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Abstract

Background: Characterization of lipoprotein particle profiles (LPPs) (including main classes and subclasses) by means of ultracentrifugation (UC) is highly requested given its clinical potential. However, rapid methods are required to replace the very labor-intensive UC method and one solution is to calibrate rapid nuclear magnetic resonance (NMR)-based prediction models, but the reliability of the UC-response method required for the NMR calibration has been largely overlooked.

Methods: This study provides a comprehensive repeatability and reproducibility study of various UC-based lipid measurements (cholesterol, triglycerides [TGs], free cholesterol, phospholipids, apolipoprotein [apo]A1 and apoB) in different main classes and subclasses of 25 duplicated fresh plasma samples and of 42 quality control (QC) frozen pooled plasma samples of healthy individuals.

Results: Cholesterol, apoA1 and apoB measurements were very repeatable in all classes (intra-class correlation coefficient [ICC]: 92.93%–99.54%). Free cholesterol and phospholipid concentrations in main classes and subclasses and TG concentrations in high-density lipoproteins (HDL), HDL subclasses and low-density lipoproteins (LDL) subclasses, showed worse repeatability

(ICC: 19.21%–99.08%) attributable to low concentrations, variability introduced during UC and assay limitations. On frozen QC samples, the reproducibility of cholesterol, apoA1 and apoB concentrations was found to be better than for the free cholesterol, phospholipids and TG concentrations.

Conclusions: This study shows that for LPPs measurements near or below the limit of detection (LOD) in some of the subclasses, as well as the use of frozen samples, results in worsened repeatability and reproducibility. Furthermore, we show that the analytical assay coupled to UC for free cholesterol and phospholipids have different repeatability and reproducibility. All of this needs to be taken into account when calibrating future NMR-based models.

Keywords: analytical variation; cholesterol; dyslipidemia; lipoproteins; quality control; triglycerides; variability.

Introduction

Lipoprotein particle profiles (LPPs) have been repeatedly shown to be a risk factor for cardiovascular disease (CVD) [1, 2]. LPs play an important role in lipid metabolism as transport vehicles of lipids (cholesterol and triglycerides [TGs]) in circulation. Commonly, LPs are classified based on density as very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL) and high-density lipoproteins (HDL). These four classes of LP, which can be further separated into subclasses, differ not only in size and density, but also in the composition of the lipids carried inside the particle and the type of apolipoproteins present in the particle membrane. LP class determines the source and function of the lipids transported inside. Therefore, determination of LP composition can serve as a snapshot of the lipid metabolism, making it possible to assess how lipid metabolism differs in health and disease states [1–3].

The classical lipid panel, which typically provides measurements for total, LDL and HDL-cholesterol and for TGs, is widely used in clinical practice as it is simple, cheap and fast to measure. In general, the classical lipid

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panel provides useful measurements and their biological variability has been extensively studied in serum and plasma [4–6]. In some cases, LDL-cholesterol values are not measured directly, but simply estimated from total cholesterol, HDL-cholesterol and total TGs using, for example, the Friedewald or Martin equations [7–11]. The main drawback of the classical lipid panel is that it provides limited lipid information, as it only focuses on the cholesterol content of two of the main LP classes. More detailed profiling of the LPPs might improve our understanding of their role in health and disease as well as their diagnostic ability for CVD [1]. Thus, clinicians have stressed the importance of LPPs for CVD risk assessment, which makes routine characterization of LPPs using separation-based technologies and clinical assays for the determination of the concentrations of components of these LPs highly desirable. The limitations of the time-consuming separation-based techniques have resulted in a quest for building NMR-based LPP prediction models. Nevertheless, a reference method is still necessary for the calibration of the rapid prediction method as well as for future maintenance of the calibration model. High-performance liquid chromatography (HPLC) and ultracentrifugation (UC) are two of the techniques used to separate the different LP fractions, containing the LP main classes and subclasses [12, 13]. UC exploits the differences in density of the different lipoprotein classes for separation and remains the classical and most commonly used method for separating lipoprotein subclasses [14]. While UC provides much more information than the classical lipid panel, it is also cumbersome, time-consuming and very much dependent on the skills and experience of the operator. In the last two decades, effort has been put into developing rapid prediction models based on ^1H nuclear magnetic resonance (NMR) spectroscopy to determine LPPs in blood samples [15–21]. NMR spectra contain sufficient physicochemical information (i.e. the intensity and position of the complex methyl and methylene signals from lipids) to extract LPPs from the spectra by calibration to an external reference method [14]. When such a calibration method is established, the LPPs of future samples can be predicted from a carefully recorded NMR spectrum by the application of an appropriate calibration model.

Regardless of whether UC combined with lipid analysis is used directly as a lipid profiling technique or as reference for NMR-based models, accurate and reliable LPPs are necessary to provide a correct picture of the lipid metabolism. Although UC is still widely used by research laboratories as part of the determination of compound concentrations in the main LP classes and subclasses,

very little attention has been given to the repeatability, reproducibility and recovery of the complete LPPs (including subclasses). Pagani and Panteghini have contributed by studying within-subject and analytical variability in TGs, cholesterol, apolipoprotein (apo)A1 and B in serum, LDL, HDL, HDL2 and HDL3 ($n=10$ healthy subjects) [22].

In the present work, we provide a thorough and comprehensive repeatability, reproducibility and recovery study of lipid measurements (cholesterol, TGs, phospholipids, free cholesterol, apoB and apoA1) in plasma, main LP classes (VLDL, IDL, HDL and LDL) and LP subclasses (HDL2a, HDL2b, HDL3, LDL1, LDL2, LDL3, LDL4, LDL5 and LDL6) separated by UC. To the best of our knowledge, this is the first paper to comprehensively describe figures of merit of measuring LPPs (including HDL and LDL subclasses) by coupling UC with colorimetric and turbidimetric assays.

Materials and methods

Participants

Male and female participants older than 20 years, with BMI ranging from 18.5 to 40 kg/m^2 , who had not received antibiotic treatment 3 months prior to the beginning of the study and who had not received pre- or probiotics 1 month prior to the beginning of the study, were included as part of the COUNTERSTRIKE (COUNTERacting Sarcopenia with proTeins and exeRcise – Screening the CALM cohort for lipoprotein biomarkers) cohort. Pregnant and lactating women, as well as individuals suffering from CVD, diabetes or chronic gastrointestinal disorders, were excluded from the study. COUNTERSTRIKE participants were recruited in the Copenhagen region via press and online announcements and gave written consent to participate in the study. The study was conducted at the Department of Nutrition, Exercise and Sports at the University of Copenhagen, Denmark and was approved by the Research Ethics Committees of the Capital Region of Denmark in accordance with the Helsinki Declaration (H-15008313) and the Danish Data Protection Agency (2013-54-0522).

Samples

In this study, LPPs of fresh fasting plasma samples from 223 individuals of the COUNTERSTRIKE cohort were determined. One individual was excluded from further analysis due to non-compliance to fasting. LPPs from 25 of the included 222 individuals were determined twice in the same UC run (UC within-run duplicates), adding up to a total of 247 measurements of the different lipid and protein compositions. Additionally, LPPs in 42 measurements of one pooled QC material (aliquots from one frozen pooled plasma from healthy adults) were measured, in different UC runs, during the span of 10 months, from November 2015 until August 2016 (UC between-run replicates).

Determination of lipoprotein particle profiles

Ultracentrifugation (UC): Main lipoprotein fractions (VLDL, IDL, HDL, LDL) and lipoprotein subfractions (HDL2a, HDL2b, HDL3, LDL1, LDL2, LDL3, LDL4, LDL5, LDL6) were separated by means of UC using a modified version of the Baumstark method [13] as illustrated in Figure 1 (see Supplementary Table 4 for densities). Several stock solutions were used for the separation steps (Supplementary Table 1). A detailed description of the UC method can be found in the Supplementary Data.

