Minireview

Diagnostic tests for Niemann-Pick disease type C (NP-C): A critical review


Abstract

Niemann-Pick disease type C (NP-C) is a neurovisceral lysosomal cholesterol trafficking and lipid storage disorder caused by mutations in one of the two genes, NPC1 or NPC2. Diagnosis has often been a difficult task, due to the wide range in age of onset of NP-C and clinical presentation of the disease, combined with the complexity of the cell biology (filipin) laboratory testing, even in combination with genetic testing. This has led to substantial delays in diagnosis, largely depending on the access to specialist centres and the level of knowledge about NP-C of the physician in the area. In recent years, advances in mass spectrometry have allowed identification of several sensitive plasma biomarkers elevated in NP-C (e.g. cholestane-3β,5α,6α-triol, lyso-SM-509, lysosphingomyelin isoforms and bile acid metabolites), which, together with the concomitant progress in molecular genetic technology, have greatly impacted the strategy of laboratory testing. Specificity of the biomarkers is currently under investigation and other biomarkers are being found to also result in elevations. Molecular genetic testing also has its limitations, notably with unidentified mutations and the classification of new variants. This review is intended to increase awareness on the currently available approaches to laboratory diagnosis of NP-C, to provide an up to date, comprehensive and critical evaluation of the various techniques (cell biology, biochemical biomarkers and molecular genetics), and to briefly discuss ongoing/future developments. The use of current tests in proper combination enables a rapid and correct diagnosis in a large majority of cases. However, even with recent progress, definitive diagnosis remains challenging in some patients, for whom combined genetic/biochemical/cytokeratin markers do not provide a clear answer. Expertise and reference laboratories thus remain essential, and further work is still required to fulfill unmet needs.

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Article history:
Received 7 April 2016
Received in revised form 3 June 2016
Accepted 3 June 2016
Available online 7 June 2016

Keywords:
Niemann-Pick disease type C
Filipin
Oxysterol
Lyso sphingomyelin
NPC1 gene
NPC2 gene

Abbreviations: 7-KC, 7-Ketocholesterol; ACAT, Acyl-CoA:cholesterol acyltransferase; ASMDs, Acid sphingomyelinase deficiencies; BMP, Bis(monoacylglyceryl)phosphate; C-triol, Cholestane-3β,5α,6α-triol; EOA, Early-onset ataxia; GC/MS, Gas chromatography–mass spectrometry; HPLC, High performance liquid chromatography; L, Lysosome; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; LDL, Low density lipoprotein; LE, Late endosome; LPDS, Lipoprotein-deficient serum; LSDs, Lysosomal storage disorders; lyso-SM-509; LysoSphingomyelin-509; MLPA, Multiplex ligation-dependent probe amplification; NGS, Next-generation sequencing; NP-A, Niemann-Pick disease type A; NP-B, Niemann-Pick disease type B; NP-C, Niemann-Pick disease type C; SI, Suspicion Index; SPC, Sphingosylphosphorylcholine; VSGP, Vertical supranuclear gaze palsy.

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http://dx.doi.org/10.1016/j.ymgme.2016.06.004
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1. Introduction

Niemann-Pick disease type C (NP-C; OMIM #257220, NPC2 #607625) is a rare, autosomal recessive neurovisceral, lysosomal cholesterol trafficking and lipid storage disorder, caused by mutations in one of the two genes, NPC1 or NPC2. It is characterised by accumulation of a broad range of lipids, including unesterified cholesterol and various sphingolipids, in the lysosomes and late endosomes [1–8]. The accumulation of lipids in NP-C is likely to be the crucial event in disease pathogenesis, but our understanding of the mechanisms leading to lipid storage, and those causing neurodegeneration, remains incomplete [7–9].

NP-C has a variable age of onset, and a range of non-specific visceral, neurological and psychiatric clinical features that can arise at different stages of disease, and progress at different rates [4,10]. Birth incidences calculated from patients diagnosed with NP-C were reported as 0.83/0.96 and 0.93/100,000 in two studies [4,11], although this has to be an underestimate, with many patients going undetected [12]. A recent analysis of massively parallel exome sequencing data sets has predicted an incidence, at conception, of 1.12/100,000 for common forms of NP-C—close to the incidence of observed cases—but suggested a higher incidence of unrecognised mild, late-onset forms of the disease [13].

There are a number of factors that delay the initial suspicion, and hence the final diagnosis of NP-C. The initial clinical presentation is non-specific which, combined with limited awareness of NP-C in general clinical practice, makes detection and clinical diagnosis difficult. Despite increased understanding of the genetics and biochemistry of NP-C, laboratory testing has remained complex, restricted to a few specialist centres, and associated with substantial costs. Although national and regional specialist centres do exist, diagnostic testing is still limited by disease awareness, test availability and cost in some parts of the world, often leading to a major delay between onset of symptoms, diagnosis, and treatment initiation.

