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Authors

Sabol, Jenny K

Wei, Wei

López-Hoyos, Marcos

et al.

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Heparan Sulfate Differences in Rheumatoid Arthritis versus Healthy Sera

Jenny K. Sabol[#†], **Wei Wei**[#‖], **Marcos López-Hoyos**[§], **Youjin Seo**[‖], **Armann Andaya**[†], and **Julie A. Leary**[*,†,‖]

[†]Department of Molecular & Cellular Biology, University of California, Davis, California 95616, USA

[‖]Department of Chemistry, University of California, Davis, California 95616, USA

[§]Immunology Section. Hospital Universitario Marques de Valdecilla-IDIVAL, Santander 39008, SPAIN

[#] These authors contributed equally to this work.

Abstract

Heparan sulfate (HS) is a complex and highly variable polysaccharide, expressed ubiquitously on the cell surface as HS proteoglycans (HSPGs), and found in the extracellular matrix as free HS fragments. Its heterogeneity due to various acetylation and sulfation patterns endows a multitude of functions. In animal tissues, HS interacts with a wide range of proteins to mediate numerous biological activities; given its multiple roles in inflammation processes, characterization of HS in human serum has significant potential for elucidating disease mechanisms. Historically, investigation of HS was limited by its low concentration in human serum, together with the complexity of the serum matrix. In this study, we used a modified mass spectrometry method to examine HS disaccharide profiles in the serum of 50 women with rheumatoid arthritis (RA), and compared our results to 51 sera from healthy women. Using various purification methods and online LC-MS/MS, we discovered statistically significant differences in the sulfation and acetylation patterns between populations. Since early diagnosis of RA is considered important in decelerating the disease's progression, identification of specific biomolecule characterizations may provide crucial information towards developing new therapies for suppressing the disease in its early stages. This is the first report of potential glycosaminoglycan biomarkers for RA found in human sera, while acknowledging the obvious fact that a larger population set, and more stringent collection parameters, will need to be investigated in the future.

Keywords

Heparan sulfate; Rheumatoid arthritis; Glycosaminoglycan; 6-O-sulfotransferase; 2-O-sulfotransferase

[*]Corresponding author at: University of California Davis, One Shields Avenue, Department of Molecular & Cellular Biology, Briggs Hall 130, Davis, California 95616, USA. jaleary@ucdavis.edu*Telephone:* (530) 752-4685.

1. Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease of unknown cause, associated with painfully stiff, swollen joints, bone and cartilage destruction, cardiovascular complications, progressive disability and early death (Aletaha et al., 2010; McInnes and Schett, 2011). RA is characterized by synovial inflammation and autoantibody production (rheumatoid factor and anti-citrullinated protein antibody [ACPA]) (McInnes and Schett, 2011). It is believed that heparan sulfate participates in the persistent inflammation of RA through its involvement in leukocyte transmigration, the essential feature of the inflammatory response (Parish, 2006). Heparan sulfate appears to be involved not only in the transmigration of leukocytes to underlying target tissue, it also plays a role in the recruitment of leukocytes from the blood, and in the activation of certain chemokines (Ferro, 2013; Santiago et al., 2012).

Heparin/heparan sulfate (HS) is a member of the glycosaminoglycan (GAG) family of polysaccharides (Rabenstein, 2002). It is a negatively charged linear polysaccharide, consisting of 50–200 repeating disaccharide units, formed of hexuronic acids (l-iduronic or d-glucuronic) which are $\alpha(1\rightarrow4)$ linked to glucosamine units, with various sulfation and acetylation patterns (Table 1) (Saad and Leary, 2003; Wei et al., 2013; Zaia and Costello, 2003). Proteoglycans are proteins to which glycosaminoglycans are covalently attached; heparan sulfate proteoglycans (HSPGs) have heparan sulfate covalently attached as their carbohydrate component (Parish, 2006). HS constitutes the most structurally complex member of the GAG family (Rabenstein, 2002), and both HSPG and free HS are ubiquitously expressed on the cell surface and in the extracellular matrix (Ferro, 2013; Parish, 2006; Wei et al., 2011).

The heterogeneous structures of HS are created through a non-template-driven biosynthetic pathway with the cooperation of several enzymes. The biosynthesis is initiated from a backbone composed of alternating GlcNAc and GlcUA residues. The N-deacetylase/N-sulfotransferase (NDST) enzymes replace N-acetates with N-sulfates, producing NS (N-sulfated domain), NA (unmodified contiguous N-acetylated domain) and NS/NA (transitional domain with alternative N-acetates and N-sulfates) domains (Kreuger et al., 2002; Sheng et al., 2011; Staples et al., 2010). Some of the GlcUA residues are replaced with IdoUA by C-5 epimerase enzymes. Further modifications involve the introduction of 2-O-sulfates, 6-O-sulfates and more rarely, 3-O-sulfates by 2-O-sulfotransferase (2OST), 6-O-sulfotransferases (6OSTs) and 3-O-sulfotransferases (3OSTs), respectively (Liu et al., 1999; Perrimon and Bernfield, 2000; Staples et al., 2010). Heparan sulfate can also be modified by editing enzymes, such as 6-O-endo-sulfatases and heparanase (Ai et al., 2003; Ilan et al., 2006; Xu et al., 2007). The extracellular 6-O-endo-sulfatases, *Sulf1* and *Sulf2*, selectively remove sulfate groups from the carbon 6 positions (Lamanna et al., 2008; Nagamine et al., 2012; Rosen and Lemjabbar-Alaoui, 2010), while heparanase cleaves HS chains from the protein core, generating free HS (Bernfield et al., 1999; Galvis et al., 2007).

