Quantification of HDL Particle Concentration by Calibrated Ion Mobility Analysis

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BACKGROUND: It is critical to develop new metrics to determine whether HDL is cardioprotective in humans. One promising approach is HDL particle concentration (HDL-P), the size and concentration of HDL in plasma. However, the 2 methods currently used to determine HDL-P yield concentrations that differ >5-fold. We therefore developed and validated an improved approach to quantify HDL-P, termed calibrated ion mobility analysis (calibrated IMA).

METHODS: HDL was isolated from plasma by ultracentrifugation, introduced into the gas phase with electrospray ionization, separated by size, and quantified by particle counting. We used a calibration curve constructed with purified proteins to correct for the ionization efficiency of HDL particles.

RESULTS: The concentrations of gold nanoparticles and reconstituted HDLs measured by calibrated IMA were indistinguishable from concentrations determined by orthogonal methods. In plasma of control (n = 40) and cerebrovascular disease (n = 40) participants, 3 subspecies of HDL were reproducibility measured, with an estimated total HDL-P of 13.4 (2.4) μmol/L. HDL-C accounted for 48% of the variance in HDL-P. HDL-P was significantly lower in participants with cerebrovascular disease (P = 0.002), and this difference remained significant after adjustment for HDL cholesterol concentrations (P = 0.02).

CONCLUSIONS: Calibrated IMA accurately determined the concentration of gold nanoparticles and synthetic HDL, strongly suggesting that the method could accurately quantify HDL particle concentration. The estimated stoichiometry of apolipoprotein A-I determined by calibrated IMA was 3–4 per HDL particle, in agreement with current structural models. Furthermore, HDL-P was associated with cardiovascular disease status in a clinical population independently of HDL cholesterol.

Plasma concentrations of HDL cholesterol (HDL-C)3 are widely used clinically to assess HDL’s cardioprotective potential. There is a robust, inverse association of HDL-C with cardiovascular disease (CVD) risk in clinical, epidemiological, and genetic studies (1). However, recent work has cast doubt on the hypothesis that the concentration of HDL-C captures its proposed cardioprotective functions (2–4). For example, genetic variations that alter concentrations of HDL-C do not always predict CVD risk (5). Strikingly, a cholesteryl ester transfer protein inhibitor and niacin, 2 interventions that increase HDL-C, failed to reduce CVD risk in statin-treated humans with established CVD (6, 7). These observations indicate that HDL-C concentrations do not always predict CVD risk and that increasing HDL-C is not necessarily therapeutic.

It is important to note that many lines of evidence strongly suggest that HDL directly protects against vascular disease. For example, a polymorphism in apolipoprotein A-I (apoA-I), the major HDL protein, associates with low HDL cholesterol concentrations and premature coronary artery disease (8). Also, humans with familial deficiency of apoA-I, the major HDL protein, suffer severe early-onset CVD (9). Furthermore, people with Tangier disease [who lack ATP-binding cassette transporter 1 (ABCA1), a key first step in cholesterol export from cells] have very low HDL-C concentrations and accumulate cholesterol-laden macrophages in many different tissues (10, 11).

These discrepancies highlight a central question: Does HDL deficiency promote human atherosclerosis, or

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is it simply a marker for other risk factors such as insulin resistance (2, 3)? To make this determination, it is critical to identify HDL metrics that truly reflect CVD risk.

HDL is a collection of macromolecular particles that contain >80 different proteins (12, 13) and range in size from <7 nm to >14 nm (14). It is therefore plausible that the plasma concentration of HDL particles (HDL-P)—or of a subset of particles—might better reflect HDL-mediated cardioprotection than surrogate measures of HDL such as cholesterol or apoA-I (14–21).

Two methods have been described for quantifying HDL-P in human plasma, one on the basis of nuclear magnetic resonance (NMR) (15), and the other, ion mobility analysis (IMA) (16). To quantify lipoproteins by NMR, the amplitudes of spectral signals emitted by lipoprotein subclasses of different sizes are measured. The data are then reduced with a proprietary algorithm. To quantify HDL by IMA, solvated lipoproteins are introduced into the gas phase by electrospray ionization (ESI). Charged HDL particles are then separated according to their electrophoretic mobility through a buffer gas. Although both approaches have helped establish HDL-P as a potentially relevant clinical metric, it will be important to resolve these discrepancies.

