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Hereditary spastic paraplegia type 56: what a mouse can tell – a narrative review

Livia Parodi[a,*], Claire Pujol[a,b,*]

Abstract

Hereditary spastic paraplegia type 56 (SPG56-HSP) is a rare autosomal recessive disorder caused by loss of function mutations in *CYP2U1*, leading to an early-onset limbs spasticity, often complicated by additional neurological or extra-neurological manifestations. Given its low prevalence, the molecular bases underlying SPG56-HSP are still poorly understood, and effective treatment options are still lacking. Recently, through the generation and characterization of the SPG56-HSP mouse model, we were able to take few important steps forward in expanding our knowledge of the molecular background underlying this complex disease. Leveraging the *Cyp2u1*$^{-/-}$ mouse model we were able to identify several new diagnostics biomarkers (vitamin B2, coenzyme Q, neopterin, and interferon-alpha), as well as to highlight the key role played by the folate pathway in SPG56-HSP pathogenesis, providing a potential treatment option. In this review, we discuss the major role played by the *Cyp2u1*$^{-/-}$ model in dissecting clinical and biological aspects of the disease, opening the way to a series of new research paths ranging from clinical trials, biomarker testing, and to the expansion of the underlying genetic and molecular, emphasizing how basic mouse model characterization could contribute to advance research in the context of rare disorders.

Keywords: folate, hereditary spastic paraplegia, mitochondria, mouse model, neurological diseases

Introduction

Hereditary spastic paraplegias (HSPs) are a group of inherited disorders caused by the progressive degeneration of the corticospinal tracts, leading to gait disorder and lower limbs spasticity, the disease hallmark.[1] HSPs are characterized by high heterogeneity concerning both their clinical manifestation and genetic background. Depending on symptoms, HSPs can be classified into pure or complex forms.[1] Pure HSPs are characterized by the presence of pyramidal signs predominantly affecting the lower limbs, causing lower limb weakness and spasticity, sometimes accompanied by sphincter disturbances.[2] The presence of additional neurological and extra neurological symptoms, such as spastic ataxia, cognitive decline, and optic atrophy, amongst many others, define the complex forms of HSPs.[1]

A wide heterogeneity characterizes not only HSPs' clinical manifestations, but also extends to the underlying genetic background. The recent introduction of next-generation sequencing (NGS) techniques in everyday diagnostic processes has greatly increased the power of genetic diagnosis, allowing to steadily increase the number of loci involved in HSPs onset, with more than 100 loci and 80 Spastic Paraplegia Genes (SPGs) reported to date.[3] Yet, despite these advances, the difficulty in connecting an observed phenotype with a specific candidate gene, and the fact that pathogenic mutations can be inherited through all known inheritance patterns or manifest sporadically, continues to make HSPs genetic diagnosis particularly challenging.[3] An additional aspect further complicating HSPs genetic landscape is the extreme phenotypic variability observed even among patients sharing the same causative mutation, suggesting the presence of additional variants acting as modifiers, as already observed in other neurological disorders.[4–6]

A direct consequence of the large number of genes and loci involved in HSPs is the extreme variety that characterizes SPG-encoded proteins and their roles in the cellular environment. Indeed, mutations affecting SPGs lead to dysregulation of a variety of essential pathways, including membrane trafficking, mitochondrial/lysosomal function, autophagy, and RNA metabolism.[7]

Along with the broadening of HSPs genetic and cellular background, remarkable advances have recently been made leveraging HSPs small animal models such as mouse, Drosophila melanogaster, and Zebrafish. The generation of such models has allowed the unraveling and refining of the biological mechanisms causing HSPs, ultimately resulting not only in a more precise and comprehensive understanding of the disease but also providing a crucial starting point for the development of new therapeutic strategies.[8]

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Among the different HSPs models available, mouse models remain the preferred one. Especially in neurological diseases such as HSPs, mouse models have allowed us to overcome the common limitations due to the difficulties in accessing target tissues of interest, such as specific subtypes of neurons or brain areas. Moreover, they have enabled the use of a wide range of tools like genome-editing through CRISPR-Cas9,[9] allowing accurate reproduction of a specific disorder. They opened the way to the exploration of the expressed phenotype leveraging complex behavioral and motor tests, providing a good read-out of neurological function, and allowing to recapitulate disorders' core characteristics. Open field, catwalk, footprint gait assessment, and Rotarod, as well as Y-maze and fear conditioning tests, are only some of the tests that could be performed leveraging HSP mice models to evaluate motor and cognitive impairments, two primary HSPs' hallmarks.[10] With the present review, we provide an overview of the results deriving from the recent characterization of the hereditary spastic paraplegia type 56 (SPG56-HSP) mouse model, which opens a new research path and highlight the important role of animal models in advancing the research of rare disorders.

