1   **Analysis of Normal Levels of Urine and Plasma Free Glycosaminoglycans**

2   **in Adults**

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Abstract

Plasma and urine glycosaminoglycans (GAGs) are long linear sulfated polysaccharides 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, the so-called GAGome, a reference study of the normal healthy GAGome is currently missing. Here, we prospectively enrolled 308 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. Of 25 blood chemistry biomarkers, we mainly observed weak correlations between the free GAGome and creatinine in urine, and hemoglobin or erythrocyte counts in plasma. We found higher free GAGome concentration – but not composition - in males. Partitioned by gender, we established reference intervals for all detectable free GAGome features in urine and plasma. We carried out a transference analysis in healthy individuals from two distinct geographical sites, including the Lifelines Cohort Study, which validated the reference intervals in urine. Our study is the first large-scale determination of normal plasma and urine free GAGomes reference intervals and represents a critical resource for physiology and 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 GAGomes have been proposed as promising biomarkers for early non-invasive diagnostics (*6*). Despite the potential role of GAGs for clinical applications, the measurements of the GAGome 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 are due to the historical lack of effective analytical methods until recently (*10–14*), which proved hard to standardize and expensive to run. As a result, the GAGome values reported in the literature for healthy subjects are widely variable and cannot be consistently used as reference for physiology and biomarker research.

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) (*15*) 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 308 self-reported healthy subjects with comprehensive demographic and blood chemistry data. We first determined the correlation of free GAGomes with demographic and blood chemistry variables. Next, we established reference intervals for the normal urine and plasma free GAGomes according to accepted guidelines (CLSI EP28-A3c). Finally, we validated the proposed references intervals by transference analysis on two independent cohorts consisting of a total of 140 healthy individuals from two distinct geographical sites.

1   **Patients and Methods.**

2   **Study design.** This study was designed and conducted in compliance with the CLSI.
3   Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory;
4   Approved Guideline—Third Edition. CLSI document EP28-A3c. Wayne, PA: Clinical and
5   Laboratory Standards Institute, 2008. The collection of specimens was planned prospectively
6   with *a priori* criteria for population sampling. The present study was approved by the Ethical
7   Committee (Etikprövningsmyndigheten) in Gothenburg, Sweden (#737-17 and #198-16).

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

23   **Transference population.** The transference analysis was performed on two cohorts (Cohorts
24   3 and 4) representative of healthy adults from two distinct and external geographical sites.
25   Cohort 3 used retrospectively archived specimens from the Lifelines Cohort study (*16*)**.**
26   Inclusion criteria were: adults older than 18 years old; self-reported healthy. Exclusion criteria
27   were: diagnosis of cancer within 18 months from sampling visit. Lifelines is a multi-
28   disciplinary prospective population-based cohort study examining in a unique three-
29   generation design the health and health-related behaviours of 167,729 persons living in the
30   North of the Netherlands. It employs a broad range of investigative procedures in assessing
31   the biomedical, socio-demographic, behavioural, physical and psychological factors which
32   contribute to the health and disease of the general population, with a special focus on multi-
33   morbidity and complex genetics. Cohort 4 was enrolled prospectively at Sahlgrenska
34   University Hospital, Göteborg, Sweden. Inclusion criteria were: adults older than 18 years
35   old; self-reported healthy. Exclusion criteria were: none.

36   **Glycosaminoglycan measurements.** Urine was collected at room temperature in a
37   polypropylene cup. EDTA-plasma was collected through venipuncture in a vacuette at room
38   temperature and next subjected to centrifugation (1100-1300 g, 20 minutes at room
39   temperature in Cohort 1, 2500 g 15 minutes at 4 °C in Cohort 2) within 15 minutes. Samples
40   could be stored refrigerated (4 °C) for 12 hours prior to transfer to a freezer (-20 °C in Cohort
41   1, -70 °C in Cohort 2). Shipment was performed at the same temperature as storage.

1   Sample preparation was performed in a single blinded GLP-compliant laboratory using the
2   MIRAM^{TM} Free Glycosaminoglycan Kit (Elypta AB, Sweden) to extract glycosaminoglycans
3   (GAGs) from urine samples. GAG detection and quantification was obtained through ultra-
4   high-performance liquid chromatography coupled with triple-quadrupole mass spectrometry
5   (Waters® Acquity I-class Plus Xevo TQ-S micro) in accordance with the instruction for use
6   in of the kit (*15*).

