To prevent hyperammonemia (and resolve growth retardation in children), a diet high in protein and lipid content and low in carbohydrates is highly recommended. This recommendation is further supported by the observed food preference of these patients. Treatment with pyruvate has also been suggested to decrease the cytosolic NADH/NAD+ ratio [79]. Moreover, in children with NICCD, supplementation with fat-soluble vitamins and use of lactose-free formula or high protein and low carbohydrate diet have also been applied [78]. In summary, treatment of NICCD requires continued dietary control and growth monitoring. Continuous monitoring of these patients is necessary to prevent a severe outcome later in life.

5.4. Amish microcephaly (MCPHA)

Amish microcephaly (MCPHA, OMIM 607196) is an inborn error of metabolism presenting severe microcephaly and 2-oxoglutaric aciduria [86]. The disorder is caused by a defect in the SLC25A19 gene which encodes a protein of 320 residues [87]. The protein was first identified as a deoxynucleotide carrier (DNC) [88] and later as a thiamine pyrophosphate carrier (TPC) [89]. In reconstituted liposomes, TPC transports the important coenzyme thiamine pyrophosphate (ThPP), thiamine monophosphate (ThMP) and deoxynucleotides in descending order of potency, dNDP→dNTP→dNMP, by an obligatory exchange mechanism. Nucleotides are also transported, though less efficiently than the corresponding deoxynucleotides. Furthermore, the deoxynucleoside triphosphates that are produced in the cytosol from deoxyxynucleosides, including antiviral and anticancer nucleoside analogs, are equally efficient substrates as dNDPs [88].

to date, Amish microcephaly has only been observed in the Old Order Amish community in Pennsylvania, U.S.A. with a high prevalence of about 1 in 500 individuals. In this community, 23 nuclear families affected with MCPHA are connected to a single ancestral couple that lived in the 1700s. The disease is characterized by severe
congenital microcephaly, elevated levels of α-ketoglutarate in urine, premature death (observed maximum survival, 14 months) and, as a result of microcephaly, nearly absent cranial convexity, frontal sloping and distorted facial features. The only non-CNS physical anomaly is moderate micrognathia. Patients manifest no orientation to sight or sound and no fine or gross motor development, have metabolic acidosis enhanced by episodic viral illnesses, in some cases mild hepatomegaly, difficulty maintaining normal body temperature and develop increasing irritability.

It was first considered that the increased levels of α-ketoglutarate might be the result of a defect in the α-ketoglutarate dehydrogenase complex which comprises three enzymes: 2-oxoglutarate dehydrogenase (OMIM 203740), dihydrolipoamide succinyltransferase (OMIM 126063) and dihydrolipoamide dehydrogenase (OMIM 246900). The genes encoding these enzymes were excluded on the basis of genetic linkage and haplotype analysis, indicating that this disorder must be associated with another genetic locus.

Through a whole-genome scan, fine mapping and haplotype analysis, the gene affected in MCPHA was localized to a region of 2 Mb, on chromosome 17q25 [87,90]. In this region, the genes encoding proteins with known mitochondrial functions were analyzed in view of the oxoglutarate abnormality in MCPHA, which suggested mitochondrial dysfunction. A homozygous nucleotide change in the coding region of TPC, c.530G→C (NM_021734), was identified in affected children. The parents of individuals affected by MCPHA are obligate heterozygotes.

The mutation, which produces a glycine-to-alanine substitution at position 177, alters a highly conserved residue in the mitochondrial carrier family that is located in the second part of the signature motif between helices h34 and H4, in proximity to one of the important salt bridges that close the substrate translocation path in the cytosolic conformation of the carrier (Fig. 5). The substitution co-segregates with the disease in the 16 families investigated [87] and is deleterious for protein function. Indeed, in reconstituted liposomes the transport activity of ThPP, ThMP or dATP mediated by the G177A mutant TPC was 25% to 30% that mediated by the wild-type TPC [89]. In addition, substitutions of G177 with bulkier residues such as valine, cysteine and leucine are not tolerated at all (F. Palmieri, unpublished data), as was also found for the equivalent glycine in other members of the mitochondrial carrier family [82] and references therein. It may be hypothesized that G177 functions as a hinge between helices h34 and H4 during the conformational changes that occur in the catalytic cycle of the carrier.

