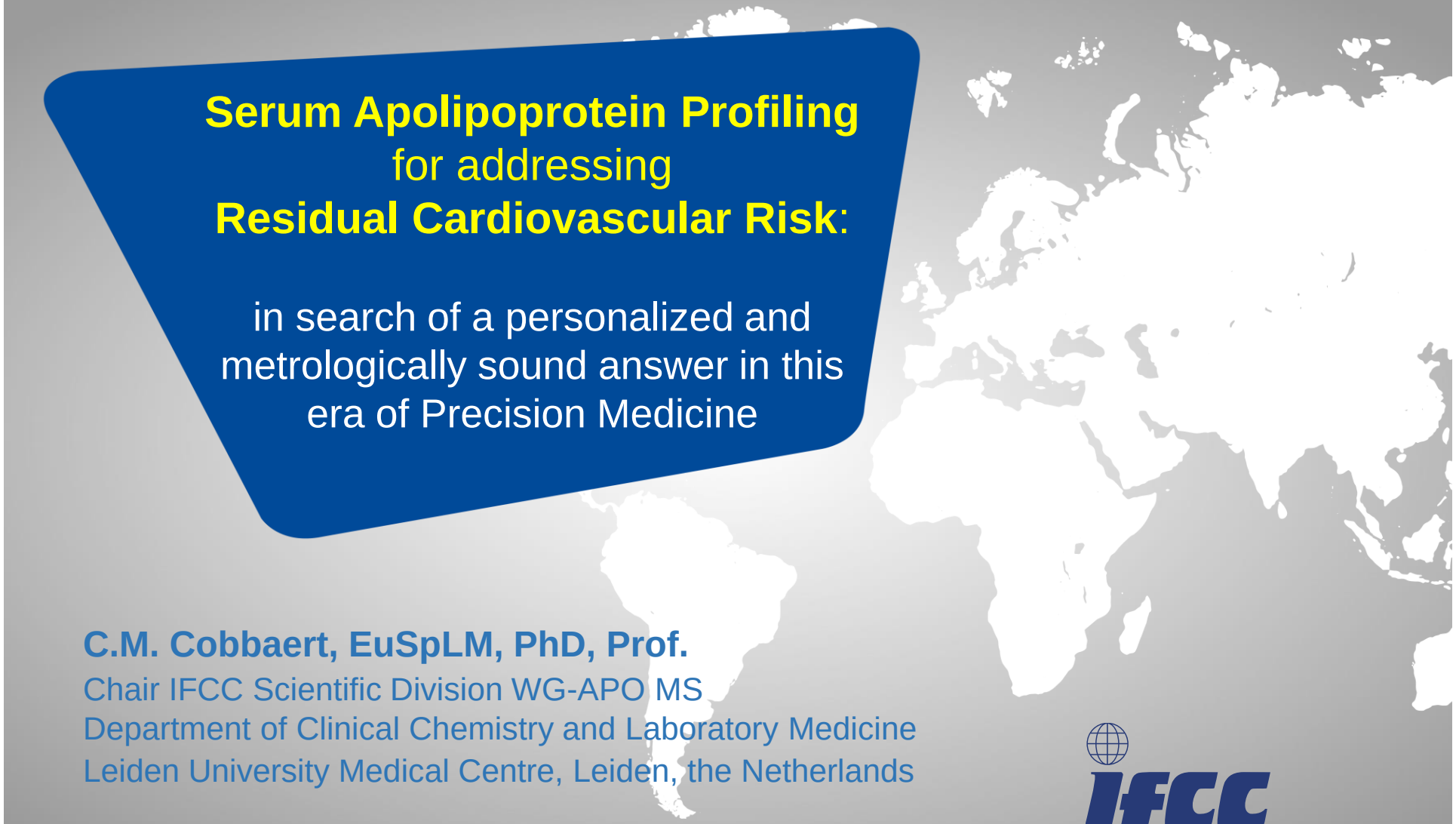
Serum Apolipoprotein Profiling
for addressing
Residual Cardiovascular Risk:

in search of a personalized and metrologically sound answer in this era of Precision Medicine



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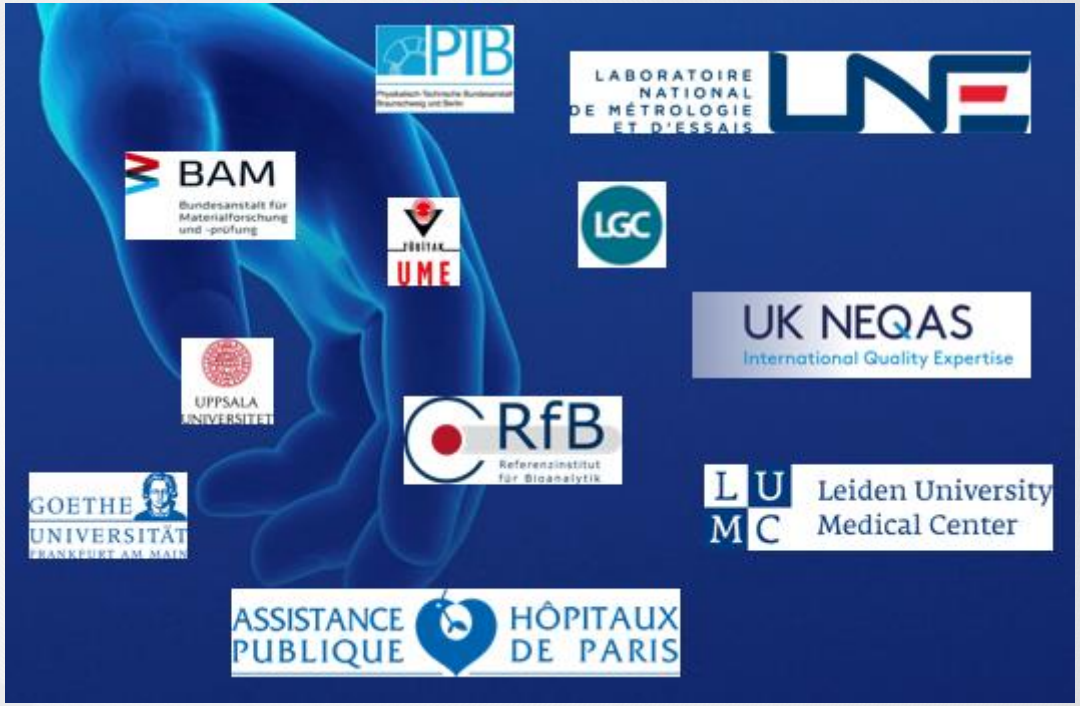
ifcc
International Federation of Clinical Chemistry and Laboratory Medicine

Disclosures

JRC
EUROPEAN COMMISSION



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CardioMet Project & Consortium:

Providing the measurement infrastructure to allow quantitative diagnostic methods for biomarkers of coronary heart diseases

Content

I. **Trends: Healthcare System development, Technological Advances & Biomarker (R)Evolution**

II. **Unmet Clinical Needs for CVRM anno 2019**

III. **How to address Unmet Clinical Needs for CVRM?**
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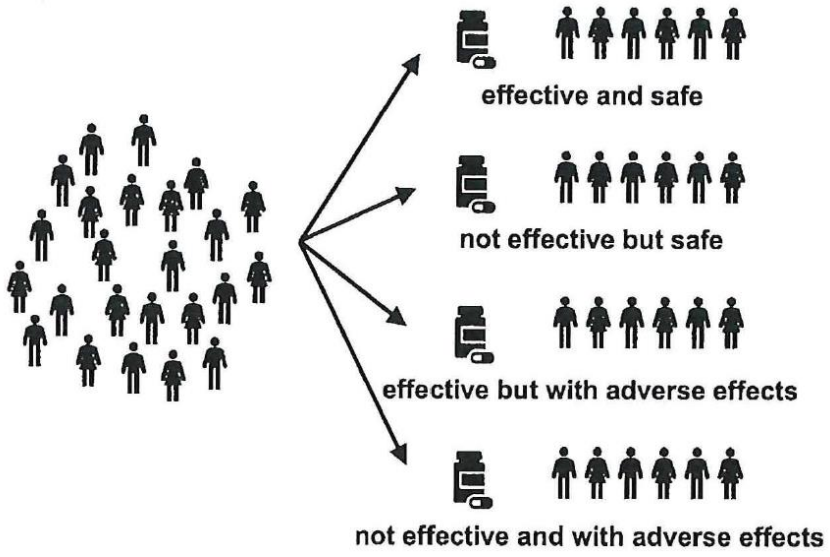
   B. Evidence for CV Precision Medicine with serum Apolipoprotein Profiling

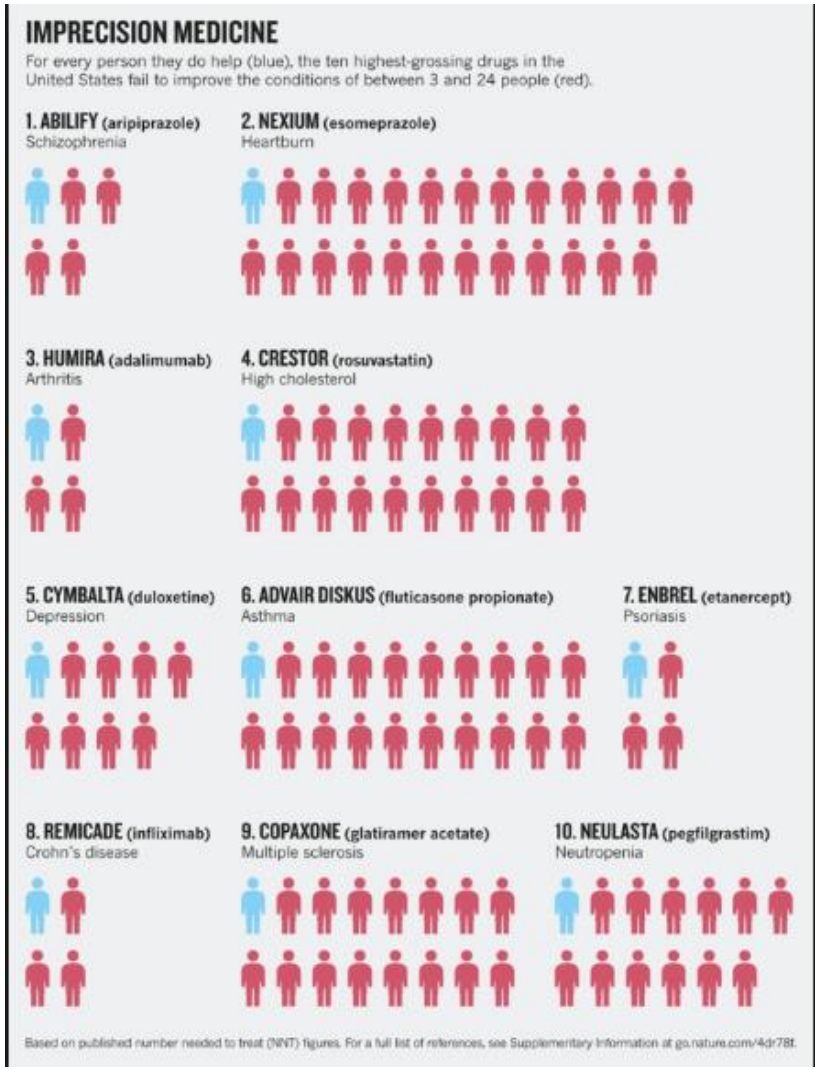
IV. **Establishing multiplexed MS-based Apolipoprotein tests**

V. **Conclusions: paving the way for Cardiovascular Precision Diagnostics**

I. Healthcare System Development

Current practice:
targeting the hypothetical average





Healthcare System Development

Current practice:

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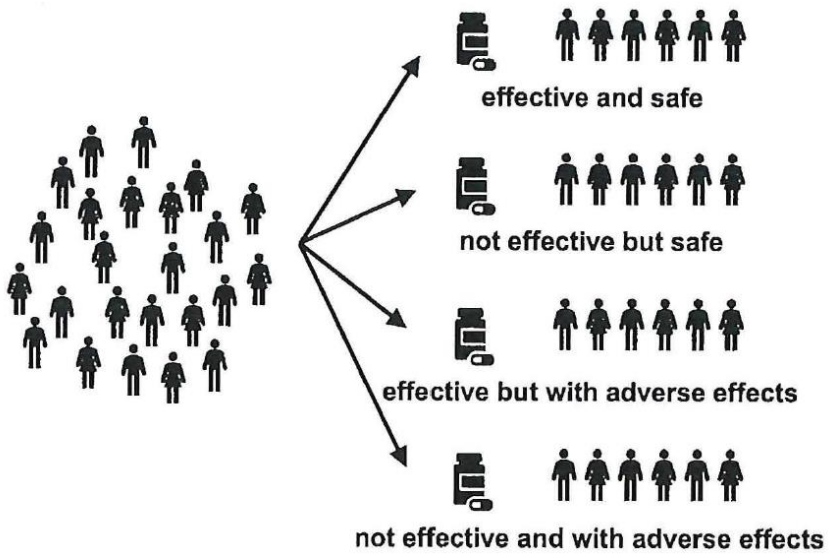
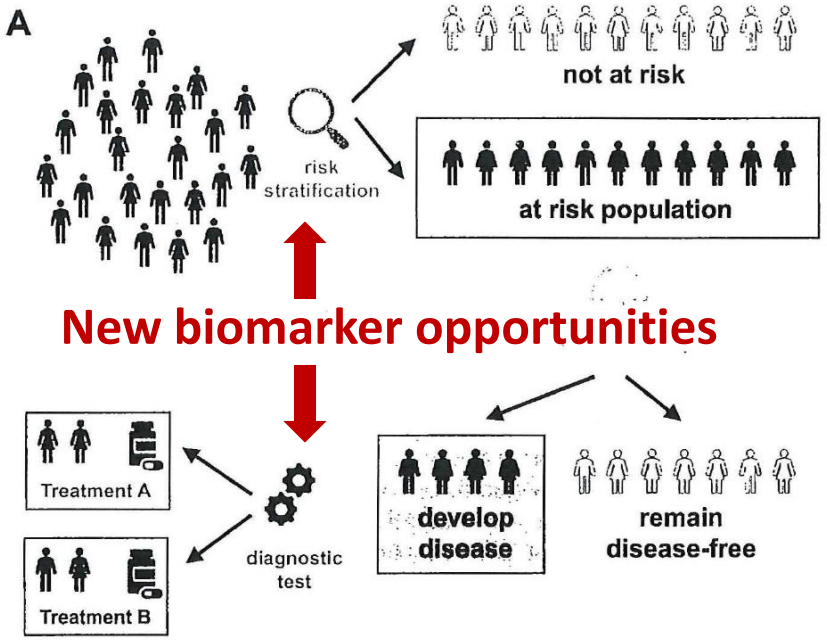


Evolving practice:

Precision Medicine



More precise definitions of health /disease & more precise characterization of populations / patients are needed with potential to translate into targeted therapeutics with improved response rate.

