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NANS-mediated synthesis of sialic acid is required for brain and skeletal development

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Abstract: We identified biallelic mutations in NANS, the gene encoding the synthase for N-acetylneuraminic acid (NeuNAc; sialic acid), in nine individuals with infantile-onset severe developmental delay and skeletal dysplasia. Patient body fluids showed an elevation in N-acetyl-D-mannosamine levels, and patient-derived fibroblasts had reduced NANS activity and were unable to incorporate sialic acid precursors into sialylated glycoproteins. Knockdown of nansa in zebrafish embryos resulted in abnormal skeletal development, and exogenously added sialic acid partially rescued the skeletal phenotype. Thus, NANS-mediated synthesis of sialic acid is required for early brain development and skeletal growth. Normal sialylation of plasma proteins was observed in spite of NANS deficiency. Exploration of endogenous synthesis, nutritional absorption, and rescue pathways for sialic acid in different tissues and developmental phases is warranted to design therapeutic strategies to counteract NANS deficiency and to shed light on sialic acid metabolism and its implications for human nutrition.

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1 **NANS-mediated synthesis of sialic acid is required for brain and skeletal**

2 **development**

3

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67 Key words: NANS gene, N-acetyl neuraminic acid synthase, CDG, skeletal dysplasia, sialic acid biosynthesis,
68 developmental delay, N-acetyl mannosamine, next generation metabolic screening

We identified bi-allelic mutations in *NANS*, the gene coding for *N*-acetyl-neuraminic acid (NeuNAc; sialic acid) synthase, in nine individuals with infantile-onset severe developmental delay and skeletal dysplasia. Patient body fluids showed an elevation of *N*-acetyl-mannosamine, and patient-derived fibroblasts had reduced NANS activity and were unable to incorporate sialic acid precursors into sialylated glycoproteins. Knockdown of *nansa* in zebrafish embryos resulted in abnormal skeletal development and exogenously added sialic acid partially rescued the skeletal phenotype. Thus, NANS-mediated synthesis of sialic acid is required for early brain development and skeletal growth. Normal sialylation of plasma proteins was observed in spite of NANS deficiency. Exploration of endogenous synthesis, nutritional absorption, and rescue pathways of sialic acid in different tissues and developmental phases is warranted to design therapeutic strategies for NANS deficiency and to shed light on sialic acid metabolism and its implications for human nutrition.

Intellectual developmental disorders (IDDs) affect 2-2.5% of children and adults worldwide[1]. The developmental origin is reflected in its definition as “substantial impairments of intellectual function and social or adaptive functioning present from early childhood”[2]. Recent advances

87 have shown that in many cases, the etiology is genetic, most frequently due to *de novo*
88 mutations[3,4]. Along with better understanding of the surrounding condition and prognosis,
89 insights into the molecular basis of neurocognitive impairment allows for the development and
90 application of targeted therapeutic strategies[5]. Although less frequent than IDD, genetic disorders
91 affecting skeletal development and growth (commonly called the "skeletal dysplasias") are a
92 group of over 500 distinct disorders[6]. Studying their molecular basis has provided precious
93 insights into the many factors necessary for skeletal development, ranging from minerals and
94 structural molecules to enzymes, to signaling molecules and transcription factors[6,7]. We report
95 here a genetic disorder presenting with a combination of severe IDD with skeletal dysplasia and
96 short stature. Our data show that its pathogenic basis is an inborn error of metabolism that
97 affects the endogenous synthesis of *N*-acetyl neuraminic acid (NeuNAc; sialic acid). Exploration
98 of the biochemical and molecular features of this disorder provides new information on the role
99 of sialic acid in the development of brain and bone.
100

101 **RESULTS**

102 **Clinical and radiographic phenotype of NANS deficiency**

103 The clinical patient reports are presented *in extenso* in the Supplementary Material. Nine patients
104 from six families were studied; patients 1, 2, and 5 have been previously described[8,9]. The main
105 clinical features are as follows: prenatal history was unremarkable in all patients except for one

106 (pt. 8), in whom prenatal hydrocephalus was diagnosed. No specific signs and symptoms were
107 present at birth except for disproportionately short limbs that were observed in three patients
108 (pts. 3, 8 and 9). In the first months of life, all patients showed muscle hypotonia, and the
109 achievement of early developmental milestones such as sitting and walking was delayed.
110 Subsequently, global developmental delay, including cognitive impairment, was the major
111 medical concern. All adult patients showed moderate to severe IDD; only one patient acquired
112 speech and none was living independently. Seizures were a prominent and early feature in one
113 patient but were infrequent in some and absent in others. Social competences were relatively
114 preserved. Body measurements at birth were normal or slightly reduced, but growth velocity
115 decreased during the first or the second year of life, and short stature with shortening of both the
116 trunk and the limbs was present in all adult patients. The facial features showed a prominent
117 forehead, mild synophrys, a sunken nasal bridge, a prominent bulbous nasal tip, and full lips
118 (Fig. 1). No endocrine anomalies were noticed and adult patients had gone through pubertal
119 development. Neuro-imaging was available in six patients: patient 8 showed prenatal-onset
120 hydrocephalus, while patient 9 showed perisylvian polymicrogyria, small basal ganglia and
121 reduced white matter (Supplementary Figure 1); the other four patients showed moderate
122 cerebral atrophy with nonspecific white matter changes. Distinct features that permitted to
123 distinguish these individuals among the large group of persons with severe IDD were the facial
124 dysmorphisms and the skeletal dysplasia with short stature, premature carpal ossification,
125 platyspondyly, longitudinal metaphyseal striations, and small epiphyses (see Fig. 1 for details).

126 For more radiographic details, see also the previous clinical reports of patients 1 and 2[8] and
127 patient 5[9] (pat. 1 in that reference).
128

129 **Identification of *NANS* mutations and functional analysis**

130 Exome sequencing was performed on genomic DNA from patients 1 to 4 (two couples of sibs).
131 After the filtering pipeline (see Methods and Supplementary Table 1), only one gene, *NANS*,
132 showed biallelic variants in all four patients (Table 1). A small insertion/deletion just 5' of exon 4,
133 c.449-10_449-5delGATTACinsATGG, was seen at heterozygosity in all four patients (reference
134 transcript: ENST00000210444, NCBI Reference Sequence: NM_018946.3). In addition to this
135 shared mutation, patients 1 and 2 (who were sisters) had a single nucleotide insertion predicted
136 to result in a frameshift with early truncation (c.389_390insT, p.Lys131Gln*fs*Ter8), while patients
137 3 and 4 (who were brother and sister) had a canonical splice donor site mutation (c.448+1G>A).
138 Computational haplotype reconstruction using exome data revealed a specific haplotype
139 encompassing a region of 1.38 MB on chromosome 9 (from 9:100388119 (rs10817858) to
140 9:101767385 (rs41305481)) in the four individuals carrying the intronic insertion/deletion,
141 indicative of a common origin. Sanger sequencing was used to confirm the mutations and to
142 verify segregation with disease in all families. To test for pathogenicity of these variants, *NANS*
143 cDNA was retrotranscribed from fibroblasts or lymphoblastoid cell RNA from patients 1, 3, and
144 4, as well as the parents of patients 3 and 4, incubated with or without cycloheximide, PCR-
145 amplified and analyzed by capillary electrophoresis (Supplementary figure 2). The c.449-10_449-

