QUOTIENT BIORESEARCH

Development of an LC-MS/MS method* to quantify plasma concentrations of the wild type and Marburg I variants of Factor VII-activating protease

Richard Kay

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*Under feasibility evaluation. Not available for sale

Overview

- Who is Quotient Bioresearch?
- What is Factor VII-activating protease (FSAP)?
- Development of an LC-MS/MS (SRM) method for both FSAP variants (WT and MRI) in human plasma.
- Analysis of 127 plasma samples for FSAP and MRI.
- Future method developments.

Quotient Bioresearch

FSAP (WT and MRI)

- A plasma serine-protease.
  - Activates coagulation factor VII
- Accumulates in unstable atherosclerotic plaques
  - thought to be involved in their destabilisation and rupture
- Studies have shown correlation to atherosclerotic / cardiovascular diseases
- 537 aa, 60 kDa protein
- Present in plasma at ~12 µg/mL
  - Should be detectable in plasma with no extraction
- Marburg I (MRI) mutant (4 – 9 % prevalence of a heterozygous genotype in Caucasian populations)
  - Has lower protease activity than WT protein
- Single amino acid difference between MRI and WT proteins

Spot the difference

WILD TYPE

FSLMSLLESLDPDWTPDQYDYSYEDYNQEEN
TSSTLTHAENPDWYYTEDQADPCQPNPCEHG
GDCLVHGSTFTCSCLAPFSGNKCQKVQNTCK
DNPCGRGQCLITQSPPYYRCVCKHPYTGPSC
SQVVPVCRPNPCQNGATCSRHKRRSKFTCAC
PDQFKGKFCEIGSDDCYVGDGYSYRGKMNRT
VNQHACLYWNSHLLLQENYNMFMEDAETHGI
GEHNFCRNPDADEKPWCFIKVTNDKVKWEYC
DVSACSAQDVAYPEESPTEPSTKLPGFDSCG
KTEIAERKIKRIYGGFKSTAGKHPWQASLQS
SLPLTISMPQGHFCGGALIHPCWVLTAAHCT
DIKTRHLKVVLGDQDLKKEEFHEQSFRVEKI
FKYSHYNERDEIPHNDIALLKLKPVDGHCAL
ESKYVKTVCLPDGSFPSGSECHISGWGVTET
GKGSRQLLDAKVKLIANTLCNSRQLYDHMID
DSMICAGNLQKPGQDTCQGDSGGPLTCEKDG
TYYVYGIVSWGLECGKRPGVYTQVTKFLNWI
KATIKSESGF

MRI

FSLMSLLESLDPDWTPDQYDYSYEDYNQEEN
TSSTLTHAENPDWYYTEDQADPCQPNPCEHG
GDCLVHGSTFTCSCLAPFSGNKCQKVQNTCK
DNPCGRGQCLITQSPPYYRCVCKHPYTGPSC
SQVVPVCRPNPCQNGATCSRHKRRSKFTCAC
PDQFKGKFCEIGSDDCYVGDGYSYRGKMNRT
VNQHACLYWNSHLLLQENYNMFMEDAETHGI
GEHNFCRNPDADEKPWCFIKVTNDKVKWEYC
DVSACSAQDVAYPEESPTEPSTKLPGFDSCG
KTEIAERKIKRIYGGFKSTAGKHPWQASLQS
SLPLTISMPQGHFCGGALIHPCWVLTAAHCT
DIKTRHLKVVLGDQDLKKEEFHEQSFRVEKI
FKYSHYNERDEIPHNDIALLKLKPVDGHCAL
ESKYVKTVCLPDGSFPSGSECHISGWGVTET
GKGSRQLLDAKVKLIANTLCNSRQLYDHMID
DSMICAGNLQKPGQDTCQGDSGGPLTCEKDG
TYYVYGIVSWGLECEKRPGVYTQVTKFLNWI
KATIKSESGF

