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## Heparan sulfate differences in rheumatoid arthritis versus healthy sera

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## 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.

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## 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 (Santiago et al., 2012; Ferro, 2013).

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  $\alpha(1 \rightarrow 4)$  linked to glucosamine units, with various sulfation and acetylation patterns (Table 1) (Saad and Leary, 2003; Zaia and Costello, 2003; Wei et al., 2013). 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 (Parish, 2006; Wei et al., 2011; Ferro, 2013).

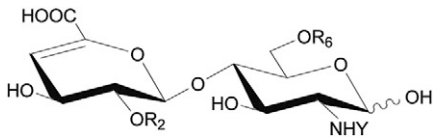
The heterogeneous structures of HS are created through a non-template-driven biosynthetic pathway with the cooperation of several enzymes. The biosynthesis is initiated from a backbone composed of alternating GlcNAc and GlcUA residues. The N-deacetylase/N-sulfotransferase (NDST) enzymes replace N-acetates 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; Staples et al., 2010; Sheng et al., 2011). Some of the GlcUA residues are replaced with IdoUA by C-5 epimerase enzymes. Further modifications involve

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**Table 1**  
Structures of 12 heparin/heparan sulfate-derived disaccharides.



Disaccharide	R2	R6	Y
$\Delta$ UA2S $\rightarrow$ GlcNAc6S	SO <sub>3</sub>	SO <sub>3</sub>	COCH <sub>3</sub>
$\Delta$ UA $\rightarrow$ GlcNAc6S	H	SO <sub>3</sub>	COCH <sub>3</sub>
$\Delta$ UA2S $\rightarrow$ GlcNAc	SO <sub>3</sub>	H	COCH <sub>3</sub>
$\Delta$ UA $\rightarrow$ GlcNAc	H	H	COCH <sub>3</sub>
$\Delta$ UA2S $\rightarrow$ GlcNS6S	SO <sub>3</sub>	SO <sub>3</sub>	SO <sub>3</sub>
$\Delta$ UA $\rightarrow$ GlcNS6S	H	SO <sub>3</sub>	SO <sub>3</sub>
$\Delta$ UA2S $\rightarrow$ GlcNS	SO <sub>3</sub>	H	SO <sub>3</sub>
$\Delta$ UA $\rightarrow$ GlcNS	H	H	SO <sub>3</sub>
$\Delta$ UA2S $\rightarrow$ GlcN6S	SO <sub>3</sub>	SO <sub>3</sub>	H
$\Delta$ UA $\rightarrow$ GlcN6S	H	SO <sub>3</sub>	H
$\Delta$ UA2S $\rightarrow$ GlcN	SO <sub>3</sub>	H	H
$\Delta$ UA $\rightarrow$ GlcN	H	H	H

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; Rosen and Lemjabbar-Alaoui, 2010; Nagamine et al., 2012), 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., 2011, 2013), 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., 2011, 2013). 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 ( $\mu$ g/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 (Fig. S1).

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 Fig. 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)  $\pm$  SEM.

In both free HS and HSPG fractions, five of the individual disaccharides ( $\Delta$ UA  $\rightarrow$  GlcNAc6S,  $\Delta$ UA  $\rightarrow$  GlcNAc,  $\Delta$ UA2S  $\rightarrow$  GlcNS6S,  $\Delta$ UA2S  $\rightarrow$  GlcNS, and  $\Delta$ UA2S  $\rightarrow$  GlcN6S) were found in significantly different percentages between healthy and RA samples, irrespective of menopausal status. The disaccharide  $\Delta$ UA  $\rightarrow$  GlcNS6S was found in significantly different percentages between healthy and RA samples for the free HS fraction only. Four disaccharides ( $\Delta$ UA2S  $\rightarrow$  GlcNAc6S,  $\Delta$ UA2S  $\rightarrow$  GlcNAc,  $\Delta$ UA  $\rightarrow$  GlcNS, and  $\Delta$ UA  $\rightarrow$  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 Fig. 2.