Database search strategy

Literature review was electronically performed using PubMed database. The following combinations of keywords were used to initially selected the articles to be evaluated: Hereditary spastic paraplegia; SPG56-HSP; *CYP2U1*; mitochondria; folate; genetic modifiers; mouse model; neurological diseases. Most of the elected studies (93% of all references) were published from 2000 to 2019. An ancient publication from 1981 was included in consideration to its relevance in the mast cell field.

Clinicogenetic aspects of hereditary spastic paraplegia type 56

SPG56-HSP is a rare autosomal recessive disorder, caused by *CYP2U1* gene mutations.[11] *CYP2U1* encodes for a member of the cytochrome P450 family involved in the conversion of various substrates, such as vitamins, into their active forms. SPG56-HSP is one of the least frequent HSP subtypes (prevalence <1/1,000,000), estimated affecting 1.5% of HSP patients.[10] So far mutations in *CYP2U1* have been detected in 54 carrier patients,[10,12–14] with an equal repartition between men (28/54, 52%) and women (25/54, 46%) (Fig. 1A). Patients carrying pathogenic mutations often present with early-onset spastic paraplegia, with a mean age at diagnosis of 3.4 ± 6.86 years. As reported in Elsayed et al,[3] patients often present with neuroregression between 0 and 3 years of age (Fig. 1B). At the neurological examination, delayed walking or gait disturbance are the most common SPG56-HSP features (49/52, 94%), often accompanied by intellectual disability or cognitive impairment (35/53, 66%).[12]

Imaging analysis highlighted that brain calcifications (13/25 patients with available CT scans) and hypomyelination (25/39 with available magnetic resonance imaging [MRI] images) appeared to be an additional frequent feature (Fig. 1C). Interestingly, *CYP2U1* mutations have also recently been implicated in a neurocutaneous syndrome,[15] and it has also been suggested that they could affect the retina, leading to macular dystrophy.[13,15–17]

As often observed in HSPs, a huge intra- and inter-familial variability characterize the severity of symptoms presented by SPG56-HSP patients, even among those sharing the same causative mutation. A clear example of this phenomenon is provided by three families sharing a stop mutation (p.Arg390X) leading to the premature termination of *CYP2U1* translation. Despite carrying the same pathogenic mutation, the phenotype expressed by the affected patients resulted in being strikingly different (Fig. 2). Three Italian patients reported by Leonardi et al[16] presented with a mild HSP, characterized by lower limb spasticity starting after 25 years of age, complicated by a maculopathy. In a French family included in our cohort,[12] the same *CYP2U1* pathogenic mutation was observed leading to an extremely severe disability affecting both lower and upper limbs, starting before the first year of age, and accompanied by intellectual disability. In contrast with the other cases reported, carrier members belonging to a Tunisian family recently reported by El Matri et al[13] did not present any symptoms at neurological examination (age at examination: 12 years of

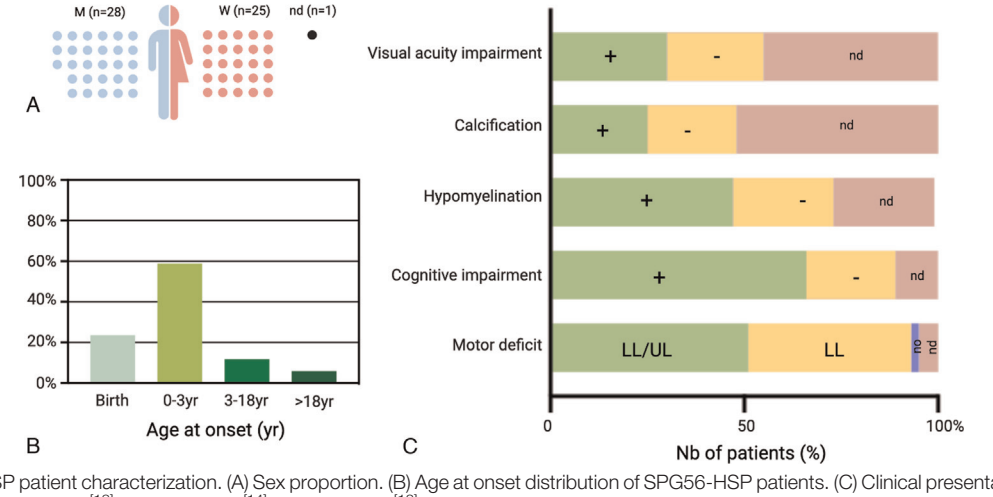

Figure 1. SPG56-HSP patient characterization. (A) Sex proportion. (B) Age at onset distribution of SPG56-HSP patients. (C) Clinical presentation of SPG56-HSP patients. Data from El Matri et al,[13] Sharawat et al,[14] and Pujol et al.[12] LL = lower limbs, M = man, Nb = number, nd = not documented, no = no motor deficits, UL = upper limbs, W = woman, yr = years.

