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HDL particle number measured on the Vantera®, the first clinical NMR analyzer

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ABSTRACT

Objectives: Nuclear magnetic resonance (NMR) spectroscopy has been successfully applied to the measurement of high-density lipoprotein (HDL) particles, providing particle concentrations for total HDL particle number (HDL-P), HDL subclasses (small, medium, large) and weighted, average HDL size for many years. Key clinical studies have demonstrated that NMR-measured HDL-P was more strongly associated with measures of coronary artery disease and a better predictor of incident cardiovascular disease (CVD) events than HDL-cholesterol (HDL-C). Recently, an NMR-based clinical analyzer, the Vantera®, was developed to allow lipoprotein measurements to be performed in the routine, clinical laboratory setting. The aim of this study was to evaluate and report the performance characteristics for HDL-P quantified on the Vantera® Clinical Analyzer.

Design and methods: Assay performance was evaluated according to Clinical and Laboratory Standards Institute (CLSI) guidelines. In order to ensure that quantification of HDL-P on the Vantera® Clinical Analyzer was similar to the well-characterized HDL-P assay on the NMR profiler, a method comparison was performed.

Results: The within-run and within-lab imprecision ranged from 2.0% to 3.9%. Linearity was established within the range of 10.0 to 65.0 μmol/L. The reference intervals were different between men (22.0 to 46.0 μmol/L) and women (26.7 to 52.9 μmol/L). HDL-P concentrations between two NMR platforms, Vantera® Clinical Analyzer and NMR Profiler, demonstrated excellent correlation ($R^2 = 0.98$).

Conclusions: The performance characteristics, as well as the primary tube sampling procedure for specimen analysis on the Vantera® Clinical Analyzer, suggest that the HDL-P assay is suitable for routine clinical applications.

Introduction

The inverse association between HDL concentrations and CVD risk is well established [1]. Results from a number of epidemiologic studies demonstrated that low HDL-C concentrations were strongly associated with CVD risk independent of LDL cholesterol (LDL-C) [2–4]. As a result of these studies, guidelines recognize low HDL-C concentration as a risk factor for CVD [5]. However, recent developments have raised doubts regarding the relationship between HDL-C and CVD risk as several clinical trials of agents that substantially raised HDL-C levels failed to show a reduction in cardiovascular event rates [6,7]. Given what we now know about the complex nature of HDL, these results are not surprising [8–13]. For example, the protein constituents on HDL particles vary tremendously depending on the individual's metabolic and inflammatory state. Additionally, the phospholipid content of the surface monolayer and the ratio of cholesteryl esters to triglycerides within the core of HDL particles differ greatly between individuals, depending on the inflammatory, nutritional and metabolic state of the patient, leaving HDL-C alone as a poor surrogate biomarker for the number of circulating HDL particles.

Results from a number of key epidemiological and clinical trials, including the Multi-Ethnic Study of Atherosclerosis (MESA) [14], the Heart Protection Study (HPS) [15] and the Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) [16], demonstrated that NMR-measured HDL-P was a

Abbreviations: NMR, nuclear magnetic resonance; HDL, high-density lipoprotein; HDL-P, HDL particle number; HDL, high-density lipoprotein; CVD, cardiovascular disease; HDL-C, high-density lipoprotein—cholesterol; LDL-C, low-density lipoprotein—cholesterol; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low-density lipoprotein; LDL-P, LDL particle number; FDA, Food and Drug Administration; CLIA, Clinical Laboratory Improvement Amendments; LOB, limit of blank; LOD, limit of detection; LOQ, limit of quantitation; CLSI, Clinical Laboratory Standards Institute; CV, coefficient of variation; CI, confidence intervals; IM, ion mobility; CIM, calibrated ion mobility.

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stronger, more independent predictor of CVD risk than HDL-C. In JUPITER, investigators evaluated the relationship of HDL-C and HDL-P with CVD risk in more than 10,000 subjects randomized to receive rosuvastatin or placebo. Similar to results from the HPS, JUPITER showed a significant, strong, inverse association between HDL-P concentrations and CVD risk. In contrast, HDL-C was not associated with CVD risk in statin-treated patients after adjustment for additional lipoprotein parameters. Therefore, NMR-measured HDL-P provides a more accurate and reliable measure of HDL quantity than HDL-C.

