Lipoprotein(a) Particle Number

A novel assay without bias from apolipoprotein(a) size isoforms

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Introduction

The major lipoproteins are classified as chylomicrons, very low-density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), lipoprotein(a) [Lp(a)], and high-density lipoprotein (HDL). Lipoproteins consist of a lipid core (triglycerides and cholesterol esters) with free cholesterol and phospholipid forming the outer layer. Lipoproteins differ in size, lipid composition, and apolipoprotein content. Table 1 summarizes the heterogeneity in size, density, and apolipoprotein content of the major lipoproteins [1].

Table 1. Characteristics of Lipoproteins

Lipoprotein	Size (nm)	Density (g/mL)	Apolipoprotein(s)
Chylomicron	75-1200	<0.950	B48, C, E
VLDL	30-80	0.950-1.006	B100, C, E
IDL	25-35	1.006-1.019	B100, E
LDL	18-25	1.019-1.063	B100
Lp(a)	25-35	1.050-1.100	B100, apo(a)
HDL	8-11	1.063-1.210	A, C, E

Lipoprotein(a), discovered in 1963 [2], is an LDL-like particle formed by the covalent addition of a unique glycoprotein, apolipoprotein(a) [apo(a)], to apolipoprotein B (apo B) of LDL (Fig. 1) [3,4]. Interestingly, Lp(a) is only found in humans and Old-World primates [5]. Several studies have found Lp(a) to be a significant, independent predictor of cardiovascular disease [6], and although the strength of association appears to be modest, the relative risk associated with Lp(a) may be attenuated by poor quality assays, especially inaccuracies associated with the use of antibodies that recognize repeating kringle 4 type 2 peptides, as described below.

Lipoprotein(a) concentration in serum is largely genetically determined [5], and therefore serum concentrations remain relatively constant over time. Concentrations are only marginally influenced by diet, exercise, and statins or other lipid-lowering drugs [7]. However, newer therapeutic agents offer hope for greater Lp(a) reduction in patients with elevated serum concentrations.

The atherogenicity of Lp(a), like LDL, is attributed to its deposition in atherosclerotic plaque and, to a prothrombotic effect related to apo(a)'s structural similarity to (and inhibition of) plasminogen [5]. Lp(a) also accumulate oxidized phospholipids, and like oxidized LDL, may be taken up by macrophages and contribute to foam cell formation [8]. A role for Lp(a) in normal physiology is unknown, but one theory is that Lp(a) binding to fibrin may deliver cholesterol to injury sites to promote wound healing [5].

Plasminogen contains an active protease domain and five highly conserved structural domains called “kringles”, so named because of its similarity with the Danish pastry.

Kringles are a loop structure formed by internal disulfide bonds. Apolipoprotein(a), the characteristic protein that defines Lp(a), shares considerable homology with plasminogen including an inactive protease domain, one copy of kringle V (KV), and multiple copies of kringle IV (KIV) [5]. The KIV domains of apo(a) are classified as ten different types: KIV-type 1 (KIV-1) to KIV-type 10 (KIV-10). It is the variable number of KIV type 2 (KIV-2) repeats between individuals that result in a significant size heterogeneity in the apo(a) protein, with up to 51 repeats and a variable molecular weight of 300 to 800 kDa [5,9]

Most of the Lp(a) in serum is found within the 1.05 to 1.10 g/mL density range [11], overlapping with the densities of HDL and LDL particles. Lp(a) density heterogeneity is attributable to particle composition.

