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Determination of Lipoprotein(a): Evaluation of Three Methods

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Summary: Lipoprotein(a) (Lp(a)) is a strong independent risk factor for premature atherosclerosis. Structurally, Lp(a) closely resembles LDL. Its protein moiety contains apolipoprotein B-100 and apolipoprotein(a). We evaluated two commercial enzyme immunoassays (EIAs) and an immunoradiometric assay (IRMA) for Lp(a). The three assays differed in their design and they used different antibodies. In the immunoradiometric assay, two different monoclonal antibodies were used. In the first EIA, monoclonal anti-apolipoprotein(a) was bound to the solid phase and Lp(a) was detected with polyclonal anti-apolipoprotein B (Lp(a):B-EIA). In the second EIA, polyclonal anti-apolipoprotein(a) was used as capturing antibody and as detecting antibody (apo(a)-EIA). Ninety three plasma samples were assayed for Lp(a) with the three methods. The best correlation was obtained between the IRMA and the Lp(a):B-EIA ($r = 0.971$). Correlations between the apo(a)-EIA on the one hand and the IRMA or the Lp(a):B-EIA on the other hand were 0.889 and 0.836, respectively. The methods significantly differed in their calibration. This resulted in different mean Lp(a) concentrations. When tested against purified Lp(a), the apo(a)-EIA appeared accurately calibrated, whereas the IRMA and the Lp(a):B-EIA overestimated Lp(a) by approximately twofold. In the Lp(a):B-EIA, the detecting antibody is directed against apolipoprotein B. The Lp(a):B-EIA is, therefore, not affected by the apolipoprotein(a) size polymorphism. This allows expression of the concentration of Lp(a):B complexes on a molar basis. In contrast, the polyclonal antibody-based apo(a)-EIA measures the concentration of apolipoprotein(a) antigen, and may, therefore, be susceptible to inter- and intra-individual polydispersity of apolipoprotein(a) and Lp(a) particles. The data underline that both design and calibration of Lp(a) immunoassays are crucial.

Introduction

Lipoprotein(a) (Lp(a)) is an LDL-like particle. Its protein moiety consists of apolipoprotein B and apolipoprotein(a) which are linked by a disulphide bond (1–5). There is ample evidence that Lp(a) is a strong independent predictor for coronary artery disease (6–11), stroke (12, 13), and retinal vascular occlusion (14). This has raised the demand for convenient and reliable means of quantifying Lp(a). So far, Lp(a) has been measured by a variety of methods such as radial immunodiffusion (15), electroimmunoassay (6, 16), zone immunoelectrophoresis (16), nephelometry (17), radioimmunoassay (18), and enzyme immunoassays (19–22).

Apolipoprotein(a) is homologous with plasminogen (23–25). This has stimulated speculation that Lp(a) has an inhibitory role in fibrinolysis (26). Apolipoprotein(a) contains a variable number of plasminogen *kringle* 4-type units, and one copy of each a *kringle* 5 and a protease-like domain. Due to variations in the number of *kringle* 4-type repeats (27), apolipoprotein(a) displays size polymorphism (28, 29). The relative masses of the apolipoprotein(a) isoforms range from M_r 400 000 to M_r 700 000. Because the *kringle* 4-type repeats share a high degree of homology, apolipoprotein(a) is expected to contain a large number of 'repetitive' epitopes. Therefore, depending on the assay type and antibody specificity, the size

polymorphism of apolipoprotein(a) may influence the measurement of Lp(a).

We here present results of a comparison of three commercial methods for the determination of Lp(a). The first method was an immunoradiometric assay (IRMA), based on two monoclonal antibodies recognizing apolipoprotein(a). The second method was an enzyme immunoassay (EIA), in which monoclonal anti-apolipoprotein(a) was used as capturing antibody, and detection was carried out with polyclonal anti-apolipoprotein B. The third method was also an EIA, which made use of polyclonal anti-apolipoprotein(a) as capturing as well as detecting antibody.