Determination of concentrations in plasma, main classes and subclasses: Colorimetric and turbidimetric assays were performed on an ABX Pentra 400 analyzer (ABX Pentra; Horiba ABX, Montpellier, France) to determine the plasma, main class and subclass concentrations of total cholesterol, TGs, apoA1 and apoB (ABX Pentra; Horiba Medical, France). Accuracy and precision testing of the assays are available from <https://toolkits.horiba-abx.com/documentation/index.php>. The total cholesterol assay has been certified by the Cholesterol Reference Method Laboratory Network (CRMLN) (https://www.cdc.gov/labstandards/pdf/crmln/MFR_TC_CRMLN-508.pdf).

Free cholesterol and phospholipids were also determined using colorimetric and turbidimetric assays (MTI Diagnostics, Germany <http://www.mti-diagnostics.com/produkte-1/index.html> and Wako Diagnostics, Wako Chemicals, USA, http://www.wakodiagnosics.com/r_free_cholesterol.html and http://www.wakodiagnosics.com/r_phospholipids.html) due to concerns with the reproducibility and repeatability of the other assays (Supplementary Table 2). Our assays showed good agreement with external control references (Supplementary Table 2). Measured concentrations were corrected for dilution effects occurring during the separation of the different LP classes. The dialysis step performed to separate the different LDL subclasses in the UC process causes a systematical bias (due to residues left in the dialysis sac) that can be corrected for when calculating LDL subclass concentrations. For a given sample i , a correction factor (c) for LDL subclass concentrations was calculated as indicated in equation 1. We calculated this factor by using cholesterol concentrations to ensure that the sum of cholesterol concentrations in the LDL subclasses adds up to the cholesterol concentration in the LDL main class (i.e. recovery of LDL cholesterol concentrations from LDL subclass cholesterol concentrations is 100%). This is done to account for potential losses as part of the dialysis step. This multiplicative factor c was then used to correct LDL subclass concentrations for all compounds.

$$c_i = \frac{[\text{LDLchol}]_i}{[\text{LDL1chol}]_i + [\text{LDL2chol}]_i + [\text{LDL3chol}]_i + [\text{LDL4chol}]_i + [\text{LDL5chol}]_i + [\text{LDL6chol}]_i} \quad (1)$$

It is important to note that the UC method followed in this study consists of many steps and is coupled to secondary analytical assays (Figure 1). This generates many possible sources of error that can contribute and accumulate to diminishing the quality of the data. It is therefore important to include replicates and QC samples to ensure that the acquired data is repeatable and reproducible.

Data analysis

Recovery of plasma concentrations from main classes and subclasses was calculated as the ratio of the sum of main classes

(and subclasses) concentrations to the total plasma concentrations for each of the compounds measured. Repeatability of LP concentration measurements was evaluated for the fresh plasma duplicates (within-run duplicates) in terms of average within-individual coefficient of variation (WCV, expressed as percentage) across replicate measurements and standard deviation (SD) of these WCV values. The single score intraclass correlation coefficient (ICC) defined by a one-way random effects model, with 95% confidence intervals (CIs), was also calculated to quantify the degree of absolute agreement of measurements [23]. For each measurement and subclass, outliers, as determined by a Grubbs test on the average of the two repeated measurements, were excluded prior to calculating WCV and ICC. A Shapiro-Wilk test was performed on the average of the two repeated measurements to test for normality. Logarithmic transformation was applied when data were not normally distributed to calculate ICCs. WCV and ICC were calculated excluding measurements below the limit of detection (LOD). Reproducibility was assessed in frozen pooled QC samples (between-run replicates) in terms of CV, also excluding measurements below the LOD.

Rationale of the approach

As there are no standards available for all LPP measurements as described, it is not possible to evaluate trueness and accuracy as figures of merit in the traditional way. The definition of recovery as we used it, is based on the facts that (i) theoretically the subclass concentrations should add up to the main class concentration and (ii) the concentration in the main class is higher and thus expected to be above LOD and reliable. It is not possible to evaluate the between-run reproducibility of the fresh samples, as the samples cannot be kept fresh for a long time. Hence, we chose to quantify reproducibility using the (frozen) QC samples. In a similar fashion, repeatability of the QC samples was not deemed representative for the repeatability of the measurements of the fresh samples. Ideally, an experimental design is used to study repeatability and reproducibility simultaneously using analysis of variance models to disentangle the different contributions, but this is not possible given the mentioned limitations.

Results

Recovery of plasma concentrations from main classes and subclasses concentrations

Overall, the participants included in the analyses had plasma lipid levels within the normal/upper normal range (see Table 1 and Supplementary Table 3). The recovery of plasma concentrations from the sum of main class or subclass concentrations (expressed as a percentage) can serve as an indicator of the consistency of UC-derived LPP concentrations. Figure 2 shows the sum of the

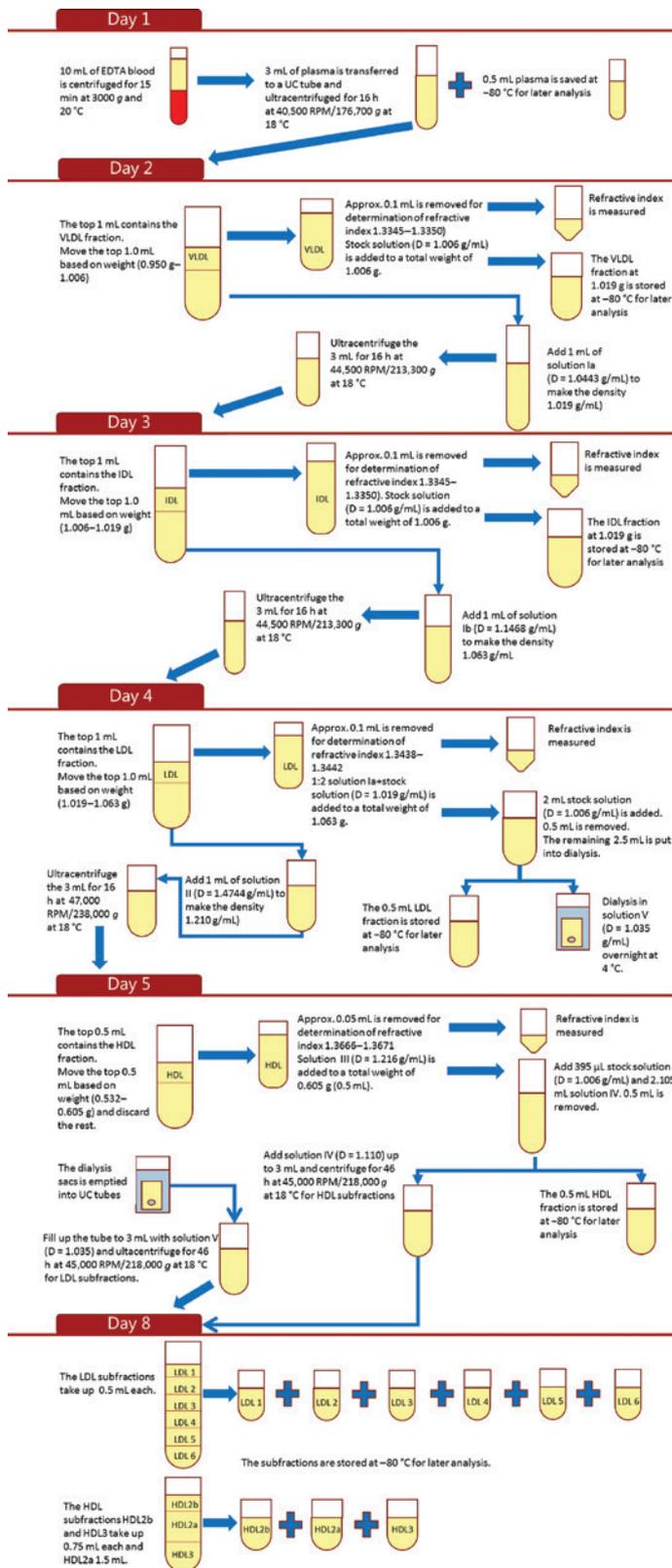


Figure 1: UC workflow followed to separate lipoprotein particle main fractions and subfractions, based on the Baumstark method [13].

concentrations in the main classes and in all subclasses vs. the plasma concentration for each of the compounds measured (recovery).