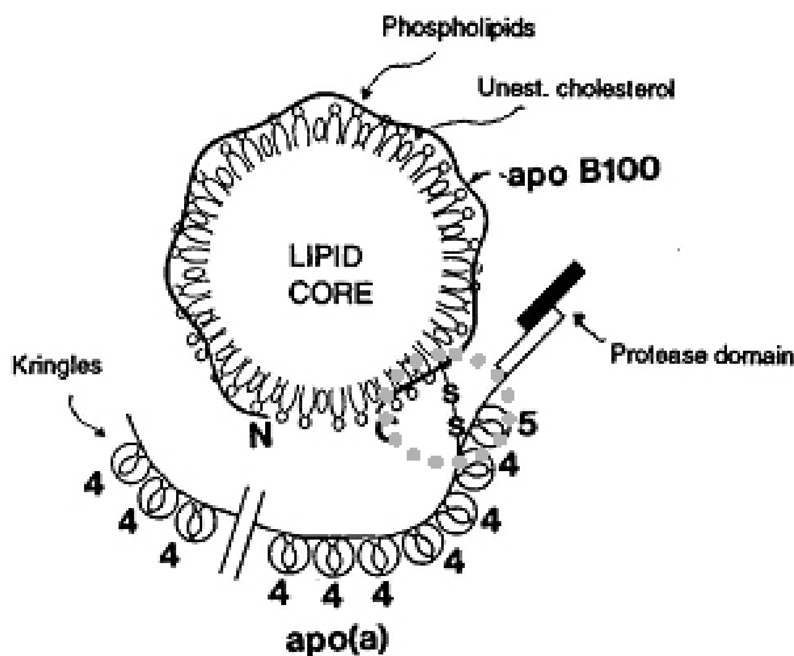


Figure 1. Structure of lipoprotein(a) [From Reference 12]

Measurement of Lipoprotein(a)

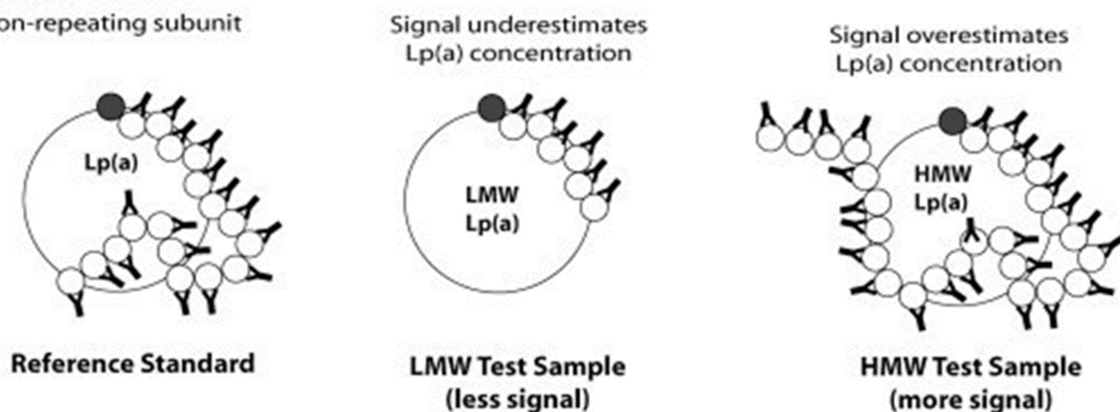
The structural homology of Lp(a) and plasminogen and the repeating KIV-2 subunits presents a challenge for the accurate measurement of Lp(a). Immunoassays are widely used for clinical measurement of Lp(a), but unfortunately, current immunoassays appear to be influenced by apo(a) size polymorphism. Most used in the clinical and research laboratories are immunoturbidimetric (ITA) and immunonephelometric (INA) assays. These methods, while relatively fast and precise, are subject to bias due to KIV-2 repeats and nonspecific light scattering.

The best design for an accurate immunoassay is a sandwich-type ELISA using monoclonal or polyclonal anti-apo(a) antibodies for capture and monoclonal or polyclonal anti-apo B for detection. This method is more specific than ITA or INA because it includes two antibodies, and the use of apo B for detection eliminates the

problem of apo(a) size polymorphisms. Antibodies generated for capturing apo(a) need not discriminate against different apo(a) size isoforms. Figure 2 illustrates how the number of kringle 4 repeats influences the strength of signal of immunoassays. A person with a greater number of k4t2 repeats relative to the calibrator will overestimate Lp(a) concentration while smaller apo(a) size will provide a falsely low concentration. Most commercially available ITAs select different calibrators for each level to try and minimize this effect; however, although there is a correlation between concentration and apo(a) size, there is considerable heterogeneity, and many results will remain biased. This is shown in Figure 3.

