A Perspective on HDL-LDL Subclass, Subspecies and Subfraction Analyses and Challenges for Standardization

December 6, 2006

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NCEP Recommendation for Reference Methods for HDL and LDL Cholesterol

”The Working Group on Lipoprotein Measurement recommends that the most prudent course at present is to measure LDL-cholesterol by methods similar to those used to establish the epidemiological database on which the relations between cardiovascular risk and LDL-cholesterol have been established”.

“The existing epidemiological database relating LDL-concentration to CHD risk includes the contribution of other potentially atherogenic particles in addition to LDL, and the methods should give results equivalent to those used to establish the database”.

“The reference method should be based on the current CDC reference methods for total cholesterol and HDL-cholesterol..”

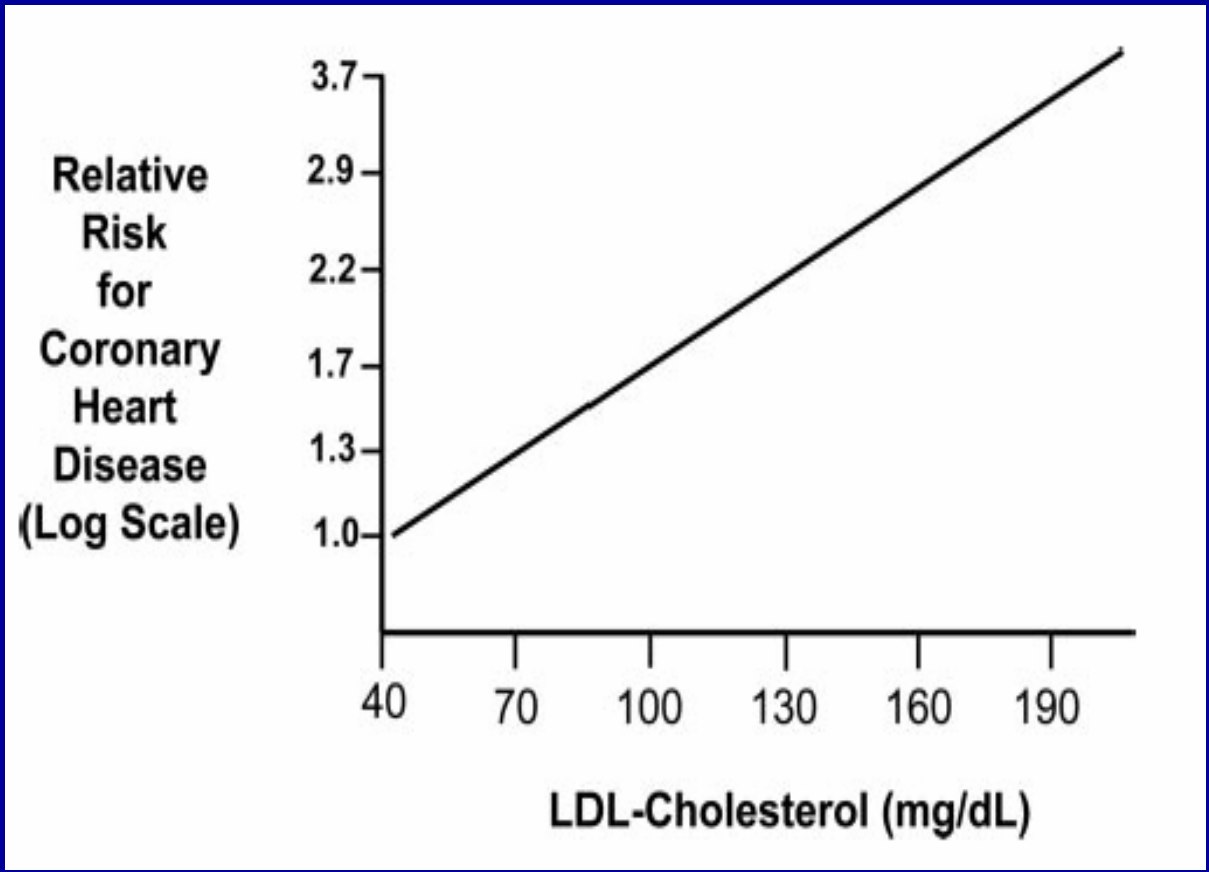
PS Bachorik and JW Ross, Clin Chem 1995; 41:1414-20

NCEP ATP III Update (2004)

High risk therapeutic option

LDL-C goal:

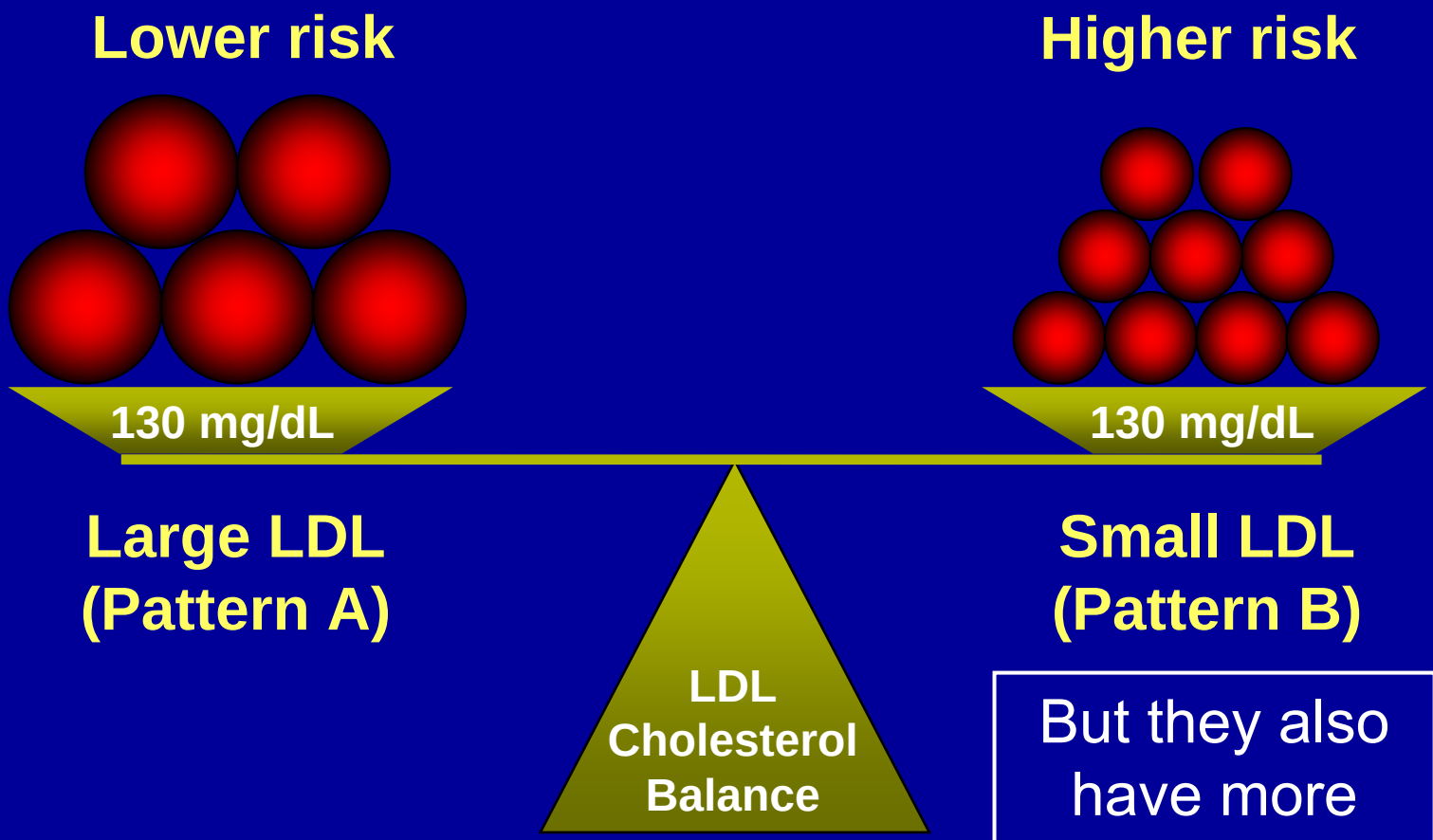
<70 mg/dL



Grundy, Circulation 2004; 110:227-239

20+ years of studies:

Patients with smaller LDL size have greater CHD risk at any given level of LDL-C

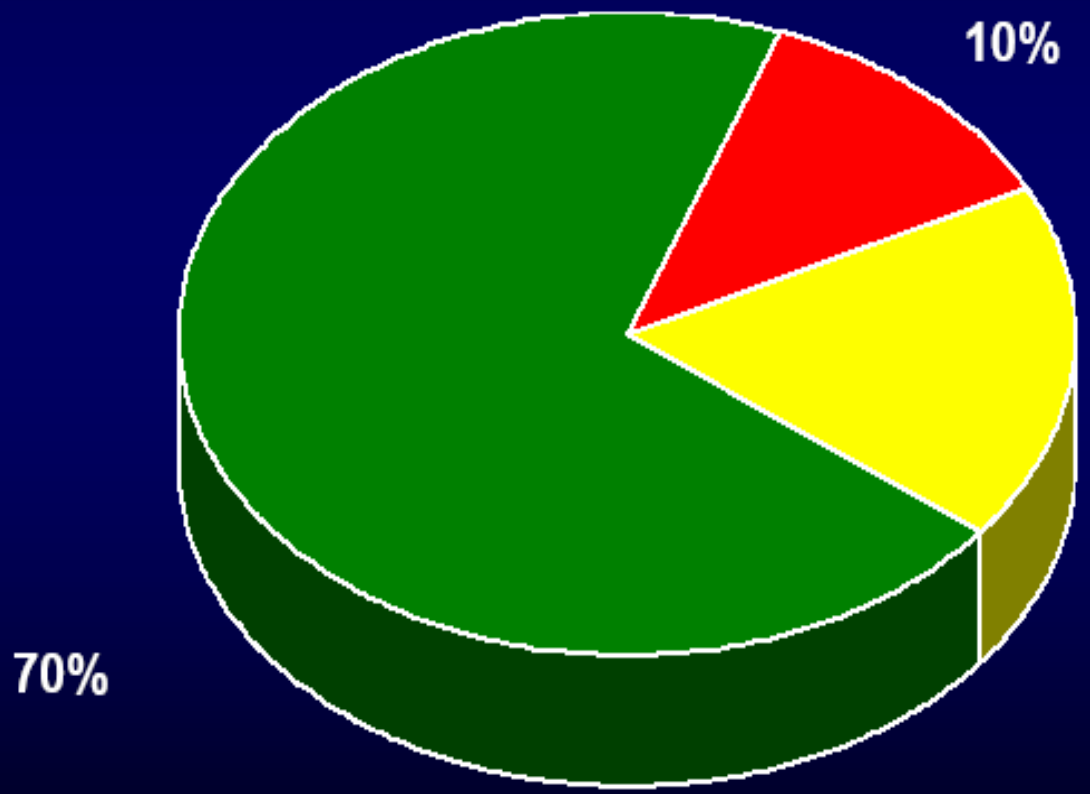


JO

How Good Is ATP III At Predicting MI?

Akosah Et al, JACC 2003:41 1475-9

222 patients with 1st acute MI, no prior CAD
men <55 y/o (75%), women <65 (25%), no DM



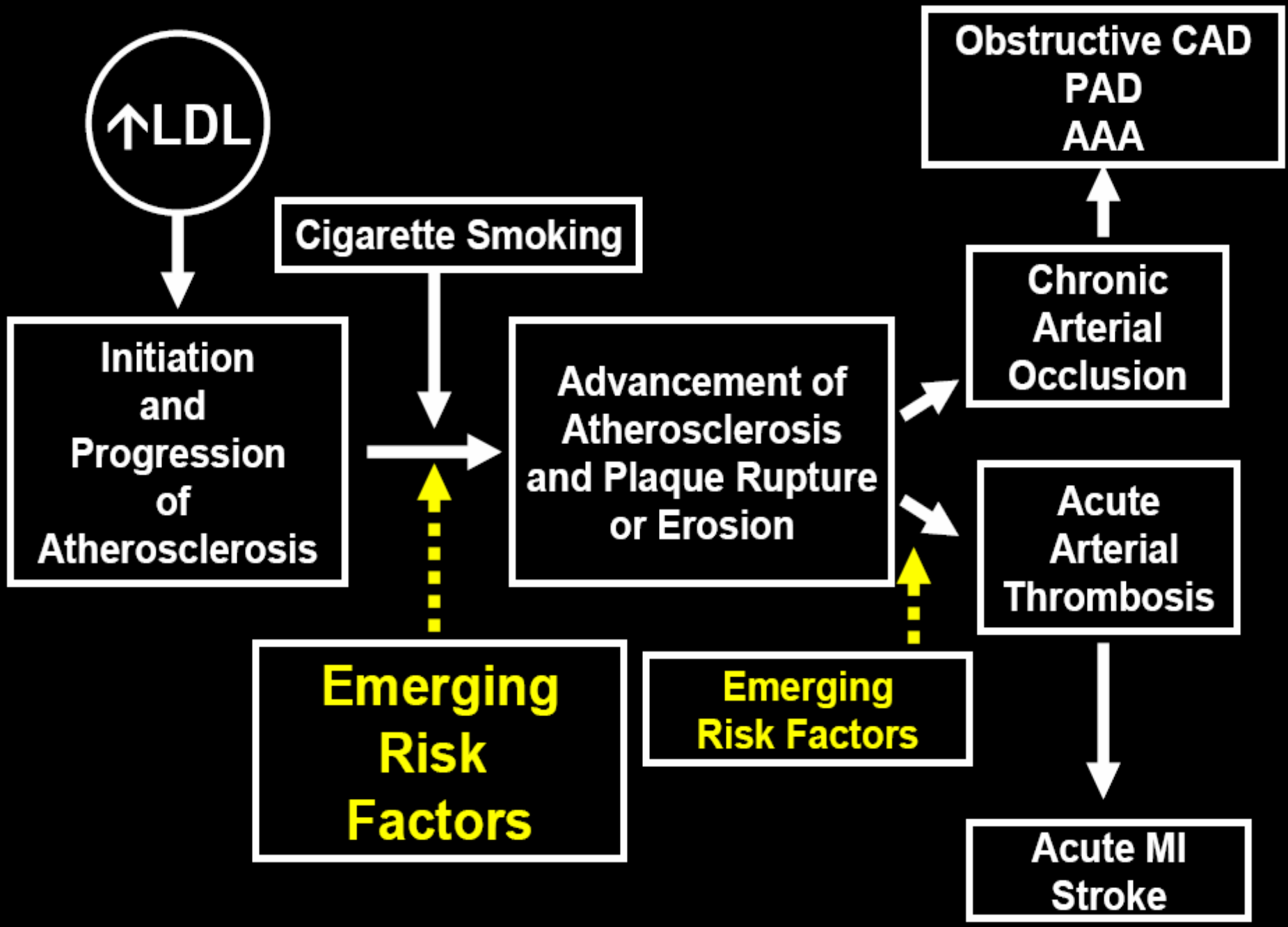
75% did not qualify for pharmcotherapy

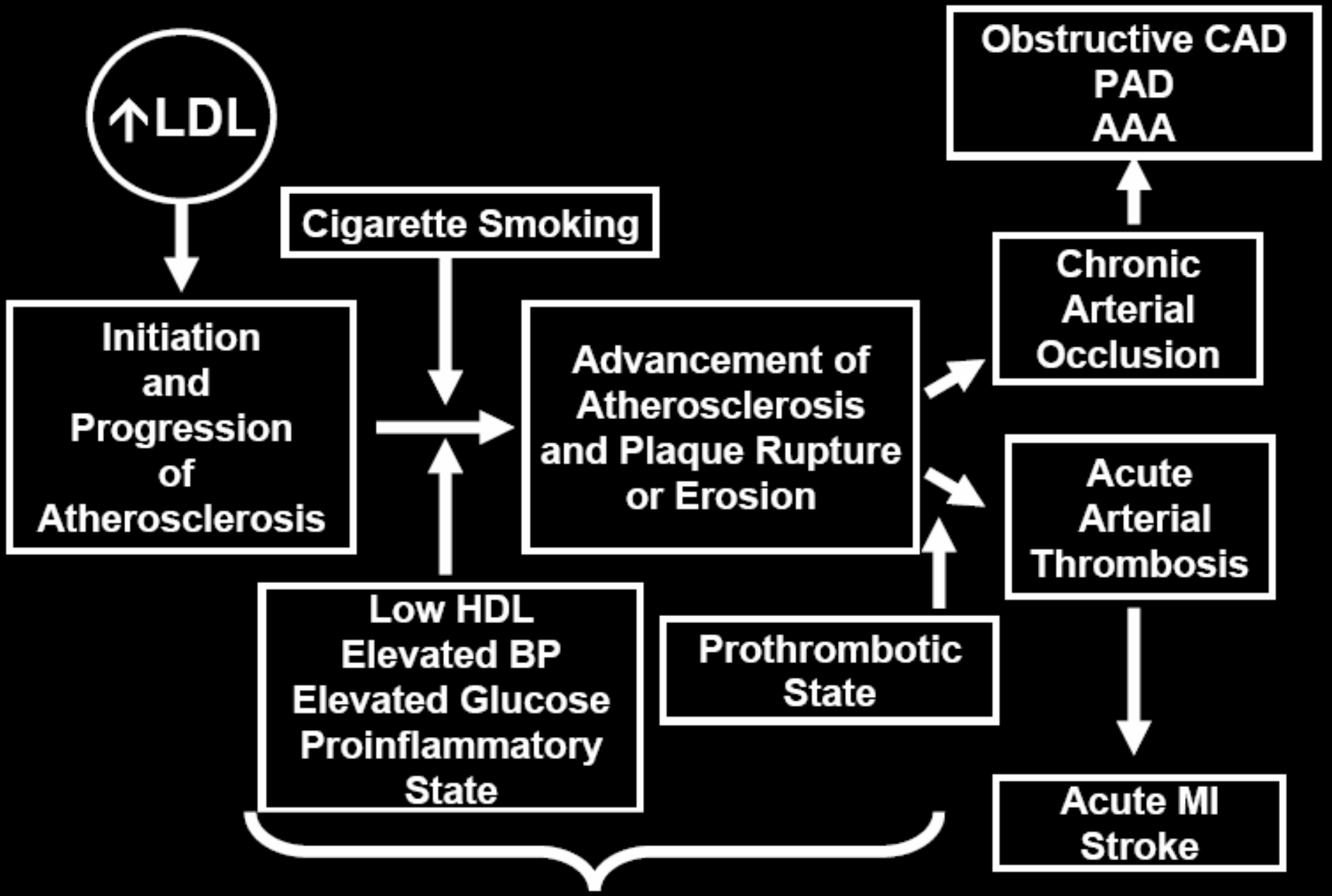
General Risk Categorization

Framingham Risk Score

<10% :	Low Risk (35% of US Population)
10-20% :	Intermediate Risk (40% of US Population)
<20% :	High Risk (25% of US Population)

