Sialylation of Thomsen–Friedenreich antigen is a noninvasive blood-based biomarker for GNE myopathy

Aim: The exact pathomechanism of GNE myopathy remains elusive, but likely involves aberrant sialylation. We explored sialylation status of blood-based glycans as potential disease markers. Methods: We employed immunoblotting, lectin histochemistry and mass spectrometry. Results: GNE myopathy muscle showed hyposialylation of predominantly O-linked glycans. The O-linked glycome of patients’ plasma compared with controls showed increased amounts of desialylated Thomsen–Friedenreich (T)-antigen, and/or decreased amounts of its sialylated form, ST-antigen. Importantly, all patients had increased T/ST ratios compared with controls. These ratios were normalized in a patient treated with intravenous immunoglobulins as a source of sialic acid. Discussion: GNE myopathy clinical trial data will reveal whether T/ST ratios correlate to muscle function. Conclusion: Plasma T/ST ratios are a robust blood-based biomarker for GNE myopathy, and may also help explain the pathology and course of the disease.

Keywords: core 1 O-linked glycan • glycosylation • hereditary inclusion body myopathy • lectin • L–MS/MS • N-acetylmannosamine (ManNAc) • sialic acid • ST-antigen

The diverse family of inclusion body myositis (IBM) is defined by the pathologic presence of rimmed vacuoles and tubulofilaments on muscle histology. This diverse disease family can be subdivided into ‘sporadic IBM (s-IBM)’, which invariably has inflammation, and ‘hereditary inclusion body myopathy (HIBM)’, which shows familial inheritance and no inflammation [1–3]. An enigmatic and often misdiagnosed disorder belonging to the h-IBM family is GNE myopathy. This rare neuromuscular disorder is historically also called HIBM, distal myopathy with rimmed vacuoles (DMRV), or quadriceps sparing myopathy (QSM) and is characterized by adult-onset, slowly progressive, distal and proximal myopathy that typically leaves patients wheelchair bound 10–20 years after onset [4–7].

GNE myopathy is caused by biallelic mutations in the GNE gene, encoding the bifunctional enzyme UDP-N-acetylgalactosamine 2-epimerase/N-acetylmansosamine kinase (GNE) [8]. GNE is the rate-limiting enzyme in the biosynthesis of 5-N-acetylneuraminic acid (Neu5Ac, Sia), the main mammalian sialic acid and precursor of most other sialic acids [9,10]. Sialic acids are terminal carbohydrate residues of most glycoconjugates, where they serve many functions, including cellular interactions and signaling [11,12].

GNE myopathy-associated GNE mutations are predominantly missense, resulting in reduced, but not absent, enzyme activities [6,13–14]. GNE null mutations have never been identified on both alleles of a patient; this would most likely be lethal since Gne ‘knockout’ mice do not survive past the embryonic stage [15]. The exact pathology of GNE myopathy remains unknown; symptoms seem to occur due to hyposialylation of a select group of (sialo-) glycans [13,16–20]. More evidence that hyposialylation is a key factor in the pathomechanism came from mouse models, in which hyposialylation and pathology could be prevented by treatment with sialic acid metabolites [21,22].
Based on the hypothesis that certain molecules could maintain or restore the structure and function of aberrantly sialylated muscle glycoproteins in GNE myopathy patients, several clinical treatment protocols were recently developed (clinical trial identifiers: NCT012536898, NCT01359319, NCT01517880 and NCT01634750) [23–25]. For these trials, informative, noninvasive biomarkers would be invaluable. In addition, such markers will foster early diagnosis of GNE myopathy, since many patients now experience a significant diagnostic delay [7].

Possible markers that aid in diagnosis of GNE myopathy have previously been suggested. Most of these markers require an invasive muscle biopsy, including analysis of glycosylation/sialylation status of muscle α-dystroglycan [17], neural crest cell adhesion molecule (NCAM) [16], nephrilysin [27], or other O-linked glycans [16]. No robust blood-based biomarkers have been identified for GNE, although serum sialylation of NCAM was suggested [28]. The historically accepted blood-based tests to identify disorders of glycosylation/sialylation, isoelectric focusing of serum transferrin for N-linked glycosylation defects and apolipoprotein C-III for O-linked glycosylation defects, show normal results in GNE myopathy patients [29,30].

