Work Package 5

Title: Biochemical and molecular diagnosis

Description: Development of widespread availability of rapid and sensitive biochemical diagnostic testing across the EU by using oxidized cholesterol assay for Niemann-Pick Disease type C (NPC); investigation of genotype phenotype relationships for all the three Niemann-Pick (NP) diseases.

Deliverable #9: Development of an EU diagnostic testing

Milestone title: Negotiation with NIH laboratory and Actelion in setting up a rapid and sensitive diagnostic test for NPC disease in most partner countries.

Introduction

A major barrier to developing new therapies for Niemann-Pick Disease Type C (NPC) disease has been the lack of widespread non-invasive and reliable diagnostic tests. The current diagnostic standard for NPC is an invasive skin biopsy and filipin staining, which is only available at a handful centers in Europe. The test is cumbersome and at times not sensitive and specific to NPC. In addition, due to limited expert centers in Europe getting result will take up to 6 months, which causes a very anxious time for the patient and families and valuable time in preventing complications to the patient is wasted while waiting for the test result. Together the complex and time-consuming processing of the biopsy and lack of sensitivity of the filipin test have contributed to significant diagnostic delays of 4-6 years to make a diagnosis of NPC disease. There is an urgent need for the development of rapid and sensitive biochemical diagnostic testing, and the test should be widely available across the EU laboratories.

Cholesterol oxidation products, specifically cholestane-$3\beta,5\alpha,6\beta$-triol ($3\beta,5\alpha,6\beta$-triol) and 7-ketocholesterol (7-KC) have been proposed as specific markers for NPC. Indeed, these oxysterols are markedly increased in the plasma of human NPC1 subjects. Two different methods have been developed by two different groups to assay $3\beta,5\alpha,6\beta$-triol and 7KC in plasma: a LC-MS/MS method published in 2011 by the group of Dr. Ory at the University of Washington (WUSTL) and a GC-MS assay developed by Dr. T. Marquat's group at the University of Munster. Both methods are able to discriminate with high sensitivity and specificity between controls and NPC1 subjects, offering a noninvasive, rapid, and highly sensitive method for the diagnosis of NPC1 disease.

I. Plasma oxysterols measurement using LC-MS/MS: method development and validation

Methods

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-□2H7□-7-ketocholesterol (□2H7□-7-KC) and 25,26,26,26,27,27,27-□2H7□-cholestan-3β,5α,6β-triol (□2H7□-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-d4), 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. Samples

Plasma samples were obtained from patients affected by NPC diagnosed 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. NPC diagnosis was confirmed by the demonstration of cholesterol accumulation in cultured skin fibroblasts through filipin staining and/or by the molecular analysis of the NPC1 and NPC2 genes.

Whenever possible, the genotype was confirmed by the analysis of the identified mutations in the patient's 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.

Plasma samples used as normal controls were obtained from healthy donors at the University Hospital Santa Maria della Misericordia, Udine. 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-K2) containing tubes.

3. Standard curves for quantification and quality control samples (QCs)

Stock solutions (1 mg/ml) were prepared in methanol (or methanol-d4 for the labeled internal standards) and stored at -80 °C. An internal standard/protein precipitation solution (40 ng/ml of □2H7□-7-KC and 40 ng/ml of □2H7□-3□,5□,6□-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.

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. [1]. Briefly, internal standard/protein precipitation solution (250 □l) was added to 50 □l 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 [1]. After quenching the reaction with 20 □l of methanol, tubes were dried with nitrogen stream, and reconstituted with 200 □L of methanol-water (4:1). As reported in [1], the derivatization procedure produced the bis-(dimethylglycinate)-derivative of 3β,5α,6β-triol (3β,5α,6β-triol DMG2) and the mono-dimethylglycinate-derivative of 7-KC (KC-DMG).

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 □g of 3β,5α,6β-triol, [2H7]-3β,5α,6β-triol, 7-KC, □2H7□-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 (Table 1).

The MRM mass transitions were m/z 591→104 (quantifier) and 591→488 (qualifier) for 3□,5□,6□-triol-DMG2, and m/z 486→104 (quantifier) and 486→383 as qualifier for 7-KC-DMG (Figure 1A).