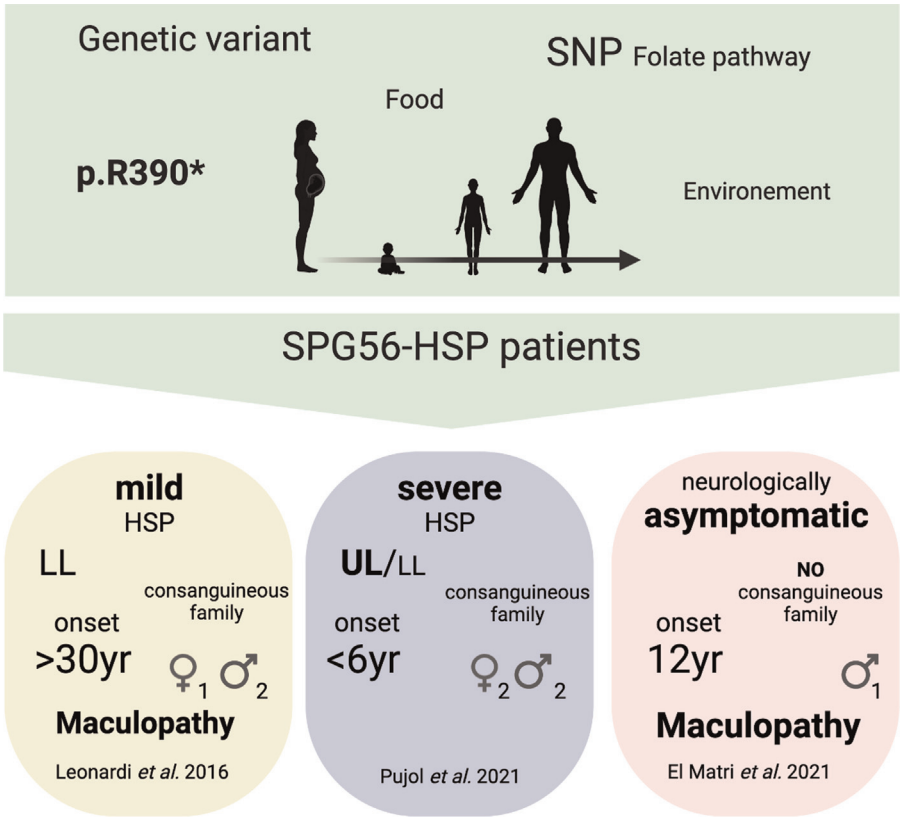

Figure 2. Example of the phenotypic variability among patients carrying the same SPG56 mutation (NM_183075.3(*CYP2U1*):c.1168C>T (p.R390X); rs772400670). Patients belonging to three different families from Leonardi et al,[16] El Matri et al,[13] and Pujol et al.[12] ♀=woman, ♂=man, HSP=hereditary spastic paraplegia, LL=lower limbs, UL=upper limbs, yr=years old.

age) but manifested visual deficits, similar to those observed in the Italian patients.

As for other forms of HSPs, the extreme phenotypic heterogeneity observed in these SPG56-HSP families cannot be entirely explained by the causative mutation itself. Further analyses aimed to identify additional genetic, epigenetic, and environmental factors acting as modifiers could help unveil the various players contributing to SPG56-HSP variability.

Cyp2u1$^{-/-}$ *mouse model: a precious tool to explore SPG56-HSP*

As in many other Mendelian disorders, animal models lacking a particular gene of interest have been generated to expand our understanding of the pathogenic mechanisms influencing HSPs onset and progression, allowing us to translate and validate genetic discoveries at the molecular and functional level. In the specific context of HSPs, behavioral analyses constitute the first set of analyses performed to investigate the presence of a motor phenotype. A large variety of tests are currently being used to evaluate gait disorders, focusing on motor coordination and strength. Despite promising results in a few select cases,[18] most HSP mice models do not recapitulate faithfully the human disease, often failing to display a motor dysfunction.[19]

Along with motor tests, and according to the manifestations and pathophysiology of the disease, a variety of additional tests evaluating cognitive capacity or nociceptive responses can be leveraged to further describe the presented phenotype.

As recently reported, we have generated and characterized the SPG56-HSP mouse model.[12] Since SPG56-HSP is caused by loss of function mutations affecting *CYP2U1* we leveraged the *Cyp2u1*$^{-/-}$ mouse from the Knockout Mouse Project,[20] which carried a ubiquitous constitutive deletion in exon 2, resulting in the complete *CYP2U1* loss (Fig. 3).[21]

Heterozygous mice were crossed to generate wild-type (*Cyp2u1*$^{+/+}$), heterozygous (*Cyp2u1*$^{+/-}$), and homozygous (*Cyp2u1*$^{-/-}$) mice. The overall phenotype of *Cyp2u1*$^{-/-}$ mice was assessed by comparing -/- mutants with wild-type and heterozygous littermates. Transgenic mice were viable and showed no gross abnormal phenotype: all mice carrying the same genotype were born at expected Mendelian frequencies and appeared normal and healthy. We did not observe any changes in lifespan nor in body mass.