In the current study, we explored blood-based glycans as possible markers for GNE myopathy. Through O-linked glycan profiling of plasma glycoproteins using mass spectrometry, we demonstrate that the ratio of the core 1 O-glycan species, Thomsen–Friedenreich (T)-antigen (Gal-GalNAc-) to its sialylated form, the ST-antigen (Sia-Gal-GalNAc-), provides an informative, reproducible plasma biomarker for diagnosis and, potentially, response to therapy for GNE myopathy.

Materials & methods

Patients

GNE myopathy patients were enrolled in either clinical protocol NCT01417533, ‘A Natural History Study of Patients With Hereditary Inclusion Body Myopathy’, or protocol NCT00369421, ‘Diagnosis and Treatment of Inborn Errors of Metabolism and Other Genetic Disorders’, approved by the Institutional Review Board of the National Human Genome Research Institute. All patients provided written informed consent. Peripheral blood samples were obtained and used for serum or plasma preparations. Genomic DNA was isolated from white blood cell pellets, and used for GNE mutation analysis for molecular validation of the GNE myopathy diagnosis (Supplementary Table 1; see online at: www.futuremedicine.com/doi/suppl/10.2217/bmm.14.12). Peripheral blood from healthy donors without clinical complaints at the time of donation were obtained from the NIH Clinical Center blood bank or from the normal serum or plasma collection at the Emory Biochemical Genetics Laboratory.

Whole blood sample preparations for immunoblotting

Serum (non-gel serum separator tube, clot activator) and plasma (K2EDTA-anticoagulant) were isolated from whole blood using standard protocols, followed by albumin and IgG depletion using a Qproteome Albumin/IgG depletion kit (Qiagen, CA, USA). Protein purification and concentration was performed with micro Ultra-0.5 ml Centrifugal Filters (EMD Millipore, MA, USA). Selected control samples were desialylated by incubation with 1 μl (50 U) neuraminidase for 1 h at a 37°C (P0720, New England Biolabs, MA, USA). This neuraminidase (cloned from Clostridium perfringens and overexpressed in Escherichia coli) catalyzes the hydrolysis of α2–3-, α2–6- and α2–8–linked N-acetyl-neuraminic acid residues from glycoconjugates.

Immunoblotting

Serum (10–40 μg) proteins were boiled at 95°C for 5 min in Laemmli Sample buffer (Bio-Rad Laboratories, CA, USA) and electrophoresed on 4–12% Tris-Glycine gels (Invitrogen, CA, USA), followed by electroblotting onto nitrocellulose membranes (Invitrogen). The membranes were incubates with Ponceau S red according to the manufacturer’s protocol (Sigma-Aldrich, MO, USA) to visualize equal loading and transfer of proteins in each lane. The membranes were either probed with primary antibodies against NCAM or with different lectins. Two antibodies against NCAM were evaluated H-300 (sc-10735) and RNL-1 (sc-53007; Santa Cruz Biotechnology, CA, USA), whose binding was visualized by IRDye 800CW conjugated secondary anti-mouse (for RNL-1) or anti-rabbit (for H-300) antibodies (Li-Cor Biosciences, NE, USA). The antigen–antibody complexes were visualized with the Li-Cor Odyssey Infrared imaging system (Li-Cor Biosciences). For lectin probing (Supplementary Figure 1), biotinylated SNA (Sambucus nigra agglutinin) and WGA (wheat germ agglutinin) were purchased from Vector Laboratories, and biotinylated Vicia villosa agglutinin (VVA) was purchased from EY Laboratories (CA, USA). IRDye 680 Streptavidin (Li-Cor Biosciences) was used to bind to biotinylated proteins and visualized with a Li-Cor Odyssey Infrared imaging system (Li-Cor Biosciences).