The values below the LOD were excluded from analysis by inserting a zero value. In Supplementary Figure 1, values below the LOD are substituted with the

LOD value for comparison of how below-LOD measurements affected the recovery. The included regression lines show that there are no systematic deviations in the recovery across the concentration range included in the cohort. Plasma cholesterol, TGs, apoB and apoA1 concentrations can be well recovered from both the main classes and the subclasses. Plasma free cholesterol and phospholipids concentrations, especially the former, are not as well recovered as the other compounds, neither from main classes nor from subclasses concentrations. In the case of free cholesterol, the recovered concentrations from main fractions and subfractions are systematically higher than the original plasma concentrations. This, together with the fact that the recoveries for different measurements are scattered around the 100% recovery line, might be an initial indicator of the presence of high measurement errors for free cholesterol (Figure 2). It is observed that the recovered concentrations for the rest of the compounds (cholesterol, TGs, phospholipids, apoA1 and apoB) are systematically lower than the plasma concentrations. This may be due to lost material during the UC process and/or during lipid and apolipoprotein analysis. An apparent inconsistency is found by the fact that, for most compounds, the sum of concentrations of the main classes is smaller than the sum of concentrations of the subclasses. This inconsistency is probably related to the HDL class, as the LDL subclass concentrations are corrected for the dialysis effect. Munroe et al. showed that the high rotor speeds (>30,000 rpm) used during the separation of the HDL fraction results in shearing of the original LP and loss of protein, and which can in turn introduce variability during the separation of classes [24]. In order to test for potential overlap of certain particle types in the different density fractions apoB in HDL and apoA1 in IDL and LDL was examined. However, we did not find significant amounts (apoB and apoA1 were well below the LOD) (data not shown) and thus this cannot explain the systematically lower observations.

Repeatability of within-run duplicate samples (fresh plasma)

In order to investigate the repeatability of UC measurements, the 25 within-run duplicates of the COUNTER-STRIKE cohort were analyzed. Most UC measurements appear to be repeatable except for free cholesterol (Table 1, Figure 3). Overall, the repeatability of cholesterol, apoA1 and apoB measurements are high and all these

measurements were above the LOD. TG measurements in some HDL subclasses (HDL2a, HDL3), the HDL main class and some LDL subclasses (LDL3, LDL4, LDL5, LDL6) were more problematic as they show low repeatability (low ICC with very broad 95% CI) and high within-run variability in terms of WCV (Table 1). It is important to note that many TGs measurements in the problematic class and subclasses (HDL, HDL2a, HDL3, LDL3, LDL4 and LDL6) are below or just slightly above the LOD of 7 mg/dL. Among others, this decreases the number of replicate measurements available for the repeatability calculations.

For free cholesterol and phospholipids, two different assays were used (MTI and Wako diagnostics). Better repeatability is observed for the Wako assays when high concentrations of phospholipids and free cholesterol are present, as is the case for plasma concentrations (WCV of 5.93 ± 10.1 and ICC of 79.85% with MTI vs. WCV of 1.5 ± 0.72 and ICC of 99.37% with Wako for plasma free cholesterol, and WCV of 1.56 ± 1.46 and ICC of 99.26% with MTI vs. WCV of 1.31 ± 0.41 and ICC of 96.77% with Wako for plasma phospholipids). From the results for free cholesterol of the main classes no clear preference for one of the two assays can be derived. Note also that in some cases the number of repeats is low, hampering a proper calculation of the figures of merit.

Reproducibility of QC samples (frozen pooled plasma)

To further investigate the long-term reproducibility of UC measurements and the factors that contribute to it, repeated between-run measurements of QC samples were analyzed. Table 2 illustrates the reproducibility in plasma, main class and subclass measurements (see also Figure 4). Between-run reproducibility in frozen pooled samples proved to be much lower than within-run repeatability in fresh plasma (Tables 1 and 2). As already observed in within-run duplicates, poor between-run reproducibility is observed for phospholipids and free cholesterol measurements when compared to cholesterol, apoA1 and apoB measurements. The reproducibility of the UC measurements for the two free cholesterol and phospholipids assays used (MTI Diagnostics and Wako Diagnostics) were analyzed separately (Table 2). CV ranges from 4.94% (LDL-cholesterol) to 75.3% (IDL phospholipids measured with the MTI Diagnostics assay) (Table 2). Possible trends in concentrations present in the QC measurements over time were also investigated (Supplementary Figure 2). For most measurements, no

Table 1: Mean and SD of within-individual means across duplicates (mg/dL), average WCV \pm SD, expressed as percentage, across duplicates and ICC (expressed as percentage) with 95% CI of the different measurements in plasma, main classes and subclasses of the duplicate plasma samples of the COUNTERSTRIKE cohort.