In animal tissues, HS interacts with a wide range of functionally diverse proteins (such as growth factors, cytokines, chemokines, proteases, and cell-adhesion molecules) (Parish, 2006) to mediate numerous biological activities, including many stages of the inflammatory

process (Ferro, 2013). Such interactions are significantly affected by variable sulfation and acetylation sequences (Bernfield et al., 1999; Perrimon and Bernfield, 2000). Through specific modification of the HS chains expressed, cells can direct their interactions with HS-binding proteins (Parish, 2006). The structural diversity of HS endows a multitude of functions and explains its contribution to a variety of disease states. Given the considerable involvement of HS in inflammation processes, characterization of HS and HSPG in serum has significant potential for elucidating disease mechanisms. Since early diagnosis of RA is considered important in decelerating the disease's progression, identification of biomolecule characterizations specific to RA may provide crucial information towards developing new therapies for suppressing RA in its early stages.

In this study, we performed compositional analysis of HS/HSPG from human serum samples, and discovered statistically significant differences in the sulfation and acetylation patterns between populations. According to compositional analysis methodology previously reported by this group (Wei et al., 2013; Wei et al., 2011), HS disaccharide profiles of 50 serum samples from women diagnosed with RA were analyzed using various chromatographic methods and online LC-MS/MS. This is the first report of potential glycosaminoglycan biomarkers found in human RA sera.

2. Results

Analytical separation and mass spectrometry methodology were used for the analysis of 50 rheumatoid arthritis (RA) serum samples, from pre- and post-menopausal women. Sera were purified using weak anion exchange chromatography, in which the eluted fraction contained both HS and HSPG, and subsequently separated using 50 kDa MWCO filters. Both HS (flow through) and HSPG (retained on filters) fractions were enzymatically digested into disaccharides, purified by SPE and analyzed by LC-MS/MS (Wei et al., 2013; Wei et al., 2011). Commercially available heparan sulfate from bovine kidney, human serum from Sigma, and a pooled sample of 50 RA sera (Freue et al., 2013) were used as control samples for daily precision and accuracy. The data from these samples were compared and analyzed statistically. The differences between healthy and RA disaccharide profiles were observed and discussed below.

2.1 Increased amounts of HS and HSPG in RA sera

Increased amounts of HS and HSPG were observed in the RA sample population compared to the healthy sample population, irrespective of menopausal status. The total HS and HSPG amounts were calculated using a modified version of a method previously published by this group (Wei et al., 2013). The median amount of free HS (μg/ml) was 1.7 times greater in the RA samples than in the healthy samples. Similarly, the median amount of HSPG was 2.5 times greater in the RA samples than in the healthy samples (Figure S2).

In consideration of menopausal status as a factor, independent sample *t* tests were performed. Changes in HS and HSPG volume according to sample group are illustrated in Figure 1. There was overwhelming evidence that menopausal status affected the amount of HSPG in healthy serum (p value 0.005, confidence interval 95%), while there was weak evidence that it affected the amount of HS in healthy serum (p value 0.059, confidence

interval 95%). There was insufficient evidence that menopausal status affected the amount of either HS or HSPG in RA serum (p values 0.456 and 0.325, respectively, confidence interval 95%). These conclusions are summarized in Table S1 of the Supplementary Information.

2.2 Significant changes in RA disaccharide profiles

Differences in the disaccharide compositions were observed between healthy and RA sera. Tables 3 and 4 show the percent composition of specific disaccharides (Table 1) from free HS fractions and HSPG fractions, respectively. The values are expressed as the mean percentage (from all samples in their respective categories) ± SEM.

In both free HS and HSPG fractions, five of the individual disaccharides (ΔUA→GlcNAc6S, ΔUA→GlcNAc, ΔUA2S→GlcNS6S ,ΔUA2S→GlcNS, and ΔUA2S→GlcN6S),were found in significantly different percentages between healthy and RA samples, irrespective of menopausal status. The disaccharide ΔUA→GlcNS6S was found in significantly different percentages between healthy and RA samples for the free HS fraction only. Four disaccharides (ΔUA2S→GlcNAc6S, ΔUA2S→GlcNAc, ΔUA→GlcNS and ΔUA→GlcN6S) were found in significantly different percentages between healthy and RA samples for the HSPG fraction only. These significantly different percentages were identified using independent sample *t* tests (p values not shown). Table S2 of the supporting information summarizes these findings. The proportional differences of the five disaccharides identified as significantly different in both free HS and HSPG fractions are illustrated in Figure 2.

Independent sample *t* tests indicated that menopausal status was only a factor for three of the disaccharides in the healthy disaccharide ΔUA→GlcNAc increased significantly post-menopause in healthy HS (p .002) and HSPG (p value .000), while there was strong evidence that disaccharide ΔUA2S→GlcNS6S decreased significantly post-menopause in healthy HS (p value .004) and HSPG (p value .013). Similarly, there was clear evidence that disaccharide ΔUA→GlcNS6S decreased significantly post-menopause in healthy HSPG (p value .001), but not in healthy HS.

