HDL3-P Activation Assay ™

Patent Pending

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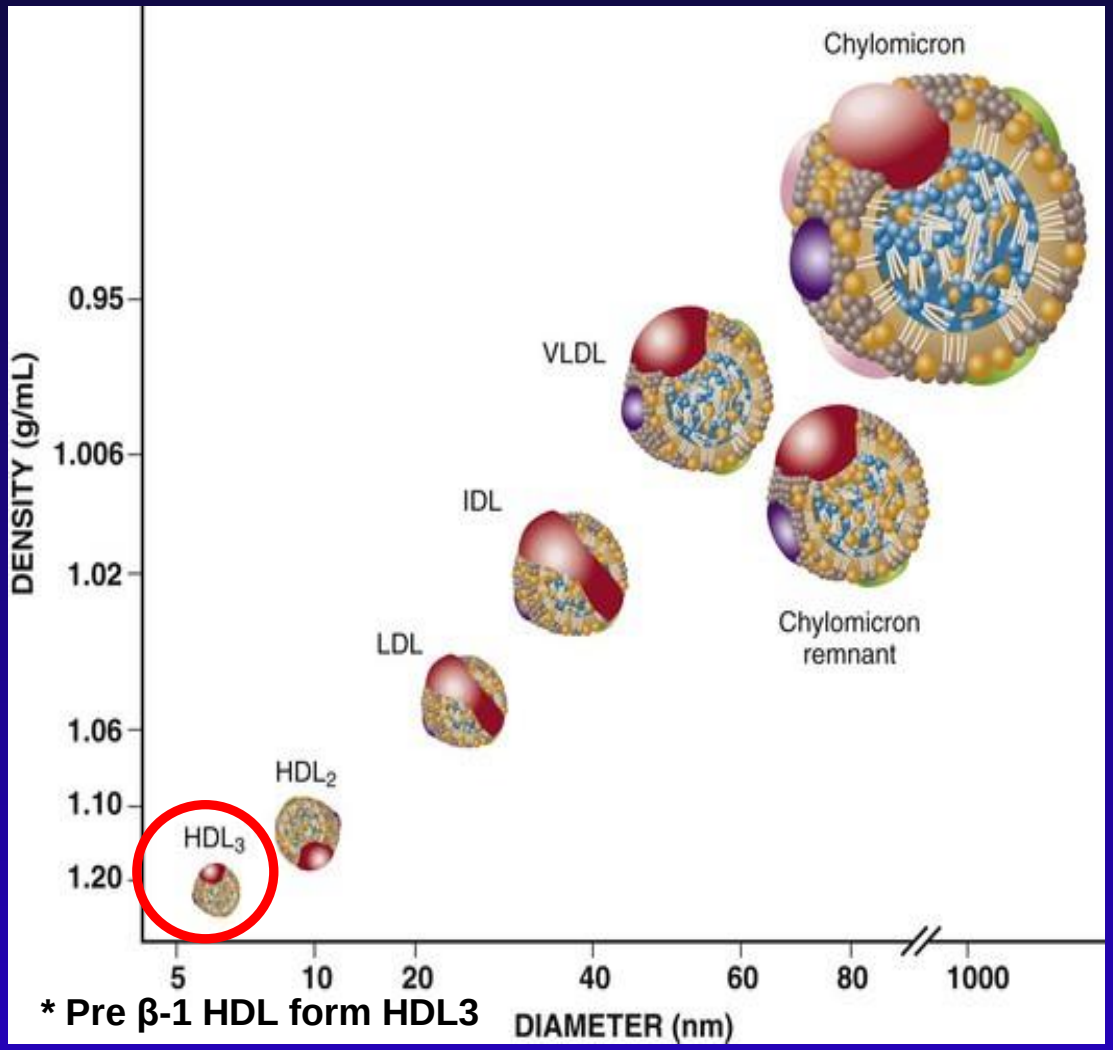
LipidRisk, LLC

HDL3-P The Risk Factor

What is HDL3-P?



