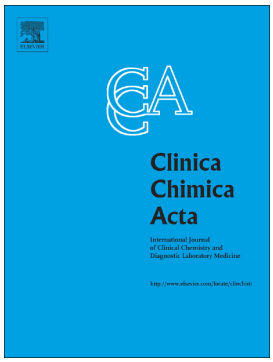


Diagnostic performance evaluation of sulfate-conjugated cholesterol metabolites as urinary biomarkers of Niemann–Pick disease type C

Masamitsu Maekawa, Aya Narita, Isamu Jinnoh, Takashi Iida, Thorsten Marquardt, Eugen Mengel, Yoshikatsu Eto, Peter T. Clayton, Hiroaki Yamaguchi, Nariyasu Mano

PII: S0009-8981(19)31715-2
DOI: https://doi.org/10.1016/j.cca.2019.03.1610
Reference: CCA 15636

To appear in: *Clinica Chimica Acta*

Received date: 2 February 2019
Revised date: 6 March 2019
Accepted date: 10 March 2019

Please cite this article as: M. Maekawa, A. Narita, I. Jinnoh, et al., Diagnostic performance evaluation of sulfate-conjugated cholesterol metabolites as urinary biomarkers of Niemann–Pick disease type C, Clinica Chimica Acta, https://doi.org/10.1016/j.cca.2019.03.1610

Diagnostic performance evaluation of sulfate-conjugated cholesterol metabolites as urinary biomarkers of Niemann–Pick disease type C

Masamitsu Maekawa [1,*], Aya Narita [2], Isamu Jinnoh [3], Takashi Iida [4], Thorsten Marquardt [5], Eugen Mengel [6], Yoshikatsu Eto [7], Peter T Clayton [8], Hiroaki Yamaguchi [1,3], and Nariyasu Mano [1,3]

[1] *Department of Pharmaceutical Sciences, Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan*

[2] *Division of Child Neurology, Tottori University Hospital, 86 Nishi-machi, Yonago, Tottori 683-8503, Japan*

[3] *Faculty of Pharmaceutical Sciences, Tohoku University, 1-1 Seiryo-machi, Aoba-Ku, Sendai 980-8574, Japan*

[4] *College of Humanities and Sciences, Nihon University, 3-25-40 Sakurajousui, Setagaya-ku, Tokyo 156-8550, Japan*

[5] *Department of Pediatrics, University Hospital of Munster, Albert-Schweitzer-Campus 1 Gebaeude A13, 48149 Muenster, Germany*

[6] *Department of Pediatric and Adolescent Medicine, University Medical Center Mainz, Langenbeckstr. 1, 55131 Mainz, Germany*

[7] *Advanced Clinical Research Center, Institute for Neurological Disorders, Furusawa-Miyako 255, Asou-ku, Kawasaki, Kanagawa 215-0026, Japan.*

[8] *Biochemistry Research Group, Clinical and Molecular Genetics Unit, UCL Institute of Child Health. 30 Guilford Street, London WC1N 1EH, UK*

* ***Corresponding author:*** *Masamitsu Maekawa, Ph.D., Department of Pharmaceutical Sciences, Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan; TEL: +81-22-717-7541, FAX: +81-22-717-7545, E-mail: m-maekawa@hosp.tohoku.ac.jp*

ABSTRACT

Background: Niemann–Pick disease type C (NPC) is an autosomal recessive inherited disorder with progressive neuronal degeneration. Because conventional diagnostic methods are complicated and invasive, biomarker tests have drawn attention. We aimed to evaluate three urinary conjugated cholesterol metabolites as diagnostic biomarkers for NPC.

Methods: Urine samples from 23 patients with NPC, 28 healthy controls, and 7 patients with inherited metabolic disorders were analyzed. 3β-Sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid and its glycine and taurine conjugates in urine were quantified by liquid chromatography-tandem mass spectrometry. The diagnostic performance of the three metabolites and their total concentration was evaluated.

Result: Creatinine-corrected concentrations of three metabolites and their total concentration were all significantly higher in NPC patients ($0.0098 < P < 0.0448$). The area under the receiver operating curve for all metabolites exceeded 0.95, the clinical specificity was 92‑100%, and the clinical sensitivity was ~95%. In the urine of patients with other inherited metabolic diseases, the concentrations of the metabolites were lower than those in the urine of patients with NPC.

Conclusion: These conjugated cholesterol metabolites in urine can serve as useful diagnostic markers for noninvasive screening of NPC.

Keywords:

Niemann–Pick disease type C, Urine, Sulfate-conjugated cholesterol metabolites, Biomarkers, Mass spectrometry, Liquid chromatography

Abbreviations:

AUC, area under the curve; Cre, creatinine; GlcNAc, *N*-acetylglucosamine; LC-MS/MS, liquid chromatography/tandem mass spectrometry; NPC, Niemann–Pick disease type C; NPC1, NPC intracellular cholesterol transporter 1; NPC2, NPC intracellular cholesterol transporter 2; SNAG-Δ^5-CA, nonamidated 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid; SNAG-Δ^5-CG, glycine-amidated 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid; SNAG-Δ^5-CT, taurine-amidated 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid.

1. Introduction

Niemann–Pick disease type C (NPC) is an autosomal recessive inherited disorder that results in progressive neuronal degeneration. This rare disease is classified as a lysosomal disease and the prevalence rate of the disease is ~1/120000. Many inherited disorders develop during the neonatal to infant period, but the onset age of NPC ranges from neonate to adult [1]. In addition, various symptoms, including visceral, neurological, and psychiatric symptoms are observed depending on the age of onset [2]. Most symptoms of the disease overlap with other inherited diseases. Therefore, the diagnosis of NPC is complicated and misdiagnosis or delay in diagnosis is common [1–4].

To date, two diagnostic techniques have been used as definitive diagnostic procedures for NPC [2]. Staining with filipin, a fluorescent antibiotic, detects free cholesterol that has accumulated in the lysosomes [5]. This method can visualize the intracellular accumulation of free cholesterol but a skin biopsy is required. Furthermore, ~15% of patients with NPC have a variant phenotype without obvious fluorescence [6]. Additionally, the fibroblast cells require a long culturing time. Thus, the diagnosis of NPC by filipin staining is slow and labour-intensive. Another test is a genetic analysis of the genes responsible for this disease: NPC intracellular transporter 1 (*NPC1*) and NPC intracellular cholesterol transporter 2 (*NPC2*). *NPC1* codes for a membrane protein located in the late endosomes/lysosomes [7], and *NPC2* codes for a lysosomal

soluble protein [8]. Because the two proteins act to export cholesterol out of the lysosome in a coordinated manner [9], mutations of either *NPC1* or *NPC2* affect their function, resulting in the accumulation of lysosomal free cholesterol. However, more than 700 different mutations have been found in the two genes, making NPC diagnosis difficult [10].

In recent years, plasma oxysterols [11–13] and plasma sphingolipids [14–15] have come to attention as potential NPC diagnostic markers. Common oxysterols for NPC diagnostic screening are cholestane-3β,5α,6β-triol and 7-ketocholesterol [12–13]. They have very high NPC diagnostic performance, but there are problems with the analytical procedures and the stability of the analytes, e.g., complicated derivatization steps [12–13]. In addition, oxysterols are produced from cholesterol in plasma at room temperature by autoxidation [13], yielding potential false positives. Lysosphingomyelin and lysosphingomyelin-509 are representative sphingolipids used for NPC screening. Lysosphingomyelin shows high clinical performance as a diagnostic marker but the ratio of the plasma concentration between patients with NPC and healthy controls is only 2.8 and increased values are seen in NPA and NPB as well as in NPC [14]. In contrast, the plasma concentration of lysosphingomyelin-509 is much higher than that of lysosphingomyelin in patients with NPC. However, the precise structure of lysosphingomyelin-509 is still unknown and there is no certified reference standard for absolute quantification [15].