7   Laboratory measurements of the GAGome included the absolute concentration in µg/mL of
8   chondroitin sulfate (CS), heparan sulfate (HS), hyaluronic acid (HA) disaccharides, resulting
9   in 17 independently measured features. Specifically, we quantified eight CS disaccharides (0s
10   CS, 2s CS, 6s CS, 4s CS, 2s6s CS, 2s4s CS, 4s6s CS, Tris CS) and eight HS disaccharides (0s
11   HS, 2s HS, 6s HS, Ns HS, Ns6s HS, Ns2s HS, 2s6s HS, Tris HS) – corresponding to different
12   sulfation patterns of CS and HS – and one HA disaccharide. A measured GAGome feature
13   was considered detectable in a fluid (plasma or urine) if its mean concentration across all
14   samples was > 0.1 ug mL^{-1}(*15*).

15   The detectable GAGome was further used to calculate additional dependent features,
16   including the total amount of CS and HS (as a sum of individually measured disaccharides),
17   the CS and HS composition (as mass fractions of individual detectable CS and HS
18   disaccharides) and the CS and HS charge (as the ratio of the sulfated disaccharides weighted
19   by their charge and total CS and HS disaccharides).

20   **Statistical analysis.** Before all analyses, GAGome features were transformed with Box-Cox
21   transformation to identify and exclude outliers. Reference intervals were established for each
22   urine and plasma free GAGome feature using a simple nonparametric method after outlier
23   identification and exclusion. The lower and upper reference limits (reference intervals) for
24   individual GAGs were estimated as the 2.5th and 97.5th percentiles of the distribution of
25   measured values for the reference population, respectively.

26   The correlation between each GAGome feature and each clinical (e.g. age) or biochemical
27   (e.g. LDL) variable was calculated by univariable linear regression of a given GAGome
28   feature as response variable on a given clinical or biochemical variable as explanatory
29   variable. We computed the statistical significance of each correlation using a likelihood ratio
30   test versus an intercept-only regression model. Multiple hypothesis testing was adjusted using
31   the Benjamini-Hochberg or Bonferroni corrections depending on if clinical or biochemical
32   variables were tested, and *FDR* values < 0.1 and *p*-values $< 1.67 \cdot 10^{-4}$ were considered
33   statistically significant, respectively. All statistical analyses were carried out in *R* (4.0.4) (*17*).

34   **Results**

35   **Subject characteristics**

36   We prospectively enrolled two cohorts of self-rated healthy adults with no history nor family
37   history of cancer (except non-melanoma skin cancer) from one site in Stockholm, Sweden
38   (Cohort 1, *N* = 292 and 2, *N* = 16), for a total of 308 participants (Table 1). Cohort 1 and 2
39   formed the reference sample group to establish reference intervals.

40   Subjects' characteristics were balanced between cohorts (Table 1). Across cohorts, the
41   median age was 57 years (range: 22 – 78) with 188 (61%) women and 120 (39%) men.

1 Virtually all subjects self-reported good (51%) or very good (45%) health status. The panel of
2 blood chemistry markers measured in Cohort 1 and 2 was the same, except for HDL/LDL
3 (measured in Cohort 1 only) and glycated hemoglobin (measured in Cohort 2 only). Complete
4 blood counts were available for Cohort 1, while Cohort 2 had available measurements for
5 erythrocyte, leukocyte and platelet counts. Blood chemistry showed normal values for all
6 biomarkers in 47% of the subjects, values outside the reference intervals for up to 2
7 biomarkers in 45% of the subjects, and more than two abnormal values in 23 subjects (8%).
8 The abnormal blood chemistry values were mostly due to elevated calcium (12.2 % subjects),
9 low sodium (10.4%), low CRP (7.5%), low EGFR (7.2%) and elevated PSA (5%). We kept
10 subjects with abnormal values in the reference sample group and performed a sensitivity
11 analysis of the reference values within this subgroup.

12 The plasma and urine free GAGomes were measured in a single GLP-compliant blinded
13 laboratory using a standardized kit on UHPLC-MS/MS in all Cohort 1 and 2 samples ($N =$
14 308).