Materials and Methods

Two site immunoradiometric assay for apolipoprotein(a)

Antibodies and reagents were obtained as components of a test kit from Pharmacia Diagnostics AB (Uppsala, Sweden). The assay was carried out according to the instructions of the supplier. It uses two monoclonal antibodies in excess. During one-step incubation, apolipoprotein(a) reacts with the capturing antibody (cl 280) coated to Sepharose-particles, as well as with the ^{125}I-labelled tracer (cl 356). The antigen/antibody sandwich is then separated from excess tracer by centrifugation and decantation, and the radioactivity in the pellet is counted. The assay was calibrated using standards provided with the kit. Results are reported in U/l. According to the manufacturer, U/l should correspond to mg/l Lp(a).

Enzyme immunoassays

Two non-competitive enzyme immunoassays were used. In the first EIA, Lp(a) reacts with monoclonal anti-apolipoprotein(a) on the solid phase and is then detected with polyclonal anti-apolipoprotein B. This method will be referred to as Lp(a):B-EIA. Reagents and microwell plates required for the Lp(a) : B-EIA were purchased from Byk Sangtec Diagnostika (Dietzenbach, Germany). The measurements were performed according to the manufacturer's instructions. In the second EIA, polyclonal anti-apolipoprotein(a) was used as the capturing antibody, and peroxidase-labelled anti-apolipoprotein(a) was used as the detecting antibody. This assay was obtained from Immuno Diagnostika (Heidelberg, Germany). It will be referred to as apo(a)-EIA. Each EIA was calibrated using standards provided by the manufacturer. Absorbances were read on a Titertek MCC 340 microplate reader (Flow Laboratories). All EIAs were run in duplicate.

Plasma samples

Analyses were carried out in specimens from outpatients referred to the Frankfurt University Hospital for lipoprotein analysis. Blood was drawn into tubes containing EDTA · K_2 (final concentration: 1.5–2 g/l). Plasma was recovered by centrifugation. Samples were stored at −20 °C, on average, for one, seven, and eight weeks before they were analysed with the IRMA, the apo(a)-EIA, and the Lp(a):B-EIA, respectively. Maximum storage times were 3 weeks, 16 weeks, and 17 weeks for the IRMA, the apo(a)-EIA, and the Lp(a):B-EIA, respectively. Previous studies in which samples with intermediate and high Lp(a) concentrations were stored at −20 °C for four weeks showed that none of the methods was significantly affected by freezing and thawing.

Purification of Lp(a)

Lp(a) was prepared from the regenerate fluid of a dextran sulphate-based LDL-apheresis system (Kanegafuchi MA 01 – Liposorber LA 15) by sequential ultracentrifugation (1.050 kg/l and 1.125 kg/l) and gel filtration (30).

Electrophoretic methods

SDS-PAGE (T = 5%, C = 2.7%) was run in a BioRad Mini Protean II cell using the buffer system of *Laemmli* (31). The samples were prepared by mixing 10 µl plasma, 50 µl electrophoresis buffer (containing 40 g/l sodium dodecyl sulphate (SDS) and 31 g/l dithiothreitol) and 5 µl β-mercaptoethanol. The mixtures were boiled for 5 minutes and 10 µl aliquots were applied to the gels. No adjustment was made for individual Lp(a) concentrations.

Electrotransfer onto nitrocellulose (32), and immunoperoxidase staining were performed as described (33). In probing the blots, monoclonal anti-apolipoprotein(a) antibodies cl 280 (IRMA capturing antibody) and cl 356 (IRMA tracer antibody, both from Pharmacia Diagnostics AB, Uppsala) were used at final concentrations of 0.66 mg/l. Biotinylated anti-mouse IgG and avidin : biotinylated horseradish peroxidase complex were from Vector Laboratories (Burlingame, CA) and used as recommended by the manufacturer.

Statistics

Linear regression analyses and *Wilcoxon*'s matched-pairs signed-ranks test were carried out with SPSS^X (release 2.0+ on UNISYS 1100/91). Non-parametric regression analysis was performed according to *Passing & Bablok* (34).

