Deciphering CAD: Structure and function of a mega-enzymatic pyrimidine factory in health and disease

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Abstract

CAD is a 1.5 MDa particle formed by hexameric association of a 250 kDa protein divided into different enzymatic domains, each catalyzing one of the initial reactions for de novo biosynthesis of pyrimidine nucleotides: glutaminase-dependent Carbamoyl phosphate synthetase, Aspartate transcarbamoylase, and Dihydroorotase. The pathway for de novo pyrimidine synthesis is essential for cell proliferation and is conserved in all living organisms, but the covalent linkage of the first enzymatic activities into a multienzymatic CAD particle is unique to animals. In other organisms, these enzymatic activities are encoded as monofunctional proteins for which there is abundant structural and biochemical information. However, the knowledge about CAD is scarce and fragmented. Understanding CAD requires not only to determine the three-dimensional structures and define the catalytic and regulatory mechanisms of the different enzymatic domains, but also to comprehend how these domains entangle and work in a coordinated and regulated manner. This review summarizes significant progress over the past 10 years toward the characterization of CAD’s architecture, function, regulatory mechanisms, and cellular compartmentalization, as well as the recent finding of a new and rare neurometabolic disorder caused by defects in CAD activities.

KEYWORDS

aspartate transcarbamoylase, carbamoyl phosphate synthetase, de novo pyrimidine biosynthesis, dihydroorotase, multienzymatic protein, nucleotide metabolism, rare diseases

1 | INTRODUCTION

Pyrimidine nucleotides are building blocks of DNA and RNA and key activators of intermediate metabolites in a number of cellular processes, including glycosylation and carbohydrate and phospholipid synthesis.1,2 The cells obtain the supply of pyrimidines through two different metabolic pathways (Figure 1). Slowly dividing or differentiated cells use preferentially salvage pathways to uptake preformed nucleosides (e.g., uridine) from the diet or from the breakdown of nucleic acids, whereas, proliferating cells, whether normal (e.g., activated lymphocytes) or tumoral, depend on de novo (“of new”) synthesis of pyrimidine nucleotides. The de novo pathway consists of six enzymatic steps yielding uridine 5-monophosphate (UMP), from which all other pyrimidine nucleotides are made (Figure 1).3 In a first step, carbamoyl phosphate synthetase (CPS; EC 6.3.5.5) makes...
carbamoyl phosphate (CP) from bicarbonate, ammonia (released from glutamine) and two ATP molecules. Then, aspartate transcarbamoylase (ATC; EC 2.1.3.2) uses CP and aspartate (Asp) to form carbamoyl aspartate. Next, dihydroorotase (DHO; EC 3.5.2.3) catalyzes the condensation of carbamoyl aspartate to dihydroorotate, which is oxidized to orotate by the dihydroorotase dehydrogenase (DHODH; EC 1.3.5.2). Orotate, a pyrimidine base, is attached to ribose by the enzyme orotate phosphoribosyltransferase (OPRT; EC 2.4.2.10), producing the nucleotide orotidine 5-monophosphate (OMP) that is subsequently decarboxylated by OMP decarboxylase (OMPDC; EC 4.1.1.23) to make UMP.

Although the six steps for de novo UMP synthesis are preserved in all living organisms, the organization of the enzymatic machinery varies greatly (Figure 2a).3–5 Whereas most prokaryotes encode individual enzymes for each step, in humans and other animals, the glutamine-dependent CPS (CPS-2), ATC and DHO activities are fused as distinct functional domains into a single ~250 kDa polypeptide named CAD (Figures 1 and 2a).6 This multifunctional protein further self-assembles into hexamers, forming a megaenzyme nearly half the size of a ribosome.7 OPRT and OMPDC are also fused into a single polypeptide named UMP synthetase (UMPS), while the remaining enzyme, DHODH, is anchored to the inner mitochondrial membrane, coupling the synthesis of pyrimidines to the respiratory chain (Figure 1). Fungi, on the other hand, have a CAD-like protein (named ura2 in S. cerevisiae) with an inactive DHO-like domain,8 and the DHO, DHODH, OPRT and OMPDC activities are performed by separated monofunctional proteins (Figure 2a). Most of these enzymes in plants and protists are also monofunctional, except for a bifunctional UMPS that in some protists (e.g., Trypanosoma) fuses the domains in reversed order (Figure 2a).5,9