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

	Cohort 1	Cohort 2	Cohort 1 + 2
N	292	16	308
Age	57 (22-78)	43 (27-51)	57 (22-78)
Gender			
Female	183	5	188
Male	109	11	120
Self-rated health			
Moderate	10	0	10
Good	153	5	158
Very good	129	11	140
Blood chemistry biomarkers			
ALAT (**µkat/L**)	0.36 (0.13-2.44)	0.49 (0.27-1.1)	0.37 (0.13-2.44)
ASAT (**µkat/L**)	0.41 (0.22-1.65)	0.41 (0.27-0.59)	0.41 (0.22-1.65)
Calcium (**mmol/L**)	2.39 (2.15-2.72)	2.42 (2.17-2.58)	2.39 (2.15-2.72)
Creatinine (**µmol/L**)	69 (46-167)	80 (59-102)	69.5 (46-167)
CRP (**mg/L**)	1.4 (0.2-54.1)	0.65 (0.27-5.5)	1.3 (0.2-54.1)
EGFR (**mL/min/1.73m^2**)	82.5 (33-90)	90 (76-90)	83 (33-90)
Glycated hemoglobin (**mmol/mol**)	-	33.5 (28-39)	33.5 (28-39)
HDL (**mg/dL**)	61.87 (29.78-123.74)	-	61.87 (29.78-123.74)
LDL (**mg/dL**)	131.48 (46.4-239.75)	-	131.48 (46.4-239.75)
Potassium (**mmol/L**)	4.2 (3.5-5.2)	4.2 (3.9-4.9)	4.2 (3.5-5.2)
Prostate specific antigen level (**ng/mL**)	0.8 (0.11-9.8)	0.7 (0.33-1.4)	0.8 (0.11-9.8)
Sodium (**mmol/L**)	39 (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 (**g/L**)	141 (116-178)	145.5 (118-163)	141 (116-178)
Mean corpuscular hemoglobin (**pg**)	30 (24-37)	30 (27-31)	30 (24-37)
Mean corpuscular hemoglobin concentration (**g/L**)	336 (304-366)	331 (315-351)	336 (304-366)
Mean corpuscular volume (**fL**)	90 (81-101)	89.5 (86-94)	90 (81-101)
Absolute neutrophil count (**10^9/L**)	3.2 (1-7.7)	-	3.2 (1-7.7)
Basophil count (**10^9/L**)	0 (0-0.2)	-	0 (0-0.2)

Eosinophil count (10^9/L)	0.1 (0-2.8)	-	0.1 (0-2.8)
Erythrocyte count (10^{12}/L)	4.6 (3.8-5.8)	4.85 (4.1-5.3)	4.6 (3.8-5.8)
Leukocyte count (10^9/L)	5.6 (2.8-10.8)	5.75 (3.5-7.7)	5.6 (2.8-10.8)
Lymphocyte count (10^9/L)	1.7 (0.7-4.5)	-	1.7 (0.7-4.5)
Monocyte count (10^9/L)	0.4 (0.2-0.9)	-	0.4 (0.2-0.9)
Platelet count (10^9/L)	261 (137-488)	239 (166-377)	261 (137-488)
Blood chemistry status			
No abnormal values	135	11	146
1-2 abnormal values	134	5	139
>2 abnormal values	23	0	23

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 groups indicative of their general health status depending on the number of abnormal values for the blood chemistry biomarkers here tested. Specifically, we grouped the subjects into three groups: 1) no abnormal values ("No abnormal value", N = 146), 2) one or two abnormal values ("1-2 abnormal values", N = 139), or 3) more than two abnormal values (">2 abnormal values", N = 23). We did not observe any statistical associations between any of GAGome features with any of these groups (Table S1).

Second, we investigated linear correlations between the concentration of each detectable GAGome feature and each of the 25 blood chemistry biomarker level (as a continuous variable) and focused on correlations that reached statistical significance after controlling for multiple testing ($p < 1.67 \cdot 10^{-4}$, Bonferroni correction, Figure S2). In urine, we observed weak to moderate positive correlations (ρ = 0.23-0.3) between multiple GAGome features with creatinine. Additionally, the urine 6s CS concentration was positively correlated with hemoglobin, hematocrit, and erythrocyte count (ρ = 0.24 - 0.25) and negatively correlated with HDL (ρ = -0.22). For the plasma free GAGome, the total plasma and 4s CS concentrations were weakly positively correlated with hemoglobin and erythrocyte count (ρ = 0.21-0.28). Additionally, plasma 4s CS was correlated with hematocrit (ρ = 0.25), while 0s CS negatively correlated with HDL at ρ = -0.22, (Figure S2).

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 (Table S2, Figure S1).

We observed statistically significant associations between gender and 10 detectable GAGome features (FDR < 0.1, Table S3, Figure 1-2). In plasma, the effect of gender was generally limited to an average 7% increase in total CS and 11% increase 4s CS concentration in males. The urine of males contained on average 31%-41% higher concentration for the major CS disaccharides (0s, 4s, 6s, and 2s6s CS), resulting in an average 34% increase in total CS; and an average 50% and 73% higher concentration for the major HS disaccharides (0s and Ns, respectively), resulting in an average 47% increase in total HS.

