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Identification of the function of the metabolite repair enzyme Nit1: the story of a collaboration with Arthur Cooper

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A B S T R A C T

Nit1 and Nit2 were initially identified in the context of cancer research, as proteins encoded by putative (anti)oncogenes. However, the presence of homologous proteins in bacteria suggested that they might be enzymes with a fundamental metabolic function. Our group, while interacting with Arthur Cooper and his collaborators, contributed to uncovering these roles: Nit2 was identified in 2009 as an ω-amidase, the enzyme that hydrolyses the 'ω' amide of α-ketoglutaramate and α-ketosuccinamate, the 'deaminated' derivatives of glutamine and asparagine produced in some irreversible transamination reactions. Later, in 2017, we showed that Nit1 functions as a metabolite-repair enzyme. Specifically, Nit1 efficiently hydrolyzes deaminated gluthathione (dGSH), a non-functional byproduct generated by a side activity of various classical transaminases. This repair function prevents the accumulation of the useless metabolite dGSH. The physiological significance of Nit1 is underscored by recent discoveries linking its deficiency in humans to a neurological disorder.

1. Nit1 and Nit2: two human members of the nitrilase family

The nitrilase family includes both authentic nitrilases, but also amidases [1]. Since vertebrates neither synthesize nor degrade nitriles, it is not surprising that the two vertebrate members of this family, nitrilase 1 (Nit1) and nitrilase 2 (Nit2), function as amidases rather than nitrilases.

Nit1 was first identified as a fusion protein with Fhit1 in *Drosophila* and *Caenorhabditis elegans* [2]. Fhit (fragile histidine triad), is a protein that belongs to the histidine triad (HIT) family of nucleotide hydrolases and cleaves substrates such as diadenosine-triphosphate and diadenosine tetraphosphate [3]. The human *FHIT* gene is located at a common fragile site (FRA3B) on chromosome 3, and its loss is implicated in the development of several cancers, including gastrointestinal carcinomas. Subsequent studies demonstrated that Nit1 also exists as an individual (non-fusion with Fhit) protein in humans, other mammals and many additional species [2]. A second family member, Nit2, sharing approximately 35 % sequence identity with Nit1, was later identified [4].

2. The Nit-Fhit fusion

The observation that Nit, in many invertebrates, formed a fusion protein with Fhit had two relevant implications. On the one hand, the inferred connection with tumor development raised a substantial interest in deciphering the physiological substrate(s) of the human Nit1 and Nit2 enzymes. On the other hand, the same fusion seemed to offer a key for understanding the function of the two Nits.

Indeed, fusion proteins usually comprise two or several domains that work together in a process. Three steps of the synthesis of pyrimidines are catalyzed by different domains of a single multifunctional enzyme in vertebrates, while they are catalyzed by distinct proteins in bacteria [5]. Finding that in some species, two putative enzymes are assembled in a bifunctional protein is highly suggestive that the two enzymes are working together in the same pathways [6]. As an example, NADHX epimerase, which helps NADHX dehydratase to repair the hydrated form of NAD(P)HX was discovered because it forms a bifunctional protein with NADHX dehydratase in bacteria, though not in eukaryotes [7]. Hence, the finding that Nit1 was often fused with Fhit suggested that the enzymatic functions of the human Nit1 and/or Nit2 were tightly connected to the function of Fhit. However, this eventually proved a false lead and the functions of the Nit enzymes were uncovered following completely different routes.

3. Nit2 is ω-amidase

The first of the two enzymes whose function was identified was Nit2.

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This discovery was made independently and almost simultaneously by Cooper's group [8] and by our group [9]. Nit2 was shown to catalyze the hydrolysis of the amide groups of α-ketoglutaramate and α-ketosuccinamate, two α-keto acids that are formed from glutamine and asparagine by transamination with glutamine transaminases (Fig. 1A). Importantly, because glutamine is far more abundant in tissues and cells than asparagine, these enzymes primarily use glutamine as their α-amino group donor.

