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Comprehensive Evaluation of Plasma 7-Ketocholesterol and Cholestan-3β,5α,6β-Triol in an Italian Cohort of Patients Affected by Niemann-Pick Disease due to *NPC1* and *SMPD1* Mutations

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ABSTRACT

Niemann-Pick C disease (NPCD) is a rare autosomal recessive neurovisceral disorder with a heterogeneous clinical presentation. Cholestan-3β,5α,6β-triol and 7-ketocholesterol have been proposed as biomarkers for the screening of NPCD. In this work, we assessed oxysterols levels in a cohort of Italian patients affected by NPCD and analyzed the obtained results in the context of the clinical, biochemical and molecular data. In addition, a group of patients affected by Niemann-Pick B disease (NPBD) were also analyzed.

NPC patients presented levels of both oxysterols way above the cut off value, except for 5 siblings presenting the variant biochemical phenotype who displayed levels of 3β,5α,6β-triol below or just above the cut-off value; 2 of them presented also normal levels of 7-KC. Both oxysterols were extremely high in a patient presenting the neonatal systemic lethal phenotype. All NPB patients showed increased oxysterols levels.

In conclusion, the reported LC–MS/MS assay provides a robust non-invasive screening tool for NPCD. However, false negative results can be obtained in patients expressing the variant biochemical phenotype. These data strengthen the concept that the results should always be interpreted in the context of the patients' clinical picture and filipin staining and/or genetic studies might still be undertaken in patients with normal levels of oxysterols if symptoms are highly suggestive of NPCD.

Both oxysterols are significantly elevated in NPB patients; thus a differential diagnosis should always be performed in patients presenting isolated hepatosplenomegaly, a common clinical sign of both NPCD and NPBD.

1. Introduction

Niemann-Pick type C disease [NPCD-MIM 257220; MIM607625] is an autosomal recessive lysosomal storage disorder due to mutations in *NPC1* (95% of patients) or *NPC2* genes, encoding two proteins involved in the intracellular trafficking of cholesterol and other lipids. The deficiency of either of them leads to the accumulation of the endocytosed unesterified cholesterol, gangliosides and other lipids within the lysosome/late endosome compartment [1].

The clinical presentation of the disease is extremely variable and the age at onset ranges from the perinatal period to adulthood. NPCD is typically characterized by visceral and neurological signs and symptoms that follow a complete independent clinical course. When present, visceral involvement, mainly characterized by hepatosplenomegaly, precedes the onset neurological signs. Besides from a small group of patients presenting a severe perinatal form leading to death due to liver or respiratory failure within the first 6 months of age and few adult

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patients reported to be free of neurological symptoms in the fifth or sixth decade of life [2,3], most patients develop progressive and fatal neurological disease. Indeed, NPCD has been classified on the basis of the age at onset of neurological symptoms [4,5].

To date, the most specific biochemical diagnostic test for NPCD is based on the demonstration of unesterified cholesterol accumulation within the lysosomes by filipin staining. Since this assay has to be performed on cultured fibroblasts obtained from skin biopsies, it is invasive, time consuming and performed only in specialized laboratories. In addition, although most patients present a massive accumulation of unesterified cholesterol, displaying a *classical biochemical phenotype*, some patients storage moderate and variable amounts of this lipid, presenting the so called *variant biochemical phenotype* [6]. In these patients, the results of filipin staining are not always conclusive. It is worth noting that this pattern of cholesterol storage can also be detected in heterozygous carriers [7]. The results of biochemical studies can be confirmed by molecular analysis of *NPC1* and *NPC2* genes. To date, 23 mutations have been reported in *NPC2* gene and more than 390 mutations have been described in *NPC1* gene (www.hgmd.cf.ac.uk), most of them are private or identified in a small number of families. Therefore, sequencing of the whole coding regions of *NPC1* (25 exons) and *NPC2* (5 exon) genes is mandatory to establish the genotype in most patients. In some cases, additional studies such as analysis of the cDNA and gene copy number by multiplex ligation probe amplification (MLPA) are needed to identify the disease causing mutation. In addition, since it is not possible to assess the functional effects of unreported missense mutations in vitro, it is difficult to determine the pathogenic nature of newly identified variants. For these reasons, the diagnosis of NPCD is not always straight forward and may be quite challenging.

Recently, two products of the non-enzymatic oxidation of cholesterol, cholestan-3β,5α,6β-triol (3β,5α,6β-triol) and 7-ketocholesterol (7-KC) have been found elevated in patients affected by NPCD. Based on these findings both 3β,5α,6β-triol and 7-KC have been proposed as NPCD specific biochemical markers [8]. Several reports have further supported the utility of plasma oxysterols as biomarkers for NPCD [9–11]. However, only few patients presenting the variant biochemical phenotype have been studied so far [11,12]. All of them presented high levels of oxysterols with values in the same range as patients with the classical phenotype. These data suggest that these biomarkers would also be useful to identify NPC patients who store moderate amounts of cholesterol leading to filipin staining results that are difficult to interpret [11]. In addition, some concerns have been raised regarding the specificity of this assay. Indeed, 7-KC seems to be a very unspecific biomarker since it has been found to be elevated in many lysosomal storage diseases and in some patients with peroxisomal or sterol disorders [11]. Although 3β,5α,6β-triol is more specific than the 7-KC, it has been recently found to be significantly elevated in patients affected by cerebrotendinous xanthomatosis, lysosomal acid lipase deficiency and acid sphingomyelinase deficiency (ASM deficiency-Niemann-Pick type A- MIM: 257200 and B-MIM: 607616) [10,13], a lysosomal storage disorder caused by mutations in the *sphingomyelin phosphodiesterase 1* (*SMPD1*) gene.

