Supplementary material to the article:

Expression of glycosaminoglycans in cirrhotic liver and hepatocellular carcinoma – A pilot study including etiology

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Checking the CS/DS digestion specificity of Chondroitinase ABC

We performed analysis on 4-4 serial sections of liver tissues with or without preceeding HA digestion. We found that no bias is caused by not performing the hyaluronan (HA) digestion. Moreover, the variance of D0a0 intensities proved to be larger when HA digestion was performed before CS digestion (Table S-2).

The method for HA digestion ws the following:

Five cycles 5 µL droplets of testicular hyaluronidase (Merck Sigma, Hungary) enzyme solution (0.16 TRU/µL hyaluronidase in the presence of 2 M ammonium acetate and 10% glycerol) was applied on the chosen spots. The resulting HA disaccharides were extracted four times by 0.3% ammonium hydroxide solution and the slides were dried. After this, CS/DS digestion was performed on the same spot as described in the Materials and Methods of the main body.

Table S-2. Intensities of the D0a0 disaccharide with and without hyaluronan (HA) digestion preceeding the CS/DS digestion.

	HA digested sample	**non-HA digested sample**
1	13915	10239
2	8969	11169
3	12379	12313
4	15497	10375
Average	12690	11024
relative standard deviation	19.03%	7.48%
t-test *p*-value	0.3262	

Investigated disaccharides

Table S-3. Structure, nomenclature, and m/z values of the CS disaccharides investigated. D0a4 and D0a6 are positional isomers and are distinguished via MS/MS measurements in the present study.

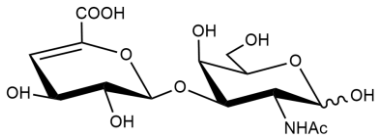
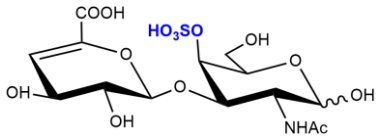
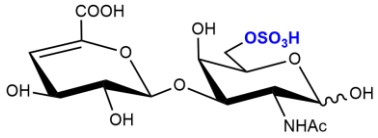
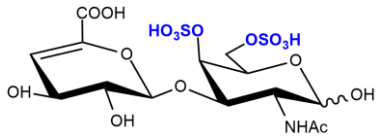
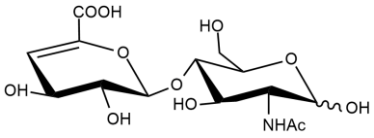
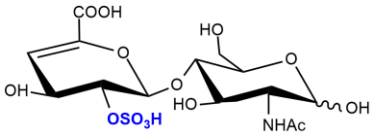
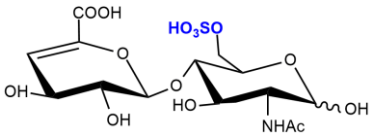
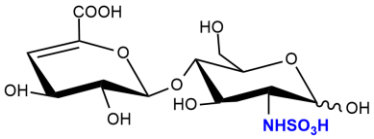
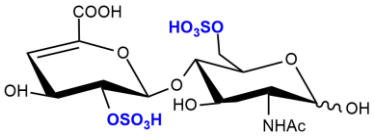
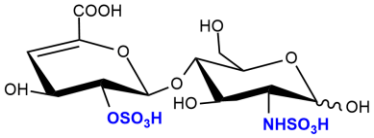
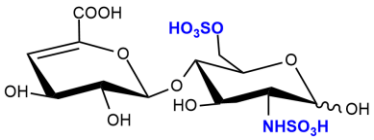
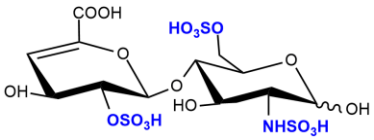
Chemical structure	Traditional name	Lawrence code	m/z (-) mode	MS/MS transition
	ΔHexA-GalNAc	**D0a0**	378.1	-
	ΔHexA-GalNAc4S	**D0a4**	458.1	300.1
	ΔHexA-GalNAc6S	**D0a6**	458.1	282.1
	ΔHexA-GalNAc4S6S	**D0a10**	538.1	-

Table S-4. Structure, nomenclature, and *m/z* values of the HS disaccharides investigated.

