Analysis of Normal Levels of Urine and Plasma Free Glycosaminoglycans in Adults

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Abstract

Plasma and urine glycosaminoglycans (GAGs), long linear sulfated polysaccharides, have been recognized as potential non-invasive biomarkers for several diseases. However, owing to the analytical complexity associated with the measurement of GAG concentration and disaccharide composition (or GAGome), a reference study of the normal healthy GAGome is currently missing. Here, we prospectively enrolled 270 healthy adults and analyzed their urine and plasma free GAGomes using a standardized ultra-high-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (UHPLC-MS/MS) method together with comprehensive demographic and blood chemistry biomarker data. Free GAGomes did not correlate with age nor with any of the 25 blood chemistry biomarkers, except for a few plasma disaccharides that correlated with hemoglobin and C-reactive protein. However, free GAGome levels were generally higher in males. Partitioned by gender, we established reference ranges for all plasma and urine free chondroitin sulfate (CS), heparan sulfate (HS), and hyaluronic acid (HS) disaccharides. Our study is the first large-scale determination of normal plasma and urine free GAGomes reference ranges and represents a critical resource for biomarker research.

Introduction

Glycosaminoglycans (GAGs) are a family of long linear polysaccharides consisting of repeating disaccharide units (*1*). Different classes of GAGs have been characterized. In humans, the most prevalent classes are chondroitine sulfate (CS) [(→3)-β-D-GalNAc(1→4)-β-D-GlcA or α-L- IdoA(1→], heparan sulfate (HS) [(→4)-α-D-GlcNAc or α-D-GlcNS(1→4)-β-D-GlcA or α-L-IdoA (1→], and hyaluronic acid (HA) [(→3)-β-D-GlcNAc(1 → 4)-β-D-GlcA(1→] where GalNAc is N-acetylgalactosamine, GlcA is glucuronic acid, IdoA is iduronic acid, GlcNAc is N-acetylglucosamine, and GlcNS is N-sulfoglucosamine. glucuronic acid, which can be further modified by sulfation in up to three sites. CS and HS disaccharides can each be further modified with O-sulfo groups in up to three positions. The resulting sulfation motifs confer GAGs highly diverse biological functions that are essential for healthy human development and physiology (*2*). The panel of GAG motifs resulting from the diversity in structure and concentration of GAGs is collectively referred to as GAGome.

Alterations in the physiological function of GAGs have been associated with several diseases ranging from mucopolysaccharidosis, a group of rare metabolic disorders caused by genetic defects in lysosomal enzymes that degrade GAGs, to complex diseases such as sepsis, rheumatoid arthrosis, and cancer (*3–6*). Plasma and urine GAGs have been routinely proposed as promising biomarkers for early non-invasive diagnostics (*6*). Despite the potential role of GAGs for clinical applications, the measurements of the GAG concentration and disaccharide composition profile – also referred to as GAGomes - has been limited to very small sample sizes (ranging from 3 healthy donors in (*7*) to 25 in (*6*)), in predominantly retrospective and selected donors, with different analytical techniques performed within academic laboratories (*3*, *4*, *6–9*). These limitations mostly arose because effective analytical methods have only been recently developed, and they proved hard to standardize and expensive to run. As a result, the reported levels in healthy subjects in literature are widely variable and cannot be consistently used to determine reference ranges.

In this study, we took advantage of a standardized analytical method using ultra-high-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (UHPLC-MS/MS) (*10*) to analyze the urine and plasma free GAGomes in a Good Laboratory Practice (GLP)-compliant blinded central laboratory in two prospectively enrolled independent cohorts of 270 self-reported healthy subjects with comprehensive demographic and blood chemistry data. After determining the correlation of free GAGomes with these demographic and blood chemistry variables, we then established reference ranges for the normal urine and plasma free GAGomes according to accepted guidelines (CLSI EP28-A3c).

Patients and Methods.

Study design. This study was designed and conducted in compliance with the CLSI. Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline—Third Edition. CLSI document EP28-A3c. Wayne, PA: Clinical and Laboratory Standards Institute, 2008. The collection of specimens was planned prospectively with *a priori* criteria for population sampling. The present study obtained ethical permission from the Ethical Committee (Etikprövningsmyndigheten) in Gothenburg, Sweden on February 8, 2018 (#737-17).