Technological Advances

New technologies change the ways health status can be assessed!

Mobile health (m-health) devices and sensors

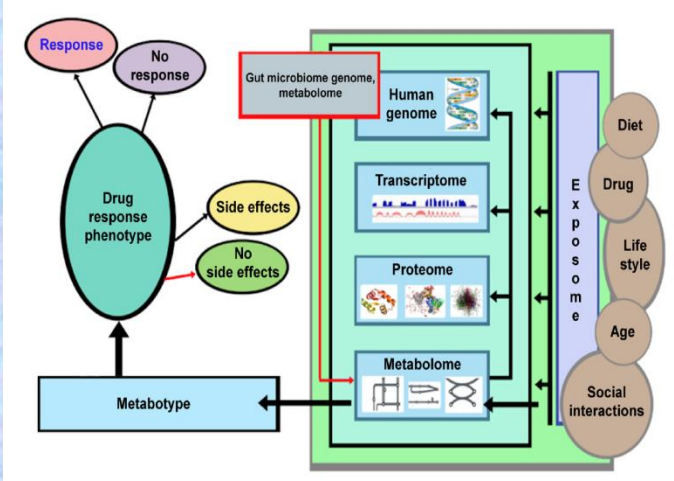
revolutionized the measurement of human dynamic physiology

e.g. BP, heart rhythm, brain waves, air quality…

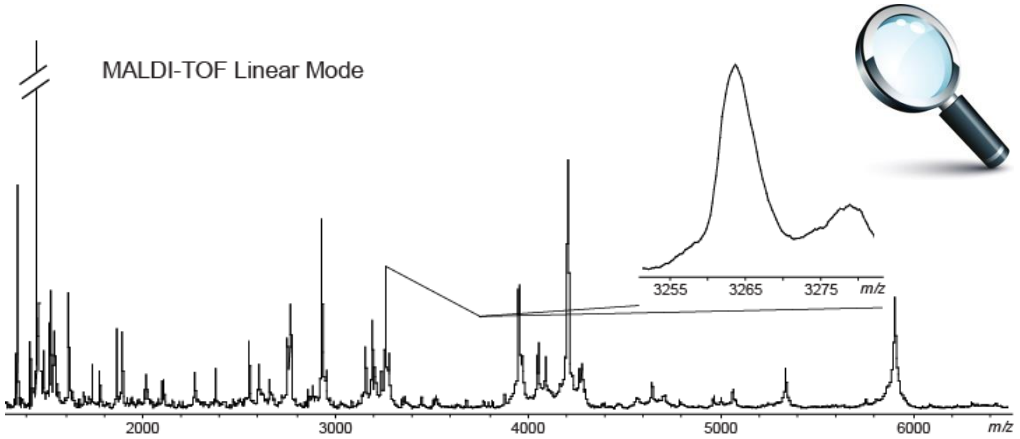
Deriving a phenotypic repertoire at scale

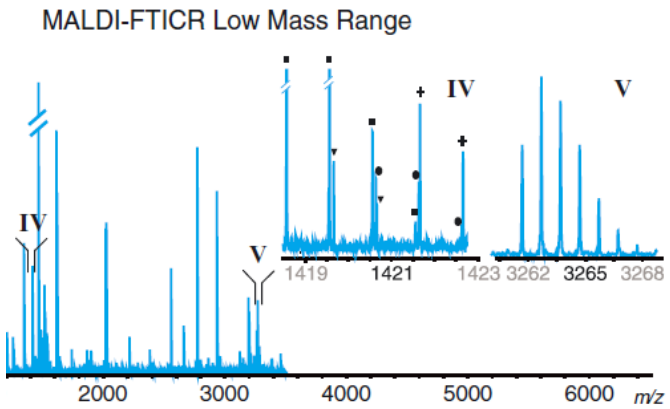
Finding novel pathways by leaving empirical science's tendency to mostly build on known paradigms.

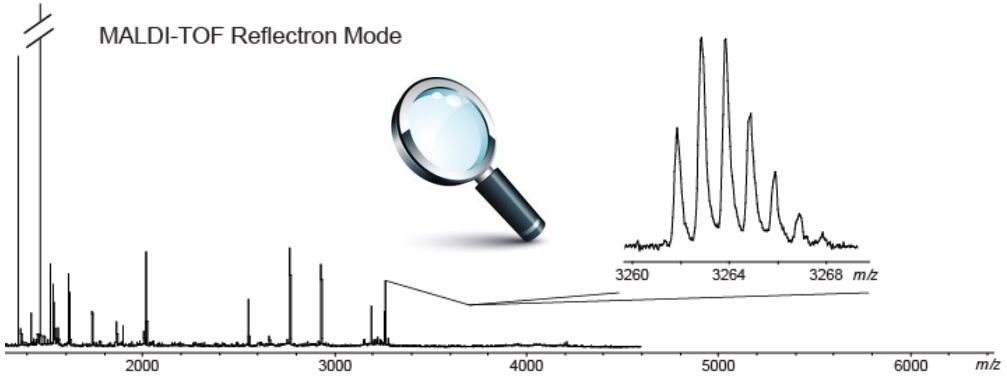
To fully redeem the promise of precision medicine, we should integrate data on all fronts from genomes to phenomes.

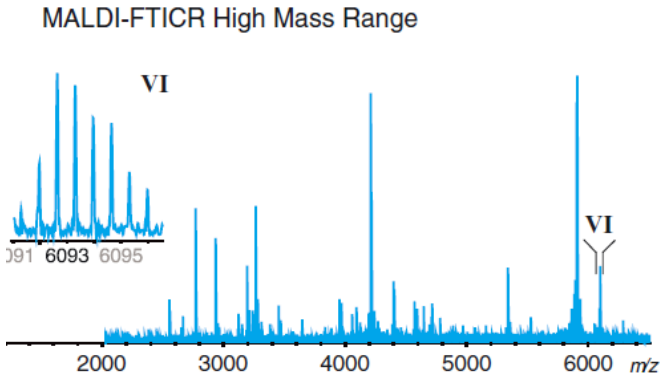


Zoom in *mass spectrum* provides new views









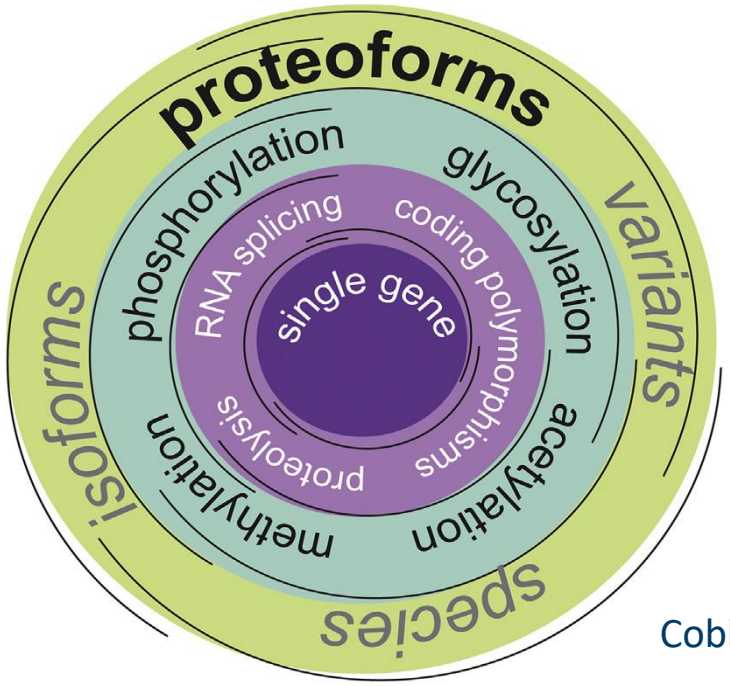
Resolving power of a mass spectrometer:
From early day hundreds (1980s) to state-of-the-art millions

Zoom provides detail and new views

from the Netherlands, to Leiden, to the LUMC, to our routine lab…

PROTEOFORM Hypothesis

The term proteoform was proposed in 2012 "**TO DESIGNATE ALL OF THE DIFFERENT FORMS IN WHICH THE PROTEIN PRODUCT OF A SINGLE GENE CAN BE FOUND**" (Neil Kelleher)



Cobbaert et al. Clin Lab Med 2018

followed in 2014 by the hypothesis that "**INTACT PROTEOFORMS REPRESENT A CLASS OF MOLECULES FOR USE AS BIOMARKERS OF DISEASE STATES**"

Biomarker (R)Evolution

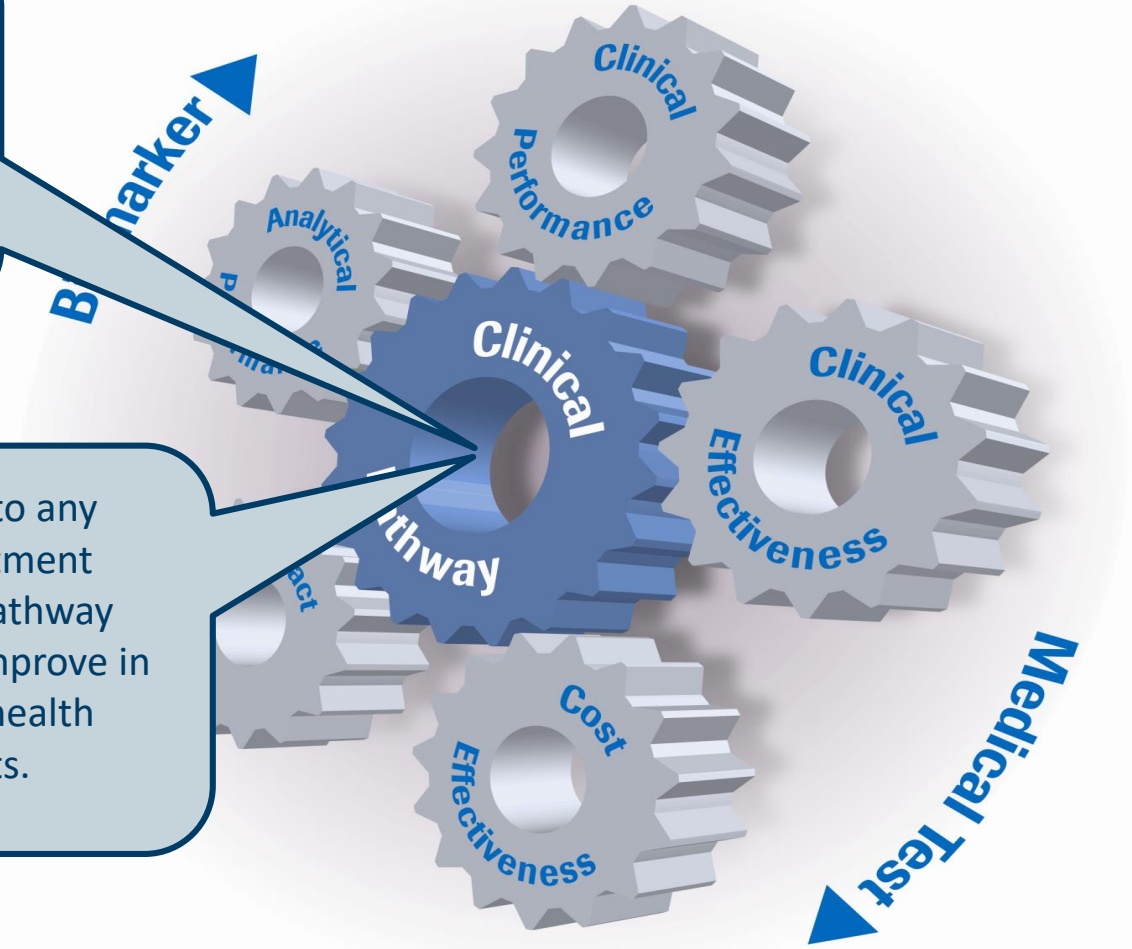
Biomarker qualification at PRESENT	Biomarker qualification in the FUTURE
Identified in **observational studies**	**Large data**, **open-discovery approach with *-omics*, big data and PM**
Association with disease	**Inter-related Molecular Alterations**, organized into **mechanistic pathways**
Unimarker tests, with heterogenous mixture of measurands.	**Multimarker PANELS** with well characterized molecular forms.
Surrogate measures at best with weak or unclear relation to patient outcome.	**Molecular Markers** reflect disease course, give mechanistic insight, or are usable as therapeutic target*.

***The closer a test is related to the pathophysiology of disease and to the mechanism of action of the proposed therapy, the better will be its precision and thereby its usefulness as stratification tool or to inform personalized approaches.**

II. Numerous Unmet Clinical Needs for CVRM!?

Is there an unmet clinical need and is there an effective intervention?

