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Comparison of four methods of analysis of lipoprotein particle subfractions for their association with angiographic progression of coronary artery disease

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ABSTRACT

Background: Compare gradient gel electrophoresis (GGE), vertical auto profile ultracentrifugation (VAP-II), nuclear magnetic resonance spectroscopy (NMR), and ion mobility for their ability to relate low- (LDL), intermediate- (IDL), very-low-density (VLDL) and high-density lipoprotein (HDL) subfraction concentrations to atherosclerotic progression.

Methods and results: Regression analyses of 136 patients who received baseline and follow-up coronary angiographies and subfraction measurements by all four methods in the HDL Atherosclerosis Treatment Study. Prior analyses have shown that the intervention primarily affected disease progression in proximal arteries with <30% stenoses at baseline.

Three-year increases in percent stenoses were consistently associated with higher on-study plasma concentrations of small, dense LDL as measured by GGE (LDLIIIb, $P = 10^{-6}$; LDLIVa, $P = 0.006$; LDLIVb, $P = 0.02$), VAP-II (LDL4, $P = 0.002$), NMR (small LDL, $P = 0.001$), and ion mobility (LDL IIb, $P = 0.04$; LDLIIIa, $P = 0.002$; LDLIIIb, $P = 0.0007$; LDLIVa, $P = 0.05$). Adjustment for triglycerides, HDL-cholesterol, and LDL-cholesterol failed to eliminate the statistical significance for on-study GGE estimated LDLIIIb ($P = 10^{-5}$) and LDLIVa ($P = 0.04$); NMR-estimated small LDL ($P = 0.03$); or ion mobility estimated large VLDL ($P = 0.02$), LDLIIIa ($P = 0.04$) or LDLIIIb ($P = 0.02$). All methods show that the effects were significantly greater for the on-study than the baseline small, dense LDL concentrations, thus establishing that the values concurrent to the progression of disease were responsible. The rate of disease progression was also related to individual VLDL, IDL, and HDL subclasses to differing extents among the various analytic methods.

Conclusion: Four methodologies confirm the association of small, dense LDL with greater coronary atherosclerosis progression, and GGE, NMR, and ion mobility confirm that the associations were independent of standard lipid measurements.

Clinical Trial Registration: clinicaltrials.gov/ct2/show/NCT00000553.

Plasma low density lipoprotein (LDL) cholesterol, along with high density lipoprotein (HDL) cholesterol and triglyceride concentrations, are recommended by European and North American guidelines for assessing and managing risk of cardiovascular disease (CVD) [1–3]. However, lipoproteins are comprised of multiple subclasses that can be distinguished by their physicochemical properties. For example, LDL-cholesterol is the sum of the cholesterol levels in at least seven particle subclasses that differ in size and density [4,5]. Although there is substantial evidence that increased levels of smaller, denser LDL particles are associated with greater risk of coronary heart disease [6], an independent effect of small dense LDL has not been established, in part due to concomitant elevations in

Abbreviations: GGE, gradient gel electrophoresis; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low-density lipoprotein; NMR, nuclear magnetic resonance spectroscopy; VAP-II, vertical auto profile; VLDL, very low density lipoprotein.

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triglycerides and total LDL particle numbers and reductions in HDL-cholesterol [6–9].

Recognition that lipoprotein heterogeneity could potentially improve risk prediction spurred technological development for its analysis. In addition to GGE, the methods include nuclear magnetic resonance spectroscopy (NMR), vertical auto profile ultracentrifugation (VAP-II), and ion mobility. The latter three methods use different principles to provide quantitative estimates of subfraction concentrations across the full spectrum of lipoprotein particles. NMR estimates are obtained from the mathematical deconvolution of spectroscopically distinct lipid methyl group NMR signals whose amplitudes are directly proportional to the numbers of subclass particles emitting the signal, irrespective of variation in particle lipid composition [10,11]. VAP-II is based on the deconvolution into subfractions of the direct cholesterol quantitation of lipoproteins separated by flotation rate (a function of size and hydrated density) [12]. Ion mobility uses an electrospray procedure to obtain direct lipoprotein particle counts as a function of particle size [13]. It is based on the principle that particles of a given size and uniform charge behave in a predictable manner when carried in a laminar airflow subjected to an electric field. GGE, VAP-II, NMR, and ion mobility may also be used to characterize relative distribution of LDL particles as a function of their particle diameter by the peak (mode) or mean of the LDL size distribution.