Spot the difference

WILD TYPE

FSLMSLLESLDPDWTPDQYDYSYEDYNQEEN
TSSTLTHAENPDWYYTEDQADPCQPNPCEHG
GDCLVHGSTFTCSCLAPFSGNKCQKVQNTCK
DNPCGRGQCLITQSPPYYRCVCKHPYTGPSC
SQVVPVCRPNPCQNGATCSRHKRRSKFTCAC
PDQFKGKFCEIGSDDCYVGDGYSYRGKMNRT
VNQHACLYWNSHLLLQENYNMFMEDAETHGI
GEHNFCRNPDADEKPWCFIKVTNDKVKWEYC
DVSACSAQDVAYPEESPTEPSTKLPGFDSCG
KTEIAERKIKRIYGGFKSTAGKHPWQASLQS
SLPLTISMPQGHFCGGALIHPCWVLTAAHCT
DIKTRHLKVVLGDQDLKKEEFHEQSFRVEKI
FKYSHYNERDEIPHNDIALLKLKPVDGHCAL
ESKYVKTVCLPDGSFPSGSECHISGWGVTET
GKGSRQLLDAKVKLIANTLCNSRQLYDHMID
DSMICAGNLQKPGQDTCQGDSGGPLTCEKDG
TYYVYGIVSWGLECGKRPGVYTQVTKFLNWI
KATIKSESGF

MRI

FSLMSLLESLDPDWTPDQYDYSYEDYNQEEN
TSSTLTHAENPDWYYTEDQADPCQPNPCEHG
GDCLVHGSTFTCSCLAPFSGNKCQKVQNTCK
DNPCGRGQCLITQSPPYYRCVCKHPYTGPSC
SQVVPVCRPNPCQNGATCSRHKRRSKFTCAC
PDQFKGKFCEIGSDDCYVGDGYSYRGKMNRT
VNQHACLYWNSHLLLQENYNMFMEDAETHGI
GEHNFCRNPDADEKPWCFIKVTNDKVKWEYC
DVSACSAQDVAYPEESPTEPSTKLPGFDSCG
KTEIAERKIKRIYGGFKSTAGKHPWQASLQS
SLPLTISMPQGHFCGGALIHPCWVLTAAHCT
DIKTRHLKVVLGDQDLKKEEFHEQSFRVEKI
FKYSHYNERDEIPHNDIALLKLKPVDGHCAL
ESKYVKTVCLPDGSFPSGSECHISGWGVTET
GKGSRQLLDAKVKLIANTLCNSRQLYDHMID
DSMICAGNLQKPGQDTCQGDSGGPLTCEKDG
TYYVYGIVSWGLECEKRPGVYTQVTKFLNWI
KATIKSESGF

Chymotryptic digestion of FSAP / MRI

WILD TYPE

FSLMSLLESLDPDWTPDQYDYSYEDYNQEEN
TSSTLTHAENPDWYYTEDQADPCQPNPCEHG
GDCLVHGSTFTCSCLAPFSGNKCQKVQNTCK
DNPCGRGQCLITQSPPYYRCVCKHPYTGPSC
SQVVPVCRPNPCQNGATCSRHKRRSKFTCAC
PDQFKGKFCEIGSDDCYVGDGYSYRGKMNRT
VNQHACLYWNSHLLLQENYNMFMEDAETHGI
GEHNFCRNPDADEKPWCFIKVTNDKVKWEYC
DVSACSAQDVAYPEESPTEPSTKLPGFDSCG
KTEIAERKIKRIYGGFKSTAGKHPWQASLQS
SLPLTISMPQGHFCGGALIHPCWVLTAAHCT
DIKTRHLKVVLGDQDLKKEEFHEQSFRVEKI
FKYSHYNERDEIPHNDIALLKLKPVDGHCAL
ESKYVKTVCLPDGSFPSGSECHISGWGVTET
GKGSRQLLDAKVKLIANTLCNSRQLYDHMID
DSMICAGNLQKPGQDTCQGDSGGPLTCEKDG
TYYVYGIVSWGLECGKRPGVYTQVTKFLNWI
KATIKSESGF