Urine collection is noninvasive but there is currently no urinary NPC biomarker testing method. Thus, we focused on the fact that many conjugated cholesterol metabolites resulting from several inherited diseases are excreted in the urine [16] and searched for characteristic peaks in the urine samples of patients with NPC using the metabolomics method [17]. Several strong, characteristic peaks were detected, some of them consistent with previously reported conjugated cholesterol metabolites [18]. We hypothesized that these unusual metabolites were possible diagnostic markers of NPC and developed a liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the determination of the multi-conjugated cholesterol metabolites 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid (SNAG-Δ^5-CA) and its glycine and taurine-conjugates (SNAG-Δ^5-CG and SNAG-Δ^5-CT, respectively) in urine. The concentrations of these biomarkers in the urine of two patients with NPC were higher than those of controls [19]. These conjugated metabolites could also be analyzed using LC-MS/MS without derivatization. Additionally, because urine sample collection is noninvasive, urine testing via LC-MS/MS would be preferable to current NPC diagnostic screening. In this study, we analyzed three metabolites in the urine from 23 patients with NPC, 28 healthy controls, and 7 patients with inherited metabolic diseases and evaluated the usefulness of the multiply conjugated cholesterol metabolites as diagnostic markers of NPC.

2. Materials and Methods

2.1.Chemicals and reagents

SNAG-Δ^5-CA, SNAG-Δ^5-CG, SNAG-Δ^5-CT, and 3β-sulfooxy-7β-hydroxy-23-*nor*-5-cholenoic acid (internal standard (IS), Supplemental Fig. 1) were synthesized as previously reported [20–21]. Ultrapure water was prepared using a PURELAB ultra apparatus (Organo Co., Ltd., Tokyo, Japan). All other chemicals were analytical grade, and the solvents were high-performance liquid chromatography (HPLC) or LC-MS grade.

2.2. Urine samples

Urine samples from patients with NPC were collected at the Tottori University Hospital (Yonago, Japan), Klinik für Kinder und Jugenmedizin (Muenster, Germany), University Medical Center of the Johannes Gutenberg University Mainz (Mainz, Germany), Osaka Red Cross Hospital (Osaka, Japan), and Southern Tohoku Research Institute for Neuroscience (Koriyama, Japan). Urine of patients with glycogen storage disease type I, citrin deficiency, and abetalipoproteinemia were donated by the Department of Pediatrics, Graduate School of Medicine, Tohoku University (Sendai, Japan). Urine samples of a patient with glycogen storage disease type II was collected at Dokkyo University Hospital (Mibu-machi, Japan). All experiments were carried out according to the protocol approved by the Ethics Committee of the Graduate School of

Medicine in Tohoku University.

2.3. Urine analysis

LC-MS/MS analysis was performed using a previously reported method *(19)*. In short, MS/MS measurements were conducted in the selected reaction monitoring and negative ion mode by using an API 5000 (SCIEX, Framingham, MA) equipped with an electrospray ion source probe. The mass-to-charge ratios (*m/z*) of 672 > 97, 364 > 433, 389 > 460, and 455 > 97 were used for the selected reaction monitoring transitions of SNAG-Δ^5-CA, SNAG-Δ^5-CG, SNAG-Δ^5-CT, and IS, respectively. A Nanospace SI-2 system (Osaka Soda Co. Ltd., Osaka) was used for liquid chromatography and online solid phase extraction was carried out by column switching. First, the specimen was mixed with the IS solution and subjected to online pretreatment. Shim-pack MAYI-C8 (4.6 mm i.d. × 10 mm, 50 μm, Shimadzu Co., Kyoto) was used as the trapping column, and 20 mM ammonium acetate buffer (pH 5.5)/methanol (9:1, v/v) was used as the mobile phase at flow rate of 1.0 mL/min. After 3 min, the pretreated fraction was loaded into the LC-MS/MS system. YMC-Pack Pro C18 (2.1 mm i.d. × 150 mm, 5 μm, YMC Co. Ltd., Kyoto, Japan) was used as the analytical column, and 20 mM ammonium acetate buffer (pH 5.5)/methanol (1:1, v/v) was used as the mobile phase at the flow rate of 0.2 mL/min. All data were analyzed with SCIEX OS-Q software (SCIEX). Analytical method validation was carried out according to the items of FDA guidance.

The urine samples were frozen immediately after collection. The frozen urine samples were shipped via a refrigerated courier service in a frozen state. The delivered urine was thawed and analyzed within 1 week of arrival. If the quantitative concentration of analytes exceeded the upper limit of quantification, the urine was diluted to one tenth or one hundredth using water and reanalyzed. Urinary creatinine was analyzed with enzymatic creatinine analysis kit (Serotec, Sapporo, Japan). The urinary concentrations of the three metabolites were corrected for the urinary creatinine concentration.

2.4. Statistical analysis

JMP Pro version 13.2.1 (SAS Institute Inc., NC, USA) was used as the statistical analysis software. Significant differential analysis of the age of each group was carried out with Student's *t*-test. Pearson's χ^2 correlation test was used for sex differential analysis, as shown in Table 1. Wilcoxon's t-test was used in the correlation of urinary concentration of cholesterol metabolites between patients with NPC and healthy controls (Fig. 1 and Supplemental Fig. 2). Single regression analysis was used for the analysis of the correlation between age and concentration (Supplemental Fig. 3). Wilcoxon's *t*-test was used in the correlation between sex and the concentration of each analyte (Supplemental Fig. 4). Receiver operating characteristic curves and the area under the curve (AUC) were generated by a logistic regression model (Supplemental

Fig. 5).

3. Results and Discussion

3.1.Analytical method validation

Because it was necessary to develop a simple diagnostic method for NPC, we investigated the usefulness of NPC diagnosis with urinary SNAG-Δ^5-CAs using an established LC-MS/MS method [19]. Before evaluating the diagnostic performance of SNAG-Δ^5-CA, SNAG-Δ^5-CG, and SNAG-Δ^5-CT as noninvasive biomarkers for NPC, we revalidated the reliability of the analytical method. Matrix effect was evaluated using matrix factor and IS normalized matrix factor [14]. The values of all analytes were in the range of 100±15% and so were measurable without being influenced by urinary components (Supplemental Table 1A). Intra-assay and inter-assay reproducibility showed the values meeting FDA criteria (Supplemental Table 1B). In addition, the analytes were stable under all storage condition (Supplemental Table 1D). There was no difference in concentration upon dilution with water, and no carryover was observed (Supplemental Table 1E and F).

