Elevated plasma free sialic acid levels in individuals with reduced glomerular filtration rates

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N-acetylneuraminic acid (Neu5Ac, sialic acid) is a negatively charged monosaccharide, and the predominant form of sialic acid in human cells. Sialic acid is typically found as the terminal monosaccharide on glycoconjugates, where it plays a role in various physiological and pathological interactions. Sialylated glycoconjugates are critical contributors to the polyanionic component of the glomerular glycocalyx, contributing to size- and charge-selectivity for plasma macromolecules. Podocyte foot process morphology is maintained by the anionic charged sialic acid residues on glycoconjugates in podocyte membranes.

Free sialic acid is filtered but not reabsorbed by the human kidney, in a fashion similar to that for creatinine, but in contrast to the handling of other monosaccharides such as glucose, mannose, galactose and fructose that are reabsorbed by tubular cells. Circulating sialic acid levels, both unbound and bound to glycoconjugates, have only been sporadically studied in different conditions, including some renal disorders, a possible causative link to reduced estimated glomerular filtration rates (eGFRs) has been seldom discussed or investigated. We designed the current study to establish a correlation between eGFR and plasma free sialic acid across a range of subjects with glomerular disorders. This would not only emphasize this often-overlooked aspect of renal filtering of free SA, but also inform our developmental program for glomerular diseases using the sialic acid precursor, N-acetylmannosamine (ManNAc), which was expected to significantly increase plasma free sialic acid levels in subjects with reduced eGFR.

Peripheral blood was obtained from 8 subjects enrolled in NIDDK natural history study 94-DK-0127 (ClinicalTrials.gov: NTC00001392), “Pathogenesis of Focal Segmental Glomerulosclerosis (FSGS)”, and from 8 subjects enrolled in NIDDK study 16-DK-0036 (ClinicalTrials.gov: NCT02639260), “A Phase 1 Multiple Ascending Dose Study to Evaluate the Safety, Tolerability, and Pharmacokinetics of ManNAc in Subjects with Primary Podocyte
Diseases”. To compare with pharmacokinetic parameters of subjects with normal kidney function, we used previously reported data that we collected with the same analytical assays from 19 subjects with GNE myopathy. All subjects provided informed consent prior to study participation. Glomerular filtration rate (eGFR) was estimated using the CKD-EPI creatinine/cystatin C equation, but in GNE myopathy subjects, eGFR was calculated using the CKD-EPI cystatin C equation, as these individuals have muscle atrophy and therefore impaired creatinine production. Quantification of free Neu5Ac (SA) in human plasma was performed using a validated liquid chromatography and tandem mass spectrometry (LC-MS/MS) method with an assay range of 25.0 to 10,000 ng/mL (Alliance Pharma, Malvern, PA). For the development of the bioanalytical method, pooled normal human plasma (n=6; EDTA-anticoagulant) was previously obtained from Bioreclamation (Westbury, NY) and was used to establish the normal range of Neu5Ac in human plasma. Among subjects with eGFR >90 mL/min/1.73m², the normal range for Neu5Ac was reported to be 100-200 ng/mL. Statistical analysis was performed using R v3.6.2 and GraphPad Prism 5.

Plasma free sialic levels were determined in 16 proteinuric subjects with different glomerular diseases and a diverse eGFR range (8-92 mL/min/1.73m²) and in 19 individuals with normal glomerular function (85-155 mL/min/1.73m²) (Tables 1 and 2). First, our study established a plasma free sialic acid range of 109-206 ng/mL (mean 151 ± 29 ng/mL) among subjects with normal eGFR (>90 mL/min/1.73 m²). This is consistent with previously reported normal free sialic acid ranges in plasma of 100-200 ng/mL (n=6), established using the same LC/MSMS method as our assays, and a range in serum of 154-309 ng/mL (0.5-1.2 nmol/mL, n=9) and a mean in serum of 68 ± 6.7 ng/mL (n=50), both established by a modified version of the thiobarbituric HPLC-based method of Warren.
Second, there was no apparent correlation of plasma free sialic acid levels to the particular glomerular diagnosis (Table 1), nor to proteinuria, sex or age of the subjects in our study (Table 2). Previous work also reported that sex or age do not influence circulating free sialic acid.\textsuperscript{14}

Third, we identified a strong inverse relationship between eGFR and plasma free sialic acid levels ($R^2 = 0.906$) (Figure 1). Plasma free sialic acid levels start accumulating in individuals with eGFR $<90$ mL/min/1.73 m$^2$ but did not rise above $\sim 500$ ng/mL in individuals with eGFR $>45$ mL/min/1.73 m$^2$ (Figure 1). In subjects with marked decreased eGFR ($<45$ mL/min/1.73 m$^2$), plasma free sialic acid levels were approximately three-fold higher (431-1260 ng/mL; mean 557-1188 ng/mL) than the values seen across subjects with normal eGFR; varying from 431 ng/mL in a subject with eGFR of 27 mL/min/1.73 m$^2$ to as high as 1260 ng/mL in a subject with eGFR of 8 mL/min/1.73 m$^2$.