Ion mobility can accurately measure the concentration of particles in the gas phase because it rests on well-established physical principles (16, 24). However, many factors affect the production of gas-phase ions during ESI (25–27). Because the generation and transmission of ions by ESI is variable, quantitative assays of aqueous particles on the basis of this approach must account for ionization efficiency.

We have developed an improved IMA method for quantifying HDL-P particles from human plasma. Calibrated IMA differs from traditional IMA in 3 critical aspects. First, it uses particles of known concentration to calibrate the assay to empirically account for ionization efficiency and other sources of signal loss. This permits the conversion of IMA signal intensity, a relative measurement, to a metric of absolute concentration. Second, IMA spectra are processed by an adaptive peak-fitting algorithm allowing reproducible deconvolution of 3 major HDL subspecies. Third, we have validated the new approach using known concentrations of monodisperse gold nanoparticles and reconstituted HDL particles. Our data provide evidence that calibrated IMA offers clinically useful information that is distinct from that provided by HDL-C.

Materials and Methods

HDL Preparation

We isolated total lipoproteins from plasma in a single ultracentrifugation step as follows: 50 μL plasma, 50 μL normal saline (with 0.5 mmol/L EDTA), and 130 μL KBr (ρ = 1.37 g/mL) were added to 7-by-20 mm ultracentrifugation tubes (final ρ = 1.21 g/mL). Tubes were centrifuged in a 72-position rotor (type 42.2 T1) at 42000 rpm (mean 214 361 g) for 12 h; 57 μL was then taken from the top of each tube and placed in a 96-well constant-flow dialyzer (Spectrum Laboratories). Samples were dialyzed for 4 h at 4°C against NH₄OAc (5 mmol/L, adjusted to pH 7.4 with NH₄OH) at a flow rate of approximately 5 mL/min. Immediately before analysis, samples were diluted 500-fold (relative to the original plasma volume) with NH₄OAc (5 mmol/L, pH 9.2).

Differential Ion Mobility

Principles of ESI and differential ion mobility (24, 28), as well as instrumentation and operation details, are given in the Supplemental Data, which accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue11. Briefly, analytes in aqueous solution are converted to gas-phase ions by ESI (see online Supplemental Fig. 1). The resulting highly charged ions are largely neutralized by α particles, yielding a small proportion of singly charged cations, which are introduced into the mobility analyzer. As the particles move through a strong electromagnetic field, they are separated according to their electrophoretic mobility and then enumerated by a particle counter.

Deconvolution of HDL Spectra

IMA spectra were expressed in units of aerosol particle concentration per size bin ([number/cm³]/size bin) with an algorithm supplied by the instrument’s manufacturer (Aerosol Instrument Manager, version 9.0.0.0, TSI) (29). We then analyzed size distribution spectra of human HDL using open-source curve-fitting software (Fityk version 1.2.0 for Macintosh) (30). With a custom script, spectra were fitted automatically with 3 Voigt probability distribution curves corresponding to the 3 HDL subspecies. The software iteratively adjusts the peak parameters to minimize the weighted sum of squared residuals, or χ². All peak parameters were unfixed but limited in range, allowing for adaptive deconvolution of the highly variable HDL size distribution profiles observed in human plasma. Finally, we converted the HDL subspecies’ peak areas into aqueous particle concentrations using glucose oxidase calibration curves.

Calibration Curves of Isolated Proteins

We prepared solutions of purified proteins gravimetrically in H₂O. Exact concentrations were determined by absorbance at 280 nm. Solutions were fur-
ther diluted in NH₄OAc (5 mmol/L, pH 9.2) before IMA. Typically, we used serial dilutions of glucose oxidase (10–1.25 μg/mL) for calibration. We calculated particle concentrations of individual protein oligomers (see online Supplemental Data) to account for the fact that total particle concentration was different than that determined by A₂₈₀ due to the presence of multiple oligomers.