Greenland, et al. Circulation. 2001;104(15):1863-7


↑LDL
Cigarette Smoking
Initiation and Progression of Atherosclerosis
Advancement of Atherosclerosis and Plaque Rupture or Erosion
Obstructive CAD
PAD
AAA
Chronic Arterial Occlusion
Acute Arterial Thrombosis
Acute MI
Stroke
Emerging Risk Factors
Emerging Risk Factors


↑LDL
Cigarette Smoking
Initiation and Progression of Atherosclerosis
Advancement of Atherosclerosis and Plaque Rupture or Erosion
Low HDL
Elevated BP
Elevated Glucose
Proinflammatory State
Prothrombotic State
Obstructive CAD
PAD
AAA
Chronic Arterial Occlusion
Acute Arterial Thrombosis
Acute MI
Stroke
Metabolic Syndrome
SG

Metabolic Syndrome and Emerging Risk Factors

Major Risk Factors

↑Apo B
↓HDL-C
↑BP
↑Glucose

Emerging Risk Factors

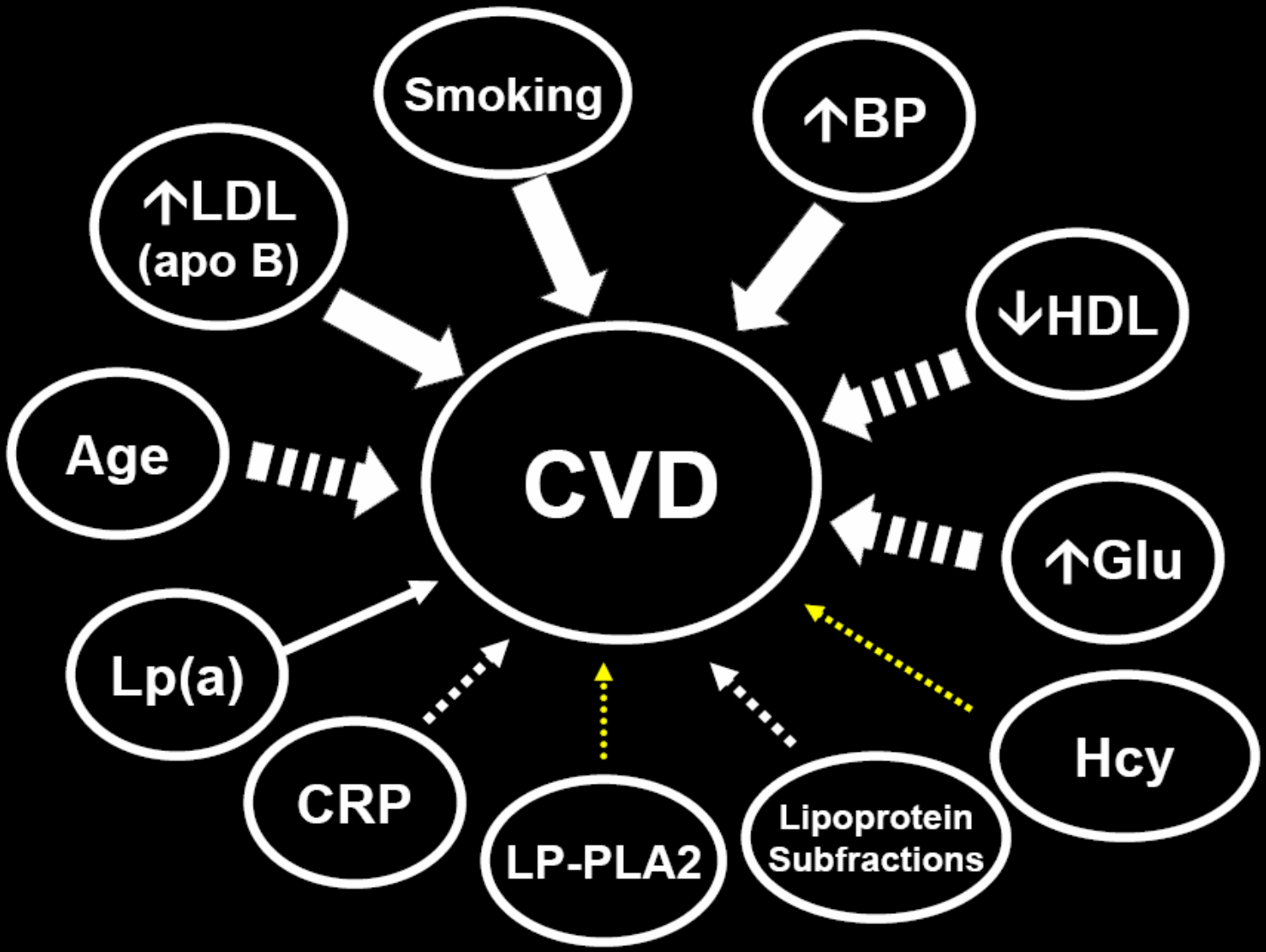
↑CRP & ↑SAA
↑sCD-40L
↑Fibrinogen
↑Small LDL
↓Small HDL
↑Remnant LP
↑IL-6; TNFα
↑PAI-1
↑Microalbuminuria
↑Insulin
Adipokines

SG

Types of Risk Factors

- Underlying risk factors
  - Causes of major and emerging risk factors
- Major independent risk factors
  - Powerful, directly causative risk factors
  - Powerful, predictive risk factors
- Emerging risk factors
  - Directly causative factors
  - Predictive factors (markers)

SG


Smoking
↑BP
↑LDL
(apo B)
↓HDL
Age
CVD
↑Glu
Lp(a)
Hcy
CRP
LP-PLA2
Lipoprotein
Subfractions

SG

Biomarkers Used for Risk Stratification Must Satisfy Several Criteria

Test Characteristics
- Widely available commercial test
- Reproducible, **standardized**, simple, inexpensive

Predicts risk in prospective studies

Adds to predictive value of traditional risk factors
- Useful in relatively low-risk patients

Improves ability to target therapy
- Alters treatment category (elevate or demote)

NCEP ATP III, Circulation 2002;106:3143-3421.

TP

Characteristics of a Marker Useful in Clinical Practice

1. Assay can be standardized with control of variability.
2. Association with CVD clinical end points in observational studies and/or clinical trials.
3. Independence from established risk factors.
4. Generalizability to various population groups.
5. Availability of population norms to guide interpretation.
6. Ability to improve overall prediction beyond that of traditional risk factors.
7. Acceptable costs of assays.

Mosca L, N. Eng. J. Med. 2002;347:1615-17

TP

Guidelines for Risk Assessment

1. Guidelines allow the latest scientific evidence to be applied to clinical practice: type and level of evidence should be used to communicate strength of recommendation.
2. A marker useful in clinical practice should be precise, independently associated with disease, generalizability to population groups, predictive of disease beyond traditional risk factors, and cost-effective.
3. Markers may have additional applications to patient behavior and to research that should be considered.

Thomas Pearson, New Risk Markers for Cardiovascular Risk: Evidence and Expectations; Emerging Cardiac Markers – Establishing Guidelines for Risk Assessment, 27th Arnold O. Beckman Conference, 2006

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American College of Cardiology/ American Heart Association

Classification of Recommendations

Class I Conditions for which there is evidence and/or general agreement that a given procedure is useful and effective

Class II Conditions for which there is conflicting evidence and/or divergence of opinion about the usefulness/efficacy of a procedure or treatment

IIa Weight and evidence/opinion is in favor of usefulness/efficacy

IIb Usefulness/efficacy is less well established

Class III Conditions for which there is evidence and/or general agreement that the procedure/treatment is not useful/effective, and in some cases may be harmful

American College of Cardiology/ American Heart Association

Levels of Evidence

Level A	Data derived from multiple randomized clinical trials
Level B	Data derived from a single randomized trial or nonrandomized studies
Level C	Consensus of experts

Lipoprotein Subclasses and Particle Size

(Draft) **Recommendation 1:**

The first step is calculation of the 10 year predicted global risk assessment using a lipid profile.

Classification of recommendation : **I**

Level of Evidence: **A**

Peter W. F. Wilson, Lipoprotein Particle Size and CHD Risk; Emerging Cardiac Markers – Establishing Guidelines for Risk Assessment, 27th Arnold O. Beckman Conference, 2006.

Lipoprotein Subclasses and Particle Size

(Draft) **Recommendation 2:**

Lipid subclasses, especially the number or concentration of small dense LDL particles, have been shown to be related to the development of initial coronary heart disease events, but the data analyses of existing studies are generally not adequate to show added benefit over standard risk assessment.

Classification of recommendation: **III** (Lipoprotein subclass determination is not recommended)

Level of Evidence: **A**

Peter W. F. Wilson, Lipoprotein Particle Size and CHD Risk; Emerging Cardiac Markers – Establishing Guidelines for Risk Assessment, 27th Arnold O. Beckman Conference, 2006.

Lipoprotein Subclasses and Particle Size

(Draft) **Recommendation 3:**

There is insufficient data that measurement of lipid subclasses over time is useful to evaluate the effects of treatments.

Classification of recommendation: **IIb**

Level of Evidence: **C**

Peter W. F. Wilson, Lipoprotein Particle Size and CHD Risk; Emerging Cardiac Markers – Establishing Guidelines for Risk Assessment, 27th Arnold O. Beckman Conference, 2006.

Lipoprotein Subclasses and Particle Size

(Draft) **Recommendation 4:**

Several methods are available to assess lipoprotein subclasses. Standardization is needed for this technology.

Classification of recommendation: **IIa**

Level of Evidence: **C**

Peter W. F. Wilson, Lipoprotein Particle Size and CHD Risk; Emerging Cardiac Markers – Establishing Guidelines for Risk Assessment, 27th Arnold O. Beckman Conference, 2006.

Most Recent NCEP Recommendation ATPIII (2001)

"… small LDL particles…are formed…as a response to elevations of triglycerides. Their presence is associated with an increased risk for CHD; however,

(1) the extent to which they predict CHD independently of other risk factors is unresolved. Moreover,

(2) standard and

(3) inexpensive methodologies are not available for their measurement. **For these reasons, ATP III does not recommend measurement of small LDL particles in routine practice.** If the clinical decision is made to detect and measure small LDL, their presence is best used as an indication for atherogenic dyslipidemia and the metabolic syndrome…."

Role of Practice Guidelines

" The practice guidelines are an essential tool for clinicians that facilitate implementation of state-of-the-art cardiovascular prevention. However due to complex interrelationships between risk factors, no single guideline takes into account all aspects of cardiovascular event risk. Thus, the essential role of a physician is to translate the guideline content into advice to an individual person, and to exercise clinical judgment in the process. The guidelines provide overall direction for clinical decisions, but by no means are they its substitute."

Dominiczak, In:Handbook of Lipoprotein Testing, 2nd Ed. AACC Press, 2000, 127-160.

Why are Lipoprotein Subclasses Measured?

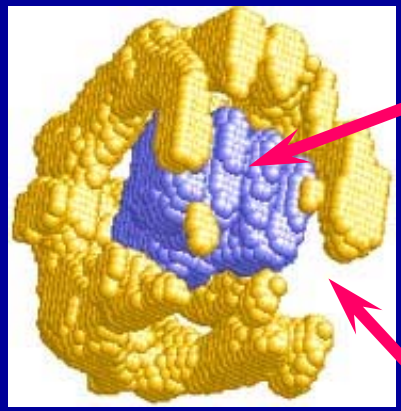
- Detect Atherogenic Dyslipidemia
  - -Small dense LDL more atherogenic? (increased apoB particles)
  - -Large HDL is more protective?
  - -TG-rich remnant lipoproteins
- Detect Metabolic Syndrome
  - -w/Elevated TG, Low HDL, small HDL
  - -w/Elevated TG, small LDL
- Clinical Judgment
  - -Measurement of subclasses could better characterize risk
  - -More information for managing treatment of patients
- Subclass measurements go beyond current NCEP guidelines
  - -NCEP ATP III does not recommend small dense LDL analysis in routine practice

Why are Lipoprotein Subclasses Measured?

" Epidemiological studies have demonstrated that small, dense LDL is associated with increased risk of CHD, and the presence of small, dense LDL precedes clinical events. These studies indicate that small, dense LDL is a component of a complex physiologic syndrome: a set of interrelated abnormalities including elevated TG, low HDL-C, insulin resistance, and increased central obesity. These factors make it difficult to determine an "independent" risk of CHD associated with small, dense LDL. Intervention studies, however, have shown that small, dense LDL predicts the angiographic changes in response to lipid-lowering therapy, and that converting small, dense LDL to buoyant LDL is associated with CHD regression. The physiologic mechanism(s) underyling these observations remains to be clearly established"

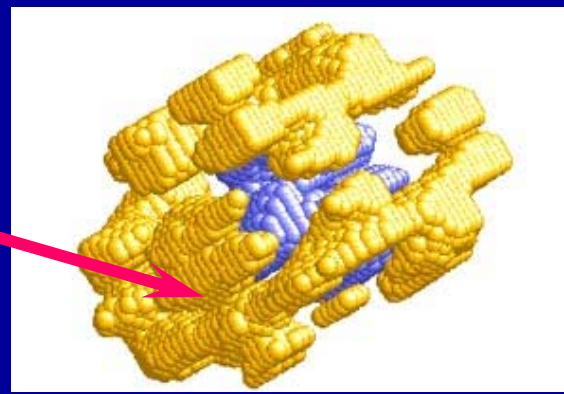
Hokanson, In:Handbook of Lipoprotein Testing, 2nd Ed. AACC Press, 2000, 311-344.

Basic Structure of Lipoproteins as shown by X-Ray Analysis



Triglyceride & cholesteryl esters

Apolipoprotein(s)



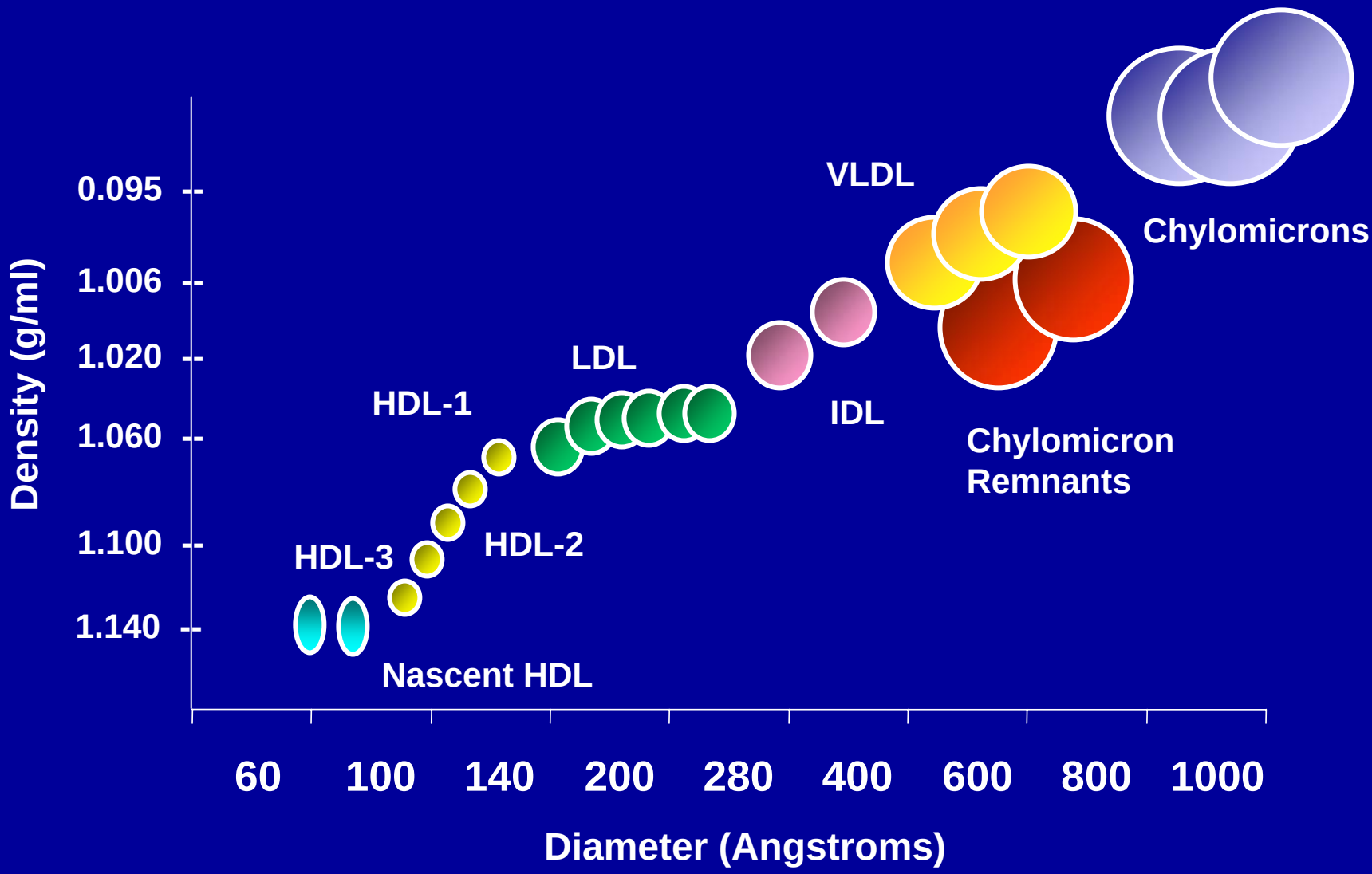
Free cholesterol & Phospholipid
(Not visible on x-rayed surface)

LDL X-ray analysis performed at the Institute of Mathematical Problems of Biology, RAS, in collaboration with the Medical University of Freiburg (Germany), Nancy University (France) and the Institute of Genetics and Molecule and Cell Biology, Strasbourg (France). Use was made of original computer methods developed in the IMPB RAS.

