Leiden University
Medical Center

Next-generation antithrombin diagnostics by mass spectrometry

M. Kruijt, M.M. Treep,
C.M. Cobbaert, L.R. Ruhaak

Department of Clinical Chemistry
and Laboratory Medicine

ECAT 2022

Antithrombin

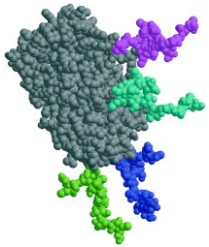
AT deficiency

- Low or dysfunctional AT
- High risk of venous thromboembolism

Causes

- Mutations (350+ reported)[1]
- Aberrant glycosylation[2]

1) Patnaik et al., Haemophilia (2008)
2) De la Morena-Barrio et al., J Thromb Haemost (2016)



McCoy et al. JMB (2003)

Diagnostics

USA

Analyte	All-Method Accuracy		All-Method Precision		Final Ranking[b]
	Bias, %	Ranking[a]	CV, %	Ranking[a]	
Antithrombin					
Activity	2.6	1	6.3	2	1
Antigen	3.8	3	7.6	3	2
Protein C					
Activity	8.5	5	6.1	1	2
Antigen	3.4	2	20.0	7	4
Protein S					
Activity	8.76	6	15.8	5	5
Total Antigen	6.2	4	15.0	4	3
Free Antigen	8.79	7	17.3	6	6

Cunningham et al., Arch Pathol Lab Med (2011)

Europe

Analyte	LCV_a (%)		Number of laboratories
	Median	95% CI	
Antithrombin (activity)	7.6	3.6–35.5	136
Protein C (activity)	8.6	3.5–25.3	132
Protein C (antigen)	10.8	4.8–33.1	48
Protein S (total antigen)	13.4	6.4–50.6	79
Protein S (free antigen)	14.1	6.5–79.1	65
Protein S (activity)	17.2	7.2–84.3	69

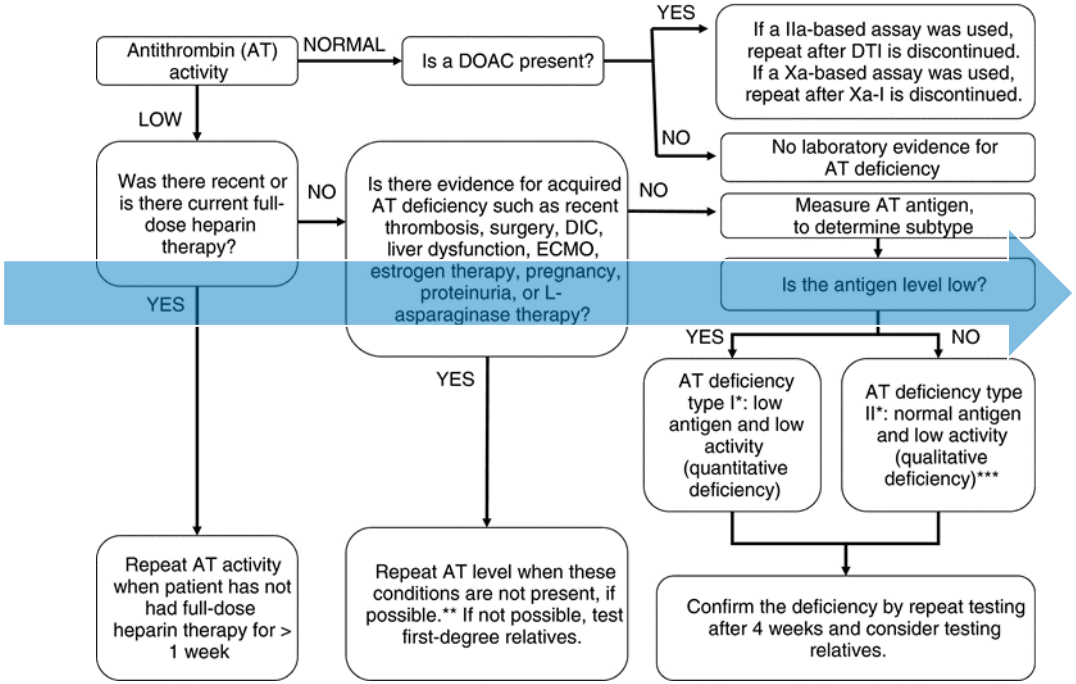
Meijer et al., J Thromb Haemost (2003)

Simple test, simple diagnosis?