146 5delGATTACinsATGG insertion/deletion (patients 1, 2, 3 and 4) resulted in very low levels of
147 mRNA, with presence of wild-type mRNA as well as an isoform lacking both exons 3 and 4 (in-
148 frame); in presence of cycloheximide, this allele appeared to produce additional abnormally
149 isoforms, that apparently were subject to NMD. The c.448+1G>A variant (patients 3 and 4)
150 produced two splicing isoforms, one lacking both exons 3 and 4 (similar to the previous
151 mutation) and expressed at levels comparable to the wild-type allele, and very low levels of an
152 out-of-frame isoform lacking exon 3 and part of exon 4. The exonic insertion c.389_390insT
153 (patients 1 and 2) triggered nonsense-mediated RNA decay (NMD), as shown by sequencing of
154 RT-PCR clones obtained from cells with or without cycloheximide. Thus, all three mutations
155 resulted either in unstable or nonfunctional *NANS* mRNA or in reduced levels of wild-type
156 transcripts. We then screened patients 5, 6, 7, and 8 by selective PCR amplification of *NANS*
157 exons from genomic DNA and direct bi-directional Sanger sequencing of the amplicons, and
158 found bi-allelic mutations in all patients, including four missense mutations and one triplet
159 insertion leading to the duplication of one amino acid (Table 1 and Fig. 2).
160
161 Investigation of patient 9, the youngest patient in our series, followed a different course; he was
162 enrolled into the TIDEX study which combines genomics and metabolomics screening[10].
163 Metabolomic screening had revealed an unusual metabolite, *N*-acetylated mannosamine
164 (ManNAc), in plasma and urine of this child (see below). Among the variants identified by
165 exome sequencing in this patient, two missense mutations in *NANS* stood out as ManNAc-

166 phosphate is the substrate of the NANS enzyme and malfunction of this enzyme would have
167 accounted for the ManNAc accumulation in body fluids. The *NANS* variants identified in patient
168 9 were confirmed via Sanger sequencing and shown to segregate correctly in the family. Of note,
169 the exome results of patients 1, 2, 3, 4 and 5 were examined for possible pathogenic mutations in
170 the *GNE* gene (see below) and none was observed. After this stage, the data of patients 1-8 and
171 those of patient 9 were combined for all subsequent studies.
172

173 Mapping of mutations on the 3D protein model of NANS

174 The NANS protein was modeled as a homo-dimer based on the *Neisseria* homolog, as well as on
175 recent results supporting dimer formation for the human protein[11]. Of the predicted amino acid
176 changes (Table 1), four mapped in or near the active site: p.Lys131Gln, p.Gly133Val,
177 p.Tyr188His, and p.Pro189Leu. It is likely that any of these would affect the catalytic activity of
178 the enzyme, either by changing the ability of the protein to bind substrate (p.Lys131 and
179 p.Tyr188 are also predicted to make H-bonds with the substrates[11], fig 2), by changing the pocket
180 shape (p.Gly133Val) or by affecting functional residues in the active site (p.Pro189 is located
181 right next to p.Tyr188). Mutations p.His29Asn and p.Arg237Cys are localized at the dimer
182 interface and likely affect the folding, protein stability and/or dimer formation. While close to
183 the dimer interface, the p.Ile327 that is duplicated is not involved in protein contacts in our
184 model and it could be affecting folding instead. Finally, p.Arg151His is located on the surface of

185 the protein and away from the dimer interface and substrate-binding site, possibly interfering

186 with folding or with a critical protein interaction.

187

188 *NANS* mutations lead to accumulation of ManNAc *in vivo*

189 Next generation metabolic screening (see Methods) was first applied to cerebro-spinal fluid

190 (CSF) of patient 9 (Figure 3 and Supplementary Table 2), and then to plasma of patients 1-4 and

191 9 (data not shown), leading to the identification of an unusual compound, *N*-acetyl mannosa-

192 mine, in all 5 patients. Quantitative NMR spectroscopy was then used to determine the

193 concentration of *N*-acetyl mannosamine in available patients' urine samples. In patients 1, 2, 3, 4

194 and 8 (all adults at the time of study), the urinary concentration of ManNAc ranged from 41 to

195 98 μmol/mmol creatinine (reference values, <10), whereas in patient 9 (age 3 years) excretion was

196 highest (295 μmol/mmol creatinine). NMR spectroscopy was applied to homogenates of cultured

197 fibroblasts to explore the intracellular sialic acid synthesis pathway. In fibroblasts from two unre-

198 lated patients (patients 3 and 9), this showed increased intracellular levels of *N*-acetylmannosa-

199 mine-6-phosphate rather than of free ManNAc. NANS can act as a *N*-acetylneuraminic acid

200 phosphate synthase (MIM 605202; EC 2.5.1.57). This may account for the accumulation of *N*-

201 acetylmannosamine in body fluids and *N*-acetylmannosamine-6-phosphate within the cell. The

202 free NeuNAc concentration in fibroblasts was normal (supplementary table ST2). Finally, we

203 evaluated whether the deficient activity of NANS would lead to systemic deficiency of sialic acid.

204 The concentration of free NeuNAc was evaluated in urine of 5 patients from 4 families (relative

205 to creatinine excretion) as well as in the CSF of patient 9. In all patients, normal values were
206 found (Supplementary Table 3) suggesting that there was no systemic depletion of free
207 neuraminic acid. Analysis of plasma transferrin- and apolipoprotein C-III isoforms, to evaluate
208 the biosynthesis of N- and O-glycans respectively, has been done repeatedly on several of the
209 patients in the clinical setting as well and given normal results (Supplementary Table 4), further
210 contributing to the notion that peripheral sialylation was not significantly affected despite NANS
211 deficiency.
212

213 ***NANS* mutations impair enzyme activity**

214 In two preliminary experiments, we explored the sialylation of proteins and lipids at the cell
215 surface of patient and control fibroblasts using FITC-labelled *Sambucus nigra* lectin (that
216 specifically binds to terminal galactose-bound sialic acid residues) and FACS analysis, and we
217 determined the cellular content of CMP-NeuNAc and total NeuNAc before and after addition of
218 10 mM ManNAc[12] . No differences between patient and control fibroblasts were seen, possibly
219 because the concentration of ManNAc used was significantly higher than what is found
220 physiologically. We then developed a method to measure NANS enzyme activity in cell lysates by
221 incubation with ManNAc-6-P and phosphoenolpyruvate (PEP) and quantification of newly
222 formed NeuNAc by mass spectrometry (Fig. 3, panel D). In comparison with five healthy
223 controls (613+/-150 nmol NeuNAc/mg protein, mean +/-SD), the three available patient
224 fibroblast lines showed reduced production of NeuNAc (163+/-111 nmol NeuNAc/mg protein,

225 mean +/-SD) at 24h incubation. Fibroblasts from the heterozygous father of patient 3 and 4

226 showed intermediate NANS activity (403 nmol NeuNAc/mg protein). Although the residual

227 activity in this assay was high, these results were in agreement with an autosomal recessive defect

228 in NANS.