These differences in the enzyme organization are accompanied by differences in the regulation of the pathway. In prokaryotes and plants, CPS makes CP both for the biosynthesis of UMP and arginine (Figure 2b,c).3 Thus, ATC becomes the first committed enzyme for pyrimidine synthesis and is the control point of the pathway, being feedback inhibited by UTP (and CTP) and activated by ATP in the case of bacteria,10 or inhibited by UMP in plants (Figure 2b,c).11 In these organisms, CPS is also inhibited by pyrimidines and activated by ornithine (a substrate for arginine synthesis). Then, when the pyrimidine pool builds up, both ATC and CPS are inhibited, and ornithine counteracts the CPS inhibition, diverting CP synthesis to the arginine pathway (Figure 2b,c). In animals and fungi, on the other hand, there are separated CP pools for pyrimidines and arginine biosynthesis (Figure 2d).3 The arginine-specific CP is
produced by a CPS (CPS-1) localized in the mitochondrial matrix, and in most terrestrial animals, this enzyme lacks glutaminase activity and funnels the circulating ammonia for its detoxification through the urea cycle (Figure 2d). In turn, the CP pool for pyrimidine synthesis is produced by CAD’s CPS-2, which is the rate-limiting and control point of the de novo pathway, being allosterically inhibited by UTP and activated by phosphoribosyl pyrophosphate (PRPP) (Figure 2d). This allosteric regulation is further modulated through phosphorylation of the CPS-2 domain. Phosphorylation by the cyclic AMP-dependent protein kinase (PKA) overcomes the inhibition by UTP, whereas phosphorylation by the mitogen-activated protein kinase (MAPK) converts UTP from an inhibitor to a modest activator and promotes the activation by PRPP (Figure 2d). Both phosphorylations are proposed to occur sequentially and to regulate the activity of CAD throughout the cell cycle.

Whereas significant progress has been made in the structural and biochemical characterization of the enzymes initiating the de novo biosynthesis of pyrimidines in bacteria, CAD, the multienzymatic protein leading this essential metabolic pathway in animals, remains surrounded by mystery. What are the structures and functional mechanisms of CAD’s enzymatic domains? What are the evolutionary advantages for their fusion? How they assemble? What is the architecture of the megaenzyme? Where is CAD within the cell? Why there are no diseases associated with defects of any of CAD’s essential activities? The last 10 years provided important answers to these questions, and we believe it is a good timing to review the efforts toward understanding CAD and its function in health and disease.

2 | GETTING CAD IN SHAPE

2.1 | Starting from the end: The ATC domain

The discovery of CAD is ligated to the rational design of PALA (N-phosphonacetyl-L-aspartate), an ATC inhibitor with structural features of the substrates CP and Asp that mimics the transition-state of the reaction. PALA proved to be a potent inhibitor of *Escherichia coli* ATC (ecATC), and also penetrated easily in mammalian cells, arresting the growth of mouse tumors and proving that de novo pyrimidine synthesis is mandatory for cell proliferation. However, a fraction of PALA-treated cells overcame the inhibitory effect by producing large amounts of ATC, and the attempt to purify the enzyme led to the striking discovery that it was covalently attached to CPS and DHO forming a single multienzymatic protein.

Forty years later, the first detailed structural information about CAD, that of the isolated human ATC domain free and bound to PALA, came to light (Table 1). The structure of the ATC domain of the CAD-like protein from *Chaetomium thermophilum* was also determined. The structures reveal a homotrimer of triangular appearance and with high similarity to bacterial ATCs (Figure 3a). Each subunit of 33 kDa is divided into two domains of comparable size (Figure 3b): an N-terminal domain that provides most contacts with the other subunits and holds the site for CP, and a C-terminal domain bearing the site for Asp. Both domains are structured by a central β-sheet of five parallel strands flanked by α-helices. The active site is formed at the cleft between the N- and C-domains and includes a loop (CP-loop) from the adjacent subunit (Figure 3a–c, colored in magenta). CP is
the substrate binding in the first place, inducing the repositioning of the CP-loop from the adjacent subunit and the closure of the C-domain, which create the pocket for Asp. Upon Asp binding, the subunit closes even more, with a large movement of a loop at the C-domain (Asp-loop) that traps and brings the substrates in close contact to favor the reaction (Figure 3b,c).19,25

Surprisingly, the human ATC trimer was shown to bind PALA with high affinity to the first two subunits, but with 100-fold lower affinity to the third one.19 Since PALA is a transition-state analog, this suggested that despite being a trimer, only two ATC subunits catalyze the reaction at a time. The idea, although bizarre, received support from the first structure of an ATC from plants. The Arabidopsis ATC was shown to bind only one PALA molecule per trimer, and thus to catalyze the reaction only in one subunit at a time.26 The mechanistic explanation resides in the CP-loop, that upon reaching the catalytic state, blocks the movement of the other CP-loops to a similar active conformation.26
In sum, CAD’s ATC domain depends on its oligomerization as a trimer to be active, and within this association there must be a communication between subunits that coordinates the sequential firing of the active sites.

