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Quantitative Lipoprotein Subclass and Low Molecular Weight Metabolite Analysis in Human Serum and Plasma by ^{1}H NMR Spectroscopy in a Multilaboratory Trial.

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ABSTRACT (one paragraph)

We report an extensive 600 MHz NMR trial of a quantitative lipoprotein and small molecule measurements in human blood serum and plasma. Five centers with eleven 600 MHz NMR spectrometers were used to analyze 98 samples including: 20 QCs, 37 commercially sourced, paired serum and plasma samples and 2 National Institute of Science and Technology, NIST, reference material 1951c replicates. Samples were analyzed using rigorous protocols for sample preparation and experimental acquisition. A commercial lipoprotein subclass analysis was used to quantify 105 lipoprotein subclasses and 24 low molecular weight metabolites from the nuclear magnetic resonance, NMR, spectra. For all spectrometers, the instrument specific variance in measuring internal quality controls, QCs, was lower than the percentage described by the National Cholesterol Education Program, NCEP, criteria for lipid testing (triglycerides<2.7%, cholesterol<2.8%; LDL-cholesterol<2.8%; HDL-cholesterol<2.3%), showing exceptional reproducibility for direct quantitation of lipoproteins in both matrices. The average RSD for the 105 lipoprotein parameters in the 11 instruments was 4.6% and 3.9% for the two NIST samples while it was 38% and 40% for the 37 commercially sourced plasmas and sera, respectively, showing negligible analytical compared to biological variation. The coefficient of variance, CV, obtained for the quantification of the small molecules across the 11 spectrometers was below 15% for 20 out of the 24 metabolites analyzed. This study provides further evidence of the suitability of NMR for high-throughput lipoprotein subcomponent analysis and small molecule quantitation with the exceptional required reproducibility for clinical and other regulatory settings.

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INTRODUCTION

Quantitative analysis of circulating lipoproteins is critical to progressing mechanistic understanding of different health status including cardiovascular diseases, metabolic syndrome, neuropathologies and other diseases which are a leading cause of worldwide mortality.[1] A simple blood lipid panel test or cholesterol testing is routinely used in clinical applications and is based on enzymatic reactions, it requires about 40 μL of sample and it takes just 2 mins.[2] However these tests are not precise and provide only total cholesterol and HDL measured levels while the rest of the parameters, normally LDL and triglycerides are obtained by calculation using the Friedewald equation.[3]

Lipoproteins are supramolecular lipid transport particles[4] stratified by density ranging from VLDL (~0.95 g/ml) to HDL (~1.2 g/ml), bound by a single outer phospholipid-cholesterol monolayer with hydrophilic functions oriented outwards (Figure 1). Embedded in the membrane are apolipoproteins whose identity determines the function of a particular lipoprotein, predominantly Apo-B and Apo-A1 and supported by Apo(a), ApoA2, ApoC1, ApoC2, ApoE, ApoH, etc. A total of 95 different proteins have been identified to date in HDL while 22 different proteins have been identified to form part of one or more LDL subclasses with ApoB100 being almost the only apo-protein subclass present.[5-6] Lipoproteins are usually broadly classified in order of decreasing density and hence increasing size into high density (HDL), low density (LDL), very low density (VLDL) lipoproteins and chylomicrons. Figure 1A illustrates the main lipoprotein subclasses with representative sizes. Chylomicrons are also major plasma lipid components composed of dietary fat triglycerides.[7]

The standard method for quantifying lipoproteins involves physical separation of lipoprotein fractions using ultracentrifugation.[8] However, this analysis is time-consuming (approx. 24 h per sample in a two-step ultracentrifugation procedure) and yields quantified values for 4 or 5 main lipoprotein density components (chylomicrons, VLDL, LDL, HDL_2 and HDL_3). The composition of the individual lipoproteins varies and so an in-depth analysis of the different subclasses to understand the function is required. Also, the measurement of lipoprotein levels can vary markedly between testing laboratories, and depending on which parameter is considered the error can lie between approximately 9-15%, and a hence a range of values is usually quoted.[9]

An alternative rapid method for quantifying lipoproteins utilizes ^{1}H NMR spectroscopy. Lipoproteins give rise to a series of broad peaks in the NMR spectrum of serum or plasma

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that are superimposed on the very broad envelope from proteins such as albumin and the sharp peaks of small molecule metabolites.[10-11] Figure 1B represents an overlay of several typical lipoprotein profiles in the 600 MHz ^{1}H NMR spectrum. In general, the peaks do not relate directly to the different lipoproteins but are due to the various positions of the hydrogen containing molecules in the fatty acyl chains of the lipids. In particular the shape of the terminal CH_3 group peak and the long chain $(CH_2)_n$ peak of the fatty acyl groups have been studied in detail. Small changes in chemical shift give rise to the peak broadening and these differences are due both to the chemical heterogeneity of the lipid and to the size of the particles themselves as this provides a magnetic susceptibility contribution to the peak position.[12] NMR signals from a variety of lipids, lipoproteins and small molecules were originally described by Nicholson et al.[10] and the application to lipidic abnormalities associated with diabetes was also described.[13]

Otvos et al.[14] found that the shape of these two peaks can be represented by a linear combination of the contributions from VLDL, LDL, HDL and proteins. Using ^{1}H 400 MHz NMR spectra acquired at 47 °C of individual sub-fractions separated on the basis of density, they formulated a model that enabled the determination of various lipoprotein parameters. Over the years the method has been refined and commercialized.[15-16] Later Ala-Korpela et al [17] also derived a peak shape fitting model using a proprietary statistical procedure.[18] Their method used a combination of measures made at ^{1}H 500 and 600 MHz NMR spectrometers operated at 37 °C and has been widely and successfully applied in various epidemiological cohorts with very large study sizes.[19-20]

Monsonis Centelles et al.[21] following the protocol that we published in 2014 for the analysis of human blood plasma samples,[22] have shown the high degree of spectra reproducibility in the same way that the spectral reproducibility had already been previously demonstrated for urine.[22] They also obtained 3 main lipoprotein parameters and 13 lipoprotein subclasses by using an existing prediction model[23] and they showed a good agreement in the results obtained among the three sites that analyzed the samples as well as with the clinical routinely measured values.

Here, we describe a new implementation of this generalized ^{1}H NMR approach based on the deconvolution of the lipid signals combined with multivariate statistical model building. This approach has been validated against both ultracentrifugation data and conventional measurements. Nevertheless, given the lack of reproducibility of the conventional

lipoprotein measurement, and the fact that both serum and plasma are often interchangeably used without validation of composition across the two matrices, we have conducted a multi-laboratory multi-spectrometer trial of the new method assessing its reproducibility and accuracy both on different NMR spectrometers within one laboratory and across several different laboratory sites. We assess the reproducibility of measurement of 105 lipoprotein particles subclasses across 11 spectrometers from five separate laboratories and extend the method to evaluate simultaneously the measurement of 24 low molecular weight metabolites present in blood plasma and serum samples at readily detectable levels in NMR spectroscopy. In addition, we have also investigated differences in metabolic concentrations between serum and plasma.

The results of this exercise, as reported here, show a high level of reproducibility and accuracy across the individual platforms underscoring the value of ^{1}H NMR spectroscopy based automated annotation and quantification of serum and plasma metabolites.