Lipoproteins are Heterogeneous

- All lipoprotein classes are heterogeneous in density, size, electrophoretic mobility, lipid composition, function(s), and binding affinity.
- Additional heterogeneity results from the metabolic delipidation cascade, as well as precursor and apolipoprotein differences.
- To explain heterogeneity we make assumptions about the existence of discrete subpopulations with unifying properties that we call subfractions, subclasses or subspecies.
- When one physicochemical property is used to separate and define a subclass, a problem arises when the other properties begin to lose correlation.

Serum Lipoprotein Size and Density



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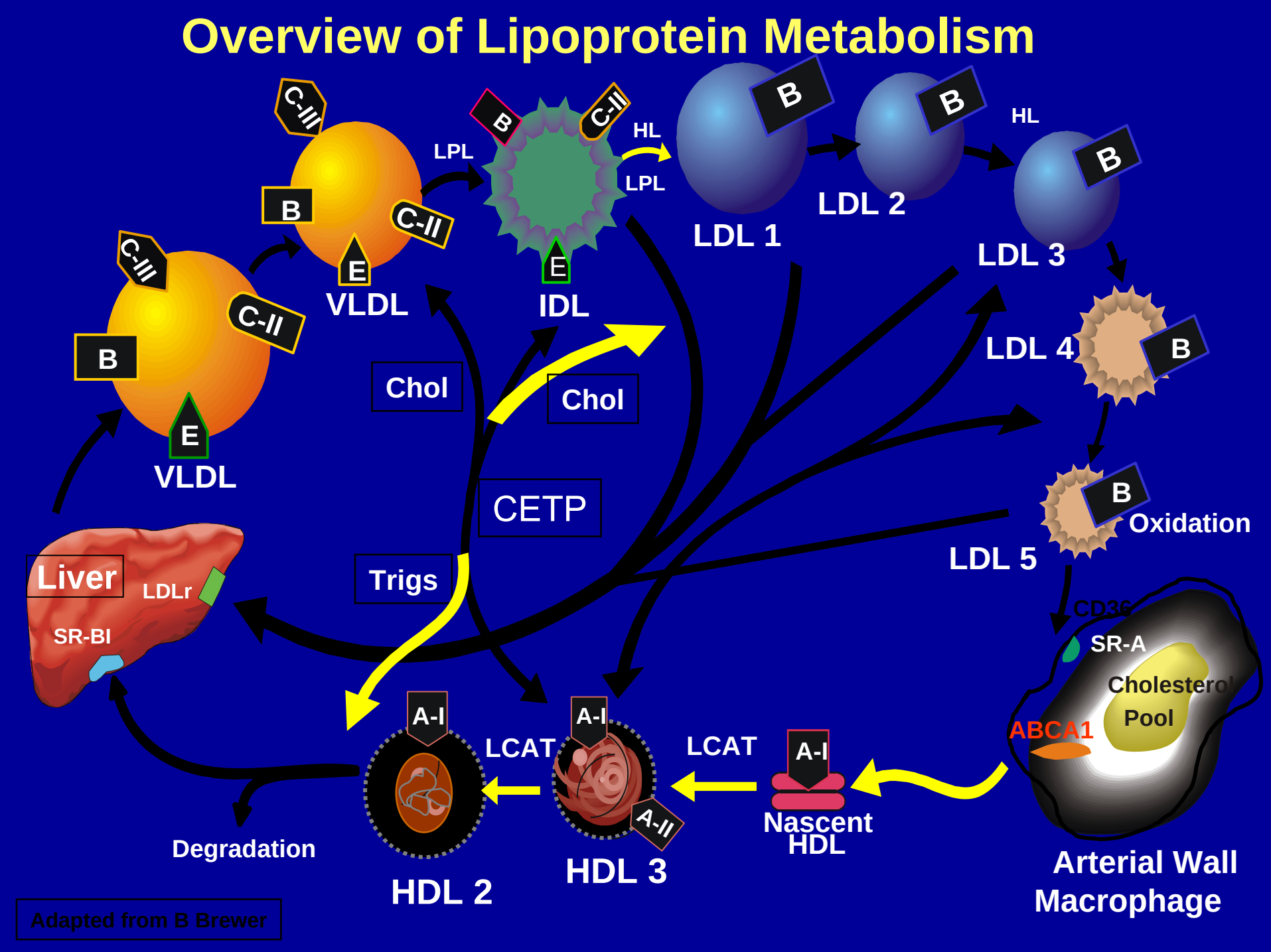
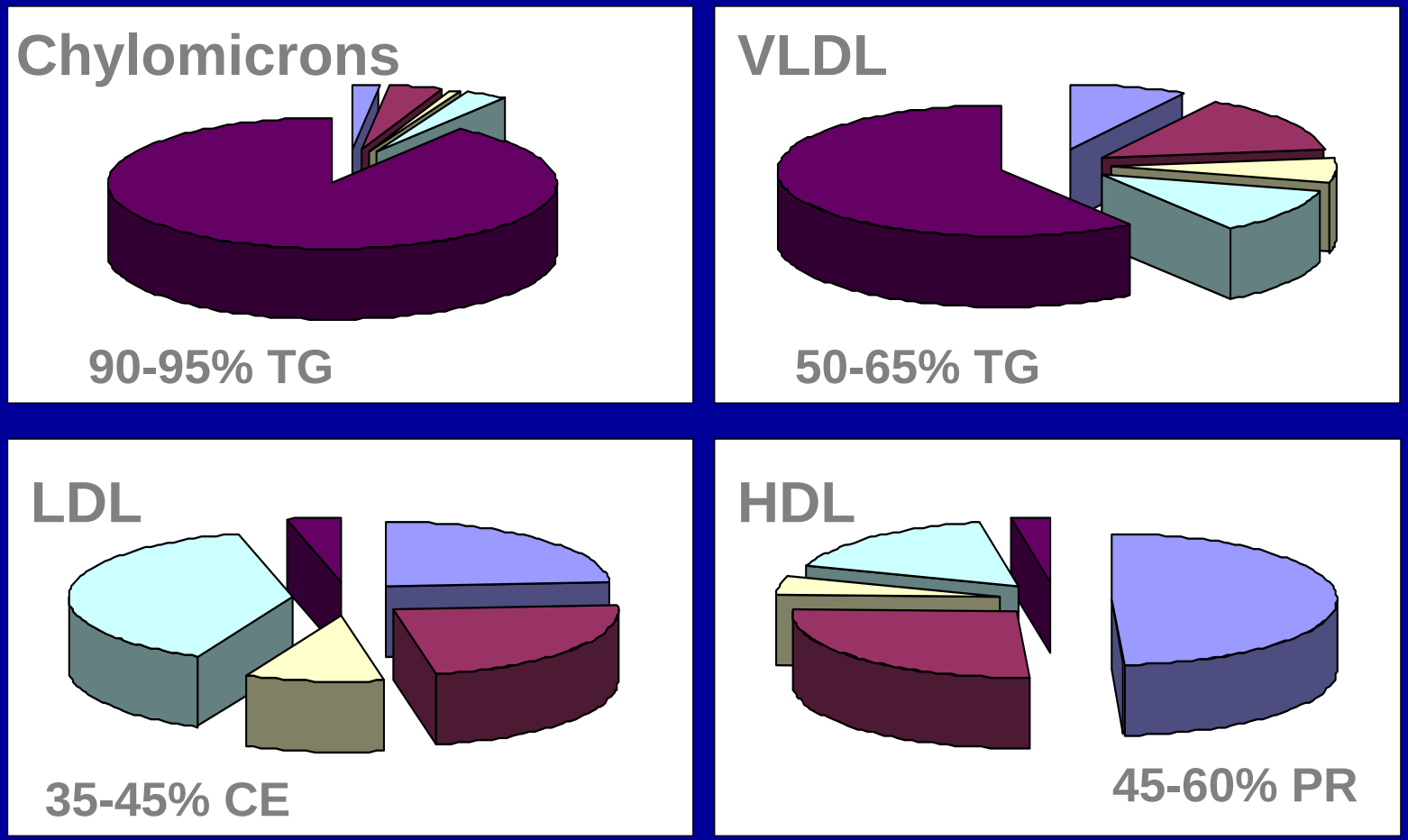

Overview of Lipoprotein Metabolism
C-III
B
C-II
E
VLDL
LPL
B
C-II
HL
LPL
E
IDL
B
LDL 1
B
LDL 2
HL
B
LDL 3
LDL 4
B
B
Oxidation
LDL 5
C-III
B
C-II
E
VLDL
Chol
Chol
CETP
Trigs
Liver
LDLr
SR-BI
Degradation
A-I
HDL 2
LCAT
A-I
A-II
HDL 3
LCAT
A-I
Nascent
HDL
CD36
SR-A
Cholesterol
Pool
ABCA1
Arterial Wall
Macrophage
Adapted from B Brewer

Lipoprotein Composition



Adapted from: McNamara et al, in Clinical Chemistry, Principles,Procedures, Correlations, ed: Bishop et al, Lippincott,Williams & Wilkins 2000, pp 235-7

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Protein components of lipoprotein metabolism

Apolipoproteins

- Apolipoprotein (a)
- Apolipoprotein A-I
- Apolipoprotein A-II
- Apolipoprotein A-IV
- Apolipoprotein A-V
- Apolipoprotein B-48
- Apolipoprotein B-100
- Apolipoprotein C-I
- Apolipoprotein C-II
- Apolipoprotein C-III
- Apolipoprotein C-IV
- Apolipoprotein D
- Apolipoprotein E

Transfer Proteins

- Cholesteryl ester transfer protein (CETP)
- Microsomal triglyceride transfer protein (MTP)
- Phospholipid transfer protein (PLTP)
- ABC1 Transporter (ABC1)

Protein components of lipoprotein metabolism

Enzymes

- Hepatic lipase (HL)
- HMG CoA reductase (HMGCR)
- Lecithin-cholesterol acyltransferase (LCAT)
- Lipoprotein lipase (LPL)
- Paraoxonase (PON1)
- Hormone sensitive Lipase (HSL)
- Cholesterol 7-alpha-hydroxylase (CYP7A1)

Receptors

- Low density lipoprotein receptor (LDLR)
- Scavenger receptor, class B (SRB1)
- Insulin receptor (INSR)
- Peroxisome proliferator-activated receptor alpha (PPARA)
- Peroxisome proliferator-activated receptor delta (PPARD)
- Peroxisome proliferator-activated receptor gamma (PPARG)
- Retinoid X receptor, alpha (RXR)

LDL Phenotype Interpretation

- Pattern A = predominantly large particles
- Pattern B = Predominantly small particles
- Intermediate pattern AB
- Peak or mean particle diameter determined
- Peak < 25.5 nm = type B phenotype
- Peak > 25.5 nm = type A phenotype
- Need to standardize methods/interpretation?

Lipoprotein particle size calibration

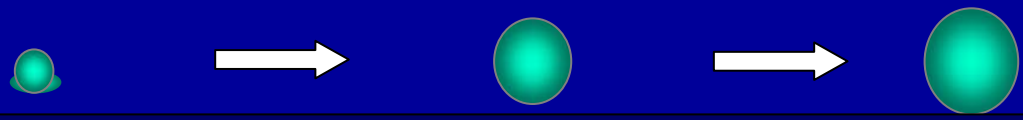
- Size standards
  - Globular proteins
    thyroglobulin (17.0 nm)
    apoferritin (12.2 nm)
    catalase (9.7 nm)
    lactate dehydrogenase (8.2 nm)
  - Plasma standard with LDL bands at 26.6nm and 27.5 nm
  - Carboxlyated polystyrene microspheres (38 nm)

Subclasses density and size

LDL subclass	Density range g/mL	Particle diameter (nm)
I	1.019-1.023	27.2-28.5
IIa	1.023-1.028	26.5-27.2
IIb	1.028-1.034	25.6-26.5
IIIa	1.034-1.041	24.7-25.6
IIIb	1.041-1.044	24.2-24.7
IVa	1.044-1.051	23.3-24.2
IVb	1.051-1.063	22.0-23.3

Clin Lab Med 2006;26: 803-846

Simplified Terminology for Lipoprotein Subclass



Segmented Gradient Gel Electrophoresis (GGE)							
LDL Particles	Pattern B			Pattern Intermediate			Pattern A
	IVb	IVa	IIIb	IIIa	IIb	IIa	I
HDL Particles	3c		3b	3a		2a	2b
VLDL Particles	VLDL subclass distribution not normally measured by this commercial method						
Nuclear Magnetic Resonance (NMR)							
LDL Particles	Pattern B						Pattern A
	L1			L2			L3
HDL Particles	H1		H2	H3		H4	H5
VLDL Particles	V1	V2		V3	V4	V5	V6
Short, single vertical automated with gradient ultracentrifugation (VAP)							
LDL Particles	Pattern B			Pattern A/B			Pattern A
	LDL 4		LDL 3		LDL 2		LDL1
HDL Particles	HDL3 (d,c,b,a)				HDL2 (a,b,c)		
VLDL Particles	VLDL 3b		VLDL 3a		VLDL 1 + 2		

PWFW

Bays *Prev Cardiol* 2003;6:179

Nomenclature for LDL Subspecies Separation

GGE	GGE	Density	Pattern	Diam	Diam	NMR
1	I	1.025-1.032		260-275	204	
2	IIA	1.030-1.038	A	255-270	200	1
3	IIB	1.035-1.040		255-270	200	
4	IIIA	1.038-1.048		247-252	196	2
5	IIIB	1.038-1.048		242-246	193	
6	IVA	1.048-1.065	B	233-242	191	3
7	IVB	1.048-1.065		218-232	190	
8	-	1.048-1.065			177	

Adapted from Campos et al, Arterioscler Thromb 1992;12:1410; McNamara et al, JLR 1996;37:1924

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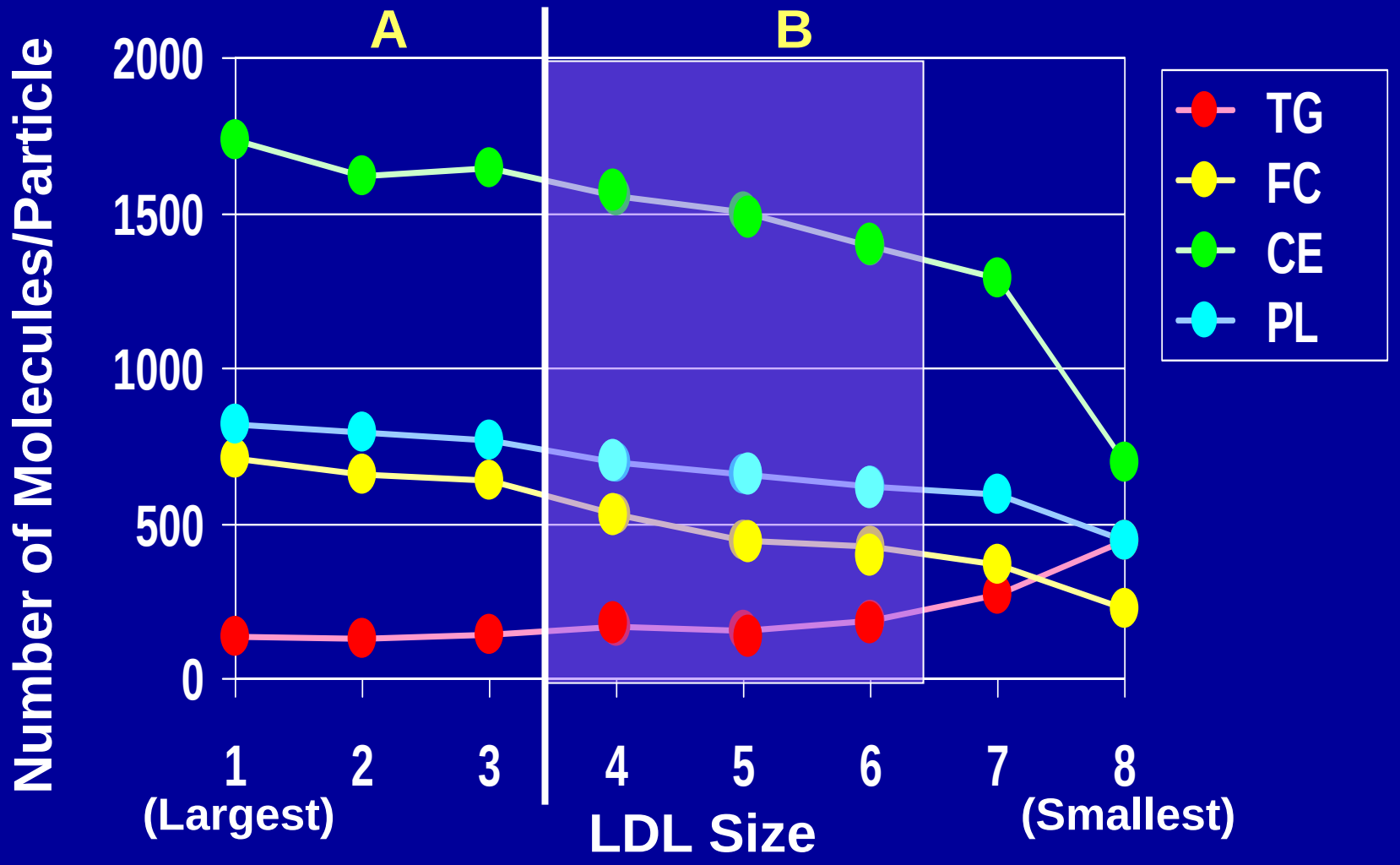
LDL Subspecies Composition