Unlike most transaminases, which catalyze readily reversible reactions, 'glutamine-transaminases', catalyze reactions that are essentially irreversible. Several factors contribute to this irreversibility. First, glutamine is present at high concentrations in cells. Second, the product of glutamine transamination, α-KGM, rapidly cyclizes to form a lactam [10], which is about 300-fold more abundant than its linear form [11,12]. Third, the open form of α-ketoglutaramate is efficiently hydrolyzed by ω-amidase, which also acts on α-ketosuccinamate, the product of asparagine deamination [11].

The cooperation between glutamine transaminases and ω-amidase is crucial for the methionine salvage pathway. During polyamine synthesis, 5′-methylthioadenosine is formed and subsequently converted in several steps into 2-keto-4-methylthiobutyrate ('ketomethionine') [13], which is then irreversibly transaminated back to methionine by a glutamine transaminase [14]. Glutamine transaminases are also involved in metabolite repair [15]: they re-aminate non-functional α-ketoacids generated by side reactions of aminotransferases such as GOT (glutamate:oxaloacetate transaminase) and GPT (glutamate:pyruvate transaminase) on phenylalanine, tryptophan, tyrosine and possibly histidine [14,16].

Cooper's group identified Nit2 as ω-amidase by purifying the enzyme to near homogeneity and sequencing it [8]. Our approach was based on database searches: in bacterial operons encoding the methionine salvage pathway [17,18], we observed, next to the gene encoding a glutamine transaminase (MtnV/YkrV) acting on ketomethylthiobutyrate [19], a gene encoding an amidase of unknown function, which we hypothesized to be ω-amidase [9]. This protein was homologous to both Nit1 and Nit2, suggesting that one of the two vertebrate enzymes was ω-amidase. Supporting this idea, the amino acid composition of rat liver ω-amidase purified by Hersh in 1971 [11] was more similar to Nit2 than to Nit1. Expression of recombinant Nit2, performed both by Cooper's group and ours, confirmed that Nit2 had indeed all the characteristics of ω-amidase, whereas Nit1 is virtually inactive on α-ketoglutaramate.

We submitted our findings to *Biochimie* in June 2009 [9] while Cooper and colleagues submitted theirs to *PNAS*, coincidentally almost on the same day. Arthur Cooper was selected as one of the reviewers for our manuscript. In his report, he wrote a positive evaluation, but informed the editor of *Biochimie,* Dr. Claude Forest, that his group had also submitted a manuscript with the same conclusions, but using a different approach, to *PNAS*. He proposed that if their manuscript was not accepted there, it would be valuable to publish both studies back-to-back in *Biochimie*. In the end, the two articles were indeed published back-to-back in *Biochimie* [8,9], which satisfied all parties.

This first interaction with Arthur Cooper and his group marked the beginning of a fruitful collaboration. Together, we later elucidated the function of Nit1, a discovery that took more time, but eventually revealed Nit1's critical role as a metabolite repair enzyme.

4. Initial collaborative work on Nit1

In August 2009, Emile Van Schaftingen (one of the co-authors of this review) exchanged emails with A. Cooper and asked a specific question: Could Nit1 hydrolyze a keto-derivative of glutathione (GSH) ? Arthur's reply was very instructive. Many years earlier, as a specialist in glutamine transaminases, he had tested whether GSH could serve as a substrate of these enzymes, but found no evidence for such activity. He also