229

230 **Incorporation of ManNAc into sialoglycoproteins is impaired**

231 We then used metabolic labeling of sialic acids using propargyloxycarbonyl(Poc)-derivatized

232 analogs of ManNAc and NeuNAc (ManNPoc and NeuNPoc, respectively) [13], a recently developed

233 technique that had been useful to confirm deficient sialic acid incorporation in cells deficient for

234 the Golgi transporter of CMP-sialic acid[14]. NeuNPoc, which enters the metabolic pathway

235 downstream of the enzymatic step catalyzed by NANS, was incorporated efficiently into

236 glycoproteins in all cell lines analyzed, while ManNPoc, which enters the pathway upstream (Fig.

237 4), was incorporated in fibroblasts from a control and from a NANS heterozygote, but not in

238 those of patients 3, 8, and 9 (Fig. 3). These data confirmed the functional impairment of NANS

239 activity on the metabolic pathway of sialic acid biosynthesis and protein sialylation. NANS

240 deficiency should therefore be included among the list of CDGs (congenital disorders of

241 glycosylation). Importantly, these data suggest that exogenous NeuNAc might be used to bypass

242 the enzymatic block (see Discussion, below).

243

244 ***nansa* knockdown perturbs zebrafish skeletal development**

245 There are two zebrafish orthologs for the human *NANS* gene, the *nansa* and *nansb* genes (see
246 Methods for detail). *Nansa* is expressed during early embryonic development including stages of
247 50% epiboly in the axis, 1-13 somites at the notochord and polster, and 14-19 somites at the
248 hatching gland. Thereafter, *nansa* is strongly expressed in the head, immature eye, myotome,
249 optic tectum and pharyngeal arch skeleton [15]. The expression pattern of *nansb* is unknown. We
250 designed splicing morpholinos to knockdown both *nansa* and *nansb* genes [16] (see Methods).
251 Microinjection of *nansa* morpholino into the newly fertilized zebrafish eggs resulted in embryos
252 with small head, pericardial edema, and developmental anomalies of the skeleton, revealed by
253 alcian blue staining, at 6dpf (Fig. 5 A, B). Interestingly, *nansa* morphants showed a complex
254 phenotype in the area of the head, including hypoplastic or absent of Meckel's cartilage, lack of
255 basihyal, shortened and abnormal ethmoid plate, trabecula, parachordal, palatoquadrate, and
256 absence of the ceratobranchial structures (Fig. 5 B) [17]. *Nansb* knockdown morphants did not
257 show an overt phenotype even at higher concentrations of morpholinos (data not shown).
258

259 **Partial rescue of skeletal development by exogenous NeuNAc**

260 Given the position of *nansa* (and NANS) in the synthetic pathway of NeuNAc (see above), we
261 tested whether the addition of sialic acid in zebrafish embryo water would rescue the head and
262 skeleton developmental phenotypes of *nansa* zebrafish morphants. Sialic acid at 200 μM resulted
263 in partial rescue of the skeletal phenotype as measured by the reappearance and development of
264 Meckel's cartilage structure; this was correctly formed in 9% of embryos in basic *nansa*

265 knockdown conditions, but in 61% of embryos when sialic acid was added to the water (Fig. 5 C,
266 D). Interestingly, the rescue effect of sialic acid was time-dependent; rescue was observed when
267 sialic acid was added to the fish water right after morpholino injection, but not when it was
268 added 24 hours post fertilization, suggesting that sialic acid plays a critical role in early
269 embryonic development, potentially in cartilage and skeleton cell lineage specification or growth.
270
271

272 **DISCUSSION**

273 We present evidence that biallelic deleterious mutations in *NANS* are associated with a severe
274 intellectual developmental disorder and skeletal dysplasia. First, we identified 10 different *NANS*
275 variants in 9 patients from 6 unrelated families, segregating according to a recessive disease
276 model. Second, the *NANS* mutations impaired the activity of the *N*-acetyl-neuraminic acid
277 synthase enzyme as evidenced by reduced enzyme activity and by the specific block of *N*-acetyl
278 mannosamine analogue incorporation in cultured cells. Third, dysfunction of NANS *in vivo* was
279 confirmed by the accumulation of substrates of the missing enzyme: *N*-acetyl mannosamine in
280 body fluids, and *N*-acetyl-mannosamine 6-phosphate in cultured cells. Finally, inactivation of the
281 enzyme activity in zebrafish embryos resulted in a complex phenotype including abnormal
282 development of skeletal structures. The conclusion that *NANS* mutations are the cause of the
283 clinical phenotype is strengthened by the fact it was reached by two independent approaches.
284 Patients 1 to 8 were ascertained because of their phenotype of intellectual disability and specific

285 skeletal dysplasia, and the genomic approach led to identification of *NANS* mutations and to
286 their validation through RNA studies. Patient 9 was subjected to metabolomic screening first in
287 order to elucidate the cause of severe IDD and dysmorphisms, leading to the identification of
288 ManNAc in body fluids, and this biochemical phenotype allowed to prioritize the *NANS*
289 mutations as the most likely pathogenic amongst the variants found subsequently on exome
290 sequencing. Although most of our patients were ascertained retrospectively and the clinical
291 assessment is heterogeneous, there are indications for different degrees of clinical severity. The
292 study of additional patients is needed to determine the clinical spectrum of NANS deficiency and
293 to establish possible genotype-phenotype correlations.
294
295 Brain contains the highest concentration of total sialic acid among human organs[18]. Sialic acid is
296 present on glycoproteins and glycolipids such as the gangliosides, that are particularly abundant
297 in nervous tissue, and NANS is highly expressed in the human brain (Supplementary Figure 3).
298 Genetic deficiency of either sialyltransferase ST3GAL3 (MIM#604402) or ST3GAL5
299 (MIM#609056), two enzymes that utilize CMP-NeuNAc to add terminal sialic acid residues to
300 the glycosidic antennae of glycoproteins and glycolipids, leads to infantile epilepsy and/or
301 developmental arrest, suggesting that appropriately sialylated glycoproteins and/or glycolipids
302 are necessary for higher brain functions[19-22]. Mutations in the CMP-NeuNAc transporter
303 SLC35A1 (MIM#605634; see Fig. 4) result in developmental disability with ataxia and bleeding
304 diathesis [23]. The observation of IDD in NANS-deficient patients and the brain dysplasia observed

305 in two of them underlines the relationship between sialylation and neurologic functions and
306 indicates that the requirements for sialic acid in the developing brain must be met at least
307 partially by endogenous synthesis of sialic acid through the NANS pathway.
308

309 The short stature and skeletal dysplasia in NANS-deficient individuals also tells us that NANS-
310 mediated sialic synthesis plays a pivotal role in skeletal development, specifically at the growth
311 plate cartilage. The skeletal anomalies seen in the zebrafish knockdown model confirmed this
312 notion. The avascular nature of cartilage may make it dependent on endogenous synthesis.
313 Several of the key players in cartilage and bone growth and development, such as chondroitin
314 sulfate proteoglycans[24], bone sialoprotein[25] and osteopontin[26], are heavily sialylated and will be
315 candidates for further studies.
316