Study population. This study prospectively enrolled self-rated healthy adult subjects in one site in Sweden forming two independent cohorts. Cohort 1 and 2 were used to form the reference sample group and they were both enrolled at Sabbatsberg Hospital, Stockholm, Sweden between May 2018 and December 2019. Inclusion criteria were: adult between 21 and 78 years old; at least moderate self-rated health; no history of cancer (except non-malignant skin cancer); no family history of cancer (first-degree relative); fit to undergo protocol procedures. Exclusion criteria were: abnormal PSA value in the last 5 years. Eligible participants were identified among volunteers based on a questionnaire by trained research nurses. Participants in each cohort formed a consecutive series. EDTA-plasma and spot-urine samples were collected in one single visit. Blood samples were also obtained by venipuncture from participants in Cohort 1 and 2 to evaluate laboratory biomarkers informative of the general health status of the subject, including the complete blood count, and the concentration of electrolytes (sodium, potassium, calcium), ALAT, ASAT, CRP, PSA, glycated hemoglobin (Cohort 2 only), LDL and HDL (Cohort 1 only). Subjects with abnormal values were referred for a doctor's appointment but were otherwise retained in the study.

Glycosaminoglycan measurements. Urine was collected at room temperature in polypropylene cup. EDTA-plasma was collected through venipuncture in a vacuette at room temperature and next subjected to centrifugation (1100-1300 g, 20 minutes at room temperature in Cohort 1, 2500 g 15 minutes at 4 °C in Cohort 2) within 15 minutes. Samples could be stored refrigerated (4 °C) for 12 hours prior to transfer to a freezer (-20 °C in Cohort 1, -70 °C in Cohort 2). Shipment was performed at the same temperature as storage.

Laboratory measurements of the GAGome included the absolute concentration in µg/mL of chondroitin sulfate (CS), heparan sulfate (HS), hyaluronic acid (HA) disaccharides, resulting in 17 independently measured features. Specifically, we quantified 8 CS disaccharides (0s CS, 2s CS, 6s CS, 4s CS, 2s6s CS, 2s4s CS, 4s6s CS, Tris CS) and 8 HS disaccharides (0s HS, 2s HS, 6s HS, Ns HS, Ns6s HS, Ns2s HS, 2s6s HS, Tris HS) – corresponding to different sulfation patterns of CS and HS – and 1 HA disaccharide. The GAGome was expanded to include the CS and HS disaccharide composition in mass fractions and the CS and HS charge. The GAGome therefore consisted of 35 features.

Sample preparation was performed in a single blinded GLP-compliant laboratory using the MIRAM™ Free Glycosaminoglycan Kit (Elypta AB, Sweden) to extract GAGs from urine samples. GAG detection and quantification was obtained through ultra-high-performance liquid chromatography coupled with triple-quadrupole mass spectrometry

(Waters® Acquity I-class Plus Xevo TQ-S micro) in accordance with the instruction for use in of the kit (*10*).

Statistical analysis. GAGome features were first transformed with Box-Cox transformation to identify outliers. Univariable linear regression was used to regress each individual GAGome feature to the clinical or biochemical variable of interest. Likelihood ratio test was used as the goodness-of-fit test for each univariable model versus an intercept-only model. Multiple testing was adjusted using the Benjamini-Hochberg or Bonferroni corrections depending on if clinical or biochemical variables were tested, and *FDR* values < 0.1 and *p*-values < $5 \cdot 10^{-5}$ were considered statistically significant, respectively. All statistical analyses were carried out in *R* (4.0.4) (*11*).

Results

Subject characteristics

We prospectively enrolled two cohorts of self-rated healthy adults with no history nor family history of cancer (except non-melanoma skin cancer) from one sites in Sweden, Stockholm (Cohort 1, *N* = 254 and 2, *N* = 16), for a total of 270 participants (Table 1). Cohort 1 and 2 formed the reference sample group to establish reference intervals.