We performed a series of behavioral tests including rotarod, open field, grip test, treadmill, and foot base angle to elucidate the possible influence of *CYP2U1* ablation on locomotor activity. No obvious phenotypic differences between the different genotypes were detected on the rotarod, open field, and grip test examination.[12] Since HSPs core symptom is spastic gait, we decided to use more specific behavioral readouts and we measured the presence of gait disturbance. We started measurements of specific angles, such as the foot-base angle during voluntary reaching pursuits. *Cyp2u1*$^{-/-}$ mice did not display any significant impairment. We next used the Clever Sys analysis system to examine the animals using an accelerating treadmill to sense the subtle changes in gait abnormalities. Again, we found no significant effect linked to a specific genotype.[12] Finally, as in other HSP mice models,[8] *Cyp2u1*$^{-/-}$ mice did not display any significant locomotor impairment in the used behavioral tests.

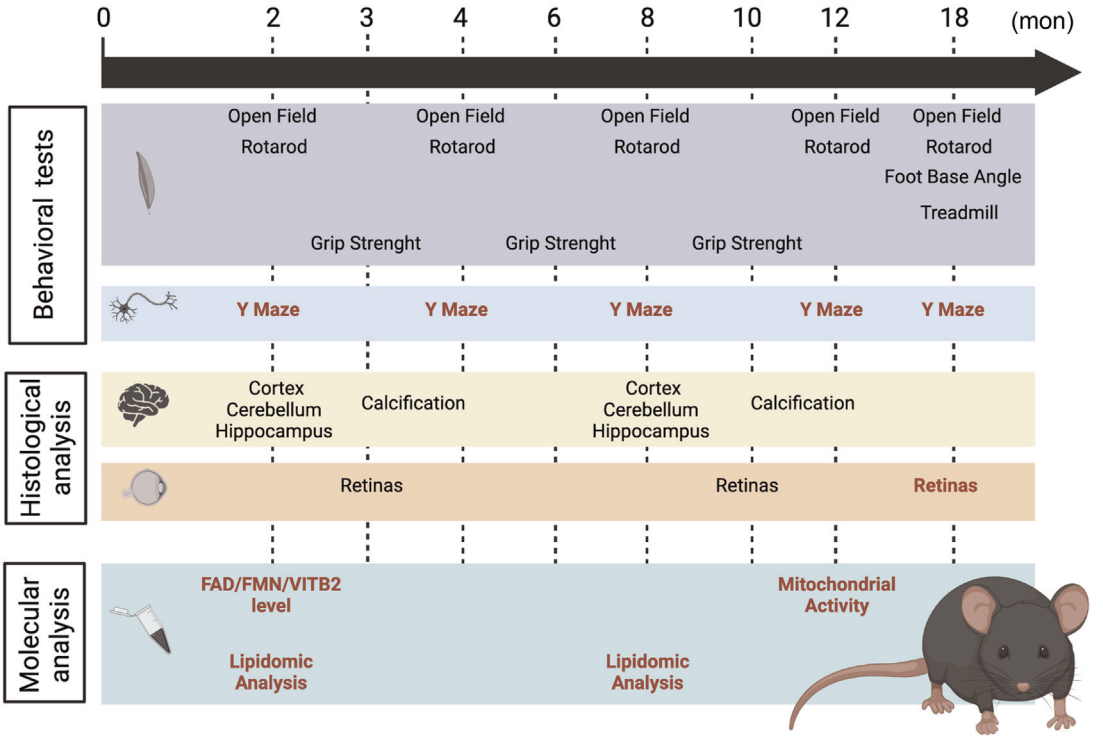

Figure 3. Phenotypic characterization of *Cyp2u1*$^{-/-}$ mice. We leveraged tests to assess the *Cyp2u1*$^{-/-}$ mice locomotor activity (violet) or cognitive ability (blue), histological studies of mouse brain (yellow) or retina (orange), and molecular analysis (green) at different time points, from 2 to 18 months old mice. Test names written in red are those whose results differ when comparing *Cyp2u1*$^{-/-}$ mice to the control littermates.

We completed this initial behavioral characterization focusing on a test to quantify cognitive deficits in transgenic mice with the Y-Maze spontaneous alternation test. Interestingly, starting from 2 months old, *Cyp2u1*$^{-/-}$ mice displayed impairment in cognitive performances.

We next determined whether *CYP2U1* loss might affect the central nervous system. We examined the potential neuronal tissue alterations by immunostaining brain sections of 2- and 8-month-old mice. Analyses of different brain structures and size measurements did not reveal any differences between genotypes, highlighting a normal brain development.

When focusing on motor abilities, the number of neurons in the motor cortex was stable, similar to that of Purkinje cells in the cerebellum, which is known to play a key role in motor coordination.

Levels of GFAP-positive cells resulted normal, indicating the absence of astrocytes activation, a response often observed in the presence of neurodegeneration. Altogether, these results highlighted the absence of neurodegeneration in *Cyp2u1*$^{-/-}$ mice brains.