317 Tests detecting hyposialylated transferrin and apolipoprotein C-III yielded normal results in our
318 NANS-deficient patients, and there was no clinical or laboratory evidence of hyposialylation of
319 plasma proteins or clotting factors, suggesting that sialylation of plasma proteins is not
320 significantly affected. How is sialylation achieved with impaired NANS activity? Several lines of
321 explanation can be considered. First, the mutations in our patients may allow for some residual
322 activity; none of our patients had a combination of two *bona fide* null alleles. Secondly,
323 endogenous NeuNAc synthesis may be rate limiting in certain tissues and at certain times, where
324 and when synthetic requirements are maximal (such as in the brain, in periods of rapid growth

325 before birth and in the first two years of life[27-29], or in cartilage during infancy and childhood),
326 but not in other tissues where synthetic requirements for sialic acid may be lower. Such a
327 mechanism has been put forward to explain the muscle-restricted phenotype of GNE myopathy
328 (MIM# 603824)[30], a disorder caused by recessive mutations in the *GNE* gene coding for UDP-*N*-
329 acetylglucosamine 2-epimerase and ManNAc kinase activities in the sialic acid synthesis pathway
330 (Fig. 4). Thirdly, some tissues may be able to rescue and recycle sialic acid derived from the
331 lysosomal breakdown of sialylated macromolecules (see Fig. 4). A fourth possibility, not mutually
332 exclusive with the others, is that nutrition-derived sialic acids may be entered into biosynthetic
333 pathways, and that this may occur in some tissues, such as the liver, but not, or to a lesser extent,
334 in brain or cartilage. The observation of normal levels of free sialic acid in the urine of our
335 NANS-deficient patients also indicates that there is no systemic depletion of sialic acid.
336
337 The role of nutrition-derived sialic acid raises the question of a possible treatment with oral sialic
338 acid in NANS-deficient individuals, in analogy to other glycosylation and sialylation defects,
339 such as CDG1B (MIM# 602579) and GNE myopathy[31], that have been amenable to treatment by
340 oral administration of specific sugars. Some of our data may indeed point in this direction. When
341 added to patients' cells in culture, the sialic acid analogue NeuNPoc (used because of its
342 detectability), but not the upstream metabolite ManNPoc, was able to bypass the enzymatic block
343 and be incorporated into macromolecules. In zebrafish embryos, exogenously added sialic acid
344 was able to partially recue the developmental phenotype caused by *NANS* knockdown. Could

345 dietary supplementation with sialic acid be beneficial for NANS deficient patients? There is
346 evidence that free sialic acid can be taken up and metabolized by cultured cells[32]; this could occur
347 through pinocytosis and release to the cytoplasm by the lysosomal sialic acid exporter (SLC17A5;
348 Fig. 4). In animal models, sialic acid injected in the peritoneum is incorporated into
349 macromolecules[33], and orally administered free sialic acid is found in plasma and subsequently in
350 the liver and the brain[29,33]. N-glycolyl-neuraminic acid, a sialic acid analogue that is widespread in
351 mammals and apes but absent in man because of an evolutionary mutation, is found in human
352 tissues as the result of dietary uptake from meat products[34,35]. Altogether, there is evidence
353 indicating that alimentary sialic acids can be taken up and incorporated into biosynthetic
354 pathways in the human. The relative contributions of endogenously synthesized, nutritionally
355 derived, and rescued sialic acid in different tissues and at different developmental stages in man
356 remain to be investigated and may explain how the consequences of NANS deficiency are
357 restricted to the developing brain and cartilage. Particularly, if the brain pathology in NANS
358 deficiency occurs in the first months of life (and perhaps even prenatally), this might be an
359 obstacle to efficient treatment. Extensive studies in cell culture and *in vivo* are needed before
360 envisaging any treatment possibility for NANS-deficient individuals. Such studies would also
361 have the potential of clarifying the role of nutritional sialic acid in the human (see below).
362
363 Human milk contains a high concentration of free oligosaccharides, most of which are
364 sialylated[27,28,36, 37], as well as free sialic acid. These oligosaccharides, that are notably absent from

365 cow's milk and infant formulas, have been attributed numerous functions, including stimulating
366 brain development and cognition in infants[27,28,37,38]. Since oral administration of sialic acid in
367 humans is considered safe and well-tolerated, nutritional supplementation with sialic acid in
368 infancy, gestation and advanced age has been proposed[27-29,37]. In view of the role and potential of
369 sialic acid in nutrition, exploring the pathogenesis of brain dysfunction and skeletal dysplasia
370 induced by NANS deficiency is worthwhile not only to elaborate therapeutic approaches for
371 NANS-deficient individuals, but also to shed light on sialic acid metabolism with its implications
372 for human nutrition.

373

374 **URLs**

375

376 The Exome Aggregation Consortium database: http://exac.broadinstitute.org/ (accessed January
377 2016)
378 The Wellderly database at Scripps Wellderly Genome Resource, The Scripps Wellderly Study, La
379 Jolla, CA: stsi-ftp.sdsc.edu (accessed January 2016)
380 The PhiloP software for mutation assessment: http://genetics.bwh.harvard.edu/pph2/
381 The Provean software for mutation assessment: http://provean.jcvi.org/index.php
382 The Zebrafish genes database (for accession numbers ENSDART0000006708 and
383 ENSDART00000169540), www.zfin.org

384 The Novoalign software, www.novocraft.com

385 The Gaslini Cell Repository and Biobank, dppm.gaslini.org/biobank/

386 The Leenaards Foundation in Lausanne, www.leenaards.ch

387 The Treatable Intellectual Disability Endeavour in British Columbia: 1st Collaborative Area of

388 Innovation, www.tidebc.org

389 The Rare Diseases Models and Mechanisms Network, www.rare-diseases-catalyst-

390 network.ca/index.php

391

392

393

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568

569

570

571 **LEGENDS TO FIGURES**

572

573

574 Fig. 1. **Morphologic and skeletal features in NANS deficiency.**

575 A, B: patient 9 at age 6 months (A) and at age 3 years (B); C,D: patient 8 at age 28 years; E,F:
576 patient 4 at age 24 years; G,H; patient 3 at age 28 years; I,J: patient 2 at age 38 years; K,L: patient 1
577 at age 40 years. Only short limbs are apparent at age 6 months; facial dysmorphism with
578 prominent forehead, saddle nose, full lips and coarsening of traits is apparent at age 3 years. All
579 adult patients have short stature (height between 130 and 150 cm). All photographs obtained and
580 published with consent. M, N, O: lateral spine films show coronal clefts at birth (M, patient 8),
581 severe vertebral body dysplasia at age 3 (N, patient 9); in adulthood, the vertebral bodies have a
582 normal shape (O, patient 2). At the knees, there are metaphyseal striations and small epiphyses
583 (epi-metaphyseal dysplasia) at age 9 years (P, patient 8); in adulthood, epiphyses remain small
584 but metaphyseal striations have disappeared (Q, patient 2). Advanced carpal ossification at age
585 21 months (R, patient 9), and metaphyseal striations (distal radius) with epiphyseal dysplasia at
586 age 9 years (S, patient 8). Short femoral necks and small, irregular capital femoral epiphyses (epi-
587 metaphyseal dysplasia) at age 3 years 7 months (T, patient 9); in the adult (U, patient 3) short
588 femoral neck and small epiphyses but bone and cartilage structure appear normal. Compare the
589 radiographic images of patients 1 and 2 and patient 5 as reported earlier[8,9].

