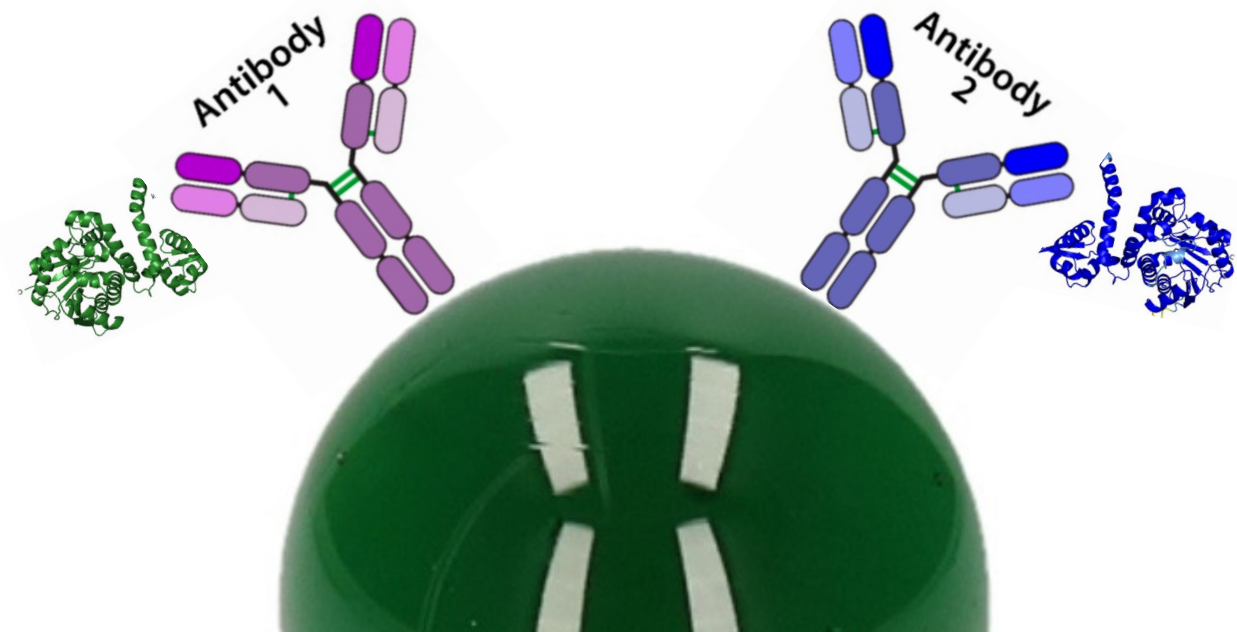


# Simultaneous quantification of protein biomarker isoforms by dual immunocapture and LC-MS/MS

Nico van de Merbel  
15 November 2023





# ACKNOWLEDGEMENT

Bas Sleumer

Rainer Bischoff

Martijn van Faassen

Michel Vos

Ido Kema

Gijs den Besten



university of  
groningen



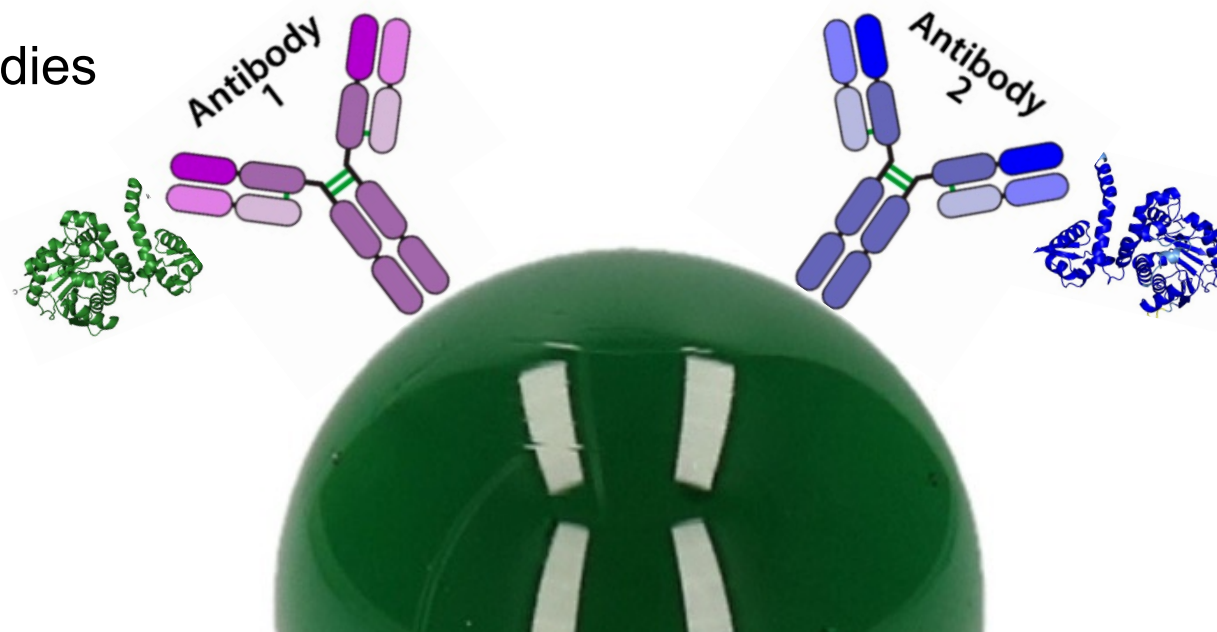
University Medical Center Groningen





# CONTENT

- Biomarker isoforms
  - growth hormone 1 and 2
- Dual immunocapture
  - two isoform-specific capture antibodies
- LC-MS/MS method
  - Special points of attention
- Application
  - Progress of pregnancy

