High Density Lipoproteins in High Resolution:

Will Proteomics Solve the Paradox for Cardiovascular Risk?

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Word count: 1,707

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Abstract

While lipid abnormalities continue to account for over 60% of the population attributable for myocardial infarction, the well-known inverse correlation between plasma high-density lipoprotein cholesterol and cardiovascular risk has failed to deliver clinically useful therapeutic interventions. Thus, there is an unmet need to better understand the functionality of different high-density lipoprotein particles. Targeted high resolution lipoproteomics provides an innovative approach to studying the kinetics of high-density lipoprotein particles. In this commentary we discuss the development of an informatics platform for increased throughput and highlight how this approach delivers the potential for novel, hybrid instrument technologies to inform clinical dyslipidaemia studies.

Elevated levels of high-density lipoprotein (HDL) cholesterol protect from cardiovascular disease (CVD) [1], but drugs that raise HDL cholesterol levels, such as cholesterylester transfer protein inhibitors, are at best ineffectual, and may even increase cardiovascular risk [2, 3]. How can HDL cholesterol be such a powerful predictor and yet such an unpredictable therapeutic target? Is it possible that HDL cholesterol is merely a marker for CVD risk but not a causal factor [4]? The strong inverse association between HDL cholesterol and cardiovascular risk can be explained in a variety of ways; it could involve an entirely different protective factor that is strongly correlated to HDL cholesterol, or it could involve a particular subspecies of HDL. In contrast, there may be an unidentified harmful factor that is associated with therapeutically raising HDL, and attenuates the protective effects of higher levels of HDL cholesterol. HDL cholesterol could also be a marker for triglyceride-rich lipoproteins that have recently been implicated in CVD [5]. These questions have led to a renewed interest in lipid metabolism, with a particular focus on the HDL proteome in the hope of finding new ways to understand the HDL-CVD relationship and hopefully harness its therapeutic potential [6-8] .

HDL particles are composed of combinations of up to 95 different lipophilic plasma proteins of which apoA-I is the major component [7], but our understanding of the assembly and organisation of HDL particles is limited to the rudimentary theory of A-class apolipoproteins and their assembly [7]. The

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liver releases pre-beta 1 particles that remove cellular cholesterol, interact with other lipoproteins, and become ever bigger particles, which are finally taken up by the liver. Thus, cholesterol is transported from the periphery back to the liver (reverse cholesterol transport). However, this entire canonical HDL pathway has not been demonstrated *in vivo*. Instead, recent evidence suggests that the pre-beta 1 particle is only one of several HDL precursors, highlighting the importance of studying the kinetics of HDL particles [8].

Kinetics studies of HDL particles use stable isotope amino acid tracers such as tri-deuterated-leucine (D3-Leu) [8-10], labelling endogenous apoA-I as a surrogate for HDL metabolism. Unfortunately, the incorporation of D3-Leu in apoA-I is very low, limiting the sensitivity and accuracy of the traditional methods of gas chromatography-mass spectrometry (GC/MS) [10] or more recently, multiple reaction monitoring (MRM) [11], to analysis of total HDL only. The use of stable isotopes to measure relative quantification has also added to the challenge due to overlapping light and heavy isotopes, or retention time shift which can cause incorrect heavy peak assignment. Detecting subtle variations in apoA-I enrichment requires confident and robust measurements, to ensure that the changes are biological and not technical, presenting a major challenge for not only the accurate measurement but also the throughput of such analysis.

The advent of the latest hybrid mass spectrometry instruments, combining high resolution accurate mass (HR/AM) with targeted analysis and methods such as parallel reaction monitoring (PRM) [12] heralds unprecedented accuracy and robustness [8]. In order to overcome the technical challenges associated with traditional techniques Singh *et al.* recently developed a HR/AM-PRM based heavy detection strategy to the study and characterisation of HDL protein metabolism [8]. The HR/AM-PRM detection makes it possible to analyse even a low enrichment of D3-peptides.

In the current issue of this Journal, the group presents an automated software tool for the detection and quantification of D3-leu tracer enrichment [9]. Using HR/AM and PRM together with their open-source software, named "extracted PRM peak intensity" (XPI) program, they were able to evaluate sources of technical noise and thus produce reliable automated quantification of very low tracer enrichment. The workflow proves to be accurate for detecting and quantifying endogenous labels, reduces analysis time, and is not sensitive to retention time shifts. While tracer studies have potential

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value in clinical trials, the workflow is only applicable to instruments capable of performing HR/AM-PRM. Such instruments are expensive and not yet accessible to most clinical laboratories. Meanwhile the PRM-XPI workflow does appear to be very useful, but we await its use for the analysis of larger cohorts.

The PRM-XPI workflow found different sized HDL fractions contained differing amounts of apoA-I. Metabolism profiles were also captured for additional HDL proteins, apoA-II, apoE and LCAT, whose abundances and enrichment profiles are distinct from that of apoA-I, suggesting that the functions of HDL may vary with particle size. The enrichment analysis of apoA-I shows a steady decrease in slope from large to small HDL fraction sizes and demonstrates that one of the smallest fractions (prebeta) enters the circulation more slowly than the larger alpha fractions. This is consistent with a recent finding that the majority of prebeta originates from the alpha3 fraction [8] and not directly from the liver as previously thought. The HDL sub-fractions from largest to smallest are alpha0, alpha1, alpha2, alpha3, and prebeta. Notably, a novel and even smaller HDL fraction named as <prebeta, suggested to be lipid-poor apoA-I, and distinct from prebeta, was newly characterised [8]. This fraction has its own unique and markedly slower metabolism, appearing in circulation the latest among all the different HDL particles. This evidence is presented as a challenge to the canonical HDL size expansion model which theorises that prebeta is released by the liver, prior to being converted to the larger sized fractions and eventually to alpha1 [1, 13]. If so, prebeta would thus have shown the greatest enrichment curve in this experiment, but in fact shows the opposite.

Lecithin–cholesterol acyltransferase (LCAT) is a key enzyme participating in reverse cholesterol transport. It is responsible for esterification of free cholesterol before internalisation into the HDL particle core [14]. According to the HDL size expansion model, the accumulation of cholesteryl esters within the HDL particle core results in a stepwise increase in the size of HDL. In this study, Lee *et al.* investigated the enrichment profile for LCAT. Its metabolism in alpha2 and alpha3 HDL fractions, where it predominantly resides, was found to be slow, similar to that of apoA-I and apoA-II, reaching its peak between 6 and 22 hours across the subjects and size fractions, in contract to the faster metabolism of apoE. The implication of the slow turnover of LCAT warrants further investigation.

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The inherent complexities of HDL metabolism require the application of more sophisticated analytical techniques. The PRM-enabled automated workflow presented by Lee and colleagues appears to be suitable for the measurement of low tracer enrichment and could be applied to other low abundant enrichment studies in future. It is clear that our current understanding of HDL metabolism is inadequate to successfully target HDL therapeutically and reduce cardiovascular risk. This method presents an example for the potential use of quantitative proteomics in clinical studies; the technology may benefit large clinical dyslipidaemia studies to further our understanding of HDL structure and function and aid the development of better HDL-based therapies in future.

FUNDING and DISCLOSURE

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