NMR spectroscopy has been successfully applied to the measurement of HDL particles for many years, providing particle concentrations for total HDL-P, HDL subclasses (small, medium, large) and weighted, average HDL size [17]. The lipoprotein profile, delivered via analysis of a single NMR spectra, also provides average size and particle concentrations for very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and low-density lipoproteins (LDL) [17–19]. Although there is value in evaluating the lipoprotein class and subclass changes that occur under various disease conditions, each lipoprotein parameter may in fact have its own unique clinical utility. For example, measurement of total LDL particle concentration via the NMR LDL-P assay is employed for the management of LDL related risk and is especially useful in patients with metabolic disease and type 2 diabetes, where LDL-C and LDL-P are often discordant [20]. The clinical utility of HDL-P information is actively being explored based on recent clinical trials, suggesting that there may be value in knowing a patient's HDL-P level, especially in cases where there is residual CVD risk [14–16]. Recently, a clinical NMR instrument, the Vantera NMR Clinical Analyzer, was developed that addresses the limiting factors of standard NMR instruments and allows lipoprotein measurements to be performed in the routine, clinical laboratory setting [20]. The Vantera Clinical Analyzer received clearance from the Food and Drug Administration (FDA) as an in vitro diagnostic device in 2012.

The aim of this study was to evaluate and report the performance that is typically observed for the HDL-P assay as implemented on the FDA-approved Vantera Clinical NMR Analyzer.

Materials and methods

Specimen collection and preparation

Serum pools and controls were purchased from Solomon Park Research Laboratories. Controls were prepared by identifying serum samples with high and low lipoprotein concentrations. Additional serum pools were prepared in-house from donor subjects recruited at LipoScience or Mayo Clinic. This study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki), cleared by an Institutional Review Board and all donors signed consent forms. Specimens were collected with NMR LipoProfile® serum separator tubes (Greiner #456293 or #455232; Greiner Bio-One; also known as LipoTubes), allowed to clot at room temperature for 30 min, centrifuged at 3,000 rpm for 15 min at room temperature and promptly stored at 4 °C. For pre-analytical collection tube studies, specimens were drawn into BD Vacutainer serum collection tubes (red-top, no gel barrier; BD Diagnostics) and allowed to clot as described above, or in K_2-EDTA plasma collection tubes (BD Diagnostics) and processed per manufacturer instruction.

Acquisition of NMR spectra and data processing

NMR spectra were acquired at the Clinical Laboratory Improvement Amendments (CLIA) approved laboratory at LipoScience (Raleigh, NC) using either the Vantera or NMR Profiler instruments as previously described [20]. Both instruments are equipped with 400 MHz NMR spectrometers. In brief, each specimen was diluted with NMR diluent and delivered into the spectrometer's homogeneous magnetic field through a heated transfer line. Typically, two levels of serum controls were included at the beginning and end of each specimen run.

Data acquisition on the NMR Profiler was performed as described previously [19] with water suppression achieved through presaturation. Data acquisition on the Vantera was accomplished in a similar fashion with the exception that water was suppressed using the WET solvent suppression technique [21]. NMR spectral data were acquired as 5 blocks of 2 scans on the NMR Profiler and 3 blocks of 4 scans on the Vantera for a total acquisition time of 40 and 48 s, respectively. For both systems, the spectra were acquired with a sweep width of 4496.4 Hz and 9024 data points. The data were processed by zerofilling to 32 K points and multiplied by a Gaussian function to provide resolution enhancement prior to Fourier transformation. The methyl signal envelope appearing between 0.718 and 0.914 ppm was further analyzed using the LipoProfile-3 algorithm, which models the signal as a combination of lipoprotein and serum protein subcomponent spectra [17]. The quantification of each subcomponent was determined by linear least-squares singular value decomposition constrained so concentrations could not be <0. Total HDL-P concentrations were calculated as the sum of the HDL subclasses (large, medium and small) with particle diameters between 7.3 and 14 nm and reported in units of μmol/L. The overall measurement time on the Vantera is less than 1 min per sample.

Vantera HDL-P trueness or drift controls

To ensure long-term stability and consistency of Vantera HDL-P measurements, a set of "trueness or drift controls" was developed. Trueness controls consisted of three serum controls with assigned HDL-P values determined across multiple Vantera NMR systems and stored at −80 °C. These were routinely used to benchmark assay performance and prevent long-term drift of HDL-P values. Assigned values and uncertainty (± 2*SD) for trueness controls were obtained using three Vantera NMR instruments, measured twice per day (5 replicates/run) for 3 days. Imprecision for trueness control value assignment was typically 3.5–4.0% CV. Trueness control values were used to qualify instruments during assignment of values for all subsequent control lots.

Assay performance testing

Sensitivity

Serum pools containing low concentrations of HDL-P (5 pools) were tested to determine limits of blank (LOB), detection (LOD) and quantitation (LOQ) according to Clinical and Laboratory Standards Institute (CLSI) guidelines [22] as previously described [20]. Mean concentration and coefficients of variation (CVs) were calculated for each pool.