Given that *CYP2U1*-mutated patients reported so far presented with brain MRI calcifications of the basal ganglia, showing age-related expansion, we tested whether the same trend could be observed in our mouse model. Various brain sections of 10-month-old *Cyp2u1*$^{-/-}$ and control mice were stained, but no calcifications were detected by Alizarin Red S staining,[22] nor leveraging the tissue clearing technique to detect post-cleaning calcium autofluorescence.[23] Neither method allowed us to spot any calcification.

We pursued the analysis of the *Cyp2u1*$^{-/-}$ model by assessing the presence of macular dystrophy, an additional manifestation due to *CYP2U1* mutations. We managed to highlight that, at a later stage (18-month-old) the analyzed mice started developing an ophthalmologic phenotype characterized by cone dystrophy, associated with microglial accumulation, features reminiscent of macular degeneration.[24]

Folate deficiency contributes to SPG56-HSP onset

The *Cyp2u1*$^{-/-}$ mouse model greatly improved our understanding of the molecular and cellular mechanisms leading to SPG56-HSP, clearly recapitulating some typical disorder's features such as cognitive deficiency and photoreceptor degeneration (Fig. 4). Thanks to the accessibility of brain tissues, such as the hippocampus, we managed to further decipher the disorder's molecular bases. Indeed metabolomic/lipidomic/proteomic analyses resulted crucial to identify alterations of the folate pathway as a possible causal mechanism underlying the disease's onset.

For the first time, our results highlighted that *CYP2U1* deficiency disrupts mitochondrial function and impacts proper neurodevelopment, suggesting that both pathologic mechanisms could be prevented by folate supplementation. Folate is essential for many cellular pathways, including mitochondrial protein translation, and methionine regeneration.[25] It has been recognized as a crucial compound in particular for the nervous system. Indeed, alterations of its uptake/metabolism can lead to severe consequences, that can be detected from the early embryonic stages, manifesting as neural tube defects (NTD), but that can appear also later in life, during infancy and childhood, impairing the correct development of the brain and/or spinal cord.[26]

One of the great advantages deriving from the use of animal models is the possibility of testing new therapeutic approaches aimed to rescue the pathologic phenotype. Leveraging the

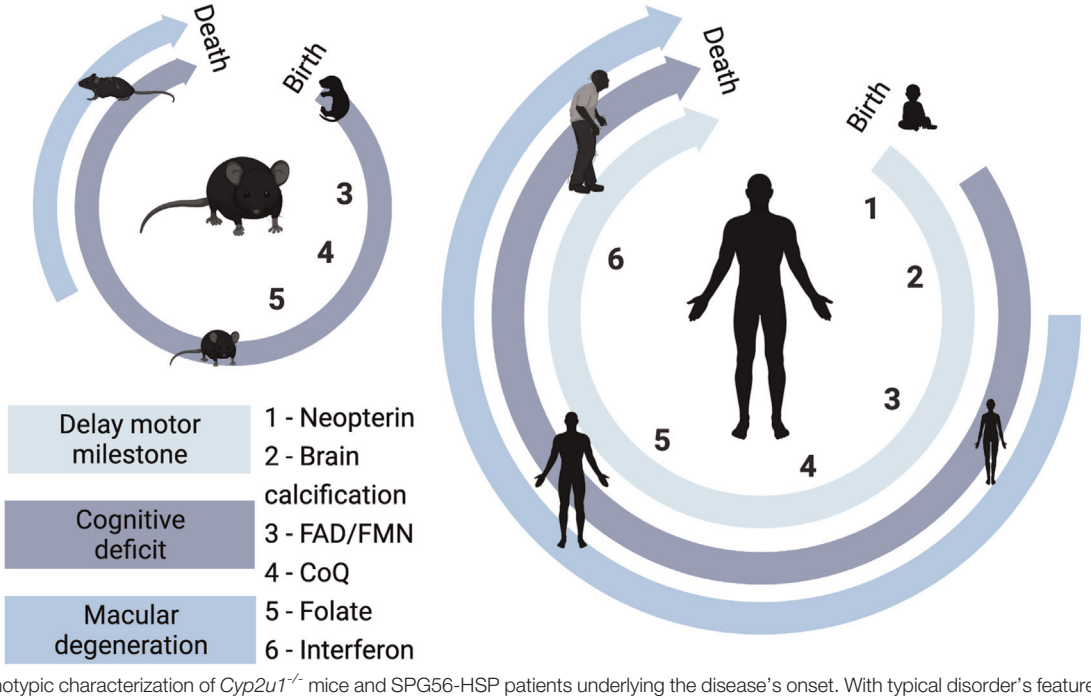

Figure 4. Phenotypic characterization of *Cyp2u1*$^{-/-}$ mice and SPG56-HSP patients underlying the disease's onset. With typical disorder's features: delay motor milestone (light blue); cognitive deficit (dark blue) and photoreceptor degeneration (blue); Metabolomic/lipidomic alterations for 1: Neopterin; 2: Brain calcification; 3: Flavin Adenine Dinucleotide (FAD)/Flavin Mononucleotide (FMN); 4: Coenzyme Q (CoQ); 5: Folate; 6: Interferon.