SLC25A19 is the only gene known to be associated with Amish microcephaly. To elucidate the pathogenic mechanism of this disease, a knock-out mouse for the SLC25A19 gene was generated, and the phenotype of both the knock-out mice and patients was studied at the cellular level [89].

All SLC25A19−/− mice died prior to 12.5 days’ gestation. The homozygous embryos at E 10.5 exhibited an abnormally developed CNS with a neural-tube closure defect and exencephaly that commonly

Fig. 5. Structural-homology model of human TPC showing the position of the mutation G177A responsible for Amish microcephaly. Cartoon representations of the G177 wild-type TPC (A) and the A177 mutated TPC (B) viewed from the cytoplasmic side. The residues G177 and A177 are in surf representation and coloured in yellow. The transmembrane α-helices are coloured as reported in Fig. 2. Purple surfaces highlight the salt bridge network between residues K241 and D140, R143 and D37, and K40 and D238. Cartoon and stick representations showing the proximity of G177 (C) and A177 (D) to the salt bridge between K241 and D140. G177 and A177 are in surf representation and coloured in yellow. In C and D, the signature motifs (P-X-D/E-X-X-K/R) of H1, H3 and H5 are coloured blue, green and orange, respectively, with the side-chains in stick representation. The salt bridges are indicated by black dotted lines.
involves the entire brain. In addition, they manifested erythropoietic failure, being nearly devoid of erythrocytes at E 9.5, and elevated oxoglutarate levels in the amniotic fluid.

In the mitochondria of knock-out mice fibroblasts and patient lymphoblasts, no differences in ribonucleoside- and deoxynucleoside-triphosphate levels, as well as no depletion or deletions in mitochondrial mtDNA abnormalities, were not detectable in the mitochondria of knock-out mice (open neural tube) (of note, ThPP and ThMP were produced in the cytosol into the mitochondria in exchange for intra-mitochondrial ThPP and ThMP), it was found that ThPP and ThMP were markedly decreased in the mitochondria of patient lymphoblasts as compared to controls. (Of note, ThPP and ThMP were increased in the cytosol.) Secondly, in the media of both cell lines Cytosolic ThPP and ThMP levels were highly increased in the cytosol (P < 0.05). Thirdly, in the media of both cell lines Mitochondrial ThPP and ThMP levels were not detectable in the mitochondria of knock-out mice (open neural tube). Lastly, the mitochondrial ThPP-dependent ketoglutarate dehydrogenase (KGDH) and pyruvate dehydrogenase (PDH) complex activities were strongly diminished in patient mitochondrial extracts as compared to controls. Interestingly, the addition of ThPP to the KGDH and PDH assay mixtures restored full activity, showing that the lower activities of KGDH and PDH in knock-out mice and patients is caused by lack of ThPP in mitochondria [89]. Taken together, these data suggest that transport of ThPP is the primary physiological function of TPC.

Table 2 summarizes and compares the phenotypes of the SLC25A19 knock-out mice and patients with Amish microcephaly. It can be observed that all the metabolic abnormalities of the human disease, starting from the decrease in ThPP level in the mitochondria, were also present in the knock-out mice. However, these abnormalities, which are caused by lack of ThPP in the mitochondria, were more severe in the knock-out mice. The only exception concerns increased oxoglutarate levels in amniotic fluid which were found to be less dramatic than those in the urine of patients. This is due to the fact that mouse embryos died prior to kidney formation and therefore did not concentrate the metabolite. The increased severity of the mouse model is also illustrated by the embryonic lethality of the SLC25A19 knock-out animals, by the arrest of brain formation at the level of the neural tube closure, probably caused by decreased fluxes through KGDH and PDH reactions, and by erythropoietic failure perhaps caused by lack of succinyl-CoA, a product of KGDH. The most likely explanation for the differences in the mouse and human phenotypes is that human point mutation retains some ThPP transport activity whereas the murine null mutation does not. In conclusion, Amish microcephaly is due to a defect of ThPP transport activity which primarily affects brain development because of the brain’s great need for oxidative metabolism.

