HPLC/MS/MS-based SRM for biomarkers of rhGH administration

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Background

- Initial biomarker discovery project
  - LC-MS of proteolytic peptides
  - Simple extraction / enrichment (crash)
  - LRG as upregulated following GH admin
  - Extended to multiplexed semi-quant analysis (50 proteins)

- Multiplexed quant analysis of GH markers possible?

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GH Biomarker targets

- IGF's (IGF-I, IGF-II, etc)
- IGF binding proteins

- LRG (leucine-rich α-2-glycoprotein)
- PIIINP

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MS approaches

- Selective
- Provide confirmatory quality data
- Ability to multiplex?
  - Minimal sample usage
- Eliminate biological reagents?
- Intact protein or proteotypic peptide?

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Analytical requirements

- Efficient (and reproducible) extraction / isolation
- Reproducible digestion
- Effective internal standard (heavy label)

- Issues
  - Binding (both selective and non-selective)
  - Blank matrix (true blank or standard addition)
  - Reference materials
  - Incurred matrices (QC's)

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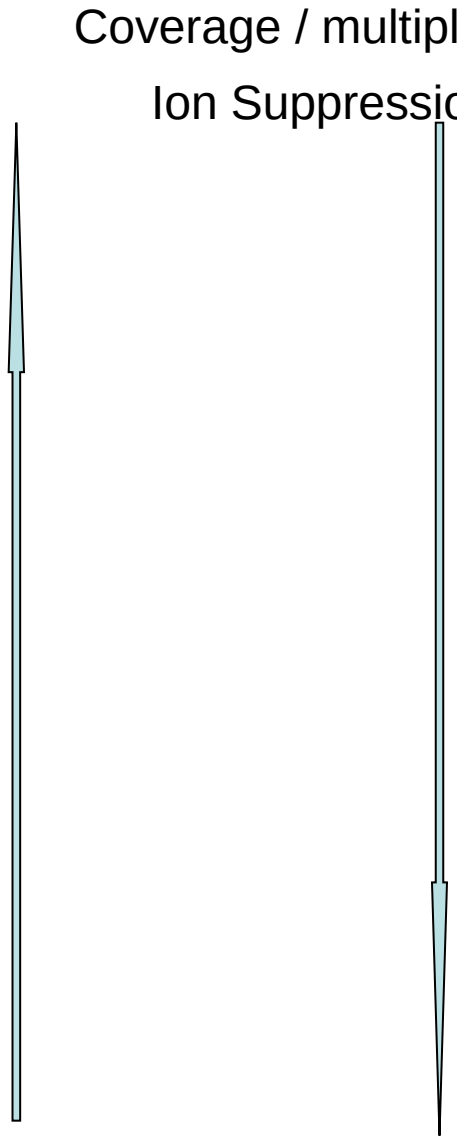

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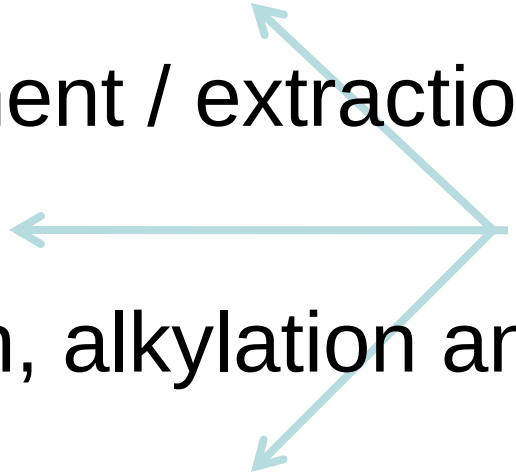
Generic approach

- Pretreatment / extraction
- Reduction, alkylation and digestion
- Quench / dilute and inject
- Integrate and quantitate