Recommendations for diagnosis and management of NP-C have been published previously [10,14]. In May 2013, a panel of experts gathered in Zurich, Switzerland, to discuss the latest advances in understanding of the genetics of NP-C. It was agreed that there was a need for a critical review of the current approaches in the laboratory diagnosis of NP-C, including emerging screening tests. A much better understanding of the advantages and limitations of the latter tests has now been achieved, making such a survey timely. This review is not intended to provide recommendations or algorithms for order of testing, but it will briefly discuss the unmet needs and expectations for the future of NP-C diagnostics.

2. Clinical examination

Clinical history and examination are the initial steps undertaken before considering further diagnostic procedures [10,14]. A detailed examination can, in some cases, help distinguish NP-C from other neurometabolic disorders [14]. However, the clinical presentation of NP-C may vary widely depending on the disease stage, and can even differ between individuals with similar genotypes, making accurate assessment particularly difficult if based on clinically presenting features alone. Therefore, similar to other inherited metabolic disorders, confirmatory laboratory tests are always required [10].

Signs and symptoms of NP-C can be broadly grouped into three categories: visceral, neurological and psychiatric [10,12,15,16]. Patients with NP-C typically present with some characteristic signs and symptoms from across these categories, which can be used to help identify the disease. These include, but are not limited to, neonatal cholestatic jaundice, splenomegaly, vertical supranuclear gaze palsy (VSGP), gelastic cataplexy, ataxia, dysphagia, dystonia, cognitive impairment and psychosis. Specific NPC1 or NPC2 mutations are associated with certain clinical subtypes of NP-C, particularly the age of onset of neurological symptoms [4]. The heterogeneity of NP-C and presence of symptoms that overlap with other diseases complicate the diagnostic process, leading to a long delay between symptom presentation and accurate diagnosis. Another working diagnosis is often not questioned until additional manifestations occur. The combined presence of visceral, neurological and/or psychiatric symptoms should lead to consideration of NP-C in the differential diagnosis; however, atypical NP-C patients are not infrequent. Examples include adult patients who have purely psychiatric symptoms and patients of any age with only visceral manifestations. In both instances there may be no evidence of neurological involvement at the time of diagnosis.

The clinical heterogeneity of initial presentation means that the patients may first be seen by generalist physicians or clinicians from different specialties [10,14]. The NP-C Suspicion Index (SI) screening tool (www.NPC-SI.com) was developed to aid clinicians in early identification of patients older than 4 years of age with suspicion of NP-C [15,17]. The tool generates a risk prediction score based on the presence of specific clinical manifestations and family history to identify patients who should be referred for further investigation of NP-C.

3. Current laboratory diagnostic techniques

Laboratory-based tests are the mainstay of diagnostic procedures in NP-C. Previously, such tests were initiated in case of a clear clinical suspicion of NP-C. Considering the often non-specific/atypical features in some patients and methodological progress, there is (and will be) an increasing trend to include NP-C in wider biochemical or molecular genetics diagnostic panels. Nevertheless, each of the current approaches has limitations, as discussed below. Diagnostic testing should therefore be...
conducted at, or in consultation with, specialised centres with extensive experience [10].

3.1. Cell biology – studying intracellular trafficking of LDL-derived cholesterol

NP-C is characterised by a unique disruption in intracellular transport of endocytosed cholesterol. Both the NPC2 and NPC1 proteins are necessary for egress of endocytosed cholesterol from late endosomes (LE)/lysosomes (L) (reviewed in [7,8]). Consequently, when either protein is dysfunctional, loading cells with low density lipoprotein (LDL) will lead to cholesterol accumulation in the LE/L compartment, which can be visualised after filipin staining [18]; this is the principle of the filipin test. Cholesterol sequestration secondarily retards all LDL-induced homeostatic responses [19], more specifically cholesteryl ester formation by acyl-CoA:cholesterol acyltransferase (ACAT) [1], which can be measured with the so-called ‘cholesterol esterification test’.

An overview of the advantages and limitations of the cell biological tests is shown in Table 1.

