Dissecting HDL: Subfractionation, Functionality, Lipidomics and Proteomics

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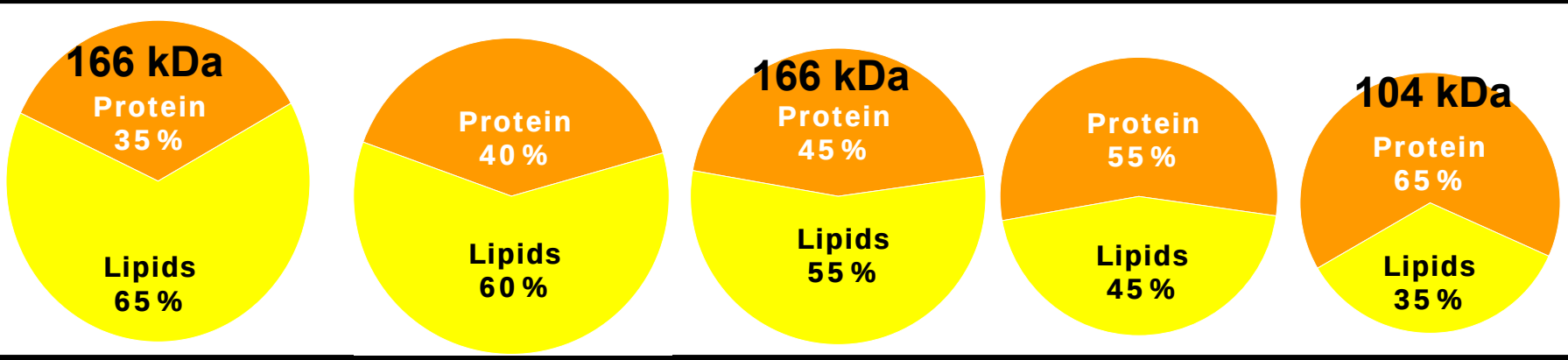
Faculty Disclosure Statement

Robert S. Rosenson, MD, FNLA

has served as a consultant and/or advisor for Abbott Laboratories, Aegerion, Amgen Inc., Kowa, LipoScience Inc., F. Hoffman LaRoche, Regeneron and Sanofi-Aventis; is a stock/shareholder of LipoScience.

HDL Particle Subspecies : physicochemical properties

	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
d (g/ml)	1.099	1.107	1.123	1.155	1.186

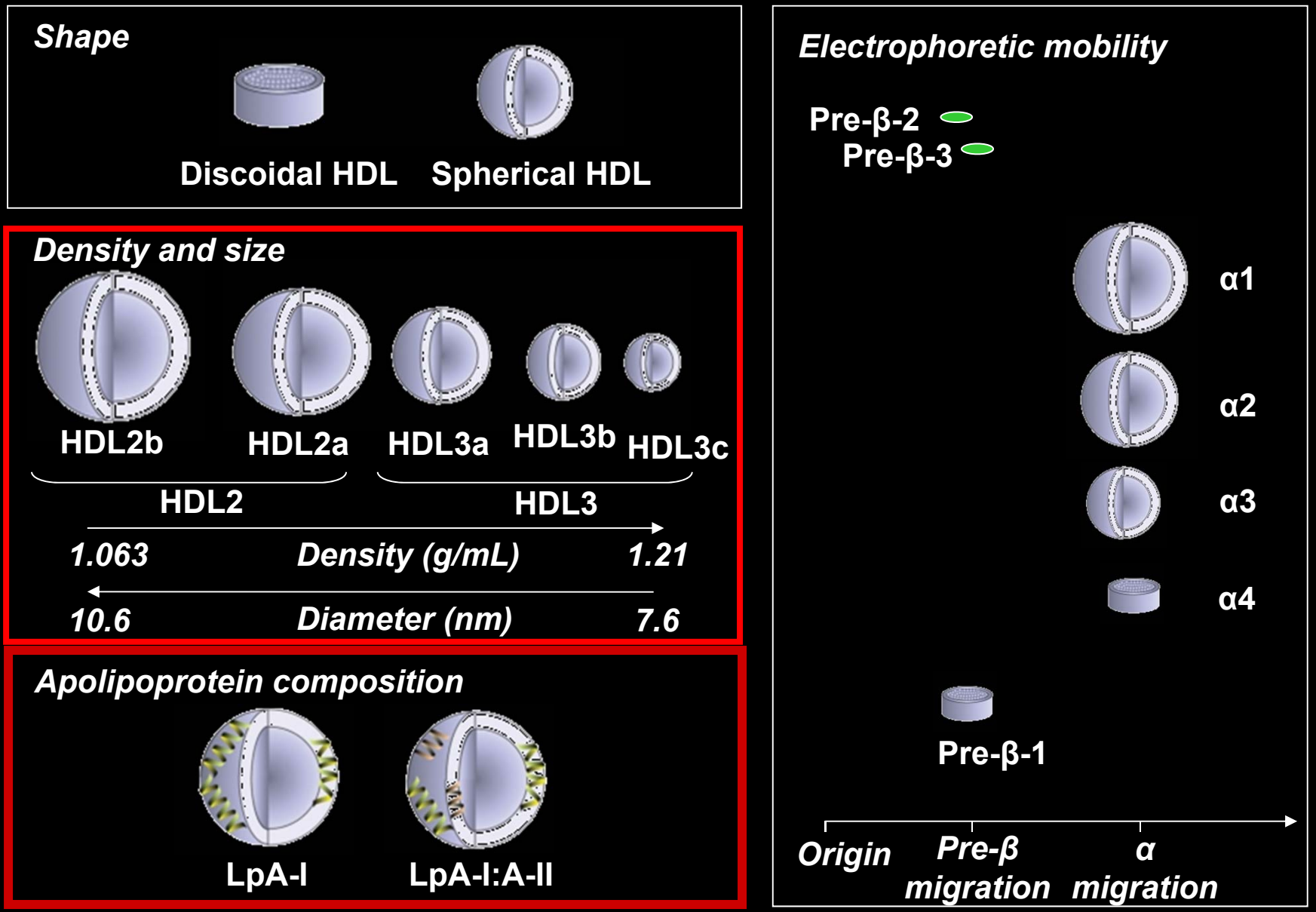


	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Diam (nm)	10.4	8.2	7.8	7.6	7.6
M_r (kDa)	499	413	370	200	160
Concn (μM)	1.7	1.8	1.9	1.3	1.0
AI/AII (mol/mol)	5.6	2.9	2.8	4.6	6.9
Surface/ Core	2.2	2.5	2.7	3.4	3.9

Kontush A, Chantepie S, Chapman MJ, Arterioscler Thromb Vasc Biol. 23: 1881-1888, 2003

Kontush A, de Faria E, Chantepie S, Chapman MJ, Arterioscler Thromb Vasc Biol. 24: 526-33, 2004

Physicochemical Heterogeneity of HDL

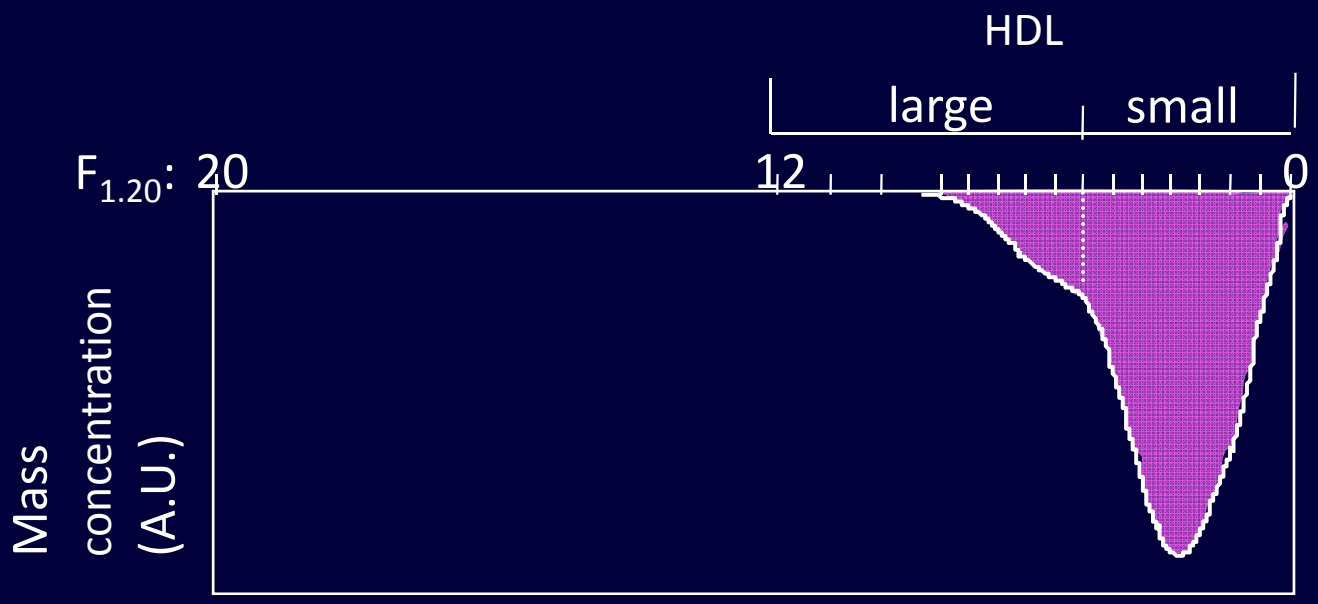


Camont L, et al. *Trends Mol Med.* 2011;17(10):594-603.

Classification of HDL by Physical Properties

- Ultracentrifuguation
- Gradient Gel Electrophoresis (GGE)
- 2-D Gel Electrophoresis
- Ion Mobility (IM)
- Nuclear Magnetic Resonance Spectroscopy (NMR)
- Vertical Auto Profile (VAP)

Measurement of Lipoprotein Subclasses by Analytical Ultracentrifugation



- **First method developed to measure lipoprotein subclass levels- "gold standard"**
- **Separation of d<1.2 g/ml lipoproteins based on flotation rates ($F^{o}_{1.2}$) of particles - function of physical properties (size, density)**
- **Method retired in 2005**

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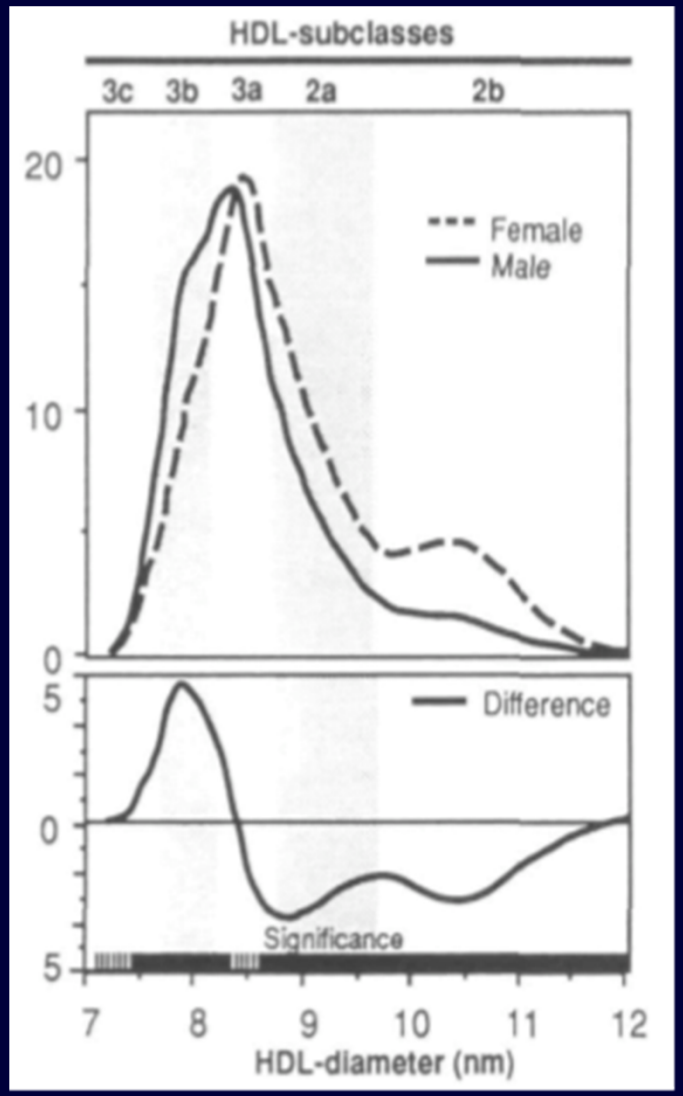
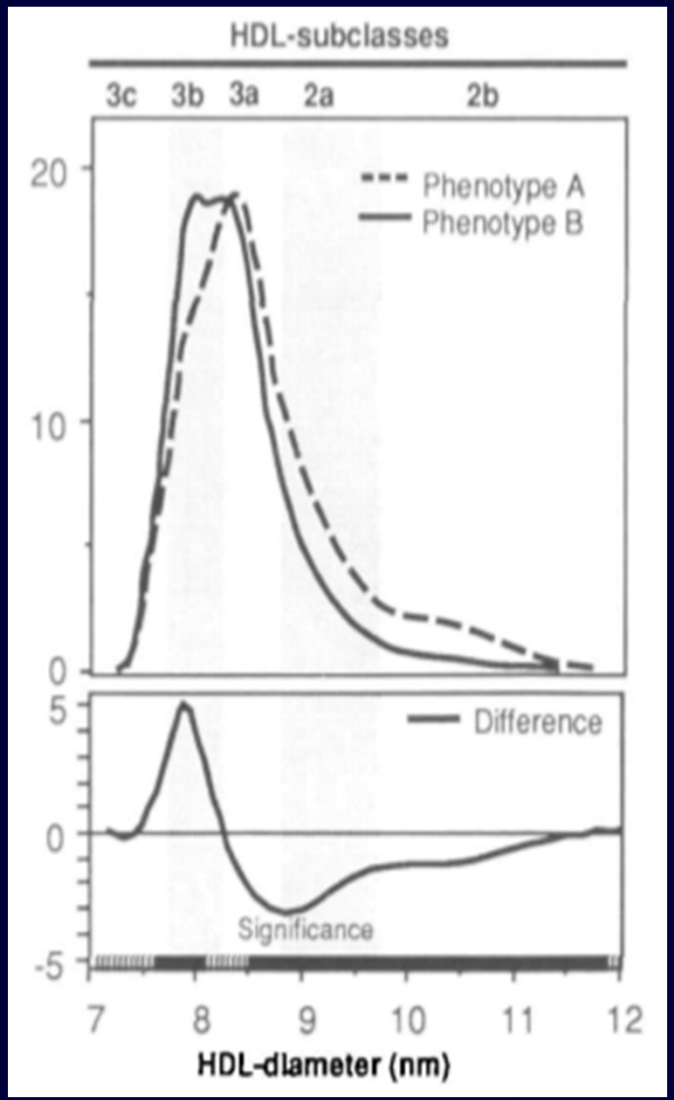
- Ion Mobility

Non-Denaturing Gradient Gel Electrophoresis

- Segmented 4-30% native pore-gradient polyacylamide gel used to separate HDL particles based on size
- Original method used d<1.21 g/mL fraction with staining for protein; other gradients and stains have been used
- Areas of five subclasses determined by densitometry after staining (protein or lipid)

HDL Subspecies by GGE





Williams, Krauss, et al..Arterioscler Thromb.;12:332-40, 1992

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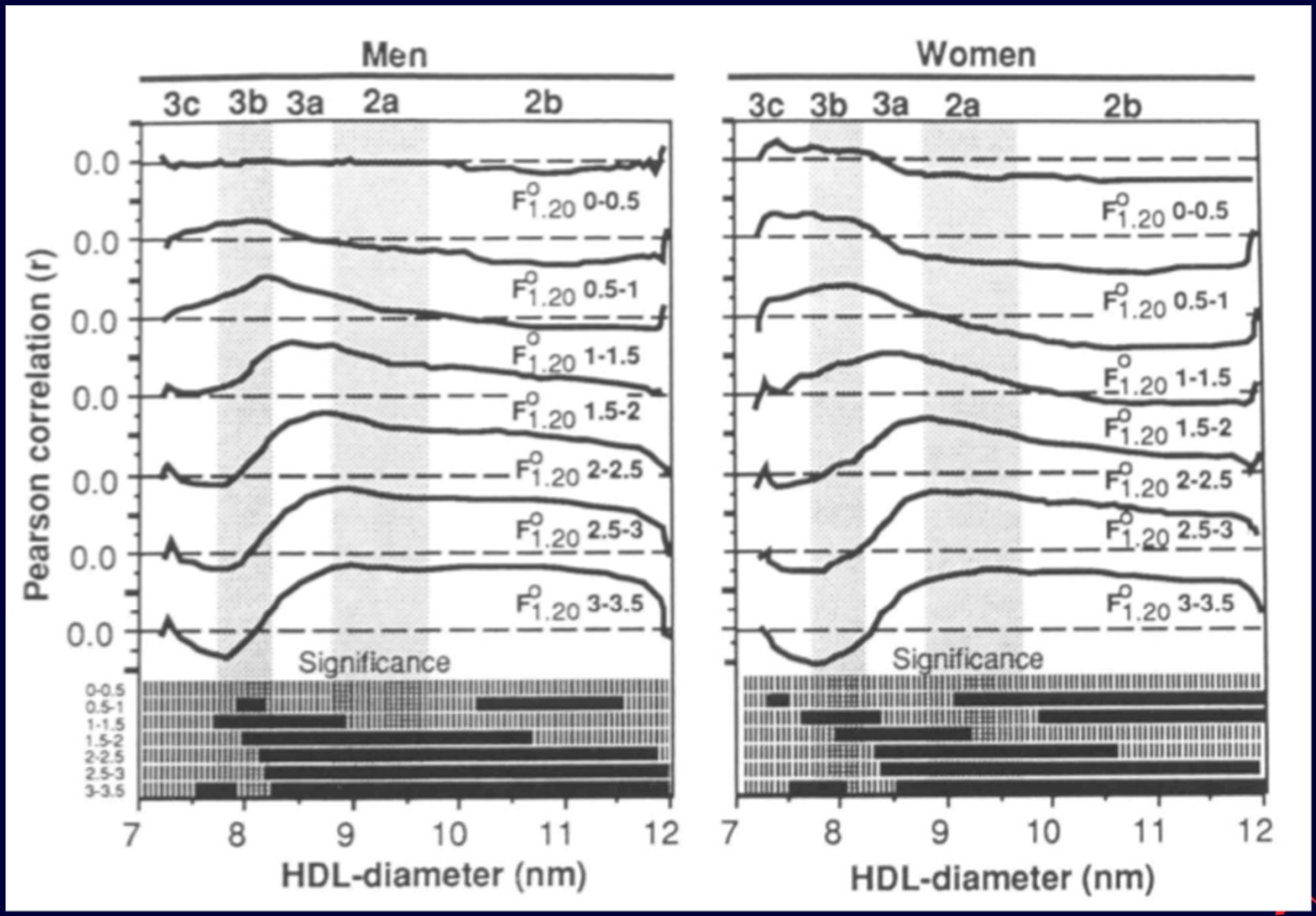
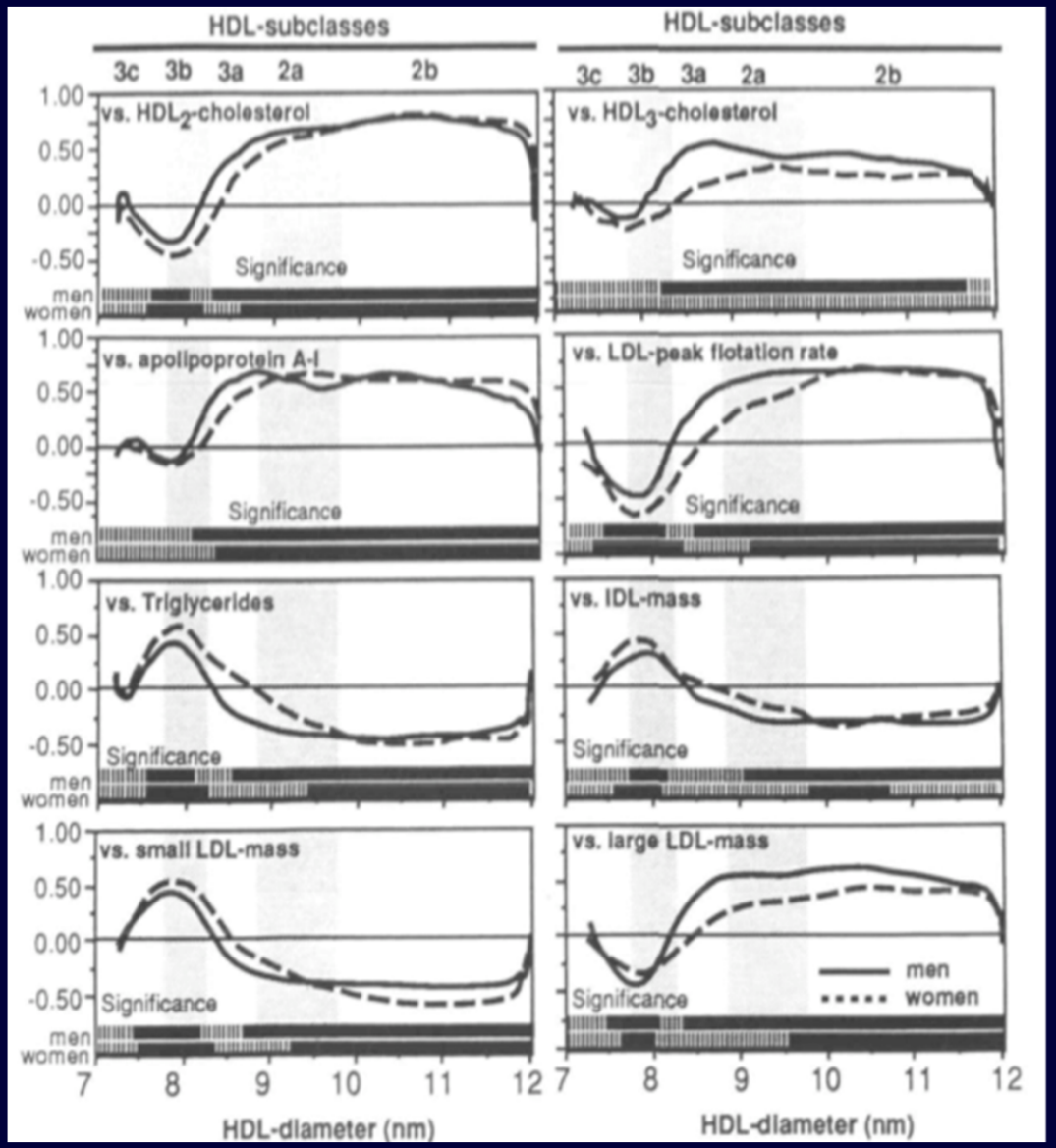
Associations of HDL fractions measured by GGE and AnUC



Williams, Krauss, et al. Arterioscler Thromb.;12:332-40, 1992

Associations of HDL subfractions with other lipoprotein measurements



Williams, Krauss et al., Arterioscler Thromb.12:332-40, 1992.

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Classification of HDL by Physical Properties

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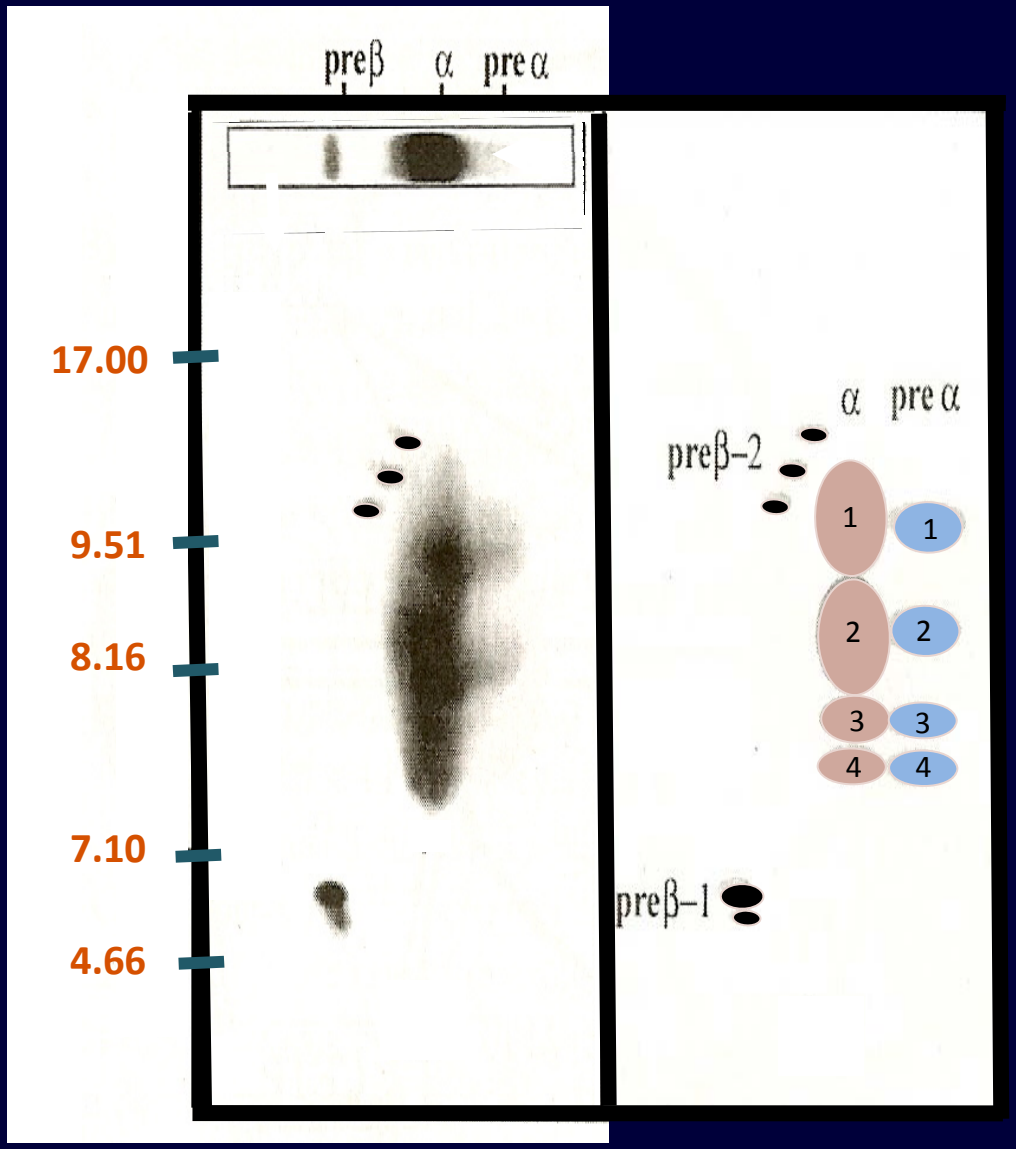
2-D Gel Electrophoresis of Plasma Lipoproteins



Asztalos BF, et al. Arterioscler Thromb Vasc Biol. 2004;24(11):2181-2187.