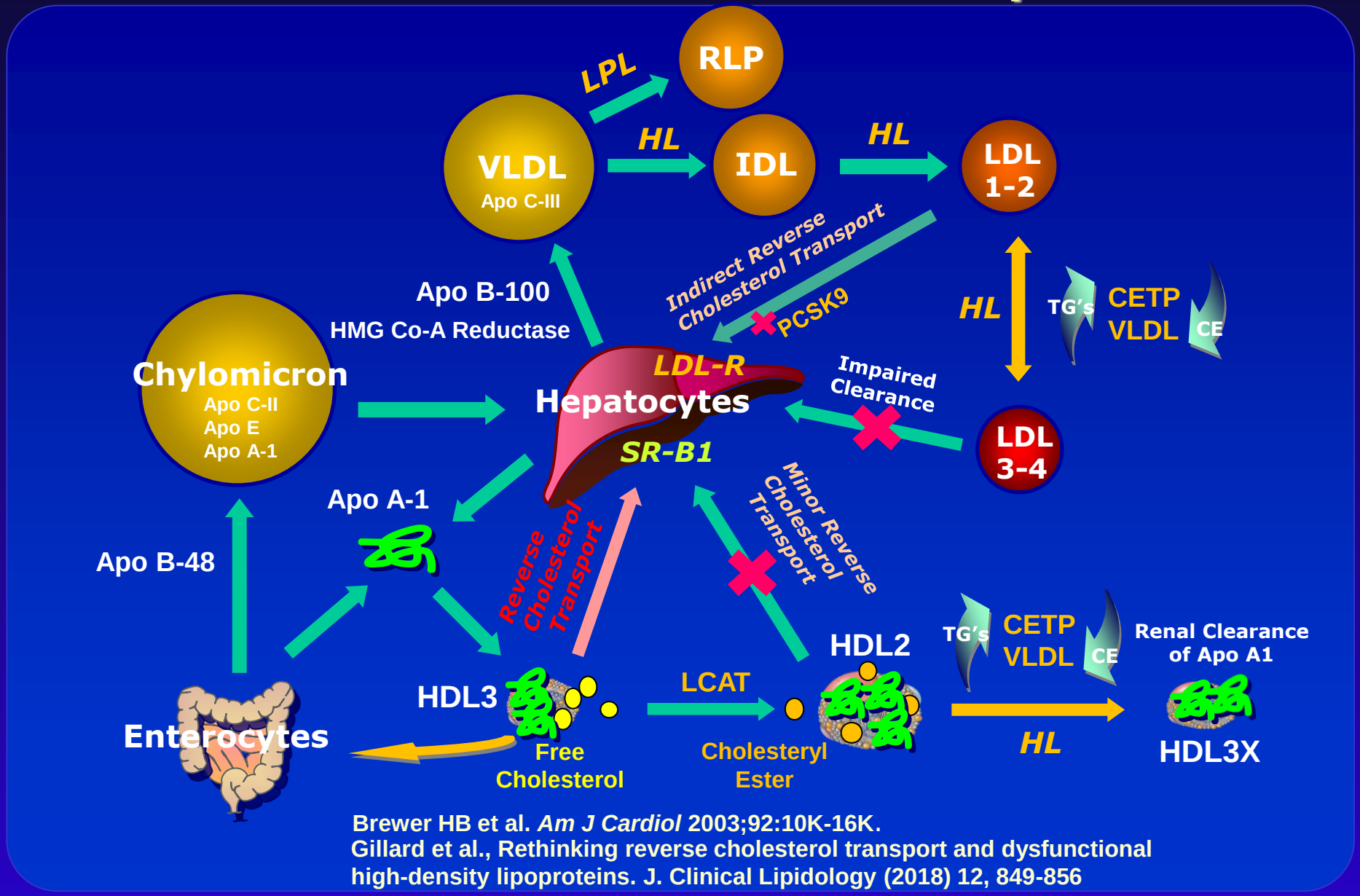
* Pre β-1 HDL form HDL3

HDL3-P, Very Small Cholesterol Containing Lipoprotein Particle*

Apo A-1 from Hepatocytes and Enterocytes Build HDL3's

HDL3-P Gathers Free Cholesterol for Reverse Cholesterol Transport and can evolve into HDL2b

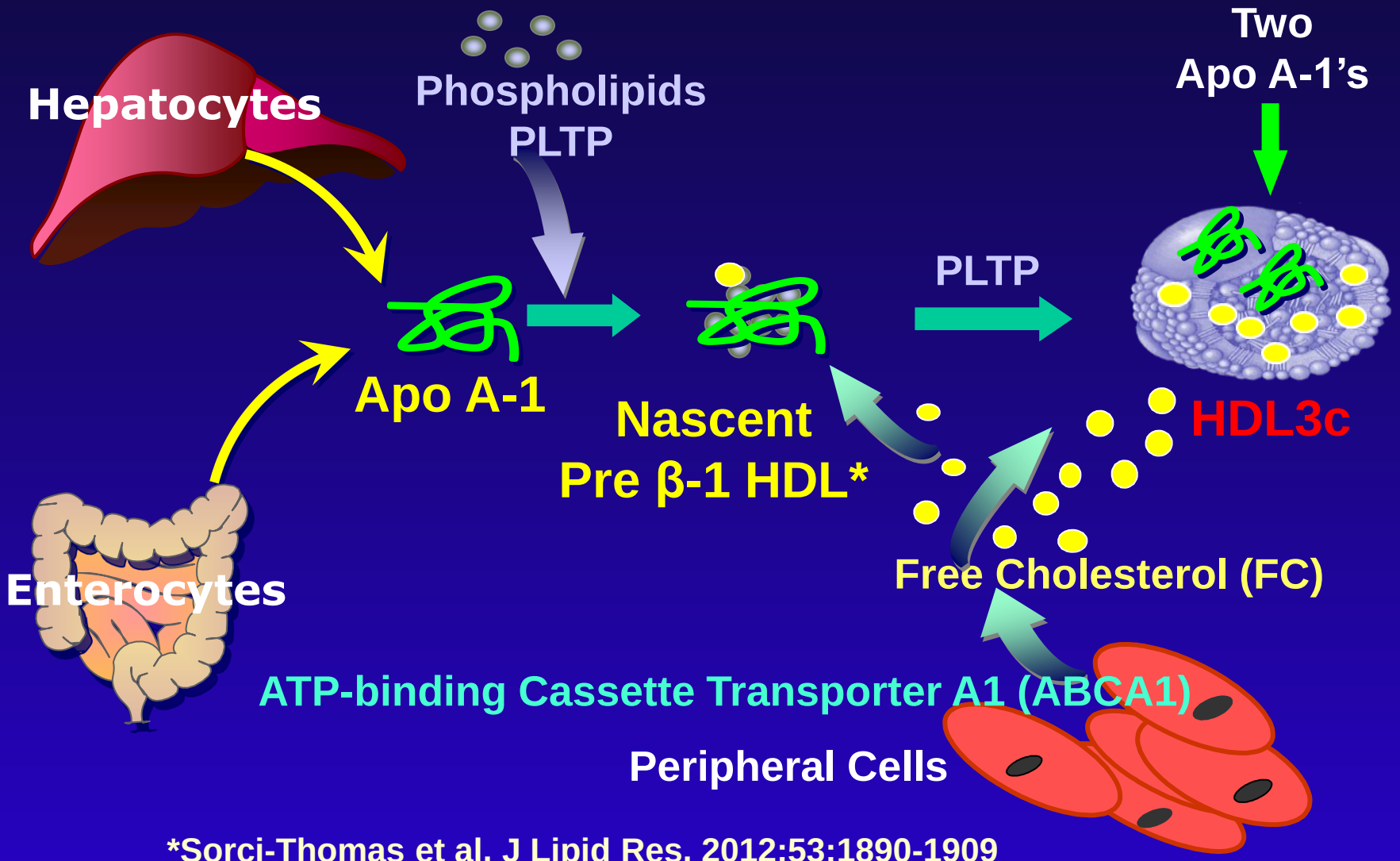
Chylomicron, VLDL, LDL & HDL Metabolism and Reverse Cholesterol Transport



Brewer HB et al. *Am J Cardiol* 2003;92:10K-16K.