<table>
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<th>Technique</th>
<th>Advantages</th>
<th>Limitations</th>
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<tr>
<td>Filipin staining of cultured skin fibroblasts (cytochemistry after cholesterol depletion and in vivo challenge with LDL; fluorescence microscopy evaluation; semi-quantitative)</td>
<td>- &gt;80% of NP-C cases display a typical (‘classical’ or ‘intermediate’) staining profile - Similar results in NP-C1 and NP-C2 - Typical staining profile is unambiguous and easy to interpret—with clinical findings it supports confident diagnosis - Longest experience in a broad clinical setting—well-defined limitations</td>
<td>- Invasive skin biopsy and cell culture - Turnaround time (5 weeks for culture, 2 weeks for testing); cost - Technical requisites: specific biological reagents; positive and negative reference cells in each assay; adequate microscopic conditions - Rigorous conditions and expertise in interpretation are critical for the following reasons: - ‘Variant’ staining profile (~15% of NP-C cases) with fewer and varied number of positive cells can be difficult to interpret - NP-C heterozygotes and other diseases (e.g. NP-A/B) may produce mildly positive results, difficult to distinguish from ‘variant’</td>
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<tr>
<td>Cholesterol esterification test (early LDL-induced cholesteryl ester formation, measured after cholesterol depletion and in vivo challenge with LDL; extract, separate and quantify radioactive product)</td>
<td>- Was very useful as second test prior to identification of NPC1/NPC2 mutations - Clear-cut results in typical NP-C cases - Differentiates NP-C from NP-A/B and some other diseases</td>
<td>- Developed 30 years ago, the filipin test has been extensively used, and until recently was considered as the gold standard assay for NP-C diagnosis [1,4,14,20]. Filipin is a fluorescent polyene antibiotic, which specifically binds to cholesterol but not to esterified sterols. It cannot be used in living cells because it perturbs the cell bilayer [21], and staining must therefore be done on fixed cells/tissue sections. In the original protocol, sparsely seeded skin fibroblasts, cultivated on slide chambers, were fed for several days with medium supplemented with 10% lipoprotein-deficient serum (LPDS) to maximise LDL-receptors, then incubated for 24 h in medium enriched with 100 μg/mL LDL. Cells were then fixed in 10% buffered formalin, before filipin staining and fluorescence microscopy examination [18]. Although minor modifications can be performed, extensive experience has shown that, globally, this relatively complex set-up is optimal. Early studies showed that in fibroblast cultures from normal subjects, fewer than 10% of cells stain positive, while in those from typical NP-C patients, nearly all cells display multiple strongly fluorescent perinuclear vesicles [18]. Studies in large series of patients [20,22–25] indicate that this typical pattern is observed in 80–85% of cases with NP-C, even when cells are challenged with a lower concentration of LDL. Subdivision of the typical staining pattern into ‘classical’ and ‘intermediate’ profiles has been proposed to reflect a variation in the percentage of positive cells (80 to 100% of cells) and their level of fluorescence, resulting in a small gradient for filipin positivity that also correlates with LDL-induced cholesteryl ester formation levels [20,23]. In both typical sub-profiles, interpretation of the test is always very easy and unambiguous. Only one condition, mucolipidosis II/III (I-cell disease) is known to mimic a typical/classical NP-C filipin profile [20], and it has been shown that the NPC2 protein is non-functional in I-cell fibroblasts [26,27]. However, in clinical practice, this is not a problem considering the widely different presentations of the diseases. In the remaining 15–20% of NP-C cases, the block in cellular cholesterol trafficking is significantly less severe (although to a variable degree), and can only be demonstrated if cells are challenged with high concentrations of pure LDL. Stringent conditions are required to observe a significant number of fluorescent perinuclear vesicles (seen in 50–80% of cells only). This pattern has been described as the ‘variant’ profile [20,23]. Confirming a diagnosis of NP-C solely from the filipin test can be particularly difficult in a proportion of cases with a ‘variant’ profile because, as discussed below, an NP-C heterozygous status [18] as well as a number of pathological conditions can also result in mild alterations in the filipin test. In case of a ‘variant’ pattern, complementary sequencing of the NPC1 and NPC2 genes is recommended before making a conclusive diagnosis [10,23]. A number of studies have shown that a ‘variant’ profile can be related to specific NPC1 mutations, and that it can prevail even when these mutations are present in a compound heterozygous status [28–30]. Notably, p.P1007A, one of the two most common NPC1 mutations, is almost invariably associated to a “variant” profile [22,28,29]. Compared with the UK and USA, Spain or France [22,24,25,31], this mutation has been described with a more than double frequency (10% of mutant alleles) in patient cohorts from Germany [32] and Czech Republic [11]. A higher proportion of “variant” patients is thus expected in these and possibly some other countries. Case studies have also indicated that the ‘variant’ profile is more common in the adult neurological onset form than in juvenile or late-infantile forms of the disease [23,33], while patients with early-infantile neurological onset show a classical pattern. Since cells from many late-onset patients also show a typical profile, no strict correlation can be made between the cholesterol trafficking abnormality and the severity of neurological involvement [20,22,23,30,33–35]. In practice, the higher prevalence of the ‘variant’ filipin profile in late-onset neurological forms of the disease adds to the difficulty of diagnosing adults with NP-C, often less typical in their clinical presentation.</td>
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Some conditions have been described to result in a mildly positive filipin test, incurring the risk of a false positive NP-C diagnosis. Among them, acid sphingomyelinase deficiencies (ASMDs; Niemann-Pick disease type A and type B [NP-A/-B])—one of the common differential diagnoses of NP-C—constitute a significant pitfall [23]. Sphingomyelin storage is likely to affect cholesterol transfer by the NPC2 protein [7], and indeed, secondary cholesterol storage occurs in most ASMD tissues. For acid lipase deficiencies (Wolman disease and cholesteryl ester storage disease) conflicting results have been reported by different laboratories. Abnormal filipin patterns have further been documented in MEGDEL syndrome [36] and in diseases likely to secondarily affect lysosomal cholesterol egress, such as Smith-Lemli-Opitz syndrome [37], and Tangier disease [38]. Following the implication of Nogo-B receptor involvement in NPC2 protein stability [39], abnormal filipin staining results were also described in a rare congenital disorder of glycosylation due to Nogo-B receptor mutations [40]. Finally, although no such mutations have been described in humans, mouse cells deficient in mannose-6-phosphate receptors MPRA6 and MPR300, showed altered filipin profiles (38% and 20% of positive cells, respectively) [26]. Implication of other cholesterol-sensor or transporter proteins closely related to NPC1, such as members of the oxysterol-binding protein-related protein family [41–43] are further candidates for false positive results in filipin testing.

