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Diagnostic workup and management of patients with suspected Niemann-Pick type C disease

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***Abstract*:** Niemann-Pick type C (NP-C) disease is a neurovisceral disorder caused by mutations in the *NPC1* and *NPC2* genes. It is characterized by lysosomal storage of a broad range of lipids as a result of abnormal intracellular lipid trafficking. Typically patients develop neurodegeneration; however, the speed of disease progression is variable. The exact functions of NPC1 and NPC2 proteins have not been determined and therefore the molecular pathophysiology of NP-C is still not clearly understood. Due to the disease's rarity and clinical heterogeneity, delays from symptom onset to diagnosis and treatment initiation are common. Current therapeutic approaches focus on multidisciplinary symptom control and deceleration (rather than reversal) of disease progression. Thus identification of cases at early stages of disease is particularly important. Recent advances in genetic and biochemical testing have resulted in the generation of relatively non-invasive, quick and cost-effective laboratory assays that are highly sensitive and specific and have the capacity to enhance the clinicians' ability to reach a diagnosis earlier. Miglustat is a compound recently licensed in many countries for the treatment of NP-C that has been shown to decelerate neurological regression, whereas many other promising drugs are currently being trialled in preclinical models or human studies. This review summarizes key clinical, genetic and biochemical features of NP-C, suggests a simple diagnostic investigation strategy and gives an overview of available therapeutic options as well as potential novel treatments currently under development.

Keywords: biochemical markers, diagnosis, genetics, miglustat, Niemann-Pick type C disease (NP-C), *NPC1*, *NPC2*

Introduction

Niemann-Pick disease type C (NP-C; OMIM: NP-C1 #257220, NP-C2 #607625) is a neurodegenerative disorder caused by mutations in *NPC1* and *NPC2* genes [Carstea *et al.* 1997; Naureckiene *et al.* 2000]. At a cellular level, it is characterized by accumulation of a broad range of lipids, including unesterified cholesterol and sphingolipids, in the lysosomes and late endosomes [Pentchev *et al.* 1985; Sokol *et al.* 1988; Vanier and Millat, 2003; Lloyd-Evans and Platt, 2010; Vanier, 2010]. The lipid accumulation and storage is associated with the multisystemic clinical manifestations of NP-C. The exact NPC1 and NPC2 protein functions are unknown [Vance and Karten, 2014; Vanier, 2015] and the mechanisms leading to abnormal storage and subsequent cellular malfunction and neurodegeneration have not, to date, been fully elucidated. A number of hypotheses have been proposed, including the suggestion that sphingosine storage in the endolysosomal compartment leads to impaired calcium homeostasis and plays an integral part in disease pathophysiology [Lloyd-Evans *et al.* 2008].

NP-C disease is very heterogeneous in its presentation. It has a variable age of onset and a range of (often nonspecific) visceral, neurological and psychiatric clinical features can arise at different disease stages and progress at different rates [Patterson *et al.* 2012]. NP-C has an estimated prevalence of 1 in 90,000–120,000 live births, although recent studies suggest this figure to be

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an underestimate, with many patients failing to reach a diagnosis [Vanier, 2010; Mengel *et al.* 2013; McKay Bounford and Gissen, 2014; Wassif *et al.* 2016]. One of the main reasons behind the diagnostic delay is believed to be the nonspecific clinical presentation, thus making detection and diagnosis at an early stage of disease difficult. Specialist biochemical and molecular genetic tests are required to confirm the diagnosis; however, the major diagnostic delay is due to the lack of awareness of NP-C among non-specialist clinical practitioners [Wraith *et al.* 2009; Patterson *et al.* 2013].

Timely and accurate NP-C diagnosis is crucial to enable prompt initiation of appropriate clinical management, especially in view of the presence of: (1) disease modifying drugs that can slow down disease progression [Patterson *et al.* 2015]; and (2) novel therapeutic approaches currently in preclinical development (or at the clinical trial stage) with promising results in animal or cell models [Kirkegaard *et al.* 2010; Kalmar *et al.* 2014; Vite *et al.* 2015]. In 2012, updated recommendations on the diagnosis and treatment of NP-C were published [Patterson *et al.* 2012]. Moreover, an increased understanding of NP-C genetics and biochemical features has recently enabled the development of laboratory tests that not only aim to aid clinicians to achieve diagnosis in a more timely fashion but are also simpler and more cost-effective than those traditionally available. However, diagnostic and management difficulties persist.

This article provides an overview of available diagnostic methods that can be utilized when NP-C is suspected, as well as their advantages and limitations, but also focuses on therapeutic strategies that can be applied once the diagnosis has been made.