Panel A: Apo(a) size-sensitive assay (using antibodies to repeating K-IV subunits)

Repeating subunit
Non-repeating subunit



Panel B: Apo(a) size-insensitive assay (using antibodies to a non-repeating K-IV subunit)

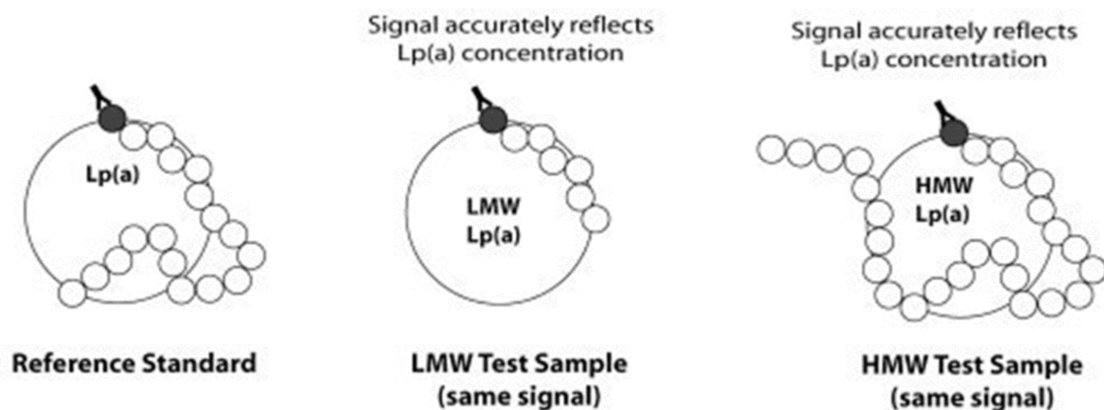


Figure 2: Bias due to apo(a) size isoforms is eliminated with apo(a) size-insensitive assay such as the Sun Diagnostics Lp(a) ELISA. From: Longenecker JC, et al. Clin Chim Acta. 2008;397:36.