Not much information exists concerning the effects of chronic exposure to high circulating free sialic acid levels, as may occur in some subjects with chronic kidney disease. Subjects with free sialic acid storage disease (FSASD) due to a defect in the lysosomal free sialic acid transporter SLC17A5,\textsuperscript{15} exhibit chronically elevated serum free sialic acid levels, i.e., 618-3059 ng/mL (n=8),\textsuperscript{3} in the same range as glomerular disease subjects with eGFR $<45$ mL/min/1.73 m$^2$. FSASD is associated with developmental delay, coarse facies, ataxia, epileptic seizures, hepatosplenomegaly, and reduced life expectancy, and other organ systems, including kidneys, are sporadically reported to be affected.\textsuperscript{15} These features appear predominantly attributed to lysosomal defects, as occurs in other lysosomal storage diseases, rather than elevated circulating free sialic acid levels. In two recent clinical trials for GNE myopathy, subjects were exposed to increased circulating free sialic acid levels. In a phase 3 trial (ClinicalTrials.gov Identifier: NCT02377921), 45 GNE myopathy subjects were exposed to 6 grams/day oral extended-release sialic acid for 48
weeks. Their mean serum free sialic acid increased from 160 ng/mL at baseline to a steady state of ~300 ng/mL up to week 48, with no drug-related serious adverse events or noteworthy differences in vital signs and laboratory findings; low-grade gastrointestinal adverse events were reported, likely due to unabsorbed gastrointestinal sialic acid. Similarly, in a phase 1 trial of single oral doses of 3, 6 or 10 grams of the sialic acid precursor ManNAc to GNE myopathy subjects (ClinicalTrials.gov Identifier: NCT01634750), plasma free sialic acid levels transiently increased up to 436 ng/mL after a single oral dose of 10 grams ManNAc, and fell back to baseline within 48 hours. This study also reported low grade gastrointestinal adverse events, likely associated with unabsorbed ManNAc in the gastrointestinal tract.

Based on these results, we consider chronically increased plasma free sialic acid levels up to ~500 ng/mL to be safe. Although no adverse events were identified related to plasma free sialic acid >500 ng/mL in our limited study population nor in the few subjects published in the literature, we recommend monitoring subjects with increased plasma free sialic acid >500 ng/mL for adverse events possibly related to increased free sialic acid. These recommendations are relevant for clinical studies that include dosing ManNAc or sialic acid to subjects with low eGFR, as in our planned clinical studies of ManNAc for subjects with glomerular diseases. We also anticipate that reducing or halting ManNAc or sialic acid supplementation will reduce free sialic acid levels in these subjects within days, based on previous pharmacokinetic parameters.

A variety of studies reported elevated circulating sialic acid levels in disorders without apparent renal involvement. Most of these studies report the total sialic acid levels, which include both sialic acid bound to glycoconjugates and free sialic acid. Elevated serum total sialic acid is often used as a marker for increased serum acute-phase response proteins, which are heavily sialylated, as reported for early-stage diabetic nephropathy, certain cancers, and rheumatic
A few reports mention renal diseases with elevated total sialic acid, but without mention of free sialic acid or glomerular function. Serum free sialic acid levels are only occasionally reported, including increased serum free sialic acid in rheumatoid arthritis and systemic lupus erythematosus, again without mention of glomerular function, which could be affected in these disorders.

This report emphasizes the role of renal function in determining circulating free sialic acid levels. When encountering increased plasma or serum total sialic acid levels in certain disease conditions, the contribution of decreased eGFR should be considered, especially in disorders associated with kidney disease. While encountering high circulating free sialic acid in subjects with reduced eGFR, some of these subjects may have reduced sialic acid on glomerular glycoconjugates. A possible correlation between circulating free sialic acid and glomerular hyposialylation remains to be investigated.

**Funding**

This work was supported by the Intramural Research Programs of the National Institute of Diabetes and Digestive and Kidney Diseases and the National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, USA.