Current diagnostic techniques

History taking and clinical examination

Clinical examination is the initial and most crucial step when assessing patients and considering the diagnosis of NP-C. Of paramount importance is the need for increased clinical awareness and suspicion, especially in view of the disease's rarity and variable, nontypical presentation. In about 90% of the patients, a progressive neurodegenerative involvement is the dominant feature [Vanier, 2015]. The combined presence of visceral, neurological and/or psychiatric symptoms should prompt clinicians to include NP-C in their differential diagnosis; however, atypical presentations in NP-C patients are common. The age at onset of neurological manifestations has been linked with prognosis, with earlier onset forms progressing faster and associated with greater mortality and morbidity [Mengel *et al.* 2013]. In each case, clinicians should look out for 'red flag' signs and symptoms that point towards the disorder and which are summarized in Figure 1. These include neonatal cholestasis, hepatosplenomegaly, vertical supranuclear gaze palsy, gelastic cataplexy, neurological regression (with or without dementia), progressive ataxia, dystonia and dysphagia as well as psychiatric symptoms (psychosis or depression).

There are currently several tools available online for clinicians which can aid in identifying patients who may warrant further investigation, such as the NP-C Suspicion Index Tool [Wijburg *et al.* 2012] (http://www.npc-si.com). The tool generates a risk prediction score based on the presence of specific clinical manifestations and family history, and provides a strong indication of those patients who should be referred for further investigations. Its application in paediatric populations has shown limited utility in younger patients (less than 4 years of life) and is unlikely to help in diagnosing disease at an early stage when symptoms are nonspecific [Wraith *et al.* 2014]. Overall, there should be a low threshold for referring suspected cases to appropriate specialist neurometabolic services where available [Wraith *et al.* 2009; Patterson *et al.* 2012], not only to establish or refute the diagnosis but also to ensure optimal utilization of diagnostic testing and appropriate multidisciplinary management if the diagnosis is reached.

Laboratory testing

Laboratory-based diagnostic tests are essential for diagnosing NP-C. Below, we focus on the genetic and biochemical diagnostic approaches that can be applied when NP-C is suspected and also provide a suggested algorithm, which is summarized in Figure 1. The algorithm is modifiable according to each test's availability, with clinicians able to prioritize some tests compared with others if more readily accessible (and/or less costly) in their practice. The appropriate use of these tests will help in prompt patient identification and treatment initiation.