Results

Two site immunoradiometric assay (IRMA)

As shown in figure 1, the two monoclonal antibodies used in the IRMA produced similar, but not identical immunoblotting patterns. Both antibodies reacted with the major apolipoprotein(a) isoforms and a large number of minor bands which are probably due to proteolytic fragmentation of apolipoprotein(a) (35, 36). Unlike the tracer antibody (cl 356), the solid phase antibody (cl 280) appeared to react with protein bands having relative molecular masses of approximately M_r 90 000. In order to study this cross-reactivity without interference from the apolipoprotein(a) proteolytic fragments, we analysed a sample virtually lacking Lp(a). In this case, cl 280 detected two bands, whereas cl 356 detected none (fig. 1, panels c and d). When purified plasminogen was analysed by immunoblotting, an identical pattern was obtained, confirming that the capturing antibody of the IRMA reacted with plasminogen (not shown). We then examined whether this cross-reactivity could cause competition of Lp(a) and plaminogen for solid phase sites. In these studies, purified Lp(a) was mixed with plasminogen at different ratios, and Lp(a) was determined in the mixtures using the IRMA. As shown in figure

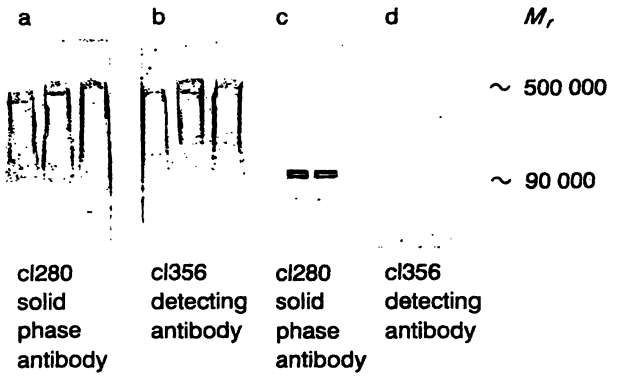

Fig. 1. Immunoblotting of apolipoprotein(a). Detection of apolipoprotein(a) was performed with monoclonal antibodies cl 280 and cl 356. In the IRMA, cl 280 and cl 356 are used as solid phase and detecting antibody, respectively.
Panel a contains analyses in three samples with 340, 420, and 520 U/l Lp(a) (from left to right) probed with cl 280.
Panel b shows the same samples probed with cl 365.
In panels c and d a so called Lp(a) 'negative' sample (Lp(a) < 10 U/l) was analysed in duplicate. c was probed with cl 280, d with cl 365.
The experiments demonstrate that cl 280 (the solid phase antibody in the IRMA), but not cl 356 (the detecting antibody) recognized plasminogen.

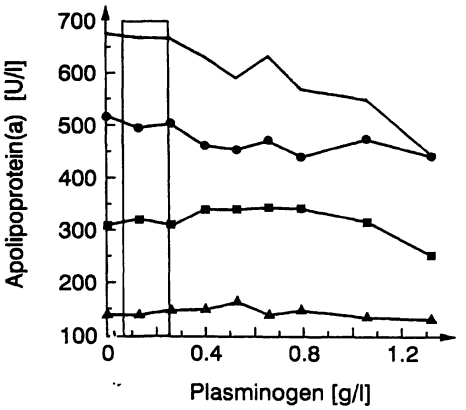

Fig. 2. IRMA for apolipoprotein(a): Interference from plasminogen. Purified Lp(a) was mixed with plasminogen at different ratios, and the mixtures were assayed for Lp(a) by IRMA (lot No. 28271). In the presence of high plasminogen concentrations, high Lp(a) concentrations were underestimated, indicating that plasminogen can displace Lp(a) from matrix binding sites.
Gray area: normal range for plasminogen.

2, Lp(a) readings decreased in the presence of abnormally high plasminogen concentrations, indicating that plasminogen can displace Lp(a) from matrix binding sites. However, up to 800 U/l Lp(a) and 400 mg/l plasminogen (which is about threefold the normal concentration) there was no displacement of Lp(a) from the solid phase.

