Atherosclerosis 289 (2019) 181–183

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Editorial

The challenges of measuring Lp(a): A fight against Hydra?

The Hydra of Lerna is a serpentine water monster in Greek mythology. The Hydra possessed many heads that had an enormous regenerative capacity: whenever a head was cut off, the Hydra would regrow two heads. Thus, it was not easy for the ancient hero Herakles to win against this constantly regenerating problem.

During recent decades, high serum levels of lipoprotein(a) (Lp(a)) turned out to be one of the strongest genetically determined risk factors for cardiovascular disease [1–4]. Lp(a) concentrations have been reported as mass of the entire particle (mass of apolipoprotein(a), apolipoprotein B-100, free cholesterol, cholesteryl ester, triglyceride, phospholipids and carbohydrate) in mg/dL, total apolipoprotein(a) particles number in nmol/L and cholesterol content in mg/dL [5]. Historically, the most common clinical reporting method has been in total mass (mg/dL), which has significant limitations in that variable amounts of each of the components may be different among patients which can then skew Lp(a) levels. In contrast, reporting values in molar concentrations of apolipoprotein(a) as nmol/L addresses many of these limitations of mass assays since it quantitates total apolipoprotein(a) particle number and is not dependent on molecular weight of Lp(a) *per se*. For this reason, the NHLBI Working group has recommended that mass assays be slowly phased out and assays reporting in molar concentrations be used to report clinical data on Lp(a) [6]. Finally, Lp(a) cholesterol assays have significant methodological issues and have not been validated to either accurately measure Lp(a)-cholesterol, particularly at low Lp(a) levels, nor to predict outcomes as well as mass or molar concentration assays, and are not currently recommended for clinical use [7,8]. Each of these assays has different "normal" values (mass < 30 mg/dL, molar concentration < 75 nmol/L and Lp(a)-cholesterol < 10 mg/dL), creating further clinical confusion.

Nonetheless, within this context of the current state of the art, additional methodological issues remain that relate to adequate manufacturer standardization of clinical assays irrespective of whether mass or molar concentration assays are used, which is the focus of this editorial and the accompanying paper [9]. Lp(a) contains multiple, identical Kringle IV type 2 repeats (KIV_2) that interfere with its accurate measurement in serum and reminds us of Hydra. The structural basis of the problem is the variable number of KIV_2 repeats, up to more than 40, encoded by the *LPA* gene [1,10]. Each of these repeats has a size of 5.6 kB, which results in a highly polymorphic copy number variation of both alleles. These repetitive structures cause the main problem for the measurement of the encoded protein: if an antibody is directed against this repetitive motif, the protein might be recognized by the antibody more than once, which makes a measurement in molar terms hardly possible. The epitopes on apolipoprotein(a) detected by most antibodies used in clinical assays are not well characterized and since almost all are polyclonal in nature almost certainly are directed against the repetitive KIV structure. This may result in a measurement bias where serum concentrations of small isoforms with a lower number of KIV_2 repeats, which are usually associated with elevated levels, are underestimated, while serum concentrations of large isoforms with a large number of KIV_2 repeats, usually associated with low levels, are overestimated (Fig. 1A). Assays having this bias are called apo(a) isoform-sensitive assays. This problem has been well recognized already a long time ago by a thorough comparison between isoform-sensitive and isoform-insensitive assays by Marcovina and colleagues [11]. They found that the relative bias can become quite high with an overestimation of 25–35% in carriers of large isoforms. However, this translates to an absolute bias in most of the samples of a few mg/dL. The relative bias for most of the carriers of small isoforms is around 10%, which translates also only to a few mg/dL. However, there are exemptions in both cases were the absolute bias can be quite high, which can create problems for the cardiovascular risk estimation. After raising this awareness, some poorly performing assays have been taken off the market. But it is still a major discussion in the field how to interpret isoform-sensitive assays. There are no widely available commercial assays that can clearly demonstrate that they use antibodies directed against a unique and only once occurring structure of apolipoprotein(a), which would allow to strictly measure Lp(a) in molar terms. In such a situation, each molecule of apo(a) is only recognized once (Fig. 1B). It is important to notice that using an antibody directed against a repetitive KIV_2 repeat does not mean a manifold overestimation in Lp(a) concentrations since the results are always put into relation to a calibrator, which also contains a multiple number of KIV_2 repeats. In addition, steric hindrance of the large antibodies will prohibit that the apo(a) molecule is overestimated manifold as not every single repeat binds an antibody molecule. It has further to be noted that even the non-repetitive "unique" kringle IV structures as type 1 or types 3 to 10 show quite substantial sequence homologies to each other which means that the (claimed) unique binding must be carefully validated [12].