590

591 **Fig. 2. 3D model of NANS protein and mapping of amino acid residues affected by mutations**

592 A : Model of the human NANS protein, showing one monomer in grey and one in blue. Mutated
593 residues are color-coded based on the patient with each allele mapped on a separate monomer.
594 Lys131 and Arg151 are shown on both monomers. B and C: Detailed view of the location of the
595 mutations. PEP: phosphoenolpyruvate.

596

597 **Fig. 3. Evidence of impaired NANS activity *ex vivo* and in cell culture. A,B,C:** 500 MHz 1D ^{1}H-
598 NMR spectra of body fluids and model compounds: **A.** CSF of patient 9 containing *N*-
599 acetylmannosamine at 180 μmol/L (reference <5). Resonances of alpha and beta forms of *N*-
600 acetylmannosamine are shown in the inserts **B.** relevant part of the CSF spectrum (a), compared
601 with the spectrum of ManNAc (b), GalNAc (c), and GlcNAc (d); **C.** Spectra obtained from
602 fibroblasts of a control (a), patient 8 (b) and model compound ManNAc-6-P. **D**: NANS enzyme
603 activity in fibroblast lysates. **E:** Metabolic labeling of sialylated proteins. Fibroblasts from a
604 control (ctr), the heterozygous father of patients 3 and 4 (father), and patients 4, 8 and 9 (see
605 table 1) were incubated with peracetylated propargyloxycarbonyl analogs of ManNAc and
606 NeuNAc - ManNPoc and NeuNPoc respectively (see Fig. 4 for their entry point in the sialic acid
607 biosynthetic pathway). Cell proteins were detected by Western blotting using streptavidin-HRP
608 (upper panels) or antibodies against GAPDH (lower panels) as internal control. In cells from the
609 control and from the heterozygous father, both ManNPoc and NeuNPoc result in strong labeling
610 of proteins. In patients' cells, ManNPoc (that enters the synthetic pathways upstream of NANS)

611 is unable to label the proteins, while NeuNPoc (that enters downstream the NANS enzyme) is
612 incorporated efficiently. This confirms a metabolic block between ManNAc and NeuNAc,
613 consistent with impaired NANS activity.
614

615 **Fig. 4. Simplified scheme of *N*-acetyl neuraminic acid metabolism in the human.** Biosynthesis
616 of NeuNAc is achieved largely in the cytoplasm, except for the CMAS (**) reaction that takes
617 place in the nucleus (nuclear membrane and its possible transporters omitted here for
618 simplicity). The synthesis of *N*-acetyl mannosamine is carried out in two steps by the
619 bifunctional enzyme, GNE/ManNAc synthase (*). *N*-Acetyl-neuraminic acid synthase (NANS) is
620 indicated in red. CMP-sialic acid is transferred by a specific transporter, SLC35A1, into the
621 medial- and trans-Golgi where it is used as a substrate for the sialylation of proteins and lipids by
622 various sialyltransferases. - When sialylated glycoproteins and lipids are degraded in the
623 lysosome, the free sialic acid released by sialidases can diffuse out of the lysosome through the
624 lysosomal sialic acid transporter (SLC17A5). Free NeuNAc (as well as the non-human sialic acid,
625 NeuNGc) can be taken up by pinocytosis and released into the cytoplasm by the SLC17A5
626 transporter. Free sialic acid can then re-enter the biosynthesis pathway (indicated as “salvage” in
627 the scheme). The relative importance of biosynthesis and salvage in different cell types and
628 tissues is largely unknown. The metabolic scheme also shows the entry points of the two
629 synthetic analogs used in this study, ManPoc and NeuNPoc (see text), the first being upstream,
630 the second downstream of the step catalyzed by NANS.

631

632

633 **Fig. 5. Abnormal skeletal development in *nansa* morpholino knockdown zebrafish is partially**

634 **rescued by exogenous sialic acid. A, B:** *nansa*-e3i3 morphants (4ng/nl) demonstrate small head,

635 pericardial edema and abnormal cartilage and skeleton development including hypoplastic or

636 absent of Meckel's cartilage (m) and lack of basihyal (bh), shortened and abnormal ethmoid plate

637 (ep), trabecula (tr), parachordal (pch), palatoquadrate (pq) and ceratobranchial (cb) structures in

638 dorsal and ventral view. Abbreviations used: lateral: m, Meckel's cartilage; bh, basihyal; dorsal:

639 ep, ethmoid plate; tr, trabecula; pch, parachordal; ventral: m, Meckel's cartilage; ch, ceratohyal

640 cartilage; cb, ceratobranchial; pq, palatoquadrate. **C, D**: 200 μM sialic acid partially rescued the

641 abnormal skeletal phenotype as measured by Meckel's cartilage analysis. P1 - complete Meckel's

642 cartilage. P2 - incomplete Meckel's cartilage, P3 - absent of Meckel's cartilage. The graph in D

643 shows the proportion of fish showing a Meckel's cartilage phenotype of P1, P2 or P3 with or

644 without exogenously added sialic acid. For each experimental condition, approx. 10-12 embryos

645 were analyzed (actual numbers shown in the figure); experiments were done in triplicate. Bars

646 represents SD between the triplicates. In the *nansa*–e313 morpholino treated animals, the

647 difference between with or without 200 μM sialic acid is statistically significant: (*) p=0.05 for P1

648 embryos, and (**) p.=0.01 for P3 embryos (two-tailed T-test).

649

Table 1. Overview of NANS mutations observed in the nine patients.

genomic DNA position (chr. 9)	cDNA position*	exon	protein	presence in databases**	*PolyPhen****	*Provean *****	observed in patient(s)
100819175	c.85C>A	1	p.His29Asn	no	probably damaging	deleterious	Pat. 8
100839236	c.389_390insT	3	p.Lys131Gln*fs*Ter8	Wellderly: 0.0008	*n.a.*	*n.a.*	Pat. 1 and 2
100839249	c.398G>T	3	p.Gly133Val	no	probably damaging	deleterious	Pat. 6 and 7
100839300	c.448+1G>A	3	aberrant splicing of exons 3 and 4 as seen in mRNA studies	ExAC=0.00002633	n.a.	n.a.	Pat. 3 and 4
100840462	c.449-10_449-5delGATTACinsATGG[§]	4	aberrant splicing of exons 3 and 4 as seen in mRNA studies	ExAC = 0.00004947	n.a.	n.a.	Pat. 1 and 2 Pat. 3 and 4
100840478	c.452G>A	4	p.Arg151His	Wellderly: 0.0008	probably damaging	neutral	Pat. 5 (homozygous)
100840588	c.562T>C	4	p.Tyr188His	no	probably damaging	deleterious	Pat. 9
100840592	c.566C>T	4	p.Pro189Leu	no	probably damaging	deleterious	Pat. 8
100843203	c.709C>T		p.Arg237Cys	no	probably damaging	deleterious	Pat. 9
100845238	c.981insATC	6	p.Ile327dup	no	probably damaging	deleterious	Pat. 6 and 7

*Transcript: ENST00000210444 (CCDS6733) NCBI Reference Sequence: NM_018946.3; ** ExAC database: http://exac.broadinstitute.org/, accessed November 2015; Wellderly database: Scripps Wellderly Genome Resource, The Scripps Wellderly Study, La Jolla, CA (URL: stsi-ftp.sdsc.edu), accessed January 2016. – *** http://genetics.bwh.harvard.edu/pph2/- **** http://provean.jcvi.org/index.php

650

651 # METHODS

652

653 **Ethics** - The studies were approved by the ethics boards of the following institutions: BC
654 Children's & Women's Hospital, University of British Columbia (#12-00067), the Ethics Board of
655 the Lausanne University Hospital. Research was performed according to the countries' ethics
656 code of conduct. Parents or guardians provided written informed consent for the biochemical
657 and genetic analysis and the publication of photographs and clinical data.