2.2 | DHO, a structural element with replaceable enzymatic activity

DHO is a metalloenzyme catalyzing the reversible interconversion of carbamoyl aspartate to dihydroorotate (Figure 1). The isolated 43 kDa DHO domain of human CAD was the first eukaryotic DHO to be structurally determined (Table 1).\textsuperscript{20,27} The protein is a homodimer in solution, with each subunit folded in a barrel of eight β-strands flanked on the outer surface by α-helices and an additional subdomain formed by the N- and the C-terminal regions (Figure 3d,e). The active site locates in a cavity shaped by the loops connecting the β-barrel with the outer α-helices and contains two catalytic Zn\textsuperscript{2+} ions coordinated by the side chain of a carboxylated Lys, four His and one Asp (Figure 3e,f). The active site is superimposable to that of \textit{E. coli} DHO,\textsuperscript{28} including a catalytic flexible loop that orients the substrate in the active site.
stabilizes the transition state of the reaction and excludes water from the active site.\textsuperscript{20,22,29} CAD’s DHO also has a third Zn\textsuperscript{2+} ion at approximately the center of the \(\beta\)-barrel that interacts with one of the catalytic metals through the side chain of a rare histidine with negative charge (histidinate anion) (Figure 3f). This third Zn\textsuperscript{2+} ion has not been described in bacterial DHOs and is proposed to increase stability and to influence the electrostatic environment of the active site.\textsuperscript{20,30}

The crystal structure of the inactive DHO-like domain of the CAD-like protein from \(C.\) \textit{thermophilum} has also been determined (Figures 2a and 3g; Table 1).\textsuperscript{23} The structure shows a protein devoid of active site but assembled in identical dimers to those observed in human DHO (Figure 3g). The dimerization involves lateral contacts of the \(\alpha/\beta\)-barrel opposite to the adjacent domain, with both subunits oriented in the same direction. Interestingly, the construction of a monomeric human DHO mutant, proved that dimerization is not required for the enzyme to be active.\textsuperscript{20}

Thus, in addition to the enzymatic activity, which is replaceable in the case of fungi, the DHO and DHO-like domains preserve an oligomeric organization that is conserved from yeast to humans, and that could be required for the assembly of the multienzymatic particle.

2.3 | Known unknowns of CPS-2 structure

The smallest intermediate in the de novo pathway, CP, is synthesized by the largest enzyme (Figures 1 and 2a). CPS-2 covers the \(\sim1.000\) aa N-terminal half of CAD and is divided in two parts: a \(\sim40\) kDa GLN domain that obtains ammonia from glutamine (Figure 1, reaction 1a), and a \(\sim120\) kDa synthetase (or SYN) domain that

![Figure 4](image-url)

\textbf{Figure 4} The structures of \(E.\) \textit{coli} CPS and human CPS-1 shed light on the structure of CAD’s CPS-2. (a) Cartoon representation of \(E.\) \textit{coli} CPS heterodimer with the small glutaminase (GLN) subunit colored magenta and the large synthetase (SYN) subunit colored in yellow (phosphorylation domains), blue (integration domain) and green (regulatory domain). The image was composed from different structures of the protein in complex with the indicated compounds that are shown as spheres (PDB ID 1T36, 1C3O, 1JBD). The internal tunnel connecting the active sites is represented as a semitransparent tube. (b–d) Detail of the glutaminase active site (b), the carbamate phosphorylation site (c) and the regulation site (d) in \(E.\) \textit{coli} CPS. The equivalent regions of human CPS-1 have been superimposed and colored in white. Some important residues are numbered in pink (b), green (d) or yellow (c) for \(E.\) \textit{coli} CPS and gray for human CPS-1. The equivalent conserved residue in CAD’s CPS-2 are indicated in between parenthesis.
2.4 | The CAD puzzle: How to put the pieces together