More clinically relevant, although not systematically reviewed, a number of lysosomal storage disorders (LSDs) could potentially result in mildly abnormal filipin patterns. Indeed, elevated free cholesterol has been found in LSD cells. This mechanism was advocated as the cause of altered trafficking of the fluorescent sphingolipid analogue, BODIPY-lactosylceramide [44], a feature shared by many LSDs including NP-C [45]. Interestingly, BODIPY-lactosylceramide trafficking was not found to be abnormal in ‘variant’ NP-C cells, unless cells were first challenged with high LDL concentrations, as required to optimise the filipin test [22].

Finally, extensive studies have been performed in parents of affected children with a ‘classical’ filipin profile. Fibroblasts from heterozygous subjects frequently display slightly abnormal filipin profiles, sometimes similar to those of patients with a ‘variant’ profile [46]. This should be kept in mind, and such an observation may lead to a dilemma whenever only one mutant NPC1 or NPC2 allele is identified in a potential NP-C patient—i.e., the individual a ‘variant’ patient with one unidentified allele or an NP-C heterozygote with another disease?

A major drawback of the filipin test is that it requires living fibroblasts cultured from a skin biopsy. This incurs invasive sampling, the need for a cell culture facility and a long (5–7 weeks) turnaround time. Attempts to use cultured lymphocytes [47] proved unsuccessful in clinical practice. Similarly, the work in a publication proposing the use of blood smears [48] has not been replicated. Other limitations include the requirement for costly LPDS and LDL reagents, the need to include positive and negative control cells in all experiments and, considering the pitfalls outlined above, the necessary experience in interpretation to ensure accurate results [23]. This restricts testing to a small number of expert centres. Results from ‘variant’ or inconclusive tests must be evaluated in light of the clinical presentation, and complemented by sequencing of the NPC1 and NPC2 genes [10,23]. When no mutations are detected, clinical data and additional NP-C biochemical tests must be reviewed, other diagnoses considered and, eventually, filipin tests repeated in another laboratory. Notwithstanding, the filipin test remains a valid tool for the laboratory diagnosis of NP-C and constitutes so far the best functional test to study the pathogenicity of novel mutations.

3.1.2. Cholesterol esterification test

The cholesterol esterification test measures the early rate of cholesteryl ester formation elicited by LDL loading of cholesterol-depleted cells [20], and evaluates the re-esterification by ACAT of cholesterol generated from LDL endocytosis after its egress from the LE/L compartment. This crucial homeostatic reaction is particularly impaired in NP-C cells [19]. Discovery of this defect constituted the initial breakthrough in the concept of NP-C as a cellular cholesterol dysregulation disease [1].

The test requires two sets of cells initially maintained for several days in a LPDS-supplemented medium, and then fed [3H]oleic acid, either in LPDS or in LPDS/LDL medium, for 4–6 h. Cells are harvested, lipids extracted and separated, radioactivity of cholesteryl oleate quantified and reported to cell protein, and the difference between the two media conditions calculated. In cells with a ‘classical’ filipin profile, the early rate of LDL-induced cholesteryl ester formation is essentially nil, while cells with an ‘intermediate’ filipin profile show a moderate deficiency. In ‘variant’ cells, cholesteryl oleate formation is often in the low normal range. In NP-C heterozygotes, cholesteryl oleate formation appears normal or mildly deficient [20].

