



The fate of orally administered sialic acid: First insights from patients with N-acetylneuraminic acid synthase deficiency and control subjects

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ABSTRACT

Background: In NANS deficiency, biallelic mutations in the N-acetylneuraminic acid synthase (NANS) gene impair the endogenous synthesis of sialic acid (N-acetylneuraminic acid) leading to accumulation of the precursor, N-acetyl mannosamine (ManNAc), and to a multisystemic disorder with intellectual disability. The aim of this study was to determine whether sialic acid supplementation might be a therapeutic avenue for NANS-deficient patients.

Methods: Four adults and two children with NANS deficiency and four adult controls received oral NeuNAc acid (150 mg/kg/d) over three days. Total NeuNAc, free NeuNAc and ManNAc were analyzed in plasma and urine at different time points.

Results: Upon NeuNAc administration, plasma free NeuNAc increased within hours ($P < 0.001$) in control and in NANS-deficient individuals. Total and free NeuNAc concentrations also increased in the urine as soon as 6 h after beginning of oral administration in both groups. NeuNAc did not affect plasma and urinary ManNAc, that remained higher in NANS deficient subjects than in controls (day 1–3; all $P < 0.01$). Oral NeuNAc was well tolerated with no significant side effects.

Discussion: Orally administered free NeuNAc was rapidly absorbed but also rapidly excreted in the urine. It did not change ManNAc levels in either patients or controls, indicating that it may not achieve enough feedback inhibition to reduce ManNAc accumulation in NANS-deficient subjects. Within the limitations of this study these results do not support a potential for oral free NeuNAc in the treatment of NANS deficiency but they provide a basis for further therapeutic approaches in this condition.

Abbreviations used

CTCAE	common Terminology Criteria for Adverse Events
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GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine

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GRAS	generally recognized as safe
HILIC	hydrophilic interaction liquid chromatography
IMS	ion mobility mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
ManNAc	<i>N</i> -acetyl mannosamine
NANSd	<i>N</i> -acetylneuraminic acid synthase deficiency
NeuNAc	<i>N</i> -acetylneuraminic acid
NMR	nuclear magnetic resonance
OMIM	Online Mendelian Inheritance in Man
RPLC-MS/MS	reversed phase liquid chromatography tandem mass spectrometry

1. Background

The concept of dietary intervention to influence genetic-metabolic disease began around seventy years ago when dietary restriction of phenylalanine was shown to have a positive influence of the neuro-cognitive outcome of children affected with phenylketonuria (1). Since then, dietary therapy has become the mainstay of treatment of many inherited metabolic diseases, proving that reduction of, or supplementation with specific nutrients can profoundly influence affected metabolic pathways and change the clinical outcome.

N-acetylneuraminic acid synthase deficiency (NANSd; OMIM #605202) is a recessively inherited disorder presenting clinically with intellectual development disorder, skeletal dysplasia and dysmorphic features (2). Biallelic mutations in the *NANS* gene cause a block in the endogenous synthesis of *N*-acetyl neuraminic acid (NeuNAc, commonly called sialic acid) and accumulation of the precursor, *N*-acetylmannosamine-6-P (ManNAc-6P). Phosphorylated sugars are not transported out of the cell, and thus, only free *N*-acetylmannosamine (ManNAc) is increased in plasma and urine. Consequently, free ManNAc accumulates in body fluid (Fig. 1).

Sialic acid is a monosaccharide with an almost ubiquitous distribution in the human body. It is particularly enriched in brain gangliosides and glycoproteins (3,4). It is an essential nutrient for brain development,

cognition and memory in animals. It seems likely that deficiency of this enzyme impairs the sialylation of glycolipids and glycoproteins in human (2,5–10). Capacity for endogenous synthesis of sialic acid has been shown to be limited when the demand is high such as during infancy when organs, and especially the brain, are growing rapidly (7). This suggests that children with NANSd could be particularly vulnerable to deficiency of this essential nutrient for brain development and cognition.

The precise metabolic fate and the relative contribution of endogenous and dietary sources of sialic acid in human remains largely unknown and extrapolation from current observations remains difficult. Nevertheless, promising evidence indicates that dietary supplementation might help patients. First, the human body is able to utilize the bound and free form of sialic acid from dietary sources, which is thus considered a safe nutrient. Second, dietary sialic acid in its free form is absorbed and incorporated into biosynthetic pathways of sialylation in human cell lines (11–19). Third, the skeletal phenotype of NANSd zebrafish embryos was partially rescued by administration of exogenous sialic acid (2). Likely a shortage of de novo synthesized NeuNAc causes the phenotype.

The aim of this study was to test the metabolic fate of orally administered free NeuNAc in NANSd patients and controls, and to assess its safety and tolerance in terms of potential gastrointestinal side effects in short-term use. The administration scheme of three doses of NeuNAc with meals during 3 days was intended to mimic supply by dietary ingestion in healthy individuals.

2. Material and Methods

2.1. Subjects

Less than 20 patients with NANSd have been identified or reported worldwide so far ((2) and unpublished observations). From these previously reported cases, four adults from two families (2 sisters, 1 brother and 1 sister) with NANSd (3 females, 1 male) and 4 controls (2 males, 2