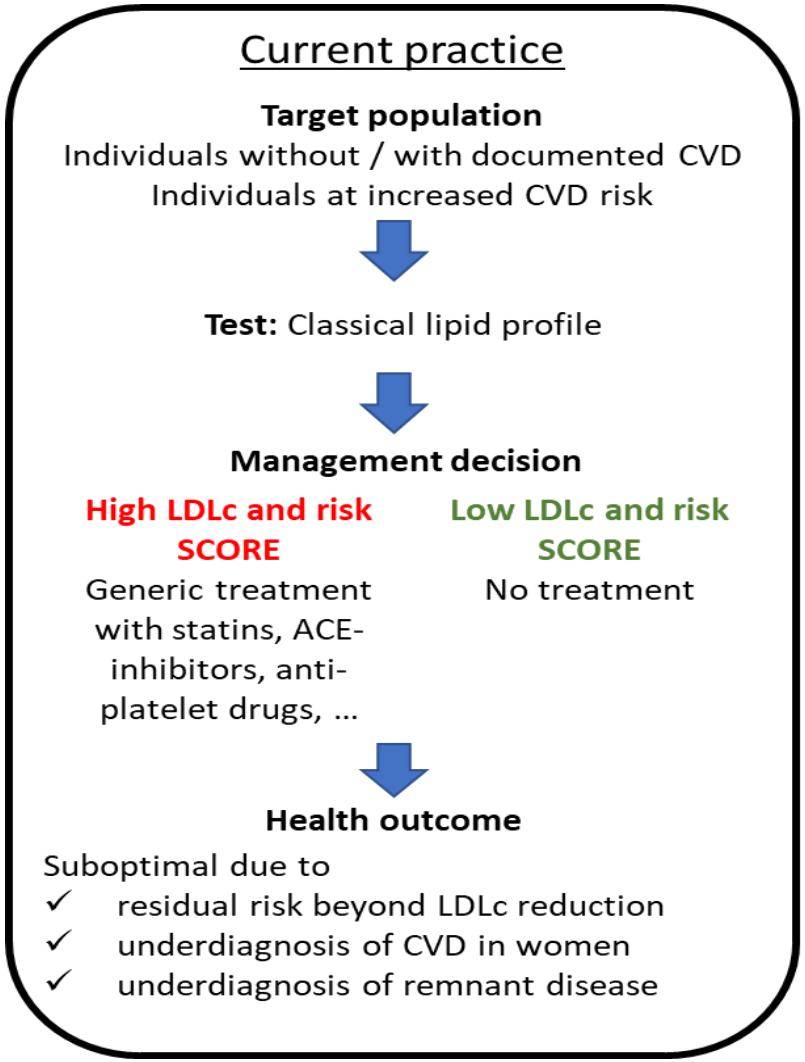
Clinical need refers to any desirable testing or treatment component of a clinical pathway where existing care could improve in order to achieve better health outcomes for patients.



EFLM EUROPEAN FEDERATION OF CLINICAL CHEMISTRY AND LABORATORY MEDICINE WG-TE

From biomarkers to medical tests: the changing landscape of Test Evaluation. Horvath AR et al., CCA 2014

Current Clinical Pathway for CVRM

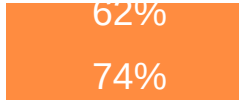


Ruhaak et al., ACB, 2019

↔ Total cholesterol
Total triglycerides
Direct HDLc
Direct or calculated LDLc

Unmet Clinical Needs with LDL-C lowering

Trial (N)	Statin treatment	Clinical events* Risk reduction vs. placebo
WOSCOPS** (6595)	Pravastatin 40 mg	31%
AFCAPS/TexCAPS** (6605)	Lovastatin 20 or 40 mg	40%
ASCOT-LLA** (10,305)	Atorvastatin 10 mg	38%
4S** (4444)	Simvastatin 20 mg	26%
CARE*** (4159)	Pravastatin 40 mg	24%
LIPID*** (9014)	Pravastatin 40 mg	24%
HPS*** (20,536)	Simvastatin 40 mg	27%
PROSPER*** (5804)	Pravastatin 40 mg	24%



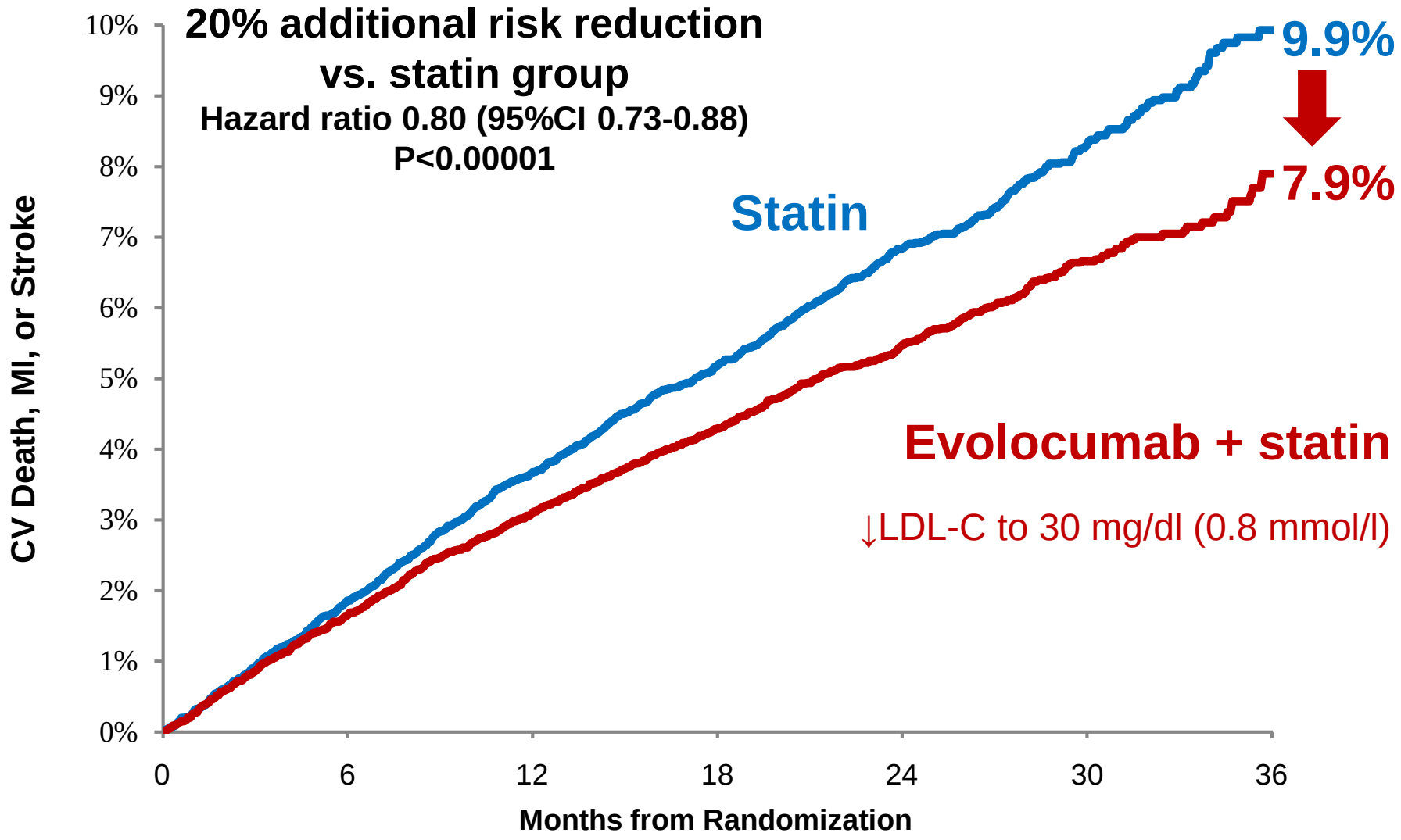
*Nonfatal and fatal myocardial infarction

**Primary prevention trial; ***Secondary prevention trial

→ Further reduce LDL-C ?

→ Further investigate and treat other factors responsible for residual risk?

Residual risk remains high on PCSK9 inhibition



Sabatine MS et al. N Engl J Med 2017;376:1713-22

Unmet Clinical Needs beyond LDLc

Ruhaak et al., ACB, 2019

Current practice

Target population

Individuals without / with documented CVD
Individuals at increased CVD risk

Test: Classical lipid profile

Management decision

High LDLc and risk SCORE	Low LDLc and risk SCORE
Generic treatment with statins, ACE-inhibitors, anti-platelet drugs, …	No treatment

Health outcome

Suboptimal due to

- ✓ residual risk beyond LDLc reduction
- ✓ underdiagnosis of CVD in women
- ✓ underdiagnosis of remnant disease

Clinical Chemistry 65:2
225-227 (2019)

Editorials

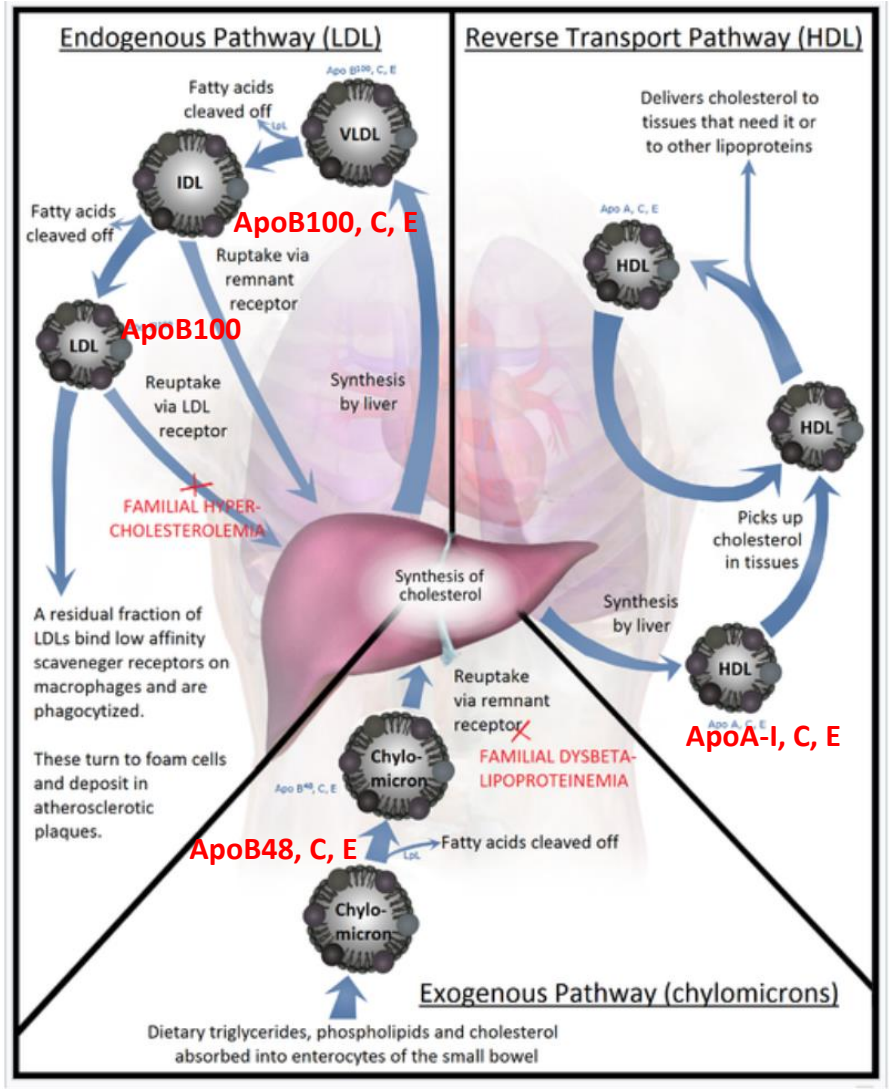
Type III Hyperlipoproteinemia: The Forgotten, Disregarded, Neglected, Overlooked, Ignored but Highly Atherogenic, and Highly Treatable Dyslipoproteinemia

Allan D. Sniderman[1*]

Cardiovascular risk is so high in type III hyperlipoproteinemia that type III, just like heterozygous familial hypercholesterolemia (FH)[2], is a treat-on-diagnosis disorder *(1–3)*. Tragically, although severe hypercholesterolemia is easy to recognize, type III hyperlipoproteinemia cannot be diagnosed using a conventional lipid panel *(4)* and the original diagnostic tools—electrophoresis and ultracentrifugation—are available in only a miniscule number of clinics *(1)*. Thus, type III cannot be diagnosed in regular clinical care using regular diagnostic tools. Moreover, while the importance of FH is recognized by all the major lipid guidelines, even the existence of type III hyperlipoproteinemia is barely acknowledged, if at all, by the same groups. Consequently, those patients with type III are lumped and dumped with all the others with mixed hyperlipidemia and the result, tragically, can be no treatment when treatment is indicated. In a wonderful paper *(3)*, Paul Hopkins and his colleagues referred to type III hyperlipoproteinemia as the "forgotten phenotype." Nothing has changed and so the extended title of this editorial.