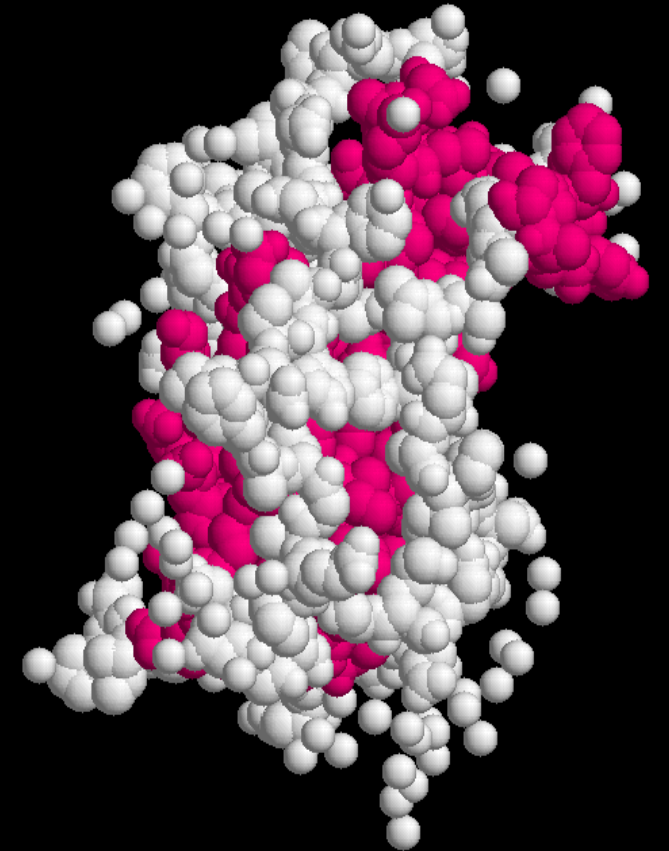




# BIOMARKER ISOFORMS

## Human growth hormone (GH)

- Four main isoforms
  - GH1-22 kDa (pituitary, ~85%) → growth disorders
  - GH1-20 kDa (pituitary, ~10%)
  - GH2-22 kDa (placental) → pregnancy
  - GH2-20 kDa (placental)
- Reference standards differ in composition
- Specificity of LBA reagents generally unknown



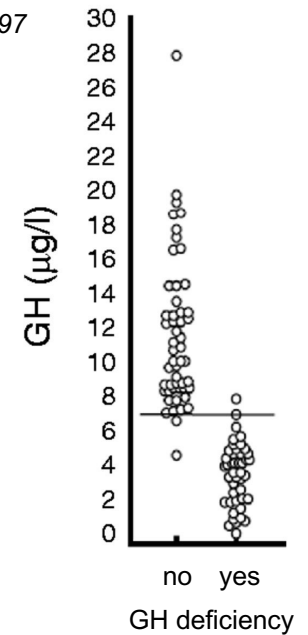
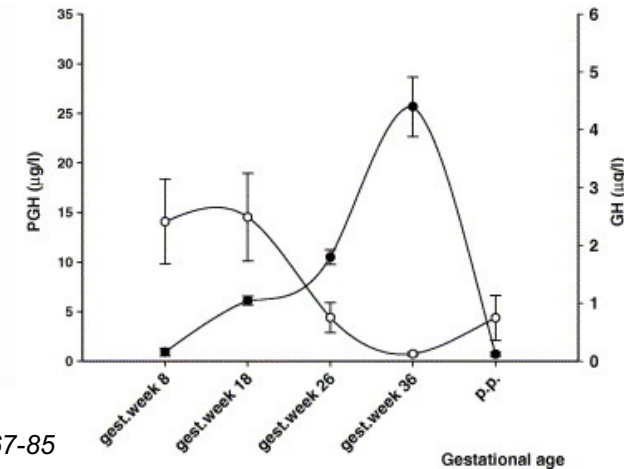


# BIOMARKER ISOFORMS

## Human growth hormone (GH)

- GH1
  - limited comparability of LBA results
- GH2
  - lack of reliable commercial LBAs

*Eur. J. Endocrinol. (2014) 171, 389-397*



*Growth Horm. IGF Res. 16 (2006) 67-85*



# BIOMARKER ISOFORMS

```

FPTIPLSRLFDNAMLRAHRLHQLAF
                                     D
STQPNQLFSYKQEKP IYAE EFEQYT
L
CFSESIPTPSNREETQQKSNLELLR
|
CYLLGYNKLLADDNHSNTDFKS L
F
R
K
D
M
DKVETFLRIVQCRS
|
DLEEGIQTLMGRLEDGSPRT P
K
L
LDYVNSDSAGYVLSNAFVSRLF

```

**GH1-22 kDa**  
(191 amino acids)  
  
childhood/puberty

```

FPTIPLSRLFDNAMLRAHRLHQLAF
                                     D
STQPN-----FEQYT
L
CFSESIPTPSNREETQQKSNLELLR
|
CYLLGYNKLLADDNHSNTDFKS L
F
R
K
D
M
DKVETFLRIVQCRS
|
DLEEGIQTLMGRLEDGSPRT P
K
L
LDYVNSDSAGYVLSNAFVSRLF

```

**GH1-20 kDa**  
(176 amino acids,  
92% similarity)  
  
childhood/puberty

```

FPTIPLSRLFDNAMLRARRRLYQLAY
                                     D
STQPNQLFSYKQEKL IYAE EFEQYT
L
CFSESIPTPSNRVKTQQKSNLELLR
|
CYLLGYNKLLADDNHSKTDFKS L
F
R
K
D
M
DKVETFLRIVQCRS
|
DLEEGIQTLMWRLEDGSPRT P
K
L
HRYVNSDSAGYVLSNAFVSRLF

```

**GH2-22 kDa**  
(191 amino acids,  
93% similarity)  
  
pregnancy

```

FPTIPLSRLFDNAMLRARRRLYQLAY
                                     D
STQPN-----FEQYT
L
CFSESIPTPSNRVKTQQKSNLELLR
|
CYLLGYNKLLADDNHSKTDFKS L
F
R
K
D
M
DKVETFLRIVQCRS
|
DLEEGIQTLMWRLEDGSPRT P
K
L
HRYVNSDSAGYVLSNAFVSRLF