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MATERIALS AND METHODS

Participant Centers

Five laboratories in 3 institutions were involved in this study including: Firstly 3 laboratories at Imperial College London: MRC NIHR-National Phenome Centre (NPC, 3 × 600 MHz Bruker Avance III HD spectrometers), Imperial Clinical Phenotyping Centre (CPC, 2 × 600 MHz Bruker Avance III spectrometers), Section of Biomolecular Medicine (BMM, 2 × 600 MHz Bruker Avance III HD spectrometers); secondly, the Phenome Centre Birmingham (PCB, 2 × 600 MHz Bruker Avance III HD spectrometers) at the University of Birmingham; and thirdly, Bruker Biospin Germany (BRK, 2 × 600 MHz Bruker Avance III HD spectrometers). The laboratories were distributed across 4 different geographical locations with a total number of 11 Bruker Avance III or Avance III HD NMR spectrometers, all working under InVitro Diagnostics research, IVDr, methods.

Each center had its own NMR team and operators were not exchanged between the different laboratories even within the same institution. There was an overall trial coordinator (Bruker Biospin Germany) and each institution had a local coordinator (NPC, CPC and BMM all belonging to Imperial College had a shared coordinator).

The overall structure of the trial is summarized in the Figure S1 included in the Supporting Information.

Reference Material

Six sets of the same National Institute of Standards and Technology (NIST) reference material 1951c (lipids in frozen human serum) were purchased (one set being sufficient to serve two spectrometers). Each NIST 1951c set was comprised of two 1mL vials of each of the two reference samples (corresponding to certified levels of total cholesterol, total triglycerides, HDL and LDL). This allowed the preparation of two aliquots of each NIST 1951c level sample per spectrometer and the acquisition of the correspondent NMR spectra.

Center QC Samples

A pool of either human blood serum or plasma samples was used as an internal quality control, QC, in each of the centers (5 different individual samples in total) and two aliquots of the sample were measured each day during the 10 days of the trial. In total 20 replicates

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of the QC sample were measured for each spectrometer. The 20 aliquots were prepared in advance to ensure that all the QCs underwent the same number of freeze/thaw cycles.

Human Serum and Plasma Samples

In total, 37 healthy volunteers (19 males and 18 females) were recruited by SeraLab (BioreclamationIVT, UK) across different sites. From their UK collection site 35 donors (18 males and 17 females) were recruited while 2 donors (1 male and 1 female) were recruited in a US collection site. Further details on gender, age and BMI are presented in Table S1.
The final groups of cholesterol ranges included:

- 37% samples in the range of 120 to 180 mg/dL (3.10 to 4.67 mmol/L)
- 46% samples in the range of 181 to 220 mg/dL (4.68 to 5.71 mmol/L)
- 14% samples in the range of 221 to 260 mg/dL (5.72 to 6.74 mmol/L)
- 3% samples in the range of 261 to 400 mg/dL (6.75 to 10.34 mmol/L).

Additional details on sample collection, trial structure and sample preparation can be found in Suplementary Material

LPD prediction method

The lipoprotein distribution (LPD) prediction method selected for the analysis of our study was the commercial **B**ruker **I**VDr **LI**poprotein **S**ubclass **A**nalysis, ***B.I.-LISA,*** method. Details for sample preparation and NMR acquisition are provided in Suporting Information. Spectra were normalized to the same quantitative scale using Bruker's QuantRef manager within TopSpin which is based on the PULCON method[24], hence spectral intensity is normalized to proton concentration in units of mmol/L. The bucket tables were mean centered using the model data mean. The chemical shift was initially calibrated to the methyl signal of Trimethylsilylpropanoic acid, TSP, using Topspin 3.5, and subsequently calibrated to the alanine doublet at 1.48 ppm. This LPD method requires the integration of the signals corresponding to the $–CH_3$ and $–CH_2–$ groups from lipoproteins appearing in the 1D ^1H general NMR profile spectrum at chemical shifts of 0.8 and 1.25 ppm, and fits them using 105 lipoprotein and lipoprotein subclass related parameters respectively using a PLS-2 regression model. The PLS-2 regression model was built combining spectra from independent blood collections in two cohorts of 100 donors, the first one including healthy volunteers only and the second one donors with some kind of lipid metabolism impairment.

Each sample of these two sets was analyzed by ultracentrifugation and 105 lipoprotein parameters were quantified using this method. Subsequently the fractions obtained by ultracentrifugation were analyzed by NMR spectroscopy in order to obtain the reference spectrum to be included in the PLS-2 model. The PLS2 model was built using bucketing parameters (size, number and exclusions) in a similar way to Okazaki et al.[25] The number of components is determined by minimizing the prediction error in a Monte-Carlo embedded Cross-Validation approach as part of the modeling procedure.

The 105 quantified parameters include concentrations of lipoprotein components related to the total plasma or serum aggregate (depending on the sample type) for the main classes (LDL, VLDL, HDL) and subdivisions of those classes: 16 subclasses in total (6 VLDL, 6 LDL and 4 HDL). For each lipoprotein subclass cholesterol, free cholesterol, phospholipids, triglycerides and apo-B and apo-A1 and A2 are calculated. The PLS model was trained and validated using ultracentrifugation measurements. The model was built with an initial cohort of 200 blood samples collected from 100 healthy donors and 100 patients under treatment for diabetes and lipid metabolism impairment. The samples were processed via ultracentrifugation to obtain quantitative information on 105 lipoprotein subfractions. These were then analyzed by NMR spectroscopy in order to obtain their metabolic profiles. NMR data as well as centrifugation data were used as input for the PLS-2 modelling. In addition to the 105 lipoprotein subfraction quantifications, the model provides 12 calculated parameters. These 12 calculated parameters include two ratios (LDL-Chol/HDL-Chol and Apo-B100/Apo-A1) and 10 particle numbers which are currently accepted as a more reliable measure of CVD than the concentration of cholesterol in different lipoprotein subfractions. (Table S2).[26-27]

Automated Quantitation Analysis of Small Molecules

A set of 24 small molecules were quantified using a simplex approach based algorithm developed in-house and implemented in MatLab R2017a (Mathworks inc.). The 1D ^{1}H general NMR profile experiment was used for the quantification of the low molecular weight molecules. 2D *J*res and CPMG spectra were used for subsequent quality control of the signal assignments and plausibility testing of the quantification results. A Gauss – Lorentz mixed line shape was used as the principal line shape for any metabolite signal

fitting. In case of multiplets a sum of Gauss-Lorentz lines was applied to reconstitute the measured line shape. These ratio was calculated for each signal and it was used as one of the constraints in the minimization. Baseline contributions were approximated by locally optimized first order polynomials with offset and slope as fit parameters which were added to the multiplet line shapes. All signals were used for the quantification of the metabolites unless they were in overlap or due to high multiplicity the limit of detection was too high for that particular signal. The algorithm takes into account up to 11 constraints for the concentration calculation such as: molecular mass, number of protons, relaxation time, multiplicity, range of signal detection, fit range, chemical shift, line width, coupling constant, percentage of Lorenzian and Gaussian used in the fitting, baseline offset, slope and curvature. A graphical example of signal fitting can be found in supporting information (Figure S2). The metabolites quantified in the serum/plasma samples included: acetoacetate, acetate, acetone, alanine, citrate, creatinine, creatine, ethanol, formate, glucose, glutamate, glutamine, glycine, glycerol, histidine, 3-hydroxybutyrate, isoleucine, lactate, leucine, phenylalanine, pyruvate, threonine, tyrosine and valine.

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RESULTS

LPD Method Accuracy

To assess the accuracy of the lipoprotein quantification method presented here, we evaluated the results obtained for the quantitation of lipoprotein parameters using the LPD method here described for the NIST standard reference material 1951c. This reference material provides certified mass concentration for triglycerides, cholesterol, LDL-cholesterol and HDL-cholesterol for two different human serum samples. Enough material was acquired to prepare two replicates of each of the two level reference material (L1, L2) per spectrometer. The correlation plots obtained for the certified NIST concentration versus each of the values obtained for the 11 instruments ranged between R=0.993, RMSE= 7.2 mg/dL for LDL-cholesterol to R=0.909, RMSE= 5.8 mg/dL for triglycerides (Figure 2).