McNamara et al, J Lipid Res 1996;37:1924

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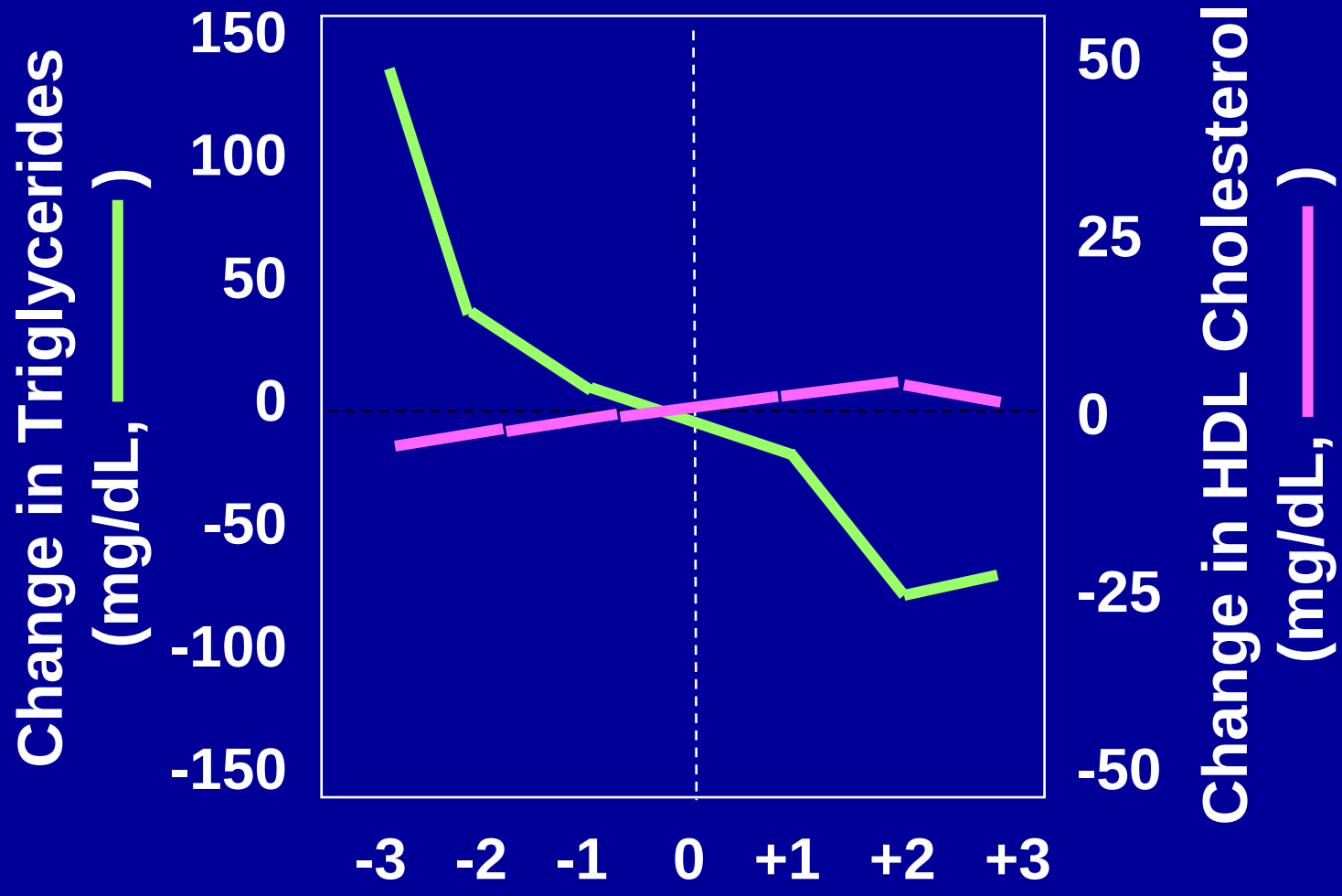
Change in Lipids and LDL Size



McNamara et al, Arterioscler Thromb 1992;12:1284

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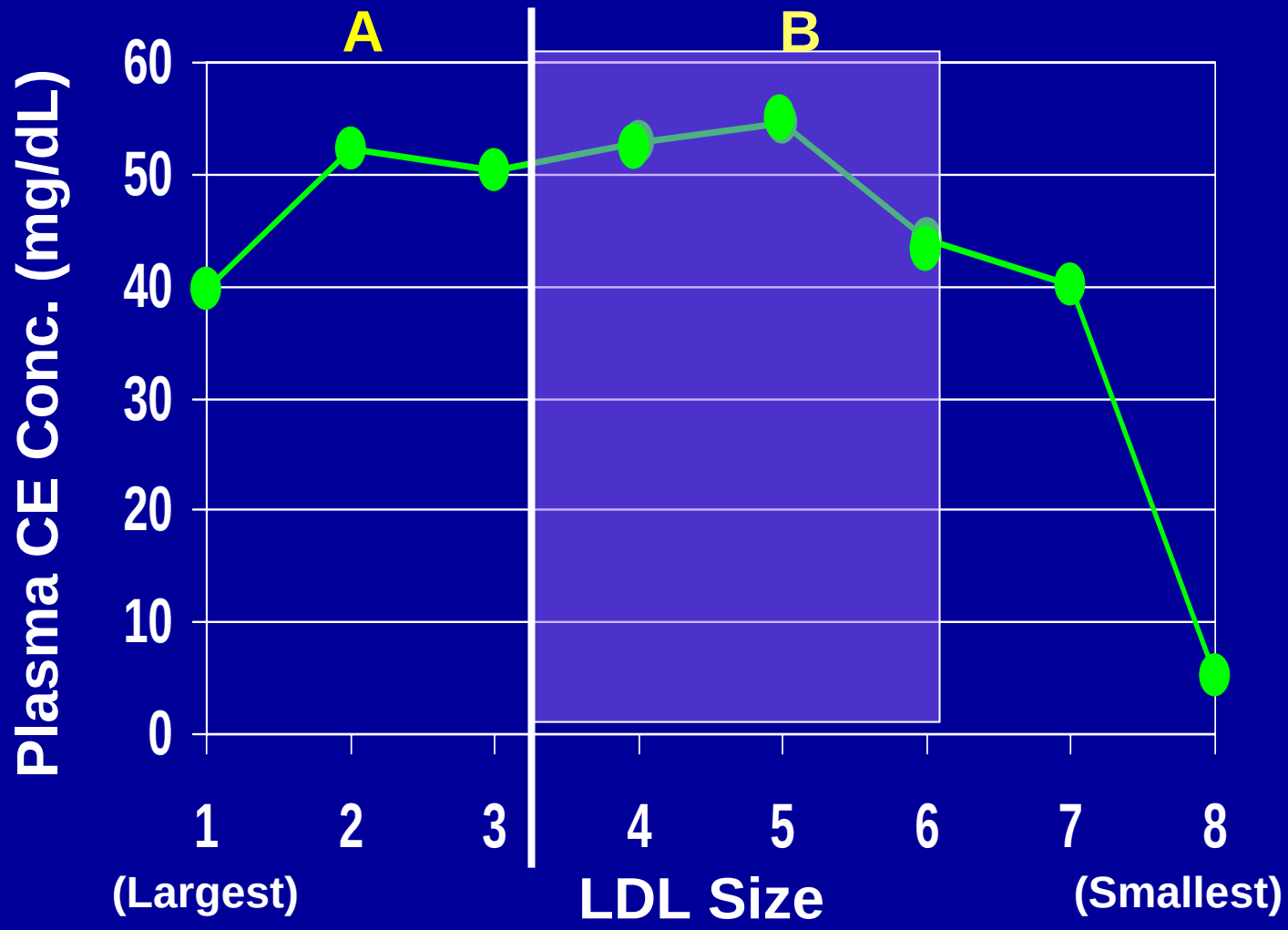
LDL Subspecies Composition



Adapted from McNamara et al, J Lipid Res 1996;37:1924

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LDL Subspecies

- In the general population, LDL size is primarily a reflection of triglyceride level.
- Change in LDL size is inversely related to change in triglyceride level.

Deckelbaum et al, Arteriosclerosis 1984;4:225;
McNamara et al, Arterioscler Thromb 1992;12:1284;
Yuan et al, Atherosclerosis 1994; 110:1;
Freedman et al, Clin Chem 2004; 50:1189

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Nomenclature for HDL Subspecies Separation

GGE	Density	Diam.	Orig.	A-I	A-II	E	2-D	IAC
1	1.063	12.5		+	–	+++	preβ-2	
2	1.068	11.7		+	–	++		
3	1.074	11.3		+	–	+		
4	1.079	11.0	HDL2b	++	–	+		LpA-I
5	1.084	10.6		++	–	+	α-1	
6	1.089	10.0		+++	+	+		
7	1.095	9.6		++	+	+		LpA-I/A-II
8	1.100	9.2		++	+	+/–		
9	1.113	8.9	HDL2a	++	+++	+/–	α-2	LpA-I/A-II
10	1.125	8.7		++	++	+/–		
11	1.147	8.5	HDL3a	+++	++	+/–		LpA-I
12	1.167	8.3	HDL3b	+++	++	+/–	α-3	
13	1.190	8.1		++	+	+/–		LpA-I/A-II
14	1.210	<7.9	HDL3c	++	+	+/–	preβ-1	

Li et al, JLR 1994;35:1698; Blanche et al, BBA 1981;665:408; Asztalos et al, BBA 1993;1169:291; Cheung & Albers, JBC 1984;259:12201

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High Density Lipoproteins

- ApoA-I, the major protein of HDL, is produced in both the liver and intestine.
- ApoA-I interacts with the ATP binding cassette transporter protein A1 (ABCA1) to pick up FC and PL from cells to form bilayers.
- FC is converted to CE via the action of lecithin cholesterol acyltransferase (LCAT), which adds fatty acid from lecithin to cholesterol to form CE.
- HDL CE is transferred to chylomicron and/or VLDL remnants by the action of CETP in exchange for TG, or is taken up directly by the liver scavenger receptor B1 (SRB1).
- HDL PL and TG are acted upon by hepatic lipase to form smaller HDL, which recycle to pick up more FC from ABCA1.

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HDL and LDL Subclass Observations

- LDL and HDL subclasses are very complex and difficult to define
- Much metabolic and mechanistic information has being gained from studying their interactions and responses to genetic mutations, genotype, life style, nutrition, and drug therapy.

Lipoprotein Subclass Methods

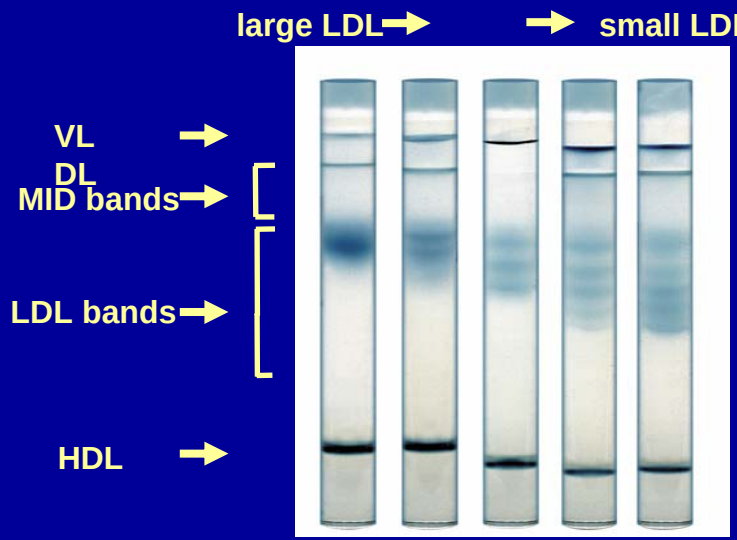
- Ultracentrifugation
  - Analytical ultracentrifuge - Model E
  - VAP (vertical auto profiler)
- Polyacrylamide electrophoresis
  - GGE (gradient gel electrophoresis)
- NMR (Nuclear magnetic resonance)
- HPLC
- Capillary isotachophoresis

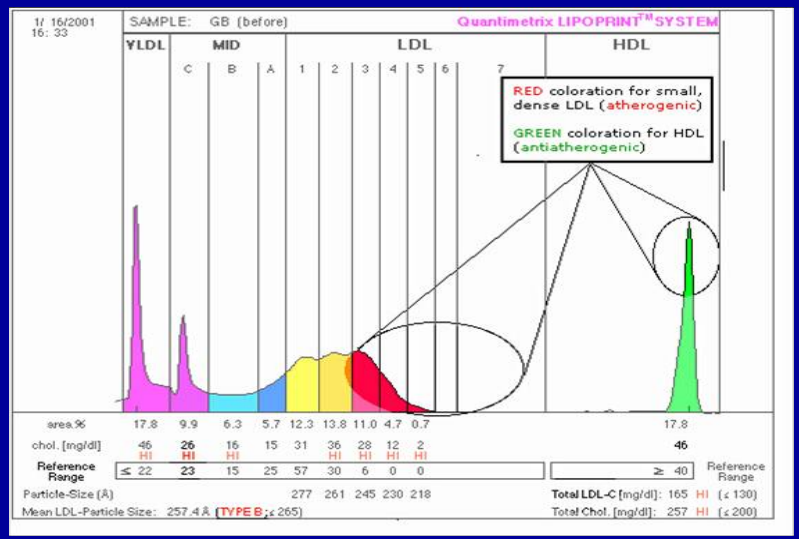
Quantimetrix Lipoprint Method

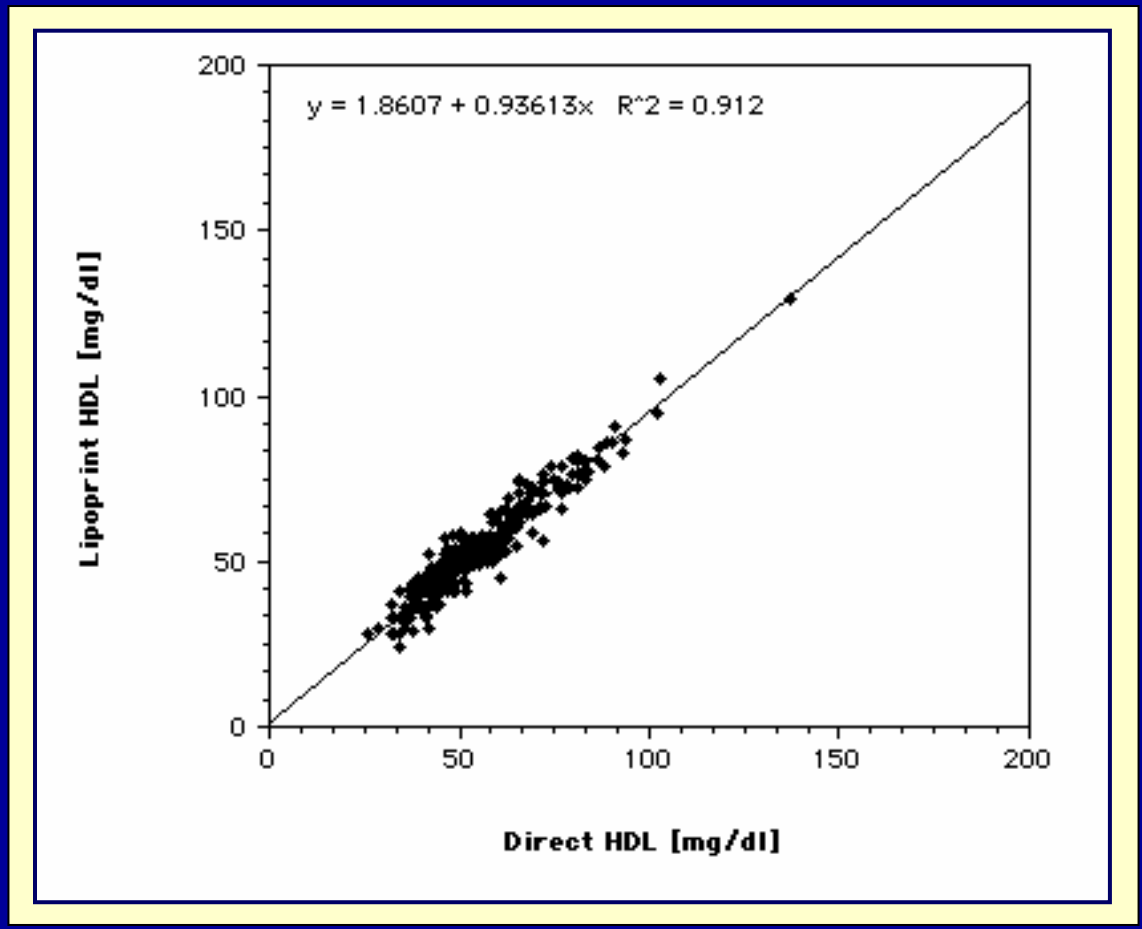
- Continuous polyacrylamide gel electrophoresis; two gel formulations available for maximum resolution of either LDLs or HDLs (research)
- Separation is based on charge and size
- Pre-staining with Sudan Black B and densitometric scanning combined with a separate total cholesterol measurement gives cholesterol concentration in each subfraction
- Quantitation of overlapping subfractions depends on mathematical “deconvolution” of area under scans
- Provides an LDL size phenotype (A, B or AB) that is based on assumed size-R_f relationship
- Report provides TC, LDL-C, HDL-C and cholesterol in subfractions