1   **Reference intervals for the normal free GAGome**

2   Given the effects of gender on the GAGome, we decided to partition reference intervals by
3   gender. We thus defined reference intervals for urine and plasma free GAGome
4   concentrations and composition for apparently healthy males and females between the ages of
5   22 and 78.

6   First, we established reference intervals for the urine free GAGome after outlier identification
7   and exclusion. For each disaccharide, *bona fide* outliers were identified according to a pre-
8   specified procedure and omitted (median % of outliers across CS disaccharides: 2% in males,
9   0% in females; across HS: 3% in males, 3% in females).

10   We then established the reference intervals partitioned by gender of all urine free CS and HS
11   features (Figure 1, Table 2).

12
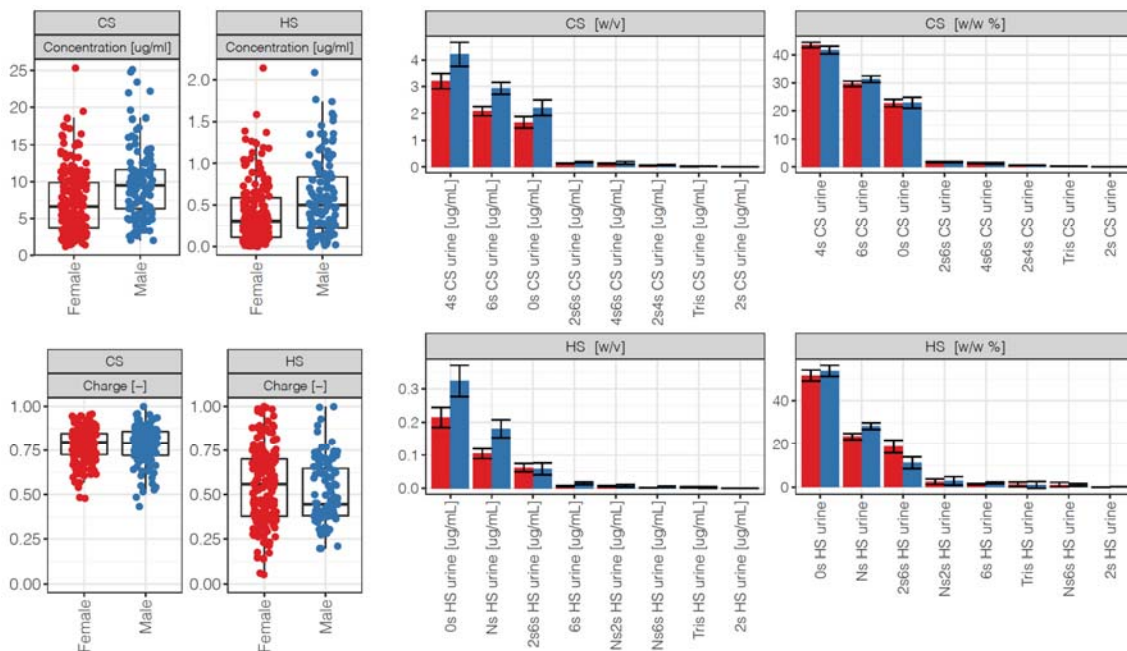
13

14   **Figure 1.** Urine free total CS and HS concentration (μg/ml), charge, and disaccharide concentration (μg/ml) and
15   composition (in mass fraction %) in the reference sample group by gender (Cohort 1 and 2, $N_{females}$ = 188 N_{males} =
16   120). Error bars indicate +/- 1.96 SEM (95% confidence interval). Key: Red – female, blue - male.

17   The average urine free total CS concentration was 8.86 μg/ml in males and 6.99 μg/ml in
18   females. The CS composition in males and females was nearly identical. The three major
19   disaccharides made up 42% and 44% (4s CS), 31% and 30% (6s CS), and 23% and 22% (0s
20   CS) of the CS fraction in urine of males and females, respectively. Of the multi-sulfated CS
21   disaccharides, the 2s6s CS and 4s6s CS cumulatively contributed approximately 3% to the CS
22   fraction in both males and females, while 2s4s CS and Tris CS were undetectable. The
23   average urine CS charge was 0.78 for males and females.