Compound	Class	Mean	SD	WCV \pm SD, %	ICC (95% CI), %	# Duplicates
Cholesterol	Plasma	181.96	38.57	1.14 \pm 1.14	99.39 (98.65, 99.73)	25
	VLDL ^a	9.35	5.02	4.9 \pm 4.77	98.37 (96.38, 99.27)	25
	IDL ^a	6.27	4.09	4.22 \pm 3.33	99.27 (98.38, 99.68)	25
	LDL	94.38	25.47	2.05 \pm 1.57	98.98 (97.72, 99.55)	25
	LDL1	27.01	9.14	3.28 \pm 3.07	98.36 (96.23, 99.3)	23
	LDL2	15.71	4.83	4.05 \pm 4.07	96.56 (92.32, 98.49)	24
	LDL3	14.09	4.79	2.42 \pm 1.78	99.13 (98.02, 99.62)	24
	LDL4	12.62	4.44	3.77 \pm 2.33	98.52 (96.66, 99.35)	24
	LDL5	10.45	3.68	3.92 \pm 2.98	97.73 (94.9, 99.01)	24
	LDL6 ^a	10.59	3.13	3.49 \pm 2.65	97.56 (94.3, 98.97)	22
	HDL ^a	58.95	18.29	4.15 \pm 2.8	97.25 (93.93, 98.77)	25
	HDL2b ^a	22.79	11.95	4.47 \pm 3.1	98.95 (97.52, 99.56)	22
	HDL2a	21.84	6.02	3.26 \pm 2.21	98.46 (96.46, 99.34)	23
	HDL3	14.39	3.26	3 \pm 2.09	97.24 (93.7, 98.81)	23
TGs	PLASMA	90.15	31.42	1.55 \pm 1.44	99.67 (99.27, 99.85)	25
	VLDL ^a	52.69	25.39	3.15 \pm 3.6	98.88 (97.51, 99.5)	25
	IDL	7.16	2.50	9.33 \pm 7.5	90.51 (79.9, 95.68)	25
	LDL	15.77	3.77	7.49 \pm 5.36	87.11 (72.82, 94.18)	24
	LDL1	5.33	1.93	7.01 \pm 7.63	96.27 (91.7, 98.36)	24
	LDL2 ^a	2.53	0.56	12.32 \pm 8.28	58.83 (17.72, 82.7)	17
	LDL3 ^a	2.23	0.39	12.07 \pm 10.01	38.09 (-19.65, 76.88)	12
	LDL4	2.31	0.30	16.71 \pm 12.65	-21.8 (-72.93, 47.34)	9
	LDL5	2.16	0.36	12.24 \pm 8.38	36.8 (-35.32, 82.77)	8
	LDL6	2.68	0.80	14.4 \pm 11.85	68.53 (20.6, 90.3)	11
	HDL ^a	13.2	5.34	8.22 \pm 6.63	91.96 (75.57, 97.59)	12
	HDL2b ^a	5.4	3.10	8.08 \pm 11.19	92.45 (78.86, 97.49)	14
	HDL2a	7.58	1.80	5.75 \pm 1.51	94.78 (39.88, 99.86)	3
	HDL3	3.12	0.59	14.4 \pm 9.85	36.77 (-30.95, 80.81)	9
Free cholesterol ^b	Plasma	57.01	11.21	5.93 \pm 10.1	79.85 (55.62, 91.67)	19
	VLDL	6.66	0.86	12.38 \pm 10.15	13.78 (-59.01, 76.51)	7
	IDL	6.8	2.06	2.43 \pm 1.03	99.29 (89.19, 99.98)	3
	LDL	38.43	8.86	8.37 \pm 11.82	72.85 (42.87, 88.51)	19
	LDL1	10.48	2.92	11.88 \pm 12.44	80.81 (56.54, 92.31)	18
	LDL2	7.38	1.65	15.31 \pm 15.69	42.72 (-7.42, 76.05)	15
	LDL3	6.11	1.41	24.48 \pm 19.78	-1.67 (-47.21, 45.18)	17
	LDL4	5.81	1.53	16.34 \pm 18.86	39.5 (-13.28, 75.33)	14
	LDL5	5.34	1.24	16.9 \pm 20.44	22.42 (-31.24, 65.89)	14
	LDL6	5	1.14	7.78 \pm 15.92	44.25 (-12.43, 79.74)	12
	HDL	27.98	8.66	9.52 \pm 9.73	78.81 (41.06, 93.74)	11
	HDL2b	12.83	6.29	15.39 \pm 19.53	92.21 (76.28, 97.67)	12
	HDL2a ^a	13.6	3.16	5.49 \pm 5.99	88.12 (65.33, 96.39)	12
	HDL3	6.31	0.67	3.8 \pm 4.2	79.01 (30.64, 95.33)	8
Free cholesterol ^c	Plasma	65.4	14.50	1.5 \pm 0.72	99.37 (96.27, 99.91)	6
	VLDL	9.43	0.00	0.17 \pm 0	NA	1
	IDL	5.68	0.61	6.83 \pm 1.29	67.06 (-51.96, 99)	3
	LDL	42	7.34	1.86 \pm 1.9	98.49 (91.31, 99.78)	6
	LDL1	12.38	6.09	11.74 \pm 8.96	91.6 (58.41, 98.75)	6
	LDL2	7.05	1.62	10.26 \pm 8.64	69.37 (-3.97, 94.95)	6
	LDL3	5.74	1.49	23.22 \pm 12.85	42.53 (-41.42, 89.07)	6
	LDL4 ^a	6.15	1.86	8.65 \pm 10.57	84.91 (10.22, 98.92)	4
	LDL5	5.42	1.64	7.13 \pm 8.59	91.73 (39.82, 99.43)	4
	LDL6	5.47	1.09	7.3 \pm 5.49	82.33 (26.57, 97.26)	6
	HDL	21.39	4.78	3.39 \pm 2.55	96.72 (78.04, 99.64)	5
	HDL2b	10.26	1.46	5.12 \pm 1.52	86.84 (17.46, 99.07)	4
	HDL2a	9.44	1.44	1.57 \pm 1.97	NA	2
	HDL3	5.23	0.57	4.82 \pm 3.42	78.21 (5.08, 97.42)	5

Table 1 (continued)

Compound	Class	Mean	SD	WCV±SD, %	ICC (95% CI), %	# Duplicates
Phospholipids ^b	Plasma	209.02	45.25	1.56±1.46	99.26 (98.12, 99.71)	19
	VLDL ^a	15.01	6.38	8.82±8.11	91.77 (77.11, 97.25)	14
	IDL	13.75	0.00	1.85±0	NA	1
	LDL	54.57	11.37	8.86±11.08	74.41 (44.51, 89.52)	18
	LDL1	16.54	5.49	6.9±5.32	93.23 (82.74, 97.47)	17
	LDL2	9.19	2.31	7.39±7.5	84.53 (59.68, 94.7)	14
	LDL3	8.04	1.58	7.51±5.99	77.76 (43.41, 92.56)	13
	LDL4	7.4	0.87	8.3±6.64	38.36 (-22.2, 78.32)	11
	LDL5	7.5	2.34	7.11±9.49	85.4 (47.44, 96.84)	8
	LDL6 ^a	10.02	5.38	7.25±7.05	94.97 (82.23, 98.71)	10
	HDL ^a	96.9	46.07	5.65±4.92	97.09 (92.76, 98.86)	19
	HDL2b ^a	43.22	35.67	8.58±9.16	97.67 (93.65, 99.17)	16
	HDL2a ^a	38.41	17.65	4.78±4.42	97.55 (92.8, 99.2)	14
HDL3	21.88	5.99	3.75±2.91	96.94 (91.95, 98.87)	17	
Phospholipids ^c	Plasma	274.98	20.91	1.31±0.41	96.77 (78.34, 99.65)	5
	VLDL	13.23	2.19	6.69±7.01	76.65 (-13.75, 98.26)	4
	IDL	10.61	1.28	5±2.19	81.21 (-24.91, 99.47)	3
	LDL	82.51	19.43	3±2.23	97.38 (85.28, 99.62)	6
	LDL1	26.75	12.80	5.71±4.67	98.55 (91.65, 99.79)	6
	LDL2	13.23	3.51	8.39±5.8	91.69 (58.8, 98.77)	6
	LDL3	12.12	3.35	7.45±6.71	89.39 (49.77, 98.41)	6
	LDL4	10.57	3.87	10.45±7.9	90.99 (55.94, 98.66)	6
	LDL5	9.4	3.71	10.4±7.03	88.55 (38.08, 98.71)	5
	LDL6	9.88	2.94	6.84±4.98	93.12 (64.84, 98.98)	6
	HDL	111.53	32.27	4.16±2.65	96.87 (82.6, 99.54)	6
	HDL2b	39.67	14.42	3±1.86	99.08 (94.61, 99.87)	6
	HDL2a	46.59	11.86	3.97±5.9	96.84 (82.47, 99.54)	6
HDL3	30.21	6.67	4.41±4.46	93.81 (67.88, 99.09)	6	
ApoB	Plasma	86.81	19.92	1.2±0.92	99.54 (98.97, 99.79)	25
	VLDL	4.64	1.81	3.91±5.69	97.04 (93.49, 98.68)	25
	IDL ^a	3.99	2.03	3.42±3.86	98.95 (97.67, 99.54)	25
	LDL	65.77	15.58	1.8±1.54	99.08 (97.96, 99.59)	25
	LDL1	16.4	4.68	5.73±4.18	92.93 (84.3, 96.92)	23
	LDL2	10.23	2.77	4.8±4.24	94.83 (88.57, 97.72)	24
	LDL3	9.49	2.83	3.55±2.53	97.63 (94.66, 98.96)	24
	LDL4	8.82	2.86	3.86±3.47	97.61 (94.63, 98.95)	24
	LDL5	7.63	2.80	4.64±3.16	96.76 (92.76, 98.58)	24
LDL6	8.44	2.73	3.08±3.04	98.18 (95.74, 99.24)	22	
ApoA1	Plasma ^a	144.82	32.50	1.19±0.99	99.48 (98.84, 99.77)	25
	HDL ^a	123.87	31.63	3.83±2.71	96.37 (91.9, 98.4)	24
	HDL2b ^a	34.19	19.67	3.91±3.46	99.2 (98.02, 99.68)	20
	HDL2a	42.62	14.13	3.14±3.26	98.49 (96.46, 99.37)	22
	HDL3	50.69	8.59	2.36±2.06	96.68 (92.29, 98.6)	22

