

# Glycosaminoglycans by LC-MS/MS

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# Glycosaminoglycans (GAGs)

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- Glycosaminoglycans or *mucopolysaccharides* because of their viscous, lubricating properties, as found in mucous secretions
- Present on all animal cell surfaces in the extracellular matrix (ECM)
- Known to bind and regulate chemokines, cytokines, growth factors, morphogens, enzymes and adhesion molecules

# Glycosaminoglycan structure

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- GAGs are long, unbranched, negatively charged heteropolysaccharide chains generally composed of a repeating disaccharide unit:



# Glycosaminoglycans properties

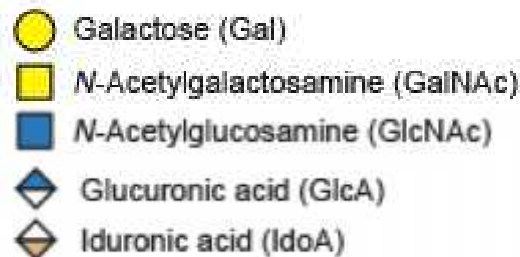
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- At neutral pH, GAGs are highly negatively charged (carboxyl and sulfate groups)
- GAGs in aqueous solution are surrounded by a shell of water molecules → occupy an enormous hydrodynamic volume in solution
- Low compressability

# Glycosaminoglycan types

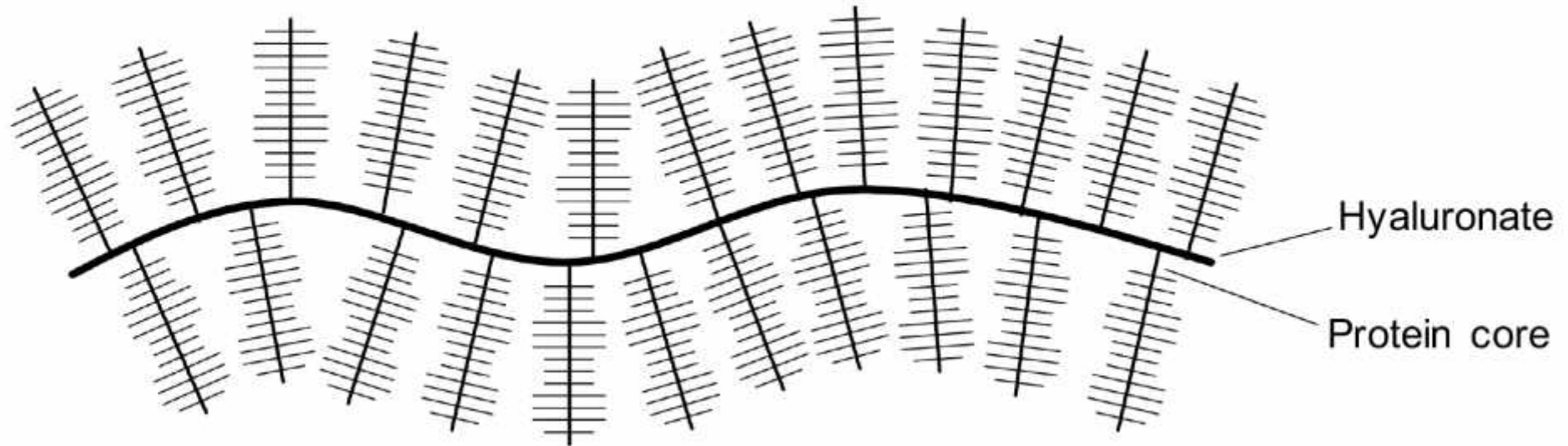
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- Heparin/Heparan sulfate
  - (N-acetyl)glucosamineglycans
- Chondroitin sulfate/Dermatan sulfate
  - (N-acetyl)galactosaminoglycans
- Keratan sulfates
  - Galactose instead of uronic acid
- Hyaluronic acid
  - Non sulfated

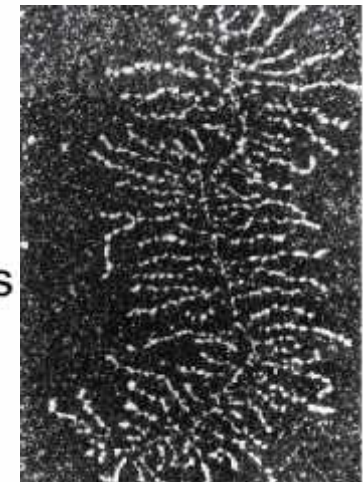
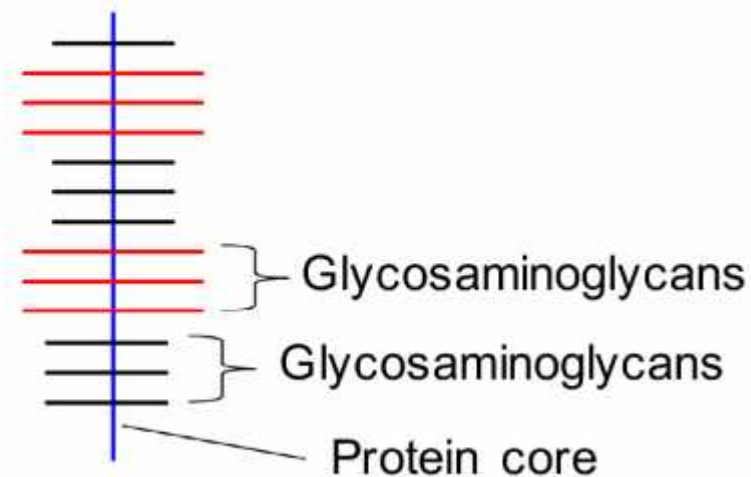