Subjects' characteristics were balanced between cohorts (Table 1). The median age across cohorts was 57 years (range: 22 – 78) with 167 (56%) women and 103 (44%) men. Virtually all subjects self-reported good (46%) or very good (41%) health status. Blood chemistry was measured in Cohort 1 and 2 with HDL/LDL only in Cohort 1 and glycated hemoglobin only in Cohort 2. About half the subjects (42%) reported no abnormal values, and 42% reported values outside the reference ranges in up to 2 biomarkers. Nineteen subjects (8%) reported more than two abnormal values. These subjects were retained in the reference sample group, but we opted to perform a sensitivity analysis of the reference values within this subgroup.

The plasma and urine free GAGomes were determined in a single GLP-compliant blinded laboratory using a standardized kit on UHPLC-MS/MS in all Cohort 1 and 2 samples (*N* = 270).

Table 1. Subject characteristics in the reference sample group (Cohort 1 and Cohort 2). Distributions are summarized as median and min-max range in brackets. Missing values were omitted.

	Cohort 1	**Cohort 2**	**Cohort 1 + 2**
N	254	16	270
Age	58 (22-78)	43 (27-51)	57 (22-78)
Gender			
Female	162	5	167
Male	92	11	103
Self-rated health			
Moderate	9	NA	9
Good	133	5	138
Very good	112	11	123
Blood biomarkers			
ALAT	0.36 (0.13-2.44)	0.49 (0.27-1.1)	0.36 (0.13-2.44)
ASAT	0.41 (0.22-1.65)	0.41 (0.27-0.59)	0.41 (0.22-1.65)
Calcium	2.39 (2.15-2.67)	2.42 (2.17-2.58)	2.39 (2.15-2.67)
Creatinine	70 (46-167)	80 (59-102)	70 (46-167)
CRP	1.4 (0.2-54.1)	0.65 (0.27-5.5)	1.3 (0.2-54.1)
EGFR	82 (33-90)	90 (76-90)	83 (33-90)

Glycated hemoglobin	-	33.5 (28-39)	33.5 (28-39)
HDL	61.87 (29.78-123.74)	-	61.87 (29.78-123.74)
LDL	131.48 (46.4-239.75)	-	131.48 (46.4-239.75)
Potassium	4.2 (3.5-5.2)	4.2 (3.9-4.9)	4.2 (3.5-5.2)
Prostate specific antigen level	0.81 (0.11-9.8)	0.7 (0.33-1.4)	0.81 (0.11-9.8)
Sodium	139 (132-145)	140.5 (138-144)	139 (132-145)
Complete blood count			
Hematocrit	0.42 (0.35-0.51)	0.44 (0.37-0.48)	0.42 (0.35-0.51)
Hemoglobin	141 (116-178)	145.5 (118-163)	141 (116-178)
Mean corpuscular hemoglobin	30 (24-37)	30 (27-31)	30 (24-37)
Mean corpuscular hemoglobin concentration	336 (304-366)	331 (315-351)	336 (304-366)
Mean corpuscular volume	90 (81-101)	89.5 (86-94)	90 (81-101)
Absolute neutrophil count	3.2 (1-7.7)	-	3.2 (1-7.7)
Basophil count	0 (0-0.2)	-	0 (0-0.2)
Eosinophil count	0.1 (0-2.8)	-	0.1 (0-2.8)
Erythrocyte count	4.6 (3.8-5.7)	4.85 (4.1-5.3)	4.6 (3.8-5.7)
Leukocyte count	5.6 (2.8-10.8)	5.75 (3.5-7.7)	5.6 (2.8-10.8)
Lymphocyte count	1.7 (0.7-4.5)	-	1.7 (0.7-4.5)
Monocyte count	0.4 (0.2-0.9)	-	0.4 (0.2-0.9)
Platelet count	261 (137-488)	239 (166-377)	261 (137-488)
Blood chemistry status			
No abnormal values	115	11	126
1-2 abnormal values	120	5	125
>2 abnormal values	19	0	19

Effect of age and gender on the normal free GAGome

We sought to identify if the urine or plasma free GAGome correlated with age and gender that could partition the reference intervals.