Chemical structure	Traditional name	Lawrence code	m/z (-) mode
	ΔHexA-GlcNAc	**D0A0**	378.1
	ΔHexA2S-GlcNAc	**D2A0**	458.1
	ΔHexA-GlcNAc6S	**D0A6**	458.1
	ΔHexA-GlcNS	**D0S0**	416.1
	ΔHexA2S-GlcNAc6S	**D2A6**	538.1
	ΔHexA2S-GlcNS	**D2S0**	496.1
	ΔHexA-GlcNS6S	**D0S6**	496.1
	ΔHexA2S-GlcNS6S	**D2S6**	576.1

Sulfation pattern of CS and HS disaccharides as shown by absolute intensities

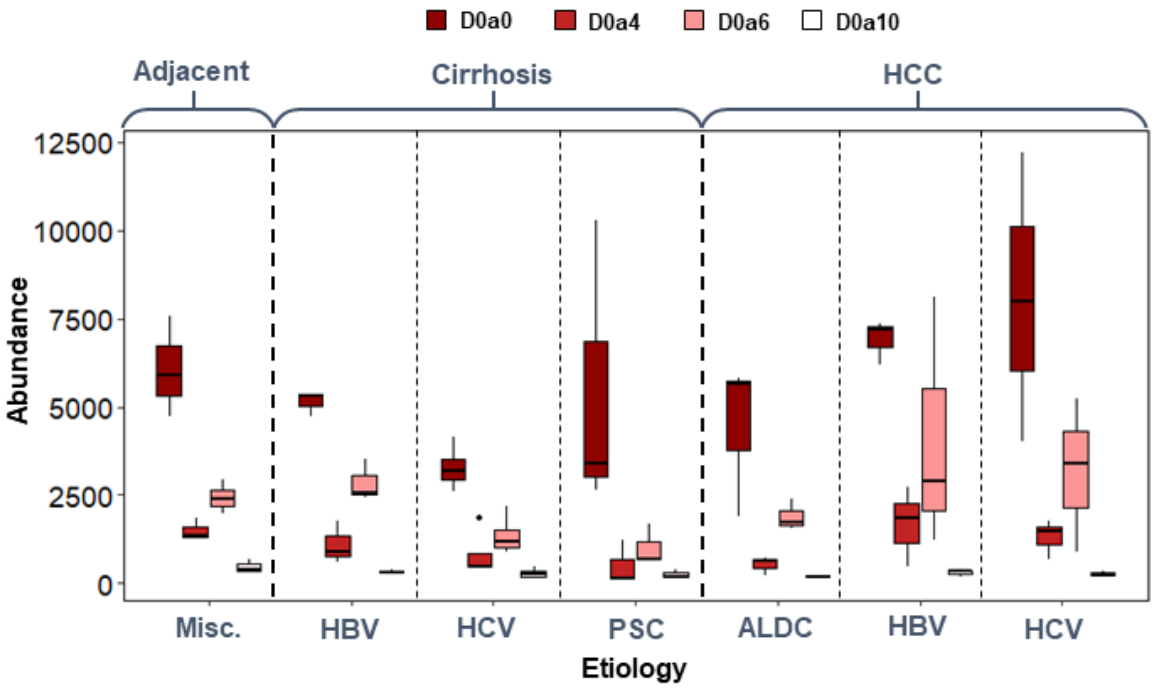


Figure S-1. Sulfation pattern of CS/DS in liver diseases concerning etiology shown by absolut intensities of disaccharides. (Cirr: cirrhosis, HCC: hepatocellular carcinoma, ALDC: alcoholic liver disease associated cirrhosis, HBV: hepatitis-B virus, HCV: hepatitis-C virus, PSC: primary sclerotizing cholangitis)

