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Phlebotomy tube interference with nuclear magnetic resonance (NMR) lipoprotein subclass analysis

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ABSTRACT

Background: Lipoprotein subclass analysis by nuclear magnetic resonance (NMR) can be used in risk assessment of atherosclerotic cardiovascular disease (ASCVD). There is little information in the literature regarding phlebotomy tube interferences with NMR testing.
Methods: Pooled human serum was exposed to phlebotomy tubes manufactured by Becton Dickinson (BD), Greiner Bio-One, or Sarstedt. Serum was analyzed on the Axinon lipoFIT by NMR assay and by conventional lipid assays performed on a Roche Cobas 8000 system. The effect of incomplete fill volume was also assessed.
Results: Analytical interference in NMR lipoprotein subclass results was observed across many different tube types. The 5 mL Greiner Bio-One Z Serum Sep Clot Activator tube correlated the best with non-gel containing serum tubes from BD and Greiner Bio-One. BD Serum Separator Tubes (SSTs) displayed strong interferences across several NMR analytes that were enhanced with decreased tube fill volumes. Interferences were also observed with different sizes of Greiner Bio-One Z Serum Sep Clot Activator tubes. Interference was generally not observed with conventional lipid testing, although minor interference was found for some tubes with lipoprotein (a) [Lp(a)].
Conclusions: NMR lipoprotein subclass analysis should be standardized by both tube type and tube size to prevent risk of analytical interference.

1. Introduction

Dyslipidemia is a major risk factor associated with the development of atherosclerotic cardiovascular disease (ASCVD) [1,2]. The most widely adopted clinical lipid assays include conventional chemical measurements of lipid panel components, including total cholesterol (CHOL), triglycerides (TRIG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) [3]. Additional advanced lipid testing methodologies are also available and include lipoprotein subclass analysis by nuclear magnetic resonance (NMR) [4]. Proton NMR methods (^{1}H NMR; "NMR" throughout this manuscript) for analysis of lipids in human plasma were developed in the 1990s. By comparing NMR spectra of human plasma – focused primarily on the methyl ($-CH_3$) and methylene ($-CH_2-$) regions – to NMR spectra derived from purified lipid and lipoprotein classes, concentrations of lipid analytes were quantifiable by NMR, including chylomicrons (CHYL), very low-density lipoprotein cholesterol (VLDL-C), LDL-C, HDL-C, CHOL, and TRIG [5–8]. Further refinement allowed for the

Abbreviations: AMR, analytical measurement range; ASCVD, atherosclerotic cardiovascular disease; BD, Becton Dickinson; $-CH_2-$, methylene; $-CH_3$, methyl; CHOL, cholesterol; CHYL, chylomicron; EDTA, ethylenediaminetetraacetic acid; EtOH, ethanol; GLY, glycerin; HDL-p, HDL particle number; HDL-s, HDL particle size; IPA, isopropyl alcohol; LDL-C-calc, calculated LDL cholesterol; LDL-C-dir, direct LDL cholesterol; LDL-p, LDL particle number; LDL-s, LDL particle size; LDT, laboratory-developed test; LHDL-p, large HDL particle number; Lp(a), lipoprotein(a); LVLDL-p, large very low-density lipoprotein particle number; NaN, no result from software due to interference; NMR, nuclear magnetic resonance; ^{1}H NMR, proton NMR; PG, propylene glycol; SLDL-p, small LDL particle number; TRIG, triglycerides; VLDL-s, VLDL particle size

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discrimination and quantification of lipoprotein subclasses [9].

A clinically available NMR-based lipoprotein subclass assay [NMR LipoProfile (LipoScience; Morrisville, NC); subsequently acquired by LabCorp (Burlington, NC)] was brought to market and received FDA 510 k clearance for LDL particle number (LDL-p), TRIG, and HDL-C by NMR [10]. Additional lipoprotein particle number and size parameters are also available in the LipoProfile assay as laboratory-developed tests (LDTs). Alternative NMR-based platforms for lipoprotein subclass analysis (e.g., Axinon lipoFIT by NMR assay; Numares AG, Regensburg, Germany) are now also available [11].

The impact of phlebotomy tube additives, anticoagulants, and/or constituents on NMR results has not been extensively investigated. Citrate and EDTA appear as strong peaks in the NMR spectra of plasma collected in corresponding anticoagulant tubes, although these peaks tend to be outside of the regions used for lipid and lipoprotein analysis [12,13]. Heparin anticoagulant has been reported as another potential interferent with NMR analysis [13–15]. Additionally, phlebotomy tube gel separators may interfere with NMR testing [13].

2. Methods

2.1. General

This project was administratively reviewed by the University of Utah Institutional Review Board, which determined that it did not meet federal definitions of human subject research. No human blood collections were performed in this investigation, which was conducted using commercially available lots of purchased human serum spiked into phlebotomy tubes as described below. Pooled normal human serum was acquired from Innovative Research (Novi, MI; product IPLASER) and stored at −80 °C upon receipt.

Phlebotomy tubes were acquired from VWR (Radnor, PA) and our hospital and clinical laboratory inventories. Complete details of all phlebotomy tubes are provided in Supplemental Table 1. For tube type experiments, 23 varieties of phlebotomy tubes from 3 vendors were acquired: Beckton Dickinson (BD; Franklin Lakes, NJ; 6 serum, 6 plasma), Greiner Bio-One (Kremsmünster; 3 serum, 3 plasma), and Sarstedt (Nümbrecht; 3 serum, 2 plasma). For tube size experiments, 7 tubes sizes of the same type [Greiner Bio-One, Z Serum Sep Clot Activator] were also acquired (see Supplemental Table 1, bottom).