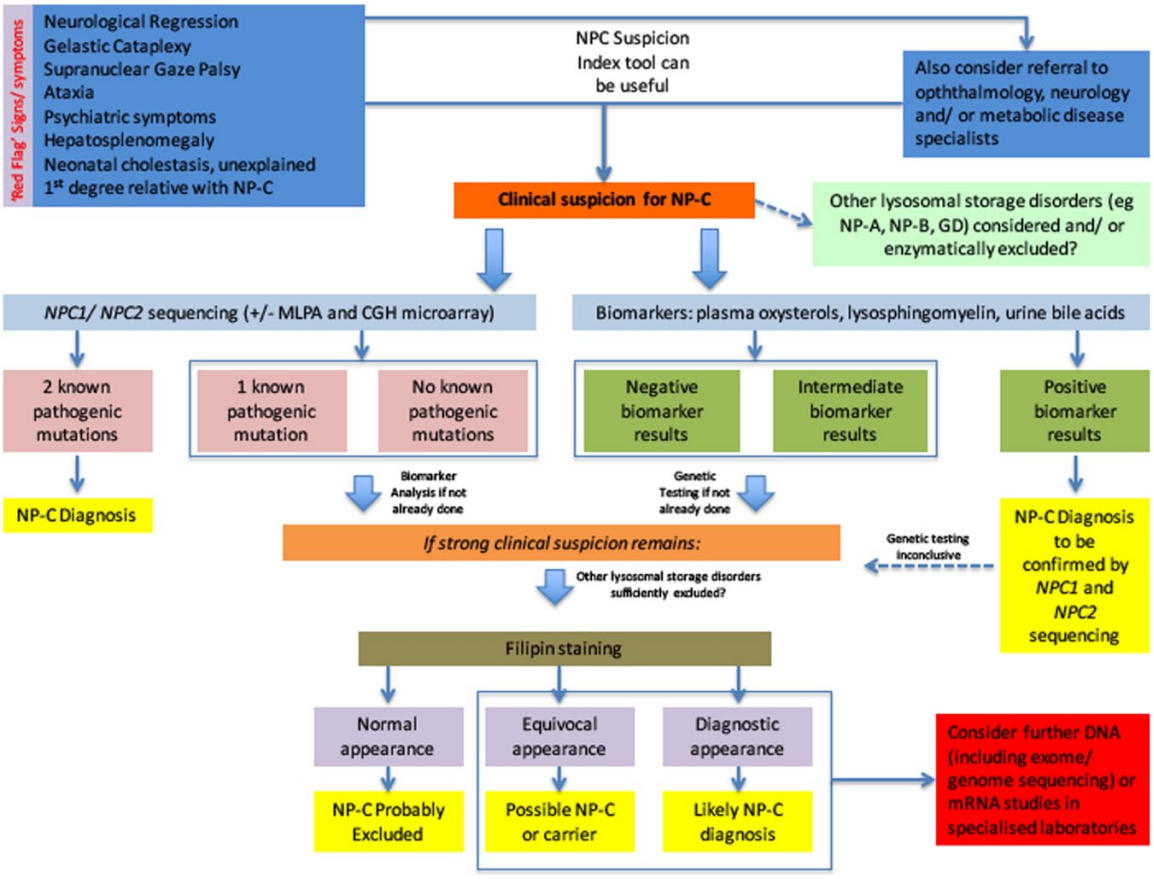

Figure 1. Proposed diagnostic algorithm for patients with suspected Niemann-Pick type C disease.
Although clinically heterogeneous, several 'red flag' signs and symptoms exist, which should alert clinicians towards suspecting and investigating for the presence of the disorder. The differential diagnosis is often broad and includes other lysosomal storage disorders that also need to be considered early on. In any case, there should be a low threshold in referring suspected cases to specialist clinics. Genetic and biomarker analysis can be performed early on in the diagnostic pathway, provided these tests are available to the clinician. Positive biomarker results should prompt *NPC1* and *NPC2* gene testing. Identification of two known pathogenic mutations (one on each allele) *via* sequencing confirms the diagnosis, while studies looking for exon or whole gene deletions might also prove diagnostic in a few cases. In clinically suspicious cases of inconclusive genotype, other lysosomal storage disorders should be excluded and other tests (such as filipin staining or advanced genotypic studies) might be considered.
+/-, test might need to be considered; CGH, comparative genomic hybridization; DNA, deoxyribonucleic acid; GD, Gaucher disease; MLPA, multiplex ligation-dependent probe amplification; mRNA, messenger ribonucleic acid; NP-A, Niemann-Pick type A; NP-B, Niemann-Pick type B; NP-C, Niemann-Pick type C.

Routine/nonspecific laboratory testing. Commonly performed laboratory routine tests in patients suspected with NP-C include full blood count, renal and liver function tests, plasma lipids and unconjugated bilirubin levels. These often yield unremarkable results. However, mild thrombocytopenia can be seen in patients with hypersplenism and liver function can be deranged in patients with cholestatic liver disease. Although plasma transaminases are generally normal, aspartate aminotransferase elevation and low high-density lipoprotein (HDL) cholesterol have been reported [Wraith *et al.* 2009].

Although the chitotriosidase assay has previously been proposed as a screening test for NP-C, this marker is, however, neither specific nor sensitive for NP-C [Ries *et al.* 2006]. Chitotriosidase is mildly elevated in many cases of NP-C but normal values are also possible [McKay Bounford and Gissen, 2014], whereas in other lysosomal storage disorders, such as Gaucher disease (GD) and Niemann-Pick type A (NB-A) and B (NP-B), very high levels are usually seen [Guo *et al.* 1995; Wajner *et al.* 2004]. The presence of elevated chitotriosidase would have to be paired with (hepato)splenomegaly and normal activities

of acid sphingomyelinase (excluding NP-A and NP-B) and β-glucocerebrosidase (excluding GD), for NP-C to be further considered [McKay Bounford and Gissen, 2014]. In addition, 10% of the population has a pseudodeficiency mutation, which makes this test uninterpretable [Ries *et al.* 2006].

Genetic testing. NP-C is inherited in an autosomal recessive manner. *NPC1* is located on chromosome 18q11-q12 [Carstea *et al.* 1997], whereas *NPC2* maps to chromosome 14q24.3 [Naureckiene *et al.* 2000]. *NPC1* gene mutations are present in 95% of cases and *NPC2* mutations are present in approximately 4%; the remainder of patients are biochemically proven cases who do not have identified mutations [Millat *et al.* 2001a; Wraith *et al.* 2009; Patterson *et al.* 2012]. More than 350 different *NPC1* and *NPC2* gene mutations have been reported in patients with confirmed diagnoses [McKay Bounford and Gissen, 2014].

Although often not done as an initial test (or traditionally requested to support suspicious biochemical/ cell biology results), genetic testing is paramount in the NP-C diagnostic process. The identification of two alleles with demonstrated disease-causing mutations in *NPC1* or *NPC2* is currently considered definitive proof of an NP-C diagnosis. For example, genetic analysis is necessary to confirm a diagnosis of NP-C following abnormal results with disease biomarkers, or inconclusive filipin staining results [Patterson *et al.* 2012; Vanier and Latour, 2015]. Identification of *NPC1* or *NPC2* mutations allows family studies, antenatal diagnosis and carrier testing to be performed [Patterson *et al.* 2012].