The «FORGOTTEN» phenotype

https://en.wikipedia.org/wiki/Lipoprotein



Simplified flowchart showing the essentials of (apo)lipoprotein metabolism

III. How to address Unmet Clinical Needs for CVRM?

Clinical Chemistry 64:7
1006–1033 (2018)

Special Report

Quantifying Atherogenic Lipoproteins: Current and Future Challenges in the Era of Personalized Medicine and Very Low Concentrations of LDL Cholesterol. A Consensus Statement from EAS and EFLM

Michel R. Langlois,[1*] M. John Chapman,[2] Christa Cobbaert,[3] Samia Mora,[4] Alan T. Remaley,[5] Emilio Ros,[6] Gerald F. Watts,[7] Jan Borén,[8] Hannsjörg Baum,[9] Eric Bruckert,[10] Alberico Catapano,[11] Olivier S. Descamps,[12] Arnold von Eckardstein,[13] Pia R. Kamstrup,[14] Genovefa Kolovou,[15] Florian Kronenberg,[16] Anne Langsted,[14] Kari Pulkki,[17] Nader Rifai,[18] Grazyna Sypniewska,[19] Olov Wiklund,[8] and Børge G. Nordestgaard,[14] for the European Atherosclerosis Society (EAS) and the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Joint Consensus Initiative

EUROPEAN FEDERATION OF CLINICAL CHEMISTRY AND LABORATORY MEDICINE

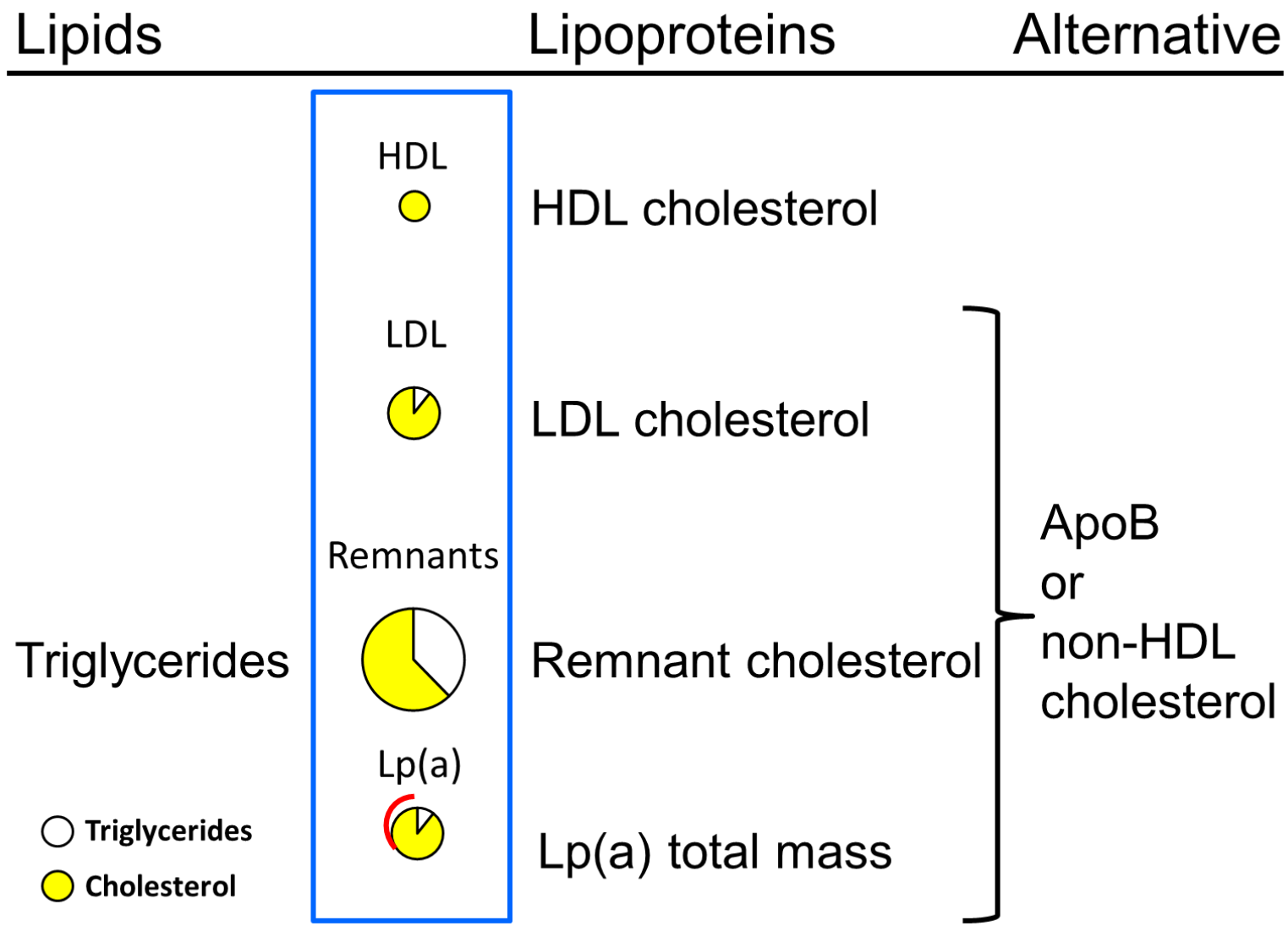
EAS-EFLM Consensus

How to address unmet clinical needs?

1. **Which atherogenic lipoproteins should be measured?**
2. Are we using the appropriate biomarker(s)?
3. Is it time to move from standard LDL-C to advanced (apo)lipoprotein testing strategies?
4. Consensus recommendations

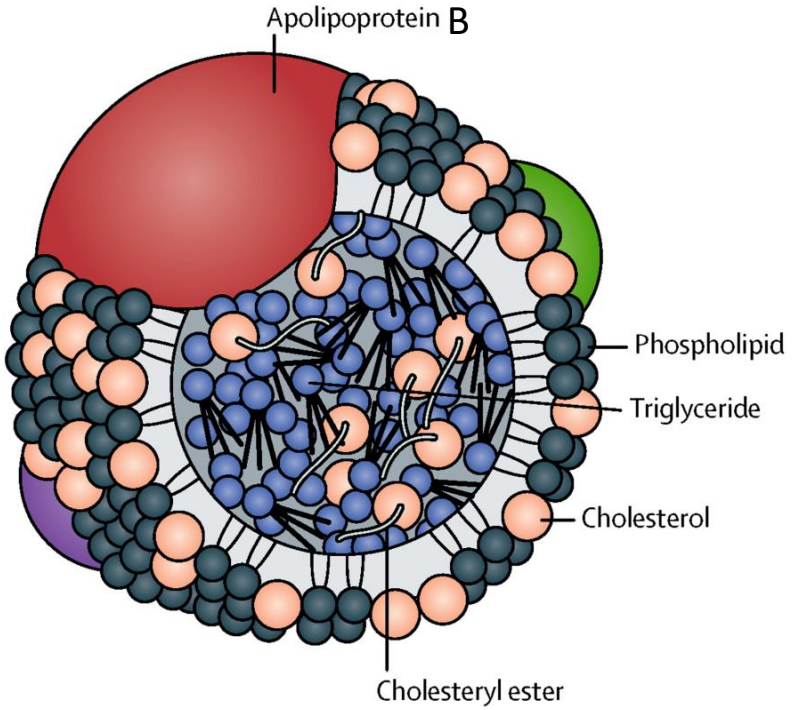
M. Langlois et al. EAS-EFLM Consensus Panel. Clin Chem 2018;64:1006-33.

Unchanged lipid profiling for decades: are we struggling with reductionism!?

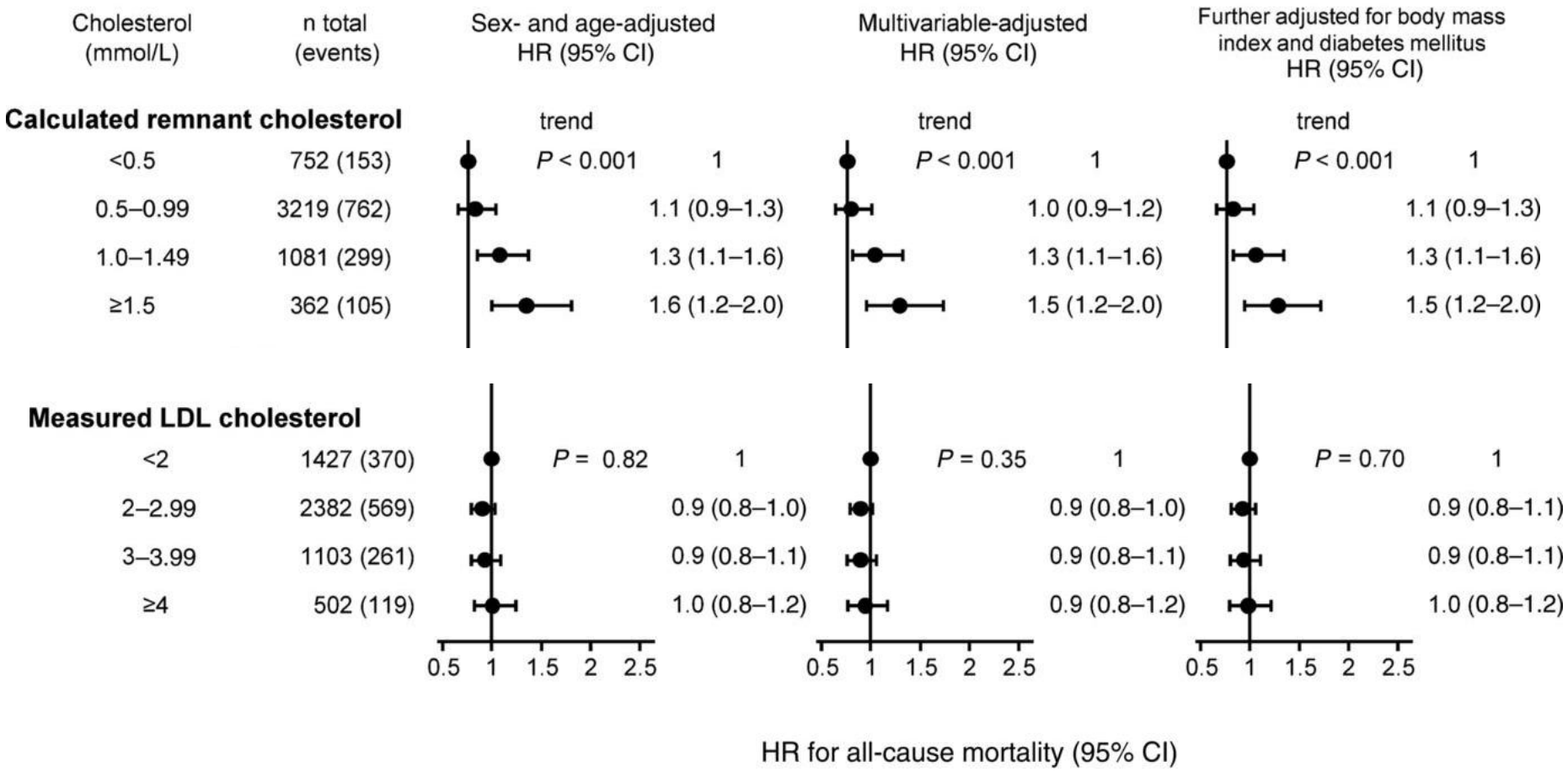


Quantifying Atherogenic Lipoproteins

- **Remnant particles?**
- **LDL-particles?**
- **Lipoprotein(a) particles?**



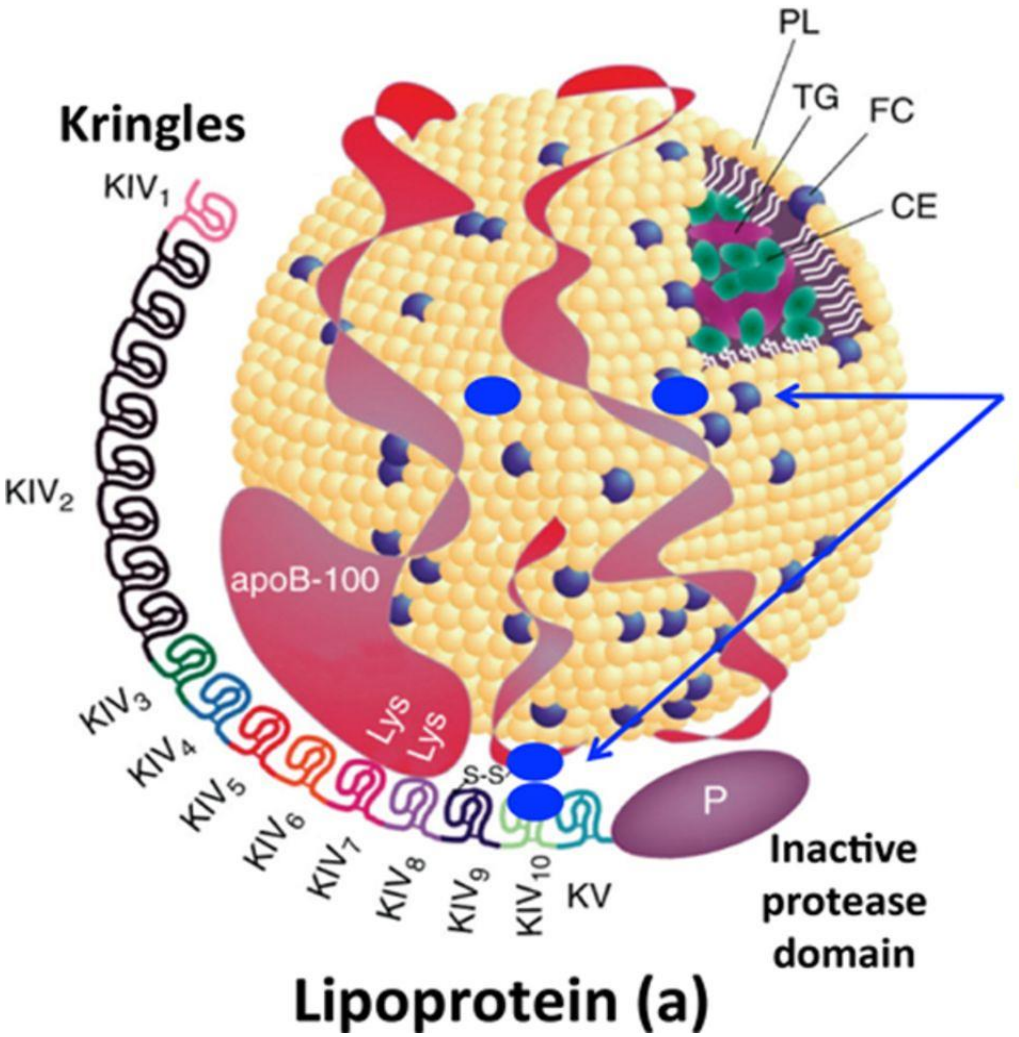
Remnant cholesterol* explains part of Residual Risk of all cause mortality in 5414 patients with IHD