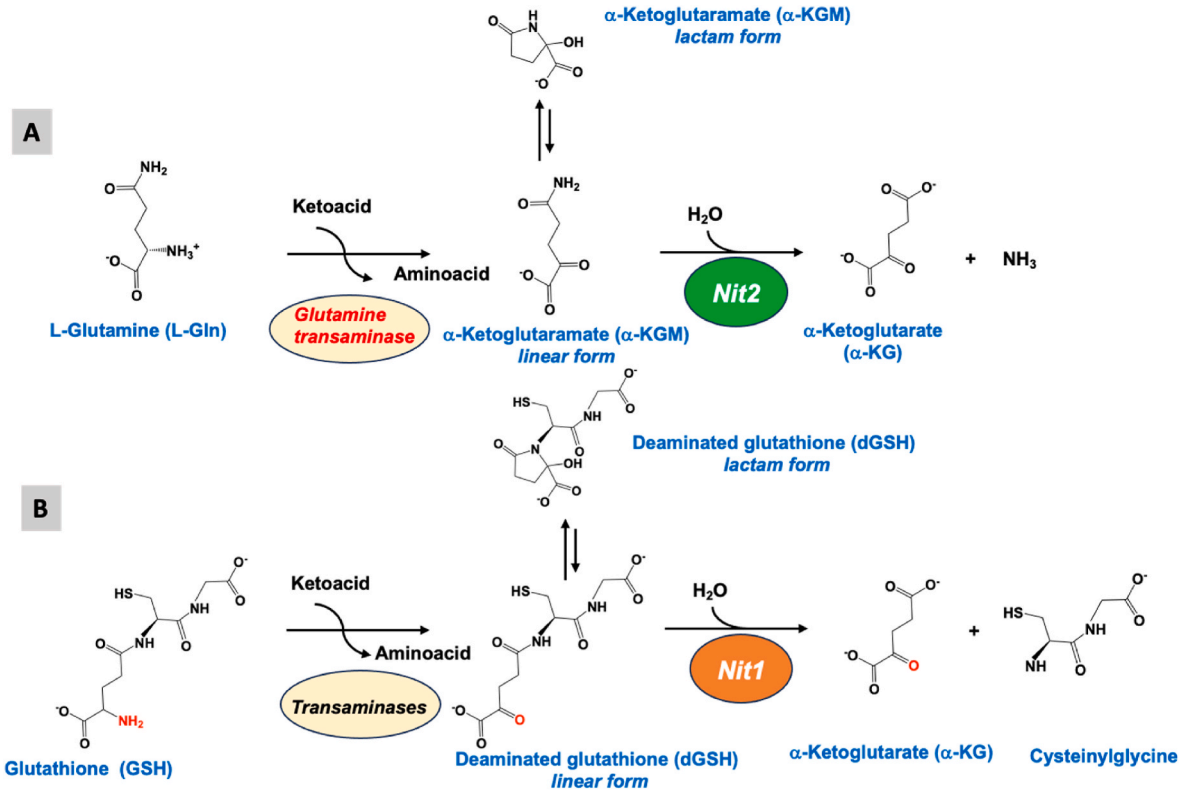

Fig. 1. The reactions catalyzed by ω-amidase (Nit2) (A) and by dGSH-amidase (Nit1) (B) in their context.
For both α-KGM and dGSH, the cyclic forms (lactams) result from an intramolecular nucleophilic attack of the amide nitrogen on the keto group. The lactam forms of both molecules, which exist as two anomers (not shown) are strongly favored due to their thermodynamic stability. In the case of α-KGM, the lactam form represents 99.7 % of the total at pH 7.2 [11,12].

drew our attention to a paper by Otani and Meister [10] showing that GSH was a substrate, albeit rather poor, for snake venom L-2-aminoacid oxidase. Having tested this himself, Arthur concluded that the reaction product had a 'masked carbonyl' (unreactive towards ceryl sulfate or H_2O_2), consistent with the lactam form of deaminated glutathione (dGSH). He suggested that snake venom L-2-aminoacid oxidase might, nevertheless, provide a convenient way of producing the putative substrate for Nit1.