We did not observe any statistically significant association between age (as a continuous variable) and any GAGome feature (FDR > 0.1, Table S1, Figure S1) except for the plasma free 6s CS concentration (% change per year of age = 1.1, FDR = 0.003). Considering that the average 6s CS concentration was relatively low to begin with, the effect of age was limited.

We observed statistically significant associations between gender and 11 (29%) GAGome features (FDR < 0.1, Table S2, Figure 1-2). In plasma, the effect of gender was generally limited to an average 7% increase in total CS and 9% increase 4s CS concentration in males (FDR = 0.042). In urine, males displayed an average 29%-41% higher concentration for the major CS disaccharides (0s, 4s, 6s, and 2s6s CS), resulting in an average 30% increase in total CS; and an average 53%-115% higher concentration for the major HS disaccharides (0s, Ns, 6s), resulting in an average 47% increase in total HS. No gender effect was observed for HA.

Effect of blood chemistry on the normal free GAGome

We sought to characterize whether other blood chemistry biomarkers were correlated with the normal urine or plasma free GAGome.

First, we classified subjects in three groups indicative of their general health status depending on whether we recorded, for any of the blood chemistry biomarkers here tested, no abnormal values ("No abnormal value", N = 126), 1 or 2 abnormal values ("1-2 abnormal values", N = 125), or more than 2 abnormal values (">2 abnormal values", N = 19). We did not observe

any statistical associations between any of GAGome feature and any of these groups (Table S3).

Secondly, we tested if there was a linear correlation between each GAGome feature and each blood chemistry biomarker level (as a continuous variable). Of 25 biomarkers, we observed no correlation with any urine free GAGome feature after controlling for multiple testing ($p > 5{\cdot}10^{-5}$, Bonferroni correction, Figure S2). For the plasma free GAGome, the total CS and 4s CS concentration were positively correlated with three biomarkers (hemoglobin, hematocrit, and erythrocyte count) with Pearson correlation coefficient ρ ranging 0.24-0.27; CRP correlated with higher total HS (ρ = 0.27) and TriS HS concentration (ρ = 0.46); and HDL was negatively correlated with total 0s CS (ρ = -0.26, Figure S3).

Reference intervals for the normal free GAGome

Given the above, we concluded that reference intervals should be partitioned by gender. We thus defined reference intervals for apparently healthy men and women between the ages of 22 and 78.

First, we established reference intervals for the urine free GAGome. For each disaccharide, *bona fide* outliers were identified according to a pre-specified procedure and omitted on the grounds of test failure (median % of outliers across CS disaccharides: 2.5% in males, 1% in females; across HS: 7.5% in males, 10.5% in females. The total CS and HS concentration, charge as well as the CS and HS disaccharide composition was then reported in both absolute (μg/ml) and relative (mass fraction %) values by gender (Figure 1).