An in-depth analysis of the accuracy of the method is presented in Table 1. The certified mass concentration values are compared with the average values obtained for the 11 spectrometers. The results are evaluated against the targets established by the National Cholesterol Education Program, NCEP, for the measurement of lipids by certified laboratories.[2] The total error incurred in the measurement of triglycerides for the 11 spectrometers remained under 4%, well below the 15% allowance by the NCEP; the measurement of total cholesterol had an associated error of 8% or below compared to the 8.9 % acceptance; the quantification of LDL by our method had the highest error associated being 10.5 % but still within acceptance; and the measurement of HDL-cholesterol fraction had an error associated of 7.5% when 13 % is the accepted criteria. The results obtained in this study show that the quantification of these four lipid species by the LPD method here presented comply with the NCEP accuracy criteria.

Intra-laboratory Reproducibility

Replicate measurements of the QC pooled sample (n=20 per spectrometer across 10 days) gave a correlation of 1 with a RMSE of 0.8 mg/dL (Figure 3, Table 2) indicating excellent reproducibility and precision of the LPD method. The average value for each lipoprotein parameter obtained for all the measurements within a center is plotted against each of the values obtained. The measurements are broken down into the main lipoprotein parameters obtained for each of the daily QCs in Figure 3.B. showing the standard deviations for each subclass measurement acquired on each spectrometer. The plotted values obtained from

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the 11 different spectrometers from the five different participating laboratories are color coded (see figure legend). The majority of the results obtained for the main lipoprotein parameters fall within just one standard deviation value with respect to the mean value obtained for the QC within the institution. A summary of the results is provided in Table 2 where the RSD for each of the main lipoprotein parameters for each of the 11 spectrometers participating in the study are compared with those recommended by the NCEP Criteria for Lipid and Lipoprotein Testing. The performances of all 11 spectrometers are well within the specified requirements for precision established by the NCEP.

Concentrations for 24 small molecule concentrations were also obtained from the ^{1}H NMR spectral profile of the QC pooled samples. In Figure 3.C the standard deviation for 6 exemplar small molecules including alanine, creatinine, glucose, glycerol, lactate and valine is presented showing the values obtained for the 11 spectrometers for the 20 QC measurements.

Inter-laboratory Reproducibility

We assessed the inter-laboratory variation and reproducibility of the LPD method in detail. The average value for each of the 105 lipoprotein subclasses obtained for the 74 commercially sourced samples (37 plasma and 37 serum), plotted against each of the 11 different values obtained for each of the spectrometers is shown in Figure 4.A. The observed correlation is exceptionally high (R=0.999, RMSE=1.6 mg/dL). An expanded panel for the HDL subclasses is provided in Figure 4.B. The correlation obtained for the individual subclasses is remarkably high as well. In the case of the HDL subclasses the correlation coefficient ranges between 0.995 for HDL-phospholipid subclass and 0.929 for HDL-free cholesterol subclass.

The precision for the quantification of the six main lipoprotein subclasses across the 74 commercially sourced samples is demonstrated in Figure S3 and Table S3 in the Supporting Information. The standard deviations of the quantification values have been plotted and color coded by center as previously described. All values for LDL-cholesterol and HDL-cholesterol were within the 99% confidence range, whilst 1 measurement for the triglyceride subfraction and 2 for the total cholesterol fell outside the 99% confidence limits.

In order to estimate the variance due to the analytical method and the contribution due to the biological difference between samples, we calculated the average of the relative

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standard deviation for the 105 lipoprotein parameters for the NIST samples (Levels 1 and 2) analyzed across all 11 instruments and the average standard deviation for these parameters in either the 37 serum or plasma commercially sourced samples. The results were 4.6% and 3.8% for NIST L1 and L2 respectively and 40% and 38% for the serum and plasma samples.

The precision for the quantification of small molecules is presented in Figure 4.C using 6 molecules as examples (alanine, creatine, creatinine, glucose, lactate and valine). The mean value calculated from the concentrations measured for each of the spectrometers for the 74 commercially sourced samples is plotted against each of the values: the correlation coefficients for the small molecules range from 0.980 and an RMSE of 0.004 mmol/L for creatine and 0.672 with RMSE of 0.057 mmol/L for glycerol. Table S4 presents the general overview of the method performance for the 24 metabolites. The mean concentrations obtained from the NMR spectra and the coefficient of variance are presented. When comparing the measurements obtained in the 11 instruments 83% of the compounds have a CV below 15% and just 1 of the compounds exceeds the 30% CV showing the high reproducibility of the method across all 11 instruments for the quantitation of small molecules. The correlation coefficients and the RMSE are also shown in Table S4. The method precision is excellent as 98% of the compounds present a correlation coefficient above 0.9. The best performance was observed for acetate, glucose, lactate, alanine and glutamine.

LPD Method Precision Overview

To give an overview of the precision of the method one sample was randomly selected out of the 74 commercially sourced samples and each of the concentrations measured on the 11 instruments were plotted. The results are shown in Figure S4. The 11 values are plotted using the color of its institution as previously described and are virtually identical for the majority of the lipoprotein parameters. The lilac shadows represent the 95% ranges of the values obtained for the 200 plasma samples used to develop the PLS model for peak fitting of the lipoprotein subclasses spectra using the B.I.Lisa method. The overall inter-lab variability is substantially less than this range, indicating inter-instrumental variability is minimal.

Applicability of the LPD Method for blood products

The values obtained for the lipoprotein subclasses for the serum samples were plotted against the values obtained for the lipoprotein subclasses of the plasma samples collected from the same donor for all the spectrometers participating in the study. Plotting of these measures demonstrates a near perfect correlation (R=0.999, RMSE=1.5 mg/dL) showing that results obtained for either plasma or serum are relevant to the other matrix, thereby broadening the potential for combining results from multiple studies (Figure S5). The average value and the relative standard deviation of the results obtained for the main lipoprotein parameters on each of the spectrometers for the 74 commercially sourced samples (37 plasma aliquots and 37 serum aliquots) are presented in Table S3. The high degree of correlation observed in Figure 4.A is apparent by the comparison of the results obtained for the plasma and serum sets presented in Table S3.

The applicability of the LPD method applies to all the different lipoprotein subclasses. The concentration of the LDL subclasses obtained for serum, plotted against those obtained for the plasma are provided as an exemplar in Figure S5.B, in which it can be seen that the majority of the values fall within the 95% range acceptance for all the different LDL subclasses as indicated by their mapping position within the green shaded regions representing percentage of variation accepted by the NCEP 1 × SD % (dark green) or 2 × SD % (light green).

In the case of small molecules, good correlation (R>0.7) between serum and plasma values for 3-hydroxybutyric acid, alanine, creatinine, creatine, glucose, isoleucine, lactate and valine were observed. However, systematically higher apparent serum concentrations were observed for some metabolites such as glutamine (R=0.786) and lactate (R=0.884), whereas pyruvate (R=0.709) showed the opposite trend with systematically higher apparent values generated for plasma (Figure S5.C).