658

659 **Identification of mutations in *NANS*** – For patients 1 to 4, fragmented genomic DNA was
660 purified with AMPure XP beads and its quality was assessed with an Agilent Bioanalyzer.
661 Preparation of the exome enriched, barcoded sequencing libraries was performed using the
662 SureSelect Human All Exon v4 kit (Agilent). The final libraries were quantified with a Qubit
663 Fluorometer (Life Technologies) and the correct size distribution was validated on the Agilent
664 Bioanalyzer. Libraries were then sequenced on a HiSeq 2000 machine (Illumina), generating 100
665 bp paired-end reads. Raw reads were aligned onto the hg19 reference genome using the
666 Novoalign software. Data cleanup and variant calling were performed according to GATK Best
667 Practices recommendations[39]. Variant filtering was made with Annovar[40] and with in-house *perl*
668 and *bash* scripts, available upon request. For patient 9, who was genotyped in the Vancouver lab,
669 similar procedures were used. To verify for the presence of a possible ancestral haplotype

670 carrying the *NANS* ins/del in patients 1 to 4, we extracted all SNP alleles that were present in the
671 region surrounding NANS and that were listed in the dbSNP database. Local genotypes were
672 then scored, and likely haplotypes were constructed. – In patients 5 to 8, all individual exons of
673 *NANS* were amplified from genomic DNA (primers available upon request) and sequenced
674 directly in both directions using the Sanger method. For patient 9, exome sequencing was
675 performed as part of the TIDEX gene discovery project using using the Agilent SureSelect kit
676 (Agilent Technologies) and Illumina HiSeq 2000 (Illumina). The sequencing reads were aligned
677 to the human reference genome version hg19 (coverage 35X) and rare variants were identified
678 and assessed for their potential to disrupt protein function. In total, we identified 19 candidate
679 genes affected by 2 rare heterozygous variants. Of these, *NANS* stood out as the most interesting
680 functional candidate because of the pre-existing next-generation metabolomics data. Sanger
681 sequencing in the patient and his parents confirmed the mutations and segregation with disease.
682
683 **Construction of a 3D protein model for NANS and mapping of the predicted mutations** - A
684 molecular model for the dimeric full-length human NANS protein was generated with I-
685 TASSER[41], using as templates the *Neisseria meningitidis* homolog[42] and the human AFPL
686 domain[43] structures. The model illustrations were generated with the PyMOL Molecular
687 Graphics System, Version 1.7.4, Schrödinger, LLC.
688

689 **mRNA studies** - Lymphoblastoid cell lines were cultured in suspension under 5% CO2 in T25
690 flasks with RPMI Medium 1640 + GlutaMAX-I (Gibco) containing 10% FBS (Gibco, ref. 10270-
691 106), 1% penicillin/streptomycin (Gibco), whereas fibroblasts were cultured in DMEM (1X) with
692 1g/L D-glucose L-glutamine (Gibco), supplemented with 10% FBS and 1%
693 penicillin/streptomycin. To test for nonsense-mediated mRNA decay (NMD), treatment with
694 cycloheximide (Sigma-Aldrich) was performed in parallel with controls by incubating 10 million
695 cells for 4 hours in the presence of medium supplemented with 28 μg/ml of this chemical,
696 according to published protocols[44]. Total RNA was isolated from both cycloheximide-treated and
697 untreated fibroblast and lymphoblast cultures by using the Direct-zol RNA MiniPrep kit (Zymo
698 Research) according to the manufacturer's instructions. cDNA was prepared following the
699 retrotranscription of 500 ng RNA, by using the PrimerScript RT-PCR kit (Clontech) and random
700 hexamers; 10 ng of the produced cDNA were then used as template for downstream experiments.
701 A specific primer pair spanning the exon-exon junctions 1-2 and 5-6 (for sequences, see
702 Supplementary Table 5) was designed to amplify the regions of the NANS cDNA containing all
703 mutations studied. RT-PCRs were performed in triplicate in a final reaction volume of 20 μl
704 containing 5X Green GoTaq reaction buffer (Promega), 100 μM dNTP mix, 200 nM of each
705 primer, and 0.1 U of GoTaq G2 DNA Polymerase (Promega). Reactions were incubated at 94°C
706 for 1 minute followed by 35 cycles at 93°C for 20 seconds, at 64.1°C for 30 seconds and 72°C for 1
707 minute. The obtained products were resolved by capillary electrophoresis with the eGene HDA-
708 GT12 Multi-Channel Genetic Analyzer (eGene Inc.), quantified, and finally ligated into the

709 pCRII-TOPO TA vector (Invitrogen). Ligation mixes were used to transform chemically
710 competent TOP-10 E. coli cells (Invitrogen), and individual clones (at least 30 clones per
711 electrophoresed sample) were sequenced by direct Sanger sequencing using BigDye terminator
712 V1.1 (Applied Biosystems) with insert-specific primers. Sequencing data were analyzed using the
713 CLC Bio software (Qiagen) and compared with the corresponding human reference sequence
714 (build hg19).
715
716 **Next generation metabolomics analysis** - High resolution untargeted metabolomics analysis of
717 body fluids was performed using UHPLC-Qtof mass spectrometry. Cerebrospinal fluid and
718 heparinised plasma samples were deproteinised in methanol/ethanol (50:50 v/v; 100 μL sample
719 plus 400 μL methanol/ethanol). Samples were analysed in duplicate. Two μL sample was applied
720 to an Acquity HSS T3 reversed phase column (100 x 2.1 mm; 100Å, 1.8 μm) and an Agilent 6540
721 UHD accurate mass UHPLC Qtof-MS with acquisition in positive and negative mode was used.
722 Buffers in positive mode consisted of A: 0.1v/v% formic acid/water, B: 0.1 vv% formic acid in
723 water/methanol (1:99 v/v) was used while in negative mode A: 10 mM acetic acid, B: 10 mM
724 acetic acid in water/ methanol (1:99 vv). After analysis and XCMS alignment, in house developed
725 bioinformatics software showed the features (exact m/z, retention time and intensity) deriving
726 from metabolites that are significantly different in intensity in the patient sample compared to
727 age- and sex-matched controls. The Human Metabolome Database 3.0 was used to putatively
728 annotate significantly different features[45] This technique, also referred to as "next generation