^aFor the calculation of the ICC, measurements were log-transformed, ensuring normality. ^bFree cholesterol and phospholipids measurements obtained with MTI assays. ^cFree cholesterol and phospholipids measurements obtained with Wako assays. ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; CI, confidence interval; ICC, intraclass correlation coefficient; HDL, high-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; VLDL, very low-density lipoproteins; WCV, within-individual coefficient of variation (WCV).

clear trends were observed, except for TGs measurements in LDL3, LDL4, LDL5, LDL6, HDL2a, HDL2b and HDL3, which are below or slightly above the LOD of 7 mg/dL. In the case of phospholipids and free cholesterol approximately the second half of the samples is closer to the mean (less deviation), which is explained

by the fact that for these samples the Wako assays (with better reproducibility) were used. In conclusion, it becomes clear that the Wako Diagnostics phospholipid and free cholesterol assays improved the measurement repeatability and reproducibility, which confirms that the choice of assay is paramount.

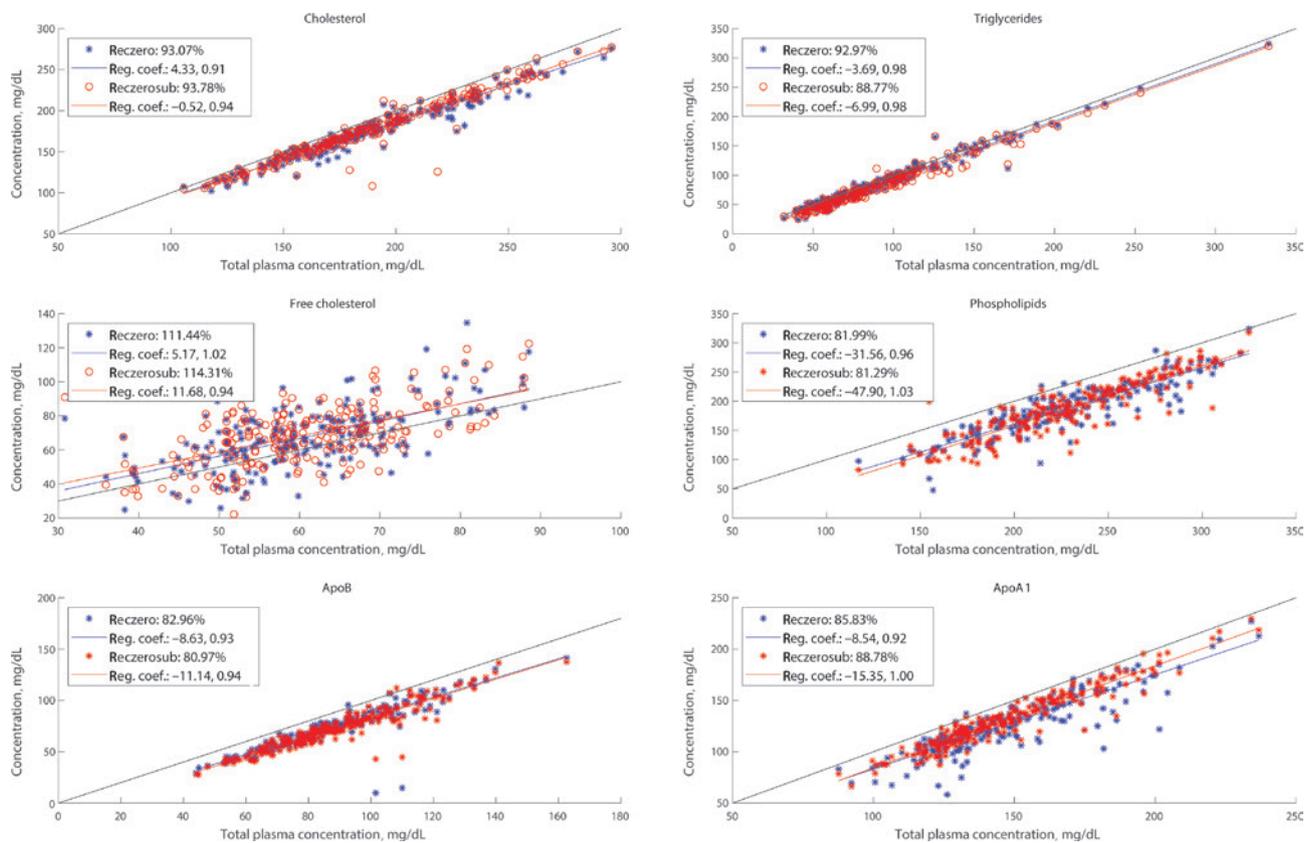


Figure 2: Sum of concentrations (mg/dL) in main classes (black) and in subclasses (red) vs. total plasma concentrations of the different compounds measured in the samples of the COUNTERSTRIKE cohort. Recovery (indicated in the legend) can only be calculated if there are no missing values in plasma, main class and subclass measurements. From the total of 247 samples, this condition was met in 224 samples for cholesterol, 210 for TGs, 223 for free cholesterol, 203 for phospholipids, 222 for apoB and 222 for apoA1. The black diagonal line represents a recovery of 100%. Legend reczero: recovery calculated substituting zero for the below LOD measurements; reg.coef: regression coefficients, intercept and slope; sub indicates subclasses.

Discussion

This study provides, for the first time, figures of merit for a method using UC coupled with colorimetric and turbidimetric assays. The study shows that cholesterol, apoB and apoA1 concentrations can be measured in a repeatable and reproducible manner in plasma, main classes and subclasses. Thus, these results can reliably be used for calibration of rapid prediction models. The results further indicate that the presence of low concentrations contributes to a decreased repeatability in fresh plasma and to lower between-run reproducibility in pooled frozen plasma, respectively. This highlights that at low concentrations, a decreased reproducibility needs to be considered when calibrating new models for predicting the LOD. This could be accommodated for by down-weighting these observations in building a calibration model. This is particularly problematic in fractions where the concentration levels are close to the LOD

of the UC-based measurements, which is the case for TGs concentrations in HDL and some LDL subclasses, as well as for free cholesterol and phospholipids concentrations in various classes. In future studies, including an analytical step for enrichment of these low concentration analytes might be of interest to better be able to determine reproducibility and improve further calibration modeling.