Heavy label internal standard

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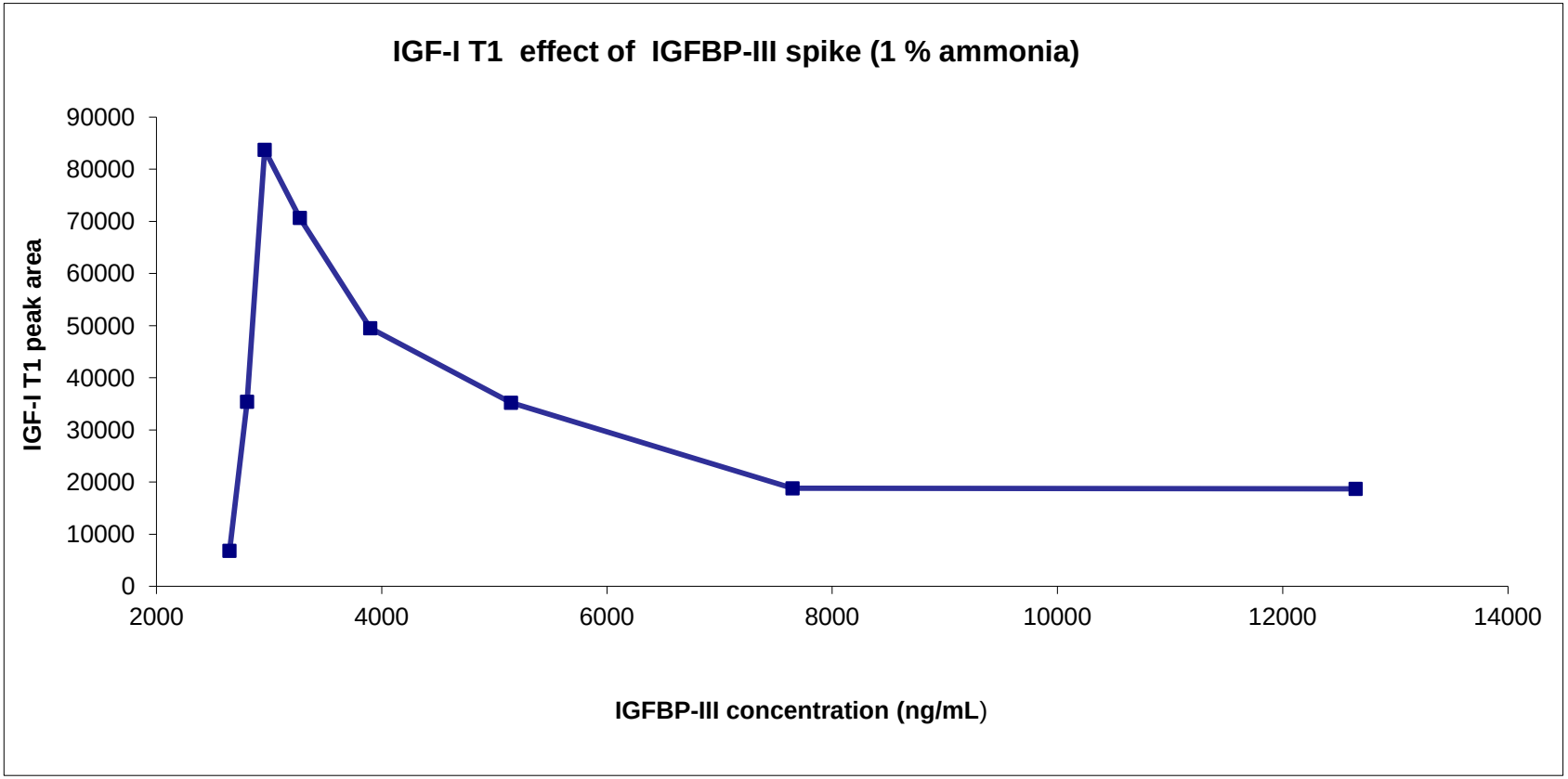
Extraction methods and the effect of binding



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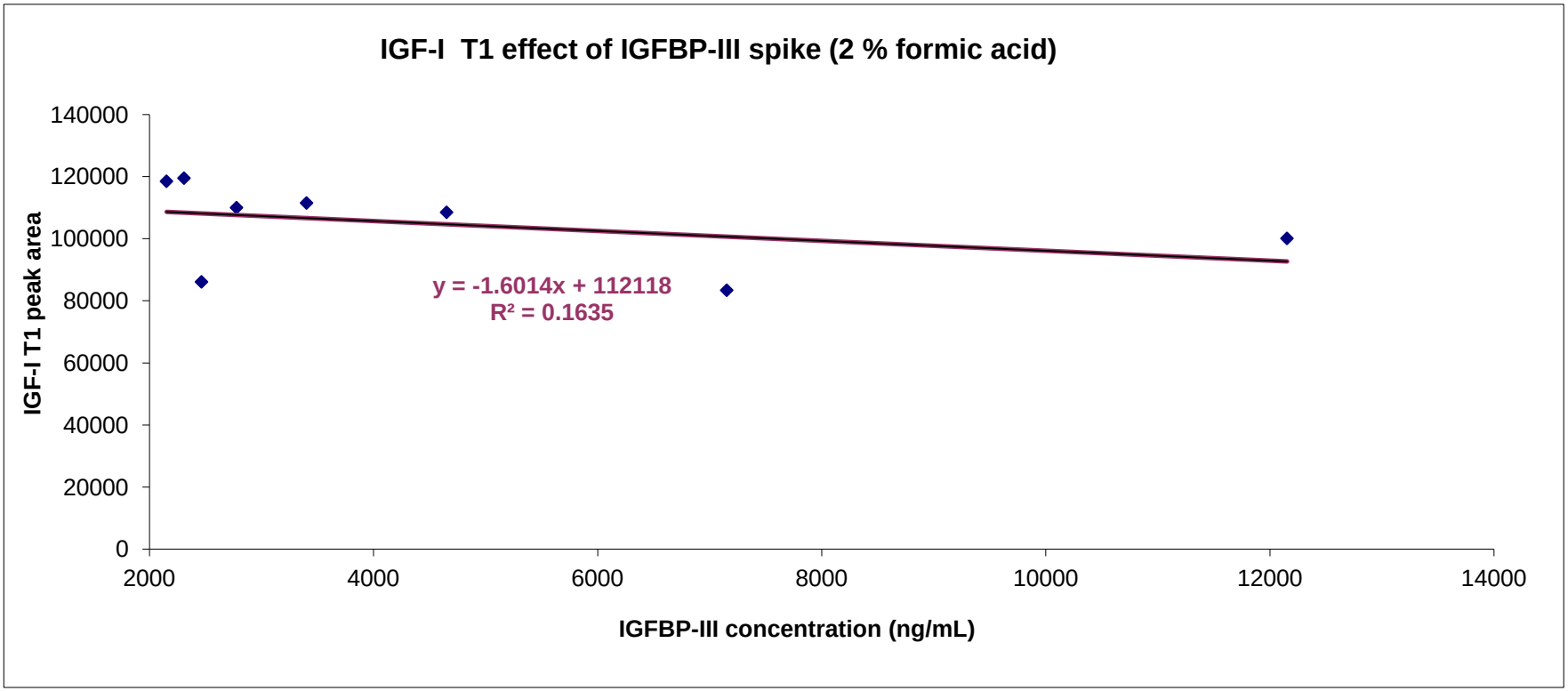
Extraction methods and the effect of binding