GGE, VAP-II, NMR, and ion mobility have been used for relating lipoprotein subfractions to various measures of CVD. However, a direct comparison of all four methods for their associations with angiographically measured coronary disease progression within the same group of patients has not been previously reported. To this end, angiographically measured coronary disease progression may be more closely related to the atherogenic properties of lipoproteins than cardiovascular events because the latter represent the consequences of both atherogenesis and factors that promote plaque rupture and thrombosis [14]. We therefore assessed whether GGE, VAP-II, NMR, and ion mobility measurements are consistent in identifying associations of specific lipoprotein subfractions with angiographically measured changes in coronary artery stenosis in the HDL-Atherosclerosis Treatment Study (HATS), a randomized placebo controlled clinical trial of simvastatin plus niacin and/or antioxidant supplements in patients with reduced HDL cholesterol [15,16]. We have reported previously that, within the LDL particle spectrum, higher on-study concentrations of LDLIIIb and LDLIVa as measured by GGE were significantly associated with greater rates of progression of coronary stenosis in HATS [16]. The current analyses test whether measurements of LDL subfractions by VAP-II, NMR, and ion mobility provide results comparable to GGE, and for the three latter methods, whether levels of subfractions within very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), or HDL also show significant associations with coronary artery disease progression. For this purpose, analyses were restricted to coronary artery regions with $<$30% stenosis since these showed the greatest benefit from intervention with drug treatment in the clinical trial [15], and also showed the strongest association with on-study LDL subfraction concentrations in subsequent analyses [16].

1. Methods

1.1. Study

The design and results of the original clinic trial have been described in detail elsewhere [15]; their summary to follow includes only those details relevant to the current analyses. The study included 160 men and women under the ages of 63 and 70 years old, respectively, who were recruited between 1995 and 1997, and who had clinical coronary disease (previous myocardial infarction, coronary interventions, or confirmed angina) and at least three stenoses $\geq$30 percent of the luminal diameter or one stenosis $\geq$50 percent. The participants were recruited for low HDL cholesterol ($\leq$0.91 mmol/L if male and $\leq$1.03 mmol/L if female), LDL-cholesterol $\leq$3.75 mmol/L and triglycerides $\leq$ 4.52 mmol/L [15]. The current analyses are restricted to the associations of lipoprotein concentrations vs. coronary disease progression, and therefore the random assignment of the patients to four different treatment arms (simvastatin-niacin with antioxidants or antioxidant placebo, antioxidants with simvastatin-niacin placebo, and placebos alone) is only relevant in producing variation in on-study lipoprotein subfraction concentrations. The protocol was approved by the human-subjects committee and the patients provided signed consent.

1.2. Arteriography

Eight views of the left and right coronary arteries at baseline and follow-up were compared side-by-side to measure the minimal luminal diameter ($\text{Diameter}_{\text{minimum}}$) and nearby normal diameters ($\text{Diameter}_{\text{normal}}$) in millimeters using the catheter for the calibration. Stenosis was expressed as a percentage (i.e., $100 \times (\text{Diameter}_{\text{normal}} - \text{Diameter}_{\text{minimum}})/(\text{Diameter}_{\text{normal}})$. The prespecified primary end point was the mean change per patient from the initial arteriogram to the final arteriogram in the percent stenosis caused by the most severe lesion in each of the nine proximal coronary segments. Those arteries exhibiting $<$30% stenosis accounted for most of intervention-related and LDL-related disease progression during the trial [15,16].

1.3. Laboratory measurements

Fasting plasma concentrations of triglycerides, total, HDL, and LDL cholesterol, and apolipoprotein B were determined by Northwest Lipid Research Laboratories [17].

Gradient gel estimates of LDL peak diameter and LDL subclass cholesterol concentrations were determined from fresh whole plasma using 2%–14% non-denaturing polyacrylamide gradient gel electrophoresis [4], The cholesterol concentrations of the subclasses were estimated by multiplying percent of the total stained areas for each subclass by the cholesterol measured in ultracentrifugally isolated LDL fractions [18].

Vertical Auto Profile (VAP-II) measurements of lipoprotein subclass cholesterol concentrations were performed at Atherotech Diagnostics Lab (Birmingham, AL) [12,19,20] on $-80\,^{\circ}$C frozen samples taken 3–6 years earlier. Plasma lipoproteins were separated by single vertical spin density-gradient ultracentrifugation of diluted plasma samples adjusted to a density of 1.21 kg/L. A density gradient was prepared by first pipetting 1.4 ml of density-adjusted diluted plasma in each tube which were then overlaid with 3.9 ml of saline/EDTA and centrifuged in tandem with a tube containing calibration plasma with a known total cholesterol concentration. A VAP-II analyzer used the direct cholesterol measurements from eluted fractions to obtain profiles of digitized absorbance units (Y-coordinate) vs. relative gradient position from the sample drain time (X-coordinate) which were decomposed into component curves corresponding to Lp(a), five HDL, four LDL, two IDL, and 3 VLDL subclasses. The analyses presented herein corresponds to HDL2, HDL3, four LDL subclasses, IDL, and buoyant (VLDL1 and VLDL2) and dense VLDL (VLDL3) concentrations. Peak LDL flotation rate was measured in time (seconds) to the mode of the LDL cholesterol concentration distribution.

NMR spectra of frozen plasma specimens were acquired at LipoScience (Raleigh, NC) in 2001 and the digitized data subsequently analyzed for lipoprotein particle concentrations and sizes using the current LipoProfile-3 spectral deconvolution algorithm.