Cyp2u1$^{-/-}$ model, we were able to test whether folate supplementation could lead to an improvement of the manifested SPG56-HSP symptoms. We leveraged the Y-maze test to evaluate *Cyp2u1*$^{-/-}$ mice phenotype after supplementation. Surprisingly, we were able to show that folate supplementation prevented cognitive deficit in mice, with no effect in control mice, strengthening our assumption that lack of folate during neuronal development underlies the cognitive alterations typical of SPG56-HSP. Our research work is the first to provide valid scientific bases for the starting of clinical trials aimed to test the effects of folate supplementation to prevent SPG56-HSP cognitive impairments. Treatment with folic acid has already been reported in treating movement disorders in adults[26] and could offer a treatment option for SPG56-HSP patients that unfortunately, like many other HSP patients, are still waiting for the introduction of effective therapeutic strategies.[27]

Along with the identification of folate as a key factor contributing to SPG56-HSP onset, the combination of various omics approaches allowed us to identify potential biomarkers of the disease, further validated leveraging the largest series of SPG56-HSP patients reported so far. We were able to detect increased levels of coenzyme Q in blood, as well as of neopterin and interferon-alpha in cerebrospinal fluid. As previously observed in the *Cyp2u1*$^{-/-}$ model, we also highlighted the systematic presence of basal ganglia calcifications, an additional imaging biomarker. Even though additional validation will be required, these novel candidate biomarkers could be extremely valuable tools, potentially boosting the SPG56-HSP diagnostic process, leading to more efficient management of affected patients.

Folate supplementation: a possible treatment for SPG56-HSP?

The *Cyp2u1*$^{-/-}$ model allowed us to focus our attention on the neurodevelopmental phase when the most significant consequences of *CYP2U1* deficit and subsequent lack of folate were observed.

Folate, also called vitamin B9, is an essential water-soluble vitamin present in leafy green vegetables, fruits, and legumes. The active form of vitamin B9 is known as tetrahydrofolic acid or tetrahydrofolate and functions as a one-carbon unit carrier in a variety of biosynthetic reactions, such as DNA methylation, synthesis of nucleic acids, amino acids, and S-adenosyl-methionine. Thus, folate metabolism is critical for cell growth and proliferation, as highlighted by many studies suggesting folate analogs and antagonists, such as methotrexate, for cancer treatments.[25]

Given that one-carbon loaded folates are unable to cross intracellular membranes, folate metabolism is comprised of cytosolic and mitochondrial pathways with nearly identical core reactions categorized into three phases: (1) integration of one-carbon units into the folate pool; (2) donation of one-carbon units from the pool; (3) interconversion of the activated one-carbon units between different oxidation states.[27] Folate metabolism is also interlinked with other essential pathways (Fig. 5): the "methylation cycle" providing the methyl groups required for all genomic and non-genomic methylation reactions in the form of S-adenosyl-methionine; the tetrahydrobiopterin cycle allowing the biosynthesis of essential cofactor required for the synthesis of neurotransmitters: dopamine, noradrenaline, or serotonin.[28]

Due to its essential role in one-carbon transfer reactions, the folate pathway has a crucial role in sustaining cellular metabolic activities and cell proliferation.[29] Folate deficiency has been linked to a variety of adverse health outcomes such as megaloblastic anemia, NTD, and coronary heart disease.[25] Among the various processes dysregulated by folate deficiency, fetal development, a complex process that requires the precise interplay between cell proliferation, migration, differentiation, and death, is one of the key examples. As previously