Another aspect of our collaboration was the use of mass spectrometry to analyze urine from Fhit, Nit1 and double Fhit/Nit KO mice, thanks to the help of Kay Huebner and colleagues at Ohio State University. In Fhit KO mice, but not in Nit1 KO mice or double KO mice (or only to a small extent), we observed accumulation of glutarate in urine from some mice. This finding, which would need to be confirmed in larger cohorts, however, did not provide much insight into the function of Nit1. Of note, Tomiko Kuhara from the Japan Clinical Metabolomics Institute detected in the urine of the Nit1 KO mice the accumulation of an unknown compound ("Unknown 1"), with a structure that none of us could identify at the time. The compound was smaller than dGSH. In hindsight, it would have been logical for us, in the de Duve Institute, in Brussels, and in Valhalla, where Arthur Cooper's laboratory was, to prepare dGSH and directly test whether it served as a substrate of Nit1, and compare it to "Unknown1". However, competing hypotheses made this option less obvious at the time, and several years passed before it was properly tested!

5. The importance of metabolite repair

As mentioned above, the eventual discovery of Nit1's activity stemmed from the hypothesis that it might function as a repair enzyme, serving to destroy one or various unusual metabolites made by one or more side reactions of central metabolic enzymes. Indeed, our earlier work on L-2-hydroxyglutaric aciduria had already led us to the conclusion that cells require enzymes specifically dedicated to eliminate such side-products. In L-2-hydroxyglutaric aciduria, L-2-hydroxyglutarate is generated as a byproduct of the side activity of L-malate dehydrogenases [20] and L-lactate dehydrogenases [21]. Although, these reactions are about 10^6 time less efficient than their canonical activities, they still lead to the formation of significant amounts of L-2-hydroxyglutaric acid, several grams per day [20]. This harmful compound is normally degraded by L-2-hydroxyglutarate dehydrogenase, an FAD-linked mitochondrial inner membrane enzyme that irreversibly re-converts L-2-hydroxyglutarate to α-ketoglutarate [22,23]. L-2-hydroxyglutaric aciduria indeed results from a defect in L-2-hydroxyglutarate dehydrogenase.

Given that many enzymes catalyze side reactions with cellular metabolites at rates much higher than those observed for malate dehydrogenase and lactate dehydrogenase, we proposed that the genome must encode numerous metabolite repair enzymes [24]. Many of the uncharacterized enzymes found in humans, and indeed in all species, including bacteria, likely belong to this class.

This concept proved highly fruitful, enabling us to assign functions to several previously uncharacterized enzymes. Examples include:

- an enzyme that destroys ethylmalonylCoA [25], a side product of acetyl-CoA carboxylase [26] and of propionyl-CoA carboxylase [27];
- a phosphatase that detoxifies 4-phosphoerythronate and L-2-phospholactate [28], two metabolites produced by side reactions of glyceraldehyde-3-phosphate dehydrogenase [28,29] and pyruvate kinase [30], respectively;
- G6PC3, an endoplasmic reticulum-localized phosphatase which, in collaboration with the glucose-6-phosphate transporter SLC37A4, dephosphorylates 1,5-anhydroglucitol-6-phosphate, a compound made by side reactions of hexokinases and which is particularly harmful for neutrophils [31].

For more information on metabolites repair and its diseases, the reader is invited to consult the reviews published on this subject [24, 32–36].

6. Structural differences between Nit1 and Nit2 suggest a metabolite repair role for Nit1

Given its high degree of identity with Nit2, a natural hypothesis was that Nit1 might also function as an efficient ω-amidase. However, as noted above, recombinant Nit1 exhibits more than 1000-fold lower activity on α-ketoglutaramate and α-succinamate compared with Nit2. Comparison of the structure of Nit2 [37] and of Nit1 (in *Caenorhabditis elegans* Nit-Fhit; [4]) revealed that while the catalytic triad and the α-ketoglutarate binding site are conserved in both enzymes, the region surrounding the amide-binding pocket is much larger in Nit1 than in Nit2 (Table 1).