Muscle lectin histochemistry

Paraffin embedded sections (5 μm) were obtained from control biceps muscle (National Disease Research Interchange [NDRI], PA, USA, right gastrocnemius
muscle from patient GNE-21 (carrying GNE mutations D378Y and A631V), and left biceps muscle from patient GNE-28 (carrying GNE mutations R129X and V696M). The sections were deparaffinized in Hematoxylin and Eosin (Scientific Safety Solvents, TX, USA), rehydrated in a series of ethanol solutions, followed by antigen retrieval (by microwaving in 0.01 M sodium citrate, pH 6.4) and blocking in Carbo-Free Blocking solution (Vector Laboratories). The slides were incubated at 4°C overnight with each fluorescein isothiocyanate (FITC)-labeled lectin aliquoted (5 μg/ml) in Carbo-Free Blocking solution. The FITC-labeled lectins VVA and WGA were purchased from purchased from EY Laboratories and SNA was purchased from Vector Laboratories. After overnight incubation, washes were performed with 0.1% Triton-X-100 in 1× Tris-buffered saline (TBS). The lectin-stained slides were incubated in 0.3% Sudan Black in 70% ethanol solution to reduce autofluorescence. Slides were mounted with Vectashield containing the nuclear dye DAPI (Vector Laboratories). The slides were imaged using a Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss, Micro Imaging Inc., NY, USA). Images were acquired using a Plan-Apochromat 40× oil DIC objective. All images are 3D projections of confocal Z-stacks.

To verify lectin specificity (Supplementary Figure 2), each lectin was incubated with its specific inhibitory carbohydrate for 1 h before overnight incubation on a slide. The inhibitory carbohydrates used were Neu5Ac (Toronto Research Chemicals, Toronto, Canada), for WGA and SNA, and GalNAc (Sigma-Aldrich) for VVA. In addition, tissue slides were desialylated by incubation with 5 μl (50 U) neuraminidase (P0720, New England Biolabs) for 1 h at a 37°C in enzyme buffer.

**Preparation & permethylation of plasma O-linked glycan species**

Blood samples for O-linked glycan analysis from all GNE myopathy patients and some controls (from NIH blood bank) were collected and processed into plasma from heparinized blood in the same laboratory at NIH. Most control plasma samples for this study came from a reference material collection at Emory Biochemical Genetics laboratory, which were all derived from heparinized blood in a similar way as the samples collected at NIH. O-linked glycan species were released from total (not albumin or IgG depleted) plasma or serum glycoproteins by β-elimination, essentially as described [31-34]. Briefly, 10 μl of plasma was mixed with raffinose (1250 pmol in 5 μl) internal standard and 65 μl water for a final volume of 100 μl. To denature the plasma proteins and release the O-linked glycan species, the sample was mixed with 100 μl 2 M sodium borate in 0.1 M sodium hydroxide (freshly prepared) and incubated at 45°C for 16 h. Next, 1.6 ml of 0.25 M acetic acid-methanol solution was dropwise added to neutralize the reaction, followed by O-glycan extraction with methanol. The extracted glycans were desalted through ion-exchange AG 50W-X8 resin (Bio-Rad, CA, USA) and lyophilized overnight.

For permethylation, four NaOH pellets (approximately 375 mg) were crushed in 10 ml anhydrous dimethyl sulfoxide (DMSO) with 0.5 μl water; 0.5 ml of this slurry and 0.2 ml CH₃I were added to the dried glycans and the mixture was shaken vigorously for 1 h, followed by five sequential chloroform/water (600 μl/200 μl) extractions from which the chloroform fractions were pooled. These combined chloroform phases were dried for 30 min under nitrogen (in chemical hood) and the permethylated O-glycan species were resuspended in 50 μl of 50% methanol and further purified through a C18 Stage Tip (Thermo Scientific, FL, USA) as described [35].