ANALYSIS OF RECONSTITUTED HDL
We prepared discoidal reconstituted HDL (rHDL) as previously described (31). The protein concentration of the rHDL particles (9.6 nm hydrated diameter) was determined by modified Lowry assay (Thermo #23240). Serial dilutions were prepared (5 mmol/L NH₄OAc, pH 9.2) and quantified by calibrated IMA. To validate calibrated IMA, we performed duplicate analyses of 2 independent rHDL preparations.

ANALYSIS OF GOLD NANOPARTICLES
Stock solutions of gold nanoparticles (10 nm; Nano-Xact from nanoComposix) were concentrated by centrifugation by use of the manufacturer’s recommended protocol. Particle concentration of the final solution was determined by absorbance at 521 nm. Serial dilutions were then prepared (5 mmol/L NH₄OAc, pH 9.2) and quantified by calibrated IMA. To validate calibrated IMA, we performed duplicate analyses of 2 independent gold nanoparticle preparations.

CLINICAL POPULATION
All participants provided signed informed consent, and all protocols were approved by the University of Washington Institutional Review Board (#32967B). Forty blood samples were randomly selected from those of 375 participants with severe carotid cerebrovascular disease enrolled in the CLEAR study (32). Forty samples were also selected from those of the study’s >1000 controls. Participants were matched by sex and diabetes status. Detailed inclusion and exclusion criteria are provided in the online Supplemental Data.

STATISTICAL ANALYSES
Statistical tests were performed with R (version 2.15.1) or Prism (version 4.0; Graphpad). All t-tests were 2-tailed and uncorrected. Correlations were evaluated by the method of Pearson. Odds ratios and their confidence intervals were extracted from generalized linear models constructed in R. For all analyses, P values <0.05 were considered significant.

Results

CALIBRATED IMA QUANTIFIES PROTEINS WITH DIFFERENT MOLECULAR WEIGHTS AND ISOELECTRIC POINTS
A key assumption of calibrated IMA is that different particles elicit similar responses when analyzed by the same instrument. To test this assumption, we first explored the linearity of the ion mobility signal response by analyzing serial dilutions of highly purified glucose oxidase [Mₘ, dimer, 160000; isoelectric point (pI), 4.2] (Fig. 1A). IMA spectral peak areas of glucose oxidase (monomers and dimers) were plotted against particle concentrations calculated from the total protein concentration determined by A₂₈₀ (Fig. 1B). Linear (r² >0.99) concentration-dependent responses were observed for the dimer, the monomer, and total particle concentration. Calibration curves routinely had r² values >0.99.

To determine how particle size and physiochemical properties (e.g., pI) affect instrument response, we interrogated 2 additional proteins in the same manner. IMA of serial dilutions of catalase (Mₘ, tetramer, 240000; pI, 5.6) and transferrin (Mₘ, monomer 80000; pI, 6.2–6.6) both yielded linear, concentration-dependent responses simi-
lar to those we obtained with glucose oxidase. Importantly, all 3 proteins produced calibration curves with essentially equivalent slopes and $y$-intercepts. Indeed, a single regression line, fit to the superimposed data (Fig. 1C), had an $r^2 = 0.98$ and passed near the origin.

These observations indicated that proteins of different molecular weights, oligomeric distributions, and isoelectric points all produced similar instrument responses. For routine analyses, we used glucose oxidase as the working calibrant due to its convenient particle diameter near the center of the HDL size distribution and its stability in aqueous solution.

**CALIBRATED IMA QUANTIFIES THE ABSOLUTE CONCENTRATION OF RECONSTITUTED HDL AND GOLD NANOPARTICLES**

We next used reconstituted discoidal HDL (9.6 nm diameter) to determine whether calibrated IMA can accurately quantify HDL-P. These particles were selected because they resemble native HDL and contain 2 apoA-I molecules per particle (31, 33), allowing us to establish the concentration of stock solutions on the basis of protein content. When particle concentrations determined by calibrated IMA were plotted against concentrations calculated from total protein (Fig. 1D), the data were linear ($r^2 = 0.98$) and had a slope close to 1 (0.99).

We similarly quantified gold nanoparticles (approximately 10-nm diameter), whose concentration we determined by absorbance at 521 nm. Once again, the 2 orthogonal methods yielded nearly identical results for particle concentration (Fig. 1E). In separate experiments, we determined the concentration of rHDL prepared in another laboratory and shipped for analysis. Particle concentrations determined in triplicate by IMA [26 (1) nmol/L] and by total protein (30.4 nmol/L) differed by $<15\%$.