Independent sample *t* tests indicated that menopausal status was only a factor for three of the disaccharides in the healthy samples. There was overwhelming evidence that disaccharide  $\Delta$ UA  $\rightarrow$  GlcNAc increased significantly post-menopause in healthy HS (*p* value .002) and HSPG (*p* value .000), while there was strong evidence that disaccharide  $\Delta$ UA2S  $\rightarrow$  GlcNS6S decreased significantly post-menopause in healthy HS (*p* value .004) and HSPG (*p* value .013). Similarly, there was clear evidence that disaccharide  $\Delta$ UA  $\rightarrow$  GlcNS6S decreased significantly post-menopause in healthy HSPG (*p* value .001), but not in healthy HS.

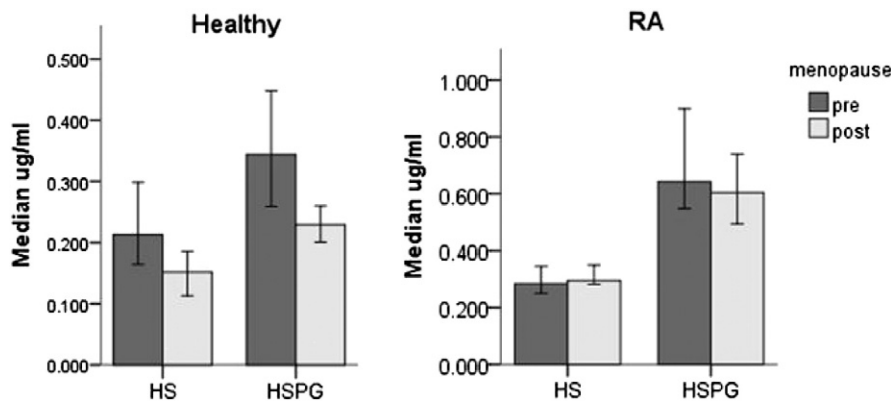


Fig. 1. Menopausal changes in median amounts ( $\mu\text{g/ml}$ ) of free HS and HSPG. Error bars represent 95% confidence intervals. Only the HSPG in healthy samples indicated significant differences due to menopausal status.

### 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, N-sulfated, 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)  $\pm$  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. Figs. 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 ( $\Delta\text{UA} \rightarrow \text{GlcNAc6S}$ ,  $\Delta\text{UA} \rightarrow \text{GlcNS6S}$ ,  $\Delta\text{UA2S} \rightarrow \text{GlcNS6S}$ , and  $\Delta\text{UA2S} \rightarrow \text{GlcN6S}$ ) 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:

$0.9 \leq \text{AUC} < 1.0$	Excellent
$0.8 \leq \text{AUC} < 0.9$	Good
$0.7 \leq \text{AUC} < 0.8$	Fair
$0.6 \leq \text{AUC} < 0.7$	Poor
$0.5 \leq \text{AUC} < 0.6$	Fail

Fig. 5 shows the ROC curves for the HS-derived disaccharides that were shown to be present in significantly greater proportions in the RA samples ( $\Delta\text{UA} \rightarrow \text{GlcNAc6S}$ ,  $\Delta\text{UA2S} \rightarrow \text{GlcNS6S}$ ,  $\Delta\text{UA} \rightarrow \text{GlcNS6S}$ , and  $\Delta\text{UA2S} \rightarrow \text{GlcN6S}$ ). Disaccharides  $\Delta\text{UA} \rightarrow \text{GlcNAc6S}$  and  $\Delta\text{UA} \rightarrow \text{GlcNS6S}$  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. Fig. 6 shows the ROC curves for the HSPG-derived disaccharides that were shown to be present in significantly greater proportions in the RA

samples ( $\Delta\text{UA} \rightarrow \text{GlcNAc6S}$ ,  $\Delta\text{UA2S} \rightarrow \text{GlcNAc}$ ,  $\Delta\text{UA} \rightarrow \text{GlcNAc}$ , and  $\Delta\text{UA} \rightarrow \text{GlcNS}$ ). In the HSPG fraction, only disaccharide  $\Delta\text{UA} \rightarrow \text{GlcNAc}$  (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 Fig. 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

Table 2  
Banked human sera samples.