3.2.Urinary concentrations of SNAG-Δ^5-CAs

Next, samples from the healthy controls and those from patients with NPC were compared. We collected urine samples from 23 patients with NPC (age 0.0274–36 y)

and 28 healthy controls (age 0.33–47 y) (Table 1). We evaluated the NPC diagnostic performance of urinary cholesterol metabolites using creatinine-corrected and uncorrected concentrations. Urinary creatinine correction is generally used for biochemical examination. However, the creatine concentration varies with age, particularly in the neonatal period because of premature renal function. The creatinine-corrected concentrations are summarized in Fig. 1, and all data are shown in Supplemental Table 2. The uncorrected concentrations are shown in Supplemental Fig. 2 and Supplemental Table 3. Although there was a slight difference in the two datasets, the trends were similar. The urinary metabolites corrected by creatinine were higher in patients with NPC (*P*-values show in Fig. 1 and Supplemental Fig. 2), and their mean concentrations in the patients with NPC were 360- to 558-times higher than those of the healthy controls (Supplemental Table 4). Early onset cases with more severe symptoms and high mortality rates are known to show high cholesterol accumulation [5]. In contrast, late onset cases with weak systemic symptoms show low cholesterol accumulation [6]. Although a detailed association between lipid accumulation and pathophysiology has not yet been elucidated, these metabolite concentrations can be related to cholesterol accumulation. In contrast, the plasma oxysterol concentrations in the patients with NPC were only eight-times higher than those in the healthy controls [12]. Oxysterols induce proteins that facilitate their metabolism and excretion, such as sulfotransferase [22], uridine 5'-diphospho (UDP)-glucuronosyltransferase (UGT) [23],

and CYP27A1 [24] via the liver X receptor. Bile acids, which are the primary metabolites of cholesterol, are conjugated with sulfuric acid and eliminated in urine, many of which are conjugated with amino acids [25]. Accordingly, the metabolism of cholesterol to SNAG-Δ^5-CAs and their excretion in urine may function as a mechanism for the elimination of abnormally accumulated cholesterol and oxysterols. As a result, the difference in urinary SNAG-Δ^5-CAs between patients with NPC and healthy controls might become larger than those of plasma oxysterols. Regarding the difference in amino acid conjugation type, the ratio of glycine conjugates to taurine conjugates was larger in patients with NPC than healthy controls (14.2 ± 12.0 vs. 8.10 ± 7.29, $P = 0.0415$). Amino acid conjugation of the 24th carboxylic acid by glycine or taurine is catalyzed by bile-acid CoA:amino acid *N*-acyltransferase in peroxisomes. The glycine/taurine ratio is also affected by diet, and a reduction in the taurine content in NPC mice brains has been reported [26]. Accordingly, the change in amino acid metabolism in NPC might affect taurine vs glycine conjugates of SNAG-Δ^5-CA. However, we could not recruit the statistically sufficient samples (more than 120 samples) for elucidating their true biomarker performances for NPC, we showed the possibility of the usefulness of urinary SNAG-Δ^5-CAs as high-performance and noninvasive diagnostic biomarkers for NPC.

On the other hand, analysis of individual cases revealed a few cases of low analyte concentrations in patients with NPC. For example, all metabolite concentrations

in the urine of patients 10 and 17 were much lower than the mean concentrations of those of the healthy controls (Supplemental Tables 2 and 3). Mazzacuva et al. reported a low urinary concentration of 3β-hydroxy-7β-GlcNAc-5-cholenoic acid in two patients with NPC who had mutations in *UGT3A1* [27]. UGT3A1 is thought to be inactivated by this mutation (p.C121G/p.C121G) [28]. Because the allele frequencies are considered to be around 30% [29], several subjects might have this type homozygous mutation. The subjects showing extremely lower concentrations of urinary SNAG-Δ^5-CAs such as patients with NPC 10 and 17 might be affected by mutations in *UGT3A1*. Therefore, it may be better to set a threshold between the concentrations of patient No. 23, who had the third lowest concentration of SNAG-Δ^5-CAs, and healthy control 19 in the case of SNAG-Δ^5-CA, SNAG-Δ^5-CG, and the total concentration or healthy control 6 in the case of SNAG-Δ^5-CT.

3.3.Sex- and age-difference of urinary SNAG-Δ^5-CAs

Subsequently, the correlation between age and the concentration of urinary metabolites was investigated (Supplemental Fig. 3). Patients are roughly divided into five phenotypes according to the age of onset. It is thought that there is a correlation between the severity of symptoms and age in patients with NPC *(2)*. Based on filipin staining, the classical type is more common in younger onset cases, and the variant type is more common in older onset cases [1]. Accordingly, the degree of cholesterol

accumulation could be related to the onset age. In this study, we collected urine specimens from 10-day-old to 36-year-old patients to cover the five NPC phenotypes. The age of the healthy controls was also in accordance with these groups (Table 1). There was an inverse correlation for all metabolites and age, and the correlation coefficients were -0.492 to -0.202 for the patients with NPC (Supplemental Fig. 3). Because SNAG-Δ^5-CAs showed the same degree of correlation as oxysterols [12], they might be markers reflecting the pathology of NPC. Interestingly, inverse correlations were only obtained for only SNAG-Δ^5-CT in the healthy controls. The supplying taurine with breast milk might influence the result.

Subsequently, the relationship between sex and urinary concentrations was investigated. While Jiang et al. reported higher plasma metabolites in male patients with NPC [30], Porter et al. reported no sex differences in plasma oxysterols [12]. In female rats, the sulfation activity and transporter proteins related to bile acids were higher than those of male rats [31]. The metabolites were not affected by the gender of the patients (Supplemental Fig. 4) at least in this study. However, because the number of samples used in this study was small, we could not confirm the sex difference regarding these metabolites.

3.4.Evaluation of diagnostic performance of SNAG-Δ^5-CAs with receiver operating characteristic analysis

Next, the diagnostic performance of urinary metabolites was evaluated using Receiver operating characteristic analysis. The AUC values of each metabolite were all more than 0.95 (Supplemental Fig. 5). However, the AUC values were slightly lower than those of the other biomarker candidates [12,14] (Supplemental Fig. 5). We think that this difference is due to the extremely low metabolite concentration (patients 10 and 17). Regarding the cut-off concentration, the concentrations with the highest value of sensitivity-(1-specificity) were selected for each metabolite. The cut-off concentrations of SNAG-Δ^5-CA and SNAG-Δ^5-CG were 90 ng/mg creatinine and 140 ng/mg creatinine, whereas that of SNAG-Δ^5-CT was much lower (18.0 ng/mg creatinine). The clinical sensitivity and specificity at those conditions for each metabolite are also shown in Supplemental Fig. 5. The sensitivities were 95.5% or 95.2%, and the specificities were 100% or 92.3%. Thus, all metabolites were useful urinary markers for NPC diagnosis. On the other hand, if the cut-off concentrations were set at lower values, the specificity dramatically decreased, and the false positive rate dramatically increased. As a result, the number of secondary tests would increase significantly, and the diagnostic test would be considered unsuitable for primary screening. We previously identified two biomarker candidates that did not have an *N*-acetylglucosamine (GlcNAc) group [32]. Thus, we consider them not affected by the polymorphism of UGT3A1, and the development as diagnostic biomarkers for NPC will also be expected in the future. However, because the number of samples used in this study was small, further

investigation will be necessary to verify the observed diagnostic performance.

3.5. Urinary concentration of SNAG-Δ^5-CA in subjects with various common symptomatic diseases

Finally, we analyzed urine specimens of patients with diseases having similar clinical symptoms to NPC, such as cholestasis, hepatomegaly, central nervous symptoms, and lipid abnormalities. All these diseases are genetic and result in abnormalities in lipids and carbohydrates. Low concentrations of urinary cholesterol metabolites were observed for these diseases (Fig.2 and Supplemental Tables 2 and 3). However, the number of samples for each disease was considerably fewer, and we could not determine the value of these metabolites for differentiating among these diseases.

4. Conclusions

In summary, we evaluated the diagnostic performance of urinary conjugated cholesterol metabolites as noninvasive diagnostic biomarkers of NPC. The concentrations of SNAG-Δ^5-CA in the urine of patients with NPC were higher than those of the healthy controls and patients with congenital diseases requiring discrimination from NPC. These urinary conjugated metabolites can serve as highly accurate diagnostic markers. Thus, we show that urinary SNAG-Δ^5-CAs are remarkably characteristic metabolites of NPC, and urinary cholesterol metabolites are useful

diagnostic biomarkers for the noninvasive diagnostic screening of NPC.

Acknowledgments

This work was supported in part by JSPS KAKENHI 16K20900 and 18K15699.