```

**GH2-20 kDa**  
(176 amino acids,  
86% similarity)  
  
pregnancy

```

VQTVPLSRLFDHAMLQAHRAHQLAI
                                     D
STQSDHLFSYKQDKPIYTEEF EQYT
F
CFSDSIPTPSNMEETQQKSNLELLR
|
CYLLGYNKLLADHNHSNTDFKS L
F
R
K
D
M
DKVETFLRMVQCRS
|
DLEEGIQTLMGRLEDGSRRT P
K
L
LHYDDSDSTDYVLNNAFMSRLF

```

**CSH**  
(191 amino acids,  
84% similarity)  
  
pregnancy



# BIOMARKER ISOFORMS

## GH1-22 kDa specific

FPTIPLSRLFDNAMLRAHRL <u>LHQLAF</u>	FPTIPLSRLFDNAMLRAHRLHQLAF
STQPNQLFSYKQEKPIYAEFEFYT	STQPN-----FEQYT
L	L
CFSESIPTPSNREETQQK <u>SNLELLR</u>	CFSESIPTPSNREETQQK <u>SNLELLR</u>
CYLLGYNKLLADDNHSNTDFKS	CYLLGYNKLLADDNHSNTDFKS
F	F
R	R
K	K
D	D
M	M
DKVETFLRIVQCRS	DKVETFLRIVQCRS
DLEEGIQTLMGRLEDGSPRT	DLEEGIQTLMGRLEDGSPRT
K	K
L	L
LDYVNSDSAGYVLSNAFVSRLF	LDYVNSDSAGYVLSNAFVSRLF

**GH1-22 kDa**  
(191 amino acids)  
  
childhood/puberty

## GH2-22 kDa specific

FPTIPLSRLFDNAMLRA <u>RRLYQLAY</u>	FPTIPLSRLFDNAMLRAHRLHQLAF
STQPNQLFSYKQEK <u>LIYAEFEFYT</u>	STQPN-----FEQYT
L	L
CFSESIPTPSNR <u>VKTQQK</u> <u>SNLELLR</u>	CFSESIPTPSNREETQQK <u>SNLELLR</u>
CYLLGYNKLLADDNHS <u>K</u> TDFKS	CYLLGYNKLLADDNHSNTDFKS
F	F
R	R
K	K
D	D
M	M
DKVETFLRIVQCRS	DKVETFLRIVQCRS
DLEEGIQTLM <u>W</u> RLEDGSPRT	DLEEGIQTLMGRLEDGSPRT
K	K
L	L
<u>H</u> RYVNSDSAGYVLSNAFVSRL	LDYVNSDSAGYVLSNAFVSRLF

**GH2-22 kDa**  
(191 amino acids,  
93% similarity)  
  
pregnancy

## total GH specific

FPTIPLSRLFDNAMLRA <u>RRLYQLAY</u>	FPTIPLSRLFDHAMLQAHRHQLAI
STQPNQLFSYKQEK <u>LIYAEFEFYT</u>	STQ <u>SD</u> HLSYKQDKPIYTEEFQYT
L	L
CFSESIPTPSNR <u>VKTQQK</u> <u>SNLELLR</u>	CF <u>S</u> DSIPTPSNMEETQQK <u>SNLELLR</u>
CYLLGYNKLLADDNHS <u>K</u> TDFKS	CYLLGYNKLLAD <u>H</u> NHSNTDFKS
F	F
R	R
K	K
D	D
M	M
DKVETFLRIVQCRS	DKVETFLR <u>M</u> VQCRS
DLEEGIQTLM <u>W</u> RLEDGSPRT	DLEEGIQTLMGRLEDG <u>S</u> RRT
K	K
L	L
<u>H</u> RYVNSDSAGYVLSNAFVSRL	<u>L</u> H <u>Y</u> DDSD <u>S</u> T <u>D</u> YV <u>L</u> N <u>N</u> AF <u>M</u> SRL