Like the filipin test, the cholesterol esterification test requires a living cell culture, but the assay is more complex, more labour intensive, and costly. Controlled conditions for all cell culture steps are essential, necessitating mandatory positive and negative controls. Even so, large inter-assay variations still occur. The radioactive precursor is a further limitation. Since the test has proved less sensitive than filipin staining, very few laboratories still perform it as part of the NP-C diagnostic process. While very useful as a complementary test before genotyping was possible, its current use is now essentially limited to research settings.

3.2. Biochemical biomarkers

To allow wider screening for NP-C, there was a clear need for convenient blood-based, cost-effective, and if possible specific, biomarkers. The study of chitotriosidase activity was for a long time the only approach. In recent years, assays for several new, more sensitive and specific metabolites have been developed. These have shown promise for NP-C and are used with increasing frequency in clinical practice as the primary screening test. An overview of these techniques, and their projected advantages and limitations, is provided in Table 2.

3.2.1. Chitotriosidase activity

Measurement of plasma chitotriosidase activity, available in many laboratories, has shown its usefulness as a biomarker of Gaucher disease. Demonstration of mild to moderate elevation is also widely used as a general and potential indicator of lysosomal storage diseases, including NP-A, -B and -C [49]. But normal chitotriosidase activity may occur in patients with NP-C, most notably in late-onset forms of the disease. Furthermore, elevated chitotriosidase activity has been noted in other conditions, including more common disorders such as stroke and type 2 diabetes mellitus [50–55]. Finally, partial or total deficiency in chitotriosidase activity due to a 24 bp duplication in the chitotriosidase gene is relatively frequent [56]. As such, the assay lacks specificity and sensitivity for NP-C.

3.2.2. Oxysterol analysis

Besides the characteristic lysosomal accumulation of unesterified cholesterol, oxidative stress leading to increased production of reactive oxygen species has been demonstrated in cultured fibroblasts from patients with NP-C [57,58], NPC1 ablated neuronal cell lines [58] and Npc1+/− mice [59].

Oxidative stress situations are known to lead to the non-enzymatic formation of cholesterol auto-oxidation products. Early work documented elevation of several oxysterols in tissues of Npc1+/− mice [60]. Reappraisal studies showed that two metabolites, cholestane-3β,5α,6β-triol (C-triol) and 7-ketocholesterol (7-KC), were of particular interest. These metabolites were found elevated in the blood plasma of Npc1+/− mice [59] and patients with NP-C, while a modest elevation was seen in NPC1 heterozygotes [61,62]. Plasma oxysterol-based tests were then proposed for rapid diagnosis of NP-C using gas chromatography–mass spectrometry (GC/MS) [61], or liquid chromatography–mass spectrometry (LC/MS) [62].
Acid lipase deficiencies [65,68,72,75,77], and cerebrotendinous xanthomatosis [65,78]. An isolated elevation of 7-KC has further been reported in Smith-Lemli-Opitz syndrome [65,77,78] and in a number of patients with peroxisomal disorders [65]. Normal results have been observed in several other lysosomal storage diseases, including Gaucher disease [61,76,77], in patients with various liver and neurologic involvement, as well as in familial hypercholesterolemia [65]. But elevated oxysterol results should be interpreted with caution in cholestatic neonates, in view of a recent publication [79].

Finally, small but significant elevations in C-triol and 7-KC have been reported in 25% and 36% of NPC1 mutation carriers, respectively [62], a finding confirmed in another study [66]. A potential discrimination problem could thus arise in subjects suspected to suffer from NP-C and showing borderline C-triol elevation, a ‘variant’ filipin profile, and only one mutated NPC1 or NPC2 pathogenic allele.

Oxysterol testing for NP-C (and NP-A/-B) is currently offered in a limited, but growing number of laboratories. As a primary screening test for NP-C it has many advantages for both patients and clinicians. The test requires frozen EDTA plasma or serum—less invasive than a skin biopsy—and plasma oxysterol levels can be quickly analysed. The more specific C-triol should be preferred. The running cost of the oxysterol assay is much less than traditional tests, and the procedure can be adapted to high-throughput testing. But it also has its limitations. The assay may require in pre-analytical handling [80], and falsely elevated results (particularly for 7-KC) can occur due to haemolysis or inadequate sample storage and transport. Access to sensitive GC/MS or LC-MS/MS machines is another potential limitation.

The utility of the C-triol measurement has recently been demonstrated through the identification of 72 new patients with NP-C1 or NP-C2 over the course of 3 years [68]. Provided other causes of elevation are kept in mind, due to its non-invasive nature, low cost, short turn-around time and good sensitivity, oxysterol testing constitutes a highly useful primary test when there is suspicion of NP-C.