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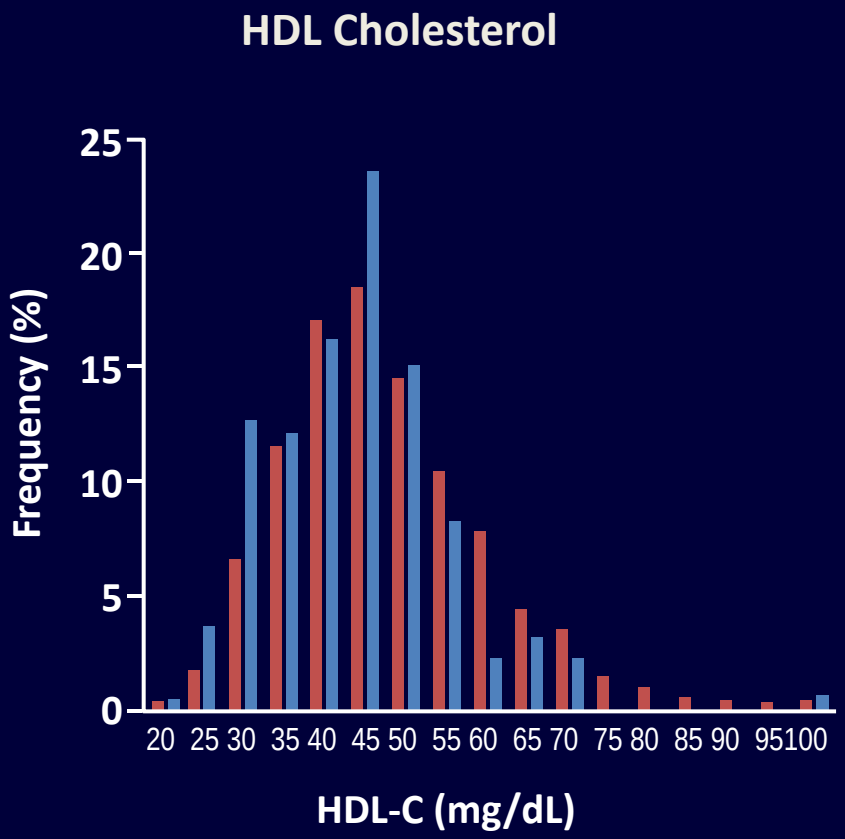
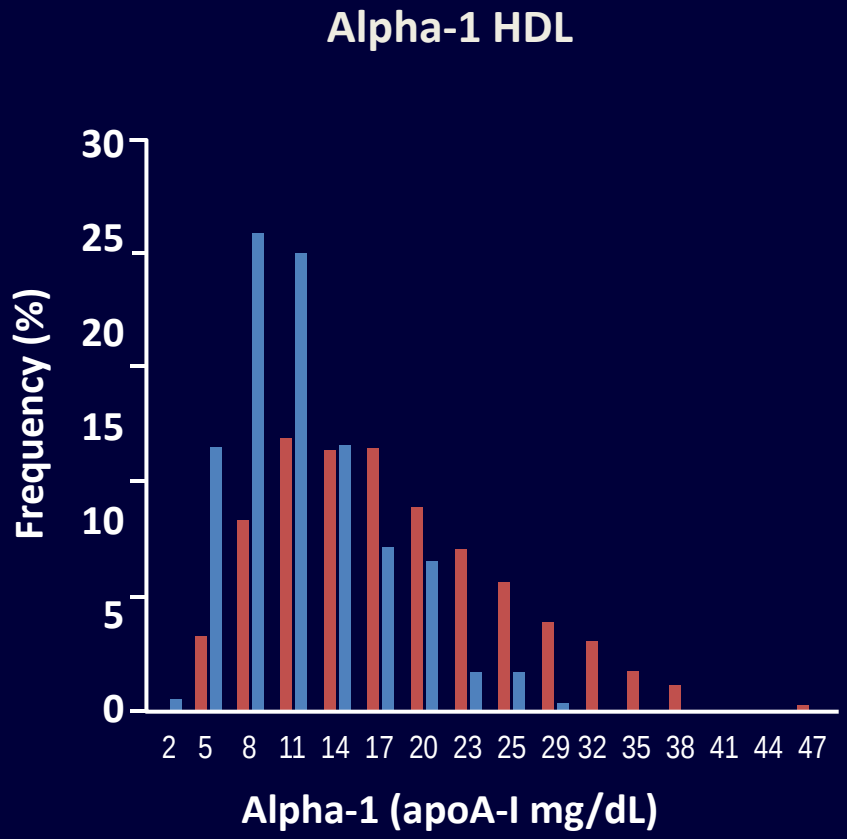
Frequency Distribution of HDL Cholesterol Levels, and Alpha-1 HDL Particle Levels of CHD Cases in FHS





All controls (n = 1277) CHD (n = 169)

Asztalos BF, et al. Arterioscler Thromb Vasc Biol. 2004;24(11):2181-2187. Copyright © 2004 Lippincott Williams & Wilkins.

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Odd ratios as calculated by a model including all HDL particles and the traditional lipid and nonlipid CHD risk factors in FHS

	All CHD vs all controls		All CHD vs HDL-C-matched controls	
	OR	*P*	OR	*P*
Pre-Beta-1	0.97	0.24	1.00	0.89
Pre-Beta-2	1.15	0.18	1.27	0.12
Alpha-1	0.73*	< 0.01	0.74*	< 0.01
Alpha-2	0.99	0.44	1.03	0.39
Alpha-3	1.15*	< 0.01	1.18*	< 0.01
Pre-Alpha-1	1.61*	< 0.01	1.68*	< 0.01
Pre-Alpha-2	1.11	0.24	1.15	0.31
Pre-Alpha-3	0.60*	< 0.01	0.62*	< 0.01
HDL-C	1.02	0.48	NA	NA

ORs were calculated for one unit increase in each determined parameter or yes or no for the other parameters. *Significantly associated with CHD prevalence; NA indicates not applicable. 169 CHD cases, 1277 controls, 358 HDL-C-matched controls.

Asztalos BF, et al. ArterioscIerThrombVasc Biol. 2004;24(11):2181-2187.

Hazard Ratios as Calculated for Alpha-2 HDL and HDL-C in Predicting CV End Points (n = 16) in the Gemfibrozil Arm of VA-HIT (n = 754)

Model[a]	HR for each 1-SD increase in alpha-2 (8.51)		HR for each 1-SD increase in HDL-C (6.37)	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Model 1	0.79 (0.68-0.93)	0.004	0.81 (0.70-0.93)	0.003
Model 2	0.81 (0.69-0.96)	0.01	0.83 (0.72-0.96)	0.01
Model 3	0.83 (0.71-0.98)	0.03	0.90 (0.76-1.07)	0.23
Model 4	0.82 (0.66-1.01)	0.06	0.93 (0.76-1.14)	0.50

[a]Model 1: data were unadjusted; model 2: data were adjusted for nonlipid CHD risk factors (age, smoking, hypertension, BMI, and diabetes); model 3: data were further adjusted for LDL-C and logTG; model 4: for alpha-2, data were further adjusted for HDL-C; and for HDL-C, data were further adjusted for alpha-2.

Asztalos BF, et al. *Metabolism.* 2008;57(1):77-83.

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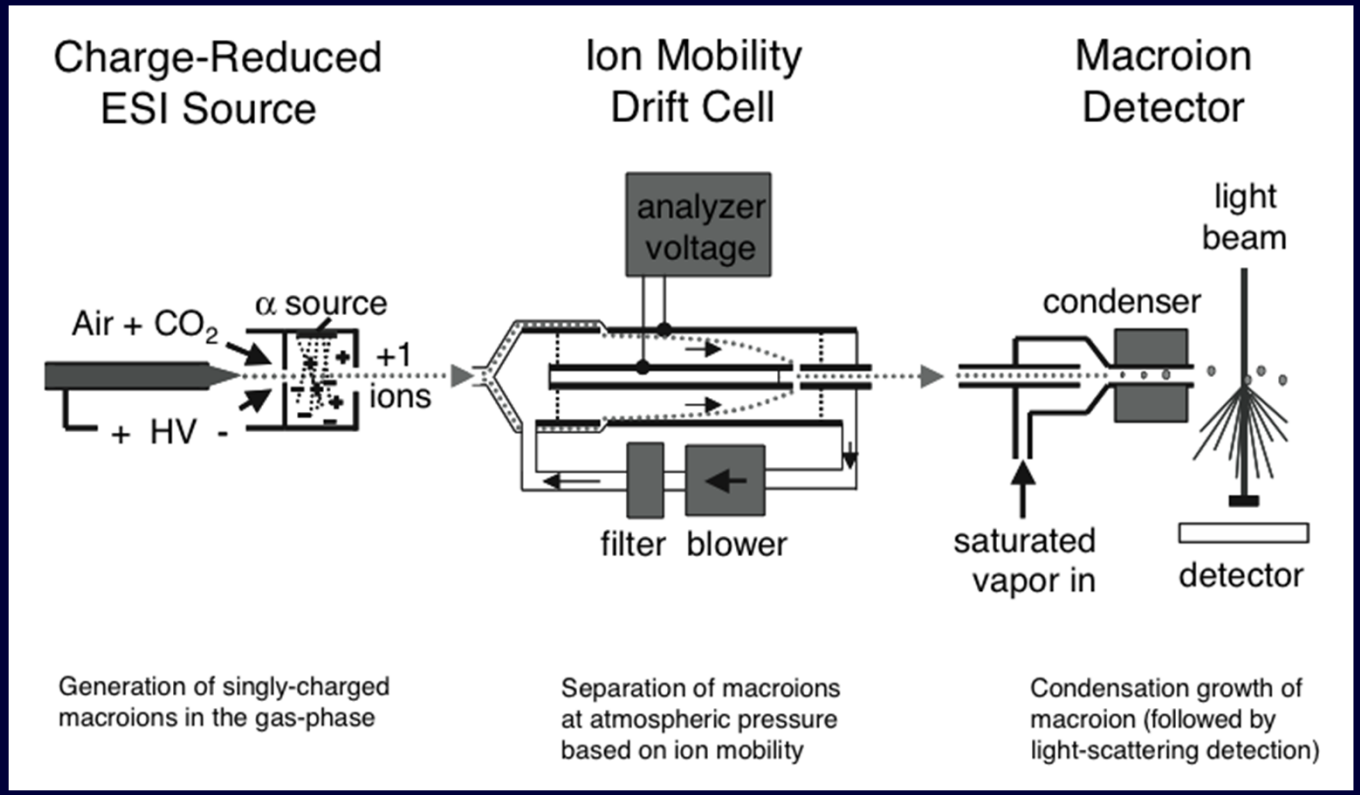
Lipoprotein Particle Measurement by Ion Mobility



- **Direct measurement of lipoprotein particle number as a function of particle size**
- **Particle concentrations can be converted to mass concentrations**

Caulfield et al. Clin Chem 54:1307, 2008

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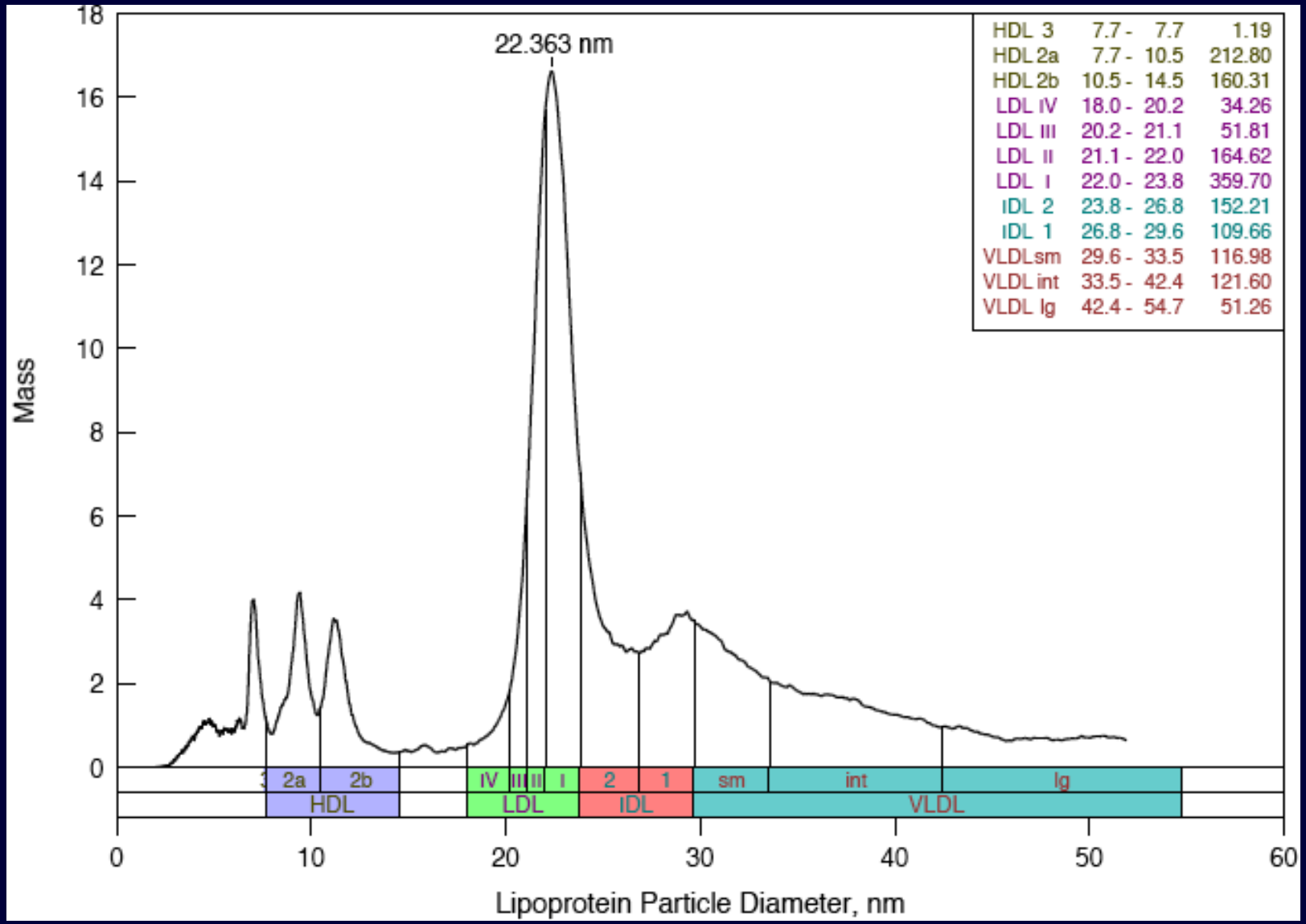
Lipoprotein Particle Analysis by Ion Mobility



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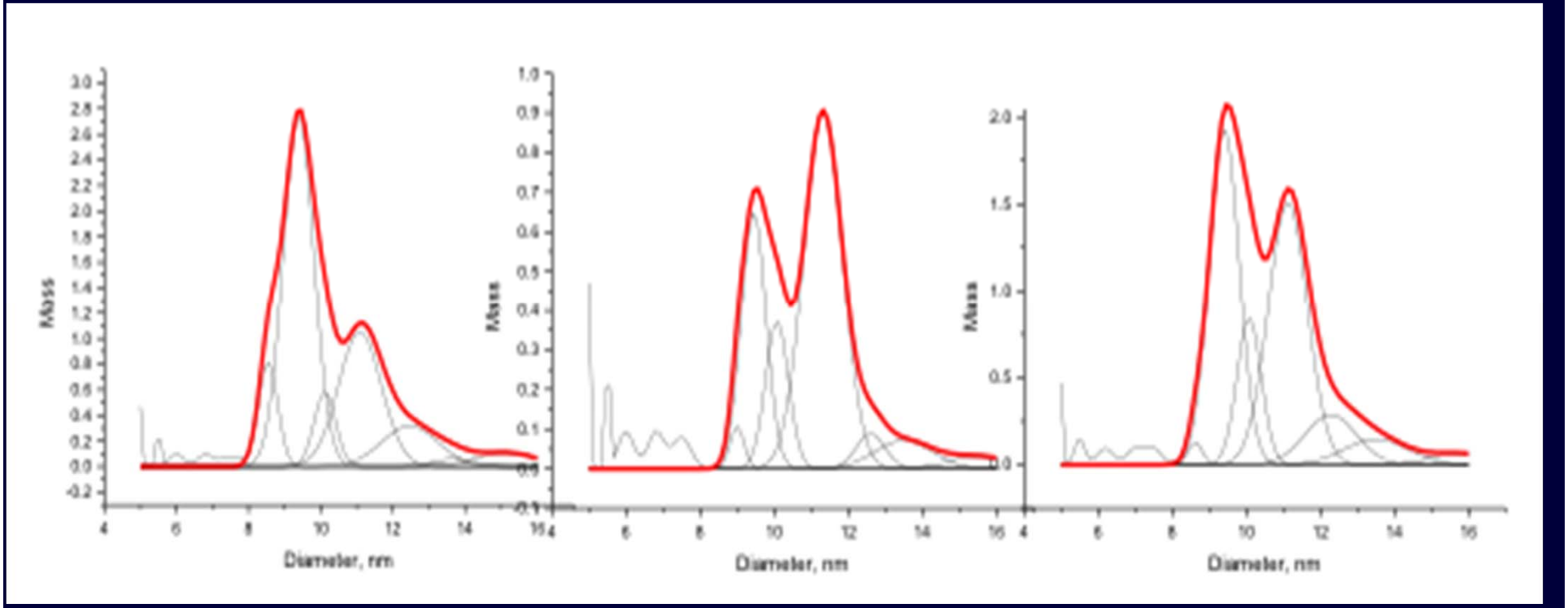
Resolution of HDL into 5 Components Based on Ion Mobility



Particle size intervals correspond to those measured by GGE

Measures particle concentrations directly in nmol/L

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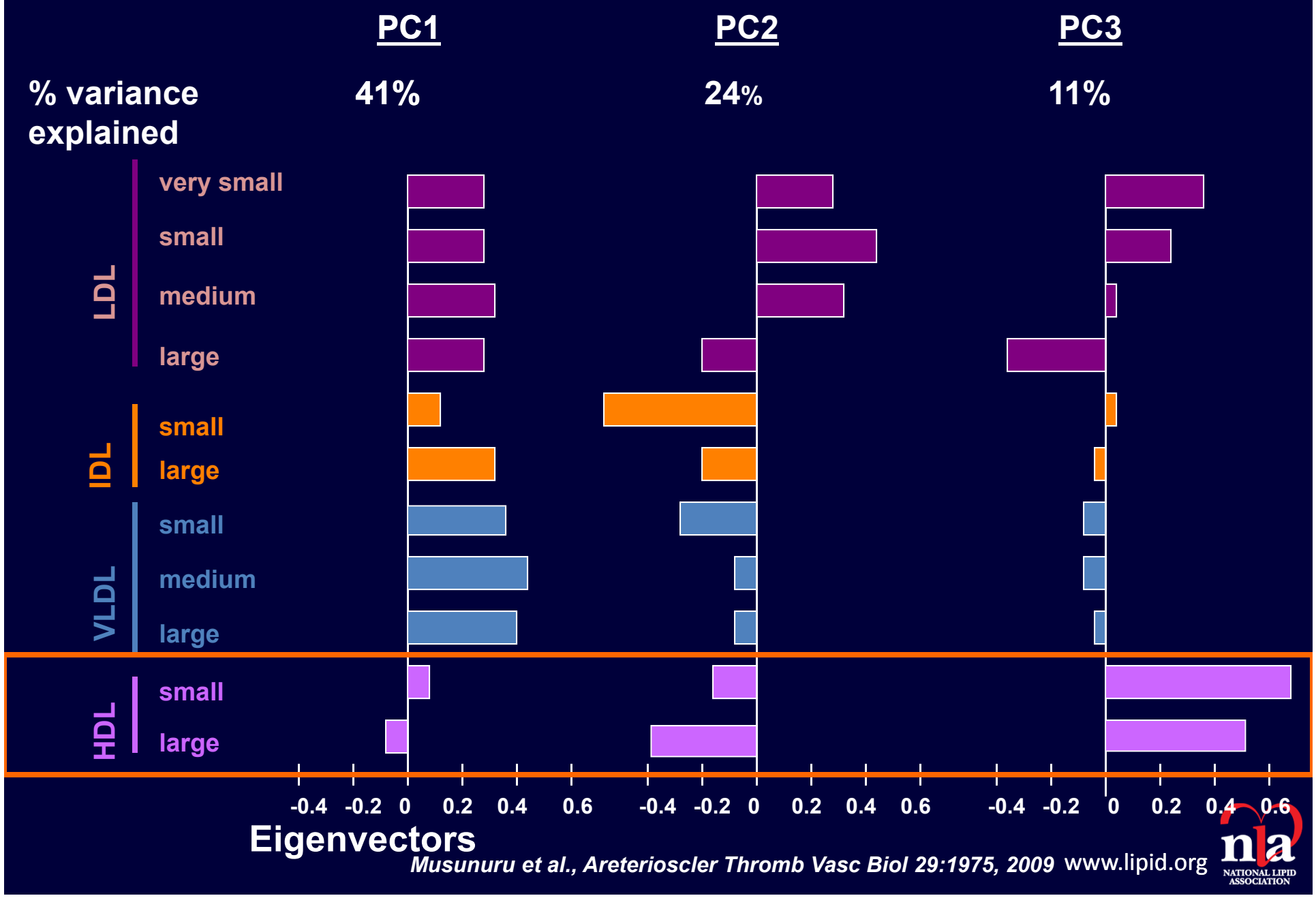
Use of Ion mobility analysis to assess relation of HDL subfractions to CHD risk

- Levels of HDL particles are strongly correlated with other lipoprotein fractions
- Can use principal component analysis to systematically analyze the correlations among these subfractions
- This was done using ion mobility measurements in a population-based prospective study of CHD risk: the Malmö Diet and Cancer Study Cardiovascular Cohort (n=4,594)

Musunuru et al., Areterioscler Thromb Vasc Biol. 29:1975, 2009

PC analysis of lipoprotein subfractions



Musunuru et al., Areterioscler Thromb Vasc Biol 29:1975, 2009 www.lipid.org

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PCs and incident cardiovascular events

Measure	HR	95% CI	*P* value
PC1	1.10	0.99 – 1.22	0.07
PC2	1.22	1.08 – 1.36	0.001
PC3	0.81	0.71 – 0.92	0.001
HDL-C	0.72	0.63 – 0.82	< 0.001
LDL-C	1.10	0.99 – 1.21	0.08
TG	1.12	1.00 – 0.25	0.04

single model

Musunuru et al., Areterioscler Thromb Vasc Biol 29:1975, 2009

Summary:
HDL subspecies defined by size and density

- Reduced levels of large HDL (HDL2b) are strongly predictive of increased CHD risk, but smaller HDL subspecies are also of importance.
- Increases in specific smaller HDL subspecies (e.g. HDL3b) are a marker of atherogenic dyslipidemia.
- There are both direct and indirect associations of HDL particles with CHD risk that may represent multiple distinct pathophysiologic processes.