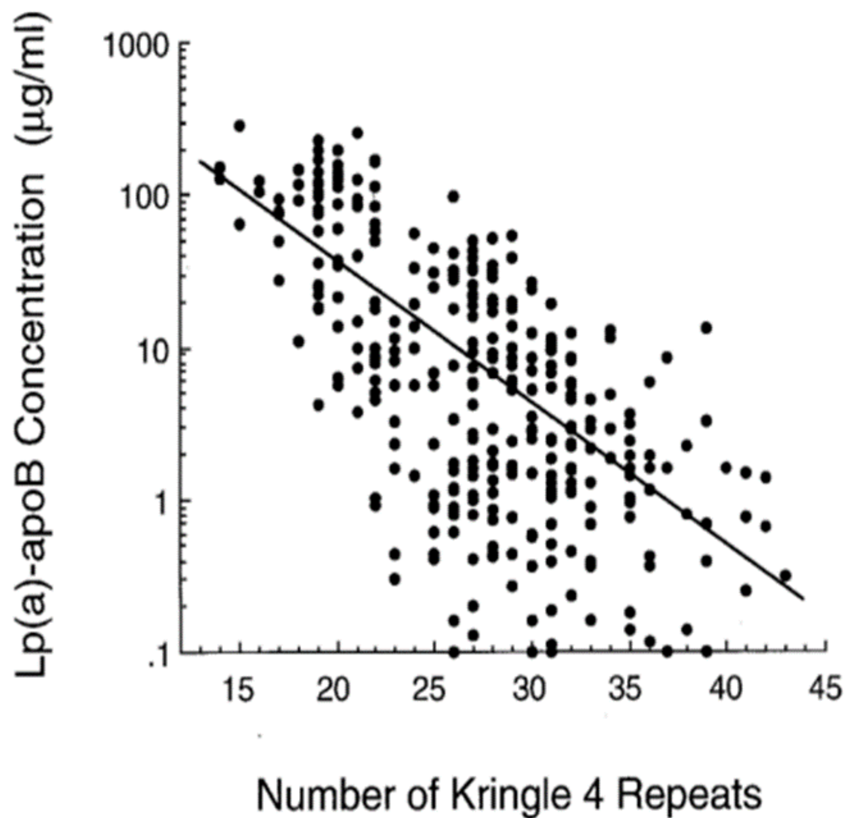
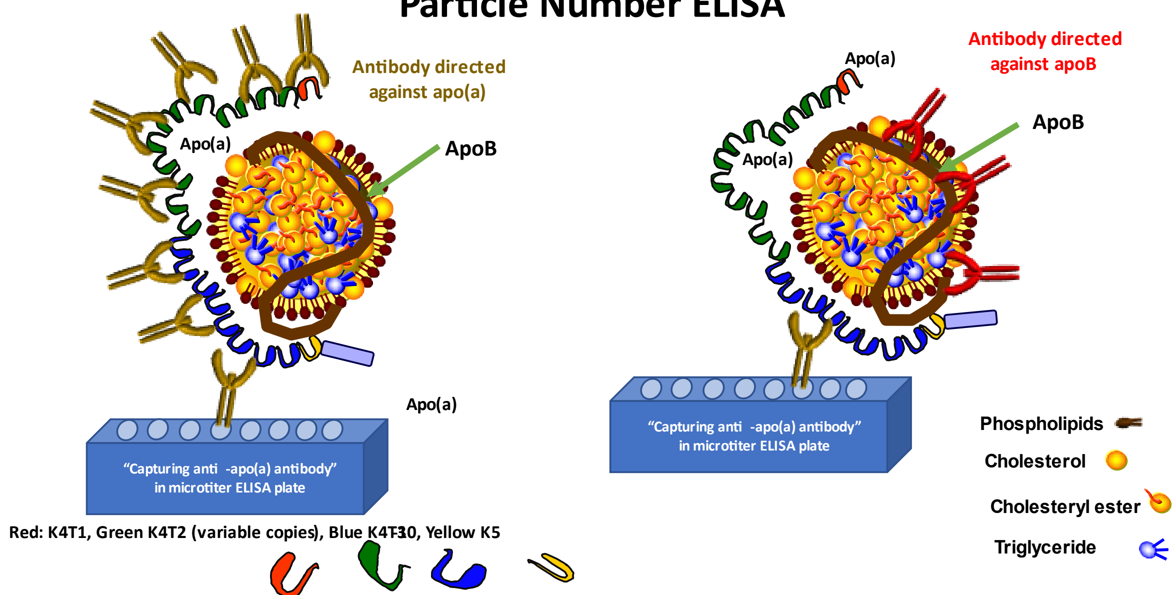


Figure 3: Association between k4t2 repeats (apo(a) size) and Lp(a)-apo B (Lp(a) Particle Number), From: Puckey LH et al. Human Molecular Genetics, 1997.

Figure 4: Lipoprotein(a) Measurement with Sun Dx Lp(a) Particle Number ELISA



Lp(a) concentration is generally expressed as total mass of Lp(a) as milligrams per deciliter (mg/dL). It is now recognized that risk is better characterized by particle number, generally measured as nanomoles per liter (nmol/L). The Sun Diagnostic Lipoprotein(a) Particle Number ELISA Kit contains reagents and microtiter plate(s) with serum-based calibrators and controls. The kit provides 1 mL vials of six liquid, ready-to-use calibrator concentrations. The target concentrations of these calibrators are approximately 0, 50, 100, 200, 300, 400 nmol/L (approximately 0, 21, 42, 83, 125, and 167 mg/dL).