3. Discussion

3.1 Significant changes in 2-O-sulfation, 6-O-sulfation, N-sulfation and N-acetylation

Tables 5 and 6 show the percent of disaccharides that are 2-O-sulfated, 6-O-sulfated, Nsulfated, and N-acetylated (Table 1) in both free HS and HSPG fractions, respectively. The values are expressed as the mean percentage (from all samples in their respective categories) ± SEM. Compared to the healthy samples, RA samples showed increased 2-O-sulfation and 6-O-sulfation in HS, but decreased 2-O-sulfation and 6-O-sulfation in HSPG. RA samples also showed increased N-sulfation in HS, but decreased N-sulfation in HSPG, while these same samples showed decreased N-acetylation in HS, but increased N-acetylation in HSPG. Statistically significant differences were observed between healthy and RA sera with p values < 0.000 in each case. Figures 3 and 4 illustrate graphical differences in 2-O-sulfation, 6-O-sulfation, N-sulfation, and N-acetylation for healthy versus RA samples.

3.2 Potential biomarkers identification

The four disaccharides (ΔUA→GlcNAc6S, ΔUA→GlcNS6S, and ΔUA2S→GlcNS6S, ΔUA2S→GlcN6S) in the HS fraction that were identified at significantly higher proportions in the RA sample population were considered as models of potential biomarkers for RA. The sensitivity and specificity of these potential biomarkers serving as diagnostic tests were evaluated using the ROC curve (Florkowski, 2008) The area under the corresponding ROC curves (AUC) represents the performances of these diagnostic tests. A guideline was employed to rate the performance (Pines et al., 2012) as follows:

0.9 ≤ AUC < 1.0	excellent
0.8 ≤ AUC < 0.9	good
0.7 ≤ AUC < 0.8	fair
0.6 ≤ AUC < 0.7	poor
0.5 ≤ AUC < 0.6	fail

Figure 5 shows the ROC curves for the HS-derived disaccharides that were shown to be present in significantly greater proportions in the RA samples (ΔUA→GlcNAc6S, ΔUA2S→GlcNS6S, ΔUA→GlcNS6S, and ΔUA2S→GlcN6S). Disaccharides ΔUA→GlcNAc6S and ΔUA→GlcNS6S, showed AUC values of 0.874 (good) and 0.928 (excellent), respectively, while the remaining two disaccharides had AUC values less than 0.80, indicating insufficiency as a diagnostic measure. Figure 6 shows the ROC curves for the HSPG-derived disaccharides that were shown to be present in significantly greater proportions in the RA samples (ΔUA→GlcNAc6S, ΔUA2S→GlcNAc, ΔUA→GlcNAc and ΔUA→GlcNS). In the HSPG fraction, only disaccharide ΔUA→GlcNAc (AUC 0.827, good) showed diagnosis predictor potential.

3.3 Implications of heparan sulfate characterization in rheumatoid arthritis

It is believed that heparan sulfate participates in the persistent synovial inflammation characteristic of RA through its involvement in leukocyte transmigration, the essential feature of the inflammatory response (Parish, 2006). Since early diagnosis of RA is considered important in decelerating the disease's progression (Aletaha et al., 2010), studies of heparan sulfate's specific characterizations in RA could provide valuable information towards development of tools that afford earlier diagnosis. Herein, we demonstrated the dramatic changes in free HS and HSPG-derived disaccharide compositions in RA sera compared to those in healthy sera.

Heparin/heparan sulfate is synthesized on protein cores through a biosynthetic pathway. The synthesis of HS is a non-template stepwise process, where various sulfation modifications depend on the activities of the corresponding enzymes. The NS and NA domains characterized by N-sulfated and the N-acetylated glucosamines are determined through the first step by NDST enzymes, as well known from previous studies by Esko and coworkers (Esko and Lindahl, 2001; Grobe et al., 2002). The following 2-O and 6-O-sulfation

modifications are affected accordingly, since 2-O-sulfotransferase and 6-O-sulfotransferases tend to add sulfate groups site specifically in the N-sulfated regions. Therefore, the variations in the disaccharide profiles can provide important information regarding the enzymes involved in the HS biosynthetic pathway along with the other editing enzymes, such as sulfatases and heparanase.

As illustrated in Figure 3, RA samples demonstrated overall increases in 6-O-sulfation, 2-O-sulfation, and N-sulfation of HS-derived disaccharides, compared to healthy samples. These variations in sulfation may be due to the increased expression and/or increased activity of 6-O-sulfotransferases, resulting in the elevated amount of 6-O-sulfation. Since the modification of the 6-O position is highly dependent on the previous N-sulfation and 2-O-sulfation distribution, sulfation might also result in the differential 6-O-sulfatransferase activities. Similarly, any deficiency in sulfatase enzymes specific to sites other than the 6-position could also cause an observed increase in those disaccharides with 6-O-sulfation. Therefore, for women with RA, there is likely to be either an increase in expression/activity of 6-O-sulfatransferases and/or deficiencies in sulfatases. Given the specific changes in 6-O-sulfation in the RA sample population, disaccharides with 6-O-sulfates may provide valuable information towards the design of future experiments that aim to elucidate disease mechanisms or develop improved diagnostic tools.