**O-linked glycan analysis by LC-MS/MS & MALDI-TOF/TOF**

HPLC separation coupled with an electrospray ionization tandem mass spectrometry (LC-MS/MS) detection of 10 μl of each sample of permethylated O-glycan species was performed on a Shimadzu Prominence 20 AD LC and a Thermo gold 3-μm C18 column (2 × 100 mm), coupled with an ABSciex API-Qtrap 5500 tandem mass spectrometer. The binary method used buffer A (acetonitrile/formic acid: water; 1:0.1:99 [v:v:v]) and buffer B (acetonitrile/formic acid: water; 99:0.1:1 [v:v:v]) with a flow rate at 0.25 ml/min under the following gradient conditions: 0–20 min, 50–80% buffer B; 20–28 min, 98% buffer B; 28–39 min, 50% buffer B. The API-QTRAP 5500 tandem mass spectrometry conditions were as follows: ion source: EPI-positive mode; curtain gas: 25; source temperature: 600. MRM transitions for core 1 T-antigen (as determined by T-antigen standard) and sialyl-T-antigen (as determined by mass and fragmentation pattern) [36] were: m/z 534/298 and m/z 895/520. Calibration curves were constructed with 6 concentrations of T-antigen (from 0.0625 to 5 μM). The ST value is based on the ratio of the ST over the internal standard raffinose peak area, times the raffinose concentration.

The permethylated O-glycans were subsequently analyzed by matrix-assisted laser desorption-ionization (MALDI) time-of-flight (TOF) mass spectrometry on an Applied Biosystems MALDI-TOF/TOF 4800 Plus (Applied Biosystems, CA, USA) as described [34].
**Results**

**NCAM immunoblotting**

Aberrantly sialylated NCAM, detected by immunoblotting of patients’ serum, is the only previously suggested blood-based marker for GNE myopathy [28]. We performed immunoblotting of GNE myopathy serum using the same conditions and NCAM (RN1-1; Santa Cruz Biotechnology) antibodies as previously employed [28], but we were unable to observe similar immunoresponsive bands (Supplementary Figure 3A). This may have been due to different sample handling or processing, or a different batch of the antibody than that used in the previous study.

However, we found that a different antibody to NCAM (H-300; Santa Cruz Biotechnology), detected all three major (~120, 140 and 180 kDa) isoforms of NCAM [37,38] in human serum samples. Compared with control serum, all GNE myopathy patient serum samples demonstrated a slight downshift of the approximately 140-kDa NCAM isoform band, similar to a desialylated (by neuraminidase treatment) control sample (Figure 1 & Supplementary Figure 3B). This downshift likely resulted from different electrophoretic mobility due to hyposialylation. The approximately 120-kDa and 180-kDa isoforms do not appear to be desialylated in GNE myopathy serum samples.

**Lectin histochemistry & lectin blotting**

Staining with lectins (i.e., sugar-binding proteins with ligand specificities for defined carbohydrate sequences [39]) was performed on normal and GNE myopathy muscle slides to examine the sialylation status. Wheat germ agglutinin (WGA) from *Triticum vulgaris* predominantly recognizes terminal sialic acid and N-acetylglucosamine (GlcNAc) on glycans [39–41], SNA (elderberry bark agglutinin from *Sambucus nigra*) predominantly recognizes terminal sialic acid in an α(2,6)-linkage with either galactose (prevalent in N-linked glycans) or with N-acetylgalactosamine (GalNAc; found in O-linked glycans) [40,42]. VVA (hairy vetch agglutinin from *Vicia villosa*) predominantly binds GalNAc O-linked to serine or threonine residues of proteins [40,43]. The results of control experiments, indicating the specificity of each lectin, are presented in Supplementary Figure 2.

GNE myopathy muscle, stained with WGA (recognizing most terminal sialic acids), showed a similar staining pattern as normal muscle (Figure 2). However, staining with SNA (binding only α(2,6)-linked sialic acid) showed a markedly decreased signal in patients’ muscle slides compared with normal, indicating that only specific sialylglycans are hyposilylated in GNE myopathy. VVA staining was almost absent in normal muscle since most glycans are sialylated, while GNE myopathy muscle showed a significant increase in staining compared with normal, indicating hyposialylation of O-linked glycans (Figure 2).