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Bond Elut Plexa polymeric restricted access cartridge

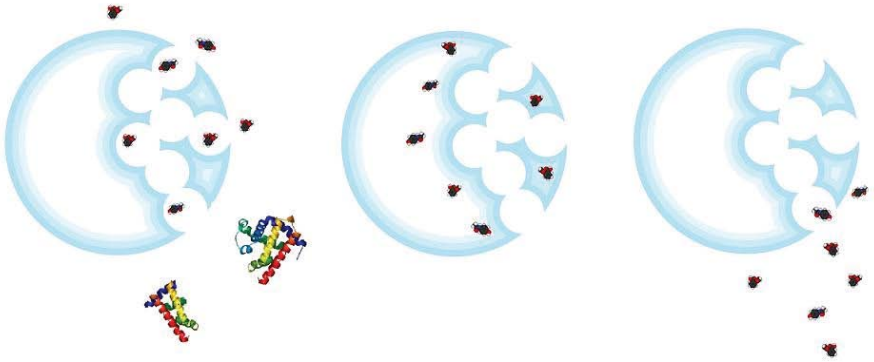
Acidic loading for IGFs (2 % formic acid)

- Breaks IGF=IGFBP interaction
- Optimal for LRG

Basic loading for IGFBPs (0.5 % ammonia)

Wash 1mL 5% acetonitrile

Elute 2 x 250mL 0.1% formic acid, 75% acetonitrile



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Incubation of sample (50µL) at appropriate pH

RT, 10 min

SPE extraction on Bond Elut Plexa 96 well plate

Evaporation to dryness overnight

Addition of 50 µL dithiothreitol (10 mM) in ammonium bicarbonate

60 °C, 1 h

Addition of 10 µL iodoacetamide (100 mM) in ammonium bicarbonate

RT, 30 min

Addition of 7.5 µL trypsin (100 µg/mL) in 50 mM acetic acid

37 °C, overnight

Addition of 15 µL formic acid (1 % v/v) in methanol

Injection (20 µL) onto LC-MS/MS system

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Why uHPLC

- High separation power (appropriate gradient)
- Fast, well suited to quantification
- Robust
- Widely employed / available
- Sensitive?

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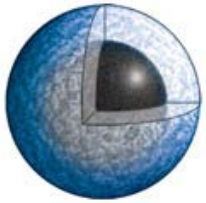
Chromatographic conditions

- 100 x 2.1 mm 1.7 µm BEH C18 or
- Ascentis Express™ C_{18} 100 x 2.1 mm, 2.7 µm fused core column



- Acetonitrile / formic acid gradient – column 50-60 ^{o}C, 700µl/min
- Sciex 5000 / 5500 Turbo V™ source

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LLOQ in plasma

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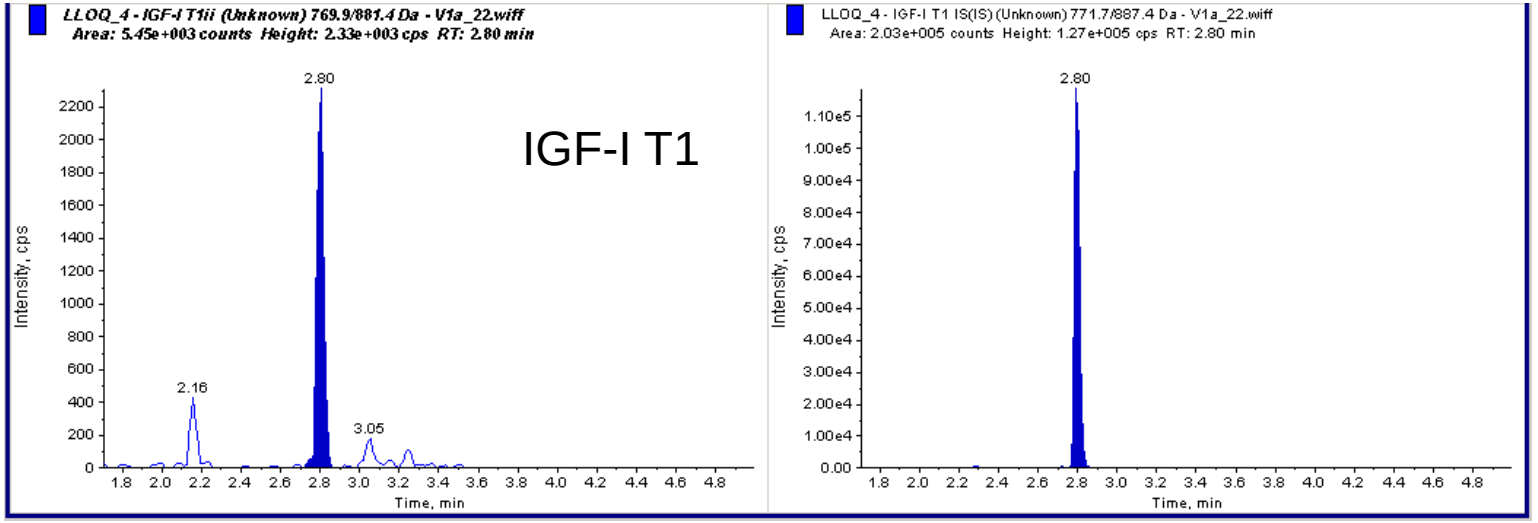