729 metabolic screening", was clinically validated using body fluids of patients with 25 known inborn
730 errors of metabolism and introduced in the Nijmegen patient care research setting.
731 Proton NMR spectroscopy of body fluids, fibroblast homogenates and model compounds
732 of *N*-acetylated sugars was performed on a 500 MHz NMR spectrometer with minor
733 modifications[46]. The NMR spectrum of fibroblasts was recorded on a homogenate obtained after
734 sonification of 7.5 10^6 cells in D_2O. The homogenate was deproteinised over a 10 kD filter and
735 trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (=TSP) was used as a chemical shift reagent in
736 the NMR spectrum. The model compounds *N*-acetylmannosamine, *N*-acetylgalactosamine, *N*-
737 acetylglucosamine were purchased from Sigma chemicals and *N*-acetylmannosamine-6-
738 phosphate from Carbosynth (disodium salt).
739
740 **Lectin staining on cultured fibroblasts** - Fibroblasts were cultured for 48 h in DMEM 10%FCS
741 supplemented with 0, 1, and 10 mM ManNAc. After incubation, fibroblasts were trypsinized,
742 washed twice in Hanks buffered saline solution (HBSS) containing 1% FCS and resuspended at 1
743 x 10^6 cells/ml. Aliquots of 10^5 cells (100 ul) were incubated with 1mM FITC-labeled
744 Sambucus nigra lectin (Vector Labs) in HBSS for 20 min on ice, washed twice in HBSS
745 containing 1% FCS, and analyzed by flow cytometry using a FACScanto II cytometer (BD
746 Biosciences).
747

748 **ManNAc incorporation in fibroblast culture** - Skin fibroblasts were cultured and expanded in
749 DMEM containing 10% FCS at 37 °C in 5% CO_2, and then for 24 hours in the absence of FCS.
750 For the incorporation experiment, mycoplasma-negative cells were incubated in DMEM, without
751 serum and antibiotics, with or without 10 mM *N*-acetylmannosamine (ManNAc), at 37°C in 5%
752 CO_2 for 48 h. After incubation, the medium was removed, cells were harvested in PBS and
753 collected by low speed centrifugation. The cell pellet was sonicated in 20 mM Tris-HCl, pH 8.0, 5
754 mM EDTA, 150 mM NaCl, 1 μg aprotinin and 1 mM PMSF and the lysate ultracentrifuged at
755 30.000 × g for 1 h at 4 °C to pellet the membrane fraction. In the clear supernatant, protein
756 content was determined with the BCA protein assay (Pierce) and aliquots of the supernatant
757 were ultrafiltered with Amicon Ultra-0.5 Centrifugal Filter Unit with a cut-off of 3kDa to
758 separate sialic acid from soluble protein. The dried filtrate was used for sialic acid analysis. The
759 CMP-sialic acid content was discriminated from free sialic acid by reduction: labelling with the
760 fluorophore 1,2-diamino-4,5-methylene-dioxybenzene (DMB) requires free keto as well as
761 carboxyl groups of the sialic acid molecule, and reduction of the keto group prior to the labelling
762 process precludes the labelling of non-activated sialic acid[12]. Each lyophilized sample was split in
763 two aliquots. To reduce free non activated monosaccharides dried samples were dissolved in 0.2
764 M sodium borate buffer, pH 8.0, containing 0.2 M sodium borohydride, incubated at 0°C o/n
765 and dried in a SpeedVac concentrator. For fluorescent labelling of sialic acid, samples were
766 hydrolyzed in 0.2 N trifluoroacetic acid (TFA) for 4 h at 80 °C, dried, redissolved twice in
767 methanol and dried again. To label sialic acids with DMB, hydrolysates were dissolved in 80 μl of

768 0.64 mg/ml DMB in 500 mM 2-mercaptoethanol, 9 mM sodium hydrosulfite, 20 mM TFA and
769 incubated for 2 h at 56 °C. Reactions were then stopped by adding 10 µl of 0.2 M NaOH. The
770 derivatized sialic acids were then quantified by HPLC on a binary pump system (1525µ Binary
771 HPLC Pump, Waters) coupled to a fluorescence detector (2475 Multy λ Fluorescence Detector,
772 Waters) set at λex 372 nm and λem 456 nm. Chromatography was carried out at room
773 temperature with a LichroCART 250-4 Superspher 100 RP18 (250 × 4 mm) column (Merck) and
774 LiChrospher 100 RP18 (25 × 4 mm) (Merck) as pre-column. Mobile phases were
775 methanol/acetonitrile/water/TFA (4:4:92:0.1) and methanol/acetonitrile/water/TFA
776 (45:45:10:0.1) and the flow rate was 0.3 ml/min[12].
777

778 **Measurement of the NeuNAc content in fibroblasts** – Fibroblasts (~2.5 millon cells) in 250 µl
779 50 mM Tris/HCl (pH 7.5) were sonicated on ice (3x 8 sec), then centrifuged (10 min, 10.000 g, 4
780 °C). To 100 µl of the supernatant (lysate) was added ^{13}C3-*N*-acetyl-neuraminic acid (± 23 µM) as
781 internal standard and the solution was applied to a 30 kDa filter cup (Amicon Ultra) with 10 µl
782 2% formic acid in the collection tube for deproteination. After centrifugation for 30 min at
783 13.000 g and 4 °C, the flow-through was used for quantification of NeuNAc by mass
784 spectrometry according to published methods[47,48]. Assays were performed in duplicate and
785 NeuNAc levels were normalized for protein level in the lysates.
786

787 **Determination on NANS activity in fibroblast cultures –** Skin fibroblasts were obtained from
788 affected individuals and healthy controls and cultured at 37.0°C under 5.0% CO_2 in culture
789 medium E199, supplemented with 10% fetal calf serum and 1% Penicillin/Streptomycin. All
790 cultures were tested for mycoplasma infection prior to cultivation. We developed an assay for
791 NANS activity as follows: fibroblasts (~7.5 million cells) in 300 μl 50 mM Tris/HCl pH (7.5) were
792 sonicated on ice (3x 8 sec), then centrifuged (10 min, 10.000 g, 4 °C). The supernatant (lysate)
793 was used for protein determination (BCA assay) and for enzyme activity assay. Incubation of *N*-
794 acetyl-D-mannosamine-6-phosphate disodium salt (Carbosynth, 2 mM) and 20 μl lysate was
795 done at 37 °C in duplicate in a total reaction volume of 100 μl. The reaction buffer consisted of 50
796 mM Tris/HCl (pH 7.5), 3 mM phosphoenol-pyruvate (PEP; Roche), and 1 mM $MgCl_2$. Control
797 incubations without ManNAc-6-P were subtracted as a blanc from the incubations. The
798 reactions were allowed to proceed for 2.5, 5, and 24 hours and then stopped by freezing (-20 °C).
799 Samples (100 μl) were deproteinized by addition of 50 μl ^{13}C3-*N*-acetyl-neuraminic acid (± 23
800 μM) as internal standard, centrifugation for 30 min at 13.000 g and 4 °C on a 30 kDa filter cup
801 (Amicon Ultra), and the flow-through was collected in 10 μl 2% formic acid. NeuNAc levels were
802 quantified by mass spectrometry as described[47,48] and normalized for protein level in the lysates.
803