We performed Western blots of controls, neuraminidase treated controls, and GNE myopathy serum proteins, and probed the blots with WGA, SNA or VVA (Supplementary Figure 1). While the neuraminidase-treated control samples showed the expected reduction (for WGA and SNA) or increase (for VVA) in lectin binding, no significant differences in lectin binding could be identified in GNE myopathy patients’ serum compared with control serum.

**T/ST ratios in GNE myopathy patients**

Plasma O-glycan species in control and GNE myopathy patients were analyzed by LC-MS/MS and MALDI-TOF/TOF. Five O-linked peaks were observed, at m/z 534, 895, 1256, 1344, and 1706 (Figure 3). The two major peaks in GNE myopathy patients represent the core 1 O-glycanspecies T-antigen (m/z 534) and the ST-antigen (m/z 895) [33,34]. The relative quantities of T and ST antigens were measured using the LC-MS/MS method by comparing their intensities to the internal standard raffinose at m/z 681 (Table 1 & Supplementary Table 1), as well as using purified T-antigen as external standard to further validate T-antigen quantities. Except for purified T-antigen, there are no purified standards of other O-glycan species (e.g., ST-antigen (m/z 895), m/z 1256, 1344 peaks) commercially available at this time. To evaluate the sialylation of core 1 O-glycan species per patient, the ratio between T- and ST-antigen was obtained. Fifty control samples (from the normal plasma collection at the Emory Biochemical Genetics Laboratory) were measured to establish a normal range for both T- and ST-antigen. Fifty control samples from the NIH blood bank were also serum samples, including samples that were collected recently previously described [33]. The results of control experiments, indicating the specificity of each lectin, are presented in Supplementary Figure 2.

In GNE myopathy plasma, one of the absolute values of either T- or ST-antigen often appeared within the normal range, but the T/ST ratio was consistently abnormal (>0.052) in all analyzed plasma samples from untreated patients (Figure 4, Table 1 & Supplementary Table 1). Importantly, the T/ST ratio of one of our untreated GNE myopathy patients was abnormal (GNE-914a; T/ST = 0.100), but shifted to the normal range 24 h after intravenous immunoglobulin (IVIG) therapy on 2 consecutive days (GNE914b; T/ST = 0.0454).

From selected patients we tested multiple plasma and also serum samples, including samples that were col-
Figure 1. NCAM (H-300) immunoblotting of serum glycoproteins. Serum samples (20 μg) from NA, control (C-1, C-2), and GNE myopathy patients (see Table 1 for details) were immunoblotted with NCAM antibodies (H-300; sc-10735). Compared with control, serum from GNE myopathy patients showed a slight downshift of the 140-kDa NCAM isoform. A similar downshift was present in NA serum. Dotted line is to aid in discerning migration. See Supplementary Figure 3B for the full gel images.

NA: Neuraminidase-treated control.

lected from the same patients at different time-points (baseline and 3, 6 and/or 9 months after baseline). These samples did not show significant differences in the T/ST ratios (Supplementary Table 2), indicating that plasma as well as serum can be used for this assay and that the assay is reproducible, as previously described [34].

Discussion

Major barriers to the diagnosis of GNE myopathy have been the rarity of the disease and the lack of an inexpensive and noninvasive diagnostic test. Most GNE myopathy patients escape diagnosis, with a typical diagnostic delay of approximately 10 years after onset of symptoms [7]. This leads to anxiety and unnecessary testing, often involving an invasive muscle biopsy [13,16–19].

As an alternative, we explored blood-based markers to aid in diagnosis and monitoring response to therapy.