Classification of HDL by Physical Properties

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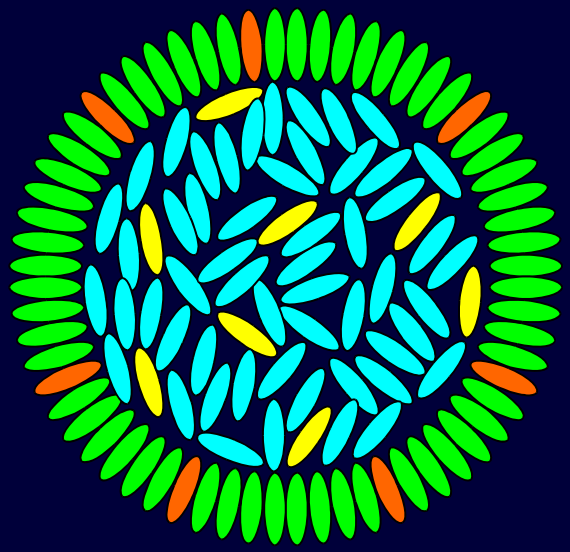
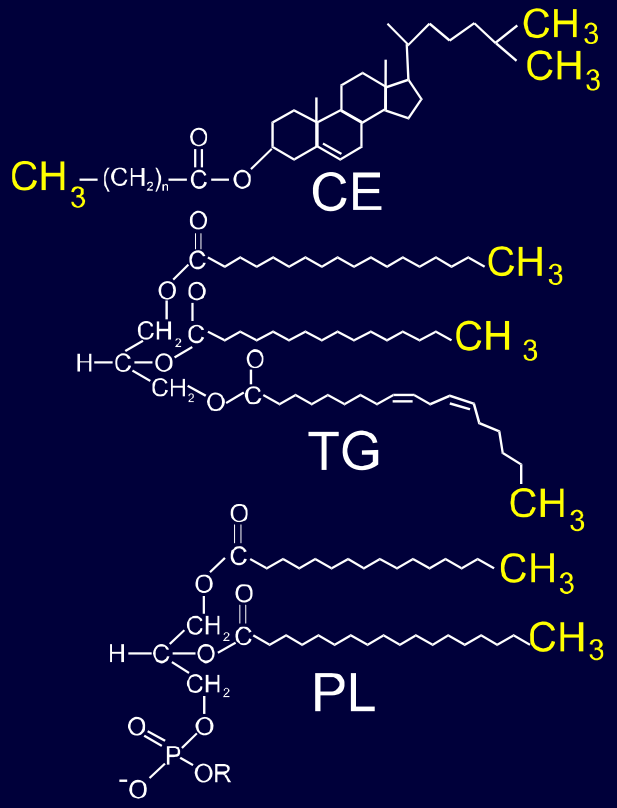
NMR Lipoprotein Particle Measurement

1. **Measure NMR spectrum of plasma or serum (~1 min)**
   - fully automated process on clinical NMR analyzer
   - primary tube sampling; no pre-analytical processing
   - auto-calibration; stable/standardized results
   - reagent-free, no kits or chemical reactant
2. **Deconvolute methyl signal envelope (<1 sec)**
   - gives subclass signal amplitudes (≈concentrations)
   - converts these into reported VLDL, LDL, HDL subclass particle concentration
3. **Validation of lipoprotein size and particle concentration**
   - highly purified lipoprotein subfractions with very narrow size distributions, a combination of ultracentrifugation and agarose gel filtration chromatography
   - Average lipoprotein diameters were derived by measuring the diameters of at least 200 lipoprotein particles quantified from two or more grids
   - Spiking experiments to determine concentration

The Measured Subclass Signals Come From the Terminal Methyl Groups on the Lipids in the Particle Shell and Core





To a close approximation, the number of these methyl groups in a particle of given size is unaffected by lipid compositional variation. The measured amplitudes of the subclass signals are thus proportional to particle concentration.

Jeyarajah EJ et al. *Clin Lab Med* 2006;26:847-70

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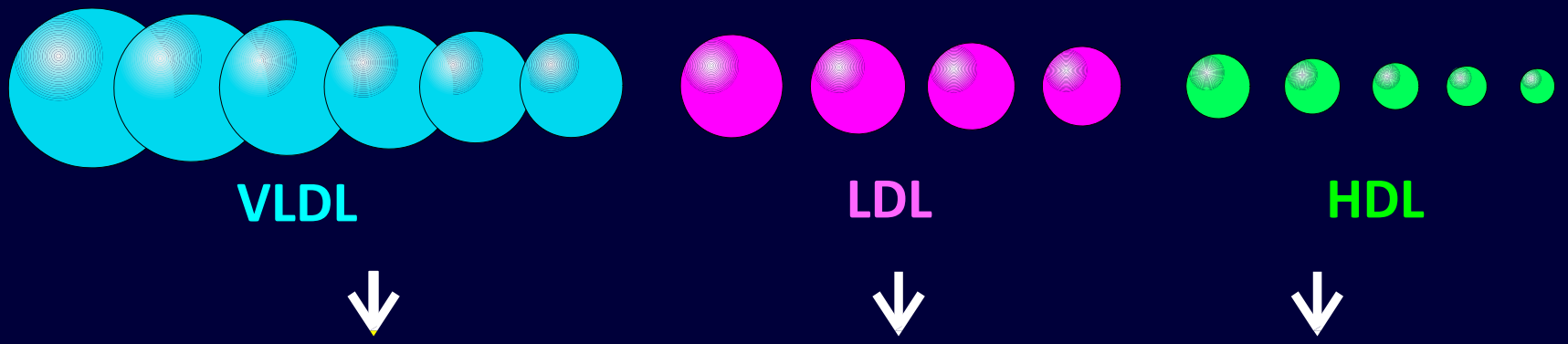
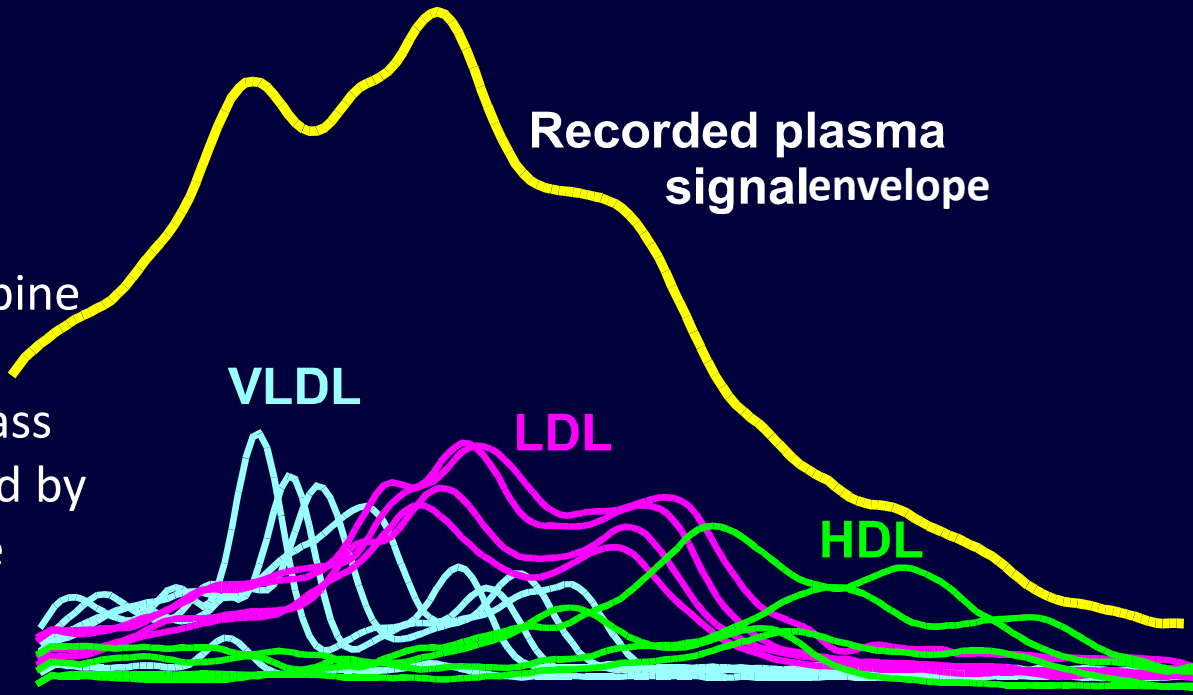
NMR Spectroscopy Measures VLDL, LDL, and HDL Particle Subclasses Simultaneously



"The whole is the sum of its parts"

The subclass signals combine to produce the measured plasma signal. The subclass signal amplitudes (derived by "deconvolution") give the subclass concentrations.



Jeyarajah EJ et al. *Clin Lab Med* 2006;26:847-70

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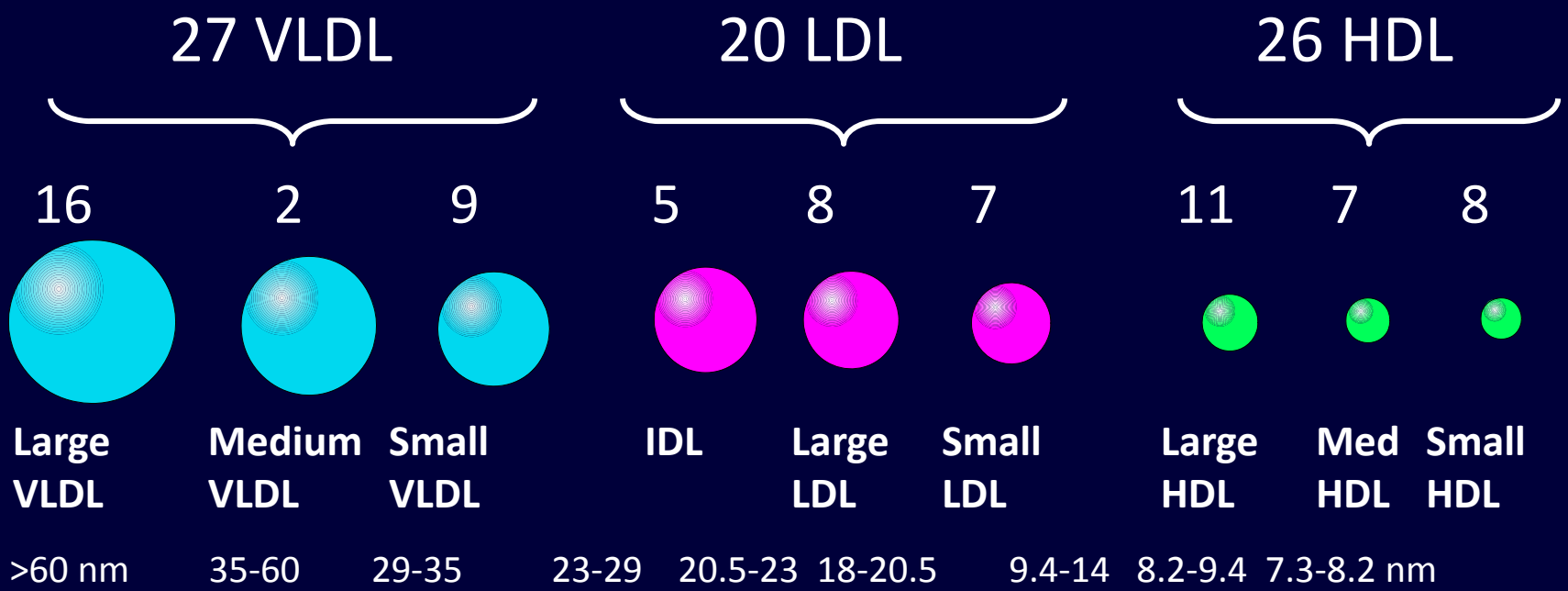
Each Measurement (Behind the Scenes) Produces Concentrations of 73 Subpopulations

These are grouped into 9 subclass categories based on functional/metabolic relatedness, as assessed by their correlations with lipid and metabolic variables



Multi-Ethnic Study of Atherosclerosis (MESA)

- NHLBI observational study of the pathogenesis and progression of subclinical atherosclerosis.
- Subjects are 6,814 asymptomatic men and women free of cardiovascular disease at entry.
- Population consists of 38% White, 28% African-American, 22% Hispanic, and 12% Asian (of Chinese descent).

Current Study

- Excluded subjects on lipid lowering medications or with missing information: n=5,714
- Outcomes:
  - Carotid IMT (cross-sectional)
  - Incident CHD events (MI, CHD death, angina) during 6.1 years of follow-up: n=231

Study Objective

Evaluate relations of HDL cholesterol (HDL-C) and HDL particle number (HDL-P) measured by NMR spectroscopy to:

1) Baseline carotid IMT, and

2) Incident CHD events, before and after adjusting for potentially confounding influences of other lipids and lipoproteins.

Spearman Correlations in MESA (n=5714)

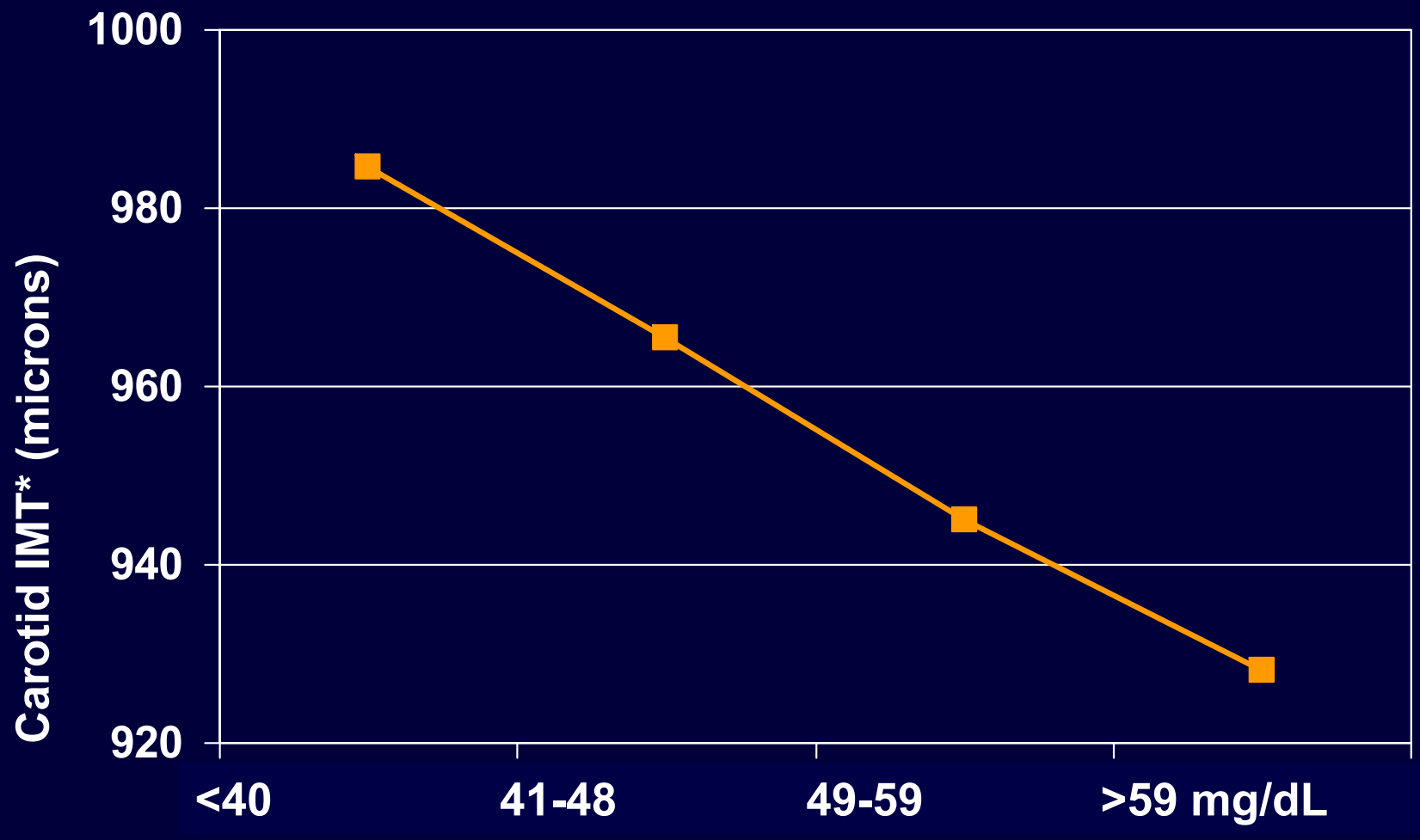
Potential for Confounding

	HDL-C	TG	LDL-C	LDL-P	Small LDL-P
HDL-C	1	-0.47	-0.05	-0.38	-0.73
HDL-P	0.73	-0.10	-0.10	-0.27	-0.46

Mackey RH, et al. J Am Coll Cardiol. 2012 Aug 7;60(6):508-16. www.lipid.org

HDL-C

Relations with Carotid IMT in MESA



*adjusted for age, sex, ethnicity, hypertension, smoking

Mackey RH, et al. J Am Coll Cardiol. 2012 Aug 7;60(6):508-16. Epub 2012 Jul 11. www.lipid.org

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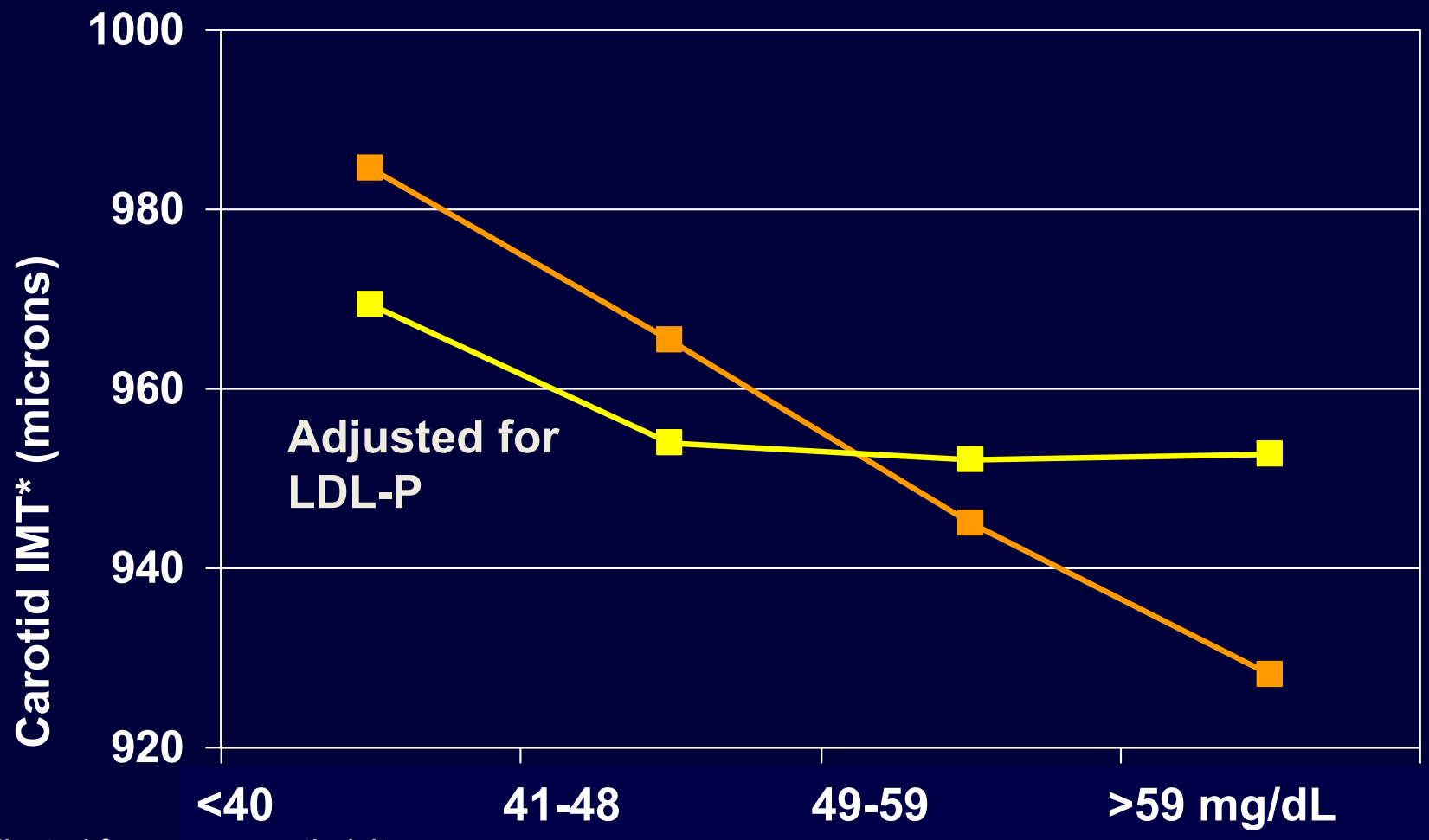
HDL-C

Relations with Carotid IMT in MESA



*adjusted for age, sex, ethnicity, hypertension, smoking

Mackey RH, et al. J Am Coll Cardiol. 2012 Aug 7;60(6):508-16. www.lipid.org

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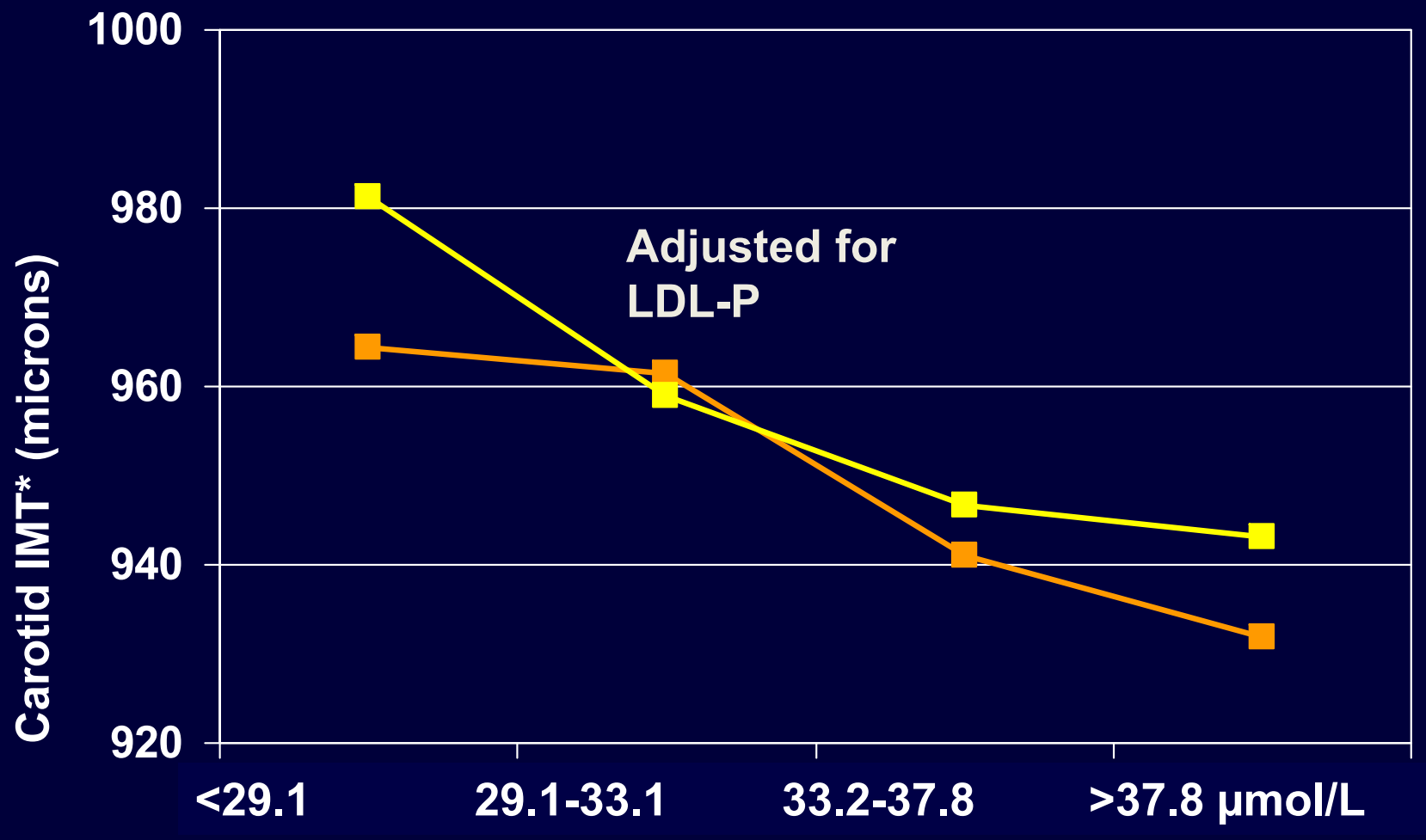
HDL-P

Relations with Carotid IMT in MESA (n=5361)



*adjusted for age, sex, ethnicity, hypertension, smoking

Mackey RH, et al. J Am Coll Cardiol. 2012 Aug 7;60(6):508-16. Epub 2012 Jul 11. www.lipid.org

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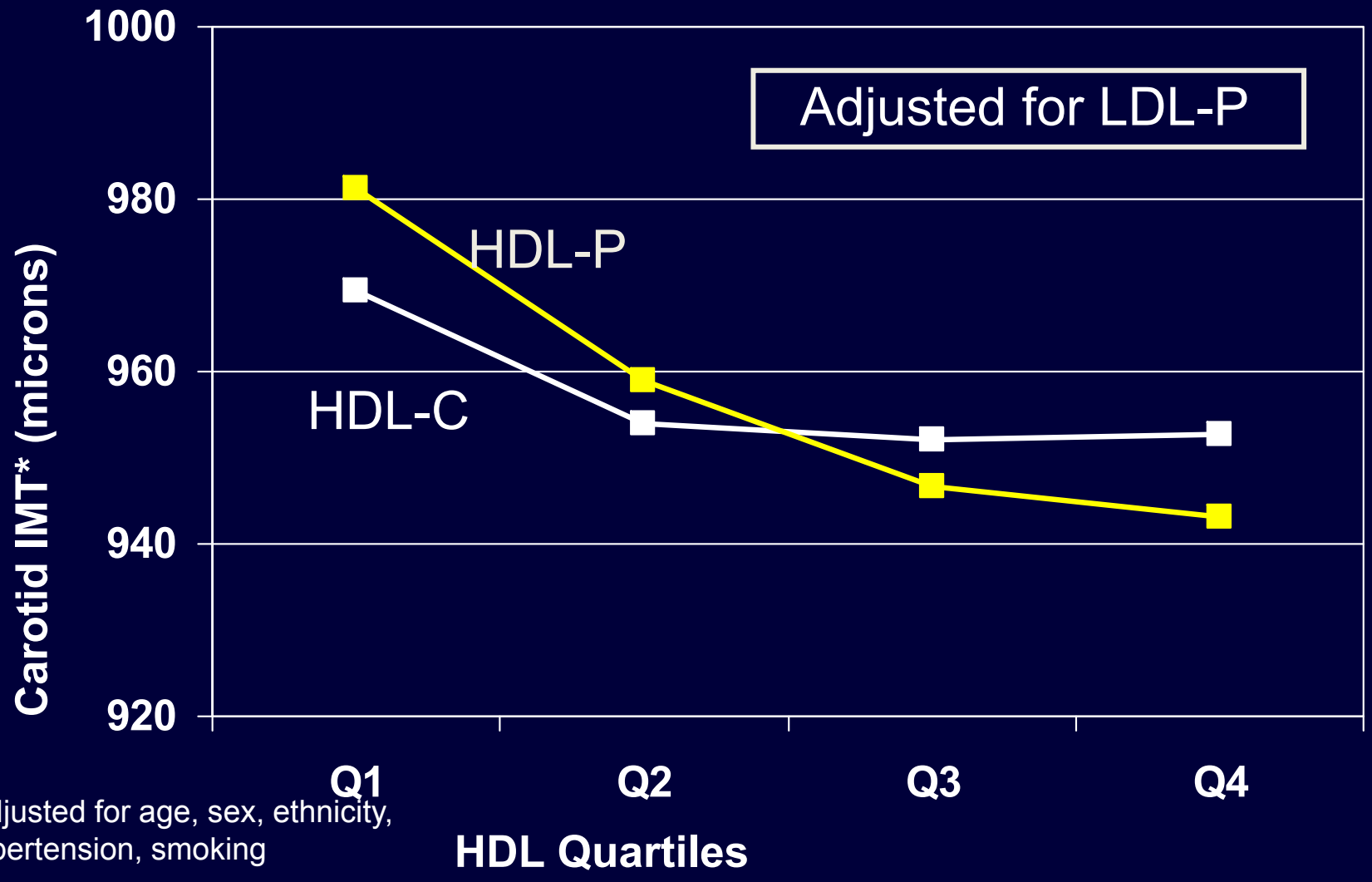
HDL-C and HDL-P

Relations with Carotid IMT in MESA



*adjusted for age, sex, ethnicity, hypertension, smoking

Mackey RH, et al. J Am Coll Cardiol. 2012 Aug 7;60(6):508-16.

