

Niemann–Pick Type C—Leaky Variants and Alternative Transcripts

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Niemann–Pick type C (NPC, ORPHA: 646) is a neuro-visceral, psychiatric disease caused predominantly by pathogenic variants in the *NPC1* gene or seldom in *NPC2*. The rarity of the disease, and its wide range of clinical phenotypes and ages of onset, turn the diagnosis into a significant challenge. Other than the detailed clinical history, the typical diagnostic work-up for NPC includes the quantification of pathognomonic metabolites.

Keywords: Niemann–Pick type C ; splicing variants ; leaky variants ; NPC1 gene ; molecular diagnosis

1. Introduction

Lysosomal storage disorders (LSDs) are a group of about 70 inherited diseases, most of which are quite rare and present with vast clinical heterogeneity, ranging from severe, early-onset diseases to milder forms, of later onset. This remarkable variability may be observed not only between different diseases from the same group but also—and most importantly—amongst patients suffering from the same exact disease. Overall, this clinical heterogeneity has a direct impact on their diagnosis. Over the past several years, the number of available treatments for patients with LSDs has rapidly increased, namely, enzyme replacement and substrate reduction therapies, the use of molecular chaperones, gene therapy, and bone marrow transplant, among others ^[1]. Nevertheless, molecular diagnosis is the ultimate and essential step to provide access to therapy. The identification of biallelic nonsense and frameshift variants, as well as missense variants in conserved regions, provides a straightforward direct gene target analysis. Nevertheless, next-generation sequencing (NGS)-targeted panels for LSD-associated genes or other NGS methodologies provide a quick way to identify the molecular defect underlying diseases with such clinical variability ^[2]. However, in specific cases, the molecular diagnosis timeline can be even longer, when such pathogenic variants affect splicing and mRNA processing. These situations represent an additional challenge, with the identification and effect prediction of abnormal transcripts. In addition, some naturally spliced forms can raise another layer of difficulty and even mimic the molecular defect and its impact on splicing. The *IDS*, *GNPTAB*, and *NPC1* genes—whose pathogenic variants underly Mucopolysaccharidosis type II, Mucopolisidosis types II or III, and Niemann–Pick type C, respectively—are some examples of LSD-associated genes with naturally occurring spliced forms already reported in the databases (<https://www.uniprot.org/uniprotkb/P22304/entry#sequences> (accessed on 28 September 2023)); (<https://www.uniprot.org/uniprotkb/Q3T906/entry#sequences> (accessed on 28 September 2023)); and (<https://www.uniprot.org/uniprotkb/O15118/entry#sequences> (accessed on 28 September 2023)).

Niemann–Pick type C (NPC, ORPHA: 646) in particular is a devastating neurodegenerative LSD, caused by loss-of-function variants in either the *NPC1* gene (in approximately 95% of cases) ^[3] or the *NPC2* gene (in 5% of cases). Analysis of next-generation sequencing (NGS) data sets indicates that the incidence rate of NPC for the classical clinical manifestations is ~1:90,000 but suggests that, for the late-onset phenotype or variant forms, the frequency might be higher ^[4].

Overall, the wide range of clinical phenotypes and the different ages of onset it may present with, together with the rarity of the disease and the fact it may be caused by mutations in two different genes, make its diagnosis a significant challenge. At a clinical level, NPC's infantile forms present varying degrees of neurologic involvement and frequently present visceral manifestations, such as splenomegaly, hepatomegaly, neonatal jaundice, and hyperbilirubinemia ^{[5][6]}. Adolescent- or adult-onset NPC, on the other hand, presents with varying combinations of progressive neurologic deficits, e.g., ataxia, dystonia and/or dementia, vertical supranuclear gaze palsy (VSGP), or major psychiatric illness, including schizophrenia, depression, and psychosis, among others ^[6].