With the exception of the N-acetylated analogs, all RA HS fractions showed an increase in 2-O-,6-O- and N-sulfation, while the corresponding HSPG fractions showed a decrease in all three from RA samples (Tables 4 and 5). Even though the role of N-acetylation is still unclear, heparan sulfate NA and NS/NA domains might also be crucial. The NA and NS/NA domains provide a degree of flexibility, which have been shown to facilitate the interactions between HS and proteins (Casu et al., 2010; Mobli et al., 2008). Heparan sulfate N-acetylation and 6-O-sulfation were reported to be involved in the interaction between natural killer cells and natural cytotoxicity receptors (Bloushtain et al., 2004). N-acetylation has also been shown to facilitate the binding with chemokines (Schenauer et al., 2007). For some diseases, such as Mucopolysaccharidosis, changes in the N-acetylation of HS were reported (Durand et al., 2010; Feldhammer et al., 2009; Holley et al., 2011). N-acetylation of HS was noticeably different in RA samples, compared to healthy samples. Figure 3 graphically demonstrates the dramatic differences that were observed in the N-acetylated disaccharides when comparing the HS and HSPG fractions. The disaccharide ΔUA→GlcNAc6S showed dramatically larger mean percentages in all comparison groups (from HSPG, both pre- and post-menopausal RA proportions were 1.4 times larger than healthy). RA samples showed significantly elevated proportions of the disaccharide ΔUA2S→GlcNAc in HSPG (2.0 times greater in pre-menopausal sera and 2.3 times greater in post-menopausal sera), implicating the potential involvement of 2-O-sulfated NA domains in the interaction between HSPG and RA-related proteins. Disaccharide ΔUA2S→GlcNAc6S was significantly decreased in HSPG from post-menopausal RA sera. This change in ΔUA2S→GlcNAc6S was specific to the post-menopausal sample population, which might suggest an explanation for the severity of RA in the post-menopausal sera. These findings raise the question of whether the importance of the N-acetylated regions of HS is underestimated.

The sulfated areas of HS GAGs provide docking sites for protein ligands such as cytokines, chemokines, and growth factors, thereby functioning in management of such processes as morphogenesis, tissue repair, and inflammation (Lindahl and Li, 2009). Heparanase is the prominent mammalian HS endoglycosidase that cleaves HS side chains at sites of low sulfation, releasing glycan products that retain the ability to form biologically active receptor-ligand complexes (Vlodavsky et al., 2012). Heparanase cleavage of HS in the ECM leads to propagation of bioactive fragments that facilitate inflammation (Barash et al., 2010). Indeed, elevated heparanase activity in the synovial fluid of RA patients has been reported (Li et al., 2008). Upregulation of heparanase in RA is a likely explanation for our results, which indicated reduced 2-O-sulfation, 6-O-sulfation, and N-sulfation in HSPG from RA sera, in concert with elevated 2-O-sulfation, 6-O-sulfation (Figure 4), and N-sulfation in free HS from RA sera.

3.4 Conclusions

Early diagnosis of RA is known to be advantageous in slowing progression of the disease (Pruijn et al., 2010), but the diagnosis is still based on clinical presentation. The current diagnostic tests for RA usually involve the evaluation of certain antibody levels in blood, such as rheumatoid factor (RF) and anti-citrullinated protein (APCA). These tests, however, have limited prognostic value, along with rather inadequate sensitivity and specificity results (Besada et al., 2012). Reliable predictive biomarker tests are needed (McInnes and Schett, 2011). Our preliminary data produced ROC curves that suggested that disaccharides ΔUA→GlcNS6S and ΔUA→GlcNAc6S in serum HS fractions deserve further attention for their potential as possible RA biomarkers.

In summary, we have applied and optimized the methodology developed in our lab for the compositional analysis of HS/HSPG-derived disaccharides from serum samples. We compared sera of 50 women with rheumatoid arthritis to sera of 51 healthy women. The comparison groups were further stratified according to menopausal status. Results show dramatic differences in the sulfation and acetylation patterns between RA and healthy. These changes implicate the corresponding increased activity of 6-O-sulfotransferases and/or decreased activity of sulfatases, and decreased activity of 2-O-sulfotransferase. The disaccharide compositions of HS reflect the activity changes of sulfatases, heparanase, and enzymes involved in the biosynthetic pathway. Therefore, analysis of the disaccharide profile and HS/HSPG concentrations in serum could be promising avenues for future development of improved RA diagnosis and prognosis methodology. To further investigate the changes of HS-editing enzymes in RA, enzyme activity studies using the corresponding 2-O- and 6-O-sulfotransferase, sulfatases and heparanase antibodies are needed, and are currently in the planning stages.

4. Materials and Methods

4.1 Materials

Heparin/heparan sulfate-derived disaccharide standards ΔUA-GlcNAc, ΔUA2S-GlcNAc, ΔUA-GlcNAc6S, ΔUA2S-GlcNAc6S, ΔUA2S-GlcNS, ΔUA-GlcNS6S, ΔUA2S-GlcNS6S, ΔUA2S-GlcN6S, ΔUA-GlcN6S, ΔUA2S-GlcN, ΔUA-GlcN, and ΔUA2S-GlcNCOEt6S

were purchased from V-Labs (Covington, LA). ΔUA-GlcNS was obtained from Iduron (Manchester, UK). Protease from *Streptomyces griseus* was purchased from Sigma-Aldrich (St Louis, MO). Heparinase I (heparinase, EC 4.2.2.7), heparinase II (heparitinase II, EC 4.2.2.8), and heparinase III (heparitinase, EC 4.2.2.8) from *Flavobacterium heparinum* were obtained from Seikagaku Corporation (East Falmouth, MA). Commercial human serum was procured from Sigma-Aldrich (St Louis, MO).

Serum samples from RA women were graciously provided by Dr. Marcos López-Hoyos from Hospital Universitario Marqués de Valdecilla. A total of 50 serum samples (Table 2) were analyzed: 25 from premenopausal women (39±7 years old) and 25 from postmenopausal women (71±10 years old). Samples were coded and randomized to minimize analytical bias. The daily accuracy and precision of the purification and analysis method was validated with a commercially available human serum control (Sigma-Aldrich St Louis, MO) using published analytical procedures (Wei et al., 2013; Wei et al., 2011).