Intermethod comparison

Figure 3 shows the results of Lp(a) determinations performed with the three methods in 93 plasma samples. There was an excellent correlation between the IRMA and the Lp(a):B-EIA (r = 0.971). Correlation coefficients were lower between the IRMA and the apo(a)-EIA, and between the Lp(a):B-EIA and the apo(a)-EIA (0.889 and 0.836, respectively).

Regression lines were calculated using the least squares method and the non-parametric method proposed by *Passing & Bablok* (34). For each regression line, the non-parametric method of *Passing & Bablok* resulted in lower absolute values for the intercepts than the least squares method (tab. 1). Regardless of whether regression lines were estimated with the least squares or the non-parametric method, their slopes consistently indicated that the Lp(a):B-EIA and the IRMA corresponded well, whereas the apo(a)-EIA resulted in significantly lower concentrations. This is also evident if means and medians are compared (tab. 2). The differences between the apo(a)-EIA and the other methods are striking. The difference between the Lp(a):B-EIA and the IRMA is smaller, but still significant when tested with *Wilcoxon*'s matched-pairs signed-ranks test (p = 0.019).

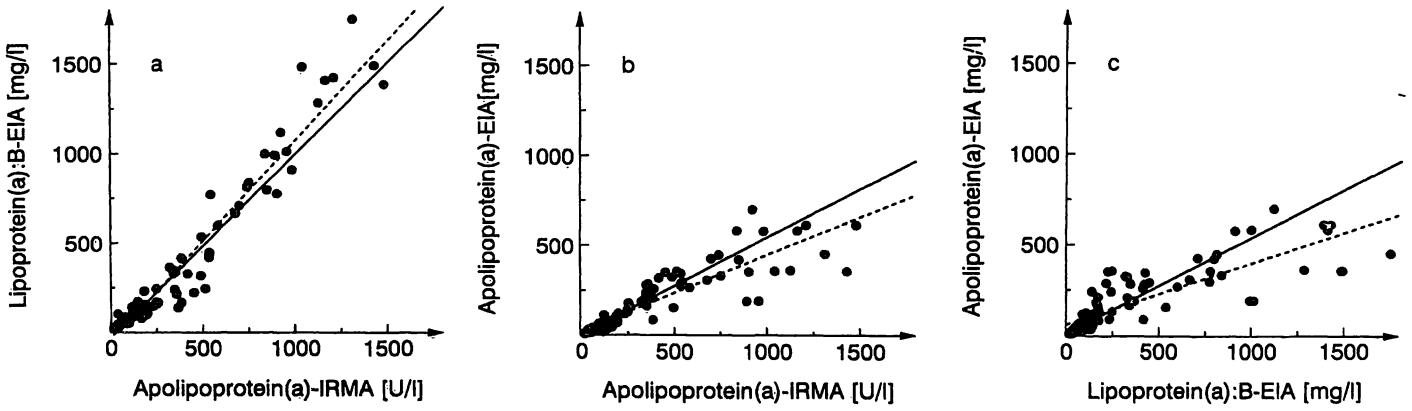

Fig. 3. Comparison of two EIAs and an IRMA for Lp(a). Determinations were carried out in parallel in 93 plasma samples. Regression lines were estimated using the least squares method (dashed) and the non-parametric method according to *Passing & Bablok* (solid). For slopes and intercepts see table 1.

Tab. 1. Comparison of three immunoassays for Lp(a). Regression lines calculated with the least squares method and with the non-parametric method according to *Passing & Bablok* (34).

Method	Least squares [mg/l]			Non-parametric [mg/l]	
	a[1]	b[1]	r[2]	a[1,3]	b[1,3]
Lp(a):B-EIA vs IRMA	1.12	−52	0.971	1.02 (0.94/1.09)	−17 (−29/−3)[4]
apo(a)-EIA vs IRMA	0.42	29	0.889	0.54 (0.50/0.60)[5]	−2 (−7/ 4)
apo(a)-EIA vs Lp(a):B-EIA	0.34	58	0.836	0.54 (0.46/0.62)[5]	4 (−1/ 14)

[1] a = slope; b = intercept
[2] *Pearson*'s correlation coefficient
[3] in parentheses: lower and upper limits of the 95% confidence intervals as calculated according to *Passing & Bablok* (34)
[4] intercept significantly different from zero ($p < 0.05$)
[5] slope significantly different from unity ($p < 0.05$)

Tab. 2. Comparison of immunoassays for Lp(a) in 93 plasma samples.

