An In-depth Comparison of the Pediatric and Adult Urinary N-Glycomes

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Abstract

We performed an in-depth characterization and comparison of the pediatric and adult urinary glycomes using a nanoLC-MS/MS based glycomics method, which included normal healthy pediatric (1-10 years, n=21) and adult (21-50 years, n=22) individuals. A total of 116 N-glycan compositions were identified, and 46 of them could be reproducibly quantified. We performed quantitative comparisons of the 46 glycan compositions between different age and sex groups. The results showed significant quantitative changes between the pediatric and adult cohorts. The pediatric urinary N-glycome was found to contain a higher level of high-mannose (HM), asialylated/afucosylated glycans (excluding HM), neutral fucosylated and agalactosylated glycans, and a lower level of trisialylated glycans compared to the adult. We further analyzed gender-associated glycan changes in the pediatric and adult group, respectively. In the pediatric group, there was almost no difference of glycan levels between males and females. In adult, the majority of glycans were more abundant in males than females, except the high-mannose, tetrasialylated and sulfated glycans. These findings highlight the importance to consider age-matching and adult sex-matching for urinary glycan studies. The identified normal pediatric and adult urinary glycomes can serve as a baseline reference for comparisons to other disease states affected by glycosylation.

INTRODUCTION

Protein N-glycosylation is one of the most common post-translational modifications,¹ which is catalyzed by the concerted actions of several glycosyltransferases and glycosidases.² About two
thirds of protein sequences are likely to be N-glycosylated.\textsuperscript{2} N-glycans play important roles in various biological processes such as protein trafficking, cell-cell interactions, signal transduction and immune responses.\textsuperscript{2} The types of N-glycans and their expression level can reflect the physiological and pathological status of the cells.\textsuperscript{3,4}

Many studies have shown that the protein glycosylation changes in various pathological conditions such as cancer,\textsuperscript{5-7} inflammation\textsuperscript{8}, congenital disorders\textsuperscript{9} and immune disorders\textsuperscript{10,11}. However, few studies have focused on the variability of glycosylation in the non-diseased, i.e. “healthy” population under a normal physiological condition, which is essential for providing a baseline reference for further studies. Those few studies mostly focused on normal human serum/plasma glycosylation.\textsuperscript{12-15} For example, Valerie et al.\textsuperscript{12} demonstrated that the serum N-glycan profile of healthy volunteers (20-99 years) changes during human aging. They found that the log of the ratio between the agalactosylated glycan (\textit{Hex$_3$HexNAc$_4$Fuc$_1$}) and the digalactosylated glycan (\textit{Hex$_3$HexNAc$_4$Fuc$_1$}), named “GlycoAgeTest”, showed a strong correlation with age in individuals more than 40 years old. Ding et al.\textsuperscript{13} reported that human serum N-glycans from 265 healthy Chinese volunteers (31-60 years) vary not only with age but also with gender. Pucic et al.\textsuperscript{14} analyzed N-glycome changes of plasma and IgG from 170 children and adolescents (6-18 years), which indicated that the pattern of age-dependent changes in children differs from changes reported in adult population and sex differences are much smaller in children than in adults and are present mainly during puberty.

Compared to serum or plasma, urine can be obtained noninvasively, is less complex, and is more stable.\textsuperscript{16} Since the urinary proteome consists of proteins from the glomerular filtrate of plasma as well as from urogenital system,\textsuperscript{17,18} urinary proteome or glycome can reflect both systemic and urogenital physiology. Despite these advantages, the urinary glycome is still relatively understudied. Variations of normal urinary proteome\textsuperscript{19,20} and the glycosylation pattern of urinary extracellular vesicles\textsuperscript{21} and exosomes\textsuperscript{22} have been reported. To date, no studies have reported the comprehensive view of the urinary glycome especially the effect of age and gender on the changes of the non-diseased human urinary glycome. In this study, we performed qualitative and quantitative comparisons of the urinary N-glycome between the
healthy pediatric and adult cohorts using a highly sensitive and reproducible nanoLC-MS/MS based glycomics method based on our previously published dual labeling of released glycans\textsuperscript{23} to investigate the age and gender-related glycome changes. This will improve our understanding of the variation of the healthy urinary glycome and provide a necessary reference for various future studies using glycan-based biomarker discovery for various diseases in both the pediatric and adult population.

