Leiden University Medical Center

Standardization of multiple serum apolipoproteins using mass-spectrometry based proteomics

on behalf of the IFCC Scientific Division WG-APO MS

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L U
M C

Disclosures: none

Content

I. **Traditional CVD diagnostics & risk models**

II. **Rationale for MS-based (Apolipo)Protein Tests?**

III. **IFCC WG on Apolipoproteins by Mass Spec & Terms of Reference**

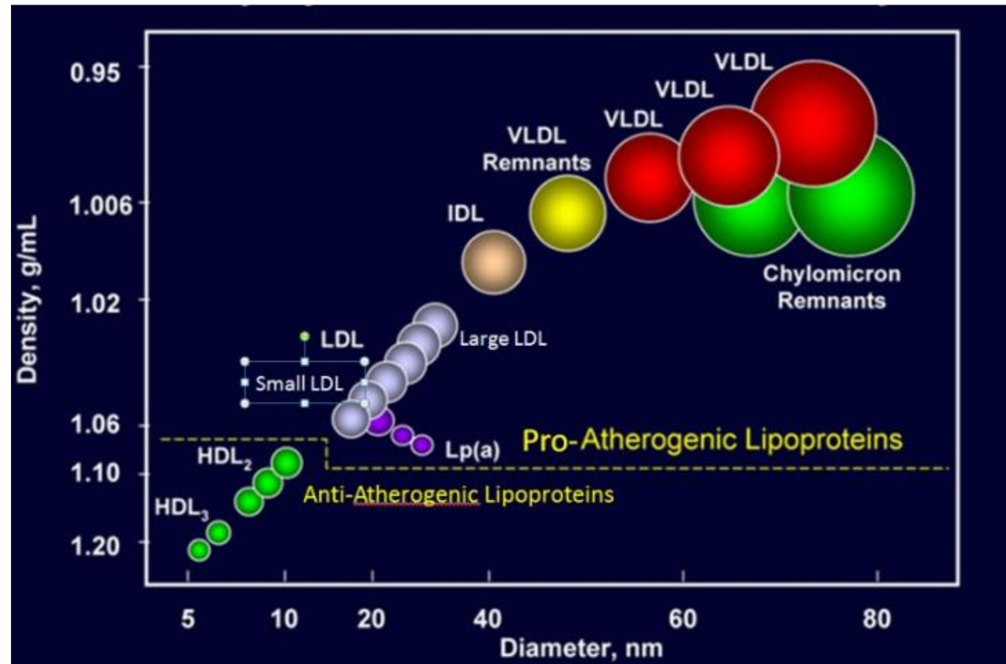
IV. **Development of a Candidate Reference Measurement System**

V. **MS-based proteomics:** potential for **ACCURATE AND SI-TRACEABLE** standardization of both well defined and heterogeneous protein analytes?

Traditional CVD risk assessment:

A. Assessment of lipoprotein composition by **measuring OVERALL blood lipids**:

- total cholesterol,
- LDL-cholesterol (LDL-c)
- HDL-cholesterol (HDL-c)
- triglycerides



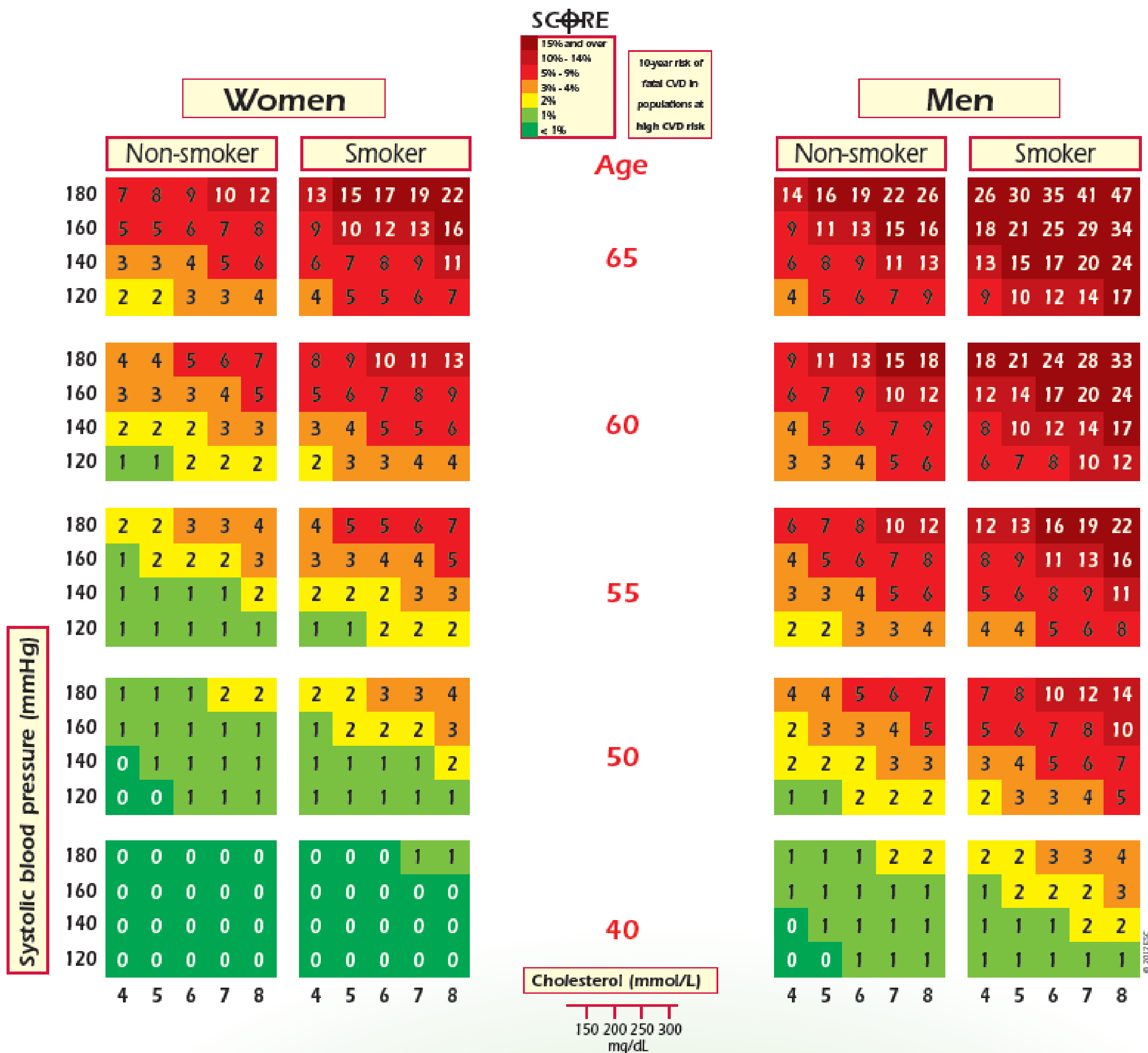
→**Classical lipids partially reflect the atherogenic burden!**