Imprecision

Serum pools targeting low, intermediate and high concentrations were used to determine within-laboratory imprecision and repeatability per CLSI guidelines [23]. Testing consisted of duplicate tests run twice per day for 20 days ($n = 80$) on one Vantera, while within-run imprecision was calculated from 20 replicates.

Linearity

A series of mixtures of high and low serum pools were created to evaluate assay linearity based on CLSI guidelines [24]. Serum pools with elevated HDL-P were obtained by pooling serum specimens with high concentrations of lipoproteins (determined by screening serum samples using both the Vantera and NMR Profiler platforms) and enriching with addition of HDL isolated by ultracentrifugation. The expected values came from measuring the concentrations for the serum pools with the highest and lowest HDL particle concentrations using the Vantera Clinical Analyzer and calculating the intermediate concentrations based on the combinations that were made between these two pools [25,26]. Specimens with intermediate HDL-P concentrations

Table 1
Within-run imprecision, repeatability, within-laboratory imprecision for HDL-P measured on the Vantera Clinical Analyzer.

	HDL-P (μmol/L)		
	Low	Intermediate	High
Within-run[b]			
Mean	27.5	36.9	47.4
SD	0.7	1.3	1.8
CV	2.4%	3.4%	3.9%
Repeatability[a]			
Mean	27.1	36.6	45.8
SD	0.5	0.7	0.5
CV	1.9%	2.0%	1.0%
Within-laboratory[a]			
Mean	27.1	36.6	45.8
SD	0.8	0.9	0.9
CV	3.0%	2.4%	2.0%

[a] Based on CLSI guidelines tested using 3 controls, 2 runs per day in duplicate, for 20 days (total $n = 80$).
[b] Based on 1 run of 20 tests.

were obtained by mixing serum pools with elevated and low lipoprotein concentrations in varying proportions. Each mixture was analyzed four times.

Method comparison

Method comparison studies consistent with CLSI guidelines were performed to ensure that the performance of the HDL-P assay on the Vantera Clinical Analyzer was similar to the assay run on the NMR Profiler [27]. Samples ($n = 1522$) were tested in singlicate on the reference (NMR Profiler) and comparator (Vantera Clinical Analyzer) NMR systems over a period of 8 days. The correlation between results generated on the two NMR platforms was evaluated using Deming regression analysis.

Reference interval studies

To determine the reference range for the HDL-P assay, samples were analyzed from healthy adult men and nonpregnant women between the ages of 18 and 84 years. A description of this normal study population has been reported [20]. The HDL-P reference range was estimated using nonparametric analyses with reference limits at the 2.5th and 97.5th percentiles according to the nonparametric method described in CLSI guidelines [28]. The reference intervals for men and women were compared by assessing their median results by a Wilcoxon rank sum test. HDL-C values were obtained using the standard chemistry method on a Beckman Coulter analyzer.

In vitro test for interfering substances

A total of 7 endogenous and 23 exogenous substances were tested for possible assay interference consistent with CLSI guidelines [29] as previously described [20].

Comparison of collection tubes

Blood from 46 donors was drawn into three different tubes: LipoTube (serum), BD Vacutainer serum tube (red-top, no gel barrier) and K_2EDTA plasma tube. In order to expand the range of measured