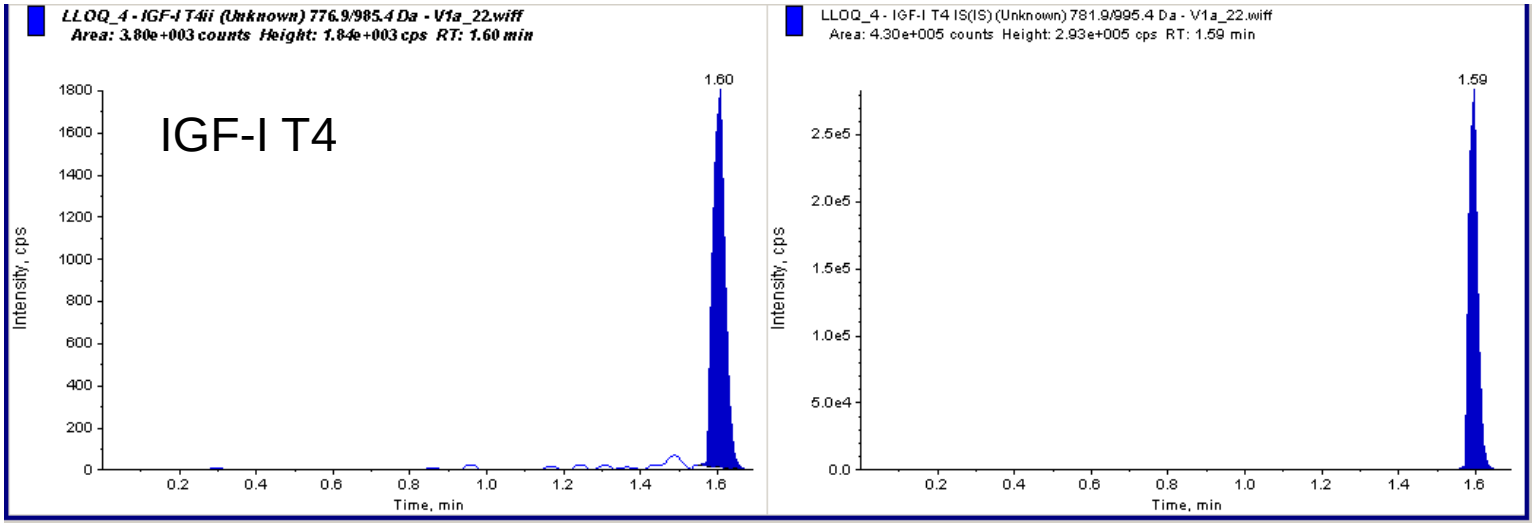

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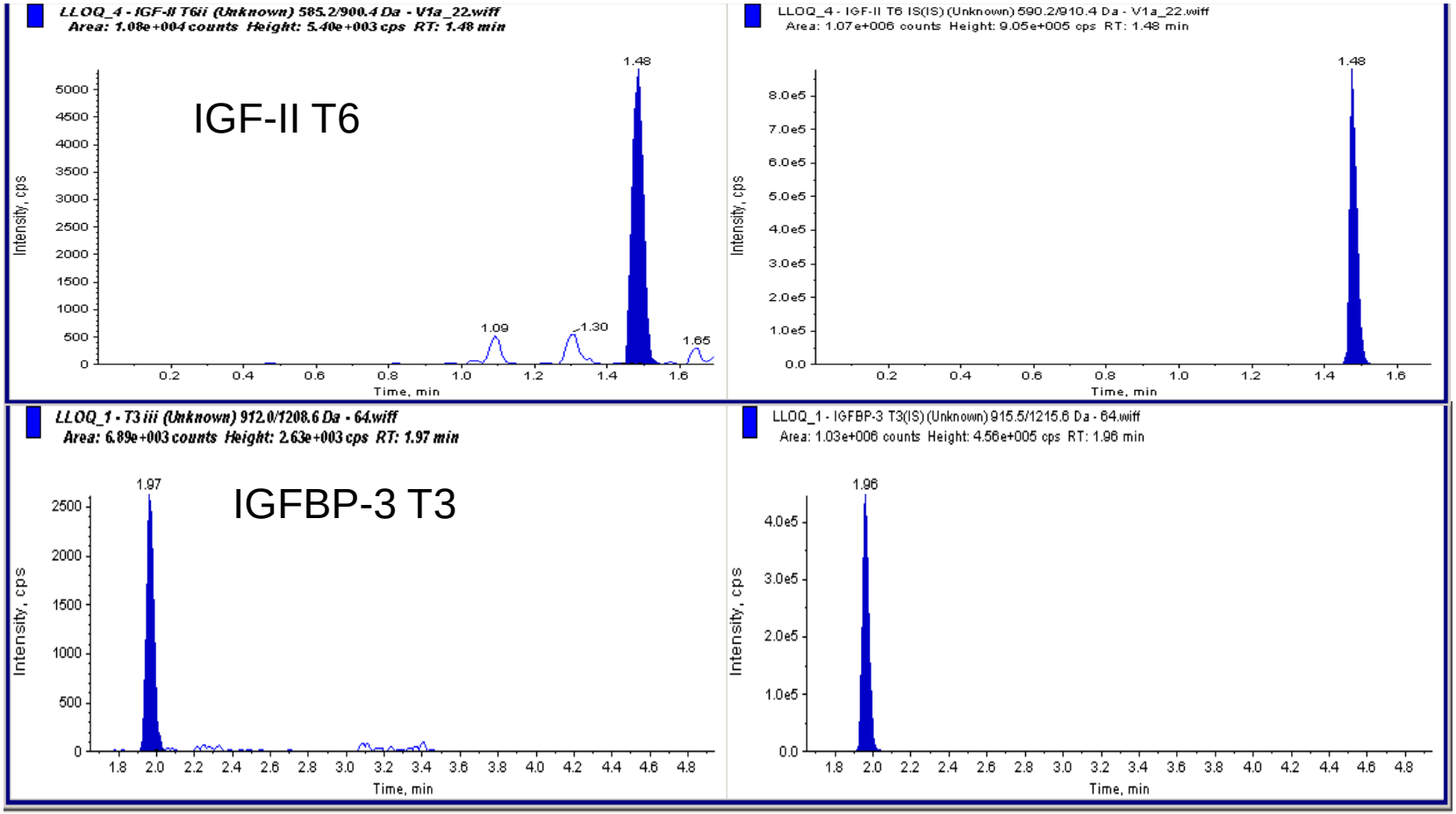
LLOQ in plasma