	Healthy		RA	
	Pre	Post	Pre	Post
Menopausal status	Female	Female	Female	Female
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)  $\pm$  SEM.

Disaccharide	Healthy (%)		RA (%)	
	Pre-menopause	Post-menopause	Pre-menopause	Post-menopause
$\Delta$ UA2S $\rightarrow$ GlcNAc6S	1.49 $\pm$ 0.18	1.92 $\pm$ 0.19	1.93 $\pm$ 0.24	1.71 $\pm$ 0.23
$\Delta$ UA $\rightarrow$ GlcNAc6S	7.31 $\pm$ 0.39	6.42 $\pm$ 0.27	12.41 $\pm$ 0.72	10.66 $\pm$ 0.74
$\Delta$ UA2S $\rightarrow$ GlcNAc	1.4 $\pm$ 0.15	1.2 $\pm$ 0.14	1.19 $\pm$ 0.19	0.99 $\pm$ 0.15
$\Delta$ UA $\rightarrow$ GlcNAc	42.47 $\pm$ 1.17	47.46 $\pm$ 1.02	31.98 $\pm$ 1.82	32.73 $\pm$ 1.51
$\Delta$ UA2S $\rightarrow$ GlcNS6S	7.49 $\pm$ 0.72	4.75 $\pm$ 0.53	9.54 $\pm$ 0.49	10.1 $\pm$ 1.53
$\Delta$ UA $\rightarrow$ GlcNS6S	9.83 $\pm$ 0.6	8.85 $\pm$ 0.47	16.75 $\pm$ 0.92	18.77 $\pm$ 1
$\Delta$ UA2S $\rightarrow$ GlcNS	13.33 $\pm$ 0.73	11.99 $\pm$ 0.58	10.35 $\pm$ 0.57	9.3 $\pm$ 0.4
$\Delta$ UA $\rightarrow$ GlcNS	13.67 $\pm$ 0.8	14.06 $\pm$ 0.61	12.84 $\pm$ 0.66	12.81 $\pm$ 0.52
$\Delta$ UA2S $\rightarrow$ GlcN6S	0.69 $\pm$ 0.08	0.82 $\pm$ 0.13	1.1 $\pm$ 0.11	1.18 $\pm$ 0.1
$\Delta$ UA $\rightarrow$ GlcN6S	1.13 $\pm$ 0.14	1.29 $\pm$ 0.15	0.95 $\pm$ 0.17	0.98 $\pm$ 0.2
$\Delta$ UA2S $\rightarrow$ GlcN	0.7 $\pm$ 0.07	0.74 $\pm$ 0.07	0.78 $\pm$ 0.17	0.62 $\pm$ 0.11
$\Delta$ UA $\rightarrow$ GlcN	0.48 $\pm$ 0.05	0.49 $\pm$ 0.05	0.54 $\pm$ 0.04	0.47 $\pm$ 0.03

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 (Mobli et al., 2008; Casu et al., 2010). 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 (Feldhammer et al., 2009; Durand et al., 2010; Holley et al., 2011). N-acetylation of HS was noticeably different in RA samples, compared to healthy samples. Fig. 3 graphically demonstrates the dramatic differences that were observed in the N-acetylated disaccharides when comparing the HS and HSPG fractions. The disaccharide  $\Delta$ UA  $\rightarrow$  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  $\Delta$ UA2S  $\rightarrow$  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  $\Delta$ UA2S  $\rightarrow$  GlcNAc6S was significantly decreased in HSPG from post-menopausal RA sera. This change in  $\Delta$ UA2S  $\rightarrow$  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 (Fig. 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