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DISCUSSION

Quantitative analysis of serum or plasma lipoprotein sub-fractions is necessary to understand the aetiopathogenesis of many diseases including cardiovascular diseases, metabolic syndrome, neuropathologies and other diseases in which disordered lipid metabolism is a feature. We evaluated an algorithm for simultaneous quantification of 105 lipoprotein fractions, 12 calculated lipoprotein ratios and particle numbers (particle numbers have recently been shown to be good predictors of vascular structure and function[26]) and 24 low molecular weight metabolites using a trial strategy in which 11 individual NMR spectrometers across five laboratories were used to generate data for serum and plasma samples. The quantitation of the lipoproteins was based on spectral deconvolution of the signals belonging to the -CH_2- and -CH_3 groups appearing at 1.25 and 0.8 ppm respectively. Comparison of the summed values of free cholesterol, triglycerides, HDL cholesterol and LDL cholesterol with NIST certified standards showed that the method was accurate and reproducible across the 11 spectrometers with total error values ranging from 3.4-10.5% (Table 1) falling well within the acceptance limits set by NCEP, ranging from 8.9-15%. All spectrometers delivered accurate measurements varying between R=0.993, RMSE= 7.2 mg/dL for LDL-cholesterol to R=0.909, RMSE= 5.8 mg/dL for triglycerides (Figure 2).

Monsonis Centelles et. al[21] already demonstrated the high reproducibility of the lipoprotein analysis performed by NMR in their 3-machine ring-test. The work here presented offers a new dimension to this initial contribution by showing finer granularity in the lipoprotein analysis obtained by using a more sophisticated model which provides quantification of 117 lipoprotein parameters. The trial has been extended to the quantification of a number of small molecules and has proven to provide very precise results.

The intra-laboratory comparisons of pooled quality control samples, conducted longitudinally over 10 days, showed excellent reproducibility for each of the 11 spectrometers and complied with the precision criteria set by the NCEP (Table 2): triglycerides <5% (measured values ranging from 1.0-2.6%); cholesterol <3% (measured values 0.75-2.14%); LDL cholesterol <4% (measured values 1.4-2.8%); HDL cholesterol <4% (measured values 0.67-2.3%).

The inter-laboratory reproducibility for the 105 measured lipoprotein fractions yielded correlation values of R>0.999 with RMSE 1.6mg/dL based on the analysis of 37 commercially

sourced serum samples and 37 matching plasma samples. Thus the method delivers highly reproducible quantitation for lipoproteins in both serum and plasma samples. On stratifying the results by lipoprotein sub-class, exemplified for the HDL subclasses (Figure 4.B), the strong reproducibility was maintained. For example, HDL of phospholipids returned a correlation value of R=0.995 with a RMSE 1.9 mg/dL, whereas the weakest correlation was found for HDL Apolipoprotein 2 with a correlation value of R=0.962 and a RMSE of 1.0 mg/dL. The correlation between serum and plasma lipoprotein values, calculated for each lipoprotein subclass, for each individual spectrometer yielded a correlation of R=0.999 with a RMSE of 1.5 mg/dL (Figure S5.A) with similar performance across the lipoprotein subclass strata, as exemplified for LDL cholesterol (Figure S5.B). This would indicate that for lipoprotein analysis, plasma and serum can be used interchangeably, which is advantageous when conducting multi-center studies and attempting to extrapolate clinical or biological inferences relating to disease.

In addition to establishing the inter-laboratory reproducibility for the lipoprotein sub-fraction analysis, we evaluated the reproducibility for quantification of selected low molecular weight metabolites. Exemplar results are provided for six metabolites selected to cover different correlation ranges in serum and plasma (Figure 4.C) and a more general overview is provided in Table S4. In general, the quantification of the low molecular metabolites precision is related to the abundance of the metabolite in blood serum or plasma, and to the spectrum signal characteristics. The best performing metabolites present normally well resolved signals with low multiplicity. In general a very high reproducibility of the results is obtained across all 11 instruments with CVs below 30% for all the metabolites but one. The relative high CVs observed for 3-Hydroxybutyrate, acetoacetate or acetone, 35.7%, 14.9% and 14.6 % respectively (Table S4) might be related to residual reactivity due to non-enzymatic interconversion. While in the case of other metabolites like threonine and glutamate 28.9 and 21.1%, respectively this relative high CV is due to technical challenges. These challenges were high multiplicity in the case of glutamate, which increases the detection limit per proton, or signal overlap in the case of threonine, whose signals are partially in overlap with those of lactate.

In the case of small molecules, systematic differences between the plasma and serum concentrations were identified for some metabolites. This is likely to relate to variations in longitudinal and transverse relaxation times between the fluids due to viscosity differences

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(serum is less viscous than plasma as it is fibrinogen depleted). Also alanine, citrate, formate and pyruvate do not bind extensively to proteins whereas this is not the case for phenylalanine[28] or lactate[29-30]. These last two molecules tend to interact with proteins which modulates their relaxation times and therefore impacts on exact quantitation measurements. Similar results in the apparent different concentration of small molecules in serum and plasma were previously described by Kaluarachchi et al.[31] The debate as to whether serum or plasma is the best biological medium to use in clinical studies is ongoing.[32] In reality, from these results, it would appear that for most low molecular weight metabolites, this automatic analytical approach will deliver high quality results and the decision as to whether plasma or serum is preferable should be based on how well the metabolites of interest are measured.

Taken together, these results indicate good reproducibility and precision for the quantification of lipoproteins in both serum and plasma samples by NMR spectroscopy, however, low molecular weight metabolites need to be considered on a metabolite by metabolite basis. A study such as the one here described can determine which metabolites can be cross compared between plasma and serum and which should not be.

In conclusion, this eleven platform trial has shown that the LPD method here evaluated is highly reproducible and fit for purpose with respect to quantifying lipoprotein fractions and allows simultaneous quantification of lipoproteins and low molecular weight metabolites. This allows inter-laboratory translatability of results and high confidence in the data regardless of where they were generated, as long as the SOPs and measurement protocols are followed carefully.

TABLES

Table 1. Accuracy of the lipoprotein analysis method from the trial: Mean and standard deviation values obtained for the quantification of the 4 lipoprotein parameters provided for the NIST standard reference material 1951c (samples L1 and L2) NIST certified values are shown in italics. The criteria established by the NCEP have been included to compare with the trial CVs.

	Total Glycerides		**Total Cholesterol**		**LDL-Cholesterol**		**HDL-Cholesterol**	
NIST Sample	**L1**	**L2**	**L1**	**L2**	**L1**	**L2**	**L1**	**L2**
NIST certified mass (mg/dL)	*152*	*145.4*	*152.4*	*241.4*	*86.4*[a]	*143.8*[a]	*41*	*64.9*
NIST certified SD (mg/dL)	*±3.2*	*±3.2*	*±1.8*	*±2.8*	*±1.4*	*±2.1*	*±0.9*	*±1.7*
Trial mean (mg/dL)	157	141.4	163.3	258.06	77.9	142.12	43.8	67.5
Trial SD (mg/dL)	±3.5	±3.7	±5.7	±7.6	±3.0	±4.5	±1.2	±1.7
NCEP total allowed error	*15*	*15*	*8.9*	*8.9*	*12*	*12*	*13*	*13*
Trial total error %	4	3.8	8.05	7.6	10.5	3.4	7.5	4.7

[a]Certified amount-of-substance concentration values for SRM 1951c. The concentrations obtained for total cholesterol determined by using Abell-Kendall reference method are 154.6 ± 1.1 (mg/dL) and 244.8 ± 1.1 (mg/dL) for L1 and L2 respectively.

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Table 2. Precision of the lipoprotein analysis method from the trial. Requirements from the National Cholesterol Education Program (NCEP) for lipoprotein measurements of the four main parameters are listed together with the precision achieved by the 11 different spectrometers participating in the trial and using the B.I.-LISA method. Percentages correspond to the variance in the measurement of total cholesterol, triglycerides, LDL-cholesterol and HDL-Cholesterol in the 5 different daily QCs used in the study for the 11 spectrometers.