Previously uncommon, sequencing of both *NPC1* and *NPC2* genes has recently become more readily available as part of the routine diagnostic process and a greater proportion of patients are now diagnosed *via* genetic testing as a primary diagnostic tool [Patterson *et al.* 2012; Bauer *et al.* 2013]. The most frequently currently employed method of genetic analysis for NP-C is Sanger sequencing, which utilizes polymerase chain reaction (PCR) to target the 30 coding exons, and intron–exon boundaries, of the *NPC1* and *NPC2* genes. Alternatively, targeted next-generation sequencing (NGS) methods may be applied. NGS has the potential to reduce costs associated with genetic testing for NP-C, particularly if the genes concerned are included on a multigene panel along with other genes considered relevant to the patient phenotype [Bruce *et al.* 2010]. In general, sequence changes that result in the introduction of premature stop codons (i.e. nonsense, frameshift and conserved splice-site mutations) produce truncated mRNA species that are usually targeted for breakdown (nonsense-mediated decay; NMD) rather than translation into proteins. Sequence changes that result in small protein sequence changes (missense and in-frame mutations) may also be disease-causing. Lastly, sequencing may identify intronic sequence variants that can disrupt RNA splicing and lead to NMD. However, missense and intronic variants are not necessarily disease-causing and several such polymorphisms exist in *NPC1* and *NPC2* [McKay Bounford and Gissen, 2014].

Despite its promise as a diagnostic tool, genetic sequencing sometimes identifies mutations clearly associated with disease on only one allele or provides negative results [Vanier, 2010; Bauer *et al.* 2013], even in patients where there is strong clinical suspicion and/or supportive biochemical test results pointing towards NP-C. Additionally, many families have 'private' sequence variants that have not been reported/published elsewhere [McKay Bounford and Gissen, 2014]. Hence, in a number of suspected patients, confirmation of a diagnosis is not possible through genetic sequencing alone.

Inconclusive genotype in the presence of convincing NP-C clinical and laboratory phenotypic features raises the possibility that mutations in other, yet unidentified, genes could cause NP-C, which would further increase the complexity of the genotype–phenotype correlation. Moreover, deep intronic mutations or mutations in regulatory/ promoter regions, not regularly identified through standard genetic sequencing techniques, may also result in aberrant protein expression. Indeed, mutations in *NPC1* introns have been identified in patients with NP-C and have been found to result in splicing defects [Di Leo *et al.* 2004]. Therefore, sequencing of only exons and exon–intron boundaries would miss such mutations. Full genomic DNA sequencing is currently possible using NGS methods but this is, in turn, also likely to identify sequence variants that are difficult to interpret [Plon *et al.* 2008; Richards *et al.* 2015]. Therefore, this strategy should be limited to patients with a suggestive clinical and/or biochemical picture where routine sequencing has not identified two known pathogenic mutations.

Investigations of such cases would sometimes also require RNA and protein studies and, as such, are often outside the scope of service laboratories. Additionally, genomic rearrangements, such as exonic deletions or whole gene deletions, are also reported to rarely cause NP-C. Although microarray comparative genomic hybridization (CGH) testing can pick up large deletions, testing for this type of mutations would generally require quantitative methodologies and is most frequently performed using multiplex ligation-dependent probe amplification (MLPA) [Patterson *et al.* 2012; McKay Bounford and Gissen, 2014]. In cases of missense or intronic changes of unknown significance, parental/familial DNA testing, the use of *in silico* protein and splicing prediction tools and adherence to recently published guidelines on variant interpretation [Houdayer *et al.* 2008; Min *et al.* 2015; Richards *et al.* 2015] may assist in assigning their pathogenicity.

Diagnostic biomarkers. Due to several scientific advances in recent years (mainly in mass spectrometry methods), a number of novel and cost-effective techniques have now become available for studying and diagnosing NP-C. These methods are much more convenient, less invasive and easier to perform compared to traditional techniques such as filipin staining, which requires a skin biopsy and specialist expertise, is time-consuming and expensive. Currently available (and perhaps other, yet to be discovered) biochemical markers could possibly substantially reduce diagnostic delays but also prove useful in establishing disease severity and monitoring disease progression and response to therapeutic interventions. Although still largely provided through specialist clinical and research mass spectrometry laboratories, these techniques are now becoming more readily available around the world. Due to their advantages, they have the potential to be used as first-line targeted tests, before genetic analysis, when a diagnosis of NP-C is suspected.

In view of the above, we propose that genotype and biochemical marker analysis (if available) may precede or, in the advent of convincing results, substitute other NP-C specific tests (like the filipin staining) in the clinicians' diagnostic pathway, a summary of which is provided in Figure 1. The practical implementation of the algorithm is dependent on each test's local availability.

