Bio SITrace

Traceability for biologically relevant molecules and entities

Funded by the European Metrology Research Programme

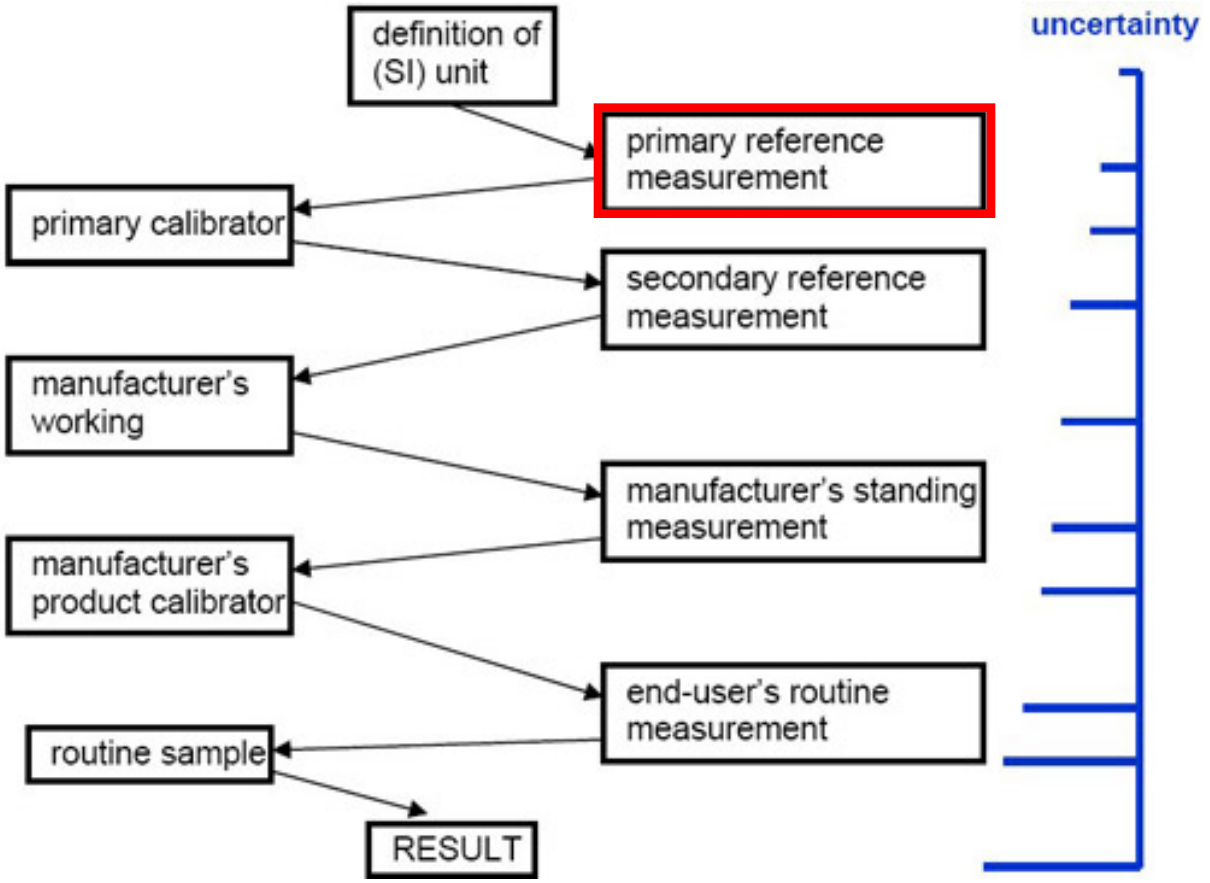
Traceable lipoprotein counting for Cardiovascular disease risk assessment

Vincent DELATOUR, PhD

LNE

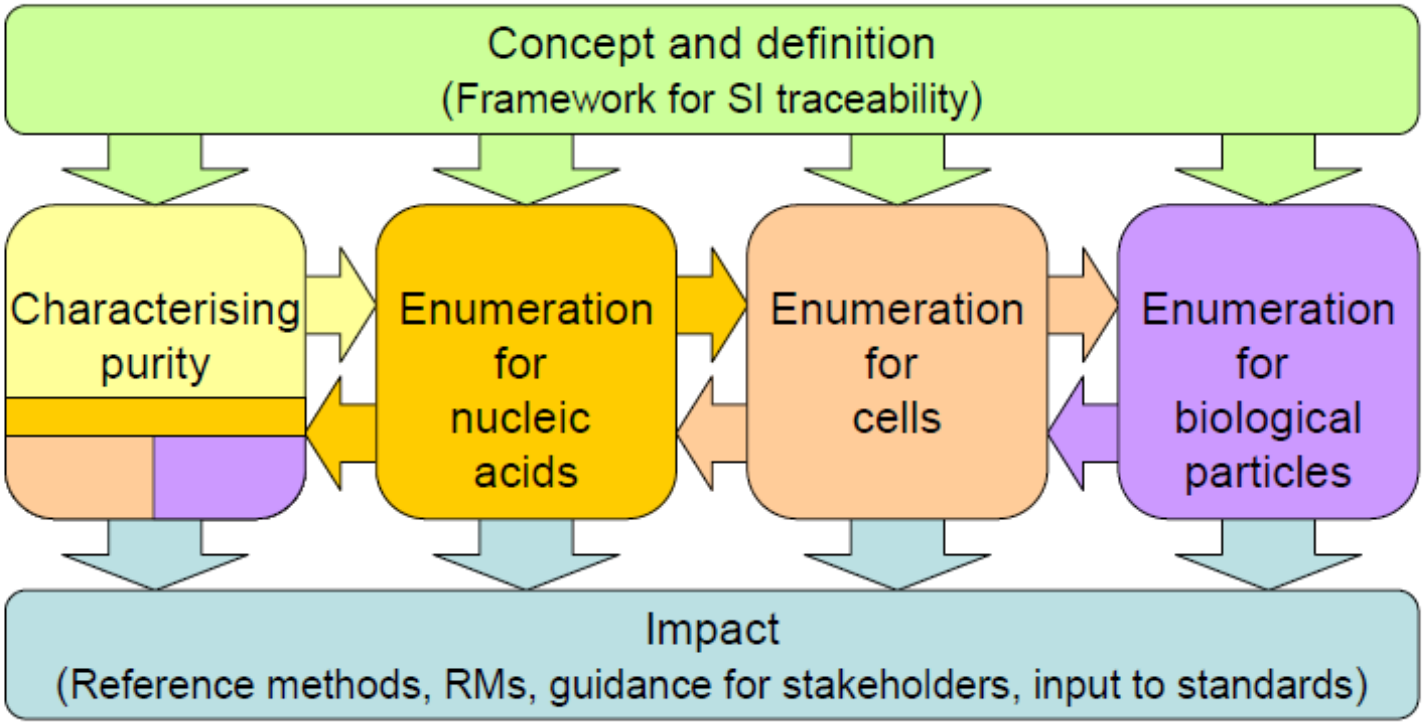
Sharing a passion for progress

The need for primary methods



VIM 2008 : Primary reference measurement procedure : "*reference measurement procedure used to obtain a measurement result without relation to a measurement standard for a quantity of the same kind*"