Knowing that DHO and ATC formed dimers and trimers, respectively, a model was proposed for the architecture of CAD, where the hexamers would actually be “dimers of trimers.” According to the model, three CAD poly-peptides could engage through their ATC domains forming a trimer, and two of these trimers could further dimerize by association of the DHO domains. A similar model had also been suggested for the Neurospora CAD-like protein, although dimerization was proposed to occur through the CPS domains. The central role of the ATC domain was proven by mutations in the predicted trimer interface, which impede the formation of the hexamers. Then, to further validate the “dimer of trimers” model, a construct spanning the DHO and ATC domains of human CAD, including the 96 aa linker, was made and shown to form hexamers in solution. In turn, similar DHO-ATC constructs bearing mutations at the ATC or DHO oligomerization interfaces were shown to assemble into dimers or trimers, respectively. Similar results were obtained for a construct spanning the DHO-like and ATC domains of *C. thermophilum* CAD-like protein. Based on these results, we constructed a model for a CAD hexameric particle with D3 symmetry—a three-fold axis with three perpendicular twofold axes—where two ATC trimers oriented face-to-face occupy apical positions separated by three DHO dimers (Figure 5a). The CPS-2 could arrange as three dimers surrounding the central DHO-ATC scaffold. Both ecCPS and huCPS-1 are known to form dimers in solution, although this does not affect their activity. Indeed, both proteins crystallized as dimers, and the dimerization interface was shown to be adjacent to the regulatory domain, as represented in Figure 5a for the putative CPS-2 dimer.

While experimental evidence is still awaiting, one can consider how this CAD model agrees with biochemical data, what could be the mechanistic advantages and how it compares to the organization of these enzymatic activities in other organisms.

3 | THE ASSEMBLY AND REGULATION OF PYRIMIDINE FACTORIES

The covalent linkage of the three enzymes leading the de novo UMP synthesis might improve the metabolic efficiency of the pathway in a number of ways. The production of a single multi-enzymatic protein grants the coordinated and stoichiometric production of the
activities acting consecutively in the pathway and favors their co-localization within the cell, minimizing the diffusion of metabolic intermediates and the interference with other pathways. Also, proximity might favor the association between the enzymes, perhaps enhancing their stability, and allowing the shuttling of intermediates between active sites. CP is very unstable and is partially channeled in CAD and CAD-like proteins between the CPS-2 and ATC active sites. In the CAD model, the exit of the CPS-2 tunnel faces the gap between DHO dimers, so that CP could be delivered into the inner space and diffuse a short distance to the ATC trimers where the high affinity for CP would facilitate its capture.

The tight association of the enzymatic domains could also favor more complex mechanisms of regulation. It is known that binding of UTP or PRPP to the CPS-2 regulatory domain affects ATC activity; whereas, binding of PALA to ATC changes the substrate affinity of CPS-2. This “reciprocal allostery” could be explained as the result of concerted movements of the CAD model around the symmetry axes (Figure 5a, red arrows). Similar to the changes induced by NAG in huCPS-1, the binding of effectors to the regulatory domain could induce a reorientation in the CPS-2 dimer, with concomitant movements of DHO dimers and ATC trimers around and along their symmetry axes; reciprocally, the closure of the ATC subunits upon substrates or PALA binding could translate into movements of the CPS-2 subunits. These concerted movements could control the affinity for the substrates, the rate and coupling of the reactions and the channeling of reaction intermediates. The equilibrium between different conformational states of the CAD particle could be further modulated by post translational...
modifications (e.g., phosphorylations\textsuperscript{12,13,49,50}) or interactions with other proteins.\textsuperscript{51}

The proposed CAD model shares some features with the association of ATC and DHO in some bacteria. In \textit{Aquifex aeolicus}, two ATC trimers assemble face-to-face with three DHO dimers in a complex with D3 symmetry needed for both enzymes to achieve maximal activity (Figure 5b).\textsuperscript{38,52} The DHO subunits dimerize through lateral contacts of the \(\beta\)-barrel like human DHO, but the subunits are oriented toward opposite ATC trimers favoring the communication between their active sites.\textsuperscript{53} In other bacteria (e.g., \textit{Pseudomonas aeruginosa}), a similar association is proposed between ATC trimers and dimers of an inactive DHO that would play a structural role similar to the DHO-like domain of fungal CAD-like protein.\textsuperscript{54}