Method	Mean	SD	Median	Range	68th percentile of distances from the median
IRMA [U/l]	353	363	191	17−1480	192
Lp(a):B-EIA [mg/l]	344	420	142	17−1748	186
apo(a)-EIA [mg/l]	176	171	104	9− 701	122

Intra-assay and inter-assay CVs are summarized in tables 3 and 4, respectively. They show that all methods worked with satisfactory precision.

In an attempt to explain the systematic differences between the methods, we analysed standards and controls included in the test kits with each of the other methods, except that the standards used in the IRMA and the Lp(a):B-EIA were not tested, because they were provided in a pre-diluted form. Results are shown in table 5. They show that the assays clearly differ in their standardization. For instance, when the calibrator of the apo(a)-EIA was analysed with the IRMA and with the Lp(a):B-EIA, results were, on average, 1.5 times and 1.6 times higher than the expected values, respectively. This was consistent with the higher Lp(a) concentrations produced by the IRMA and the Lp(a):B-EIA, compared with the apo(a)-EIA.

To determine which of the assays was the most accurate, two highly purified Lp(a) preparations were analysed with the three methods. The Lp(a) concentrations in these preparations were calculated from their cholesterol content, assuming cholesterol to constitute 44.5% of the Lp(a) particle mass. This resulted in Lp(a) concentrations of 438 mg/l and 417 mg/l, respectively. With the IRMA, we found 1102 mg/l

Tab. 3. Intra-assay precision (n = 8) of three immunoassays for the determination of Lp(a).

Method	Mean	SD	CV
IRMA [U/l]			
sample 1	71.1	7.0	9.85
sample 2	123.9	7.5	6.05
sample 3	552.6	16.4	2.97
Lp(a):B-EIA [mg/l]			
sample 1	37.1	2.6	6.95
sample 2	69.6	1.6	2.36
sample 3	358.0	8.1	2.27
Apo(a)-EIA [mg/l]			
sample 1	50.6	3.1	6.18
sample 2	106.6	8.6	8.10
sample 3	264.6	14.1	5.34

Tab. 4. Inter-assay precision of three immunoassays for the determination of Lp(a).

Method	n	Mean	SD	CV
IRMA [U/l]				
Control Low[1]	15	129.9	9.0	6.9
Control High[1]	15	393.8	29.8	7.6
Lp(a):B-EIA [mg/l]				
sample A[2]	9	96.0	5.1	5.3
sample B[2]	9	363.0	22.2	6.1
sample C[2]	9	539.7	34.5	6.4
Apo(a)-EIA [mg/l]				
Low Level Control[3]	7	306.2	24.7	8.1
High Level Control[3]	7	414.1	31.2	7.5

[1] purchased from Pharmacia AB
[2] Because control sera were not available from Byk Sangtec Diagnostika, frozen plasma samples were used to estimate the inter-assay precision of the Lp(a):B-EIA.
[3] purchased from Immuno Diagnostika

Tab. 5. Standardization of Lp(a) immunoassays. Analyses of commercial standards, controls, and of purified Lp(a) with three different assays.