**Disclosures**

All authors have nothing to disclose.
**Author Contributions**

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The final version of the manuscript was approved by all authors.
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Table 1: Summary of Studied Subjects

| Condition                        | Number of subjects | eGFR range (mL/min/1.73m²) | Plasma Neu5Ac (ng/mL) |
|----------------------------------|--------------------|-----------------------------|-----------------------|
| Focal segmental glomerulosclerosis | 11                 | 8 - 82                      | 310 - 1260            |
| Minimal change disease           | 1                  | 92                          | 204                   |
| Mesangial proliferative          | 1                  | 60                          | 353                   |
| Glomerulonephritis               | 1                  | 46                          | 317                   |
| p-ANCA vasculitis                | 1                  | 28                          | 819                   |
| Membranous nephropathy           | 1                  | 20                          | 546                   |
| IgA nephropathy                  | 1                  | > 90                        | 109 - 206             |
| GNE myopathy<sup>11</sup>        | 19                 | 85 - 155                    | 109 - 206             |
| Normal control<sup>13</sup>      | 6                  | > 90                        | 100 - 200             |

Subjects included those with kidney disease who participated in the present study (n=16) and those with GNE myopathy (n=19) and controls (n=6) with normal kidney function who participated in our previously-published studies. The same Neu5Ac bioanalytical LC/MSMS assay was employed. (Neu5Ac = sialic acid)
Table 2: Plasma Free Sialic Acid (Neu5Ac) Levels in Subjects with Diverse eGFR

| Subject | Age  | Sex | eGFR’ (y) | Plasma free Neu5Ac (ng/mL) | Serum Creatinine (µM/L) | Serum Albumin (g/dL) | Urine Creatinine (mg/dL) | Urine Protein (mg/dL) | Urine Albumin (mg/L) | UPCR (mg/mg) | UACR (mg/g) |
|---------|------|-----|-----------|---------------------------|------------------------|----------------------|------------------------|-----------------------|---------------------|--------------|-------------|
| MCD1    | 28   | M   | 92        | 204                       | 0.660                  | 1.09                 | 5.1                    | 158                   | 12                  | 14.9         | 0.076       | 9.4         |
| FSGS1   | 47   | F   | 82        | 310                       | 1.002                  | 0.84                 | 3.5                    | 176                   | 673                 | 5030         | 3.824       | 2858        |
| FSGS2   | 36   | M   | 79        | 369                       | 1.193                  | 1.32                 | 3.6                    | 104                   | 358                 | 3034         | 3.442       | 2917        |
| FSGS3   | 58   | M   | 61        | 345                       | 1.116                  | 1.28                 | 3.3                    | 33                    | 117                 | 847          | 3.545       | 2567        |
| GN1²    | 46   | M   | 60        | 353                       | 1.141                  | 1.39                 | 4.4                    | 39                    | 14                  | 85.9         | 0.359       | 220.3       |
| FSGS4   | 42   | F   | 56        | 344                       | 1.112                  | 1.35                 | 3.5                    | 34                    | 44                  | 293          | 1.294       | 862         |
| ANCA1³  | 27   | F   | 46        | 317                       | 1.025                  | 1.52                 | 4.1                    | 223                   | 327                 | 3188         | 1.668       | 1429.6      |
| FSGS5   | 74   | M   | 35        | 629                       | 2.034                  | 1.85                 | 3.6                    | 44                    | 381                 | 2670         | 8.659       | 6068        |
| FSGS6   | 37   | M   | 30        | 677                       | 2.189                  | 2.91                 | 4.3                    | 84                    | 175                 | 1361         | 2.083       | 1620.2      |
| FSGS7⁴  | 45   | M   | 29        | 603                       | 1.950                  | 2.75                 | 3.7                    | 121                   | 509                 | 3776         | 4.207       | 3120        |
| MN1     | 65   | M   | 28        | 819                       | 2.648                  | 2.87                 | 1.5                    | 170                   | 1810                | 12662        | 10.647      | 7448.2      |
| FSGS8   | 39   | M   | 27        | 431                       | 1.394                  | 2.34                 | 3.6                    | 71                    | 673                 | 4654         | 9.479       | 6555        |
| FSGS9   | 49   | M   | 26        | 509                       | 1.646                  | 2.68                 | 3.9                    | 45                    | 131                 | 923          | 2.911       | 2051.1      |
| IgA1    | 68   | F   | 20        | 546                       | 1.765                  | 2.35                 | 4.5                    | 48                    | 49                  | 293.4        | 1.021       | 611.3       |
| FSGS10  | 74   | F   | 14        | 851                       | 2.752                  | 3.48                 | 4.5                    | 95                    | 305                 | 1934         | 3.211       | 2035.8      |
| FSGS11  | 53   | M   | 8         | 1260                      | 4.074                  | 8.36                 | 4.8                    | 61                    | 74                  | 403.4        | 1.213       | 661.3       |
| Normal Range |            |            |            |              |                        |                      |                        |                      |                    |              |             |