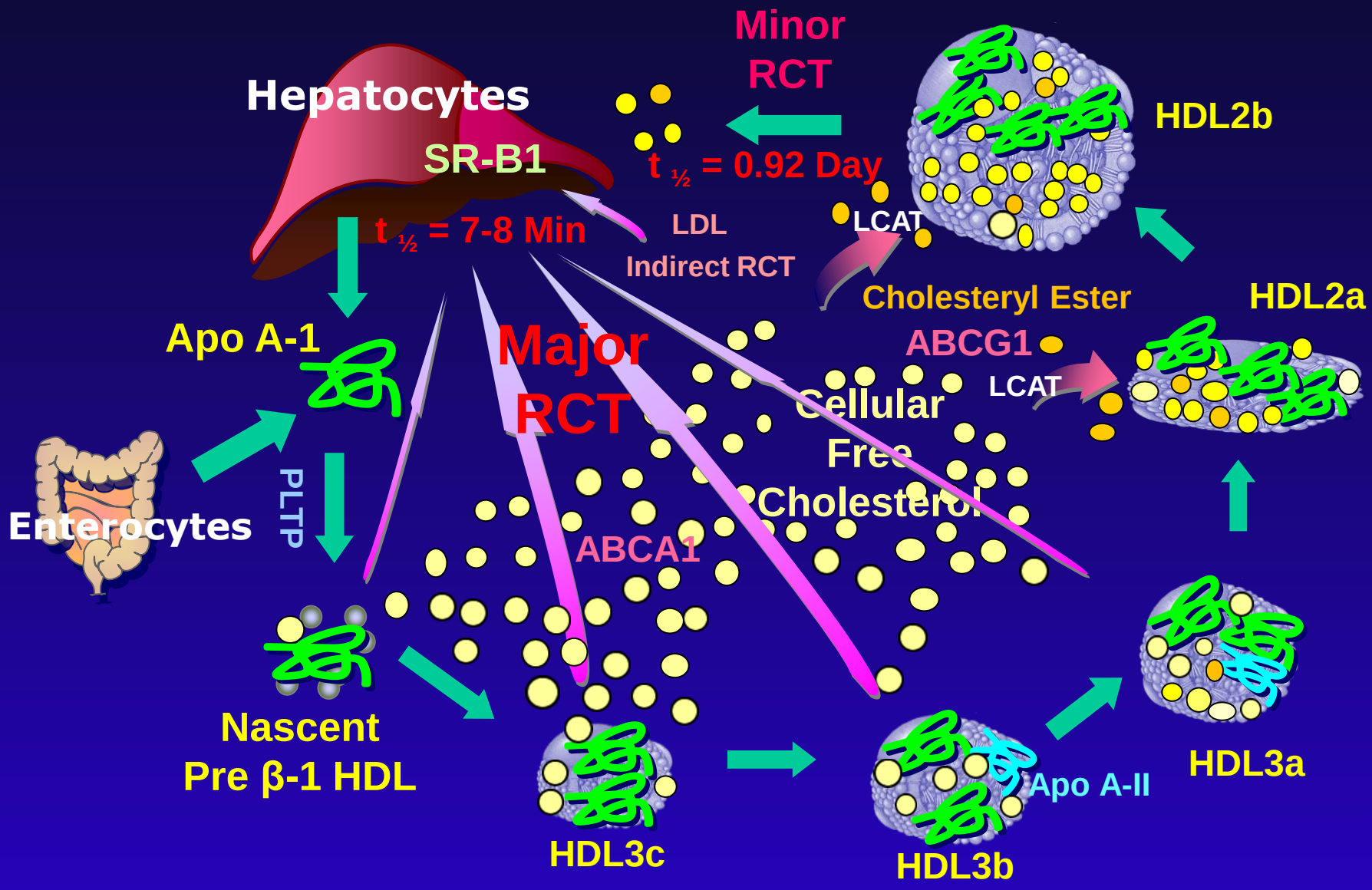
Gillard et al., Rethinking reverse cholesterol transport and dysfunctional high-density lipoproteins. J. Clinical Lipidology (2018) 12, 849-856

Smallest HDL - HDL3c Formation



*Sorci-Thomas et al. J Lipid Res. 2012;53:1890-1909

Reverse Cholesterol Transport (RCT)



Gillard et al., Rethinking reverse cholesterol transport and dysfunctional high-density lipoproteins. J. Clinical Lipidology (2018) 12, 849-856

HDL3 – A Powerful Antioxidant

HDL is Known to Have Antioxidant and Anti-inflammatory Properties

Properties are Most Likely From the Association with

Paraoxonase-1 (PON1)
Protecting LDL from Oxidation

Handrean Soran, Jonathan D.Schofield and Paul N.Durrington,
Frontiers in Pharmacology (2005) *6:222*

HDL3 is a More Powerful Antioxidant than HDL2

Yoshhikawa M, Sakuma N, Hibino T, Sato T, Fujinami T,
Clinical Biochemistry (1997) Apr;30(3):221-5

Kontush A, Chapman MJ, Nat Clin Pract Cardiovasc Med. (2006) Mar;3(3):144-53

HDL3 is a Potent Protector of LDL

Reduces Lipid Hydroperoxides to Hydroxides

HDL3 is a Future Therapeutic Target

Brites F, Martin M, Guillas I, Kontush A, BBA Clinical 8 (2017) 66–77
Zerrad-Saadii et al., Arterioscler Thromb Vasc Biol. (2009)29:2169-2175

HDL Lipoprotein Subgroups

Approximate Densities and Sizes for Various Lipoprotein Testing Technologies:

Ultracentrifugation (LPP–P, VAP-C)			2-Dimensional		
Electrophoresis-%	NMR-P	Ion Mobility-P	Electrophoresis-A	Density (g/ml)	Size (nm)
Nascent HDL			Pre β-1 HDL		5.6
HDL3a, 3b, 3c	Sm – Med	Small	α-3, α-4	1.125 – 1. 21	7 – 8.8
HDL2a	Large	Small	α-2	1.100 – 1.125	8.8 – 10
HDL2b	Large	Large	α-1	1.063 – 1.100	10 – 14.5

(-P) is measured as particles, (-C) is measured as cholesterol,
(-%) is measured as a percent, (-A) is measured as Apo A-1

HDL3-P - An Independent Risk Factor

Pearson Correlation Coefficients for 600 Random Fasting Specimens Measured with LPP

	Subgroup or Analyte	r =
HDL3-P	Triglycerides	-0.10
HDL3-P	Total LDL-P	0.00
HDL3-P	LDL III-P	0.18
HDL3-P	Total HDL-P	0.48
HDL3-P	HDL2b-P	-0.23
HDL3-P	HDL2a-P	0.15
HDL3-P	HDL-C	-0.09
HDL3-P	hs-CRP	-0.15
HDL3-P	HA1c	-0.10
Apo A-1	HDL2b-P	0.86
Apo A-1	HDL2a-P	0.26
Apo A-1	HDL3-P	-0.11

New HDL3-P Activation Assay

Assay to Measure the Activation and Cardiovascular Risk from the

Postprandial Changes in HDL3-P

HDL3 Activation Index ™, (HDL3-PA ™)

A Study of Postprandial Lipoprotein Particle Numbers Using LPP

Effect of meal composition on postprandial lipid concentrations and lipoprotein particle numbers: A randomized cross-over study

Meena Shah, Manall Jaffery, Beverley Adams-Huet, Brian Franklin, Jonathan Oliver, Joel Mitchell

PLOS ONE | DOI:10.1371/journal.pone.0172732 February 21, 2017)

Most of the Day a Person is in a Non-Fasting State

What Have We Been Missing?