This raised the possibility that that Nit1 might act on a bulkier substrate that could be formed as a side-product of enzyme activity. Two candidates were considered:

1 – dGSH - potentially generated by transamination involving the α-amino group of the glutamyl moiety of GSH. Given the high concentration of GSH in cells, compared to most amino acids, this was a strong possibility. In support of this idea, crystallization of an inactive form of the yeast homolog of Nit1 (in which the catalytic cysteine, Cys169, was replaced by a serine) revealed a large ligand, of approximately the size of GSH, bound in the catalytic site [38]. However, the authors did not test this hypothesis directly and the possibility of the unknown ligand being dGSH was mentioned only in the discussion section, not in the results or in the summary.

2 – α-Ketoglutaryl-CoA - a hypothetical compound that could be produced as a side reaction of the enzyme that transfers CoASH from succinyl-CoA onto dicarboxylic acids (encoded by the C7ORF10/SUGCT gene [39]). The main physiological function of this enzyme is to allow the conversion of glutarate to glutarylCoA, which can then be metabolized by glutaryl-CoA dehydrogenase. However, it can also act on hydroxymethylglutarate, a compound with similar size to α-ketoglutarate, producing its CoA ester, as well as showing several other side activities [39].

7. Nit1 is a dGSH-amidase

We prepared dGSH and its structural analog deaminated ophthalmic acid (dOA) using snake venom L-amino acid oxidase. Purification of these compounds revealed two peaks corresponding to the α and β anomers of the cyclic (lactam) form, whereas the linear form was not detectable by this method. Both dGSH and dOA proved to be excellent substrates for mouse Nit1 and its yeast ortholog (ScNit1), which displayed only a very weak, but significant, activity on α-ketoglutaramate (more than 1000-fold lower than on dGSH) [40].

Nit1 itself has only minimal hydrolase activity on GSH, just as ω-amidase shows only very low activity on glutamine. This likely reflects structural constraints: in both Nit1 and Nit2, the keto group on carbon 2 of the ketoglutaramate moiety forms π-π interactions with Phe131 and Phe195 (in ScNit1). These residues are highly conserved in mammalians Nit1 and Nit2, with the notable exception of human Nit1, where Phe195 is replaced by a cysteine (Table 1).

Reaction products confirmed hydrolysis of the γ-amide bond of the substrates, as expected (Fig. 1B). Additional experiments with dGSH showed that the true substrates were the minor open chain forms rather than the more abundant cyclic ones. Based on this, the calculated catalytic efficiency (Kcat/Km) for dGSH largely exceeds 10^6 M^{-1} s^{-1}, placing Nit1 in the high range for metabolic enzymes [41].

Functional studies [40] reinforced this assignment. Nit1 KO Hap1 cell lines generated using the CRISPR/Cas9 technology accumulated 6-fold more dGSH than control cells. Similarly, yeast cells deficient in

Table 1
Specific function of substrate-binding residues in Nit1 (dGSH-amidase) and Nit2 (ω-amidase) in various species. The assignments for the interaction of residues derives from the structure of *S. cerevisiae* Nit2 [38], which is shown to bind an unknown molecule hypothesized to be dGSH. Residues in ω-amidase shown in red cannot interact with dGSH. The protein sequences shown are referenced in the Uniprot database (https://www.uniprot.org): *S. cerevisiae* Nit2, P47016; H. sapiens Nit1, Q86X76; C. elegans NITFHIT, O76463; H. sapiens Nit2, Q9NQR4; S. cerevisiae Nit3, P49954. Interaction of the relevant substrate groups with the indicated amino acid residues: SC - interaction with the side chain; MC - interaction with the main chain. (1) Binding of this arginine residue to the carboxylate of the cysteinylglycine makes more sense than the proposed binding to the carbonyl of the cysteine moiety. The incorrect attribution may be due to the low resolution of the density attributed to dGSH [38].