MRI

FSLMSLLESLDPDWTPDQYDYSYEDYNQEEN
TSSTLTHAENPDWYYTEDQADPCQPNPCEHG
GDCLVHGSTFTCSCLAPFSGNKCQKVQNTCK
DNPCGRGQCLITQSPPYYRCVCKHPYTGPSC
SQVVPVCRPNPCQNGATCSRHKRRSKFTCAC
PDQFKGKFCEIGSDDCYVGDGYSYRGKMNRT
VNQHACLYWNSHLLLQENYNMFMEDAETHGI
GEHNFCRNPDADEKPWCFIKVTNDKVKWEYC
DVSACSAQDVAYPEESPTEPSTKLPGFDSCG
KTEIAERKIKRIYGGFKSTAGKHPWQASLQS
SLPLTISMPQGHFCGGALIHPCWVLTAAHCT
DIKTRHLKVVLGDQDLKKEEFHEQSFRVEKI
FKYSHYNERDEIPHNDIALLKLKPVDGHCAL
ESKYVKTVCLPDGSFPSGSECHISGWGVTET
GKGSRQLLDAKVKLIANTLCNSRQLYDHMID
DSMICAGNLQKPGQDTCQGDSGGPLTCEKDG
TYYVYGIVSWGLECEKRPGVYTQVTKFLNWI
KATIKSESGF

LC-MS/MS quantitative approach

- Synthesised labelled and unlabelled forms of the chymotryptic peptides (pure protein currently unavailable):
  - **GLECGKRPGVY (WT)** **V labelled with ^{13}C and ^{15}N**
  - **GLECEKRPGVY (MRI)**

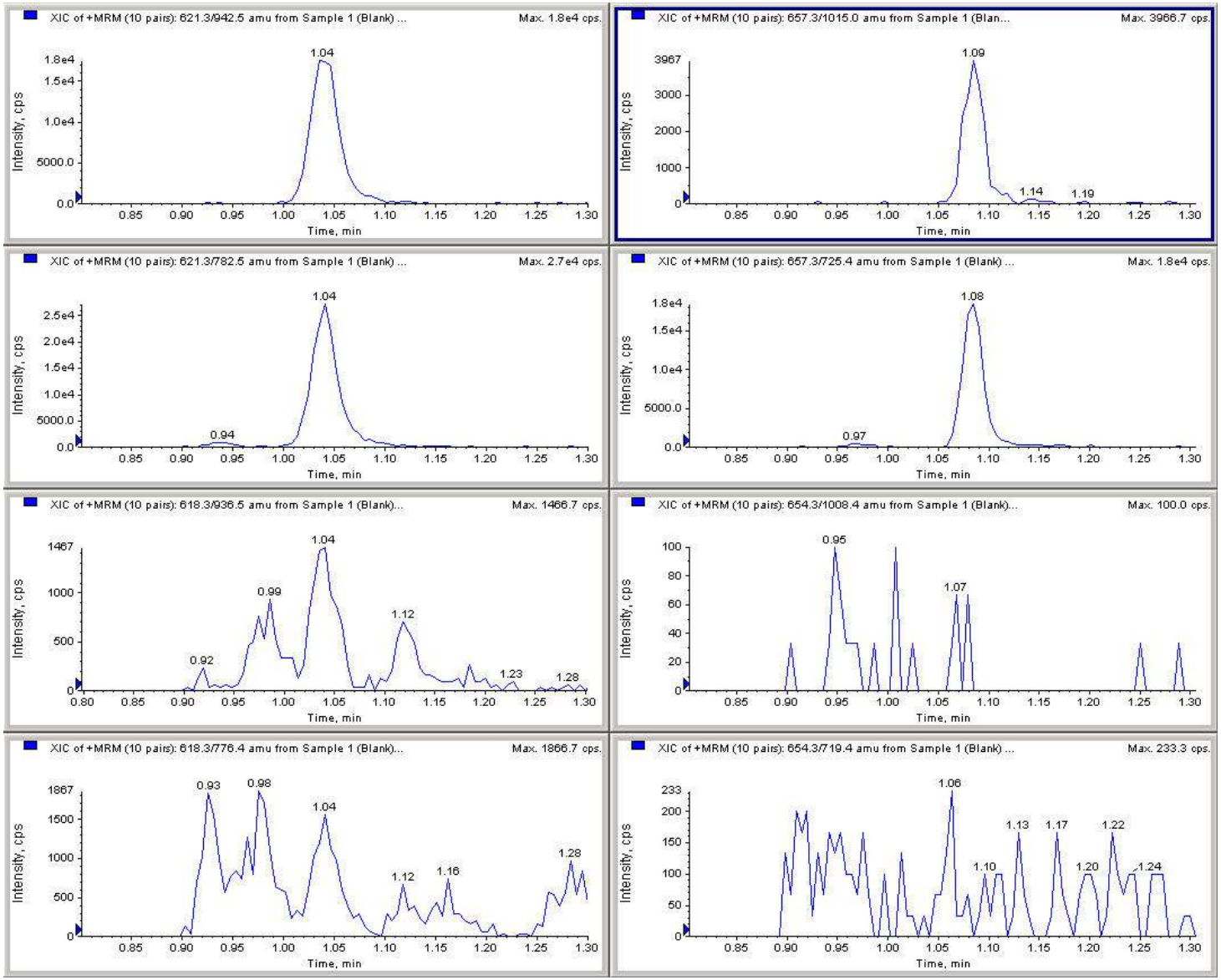
- Generated standard curves using the unlabelled peptides (1 – 40 µg/mL) in wild type plasma (no MRI).
- Added arbitrary amount of labelled peptides to all samples