→**Limited clues to underlying molecular defects!**

B. **Risk prediction models**: US Framingham score, European SCORE, …

SCORE: European High Risk Chart

10 year risk of fatal CVD in high risk regions of Europe by gender, age, systolic blood pressure, total cholesterol and smoking status

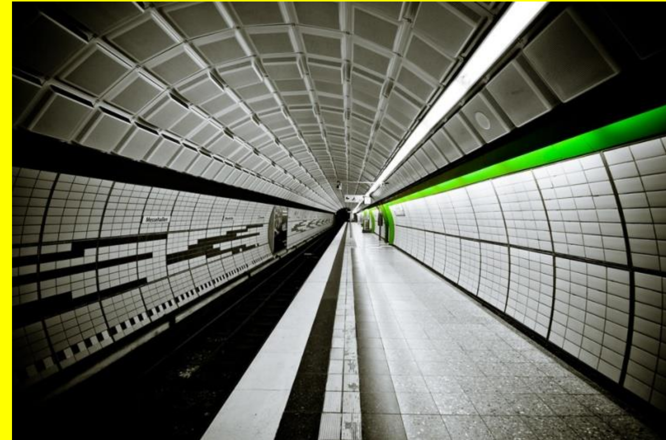


Treatment of CVD anno 2017 is based on:

A. <u>OVERALL blood lipids</u>:

Unchanged Medical Test Practice since 1960s

Tunnel vision?

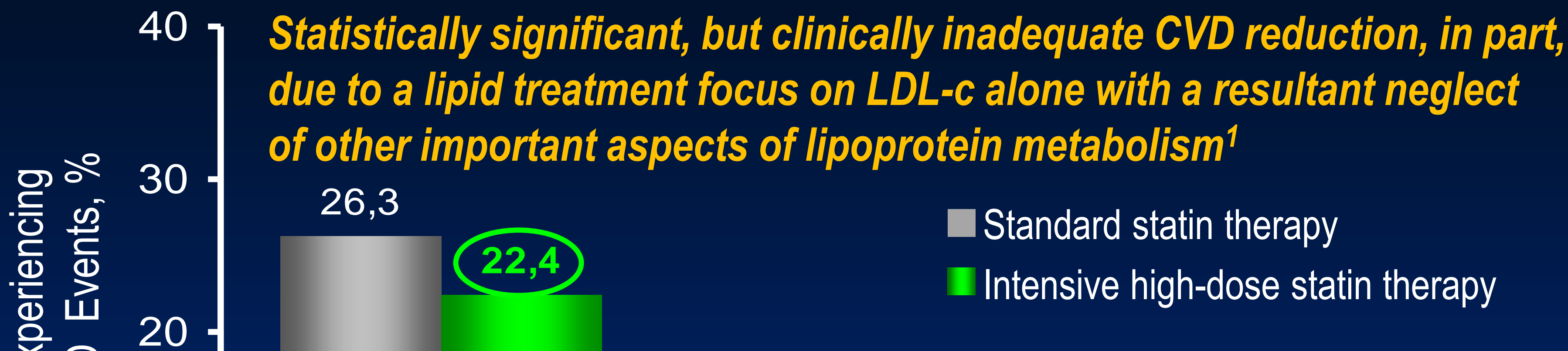


B. Risk prediction models: US Framingham score, European SCORE, …

*cholesterol-efflux-capacity: low CEC is an independent risk factor for CVD

Residual CVD Risk with *Intensive* Statin Therapy

Less, but Still Unacceptably High



Are we measuring the wrong targets!?

	PROVE IT-TIMI 22[2]		IDEAL		TNT	
n	4162		8888		10,001	
LDL-C* mg/dL	95	62	104	81	101	77

[1]Superko HR. *Br J Cardiol.* 2006;13:131-136.
[2]Cannon CP et al. *N Engl J Med.* 2004;350:1495-1504.
[3]Pedersen TR et al. *JAMA.* 2005;294:2437-2445.
[4]LaRosa JC et al. *N Engl J Med.* 2005;352:1425-1435.

*Mean or median LDL-C after treatment

Apolipoproteins: holy grail for unraveling dyslipidemia?

Ignored in most clinical guidelines for CVRM so far!

If available: uniplex immunoassays for RUO

Apolip				
Apo A-				es
Apo A-				es
Apo A-				
Apo A-				is
Apo B-				
Apo B-				
Apo C-			HDL	
Apo C-II	8,800	Liver	Chylomicrons, VLDL, HDL	Co-factor for LPL
Apo C-III	8,800	Liver	Chylomicrons, VLDL, HDL	Inhibits LPL and uptake of lipoproteins
Apo E	34,000	Liver	Chylomicron remnants, IDL, HDL	Ligand for LDL receptor
Apo (a)	250,000-800,00	Liver	Lp (a)	Inhibits plasminogen activation

II. Drivers for Mass Spectrometry-based (Apolipo)Protein Tests!?

A. The era of Precision Medicine

B. Technological Revolution enabling Precision Medicine

A. The Human Proteome Project & the era of Precision Medicine

2001: publication of the Human Genome by HUGO

i. *Surprise: lower-than-anticipated number of genes identified*
(~20.300 protein-coding genes rather than 100.000 estimated; ~3% genome).

ii. *ENCODE project: 4 * 10^6 locations within the genome that serve as switches to control transcriptional activity of the ~20.300 genes.*

iii. *Recognition that complexity of biological machinery is at the level of protein variation.*

2003: founding of the **Human Proteome Organization (HUPO)**

2010: announcement of the **Human Proteome Project (HPP)**

i. *To identify and characterize all proteins encoded by each of the ~20.300 human protein-coding genes, as well as their co- and posttranslational modifications.*

2015: Obama announces a **new era of medicine, called Precision Medicine**,

i. *One that delivers the right treatment at the right time.*

The Human Proteome Project

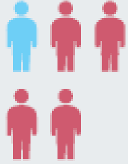
Collaborative efforts providing the **foundation for proteomics-based precision medicine** initiatives are highlighted. Data repositories such as Peptide Atlas and knowledgebases like neXtProt and TopFIND are key to disseminate data and knowledge to the broader scientific community, providing the foundation for the development of diagnostic, prognostic, therapeutic, and preventive medical applications.

The drugs don't work .....

IMPRECISION MEDICINE

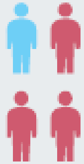
For every person they do help (blue), the ten highest-grossing drugs in the United States fail to improve the conditions of between 3 and 24 people (red).