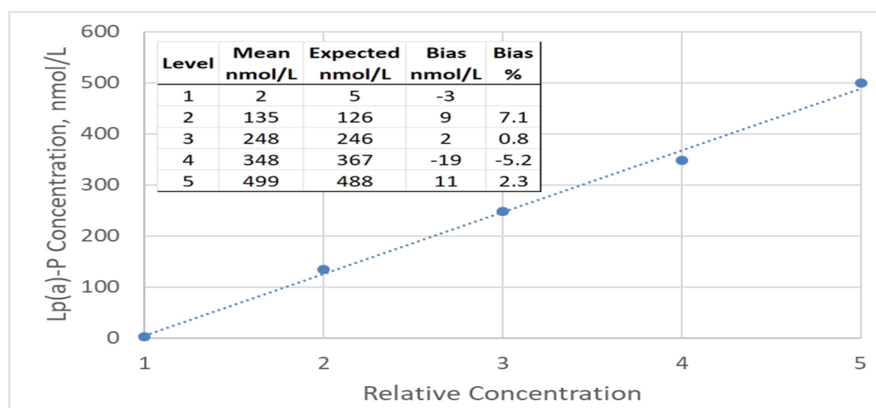
In the Sun Diagnostics Lp(a) assay, sample is diluted with buffer, transferred to the microtiter plate (coated with a proprietary anti-apo(a) monoclonal antibody) along with calibrators and quality controls, and incubated for one hour at room temperature. The plate is then washed and anti-apoB-HRP conjugate is added. After incubation at room temperature for one hour the plate is again washed and substrate is added to activate the HRP. The reaction is stopped after 30 minutes, and absorbance is read at 450/650 nm. Lipoprotein (a) particle concentration is determined from a standard curve.

Validation Data

Total Precision was assessed by measuring 10 replicates each of five serum pools over five plates over three days. The imprecision at the extreme low end of the analytical range was 12.1%; the imprecision in the midrange of the assay varied from 6.9% to 7.7%.

	Very Low Pool	Low Pool	Medium Pool	High Pool
Mean	25.0	76.9	113	318
SD	3.03	5.33	8.74	24.5
CV	12.1%	6.9%	7.7%	7.7%

A linearity study across the entire analytical measurement range was assessed using five samples prepared from high and low serum pools by intermixing. The range of samples tested was from 5 to 500 nmol/L (2-208 mg/dL). The observed values were compared to the expected values.



Level	Mean nmol/L	Expected nmol/L	Bias nmol/L	Bias %
1	2	5	-3	
2	135	126	9	7.1
3	248	246	2	0.8
4	348	367	-19	-5.2
5	499	488	11	2.3

The estimations of the Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantitation (LoQ) were performed according to CLSI guideline EP17-A, Protocols for Determination of Limits of Detection and Limits of Quantitation, by running 38 to 50 replicates of "blank" serum pool and two serum pools with low Lipoprotein(a) concentration.

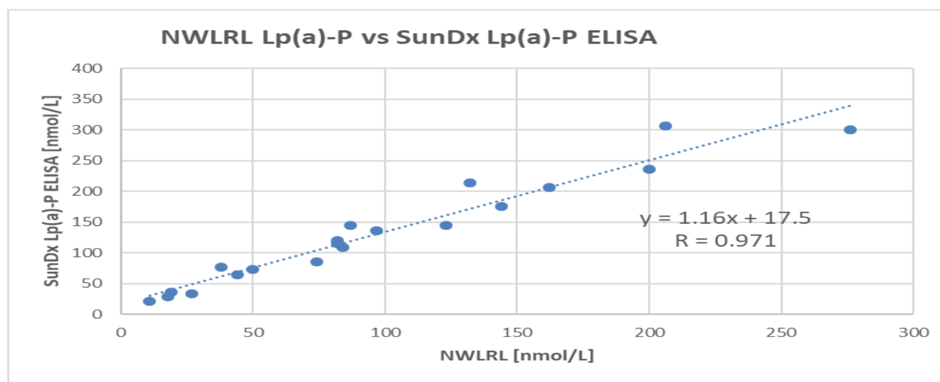
The limit of the blank (LOB) is the concentration of a sample that does not contain any analyte. Delipidized human serum (Golden West Biologicals) was assayed 50 times (10 replicates per plate over five days). The LOD is the lowest measurable concentration of analyte by an assay. Two human serum samples with low concentrations were assayed ~40 times over four days.