FUNCTION	dGSH-AMIDASE			ω-AMIDASE	
PROTEIN	**scNit1** Nit2 *Saccharomyces cerevisiae*	**Nit1** *Homo sapiens*	**Nit-Fhit** *Caenorhabditis elegans*	**Nit2** *Homo sapiens*	**scNit2** Nit3 *Saccharomyces cerevisiae*
SUBSTRATE MOIETY :					
Catalytic triad - SC	Cys169	Cys203	Cys169	Cys153	Cys169
Catalytic triad - SC	Lys127	Lys161	Lys126	Lys112	Lys128
Catalytic triad - SC	Glu45	Glu86	Glu54	Glu43	Glu53
α-ketoglutaryl: C1 carboxylate - SC	Arg173	Arg207	Arg173	Arg157	Arg173
α-ketoglutaryl: C1 carboxylate - SC	Thr199	Thr233	Thr199	Thr183	Thr197
α-ketoglutaryl: C2 carbonyl - SC	Phe195	Phe229	Phe195	Phe179	Phe195
α-ketoglutaryl: C2 carbonyl - SC	Phe131	Cys165	Phe131	Phe116	Phe132
α-ketoglutaryl-Cys amide bond - MC	Ala194	Ala228	Ala194	Ala178	Ala192
Cysteinylglycine: carboxylate (1)	Arg250	Arg266	Arg232	Tyr216	Tyr232
Keep the substrate binding subpocket open in dGSH-amidase, or fill it in ω-amidase	Ser51	Ala92	Gly60	Tyr49	Tyr59

scNit1 displayed markedly increased levels of dGSH. In mice, Nit1 deficiency led to urinary dGSH concentrations about 15-fold higher than in controls. Furthermore, GC-MS analysis revealed that the compound previously identified as "Unknown 1" by Tomiko Kuhara corresponded to a dehydrated, dethiolated derivative of dGSH, explaining why its structure could not be elucidated in the absence of a dGSH standard.

The most plausible route for dGSH formation is transamination (see Fig. 1B). This was confirmed experimentally: five recombinant mammalian transaminases, from both cytosol and mitochondria were tested and all showed some capacity of forming dGSH. Additional evidence came from intact-cell studies, where aminooxyacetate, a general inhibitor of pyridoxal-phosphate-dependent aminotransferases, inhibited the accumulation of dGSH in Nit1 KO cells [40].

Subcellular localization studies revealed that Nit1 is present in both cytosol and mitochondria, consistent with the existence of two types of transcript variants encoding proteins with or without a mitochondrial targeting sequence. Nit1 is therefore capable of hydrolyzing dGSH in both cellular compartments where it is expected to be formed.

Finally, we explored the second hypothesis concerning another potential substrate of Nit1, α-ketoglutarylCoA. Both Nit1 and Nit2 were found to hydrolyze this compound at similar rates, suggesting a possible auxiliary role of Nit1 in eliminating this non-canonical metabolite. Nevertheless, evolutionary conservation of Nit1 is most likely explained by its unique and efficient ability to hydrolyze dGSH [40].

8. dGSH-amidase is widely distributed among organisms that produce GSH

Nit1 and Nit2 orthologs are present in animals, yeast and plants. The ortholog from *Arabidopsis thaliana* (now referred to as NIT1) has been characterized and shown to also function as an excellent dGSH-amidase. Its inactivation in plants leads to the accumulation of dGSH in plant tissues [42].

Many prokaryotes also contain homologues of Nit1 and of Nit2. However, in most cases, it is not possible to predict their specificity based solely on higher sequence identity to either Nit1 or Nit2. In *E. coli,* the homologues Yafv and Ybem have been expressed and characterized, as an ω-amidase and a dGSH-amidase, respectively. Interestingly, YafV shows similar identities to both Nit proteins (23 % to mouse Nit1 and 21.9 % to Nit2) whereas YbeM shows a 34.7 % identity to mouse Nit1 and 25.6 % identity to mouse Nit2 [40].