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HDL-C and HDL-P

Associations with Carotid IMT

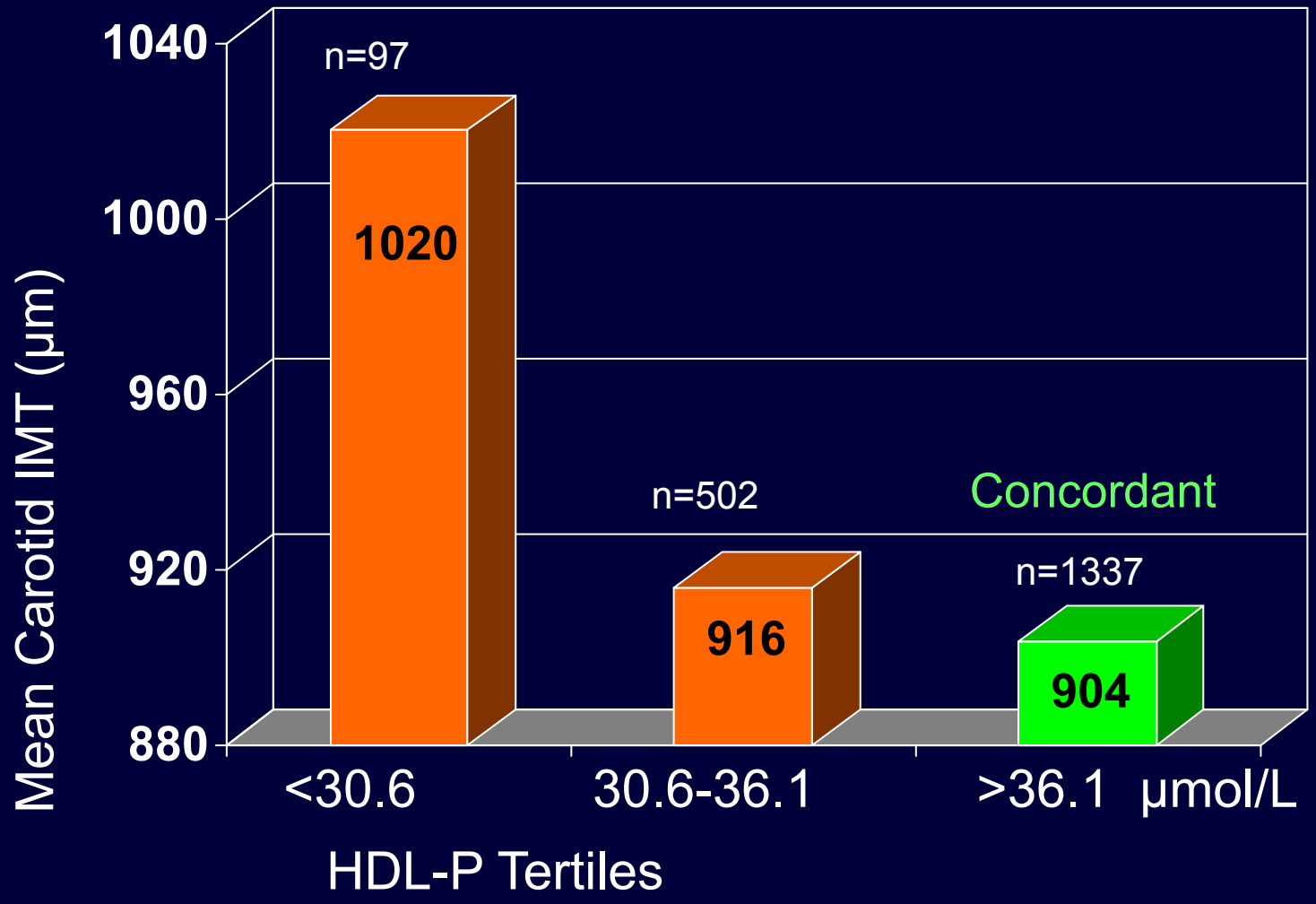
	Carotid IMT Difference (95% CI) in μm	
	HDL-C	HDL-P
	HDL-C and HDL-P in separate models	
Adjusted*	-24.2 (-32.9, -15.6)	-28.1 (-36.8, -19.4)
Adj* + LDL-P	-8.7 (-17.9, 0.5)	-18.2 (-27.1, -9.3)
	HDL-C and HDL-P in joint model	
Adj* + LDL-P	5.1 (-6.7, 16.8)	-21.2 (-32.6, -9.8)

*From linear regression models adjusted for age, sex, ethnicity, hypertension, and smoking. Results are for 1 SD difference

Mackey RH, et al. J Am Coll Cardiol. 20127;60:508-16. www.lipid.org

Discordance Between HDL-C and HDL-P

Subgroup with High HDL-C (≥ 55 mg/dL)



Mackey RH, et al. J Am Coll Cardiol. 2012 ;60:508-16. www.lipid.org

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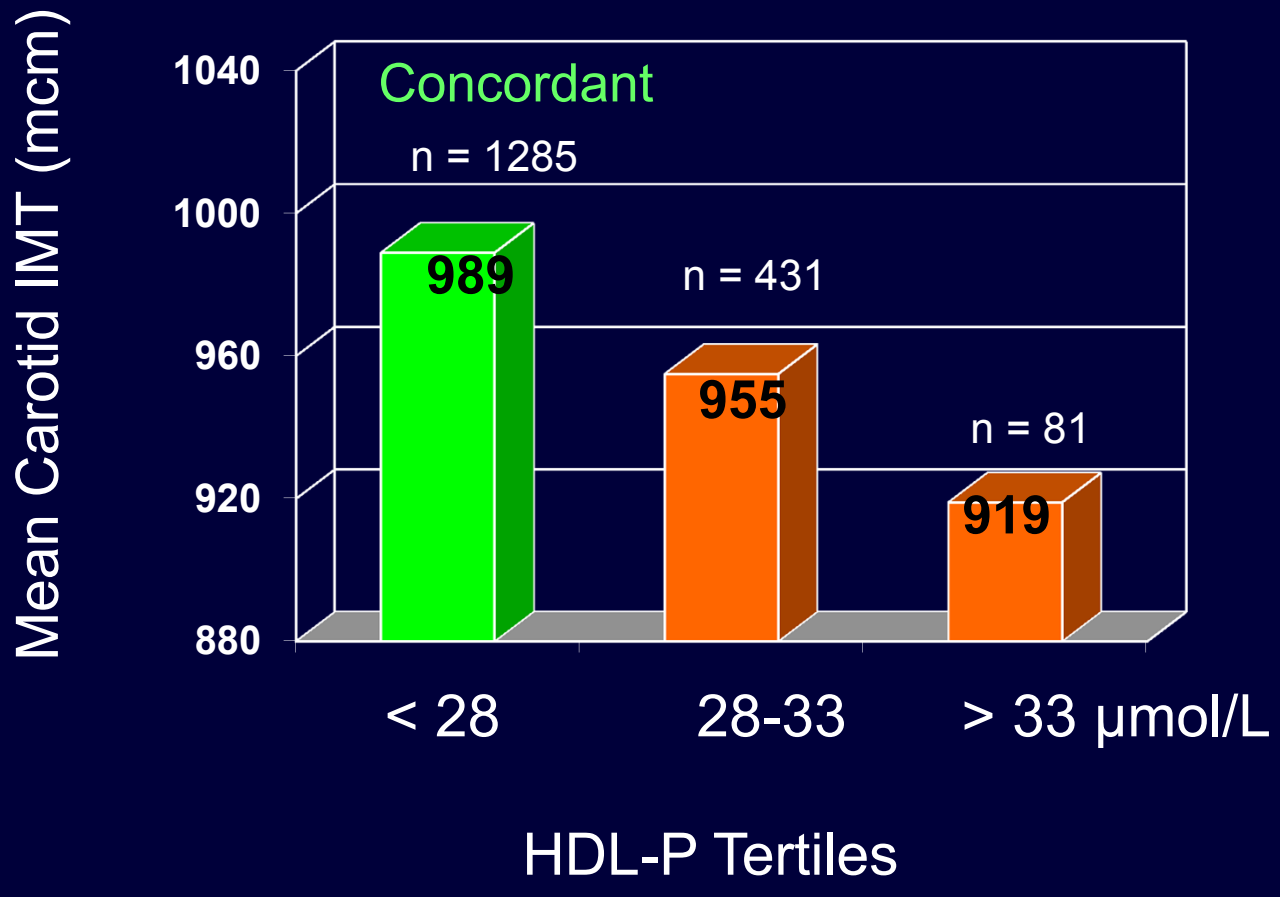
Discordance Between HDL-C and HDL-P

Subgroup with low HDL-C (≤ 42 mg/dL)



Mackey RH, et al. J Am Coll Cardiol. 2012 Aug 7;60(6):508-16. Epub 2012 Jul 11. www.lipid.org

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HDL-C and HDL-P

Associations with Incident CHD Events

	Hazard Ratio (95% CI)	
	HDL-C	HDL-P
	HDL-C and HDL-P in separate models	
Adjusted*	0.73 (0.62, 0.86)	0.70 (0.60, 0.82)
Adj* + LDL-P	0.80 (0.67, 0.95)	0.75 (0.64, 0.89)
	HDL-C and HDL-P in joint model	
Adj* + LDL-P	0.95 (0.76, 1.19)	0.77 (0.63, 0.96)

*From Cox regression models adjusted for age, sex, ethnicity, hypertension, and smoking. Results are for 1 SD difference

Mackey RH, et al. J Am Coll Cardiol. 2012;:508-16. www.lipid.org

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Lipoprotein Subclass Analyses in VA-HIT

- Nested case-control design
- 362 men with nonfatal MI or CHD death during 5-year follow-up
- 694 age-matched controls
- Lipids/lipoproteins measured on baseline and on-trial
- (7-12 month) plasma specimens
- Lipids/apolipoproteins by std. chemical methods
- LDL and HDL particle subclasses by NMR
- All data analysis performed at VA Medical Center
- Cooperative Studies Program Coordinating Center

Otvos JD, et al. *Circulation* 2006;113:1556-1563.

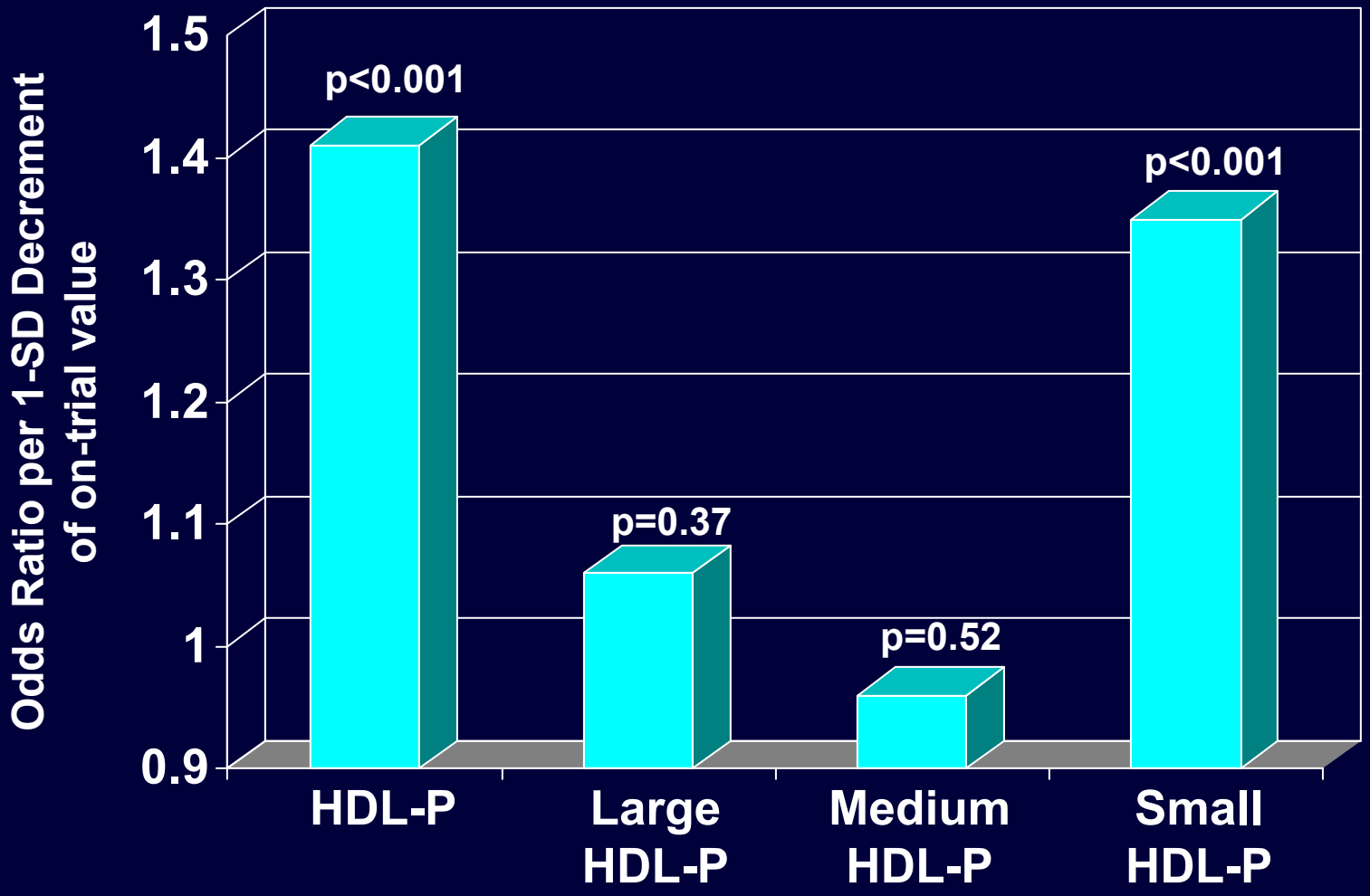
Lipoprotein Particles as Predictors of CHD Events in VA-HIT

Variable	Baseline RR (1 SD Change)	On-Trial RR (1 SD Change)
LDL-C	1.08	1.08
HDL-C	0.92	0.95
Triglycerides	1.07	1.02
HDL_2-C	0.94	0.98
HDL_3-C	0.88*	0.95
Apo B	1.07	1.08
Apo A1	0.87**	0.94

Adjusted for treatment, age, diabetes, hypertension, smoking, and BMI.
*P<0.05; **P<0.01; ***P<0.001

Otvos JD, et al. *Circulation* 2006;113:1556-1563.

HDL Subclasses as Predictors of CHD Events in VA-HIT



Adjusted for treatment, age, hypertension, smoking, BMI, and diabetes

Otvos J, et al. *Circulation* 2006;113:1556-63

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NMR Lipoprotein Subclass Particle Parameters as Multivariable Predictors of CHD Events in VA-HIT

	Baseline		On-Trial	
Variable	**OR* (95% CI)**	**P**	**OR (95% CI)**	**P**
Large LDL particles	**1.31 (1.09-1.57)**	**0.003**	**1.34 (1.11-1.62)**	**0.002**
Small LDL particles	**1.44 (1.20-1.73)**	**<0.0001**	**1.41 (1.14-1.73)**	**0.001**
IDL Particles	**0.98 (0.86-1.12)**	**0.78**	**1.13 (0.97-1.30)**	**0.11**
Large HDL particles	**0.95 (0.82-1.11)**	**0.53**	**0.92 (0.79-1.07)**	**0.30**
Medium LDL particles	**0.82 (0.70-0.96)**	**0.02**	**0.82 (0.69-0.97)**	**0.02**
Small HDL particles	**0.71 (0.60-0.84)**	**<0.0001**	**0.67 (0.57-0.79)**	**<0.0001**

*Ors (95% CIs) were calculated for a 1-SD increment in each lipoprotein in each lipoprotein subclass parameter at baseline and on-trial with the use of logistic regression models that included all lipoprotein particle parameters in 1 model. All models were additionally adjusted for treatment group, age, hypertension, smoking, body mass index, and diabetes.

Otvos JD et al. *Circulation*. 2006;113:1556-63.

Classification of HDL by Physical Properties

- Ultracentrifuguation
- Gradient Gel Electrophoresis
- 2-D Gel Electrophoresis
- Ion Mobility
- Nuclear Magnetic Resonance Spectroscopy (NMR)
- Vertical Auto Profile (VAP)

VAP Description

- VAP is a micro-sample, automated, ultracentrifugation technique for lipoprotein subclass quantification
- Measures all 3 major lipoprotein classes (VLDL-C, LDL-C, HDL-C)
- 21 subclasses [$VLDL_{1-3}$,Remnant Lp,LDL_{1-4} (density pattern A/B), IDL-C, Lp(a)-C, HDL_{2-3}]
- Non-FDA approved
- Available from Atherotech, a lipoprotein specialty reference lab in Birmingham, AL

VAP Description

- Uses a vertical rotor, which allows high resolution separation of lipoproteins by density
- In 47 min, the VLDL, LDL, and HDL and their subfractions are layered one on top of each other according to their densities by the high-speed centrifuge
- The bottom of the tube is punctured and the cholesterol in each layer is measured enzymatically
- Cholesterol values form each layer are then integrated with the computer to obtain a profile of each lipoprotein subfraction pattern
- Subcurves corresponding to each lipoprotein subfraction are deconvoluted and the cholesterol content of each fraction is determined by measuring the area under the subcurve

Basic Principles of the VAP

100 uL Specimen is added to KBr solution (1.21 g/mL density)

Sample overlaid in UC tube containing saline (1.006 g/mL density)

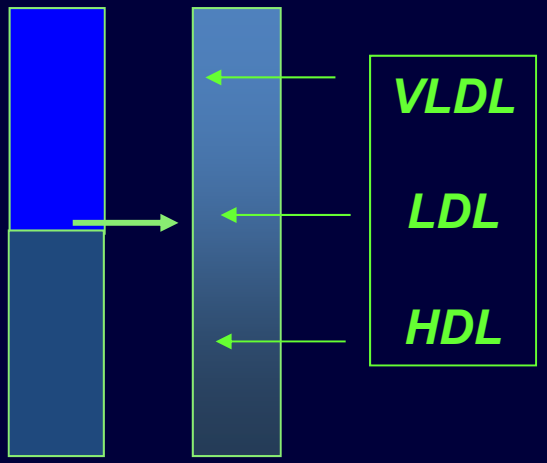
Spin in u/c at 65K rpm for 47 min

Use enzymatic reagent to "stain" cholesterol

In controlled manner run contents of tube (bottom to top) thru a spectrophotometer

Digitize & deconvolute into 5 Gaussian distributions *(HDL, Lp(a), LDL, IDL, and VLDL)*

Deconvolute individual curves into sub-fractions



VAP-II Subfractions

- LDL into 4 density subfractions
  - also reported as Pattern A, B, or AB
- HDL into 7 density subfractions
  - also reported as $HDL_2$and HDL_3
- VLDL into multiple subfractions
  - also reported as $VLDL_3$ subfraction

Performance Characteristics: Precision of VAP

	% CV (Variation)
Cholesterol (mg/dL)	1.3%
HDL-C (mg/dL)	2.5%
LDL-C^{Real} (mg/dL)	1.9%
VLDL-C^{Total} mg/dL	5.8%
LDL-C (mg/dL)	5.9%
Lp(a)-C (mg/dL)	4.3%
HDL_2-C (mg/dL)	7.9%
HDL_3-C (mg/dL)	2.3%

VAP Summary

- Relatively good analytical performance
- Not amenable for routine clinical lab
- Limited studies comparing it to alternative methods
- Most likely useful for intermediate risk subjects
- Limited studies showing clinical utility

Classification of HDL by Physical Properties

Proposed term	Very large HDL (HDL-VL)	Large HDL-V (HDL-L)	Medium HDL (HDL-M)	Small HDL (HDL-S)	Very small HDL (VS-HDL)
Density range, g/mL	1.063-1.087	1.088-1.110	1.110-1.129	1.129-1.154	1.154-1.21
Size range, nm	12.9-9.7	9.7-8.8	8.8-8.2	8.2-7.8	7.8-7.2
Density gradient ultracentrifugation	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Density range, g/mL	1.063-1.087	1.088-1.110	1.110-1.129	1.129-1.154	1.154-1.170
Gradient gel electrophoresis	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Size range, nm	12.9-9.7	9.7-8.8	8.8-8.2	8.2-7.8	7.8-7.2
2D gel electrophoresis	Alpha-1	Alpha-2	Alpha-3	Alpha-4	Preβ-1 HDL
Size range, nm	11.2-10.8	9.4-9.0	8.5-7.5	7.5-7.0	6.0-5.0
NMR	Large HDL-P	Medium HDL-P		Small HDL-P	
Size range, nm	12.9-9.7	9.7-8.8	8.8-8.2	8.2-7.8	7.8-7.2
Ion mobility	HDL 2b	HDL 2a + 3			
Size range, nm	14.5-10.5	10.5-7.65			

Rosenson RS, Brewer HB Jr, Chapman MJ, Fazio S, Hussain M, Kontush A, Krauss R, Otvos J, Remaley A, Schaefer A. HDL Measures, HDL Particle Heterogeneity, Proposed HDL Nomenclature and Relation to Atherosclerotic Cardiovascular Events. *Clin Chem.*2011;57:392-410.

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Conclusions

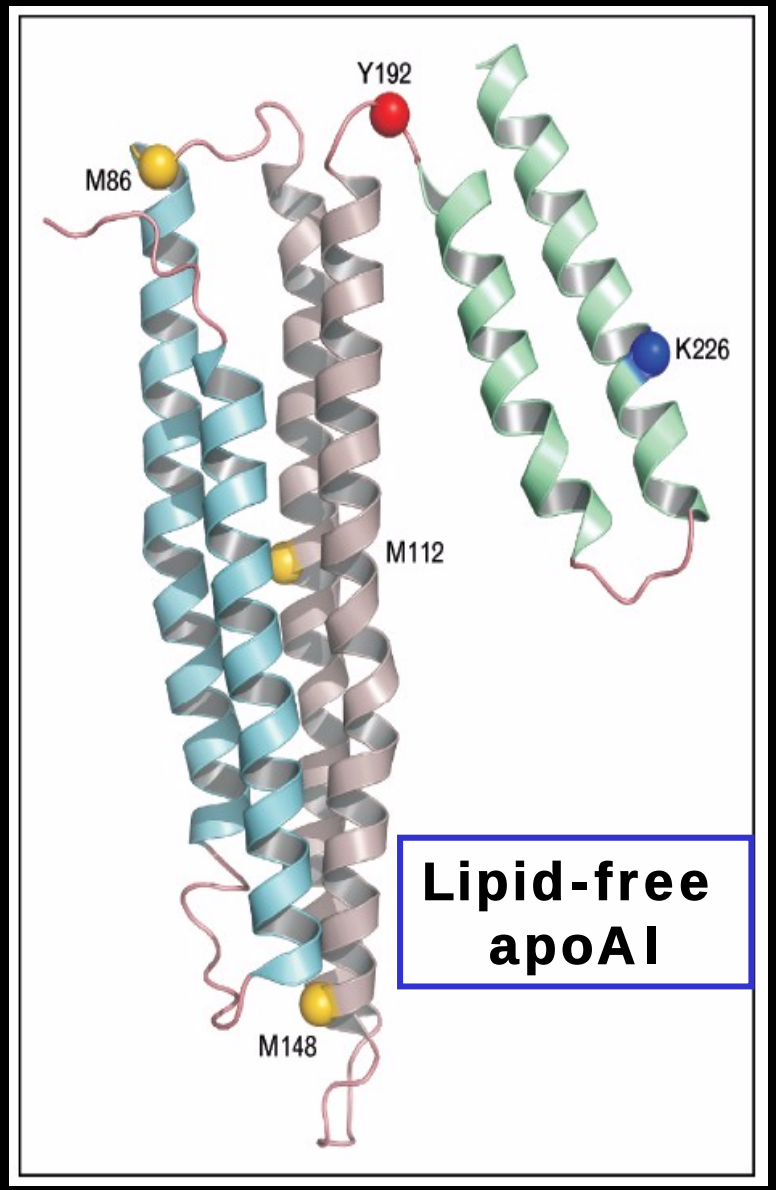
- Relations of HDL-C with CVD risk are partially confounded by the association of low HDL-C with elevated LDL-P
- Apolipoprotein A-I provides comparable information on cardiovascular risk as HDL-C
- On lipid-altering therapy, on-trial measures of HDL-C and apoA-I provide equal information on residual cardiovascular risk
- HDL particle measures provide statistically significantly more information on cardiovascular risk than HDL-C or apoA-I
- HDL particle concentration is a quantitative measure and not a qualitative measure of HDL function

Conclusions (cont.)