The column for chromatographic separation was a Betasil C18, 100 mm × 2.1 mm, 4 □m 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 [1]. A 15 □L sample injection was used. The autosampler wash solvent was methanol.

6. Ion suppression experiments

A T-junction was placed between the HPLC and the MS/MS; 10 □g/ml of dimethylglycinates oxysterols, previously extracted with diethyl ether, was infused continuosly at a flow rate of 10 □l/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, was injected into the HPLC system and was monitored by MS/MS in the MRM mode. In this way, we controlled if a drop in the baseline was present.

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 □1□.

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.

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 indepedent 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).

Results

Selection of ions for Multiple Reaction Monitoring experiments

The full scan analysis experiments showed the presence of 3β,5α,6β-triol -DMG2 at □M+H□+ 591 m/z, and of KC-DMG at □M+H□+ 486 m/z. As previously published [1], the fragmentation of the □M+H□+ ions of 3β,5α,6β-triol-DMG2 and KC-DMG showed the predominant product ions at m/z 104 and 488 for □3□,5□,6□-triol-DMG2+H□+ and at m/z 104 and 383 for □KC-DMG+H□+ (Figure 1B).

Likewise, □D7-3□,5□,6□-triol-DMG2+H□+ at □M+H□+ 598 m/z gave the major product ions at m/z 104 and 495, and □D7-KC-DMG+H□+ at □M+H□+ 493 m/z yielded m/z 104 and 390 ions (Table 1). The MRM transitions we chose for 3β,5α,6β-triol-DMG2 and KC-DMG were 591/104 m/z and 486/104 m/z, respectively.

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 Figure 2A, 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. Chromatograms of the second mass transitions 591→488 (3β,5α,6β-triol) and 486→383 (7-KC) were used for confirmation purposes (Figure 2B).

Evaluation of of oxysterol extraction efficiency and matrix suppression effects

The recovery of the compounds was assessed using deuterated internal standards (□2H7□3□,5□,6□-triol and □2H7□7-KC) as reported [1], 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 □2H7□3□,5□,6□-triol and □2H7□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 □2H7□3□,5□,6□-triol and □2H7□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 □2H7□3□,5□,6□-triol and □2H7□-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 [1].

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) or in methanol-water (1:1), in addition to human plasma as previously described [1]. The representative slope, intercept, and coefficient of linear regression (r2) 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.

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 (Figure 3). A typical chromatogram at the LLOQ concentration is shown in Figure 2C.

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 C-triol and 5%, and 5% for 7-KC, respectively (Table 2).

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 3). 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.

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 [1-3]. 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 (Figure 4). Therefore, whole blood samples can be shipped at room temperature but must reach the laboratory by maximum 48 h from sampling.

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 NPC due to NPC1 gene mutations (0-38 years; mean age 18 years) and 27 NPC1 heterozygotes (parents and siblings of NPC1 subjects) (3-69 years, mean age 43 years).

In line with previous reports [1-5], plasmatic levels of 3β,5α,6β-triol (control median 9.03 ng/ml, IQR: 7.38 to 11.34, range 5.28-16.98 ng/ml; NPC1 median 48.44 ng/ml, IQR: 24.86 to 60, range 8.13-483.52 ng/ml; p= 0.0000) and 7-KC (control median 27.08 ng/ml, IQR: 24.31 to 30.66, range 13.76-40.32 ng/ml; NPC1 median 137.95 ng/ml, IQR: 78.16 to 192.22, range 31.41-949.91 ng/ml; p= 0.0000) were significantly elevated in the NPC1 subjects with respect to normal controls (Figure 5). Both oxysterols were also significantly elevated in NPC1 heterozygotes (Hets) (3β,5α,6β-triol median 12.4 ng/ml, IQR: 9.5 to 15.08, range 4.32-42.35; p= 0.0003; 7-KC median 37.8 ng/ml, IQR: 30.44 to 49.31, range 17.41-109.92 ng/ml; p= 0.0000), as compared with healthy subjects (Figure 5).