**EXPERIMENTAL PROCEDURES**

**Experimental Design and Statistical Rationale**

Urine samples and demographic data were obtained from a urine specimen registry at Boston Children's Hospital using an IRB approved protocol. All infant urine samples (10-90 mL) were obtained via a sterile, 5 French (1.67 mm) pediatric feeding tube utilizing standardized protocols at Boston Children's Hospital.\textsuperscript{19} All the other urine samples (30-250 mL) were obtained via midstream, clean-catch donation into urine specimen cups and stored in ice boxes followed by centrifuging at 4000 rpm for 10 min at 4 °C to remove debris and stored at -80 °C prior to further processing. Forty three healthy individuals including 22 adults and 21 children were included (Table 1). Pooled urine from 8 adults (4 females and 4 males) with an average age of 33 years old was used to isolate N-glycans as a pooled internal standard. Two technical replicates were measured for the analysis of glycan changes. All samples were quantified against the pooled internal standard of urinary glycans, to minimize technical variations between runs and normalize samples for cohort-wide evaluation. Fold change for each glycan composition or glycan group was determined using the ratio of the normalized peak area values between the cohorts. T-test (two-tailed, homoscedastic) was used to compare the data and p<0.05 was considered significant.

**Chemicals and Materials**

Dimethyl sulfoxide (DMSO), \textsuperscript{12}C\textsubscript{6}- and \textsuperscript{13}C\textsubscript{6}-2-aminobenzoic acid, (7-azabenzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate (PyAOP) and cellulose (fibrous,
medium), were obtained from Sigma-Aldrich (St. Louis, MO). PNGase F (glycerol free) was purchased from New England Biolabs (Ipswich, MA). The Viva Spin 2 series of spin filters (Polyethersulfone-type membrane) were purchased from Sartorius Stedim Biotech (Aubagne, France). All solvents used were HPLC grade. All other chemicals were purchased from Sigma-Aldrich.

**Preparation of N-Glycans**

Urine samples were initially centrifuged at 4,000 rpm (680g) for 10 min to remove particles and cell debris, further concentrated by a 10 kDa cut-off centrifuge filters (Millipore, US) followed by washing twice with 2mL 8M urea in 100 mM triethylammonium bicarbonate. Urine proteins remaining in the filter were further reduced and alkylated. The proteins were subjected to on-filter deglycosylation by PNGase F, and the released N-glycans were eluted into the collecting chamber of the filter device by centrifugation at 4,000g for 10 minutes, followed by repeated centrifugation with 2x0.5 mL of H$_2$O and 0.5 mL of ice-cold 0.1% formic acid successively. All the flow-through fractions were combined, and dried completely using a Speedvac concentrator, and stored at -20°C before use.

**2-AA Labeling**

Released N-glycans from 25 μg of urinary protein were labeled with $^{12}$C$_6$-2-AA (or $^{13}$C$_6$-2-AA) as previously reported$^{25}$ with slight modifications. First, a solution of 4% sodium acetate (w/v) and 2% boric acid (w/v) in methanol was prepared. Then, the labeling reagent was prepared fresh by dissolving 30 mg of $^{12}$C$_6$- or $^{13}$C$_6$-2-AA and 25 mg of sodium cyanoborohydride in 2.0 mL of the above prepared solution. Dried N-glycan samples were dissolved in 0.1 mL of labeling reagent solution followed by incubation at 60°C in a thermomixer shaking constantly at 1200 rpm for 90 minutes. The $^{12}$C$_6$-2-AA labeled glycans from each urine sample and $^{13}$C$_6$-2-AA labeled pooled internal standard of urinary N-glycans were combined after the reactions.

**Methylamidation**
The pooled and purified 2-AA labeled glycan samples were further methylamidated as previously described\textsuperscript{26} with slight modifications. Briefly, the dried samples were dissolved in 50 μL of DMSO solution containing 2.5 M methylamine hydrochloride, followed by the addition of 25 μL of PyAOP (250 mM in 30% 4-methylmorpholine/DMSO). The reaction mixture was incubated at room temperature for 1 h with constant shaking, followed by purification. The purified glycans were dried in a vacuum centrifuge and stored at -20 °C prior to MS analysis.

**Purification of Labeled N-glycans**

The reaction mixture was dissolved in 1 mL of 80% ACN containing 50 mg of cellulose powder, followed by washing with 80% ACN. The labeled glycans were finally eluted after incubation with 50% ACN and dried prior to LC-MS analysis.