***Calculated as TC – HDL-C – LDL-C**

Jepsen AK et al. Clin Chem 2016;62:593-604

Lipoprotein (a) particle

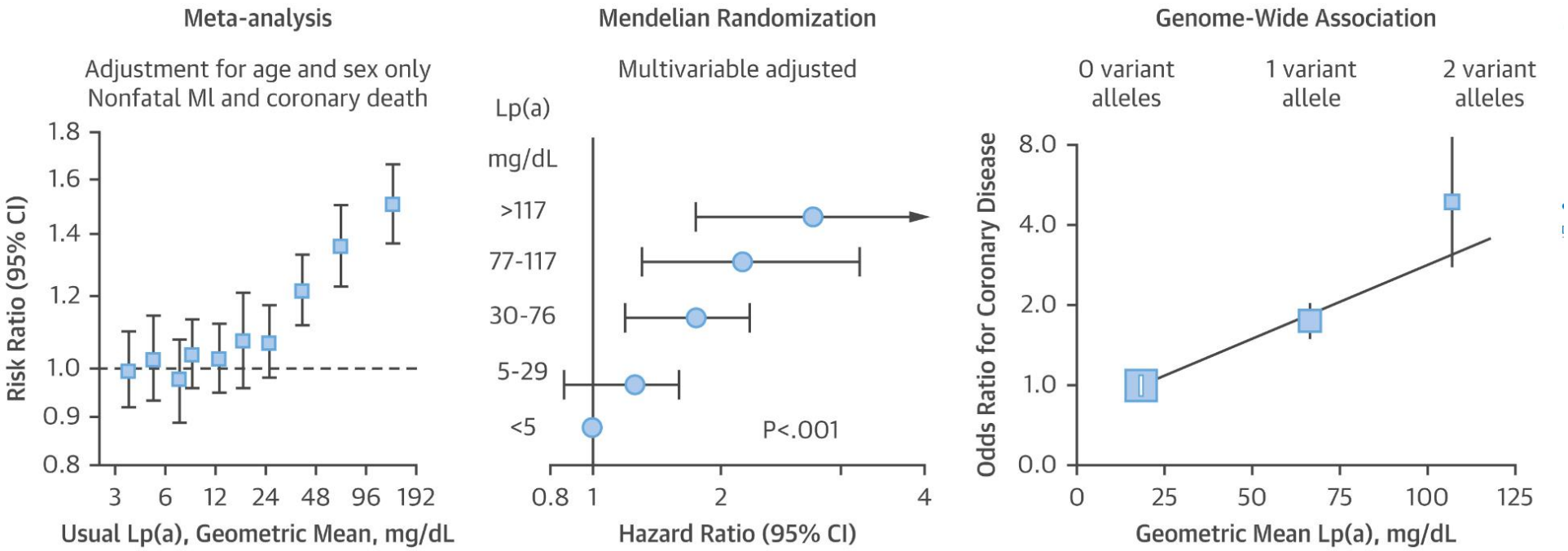


LDL particle + apo(a)

American Heart Association.

Nordestgaard BG et al. Eur Heart J 2010;31:2844-53.

Evidence for Lp(a) as an independent GENETIC CV risk factor



Tsimikas S et al. J Clin Lipidology 2018

EAS-EFLM Consensus

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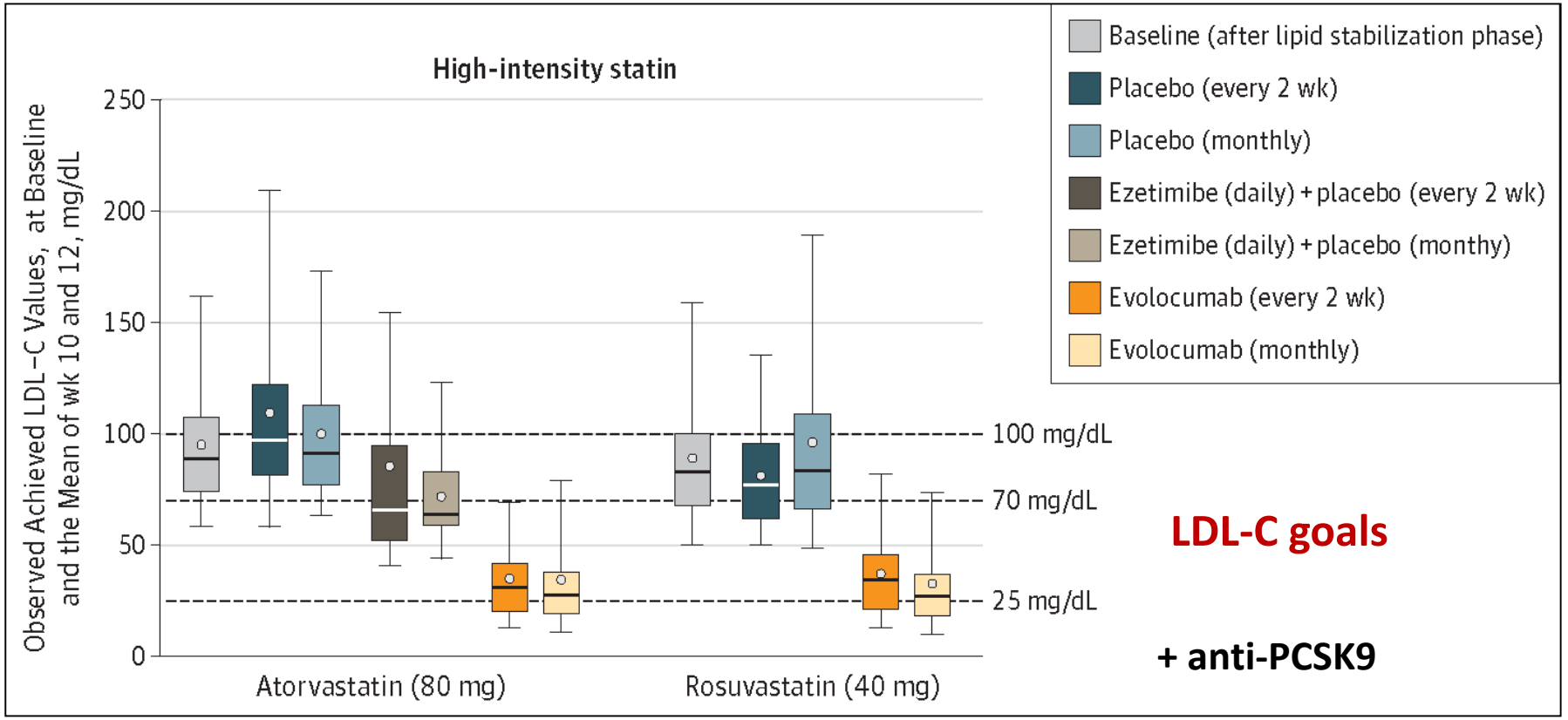
4. Consensus recommendations

M. Langlois et al. EAS-EFLM Consensus Panel. Clin Chem 2018;64:1006-33.

Novel therapies reduce LDL-C to very low concentrations



LDL-C goals

+ anti-PCSK9

The JAMA Network

From: **The LAPLACE-2 Randomized Clinical Trial** JAMA 2014;311:1870-83

Friedewald formula:

fit for the new era of very low LDLC ?

LDLC = TC – HDLC – VLDLC

LDLc: not fit-for-clinical purpose in the low LDLc era

Estimated as TG/5 (mg/dl) or TG/2.2 (mmol/l)

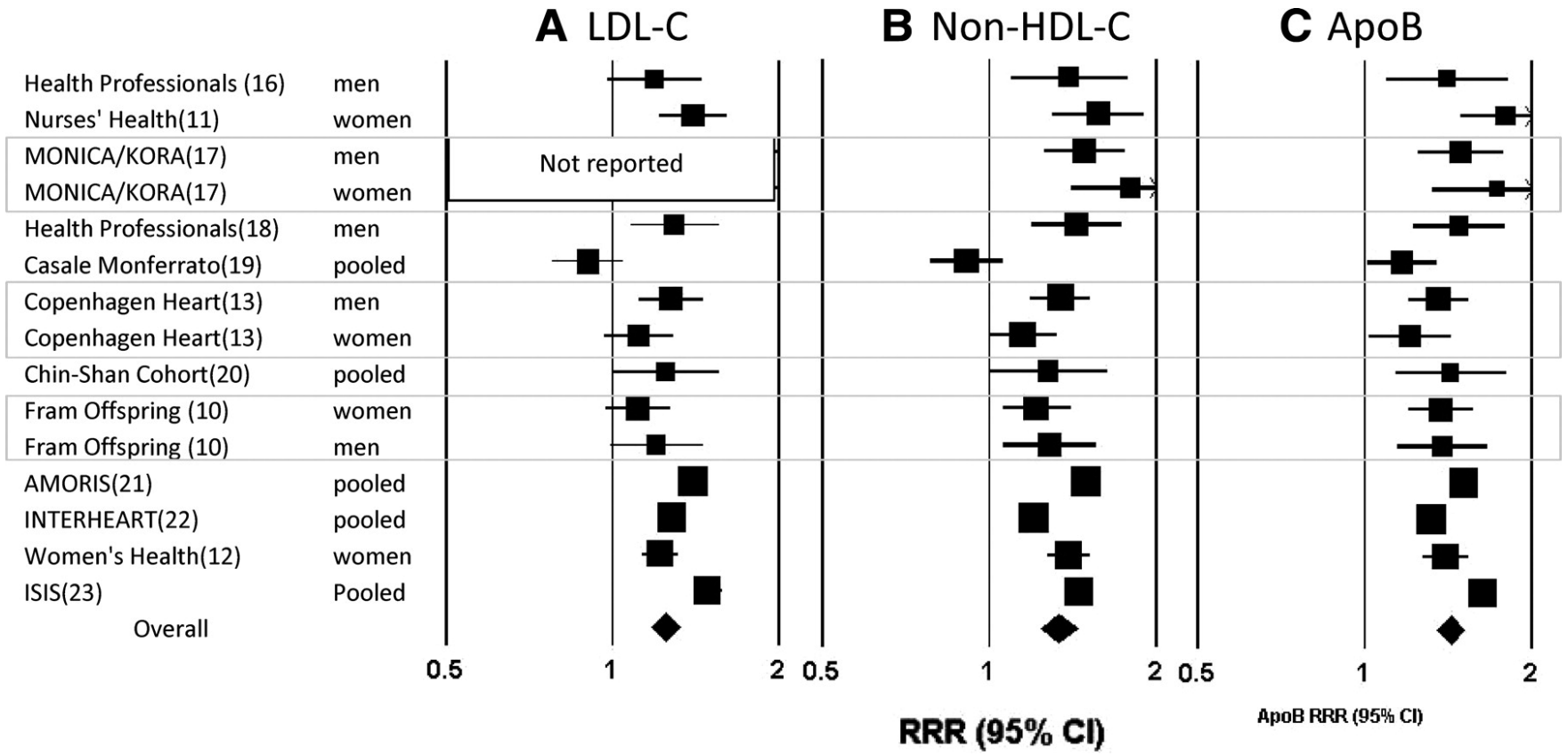
TG/VLDLC ratio >5–10 in TG-rich VLDL

VLDLC overestimated in hypertriglyceridemia

Friedewald WT et al. Clin Chem 1972;18:499-502.

Relative Risk Ratios for LDL-C, non-HDL-C, and apoB in 12 fasting and non-fasting population studies

META-ANALYSIS



LDL-C 1.25 (1.18-1.33) **non-HDL-C 1.34 (1.24-1.44)** **ApoB 1.43 (1.35-1.51)**

Sniderman A. et al. Circ Cardiovasc Qual Outcomes 2011;4:337-345

EAS-EFLM consensus & Cardiovascular Test Performance Characteristics

TEST CHARACTERISTICS	LDL-C	non-HDL-C	ApoB
Analytical performance			
Accurate assays (method independency)	**No**	**No**	**Yes**
Nonfasting measurement possible	With TG<4.5 mmol/l	Yes	Yes
Widely accessible, automated assays	Yes	Yes	Yes
Reasonable operational costs	Yes	No extra cost	Yes
Clinical performance			
Robust associations with incident CVD?	Yes	Yes	Yes
Novel information beyond existing markers?	(Reference)	Yes	Yes
Validated decision limits?	No	No	No
Clinical effectiveness			
Superiority to existing tests?	(Reference)	Probably	Probably
Modifiable risk association (treatment target)?	Yes	Yes	Yes
Biomarker-guided treatment reduces CVD risk?	Yes	Probably	Probably
Cost effectiveness			
Biomarker-guided treatment saves healthcare costs?	Yes	Unknown	Unknown

M. Langlois et al. EAS-EFLM Consensus Panel. Clin Chem 2018;64:1006-33.

EAS-EFLM Consensus

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M. Langlois et al. EAS-EFLM Consensus Panel. Clin Chem 2018;64:1006-33.