We are grateful to the doctors who collected urine samples. We would like to thank Editage by Cactus Commununiations Co., Ltd. (Tokyo) for English language editing.

References

[1] M.T. Vanier, Niemann–Pick disease type C, Orphanet J. Rare Dis. 5 (1) (2010) 16–33.

[2] M.C. Patterson, C.J. Hendriksz, M. Walterfang, F. Sedel, M.T. Vanier, F. Wijburg, Recommendations for the diagnosis and management of Niemann–Pick disease type C: An update, Mol. Genet. Metab. 106 (2012) 330–344.

[3] J.E. Wraith, M.R. Baumgartner, B. Bembi, A. Covanis, T. Levade, E. Mengel, et al., Recommendations on the diagnosis and management of Niemann–Pick disease type C, Mol. Genet. Metab. 98 (2009) 152–165.

[4] S.M. Lo, J. McNamara, M.R. Seashore, P.K. Mistry, Misdiagnosis of Niemann–Pick disease type C as Gaucher disease, J. Inherit. Metab. Dis. 33 (2010) 429–433.

[5] E.J. Blanchette-Mackie, N.K. Dwyer, L.M. Amende, H.S. Kruth, J.D. Butler, J. Sokol, et al., Type-C Niemann–Pick disease: low density lipoprotein uptake is associated with premature cholesterol accumulation in the Golgi complex and excessive cholesterol storage in lysosomes, Proc. Natl. Acad. Sci. USA. 85 (1988) 8022–8026.

[6] M.T. Vanier, P. Latour, Laboratory diagnosis of Niemann–Pick disease type C: The filipin staining test, Methods Cell. Biol.126 (2015) 357–375.

[7] E.D. Carstea, M.H. Polymeropoulos, C.C. Parker, S.D. Detera-Wadleigh, R.R. O'Neill, M.C. Patterson, et al., Linkage of Niemann–Pick disease type C to human chromosome 18, Proc. Natl. Acad. Sci. USA.90 (1993) 2002–2004.

[8] S.J. Steinberg, C.P. Ward, A.H. Fensom, Complementation studies in Niemann–Pick disease type C indicate the existence of a second group, J. Med. Genet. 31 (1994) 317–320.

[9] H.J. Kwon, L. Abi-Mosleh, M.L. Wang, J. Deisenhofer, J.L. Goldstein, M.S. Brown, et al., Structure of N-terminal domain of NPC1 reveals distinct subdomains for binding and transfer of cholesterol, Cell 137 (2009) 1213–1224.

[10] K. Bounford, P. Gissen, Genetic and laboratory diagnostic approach in Niemann–Pick disease type C, J. Neurol. 261 (2014) 569–575.

[11] R.C. Murphy, K.M. Johnson, Cholesterol, reactive oxygen species, and the formation of biologically active mediators, J. Biol. Chem. 283 (2008) 15521–15525.

[12] F.D. Porter, Scherrer DE, Lanier MH, Langmade SJ, Molugu V, Gale SE, et al., Cholesterol oxidation products are sensitive and specific blood-based biomarkers for Niemann–Pick C1 disease, Sci. Transl. Med. 2 (2010) 56ra81.

[13] X. Jiang, R. Sidhu, F.D. Porter, N.M. Yanjanin, A.O. Speak, D. Taylor-te Vruchte, et al., A sensitive and specific LC-MS/MS method for rapid diagnosis of Niemann–Pick C1 disease from human plasma, J. Lipid Res.52 (2011) 1435–1445.

[14] R.W.D. Welford, M. Garzotti, C. Marques Lourenço, E. Mengel, T. Marquardt, J. Reunert, et al., Plasma lysosphingomyelin demonstrates great potential as a diagnostic biomarker for Niemann–Pick disease type C in a retrospective study, PLoS One9 (2014) e114669.

[15] A. Giese, H. Mascher, U. Grittner, S. Eichler, G. Kramp, J. Lukas, et al., A novel, highly sensitive and specific biomarker for Niemann–Pick type C1 disease, Orphanet J. Rare Dis. 10 (2015) 1–23.

[16] P.T. Clayton, Disorders of bile acid synthesis, J. Inherit Metab. Dis. 34 (2011) 593–604.

[17] M. Maekawa, M. Shimada, K. Ohno, M. Togawa, H. Nittono, T. Iida, et al., Focused metabolomics using liquid chromatography/electrospray ionization tandem mass spectrometry for analysis of urinary conjugated cholesterol metabolites from patients with Niemann–Pick disease type C and 3β-hydroxysteroid dehydrogenase deficiency, Ann. Clin. Biochem. 52 (2015) 576–587.

[18] G. Alvelius, O. Hjalmarson, W.J. Griffiths, I. Björkhem, J. Sjövall, Identification of unusual 7-oxygenated bile acid sulfates in a patient with Niemann–Pick disease, type C, J. Lipid Res. 42 (2001) 1571–1577.

[19] M. Maekawa, Y. Misawa, A. Sotoura, H. Yamaguchi, M. Togawa, K. Ohno, et al., LC/ESI-MS/MS analysis of urinary 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid and its amides: New biomarkers for the detection of Niemann–Pick type C disease, Steroids 78 (2013) 967–972.

[20] T. Iida, G. Kakiyama, Y. Hibiya, S. Miyata, T. Inoue, A.F. Hofmann, Chemical synthesis of the 3-sulfooxy-7-*N*-acetylglucosaminyl-24-amidated conjugates of 3,7-dihydroxy-5-cholen-24-oic acid, and related compounds: Unusual, major metabolites of bile acid in a patient with Niemann–Pick disease type C1, Steroids 1 (2005) 18–29.

[21] G. Kakiyama, A. Muto, M. Shimada, N. Mano, J. Goto, A.F. Hofmann, et al., Chemical synthesis of 3β-sulfooxy-7β-hydroxy-24-*nor*-5-cholenoic acid: An internal standard for mass spectrometric analysis of the abnormal Δ^5-bile acids occurring in Niemann–Pick disease, Steroids 74 (2009) 766–772.

[22] M. Runge-Morris, T.A. Kocarek, C.N. Falany, Regulation of the cytosolic sulfotransferases by nuclear receptors, Drug Metab. Rev. 45 (2013) 15–33.

[23] O. Barbier, J. Trottier, J. Kaeding, P. Caron, M. Verreault, Lipid-activated transcription factors control bile acid glucuronidation, Mol. Cell Biochem. 326 (2009) 3–8.

[24] M. Crestani, E. De Fabiani, D. Caruso, N. Mitro, F. Gilardi, A.B. Vigil Chacon, et al., LXR (liver X receptor) and HNF-4 (hepatocyte nuclear factor-4): Key regulators in reverse cholesterol transport. Biochem. Soc. Trans. 32 (2004) 92–96.

[25] S.P.R. Bathena, S. Mukherjee, M. Olivera, Y. Alnouti, The profile of bile acids and their sulfate metabolites in human urine and serum, J. Chromatogr. B 942–943 (2013) 53–62.

[26] J.W. Totenhagen, A. Bernstein, E.S. Yoshimaru, R.P. Erickson, T.P. Trouard, Quantitative magnetic resonance imaging of brain atrophy in a mouse model of Niemann–Pick type C disease, PLoS One 12 (2017) 1–11.

[27] F. Mazzacuva, P. Mills, K. Mills, S. Camuzeaux, P. Gissen, E.R. Nicoli, et al., Identification of novel bile acids as biomarkers for the early diagnosis of Niemann–Pick C disease, FEBS Lett. 590 (2016) 1651–1662.