The interaction between ATC and DHO is not mandatory. In some bacteria, like \textit{E. coli}, two catalytic ATC trimers associate through three dimers of regulatory subunits (Figure 5c), forming a dodecameric holoenzyme with no interaction with DHO.\textsuperscript{10,39} Within this complex,

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{CAD_function_in_health_and_disease.png}
\caption{CAD function in health and disease. (a) Expression of CAD in human tissues based on RNA levels (in transcripts per million, TPM) and protein expression data obtained from the Human Protein Atlas database (www.proteinatlas.org). (b) Functional assay to validate the pathogenicity of CAD variants using patient-derived fibroblast and HPLC analysis of nucleotide levels. The graph is adapted from Koch et al.\textsuperscript{58} (c) Growth complementation assay using CAD-knockout (CAD-KO) cells unable to grow in absence of uridine. Transfection with a functional CAD variant, rescues the growth phenotype in absence of uridine. If transfected cells do not grow, the variant is identified as pathogenic. Graph adapted from del Caño-Ochoa et al.\textsuperscript{59} (d) Representation of the 44 exons in the \textit{CAD} gene, indicating all the clinical variants described up today, colored according to the result of the cellular complementation assay: pathogenic (red), benign (cyan) or not tested (grey).}
\end{figure}
the substrate affinity and activity of the ATC trimers is modulated by binding of ATP (activator) or UTP and CTP (inhibitors) to the regulatory dimers (Figures 2b and 5c). The allosteric mechanism involves the rotation and translation of the catalytic trimers and regulatory dimers around and along their symmetry axes, resulting in a conformational equilibrium between active (R-state) and inactive (T-state) forms (Figure 5c). The E. coli DHO, on the other hand, is an independent homodimer formed by subunits interacting through the loops above the active sites and not by lateral contacts as in human or A. aeolicus DHOs.28,29 The E. coli DHO structure and dimeric association is shared by the active yeast DHO, which replaces the inactive DHO-like domain of the CAD-like protein,66 and is also probably shared by the DHO in plants.20 Intriguingly, in plants, ATC was long known to be a trimer without regulatory subunits but yet still inhibited by UMP (Figure 2c). This observation was puzzling, since the isolated bacterial ATC or CAD’s ATC domains lack regulatory properties.19 But recently, the crystal structure of Arabidopsis ATC bound to UMP revealed a unique mechanism of regulation, where the nucleotide binds to the active sites rather than to an allosteric pocket, and directly competes with the binding of the substrates (Figure 5d).26

In sum, it is fascinating how homologous enzymes with overall structural similarity and virtually identical active sites combine into assemblies endowed with different regulatory properties (Figure 2b–d). It is even more fascinating to observe how macromolecular complexity builds up from small—sometimes subtle—changes in protein sequence.

4 | LOCALIZATION OF CAD IN THE CELL

The architecture is not the only elusive aspect of CAD. Its cellular location has been controversial and attracted much attention. CAD is ubiquitously expressed in all cells (Figure 6a). Early studies using differential centrifugation, located the CPS-2, ATC and DHO activities exclusively in the microsomal fraction of rat tissues,50 whereas others found a significant activity (20–30%) in crude nuclear fractions of rat ascites cellular carcinoma.61 The use of immunolocalization techniques showed CAD exclusively at the cytoplasm of baby hamster kidney (BHK) cells,62 but other study using antibodies against synthetic CAD peptides found the protein in the nucleus and cytoplasm of BHK cells, and also reported its localization throughout the nuclear matrix and outside the mitochondria of spermatozoa.63 CAD has also been reported in the nuclear matrix of adenovirus-infected HeLa cells, at sites of active viral replication.64

Parallel studies in yeast, proved by immunocytochemistry and using GFP-fused constructs, that yeast CAD-like protein (Ura2) is cytoplasmic and excluded from the nucleus.65 Nonetheless, using a similar GFP-hybrid, Sigoillot et al. reported the translocation of a significant fraction (20–30%) of hamster CAD to the nucleus during S phase.14,66 Attracted by the possible function of CAD in the nucleus, our group used GFP- and Cherry-tagged human and hamster CAD hybrids, antibodies against the endogenous protein, life imaging and subcellular fractionation, but repeatedly failed to detect the nuclear translocation of CAD throughout the entire cell cycle.67 Even the addition of a strong nuclear trafficking signal failed to change the cytosolic distribution of the protein. Instead, we found that the antibody used by others recognized at least one nuclear protein other than CAD,67 a result that was confirmed using CAD-knockout human cells (unpublished), and that reassured our conclusions that CAD localizes exclusively in the cytoplasm.