# Glycosaminoglycan structure

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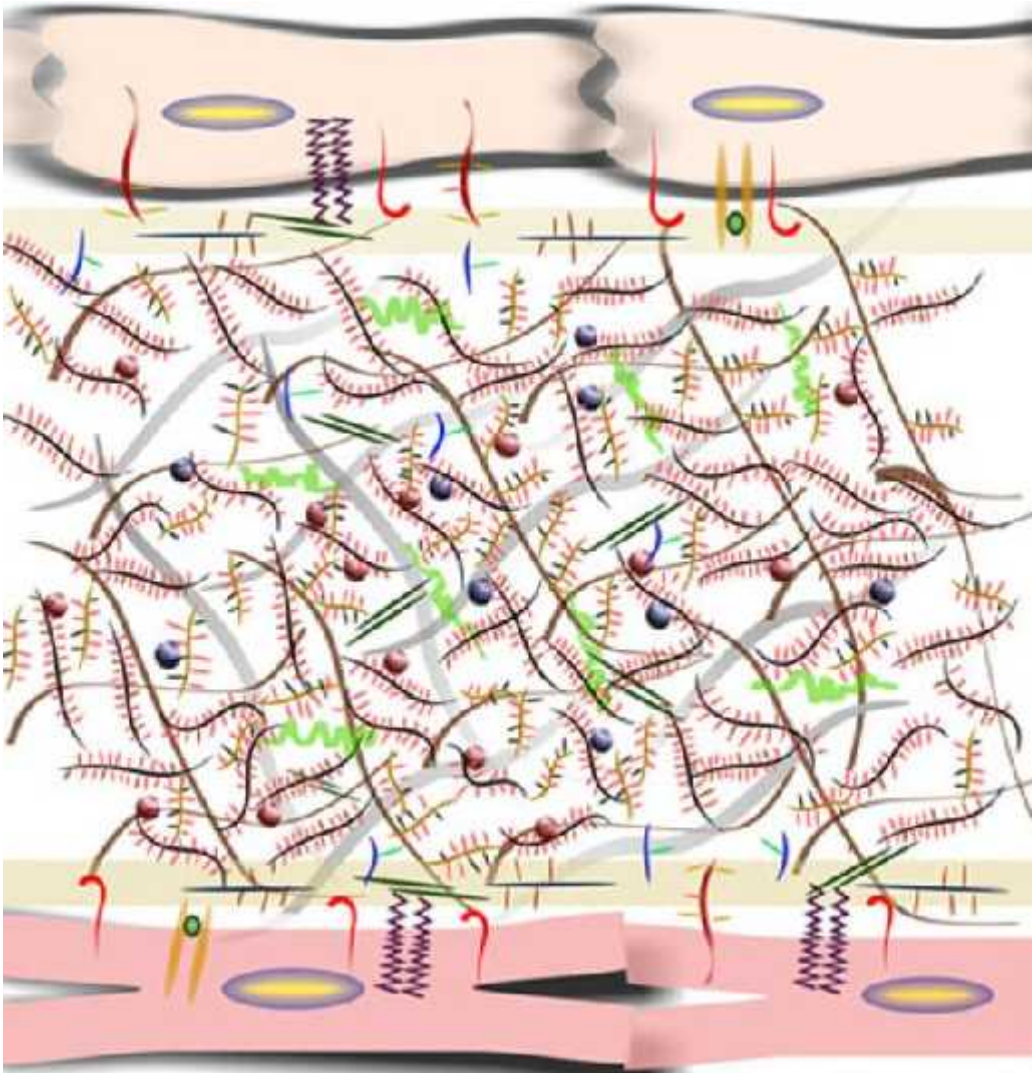


GAGs are linked to core proteins (except for hyaluronic acid) = proteoglycans or **mucopolysaccharides**



# GAGs in the ECM

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	Endothelial cell		Cardiac myocyte		CD44
	Elastin		Collagen		Hyaluronan
	TLR4		Versican		Decorin
	Integrin		Aggrecan		Fibronectin
	Perlecan		Biglycan		Growth factor
	Syndecan		Cytokine/ chemokine		DS \ KS
			HS - CS		

# Glycosaminoglycans properties

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- Heparin/Heparan sulfate (HSGAGs)
- Chondroitin sulfate/Dermatan (CSGAGs)
  - O-linked glycans
- Keratan sulfate
  - N-linked or O-linked glycans

*Synthesised in the golgi*
- Hyaluronic acid
  - Direct secretion in extracellular matrix from plasma membrane



# Glycosaminoglycans functions

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- Many functions:
  - Heparin/Heparan sulfate (HS)
    - Histamine storage in mast cells (heparin)
    - Anticoagulant, LPL release (heparin)
    - Cell adhesion, regulation of cell growth
  - Chondroitin sulfate (CS)
    - Most abundant GAG in the body
    - Found in cartilage, tendon, ligament, aorta
  - Dermatan sulfate (DS)
    - Found in skin, blood vessels, heart valves
  - Keratan sulfate (KS)
    - tissue hydration, anti-adhesive
    - Found in cartilage and cornea
  - Hyaluronic acid
    - Major component of synovial tissues and fluid, vitreous body (eye)
    - Excellent lubricator and shock absorber

# Mucopolysaccharidoses and mucopolipidoses

Type	Eponym	Enzyme deficiency	Storage product*
MPS I	Hurler,	$\alpha$ -L-iduronidase	DS, HS
	Hurler/Scheie, Scheie		
MPS II	Hunter	Iduronate-2-sulfatase	DS, HS
MPS III A	Sanfilippo A	Heparan-N-sulfatase	HS
MPS III B	Sanfilippo B	N-acetyl- $\alpha$ -glucosaminidase	HS
MPS III C	Sanfilippo C	Acetyl-CoA: $\alpha$ -glucosaminide N-acetyltransferase	HS
MPS III D	Sanfilippo D	N-acetylglucosamine 6-sulfatase	HS
MPS IVA	Morquio A	Galactose-6-sulfatase	KS, CS
MPS IVB	Morquio B	$\beta$ -galactosidase	KS
MPS VI	Maroteaux-Lamy	N-acetylgalactosamine-4-sulfatase	DS
MPS VII	Sly	$\beta$ -glucuronidase	DS, HS, CS
MPS IX	-	Hyaluronidase	Hyaluronan
ML II	I-cell disease	N-acetylglucosaminyl-1-phosphotransferase	GAGs, sphingolipids
ML III	Pseudo-Hurler polydystrophy		

