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Validation of a lipoprotein(a) particle concentration assay by quantitative lipoprotein immunofixation electrophoresis

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ABSTRACT

Background: Low-density lipoprotein (LDL) particle (P, or molar) concentration has been shown to be a more sensitive marker of cardiovascular disease (CVD) risk than LDL cholesterol. Although elevated circulating lipoprotein(a) [Lp(a)] cholesterol and mass have been associated with CV risk, no practicable method exists to measure Lp(a)-P. We have developed a method of determining Lp(a)-P suitable for routine clinical use.
Methods: Lipoprotein immunofixation electrophoresis (Lipo-IFE) involves rigidly controlled electrophoretic separation of serum lipoproteins, probing with polyclonal apolipoprotein B antibodies, then visualization after staining with a nonspecific protein stain (Acid Violet). Lipo-IFE was compared to the Lp(a) mass assay for 1086 randomly selected patient samples, and for 254 samples stratified by apo(a) isoform size.
Results: The Lipo-IFE method was shown to be precise (CV <10% above the 50 nmol/l limit of quantitation) and linear across a 16-fold range. Lipo-IFE compared well with the mass-based Lp(a) assay ($r = 0.95$), but was not affected by variations in apo(a) isoform size. With a throughput of 100 samples in 90 min, the assay is suitable for use in the clinical laboratory.
Conclusions: The Lipo-IFE method will allow Lp(a)-P to be readily tested as a CVD risk factor in large-scale clinical trials.

1. Introduction

It is well-established that increased serum concentrations of apolipoprotein B (apoB)-containing lipoproteins are associated with an increased risk of developing cardiovascular disease (CVD) [1–5]. In particular, low density lipoproteins (LDL) and lipoprotein(a) [Lp(a)] are significant prognostic risk factors for future atherosclerotic disease and associated adverse events [5–12]. Lp(a) is an LDL particle to which apolipoprotein(a) [apo(a), a polypeptide comprised of multiple loop domains called "kringles," K] is covalently attached to apoB (Fig. 1). It is the apo(a) portion that is thought to imbue the particle with antifibrinolytic properties owing to the structural similarity of apo(a) to plasminogen [13]. Other proatherogenic properties are thought to arise from the ability of Lp(a) to traffic oxidized phospholipids and induce inflammatory processes in the arterial intima [14], which can lead to endothelial dysfunction, plaque development, and subsequent rupture. Indeed, loss-of-function variants in the *LPA* gene that lower circulating Lp(a) levels have also been shown to confer protection from CVD [15]. Apo(a) consists of two kringle types—KIV, with ten subtypes, and KV. Genetic variation in the length of the KIV type 2 (KIV-2) repeat region of apo(a) is largely responsible for the interindividual variability in Lp(a) size (i.e., molecular mass) [16,17] and, to some extent, plasma Lp(a) levels [18]. These multiple molecular isoforms [which vary from about 300 to 800 kDa) [19]] have presented a challenge for standardization of the measurement of Lp(a) mass in plasma [20–24], and variability in Lp(a) values obtained from different measurement methods has also made it difficult to interpret and compare published data between clinical studies [25].

Over recent years, LDL particle (or more properly, molar) concentration (LDL-P, nmol/l) has been proposed as a more robust measure of LDL levels than LDL-cholesterol (LDL-C), owing to the fact that LDL particles can carry variable amounts of cholesterol (and other lipids). Indeed, LDL-C varies widely among individuals with the same LDL-P concentration [5–7]. Moreover, for individuals with discordant LDL-C and LDL-P levels (i.e., increased for one metric but not the other), the LDL-attributable CVD risk is better indicated by LDL-P [2,26]. Similar discordance could theoretically apply to Lp(a)-P, owing to variations in apo(a) mass as noted above.

Abbreviations: P, particle concentration; CVD, cardiovascular disease; Lp(a), lipoprotein(a); Lipo-IFE, lipoprotein immunofixation electrophoresis; K, kringle; SPIFE, serum protein immunofixation electrophoresis; IDL, intermediate density lipoprotein.

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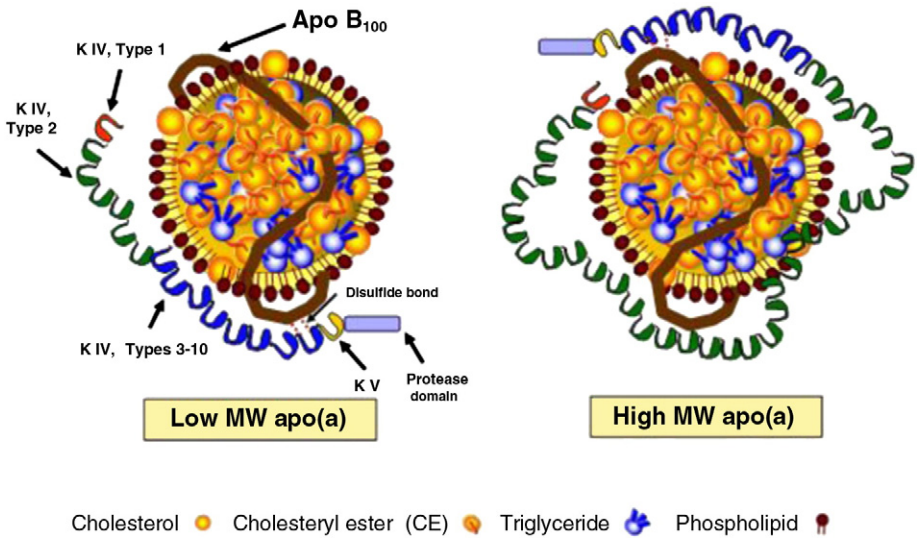

Fig. 1. Lp(a) particles of low and high molecular mass due to differences in apo(a) isoform size. Higher mass isoforms have more Kringle (K) IV-2 repeats than do lower mass isoforms. Apo(a) with 6 (left) and 35 (right) KIV-2 repeats are shown here; 3 to 43 repeats have been reported [17], with resulting molecular masses varying from 300 to 800 kDa [16]. KIV-1 and KIV-3-10 and KV are identical in all apo(a) isoforms. Labels on the lower mass isoform apply to the high mass isoform as well.