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, (Figure 6). 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 are siblings and displayed a variant biochemical phenotype when the intracellular accumulation of unesterified cholesterol was evaluated by filipin staining. In this 2 patients also the levels of 7-KC were quite low. These data suggest that patients who accumulate moderate amounts of cholesterol may present lower levels of plasmatic oxysterols, in particular 3β,5α,6β-triol.

In general, patients presenting a variant biochemical phenotype, represent approximately 10-15 % of total cases of NPC, while in our cohort they account for 35% of cases (6 out of 17). Therefore, the lower sensitivity of the described assay with respect to the sensitivity described when the same method was applied to other populations (87.5% vs. 97.3% from [1]) is likely to be the consequence of the relative high number of patients presenting a variant biochemical phenotype in the present series.

Conclusions

The reported LC-MS/MS assay provides a robust non-invasive screening tool for NPCD. However, false negative results can be obtained in some patients expressing the variant biochemical phenotype. These data strengthen the concept that the results should always be interpreted in the context of the patient 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 NPC.

Table 1. Compound parameters optimized for each MRM transition

DMG-derivative	Mass transition Q1/Q3	DP	CE	EP	CXP
3□,5□,6□-triol	591.3→104	110	50	10	6
	591.3→488.3	115	27	10	20
7-KC	486.5→104	80	35	10	15
	486.5→383.2	80	27	10	12
□2H7□-3□,5□,6□-triol	598.3→104	95	50	10	17
	598.3→495.3	95	30	10	28
□2H7□-7-KC	493.5→104	85	37	6	17
	493.5→390.2	85	27	6	28

Table 2. Precision data for the LC-MS/MS assay

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

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

	Mean ± SD, ng/ml	CV, %	Recovery, %
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	1.2%	n.c.
150 ng/ml	171.18 ± 5.01	2.9%	96.1
300 ng/ml	327.74 ± 7.62	2.3%	100.0

Figure legends
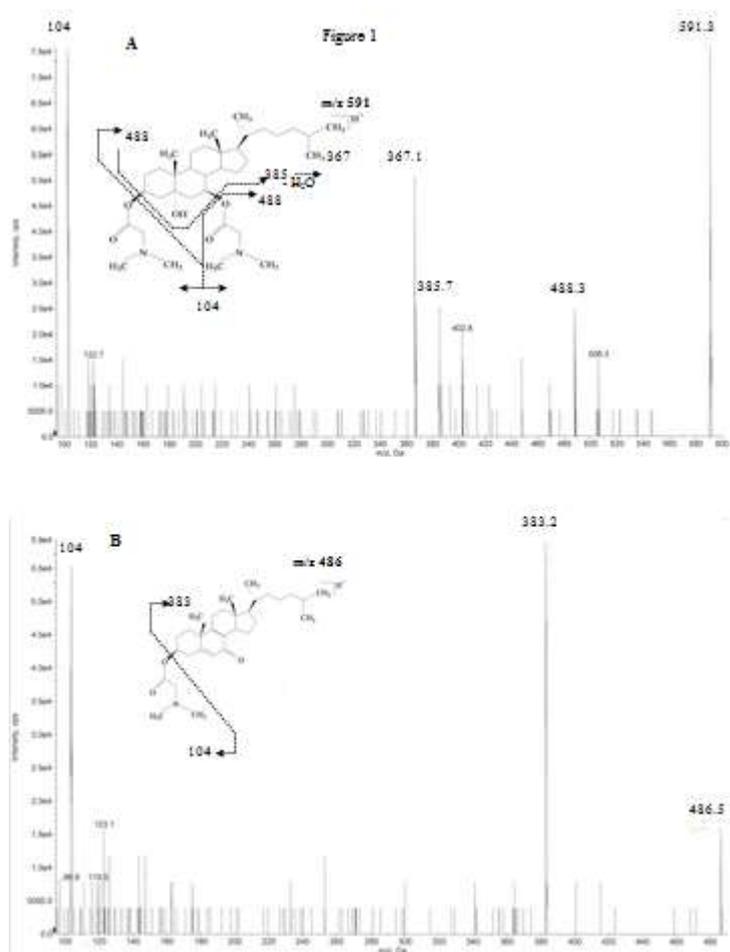
Figure 1. Product ion spectra and collision-activated dissociation of dimethylglycine derivatives of 3β,5α,6β-triol and 7-KC. Spectra for the bis-(dimethylglycinate)-derivative of 3β,5α,6β-triol