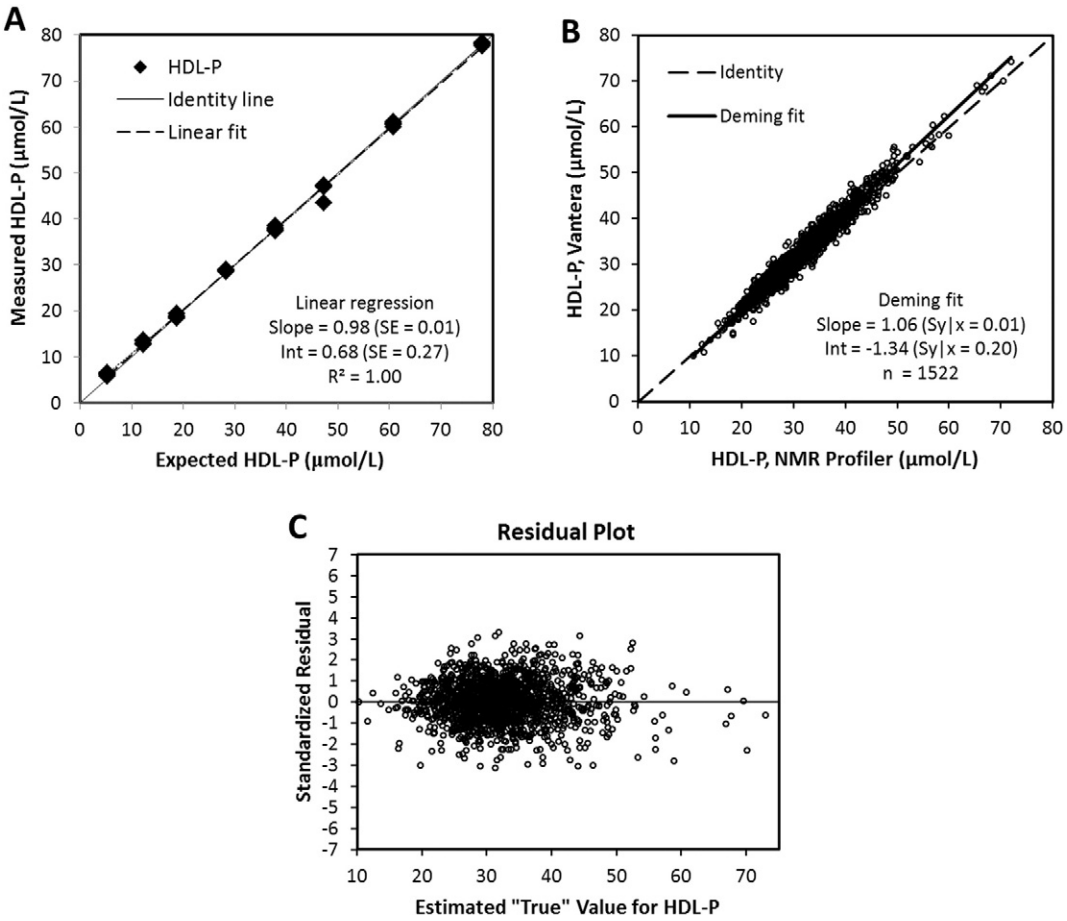


Fig. 1. (A) Results of linearity testing for the Vantera HDL-P assay. (B) Comparison of Vantera and NMR Profiler methods for detection and quantification of HDL-P. (C) Residual plot.

values, 2 specimens with low analyte concentrations were diluted (≤50%) and 2 specimens with elevated analytes were spiked with fractionated HDL (≤10% by volume). Therefore, HDL-P was measured on a total of 50 specimens. Results for conventional serum and EDTA plasma tubes were compared to results for the LipoTube by linear regression.

Stability testing

Samples drawn in LipoTubes obtained from three separate studies of 10 donors each ($n = 30$) were used to assess stability of HDL-P. Samples were stored at 4 °C and aliquots were tested daily for 7 days. Daily mean results for all donors were evaluated with acceptable differences falling within ±10% of the day 0 (draw day) mean.

Results

Assay performance

The analytical performance of the NMR-based HDL-P assay, as measured on the Vantera Clinical Analyzer, was evaluated for the ability to reliably detect and accurately quantify HDL particles in serum. The average value obtained when testing replicate blanks (LOB) was 4.2 μmol/L while the analytical sensitivity or limit of detection (LOD) was 4.6 μmol/L. Testing of nine serum pools, with HDL-P ranging from 4.8 to 7.4 μmol/L, gave a functional sensitivity or limit of quantitation (LOQ) of 4.8 μmol/L with a CV of <10%. Controls with three varying concentrations of HDL analytes (low, intermediate and high) were tested for repeatability, intra-assay (within-run) precision, inter-assay (within-lab) precision. The CV for the HDL-P assay ranged from 2.4% to 3.9% for within-run imprecision, from 1.0% to 2.0% for repeatability and from 2.0% to 3.0% for within-lab imprecision (Table 1).

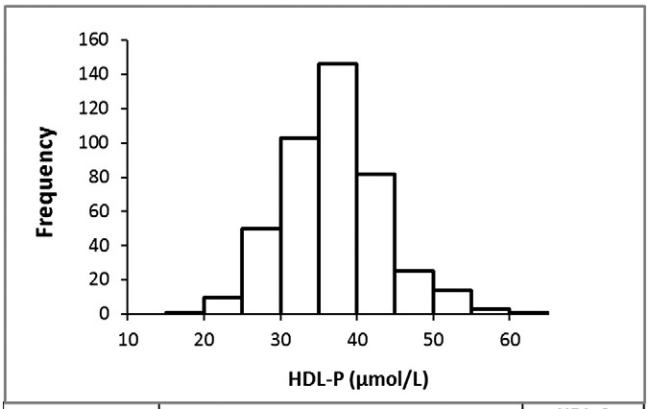
In order to test the ability of the assay to detect HDL particles, the measured values were plotted against the actual or expected concentrations. Linearity of HDL-P was demonstrated throughout the reportable range of 10 to 65 μmol/L with a correlation coefficient of $R^2 = 1.0$ (Fig. 1A).