4.2 Purification of Free HS and HSPG Sugar Chains from Human Serum

The original method for free HS and HSPG purification has been published previously. (Wei et al., 2013) However, some changes have been made for this study. Briefly, each of the 50 RA serum samples (70 μL) was centrifuged at 12,000 × g for 15 min. The supernatant was diluted using 0.2 M NaCl and applied to a HiTrap DEAE FF column (GE Healthcare, Pittsburgh, PA). The column was washed exhaustively with a low concentration of 0.3 M NaCl. HS and HSPGs were eluted using a high concentration of 2 M NaCl. The HS were separated from HSPGs using a 50-kDa molecular weight cut-off (MWCO) filter (Millipore, Billerica, MA). The HSPG fraction was digested by pronase (0.1 mg/mL) overnight at 37°C using gentle agitation. The HS chains released from HSPG protein cores were purified using the same DEAE method. All the DEAE-purified HS fractions were desalted using two HiTrap desalting columns (GE Healthcare, Pittsburgh, PA) connected in series and dried *in vacuo*.

4.3 Digestion and Sample Clean-up

The HS and HSPG fractions were resuspended in 25 μL of digestion buffer, containing 20 mM NH_4OAc (pH 7.5) and 1 mM $Ca(OAc)_2$. A combination of heparinase enzymes I, II and III (1 mU each) was added to each HS sample and incubated at 37°C for 20 hours with gentle agitation. After digestion, each sample was spiked with 2 μL of 20 mM internal standard I-P (ΔUA2S→GlcNCOEt6S) (Saad et al., 2005; Zaia and Costello, 2001) and purified using a C18+carbon SPE TopTip cartridge (Glygen, Columbia, MD). The cartridge was conditioned 2 times with 50 μL of 0.1% trifluoroacetic acid (TFA) in 80% (v/v) ACN/H_2O, followed by 100 μL of Milli-Q water and 100 μL of 0.5 M NaCl. Each digested sample was diluted five-fold with 0.5 M NaCl, and then applied to the cartridge. Subsequently, each cartridge was washed 5 times with 100 μL of Milli-Q water. Heparin/HS-derived disaccharides retained by the cartridge were eluted with 250 μL of 0.05% TFA in 40% (v/v) ACN/H_2O. All steps were performed in a swinging platform centrifuge at 2000 rpm for 1 min. The eluted fractions were collected and dried *in vacuo*. Finally, samples were redissolved in 20 μL of Milli-Q water before LC-MS/MS analysis.

4.4 LC-MS/MS Analysis

Mass spectra were acquired using a LTQ 2-D linear ion trap mass spectrometer equipped with an electrospray ionization (ESI) source and directly coupled to an HPLC system (Thermo Electron, San Jose, CA). The LC-MS/MS method was described previously (Wei et al., 2011; Zaia, 2013). Briefly, the purified disaccharide samples were loaded onto a Hypercarb column (2.1 mm ID × 150 mm, 5 μm) (Thermo Fisher Scientific, Waltham, MA) at a flow rate of 200 μL/min. The sample was desalted with 100% solvent A (5 mM NH_4HCO_3/H_2O) for 4 min, then eluted in 38% solvent B (5 mM NH_4HCO_3 in 80% ACN). The MS analysis was performed in the negative ion mode using a capillary temperature of 250°C. The instrument was tuned using disaccharide standards for optimal performance before sample analysis. For MS/MS experiments, the precursor ions were selected using an isolation width of 3 Da and activated using 18–21% normalized collision energy for 100 ms. Data acquisition and analysis were performed using Xcalibur 2.0 software.

4.5 Statistical Analysis

Statistical tests were performed using IBM SPSS Statistics 20. Comparisons between groups were performed using independent two-sample *t* tests, assuming normal distribution of the larger population. The evaluation of the potential RA diagnostic variables was performed using receiver operating characteristic (ROC) curves (Florkowski, 2008). A 95% confidence interval was used with a p value less than 0.05 being considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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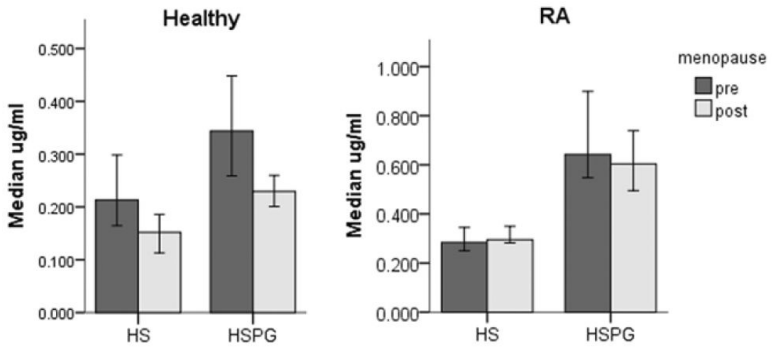


Figure 1.

Menopausal changes in median amounts (μg/ml) of free HS and HSPG. Error bars represent 95% confidence intervals. Only the HSPG in healthy samples indicated significant differences due to menopausal status.