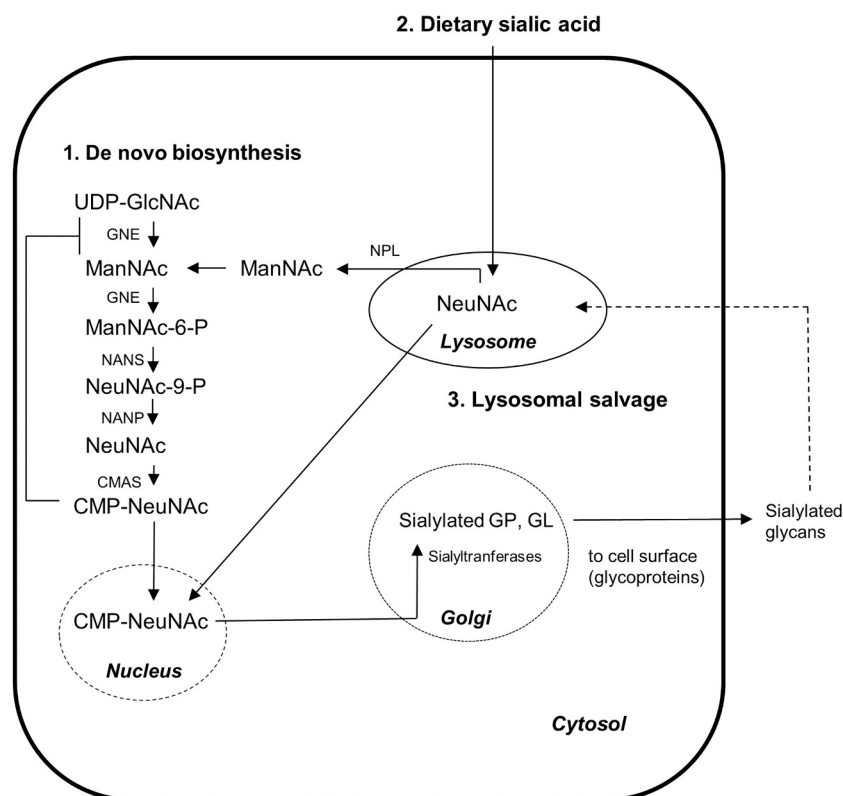


Fig. 1. Sialic acid metabolism.

Sialic acid (NeuNAc) synthesis is catalyzed by several enzymes; UDP-GlcNAc 2 epimerase/ManNAc-6-kinase (GNE), NeuNAc synthase (NANS) and NeuNAc phosphatase (NANP). NeuNAc is converted to CMP-NeuNAc by CMP-sialic acid synthase (CMAS). CMP-NeuNAc is transported to the Golgi apparatus for sialylation of glycoproteins (GP) and glycolipids (GL). Within the cytoplasm, CMP-NeuNAc binds to GNE providing negative feedback regulation of sialic acid de novo biosynthesis. Released NeuNAc from lysosomes (lysosomal salvage) are pumped back to the cytosol and reenter another cycle of sialylation in the Golgi apparatus or can be converted to ManNAc by NeuNAc pyruvate lyase (NPL). Sialylated glycans made in the Golgi apparatus require sialic acid (NeuNAc) derived from 1. De novo biosynthesis, 2. diet and 3. recycled glycans (lysosomal salvage). Variants in *NANS* impair de novo biosynthesis and suggest that dietary NeuNAc might supplement the impaired de novo biosynthesis. GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetyl mannosamine; NeuNAc, *N*-acetylneuraminic acid. Adapted from (2,30,43).

females) were included in the study. In addition, two children (brother and sister) with NANS deficiency (NANSd, 1 female, 1 male) were included in the study and analyzed separately. The NANS genotype of all NANSd subjects was established in the Division of Genetic Medicine (Lausanne University Hospital). The variants found in the four adult patients were published previously (2), while the previously unpublished pediatric patients had one previously published variant and a novel missense variant. Adult control subjects were recruited from the general population of Lausanne (Switzerland). The controls were in good health with a normal body mass index ($BMI < 30 \text{ kg/m}^2$) and did not take any medication. Before inclusion, they underwent a physical examination to ensure good physical health. All participants (or their legal representative) provided written informed consent. This pilot study was approved by the Swiss Ethics Committee on research involving humans (Approval # 2018-00284), the Ethics Committee of Area Vasta Emilia Nord, Italy (Approval # 2018/0112727) and was registered at the U.S Clinical Trials Registry as NCT03545568 (first posted 04/06/2018). The study was conducted in accordance with the declaration of Helsinki.

2.2. Study Design

During the 3 days preceding the first visit, subjects were asked to consume a low sialic acid diet ($<10 \text{ g/day}$, *counseled* by a dietitian). On the fourth day (day 0), adult NANSd, control and pediatric NANSd subjects reported to the different study sites for a screening visit. From day 1 to day 4, participants reported to the study sites at 8:00 AM after a 12-h fast. Upon arrival, their weight, blood pressure and heart rate were recorded. NeuNAc dosing occurred 3× daily for 3 consecutive days. They received 50 mg/kg free NeuNAc t.i.d (total 150 mg/kg/d, max 12 g/d). Free NeuNAc was provided as a powder to be dissolved in water flavored with conventional syrup to a final concentration of 5% (e.g. 10 g NeuNAc/200 mL flavored water). On day 1, participants were studied over 6 h after ingestion of free NeuNAc. Blood and urine were collected at baseline, 2, 4 and 6 h after ingestion of the 1st free NeuNAc dose for quantitative measurements of metabolites. On day 2 and 3 blood was collected 2 h after the first free NeuNAc dose of the day and urine was collected at baseline, 2 h and 6 h after the first free NeuNAc dose in adult participants only. For practical reasons, three random urine samples were collected in children. Last dose of free NeuNAc was given in the evening of day 3. Adult individuals and the legal representative of pediatric NANSd patients recorded all food and beverages consumed from day 1 to day 3 in a dietary diary. A dietitian estimated the sialic acid and energy intake from the records of each participant. On day 4 (follow-up visit), no supplementation was given. Blood and urine were collected after an 8-h overnight fast in adults and children (Fig. 2).

2.3. Product Description

The monosaccharide *N*-Acetyl-D-neuraminic acid (NeuNAc, Neu5Ac,

“sialic acid”) is a 9-carbon sugar found in mammals. The IUPAC name is 5-acetamido-3,5-dideoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid and the CAS registry number is 131-48-6. The predominant form is a dehydrate form and presents as a water soluble white crystalline powder. NeuNAc is “generally recognized as safe” (GRAS) (20) for use as a food ingredient for children above 10 years by EFSA (21) and also in term formulas for infant by the FDA (17,20). NeuNAc is produced by Glycom (Denmark) and has been provided by the Nestlé Research Center, Lausanne Switzerland.