Plasma oxysterols. In NP-C, unesterified cholesterol accumulates in the lysosomes but increased production of reactive oxygen species (ROS) secondary to oxidative stress also occurs [Tint *et al.* 1998; Reddy *et al.* 2006; Zhang *et al.* 2008; Zampieri *et al.* 2009]. ROS leads to conversion of unesterified cholesterol into cholesterol oxidation products (mainly cholestane-3β,5α,6β-triol and 7-ketocholesterol). After having been noted to be elevated in *NPC1* mice models [Zhang *et al.* 2008], various studies have shown that plasma oxysterol levels are high in patients with *NPC1* and *NPC2* mutations [Porter *et al.* 2010; Jiang *et al.* 2011; Boenzi *et al.* 2014; Zhang *et al.* 2014; Reunert *et al.* 2015]. There seems to be clear discrimination between NP-C patients and controls, providing a high sensitivity for this assay, and cholestane-3β,5α,6β-triol might be better than 7-ketocholesterol in discriminating between the two groups [Porter *et al.* 2010; Jiang *et al.* 2011; Boenzi *et al.* 2014; Klinke *et al.* 2015]. However, high plasma oxysterol concentrations can also be found in unaffected *NPC1* heterozygotes [Porter *et al.* 2010; Jiang *et al.* 2011] and in other disorders, including NP-A and NP-B [Lin *et al.* 2014; Klinke *et al.* 2015], lysosomal acid lipase deficiency [Reunert *et al.* 2014], peroxisomal disorders and Smith–Lemli–Opitz syndrome [Pajares *et al.* 2015]. Other difficulties encountered include the assay's limited availability to general clinical practice; at present, oxysterol testing is only successfully performed by a limited number of specialized laboratories. Sample exposure to high temperatures, haemolysis and long transportation times from collection to the laboratory could result in erroneous (falsely elevated) test results due to oxidation [Helmschrodt *et al.* 2014].

Plasma lysosphingolipids. Apart from cholesterol, various other types of lipids accumulate in NP-C, including sphingomyelin, multiple glycosphingolipids and sphingosine [Lloyd-Evans and Platt, 2010]. Plasma lysosphingolipids, previously proven useful as disease biomarkers in Fabry disease and GD [Aerts *et al.* 2008; Dekker *et al.* 2011], have recently also been identified as potential diagnostic markers in NP-C [Bauer *et al.* 2013; Welford *et al.* 2014; Giese *et al.* 2015]. Plasma lysosphingomyelin (SPC) has been reported to have very high sensitivity and specificity in distinguishing between treatment-naïve NP-C patients and control subjects [Welford *et al.* 2014] and seems to be elevated in NP-C patients independent of age. However, some overlap between patients and controls can be seen in very few cases. More recently, another biomarker (lysosphingomyelin-509, with a similar structure

to SPC) was suggested to be superior to SPC and oxysterols at differentiating patients with NP-C from healthy controls and *NPC1* carriers [Giese *et al.* 2015]. Plasma oxysterols are similarly elevated in NP-A, NP-B and NP-C [Lin *et al.* 2014], whereas lysosphingolipids (and especially lysosphingomyelin-509) might be better in differentiating these disease pathologies. Therefore, lysosphingolipids pose as potentially very useful NP-C biochemical markers, while the prospect of screening for other sphingolipidoses (such as GD and Fabry disease) with the same assay makes their use even more alluring. However, data regarding the utility of lysosphingolipids in NP-C are still scarce and mostly based on retrospective studies; hence, the assay's exact sensitivity and specificity still needs to be established.

Urine bile acids. High urinary concentrations of 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid (SNAG-Δ^5−CA) (and its glycine- and taurine- amides) in NP-C patients compared with healthy controls have recently been identified [Alvelius *et al.* 2001; Maekawa *et al.* 2013]. Other studies have demonstrated that di-docosahexaenoyl (22:6) bis(monoacylglycerol) phosphate (di-22:6-BMP), a marker of drug-induced phospholipidosis [Baronas *et al.* 2007; Thompson *et al.* 2012], also appears in high urinary concentrations in NP-C patients compared with controls [Liu *et al.* 2014]. These findings suggest that urinary bile acids may be another potentially powerful biomarker in the detection of NP-C. Again, potential benefits include the test's noninvasive nature and cost-effectiveness compared to more traditional diagnostic methods. At present, though, minimal data is available regarding these markers' sensitivity, specificity and ability to discriminate between NP-C patients, controls and carriers (as well other disorders) and further research into urinary (and plasma) bile acids is warranted.