The “Bio-SITrace” Project



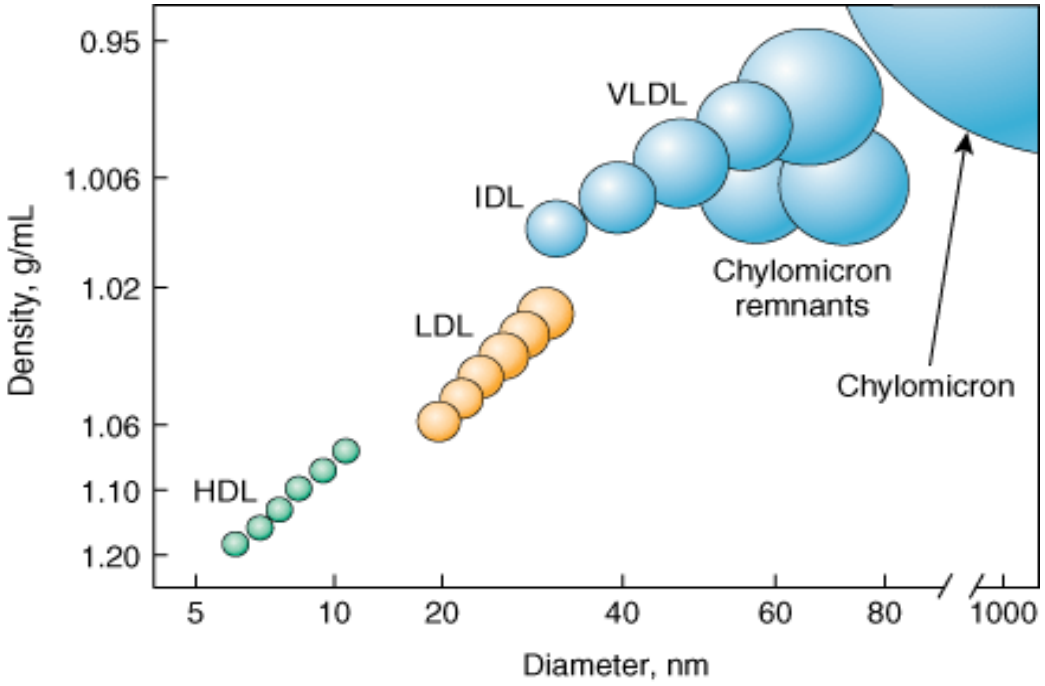
COUNTING BIOLOGICAL ENTITIES

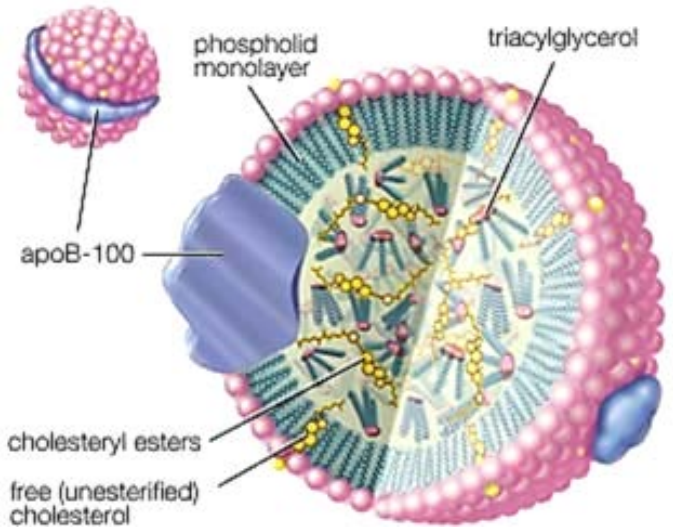
For all biological entities covered in the project, objectives are to :

- **Develop methods for direct and absolute counting of biological entities**
  - → **Commonalities between counting of different biological entities?**
  - → **Potential of dPCR, flow cytometry & ES-DMA to be primary methods**
- **Develop purification & characterization techniques to determine / confirm what is really counted**
- **Through cross-platform comparisons, identify sources of bias that could explain potential discrepencies between different methods and samples**
- **Propose international guidelines for SI-traceable enumeration results**

What are lipoproteins?

Measurand definition is complex because lipoproteins are nanobioparticles / supramolecular assemblies of heterogeneous size and constitution that can be defined as function of their density, size, NMR resonnance, electrophoretic mobility, apolipoprotein content…

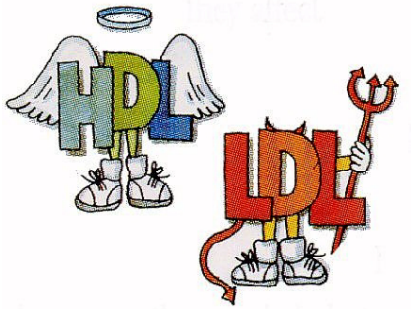




CVD risk assessment in clinical practice

Although controversy exists among national guidelines, CVD risk is often estimated through lipids measurements that are routinely performed in medical labs with fully automated methods / analyzers :

- ⇨ **Triglycerides (TG)**
- ⇨ **Total cholesterol (TC)**
- ⇨ **HDL-cholesterol (HDL-C : *« Good Cholesterol »)***
- ⇨ **LDL-cholesterol (LDL-C : *« Bad Cholesterol »)***

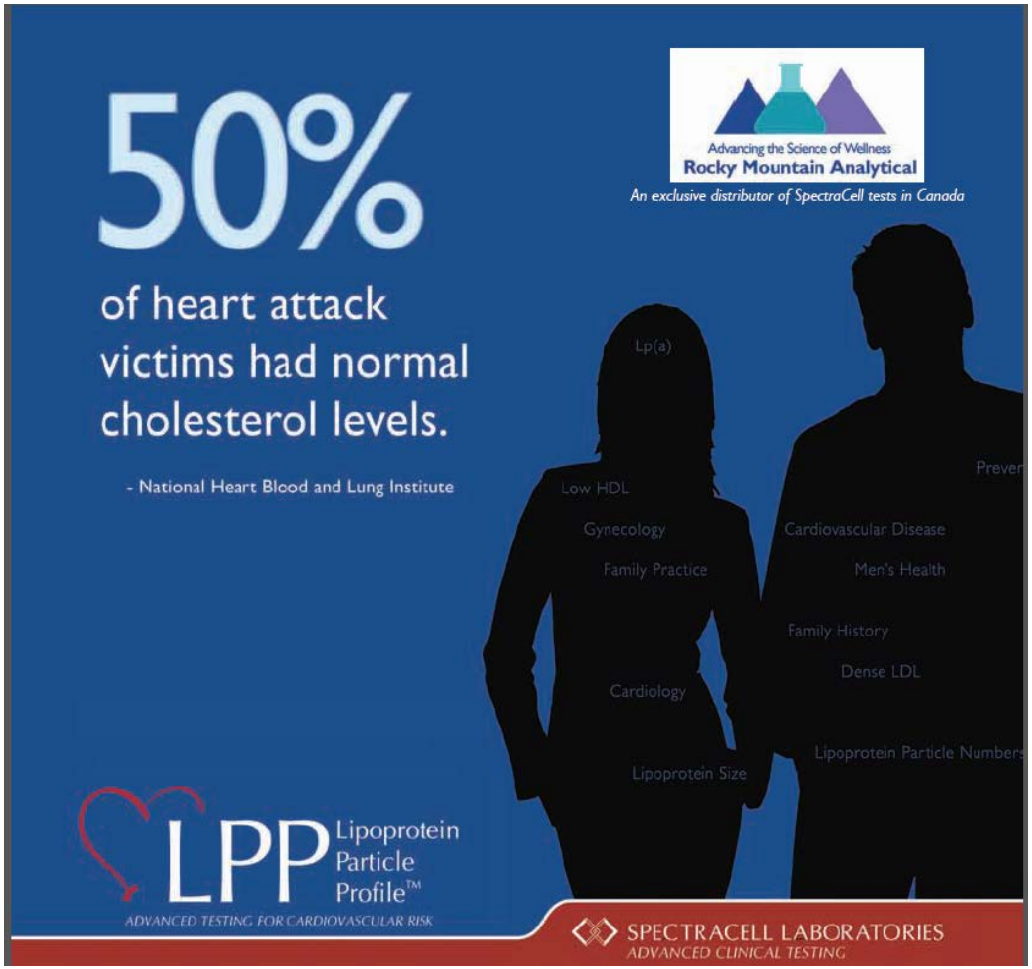


In France and many other countries, therapeutic intervention thresholds are based on LDL-C targets that depend on the nb of CVD risk factors (smoking, etc…)

Risk factors	Target LDL-C
0	2,20 g/L (5,7 mmol/L)
1	1,90 g/L (4,9 mmol/L)
2	1,60 g/L (4,1 mmol/L)
> 2	1,30 g/L (3,4 mmol/L)
Past CVD event	1,00 g/L (2,6 mmol/L)

French guidelines (2008)

Are lipid measurements enough to estimate CVD risk?