**Nano-LC MS/MS Analysis of Labeled N-Glycans**

The labeled N-glycans were resuspended in water and analyzed on a Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA) equipped with a NanoLC 415 system (Eksigent, Dublin, CA). Glycans were separated by a ProteoPepII C18 column (New Objective) at 45°C. The mobile phases consisted of 0.1 % formic acid in water (Solvent A) and 0.1 % formic acid in 100 % ACN (Solvent B). The glycans were eluted using a gradient from 12 to 22% of mobile phase B over 33 min at 300 nL/min. Each sample was run in replicate. The mass spectrometer was operated in positive-ion mode with a spray voltage at 2.5 kV and capillary temperature at 300 °C. The full MS scans (m/z 400 to 2,000) were acquired at a resolution of 70,000 with automatic gain control (AGC) target of 3 \times 10^6 ions and maximum ion transfer time (IT) of 20 ms. HCD MS/MS acquisitions were performed in a data-dependent mode, at a resolution of 35,000 at m/z 200. AGC target was 2 \times 10^5 ions and maximum IT was 120 ms for MS/MS acquisitions and underfill ratio was 2.5%. The 6 most abundant precursor ions with charge state from 1 to 4 were selected for MS/MS. Precursor isolation window was 1.6 m/z. Monoisotopic precursor selection and dynamic exclusion (14s duration) were enabled. HCD fragmentation was with stepped normalized collision energy from 10-25%.

**Data Analysis**
Glycan identification was performed semi-manually assisted with our in-house software. The compositions of N-glycans were assigned on the basis of accurate mass and MS/MS fragmentation. The theoretical mass for 2-AA labeled and methylamidated glycans were calculated as follows: the mass of modified glycans = the mass of unmodified glycans + the mass of methylamidated 2-AA (134.0844 for $^{12}$C$_6$-2-AA or 140.1045 for $^{13}$C$_6$-2-AA) + the number of sialic acids x 13.0316. Mass accuracy within 10 ppm was required for compositional assignment. The GlycoWorkbench 2.1 software was additionally employed to assist in putative glycan structure annotation and in silico fragmentation analysis. Glycan quantification was performed semi-manually assisted with Xcalibur 3.0.63 (Thermo Fisher Scientific). The quantitative measurement was calculated based on the summed peak areas from extracted ion chromatographs (EIC) of the first three isotopic peaks of each glycan composition. If the glycan had multiple precursor ions, such as different charge states, the peak areas from all ions were added. For the relative quantification of each glycoform in the individual sample, the percentage of each glycoform towards the total glycome was calculated. Glycan compositions were abbreviated as follows: hexose (Hex), N-acetylhexosamine (HexNAc), fucose (Fuc), N-acetylneuraminic acid (NeuAc), and Sulfate (S).

**RESULTS**

**Identifications of Urinary Glycans**

A total of 116 N-glycan compositions were identified from all the urine samples (Table S1), and 7 of them were high-mannose type (Hex$_3$-9HexNAc$_2$), 11 were asialylated/afucosylated glycans (excluding HM) (Hex$_3$-7HexNAc$_4$-7), 23 were neutral fucosylated (Hex$_3$-6HexNAc$_2$-5Fuc$_1$-4), 29 were sialylated with no fucose (Hex$_3$-8HexNAc$_3$-7NeuAc$_1$-4S$_0$-2), 42 were both sialylated and fucosylated (Hex$_3$-9HexNAc$_4$-8Fuc$_1$-2NeuAc$_1$-4S$_0$-2). Interestingly, we found 19 sulfated glycans. The relative abundance of each glycan group was calculated based on the average of two samples (Figure 1). The majority of urinary glycans were sialylated and fucosylated, and sulfated glycans were a minor species, accounting for approximately 1.0% of the total glycome. Since only the sialylated
glycans were neutralized by methylamidation in this study, the percentages of negatively-charged sulfate glycans could be underestimated in this comparison.