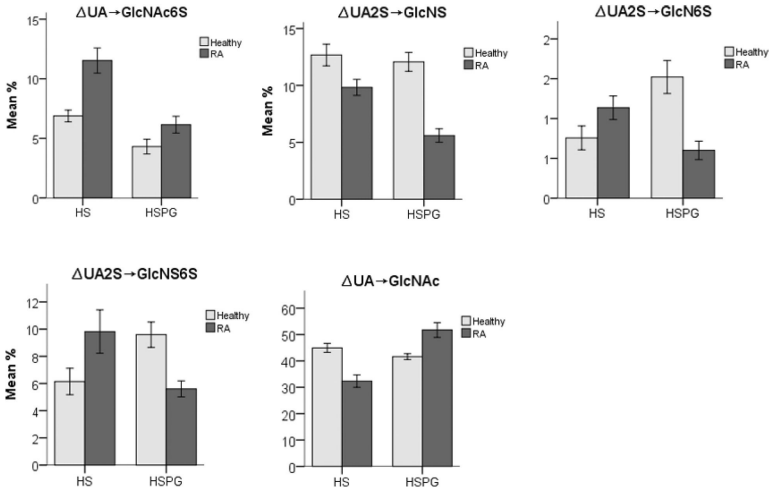


Figure 2.

Five disaccharides were found in significantly different proportions between healthy and RA samples, in both free HS and HSPG fractions. Error bars represent 95% confidence intervals.

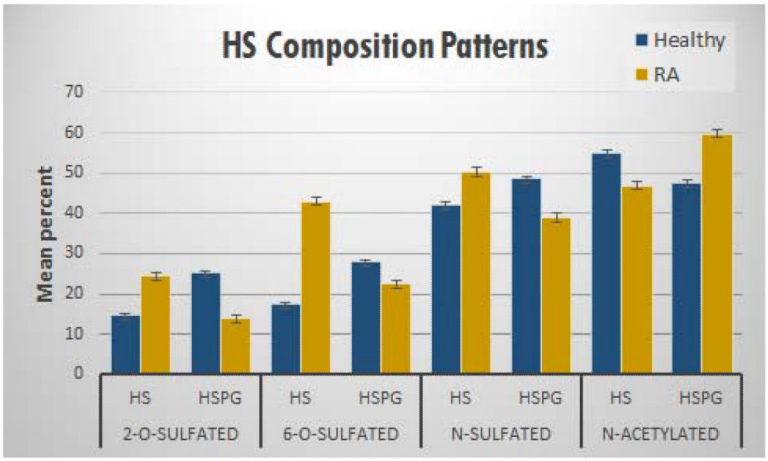


Figure 3.

Amounts of 2-O-sulfation, 6-O-sulfation, N-sulfation, and N-acetylation were considerably different in RA samples compared to healthy. The chart values reflect mean percentages of total HS/HSPG composition. Error bars represent 95% confidence intervals.

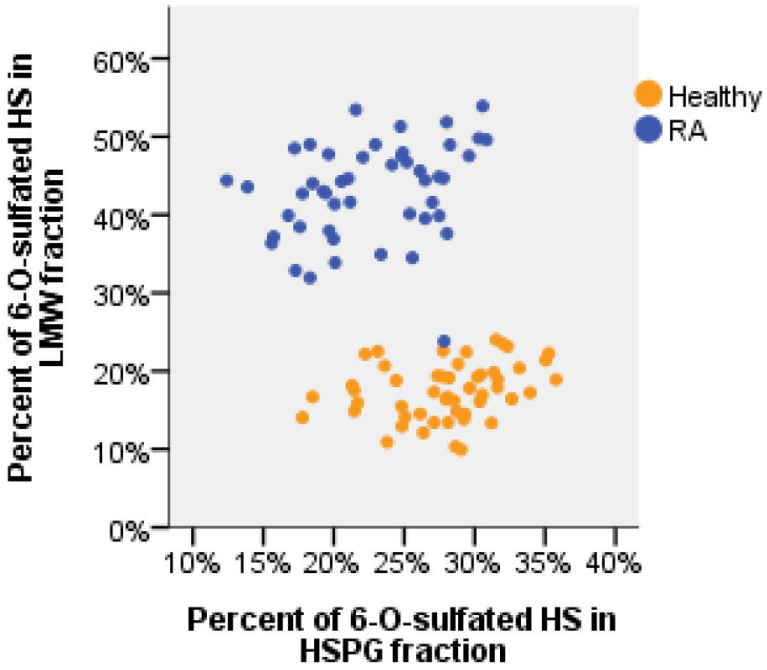


Figure 4.

Scatter plot comparison of 6-O-sulfation in serum samples indicates drastic differences between healthy and RA populations. For each data point, the percentage identified in the high molecular weight fraction (“HSPG”) is plotted against the percentage identified in the free HS fraction (“LMW fraction”).