Quantimetrix Lipoprint LDL

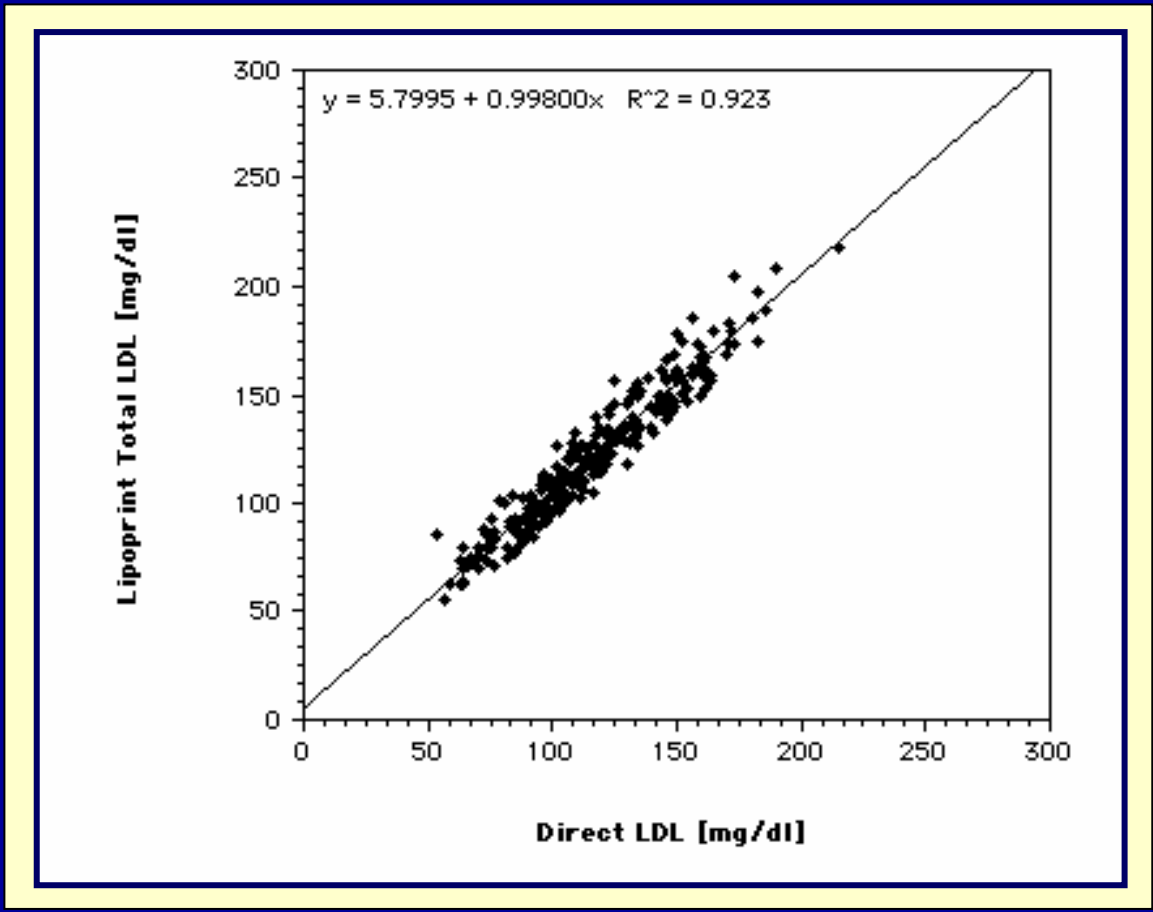
- **Consumables:**
  - **Precast PAG tubes**
  - **Loading gel**
  - **Electrophoresis buffer**
- **System components:**
  - **Chamber + Rack**
  - **Light + Power Supply**
  - **Digital Scanner**
  - **iMac + software + printer**







Quantimetrix Corporation
LIPOPRINT® SYSTEM

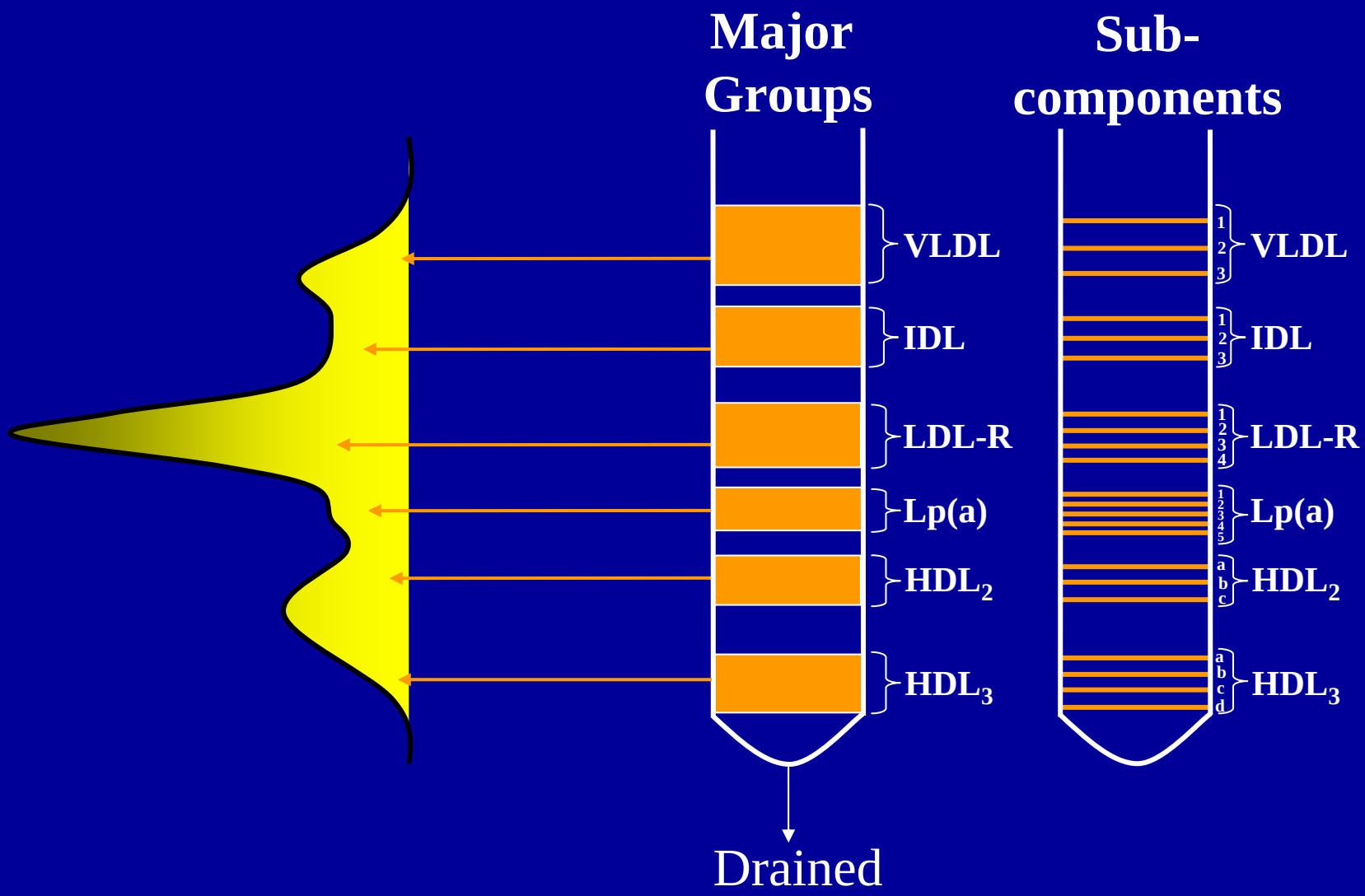


Quantimetrix Corporation
LIPOPRINT® SYSTEM

Atherotech VAP Method

- Fractionation based on density gradient ultracentrifugation
- Rapid separation on short vertical axis, re-orients
- Cholesterol analysis of effluent from tubes provides particle density profile
- Overlapping subclasses quantitation depends on proprietary software for “deconvolution” of profile using algorithm based on purified UC fractions
- Provides a size phenotype, A, B or AB
- Measures Lp(a) cholesterol content
- Provides non-HDL cholesterol
- Separate tests for TG, hsCRP and homocysteine

VAP Profile


Major Groups
Sub-components
VLDL
IDL
LDL-R
Lp(a)
HDL2
HDL3
1
2
3
1
2
3
1
2
3
4
1
2
3
4
5
a
b
c
a
b
c
d
Drained

Atherotech VAP Method

Correlates with Beta-Quantification Reference Method and sequential density gradient UC

Lipoprotein	Correlation coefficient
TC	0.990
HDL	0.990
LDL	0.980
VLDL	0.976
HDL_2	0.936
HDL_3	0.913
Lp(a)	0.77
IDL	0.78

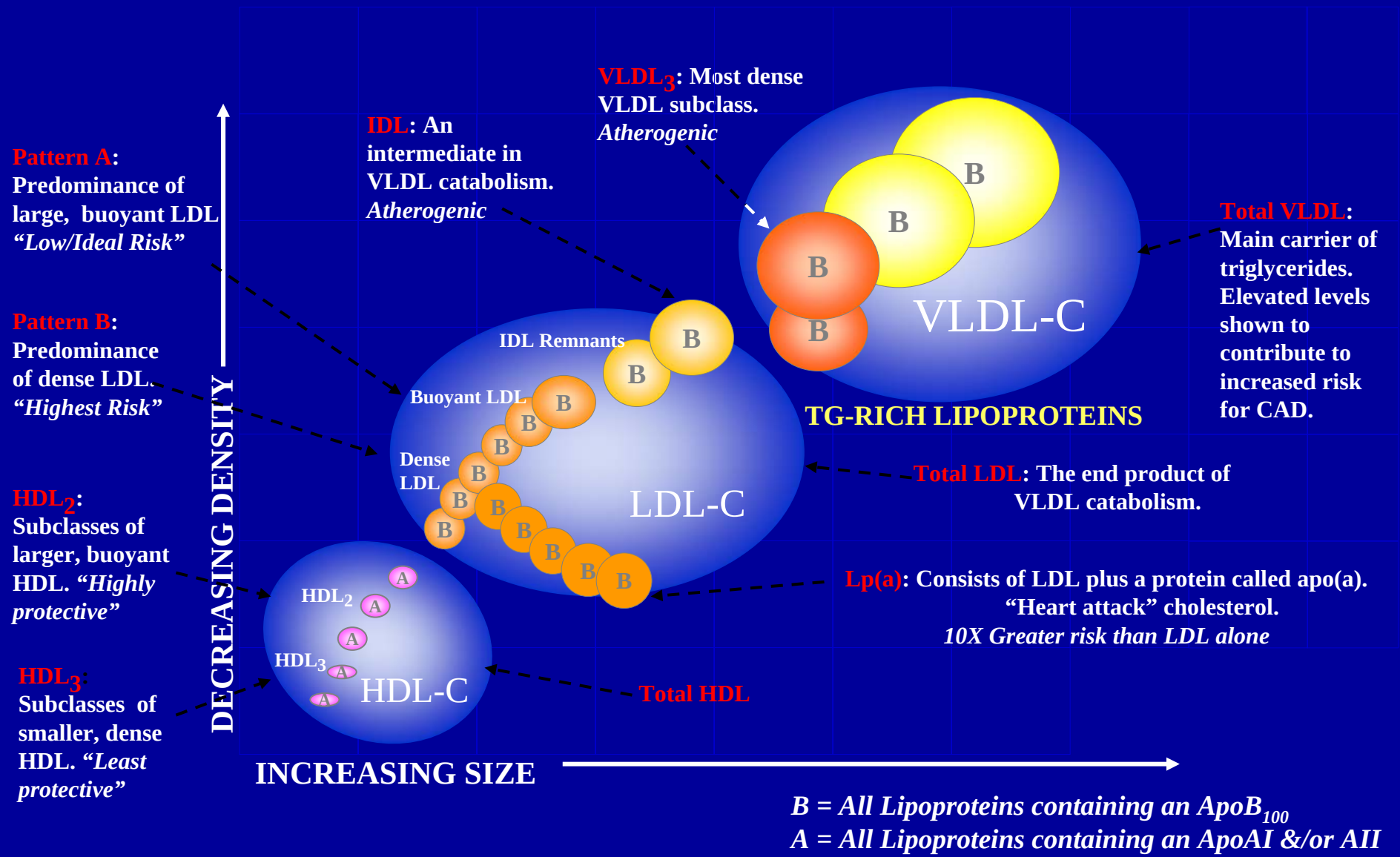
Kulkarni, Clin Lab Med 2006;26: 787-802

VAP Method Reproducibility

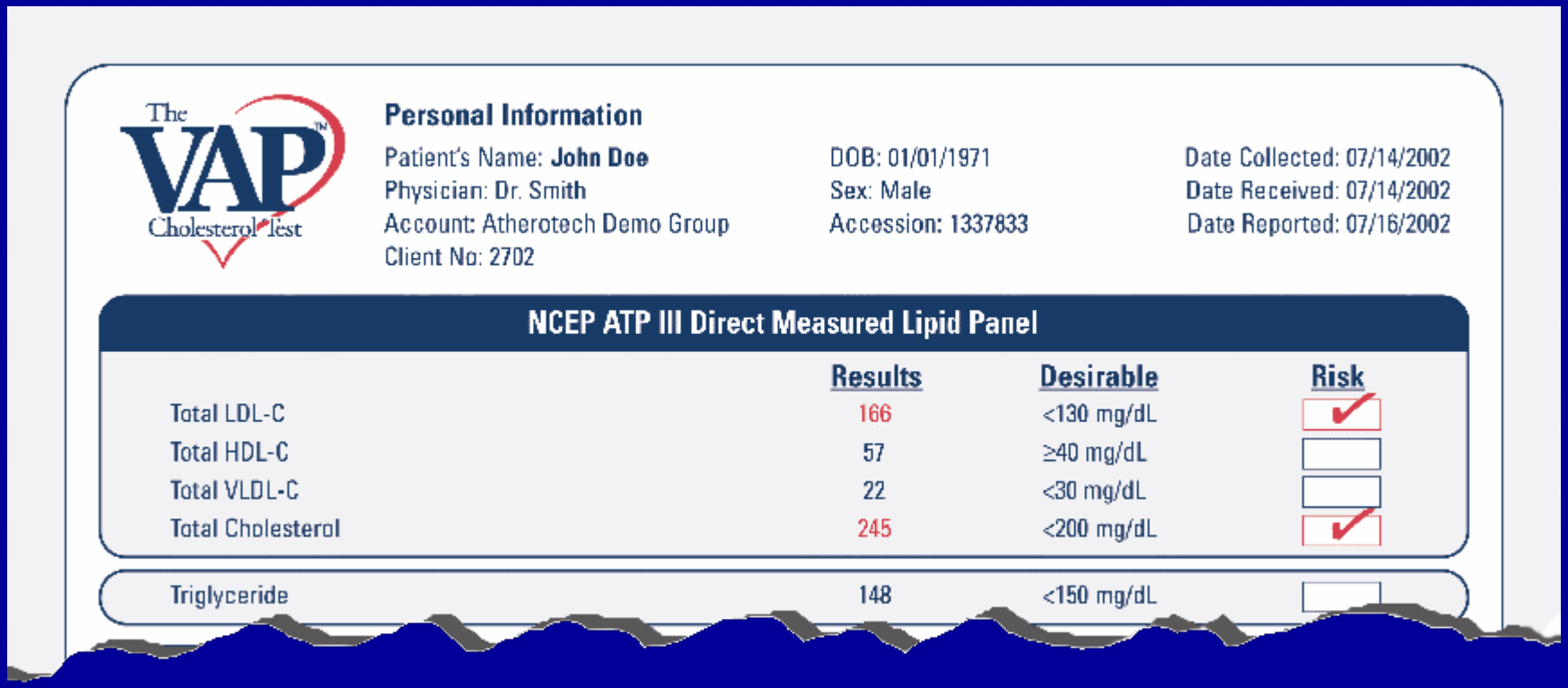
Lipoprotein	Within rotor % CV	Within day % CV	Between day % CV
TC	1.1	1.3	2.0
HDL	2.4	2.5	2.9
LDL	1.0	1.3	2.1
VLDL	1.6	3.0	2.8
IDL	4.1	6.3	8.2
Lp(a)	4.6	9.7	9.1
LDL-R	0.7	1.2	2.1
HDL_2	4.2	6.5	9.2
HDL_3	4.4	3.6	2.5

Kulkarni, Clin Lab Med 2006;26: 787-802

VAP Subclasses and Risk



VAP Direct-Measured Lipid Panel is accurate independent of Triglyceride values*



The VAP™ Cholesterol Test

Personal Information

Patient's Name: **John Doe**
Physician: Dr. Smith
Account: Atherotech Demo Group
Client No: 2702

DOB: 01/01/1971
Sex: Male
Accession: 1337833

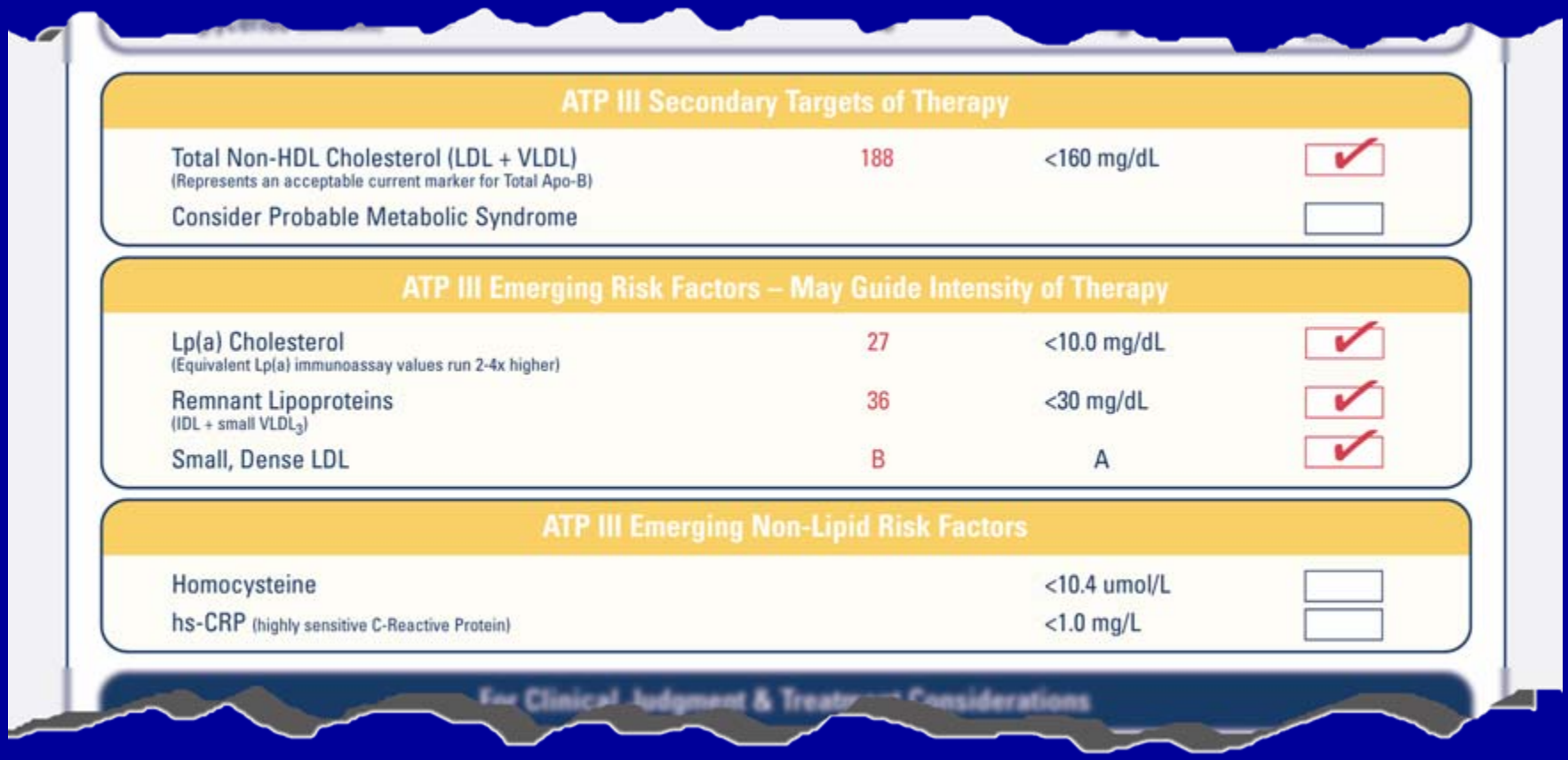
Date Collected: 07/14/2002
Date Received: 07/14/2002
Date Reported: 07/16/2002

NCEP ATP III Direct Measured Lipid Panel

	Results	Desirable	Risk
Total LDL-C	166	<130 mg/dL	✓
Total HDL-C	57	≥40 mg/dL	
Total VLDL-C	22	<30 mg/dL	
Total Cholesterol	245	<200 mg/dL	✓
Triglyceride	148	<150 mg/dL	

*Test report starts with basic standard lipid panel.