1. ABILIFY (aripiprazole)
Schizophrenia



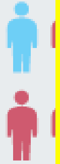
2. NEXIUM (esomeprazole)
Heartburn

3. HUMIRA (adalimumab)
Arthritis

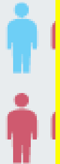


4. CRESTOR (rosuvastatin)
High cholesterol

5. CY
Depre



8. RE
Crohr



Based on published number needed to treat (NNT) figures. For a full list of references, see Supplementary Information at go.nature.com/4dr78f.

Recognition that doctors need to take **INDIVIDUAL VARIABILITY** into account is driving huge interest in Precision Medicine.

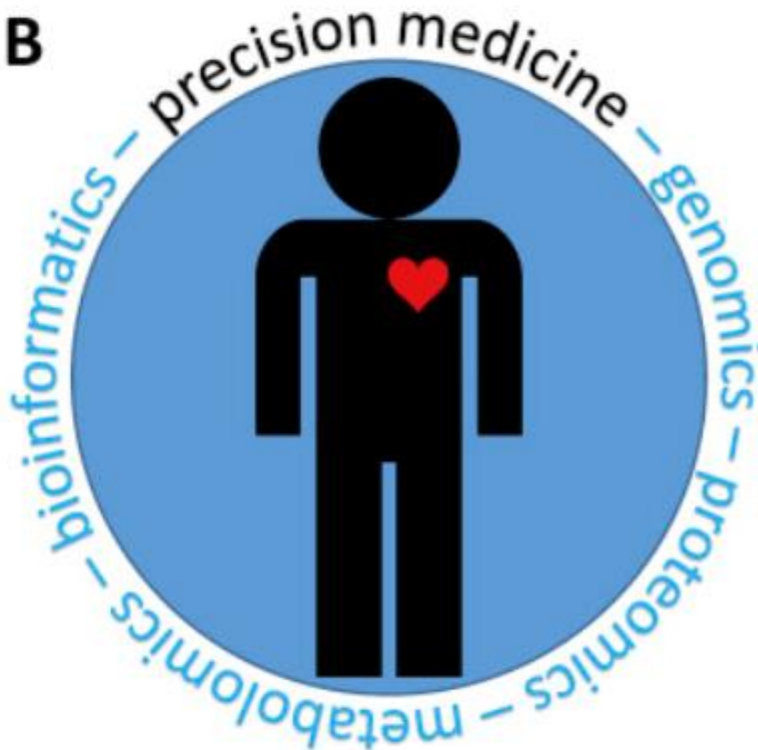
Urgent need for (protein) tests that enable patient **STRATIFICATION**!

Opportunities for medical labs!

Imprecision Medicine and Prescription Roulette

5; 520: 609-611

Precision Medicine: away from one size fits all!



Tier 1:
- Genomics
- Pharmacogenomics

STATIC

Tier 2:
- Proteomics
- Positional Proteomics

DYNAMIC

Tier 3:
- Metabolomics
- Microbiomics

P4

personalized: customized diagnosis and treatment

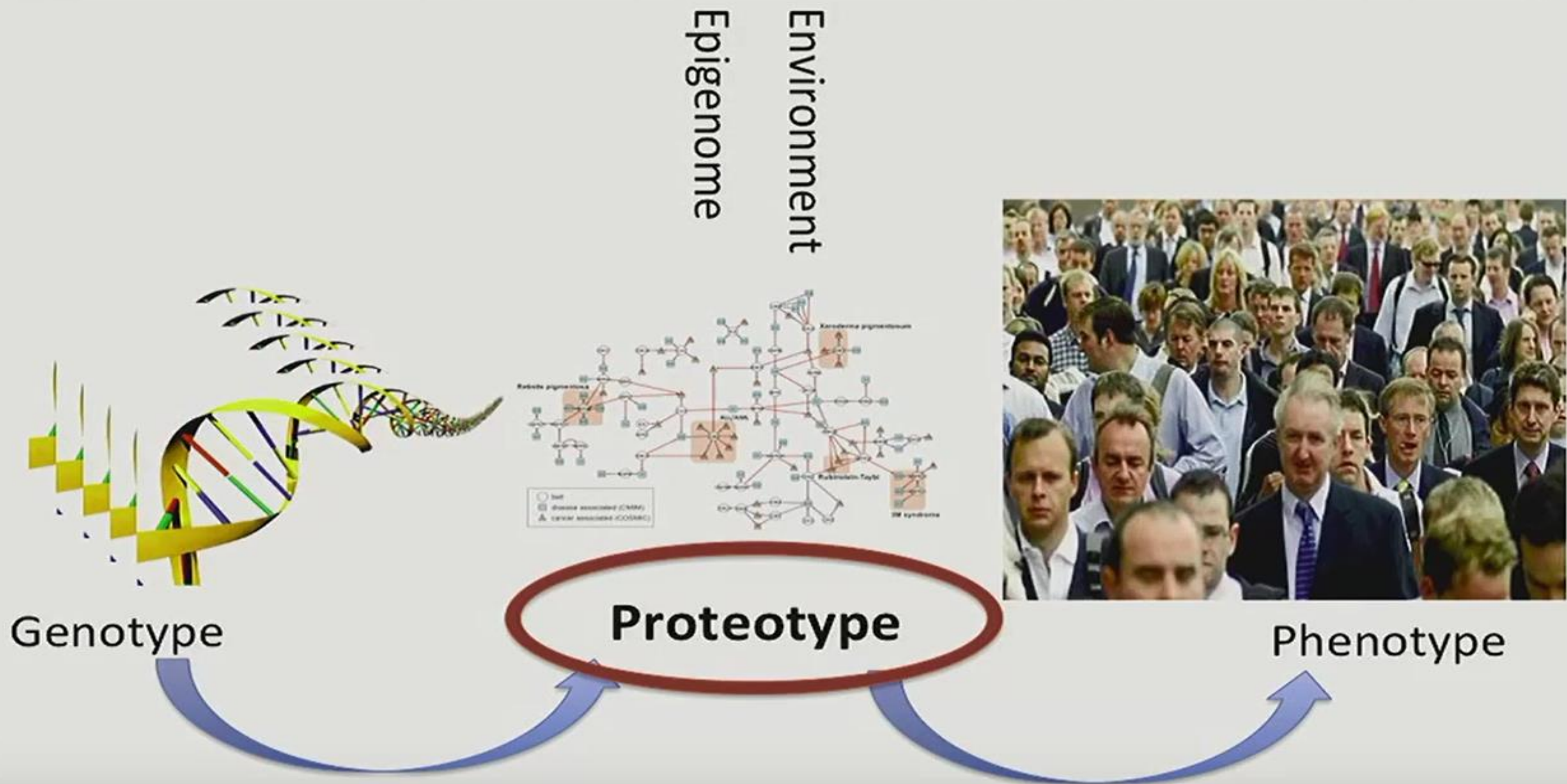
predictive: profile based prediction of disease and therapy outcomes

preventive: prevention and early diagnosis instead of disease curation

participatory: empowerment and involvement of patients

"Precision Medicine" rests upon genomics, proteomics, metabolomics and bioinformatics.