That is why a definitive NPC diagnosis must rely on additional laboratorial analyses. The classical method of establishing a NPC diagnosis relies on the filipin staining of cultured fibroblasts from skin biopsies ^[7]. This is a microscopy-based test that takes advantage of the fact that filipin specifically binds to unesterified cholesterol, allowing the evaluation of

cholesterol accumulation in the perinuclear vesicular compartments [8]. This rationale is consistent with the current assumption that the impaired egress of cholesterol from the late endosome/lysosome (LE/L) is a key element of NPC pathogenesis. Nevertheless, even the most severely affected patients may fail to be diagnosed through this method [9][10]. In fact, patients with proven NPC disease may present with variable filipin patterns, from typical “classical” or “intermediate” to “atypical” or “variant” ones, which fail to be classified as a NPC by filipin staining alone. Recent advances in the field are actively contributing to an increase in the detection of NPC patients. Among those advances is the development of rapid and reliable biomarkers, including oxysterols [11][12][13], lysosphingomyelin derivatives [14][15], and bile acids [16][17], even though none of them are specific to NPC [18]. However, *N*-palmitoyl-*O*-phosphocholineserine, (PPCS, previously known as lysosphingomyelin-509) has been shown to be elevated in the plasma and dried blood spots of NPC patients [19][20]. But, these novel biomarkers are not the sole contributors to the increased recognition of this disorder and its more expedited diagnosis. The increased availability of NGS has also contributed to the update of the overall NPC diagnostic algorithm while actively contributing to an increase in the number of positive molecular NPC diagnoses. Currently, there are a number of fully described diagnostic workflows for NPC [18], which may slightly vary between different labs depending on the tests each of them has available. However, *NPC1* and *NPC2* molecular analysis is mandatory in all of them and usually represents the ultimate step towards diagnosis [21]. Indeed, a rapid molecular diagnosis of a potential NPC patient is essential, not just for swift access to available therapies (currently miglustat is the only one approved within the European Union) but also to slow the progression of the disease and ultimately because it is the sole method of offering prenatal diagnosis to affected families [22].

2. A Brief Overview of the Diagnostic Workflow for Niemann-Pick Type C

2.1. The Picture in Black and White: Standard Workflows and Straightforward Diagnoses

In general, following a suspicious timeline of clinical manifestation and/or a biomarker profile consistent with NPC, the next step is the *NPC1* and *NPC2* sequencing of the index case (**Figure 1**) and subsequent segregation studies of the parents [18]. The *NPC1* gene (MIM# 607623) comprises 25 exons and over 600 disease-causing variants have been reported to date [23], most of which encode missense alleles. For the *NPC2* gene (MIM# 601015), thirty-four disease-causing variants have been described and four of them are splicing (<https://my.giagendigitalinsights.com/>, HGMD Professional 2023.3, accessed on 17 October 2023). Among the most common *NPC1* pathogenic variants are p.Ile1061Thr, found in 20% of patients of Western European descent [24], and p.Pro1007Ala, associated with milder forms of the disease [25][26]. In Portugal, however, the most frequent disease-causing variant is the missense p.Ala1035Val, which accounts for 15–20% of the affected cases (unpublished data transmitted by Quelhas D and Ribeiro I); it was recently reported as the most common in patients from Latin America [27].