804 **Metabolic labeling of sialic acids and glycoproteins in fibroblast cultures**
805 Skin derived fibroblasts were cultured in M199 medium (PAN biotech) supplied with 10% fetal
806 bovine serum (PAA), non essential amino acids (NEAA) (Gibco), 100 U/mL

807 Penicilline/streptomycine (Gibco) and tested for mycoplasma contamination. Eighty percent
808 confluent cells were further grown for 5 days in medium containing 15 μM of Ac5NeuNAc,
809 Ac4ManNPoc, or Ac5NeuNPoc. Cells were collected in PBS by scraping and lysed in 150 mM
810 NaCl, 50 mM TRIS-HCl, pH 7.5, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1× cOmplete®
811 protease inhibitor cocktail (Roche). To biotinylate propargyloxycarbonyl-containing
812 glycoproteins, cell lysates were resolved on 10% SDS gels, blotted on polyvinylidene fluoride
813 (PVDF) membrane and after blocking of unoccupied membrane sites with 5% dry milk in PBS,
814 incubated with 500 μM CuSO4, 250 μM l-histidine, 100 μM azide-PEG3-biotin, 500 μM sodium
815 ascorbate in PBS for 1h at 37°C. Biotinylated sialoglycoproteins were visualized by incubation
816 with Horseradish Peroxidase conjugated Streptavidin (GE Healthcare), followed by signal
817 development with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermofisher
818 Scientific). As a loading control an immunostaining with Anti-GAPDH (Abcam Cat N ab8245)
819 of the same western blots was used. Based on this protocol, we found that NeuNPoc at 15 μM
820 concentration would readily label cellular sialoglycans after 5 days of incubation. For ManNPoc
821 at the same concentration of 15 μM, longer incubation times were required to obtain strong
822 labeling of glycoconjugates in fibroblasts from healthy donors (Figure 5).
823
824 **Determination of glycosylation of plasma proteins** - Analysis of plasma transferrin N-
825 glycosylation was done by isoelectric focusing[49] as well as by nanochip QTOF mass

826 spectrometry50. The mucin-type O-glycosylation of plasma apolipoprotein CIII was done by
827 isoelectric focusing[49].
828
829 **Zebrafish studies** - Zebrafish were maintained at 28.5 C in a 10/14-h dark/light cycle. Protocols
830 for experimental procedures were approved by the Research Ethics Board of St. Michaels
831 Hospital, Toronto, Canada (protocol number ACC660). To knock down gene expression, we
832 used splicing blocking morpholino oligonucleotides (MO) for *nansa* and *nansb* knockdown plus
833 a standard control MO (ctrl MO) (for sequences, see Supplementary Table 5). The zebrafish
834 *nansa* gene has six exons with the translation start codon in exon 1 (access number
835 ENSDART0000006708; chr. 1). we designed a splicing blocking morpholino *nansa-e3i3* that can
836 block the splicing donor of exon 3, resulting in intron 3 retention and a truncated protein in
837 translation (data not shown). *Nansb* also has 6 exons with translation start codon in exon 1
838 (access number ENSDART00000169540; chr. 25) and we designed a morpholino *nansb-e1i1* to
839 block the splicing donor site of exon 1 of *nansb*, resulting in intron 1 retention and a truncated
840 *nansb* protein (data not shown). MOs were synthesized by Gene Tools, LLC (for sequences, see
841 supplementary material) and microinjected individually or in combination into 1 cell stage
842 zebrafish embryos. We injected individually *nansa*-e3i3 (4ng/nl) and *nansb*-e1i1 (4 ng/nl) MOs,
843 and co-injected *nansa*-e3i3 (4 ng/nl)/*nansb*-e1i1 (4 ng/nl) MOs. Each injection was repeated at
844 least three times. Knock-down of *nansa* and *nansb* was confirmed by RT-PCR to confirm
845 splicing defect and retention of the intron (data not shown). For control MO, embryos were

846 injected at 1 cell stage with 4 ng/nl. At least 30 embryos were injected per condition and included
847 in the analysis. At 24 hpf, embryos were manually dechorionated. Total RNA was extracted from
848 embryos at 48 hpf using TRIzol (Invitrogen). The RNA concentration of each sample was
849 quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA
850 integrity was verified in 1% agarose gel electrophoresis. The RNA template was converted into
851 cDNA using Superscript II reverse transcriptase (Invitrogen), and used to amplify a *nansa*
852 specific cDNA (for primer sequences, see Supplementary Table 5).
853

854 To visualize the cartilaginous structures, Alcian blue (Sigma) was dissolved in 70% ethanol and
855 1% hydrochloric acid. The zebrafish embryos (6 dpf) were fixed in 4% paraformaldehyde
856 overnight at 4 C, and maintained in 100% methanol at -20 ^{0}C until processing. The embryos were
857 washed with phosphate-buffered saline with 0.1% Tween-20 (PBST). The embryos were bleached
858 in 30% hydrogen peroxide for 2 hours, washed with PBST and transferred into Alcian blue
859 solution. Embryos were stained overnight at room temperature. The embryos were rinsed 4 times
860 with acidified ethanol (HCl–EtOH) 5% hydrochloric acid, and 70% ethanol. Embryos were
861 rinsed for 20 min in HCL-EtOH, and re-hydrated by washing 10 min in a HCL-EtOH/ H2Od
862 series (75%, 25%, 50%, 50%, 25%, 75%) and 100% H2Od. Embryos were stored in 1 ml of
863 glycerol-KOH.
864
865

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TIM barrel domain
AFPL domain
B
AFPL domain
Ile327*ins*
Gly133
Tyr188
ManNAc-6-P
TIM barrel domain
PEP
Pro189
Lys131
Arg151
C
Arg237
PEP
His29
TIM barrel domain

A
B
C
a
b
c
d
D
Ctr.1
Ctr.2
Ctr.3
Ctr.4
Ctr.5
Father
Pat.9
Pat.8
Pat.4
incubation time (hrs)
E
Ctr
Pat.9
Pat.4
Father
Pat.8
ManNPoc
NeuNPoc
Streptavidin
-HRP
GAPDH
250
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biosynthesis
uridine diphosphate-GlcNAc
(UDP-GlcNAc)
UDP
N-acetyl-D-mannosamine
(ManNAc)
UDP-GLcNAc 2-epimerase
(GNE) / ManNAc 6-kinase*
ATP
ADP
ManNPoc
ManNAc 6-P
PEP
Pi
N-acetyl neuraminic
acid synthase (NANS)
NeuNAc 9-P
NeuNAc 9-phosphate
phosphatase (NANP)
Pi
NeuNAc
CTP
PPi
CMP-sialic acid synthase
(CMAS)**
CMP-NeuNAc
SLC35A1
medial and trans-Golgi
CMP-NeuNAc
CMP
sialylation
glycoproteins
glycolipids
sialoglycoproteins
sialoglycolipids
sialyltransferases
salvage
free
NeuNAc
pinocytosis
lysosome
NeuNAc
SLC17A5
NeuNAc
sialoglycoproteins
sialoglycolipids
glycoproteins
glycolipids
sialidases
de-sialylation

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70%
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30%
20%
10%
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