In the present issue of *Atherosclerosis*, Scharnagl and colleagues [9] have made a major attempt to compare six widely used commercially available assays by measuring Lp(a) in 144 serum samples. All assays used five-point calibrators provided by the manufacturers. Important biases were noted by most assays which differed significantly across the clinically relevant concentration range in a non-linear manner and were highest at high Lp(a) concentrations, including comparing the results to a reference material with known Lp(a) value. Furthermore, these

DOI of original article: https://doi.org/10.1016/j.atherosclerosis.2019.08.015

https://doi.org/10.1016/j.atherosclerosis.2019.08.019
Received 23 August 2019; Accepted 29 August 2019
Available online 31 August 2019

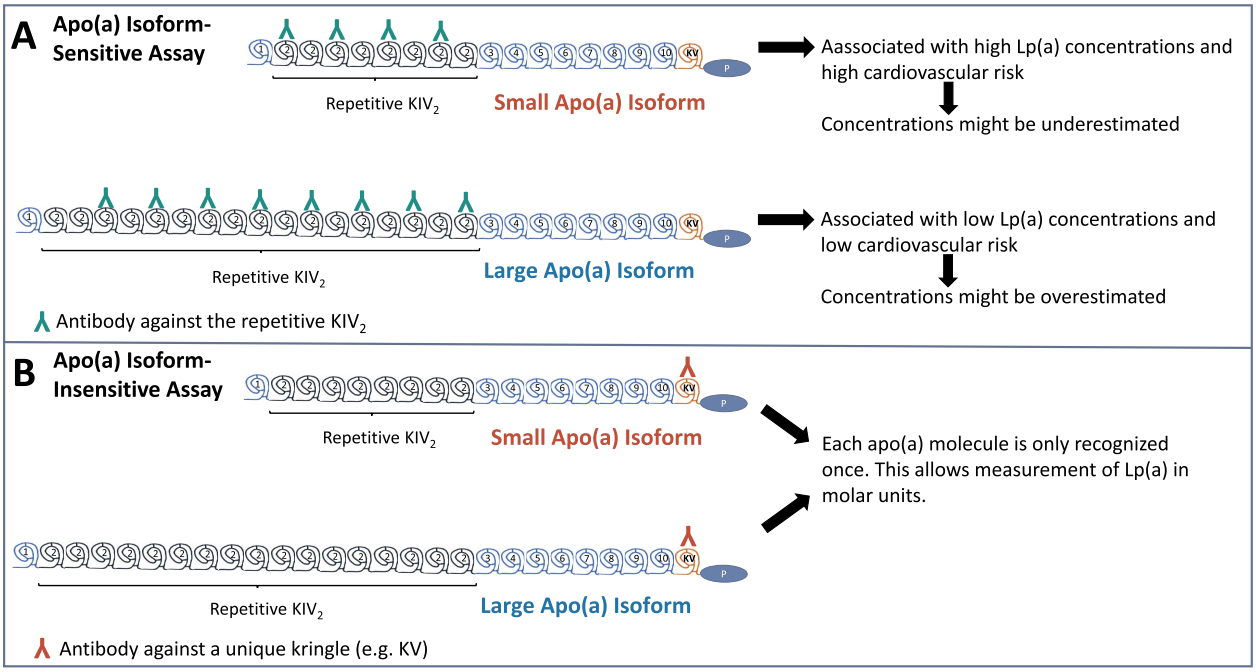

Fig. 1. (A) Schematic illustration of the apo(a) isoform-dependent measurement of Lp(a) concentrations when using antibodies directed against the repetitive KIV type 2 (KIV_2).
(B) This illustration shows the situation in case of an apo(a) isoform-independent assay that uses antibodies that are directed against a unique kringle structure (in this case against the kringle V). For explanation, see text.

differences were not conclusively explained by apolipoprotein(a) phenotypes. This study raises concerns since it shows absolute differences in Lp(a) measurements for single samples between two assays of up to almost 80 mg/dL. The measurements of some of the assays were over a wide range highly correlated but this was not the case for others that showed a pronounced scattering of the same samples. Unfortunately, the authors could not compare the results to a gold standard assay, which is able to measure Lp(a) in molar terms by using antibodies directed against a unique structure of apo(a) as this have been done by an ELISA from Dr. Marcovina's lab [11] or by a mass-spectrometric approach [13]. Instead they used for the main comparisons (without making it to the “gold standard”) an assay from Denka Seiken that is often claimed to measure apo(a) isoform-independent. However, it has recently been shown that this assay compared to Dr. Marcovina's assay also overestimates Lp(a) at low concentrations and underestimates Lp (a) in high concentrations which is probably related to the apo(a) isoform size [14].

There are some widespread misconceptions on the measurement of Lp(a). It is often believed that using a monoclonal antibody against apo (a) would result in an isoform-independent measurement. By binding always to the same epitope of the protein, monoclonal antibodies might have a monovalent affinity. However, if the epitope is located on a repetitive structure such as the KIV_2 of apo(a), it will recognize one apo (a) particle several times and will therefore again not accurately measure in molar terms. Experience in raising antibodies against apo(a) shows that most antibodies will recognize one of the repetitive structures which are present in excess compared to the unique structures.