(3β,5α,6β-triol -DMG2) (A) and the mono-dimethylglycinate-derivative of 7-KC (KC-DMG) (B) are presented. Structure of each analyte with their fragmentation patterns are shown above spectra.
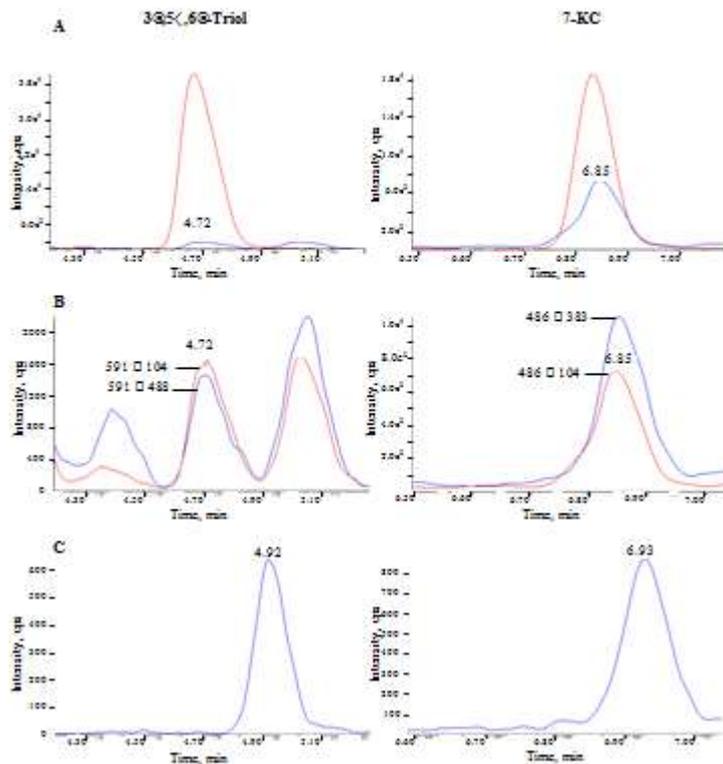
Figure 2

Figure 2. Chromatograms of oxysterols dimethylglycinates. Extract ion chromatograms of dimethylglycinate derivatives of 3β,5α,6β-triol (retention time 4.72 min) and 7-KC (retention time 6.85) in control plasma (A) with analytes in blue and internal standard in red; in control plasma in 2 MRM transitions (B); and in LLOQ prepared in methanol-water (1:1) (C).
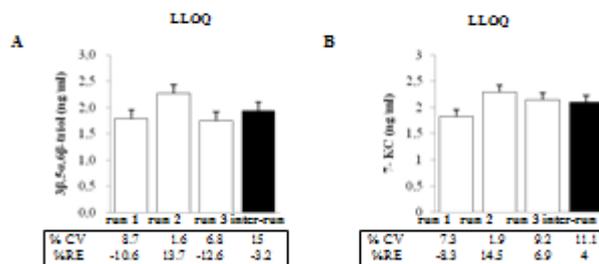
Figure 3

Figure 3. Intra- and inter-assay accuracy and precision of measurement of plasma oxysterols. Assay accuracy and precision was determined for 3β,5α,6β-triol (A) and 7-KC (B) measurements at LLOQ. %CV and %RE were based on three independent runs (six replicates/run) and are shown below each graph.
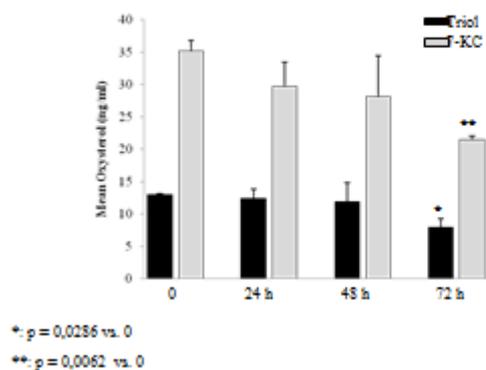
Figure 4

*: p = 0,0286 vs. 0
**: p = 0,0062 vs. 0

Figure 4. Stability of oxysterols. Stability of 3β,5α,6β-triol and 7-KC in whole blood at room temperature. Each data point represents n=3 replicates. *: p= 0.0286 vs. 0; **: p= 0.0062 vs. 0.