2.3.1. Dose Calculation and Administration

In our protocol, we administered free NeuNAc at a dose of $\sim 150 \text{ mg/kg/day}$ (maximum 12 g/d). By analogy with previous studies using a slow-release form of NeuNAc (SA-ER: sialic acid extended release (22) or ManNAc supplementation (23) including a completed Open-Label phase 2 study (NCT02346461) where 6 g/d and 12 g/d, respectively, were considered as safe for GNE myopathy, we chose to limit our dose of free NeuNAc to a maximal dose of 12 g/d.

2.3.2. Safety

Safety was assessed by physical examination, body weight, vital signs and clinical laboratory tests. The severity of adverse events was graded according to the Common Terminology Criteria for Adverse Events (CTCAE), version 5.0. Vital signs were collected during screening visit (day 0), pre-dose on day 1 and before the first drug administration on day 2, 3 and at the end of the test (day 4). Clinical laboratory tests were performed at baseline and at the end of the study (day 4) and included alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and glucose. Glomerular filtration rate was estimated (eGFR) from CKD-EPI equation (24).

2.4. Analytical Procedures

Blood for kinetic analysis was collected in lithium heparin coated tubes. After blood collection, samples were kept on ice and centrifuged (4°C for 15 min at 3000g) within 30 min to obtain plasma samples. Each plasma was aliquoted in cryogenic vials that were immediately frozen at -80°C . Samples were shipped on dry ice to the analytical lab (Metabolomic Platform, UNIL) for free NeuNAc, total NeuNAc and ManNAc measurements.

2.4.1. Sample Preparation for Measurement of Free NeuNAc and ManNAc

Plasma samples (100 μL) were extracted by the addition of ice-cold MeOH (415 μL) spiked with internal standards ($^{13}\text{C}_3$ -NeuNAc, ManNAc- d_3 ; Toronto Research Chemicals, North York, ON, Canada). Samples were then centrifuged for 15 min at 21000g at 4°C and the resulting supernatants were transferred to 96-well plates and dried under Nitrogen at 25°C (TurboVap96, Biotage, Uppsala, Sweden). Dried extracts were re-suspended in water (100 μL), vortexed, sonicated for 1 min and centrifuged for 15 min at 21000g at 4°C . Urine samples (10 μL) were

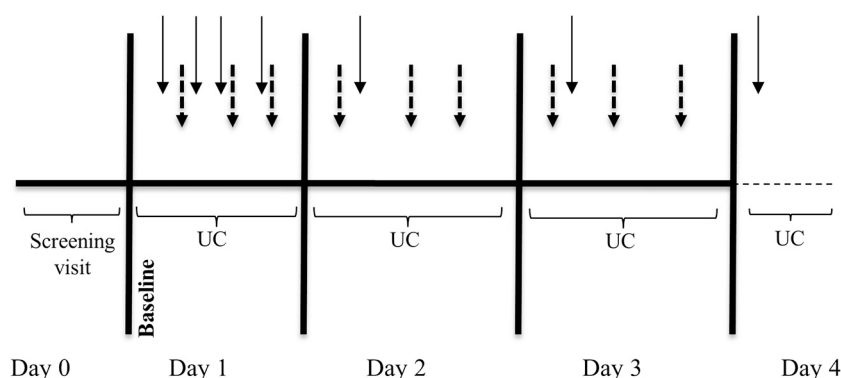


Fig. 2. Experimental setting of the metabolic test.

Each subject was studied over 4 days. From day 1 to day 3, each subject was supplemented with 150 mg/kg/d NeuNAc divided in 3 doses (1st dose at 8.30 am, 2nd dose at 12.30 am and 3rd dose at 5.30 pm). Symbols: ↓ = Blood sampling in children and adults. Blood sampling on day 1 was done 4× (baseline, +2 h, +4 h and +6 h after 1st dose of NeuNAc supplementation), on day 2 and 3, 1× (+2 h after NeuNAc supplementation) and on day 4, 1× (fasting). UC = Urine collection, UC in adults: UC on day 1 was done 3× (baseline, +2 h and +6 h after NeuNAc supplementation), on day 2 and 3, 3× (fasting, +2 h, +6 h after NeuNAc supplementation) and on day 4, 1× (fasting). UC in children: 2–3 random urine samples/day. UC was collected 1× on day 4 (fasting). ↓ = administration of 50 mg/kg free *N*-acetylneuraminic acid (NeuNAc).

diluted 1/10 with water containing internal standards (90 μL), centrifuged for 15 min at 21000g at 4 °C. The resulting supernatants of plasma and of urine extracts were transferred to LC-MS vials for the LC-MS/MS analysis.

2.4.2. Sample Preparation for the Measurement of Total NeuNAc

Plasma and urine samples (25 μL) were extracted by the addition of water (15 μL) spiked with internal standard ($^{13}\text{C}_3$ -NeuNAc) and 60 μL of sulfuric acid at 63 mM. Samples were then incubated at 80 °C for one hour and cooled-down at room temperature for 10 min prior to centrifugation for 15 min at 21000g at 4 °C. The resulting supernatant was transferred to LC-MS vials for the LC-MS/MS analysis.