Instrument	**Total Triglycerides**	**Total Cholesterol**	**LDL-Cholesterol**	**HDL-Cholesterol**
NCEP Target Imprecision	*< 5 %*	*< 3 %*	*< 4 %*	*< 4 %*
Lab1A HD	2.0 %	1.4 %	1.6 %	1.6 %
Lab1B HD	1.4 %	1.3 %	2.3 %	1.4 %
Lab1C HD	1.01 %	1.8 %	1.9 %	1.8 %
Lab2A	2.7 %	2.14 %	2.4 %	1.9 %
Lab2B	1.5 %	0.75 %	1.07 %	0.67 %
Lab3A	1.8 %	2.8 %	2.8 %	2.3 %
Lab3B HD	1.06 %	1.4 %	1.5 %	1.4 %
Lab4A HD	2.6 %	1.6%	2.3%	2.2 %
Lab4B HD	2.4 %	2.12 %	2.5 %	1.6 %
Lab5A HD	1.0 %	1.3 %	1.5 %	1.14 %
Lab5B HD	1.5 %	1.5 %	1.4 %	1.6 %
Combined Imprecision 11 Instruments	*1.6 %*	*1.6 %*	*2.0 %*	*1.6 %*

FIGURE LEGEND

Figure 1. A) ChemDraw cartoon representing lipoprotein composition size and density[33] (ChemDraw Professional v. 16.0 (Perkin-Elmer Inc.)). B) ^{1}H NMR spectrum of a serum sample. C) Insert of several ^{1}H NMR spectra of several sera samples showing the region where the lipoprotein signals appear and different profiles that the lipoproteins show in different samples due to differing compositions. D) ^{1}H NMR spectra of the 24 small molecules quantified in this study. The spectra have been color coded: 3-hydroxybutyrate (1, black), acetoacetate (2, blue), acetate (3, dark green), alanine (4, dark red), citrate (5, orange), creatinine (6, purple), creatine (7, dark brown), ethanol (8, cyan), formate (9, grey), glucose (10, light green), glutamine (11, pink), glycerol (12, lilac), isoleucine (13, light blue), lactate (14, medium green), leucine (15, red), phenylalanine (16, light orange), pyruvate (17, fuchsia), threonine (18, light brown), valine (19, dark grey), glycine (20, dark duck egg), acetone (21, turquoise), tyrosine (22, maroon), histidine (23, light duck egg), glutamate (24, dark purple). E) Expansion of the most crowded region (0.75-4 ppm) of the spectra shown in D). Amix-Viewer version 3.9.14 and S-BASE library (Bruker Ltd.) have been used to generate D) and E).

Figure 2. Method Accuracy. Regression curves showing the method performance for A) triglycerides RMSE=5.8 mg/dL and R=0.909, B) total cholesterol RMSE=13.1 mg/dL and R=0.990, C) LDL cholesterol RMSE=7.2 mg/dL and R=0.993 and D) HDL cholesterol RMSE=3.1 mg/dL and R=0.992 in serum. The certified concentration values for the NIST 1951c samples are plotted against each of the measures obtained for each sample in the 11 spectrometers (4 sets of NIST samples were measured in each spectrometer, 2 replicates × samples L1 and L2); The green shaded regions show the allowance in imprecision stablished by the NCEP for the analysis of lipoproteins, where dark green: 66% range of samples (1 × RSD), light green + dark green: 95 % range of samples (2 × STD), yellow + light green + dark green: 99% range of samples (3 × STD). See Table 2 for the specific NCEP target imprecision.

Figure 3. Intra-laboratory Reproducibility: A) Regression curve where the mean value of each lipoprotein subclass is plotted against the values obtained for each of the 105 lipoprotein parameters in each of the measurements (R^2=1.000, RMSE=0.8 mg/dL). Twenty independent values (2 QC replicates x 10 days of analysis) are used per instrument (11

spectrometers); B) Instrument-specific variability for lipoprotein quantification for six selected parameters (triglycerides, cholesterol, apo-A1, LDL-cholesterol, HDL-cholesterol and apo-B). Each plot represents the percentage of error ((x-ẋ)/ẋ) for the main lipoprotein parameters obtained for each of the QC samples obtained daily. The five different participating laboratories are color coded. The green shaded regions show the limits represent the percentage of variation as explained in Figure 2; the light blue patches represent the sample respective standard deviation and twice the standard deviation, ie. the 66% and the 95% range as there is no NCEP imprecision target stablished for apo-lipoproteins. C) Instrument specific variability for small molecule quantification. Plot representing the standard deviation values for selected small molecules quantified in the daily QCs. Color codes and blue shadows as per b)

Figure 4. Inter-laboratory Reproducibility. Analysis of commercially sourced samples A) Regression curve where the mean value of each lipoprotein parameter, calculated for the different acquisitions of each serum or plasma spectrum (74 measures in total for 11 instruments), is plotted against each of the values obtained for the lipoprotein parameters in each individual measurements (R=0.999, RMSE=1.6 mg/dL); B) Example of real sample analysis for one lipoprotein class. Regression curve showing the relationship between the mean concentration values of main parameters of the HDL lipoprotein obtained for the 74 samples with respect to the values obtained for the 74 samples analyzed in the 11 different spectrometers [HDL triglycerides (R=0.987, RMSE=0.7 mg/dL), HDL phospholipids (R=0.995, RMSE=1.9 mg/dL), HDL cholesterol (R=0.989, RMSE=1.8 mg/dL), HDL free cholesterol (R=0.999, RMSE=1.6 mg/dL), HDL Apo-1 (R=0.987, RMSE=4.5 mg/dL) and HDL Apo-2 (R=0.962, RMSE=1.0 mg/dL) parameters]; C) Regression curves where the mean value of the concentration obtained for 6 small molecules for the commercially sourced samples, is plotted against each of the concentrations obtained for each of the 74 samples in the 11 spectrometers, namely: alanine (R=0.980, RMSE=0.019 mmol/L), creatine (R=0.9612, RMSE=0.052 mmol/L), creatinine (R=0.854, RMSE=0.010 mmol/L), glucose (R=0.970, RMSE=0.064 mmol/L), lactate (R=0.987, RMSE=0.155 mmol/L) and valine (R=0.930, RMSE=0.018 mmol/L)

ACKNOWLEDGEMENTS

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REFERENCES

1. Wang, H. D.; Naghavi, M.; Allen, C.; Barber, R. M.; Bhutta, Z. A.; Carter, A. et al. Mortality and Causes of Death Collaborators. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **2016,** *388* (10053), 1459-1544.

2. Campbell, M.; Humanki, J.; Zierhut, H., A novel approach to screening for familial hypercholesterolemia in a large public venue. *J Community Genet* **2017,** *8* (1), 35-44.

3. Friedewald, W. T.; Levy, R. I.; Fredrickson, D. S., Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* **1972,** *18* (6), 499-502.

4. *Biochemistry of Lipids, Lipoproteins and Membranes*. 6th ed.; Elsevier Science: 2016; p 612.

5. Rosales, C.; Davidson, W. S.; Gillard, B. K.; Gotto, A. M., Jr.; Pownall, H. J., Speciated High-Density Lipoprotein Biogenesis and Functionality. *Curr Atheroscler Rep* **2016,** *18* (5), 25.

6. Zychlinski, A.; TorstenKleffmann, Dissecting the proteome of lipoproteins: New biomarkers for cardiovascular diseases? *Translational Proteomics* **2015,** *7*, 30-39.

7. Cox, R. A.; Garcia-Palmieri, M. R., Cholesterol, Triglycerides, and Associated Lipoproteins. In *Clinical Methods: The History, Physical, and Laboratory Examinations*, rd; Walker, H. K.; Hall, W. D.; Hurst, J. W., Eds. Boston, 1990.

8. Chapman, M. J.; Goldstein, S.; Lagrange, D.; Laplaud, P. M., A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *J Lipid Res* **1981,** *22* (2), 339-58.