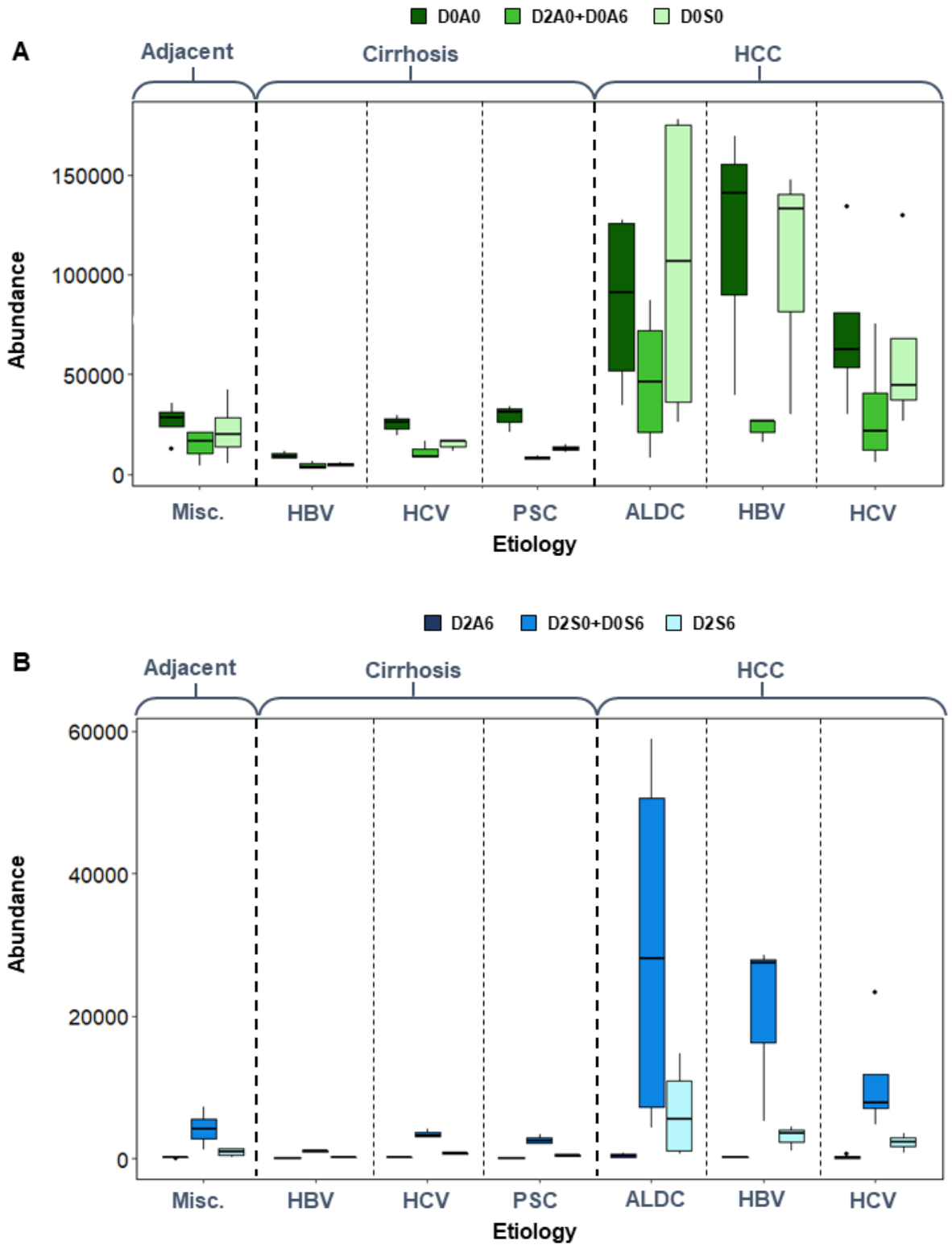


Figure S-2. Sulfation pattern of heparan sulfate in liver diseases concerning etiology shown by absolut intensities of disaccharides. **A:** non-sulfated and monosulfated HS disaccharides, **B:** doubly and triply sulfated HS disaccharides. (Cirr: cirrhosis, HCC: hepatocellular carcinoma, ALDC: alcoholic liver disease associated cirrhosis, HBV: hepatitis-B virus, HCV: hepatitis-C virus, PSC: primary sclerotizing cholangitis)

Shotgun proteomics experiments

1. On-surface digestion

After CS and HS surface digestion, the same tissue spots were used for proteomic digestion. First, the proteins were reduced using RapiGest and DTT in 5 µL of 20% glycerol for 20 minutes at 55 °C, then alkylated using IAA in 5 µL of 25 mM ammonium bicarbonate (ABC) puffer and 20% glycerol for 20 minutes at room temperature in the dark. The digestion was done cyclically, each one lasting for 40 minutes at 37 °C in a humidified box, 5 cycles in total. In the first two cycles, LysC-Trypsin mixture was added in a 1:25 ratio, in 5 µL 50 mM ABC, and 20% glycerol. Subsequently, in the last three cycles, Trypsin was added in a 1:10 ratio, in 5 µL 50 mM ABC, and 20% glycerol. After digestion, proteins were extracted from the surface with 5x5 µL of 10% acetic acid. Peptide extracts were then dried down, and clean-up was performed using C18 spin columns (Thermo Scientific) using the manufacturer's protocol. The cleaned peptide extracts were dried down and stored at -20°C for further usage.