- New HDL measures have been investigated in randomized clinical trials; of these measures, 2-dimensional gradient gel electrophoresis has been reported to incremental information on risks of atherosclerosis and cardiovascular events beyond standard lipida
- Assays of cholesterol efflux quantify 1 important aspect of HDL function
- Regardless of the HDL measure, all of the HDL methods require validation in randomized, placebo-controlled clinical trials

Characterizing HDL by Surface Proteins

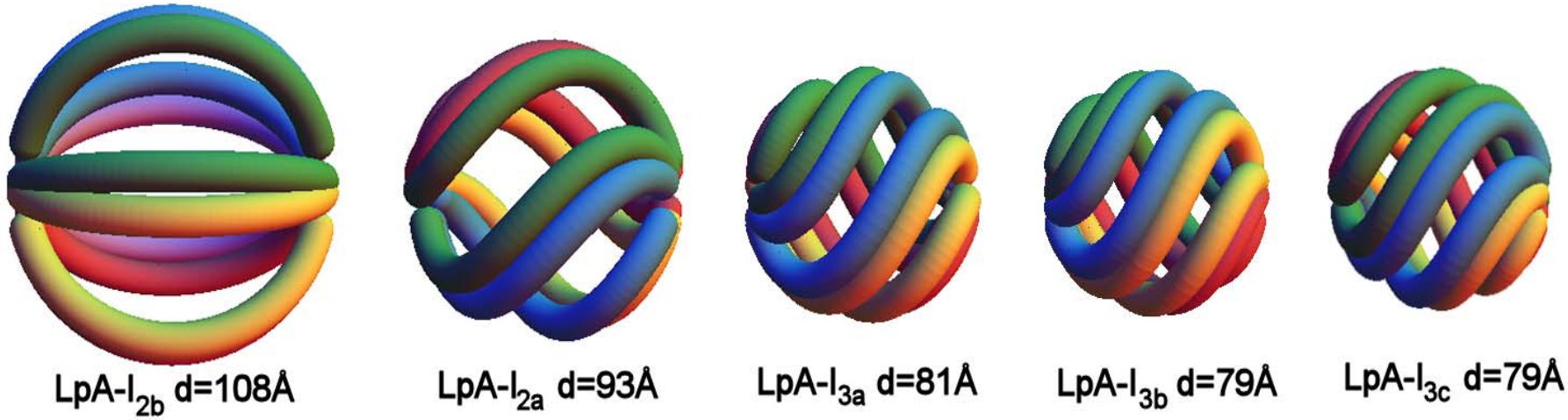
Proteomics



The HDL Building Block

Plasma HDL subpopulations :

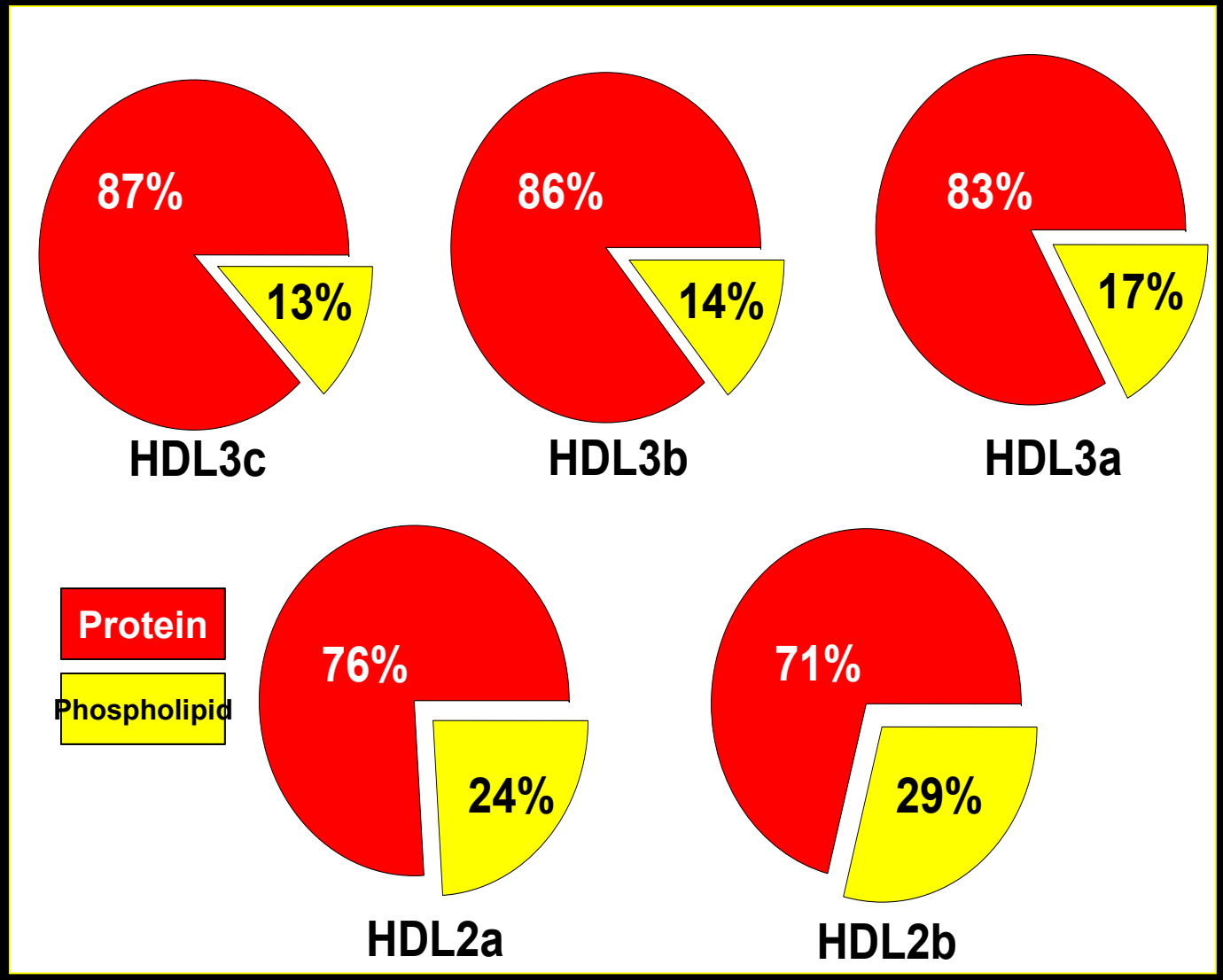
symmetrical cage-like structure with 4 apoAI copies per particle



Progressive Lipidation

Huang et al, Nature Struct Mol Biol, 2011

HDL particle surface is occupied primarily by Protein



Courtesy of S Davidson, unpublished data

How might combinations of these diverse proteins be associated, and in turn distributed, in the form of HDL subpopulations ?

Determinants of quantitative and qualitative protein segregation among subpopulations:

- HDL isolation procedure
- HDL particle surface pressure and surface properties
- Lipid-protein interaction
- Protein-protein interaction (electrostatic; covalent; vdW; mixed)
- Protein structure and conformation
- Relative abundance

HDL Particle Heterogeneity : Structure and Function

Working hypothesis

Specific biological functions of HDL might be mediated by distinct particle subspecies defined by specific cluster(s) of bound proteins.

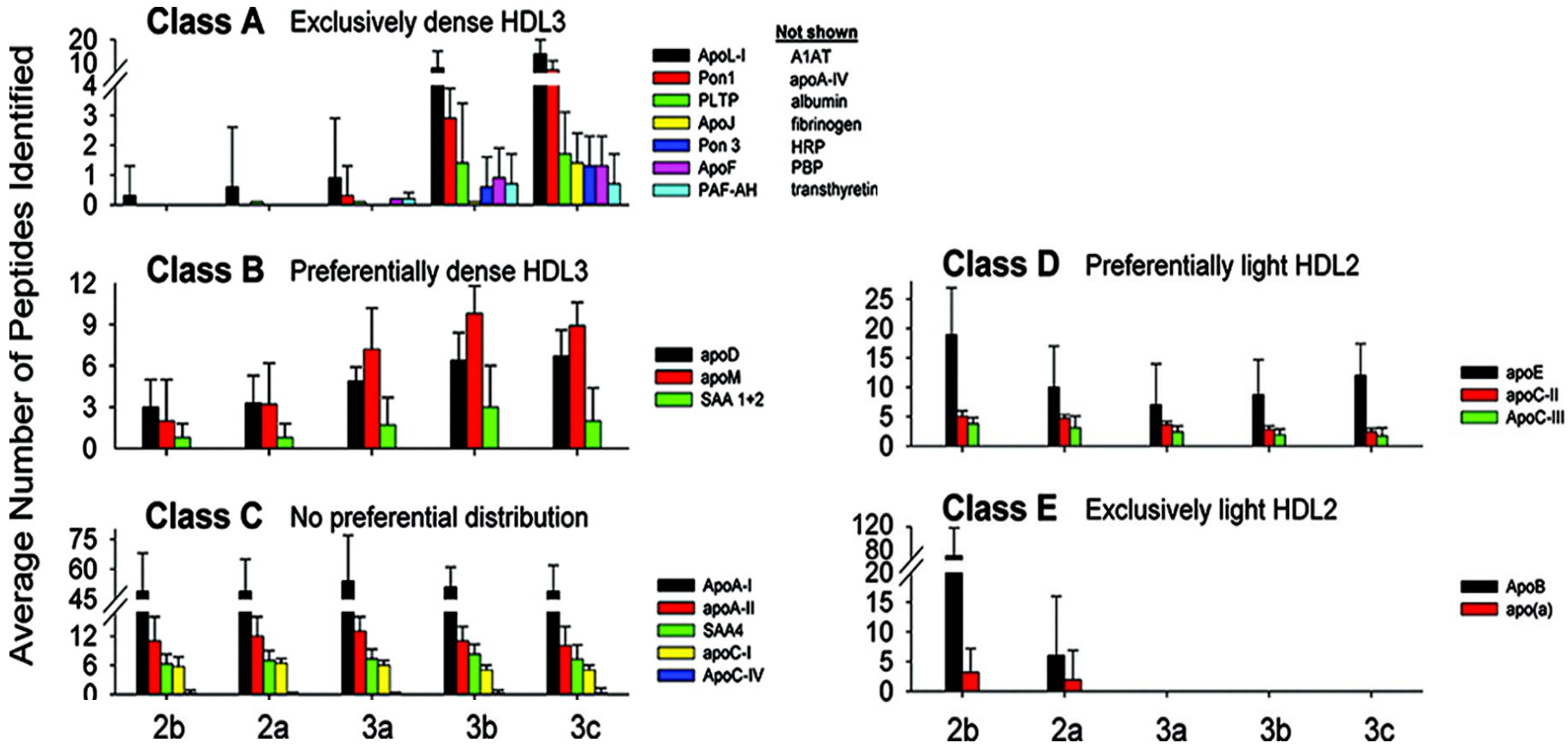
Each protein cluster may be associated with a distinct subset of lipid constituents (lipidome).

Isolation / subfractionation strategy ?

Analytical proteomic strategy ?

Relative protein abundance ?
Quantitation :low vs high abundance ?
Particle distribution frequency ?

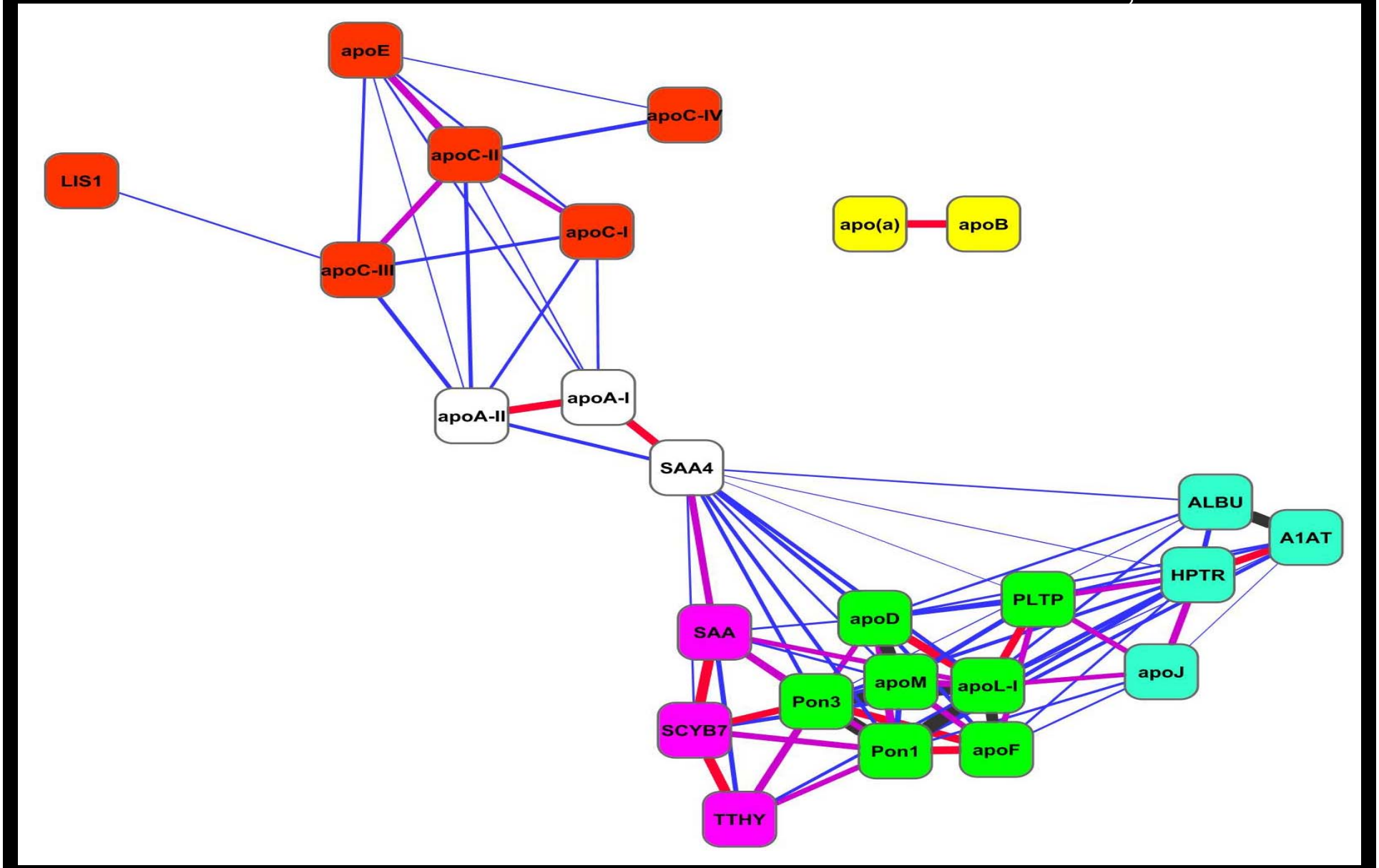
DGUC : Evidence for stable HDL subspeciation



Davidson, W. S. et al., Arterioscler Thromb Vasc Biol 2009;29:870-876

DGUC : Correlational network of HDL proteins

Davidson et al, ATVB 2009

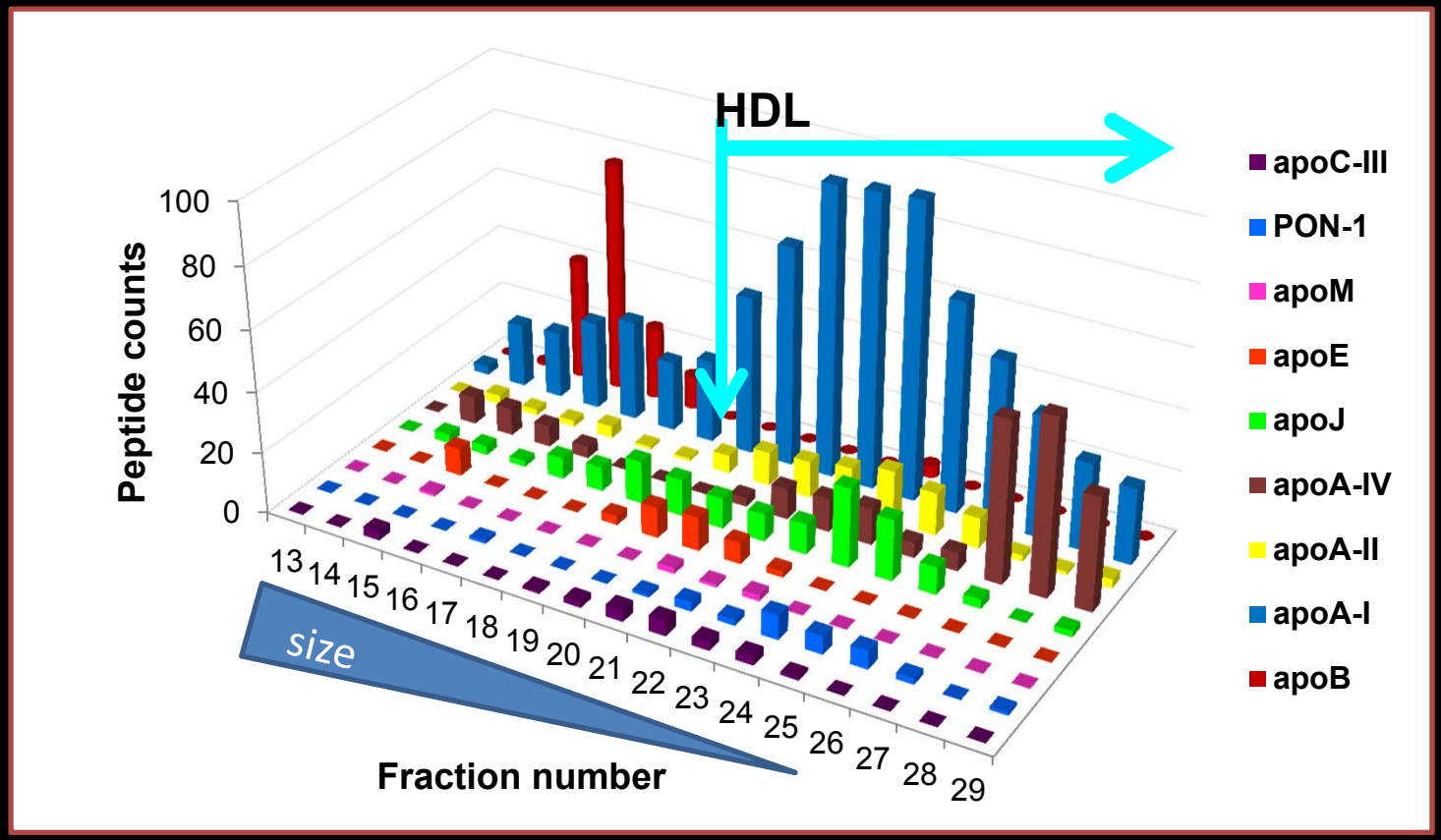


When studying the HDL proteome, can

Isolation-specific bias
and / or
Isolation-specific artefacts
be involved ?

Comparison of the proteome of HDL isolated by different technologies is of critical importance !

Distribution patterns of common HDL-associated proteins across gel filtration fractions : normal human plasma



Gordon SM et al. J Proteome Res 2010, 9, 5239–5249

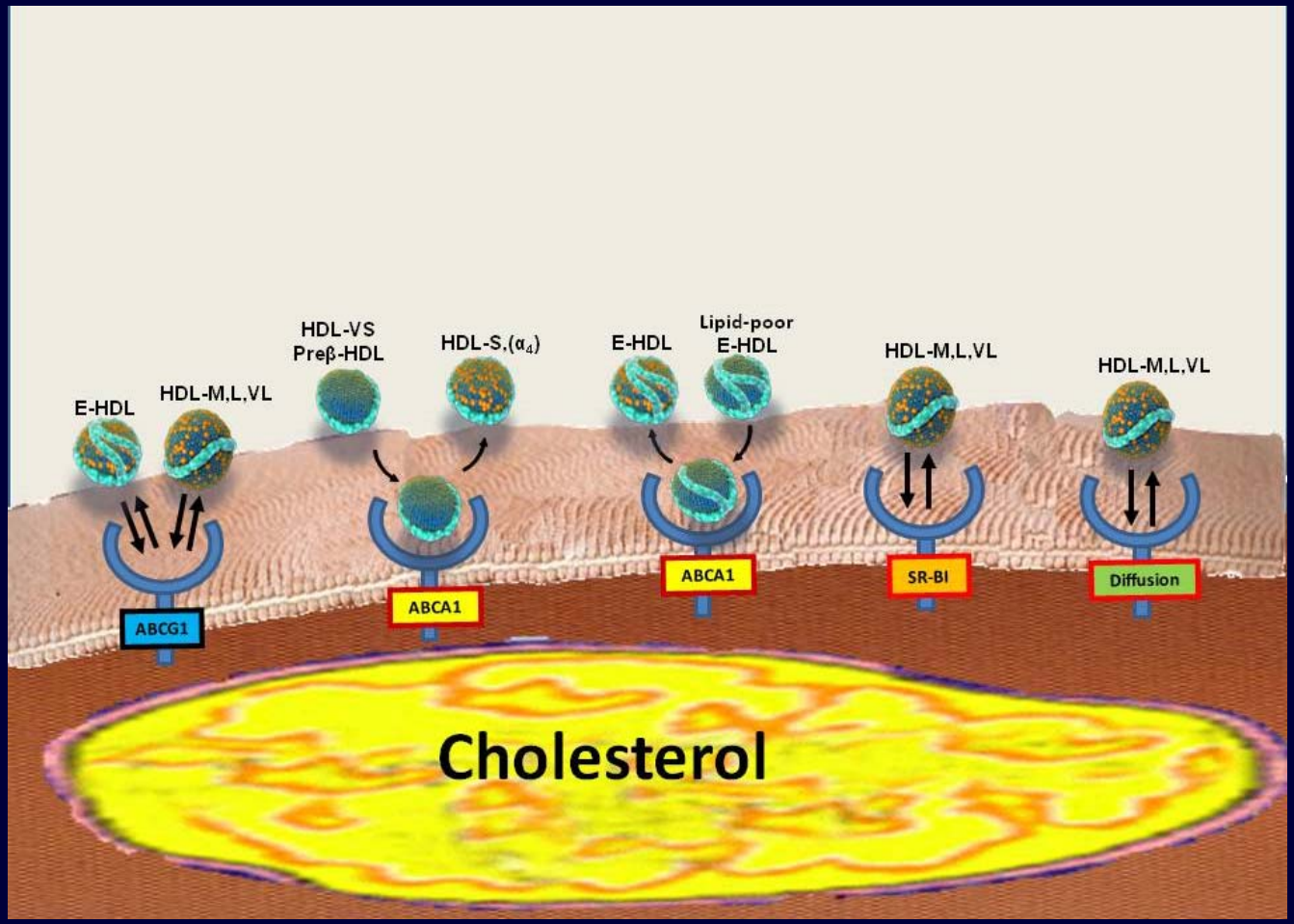
Phospholipid binding of FPLC HDL subfractions

- Isolation and identification of lipid-bound proteins in HDL subfractions derived from FPLC
- Calcium silicate hydrate (CSH) binding of PL-containing HDL in each HDL subfraction
- Control expts: ether delipidation
- Direct proteolysis of bound proteins
- ESI-MS/MS analysis of tryptic peptides
- 47 proteins identified : 17 new
  - 8 implicated in the complement cascade
  - 2 SERPINS : AACT, PEDF

Gordon et al, J Proteome Res, 2010

- Do HDL subpopulations exist demonstrating structural and functional subspeciation ?
- Do we have definitive evidence for the role of one or more specific HDL proteins in a given biological function ?
- Do we have evidence for synergy between components of the HDL proteome in function ?

Summary of the Major Lipoproteins
Particles That Participate in Cholesterol Efflux From Cholesterol-Loaded Cells



Rosenson RS, Brewer HB, Jr., Davidson WS, Fayad ZA, Fuster V, Goldstein J, Hellerstein M, Jiang XC, Phillips MC, Rader DJ, Remaley AT, Rothblat GH, Tall AR, Yvan-Charvet L. *Circulation*. 2012;125(15):1905-1919. Copyright © 2012, Wolters Kluwer Health.