VAP Report follows with ATP-III Secondary and Emerging Risk Factors*



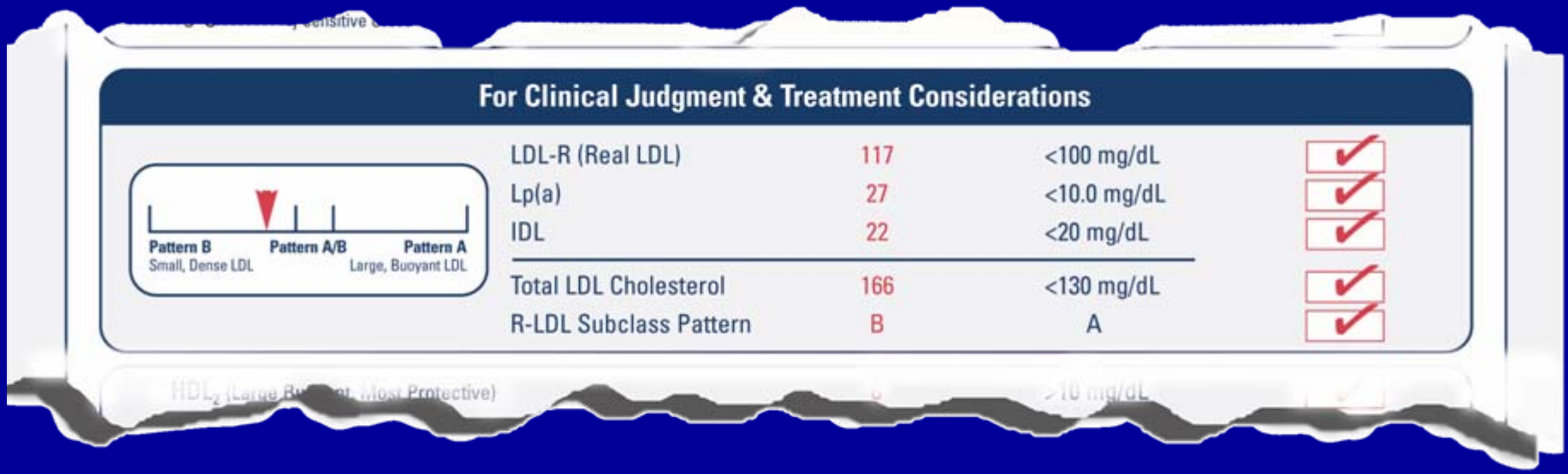
ATP III Secondary Targets of Therapy			
Total Non-HDL Cholesterol (LDL + VLDL) (Represents an acceptable current marker for Total Apo-B)	188	<160 mg/dL	✓
Consider Probable Metabolic Syndrome			☐

ATP III Emerging Risk Factors – May Guide Intensity of Therapy			
Lp(a) Cholesterol (Equivalent Lp(a) immunoassay values run 2-4x higher)	27	<10.0 mg/dL	✓
Remnant Lipoproteins (IDL + small $VLDL_3$)	36	<30 mg/dL	✓
Small, Dense LDL	B	A	✓

ATP III Emerging Non-Lipid Risk Factors		
Homocysteine	<10.4 umol/L	☐
hs-CRP (highly sensitive C-Reactive Protein)	<1.0 mg/L	☐

*Homocysteine and hs-CRP are optional add-on tests.

Initial Treatment Optimized by Stratifying LDL into its Three Treatment Components*



For Clinical Judgment & Treatment Considerations

LDL-R (Real LDL)	117	<100 mg/dL
Lp(a)	27	<10.0 mg/dL
IDL	22	<20 mg/dL
Total LDL Cholesterol	166	<130 mg/dL
R-LDL Subclass Pattern	B	A

*Note: all three components are listed as LDL-C on the standard lipid panel and are not treatment specific.

VAP Risk Stratification: HDL_2 and $VLDL_3$

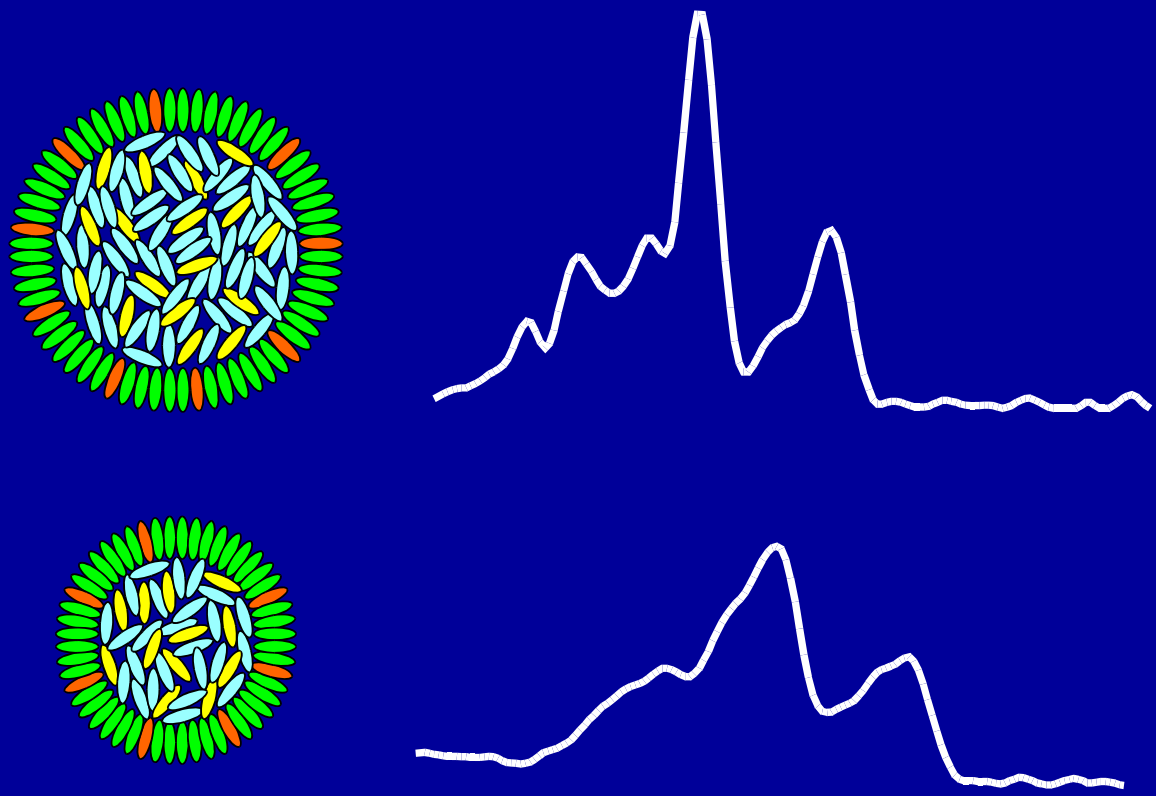
HDL_2 (Large Buoyant, Most Protective)	6	>10 mg/dL	✓
HDL_3 (Small Dense, Less Protective)	51	>30 mg/dL	
Total HDL Cholesterol	57	≥40 mg/dL	
$VLDL_{1+2}$ (Large Buoyant)	8	<20 mg/dL	
$VLDL_3$ (Small Remnant)	14	<10 mg/dL	✓
Total VLDL Cholesterol	22	<30 mg/dL	

Atherotech VAP Method

- Test and report consonant with NCEP ATP III
  - Emerging risk factors
  - Metabolic syndrome
- Total cholesterol calibrator traceable to CDC Lipid Reference Laboratory through the CDC-CRMLN
- BQ LDL-C and HDL-C used to evaluate and monitor VAP versus CDC-CRMLN laboratory
- Subfractions evaluated vs UC-prepared subfractions with a CDC-CRMLN laboratory

How NMR Lipoprotein Analysis Works

Lipoprotein subclasses of different size broadcast lipid NMR signals that are naturally distinguishable. The measured amplitudes of these signals provide subclass quantification.



Clin Lab 2002;48:171-80

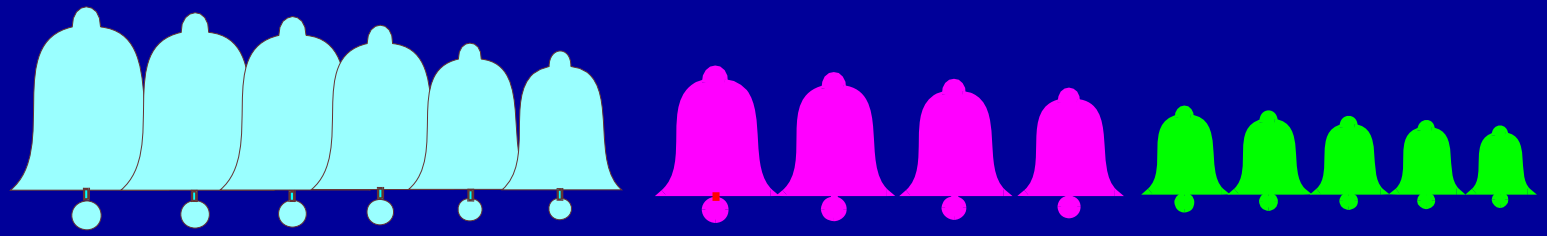
Liposcience NMR Method

- Requires small sample (200 µL) with no pretreatment and ~1 minute analysis time; therefore is practical to analyze much larger sample numbers in studies
- Lipoprotein subclasses are quantified without being fractionated
- Provides lipoprotein subclass particle concentration (nmol/L) and weighted average particle size (nm)

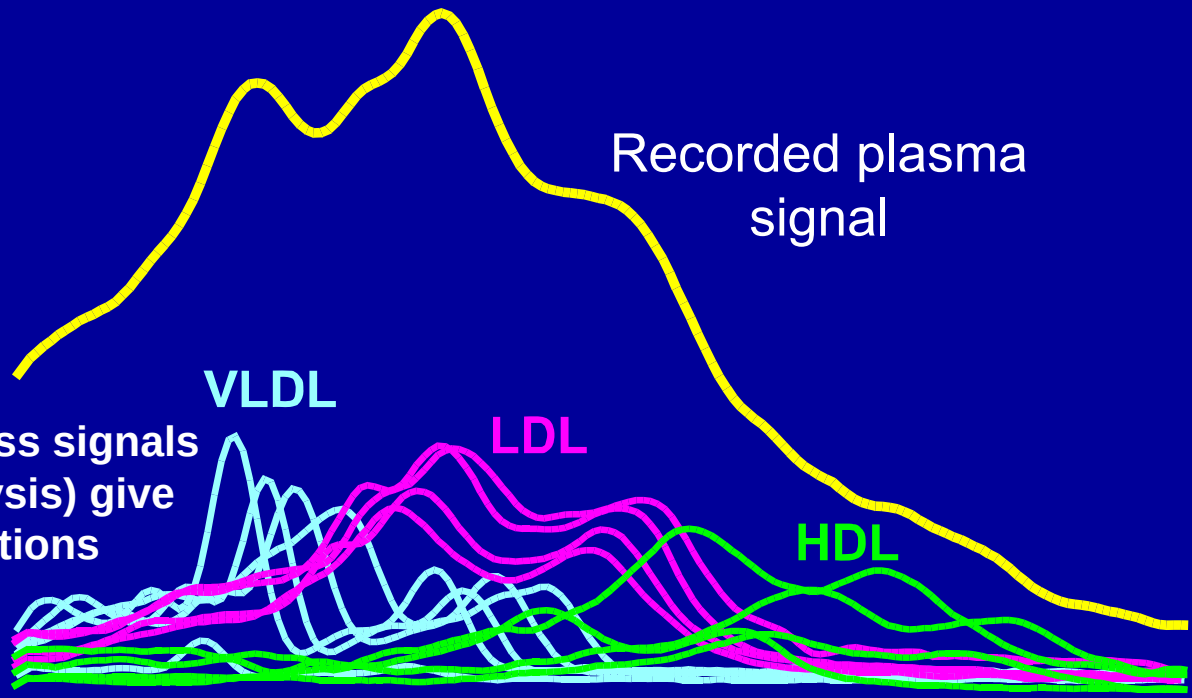
Liposcience NMR Method
Particle Number

- Determines particle numbers from terminal methyl group proton shifts by NMR in EDTA plasma or EDTA-spiked serum
- Peaks are quantified by deconvolution of signal envelope using a library of more than 30 methyl signals representing every spectrally distinct subclass likely to be encountered
- Method “calibrated” vs more than 30 isolated VLDL, LDL and HDL subfractions that were characterized for lipid composition (chemical analysis) and for size (electron microscopy or GGE)
- Neighboring subclasses are grouped empirically into a smaller number of categories (small, medium, large) so the summed amplitudes of these subpopulation signals provide acceptable measurement precision
- Constancy of the relationship between subclass signal amplitude and particle concentration is what gives NMR ability to quantify lipoprotein particles even with significant variation in cholesterol in subclass particles

Each lipoprotein subclass broadcasts a unique NMR “sound”



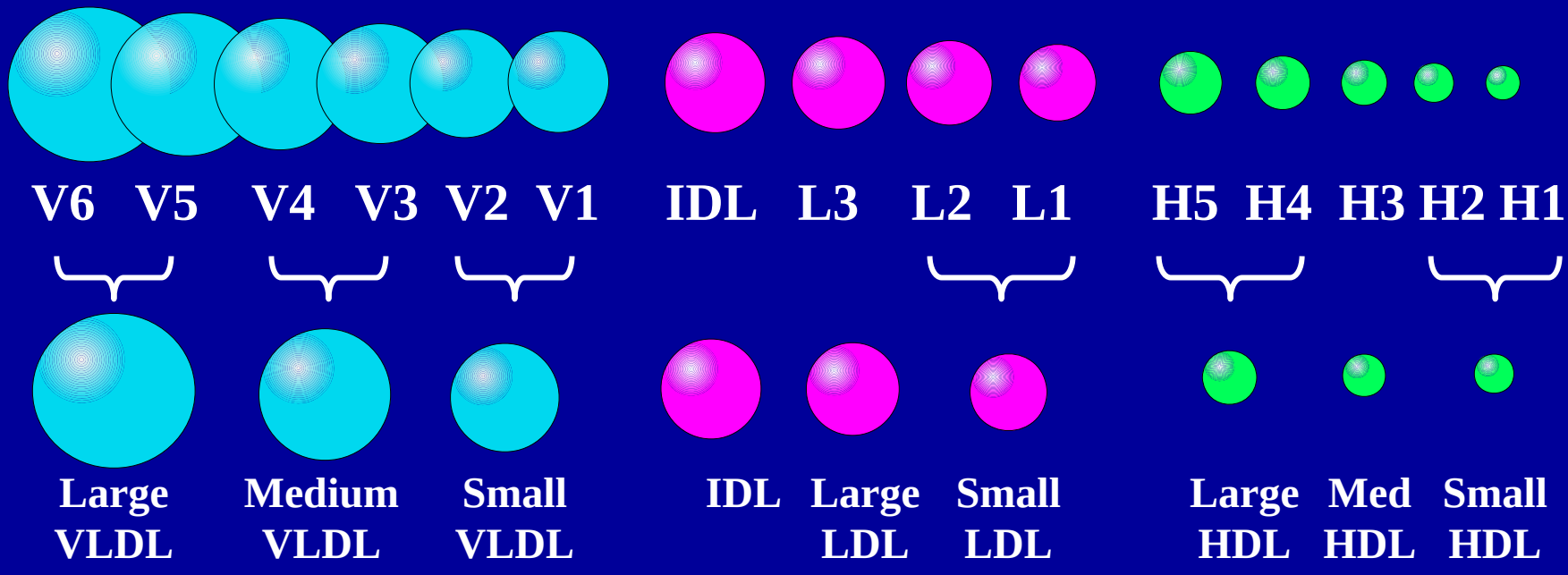
Simultaneous “ringing” of the plasma lipoproteins produces a recorded signal



Sizes of contributing subclass signals (derived by computer analysis) give the subclass concentrations

***Clin Lab* 2002;48:171-80**

NMR Lipoprotein Particle Number Analysis



Subclass Particle Numbers (nmol/L)

Weighted Ave VLDL, LDL, HDL Particle Sizes (nm)