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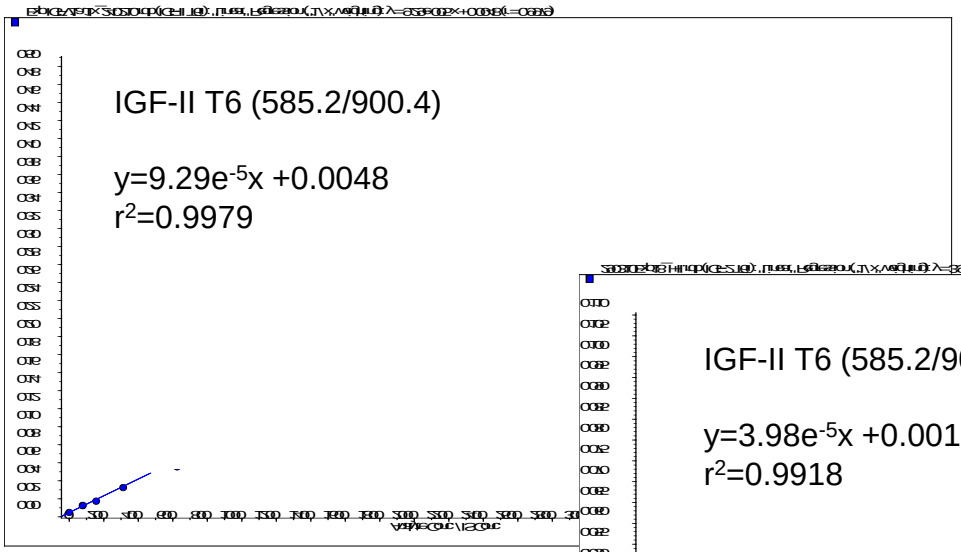
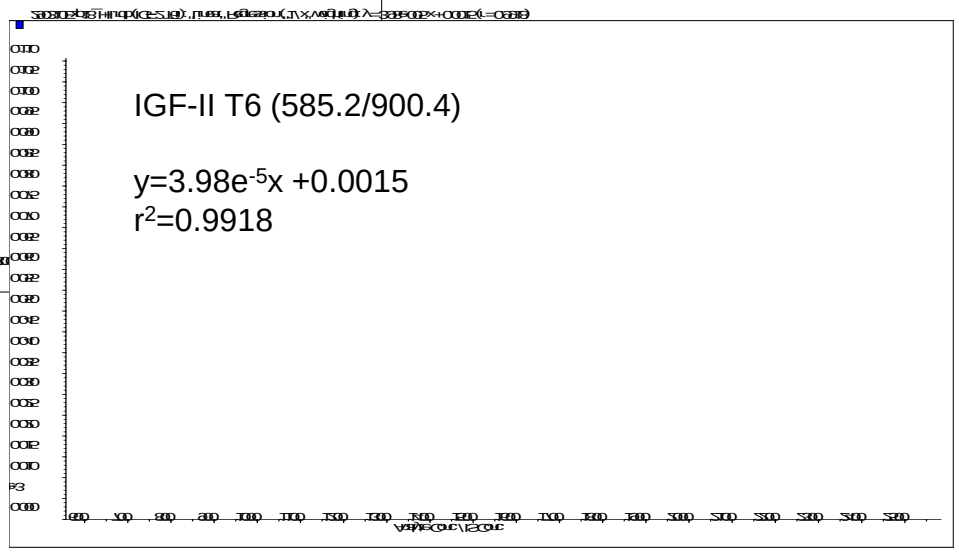
IGF-II (T6) in buffer and plasma