Genotype – Proteotype - Phenotype



B. Technological Revolution: Targeted MS-based Proteomics

METHOD OF THE YEAR **2012** NEWS FEATURE | SPECIAL FEATURE

Targeted proteomics

Analysis of a preselected group of proteins delivers more precise, quantitative, sensitive data to more biologists. Vivien Marx reports.

Although the number and identity of protein-coding genes in humans and many other organisms are known to a certain level of approximation, the numbers of proteins produced by each of these genes remains a mystery. Further complicating matters, given the many possible splice forms and post-translational modifications, the potential number of proteins is "staggering," says Arizona State University researcher Josh LaBaer, who is also president-elect of the US Human Proteome Organization. A protein is also dynamic. "It's phosphorylated this minute; it's not phosphorylated the next

"I personally can't wait until we stop hearing about someone describing how big of a list of proteins, peptides or phosphopeptides they detected," says one researcher critical of discovery proteomics who did not wish to be identified. Proteomics has been doing "my list is bigger than your list" for far too long. "It is way more important to measure the one right protein than 10,000 wrong ones."

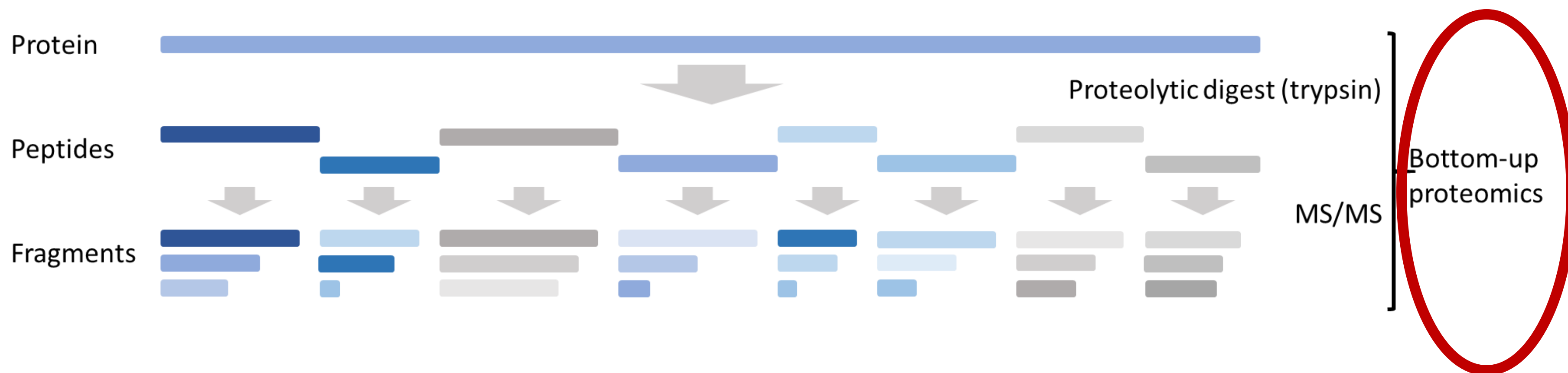
Scientists wanting to follow well-founded hunches about dozens or hundreds of proteins seek a focused, reproducible, quantitative view of a small subset of the whole

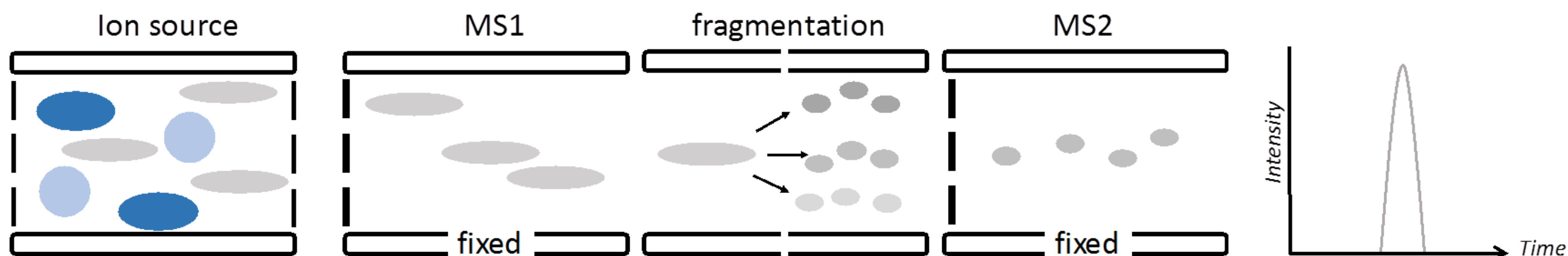
Focuses on a specific set of protein(s) of interest

- Measure disease related changes in proteins
- Obtain knowledge on molecular pathophysiology or defects in signalling pathways
- Identify potential therapeutic targets
- Assess efficacy and safety of therapy
- Highly multiplexed alternative method to western blots/antibodies
- **Hypothesis driven questions!**

Protein Quantification with LC-MRM-MS

QqQ-MS: riding the workhorse





Adjusted from Domon and Aebersold, Science, 2006

III. IFCC WG on Apolipoproteins by Mass Spectrometry

IFCC WG on Apolipoproteins by Mass Spectrometry (WG-APO MS)

Terms of Reference

1. To achieve **standardization** of a panel of clinically relevant serum apolipoproteins (apo) A-I, B, C-I, C-II , C-III, E and apo (a) (including qualitative phenotyping where needed).
   - Standardization will be done in such a way that measurement results become **traceable to SI as outlined in ISO 17511**.
   - Other traceability chains will be used in cases where traceability to SI cannot be achieved.

2. To evaluate **clinical performance and clinical utility** of serum apolipoprotein panel(s) for improved CVD risk stratification and treatment, in comparison to or together with contemporary blood lipids.