In this work, we report the analysis of 3β,5α,6β-triol and 7-KC in healthy controls, a group of 17 patients affected by NPCD, including 6 patients displaying a variant biochemical phenotype and 8 patients affected by Niemann-Pick type B disease (NPBD). Based on the obtained results the drawbacks, advantages and possible new applications of plasma oxysterols assessment are discussed.

2. Materials and methods

2.1. Chemicals and reagents

3β,5α,6β-triol and 7-KC were obtained from Steraloids (Rhode Island, USA). Labeled internal standards, 25,26,26,26,27,27,27-[$^2H_7$]-7-ketocholesterol ([$^2H_7$]-7-KC) and 25,26,26,26,27,27,27-[$^2H_7$]-cholestan-3β,5α,6β-triol ([$^2H_7$]-3β,5α,6β-triol) were obtained from Avanti Polar Lipids (Alabama, USA). Dimethylglycine hydrochloride (DMG), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), 4-(dimethylamino)pyridine (DMAP), tetradeuteromethanol (methanol-d_4), tricholoroacetic acid (TCA), acetic acid and diethyl ether were obtained from Sigma Aldrich, St Louis, MO. All HPLC solvents (methanol and acetonitrile) were CHROMASOLV® HPLC Grade (Sigma Aldrich, St Louis, MO).

2.2. Samples

Plasma samples were obtained in the course of diagnostic work-up from patients affected by NPBD and NPCD at the Regional Coordinator Centre for Rare Diseases. The research has been performed in accordance with the Declaration of Helsinki and written consent was obtained from subjects or carers/guardians on the behalf of the minors involved in the study. This study was approved by an independent Ethical Committee of the institution. The diagnosis of NPB disease was confirmed by the demonstration of reduced levels of ASM activity in peripheral blood leucocytes or fibroblasts [14], followed by the molecular analysis of the *SMPD1* gene. NPCD diagnosis was confirmed by the demonstration of cholesterol accumulation in cultured skin fibroblasts through filipin staining [15] and/or by the molecular analysis of the *NPC1* and *NPC2* genes [16].

Whenever possible, the genotype was confirmed by the analysis of the identified mutations in the patients' parents.

The pathological nature of novel *NPC1* sequence alterations detected was addressed by (i) searching dbSNP (http://www.ncbi.nlm.nih.gov/SNP) for their presence, (ii) screening 100 alleles from healthy control subjects for each alteration.

Anonymized residual plasma samples from healthy donors were collected from routine analysis at the University Hospital Santa Maria della Misericordia, Udine and used as normal controls. Control human plasma, which was used to prepare quality control (QC) samples and standard curves, was obtained commercially from GENTAUR (Bergamo, Italy). All plasma samples were collected in ethylenediamine tetraacetic acid dipotassium salt (EDTA-K_2) containing tubes.

2.3. Standard curves for quantification and quality control samples

Stock solutions (1 mg/ml) were prepared in methanol (or methanol-d_4 for the labeled internal standards) and stored at −80 °C. An internal standard/protein precipitation solution (40 ng/ml of [$^2H_7$]-7-KC and 40 ng/ml of [$^2H_7$]-3β,5α,6β-triol) was prepared in methanol.

The standard curve was prepared by serial dilution of the 3β,5α,6β-triol and 7-KC working solution spiked into methanol–water (1:1) to obtain standard solutions of 2, 4, 10, 20, 50, 100, 200, and 400 ng/ml. Standards were analyzed with the same procedure of plasma samples. Methanol–water (1:1) served as blank. In addition, because of the endogenous presence of 3β,5α,6β-triol and 7-KC in human plasma and to quantitatively estimate the ion suppression, the same standard solutions were prepared in human plasma to compare the slopes of the curves. The acquired data were processed using MultiQuant version 2.1 software (ABSciex, Framingham, MA,USA). Calibration curves were constructed using a linear least-square regression (weighting type 1/x).

A pooled-plasma sample (Gentaur, London, UK) was analyzed to establish the mean concentration of endogenous 3β,5α,6β-triol and 7-KC and used as the low plasma quality control (LQC). The medium and high plasma quality control (MQC and HQC) samples were prepared by spiking 3β,5α,6β-triol and 7-KC working solution to obtain endogenous level + 150/150 and +300/300 ng/ml, respectively. A solution at a concentration higher than the upper limit of quantification (ULOQ) (the endogenous level + 800/800 ng/ml QC), was diluted 1:5 with water prior to extraction. The lower limit of quantification (LLOQ) sample was prepared in methanol–water (1:1) and was considered as the lowest concentration for which the signal-to-noise ratio (S/N) was

at least 10. The limit of detection (LOD) was calculated as three times S/N ratio.