Although we were not able to reveal complete structural information, partial structural information can be deduced based on a combination of accurate precursor masses, MS² fragment ions, known biosynthesis pathway and published structures. With the 2-AA label at the reducing end, it is easy to differentiate between fragment ions containing non-reducing and reducing end residues. A list of characteristic fragment ions resulting from glycosidic cleavages was generated, which allowed for rapid screening and identification of different glycan species (Table S2). For example, sialic acid-containing ions can be used as diagnostic ions for sialylated glycans and locations of sialic acids. The fragment ions of m/z 508.21 (HexNAC₁NeuAc₁) and m/z 711.29 (HexNAC₂NeuAc₁) indicated that sialic acid was linked to GlcNAc (Figure 2A). It has been previously reported that the non-reducing terminal residue of glycans in mammalian glycoproteins, GalNAC(β1-4) GlcNAc(β-), can be sialylated in the form of GalNAC(β1-4)[NeuAc(α2-6)]GlcNAc(β-).²⁹ The ions of m/z 670.27 (Hex₁HexNAC₁NeuAc₁) and m/z 873.35 (Hex₁HexNAC₂NeuAc₁) (Figure 2B) suggested the possible presence of the Sd² antigen determinant (NeuAc(α2-3)[GalNAC(β1-4)]Gal(β1-4)GlcNAc(β-)) which has been reported present on the antennae of N-glycans from the most abundant urinary protein, uromodulin.³⁰

A series of fucose-containing fragment ions were used to determine the presence of fucose residues and distinguish between core and terminal fucose-containing N-glycans. In this way, we identified two isomeric fucosylated glycans with the composition of Hex₆HexNAC₅Fuc₁NeuAc₃ that were eluted at different times (Figure 3A). The fragment ions of m/z 512.20 (Hex₁HexNAC₁Fuc₁) and m/z 816.33 (Hex₁HexNAC₁Fuc₁NeuAc₁) (Figure 3B) indicated antennal fucose while the ions at m/z 502.24 (Hex₁HexNAC₁(2-AA)Fuc₁) and m/z 1029.43 (Hex₂HexNAC₂(2-AA)Fuc₁) indicated that the fucose was attached to the reducing end GlcNAc as a core-fucosylation (Figure 3C). The majority of the fucose-containing glycans identified in this study were core-fucosylated. The presence of bisecting GlcNAc can be determined based on the bisecting GlcNAc-containing fragment ions at m/z 924.39 (Hex₁HexNAC₃(2-AA)) and 1070.45 (Hex₁HexNAC₃(2-AA)Fuc₁) (Figure S1).
Sulfated N-glycans were determined based on the presence of sulfate-containing ions such as m/z 446.10 (Hex₁HexNAc₁S₁), m/z 1490.51 (Hex₄HexNAc₃S₁) (Figure 4A) and m/z 487.12 (HexNAc₂S₁) (Figure 4B). Although the monoisotopic mass of sulfate (79.9568) is very close to that of phosphate (79.9663), they can be differentiated by the high resolution accurate MS used in this study. The location of sulfate was assigned putatively based on the study of Van Rooijen et al., which has reported sulfated di-, tri- and tetra-antennary N-glycans in uromodulin where the sulfate groups were shown to be present as 3-O-sulfated Gal (Gal3S(β1-4)) and 4-O-sulfated GalNAc (GalNAc4S(β1-4)) using NMR spectroscopy.³¹

N-glycome from Healthy Human Urine is Age and Sex Dependent

In this study, we have included both pediatric (males: 1-10 years; female: 2-9 years) and adult groups (males: 21-50 years; female: 21-33 years). We identified a total of 116 glycan compositions. At the compositional level, there was no difference between the two age and sex groups. Among the total identified glycans, 46 of them were present across all the biological samples and technical replicates, and could be reproducibly quantified (coefficient of variation (CV) < 15% between technical replicates). These 46 glycans accounted for approximately 90% of the total glycome. We performed quantitative comparisons of the 46 glycan compositions between different age and sex groups, and 42 of them were up- or down-regulated (≥1.2 fold change, p<0.05) (Table S3). These 42 glycans were divided into different groups based on their compositions and were further quantitatively compared based on age and sex, respectively.

Quantitative differences of N-glycans between Pediatric and Adult groups

We first compared the urinary glycan differences between the pediatric and adult groups. Table 2 shows glycan groups with significant changes (≥1.2 fold change, p<0.05). The results showed that total glycan level remain unchanged between the pediatric and adults. However, many groups of glycans sharing specific structural elements changed significantly. For example, high-mannose, α- and mono-galactosylated, bisecting GlcNAc and neutral fucosylated glycans were
all down-regulated, while trigalactosylated and trisialylated glycans were up-regulated in adults compared to the pediatric cohort.

Age-related changes were further analyzed in males and females separately, and the pattern of changes has similarities and showed the same change trend in both gender. It can be seen that the high-mannose, asialylated/afucosylated (excluding HM), neutral fucosylated and agalactosylated glycans were down-regulated while the trisialylated glycans were up-regulated in both adult males and adult females.