WG-APO MS & members

Apolipoproteins by Mass Spectrometry (WG-APO MS)

Membership

Name	Position	Country	Term	Time in Office
C. Cobbaert	Chair	NL	1st	2017 01 - 2019 12
U. Ceglarek	Member	DE		
V. Delatour	Member	FR		
J. Dittrich	Member	DE		
C. Hirtz	Member	[illegible]		
A. Hoofnagle	Member	[illegible]		
Z. Kuklenyik	[illegible]	[illegible]		
L.R. Ruha[illegible]	[illegible]	[illegible]		
[illegible]	[illegible]	[illegible]		
[illegible]	[illegible]emens	DE		
[illegible]	[illegible]epresentative/Roche	DE		
[illegible]	Consultant	AT		
[illegible]chimmel	Consultant	BE		
I. Zegers	Consultant	BE		

Global representation of different stakeholders of the traceability chain

WG-APO MS & stepwise approach

1. Define the analytes / measurands intended to be measured.

2. Development of primary and secondary **Reference Materials**, including evaluation of commutability.

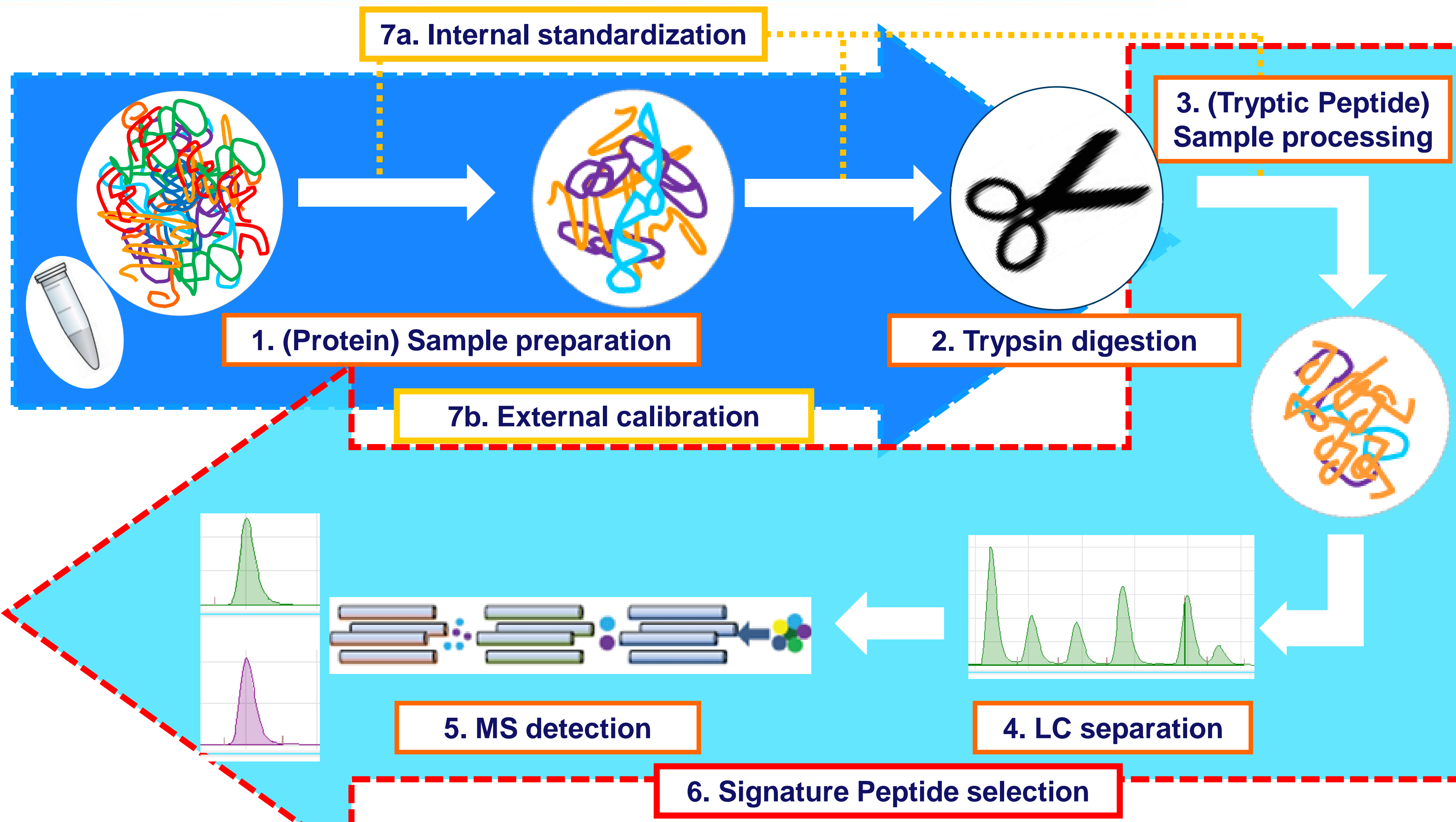
3. Development of a candidate **LC-MS/MS-based Reference Method** that is unaffected by genetic variants, post-translational modifications and other factors. The reference method should meet relevant ISO standards (i.e., ISO 15193, 15194 and 15195).

4. Validation of the analytical performance of the LC-MS/MS Reference Measurement System.
   - ✓ Assessment of the performance of commercially available apolipoprotein tests compared to the reference method using commutable reference materials as well as single donation samples.

5. Any Reference Materials and Reference Measurement Procedures developed will be submitted to JCTLM for review and listing on the JCTLM database.

IV. Development of a candidate MS-based Apolipoprotein Reference System

MS-based Protein Tests:
Critical Factors for Standardization



Specific challenge in bottum up proteomics: changing measurands!

13) 161-179

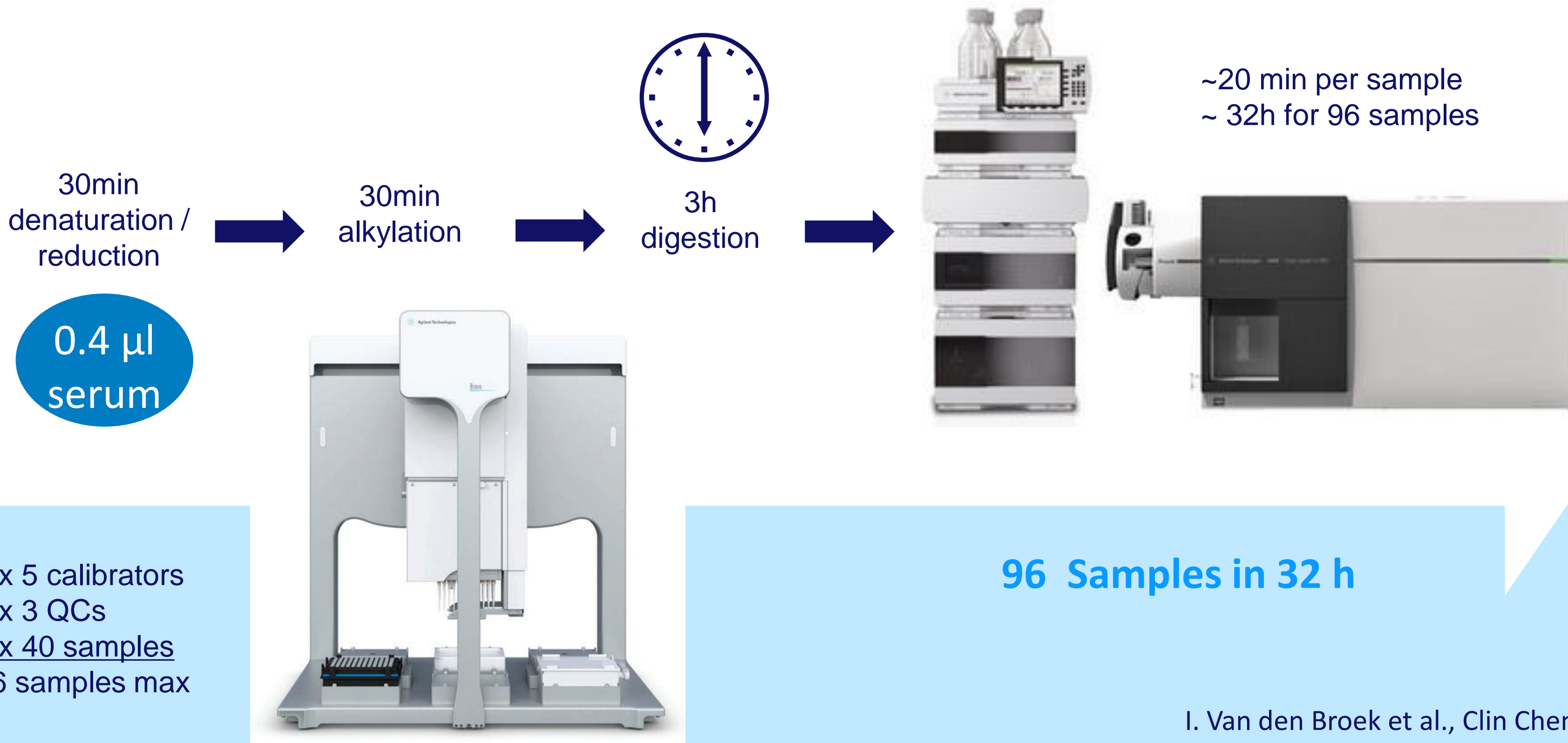
A. Development of a common accuracy base by IFCC WG APO-MS