Study on Dietary Response of Lipoproteins

Shah et al.* showed that High Protein and High Mono Unsaturated Fat provide:

No significant change in HDL-C but Total HDL-P and (HDL2a-P + HDL3-P) show a Significant Increase

Result was interpreted as a "less favorable medium and small HDL-P" response

This followed the lead of the NCEP guidelines and many studies showing that:

Increases in Total HDL-C and Large HDL2b-C are Favorable

** Meena Shah et al., Effect of meal composition on postprandial lipid concentrations and lipoprotein particle numbers: A randomized cross-over study, PLOS ONE | DOI:10.1371/journal.pone.0172732 February 21, 2017)*

Dietary Response of HDL-P

The 120-minute Postprandial Response Showed a Large Preferential Increase in Medium and Small HDL in the High Protein Meal over a High Mono-Unsaturated Fat Meal (p=0.01)*.

Our small internal study was used to determine if a complete meal of Fats and Carbohydrates is necessary or if Protein Alone would give the response.

It was Determined that the Response is Primarily Due to Protein

Therefore, Commercially Available High Protein, Low Fat and Low Carbohydrates Drinks are Perfect for the Assay

** Meena Shah et al., Effect of meal composition on postprandial lipid concentrations and lipoprotein particle numbers: A randomized cross-over study, PLOS ONE | DOI:10.1371/journal.pone.0172732 February 21, 2017)*

Dietary Response of HDL-P

Additionally, most of the increase in
HDL2a-P + HDL3-P (medium and small HDL)
is due to the HDL3-P increase,
the smallest HDL particle

A Postprandial time study was undertaken to see if times shorter than 120 to 180 minutes would give a similar response

Postprandial response at
60, 75, 90, 100, 120 and 180 minutes were tested.
Postprandial times of 90 to 180 minutes
were not significantly different so a reasonable time for a laboratory assay was selected of 90 minutes

Dietary Requirement for HDL-P Response

The Recommended Dietary Allowance (RDA) for a Healthy Adult is:

0.8 g of Protein per kg of Body Weight per Day

A person with moderate physical activity the amount is 1.3 grams of protein per kg of body weight or 0.6 grams per pound per day.*

A good high estimate of a persons meal requirement is ½ of the dietary allowance:

0.3 g of Protein per Pound of Body Weight

(a similar amount of protein to the Shah study)

*Wu, G., Food Function, 2016 Mar;7(3):1251-65

HDL3-P Activation Assay

(A) Choose a **High Protein**, Low Carbohydrate, Low Fat supplement with known grams of protein per ounce

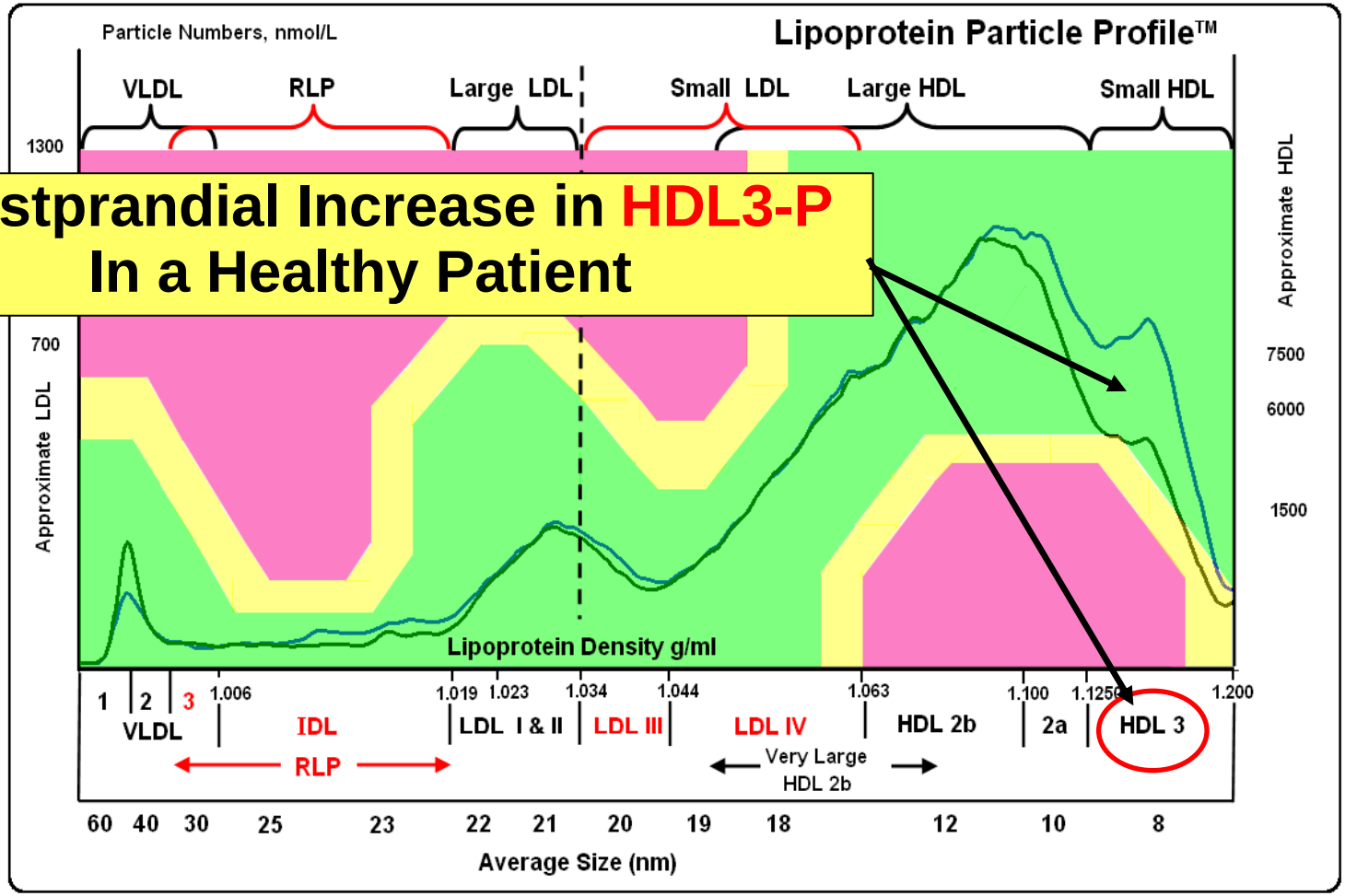
(B) Calculate the Supplement **Volume** Based on Weight:
0.3 Grams of Protein per Pound
150 lb person x 0.3 = 45 (+/- 5) grams of protein