2.4.3. Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Measurement

Free NeuNAc, ManNAc, and total NeuNAc, in both plasma and urine, were measured by quantitative stable-isotope dilution assisted assay where reversed phase liquid chromatography was coupled with tandem mass spectrometry (RPLC-MS/MS) in positive ionization mode using a 6495 triple quadrupole system (QqQ) interfaced with 1290 UHPLC system (Agilent Technologies, Santa Clara, US). The chromatographic separation was carried out in an Acquity UPLC HSS T3, 1.7 μm , 100 mm \times 2.1 mm I.D. column (Waters, Massachusetts, US). Mobile phases were composed of A = 0.2% formic acid in water and B = 0.2% formic acid in MeOH. The linear gradient elution from 100% A (0–2 min) to 50% A (2 min - 4 min) and then down to 10% A (4 min - 5 min) was applied and held for 2 min, followed by the initial chromatographic conditioning during the 3 min post-run for column re-equilibration. The flow rate was 400 $\mu\text{L}/\text{min}$, column temperature 25 °C and sample injection volume 2 μL . ESI source conditions were set as follows: dry gas temperature 250 °C, nebulizer 35 psi and flow 15 L/min, sheath gas temperature 400 °C and flow 8 L/min, nozzle voltage 1000 V, and capillary voltage 3000 V. The data were acquired in a dynamic Multiple Reaction Monitoring (dMRM) mode with a total cycle time of 500 ms. Two transitions were used to monitor each compound, and quantifiers m/z 310 \rightarrow 274 and m/z 222 \rightarrow 126 were used for the quantification of NeuNAc and ManNAc, respectively.

2.4.4. Data Processing

Raw LC-MS/MS data were processed using the Agilent Quantitative analysis software (B.07.00, MassHunter Agilent technologies). For absolute quantification, calibration curves and the stable isotope-labeled internal standards (IS) were used to determine the response factor.

2.5. Statistics

Baseline clinical characteristics, plasma and urine data were summarized by their mean (SD). Summaries were reported by group as well as by group and day. Groups differences of baseline patient's characteristics were tested using an unpaired Mann-Whitney test (25). The outcomes (plasma and urine data) distribution were visually inspected using the Quantile-quantile plots and histograms. The normality assumption was clearly violated for all outcomes. A Zero-skewness log transform was then applied for each outcome. Linear Mixed models using Time (T), Condition (C) and their interaction were used to test for the difference between the two groups, the changes over time and the difference in the dynamic of change between groups (TxC). Data from pediatric patients were not included in the statistics due to limited number of patients and absence of controls, but were described individually. Analyses were performed using Stata 16 software (StataCorp. 2019. Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC).

3. Results

3.1. Adult Subjects' Characteristics and Safety Biological Variables at Baseline

Clinical characteristics and blood metabolites of adult controls and NANSd are summarized in Table 1. Eight adults (5 females and 3 males) and two pediatric subjects (1 female and 1 male, siblings) were enrolled in the study. The mean age of NANSd and control subjects was 39.70 years (± 5.54) and 35.42 years (± 7.48), respectively; mean body mass index (BMI) was 20.19 kg/m^2 (± 1.64) and 24.70 kg/m^2 (± 3.50) kg/m^2 , respectively. All subjects had normal creatinine, eGFR, AST, ALT, glucose and blood count. Among NeuNAc metabolites, total plasma and urine free NeuNAc did not differ between both groups (all $P > 0.1$) and were within normal values for reference ranges in healthy humans, suggesting that there was no systemic depletion of free NeuNAc in NANSd (26–28). However, plasma and urine ManNAc were higher in NANSd compared to controls (all $P < 0.05$) thus confirming the enzymatic block in NANSd leading to precursor accumulation.

3.2. Free and Total NeuNAc Concentrations in Plasma and Urine (From Day 1 to Day 4)

Individual plasma concentrations across time of free and total NeuNAc during day 1 are shown in Fig. 3A,B. Oral doses of NeuNAc supplementation significantly increased mean plasma free NeuNAc over time (T effect: $P < 0.001$) and this increase was higher in controls than NANSd at time T + 6 h (T \times C effect: $P < 0.05$). However, acute supplementation did not affect plasma total NeuNAc (T effect: $P > 0.1$). On day 2 to 3, plasma free and total NeuNAc did not differ between groups (Fig. 3C,D). On day 4, free NeuNAc did not differ between groups ($P > 0.1$) while total NeuNAc was higher in controls compared to NANSd ($P < 0.05$).

Individual urinary concentrations across time of free and total

Table 1
Baseline clinical characteristics and plasma variables of adult study participants.

	Controls	NANSd
Clinical characteristics		
Age, y	35.42 \pm 7.48	39.70 \pm 5.54
Weight, kg	77.37 \pm 7.97	34.37 \pm 2.89*
BMI, kg/m^2	24.70 \pm 3.50	20.19 \pm 1.64
Systolic BP, mmHg	132.5 \pm 10.25	101.25 \pm 2.50*
Diastolic BP, mmHg	80.5 \pm 5.26	70.75 \pm 6.50
Heart rate, beats/min	69.75 \pm 1.26	80.5 \pm 6.61*
Analysis in Plasma		
Fasting glucose (mmol/L)	5.05 \pm 0.21	4.90 \pm 0.22
Creatinine ($\mu\text{mol}/\text{L}$)	77.5 \pm 21.70	49.34 \pm 4.77
CKD-EPI_noBSA (mL/min)	110 \pm 8.87	77.25 \pm 7.54
AST (U/L)	23.75 \pm 3.40	20.00 \pm 4.97
ALT (U/L)	17.75 \pm 2.63	14.25 \pm 3.10
Hemoglobin (g/dL)	14.72 \pm 8.06	12.63 \pm 6.81
Platelet count ($\times 10^4/\mu\text{L}$)	24.62 \pm 50.95	22.00 \pm 67.09
WBC (G/L)	5.02 \pm 1.43	4.48 \pm 0.72
Free NeuNAc ($\mu\text{mol}/\text{L}$)	0.65 \pm 0.11	0.65 \pm 0.08
Total NeuNAc ($\mu\text{mol}/\text{L}$)	1977.17 \pm 111.36	1882.14 \pm 261.92
ManNAc ($\mu\text{mol}/\text{L}$)	1.51 \pm 0.24	2.78 \pm 0.58*
Analysis in Urine		
Free NeuNAc ($\mu\text{mol}/\text{mmol creatinine}$)	5.92 \pm 1.57	9.09 \pm 1.72
Total NeuNAc ($\mu\text{mol}/\text{mmol creatinine}$)	20.96 \pm 4.87	26.29 \pm 2.25
ManNAc ($\mu\text{mol}/\text{mmol creatinine}$)	85.01 \pm 8.03	183.45 \pm 9.0*