Due to the limitations of the available antibodies, some companies tried to mitigate the problem of isoform-dependent measurements by using different calibrators for each of the expected Lp(a) concentration strata. This means, a serum sample with high Lp(a) concentrations is expected to carry a small isoform and therefore a calibrator for this concentration stratum is used from the serum of a carrier with a small isoform. This sounds plausible but the mysterious world of Lp(a) does not always follow plausible rules: for example Lp(a) values of individuals with the same apo(a) isoform can still vary by up to 200-fold [15]. That means that someone can carry a small-sized apo(a) isoform for which on average high Lp(a) concentrations are expected but this person has low concentrations of a few mg/dL. The sample from that person would now meet in the assay the calibrator that is mentioned for a large isoform which might even enforce the problem of the isoform-sensitivity of the assay. This becomes more and more obvious since recently very frequent mutations in the KIV_2 region have been identified that dramatically lower Lp(a) concentrations despite a low number of KIV_2 repeats due to a reduction of splicing efficiency [16]. Interestingly, the assay companies do not disclose what isoforms their multipoint calibrators have. It is also not disclosed whether companies, which have once compared their results with an isoform-insensitive assay, perform this comparison each time they make any changes in their assay in terms of antibodies or batches of calibrators they are using.

Fortunately, Scharnagl and colleagues [9] did not follow the attempt to deduce from the data some conversion factors to recalculate results from one assay to the other to make results comparable. Simply from looking at the scattering of the measured values it becomes clear that this would result in a large imprecision.

What are the clinical implications of this bias and imprecision of currently available assays? If a patient has concentrations clearly in a range that are considered to be associated with either a low (< 30 mg/dL or $< \sim 75$ mmol/L)), an elevated risk (> 50 mg/dL or $> \sim 125$ nmol/L), or a very high risk (> 100 mg/dL or $> \sim 250$ nmol/L), then this level of precision might be enough from a clinical standpoint. However, for the grey zones around the proposed threshold of Lp(a) concentrations, this might result in a misclassification of risk [17]. Or would it be even more appropriate to define assay specific cut-offs, e.g. the 80th percentiles of Lp(a) levels measured in a large number of individuals from a population-based study? This might probably introduce further confusion to the field. The clinical need is pressing as physicians will increase measuring Lp(a) significantly since the evidence for a causal association of high Lp(a) levels and outcomes has become strong [1,18]. Upcoming phase 3 trials for lowering Lp(a) will start soon which strongly increases the interest in Lp(a) [19] (see

NCT04023552: Assessing the Impact of Lipoprotein (a) Lowering With TQJ230 on Major Cardiovascular Events in Patients With CVD (Lp(a) HORIZON). Reporting of Lp(a) concentrations in nmol/L instead of the widely used mg/dL has created a lot of confusion although, from a puristic point of view, many assays often cannot measure in molar terms by definition but report results in molar terms nevertheless. We have to keep in mind that a good physician will base the judgement on an individual risk for a single patient not only based on one lab value but many other factors.

There is no doubt, the Hydra is there and endangers the field, but it can be overcome and defeated by additional efforts from all stakeholders, including academia, manufacturers of Lp(a) assays, clinical laboratories, regulatory bodies in laboratory medicine and clinical trials and government bodies in charge of public health. The effort should be greatly rewarded as it is estimated that there are 1.4 billion subjects with elevated Lp(a) globally, and having an accurate method to quantitate their Lp(a) levels should lead to more accurate diagnosis of this risk factor. All efforts should be supported which result in a standardization of the Lp(a) assays [6] which includes not only the use of appropriate antibodies but also of calibrators as well as widely available reference materials. Ultimately, Herakles defeated the Hydra by not only cutting off the head, but also cauterizing the stump to prevent regrowth. With the emergence of effective Lp(a) lowering therapies [19,20] and the beginning of international phase 3 clinical trials, it will be even more important in the near future to standardize Lp(a) levels in a global platform.

Conflicts of interest

Dr. Kronenberg has received honoraria related to consulting or speaker activities from: Amgen, Fresenius, Kaneka and Miltenyi Biotech. Dr. Tsimikas is a co-inventor and receives royalties from patents owned by UCSD on oxidation-specific antibodies and of biomarkers related to oxidized lipoproteins, has a dual appointment at UCSD and Ionis Pharmaceuticals, is a co-founder of Oxitope, Inc and Kleanthi LLC, and is a consultant to Boston Heart Diagnostics.

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Florian Kronenberg*

Institute of Genetic Epidemiology, Department of Genetics and Pharmacology, Medical University of Innsbruck, Schöpfstr, 41, A-6020, Innsbruck, Austria

E-mail address: Florian.Kronenberg@i-med.ac.at.

Sotirios Tsimikas**

Vascular Medicine Program, Sulpizio Cardiovascular Center, Division of Cardiovascular Diseases, University of California San Diego, 9500 Gilman Drive, La Jolla, CA, USA

E-mail address: stsimikas@ucsd.edu.

* Corresponding author. Institute of Genetic Epidemiology, Department of Genetics and Pharmacology, Medical University of Innsbruck, Schöpfstr, 41, A-6020, Innsbruck, Austria.

** Corresponding author. Division of Cardiovascular Diseases, University of California San Diego, 9500 Gilman Drive, La Jolla, CA, 92093-0682, USA.