

Letters to the Editor

DOI: 10.1373/clinchem.2008.113795

apo A-I concentration of about 60 $\mu\text{mol/L}$ (i.e., 60 000 nmol/L). The ratio yields a stoichiometry of about 20 apo A-I molecules per HDL particle instead of the expected stoichiometry of approximately 2.5. Thus, IM-measured values for the HDL particle concentration appear to be almost an order of magnitude too low.

Caulfield et al. suggest that IM lipoprotein analysis has an inherent advantage over other methods that "require some form of algorithm" because it counts the particles directly; however, from our reading of the description of the methodology, the authors have made many assumptions in calculating IM-measured particle concentrations from the particle numbers "counted" by the detector. These assumptions may or may not be valid, and the incorrectness of one or more of these assumptions could have led to the discrepancies we have identified. Without better agreement between lipoprotein concentrations measured by IM and those obtained with more established, clinically validated methods, the value of the IM-analysis method as presented remains uncertain. Once validation of the IM method has been accomplished, collaborative standardization efforts between groups that perform particle-concentration measurements will be required to enable its broader use in clinical practice.

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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.

Authors' Disclosures of Potential Conflicts of Interest: Upon submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: J.D. Otvos is Chief Scientific Officer of Liposcience, Inc. **Consultant or Advisory Role:** None declared.

Stock Ownership: None declared.

Honoraria: L.L. Rudel has accepted honoraria from Glaxo SK.

Research Funding: None declared.

Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

James D. Otvos^{2*}
Lawrence L. Rudel³
Joseph P. McConnell⁴

² Liposcience, Inc.
Raleigh, NC

³ Department of Pathology
Section on Lipid Sciences
Wake Forest University School of Medicine
Winston-Salem, NC

⁴ Department of Laboratory Medicine and
Pathology
Mayo Clinic
Rochester, MN

* Address correspondence to this author at:
LipoScience, Inc.
2500 Sumner Blvd.
Raleigh, NC 27616
E-mail jotvos@liposcience.com

Protein Microarrays: Before the Elephant Got in the Room

To the Editor:

I read with great interest the recent editorial in *Clinical Chemistry* on protein microarrays (1). "The Elephant in the Room" in its title refers to a long-ignored issue, namely the problems involved in standardization and normalization of data from immunoassays on protein microarrays. I would like to take this opportunity to review approaches to those issues in the first publications on protein arrays, hence my title. Kattah et al. (1) referred to the wide use of normalization via total immunoglobulin for antigen arrays, and the inability to correct for differences in the spotting of individual features. This imperfection notwithstanding, a 1982 publication on protein arrays (2) already grappled with this issue and showed a calibration curve of immunoglobulin concentration against a densitometric scan intensity. This was further elaborated in a subsequent publication (3), in which empirical equations were used to fit the calibration curves and a computer program was used in extraction of the data.

Indeed, there is a body of literature from that period which foreshadowed current approaches, and which is now largely forgotten or ignored. Reasons for this include the fact that the current terminology was not in use then, so key term searches would not find these publications, and work in the patent literature is not widely read by the scientific community. I therefore consider it of interest to look back at this literature from a current perspective.

An example is a publication of an approach to the diagnosis and management of autoimmune disease using libraries of potential antigens on single patient samples (4). In addition to showing distinguishable autoantibody responses to native or denatured DNA, this early work attempted an "omics"-like approach by fractionating generic human cells into empirical compartments and using each compartment as a distinct site on an array. The term "array" was not used in those publications, since individual features were dots on strips (so these were linear arrays). Marinkovich (5) also described a method of linear array fabrication for allergens for simultaneous analysis of IgE reactivity of patient sera. The demonstration of the feasibility of higher-density arrays, true microarrays, was described, but not claimed, in a patent family that was first published as a European Patent Application in 1982 (6). There, the possibility of 10^5 features per array was shown to be feasible. This patent was eventually issued in the US but never enforced.

Kattah et al. (1) referred to their unpublished comparison of many commercially available slide surfaces, finding that nitrocellulose proved promising. In the patent (6), a survey was made of a set of then-available supports, with the same conclusion. Further, generic arrays for infectious disease diagnosis were demonstrated in that patent application. The demonstration made use of antigens and control sera that were commercially available at the time. With such arrays, there was the opportunity to do a matrix of tests with all features challenged with all positive controls. Strikingly, each individual positive control showed a characteristic fingerprint of reactivity against all of the features. From this, arose another -omics concept: the idea of creating very

large arrays of arbitrary antigenic features that could be used to generate empirical patterns of reactivity to be correlated with different disease states.

In conclusion, precursors to many current concepts have been in the published literature for >2 decades.

Author Contributions: The author confirmed he has contributed to the intellectual content of this paper and has 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.

Authors' Disclosures of Potential Conflicts of Interest: Upon manuscript submission, the author completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: FIND Diagnostics.

Stock Ownership: Abbott Laboratories.

Honoraria: None declared.

Research Funding: None declared.

Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: I gratefully acknowledge David Kelso for his review and input concerning the manuscript.

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Julian Gordon

Department of Biomedical Engineering
McCormick School of Engineering
and Applied Science
Northwestern University
Evanston, IL

Address correspondence to the author at:
Department of Biomedical Engineering
McCormick School of Engineering
and Applied Science
Northwestern University
2145 Sheridan Road
Evanston, IL 60208
E-mail julian-gordon@northwestern.edu

DOI: 10.1373/clinchem.2008.113852

In Reply

Otvos et al. raise concerns regarding the results we reported for lipoprotein particle measurements using ion mobility (IM)¹ in comparison to assessment of lipoprotein particle concentrations based on levels of apolipoproteins B (non-HDL) and A1 (HDL) (1). We have recently made corrections for particle recovery in the IM instrument: a diffusional loss factor (corrects for the loss of particles onto the inner wall of tubing in the instrument) and an efficiency factor for particle aerosolization in the electrospray interface. The diffusional loss had the greatest effect, especially on the smaller particles in the HDL size range. Data generated from reanalysis of the original data presented in the article (1) (Fig. 4C for apoB and IM non-HDL and supplemental Fig. 3B for

¹ Nonstandard abbreviations: IM, ion mobility; apoB, apolipoprotein B; apoA1, apolipoprotein A1.

Table 1. Linear regression analysis of IM vs biochemical parameters.^a

	Range	Slope	Intercept	R
Reported in original article ^b				
IM non-HDL vs apoB, nmol/L	433–2594	1.05	624	0.92
IM HDL vs apoA1, μ mol/L	1.6–6.45	7.9	37.6	0.67
Recalculated from original data				
IM non-HDL vs ApoB, nmol/L	678–2938	1.01 ^c	158 ^c	0.91
IM HDL vs ApoA1, μ mol/L	5.2–21.3	3.52	23.9	0.86

^a (1).^b We ran 79 samples in the IM and in commercial apoA1 and apoB assays. Molecular weights used to convert apolipoproteins from mg/dL to mol/L were 28 300 and 500 000 for apoA1 and apoB, respectively.^c Slope and intercept were not significantly different from 1 and 0, respectively.

IM HDL and apoA1) are given in Table 1.

For IM non-HDL vs apoB, there were minor changes in the slope and correlation coefficient, and the intercept changed from 624 to 158 nmol/L. The intercept of 158 nmol/L was not significantly different from zero by Deming analysis. The effect on the calculation for HDL was more dramatic: the *r* value for the correlation of IM HDL particle concentration to apoA1 improved significantly, with the slope <50% of that reported previously. We have not yet attempted, as suggested by Otvos et al., to improve this fit further by accounting for the number of apoA1 molecules present in differently sized particles, because the full range of heterogeneity of apoA1 content among HDL particles cannot be simply mapped to the size intervals determined by IM. This will require further studies of HDL subfractions that are currently in progress.

The inclusion of the new factors to account for particle recovery, therefore, corrects the measurement of concentrations of both non-HDL and HDL particles. The reference ranges reported for individual subfraction concentra-

tions (1) have been modified accordingly, and these will be updated and published separately.

It is important to point out that IM is a novel method for measuring size and concentration of lipoprotein particles; because it determines these directly and independently of the particles' constituents, its use will allow assessment of how well other assays serve as surrogate measures of concentrations of particles in various size intervals. In this regard we agree with Otvos et al. that the methods available for the measurement of lipoprotein size and concentration should be carefully compared and efforts should be made to standardize the reporting of results.

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.

Authors' Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of

Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: Quest Diagnostics Inc.: M.P. Caulfield, scientific director, R&D; W.A. Salameh, medical director; R.E. Reitz, acting vice president, Science & Innovation.

Consultant or Advisory Role: W.H. Benner, Quest Diagnostics Inc.

Stock Ownership: M.P. Caulfield, W.A. Salameh, and R.E. Reitz, Quest Diagnostics Inc.

Honoraria: None declared.

Research Funding: R.M. Krauss, Quest Diagnostics Inc.

Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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- Caulfield MP, Li S, Lee K, Salameh WA, Blanche P, Benner WH, Reitz RE, Krauss RM. Direct determination of lipoprotein particle sizes and concentrations by ion mobility. *Clin Chem* 2008;54:1307–16.

Michael P. Caulfield^{2*}
Shuguang Li²
Gloria Lee²
Patricia A. Blanche³
Wael A. Salameh²
W. Henry Benner⁴
Richard E. Reitz²
Ronald M. Krauss³

² Quest Diagnostics Nichols Institute
 San Juan Capistrano, CA

³ Children's Hospital
 Oakland Research Institute
 Oakland, CA

⁴ Lawrence Livermore
 National Laboratory
 Livermore, CA

*Address correspondence to this author at:
 33608 Ortega Highway
 San Juan Capistrano, CA 92675
 Fax 949-728-4872
 E-mail
 michael.p.caulfield@questdiagnostics.com

DOI: 10.1373/clinchem.2008.115667