**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)  $\pm$  SEM.

Disaccharide	Healthy (%)		RA (%)	
	Pre-menopause	Post-menopause	Pre-menopause	Post-menopause
$\Delta$ UA2S $\rightarrow$ GlcNAc6S	0.95 $\pm$ 0.11	1.27 $\pm$ 0.14	0.81 $\pm$ 0.1	0.7 $\pm$ 0.13
$\Delta$ UA $\rightarrow$ GlcNAc6S	4.24 $\pm$ 0.33	4.38 $\pm$ 0.52	5.97 $\pm$ 0.53	6.33 $\pm$ 0.46
$\Delta$ UA2S $\rightarrow$ GlcNAc	0.51 $\pm$ 0.08	0.49 $\pm$ 0.11	1.02 $\pm$ 0.16	1.11 $\pm$ 0.19
$\Delta$ UA $\rightarrow$ GlcNAc	39.44 $\pm$ 0.57	43.92 $\pm$ 0.74	53.42 $\pm$ 2.12	50.03 $\pm$ 1.79
$\Delta$ UA2S $\rightarrow$ GlcNS6S	10.71 $\pm$ 0.6	8.44 $\pm$ 0.65	5.48 $\pm$ 0.48	5.73 $\pm$ 0.34
$\Delta$ UA $\rightarrow$ GlcNS6S	11.47 $\pm$ 0.42	8.78 $\pm$ 0.67	9.21 $\pm$ 0.54	8.97 $\pm$ 0.46
$\Delta$ UA2S $\rightarrow$ GlcNS	12.58 $\pm$ 0.61	11.54 $\pm$ 0.54	5.15 $\pm$ 0.43	6.04 $\pm$ 0.39
$\Delta$ UA $\rightarrow$ GlcNS	16.33 $\pm$ 0.74	17.29 $\pm$ 0.72	17.34 $\pm$ 1.09	19.81 $\pm$ 0.86
$\Delta$ UA2S $\rightarrow$ GlcN6S	1.72 $\pm$ 0.16	1.31 $\pm$ 0.12	0.56 $\pm$ 0.06	0.64 $\pm$ 0.1
$\Delta$ UA $\rightarrow$ GlcN6S	1.11 $\pm$ 0.15	1.47 $\pm$ 0.16	0.54 $\pm$ 0.19	0.18 $\pm$ 0.08
$\Delta$ UA2S $\rightarrow$ GlcN	0.41 $\pm$ 0.06	0.53 $\pm$ 0.05	0.4 $\pm$ 0.07	0.35 $\pm$ 0.05
$\Delta$ UA $\rightarrow$ GlcN	0.53 $\pm$ 0.07	0.59 $\pm$ 0.07	0.44 $\pm$ 0.08	0.43 $\pm$ 0.07