IGF-II T6 (585.2/900.4)

$y=9.29e^{-5}x +0.0048$

$r^2=0.9979$



IGF-II T6 (585.2/900.4)

$y=3.98e^{-5}x +0.0015$

$r^2=0.9918$

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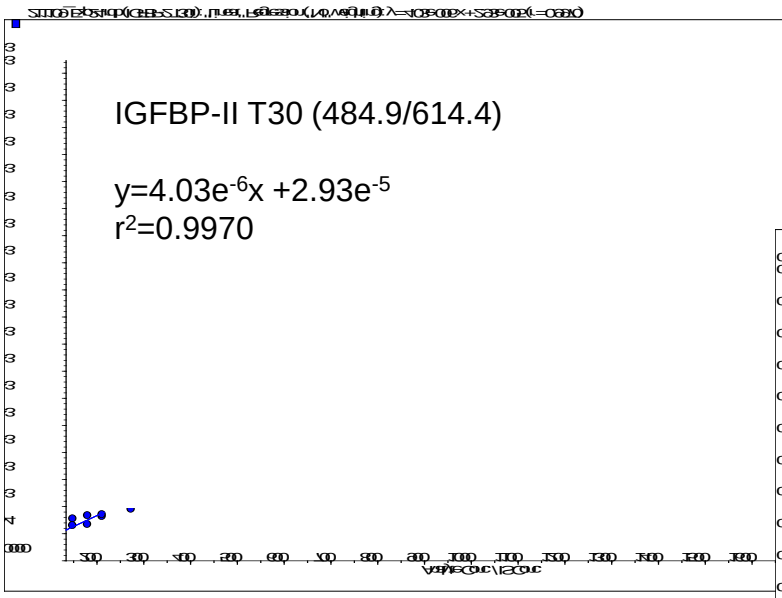
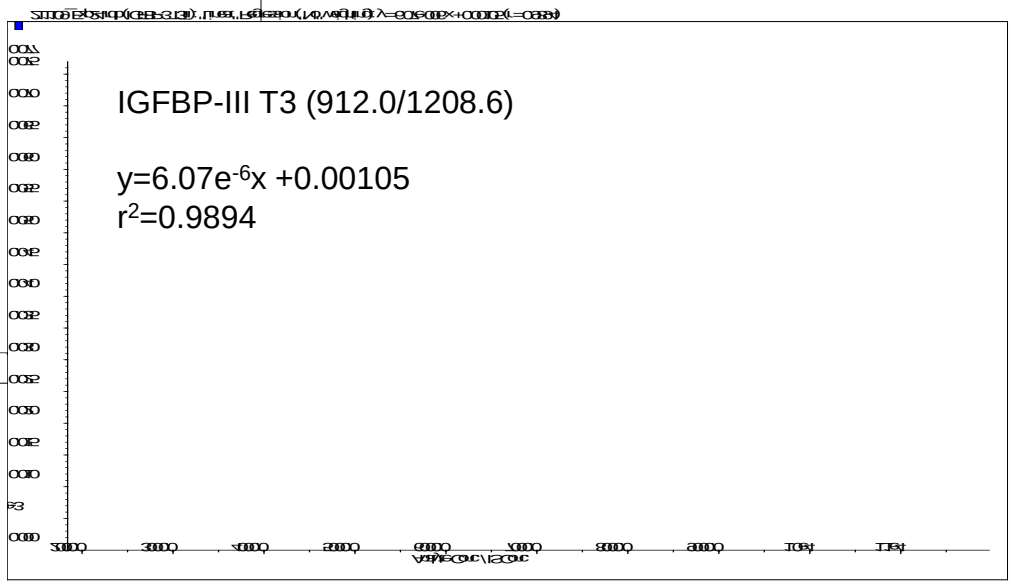
IGFBP-II (T30) IGFBP-III (T3) in plasma



IGFBP-II T30 (484.9/614.4)