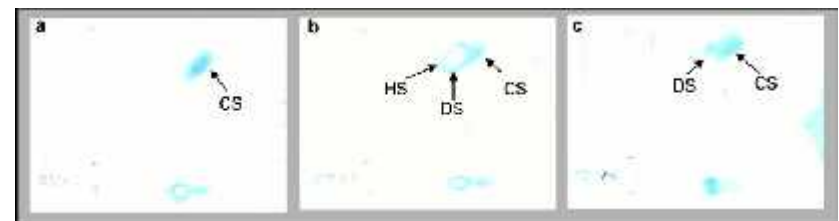
\* DS: Dermatan sulfate, HS: Heparan sulfate, CS: Chondroitin sulfate, KS: Keratan sulfate, GAGs: glycosaminoglycans.

- Deficiency of lysosomal enzymes of GAGs degradation cause mucopolysaccharidoses and mucopolipidoses
- Accumulation of GAGs in urine is a diagnostic marker

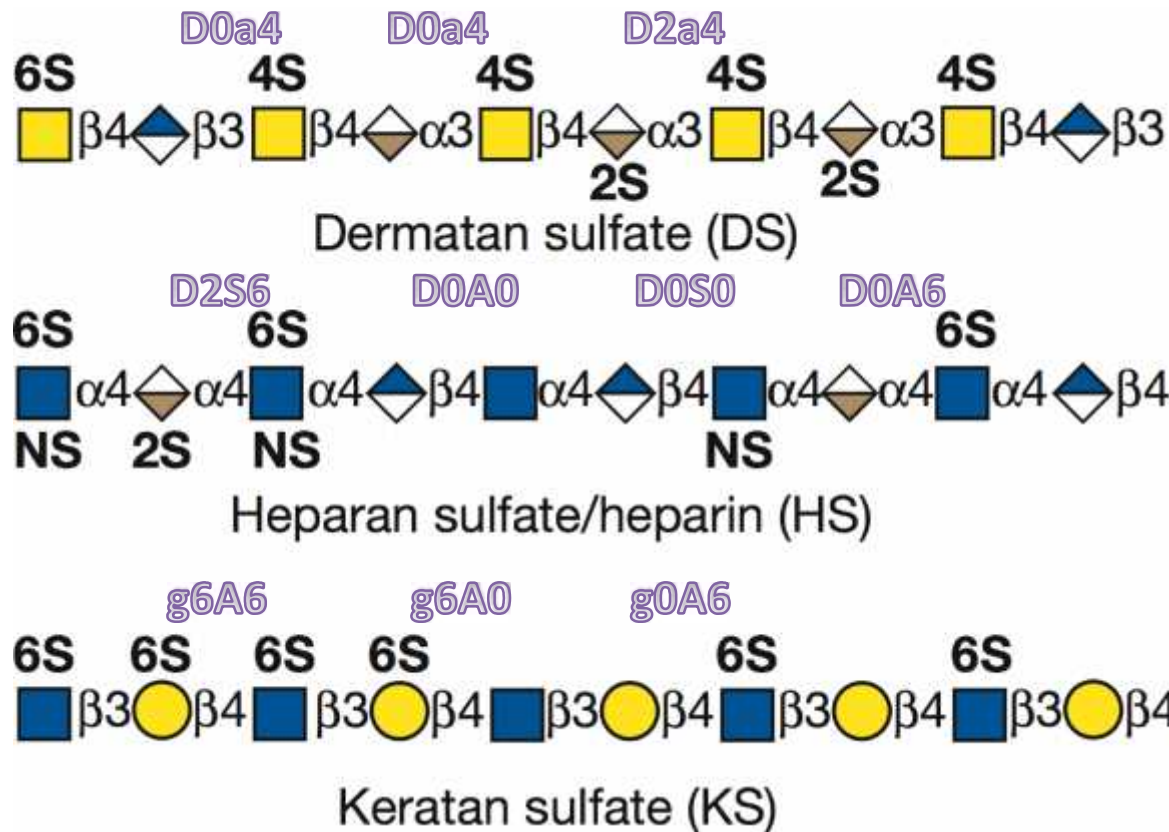
# Mucopolysaccharidoses and mucopolipidoses

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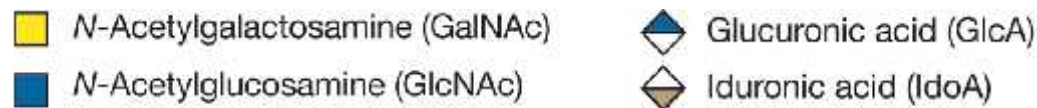
- Main screening test is dimethylmethylene blue (DMB) test
- If elevated → mucopolysaccharide electrophoresis
  - Reagent increasingly difficult to obtain
  - Not specific
  - Many false positives
  - False negatives (MPS III and IV and mild patients)
- New assay needed
  - Methanolysis → only HS and DS (not KS!)
  - Enzymatic digestion of GAGs to disaccharides → analyse