(C) Draw a Fasting Blood Specimen

(D) Drink Supplement (Muscle Milk was used)
90 minutes before the Postprandial Blood Draw

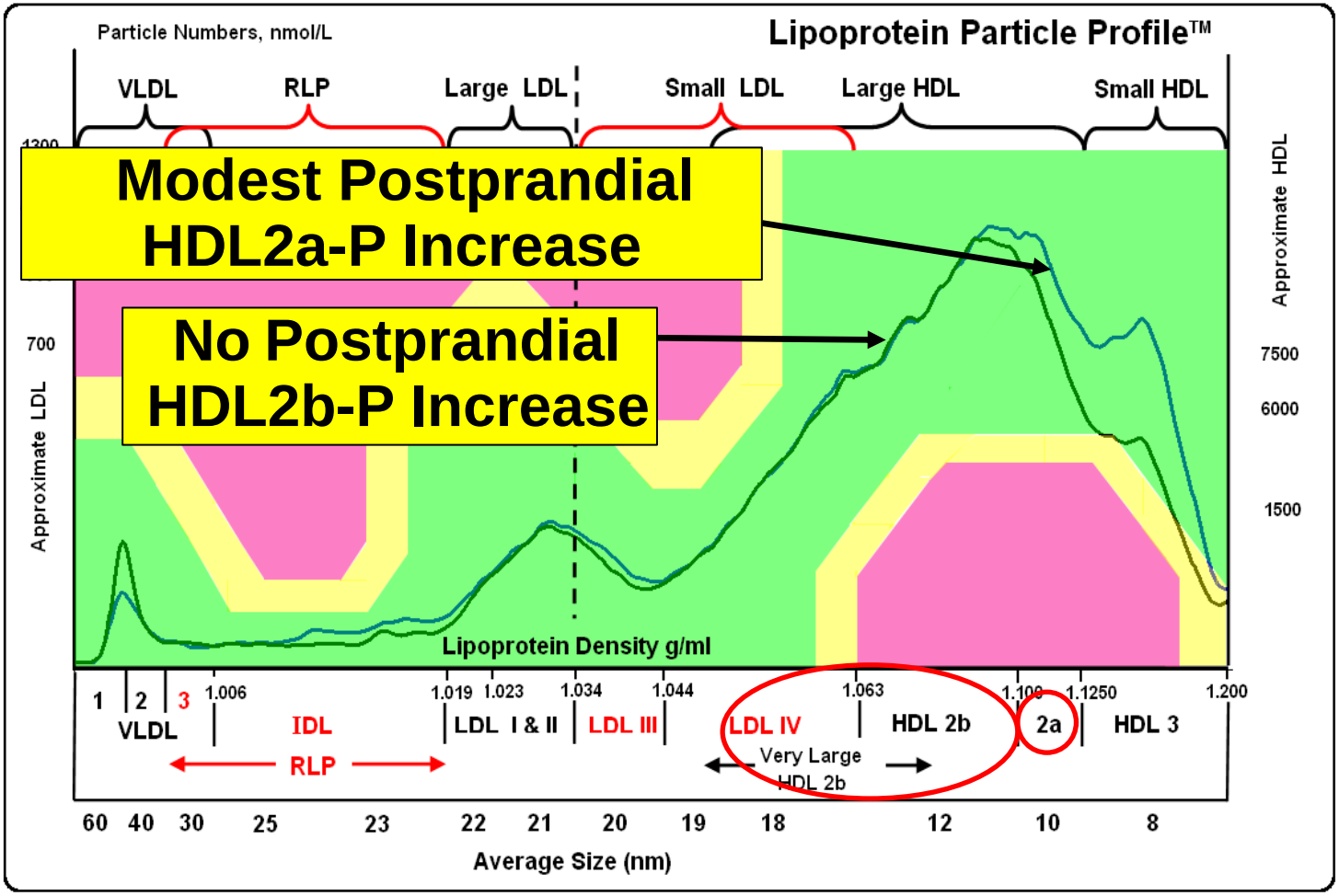
(E) Measure the Fasting and Postprandial Specimens for HDL3 Particle Numbers using **LPP Technology**
The increase in HDL3 is the **"HDL3 Activation Index ™ "**

HDL3-P Activation Index ™



HDL3-P Activation Index ™ = 1509 nmol/L
Postprandial HDL3-P minus Fasting HDL3-P
After the Specified High Protein Drink

HDL3-P Activation Index ™



Risk Factor

Borderline

Normal

HDL-C and HDL2b-P remain at fasting levels

HDL3-P is the primary lipoprotein increased for Improved Macrophage and Cellular Free Cholesterol Efflux

Postprandial HDL3-P Activation

Lipoprotein Particle Numbers (nmol/

	Fasting Value	Postprandial	Percent Change	Reference Value
VLDL Particles	41	33	-20	<85
Total LDL Particles	553	603	9	<900
Non - HDL Particles	594	636	7	
RLP (Remnant Lipoprotein)	102	127	24	
Small - Dense LDL III	163	189	16	
Small - Dense LDL IV	122	116	-5	
Total HDL Particles	7974	9654	21	
Large - Buoyant HDL 2b	3525	3543	1	>1500
Small - Dense HDL 3	3082	4591	49	>3800

HDL2b-P	1% Increase
HDL3-P	49% Increase
Total HDL-P	21% Increase

Biomarkers and Risk Factors

	Value		Reference Value
Apo B-100 (mg/dl)	65	64	40 - 100
Apo A-1 (mg/dL)	209	210	>115

Lipid Panel (mg/dL)

	Value		Reference Value
Total Cholesterol	160	160	
LDL - Cholesterol	61	62	
HDL - Cholesterol	78	78	
Triglycerides	164	76	
Non - HDL- Chol (calc)	82	82	

HDL-C and LDL-C
No Change

Fasting and 90 Minute postprandial specimen changes from a 64 gram protein, low fat and low carbohydrate supplement

Postprandial HDL3-P Increase

HDL3 Activation Index ™ (HDL3-PA™) has been observed to increase between 0% and 100% in a small study and appears to be correlated to Cardiovascular Health

Up to a 100% Increase in Previously Unknown Reverse Cholesterol Transport Potential