**GH2-20 kDa**  
(176 amino acids,  
86% similarity)  
  
pregnancy

**CSH**  
(191 amino acids,  
84% similarity)  
  
pregnancy

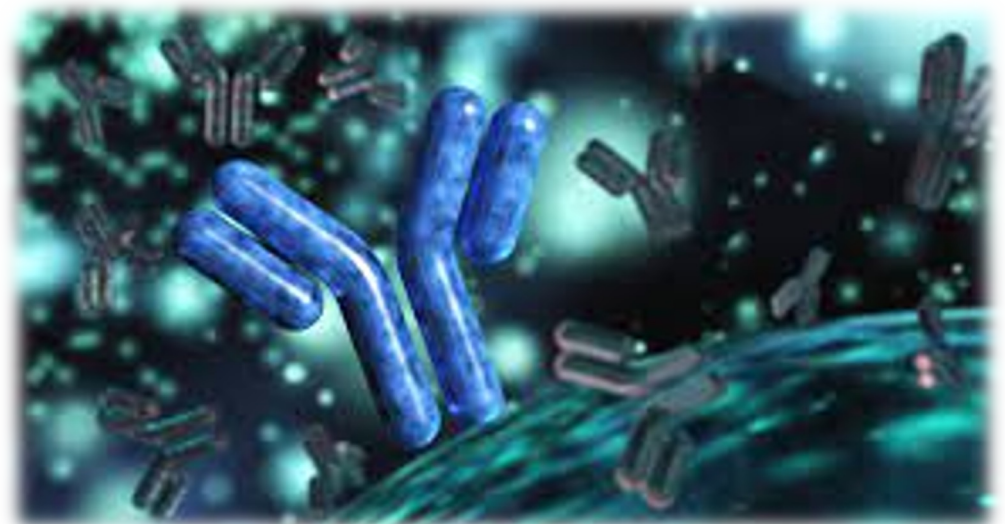


# IMMUNOCAPTURE

## Relevant plasma concentrations:

- GH1-22 kDa: ~0.5-30 ng/mL
- GH2-22 kDa: ~2-50 ng/mL
- total GH: unknown

} immunocapture needed for  
 analyte enrichment

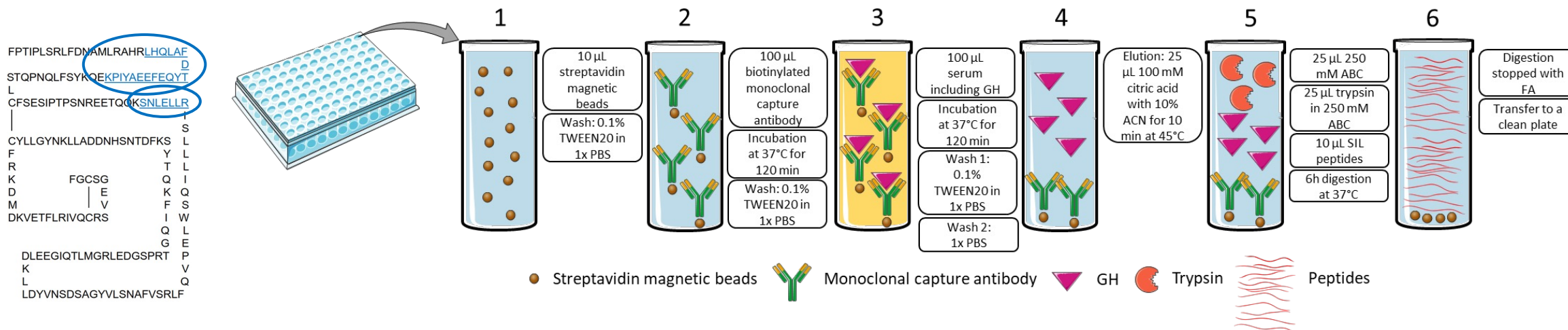




# IMMUNOCAPTURE

## Analytical approach single analyte:

- Reference standard: recombinant human GH1-22 kDa
- Internal standards: two stable isotope labeled signature peptides
- Capture by mouse **anti-human GH** antibody, specificity and binding epitope unknown
- Surrogate matrix: rat plasma





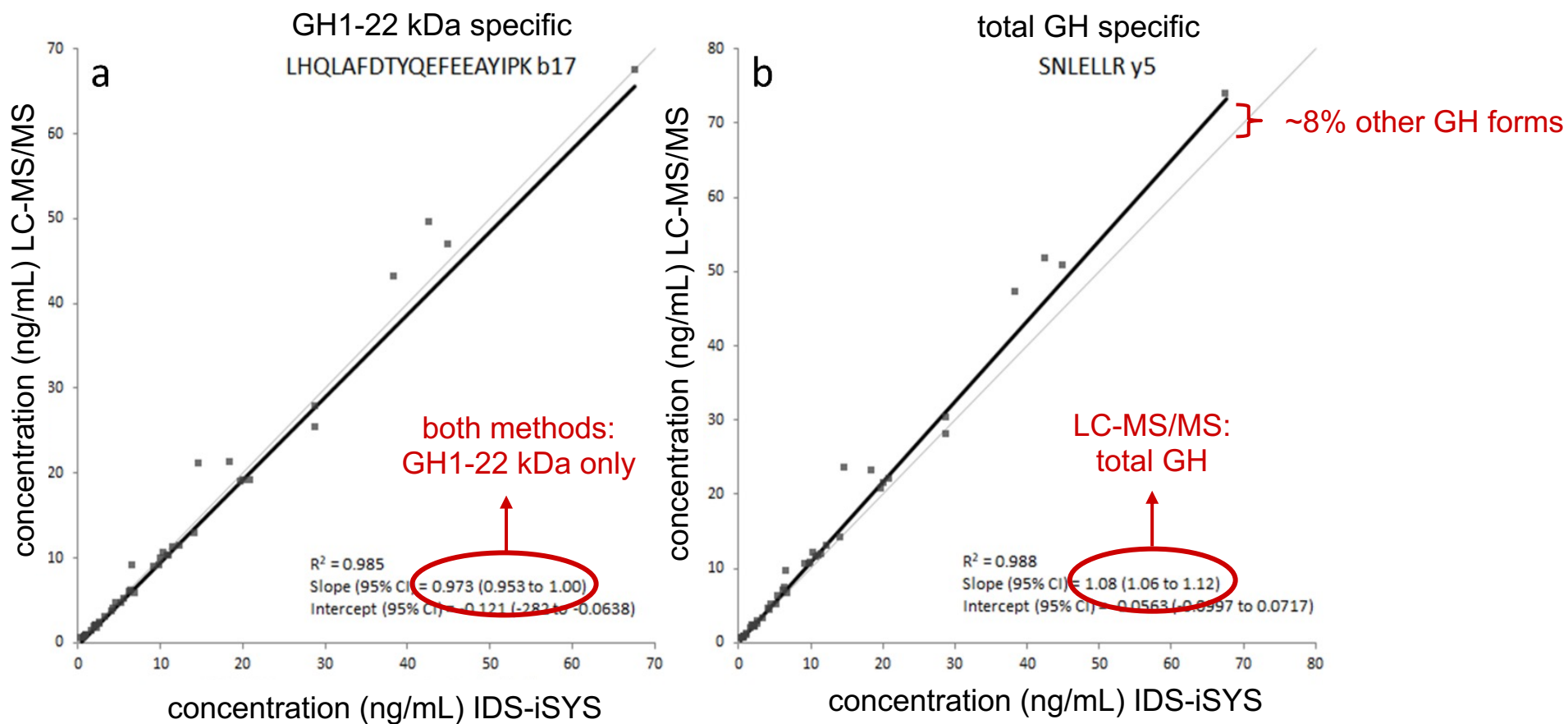
# IMMUNOCAPTURE

## Results single analyte:

- Anti-GH capture antibody shows cross-reactivity
- Extraction recovery GH1-22 kDa: ~70%, reproducible across three batches of capture reagent
- Extraction recovery GH2-22 kDa: ~30-60%, fluctuating across three batches of capture reagent
- Method validated for GH1-22 kDa quantification (monitoring growth disorders and treatment)