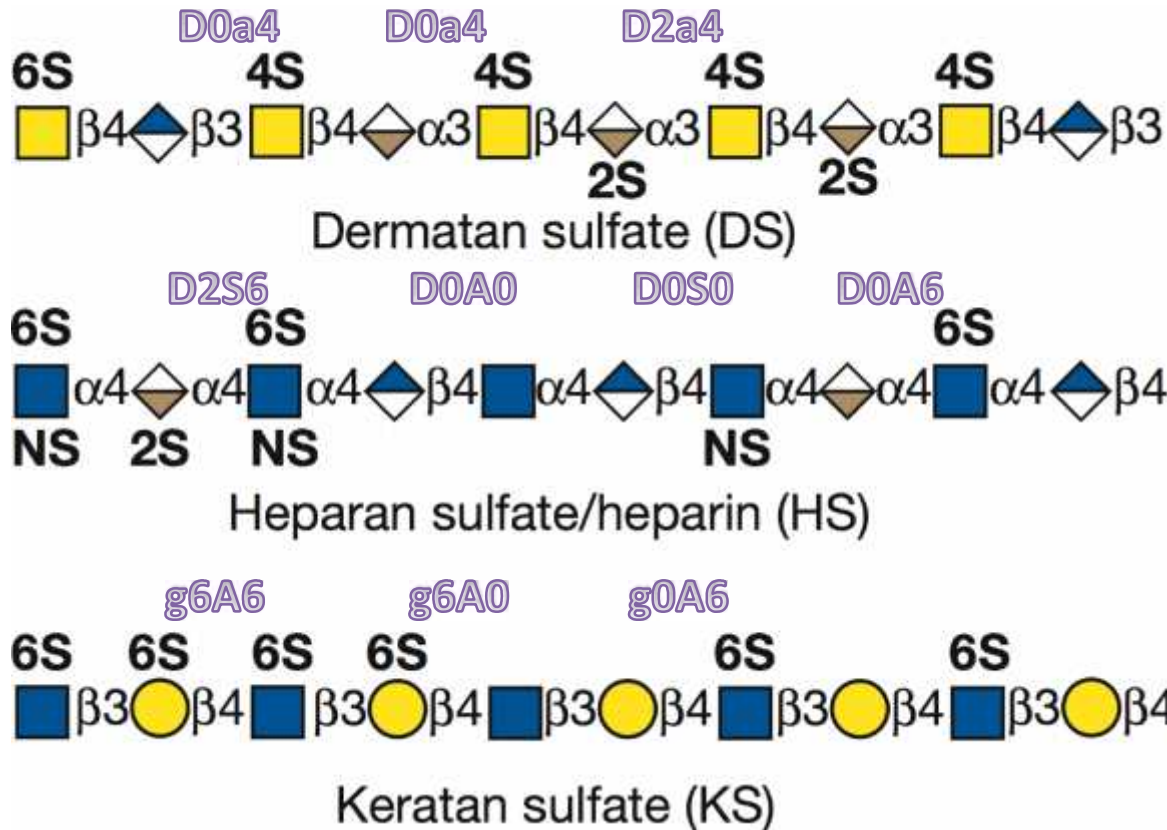
# Disaccharide nomenclature



- Abbreviated disaccharide nomenclature
- Easier than old (and long) system
- Cryptic at first but useful



# Disaccharide nomenclature



- N-Acetylgalactosamine (GalNAc)
- N-Acetylglucosamine (GlcNAc)
- ◊ Glucuronic acid (GlcA)
- ◊ Iduronic acid (IdoA)

**Non-reducing end descriptor**  
 U = undesignated uronic acid  
 D = Δ<sup>4,5</sup>-unsaturated uronic acid  
 G = glucuronic acid  
 I = iduronic acid  
 g = galactose

**Hexosamine descriptor**  
 A = glucosamine  
 a = galactosamine  
 M = anhydromannose  
 T = anhydrotalose  
**Amine substitution**  
 H = free amine  
 A = N-acetylated  
 S = N-sulfated  
 R = amino-tagged

DOA0

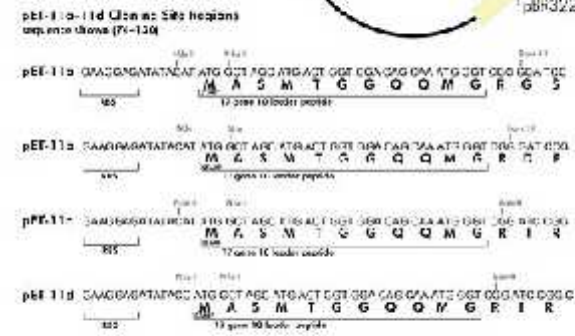
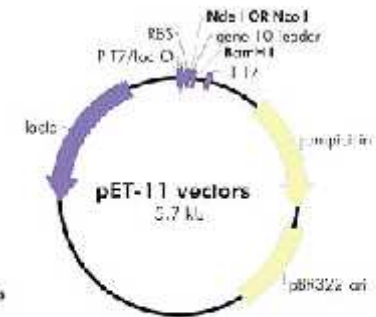
**Hexosamine O-sulfation**  
 0 = No sulfation  
 3 = 3-O-sulfation  
 4 = 4-O-sulfation  
 6 = 6-O-sulfation  
 9 = 3,6-O-disulfation  
 10 = 4,6-O-disulfation

**Non-reducing end O-sulfation**  
 0 = No sulfation  
 2 = 2-O-sulfation  
 3 = 3-O-sulfation  
 6 = 6-O-sulfation

Lawrence, R et al, 2008. Disaccharide structure code for the easy representation of constituent oligosaccharides from glycosaminoglycans. PMID: 18376390

# Financial issue

- GAG degrading enzymes are REALLY expensive
  - One incubation = ~100 euro!!
- Express your own!