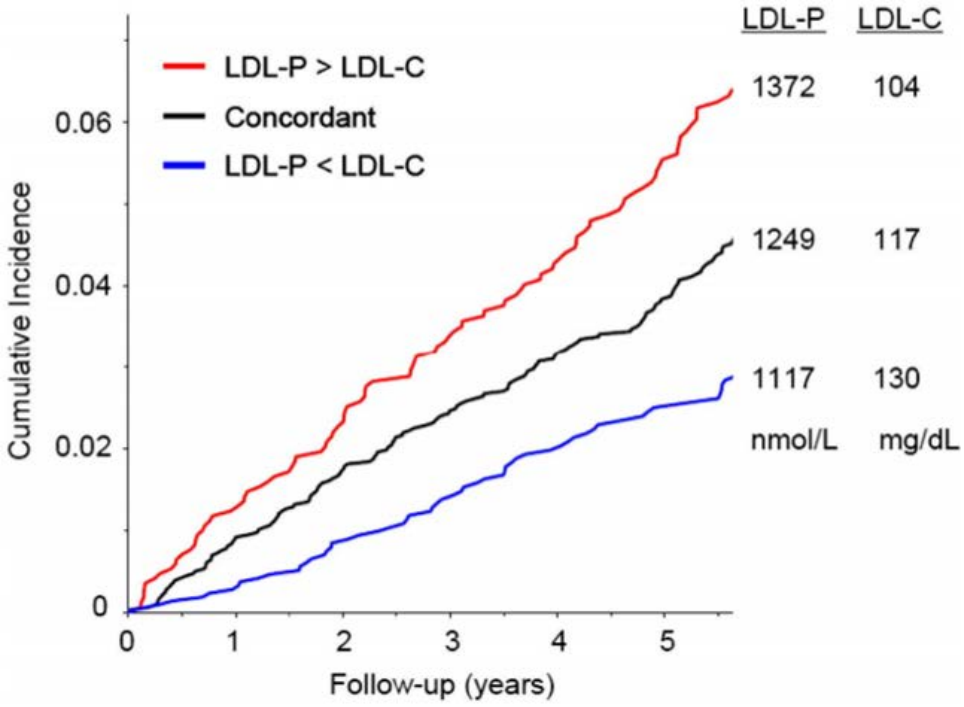


High residual risk!

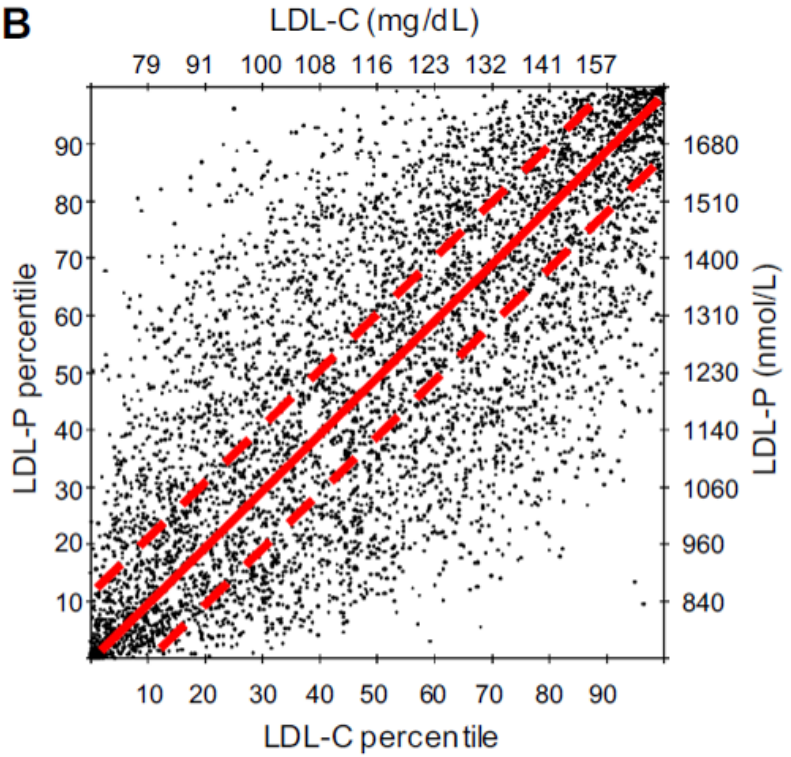
LDL-C testing not such a good screening tool?