The best predictor of Nit1/2 homolog specificity is the set of residues that define the size of the subpocket accommodating either the cysteinylglycine moiety of dGSH or the terminal amine of α-ketoglutaramate. In mouse Nit2, the highly conserved Tyr87 and Tyr254 reduce the size of this subpocket [38,40]. In multiple alignments, the first of these residues is replaced by a glycine (or another small residue) in Nit1 and other dGSH-amidases, while the second one is replaced by an arginine or a lysine, which can form an ion pair with the carboxylic group of the glycine moiety (see Table 1). We proposed this criterion to predict the specificity of Nit1/2 homologues from bacteria [40].

dGSH-amidase seems to be absent from virtually all bacteria that lack GSH, while it is present in most, but not all, of the bacteria that synthesize GSH. Importantly, GSH is produced by a diverse set of enzymes, suggesting that this antioxidant was acquired on distinct occasions during evolution [43,44]. Sequence comparisons further suggest that dGSH-amidases themselves arose multiple times during evolution. It is also possible that, in some GSH-producing species lacking Nit1/2, dGSH-amidase activity is provided by an unrelated family of enzymes.

9. Why is dGSH-amidase important?

In mice, Nit1 deficiency leads to a substantial elimination of dGSH in urine (1.26 mmol/mmol creatinine i.e. 4.65 μmol/day assuming an excretion of 418 ± 100 μg creatinine/day; [45]). This corresponds to roughly 10 % of the total GSH pool per day [40].

Nit1 deficiency in mice does not cause overt phenotypic defects, nor does it affect embryogenesis, development, or fertility [46]. Because of Nit1's potential role in oncogenesis, studies have focused on tumor incidence. In mice, no spontaneous tumors were observed during one year, but Nit1 deficiency was associated with an approximate 2-fold increase in the incidence of NMBA-(N-nitroso-N-methylbenzylamine)-induced forestomach tumors. The overall phenotype has not been extensively studied.

Interestingly, humans with Nit1 deficiency develop neurological symptoms (see below), but understandably the phenotype found in humans, appearing mostly at a late age, was not reported for mice. Given the significant metabolic impact of Nit1 deficiency in plants, metabolomic profiling of Nit1 KO mice would be of considerable interest.

In plants, loss of dGSH-amidase causes a marked reduction of 22

different triglycerides [42]. The underlying mechanism remains unclear. The authors suggested that dGSH might inhibit GSH peroxidases, leading to enhanced lipid peroxidation. Although GSH levels were not decreased, Nit1-deficient cells showed a >10-fold reduction in sinapine, an antioxidant specific to Brassicaceae, consistent with elevated oxidative stress. Despite these biochemical changes, no impairment in plant growth was observed.

In *E. coli*, the commonly studied K-12 strain contains a mutation in the ω-amidase gene, *ybeM*, that introduces a frameshift, producing a truncated 76-residue protein instead of the full length 263-residue enzyme. In contrast, the *E. coli* BL21(DE3) strain carries an intact *ybeM* gene, which was used for overexpression studies and functional characterization [40]. Whether dGSH accumulates in the K-12 strain remains unknown.

10. Deficiency of Nit1

The physiological importance of dGSH-amidase has been highlighted by three recent studies describing patients with inactivating mutations in both alleles of the *NIT1* gene. All affected individuals presented with neurological symptoms, specific brain lesions, and were diagnosed in adulthood.

Rendu et al. [47] described a family in which 3 out of 9 siblings were affected. The index case had mild intellectual disability, experienced an acute psychotic episode at the age of 32, followed by a generalized seizure, and two years later developed sudden left hemiparesis. Brain imaging revealed bilateral cystic lesions affecting the basal ganglia. Similar brain abnormalities were observed in two brothers. One suffered a sudden coma with cardiovascular collapse at age 58, while the other was diagnosed with schizophrenia, at age 30. Exome sequencing identified two mutations: p.Gly153Arg and a c.670dupA, which truncates Nit1 after Thr224. Gly153 is highly conserved in Nit1 (and Nit2), and both mutations are most likely null alleles, consistent with the absence of Nit1 protein in patient's fibroblasts, as seen by western blotting. These mutations were not detected in unaffected siblings. All three patients showed a positive urinary sulfite test, confirmed by GC-MS to result from elevated dGSH [47].