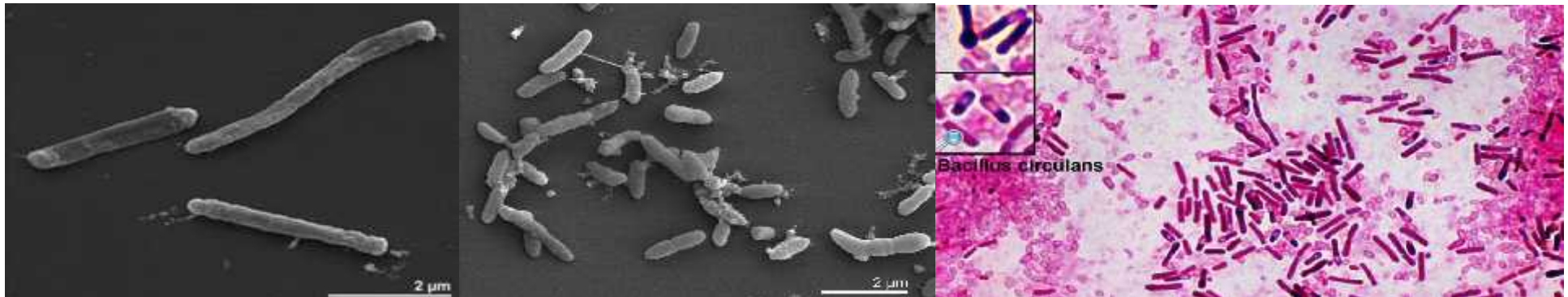


Features	Nucleotide Position			
	pET-11a	pET-11b	pET-11c	pET-11d
T7 promoter with lac operon	1-13	1-13	1-13	1-13
ribosome binding site (RBS)	71-80	74-80	74-80	74-80
leader pET-11a-lacI or leader pET-11b-lacI only via	85-91	86-91	76-91	86-91
T7 gene 10 basal/total leader	87-121	89-121	79-121	88-120
BamHI cloning site	125-130	124-125	123-125	122-127
T7 terminator	191-195	192-214	197-213	196-212
ampicillin resistance (bla <sub>CRF</sub> )	437-1511	455-1513	455-1512	454-1511
pBR322 origin of replication	665-2830	1654-2931	1655-2931	1663-2929
kanamycin resistance (kan <sub>CRF</sub> )	4219-5291	4211-5292	4212-5292	4209-5290

# Expression of GAG degrading enzymes

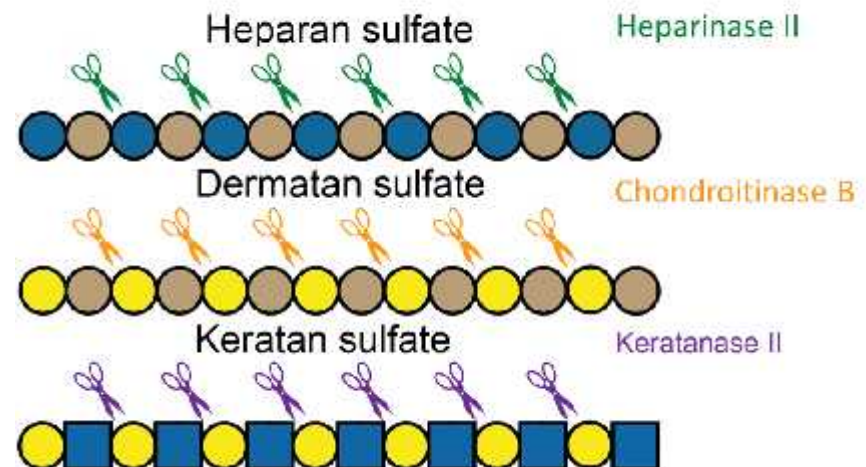
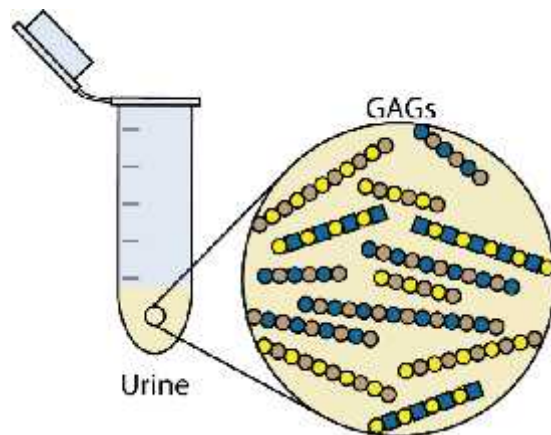
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- All enzymes were expressed as His-tagged fusion proteins in *E. coli*
  - Keratanase II: *Bacillus circulans* **KS**
  - Chondroitinase B: *Pedobacter heparinus* **DS**
  - Heparinase II: *Flavobacterium heparinum* **HS**
- Purify enzymes on HisLink Protein Purification Resin
- After dialysis, snap-freeze in liquid nitrogen and store at -80°C.



# Enzymatic hydrolysis of GAGs

- Enzymatic hydrolysis of GAGs to disaccharides:
  - 50  $\mu\text{L}$  urine diluted to 2 mM creatinine
  - Incubate with heparinase II, chondroitinase B and keritinase II for 2 h at 30°C
  - Add 15  $\mu\text{L}$  of 150 mM EDTA (pH7.0)
  - Add 125 ng of the internal standard, 4UA- 2S-GlcNCOEt-6S
  - Boil for 5 min to precipitate proteins, centrifuge
  - Apply supernatant to Amicon Ultra 10 kD centrifugal filter (Millipore) and centrifuge
  - Analyse filtrate by UPLC-MS/MS





# UPLC-MS/MS analysis

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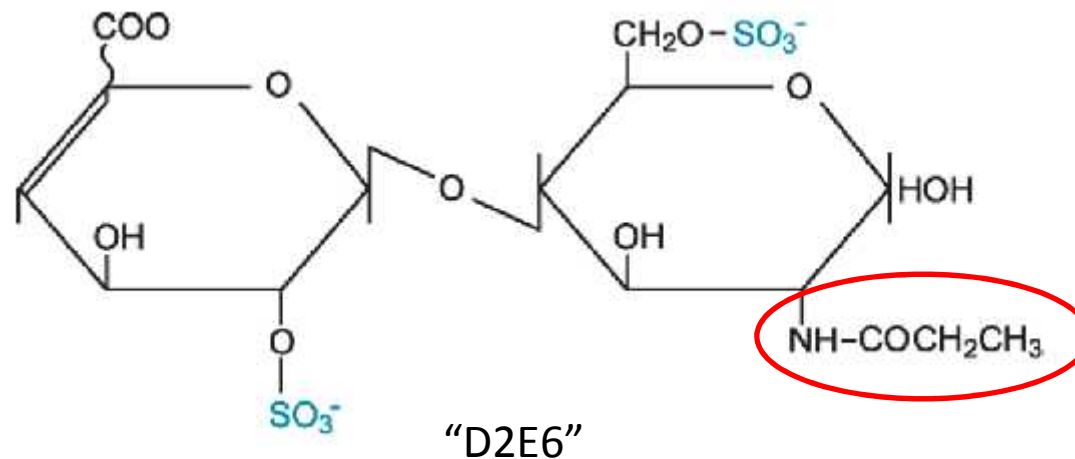
- Waters Quattro Premier XE (tandem) mass spectrometer with Acquity UPLC system
- Thermo Hypercarb HPLC column (100 × 2.1 mm, 5 μm)
- Buffer A: 10 mM  $\text{NH}_4\text{HCO}_3$  (pH 10)
- Elute with buffer B: acetonitrile gradient of 0% to 20%
- Total run time 7 minutes