Sialylation on NCAM detected by immunoc hemistry was suggested as a muscle- [26] and blood-based marker for GNE myopathy patients [28], but results may vary with the antibodies used, since NCAM has several membrane bound and soluble tissue-specific isoforms [37,38]. Our application of a reported informative NCAM antibody (RNL-1 [28]) on GNE myopathy serum samples did not show reproducible data (Supplementary Figure 3), possibly related to differences in sample processing or antibody batch. However, our tests with another NCAM antibody (H-300) showed reactivity for all three major NCAM isoforms in human serum samples. GNE myopathy patients’ sera showed a slight down-shift of the 140-kDa NCAM isoform, indicating a possible difference of sialylation on NCAM, resulting in different gel mobility in GNE myopathy patients (Figure 1). Interestingly, a downshift of the approximately 140-kDa NCAM isoform was previously reported in muscle extracts of GNE myopathy patients [19,26], indicating a possible link of this isoform to the disease. The approximately 120-kDa and 180-kDa isoforms of serum NCAM appeared not informative for diagnosis of GNE myopathy. Optimizing specificity and sensitivity of the immunoreactive approximately 140-kDa NCAM band in human serum may in the future proof informative for GNE myopathy.
Based on the presence of predominantly hyposialy-lylated O-linked glycans in GNE myopathy [16–17,25,44], we explored plasma analysis of O-linked glycan structures by a recently developed semi-quantitative method that determines the ratio of the T- and ST-antigens (T/ST) [34]. Using this method, we demonstrated mild undersialylation of plasma O-linked glycan species in all tested GNE myopathy patients, resulting in abnormally high T/ST ratios (>0.052; Table 1). Determining the T/ST ratios in GNE myopathy proved robust and superior to solely semi-quantifying and comparing only the individual T- and ST-antigen values; while individual T- and ST-antigen values can be in the normal range in some GNE myopathy patients (Table 1), the T/ST ratio was abnormal (>0.052) in all untreated patients. Serum samples from selected GNE myopathy patients showed similar T/ST ratios (results not shown) to the corresponding plasma samples, indicating that either serum or plasma can be used for this assay.

**Figure 2. Muscle lectin histochemistry.** Paraffin-embedded muscle sections from biceps (control and GNE-28) and gastrocnemius (GNE-21) were stained with three lectins (green) informative for sialylation status and co-stained with the nuclear dye DAPI (blue). GNE myopathy muscle specimens show selective hyposialylation compared with control muscle, demonstrated by apparent normal staining of WGA (binding to most sialic acid groups), but decreased staining of SNA (predominantly binding terminal α(2,6)-linked sialic acid on all glycans). In addition, staining of VVA (predominantly binding terminal GalNAc, without sialic acid attached, O-linked to serine or threonine residues of glycoproteins) was increased in GNE myopathy muscle specimen compared with control, indicating hyposialylation of O-linked glycans.

Please see color figures online at www.futuremedicine.com/doi/pdf/10.2217/bmm.14.2.

SNA: *Sambucus nigra* agglutinin; VVA: *Vicia villosa* agglutinin; WGA: Wheat germ agglutinin.
The fact that some GNE myopathy patients have normal values of T- or ST-antigen indicates that their undersialylation of O-linked glycan species is likely mild. It is credible that due to defects in GNE enzyme activities [13,14], a gradual defect in de novo sialic acid production occurs in GNE myopathy patients. Some glycans may be preferentially (under)sialylated, perhaps based on (tissue-specific) substrate affinity, protein-

Figure 3. Plasma O-glycan MALDI-TOF/TOF profiles. Human control and GNE myopathy plasma O-glycan species were released by β-elimination and permethylated before MALDI-TOF/TOF analysis. Measured m/z and % intensity compared with the internal standard (IS) raffinose of the major detected small O-glycan species are shown as well as their structures (yellow squares, GalNAc; yellow circles, Gal; purple diamonds, Sia; blue squares, GlcNAc).