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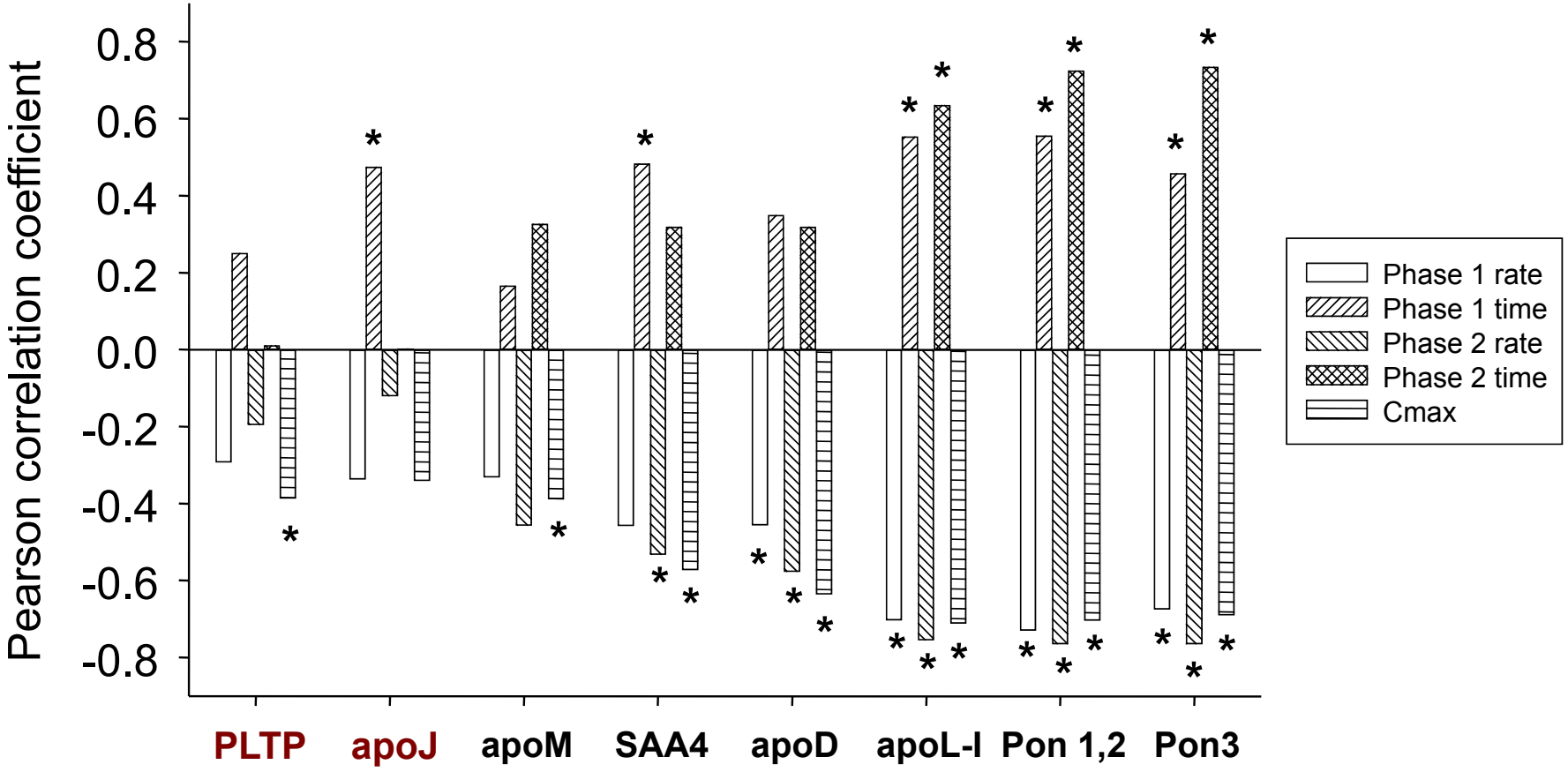
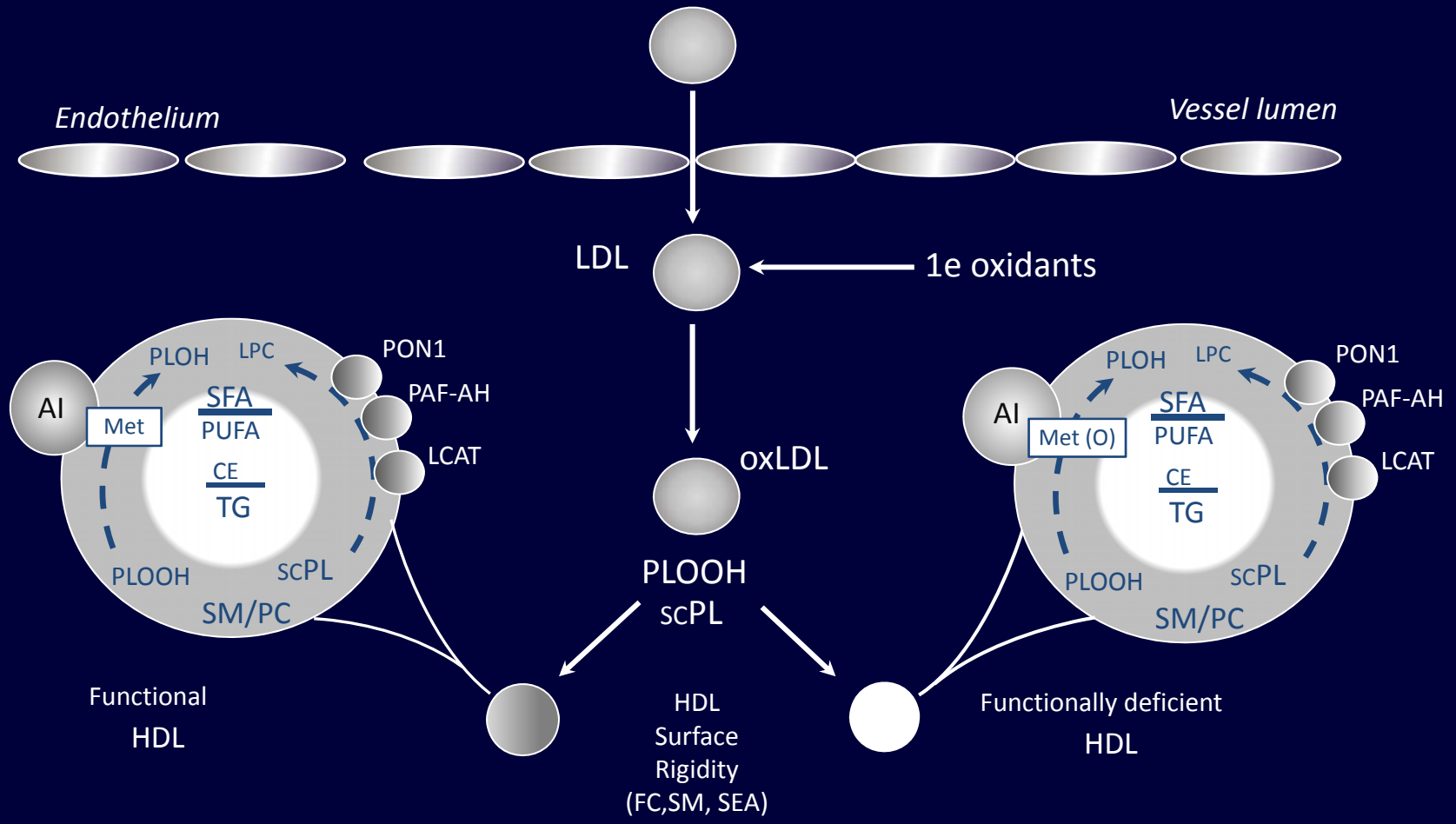
Anti-oxidative activity :

HDL3 proteins with the capacity to attenuate LDL oxidation



Davidson et al, ATVB 2009

HDL3 Mediated Protection of LDL against Oxidative Damage from Free Radicals



Kontush A, et al. *CurrOpinLipidol*2010;21:312-318.

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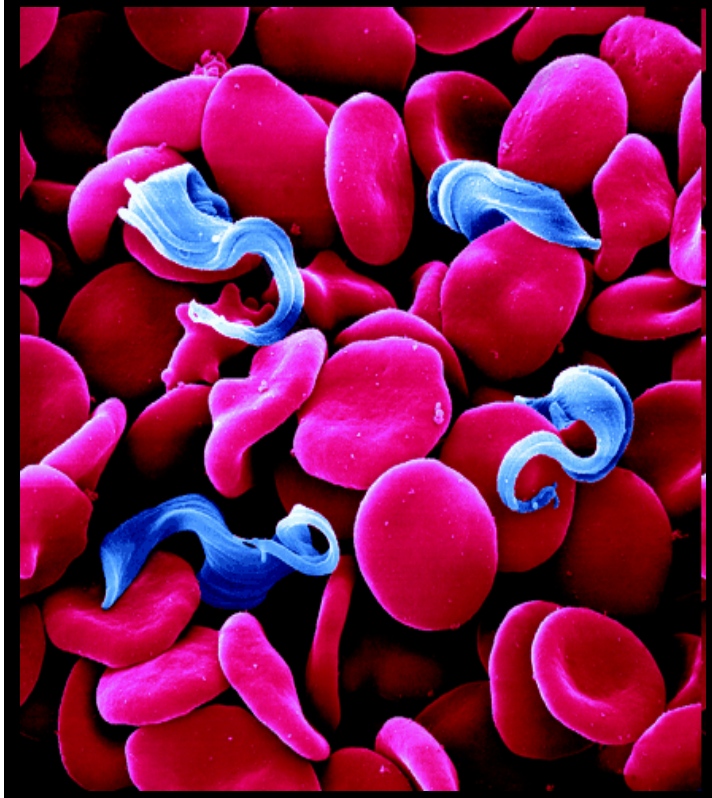
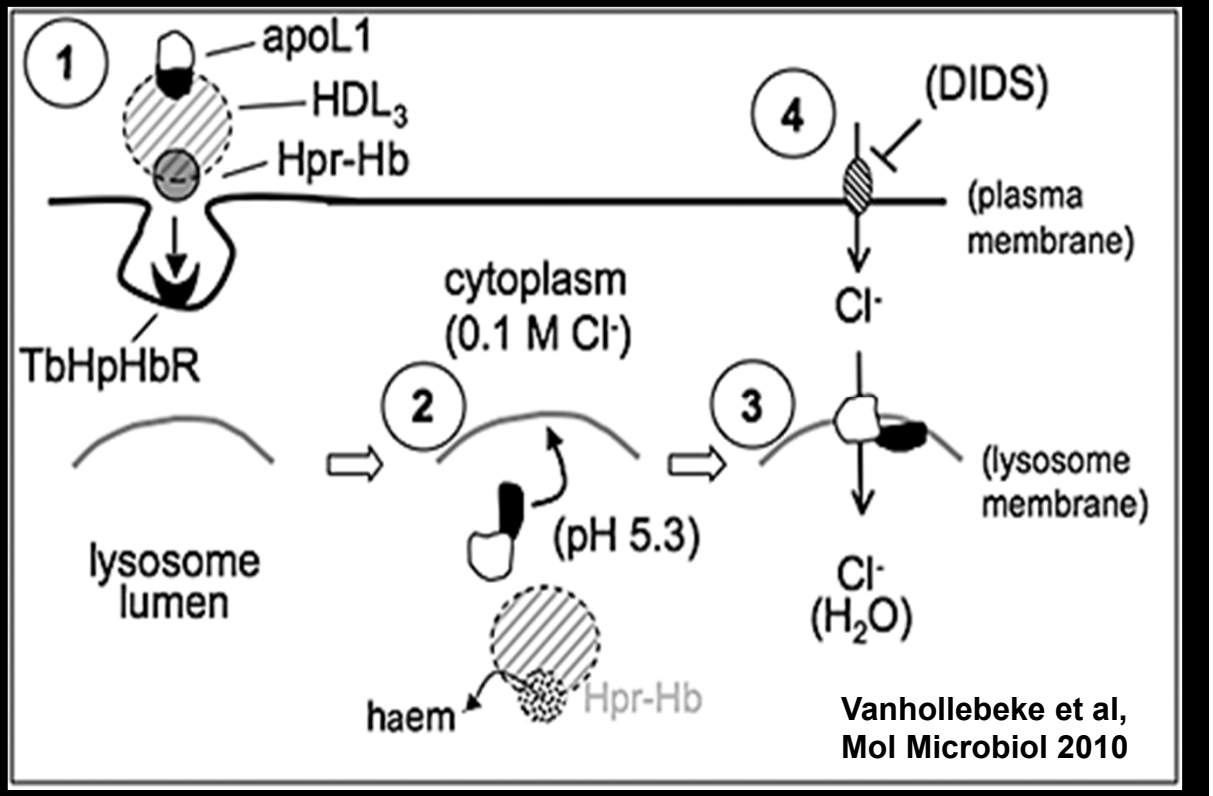
Anti-infectious activity :

HDL subspecies with highly specialized function

Trypanosome lytic factor





ApoL-1, haptoglobin-related protein and apoAI

Concept

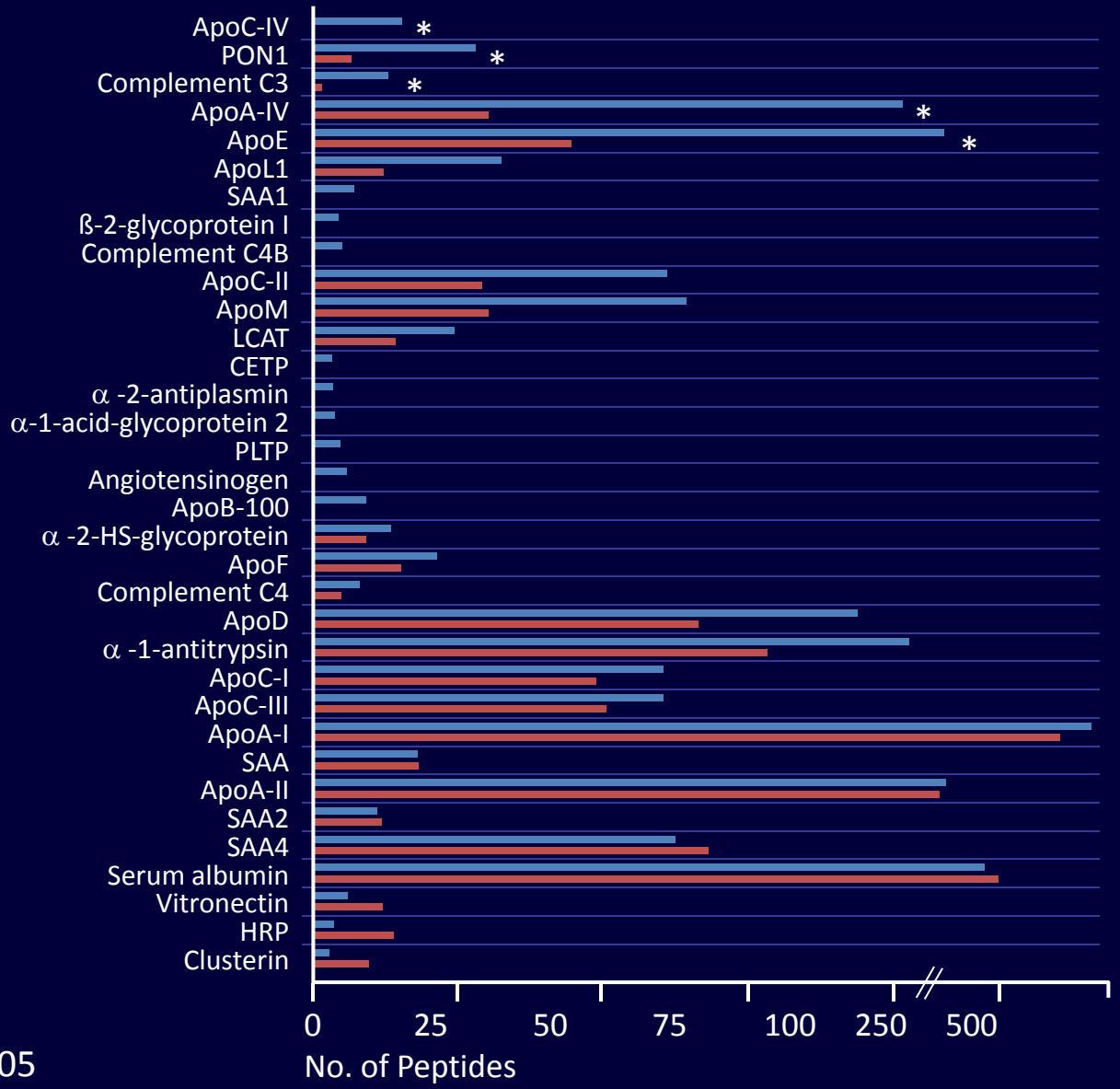
The dynamic intravascular metabolism of the lipid and protein components of HDL may be perturbed in cardiometabolic diseases associated with dyslipidemia, inflammation, and premature CVD, resulting in altered atheroprotective function(s).

Kontush, A., and Chapman, MJ, Pharmacol Rev 2006

1) Does HDL in CAD Subjects Carry a Unique Protein Cargo?

- Subjects
  - Healthy Controls
  - CAD Subjects
- HDL Levels Matched
- Age- and Sex-matched
- Shotgun proteomics of HDL_3
  - Apo B, albumin present in total HDL

Peptide Number and Number of Subjects with Detectable Peptides in HDL_3 Isolated from Control Subjects and CAD Subjects



*$P<0.05$

Vaisar T, et al. *J Clin Invest.* 2007;117(3):746-756.

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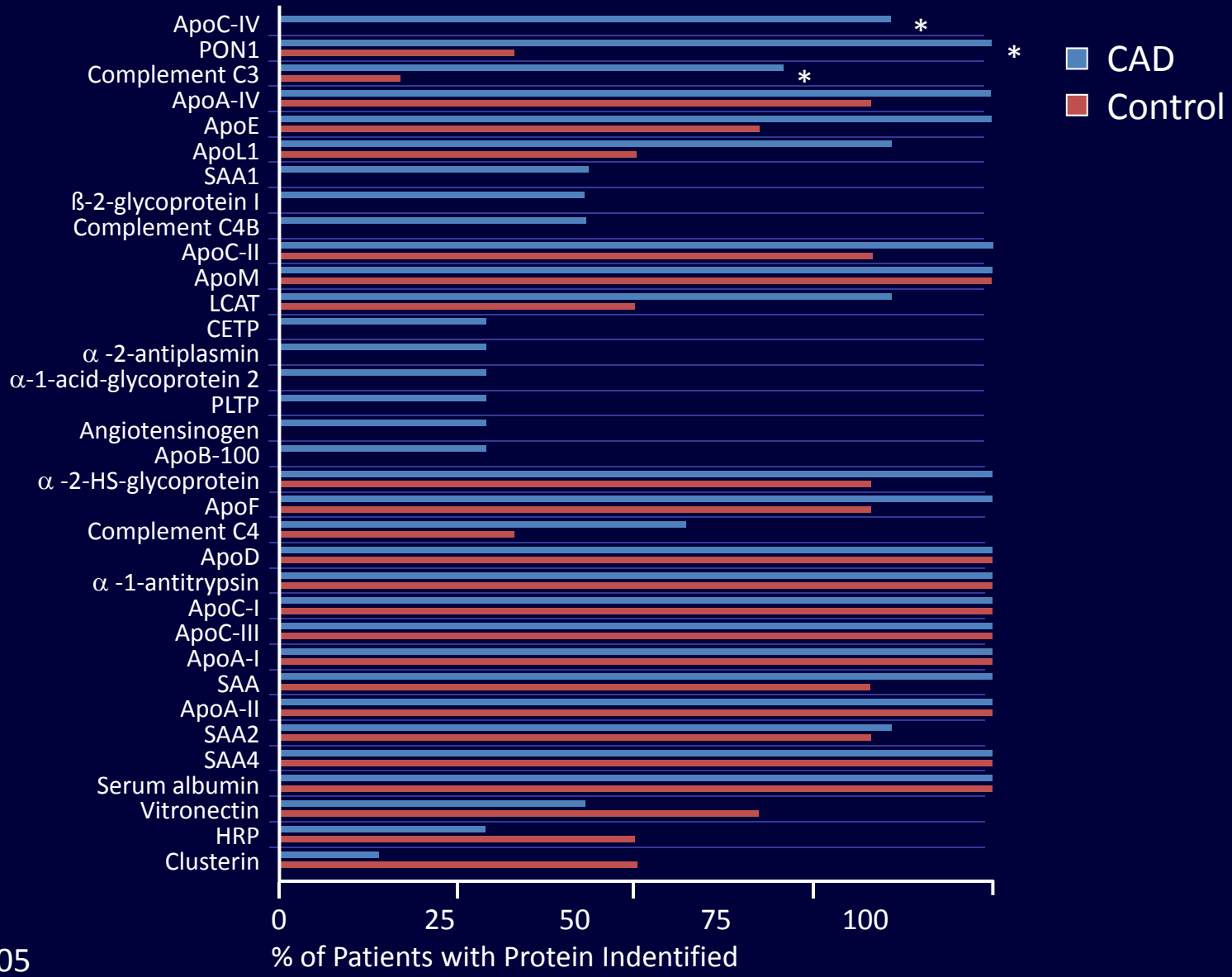
Peptide Number and Number of Subjects with Detectable Peptides in HDL_3 Isolated from Control Subjects and CAD Subjects



*P<0.05

Vaisar T, et al. *J Clin Invest.* 2007;117(3):746-756.

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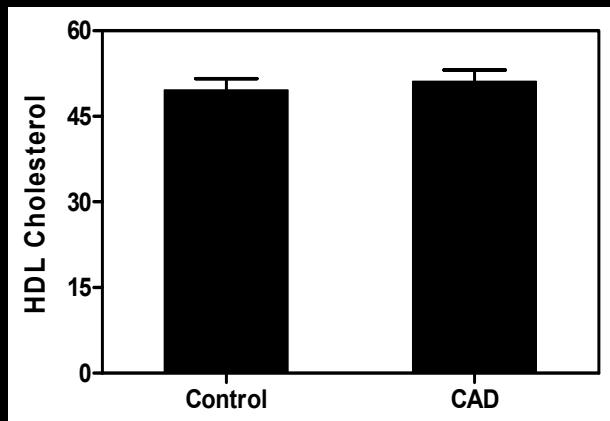
Validation of Spectral Index

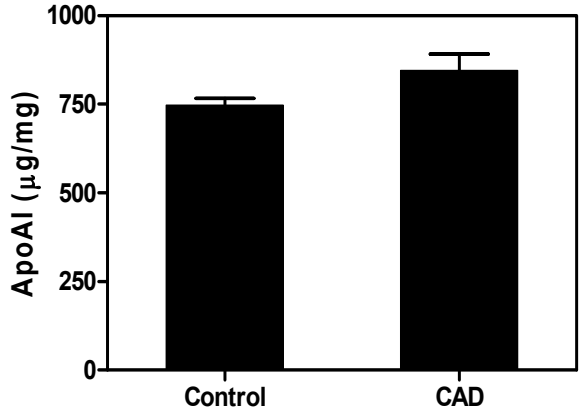
- PRINCE Study (PI : P.Ridker)
  - Effect of pravastatin on CRP levels
- 64 Subjects
  - 32 Healthy controls
  - 32 Newly diagnosed CAD subjects
- Matched
  - Age, Sex
  - Triglycerides, HDL-C

Composition of HDL_3 in Control and CAD Subjects

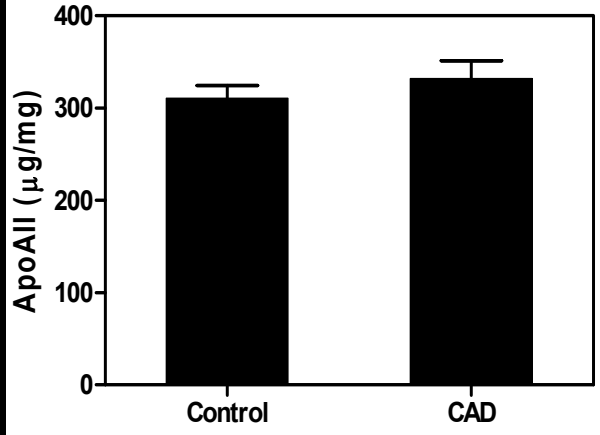
HDL-C



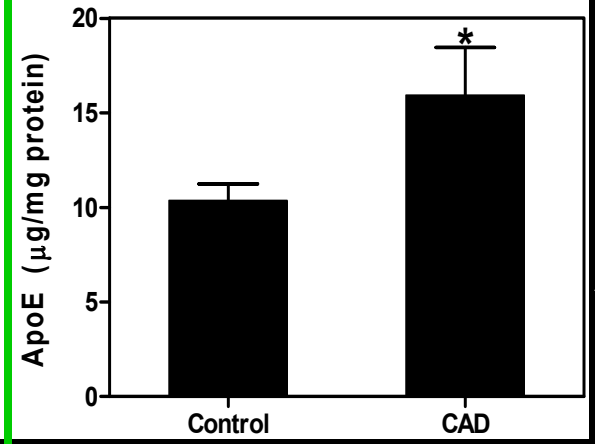
ApoAI



ApoAII



ApoE



*P = 0.02

HDL carries a unique protein cargo in CAD subjects

The CARE Study

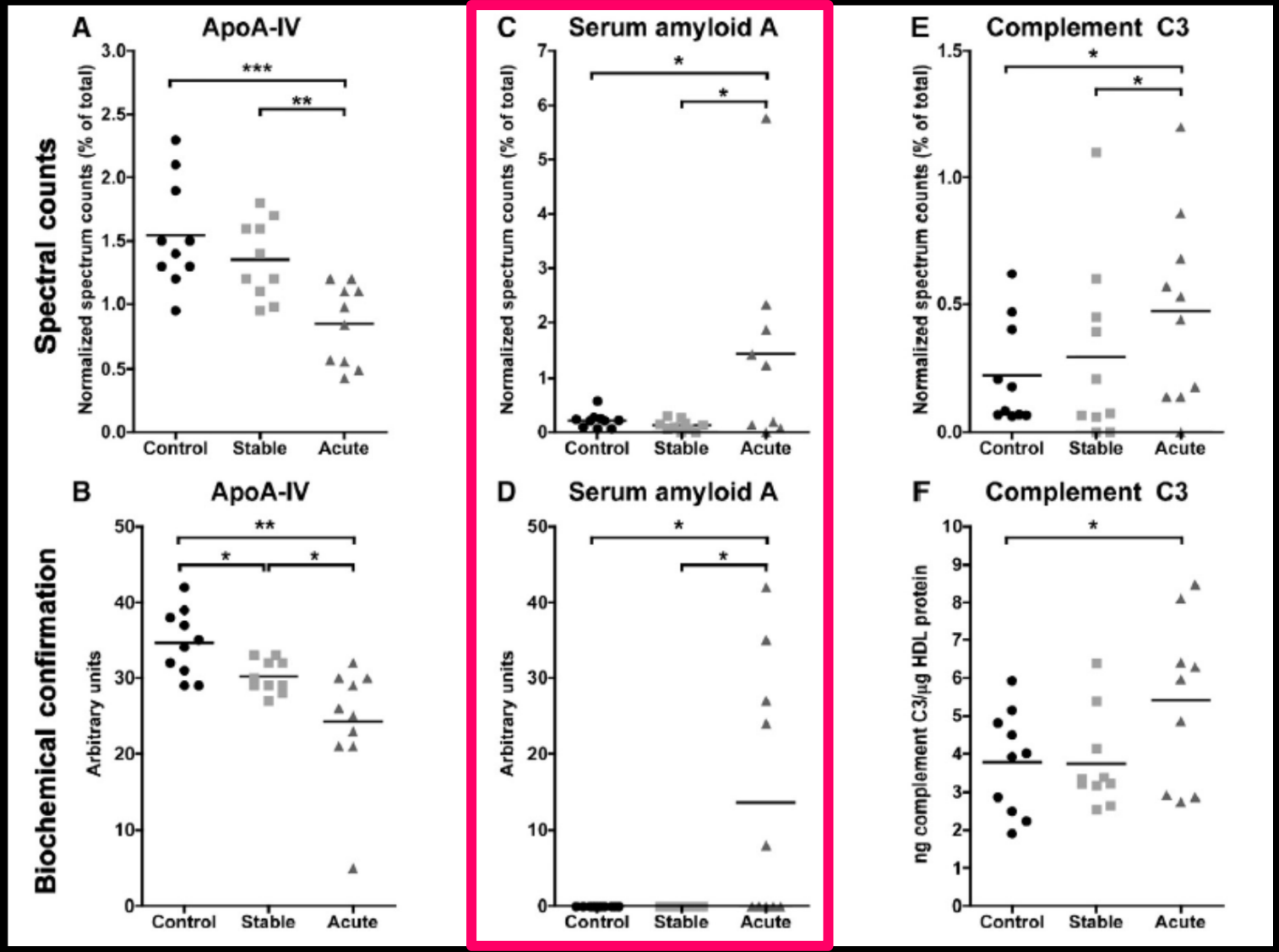
- Randomized placebo-controlled trial of pravastatin in 4159 patients with myocardial infarction and average LDL concentrations at baseline.
- 5 years of follow-up.
  - Patients who subsequently had MI/sudden death (cases, n=418) or those who did not have a cardiovascular event (control subjects, n=370)