- Experimental procedure:
  - Take 5 µL of plasma
  - Reduce, alkylate and chymotryptically digest overnight
- Analyse by LC-MS/MS (Acquity + API5000 QqQ)
  - 3 minute method, 700 µL/min
  - 2 transitions per peptide (8 in total)

Example chromatogram (WT blank)

WILD TYPE

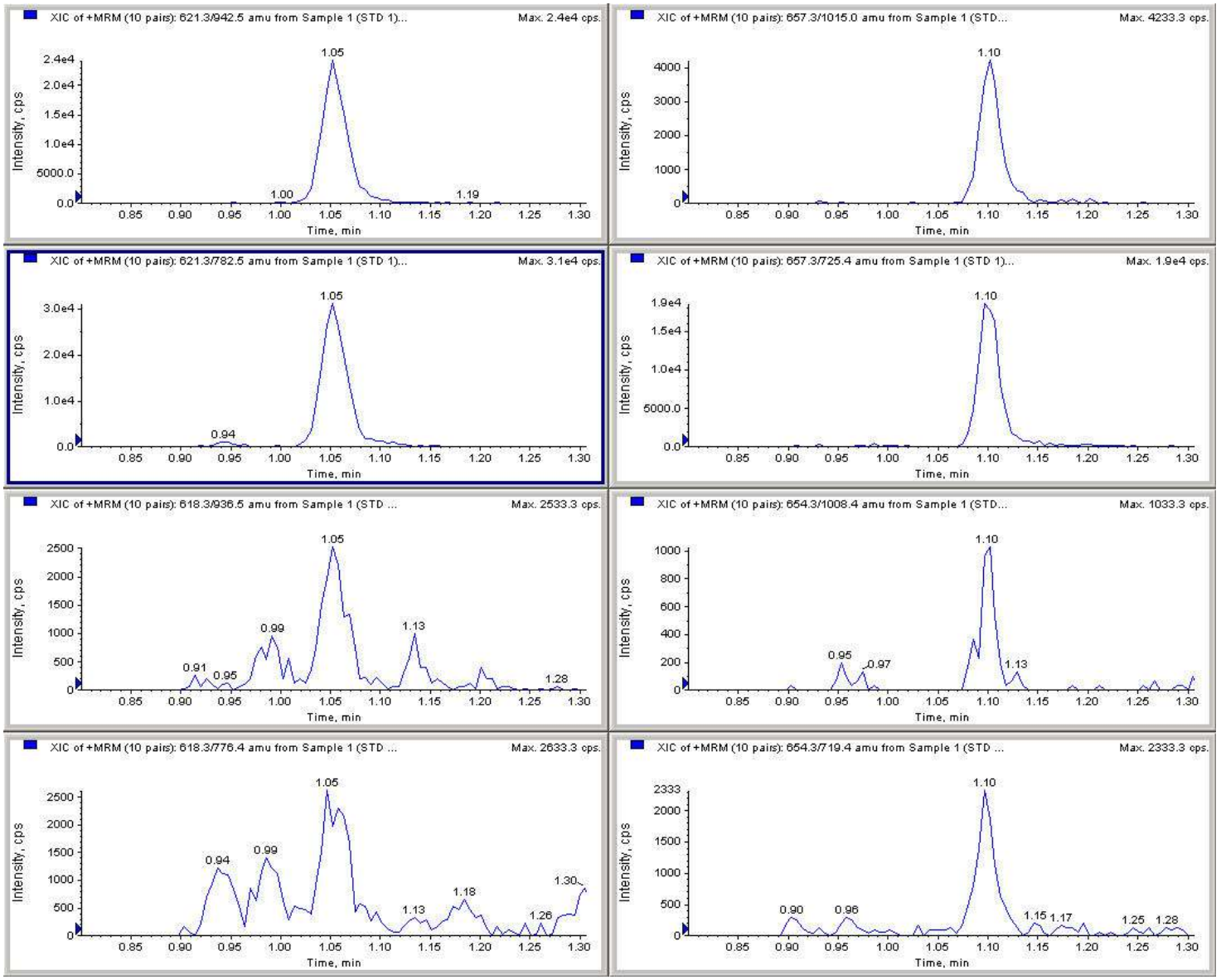
MRI



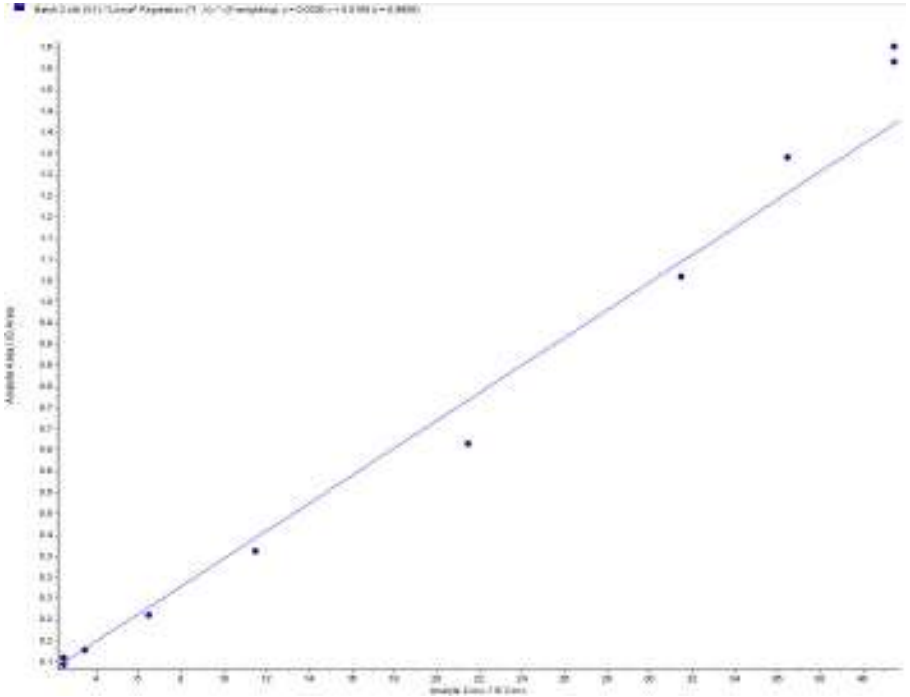
Example chromatogram (1 µg/mL)