Please see color figures online at www.futuremedicine.com/doi/pdf/10.2217/bmm.14.2.
For additional information see Table 1 & Supplementary Table 1.
specific transport pathways through the Golgi-complex for sialylation, expression of certain sialyltransferases or neuraminidases, or other mechanisms [45–47]. The gradual shortage of tissue-, protein-, or sialyl linkage-specific sialylation of predominantly O-linked glycans may play a role in the adult onset and muscle specific symptoms of GNE myopathy. Proteins with significant O-linked glycosylation, most of which remain to be identified, may largely be affected and contribute to the phenotype. In our cohort of GNE myopathy patients, there was no direct correlation of T/ST plasma ratios to severity and onset of the disease, nor to GNE gene mutations (Table 1). Testing more patients and analysis of natural history data will reveal whether T/ST ratios can be correlated to muscle function. Unfortunately, it is difficult to identify GNE myopathy patients before the onset of symptoms, but the evaluation of T/ST ratios in such non-symptomatic patients may indicate the usefulness of T/ST ratios as an early diagnostic tool for the disease.

Abnormal plasma T/ST ratios are not unique to GNE myopathy patients. Historically, the presence of T-antigen, Tn-antigen and STn-antigens are utilized as markers for certain cancers. Because absolute T-, ST-, Tn- and STn-antigen values are often significantly altered in different forms or stages of cancers [48-51], their ratios (including T/ST) are rarely used in cancer research. For some other recently reported disorders, T/ST ratios were informative, including abnormal T/ST values in patients with classic galactosemia (galactose-1-phosphate uridylyltransferase [GALT]-deficiency [51]), deficiency in Conserved Oligomeric Golgi complex 4 (COG4), COG7, transmembrane protein 165 (TMEM165), or phosphoglucomutase 1 (PGM1). Most such glycosylation disorders present with severe congenital clinical phenotypes, much different from adult-onset GNE myopathy. Early clinical symptoms of GNE myopathy (waddling gait, footdrop) are non-specific features of various neurological/muscular disorders and contribute to the delayed diagnosis of patients. Such early symptoms in combination with abnormal plasma T/ST ratios may be future indicators for GNE mutation testing, which will ultimately confirm the diagnosis of GNE myopathy. However, to assess specificity of abnormal T/ST ratios for GNE myopathy diagnosis, it is of importance to evaluate this ratio in plasma samples of patients with clinically similar distal myopathies [52] and/or other glycosylation-affected myopathies, such as dystroglycanopathies [53].

These findings beg the question whether sialylation-increasing therapies could normalize the plasma T/ST ratios in GNE myopathy patients, and possibly indicate response to therapy. Unfortunately, no therapies are currently approved for GNE myopathy. We acquired plasma samples from one GNE myopathy patient who was part of a previously conducted pilot clinical trial of intravenous supplementation of sialylated compounds in the form of immune globulins (IVIG; NCT00195637) [23]. The sialic acid residues on IgG (∼8 μmol of sialic acid/g) could presumably be recycled to sialylate other glycans. While this study showed improvement in strength of different muscle groups and notable subjective improvement reported by the patients, no biochemically relevant evidence of re-sialylation could be detected at that
time [23]. Plasma from the patient before therapy had an abnormal T/ST value (0.100), while a plasma sample acquired 24 h after 1 g/kg IVIG loading on 2 consecutive days showed a normalized T/ST ratio (0.045). Human IVIG is N-glycosylated and does not contain O-linked glycans [54]. Therefore, the potential presence of residual, non-degraded IgG in the patient’s plasma did not directly contribute to the ST- value after therapy. The increased ST values and decreased T/ST ratios after therapy suggest that sialic acids on the loaded IgG were processed/recycled to create sialylation of T-antigens on other glycans. These findings offer prospects for further exploring plasma T/ST ratios for response to therapy in GNE myopathy patients. Please discuss whether this result indicates only the presence of loaded IgG in patient’s blood, or sialic acids on loaded IgG might be processed to ST antigen of the other glycoproteins.