Material	Expected concentrations	Measured concentrations					
		IRMA		Lp(a):B-EIA		apo(a)-EIA	
			m/e[1]		m/e[1]		m/e[1]
IRMA Control High	437	382	0.87	218	0.50	165	0.38
IRMA Control Low	132	126	0.95	103	0.78	60	0.45
Apo(a)-EIA Low Level Control	325	515	1.58	569	1.75	332	1.02
Apo(a)-EIA High Level Control	425	661	1.56	535	1.26	467	1.10
Apo(a)-EIA calibrator 2	70	104	1.49	155	2.21	–	n. d.[2]
Apo(a)-EIA calibrator 3	160	233	1.46	288	1.80	–	n. d.
Apo(a)-EIA calibrator 4	360	496	1.38	437	1.21	–	n. d.
Apo(a)-EIA calibrator 5	700	1125	1.61	837	1.20	–	n. d.
Purified Lp(a) preparation 1	438	1102	2.52	760	1.74	460	1.05
Purified Lp(a) preparation 2	417	1087	2.61	710	1.70	428	1.03

Standards and controls provided by the manufacturers of three Lp(a) assays and two different Lp(a) preparations were analysed with each method. The expected concentrations are those specified by the manufacturer of the respective standard or control. In the case of purified Lp(a), the expected value was calculated from cholesterol, assuming that cholesterol constitutes 44.5% of the Lp(a) particle mass.

[1] m/e is the ratio of the measured to the expected concentration. For instance, the Low Control supplied with the IRMA test kit was specified to contain 132 U/l apolipoprotein(a). When this control was analysed with the Lp(a):B-EIA, 103 mg/l Lp(a) were found, and the m/e ratio was 0.78. Data for the calibrators provided with the IRMA and the Lp(a):B-EIA are not presented because these materials were provided in a pre-diluted form.

[2] n. d. = not determined

and 1087 mg/l Lp(a) in the two isolates which is more than 2.6 times greater than the expected concentration (cf. tab. 5). With the Lp(a):B-EIA, we obtained 760 mg/l and 710 mg/l Lp(a) which is 1.7 times more than expected. Finally, according to the apo(a)-EIA, the two Lp(a) preparations contained 460 mg/l and 428 mg/l Lp(a), respectively. Thus, the IRMA and the Lp(a):B-EIA considerably overestimated Lp(a), whereas the apo(a)-EIA was accurately calibrated.

Discussion

We evaluated three commercial methods for the determination of Lp(a): an IRMA, and two non-competitive EIAs. Precision was satisfactory for all methods. The Lp(a):B-EIA worked with slightly lower CVs than the other methods.

The apo(a)-EIA yielded markedly lower Lp(a) values than the IRMA and the Lp(a):B-EIA. The differences between the methods were attributable to different calibrations of the assays. Whereas both the IRMA and the Lp(a):B-EIA overestimated their analyte, the apo(a)-EIA appeared accurately calibrated when tested against highly purified Lp(a). This is in line with earlier results from our laboratory in which the specification of a standard obtained from the manufacturer of the apo(a)-EIA was in excellent agreement with our own estimates (37). According to current opinion, Lp(a) concentrations above 300 mg/l are considered to be associated with a 2–3 fold increase in coronary risk. If one applies this threshold value to the current study, 17 out of 93 samples would be classified differently using either the apo(a)-EIA or the IRMA (cf. tab. 6). Similarly, 15 out of the 93 samples would be classified ambiguously with the apo(a)-EIA or with the Lp(a):B-EIA. This underlines that a consensus on the standardization of Lp(a) immunoassays is regardly needed.

Tab. 6. Classification of 93 plasma samples according to Lp(a) concentrations obtained with different Lp(a) immunoassays.

Method	IRMA [U/l]		Lp(a):B-EIA [mg/l]	
	< 300	≥ 300	< 300	≥ 300
Apo(a)-EIA < 300 mg/l	55	17	59	13
Apo(a)-EIA ≥ 300 mg/l	0	21	2	19

The three assays evaluated in this study differ in their design and the antibodies used. The IRMA is based on two monoclonal antibodies. The capturing antibody cross-reacts with plasminogen. However, because the detecting antibody does not recognize plasminogen, the assay is specific for Lp(a). Moreover, we have ruled out the possibility that, under normal conditions, competition of plasminogen and Lp(a) for the solid phase resulted in falsely low Lp(a) concentrations. Three to four times the normal plasminogen concentration and high Lp(a) concentrations have to be present, before any relevant interference occurs.