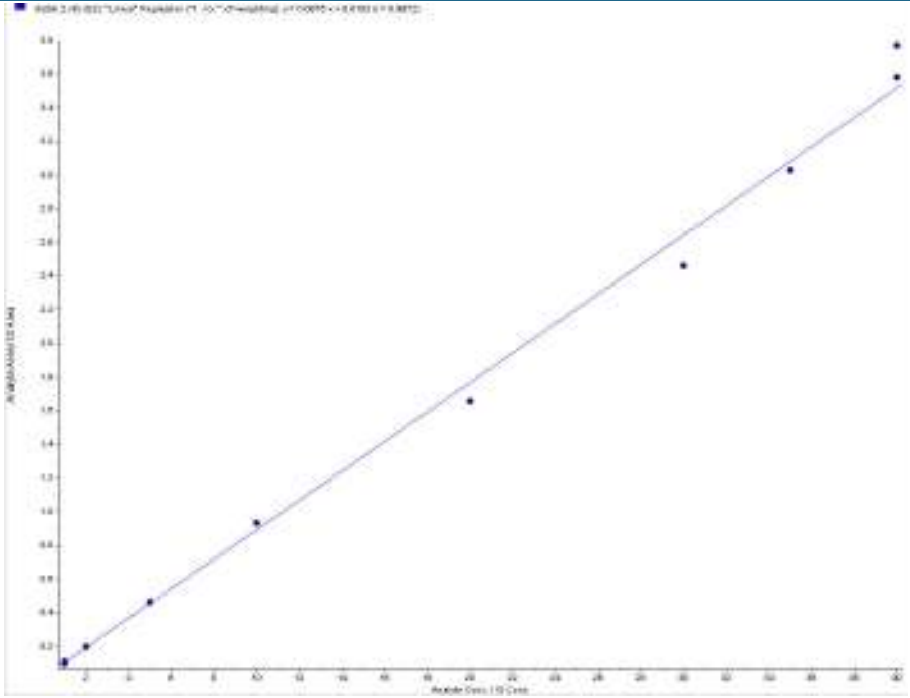
WILD TYPE

MRI



Calibration lines (WT and MRI)





WILD TYPE (Standard addition)

MRI

- Calibration line parameters:
  - R^2 of 0.9906, 0.9972 (WT and MRI)
  - All points within ± 20% (±25 at LLOQ) precision
  - All points within ± 20% (±25 at LLOQ) accuracy

Quality control samples

Four levels (n=6) (spiked peptide)

LLOQ	1 µg/mL
LOW QC	2 µg/mL
MED QC	10 µg/mL
HIGH QC	35 µg/mL

WILD TYPE

	Concentration	SD	%CV	Accuracy
LLOQ	2.15	0.17	8.63	94.1
LOW QC	3.15	0.14	5.21	85.8
MED QC	11.15	0.59	5.83	90.2
HIGH QC	31.15	1.58	4.40	115.7

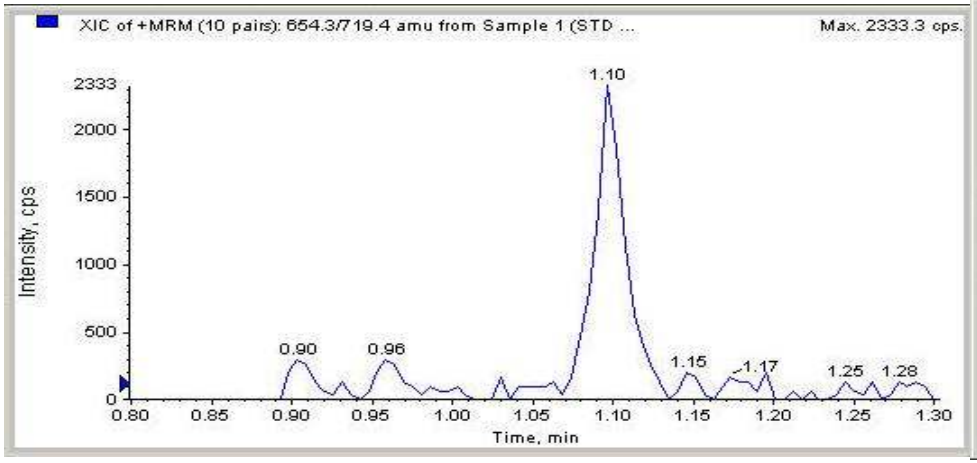
MRI

	Concentration	SD	%CV	Accuracy
LLOQ	1	0.11	11.72	89.7
LOW QC	2	0.15	8.32	87.7
MED QC	10	0.75	7.48	100.8
HIGH QC	35	1.80	5.49	93.5