**Sex Differences of N-glycans are Much Smaller in Pediatric than in Adult**

We compared the glycan profiles between male and female groups, and found that males in general had higher abundance of glycans (Table 3). We further analyzed gender-associated glycan changes in the pediatric and adult group respectively. In the pediatric group, there was almost no difference of glycan levels between males and females. In adult, the majority of glycans was more abundant in males than females, except high-mannose and tetrasialylated glycans.

**DISCUSSION**

This study reports 1.5 fold more glycan compositions than previously reported in human plasma using an LC-MS based method. Among the 116 urinary glycan compositions identified in this study, 57 were shared with the published N-glycome of human serum/plasma while half of them have not been reported in the serum/plasma. There were significant age- and gender-associated glycan differences in plasma, however such changes have not been studied in urine. Most of the studies focused on the adult population (age >20 years) while only one study focused on the younger population (6-18 years). In this study, we are the first to have included both pediatric and adult urine, and found significant changes of the different glycan patterns between pediatric and adult cohorts and with similar change trend between genders. We also found that sex differences of N-glycans were much smaller in pediatric than in adult cohort. Adult males had a higher abundance of glycans than adult females. The alteration of these glycan features could be caused by the changes in the expression level of their carrier...
glycoproteins and/or glycosyltransferases and glycosidases, and the sugar nucleotide donors involved in the glycosylation pathway.\textsuperscript{36}

The various urinary N-glycans found in this study are critical for many biological functions. Those additional urinary N-glycans such as sulfated glycans not reported in serum may have been derived from urogenital system-originated glycoproteins. Sulfation is an N-glycan modification that is catalyzed by sulfotransferases.\textsuperscript{37} Sulfate can be added to the core or the antennae of hybrid and complex N-glycan chains, and it has been found in a variety of urinary glycoproteins including uromodulin and podocalyxin.\textsuperscript{38} It has been shown that the Sd\textsuperscript{a} antigen can prevent the binding of NeuAc(\alpha2-3)Gal-recognizing type-S fimbriated \textit{E.coli} to the endothelium of the kidney and the intestine.\textsuperscript{39} Removal of the sialic acids from uromodulin promotes crystal aggregation of calcium oxalate and calcium phosphate and hence stone formation.\textsuperscript{40,41} N-glycans terminated with GalNAc and sulfate residues have interleukin-1 binding activities and inhibit T-cell proliferation.\textsuperscript{42,43} Sialylation and sulfation of podocalyxin enhances the net negative charge on the surface of podocytes, which is essential for maintaining the normal podocyte morphology and the efficient filtration functions of the glomerular basement membrane.\textsuperscript{44,45}

Similar to the plasma reports, we also found significant age-associated glycan changes in urine. Our previous urinary proteome study\textsuperscript{19} on healthy pediatric and adult males showed that the most abundant urinary protein, uromodulin, which carries around 30% high-mannose glycans, was down-regulated in the adult males. In addition, \textalpha\text-mannosidase II, which controls the conversion of high-mannose to complex N-glycans, was up-regulated in the adult male urinary proteome. Therefore, the decrease in high-mannose and increase in complex glycans (Trigalactosylated and Trisialylated glycans) we observed in this study could be partially due to the lower expression level of uromodulin and higher expression level of \textalpha\text-mannosidase II in the adult.

The increase of agalactosylated glycans with age has been frequently reported in the serum of adults older than 40 years\textsuperscript{12,13,35} while the opposite change trend was reported in the age group of 6 to 18 years\textsuperscript{14}. Our patient cohort was not designed to verify changes in middle age, but our
results were consistent with the latter study in which we found less agalactosylated glycans in adults versus children. The decrease in α- and mono-galactosylated glycans and increase in tri-galactosylated glycans could be a result of decreased activity of β-galactosidase or increased activity of β-1,4-galactosyltransferase (B4GALT) in the adult. It has been shown that plasmatic B4GALT activity exhibited a linear increase from infancy to centenarians.46