# Analysis of disaccharides

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- Calibration curve of each disaccharide with 4UA-2S-GlcNCOEt-6S as internal standard



- Sum all disaccharides and report as total HS, DS and KS

# Disaccharides

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- D0A0
- D0S0
- D0A6 and D2A0
- D2S0 and D0S6
- D0a4
- D0a10
- g0A6
- g6A6
  
- D2S6

# Disaccharides

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- D0A0
- D0S0
- D0A6 and D2A0
- D2S0 and D0S6 **HS**
- D0a4
- D0a10
- g0A6
- g6A6
  
- D2S6

# Disaccharides

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- D0A0
- D0S0
- D0A6 and D2A0
- D2S0 and D0S6 **HS**
- D0a4 **DS**
- D0a10 **DS**
- g0A6
- g6A6
- D2S6

# Disaccharides

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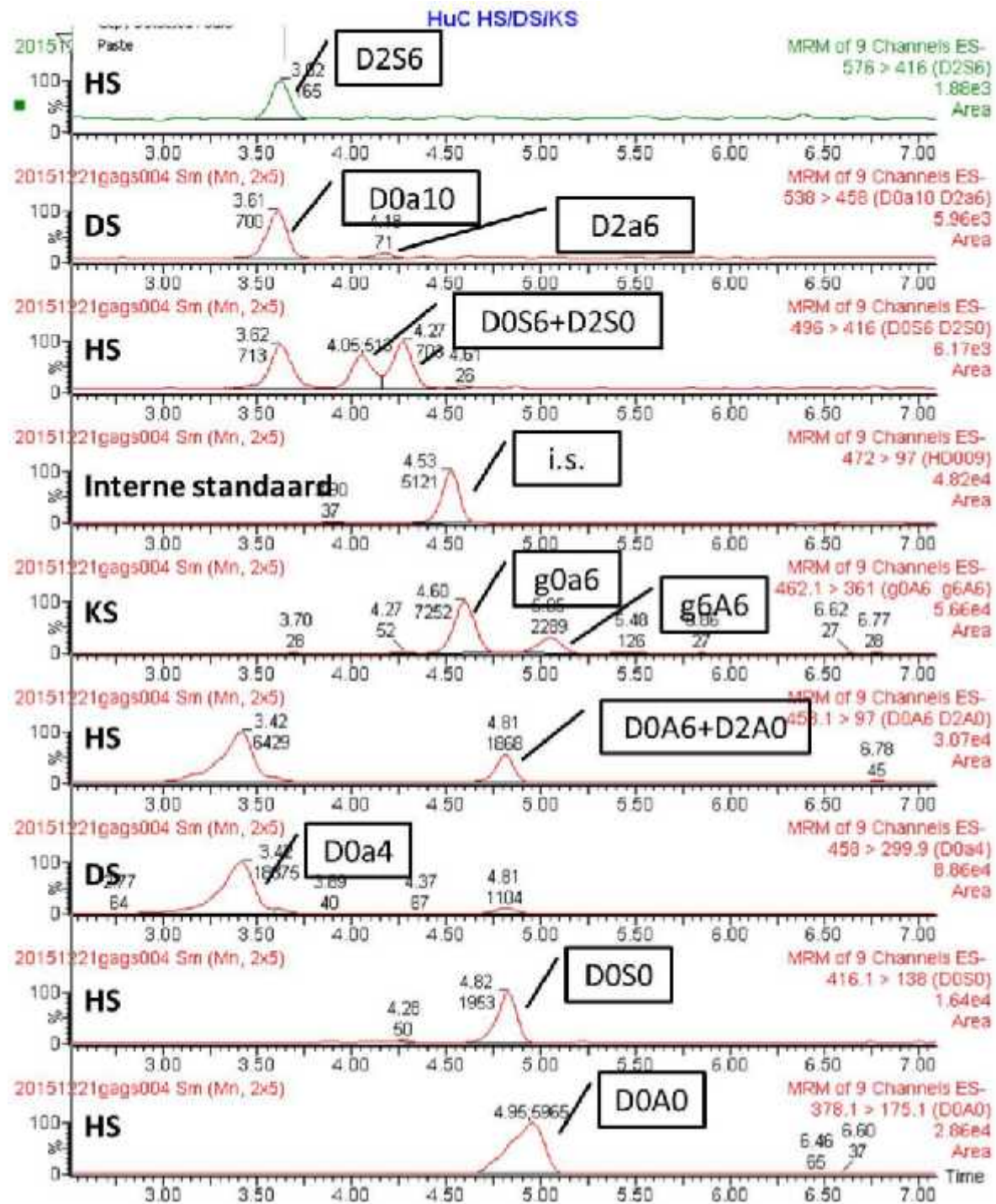
- D0A0
- D0S0
- D0A6 and D2A0
- D2S0 and D0S6 **HS**
- D0a4 **DS**
- D0a10 **DS**
- g0A6 **KS**
- g6A6 **KS**
  
- D2S6

# Disaccharides

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- D0A0
  - D0S0
  - D0A6 and D2A0
  - D2S0 and D0S6 **HS**
  - D0a4
  - D0a10 **DS**
  - g0A6
  - g6A6 **KS**
- 
- D2S6 **HS** and Heparin

If  $D2S6/D0A0 > 0.8$   
exogenous heparin is  
possible





# Practicalities

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- Despite sample cleanup, after 20-30 urine samples peaks start shifting (less retention)
- Column needs to be reequilibrated by rinsing with buffer A
- Not a problem with plasma/CSF (under development)