Some studies have used research-based, isoform-insensitive immunoassays to measure Lp(a)molar concentrations, but, if done properly (i.e., with direct analysis of apo(a) mass), these methods are time-consuming and labor-intensive [18,21,24]. Nuclear magnetic resonance spectroscopy, which can measure LDL particle concentrations, cannot distinguish Lp(a) from other lipoprotein families. Some assays that measure Lp(a) mass cannot distinguish between high and low molecular weight apo(a) isoforms, and thus cannot be translated into Lp(a)-P concentrations. There are antibodies against nonvariable epitopes of apo(a) (i.e., not on KIV-2) that form the basis for common Lp(a) assays that should, in theory, be insensitive to variations in isoform mass [18], but turbidometric assays utilizing this antibody might still be influenced by differences in total particle mass [25]. Because of the inherent atherogenicity of Lp(a) particles, being able to measure Lp(a)-P concentrations may have important clinical utility, facilitating risk assessment and optimizing lipid-modulating therapies [8,9]. To our knowledge, there are no Lp(a)-P assays that are both isoform independent and suited for use in the clinical laboratory. The purpose of this study was to utilize recent advances in electrophoretic methodologies, coupled with immunofixation detection, to develop and validate a novel, high-throughput, high-sensitivity technique to determine serum Lp(a)-P concentrations.

2. Materials and methods

2.1. Sample treatment procedure

We analyzed serum samples submitted to Health Diagnostic Laboratory, Inc. IRB approval for studies using de-identified and aggregated laboratory data was obtained from the Copernicus Group. At collection, the blood sample was drawn into an 8.5 ml BD Vacutainer® SST™ "Tiger Top" serum-separator tube (Becton, Dickinson), immediately inverted 8–10 times, and allowed to clot for 30 min in an upright position. After 15 min of centrifugation at 3000 rpm, the tube was then placed in the biohazard bag provided with absorbent material, placed in the refrigerator, and shipped to HDL, Inc. within 24 h. Serum samples were kept at 4 °C and were analyzed within 4 days of collection. Lipo-IFE (lipoprotein immunofixation electrophoresis) was performed using agarose electrophoresis [Serum Protein-IFE (SPIFE®) Helena Laboratories, Beaumont, TX] followed by immunofixation for apoB (anti-apoB Goat pAb, CalBiochem), protein staining (Acid Violet), and densitometric scanning (QuickScan 2000 WIN V2 software). The protocol was a modification of the SPIFE® Cholesterol-Vis System (Helena) [18]. Following electrophoresis, the gel blocks were removed and a rigid antisera template was placed on the gel. The antibody was diluted 1:4 with normal saline and administered through the template onto the gel for 2 min. Excess antibody was removed by blotting and pressing. Residual matrix antibody was removed by rehydration of the gel in a tris-buffered saline bath for 1 min. These steps were performed three times. The gel was subsequently dried at 56° for 8 min, then stained with Acid Violet and scanned.

2.2. Quantification of serum apoB and lipoprotein particles

Quantification of serum apoB was performed by immunoturbidimetry (Roche Diagnostics). Areas under the Lipo-IFE tracing corresponding to apoB-containing lipoproteins [very low and intermediate density lipoproteins (VLDL, IDL); LDL and Lp(a)] were converted into apoB concentrations and then into particle concentrations (nmol/l) based on the following equation using Lp(a)-P as an example:

$$Lp(a)\ Particle\ Number\ \left(\frac{nmol}{l}\right) = Total\ Serum\ ApoB\ \left(\frac{mg}{dl}\right) \times Area\ \%\ of\ Lp(a)\ band \times 18.52$$

(The factor 18.52 was derived as follows: [ApoB (mg/dl) × 10 dl/l × 10^6 nmol/mmol]/[molecular mass of apoB (540,000 mg/mmol)]

2.3. Accuracy, linearity, precision

The Lp(a)-P test accuracy (trueness) was determined by using Lipo-IFE to measure Lp(a)-P concentrations in standards that had been pre-assigned a concentration using a research immunoassay insensitive to apo(a) polymorphisms (10 standards with a range of 50 to 400 nmol/l; kindly provided by S. Marcovina, Northwest Lipid Metabolism and Diabetes Research Laboratories) [18]. Linearity was assessed across a range of 50 to 800 nmol/l by serial dilution. Precision was determined using five serum samples run in 10 replicates (at 50 nmol/l and above) on 20 separate gels, on 2 separate instruments over 3 days (i.e., 200 replicates of each of the five samples).

2.4. Specificity and interference

Specificity (i.e., insensitivity to apo(a) isoform size) was evaluated by comparing the particle concentrations derived from two assays