3.2.3. Lysosphingolipids

Lysosphingolipids are sphingolipids lacking their N-acyl group, and the lysosom-counterpart of the primary stored compound has been found to be elevated in tissues of a number of sphingolipidoses. Lysosphingolipids are thought to be ‘toxic metabolites’ that contribute to disease pathophysiology [81,82]. Plasma globotriaosylsphingosine and glucosylsphingosine have been found to be excellent plasma biomarkers in Fabry disease [83] and Gaucher disease [84], respectively, with levels in these patients more than 10-fold above normal. Measurement of increases in galactosylsphingosine and sphingosylphosphorylcholine (SPC; lysosphingomyelin) in dried blood spots have also been proposed for screening of Krabbe disease and NP-B [85,86]. Currently, the measurement of lysosphingolipids is performed using LC-MS/MS.

NP-C is characterised by tissue-specific accumulation of several lipids, including cholesterol, sphingomyelin, multiple glycosphingolipids and free sphingoid bases (reviewed in [7]). Although the mechanism for sphingolipid storage is complex and different from that seen in primary sphingolipidoses [87], the observed tissue lipid profile accumulation led to the hypothesis that plasma lysosphingolipids might also be biomarkers for NP-C [74,88].

An initial prospective study indicated that SPC could be elevated in plasma in patients with NP-C [74]. A retrospective study from the same team, applying a validated method to 70 control subjects and 22 treatment-naïve patients with NP-C, found a 2.8-fold median elevation of SPC in the NP-C group, with only minor overlap with control values [88]. The SPC increase in patients with NP-C was also found to be independent of age [88]. Other laboratories have since implemented measurement of plasma SPC and presented preliminary findings [89,90]. SPC was found to be elevated more than 10-fold in NP-A and NP-B, while the increase in NP-C was modest, with variable overlap with controls [91,92].
Finally, another group recently reported on an ill-defined SPC derivative (MW 508), currently referred to as lysophosphingomyelin-509 (lyso-SM-509). This particular isoform showed a very large increase in patients with NP-C, with even higher levels in NP-A and NP-B [93], and preliminary data from two additional laboratories confirmed these findings [89,90]. Due to the absence of an authentic standard for lyso-SM-509, calculated concentrations probably deviate from the true concentration; however, relative quantitation of lyso-SM-509 clearly offers a powerful tool for differentiating patients with NP-C and NP-A/-B from control subjects. As there is some overlap between lyso-SM-509 levels observed in NP-C and NP-A/-B, co-measurement of the classical SPC isoform may allow clear differentiation of NP-A/-B from NP-C. Similar to the oxysterol markers, in the coming years we will gain a better understanding of which disorders outside of classical LSDs may affect the level of lysosphingolipids. Metabolic syndrome has been found to lead to elevated plasma SPC, although a differential diagnosis with NP-C is highly unlikely given the differing symptomatology [94]. The recent observation that patients with action myoclonus-renal failure syndrome and elevated plasma SPC, although a differential diagnosis with NP-C is highly unlikely given the differing symptomatology [94].

3.2.4. Bile acid metabolites

A unique C3, bile acid, 3β-sulfoxy-7β-N-acetylgulosaminyl-5-cholen-24-oic acid (SNAC-5β,CA) and its glyco- and taurine-conjugates were identified in high concentrations in the urine of a patient with NP-C [97]. It was subsequently hypothesized that the altered distribution of cholesterol in patients with NP-C may result in utilisation of different bile acid synthesis pathways compared with healthy individuals. Using an LC-MS/MS assay, concentrations of these urinary bile acids were found to be elevated in 2 patients with NP-C compared with healthy controls [98], suggesting urinary bile acids as potential biomarkers of NP-C.

Active research has further been conducted in two independent laboratories. Two bile acid metabolites elevated in NP-C, 3β-hydroxy-7β-N-acetylgulosaminyl-5-cholen-24-oic acid (SNAC-5β,CA) and its glycine- and taurine-conjugates were identified in high concentrations in the urine of a patient with NP-C [97]. It was subsequently hypothesized that the altered distribution of cholesterol in patients with NP-C may result in utilisation of different bile acid synthesis pathways compared with healthy individuals. Using an LC-MS/MS assay, concentrations of these urinary bile acids were found to be elevated in 2 patients with NP-C compared with healthy controls [98], suggesting urinary bile acids as potential biomarkers of NP-C.

3.2.5. Bis(monoacylglycero)phosphate

Bis(monoacylglycero)phosphate (BMP or LBPA) is an atypical phospholipid, particularly enriched in the late endosomes, which plays a major role in both cholesterol processing and sphingolipid degradation in the LE/L (reviewed in [7]). A pathological increase in BMP in several tissues has been described in NP-A, -B and -C and drug-induced phospholipidosis (reviewed in [101]), and in plasma and fibroblasts in various lysosomal disorders [102]. Non-clinical studies in urine [103,104] and plasma [105] have also shown its potential in monitoring drug-induced phospholipidosis. Recently, Liu and colleagues demonstrated a 50-fold increase of di-22:6-BMP in the urine of patients with NP-C [106]. Whether this will be of clinical utility in NP-C is still unknown.