Filipin staining (and cholesterol esterification test). The demonstration of impaired intracellular cholesterol transport and homeostasis has traditionally been considered the 'gold standard' diagnostic test for NP-C [Pentchev *et al.* 1985; Vanier *et al.* 1991; Wraith *et al.* 2009; Vanier, 2010]. The test involves a skin biopsy, after which cultured skin fibroblasts are exposed to an low-density lipoprotein (LDL) enriched medium for 24 hours. Filipin, a fluorescent chemical compound isolated from *Streptomyces filipinensis* that specifically binds to unesterified cholesterol [Bornig and Geyer, 1974], is subsequently used to measure sequestration and accumulation of cholesterol in the fibroblasts using fluorescence microscopy. In normal fibroblasts, fewer than 10% of cells would be expected to stain positive for filipin. In contrast, due to cholesterol accumulation, skin fibroblasts from 80–85% of patients with NP-C demonstrate numerous strongly fluorescent (cholesterol-filled) perinuclear vesicles in more than 80% of cells. A smaller proportion of NP-C patients are found to have less obvious filipin staining and are described as having a 'variant' phenotype. 'Variant' profiles have been linked to specific mutations in *NPC1* [Millat *et al.* 2001b; Sevin *et al.* 2007]. It has also been suggested that 'variant' filipin staining is more commonly associated with adult-onset disease or might point towards overall milder disease severity and slower progression rate [Sevin *et al.* 2007; Walterfang And Velakoulis, 2010]. Moreover, an abnormal filipin test can also be encountered in heterozygote carriers and in a number of other disorders (including MEGDEL syndrome, Smith–Lemli–Opitz syndrome and Tangier disease), whereas a variant phenotype, suggestive of 'possible NP-C', can also be seen in patients with NP-A or NP-B [Vanier, 1997, 2010; Wortmann *et al.* 2012; Platt *et al.* 2014; Sechi *et al.* 2014; Vanier and Latour, 2015]. All the above can complicate the diagnostic process.

The Cholesterol Esterification Test is based on the inability of NP-C cells to traffic cholesterol from the endolysosomal compartment to the endoplasmic reticulum where cholesterol is esterified. Deficient cholesterol esterification can be detected by measuring cholesteryl oleate formation after the LDL challenge, in the presence of [^{3}H]oleic acid [Vanier *et al.* 1991]. Overall, the assay is labour-intensive and has reduced sensitivity compared with filipin staining; thus it is no longer performed in most diagnostic laboratories.

Currently available treatments for NP-C

In the absence of a disease cure, management approaches in NP-C focus on trying to delay the onset and progression of symptoms, but also on their early recognition and appropriate multidisciplinary management. Due to the disease's rarity, care is often led or coordinated by large academic centres with the appropriate staff and expertise; clinicians are encouraged to refer NP-C patients to such establishments, especially when there is lack of resources to deal with the

multitude of issues these patients are likely to encounter. Management strategies are similar to other progressive neurodegenerative disorders and based on close collaboration between healthcare professionals to achieve holistic care. This includes: neurological management of extrapyramidal and pyramidal disorders, seizures and sleep disturbance; appropriate psychiatric input for behavioural concerns and neuropsychiatric symptoms; pain management; management of gastrointestinal (GI) issues such as constipation, gastro-oesophageal reflux and swallowing difficulties; regular assessment of feeding and nutritional status; appropriate orthopaedic management of secondary complications (contractures, hip dislocation, kyphoscoliosis); preventative measures and prompt treatment of respiratory complications; physiotherapy, occupational therapy, speech and language therapy; input from psychology and social services when needed; and genetic counselling for family members, as appropriate.

Substrate reduction therapy

N-butyldeoxynojirimycin (miglustat) (Zavesca®, Actelion Pharmaceuticals Ltd) is a small iminosugar that partially inhibits glucosylceramide synthase and the synthesis of all glucosylceramide-based glycosphingolipids. Miglustat was initially approved for the treatment of GD type I [Elstein *et al.* 2007; Abian *et al.* 2011; Shemesh *et al.* 2015]. After evidence of benefit in animal models of NP-C [Zervas *et al.* 2001; Stein *et al.* 2012], and following several clinical trials [Patterson *et al.* 2007, 2010; Wraith *et al.* 2010], miglustat has been approved for the treatment of neurological manifestations in paediatric and adult NP-C patients in the European Union and a number of other countries. However, its use for NP-C disease remains off-label in the USA. Recent studies suggested that miglustat has a beneficial effect on dysphagia, a major factor of mortality and morbidity in NP-C [Walterfang *et al.* 2012]. In a longitudinal study examining data from the International Registry for NP-C disease, the clinical severity composite scale (a disease-specific scale based on ambulation, manipulation, language and swallowing which is used as a marker of disease severity and neurodisability status in many centres) [Iturriaga *et al.* 2006; Yanjanin *et al.* 2010] remained stable or even improved in the majority of patients who received continuous miglustat therapy for a period of approximately 2 years [Patterson *et al.* 2015]. This effect was more pronounced in patients with later onset of neurological manifestations compared with others with onset during infancy or childhood.