# Reproducibility

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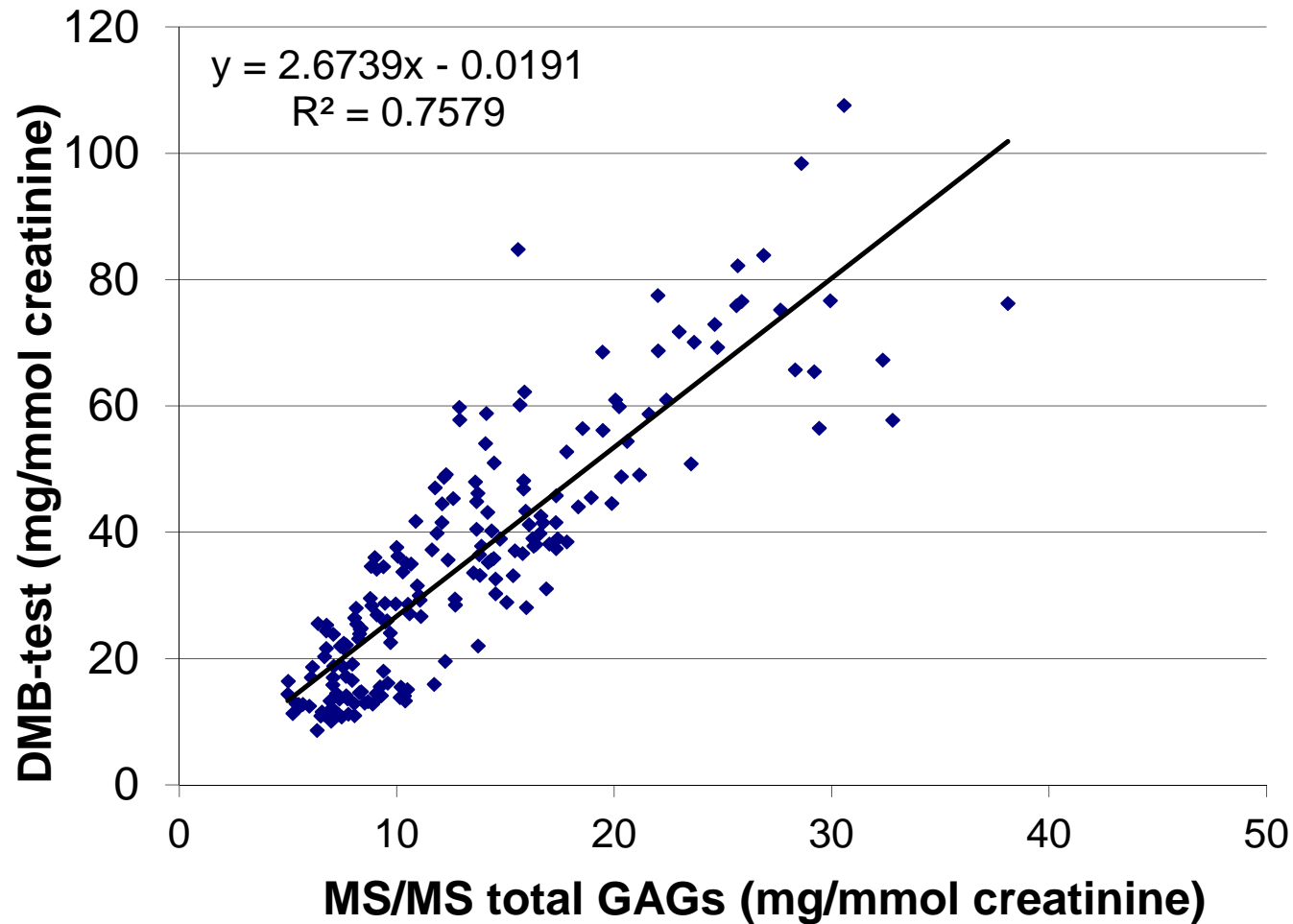
	HS	DS	KS
<b>Level 1</b>	ng/ml	ng/ml	ng/ml
<b>Average</b>	833	175	1017
<b>SD</b>	98	19	111
<b>vc %</b>	11.8	10.9	10.9
<b>Level 2</b>	ng/ml	ng/ml	ng/ml
<b>Average</b>	1592	1263	2552
<b>SD</b>	160	153	236
<b>vc %</b>	10.0	12.1	9.3

- Control samples
- Two levels
- N=30

- Variation is quite high but differences between patients and controls are much larger