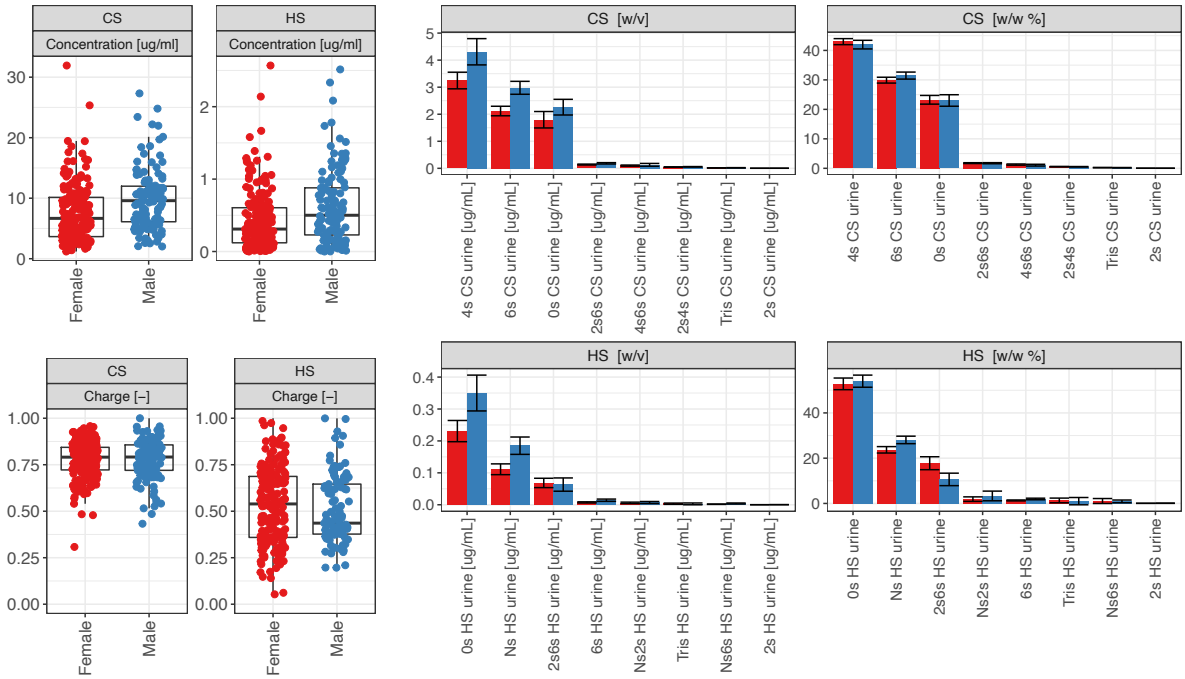

Figure 1. Urine free total CS and HS concentration (μg/ml), charge, and disaccharide concentration (μg/ml) and composition (in mass fraction %) in the reference sample group by gender (Cohort 1 and 2, $N_{females}$ = 167, N_{males} = 103). Error bars indicate +/- 1.96 SEM (95% confidence interval). Key: Red – female, blue - male.

The average urine free total CS concentration was 8.99 μg/ml in males and 7.02 μg/ml in females. The CS composition in males and females was nearly identical. Respectively, 43%-45% 4s CS, 33%-29% 6s CS, and 23% 0s CS, 2% 2s6s CS, 1% 2s4s CS, and 1% 4s6s CS. The fraction of 2s CS and TriS CS were undetectable. Thus, the average CS charge was 0.78 for both genders. The average total free HS concentration was 0.50 μg/ml in males and 0.29 μg/ml in females. The HS composition in males and females was 62% 0s HS, and, respectively, 34% and 28% Ns CS, 4% and 14% 2s6s HS, 2% and undetectable 6s HS. The fractions of Ns2s HS, Ns6s HS, 2s HS and TriS HS were undetectable. The average HS charge was 0.42 in males and 0.50 in females. We noticed that in general the HS fraction resulted in higher variability than the CS fraction.

Reference intervals were established for each urine free GAGome feature using the simple nonparametric method after outlier identification and exclusion. We reported in Table 2 the reference intervals of all urine free CS and HS features using the corresponding central 95% distribution of values.

Table 2. Reference intervals of urine free total CS and HS concentration (μg/ml) and disaccharide concentration (μg/ml) by gender. Outliers were excluded.