24   The average urine total free HS concentration was 0.46 μg/ml in males and 0.25 μg/ml in
25   females. The only detectable free HS disaccharides were 0s HS and Ns HS. The HS urine

1   composition was 55% 0s HS and 30% Ns HS in males, and 53% 0s HS and 23% Ns HS in
2   females. The average HS charge was 0.35 in males and 0.30 in females.
3

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

	Female (N = 188)			Male (N = 120)		
	Outliers	Mean	Reference Interval	Outliers	Mean	Reference Interval
CS						
Concentration						
Total CS [µg/mL]	-	6.99	[1.77-16.04]	-	8.86	[2.62-17.17]
4s CS [µg/mL]	0 (0.0%)	3.19	[0.68-7.42]	2 (2.0%)	4.04	[0.84-10.14]
6s CS [µg/mL]	0 (0.0%)	2.07	[0.54-4.68]	1 (1.0%)	2.90	[0.87-5.67]
0s CS [µg/mL]	2 (1.0%)	1.59	[0.20-5.18]	5 (4.0%)	1.99	[0.36-4.86]
2s6s CS [µg/mL]	0 (0.0%)	0.13	[0.00-0.37]	1 (1.0%)	0.18	[0.00-0.43]
4s6s CS [µg/mL]	9 (5.0%)	0.09	[0.00-0.41]	8 (7.0%)	0.10	[0.00-0.44]
Composition						
4s CS [%]	2 (1.0%)	43.66	[32.00-55.26]	4 (3.0%)	42.10	[29.31-53.73]
6s CS [%]	0 (0.0%)	29.76	[16.38-43.89]	2 (2.0%)	31.19	[19.44-42.51]
0s CS [%]	2 (1.0%)	22.28	[6.57-40.65]	2 (2.0%)	22.62	[6.32-46.47]
2s6s CS [%]	0 (0.0%)	1.73	[0.00-3.31]	0 (0.0%)	1.75	[0.00-3.24]
4s6s CS [%]	0 (0.0%)	1.41	[0.00-4.25]	2 (2.0%)	1.20	[0.00-4.43]
Charge CS	2 (1.0%)	0.78	[0.60-0.93]	1 (1.0%)	0.78	[0.57-0.94]
HS						
Concentration						
Total HS	-	0.25	[0.00-0.68]	-	0.46	[0.00-1.23]
0s HS [µg/mL]	10 (5.0%)	0.18	[0.00-0.55]	2 (2.0%)	0.31	[0.00-0.84]
Ns HS [µg/mL]	13 (7.0%)	0.08	[0.00-0.24]	4 (3.0%)	0.16	[0.00-0.42]
Composition						
0s HS [%]	5 (3.0%)	52.57	[18.65-83.45]	5 (4.0%)	55.19	[32.97-73.63]
Ns HS [%]	2 (1.0%)	22.96	[1.45-40.31]	8 (7.0%)	29.72	[13.27-41.34]
Charge HS	0 (0.0%)	0.30	[0.06-0.47]	0 (0.0%)	0.35	[0.21-0.44]

4

1   We repeated the procedure above for the plasma free GAGome (median % of outliers across
2   CS disaccharides: 0 % in males or 1% in females, Figure 2, Table 3). The plasma HS fraction
3   was largely undetectable (mean total HS < 0.001 µg/ml) and we therefore omitted it from
4   further analyses.

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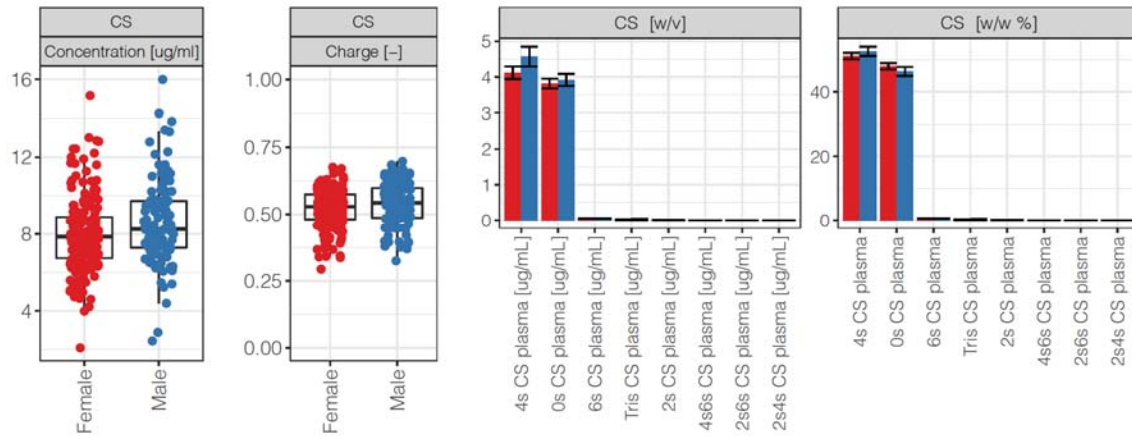
Figure 2. Plasma free total CS concentration (µg/ml), charge, and disaccharide concentration (µg/ml) and composition (in mass fraction %) in the reference sample group (Cohort 1 and 2). Plasma free HS was undetectable. Error bars indicate +/- 1.96 SEM (95% confidence interval). Key: Red – female, blue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