2.4. Sample treatment procedure

Plasma was immediately separated from blood samples and stored frozen at −80 °C until analysis. The samples were prepared as described in Jiang et al. [8]. Briefly, internal standard/protein precipitation solution (250 μl) was added to 50 μl of standards, QCs, blank or study samples to extract the metabolites. Each tube was mixed by a vortex, centrifuged for 10 min at 13,000 rpm and the supernatant fluid was transferred to 1.2 ml Corning polypropylene cluster tubes, dried under a stream of nitrogen in a Multi-Well Evaporation Systems (VWR International PBI, Milan, Italy) and then derivatized with DMG for 1 h at 45 °C [8]. After quenching the reaction with 20 μl of methanol, tubes were dried with nitrogen stream, and reconstituted with 200 μL of methanol–water (4:1). As reported in [8], the derivatization procedure produced the bis-(dimethylglycinate)-derivative of 3β,5α,6β-triol (3β,5α,6β-triol DMG_2) and the mono-dimethylglycinate-derivative of 7-KC (KC-DMG).

2.5. LC–MS/MS analysis

Samples analysis was performed by HPLC-MS using a Prominence UFLCXR system (Shimadzu Scientific Instruments, Columbia, MD, USA), and a 4000 Qtrap MASS SPECTROMETER (ABSciex, Framingham, MA,USA).

Mass spectrometry was performed in the positive ionization mode using an atmospheric pressure chemical ionization (APCI) source. The partially purified DMG derivatives prepared from 10 μg of 3β,5α,6β-triol, [$^2H_7$]-3β,5α,6β-triol, 7-KC, [$^2H_7$]-7-KC which were isolated from the organic phase from partition of the crude reaction mixture between diethyl ether and water, were dissolved in 1 ml of methanol–water (4:1). Mass spectrometric parameters were optimized by infusing these solutions into mobile phase flow via a T-union and manually adjusting mass spectrometric settings to achieve maximum response. The optimized heated nebulizer source and MS/MS conditions were as follows. The source temperature was 500 °C. Collision activated dissociation, ion source gas 1 and curtain gases were set at medium, 60 and 30, respectively. Needle current was set at 5.00. Optimized voltages and mass transitions are shown in (S1 Table).

The MRM mass transitions were m/z 591 → 104 (quantifier) and 591 → 488 (qualifier) for 3β,5α,6β-triol-DMG_2, and m/z 486 → 104 (quantifier) and 486 → 383 as qualifier for 7-KC-DMG (S1).

The column for chromatographic separation was a Betasil C_{18}, 100 mm × 2.1 mm, 4 μm particle size (Thermo Fisher Scientific, Waltham, MA USA). The mobile phase consisted of solvent A = 0.015% TCA, 0.5% acetic acid in water, and solvent B = 0.015% TCA, 0.5% acetic acid in acetonitrile; the flow rate was 1 ml/min. Chromatographic separation of metabolites was obtained with the same step gradient previously reported [8]. A 15 μL sample injection was used. The autosampler wash solvent was methanol.

Table 1
Precision data for the LC–MS/MS assay.

	Mean ± SD, ng/ml	CV, %
Intra-day (n = 12)		
3β,5α,6β-triol	6.62 ± 0.51	7.7%
7-KC	27.08 ± 1.35	5%
Inter-assay (n = 36)		
3β,5α,6β-triol	6.7 ± 0.081	1.2%
7-KC	28.26 ± 1.45	5%

Table 2
Accuracy data for 3β,5α,6β-triol and 7-KC.

	Mean ± SD, ng/ml	CV, %	Recovery, %
3β,5α,6β-triol added (n = 12)			
0 ng/ml	6.62 ± 0.51	7.7%	n.c.
150 ng/ml	162.71 ± 2.99	1.8%	104.1
300 ng/ml	304.48 ± 13.67	4.5%	99.3
7-KC added (n = 12)			
0 ng/ml	27.08 ± 1.35	5%	n.c.
150 ng/ml	171.18 ± 5.01	2.9%	96.1
300 ng/ml	327.74 ± 7.62	2.3%	100.0

2.6. Ion suppression experiments

A T-junction was placed between the HPLC and the MS/MS; 10 μg/ml of dimethylglycinates oxysterols, previously exctracted with diethyl ether, were infused continously at a flow rate of 10 μl/min into the post-column HPLC effluent (1 ml/min of mobile phase), creating a constant baseline. At the same time, a blank solvent solution (methanol), a blank (reference signal) and then a matrix, both after derivatization with DMG, were injected into the HPLC system and were monitored by MS/MS in the MRM mode. In this way, we controlled if a drop in the baseline was present.

2.7. Linearity, precision, and accuracy

The linearity of the response of each analyte was assessed over their respective calibration range for three analytical batch runs. The precision and accuracy of the assay was determined for each analyte at three QC concentration levels in human plasma over three batch runs. For each QC concentration, analysis was performed in six replicates on each day. Precision was denoted by a percent coefficient of variance (%CV). The accuracy was denoted by a percent relative error (%RE), calculated by subtracting the nominal level from the mean amount divided by the theoretical amount and then multiplied by 100 [((Mean - Nominal)/(Nominal)) × 100] as reported in [8].

2.8. Sample stability

We assessed the stability of oxysterols in whole blood at room temperature, after separating plasma from blood samples at different times: 0 h, 24 h,48 h and 72 h. Plasma was then stored frozen at −80 °C until analysis.

2.9. Statistics

Continuous variables were described with median and interquartile range. Due to small sample size, non-parametric tests were applied: sum rank test was used for comparisons between two independent groups while Kruskall Wallis test was performed when more than two groups were to be compared.

A ROC curve was plotted used the “roctab” command. All the analyses were performed using the statistical package Stata Version 11.0 for Windows (StataCorp LP, College Station, TX, USA).