Van Cott et al.,
J Thromb Haemost (2019)

Recommendations for clinical laboratory testing for antithrombin deficiency; Communication from the SSC of the ISTH

The activity results



The diagnosis

Vermeer, Girl with a pearl earring (1665)

It’s all in the details…

The activity results

The diagnosis?

Vermeer, Girl with a pearl earring (1665)

The mutation

Herbert, A Compendium of Cultured Cats (2015)

Limitations of traditional diagnostics

- Risk of underdiagnosis using activity tests

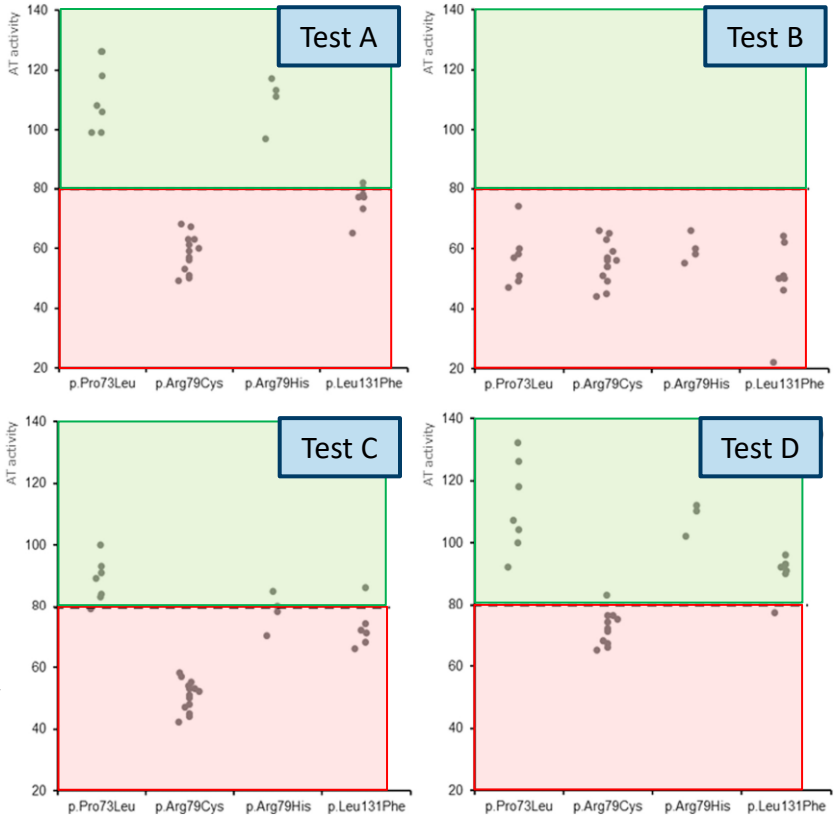
- Belief: lower activity = more severe disease

activity *in vitro* ≠ functionality *in vivo*

ISTH SSC:

"Molecular testing […] will identify mutations that can be missed by traditional activity assays."