$y=4.03e^{-6}x +2.93e^{-5}$

$r^2=0.9970$



IGFBP-III T3 (912.0/1208.6)

$y=6.07e^{-6}x +0.00105$

$r^2=0.9894$

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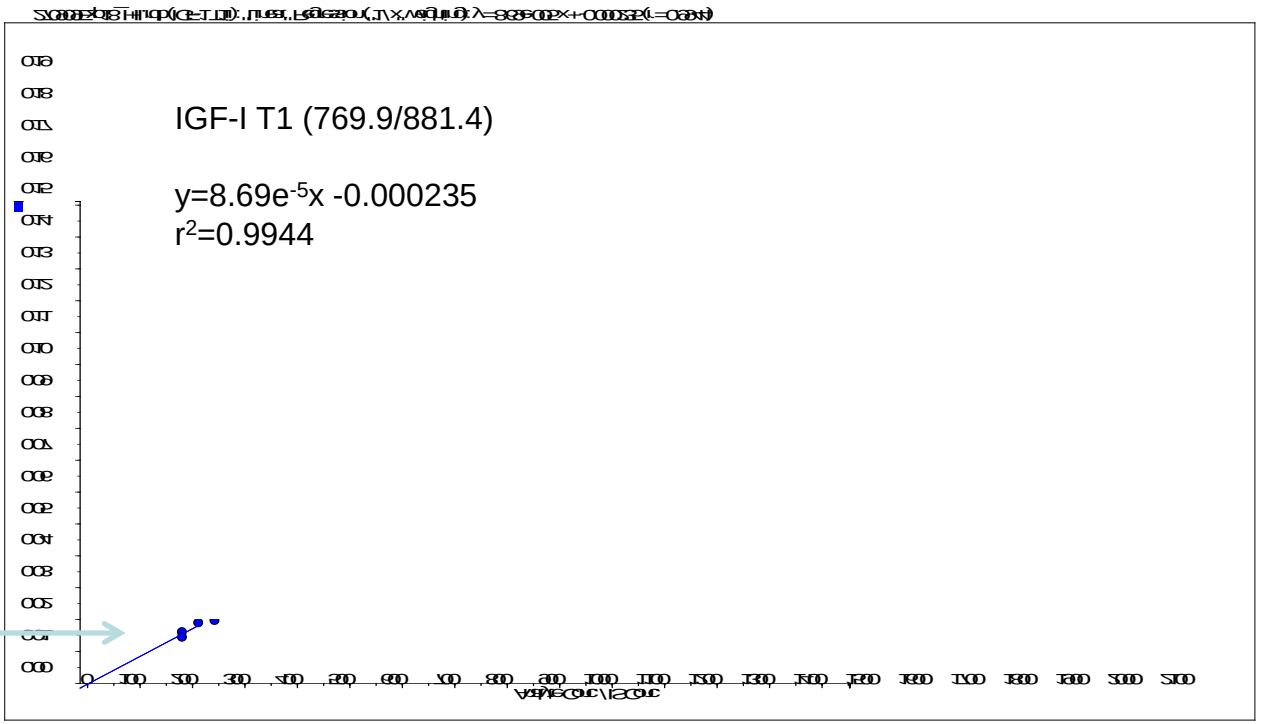
IGF-I (T1) in plasma

Incurred level corrected



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Clinical serum samples

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- Addenbrookes patient samples
- Monitored T1 and T4 peptide surrogates of IGF-I
- Good correlation between methodologies

Reference matrix	Immulite™ (ng/mL)	LC-MS/MS (ng/mL)	RE (%)
Plasma	119	117	1.7
Serum 1	171	172	0.6
Serum 2	346	336	2.9