2.2. NMR analysis

NMR lipoprotein and subclass analyses were performed using the Axinon lipoFIT by NMR assay with an Avance III HD NMR spectrometer (Bruker; Billerica, MA), an Ascend 600 MHz magnet (Bruker), and using TopSpin 3.2 (Bruker) and Axinon Suite 1.0.0.1 (Numares) software. The NMR lipoprotein subclass parameters clinically reported using the lipoFIT assay in our laboratory are LDL particle number (LDL-p; in nmol/L), small LDL particle number (SLDL-p; in nmol/L), large VLDL particle number (LVLDL-p; in nmol/L), HDL particle number (HDL-p; in μmol/L), large HDL particle number (LHDL-p; in μmol/L), LDL particle size (LDL-s; in nm), VLDL particle size (VLDL-s; in nm), and HDL particle size (HDL-s, in nm). The NMR platform can also calculate additional lipid parameters, which include CHOL (mg/dL), TRIG (mg/dL), HDL-C (mg/dL), and LDL-C (mg/dL). CHOL, TRIG, HDL-C, and LDL-C were evaluated by both NMR and conventional methods in the present report for comparative purposes.

2.3. Tube type and tube size studies

Serum was thawed overnight at 4 °C and mixed thoroughly prior to use. Serum was added to phlebotomy tubes at 55% of the tube fill volume specified by the manufacturer (see Supplemental Table 1 for manufacturer-listed volumes). This was performed to account for the amount of serum or plasma expected in a completely filled phlebotomy tube when blood is collected.

The protocol for specimens in the tube type and tube size studies was designed to approximate exposure to tube components expected with typical outpatient collections and transportation to centralized laboratory facilities. Phlebotomy tubes were uncapped and serum was added to each tube type (n = 10 replicates) per fill volumes as described above. The tubes were recapped and remained on a rocker at ambient temperature for one hour. Tubes were then centrifuged (5 min at 3000 × *g*; Allegra X-12 Centrifuge; Beckman Coulter; Brea, CA) and then placed upright in test tube racks for four hours of ambient incubation. Specimens were then poured into standard transport tubes (polypropylene; #62.612; Sarstedt) and delivered to the clinical laboratory section for analysis.

Results are shown as percent (%) interference, calculated as:

$$\left(\left(\frac{\text{Average Result}}{\text{Control Tube Average Result}}\right)\times 100\right)-100$$

For the initial tube type study, the control tube was established as tube GSC [Supplemental Table 1; Greiner Bio-One Z Serum Sep Clot Activator (black cap/yellow ring), 5 mL, LipoScience LipoTube], as this tube had previously been validated for use in the NMR LipoProfile assay and is in widespread clinical use. Subsequent analyses used an equivalent tube type as control [GSB, see Supplemental Table 1; Greiner Bio-One Z Serum Sep Clot Activator (red cap/yellow ring), 5 mL]. The GSB tube appears identical to GSC, but with a different cap color and is commercially available from Greiner Bio-One.

2.4. Gel ratio Study

To determine the volumes of separator gel in the seven sizes of Greiner separator tubes (2.5, 3.5, 4.0, 5.0, 7.0, 8.0, and 9.0 mL tube sizes), the separator gel was removed from 1 tube of each type by repeated swabbing with cotton-tipped wooden applicators (Puritan; Guilford, ME) wrapped in strips of paper towels (Bounty; Proctor & Gamble; Cincinnati, OH). Uncapped, empty (gel-removed) tubes were placed individually on an analytical balance (AG285; Mettler-Toledo; Columbus, OH), tared, and filled to their maximum capacity (top of the tube) with demineralized, distilled water (ddH_2O). The mass of ddH_2O added was recorded and the tubes were then dried. This procedure was repeated twice for a total of 3 measurements per tube size and an average was calculated. Subsequently, three tubes of each size (with original gel) were individually weighed, tared, maximally filled with ddH_2O, and the mass of the added ddH_2O was recorded for each. An average mass of ddH_2O was calculated for each gel-containing tube size. The difference in mass of ddH_2O between maximally filled gel-removed and gel-containing tubes (given 1 g/mL density of ddH_2O) is equal to the volume of gel in mL present in each original tube size. A ratio of the serum volume added in mL (as per the prior tube-size studies) to the volume of gel in mL could then be calculated.

2.5. Fill volume study

Pooled human serum was added to BD Vacutainer SST Tubes (Hemogard™ Closure) (BSD; see Supplemental Table 1; 5 mL tube size) at six different volumes: 2.75, 2.48, 2.20, 1.93, 1.65, and 1.38 mL (n = 3 replicates each). These volumes correspond to 100%, 90%, 80%, 70%, 60%, and 50% of the plasma volume that would be contained in a maximally filled whole blood draw (55% of whole blood volume). Additionally, 5 mL GSB tubes (n = 3) filled with 2.75 mL of serum served as controls. Specimen processing was then identical to the studies noted above.

2.6. Conventional lipid testing

After NMR testing was complete, specimens were refrozen at

−80 °C. At the conclusion of NMR experiments, specimens were thawed, mixed on rockers at ambient temperature, and tested using conventional chemistry assays. Conventional assays were performed on a cobas 8000 system (c502 and c702; Roche Diagnostics; Indianapolis, IN) and included TRIG (Roche), CHOL (Roche), HDL-C (Roche), direct LDL-C (LDL-C-dir; N-geneous LDL; Sekisui Diagnostics; Lexington, MA), and lipoprotein(a) (Lp(a); Pointe Scientific; Canton, MI). Calculated LDL (LDL-C-calc) was derived using the Friedewald equation [LDL-C-calc = CHOL-(HDL-C)-(TRIG/5)]. Results from conventional testing were analyzed as percent interference using the GSC and GSB tubes as the controls for tube type and tube size studies, respectively.

2.7. Organic substances spiking study

Ethanol (EtOH; Avantor Macron Fine Chemicals; from VWR), isopropyl alcohol (IPA; Avantor Macron Fine Chemicals), propylene glycol (PG; Acros Organics; Geel, Belgium; from Thermo Fisher; St. Louis, MO), and glycerin (GLY; Spectrum Chemical Manufacturing; New Brunswick, NJ; from VWR) were acquired. Tubes of normal human serum (Innovative Research) were spiked [n = 5 for each chemical; in standard transport tubes (#62.612, polypropylene; Sarstedt)], and then serially diluted 6 times in normal human serum to acquire the final concentrations. The fourth and fifth serial dilutions were neither tested nor used for analysis, in order to keep the total tube count within a single NMR run. NMR testing and subsequent analysis was conducted per the methods described in the manuscript. Results are shown as percent interference with unspiked human serum serving as the control.