The neutral fucosylated glycans were all found to be core-fucosylated. Core-fucosylation, catalyzed by fucosyltransferase, FUT8, is vital for normal development and regulation of the immune system. About 70% of FUT8 knock-out mice died within three days after birth due to major defects in developmental growth and the respiratory system, while the survivors showed severe growth retardation and emphysema-like changes in the lungs.47,48 Kreidberg et al. reported that core fucosylation of α3β1 integrin plays a critical role in kidney and lung organogenesis.49 Lack of core fucosylation can disrupt signaling mediated by epidermal growth factor receptor50 and vascular endothelial growth factors.51 The glycoprotein, epidermal growth factor (EGF), has the highest level in urine compared to other tissues and body fluids and is one of the more abundant urinary proteins.52 EGF stimulates epithelial cell growth and metabolism53 and is 9.8 times higher in the urine of pediatric males compared to adult males.19 This suggests that increased urinary EGF could be one factor that contributes to the up-regulation of the neutral fucosylated glycans and other glycans in the urine of children.

We found down-regulation of bisecting GlcNAc in female adults. Bisecting GlcNAc biosynthesis is catalyzed by the N-acetylgalcosaminyltransferase, GnT-III, by introducing of GlcNAc in a β1-4 linkage to the mannose residue at the base of the trimannosyl core of the N-glycan.54 Thus, the decreased bisecting GlcNAc in female adults could be due to the decreased activity of GnT-III. Bisecting GlcNAc is expressed highly in the brain and kidney under normal condition55,56 and it is involved in the maintenance of kidney homeostasis57 and onset of Alzheimer's disease and/or progression in aging.58 However, GnT-III-deficient mice were found to be viable and able to reproduce normally, suggesting that bisecting GlcNAc is dispensable for normal growth and development.
Our results were consistent with the previous plasma N-glycome study in children and adolescents aged from 6 to 18 years which showed that sex differences are much smaller in children than in adults.\textsuperscript{14} Shao \textit{et al}\textsuperscript{20} have analyzed gender-related urinary proteins from healthy adult donors (20-69 years), which showed that prostate-secreted glycoproteins including prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) were much more abundant in the male urine samples than in the females. PSA and PAP from normal seminal plasma was reported to possess high-mannose, biantennary, galactosylated, mono- and di-sialylated glycans and core fucosylated glycans.\textsuperscript{59-61} These upregulated prostate-originated glycoproteins could contribute to the higher abundance of the N-glycans in adult males compared to adult females found in this study.

Men and women have different levels of certain hormones which are found in urine, and many of them are N-glycosylated, such as luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone and human chorionic gonadotrophin (hCG).\textsuperscript{62} N-glycans affect the folding, assembly, secretion and biological activity of these hormones and binding to and activation of the hormone receptors.\textsuperscript{63-66} N-glycans of FSH and hCG are completely sialylated while those of LH are terminated with 4-O-sulfated-GalNAc instead.\textsuperscript{67,68} These differences in glycosylation are critical in determining the half-life of hormones in circulation and regulating levels of circulating hormones.\textsuperscript{68} Thus, the gender differences of N-glycans could also be attributed to the different sex hormone levels and regulation in adult males and females.

\textbf{CONCLUSIONS}

This is the first in-depth study of the normal pediatric and adult urinary glycomes. Our results demonstrated that urinary glycan composition is age independent while quantitatively is age-dependent. In the meantime, urinary glycome is sex independent in the pediatric cohorts and sex-dependent in the adult cohorts. Age- and sex-specific quantitative differences between these glycomes further highlight the importance of understanding the variation among normal, \textit{i.e.} non-diseased samples to better understand the urinary glycome. These findings also strongly emphasized the need to consider age matching and adult sex-matching for urinary
glycan marker discovery. Based on previous reports, the reported differences between pediatric and adult samples could be due to complex underlying causes, including differential expression of a distinct class of glycoproteins, differential glycosidase and/or glycosyltransferase activity, and differences in growth and cell metabolism. The identified normal pediatric and adult urinary glycomes are helpful and highly impactful for future studies by serving as a baseline reference for comparisons to other disease states affected by glycosylation in both pediatric and adult populations.

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Notes
The authors declare no competing financial interest.