[28] P.I. Mackenzie, A. Rogers, J. Treloar, B.R. Jorgensen, J.O. Miners, R. Meech, Identification of UDP glycosyltransferase 3A1 as a UDP *N*-acetylglucosaminyltransferase, J. Biol. Chem. 283 (2008) 36205–36210.

[29] International HapMap Consortium, A haplotype map of the human genome, Nature 437 (2005) 1299–1320.

[30] X. Jiang, R. Sidhu, L. Mydock-McGrane, F. Hsu, D.F. Covey, D.E. Scherrer, et al., Development of a bile-acid-based newborn screen for Niemann–Pick disease type C, Sci. Transl. Med. 8 (2016) 1–11.

[31] W.F. Balistreri, L. Zimmer, F.J. Suchy, K.E. Bove, Bile salt sulfotransferase: Alterations during maturation and non-inducibility during substrate ingestion, J. Lipid Res. 25 (1984) 228–235.

[32] M. Maekawa, K. Omura, S. Sekiguchi, T. Iida, D. Saigusa, H, Yamaguchi, et al., Identification of two sulfated cholesterol metabolites found in the urine of a patient with Niemann–Pick disease type C as novel candidate diagnostic markers, Mass Spectrom. (Tokyo) 5 (2016) S0053.

Table 1. Demographics of healthy controls and patients with NPC.

Group	Healthy	Patients with	*P*-value

	controls	NPC	
Number of values	28	23	
Male (%)	71.4	39.1	0.0261
Age (y) minimum	0.33	0.0274	
Age (y) 25 percentile	1.25	4	
Age (y) median	21.5	12	0.4074
Age (y) 75 percentile	29.75	22	
Age (y) maximum	47	36	

NPC, Niemann–Pick disease type C.

Fig. 1. Creatinine-corrected concentrations of SNAG-Δ^5-CA (A), SNAG-Δ^5-CG (B), SNAG-Δ^5-CT (C), and the total concentration (D) in the urine of healthy controls and patients with NPC. All metabolite concentrations and their total concentration in the urine of patients with NPC were significantly higher than those in the healthy controls. *P*-values of SNAG-Δ^5-CA, SNAG-Δ^5-CG, SNAG-Δ^5-CT, and the total concentration were 0.0098, 0.0440, 0.0448, and 0.0163, respectively.

NPC, Niemann–Pick disease type C; SNAG-Δ^5-CA, nonamidated 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid; SNAG-Δ^5-CG, glycine-amidated 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid; SNAG-Δ^5-CT, taurine-amidated 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid.

Fig. 2. Selected reaction monitoring chromatograms of SNAG-Δ^5-CA, SNAG-Δ^5-CG, and SNAG-Δ^5-CT in urine samples from an patient with NPC (A), cerebrotendinous xanthomatosis (B), abetalipoproteinemia (C), citrin deficiency (D), glycogen storage disease type 1 (E), and glycogen storage disease type 2 (F). High-intensity peaks were observed in the chromatograms of patients with (A), but low-intensity peaks were observed in the urine of the other controls (B–F).

NPC, Niemann–Pick disease type C; SNAG-Δ^5-CA, nonamidated 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid; SNAG-Δ^5-CG, glycine-amidated 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid; SNAG-Δ^5-CT, taurine-amidated 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid.

Graphical abstract:

Highlights:

- Urinary conjugated cholesterol metabolites are expected as biomarkers for NPC.
- They are analyzed by liquid chromatography/tandem mass spectrometry.
- The analytical method was verified according to the FDA guideline.
- The urinary metabolites were significant higher in patients with NPC.
- They all showed good biomarker performances with ROC analysis.

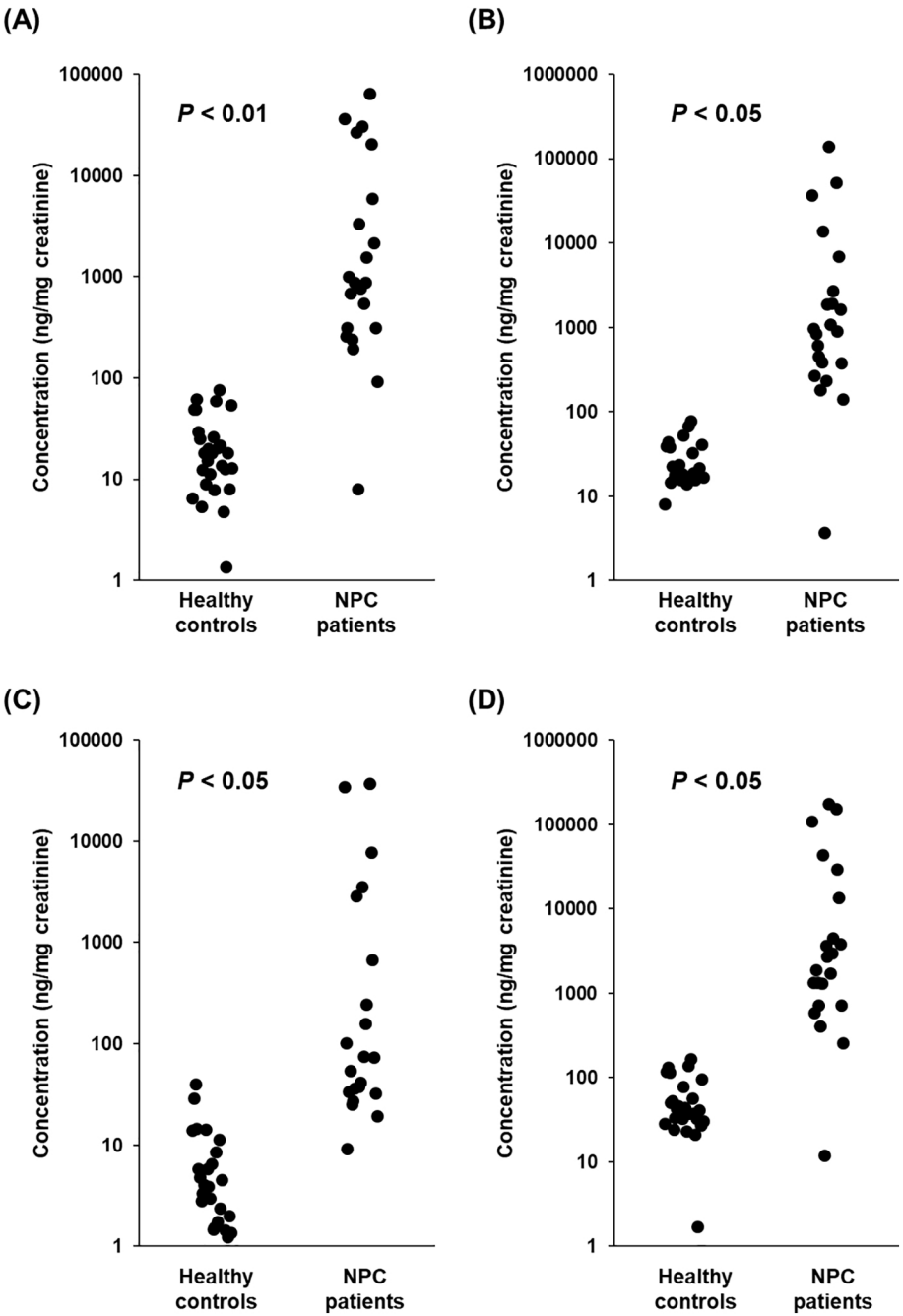

(A)
P < 0.01
Concentration (ng/mg creatinine)
100000
10000
1000
100
10
1
Healthy controls
NPC patients
(B)
P < 0.05
Concentration (ng/mg creatinine)
1000000
100000
10000
1000
100
10
1
Healthy controls
NPC patients
(C)
P < 0.05
Concentration (ng/mg creatinine)
100000
10000
1000
100
10
1
Healthy controls
NPC patients
(D)
P < 0.05
Concentration (ng/mg creatinine)
1000000
100000
10000
1000
100
10
1
Healthy controls
NPC patients