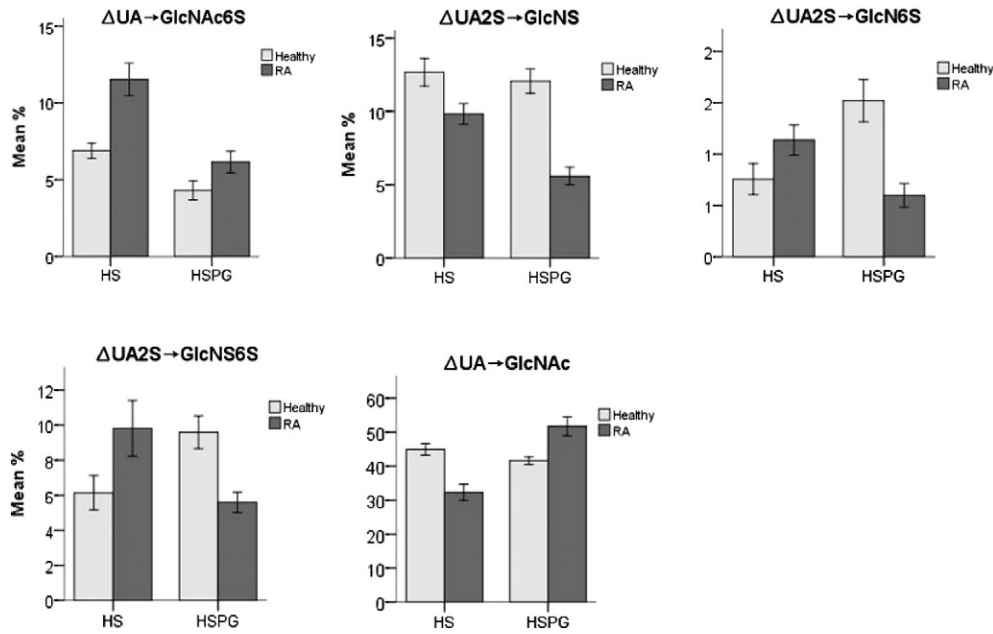


Fig. 2. Five disaccharides were found in significantly different proportions between healthy and RA samples, in both free HS and HSPG fractions. Error bars represent 95% confidence intervals.

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  $\Delta\text{UA} \rightarrow \text{GlcNS6S}$  and  $\Delta\text{UA} \rightarrow \text{GlcNAc6S}$  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 the 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  $\Delta\text{UA-GlcNAc}$ ,  $\Delta\text{UA2S-GlcNAc}$ ,  $\Delta\text{UA-GlcNAc6S}$ ,  $\Delta\text{UA2S-GlcNAc6S}$ ,  $\Delta\text{UA2S-GlcNS}$ ,  $\Delta\text{UA-GlcNS6S}$ ,  $\Delta\text{UA2S-GlcNS6S}$ ,  $\Delta\text{UA2S-GlcN6S}$ ,  $\Delta\text{UA-GlcN6S}$ ,  $\Delta\text{UA2S-GlcN}$ ,  $\Delta\text{UA-GlcN}$ , and  $\Delta\text{UA2S-GlcNCOEt6S}$  were purchased from V-Labs (Covington, LA).  $\Delta\text{UA-GlcNS}$  was obtained from Iduron (Manchester, UK). Protease from *Streptomyces griseus* was purchased from Sigma-Aldrich (St. Louis, MO). Heparinase I (heparinase, EC

Table 5

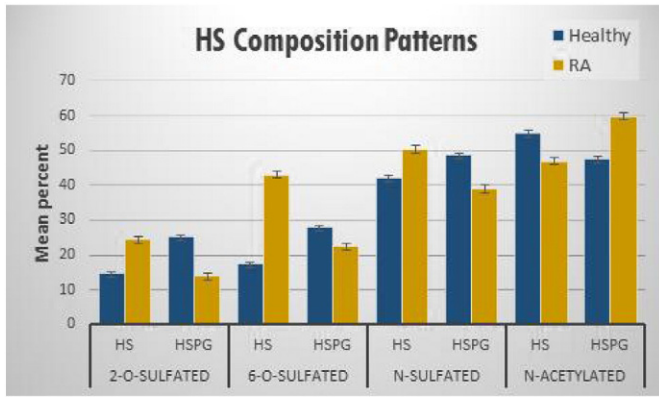
Free HS compositions from healthy and RA sera. The values are expressed as the mean percentage (from all samples in their respective categories)  $\pm$  SEM.

	Healthy (%)		RA (%)	
	Pre-menopause	Post-menopause	Pre-menopause	Post-menopause
% 2-O-sulfated	15.43 $\pm$ 0.78	13.93 $\pm$ 0.62	24.89 $\pm$ 0.77	23.9 $\pm$ 1.56
% 6-O-sulfated	18.27 $\pm$ 0.76	16.56 $\pm$ 0.61	42.68 $\pm$ 1.49	43.39 $\pm$ 1.45
% N-sulfated	44.31 $\pm$ 1.03	39.65 $\pm$ 0.93	49.48 $\pm$ 1.58	50.97 $\pm$ 1.43
% N-acetylated	52.68 $\pm$ 1.06	57 $\pm$ 0.87	47.51 $\pm$ 1.49	46.09 $\pm$ 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)  $\pm$  SEM.