# IMMUNOCAPTURE

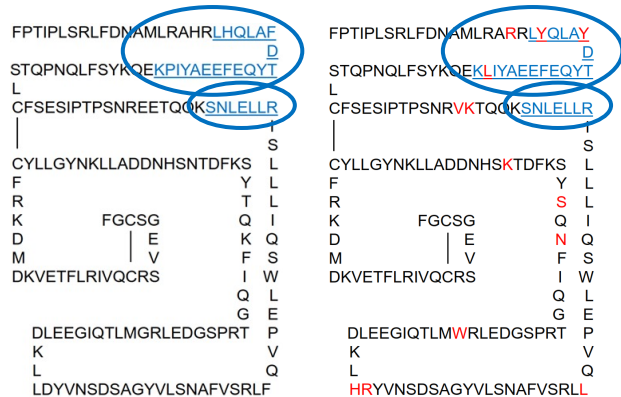




# DUAL IMMUNOCAPTURE

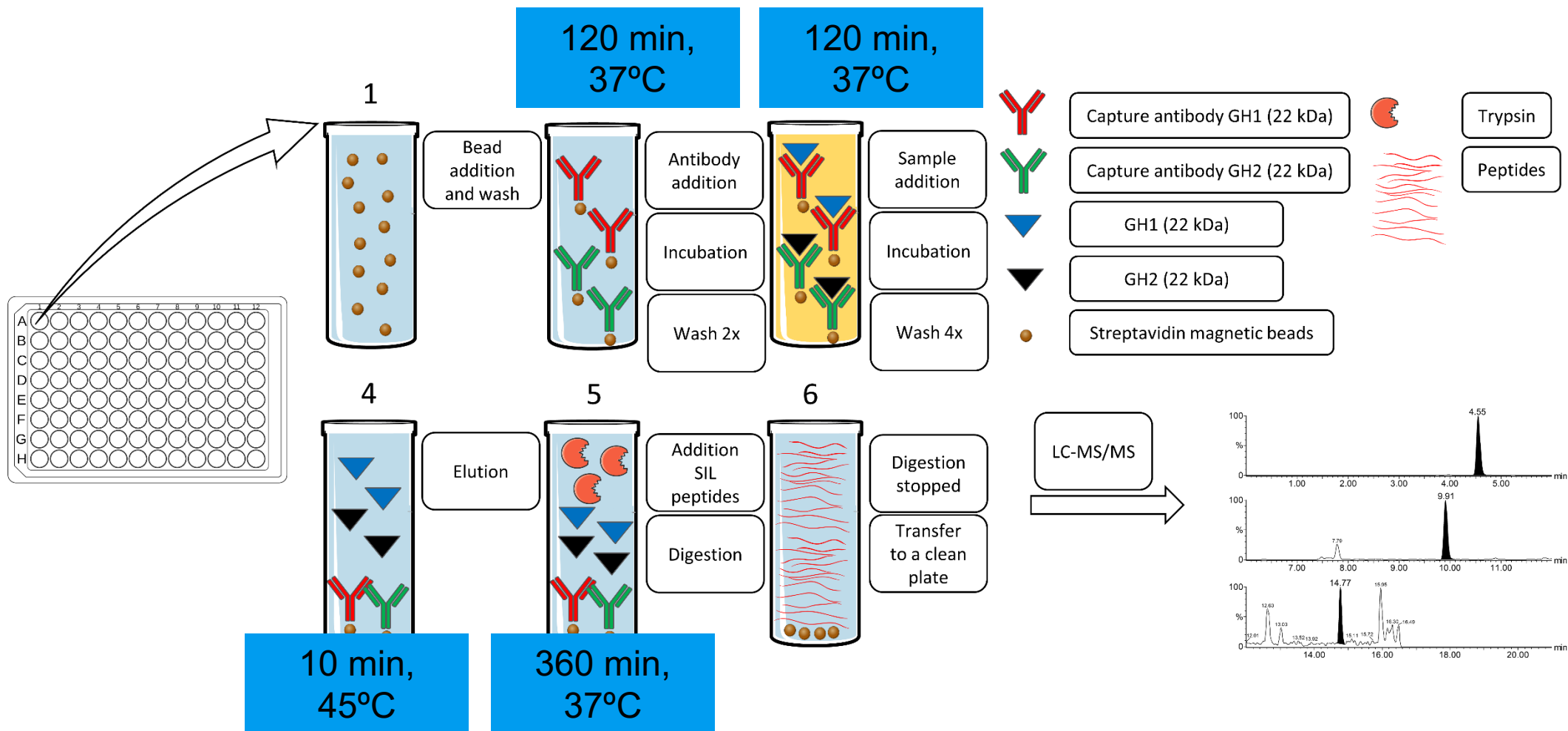
## Analytical approach two analytes:

- Reference standards: recombinant human GH1-22 kDa and GH2-22 kDa
- Internal standards: three stable isotope labeled signature peptides
- Capture by (1) mouse **anti-human GH** antibody, specificity and binding epitope unknown and (2) mouse **anti-human GH2** antibody, no cross-reactivity towards GH1 or CSH
- Surrogate matrix: rat plasma





# DUAL IMMUNOCAPTURE



# DUAL IMMUNOCAPTURE

## Results two analytes:

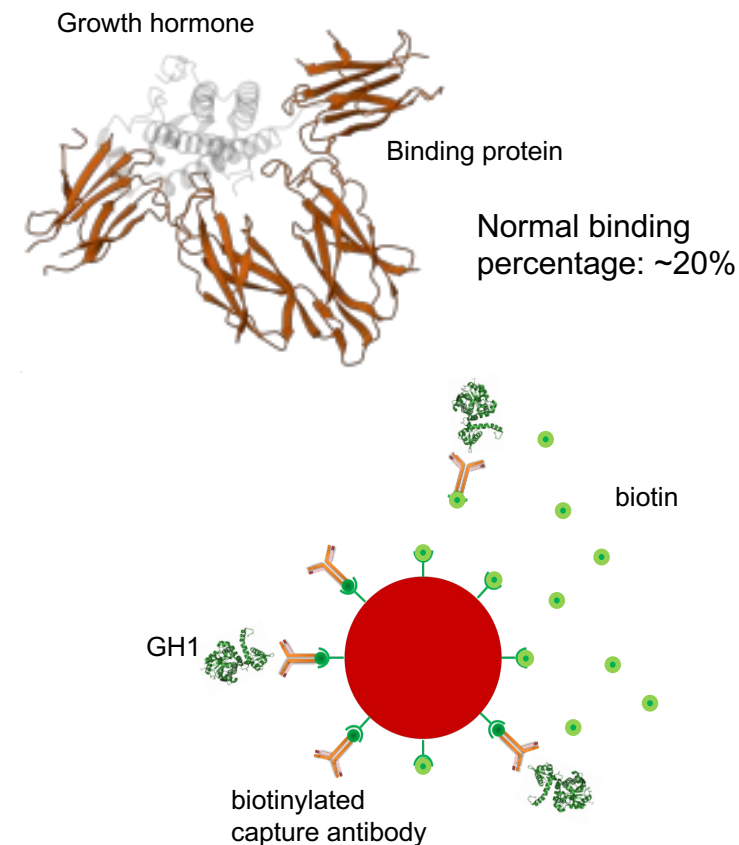
- Extraction recovery GH1-22 kDa: ~70%
- Extraction recovery GH2-22 kDa: ~90%