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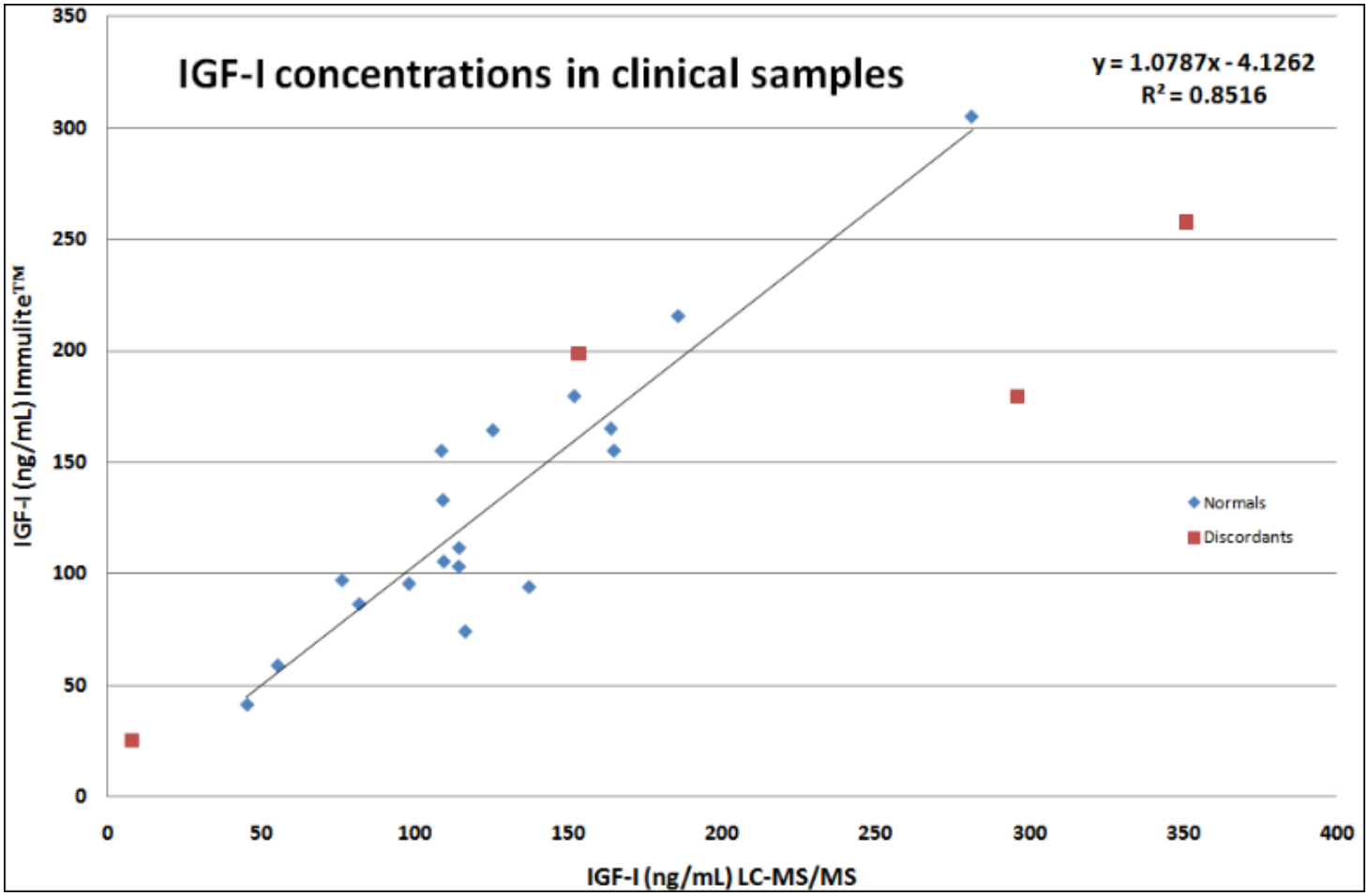
Clinical serum samples



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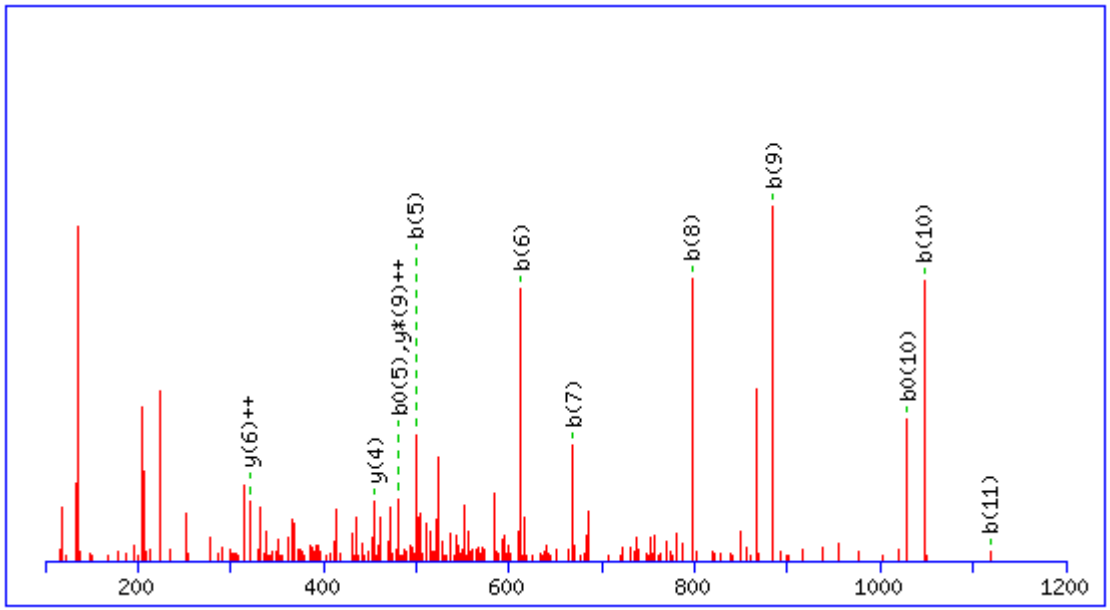
PIIINP



No suitable tryptic fragment

42 kDa

Low levels in plasma (~ 3 ng/mL)

Possible target - endoproteinase Glu-C G3 peptide GGCSHLGQSYAD

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Summary

- LC-MS quantification of IGF's and IGFBP's √
- Generic extraction and uHPLC √
- Multiplex analysis?
- Confirmatory quality data √
- Powerful tool to validate immunoassays √
- Transferable?
- Worthy of further investigation

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