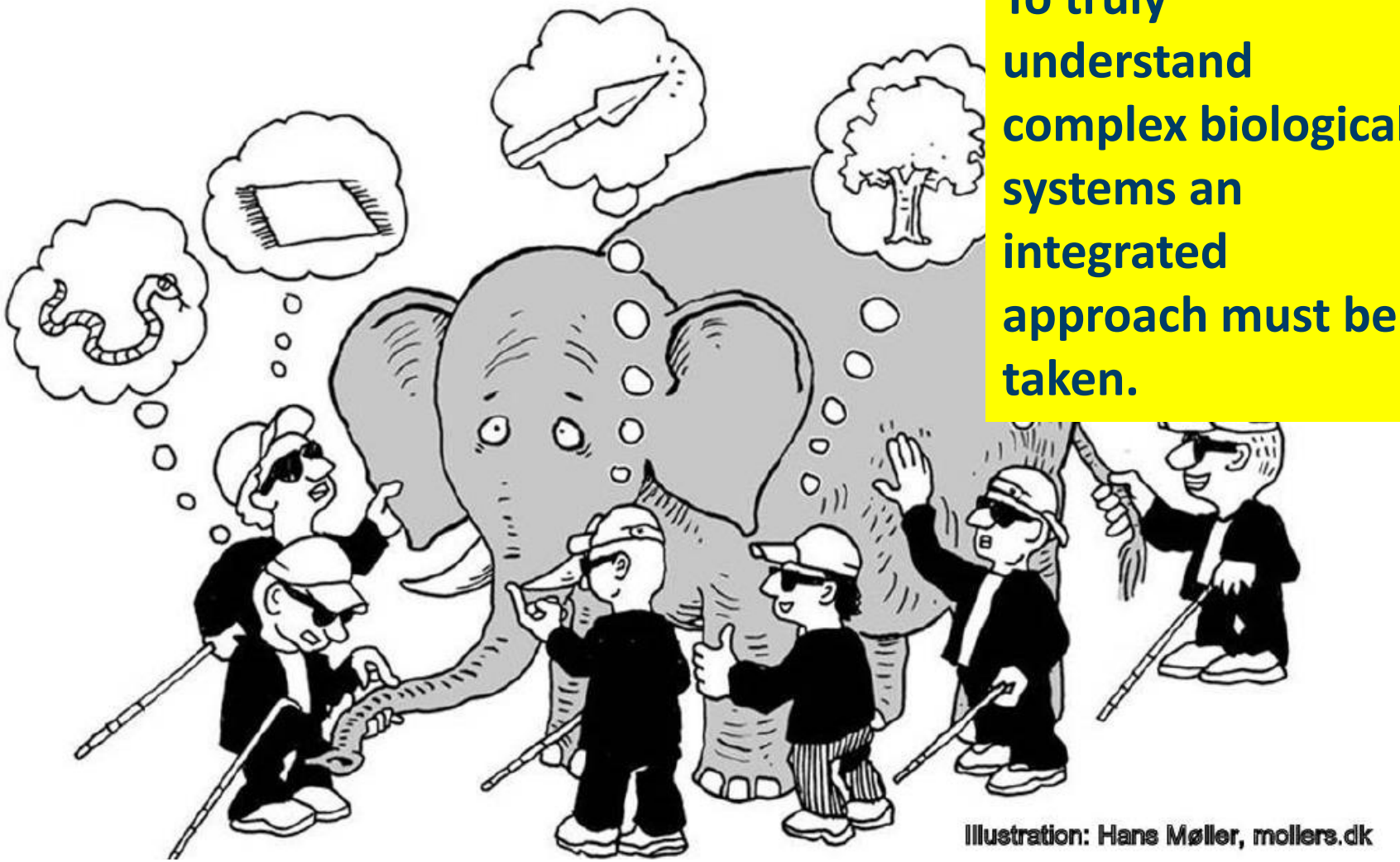
Apolipoproteins:
holy grail for unraveling dyslipidemia?

Apolipoprotein	MW	Primary Source	Lipoprotein Association	Function
Apo A-				
Apo A-				
Apo A-				
Apo A-				
Apo B-				
Apo B-				
Apo C-I	6,600	Liver	Chylomicrons, VLDL, HDL	Activates LCAT
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

No or limited use in clinical guidelines for CVRM so far!

Systems Medicine & the complexity of chronic diseases

Story of 7 blind men and an elephant



To truly understand complex biological systems an integrated approach must be taken.

i. Plasma Apolipoprotein PANEL predicts incident CVD

JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY
VOL. 69, NO. 7, 2017

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PUBLISHED BY ELSEVIER
http://dx.doi.org/10.1016/j.jacc.2016.11.066

EDITORIAL COMMENT

Deep Apolipoprotein Proteomics to Uncover Mechanisms of Coronary Disease Risk*

Bruneck study

Daniel J. Rader, MD,[a,b,c,d,e] Archna Bajaj, MD,[a,b,d,e] Sumeet A. Khetarpal, PhD[a,b,d,e]

Bruneck Population Study

1. **Prospective, population-based survey** of the epidemiology and pathogenesis of atherosclerosis and CVD.

2. Age and sex-stratified random sample of inhabitants of Bruneck, Italy, all of Caucasian descent.

3. Detailed information on **fatal and nonfatal CVD** after **10 year of follow-up**, with follow-up 100% complete for clinical outcomes (N = 688). Clinical outcomes were adjudicated by 1 senior researcher blinded to baseline data.

4. Multiplex measurement of 13 plasma apolipoproteins with **MS-based bottom-up quantitative proteomics**, beyond classical serum lipids.

5. Associations of 13 plasma apolipoproteins and lipids with **incident CVD over 10 years** were studied.

Serum apolipoprotein PANEL predicts incident CVD

JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY VOL. 69, NO. 7, 2017

ISSN 0735-1097
http://dx.doi.org/10.1016/j.jacc.2016.11.065

Very-Low-Density Lipoprotein–Associated Apolipoproteins Predict Cardiovascular Events and Are Lowered by Inhibition of APOC-III

Bruneck study

Raimund Pechlaner, MD, PhD,[a] Sotirios Tsimikas, MD,[b,c] Xiaoke Yin, PhD,[d] Peter Willeit, MD, PhD,[a,e] Ferheen Baig, MSc,[d] Peter Santer, MD,[f] Friedrich Oberhollenzer, MD,[f] Georg Egger, MD,[g] Joseph L. Witztum, MD,[b] Veronica J. Alexander, PhD,[c] Johann Willeit, MD,[a] Stefan Kiechl, MD,[a] Manuel Mayr, MD, PhD[d]

Conclusions from the Bruneck Population Study

1. Apolipoprotein profiling provides **strong epidemiological support to the concept that TRLs contribute to atherosclerosis**.

2. **ApoC-II, apoC-III, and apoE are abundant on VLDL**, which may represent **underappreciated RISK FACTORS for CVD**.

3. Intervention trials with **volanesorsen** demonstrate that targeting apoC-III favorably affects apolipoprotein and lipid profiles. Thus, **lowering VLDL,** in addition to LDL and lipoprotein(a), might represent **a novel strategy to further reduce CVD risk in the statin era,** and should be tested by appropriately designed outcome trials.

ii. MS-based apolipoprotein profiling in a clinical study of patients with STEMI

Journal of Clinical Lipidology (2017) ■, ■–■

Journal of Clinical Lipidology

Original Article

Low levels of apolipoprotein-CII in normotriglyceridemic patients with very premature coronary artery disease: Observations from the MISSION! Intervention study-R2

Maaike P. J. Hermans, MD, Mathijs C. Bodde, MD, Wouter J. Jukema, MD, PhD*, Martin J. Schalij, MD, PhD, Arnoud van der Laarse, PhD, Christa M. Cobbaert, PhD, Pharm, EurSpLM

Secondary prevention trial in ACS patients

MISSION! INTERVENTION STUDY

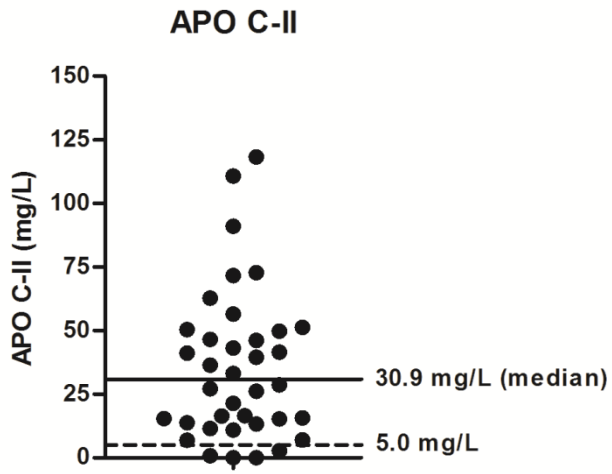
BACKGROUND: while the overall AMI rates declined in women and men, however premature AMI rates remained stable in men and increased in women.

OBJECTIVE: to assess whether baseline apolipoprotein (apo) levels, clinical characteristics, and follow-up of **patients with very premature coronary artery disease (CAD)** could provide novel clues for the identification of high-risk individuals.

METHODS: serum apo panel measured **with a validated quantification LC-MS method** in a well-defined cohort of **patients aged ≤ 45 years admitted with acute STEMI**.

PATIENTS: 1st STEMI who were initially included in the MISSION! Intervention Trial and had 10 years of follow-up

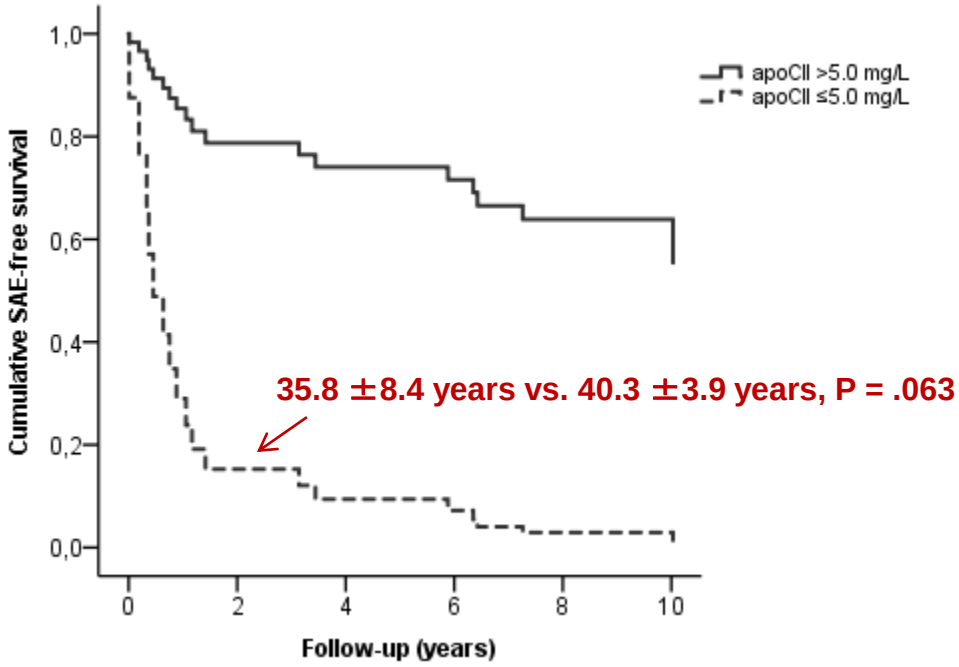
Apo CII “deficient” subgroup with deleterious outcome



Mean age: 39.8 ± 4.6 years and 24% was female.

ApoCII is primarily synthesized in the liver and serum concentrations normally range from 22 to 55 mg/L, which corresponds with a median level of apoCII of 30.9 mg/L in our cohort of patients with very premature CAD.

Apo CII is an activator of LPL.



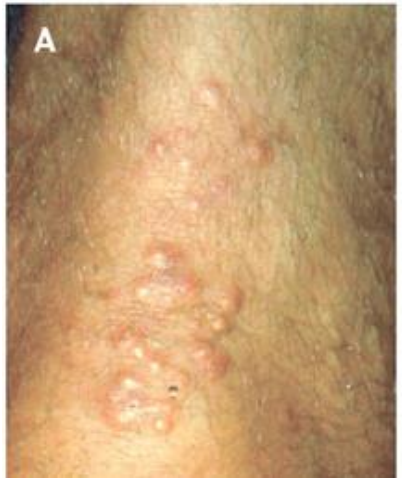
Estimated adverse event free survival (free of re-infarction or revascularization) in premature CAD patients stratified by baseline apo C-II levels.

Classical apo CII deficiency acc. to textbooks

Apo CII is an apoprotein component of VLDL that activates the enzyme LPL, which hydrolyzes TG and thus provides free fatty acids for cells.

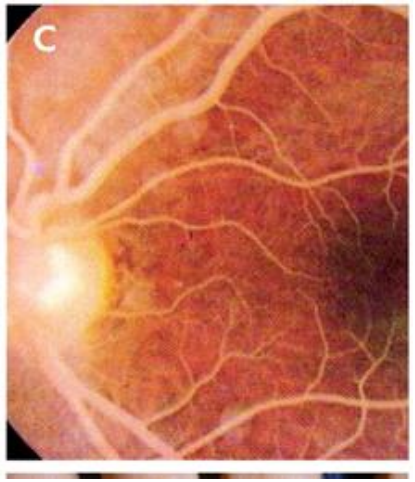
Total lack of apoCII (1:10^6) is assumed to result in **intravascular TG accumulation** because of non-activation of LPL!

Classical apoCII-deficient patients present with eruptive xanthomas, chronic pancreatitis and hepatosplenomegaly, early atherosclerosis as a consequence of fasting chylomicronemia and extremely high levels of TG.

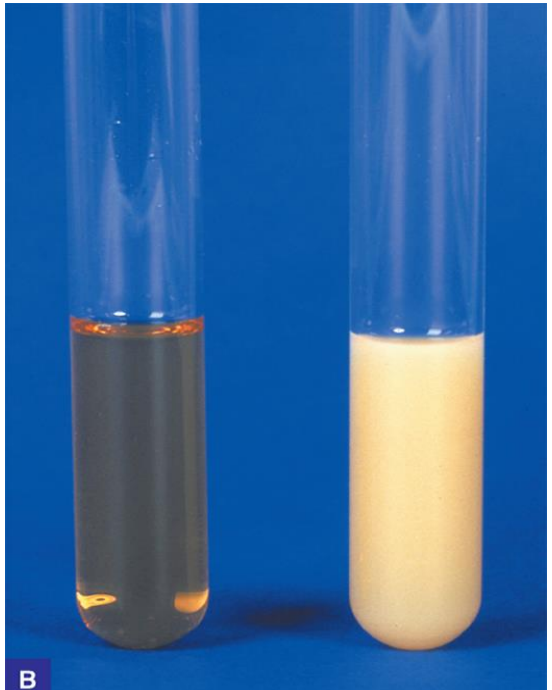


Eruptive xanthomas

Milky plasma



Lipemia retinalis



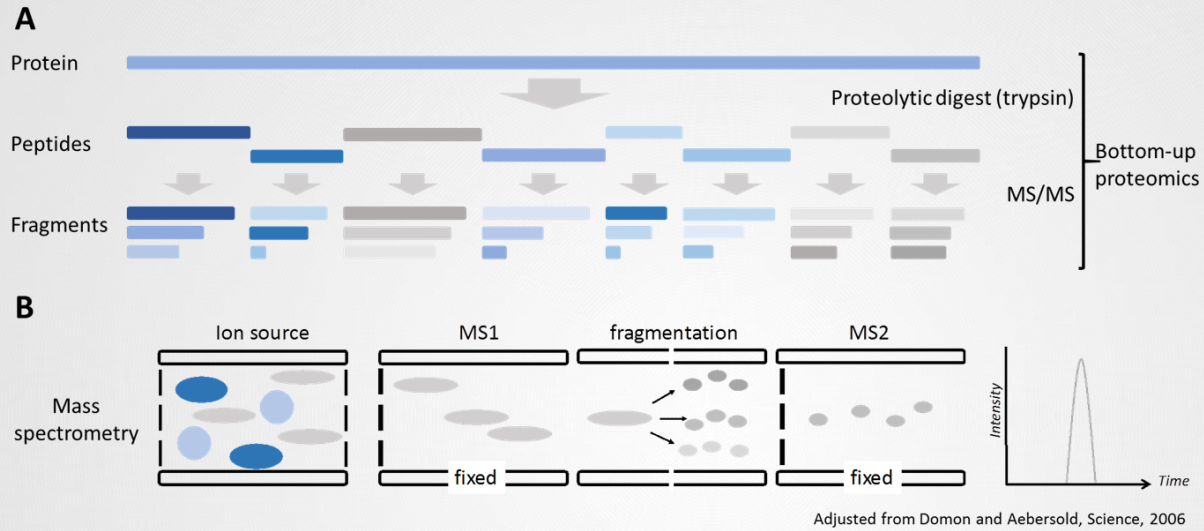
Apo profiling reveals a NEW apo CII deficient phenotype

1. In 38 patients with **very premature CAD**, 4 (11%) were found to have **low apoCII levels (≤5.0 mg/L; undetectable) with normal triglyceride levels.**

2. Despite a **misleading low a priori risk for CAD**, these patients presented with ST-segment elevation myocardial infarction and had a **high relative risk of 10-year reinfarction or revascularization**.