2.8. Data analysis

Analysis was conducted using Excel 2016 (Microsoft; Redmond, WA), SigmaPlot 13 (Systat; San Jose, CA), and SPSS Statistics 25 (IBM; Armonk, NY). One-way analysis of variance (ANOVA) was performed in SPSS using the Scheffé post-hoc method, chosen as a conservative approach given the large number of comparisons [16]. P values < 0.05 were considered significant. Results that were significantly different from the control tube are indicated with an asterisk (*) in the tables. Data is presented as mean ± SD unless otherwise indicated.

3. Results

The percent interference in NMR lipoprotein subclass results across the twenty-three different tube types is shown in Table 1 (top). A wide range of potential interference was observed (−60.7% to 128.7%; with some analytes unable to be quantified by the software, e.g., NaN errors). In general, equivalent results were seen for most analytes with Greiner Bio-One serum tubes (GSA, GSB, and GSC), although increased LVLDL-p was observed in the Greiner Bio-One tube without separator gel (GSA). Similar results were also observed for BD serum tubes that did not contain separator gel (BSA and BSB) and the BD Rapid Serum Tube (BSE). A different color/opacity separator gel was observed in the BSE versus the other BD tubes studied (BSC, BSD, BPE, BPF). These other BD tubes (BSC, BSD, BPE, BPF) showed strong interference for SLDL-p precluding the ability of the software to obtain an accurate result, negative interference for LVLDL-p and VLDL-s, and positive interference for LHDL-p and HDL-s (Table 1, top).

Table 1
Lipoprotein particle number and size - % interference versus control tubes.

	Tube (abbreviated name)	Code	LDL-p (%)	SLDL-p (%)	LVLDL-p (%)	HDL-p (%)	LHDL-p (%)	LDL-s (%)	VLDL-s (%)	HDL-s (%)
Tube Type Study	Greiner, Serum Sep	GSC[a]	0.0 ± 2.4	0.0 ± 2.7	0.0 ± 2.9	0.0 ± 2.1	0.0 ± 1.9	0.0 ± 0.1	0.0 ± 0.3	0.0 ± 0.1
	Greiner, Serum Sep	GSB	−1.0 ± 3.4	1.0 ± 4.5	−1.0 ± 3.7	−0.9 ± 3.2	−1.4 ± 4.1	0.0 ± 0.1	−0.3 ± 0.5	0.0 ± 0.2
	Greiner, Serum	GSA	−2.8 ± 3.6	1.4 ± 4.2	15.0 ± 3.0*	−2.6 ± 3.0	−8.1 ± 3.0	0.2 ± 0.1	1.6 ± 0.4	−0.4 ± 0.2
	BD, Vacut. Serum (Hemogard)	BSA	−2.4 ± 3.4	−3.5 ± 3.0	8.5 ± 3.3	−1.1 ± 2.9	−2.2 ± 3.6	0.2 ± 0.1	1.1 ± 0.3	0.0 ± 0.1
	BD, Vacut. Serum (Rubber)	BSB	−2.6 ± 2.4	−9.0 ± 5.2	−8.6 ± 3.2	0.2 ± 2.2	3.6 ± 3.1	0.4 ± 0.1	0.1 ± 0.5	0.4 ± 0.2
	BD, Vacut. RST (Hemogard)	BSE	−1.7 ± 2.2	−10.5 ± 4.6	−4.2 ± 4.1	−4.2 ± 1.8	0.0 ± 2.2	0.6 ± 0.2*	−0.3 ± 0.6	0.3 ± 0.2
	BD, Vacut. Trace Ele (Hemogard)	BSF	−1.8 ± 2.3	0.1 ± 4.9	17.3 ± 3.4*	−2.2 ± 2.0	−6.9 ± 2.2	0.1 ± 0.1	1.6 ± 0.6	−0.4 ± 0.2
	BD, Vacut. SST (Hemogard)	BSD	7.2 ± 2.3*	NaN[c]	−25.4 ± 3.0*	−1.2 ± 1.4	89.4 ± 5.1*	2.6 ± 0.3*	−18.4 ± 1.2*	5.0 ± 0.3*