Beyond LDL-C in CVD risk assessment

CVD risk is more strongly associated with LDL-P than LDL-C

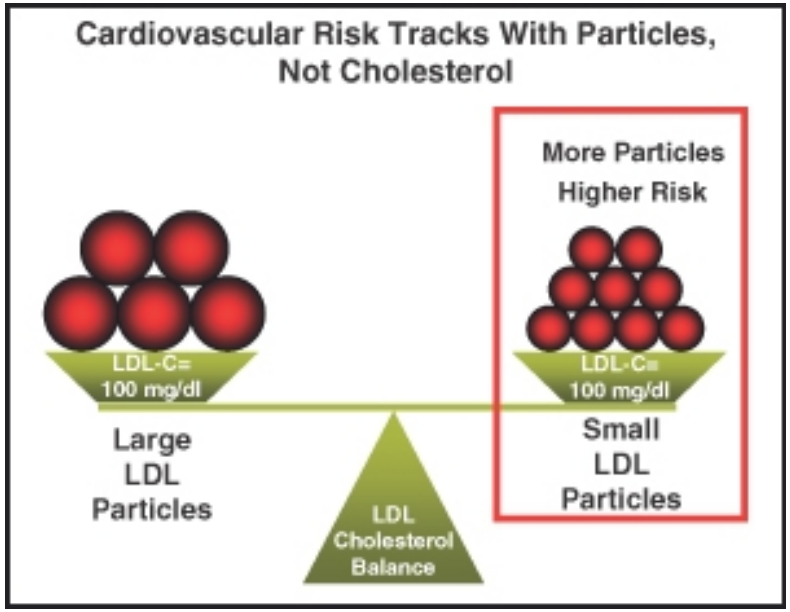


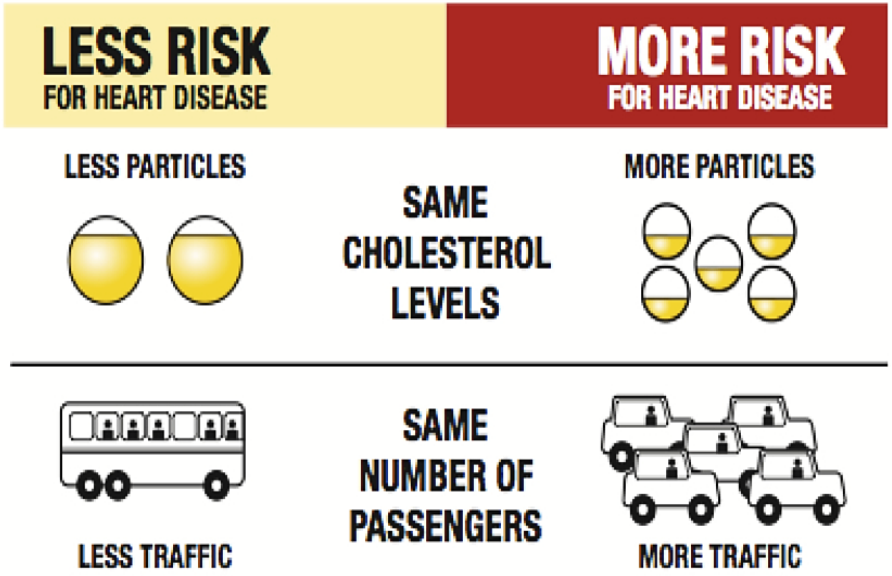
LDL-P poorly correlates with LDL-C



Otvos et al. J Clin Lipidol. 2011;5(2):105-113

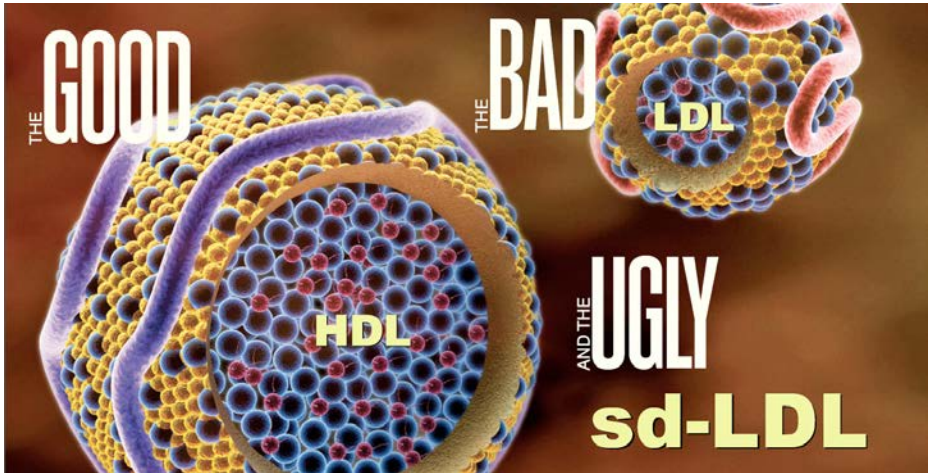
Beyond LDL-C in CVD risk assessment



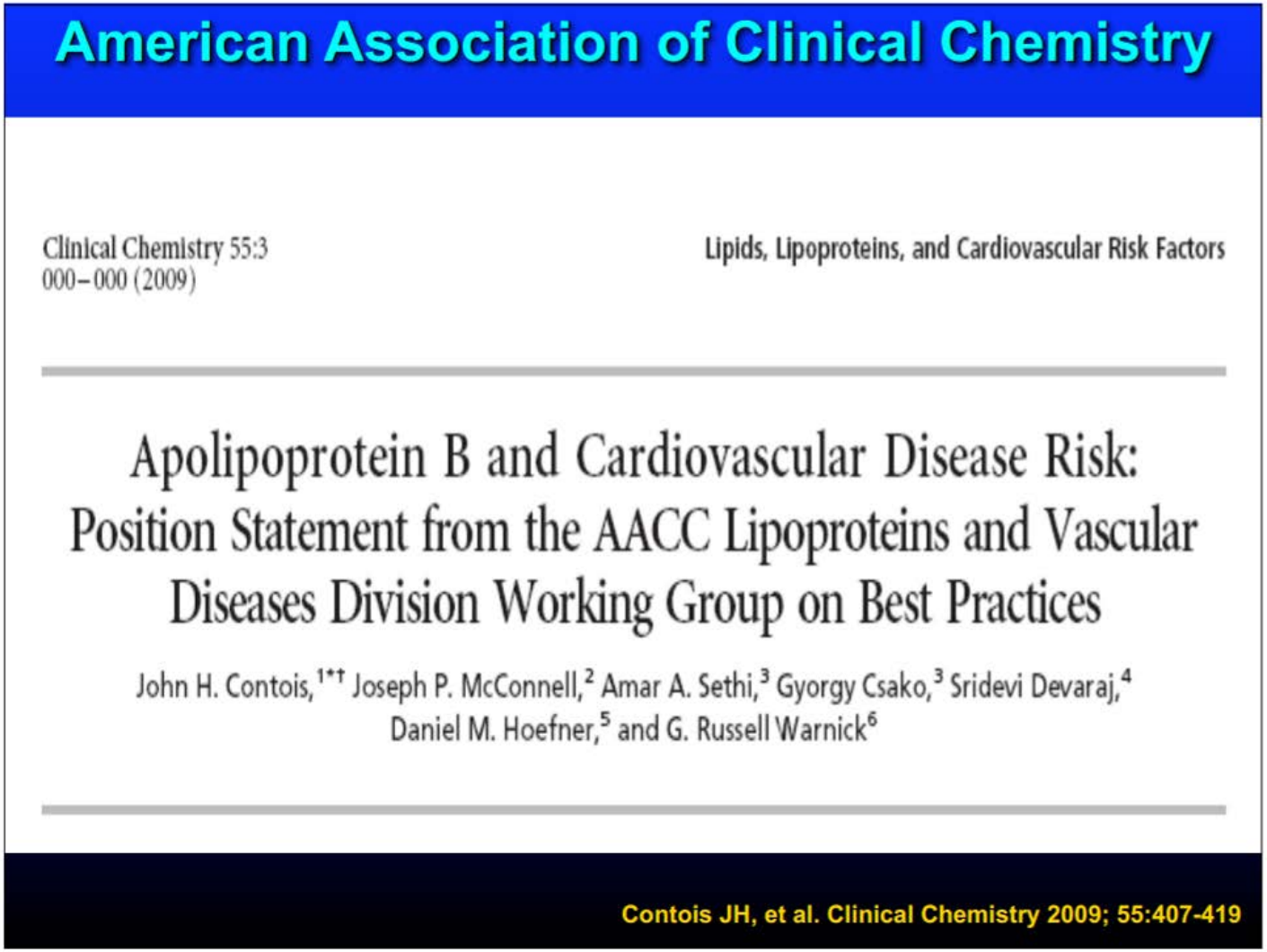


In addition to particle number, particle size also matters :

small dense LDLs (sd-LDL) are more atherogenic than Large Buoyant LDLs



Beyond LDL-C in CVD risk assessment



American Association of Clinical Chemistry

Clinical Chemistry 55:3
000–000 (2009)

Lipids, Lipoproteins, and Cardiovascular Risk Factors

Apolipoprotein B and Cardiovascular Disease Risk: Position Statement from the AACC Lipoproteins and Vascular Diseases Division Working Group on Best Practices

John H. Contois,[1*†] Joseph P. McConnell,[2] Amar A. Sethi,[3] Gyorgy Csako,[3] Sridevi Devaraj,[4] Daniel M. Hoefner,[5] and G. Russell Warnick[6]

Contois JH, et al. Clinical Chemistry 2009; 55:407-419

Beyond LDL-C in CVD risk assessment

Recommendations from AACC Lipoproteins and Vascular Diseases Division Working Group on Best Practices

"In light of the mounting evidence, the members of this working group of the Lipoproteins and Vascular Diseases Division of the AACC believe that **apoB and alternate measures of LDL particle concentration should be recognized and included in guidelines, rather than continuing to focus solely on LDL-C.**"

Contois JH, et al. Clinical Chemistry 2009; 55:407-419

Beyond LDL-C in CVD risk assessment

Clinical Chemistry 59:5
752–770 (2013)

Special Report

Association of Apolipoprotein B and Nuclear Magnetic Resonance Spectroscopy–Derived LDL Particle Number with Outcomes in 25 Clinical Studies: Assessment by the AACC Lipoprotein and Vascular Diseases Division Working Group on Best Practices

Thomas G. Cole,[1*] John H. Contois,[2] Gyorgy Csako,[3] Joseph P. McConnell,[4] Alan T. Remaley,[3] Sridevi Devaraj,[5] Daniel M. Hoefner,[4] Tonya Mallory,[4] Amar A. Sethi,[6] and G. Russell Warnick[4]

CONCLUSIONS: In most studies, both apo B and LDL-P were comparable in association with clinical outcomes. The biomarkers were nearly equivalent in their ability to assess risk for CVD and both have consistently been shown to be stronger risk factors than LDL-C. We support the adoption of apo B and/or LDL-P as indicators of atherogenic particle numbers into CVD risk screening and treatment guidelines. Currently, in the opinion of this Working Group on Best Practices, apo B appears to be the preferable biomarker for guideline adoption because of its availability, scalability, standardization, and relatively low cost.

Beyond LDL-C in CVD risk assessment

Clinical Chemistry 59:5
723–725 (2013)

Editorials

Beyond LDL Cholesterol in Assessing Cardiovascular Risk: apo B or LDL-P?