3.3. Genetic testing

NP-C is caused by autosomal recessive mutations in either the NPC1 (~95% of cases) or NPC2 genes (~5% of cases) [10]. About 380 distinct NPC1 and 22 NPC2 mutations are already known and are recorded in mutation databases [107–109], with only a few of them being common or recurrent. Although nonsense and nonsense mutations constitute the vast majority of known NP-C mutations, small and large genomic deletions, as well as deep intrinsic mutations, have also been described [25, 29, 109,110].

Genetic analysis is necessary to confirm a diagnosis of NP-C following abnormal results with disease biomarkers or inconclusive filipin staining results [10,23], but identification of NPC1 or NPC2 mutations should also optimally complement even clear-cut results from filipin staining [10]. Although, until recently, it was predominantly used as a confirmatory diagnostic method in NP-C, rather than as an initial diagnostic procedure, with the much better availability of newest sequencing methods, primary exon and junction sequencing of both the NPC1 and NPC2 genes is becoming increasingly common as part of the routine diagnostic process, and has been recommended to be performed in all patients [10,108].

The identification of two alleles with demonstrated disease-causing mutations in NPC1 or NPC2 confirms the diagnosis of NP-C, with supporting mutation analysis in at least one parent. Attention needs however to be paid to the source of genotype–phenotype verification, as some polymorphisms (e.g. p.P2275S in NPC1 [111]) are known to be erroneously registered in mutation databases as disease causing mutations. As with other testing methods, routine genetic sequencing techniques sometimes fail to identify mutations or may provide inconclusive results [74]. Sanger sequencing of gDNA and cDNA is currently the most widely used technique for genetic analysis in NP-C [108], allowing targeted sequencing, validation of diagnoses based on other positive tests, and identification of hard-to-find mutations [25]. Next-generation sequencing (NGS) techniques are becoming increasingly more cost-effective and accessible for genetic analysis in NP-C [108], and can be highly valuable for screening in patients with specific phenotypes.

Gene panels appear to be an appropriate NGS methodology for directed diagnosis. These panels must be designed to include both the NPC1 and NPC2 genes with complete exons and exon-intron boundaries. As genomic deletions and deep intronic mutations are not exceptional in this disease [25], in complex cases more detailed molecular characterisation is required, and the need for sequencing of promoters and deeper intronic regions should be assessed [4,10]. Sequencing of only exons and exon–intron boundaries does not allow the identification of deep intronic mutations, mutations in regulatory regions, or large structural variants that may result in aberrant protein expression. Indeed, mutations in NPC1 introns have been identified in patients with NP-C and were found to result in splicing defects [25,112,113]. Large structural variants that encompass NPC1 and the flanking genes have also been described [114], as have whole gene deletions [25]. cDNA sequencing is helpful to detect intrinsic problems and for identification of splice mutations; several early NPC1 mutational studies utilised cDNA sequencing [28,115,116], and in some instances revealed the effect of complex genomic mutations. Multiplex ligation-dependent probe amplification (MLPA) and quantitative PCR can be used to detect genomic rearrangements (exonic or whole gene deletions) [10,108,113,117], and assessment of mRNA degradation (inhibition of nonsense-mediated mRNA decay processes) has also been used to investigate mutations resulting in aberrant splicing [10,25,118].

NGS methodologies can be used for gene panel sequencing, large-scale genetic analysis, and whole exome and whole genome analyses, and have recently been employed in NP-C screening and diagnosis. In
Beyond diagnosis, identification of NPC1 and/or NPC2 mutations combined with descriptions of clinical phenotype, will improve our knowledge of pathogenic mutations and our understanding of genotype-phenotype correlations. Mutational analysis is also the only method that supports reliable screening for heterozygous status in blood relatives, and safe prenatal diagnosis in instances with at-risk parents [4,10]. Together, this highlights the importance of conducting comprehensive genetic analyses in every patient.

4. Concluding remarks

This review is intended to increase awareness of the currently available approaches to laboratory diagnosis of NP-C, and to provide an up-to-date, comprehensive and critical evaluation of the various methods. Significant advances, anticipated to have a positive impact on NP-C diagnosis, have been achieved in recent years with the development of sensitive tests for NP-C using plasma samples, and the huge progress in molecular genetic techniques.

Plasma biomarkers, currently C-triol and the lysosphingomyelin isoforms (and likely very soon, bile acid metabolites) appear today as rapid, cost-effective and efficient tests for first screening. They are relatively easy to implement in laboratories specialised in inherited metabolic diseases, since many of those are already using LC-MS/MS techniques. Cross-validation with standard samples is important; of note, C-triol measurement is already included in some international quality control programmes (e.g., ERNDIM). Nevertheless, these methods are not without limitations: 1) elevated levels occur in diseases other than NP-C; 2) normal levels do not necessarily exclude a diagnosis of NP-C; and 3) insufficient data are available with respect to these biomarkers in NP-C heterozygous individuals. A combination of C-triol and lysosphingolipid measurements will likely decrease the proportion of false negative diagnostic results; however, particularly for lysosphingolipids, more experience within a clinical setting is still required to fully ascertain their potential.