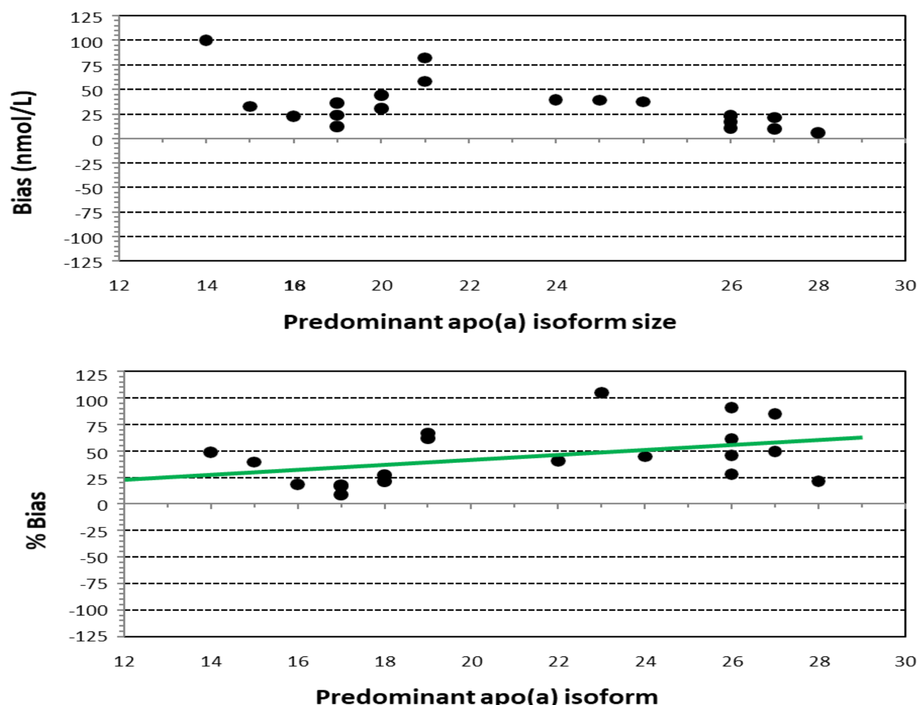
The LOB was 1 nmol/L; The LoD for Lp(a)-P ELISA was 13 nmol/L;

The LOQ is the lowest measurable concentration of analyte that is measured with reasonable precision (<20%CV). The two human serum samples with low Lp(a) concentrations used for the LoD experiment both had CVs <20%. Therefore, the LOQ is equal to the LOD: 13 nmol/L.

Method Comparison

In order to assess accuracy and bias due to k4t2 size isoforms we purchased 20 samples from the Northwest Lipid Metabolism and Diabetes Research Laboratory at the University of Washington (NWLDRL; Seattle, WA). These samples were selected to provide a range of Lp(a) concentrations and apo(a) size isoforms. The 0.5 mL samples were stored and handled as instructed. An accuracy-based target value in nmol/L was provided for each sample by the NWLDRL using their ELISA reference method calibrated with WHO/IFCC SRM-2B Reference Material. Apo(a) isoform size was also determined by NWLDRL using agarose gel electrophoresis followed by immunoblotting; the isoforms were expressed as total number of kringle 4 type 2 repeats. Data were submitted to NMLDRL for statistical evaluation, Linear regression analysis for our Lp(a)-P ELISA (Y) versus the NWLDRL comparison method (X) produced a line with a slope of 1.16 and an intercept of 18 nmols/L, demonstrating a calibration bias that was corrected by adjusting calibrator values. Bias plot of Sun Diagnostic Lp(a)-P concentration minus NWLDRL concentration(Y), versus number of k4t2 repeats (X) was not statistically significant ($r^2 = 0.174$, $p>0.05$), demonstrating that the assay was not biased by k4t2 repeats.





Interferences from icterus, lipemia, hemolysis, proteins, rheumatoid factor (RF), human anti-mouse antibodies (HAMA), apoB, and plasminogen were evaluated according to CLSI guidelines. Briefly, screening experiments were first conducted comparing high versus low interference pools at low and high Lp(a) concentrations. No significant interference was found at unconjugated or unconjugated bilirubin concentrations up to 40 mg/dL nor with hemolysis up to 1000 mg/dL hemoglobin. No significant lipemia interference was found at triglyceride concentrations up to 1500 mg/dL in samples with low (82 nmol/L) Lp(a) but significant interference was observed in a sample with an Lp(a)-P of 213 nmol/L at an estimated triglyceride concentration of 580 mg/dL. No significance interference was seen with HAMA at a titer up to 1:640, RF up to 982 IU/mL, plasminogen up to 80 mg/dL, and apo B (LDL) up to 200 mg/dL.

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