Figure 1

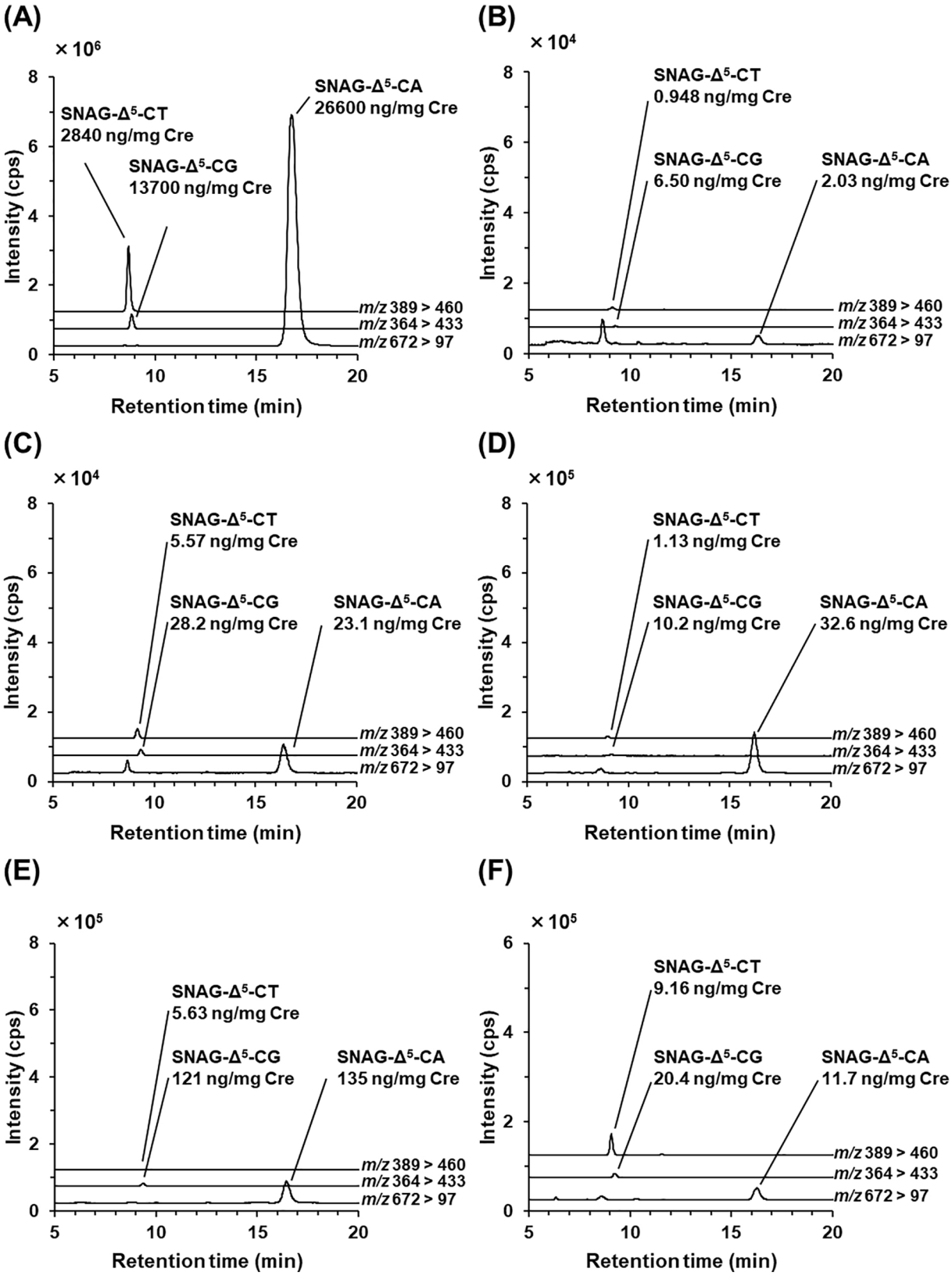
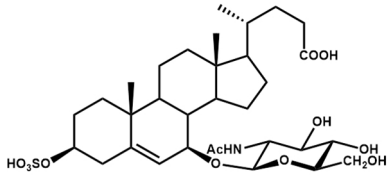
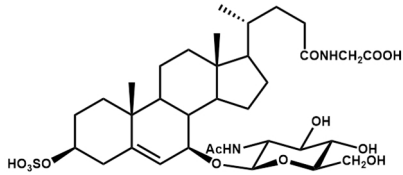

(A)
× 10^6
SNAG-Δ5-CT
2840 ng/mg Cre
SNAG-Δ5-CG
13700 ng/mg Cre
SNAG-Δ5-CA
26600 ng/mg Cre
m/z 389 > 460
m/z 364 > 433
m/z 672 > 97
Intensity (cps)
Retention time (min)
(B)
× 10^4
SNAG-Δ5-CT
0.948 ng/mg Cre
SNAG-Δ5-CG
6.50 ng/mg Cre
SNAG-Δ5-CA
2.03 ng/mg Cre
m/z 389 > 460
m/z 364 > 433
m/z 672 > 97
Intensity (cps)
Retention time (min)
(C)
× 10^4
SNAG-Δ5-CT
5.57 ng/mg Cre
SNAG-Δ5-CG
28.2 ng/mg Cre
SNAG-Δ5-CA
23.1 ng/mg Cre
m/z 389 > 460
m/z 364 > 433
m/z 672 > 97
Intensity (cps)
Retention time (min)
(D)
× 10^5
SNAG-Δ5-CT
1.13 ng/mg Cre
SNAG-Δ5-CG
10.2 ng/mg Cre
SNAG-Δ5-CA
32.6 ng/mg Cre
m/z 389 > 460
m/z 364 > 433
m/z 672 > 97
Intensity (cps)
Retention time (min)
(E)
× 10^5
SNAG-Δ5-CT
5.63 ng/mg Cre
SNAG-Δ5-CG
121 ng/mg Cre
SNAG-Δ5-CA
135 ng/mg Cre
m/z 389 > 460
m/z 364 > 433
m/z 672 > 97
Intensity (cps)
Retention time (min)
(F)
× 10^5
SNAG-Δ5-CT
9.16 ng/mg Cre
SNAG-Δ5-CG
20.4 ng/mg Cre
SNAG-Δ5-CA
11.7 ng/mg Cre
m/z 389 > 460
m/z 364 > 433
m/z 672 > 97
Intensity (cps)
Retention time (min)