Moreover, the choice of assay can have a big impact on the repeatability and reproducibility of concentrations and our study provides a useful example for future experiments within LPP analysis. In this study, it was found that the phospholipids and free cholesterol measurements acquired with the Wako Diagnostics assays are more repeatable and reproducible than those acquired with the MTI Diagnostics assay. It is paramount to choose assays that can handle relatively wide ranges of concentrations and that are able to measure low concentrations accurately. Despite the fact that the isolated subfraction

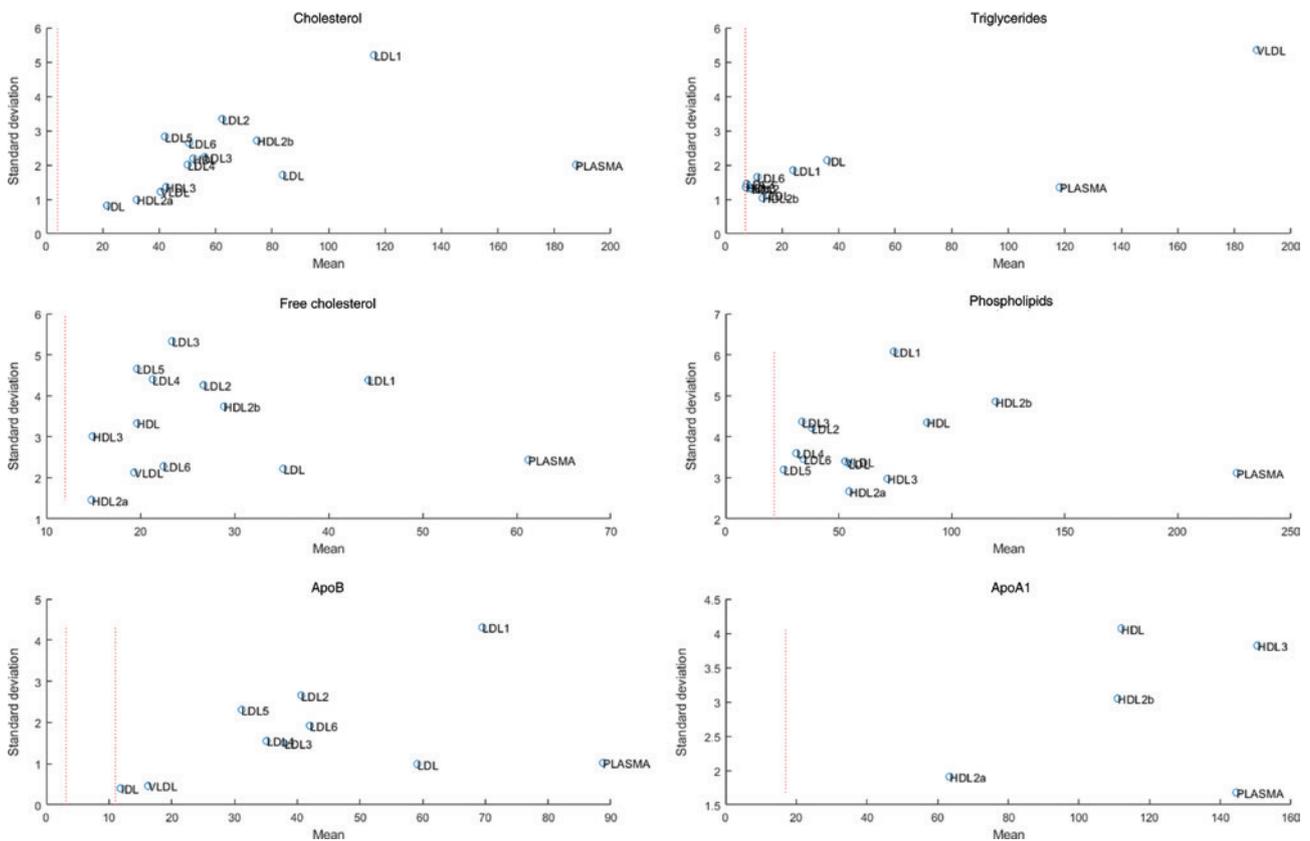


Figure 3: Average within-individual standard deviation vs. average within-individual mean (mg/dL) across the 25 COUNTERSTRIKE duplicates for each measured compound.

Here, concentrations refer to raw measurements (without correcting for dilution/dialysis effects). The red dotted line indicates the lower the LOD. Two different dilutions were made for apoB (in VLDL and IDL) thus two lower LOD lines are present.

samples are very different from regular plasma samples and that the assays used for analysis were designed for regular plasma samples, no aberrant, abnormal or obviously erroneous assay behavior or results were observed and thus we assume that accurate and reliable measurements were obtained. This is in line with the manufacturers testing of possible interfering agents, which also show no significant impact on the assay.

Our results show that repeatability in within-run duplicates (fresh plasma) is, as expected, better than the long-term reproducibility in the between-run QC samples (frozen pooled plasma) (Tables 1 and 2). Several factors might be contributing to this worse reproducibility in the case of the QC samples, one of them being the analysis of samples on different days. In future studies, including a repeatability test with frozen pooled plasma could be used in addition to the fresh plasma samples. However, another source of variability that is of special concern with the current method is the freeze-thaw cycle that the samples undergo prior to UC. This is in line with earlier findings of Castile and Taylor which already suggest that the

freeze-thaw cycle results in an aggregation of liposomes [25]. In addition, the freeze-thaw cycle has been shown in the literature to introduce variability in the measurements of cholesterol and TG concentrations of serum main classes [26]. This study indicates that it is advisable to obtain UC LPPs on fresh blood samples when possible. Unfortunately, it is not possible to determine long-term reproducibility in fresh plasma. As the QC measurements were taken during the span of 10 months, apart from the freeze-thaw cycle, storage time, change of calibration kits and slight room temperature variations might explain the apparent trends in the measurements and higher CVs. In spite of the lower reproducibility of measurements in frozen QC samples when compared to the repeatability of duplicate fresh plasma samples, the variability in main classes measurements was found to be lower than in other studies that also used UC to separate LPs according to density [4]. This study used a modified version of the Baumstark method, which utilized serum to perform the separation. As both serum and plasma samples are routinely used for lipoprotein measurements these results