DATA AVAILABILITY
All the data that support the findings of this study are available from the corresponding author on reasonable request. Glycomic LC-MS/MS raw data and all the Glycoworkbench annotated representative MS2 spectra (.gwp file) of the 116 glycan compositions identified in pediatric and adult urine samples were deposited to the GlycoPOST (URL: https://glycopost.glycosmos.org/preview/13555095465ef17c7ae07b4, PIN CODE: 3636) (ID: GPST000109).
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**Table 1. The demographic information of analyzed samples**

| Samples    | Adult (22) | Children (21) |
|------------|------------|---------------|
|            | No.        | Age (yr)      | No.        | Age (yr)      |
| Male (24)  | 11         | range 21-50   | 13         | range 1-10    |
|            |            | mean 28       |            | mean 5       |
|            |            | median 25     |            | median 7     |
| Female (19)| 11         | range 21-33   | 8          | range 2-9    |
|            |            | mean 25       |            | mean 5       |
|            |            | median 24     |            | median 5     |
Table 2. Up- or down-regulated glycans in different age groups. Numbers indicate the fold change between the cohorts (p<0.05). Values <0 are down-regulated in the adults and >0 are up-regulated in the adults. ‘-' means no significant change.

| Glycans                        | Adult: Ped | Males (Adult: Ped) | Females (Adult: Ped) |
|-------------------------------|------------|--------------------|----------------------|
|                               | Fold change| p value            | Fold change          | p value          | Fold change | p value          |
| Total                         | -          | -                  | -                    | -                | -           | -                |
| High mannose                  | -2.23      | 1.09E-08           | -2.79                | 4.34E-07         | -1.75       | 6.17E-03         |
| Asialylated/afucosylated (excluding HM) | -1.54      | 3.26E-14           | -1.41                | 3.23E-07         | -1.67       | 1.12E-08         |
| Neutral fucosylated           | -1.47      | 8.49E-13           | -1.32                | 6.10E-09         | -1.66       | 2.39E-06         |
| Agalactosylated               | -1.88      | 1.46E-15           | -1.75                | 5.39E-09         | -2.04       | 1.64E-07         |
| Monogalactosylated            | -1.38      | 3.71E-08           | -                    | -                | -1.64       | 7.02E-07         |
| Digalactosylated              | -          | -                  | -                    | -                | -1.22       | 1.15E-02         |
| Trigalactosylated             | 1.23       | 6.96E-05           | 1.32                 | 8.73E-06         | -           | -                |
| Tetragalactosylated           | -          | -                  | -                    | -                | -           | -                |
| Bisecting GlcNAc              | -1.32      | 1.15E-05           | -                    | -                | -1.55       | 2.22E-04         |
| Sialylated (no fucose)        | -          | -                  | -                    | -                | -1.26       | 6.68E-03         |
| Fucosylated & Sialylated      | -          | -                  | -                    | -                | -           | -                |
| Monosialylated                | -          | -                  | -                    | -                | -1.29       | 2.75E-04         |
| Disialylated                  | -          | -                  | 1.27                 | 1.73E-03         | -           | -                |
| Trisialylated                 | 1.33       | 2.50E-07           | 1.40                 | 2.26E-07         | 1.27        | 7.66E-03         |
| Tetrosialylated               | -          | -                  | -1.31                | 7.05E-03         | -           | -                |

Table 3. Up- or down-regulated glycans in different sex groups. Numbers indicate the fold change between the cohorts (p<0.05). Values <0 are down-regulated in the males and >0 are up-regulated in the males. ‘-' means no significant change.

| Glycans                        | Males: Females | Ped (Males: Females) | Adult (Males: Females) |
|-------------------------------|---------------|----------------------|------------------------|
|                               | Fold change   | p value              | Fold change            | p value          | Fold change | p value          |
| Total                         | 1.25          | 1.53E-06             | -                      | -                | 1.38        | 2.04E-06         |
| High mannose                  | -             | -                    | -                      | -                | -1.36       | 2.16E-02         |
| Asialylated/afucosylated (excluding HM) | 1.23          | 1.36E-03             | -                      | -                | 1.30        | 1.07E-03         |
| Neutral fucosylated           | 1.20          | 2.98E-03             | -                      | -                | 1.31        | 1.18E-04         |
| Agalactosylated               | -             | -                    | -                      | -                | -           | -                |
| Monogalactosylated            | 1.28          | 7.04E-05             | -                      | -                | 1.48        | 8.33E-07         |
| Digalactosylated              | 1.34          | 3.89E-07             | -                      | -                | 1.59        | 1.19E-07         |
| Trigalactosylated             | -             | -                    | -                      | -                | 1.24        | 2.32E-03         |
| Tetragalactosylated           | -             | -                    | -                      | -                | -           | -                |
| Bisecting GlcNAc              | 1.29          | 8.78E-05             | -                      | -                | 1.48        | 1.08E-06         |
| Sialylated (no fucose)        | 1.36          | 2.09E-05             | -                      | -                | 1.66        | 4.72E-06         |
| Fucosylated & Sialylated      | -             | -                    | -                      | -                | 1.28        | 1.26E-04         |
| Monosialylated                | 1.26          | 3.80E-07             | -                      | -                | 1.39        | 1.70E-06         |
| Disialylated                  | 1.36          | 2.39E-06             | -                      | -                | 1.64        | 2.10E-07         |
| Trisialylated                 | -             | -                    | -                      | -                | 1.20        | 9.54E-03         |
| Tetrosialylated               | -             | -                    | -                      | -                | -1.17       | 8.96E-03         |
**Figure 1.** Relative abundance of the 6 glycan types found in urine