| GNEM-1  | 37   | M   | 155       | 179                       | 0.579                  | -                    | -                      | -                     | -                   | -            | -           |
| GNEM-2  | 32   | M   | 149       | 114                       | 0.369                  | -                    | -                      | -                     | -                   | -            | -           |
| GNEM-3  | 47   | M   | 146       | 138                       | 0.446                  | -                    | -                      | -                     | -                   | -            | -           |
| GNEM-4⁵ | 36   | F   | 143       | 121                       | 0.391                  | -                    | -                      | -                     | -                   | -            | -           |
| GNEM-5⁵ | 36   | F   | 143       | 175                       | 0.566                  | -                    | -                      | -                     | -                   | -            | -           |
| GNEM-6  | 32   | M   | 141       | 134                       | 0.433                  | -                    | -                      | -                     | -                   | -            | -           |
| GNEM-7⁵ | 49   | M   | 137       | 129                       | 0.417                  | -                    | -                      | -                     | -                   | -            | -           |
|    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|
| GNEM-8 | 32 | M | 135 | 116 | 0.375 | - | - | - | - | - | - |
| GNEM-9 | 30 | F | 131 | 129 | 0.417 | - | - | - | - | - | - |
| GNEM-10 | 47 | F | 119 | 109 | 0.352 | - | - | - | - | - | - |
| GNEM-11 | 51 | F | 116 | 206 | 0.666 | - | - | - | - | - | - |
| GNEM-12 | 51 | F | 110 | 167 | 0.534 | - | - | - | - | - | - |
| GNEM-13 | 53 | F | 108 | 173 | 0.559 | - | - | - | - | - | - |
| GNEM-14 | 54 | M | 105 | 140 | 0.453 | - | - | - | - | - | - |
| GNEM-15 | 49 | M | 105 | 141 | 0.456 | - | - | - | - | - | - |
| GNEM-16 | 35 | F | 105 | 166 | 0.537 | - | - | - | - | - | - |
| GNEM-17 | 30 | F | 95 | 121 | 0.391 | - | - | - | - | - | - |
| GNEM-18 | 65 | M | 95 | 180 | 0.582 | - | - | - | - | - | - |
| GNEM-19 | 50 | F | 95 | 200 | 0.647 | - | - | - | - | - | - |
| GNEM-20 | 44 | F | 92 | 142 | 0.459 | - | - | - | - | - | - |
| GNEM-21 | 39 | F | 90 | 182 | 0.588 | - | - | - | - | - | - |
| GNEM-22 | 52 | M | 85 | 164 | 0.530 | - | - | - | - | - | - |

**Abbreviations:** p-ANCA: perinuclear antineutrophil cytoplasmic antibodies; FSGS: focal segmental glomerulosclerosis; GN: glomerulonephritis; GNEM: GNE myopathy; IgA: IgA nephropathy; MCD: minimal change disease; MN: membranous nephropathy; UACR: urine albumin to creatinine ratio; UPCR: urine protein to creatinine ratio.

1 Note that eGFR was estimated using the CKD-EPI creatinine/cystatin C equation for glomerular disease subjects, and using the CKD-EPI cystatin C equation for subjects without kidney disease.12
2 Mesangial proliferative GN.
3 p-ANCA vasculitis.
4 Collapsing FSGS.
5 In 3 subjects, plasma free Neu5Ac and eGFR were assessed twice, at least 3 months apart. Although these 3 samples were from the same subjects, they were included in our assessment because they were collected under different circumstances. The following subject numbers represent the same individual: GNEM-4 and GNEM-5; GNEM-11 and GNEM-12; GNEM-7 and GNEM-15.
Figure 1: Plasma free sialic acid levels in relation to eGFR

Plasma free sialic acid (Neu5Ac) levels are shown for subjects with glomerular disease (filled circles) and subjects without kidney disease (open circles), showing a significant inverse relationship between estimated glomerular filtration rate (eGFR) and plasma free sialic acid among subjects with glomerular disease ($R^2 = 0.9061$). Note that eGFR was estimated using the CKD-EPI creatinine/cystatin C equation for glomerular disease subjects, and using the CKD-EPI cystatin C equation for subjects without kidney disease.$^{11}$