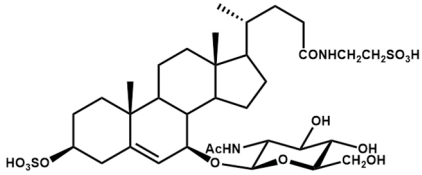
Figure 2



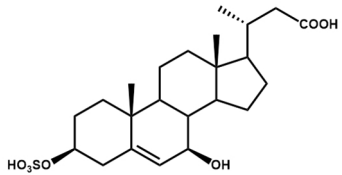
(A) SNAG-Δ^5-CA



(B) SNAG-Δ^5-CG



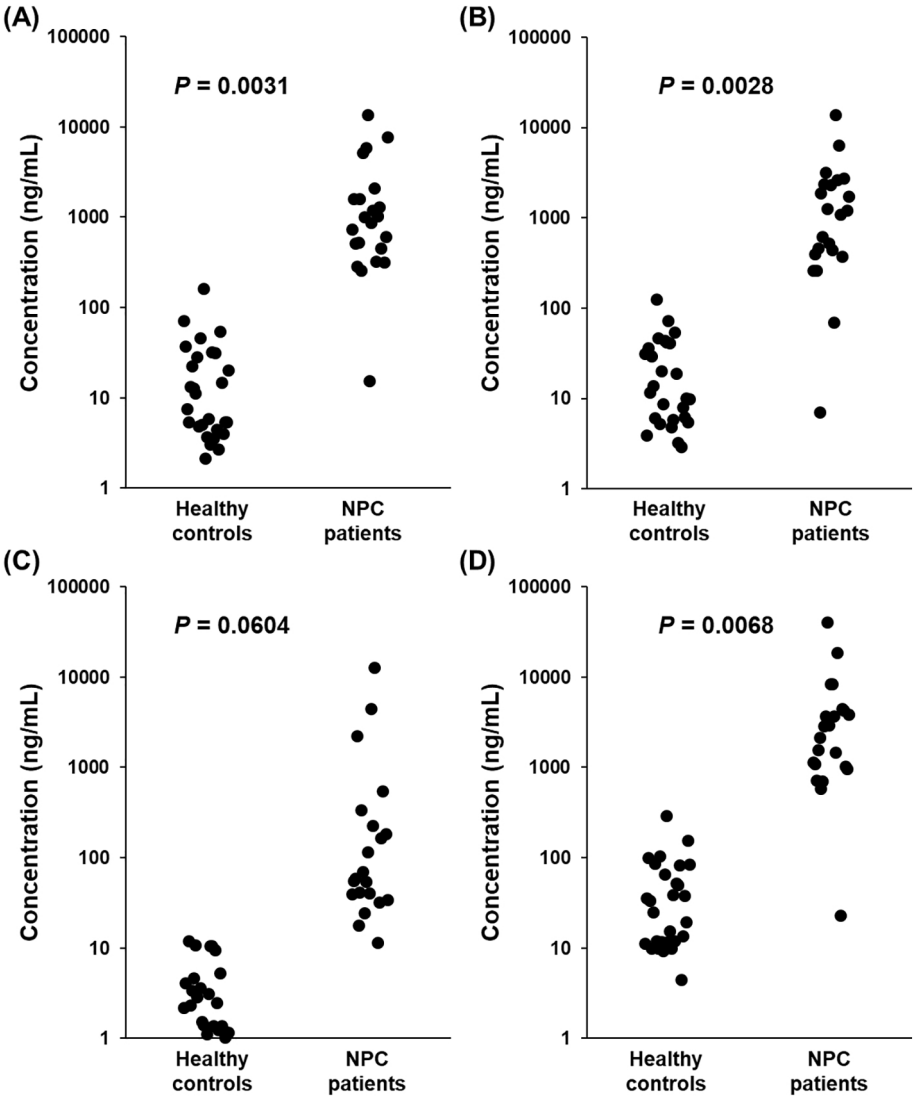
(C) SNAG-Δ^5-CT



(D) IS

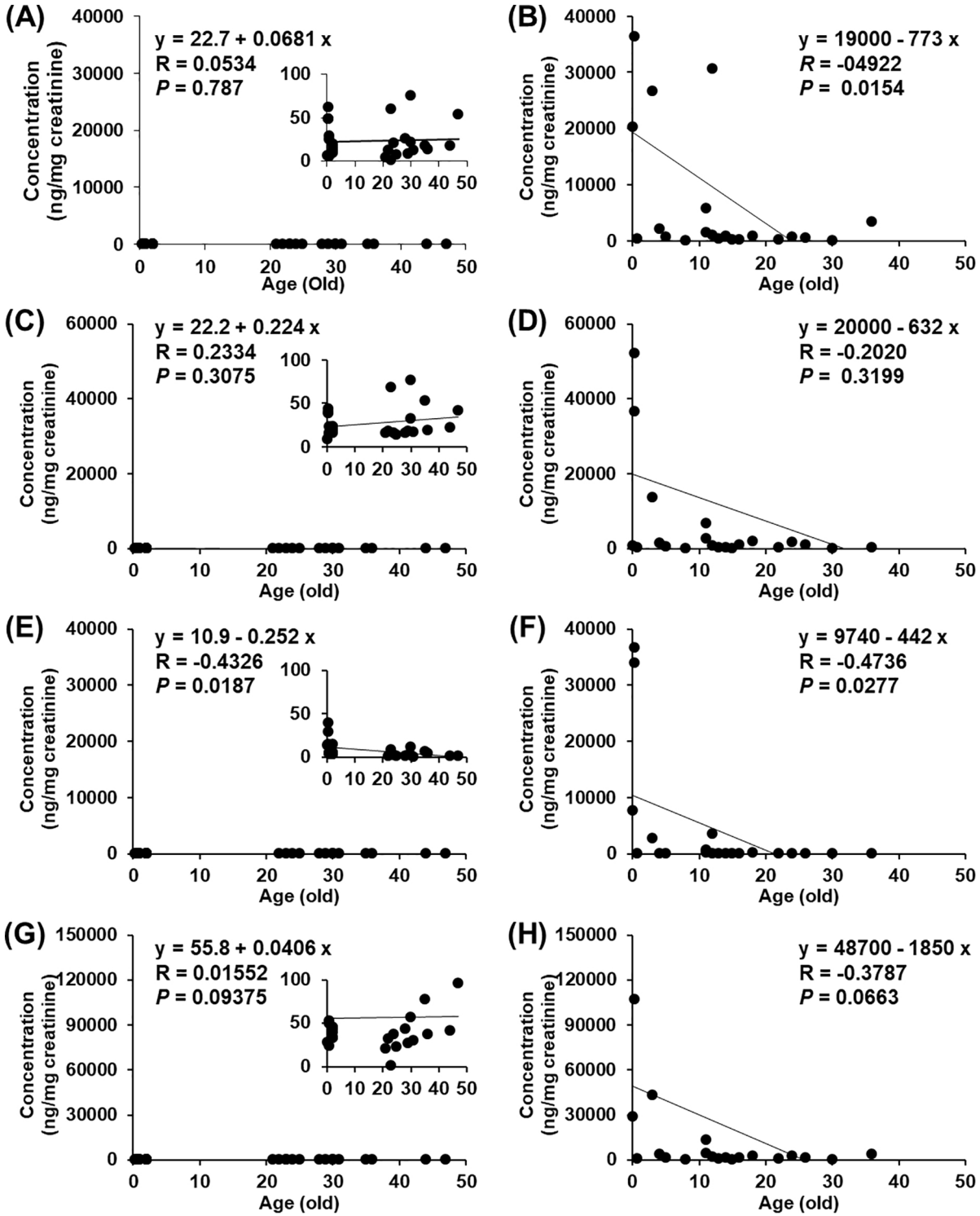
Chemical structure of analytes and internal standard (IS). (A) SNAG-Δ^5-CA, (B) SNAG-Δ^5-CG, (C) SNAG-Δ^5-CT, and (D) IS.