The increase in HDL3-P corresponds to an increase in Total HDL-P of over 30%

Small Study on HDL3 Activation

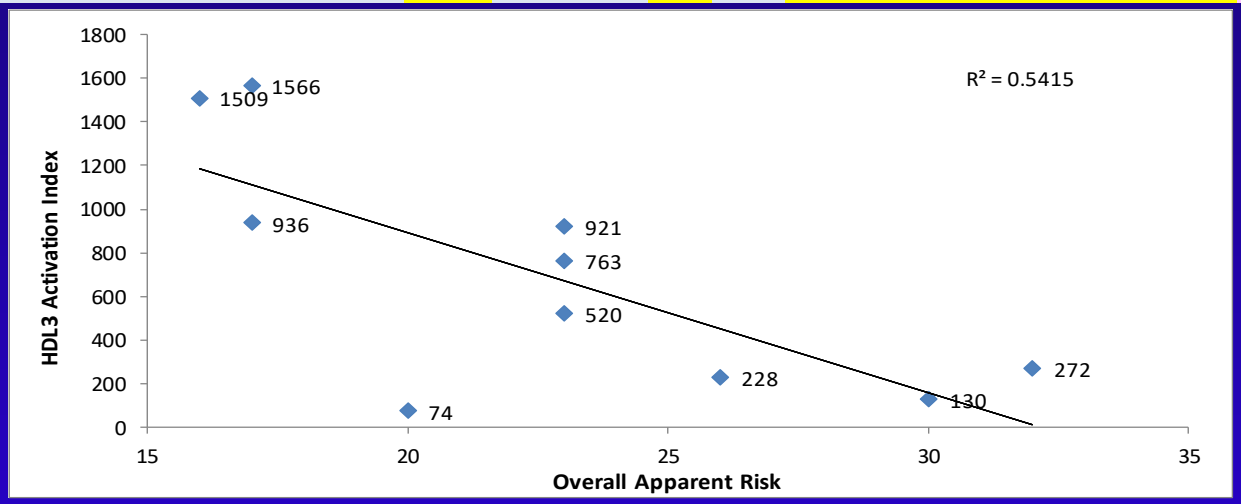
HDL3-P Activation is inversely correlated to CVD Risk and Diet

HDL3-P Study - Risk Totals

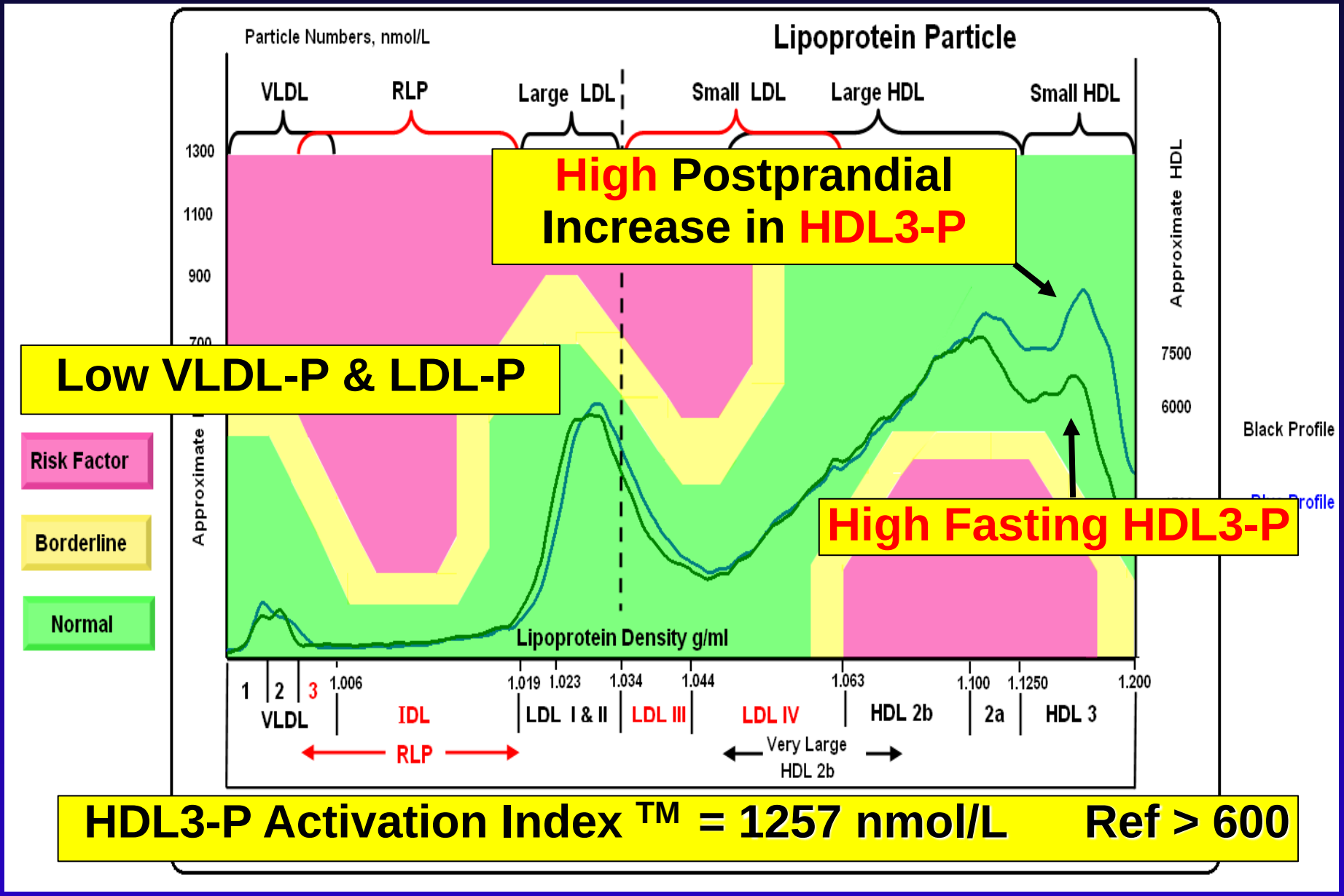
Fasting patients consumed 0.3 grams of protein per pound of body weight in the form of a high protein, low carbohydrate, low fat drink. Activated serum was drawn 90 minutes after the drink was consumed and the LPP ® lipoprotein particle number assays were performed.