3. This particular phenotype of relatively **young female patients** with CAD is not recognized earlier and deserves further study.

IV. Challenges for Quantification of MULTIPLEXED Apolipoprotein Tests using bottum-up Proteomics



Adjusted from Domon and Aebersold, Science, 2006

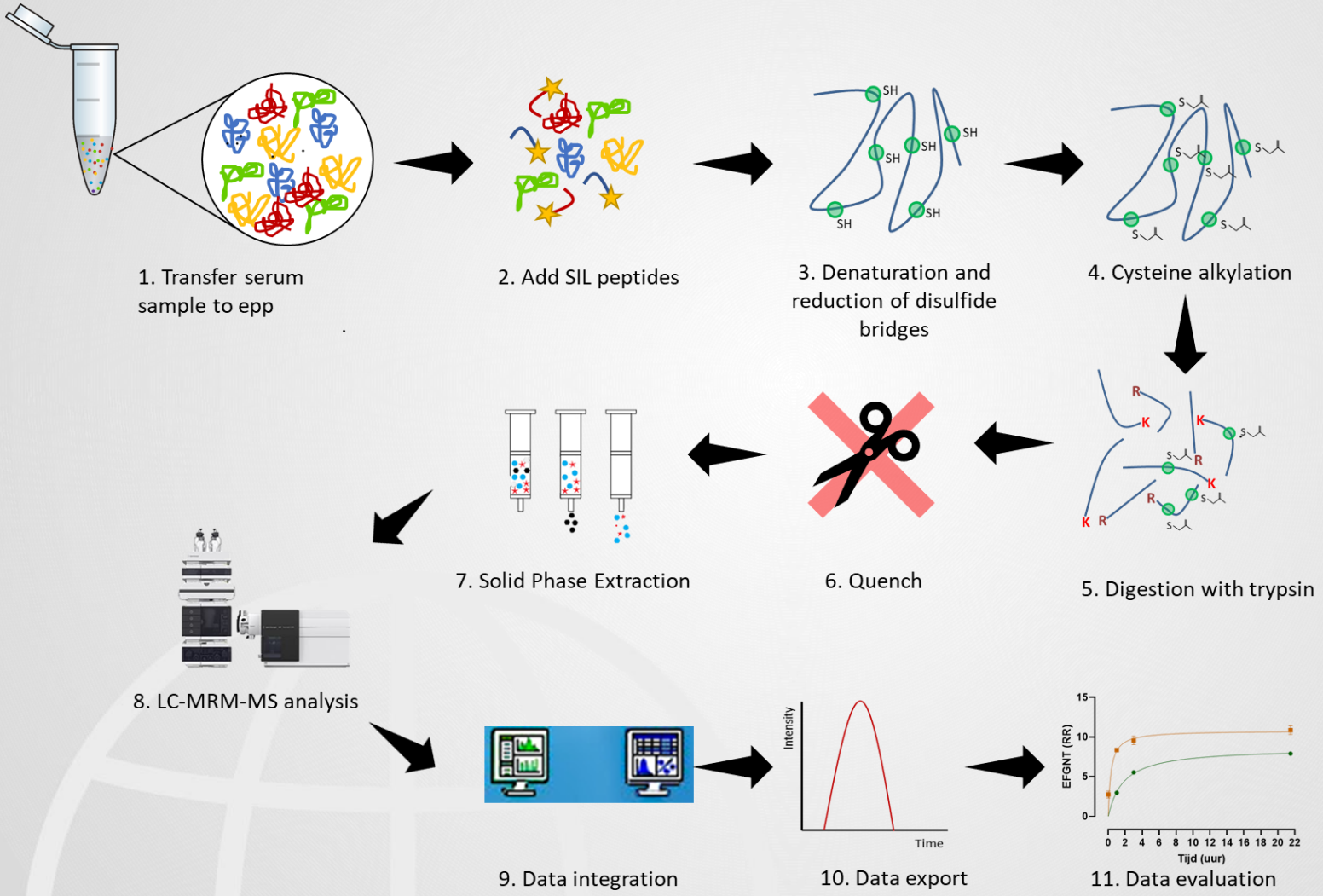
ADVANTAGES of bottom-up proteomics:

- Antibody independent
- Enables multiplexed tests
- 'lower' production costs
- Allows for molecular characterization of the measurand

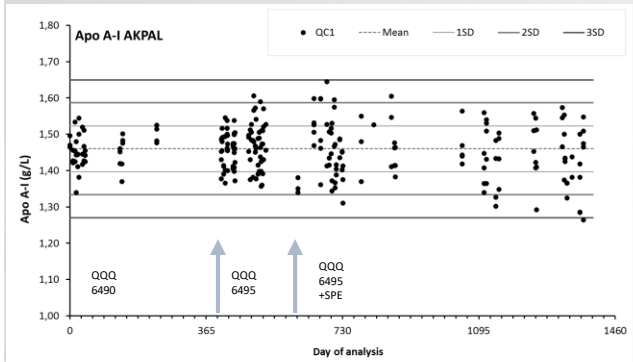
ASSUMPTIONS of bottom-up proteomics:

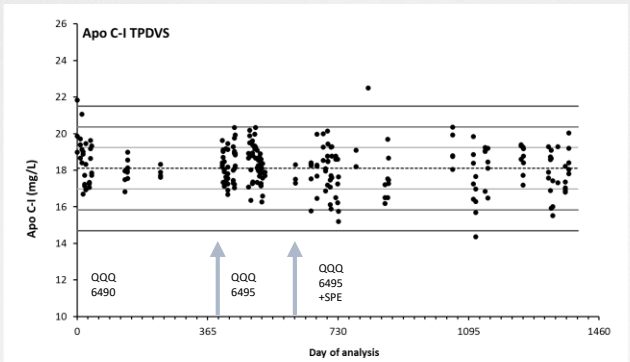
- Intact protein present in matrix
- No modifications in quantifying peptides (unless these are targeted)
- Equimolar or at least stable digestion of proteins, independent of matrix

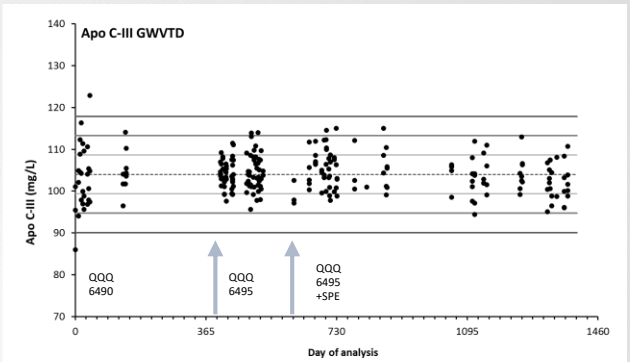
MS-based workflow for (apolipo)protein quantitation

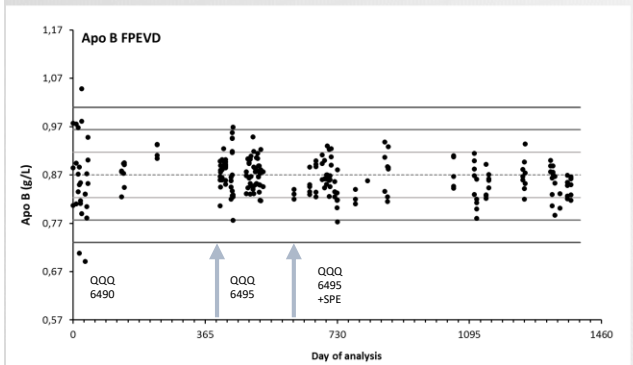


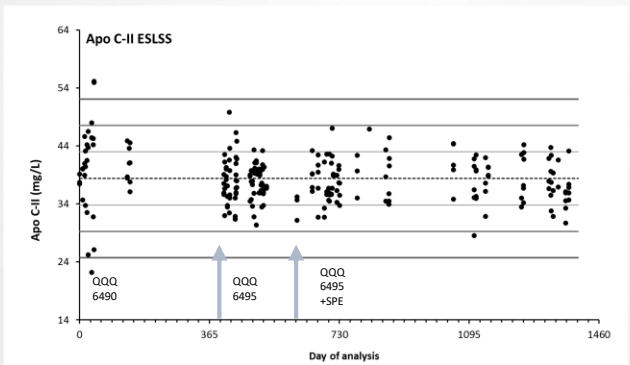
Long term stability of *in house* multiplex MS-based apo test

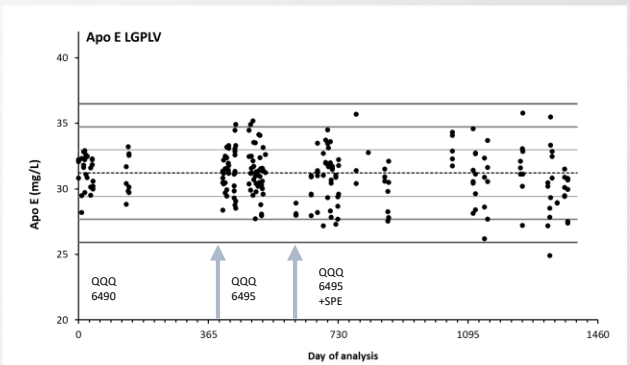












% CV	Apo A-I	Apo B	Apo C-I	Apo C-II	Apo C-III	Apo E
Year	AKPAL	FPEVD	TPDVS	ESLSS	GWVTD	LGPLV
1st	3,0	8,1	6,1	17,5	6,9	4,2
2nd	4,5	4,1	5,6	9,2	4,0	5,8
3rd	5,0	5,1	9,4	10,8	4,2	6,6
4th	5,8	3,9	7,0	9,7	4,3	7,5

Ruhaak et al. Clin Chem, 2018, adapted with additional data

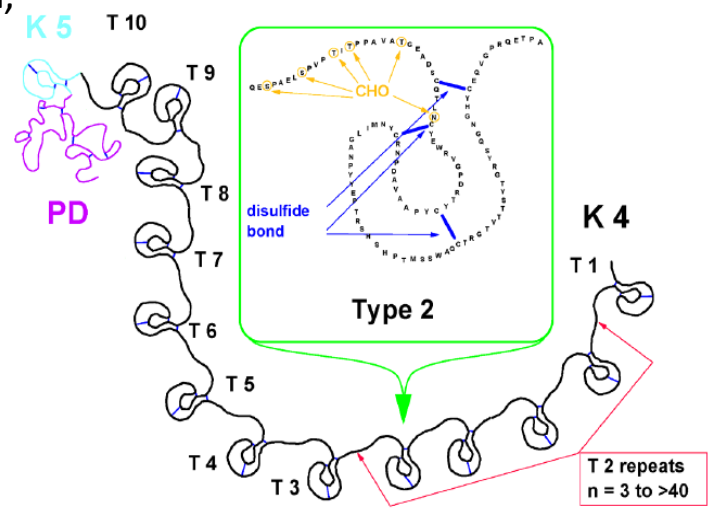
Lp(a) structural characteristics and their impact on Lp(a) measurement

Lp(a) particle:

- ✓ the most complex and polymorphic of all serum lipoproteins
- ✓ Highly prevalent
- ✓ 50 yrs of intense research and still poorly understood

Presence of a unique, hydrophilic, highly glycosylated protein,

- ✓ covalently attached to apoB in Lp(a)
- ✓ ~80% AA homology with plasminogen (PLG)
- ✓ multiple copies of PLG-like kringle IV Type 2 (K4): 3 to >40