Other substrate replacement therapies for GNE myopathy patients are currently in exploratory stages, and include oral supplementation of sialic acid itself (NCT01634750, NCT01236898, and NCT01517880) and oral supplementation of the sialic acid precursor N-acetylmannosamine (ManNAc; NCT01634750). Once patients’ plasma samples from these trials become available, it would be of great interest to analyze their T/ST ratios to verify whether this ratio is informative for gauging response to therapy.

Conclusion & future perspective
In this study we demonstrate that the ratio of the Thomsen–Friedenreich (T)-antigen to its sialylated form, ST-antigen, detected by semi-quantitative LC-MS/MS and MALDI-TOF/TOF, is a robust blood-based (serum or plasma) biomarker informative for diagnosis and possi-
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likely for response to therapy for GNE myopathy. In addition, the specific hyposialylation of core 1 O-linked glycan species may aid in further elucidating the pathology and adult onset clinical symptoms of GNE myopathy.

Financial & competing interests disclosure
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Executive summary

Background
• GNE myopathy is a recessive inherited, adult-onset, rare neuromuscular disorder.
• Mutations in GNE, encoding the key enzyme of sialic acid synthesis, are associated with GNE myopathy.
• Clinical trials for sialylation-increasing therapies for GNE myopathy are currently ongoing.
• A noninvasive biomarker for GNE myopathy diagnosis and response to therapy is highly desirable.

Materials & methods
• Blood samples and selected muscle biopsies from GNE myopathy patients enrolled in clinical protocols at the NIH (MD, USA) were investigated.
• Lectin histochemistry, western blotting and mass spectrometry-based glycan profiling studies were performed to assess sialylation status in plasma, serum or muscle from control individuals and GNE myopathy patients.

Results
• Immunoblotting of serum glycoproteins with NCAM antibodies (H-300, Santa Cruz Biotechnology, CA, USA) showed a slight downshift of the 140-kDa NCAM isoform in all GNE myopathy patients’ samples compared with control samples.
• Lectin histochemistry on paraffin-embedded slides from GNE myopathy patients’ muscle biopsies demonstrated hyposialylation of predominantly O-linked muscle glycans.
• Plasma O-glycan semi-quantitative LC-MS/MS and MALDI-TOF/TOF mass spectrometry analysis demonstrated that the ratio of the Thomsen–Friedenreich (T)-antigen (Gal-GalNAc-) to its sialylated form, ST-antigen (Sia-Gal-GalNAc-) is abnormal high (>0.052) in all tested, untreated GNE myopathy patients when compared with 50 unaffected control samples (normal range: 0.013–0.052).
• Plasma T/ST values of one untreated GNE myopathy patient was abnormal high (T/ST=0.100), but shifted to the normal range (T/ST = 0.0454) after sialylation-increasing therapy in the form of intravenous immunoglobulins.

Discussion
• Plasma T/ST values, measured by LC-MS/MS and MALDI-TOF/TOF provide an informative, reproducible, blood-based biomarker for diagnosis, and, potentially, response to therapy for GNE myopathy.
• Hyposialylation of core 1 O-linked glycan species (such as the T-antigen) may aid in elucidating the still obscure pathomechanism of GNE myopathy.

References
Papers of special note have been highlighted as:
• of interest; •• of considerable interest


• Extensive, most recent review of clinical, genetic, biochemical, cellular and therapeutic aspects of GNE myopathy.


• The first report demonstrating that mutations in the GNE gene underlay GNE myopathy (hereditary inclusion body myopathy).


• Reporting biochemical and cellular consequences of GNE mutations that underly GNE myopathy, including GNE enzyme activity measurements, assessment of sialylation status and lectin stainings.


• Reporting hyposialylation of predominantly O-linked glycans in GNE myopathy muscle tissues.


• Presenting preclinical evidence of utilizing the sialic acid precursor ManNAc as substrate replacement therapy in a mouse model of GNE myopathy.


• Presents preclinical evidence of applying sialic acid metabolite therapy in a mouse model of GNE myopathy.


** Describing the full methods, sensitivity and specificity and applicability of determining TST ratios in plasma or serum by LC-MS/MS.


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