	BD, Vacut. SST (Rubber)	BSC	6.7 ± 2.5	NaN[d]	−40.2 ± 2.5*	−0.8 ± 1.8	102.7 ± 5.9*	3.0 ± 0.2*	−20.2 ± 1.5*	5.9 ± 0.3*
	Sarstedt, S-Monovette Z	SSA	−3.9 ± 1.4	−12.9 ± 3.7*	−9.5 ± 2.0	−3.1 ± 1.7	6.3 ± 2.5	0.7 ± 0.1*	0.2 ± 0.4	1.0 ± 0.2*
	Sarstedt, S-Monovette Z-Gel	SSB	−2.6 ± 2.2	−15.0 ± 5.4*	−15.0 ± 3.0*	−1.3 ± 2.1	10.7 ± 3.1	0.6 ± 0.2*	−0.2 ± 0.7	1.1 ± 0.2*
	Greiner, LH Li Hep.	GPB	−8.2 ± 1.8*	−3.7 ± 2.5	−2.5 ± 5.1	0.1 ± 1.8	−5.8 ± 1.4	0.1 ± 0.1	1.9 ± 0.5	−0.2 ± 0.1
	Greiner, LH Lit Hep. Sep	GPA	−6.3 ± 3.0	−3.2 ± 4.9	−25.7 ± 4.9*	1.6 ± 2.5	0.4 ± 2.4	−0.2 ± 0.1	−0.5 ± 0.4	0.1 ± 0.2
	Greiner, K2E K2EDTA	GPC	−8.9 ± 1.9*	−10.1 ± 3.9	20.3 ± 3.3*	−3.0 ± 1.8	−6.1 ± 3.4	0.8 ± 0.2*	2.5 ± 0.6	0.0 ± 0.2
	BD, Vacut. Hep. (Hemogard)	BPA	−8.3 ± 2.3*	−2.8 ± 3.4	3.9 ± 3.0	−0.9 ± 1.5	−9.1 ± 2.2	0.1 ± 0.1	2.1 ± 0.6	−0.5 ± 0.1
	BD, Vacut. Barricor Plasma	BPD	−7.4 ± 1.6*	−5.4 ± 8.6	−15.3 ± 13.0*	0.8 ± 2.3	−3.4 ± 6.7	0.0 ± 0.1	0.5 ± 1.3	−0.2 ± 0.4
	BD, Vacut. EDTA (Hemogard)	BPC	−9.5 ± 2.3*	−11.0 ± 3.8*	19.8 ± 2.6*	−5.9 ± 2.3	−8.8 ± 2.2	0.9 ± 0.1*	2.4 ± 0.5	−0.1 ± 0.1
	BD, Vacut. Hep. (Rubber)	BPB	−11.3 ± 3.1*	−18.0 ± 3.2*	−32.8 ± 7.1*	−0.1 ± 2.4	2.0 ± 3.5	0.3 ± 0.1	0.4 ± 0.5	0.4 ± 0.1
	BD, Vacut. PST (Rubber)	BPF	−0.9 ± 3.6	NaN[d]	< AMR[e]	0.7 ± 2.9	98.8 ± 9.3*	2.7 ± 0.2*	−20.2 ± 2.0*	5.7 ± 0.5*
	BD, Vacut. PST (Hemogard)	BPE	1.1 ± 3.1	NaN[d]	< AMR[e]	0.7 ± 1.6	128.7 ± 10.8*	3.5 ± 0.4*	−25.7 ± 2.6*	7.5 ± 0.6*
	Sarstedt, S-Monovette LH	SPA	−12.8 ± 2.9*	−34.3 ± 3.8*	−43.9 ± 5.8*	−4.3 ± 2.5	19.7 ± 3.1*	1.0 ± 0.2*	−0.4 ± 0.8	2.3 ± 0.2*
	Sarstedt, S-Monovette LH-Gel	SPB	−10.9 ± 2.6*	−25.6 ± 4.2*	−60.7 ± 21.0*[f]	1.3 ± 2.3	11.4 ± 3.3	0.5 ± 0.1	−1.1 ± 0.7	1.1 ± 0.2*
	Sarstedt, S-Monovette K3E	SPC	−11.8 ± 2.6*	−30.2 ± 2.6*	−4.4 ± 3.0	−8.9 ± 2.3*	1.8 ± 2.8	1.4 ± 0.1*	1.7 ± 0.3	0.9 ± 0.1*
Tube Size Study	Greiner, Serum Sep 2.5 mL	GSE	2.6 ± 3.8	−2.2 ± 7.9	−36.5 ± 2.9*	4.8 ± 4.2*	18.2 ± 3.9*	−0.2 ± 0.4	−2.4 ± 0.7*	1.2 ± 0.2*
	Greiner, Serum Sep 3.5 mL	GSF	2.1 ± 1.7	−9.0 ± 4.5*	−31.3 ± 3.0*	5.3 ± 2.2*	16.4 ± 2.1*	0.2 ± 0.1	−2.0 ± 0.5*	1.0 ± 0.1*
	Greiner, Serum Sep 4 mL	GSG	0.2 ± 2.4	4.2 ± 4.7	−6.0 ± 3.1*	−0.4 ± 2.6	1.5 ± 1.5	−0.3 ± 0.3	−0.3 ± 0.5	0.1 ± 0.1
	Greiner, Serum Sep 5 mL	GSB[b]	0.0 ± 1.6	0.0 ± 4.2	0.0 ± 4.0	0.0 ± 1.4	0.0 ± 2.5	0.0 ± 0.2	0.0 ± 0.5	0.0 ± 0.1
	Greiner, Serum Sep 7 mL	GSD	3.9 ± 1.8*	−2.3 ± 4.4	−37.0 ± 2.5*	4.9 ± 2.1*	18.5 ± 2.0*	−0.2 ± 0.2	−3.1 ± 0.5*	1.1 ± 0.1*
	Greiner, Serum Sep 8 mL	GSI	−3.7 ± 1.6*	−20.9 ± 2.9*	−34.8 ± 2.7*	0.3 ± 1.4	11.1 ± 3.1*	0.7 ± 0.2*	−1.7 ± 0.4*	0.8 ± 0.2*
	Greiner, Serum Sep 9 mL	GSJ	1.9 ± 2.0	−4.0 ± 2.9	−27.3 ± 4.4*	3.6 ± 2.1	12.6 ± 2.8*	−0.1 ± 0.2	−2.2 ± 0.6*	0.7 ± 0.1*