9   The average plasma free total CS concentration was 8.52 µg/ml in males and 7.79 µg/ml in
10 females. The CS composition was nearly identical across genders, 53% and 51% 4s CS and
11 46% and 48% 0s CS (Table 3) for males and females, respectively. The remaining free CS
12 disaccharides were undetectable. The average plasma CS charge was 0.53 in men and 0.52 in
13 women.

14 **Table 3.** Reference intervals of plasma free total CS concentration (µg/ml) and disaccharide
15 concentration (µg/ml) and composition (% w/w) by gender. Plasma free HS was undetectable.
16 Outliers were excluded.

	Female (N = 188)			Male (N = 120)		
	Outliers	Mean	Reference Interval	Outliers	Mean	Reference Interval
CS						
Concentration						
Total CS		7.79	[4.91-11.28]		8.52	[5.93-12.78]
4s CS [µg/mL]	6 (3.0%)	4.07	[2.07-6.37]	2 (2.0%)	4.55	[2.25-8.02]
0s CS [µg/mL]	4 (2.0%)	3.78	[2.26-5.48]	4 (3.0%)	3.96	[2.56-5.82]
Composition						
4s CS [%]	2 (1.0%)	51.41	[36.16-62.06]	0 (0.0%)	52.64	[36.64-65.46]
0s CS [%]	1 (1.0%)	48.02	[37.77-64.12]	0 (0.0%)	46.37	[33.92-62.09]

	Female (N = 188)			Male (N = 120)		
	Outliers	Mean	Reference Interval	Outliers	Mean	Reference Interval
Charge CS	0 (0.0%)	0.52	[0.36-0.62]	0 (0.0%)	0.53	[0.37-0.66]

1

Transference of reference intervals in an independent population

We validated the transference of the above established reference intervals for each GAGome feature in two independent populations from two distinct geographical sites (Cohort 3 and 4) by determining the complete urine and plasma free GAGome in Cohort 3 (N = 110, 60 males and 50 females) and Cohort 4 (N = 30, 15 males and 15 females). The average age was 59 years old (range 30-84) for Cohort 3 and 45 years old (range 22-66) for Cohort 4. We observed that Cohort 3 and 4 had largely similar GAGome measurements. Therefore, we opted to carry out the transference analysis in a group combining Cohort 3 and Cohort 4, partitioned by gender (N = 140, 65 females and 75 males, see Tables S4 and S5 for transference analyses on separate cohorts).

Across all urine free GAGome features, we excluded 0-9.2 % (median = 1.5%) outliers in females and 0-12 % (median = 3.3%) in males. Across all plasma free GAGome features, we excluded 0-4.6 % (median = 1.5%) outliers in females and 0-1.3 % (median = 0%) in males. We next determined the percentage of values outside the established reference limits for each urine and plasma GAGome feature, where <5% was considered acceptable for transference validation.

In urine, we observed that the transference of reference intervals was validated in both genders for the concentration of all detectable free GAGome features (Table 4). The total urine CS was outside the reference interval in 5.6% samples, while all samples had total HS within the reference interval. As regards composition, we observed a shift towards a higher 4s CS % (mean in reference sample group: 44% in females, 42% in males; in transference group: 50% in females, 47% in males) and concomitantly other smaller shifts towards lower values in the remaining CS disaccharides.

In plasma, we could not validate the transference of reference intervals for either of the two detectable plasma free GAGome features (0s CS and 4s CS) since 5 to 29% of the transference group had increased concentration (Table 5). The discrepancy was less pronounced for composition, where 11-22% had values outside the reference intervals.

Table 4. Transference of reference intervals of urine free CS and HS in an independent population (Cohorts 3 and 4). Outliers were excluded.