Starting point: Lab-developed Multiplex Serum Apolipoprotein Tests

3 calibration labs:
CDC, Leipzig and Leiden UMC

Protocol Leiden method:

- ✓ Direct measurement of apo A-I, B, Cs and E (µmol/L range)
- ✓ DOC/TRIS/TCEP
- ✓ Semi-automated BRAVO LH platform
- ✓ 3 hrs tryptic digestion 37 degrees Celsius



2 x 5 calibrators
2 x 3 QCs
2 x 40 samples
96 samples max

96 Samples in 32 h

I. Van den Broek et al., Clin Chem, 2016

Multiplex apolipoprotein test

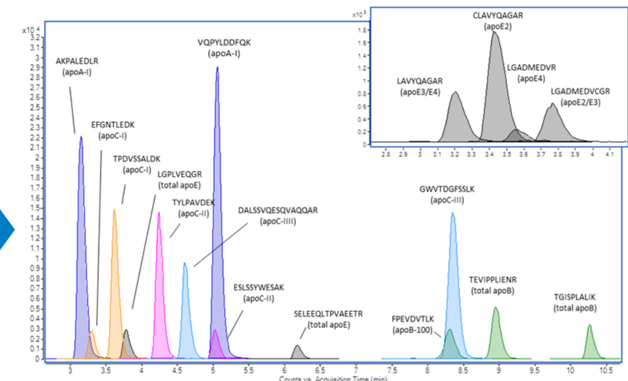
Apolipoproteins
A-I
B100/B48
C-I
C-II
C-III
E (E3, E2 and E4)

Total of 17 peptides
6 Quantification peptides
6 Confirmation peptides
2 differential peptides
14(SIL)-peptides
3 phenotyping peptides

Total of 84 MRM
3 ions/peptide
1 quantifier + 2 qualifiers

Dynamic MRM
14 peptides within their Rt window during chromatographic run (total run time 17 min)

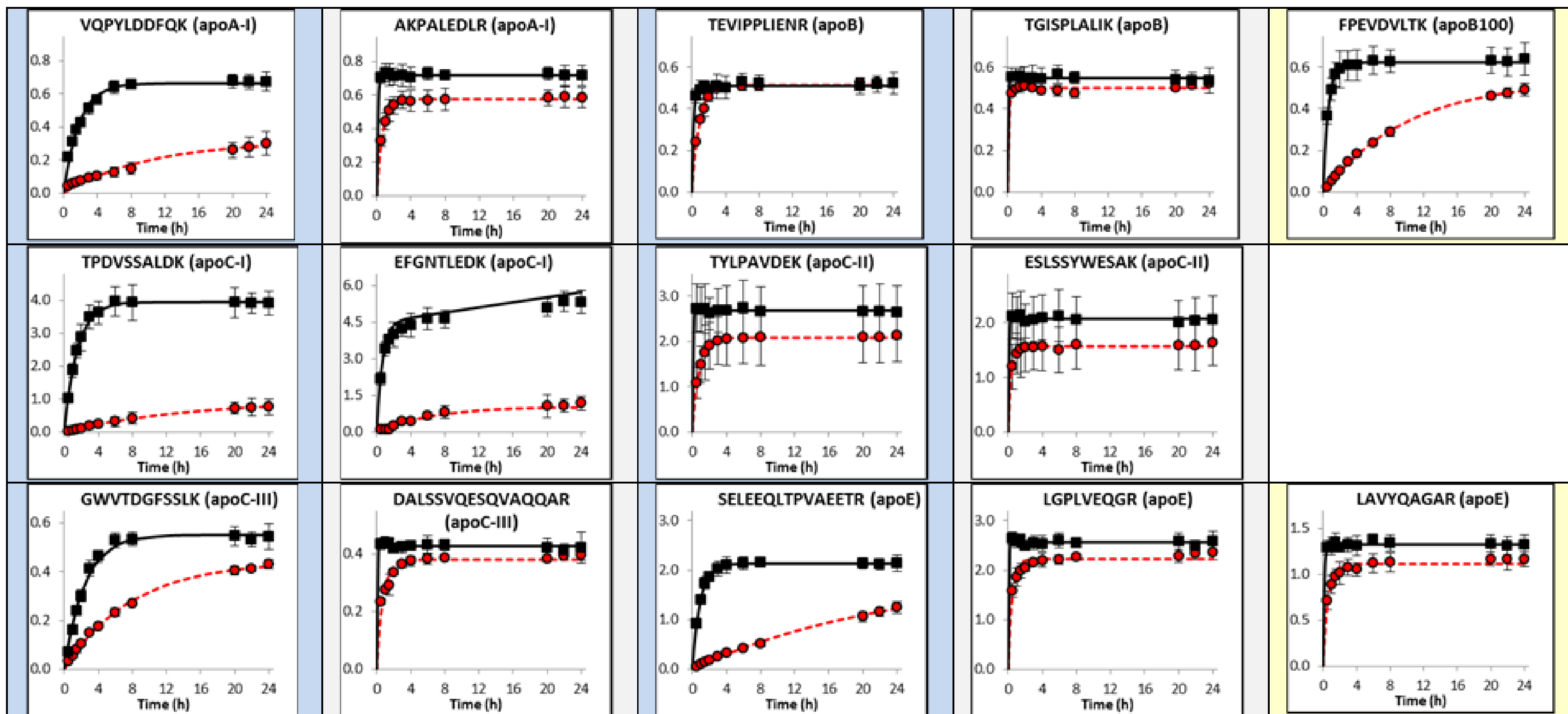
Protein	Peptide	Precursor (m/z)	CE (eV)	Ion 1 quantifier	Ion 2 qualifier 1	Ion 3 qualifier 2
apoA-I	VQPYLDDFQK*	626.8	18	1025.5	765.4	228.1
	AKPALEDLR	506.8	14	813.4	716.4	288.2
apoB	TEVIPPLIENR*	640.9	15	838.5	951.6	443.2
	TGISPLALIK	506.8	13	741.5	854.6	654.5
apoB-100	FPEVDVLTK*	524.3	20	450.8	900.5	803.5
apoC-I	TPDVSSALDK*	516.8	19	466.2	834.4	620.3
	EFGNTLEDK	526.7	15	504.3	605.3	391.2
apoC-II	TYLPAVDEK*	518.3	12	265.1	771.4	658.3
	ESLSSYWESAK	643.8	19	957.4	870.4	620.2
apoC-III	GWVTDGFSSLK*	598.8	18	854.4	953.5	244.1
	DALSSVQESQVAQQAR	858.9	26	1144.6	887.5	573.3
apoE	SELEEQLTPVAEETR*	865.9	28	801.4	1015.3	902.5
	LGPLVEQGR	484.8	20	588.3	701.4	489.2
apoE3 and E4	LAVYQAGAR*	474.8	14	764.4	665.3	502.3
apoE2	$C_{[CM]}$LAVYQAGAR	554.8	20	835.4	764.4	665.3
apoE2 and E4	LGADMEDV$C_{[CM]}$GR	611.8	20	735.3	981.4	866.3
apoE4	LGADMEDVR	503.2	20	764.3	649.3	518.3