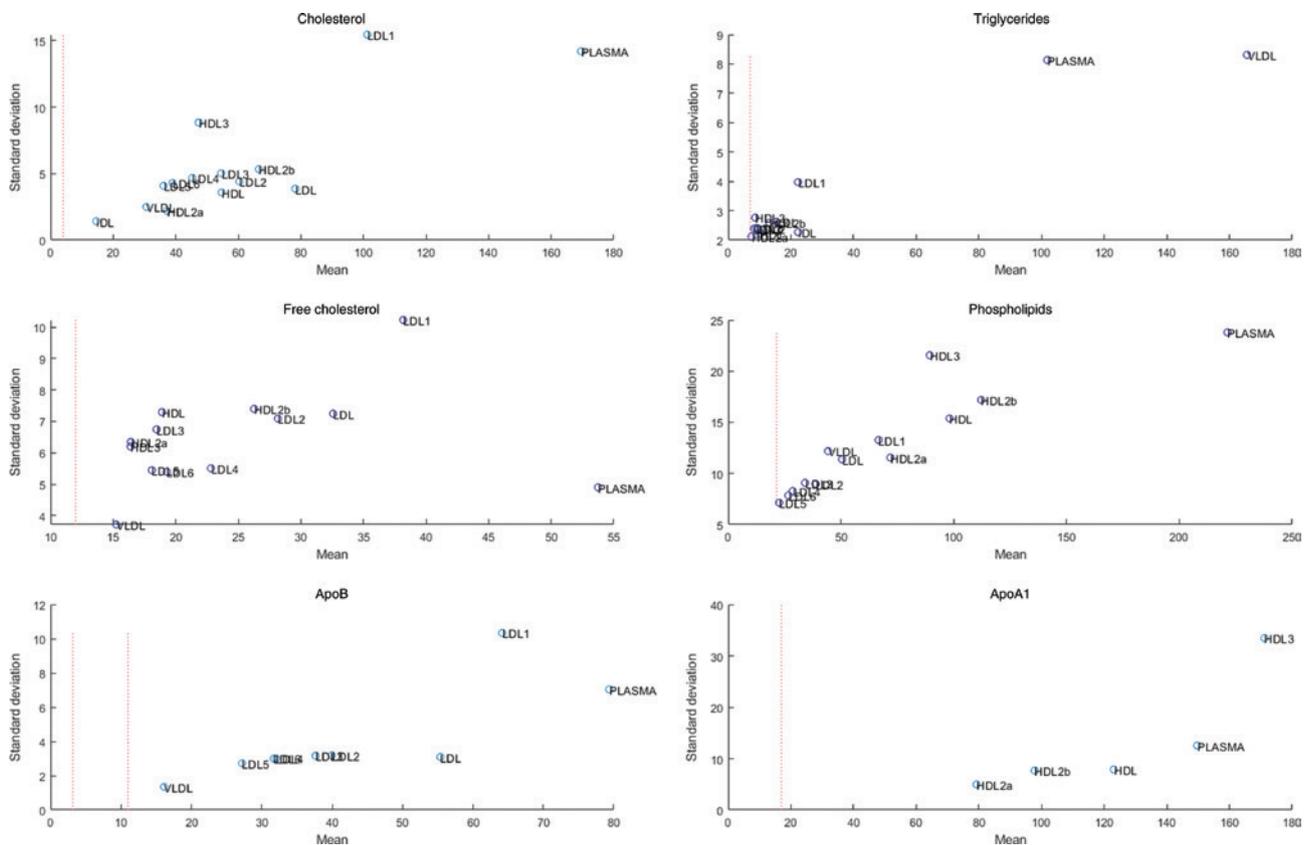


Figure 4: SD vs. average concentration (mg/dL) in the 42 QC samples for each measured compound.

Here, concentrations refer to raw measurements (without correcting for dilution/dialysis effects). Two different dilutions were made for apoB (in VLDL and IDL) thus two lower LOD lines are present.

are most likely also be applicable for serum samples. However, further investigations are required to establish this with confidence [14].

The literature suggests that UC coupled with analytical assays is the preferred reference value for the calibration of NMR-based models for LPP determination [14]. Bathen et al., Petersen et al. and Ala-Korpela et al. reported difficulties when modeling TG measurements in UC-derived HDL classes from plasma samples [17, 18, 27]. Bathen et al. already attributed this difficulty to the presence of low concentrations and to the small range of concentrations covered in the different samples, as well as to the low accuracy of the clinical assay used as a reference [18]. While acknowledging that the UC protocol, rotor and analyzer used in previous studies and the present one might differ, our results suggest that, apart from the presence of low concentrations and the variability introduced during the separation of these classes in UC [24], additional factors contributing to the difficulties in modeling the HDL main class and subclasses in some of the previous studies [17] might have been the variability introduced during the

freeze-thaw cycling (Table 2). Other studies have shown that using UC as part of the beta quantification method for LDL- and HDL-cholesterol determination is highly accurate across laboratories and stable over time, however, similar comparisons for subclasses are lacking [28].

In conclusion, there are several factors that can affect repeatability and reliability of LPPs obtained by UC and these should be considered when building calibration NMR-based models for LPP determination. It is paramount to acknowledge and understand unwanted variability in LPPs prior to building predictive models, as measurement errors introduced during separation by UC and during lipid or protein analysis can be propagated into complex regression models. For this reason, it is advisable in future studies to include within-run duplicate and QC LPP measurements to the experimental design for the acquisition of UC data from large cohorts that are going to be used to build calibration models. To exploit all benefits from fast, highly repeatable and reproducible secondary analytical prediction methods (e.g. NMR), it is important to strive to improve

Table 2: Mean (mg/dL), SD and CV (expressed as percentage) of the different measurements in plasma, main classes and subclasses of the 42 QC aliquots of the same frozen pooled plasma.

Compound	Class	Mean	SD	CV (%)	# Samples
Cholesterol	Plasma	169.55	14.21	8.38	42
	VLDL	11.34	0.92	8.14	42
	IDL	5.41	0.53	9.7	42
	LDL	86.9	4.29	4.94	41
	LDL1	26.12	3.6	13.79	39
	LDL2	15.57	0.96	6.16	39
	LDL3	14.08	1.16	8.23	39
	LDL4	11.68	1.05	9.03	39
	LDL5	9.31	0.89	9.61	39
	LDL6	10.05	0.97	9.69	39
	HDL	60.75	3.98	6.55	42
	HDL2b	22.18	1.78	8.01	39
	HDL2a	24.78	1.47	5.93	39
HDL3	15.78	2.95	18.68	39	
TGs	Plasma	101.86	8.13	7.99	42
	VLDL	61.33	3.08	5.02	42
	IDL	8.27	0.84	10.16	42
	LDL	17.16	2.92	17.01	41
	LDL1	5.75	0.96	16.71	39
	LDL2	2.53	0.62	24.45	36
	LDL3	2.4	0.44	18.53	29
	LDL4	2.25	0.45	20.09	17
	LDL5	2.11	0.37	17.43	16
	LDL6	2.49	0.54	21.68	32
	HDL	11.47	2.28	19.9	41
	HDL2b	4.38	0.86	19.69	39
	HDL2a	5.81	0.96	16.51	25
HDL3	3.21	0.82	25.42	29	
Free cholesterol	Plasma	55.44 (51.26)	4.39 (4.64)	7.92 (9.05)	25 (17)
	VLDL	5.62 (5.7)	0.94 (1.88)	16.66 (32.98)	25 (17)
	IDL	3.15 (2.52)	1.07 (0.65)	33.83 (25.93)	25 (16)
	LDL	39.24 (31.44)	8.66 (3.66)	22.06 (11.64)	25 (16)
	LDL1	11.02 (8.01)	2.51 (1.38)	22.79 (17.29)	24 (15)
	LDL2	8.16 (5.93)	1.85 (1)	22.67 (16.79)	24 (15)
	LDL3	4.8 (4.7)	2.02 (1.14)	42.07 (24.27)	24 (15)
	LDL4	6.59 (4.83)	1.27 (1.17)	19.2 (24.13)	24 (15)
	LDL5	4.96 (4.28)	1.72 (0.86)	34.67 (20.04)	24 (15)
	LDL6	5.52 (4.15)	1.47 (0.96)	26.56 (23.06)	24 (15)
	HDL	22.98 (18.09)	9.34 (4.71)	40.66 (26.06)	25 (17)
	HDL2b	9.51 (7.66)	2.82 (1.26)	29.65 (16.42)	23 (16)
	HDL2a	12.66 (8.49)	4.57 (1.95)	36.07 (23.02)	23 (16)
HDL3	5.87 (4.88)	2.48 (1.08)	42.24 (22.11)	23 (16)	
Phospholipids	Plasma	214.68 (231.08)	23.4 (21.43)	10.9 (9.27)	25 (17)
	VLDL	14.39 (19.33)	4.67 (1.99)	32.48 (10.31)	25 (17)
	IDL	5.17 (5.19)	3.66 (–)	70.86 (–)	6 (1)
	LDL	51.47 (63.32)	13.6 (6.23)	26.42 (9.83)	25 (16)
	LDL1	16.68 (18.05)	3.68 (2.42)	22.08 (13.41)	24 (15)
	LDL2	9.29 (11.38)	2.40 (0.81)	25.85 (7.1)	22 (15)
	LDL3	8.01 (10.37)	2.32 (0.74)	29.02 (7.09)	22 (15)
	LDL4	7.03 (8.94)	1.56 (0.62)	22.2 (6.92)	18 (15)
	LDL5	5.86 (6.98)	1.42 (0.87)	24.2 (12.43)	14 (15)
	LDL6	6.67 (8.34)	1.53 (0.56)	22.96 (6.74)	17 (15)
	HDL	102.58 (118.62)	18.6 (8.17)	18.13 (6.89)	25 (17)
	HDL2b	34.62 (41.37)	5.87 (2.01)	16.94 (4.87)	23 (16)
	HDL2a	44.63 (52.63)	8.45 (2.28)	18.94 (4.33)	23 (16)
HDL3	26.81 (34.06)	4.1 (8.55)	15.3 (25.1)	23 (16)	