Method comparison

The performance of the HDL-P assay on the Vantera Clinical Analyzer was found to be similar to the well-characterized HDL-P assay on the NMR Profiler. The linear regression (data not shown) for the HDL-P data, with the NMR Profiler results as the reference method, produced a slope ±95% confidence intervals (CI) of 1.04 (95% CI: 1.03 to 1.05), intercept of −0.58 (95% CI: −0.94 to −0.22) and correlation coefficient (R^2) of 0.98; Deming regression produced a slope of 1.06 (95% CI: 1.05 to 1.08) and an intercept of −1.34 (95% CI: −1.73 to −0.95) (Fig. 1B). Differences between the HDL-P values and the regression line (residuals) were plotted against the estimated concentration of HDL-P. The points on the plot were randomly dispersed around the horizontal axis, suggesting that the results of the two assays were linearly related with no significant bias (Fig. 1C). There also did not appear to be outliers.

Reference interval determination

A population of healthy individuals ($n = 452$) was used to determine the reference interval for the HDL-P assay. Fig. 2 shows the distribution of HDL-P values in this population. The mean HDL-P value was 37.0 ± 6.7 μmol/L, the median was 36.6 μmol/L and the reference interval was 24.4–51.4 μmol/L. The reference interval for the HDL-P assay showed significant differences between genders (men = 22.0–46.0 and women = 26.7–52.9 μmol/L) as did the medians (men = 34.4 and women 37.8 μmol/L) and the means (men = 34.6 ± 6.2 and women 38.2 ± 6.6 μmol/L) ($p = 0.001$). The HDL-C results from the same reference population were compared to the HDL-P results (Fig. 2). The mean HDL-C was 57 ± 14 mg/dL. HDL-C concentrations below 40 mg/dL are classified by National Cholesterol Education Panel's Adult Treatment Panel (ATP) III guidelines [30] as low, signifying that a patient may be at higher risk for CVD. In addition, an HDL-C value of <40 mg/dL has been recommended as a factor used to assess an individual's risk for atherosclerotic cardiovascular disease and need for lipid-lowering therapy (AHA/ACC guidelines). In this population, 40 mg/dL corresponded to the 10th percentile of the HDL-C values and an HDL-P concentration of 28.7 μmol/L (Fig. **2**).



	HDL-P (μmol/L)			HDL-C (mg/dL)
Percentile	All (n=452)	Men (n=158)	Women (n=294)	All (n=452)
5	26.8	24.2	27.7	37
10	28.7	27.2	30	40
20	31.3	29.8	32.6	45
30	33.1	31.1	35.2	49
40	35.3	32.5	36.3	53
50	36.6	34.4	37.8	56
60	38.4	36.3	39.5	59
70	39.9	37.9	40.7	64
80	41.9	40.0	43.3	69
90	45.0	42.6	46.5	77
95	48.6	44.1	50.8	84
Median	36.6	34.4	37.8	56
Mean (SD)	37.0 (6.7)	34.6 (6.2)	38.2 (6.6)	57 (14)
95% Reference Interval	24.4-51.4	22.0-46.0	26.7-52.9	34-88

Fig. 2. Distribution of HDL-P values for reference range study participants.

In vitro test for interfering substances

Thirty substances were tested in vitro for interference with HDL-P quantitation. As per CLSI standards for in vitro interference testing, drug concentrations tested are representative of parent concentrations and do not reflect therapeutic concentrations of the active or inactive metabolites of these agents. Results (Table 2) demonstrated no interference or differences that were >10% at the highest level tested for 27 of the 30 substances tested. Three substances (acetylsalicylic acid, nicotinic acid and clopidogrel hydrogen sulfate) showed potential interference with the HDL-P assay in the initial screening and were tested at multiple concentrations to identify levels at which interference was significant (Table 2). Acetylsalicylic acid at concentrations >1.7 mM (>1536 mg) (therapeutic range = 0.7–2.2 mM or 630–1980 mg) resulted in HDL-P values that were 10–20% lower, while nicotinic acid at concentrations >2.6 mM (>1850 mg) resulted in HDL-P values that were 10–15% lower (therapeutic range ≤2.8 mM or ≤2050 mg). Upon retesting, however, neither substance produced an effect >10% on the HDL-P

Table 2
Summary of interference testing for the NMR HDL-P assay measured on the Vantera Clinical Analyzer.