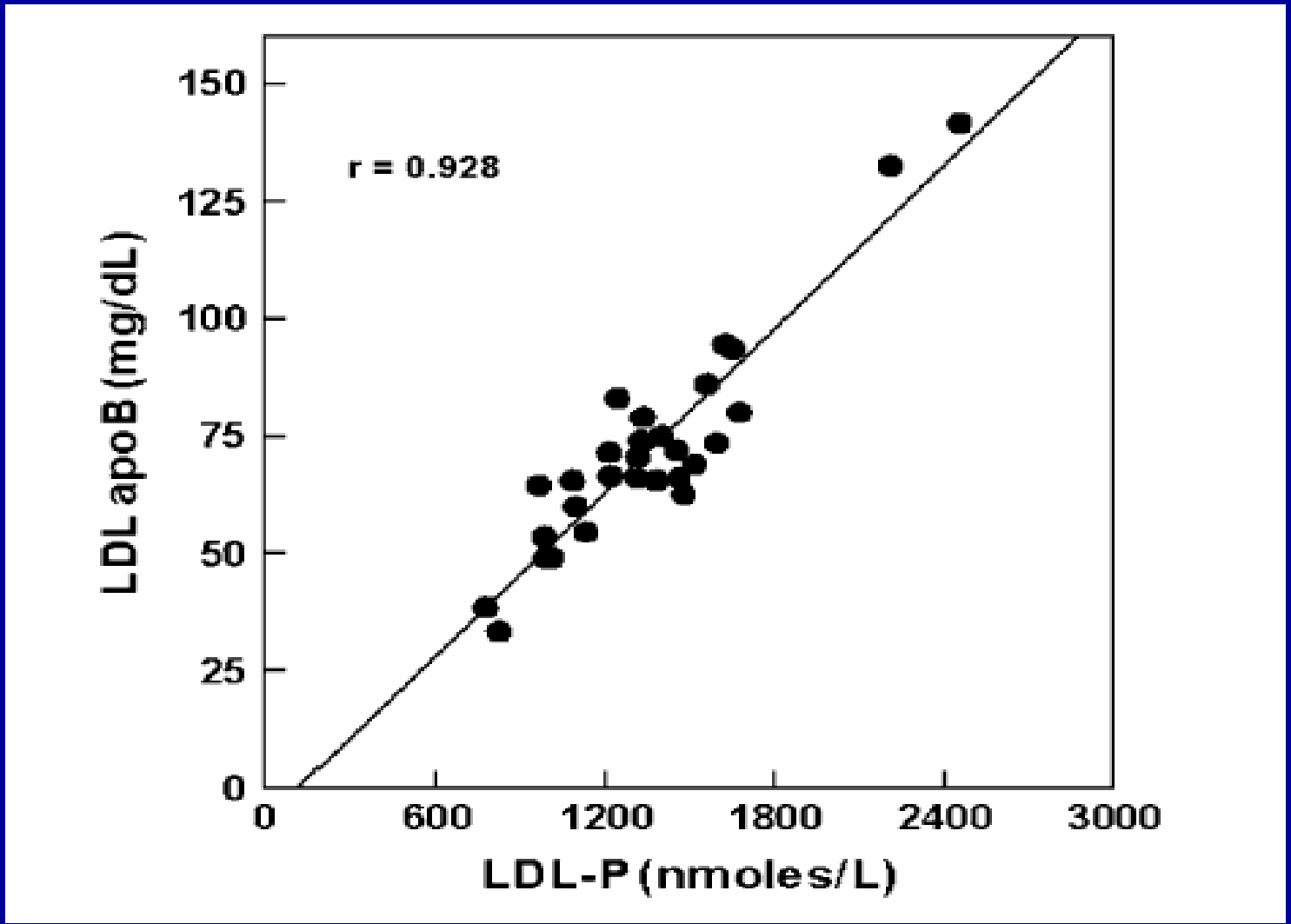
Clin Lab 2002;48:171-80

NMR Method Reproducibility

Table 2
Intra-assay and interassay measurement precision: pool A

NMR lipoprotein	Intra-assay precision[a]			Interassay precision[b]		
Parameter (Units)	Mean	SD	%CV	Mean	SD	%CV
VLDL (nmol/L)						
VLDL particles (total)	94.2	1.3	1.4	96.5	3.0	3.1
Large VLDL/chylomicrons	10.1	0.2	2.4	10.0	0.5	5.1
Medium VLDL	47.5	1.5	3.2	48.6	2.0	4.1
Small VLDL	36.6	2.0	5.4	37.9	2.7	7.1
LDL (nmol/L)						
LDL particles (total)	1876	44.3	2.4	1913	39.4	2.1
IDL	94	9.7	10.3	89	11.6	13.1
Large LDL	509	32.4	6.4	522	33.1	6.3
Small LDL (total)	1273	70.8	5.6	1301	60.8	4.7
Medium small LDL	233	12.7	5.4	238	10.9	4.6
Very small LDL	1039	59.4	5.7	1063	50.8	4.8
HDL (μmol/L)						
HDL particles (total)	33.2	0.4	1.2	33.6	0.5	1.5
Large HDL	7.7	0.4	5.6	7.6	0.4	5.9
Medium HDL	2.5	1.0	**	2.8	0.9	**
Small HDL	23.0	0.9	4.1	23.1	0.8	3.7
Mean particle sizes (nm)						
VLDL size	63.9	0.5	0.8	63.1	1.1	1.8
LDL size	20.53	0.10	0.5	20.54	0.09	0.4
HDL size	8.57	0.04	0.5	8.56	0.05	0.6
Calculated lipids (mg/dL)						
Total triglycerides	229	1.3	0.6	229	2.4	1.1
VLDL triglycerides	180	0.7	0.4	180	2.7	1.5
HDL cholesterol	46	0.5	1.1	46	0.8	1.8

Liposcience NMR Method

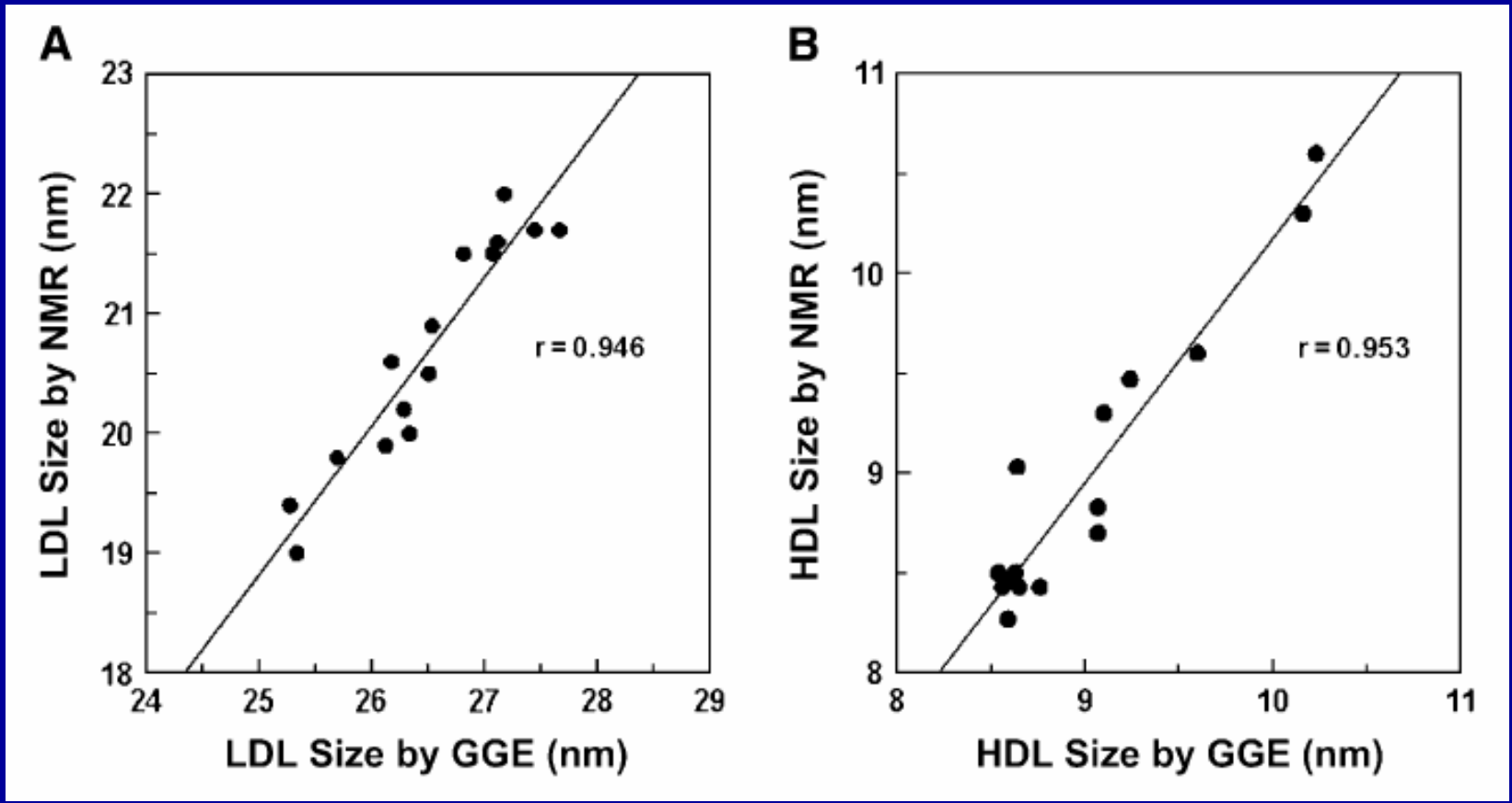


Liposcience NMR Method
Particle Size

- Weighted average VLDL, LDL and HDL particle sizes are calculated from subclass concentrations
- Calculation uses (assumes) fundamental relationship between particle diameter of lipoproteins and total core lipid (cholesterol ester + triglyceride) content using a spherical model
- Method “calibrated” versus more than 30 isolated VLDL, LDL and HDL subfractions that were characterized for size by electron microscopy or size standards on GGE
- The 5 to 6 nm smaller diameters determined for LDL by NMR versus GGE are because of the early in development choice of electron microscopy-based size calibration of LDL
- NMR HDL sizes closely agree with GGE

NMR Method

Particle size correlates with GGE



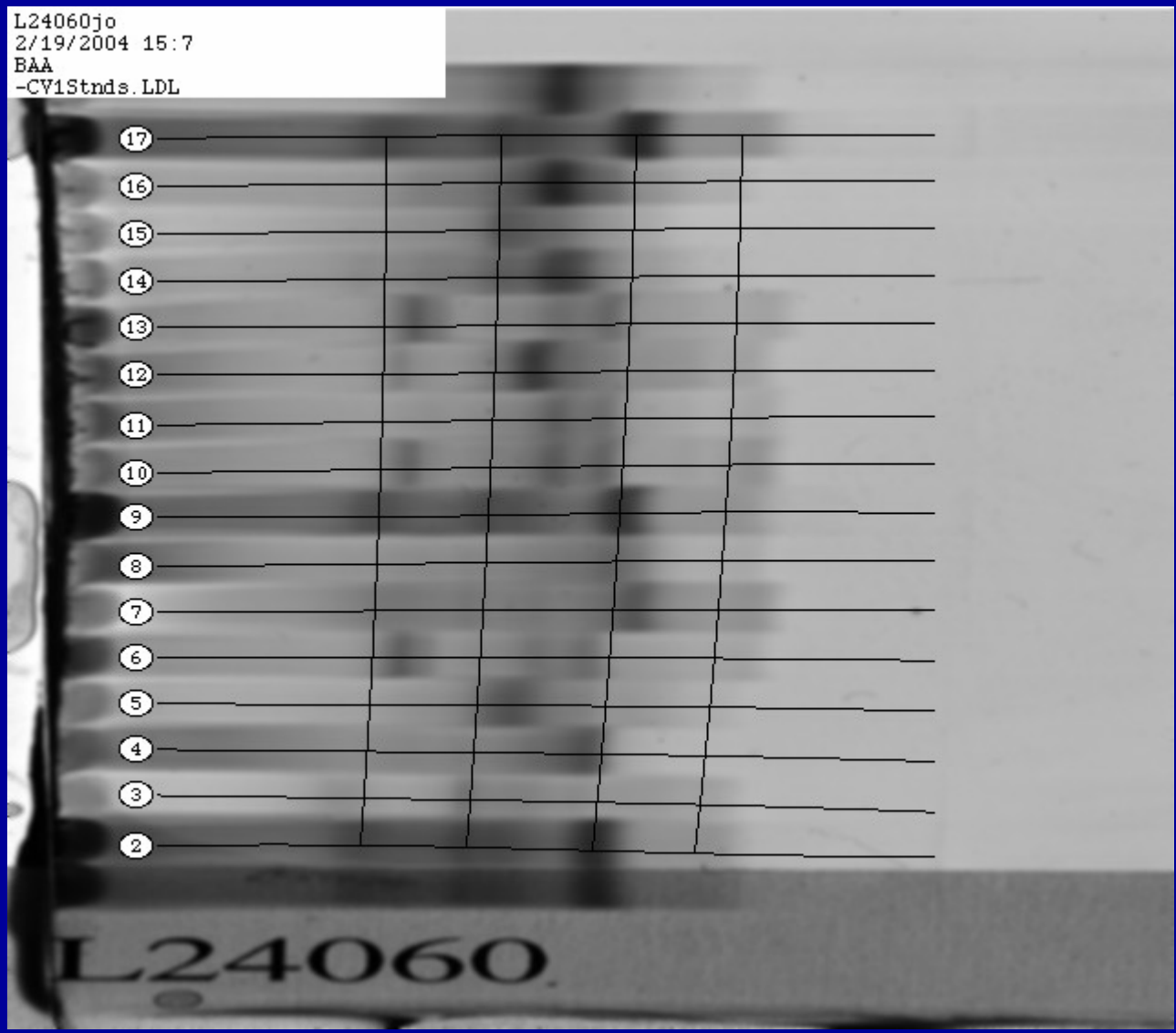
Liposcience NMR Method

- Small sample requirement (200 µL) with no pretreatment and ~1 minute analysis time means it is practical to analyze much larger sample numbers in studies, not subsets
- More than 200 clinical studies completed and 150 are ongoing. NMR lipoprotein particle data reported in more than 110 publications
- Result: NMR method dominates the database of recent clinical studies involving measurement of lipoprotein subclasses

Berkeley HeartLab Method

- Segmented non-linear gradient gel electrophoresis; two gel formulations available for maximum resolution of either LDLs or HDLs
- Gel separations similar to research grade gels used in studies
- Separation based on size and charge
- Sizes and fractions closely linked to original analytical ultracentrifugation method
- Staining with Oil red O and densitometric scanning gives a profile with relative cholesterol concentration in each subfraction
- Overlap of subfractions in the profile requires use of proprietary software for mathematical “deconvolution” to give % area for each subfraction
- Provides a reproducible LDL size that is based on standards incorporated into the gels
- Subfraction information in the Patient Progress Summary is only part of an overall report of CVD risk factors

Berkeley HeartLab Method



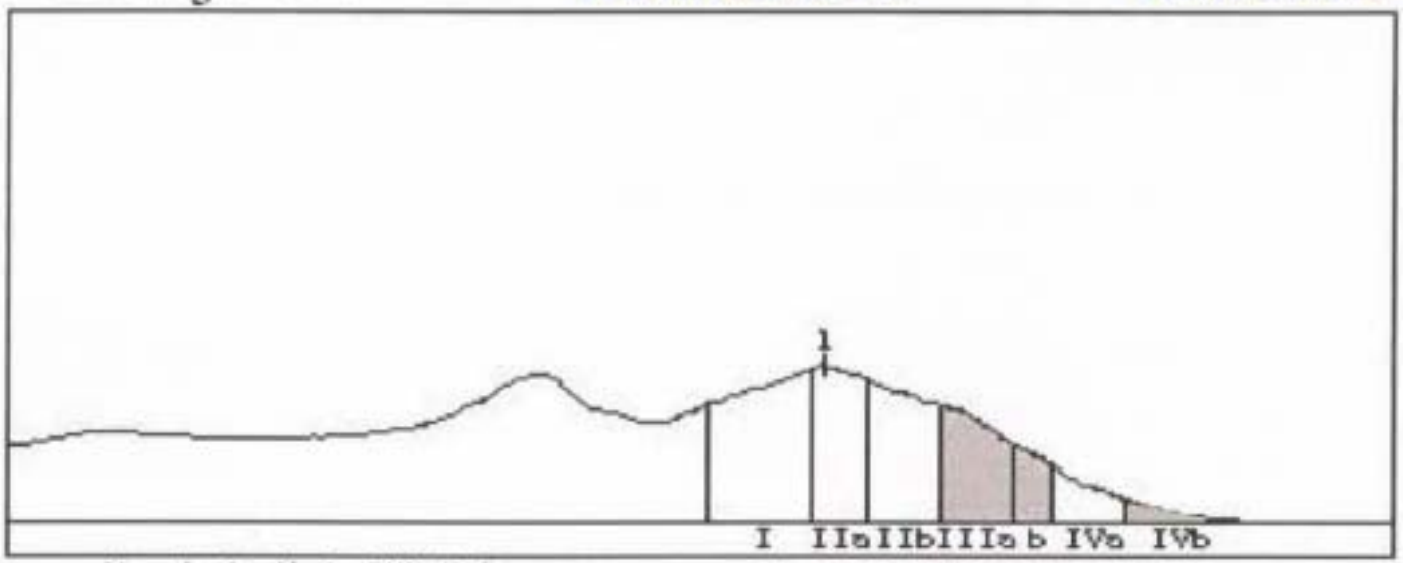
Berkeley HeartLab Method

LDL-S_3GGE®

LDL IIIa+b: 23.6%

LDL IVb: 3.0%

LDL I (%)	28.1
LDL IIa (%)	18.5
LDL IIb (%)	20.2
LDL IIIa (%)	17.1
LDL IIIb (%)	6.5
LDL IVa (%)	6.7
LDL IVb (%)	3.0



	Normal	Inter-mediate	At Risk	Last Visit	Alert Value	BHL Goal	Reference Range
LDL IIIa+b (%)			23.6	18.70	>=20	<=15	11.2 - 39.2*
LDL IVb (%)	3.0			5.9	>=10	<=5	1.2 - 8.7*