Miglustat side effects include GI disturbances such as diarrhoea, flatulence, abdominal discomfort, nausea and vomiting. These are the most frequent adverse effects associated with miglustat therapy and are present in up to 80% of patients during the first 6 months compared with 50–60% in the ensuing period [Remenova *et al.* 2015]. GI disturbances have been reported as the most common reason for miglustat discontinuation [Hollak *et al.* 2009; Belmatoug *et al.* 2011]. These are caused by inhibition of intestinal disaccharidase enzymes leading to suboptimal hydrolysis of carbohydrates and osmotic diarrhoea, whereas subsequent bacterial fermentation of unabsorbed material leads to flatulence, abdominal distension and discomfort. Transient weight decrease, presumably due to carbohydrate malabsorption, is also often reported in the first few weeks of treatment. Dietary modifications such as reduced sucrose, maltose and lactose consumption have been shown to improve the drug's GI tolerability and ameliorate changes in body weight, particularly if initiated at or before the start of therapy. Gradual dose escalation at treatment initiation may also reduce GI disturbances. Recently, a randomized double-blind, placebo-controlled study pointed towards increased miglustat GI tolerability when administered in conjunction with *Saccharomyces bulardii* probiotics, as the yeast produces an enzyme that breaks down sucrose [Remenova *et al.* 2015]. Other, less commonly reported miglustat-associated side effects include tremor and peripheral neuropathy; however, these can also feature in the disease even without miglustat administration [Hollak *et al.* 2009; Patterson *et al.* 2015].

Acetyl-DL-leucine

Acetyl-DL-leucine, a modified amino acid was administered to 12 NP-C patients aged from 13 to 32 years in a recent prospective, open-label study [Bremova *et al.* 2015]. A month after treatment, the Assessment and Rating of Ataxia (SARA) scale [Schmitz-Hubsch *et al.* 2006; Subramony, 2007], as well as dexterity in the dominant hand, showed an improvement of statistical significance. This improvement in cerebellar signs and symptoms led to reduction in the composite disease severity score used [Pineda *et al.* 2010], but also improvement in quality of

life for patients and family members, as assessed by a questionnaire. Adverse effects in the form of transient dizziness were reported in 1/12 patients. Hence, acetyl-DL-leucine has the potential of being a well-tolerated adjunctive therapy for cerebellar signs and symptoms in NP-C.

Other therapeutic compounds with no established benefit and treatments under development

Various compounds have been tested in NP-C animal and cell models, a few having shown potential that has not translated to clinical practice.

Cholesterol-lowering agents such as cholestyramine, lovastatin and nicotinic acid, as well as a low cholesterol diet, are ineffective in altering the neurological course in NP-C disease [Patterson *et al.* 1993; Somers *et al.* 2001].

Curcumin, the active ingredient of turmeric, has been shown to elevate cytosolic calcium *in vitro*, normalize *NPC1* disease cellular phenotypes and prolong survival of NP-C mice [Lloyd-Evans *et al.* 2008]. However, other studies suggested the lack of curcumin's efficiency in preventing neurodegeneration in mice [Borbon *et al.* 2012].

Histone deacetylase (HDAC) inhibitors have shown promise in correcting cholesterol storage defects in human *NPC1* mutant cells by increasing expression of the low activity mutant *NPC1* protein [Munkacsi *et al.* 2011; Pipalia *et al.* 2011]. HDACs are part of a vast family of enzymes that have crucial roles in numerous biological processes, largely through their repressive influence on transcription; hence, HDAC inhibitors have long been used in psychiatry and various brain disorders and are being investigated as possible treatments for several neurological diseases[Kazantsev and Thompson, 2008; Haberland *et al.* 2009]. LBH589 (panobinostat) and vorinostat, orally available HDAC inhibitors that cross the blood–brain barrier and have been trialled for oncological disorders, seem to restore cholesterol homeostasis in cultured *NPC1* mutant fibroblasts [Pipalia *et al.* 2011; Helquist *et al.* 2013]. Again, no data deriving from human studies are currently available; however a phase I/II clinical trial of vorinostat is currently in progress [ClinicalTrials.gov identifier: NCT02124083].

Arimoclomol induces expression of a group of chaperone proteins called heat shock proteins (HSPs). The heat shock response is an endogenous cytoprotective mechanism responsible for inducing the synthesis of HSPs. Under stress conditions, upregulation of certain classes of HSPs has been shown to protect cells from excessive cellular damage, thereby providing a natural cellular protection system. HSP-enabled prevention of protein misfolding, aggregation and abnormal degradation has been found to be neuroprotective in a number of neurodegenerative disease models including amyotrophic lateral sclerosis [Kalmar *et al.* 2014]. HSP upregulation was also seen to ameliorate intracellular lipid accumulation in NP-C cells [Kirkegaard *et al.* 2010]. A prospective non-therapeutic observational study in NP-C patients is currently underway and will be followed by enrolment into a phase II/III study [ClinicalTrials.gov identifier: NCT02435030].