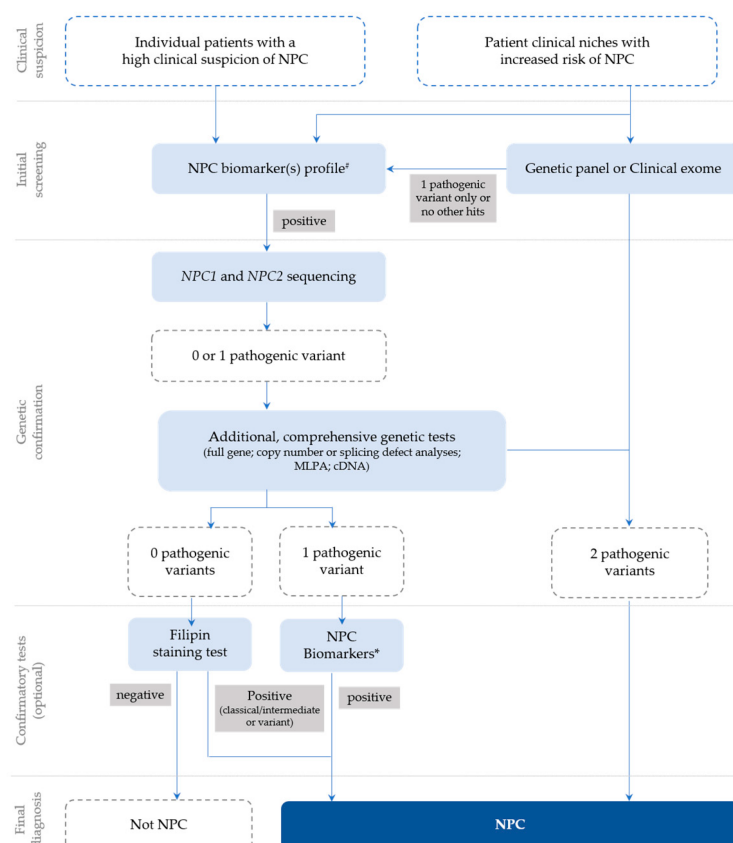


Figure 1. Recommendations for the detection and diagnosis of NPC, based on Patterson et al. [18] with slight updates to accommodate the most recent technologies, which are now commonly used for diagnostic purposes (e.g., clinical exome), as well as the current nomenclature. # Negative biomarkers may be suggestive that the diagnosis is not NPC; * Biomarker(s) profiling (if not initially conducted) or extended biomarker(s) profiling (in addition to those already conducted).

However, the highly polymorphic nature of *NPC1* can muddle diagnostic conclusions and turn the interpretation of novel variants of unknown significance (VUSs) into a challenge. In addition, cDNA sequencing is necessary to address mRNA processing in the presence of silent variants, or other VUSs, including missense variants near the (exonic or intronic) splicing regions.

More specifically, the cDNA analysis of exonic variants may help confirm the pathogenic effect of variants predicted to affect splice sites [28]. Several splice-site pathogenic variants have been identified in NPC and in many other LSDs [29]. In some instances, these variants do not allow the generation of functional mRNAs [30]. However, they are leaky and frequently produce a small percentage of correctly spliced and translated transcripts, leading to attenuated phenotypic expression of the disease [31].

Whenever conventional gDNA analysis leads to a single variant identification, the genetic study focuses on detecting the second damaging variant. For this reason, complementary studies, such as multiplex ligation-dependent probe amplification (MLPA) in gDNA to cover intragenic deletions or duplications or cDNA sequencing, may also be required for proper diagnosis of NPC [18][22]. As straightforward as this approach may sound, reaching a conclusive molecular diagnosis of NPC may, in some cases, be harder than it seems.

2.2. The Grayscale Image

Among the confounding factors that can either hinder or delay a definitive diagnosis of NPC is the presence of genetic variants affecting the normal *NPC1* and *NPC2* splicing patterns.

Several pathogenic variants affecting both *NPC1* and *NPC2* mRNA splicing, occurring in intronic and exonic regions, have already been described [32]. Although quite rare, three pathogenic intronic variants have been described in the *NPC2* gene [12][28][33]. One additional variant affecting splicing was found in both healthy controls and patients [4].

Interestingly, missense variants, such as the c.1553G>A (p.Arg518Gln), were proven to have an additional impact on the splicing mechanism in the *NPC1* gene, as long as they occur in the coding exons' splicing regulatory sequences [28][34][35][36].

Following a combined approach (gDNA and cDNA studies), the researchers have previously proven the impact of a silent variant in the *NPC1* gene that leads to exon skipping—p.Val562= (Figure 2) [37]. This variant is located in Exon 11 and was initially reported in Spanish NPC patients and classified, at that time, as a VUS or polymorphism, after a genomic DNA study [38].



Figure 2. Schematic representation of the silent variants in the *NPC1* exonic region affecting splicing and the effect on splicing based on in silico predictions (Human Splicing Finder—HSF and EX-SKIP tools and Maxent). P.Val562= localization on Exon 11 (red) and the effect on splicing based on in silico predictions. EX-SKIP compares the Exonic Splicing Enhancer (ESE)/Exonic Splicing Silencer (ESS) profile of a wild type (WT) and a mutated allele to determine if a specific exonic variant increases the chance of exon skipping. It calculates the total number of ESSs, ESEs, and their ratio. The p.Val562= mutant is associated with a change in the ESE/ESS ratio, which is compatible with a higher chance of exon skipping than in the WT allele. In addition, the HSF (a tool to predict the effects of pathogenic variants on splicing signals or to identify splicing motifs in any human sequence) predicts that the p.Val562= mutant leads to the creation of an ESS site. It involves the cDNA sequences CTTGTAAT (orange) [39] and CTTGTA (yellow) [40], which might be associated

with a potential alteration of splicing. In the case of the silent variant p.Val562=, functional cDNA analysis was performed [19], confirming the bioinformatic prediction of Exon 11 skipings.