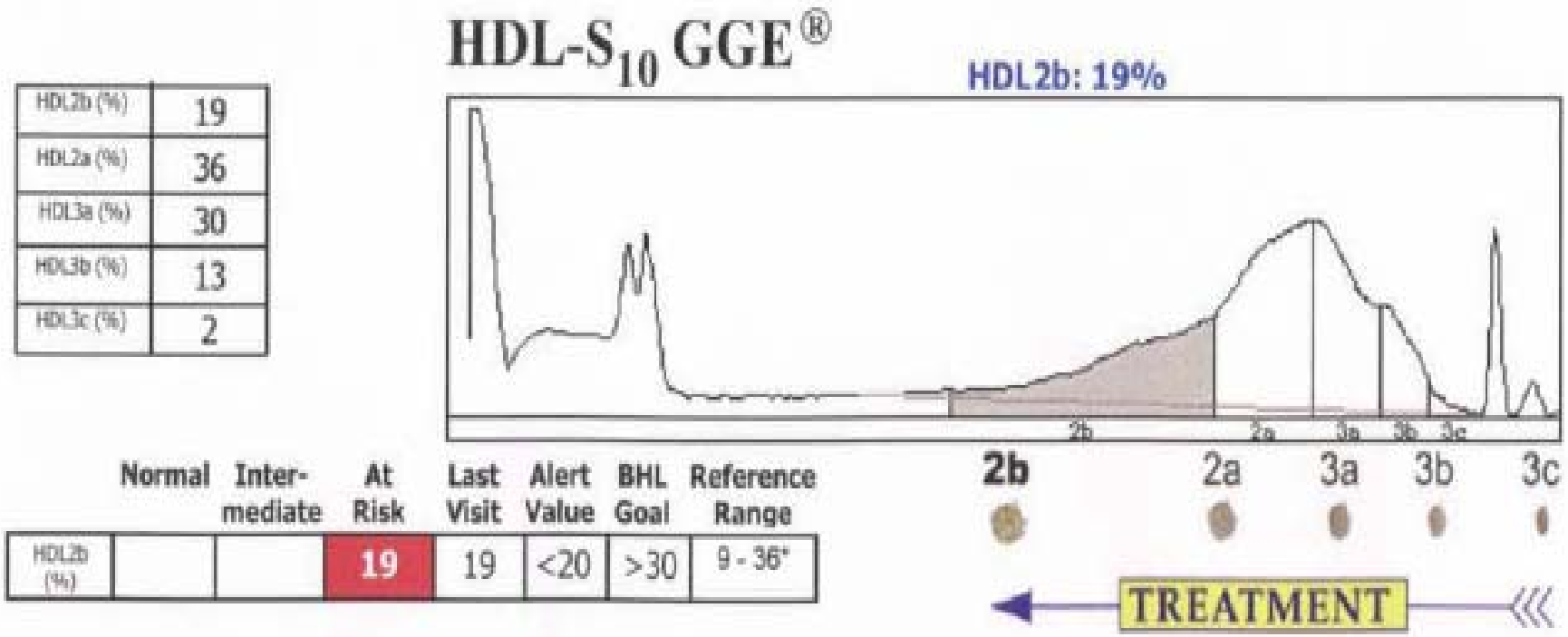
TREATMENT

Atherogenic Subclass Quantitation

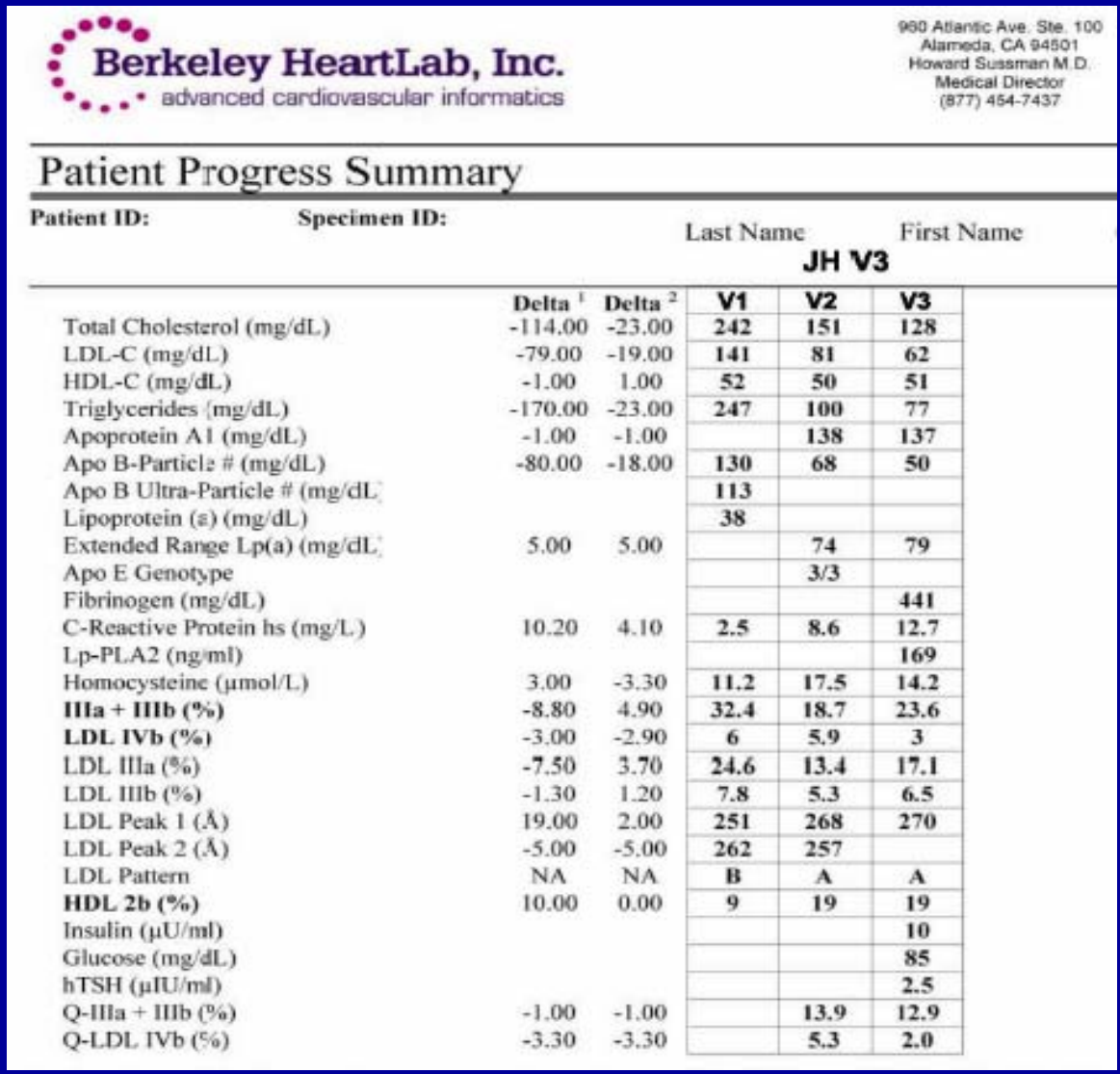
	Value	Last Visit	Reference Range*
LDL IIIa+b (mg/dL)	12.9	13.9	12.0 - 32.1
LDL IVb (mb/dL)	2.0	5.3	1.5 - 11.2

	Pattern A Large LDL 263.5 - 285	Pattern I Int. LDL 257.5 - 263.4	Pattern B Small LDL 220 - 257.4
LDL Peak 1 (Å)	270		
LDL Peak 2 (Å)			

Berkeley HeartLab Method



Berkeley HeartLab Method



Berkeley HeartLab, Inc.
advanced cardiovascular informatics

960 Atlantic Ave. Ste. 100
Alameda, CA 94501
Howard Sussman M.D.
Medical Director
(877) 454-7437

Patient Progress Summary

Patient ID: **Specimen ID:** Last Name First Name

JH V3

	Delta [1]	Delta [2]	V1	V2	V3
Total Cholesterol (mg/dL)	-114.00	-23.00	242	151	128
LDL-C (mg/dL)	-79.00	-19.00	141	81	62
HDL-C (mg/dL)	-1.00	1.00	52	50	51
Triglycerides (mg/dL)	-170.00	-23.00	247	100	77
Apoprotein A1 (mg/dL)	-1.00	-1.00		138	137
Apo B-Particle # (mg/dL)	-80.00	-18.00	130	68	50
Apo B Ultra-Particle # (mg/dL)			113		
Lipoprotein (a) (mg/dL)			38		
Extended Range Lp(a) (mg/dL)	5.00	5.00		74	79
Apo E Genotype				3/3	
Fibrinogen (mg/dL)					441
C-Reactive Protein hs (mg/L)	10.20	4.10	2.5	8.6	12.7
Lp-PLA2 (ng/ml)					169
Homocysteine (µmol/L)	3.00	-3.30	11.2	17.5	14.2
IIIa + IIIb (%)	-8.80	4.90	32.4	18.7	23.6
LDL IVb (%)	-3.00	-2.90	6	5.9	3
LDL IIIa (%)	-7.50	3.70	24.6	13.4	17.1
LDL IIIb (%)	-1.30	1.20	7.8	5.3	6.5
LDL Peak 1 (Å)	19.00	2.00	251	268	270
LDL Peak 2 (Å)	-5.00	-5.00	262	257	
LDL Pattern	NA	NA	B	A	A
HDL 2b (%)	10.00	0.00	9	19	19
Insulin (µU/ml)					10
Glucose (mg/dL)					85
hTSH (µIU/ml)					2.5
Q-IIIa + IIIb (%)	-1.00	-1.00		13.9	12.9
Q-LDL IVb (%)	-3.30	-3.30		5.3	2.0

Berkeley HeartLab Method

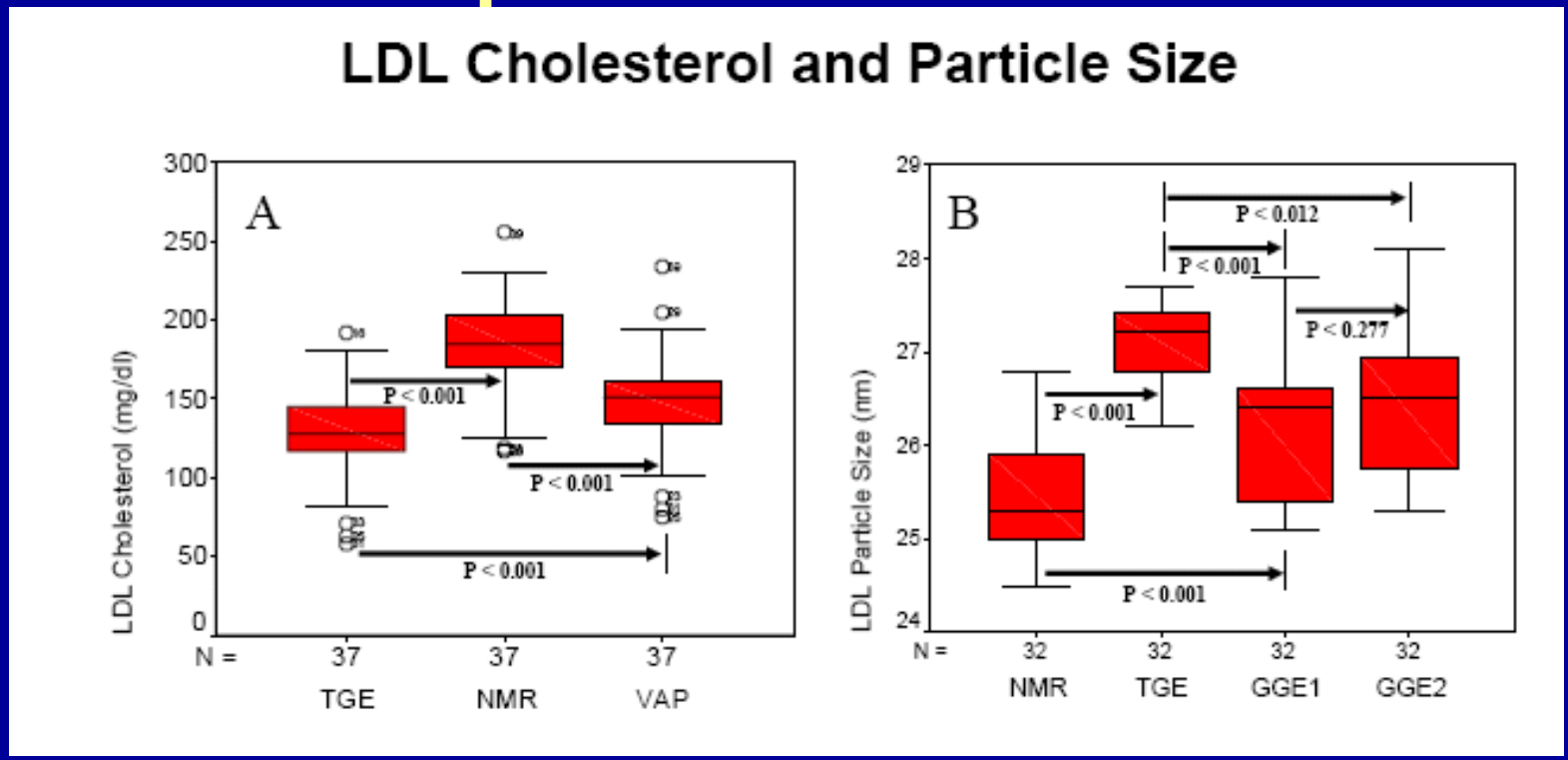
- Provides personalized information to patient at 4myheart.com
- Lab reports show progress over time
- Diet
- Exercise
- Medications

Comparison of LDL Subclass Methods

- Assessed differences between leading technologies for LDL subclasses; GGE, VAP, NMR and TGE
- Collected 4 simultaneous samples from 40 subjects and sent to labs performing each assay Berkley Heart Lab, Atherotech, LipoScience and Kronos Science (Quantimetrix) Lipoprint respectively
- LDL subfractions evaluated for particle size and LDL phenotype (pattern A or B)
- Complete agreement with respect to LDL subclass pheotypes in 8% (3 of 40) subjects

Ensign W et al Clin Chem 2006;52:1722-7

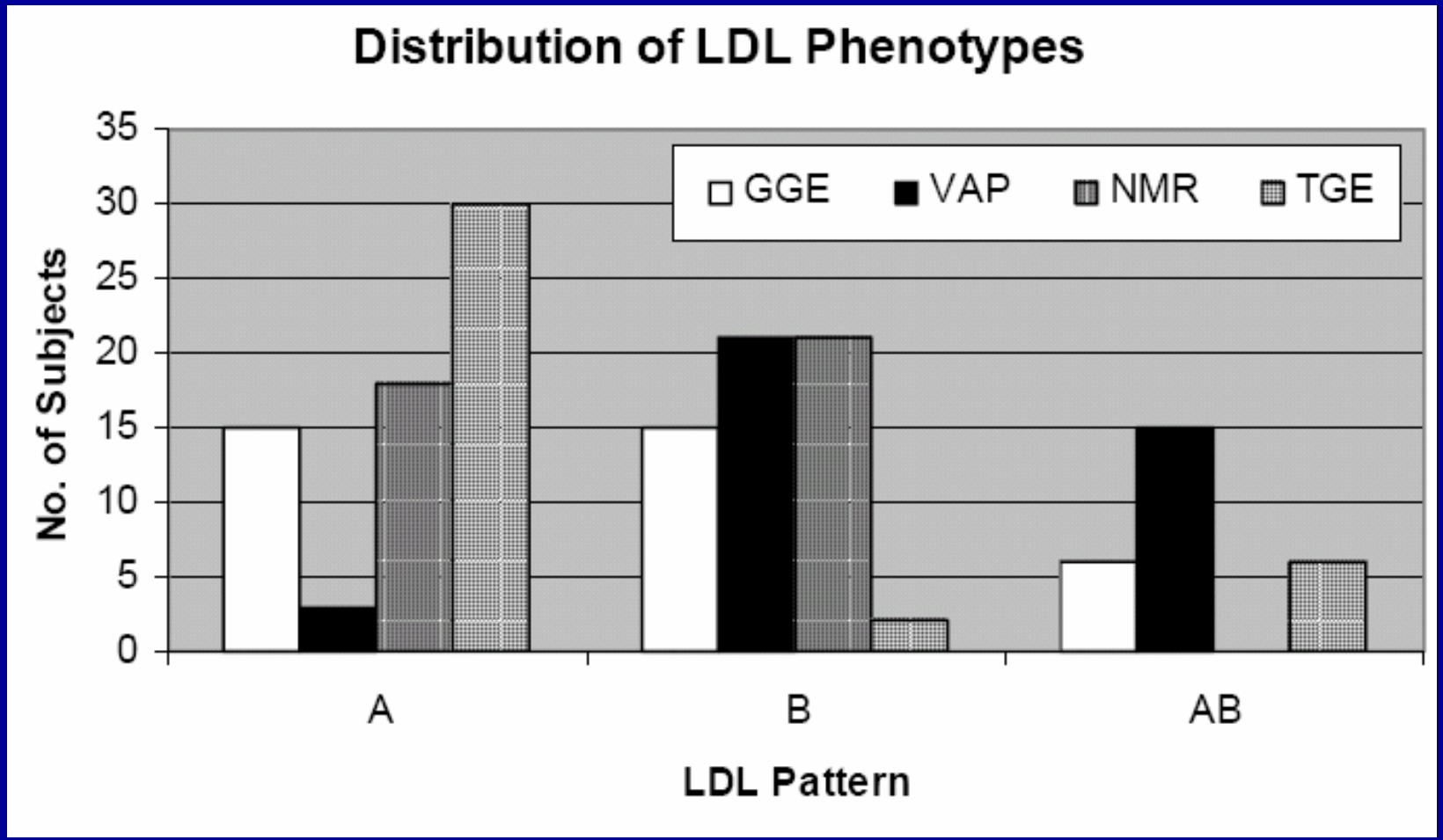
Distribution of (A) LDLc Estimates for 3 methods where LDLc content estimated; (B) LDL particle sizes for main LDL fraction used for patient classification



A B

Ensign W et al Clin Chem 2006;52:1722-7

Number of subjects in each of 3 LDL pattern phenotypes (A, B or A/B)



Ensign W et al Clin Chem 2006;52:1722-7

Comparison study Conclusions

- Measurement of subclasses not standardized
- Laboratories provide less than optimal measurement of LDLC
- Predicting pattern A or B can be done as reliably using triglyceride cut point of < or > 150 mg/dL
- “Variation among currently available methods renders them unreliable and limits their clinical usefulness”

Ensign W et al Clin Chem 2006;52:1722-7

Subclass Standardization Conclusions

- Method-dependent results make it difficult to compare results among studies. Each method is probably defining a different sub-population of lipoproteins as they take a different slice of the continuum of properties that do not correlate perfectly.
- The choice of the best reference method to standardize lipoprotein subfraction methods is not obvious. A method based on density gradient ultracentrifugation for example may require arbitrary modifications of a method separating bases on size in order to be standardized.
- Direct comparison studies among methods could identify the basis for potential differences among methods. The commonly defined analytes subfractions should be defined. It may not be possible to use standardization harmonize the methods because the measurement principles are so different
- Standardization of defined sub-populations of atherogenic and anti-atherogenic lipoprotein particle concentrations should be the goal.

Slide Acknowledgments

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• Berkeley HeartLab	
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• Quantimetrix	