**CALIBRATED IMA QUANTIFIES TOTAL HDL-P AND 3 SUBSPECIES IN HUMAN PLASMA**

The workflow for determining HDL-P by calibrated IMA is shown in Fig. 2A. To summarize, we isolated total lipoproteins from plasma by a single ultracentrifugation ($\rho = 1.21 \text{ g/mL}$) step (34) and then dialyzed the preparation to remove salts (which interfere with
IMA). After diluting the samples, we used differential mobility analysis to determine the size distribution and uncorrected particle concentration of the isolated HDL species. Because electrophoretic mobility depends chiefly on size, IMA data are expressed in terms of particle diameter, which corresponds to the calculated diameter of a singly charged, spherical particle with the same electrophoretic mobility.

For each spectrum, 3 HDL subspecies (small, medium, and large) were deconvoluted by unsupervised, iterative curve-fitting (Fig. 2, B–D). Finally, HDL peak areas were directly converted to HDL-P by use of the calibration curve. Using this approach, we determined HDL-P in 40 control participants (<15% carotid intimal thickening) and 40 participants with severe carotid cerebrovascular disease (CCVD; >80% carotid stenosis by MRI) enrolled in the CLEAR study. The clinical characteristics of the 2 groups are presented in online Supplemental Table 1. The mean (SD) total HDL-P obtained in all 80 participants by calibrated IMA was 13.4 (2.4) μmol/L with a mean value for plasma apoA-I of 48.8 μmol/L determined by a clinical laboratory. Calibrated IMA consistently identified 3 major HDL subspecies in plasma from the 80 participants. They were small HDL (S-HDL, average diameter 7.9 mm), medium HDL (M-HDL, 8.6 mm), and large HDL (L-HDL, 10.4 mm)

The observed diameters of reference proteins were plotted against their molecular masses (A). For all points, SDs were smaller than the dots. A best-fit curve (power series; black curve) was used to interpolate the apparent molecular mass of HDL subspecies. Mean diameters, sizespans, and corresponding apparent molecular masses are tabulated (B).

SUBSPECIES DISTRIBUTIONS EXPLAIN DISCORDANT VALUES FOR HDL-P AND HDL-C
We next determined the relationship between HDL-P and HDL-C in all 80 participants (Fig. 4, A–D). The concentration of HDL-C was determined on plasma by a clinical laboratory. HDL-C predicted >60% of the variance in L-HDL-P (r = 0.78, P < 0.0001), whereas it predicted <30% of the variance in M-HDL-P (r = 0.53, P < 0.0001). The concentration of S-HDL did not correlate with HDL-C but trended inversely (r = −0.22). Total HDL-P correlation with HDL-C was moderate (r = 0.69, P < 0.0001). The relationships between HDL-P (total and subspecies) and plasma apoA-I were similar to the HDL-C correlations de-
There was little correlation of HDL-P with concentration of LDL cholesterol or other lipids (see online Supplemental Table 2).

HDL-C explained only approximately 50% of the variation in total HDL-P (Fig. 4D). Consistent with this observation, certain participants showed discordant values of HDL-P and HDL-C. The variable cholesterol content of individual HDL particles (22, 23) suggested that subspecies’ distributions might explain the 2 metrics’ conflicting values. We therefore compared the subset of participants (n/H11005/5) with both high HDL-P (mean) and low HDL-C (mean) with those (n/H11005/10) who had both low HDL-P (mean) and high HDL-C (mean) (Fig. 4, D and E). The latter had twice the concentration of L-HDL particles (2.2 vs 1.0 μmol/L; P/H11005/0.02). Conversely, the participants with high HDL-P/low HDL-C had nearly twice the concentration of S-HDL particles (7.5 vs 3.8 μmol/L; P/H11005/0.0003). Although the 2 groups had markedly different HDL-C (P/H11005/0.0002), they had similar concentrations of M-HDL particles. Calibrated IMA spectra of representative participants from each group are shown in Fig. 4F.