9. Contois, J. H.; Warnick, G. R.; Sniderman, A. D., Reliability of low-density lipoprotein cholesterol, non-high-density lipoprotein cholesterol, and apolipoprotein B measurement. *J Clin Lipidol* **2011,** *5* (4), 264-72.

10. Nicholson, J. K.; Buckingham, M. J.; Sadler, P. J., High resolution 1H n.m.r. studies of vertebrate blood and plasma. *Biochem J* **1983,** *211* (3), 605-15.

11. Nicholson, J. K.; Foxall, P. J.; Spraul, M.; Farrant, R. D.; Lindon, J. C., 750 MHz 1H and 1H-13C NMR spectroscopy of human blood plasma. *Analytical chemistry* **1995,** *67* (5), 793-811.

12. Lounila, J.; Ala-Korpela, M.; Jokisaari, J.; Savolainen, M. J.; Kesaniemi, Y. A., Effects of orientational order and particle size on the NMR line positions of lipoproteins. *Phys Rev Lett* **1994,** *72* (25), 4049-4052.

13. Nicholson, J. K.; O'Flynn, M. P.; Sadler, P. J.; Macleod, A. F.; Juul, S. M.; Sonksen, P. H., Proton-nuclear-magnetic-resonance studies of serum, plasma and urine from fasting normal and diabetic subjects. *Biochem J* **1984,** *217* (2), 365-75.

14. Otvos, J. D.; Jeyarajah, E. J.; Bennett, D. W., Quantification of plasma lipoproteins by proton nuclear magnetic resonance spectroscopy. *Clin Chem* **1991,** *37* (3), 377-86.

15. Otvos, J. D.; Jeyarajah, E. J.; Bennett, D. W.; Krauss, R. M., Development of a proton nuclear magnetic resonance spectroscopic method for determining plasma lipoprotein concentrations and subspecies distributions from a single, rapid measurement. *Clin Chem* **1992,** *38* (9), 1632-8.

16. Freedman, D. S.; Otvos, J. D.; Jeyarajah, E. J.; Shalaurova, I.; Cupples, L. A.; Parise, H.; D'Agostino, R. B.; Wilson, P. W.; Schaefer, E. J., Sex and age differences in lipoprotein subclasses measured by nuclear magnetic resonance spectroscopy: the Framingham Study. *Clin Chem* **2004,** *50* (7), 1189-200.

17. Soininen, P.; Kangas, A. J.; Wurtz, P.; Suna, T.; Ala-Korpela, M., Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics. *Circ Cardiovasc Genet* **2015,** *8* (1), 192-206.

18. Wurtz, P.; Kangas, A. J.; Soininen, P.; Lawlor, D. A.; Davey Smith, G.; Ala-Korpela, M., Quantitative Serum Nuclear Magnetic Resonance Metabolomics in Large-Scale Epidemiology: A Primer on -Omic Technologies. *Am J Epidemiol* **2017,** *186* (9), 1084-1096.

19. Wurtz, P.; Wang, Q.; Soininen, P.; Kangas, A. J.; Fatemifar, G.; Tynkkynen, T.; Tiainen, M.; Perola, M.; Tillin, T.; Hughes, A. D.; Mantyselka, P.; Kahonen, M.; Lehtimaki, T.; Sattar,

N.; Hingorani, A. D.; Casas, J. P.; Salomaa, V.; Kivimaki, M.; Jarvelin, M. R.; Smith, G. D.; Vanhala, M.; Lawlor, D. A.; Raitakari, O. T.; Chaturvedi, N.; Kettunen, J.; Ala-Korpela, M., Metabolomic Profiling of Statin Use and Genetic Inhibition of HMG-CoA Reductase. *J Am Coll Cardiol* **2016,** *67* (10), 1200-1210.

20. Kaikkonen, J. E.; Kresanov, P.; Ahotupa, M.; Jula, A.; Mikkila, V.; Viikari, J. S.; Juonala, M.; Hutri-Kahonen, N.; Kahonen, M.; Lehtimaki, T.; Kangas, A. J.; Soininen, P.; Ala-Korpela, M.; Raitakari, O. T., Longitudinal study of circulating oxidized LDL and HDL and fatty liver: the Cardiovascular Risk in Young Finns Study. *Free Radic Res* **2016,** *50* (4), 396-404.

21. Monsonis Centelles, S.; Hoefsloot, H. C. J.; Khakimov, B.; Ebrahimi, P.; Lind, M. V.; Kristensen, M.; de Roo, N.; Jacobs, D. M.; van Duynhoven, J.; Cannet, C.; Fang, F.; Humpfer, E.; Schafer, H.; Spraul, M.; Engelsen, S. B.; Smilde, A. K., Toward Reliable Lipoprotein Particle Predictions from NMR Spectra of Human Blood: An Interlaboratory Ring Test. *Analytical chemistry* **2017,** *89* (15), 8004-8012.

22. Dona, A. C.; Jimenez, B.; Schafer, H.; Humpfer, E.; Spraul, M.; Lewis, M. R.; Pearce, J. T.; Holmes, E.; Lindon, J. C.; Nicholson, J. K., Precision high-throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale metabolic phenotyping. *Analytical chemistry* **2014,** *86* (19), 9887-94.

23. Mihaleva, V. V.; van Schalkwijk, D. B.; de Graaf, A. A.; van Duynhoven, J.; van Dorsten, F. A.; Vervoort, J.; Smilde, A.; Westerhuis, J. A.; Jacobs, D. M., A systematic approach to obtain validated partial least square models for predicting lipoprotein subclasses from serum NMR spectra. *Analytical chemistry* **2014,** *86* (1), 543-50.

24. Wider, G.; Dreier, L., Measuring protein concentrations by NMR spectroscopy. *J Am Chem Soc* **2006,** *128* (8), 2571-6.

25. Okazaki, M.; Usui, S.; Ishigami, M.; Sakai, N.; Nakamura, T.; Matsuzawa, Y.; Yamashita, S., 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* (3), 578-84.

26. Urbina, E. M.; McCoy, C. E.; Gao, Z. Q.; Khoury, P. R.; Shah, A. S.; Dolan, L. M.; Kimball, T. R., Lipoprotein particle number and size predict vascular structure and function better than traditional lipids in adolescents and young adults. *Journal of Clinical Lipidology* **2017,** *11* (4), 1023-1031.

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27. Cromwell, W. C.; Otvos, J. D., Low-density lipoprotein particle number and risk for cardiovascular disease. *Curr Atheroscler Rep* **2004,** *6* (5), 381-7.

28. Nicholson, J. K.; Gartland, K. P., 1H NMR studies on protein binding of histidine, tyrosine and phenylalanine in blood plasma. *NMR Biomed* **1989,** *2* (2), 77-82.

29. Bell, J. D.; Brown, J. C.; Kubal, G.; Sadler, P. J., NMR-invisible lactate in blood plasma. *FEBS Lett* **1988,** *235* (1-2), 81-6.

30. Chatham, J. C.; Forder, J. R., Lactic acid and protein interactions: implications for the NMR visibility of lactate in biological systems. *Biochim Biophys Acta* **1999,** *1426* (1), 177-84.

31. Kaluarachchi, M.; Boulangé, C. L.; Karaman, I.; Lindon, J. C.; Ebbels, T. M. D.; Elliott, P.; Tracy, R. P.; Olson, N. C., A comparison of human serum and plasma metabolites using untargeted ^{1}H NMR spectroscopy and UPLC-MS. *Metabolomics* **2018,** *14* (32),.

32. Frank, E. A.; Shubha, M. C.; D'Souza, C. J., Blood glucose determination: plasma or serum? *J Clin Lab Anal* **2012,** *26* (5), 317-20.