	Healthy (%)		RA (%)	
	Pre-menopause	Post-menopause	Pre-menopause	Post-menopause
% 2-O-sulfated	26.88 $\pm$ 0.64	23.58 $\pm$ 0.79	13.42 $\pm$ 0.99	14.57 $\pm$ 0.86
% 6-O-sulfated	30.2 $\pm$ 0.59	25.64 $\pm$ 0.81	22.56 $\pm$ 1.02	22.55 $\pm$ 0.87
% N-sulfated	51.08 $\pm$ 0.7	46.04 $\pm$ 0.92	37.18 $\pm$ 1.77	40.55 $\pm$ 1.48
% N-acetylated	45.14 $\pm$ 0.73	50.06 $\pm$ 0.89	61.21 $\pm$ 1.8	58.17 $\pm$ 1.54



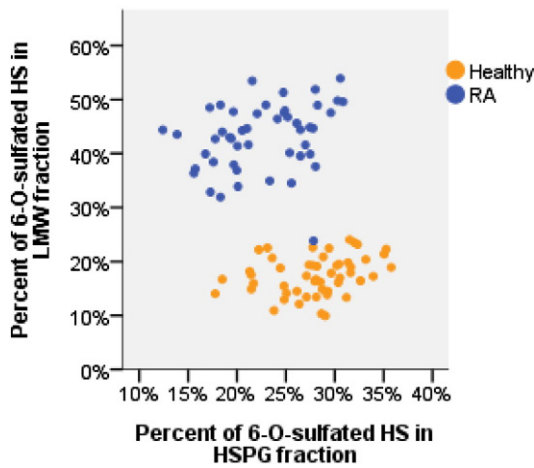
**Fig. 3.** Amounts of 2-O-sulfation, 6-O-sulfation, N-sulfation, and N-acetylation were considerably different in RA samples compared to healthy samples. The chart values reflect mean percentages of total HS/HSPG composition. Error bars represent 95% confidence intervals.

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).

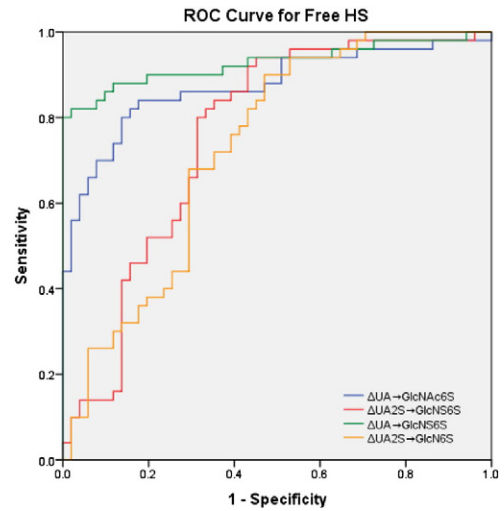
Serum samples from RA women were graciously provided by Dr. Marcos López-Hoyos from Hospital Universitario Marqués de Valdecilla. A total of 50 serum samples (Table 2) were analyzed: 25 from premenopausal women ( $39 \pm 7$  years old) and 25 from postmenopausal women ( $71 \pm 10$  years old). Samples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

**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  $\mu$ L) was centrifuged at 12,000  $\times$ 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



**Fig. 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 HSPG fraction is plotted against the percentage identified in the free HS fraction.