Risk Scored from 1 to 4 for Each Factor

#	Sex	Diet Risk	Exercise Risk	Cardiovascular Related Disease, Age Adjusted	Health Risk	Age	Weight lbs	BMI Risk	HA1c %	HA1c Risk	CRP Risk	Insulin Risk	VLDL Risk	LDL-P Risk	HDL-P Risk	Overall Risk	HDL3 Activation Index ™ (HDL3-PA) nmol/L < 600
1	F	3	2	Weight Control Problem, Lp(a)	3	28	160	3	4.9	1	1	1	1	3	2	20	74
2	M	4	4	Advanced Diabetes Mellitus, Lp(a)+	4	53	175	2	6.9	4	2	4	1	3	2	30	130
3	M	2	4	Weight Control Problem	2	64	247	3	5.6	3	4	1	1	4	2	26	228
4	M	4	4	CVD, By-pass Surgery, Lp(a)	4	80	250	4	5.7	4	2	2	1	3	4	32	272
5	M	4	2	Very High Carbohydrate Diet	3	15	150	2	5.3	2	1	4	1	1	3	23	520
6	F	2	3	Weight Control Problem	3	44	189	4	5.2	1	3	1	1	4	1	23	763
7	F	2	3	Gastric Sleeve Surgery	2	75	162	3	5.6	3	2	2	1	3	2	23	921
8	M	1	2	Weight Control Problem, Lp(a)	2	72	234	3	5.0	1	3	1	1	2	1	17	936
9	M	1	2	Atrial Flutter, Ablation Surgery, Lp(a)	2	71	228	3	5.0	1	2	1	1	2	1	16	1509
10	F	1	1	Tachycardia	1	66	128	1	5.5	3	3	1	1	4	1	17	1566