Complete details of tube types is present in Supplemental Table 1.
[a] GSC used as control tube for Tube Type Study.
[b] GSB used as control tube for Tube Size Study.
[c] 9 NaN (no result from software due to interference), one < AMR (analytical measurement range).
[d] All results NaN, cannot calculate.
[e] Results < AMR, cannot calculate average.
[f] Two results < AMR, calculation performed n = 8.
* P < 0.05.

Table 2
Effect of Tubes on General Lipid Measurements (NMR-Methods) - % Interference Versus Control Tubes.

	Tube (Abbreviated Name)	Code	CHOL (%)	TRIG (%)	HDL-C (%)	LDL-C (%)
Tube Type Study	Greiner, Serum Sep	GSC[a]	0.0 ± 1.5	0.0 ± 1.5	0.0 ± 1.5	0.0 ± 1.6
	Greiner, Serum Sep	GSB	0.0 ± 2.6	-0.4 ± 2.5	-0.6 ± 2.6	0.3 ± 2.7
	Greiner, Serum	GSA	-2.4 ± 2.9	4.0 ± 2.7	-2.3 ± 2.4	-3.3 ± 3.3
	BD, Vacut. Serum (Hemogard)	BSA	-1.4 ± 2.7	2.0 ± 2.3	-0.8 ± 2.2	-2.6 ± 3.1
	BD, Vacut. Serum (Rubber)	BSB	-0.5 ± 2.2	-2.0 ± 1.4	1.0 ± 1.7	-1.4 ± 2.6
	BD, Vacut. RST (Hemogard)	BSE	-1.1 ± 1.6	0.4 ± 2.2	-1.3 ± 1.2	-1.3 ± 2.2
	BD, Vacut. Trace Ele (Hemogard)	BSF	-2.0 ± 1.8	5.3 ± 1.9	-2.9 ± 1.5	-3.2 ± 2.1
	BD, Vacut. SST (Hemogard)	BSD	3.5 ± 2.3	-4.4 ± 1.9	-1.3 ± 1.3	3.9 ± 3.4
	BD, Vacut. SST (Rubber)	BSC	2.8 ± 2.1	-7.9 ± 1.6*	-0.4 ± 1.6	2.7 ± 2.7
	Sarstedt, S-Monovette Z	SSA	-0.4 ± 1.5	-0.4 ± 1.2	0.8 ± 1.2	-1.5 ± 1.7
	Sarstedt, S-Monovette Z-Gel	SSB	0.5 ± 1.7	-3.8 ± 1.9	1.6 ± 1.7	-0.1 ± 1.8
	Greiner, LH Li Hep.	GPB	-5.4 ± 1.8	-8.5 ± 2.1*	-0.2 ± 1.5	-7.3 ± 2.2*
	Greiner, LH Lit Hep. Sep	GPA	-3.6 ± 2.4	-14.1 ± 3.5*	1.2 ± 1.9	-3.7 ± 2.6
	Greiner, K2E K2EDTA	GPC	-3.3 ± 1.7	7.8 ± 1.7*	2.1 ± 1.7	-6.2 ± 1.8
	BD, Vacut. Hep. (Hemogard)	BPA	-6.3 ± 1.7*	-6.4 ± 1.5	-1.5 ± 1.1	-8.4 ± 2.2*
	BD, Vacut. Barricor Plasma	BPD	-4.6 ± 1.4	-9.9 ± 4.5*	0.1 ± 2.1	-5.7 ± 1.6
	BD, Vacut. EDTA (Hemogard)	BPC	-4.3 ± 2.1	8.0 ± 1.4*	-0.6 ± 1.8	-7.2 ± 2.5*
	BD, Vacut. Hep. (Rubber)	BPB	-6.7 ± 2.9*	-17.4 ± 4.3*	0.4 ± 1.8	-8.3 ± 3.1*
	BD, Vacut. PST (Rubber)	BPF	-1.4 ± 3.1	-25.7 ± 4.3*	0.7 ± 2.1	-1.4 ± 3.7
	BD, Vacut. PST (Hemogard)	BPE	-0.4 ± 2.5	-27.8 ± 3.1*	0.9 ± 1.4	-0.7 ± 3.6
	Sarstedt, S-Monovette LH	SPA	-4.6 ± 1.7	-20.5 ± 3.4*	4.2 ± 1.6	-6.9 ± 2.0*
	Sarstedt, S-Monovette LH-Gel	SPB	-5.4 ± 2.4	-23.1 ± 4.0*	3.1 ± 1.7	-6.9 ± 2.7*
	Sarstedt, S-Monovette K3E	SPC	-6.8 ± 2.3*	-1.5 ± 2.1	-4.6 ± 1.8	-9.7 ± 2.6*
Tube Size Study	Greiner, Serum Sep 2.5 mL	GSE	3.8 ± 3.3*	-12.6 ± 1.9*	5.6 ± 3.3*	6.2 ± 3.7*
	Greiner, Serum Sep 3.5 mL	GSF	3.0 ± 1.5	-10.0 ± 1.8*	5.8 ± 1.4*	4.2 ± 1.8*
	Greiner, Serum Sep 4 mL	GSG	0.2 ± 2.1	-3.0 ± 1.5*	-0.4 ± 2.3	0.9 ± 2.5
	Greiner, Serum Sep 5 mL	GSB[b]	0.0 ± 1.2	0.00 ± 1.3	0.0 ± 0.9	0.0 ± 1.5
	Greiner, Serum Sep 7 mL	GSD	4.6 ± 1.6*	-10.6 ± 1.2*	5.1 ± 1.4*	7.2 ± 1.9*
	Greiner, Serum Sep 8 mL	GSI	-1.9 ± 1.2	-10.3 ± 1.7*	1.9 ± 1.0	-1.9 ± 1.5
	Greiner, Serum Sep 9 mL	GSJ	3.0 ± 1.7	-8.9 ± 1.7*	3.1 ± 2.0	4.8 ± 2.0*

[a] GSC used as control tube for Tube Type Study.
[b] GSB used as control tube for Tube Size Study.
* $P < 0.05$.

Negative interference with lithium heparin were observed for LDL-p with some varieties of tubes (GPB, BPA, BPB, BPD, SPA, SPB), but not others (GPA, BPE, BPF) (Table 1, top). Negative interference was observed for SLDL-p and LVLDL-p with Sarstedt serum tubes (SSA and SSB). Negative interference was also observed for LVLDL-p in the gel-containing SSB tube.

Interference for NMR-based determinations of CHOL, TRIG, HDL-C, and LDL-C (Table 2, top) were compared to those obtained using conventional chemistry assays (Supplemental Table 2, top). Negative interference was observed for NMR-based TRIG measurements in all lithium heparin-containing tubes (Table 2; GPA, GPB, BPB, BPD, BPE, PBF, SPA, and SPB). Positive interference was observed for NMR-based TRIG measurements in the EDTA-containing Greiner Bio-One (GPC) and BD (BPC) tubes, but not the Sarstedt (SPC) tubes. Negative interferences were observed for other select NMR-based analyte/tube combinations (see LDL-C and CHOL; Table 2). However, for conventional chemistry-based lipid determinations, significant interference greater than ± 5% was rarely observed for any of the tube types (Supplemental Table 2, top). Negative interference was only observed with the SPC and BPA tubes for Lp(a).

Experiments were then conducted to determine whether different sizes of the GSB tube produced equivalent lipoprotein subclass results (Table 1, bottom). Surprisingly, LVLDL-p showed negative interference in all non-5 mL tube sizes, whereas positive interference for LHDL-p was observed for 2.5, 3.5, 7, 8, and 9 mL tube sizes (Table 1, bottom). Negative interference was also observed for SLDL-p in 3.5 and 8 mL tubes, whereas positive interference > 5% was observed for HDL-p in 3.5 mL tubes (and nearly that magnitude in 2.5 and 7 mL tubes). Interferences were also observed for NMR-based TRIG for all tube sizes except the 4 mL tube (Table 2, bottom), whereas minor interferences were also observed across tube sizes for HDL-C and LDL-C. A positive interference was also observed for Lp(a) across tube sizes (Supplemental Table 2, bottom).