Values presented as mean \pm SD ($n = 4$ subjects per group). Changes were assessed by a Mann-Whitney unpaired test. * $P < 0.05$ vs Controls. AST, aspartate aminotransferase; ALAT, alanine aminotransferase; BMI, body mass index; BP, blood pressure; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration eGFR equation; CKD-EPI_noBSA, CKD-EPI equation without BSA normalization; NANSd, N-acetylneuraminic acid synthase deficiency; ManNAc, N-acetylmannosamine; NeuNAc, N-acetylneuraminic acid; WBC, white blood count.

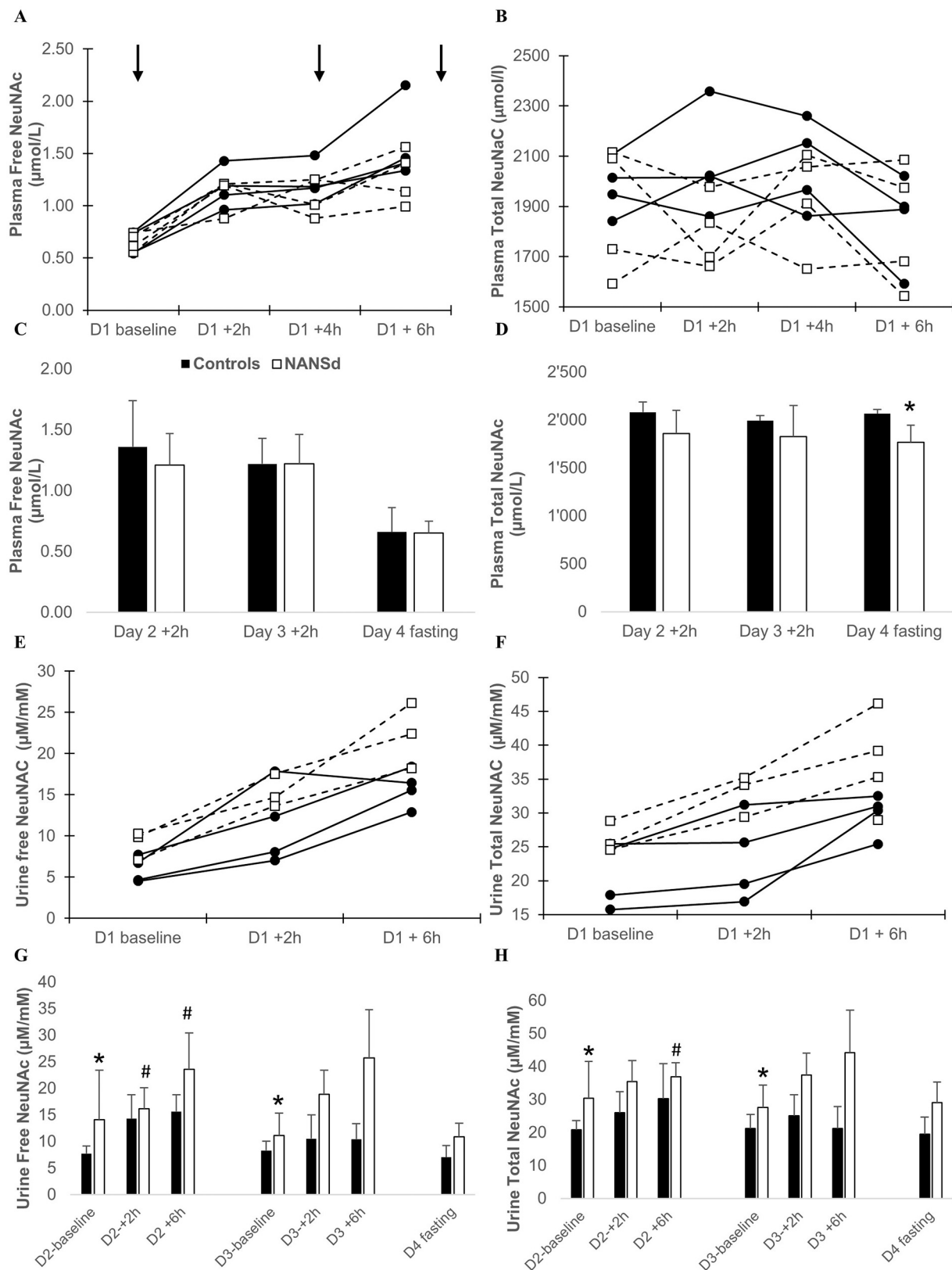


Fig. 3. Concentrations of plasma and urinary free and total NeuNAc in adults after ingestion of 150 mg/kg/d N-Acetyl-D-neuraminic acid (NeuNAc) from day 1 to day 4.

Day 1: individual plasma (A-B) and urinary (E-F) concentrations of total NeuNAc and free NeuNAc across time points after NeuNAc supplementation (*n* = 4 per group). Metabolites in urine are expressed relative to creatinine concentrations.