3. Results and discussion

3.1. Selection of ions for multiple reaction monitoring experiments

The full scan analysis experiments showed the presence of 3β,5α,6β-triol-DMG_2 at $[M + H]^+$ 591 m/z, and of KC-DMG at $[M + H]^+$ 486 m/z. As previously published [8], the fragmentation of the $[M + H]^+$ ions of 3β,5α,6β-triol-DMG_2 and KC-DMG showed the predominant product ions at m/z 104 and 488 for [3β,5α,6β-triol-

Table 3
Clinical phenotype, genotype, plasma oxysterols and filipin test in NPC1 patients.

Patient	Clinical Phenotype	NPC1 Genotype*	3β,5α,6β-triol (ng/ml)	7-KC (ng/ml)	Biochemical phenotype
1§	Juvenile	[c.1351G > A (p.E451K)] + [c.2974G > T (p.G992W)]	13.4	45.58	Variant
2§	NC	[c.1351G > A (p.E451K)] + [c.2974G > T (p.G992W)]	24.86	78.16	Variant
3§	NC	[c.1351G > A (p.E451K)] + [c.2974G > T (p.G992W)]	8.13	31.42	Variant
4§	NC	[c.1351G > A (p.E451K)] + [c.2974G > T (p.G992W)]	20.45	72.2	Variant
5§	NC	[c.1351G > A (p.E451K)] + [c.2974G > T (p.G992W)]	20.81	63.54	Variant
6	Early infantile	[c.2800C > T (p.R934X)] + [**c.3235 T > C (p.F1079L**)]	77.02	369.05	Classical
7	Adult	[c.2130dupG (p.R711EfsX3)] + [c.2974G > C (p.G992R)]	60.0	137.95	Classical
8	Neonatal systemic lethal forms	[**c.1029dupG (p.S344VfsX36)**] + [**c.1029dupG (p.S344VfsX36)**]	483.52	949.91	ND
9	NC	[c.665 A > G (p.N222S)] + [**c.2689C > A (p.H897N)**]	47.65	147.51	Variant
10#	Juvenile	[c.2662C > T (p.P888S)] + [c.2662C > T (p.P888S)]	40.02	118.05	Classical
11#	Juvenile	[c.2662C > T (p.P888S)] + [c.2662C > T (p.P888S)]	48.82	150.94	ND
12	Juvenile	[c.3467 A > G (p.N1156S)] + [c.3467 A > G (p.N1156S)]	48.44	135.17	ND
13	Adult	[c.3019C > G (p.P1007A)] + [c.3019C > G (p.P1007A)]	54.84	192.22	ND
14	Juvenile	[c.1421 C > T (p.P474L)] + [c.1501G > T (p.D501Y)]	45.64	154.8	Classical
15	Juvenile	[c.2291C > T (p.A764V)] + [c.2819C > T (p.S940L)]	32.75	107.02	Classical
16	Early infantile	[c.2761C > T (p.Q921X)] + [c.2761C > T (p.Q921X)]	63.4	264.35	ND
17	NC	[c.2972_2973delAG (p.Q991RfsX15)] + [c.3182 T > C (p.I1061T)]	127.93	674.22	Classical

§,# Siblings; ND: Not Determined. According to Millat et al. (ref), patients phenotype classification was based on the age of appearance of first neurological signs: severe infantile (age of onset <2 years), late-infantile (age of onset 3–5 years), juvenile (age of onset 5–16 years), and adult phenotype (age of onset >16 years). Patients who died during the first months of life, due to liver insufficiency without signs of neurological involvement, were classified as *neonatal systemic lethal forms*. NC: non classificable since they did not present neurological symptoms at last follow up. Novel mutation are indicated in bold. *RefSeq cDNA: NM_000271.4. For cDNA numbering +1 corresponds to the A of the first ATG translation initiation codon. RefSeq protein: NP_000262.2.

$DMG_2 + H]^+$ and at m/z 104 and 383 for $[KC\text{-}DMG + H]^+$ (S1), deriving from the loss of DMG via McLafferty rearrangement [8].

Likewise, $[D_7\text{-}3\beta,5\alpha,6\beta\text{-triol-}DMG_2 + H]^+$ at $[M + H]^+$ 598 m/z gave the major product ions at m/z 104 and 495, and $[D_7\text{-KC-}DMG + H]^+$ at $[M + H]^+$ 493 m/z yielded m/z 104 and 390 ions (S1 Table). The MRM transitions we chose for $3\beta,5\alpha,6\beta\text{-triol-}DMG_2$ and KC-DMG were 591/104 m/z and 486/104 m/z, respectively. The second mass transitions were used for confirmation purposes.

3.2. Chromatography and selectivity of the method

Multiple Reaction Monitoring (MRM) was used for the detection of the specific transitions in the positive ion mode for monitoring of either the analyte or the internal standards. Typical chromatograms of human plasma are shown in S2A, which shows that 3β,5α,6β-triol and 7-KC were eluted with their respective labeled internal standard at the retention times of 4.72 min and 6.85 min, respectively. The chromatographic separation allowed to discriminate 3β,5α,6β-triol, eluting at 4.72 min, from an unknown compound present in plasma samples with the same 591/104 m/z transition and eluting at the retention time 5.05 min. Chromatograms of the second mass transitions 591 → 488 (3β,5α,6β-triol) and 486 → 383 (7-KC) were used for confirmation purposes (S2B).