Absolute concentration	**Male (N = 103)**				**Female (N = 167)**			
	N outliers	**Mean**	**Lower reference limit (2.5th perc.)**	**Upper reference limit (97.5th perc.)**	**N outliers**	**Mean**	**Lower reference limit (2.5th perc.)**	**Upper reference limit (97.5th perc.)**
Total CS		8.99	2.69	18.26		7.02	1.67	16.45
4s CS	2 (2%)	3.86	0.79	9.14	0 (0%)	3.13	0.64	7.29
6s CS	0 (0%)	2.93	0.9	5.75	0 (0%)	2.07	0.53	4.56
0s CS	3 (3%)	2.08	0.31	5.13	1 (1%)	1.61	0.2	5.61
2s6s CS	1 (1%)	0.17	0	0.39	0 (0%)	0.13	0	0.38
4s6s CS	6 (6%)	0.07	0	0.42	8 (5%)	0.08	0	0.38
2s4s CS	0 (0%)	0.06	0	0.17	2 (1%)	0.05	0	0.15
Tris CS	10 (10%)	0.01	0	0.04	14 (8%)	0.01	0	0.04
2s CS	14 (14%)	0	0	0	23 (14%)	0	0	0
Total HS		0.50	0.01	1.26		0.29	0.01	0.79
0s HS	2 (2%)	0.31	0	0.82	10 (6%)	0.18	0.00	0.52
Ns HS	2 (2%)	0.17	0	0.42	11 (7%)	0.08	0.00	0.24
2s6s HS	17 (17%)	0.02	0	0.1	13 (8%)	0.04	0.00	0.20
6s HS	8 (8%)	0.01	0	0.04	16 (10%)	0.00	0.00	0.02
Ns2s HS	14 (14%)	0	0	0.01	23 (14%)	0.00	0.00	0.01
Ns6s HS	18 (17%)	0	0	0	16 (10%)	0.00	0.00	0.00
2s HS	6 (6%)	0	0	0	19 (11%)	0.00	0.00	0.00
Tris HS	7 (7%)	0	0	0	25 (15%)	0.00	0.00	0.00

We repeated the procedure above for the plasma free GAGome (median % of outliers across CS disaccharides: 9% in males or females, Figure 2). The HS fraction was largely undetectable (mean total HS < 0.001 μg/ml) and we therefore omitted it.

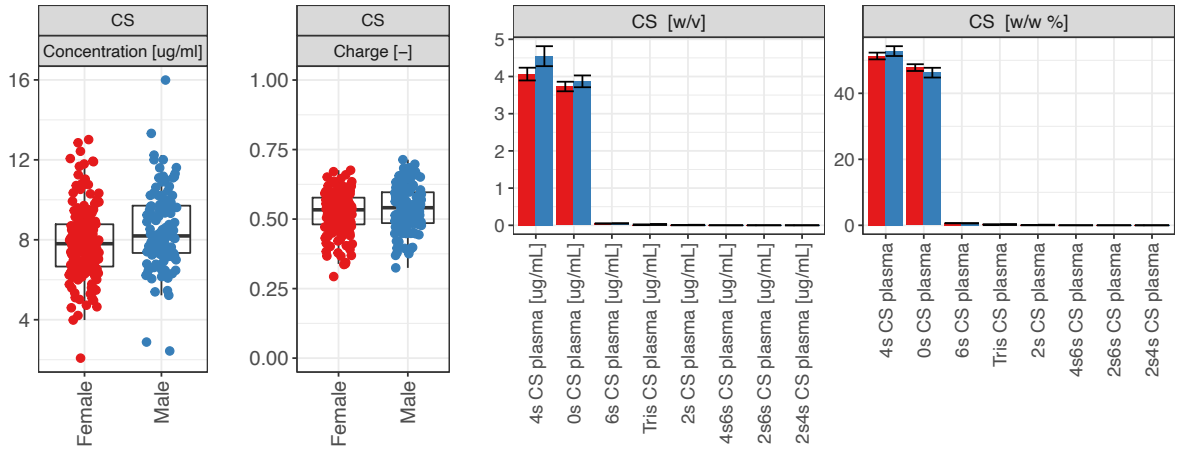

Figure 2. Plasma free total CS concentration (µg/ml), charge, and disaccharide concentration (µg/ml) and composition (in mass fraction %) in the reference sample group (Cohort 1 and 2). Plasma HS was undetectable. Error bars indicate +/- 1.96 SEM (95% confidence interval). Key: Red – female, blue - male.

The average plasma free total CS concentration was 8.22 µg/ml in males and 7.67 µg/ml. The CS composition was nearly identical across genders, 52% 4s CS, 48% 0s CS, and 0.6% 6s CS. The fractions of the remaining CS disaccharides were undetectable. The average CS charge was 0.52.

Table 3. Reference intervals of plasma free total CS concentration (µg/ml) and disaccharide concentration (µg/ml) by gender. Plasma free HS was undetectable. Outliers were excluded.