Immuno-nephelometry
Immuno-turbidimetry
LC/MS/MS
NMR
ES-DMA

Stephen R. Master[1,3*] and Daniel J. Rader[2,3*]

Plasma concentrations of LDL cholesterol (LDL-C)[4] are positively associated with increased risk of atherosclerotic cardiovascular disease. There is a variety of robust evidence indicating that this association is causal in nature. First, rare and common genetic variants that specifically influence LDL-C concentrations are also strongly associated with cardiovascular risk *(1)*. Second, interventions that reduce LDL-C, especially but not exclusively statin therapy, reproducibly reduce cardiovascular events *(2)*. In fact, the data with statins are so strong that they are often used in patients whose LDL-C concentrations are not particularly increased, a setting in which statins have still been shown to reduce cardiovascular risk. Thus there is substantial interest in lipoprotein-related biomarkers that provide information about future cardiovascular risk above and beyond LDL-C itself.

Several methods have emerged that allow a more direct quantification of the number of LDL particles. Because an LDL particle contains a single molecule of apo B, it is possible to directly estimate the number of particles through a simple measurement of apo B concentration (particularly when expressed in molar units). apo B is typically measured by immunonephelometry or immunoturbidimetry, and reagents are available from a wide variety of manufacturers. Standardization of these measurements has been facilitated by the availability of WHO-IFCC reference materials (SP3–07, SP3–08) *(4, 5)*. apo B analytical measurements have shown good reproducibility across laboratories (6%–8% CV in 2012 College of American Pathologists survey), although a number of preanalytical biological confounders, including diurnal and seasonal effects, have been described *(6)*.

Lipoprotein enumeration by Differential Mobility Analysis

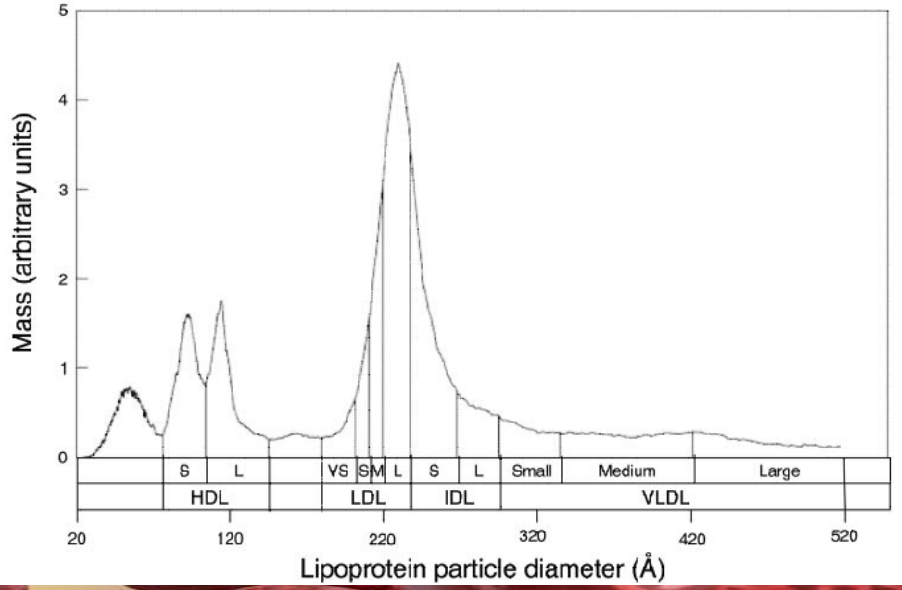
Clinical Chemistry 54:8
1307–1316 (2008)

Direct Determination of Lipoprotein Particle Sizes and Concentrations by Ion Mobility Analysis

Michael P. Caulfield,[1*] Shuguang Li,[1] Gloria Lee,[1] Patricia J. Blanche,[2] Wael A. Salameh,[1] W. Henry Benner,[3] Richard E. Reitz,[1] and Ronald M. Krauss[2]

CONCLUSIONS: The IM method provides accurate, reproducible, direct determination of size and concentration for a broad range of lipoprotein particles. Use of this methodology in studies of patients with cardiovascular disease and other pathologic states will permit testing of its clinical utility for risk assessment and management of these conditions.

Has Differential Mobility Analysis the potential to be a primary method?



Electrospray Differential Mobility Analysis (ES-DMA)