**GH1**

Interference test for LHQLAFDTYQEFEEAYIPK, n=3				
Concentration (ng/mL)	Low QC (2.37 ng/mL)			
Interference	GHBP (100-fold)	Biotin (750 ng/mL)	Biotin (1000 ng/mL)	Biotin (1500 ng/mL)
Bias (%)	+8.1	+4.2	-14.5	-24.1
CV (%)	1.9	10.8	9.3	2.0

**GH2**

Interference test for LYQLAYDTYQEFEEAYILK, n=3				
Concentration (ng/mL)	Low QC (4.43 ng/mL)			
Interference	GHBP (50-fold)	Biotin (500 ng/mL)	Biotin (1000 ng/mL)	Biotin (1500 ng/mL)
Bias (%)	+0.9	9.6	-0.7	-6.0
CV (%)	0.1	8.6	12.5	13.5



# DUAL IMMUNOCAPTURE

## Results two analytes:

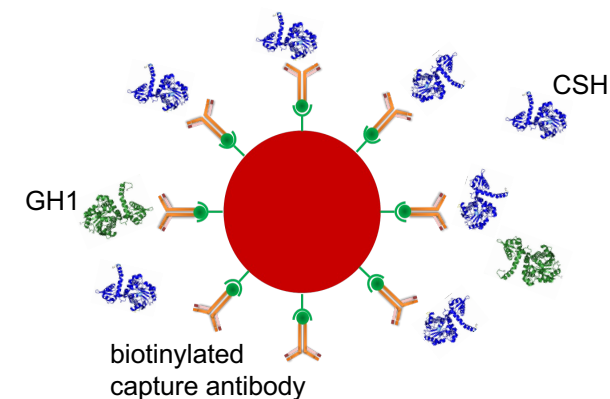
- Extraction recovery GH1-22 kDa: ~70%
- Extraction recovery GH2-22 kDa: ~90%

GH1

Interference test for LHQLAFDTYQEFEEAYIPK, n=3				
Concentration (ng/mL)	Low QC (2.37 ng/mL)		Low QC (1.50 ng/mL)	
Interference	GH2-22 kDa (35 ng/mL)	20 kDa forms (6 ng/mL)	CSH (~100 ng/mL)	
Bias (%)	+6.3	+6.4	-19.8	
CV (%)	2.1	3.2	7.6	

GH2

Interference test for LYQLAYDTYQEFEEAYILK, n=3				
Concentration (ng/mL)	Low QC (4.43 ng/mL)		Low QC (5.00 ng/mL)	
Interference	GH1-22 kDa (35 ng/mL)	20 kDa forms (6 ng/mL)	CSH (~20 ng/mL)	
Bias (%)	-3.3	+3.0	+15.0	
CV (%)	5.7	8.8	10.2	





# LC-MS/MS METHOD

## Concentration of stock solution

Reference standards typically provided as lyophilized dry powder (down to low  $\mu\text{g}$  quantities)

Confirm concentration by reconstitution, digestion and comparison to equimolar amount of signature peptide

GH1-22 kDa:

- WHO standard, lyophilized aqueous solution containing 1.95 mg GH, 20 mg glycine, 2 mg mannitol, 2 mg lactose, 2.5 mg sodium bicarbonate
- Advice: redissolve content of vial in suitable solution, for low concentrations include carrier protein
- Reconstitution in 1.95 mL of water ( $\rightarrow$  1 mg/mL) gives **100% of label claim**







# LC-MS/MS METHOD

## Concentration of stock solution

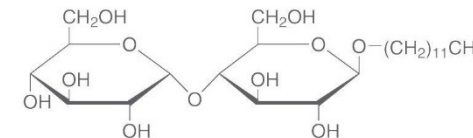
Reference standards typically provided as lyophilized dry powder (down to low  $\mu\text{g}$  quantities)

Confirm concentration by reconstitution, digestion and comparison to equimolar amount of signature peptide

GH2-22 kDa:

- Commercial provider, lyophilized aqueous sodium bicarbonate solution pH8-9, containing 50  $\mu\text{g}$  GH
- Advice: redissolve content of vial in 0.4%  $\text{NaHCO}_3$  or water pH 8-9, not below 100  $\mu\text{g}/\text{mL}$
- Reconstitution in 0.5 mL of water pH 9 ( $\rightarrow$  100  $\mu\text{g}/\text{mL}$ ) gives **<50% of label claim**
- Reconstitution in 0.5 mL of water containing 0.1% BSA and 0.1% DDM ( $\rightarrow$  100  $\mu\text{g}/\text{mL}$ ) gives **100% of label claim**