Absolute concentration	**Male (N = 103)**				**Female (N = 167)**			
	N outliers	**Mean**	**Lower reference limit (2.5th perc.)**	**Upper reference limit (97.5th perc.)**	**N outliers**	**Mean**	**Lower reference limit (2.5th perc.)**	**Upper reference limit (97.5th perc.)**
Total CS		8.22	5.7	11.16		7.67	5.01	10.76
4s CS	2 (2%)	4.29	2.24	6.54	4 (2%)	3.93	2.06	5.61
0s CS	4 (4%)	3.89	2.58	5.61	4 (2%)	3.7	2.39	5.28
6s CS	3 (3%)	0.05	0.02	0.09	2 (1%)	0.05	0.03	0.09
2s CS	13 (13%)	0	0	0	22 (13%)	0	0	0
4s6s CS	14 (14%)	0	0	0	23 (14%)	0	0	0
2s4s CS	6 (6%)	0	0	0	11 (7%)	0	0	0
2s6s CS	17 (17%)	0	0	0	37 (22%)	0	0	0
Tris CS	12 (12%)	0	0	0	19 (11%)	0	0	0

Discussion

Glycosaminoglycan metabolism has been implicated in multiple pathways regulating development of healthy tissues and pathogenic states, and particularly sulfation patterns of GAGs, which establish a relationship between the structure and function (*1*, *2*) (*12*). Their relevance to different disease states underscores the role of GAGs as potential biomarkers with several studies focusing on plasma and urine GAGs for non-invasive diagnostic and prognostic applications (*4*, *6*, *8*, *9*, *13*). However, these studies had very limited sample sizes.

Critical information such as the influence of age, gender, and other biomarkers on the GAGs is imperative for biomarker characterization. Here, we established such correlations and established reference ranges taking advantage of standardized high throughput UHPLC-MS/MS method to profile free GAGomes in a large adult population (*10*).

We found that age correlated with an increase in free 6s CS in plasma, but no other GAGome feature showed any notables differences with respect to age in adults. While this finding in urine agree with previous studies analyzing total GAGs (*14*), it contradicts similar studies in plasma total GAGs (*15*). In contrast to age, we found that the concentrations of several CS and HS disaccharides were higher in males than females with a larger effect in urine than plasma. Previous studies noted gender differences in total GAGs, although in the opposite directions for both urine and plasma (*14*, *15*). The origin of these discrepancies remains to be ascertained, but we speculate that they could be attributed to the focus on the total rather than the free GAG fraction in previous studies, as well as older analytical techniques for GAG measurements. We also observed that urine free GAGomes appeared independent of other blood chemistry biomarkers, including markers of inflammation, glucose metabolism, and liver functions – underscoring that they may reflect an independent physiological state in adults. In contrast, plasma free CS and HS had weak to moderate correlations with markers related to circulating oxygenation and inflammation, respectively.

We observed that the reference ranges established for urine and plasma free GAGomes were remarkably tight for most plasma disaccharides, deviating by ~50% at most from the mean, and quite stable also for most urine disaccharides, with ~2-fold maximum deviation from the mean. Overall, these findings suggest that urine and plasma free GAGomes have independent and stable levels in the present adult population. Therefore, free GAGs are suitable as biomarker candidates, if deviations from aforementioned reference ranges are determined in association with a certain disease state. While it remains to be ascertained whether the here established reference ranges and correlations are applicable to other populations, we believe that this study represents a critical resource for biomarker research using biofluidic GAGomes.

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Author contributions

Fr.G., J.N. conceived, designed, and coordinated the study. S.B., A.L., and Fr.G. performed data analysis and statistics. N.V., Fa.G., and F.M. assisted with the laboratory method. S.B., A.L., and Fr.G. drafted the manuscript. All authors edited and approved the manuscript in its final form.

Conflict of interest

At study start, Fr.G. and J.N. were listed as co-inventors in patent applications related to the biomarkers described in this study. At the time of submission, Fr.G. and J.N. were

shareholders in Elypta AB, which owned the above-mentioned patent applications, Fr.G. was an employee in Elypta AB and J.N. was member of the board. Fr.G. and J.N. declare no further conflict of interest. All other authors declare no conflict of interest.

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