To determine whether the relative volume of serum to gel across different GSB tube sizes may contribute to the differences in NMR lipoprotein subclass results observed, the ratio of serum to gel volume was plotted versus percent interference for each NMR parameter (see Fig. 1 and Supplemental Fig. 1). Clusters of results corresponding to tube sizes (e.g., 4 and 5 mL) could be observed for LVLDL-p (Fig. 1C), LHDL-p (Fig. 1E), and TRIG (Supplemental Fig. 1B). Other parameters did not show result variability across serum to gel volume ratios (e.g., LDL-p, Fig. 1A; LDL-s, Fig. 1F; VLDL-s, Fig. 1G; HDL-s, Fig. 1H).

Fig. 2 shows a representative NMR spectrum from control serum (A), as well as an NMR spectrum from serum incubated at 50% expected fill volume in a BSD tube (B). An interference peak can be observed in the corresponding $-CH_3$ region of analysis (Fig. 2B; inset). To determine whether the relative fill volume changes the magnitude of observed interference, BSD tubes were filled to decreasing relative volumes with serum, processed in the same manner as prior experiments, and then analyzed for NMR lipoprotein subclasses. As shown in Fig. 3 (and Supplemental Fig. 2), decreasing the fill volume produced strong positive interference across 10 of 12 analytes. For the remaining 2 analytes (SLDL-p and LVLDL-p), interference either completely precluded any NMR-determination of that analyte (NaN errors) (SLDL-p, Fig. 3B) or prevented measurement at ≤70% fill volume (LVLDL-p, Fig. 3C).

Lastly, additional studies were conducted to investigate potential interference due to the presence of other organic substances that may be found in patient specimens (e.g., ethanol; EtOH), or that could be introduced as contaminants during phlebotomy [e.g., isopropyl alcohol (IPA) as an antiseptic; glycerin (GLY) and propylene glycol (PG) in skin creams, lotions, soaps, and/or cosmetics]. While each of these four

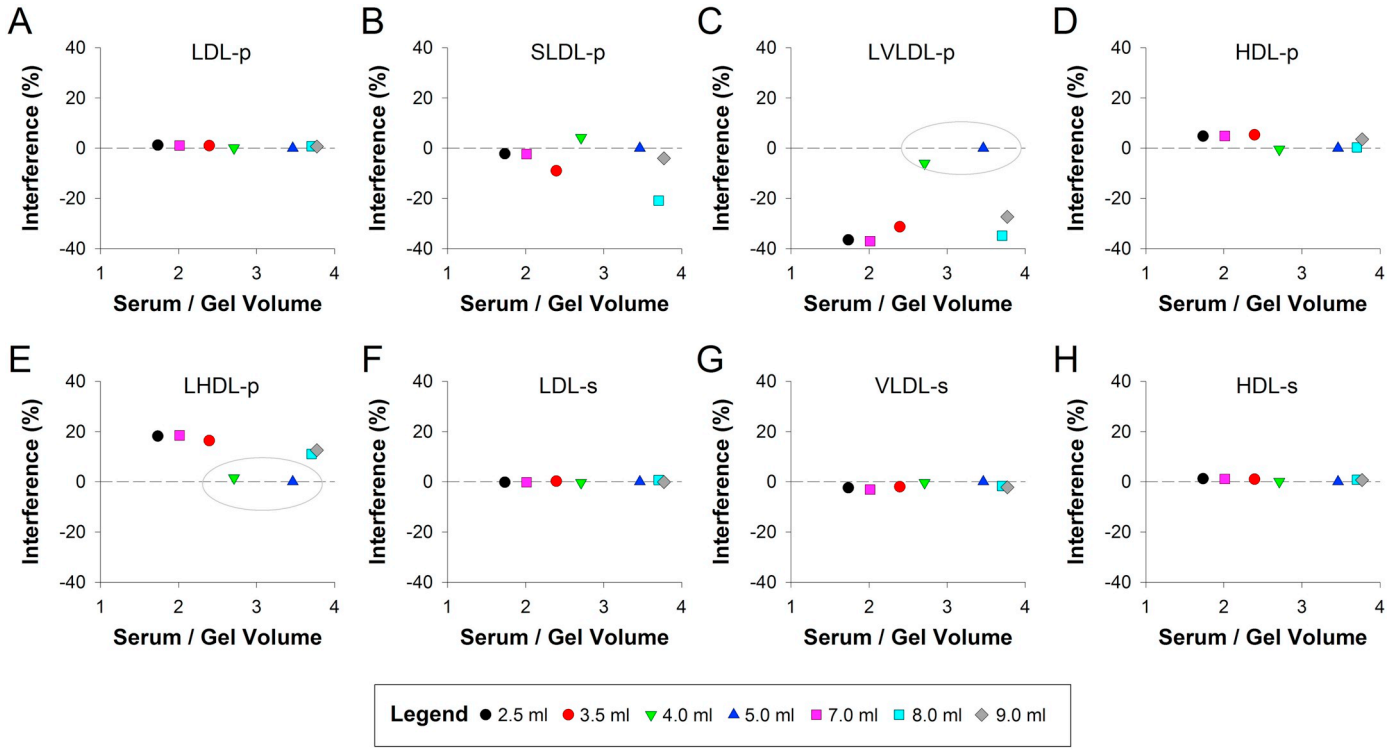

Fig. 1. Gel ratio study – Greiner Bio-One Z Serum Sep Clot Activator Tubes. The % interference (as compared to GSB control tube) is plotted versus the ratio of serum to gel volume for seven different sizes of Greiner Bio-One Z Serum Sep Clot Activator Tubes (GSE, 2.5 mL, black circle; GSF, 3.5 mL, red circle; GSG, 4 mL, green downward triangle, GSB, 5 mL, royal blue upward triangle; GSD, 7 mL, lavender square; GSI, 8 mL, turquoise square; GSJ, 10 mL, gray diamond) for (A) LDL-p, (B) SLDL-p, (C) LVLDL-p, (D) HDL-p, (E) LHDL-p, (F) LDL-s, (G) VLDL-s, and (H) HDL-s as determined by NMR analysis. Clustering of results for GSG (4 mL) and GSB (5 mL) tubes are indicated by gray outline ellipses in LVLDL-p (C) and LHDL-p (E). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