- Specific mutations may have specific risks
  - Venous Thromboembolism (VTE)
  - Arterial Thromboembolism (ATE)
  - Recurrent Pregnancy Loss (RPL)



Adapted from Orlando C et al, J Thromb Haemost (2015)

Clinical example

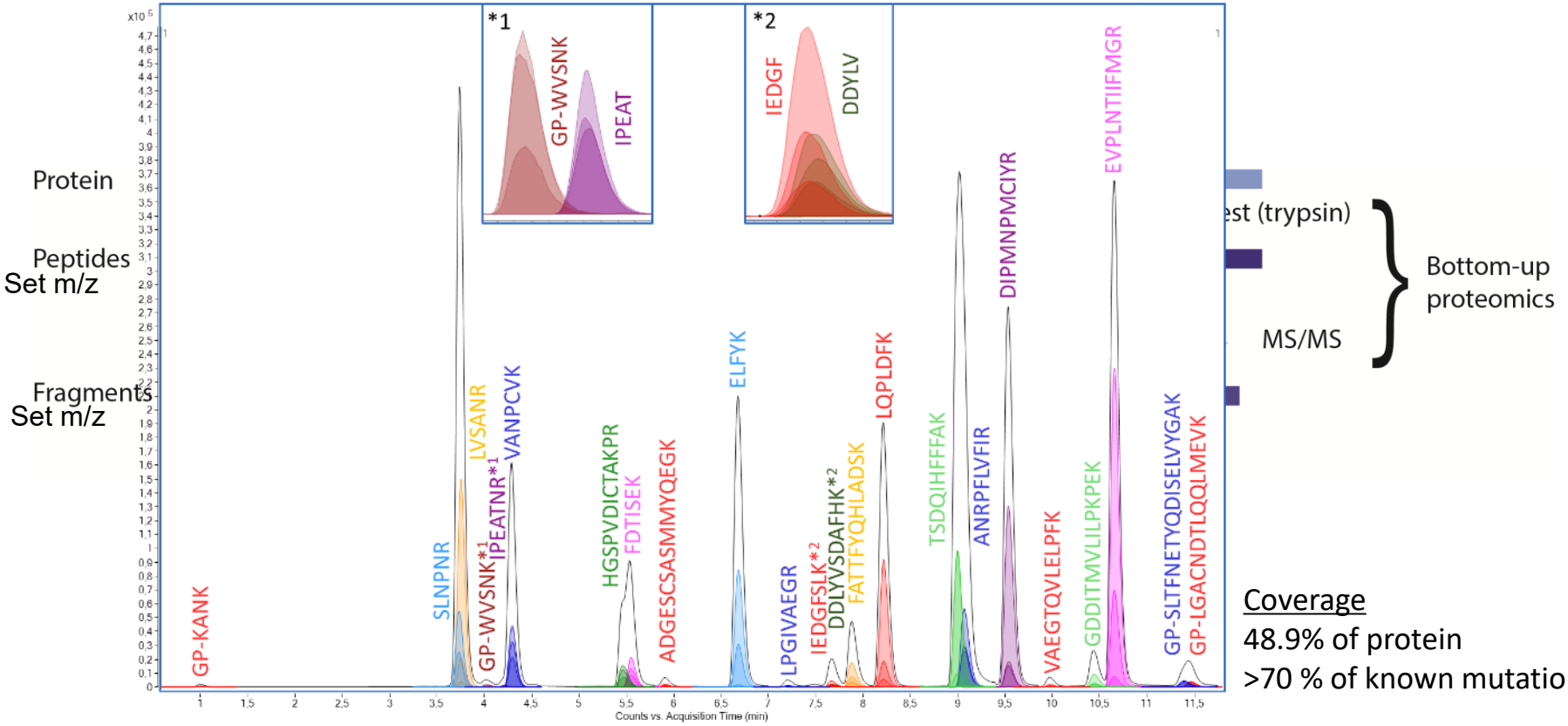
Kruijt et al.,
J Thromb Haemost (2021)

Woman with unexplained recurrent pregnancy loss

- No (familial) history of VTE
- Thrombophilia screening due to study eligibility