In the previous study, cDNA analysis of the affected patient and his mother (heterozygous carrier) made it possible to identify a transcript with the skipping of Exon 11. This caused a shift in the reading frame and the emergence of a premature termination codon [37], leading to its reclassification as a disease-causing variant. Importantly, the p.Val562= variant was found not only in three independent Portuguese families but also in previously reported Spanish [38] and French patients [41]. In a French cohort, this variant was reported in heterozygosity with the p.Ile1061Thr in two siblings [41] [42]; however, its functional consequence was not ascertained. Despite the sequencing of five overlapping *NPC1* cDNA fragments in the two siblings carrying the p.Val562=, the pathogenic effect of the variant was considered unknown [41]. The most likely explanation is the degradation of the aberrant transcript by NMD. There is no information about the frequency of this variant in gnomAD. Looking to other repositories, this variant is only reported in a database from Tübingen University (NPC-db2; <https://medgen.medizin.uni-tuebingen.de/NPC-db2/search.php> (accessed on 28 September 2023))—it was found in one patient but not in the controls. No information was provided regarding the homo- or heterozygosity of that patient; however, both in the literature and in the researchers' cohort, only heterozygous patients were identified.

Another example of a NPC-causing variant associated with complex mRNA processing is the c.190+5G>A variant. This particular variant is located not in *NPC1*, the most obvious candidate to harbor a disease-causing mutation, but in Intron 2 of the *NPC2* gene. Again, this variant seems to be associated with a milder clinical course since both reported patients—two siblings homozygous for this variant—presented with a juvenile onset of the neurological disease and prolonged survival. A more detailed study showed that this splice variant generated multiple abnormal mRNAs [43]. However, in fibroblasts, a very small proportion of the correctly spliced transcript was also observed. Although this was not sufficient in producing enough *NPC2* protein for Western Blot detection, the presence of low levels of functional protein presumably accounts for the milder clinical course. The question of whether different tissues could display variable levels of abnormally/normally spliced RNA transcribed from the c.190+5G>A variant can also be raised.

Ideally, however, these variants should be easily detected whenever an adequate cDNA analysis of any of the involved genes is performed. Still, that is not always the case, as the researchers will demonstrate with a few practical examples.

3. Variants in the *NPC1* Gene That Affect Splicing

Pathogenic variants that affect pre-mRNA splicing account for at least 15% of disease-causing mutations [44]. Most of these variants affect 5' and 3'ss, the polypyrimidine tract, the branch-point sequence, and also *cis*-acting elements (exonic/intronic splicing enhancers and silencers). Other variants create novel splicing sequences deeply within introns, causing the abnormal inclusion of intron sequences. All of these variants lead to the production of abnormal transcripts that usually contain PTCs and are degraded by nonsense-mediated mRNA decay (NMD) [45]. Even exonic variants (missense and synonymous) may affect splicing, having a completely different effect from what was expected [46]. Therefore, both gDNA and cDNA should be analyzed.

The *NPC1* gene contains 25 exons and spans 55 kb from the base pairs (23, 531, 442) to 23, 586, 506 on the reverse strand of chromosome 18 at 18q11.2 (NC_000018.10); here the researchers report on 53 published pathogenic variants affecting splicing.

Seven of them are exonic, two are synonymous, five are missense, and forty-six are intronic variants, mainly affecting the 3'ss and the 5'ss; however, there are also variants reported in the branch point as well as deep-intronic variants.

Altogether, these observations call attention to the need for extensive mRNA studies in *NPC1*, or even *NPC2*, to establish a definitive NPC diagnosis. In this context, the presence of an alternatively spliced transcript may be somewhat confusing and even mask or mimic a real pathogenic variant that impacts splicing only in NPC patients and *NPC1* variant carriers.

As for tissue-specific differences in the relative abundance of the two *NPC1* splice isoforms, the researchers observed a higher expression of the spliced isoform in fibroblasts than in blood. In fact, in the genotype-tissue expression project (GTEx), which studies tissue-specific gene expression and regulation in fibroblasts, they quantified 30 fragments per kilobase of exon per million fragments mapped; meanwhile, in whole blood, only 17 fragments were quantified (<https://www.genecards.org/cgi-bin/carddisp.pl?gene=NPC1&keywords=npcl1#expression> (accessed on 28 September 2023)).

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