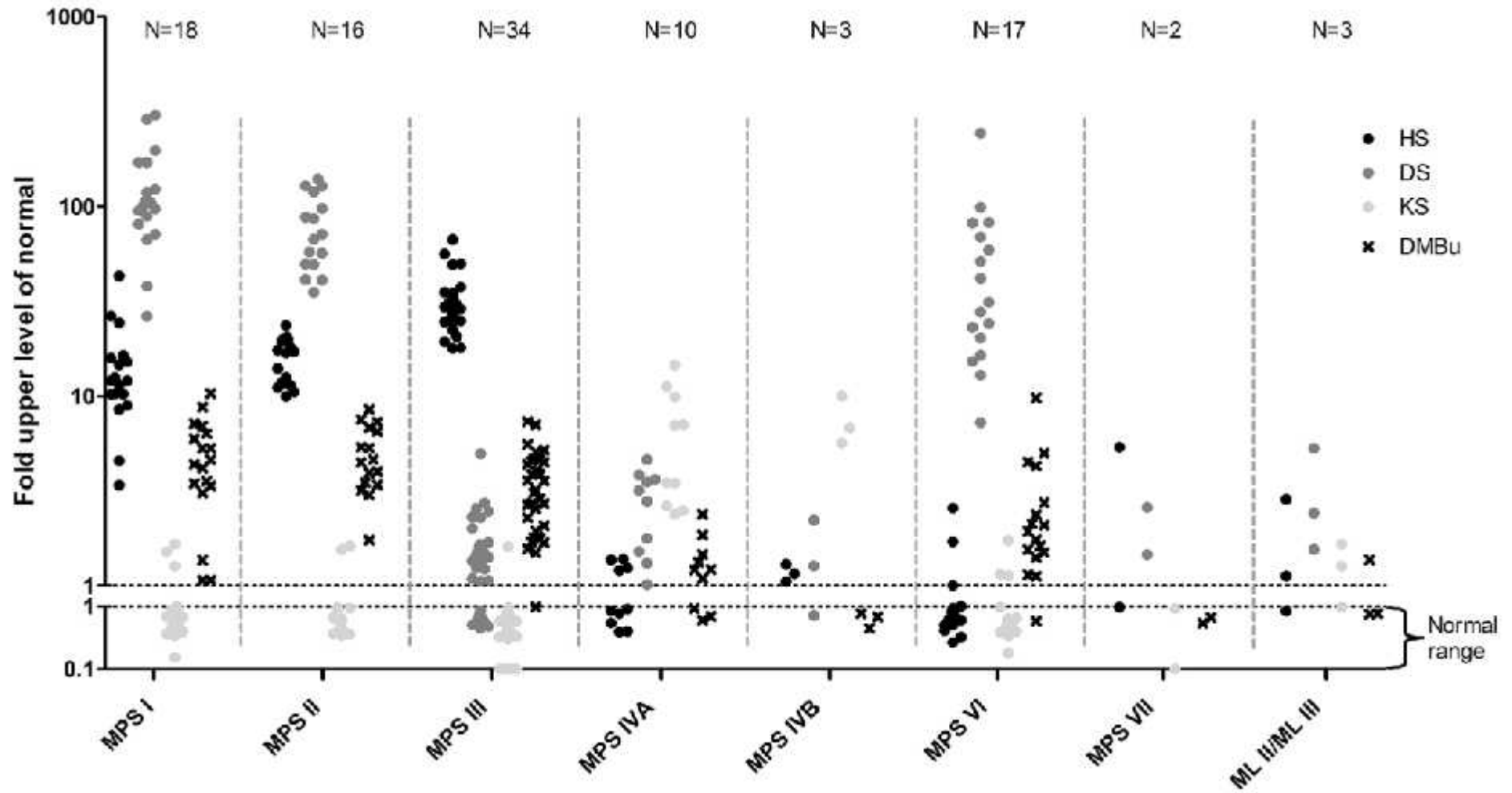
# Total GAGs vs DMB test

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- Cleavage of GAGs is not complete
- About 40%

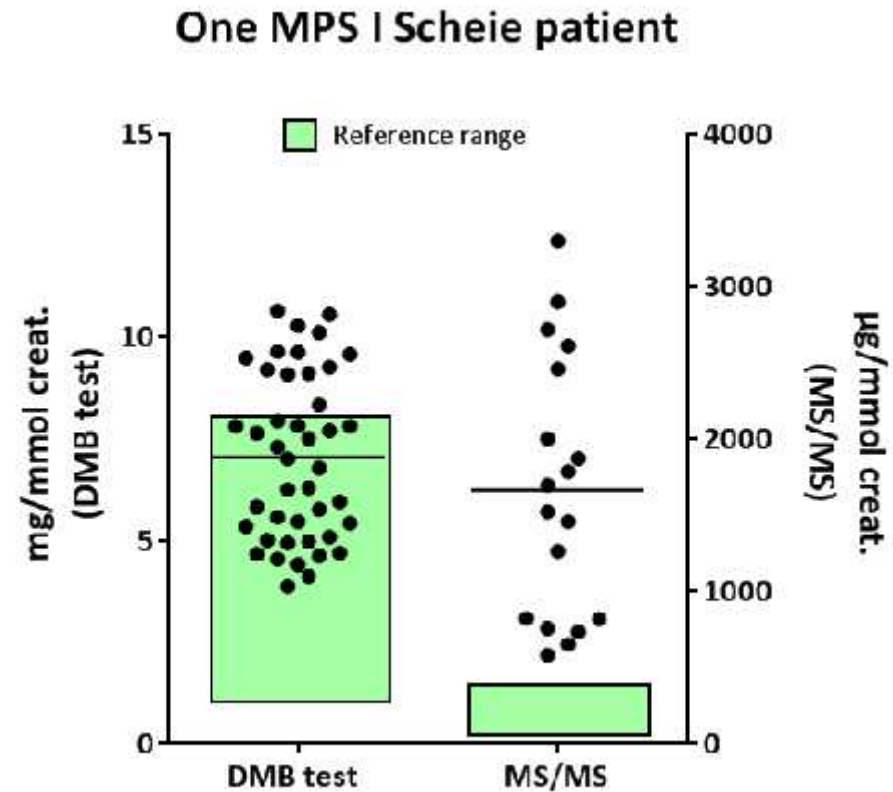
# Clinical validation



# Performance

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- Test is much better, especially for therapy monitoring



# Conclusion

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- Developed screening assay for mucopolysaccharidoses and mucopolipidoses
- Better sensitivity and specificity than DMB-test + replaces GAGs electrophoresis
- Well suited as a first diagnostic test for all MPS subtypes

# Acknowledgements

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## A Multiplex Assay for the Diagnosis of Mucopolysaccharidoses and Mucopolipidoses

Eveline J. Langereis<sup>1</sup>, Tom Wagemans<sup>1,2</sup>, Wim Kulik<sup>2</sup>, Dirk J. Lefeber<sup>3</sup>, Henk van Lenthe<sup>2</sup>,  
Esmee Oussoren<sup>4</sup>, Ans T. van der Ploeg<sup>4</sup>, George J. Ruijter<sup>5</sup>, Ron A. Wevers<sup>3</sup>, Frits  
A. Wijburg<sup>1\*</sup>, Naomi van Vlies<sup>1,2</sup>



Heleen  
ten Brinke

PMID: 26406883

# Questions?

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Thank you for your attention!