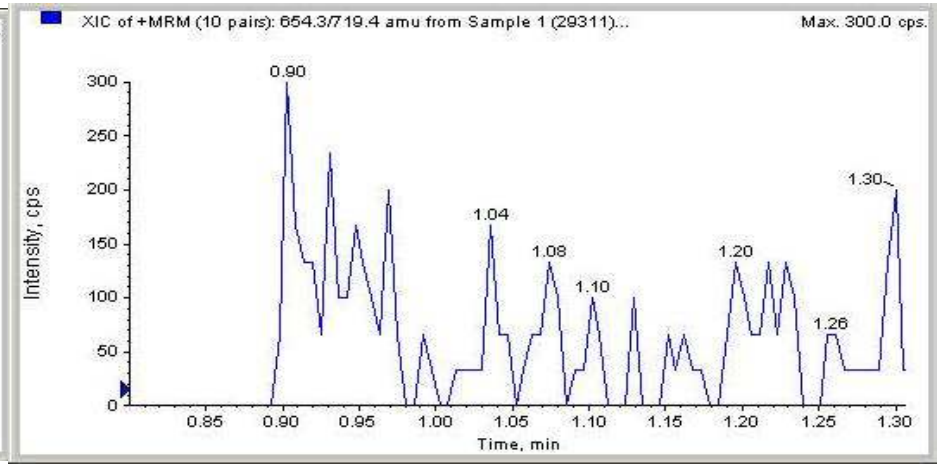
Clinical sample analysis

- 127 human plasma samples (supplied by Siemens)
  - Blinded for WT or MRI status
  - Digestions performed in 2 x 96 well plates
  - LC-MS/MS analysis took <10 hrs (with standards and QC's)
- WT concentrations were between 1.2 and 2.0 µg/mL
- Unfortunately, all samples containing MRI peptide were assigned as BLQ (<1 µg/mL)
- However, not all is lost!!
- We can obtain information from the peak areas obtained during the assay…