Figure 5

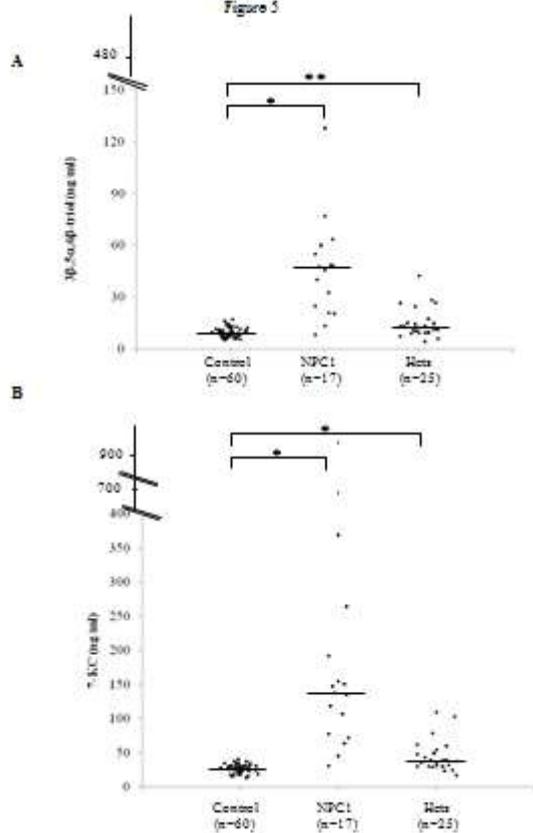Figure 5. Plasma 3β,5α,6β-triol and 7-KC concentrations in human subjects. (A) 3β,5α,6β-triol and (B) 7-KC concentrations in control, NPC1, and NPC1 heterozygote (Hets). *: p=0.0000; **: p= 0.0003. Line: median.

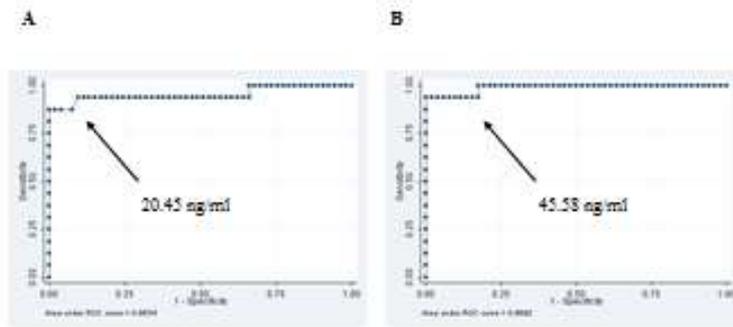Figure 6. Sensitivity and specificity of the method. (A) 3β,5α,6β-triol and (B) 7-KC assay. ROC curve demonstrates 0.9534 and 09892 area under the curve for 3β,5α,6β-triol and 7-KC, respectively. For 3β,5α,6β-triol, using a cut-off value of 20.45 ng/ml specificity is 100 % and sensitivity 87.5%. For 7-KC, using a cut-off value of 45.58, specificity is 100% and sensitivity 93.75%.

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II. Implementation of oxysterols assay among EU laboratories

To facilitate the implementation of oxysterols assay among EU laboratories, in March 2014 a "Workshop on the current state of oxysterols analytics and further lab parameters for NP-C" was organized in Munster, Germany to discuss the development of oxysterols measurements for the diagnosis of NPC disease.

Forty two researcher from 13 different countries participated in the workshop.