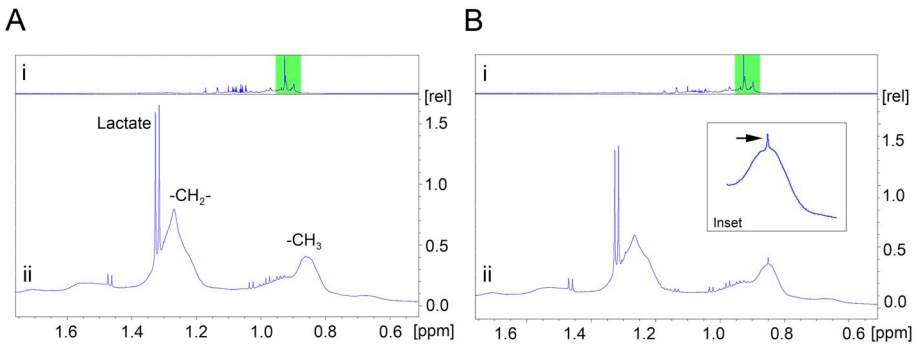

Fig. 2. NMR spectra – normal serum and bd serum separator tube (SST) interference. Each panel shows the entire NMR spectra (top, i), and a region of interest as indicated by green shading (i) and magnified below (ii). A, NMR spectrum of a normal control serum. Labeled are regions representing methyl groups ($-CH_3$) where lipoprotein particle numbers and sizes are measured, the methylene groups ($-CH_2-$), and the lactate standard. *B*, NMR spectrum of normal human serum incubated with 50% of the expected fill volume in a BD Vacutainer Serum Separator Tube (SST) Hemogard Closure (Supplemental Table 1; see BSD). An interfering peak is observed in the $-CH_3$ region (arrow, inset, magnified view). x-axes are in parts per million (ppm); y-axes are in relative units.

substances could be observed in NMR spectra when spiked into serum (see Supplemental Fig. 3), only slight interferences in NMR lipoprotein subclasses was observed for SLDL-p (see Supplemental Fig. 4). All other analytes were insensitive to interference, even at extremely elevated spiking concentrations.

4. Discussion

The present experiments demonstrate that NMR-based lipoprotein assays are more vulnerable to tube type and tube size differences than has previously been described. Clinical and analytical studies evaluating NMR-based lipoprotein subclasses should clearly describe the specific tube types and sizes used in their investigations. Standardization around a single tube type and size should also be considered for clinical testing and study-related purposes, as tube-related interferences may be difficult (if not impossible) to identify, particularly in settings that use sample aliquots for testing.

The LipoProfile assay recommends use of LipoTubes [17]. Plain red top serum, EDTA plasma (no gel), and heparin plasma (no gel) are also considered as acceptable tubes, while specimens collected in gel separator tubes other than LipoTubes are not acceptable for this assay [17]. Minor decreases in LDL-p and HDL-p have been described using the LipoProfile assay on the Vantera NMR platform with EDTA plasma [18,19]. It is unknown whether the tube type and size interferences observed in the present report would also be observed using the LipoProfile assay.

The Axinon lipoFIT by NMR assay serum kit instructions for use state that specimens should not contain anti-coagulant additives, that the S-Monovette serum tubes are appropriate for use, and that other tube types would need to be qualified (i.e. validated) [20]. This assay performed in our laboratory was validated as an LDT using the commercially available 5 mL GSB tubes. Serum red top tubes are also accepted for this clinical assay, although plasma specimens or specimens collected in any other gel-separator tubes are not [21].