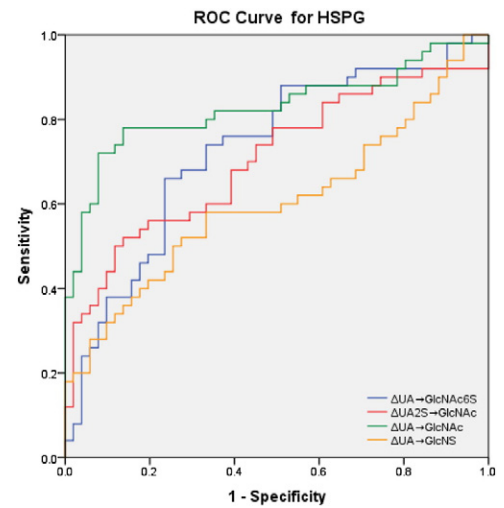


**Fig. 5.** ROC curves for HS-derived disaccharides  $\Delta$ UA  $\rightarrow$  GlcNAc6S,  $\Delta$ UA2S  $\rightarrow$  GlcNS6S,  $\Delta$ UA  $\rightarrow$  GlcNS6S, and  $\Delta$ UA2S  $\rightarrow$  GlcN6S. The areas under the curves are 0.874, 0.759, 0.928, and 0.730, respectively. As indicated in the figure, disaccharides  $\Delta$ UA  $\rightarrow$  GlcNAc6S and  $\Delta$ UA  $\rightarrow$  GlcNS6S showed high accuracy (AUC > 0.8) as potential diagnostic measures of RA.

using a high concentration of 2 M NaCl. The HS was separated from HSPG 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  $^{\circ}$ 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  $\mu$ L of digestion buffer, containing 20 mM  $\text{NH}_4\text{OAc}$  (pH 7.5) and 1 mM  $\text{Ca}(\text{OAc})_2$ . A combination of heparinase enzymes I, II and III (1 mU each) was added to each HS sample and incubated at 37  $^{\circ}$ C for 20 h with gentle agitation. After digestion, each sample was spiked with 2  $\mu$ L of 20 mM internal standard I-P ( $\Delta$ UA2S  $\rightarrow$  GlcNCOEt6S) (Zaia and Costello, 2001; Saad



**Fig. 6.** ROC curves for HSPG-derived disaccharides  $\Delta$ UA  $\rightarrow$  GlcNAc6S,  $\Delta$ UA2S  $\rightarrow$  GlcNAc,  $\Delta$ UA  $\rightarrow$  GlcNAc, and  $\Delta$ UA  $\rightarrow$  GlcNS. The areas under the curves are 0.728, 0.704, 0.827, and 0.603, respectively. As indicated in the figure, disaccharide  $\Delta$ UA  $\rightarrow$  GlcNAc showed high accuracy (AUC > 0.8) as a potential diagnostic measure of RA.

et al., 2005) and purified using a C18 + carbon SPE TopTip cartridge (Glygen, Columbia, MD). The cartridge was conditioned 2 times with 50  $\mu\text{L}$  of 0.1% trifluoroacetic acid (TFA) in 80% (v/v) ACN/ $\text{H}_2\text{O}$ , followed by 100  $\mu\text{L}$  of Milli-Q water and 100  $\mu\text{L}$  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  $\mu\text{L}$  of Milli-Q water. Heparin/HS-derived disaccharides retained by the cartridge were eluted with 250  $\mu\text{L}$  of 0.05% TFA in 40% (v/v) ACN/ $\text{H}_2\text{O}$ . 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  $\mu\text{L}$  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  $\times$  150 mm, 5  $\mu\text{m}$ ) (Thermo Fisher Scientific, Waltham, MA) at a flow rate of 200  $\mu\text{L}/\text{min}$ . The sample was desalted with 100% solvent A (5 mM  $\text{NH}_4\text{HCO}_3/\text{H}_2\text{O}$ ) for 4 min, then eluted in 38% solvent B (5 mM  $\text{NH}_4\text{HCO}_3$  in 80% ACN). The MS analysis was performed in the negative ion mode using a capillary temperature of 250  $^\circ\text{C}$ . 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.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.matbio.2014.08.016>.

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