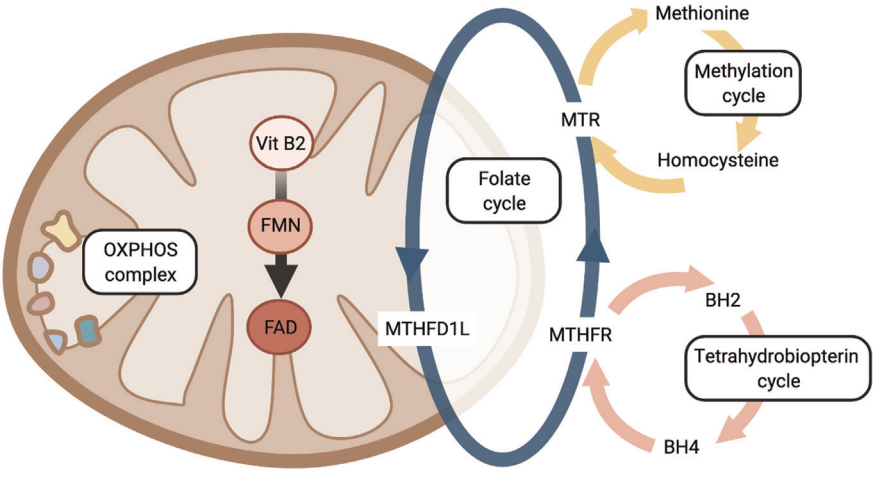

Figure 5. Folate cycle and interlinked pathways. Dysregulation of the Folate cycle is interlinked with other essential pathways: the methylation cycle providing the methyl groups required for all methylation reactions; the tetrahydrobiopterin cycle allowing the biosynthesis of essential cofactor required for the synthesis of neurotransmitters. BH2 = dihydrobiopterin, BH4 = tetrahydrobiopterin, FAD = flavin adenine dinucleotide, FMN = flavin mononucleotide, MTHFD1L = methylenetetrahydrofolate dehydrogenase 1 like, MTHFR = methylenetetrahydrofolate reductase, MTR = methionine synthase reductase, OXPHOS = oxidative phosphorylation.

observed,[30] proper function of folate metabolism is critical during embryogenesis, and folate deficiency is well known to cause NTD, arising during the neurulation phase when the brain and spinal cord precursors fail to close properly. Leveraging the >250 NTD mice models generated to characterize the disease; it was possible to prove that maternal folic acid supplementation reduced the frequency of neural tube closure defects.[29] Further investigations highlighted that the recommended daily amount of folate intake varies from 85 μg in infants, to 400 μg in adults,[31] Nowadays, folic acid supplementation is indicated in all those conditions in which its deficiency is detected, either because of a reduced intake, or a decreased absorption,[32] or when it is caused by an increased need that can occur for instance in presence of high alcohol consumption, malabsorption disorders, or specific mutations in genes involved in folic acid metabolism.[33] An example of successful supplementation is provided by the Riboflavin transporters that, if mutated, can lead to severe neurologic conditions characterized by neuronopathy with consequent muscle weakness, vision loss, deafness, and sensory ataxia.[30] For this genetic deficiency, oral supplementation of riboflavin was observed to directly improve symptoms and signs at clinical examination.[33] It is important to highlight that the earlier the supplementation is given, the stronger is the response, emphasizing the importance of treating patients as soon as the initial manifestations appear.[33]

Altogether, these findings opened the way to the introduction of NTDs preventive strategies leveraging folic acid dietary supplementation. More than 80 countries worldwide have recently started implementing mandatory folic acid fortification of foods, such as non-whole meal wheat flour, as newly established in the UK.[34]

Folate pathway: a perfect target to unravel SPG56-HSP modifying factors

As previously introduced, the increased use of NGS approaches in everyday diagnostic routine has not only greatly boosted the identification of new genes implicated in disorders' onset, but, more importantly, it has allowed the progressive overcoming of the classical monogenic paradigm, "one gene, one phenotype," that traditionally defined many Mendelian disorders.[35] Indeed, the identification of variants acting as genetic modifiers that modulate the expressed phenotype in combination with a primary causal allele has gradually led toward a more oligogenic inheritance mechanism.[35–37] Modifiers alleles having an impact on diseases' key features such as age at onset and progression/severity have been identified in various neurological disorders including Parkinson disease,[4] Alzheimer disease,[6] Frontotemporal Lobar Degeneration,[38] and Huntington Disease[5,39] amongst others.

HSPs' low incidence makes the search for modifiers particularly challenging. Advances in the identification of variants acting as age at onset and disease severity modifiers have recently been made in the context of SPG4-HSP, the most common HSP subtype due to *SPAST* gene mutations.[40] The analysis of the largest SPG4-HSP patient cohort has allowed to highlight that both, the nature of the causative mutation and sex, influence its age at onset.[41] Furthermore, intragenic variants affecting *SPAST* exon 1,[42] and deletions extending from the neighboring gene *DPY30*,[43] have been associated with earlier age at onset and greater disorder severity, when detected in combination with a major *SPAST* mutation. Even though the identification of genetic modifiers remains arduous in other, less frequent HSPs subtypes, the use of NGS approaches and the constant development of tools improving variants' analysis give high hopes for future discoveries.

The precise characterization of the genes/pathways potentially involved in the pathogenesis of the disease, deriving from previous experimental knowledge or animal models, offers the ideal starting point for the detection of candidate modifier variants. Indeed, the hypothesis-based candidate gene approach is one of the various analytical strategies used to identify genetic modifiers.[35]

Our recent findings leveraging the *Cyp2u1*$^{-/-}$ mouse model point towards variations in folate intake and metabolism, as primary candidates to explain the residual phenotypic variability among patients carrying *CYP2U1* mutation As already mentioned, insufficient folate intake, or genetic polymorphisms affecting folate-dependent genes, encoding a wide number of transporters and enzymes, can lead to a higher risk of developing NTD, cancer, and cardiovascular diseases, amongst others.[44]