NeuNAC relative to creatinine concentrations during day 1 are shown in Fig. 3E,F. For one patient, only one urinary measure was available (day 1, T + 6 h). Mean urinary creatinine was not affected in both groups in the first 6 h (T effect: $P > 0.1$; T x C effect: $P > 0.1$). Urinary free and total NeuNAC increased in the first 6 h after supplementation (both T effect: $P < 0.01$), yet similarly between both groups (both T x C effect: $P > 0.1$; Fig. 3E,F). On day 2, free and total NeuNAC were higher in NANSd (both C effect: $P < 0.05$) and increased in the first 6 h after supplementation (both T effect: $P < 0.01$), yet similarly between groups (both T x C effect: $P > 0.1$; Fig. 3G,H). On day 3, urinary free and total NeuNAC were higher in NANSd but were not affected over time (both T effect: $P > 0.05$; T x C effect: $P > 0.05$; Fig. 3G,H). On day 4, urinary concentrations did not differ between groups (both $P > 0.1$; Fig. 3G,H).

3.3. ManNAC in Plasma and Urine (From Day 1 to Day 4)

Individual plasma concentrations across time of ManNAC during day 1 are shown in Fig. 4A. Plasma ManNAC was higher in NANSd than in controls throughout the 6 h but was not affected by supplementation over time (C effect: $P < 0.001$; T and T x C effect: both $P > 0.1$). On day 2 to 4, plasma ManNAC remained higher in NANSd compared to controls ($P < 0.05$; Fig. 4B).

Individual urinary concentrations across time of ManNAC relative to creatinine concentrations during day 1 are shown in Fig. 4C. For one patient, only one urinary measure was available (day 1, T + 6 h). Mean urinary ManNAC was not affected over time (T effect: $P > 0.1$; T x C effect: $P > 0.1$) and remained higher in NANSd (C effect: $P < 0.01$). On

day 2 and 3 urinary ManNAC was not affected over time (both T effect: $P > 0.1$) and remained higher in NANSd (both C effect: $P < 0.01$; Fig. 4D). A slight decrease of ManNAC was observed in NANSd only during day 2 at time T + 2 h and T + 6 h (T x C: $P < 0.05$). On day 4, ManNAC tended to remain higher in NANSd ($P = 0.057$), with concentration close to the baseline value prior to supplementation (day 1 baseline).

Statistical analyses for linear mixed models are summarized in Table S1.

3.4. Dietary Records During NeuNAC Supplementation

A 3-day dietary record was taken during the experiments to measure sialic acid intake (mg/kg) and energy intake (kcal/d) from regular diet. Control and NANSd participants had similar sialic acid and energy intake over the 3 days (both $P > 0.05$). Sialic acid ingested from diet (mg/kg/d) during day 1 to day 3 was 0.54 and 0.42, respectively.

3.5. Experiment With Pediatric NANSd

Clinical characteristics, plasma and urinary metabolic variables of pediatric patients at baseline (day 1) and end of the test (day 4) are summarized in Table S2. Among NeuNAC metabolites in plasma and urine, total and free NeuNAC concentrations were within reference ranges while plasma and urinary ManNAC were elevated (2,26–28). Oral administration of free NeuNAC increased plasma free NeuNAC over 2 h after supplementation and decreased rapidly within the same day (Day 1; Fig. S1A). Urinary free and total NeuNAC increased 2 h after the first