Metabolic screening and eventual imaging studies are warranted for all children with congenital microcephaly. No intervention or vitamin therapy has proven to be effective for treating and/or improving the disorder. Only supportive therapy is available for MCPHA.

Table 2 Phenotypes of the knock-out mice and patients with Amish microcephaly

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Knock-out mice</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPC activity</td>
<td>Absent</td>
<td>Reduced to 25–30%</td>
</tr>
<tr>
<td>Lethality</td>
<td>Embryonic</td>
<td>1–14 months</td>
</tr>
<tr>
<td>CNS defects</td>
<td>Arrest of brain formation</td>
<td>Microcephaly</td>
</tr>
<tr>
<td>Mitochondrial ThPP and ThMP</td>
<td>Highly increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Cytosolic ThPP and ThMP</td>
<td>Not detectable</td>
<td>Decreased</td>
</tr>
<tr>
<td>KGDH and PDH activities</td>
<td>Highly decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>KGC</td>
<td>Increased</td>
<td>Highly increased</td>
</tr>
<tr>
<td>Lactate</td>
<td>Highly increased</td>
<td>Increased</td>
</tr>
<tr>
<td>mtDNA abnormalities</td>
<td>Absent</td>
<td>Normal</td>
</tr>
<tr>
<td>Mitochondrial (d)NTP levels</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

*Data obtained from SLC25A19−/− mouse fibroblasts and patient lymphoblasts.

In amniotic fluid.

Fig. 6. Partial sequence alignment of the glutamate and aspartate/glutamate carriers in man and other species displaying high conservation of P206 and G236 of the human glutamate carrier isoform 1. Abbreviations: Hs, Homo sapiens; Mn, Mus musculus; Br, Bos taurus; Ra, Rattus norvegicus; Xl, Xenopus tropicalis; Dl, Danio rerio; Cg, Gallus gallus; Dm, Drosophila melanogaster; Ag, Anopheles gambiae; Nv, Nasonia vitripennis; Sc, Saccharomyces cerevisiae; Nf, Neosartorya freschi; Nf, Aspergillus fumigatus; Nc, Neurospora crassa; Dd, Dictyostelium discoideum.
affected patients. Phenobarbital has been administered for the treatment of seizures, and physical therapy is encouraged for alleviating contractures or other secondary neurologic manifestations. Genetic risk of MCPHA is best determined in the pre-gestational phase.

5.5. Neonatal myoclonic epilepsy

Mutations in the SLC25A22 gene encoding isoform 1 of the glutamate carrier (GC1) cause a form of early myoclonic epilepsy (EME), named neonatal myoclonic epilepsy (OMIM 609304) [91]. The SLC25A22 gene maps to chromosome 11p15.5 and contains 8 coding exons. GC1 and its isoform GC2, which is encoded by SLC25A18, were identified upon gene expression in E. coli as glutamate carriers by their transport properties in reconstituted liposomes [92]. Both isoforms are located in the mitochondrial membrane (M. Lasorsa and F. Palmieri, unpublished data) and catalyze a glutamate/H+ symport with high specificity. However, GC1 has a high Km for glutamate (∼5 mM), whereas the Km value of GC2 is low (∼0.22 mM) and the Vmax value of GC1 is higher than that of GC2. Furthermore, isoform 1 is expressed at higher levels than isoform 2 in all the tissues investigated, particularly in liver, pancreas and brain. The differences in expression levels and kinetic parameters of the two isoforms suggest that isoform 2 matches the basic requirement of all tissues, especially with respect to amino acid degradation, and that isoform 1 becomes operative to accommodate higher demands associated with specific metabolic functions. Because glutamate is co-transported with an H+ by the GC, and therefore its distribution across the mitochondrial membrane is dependent on ΔpH, entry of glutamate is favoured in energized mitochondria. However, when glutamate is generated intramitochondrially (e.g., by proline oxidation), the GC may operate in the reverse direction to limit intramitochondrial accumulation of glutamate.