Significant independent predictor of subsequent CAD events:

ApoE in HDL (RR 1.8, P=0.02)

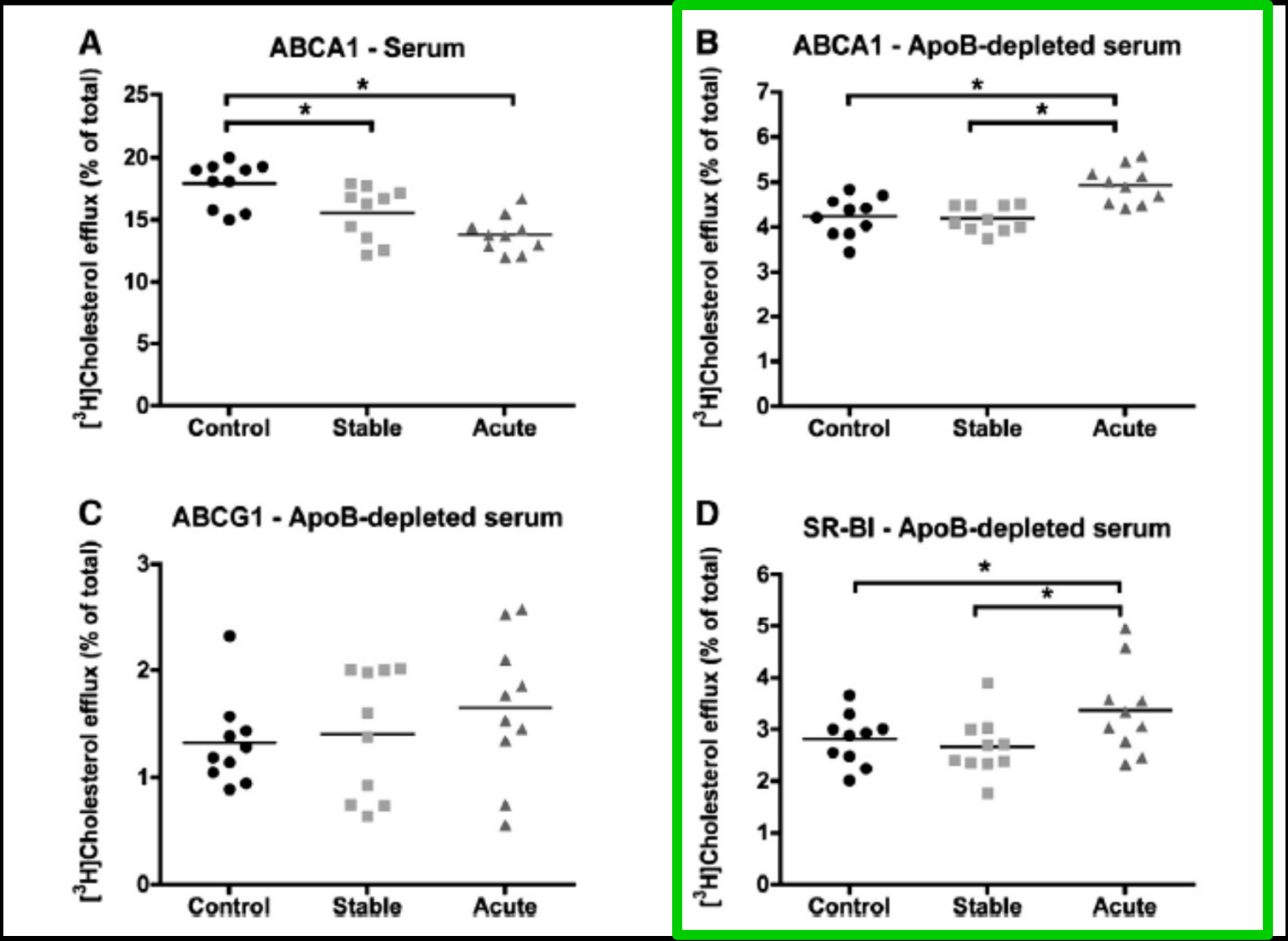
Sacks et al. Risk of recurrent coronary events in the Cholesterol and Recurrent Events (CARE) trial. ***Circulation.*** 2000;102:1886.

ApoA-IV, SAA, and C3 levels in HDL isolated from control, CAD, and ACS subjects



Alwaili K,et al. Biochim Biophys Acta. 2011 Jul 23. [Epub ahead of print]

Functional capacity of total serum or apoB-depleted serum from control, stable CAD, and ACS subjects to promote cholesterol efflux

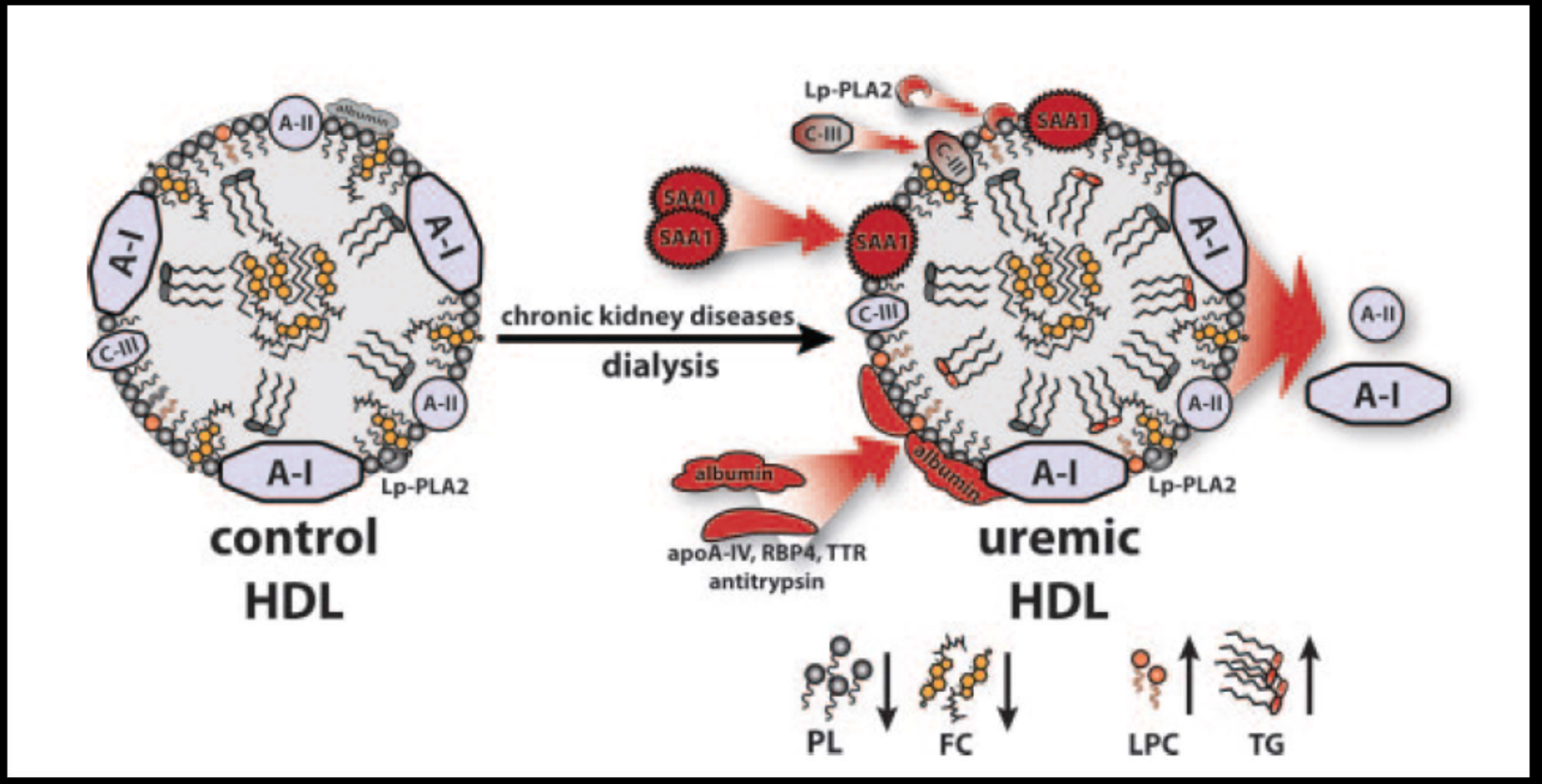


Alwaili K,et al. Biochim Biophys Acta. 2011 Jul 23. [Epub ahead of print]

Cardiometabolic diseases in which HDL metabolism is perturbed, and in which the proteome and functionality of HDL particles may be altered :

1) CAD / ACS
2) Acute Systemic inflammation / Sepsis / Endotoxemia
3) Uremia

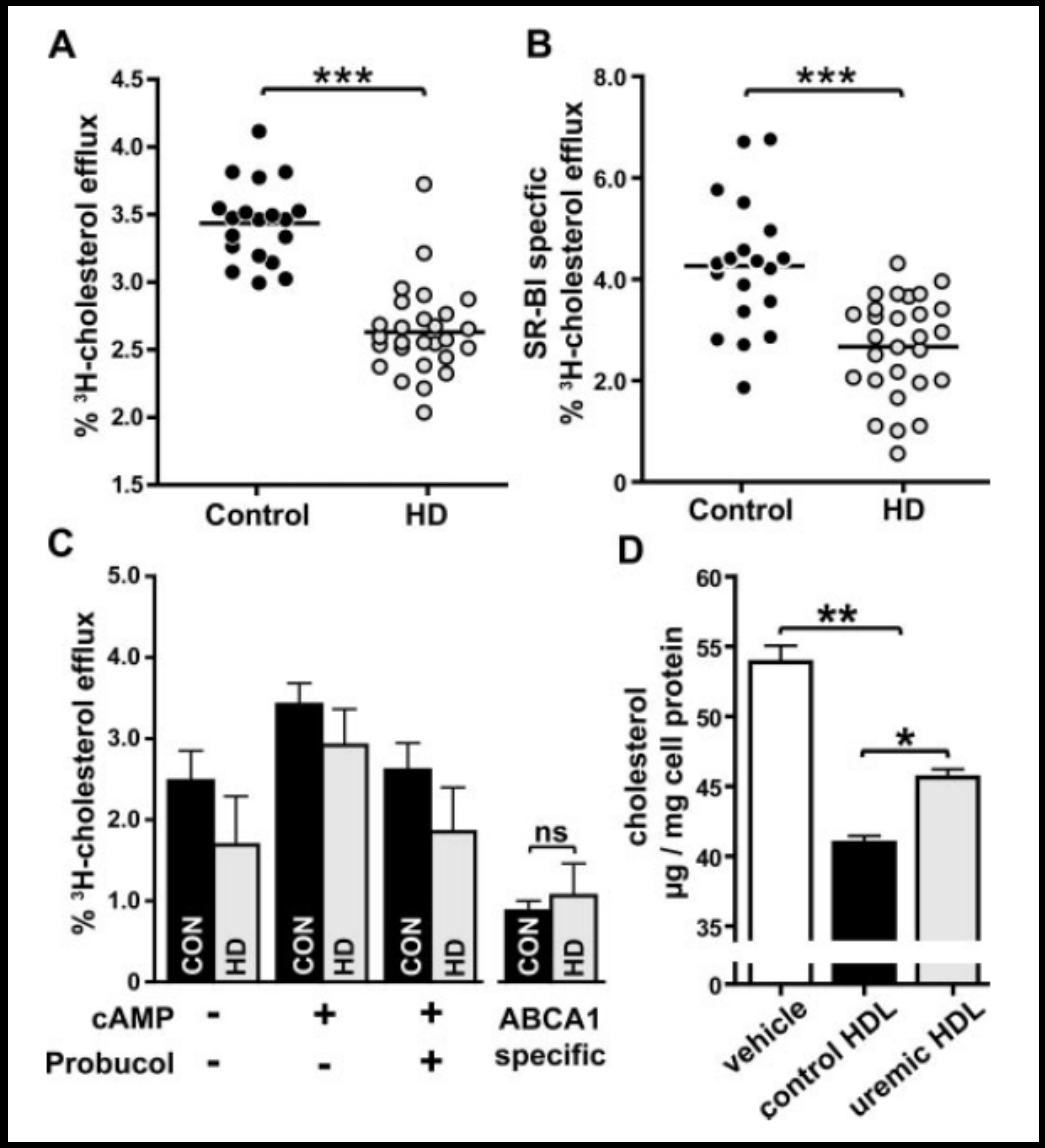
3. HDL remodelling in uremic patients



End stage renal disease

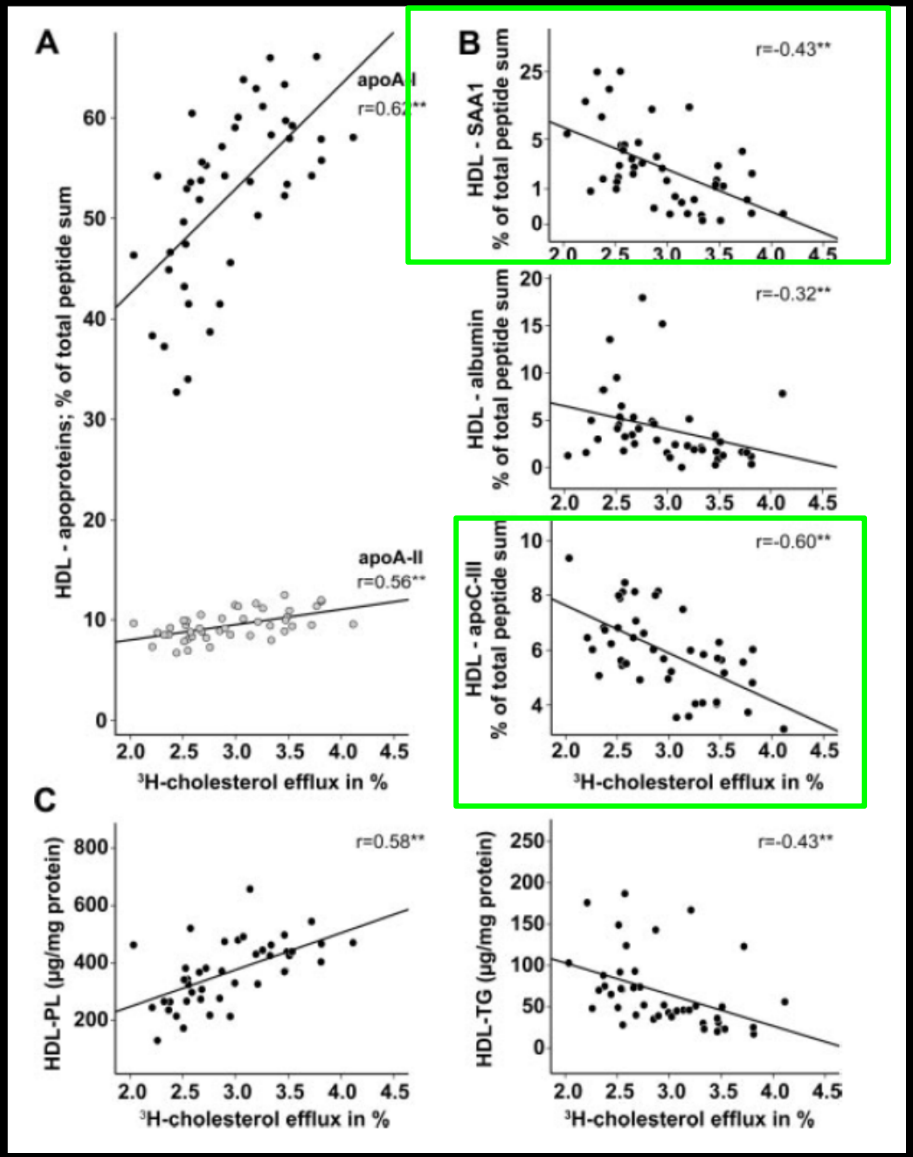
Holzer M et al. J Am Soc Nephrol. 2011 22:1631-41

Uremia impairs cholesterol efflux capability of HDL



Holzer M et al. J Am Soc Nephrol. 2011 22:1631-41

Determinants of cholesterol efflux potential of HDL



HDL-SAA1 and –CIII content are negatively correlated with cholesterol efflux capacity

Holzer M et al. J Am Soc Nephrol. 2011 22:1631-41

Weaknesses in Published studies

•Lack of insight into the complexity of authentic HDL particle subspecies; clear definition of composition , structure and functionality of individual subspecies lacking. Inadequate methodologies.

•Lack of coherence across proteomic analyses of HDL when compared on the basis of the “same” clinical state .

- Insufficient clinical and biological phenotyping of study subjects. (eg. CAD patients).

- Wide variation in degree of inflammation and stage of clinical recovery across studies.

•Failure to consider dynamic aspects of the inflammatory process.

•Need for in vivo turnover studies of HDL proteins and lipid components in protein-defined HDL subspecies in inflammatory states.

BURNING QUESTIONS

- Can we impute HDL-associated functions to unique HDL subspecies as defined by their proteome ?
- Can distinct functions of HDL subspecies be accounted for by specific protein components ?
- If the HDL proteome is perturbed by metabolic/inflammatory background, can we relate such changes to alteration in function ?
- Can we pharmacologically target altered HDL proteome and function in cardiometabolic disease states, and in consequence, normalise them ?
- Is such normalization accompanied by reduced CVD risk ?

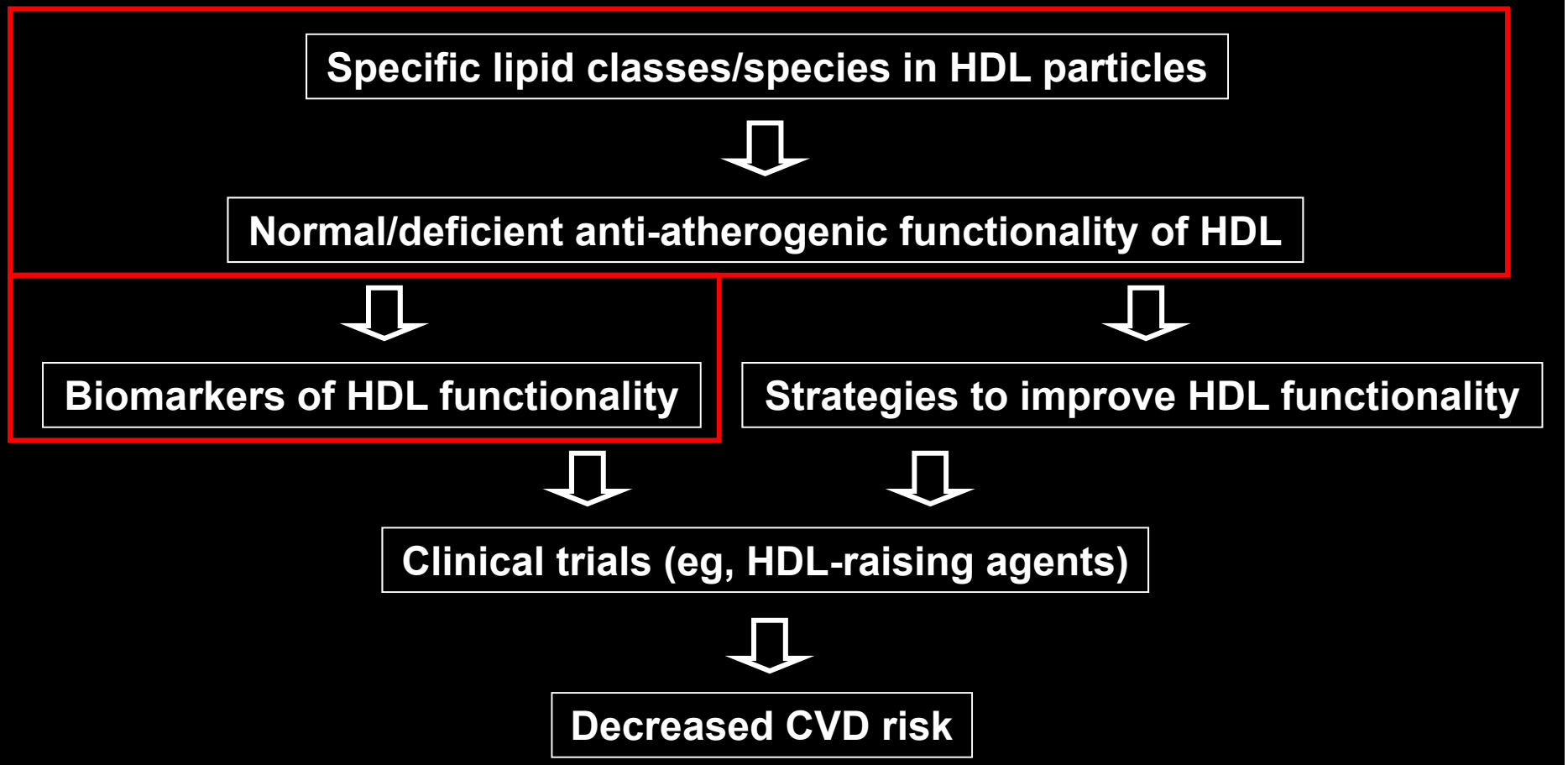
BURNING NEEDS : Methodological approaches

1)New highly resolutive, non-denaturing reproducible preparative methods for fractionation of HDL subspecies on the basis of specific associations of protein components; international validation and standardisation

2) Standardisation and calibration of MS/MS methodologies for HDL proteome analysis at an international level

HDL Functionality: Insight from Lipidomics into Antioxidative / Anti-inflammatory Activities

HDL Lipidomics : Experimental Strategy



Lipidomics by LC/MS/MS

1) Optimization of ionization and fragmentation conditions of individual lipid species
2) Choice of internal standards: one per lipid class

Lipid class	Fragmentation	Internal Standard	MS Conditions
PtdCholine, LPC	PIS + 184	PC d9 16:0/16:0	CE: +37
PtdEthanolamine, LPE	NL 141	PE 12:0/13:0	CE: +44
PtdGlycerol	NL 172	PG 12:0/13:0	CE: +25
PtdSerine	NL 185	PS 12:0/13:0	CE: +29
PtdAc	NL 115	PA 12:0/13:0	CE: +21
PtdInositol	PIS - 241	PI 12:0/13:0	CE: -56
Sphingomyelin	PIS + 184	PC d9 16:0/16:0	CE: +39
Ceramide	NL 262, 264	Cer 17:0	CE: +40

PIS=product ion scan; NL=neutral loss; CE=collision energy

Lhomme M, Camont L, Chapman MJ, Kontush A. unpublished data.