	Female (N = 65)					Male (N = 75)				
	N	Mean	Ref. Interval	Cohort Range	Outside Ref. Interval	N	Mean	Ref. Interval	Cohort Range	Outside Ref. Interval
CS										
Concentration										

	Female (N = 65)					Male (N = 75)				
	N	Mean	Ref. Interval	Cohort Range	Outside Ref. Interval	N	Mean	Ref. Interval	Cohort Range	Outside Ref. Interval
Total CS [µg/mL]	62	6.2	1.8-16.0	2.3-18.8	2 (3.2%)	72	8.7	2.6-17.2	2.5-26.7	4 (5.6%)
4s CS [µg/mL]	65	3.1	0.7-7.4	1.0-10.2	2 (3.1%)	75	4.1	0.8-10.1	1.4-14.0	2 (2.7%)
6s CS [µg/mL]	65	1.6	0.5-4.7	0.7-6.9	1 (1.5%)	74	2.2	0.9-5.7	0.7-7.3	3 (4.1%)
0s CS [µg/mL]	62	1.2	0.2-5.2	0.3-4.9	0 (0.0%)	73	1.9	0.4-4.9	0.5-5.2	2 (2.7%)
2s6s CS [µg/mL]	64	0.1	0.0-0.4	0.0-0.4	2 (3.1%)	70	0.2	0.0-0.4	0.0-0.4	1 (1.4%)
4s6s CS [µg/mL]	65	0.1	0.0-0.4	0.0-0.4	0 (0.0%)	70	0.2	0.0-0.4	0.0-0.4	0 (0.0%)
Composition										
4s CS [%]	60	49.4	32.0-55.3	32.4-61.8	13 (21.7%)	72	46.9	29.3-53.7	35.4-57.4	10 (13.9%)
6s CS [%]	62	25.4	16.4-43.9	14.2-33.2	2 (3.2%)	73	25.5	19.4-42.5	17.1-33.7	5 (6.8%)
0s CS [%]	64	19.6	6.6-40.6	5.5-44.1	3 (4.7%)	75	22.2	6.3-46.5	6.9-50.6	1 (1.3%)
2s6s CS [%]	64	2.0	0.0-3.3	0.5-3.4	2 (3.1%)	68	2.0	0.0-3.2	0.6-3.6	2 (2.9%)
4s6s CS [%]	65	1.9	0.0-4.2	0.0-4.6	2 (3.1%)	75	2.0	0.0-4.4	0.0-4.8	1 (1.3%)
Charge CS	64	0.8	0.6-0.9	0.6-0.9	6 (9.4%)	74	0.8	0.6-0.9	0.6-0.9	0 (0.0%)
HS										
Concentration										
Total HS [µg/mL]	59	0.3	0.0-0.7	0.0-0.7	1 (1.7%)	66	0.3	0.0-1.2	0.0-0.8	0 (0.0%)
0s HS [µg/mL]	61	0.2	0.0-0.6	0.0-0.7	2 (3.3%)	70	0.2	0.0-0.8	0.0-0.8	0 (0.0%)
Ns HS [µg/mL]	61	0.1	0.0-0.2	0.0-0.2	1 (1.6%)	68	0.1	0.0-0.4	0.0-0.2	2 (2.9%)
Composition										
0s HS [%]	65	55.5	18.6-83.5	6.4-80.6	3 (4.6%)	75	52.7	33.0-73.6	2.3-74.7	9 (12.0%)
Ns HS [%]	62	22.6	1.4-40.3	8.8-30.7	0 (0.0%)	71	23.4	13.3-41.3	10.9-34.9	6 (8.5%)
Charge HS	65	0.5	0.1-0.5	0.2-1.0	33 (50.8%)	75	0.5	0.2-0.4	0.3-1.0	41 (54.7%)

1
2

1 **Table 5.** Transference of reference intervals of plasma free CS in an independent population
2 (Cohorts 3 and 4). Outliers were excluded.

	Female (N = 65)					Male (N = 75)				
	N	Mean	Ref. Interval	Cohort Range	Outside Ref. Interval	N	Mean	Ref. Interval	Cohort Range	Outside Ref. Interval
CS										
Concentration										
Total CS [µg/mL]	62	10.0	4.9-11.3	6.7-14.9	18 (29.0%)	74	10.1	5.9-12.8	6.7-15.9	4 (5.4%)
4s CS [µg/mL]	63	5.6	2.1-6.4	3.2-8.5	18 (28.6%)	75	5.5	2.3-8.0	2.9-11.0	4 (5.3%)
0s CS [µg/mL]	64	4.4	2.3-5.5	2.6-8.5	7 (10.9%)	74	4.5	2.6-5.8	2.4-8.2	12 (16.2%)
Composition										
4s CS [%]	65	56.3	36.2-62.1	30.0-72.3	14 (21.5%)	75	54.6	36.6-65.5	33.7-72.8	11 (14.7%)
0s CS [%]	64	42.8	37.8-64.1	26.9-59.1	14 (21.9%)	75	44.7	33.9-62.1	26.4-65.9	11 (14.7%)
Charge CS	65	0.6	0.4-0.6	0.3-0.7	15 (23.1%)	75	0.6	0.4-0.7	0.3-0.7	12 (16.0%)