3.3. Evaluation of of oxysterol extraction efficiency and matrix suppression effects

The recovery of the compounds was assessed using deuterated internal standards ($[^2H_7]3\beta,5\alpha,6\beta$-triol and $[^2H_7]$7-KC) as reported [8], by comparison of the mean peak areas of internal standard added to the control plasma before protein precipitation (extraction) (C) with those of internal standard spiked into the plasma after the extraction (B). The recoveries (C/B) of $[^2H_7]3\beta,5\alpha,6\beta$-triol and $[^2H_7]$7-KC were 93.22% (10.48% CV, n = 3) and 89.75% (2.73% CV, n = 3), respectively. To evaluate the matrix ion suppression effects (ME %), we assessed the suppression coefficients for $[^2H_7]3\beta,5\alpha,6\beta$-triol and $[^2H_7]$7-KC by the ratio of the average peak area for the deuterated standards spiked in the plasma after protein precipitation (B) to the average peak area of the deuterated standards in methanol–water (1:1) samples (A). The matrix effects (B/A = ME%) of $[^2H_7]3\beta,5\alpha,6\beta$-triol and $[^2H_7]$-KC were 90.39% (10.8% CV, n = 3) and 87.9% (12.85 CV, n = 3), respectively, indicating similar matrix effects in plasma and non-biological matrix, as previously reported [8].

3.4. Calibration curves

The calibration curves were performed using eight standards of different concentrations, each in duplicate, ranging from 2 to 400 ng/ml of 3β,5α,6β-triol and 7-KC in methanol–water (1:1), in addition to human plasma as previously described [8]. The representative slope, intercept, and coefficient of linear regression (r^2) were respectively: 0.0027, −0.0246, and 0.9969 for 3β,5α,6β-triol and 0.0027, 0.0301, and 0.9997 for 7-KC in methanol–water; 0.0027, 0.0301, and 0.9997 for 3β,5α,6β-triol and 0.0027, 0.1071, and 0.9998 for 7-KC in plasma. The slopes of calibration curves were nearly identical in methanol–water and plasma, indicating that methanol–water (1:1) was a good surrogate matrix.

3.5. Sensitivity, precision and recovery

The sensitivity of the assay, as defined by the LLOQ, was determined for both 3β,5α,6β-triol and 7-KC using methanol–water (1:1) samples at 2 ng/ml. At the LLOQ, the intra-run precision (CV) was <10% for 3β,5α,6β-triol and 7-KC, and the intra-run accuracies (RE, relative error) were within ±15% for 3β,5α,6β-triol and 7-KC (S3). A typical chromatogram at the LLOQ concentration is shown in S2C.

The precision was assessed by analyzing QC samples on different days. The intra-assay variation was assessed from 12 replicates of pooled plasma control within one day (n = 12) and inter-assay from 12 replicates on three different days (n = 36). Intra-day and inter-day coefficients of variation (CV) were 7.7% and 1.2% for 3β,5α,6β-triol and 5%, and 5% for 7-KC, respectively (Table 1).

Recovery experiments were performed on spiked QCs at two different concentrations of both 3β,5α,6β-triol and 7-KC spiked into pooled plasma (150 and 300 ng/ml). The 3β,5α,6β-triol recovery was 104.1% and 99.3%, respectively, of the expected amount; 7-KC recovery was 96.1 and 100%, respectively (Table 2). These data demonstrated that the LC–MS/MS method successfully resolved the oxysterols derivatives with high sensitivity and enabled accurate quantification of 3β,5α,6β-triol and 7-KC levels in human plasma.

