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A comparative study of four independent methods to measure LDL particle concentration

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ABSTRACT

Background: Low-density lipoprotein particle concentration (LDL-P) is generally more predictive of clinical cardiovascular endpoints than LDL cholesterol (LDL-C). Few studies have directly compared multiple LDL-P methods, particularly with ultracentrifugation.
Objective: Examine comparability and precision of 4 LDL-P methods.
Methods: We divided serum from 48 subjects into blinded triplicates and measured LDL-P in 3 separate laboratories by 4 methods: ultracentrifugation (reference method), a novel electrophoretic method, and nuclear magnetic resonance spectroscopy (NMR) by 2 independent methods: a 400 MHz Vantera® instrument supplied by Liposcience (LS-NMR) and operated at ARUP Laboratories, and a 600 MHz Bruker instrument (ASCEND 600) operated at Health Diagnostic Laboratory (HD-NMR).
Results: Of the 4 methods, ultracentrifugation was the most precise and LS-NMR the least; the latter had a significantly greater CV ($p < 0.0001$) as compared with all 3 of the other methods, although all CVs were clinically acceptable. The electrophoretic method showed similar precision to ultracentrifugation, while HD-NMR was intermediate. The HD-NMR had the slope closest to 1 (0.90, 95% CI 0.71 to 1.09) and the intercept closest to 0 (−48, −353 to 256) compared to the ultracentrifugation method in Deming regression models. While the two NMR methods correlated well ($r = 0.95$) with each other and had a slope equivalent to 1 (1.08, 0.98 to 1.19), their intercept in Deming regression excluded 0 (194, 53 to 335) indicating a vertical shift between the two methods.
Conclusions: This LDL-P method comparison may prove useful for future research and clinical applications.

1. Introduction

Increased levels of low-density lipoproteins (LDL) are causally related to greater risk of coronary heart disease (CHD) [1–4]. An association between CHD and measured LDL particles was perhaps first shown using analytical ultracentrifugation [5–7]. A few studies have examined CHD risk with LDL cholesterol (LDL-C) measured in all participant samples by preparative ultracentrifugation – either by β-quantification [8–11] or density gradient ultracentrifugation [12]. Most commonly, LDL has been estimated as LDL-C by the Friedewald equation [13], with numerous prospective studies showing a positive association between LDL-C and CHD risk [1], and a substantial number of intervention trials showing reduction of CHD risk when LDL-C was lowered by various interventions, particularly statin drugs [3]. As apolipoprotein (apo) B is the main structural protein of all atherogenic lipoproteins and because non-high-density lipoprotein cholesterol (non-HDL-C) should reflect the cholesterol content of these particles, apo B and non-HDL-C have been suggested as better indicators of risk than LDL-C. Indeed, in studies where LDL-C, non-HDL-C, and apo B have been measured together, apo B tends to be the most predictive of CHD risk, LDL-C the least, and non-HDL-C intermediate [14].

More recently, estimates of LDL particle number or concentration (LDL-P), most often by nuclear magnetic resonance (NMR) methods, have also generally been found to provide better predictions of risk than LDL-C [15–24] or non-HDL-C [25]. These findings are consistent with those of animal studies, which suggest

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that a larger number of smaller LDL particles are more atherogenic than fewer but larger lipoprotein particles (including triglyceride-rich remnants) for a given total cholesterol concentration [26,27]. Indeed, even among the LDL class, small dense LDL may be more atherogenic than larger particles [17,28–30], though whether this relationship is independent of LDL-P remains controversial [31–33]. Recognition that LDL-P is disproportionately elevated (as compared to LDL-C) among diabetic individuals or persons with elevated triglycerides (TG), low HDL, insulin resistance, or prior CHD, and may provide additional guidance for treatment among certain intermediate or high-risk patients, has led to the suggestion that measurement of LDL-P is reasonable for many such patients [34]. Moreover, discordance between apo B and NMR-determined LDL-P measures is not uncommon in clinical practice, with evidence suggesting utility of both in the assessment of cardiovascular risk [35,36].

Very few studies have compared NMR particle numbers with traditional ultracentrifugation methods [37,38]. On the other hand, several studies have examined agreement between NMR, density gradient ultracentrifugation, and gradient gel electrophoresis for LDL particle *size*, with results generally showing only moderate concordance [32,39–42]. We have measured LDL-P by four independent methods to examine comparability and precision for each of the methods.

2. Materials and methods

2.1. Sample collection and distribution

Fasting blood was drawn at Health Diagnostic Laboratory (HD Lab) (Richmond, VA) from 48 ambulatory, currently asymptomatic adult volunteers. Blood samples were allowed to clot at room temperature for 30 min, and then centrifuged at 4 °C for 15 min at 3000 rpm. After aliquoting each serum sample into 9 polypropylene screw-capped cryovials (3 sets of identical triplets), each triplet was de-identified and assigned a randomly generated fictitious name, demographics, and ID number. While no demographic, diagnostic, anthropometric, or blood pressure data was recorded due to the fictitious names used in the blinding process, these volunteers had no acute medical problems, a mean age in the 40s, and a fairly even gender distribution. Thereafter, specimens were distributed within 24 h by refrigerated transport to the three participating laboratories [HD Lab, Cardiovascular Genetics (CVG), and Associated Regional and University Pathologists (ARUP)]. Each of the 3 laboratories treated all samples as unique specimens. Thus, all samples had the same history at the time of testing and all analysts were kept uninformed throughout as to the origin of the matching triplets. Analyses at all three sites were completed within 7 days of blood collection. All participants gave informed consent to participate in this study, which was approved by the Copernicus Group Institutional Review Board, Durham, NC.