| Glycan Type                        | Percentage (%) |
|------------------------------------|----------------|
| High-mannose (HM)                  | 42.1 (±3.0)    |
| Asialylated/afucosylated (excluding HM) | 8.4 (±1.6)    |
| Neutral fucosylated                | 4.0 (±2.2)     |
| Sialylated (no fucose)             | 6.6 (±1.1)     |
| Sialylated & fucosylated           | 1.7 (±0.2)     |
| Sulfated                           | 6.6 (±1.1)     |

**Figure 2.** Representative HCD-MS/MS spectra of two sialylated N-glycans released from urine. (A) MS/MS spectrum of the $^{12}$C$_6$-2-AA labeled Hex$_3$HexNAc$_3$Fuc$_1$NeuAc$_1$ (m/z 951.3844, z=2). (B) MS/MS spectrum of the $^{12}$C$_6$-2-AA labeled Hex$_6$HexNAc$_6$NeuAc$_3$ (m/z 1086.0970, z=3). All of the annotated peaks were single proton adducts. The peaks were assigned a putative topology based on their m/z values, MS$^2$ fragments and known N-glycosylation biosynthetic pathway. Further structural details, such as inter-residue linkage, branching pattern and anomericity,
were not determined. The illustrations of the representative monoisotopic fragment ions were edited by the software GlycoWorkbench 2.1.\textsuperscript{28} Symbols: blue square: N-acetylglucosamine; yellow square: N-acetylgalactosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose; purple diamond: N-acetylneuraminic acid; AA: 2-aminobenzoic acid.

Figure 3. LC-MS/MS spectra of two fucosylated glycan isomers released from urine. (A) EIC of the $^{12}$C$_6$-2-AA labeled Hex$_6$HexNAC$_5$Fuc$_1$NeuAc$_3$ (m/z 1067.0907, z=3) from urine. The peaks are indicated by the numbers. (B) HCD-MS/MS spectrum of peak 1. (C) HCD-MS/MS spectrum of peak 2. All of the annotated peaks were single proton adducts. The peaks were assigned a putative topology based on their m/z values, MS$^2$ fragments and known N-glycosylation biosynthetic pathway. Further structural details, such as inter-residue linkage, branching pattern and anomericity, were not determined. The illustrations of the representative monoisotopic fragment ions were edited by the software GlycoWorkbench 2.1.\textsuperscript{28} Symbols: blue square: N-acetylglucosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose; purple diamond: N-acetylneuraminic acid; AA: 2-aminobenzoic acid.
Figure 4. Representative HCD-MS/MS spectra of two sulfated N-glycans released from urine. (A) MS/MS spectrum of the $^{12}\text{C}_6\text{-2-}2\text{-AA labeled Hex}_5\text{HexNAc}_4\text{NeuAc}_2\text{S}_1$ (m/z 821.9698, z=3). (B) MS/MS spectrum of the $^{13}\text{C}_6\text{-2-}2\text{-AA labeled Hex}_4\text{HexNAc}_5\text{Fuc}_1\text{NeuAc}_1\text{S}_1$ (m/z 1176.9400, z=2). All of the annotated peaks were single proton adducts. The peaks were assigned a putative topology based on their m/z values, MS$^2$ fragments and known N-glycosylation biosynthetic pathway. Further structural details, such as inter-residue linkage, branching pattern and anomericity, were not determined. The illustrations of the representative monoisotopic fragment ions were edited by the software GlycoWorkbench 2.1. Symbols: blue square: N-acetylglucosamine; yellow square: N-acetylgalactosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose; purple diamond: N-acetylneuraminic acid; S: sulfate; AA: 2-aminobenzoic acid.