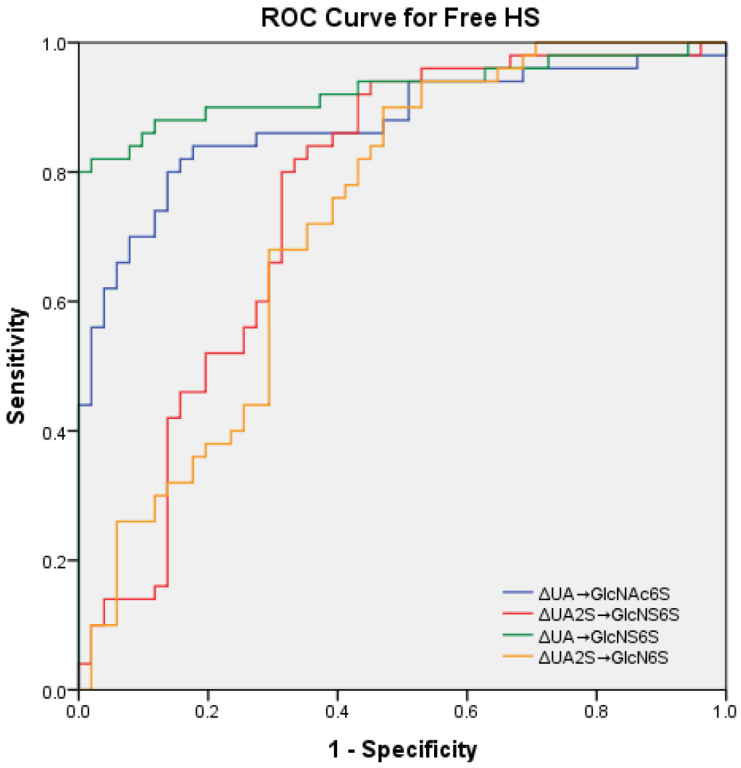


Figure 5.

ROC curves for HS-derived disaccharides ΔUA→GlcNAc6S, ΔUA2S→GlcNS6S, ΔUA→GlcNS6S, and ΔUA2S→GlcN6S. The areas under the curves are 0.874, 0.759, 0.928, and 0.730, respectively. As indicated in the figure, disaccharides ΔUA→GlcNAc6S and ΔUA→GlcNS6S showed high accuracy (AUC > 0.8) as potential diagnostic measures of RA.

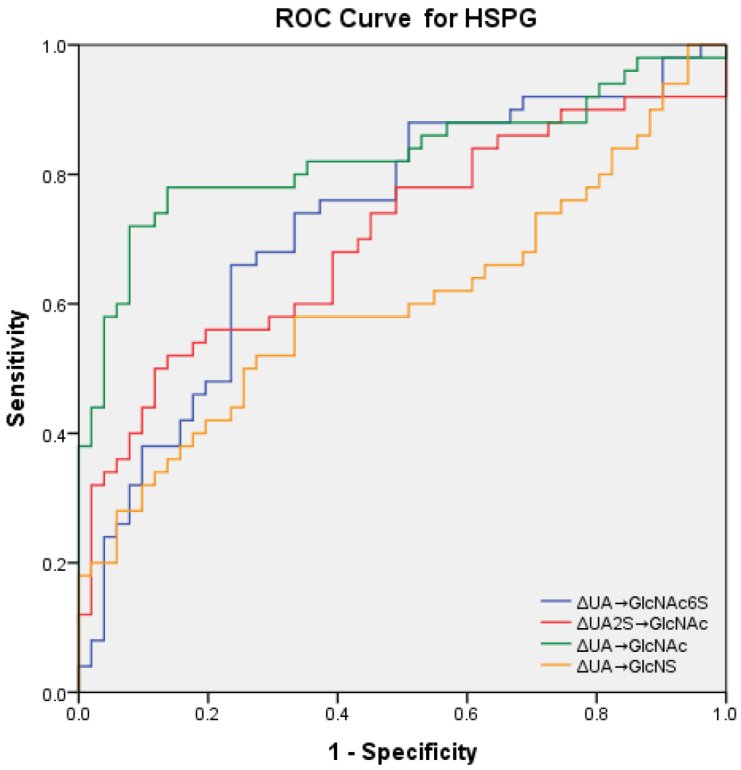
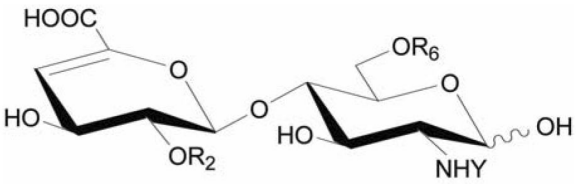


Figure 6.

ROC curves for HSPG-derived disaccharides ΔUA→GlcNAc6S, ΔUA2S→GlcNAc, ΔUA→GlcNAc, and ΔUA→GlcNS. The areas under the curves are 0.728, 0.704, 0.827, and 0.603, respectively. As indicated in the figure, disaccharide ΔUA→GlcNAc showed high accuracy (AUC > 0.8) as a potential diagnostic measure of RA.

Table 1

Structures of 12 heparin/heparan sulfate-derived disaccharides



Disaccharide	R2	R6	Y
ΔUA2S→GlcNAc6S	SO_3	SO_3	$COCH_3$
ΔUA→GlcNAc6S	H	SO_3	$COCH_3$
ΔUA2S→GlcNAc	SO_3	H	$COCH_3$
ΔUA→GlcNAc	H	H	$COCH_3$
ΔUA2S→GlcNS6S	SO_3	SO_3	SO_3
ΔUA→GlcNS6S	H	SO_3	SO_3
ΔUA2S→GlcNS	SO_3	H	SO_3
ΔUA→GlcNS	H	H	SO_3
ΔUA2S→GlcN6S	SO_3	SO_3	H
ΔUA→GlcN6S	H	SO_3	H
ΔUA2S→GlcN	SO_3	H	H
ΔUA→GlcN	H	H	H

Table 2

Banked human sera samples

	Healthy		RA	
Menopausal status	Pre	Post	Pre	Post
Gender	Female	Female	Female	Female
Numbers of samples	26	25	25	25
Age	30 – 46	49 – 65	32 – 46	61 – 81
RF range (IU/ml)			40 – 2480	42 – 2690
Anti-CCP range (U/ml)			63 – 1206	61 – 1189

Table 3

Percent composition of specific disaccharides in free HS from healthy and RA sera. The values are expressed as the mean percentage (from all samples in their respective categories) ± SEM.