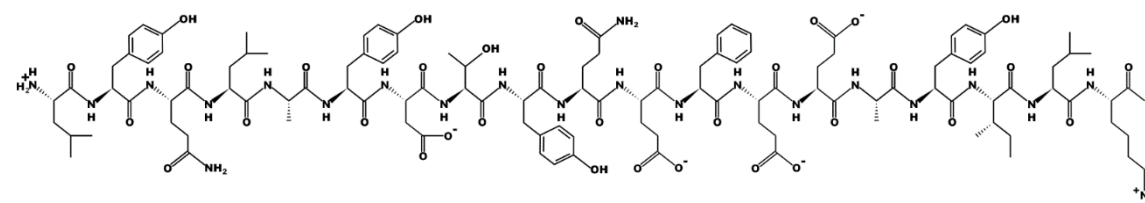
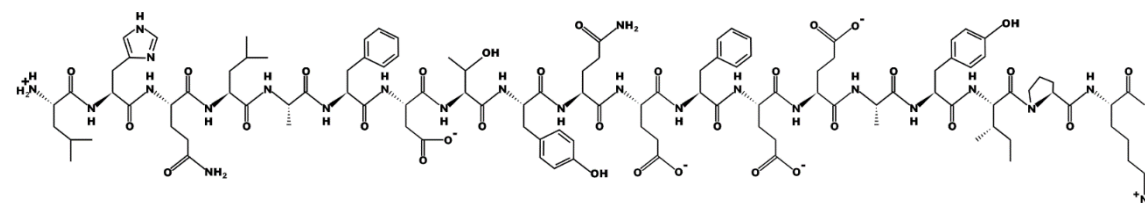
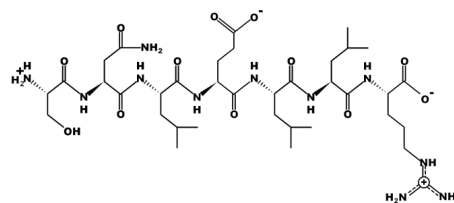
Dodecyl- $\beta$ -D-maltoside (DDM)



# LC-MS/MS METHOD

## Signature peptides:

- total GH: SNLELLR
  - m/z: 422.7 [M+2H]<sup>2+</sup> > 530.3 (y<sub>4</sub><sup>+</sup>)
  - pl: 7.0; hydrophobicity: 26.3
- GH1-22 kDa: LHQLAFDTYQEFEEAYIPK
  - m/z: 781.4 [M+3H]<sup>3+</sup> > 993.4 (b<sub>16</sub><sup>2+</sup>)
  - pl: 4.2; hydrophobicity: 44.7
- GH2-22 kDa: LYQLAYDTYQEFEEAYILK
  - m/z: 800.7 [M+3H]<sup>3+</sup> > 824.9 (y<sub>13</sub><sup>2+</sup>)
  - pl: 3.7; hydrophobicity: 48.9



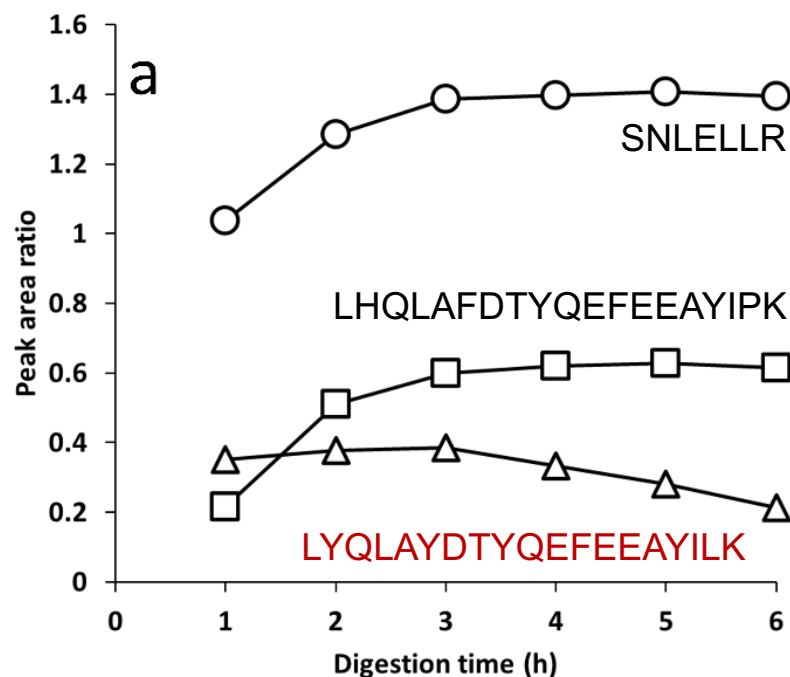




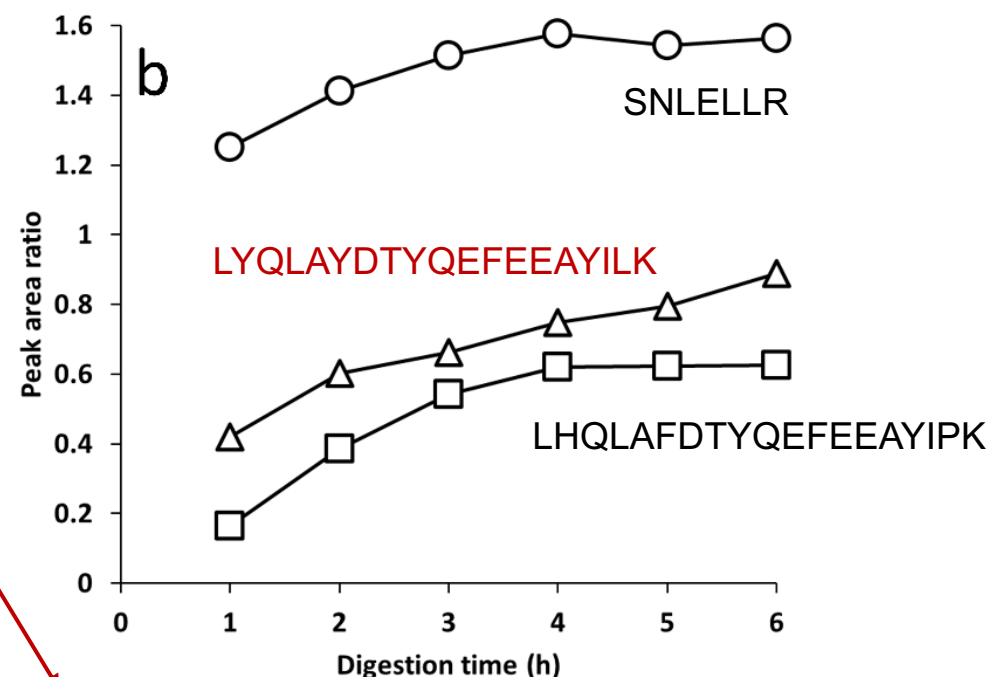
# LC-MS/MS METHOD

## Digestion:

crude trypsin (16  $\mu\text{g/mL}$ )



proteomics-grade trypsin (32  $\mu\text{g/mL}$ )