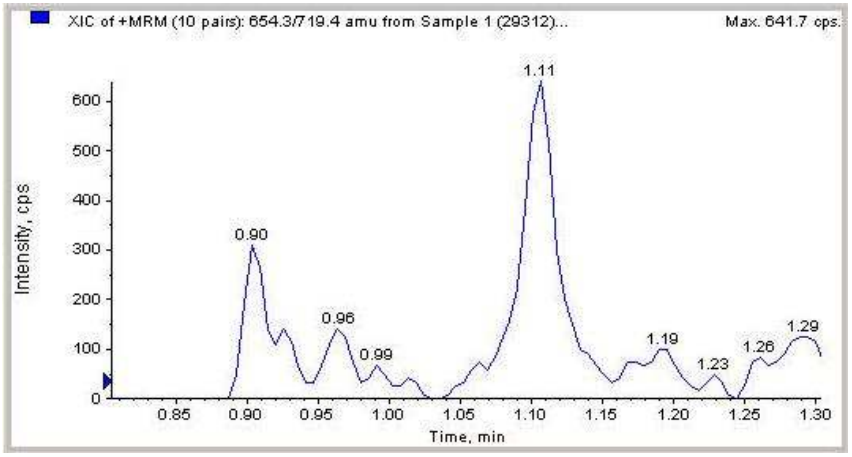
MRI peptide peaks in samples



1 µg/mL STD MRI peptide



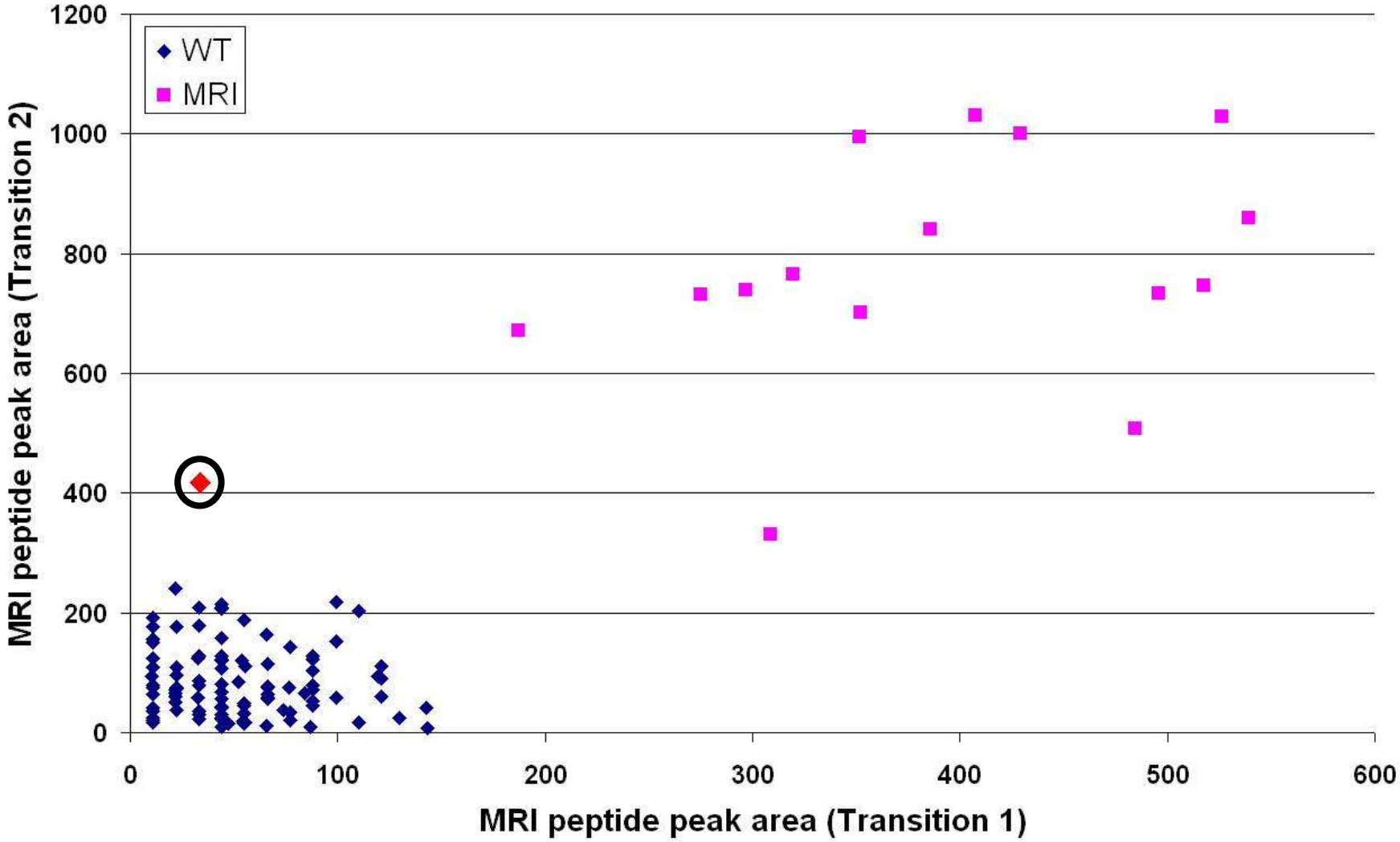
No MRI peptide in WT sample



MRI peptide in real sample

MRI peptide peak areas

Plotted peak areas of both MRI peptide transitions



Clinical sample analysis

- Unblinding of samples:
  - 17 MRI
  - 110 WT
- LC-MS/MS identified 15 of the 17 MRI samples
- 2 MRI samples and 11 WT samples demonstrated absence of peaks for both peptide variants.
  - Digestion failure?
  - Old (degraded) samples?
- Specificity = 100% (no false +ve's)
- Sensitivity = 100% *88%

Areas for method development

- Can we increase chymotryptic release?
  - Use of detergents / organic solvents during digestion?
- Obtain completely blank plasma
  - Analyte free matrix will make quantitation of WT FSAP easier, as standard addition approach won't be required
- Obtaining pure FSAP and MRI reference standards
  - This would mitigate chymotryptic digestion probles
  - Similar digestion efficiency for standards and samples
- Targeting additional FSAP (common) peptides
  - Total FSAP plasma concentrations

Summary

- LC-MS/MS was capable of detecting two different FSAP isoforms in clinical samples
- Truly high throughput approach (3 minute method)
  - LC-MS/MS systems are present in clinical laboratories
- Peptide surrogate quantitation approach demonstrated good precision and accuracy
- Application of methodology to real clinical samples resulted in lower that expected FSAP and MRI concentrations
  - Believed to be due to less than optimal chymotryptic digestion
  - Inherent problem with peptide surrogate approach
  - Best approach is to have intact protein (poster 48)

- Further work is planned to improve chymotryptic peptide release and improve on quantitative approach

Acknowledgements / Thanks

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  - Ian Ward, Dr. Ellen Vringer-Stockvis, Dr. Steve Pleasance
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  - Dr. Frank Vitzthum, Herbert Schwarz
- Peptide chemists at CRB
  - Sorry for the “Friday afternoon” peptides!
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  - PhD supervisor
- Professor Rob Beynon
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ANY QUESTIONS?