Substance	Drug name	Test concentration	Concentration eliciting interference
Bilirubin, unconjugated	–	200 μg/mL (342 μM)	
Bilirubin, conjugated	–	289 μg/mL (342 μM)	
Creatinine	–	50 μg/mL (442 μM)	
Hemoglobin	–	2 mg/mL	
Protein/albumin[a]	–	0–60 mg/mL (6.0 g/dL)	
Urea	–	2.6 mg/mL (43 mM)	
Uric acid	–	235 μg/mL (1.4 mM)	
Acetaminophen	Tylenol	200 μg/mL (1.32 mM)	
Acetylsalicylic acid	Aspirin	0–660 μg/mL (3.62 mM)	>1.7 mM (1536 mg)[b]
Atorvastatin	Lipitor	600 μg Eq/mL	
Clopidogrel hydrogensulfate	Plavix	0–360 μg/mL	>107 μg/mL (535 mg)
Enalaprilat dihydrate	Vasotec	0.33 μg/mL (0.86 μM)	
Fenofibrate	Tricor	0–45 μg/mL (125 μM)	
Furosemide	Lasix	60 μg/mL (181 μM)	
Glipizide	Glucotrol	2.0 μg/mL (4.48 μM)	
Heparin	Lovenox	3000 U/L	
Hydralazine hydrochloride	Apresoline	180 μg/mL	
Ibuprofen sodium salt	Advil	0–560 μg/mL (2425 μM)	
Isosorbide dinitrate	Isordil	150 ng/mL (636 nM)	
Menhaden oil	Fish oil	2.4 mg/mL	
Metformin hydrochloride	Glucophage	600 μg/mL	
Metoprolol tartrate	Lopressor	13 μg/mL (18.7 μM)	
Naproxen sodium	Aleve	550 μg/mL (2170 μM)	
Nicotinic acid sodium salt	Vitamin B3, Niacin	0–1.2 mg/mL	>2.6 mM (1850 mg)[b]
Nifedipine	Adalat	0.4 μg/mL (1.16 μM)	
Pioglitazone hydrochloride	Actos	27 μg/mL	
Piroxicam	Feldene	60 μg/mL (181 μM)	
Pravastatin	Pravachol	48 μg/mL	
Salicylic acid	–	0–599 μg/mL (4.34 mM)	
Simvastatin	Zocor	48 μg/mL	

Test concentrations were obtained from CLSI EP7-A2 guidelines Appendix C, where available.
[a] Concentration range reflects added albumin not including endogenous albumin.
[b] Borderline effect that does not reproducibly exceed interference criteria of 10% difference from control.

values even at the highest concentrations. Clopidogrel hydrogen sulfate at concentrations >107 μg/mL (535 mg) resulted in a 10–15% increase in HDL-P (therapeutic range ≤120 μg/mL or ≤600 mg).

Comparison of specimen collection tubes

Several types of specimen collection tubes were compared to LipoTubes for their suitability in the HDL-P assay. The results performed on specimens collected in LipoTubes were plotted against results obtained in conventional red-top serum tubes (no gel barrier) (Fig. 3A). A linear regression analysis was performed, and the resulting line gave a slope of 0.99 ($R^2 = 0.99$). Moreover, red-top serum tubes showed no significant bias based on either 95% confidence intervals around the correlation slope and intercept, or estimation of bias from Bland–Altman residual plots (data not shown). For the HDL-P assay performed on specimens collected in EDTA plasma tubes (Fig. 3B), the resulting linear regression produced a slope of 0.97 ($R^2 = 0.99$). The HDL-P assay

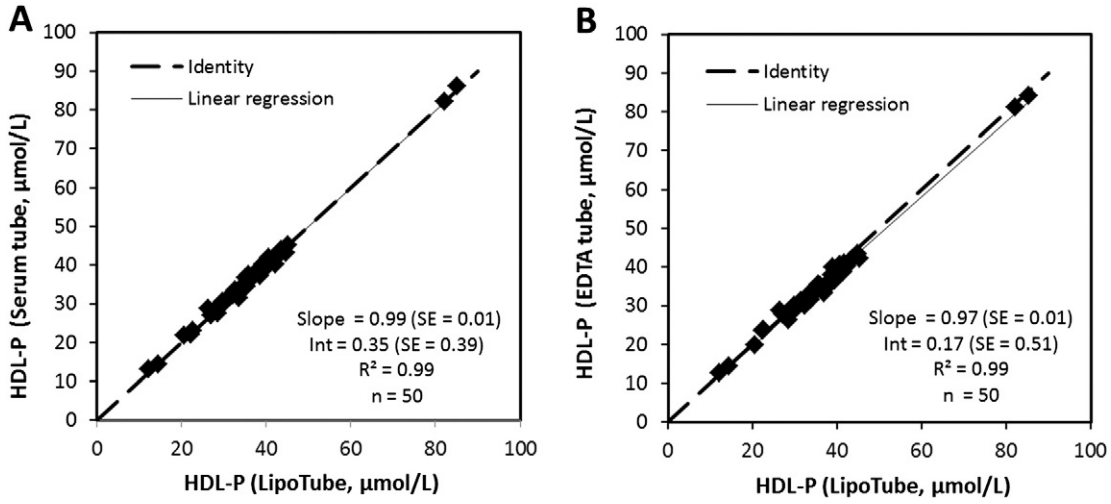


Fig. 3. Comparison of collection tubes. (A) Results for BD Vacutainer serum tubes (no gel barrier) compared to NMR LipoProfile test serum tube (gel barrier). (B) Results for EDTA plasma tubes compared to NMR LipoProfile test serum tube.