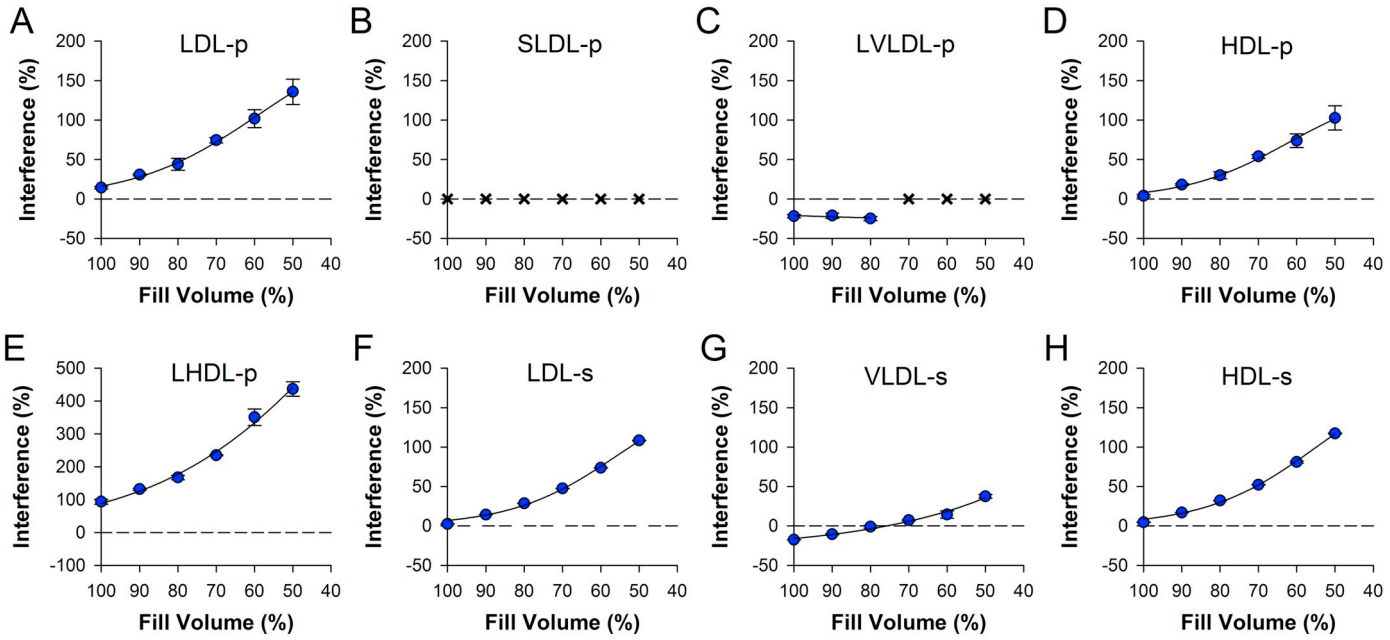

Fig. 3. BD serum separator tube (SST) interference in NMR lipoprotein analysis – impact of decreasing fill volumes. % interference in BSD tubes (as compared to the GSB control) is plotted versus the % fill volume of BSD tubes for (A) LDL-p, (B) SLDL-p, (C) LVLDL-p, (D) HDL-p, (E) LHDL-p, (F) LDL-s, (G) VLDL-s, and (H) HDL-s as determined by NMR analysis. % interference at 100% BSD fill volume is shown on the Y-axis and corresponds to BSD results in Table 1. Scenarios where no result was obtainable due to analytical error flags (NaN) are indicated by "x" (see B, SLDL-p, all fill volumes; C, LVLDL-p, 70%, 60%, and 50% fill volumes). Curve fit for G is Sigmoidal, Sigmoid, 4 Parameter; curve fit for all other graphs are Sigmoidal, Sigmoid, 3 Parameter. Note: y-axis scale is different for E to show maximal interference.

Blood collection tube interferences are a widely recognized source of potential pre-analytical error in clinical laboratory testing [22,23]. While use of incorrect tubes may be easy to identify in certain circumstances [e.g., markedly low calcium (Ca^{2+}) if an EDTA-containing tube was collected], more subtle interferences are often unrecognized. Tube type interferences have previously been identified in several mass spectrometry and metabolomics studies [24–28]. While tube type interferences described with therapeutic drug monitoring are often associated with absorption of drugs into separator gels [29], analytical interferences may also be due to additives and/or gel components leaching into the specimen and affecting subsequent testing.

Phlebotomy tubes may contain anticoagulants (e.g., EDTA, heparin, citrate) or clotting agents (e.g., silica / silicone), but also a myriad of additional components including rubber stoppers, plasticizers, lubricants, wall materials, surfactants, polymers, and gels [22,23,30]. These exact components are often proprietary, and they are only rarely specifically identified as causative interferents [31]. Unfortunately, we do not know the definitive constituents responsible for the interferences observed in the present report.

The clustering of results between 4 mL and 5 mL Greiner Bio-One tubes (versus other tube sizes; see Fig. 1; Table 1, bottom; and Table 2, bottom) suggests that tube composition differences may exist, even within different size tubes of the same tube type. Similar magnitude differences were generally not observed for most conventional chemistry assays across these tube sizes (Supplemental Table 2, bottom), although tube size differences were observed for Lp(a), suggesting that tube size differences may also be under-recognized for certain assays. Published guidelines regarding manufacturer and clinical laboratory tube validations do not specifically address this possibility [30].

Given the lipophilic nature of lipoprotein constituents, we cannot definitively exclude the possibility that such lipids may absorb into organic (and/or lipophilic) components of phlebotomy tubes. The lack of interference with conventional chemistry assays (Supplemental Table 2), and the positive interference observed with several analytes (Table 1), contradicts the idea that absorption is the primary mechanism of interference across the present studies. Instead, our results suggest the interfering substances may be leaching into the sample affecting sample analysis by NMR.

LVLDL-p (which likely includes chylomicrons) [14] was often not present in commercially obtained serum. Per vendor specifications, the serum used in the present study was pooled, normal donor serum [32]. We do not know the fasting status of such donors. Previous studies from our laboratory have demonstrated limited frozen (−20 °C) stability for LVLDL-p [33], which may have contributed to the difficulty in obtaining material with quantifiable analyte. Additionally, interference patterns for LVLDL-p – a TRIG-rich lipoprotein – appear to correlate to interference patterns for NMR-based TRIG determinations, as 10 of the 14 tubes with LVLDL-p interference also show TRIG interference (Tables 1 and 2). This may reflect an analytical relationship in how these NMR-derived results are obtained from the similar regions of the NMR spectra.

The interference observed with Lp(a) (albeit relatively minor) was not expected. Review of the manufacturer package insert, however, suggested that a slightly decreased measured Lp(a) had previously been observed with sodium and lithium heparin specimens with this Lp(a) assay [34]. The exact specifications for the tubes used for those studies were not stated by the manufacturer, precluding further comparison across our datasets.

Regarding limitations of this research, it is important to emphasize that the interferences observed in the present investigation were derived using pooled serum incubated in a variety of tubes. These tubes were designed to collect whole blood, and for the purpose of obtaining serum or plasma for subsequent analyses. The present studies can therefore not distinguish serum versus plasma matrix differences in any measured analyte. Additionally, the potential impact of varying centrifugation speed or time was also not investigated. While conventional lipid testing was conducted after an additional freeze thaw cycle (versus prior NMR testing), results were all normalized to control tubes (GSB or GSC) that had undergone the same corresponding number of freeze thaw cycles. This limited the impact of potential analyte instability on result interpretation. Strengths of this study include the large number of different sample tubes and potential interferences investigated. This

research has also revealed under-filling of tubes as a previously unrecognized source of NMR lipoprotein subclass interference.

Investigators often use general terms (e.g., serum, heparin plasma) to describe specimen types used in published reports. If consistent tube types are used within an investigation, the relative differences in results across test conditions should still be valid. However, comparison of absolute differences across studies may be problematic. Small differences in analyte concentrations could affect the clinical interpretation of individual patient results in the context of ASCVD risk assessment. Standardization toward a common tube type for NMR lipoprotein testing should, therefore, be considered for future clinical trial and patient care purposes.

Declarations of interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.11.010.

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