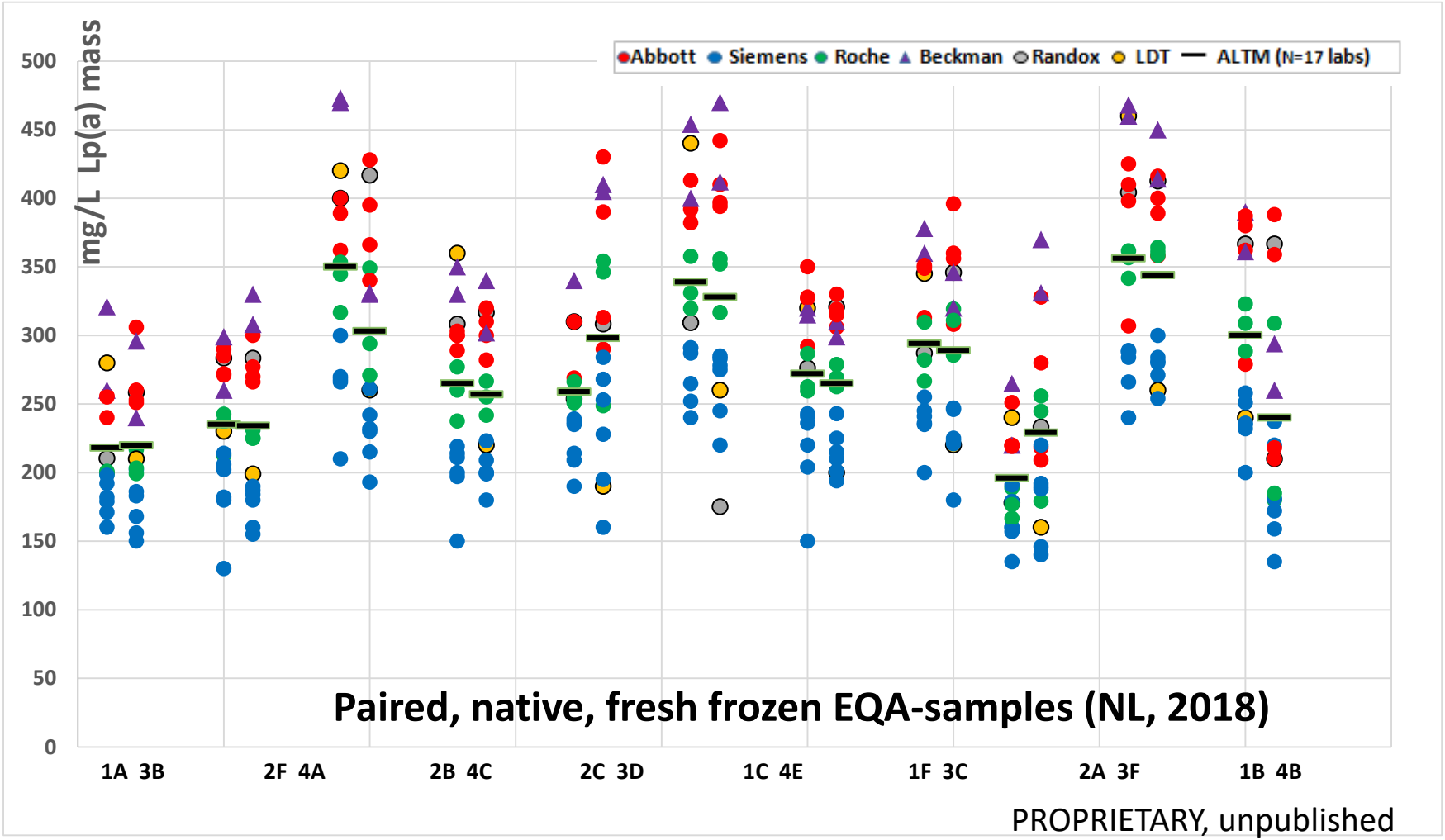
Variety of **IMMUNOCHEMICAL METHODS**, CE-IVD approved:

- ✓ ELISA
- ✓ Nephelometry
- ✓ Immunoturbidimetry
- ✓ DELFIA

mostly expressing the measured apo(a) in Lp(a) mass units (mg/L or mg/dL)!

Lp(a) mass tests:
interlaboratory variation in the Netherlands anno 2018

Unharmonized Lp(a) test results!



PROPRIETARY, unpublished

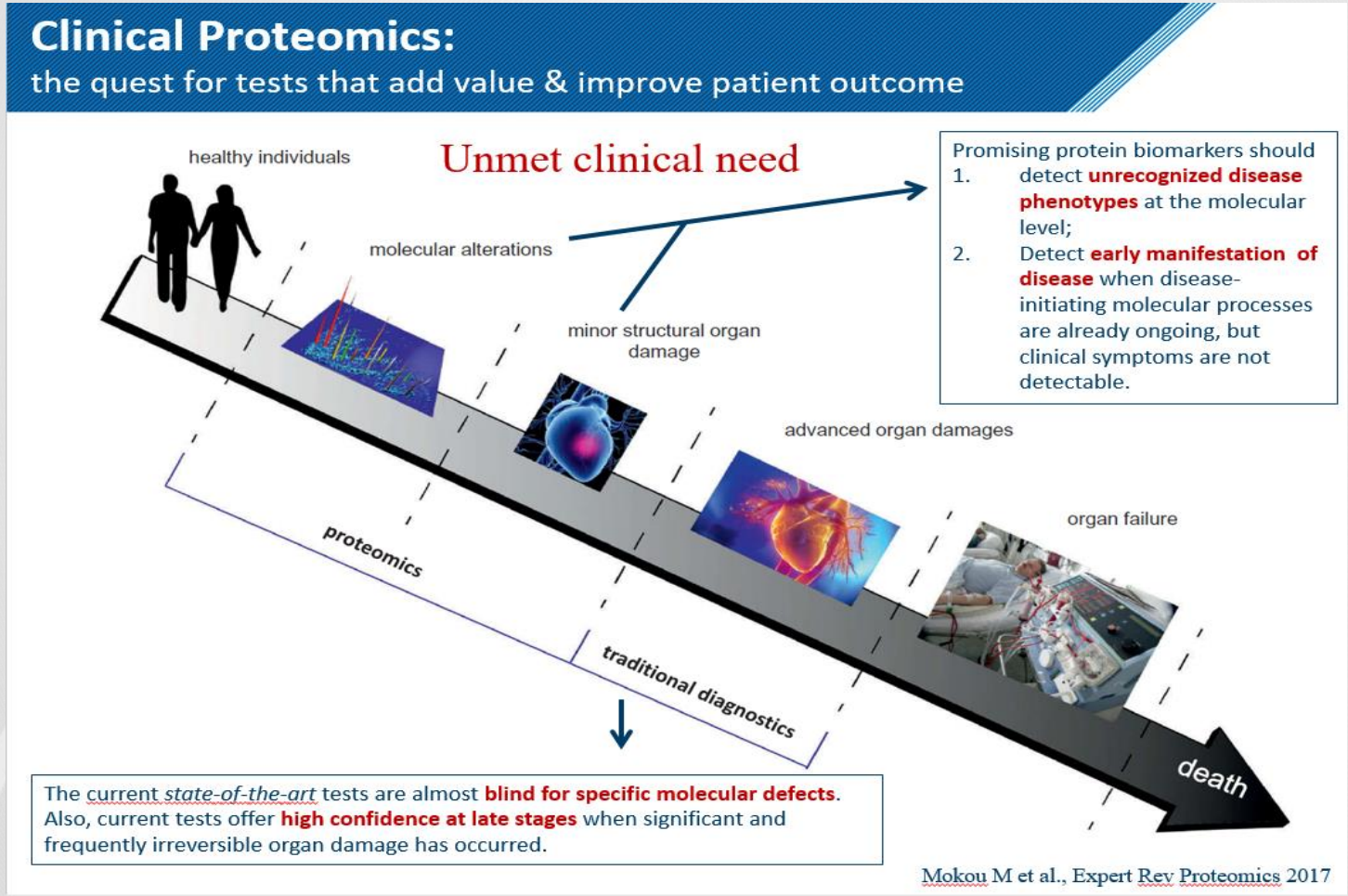
Establishment of IFCC Working Group on APO-MS:
proof-of-principle for standardizing **MS-based** proteomics tests

Terms of Reference:

- To **use MS for 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 are **traceable to SI as outlined in ISO 17511**. Other traceability chains will be used in cases where traceability to SI cannot be achieved.

- To evaluate **clinical performance and clinical utility** of serum apolipoprotein panel(s) for CVRM, in comparison to or together with contemporary blood lipids, in order **to understand their added value for addressing unsolved residual CV risk.**

Potential of multiplex **MS-based apolipoprotein profiling for CV Precision Medicine**



Proposed new Clinical Pathway for CVRM

Current practice

Target population
Individuals without / with documented CVD
Individuals at increased CVD risk

Test: Classical lipid profile

Management decision

High LDLc and risk SCORE
Generic treatment with statins, ACE-inhibitors, anti-platelet drugs, …

Low LDLc and risk SCORE
No treatment

Health outcome

Suboptimal due to
- ✓ residual risk beyond LDLc reduction
- ✓ underdiagnosis of CVD in women
- ✓ underdiagnosis of remnant disease

New practice

Target population
Individuals without / with documented CVD
Individuals at increased CVD risk

Tests: Classical lipid profile AND apolipoprotein profile

Management decision

Improved characterization of dyslipidemia and/or high risk SCORE
Targeted treatment based on individual apo levels

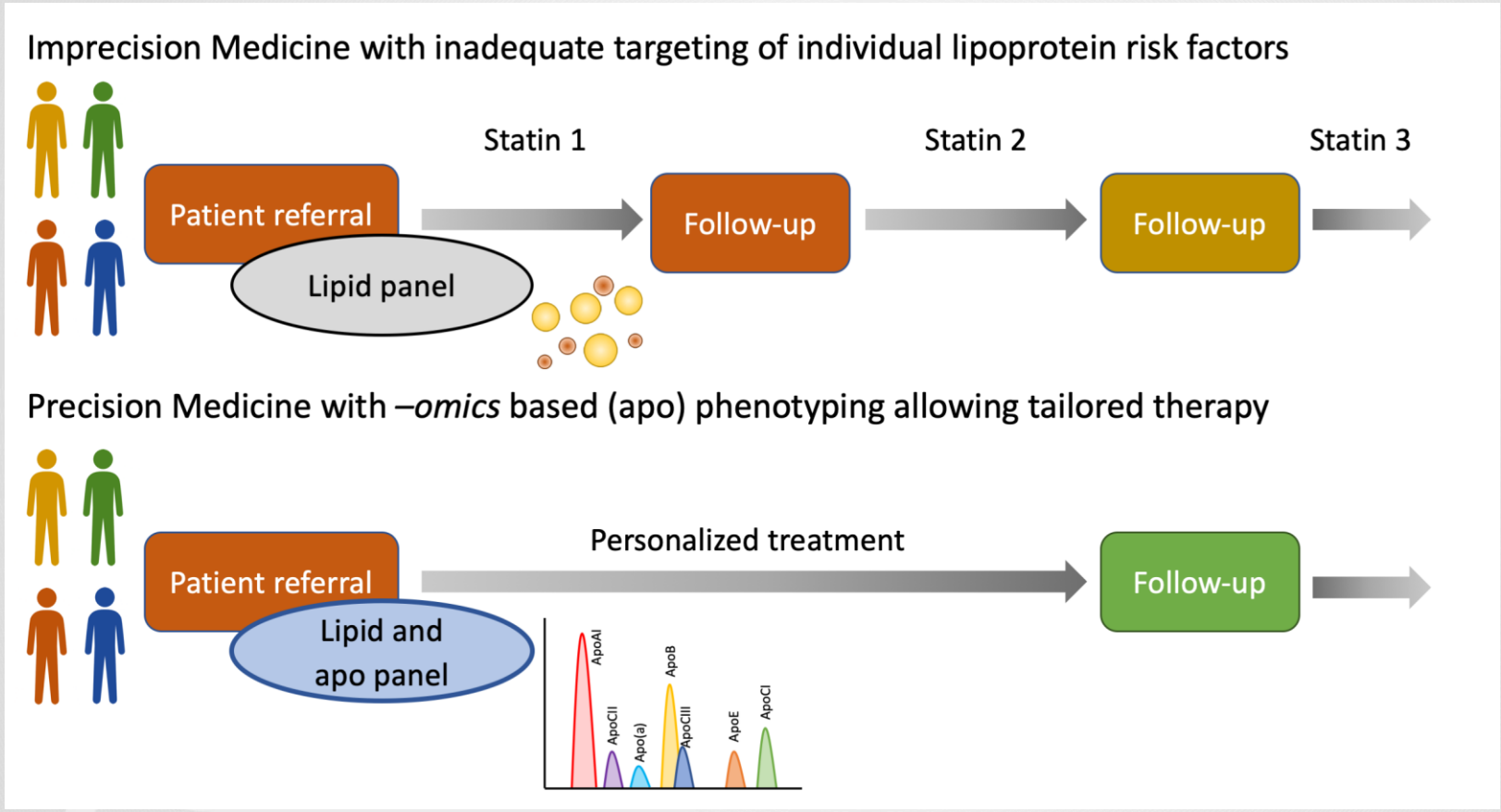
Low risk SCORE
No treatment

Health outcomes

Improved due to
- ✓ Biology-based diagnosis/treatment
- ✓ Reduced residual risk
- ✓ Personalized and more effective treatment

Ruhaak, vd Laarse and Cobbaert, ACB, 2019

Personalized treatment is needed to achieve more effective healthcare

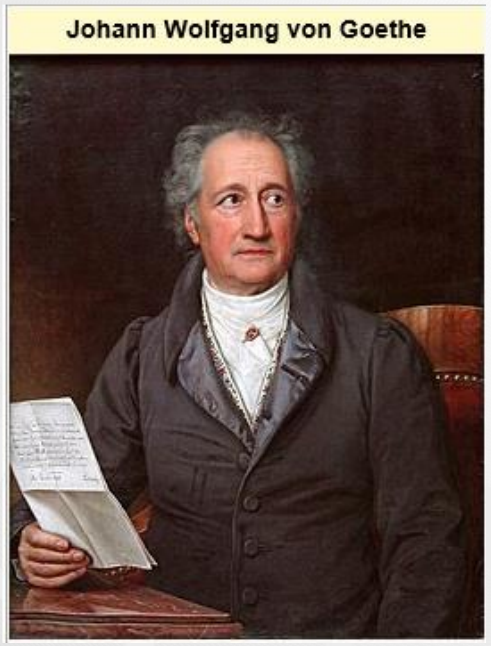


Ruhaak, vd Laarse and Cobbaert, ACB, 2019

Conclusions regarding CV Precision Medicine

Knowing is not enough, we must apply.

Willing is not enough, we must do.



JW von Goethe

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EAS-EFLM Joint Consensus Panel

Michel Langlois

Cardiomet Consortium