2. Mass spectrometry and chromatography analysis

Samples were dissolved in 8 µL solvent (98% water, 2% acetonitrile, and 0.1% formic acid) out of which 6 µL was subjected to nanoLC-MS/MS analysis using a Dionex Ultimate 3000 RSLC nanoLC (Dionex, Sunnyvale, CA, USA) coupled to a Bruker Maxis II Q-TOF (Bruker Daltonik GmbH, Bremen, Germany) via CaptiveSpray nanoBooster ionization source. Peptides were separated on an Acquity M-Class BEH130 C_{18} analytical column (1.7µm, 75µm x 250mm Waters, Milford, MA) using gradient elution (isocratic hold at 4% for 11 min, then elevating B solvent content to 25% in 75 min, and 40% in 15 min) following trapping on an Acclaim PepMap100 C18 (5µm, 100µm x 20mm, Thermo Fisher Scientific, Waltham, MA) trap column. Solvent A consisted of water + 0.1% formic acid, while Solvent B was acetonitrile + 0.1% formic acid. Spectra were collected using a fixed cycle time of 2.5 sec and the following scan speeds: MS spectra at 3 Hz, while CID was performed on multiply charged precursors at 16 Hz for abundant ones and at 4 Hz for low abundance ones. Internal calibration was performed by infusing sodium formate and data were automatically recalibrated using the Compass Data Analysis software 4.3 (Bruker Daltonik GmbH, Bremen, Germany).

3. Data analysis

Database search was performed with Byonic 3.8 software. 10ppm internal and 20 ppm external mass accuracy, 2 missed cleavages, carbamidomethylation of cysteines as fixed modification, deamidation (NQ) and oxidation (M) as variable modifications) and proteins were identified using 1% FDR limit. A focused FASTA file was created from the proteins identified in all the samples and that was used as the database for label-free quantitation using MaxQuant 2.0.3.0 using default setting for Bruker QTOF [1]. The MaxQuant output was then loaded into Perseus 1.6.5.0, missing values were imputed from a normal distribution with the default settings for the whole matrix (down shift of 1.8 and width of 0.3). Statistical analysis was then performed, Kruskal-Wallis test.

4. Results

The proteoglycans identified in the samples were specifically searched for if they showed significant quantity differences among the samples. The shotgun proteomics analysis determined, that no proteoglycans exerted significant changes, thus the difference in GAG composition is due to the alteration in the polysaccharide synthesis. The Kruskal-Wallis p-values of the quantified proteoglycans are shown in Table S-4.

Table S-5. Kruskal-Wallis *p*-values of proteoglycans among the cirrhotic groups according to shotgun proteomics experiments.

Uniprot ID	Proteoglycan	KW *p*-values
P20774	Mimecan	0.3011
P07585	Decorin	0.3454
P21810	Biglycan	0.5210
P51888	Prolargin	0.5407
Q99715	Collagen alpha-1(XII) chain	0.7365
P16070	CD44	0.7423
P98160	Perlecan	0.7466
P13611	Versican	0.7845
O00468	Agrin	0.7926
P51884	Lumican	0.8214

1. J. Cox, M. Mann, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification, Nature Biotechnology. 26 (2008). 1367-1372. DOI: 10.1038/nbt.1511.

Table S-6. Estimation of minimum sample size for a future large-scale study based on HS and CS sulfation pattern.

HS sulfation pattern						
	D0A0	**D2A0/D0A6**	**D0S0**	**D2A6**	**D2S0/D0S6**	**D2S6**
Adjacent	12	14	11	353	42	33
Cirr_HBV	417	75	1341	41	75	119
Cirr_HCV	308	32	109	32	393	179
Cirr_PSC	114	276	378	52	306	2957
HCC_ALDC	8	34	30	278	5	5
HCC_HBV	728	23	65	203	54	287
HCC_HCV	41	14	63	106	114	8

Min 20 samples discriminate between	**8**	comparisons
Min 25 samples discriminate between	**9**	comparisons
Min 30 samples discriminate between	**10**	comparisons
Min 35 samples discriminate between	**14**	comparisons
Min 40 samples discriminate between	**14**	comparisons
Min 45 samples discriminate between	**17**	comparisons

CS sulfation pattern				
	D0a0	**D0a4**	**D0a6**	**D0a10**
Adjacent	68	29	60	19
Cirr_HBV	94	33	499	94434
Cirr_HCV	41	13	264	22
Cirr_PSC	6611	62	236	61
HCC_ALDC	17	19	32	17
HCC_HBV	10	25	8	147
HCC_HCV	6	13	6	240

Min 20 samples discriminate between	**10**	comparisons
Min 25 samples discriminate between	**12**	comparisons
Min 30 samples discriminate between	**13**	comparisons
Min 35 samples discriminate between	**15**	comparisons
Min 40 samples discriminate between	**15**	comparisons
Min 45 samples discriminate between	**16**	comparisons