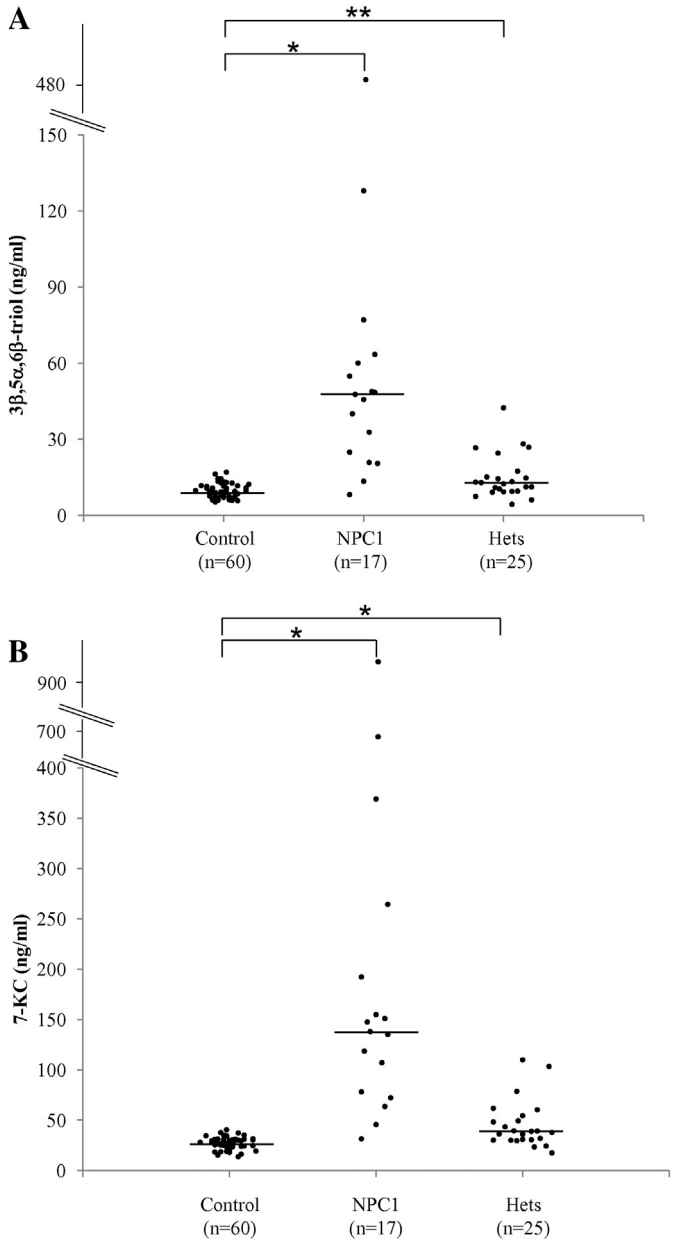

Fig. 1. Plasma 3β,5α,6β-triol and 7-KC concentrations in human subjects. (A) 3β,5α,6β-triol and (B) 7-KC concentrations in control, NPC1, and NPC1 heterozygote (Hets). *: p = 0.0000; **: p = 0.0003. Line: median.

3.6. Sample stability

The stability of 3β,5α,6β-triol and 7-KC in methanol stock solution and in plasma under a variety of conditions has already been reported in literature [8–10]. Since we frequently receive whole blood samples from other Centers, we assessed the stability of oxysterols in whole blood samples at room temperature. The bench-top stability study showed that the 3β,5α,6β-triol and 7-KC were stable in human whole blood at room temperature for 48 h (S4). Therefore, whole blood samples can be shipped at room temperature but must reach the laboratory by maximum 48 h from sampling.

3.7. Measurement of 3β,5α,6β-triol and 7-KC in NPC1 patients and healthy controls

The LC–MS/MS method described above was used to analyze 3β,5α,6β-triol and 7-KC concentrations in plasma samples from 60 healthy subjects from 0 to 67 years old (mean age 34 years), 17 subjects affected by NPCD due to *NPC1* gene mutations (0–38 years; mean age 18 years) (Table 3) and 27 NPC1 heterozygotes (parents and siblings of NPC1 subjects) (3–69 years, mean age 43 years). In line with previous reports [8–12,17,18], plasma levels of 3β,5α,6β-triol and 7-KC were significantly elevated in the NPC1 subjects in comparison with normal controls (Fig. 1, Table 4). Both oxysterols were also significantly elevated in NPC1 heterozygotes (Hets), as compared with healthy subjects (Fig. 1, Table 4).

In order to measure the performance of the method in discriminating NPC patients from normal subjects, a receiver operating characteristic (ROC) analysis was performed. The area under curve (AUC) was 0.9534 for 3β,5α,6β-triol, and 0.9892 for 7-KC, (Fig. 2). An optimal upper limit of 20.45 ng/ml for the 3β,5α,6β-triol, resulted in 97.1% of cases to be correctly classified, and yielded a specificity of 100% and a sensitivity of 87.5%. For 7-KC, a cut-off value of 45.58 ng/ml, resulted in 98.53% of cases to be correctly classified and yielded a specificity of 100% and sensitivity of 93.75%. Although these results indicate a good overall performance of both oxysterols to discriminate NPC patients from healthy controls, a quite low sensitivity was obtained for the 3β,5α,6β-triol assay, meaning that 2 out of 17 NPC1 patients displayed 3β,5α,6β-triol levels below the cut-off value. The use of a lower cut off was evaluated. However, in order to significantly improve the sensitivity, the cut-off should have been set at 13.4 ng/ml (sensitivity 93.75%), which would result in a significant decrease of specificity (90.57%), with the consequent misclassification of 7 normal subjects as affected.

It is worth noting that the 2 patients presenting 3β,5α,6β-triol levels below the cut-off value of 20.45 ng/ml (patients 1 and 2, Table 3) are siblings and displayed a variant biochemical phenotype when the intracellular accumulation of unesterified cholesterol was evaluated by filipin staining. In these 2 patients also the levels of 7-KC were quite low. Furthermore, among the analyzed NPC population, other 3 patients belonging to the same family presented the same pattern of cholesterol storage (patients 3–5, Table 3) and showed levels of 3β,5α,6β-triol equal or just above the cut-off value, way below the levels found in patients presenting the classical phenotype. Conversely, in these 3 siblings the levels of 7-KC were clearly above the cut off value. These data suggest that some patients who accumulate moderate amounts of cholesterol may present lower levels of plasma oxysterols, in particular 3β,5α,6β-triol.

In general, patients presenting a variant biochemical phenotype, represent approximately 10–15% of total cases of NPC [5,6], while in our cohort they account for 35% of cases. Therefore, the lower sensitivity

Table 4
Median, interquartile and reference ranges in the different groups analyzed.

	3β,5α,6β -triol (ng/ml)			7-KC (ng/ml)		
	median	IQR	range	median	IQR	range
Ctrl	9.03	7.38–11.34	5.28–16.98	27.08	24.31–30.66	13.76–40.32
NPC1	48.44*	24.86–60	8.13–483.52	137.95*	78.16–192.22	31.41–949.91
NPC1 Hets	12.4**	9.5–15.08	4.32–42.35	37.8*	30.44–49.31	17.41–109.92
NPB	35.21*	26.12–60.39	23.15–71.7	120.22*	78.69–165.2	75.67–195.21

*: p = 0.0000 vs. Ctrl; **: p = 0.0003 vs. Ctrl.