2.5 – 11 min
chromatogram

I. Van den Broek et al., Clin Chem, 2016

Optimized digestion efficiency with DOC in 3 hrs



Digestion time curves: previous (red) and optimized (black) conditions

Blue: quantification peptides; Gray: confirmation peptides; Yellow: Differential peptides.

Protocol:	previously described (1-3)	after optimization
	----	——
Buffer:	50 mmol/L ABC pH 8.0	100 mmol/L Tris pH 8.1
Denaturation[1]:	6 mol/L urea	0.33% DOC
Reduction[2]:	1150 pmol DTT	230 pmol TCEP
Alkylation[2]:	3450 pmol IAM	230 pmol IAM

[1] Concentration during incubation; [2] Amount per µl serum

Digestion time curves representing the average relative response (light-to-heavy ratio) of triplicate preparations at 11 time points.
Note that although scaling of the y-axis is arbitrary (absolute peptide recovery is unknown), the intensities between both conditions are proportional.

Van den Broek I. et al., Clin Chem, 2016

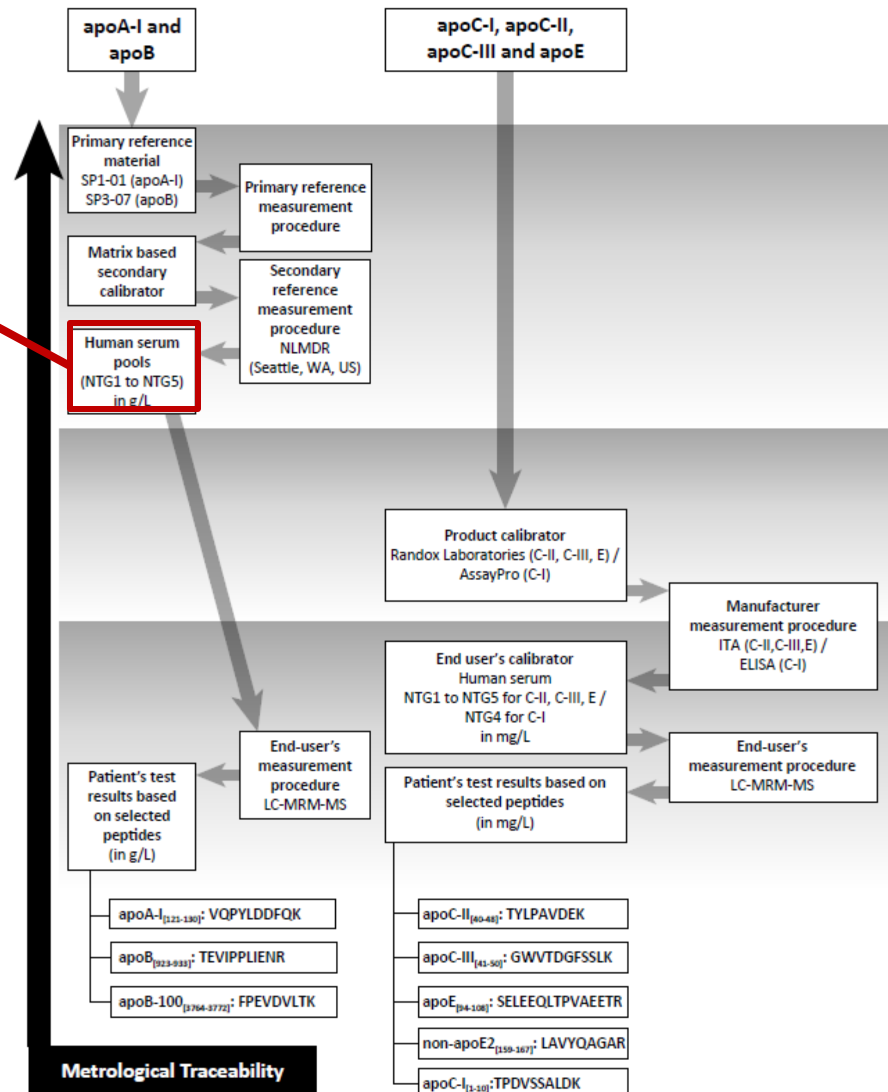
Implementing the Concept of Metrological Traceability

Prerequisites for traceability of apo results:

1. Well defined measurands;
2. **Calibration standards:** CLSI-C37A prepared, value-assigned **native PROTEIN** calibrators traceable to WHO-IFCC RMs;
3. **IS: SIL-PEPTIDES** undergoing the entire workflow, incl. tryptic digestion;
4. **Equimolarity between quantifying peptides and apolipoproteins.**
5. Predefined Analytical Performance Specs.