Representatives from Austria, Germany, Italy, Switzerland and United Kingdom have presented the results of the implementation and use of oxysterols assay in their laboratories.

During the **first session** of the workshop the results of the **validation of oxysterols assay in different laboratories across EU** were presented**.**

Dr. Polo (Padova , Italy), Dr. Boenzi (Rome, Italy), Dr. Wu (Manchester, UK) and Dr. Klinke (Zurich, CH) presented the results of the validation of the LC-MS/MS method.

Dr. Kannenberg (Munster, Germany) and Dr. Fauler (Graz, Austria) presented the results of the GC-MS method validation.

The **following conclusions** can be drawn from these presentations:

- Both methods (LC-MS/MS and GC-MS) were able to discriminate between healthy controls and NPC1 patients.

- The cholestane-3β,5α,6β-triol is stable at room temperature for 2-3 days, the measurement should be done within a week. Storage at -20°C is acceptable for short term storage but for long term storage -80°C is recommended.

- Cholestane-3β,5α,6β-triol was preferred as a biomarker and 7 KC can be used to control correct storage of the sample.

- Cholestane-3β,5α,6β-triol is not increased in other neurological inherited diseases such as Smith-Lemli-Opitz-syndrome, sitosterolemia and Chorea di Huntington. However, a patient with cerebrotendinous xanthomatosis and patients affected by NPA and NPB showed elevated levels of this oxysterol.

- Some concerns have been raised regarding the LC-MS/MS method since the derivatization of the sample is not always reproducible.

During the **second session** of the workshop the results of the **implementation and use of the oxysterols assay in clinical settings were discussed.**

Dr Dardis and Dr. Reunert presented the experience at the Regional Coordinator Centre for Rare Diseases (Udine, Italy) and at the University of Munster (Munster, Germany), respectively.

Both presentations showed:

- The utility of the Cholestane-3β,5α,6β-triol as a biomarker not only for NPC1 but also for NPC2 patients in clinical settings.

- However, false negative results could be obtained in particular patients presenting a "variant biochemical phenotype."

- The need for performing confirmation tests (molecular analysis) has been stressed.

Data obtained at the University of Munster on 890 samples showed:

- Low rates of false positives and false negatives

- A combination of oxysterols and chitotriosidase activity is more specific for detection of NPC

- Problems may result from: wrong shipping or storage conditions, problems at customs, missing feedback on false negative samples; wrong sampling (serum instead of plasma, no EDTA blood for genetic analysis).

Future prospective:

- Nine laboratories expressed interest in participating in trials to harmonize the measurement. Normal non-frozen serum patients' samples should prefer for survey.
- Membership to the NPC-group within ENOR (European Network for Oxysterol Research: http://oxysterols.com/) was encouraged.
- Further experiments are necessary to analyze inter- and intra-individual variation, circadian rhythm, analyte stability, sample shipment and optimal storage conditions.
- On-site training in Münster/GC-MS and Padova/HPLC-MS/MS will be provided for participants.

A summary of the current status of oxysterols assay development in Europe is provided in the table below

European centers currently performing oxysterols assay in clinical settings:

Country	Center	Method
France	Hospices Civils de Lyon	LC-MS/MS
Germany	University of Munster	GC-MS
Italy	University Hospital Santa Maria della Misericordia , Udine	LC-MS/MS

	University Hospital of Padova	LC-MS/MS
	Pediatric Hospital "Bambino Gesù", Roma	LC-ESI-MS/MS
Spain	Institut de Bioquímica Clínica. Servei de Bioquímica I Genètica Molecular	LC-MS/MS
Switzerland	University Childern's Hospital, Zurich	LC-MS/MS
UK	St. Mary's Hospital	LC-MS/MS

European centers currently developing oxysterols assay:

Country	**Center**	**Method**
Austria	University of Graz	GC-MS
UK	University Hospital Birmingham	LC-MS/MS
UK	University Hospital London	LC-MS/MS

Summary

We have achieved a development of widespread availability of rapid and sensitive biochemical diagnostic testing across at least six EU countries by using oxidized cholesterol assay for Niemann-Pick Disease type C.