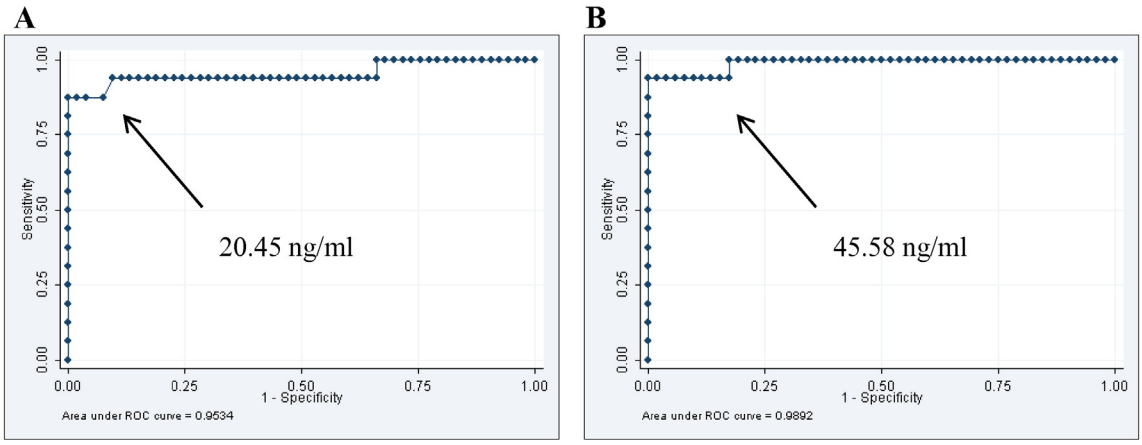

Fig. 2. Sensitivity and specificity of the method. (A) 3β,5α,6β-triol and (B) 7-KC assay. ROC curve demonstrates 0.9534 and 0.9892 area under the curve for 3β,5α,6β-triol and 7-KC, respectively. For 3β,5α,6β-triol, using a cut-off value of 20.45 ng/ml specificity is 100% and sensitivity 87.5%. For 7-KC, using a cut-off value of 45.58, specificity is 100% and sensitivity 93.75%.

of the described assay in comparison with the sensitivity described when the same method was applied to other populations (87.5% vs. 97.3% from [8]) is likely to be the consequence of the relative high number of patients presenting a variant biochemical phenotype in the present series. Therefore, we decided to establish the value of 20.45 ng/ml as the optimal upper limit for the 3β,5α,6β-triol, even knowing that these cut-off may lead to the misclassification of some patients with the variant biochemical phenotype.

In spite of the results obtained in patients 1–5, it is worth noting that the analysis of 3β,5α,6β-triol was still useful to complete the diagnostic picture of patient 9 (non related with patients 1–5, Table 3). This patient is a 36 year old woman who presented with splenomegaly, mild thrombocytopenia and no signs of neurological involvement. After excluding Gaucher and Niemann-Pick B disease, a diagnostic work up for NPCD was undertaken. The analysis of intracellular accumulation of cholesterol by filipin staining showed a variant biochemical phenotype. Two mutations were identified in the *NPC1* gene, one of which was novel and its pathogenetic nature was unknown. However, this variant was not found among 100 control alleles. Taken together, these findings were highly suggestive of NPCD and indeed, the plasma levels of oxysterols resulted way above the cut off value (Table 3).

Plasma levels of both oxysterols were extremely high in patient 8 (Table 3), who presented a severe neonatal cholestatic form of the disease, leading to death during the first months of life due to liver failure, without signs of neurological involvement. Recently, similar high levels of 3β,5α,6β-triol have been reported in an NPC2 patient [19], who also showed a fatal perinatal phenotype. Although the previously reported patient died at 4 months of age due to respiratory insufficiency, he presented with prolonged neonatal cholestatic jaundice, severe liver dysfunction and hepatosplenomegaly. Taken together, these data suggest that plasma oxysterols may be particularly elevated in patients presenting the neonatal systemic lethal phenotype.

Using the parameters described above, 20% and 32% of the Hets displayed plasma 3β,5α,6β-triol and 7-KC concentrations above the cut-off, respectively (Fig. 1, Table 4).

3.8. Measurement of 7-KC and 3β,5α,6β-triol in NPB patients

Increased levels of 7-KC and 3β,5α,6β-triol have been recently reported in patients affected by ASM deficiency (NPA and B disease) [10]. To further confirm these data, we analyzed both 3β,5α,6β-triol and 7-KC in a group of 8 patients affected by NPB disease previously diagnosed by enzymatic and molecular analysis (Table 5). As shown in Fig. 3 and Table 4, both metabolites were significantly elevated in NPB patients as compared with healthy subjects. Instead, plasma levels of both oxysterols were not significantly different between NPB and NPC patients (Table 4).

In light of these data, elevated levels of plasma oxysterols should be interpreted with caution, in particular when this assay is used in patients affected by isolated hepatosplenomegaly, a common presentation of both NPC and NPB patients. In these patients, the assessment of ASM activity is mandatory to exclude NPB disease.

Besides these considerations, this finding may open new opportunities for monitoring the efficacy of therapeutic interventions in NPB patients. Enzyme replacement therapy (ERT) with human recombinant ASM has been developed for the treatment of NPB and a phase 1 clinical trial has been recently completed. The availability of ERT increases the need for biochemical markers of treatment efficacy and oxysterols may represent a good candidate.