### HDL-P Associates with Carotid Cerebrovascular Disease Independently of HDL-C

To explore whether calibrated IMA might be a clinically useful alternative to HDL-C measurements, we compared HDL-P in control participants (n = 40) and participants with severe carotid cerebrovascular disease (CCVD; n = 40), a major risk factor for stroke. The participants’ characteristics are summarized in online Supplemental Table 1.

Compared with the controls, the participants with carotid disease had significantly lower concentrations of HDL-C, apoA-I, M-HDL-P, and total HDL-P (P/H11005/0.04, 0.03, 0.004, and 0.002, respectively) (Fig. 5). Unadjusted odds ratios (Fig. 5D) revealed that total HDL-P and M-HDL-P were the strongest predictors of CCVD, followed by HDL-C and apoA-I; no other traditional lipid risk factors quantified by a clinical laboratory were significant predictors in this population.

Importantly, differences in total HDL-P and M-HDL-P remained significant after adjustment for HDL-C (P/H11005/0.02 and 0.04, respectively). After adjustment for LDL and triglycerides, HDL-C no longer differed significantly between groups (P/H11005/0.06), whereas both M-HDL and total HDL-P remained strong predictors of CCVD (P/H11005/0.003 and 0.009, respectively). Adding age and sex to this model did not affect the significance of HDL-P. Collectively, these observations indicate that HDL-P might provide clinical information about CVD risk that is independent of other traditional lipid risk factors.

### Discussion

The concentration and size of HDL particles in plasma, HDL-P, might represent a metric that more accurately assesses CVD risk than HDL-C. We therefore determined whether calibrated IMA—our modification of the standard technique—could quantify HDL-P. Because of variability in ionization efficiency during IMA, an unresolved issue was whether this approach could provide an absolute, quantitative measure.

IMA of proteins of different sizes and physiochemical properties yielded linear calibration curves that were essentially superimposable, suggesting that protein standards could be used to quantify other particles of unknown concentration. Consistent with this proposal, the concentrations of reconstituted HDL particles and gold nanoparticles determined by calibrated IMA were in excellent agreement with concentrations determined by orthogonal methods. Taken together, these observations strongly suggest that calibrated IMA can quantify particles in aqueous solution that range widely in size and composition.

We next used calibrated IMA to investigate the size and concentration of HDL particles in human plasma.

### Table 1. Precision of calibrated IMA.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Analytical CV (%)a</th>
<th>Intra-assay CV (%)b</th>
<th>Interassay CV (%)c</th>
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<tbody>
<tr>
<td>Determination</td>
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<tr>
<td>Samples</td>
<td>1</td>
<td>12</td>
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</tr>
<tr>
<td>Analyses/sample</td>
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<td>3</td>
<td>3</td>
</tr>
<tr>
<td>HDL-P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.8</td>
<td>6.2</td>
<td>11.4</td>
</tr>
<tr>
<td>Small</td>
<td>11.9</td>
<td>18.8</td>
<td>19.7</td>
</tr>
<tr>
<td>Medium</td>
<td>5.9</td>
<td>12.8</td>
<td>15.0</td>
</tr>
<tr>
<td>Large</td>
<td>8.0</td>
<td>7.1</td>
<td>19.8</td>
</tr>
<tr>
<td>HDL-P subspeciesd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% small</td>
<td>6.6</td>
<td>20.1</td>
<td>20.6</td>
</tr>
<tr>
<td>% medium</td>
<td>3.0</td>
<td>9.4</td>
<td>10.2</td>
</tr>
<tr>
<td>% large</td>
<td>9.9</td>
<td>6.2</td>
<td>10.1</td>
</tr>
<tr>
<td>HDL sized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small</td>
<td>0.6</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Medium</td>
<td>0.4</td>
<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Large</td>
<td>0.7</td>
<td>0.7</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Repeated analysis of a single HDL isolate.
* Parallel isolations and analyses of plasma samples.
* Serial isolations and analyses of plasma samples.
* Based on calibration and deconvolution of IMA spectra into 3 HDL subspecies.
The 3 subspecies closely matched the sizes of HDL particles defined by ultracentrifugal Schlieren patterns and nondenaturing 2-dimensional gradient gel electrophoresis (14, 36, 37). Thus, S-HDL, M-HDL, and L-HDL likely correspond to \( \alpha_3 \)-, \( \alpha_2 \)-, and \( \alpha_1 \)-HDL, respectively. In contrast, noncalibrated IMA detected only 2 subspecies: large HDL and small HDL (16). Our ability to quantify 3 subpopulations of HDL likely reflects differences in the methods used to isolate the HDL and the adaptive curve-fitting algorithm, which permits deconvolution of partially overlapping HDL subspecies.