Table 2 (continued)

Compound	Class	Mean	SD	CV (%)	# Samples
ApoB	Plasma	79.35	7.04	8.88	41
	VLDL	5.97	0.5	8.39	41
	IDL	3.96	0.34	8.5	41
	LDL	61.53	3.44	5.59	40
	LDL1	16.54	2.52	15.21	38
	LDL2	10.31	0.8	7.79	38
	LDL3	9.71	0.75	7.78	38
	LDL4	8.26	0.69	8.35	38
	LDL5	7.01	0.63	8.91	38
	LDL6	8.17	0.75	9.15	38
ApoA1	Plasma	149.66	12.55	8.39	42
	HDL	136.72	8.75	6.4	42
	HDL2b	32.61	2.56	7.85	39
	HDL2a	52.84	3.32	6.29	39
	HDL3	57.06	11.16	19.55	39

Mean, SD and CV of the aliquots measurements acquired with the free cholesterol and phospholipids Wako assays are indicated in parenthesis. ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; CV, coefficient of variation; HDL, high-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; SD, standard deviation; VLDL, very low-density lipoproteins.

the indispensable reference measurements of LPPs by standardizing protocols and choosing appropriate analytical assays that are able to measure reliably within the ranges of values covered in the population of interest.

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References

- Superko HR. Advanced lipoprotein testing and subfractionation are clinically useful. *Circulation* 2009;119:2383–94.
- Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am J Med* 1977;62:707–14.
- Fredrickson DS, Levy RI, Lees RS. Fat transport in lipoproteins – an integrated approach to mechanisms and disorders. *N Engl J Med* 1967;276:215–25.
- Han S, Flattery AM, McLaren D, Raubertas R, Lee SH, Mendoza V, et al. Comparison of lipoprotein separation and lipid analysis methodologies for human and cynomolgus monkey plasma samples. *J Cardiovasc Transl Res* 2012;5:75–83.
- Smith SJ, Cooper GR, Myers GL, Sampson EJ. Biological variability in concentrations of serum lipids: sources of variation among results from published studies and composite predicted values. *Clin Chem* 1993;39:1012–22.
- Marcovina SM, Gaur VP, Albers JJ. Biological variability of cholesterol, triglyceride, low- and high-density lipoprotein cholesterol, lipoprotein(a), and apolipoproteins A-I and B. *Clin Chem* 1994;40:574–8.
- Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;18:499–502.
- Schectman G, Patsches M, Sasse EA. Variability in cholesterol measurements: comparison of calculated and direct LDL cholesterol determinations. *Clin Chem* 1996;42:732–7.
- Fukuyama N, Homma K, Wakana N, Kudo K, Suyama A, Ohazama H, et al. Validation of the Friedewald equation for evaluation of plasma LDL-cholesterol. *J Clin Biochem Nutr* 2008;43:1–5.
- Meeusen JW, Snozek CL, Baumann NA, Jaffe AS, Saenger AK. Reliability of calculated low-density lipoprotein cholesterol. *Am J Cardiol* 2015;116:538–40.
- Martin SS, Blaha MJ, Elshazly MB, Toth PP, Kwiterovich PO, Blumenthal RS, et al. Comparison of a novel method vs. the Friedewald equation for estimating low-density lipoprotein cholesterol levels from the standard lipid profile. *J Am Med Assoc* 2013;310:2061–8.
- Okazaki M, Usui S, Ishigami M, Sakai N, Nakamura T, Matsuzawa Y, et al. Identification of unique lipoprotein subclasses for visceral obesity by component analysis of cholesterol profile in high-performance liquid chromatography. *Arterioscler Thromb Vasc Biol* 2005;25:578–84.
- Baumstark MW, Kreutz W, Berg A, Frey I, Keul J. Structure of human low-density lipoprotein subfractions determined by X-ray

- small-angle scattering. *Biochim Biophys Acta – Protein Struct Mol Enzymol* 1990;1037:48–57.
14. Aru V, Lam C, Khakimov B, Hoefsloot HC, Zwanenburg G, Lind MV, et al. Quantification of lipoprotein profiles by nuclear magnetic resonance spectroscopy and multivariate data analysis. *TrAC – Trends Anal Chem* 2017;94:210–9.
 15. Mihaleva VV, Van Schalkwijk DB, De Graaf AA, Van Duynhoven J, Van Dorsten FA, Vervoort J, et al. A systematic approach to obtain validated partial least square models for predicting lipoprotein subclasses from serum nmr spectra. *Anal Chem* 2014;86:543–50.
 16. Jeyarajah EJ, Cromwell WC, Otvos JD. Lipoprotein particle analysis by nuclear magnetic resonance spectroscopy. *Clin Lab Med* 2006;26:847–70.
 17. Petersen M, Dyrby M, Toubro S, Engelsen SB, Nørgaard L, Pedersen HT, et al. Quantification of lipoprotein subclasses by proton nuclear magnetic resonance-based partial least-squares regression models. *Clin Chem* 2005;51:1457–61.
 18. Bathen TF, Krane J, Engan T, Bjerve KS, Axelson D. Quantification of plasma lipids and apolipoproteins by use of proton NMR spectroscopy, multivariate and neural network analysis. *NMR Biomed* 2000;13:271–88.
 19. Ala-Korpela M, Hiltunen Y, Bell JD. Quantification of biomedical NMR data using artificial neural network analysis: lipoprotein lipid profiles from ¹H NMR data of human plasma. *NMR Biomed* 1995;8:235–44.
 20. Dona AC, Jimenez B, Schafer H, Humpfer E, Spraul M, Lewis MR, et al. Precision high-throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale metabolic phenotyping. *Anal Chem* 2014;86:9887–94.
 21. Monsonis Centelles S, Hoefsloot HC, Khakimov B, Ebrahimi P, Lind MV, Kristensen M, et al. Toward reliable lipoprotein particle predictions from NMR spectra of human blood: an interlaboratory ring test. *Anal Chem* 2017;89:8004–12.
 22. Pagani F, Panteghini M. Significance of various parameters derived from biological variability for lipid and lipoprotein analyses. *Clin Biochem* 1993;26:415–20.
 23. McGraw KO, Wong SP. Forming inferences about some intraclass correlation coefficients. *Psychol Methods* 1996;1:30–46.
 24. Munroe WH, Phillips ML, Schumaker VN. Excessive centrifugal fields damage high density lipoprotein. *J Lipid Res* 2015;56:1172–81.
 25. Castile JD, Taylor KM. Factors affecting the size distribution of liposomes produced by freeze-thaw extrusion. *Int J Pharm* 1999;188:87–95.
 26. Zivkovic AM, Wiest MM, Nguyen UT, Davis R, Watkins SM, German JB. Effects of sample handling and storage on quantitative lipid analysis in human serum. *Metabolomics* 2009;5:507–16.
 27. Ala-Korpela M, Korhonen A, Keisala J, Hökkö S, Korpi P, Ingman LP, et al. ¹H NMR-based absolute quantitation of human lipoproteins and their lipid contents directly from plasma. *J Lipid Res* 1994;35:2292–304.
 28. Nakamura M, Kayamori Y, Iso H, Kitamura A, Kiyama M, Koyama I, et al. LDL cholesterol performance of beta quantification reference measurement procedure. *Clin Chim Acta* 2014;20:288–93.
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