Given its implication in a wide range of disorders, the identification of variants impairing the activity of the numerous

proteins involved in folate metabolism and uptake is well documented.[45–55] Among the various polymorphisms altering folate levels, special attention has been given to two common variants affecting methylene tetrahydrofolate reductase (*MTHFR*), a gene encoding a key enzyme responsible for the production of the biologically active form of folate.[44] The C677T variant (Ala222Val, rs1801133) leads to a drastic reduction of MTHFR enzymatic activity, with 70% of residual activity in heterozygous CT carriers, and only 30% in homozygous TT carriers.[56] As a consequence, TT carriers have been reported presenting with lower folate and vitamin B12 levels, and higher homocysteine levels.[57] Similarly, the A1298C substitution (Glu429Ala, rs1801131), is accompanied by lower folate levels in CC carriers.[57]

Variants affecting the numerous genes involved in folate intake/metabolism pathways[58] could therefore be prioritized to start exploring the genetic causes underlying the phenotypic variability often observed among SPG56-HSP patients. Detecting variants acting as genetic modifiers could not only expand our knowledge of the biological processes underlying SPG56-HSP but, more importantly, it could also lead to the identification of new biomarkers and new therapeutic targets, potentially resulting in novel diagnostic tools and treatment options.

The combination of whole exome sequencing and subsequent analysis of both common and rare variants adopting a candidate gene approach could be ideal for this purpose, especially considering the increased accessibility of NGS technologies in everyday diagnostic routines.

Limitations

The present mini-review is mostly revolving around the *Cyp2u1*$^{-/-}$ mouse model described in our recent publication.[12] We focus on its role in expanding our knowledge of SPG56-HSP clinical and biological background, as well as on its impact in proposing new therapeutic strategies. One of the major findings highlighted by our study is the detection of folate deficiency as a hallmark of SPG56-HSP and, most importantly, as a new therapeutic target. It is important to underline that even though the folate pathway seems to be a very promising candidate for the development of new therapeutic strategies, additional experiments will be needed to test other molecular mechanisms and compounds such as antioxidants, methylation cycle, and tetrahydrobiopterin (Fig. 5) that could as well be identified as potential drug targets. Additional analyses will also be required to validate any significant biological overlap between SPG56-HSP and other HSPs, allowing extending our findings and further contributing to unravel the complex pathophysiological background characterizing such rare disorders.

Conclusions

Animal models have a fundamental role in biomedical research, especially in the context of complex diseases like HSPs. Despite some limitations, our *Cyp2u1*$^{-/-}$ mouse model could clearly recapitulate some typical SPG56-HSP features such as cognitive deficiency and photoreceptor degeneration. The *Cyp2u1*$^{-/-}$ mice represent the first model for SPG56-HSP and offer key knowledge on the molecular processes occurring when lacking *CYP2U1*. This work allowed us to clearly demonstrate the crucial role of folate in disease pathology, as well as to identify several robust biomarkers. Our work opens the way to the exploration of new research paths in SPG56-HSP clinical and genetic research.

First, they provide solid bases and extremely optimistic preliminary results in support of the introduction of folate supplementation as a potential treatment option for SPG56-HSP patients. Indeed, this already well-established supplementation raises hope for SPG56-HSP patients providing, as supported by our results in our mouse model, a possible treatment option to delay the disease progression.

Furthermore, the central role played by folate pathways in SPG56-HSP pathogenesis lays the bases for new functional and genetic analyses, providing new molecular targets and potentially leading to the unraveling of additional key members contributing to the disease onset. The numerous proteins involved in pathways affected by folate deficiency are the perfect candidates for additional analyses aimed to identify modifying factors. Indeed, the detection of variants harbored in targeted genes could refine SPG56-HSP genetic diagnosis, potentially leading to the identification of new drug targets, as well as to stratify patients based on their genetic background, setting up personalized therapeutic strategies ultimately resulting in better patient management and care. Another extremely important consequence deriving from *Cyp2u1*$^{-/-}$ mouse model characterization is the expansion of the SPG56-HSP phenotypic spectrum. Macular degeneration or the presence of brain calcifications, previously not included as typical signs of SPG56-HSP, should be now considered by clinicians among the symptoms arising following *CYP2U1* mutations, potentially leading to HSP. A precise clinical characterization is extremely important for different reasons. It could increase the monitoring of those patients carrying *CYP2U1* mutations but not yet presenting with the full SPG56-HSP clinical picture, allowing to set up preventive measures aimed to delay or treat the manifestation of additional symptoms. In addition, as often observed in HSPs, it could allow the identification of new clinical overlaps between SPG56-HSP and other disorders, possibly leading to the repurposing of treatment options.

In conclusion, our research work shows the crucial role of animal models' detailed characterization in advancing our knowledge of diseases, from their mechanistic and molecular features to their phenotypic manifestations. Our findings are proof that impactful advances can be made even in rare and complex diseases such as SPG56-HSP, raising hope for the development of new, long-awaited, therapeutic strategies.

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Author contributions

Both authors designed, wrote and edited the manuscript and approved the final version of the manuscript.

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Conflicts of interest

The authors have no conflicts of interest to disclose.

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