But the location of CAD is not an ended question. Different groups reported an intriguing punctuated patterns of CAD in the cytoplasm that does not correlate with mitochondria, cytoskeleton, lysosomes or other subcellular structures.14,50,62,67–69 Remarkably, this dotted distribution is enhanced upon activation of de novo pyrimidine synthesis. In addition to MAPK and PKA phosphorylation, CAD is phosphorylated at the DHO-ATC linker by the S6 kinase in the downstream pathway of mTORC1.50,51 The phosphorylation activates CAD and enhances its punctuated distribution. These enlarge CAD foci have been related to protein oligomerization,50 but other factors could be involved, including the interactions with other proteins of the mTORC pathways, like Rheb or mLST8.59,70 This punctuated CAD pattern resembles the foci observed upon activation of the de novo biosynthesis of purine nucleotides. Under conditions of high purine demand, the six enzymes involved in this pathway (three are multienzymatic like CAD) cluster into a dynamic multienzyme complex named “purinosome,” where they might find a favorable microenvironment for enhanced stability and transfer of intermediates without interference of other metabolic pathways.71 The possibility that CAD assembles into a supramolecular complex or “pyrimidinosome” is of great relevance to understand the regulation of de novo UMP synthesis and deserves further investigation.

5 | DEFECTS IN CAD CAUSE A RARE NEUROMETABOLIC DISEASE

Partial or total loss of function of any of the three enzymes involved in de novo pyrimidine biosynthesis cause severe congenital metabolic disorders. UMPS
deficiency or orotic aciduria (OMIM #258900) is a rare autosomal recessive disease characterized by excretion of orotic acid, anemia, seizures and intellectual and motor impairment. DHODH defects, on the other hand, cause Miller syndrome (OMIM #263750), a rare malformation disorder with no intellectual damage. However, the fact that no diseases were associated with defects in CAD led to the belief that mutations compromising any of its enzymatic activities would be lethal. Then, in 2015, the first case of a child with a rare inborn error of metabolism and biallelic mutations in CAD was reported, and was shortly followed by a second study describing four similar cases from three unrelated families. This CAD-deficiency (OMIM #616457), also known as early infantile epileptic encephalopathy-50 (EIEE-50) or CAD congenital disorder of glycosylation, typically manifests at an early age as a progressive refractory epilepsy with loss of skills, global developmental delay, intellectual disability and movement disorders, anemia and abnormal red blood cells. The course of the disease can be lethal, with most untreated cases not surviving beyond 4.5–9 years of age. However, oral supplementation with uridine or UMP replenish the pyrimidine levels through the salvage pathway (Figure 1) and leads to significant improvement, with immediate cessation of seizures, and progression from a minimally conscious state to communicate and move without assistance after several weeks of treatment.

An early treatment can prevent irreversible damage, but the diagnosis is difficult since there are no biomarkers for screening and symptoms are easily confused or masked by nutritional factors. So far, all 31 CAD-deficient patients reported worldwide were diagnosed by exome sequencing and validation of CAD variants. However, the large size of CAD and our still limited knowledge about how it functions impose great difficulties to interpret the pathogenic potential of clinical missense mutations. To test the functionality of these variants it is possible to use patient-derived fibroblast and measure the levels of nucleotides and UDP-sugars (Figure 5b), as well as the incorporation of labelled Asp into DNA and RNA. A complementary cellular assay has been developed using human CAD-knockout cells that only proliferate in media supplemented with uridine or upon transfection with a functional CAD variant (Figure 5c). This assay is used to distinguish between “benign” variants that restore cell proliferation in absence of uridine from those pathogenic variants that damage CAD activity and impair de novo pyrimidine synthesis and cell proliferation (Figure 5d).

In the future, the advent of affordable next generation sequencing for genetic testing, the automation of functional assays and an in-depth knowledge of CAD structure and function will improve the diagnosis and treatment of children affected by this rare neuro-metabolic disease.

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CONFLICT OF INTEREST
The authors declare no competing financial interests.

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Francisco del Caño-Ochoa: Writing - original draft; writing-review & editing. Santiago Ramón-Maiques: Funding acquisition; visualization; writing - original draft; writing-review & editing.

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