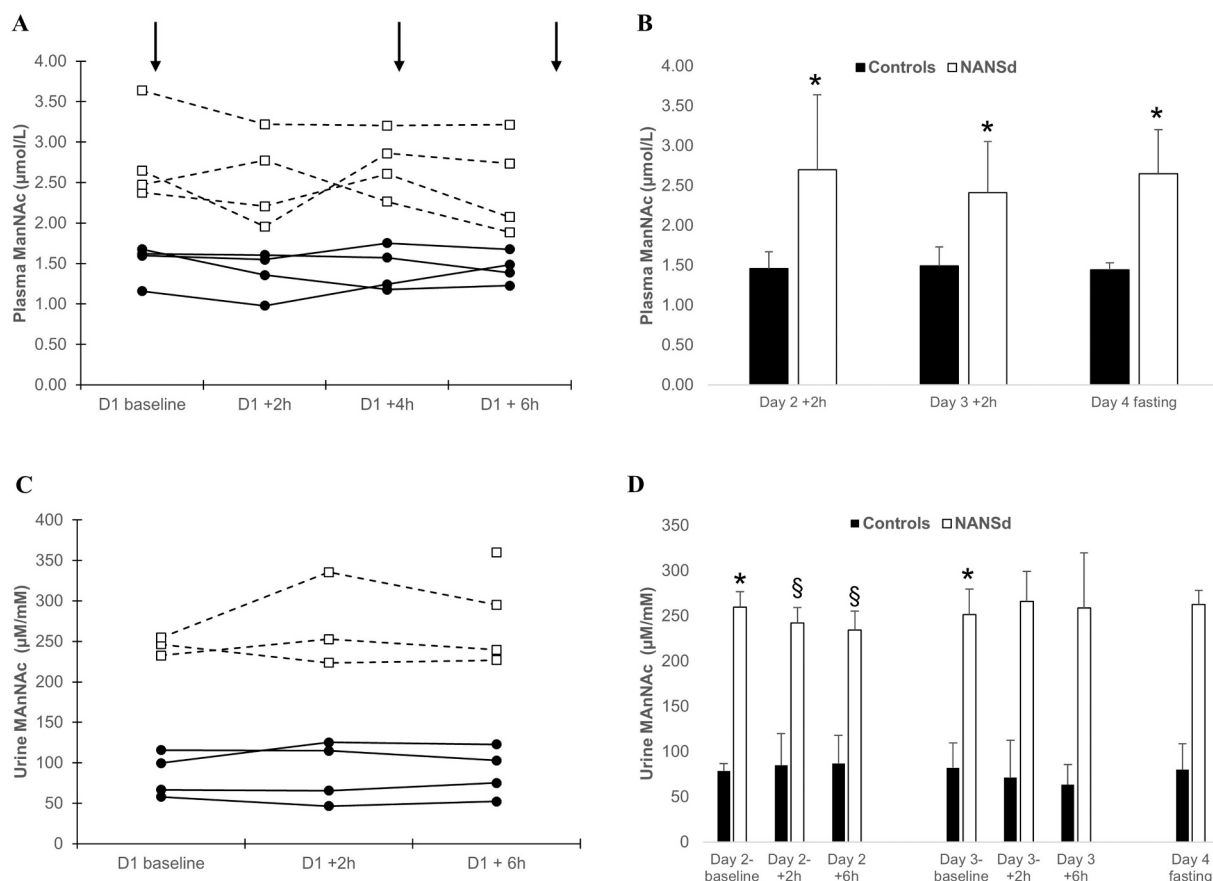


Fig. 4. Concentrations of plasma and urinary ManNAC in adults after ingestion of 150 mg/kg/d N-Acetyl-D-neuraminic acid (NeuNAC) from day 1 to day 4. *Day 1:* individual plasma (A) and urinary (C) concentrations of ManNAC across time points after NeuNAC supplementation ($n = 4$ per group). Metabolites in urine are expressed relative to creatinine concentrations. *Day 2 to day 4:* Values of plasma (B) and urinary (D) ManNAC are presented as mean \pm SD ($n = 4$ subjects per group). *Statistics:* same as for Fig. 3. *Symbols:* $\downarrow = 50$ mg/kg N-Acetyl-D-neuraminic acid (NeuNAC). D, Day; NANSd, N-acetylneuraminic acid synthase deficiency; NeuNAC, N-acetylneuraminic acid; ManNAC, N-acetyl-D-mannosamine. For all panels, white squares are patients, black circles are controls.

dose of supplementation (Fig. S1D-E). Sialic acid ingested from the diet (mg/kg/d) was 11.19 ± 2.25 for patient 1 and 13.66 ± 3.38 for patient 2 over the three days of the test.

3.6. Safety and Tolerability

All subjects (adults and children) completed study treatment. Free NeuNAc administered orally was well tolerated with no serious or severe adverse event reported. A self-resolving episode of flatulence, which was not associated with abdominal pain or other gastrointestinal symptoms (Grade 1: mild symptoms) was reported in one adult control subject shortly after ingestion of free NeuNAc. No other relevant changes in clinical parameters, vital signs including heart rate or blood pressure, or laboratory values (creatinine, eGFR, ALT, AST and glucose) were observed during the study (Table S3).

4. Discussion

Given the experimental evidence that alimentary sialic acids can be absorbed and incorporated into the sialylation pathway (6), the aim of our study was to explore the short term fate of oral free NeuNAc as a potential therapeutic approach for NANS deficiency.

4.1. Kinetics of Absorption and Excretion of Free NeuNAc

Ingestion of free NeuNAc was safe during short-term administration and well tolerated in all individuals (adults and children). NeuNAc administration was followed by an increase of free NeuNAc concentrations in both, plasma and urine samples of NANSd and controls in a matter of hours, suggesting that oral doses are rapidly absorbed in the systemic circulation and excreted in urine, which is in line with results from animal studies (10,12). Thus, oral NeuNAc is rapidly absorbed and rapidly excreted, with no signs of toxicity or significant clinical side effects after short-term administration.

4.2. Metabolic Fate of Oral Free NeuNAc

Under normal conditions, NeuNAc derived from endogenous synthesis is converted to CMP-NeuNAc in the nucleus and/or cytosol and transported in the Golgi complex where it is used for the sialylation of glycans and, at the same time, reduces ManNAc concentration by feedback inhibition on UDP-GlcNAc epimerase 2 (29). Thus, one of the aims of this study was to ascertain whether oral NeuNAc was able to exert negative feedback on the synthesis of ManNAc. Our results showed that short-term (3 days) NeuNAc administration did not reduce ManNAc concentrations in plasma or urine of NANSd. We cannot exclude that the administered free NeuNAc may have been partially hydrolyzed to ManNAc by *N*-acetylneuraminidase pyruvate lyase (NPL) using the catabolic pathway (16,30) (Fig. 1). This might be responsible for the failure of reducing ManNAc levels. The relative importance of the two pathways (direct conversion of free NeuNAc to CMP-NeuNAc vs conversion to ManNAc and reentry to the biosynthesis pathway) is unknown and worth investigating. Thus, we must conclude that at least in this short-term experimental setting, oral NeuNAc supplementation failed to exert a significant feedback inhibition and to lower ManNAc concentrations in plasma and urine, indicating that it might have not reached the NeuNAc-synthesizing cells and their Golgi apparatus. This observation could be explained by the rapid urinary excretion of free NeuNAc in excess. Animal studies have shown that the majority of oral NeuNAc (~90%) was efficiently absorbed through the gastrointestinal tract, reaching the systemic circulation within 30 min to 1 h of administration, and peak plasma concentrations attained within 1.5 to 4 h. Only a tiny proportion of the absorbed NeuNAc was distributed to other organs, mainly liver and brain, within 6 h (max 3–4% of the administered dose, respectively) and the majority (at least 60–90%) of ingested NeuNAc was excreted unchanged in the urine after 6 h (10–12). Cellular and

animal studies have shown an increase in cerebral and cerebellar gangliosides and glycoprotein NeuNAc concentrations upon administration of intravenous, intraperitoneal, and/or oral NeuNAc (14,19,31,32). While the majority is excreted in the urine, a still significant proportion of bolus dose of free NeuNAc was incorporated into sialylglycoconjugates.