Table 3
Stability data for HDL-P assay measured on the Vantera Clinical Analyzer.

Day	*N*	Mean HDL-P (μmol/L)	% Bias
0	30	36.1	n/a
1	30	35.3	−2.2
2[a]	20	35.1	−1.9
3[a]	20	34.7	−3.0
4	30	34.8	−3.7
5	30	34.2	−5.4
6	30	34.2	−5.3
7[b]	29	33.9	−5.2

[a] Days 2 and 3 not observed in 1 of 3 studies ($n = 20$). % Bias calculated relative to mean day 0 HDL-P of 35.8 μmol/L.
[b] Day 7 missing 1 subject due to short draw ($n = 29$). % Bias calculated relative to mean day 0 HDL-P of 35.8 μmol/L.

results in EDTA plasma tubes, however, were modestly (3–7%) lower than results from the same specimens collected in LipoTubes.

Stability testing

The stability of HDL-P as measured on the Vantera Clinical Analyzer was evaluated in thirty serum samples stored for up to 7 days at 4 °C. Measurements were deemed acceptable if they were within 10% of the day 0 mean HDL-P. Results demonstrated that HDL-P was stable out to day 7 at 4 °C with changes ≤5.4% (Table 3).

Discussion

Several epidemiological studies have served the role of establishing the inverse association between HDL-C and the risk of experiencing a CVD event [2–4]. The association of HDL-C with CVD has been attributed to several anti-atherogenic roles that HDL particles play, including the promotion of cholesterol efflux from macrophages in the arterial wall [31]. However, recent developments have raised doubts regarding the relationship between HDL-C and CVD risk. Clinical trials of agents that substantially raised HDL-C levels failed to show a reduction in CVD event rates [6,7]. The failures of these early agents highlighted the fact that investigators had not fully comprehended the complex nature of HDL particles at the time the targets for HDL-C raising therapies were chosen [8–12]. Recent scientific evidence has shown that HDL particles are highly diverse in molecular composition (e.g., proteome, lipidome) and function, both of which vary depending on the inflammatory, nutritional, metabolic or disease state of the patient. This new appreciation for HDL particle diversity has led many to believe that HDL-C is a poor surrogate for the number of circulating HDL and that raising HDL particle numbers and enhancing their function are the more relevant end points to target with an intervention aimed at reducing risk of CVD events, especially in patients whose LDL levels have already been lowered by statin treatment [6,7,11,12,32]. In support, several recent epidemiological and clinical trials showed that NMR-measured HDL-P is a better measure of CVD risk than HDL-C [14–16]. Consideration of these facts have led the developers of newer HDL modulating agents to measure HDL-P and HDL function rather than relying solely on HDL-C. Here we report the analytical performance characteristics for the HDL-P assay on the Vantera Clinical Analyzer. Based on this analysis, NMR HDL-P exhibited acceptable sensitivity, precision and stability, making it a reliable assay for quantification of HDL particle number in the routine clinical laboratory.

Besides HDL-C and NMR-derived HDL-P, two additional measures have been used to estimate circulating concentrations of HDL particles: (1) apoA-I by ELISA or nephelometry and (2) HDL-P by ion mobility spectrometry. Unlike LDL particles that contain one apolipoprotein B (apoB) molecule per particle, allowing apoB to be a fairly good surrogate marker for circulating LDL-P, HDL particles contain varying numbers of apoA-I molecules per particle. The number of apoA-I particles depends on the size of the particle as well as the number of auxiliary proteins carried per particle under various inflammatory or metabolic conditions [8–13]. Therefore, apoA-I is not a good surrogate marker for HDL-P. Ion mobility (IM) spectrometry, where ionized lipoproteins are separated by size and charge, measures HDL particles that have been isolated by ultracentrifugation [33]. Recently, a new calibrated method for measuring HDL-P via ion mobility (CIM) was reported [34]. CIM appears to be an advancement over IM as the estimated stoichiometry of apoA-I molecules per HDL particle was reported to be on average 3–4, which is in closer agreement with current structural models than the average estimated by the original method (13 apoA-I per particle) [35,36]. NMR, on the other hand, estimates <2.0 copies of apoA-I per particle, which is closer to the original IM method, but slightly lower than what the structural models suggest may be the true average. As the editorial that accompanied the journal article describing CIM illustrated, there are strengths and limitations to both NMR and CIM with respect to estimating circulating concentrations of HDL particles (Table 4) [37]. One of the limitations for either NMR or CIM measurement of HDL-P is that to date there has been no reference method with standard calibration of HDL particles in a complex solution such as serum to which an HDL-P assay could be compared for accuracy. Practically speaking, however, NMR is currently the only HDL-P assay with results that have repeatedly been shown to be associated with CVD outcomes and are available in the clinical laboratory setting.