Test	Result	Reference
Protein C (% activity)	106	>66
Protein C (% antigen)	69	>64
Factor II (% antigen)	88	60-137
Factor X (% antigen)	65	65-121
Free Protein S (IU/mL)	1.00	0.53-1.51
APC resistance (ratio)	5.56	>2.90
Factor II mutation	ND	
Antithrombin (% activity)	69-72	84-116

Mass spectrometry for precision diagnostics



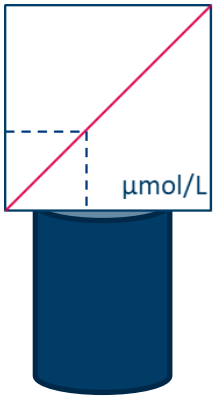
Coverage

48.9% of protein

>70 % of known mutations

Molecular characterization by peptide monitoring

Quantitate

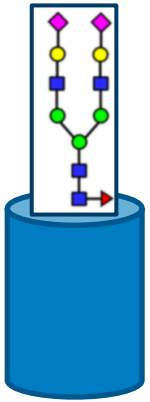


3 non-mutation-prone peptides

Mutations

16 qualifying peptides

Glycosylation



4 glycopeptides

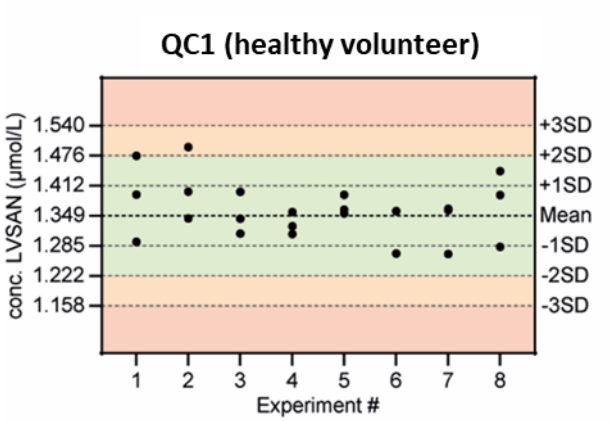
Which proteoform is present in the patient?

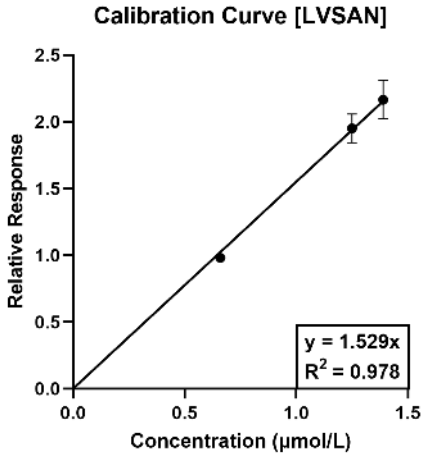
Quality Control & Calibration

synthetic peptides

- Stable-Isotope-Labelled Peptide spike (internal control)
- System Suitability Test (monitor system performance)