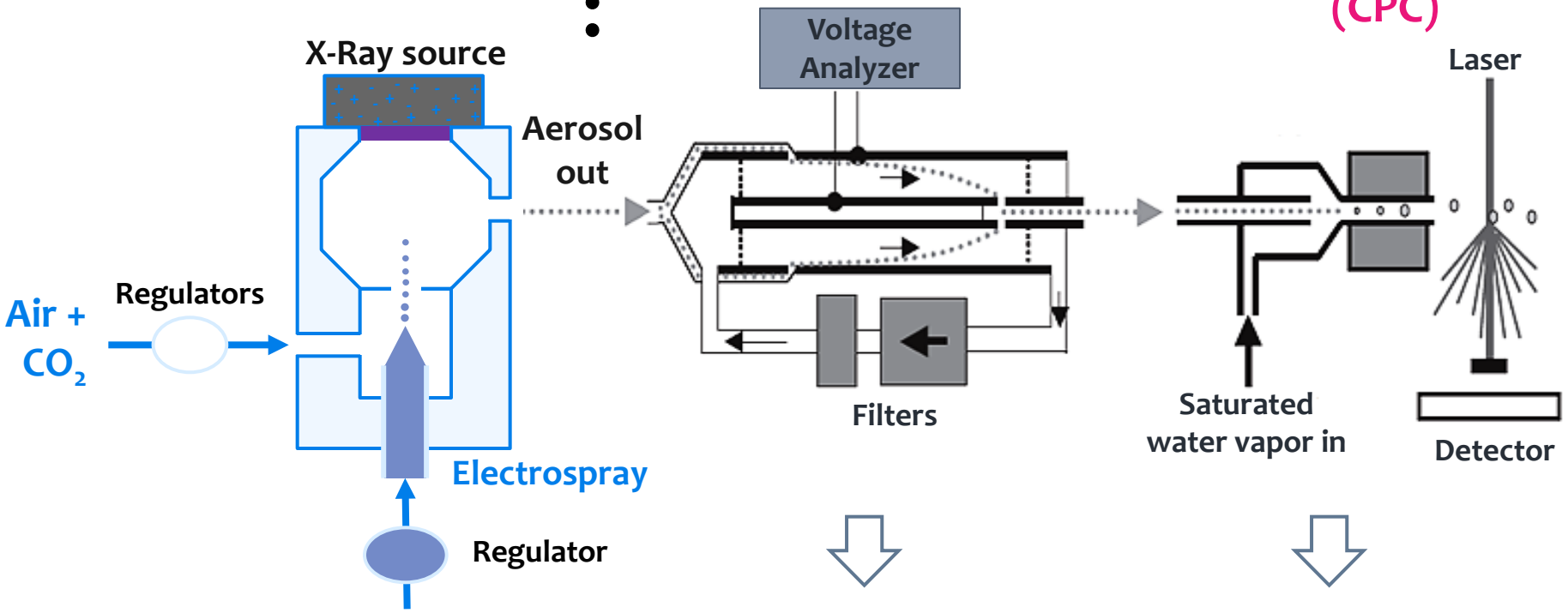
Liquid Phase

Aerosol Phase

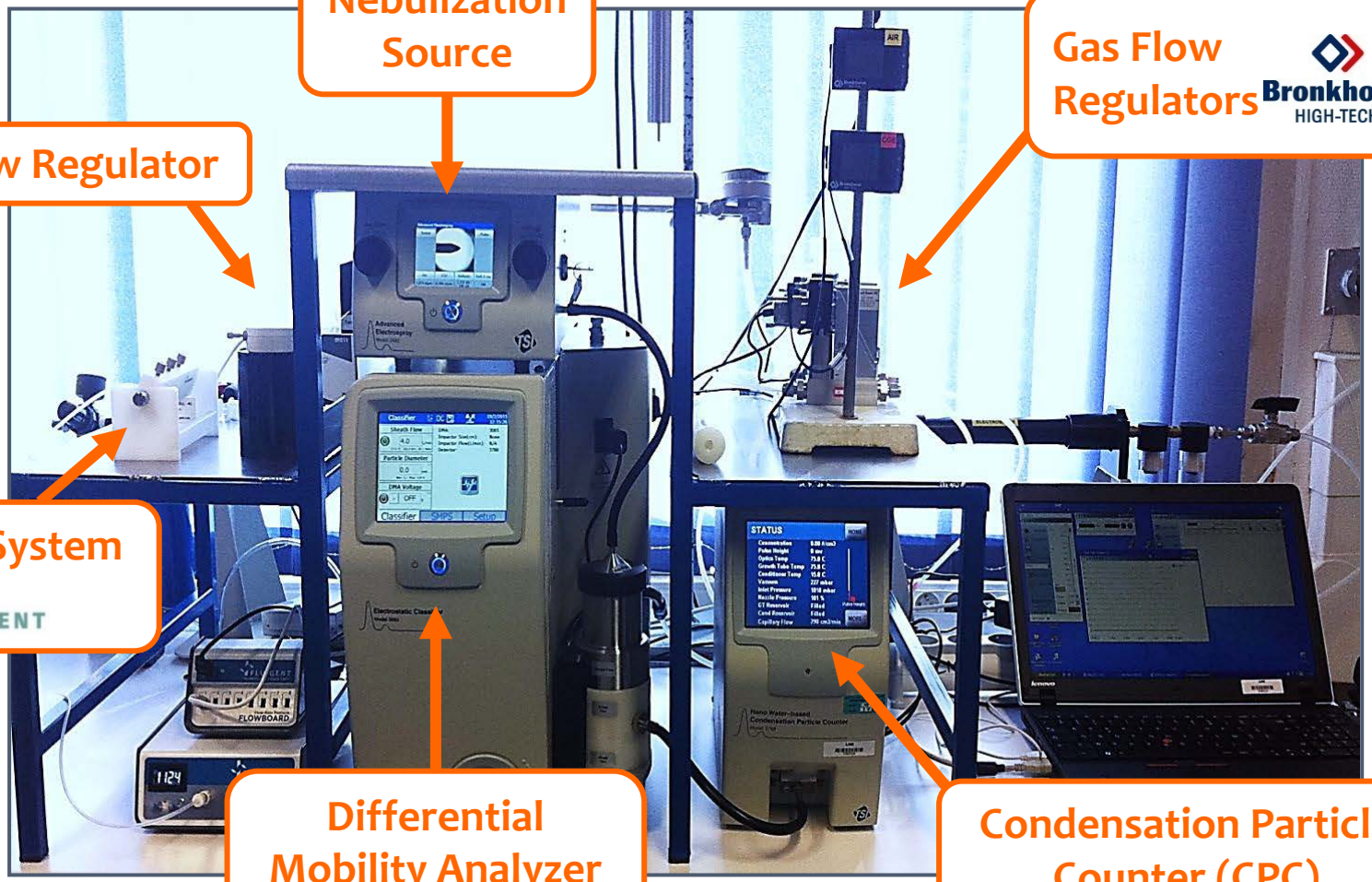
Nebulization source

Differential Mobility Analyzer (DMA)

Condensation Particle Counter (CPC)



LNE's ES-DMA Platform



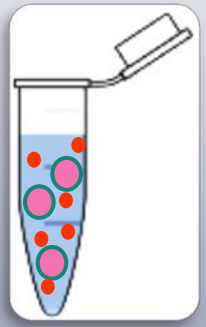
Lipoprotein enumeration by ES-DMA

ES-DMA Analysis

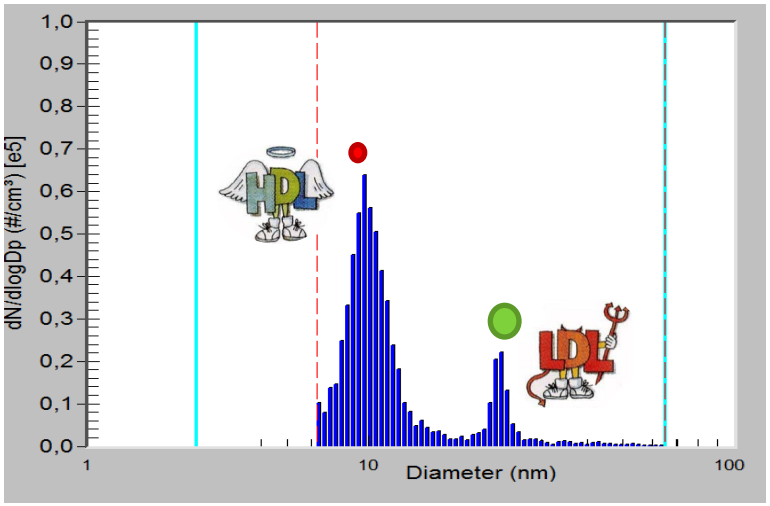
Sample Preparation

Sample in Liquid Phase

- *LDL*
- *HDL*



Number Size Distribution



Particle number Concentration in Aerosol Phase P_n

Simplified Equation

$$C_{\bullet} = E \times \frac{P_n \times g}{L}$$

Particle number Concentration in the Liquid Phase $C_{\bullet}$

Establishing SI-Traceability of results

- **Liquid and gas flows : calibrated flow-meters**

  - **Liquid flow meter** calibrated in METAS ⇨ **L ± ΔL**
  - **Gas flow meters** calibrated in LNE ⇨ **g ± Δg**

$$C = E \times \frac{P_n \times g}{L}$$

- **Uncertainties associated with P_n : Monte Carlo simulations**
  - ⇨ Correction of the different loss sources *(diffusion, efficiencies, system's performances…)*
    - ⇨ **Software developped in LNE**

$$C = E \times \frac{P_n \times g}{L}$$

Establishing SI-Traceability of results

- **Electrospray efficiency E**

analytical chemistry

Anal Chem. 2014;86(24):12130-7 Article

pubs.acs.org/ac

Absolute Quantification Method for Protein Concentration

Mingdong Li,[†,‡,§] Jiaojie Tan,[†,‡,§] Michael J. Tarlov,[§] and Michael R. Zachariah*[,†,‡,§]

[†]Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, Maryland 20742, United States

[‡]Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, United States

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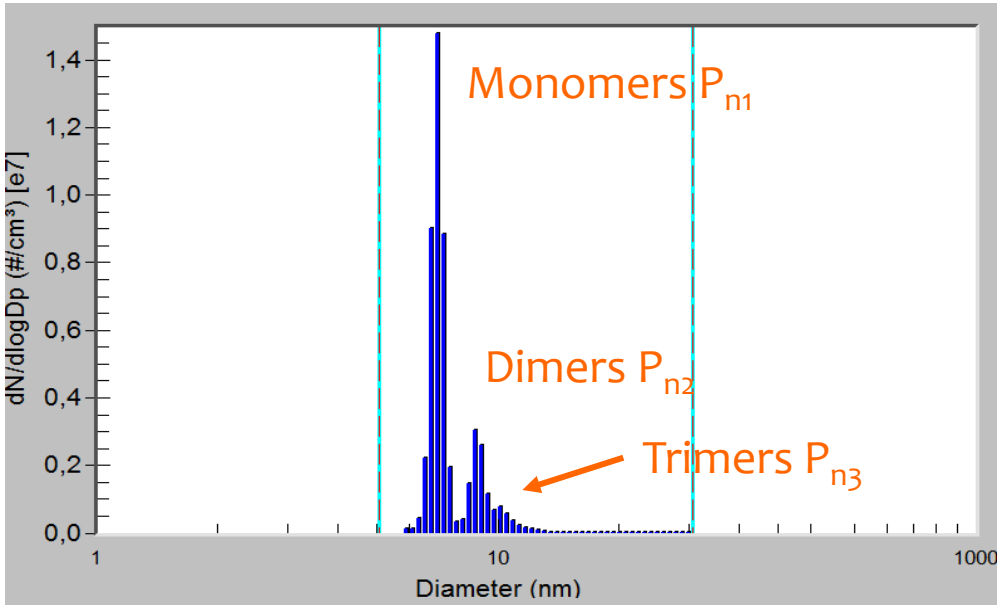

- ⇨ **Serial dilutions of BSA solutions (NIST SRM 927e certified by AAA)**
- ⇨ **BSA concentration in liquid phase calculated from : peak area ratio, dilution factor and droplet size**
- ⇨ **Droplet size measured using 0,063% sucrose solutions**
- ⇨ **E = ratio between the measured and the certified concentration of BSA**

Robusteness & stability of E?