A key issue was whether our approach recovered HDL quantitatively from plasma. Immunoblot analysis of material prepared by ultracentrifugation from 4 individuals indicated that we recovered approximately 80% of the apoA-I in the HDL fraction (see online Supplemental Data). It is noteworthy that 5–10% of plasma apoA-I is unassociated with lipoproteins (38). Assuming that 10% of apoA-I is indeed not associated with HDL, we estimate that our recovery of small, medium, and large HDLs—the particles quantified by calibrated IMA—approaches 90%.

A fundamental unresolved issue is the concentration of HDL particles in blood, which, along with subspecies distribution, is likely to impact HDL’s functions. In 7 independent studies, the mean total HDL-P reported by noncalibrated IMA studies was 5.3 \( \mu \text{mol/L} \), and the average plasma apoA-I concentration was 51 \( \mu \text{mol/L} \) (see online Supplemental Table 3). These values imply a mean stoichiometry of almost 10 apoA-I molecules per HDL particle. In contrast, HDL particle concentrations derived from NMR analyses were approximately 30 \( \mu \text{mol/L} \) (see online Supplemental Table 3), indicating a stoichiometry of approximately 1.6 apoA-I molecules per HDL particle. The mean total HDL-P obtained by calibrated IMA was 13.4 \( \mu \text{mol/L} \), with a mean plasma apoA-I value of 48.8 \( \mu \text{mol/L} \), implying 3.6 apoA-I per HDL if all HDL particles contain apoA-I. This stoichiometry is in excellent agreement with abundant biochemical data suggesting a mean of 3–4 apoA-I/HDL and with our current understanding of HDL structure (22, 23). Importantly, this observation further supports the proposal that we recovered HDL in near-quantitative yield from plasma.

A striking feature of the clinical data was the marked variability in the abundance of HDL subspecies in different participants. Among individual participants, for example, the percentage of M-HDL ranged from <15% to >70%; S-HDL and L-HDL showed...
similar variation. This HDL heterogeneity highlights the need for a flexible data processing approach.

It is noteworthy that approximately 20% of the participants in our clinical population had high HDL-P concentrations (>mean) and low HDL-C values (<mean) or low HDL-P (<mean) and high HDL-C (>mean) HDL-C values. These differences in turn reflected major differences in the relative abundance of S-HDL and L-HDL particles. These results support the notion that HDL-P can vary independently from HDL-C and that differences in the proportions of sub-species could account for the discrepancy.

In a clinical population, low total HDL-P associated strongly and inversely with severe carotid cerebrovascular disease (>80% stenosis). Notably, M-HDL particles were selectively depleted, suggesting that the abundance of a specific HDL subpopulation was reduced in this clinical population. M-HDL only moderately correlated with HDL-C, strongly suggesting that quantifying specific subpopulations of HDL particles might offer information distinct from HDL-C. Importantly, differences in total HDL-P and M-HDL-P remained significant after adjustment for HDL-C, suggesting that HDL-P can offer clinically relevant information beyond HDL-C. The association of low HDL-P with carotid disease persisted after adjustment for other risk factors, including LDL-C, triglycerides, age, and sex. In future studies, it will be critical to extend these observations to larger numbers of participants, to validate calibrated IMA for quantification of other lipoproteins (both human and mouse; see online Supplemental Data), and to assess whether HDL-P can predict risk in prospective studies.

In conclusion, we describe a method for determining the size and concentration of HDL in human plasma. The method leverages empiric calibration and was validated by measuring particles of known concentration. Quantifying HDL-P yielded a value for the stoichiometry of apoA-I per HDL particle that fits well with our current understanding of HDL structure. HDL-P was also a strong and independent predictor of CCVD status in a clinical population.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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