The first GC1 mutation, a substitution of proline 206 with leucine, was found in a consanguineous Arab Muslim family with 4 affected children among 8 [91]. A second mutation (a change from glycine 236 to tryptophan) was identified in a consanguineous family from Algeria with 1 affected child among 3 (L. Colleaux and F. Palmieri, unpublished data). All affected individuals were homozygous for the above-mentioned mutations, whereas the parents were heterozygous.

The symptoms of the affected children in both families were similar: very-early-onset intractable myoclonic seizures, hypotonia, progressive microcephaly, abnormal visual nerve conduction, and rapid evolution into encephalopathy and spasticity. Concerning laboratory features, neonatal myoclonic epilepsy is characterized by a typical EEG pattern with suppression burst and by an abnormal evoked potential with low amplitude signal and slow response.

Both the P206L and G236W substitutions, which are located in H4 and H5 two and three helix turns from the cytosolic membrane side, respectively, alter highly conserved residues in the glutamate and aspartate/glutamate carriers found in man and other species (Fig. 6) and abolish glutamate transport in reconstituted liposomes. In digitonin-permeabilized fibroblasts from patients, glutamate oxidation was also strongly defective [91] and unpublished data). These results have provided the first evidence that, despite normal oxidative phosphorylation, impaired mitochondrial glutamate import/metabolism leads to an alteration of neuronal excitability.

Although the pathogenesis of neonatal myoclonic epilepsy remains an open question, the following information should be stressed: i) during fetal development, GC1 is expressed first in brain, specifically within territories that contribute to the genesis and control of myoclonic seizures [91]; ii) GC1 expression is much greater in astrocytes than in neurons [93]; iii) astrocyte mitochondria do not contain the aspartate/glutamate carrier [94] and thus take up glutamate only via the glutamate carrier; iv) glutamate is a major excitatory neurotransmitter; and v) after its release into the synapse glutamate is taken up by astrocytes. It may be that GC1-dependent epilepsy is due to a defect of mitochondrial glutamate transport in astrocytes, which would consequently lead to an increase in intrasynaptic glutamate concentration.

There is no effective treatment for neonatal myoclonic epilepsy; affected children die within 1 to 2 years after birth or survive in a vegetative state. Some severe cases of the disorder may be confused with febrile seizures, which are of shortened duration and recur less frequently. It is also important to distinguish neonatal myoclonic epilepsy from benign neonatal sleep myoclonus. The latter condition is seen in healthy infants, occurs only in sleep, and EEG is normal. This is the most common cause of neonatal myoclonus and may be confused with epileptiform activity. The seizures are often brief and self-limiting, and the child may be able to sleep through them. However, some cases may require treatment with anticonvulsant drugs.

6. Conclusions

Since 1997, when carnitine–acycarnitine carrier deficiency was associated with the nuclear SLC25A20 gene, the number of nuclear gene defects impairing the function of mitochondrial metabolite transporters has been rapidly increasing. To date, eight mitochondrial carrier-related diseases have been well characterized biochemically and genetically. This new group of diseases contributes to the current expansion of the field of mitochondrial disorders which were first thought to be limited to those related to respiratory chain dysfunction. In the last decade, the number of mitochondrial diseases caused by defects of the nuclear-coded components of the mitochondrial proteome has been growing at a booming rate to justify what is now known as “mitochondrial medicine”.

Despite the substantial progress that has been made in our understanding of the molecular bases of mitochondrial carrier-associated diseases, etiologic therapy is not yet available. Current therapy is symptomatic and also addresses disease complications. However, some mitochondrial carrier-associated diseases, like other metabolic disorders, benefit enormously from appropriate dietary measures. Effective disease management should include wider diffusion of newborn screening and close collaboration among physicians, geneticists and metabolic disease experts in specialized centers. Preventive therapy through genetic counseling and prenatal diagnosis is essential and already available. As we wait for gene therapy to become available for practical application, further understanding of pathogenetic mechanisms through a multidisciplinary approach combining in vitro assays with in vivo studies on patients, cellular and animal models will help to develop new therapeutic strategies that may prove useful to this group of metabolic disorders. Further studies are likely to reveal additional mitochondrial carrier-related diseases (particularly in view of the fact that the function of about 20 human genes of the SLC25 family has yet to be identified) and provide new insight into this exciting field.

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