Standardization of Measurements for Cholesterol, Triglycerides, and Major Lipoproteins

G. Russell Warnick, MS, MBA,[1] Mary M. Kimberly, PhD,[2] Parvin P. Waymack, PhD,[2] Elizabeth T. Leary, PhD,[3] Gary L. Myers, PhD[2]

LabMedicine (2008) 39, 481-490

Table 2_Lipid and Lipoprotein Reference Systems

Analyte	1° Reference Measurement Procedure	1° Reference Material	2° Reference Measurement Procedure	2° Reference Material
Cholesterol	ID-MS (NIST)	NIST SRM 911c Pure cholesterol	Abell-Kendall (CDC)	CDC Frozen Pools NIST SRM 909 NIST SRM 1951b
HDL-C	Not available	Not available	UC/Heparin-Mn2+-Abell-Kendall (CDC) Recommended by NCEP	CDC Frozen Pools NIST SRM 1951b
LDL-C	Not available	Not available	Beta-quantification (CDC) Recommended by NCEP	CDC Frozen Pools NIST SRM 1951b
Triglyceride	ID-MS (NIST)	NIST SRM 1595 Tripalmitin	Methylene chloride Silicic acid-chromotropic acid (CDC). Recommended by NCEP	CDC Frozen Pools NIST SRM 1951b
Lipoprotein(a)	Not available	Lyophilized purified Lp(a)	Consensus ELISA method	WHO/IFCC SRM 2B
ApoA-1	HPLC-MS (CDC) (primary standard only) (Candidate)	BCR-CRM 393 (Purified ApoA-1)	Not available	WHO Reference Reagent SP1-01 (for manufacturers). Value-assigned by CDC-RIA comparison method.
ApoB	Not available	d = 1.030–1.050 (UC purified LDL)	Not available	WHO Reference Reagent SP3-08 (for manufacturers). Value-assigned by NWLMDRL-Immunonephlometry comparison method.

CDC = Centers for Disease Control and Prevention; NCEP = National Cholesterol Education Program; WHO = World Health Organization; NWLMDRL = Northwest Lipid Metabolism and Diabetes Research Laboratories.

Apolipoprotein A-I and B

Standardization of apoA-I and apoB in routine laboratories is hampered by the lack of availability of 2° RMP and a comprehensive standardization program. The program available through NWLMDRL serves as an interim solution.

Standardization of advanced lipoperotein testing

Circulation

American Heart Association.

CONTROVERSIES IN CARDIOVASCULAR MEDICINE

Are advanced lipoprotein testing and subfractionation clinically useful?

Advanced Lipoprotein Testing and Subfractionation Are Not (Yet) Ready for Routine Clinical Use

Samia Mora, MD, MHS

Mora et al. Circulation 2009; 119: 2396-2404

Standardization of advanced lipoperotein testing

Comparisons

Direct comparisons of these techniques are limited. The correlation for LDL size between NMR and GGE was 0.86 in a small study of men.[20] In another study (n=324 individuals), LDL size by GGE and NMR was only moderately correlated (Spearman correlation 0.4), and the chance-adjusted κ statistic was moderate (0.3).[21] A more recent study by Ensign et al[22] (n=40 individuals) found the agreement between GGE and NMR to be 70%. However, when results were compared across 4 methods that are used to determine LDL size, complete agreement among the 4 methods examined (GGE, NMR, VAP, and tube gel electrophoresis) for LDL size phenotype was only 8% (Figure 1). This highlights the important need for standardization if these measurements are to be more widely used in clinical practice, especially given the fact that the methods use different principles for subfractionation of lipoproteins.[23]

Mora et al. Circulation.2009; 119: 2396-2404

Standardization of advanced lipoperotein testing

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STATE-OF-THE-ART PAPER AND COMMENTARY

What Is the Role of Advanced Lipoprotein Analysis in Practice?

Jennifer G. Robinson, MD, MPH

Iowa City, Iowa

Some practitioners use advanced lipoprotein analysis with the goal of better predicting risk and individualizing lifestyle and drug therapy for cardiovascular prevention. Unfortunately, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) particle number and size, other lipoprotein subfractionation, apolipoproteins B and A, and lipoprotein(a) have not yet met current standards for biomarker evaluation, and it remains to be determined whether these tests incrementally add to cardiovascular risk predicted by traditional risk factors. More importantly, it has yet to be determined whether treatment strategies guided by, or targeting, these measures improve cardiovascular outcomes. Drug therapies known to alter advanced lipoprotein analysis parameters, specifically niacin and fenofibrate, have not been shown to additionally reduce cardiovascular risk in recent randomized trials of high-risk patients treated with statin therapy. These findings suggest advanced lipoprotein analysis–guided strategies may not further reduce cardiovascular events and could lead to increased adverse effects and costs; this approach needs further research to establish its role in individualizing therapies for cardiovascular prevention. In contrast, a large body of evidence supports focusing on LDL cholesterol reduction and intensification of statin therapy to reduce cardiovascular risk. (J Am Coll Cardiol 2012;60:2607–15) © 2012 by the American College of Cardiology Foundation

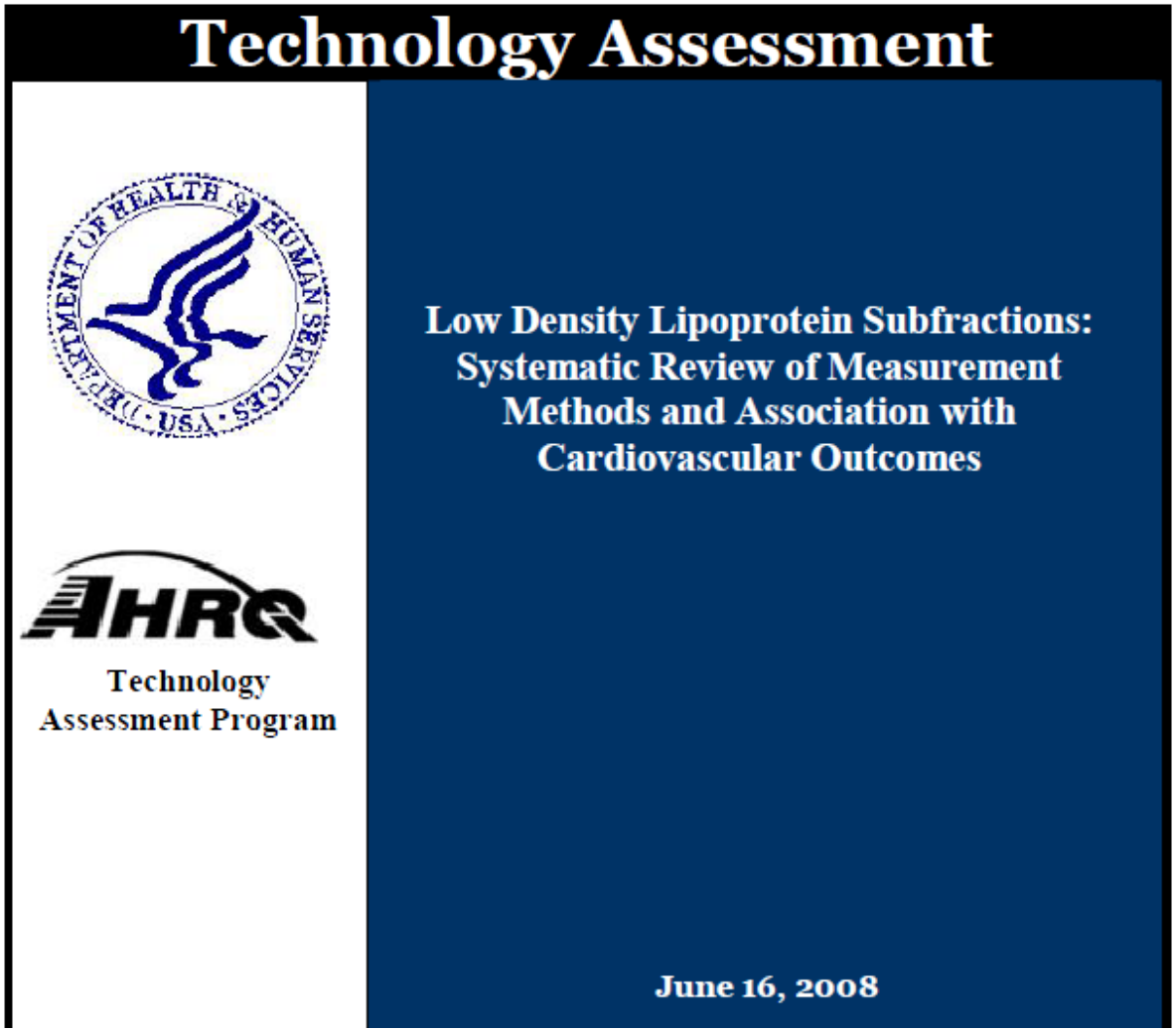
Standardization of advanced lipoperotein testing