2.2. Ultracentrifugation

Ultracentrifugation and subsequent analyses were performed at the CVG biochemistry laboratory at the University of Utah, Salt Lake City, UT. To simultaneously "float" and "wash" very-low-density lipoprotein (VLDL), 200 μL whole serum at its natural density was layered beneath a cushion of 750 μL buffer (150 mM NaCl, 1 mg/mL EDTA, 1 mg/mL azide, pH 7.4) in a 11 × 35 mm thick-walled polycarbonate tube. For isolation of combined VLDL and intermediate-density lipoprotein (IDL), 300 μL serum, adjusted to a background density of D = 1.019 g/mL (2 volumes of serum plus 1 volume of D = 1.045 g/mL buffer), was layered beneath a cushion of 650 μL buffer of D = 1.019 g/mL. Both tubes were then spun at 100,000 rpm in a TL-100.2 fixed-angle rotor and Beckman TL-100 tabletop ultracentrifuge for 4 h at 4 °C. After gentle braking, approximately 400 μL supernatant from each tube was carefully needle aspirated and saved for subsequent chemical analysis; exact recovery was determined by weighing the centrifuged tubes before and after aspiration.

The chemical content of the D < 1.006 g/mL supernatant was considered to reflect VLDL. Constituents in IDL were calculated as the difference between the D < 1.019 g/mL and D < 1.006 g/mL supernatants. Constituents in LDL [including Lp(a)] were calculated as whole serum minus the D < 1.019 g/mL supernatant for apo B and minus the HDL fraction (see below) for lipid fractions.

2.3. Lipids and apolipoproteins (CVG, Salt Lake City, UT)

Cholesterol and TG were quantified in whole serum and D < 1.006 g/mL and D < 1.019 g/mL ultracentrifugal subfractions using Trinder-based enzymatic reagents (Sigma, Wako, Sekisui, and Research Organics) and a Thermo Multiskan microtiter plate spectrophotometer as previously described [43]. Precinorm L® (Roche Diagnostics) was used as the calibrator. HDL lipids were quantified in supernatants following precipitation of apo B-containing lipoproteins with dextran sulfate (50 kD Mol Wt, Sigma–Aldrich) and magnesium chloride (final concs 0.9 mg/mL and 45 mmol/L, respectively) [44]. All assays were performed in duplicate with intra-assay coefficients of variation (CVs) < 3%. Apo A-I and apo B concentrations in whole serum and D < 1.006 g/mL and D < 1.019 g/mL subfractions were quantified by liquid-phase double-antibody radioimmunoassays as previously described [45]. Determinations were performed in duplicate with intra-assay CVs <6%.

In calculations of particle number and diameter it was assumed that each VLDL, IDL, or LDL particle is a sphere with a core composed of cholesteryl ester (CE) and TG only, has a constant thickness of the polar shell of 20.2 Å, and contains a single molecule of apo B with molecular weight 547 kD [46]. Partial specific volumes of CE and TG are 1068 and 1556 cubic angstroms, respectively.

2.4. LS-NMR analysis (LipoScience NMR on Vantera Instrument, ARUP Labs, Salt Lake City, UT)

400 MHz proton NMR spectra of sera were acquired at ARUP Laboratory (Salt Lake City) on the Vantera® Clinical Analyzer. The digitized composite signals at ~0.8 ppm were subsequently analyzed at LipoScience (Raleigh, NC) to give lipoprotein particle concentrations and sizes using the LipoProfile-3 spectral deconvolution algorithm [47]. Lipoprotein measures provided by this method included LDL particle number, HDL particle number, small LDL particle number, LDL size, large VLDL particle number, large HDL particle number, VLDL size, and HDL size.

2.5. HD-NMR analysis (Bruker Instrument, HD Lab, Richmond, VA)

LDL-P was also measured by NMR using a 600 MHz instrument (ASCEND 600, Bruker Biospin GmbH, Germany) at HD Lab, Richmond VA. This method (HD-NMR), developed in collaboration with the Numares Group, Regensburg, Germany, employs internal standards to compensate for environmental effects and discrete sample tubes to avoid carryover. This method provides LDL particle number, HDL particle number, and small LDL particle number. In prior development testing, intra- and inter-assay coefficients of variation were <5% and <9%, respectively. HD Lab also determined a standard lipid panel (total cholesterol, HDL-C, TG, and LDL-C calculated by the Friedewald method), and apo B and Lp(a) mass by turbidimetric assays.