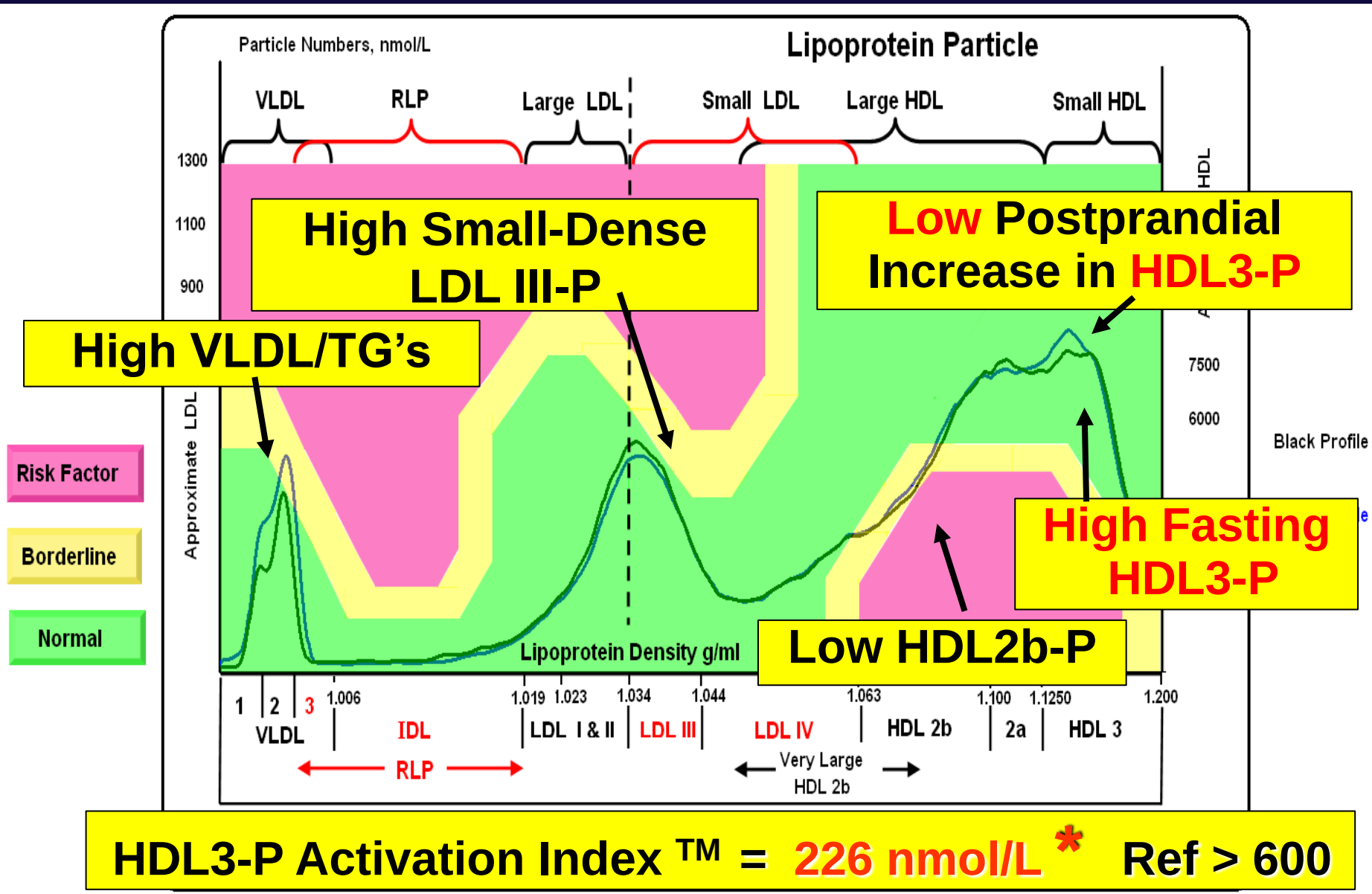


Healthy Patient



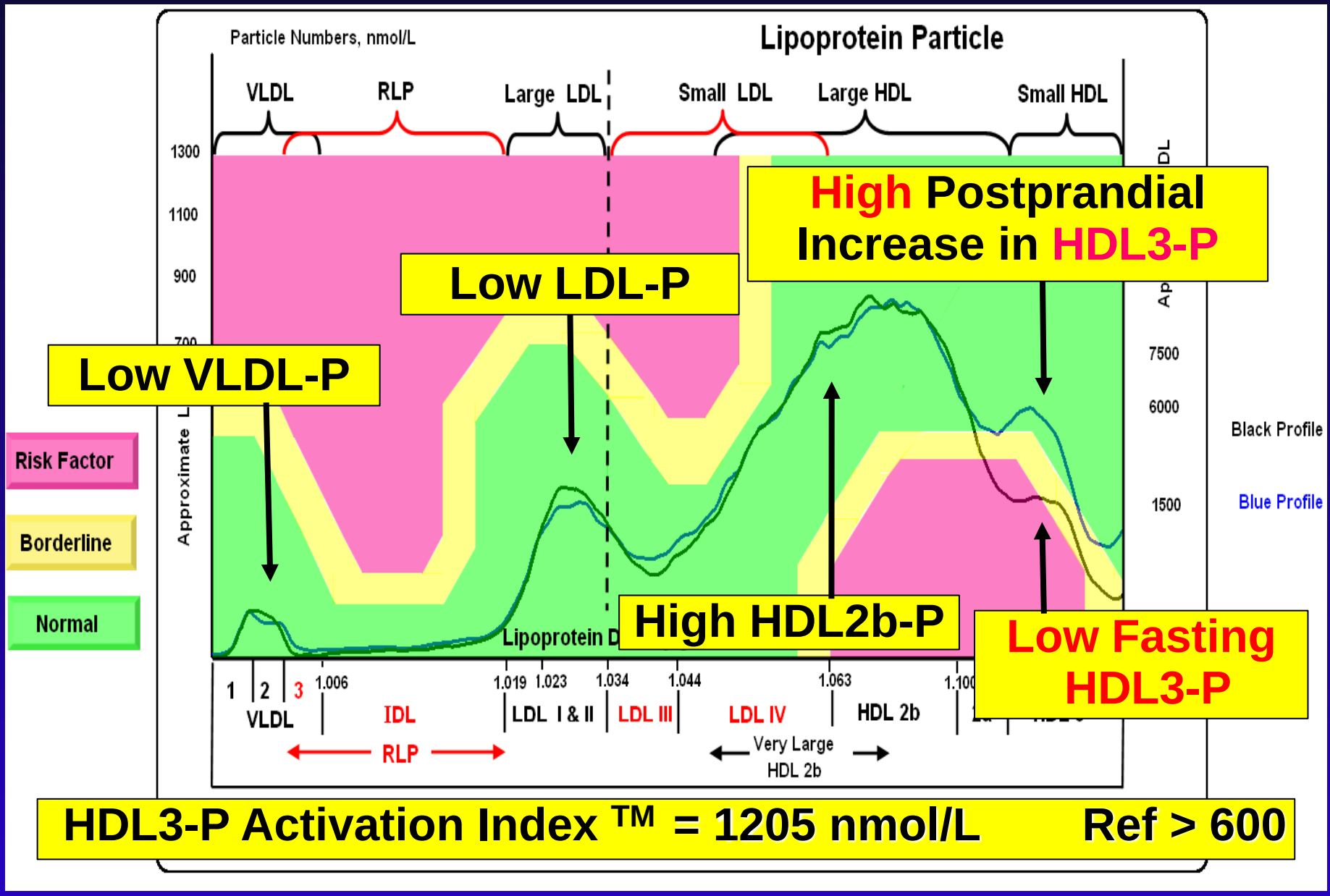
HDL3-P Activation Index ™ = 1257 nmol/L Ref > 600

Metabolic Syndrome Patient – Pre T2D



HDL3-P Activation Index ™ = 226 nmol/L * Ref > 600

Low Fasting HDL3-P



HDL3-P Activation Results

HDL3-C values are unchanged from fasting to postprandial levels

Patients with high fasting HDL3-P often show postprandial increases however some show very little increase such as in Metabolic Syndrome.

Fasting HDL3-P values are not an indication of the activation of HDL3-P

A patient with low fasting HDL3-P is not an indication of poor activation

Postprandial insulin increased as expected however, healthier patients had a larger insulin increase over fasting values than CVD patients.

The postprandial insulin increase may be a better indicator of insulin resistance than fasting insulin alone.

The Tale of Two HDL3's

HDL3 can be made by Two Different Pathways

One is newly formed HDL3(c,b,a) that are derived from Apo A-1 and Pre β-1 HDL's
These particles participate in Rapid Reverse Cholesterol Transport that has a half-life of 7-8 minutes

The other HDL3, call it HDL3X, is the product of CETP (cholesterol ester transport protein) and HL (hepatic lipases) action on HDL2's in the presence of elevated Triacylglycerols. This enzymatic activity reduces the size and density of an HDL2 to that of an HDL3.

HDL3X is produced in Metabolic Syndrome and Diabetic Patients and appears to not be recognized by hepatic SR-B1 (scavenger receptor B1) for Functional Reverse Cholesterol Transport

In Metabolic Syndrome and Diabetic Patients the majority of HDL particles are HDL3X with very little HDL2b. This gives Poor HDL Functionality in spite of reasonable total HDL particle numbers.

Understanding the HDL-3 Activation Assay™

The LPP profile is optimized in the HDL area to clearly show changes in the HDL-P subgroups of HDL2b, HDL2a and HDL3

A High Protein Supplement provides the amino acid nutrients for the creation of Apo A-1, Nascent HDL and HDL3 particles.

HDL3 has two Apo A-1's with a unique folding pattern that is different from HDL2. The folding creates a spherical particle capable of rapid reverse cholesterol transport. $t_{1/2}$ = 7-8 min

HDL2a and HDL2b have three and four Apo A-1's with discoidal and spherical shapes respectively. These particles proceed to reverse cholesterol transport by a slower mechanism. $t_{1/2}$ = 0.92 day

The short life of newly formed HDL3 particles eluded detection in fasting specimens and non-particle measurements technologies.

Fasting lipoprotein profiles with High or Low HDL3-P or HDL3-C are Not Useful in Understanding the Functionality of HDL.

Poor HDL3-P Activation

Conditions Associated with Poor HDL3-P Activation

Pre-Diabetic or Diabetic Patient

High Triacylglycerols, HA1c, Glucose or Insulin Resistance

Early Signs of Atherosclerosis

Poor – CIMT, Calcium Score, Stress Test or Lipid Panel

Existing Atherosclerosis

Stent, Cardiac Bypass or Heart Valve Replacement

Obesity or Poor Physical Condition

Poor Diet and/or Exercise Routine

Family History

Genetic Predisposition to Atherosclerosis

What to do with the Results?

Improve HDL3-P Activation to Reduce Risk.

Pre-Diabetic or Diabetic Patient

Diet Modification (Reduced Carbohydrates, Increase Omega-3's), **Exercise and/or Medication**

Existing Atherosclerosis

LDL Reduction (Statins and PCSK9 Inhibitors),

Reduce Triacylglycerols (Reduce Carbohydrates, Increase Omega-3's), **Exercise**

Obesity, or Poor Physical Condition

Reduce Triacylglycerols (Reduce Carbohydrates, Increase Protein and Omega-3's), **Exercise**

Summary

HDL3 is a Powerful Antioxidant through association with PON1 which protects LDL from oxidation

Fasting HDL lipoproteins reflect the end result of HDL metabolism but do not determine if the process is stagnant or active

The functionality of HDL2 and HDL3 are quite different. HDL3 proceeds through Reverse Cholesterol Transport 200 Times Faster.

Functional Reverse Cholesterol Transport involves the activated production of Pre β-1 HDL which become HDL3(c,b,a) Particles.

Patients are Not Fasting most of the day so the true active levels of HDL-3 are not known

Risk should be determined with HDL3-P in the Activated Postprandial State when Compared to Fasting Specimens

In our small study the HDL3 Activation Index ™ was Predictive of Overall Cardiovascular Risk

Final Thought

Based on the data:

There should be a shift in the human diet

from

Carbohydrate Calories

to More Good Protein Calories