	Biological Variation		Desirable Performance (%)		
	CV_I	CV_G	I	B	TE_a
Apo A-I	6.5	13.4	**3.3**	3.7	9.1
Apo B	6.9	22.8	**3.5**	6.0	11.6

www.westgard.com



Additional challenges for Apo(a) /Lp(a) standardization

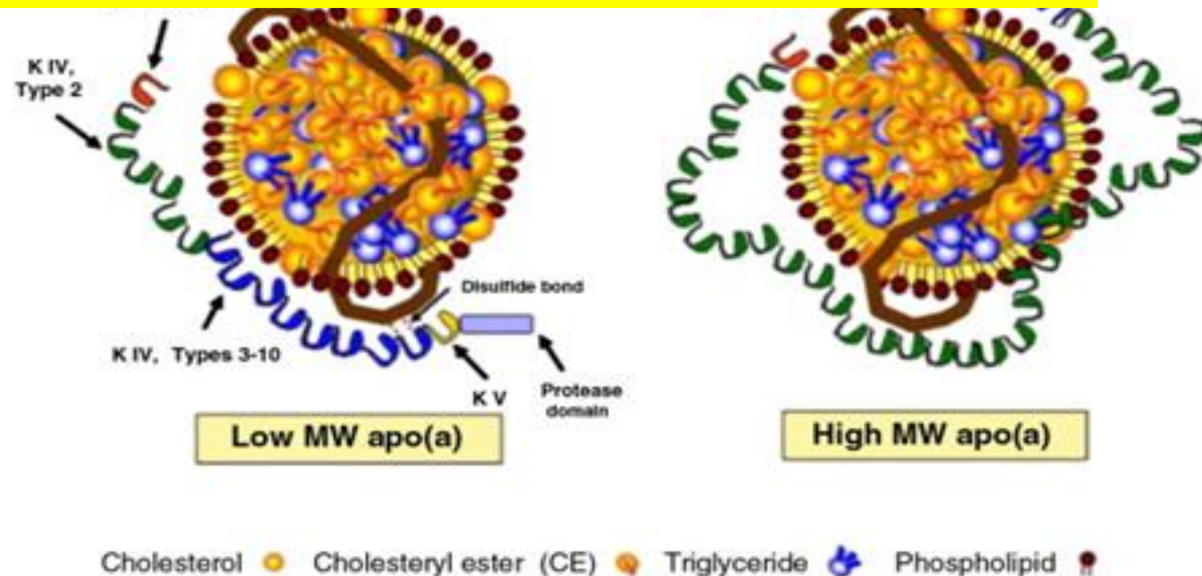
1. **LDL particle with apoB-100 linked with apo(a).**
2. **Lp(a) concentration dependent on apo(a) size, highly dependent on genetics.**
3. **Biologic function uncertain; thought to be thrombogenic given homology to plasminogen and/or to be atherogenic given homology with LDL**

Lp(a) mass: a massively misunderstood metric!

McConnell et al., JCL 2014

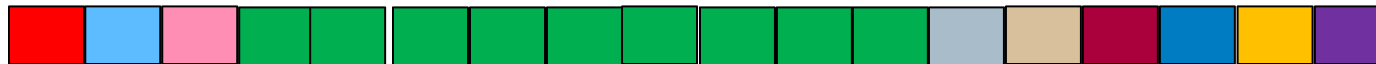
6 (left) and 35 (right) KIV-2 repeats. KIV-1 and KIV-3-10 are identical in all apo (a) isoforms. Guadagno et al.

Smaller apo (a) with lower KIV-2 repeats is associated with higher Lp(a) concentration and increased risk for CVD and calcific aortic valve stenosis (CAVS) (Tsimikas S., JACC, 2017).



Apo(a) size polymorphism: requirements for accurate **MOLAR** quantitation

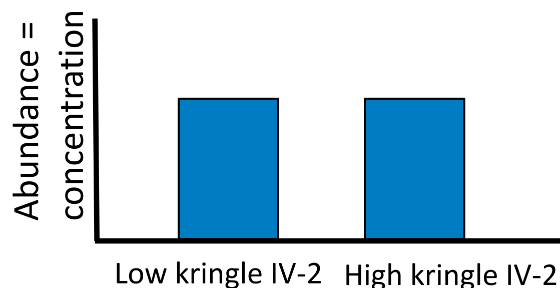
Apo(a) with few kringle IV-2 repeats



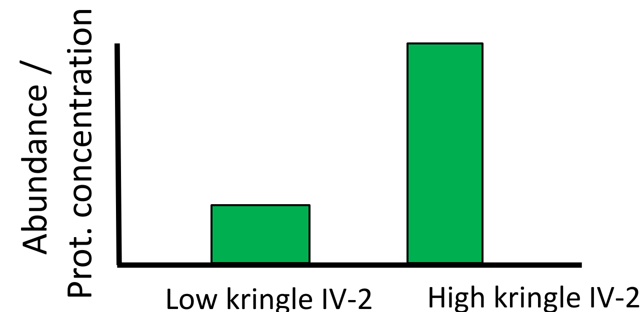
Apo(a) with many kringle IV-2 repeats

1. Quantification of serum apo(a) should be performed on a non-kringle IV-2 peptide (non-green), while the number of kringles might be indicated by a kringle IV-2 peptide (green).
2. The non-kringle IV-2 peptide reflects the apo(a) concentration, while the relative abundance of the kringle IV-2 peptide to the concentration reflects the number of kringles.

Non-kringle IV-2 specific signature peptide



Kringle IV-2 specific signature peptide



B. Development of Reference Materials by IFCC WG APO-MS

Starting point: striving for SI-traceability with molar concentrations!

Under investigation in the WG:

a. General calibration strategy:

Peptide-based calibration (CDC) versus native protein-based calibration (LUMC) of MS-based multiplex apo test, beyond SIL-peptide internal standardization?

Required: complete digestion and commutability of matrix-based RMs

b. Apo(a) specific calibration strategy:

Apo(a) specific transgenic pig apo (a) with defined number of KIV-2 repeats

V. MS-based proteomics:

potential for **SI-TRACEABLE** standardization of both

well defined and heterogeneous protein analytes?

Conclusions so far

1. Well defined apoprotein analytes in the top of the traceability chain, including apo E isoforms.

2. Selective, direct and mass-independent measurement of specific apolipoproteins by LC-MRM-MS.

3. Multiplexing capabilities, theoretically enabling the develpment of one candidate RMP.

4. Co-development of well characterized secondary Reference Materials, value-assigned by peptide-based methods, and providing an anchor for SI-traceability of serum apo tests.

Leiden University Medical Center

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IFCC WG APO STANDARDIZATION BY MASS SPEC

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Manfred Wuhrer

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Leiden University Medical Center

Thanks for your attention

QUESTIONS?