4.3. Pharmacological Considerations: Is Free NeuNAc the Best Way to Stimulate Sialylation In Vivo?

There is experimental evidence from animal studies that glycoconjugates of sialic acid are incorporated to a higher extent than free NeuNAc. The fact that free NeuNAc is excreted in the urine almost as rapidly as it is absorbed may partially explain this observation. Sialic acid bound in a glycoside might be absorbed more slowly and/or escape urinary excretion, thereby being a more efficient source for metabolic incorporation (11,33,34). A nutritional intervention study compared a sustained-release preparation of sialic acid (6'-sialyllactose) to free NeuNAc in a mouse model of UDP-*N*-acetylglucosamine 2-epimerase (GNE) myopathy (35), in which endogenous synthesis of sialic acid is compromised by variants in the *GNE* gene (OMIM 603824). In this study, 6'-sialyllactose was shown to have a significant advantage over free NeuNAc in increasing the concentration of sialic acid in muscle. Unfortunately, a phase 3 study evaluating slow release sialic acid (Ultragenyx) in patients with GNE myopathy did not find any improvement in muscle strength, resulting in the discontinuation of the product's development by the company (36,37). This suggests that even if a slowly metabolized form of sialic acid allows a better incorporation in tissues, it may be not be sufficient to clinically improve their function. Thus, in order to further explore the possibility of an oral therapy with NeuNAc in NANSd patient, attention must be paid to the pharmacokinetics and tissue distribution of the different forms and dosing regimens of NeuNAc in different age groups (i.e. immediate, sustained, crossing the blood-brain barrier) as well as its conversion to CMP-sialic acid and incorporation into glycoconjugates. Sugars required for protein glycosylation can be made de novo, salvaged from degraded glycans or captured from diet. The problem is that we do not know how much each pathway contributes to glycosylation in different cells. Thus, we cannot formally predict at this stage whether a sugar supplement will be beneficial and in which form (38). To date, there have been no bio-analytical human studies investigating accumulation of NeuNAc in the brain upon administration of exogenous NeuNAc. Studies with glycomic and stable isotope-labeled forms of sialic acid in human would be of particular interest in this context (16,39).

Research in NANS deficiency has been hampered by the lack of an animal model. It would be of interest to generate NANS mutated mice although the metabolism of sialic acid in humans may be different than in mice, studies of labeled substrates performed on mice might answer several questions raised by our study and others and inform the design of subsequent human studies.

4.4. Limitations

From a statistical point of view, our study present some important limitations. *First*, the small sample size; it is however a limitation that cannot be improved (related to the number of cases identified worldwide who are less than 20). *Second*, individuals affected by the disease are genetically related (sisters or sister-brother). As recessive genetic disorders tend to recur in sibs, taking into account the genetic link between subjects would have required require a two-level model (level-1 = individual, level-2 = family) and reducing the number of patients to a single one per family would have halved the size of the patient samples. Further, our observation window of three days is short. If NANS deficiency entails compensatory mechanisms, such as an upregulation of ManNAc synthesis, a period of three days may not have been enough to observe a significant downregulation. In spite of our negative results

over a short period, further studies with longer observation span and/or other NeuNAc formulations may still be warranted. It is important to consider that sialic acids are present in diverse isoforms in human blood and urine. In the present study, using the applied reversed phase liquid chromatography tandem mass spectrometry (RPLC-MS/MS) methodology, ManNAc could not be distinguished from its stereoisomers, *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc), due to the lack of chromatographic resolution. A complete chromatographic separation of specific isomers could not have been reached either with hydrophilic interaction liquid chromatography (HILIC), using columns with amide and zwitterionic stationary phases in different gradient conditions. However, the partial separation of these isomers in HILIC-assays allowed us to conclude that the major isoform detectable in plasma and urine was ManNAc. Other isoforms have also likely contributed to ManNAc measurement which, as a result, might have been overestimated. More specific strategies for carbohydrate analysis, such as ion mobility mass spectrometry (IMS), capillary electrophoresis – mass spectrometry (CE-MS) and/or chiral chromatography – mass spectrometry need to be explored for future studies on sialic acid metabolism (39–42). Studies in NANS deficient patients have so far not measured a significant difference in the sialylation pattern of plasma glycoproteins; therefore we did not include this aspect in our present study. It is possible that newer more sensitive technologies may uncover such differences that might be used to monitor therapeutic interventions.

5. Conclusions

In spite of sialic acid playing such an important role in human nutrition, there seem to be no studies on the fate of orally administered free NeuNAc in healthy humans, and neither in NANS-deficient patients. Thus, the originality of our study and the insights it offers seem to be interesting and worthwhile even in spite of the small number of individuals. We investigated the effect and safety of oral free NeuNAc supplementation on endogenous synthesis of NeuNAc metabolism in patients with NANSd compared to controls. Over a short-term period of 3 days, oral free NeuNAc was well tolerated, rapidly absorbed in the systemic circulation but (unfortunately), also rapidly excreted in urine. Oral free NeuNAc administration had no apparent effect on ManNAc concentration which remained higher in NANSd compared to control individuals. This observation suggests that free NeuNAc, at least in the short term observed in this study, may be unable to enter the biosynthetic pathways and exert enough feedback inhibition to reduce ManNAc levels. As mentioned above, our study has limitations and it is therefore essential that our results and the subsequent hypotheses be validated in the future using a larger sample sizes for patients and controls and possibly a longer period of observation.

In order to make progress on the path to a possible oral therapy with NeuNAc in NANSd, attention should also be given to pharmacokinetics of various forms and doses of NeuNAc and its eventual adverse health effects. More generally, the quest for nutritional therapy for NANSd requires further exploration of the nutritional and endogenous contributions of sialic acid to glycosylation in humans. This exploration will undoubtedly advance our understanding on sialic acid biology and possibly have implications far beyond the treatment of a rare genetic disorder.

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Statement of authors' contributions to manuscript

1) C.T., D.B., S.C.B. and A.S.F. conceptualization; 2) C.T., D.B., I.I., S.G.C., S.G. and L.G. project administration; 3) T.T., H.G.A., J.I., D.L., S.G.C. and L.G. methodology; 4) T.T., H.G.A., J.I., D.L., C.T. and A.S.F. formal analysis; 5) C.T. and A.S.F. original draft; 6) All authors: writing-review, editing and validation of the final manuscript.

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Declaration of Competing Interest

The authors declare no potential conflict of interest.

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