3

4 **Discussion**

5 In this study, we established reference intervals for the urine and plasma free GAGomes in a
6 large adult healthy population by taking advantage of standardized high throughput UHPLC-
7 MS/MS method (*15*). In addition, the extensive demographic and biochemical
8 characterization of subjects allowed us to assess novel correlations with the free GAGome.

9 We found that no free GAGome feature showed any notables differences with respect to age
10 in adults. While this finding in urine agree with previous studies analyzing total – as opposed
11 to free - GAGomes (*18*), it contradicts similar studies in plasma, where an increase with age
12 was reported in males only (*19*). In contrast to age, we found that the concentrations of
13 several CS and HS disaccharides were higher in males than females with a larger effect in
14 urine than plasma. Previous studies noted gender differences in total GAGomes, although in
15 the opposite directions for both urine and plasma (*18*, *19*). The origin of these discrepancies
16 remains to be ascertained, but we speculate that they could be attributed to the focus on the
17 total rather than the free GAGome fraction in previous studies, as well as older analytical
18 techniques for GAG measurements and smaller sample sizes. We also observed that urine free
19 GAGomes appeared independent of other blood chemistry biomarkers, including markers of
20 inflammation, glucose metabolism, and liver functions – underscoring that they may reflect an
21 independent physiological state in adults. The only exception was serum creatinine, which in
22 the context of its correlation with urine free GAGomes may indicate a dependency on urine

1   excretion rather than renal function. Thus, we speculate that normalization by urinary
2   creatinine could render urine free GAGome measurements more robust. In contrast, plasma
3   free CS had weak correlations with markers related to erythrocytes, particularly 4s CS. This
4   association has been previously described for platelets where their activation can lead to rapid
5   increases of circulating 4s CS (*20*), but not in the context of other blood cells such as
6   erythrocytes.

7   We observed that the reference intervals established for free GAGomes were remarkably tight
8   in the reference sample group. In plasma, each free GAGome feature deviated by ~75% at
9   most from the mean. Similarly, in the urine each feature deviated by a maximum of ~3-fold
10   from the mean. These findings suggest that urine and plasma free GAGomes have stable and
11   predictable levels in a healthy adult. This conclusion appears corroborated by the transference
12   analysis that validated all reference intervals established for urine in independent adult
13   samples from two different geographical sites. Even among the free GAGome features that
14   failed to validate the reference intervals, chiefly in plasma, the deviation from the reference
15   sample group was limited. For example, the mean plasma 4s CS concentration was 4.1 ng mL$^-$
16   ^{1} in the female reference sample group and 5.6 ng mL in the transference group, that is 36%
17   higher. In comparison, the reference interval for the platelet count in the reference sample
18   group spans 137 to 488•10^9 L^{-1}, a 3-fold difference between the reference limits. Considering
19   this, we speculation that if a substantial deviation of a free GAGome feature from the here
20   reported reference interval is observed in an adult, then this deviation may be more indicative
21   of a disease state than physiological variability. This makes free GAGomes suitable
22   candidates for biomarker studies.

23   In conclusion, this study established and validated reference intervals for plasma and urine
24   free GAGomes in the largest adult population to date. As such, we believe that this study
25   represents a critical resource for physiology and biomarker research using biofluidic free
26   GAGomes.

27   **Acknowledgments**

38   **Author contributions**

39   Fr.G., J.N conceived, designed, and coordinated the study. S.B., A.L., and Fr.G. performed
40   data analysis and statistics. N.V., Fa.G., and F.M. assisted with the laboratory method. M.L.

1 contributed to subject enrollment. S.B., A.L., and Fr.G. drafted the manuscript. All authors
2 edited and approved the manuscript in its final form.

Conflict of interest

4 At study start, Fr.G. and J.N. were listed as co-inventors in patent applications related to the
5 biomarkers described in this study. At the time of submission, Fr.G. and J.N. were
6 shareholders in Elypta AB, which owned the above-mentioned patent applications, Fr.G. was
7 an employee in Elypta AB and J.N. was member of the board. Fr.G. and J.N. declare no
8 further conflict of interest. All other authors declare no conflict of interest.

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