HDL Lipidomics: Multiple Step Analysis

1 HDL: 30 mcg phospholipid

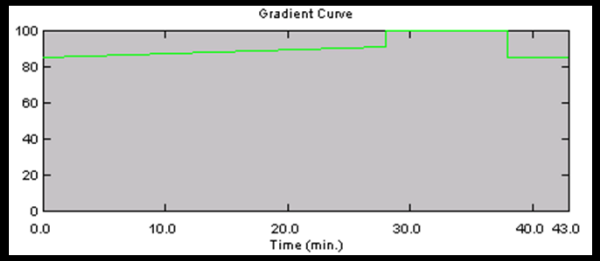
2 Addition of internal standards: PC d9 32:0, PE, PS, PG, PA, PI 12:0/13:0

3 Liquid-liquid extraction ($CHCl_3$/MeOH/H_2O)

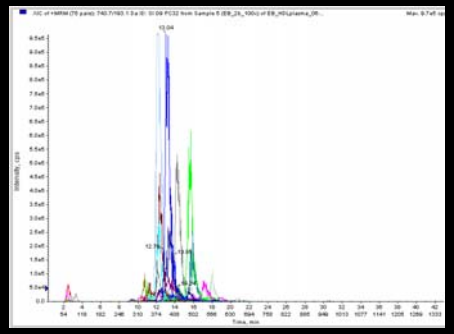
4 Residue re-suspended in ISP/hexane/H_2O

5 Sample dilution 1x, 10x, and 100x

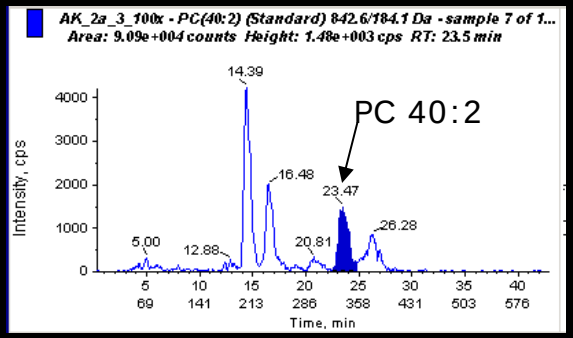
6 Separation by HPLC (C_8 column)



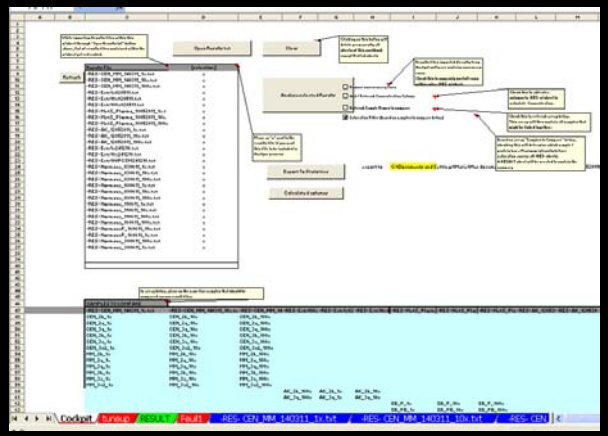
7 Detection of 160 lipid species by MS/MS using MRM



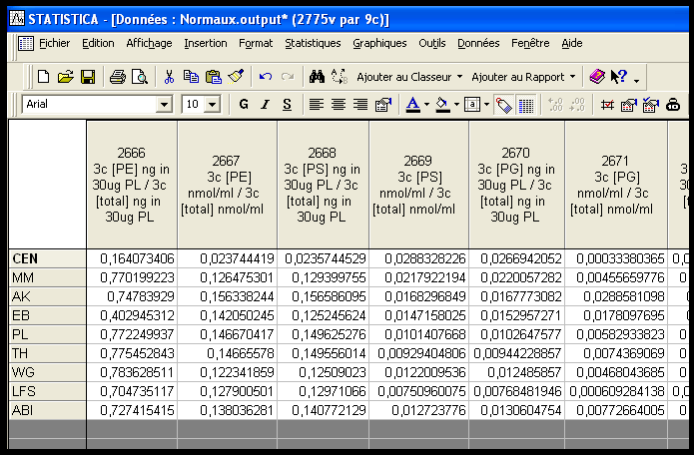
8 Identification and integration of chromatographic peaks



9 Automatic calculation of concentrations



10 Data conversion and export into Statistica program

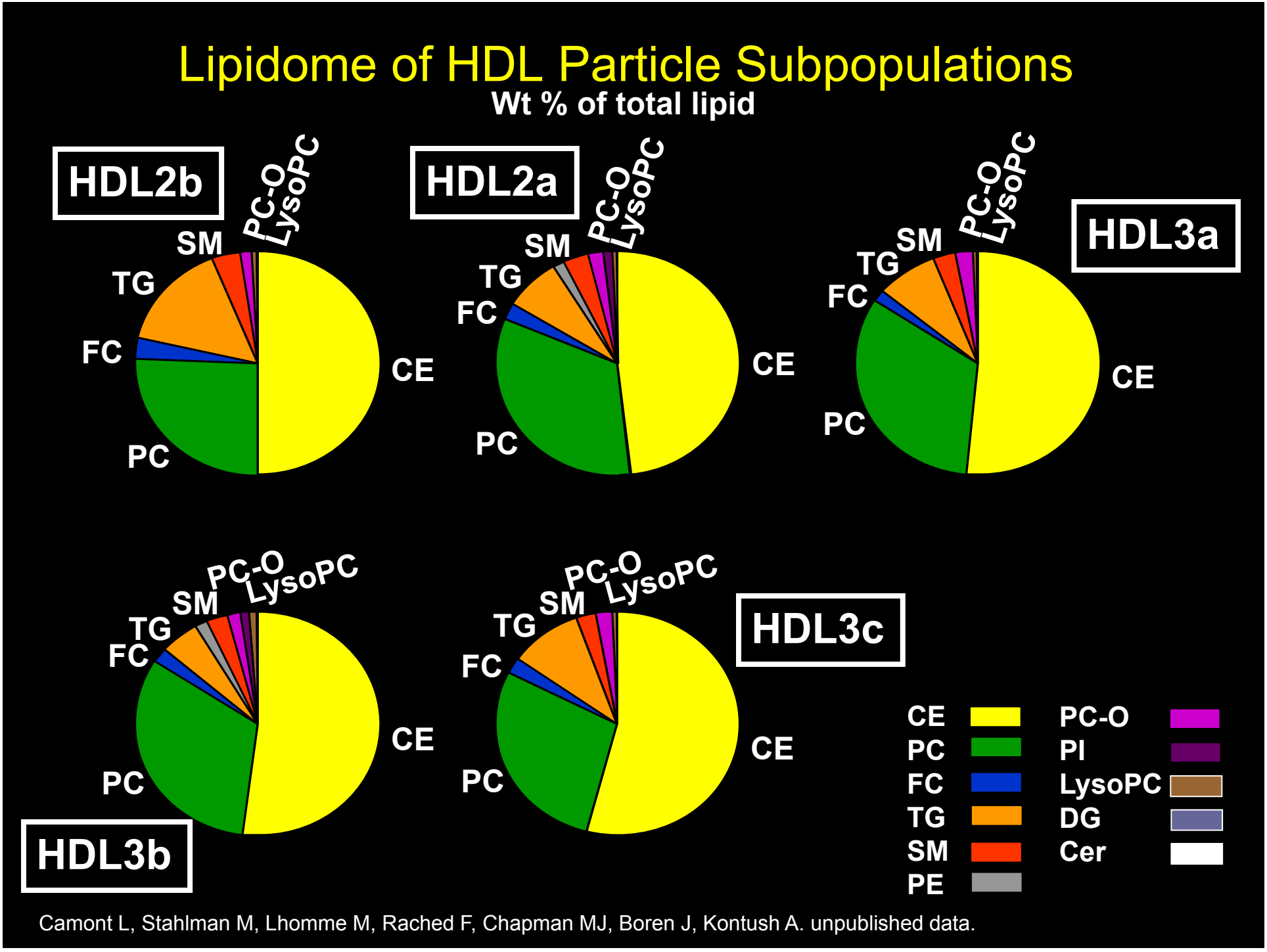


	2666 3c [PE] ng in 30ug PL / 3c [total] ng in 30ug PL	2667 3c [PE] nmol/ml / 3c [total] nmol/ml	2668 3c [PS] ng in 30ug PL / 3c [total] ng in 30ug PL	2669 3c [PS] nmol/ml / 3c [total] nmol/ml	2670 3c [PG] ng in 30ug PL / 3c [total] ng in 30ug PL	2671 3c [PG] nmol/ml / 3c [total] nmol/ml
CEN	0,164073406	0,023744419	0,0235744529	0,0288328226	0,0266942052	0,00033380365
MM	0,770199223	0,126475301	0,129399755	0,0217922194	0,0220057282	0,00455659776
AK	0,74783929	0,156338244	0,156586095	0,0168296849	0,0167773082	0,0288581098
EB	0,402945312	0,142050245	0,125245624	0,0147158025	0,0152957271	0,0178097695
PL	0,772249937	0,146670417	0,149625276	0,0101407668	0,0102647577	0,00582933823
TH	0,775452843	0,14665578	0,149556014	0,00929404806	0,00944228857	0,0074369069
WG	0,783628511	0,122341859	0,12509023	0,0122009536	0,012485857	0,00468043685
LFS	0,704735117	0,127900501	0,12971066	0,00750960075	0,00768481946	0,000609284138
ABI	0,727415415	0,138036281	0,140772129	0,012723776	0,0130604754	0,00772664005

Kontush A, Camont L, Chapman MJ. J Lipid Res 2013 Mar 30 [Epub ahead of print].

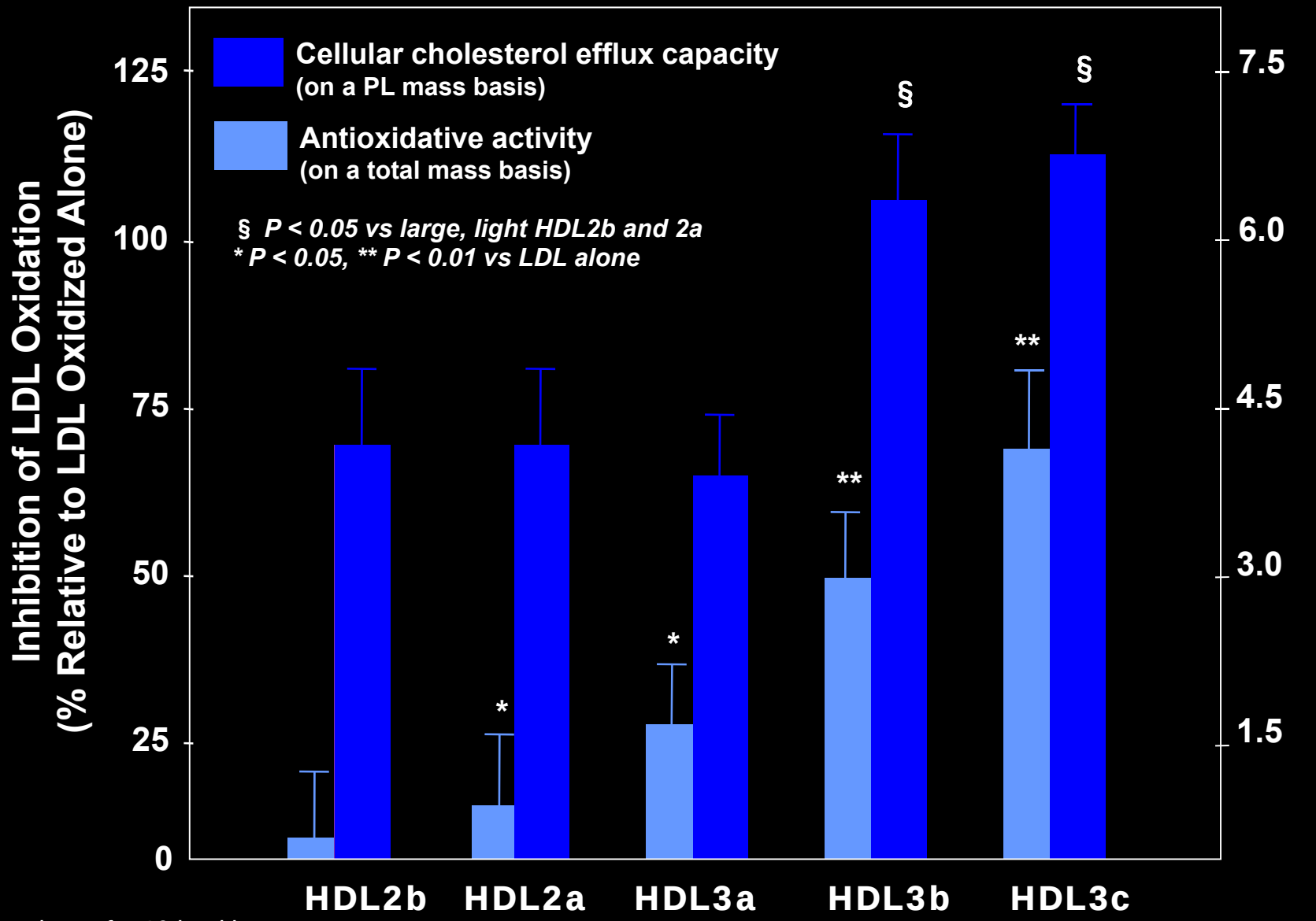
Lipidome of HDL Particle Subpopulations

Wt % of total lipid



Camont L, Stahlman M, Lhomme M, Rached F, Chapman MJ, Boren J, Kontush A. unpublished data.

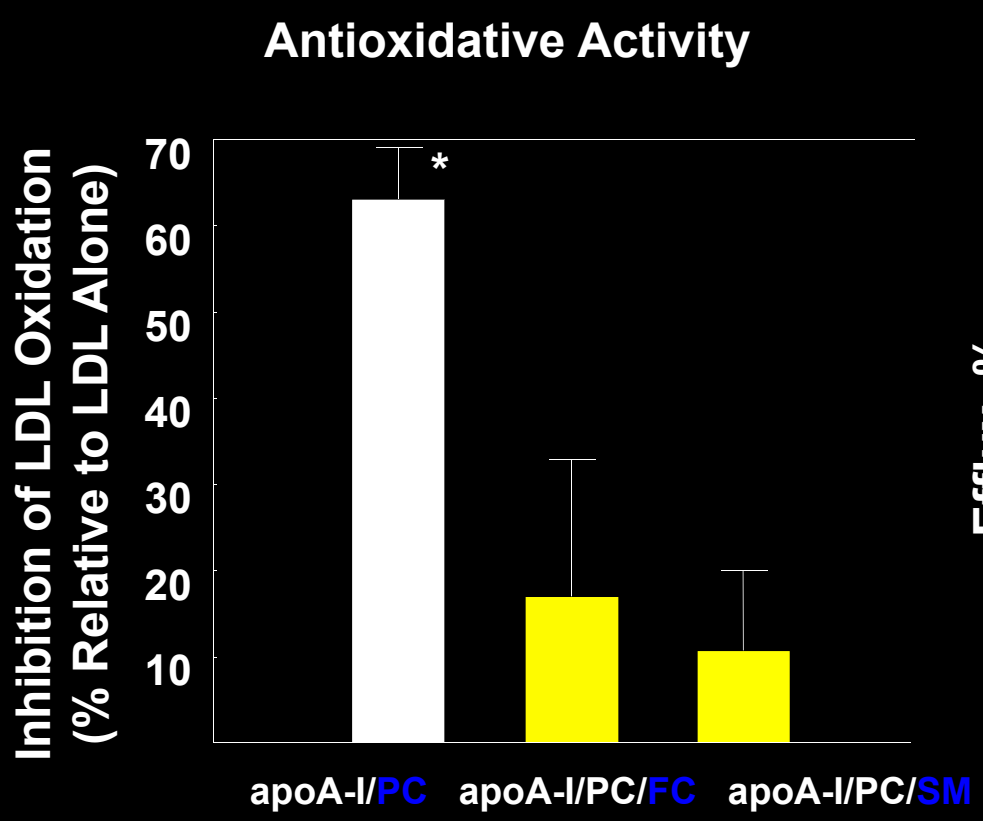
Both Cholesterol Efflux Capacity and Antioxidative Activity Are Enhanced in Small, Dense HDL3 Particles

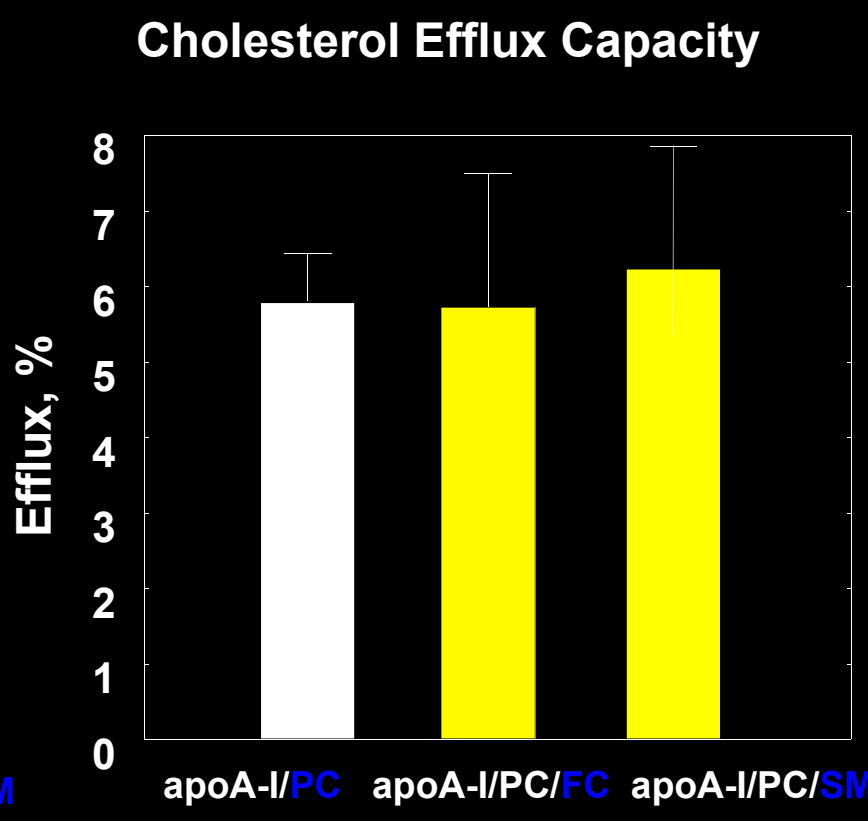


Data are shown for 10 healthy normolipidemic subjects.

Camont L, Lhomme M, Rached F, Chapman MJ, Kontush A. unpublished data.

Dissection of Functional Roles of PC, FC, and SM: Experiments With Reconstituted HDL

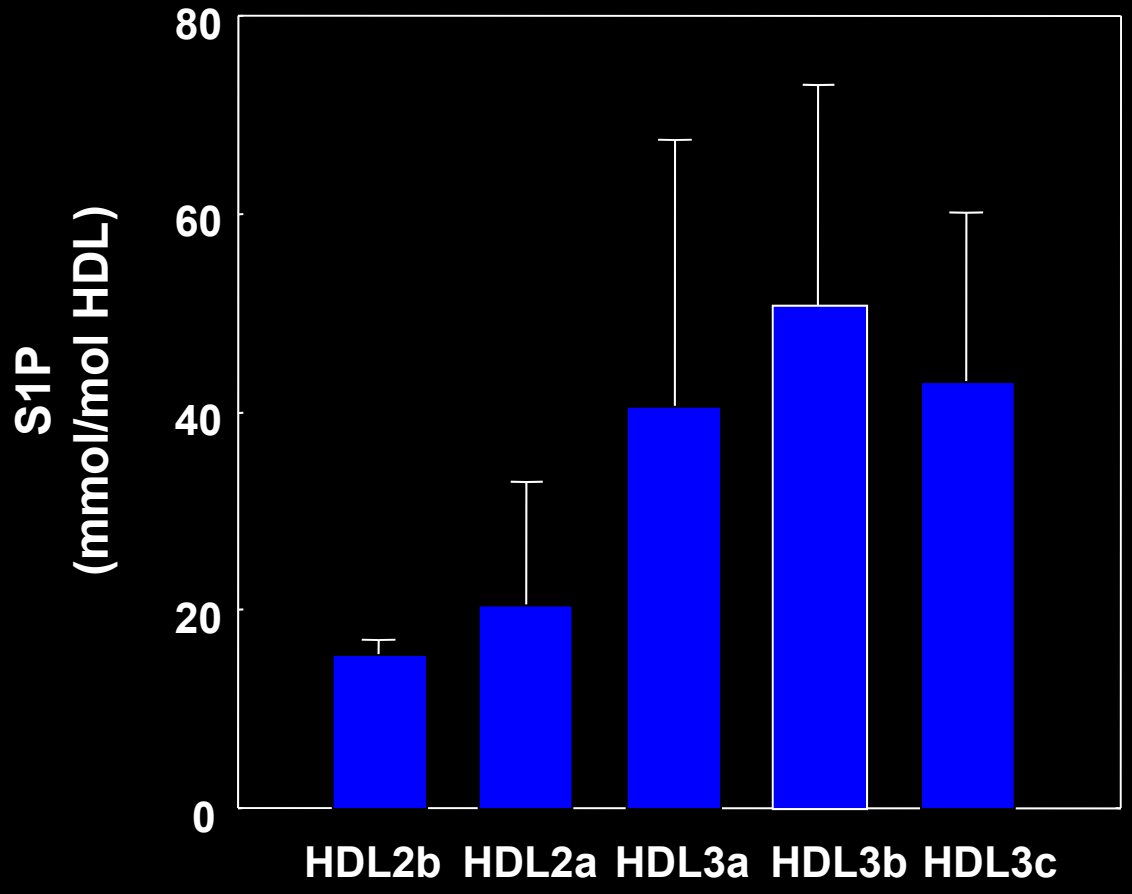




****P* < 0.05; FC=free cholesterol; SM=sphingomyelin; PC=phosphatidylcholine**

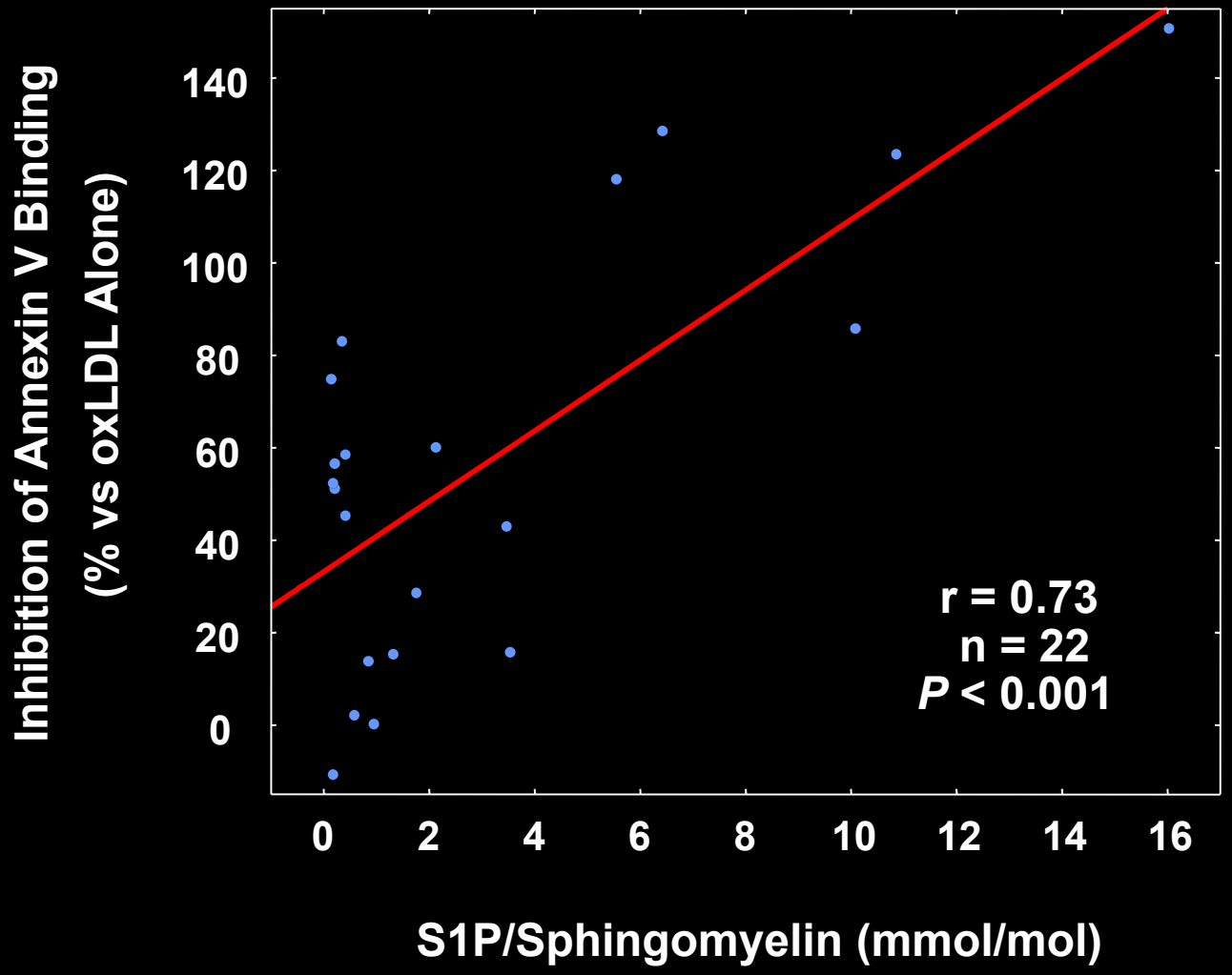
Camont L, Lhomme M, Rached F, Chapman MJ, Kontush A. unpublished data.

Small, Dense HDL3 Are Enriched in Sphingosine-1-Phosphate (S1P), a Minor Bioactive Lipid



Kontush A, et al. *Arterioscler Thromb Vasc Biol.* 2007;27(8):1843-1849.

Correlations Between the Capacity of HDL Subpopulations to Inhibit HMEC-1 Apoptosis and the S1P/SM Molar Ratio



Kontush A, et al. *Arterioscler Thromb Vasc Biol.* 2007;27(8):1843-1849.

Conclusions and Perspectives

- Lipidomic analysis can identify compositional determinants of HDL functionality in healthy normolipidemic subjects
- PC, SM, FC, ceramide, and S1P represent plausible candidates for lipidomic biomarkers of HDL function/dysfunction
- Lipidomic studies in patients at elevated CV risk and in those with established CV disease are required to assess biological and clinical value of such biomarkers

Lipidomic analysis of whole plasma recently has identified potential biomarkers of CVD risk

(Meikle PJ, et al. *Arterioscler Thromb Vasc Biol.* 2011;31(11):2723-2732.)

Are they related to HDL?