Neurosteroids, particularly **allopregnanolone**, were shown in mouse models to increase lifespan, delay the onset of neurological symptoms, and result in neuronal survival and reduction of cholesterol and ganglioside accumulation [Griffin *et al.* 2004]. Enhanced myelination and improvement of autophagic/lysosomal function were also shown [Mellon *et al.* 2008; Liao *et al.* 2009]. Studies on human fibrobasts suggested that allopregnanolone's effect might be due to reduction of oxidative stress [Zampieri *et al.* 2009]. However, no data on the effect of neurosteroids in human subjects currently exist.

2-Hydroxypropyl-beta-cyclodextrin (HPBCD) is a cyclic oligosaccharide with a hydrophilic exterior and a hydrophobic interior, enhancing the solubility of poorly water-soluble compounds (such as cholesterol) *via* formation of compound-cyclodextrin complexes. Through this structure, it acts as an excipient [Loftsson and Brewster, 2012], helping molecules cross membranes. Initially, allopregnanolone, complexed with hydroxypropyl-β-cyclodextrin (HPBCD), was deemed beneficial in *Npc1*−/− mice [Griffin *et al.* 2004]. However, subsequent studies showed that HPBCD alone was effective in reducing neurodegeneration, cholesterol and ganglioside storage and prolonging lifespan [Davidson *et al.* 2009; Liu *et al.* 2010]. Hence, cyclodextrins emerged as possible treatments for NP-C, as they have been shown in cell-based systems to mediate efflux of cholesterol from within the cell [Yancey *et al.* 1996; Atger *et al.* 1997]. The mechanism by which HPBCD decelerates the neurodegenerative process is unclear. HPBCD does not cross

the blood–brain barrier [Pontikis *et al.* 2013] and is most active in slowing disease progression and clearing cholesterol from the brain if injected intraventricularly in mice [Aqul *et al.* 2011]. Lately, studies on a feline model [Vite *et al.* 2015] showed that subcutaneous HPBCD administration ameliorated hepatic disease, but doses sufficient to affect the central nervous system resulted in pulmonary toxicity. In contrast, when administered into the cisterna magna, HPBCD prevented the onset of cerebellar dysfunction in presymptomatic cats for more than 12 months and resulted in a reduction in Purkinje cell loss and near normal concentrations of cholesterol and sphingolipids. Administration of intracisternal HPBCD to NP-C cats with ongoing cerebellar dysfunction slowed disease progression, increased survival time and decreased the accumulation of brain gangliosides. An increase in hearing threshold was identified as an adverse effect. Intravenous HPBCD was trialled on two NP-C patients [Matsuo *et al.* 2013] and had little or no effect in controlling or delaying neurological symptoms, although it resulted in improvement of liver size and function. A 12-year old patient with *NPC1* was recently given HPBCD intrathecally every 2 weeks and response was assessed after 27 injections [Maarup *et al.* 2015]. The NP-C severity scale score improved over 18 months, with all neurological functions remaining stable and a 1-point improvement shown in eye movements. High frequency hearing loss was observed bilaterally, investigators attributing this to the study drug rather than disease progression. More studies to further evaluate the safety and efficacy of HPBCD in NP-C patients are underway [ClinicalTrials.gov identifier: NCT01747135].

Conclusion

NP-C is a devastating neurodegenerative disorder with heterogeneous presentation. Its rarity, heterogeneity and, at least until recently, the lack of easy-to-perform, accurate and cost-effective diagnostic tests, often lead to severe delays from symptom onset to diagnosis. Diagnosing NP-C early is critical in view of substrate reduction therapies that can decelerate disease progression, but also because of promising new therapeutic approaches currently being developed. Recent advances in gene sequencing and mass spectrometry methods have resulted in tests that are highly specific, sensitive and less invasive than others previously considered to be gold standards for diagnosing NP-C. These assays promise to reduce diagnostic delays and enable instigation of treatment as soon as possible. However, these techniques are not readily available to all clinicians around the world or can only be accessed through research laboratories. Hence, raised clinical awareness, as well as low threshold for referring patients with suspected NP-C to specialized centres, is warranted, whereas consulting available diagnostic and management recommendations [Patterson *et al.* 2012] should also be considered. In the absence of a cure, therapeutic approaches focus on symptom control and aim to maintain neurological function as long as possible; however, eventual neurological regression and a limited lifespan are currently inevitable. While many clinical trials are currently underway, more research is needed in elucidating the disease's underlying pathogenetic mechanisms, identifying potential novel therapeutic compounds and also discovering new biochemical markers that can enhance the monitoring of disease progression and response to therapeutic interventions.

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