Advanced Lipoprotein Analysis Methods and Performance

No standardized laboratory methods for lipoprotein subclass distribution and quantitation have been established (12,14,15). The currently available commercial laboratory methods use a variety of methods to measure lipoprotein subfractions: gradient gel electrophoresis (Berkeley Heart Lab, Inc., Berkeley, California), nuclear magnetic resonance (NMR) (Liposcience, Inc, Raleigh, North Carolina), density gradient rapid ultracentrifugation (termed the "vertical auto profile" [VAP]; Atherotec, Birmingham, Alabama), and most recently, microfluidic gel electrophoresis using a chip technology (Quest Diagnostics Inc., Madison, New Jersey). Each method measures different physiochemical properties, such as size, charge, distribution of cholesterol, or magnetic resonance to estimate lipoprotein subclass distribution. An Agency for Healthcare Research and Quality–funded systematic review of reports published through June 2008 found widely varying agreement among methods (ranging from 7% to 94% concordance) for measuring LDL subfractions, such that measurements using different methods were not directly comparable (14).

Robison et al. J Am Coll Cardiol. 2012;60(25):2607-15

Standardization of advanced lipoperotein testing



Standardization of advanced lipoperotein testing

As described in the results sections for Questions 1, 2 and 3, there is not yet a standard method of subfraction measurement that can be used as a reference standard, has been demonstrated to be superior to other methods, or has been demonstrated to be accurate and reliable. Each of the three major methods for measuring LDL subfractions – GE, NMR, and ultracentrifugation – describes and measures the subfractions differently.

It is important to note, though, that comparisons of methods based on agreement in size or phenotypes are necessary, but not sufficient, to evaluate whether the different methods are measuring the same LDL subfraction analytes. Since different combinations of physicochemical properties are used to separate lipoproteins with different methods (eg, density, size, electrophoretic mobility) the correlation between methods will inevitably be imperfect.

Development of reference materials are necessary to allow for descriptions of the similarities and differences of the various measurements produced by the different methods. A reference method needs to be widely accepted as appropriate, accurate and reliable. However, even with a consensus reference method, it may not be possible to standardize or harmonize all of the methods because their measurement principles are so different. Possible approaches to reference measurements would include developing reference materials that are at a minimum are characterized and defined by composition, density and size.

Standardization through REFERENCE MATERIALS

Standardization of advanced lipoperotein testing

Clinical Chemistry 59:5
752–770 (2013)

Special Report

Association of Apolipoprotein B and Nuclear Magnetic Resonance Spectroscopy–Derived LDL Particle Number with Outcomes in 25 Clinical Studies: Assessment by the AACC Lipoprotein and Vascular Diseases Division Working Group on Best Practices

Thomas G. Cole,[1*] John H. Contois,[2] Gyorgy Csako,[3] Joseph P. McConnell,[4] Alan T. Remaley,[3] Sridevi Devaraj,[5] Daniel M. Hoefner,[4] Tonya Mallory,[4] Amar A. Sethi,[6] and G. Russell Warnick[4]

RECOMMENDATIONS

Based on the preceding observations, we make the following recommendations:

1. The measurement of particle number, either as concentration of apo B or LDL-P should be incorporated into the guidelines for the assessment of CVD risk.
2. Manufacturers of analytical systems for measurement of apo B concentration or particle number should produce well-characterized, robust assays with disclosure of analytical properties, such as antibody specificity, and information regarding standardization.
3. All manufacturers should standardize their assays according to WHO-IFCC reference materials by the currently available standardization program at the NWLMDRL using the apo B DCM.
4. Researchers and laboratories using these assays in clinical studies should calibrate or verify the accuracy through the use of frozen serum samples from NWLMDRL.
5. Performance goals (precision, bias, total error) for LDL-P assays should be determined by expert consensus, as was done for other lipid/lipoprotein biomarkers.
6. Additional studies should be performed to determine the optimum specificities for apo B antibodies (e.g. apo B-100, apo B-48, apo [a]), and to the various apo B–carrying particles, to best characterize CVD risk and monitor therapy.
7. Further studies should be performed to compare apo B to LDL-P using a variety of representative specimens to better understand the inherent differences and contributors to discordance, as well as relative advantages and disadvantages of the 2 assays.

Otvos et al. clin chem 2008;54(12):2086-7

« Collaborative standardization efforts between groups that perform particle-concentration measurements will be required to enable its broader use in clinical practice »

Bio-SITrace crossplatform comparison of lipoprotein enumeration techniques

Objectives : 1/ assess comparability of lipoprotein enumeration techniques, 2/ identify what parameters cause discrepancies & assess impact of freezing in order to establish requirements specification of candidate RMs

Samples :

- 25 patient samples measured before & after freezing (ie. 25 fresh + 25 frozen)
- 3 candidate RM (frozen serum pools CLSI C37-A)
- WHO reference reagent SP3-08 (used to calibrate ApoB routine assays)

Methods / Participants :

- NMR @ NIH (Alan Remaley) & LipoScience / LabCorp (Jim Otvos)
- ES-DMA @ LNE, CHORI (Ron Krauss), Quest Diagnostics (Mike Caulfield)
- ApoB immuno-nephelometry @ Univ. Washington (Santica Marcovina)
- Apo B IDMS @ CDC (John Barr), Univ. Leiden (Christa Cobbaert), Univ. Washington (Andy Hoofnagle)

Other methods : Density Gradient Ultracentrifugation / VAP (Kris Kulkarni), Tube Gel Electrophoresis / LipoPrint (Nehemias Muniz), Gradient Gel Electrophoresis (Ron Krauss), lipids measurements (TC, TG, HDL-C & LDL-C)

Conclusions & discussion

- ✓ **Given the difficulty to (re)define the measurand, establishing (SI) traceability in lipoprotein testing is extremely challenging**
- ✓ **Potential of ES-DMA to be a primary method still under evaluation**
- ✓ **If traceability to the SI is not achievable, should we better go for standardization or harmonization?**
- ✓ **Consensus needed before new traceability chains can be implemented**
- ✓ **BioSITrace cross-platform comparison will be a valuable tool to :**
  - **assess comparability of enumeration methods**
  - **identify what parameter(s) hamper comparability**
  - **qualify candidate international standards**

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Programme of EURAMET

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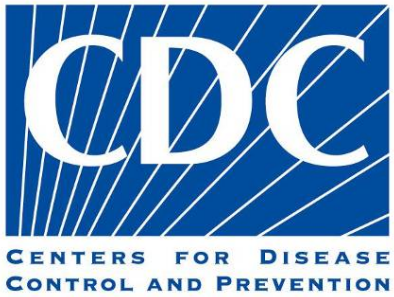
Stakeholders and partners (WP3 on Lipoproteins)

BioSITrace



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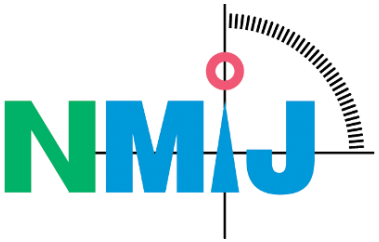
LIPOSCIENCE

CHU DE REIMS



Thank you for you attention!

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