Besides measurements of lipoprotein particle numbers and size, NMR is capable of simultaneously profiling a range of biological molecules and metabolites from a single sample, making it an attractive tool for applications related to personalized diagnostics. The ability to profile complex clinical specimens requires that interference from drugs and other substances be kept at a minimum. NMR technology is not influenced by the type of interferences common to chemical methods. Since all the NMR-based lipoprotein information are extracted

Table 4
Comparison of NMR and CIM HDL-P assays.

HDL-P assay	Assay characteristics	Strengths	Limitations
NMR	• Intra-assay CV 3.2% • Inter-assay CV 2.5%	• Direct measurement without HDL isolation or sample manipulation • Fully automated platform • High throughput	• Measures particle size using the lipid component only
CIM	• Intra-assay CV 6.2% • Inter-assay CV 11.4%	• Measures particle size using all components • Increased accuracy over original IM method due to calibration	• HDL must be isolated by ultracentrifugation which can lead to loss of apolipoproteins therefore redistribution of sizes may occur • Extrapolation of CIM values back to circulating HDL-P concentrations requires careful accounting of volumes, dilutions and sample loss • Labor intensive; low throughput

from the terminal methyl lipid signals, only substances that contain a methyl signal that appears in the lipid region of 0.7–0.9 ppm can potentially interfere with the lipoprotein analysis. Dramatic changes in the matrix including ionic strength and pH can have an effect on the chemical shift of the lipid signal, though this is unlikely to occur with samples obtained under normal specimen handling conditions. However, the in vitro method recommended by CLSI for evaluating substances for assay interference, which entails spiking substances into plasma or serum, may introduce artificial changes to the spectra that are unlikely to occur with circulating concentrations of drug after oral administration. In the current study, in vitro addition of substances to serum revealed that nicotinic acid, acetylsalicylic acid and clopidogrel affected the HDL-P results. The effects seen with nicotinic acid and acetylsalicylic acid were borderline and did not repeat upon further testing; therefore they are not of concern. Furthermore, the concentrations of these drugs that interfered with the HDL-P values in vitro are not expected to be observed in blood samples obtained from patients taking therapeutic doses. For example, nicotinic acid is normally taken in the evening and is rapidly metabolized after oral administration (half-life = 20–45 min). Therefore, circulating concentrations of nicotinic acid are not likely to be high when a patients serum samples is drawn for a lipoprotein analysis, in the morning after overnight fasting. Similarly, the recommended doses of acetylsalicylic acid or aspirin are 81–160 mg for prevention of CVD and 325–650 mg for various other indications. The results of this study revealed that a patient would have to achieve a circulating concentration of acetylsalicylic acid >1.73 mM in order to elicit an effect on the HDL-P results. This is not likely to occur because aspirin is also rapidly metabolized (half-life = 15 min) and the major metabolite of aspirin, salicylic acid, did not affect HDL-P values (Table 2). Clopidogrel is an inactive pro-drug that is rapidly converted to the active form (30–60 min) after oral administration [38]. The clopidogrel metabolites do not contain methyl groups that would arise within the lipid region of the NMR spectrum nor are they hydrophobic enough to bind to the lipoprotein particles and affect their structures. Therefore, they are unlikely to interfere with lipoprotein particle quantitation in vivo. Unfortunately, the clopidogrel metabolites were not available for testing at the time this study was conducted. In conclusion, although in vitro testing in this study indicated some sources of potential interference, this does not prohibit reliable HDL-P quantitation since most changes were observed at concentrations above the therapeutic window or were not likely to affect NMR spectra obtained from patients taking therapeutic doses of these substances.

The successful development of a method to measure HDL-P on a fully automated NMR platform allows decentralized testing in the clinical laboratory setting and creates the opportunity for NMR-based testing across a broader range of clinical applications. The Vantera Clinical Analyzer has been successfully integrated into routine clinical laboratory operations within several national reference laboratories and large healthcare systems within the United States. Current and future efforts are focused on expanding the menu of NMR-based tests that are available for physicians to make informed decisions on patient care, especially in an era when personalized medicine, using tests that are inexpensive and widely available, is becoming highly desirable.

Conflict of interest

S.A.M., P.J.B, J.D.-W., E.J.J., I.S., S.M.W., T.J.F and M.A.C. were employees of LipoScience, Inc. at the time the studies were conducted. AKS had no conflicts of interest.

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