	Healthy (%)		RA (%)	
Disaccharide	**pre-menopause**	**post-menopause**	**pre-menopause**	**post-menopause**
ΔUA2S→GlcNAc6S	1.49 ± 0.18	1.92 ± 0.19	1.93 ± 0.24	1.71 ± 0.23
ΔUA→GlcNAc6S	7.31 ± 0.39	6.42 ± 0.27	12.41 ± 0.72	10.66 ± 0.74
ΔUA2S→GlcNAc	1.4 ± 0.15	1.2 ± 0.14	1.19 ± 0.19	0.99 ± 0.15
ΔUA→GlcNAc	42.47 ± 1.17	47.46 ± 1.02	31.98 ± 1.82	32.73 ± 1.51
ΔUA2S→GlcNS6S	7.49 ± 0.72	4.75 ± 0.53	9.54 ± 0.49	10.1 ± 1.53
ΔUA→GlcNS6S	9.83 ± 0.6	8.85 ± 0.47	16.75 ± 0.92	18.77 ± 1
ΔUA2S→GlcNS	13.33 ± 0.73	11.99 ± 0.58	10.35 ± 0.57	9.3 ± 0.4
ΔUA→GlcNS	13.67 ± 0.8	14.06 ± 0.61	12.84 ± 0.66	12.81 ± 0.52
ΔUA2S→GlcN6S	0.69 ± 0.08	0.82 ± 0.13	1.1 ± 0.11	1.18 ± 0.1
ΔUA→GlcN6S	1.13 ± 0.14	1.29 ± 0.15	0.95 ± 0.17	0.98 ± 0.2
ΔUA2S→GlcN	0.7 ± 0.07	0.74 ± 0.07	0.78 ± 0.17	0.62 ± 0.11
ΔUA→GlcN	0.48 ± 0.05	0.49 ± 0.05	0.54 ± 0.04	0.47 ± 0.03

Table 4

Percent composition of specific disaccharides in HSPG from healthy and RA sera. The values are expressed as the mean percentage (from all samples in their respective categories) ± SEM.

	Healthy (%)		RA (%)	
Disaccharide	**pre-menopause**	**post-menopause**	**pre-menopause**	**post-menopause**
ΔUA2S→GlcNAc6S	0.95 ± 0.11	1.27 ± 0.14	0.81 ± 0.1	0.7 ± 0.13
ΔUA→GlcNAc6S	4.24 ± 0.33	4.38 ± 0.52	5.97 ± 0.53	6.33 ± 0.46
ΔUA2S→GlcNAc	0.51 ± 0.08	0.49 ± 0.11	1.02 ± 0.16	1.11 ± 0.19
ΔUA→GlcNAc	39.44 ± 0.57	43.92 ± 0.74	53.42 ± 2.12	50.03 ± 1.79
ΔUA2S→GlcNS6S	10.71 ± 0.6	8.44 ± 0.65	5.48 ± 0.48	5.73 ± 0.34
ΔUA→GlcNS6S	11.47 ± 0.42	8.78 ± 0.67	9.21 ± 0.54	8.97 ± 0.46
ΔUA2S→GlcNS	12.58 ± 0.61	11.54 ± 0.54	5.15 ± 0.43	6.04 ± 0.39
ΔUA→GlcNS	16.33 ± 0.74	17.29 ± 0.72	17.34 ± 1.09	19.81 ± 0.86
ΔUA2S→GlcN6S	1.72 ± 0.16	1.31 ± 0.12	0.56 ± 0.06	0.64 ± 0.1
ΔUA→GlcN6S	1.11 ± 0.15	1.47 ± 0.16	0.54 ± 0.19	0.18 ± 0.08
ΔUA2S→GlcN	0.41 ± 0.06	0.53 ± 0.05	0.4 ± 0.07	0.35 ± 0.05
ΔUA→GlcN	0.53 ± 0.07	0.59 ± 0.07	0.44 ± 0.08	0.43 ± 0.07

Table 5

Free HS compositions from healthy and RA sera. The values are expressed as the mean percentage (from all samples in their respective categories) ± SEM.

	Healthy (%)		**RA (%)**	
	pre-menopause	**post-menopause**	**pre-menopause**	**post-menopause**
% 2-O-Sulfated	15.43 ± 0.78	13.93 ± 0.62	24.89 ± 0.77	23.9 ± 1.56
% 6-O-Sulfated	18.27 ±0.76	16.56 ± 0.61	42.68 ± 1.49	43.39 ± 1.45
% N-Sulfated	44.31 ± 1.03	39.65 ± 0.93	49.48 ± 1.58	50.97 ± 1.43
% N-Acetylated	52.68 ±1.06	57 ± 0.87	47.51 ± 1.49	46.09 ± 1.55

Table 6

HSPG compositions from healthy and RA sera. The values are expressed as the mean percentage (from all samples in their respective categories) ± SEM.

	Healthy (%)		**RA (%)**	
	pre-menopause	**post-menopause**	**pre-menopause**	**post-menopause**
% 2-O-Sulfated	26.88 ± 0.64	23.58 ± 0.79	13.42 ± 0.99	14.57 ± 0.86
% 6-O-Sulfated	30.2 ±0.59	25.64 ± 0.81	22.56 ±1.02	22.55 ± 0.87
% N-Sulfated	51.08 ±0.7	46.04 ± 0.92	37.18 ±1.77	40.55 ± 1.48
% N-Acetylated	45.14 ±0.73	50.06 ± 0.89	61.21 ±1.8	58.17 ±1.54