Figure 3

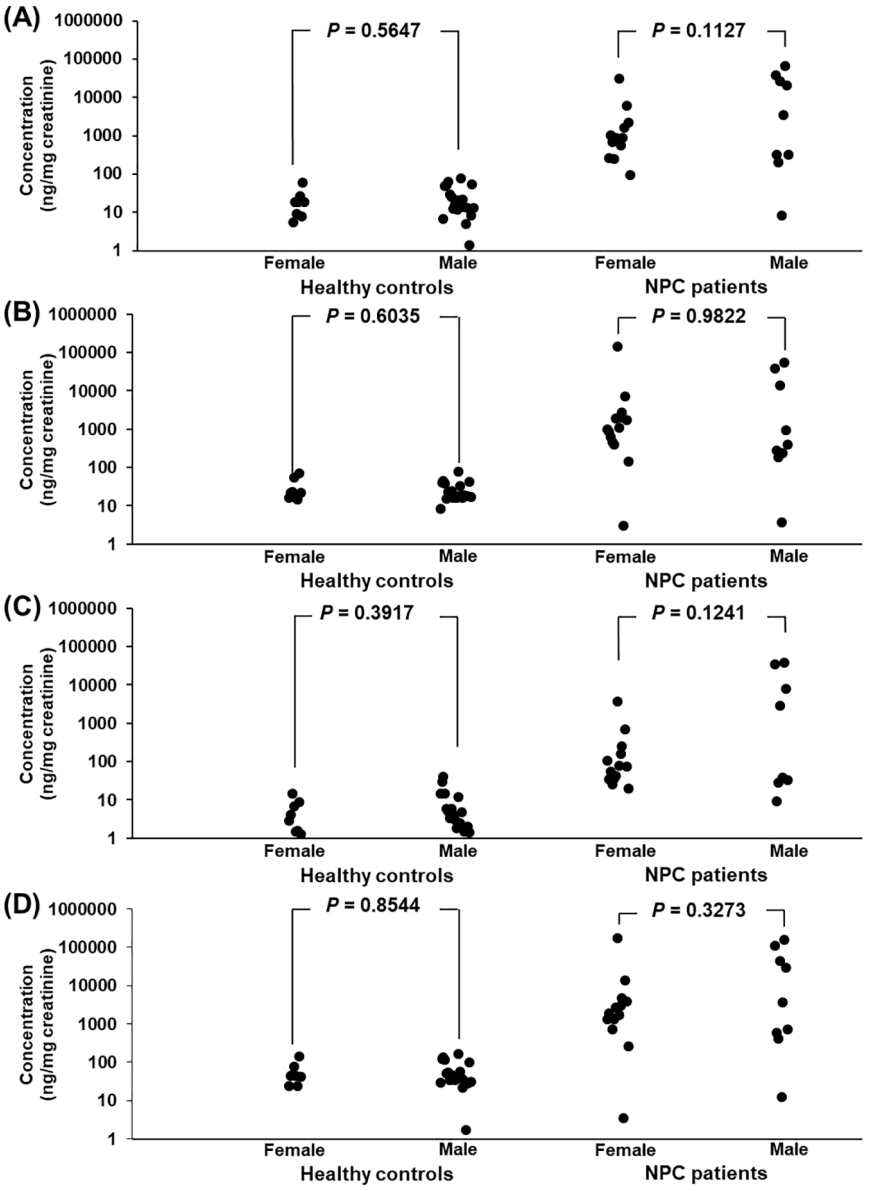


Uncorrected concentrations of SNAG-Δ^5-CA (A), SNAG-Δ^5-CG (B), SNAG-Δ^5-CT (C), and their total concentration (D) in the urine of healthy controls and NPC patients. SNAG-Δ^5-CA, SNAG-Δ^5-CG, and their total concentration in the urine of NPC patients were significantly higher than those in healthy controls.

Figure 4

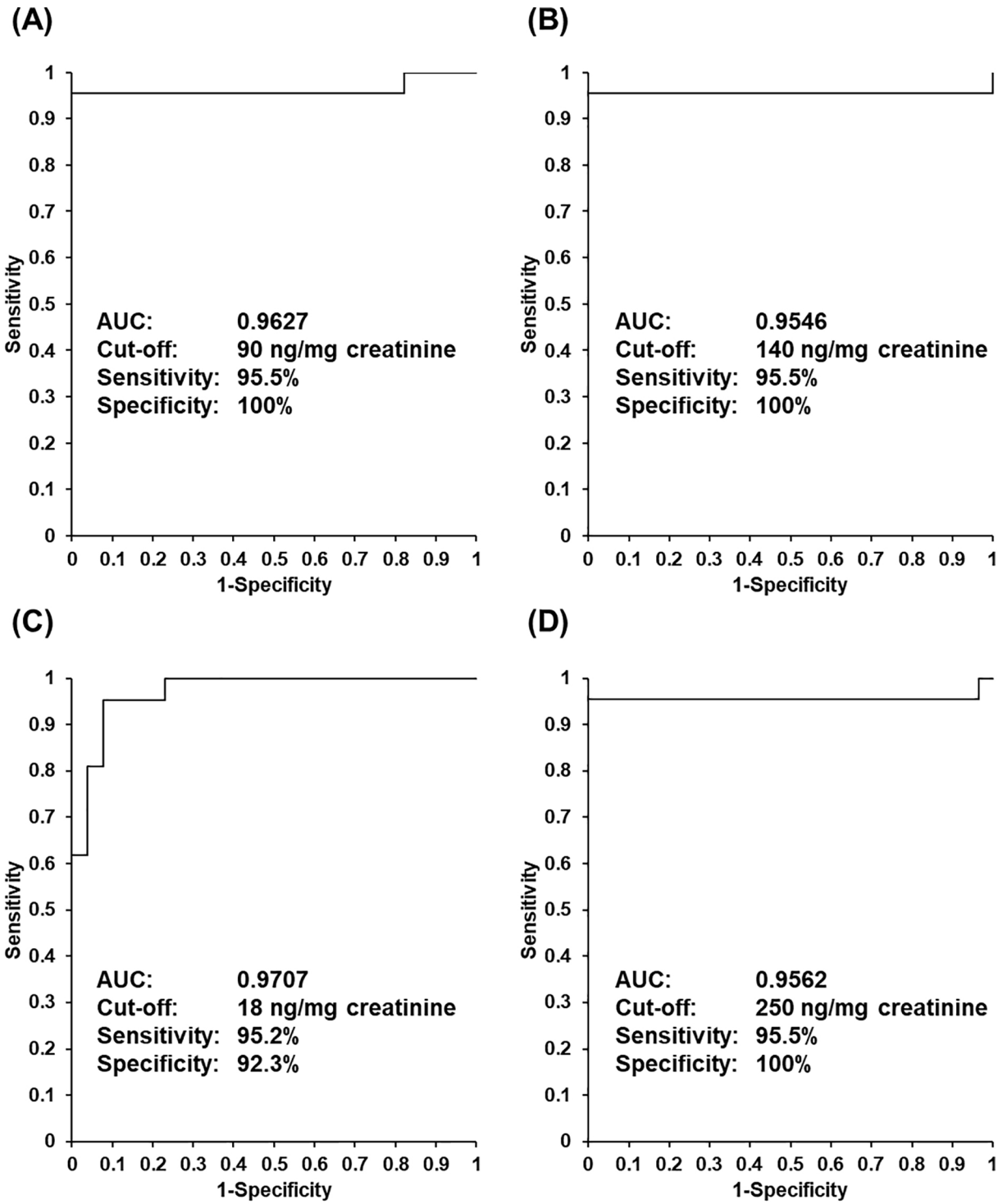


The correlation of age and the concentrations of SNAG-Δ^5-CA (A and B), SNAG-Δ^5-CG (C and D), SNAG-Δ^5-CT (E and F), and their total concentration (G and H) in the urine of healthy controls (A, C, E, G) and those of NPC patients (B, D, F, H).



The correlation between sex and the concentrations of SNAG-Δ^5-CA (A), SNAG-Δ^5-CG (B), SNAG-Δ^5-CT (C), and their total concentration (D) in the urine of healthy controls and those of NPC patients. There was no significant difference between all analytes.

Figure 6



Receiver operating characteristic analysis results for the urinary concentrations of SNAG-Δ^5-CA (A), SNAG-Δ^5-CG (B), SNAG-Δ^5-CT (C), and the total concentration (D).