**residual chymotrypsin activity**



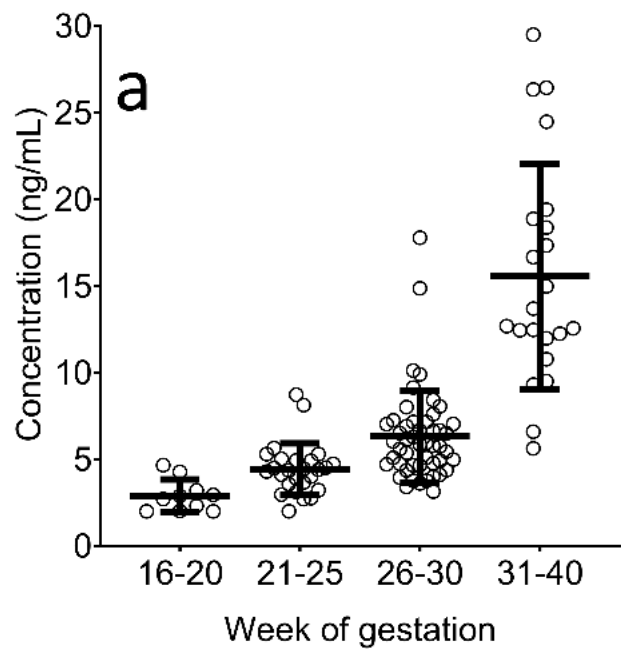
# APPLICATION

## Sample set:

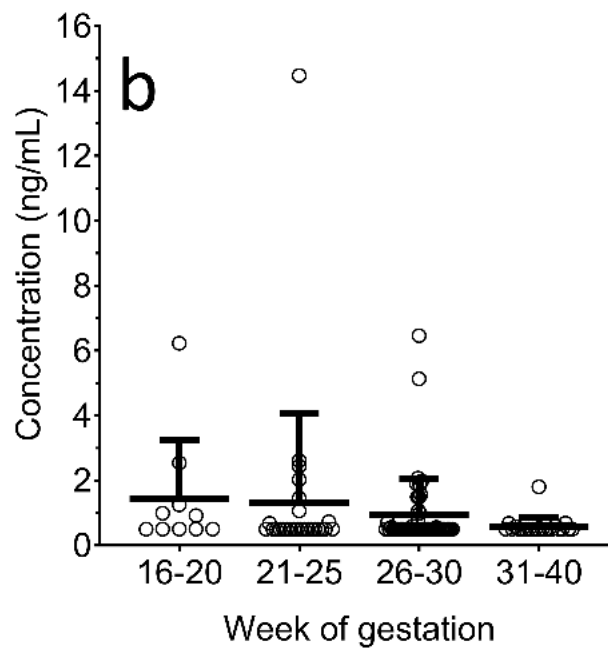
- 107 plasma samples from pregnant individuals (weeks of gestation: 16-37)
- GH2-22 kDa levels (based on peptide LYQLAYDTYQEFEEAYILK) **increase during pregnancy:**
  - average of 3 ng/mL in weeks 16-20 of pregnancy
  - average of 15 ng/mL in weeks 31-40 of pregnancy
- GH1-22 kDa levels (based on peptide LHQLAFDTYQEFEEAYIPK) **almost completely suppressed:**
  - 50% have quantifiable (>0.5 ng/mL) levels in weeks 16-20 of pregnancy
  - 20% have quantifiable (>0.5 ng/mL) levels in weeks 31-40 of pregnancy
- total GH levels (based on peptide SNLELLR) also estimated to **increase during pregnancy:**
  - average of 10 ng/mL in weeks 16-20 of pregnancy
  - average of 60 ng/mL in weeks 31-40 of pregnancy



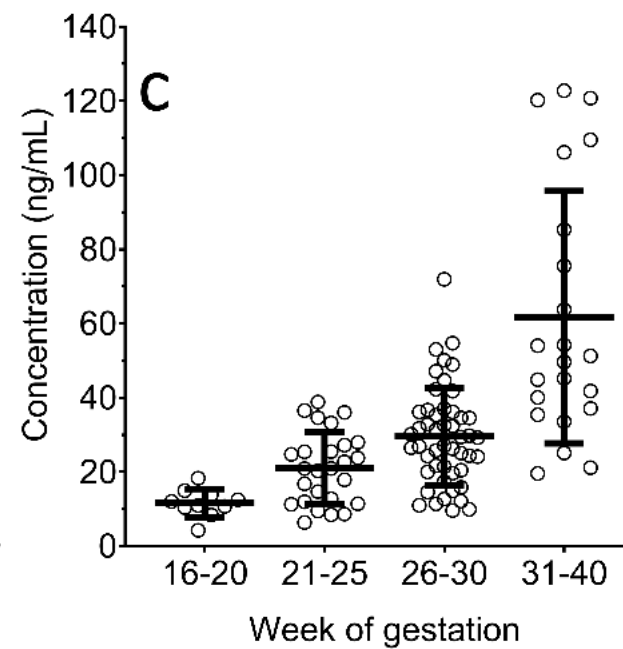
# APPLICATION



GH2-22 kDa



GH1-22 kDa



total GH



# CONCLUSION

- Simultaneous quantification of low to sub-ng/mL plasma levels **protein biomarkers isoforms** of high structural similarity can be achieved
- By the combination of **multiplexed immunocapture**, enzymatic digestion and LC-MS/MS determination of an isoform-specific signature peptide for each isoform
- Exemplified by human **growth hormone** isoforms GH1-22 kDa and GH2-22 kDa (93% similarity) with an additional readout for total GH
- Next to other method performance parameters, it is essential to confirm the **absence of interference** of other isoforms, binding protein(s) and biotin