native citrate plasma

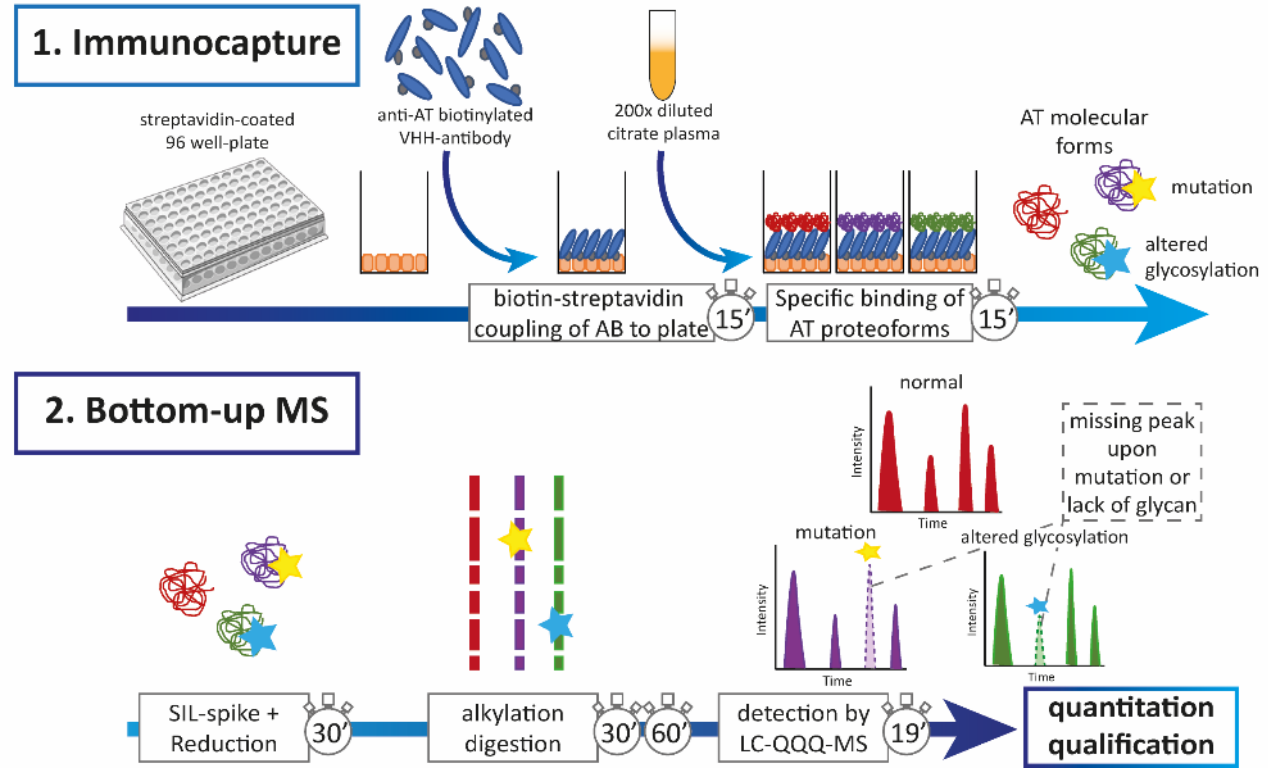




$$\%\ glycosylation = \frac{conc.\ glycopeptide}{conc.\ quantifier} x\ 100\%$$

Precision diagnostics by LC-MRM-MS

Agilent BRAVO
liquid handling platform

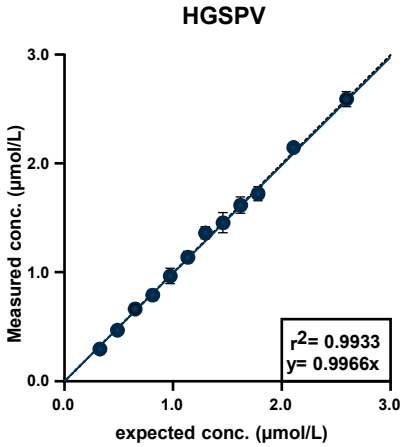


Agilent 6495C
LC-QQQ-MS

Analytical validation

Kruijt et al., manuscript in preparation

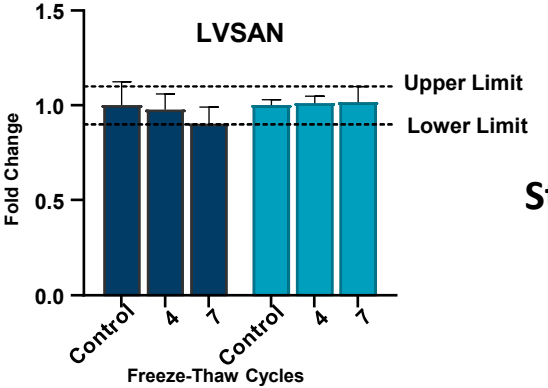
Linearity



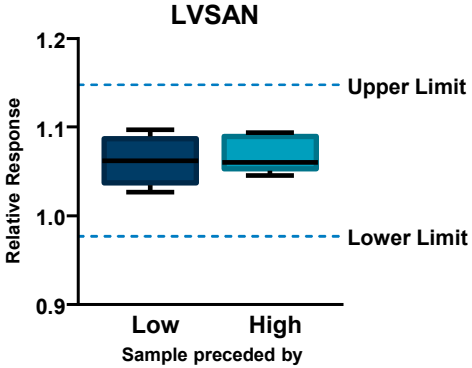
Precision

Quantifiers:	5.9 - 7.8%
Qualifiers:	5.5 – 17.3 %
Glycopeptides:	6.4 – 10.3%

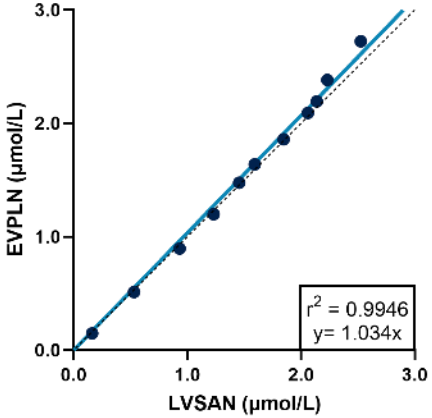
Stability



Carry-over



Interpeptide agreement

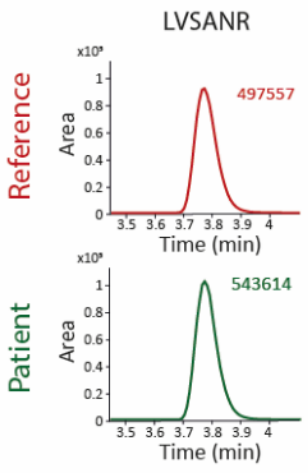


Bringing the test into clinical practice

Kruijt et al.,
J Thromb Haemost (2021)

Woman with unexplained recurrent pregnancy loss

- Thrombophilia screening: activity AT **69-72%**



Peptide	Result	Reference
LVSANR (µmol/L)	1.91	1.33 - 1.91

Clear diagnosis of AT deficiency caused by heterozygous Pro73Leu mutation Associated with pregnancy complications (Puurunen et al. , J Thromb Haem (2013)

Next steps