The use of monoclonal antibodies in Lp(a) immunoassays may entail several problems. More than 20 genetically determined apolipoprotein(a) isoforms have been described so far. Their relative masses range from M_r 400 000 to M_r 700 000. Structurally, apolipoprotein(a) isoforms differ by the number of *kringle* 4 elements, which are homologous, but not identical. Only one isoform of apolipoprotein(a) containing 37 *kringle* 4 repeats has been cloned. It is, therefore, not known which types of *kringle* 4 are repeated or whether some of them are missing in smaller apolipoprotein(a) isoforms. Furthermore, there may be sequence heterogeneity in *kringle* 5 or the protease domain. Thus, it is conceivable that certain apolipoprotein(a) isoforms are not recognized by monoclonal antibodies. However, these considerations do obviously not apply to the IRMA used in this study. First, the good correlation between the IRMA and the Lp(a):B-EIA makes it unlikely that one of the two assays fails to recognize a particular apolipoprotein(a) isoform. Second, among 93 samples studied none was found which gave a substantially lower signal in the IRMA (two monoclonal antibodies) than in the apo(a)-EIA (two polyclonal antibodies). Third, when antibodies cl 280 and cl 356 were used in immunoblotting experiments with different plasma samples they produced patterns identical to those obtained with polyclonal antisera (*März, W.*, unpublished).

In this study, the Lp(a):B-EIA correlated better with the IRMA than the apo(a)-EIA. Most likely this is due to differences in design of the EIAs. The apo(a)-EIA uses the same polyclonal anti-apolipoprotein(a) as tracer and as capturing antibody. Strictly speaking, this assay measures apolipoprotein(a) mass concentration. Hence it may be influenced by the apolipoprotein(a) genetic polymorphism. Beyond this polymorphism, there is a tremendous intra-individual heterogeneity of lipoprotein(a) particles (35). It is, therefore, not very likely that apolipoprotein(a) can be measured on a molar basis, when only polyclonal anti-apolipoprotein(a) is used. In contrast, because it is suggested that there is one molecule of apolipoprotein B per Lp(a) particle, the Lp(a):B-EIA will reflect the molar concentration of intact Lp(a):B complexes, irrespective of the apolipoprotein(a) isoform. A potential drawback of assays using anti-apolipoprotein B as detecting antibody is that Lp(a) may be overestimated due to the presence of complexes between Lp(a) and LDL (38, 39). However, in solid phase immunoassays, this may not be a major problem, because incubations are performed in the presence of non-ionic detergents which may dissociate Lp(a)-LDL complexes.

As mentioned before, the IRMA excellently correlated with the Lp(a):B-EIA. This suggests that not only the Lp(a):B-EIA, but also the IRMA estimated Lp(a):B particle concentration rather than apolipoprotein(a) mass concentration. Since two monoclonal antibodies for apolipoprotein(a) are used in the IRMA, the tracer antibody must recognize an epitope present once per apolipoprotein(a) molecule. Such an epitope is more likely to be located in *kringle* 5 or the protease-like domain of apolipoprotein(a) than in the repetitive *kringle* 4. As a consequence, the IRMA should be insensitive to variations in the number of *kringle* 4 repeats, i. e. apolipoprotein(a) size polymorphism.

In summary, these data show that there are still many unresolved problems concerning the measurement of Lp(a). Design and antibodies have strong impacts on Lp(a) assays. Methods reflecting particle concentration such as the IRMA or the Lp(a):B-EIA are insensitive to apolipoprotein(a) heterogeneity. In solid phase binding assays using anti-apolipoprotein(a) as capturing antibody and anti-apolipoprotein B as detecting antibody, cross-reactivity with plasminogen is not a major concern. In further attempts to standardize the measurement of Lp(a), major efforts should therefore be devoted to this type of assay. As results of Lp(a) assays may differ by more than 100% these attempts should be made soon.

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Note added in proof

On the basis of the results presented in this article, the calibration of the Lp(a):B-EIA will be revised by the manufacturer.