33. Ridker, P. M., LDL cholesterol: controversies and future therapeutic directions. *Lancet* **2014,** *384* (9943), 607-617.

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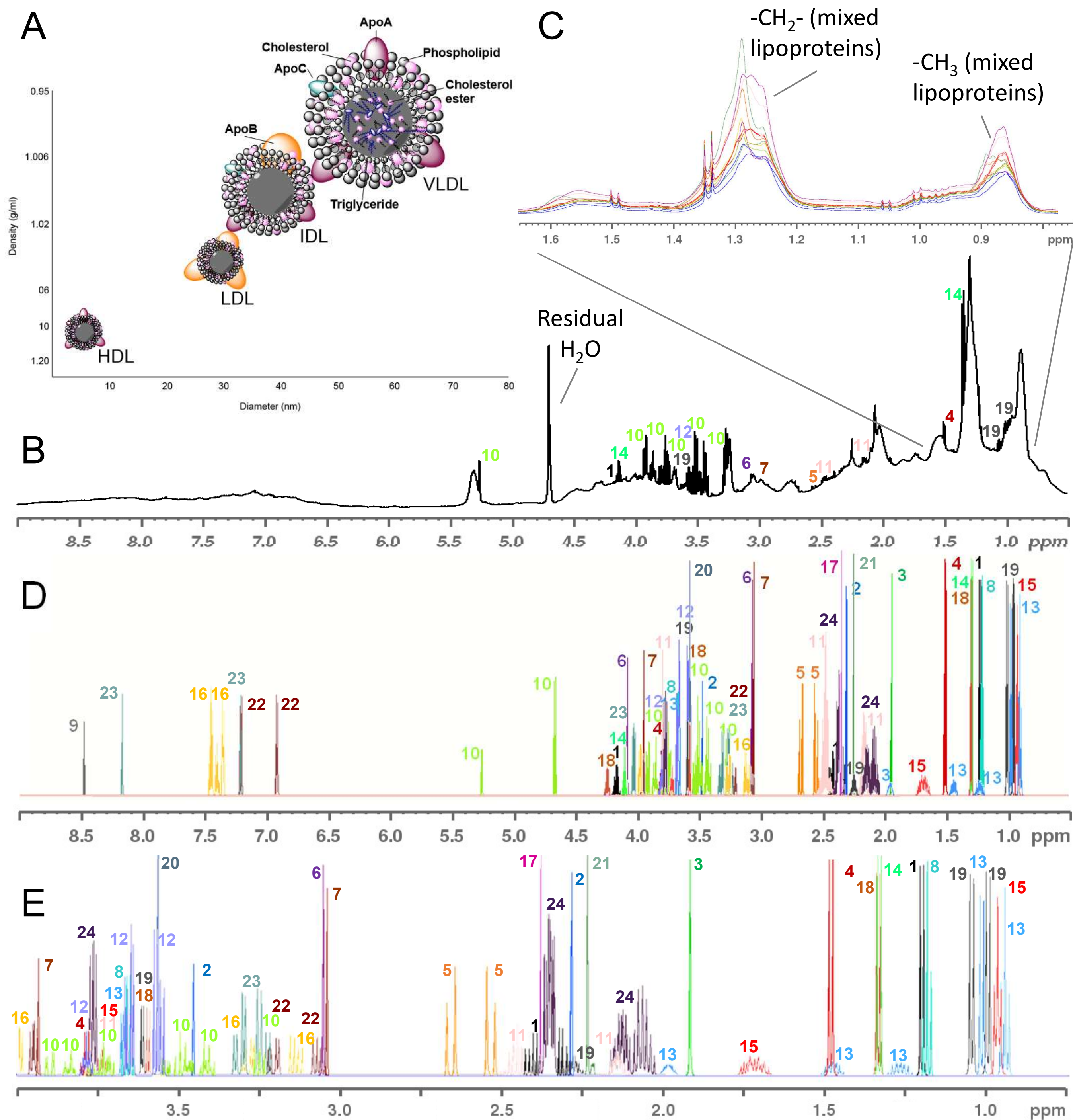

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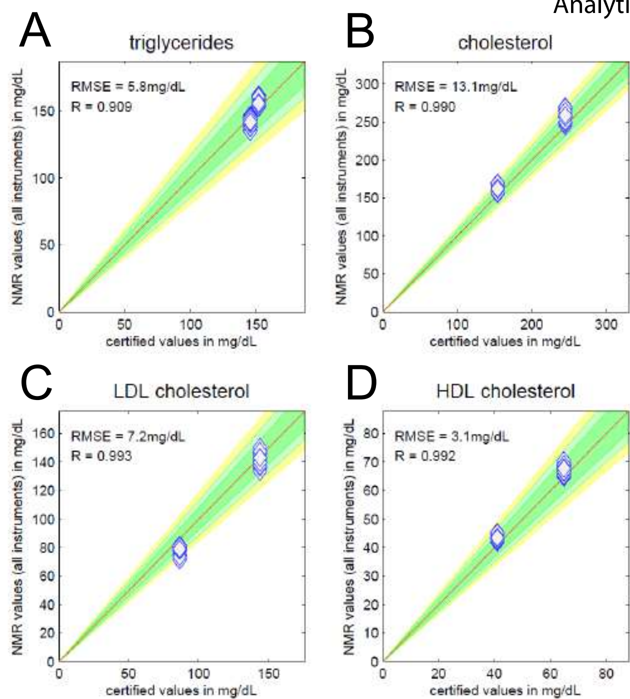
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RMSE = 7.2mg/dL
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NMR values (all instruments) in mg/dL
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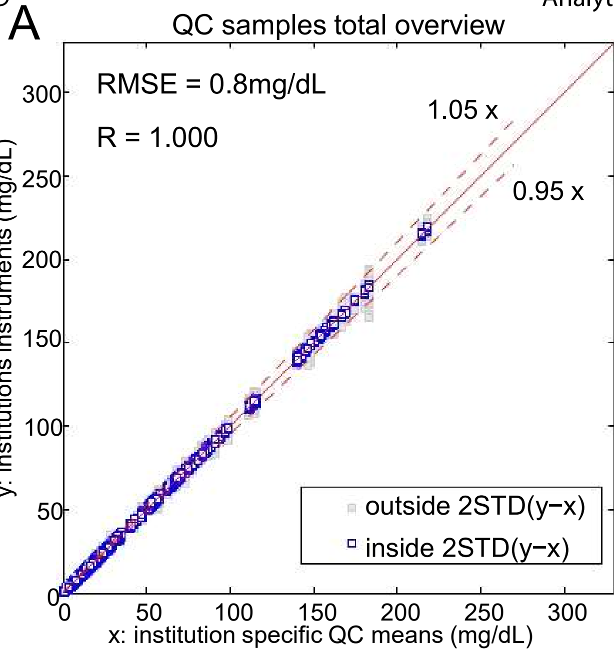