Horvath et al.,
Clin Chim Acta. (2014)

investigate potential of the test in RPL population
(M.P. van der Helm, kcio)

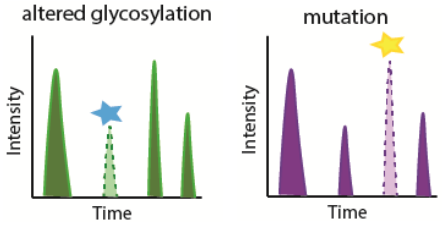
Conclusion

Correct test, correct result, correct interpretation?

- Should we (only) look at the (average) activity?
- Do underlying proteoforms tell us more about patient risks?

Alternative / Add-on: AT proteoforms by mass spectrometry

- Molecular characterization in an all-in-one test
- Analytical performance according to pre-set specifications



Next step: clinical performance / effectiveness

- Which patients may benefit most from a more personalized approach?
- Can we use the test to evolve into precision/personalized medicine?

Leiden University Medical Center

Thank you for your time

Department of Clinical Chemistry and Laboratory Medicine

T.T. van Duijl
M.M. Treep
N.P.M. Smit
C.M. Cobbaert
M.P. van der Helm
E. Reijnders
M.M. Pieterse
F.P.H.T.M. Romijn
L.R. Ruhaak

Department of Thrombosis and Haemostasis

L.M. van der Pol
J. Eikenboom

Department of Obstetrics

H.J. Verburg

M. Kruijt (m.kruijt@lumc.nl)