Rutten et al. [48] in a study on genetic causes of intracerebral hemorrhage (ICH), reported seven additional patients with Nit1 deficiency from six families. Patients were either compound heterozygotes for a p.Arg243Trp variant (affecting a highly conserved residue in Nit1 and Nit2) and a deletion variant truncating the protein after Ala68, or homozygotes for p.Arg243Trp. Patient fibroblasts showed complete absence of Nit1 protein, and urinary dGSH was elevated, confirming Nit1 inactivation. Clinically, patients developed progressive movement disorders, gait instability, dysarthria, and slowly progressive cognitive decline. Two of them died of massive non-lobar ICH between age 45 and 60, while a third also had a history of ICH. Neuroimaging revealed a consistent pattern: dilated perivascular spaces, particularly in the basal ganglia and thalamus, producing a honeycomb appearance. Hemorrhagic foci were visible in the basal ganglia in three patients. Pathological analysis showed vessel wall abnormalities, including thickened media with abundant hyalin and fibrin. Electron microscopy revealed pronounced electron-dense deposits in the media and the adventitia, reminiscent of the osmiophilic granules seen in CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts) [49,50].

John et al. 2025 [51] reported an additional patient with Nit1 deficiency in his 50s, who presented with recurrent subcortical ischemic strokes. Imaging again showed numerous dilated perivascular spaces with a honeycomb appearance, along with chronic lacunar infarcts.

The mechanism by which Nit1 deficiency causes this novel disease remains unknown. Interestingly, Nit1 deficiency shares certain features with CADASIL [52], a small brain blood vessels disease that predisposes to ischemic strokes. CADASIL is caused by dominant heterozygous mutations of *NOTCH3* that abolish conserved cysteine residues within EGF-like repeats, or introduce new ones, leading to protein misfolding and aggregation [53]. One intriguing hypothesis is that dGSH, in Nit1 deficiency, which appears in urine and must therefore escape the cells where it is formed, may disrupt the disulfide bridges in Notch3 and/or other proteins. Alternatively, dGSH could diffuse into the ER and the secretory pathway, interfering with protein folding. These hypotheses certainly deserve to be tested when further exploring the pathophysiological mechanism of Nit deficiency.

11. Conclusion

The collaboration with Arthur Cooper and his team was important in defining the metabolic roles of NIT amidases. Cooper's group independently identified Nit2 as ω-amidase, an enzyme that hydrolyzes α-ketoglutaramate and α-ketosuccinamate, linking Nit2 to the glutamine transaminase pathway and methionine salvage metabolism. This discovery [8], published back-to-back with our own findings [9], firmly established Nit2's role in preventing accumulation of harmful intermediates from irreversible transamination reactions. Building on this foundation, joint discussions and shared insights and experiments led us to identification of the biochemical function of Nit1 as a dGSH amidase [40].

The discovery of the function of Nit1 provides a new example of metabolite repair and once again, a defect in metabolite repair has been linked to a human disease. A key challenge now is to identify the pathophysiological mechanisms, which most likely involve one or more toxic effects of dGSH. Several possibilities can be considered: Could dGSH, through its reducing power, interfere with the proper formation of disulfide bridges? Might it inhibit enzymes that normally use GSH as a substrate, such as GSH S-transferase and/or glutaredoxins, due to its structural similarity with GSH? Could it instead bind metals such as iron or copper with high affinity, thereby disrupting cellular metal homeostasis?

Another intriguing question concerns the *NIT-FHIT* fusion. At first glance, this fusion is puzzling: Nit1 functions in GSH metabolism, whereas Fhit hydrolyzes unusual nucleotides such as diadenosine-tri and tetra-phosphates. Does this fusion simply reflect chance, or are we overlooking an additional, evolutionary conserved function of one of these enzymes that could explain the persistence of such an ancient fusion?

CRediT authorship contribution statement

Emile Van Schaftingen: Writing – original draft, Conceptualization. **Alessio Peracchi:** Writing – review & editing. **Maria Veiga-da-Cunha:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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