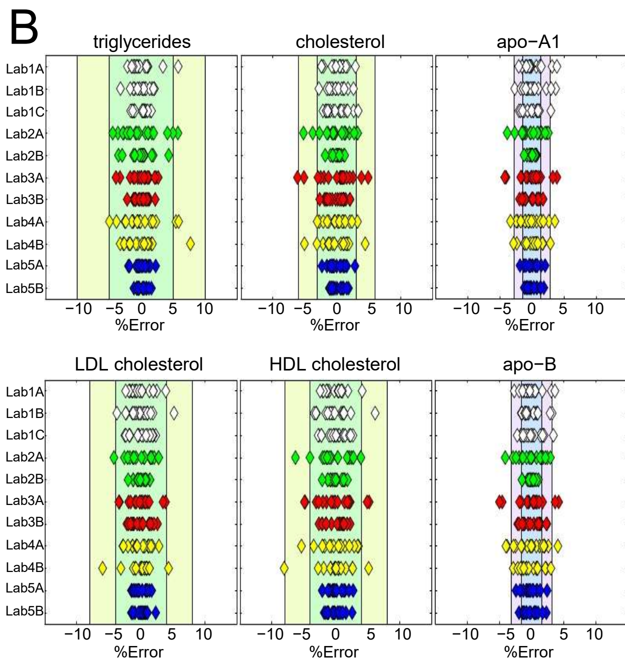

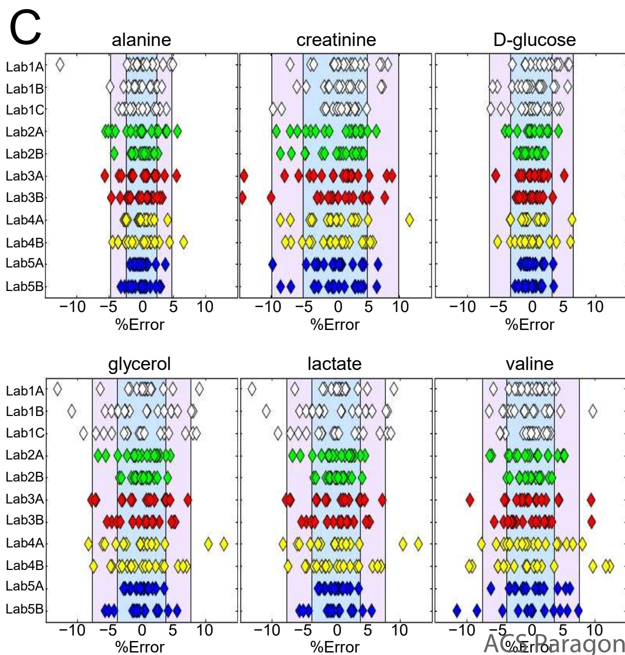

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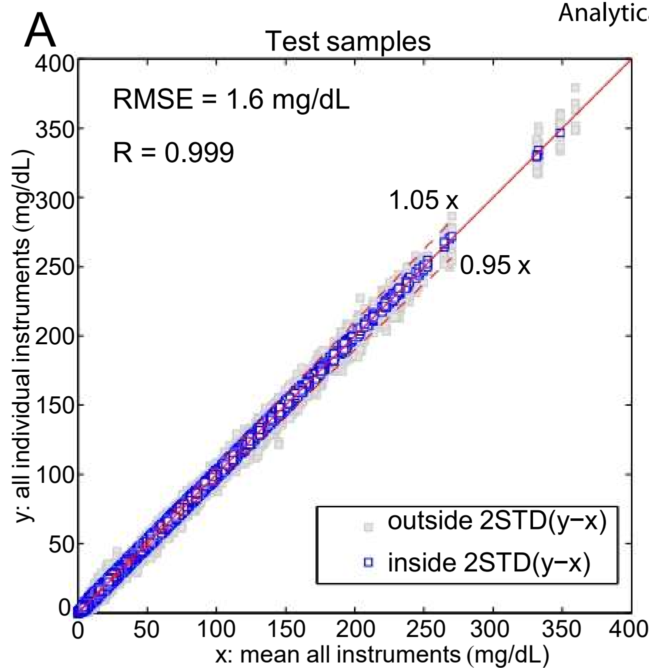

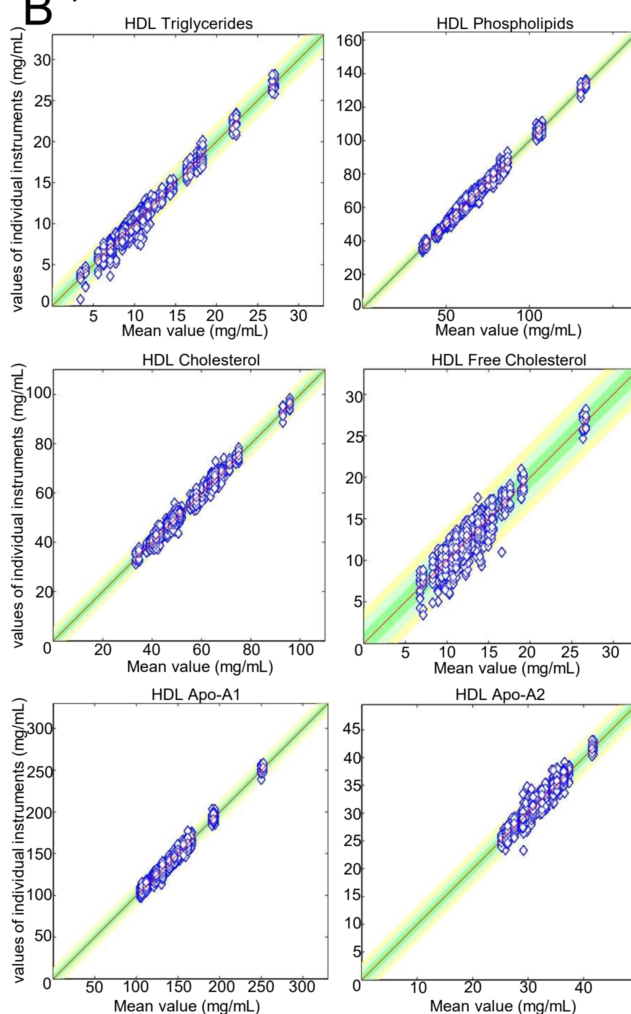

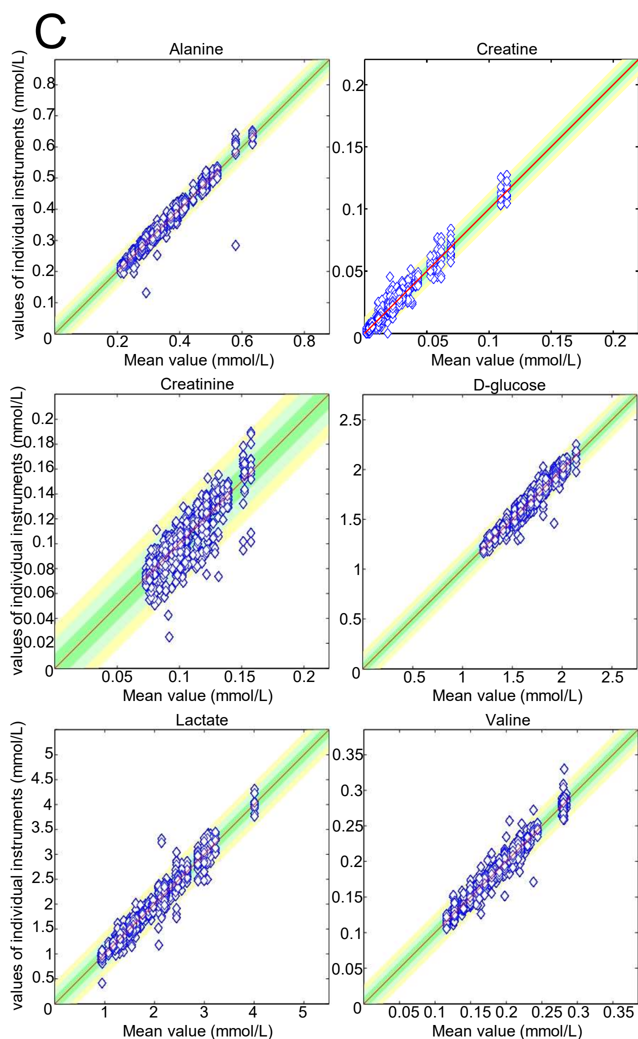

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