4. Conclusions

Several conclusions can be drawn from the present study:

In agreement with previous data, the measurement of plasma oxysterols, 3β,5α,6β-triol and 7-KC, represents a rapid and non invasive

Table 5
Genotype and plasma oxysterols in NPB patients.

Patient	*SMPD1* Genotype*	3β,5α,6β -triol (ng/ml)	7-KC (ng/ml)
1	[c.96G > A (p.W32X)] + [c.96G > A (p.W32X)]	59.61	160.25
2	[c.2 T > G (p.M1_W32del)] + [c.2 T > G (p.M1_W32del)]	23.15	75.67
3	[c.1493G > T (p.R498L)] + [c.1829_1831delGCC (p.R610del)]	35.56	146.13
4	[c.96G > A (p.W32X)] + [c.96G > A (p.W32X)]	62.74	180.03
5	[c.1675_1676delGT (p.V559IfsX19)] + [c.1829_1831delGCC (p.R610del)]	71.7	195.21
6	[c.573delT (p.S192Afs*65)] + [c.1828C > T (p.R610C)]	24.18	79.42
7	[c.581dupC (p.A195Sfs*14)] + [c.1805G > T (p.R602P)]	26.77	76.48
8	[c.1829_1831delGCC (p.R610del)] + [c.1829_1831delGCC (p.R610del)]	34.85	94.3

* RefSeq cDNA: NM_000543.4. For cDNA numbering +1 corresponds to the A of the first ATG translation initiation codon. RefSeq protein: NP_000534.3.

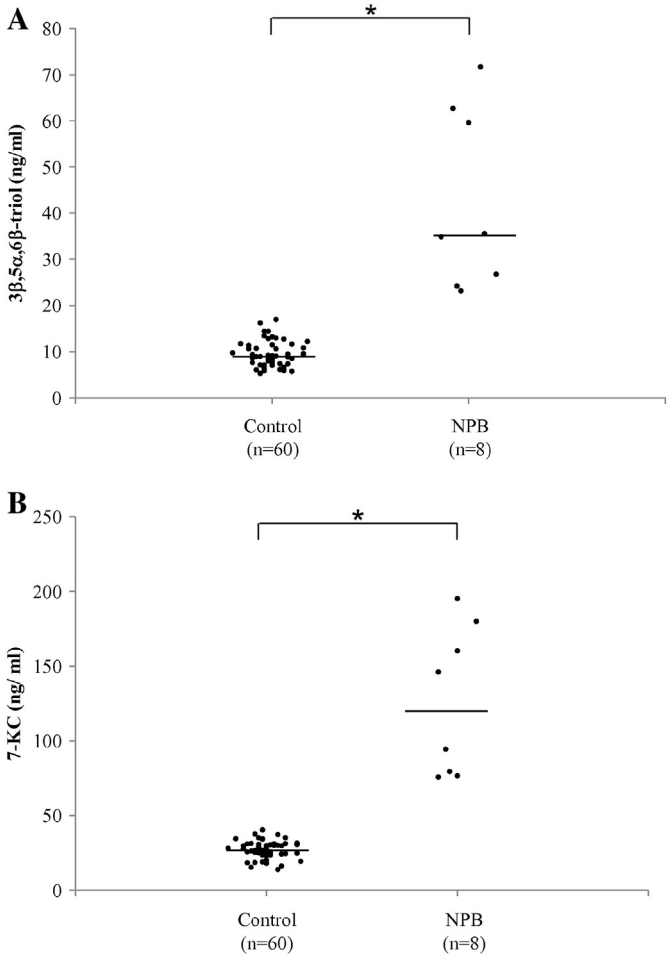

Fig. 3. Plasma 3β,5α,6β-triol and 7-KC concentrations in human subjects. (A) 3β,5α,6β-triol and (B) 7-KC concentrations in control and NPB subjects. *: p = 0.0000. Line: median.

method for the screening of NPCD [8–12]. However, positive results must be always confirmed by molecular analysis of *NPC1* and *NPC2* genes and when novel mutations are identified, by filipin staining. False negative results can be obtained in some patients affected by NPCD who accumulate moderate amounts of cholesterol resulting in a variant biochemical phenotype. These patients may display levels of oxysterols in the range of heterozygous or even healthy controls. Therefore, the results should always be interpreted in the context of the clinical picture. As it was suggested for patients testing negative for filipin staining, genetic studies may still be undertaken in patients with negative oxysterols if the symptoms are highly suggestive of NPCD.

We confirmed data from Klinke et al. [10], showing that both 7-KC and 3β,5α,6β-triol are also elevated in patients affected by NPB. Therefore, a differential diagnosis should be performed, in particular in patients presenting with isolated hepatosplenomegaly. This finding suggests that oxysterols levels may be a useful biomarker for NPB disease and their possible use in evaluating the therapeutic efficiency of the forthcoming ERT should be considered.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2016.01.003.

Competing interests

MR, SZ, NB, LD, AS, RP, BBo, FB, ABr, CVR, ABo declare that they have no competing interests. This work was partially supported by Actelion Pharmaceuticals Ltd. The funding source had no involvement in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication. AD has received travel reimbursements and speaker honoraria from Actelion Pharmaceuticals Ltd. AF has received travel reimbursements from Actelion Pharmaceuticals Ltd. BBe has received travel reimbursements and speaker honoraria from Actelion Pharmaceuticals Ltd. and took part to advisory boards organized by the same company.

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