Newer tests recently validated but currently still in development, such as bile acid derivatives, may complement or possibly replace the current techniques. Nevertheless, it should be kept in mind that these biochemical tests alone do not provide a definitive diagnosis.

Identifying two alleles with NPC1 or NPC2 disease-causing mutations will confirm a diagnosis of NP-C. Genomic DNA sequencing has become easier, faster and cheaper. The increased use of targeted NGS (e.g. ataxia genes) or exome sequencing, has already led to the diagnosis of new cases. But, as discussed in this review, sequencing of exons and exon-intron boundaries will not allow the identification of all mutations, and complementary studies (e.g., MLPA, cDNA sequencing or more complex techniques) may still be necessary. Further, parental studies are essential to avoid erroneous interpretation of data.

A major benefit of genotyping is to allow prenatal diagnosis in at-risk couples, and reliable genetic counselling in blood relatives. Achieving genotyping for all index cases (including those where diagnosis was established with filipin staining), is therefore of utmost importance. Prenatal diagnosis of NP-C is currently achieved by mutation analysis [4,10]. Notable exceptions can be pregnancies with echographic signs of NP-C, but no index case. In such cases, a filipin test on cultured amniocytes could be considered, as molecular genetic analysis may incur pitfalls.

When properly conducted, the filipin test allows diagnostic conclusions in the majority of cases, but it has many disadvantages and, like mutational analysis, results may be inconclusive. In practice, the two approaches are often complementary. A diagnosis may be supported by the finding of known or certainly pathological mutations in a patient with a ‘variant’ filipin profile; likewise, mutations of uncertain pathogenicity associated with clearly positive filipin staining will confirm a diagnosis.

In summary, all current NP-C diagnostic tests have their limitations, but when used in combination, they lead to a correct diagnosis in the
vast majority of cases. Biomarker studies and the filipin test allow individual testing. Genetic testing should include analysis of at least one of the parents, which may be a limitation in adult cases. Following clinical orientation, it seems logical today to first enlist plasma biomarkers (if possible a panel), usually followed by genetic testing in case of an “NPC” profile, with filipin test mandatory only in the some 15% of cases in which molecular results remain inconclusive. Mutation analysis is essential in all cases, as ultimate confirmation of the diagnosis, and as a requisite for prenatal diagnosis and carrier detection. Diagnosis can however remain a difficult task in some patients. Expertise and reference laboratories thus remain essential, and further work is still needed to develop a definitive and rapid NPC test. World-wide, the current strategy used for laboratory diagnosis of NPC may however differ from country to country, depending on local facilities, resources and healthcare constraints. Particularly in the case of practical limitations, but also because improved approaches are likely to be developed, it is advisable to keep samples from suspected patients in biobanks for use in future studies.

For many patients, the main problem today is still not the months that may be required to establish the diagnosis, but the years during which NP-C was not suspected, and no disease-specific test was available. To introduce and establish therapy early in the disease course, and to provide appropriate genetic counselling, a prompt diagnosis is essential. This requires better recognition of the symptoms in clinical practice and referral of samples for appropriate diagnostic testing. Efforts to increase awareness of NP-C among clinicians are still needed, but the recent development of rapid and relatively simple laboratory tests should be of great help. Such assays could become part of wider screening panels used in target populations, leading to more specific tests in case of abnormal results.

Finally, systematic genotyping and the reappraisal and updating of the NP-C gene variation database [109], as part of an ongoing EU project, will provide a useful prospective tool. This database should help to discriminate between pathogenic and non-pathogenic gene variations and — potentially in combination with existing or new biomarkers—, expand our currently limited knowledge of genotype-phenotype correlations.

Conflicts of interest

All authors qualify for authorship of this minireview, as stated in the International Committee of Medical Journal Editors (ICMJE) criteria.

The authors declare the following potential conflicts of interest:

MTV, PG, PB, MJC, AB, CJH, PL, CG, TM have received travel reimbursements and consulting fees or honoraria from Actelion Pharmaceuticals Ltd.

PG, PB, CJH, and TM were also the recipient of research grants from Actelion Pharmaceuticals Ltd.

RWDW and SAK are full time employees of Actelion Pharmaceuticals Ltd.

Since January 2016 PB is Chief Operating Officer at Centogene AG (present address: Schillingallee 68, Rostock, Germany) but still works for Actelion Pharmaceuticals Ltd.


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